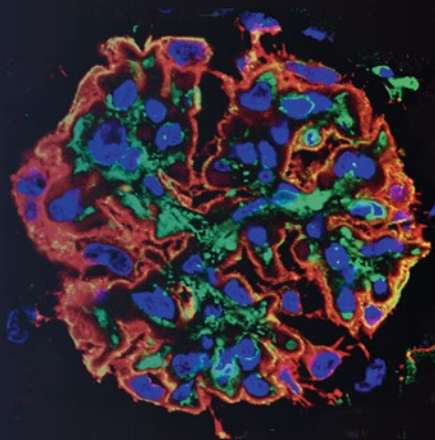


Methods in
Molecular Biology 1560

Springer Protocols



Carlo Pellicciari
Marco Biggiogera *Editors*

Histochemistry of Single Molecules

Methods and Protocols

 Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor

John M. Walker

School of Life and Medical Sciences

University of Hertfordshire

Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:

<http://www.springer.com/series/7651>

Histochemistry of Single Molecules

Methods and Protocols

Edited by

Carlo Pellicciari and Marco Biggiogera

Department of Biology and Biotechnology, University of Pavia, Pavia, Italy

 **Humana Press**

Editors

Carlo Pellicciari
Department of Biology and Biotechnology
University of Pavia
Pavia, Italy

Marco Biggiogera
Department of Biology and Biotechnology
University of Pavia
Pavia, Italy

ISSN 1064-3745

ISSN 1940-6029 (electronic)

Methods in Molecular Biology

ISBN 978-1-4939-6787-2

ISBN 978-1-4939-6788-9 (eBook)

DOI 10.1007/978-1-4939-6788-9

Library of Congress Control Number: 2016962655

© Springer Science+Business Media LLC 2017

This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.

The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made.

Cover Illustration: Double staining of claudin-5 (red fluorescence) and beta-actin (green) in the glomerulus of mouse kidney in a formalin-fixed paraffin-embedded tissue. Beta-actin and claudin-5 colocalize along the capillary walls showing yellow to orange color, which might correspond to pedicels of podocytes. Beta-actin is also demonstrated in the mesangial cells. Nuclei were stained with the DNA-specific dye DAPI (blue fluorescence). (Courtesy of Shuji Yamashita and Osamu Katsumata.)

Printed on acid-free paper

This Humana Press imprint is published by Springer Nature

The registered company is Springer Science+Business Media LLC

The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

The story of histochemistry goes a long way back and, in the last 150 years, has evolved in parallel with the operating instruments, from bright field microscopy to super-resolution microscopy. In this evolution resides the uniqueness of this science. Compared to the biochemical and molecular methods, the distinctive feature of histochemical techniques is the ability to localize chemical species in the very place (in a tissue, or a cell or an organelle) where they exist, are produced, or operate *in vivo*; this makes histochemistry an irreplaceable tool in basic and applied biomedical research.

Actually, over the last fifteen years, about 400,000 “histochemical” articles have been published in qualified international journals (according to the *Scopus* database). This demonstrates the impact of histochemistry on a wide variety of research subjects (from cell and tissue biology to anatomy and pathology, from zoology to botany, from ecology to nanotechnology), where histochemistry is essentially used for localizing (and often quantifying) *in situ* single molecules or molecular complexes to relate structural organization and function.

This book aims at providing an (certainly nonexhaustive) overview of histochemical techniques, through a series of lab-tested protocols for the detection of specific molecules or metabolic processes, both at light and electron microscopy. More in detail, the book is divided into six parts covering a variety of chemical targets.

The first part is on *vital histochemistry*, including overviews on single-cell histochemistry and autofluorescence. In these chapters, the detection of enzymatic activities is shown through the protocols for detection of peptidases, and a series of enzyme-histochemical methods are described for investigating functional histology in different invertebrate taxa.

Lectin histochemistry is represented by an overview followed by the use of lectins to detect glycosylation-specific cell types, cancer cells, or apoptotic cells.

Histochemistry of proteins used to be the hardest part of the histochemical course for a student in the 1970s, with a huge number of reactions for different chemical groups; nowadays antibodies have the stage, and in the third part, they are used to detect proteins marking neuronal differentiation, the myogenic progenitors, or autophagy. An essential prerequisite for all these techniques is antigen preservation and detection, which is often made problematic by sample fixation and embedding; a chapter is therefore devoted to the most suitable protocols for retrieving antigens in formalin-fixed paraffin-embedded specimens and frozen sections.

The fourth part on *lipid histochemistry* offers basics in fixation and tissue processing, staining for myelin, lipids in yeast, and single lipid droplets.

Nuclear histochemistry, after an overview of DNA fluorochromes, contains protocols for staining and labeling DNA and RNA at electron microscopy as well as for single- and double-strand breaks detection.

The last part is on *plant histochemistry* and deals with nuclear proteins, plant secretory structures, and acetogenins.

From this short list of contents, covering molecules, tissues, and species very far from each other, a simple conclusion may be drawn: histochemistry can be, and actually is, applied in *all* these cases and with successful results. With their specificity and resolution, histochemical and cytochemical methods are more than alive today and can effectively help scientists in very different research fields to elucidate biological issues through a unique approach to molecular biology in situ.

Pavia, Italy

*Carlo Pellicciari
Marco Biggiogera*

Contents

<i>Preface</i>	<i>v</i>
<i>Contributors</i>	<i>xi</i>

PART I VITAL HISTOCHEMISTRY

1 Single Cell Cytochemistry Illustrated by the Demonstration of Glucose-6-Phosphate Dehydrogenase Deficiency in Erythrocytes	3
<i>Anna L. Peters and Cornelis J.F. van Noorden</i>	
2 Autofluorescence Spectroscopy for Monitoring Metabolism in Animal Cells and Tissues	15
<i>Anna C. Croce and Giovanni Bottioli</i>	
3 Enzyme-Histochemistry Technique for Visualizing the Dipeptidyl-Peptidase IV (DPP-IV) Activity in the Liver Biliary Tree	45
<i>Vittorio Bertone, Eleonora Tarantola, and Isabel Freitas</i>	
4 Histochemical Demonstration of Tripeptidyl Aminopeptidase I	55
<i>Mashenka B. Dimitrova, Dimitrinka Y. Atanasova, and Nikolai E. Lazarov</i>	
5 Enzyme Histochemistry for Functional Histology in Invertebrates	69
<i>Francesca Cima</i>	

PART II LECTIN HISTOCHEMISTRY

6 Lectin Histochemistry: Historical Perspectives, State of the Art, and the Future	93
<i>Susan A. Brooks</i>	
7 Isolation of Viable Glycosylation-Specific Cell Populations for Further In Vitro or In Vivo Analysis Using Lectin-Coated Magnetic Beads	109
<i>Ellie-May Beaman, David R.F. Carter, and Susan A. Brooks</i>	
8 Lectin Histochemistry for Metastasizing and Non-metastasizing Cancer Cells	121
<i>Gerrit Wolters-Eisfeld and Udo Schumacher</i>	
9 The Use of Lectin Histochemistry for Detecting Apoptotic Cells in the Seminiferous Epithelium	133
<i>Vicente Seco-Rovira, Ester Beltrán-Frutos, Jesús Hernández-Martínez, Concepción Ferrer, and Luis Miguel Pastor</i>	

PART III PROTEIN HISTOCHEMISTRY

10 Heat-Induced Antigen Retrieval in Immunohistochemistry: Mechanisms and Applications	147
<i>Shuji Yamashita and Osamu Katsumata</i>	

- 11 Detecting Neuronal Differentiation Markers in Newborn Cells
of the Adult Brain 163
Sara Bonzano and Silvia De Marchis
- 12 Characterizing Satellite Cells and Myogenic Progenitors
During Skeletal Muscle Regeneration 179
Nicolas A. Dumont and Michael A. Rudnicki
- 13 Immunohistochemical Detection of the Autophagy Markers LC3
and p62/SQSTM1 in Formalin-Fixed and Paraffin-Embedded Tissue 189
Sabina Berezowska and José A. Galván

PART IV LIPID HISTOCHEMISTRY

- 14 Tissue Fixation and Processing for the Histological Identification of Lipids . . . 197
*Víctor Carriel, Fernando Campos, José Aneiros-Fernández,
and John A. Kiernan*
- 15 Staining Methods for Normal and Regenerative Myelin
in the Nervous System 207
*Víctor Carriel, Antonio Campos, Miguel Alaminos, Stefania Raimondo,
and Stefano Geuna*
- 16 Nile Red Staining of Neutral Lipids in Yeast 219
Kerry Ann Rostron and Clare Louise Lawrence
- 17 Staining of Lipid Droplets with Monodansylpentane 231
*Bo-Hua Chen, Huei-Jiun Yang, He-Yen Chou, Guang-Chao Chen,
and Wei Yuan Yang*

PART V NUCLEAR HISTOCHEMISTRY

- 18 Fluorochromes for DNA Staining and Quantitation 239
Giuliano Mazzini and Marco Danova
- 19 Osmium Ammine for Staining DNA in Electron Microscopy 261
Irene Masiello and Marco Biggiogera
- 20 DNA Labeling at Electron Microscopy 269
Nicolas Thelen and Marc Thiry
- 21 Visualizing RNA at Electron Microscopy by Terbium Citrate 277
Marco Biggiogera and Irene Masiello
- 22 Two-Tailed Comet Assay (2T-Comet): Simultaneous Detection
of DNA Single and Double Strand Breaks 285
*Elva I. Cortés-Gutiérrez, José Luis Fernández, Martha I. Dávila-Rodríguez,
Carmen López-Fernández, and Jaime Gosálvez*

PART VI PLANT HISTOCHEMISTRY

- 23 Detection of Endogenous Nuclear Proteins in Plant Cells:
Localizing Nuclear Matrix Constituent Proteins (NMCPs),
the Plant Analogs of Lamins 297
Malgorzata Ciska and Susana Moreno Díaz de la Espina

24	Histochemical Analysis of Plant Secretory Structures	313
	<i>Diego Demarco</i>	
25	A Histochemical Technique for the Detection of Annonaceous Acetogenins	331
	<i>Guillermo Laguna-Hernández, Alicia Enriqueta Brechú-Franco,</i> <i>Iván De la Cruz-Chacón, and Alma Rosa González-Esquinca</i>	
	<i>Index</i>	339

Contributors

- MIGUEL ALAMINOS • *Tissue Engineering Group, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain; Instituto de Investigación Biosanitaria Ibs, Granada, Spain*
- JOSÉ ANEIRO-S-FERNÁNDEZ • *Pathology Unit, Hospital Universitario Virgen de las Nieves, Granada, Spain*
- DIMITRINKA Y. ATANASOVA • *Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria; Department of Anatomy, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria*
- ELLIE-MAY BEAMAN • *Department of Biological & Medical Sciences, Oxford Brookes University, Headington, Oxford, UK*
- ESTER BELTRÁN-FRUTOS • *Department of Cell Biology and Histology, School of Medicine, IMIB-Arrixaca, University of Murcia, Espinardo, Murcia, Spain*
- SABINA BEREZOWSKA • *Institute of Pathology, University of Bern, Bern, Switzerland*
- VITTORIO BERTONE • *Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Pavia, Italy*
- MARCO BIGGIOGERA • *Department of Biology and Biotechnology, Laboratory of Cell Biology and Neurobiology, University of Pavia, Pavia, Italy*
- SARA BONZANO • *Department of Life Sciences and Systems Biology (DBIOS), University of Turin, Turin, Italy; Neuroscience Institute Cavalieri Ottolenghi (NICO), Orbassano, Turin, Italy*
- GIOVANNI BOTTIROLI • *Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Pavia, Italy*
- ALICIA ENRIQUETA BRECHÚ-FRANCO • *Facultad de Ciencias, Universidad Nacional Autónoma de México, Colonia Universidad Nacional Autónoma de México, Coyoacán, Ciudad de México, Mexico*
- SUSAN A. BROOKS • *Department of Biological & Medical Sciences, Oxford Brookes University, Headington, Oxford, UK*
- ANTONIO CAMPOS • *Tissue Engineering Group, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain; Instituto de Investigación Biosanitaria Ibs, Granada, Spain*
- FERNANDO CAMPOS • *Tissue Engineering Group, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain*
- VÍCTOR CARRIEL • *Tissue Engineering Group, Department of Histology, Faculty of Medicine, University of Granada, Granada, Spain; Instituto de Investigación Biosanitaria Ibs, Granada, Spain*
- DAVID R.F. CARTER • *Department of Biological & Medical Sciences, Oxford Brookes University, Headington, Oxford, UK*
- BO-HUA CHEN • *Chemical Biology and Molecular Biophysics Program, Taiwan International Graduate Program, Taipei, Taiwan; Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan; Institute of Bioinformatics and Structural Biology, National Tsing Hua University, Hsinchu, Taiwan*

- GUANG-CHAO CHEN • *Chemical Biology and Molecular Biophysics Program, Taiwan International Graduate Program, Taipei, Taiwan; Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan; Institute of Biochemical Sciences, College of Life Sciences, National Taiwan University, Taipei, Taiwan*
- HE-YEN CHOU • *Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*
- FRANCESCA CIMA • *Department of Biology, University of Padova, Padova, Italy*
- MALGORZATA CISKA • *Department of Cell and Molecular Biology, Biological Research Center, CSIC, Madrid, Spain*
- ELVA I. CORTÉS-GUTIÉRREZ • *Department of Genetics, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, Mexico*
- ANNA C. CROCE • *Institute of Molecular Genetics (IGM), CNR, Pavia, Italy*
- IVÁN DE LA CRUZ-CHACÓN • *Instituto de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas, Mexico*
- MARCO DANOVA • *Department of Medicine, Azienda Socio-Sanitaria Territoriale, Pavia, Italy*
- MARTHA I. DÁVILA-RODRÍGUEZ • *Department of Genetics, Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Monterrey, Mexico*
- DIEGO DEMARCO • *Laboratório de Anatomia Vegetal, Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil*
- MASHENKA B. DIMITROVA • *Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria*
- NICOLAS A. DUMONT • *Centre for Stem Cell Research, Ottawa Hospital Research Institute, Ottawa, ON, Canada; Faculty of Medicine, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada; Sainte-Justine Hospital Research Center and Department of Rehabilitation, Faculty of Medicine, University of Montreal, Montreal, QC, Canada*
- SUSANA MORENO DÍAZ DE LA ESPINA • *Department of Cell and Molecular Biology, Biological Research Center, CSIC, Madrid, Spain*
- JOSÉ LUIS FERNÁNDEZ • *Genetics Unit, INIBIC, Complejo Hospitalario Universitario A Coruña and Laboratory de Genética Molecular y Radiobiología, Centro Oncológico de Galicia, La Coruña, Spain*
- CONCEPCIÓN FERRER • *Department of Cell Biology and Histology, School of Medicine, IMIB-Arrixaca, University of Murcia, Espinardo, Murcia, Spain*
- ISABEL FREITAS • *Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Pavia, Italy*
- JOSÉ A. GALVÁN • *Institute of Pathology, University of Bern, Bern, Switzerland*
- STEFANO GEUNA • *Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Torino, Italy*
- ALMA ROSA GONZÁLEZ-ESQUINCA • *Instituto de Ciencias Biológicas, Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas, Mexico*
- JAIME GOSÁLVEZ • *Unit of Genetics, Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain*
- JESÚS HERNÁNDEZ-MARTÍNEZ • *Department of Cell Biology and Histology, School of Medicine, IMIB-Arrixaca, University of Murcia, Espinardo, Murcia, Spain*
- OSAMU KATSUMATA • *Department of Anatomy, Kitasato University School of Medicine, Kanagawa, Japan*

- JOHN A. KIERNAN • *Department of Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada*
- GUILLERMO LAGUNA-HERNÁNDEZ • *Facultad de Ciencias, Universidad Nacional Autónoma de México, Colonia Universidad Nacional Autónoma de México, Coyoacán, Ciudad de México, Mexico*
- CLARE LOUISE LAWRENCE • *School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, Lancashire, UK*
- NIKOLAI E. LAZAROV • *Institute of Neurobiology, Bulgarian Academy of Sciences, Sofia, Bulgaria; Department of Anatomy and Histology, Medical University of Sofia, Sofia, Bulgaria*
- CARMEN LÓPEZ-FERNÁNDEZ • *Unit of Genetics, Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain*
- SILVIA DE MARCHIS • *Department of Life Sciences and Systems Biology (DBIOS), University of Turin, Turin, Italy; Neuroscience Institute Cavalieri Ottolenghi (NICO), Orbassano, Turin, Italy*
- IRENE MASIELLO • *Department of Biology and Biotechnology, Laboratory of Cell Biology and Neurobiology, University of Pavia, Pavia, Italy*
- GIULIANO MAZZINI • *Institute of Molecular Genetics, CNR, Pavia, Italy; Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Pavia, Italy*
- CORNELIS J.F. VAN NOORDEN • *Department of Cell Biology and Histology, Academic Medical Centre, Amsterdam, The Netherlands*
- LUIS MIGUEL PASTOR • *Department of Cell Biology and Histology, School of Medicine, IMIB-Arrixaca, University of Murcia, Espinardo, Murcia, Spain*
- ANNA L. PETERS • *Department of Intensive Care, Academic Medical Centre, Amsterdam, The Netherlands*
- STEFANIA RAIMONDO • *Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Turin, Italy*
- KERRY ANN ROSTRON • *School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, Lancashire, UK; School of Biological Sciences, University of Reading, Reading, Berkshire, UK*
- MICHAEL A. RUDNICKI • *Centre for Stem Cell Research, Ottawa Hospital Research Institute, Ottawa, ON, Canada; Faculty of Medicine, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada*
- UDO SCHUMACHER • *Department of Anatomy and Experimental Morphology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*
- VICENTE SECO-ROVIRA • *Department of Cell Biology and Histology, School of Medicine, IMIB-Arrixaca, University of Murcia, Espinardo, Murcia, Spain*
- ELEONORA TARANTOLA • *Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Pavia, Italy*
- NICOLAS THELEN • *Giga Neurosciences, Department of Life Sciences, Laboratory of Cell Biology, University of Liège, Liège, Belgium*
- MARC THIRY • *Giga Neurosciences, Department of Life Sciences, Laboratory of Cell Biology, University of Liège, Liège, Belgium*
- GERRIT WOLTERS-EISEFELD • *Medical Glycobiology Group, Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany*

SHUJI YAMASHITA • *Department of Pathology, School of Medicine, Keio University, Tokyo, Japan*

HUEI-JIUN YANG • *Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*

WEI YUAN YANG • *Chemical Biology and Molecular Biophysics Program, Taiwan International Graduate Program, Taipei, Taiwan; Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan; Institute of Biochemical Sciences, College of Life Sciences, National Taiwan University, Taipei, Taiwan*

Part I

Vital Histochemistry

Chapter 1

Single Cell Cytochemistry Illustrated by the Demonstration of Glucose-6-Phosphate Dehydrogenase Deficiency in Erythrocytes

Anna L. Peters and Cornelis J.F. van Noorden

Abstract

Cytochemistry is the discipline that is applied to visualize specific molecules in individual cells and has become an essential tool in life sciences. Immunocytochemistry was developed in the sixties of last century and is the most frequently used cytochemical application. However, metabolic mapping is the oldest cytochemical approach to localize activity of specific enzymes, but in the last decades of the previous century and the first decade of the present century it almost became obsolete. The popularity of this approach revived in the past few years. Metabolism gained interest as player in chronic and complex diseases such as cancer, diabetes, neurodegenerative diseases, and vascular diseases and both enzyme cytochemistry and metabolic mapping have become important tools in life sciences.

In this chapter, we present glucose-6-phosphate dehydrogenase (G6PD) deficiency, the most prevalent enzyme deficiency worldwide, to illustrate recent developments in enzyme cytochemistry or metabolic mapping. The first assays which were developed quantified enzyme activity but were unreliable for single cell evaluation. The field has expanded with the development of cytochemical single cell assays and DNA testing. Still, all assays—from the earliest developed tests up to the most recently developed tests—have their place in investigations on G6PD activity. Recently, nanoscopy has become available for light and fluorescence microscopy at the nanoscale. For nanoscopy, cytochemistry is an essential tool to visualize intracellular molecular processes. The ultimate goal in the coming years will be nanoscopy of living cells so that the molecular dynamics can be studied. Cytochemistry will undoubtedly play a critical role in these developments.

Key words Cytochemistry, History, Metabolic mapping, Glucose-6-phosphate dehydrogenase deficiency

1 Introduction

Cytochemistry is the discipline that is applied to visualize specific molecules in individual cells and has become an essential tool in life sciences. Most users of cytochemical techniques do not realize nowadays that they are performing cytochemistry when they are labeling specific cellular proteins for flow cytometry and cell sorting, because this has become routine. Flow cytometry and cell

sorting are undoubtedly the most frequently-used applications of cytochemistry these days. Besides, cytochemistry has been an essential tool in electron microscopical analysis of specific molecules in cells to understand functioning of subcellular compartments. Here, electron dense markers have to be used instead of fluorescence markers. Recently, nanoscopy has become available for light and fluorescence microscopy at the nanoscale using approaches such as STED, SIM, PALM, and STORM [1–3]. These techniques do not obey the law of Abbe with respect to the limit of spatial resolution when using light to obtain images because of the wavelength character of light. For nanoscopy, cytochemistry is essential to visualize intracellular molecular processes. The ultimate goal in the coming years will be nanoscopy of living cells so that intracellular molecular dynamics can be studied. Text books will then be rewritten in a similar way as in the past decades on the basis of living cell imaging in the micrometer range using cytochemistry and confocal microscopy.

Cytochemistry as it is applied these days is almost exclusively based on immunocytochemistry to visualize specific proteins. It should be realized that the presence of a protein in (sub)cellular compartments does not automatically mean that this protein exerts the activity the researcher thinks that he/she investigates. It becomes more and more clear that epigenetic and posttranslational modifications of a protein, its microenvironment and/or its moonlighting (i.e., exerting different functions) are essential aspects of cellular proteins. Therefore, tools to visualize activity of proteins *in situ* are urgently needed to increase our understanding of cellular functions in health and disease. One such cytochemical approach is the visualization of the activity of a specific type of protein, enzymes, using enzyme cytochemistry or metabolic mapping. In fact, it is the oldest cytochemical approach of single molecule cytochemistry since Gomori published his cytochemical study on phosphatase activity in 1939 [4]. Immunocytochemistry was developed in the 1960s of last century and *in situ* hybridization for the visualization of specific mRNAs as measure of expression of a specific gene even later. Nevertheless, enzyme cytochemistry or metabolic mapping never grew into a position in cytochemistry that it deserves. In fact, in the last decades of the previous century and the first decade of the present century it almost became obsolete. However, since metabolism has gained novel interest as players in chronic and complex diseases such as cancer, diabetes, neurodegenerative diseases, and vascular diseases, enzyme cytochemistry or metabolic mapping has revived tremendously [5, 6].

In this chapter, we present the cytochemical visualization of glucose-6-phosphate dehydrogenase (G6PD) deficiency, the most prevalent enzyme deficiency worldwide, to illustrate recent developments in enzyme cytochemistry or metabolic mapping. G6PD is the key enzyme in the oxidative pentose phosphate pathway (PPP).

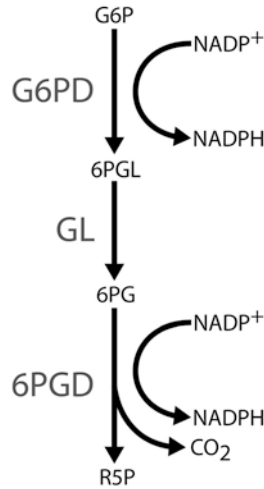


Fig. 1 The NADPH-producing steps of the oxidative pentose phosphate pathway. *6PG* 6-phosphogluconate, *6PGD* 6-phosphogluconate dehydrogenase, *6PGL* 6-phosphogluconolactone, *G6P* glucose-6-phosphate, *G6PD* glucose-6-phosphate dehydrogenase, *GL* gluconolactonase, *R5P* ribulose-5-phosphate

This pathway is one of the major pathways in which nicotinamide adenine dinucleotide phosphate (NADP^+) is converted into its reduced form NADPH, which is essential for the protection against reactive oxygen species (ROS) in cells and in particular erythrocytes (Fig. 1) [7, 8]. G6PD deficiency can lead to acute hemolytic anemia (AHA), chronic non-spherocytic hemolysis and hyperbilirubinemia resulting in neonatal kernicterus [9]. G6PD deficiency is the most common enzyme deficiency and worldwide an estimated 300–400 million people carry at least one deficient G6PD gene [10, 11]. The deficiency is mainly found in Africa, Asia, and Mediterranean Europe, areas where malaria is endemic or has been endemic [10, 12]. In these areas, G6PD deficiency has been found to protect against severe malaria infection [13].

2 Early History of G6PD Deficiency

Accounts of G6PD deficiency can be traced back to Pythagoras. The ancient Greek philosopher and mathematician reportedly forbade his followers to eat fava beans (*Vicia faba*), possibly because of their potential to induce favism [14, 15]. Favism is a pathological disorder in which a G6PD-deficient individual develops AHA after ingestion of fava beans. Reports of favism date back centuries and investigations into its pathogenesis intensified in the first half of the twentieth century. In the early decades, several reports of favism were published which coincided with cases of AHA after ingestion of 6-methoxy-8-aminoquinoline drugs for the treatment of malaria

[16]. Detailed research was performed on the metabolism of the erythrocyte, and in 1954 induction of AHA by primaquine (a similar compound as 6-methoxy-8-aminoquinoline) was attributed to G6PD deficiency [17]. Some years later, the similarities between AHA induced by primaquine and by favism became apparent and the link between favism and G6PD deficiency was established [18, 19]. It was observed that G6PD deficiency did not transfer from father to son and that all males who carry the gene show full expression. It thus was concluded that G6PD deficiency is transferred X-chromosomally. Males can be hemizygotously deficient, while females can be homozygotously deficient or heterozygotously deficient. Heterozygotously-deficient women have a mixed population of G6PD-sufficient and G6PD-deficient erythrocytes, owing to random inactivation of one of the two X-chromosomes. This process is known as lyonization [20].

3 Enzymology

G6PD is the rate-limiting enzyme of the PPP. The enzyme is a monomer of 515 amino acids and has a molecular mass of over 59 kDa [21]. Over 300 variants have been identified based on enzyme kinetics and physicochemical characteristics [22]. G6PD is activated by formation of a dimer or tetramer that contains tightly bound NADP⁺ [23]. Active G6PD initiates the PPP in which NADP⁺ is converted into NADPH, and G6P is converted into a pentose sugar, ribulose-5-phosphate, precursor of DNA, RNA, and ATP. NADPH is the most important reducing agent in the cytoplasm (Fig. 1) [7, 8]. During aging of the erythrocyte, the quantity of active G6PD decreases. The cells are not able to synthesize new proteins as mature erythrocytes do not have a nucleus, mitochondria, or ribosomes [24]. This results in increased susceptibility to oxidative stress in senescent erythrocytes. This natural process occurs more rapidly in G6PD deficiency. The deficiency causes increased susceptibility of erythrocytes to H₂O₂ and other ROS that can lead to AHA. Still, in daily life, G6PD deficiency is usually clinically silent because only 1–2% of the total NADPH production capacity is used in healthy erythrocytes, even in episodes of hemolytic stress [25]. However, numerous drugs and chemicals, such as primaquine and dapsone, ingestion of fava beans, and stress (for example, infection) can induce AHA in G6PD-deficient individuals [12].

The severity of G6PD deficiency is usually measured in four classes in which class I is the most severe form of deficiency and class IV has near to normal function (Table 1) [25, 26]. Mutations in the region of the enzyme where NADP⁺ or G6P bind cause severe loss of G6PD activity and are associated with class I deficiencies. These deficiencies are rare but can induce transfusion

Table 1
Classes of G6PD deficiency

WHO class	Severity	Percentage of normal G6PD function, %
Class I	Severe mutations with chronic non-spherocytic hemolytic anemia	≤10
Class II	Intermediate	10
Class III	Mild	10–60
Class IV	Asymptomatic	60–100
Class V	Increased function	>100

The WHO designates four classes of G6PD deficiency [25]. Sometimes a fifth class of mutations causing increased G6PD activity is included [26]

dependency as patients in this class usually suffer from chronic non-spherocytic hemolytic anemia. The other classes of deficiency are usually asymptomatic unless patients are in contact with agents that induce hemolytic stress.

4 Investigations on G6PD Activity

In 1967, the World Health Organization (WHO) published recommendations on the standardisation of investigations of G6PD deficiency [27]. At this time, several G6PD-enzyme variants had been detected and in this bulletin it was recommended that all investigations should include spectrophotometric estimations of erythrocyte G6PD activity, electrophoretic enzyme migration, the Michaelis constant for G6P, thermal stability and the relative rate of utilization of G6P. The gold standard for detection of G6PD deficiency was spectrophotometry.

Several tests had already been developed on the basis of dye decolorization methods (brilliant cresyl blue), methemoglobin reduction and reoxygenation, spot testing with tetrazolium salts, and fluorescent spot tests. However, spectrophotometry was the only assay in which G6PD activity could be quantified, thus it became the golden standard.

5 Diagnosis of G6PD Deficiency

Diagnosing G6PD deficiency in hemizygous males is (usually) straight forward. In hemizygous males, all erythrocytes express the deficiency and both qualitative and quantitative assays can detect this type of deficiency. The same holds for homozygously deficient

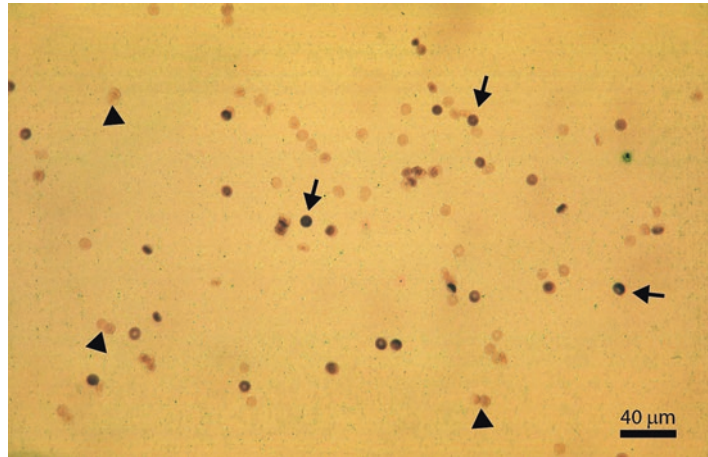


Fig. 2 Erythrocytes after cytochemical staining of glucose-6-phosphate dehydrogenase (G6PD) activity showing stained G6PD-containing erythrocytes (*arrows*) and unstained G6PD-deficient erythrocytes (*arrowheads*). The figure illustrates heterozygous deficiency with a mixed population of G6PD-containing erythrocytes and G6PD-deficient erythrocytes. Bar: 40 μm

females. However, detection of heterozygously deficient females can be challenging as heterozygously deficient women have a mixed population of G6PD-sufficient and G6PD-deficient erythrocytes, owing to random inactivation of one of the two X chromosomes (Fig. 2) [20]. Diagnosing G6PD deficiency in these women can be difficult as many assays are based on quantitative methods that can miss females with favourable lyonization [28, 29]. Analysis of patients who recently suffered from AHA can also result in false negative test results, because young erythrocytes express normal G6PD activity and can thus mask G6PD deficiency. However, repeating of the test after a few weeks circumvents this problem.

6 Early Quantitative Assay: Spectrophotometry

Spectrophotometry is a technique in which the light reflecting and/or light-absorbing potential of a molecule is quantified. To detect G6PD deficiency, the light transmittance of a solution with a hemolysate of blood is measured. The assay is based on the difference in absorbance of NADPH and NADP^+ when excited with light of a wavelength of 340 nm. The assay is more reliable than most qualitative assays for detection of heterozygous females. However, it still can miss heterozygously deficient females with favorable lyonization. An alternative spectrophotometric method used for the detection of G6PD deficiency is based on inhibition by chromate of glutathione reductase (GSSG-R) activity in normal

erythrocytes, but not in G6PD deficient cells [30]. This assay increases the sensitivity for heterozygously-deficient females. However, the excess activity of G6PD-sufficient erythrocytes in these patients can mask G6PD deficiency and even with chrome inhibition, a substantial number of patients are missed [30].

7 Qualitative Assays

After the discovery of G6PD deficiency, several assays were developed which could be used for qualitative detection of G6PD deficiency. These assays are of limited value for detection of heterozygously deficient female patients with favorable lyonization but detect homozygous and hemizygous G6PD-deficiency very accurately [31]. The simplicity of the assays makes them very useful for rapid screening in low-income areas. Examples of these assays are dye decolorization methods such as the brilliant cresyl blue test. Brilliant cresyl blue is reduced to a colorless solution by NADPH. Another test is the methemoglobin reduction test which is based on the difference in color of oxyhemoglobin (red) and methemoglobin (brown). In the first step of the test full blood is exposed to sodium nitrite which converts all oxyhemoglobin into methemoglobin. The color of the tubes containing blood changes from clear red to brown. This is followed by an incubation period in the presence of an electron carrier, for example Nile blue. This facilitates the conversion of methemoglobin back to oxyhemoglobin in G6PD normal blood, producing red colored blood. G6PD deficient blood retains the brown color [27].

The most frequently-applied qualitative assay is the fluorescent spot test. After addition of G6P and NADP⁺ to a spot of full blood, the spot fluoresces when excited at 340 nm. The test has a high sensitivity and specificity for hemizygous and homozygous G6PD deficiency and is commonly used for screening of large groups of patients [12].

8 Single Cell Assay

Already in the 1960s of the previous century, the first single cell assay to detect G6PD deficiency was developed. In this test, G6PD activity in individual erythrocytes is determined. The cytochemical assay is based on staining of individual erythrocytes by the conversion of exogenous G6P and NADP⁺ by G6PD in the individual erythrocyte. Unstained erythrocytes have little or no G6PD activity. In theory, the assay can assess G6PD function very precisely as the percentage of stained and unstained cells can be determined by light microscopy (Fig. 2). However, leakage of colored end products from healthy erythrocytes into the medium and unstained cells

makes the test unreliable. In the early 1980s the staining procedure was optimized [32]. The cytochemical assay is based on the reduction of water-soluble colorless tetranitro blue tetrazolium via the electron carrier 1-methoxyphenazine methosulfate in its water-insoluble dark-colored formazan by NADPH [32, 33]. Dark-purple granules are present in erythrocytes that contain G6PD activity, whereas G6PD deficient erythrocytes remain unstained (Fig. 2). The assay was compared with spectrophotometry and chrome inhibition, and was found to be equally reliable for detection of hemizygous and homozygous G6PD deficiency. The detection of heterozygous-deficient females was much improved when compared to the other assays [30]. Thus, the first reliable single cell assay was developed with which all types of deficiency could reliably be detected. However, the test was not fully adopted in diagnostic laboratories as it is a time-consuming and technically difficult test.

9 A New Era: Genetics

In 1986, researchers reported the entire G6PD gene structure [34] and in 1991, the location of the gene on the distal long arm of the X-chromosome was discovered [35]. The G6PD gene is 18 kb long and contains 13 exons and 12 introns, of which the length varies between 12 and 236 bp [36]. After sequencing of the G6PD gene, investigations of the genetics progressed rapidly. At this moment, more than 180 mutations of the gene have been described and new variants are discovered annually [12, 37]. DNA analysis is highly sensitive for detection of all types of deficiency, but it cannot be used to diagnose unknown mutations. It can also not be used to quantify the effect of mutations on enzyme activity. With the introduction of multiplex polymerase chain reaction (PCR) and microarray analysis DNA investigations has become less costly and time-consuming, but still DNA analysis is not suitable for routine diagnostics [38].

10 Expansion of Single Cell Assays

With the introduction of advanced methods for automated fluorescence-activated cell sorting (FACS), the cytochemical single cell assay was further optimized (Fig. 3) [39]. Use of FACS now permitted automated calculations of percentages of G6PD negative erythrocytes but the assay remained a difficult procedure, prone to error. In 2012, 60 years after discovery of G6PD deficiency, a new single cell assay was introduced. This assay is based on the fluorescent properties of oxyhemoglobin. First, all oxyhemoglobin is converted to methemoglobin by exposure to sodium nitrite.

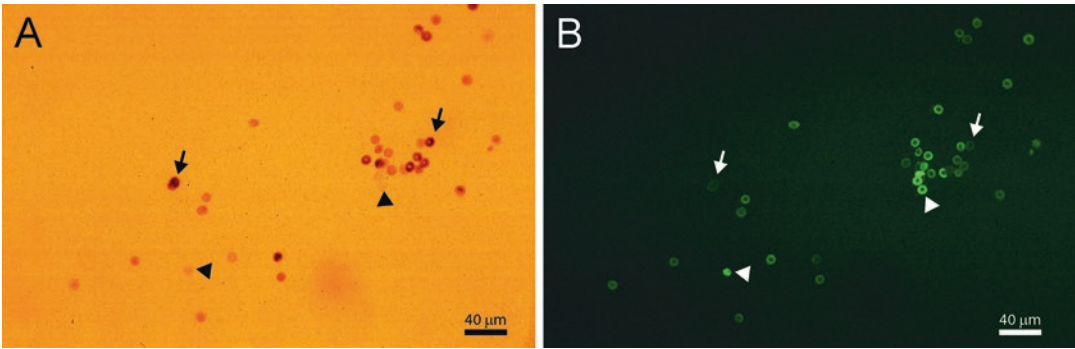


Fig. 3 Erythrocytes of a heterozygously G6PD-deficient patient after cytochemical staining. The erythrocytes marked with *arrows* contain G6PD activity; the erythrocytes marked with *arrowheads* are G6PD deficient. (a) Light microscopical image. (b) Fluorescence image. Fluorescence is quenched by formazan, and thus G6PD-deficient erythrocytes fluoresce strongest. Bar: 40 µm

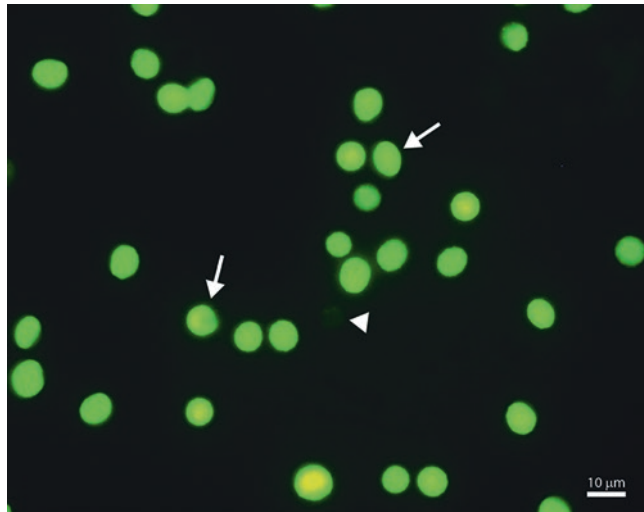


Fig. 4 Erythrocytes of a healthy volunteer without G6PD deficiency after cytofluorometric staining according to the method by Shah et al. [40]. The erythrocytes marked with *arrows* contain G6PD activity; the *arrowhead* marks a senescent erythrocyte with reduced G6PD activity. Bar: 10 µm

Incubation with glucose and methylene blue induces reduction of methemoglobin back to oxyhemoglobin. The erythrocytes are then exposed to hydrogen peroxide which produces a fluorescent product when it interacts with oxyhemoglobin (Fig. 4). The product of the assay can be measured with FACS. The reduction of methemoglobin to oxyhemoglobin is G6PD dependent and the assay is thus very sensitive to detect G6PD deficiency. At the moment, this assay is the easiest and most reliable cytochemical assay to detect heterozygous G6PD deficiency [40].

11 Conclusion

The field of enzyme activity evaluation has developed rapidly in the past century. The first assays which were developed quantified enzyme activity but were unreliable for single cell evaluation. The field has expanded with the development of cytochemical single cell assays and DNA testing. Still, all assays—from the earliest developed tests up to the most recently developed tests—have their place in investigations on enzyme activity. In this chapter we use G6PD as example: spectrophotometry is cheap, easy, and reliable for detection of G6PD-deficient males and can be used to estimate G6PD function. Qualitative assays are useful for rapid screening of G6PD deficiency while the newest single cell assays can be used to detect heterozygous G6PD-deficient females. DNA analysis is useful for detection of all types of deficiencies and mutations, but is limited to known mutations although entire gene sequencing is becoming cheaper and easier to use. However, the quantitative cytochemical assay as developed by Shah et al. [40] is the best option to detect all forms of G6PD deficiency.

References

1. Heilemann M (2010) Fluorescence microscopy beyond the diffraction limit. *J Biotechnol* 149(4):243–251. doi:[10.1016/j.jbiotec.2010.03.012](https://doi.org/10.1016/j.jbiotec.2010.03.012)
2. Galbraith CG, Galbraith JA (2011) Super-resolution microscopy at a glance. *J Cell Sci* 124(Pt 10):1607–1611. doi:[10.1242/jcs.080085](https://doi.org/10.1242/jcs.080085)
3. Hell SW (2009) Microscopy and its focal switch. *Nat Methods* 6(1):24–32. doi:[10.1038/nmeth.1291](https://doi.org/10.1038/nmeth.1291)
4. Gomori G (1939) Microtechnical demonstration of phosphatase in tissue sections. *Exp Biol Med* 42(1):23–26
5. Van Noorden CJ (2010) Imaging enzymes at work: metabolic mapping by enzyme histochemistry. *J Histochem Cytochem* 58(6):481–497. doi:[10.1369/jhc.2010.955518](https://doi.org/10.1369/jhc.2010.955518)
6. Van Noorden CJ (2014) Metabolic mapping by (quantitative) enzyme histochemistry. In: McManus L, Mitchell R (eds) *Pathobiology of human disease*. Elsevier, San Diego, CA, pp 3760–3774
7. Bleeker FE, Atai NA, Lamba S et al (2010) The prognostic IDH1(R132) mutation is associated with reduced NADP⁺-dependent IDH activity in glioblastoma. *Acta Neuropathol* 119(4):487–494. doi:[10.1007/s00401-010-0645-6](https://doi.org/10.1007/s00401-010-0645-6)
8. Koehler A, Van Noorden CJ (2003) Reduced nicotinamide adenine dinucleotide phosphate and the higher incidence of pollution-induced liver cancer in female flounder. *Environm Toxicol Chem* 22(11):2703–2710
9. Luzzatto L (2006) Glucose 6-phosphate dehydrogenase deficiency: from genotype to phenotype. *Haematologica* 91(10):1303–1306
10. Howes RE, Piel FB, Patil AP et al (2012) G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geo-statistical model-based map. *PLoS Med* 9(11), e1001339. doi:[10.1371/journal.pmed.1001339](https://doi.org/10.1371/journal.pmed.1001339)
11. Nkhoma ET, Poole C, Vannappagari V et al (2009) The global prevalence of glucose-6-phosphate dehydrogenase deficiency: a systematic review and meta-analysis. *Blood Cell Mol Dis* 42(3):267–278. doi:[10.1016/j.bcmd.2008.12.005](https://doi.org/10.1016/j.bcmd.2008.12.005)
12. Peters AL, Van Noorden CJ (2009) Glucose-6-phosphate dehydrogenase deficiency and malaria: cytochemical detection of heterozygous G6PD deficiency in women. *J Histochem Cytochem* 57(11):1003–1011. doi:[10.1369/jhc.2009.953828](https://doi.org/10.1369/jhc.2009.953828)
13. Guindo A, Fairhurst RM, Doumbo OK et al (2007) X-Linked G6PD deficiency protects hemizygous males but not heterozygous females against severe malaria. *PLoS Med* 4(3), e66. doi:[10.1371/journal.pmed.0040066](https://doi.org/10.1371/journal.pmed.0040066)
14. Cappellini MD, Fiorelli G (2008) Glucose-6-phosphate dehydrogenase deficiency. *Lancet* 371(9606):64–74. doi:[10.1016/S0140-6736\(08\)60073-2](https://doi.org/10.1016/S0140-6736(08)60073-2)
15. Meletis J, Konstantopoulos K (2004) Favism—from the “avoid fava beans” of Pythagoras to the present. *Haematology* 7(1):17–21

16. Beutler E (2008) Glucose-6-phosphate dehydrogenase deficiency: a historical perspective. *Blood* 111(1):16–24. doi:[10.1182/blood-2007-04-077412](https://doi.org/10.1182/blood-2007-04-077412)
17. Dern R, Weinstein IM, Leroy G et al (1954) The hemolytic effect of primaquine. I. The localization of the drug-induced hemolytic defect in primaquine-sensitive individuals. *J Lab Clin Med* 43(2):303–309
18. Crosby WH (1956) Favism in Sardinia (news-letter). *Blood* 11(1):91–92
19. Sansone G, Segni G (1958) New aspects of the biochemical alterations in the erythrocytes of patients with favism; almost complete absence of glucose-6-phosphate dehydrogenase. *Boll Soc Ital Biol Sper* 34(7):327
20. Lyon MF (1961) Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* 190:372–373
21. Rattazzi MC (1968) Glucose 6-phosphate dehydrogenase from human erythrocytes: molecular weight determination by gel filtration. *Biochem Biophys Res Commun* 31(1):16–24
22. Luzzatto L, Battistuzzi G (1985) Glucose-6-phosphate dehydrogenase. *Adv Hum Genet* 14:217–329
23. Wrigley NG, Heather JV, Bonsignore A et al (1972) Human erythrocyte glucose 6-phosphate dehydrogenase: electron microscope studies on structure and interconversion of tetramers, dimers and monomers. *J Mol Biol* 68(3):483–499
24. Marks PA, Johnson AB, Hirschberg E (1958) Effect of age on the enzyme activity in erythrocytes. *Proc Natl Acad Sci U S A* 44(6):529–536
25. WHO Working Group (1989) Glucose-6-phosphate dehydrogenase deficiency. *B World Health Organ* 67(6):601–611
26. Beutler E (1994) G6PD deficiency. *Blood* 84(11):3613–3636
27. World Health Organization (1967) Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group. *W Health Organ Tech Rep Ser* 366:1–53
28. LaRue N, Kahn M, Murray M et al (2014) Comparison of quantitative and qualitative tests for glucose-6-phosphate dehydrogenase deficiency. *Am J Trop Med Hyg* 91(4):854–861. doi:[10.4269/ajtmh.14-0194](https://doi.org/10.4269/ajtmh.14-0194)
29. Nantakomol D, Paul R, Palasuwan A et al (2013) Evaluation of the phenotypic test and genetic analysis in the detection of glucose-6-phosphate dehydrogenase deficiency. *Malar J* 12:289. doi:[10.1186/1475-2875-12-289](https://doi.org/10.1186/1475-2875-12-289)
30. Jonges GN, Hagen H, Van Noorden CJ et al (1989) Comparison between the chromate inhibition test and a cytochemical method for the determination of glucose-6-phosphate dehydrogenase deficiency in erythrocytes. *Clin Chim Acta* 181(2):135–141
31. Wolf BH, Weening RS, Schutgens RB et al (1987) Detection of glucose-6-phosphate dehydrogenase deficiency in erythrocytes: a spectrophotometric assay and a fluorescent spot test compared with a cytochemical method. *Clin Chim Acta* 168(2):129–136
32. Van Noorden CJ, Vogels IM, James J et al (1982) A sensitive cytochemical staining method for glucose-6-phosphate dehydrogenase activity in individual erythrocytes. I. Optimization of the staining procedure. *Histochemistry* 75(4):493–506
33. Van Noorden CJ, Vogels IM (1985) A sensitive cytochemical staining method for glucose-6-phosphate dehydrogenase activity in individual erythrocytes. II. Further improvements of the staining procedure and some observations with glucose-6-phosphate dehydrogenase deficiency. *Br J Haematol* 60(1):57–63
34. Persico MG, Viglietto G, Martini G et al (1986) Isolation of human glucose-6-phosphate dehydrogenase (G6PD) cDNA clones: primary structure of the protein and unusual 5' non-coding region. *Nucleic Acids Res* 14(6):2511–2522
35. Trask BJ, Massa H, Kenwrick S et al (1991) Mapping of human chromosome Xq28 by two-color fluorescence *in situ* hybridization of DNA sequences to interphase cell nuclei. *Am J Hum Genet* 48(1):1–15
36. Martini G, Toniolo D, Vulliamy T et al (1986) Structural analysis of the X-linked gene encoding human glucose 6-phosphate dehydrogenase. *EMBO J* 5(8):1849–1855
37. Minucci A, Moradkhani K, Hwang MJ et al (2012) Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the “old” and update of the new mutations. *Blood Cell Mol Dis* 48(3):154–165. doi:[10.1016/j.bcmd.2012.01.001](https://doi.org/10.1016/j.bcmd.2012.01.001)
38. Von Seidlein L, Auburn S, Espino F et al (2013) Review of key knowledge gaps in glucose-6-phosphate dehydrogenase deficiency detection with regard to the safe clinical deployment of 8-aminoquinoline treatment regimens: a workshop report. *Malar J* 12:112. doi:[10.1186/1475-2875-12-112](https://doi.org/10.1186/1475-2875-12-112)
39. Van Noorden CJ, Dolbear F, Aten J (1989) Flow cytofluorometric analysis of enzyme reactions based on quenching of fluorescence by the final reaction product: detection of glucose-6-phosphate dehydrogenase deficiency in human erythrocytes. *J Histochem Cytochem* 37(9):1313–1318
40. Shah SS, Diakite SA, Traore K et al (2012) A novel cytofluorometric assay for the detection and quantification of glucose-6-phosphate dehydrogenase deficiency. *Sci Rep* 2:299. doi:[10.1038/srep00299](https://doi.org/10.1038/srep00299)

Chapter 2

Autofluorescence Spectroscopy for Monitoring Metabolism in Animal Cells and Tissues

Anna C. Croce and Giovanni Bottiroli

Abstract

Excitation of biological substrates with light at a suitable wavelength can give rise to a light emission in the ultraviolet (UV)-visible, near-infrared (IR) spectral range, called autofluorescence (AF). This is a widespread phenomenon, ascribable to the general presence of biomolecules acting as endogenous fluorophores (EFs) in the organisms of the whole life kingdom. In cytochemistry and histochemistry, AF is often an unwanted signal enhancing the background and affecting in particular the detection of low signals or rare positive labeling spots of exogenous markers. Conversely, AF is increasingly considered as a powerful diagnostic tool because of its role as an intrinsic biomarker directly dependent on the nature, amount, and microenvironment of the EFs, in a strict relationship with metabolic processes and structural organization of cells and tissues. As a consequence, AF carries multiple information that can be decrypted by a proper analysis of the overall emission signal, allowing the characterization and monitoring of cell metabolism in situ, in real time and in the absence of perturbation from exogenous markers. In the animal kingdom, AF studies at the cellular level take advantage of the essential presence of NAD(P)H and flavins, primarily acting as coenzymes at multiple steps of common metabolic pathways for energy production, reductive biosynthesis and antioxidant defense. Additional EFs such as vitamin A, porphyrins, lipofuscins, proteins, and neuromediators can be detected in different kinds of cells and bulk tissues, and can be exploited as photophysical biomarkers of specific normal or altered morphofunctional properties, from the retinoid storage in the liver to aging processes, metabolic disorders or cell transformation processes. The AF phenomenon involves all living system, and literature reports numerous investigations and diagnostic applications of AF, taking advantage of continuously developing self-assembled or commercial instrumentation and measuring procedures, making almost impossible to provide their comprehensive description. Therefore a brief summary of the history of AF observations and of the development of measuring systems is provided, along with a description of the most common EFs and their metabolic significance. From our direct experience, examples of AF imaging and microspectrofluorometric procedures performed under a single excitation in the near-UV range for cell and tissue metabolism studies are then reported.

Key words Spectrofluorometry, Endogenous fluorophores, Curve fitting analysis, Energy metabolism, Lipofuscin

1 Introduction

The common presence of biomolecules acting as endogenous fluorophores (EFs) in the organisms of the whole life kingdom accounts for the emission of light in the ultraviolet (UV)-visible, near-infrared

(IR) spectral range under excitation at a suitable wavelength. A lot of literature has been produced on this ubiquitous phenomenon, known as autofluorescence (AF), and its negative or increasingly considered positive implications in biomedical investigations [1–3].

In fluorescence microscopy and cytometry, AF is often perceived as a nuisance since it increases the background signal, hindering the detection of exogenous fluorochromes. This disturbance can be particularly adverse in the case of exogenous dyes with relatively low emission signals or labeling only rare and sporadic substrates, in the presence of EFs with a high amplitude and broad emission, such as lipofuscins and the fibrous proteins, collagen and elastin. Strategies for avoiding or lowering unwanted fluorescence before staining procedures have been proposed, consisting for example in the chemical reduction of lipofuscins and elastin to decrease their bright emission signals, or simply in AF bleaching by pre-irradiation [4, 5]. As to the bleaching phenomenon, anyway, it is to remind that it can be conveniently used for other different applications, such as an engineering characterization of mechanical properties and responses to pressure of biological microstructures, for example the liver capsule, or an improved differentiation of EFs depending on their photosensitivity [6–8]. Coming back to AF as an unwanted signal, it can be minimized also by choosing proper optical filters and dyes with suitable spectral properties. This approach is facilitated by the continuously increasing commercial offer of exogenous fluorophores, with elevated emission quantum yield and spectra as much as possible different from those of the EFs of the biological substrate under study, in particular as to excitation far from the absorption of EFs often prevailing in the near-UV-blue spectral region. Attention to fixatives is also recommended [9]. Aldehyde derivatives, in particular, are well known to undergo condensation reactions with amines and proteins when used to preserve cell and tissue structures. Fluorescent products are generated, enhancing the background signal particularly undesirable when using antibodies, lectins, or receptor probes to detect rare specific targets in fixed material.

Apart these problems, an increasing attention is deserved to AF as a powerful diagnostic resource to characterize and monitor in real time the morphofunctional properties of a biological substrate under normal, physiologically altered and diseased conditions. AF can thus act as an intrinsic, photophysical biomarker since its properties in terms of lifetime, amplitude, and spectral shape depend on the nature, amount, and microenvironment of the EFs present in cells and tissues, in a close relationship with their structural and metabolic properties. Therefore the overall AF signal carries multiple bio-metabolic information, that can be interpreted by means of a suitable data processing. The relatively low number of EFs involved in the main metabolic functions and biological structures helps the achievement of the

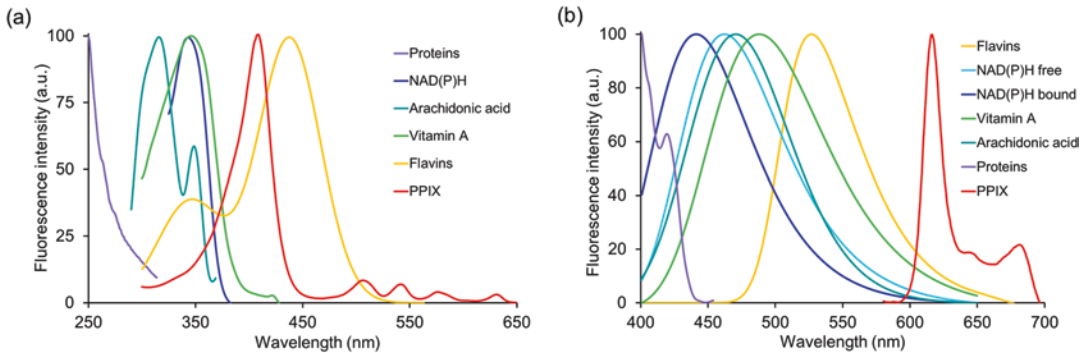


Fig. 1 Excitation (a) and emission spectra (b) of the most common EF encountered in animal cells and tissues. Spectra have been recorded from pure compound solutions or from extracts or selected tissue regions of liver tissue specimens, as described in the “AF characterization of EFs as pure compounds in solution,” and the “AF characterization of EFs from cells and tissues” sections. Note that absorption of all EFs is below 400 nm, with the few exceptions of flavins, exhibiting two absorption bands centered at about 360 and at 440 nm, and porphyrins, exhibiting a main absorption just around 400 nm and additional minor bands in the 500–700 nm, respectively the Soret and the Q bands (a). Emission spectra are more dispersed in the 400–700 nm range, helping to distinguish the AF signal of the different EFs under a single near UV-excitation (b). This is particularly favorable to estimate simultaneously pairs of metabolically related EFs, such as fluorescing fatty lipids and lipofuscin-like lipopigments as their oxidized products, or NAD(P)H in the bound/free state and flavins

diagnostic information in the absence of perturbations from fixation or administration of exogenous dyes, and even in living conditions. An advantage comes also from the spectral properties of the main EFs, usually excited in the near-UV spectral range up to 400 nm, and giving rise to emission spectra dispersed in the 400–700 nm range (Fig. 1a, b). These features make it possible to excite the EFs simultaneously in the near-UV spectral region, and to estimate the contribution of each EF to the overall AF emission by a proper spectral fitting analytical procedure. This chance, as remarked in more detail below (in Subheading 1.4), is particularly useful to simultaneously investigate the reciprocal behavior of pairs of EFs significant of dynamic metabolic functions.

1.1 Short Historical Notes

Before describing the procedures for AF detection and analysis, here we briefly summarize the main steps in AF uncovering, and the most significant EFs that have been up to now investigated to assess cell and tissue composition, metabolic engagement, and their changes. Following the first observations by David Brewster of light emission from organic compounds early in 1838, and called “fluorescence” by George Stokes, the attention from the scientific community on the fluorescence properties of biological substrates was remarkably enhanced by the parallel development of the instrumentation suitable for its detection. In 1911, Hans Stübel, a physiologist at Jena University reported on the first observations at microscope of fluorescence emission from single unicellular organisms or animal tissues [1, 2].

With more specific reference to metabolic processes and related disorders, already in 1944 Hans Popper and coworkers [10] described a yellowish fluorescence in a liver with induced cirrhosis. Later, in 1973 Al L. Tappel demonstrated the oxidized amino phospholipid products as the responsible for that fluorescence, similar to the signal arising from lipid extracts of peroxidized cell membranes [11, 12]. A few studies followed on the AF of lipids and their oxidized products, concerning their in situ localization of atherosclerotic plaques [13, 14], or their involvement in pathomechanisms, such as in ceroid lipofuscinosis in the nervous tissues or in the macular degeneration in retinal pigment epithelium [15, 16]. Only more recently, a renewed interest in fluorescing fatty acids and lipofuscin-like lipopigments in the liver focused on metabolic disorders, with particular reference to oxidative stress and the lipotoxicity phenomena [17–21]. On the other hand, unrivaled autofluorescence-based studies concerned NAD(P)H and flavins. Their presence is unavoidable, due to the essential roles in energetic metabolism, antioxidant defense, reductive biosynthesis and signal transduction processes: this makes NAD(P)H and flavins mainly responsible for the AF signals in the cell cytoplasm, and extremely powerful intrinsic photophysical biomarkers of biometabolic processes. In this regard, it is worth recalling that the initial observations of L.N. Duysen and J. Ames in 1957 [22] and the massive insights from Britton Chance and coworkers [23–25] on the AF intracellular detection of NAD(P)H and flavin established a borderline between the mere AF observation and its applications in dynamic metabolic studies, paving the way to unceasing application of AF analysis in the biomedical field [26–33].

1.2 Techniques for AF Spectral Studies and Their Evolution

As reviewed by Frederick H. Kasten [1], fluorescence microscopy started with an UV absorption microscope, invented by August Kohler from Carl Zeiss/Jena, in 1904. The use of quartz monochromatic objectives and coupling with a photographic system allowed to detect fluorescence emission from barium platinum cyanide under UV excitation, UV being used to exploit the cell absorption in this region. Subsequently different scientists, including Robert Wood at the Johns Hopkins University, Heinrich Lehmann from Carl Zeiss, and Carl Reichert from Vienna, gave improvements, particularly as to the shift from UV excitation at 275 nm from a cadmium spark to the 300–400 nm band provided by an arc lamp source and dark field quartz condenser. A new Reichert fluorescence microscope in 1911 was thus assembled, which was commercialized in 1912. Simultaneously, Heinrich Lehmann revealed a new Carl Zeiss *Lumineszenzmikroskop* in Munster, next to talk over the state of the art and perspectives in fluorescence microscopy. Both microscope models used very high power light sources, with possible severe electrical and UV hazards for the users. Anyway, as above reminded, the newly invented

microscopes allowed in 1911 the first observation reported by Hans Stubel in Jena on fluorescence rising from various biological substances. The properties of natural fluorescence, or AF, were then studied by different scientists aiming to discriminate biological substances, organisms, and diseased conditions. For example Carl Kaiserling, in 1917–1938, attempted to distinguish bacterial pathogens, and researchers such as Eugene Derrien, Jean Turchini, Albert Policard, and Karl A. Seggel extensively studied animal porphyrins, which were easily detectable because of their emission in the spectral red region. The studies on porphyrins were then supported by spectroscopy, the validation of porphyrin fingerprints having been reported early in 1929 by Max Borst and H. Konigsdorffer in Munich, by using a fluorescence microscope adapted for microspectrofluorometric measurements. Much greater perspectives for microspectrofluorometry application were however provided later by Britton Chance and coworkers, demonstrating that “the localization of activities of respiratory and glycolytic enzymes in cells affords a new approach to the dynamic aspects of metabolic reactions” [29].

A rapid advancement in excitation sources, devices for AF imaging and spectral detection, including fiber optic probes for both excitation and AF signal collection directly in living organs, data processing and analysis boosted the application in both the experimental biomedical and the clinical fields.

EFs absorb mainly in the near-UV spectral region, requiring excitation conditions which are more likely to cause negative effects such as photobleaching and damages to cell biomolecules, than the less energetic, longer wavelengths. Therefore, when using directly the near-UV excitation region attention is to be given to the light doses, lowering the power and shortening the exposure time, possibly compensating by an improved detection sensitivity for a reliable signal measurement. Apart cases of substrates with a high quantum yield, such as the application in dermatology of reflectance confocal microscopy [34], the requirement of a near-UV excitation for AF studies hindered its direct application in confocal microscopy, due to the high energy delivered at the measuring spot. Anyway, the problem was successfully overcome by multiple photon excitation, allowing to take advantage of the high topographical resolution of confocal microscopy also in AF investigations. The apparatus will obviously require suitable optics and devices for the signal collection and processing. In this regard, a comparative analysis of advantages and limitations of standard, confocal, and two-photon microscopy has been extensively considered, as for example reviewed by Irene Georgakoudi and Kyle P. Quinn [35]. Here we only briefly remind that the high damaging risk of the single excitation at the near-UV wavelengths are minimized when multiple photons at longer wavelength are absorbed, so that the sum of their energy is equivalent to that

required to excite the EF. The double absorption phenomenon occurring preferentially in the focus of the excitation laser beam will decrease the risk to damage the surrounding area. However, the low probability of the simultaneous absorption of two photons by the EF will require a high power laser beam to obtain sufficient excitation, and the consequent risk of heating and damaging at the measuring point will be minimized by delivering short pulses. A valuable advantage of multiphoton excitation will be also the sampling of deeper layers when analyzing bulk tissues, since the biological material is in general more transparent to the long wavelength light, improving the localization of the signal [36, 37]. An example is the depth-resolved NADH AF images which discriminate normal and precancerous engineered cells, with potential diagnostic insights on the morphological changes in precancerous lesions [38].

The separation of the signals rising from each EF is mandatory when studying cell and tissue metabolism, and this can be achieved depending on the spectral properties or the EF time decay [39–43].

Time resolved fluorescence lifetime imaging relies on time-domain rather than on frequency-domain analysis. However, data processing and EF identification and quantification by exponential analysis can be quite complex when multiple EFs, each one with more than one exponential decay, are present [44]. An easier and quite intuitive fluorescence lifetime data interpretation has been achieved by processing the time delay data from each pixel in a phasor. This is similar to a vector, and a two-dimensional histogram (phasor plot) is given by points assigned by the pixel data processing to a spectral position typical of each EF. Each point of the phasor plot will be then derived from, and related to each pixel of the image. Each EF, in turn, will be identified depending on its position in the phasor [45].

A separation of EFs depending on their spectral properties can be performed in imaging analysis. Multispectral imaging, in particular, is based on the acquisition of pixels with a spectrally resolved information, which can be achieved by using different technologies, from microscopy to AF investigation in vivo [46, 47]. Multispectral imaging can represent either a typical case of AF perceived as both a disturbing signal to be removed [48], or a valuable photophysical biomarker with diagnostic meaning [49]. In this view, the advantages of multiphoton excitation combined by multispectral AF imaging surely improve metabolic investigation in living cells. For example, the acquisition of images relative to dominant EFs from the same microscope field, as it is the case of NAD(P)H- and flavin-based ratiometric redox imaging, allows to assess in situ mitochondrial metabolic states and monitor cell differentiation [42, 43]. A new frontier in AF spectroscopy is also the translation of the high-speed multispectral confocal

imaging to endoscopy, as an advancement of confocal laser endoscopy or endomicroscopy [50, 51].

Confocal, two-photon excitation, lifetime, and multispectral imaging microscopy can thus allow a sensitive and thoughtful AF analysis. However these methods are based on sophisticated and expensive instrumentation, and on complex data processing procedures as compared with standard fluorescence microscopy. This latter is of simpler implementation and relatively lower cost, including standard light sources such as high pressure Hg lamp, or light emitting diodes (LEDs) to provide excitation in the near-UV region. The balance between advantages and limitations, including the simultaneous detection of different EFs and restricted spatial, time, and depth resolution, makes anyway the standard fluorescence microscopy a reliable tool for AF spectrofluorometric studies in the biomedical field. The contribution of each EF to overall emission spectra recorded from cells or tissues can be estimated on the basis of its typical AF spectral properties, as described in more detail in the next section “Spectrofluorometric studies of EFs and curve fitting analysis.”

1.3 Endogenous Fluorophores

1.3.1 NAD(P)H and Flavins and Redox Metabolic Functions

The enrollment in redox metabolism can influence the AF emission properties of NAD(P)H and flavins, making them sensitive biomarkers of single cells and organ vitality and function [52–55]. In fact, flavins are fluorescent in the oxidized state and NAD(P)H in the reduced one, and exhibit differences in emission amplitude, spectral positions, and time decay depending also on their bound/free condition. Fluorescence lifetimes of NAD(P)H in the free and bound state and flavins, for example, are respectively of about 0.4–0.5, 2.0–2.5, and 6 ns [39].

The free derivatives flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) show similar excitation/emission profiles in aqueous solution (pH 7), their absorption/excitation and emission peak positions being found at about 440–450 and 525 nm, respectively [56]. In isolated mitochondria it was demonstrated that flavins contribute at different extents to the organelle overall AF emission upon binding to the flavoproteins engaged in energetic metabolism. Electron transfer flavoprotein and α -lipoamide dehydrogenase are responsible for about 25 and 50% of the total signal, their emission being found respectively around 530 and 480 nm position maxima [27, 57]. These findings and evidence from isolated liver model indicated that flavin AF in cells and tissues commonly rises from mitochondria [58]. Therefore, apart from some cases of AF contribution from flavins related to other intracellular components [59], flavins are the complementary biomarker of NAD(P)H in studying energy metabolism and the compartmentalization of the intracellular metabolic redox system.

The NAD(P)H AF depends on the nicotinamide group, absorbing at about 364 nm and fluorescing in the 420–490 nm region.

No appreciable differences in the spectral shape can be detected between NADH and NADPH, while upon binding to the specific enzymes spectra undergo a blue shift, quantum yield efficiency increases (free-bound ratio: 1:3) and time decay lengthens [26, 60]. Therefore, common fluorescence properties are given by NADPH and NADH, which however differ for their metabolic engagement as electron donor/acceptors: NADPH participates to reductive biosynthesis and antioxidant defense, while NADH participates mainly to energy production reactions. Of these, anaerobic glycolysis taking place in the cytoplasm and the citric acid Krebs cycle in mitochondrial matrix produce the reduced form of NADH, which is mainly reoxidized in the respiratory chain by oxidative phosphorylation to produce ATP and heat. Shuttle systems and transhydrogenases then ensure an equilibrium between cytoplasm and mitochondria and between NAD(P)H and NAD(P)⁺ (Fig. 2).

The numerous reaction pathways and their complex equilibria can drive to different kinds of reciprocal changes in the redox state of NAD(P)H and flavin in dependence of the preceding metabolic steady state of the cells undergoing metabolic alterations, in a “bistable” effect [61] Anyway, the changes in AF signals from

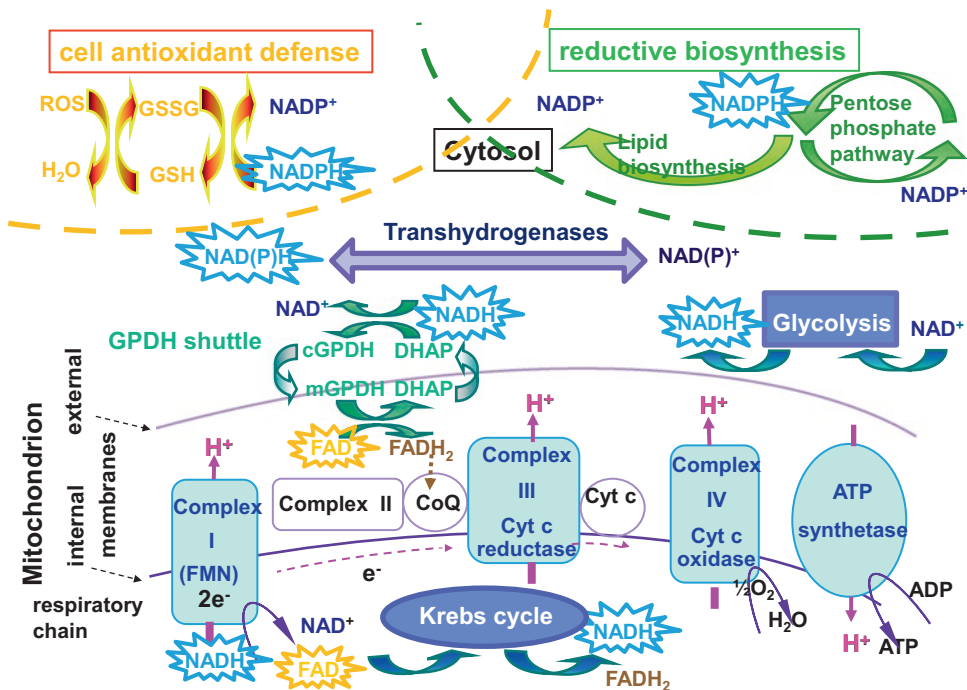


Fig. 2 Representation summarizing the main roles of the coenzymes NAD(P)H and flavins as donor/acceptors of reductive equivalents in the main metabolic oxido-reductive reaction pathways. Star frames indicate fluorescence rising from NAD(P)H and flavins in the respective reduced and oxidized states. Modified from Croce AC & Bottioli G (2014) Eur J Histochem [3]

NADH and flavins mainly related to energy metabolism are in general dominating on the NADPH contribution [62], and the redox state of the cells can be represented by means of a very simplified equation (Fig. 3), according to Haung et al. [42].

FP5 of the dehydrogenase multienzyme complex is responsible for the electron transfer from pyruvate to NAD⁺, as a point of electron entry in the respiratory chain. This simplification accounts, for example, for the frequent use of the mere signal amplitude of NAD(P)H and flavin fluorescence in experimental studies on different organs such as heart, kidney, and liver for an effective real-time monitoring of functional responses to ischemia and oxygenation, or when investigating strategies aiming to monitor and counteract possible injuries from ischemic conditions during surgery or transplantation [63–65].

The $\text{NAD(P)H}_{\text{bound/free}}$ ratio or redox ratio ($(\text{flavin})/(\text{NAD(P)H}_{\text{total}} + \text{flavin})$) can reflect specifically the aerobic metabolic activity and redox state of both tissue or single cell undergoing physiological, induced, or disease dependent alterations. For example, the $\text{NAD(P)H}_{\text{bound/free}}$ ratio depends on the efficiency and functional integrity of the mitochondrial machinery, considering that interaction steps between NAD(P)H and related enzymes are more numerous in aerobic than in anaerobic metabolism, and that mitochondrial flavoproteins provide the major contribution to overall cell flavin AF emission, as reminded above [57, 65–67]. A lowering of the $\text{NAD(P)H}_{\text{bound/free}}$ ratio was for example related to the cell stemness or transformation condition [68–70], or to a rising of metabolic disorders, such as in the case of liver accumulating lipids [71], in agreement with a switch to the prevalence of anaerobic on aerobic metabolism. In this concern, it is worth to remind that the dominance of anaerobic over aerobic energetic metabolism as a typical effect of neoplastic transformation was observed by Warburg already in 1956 [72]. Actually, in the tumor cells the pentose-

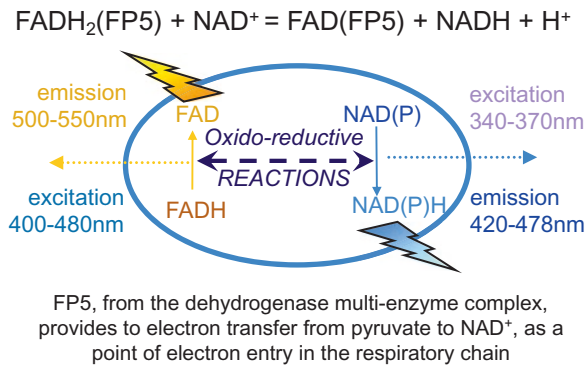


Fig. 3 Simplified scheme illustrating the reciprocal redox state balance between NAD(P)H and flavins, influencing the cell AF emission depending on oxido-reductive reactions

phosphate pathway fueling the reductive biosynthesis can prevail on a weakened mitochondrial respiratory activity, favoring cell growth, invasiveness, and survival in poorly oxygenated sites. Such metabolic condition is therefore consistent with a common lowering of the $\text{NAD(P)H}_{\text{bound/free}}$ ratio values in neoplastic as compared with nonneoplastic conditions of cells and tissues [26, 66, 67, 69, 73–75]. The fewer interaction steps between NAD(P)H and related enzymes characterizing anaerobic as compared with aerobic pathways metabolism are thus accounting for the decrease in the relative fraction of bound NADH . On the other hand, the greater variability in this parameter more relevant to cancerous than to cells with standard metabolic conditions is consistent with the differences in the actual metabolic engagement [76].

Indications even more specifically linked to the mitochondrial activity can be provided by calculation of redox ratios. The potential of this AF parameter has been demonstrated at both cell and tissue levels. Redox ratio values were shown to change along with the cell layers of normal epithelia, indicating changes in their metabolic activity, as compared with the absence of remarkable changes in the different layers of precancerous tissue, providing interesting insights for early diagnosis of neoplasia [77], while in the gerbil, as an animal model of the central nervous system, redox ratio values were suggested to highlight regional differences in tissue energy metabolism [78]. More numerous applications of redox ratios concerned liver metabolism, for which the values ranging between 0.212 and 0.639 were indicated to reflect respectively the maximal reduced or oxidized state in frozen liver samples, providing a valuable tool for a direct detection of liver redox changes [71, 79, 80]. At the single cell level, redox ratios have been found to be inversely related with the metabolic activity in cultured human mesenchymal stem cells undergoing adipogenic differentiation [43]. This study highlighted also the role of lipofuscins as AF biomarkers of differentiation.

1.3.2 Lipofuscins, Vitamin A, Porphyrins and More

Lipofuscins are EFs detected occasionally as brightly fluorescing particles in the cell cytoplasm; they are marker of undigested material from phagocytotic or autophagic processes, and accumulate during cell differentiation and aging, as it occurs in the central nervous system or in the liver [81–83]. Recent observation suggests lipofuscins to depend on mitochondrial number regulation to preserve the anaerobic metabolism characterizing stem cells [43, 84]. Lipofuscin emission in the yellow-reddish range varies depending on their heterogeneous components, including lipids, proteins, and carotenoids in variable fractions, and cross-links and oxidation degree [85]. Similar AF properties belong to lipofuscin-like lipopigments or ceroids, which are however distinguished from lipofuscins because of their origin from oxidative damages and pathological conditions [16, 86, 87]. In ophthalmology a constant attention is

stimulated by the presence of lipofuscins and retinoid derivatives in the retinal pigment epithelial (RPE) cells: within the huge amount of work based on their AF properties, we remind their supportive role to study the etiology of many retinal disorders, and to develop diagnosis and follow-up procedures [88, 89]. As to more particular cases of intracytoplasmic fluorescing particles, it is worth reminding eosinophils and their brightly emitting granules, reported to be enriched in FAD and to undergo an emission increase and a longer wavelength shift when the cells become resident in the tissues [90–93]. Another specific case concerns the lipofuscin-like cell pigments in the amphibian liver (likely involving also melanin as an additional component): these pigments undergo changes linked to the plasticity of liver and to its engagement in compensating seasonal-dependent changes in oxidative conditions [94].

AF emission of vitamin A is typically detected in the liver, rich in retinoids because of its role in their storage, metabolism and mobilization to the blood plasma. These functions involve the activity of both hepatocytes and hepatic stellate cells, to maintain the retinoid homeostasis in the organism [95]. Vitamin A is characterized by a high photolability which can be exploited both to bleach its unwanted signal from the Ito cells when selectively studying the AF of NADH under living condition [96], or to assess its amount through the signal decrease under continuous excitation [7, 97].

Photolability is also typical of porphyrins, chemically based on a tetrapyrrolic ring structure. Protoporphyrin IX, in particular consists in the heme group deprived of iron, and its presence in cells and tissues is linked to the synthesis of heme [98, 99], depending on its multiple roles as a prosthetic group in essential activities, such as transport of oxygen and energy production, catalysis and pigmentation. As a consequence, while the physiological presence of porphyrins in rodent Harderian glands was useful to study control mechanisms of porphyrin biosynthesis [100], a tissue specific or systemic enhancement of porphyrins can reflect pathological conditions affecting the availability of iron and/or the activities of the enzymes involved in the synthesis of heme [8, 101]. In this respect, it is to remind that the peculiar ability of porphyrins to fluoresce in the red region under near-UV excitation was at the basis of the early observations of AF emission in animal tissues and its ascription to a diseased condition, such as congenital porphyria or neoplasia. Actually, these studies inspired the huge, enduring work on the photo-dynamic diagnosis (PDD) and photo-dynamic therapy (PDT) strategies, for the detection and cure of cancer based on the administration of exogenous photosensitizers. Porphyrin derivatives have been the first photosensitizers used in clinics, and they are currently approved for PDD and PDT applications. Interestingly, the strict relationship between the aggregation degree of porphyrins and their persistence in the target tissue, photodynamic activity

and emission properties resulted in the use of spectrofluorometry to optimize the treatment conditions [102–106]. More recently a reevaluated attention has been paid to the systemic increase of endogenous porphyrins as a supportive diagnostic tool in tumor bearing subjects [107, 108].

Proteins in general give rise to AF emission depending on the presence of the aromatic amino acids tryptophan, tyrosine, and phenylalanine [109, 110], which however are excited below 280 nm and fluorescence at wavelengths shorter than 400 nm. Although the aromatic amino acids exhibit differences in their spectral positions and emission quantum yield, the detection of their AF inside cells and tissues does not comply with excitation, filtering, and optic conditions usually applied in fluorescence microscopy, as described more extensively below. However, unlike the majority of globular proteins, the fibrous proteins such as keratins [111, 112], elastin, and collagen are characterized by remarkable AF emission in the visible interval. This property is likely due to their polymeric and cross-linked nature, and to the presence of tricarboxylic triamino pyridinium ring derivative, 4-hydroxyphenylalanine, or hydroxylysyl pyridinoline and lysyl pyridinoline respectively in elastin and collagen [113, 114]. Elastin and collagen therefore received much greater attention than globular proteins in AF-based investigations. The value of collagen detection for widespread diagnostic application is also enforced by the second harmonic generation (SHG), ascribable to the birefringence properties of fibrillary collagen [115]. Collagen gives rise to an AF emission whose amplitude and spectral shape may vary depending on both collagen type and aging [116]. Early findings on its diagnostic meaning concerned the derangement of the submucosal architecture and the loss of collagenous components consequent to neoplastic transformation in multilayered epithelial tissues, causing a decrease of the signal detected at the mucosal surface exploitable for a guided collection of biopsies and early diagnosis of cancer at endoscopy [117–120]. Ensuing works ranged from intracellular and extracellular imaging of collagen to monitor the differentiation of human mesenchymal stem cells in culture, to the detection of liver disorder progression in terms of new collagen production when Ito cells are switched by damaging stimuli from the vitamin A deposit to the fibrogenic role, and to the detection of skin photodamage alterations [43, 121–124].

Keratins were early demonstrated to provide a noticeable contribution to the epithelium AF emission. However despite their recognized importance to distinguish health and diseased tissues, their AF has been up to now poorly considered [112, 125]. As to the skin, it is to underline also the emerging importance given to the AF detection of the advanced glycation end products (EGP), as biomarkers for an easy and cost-effective follow-up of diabetes and inflammation, as well as of renal and cardiovascular disease [126–128].

Finally, neuromediators have received a recent renewed interest as to their direct, in situ detection in cells and tissues of the central nervous system. The favorable AF properties of serotonin, in particular, are usefully exploitable in pharmacological studies [129–132].

A summary of the main EFs reminded here, their metabolic meaning and AF properties is given in Table 1.

1.4 AF Metabolic Investigation in Living Conditions

The detection of the EFs significant of metabolic processes in a living system requires care to preserve metabolic equilibria or to induce perturbation by purposely applied stimuli. Incorrect procedures can easily result in misleading data on cell and tissue metabolic state. Energy metabolism is extremely sensitive to changes in oxygenation and temperature which lead to immediate alterations of NAD(P)H and flavin AF values. The modulation of atmosphere oxygenation and temperature when required by the experimental purposes can be obtained by the use of chambers for cultured cells, suitable for observation at fluorescence microscopy while ensuring the required environmental conditions and the ability to change them, as illustrated for example already by Obi Tabot et al. [133].

AF analysis at the tissue level, with the exception of skin and the sites accessible via endoscopy, in general entails the exposure or isolation of the organ. The latter cases are particularly relevant for biomedical research, and require a careful control of the vital parameters and their preservation, for example by monitoring the animal model sedation or the organ preservation. Vitality and metabolic homeostasis of isolated organs are typically maintained by perfusion with proper solutions, through the vascular accesses [134].

AF based metabolic studies are commonly designed to provide a comparative analysis of physiological and altered or diseased states. Therefore AF data are to be collected from cells or tissue in a basal state, as the condition to which refer data collected upon purposely applied interventions or in a diseased condition for their correct diagnostic interpretation. Examples are reported in the literature on intravital microscopy, which demonstrated to allow a direct visualization and quantitative analysis of NADH by means of its AF signals in the exposed rat liver, for an in vivo comprehensive analysis of hepatic changes depending on disease progression, oxygenation or regeneration [53, 135]. A videofluorometer was for example used to directly image and perform a semiquantitative analysis of the AF emission of NADH from a blood perfused rat heart [136]. In vivo AF analysis, however, relies mainly on the use of fiber optic probes, from the mere collection of AF signal due to NADH and its changes, to the monitoring of tissue and organ oxidation–reduction states, and more comprehensive AF spectral investigations [55].

The latter studies are based on a proper awareness of the kind of EFs expected to reside in the cells or tissues of interest, and on

Table 1
Metabolic (physiological and pathological) functions, and autofluorescence properties of endogenous fluorophores

Metabolic functions		Endogenous fluorophore	Autofluorescence Exc./Em. peak ranges	Photophysical correlates vs normal parenchyma
Normal/physiological	Pathological alteration			
Energy metabolism/ reductive biosynthesis/ antioxidant defense	Redox state/WBC recruitment/Flogosis/ carcinogenesis	NAD(P)H/flavins [26–28, 41–43, 49, 52, 57, 58, 65, 69, 73–77, 80]	(330–380 nm)/(440 nm, bound 465 nm, free) (360–445 nm)/(480/540 nm)	Spectral shape Emission amplitude
Lipid metabolism	Metabolic syndrome/liver steatosis	Free Fatty acids (arachidonic acid) [71, 75, 137]	(330–350 nm)/(470–480 nm)	Spectral shape Photofading
Proteins/extracellular matrix/connective tissue	Fibrosis/necroinflammation	Collagen [97, 115, 116, 119] Cytokeratins [112] Elastin [114]	(330–340 nm)/(400–410 nm) (280–325 nm)/(495–525 nm)	Spectral blue shift Amplitude increase
Heme catabolic products/ bile components	Iron metabolism derangement/liver injury	Porphyrins [8, 104, 105] Bilirubin/biliary salts [141, 142]	(405 nm)/(615–670 nm) (366–450 nm)/(540–600 nm) 366 nm/(400–490 nm)	Spectral red bands Photofading Spectral green band
Oxidizing events	Oxidative stress	Lipofuscins [85] Vitamin A [7, 75, 96]	Near UV/<460 nm (370–380 nm)/ (490–510 nm)	Spectral red shift Photofading
CNS neuromediators	Uptake disorder at synapsis	5-hydroxytryptamine (5-HT) [130, 132]	Near UV/<400 nm	Spectral shape Emission amplitude

the availability of a library of their spectral properties. As already reminded, NAD(P)H and flavins are ubiquitous, and thus their presence inside cells is almost certain. The presence of other fluorophores is to be solved depending on the type of cell or tissue to be analyzed, and preliminary biochemical and morphological inspection can be necessary for novel biological substrates, to focus on the right EFs. Anyway, this task is made easy by the relatively low number of EFs effectively responsible for the AF emission of animal cells and tissues, as described from the preceding sections. A valuable support may also come from the literature and the personal prior experience.

As reminded above, the possibility to simultaneously excite the EFs in the near-UV region, and estimate their relative contribution to the overall AF emission in the 400–700 nm range by means of curve fitting analysis, is particularly useful to investigate pairs of EFs significant of dynamic biological functions. In fact, the analysis of AF data pairs in terms of ratio values can provide valuable parameters of metabolic functions, avoiding artifacts from the sequential measurements of separate single fluorophores, which entail negative aspects from photobleaching and changes in topological sampling. Valuable examples of EF pair analysis regard fluorescing fatty lipids and lipofuscin-like lipopigments (to assess the yield of lipid oxidation and thus the yield of oxidizing events in cells and tissues), and NAD(P)H in the bound/free state and flavins (reflecting mitochondrial functionality and the cell and tissue redox state) [137, 138]. As to the detection of the pair NAD(P)H-flavins in the near-UV excitation region, the advantages described can even reward the lower yield of flavin detection as compared with its optimal excitation at a longer wavelength.

2 Spectrofluorometric Studies of EFs and Curve Fitting Analysis

2.1 Instrumentation

Apart the brief note on the evolution of the techniques used for AF spectral studies given above, a report on instrumentation is beyond the aim of this text. Here we only remind that several kinds of sophisticated instrumental equipment and strategies for data processing are described in the literature, to which we therefore refer to for more specific information and details. Often appliances are self-assembled, and their setup is customized to meet the requirement of a specific research, taking advantage of complementary biological and engineering expertise of the research group. A wide offer of instrumentation, light sources, and optical material is also given nowadays on the market.

Here we want only to remind that lenses, objectives, optical filters and their setup, fiber optics, and spectral sensitivity of light

detectors and related correction are to be carefully chosen taking into due account the biological material and related AF properties to be investigated.

2.2 AF Investigation Based on the Spectral Shape

AF analysis can be performed on substrates varying from pure biomolecules to living systems. A preliminary characterization of the single EFs is in principle needed to provide parameters describing their typical excitation/emission features. Reference spectra will be thus obtained, to be next used to process AF overall signals from fluids, cells and tissues. In this regard, it is to remind that AF spectral shape and emission yield of an EF are influenced by the biological environment, which is to be taken into due account to achieve AF parameters for their reliable analytical application. As to a pure EF in solution, attention is to be paid to match as much as possible their usual environment, such as pH and solutes preserving or affecting the AF properties. Depending on the EF and its related biological substrate, an experimental countercheck can be the biochemical extraction of EFs to confirm their presence and their AF properties in solution. In the case of components such as collagen or lipofuscins, which are barely soluble and heterogeneous in their composition, the AF native properties can be assessed by a direct in situ analysis in a biological substrate where these components are particularly present. This is for example the case of collagen-rich connective tissue, or of lipofuscin granules easily recognizable in cells and tissues, e.g., in the liver [69, 97]. Data mining from the literature is also helpful to confirm self-built AF spectral libraries.

A spectral shape adjustment might be then required when performing a curve fitting analysis of an overall AF spectrum from a biological substrate, to optimize the combination between the measured and calculated curves. This could be the case of EFs with heterogeneous composition, such as proteins and lipofuscins. In our experience on different substrates, from single cells to the brain and liver tissue, for the AF of single molecules, such as bound and free NAD(P)H, flavins, arachidonic acid and vitamin A and porphyrins, the adjustment were always within the limit of the 5 % of their spectral area value, considered as the statistical limit.

2.3 AF Characterization of EF Spectral Parameters from Pure Compounds in Solution

Solutions of the main EFs as pure compounds can be obtained as follows:

- NAD(P)H: only the reduced form is fluorescent and no remarkable differences occur between AF spectra of NADH and NAD(P)H. A suitable solution for each of the two compounds is obtained by dissolving them in tris(hydroxymethyl)amino-methane (TRIS) buffer (0.1 M, pH 8), the basic condition being necessary to preserve the stability of the reduced form.

The bound form is obtained by mixing the solution of the pure compound with a solution of a specific enzyme. For example, for

NADH a solution of alcohol dehydrogenase (E.C. 1.1.1.1) diluted in the same buffer can be used, adjusting the stock solutions to the respective concentrations of 10 and 20 mg/ml to obtain the respective final concentrations of 5 and 10 mg/ml, in the mixed solution.

- FAD, or FMN, dissolved in phosphate buffer (0.05 M, pH 7.4, at concentration of 5 mg/ml).
- Protoporphyrin IX diluted in PBS (25 µg/ml).
- Bovine albumin, as an example of soluble protein, dissolved in PBS (5 µg/ml).
- Vitamin A (All-trans-retinoic acid) and arachidonic acid, as hydrophobic compounds, can be dissolved in chloroform (25 µg/ml).

Excitation and emission spectra of pure compounds in solution can be conventionally characterized by fluorometry, at the respective maximum emission and excitation peaks adjusting, if necessary, their concentrations depending on the instrument sensitivity.

The shape of the emission spectra and related parameters to be used for curve fitting analysis will be then validated by collecting spectra from pure compound solutions under the same apparatus used for the spectrofluorometric analyses of cells and tissues, i.e., under 366 nm excitation in epi-illumination.

2.4 AF

Characterization of EFs Extracted from Cells and Tissues

EF characterization of cell and tissue extracts in solution responds to two main requirements: verifying the kind of EFs actually present in the biological substrate and validating their AF properties.

A reliable characterization of EFs from cells and tissue depends on their proper preservation, ensured by a careful management of the biological substrate. This can be achieved by a quick collection and immediate block of metabolic functions without addition of external agents, i.e., by freezing the samples in liquid nitrogen and preserving them at -80°C . EFs will be then brought to solution by means of homogenization procedures. The homogenization medium must favor the solubilization of EFs and preserve their chemical features.

A suitable homogenization medium to solubilize hydrophilic EFs can be: phosphate buffer (pH 7.4, 0.005 M) containing: dl-dithiothreitol (20 mM) as antioxidant, phenylmethane sulfonyl fluoride (1 mM) as proteolytic inhibitor, EDTA (5 mM) as chelating agent and Triton X-100 (0.01 % w/v) as detergent to improve EF solubilization [119]. Cell or tissue samples will be immersed in the homogenizing medium, homogenized and then centrifuged to collect the supernatant, containing the EFs soluble in aqueous medium. The pellet is to be recovered in an organic solvent, to extract lipophilic EFs.

In more detail, we report an example from our experience on EF characterization in the liver: tissue samples were taken from rat anesthetized (i.p. injection of pentobarbital, 40 mg kg⁻¹) to expose the liver which was immersed in the homogenizing medium (ratio of 150 mg of tissue: 1 ml of medium) and kept in an ice bath for homogenization (UltraTurrexT25 tissue stirrer, Janke and Kunkel, Stauffen, Germany); the homogenate was then centrifuged at 5000×*g* for 15 min. The supernatant aqueous phase was diluted 1:100 in the same buffer in the absence of Triton, to make negligible the fluorescence signals of the buffer components. The remnant pellet was then resuspended and strongly shaken in a mixture of chloroform–water (2:1), to be then centrifuged to recover the organic phase (containing vitamin A and fatty acids) and a residual pellet. This was submitted to additional organic extraction in the attempt to recover a soluble fraction from lipofuscins or lipopigments-like lipofuscins.

For a comparative analysis with pure compounds, excitation and emission spectra were recorded from homogenate solutions by using both a conventional spectrofluorometer, and the microspectrofluorometer used for the AF analysis of cell and tissues, as already described. Microspectrofluorometry and spectrofluorometry confirmed the spectral shapes of single EFs and their distinctive parameters, with the exception of proteins and lipofuscins, limited by low solubility, heterogeneity and consequent lack of single pure reference compounds. Excitation and emission peak wavelengths for soluble proteins were below the spectral range capacity of the microspectrofluorometric apparatus, able to detect only the long wavelength tail of their emission spectrum. Fully consistent with their heterogeneity, lipopigment-like lipofuscins as the fraction recovered from the remnant pellet after both aqueous and organic extraction showed a broad emission band in the yellow-red region, while the residual pellet submitted to the microspectrofluorometric analysis resulted in an emission shape with a great profile variability, depending on excitation wavelength.

2.5 Curve Fitting Analysis to Estimate the EF Contribution to Overall Cell and Tissue AF Spectra

The overall, wide AF emission rising from cells and tissue as the sum of the AF signals of the different EFs present in the biological substrate can be decrypted on the basis of the specific spectral properties of each EF, estimating their contributions by means of curve fitting analysis procedures. To this end, the procedure based on Marquardt–Levenberg algorithm to minimize χ^2 is in general preferred, since it is recognized to be successful even for a less than optimum prediction of the initial peak parameter and it is well-documented [139, 140]. This algorithm is at the basis of some commercial analysis packages proposed for nonlinear curve fitting analysis, with an accurate reduction of noise and quantification of peaks.

In our experience, fitting analysis based on Half-Gaussian Modified Gaussian (GMG) spectral function parameters allows to respect the asymmetric shape of spectra, which is usually broader towards longer wavelengths.

Each EF spectrum is identified by spectral parameters, in terms of peak center wavelength position (λ), full width at half intensity maximum and asymmetry. These parameters can be obtained empirically by a peak fit analysis of the real spectra measured from each pure compound. Practically, a GMG function is created and, after a first manual adjustment, further automatic adjustments are performed until the calculated curve fits at the best with the measured curve, that is when errors in terms of χ^2 values are minimized. The procedure will be performed for each pure compound representing the EFs expected to be present in the investigated cell or tissue. A library of curves will be thus created, for their combined use when analyzing experimental spectra. Once recovered the measured spectrum, curves representing the EFs expected to contribute to the overall emission will be created by using the specific parameters, and following a first a manual adjustment operating only on area values a further automatic adjustments will be performed until the calculated curve fits at the best with the experimental one. The goodness of fit will be verified by residuals and coefficient of correlation (r^2) values, and the relative contribution of each EF to the overall AF emission provided in terms of area values (in arbitrary units). In some cases, as for proteins and lipofuscin-like lipopigments, the curves will be made free to adapt to achieve a satisfying spectral combination, considering the variability of their emission signals depending on their heterogeneous chemical composition, oxidation, and cross-link degree.

For a rewarding and easier analysis, preliminary treatment of spectral data is to be performed by: (1) normalizing the maximum peak value to 100, to avoid additional variability from differences in the real amplitude (only the relative contribution of each EF to the overall spectrum is estimated and given as percentage of the overall AF emission area: when necessary, the actual, real contribution can be then calculated by a next simple correction for the real measured area values); (2) wavelengths are to be converted in wavenumbers, since the algorithm and the theoretical models being developed for line spreading are based upon frequency (a reversal operation will be then given on the resulting curves, for their more familiar representation in wavelengths); (3) since AF spectra do not provide absolute quantitative values, a reliable result interpretation in AF metabolic studies will be conducted by assessing relative changes against an initial reference, or control condition.

3 Examples of AF Spectra Curve Fitting Analysis

The use of AF spectroscopy for estimating and monitoring cell and tissue metabolism entails the assessment of a reference condition before subsequently investigating metabolic changes, depending on purposely induced physiological alterations or disorders or pathologies of interest. These studies essentially rely on the correct detection and interpretation of AF signal as to the actual EFs present in the biological substrate, and the proper assessment of the control condition to be used as a reference. Therefore, and considering the richness in studies reporting of AF based monitoring of metabolic changes that can be found in the literature, here we privilege the demonstration of AF characterization of single cultured cells and liver tissue under near-UV excitation. Apart the already reminded importance of a prior knowledge of the biochemical components occurring in the biological substrate under study, here we aim to highlight AF imaging as an essential support to set up AF spectral analysis and validate its diagnostic interpretation.

In single cultured cells, AF rises essentially from the cytoplasm, and surrounds darker areas easily identifiable as the nuclei. The cytoplasm shows a faint and diffuse signal, along with brighter particles characterized by an even intensity. The distinctive distribution patterns of these organelles, recalling the common organization of mitochondria, the typical role of NAD(P)H as the main EF in single cells due to its engagement in redox reactions, primarily in energy metabolism, and the minor contribution of globular proteins under the conditions here used for AF analysis (exc 366 nm; em 410–600 nm) support the detection of diffuse and mitochondrial NAD(P)H. An occasional presence of lipofuscins in some cells can be discriminated depending on the uneven intracytoplasmic distribution of much brighter yellowish granules. In full agreement with the literature, these findings, and data from spectral library allow to plan a curve fitting analysis of AF spectra recorded from the cells based essentially on the contribution of bound and free NAD(P)H, proteins, flavins and lipopigments, as from the example shown in Fig. 4.

Additional EFs contributing to the overall emission signal will need to be added for a correct AF analysis of more complex biological substrates. This can be the case of liver tissue, whose multiple functional activities result in the presence of additional EFs, providing an useful example to demonstrate the potential of AF analysis. In the normal liver parenchyma, in addition to NAD(P)H and flavins, more EFs to be taken into account are the commonly present vitamin A and fluorescing fatty acids. Metabolic disorders or progression to pathology can alter the presence of fatty acids, proteins, and porphyrins, as well as bilirubin in the bile [97, 141, 142].

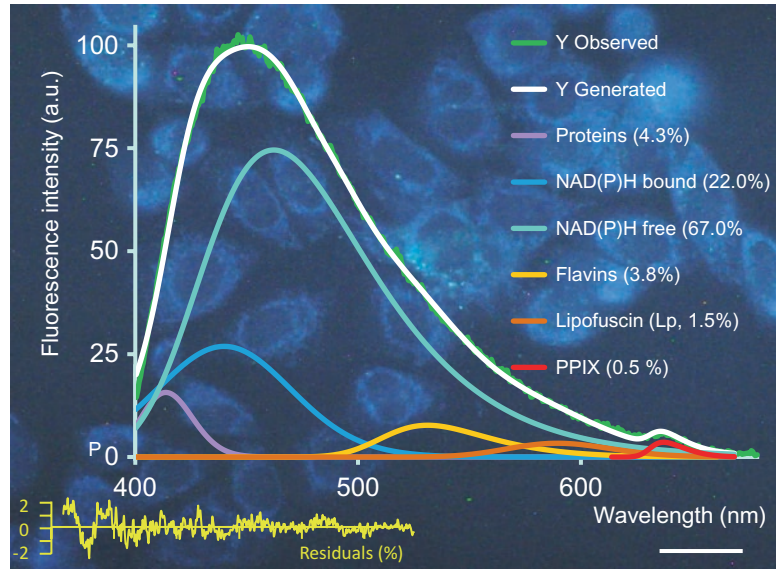


Fig. 4 In single cultured cells, the combined analysis of AF microspectroscopy and imaging supports the identification of mitochondria and the detection of NAD(P)H. This is demonstrated by AF patterns of HeLa cultured cells recorded under living conditions (360–380 nm excitation, dichroic mirror 390 nm, barrier filter 400 nm) and a related AF spectral curve fitting analysis (366 nm excitation, dichroic mirror 390 nm, barrier filter 400 nm). AF imaging allows to appreciate the cytoplasmic faint diffuse emission, overimposed by brighter particles reminding a distribution pattern typical of mitochondria. One distinct cell shows much brighter granules that, because of their yellowish emission and unevenly distributed patterns are likely ascribable to lipofuscins. The AF spectrum recorded from a single cell is represented along with the combination of spectral functions describing the emission profile and the contribution from each EF to the overall emission. EFs are defined by colors on the right. The goodness of fitting is verified in terms coefficient of determination ($r^2 \leq 0.985$, in this case) and inspection of residuals, shown in the Figure. Bar: 20 μm

Accumulation of lipofuscins and/or lipofuscin-like lipopigment in the liver parenchyma may depend on normal physiological aging, or these compounds may increase following oxidizing events, for example as products of oxidative stress.

With reference to lipofuscins accumulation in aging liver, we show an example of a reciprocal validation of AF imaging and spectroscopy (Fig. 5a–c). It is stressed in particular the ability of microspectroscopy and fitting analysis to discriminate a defined EF (lipofuscin in this case) among other EFs, by using a single excitation condition, while imaging may need more specific excitation/emission conditions to evidence selectively the EF.

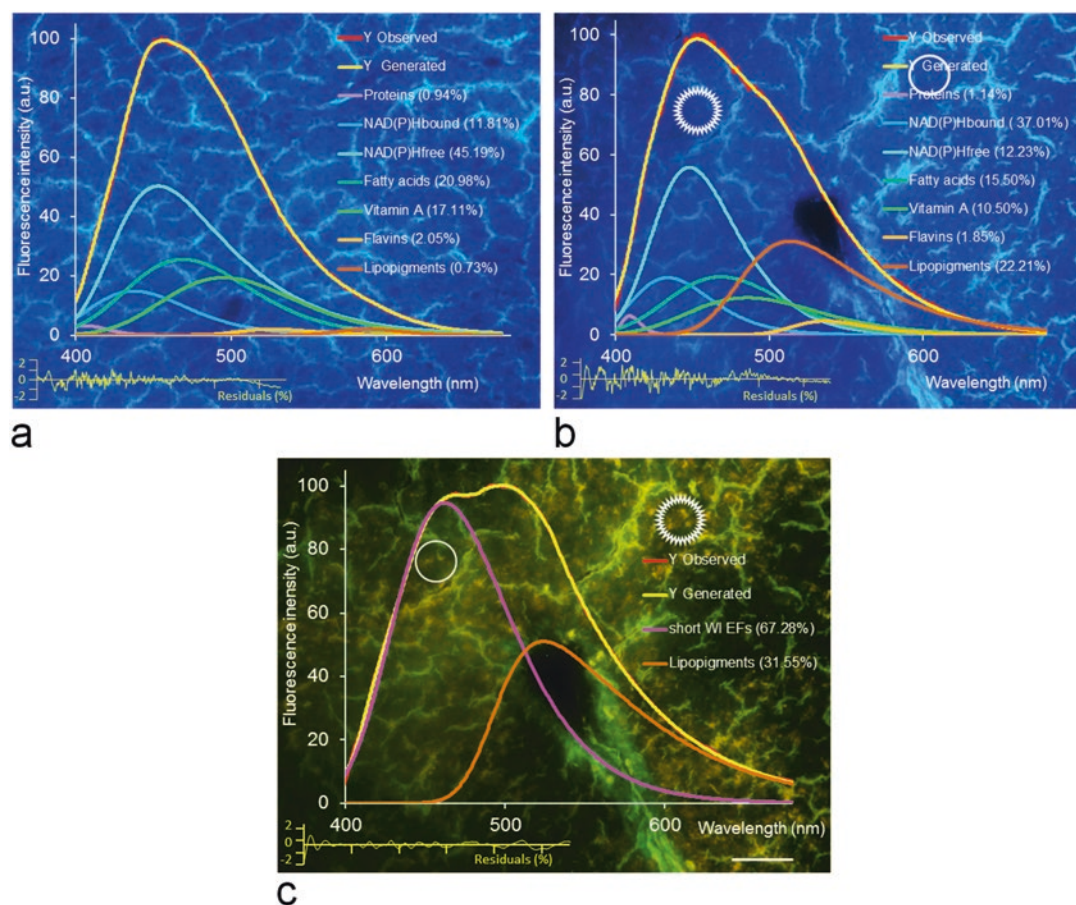


Fig. 5 AF images and AF emission spectra from unfixed, unstained, cryostatic liver tissue sections (thickness 25 μm) of normal young (**a**, 3 months), and old (**b**, **c**, 16 months) rat models. The influence of lipofuscins on liver AF emission depending on their richness in the measured area is highlighted by the combined analysis of AF spectra and images. All AF spectra have been recorded under 366 nm excitation (dichroic mirror 390 nm, barrier filter 400 nm). Each EF is represented by a GMG curve as defined by the *colors* on the *right*, where the relative contribution to the overall AF emission area is also reported. Images (**a**, **b**) have been recorded under 360–380 nm excitation, dichroic mirror 390 nm, barrier filter 400 nm, similarly to spectral acquisition; image (**c**) has been recorded from the same area as (**b**), under 440–480 nm excitation, dichroic mirror 500 nm, barrier filter 500 nm to favor lipofuscin observation. In (**b**, **c**) star shaped circles indicate the areas where each AF spectrum was collected. AF image levels have been adjusted to optimize observation. Bars: 25 μm

4 Concluding Remarks

The value of EFs as intrinsic biomarkers of cell and tissue metabolism accounts for the long-time, enduring attention of the scientific community to improve their detection and to advance the knowledge on their diagnostic potential. The continuous progress in technology and in AF data processing led to the emergence of several excellent laboratories, as well as in a growing offer of

instrumentation on the market. As a consequence, numerous AF studies rely on different technologies, and an alignment of the methods up to now used is hard to be foreseen at least in a short time. On the other hand, it is to remark that AF studies rely on common issues, consisting in the widespread presence of EFs in cell and tissue, and precautions to be taken for the EF reliable detection, from the exact assessment of AF parameters to the preservation of the AF signal by preventing photobleaching phenomena and even the EF loss. Therefore, independently from the kind of the techniques used, a correct EF detection and the comparative interpretation their related data is to be expected to further expand AF analysis applications in biomedicine.

Acknowledgments

We wish to thank all the the colleagues contributing to our autofluorescence studies, as referred to in this chapter.

References

1. Kasten FH (1989) Cell Structure and Function by Microspectrofluorometry. *Cell Struct Funct by Microspectrofluorometry*. doi:[10.1016/B978-0-12-417760-4.50008-2](https://doi.org/10.1016/B978-0-12-417760-4.50008-2)
2. Rost BFW (1995) Fluorescence microscopy, volume II. By F.W.D. Rost Cambridge University Press, Cambridge and New York. DOI: [10.1002/sca.4950180810](https://doi.org/10.1002/sca.4950180810)
3. Croce AC, Bottiroli G (2014) Autofluorescence spectroscopy and imaging: a tool for biomedical research and diagnosis. *Eur J Histochem* 58:2461. doi:[10.4081/ejh.2014.2461](https://doi.org/10.4081/ejh.2014.2461)
4. Wessendorf M (2004) Autofluorescence: Causes and Cures. <http://www.uhnres.utoronto.ca/facilities/wcif/PDF/Autofluorescence.pdf> 1–8.
5. Johnston NW, Bienenstock J (1974) Abolition of non-specific fluorescent staining of eosinophils. *J Immunol Methods* 4:189–194. doi:[10.1016/0022-1759\(74\)90060-X](https://doi.org/10.1016/0022-1759(74)90060-X)
6. Jayyosi C, Coret M, Bruyère-Garnier K (2016) Characterizing liver capsule microstructure via in situ bulge test coupled with multiphoton imaging. *J Mech Behav Biomed Mater* 54:229–243. doi:[10.1016/j.jmbbm.2015.09.031](https://doi.org/10.1016/j.jmbbm.2015.09.031)
7. Croce AC, De Simone U, Vairetti M et al (2008) Liver autofluorescence properties in animal model under altered nutritional conditions. *Photochem Photobiol Sci* 7:1046–1053. doi:[10.1039/B804836C](https://doi.org/10.1039/B804836C)
8. Croce AC, Santamaria G, De Simone U et al (2011) Naturally-occurring porphyrins in a spontaneous-tumour bearing mouse model. *Photochem Photobiol Sci* 10:1189–1195. doi:[10.1039/c0pp00375a](https://doi.org/10.1039/c0pp00375a)
9. Neumann M, Gabel D (2002) Simple Method for Reduction of Autofluorescence in Fluorescence Microscopy. *J Histochem Cytochem* 50:437–439. doi:[10.1177/002215540205000315](https://doi.org/10.1177/002215540205000315)
10. Popper H, Gyorgy P, Goldblatt H (1944) Fluorescent material (ceroid) in experimental nutritional cirrhosis. *Arch Path* 37:161–168
11. Tappel AL (1973) Lipid peroxidation damage to cell components. *Fed Proc* 32:1870–1874
12. Bidlack WR, Tappel AL (1973) Fluorescent products of phospholipids during lipid peroxidation. *Lipids* 8:203–207
13. Verbunt RJ, Fitzmaurice MA, Kramer JR et al (1992) Characterization of ultraviolet laser-induced autofluorescence of ceroid deposits and other structures in atherosclerotic plaques as a potential diagnostic for laser angioplasty. *Am Heart J* 123:208–216
14. Phipps JE, Hatami N, Galis ZS et al (2011) A fluorescence lifetime spectroscopy study of matrix metalloproteinases-2 and -9 in human atherosclerotic plaque. *J Biophotonics* 4:650–658. doi:[10.1002/jbio.201100042](https://doi.org/10.1002/jbio.201100042)
15. Ottis P, Koppe K, Onisko B et al (2012) Human and rat brain lipofuscin proteome.

- Proteomics 12:2445–2454. doi:[10.1002/pmic.201100668](https://doi.org/10.1002/pmic.201100668)
16. Palmer DN, Husbands DR, Winter PJ et al (1986) Ceroid lipofuscinosis in sheep. I. Bis(monoacylglycerol)phosphate, dolichol, ubiquinone, phospholipids, fatty acids, and fluorescence in liver lipopigment lipids. *J Biol Chem* 261:1766–1772
 17. Grattagliano I, Caraceni P, Calamita G et al (2008) Severe liver steatosis correlates with nitrosative and oxidative stress in rats. *Eur J Clin Invest* 38:523–530. doi:[10.1111/j.1365-2362.2008.01963.x](https://doi.org/10.1111/j.1365-2362.2008.01963.x)
 18. Reiniers MJ, van Golen RF, van Gulik TM, Heger M (2014) Reactive oxygen and nitrogen species in steatotic hepatocytes: a molecular perspective on the pathophysiology of ischemia-reperfusion injury in the fatty liver. *Antioxid Redox Signal* 21:1119–1142. doi:[10.1089/ars.2013.5486](https://doi.org/10.1089/ars.2013.5486)
 19. Neuschwander-Tetri BA (2010) Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* 52:774–788. doi:[10.1002/hep.23719](https://doi.org/10.1002/hep.23719)
 20. Leamy AK, Egnatchik RA, Young JD (2013) Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. *Prog Lipid Res* 52:165–174. doi:[10.1016/j.plipres.2012.10.004](https://doi.org/10.1016/j.plipres.2012.10.004)
 21. Peverill W, Powell LW, Skoien R (2014) Evolving concepts in the pathogenesis of NASH: beyond steatosis and inflammation. *Int J Mol Sci* 15:8591–638. doi:[10.3390/ijms15058591](https://doi.org/10.3390/ijms15058591)
 22. Duysen LN, Ames J (1957) Fluorescence spectrophotometry of reduced phosphopyridine nucleotide in intact cells in the near-ultraviolet and visible region. *Biochim Biophys Acta* 24:19–26
 23. Chance B, Legallais V (1951) Rapid and Sensitive Spectrophotometry. II. A Stopped-Flow Attachment for a Stabilized Quartz Spectrophotometer. *Rev Sci Instrum* 22:627. doi: [10.1063/1.1746020](https://doi.org/10.1063/1.1746020)
 24. Chance B, Legallais V, Schoener B (1962) Metabolically linked changes in fluorescence emission spectra of cortex of rat brain, kidney and adrenal gland. *Nature* 195:1073–1075
 25. Chance B, Legallais V (1959) Differential Microfluorimeter for the Localization of Reduced Pyridine Nucleotide in Living Cells. *RevSciInstrum*30:732. doi:[10.1063/1.1716736](https://doi.org/10.1063/1.1716736)
 26. Salmon JM, Kohen E, Viallet P et al (1982) Microspectrofluorometric approach to the study of free/bound NAD(P)H ratio as metabolic indicator in various cell types. *Photochem Photobiol* 36:585–593. doi:[10.1111/j.1751-1097.1982.tb04420.x](https://doi.org/10.1111/j.1751-1097.1982.tb04420.x)
 27. Kunz WS, Kunz W (1985) Contribution of different enzymes to flavoprotein fluorescence of isolated rat liver mitochondria. *Biochim Biophys Acta* 841:237–246
 28. Chance B, Legallais V (1963) A spectrofluorometer for recording of intracellular oxidation-reduction states. *IEEE Trans Biomed Eng* 10:40–47
 29. Chance B, Thorell B (1959) Localization and kinetics of reduced pyridine nucleotide in living cells by microfluorometry. *J Biol Chem* 234:3044–3050
 30. Pollak N, Dölle C, Ziegler M (2007) The power to reduce: pyridine nucleotides--small molecules with a multitude of functions. *Biochem J* 402:205–18. doi:[10.1042/BJ20061638](https://doi.org/10.1042/BJ20061638)
 31. Nakamura M, Bhatnagar A, Sadoshima J (2012) Overview of pyridine nucleotides review series. *Circ Res* 111:604–610. doi:[10.1161/CIRCRESAHA.111.247924](https://doi.org/10.1161/CIRCRESAHA.111.247924)
 32. Reinert KC, Gao W, Chen G et al (2011) Cellular and metabolic origins of flavoprotein autofluorescence in the cerebellar cortex in vivo. *Cerebellum* 10:585–599. doi:[10.1007/s12311-011-0278-x](https://doi.org/10.1007/s12311-011-0278-x)
 33. Wyroba E, Bottioli G, Giordano P (1981) Autofluorescence of axenically cultivated *Paramecium aurelia*. *Acta Protozool* 20:165–170
 34. Nwaneshiudu A, Kuschal C, Sakamoto FH et al (2012) Introduction to confocal microscopy. *J Invest Dermatol* 132, e3. doi:[10.1038/jid.2012.429](https://doi.org/10.1038/jid.2012.429)
 35. Georgakoudi I, Quinn KP (2012) Optical imaging using endogenous contrast to assess metabolic state. *Annu Rev Biomed Eng* 14:351–367. doi:[10.1146/annurev-bioeng-071811-150108](https://doi.org/10.1146/annurev-bioeng-071811-150108)
 36. König K (2000) Multiphoton microscopy in life sciences. *J Microsc* 200:83–104. doi:[10.1046/j.1365-2818.2000.00738.x](https://doi.org/10.1046/j.1365-2818.2000.00738.x)
 37. Williams RM, Piston DW, Webb WW (1994) Two-photon molecular excitation provides intrinsic 3-dimensional resolution for laser-based microscopy and microphotochemistry. *FASEB J* 8:804–813
 38. Levitt JM, Hunter M, Mujat C et al (2007) Diagnostic cellular organization features extracted from autofluorescence images. *Opt Lett* 32:3305–3307. doi:[10.1364/OL.32.003305](https://doi.org/10.1364/OL.32.003305)
 39. Koenig K, Schneckeburger H (1994) Laser-induced autofluorescence for medical diagnosis. *J Fluoresc* 4:17–40. doi:[10.1007/BF01876650](https://doi.org/10.1007/BF01876650)
 40. Bird DK, Yan L, Vrotsos KM et al (2005) Metabolic mapping of MCF10A human breast cells via multiphoton fluorescence lifetime imaging of the coenzyme NADH. *Cancer*

- Res 65:8766–73. doi:[10.1158/0008-5472.CAN-04-3922](https://doi.org/10.1158/0008-5472.CAN-04-3922)
41. Skala MC, Riching KM, Gendron-Fitzpatrick A et al (2007) In vivo multiphoton microscopy of NADH and FAD redox states, fluorescence lifetimes, and cellular morphology in precancerous epithelia. *Proc Natl Acad Sci U S A* 104:19494–9. doi:[10.1073/pnas.0708425104](https://doi.org/10.1073/pnas.0708425104)
 42. Huang S, Heikal AA, Webb WW (2002) Two-photon fluorescence spectroscopy and microscopy of NAD(P)H and flavoprotein. *Biophys J* 82:2811–2825. doi:[10.1016/S0006-3495\(02\)75621-X](https://doi.org/10.1016/S0006-3495(02)75621-X)
 43. Rice WL, Kaplan DL, Georgakoudi I (2010) Two-photon microscopy for non-invasive, quantitative monitoring of stem cell differentiation. *PLoS One* 5, e10075. doi:[10.1371/journal.pone.0010075](https://doi.org/10.1371/journal.pone.0010075)
 44. Gratton E, Breusegem S, Sutin J et al (2003) Fluorescence lifetime imaging for the two-photon microscope: time-domain and frequency-domain methods. *J Biomed Opt* 8:381–390. doi:[10.1117/1.1586704](https://doi.org/10.1117/1.1586704)
 45. Digman MA, Caiolfa VR, Zamai M, Gratton E (2008) The Phasor Approach to Fluorescence Lifetime Imaging Analysis. *Biophys J* 94: L14–16. doi:[10.1529/biophysj.107.120154](https://doi.org/10.1529/biophysj.107.120154)
 46. Levenson RM, Mansfield JR (2006) Multispectral imaging in biology and medicine: slices of life. *Cytometry A* 69:748–758. doi:[10.1002/cyto.a.20319](https://doi.org/10.1002/cyto.a.20319)
 47. Levenson RM, Fornari A, Loda M (2008) Multispectral imaging and pathology: seeing and doing more. *Expert Opin Med Diagn* 2:1067–1081. doi:[10.1517/17530059.2.9.1067](https://doi.org/10.1517/17530059.2.9.1067)
 48. Mansfield JR, Gossage KW, Hoyt CC, Levenson RM (2005) Autofluorescence removal, multiplexing, and automated analysis methods for in vivo fluorescence imaging. *J Biomed Opt* 10:41207. doi:[10.1117/1.2032458](https://doi.org/10.1117/1.2032458)
 49. Rigacci L, Alterini R, Bernabei PA et al (2000) Multispectral imaging autofluorescence microscopy for the analysis of lymph-node tissues. *Photochem Photobiol* 71:737–742. doi:[10.1562/0031-8655\(2000\)0710737MIAMFT2.0.CO2](https://doi.org/10.1562/0031-8655(2000)0710737MIAMFT2.0.CO2)
 50. Carver GE, Locknar SA, Morrison WA et al (2014) High-speed multispectral confocal biomedical imaging. *J Biomed Opt* 19:36016. doi:[10.1117/1.JBO.19.3.036016](https://doi.org/10.1117/1.JBO.19.3.036016)
 51. Rey JW, Kiesslich R, Hoffman A (2014) New aspects of modern endoscopy. *World J Gastrointest Endosc* 6:334–344. doi:[10.4253/wjge.v6.i8.334](https://doi.org/10.4253/wjge.v6.i8.334)
 52. Monici M, Pratesi R, Bernabei P et al (1995) Natural fluorescence of white blood cells: spectroscopic and imaging study. *J Photochem Photobiol B* 30:29–37. doi:[10.1016/1011-1344\(95\)07149-V](https://doi.org/10.1016/1011-1344(95)07149-V)
 53. Klauke H, Minor T, Vollmar B et al (1998) Microscopic analysis of NADH fluorescence during aerobic and anaerobic liver preservation conditions: A noninvasive technique for assessment of hepatic metabolism. *Cryobiology* 36:108–114. doi:[10.1006/cryo.1997.2068](https://doi.org/10.1006/cryo.1997.2068)
 54. Mayevsky A, Walden R, Pewzner E et al (2011) Mitochondrial function and tissue vitality: bench-to-bedside real-time optical monitoring system. *J Biomed Opt* 16:067004. doi:[10.1117/1.3585674](https://doi.org/10.1117/1.3585674)
 55. Mayevsky A, Chance B (2007) Oxidation-reduction states of NADH in vivo: from animals to clinical use. *Mitochondrion* 7:330–339. doi:[10.1016/j.mito.2007.05.001](https://doi.org/10.1016/j.mito.2007.05.001)
 56. Thomson A (1981) An Introduction to Spectroscopy for Biochemists. *Biochem Educ* 9:35. doi:[10.1016/0307-4412\(81\)90077-7](https://doi.org/10.1016/0307-4412(81)90077-7)
 57. Kunz WS (1986) Spectral properties of fluorescent flavoproteins of isolated rat liver mitochondria. *FEBS Lett* 195:92–96. doi:[10.1016/0014-5793\(86\)80137-5](https://doi.org/10.1016/0014-5793(86)80137-5)
 58. Scholz R, Thurman RG, Williamson JR et al (1969) Flavin and pyridine nucleotide oxidation-reduction changes in perfused rat liver. I Anoxia and subcellular localization of fluorescent flavoproteins. *J Biol Chem* 244:2317–2324
 59. Croce AC, Bottiroli G (2015) New light in flavin autofluorescence. *Eur J Histochem* 59:2576. doi:[10.4081/ejh.2015.2576](https://doi.org/10.4081/ejh.2015.2576)
 60. Lakowicz JR, Szmacinski H, Nowaczyk K, Johnson ML (1992) Fluorescence lifetime imaging of free and protein-bound NADH. *Proc Natl Acad Sci U S A* 89:1271–1275
 61. Hess B (1973) Organization of glycolysis: oscillatory and stationary control. *Symp Soc Exp Biol* 27:105–131
 62. Vishwasrao HD, Heikal AA, Kasischke KA, Webb WW (2005) Conformational dependence of intracellular NADH on metabolic state revealed by associated fluorescence anisotropy. *J Biol Chem* 280:25119–25126. doi:[10.1074/jbc.M502475200](https://doi.org/10.1074/jbc.M502475200)
 63. Vollmar B, Burkhardt M, Minor T et al (1997) High-resolution microscopic determination of hepatic NADH fluorescence for in vivo monitoring of tissue oxygenation during hemorrhagic shock and resuscitation. *Microvasc Res* 54:164–173. doi:[10.1006/mvre.1997.2028](https://doi.org/10.1006/mvre.1997.2028)
 64. Croce AC, Ferrigno A, Vairetti M et al (2005) Autofluorescence spectroscopy of rat liver during experimental transplantation procedure. An approach for hepatic metabolism

- assessment. *Photochem Photobiol Sci* 4:583–590. doi:[10.1039/b503586d](https://doi.org/10.1039/b503586d)
65. Thorniley MS, Simpkin S, Fuller B et al (1995) Monitoring of surface mitochondrial NADH levels as an indication of ischemia during liver isograft transplantation. *Hepatology* 21:1602–1609. doi:[10.1002/hep.1840210619](https://doi.org/10.1002/hep.1840210619)
66. Meixensberger J, Herting B, Roggendorf W, Reichmann H (1995) Metabolic patterns in malignant gliomas. *J Neurooncol* 24:153–161
67. Long Z, Maltas J, Zatt MC et al (2015) The real-time quantification of autofluorescence spectrum shape for the monitoring of mitochondrial metabolism. *J Biophotonics* 8:247–257. doi:[10.1002/jbio.201300207](https://doi.org/10.1002/jbio.201300207)
68. Stringari C, Cinquin A, Cinquin O et al (2011) Phasor approach to fluorescence lifetime microscopy distinguishes different metabolic states of germ cells in a live tissue. *Proc Natl Acad Sci U S A* 108:13582–7. doi:[10.1073/pnas.1108161108](https://doi.org/10.1073/pnas.1108161108)
69. Croce AC, Spano A, Locatelli D et al (1999) Dependence of fibroblast autofluorescence properties on normal and transformed conditions. Role of the metabolic activity. *Photochem Photobiol* 69:364–374. doi:[10.1111/j.1751-1097.1999.tb03300.x](https://doi.org/10.1111/j.1751-1097.1999.tb03300.x)
70. Santin G, Paulis M, Vezzoni P et al (2013) Autofluorescence properties of murine embryonic stem cells during spontaneous differentiation phases. *Lasers Surg Med* 45:597–607. doi:[10.1002/lsm.22182](https://doi.org/10.1002/lsm.22182)
71. Croce AC, Ferrigno A, Piccolini VM et al (2014) Integrated autofluorescence characterization of a modified-diet liver model with accumulation of lipids and oxidative stress. *Biomed Res Int* 2014:803491. doi:[10.1155/2014/803491](https://doi.org/10.1155/2014/803491)
72. Warburg O (1956) On the origin of cancer cells. *Oncol* 9:75–83. doi:[10.1136/bmj.1.4082.694-a](https://doi.org/10.1136/bmj.1.4082.694-a)
73. Villette S, Pigaglio-Deshayes S, Vever-Bizet C et al (2006) Ultraviolet-induced autofluorescence characterization of normal and tumoral esophageal epithelium cells with quantitation of NAD(P)H. *Photochem Photobiol Sci* 5:483–492. doi:[10.1039/b514801d](https://doi.org/10.1039/b514801d)
74. Galeotti T, van Rossum GD, Mayer DH, Chance B (1970) On the fluorescence of NAD(P)H in whole-cell preparations of tumours and normal tissues. *Eur J Biochem* 17:485–496. doi:[10.1111/j.1432-1033.1970.tb01191.x](https://doi.org/10.1111/j.1432-1033.1970.tb01191.x)
75. Croce AC, Ferrigno A, Vairetti M et al (2004) Autofluorescence properties of isolated rat hepatocytes under different metabolic conditions. *Photochem Photobiol Sci* 3:920–926. doi:[10.1039/B407358D](https://doi.org/10.1039/B407358D)
76. Viallet P, Salmon JM, Vigo J (1989) Cell Structure and Function by Microspectrofluorometry. *Cell Struct Funct by Microspectrofluorometry*. doi:[10.1016/B978-0-12-417760-4.50020-3](https://doi.org/10.1016/B978-0-12-417760-4.50020-3)
77. Skala MC, Riching KM, Bird DK et al (2007) In vivo multiphoton fluorescence lifetime imaging of protein-bound and free nicotinamide adenine dinucleotide in normal and precancerous epithelia. *J Biomed Opt* 12:024014. doi:[10.1117/1.2717503](https://doi.org/10.1117/1.2717503)
78. Shiino A, Haida M, Beauvoit B, Chance B (1999) Three-dimensional redox image of the normal gerbil brain. *Neuroscience* 91:1581–1585. doi:[10.1016/S0306-4522\(98\)00670-8](https://doi.org/10.1016/S0306-4522(98)00670-8)
79. Sato B, Tanaka A, Mori S et al (1995) Quantitative analysis of redox gradient within the rat liver acini by fluorescence images: effects of glucagon perfusion. *Biochim Biophys Acta* 1268:20–26
80. Chance B, Schoener B, Oshino R et al (1979) Oxidation-reduction ratio studies of mitochondria in freeze-trapped samples. NADH and flavoprotein fluorescence signals *J Biol Chem* 254:4764–71
81. Rattan SI, Keeler KD, Buchanan JH, Holliday R (1982) Autofluorescence as an index of ageing in human fibroblasts in culture. *Biosci Rep* 2:561–567
82. Riga D, Riga S (1995) Lipofuscin and ceroid pigments in aging and brain pathology. A review. I. Biochemical and morphological properties. *Rom J Neurol Psychiatry* 33:121–136
83. Jung T, Bader N, Grune T (2007) Lipofuscin: formation, distribution, and metabolic consequences. *Ann N Y Acad Sci* 1119:97–111. doi:[10.1196/annals.1404.008](https://doi.org/10.1196/annals.1404.008)
84. Santin G, Bottone MG, Malatesta M et al (2013) Regulated forms of cell death are induced by the photodynamic action of the fluorogenic substrate, Hypocrellin B-acetate. *J Photochem Photobiol B Biol* 125:90–97. doi:[10.1016/j.jphotobiol.2013.05.006](https://doi.org/10.1016/j.jphotobiol.2013.05.006)
85. Wolman M (1980) Lipid pigments (chromolipids): their origin, nature, and significance. *Pathobiol Annu* 10:253–267
86. Brunk UT, Terman A (2002) Lipofuscin: mechanisms of age-related accumulation and influence on cell function. *Free Radic Biol Med* 33:611–619. doi:[10.1016/S0891-5849\(02\)00959-0](https://doi.org/10.1016/S0891-5849(02)00959-0)
87. Patková J, Vojtříšek M, Tůma J, et al. (2012) Evaluation of lipofuscin-like pigments as an index of lead-induced oxidative damage in the brain. *Exp Toxicol Pathol Off J Gesellschaft für Toxikologische Pathol* 64:51–56. doi:[10.1016/j.etp.2010.06.005](https://doi.org/10.1016/j.etp.2010.06.005)

88. Sparrow JR, Gregory-Roberts E, Yamamoto K et al (2012) The bisretinoids of retinal pigment epithelium. *Prog Retin Eye Res* 31:121–135. doi:[10.1016/j.preteyeres.2011.12.001](https://doi.org/10.1016/j.preteyeres.2011.12.001)
89. Guha S, Liu J, Baltazar G et al (2014) Rescue of compromised lysosomes enhances degradation of photoreceptor outer segments and reduces lipofuscin-like autofluorescence in retinal pigmented epithelial cells. *Adv Exp Med Biol* 801:105–111. doi:[10.1007/978-1-4614-3209-8_14](https://doi.org/10.1007/978-1-4614-3209-8_14)
90. Grossi E, Zaccheo D (1963) On the autofluorescence of specific granulations of eosinophilic leukocytes. *Boll della Soc Ital di Biol Sper* 39:421–424
91. Mayeno AN, Hamann KJ, Gleich GJ (1992) Granule-associated flavin adenine dinucleotide (FAD) is responsible for eosinophil autofluorescence. *J Leukoc Biol* 51:172–175
92. Barnes D, Aggarwal S, Thomsen S et al (1993) A characterization of the fluorescent properties of circulating human eosinophils. *Photochem Photobiol* 58:297–303. doi:[10.1111/j.1751-1097.1993.tb09565.x](https://doi.org/10.1111/j.1751-1097.1993.tb09565.x)
93. Dorward DA, Lucas CD, Alessandri AL et al (2013) Technical advance: autofluorescence-based sorting: rapid and nonperturbing isolation of ultrapure neutrophils to determine cytokine production. *J Leukoc Biol* 94:193–202. doi:[10.1189/jlb.0113040](https://doi.org/10.1189/jlb.0113040)
94. Barni S, Vaccarone R, Bertone V et al (2002) Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of *Rana esculenta* L. *J Anat* 200:185–94. doi:[10.1046/j.0021-8782.2001.00011.x](https://doi.org/10.1046/j.0021-8782.2001.00011.x)
95. D'Ambrosio DN, Clugston RD, Blaner WS (2011) Vitamin A metabolism: an update. *Nutrients* 3:63–103. doi:[10.3390/nu3010063](https://doi.org/10.3390/nu3010063)
96. Vollmar B, Burkhardt M, Minor T et al (1997) In vivo analysis of hepatic NADH fluorescence. Methodological approach to exclude Ito-cell vitamin A-derived autofluorescence. *Microvasc Res* 45:83–89. doi:[10.1006/mvre.1997.2028](https://doi.org/10.1006/mvre.1997.2028)
97. Croce AC, De Simone U, Freitas I et al (2010) Human liver autofluorescence: an intrinsic tissue parameter discriminating normal and diseased conditions. *Lasers Surg Med* 42:371–378. doi:[10.1002/lsm.20923](https://doi.org/10.1002/lsm.20923)
98. Taketani S, Ishigaki M, Mizutani A et al (2007) Heme synthase (ferrochelatase) catalyzes the removal of iron from heme and demetalation of metalloporphyrins. *Biochemistry* 46:15054–61. doi:[10.1021/bi701460x](https://doi.org/10.1021/bi701460x)
99. Sakaino M, Ishigaki M, Ohgari Y et al (2009) Dual mitochondrial localization and different roles of the reversible reaction of mammalian ferrochelatase. *FEBS J* 276:5559–70. doi:[10.1111/j.1742-4658.2009.07248.x](https://doi.org/10.1111/j.1742-4658.2009.07248.x)
100. Spike RC, Johnston HS, McGadey J et al (1986) Quantitative studies on the effects of hormones on structure and porphyrin biosynthesis in the harderian gland of the female golden hamster. II The time course of changes after ovariectomy *J Anat* 145:67–77
101. Freitas I, Boncompagni E, Vaccarone R et al (2007) Iron accumulation in mammary tumor suggests a tug of war between tumor and host for the microelement. *Anticancer Res* 27:3059–3065
102. Dougherty TJ, Gomer CJ, Henderson BW et al (1998) Photodynamic therapy. *J Natl Cancer Inst* 90:889–905
103. Berg K, Selbo PK, Weyerang A et al (2005) Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. *J Microsc* 218:133–147. doi:[10.1111/j.1365-2818.2005.01471.x](https://doi.org/10.1111/j.1365-2818.2005.01471.x)
104. Bottiroli G, Ramponi R, Croce AC (1987) Quantitative analysis of intracellular behaviour of porphyrins. *Photochem Photobiol* 46:663–667. doi:[10.1111/j.1751-1097.1987.tb04829.x](https://doi.org/10.1111/j.1751-1097.1987.tb04829.x)
105. Dal Fante M, Bottiroli G, Spinelli P (1988) Behaviour of haematoporphyrin derivative in adenomas and adenocarcinomas of the colon: a microfluorometric study. *Lasers Med Sci* 3:165–171. doi:[10.1007/BF02593808](https://doi.org/10.1007/BF02593808)
106. Agostinis P, Berg K, Cengel KA et al (2011) Photodynamic therapy of cancer: an update. *CA Cancer J Clin* 61:250–281. doi:[10.3322/caac.20114](https://doi.org/10.3322/caac.20114)
107. Al-Salhi M, Masilamani V, Vijmasi T et al (2011) Lung cancer detection by native fluorescence spectra of body fluids--a preliminary study. *J Fluoresc* 21:637–645. doi:[10.1007/s10895-010-0751-9](https://doi.org/10.1007/s10895-010-0751-9)
108. Lualdi M, Colombo A, Leo E et al (2007) Natural fluorescence spectroscopy of human blood plasma in the diagnosis of colorectal cancer: feasibility study and preliminary results. *Tumori* 93:567–571
109. Lakowicz JR (2006) Principles of Fluorescence Spectroscopy. doi: [10.1007/978-0-387-46312-4](https://doi.org/10.1007/978-0-387-46312-4)
110. Teale FW, Weber G (1957) Ultraviolet fluorescence of the aromatic amino acids. *Biochem J* 65:476–482
111. Schweizer J, Bowden PE, Coulombe PA et al (2006) New consensus nomenclature for mammalian keratins. *J Cell Biol* 174:169–174. doi:[10.1083/jcb.200603161](https://doi.org/10.1083/jcb.200603161)

112. Beuthan J, Minet O, Müller G (1998) Optical biopsy of cytokeratin and NADH in the tumor border zone. *Ann N Y Acad Sci* 838:150–710. doi:[10.1111/j.1749-6632.1998.tb08196.x](https://doi.org/10.1111/j.1749-6632.1998.tb08196.x)
113. Eyre DR, Paz MA, Gallop PM (1984) Cross-linking in collagen and elastin. *Annu Rev Biochem* 53:717–48. doi:[10.1146/annurev.bi.53.070184.003441](https://doi.org/10.1146/annurev.bi.53.070184.003441)
114. Thornhill DP (1975) Separation of a series of chromophores and fluorophores present in elastin. *Biochem J* 147:215–219
115. Mather SJ (2015) What can biophotonics tell us about the 3D microstructure of articular cartilage? *Quant Imaging Med Surg* 5:143–158. doi:[10.3978/j.issn.2223-4292.2014.12.03](https://doi.org/10.3978/j.issn.2223-4292.2014.12.03)
116. Marcu L (2000) Characterization of type I, II, III, IV, and V collagens by time-resolved laser-induced fluorescence spectroscopy. *Proc SPIE* 3917:93–101. doi:[10.1117/12.382720](https://doi.org/10.1117/12.382720)
117. Fiarman GS, Nathanson MH, West AB et al (1995) Differences in laser-induced autofluorescence between adenomatous and hyperplastic polyps and normal colonic mucosa by confocal microscopy. *Dig Dis Sci* 40:1261–1268
118. Banerjee B, Rial NS, Renkoski T et al (2013) Enhanced visibility of colonic neoplasms using formulaic ratio imaging of native fluorescence. *Lasers Surg Med* 45:573–581. doi:[10.1002/lsm.22186](https://doi.org/10.1002/lsm.22186)
119. Bottioli G, Croce AC, Locatelli D et al (1995) Natural fluorescence of normal and neoplastic human colon: a comprehensive “ex vivo” study. *Lasers Surg Med* 16:48–60. doi:[10.1002/lsm.1900160107](https://doi.org/10.1002/lsm.1900160107)
120. Sturm MB, Wang TD (2015) Emerging optical methods for surveillance of Barrett’s oesophagus. *Gut* 64:1816–23. doi:[10.1136/gutjnl-2013-306706](https://doi.org/10.1136/gutjnl-2013-306706)
121. Nazeer SS, Sandhyamani S, Jayasree RS (2015) Optical diagnosis of the progression and reversal of CCl₄-induced liver injury in rodent model using minimally invasive autofluorescence spectroscopy. *Analyst* 140:3773–3780. doi:[10.1039/c4an01507j](https://doi.org/10.1039/c4an01507j)
122. Thorling CA, Crawford D, Burczynski FJ et al (2014) Multiphoton microscopy in defining liver function. *J Biomed Opt* 19:090901. doi:[10.1117/1.JBO.19.9.090901](https://doi.org/10.1117/1.JBO.19.9.090901)
123. Thimm TN, Squirrell JM, Liu Y et al (2015) Endogenous Optical Signals Reveal Changes of Elastin and Collagen Organization During Differentiation of Mouse Embryonic Stem Cells. *Tissue Eng Part C Methods* 21:995–1004. doi:[10.1089/ten.TEC.2014.0699](https://doi.org/10.1089/ten.TEC.2014.0699)
124. Lutz V, Sattler M, Gallinat S et al (2012) Characterization of fibrillar collagen types using multi-dimensional multiphoton laser scanning microscopy. *Int J Cosmet Sci* 34:209–15. doi:[10.1111/j.1468-2494.2012.00705.x](https://doi.org/10.1111/j.1468-2494.2012.00705.x)
125. Toivola DM, Boor P, Alam C, Strnad P (2015) Keratins in health and disease. *Curr Opin Cell Biol* 32:73–81. doi:[10.1016/j.ceb.2014.12.008](https://doi.org/10.1016/j.ceb.2014.12.008)
126. Yamagishi S-I, Fukami K, Matsui T (2015) Evaluation of tissue accumulation levels of advanced glycation end products by skin autofluorescence: A novel marker of vascular complications in high-risk patients for cardiovascular disease. *Int J Cardiol* 185:263–268. doi:[10.1016/j.ijcard.2015.03.167](https://doi.org/10.1016/j.ijcard.2015.03.167)
127. Hu H, Jiang H, Ren H et al (2015) AGEs and chronic subclinical inflammation in diabetes: disorders of immune system. *Diabetes Metab Res Rev* 31:127–137. doi:[10.1002/dmrr.2560](https://doi.org/10.1002/dmrr.2560)
128. Arsov S, Graaff R, van Oeveren W et al (2014) Advanced glycation end-products and skin autofluorescence in end-stage renal disease: a review. *Clin Chem Lab Med* 52:11–20. doi:[10.1515/cclm-2012-0832](https://doi.org/10.1515/cclm-2012-0832)
129. Kaushalya SK, Nag S, Ghosh H et al (2008) A high-resolution large area serotonin map of a live rat brain section. *Neuroreport* 19:717–721. doi:[10.1097/WNR.0b013e3282fd6946](https://doi.org/10.1097/WNR.0b013e3282fd6946)
130. Crespi F, Croce AC, Fiorani S et al (2004) Autofluorescence Spectrofluorometry of Central Nervous System (CNS) Neuromediators. *Lasers Surg Med* 34:39–47. doi:[10.1002/lsm.10240](https://doi.org/10.1002/lsm.10240)
131. Balaji J, Desai R, Maiti S (2004) Live cell ultra-violet microscopy: a comparison between two- and three-photon excitation. *Microsc Res Tech* 63:67–71. doi:[10.1002/jemt.10426](https://doi.org/10.1002/jemt.10426)
132. Botchway SW, Parker AW, Bisby RH, Crisostomo AG (2008) Real-time cellular uptake of serotonin using fluorescence lifetime imaging with two-photon excitation. *Microsc Res Tech* 71:267–73. doi:[10.1002/jemt.20548](https://doi.org/10.1002/jemt.20548)
133. Obi-Tabot ET, Hanrahan LM, Cachecho R et al (1993) Changes in hepatocyte NADH fluorescence during prolonged hypoxia. *J Surg Res* 55:575–80. doi:[10.1006/jsre.1993.1187](https://doi.org/10.1006/jsre.1993.1187)
134. Ferrigno A, Richelmi P, Vairetti M (2013) Troubleshooting and improving the mouse and rat isolated perfused liver preparation. *J Pharmacol Toxicol Methods* 67:107–114. doi:[10.1016/j.vascn.2012.10.001](https://doi.org/10.1016/j.vascn.2012.10.001)
135. Abshagen K, Eipel C, Menger MD, Vollmar B (2006) Comprehensive analysis of the regenerating mouse liver: an in vivo fluorescence microscopic and immunohistological study. *J Surg Res* 134:354–62. doi:[10.1016/j.jss.2006.01.002](https://doi.org/10.1016/j.jss.2006.01.002)

136. Coremans JM, Ince C, Bruining HA, Puppels GJ (1997) (Semi-)quantitative analysis of reduced nicotinamide adenine dinucleotide fluorescence images of blood-perfused rat heart. *Biophys J* 72:1849–1860. doi:[10.1016/S0006-3495\(97\)78831-3](https://doi.org/10.1016/S0006-3495(97)78831-3)
137. Croce AC, Ferrigno A, Di Pasqua LG et al (2016) Autofluorescence discrimination of metabolic fingerprints in nutritional and genetic fatty liver models. *J Photochem Photobiol B* 164:13–20. doi: [10.1016/j.jphotobiol.2016.09.015](https://doi.org/10.1016/j.jphotobiol.2016.09.015)
138. Croce AC, Ferrigno A, Bertone V et al (2016) fatty liver oxidative events monitored by autofluorescence optical diagnosis: Comparison between subnormothermic machine perfusion and conventional cold storage preservation. *Hep Res*. doi: [10.1111/hepr.12779](https://doi.org/10.1111/hepr.12779)
139. Marquardt DW (1963) An Algorithm for Least-Squares Estimation of Nonlinear Parameters. *J Soc Ind Appl Math* 11:431–441. doi:[10.1137/0111030](https://doi.org/10.1137/0111030)
140. Hesse R, Chassé T, Szargan R (1999) Peak shape analysis of core level photoelectron spectra using UNIFIT for WINDOWS. *Fresenius J Anal Chem* 365:48–54. doi:[10.1007/s002160051443](https://doi.org/10.1007/s002160051443)
141. Croce AC, Ferrigno A, Santin G et al (2014) Bilirubin: an autofluorescence bile biomarker of liver functionality monitoring. *J Biophotonics* 7:810–817. doi: [10.1002/jbio.201300039](https://doi.org/10.1002/jbio.201300039)
142. Croce AC, Ferrigno A, Santin G et al (2014) Autofluorescence of liver tissue and bile: organ functionality monitoring during ischemia and reoxygenation. *Lasers Surg Med* 46:412–421. doi:[10.1002/lsm.22241](https://doi.org/10.1002/lsm.22241)

Enzyme-Histochemistry Technique for Visualizing the Dipeptidyl-Peptidase IV (DPP-IV) Activity in the Liver Biliary Tree

Vittorio Bertone, Eleonora Tarantola, and Isabel Freitas

Abstract

Dipeptidyl-peptidase IV is an enzyme involved in a lot of biochemical processes, where it modifies a number of regulatory proteins by removing the terminal peptides by hydrolysis. Here we describe a histochemical method to demonstrate with accuracy and precision its in situ activity on cryostatic section of Wistar rat liver by means of a simultaneous azo-coupling method.

Key words Dipeptidyl-peptidase IV, Enzyme histochemistry, In situ enzyme activity, Cryostatic samples

1 Introduction

Dipeptidyl-peptidase IV (DPP-IV, E.C. 3.4.14.5) is an enzyme belonging to the family of peptidase, able to hydrolyse terminal peptide bonds. It can be identified with different denominations, such as DPP-IV, Gly-Pro naphthylamidase, Post-proline dipeptidyl aminopeptidase IV, Xaa-Pro-dipeptidylaminopeptidase, CD26, adenosine deaminase complexing protein 2, ADABP, ADCP2, DPPIV, TP103.

This enzyme is a membrane glycoprotein and a serine exopeptidase that cleaves X-proline dipeptides from the N-terminus of polypeptides.

DPP-IV (CD26) has an essential role in immune regulation as a T cell activation molecule and a regulator of chemokine function [1, 2]. It associates with CXCR4 and gp120 and may influence the pathophysiology of HIV infection [1]. Adenosine deaminase (ADA) co-localizing with adenosine receptors on dendritic cells is able to interact with CD26 expressed on lymphocytes [3]. This costimulatory signal in the immunological synapse leads to an increase in the production of the T helper 1 and proinflammatory

cytokines IFN- γ , TNF α , and IL-6. CD26 plays a role in the pathogenesis and behavior of human cancers, including solid tumors and hematological malignancies. CD26–caveolin-1 interaction plays a role in the upregulation of CD86 on TT-loaded monocytes and subsequent engagement with CD28 on T cells, leading to antigen-specific T cells activation [4].

As regarding in particular the functions in the hepatic context, DPP-IV is demonstrated to be a good *in situ* marker of biliary functionality in normal and fatty rat liver [5]. DPP-IV cleaves N-terminal dipeptides of proline or alanine-containing peptides including incretins [e.g., Glucagon-like peptide 1 (GLP1), Gastric Inhibitory Polypeptide (GIP)], appetite-suppressing hormones [neuropeptide such as P substance, Vasoactive Intestinal Peptide (VIP), Neuropeptide Y], and chemokines [6–10]. The activity/ expression of DPP-IV are strongly correlated to pathologies such as nonalcoholic fatty liver diseases (NAFLD), nonalcoholic steatohepatitis (NASH) and type II diabetes mellitus (T2D) [11, 12]. An altered regulation of the DPP-IV substrates incretins and neuropeptides plays an important role in the pathogenesis of obesity and insulin-resistance [13]. Since GLP-1 is a potent anti-hyperglycemic hormone, DPP-IV inhibitors are promising oral hypoglycemic and insulin-sensitizing drugs used in T2D [14–17]. Besides the enzymatic role, DPP-IV also function as a ligand; in particular, it interacts with several extracellular matrix (ECM) proteins, preferentially collagens I and III, and fibronectin [18–20]. DPP-IV can also contribute to ECM degradation [10] by binding to adenosine deaminase and by activating plasminogen-2, leading to an increase in plasmin levels [21]; this in turn causes degradation of type IV collagen, fibronectin, laminin, and proteoglycan, and induces matrix metalloproteinases activation [9, 22]. DPP-IV is currently been investigated as a target of warm ischemia–reperfusion (I/R) injury in organs such as lungs, heart and kidney [23]. Finally, DPP-IV was recently shown to regulate stem/progenitor and more differentiated hemopoietic and other cell behavior by truncating chemokines, colony stimulating factors, and interleukins [24].

DPP-IV is involved in the inactivation of incretins and neuropeptides, plays an important role in glucose metabolism rapidly inactivating insulin secreting hormones (incretins) that are important regulators of glucose metabolism. Neuropeptides regulating bile transport and composition are further important substrates of DPP-IV which is a good marker of the biliary tree in rat liver, as demonstrate here.

Activity of this enzyme can be histochemically demonstrated by a simultaneous azo-coupling method using derivatives of 1-naphthylamine, 2-naphthylamine, or 4-methoxy-2-naphthylamide as substrates and Fast Blue BB as chromogen.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at 4 °C (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

1. 0.1 M Tris maleate buffer, pH 8: prepare first 0.2 M Solution A dissolving 6.05 g of Tris(hydroxymethyl)aminomethane and 5.8 g of maleic acid in 250 mL distilled water, then 0.2 M Solution B dissolving 2 g sodium hydroxide (NaOH) in 250 mL distilled water. Use graduated cylinder to precisely measure the quantities of water. To obtain a 0.2 M buffer solution, put in a 200 mL beaker 50 mL of solution A, a magnetic stirring bar and add with a glass Pasteur pipette a variable quantity of solution B in drops while monitoring the pH value under continuous stirring, until the value of pH 8.0 is reached. To obtain the 0.1 M final concentration dilute the volume of 0.2 M solution obtained with the same quantity of distilled water. Check again the pH value and be sure the value is 8.0.
2. The day before the experiment, prepare the 18% polyvinyl alcohol (PVA) solution in 0.1 M Tris-maleate buffer pH 8.0: put 9 g of PVA (*see Note 1*) in a 100 mL Pyrex glass bottle with hermetic sealing cap. Gently add 50 mL of Tris maleate buffer and put a magnetic stirring bar into the bottle; then prepare a bain-marie in a 250 mL beaker and immerge the Pyrex bottle with the cap leaning on top (*see Note 2*). Set the temperature of a hot plate stirrer to about 120 °C and stir it all at slow speed for 20 min, until the solution becomes transparent (*see Note 3*). Close firmly the cap. The solution must be stored almost one night and for a maximum of 4 weeks, in a thermostatic chamber at 60 °C until use. During this period the small bubbles inside will disappear and the solution will become clear.
3. Acetone.
4. 96% ethanol.
5. Dimethylformamide (DMF).
6. 100 mM NaCl (*see Note 4*).
7. 1 mM Gly-Pro-methoxy- β -naphthylamide: dissolve the substrate in an adequate quantity of dimethylformamide in a glass tube (*see Notes 5 and 6*), according to Table 1.
8. 2 mM Fast-Blue BB solution: dissolve the chemical in an adequate amount of DMF in a glass tube according to Table 1 (*see Notes 6 and 7*).

Table 1

Preparation of incubation medium (it is assumed that the addition of small quantities of diluent, such as H₂O or DMF, to some chemical does not alter the final concentration of the substances)

Chemicals	Final concentration	Reference doses for 1 mL of medium	Doses for 2 mL medium (for 4 sections)	Doses for 4 mL medium (for 8 sections)	Control reaction 1 (without substrate)	Control reaction 2 (with Diprotin-A inhibitor added)
PVA	18 %	1 mL	2 mL	4 mL	yes	yes
Substrate (Gly-Pro-Methoxy- β -naphthylamide)	1 mM	0.33 mg dissolved in 50 μ l DMF (glass tube)	0.66 mg dissolved in 100 μ l DMF (glass tube)	1.32 mg dissolved in 200 μ l DMF (glass tube)	NO (TRIS-maleate buffer, instead)	yes
NaCl	100 mM	5.85 mg dissolved in 25 μ l H ₂ O	11.7 mg dissolved in 50 μ l H ₂ O	23.4 mg dissolved in 100 μ l H ₂ O	yes	yes
FAST-Blue BB	2 mM	0.95 mg dissolved in 50 μ l DMF (glass tube)	1.9 mg dissolved in 100 μ l DMF (glass tube)	3.8 mg dissolved in 200 μ l DMF (glass tube)	yes	yes
		Only in the control reaction 2 0.34 mg dissolved in 50 μ l DMF (glass tube)	Only in the control reaction 2 0.68 mg dissolved in 100 μ l DMF (glass tube)	Only in the control reaction 2 1.36 mg dissolved in 200 μ l DMF (glass tube)	NO	Diprotin-A (same concentration of the substrate 1 mM)

9. Incubation medium (*see* Table 1).
10. Control reaction #2 with inhibitor Diprotin-A (Ile-Pro-Ile): dissolve the chemical in an adequate quantity of DMF in a glass tube according to Table 1 (*see* Note 6).
11. Slide staining racks.
12. Glycerine jelly (*see* Note 8).

3 Methods

The reaction is performed on cryostatic sections (8–10 μm thick). It is possible to utilize both fresh and previously frozen tissue. In either case, put the sample on the cryostatic plate inside the cabinet of the cryostat, blocking it with Tissue-Tek®-O.C.T.™ Compound (Sakura Finetek Europe B.V., 2382 AT, Zoeterwoude, NL), and go to the cutting procedure.

3.1 Tissue Preparation

It is possible to perform this method using fresh tissue or, alternatively, previously frozen tissue.

In the latter case put small blocks of fresh tissue (approximately $0.5 \times 0.5 \times 0.5$ cm) in cryogenic vials, close firmly the cap, then immerse in liquid nitrogen for almost 15–20 s. After that store vials in -80 °C freezer until use (*see* **Note 9**).

3.2 Tissue Cutting

Prepare cryostatic sections from fresh liver samples, or using previously stored (in -80 °C freezer for some weeks) tissue. Put a small amount of Tissue-Tek®-O.C.T.™ Compound over a horizontal cryostatic plate and immediately put the block of fresh or frozen tissue on top. The glycolic compound solidifies within seconds blocking the sample. Put the plate with the sample in position of cutting (*see* **Note 10**). Cut 8–10 μm thick sections and collect on slides (*see* **Note 11**). Slides with sections can be used immediately for the histochemical method, or stored in hermetic boxes in -24 °C freezer for some days until use.

3.3 Pre-fixation of Sections

In order to better and specifically visualize the enzymatic activity, a mild pre-fixation is performed on sections [25–27]. Carry out all procedures at room temperature unless otherwise specified.

1. Extract sections from -24 °C freezer (or use the fresh sections), leave them for 15 min on the bench until reach the room temperature.
2. Fix the sections by consecutive immersion of slides first in cold acetone (4 °C) for 5 min, then in cold ethanol 95 % (4 °C) for 5 min.
3. Air-dry for 5 min at room temperature.

3.4 Incubation

1. Prepare the incubation medium for visualizing the enzymatic activity according to the method of simultaneous azo-coupling [28, 29]. Refer to Table 1 for reagents quantities, according to the number of sections to be treated.
2. First add the 18% PVA solution in Tris-maleate buffer 0.1 M pH 8 in a 15 mL graduated plastic tube marked with a pen on the level that PVA must reach. Because of its density, it should be taken with a 5 mL micropipette equipped with a tip in which the

end parts had been cut. Add PVA solution until the right level is reached. Be very careful not to form bubbles (*see Note 4*).

3. Add every single component in the 15 mL centrifuge tube using an automatic micropipette, *in the order and amounts indicated in Table 1*, while *gently* mixing with a spatula every time you add a single chemical (*see Note 12*).
4. Place slides in an moist chamber, then use a 4 mL polyethylene plastic dropper with the terminal part cut (because of the density of the medium) to gently put a drop of the incubation medium over the sections. *Take extreme care not to form bubbles on sections*, then close the chamber.
5. Incubate at 37 °C for 30 min.
6. After the incubation put slides vertically in a slide staining rack for histology and wait 1 min until most of the medium had slid away. Then rinse it all vigorously by immersion in a glass beaker containing water at 45–50 °C for 3–4 min, to completely remove the incubation medium, until the slides appear clean and the surface of sections opaque when observed in oblique light.
7. Mount the slides with glycerine jelly.
8. As usual in enzyme histochemistry, in order to assess the specificity of the reaction it is necessary to perform also a first control reaction (control #1) with an incubation medium free of the enzyme substrate Gly-Pro-methoxy- β -naphthylamide. Replace the substrate with the same amount of Tris maleate buffer according to Table 1. Then perform a reaction #2 with an incubation medium enriched with the selective competitive inhibitor Diprotin-A (Ile-Pro-Ile) at the same concentration of the substrate, according to Table 1 [6, 28]. Control reactions must be negative.

3.5 Observation

With this azo-coupling method the DPP-IV activity is demonstrated by an orange-red final reaction product selectively located on the cellular sites. This method applied on Wistar rat liver shows in particular the activity of dipeptidyl-peptidase IV at biliary tree level, from canalicula to portal duct, as shown in the images in Fig. 1.

The technique uses a polyvinil alcohol (PVA) solution with opportune density as tissue protectant added to the incubation medium. The PVA creates a kind of three-dimensional network inside the incubation medium in which the reagents are free to move, while the reaction product remains precisely localized at subcellular sites level where the enzyme is present and catalyzes the reaction with the substrate. This method ensures a precise and accurate localization of the enzyme activity [29, 30].

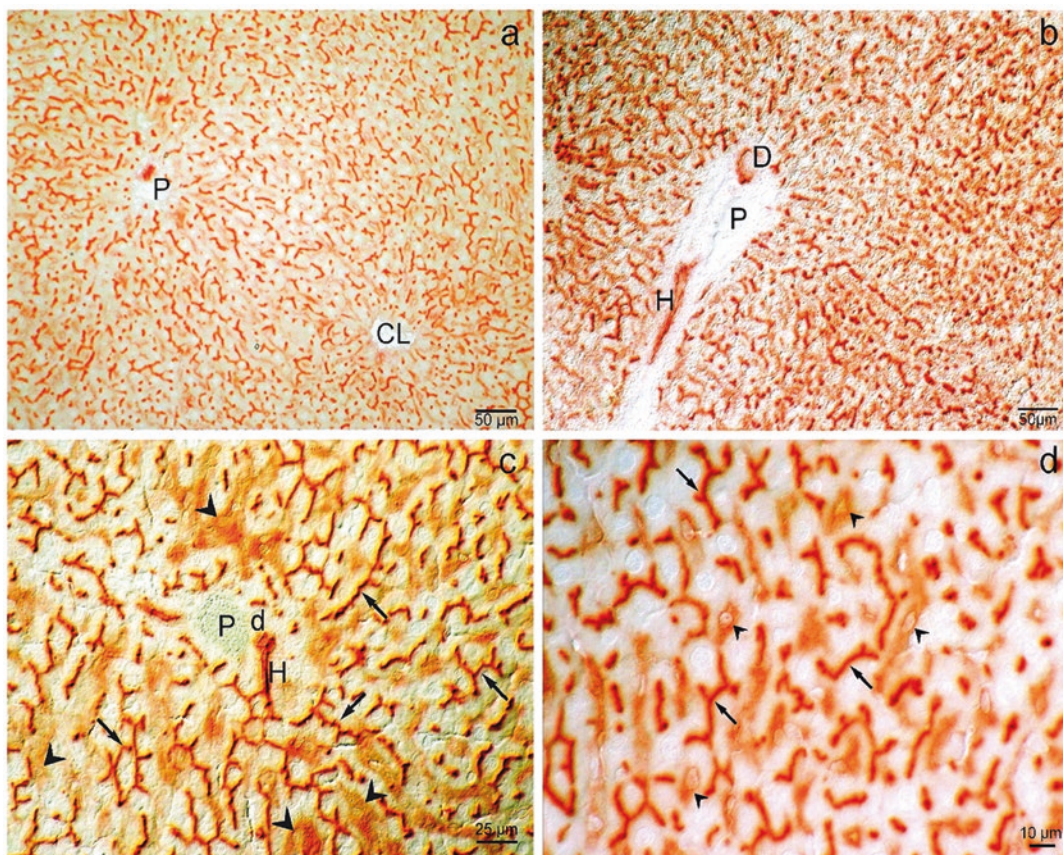


Fig. 1 Wistar rat liver. Enzyme activity of DPP-IV: a) Survey view under low magnification of a wide area of liver parenchyma; the reaction is very intense in canaliculi from the portal to the centrolobular zone; brightfield. b) Besides in canaliculi the reaction is also present in a Hering canal (H) and a bile duct (D); differential interference contrast (DIC). c) Particular of anastomotic network of branched channels and of hexagonal profiles restricted to regions between adjacent cells, representing the typical chicken-wire pattern of bile canaliculi (arrows); the reaction is furthermore present, though less intense, in sinusoidal endothelial cells (arrowhead) in the portal zone; a Hering canal (H) and a small bile duct (d) are also apparent; DIC. d) Details, under higher magnification, of the reaction in bile canaliculi (arrows) and in sinusoidal endothelial cells (arrowhead); DIC. P, portal vein; CL, centrolobular vein. (Reproduced with permission from [5].)

4 Notes

1. Be careful: avoid breathing powder wearing a protective mask.
2. Do not completely leave open the bottle, otherwise the content evaporates and the PVA solution will not be 18 % but more dense.
3. Be careful to start the stirrer at very low speed, otherwise the powder of PVA will rise along the walls of the bottle and not come down more, altering the title of the solution.

4. Prepare at the moment of the experiment the 100 mM NaCl solution, dissolving an adequate amount of chemical in the corresponding amount of distilled water, according to Table 1.
5. Prepare at the moment of the experiment the substrate Gly-Pro-methoxy- β -naphthylamide 1 mM dissolving the chemical in an adequate amount of dimethylformamide (DMF), according to the Table 1 (*see Note 6*).
6. Do not use plastic tube, as DMF is corrosive.
7. Prepare at the moment of the experiment the 2 mM Fast-Blue BB solution, dissolving the chemical in an adequate amount of dimethylformamide (DMF) in a glass tube according to Table 1 (*see Note 6*).
8. Glycerine jelly is commercially available, but it can be easily prepared as follows.

Weigh 7 g of edible gelatin in 42 ml distilled water and leave overnight at room temperature. Mix with 50 ml glycerol (glycerin) in a 70 °C bain-marie under gentle stirring, and add 0.5 g of phenol (carbolic acid). After 15 min, when the jelly has melted, filter with paper or fiberglass and prepare aliquotes of 5-10 ml in glass vials. Close firmly the cap and wait until room temperature is reached. Stock in -24 °C freezer for months. A few hours before use put it in 37 °C thermostat and mount slides with a drop of the compound. After some week in 37°C thermostat the color of the compound tends to change from pale yellow to brown, and it increases the tendency to make bubbles, and so it should be replaced.

9. 2 mL vials with external thread are particularly indicated, because the sample can modify his volume and shape during the freezing procedure and interfere with an inner thread during the extraction.
10. If a sample coming from -80 °C freezer is used, you have to wait some minutes until the -24 °C temperature is reached.
11. 8 μ m thick section results in a little bit better “sharpness” of the samples, especially if it will be observed and photographed at high magnifications, using 60, 63, or 100 $\times$ oil immersion objectives.
12. It is particularly important not to form bubbles, in order to homogeneously cover sections with the incubation medium, so be careful with the (micro)pipette and mix *gently* with the spatula

References

1. Pro B, Dang NH (2004) CD26/dipeptidyl peptidase IV and its role in cancer. *Histol Histopathol* 19:1345–1351
2. Ohnuma K, Hosono O, Dang NH, Morimoto C (2011) Dipeptidyl peptidase in autoimmune pathophysiology. *Adv Clin Chem* 53:51–84
3. Gorrell MD, Abbott CA, Kähne T et al (2000) Relating structure to function in the beta-propeller domain of dipeptidylpeptidase IV. Point mutations that influence adenosine deaminase binding, antibody binding and enzyme activity. In: Langner J, Ansorge S (eds)

- Cellular peptidases in immune functions and diseases 2. Kluwer, New York, NY, pp 89–95
4. Ohnuma K, Inoue H, Uchiyama M, Yamochi T, Hosono O, Dang NH, Morimoto C (2006) T-cell activation via CD26 and caveolin-1 in rheumatoid synovium. *Mod Rheumatol* 6(1):3–13
 5. Tarantola E, Bertone V, Milanese G et al (2012) Dipeptidylpeptidase IV, a key enzyme for the degradation of incretins and neuropeptides: activity and expression in the liver of lean and obese rats. *Eur J Histochem* 56:e41
 6. Mentlein R (1999) Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. *Regul Pept* 85:9–24
 7. Mentlein R (2009) Mechanisms underlying the rapid degradation and elimination of the incretin hormones GLP-1 and GIP. *Best Pract Res Clin Endocrinol Metab* 4:443–452
 8. Hildebrandt M, Rose M, Mayr C et al (2000) Dipeptidyl peptidase IV (DPP IV, CD26) in patients with mental eating disorders. *Adv Exp Med Biol* 477:197–204
 9. Gorrell MD (2005) Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin Sci* 108:1–16
 10. Itou M, Kawaguchi T, Taniguchi E, Sata M (2013) Dipeptidyl peptidase-4: a key player in chronic liver disease. *World J Gastroenterol* 19:2298–2306
 11. Miyazaki M, Kato M, Tanaka K et al (2012) Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism. *Mol Med Report* 5:729–733
 12. Williams KH, Shackel NA, Gorrell MD et al (2013) Diabetes and nonalcoholic fatty liver disease: a pathogenic duo. *Endocr Rev* 34:84–129
 13. Kestly NC, Roth JD, Maggs D (2008) Hormone based therapies in the regulation of fuel metabolism and body weight. *Expert Opin Biol Ther* 8:1733–1747
 14. Chyan YJ, Chuang LM (2007) Dipeptidyl peptidase-IV inhibitors: an evolving treatment for type 2 diabetes from the incretin concept. *Recent Pat Endocr Metab Immun Drug Discov* 1:15–24
 15. DiStefano JK, Watanabe RM (2010) Pharmacogenetics of anti-diabetes drugs. *Pharmaceuticals (Basel)* 3:2610–2646
 16. Martin JH, Deacon CF, Gorrell MD, Prins JB (2011) Incretin-based therapies-review of the physiology, pharmacology and emerging clinical experience. *Intern Med J* 41:299–307
 17. Bergmann K, Sypniewska G (2013) Diabetes as a complication of adipose tissue dysfunction. Is there a role for potential new biomarkers? *Clin Chem Lab Med* 51:177–185
 18. Piazza GA, Callanan HM, Mowery J et al (1989) Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. *Biochem J* 262:327–334
 19. Balaban YH, Korkusuz P, Simsek H et al (2007) Dipeptidyl peptidase IV (DDP IV) in NASH patients. *Ann Hepatol* 6(4):242–250
 20. Wang XM, Yao TW, Nadvi NA et al (2008) Fibroblast activation protein and chronic liver disease. *Front Biosci* 13:3168–3180
 21. Gorrell MD, Gysbers V, McCaughan GW (2001) CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. *Scand J Immunol* 54:249–264
 22. Gonzalez-Gronow M, Hershfield MS, Arredondo-Vega FX et al (2001) Cell surface adenosine deaminase binds and stimulates plasminogen activation on 1-LN human prostate cancer cells. *J Biol Chem* 279:20993–20998
 23. Matheeußen V, Jungraithmayr W, De Meester I (2012) Dipeptidyl peptidase 4 as a therapeutic target in ischemia/reperfusion injury. *Pharmacol Ther* 136:267–282
 24. Ou X, O’Leary HA, Broxmeyer HE (2013) Implications of DPP4 modification of proteins that regulate stem/progenitor and more mature cell types. *Blood* 122:161–169
 25. Batra S, Kuo C, Rakusan K (1989) Spatial distribution of coronary capillaries: A-V segment staggering. *Adv Exp Med Biol* 248:241–247
 26. Batra S, König MF, Cruz-Orives LM (1995) Unbiased estimation of capillary length from vertical slices. *J Microscopy* 178(Pt 2):152–159
 27. Dimitrova D, Tashkova S, Dikov A et al (2002) Enzyme activities of human milk cells. *Comptes rendus de l’Academie Bulgare des Sciences, Tome*, p 55
 28. Lojda Z, Gossrau R, Schiebler TH (1979) *Enzyme histochemistry. A laboratory manual*. Springer, Berlin
 29. Van Noorden CJF, Fredericks WM (1992) *Enzyme histochemistry. A laboratory manual of current methods*. Oxford Science Publications. Oxford University Press – Royal Microscopy Society, Oxford
 30. Horobin RW (1982) *Histochemistry*. Pag 135, Fig. 47. Fisher & Butterworths eds

Histochemical Demonstration of Tripeptidyl Aminopeptidase I

Mashenka B. Dimitrova, Dimitrinka Y. Atanasova, and Nikolai E. Lazarov

Abstract

Enzyme histochemical methods are valuable for the studies on the enzyme involvement in different pathological processes. Here we describe two protocols for chromogenic and fluorogenic histochemical demonstration of tripeptidyl aminopeptidase I (TPPI), a protease that is crucial for neuronal functions. The procedures are based on newly synthesized substrates for TPPI—glycyl-L-prolyl-L-metionyl-5-chloro-1-anthraquinonylhydrazide (GPM-CAH) and glycyl-L-prolyl-L-metionyl-4-hydrazido-*N*-hexyl-1,8-naphthalimide (GPM-HHNI). Using such protocols, precise enzyme localization can be obtained in tissue sections of mammalian organs.

Key words Enzyme histochemistry, Chromogenic and fluorogenic substrates, Brightfield microscopy, Fluorescence microscopy, Mouse, Rat, Tripeptidyl peptidase I

1 Introduction

Different methods for the enzyme visualization exist, amongst which enzyme histochemistry has its special place not only because it is one of the oldest but also because of its undoubted advantages. Indeed, enzyme histochemistry allows localization of enzymes in the heterogeneous structures of organs and tissues through their activities by using specific enzyme substrates. Moreover, fluorescent enzyme imaging is especially useful for at least three reasons: (1) Its high sensitivity allows enzyme visualization also in places of very low enzyme activity; (2) The use of confocal microscopy makes it possible to specify enzyme locations even at subcellular levels; (3) It permits the application of modern software for quantitative assays depending on the intensity of the fluorescent signal.

Tripeptidyl aminopeptidase I (TPPI; EC 3.4.14.9) is presently a subject of substantial scientific interest due to its wide tissue distribution and multiple functions in Mammals, including humans. TPPI is a lysosomal enzyme belonging to the S53 sedolisin family of serine-carboxyl peptidases [1]. It hydrolyzes tripeptides from the N-terminus of oligopeptides and polypeptides at the optimum

pH 4.5. It has also been shown that at lower pH values (3.0–3.5) it possesses endopeptidase activity [2]. TPPI is a highly conservative glycoprotein, widely distributed in all the mammalian species and humans [3]. The enzyme gene in humans has been carted at 11p15 [4]. The mature enzyme molecule is 46 kDa and may form clusters of different molecular weights [5]. TPPI participates in the hydrolysis of collagen type I [6] and is known to regulate the levels of biologically active peptides such as angiotensins of different lengths [7], neuromedin B [8], substance P [9], and cholecystokinin-5 [10]. The gene encoding TPPI is identical to the *cln2* gene [11], whose mutations lead to the fatal autosomal recessive disease late infantile neuronal ceroid lipofuscinosis (LINCL), associated with severe symptoms and death in adolescence. Altered expression levels of TPPI have been observed in different malignant diseases (reviewed in [12]), including breast cancer [13], squamous cell carcinoma of the esophagus [14], and colorectal carcinoma [15]. It has been supposed that the enzyme is involved in tumor progression and metastasis. Recently, it has been also shown that TPPI participates in receptor-mediated apoptosis [16, 17].

Most of the in situ studies on TPPI have been performed by immunohistochemical methods using both polyclonal [18, 19] and monoclonal [20, 21] antibodies. Immunohistochemistry actually reveals tissue localization of the enzyme molecule but does not provide information whether the enzyme is active or not in a particular location. Moreover, using different antibodies, dissimilar results may be obtained [20].

Studies on the involvement of TPPI in different physiological and pathological processes would favor a specific histochemical method for the enzyme visualization. Recently, we synthesized chromogenic and fluorogenic specific substrates for TPPI, glycyl-L-prolyl-L-metionyl-5-chloro-1-anthraquinonylhydrazide (GPM-CAH) and glycyl-L-prolyl-L-metionyl-4-hydrazido-*N*-hexyl-1,8-naphthalimide (GPM-HHNI), respectively, and on the basis of those substrates we developed methods for the enzyme histochemical detection [22, 23]. The histochemical principles implicating the two substrates are quite similar. The enzyme hydrolyzes the tripeptide glycyl-L-prolyl-L-metionine from the respective substrate, thus releasing the corresponding hydrazine, i.e., 5-chloro-1-anthraquinonyl hydrazine (CAH) or 4-hydrazino-*N*-hexyl-1,8-naphthalimide (HHNI). Those compounds react quickly with 4-nitrobenzaldehyde (CAH) or piperonal (HHNI) so as to obtain an insoluble colorful or fluorescent final reaction product which precipitates on the enzyme original location sites, and thus marking them. Here we describe two protocols for demonstrating TPPI activity in various animal tissues and organs. In our laboratory these protocols are used to demonstrate the histochemical localization of TPPI in the rodent central nervous system (CNS) [24], reproductive organs [25], and the carotid body [26]. The detection procedures utilize chromogens or fluorogens that respectively permit

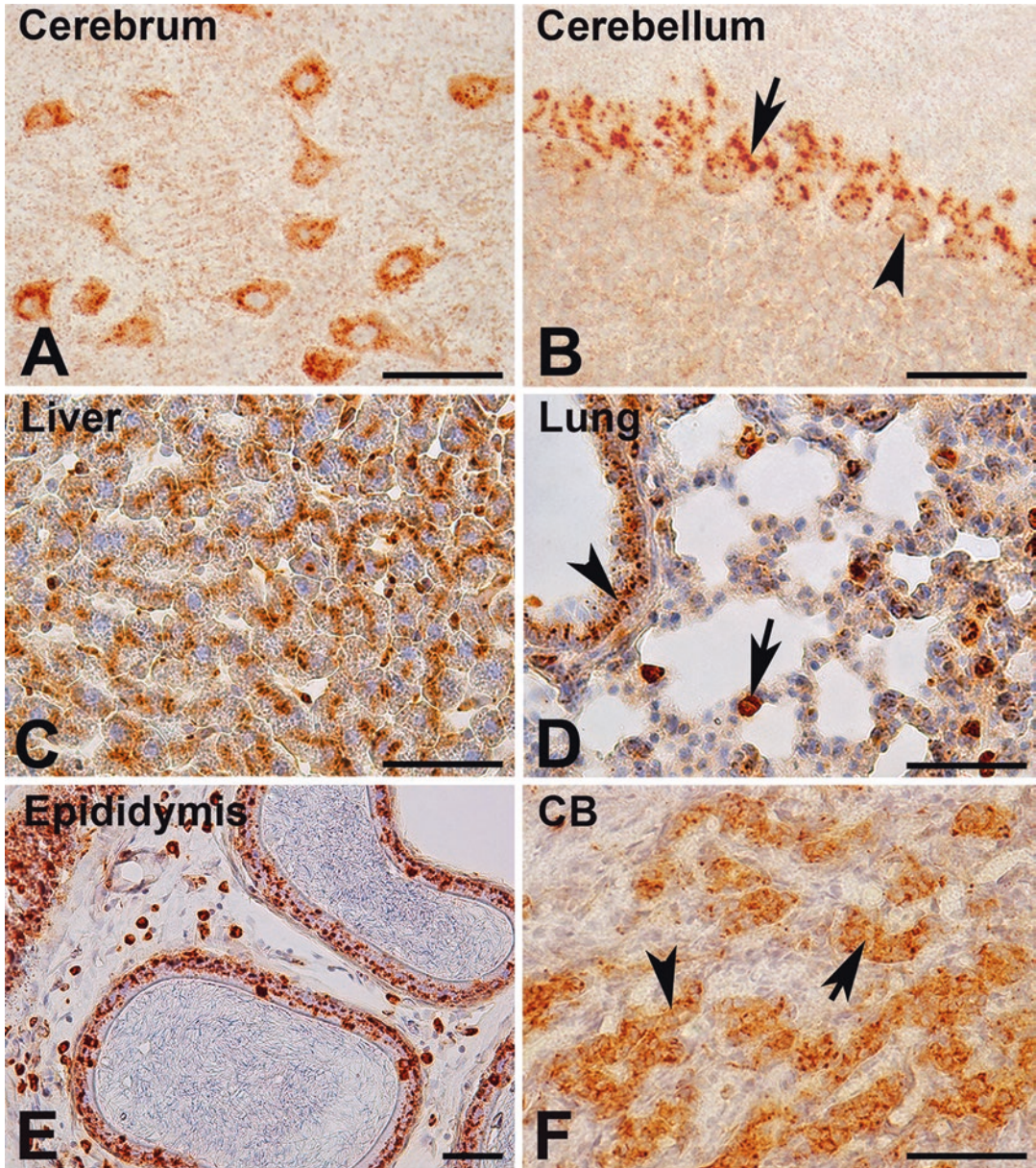


Fig. 1 Localization of TPPI activity in various organs of the rat using the chromogenic histochemical substrate GPM-CAH. The reaction product of the enzyme activity can be observed in cortical neurons of the cerebral cortex (a), in the Purkinje cells (*arrowhead*) and Bergmann glial cells (*arrow*) of the cerebellar cortex (b), in the hepatocytes in the liver (c), in the bronchial epithelial cells (*arrowhead*) and alveolar macrophages (*arrows*) in the lung (d), in the epithelial and interstitial cells of the epididymis (e) as well as in the glomus (*arrow*) and sustentacular cells (*arrowhead*) of the carotid body (CB) (f). Scale bars = 50 μm

observation of the detected enzyme by bright-field microscopy (Fig. 1), or epifluorescence (Fig. 2). Our results are largely consistent with those obtained by other authors using immunohistochemical methods for enzyme visualization [18, 19]. Since the enzyme is

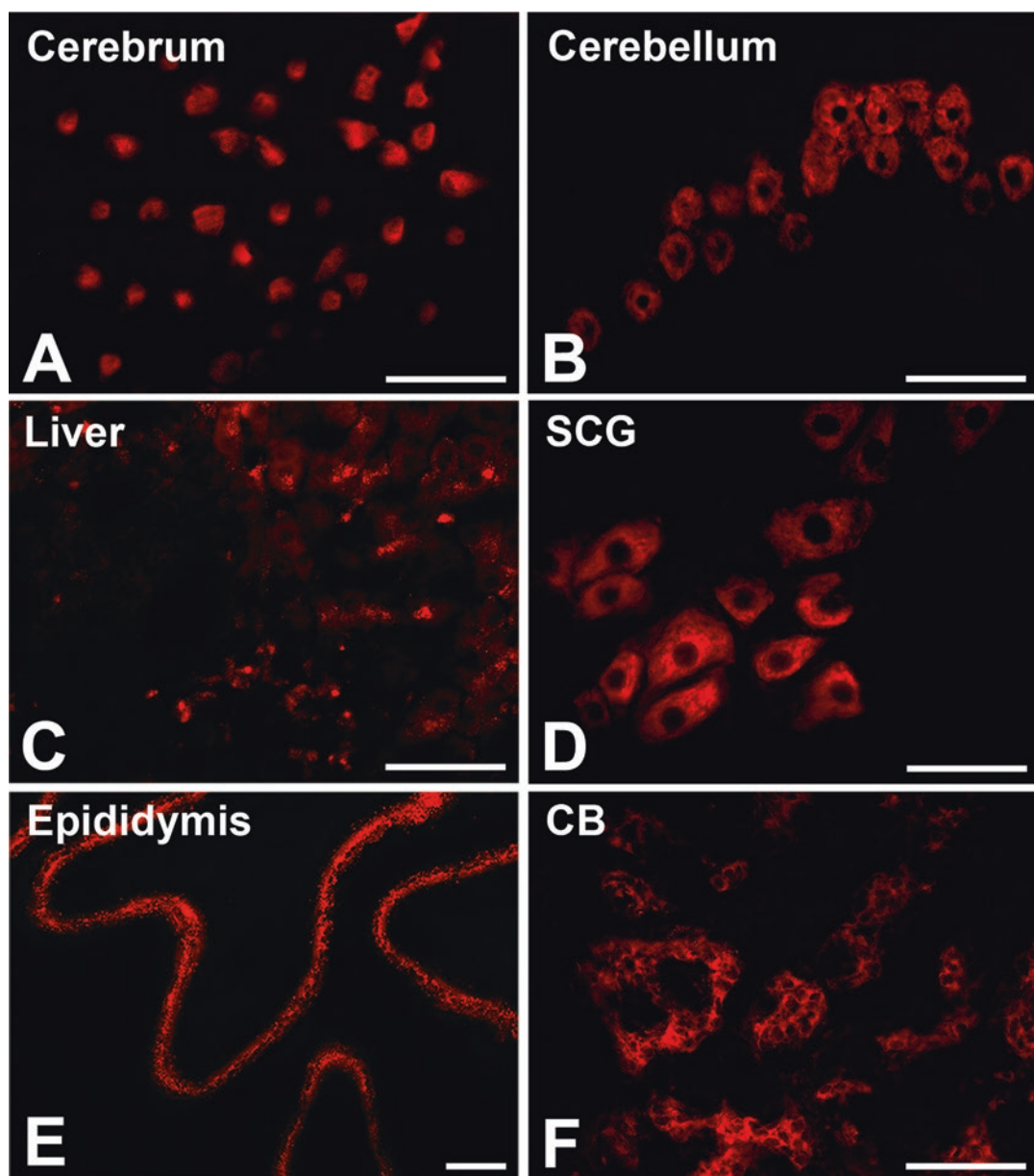


Fig. 2 Localization of TPPI activity in various organs of the rat using the fluorogenic histochemical substrate GPM-HHNI. Enzyme histochemical reaction product is seen in certain motoneurons in the brain (a), the Purkinje cells of the cerebellar cortex (b), the hepatocytes in the liver (c), the superior cervical ganglion (SCG) cells (d), the epithelial cells of the epididymis (e), the glomus and sustentacular cells of the carotid body (CB) glomeruli (f). Scale bars = 50 μ m

developmentally regulated in laboratory animals such as rats [27, 28] and mice [29], as well as in humans [20, 21], the present protocols can be employed for histochemical studies not only in adult tissues but also during development.

2 Materials

Animal care and treatment should be in accordance with international animal care guidelines for the use of experimental animals in research and to be approved by local authorities. Laboratory supplies and equipment do not differ from the ones usually used in common histochemical studies, and include glass slides and coverslips, glassware for incubation, washing, and staining of the sections. A cryostat to maintain a temperature from -25 to -30 °C is needed for the preparation of tissue sections from frozen tissue samples. Additionally, a pH meter and magnetic stirrer are to be used for the preparation of buffers and other solutions. For the incubation process, a thermostat keeping a constant temperature of 37 °C is necessary. Bright-field and fluorescence microscopes are needed for specimen observations. Fluorescent samples may also be observed under a confocal laser scanning microscope. The excitation and emission wavelengths of the yielded fluorescent end product are 460 and 580 nm respectively, meaning that a green excitation filter (or argon laser) should be used and the fluorescence would be observed in the orange-red to red area of the spectrum.

All the buffers and other aqueous solutions are to be prepared with distilled water. The buffers used have to be stored either at room temperature or at 4 °C in the refrigerator (as indicated below).

2.1 Chemicals, Buffers, and Other Solutions

1. 0.1 M phosphate buffer (PB), pH 7.2: Dilute 1.42 g anhydrous disodium hydrogen phosphate (Na_2HPO_4) in 70 mL distilled water and 1.36 g potassium dihydrogen phosphate (KH_2PO_4) in 30 mL distilled water. Mix well the two solutions and adjust pH on a pH meter to 7.2, pH 7.3–7.4 is also fine.
2. 0.1 M acetate buffer, pH 4.5: Dilute 1.64 g sodium acetate ($\text{C}_2\text{H}_3\text{NaO}_2$) in 74 mL distilled water and 1.2 mL acetic acid (CH_3COOH) in 126 mL distilled water. Mix well the two solutions and adjust pH to 4.5 on a pH meter.
3. Fixative solution: For 100 mL 2% paraformaldehyde solution, dissolve 2 g paraformaldehyde in 98 mL 0.1 M PB, pH 7.2, heat to 70 °C while stirring on a hot plate under a fume hood for several minutes. Be careful not to boil the solution (*see Note 1*). Once the solution has cleared (it should take 5 min or less), leave it at room temperature for an hour or cool it in ice, and later place it in the refrigerator at 4 °C. In our laboratory we prefer to prepare the fixative extempore each time and keep it cold until used (*see Note 2*).
4. Tissue washing solution: Mix well in a dry flask 60 g sucrose and 1 g gum arabic by agitating the flask by hand. Then add 140 mL of 0.1 M PB, pH 7.2 and stir the solution on a magnetic stirrer until the substances are dissolved (*see Note 3*). Store this Holt's solution in the refrigerator at 4 °C and use it cold.

5. Collodion solution: For the preparation of 100 mL 0.5 % collodion solution, pour 25 mL of collodion solution in 2 % amyl acetate into a previously prepared mixed solvent, consisting of 4 parts (30 mL) dry acetone, 3 parts (22.5 mL) absolute ethanol and 3 parts (22.5 mL) dry diethyl ether. Stir gently the solution on a magnetic stirrer for a few minutes at room temperature. Then, place it in a refrigerator at 4 °C, to be stored cold before use (*see* **Note 4**).
6. TPPI substrate for chromogenic detection: glycyl-L-prolyl-L-metionyl-5-chloro-1-anthraquinonylhydrazide (GPM-CAH), synthesized exactly following the procedure, described previously (*see* ref. **22**).
7. TPPI substrate for fluorogenic detection: glycyl-L-prolyl-L-metionyl-4-hydrazido-*N*-hexyl-1,8-naphthalimide (GPM-HHNI) synthesized exactly following the procedure, described previously (*see* ref. **23**).
8. TPPI specific inhibitor: Ala-Ala-Phe-chloromethylketone (Bachem Holding AG, Bubendorf, Switzerland).
9. Incubation solution for chromogenic detection: For preparation of 100 mL incubation solution, place 30 mg of the TPPI chromogenic substrate GPM-CAH and 50 mg 4-nitrobenzaldehyde in a beaker, add dimethylformamide (DMF) dropwise (usually no more than 0.3 mL are needed) and stir the contents constantly with a glass rod until the solutes are completely dissolved. Thereafter, add 100 mL of 0.1 M acetate buffer, pH 4.5 and stir the solution for a few minutes on a magnetic stirrer. Prior to use, filter the resulting solution and transfer it to a cuvette in a thermostat at 37 °C before incubation. Do not prepare stock incubation solutions (*see* **Note 5**).
10. Incubation solution for fluorogenic detection: For the preparation of 100 mL incubation solution, put 32 mg of the fluorogenic substrate GPM-HHNI and 50 mg piperonal in a beaker, add DMF in drops to the substances and agitate the content energetically with a glass rod until they are completely dissolved (*see* **step 9** above). Subsequently, add 100 mL of 0.1 M acetate buffer, pH 4.5 and stir the solution for a couple of minutes using a magnetic stirrer. Before use, filter the solution and place it in a cuvette in a thermostat at 37 °C before incubation. Do not prepare stock incubation solutions (*see* **Note 5**).
11. Post-fixation solution: 4 % formaldehyde solution in 0.1 M PB, pH 7.2: For 100 mL solution, dissolve 4 g paraformaldehyde in 96 mL 0.1 M PB, pH 7.2, heat to 70 °C while stirring on a hot plate under a fume hood for several minutes. Be careful not to boil the solution (*see* **Note 1**). Cool down the solution to room temperature and store it maximum for 2 months. The solution can be reused and is to be prepared again only if it

changes from transparent colorless to yellowish (for the disposal, *see* **Note 2**).

12. Inhibitor solution: Immediately before use, dissolve 1 mg of the TPPI specific inhibitor L-alanyl-L-alanyl-L-metionyl-chloromethylketone trifluoroacetic salt (Bachem, Switzerland) in 200 mL acetate buffer, pH 4.5. The inhibitor solution should be used once only and cannot be stored for future use. Stock inhibitor solutions are not to be made, either.
13. Incubation media for the negative controls: 1 mg of the inhibitor is added to 200 mL incubation solutions prepared as described in **steps 9** or **10** from Subheading **2.1**.
14. Mayer's hematoxylin solution: Dissolve 1 g of hematoxylin powder (dark brown in color) in 50 mL ethanol using a stir bar. Warm up to 60–65 °C while stirring. Completely dissolve 50 g ammonium aluminum sulfate in 900 mL distilled water by heating. Remove the container from the heat; mix the two solutions slowly adding hematoxylin by trickling alongside the beaker. Then on low heat add 200 mg of potassium iodide and 100 mg of citric acid. Take care to add hematoxylin dissolved in ethanol, otherwise there is a chance for fire or bursting of the potash alum solution. Reheat on low temperature until completely dissolved. Allow to cool and filter using filter paper. Store this solution in a brown bottle at room temperature because hematoxylin is light sensitive and subject to oxidation. When stored like this it should be stable for 2 months (*see* **Note 6**).
15. Glycerol-jelly: Dissolve 7 g gelatin in 42 mL of distilled water. Stir at low temperature until dissolved. Add 50 mL glycerin and 1 g phenol (*see* **Note 7**); continue to stir until entirely dissolved. The opaqueness usually disappears within 10–15 min. Filter through cotton wool. The glycerol-jelly becomes a firm gel at room temperature and is to be dissolved by heating in a water bath before use.
16. Water-based mounting medium with an anti-fading agent (for the fluorescent-stained sections): e.g., Fluoro Gel with DABCO™ anti-fadent (Electron Microscopy Sciences, Hatfield PA, USA).
17. Tissue-Tek® O.C.T. (optimal cutting tissue) compound (Sakura Finetek, Zoeterwoude, the Netherlands).

3 Methods

Carry out all procedures at room temperature, unless otherwise specified.

3.1 Animals and Tissue Processing

1. Use mature experimental animals (*see* **Note 8**). We apply perfusion fixation for adult Wistar rats and immersion fixation for BalbC mice.

2. Prepare a fresh fixative solution (*see Note 2*). For adult Wistar rats (250–300 g body weight) make 400 mL of fixative and for adult BalbC mice (weighing 25–30 g) 250 mL of fixative. Cool the solution in ice before use for perfusion fixation.
3. For immersion fixation, first deeply anesthetize the mouse in a closed glass chamber in the presence of cotton pads soaked in diethyl ether (*see Note 4*) and dissect out the tissues.
4. Immerse the trimmed tissue blocks in a cold fixation solution in appropriate containers and leave them for fixation in the refrigerator at 4 °C for 18–24 h (usually overnight). The volume of the fixative should be at least 20 times larger than the volume of the tissue.
5. For perfusion fixation, first fill the perfusion needle with buffer to eliminate the possibility of air bubbles. Open the abdomen and thorax so that the heart is well exposed. Cannulate the ascending aorta via the left ventricle. Start perfusion with a vascular wash-out, with washing solution to remove blood, and then perfuse with the fixative.
6. Following perfusion, decapitate the animal and quickly remove the brain, spinal cord and the desired organs. Dissect out tissue pieces and slice them in cube blocks no more than 0.5 cm thick.
7. After fixation, lightly wipe immersion- or perfusion-fixed tissue pieces with a gauze or paper tissue, and then wash them in several changes (usually 4–5 times) of washing solution for about 48 h in the refrigerator at 4 °C until they sink.
8. Place specimens on cork mats, embed the tissue in Tissue-Tek® O.C.T., wrap it in a layer of plastic wrap and carefully immerse in liquid nitrogen (*see Note 9*).

3.2 Histochemical Reaction

Perform the histochemical reaction according the procedure described (*see refs. 22, 23 and Note 10*) as follows:

1. Cut 8–10 µm thick sections on a cryostat at –26 to –28 °C and mount them on non-precooled gelatinized glass slides. Then dry the mounted samples with a ventilator for 10–15 min at room temperature. Enzyme activity in cryostat sections is usually preserved for at least 2 months provided they are stored in cuvettes in a freezer at –20 °C. Prior to incubation, immerse the sections for 60 s in cold collodion solution in a cuvette, carefully drain and leave them to dry out in front of a ventilator for 10 min.
2. Incubation process: Immerse the tissue sections covered with collodion in the incubation solution for either the chromogenic or the fluorogenic detection. Incubate them in a thermostat at 37 °C for 1.5 h (chromogenic substrate), or 4 h to overnight, depending on the enzyme activity in the different organs (fluorogenic substrate) (*see Note 10*).

3. Post-incubation treatment: Take away the samples from the incubation solution and rinse them in several changes of distilled water, 30 s to 1 min each. Afterwards, remove the collodion film from the back side of the glass slides by thoroughly wiping with a gauze or paper tissue (*see Note 11*). Finally, post-fix the samples by immersion in the post-fixation solution for 15 min at room temperature and wash again by immersing them in distilled water.
4. Counterstain for 1–2 min in hematoxylin, a general-purpose blue nuclear stain (*see Note 12*).
5. Coverslip with an aqueous mounting medium, i.e., glycerol jelly for the permanent mounting of the chromogen-stained tissues. For this purpose, place the storage container in boiling water to melt gelatin, apply one drop on each slide and then place a coverslip on it. For the fluorescent-stained sections, use a water-based mounting medium with an anti-fading agent for preserving fluorescence of tissues. Apply 3–4 drops of mounting medium on each slide, let them stay at room temperature for about 5 min and carefully put coverslips avoiding air bubbles. For a prolonged storage, these slides should always be stored in the dark, away from light, usually in a refrigerator at 4 °C, to minimize fading and retain fluorescence.

3.3 Observation and Imaging

1. Observe the chromogen-stained slides under a brightfield microscope. Result: the enzyme activity location sites in bright-field imaging are visible as dark orange to brown granules around the cell nuclei (Fig. 1). In the cells with a low enzyme activity, the final reaction product is observed as an almost homogenous yellowish precipitate (*see Note 13*).
2. Observe the fluorescent-stained sections with an epifluorescence microscope: Result: The final enzyme reaction product can be observed in brightfield as well as in fluorescence field (Fig. 3). In the brightfield images it appears as orange-red to brownish granules around the cell nucleus. In the cells of low enzyme activity, the reaction product may be seen as nearly homogenous yellowish precipitates as outlined above. In fluorescent images, the reaction product is very clearly visible as orange-red to bright red granules (Fig. 2). In some cases, fluorescence around the cells may also be seen. Translocation of the final reaction product (precipitates outside the cells or even outside the samples) may appear in the case of incompletely present collodion film (*see Note 14*).

3.4 Controls

1. Inhibitor controls: The procedure is principally the same, except that before the incubation, the sections should be immersed in the inhibitor solution and left in it for 40 min at room temperature. Thereafter, incubate them in the full substrate medium in the presence of the inhibitor, as described

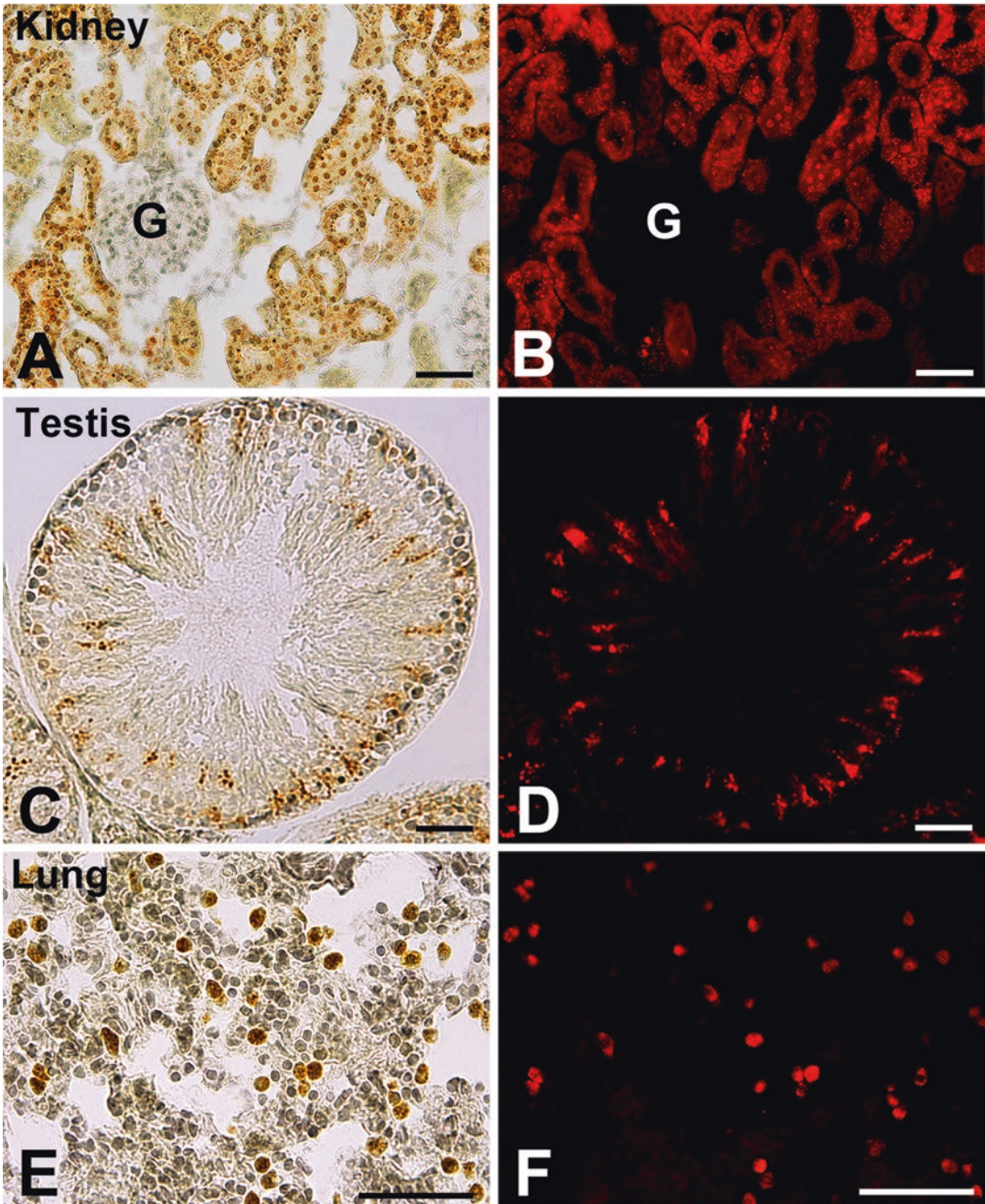


Fig. 3 Bright-field (**a, c, e**) and fluorescence (**b, d, f**) images showing the TPPI activity in various organs of the rat using the GPM-HHNI substrate. Note the final reaction product in the epithelium of convoluted renal tubules (**a, b**), while the glomeruli (G) in the kidney are negative. The Sertoli cells of a seminiferous tubule in the testis (**c, d**) and the alveolar macrophages in the lung (**e, f**) are highly TPPI-positive as well. Scale bars = 50 μ m

above. After incubation, the control samples are treated exactly the same way as all the other samples.

Result: No reaction product is observed in the control sections under both the bright-field and fluorescence microscopes.

4 Notes

1. Paraformaldehyde is toxic. Wear personal protection and work under a fume hood.
2. In order to avoid loss of enzyme activity, the fixative should never be left or used at room temperature, but refrigerated at 4 °C. It can be stored in these conditions for up to 2 weeks; nonetheless we recommend for each experiment to prepare a fresh fixative solution. After use, it should be disposed of according to the locally accepted rules.
3. Tissue washing solution is necessary for the recovery of the enzyme activity after the fixation. Mixing in dry of sucrose and gum arabic is important, because the gum arabic powder is poorly soluble in aqueous media and so, this facilitates its dissolution. It is better to use freshly prepared tissue washing solution. In any case, it cannot be stored for more than a week.
4. Remember to be careful when handling diethyl ether so as to avoid naked flames. All the solvents for the preparation of the collodion solution are to be water free (i.e., absolute solvents), since collodion is insoluble in aqueous media and even if minimal amounts of water are present, it precipitates to make the mixture unusable. All the precautions, given in the collodion certificate, should be followed strictly and thereafter the solution should be disposed of according to the locally accepted rules. The collodion solution can be reused many times. It is stable in a refrigerator and can be stored a long time depending on the number of applications. If it is frequently applied, its concentration increases due to the volatility of solvents. This should always be kept in mind and the solution should be renewed when it thickens.
5. Preparation of stock incubation solutions is highly inadvisable for at least two reasons, even if they are to be used at short intervals. First, they can precipitate and decrease the concentration of the substrate in the incubation solution (even if the precipitates are invisible to the naked eye). Second, the peptide substrates in a solution are usually unstable and inclined to self-hydrolysis.
6. There are numerous commercially available hematoxylin formulations, but four of them are the main ones employed in nuclear staining, i.e., Ehrlich's hematoxylin, Harris's hematoxylin, Gill's 1, 2, or 3 hematoxylin, and Mayer's hematoxylin. However, based on our experience, it is most economical to prepare these staining solutions in the lab rather than purchase commercial ones.
7. Since phenol is toxic and readily absorbed through the skin, it should be handled with care to avoid contact and inhalation. Thus, when preparing the glycerol jelly medium wear gloves,

goggles, and lab coat, and work in a well-ventilated area under a fume hood. This water-based mounting medium is also commercially available as ready-to-use Kaiser's glycerol jelly® (Merck, Darmstadt, Germany).

8. If newborn or very young animals are used, the enzyme reaction especially in the CNS, but also in some other organs (e.g., pancreas) is very low.
9. O.C.T. compound prevents the tissue blocks from making large crystals during the freezing process, whereas the plastic wrap prevents them from drying. Tissue blocks should be placed first in the vapors of liquid nitrogen and then slowly immersed in it, thus preventing clogging of the blocks in the nitrogen. Tissue blocks can be stored in liquid nitrogen for more than a year. Otherwise, after staying for an hour in the liquid nitrogen, they may be placed in a plastic box and stored in a freezer at -30°C for 3–4 months.
10. The fluorogenic reaction is much slower than the chromogenic one and needs about 4–5 h for the visualization of TPPI even in organs of a considerable enzyme activity (such as kidney, spleen, and epididymis), and an overnight incubation for the CNS and other internal organs.
11. The thin collodion film is formed during the drying process as described in **step 1** from Subheading 3.2 of the Methods section on both sides of the glass slides. It is necessary during the incubation to prevent translocation of the reaction products. However, if it is left longer on the back side of the sample, it may adversely affect the quality of microscopic images.
12. Nuclear counterstaining with hematoxylin is not obligatory, however it permits a better differentiation between the cell types in which the enzyme is visualized. It should be kept in mind that the penetration of the nuclear stain through the collodion film is complicated to some extent and the staining procedure might need more time than usual. The best way would be to use one or two collodion covered samples to determine the optimal time in the dye before the enzyme visualization procedure. Besides, apart from Gill hematoxylin, all other hematoxylin develop a surface metallic sheen that appears as a precipitate on the slide. To avoid this, we recommend daily filtering of the hematoxylin solution.
13. In some cases, a yellowish veil is formed around cells with a very high TPPI activity, whose cytoplasm is filled with granulated brown product. It should be kept in mind that, like many other lysosomal enzymes, some quantities of TPPI may be sorted for secretion in cells overexpressing the enzyme.
14. Sometimes, for unknown reasons, a collodion film simply does not form over the glass slides with tissue sections. We advise to

incubate only samples with a collodion film. Otherwise, translocation of fluorescent precipitates usually occurs and the original enzyme sites are not properly marked.

Acknowledgments

The authors greatly thank Prof Dr. Michail Davidoff for protocol consultation.

References

1. Wlodawer A, Li M, Gustchina A, Oyama H et al (2003) Structural and enzymatic properties of the sedolisin family of serine-carboxyl peptidases. *Acta Biochim Polon* 50:81–102
2. Ezaki J, Takeda-Ezaki M, Oda K et al (2000) Characterization of endopeptidase activity of tripeptidyl peptidase-I/CLN2 protein which is deficient in classical late infantile neuronal ceroid lipofuscinosis. *J Biochem Biophys Res Commun* 268:904–908
3. Wlodawer A, Durell SR, Li M et al (2003) A model of tripeptidyl peptidase I (CLN2), a ubiquitous and highly conserved member of the sedolisin family of serine-carboxyl peptidases. *BMC Struct Biol* 3:8–18
4. Sharp JD, Wheeler RB, Lake BD et al (1997) Loci for classical and a variant late infantile neuronal ceroid lipofuscinosis map to chromosomes 11p15 and 15q21–23. *Hum Mol Genet* 6:591–595
5. Lin L, Lobel P (2001) Production and characterization of recombinant human CLN2 protein for enzyme-replacement therapy in late infantile neuronal ceroid lipofuscinosis. *Biochem J* 357:49–55
6. Page AE, Fuller K, Chambers TJ et al (1993) Purification and characterization of a tripeptidyl peptidase I from human osteoclastomas: evidence for its role in bone resorption. *Arch Biochem Biophys* 306:354–359
7. Warburton MJ, Bernardini F (2001) The specificity of lysosomal tripeptidyl peptidase-I determined by its action on angiotensin-II analogues. *FEBS Lett* 500:145–148
8. Kopan S, Sivasubramaniam U, Warburton MJ (2004) The lysosomal degradation of neuro-medin B is dependent on tripeptidyl peptidase-I: evidence for the impairment of neuropeptide degradation in late-infantile neuronal ceroid lipofuscinosis. *Biochem Biophys Res Commun* 319:58–65
9. Junaid M, Wu G, Pullarkat R (2000) Purification and characterization of bovine brain lysosomal pepstatin-insensitive proteinase, the gene product deficient in the human late-infantile neuronal ceroid lipofuscinosis. *J Neurochem* 74:287–294
10. Bernardini F, Warburton MJ (2002) Lysosomal degradation of cholecystokinin-(29–33)-amide in mouse brain is dependent on tripeptidyl peptidase-I: implications for the degradation and storage of peptides in classical late-infantile neuronal ceroid lipofuscinosis. *Biochem J* 366:521–529
11. Sleat DE, Donnelly JR, Lackland H et al (1997) Association of mutations in a lysosomal protein with classical late-infantile neuronal ceroid lipofuscinosis. *Science* 277:1802–1805
12. Golabek A, Kida E (2006) Tripeptidyl-peptidase I in health and disease. *Biol Chem* 387:1091–1099
13. Junaid MA, Clark GM, Pullarkat RK (2000) A lysosomal pepstatin-insensitive proteinase as a novel biomarker for breast carcinoma. *Int J Biol Markers* 15:129–134
14. Altorjay A, Paal B, Sohar N et al (2005) Significance and prognostic value of lysosomal enzyme activities measured in surgically operated adenocarcinomas of the gastroesophageal junction and squamous cell carcinomas of the lower third of esophagus. *World J Gastroenterol* 11:5751–5756
15. Tsukamoto T, Lida J, Dobashi Y (2006) Overexpression in colorectal carcinoma of two lysosomal enzymes, CLN2 and CLN1, involved in neuronal ceroid lipofuscinosis. *Cancer* 106:1489–1497
16. Autefage H, Albinet V, Garcia V et al (2009) Lysosomal serine protease CLN2 regulates tumor necrosis factor- α -mediated apoptosis in a Bid-dependent manner. *J Biol Chem* 284:11507–11516
17. Van Beersel G, Tihon E, Demine S et al (2013) Different molecular mechanisms involved in spontaneous and oxidative stress-induced mitochondrial fragmentation in tripeptidyl peptidase-1 (TPP-1)-deficient fibroblasts. *Biosci Rep* 33:243–258

18. Koike M, Shibata M, Ohsawa Y et al (2002) The expression of trypsinogen in various tissues of rats and mice. *Arch Histol Cytol* 65:219–232
19. Yayoi Y, Ohsawa Y, Koike M et al (2001) Specific localization of lysosomal aminopeptidases in type II alveolar epithelial cells of the rat lung. *Arch Histol Cytol* 64:89–97
20. Kurachi Y, Oka A, Itoh M et al (2001) Distribution and development of CLN2 protein, the late-infantile neuronal ceroid lipofuscinosis gene product. *Acta Neuropathol* 102:20–26
21. Kida E, Golabek A, Walus M et al (2001) Distribution of trypsinogen in human tissues under normal and pathological conditions. *J Neuropathol Exp Neurol* 60:280–292
22. Dikov A, Dimitrova M, Ivanov I et al (2000) Original method for the histochemical demonstration of trypsinogen. *Cell Mol Biol* 46:1219–1225
23. Ivanov I, Tasheva D, Todorova R et al (2009) Synthesis and use of 4-peptidylhydrazido-N-hexyl-1,8-naphthalimides as fluorogenic histochemical substrates for dipeptidyl peptidase IV and trypsinogen. *Eur J Med Chem* 44:384–392
24. Dimitrova M, Ivanov I, Deleva D (2009) Distribution of trypsinogen activity in the rat brain and spinal cord. *Compt Rend Acad Bulg Sci* 62:729–734
25. Dimitrova M, Ivanov I, Todorova R et al (2008) Fluorescent localization of trypsinogen activity in tissue sections of Balb/c mice reproductive organs. *Compt Rend Acad Bulg Sci* 61:349–356
26. Atanasova D, Lazarov N (2015) Histochemical demonstration of trypsinogen in the rat carotid body. *Acta Histochem* 117:219–222
27. Dimitrova M, Deleva D (2009) Histochemical study of the changes in trypsinogen activity in developing rat brain and spinal cord. *Compt Rend Acad Bulg Sci* 62:1407–1412
28. Suopanki J, Partanen S, Ezaki J et al (2000) Developmental changes in the expression of neuronal ceroid lipofuscinoses-linked proteins. *Mol Genet Metab* 71:190–194
29. Dimitrova M, Deleva D, Pavlova V et al (2011) Developmental study of trypsinogen activity in the mouse central nervous system and peripheral organs. *Cell Tissue Res* 346:141–149

Enzyme Histochemistry for Functional Histology in Invertebrates

Francesca Cima

Abstract

In invertebrates, enzyme histochemistry has recently found a renaissance regarding its applications in morphology and ecology. Many enzyme activities are useful for the morphofunctional characterization of cells, as biomarkers of biological and pathologic processes, and as markers of the response to environmental stressors. Here, the adjustments to classic techniques, including the most common enzymes used for digestion, absorption, transport, and oxidation, as well as techniques for azo-coupling, metal salt substitution and oxidative coupling polymerization, are presented in detail for various terrestrial and aquatic invertebrates. This chapter also provides strategies to solve the problems regarding anesthesia, small body size, the presence of an exo- or endoskeleton and the search for the best fixative in relation to the internal fluid osmolarity. These techniques have the aim of obtaining good results for both the pre- and post-embedding labeling of specimens, tissue blocks, sections, and hemolymph smears using both light and transmission electron microscopy.

Key words Azo-coupling, Electron microscopy, Hemolymph smears, Invertebrates, Metal salt substitution, Oxidative coupling polymerization

1 Introduction

Enzyme histochemistry is still very important as a histochemical method for the intracellular localization of specific enzyme activities through the formation of an insoluble reaction product in tissue sections and/or whole organisms. It is distinguished from both in situ hybridization, which can provide features of gene expression (mRNA), and immunohistochemistry, which can demonstrate the presence of an enzyme, even when it is in its inactive form, i.e., as a proenzyme. Enzyme histochemistry can simultaneously detect enzyme localization in situ using both light and—after osmication—electron microscopy and directly characterizes the functionality of a cell or tissue under normal and pathological conditions. Although unfixed cryostat sections represent the best and most widely used technique to analyze total enzyme activity in various

tissues from mammals and other vertebrates, it is not recommended for most invertebrates that have hard endoskeletons or exoskeletons and cuticles. These samples require long decalcification and dechitinization treatments to remove the endoskeleton and exoskeleton or cuticles and allow better penetration of the dyes. Other invertebrates have large amounts of water (more than 90 % in cnidarians and tunicates), which facilitates the formation of ice crystals inside the cells, despite the addition of an antifreeze solution. Many classical procedures for pre- and post-embedding labeling are based on azo-coupling and metal salt methods, in which visualization is accomplished with an insoluble dye product [1–3]. These methods can also be applied to invertebrates, with suitable variations, as reported for cnidarians [4, 5], flat worms [6], nematodes [7], arthropods [8–10], bivalves [11, 12], gastropods [13], annelids [14, 15], sipunculid worms [16], echinoderms [17], and tunicates [18, 19].

In this chapter, a series of useful microscopic techniques for invertebrate tissues are presented and described in detail to allow researchers to properly reproduce the experiments. They are based on assays to detect the activity of common enzymes involved in main physiological functions, such as digestion, transport, absorption, and oxidation. Other procedures for the histochemical detection of particular enzyme activities, such as nitric oxide synthase, NADPH dehydrogenase [20], acetylcholinesterase [21], and carbonic anhydrase [22], are rarely reported in the invertebrate literature. For the histochemical detection of enzymes, it is fundamental to obtain and visualize a product of the enzymatic reaction at the location of the enzyme. Fixation is the most important step in the entire process because it preserves the structural integrity of cells and tissues. The choice of fixative and the fixation time are dependent on the size and the thickness of the sample, as well as the type of histoenzymatic survey to be performed. Because fixation in aldehydes inhibits the activities of many enzymes, the recommended concentrations of the fixative mixtures and incubation times must be strictly observed. It is also important to consider that the exposure time, the reaction temperature and the reagent concentrations could be modified depending on the species being considered. For some enzymes, such as glycosidases, the product of the enzymatic reaction is very soluble in aqueous solutions and is lost after rinsing. In this case, immunohistochemistry is the only method to localize this type of enzymes *in situ*, whereas the activity can be assessed indirectly through digestion or by staining a substrate-containing gelatine film that covers the tissue sections [23]. The digested zones in gelatine film must then be compared with their localization in adjacent nuclear-stained sections for establishing the tissue involved in the specific enzyme activity. In conclusion, enzyme histochemistry is a simple, rapid and inexpensive method with a broad range of sensitive dynamic applications,

and it represents a link between biochemistry and morphology, together with conventional histology, immunohistochemistry and in situ hybridization. In invertebrates, the choice of key enzymes not only appears to be particularly useful to identify and characterize cell populations but also to investigate their functional roles and the effects of environmental pollutants, pathogens and stressors related to anthropic impact and climate change on these biomarkers.

2 Materials

This section primarily presents a list of carefully chosen anesthetic solutions for each type of invertebrate that can obtain the best narcotic effects without killing the animals. Treatments for softening or dissolving the siliceous and calcareous skeletons as well as cuticles are essential before the histological processing. Anticoagulant solutions are fundamental for collecting the hemolymph and preventing clotting, but the use of EDTA or EGTA is not advisable because these chelating agents negatively affect the proteins of cytoskeleton causing the loss of the amoeboid shape. The choice of fixative mixture is very important for preserving the proteins, particularly enzyme activities, some of which are negatively affected by aldehydes in a dose- and time-dependent manner. In any case, for the success of preparations, aqueous solutions of anticoagulants and fixative solutions must be prepared in freshwater, buffer (saline solution) or seawater, depending on the fluid osmolarity of the animals; avoid the use of distilled or deionized water. The reaction mixtures for the histochemical detection of enzymes must be prepared immediately before use and be checked for change of color and formation of precipitates.

2.1 Anesthetic Solutions

1. For aquatic invertebrates (alternative solutions): 0.1 % ethyl 3-aminobenzoate methanesulfonic acid salt (MS222) solution (*see ref. 24*); 7–30 % magnesium chloride or magnesium sulfate solution (*see ref. 25, 26*); a few crystals of menthol (for 2–3 h).
2. For large aquatic arthropods (crustaceans): place on ice for 10–15 min (*see ref. 27*).
3. For terrestrial annelids: 10 % ethanol in freshwater by slowly adding a 50 % ethanol solution in a dropwise manner.
4. For terrestrial arthropods: chloroform or ether vapors from a soaked cotton ball placed in a glass container with a sealed lid.
5. For terrestrial snails: isoflurane administration in an anesthetic chamber with the ability for fresh gas inflow and waste gas scavenging (*see ref. 28*).

2.2 Saline Solutions**2.2.1 For Marine Invertebrates (Isotonic with Seawater)**

1. 0.2 M sodium cacodylate, pH 7.4, containing 1 % NaCl and 1 % sucrose.
2. 20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl.

2.2.2 For Earthworms

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 10 mM NaH₂PO₄, pH 7.3.

2.3 Desilicification Solutions

1. Poriferans: 80 % ethanol plus 5 % hydrofluoric acid, gradually added to a glass dish coated with paraffin. After a few hours, transfer the specimens to 80 % ethanol.

2.4 Decalcification Solutions

1. Arthropods and some poriferans: 5 % formic acid.
2. Stony corals and echinoderms: 10 % sodium citrate, 20 % formic acid (Morse's solution).

2.5 Dechitinization Solution

1. Arthropods: carbolic acid (three parts), lactic acid (one part), glycerine (two parts), distilled water (one part).
2. Enzymatic method: 8 U/mg chitinase from *Streptomyces* sp. in an aqueous solution, pH 5.5, at 37 °C (*see ref.* 29).

2.6 Anticoagulant Solutions (for Hemolymph Collection)

1. 0.38 % sodium citrate, pH 7.5.
2. 10 mM l-cysteine, pH 7.0–7.2.
3. 50 mM 2-mercaptoethanol, 3 mM anhydrous caffeine, 2 mM *p*-toluene sulfonyl-l-arginine methyl ester (TAME), pH 7.4.
4. Citrate buffer/EDTA for crustaceans: 0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6 (*see Note 1*).

2.7 Fixative Mixtures

1. 10 % buffered formalin (aqueous solution).
2. Bouin's fixative: 15 mL of a saturated aqueous solution of picric acid (*see Note 2*), 5 mL of buffered formaldehyde, 1 mL of glacial acetic acid. Mix the parts immediately before use. Directly transfer the sample into 50 % ethanol, which must be changed several times until the sample does not release a yellow color.
3. Fixative mixture for marine invertebrates (1 L): dissolve 40 g of paraformaldehyde in 500 mL of distilled water. Mix well in a well-stoppered bottle and heat in a 60 °C water bath (fume cupboard) until the solution appears opalescent. Add a few drops of concentrated NaOH with continuous stirring at 60 °C until the solution appears clear and transparent. Allow the solution to cool to room temperature (20–25 °C). Add 4 mL of 25 % -commercial glutaraldehyde and then 500 mL of double-concentrated saline solution for marine invertebrates (*see item 1* in Subheading 2.2.1). Filter the mixture and make

sure that the pH is 7.4. The final aldehyde concentration is 4 % paraformaldehyde and 0.1 % glutaraldehyde. Divide the solution into 50 mL aliquots and store them at -20°C (*see* ref. 30).

4. Fixative for detecting enzyme activities that are sensitive to aldehydes: cold acetone (4°C). For the correct temperature, place the bottle in the refrigerator the day before the fixation. Because acetone is a coagulant fixative for proteins and causes the coarctation and hardening of tissues, this procedure is not suitable for electron microscopy because it does not maintain the ultrastructural quality required to observe the tissue morphology.

2.8 Agar Solution for Pre-embedding

1. Add 2 g of dry powdered agar to 23 mL of distilled water in a small flask. The solution is heated while stirring by placing the flask in a 45°C water bath (*see* Note 3).

2.9 Buffered Hexazonium *p*-Rosaniline or Hexazotized New Fuchsin Solution

1. Solution A: 0.4 g of new fuchsin dissolved in 2 mL of 36 % HCl, and 8 mL of distilled water. Mix well until dissolved (*see* Note 4).
2. Solution B: 4 % NaNO_2 in distilled water (*see* Note 5).
3. In a 10-mL tube, thoroughly mix 200 μL of solution A and 200 μL of solution B immediately before use.
4. Add 5 mL of the buffer (the type of buffer depends on the enzymatic assay, *see* Subheadings 2.10.1, 2.10.7, 2.10.8) and mix well.
5. Add the solution to the final volume of the buffer (the volume depends on the enzymatic assay, *see* Subheadings 2.10.1, 2.10.7, 2.10.8). Note that a yellow color of the final solution indicates good hexazotization, whereas a brown or dark reddish solution should not be used because the results are unsatisfactory. The yellow background must be considered when evaluating the enzyme reaction (red staining) on sections and hemolymph smears under a light microscope, particularly after fixation in glutaraldehyde.

2.10 Reaction Mixtures for the Histochemical Detection of Enzymes

2.10.1 For Acid Phosphatase (EC 3.1.3.2)

1. Dissolve 5 mg of naphthol AS-BI phosphate in 200 μL dimethylformamide (DMF). Add dropwise the naphthol AS-BI phosphate solution to buffered hexazonium *p*-rosaniline (*see* Subheading 2.9), where the buffer is 10 mL of 0.1 M sodium acetate buffer, pH 5.2.
2. For the assay of enzyme inhibition: 0.1 M sodium fluoride in 0.1 M sodium acetate buffer, pH 5.2.

2.10.2 For 5'-Nucleotidase (EC 3.1.3.5)

1. Dissolve 20 mg of adenosine-5'-monophosphate (AMP) in 22 mL of distilled water, 20 mL of 0.2 M Tris-maleate buffer, pH 7.2, 5 mL of 2.5 % MgSO_4 , and add dropwise 3 mL of a 2 % aqueous $\text{Pb}(\text{NO}_3)_2$ solution as capture agent.

2. 1 % aqueous ammonium sulfide solution (from a 21 % commercial solution).
3. For the assay of enzyme inhibition: 0.2 M levamisole in 0.2 M Tris-maleate buffer, pH 7.2.

2.10.3 For
Aminopeptidase M (EC
3.4.11.2)

1. Dissolve 25 mg of l-leucyl-4-methoxy-2-naphthylamide in 1 mL of DMFA. Add dropwise this solution to 49 mL of 0.1 M citric acid-phosphate buffer, pH 6.7 containing 50 mg of Fast Blue B.
2. 2 % aqueous CuSO₄ solution.
3. For the assay of enzyme inhibition: add 0.02 % 1,10-phenanthroline previously dissolved in a small amount of DMFA to 0.1 M phosphate buffer, pH 6.7.

2.10.4 For Serine
Protease (Trypsin, EC
3.4.21.59)

1. Dissolve 4 mg of Z-Ala-Ala-Lys-4-methoxy-2-naphthylamide (Z-AAK-mna) in 0.5 mL of DMFA. Add dropwise the Z-AAK-mna solution to 10 mL of 0.1 M Tris-HCl, pH 7.0, containing 10 mg of Fast Blue B.
2. 1 % aqueous CuSO₄ solution.
3. For the assay of enzyme inhibition: dissolve 100 µM chloromethylketone (Tosyl-Lys-CMK) in 0.5 mL of DMFA and add this solution to 0.1 M Tris-HCl, pH 7.0.

2.10.5 For Nonspecific
Esterases (EC 3.1.1.x)

1. Dissolve 10 mg of 1-naphthyl acetate in 1 mL of acetone. Add dropwise the 1-naphthyl acetate solution to 49 mL of 0.1 M phosphate buffer, pH 7.2, containing 80 mg of Fast Blue B.
2. For the assay of enzyme inhibition: 0.1 M sodium fluoride in 0.1 M phosphate buffer, pH 7.2.

2.10.6 For lipase (EC
3.1.1.3)

1. Stock solution: dissolve 5 mL of Tween 20 in 100 mL of 0.1 M Tris-HCl buffer, pH 7.3, and add 0.5 g of CaCl₂.
2. Working solution: 46 mL of 0.05 M Tris-HCl buffer, pH 7.3, 3 mL of 2 % CaCl₂ and 1 mL of the stock solution.
3. 2 % aqueous Pb(NO₃)₂ solution as capture agent.
4. 1 % aqueous ammonium sulfide solution (from a 21 % commercial solution).
5. For the assay of enzyme inhibition: 0.1 M carnosic acid in 0.1 M Tris-HCl buffer, pH 7.3.

2.10.7 For
β-Glucuronidase (EC
3.2.1.31)

1. Dissolve 4 mg of naphthol AS-BI β-glucuronide in 250 µL of DMFA. Add dropwise the naphthol AS-BI β-glucuronide solution to buffered hexazonium *p*-rosaniline (*see* Subheading 2.9), where the buffer is 20 mL of 0.1 M sodium acetate buffer, pH 5.2.

2. For the assay of enzyme inhibition: 0.1 M mucic acid in 0.1 M sodium acetate buffer, pH 5.2.

2.10.8 For β -N-Acetylglucosaminidase (EC 3.2.1.30)

1. Dissolve 3 mg of naphthol AS-BI β -N-acetylglucosaminide in 250 μ L of DMFA. Add dropwise the naphthol AS-BI β -N-acetylglucosaminide solution to buffered hexazonium-*p*-rosaniline (*see* Subheading 2.9), where the buffer is 10 mL of 0.1 M citric acid-phosphate buffer, pH 6.
2. For the assay of enzyme inhibition: 0.25–1 M acetamide in 0.1 M citric acid-phosphate buffer, pH 6.

2.10.9 For Ca^{2+} -ATPase (EC 3.6.3.8)

1. 1 % aqueous CaCl_2 solution (pre-incubation solution).
2. Dissolve 76 mg of adenosine-5'-triphosphate (ATP) in 10 mL of 0.1 M barbital sodium buffer, pH 7.2. Add 5 mL of 0.25 % CaCl_2 , 30 mg of 2,4-dinitrophenol (DNP), and 35 mL of distilled water.
3. 1 % aqueous $\text{Co}(\text{NO}_3)_2$ solution as capture agent.
4. 1 % aqueous ammonium sulfide solution (from a 21 % commercial solution).

2.10.10 For Mg^{2+} -ATPase (EC 3.6.3.2)

1. Dissolve 25 mg of ATP in 20 mL of distilled water. Add 20 mL of 0.2 M Tris-maleate buffer, pH 7.2, 3 mL of a 2 % aqueous $\text{Pb}(\text{NO}_3)_2$ solution (to be added in a dropwise manner), 5 mL of 0.25 % MgSO_4 , 40 mg of DNP, 0.012 g of NaCl, and 2 mL of distilled water.
2. 1 % aqueous ammonium sulfide solution (from a 21 % commercial solution).

2.10.11 For Alkaline Phosphatase (EC 3.1.3.1)

1. Dissolve 10 mL of 3 % sodium 2-glycerophosphate in 10 mL of 10 % barbital sodium. Add 15 mL of 2 % CaCl_2 , 10 mL of 2 % MgSO_4 , 5 mL distilled water.
2. 2 % aqueous $\text{Co}(\text{NO}_3)_2$ solution.
3. 1 % aqueous ammonium sulfide solution (from a 21 % commercial solution).
4. For the assay of enzyme inhibition: 0.1 M levamisole in 10 % barbital sodium.

2.10.12 For Peroxidase (EC 1.11.1.x)

1. Dissolve 5 mg of 3,3'-diaminobenzidine (DAB) in 0.1 M phosphate buffer, pH 7.2. Add 5 μ L of 30 % (w/v) H_2O_2 immediately before use.
2. For the assay of enzyme inhibition: 0.065 % KCN in 0.1 M phosphate buffer, pH 7.2.

2.10.13 For *d*-Amino Acid Oxidase (EC 1.4.3.3)

1. Dissolve 0.015 g of nitro blue tetrazolium (NBT) in 0.5 mL DMFA and add 4.5 mL distilled water. Add 5 mL of 0.1 M

phosphate buffer, pH 7.8, 0.89 g d-alanine and 10 mg of phenazine methosulfate.

2. For the assay of enzyme inhibition: 0.1 M 3-hydroxyquinolin-2-(1H)-one in 0.1 M phosphate buffer, pH 7.8.

2.10.14 For
Phenoloxidase (EC
1.14.18.1)

1. Prepare a saturated solution of 3-(3,4-dihydroxyphenyl)-l-alanine (L-DOPA) in 0.1 M cacodylate buffer, pH 7.0.
2. For the assay of enzyme inhibition: 0.1 M Na-diethyldithiocarbamate or 20 mM Na-benzoate or 2 mM tropolone or 1 mM phenylthiourea in 0.1 M cacodylate buffer, pH 7.0 (alternative solutions).

2.10.15 For
Cytochrome-c Oxidase,
COX (EC 1.9.3.1)

1. Dissolve 0.2 % DAB in a 1 % aqueous MnCl_2 solution containing 0.01 % H_2O_2 , the latter added immediately before use.
2. For the assay of enzyme inhibition: 0.01 M KCN in distilled water or phosphate buffer, pH 7.2.

3 Methods

Anesthesia/sedation prior to fixation is strongly encouraged, and the animal should be left in the solution until there is no movement and/or contraction. Then, pour off the solution and slowly add the fixative by totally immersing the animal in the fixative mixture (small aquatic invertebrates), adding it dropwise (small terrestrial invertebrates), adding it directly to the oral cavity (large cnidarians) or injecting it (large arthropods). Ideally, the fixative should be replaced after a few minutes, particularly for marine invertebrates, to remove the seawater. Pre-embedding in agar is desirable for very small specimens (e.g., nematodes, tardigrades, rotifers, appendicularians, embryonic and larval stages, etc.) and is useful for their orientation in the capsule for resin/paraffin embedding. The hemolymph can be directly collected and smeared on a glass slide before fixation or centrifuged to obtain a pellet that can be placed in a tube and treated as a tissue block. Two methods for detecting the enzyme activity can be performed. In the first method, the assays can be directly performed on the fixed hemolymph smears/pellets, sections of specimens, and tissue blocks that were previously wax-embedded in Paraplast® X-tra, a refined mixture of highly purified paraffin containing plastic polymers, which is recommended for lower temperature infiltration to preserve enzyme activity (melting point 53–54 °C), for light microscopy. For both light and electron microscopy, the samples can be embedded in a hydrophilic acrylic resin, e.g., LR® White or Gold, GMA®, JB-4®, Technovit®, according to the manufacturer's instructions. In the second method, fixed specimens and tissue blocks are incubated in the reaction mixtures to detect the enzyme activity and then embedded in Paraplast®

X-tra or Plus for light microscopy or, after a post-incubation in osmium tetroxide, in a hydrophobic epoxy resin for both light and electron microscopy. All solutions and mixtures must be freshly prepared. To obtain the best results and avoid the formation of flaky precipitates, preparation of the reaction mixtures following the procedures step-by-step is recommended. For cover glass mounting on slides with sections and hemolymph, glycerine must be avoided because it progressively bleaches the sample. The use of a commercial aqueous medium or a synthetic mounting resin, the latter after dehydration in alcohol and clarification in xylene of the sample, is therefore strongly advised.

3.1 Anesthetization

1. See Subheading 2.1 for the choice of the optimal anesthetic solution.
2. Place the animals directly in the solution or on ice (aquatic invertebrates) or enclose them in a vapor-saturated environment (terrestrial invertebrates) until they are adequately anesthetized.
3. Check the reactivity (body, siphons or tentacle withdrawal response, antenna and leg movements are absent) by gently stimulating the organisms with a needle.

3.2 Tissue/Organ/ Organism Fixation

1. Fix the samples by incubating them in the appropriate fixative mixture (*see* Subheading 2.7) for 2–15 h at 4 °C, depending on the sample volume (do not exceed these recommended times) (*see* **Note 6**). In the case of large invertebrates, such as crustaceans fixed by injection through the arthrodial membranes, which are the flexible and soft cuticles at the suture lines of the sclerotized elements and the base of the legs, the incubation time can be extended to 48 h.
2. Wash well, preferably in the buffer that will be used in the subsequent enzymatic assay, to prepare the samples to the reagents employed in the assay and to remove the excess of fixative, which could interact with the reagents themselves. In the case of electron microscopy, avoid post-fixation in osmium tetroxide.
3. For some invertebrates, desilicification, decalcification and/or dechitinization of the exoskeleton with appropriate solutions, depending on the taxon (*see* Subheading 2.3–2.5), is recommended before dehydration and embedding. Check the reaction to avoid tissue disruption and stop it as soon as the tissue/specimen begins to soften enough to be sectioned (*see* **Note 7**).

3.3 Agar Pre- embedding for Small Specimens

1. After fixation, the organisms are concentrated by gentle centrifugation in a centrifuge tube. Do not discharge the supernatant.
2. Add an equal volume of agar solution at 45 °C, resulting in a final 4 % agar solution.

3. The pellet–agar mixture is hardened into a block by placing the tube at 4 °C.
4. Remove the block with a needle after adding a few drops of 70 % ethanol and cut it with a lancet forming small cubes (*see Note 8*).
5. The cubes can be processed for enzyme detection, dehydrated, and embedded in Paraplast® X-tra or in a resin.

3.4 Hemolymph Collection and Smearing

Before fixation and the subsequent enzyme assays, the hemolymph can be collected after the animal has been anesthetized (*see Subheadings 2.1 and 3.1*). Rinse the syringe or pipette twice in a freshly prepared anticoagulant solution (*see Subheading 2.6*), collect the hemolymph, place it in Eppendorf tubes on ice, and dilute it 1:2 or 1:4 in the same anticoagulant solution. After collecting the hemolymph with the following methods for various animals, let the suspension in the tube on ice for about 5–10 min to allow the slow fall down of the tissue debris. Transfer the suspension in another tube being careful to avoid to resuspend the tissue debris in the bottom of the first tube. Centrifuge the sample at $780 \times g$ for 10 min, discharge entirely the supernatant. Gently resuspend the cells with a micropipette in a quantity of appropriate isosmotic solution (*see Subheading 2.2*) corresponding to the final volume that will be used in the histological experiments. In order to maximize the concentration of hemocytes, consider that each smear will be formed of one drop of 50 μL of the hemocyte suspension. Determine the number of hemocytes in the final suspension using a Bürker hemocytometer. Put the drops of 50 μL of the hemocyte suspension on a poly-l-lysine-coated microscope slide or an industrially processed slide with a permanent positive charge on its surface and incubate the slide horizontally for 30 min to allow permanent adhesion of the living cells before fixation and the treatments used to detect the enzyme activities. Labeling with a small circle the opposite side of the slides with a marking-pen where the drop will be placed is recommended for finding easily the smears during the treatments and the observation under the light microscope.

3.4.1 Large Arthropods

1. After anesthetization, collect the hemolymph from the pericardial sinus or the coxal sinus, the latter of which is located under the unsclerotized coxal membrane of the walking legs, using a 1-mL plastic syringe.

3.4.2 Small Insects

1. After anesthetization, collect the hemolymph by puncturing with a microinjection needle between the ventral segments, extract it from a small incision in the ptilinum at the front of the head with a pipette by softly squeezing the abdomen, or extract it from the tip of an amputated antenna using an air-flow/pressure device (*see ref. 31*).

- 3.4.3 *Gastropods*
1. After anesthetization, exert a slight pressure on the animal's foot and collect the hemolymph with a Pasteur pipette.
- 3.4.4 *Bivalves*
1. No anesthetization needed.
 2. Open the valves with a lancet and insert a micropipette tip to block their closure. Insert a 2-mL sterile syringe containing 300 μ L of an anticoagulant (*see* Subheading 2.6) in the anterior adductor muscle being careful not to pierce it from side to side and not to lacerate it, and gently aspirate the hemolymph by maintaining a slight but constant pressure until the hemolymph starts to flow into the syringe (*see* Note 9).
- 3.4.5 *Earthworms*
1. Place the animals in a sterile plastic petri dish with cold PBS (*see* Subheading 2.2.2) diluted 1:1 with absolute ethanol. After a few seconds, the earthworms vigorously extrude their coelomocytes through their dorsal pores.
- 3.4.6 *Aquatic Worms*
1. After anesthetization, collect the coelomic fluid from the coelomic cavity with a 1-mL plastic syringe.
- 3.4.7 *Sea Urchins*
1. No anesthetization needed.
 2. Coelomocytes can be obtained through a cut in the peristomal membrane in an anticoagulant solution or following removal of Aristotle's lantern.
- 3.4.8 *Tunicates*
1. After anesthetization, place the animals in the anticoagulant solution for 5 min.
 2. For solitary ascidians, incise the incurrent siphon and collect the exuding hemolymph or insert a syringe directly in the heart.
 3. For colonial ascidians without previous anesthetization, collect the hemolymph with a glass micropipette after puncturing the tunic marginal vessel of the colonies with a fine tungsten needle, maintaining the colonies in seawater. In the case of species without tunic vessels, collect the hemolymph which flows around and below the colony with a glass micropipette after the colonies are gently dried with a blotting paper and the zooids have been disrupted with a needle beginning from the colonial periphery. Add 1–2 drops of anticoagulant solution on the colony for collecting entirely the hemolymph come out.
- 3.5 Assays for Digestive Enzymes**
- 3.5.1 *Acid Phosphatase*
(EC 3.1.3.2)
1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 1 h at 37 °C in the reaction mixture (*see* Subheading 2.10.1) based on the azo-coupling method [33].
 2. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are

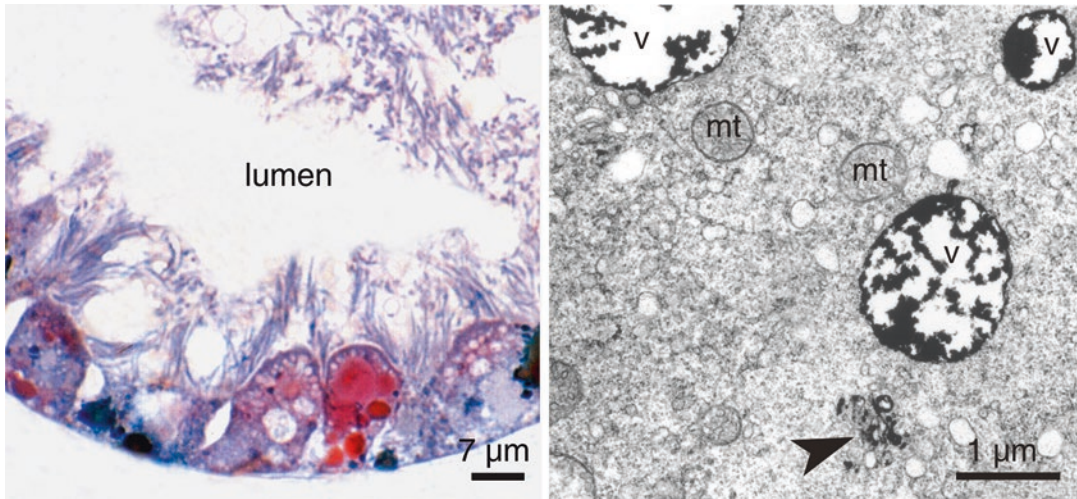


Fig. 1 Acid phosphatase in tunicate gut. On the *left*: pre-epoxy resin embedding procedure on *Oikopleura dioica* whole specimen. Thick section showing red-stained reaction for the enzyme (hexazonium *p*-rosaniline as diazonium salt) in distal intestine, and toluidine blue as a counterstain for masking *yellow* background. On the *right*: enzyme reaction viewed at TEM in apical vesicles (v) and inside Golgi apparatus (*arrowhead*) of a globular cell involved in intracellular digestion. *mt* mitochondrion

stained red under a light microscope and a fine granular precipitate is revealed under an electron microscope (Fig. 1).

3. Control: incubation without naphthol AS-BI phosphate as the specific enzyme substrate.
4. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 2* in Subheading 2.10.1) for 15–30 min at 20–25 °C.

3.5.2 5'-Nucleotidase (EC 3.1.3.5)

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 2 h at 37 °C in the reaction mixture (*see item 1* in Subheading 2.10.2) based on the metal salt procedure [34].
2. Rinse twice in distilled water and then incubate the samples in a 1 % aqueous ammonium sulfide solution for 2 min (sections and hemolymph smears) or 30 min (specimens and blocks).
3. Rinse the specimens/sections in tap water for 30 min.
4. Proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained brown under a light microscope and show a strong electron-dense signal under an electron microscope.
5. Control: incubation without AMP as the specific enzyme substrate.
6. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 3* in Subheading 2.10.2) for 15–30 min at 20–25 °C.

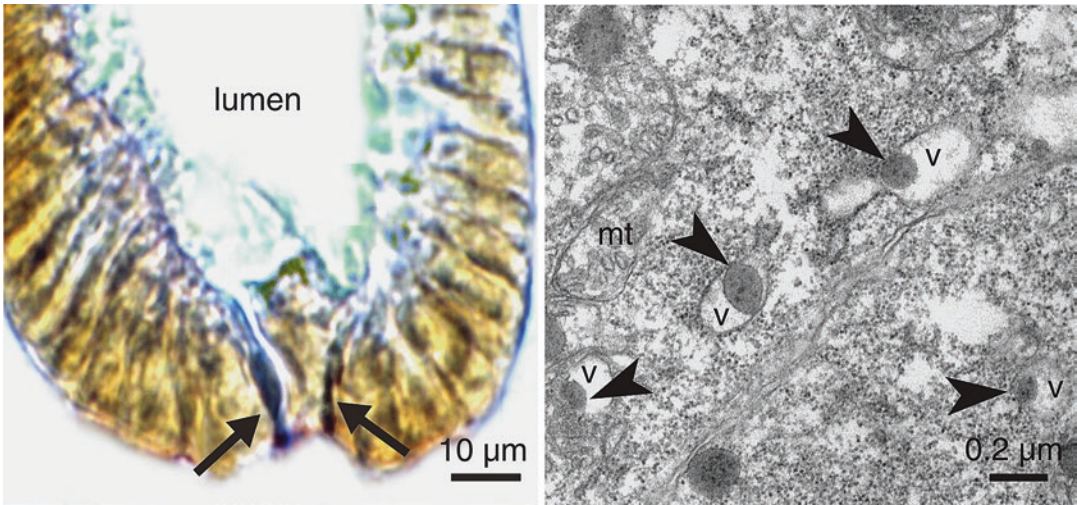


Fig. 2 Aminopectidase M in tunicate gut. On the *left*: post-paraffin embedding procedure on transversal section of a *Botryllus schlosseri* gastric ansa, in which the enzyme activity is revealed as blue staining in the zymogenic cells (*arrows*), placed in the crypt ground. Note *yellow* background due to diazonium salt used (Fast Blue B) at pH 6.7. On the *right*: pre-epoxy resin embedding procedure on whole specimen. Thin section, viewed at TEM, of two adjacent zymogenic cells involved in extracellular digestion showing drop-like electron-dense product of enzyme activity (*arrowheads*) inside apical vesicles (*v*). *mt* mitochondrion

3.5.3 Aminopeptidase M (EC 3.4.11.2)

1. Incubate the specimens, blocks, sections, and hemolymph smears for 12 h at 4 °C in the reaction mixture (*see item 1* in Subheading 2.10.3) based on azo-coupling method [35].
2. Rinse the samples in distilled water for 30 min and incubate them in a 2 % aqueous CuSO₄ solution for 5 min (sections and hemolymph smears) or 30 min (specimens and blocks). For electron microscopy, treatment with 1 % OsO₄ is recommended instead of chelation with CuSO₄.
3. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained blue under a light microscope, and small electron-dense drops are revealed under an electron microscope (Fig. 2).
4. Control: incubation without l-leucyl-4-methoxy-2-naphthylamide as the specific enzyme substrate.
5. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 3* in Subheading 2.10.3) for 15–30 min at 20–25 °C.

3.5.4 Serine Protease (Trypsin, EC 3.4.21.59)

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 1 h at 37 °C in the reaction mixture (*see item 1* in Subheading 2.10.4) based on the azo-coupling method [36].

2. Rinse the samples in distilled water for 30 min and incubate them in a 1 % aqueous CuSO_4 solution for 10 min (sections and hemolymph smears) or 30 min (specimens and blocks). For electron microscopy, treatment with 1 % OsO_4 is recommended instead of chelation with CuSO_4 .
3. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained dark blue to black under a light microscope, and small electron-dense drops are revealed under an electron microscope.
4. Control: omit the substrate.
5. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see* **item 3** in Subheading 2.10.4) for 15–30 min at 20–25 °C.

3.5.5 Nonspecific Esterases (EC 3.1.1.x)

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 3 h at 4 °C in the reaction mixture (*see* **item 1** in Subheading 2.10.5) based on the azo-coupling method [37].
2. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained blue under a light microscope and show small electron-dense drops under an electron microscope.
3. Control: incubation without naphthyl acetate as the specific enzyme substrate.
4. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see* **item 2** in Subheading 2.10.5) for 15–30 min at 20–25 °C.

3.5.6 Lipase (EC 3.1.1.3)

1. Use acetone-fixed specimens/blocks because aldehydes inhibit the enzyme activity, even at low concentrations.
2. Incubate the specimens, blocks and sections for 12 h at 37 °C in the reaction mixture (*see* **item 2** in Subheading 2.10.6) based on the metal salt procedure [38].
3. Rapidly rinse the samples in distilled water and incubate them in a 2 % aqueous $\text{Pb}(\text{NO}_3)_2$ solution for 10 min at 60 °C (*see* **Note 10**).
4. Rinse the samples in distilled water repeatedly for 5–10 min, and then incubate them in a 1 % aqueous ammonium sulfide solution for 10 min.
5. Rinse the samples in tap water 10 min, and dehydrate them in ethanol, but use tetrachloroethylene instead of xylene to avoid discoloration. The positive sites are stained black under a light microscope. This technique cannot be performed for electron microscopy.

6. Control: incubation without Tween 20 as the specific enzyme substrate.
7. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see* **item 4** in Subheading 2.10.6) for 15–30 min at 20–25 °C.

3.5.7 β -Glucuronidase
(EC 3.2.1.31)

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 2 h at 37 °C in the reaction mixture (*see* **item 1** in Subheading 2.10.7) based on the azo-coupling method [39].
2. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained red under a light microscope and a fine granular precipitate is revealed under an electron microscope.
3. Control: incubation without naphthol AS-BI β -glucuronide as the specific enzyme substrate.
4. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see* **item 2** in Subheading 2.10.7) 15–30 min at 20–25 °C.

3.5.8 β -N-Acetylglucosaminidase (EC 3.2.1.30)

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 3 h at 37 °C in the reaction mixture (*see* **item 1** in Subheading 2.10.8) based on the azo-coupling method [40].
2. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained red under a light microscope and a fine granular precipitate is revealed under an electron microscope.
3. Control: incubation without AS-BI β -N-acetylglucosaminide as the specific enzyme substrate.
4. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see* **item 2** in Subheading 2.10.8) for 15–30 min at 20–25 °C.

**3.6 Assays
for Transport/
Absorption Enzymes**

3.6.1 Ca^{2+} -ATPase (EC 3.6.3.8)

1. Pre-incubate the specimens, blocks, sections, and hemolymph smears in a 1 % aqueous CaCl_2 solution for 10 min.
2. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 30 min at 37 °C in the reaction mixture (*see* **item 2** in Subheading 2.10.9) based on the metal salt procedure [41].
3. Rinse the samples in a 1 % CaCl_2 solution in distilled water twice for 5 min.

4. Rinse the samples in 1 % $\text{Co}(\text{NO}_3)_2$ solution in distilled water for 5 min (sections and hemolymph smears) or 30 min (specimens and blocks), followed by a rinse in tap water for 10 min.
5. Incubate the samples for 2 min (sections and hemolymph smears) or 30 min (specimens and blocks) in a 1 % aqueous ammonium sulfide solution.
6. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained black under a light microscope and show a strong electron-dense signal under an electron microscope.
7. Controls: incubation without ATP as the specific enzyme substrate or in distilled water heated to 80 °C for 10 min (heat-inactivation).

3.6.2 *Mg²⁺-ATPase (EC 3.6.3.2)*

Because this enzyme is coupled with the $\text{Na}^+\text{-K}^+\text{-ATPase}$, its activity can be assessed as an indirect method to detect the latter.

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 2 h at 37 °C in the reaction mixture (*see item 1* in Subheading 2.10.10) based on the metal salt procedure [42].
2. Rinse the samples in tap water for 10 min, and then incubate them in a 1 % aqueous ammonium sulfide solution for 2 min (sections and hemolymph smears) or 30 min (specimens and blocks).
3. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained brown under a light microscope and show a spot-like electron-dense signal under an electron microscope (Fig. 3).
4. Controls: *see item 7* in Subheading 3.6.1 ($\text{Ca}^{2+}\text{-ATPase}$).

3.6.3 *Alkaline Phosphatase (EC 3.1.3.1)*

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 1 h at 37 °C in the reaction mixture (*see item 1* in Subheading 2.10.11) based on the metal salt procedure [38].
2. Rinse the samples in tap water for 1 min, in a 2 % aqueous $\text{Co}(\text{NO}_3)_2$ solution for 5 min (sections and hemolymph smears) or 1 h (specimens and blocks), and in tap water for 10 min.
3. Incubate the samples in a 1 % aqueous ammonium sulfide solution for 30 min (sections and hemolymph smears) or 2 min (specimens and blocks).
4. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are

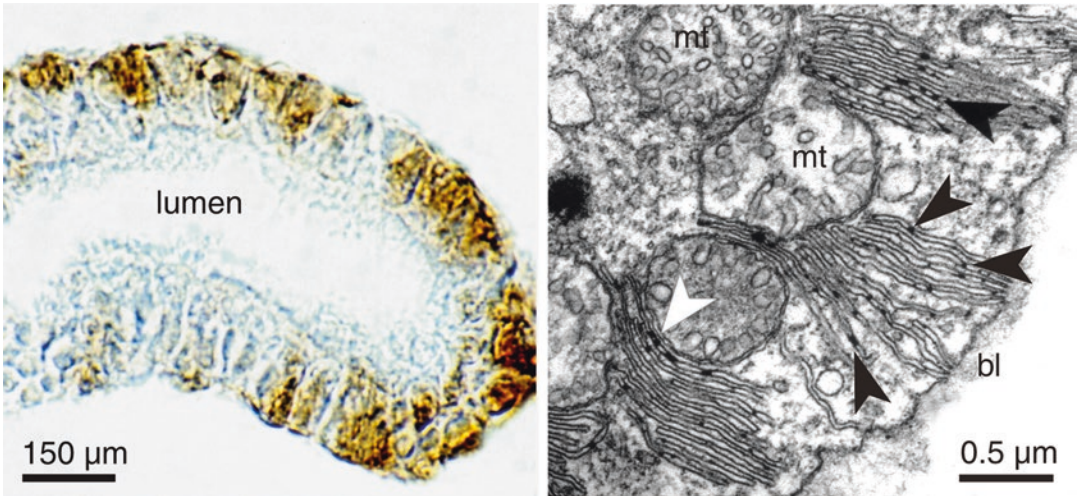


Fig. 3 Mg^{2+} -ATPase in tunicate gut. Post-paraffin embedding procedure on transversal section of *Botryllus schlosseri* stomach, in which the enzyme activity is revealed as brown staining in the basal region of the plicated cells of the pyloric caecum after metal salt method. On the *right*: pre-epoxy resin embedding procedure on whole specimen. At TEM, spot-like strong electron-dense product of enzyme activity is visible on basolateral membrane infoldings (*arrowheads*) associated with mitochondria (*mt*) of plicated cells involved in excretion and regulation of internal fluid osmolarity. *bl*/basal lamina

stained black under a light microscope and show a strong electron-dense signal under an electron microscope (Fig. 4).

5. Control: incubation without sodium 2-glycerophosphate as the specific enzyme substrate.
6. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 4* in Subheading 2.10.11) for 15–30 min at 20–25 °C.

3.7 Assays for Oxidative Enzymes

3.7.1 Peroxidase (EC 1.11.1.x)

1. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 10–30 min at 37 °C in the reaction mixture (*see item 1* in Subheading 2.10.12) based on the oxidative coupling polymerization procedure [43]
2. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained brown under a light microscope and show a strong electron-dense signal under an electron microscope.
3. Control: incubation without H_2O_2 as the specific enzyme substrate or in distilled water heated to 80 °C for 10 min (heat-inactivation).
4. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 2* in Subheading 2.10.12) for 15–30 min at 20–25 °C.

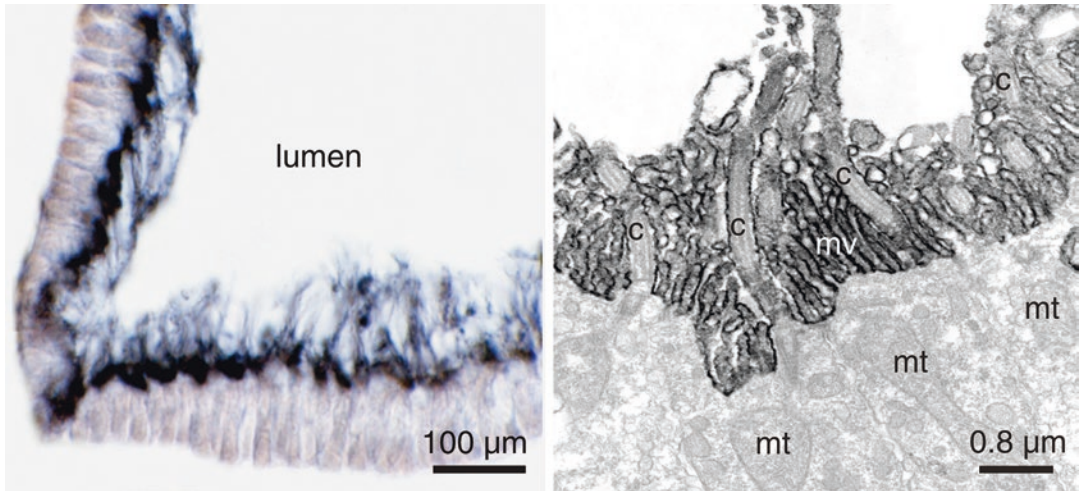


Fig. 4 Alkaline phosphatase in tunicate gut. On the *left*: post-paraffin embedding procedure on transversal section of *Botryllus schlosseri* proximal intestine, in which the enzyme activity is revealed as black staining at the epithelial apex after metal salt method. On the *right*: pre-epoxy resin embedding procedure on whole specimen. At TEM, strong electron-dense reaction labels profiles of cilia (c) and microvilli (mv) at the apex of both ciliated microvillar cells and vacuolated cells, involved in uptake of nutrients. *mt* mitochondria

3.7.2 *d*-Amino Acid Oxidase (EC 1.4.3.3)

1. Briefly fix the samples (10–15 min at 4 °C).
2. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 30 min at 37 °C in the dark in the reaction mixture (*see item 1* in Subheading 2.10.13) based on the oxidative coupling polymerization procedure [44].
3. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained blue under a light microscope and a fine granular electron-dense precipitate is revealed under an electron microscope.
4. Control: incubation without *d*-alanine as the specific enzyme substrate.
5. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 2* in Subheading 2.10.13) for 15–30 min at 20–25 °C.

3.7.3 Phenoloxidase (EC 1.14.18.1)

1. Incubate the specimens, blocks, sections, and hemolymph smears for 12 h at 4 °C in the reaction mixture (*see item 1* in Subheading 2.10.14) based on polyphenolic oxidation [45].
2. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting

(for sections and hemolymph smears). The positive sites are stained black or brown under a light microscope, and strong electron-dense precipitates are revealed under an electron microscope.

3. Control: incubation without 3-(3,4-dihydroxyphenyl)-l-alanine (L-DOPA) as the specific enzyme substrate.
4. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 2* in Subheading 2.10.14) for 15–30 min at 20–25 °C.

3.7.4 Cytochrome-c Oxidase, COX (EC 1.9.3.1)

1. Preincubate the samples in 0.1 M sodium acetate buffer, pH 5.5, for 10 min.
2. Incubate the specimens and blocks for 12 h or the sections and hemolymph smears for 4 h at 37 °C in the reaction mixture (*see item 1* in Subheading 2.10.15) based on the oxidative coupling polymerization procedure [46].
3. Rinse the specimens/sections in tap water for 30 min and proceed with embedding (for specimens and blocks) or mounting (for sections and hemolymph smears). The positive sites are stained brown under a light microscope and show a strong electron-dense signal under an electron microscope.
4. Control: incubation without H₂O₂ as the specific enzyme substrate.
5. Enzyme inhibition (negative control): preincubation in the inhibition solution (*see item 2* in Subheading 2.10.15) for 15–30 min at 20–25 °C.

3.8 Transmission Electron Microscopy (TEM)

Osmication is a procedure that makes the reaction product more electron-dense. After incubation in the reaction (e.g., azo-coupling and oxidative coupling polymerization procedures) and/or post-incubation mixture (e.g., metal salt methods), the thin sections, specimens and blocks must be postfixed in 1 % OsO₄ in 0.2 M cacodylate buffer, pH 7.4, for 30 min. Then, the thin sections are ready for treatment with uranyl acetate and lead citrate prior to examination under an electron microscope, whereas the specimens and blocks must be dehydrated and embedded in an epoxy resin prior to sectioning with an ultramicrotome.

For electron microscopy, the hemolymph is collected in a tube as previously described, the anticoagulant solution is removed by centrifugation at $780 \times g$ for 10 min, and the pellet is resuspended in an isosmotic solution. Pellets are obtained by a second centrifugation, and then they are processed similar to tissue blocks. Embed the pellets treated with assays for enzymatic activity in an epoxy resin and the untreated pellets in an acrylic resin.

4 Notes

1. Note that EDTA strongly interacts with Ca^{2+} and cytoskeletal proteins, thus changing the cell morphology, e.g., loss of pseudopodia.
2. Handle with care because picric acid could explode following accidental bumps. Moreover, all solutions must be produced and mixed under a fume cupboard.
3. This is an 8 % agar solution. For marine invertebrates, buffers or saline solutions must be used to prepare the agar solution instead of distilled water.
4. This solution is stable for months if it is stored at 4 °C.
5. This solution must be prepared immediately before use.
6. To fix the samples to determine the lipase activity, two changes of fixative are recommended after 30 min to remove the water. In some cases, it is possible to incubate the specimens for 15 h. To obtain the best results, an important step is to change the cold acetone frequently, particularly at the beginning of the fixation. It should be transparent and not opalescent.
7. It is not advisable to incubate the tissues or specimens in the decalcification solution for long periods because there are some indications of adverse effects on the staining reactions in tissues and the maintenance of the enzymatic activities.
8. If the agar block is too soft, it can be hardened with 5 % formaldehyde. If necessary, the agar can be stained with 0.5 % basic fuchsin in distilled water after fixation to make the small cubes more visible after embedding in epoxy resin or paraffin.
9. For mussels, the hemolymph can be directly collected by inserting a syringe between the valves, just beneath the exit-point of the byssus. This method is useful when a large amount of fluid is needed, but it is of extreme importance to check for the quality of the hemolymph immediately after withdrawal to ensure that neither the gonads nor the digestive gland have been touched or damaged by the needle [32].
10. Incubate this solution at 60 °C for several hours before use so that it will be at the recommended temperature at the time of use.

References

1. Lojda Z, Gossrau R, Schiebler TH (1979) Enzyme histochemistry: a laboratory manual. Springer, Berlin
2. Stoward PJ, Pearse AGE (eds) (1991) Histochemistry: theoretical and applied, 4th ed, vol. 3, enzyme histochemistry. Churchill-Livingstone, Edinburgh
3. Bancroft JD, Gamble M (2002) Theory and practice of histological techniques. Churchill Livingstone, London
4. Lentz TL, Barnett RJ (1961) Enzyme histochemistry of *Hydra*. J Exp Zool 147:125–149
5. Menzel LP, Bigger CH (2015) Identification of unstimulated constitutive immunocytes, by

- enzyme histochemistry, in the coenenchyme of the octocoral *Swiftia exserta*. Biol Bull 229:199–208
6. Matskási I, Hajdú É (1983) Studies on the lipase activity of parasitic platyhelminthes. Parasitol Hung 16:53–57
 7. Omar MS, Raoof AM, Alò Amari OM (1996) *Onchocerca fasciata*: enzyme histochemistry and tissue distribution of various dehydrogenases in the adult female worm. Parasitol Res 82:32–37
 8. Ashrafi SH, Fisk FW (1961) Histochemical localization of phosphatases in the stable fly, *Stomoxys calcitrans* (L.), using naphthol AS-phosphate. Ohio J Sci 61:7–13
 9. Weber G (1974) Glycol methacrylate embedding in enzyme histochemistry: application to arthropod tissue incubated for demonstration of unspecific esterase and succinic dehydrogenase activity. Histochemistry 39:155–161
 10. Stokes DR, Vitale AJ, Morgan CR (1979) Enzyme histochemistry of the mesocoel muscles of *Periplaneta americana*. Cell Tissue Res 198:175–189
 11. Jansen HH (1981) On the enzyme histochemistry and ultrastructure of the stomach and mid-gut gland in some species of mussels. Zool Anz 202:54–69
 12. Cima F, Matozzo V, Marin MG et al (2000) Haemocytes of the clam *Tapes philippinarum* (Adams & Reeve, 1850): morphofunctional characterisation. Fish Shellfish Immunol 10:677–693
 13. Sminia T (1972) Structure and function of blood and connective tissue cells of the freshwater pulmonate *Lymnaea stagnalis* studied by electron microscopy and enzyme histochemistry. Z Zellforsch Mikrosk Anat 130:497–526
 14. Michel C (1971) Histochemical study of pharyngeal epithelium of *Nephtys hombergii* (Polychaetes, Annelida). Ann Histochim 16:329–337
 15. Gelder SR (1984) Diet and histophysiology of the alimentary canal of *Lumbricillus lineatus* (Oligochaeta, Enchytraeidae). Hydrobiologia 115:71–81
 16. Matozzo V, Cima F, Perin L et al (2001) Phagocytic and enzymatic activities of cells and cell complexes in the coelomic fluid of the marine worm *Sipunculus nudus* (Sipuncula). It J Zool 68:273–280
 17. Van der Plas AJ, Voogt PA (1983) Histochemical observations on the pyloric caeca of *Asterias rubens* (Echinodermata, Asteroidea) in relation to the reproductive cycle. J Morphol 178:179–186
 18. Ballarin L, Cima F (2005) Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-functional characterisation. Eur J Histochem 49:255–264
 19. Cima F, Caicci F, Sordino P (2014) The haemocytes of the salp *Thalia democratica* (Tunicata, Thaliacea): an ultrastructural and histochemical study in the oozoid. Acta Zool 95:375–391
 20. Martínez A (1995) Nitric oxide synthase in invertebrates. Histochem J 27:770–776
 21. Mansueto V, Cangialosi MV, Augustine Arukweb A (2012) Acetylcholinesterase activity in juvenile *Ciona intestinalis* (Ascidiacea, Urochordata) after exposure to tributyltin. Caryologia 65:18–26
 22. Da Silva SF, Taffarel M, Allodi S (2001) Crustacean visual system: an investigation on glial cells and their relation to extracellular matrix. Biol Cell 93:293–299
 23. Cunningham L (1967) Histochemical observations of the enzymatic hydrolysis of gelatin films. J Histochem Cytochem 51:368–380
 24. Ross LG, Ross B (1999) Anaesthetic and sedative techniques for aquatic animals. Blackwell Science, Oxford
 25. Pennak RW (1953) Fresh-water invertebrates of the United States. The Ronald Press, New York, NY
 26. Berzins IK, Smolowitz RM, Lewbart GA (2012) Diagnostic techniques and sample handling. In: Lewbart GA (ed) Invertebrate medicine, 2nd edn. John Wiley & Sons, Chichester, pp 389–400
 27. Matozzo V, Marin MG (2010) First cytochemical study of haemocytes from the crab *Carcinus aestuarii* (Crustacea, Decapoda). Eur J Histochem 54:44–49
 28. Gunkel C, Lewbart GA (2012) Invertebrates. In: West G, Heard D, Caulkett N (eds) Zoo animals and wildlife immobilization and anesthesia. Blackwell Publishing, Oxford, pp 147–158
 29. Gorb SN (1997) Porous channels in the cuticle of the head-arrester system in dragon/damselflies (Insecta: Odonata). Microsc Res Tech 37:583–591
 30. Cima F (2010) Microscopy methods for morpho-functional characterisation of marine invertebrate haemocytes. In: Mendez-Vilas A, Alvarez JD (eds) Microscopy: science, technology, applications and education, microscopy book series, no. 4, vol 2. Formatex Research Center, Badajoz, pp 1100–1107, <http://www.formatex.org/microscopy4/index.html>
 31. MacMillan HA, Hughson BN (2014) A high-throughput method of hemolymph extraction from adult *Drosophila* without anesthesia. J Insect Physiol 63:27–31

32. Malagoli D, Casarini L, Sacchi S et al (2007) Stress and immune response in the mussel *Mytilus galloprovincialis*. Fish Shellfish Immunol 23:171–177
33. Barka T, Anderson PJ (1962) Histochemical methods for acid phosphatase using hexazonium pararosaniline as coupler. J Histochem Cytochem 10:741–753
34. Wachstein M, Meisel E (1957) Histochemistry of hepatic phosphatases at a physiological pH with special reference to the demonstration of bile canaliculi. Am J Clin Pathol 27:13–23
35. Nachlas MM, Monis B, Rosenblatt D et al (1960) Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, L-leucyl-4-methoxy-2-naphthylamide. J Biophys Biochem Cytol 7:261–264
36. Kurokawa M, Ogata K, Uchiyama F et al (1998) Reappraisal of the expression of mast cell proteases of Mongolian gerbils (*Meriones unguiculatus*). APMIS 106:727–735
37. Gomori G (1948) Histochemical demonstration of sites of choline esterase activity. Proc Soc Exp Biol Med 68:354–358
38. Lison L (1960) Histochimie et cytochimie animales. Principes et methodes. Gauthier-Villars, Paris
39. Hayashi M, Nakajima Y, Fishman WH (1964) The cytologic demonstration of β -glucuronidase employing naphthol AS-BI glucuronide and hexazonium pararosanilin; a preliminary report. J Histochem Cytochem 12:293–297
40. Hayashi M (1965) Histochemical demonstration of N-acetyl- β -glucosaminidase employing N-acetyl- β -glucosaminide as substrate. J Histochem Cytochem 13:355–360
41. Chayen J, Bytensky L, Butcher RG, Poulter LW (1969) A guide to practical histochemistry. Oliver and Boyd, Edinburgh, pp 129–132
42. Wachstein M, Meisel E (1956) On the histochemical demonstration of glucose-6-phosphatase. J Histochem Cytochem 4:592
43. Graham RC, Karnovsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302
44. Shnitka TK, Talibi GG (1971) Cytochemical localization by ferricyanide reduction of α -hydroxy acid oxidase activity in peroxisomes of rat kidney. Histochemie 27:137–158
45. Hose JE, Martin GG, Nguyen VA, Lucas J, Rosenstein AT (1987) Cytochemical features of shrimp hemocytes. Biol Bull 173: 178–187
46. Novikoff AB, Goldfischer S (1969) Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. J Histochem Cytochem 17:675–680

Part II

Lectin Histochemistry

Lectin Histochemistry: Historical Perspectives, State of the Art, and the Future

Susan A. Brooks

Abstract

Lectins, discovered more than 100 years ago and defined by their ability to selectively recognize specific carbohydrate structures, are ubiquitous in living organisms. Their precise functions are as yet underexplored and incompletely understood but they are clearly involved, through recognition of their binding partners, in a myriad of biological mechanisms involved in cell identity, adhesion, signaling, growth regulation, in health and disease. Understanding the complex “sugar code” represented by the glycome is a major challenge and at the forefront of current biological research. Lectins have been widely employed in histochemical studies to map glycosylation in cells and tissues. Here, a brief history of the discovery of lectins and early developments in their use is presented along with a selection of some of the most interesting and significant discoveries to emerge from use of lectin histochemistry. Further, an evaluation of the next generation of lectin-based technologies is presented, including the potential for designing recombinant lectins with more precisely defined binding characteristics, linking lectin-based studies with other technologies to answer fundamental questions in glycobiology, and approaches to exploring the interactions of lectins with their binding partners in more detail.

Key words Lectin histochemistry, History, Carbohydrates, Glycosylation, Review, Methodology, Glycomics

1 Historical Perspectives: What Are Lectins?

The term “lectin,” first suggested by Boyd and Shapleigh in 1954 [1], is derived from the Latin *legere* which means to select or to choose. It was originally applied to a group of proteins, mainly derived from plants, that had the property of being able to bind to specific carbohydrates and which agglutinated cells. Subsequently, lectins have been described from a diversity of biological sources as wide ranging as mammals, slime molds, fungi, invertebrates, and bacteria and are recognized as being ubiquitous in living organisms. They are very heterogeneous in structure, usually multivalent, and are distinct from immunoglobulins that recognize carbohydrate-based antigens or enzymes that have carbohydrates as their substrate [2].

The function of many lectins within the organism that produces them is mostly unknown but it is increasingly being recognized that lectin–carbohydrate binding interactions are functional in a diversity of biological processes involving cell–cell recognition, adhesion, and signaling and that this is an area of research that is under-explored and potentially extremely revealing.

2 Lectins: A Brief Historical Introduction

Lectins first aroused interest at the end of the nineteenth century and during the early years for the twentieth century and were first described during investigations into toxic plant extracts that were at that time used in medicine. During this time, the ability of “hemagglutinins,” as they were known initially, to selectively agglutinate cells and precipitate glycoconjugates and polysaccharides was established, as was the inhibition of these reactions by certain carbohydrates and carbohydrate-containing substances.

The lectin “story,” which is well reviewed by [3, 4], begins with Stillmark who in 1888 and 1889 purified a protein from a toxic extract of *Ricinus communis*, castor oil, seeds which he named “ricin.” He tested its effects upon various different cells and noted that sometimes he saw an agglutination reaction “like in clotting.” Meanwhile, descriptions of toxic extracts from other plants including *Abrus precatorius*, the jequirty bean (named “abrin”) and *Robinia pseudoacacia*, the black locust (named “robin”) that could also agglutinate cells emerged.

Over the twenty years following Stillmark’s original description of ricin, a great many studies were published describing dozens of new hemagglutinins from diverse sources. Owing to ease of availability, most were derived from plant sources and it became apparent that seeds of the *Leguminosae*, *Euphorbiaceae*, and *Solanaceae* were particularly rich sources. However, lectins were also described in fungi, invertebrates, and the venom of snakes. Over time, it became clear that lectins are ubiquitous in nature.

As described by [3, 4], Stillmark, in his original description in 1889, noted that the presence of serum effectively inhibited the agglutination of erythrocytes by ricin, and later workers also noted that various biological substances such as gastric mucus could sometimes inhibit or reverse agglutination. At the time, the significance of these observations was not realized, but they were the first indications of the carbohydrate-binding properties of lectins. Watkins and Morgan [5] were the first to show that simple sugars (monosaccharides) are able to inhibit lectin binding. While there were reports from the early years of the twentieth century that “hemagglutinins” did not always agglutinate erythrocytes from different human individuals to an equal extent, the ability of some lectins to distinguish between erythrocytes of different blood

groups was not realized until in 1949 Boyd and Reguera [6] reported that an extract from *Phaseolus limensis*, Lima beans, agglutinated human blood group A erythrocytes, but not blood group B or O cells. An enormous amount of interest in the search for blood group-specific lectins followed and the literature of the 1950s and 1960s reports the results of screening of hundreds of plant extracts, in particular, although extracts from other, often exotic, sources, for example, fish sera and snake venom, were also tested. By 1964, more than 100 blood group-specific lectins had been described. Morgan and Watkins [7] were first to demonstrate that the blood group specificity of lectins resulted from their carbohydrate-binding preferences. They showed that agglutination by the blood group A specific lectin from *Phaseolus limensis* could be inhibited by the presence of *N*-acetyl-D-galactosamine (GalNAc), whilst agglutination by *Lotus tetragonolobus* lectin, specific for blood group O erythrocytes, could be inhibited by alpha-methyl-L-fucose (Fuc). This is some of the first evidence of the presence of carbohydrates on the cell surface.

The first mammalian lectin to be described was the galactose (Gal)-binding liver lectin which is responsible for uptake and subsequent degradation of asialylated glycoproteins [8] and this was followed by the first description of a member of the galactoside-binding lectin family, the galectins [9]. Perhaps one of the best studied families of mammalian lectins is the selectins, whose role in lymphocyte migration and homing in immune surveillance is understood in some detail (*see* [10] for review). Moreover, lectin-carbohydrate recognition is a fundamental element of the mammalian innate immune response with, for example, a mannose (Man)-binding lectin functioning in pathogen recognition and initiating the complement cascade (reviewed by [11]). It is now well established that lectin-carbohydrate recognition is a fundamental signaling and recognition system in virtually all living things, including humans, and research into animal lectins is intense.

3 Lectins as Tools for Histochemistry: The Range of Lectins Available

As far as lectin histochemistry is concerned, today, purified lectins from a diversity of sources are available for use in research. Still, the majority are from plant sources, mostly derived from seeds (e.g., *Phaseolus vulgaris*, kidney bean; *Dolichos biflorus*, horsegram), but also bulbs (e.g., *Galanthus nivalis*, snowdrop), tubers (e.g., *Solanum tuberosum*, potato), fruit (e.g., *Lycopersicon esculentum*, tomato), and bark (e.g., *Sambucus nigra*, elderberry). However, lectins from fungi (e.g., *Aleuria aurantia*, mushroom), invertebrates (e.g., *Helix pomatia*, Roman snail; *Limulus polyphemus*, horseshoe crab), fishes (*Anguilla anguilla*, eel), snakes (e.g., *Naja mocambique mocambique*, Mozambique cobra), and marine algae (e.g., *Codium fragile*, green marine algae) also feature.

4 Lectin Binding Carbohydrate Specificity

The carbohydrate-binding specificity of a lectin is most usually expressed in terms of the simple monosaccharide, or sometimes disaccharide or trisaccharide, that has been demonstrated to most effectively inhibit its binding. For example, *Dolichos biflorus* lectin is said to be alpha-GalNAc-binding or alpha-GalNAc-specific; *Arachis hypogaea* (peanut) lectin is specific for beta-Gal(1 → 3)GalNAc, the T (or TF, Thompson-Friedenreich) antigen. However, it is important to appreciate that the lectin is usually recognizing a structure much larger and more complex than a single monosaccharide or disaccharide, and in three dimensions, taking into account specific glycosidic linkages and potentially hydrophobic and electrostatic interactions [12]. Thus, two lectins with apparently identical simple carbohydrate-binding specificity may actually recognize quite distinct profiles when used as tools in lectin histochemistry. A good example is Concanavalin A (Con A, the lectin from *Canavalia ensiformis*) and *Lens culinaris* lectins, both of which are generally defined as being Man- and Fuc-binding, and, indeed their binding can be effectively inhibited in the presence of free Man or Fuc. However, their binding patterns in histochemical studies are quite distinct [12]. We have reported quite distinct tissue-labeling patterns achieved using a panel of 26 different lectins all reported to be specific for GalNAc [13]. Furthermore, for the majority of commercially available lectins used in lectin histochemistry studies, the actual binding partner(s) present in tissues, or indeed their carbohydrate binding partners in the organisms from which they were isolated, is generally poorly defined. The complexity involved in understanding the detailed carbohydrate-binding properties of lectins is exemplified by research into the characterization of *Helix pomatia* lectin (HPA), which is of interest because lectin histochemistry using this lectin identifies cancers that have metastasized and therefore have a poor prognosis (see the Subheading 6). Extensive inhibition studies in the 1970s and 1980s first demonstrated that alpha-GalNAc is the best monosaccharide inhibitor of its binding but that its binding can also be inhibited to a lesser extent by alpha-N-acetylglucosamine (GlcNAc) and poorly also by alpha-Gal. Its binding preferences can be summarized as:

GalNAc-alpha(1 → 3)GalNAc > alpha-GalNAc > alpha-GlcNAc >> alpha Gal (reviewed by [14]).

However, even when their precise binding partners are not fully elucidated, lectins can still be effectively be used to define the distribution of very specific glycan structures in lectin histochemistry. For example, several lectins can be used to map the distribution of specific sialylated glycans. Sialic acid is a common terminal monosaccharide in glycan chains. *Limax flavus* lectin will recognize terminal sialic acid residues on N- and O-linked glycans, regardless of

linkage, and which are commonly attached to a subterminal Gal residue [15], and is therefore useful to map the distribution of sialylated glycans overall. *Maackia amurensis* lectin preferentially recognizes alpha 2,3 linked sialic acid. *Sambucus nigra* and *Polyporus squamosus* lectins both preferentially recognize alpha 2,6 linked sialic acid but *Polyporus squamosus* preferentially recognizes sialic acids terminating the chains of N-linked glycans, while *Sambucus nigra* lectin not only recognizes sialic acid terminating O-linked glycans chains too, but also has some preference for binding terminal GalNAc giving it a much broader binding specificity [16–18]. As yet more lectins are isolated and made available commercially, and as the fine carbohydrate-binding preferences of lectins are better defined, thus the capacity for lectin histochemistry to reveal specific aspects of glycosylation will increase.

5 The Development of Lectin Histochemistry

During the 1980s the technique of immunohistochemistry gained great popularity and was widely employed to study the distribution of numerous markers in normal and diseased tissues. Immunohistochemistry uses antibodies as reagents to detect the cell or tissue localization of a specific antigen through identification by a label that can be identified using microscopy. It originated in the 1940s and 1950s when Coons and colleagues [19–21] employed directly fluorescently labeled antibodies to localize antigens in tissue sections using microscopy. Significant developments then emerged, including the use of a range of fluorescent, enzyme-based, radioactive and electron-dense labels and various direct and indirect detection systems to enhance specificity and sensitivity (see [22] for review). It was soon realized that the basic concept underlying immunohistochemistry, that of detecting the binding of an antibody directed against a target antigen, could be applied to the analogous situation of detecting a lectin binding to carbohydrate structures using lectin histochemistry. With some technical modifications, approximately similar methodologies apply and technical aspects have been recently reviewed for both light and electron microscopy [12]. Significantly, the development of lectin histochemistry opened the way for far more specific, detailed and revealing mapping of carbohydrate structures in tissues than was previously possible using traditional histochemical techniques such as periodic acid Schiff or Alcian blue staining.

While conditions of fixation and tissue processing for lectin histochemistry of cells and animal tissues may be specially adapted to optimize labeling results, lectin histochemistry works well on routinely formalin-fixed and paraffin wax embedded specimens, such as those produced in routine cellular pathology hospital laboratories [12]. This means that clinical studies associating

carbohydrate-profiling of tissues with disease states or with disease progression can be very informative. Moreover, archival paraffin wax embedded tissues retain their carbohydrate profiles, facilitating powerful retrospective studies with long patient follow up. Examples of such studies are featured in the next section.

6 Glycosylation Changes Associated with Progression of Malignancy Revealed by Lectin Histochemistry

One area of lectin histochemical research that has proved especially fruitful has been the exploration of changes in cellular glycosylation associated with transformation of normal cells to malignant, and changes associated with cancer progression. Interest began with early cell agglutination studies. Aub et al. [23] were amongst the first to investigate the idea that one of the differences between normal and malignant cells was related to aspects of the cell surface. They incubated malignant and normal cells with a range of enzyme preparations and noted that a crude preparation of lipase from wheat germ agglutinated malignant cells and left normal cells unaffected. Later work proved that it was not the lipase that was responsible for the selective cell clumping, but was the action of a lectin, now referred to as wheat germ agglutinin (WGA), and much interest in WGA as a marker of malignancy resulted. Subsequent studies, such as [24, 25] also reported that other lectins, such as Con A and soyabean agglutinin (*Glycine max*) also could preferentially bind to, and agglutinate, cancer cells (e.g., see [26] for review).

With increasing popularity of lectin histochemistry, and, as indicated in the Subheading 5, its effective application to routinely fixed and processed archival clinical specimens, many studies in the 1980s and 1990s, in particular, investigated the ability of lectins to distinguish normal from malignant cells, or stratify tumors into different histological types or with different clinical outcomes. As a result, it is now well established that fundamental changes in cellular glycosylation are associated with cancer, and that some hold prognostic and functional significance. Some commonly described glycosylation changes associated with cancer are reviewed in [27]. Of special note, as far as lectin histochemistry is concerned, are truncation of O-linked glycans and an increase in beta-1,6 branching of N-glycans (fuller descriptions of glycosylation mechanisms underlying these changes are given in [27–29]).

Failure in normal carbohydrate chain extension results in truncation of glycan chains, and this has emerged as a common feature of many cancer types. Mucin type O-linked glycosylation of proteins begins with the attachment of a GalNAc monosaccharide to a serine or threonine residue of the polypeptide. The resulting structure, GalNAc- α -O-Ser/Thr, referred to as Tn antigen, is then always extended in normal adult human cells to form a range of basic core structures, or is capped by sialic acid to yield sialyl-Tn

which terminates chain extension. Interestingly, Tn, which is detected by binding of the lectin HPA (the lectin has a nominal binding preference for GalNAc and will recognize glycans with terminal GalNAc including, but not exclusively, Tn [30] and Subheading 4) and *Vicia villosa* isolectin B4, is frequently unelaborated in cancers and cancers exhibiting high levels of Tn antigen are often more aggressive. For example, there have been numerous studies showing that HPA binding is a powerful marker of poor prognosis in many cancer types, most notably breast and colorectal cancer (for review see [27, 31]). As Tn is normally cryptic, its exposure on cancer cells results in an immune response in cancer patients and it has been explored as a potential target for immunotherapy [32, 33]. T antigen, formed by the attachment of a beta 1,3 linked Gal to Tn to yield beta-Gal(1→3)GalNAc and detected by binding of peanut lectin, has also been described to be associated with poor prognosis and aggressive biological behavior in colorectal and other cancers (e.g., see [34] for review).

Beta 1,6 branched N-linked glycans can be detected using histochemistry for the binding of *Phaseolus vulgaris* leucoagglutinin (PHA-L) and an increase in this feature is associated with aggressive biological behavior and poor prognosis in cancers such as breast and colorectal cancer [35, 36]. The reason for the glycosylation change is increased activity of the glycosyltransferase that underlies the synthesis of these structures (GlcNAc transferase V [37]). The association between the transferase activity, the synthesis of beta 1,6 branched N-linked glycans and metastatic potential is confirmed in animal models of metastasis (reviewed in [27]) and the mechanisms by which this change influences cancer behavior have been elucidated: the altered glycosylation of matriptase, an enzyme that is key to activating a cascade of protease activity involved in cancer cell invasion, renders it more resistant to degradation, thereby upregulating the entire cascade. Swainsonine, an inhibitor of one of the early stages in the synthesis of N-linked glycosylation, has been tested in clinical trials as a potential antitumor therapy, although it was not well tolerated by patients [38].

Thus, as exemplified by detection of beta 1,6 branching N-linked glycans by PHA-L and detection of the normally cryptic Tn antigen by HPA, described previously, lectin histochemistry to detect alterations in glycosylation may have potential in identifying further novel targets for innovative treatment approaches.

7 Mapping Glycosylation Changes in Normal Human and Animal Tissues

The facility of lectin histochemistry to reveal glycosylation profiles of cells and tissues has resulted in a substantial literature of papers describing histochemical studies of various normal animal and human tissues. Some describe lectin binding characteristics of a particular tissue or tissues in selected species and compare results

to what has been described for the same tissues in other species. Others describe changes in lectin binding with embryogenesis or differentiation. A profile of such studies from the 1990s when these types of studies were most prolific, for example, is summarized in [39]. The majority of studies have been simple descriptive ones and such investigations have generally not been carried out in any systematic way or in response to a well-defined research question. Thus, there remains much that lectin histochemistry can reveal regarding the glycosylation profiles of normal healthy tissues and, as described in the Subheading 8, there is currently much interest in exploring the glycomes (the profile of glycoconjugates synthesized by cells or tissues) of species, including humans, in development, health, and disease.

8 Lectins to Decipher the Complexities of the “Sugar Code”

The chemistry and biology of glycosylation are topics that lie outside the scope of this chapter and are well described by [28, 29]. The chemistry of carbohydrates is such that the “sugar code,” as it is increasingly named, has extraordinary capacity for building glycan structures with a degree of diversity several orders of magnitude higher than that achieved by proteins or nucleic acids (see [40, 41] for review). Glycoconjugates, carrying this rich code, are positioned on eukaryotic cell surfaces and are ideally placed to function in diverse biological mechanisms in all living things including cell identity, recognition, signaling, growth regulation, adhesion, and in development, health, and disease, and their significance is only just beginning to be appreciated and explored. This is a topic that is at the forefront of biology, but which has lagged behind exploration of proteins and nucleic acids because the tools to enable exploration are only just really becoming available. In many ways, lectin histochemistry, whilst a long-established and commonly employed technique to map glycosylation in cells and tissues, has yet to reveal its full potential because the carbohydrate binding partners recognized by lectins are often not fully characterized and their biological functions are still poorly understood. However, exciting developments indicate that this may soon change and there is increasing interest in exploring the glycome and its functional significance.

9 The Next Generation of Lectin-Based Technologies: A Wider Range of Tools Become Available

As indicated in the Subheading 3, a wide selection of purified lectins, supplied in their native form and conjugated to fluorescent labels such as FITC and TRITC, enzyme labels such as peroxidase

and alkaline phosphatase, biotin and electron dense labels such as gold for lectin histochemistry using a range of detection systems, are commercially available. While, as indicated in the Subheading 4, the precise binding partners of many remain incompletely defined, still progress is being made to elucidate their properties in more detail. Moreover, advances in molecular biology mean that the concept of engineering lectins with clearly defined, altered or improved carbohydrate-binding specificity is becoming an exciting reality, potentially circumventing the problems of batch to batch variation in the activity and properties, weak binding affinity, and the often broad and incompletely defined binding repertoire of the naturally occurring product. Moreover, potentially, lectins can be engineered that bind to less common glycan structures where no naturally occurring lectin partner is known. This topic is reviewed by [42]. One good example is that of HPA which, as described in the Subheading 6, can be used to identify cancers that have metastasized. Markiv et al. [43] describe the various studies from the 1960s onwards aimed at solving the structure and elucidating the binding site of the lectin. They finally isolated two closely related and GalNAc-binding isolectins from *Helix pomatia* and expressed recombinant forms of the lectins that had a slightly higher affinity for GalNAc than the native lectins. They propose that the recombinant form, being more homogeneous and well characterized, might provide a more robust tool for cancer prognostication than the naturally variable native product.

Advances too have been made in development of technology to enable analysis of complex carbohydrates, a field that has been hampered for many years by the technical challenges imposed by the unique chemistry of this group of molecules, although detailed analysis of these complex structures remains a specialist and complex field (see [42, 44] for review). There have been great advances in development of high throughput screening technologies, exemplified by lectin and carbohydrate microarrays that facilitate the rapid screening of multiple glycan–lectin interactions. For example, for profiling global changes in mammalian [45, 46] and bacterial [47] cell surface glycomes.

10 Lectin Histochemistry Complements Other Methods of Exploration

While the literature continues to reveal that simple lectin histochemical mapping studies of tissues remain popular, increasingly studies are being designed where lectin histochemistry is just one of a range of techniques employed to address a specific and carefully formulated research question regarding the functionality of the glycome. A good example of this is the investigation of the functional significance of altered O-linked glycosylation in cancer, described in the Subheading 6. Simple lectin histochemical studies in the late

1980s and 1990s first established that increased binding of HPA preferentially recognizes glycans synthesized by cancers that are more biologically aggressive, and it is therefore a useful prognostic marker (see [27, 30] for review). However, the precise identity of the binding partner(s) recognized by HPA in cancers has still not been completely established, nor has the functional significance of their glycosylation. Initially, lectin histochemistry was combined with lectin affinity chromatography to isolate HPA-binding glycoproteins isolated from cancer and normal cells and tissues and characterization was attempted using SDS-PAGE and western blotting [30, 48]. This revealed that the lectin was recognizing a heterogeneous range of proteins bearing GalNAc-moieties, including, but not exclusively the Tn antigen. Studies have also been conducted where the HPA-binding glycans have been cleaved from HPA lectin affinity-purified glycoproteins and analyzed using a range of chromatography approaches [49]. Most recently, Peiris et al. [50] employed an elegant glycoproteomic approach to identify specific HPA-binding glycoproteins that were increased in colorectal cancer specimens. Here, studying human cancer samples where clinico-pathological features of the patients were known, a combination of traditional lectin histochemistry was combined with HPA affinity chromatography to isolate proteins of interest, then their analysis by SDS-PAGE and western blotting, two dimensional electrophoresis (2-DE) and identification by matrix assisted laser desorption ionization mass spectroscopy. Moreover, prediction of potential glycosylation sites on the proteins was performed using an *in silico* bioinformatics approach, and correlations were also made with the status of cancer-related genes p53, using immunohistochemistry, and KRAS, using polymerase chain reaction (PCR). Such studies illustrate the potential for using lectin histochemistry, alongside other techniques, to answer a defined question related to glycosylation and (in this example, where annexin 4, annexin 5 and calcium activated chloride channel protein 1 (CLCA1) were highlighted as being of particular interest), to more clearly define biomarkers as novel targets for disease treatment or to more fully understand disease processes at a molecular level.

11 Animal and Human Lectins

A topic that is of great interest and is yet very incompletely explored is that of endogenous animal/human lectins. This area of research appears to be expanding faster than any other type of lectin study. Traditional lectin histochemical approaches elegantly reveal the distribution of specific glycoconjugates on the surface of cells or within tissues and changes with development and in health and disease. Such lectin histochemistry commonly employs lectins derived from plants and invertebrates, but their selective binding

to carbohydrate partners in animal or human tissues poses the question of what endogenous binding receptors (lectins) they are designed to complement, and what cellular responses are elicited *in vivo* by their interaction with such partners. One approach to identifying and isolating such endogenous tissue lectins is through the application of neoglycoproteins or neoglycoconjugates. Briefly, specific glycans or glycoconjugates are linked to a protein or synthetic polymer scaffold and their binding to cell- or tissue-bound lectins detected through techniques analogous to lectin- or immunohistochemistry, termed glycohistochemistry (see [41] for review). For example, Gabius et al. [51] synthesized a neoglycoprotein based on the T antigen (see the Subheading 6) and labeled cancer cell lines and tissue sections of breast cancer to seek endogenous complementary lectin partners. Neoglycoproteins/neoglycoconjugates can also be employed to affinity purify their tissue lectin partners for further functional analysis to begin to explore in more depth their role in glycan-based cellular communication. There is clearly potential also, once endogenous lectins are sequenced, to produce recombinant human lectins which themselves could be employed in more specific, selective and targeted lectin histochemical studies. A good example of this is described by Habermann et al. [52] who labeled *zona pellucida* of bovine eggs for binding of recombinant galectins.

12 Exploring Lectin Interactions with Their Carbohydrate Binding Partners in More Depth

Since carbohydrate recognition by lectins plays a role in such a diversity of biological processes, there is interest to more fully understand the nature of their binding interaction. The chemistry of carbohydrates (reviewed by [28, 29]), while it underlies the extraordinary potential for diversity within the glycome, also features aspects that make understanding their specific recognition sites challenging. Monosaccharides, the basic building blocks of carbohydrate chains, are often chemically very similar to each other – for example, both Gal and Man are epimers of glucose (Glc); Fuc is the deoxy-form of Gal. Moreover, the complexity of the structures built from them depends not only on the sequence of the monosaccharide building blocks, but differences in the glycosidic linkages between them, in terms of their anomeric character (ie alpha or beta linkages) and the carbon atoms involved in the linkage (1 → 3, 1 → 6 etc). As reviewed by [53], this makes isolation of a pure sample of a particular carbohydrate/glycoconjugate for study technically difficult and lectin interaction with a potential carbohydrate binding partner can thus only be examined in the presence of heterogeneous, similar, but subtly different glycoforms. The study of such interactions is further complicated

because lectin–carbohydrate binding interactions are weak, but lectins are usually multivalent, thus enhancing binding through cooperation where binding characteristics at one site are influenced by the binding status of others. One approach to overcoming these technical difficulties, as described by Johnson et al. [53], is through computer simulation. Alternatively, lectin–carbohydrate binding interactions have been studied using libraries of carbohydrate structures immobilized as microarrays (for example, [54, 55]) or, alternatively, lectin microarrays (for example, [56]). Highly sophisticated approaches have been developed to study single molecule lectin–carbohydrate interactions. For example, Zhang and Yadavalli [57] described how a carbohydrate or lectin array could be fabricated based on a self-assembling monolayer and lectin–carbohydrate interactions interrogated at the single molecule level using atomic force microscopy. They imaged and measured interactions between the lectin Con A and mannosyl groups; specificity of binding was confirmed by inhibition of binding by free Man, and investigations at different pH values facilitated exploration of multivalent interactions. Atomic force microscopy has been employed in many studies to investigate carbohydrate–lectin interactions. For example, by Yan et al. [58] to study interactions of glycophorin A, immobilized on a mica surface or presented in the erythrocyte membrane, with *Psathyrella velutina* lectin and by Gour and Verma [59] to study interaction of Con A with carbohydrate microarrays. Investigations into the functions of lectin–glycan binding interactions in vivo are being facilitated too by the development of lectin-deficient animals. For example, Stubke et al. [60] studied metastasis in E- and P-selectin deficient mice and demonstrated that spontaneous metastasis to bone marrow and lungs was reduced, but not completely abrogated, in their absence, indicating that while they do play a role in metastatic mechanisms, additional cell adhesion molecules are also involved.

13 Conclusion

Lectins first elicited the interest of researchers more than 100 years ago owing to their curious ability to agglutinate living cells. When it became apparent that they could do so through their selective recognition of cell-surface carbohydrates, and furthermore that different types of cells exhibited different patterns of glycosylation and, thus, different lectin-binding profiles, they became, and remain, the tool of choice for exploring the glycome. Lectin histochemistry has been instrumental in mapping cellular glycosylation in different species, different cell types and in normal and disease processes. In recent years, the remarkable potential of the “sugar code,” its complexity, and its as yet incompletely explored functional importance in all aspects of biology has given new impetus

to the development of tools to decipher it. Traditional lectin histochemistry still has a place, alongside developing technologies, in revealing fundamental aspects of glycosylation, while new developments hold promise of a new generation of lectin-based research approaches.

References

1. Boyd WC, Shapleigh E (1954) Separation of individuals of any blood group into secretors and non-secretors by use of a plant agglutinin (lectin). *Blood* 9:1195–1198
2. Goldstein IJ, Hughes RC, Monsigny M et al (1980) What should be called a lectin? *Nature* 285:66
3. Kocourek J (1986) Historical background. In: Liener IR, Sharon N, Goldstein IJ (eds) *The lectins: properties, functions and applications in biology and medicine*. Academic Press, London, pp 3–33
4. Sharon N, Lis H (2004) History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology* 14:53R–63R
5. Watkins WM, Morgan WT (1952) Neutralization of the anti-H agglutinin in eel serum by simple sugars. *Nature* 169:825–826
6. Boyd WC, Reguera RM (1949) Haemagglutinating substances for human cells in various plants. *J Immunol* 62:333–339
7. Morgan WTJ, Watkins WM (1959) The inhibition of the haemagglutinins in plant seeds by human blood group substances and simple sugars. *Br J Exp Pathol* 34:94–103
8. Hudgin RL, Pricer WE Jr, Ashwell G et al (1974) The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. *J Biol Chem* 249:5536–5543
9. Teichberg VI, Silman I, Beitsch DD et al (1975) A α -D-galactoside binding protein in the electric organ tissue of *Electrophorus electricus*. *Proc Natl Acad Sci USA* 72:1383–1387
10. Ley K (2003) The role of selectins in inflammation and disease. *Trends Mol Med* 9:263–268
11. Dommert RM, Klein N, Turner MW (2006) Mannose-binding lectin in innate immunity: past, present and future. *Tissue Antigens* 68:193–209
12. Roth J (2011) Lectins for histochemical demonstration of glycans. *Histochem Cell Biol* 136:117–130
13. Khan S, Brooks SA, Leathem AJC (1994) GalNAc-type glycoproteins in breast cancer – a 26 lectin study. *J Pathol* 172(Suppl):134A
14. Goldstein IJ, Poretz RD (1986) Chapter 2: isolation, physiochemical characterization, and carbohydrate-binding specificity of lectins. In: Liener IR, Sharon N, Goldstein IJ (eds) *The lectins: properties, functions and applications in biology and medicine*. Academic Press, London, pp 35–250
15. Miller R, Collowan J, Fish W (1982) Purification and molecular properties of a sialic acid-specific lectin from the slug *Limax flavus*. *J Biol Chem* 257:7574–7580
16. Mo HQ, Winter HC, Goldstein IJ (2000) Purification and characterisation of a Neu5Ac α 2-6Gal β 1-4Glc/GalNAc-specific lectin from the fruiting body of the polypore mushroom *Polyporus squamosus*. *J Biol Chem* 275:10623–10629
17. Wang WC, Cummings RD (1988) The immobilised leucoagglutinin from the seeds of *Maackia amurensis* binds with high affinity to complex type asn-linked oligosaccharides containing terminal sialic acid α 2,3 linked to penultimate galactose residues. *J Biol Chem* 263:4576–4585
18. Taatjes DJ, Roth J, Peumans W et al (1988) Elderberry bark lectin-gold techniques for the detection of NeuAc (α 2,6) Gal/GalNAc sequences: applications and limitations. *Histochem J* 20:478–490
19. Coons AH, Creech HJ, Jones RN (1941) Immunological properties of an antibody containing a fluorescent group. *Proc Soc Exp Biol Med* 47:200–202
20. Coons AH, Leduc EH, Connolly JM (1955) Studies on antibody production. I: a method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J Exp Med* 102:49–60
21. Coons AH, Kaplan MH (1950) Localisation of antigen in tissue cells. *J Exp Med* 91:1–13
22. Polak JM, Van Noorden S (1997) Introduction to immunocytochemistry, 2nd edn, Chapter 1 introduction. Royal Microscopical Society Handbooks number 37. Bios Scientific Publishers Ltd, Oxford, pp 1–4
23. Aub JC, Tieslau C, Lankester A (1963) Reactions of normal and tumour cell surfaces to enzymes. I wheat-germ lipase and associated mucopolysaccharides. *Proc Natl Acad Sci USA* 50:613–619

24. Inbar M, Sachs L (1969) Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. *Proc Natl Acad Sci USA* 63:1418–1425
25. Sela BA, Lis H, Sharon N et al (1970) Different locations of carbohydrate-containing sites at the surface membrane of normal and transformed mammalian cells. *J Membr Biol* 3:267–279
26. Sharon N (1977) Lectins. *Sci Am* 236:108–119
27. Brooks SA, Carter TM, Royle L et al (2008) Altered glycosylation of proteins in cancer: what is the potential for new anti-tumour strategies? *Anticancer Agents Med Chem* 8:2–21
28. Varki A, Cummings R, Esko J et al (1999) *Essentials of glycobiology*. Cold Spring Harbour Laboratory Press, New York
29. Brooks SA, Dwek MV, Schumacher U (2002) *Functional and molecular glycobiology*. Bios Scientific Publishers Ltd, Oxford
30. Brooks SA, Leathem AJC (1995) Expression of GalNAc glycoproteins by breast cancers. *BJC* 71:1033–1038
31. Brooks SA (2000) The involvement of *Helix pomatia* lectin (HPA) binding N-acetylgalactosamine glycans in cancer progression. *Histol Histopathol* 15:143–158
32. Springer GF, Desai PR, Tegtmeier H et al (1994) T/Tn antigen vaccine is effective and safe in preventing recurrence of advanced human breast carcinoma. *Cancer Biother* 9:5–15
33. Lo-Man R, Vichier-Guerre S, Bay S et al (2001) Anti-tumor immunity provided by a synthetic multiple antigenic glycopeptide displaying tri-Tn glycotope. *J Immunol* 166:2849–2854
34. Hanisch FG, Baldus SE (1997) The Thompson-Friedenreich (TF) antigen: A critical review on the structural, biosynthetic and histochemical aspects of a pancarcinoma-associated antigen. *Histol Histopathol* 12:263–281
35. Fernandes B, Sagman U, Auger M et al (1991) Beta 1–6 branched oligosaccharides as a marker of tumour progression in human breast and colon neoplasia. *Cancer Res* 51:718–723
36. Dennis JW, Laferte S, Waghorne C et al (1987) Beta 1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science* 236:582–585
37. Yousefi S, Higgins E, Daoling Z et al (1991) Increased UDP-GlcNAc:Gal beta 1–3 GalNAc-R (GlcNAc to GalNAc) beta 1–6 N-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. Control of polyactosamine synthesis. *J Biol Chem* 266:1772–1782
38. Goss PE, Cl R, Bailey D et al (1997) Phase I clinical trial of the oligosaccharide processing inhibitor swainsonine in patients with advanced malignancies. *Clin Cancer Res* 3:1077–1086
39. Brooks SA, Leathem AJC, Schumacher U (1997) *Lectin histochemistry, a concise practical handbook*. Royal Microscopical Society handbook series number 36. Bios Scientific Publishers Ltd, Oxford
40. Laine RA (1997) The information-storing potential of the sugar code. In: Gabius H-J, Gabius S (eds) *Glycosciences: status and perspectives*. Chapman and Hall, London, pp 1–4
41. Gabius H-J, Kayser K (2014) *Introduction to glycopathology: the concept, the tools and the perspectives*. *Diagn Pathol* 9:4
42. Hu D, Tateno H, Hirabayashi J (2015) Lectin engineering, a molecular evolutionary approach to expanding lectin utilities. *Molecules* 20:7637–7656
43. Markiv A, Peiris D, Curley GP et al (2001) Identification, cloning, and characterization of two N-acetylgalactosamine-binding lectins from the albumen gland of *Helix pomatia*. *J Biol Chem* 286:20260–20266
44. Adamczyk B, Tharmalingam T, Rudd PM (2012) Glycans as cancer biomarkers. *Biochim Biophys Acta* 1820:1347–1353
45. Tateno H, Uchiyama N, Kuno A et al (2007) A novel strategy for mammalian cell surface glycome profiling using lectin microarray. *Glycobiology* 17:1138–1146
46. Pilobello K, Slawek DE, Mahal LK (2007) A ratiometric lectin microarray approach to analysis of the dynamic mammalian glycome. *Proc Natl Acad Sci USA* 104:11534–11539
47. Hsu K-L, Pilobello KT, Mahal LK (2007) Analysing the dynamic bacterial glycome with a lectin microarray approach. *Nat Chem Biol* 2:153–157
48. Schumacher U, Adam E, Brooks SA et al (1995) Lectin binding properties of human breast cancer cell lines and human milk with particular reference to *Helix pomatia* agglutinin. *J Histochem Cytochem* 43:275–281
49. Dwek MV, Lacey HA, Streets AJ et al (2001) *Helix pomatia* agglutinin lectin-binding oligosaccharides of aggressive breast cancer. *Int J Cancer* 95:79–85
50. Peiris D, Ossondo M, Fry S et al (2015) Identification of O-linked glycoproteins binding to the lectin *Helix pomatia* agglutinin as markers of metastatic colorectal cancer. *PLoS One* 10(10), e0138345. doi:[10.1371/journal.pone.0138345](https://doi.org/10.1371/journal.pone.0138345)

51. Gabius H-J, Schroter C, Gabius S et al (1990) Binding of T-antigen bearing neoglycoprotein and peanut agglutinin to cultured tumour cells and breast carcinomas. *J Histochem Cytochem* 38:1625–1631
52. Habermann FA, Andre S, Kaltner H et al (2011) Galectins as tools for glycan mapping in histology: comparison of their binding profiles to bovine *zona pellucida* by confocal scanning electron microscopy. *Histochem Cell Biol* 135:539–552
53. Johnson QR, Lindsay RJ, Petridis L et al (2015) Investigation of carbohydrate recognition via computer simulation. *Molecules* 20:7700–7718
54. Park S, Lee MR, Pyo SJ et al (2004) Carbohydrate chips for studying high-throughput carbohydrate-protein interactions. *J Am Chem Soc* 126:4812–4819
55. Fukui S, Feizi T, Galustian C et al (2002) Oligosaccharide microarrays for high throughput detection and specificity assignments of carbohydrate-protein interactions. *Nat Biotechnol* 20:1011–1107
56. Jelinek R, Kolusheva S (2004) Carbohydrate biosensors. *Chem Rev* 104:5987–6015
57. Zhang X, Yadavalli VK (2009) Functionalized self-assembled monolayers for measuring single molecule lectin carbohydrate interactions. *Anal Chim Acta* 649:1–7
58. Yan C, Yersin A, Afrin R et al (2009) Single molecular dynamic interactions between glycophorin A and lectin as probed by atomic force microscopy. *Biophys Chem* 144:72–77
59. Gour N, Verma S (2008) Synthesis and AFM studies of lectin-carbohydrate self-assemblies. *Tetrahedron* 64:7331–7337
60. Stubke K, Wicklein D, Herich L et al (2012) Selectin-deficiency reduces the number of spontaneous metastases in a xenograft model of human breast cancer. *Cancer Lett* 321:89–99

Isolation of Viable Glycosylation-Specific Cell Populations for Further In Vitro or In Vivo Analysis Using Lectin-Coated Magnetic Beads

Ellie-May Beaman, David R.F. Carter, and Susan A. Brooks

Abstract

The glycans displayed on the cell surface are highly heterogeneous and their function in cell recognition, identity, signaling, adhesion, and behavior is increasingly recognized. Moreover, as it is yet incompletely understood, it is a topic of significant current interest. Lectins (naturally occurring carbohydrate-binding proteins) are very useful tools for exploring cellular glycosylation. Cell populations, within or between different tissues or species, and in development, health and disease, exhibit different glycosylation and thus distinct lectin-binding characteristics. Even monoclonal cell populations of established cell lines feature subpopulations with strikingly different glycosylation characteristics, and these differences may reflect differences in behavior or function. By separating cell populations on the basis of their cell surface glycosylation, the functional significance of glycosylation can be investigated in in vitro or in vivo models. Also, factors affecting glycosylation, which are also incompletely understood, can be explored or manipulated. In the protocol given here, cells can be separated into subpopulations on the basis of their recognition by a specific biotinylated lectin of choice immobilized on avidin-coated magnetic beads. Importantly, the protocol has been optimized such that lectin-binding and non-binding cells remain viable such that they can be further cultured, if necessary, for subsequent investigations.

Key words Magnetic beads, Cell separation, Glycosylation, Lectin binding

1 Introduction

1.1 Lectins Are Versatile Tools to Identify Cells That Have Distinct Glycosylation Profiles

The ability of lectins to recognise cell surface glycoconjugates is a useful tool to investigate the glycosylation of tissues and cells [1]. Lectin labeling often reveals surprising heterogeneity in the glycome, that is, the array of glycosylated structures, of cells—even those derived from the same tissue or cell culture. Early cell agglutination studies, for example, established that some lectins were able to distinguish between erythrocytes taken from individuals of different blood groups—both the familiar ABO blood groups and also other, less commonly used blood grouping systems—owing to the blood group substances being carbohydrates [2, 3]. As early as

the 1960s, studies were being undertaken to attempt to distinguish between normal and malignant cells on the basis of their cross-linking and agglutination by lectins that recognized cancer-related cell surface glycoconjugates [4–6]. In more recent years, lectin histochemistry has become a widely used, versatile and convenient approach to map glycosylation patterns in cells within different tissue types, between species, or in disease states such as cancer.

A single population of cells, even a monoclonal population such as those of an established cell line, usually contains subpopulations of cells with different glycosylation profiles and they may exhibit different behaviors or properties. For example, we have previously reported subpopulations of cells exhibiting different levels of synthesis of glycans terminating in N-acetylgalactosamine (GalNAc) in established breast cancer cell lines, revealed through lectin histochemistry using the lectin from *Helix pomatia* (HPA) [7], and this is also illustrated in Fig. 1. Here, different cell lines exhibited different typical profiles of glycosylation which were stable over repeated passages and reflected the derivation and phenotype of the cell line, being consistent with clinical studies on human tumours that had established that HPA recognises glycans associated with metastatic potential [8]. Schumacher et al. [9] reported the lectin binding profiles of seven different breast cancer cell lines labeled for binding of 15 lectins with wide ranging carbohydrate-specificity and reported marked heterogeneity in glycosylation both within, and between, cell lines. This situation is comparable to that seen in human tumours, where marked subpopulations of cancer cells with noticeably different glycosylation profiles, revealed by lectin histochemistry, are often seen; and the glycosylation of the tumour cells is distinct from that of the adjacent healthy tissue ([7, 10]; reviewed in [8, 9]). In normal human and animal tissues, lectin histochemistry reveals very marked differences in glycosylation between different cell populations, often reflecting differences in differentiation, position or function.

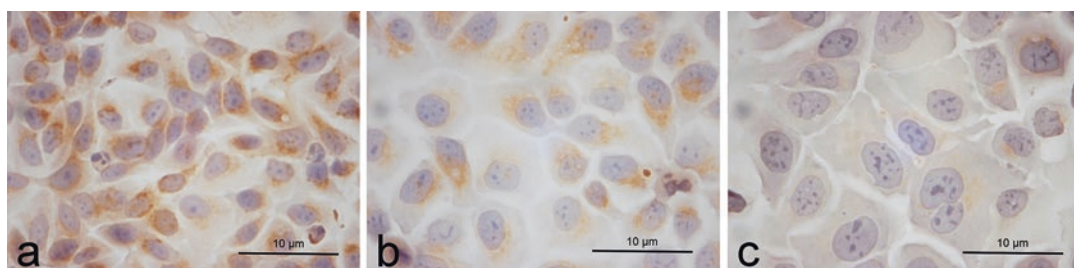


Fig. 1 Even within the monoclonal populations of cells of an established cell line, populations featuring markedly different glycosylation, revealed by lectin labeling, are seen. Here, breast cancer cell lines are labeled for the binding of *Helix pomatia* agglutinin (HPA), which preferentially recognizes glycans terminating in N-acetylgalactosamine. While, overall, (a) MCF7 cells are strongly HPA-binding, (b) ZR 75 1 show intermediate levels of HPA-binding, and (c) BT 474 are weakly HPA-binding, within each cell line, there is clear heterogeneity within the cell population with some cells labeling much more strikingly than others

1.2 The Regulation and Functional Significance of Cellular Glycosylation Is Poorly Understood

The mechanisms that control cellular glycosylation and underlie the regulation of the cellular glycome, are not well understood. Glycosylation is not under direct genetic control, but is instead regulated by changes in expression, activity and location of glycosyltransferases within the secretory pathway, availability of sugar donors and transporters, as well as many other, complex factors; for example, some factors regulating O-glycosylation have recently been reviewed by [11]. Thus, the glycome of a cell or population of cells is dynamic and can subtly change in response to changes in environment. The functional significance of glycosylation is also under-explored and mostly poorly understood. However, the potential of the glycome to encode a wealth of information that is fundamental to cell recognition, identity, signaling, adhesion, and behavior, that changes in development, health and disease, is becoming increasingly apparent, as described in [1], and this is a topic that is at the forefront of cell biology research. Thus, a technique to separate populations of cells based on their glycosylation for potential further functional or regulatory studies, as presented here, can be very valuable.

By separating cells from a parental population, for example, a cultured cell line, on the basis of their lectin-binding profile it is possible to begin to investigate the functional significance of glycosylation and its regulation. This can be approached by examining the properties of glycosylation-distinct subpopulations of cells in in vitro assays of cell behavior or in vivo in animal models, or by manipulating culture conditions or cell environment to assess the effect that this has on the glycome.

1.3 A Method for Separating Viable Cells on the Basis of Their Glycosylation Profiles

The method described in this chapter, if performed under sterile conditions, can be used to separate cells into different populations based on their glycosylation profiles which can then be subcultured for further assays or analysis. Maintaining sterility is, however, not necessary if cells are to be used for immediate analysis post-separation, for example, genetic profiling.

The technique, illustrated in Fig. 2, employs magnetic beads bearing avidin (we use DynaBeads® although comparable products are available from other manufacturers). Biotin-labeled lectin can be immobilized on the beads, taking advantage of the tight binding of avidin with biotin. The immobilisation step involves simply mixing the avidin-coated beads with biotinylated lectin at optimum concentration and in an appropriate buffer. A very wide range of biotinylated lectins with different carbohydrate-binding specificities are available commercially. In the protocol given here, a small volume of beads are used, such that the cell separation can be performed on a small scale, making this a very cost-effective approach. However, it can be readily scaled up if larger quantities of separated cells are required. Once the lectin is immobilized on the beads, they are very gently (to minimise physical trauma to the

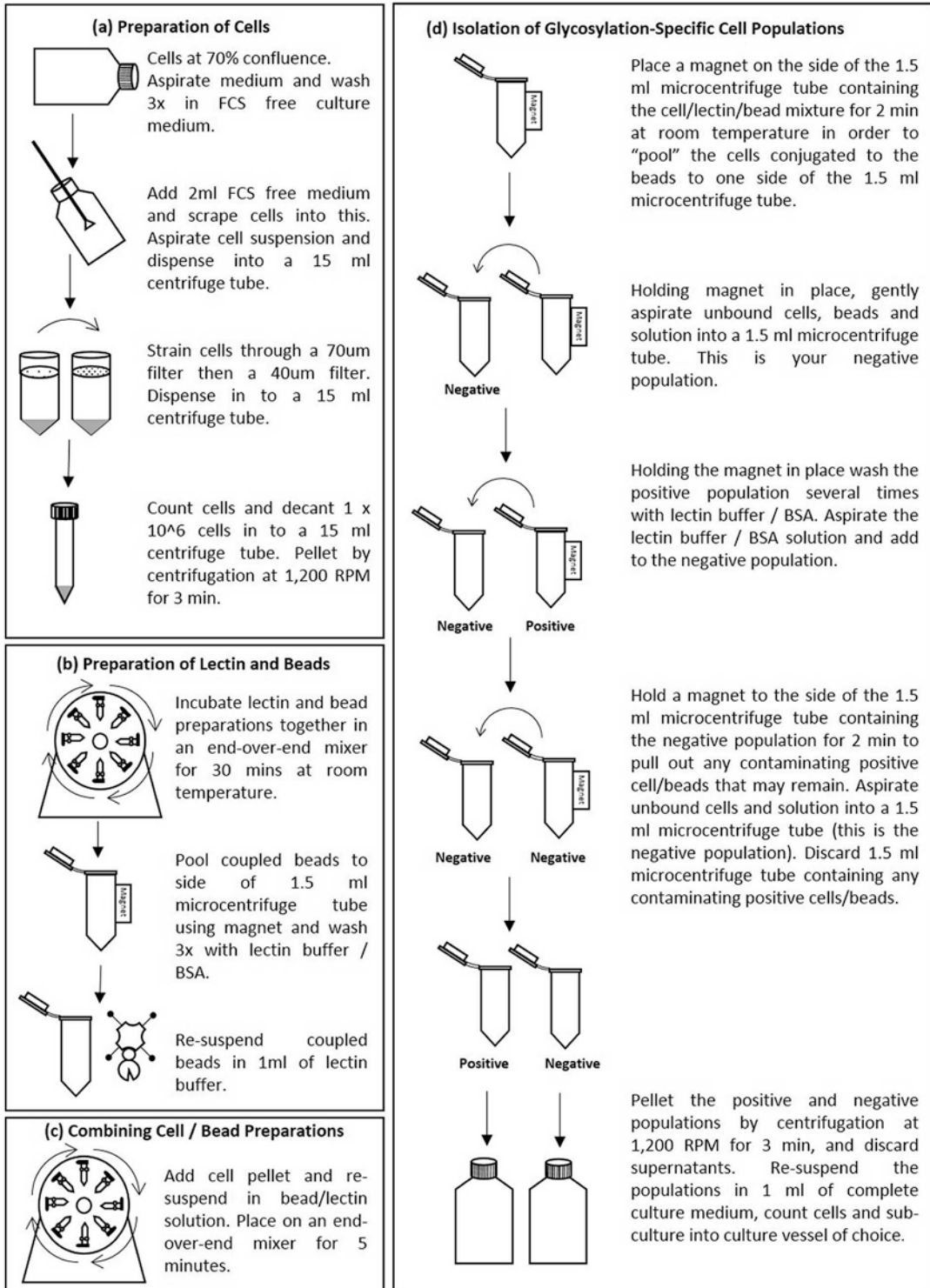


Fig. 2 Summary of the cell separation method

cells) mixed with a suspension of cells. The mixing step is kept brief so that it can be conveniently carried out on the bench-top, rather than in a 37 °C/CO₂ incubator, without being detrimental to cell viability. During the mixing step, cells that exhibit cell surface glycosylation profiles recognized by the immobilized lectin will bind to the beads; those that do not will remain free in suspension. Once the lectin-binding cells are attached to the beads, they can be very easily separated from the remaining unbound cell population, those with a glycosylation profile not recognized by the lectin, using a magnet. Thus two separated cell populations (*see* Fig. 3) are available for further culturing and/or analysis.

In order for cells to remain viable after separation, such that they can be cultured on as lectin-positive and lectin-negative populations, it is imperative that conditions are kept sterile throughout. This is not an issue if, on the other hand, the cells are instead destined for immediate analysis, for example, genetic profiling. Viability is also enhanced if physical trauma to the cells is kept to a minimum and this is ensured by very gentle mixing using an end-over-end mixer during the lectin-binding step, gentle handling during removal of the beads and washing, and keeping the time that cells are out of the 37 °C/CO₂ incubator to a minimum.

Once cells have bound to the beads, it is not necessary to release/dissociate the cells. In our experience, the presence of cell-bound beads (*see* Fig. 3a) does not adversely affect cell viability and during subsequent culture the presence of beads naturally diminishes. However, we have found when we have subjected separated cells to further lectin cytochemistry, using the same lectin that was employed to separate them in order to assess their glycosylation profiles post-separation, the presence of cell-bound beads does reduce their ability to label. We interpret this as being a result of either steric hindrance due to the physical presence of the beads, or to unavailability of

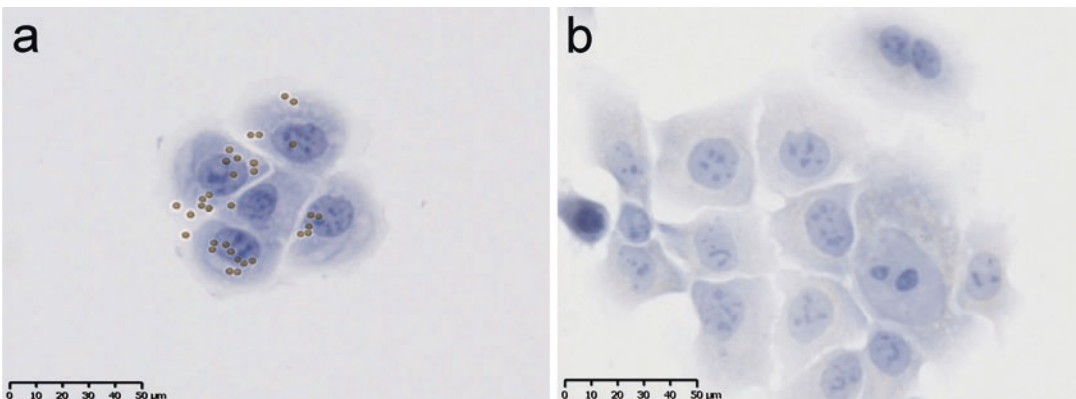


Fig. 3 ZR 75 1 cells after separation using *Helix pomatia* agglutinin (HPA) immobilized on magnetic beads. (a) HPA-binding cells. Lectin-coated magnetic beads attached to the cells are readily visible. (b) HPA-non-binding cells derived from the same original cell culture

lectin-binding sites as they are occupied by binding to lectin immobilized on the beads. If this is an issue, it is possible to dissociate the beads from the separated cells. This can be achieved using competitive inhibition in a solution of the simple sugar for which the lectin shows greatest specificity or by using EDTA/trypsin. This step may not be necessary for many applications.

1.4 A Versatile Technique Appropriate for Many Applications

In our hands, this method has proved to be very simple and straightforward. It has allowed us to separate and study the behavior and properties of cells separated from an apparently uniform parental population on the basis of their fine glycosylation profiles and to explore changes in cellular glycosylation over time and under different culture conditions. The same approach could be used for cells released from fresh, solid tissue specimens. Here, we have presented a technique for separating cells in a viable condition and under sterile conditions appropriate for further culture but this aspect may not be necessary if, for example, immediate genetic or other analysis is planned and the protocol can then be simplified. It could also easily be adapted for the separation of cells on the basis of their binding to immobilized biotinylated antibody or neoglycoprotein, rather than lectin, making this technique very widely applicable in cell biology research.

2 Materials

2.1 Preparation of Cells

1. Cells cultured under standard conditions, use a 70% confluent T75 flask.
2. Foetal calf serum (FCS) free cell culture medium (*see Note 1*).
3. Rubber cell scraper.
4. 15 and 50 ml centrifuge tubes.
5. 70 and 40 μm cell culture filter.

2.2 Buffers (See Note 2)

1. Lectin buffer: 60.57 g Tris, 87.0 g NaCl, 2.03 g MgCl_2 , 1.11 g CaCl_2 dissolved in 1 l distilled water. pH to 7.6 using concentrated HCl. Make up to a total volume of 10 l using distilled water (*see Notes 3 and 4*).
2. Lectin buffer/ BSA: 3% w/v bovine serum albumin (BSA) in lectin buffer (*see Note 5*).
3. 0.22 μm sterile cell culture filter.

2.3 Preparation of Lectin and Streptavidin-Coupled DynaBeads®

1. Lectin solution: biotin-labeled lectin (kept sterile by only opening in a class II cell culture hood) at 10 $\mu\text{g}/\text{ml}$ in lectin buffer/BSA (*see Subheading 2.2*).
2. Bead preparation: streptavidin-DynaBeads® (kept sterile by opening in a class II cell culture hood, mixing well before use) at 5 $\mu\text{g}/\text{ml}$ in lectin buffer/BSA (*see Subheading 2.2*).

3. End-over-end mixer.
4. Magnet (*see* **Note 5**).
5. Lectin buffer (*see* Subheading 2.2).
6. Lectin buffer/BSA (*see* Subheading 2.2).
7. 1.5 ml microcentrifuge tubes.

2.4 Combining Cell and Lectin Streptavidin-Coupled DynaBeads® Preparations

1. Lectin buffer/BSA (*see* Subheading 2.2).
2. End-over-end mixer.

2.5 Isolation of Glycosylation-Specific Cell Populations

1. Lectin buffer (*see* Subheading 2.2).
2. Complete culture medium.
3. Magnet (*see* **Note 5**).
4. 1.5 ml microcentrifuge tubes.

2.6 Removal of Lectin Streptavidin-Coupled DynaBeads®

2.6.1 Dissociation of Cells from Beads Using Competitive Inhibition of Lectin-Binding

1. 0.1 M appropriate simple sugar prepared in lectin buffer/BSA (*see* Subheading 2.2) (*see* **Note 6**).
2. 1.5 ml microcentrifuge tubes.
3. End-over-end mixer.
4. Magnet (*see* **Note 5**).

2.6.2 Dissociation of Cells from Beads Using 0.05 % Trypsin–EDTA

1. Phosphate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ dissolved in distilled water, pH to 7.4 using concentrated HCl, then make up to 1 l using distilled water.
2. 0.05 % trypsin–EDTA made up in PBS.
3. 1.5 ml microcentrifuge tubes.
4. End-over-end mixer.
5. Magnet (*see* **Note 5**).
6. 15 ml centrifuge tubes.
7. Complete culture medium.

3 Methods

All equipment should be sterile and all procedures where cells are not housed within sterile sealed containers should be performed in a class II cell culture hood. The method is illustrated in Fig. 2.

3.1 Preparation of Cells

See Fig. 2a.

1. Wash cultured cells 3× in fresh FCS free culture medium.
2. Aspirate and discard the medium.
3. Scrape cells from the culture flask using a rubber scraper (*see Note 7*) and decant into 2 ml of fresh FCS free culture medium in a 15 ml centrifuge tube.
4. Strain cells through a 70 µm cell culture filter placed on a 50 ml centrifuge tube and then a 40 µm culture filter placed on a 50 ml centrifuge tube (*see Note 8*).
5. Count cells, and decant 1×10^6 cells into a 15 ml centrifuge tube.
6. Pellet cells by centrifugation at $1100 \times g$ for 3 min and discard supernatant.

3.2 Preparation of Lectin and Streptavidin-Coupled DynaBeads®

See Fig. 2b.

1. Incubate 500 µl each of lectin solution and bead preparations together in a 1.5 ml microcentrifuge tube on an end-over-end mixer for 30 min at room temperature.
2. Trap the magnetic beads at the side of the 1.5 ml microcentrifuge tube using a magnet and aspirate the lectin buffer/BSA.
3. Wash bound beads 3× with lectin buffer to remove any unbound biotinylated-lectin.
4. Remove the magnet to release the beads.
5. Add 1 ml of lectin buffer/BSA and resuspend lectin-bound beads.

3.3 Combining Cell and Lectin Streptavidin-Coupled DynaBeads® Preparations

All reagents to be warmed to 37 °C and all procedures with cells at room temperature to be performed swiftly to reduce stress upon cells. See Fig. 2c.

1. Take the cell pellet (described in Subheading 3.1) and resuspend cells in the lectin-bound bead preparation (described in Subheading 3.2).
2. Place on an end-over-end mixer for 5 min at room temperature (*see Note 9*).

3.4 Isolation of Glycosylation-Specific Cell Populations

See Fig. 2d.

1. Place a magnet on the side of the 1.5 ml microcentrifuge tube containing the cell/lectin/bead mixture (described in Subheading 3.3) for 2 min at room temperature in order to trap the cells conjugated to the beads at one side of the 1.5 ml microcentrifuge tube (*see Note 10*).
2. Holding magnet in place, gently aspirate (*see Note 11*) unbound cells, beads and solution into a 1.5 ml microcentrifuge

tube (this is the negative population, i.e., the population of cells without the selected glycosylation profile).

3. Holding the magnet in place, wash (*see Note 12*) the positive population (i.e., the population of cells with the selected glycosylation profile) several times with lectin buffer to ensure that only cells bound to the lectin/beads remain. Aspirate the wash solution and add to the negative population.
4. Repeat **step 3** twice more.
5. Pellet the positive population by centrifugation at $1100\times g$ for 3 min, and discard supernatant.
6. Resuspend the positive population in 1 ml of complete culture medium (*see Note 13*), count cells and subculture into a culture vessel of choice.
7. To further purify the negative population hold a magnet to the side of the 1.5 ml microcentrifuge tube for 2 min to trap any contaminating positive cell/beads that may remain.
8. Aspirate the remaining pure population of free, unbound cells and solution into a fresh 1.5 ml microcentrifuge tube (this is the negative population).
9. Discard the 1.5 ml microcentrifuge tube containing any contaminating positive cells/beads (*see step 7*).
10. Repeat **step 7** twice more.
11. Pellet the negative population (*see step 8*) by centrifugation at $1100\times g$ for 3 min, and discard supernatant (*see Note 14*).
12. Resuspend the negative population in 1 ml of complete culture medium (*see Note 13*), count cells and subculture into culture vessel of choice.
13. Allow cells to proliferate for 24 h at 37 °C under 5 % CO₂ and then feed or subculture as appropriate.

3.5 Removal of Lectin Streptavidin- Coupled DynaBeads®

If downstream applications of the isolated population which is positive for the desired glycosylation requires the cells to be free from the lectin-coupled beads, then it is possible to remove them with either of the below detailed methods. The choice of approach will be dependent on the down-stream application. Continue from Subheading **3.4 step 5**.

3.5.1 Dissociation of Cells from Beads Using Competitive Inhibition of Lectin Binding

1. Resuspend the positive population in 1 ml of 0.1 M sugar solution in lectin buffer/BSA in a 1.5 ml microcentrifuge tube.
2. Place on an end-over-end mixer for 5 min at room temperature.
3. Place a magnet on the side of the 1.5 ml microcentrifuge tube to trap the beads to one side.

4. Aspirate the unbound cells into a fresh 1.5 ml microcentrifuge tube.
5. Repeats **steps 3** and **4** twice more.
6. Continue from **step 6** of Subheading [3.4](#).

**3.5.2 Dissociation
of Cells from Beads Using
0.05 % Trypsin-EDTA**

1. Resuspend the positive population in 1 ml of 0.05 % trypsin-EDTA in PBS in a 1.5 ml microcentrifuge tube.
2. Place on an end-over-end mixer for 5 min at room temperature.
3. Place a magnet on the side of the 1.5 ml microcentrifuge tube to trap the beads to one side.
4. Aspirate the unbound cells into a 15 ml centrifuge tube containing 1 ml of complete culture medium.
5. Repeat **steps 3** and **4** twice more.
6. Continue from **step 6** of Subheading [3.4](#).

4 Notes

1. Foetal calf serum contains glycan moieties and so must be washed away prior to the cells interacting with the lectin-bound beads to prevent it from competitively inhibiting lectin binding.
2. Buffers should be filter sterilized by passing through a 0.22 µm cell culture filter.
3. Lectin buffer is Tris buffered saline (TBS) with CaCl₂ and MgCl₂ added, and is appropriate for all lectin histochemistry applications. We recommend it for all dilutions and washes. Many lectins are known to require Ca²⁺ and Mg²⁺ to stabilize their carbohydrate-binding site(s). The requirements of other lectins remain unknown. As a starting point, it is inadvisable to use phosphate-buffered saline (PBS) for lectin histochemistry as the phosphate ions may bind with and sequester metal ions from lectin-binding sites.
4. Buffers can be stored at 4 °C for up to a month. Containers must be opened in a class II cell culture hood to retain sterility.
5. A commercially available magnetic separator stand can be used. Alternatively, use masking tape to secure a conveniently sized magnet to the side of the microcentrifuge tube but take care that the inside of the tube remains clearly visible.
6. The simple sugar should usually be the monosaccharide for which the lectin has greatest reported binding affinity. For example, for *Helix pomatia* agglutinin (HPA), 0.1 M N-acetylgalactosamine would be used.

7. During cell preparation cells are scraped instead of using trypsin, as trypsinisation would cleave surface proteins.
8. Cells are passed through a cell culture filter to ensure they are in a single cell suspension. Clumped cells can result in incomplete separation of glycosylation-specific populations.
9. Ensure the mixing of the lectin/beads with cells is gentle and thorough. There should be room in the microcentrifuge tube for the movement of the liquid to allow for optimal interaction of the lectin and beads.
10. The beads will be visible to the naked eye as a brown mass at the side of the microcentrifuge tube.
11. When aspirating a solution whilst the positive population is trapped by the magnet, use a small pipette tip and slowly aspirate in order not to disturb the trapped cells. It may be most effective to leave some unaspirated unbound cells in the solution in the microcentrifuge tube because they will be removed during subsequent steps.
12. When washing the cells, be gentle in order not to damage the cells but forceful enough as to ensure that non-bound cells are removed into the negative population.
13. The volume that the cells are resuspended in will be dependent on the applications of the populations post-separation.
14. If positive cells remain, the pellet will appear visibly brown to the naked eye; if this is the case then repeat **step 7**.

References

1. Brooks SA (2017) Lectin histochemistry: historical perspectives, state of the art and the future. *Methods Mol Biol* 1560:93–107
2. Goldstein IJ, Poretz RD (1986) Isolation, physiochemical characterization, and carbohydrate-binding specificity of lectins. In: Liener IR, Sharon N, Goldstein IJ (eds) *The lectins: properties, functions and applications in biology and medicine*. Academic Press, London, pp 35–250
3. Brooks SA, Dwek MV, Schumacher U (2002) Carbohydrate-binding proteins (lectins) in functional and molecular glycobiology. Bios Scientific Publishers Ltd, Oxford, pp 227–247
4. Aub JC, Tieslau C, Lankester A (1963) Reactions of normal and tumour cell surfaces to enzymes. I wheat-germ lipase and associated mucopolysaccharides. *Proc Natl Acad Sci U S A* 50:613–619
5. Inbar M, Sachs L (1969) Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. *Proc Natl Acad Sci U S A* 63:1418–1425
6. Sela BA, Lis H, Sharon N et al (1970) Different locations of carbohydrate-containing sites at the surface membrane of normal and transformed mammalian cells. *J Membr Biol* 3:267–279
7. Brooks SA, Hall DMS, Buley I (2001) GalNAc glycoprotein expression by breast cell lines, primary breast cancer and normal breast epithelial membrane. *BJC* 85:1014–1022
8. Brooks SA (2000) The involvement of *Helix pomatia* lectin (HPA) binding N-acetylgalactosamine glycans in cancer progression. *Histol Histopathol* 15:143–158
9. Schumacher U, Adam E, Brooks SA et al (1995) Lectin-binding properties of human breast cancer cell lines and human milk with particular reference to *Helix pomatia* agglutinin. *J Histochem Cytochem* 43:275–281
10. Leatham A, Dokal I, Atkins N (1983) Lectin binding to normal and malignant breast tissue. *Diagn Histopathol* 6:171–180
11. Bard F, Chia J (2016) Cracking the glycome encoder: signalling, trafficking, and glycosylation. *Trends Cell Biol* 26:379–388

Lectin Histochemistry for Metastasizing and Non-metastasizing Cancer Cells

Gerrit Wolters-Eisfeld and Udo Schumacher

Abstract

Changes in glycosylation of the cancer cell glycocalyx are a hallmark of metastasizing cancers and critically contribute to distant metastasis. In this chapter we concentrate on two lectins capable of specifically binding tumor-associated glycans in cryostat or formalin-fixed, paraffin-embedded tissue sections derived from primary clinical material, genetically engineered mouse models with endogenous cancer formation or xenograft mouse models. The snail lectin of *Helix pomatia* (HPA) binds N-acetylgalactosamine (GalNAc) that is expressed among others as Tn antigen (O-linked GalNAc) in primary tumors and metastases in several human adenocarcinomas. Another lectin, *Phaseolus vulgaris* leucoagglutinin (PHA-L) binds to complex β 1-6 branched N-linked oligosaccharides associated with increased metastatic potential in breast, colon, and prostate cancer. Using these two lectins both O- and N-linked alterations in the glycocalyx of cancer cells can be monitored. As they are commercially available in a biotinylated or fluorescence-labeled form they can be readily used in cancer metastasis studies.

Key words Cancer cell glycosylation, Clinical studies, *Helix pomatia agglutinin* (HPA), Lectin histochemistry, Metastasis formation, *Phaseolus vulgaris* leucoagglutinin (PHA-L), Tn antigen, Xenograft models

1 Introduction

Every fourth citizen in the developed world dies of cancer and more than 90 % of the cancer patients die from distant metastases and not from the primary tumor [1]. Sadly, this situation has not much changed since 1971 when US President Richard Nixon declared “war on cancer” [2]. The main efforts in the last two decades have focused on detecting genetic alterations associated with metastasis, however, “despite intensive efforts, no genetic alterations have been shown to be required to convert a malignant primary tumor into a metastatic lesion” [3]. Cancer cell glycosylation may be one important aspect of metastasis formation and as carbohydrate residues are not encoded in the genome directly as proteins are, the functional role of glycosylation might have been

overlooked. As no genetic alteration is typical for metastasis formation, it must depend on the cellular context under which the cancer cell operates and therefore it is not astonishing that the tumor microenvironment has come into the focus of metastasis research [4]. The interaction of the cancer cell with its neighboring cells and its surrounding extracellular matrix is mediated by the outer carbohydrate rich coat of mammalian cells called the glycocalyx. The carbohydrate residues of this glycocalyx can be characterized by lectins or human glycoreceptors, carbohydrate binding proteins [5, 6].

The carbohydrate residues of the glycocalyx are covalently linked to the transmembrane glycoproteins. In principle, two different types of linkages exist, namely N- and O-linked glycosylation [7]. N-linked glycosylation starts in the endoplasmic reticulum, where a complex branched oligosaccharide “tree” is covalently linked to an N-atom of an asparagine side chain. This oligosaccharide is further processed both within the lumen of the endoplasmic reticulum and in the Golgi apparatus. In the last trans-cistern of the Golgi apparatus often a terminal sialic acid residue is added as the final step of N-glycosylation. In contrast, O-glycosylation starts and finishes in the Golgi apparatus. Here the carbohydrate side chains are less branched and shorter than those of the N-linked glycosylation and are covalently linked via an O-atom of a serine or threonine residue. Again, sialic acid can be the terminal carbohydrate residue of O-linked glycosylation. As this form of glycosylation is predominantly found in mucins, they are often called mucin-type glycoproteins. And as mucins are generally secreted by epithelial cells, O-linked glycoproteins are typically expressed on epithelial cells and in their neoplasms, the carcinoma. Conversely, N-linked glycoproteins are a hallmark of mesenchymal cells and their neoplasms, the sarcomas. However, this dichotomy of epithelial versus mesenchymal O- and N-linked glycosylation has not to be viewed as an absolute one. Indeed, changes of the glycosylation pattern occur during epithelial to mesenchymal transition (EMT). During EMT cancer cells change their epithelial sessile phenotype to a motile mesenchymal one. Therefore changes in both O- and N-linked glycosylation are typically observed during EMT [8].

Changes in glycosylation can therefore be viewed as a “hallmark of metastasizing cancers” which could also be used as drug targets [9, 10]. In particular, two lectins, namely *Helix pomatia* Agglutinin (HPA) and phythaemagglutinin (PHA-L) have found interest in metastasis research.

HPA rose to prominence in the landmark studies of Leathem and Brooks in which they showed that HPA binding to primary breast cancers in wax tissue sections was correlated with the prognosis in breast cancer patients [11, 12]. This finding was later confirmed in many other clinic-pathological studies [13], however, it is

critically important that the methodology used by Leathem and Brooks is followed. They used an indirect method with the native lectin followed by an anti-lectin antibody. If the lectin is directly coupled to horseradish peroxidase, the correlation does not hold true as covalently linking horseradish with HPA obviously changes its carbohydrate specificity [14]. Using the indirect method, HPA has been shown to be a prognostic indicator in many other entities including among others colon cancer [15], adenocarcinoma of the lung [16] and malignant melanoma [17]. HPA has not only been a useful marker of metastases in clinical studies, but it also holds true for human breast and colon cancer cells transplanted into immunodeficient mice making it a useful tool for experimental cancer research by linking clinical and experimental studies thus giving the latter one a clinical relevance [18, 19] (Fig. 1). The carbohydrate binding specificity of HPA is complex as GalNAc residues can be found as part of the blood group substance A or the Tn-antigen. However, applying both antibodies against the two glycotopes as well as HPA

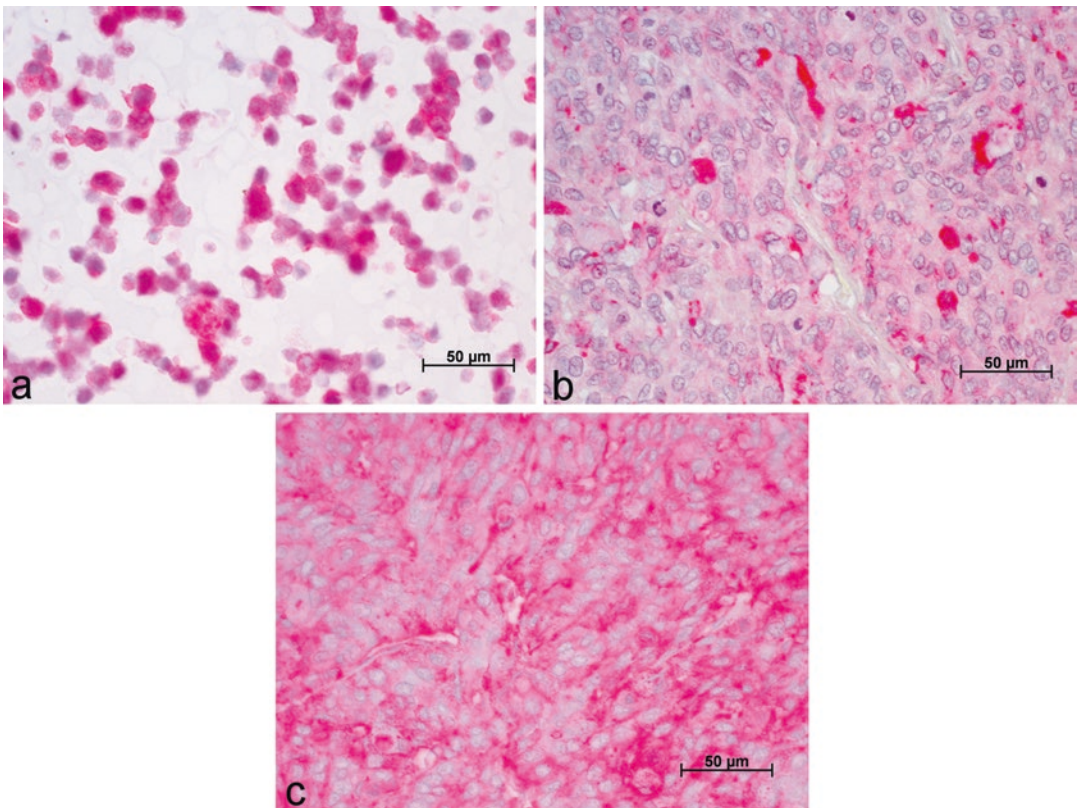


Fig. 1 Lectin histochemistry of the human colon cancer cell line HT 29 grown in cell culture and grown as primary tumors in severe combined immunodeficient mice. (a) Cells grown in vitro, formaldehyde-fixed, paraffin wax-embedded, and HPA stained. (b) Primary tumor stained with HPA and (c) primary tumor stained with PHA-L. Note that HPA staining is much more intense and uniform if cells were grown in vitro as compared to those cells grown in mice

to a series of breast cancer samples revealed that the binding pattern were distinctive and different [20]. Applying a series of lectins including N-acetylgalactosamine and N-acetylgalactosamine ones revealed that HPA shows a distinct binding pattern and carbohydrate specificity in tissue sections which also might in part include N-acetylglucosamine as a terminal sugar as well [21]. From the protein backbone carrying the HPA positive glycotopes, several proteins have been identified including integrin alpha 6, heat shock protein 27, annexin IV and V [22, 23].

From a functional point of view, HPA positive cancer cells interact with the endothelium via their carbohydrate residues in breast cancer [24] and colon cancer with selectins as a likely binding partner [25].

Next to HPA, PHA-L has been used as a marker of metastasis formation in breast and colon cancer [26]. Although both lectins are indicative of metastasis formation, their binding pattern is markedly different [27] which is not astonishing as PHA-L recognizes β 1-6 branched oligosaccharides typical for N-glycosylation while HPA recognizes O-linked glycans (*see above*). This finding is well illustrated in prostate cancer where HPA was not associated with metastasis formation while PHA-L was [28, 29].

In summary both lectins have been applied in clinical and experimental studies of distant metastasis formation. If cancer cell adhesion to endothelium is one important role of the HPA and PHA-L binding cell surface oligosaccharides this finding implies that both O- and N-linked glycoproteins are involved in this adhesive process. Therefore research using these two lectins still has some mileage to go.

2 Materials

2.1 Tissue Preparation

2.1.1 Formalin-Fixed, Paraffin-Embedded Sections (FFPE)

1. Paraffin-embedded tissue blocks (*see Note 1*).
2. 20 % v/v ethanol in distilled water.
3. Silane-coated glass microscope slides (*see Note 2*).
4. Xylene.
5. Absolute ethanol.
6. 70 % v/v ethanol in distilled water.
7. Distilled water.

2.1.2 Cryostat Sections

1. Fresh frozen tissue specimens approximately 0.5 cm³ in size.
2. OCT embedding medium.
3. Clean glass microscope slides (*see Note 1*).
4. Acetone.

5. Tris-buffered saline (TBS), pH 7.6: Dissolve 60.57 g of Tris, 87.0 g of NaCl in 1 L of distilled water. Adjust pH to 7.6 using concentrated HCl. Make up to a volume of 10 L using distilled water.
6. Disposable embedding molds.

2.2 Antigen Retrieval

2.2.1 Carbohydrate/ Glycoprotein Retrieval by Microwave Treatment

1. Citrate buffer, pH 6.0: Dissolve 2.1 g of citric acid in 1 L of distilled water. Adjust pH to 6.0 using concentrated HCl.
2. Distilled water.

2.2.2 Carbohydrate/ Glycoprotein Retrieval by Trypsinisation

1. Tris-buffered saline (TBS), pH 7.6 (*see* Subheading 2.1.2, **step 5**).
2. Trypsin solution: 1 mg/mL of crude, type II trypsin from porcine pancreas in TBS, pH 7.6 (*see* Note 3).

2.3 Blocking Reagents

2.3.1 Quenching Endogenous Peroxidase

1. Methanol-hydrogen peroxide solution. 3% v/v hydrogen peroxide in methanol or commercial reagent (*see* Note 4).

2.3.2 Blocking of Nonspecific Binding

1. Use non-glycosylated blocking reagents, e.g., Carbo-Free Blocking Solution (Vector Laboratories) to reduce nonspecific lectin binding (*see* Note 5).

2.3.3 Streptavidin/Biotin Blocking

1. We recommend using a commercial kit blocking all endogenous biotin, biotin receptors, and streptavidin binding sites present in tissues.

2.4 Histochemical Staining Techniques

2.4.1 Biotin-Labeled Lectin Chromogen

1. Biotin-labeled lectin.
2. Lectin buffer: 24.23 g Tris, 87.66 g NaCl, 4.07 g MgCl₂ (hexahydrate), 2.19 g CaCl₂ (hexahydrate), 0.02 g MnCl₂ (tetrahydrate). Adjust pH to 7.6 using concentrated HCl. Make up to a total volume of 10 L (*see* Note 6).
3. Streptavidin labeled with horseradish peroxidase.
4. Chromogen solution 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) (*see* Note 7).
5. Mayer's hematoxylin (*see* Note 8).

2.4.2 Biotin-Labeled Lectin Fluorescence

1. Biotin-labeled lectin.
2. Lectin buffer (*see* Subheading 2.4.1, **step 2**, and Note 6).
3. Streptavidin labeled with fluorescent dye (*see* Note 9).
4. DAPI (4',6-diamidino-2-phenylindole) or other fluorescent DNA stains (*see* Note 10).

2.4.3 Fluorescence-Labeled Lectin

1. Fluorescence-labeled lectin, for example, HPA Alexa Fluor 488 (Sigma-Aldrich).
2. Lectin buffer (*see* Subheading 2.4.1, step 2, and Note 6).
3. DAPI (4',6-diamidino-2-phenylindole) or other fluorescent DNA stains (*see* Note 10).

2.5 Dehydration, Clearing, and Mounting Slides for Viewing by Microscopy

1. 70 % v/v ethanol in distilled water.
2. 100 % ethanol.
3. Xylene.
4. Xylene-based mounting medium, for example, Eukitt for enzyme-labeled preparations, or anti-fade mount, CitiFluor or other fluorescence mounting medium for fluorescent-labeled preparation.
5. Coverslips.

3 Methods

3.1 Tissue Preparations

3.1.1 FFPE Sections

1. Cool paraffin-embedded tissue blocks on ice approximately 15 min.
2. Cut 5 µm thick sections by microtome (*see* Note 11).
3. Carefully transfer sections onto the surface of a water bath heated to 40 °C. Sections should stretch and become flat (*see* Note 12).
4. Separate out sections very gently, using the tips of either fine forceps or brushes.
5. Pick up sections on clean, silane-coated glass microscope slides (*see* Note 2).
6. Dry slides either in a 37 °C incubator overnight, or on a hot-plate at 60 °C for 20 min. Slides may then be stored at room temperature until required.
7. When required, soak slides in xylene for 10 min to remove paraffin wax.
8. Transfer slides through a descending alcohol series of 100, 96, 80, and 70 % ethanol for 5 min each. Transfer slides to distilled water or PBS.

3.1.2 Cryostat Sections

1. Place the frozen OCT-embedded tissue on a precooled OCT-coated cryostat chunk (*see* Note 13).
2. Place the frozen chunk in the cabinet of the cryostat and equilibrate for approximately 30 min.
3. Cut 5 µm thick sections and pick them up on clean glass microscope slides (*see* Note 2). Allow to air dry for 10 min.

4. Sections may then be stored at -20°C until required, or may be used at once. If stored frozen, allow to thaw and equilibrate to room temperature before use.
5. Fix tissue in ice-cold acetone for 5–10 min (*see* **Note 14**).
6. Wash with PBS three times.

3.2 Antigen Retrieval (*See* **Note 15**)

3.2.1 Carbohydrate/ Glycoprotein Retrieval by Microwave Treatment

1. Immerse slides in citrate buffer pH 6.0 in any suitable microwaveable container with lid (*see* **Note 16**).
2. Place container in microwave (600 W) and boil for 10 min.
3. Allow slides to cool to room temperature for 30 min (*see* **Note 17**).
4. Wash slides carefully under running tap water for 5 min.

3.2.2 Carbohydrate/ Glycoprotein Retrieval by Trypsinisation

1. Immerse slides in trypsin solution at 37°C , in an incubator or water bath for 5–30 min (*see* **Notes 3** and **18**).
2. Wash slides carefully under running tap water for 5 min.

3.3 Blocking Reagents

3.3.1 Quenching Endogenous Peroxidase

1. Immerse slides in methanol–hydrogen peroxide solution for 15 min.
2. Carefully wash under running tap water for 5 min.

3.3.2 Blocking of Nonspecific Binding

1. Block nonspecific binding by incubating sections with Carbo-Free Blocking Solution (Vector Laboratories) for 30 min at room temperature (*see* **Note 5**). Blot excess blocking solution from the sections.

3.3.3 Streptavidin/Biotin Blocking

1. Incubate sections with streptavidin solution for 15 min at room temperature.
2. Rinse briefly with PBS.
3. Incubate sections with biotin solution for 15 min at room temperature.

3.4 Histochemical Staining Techniques

3.4.1 Biotin-Labeled Lectin Chromogen

1. Incubate section with biotin-labeled lectin at a concentration of $10\text{ }\mu\text{g/mL}$ lectin in lectin buffer. Perform incubation in a humid chamber for 1 h (*see* **Note 19**).
2. Wash sections three times with lectin buffer for 5 min (*see* **Note 20**).
3. Incubate with streptavidin-HRP in lectin buffer in a humid chamber for 30 min.
4. Wash sections three times with lectin buffer for 5 min.
5. Incubate with freshly prepared chromogen solution for 2–20 min. Monitor intensity of staining under a microscope to ensure proper intensity of tissue staining.
6. Stop the reaction with tap water.

7. Counterstain in Mayer's hematoxylin for 10 s (*see* **Note 8**).
8. "Blue" under tap water for approximately 5 min (*see* **Note 21**).
9. Mount slides (*see* Subheading 3.5).

3.4.2 Biotin-Labeled Lectin Fluorescence

1. Incubate section with biotin-labeled lectin at a concentration of 10 µg/mL lectin in lectin buffer. Perform incubation in a humid chamber for 1 h (*see* **Note 19**).
2. Wash sections three times with lectin buffer for 5 min (*see* **Note 20**).
3. Incubate with fluorescence-labeled streptavidin in lectin buffer in a humid chamber for 30 min in the dark.
4. Wash sections three times with lectin buffer for 5 min.
5. Wash sections with lectin buffer for 5 min.
6. Perform DAPI (1 µg/mL) staining of cell nuclei for 5 min (*see* **Note 10**).
7. Wash two times with lectin buffer for 5 min.
8. Mount slides (*see* Subheading 3.5).

3.4.3 Fluorescence- Labeled Lectin

1. Incubate section with fluorescence-labeled lectin at a concentration of 10 µg/mL lectin in lectin buffer. Perform incubation in a humid chamber for 1 h in the dark (*see* **Note 19**).
2. Wash sections with lectin buffer for 5 min.
3. Perform DAPI (1 µg/mL) staining of cell nuclei for 5 min.
4. Wash two times with lectin buffer for 5 min (*see* **Note 20**).
5. Mount slides (*see* Subheading 3.5).

3.5 Dehydration, Clearing, and Mounting FFPE Slides for Viewing by Microscopy

1. Dehydrate by passing through 70 % ethanol, then two changes of 100 % ethanol, than clear in xylene. Gently shake slides for 1–2 min at each stage to equilibrate.
2. Chromogen labeled slides should be mounted in a xylene-based mounting medium, for example Eukitt. Fluorescence-labeled slides should be mounted in a commercially available fade-resistant mounting medium.

3.6 Mounting of Cryostat Slides for Viewing by Microscopy

1. Slides can be directly mounted using aqueous mounting media like Aqua-Poly/Mount (Polysciences).

4 Notes

1. Paraffin-embedded tissue blocks are often the most convenient source of material for clinical studies as tumor specimens are routinely fixed in formalin and processed to paraffin embedding

for sectioning, staining and pathological diagnosis. Archived FFPE tissue samples are invaluable resources for studying glycosylation in cancer and metastasis.

2. High quality treated glass slides are available from several suppliers. Those are key to success especially if the slides are to be microwave treated. The aggressiveness of the treatment will otherwise dislodge the tissue preparation.
3. Crude, type II trypsin from porcine pancreas works best as the presence of impurities (e.g., chymotrypsin) assists its effect.
4. Prepare methanol-hydrogen peroxide solution freshly every 2–3 days or use commercially available solutions in dropper bottles.
5. Carbo-Free Blocking Solution (Vector Laboratories) is a blocking reagent containing no glycans, generally reducing background staining.
6. The lectin buffer formula is related to TBS containing CaCl_2 , MgCl_2 and MnCl_2 and works for all lectin histochemistry applications. Molarities are 20 mM Tris, 150 mM NaCl, 2 mM MgCl_2 , 1 mM CaCl_2 , and 0,01 mM MnCl_2 . Many lectins are known to require cofactors such as Ca^{++} or Mg^{++} for binding; the requirements of other lectins remain unknown. Do not use phosphate-buffered saline (PBS) for lectin histochemistry as the phosphate ions bind and sequester the metals.
7. DAB and AEC are available in dropper bottle kits from major suppliers. This is a more expensive option than preparing the buffers oneself but it is more convenient and safer.
8. A number of hematoxylin solutions are commercially available and work equally effective.
9. Many fluorescent dyes couples to streptavidin or avidin are available by major suppliers. Sequential staining of lectins with different fluorescent dyes is possible.
10. DAPI (10 mg/mL stock solution) can be diluted up to 10,000-fold. There are fluorescence mounting media containing DAPI available.
11. Most tissues cut most effectively when very cold. Ice should be applied to the surface of the tissue block every few min during cutting. The composition of the paraffin also critically contributes to the outcome of sectioning.
12. If the sections remain wrinkled, it may indicate the water is too cold. If the wax begins to melt, it is too hot.
13. OCT acts as a support for the tissue block during sectioning. Be generous with it and embed the tissue block in a copious amount of it. Align the tissue block, in the OCT, with one of the straight edges parallel with the cutting edge of the chunk, as it will make sectioning easier.

14. Acetone will also permeabilize. Consequently, no further permeabilization step is required.
15. Carbohydrate retrieval methods: In some cases, if lectin histochemistry results on formalin-fixed, paraffin-embedded (FFPE) tissues are disappointing, it may be because carbohydrate moieties have been sequestered during tissue fixation and processing to paraffin. Results can often be dramatically improved by carbohydrate retrieval techniques including trypsinization or microwave treatment. It is not possible to predict if carbohydrate retrieval methods will be effective, or which to choose. Try both and select which conditions work best for your application.
16. Slides can be conveniently be placed in commercially available slide carriers, which hold typically hold up to 25 slides, or upright Coplin jars.
17. This cooling down period is part of the retrieval method and should not be skipped.
18. Make trypsin solution up fresh immediately before use, as it loses activity over time. Initially try a range of trypsinization time. Do not exceed 30 min, as visible damage to the tissue morphology becomes apparent.
19. Lectins are usually purchased in powder form. We routinely dissolve the powder in lectin buffer to give a stock solution of 1 mg/mL lectin, which is stable in the refrigerator for several months. Lectin stock solution can then be diluted to the optimum working concentration as required. A working concentration of 10 µg/mL lectin is optimal for most lectins in histochemistry. It may be a good idea to try a range of concentrations, 2.5, 5, 10, 20, 40 µg/mL lectin to assess which lectin concentration give the best result.
20. We recommend vigorous washing in the changes of lectin buffer. Insufficient washing, in particular omitting one or more changes of buffer, can result in unacceptably high levels of background staining.
21. Mayer's hematoxylin is deep red in color and tissue preparations will stain deep red after immersion in it. The hematoxylin changes to a deep navy blue when exposed to mildly alkaline conditions, known as "blueing." If blueing is unsuccessful owing to unusually acidic tap water, soak slides instead of tap or distilled water to which a few drops of ammonia or sodium chloride have been added.

Acknowledgment

This work was supported by the German Research Foundation (DFG) grant WO 1967/1-1 to G.W.-E.

References

- Valastyan S, Weinberg RA (2011) Tumor metastasis: molecular insights and evolving paradigms. *Cell* 147:275–292
- Editorial (2013) Conquering cancer. *Lancet* 382:2039
- Vogelstein B, Kinzler KW (2015) The path to cancer – three strikes and you're out. *N Engl J Med* 373:1895–1898
- Mlecnik B, Bindea G, Kirilovsky A et al (2016) The tumor microenvironment and Immunoscore are critical determinants of dissemination to distant metastasis. *Sci Transl Med* 8:327ra326
- Mitchell BS, Vernon K, Schumacher U et al (1995) Ultrastructural localization of Helix pomatia agglutinin (HPA)-binding sites in human breast cancer cell lines and characterization of HPA-binding glycoproteins by western blotting. *Ultrastruct Pathol* 19:51–59
- Nollau P, Wolters-Eisfeld G, Mortezaei N et al (2013) Protein domain histochemistry (PDH): binding of the carbohydrate recognition domain (CRD) of recombinant human glyco-receptor CLEC10A (CD301) to formalin-fixed, paraffin-embedded breast cancer tissues. *J Histochem Cytochem* 61:199–205
- Brooks SA, Dwek MV, Schumacher U (2002) Functional and molecular glycobiology. BIOS Scientific, Oxford
- Lange T, Samatov TR, Tonevitsky AG et al (2014) Importance of altered glycoprotein-bound N- and O-glycans for epithelial-to-mesenchymal transition and adhesion of cancer cells. *Carbohydr Res* 389:39–45
- Brooks SA, Carter TM, Royle L et al (2008) Altered glycosylation of proteins in cancer: what is the potential for new anti-tumour strategies. *Anticancer Agents Med Chem* 8:2–21
- Dwek MV, Brooks SA (2004) Harnessing changes in cellular glycosylation in new cancer treatment strategies. *Curr Cancer Drug Targets* 4:425–442
- Brooks SA, Leatham AJ (1991) Prediction of lymph node involvement in breast cancer by detection of altered glycosylation in the primary tumour. *Lancet* 338:71–74
- Leatham AJ, Brooks SA (1987) Predictive value of lectin binding on breast-cancer recurrence and survival. *Lancet* 1:1054–1056
- Brooks SA (2000) The involvement of helix pomatia lectin (HPA) binding N-acetylgalactosamine glycans in cancer progression. *Histol Histopathol* 15:143–158
- Brooks SA, Lymboura M, Schumacher U et al (1996) Histochemistry to detect Helix pomatia lectin binding in breast cancer: methodology makes a difference. *J Histochem Cytochem* 44:519–524
- Schumacher U, Higgs D, Loizidou M et al (1994) Helix pomatia agglutinin binding is a useful prognostic indicator in colorectal carcinoma. *Cancer* 74:3104–3107
- Laack E, Nikbakht H, Peters A et al (2002) Lectin histochemistry of resected adenocarcinoma of the lung: helix pomatia agglutinin binding is an independent prognostic factor. *Am J Pathol* 160:1001–1008
- Thies A, Moll I, Berger J et al (2001) Lectin binding to cutaneous malignant melanoma: HPA is associated with metastasis formation. *Br J Cancer* 84:819–823
- Schumacher U, Adam E (1997) Lectin histochemical HPA-binding pattern of human breast and colon cancers is associated with metastases formation in severe combined immunodeficient mice. *Histochem J* 29:677–684
- Valentiner U, Hall DM, Brooks SA et al (2005) HPA binding and metastasis formation of human breast cancer cell lines transplanted into severe combined immunodeficient (scid) mice. *Cancer Lett* 219:233–242
- Brooks SA, Leatham AJ (1995) Expression of alpha-GalNAc glycoproteins by breast cancers. *Br J Cancer* 71:1033–1038
- Brooks SA, Leatham AJ (1998) Expression of N-acetyl galactosaminylated and sialylated glycans by metastases arising from primary breast cancer. *Invasion Metastasis* 18:115–121
- Peiris D, Ossondo M, Fry S et al (2015) Identification of O-linked glycoproteins binding to the lectin helix pomatia agglutinin as markers of metastatic colorectal cancer. *PLoS One* 10, e0138345
- Rambaruth ND, Greenwell P, Dwek MV (2012) The lectin helix pomatia agglutinin recognizes O-GlcNAc containing glycoproteins in human breast cancer. *Glycobiology* 22:839–848
- Bapu D, Runions J, Kadhim M et al (2016) N-Acetylgalactosamine glycans function in cancer cell adhesion to endothelial cells: a role for truncated O-glycans in metastatic mechanisms. *Cancer Lett* 375(2):367–374
- Kohler S, Ullrich S, Richter U et al (2010) E-/P-selectins and colon carcinoma metastasis: first in vivo evidence for their crucial role in a clinically relevant model of spontaneous metastasis formation in the lung. *Br J Cancer* 102:602–609

26. Fernandes B, Sagman U, Auger M et al (1991) Beta 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. *Cancer Res* 51:718–723
27. Mitchell BS, Brooks SA, Leathem AJ et al (1998) Do HPA and PHA-L have the same binding pattern in metastasizing human breast and colon cancers? *Cancer Lett* 123:113–119
28. Lange T, Kupfernagel M, Wicklein D et al (2014) Aberrant presentation of HPA-reactive carbohydrates implies selectin-independent metastasis formation in human prostate cancer. *Clin Cancer Res* 20:1791–1802
29. Lange T, Ullrich S, Muller I et al (2012) Human prostate cancer in a clinically relevant xenograft mouse model: identification of beta(1,6)-branched oligosaccharides as a marker of tumor progression. *Clin Cancer Res* 18:1364–1373

The Use of Lectin Histochemistry for Detecting Apoptotic Cells in the Seminiferous Epithelium

Vicente Seco-Rovira, Ester Beltrán-Frutos, Jesús Hernández-Martínez, Concepción Ferrer, and Luis Miguel Pastor

Abstract

Lectin histochemistry is commonly used to characterize the pattern of glycoconjugates in cells and tissues. Recent studies show that alterations in these glycoconjugates are associated with the entry of cells into apoptosis. A widely used technique for the detection of apoptotic cell death is TUNEL. In this chapter, we study the sensitivity of both techniques to identify apoptotic cells in the testis of photo-inhibited Syrian hamster.

Key words Lectin histochemistry, TUNEL, Apoptosis, Testis, Germ cell, Syrian hamster, Sensitivity

1 Introduction

The cell balance within a tissue is determined by the maintenance of a stable cell number. In this balance, both cell proliferation and apoptosis (programmed cell death) play an essential role, both processes forming part of the so-called cell cycle.

The morphological changes that occur during apoptosis can be seen microscopically using specific staining for light microscopy and using transmission electron microscopy to observe ultrastructural changes. Unlike in necrosis, apoptosis is initially manifested as changes in the condensation of nuclear chromatin at the nuclear perimeter, the separation of neighboring cells and the loss of extracellular matrix connections. Moreover, in advanced stages of apoptosis, protrusions of the plasma membrane or “blebs” appear and the fully condensed nucleus is divided into several fragments [1]. At cytoplasmic level, intact organelles are usually observed although dilation of the endoplasmic reticulum, the loss of ribosomes and the presence of cytoplasmic vacuoles, ending with the disintegration of the cell and the appearance of apoptotic bodies [2] which are phagocytosed by neighboring cells, have also been described.

The cell balance may be changed as a result of very different physiological conditions, for example, follicular atresia [3] or germ cell apoptosis [4, 5] and in pathological conditions, such as cancer [6], autoimmune and neurodegenerative disorders (Alzheimer's or Parkinson) and viral infections (AIDS) [7]. The histochemical TUNEL technique (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling in situ) is frequently used to study apoptosis, based on the detection of DNA fragmentation in apoptotic cells [8].

Cell apoptosis is associated with changes in glycoconjugates (Glyc) (residues of oligosaccharides linked to proteins by N- or O-glycosidic bonds) in various cell types. Glyc, either alone or complexed with glucan-binding proteins, are related to intracellular signals or control extracellular processes that lead to programmed cell death [9].

For Glyc pattern identification in tissue sections, lectins have been widely used. These are proteins of plant, fungal, microbial, or animal origin that specifically recognize sugar residues of glycoproteins and glycolipids, thereby identifying a characteristic pattern that defines the Glyc of an organ or tissue [10]. The use of lectins for the histochemical detection of glycans in tissue sections (lectin histochemistry) is an important area of application for these molecules. The interaction of lectins with their binding sites on the cell surface of both cells and tissue can be visualized using different markers commonly used in immunohistochemistry. For example, lectin histochemistry has been used to study the respiratory [11–13], gastrointestinal [14, 15] and male [16–18] and female [19, 20] reproductive systems, in addition to various pathological situations [21]. This histochemical technique makes it possible to identify changes in the pattern of Glyc characteristic of an organ or tissue both in physiological and pathological conditions [22].

Another application of lectins proposed several years ago is for the identification of apoptotic cells [23]. In this regard, studies that relate death by apoptosis with variations in the pattern of Glyc in these cells are not very numerous and mainly focus on primarily cultured cell lines in vitro [24–28] and, in some cases, in vivo [29]. In these studies, it has been seen that cells expressing glycoproteins containing α -D-mannose and β -D-galactose may be in a state of apoptosis [24, 25]. Other studies conclude that the cells, which will enter apoptosis, show increased exposure of Glyc rich in N-acetylglucosamine, N-acetylgalactosamine, D-mannose and L-fucose [26, 27]. Furthermore this is a good technique to identify membrane Glyc in pathological conditions since it has been observed that apoptotic cells release various types of membrane vesicles manifesting “eat-me” signals on their surface, whose presence promotes the phagocytic activity of adjacent macrophages [28]. However, these studies were performed using isolated cells separated by flow cytometry or smear focusing on glycocalyx of apoptotic cells.

To date, only two lectin histochemical studies have been made in organ and tissue sections to identify cells in apoptosis [30, 31]. These studies show that the increased expression of Glyc bound to β -D-galactose in spermatoblasts [30] and both β -D-galactose and α -D-mannose, and in some cases, L-fucose, are characteristic of germ cells in apoptosis in the seminiferous epithelium [31]. This latter study shows the usefulness of the application of lectin histochemistry to identify germ cells in apoptosis, and to identify in which phase of apoptosis the different cell types studied are because they have been identified from very early stages of apoptosis to very advanced stages of it. This point to that probably the lectin histochemistry could be a useful tool for identifying cell death by apoptosis in tissue and organ sections. This chapter explains how to use lectin histochemistry to identify apoptotic cells in the seminiferous epithelium of the Syrian hamster during its regression due to a short photoperiod, when there is an increase in apoptosis of spermatocytes and spermatids.

The described protocol may be also performed after processing the slides for the TUNEL technique: this allows comparing the sensitivity of the two histochemical techniques for the detection of apoptotic cells in the different phases of the apoptosis process.

2 Materials

1. Methacarn fixative: chloroform, methanol and acetic acid at a ratio of 6:3:1.
2. Graded series of ethanol (100°, 90°, 70°).
3. O-xylene for dewaxing sections.
4. Poly-L-lysine-coated glass slides: Dilute poly-L-lysine solution 1:10 with deionized water prior to coating slides and store it at room temperature prior use it or in fridge during 2–3 months. Place clean slides in diluted working poly-L-lysine solution for 5 min. Drain slides and dry in 60 °C oven for 1 h or at room temperature (18–26 °C) overnight.
5. Coplin Jars.
6. Humidity chambers (see **Note 1**) for the incubation of samples.
7. Tris Buffered Saline (TBS): For 10 l of final volume, firstly mix 60.57 g of Tris, 87 g of NaCl in 10 l of distilled water. Once totally diluted, adjust the pH until 7.4 adding HCl 0.1 M. Then, add 1.11 g of CaCl_2 or (1.47 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 2.03 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 1.9 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$. Never add the last three products before you correct to pH 8.
8. 0.05 % 3,3' diaminobenzidine (DAB) for visualization of the positivity in lectin histochemistry.

9. HRP-conjugated lectins: PNA and Concanavalin-A lectins at a concentration of 12 µg/ml (*see Note 2*).
10. Hansen's Heamatoxilin for counterstaining the samples.
11. DNase-Free H₂O for the preparation of all solutions (*see Note 3*).
12. Phosphate buffered saline (PBS): made using DNase-free H₂O. In our lab, we prepare it at a concentration of 10× and, when necessary, we dilute it to a concentration of 1×. For 1 l of 10× PBS: Add 87 g of NaCl, 2.72 g of KH₂PO₄, 11.36 g of Na₂PO₄ (or 26.9 g of NaHPO₄•12H₂O) to 1 l of DNase-free H₂O. When everything is completely diluted, adjust the pH to 7.4.
13. Cardio TACS In Situ Apoptosis Detection Kit (R&D Systems, Catalogue number: TA5353). The kit contains all the reagents and buffers for detecting apoptotic cells by the TUNEL technique. In this kit, Cytonin to permeabilize membranes and Nuclear Fast Red for counterstaining the samples are provided.
14. 30 % H₂O₂.
15. TBS+H₂O₂: 100 ml of TBS plus 1 ml of 30 % H₂O₂.
16. 1.5 ml microtubes.
17. Light microscope equipped with a camera for digital image acquisition.

3 Methods

For this study, testicular samples of Syrian hamster (*Mesocricetus auratus*) that had been exposed to a short photoperiod of 8 h light and 16 h dark for 12 weeks were used. During this period, the animals were kept at the animal facility of the University of Murcia at a constant temperature of 21 ± 1 °C and with food and water provided ad libitum. The animals were killed by CO₂ overdose in a closed chamber. Then, the testes were fixed in methacarn, for 8 h (*see Note 4*). Once the samples were properly fixed, they were embedded in paraffin using an automatic tissue processor (*see Note 5*) before preparing paraffin blocks for slicing (5 µ thickness) using a microtome (*see Note 6*). Tissue sections were placed on slides (*see Note 7*) for later use in the various histochemical techniques applied in this chapter.

As a general rule, all steps should be performed at room temperature unless otherwise specified.

3.1 Lectin Histochemical Technique

1. Deparaffinize the slides in o-xylene, 3 × 5 min (*see Note 8*).
2. Rehydrate with ethanol of decreasing gradation (100°, 90°, 70°), 3 × 5 min each.

3. Wash in distilled water, 2×2 min.
4. Wash in TBS, 5 min.
5. Quench the endogenous peroxidase activity by incubating the samples in TBS + H_2O_2 for 30 min (*see Note 9*).
6. Wash in TBS, 3×5 min.
7. Incubate the samples with the lectin at the concentration of $12 \mu\text{g}/\text{ml}$ for 90 min in humidity chamber at room temperature.
8. Wash in TBS, 3×5 min.
9. Immerse in TBS containing 0.05 % DAB and 0.015 % H_2O_2 to demonstrate peroxidase-containing sites.
10. Counterstain with Haematoxylin for 5 s (*see Note 10*).
11. Dehydrate with alcohols of increasing gradation (100° , 90° , 70°), 3×5 min each.
12. Clarify with o-xylene, 3×1 min (*see Note 11*).
13. Mount with an appropriate mounting medium (e.g., DPX), and coverslip (*see Note 12*).

3.2 Observation of Apoptotic Cells by Lectin Histochemistry

In the case of the Syrian hamster testis, an increased positivity of some lectins as PNA and Concanavalin-A (Con-A) both at the plasma membrane and the cytoplasm in spermatocytes and round spermatids entering apoptosis is observed (Fig. 1).

3.3 TUNEL Histochemical Technique

This procedure may be performed before processing the slides for the lectin protocol described in Subheading 3.1. It is very important to use DNase-free H_2O with this technique for both the buffers and whenever the use of water is specified.

1. Deparaffinize and rehydrate the slides in a Coplin jar, as described in Subheading 3.1, steps 1–3.
2. Wash in PBS ($1\times$) for 10 min.
3. Permeabilize with Cytonin: put a drop of $50 \mu\text{l}$ of Cytonin per section and incubate the slides in a humidity chamber for 30 min at room temperature.
4. In a Coplin jar, wash in DNase free H_2O , 2 min
5. Incubate the samples in 3 % H_2O_2 for 5 min, to block endogenous peroxidases (*see Note 13*).
6. Wash in DNase-free H_2O 1 min
7. Wash in $1\times$ TdT Labeling Buffer for 5 min at room temperature (*see Note 14*).
8. Prepare in a 1.5 ml microtube the Labeling Reaction Mix containing $1 \mu\text{l}$ of TdT dNTP Mix (*see Note 15*), $1 \mu\text{l}$ of $50\times \text{Mn}^{+2}$ (*see Note 15*), $50 \mu\text{l}$ of $1\times$ TdT Labeling Buffer (*see step 7*), $1 \mu\text{l}$ of TdT Enzyme (taken directly from the freezer) per sample.

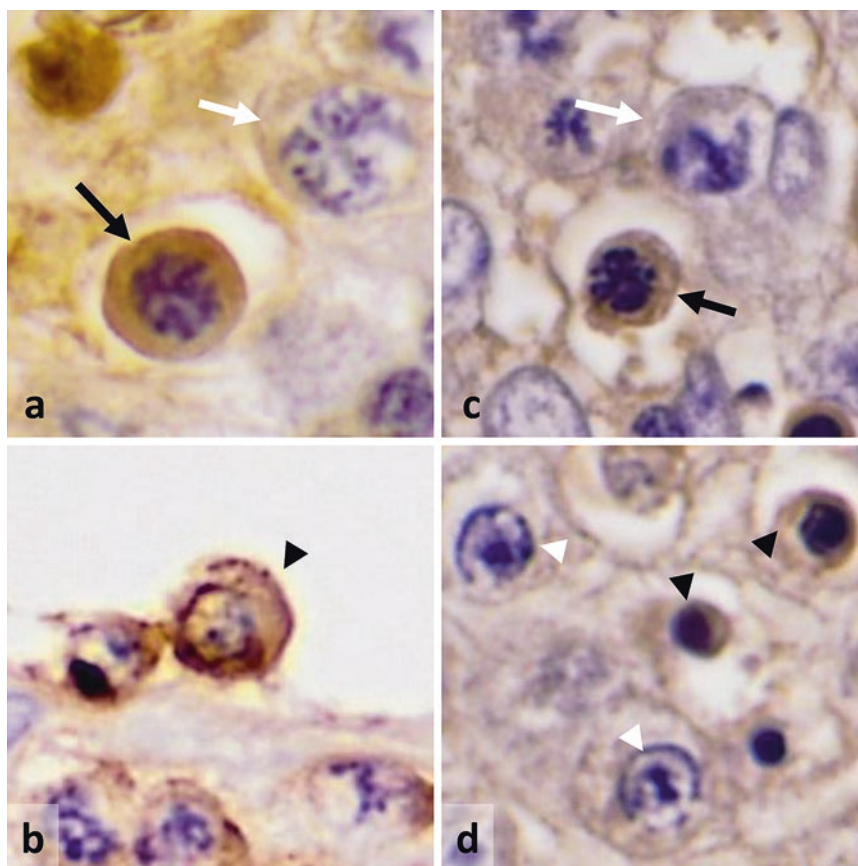


Fig. 1 Apoptotic spermatocytes and spermatids detected by using the lectin histochemistry technique counterstained with haematoxylin. (a–b) PNA lectin histochemistry showing in (a) an apoptotic spermatocyte (arrow) near a normal spermatocyte (white arrow) and in (b) an apoptotic round spermatid with evident signs of apoptosis strongly labeled (arrowhead). (c–d) Con-A lectin histochemistry showing in (c) an apoptotic spermatocyte (arrow) near a normal spermatocyte (white arrow) and in (d) apoptotic round spermatids labeled (arrowheads) near normal round (white arrowheads). Objective magnification $\times 40$

9. Put a drop of 50 μ l of the Labeling Reaction Mix on each section and incubate in a humidity chamber for 1 h at 37 °C.
10. In a Coplin jar, dip the slides for 5 min at room temperature in the stop solution (5 ml TdT Stop Buffer + 45 ml DNase free H₂O).
11. Wash in PBS (1 $\times$), 2 $\times$ 5 min.
12. Prepare the Streptavidin-HRP working solution: 1 μ l Streptavidin-HRP + 750 μ l blue Streptavidin-HRP diluents.
13. Incubate with 50 μ l Streptavidin-HRP working solution per sample in the humidity chamber for 10 min.
14. Wash in PBS (1 $\times$), 2 $\times$ 5 min.

15. To reveal TUNEL-positive cells, add 50 μ l of TACS Blue Label to each section and incubate in a humidity chamber at room temperature for 2–10 min (*see Note 16*).
16. Wash the samples in distilled H₂O for a few seconds in a Coplin jar.
17. Counterstain with Nuclear Fast Red for 90 s (*see Note 17*) in a Coplin jar.
18. Wash the samples in distilled H₂O for a few seconds in a Coplin jar.
19. Dehydrate and mount the slides as described in Subheading 3.1, steps 11–13.

3.4 Comparison of the Sensitivity of the Two Techniques for Detecting Germ Cell Apoptosis

Once the apoptotic cells have been characterized with the different lectins, the TUNEL technique can be applied in order to confirm that these cells are in apoptosis and to compare this last results obtained in the lectin histochemistry.

For the comparative study of the sensitivity between TUNEL and lectin techniques, the TUNEL technique must be first performed and microscope images of complete tubular sections acquired (*see Note 18*). The slides are then dismounted by immersing in xylene until the coverslip separates (*see Note 19*), and after, the lectin histochemical technique is applied (*see Note 20*). Images exactly corresponding to those obtained in the TUNEL technique are acquired to carry out a comparative study in the same tubular section.

In the example given (*see Table 1*) with both images taken, the following counts were made:

- Total number of each germ cell type per tubular section.
- Total number of each TUNEL-positive germ cell type.
- Total number of each lectin-positive germ cell type.
- Total number of each TUNEL-positive and lectin-positive germ cell type.

For the correct identification of each cell type, using the image analysis software Image J, both images were merged (Fig. 2). For the statistical analysis, a one-way ANOVA was conducted followed by a *post hoc* test contrasting equality between pairs of means, using the least significance difference test and Bonferroni method. The results, which are presented as average ($\pm$ SEM) in Table 1, were considered statistically significant when the P value was less than 0.05. The SPSS 19 statistical software package was used for these analyses.

The results obtained for each lectin studied showed that the histochemical technique for the PNA and Con-A lectins had significantly higher sensitivity ($P < 0.05$) than the TUNEL technique for identifying apoptotic germ cells (Table 1). In addition, for both

Table 1
Semiquantitative study of the apoptotic cell types per tubular section using the TUNEL histochemical technique and lectin histochemical technique

Histochemical technique for TUNEL, PNA and Con-A lectins			
	TUNEL+	PNA+	TUNEL+/PNA+
% of round spermatids	14.88 ± 4.81 ^a	94.34 ± 5.26 ^b	10.70 ± 3.32 ^a
% of spermatocytes	4.85 ± 1.20 ^a	15.66 ± 1.94 ^b	1.80 ± 0.46 ^a
	TUNEL+	Con-A+	TUNEL+/Con-A+
% of round spermatids	2.42 ± 0.94 ^a	12.84 ± 3.32 ^b	2.42 ± 0.94 ^a
% of spermatocytes	3.84 ± 0.71 ^a	16.31 ± 2.04 ^b	3.65 ± 0.73 ^a

a and b represent significant differences between techniques when *P* value <0.05

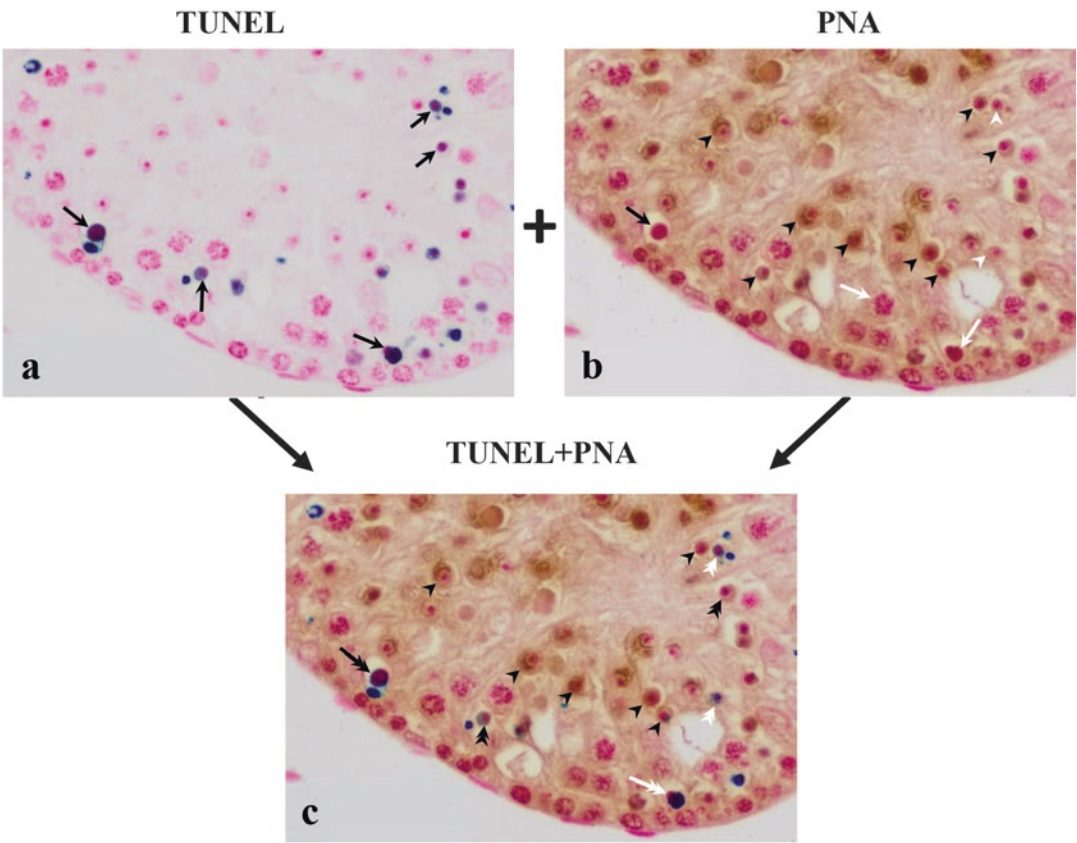


Fig. 2 Operational chart for merging the images studied using an image analysis software. **(a)** Image taken with TUNEL histochemistry where TUNEL + germ cells (*arrows*) can be seen. **(b)** Image taken with the lectin histochemical technique and counterstained with Nuclear Fast Red. A large number of round spermatids (*arrowheads*) and a spermatocyte (*arrow*) PNA + alongside round spermatids (*white arrowheads*) and spermatocytes (*white arrow*) PNA- are identified. **(c)** Result of merging the images **(a)** and **(b)**. Note that in TUNEL + cells image **(a)** there are as many spermatocytes (*double arrow*) as round spermatids (*double arrowheads*) both TUNEL +/PNA +. Also round spermatids TUNEL–/PNA + (*arrowheads*) and round spermatids (*double white arrowheads*) and a spermatocyte (*double white arrow*) TUNEL +/PNA- can be observed. Objective magnification ×40

lectins, the apoptotic cell population identified as TUNEL-positive was similar to that seen by adding the TUNEL-positive and Lectin-positive populations.

In conclusion, lectin histochemistry is a very useful tool for the histological identification of apoptotic cells in the seminiferous epithelium as it is able to detect germ cells from very early until very advanced stages of apoptosis, making it a technique with great sensitivity to this type of study. However, in order to determine which of the lectins is the most effective for revealing the apoptotic cell population of a given tissue, previous histochemical studies using the TUNEL technique are always advisable.

4 Notes

1. The humidity chambers can be made using petri plates. Place paper on the base and two glass rods to prevent the slides from directly touching the paper. Before use, wet the paper with water and remove the excess.
2. Dilute the lectins in TBS at a concentration of 100 µg/ml and store frozen in 1 ml microtubes. When needed, thaw one microtube and make the necessary dilution.
3. The DNase-free H₂O can be purchased commercially, or ultra-pure water can be used. In our lab, we use distilled water boiled for 30 min. When it has cooled to room temperature, it can be used as solvent for buffers and reagents.
4. After about 30 min, cut the testis into two or three pieces to allow proper fixation of the central portion. If sample processing is not continued, change the fixative for PBS buffer in order to avoid overfixed tissue.
5. The times of paraffin embedding may vary with the model of tissue processor. We recommend a minimum time of 15 h for this step to ensure proper inclusion.
6. In our tissue used, testis, sections should not be more than 5 µm thick to prevent overlapping of cell nuclei and elements that could be positive in the techniques used but belonging to cells that are at different depths.
7. The slides must be previously dipped in poly-L-lysine to prevent the tissue section from coming off the slide when the histochemical techniques are being carried out. Moreover, once the tissue sections have been placed on the slides it is strongly recommended to wait at least 24 h for correct fixing to the slide.
8. Dewaxing is also possible in o-xylene for 15 min in a Coplin jar. This o-xylene can be reused several times (5-8 times) according to how many slide have been dewaxed.

9. For this, mix 1 ml of 30 % H_2O_2 in 100 ml of TBS.
10. Normally, the lectin histochemistry is counterstained with haematoxylin. If you want to study the sensitivity of lectin histochemistry against the TUNEL counterstain with Nuclear Fast Red as described in Subheading 3.2, **step 17**.
11. For the step from 100% alcohol to xylene, it is necessary to drain the slides well by vigorous stirring without letting any sections dry out. Then put the slides in the xylene and shake gently to generate a current to remove any traces of alcohol in the tissue section. If this is not done properly, the tissue will dry and the sample may be lost.
12. Check that there are no air bubbles in the tissue. If there are any, press the tissue gently with the end of tweezers next to the bubble to remove it. Never move the coverslip because you can tear the tissue.
13. Mix 5 ml of 30 % H_2O_2 + 45 ml of Methanol.
14. Prepare 45 ml DNase-free H_2O + 5 ml 10× TdT labeling buffer. Important! Take 50 μl per sample for the next step. For example, if you have 10 samples, use a microtube to take 500 μl of 1× TdT Labeling Buffer for **step 8**.
15. Both the TdT dNTP Mix and 50× Mn^{+2} are frozen. Remove from freezer few minutes before use.
16. For development with TACS Blue Label it is necessary to wait for 5 min after placing the 50ul in each of the samples. When using other tissues it may be necessary to adjust the time. This can be done by directly observing the reaction on a light microscope. An excessive exposure time to TACS Blue Label will probably lead to the appearance of a black-colored background in the sample.
17. Time contrast with the Nuclear Fast Red is indicative. The staining does not lose positivity so it is better to leave it a little longer than necessary rather than to remove it before time.
18. Because the positivity of TUNEL histochemistry is progressively lost with the passage of time it is recommended to acquire the necessary images as soon as possible.
19. The time required for the coverslip to separate from the slides will depend on the time that the slides were mounted. For removing slides mounted a week earlier, the time required to separate the coverslips should not exceed 24 h.
20. In this case, a wash is performed in xylene to remove traces of the mounting medium that may be attached to that tissue.

Acknowledgment

This work was supported by GERM 19892/15 from Fundación Seneca CARM.

References

- Häcker G (2000) The morphology of apoptosis. *Cell Tissue Res* 301:5–17
- Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239–257
- Tilly JL, Kowalski KI, Johnson AL et al (1991) Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 129:2799–2801
- Pastor LM, Zuasti A, Ferrer C et al (2011) Proliferation and apoptosis in aged and photoregressed mammalian seminiferous epithelium, with particular attention to rodents and humans. *Reprod Domest Anim* 46:155–164
- Morales E, Ferrer C, Zuasti A et al (2007) Apoptosis and molecular pathways in the seminiferous epithelium of aged and photoinhibited Syrian hamsters (*Mesocricetus auratus*). *J Androl* 28:123–135
- Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456–1462
- Carson DA, Ribeiro JM (1993) Apoptosis and disease. *Lancet* 341:1251–1254
- Bonda-Ostaszewska E, Włostowski T (2015) Apoptosis, proliferation, and cell size in seasonal changes of body and organ weight in male bank voles *Myodes glareolus*. *Mamm Res* 60:255–261. doi:10.1007/s13364-015-0224-2
- Lichtenstein RG, Rabinovich GA (2013) Glycobiology of cell death: when glycans and lectins govern cell fate. *Cell Death Differ* 20:976–986
- Spicer SS (1993) Advantages of histochemistry for the study of cell biology. *Histochem J* 25:531–547
- Pastor LM, Frutos MJ, Graña L et al (1992) Histochemical study of glycoconjugates in the nasal mucosa of the rat and guinea pig. *Histochem J* 24:727–736
- Pastor LM, Ferran A, Calvo A et al (1994) Morphological and histochemical study of human submucosal laryngeal glands. *Anat Rec* 239:453–467
- Kasper M, Singh G (1995) Epithelial lung cell marker: current tools for cell typing. *Histol Histopathol* 10:155–169
- Madrid JF, Hernández F, Ballesta J (1997) Characterization of glycoproteins in the epithelial cells of human and other mammalian gallbladder. A review. *Microsc Res Tech* 38:616–630
- Madrid JF, Leis O, Díaz-Flores L et al (1998) Lectin-gold localization of fucose residues in human gastric mucosa. *J Histochem Cytochem* 46:1311–1320
- Calvo A, Pastor LM, Horn R et al (1995) Histochemical study of glycoconjugates in the epididymis of the hamster (*Mesocricetus auratus*). *Histochem J* 27:670–680
- Calvo A, Pastor LM, Bonet S et al (2000) Characterization of the glycoconjugates of boar testis and epididymis. *J Reprod Fertil* 120:325–335
- Morales E, Polo LA, Pastor LM et al (2005) Characterization of corpora amylacea glycoconjugates in normal and hyperplastic glands of human prostate. *J Mol Histol* 36:235–242
- Avilés M, El-Mestrah M, Jaber L et al (2000) Cytochemical demonstration of modification of carbohydrates in the mouse zona pellucida during folliculogenesis. *Histochem Cell Biol* 113:207–219
- Pastor LM, Lucas X, Pallares J et al (2008) Characterization of glycoside residues of porcine zona pellucida and ooplasm during follicular development and atresia. *Mol Reprod Dev* 75:1473–1483
- Roth J, Zuber C, Komminoth P et al (1996) Applications of immunogold and lectin-gold labeling in tumor research and diagnosis. *Histochem Cell Biol* 106:131–148
- Roth J (2011) Lectins for histochemical demonstration of glycans. *Histochem Cell Biol* 136:117–130
- Taatjes DJ, Sobel BE, Budd RC (2008) Morphological and cytochemical determination of cell death by apoptosis. *Histochem Cell Biol* 129:33–43
- Bilyy RO, Antonyuk VO, Stoika RS (2004) Cytochemical study of role of alpha-d-mannose- and beta-d-galactose-containing glycoproteins in apoptosis. *J Mol Histol* 35:829–838
- Bilyy RO, Stoika RS (2003) Lectinocytochemical detection of apoptotic murine leukemia L1210 cells. *Cytometry A* 56:89–95

26. Franz S, Frey B, Sheriff A et al (2006) Lectins detect changes of the glycosylation status of plasma membrane constituents during late apoptosis. *Cytometry A* 69:230–239
27. Malagolini N, Chiricolo M, Marini M et al (2009) Exposure of alpha 2,6-sialylated lactosaminic chains marks apoptotic and necrotic death in different cell types. *Glycobiology* 19:172–181
28. Azuma Y, Inami Y, Matsumoto K (2002) Alterations in cell surface phosphatidylserine and sugar chains during apoptosis and their time-dependent role in phagocytosis by macrophages. *Biol Pharm Bull* 25:1277–1281
29. Bilyy R, Stoika R (2007) Search for novel cell surface markers of apoptotic cells. *Autoimmunity* 40:249–253
30. Liguoro A, Prisco M, Mennella C et al (2004) Distribution of terminal sugar residues in the testis of the spotted ray *Torpedo marmorata*. *Mol Reprod Dev* 68:524–530
31. Seco-Rovira V, Beltrán-Frutos E, Ferrer C et al (2013) Lectin histochemistry as a tool to identify apoptotic cells in the seminiferous epithelium of Syrian hamster (*Mesocricetus auratus*) subjected to short photoperiod. *Reprod Domest Anim* 48:974–983

Part III

Protein Histochemistry

Heat-Induced Antigen Retrieval in Immunohistochemistry: Mechanisms and Applications

Shuji Yamashita and Osamu Katsumata

Abstract

Heat-induced antigen retrieval (HIAR) is an essential technique for current immunohistochemistry. We describe the mechanisms of HIAR and its protocols for formalin-fixed, paraffin-embedded specimens and frozen sections, which are the most popular materials for immunohistochemistry using light microscopy. In addition, we describe the antigen retrieval method for highly masked epitopes and double immunostaining using the horseradish peroxidase (HRP)-labeled antibody method and immunofluorescence method in this chapter.

Key words Heat-induced antigen retrieval, Formalin-fixed, paraffin-embedded tissues, Frozen tissues, Highly masked epitopes, HRP-labeled antibody method, Immunofluorescence method, Double immunostaining

1 Introduction

Since immunohistochemistry is used for identifying the localization of cellular or tissue constituents (antigens) based on antigen–antibody interactions by using labeled antibodies that can be visualized under a microscope, the use of specific primary antibodies and tissue preparation techniques that conserve tissue structure and antigenicity are essential. The conservation of protein conformation was thought to be important for the immunohistochemistry of protein antigens until the end of the 1980s, and many kinds of fixatives, fixation protocols, and tissue preparation techniques were used for immunohistochemical studies. On the other hand, antigen retrieval techniques using enzyme digestion and treatment with protein denaturants were often applied for formalin-fixed and paraffin-embedded (FFPE) materials, particularly in the field of pathology. The development of heat-induced antigen retrieval (HIAR), as reported by Shi et al. in 1991 [1], for FFPE specimens greatly changed the concept of immunohistochemistry, and several investigators have now made references “post-antigen-epitope

retrieval" era. HIAR is now applied to FFPE sections [1, 2], frozen sections [3], cultured cells, physically fixed materials, and plastic embedded specimens for both light and electron microscopy studies [4, 5].

The mechanism of fixation using formaldehyde is thought to be as follows [6, 7]. Formaldehyde forms an adduction of hydroxyl methyl/methylol (CH_2OH) with functional groups of amino acids such as lysine and cysteine and with an N-terminal of polypeptides and bases of RNA and single strand DNA. A part of the methylol group then forms imines (Schiff base) through the removal of H_2O , and the imines then combine with the side chains of several kinds of amino acids to form methylene bridges ($-\text{CH}_2-$). These adductions, imine formations and crosslinkings progress in a time-dependent and temperature-dependent manner. FFPE specimens are assumed to be highly crosslinked, compared with formalin-fixed frozen sections for the same period. Since ethanol accelerates the imine formation of methylol groups and causes the rearrangement of the β -sheet and exposure of hydrophobic amino acids, which are hidden in aqueous solutions, the crosslinking may advance during dehydration with ethanol [8]. Immersion in paraffin at around 60°C may facilitate further intramolecular and intermolecular crosslinking in proteins and nucleic acids. Thus, these crosslinks interfere with the access of antibodies to antigens even if the epitopes do not have functional groups of amino acids that are directly modified by formaldehyde.

Heat treatment cleaves the crosslinks and methylol groups in polypeptides and nucleic acids and unfolds the polypeptides in tissues [2, 9]. The unfolded polypeptides expose epitopes hidden in the inner portion of antigens or surface epitopes covered by adjacent macromolecules. During the cooling process, the polypeptide chains rapidly refold; the epitopes are presumably concealed in solutions with a neutral pH or high ionic strength because a high concentration of ions may counteract the electrostatic repulsion force and strong hydrophobic attractive forces would entangle the neighboring polypeptides randomly. In solutions with basic or acidic pH values and low ionic strength, however, the majority of the extended polypeptides would be charged negatively or positively, and an electrostatic repulsive force would act to prevent random aggregation and the entanglement of neighboring polypeptides by the hydrophobic force, thereby exposing the epitopes for interaction with antibodies [9]. Some macromolecules should be extracted from the gel-like tissues, enabling the antibodies to penetrate the tissues easily and to interact with the antigens; this process is similar to the effects of digestion with proteinases.

Antibodies recognize epitopes that are composed of 5–20 amino acids but not the entire antigen molecule. There are two types of epitopes, i.e., conformational and linear epitopes. Conformational epitopes are composed of amino acids that are located far apart in their linear sequence but become juxtaposed

when the protein is folded in its native shape. Therefore, heating may be unsuitable for the antigen retrieval of conformational epitopes because of the destruction of protein folding, and proteinase treatment might be effective for these epitopes. Linear epitopes are composed of a particular stretch of consecutive amino acids. Epitopes located on the surface of protein molecules might react with antibodies independent of whether the protein is in its native or denatured state. Meanwhile, internal linear epitopes that might not bind to antibodies when the protein is in its native or fixed state may bind to antibodies when the protein is denatured and extended after heating [9].

We describe the HIAR protocols for FFPE specimens and frozen sections, which are the most popular materials for immunohistochemistry using light microscopy. In addition, we describe the antigen retrieval method for highly masked epitopes and double immunostaining using the horseradish peroxidase (HRP)-labeled antibody method and immunofluorescence method in this chapter.

2 Materials

2.1 Antigen Retrieval Solutions

1. 0.2 M Tris-HCl buffer, pH 9.0: Store at room temperature. Dilute tenfold to use (*see Note 1*).
2. 0.1 M citrate buffer, pH 6.0: Prepare 0.1 M citric acid and 0.1 M sodium citrate. Adjust pH 6.0 by adding 0.1 M citric acid into 0.1 M sodium citrate. Store at 4 °C. Dilute tenfold to use.
3. 0.1 M citrate buffer, pH 3.0: Adjust pH 3.0 by adding 0.1 M sodium citrate into 0.1 M citric acid. Store at 4 °C. Dilute tenfold to use.

2.2 Immunostaining for FFPE

1. 0.1 M phosphate buffer, pH 7.4: Prepare 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 . Adjust pH 7.4 by adding 0.1 M NaH_2PO_4 into 0.1 M Na_2HPO_4 .
2. Phosphate buffered-saline (PBS), 10 mM phosphate buffer, pH 7.4 containing 0.15 M NaCl: Dissolve 86.6 g of NaCl in 1 L of 0.1 M phosphate buffer, pH 7.4. Dilute tenfold to use.
3. Tris-buffered saline (TBS), 10 mM Tris-HCl, pH 7.4 containing 0.15 M NaCl (*see Note 2*): Dissolve 86.6 g of NaCl in 1 L of 0.1 M Tris-HCl buffer, pH 7.4. Dilute tenfold to use.
4. Blocking solution: TBS containing 1 % bovine serum albumin (BSA, Fraction V) and 0.05 % sodium azide (*see Note 3*).
5. Primary antibody: Dilute to appropriate concentration using the blocking solution (*see Note 4*).
6. Normal IgG from the same species as the primary antibody: Dilute to the same concentration as the primary antibody using the blocking solution.

7. Envision Plus System-HRP labeled polymer (anti-rabbit or anti-mouse) (DakoCytomation, Glostrup, Denmark) (*see Note 5*).
8. Buffer for DAB (3,3' diaminobenzidine tetra hydrochloride) reaction (0.1 M imidazole/0.5 M Tris-HCl buffer, pH 7.4): Dissolve 60.5 g of Tris hydroxylaminomethane and 6.8 g of imidazole in about 700 mL of distilled water and adjust pH to 7.4 by adding concentrated HCl. Make up to 1 L with distilled water. Store at 4 °C.
9. 1 % hydrogen peroxide: Dilute 35 % hydrogen peroxide with distilled water. Store at 4 °C.
10. Imidazole-DAB solution: Mix 10 mL of buffer for the DAB reaction and 90 mL of distilled water and dissolve 40 mg of DAB. Add 1.5 mL of 1 % hydrogen peroxide just before the start of the reaction (*see Note 6*).
11. Ni-Co-DAB solution: Dissolve 50 mg of DAB, 25 mg of CoCl_2 , and 20 mg of $\text{NiSO}_4 (\text{NH}_4)_2$ in 100 mL of 0.1 M phosphate buffer, pH 7.4. Add 1 mL of 1 % hydrogen peroxide just before the start of the reaction (*see Note 6*).
12. Xylene.
13. 100 % ethanol, 70 % ethanol.
14. Meyer's hematoxylin.
15. 0.05 % lithium carbonate.
16. Embedding medium for light microscopy (e.g., Malinol).
17. High-pressure steam sterilizer (e.g., Tomy BS-235, Tomy Seiko Co., Tokyo, Japan) (*see Note 7*).
18. Silan coated glass slides.
19. Coverslips.
20. Metal slide holder.
21. Square glass staining box.
22. 500 mM Griffin beaker.
23. Moisture chamber.
24. Styrol box.
25. Filter paper or kitchen towel.
26. PAP pen.
27. Light microscope.

2.3 HIAR for Fresh Frozen Sections

1. 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH buffer, pH 7.4.
2. Formalin.
3. Fixative, 10 % formalin containing 25 mM CaCl_2 /0.1 M HEPES buffer, pH 7.4 (*see Note 8*): Mix 400 mL of distilled water, 50 mL of formalin, 50 mL of 1 M HEPES buffer, and 1.38 g of CaCl_2 . Store at room temperature.

4. Cryostat.
5. Hair dryer or electric fan.
6. Microwave oven.

2.4 Antigen Retrieval for Highly Masked Antigens

1. 0.1 M dithiothreitol (DTT)/20 mM Tris-HCl, pH 9.0.
2. 0.1 M iodoacetamide/0.1 M Tris-HCl, pH 9.0.

2.5 Immuno-fluorescence Antibody Method

1. Alexa Fluor donkey anti-mouse IgG 549 (Molecular Probes, Eugene, OR).
2. Alexa Fluor donkey anti-rabbit IgG 488 (Molecular Probes, Eugene, OR).
3. DAPI (4',6-diaminidino-phenylindole dihydrochloride) solution: Dissolve 1 mg of DAPI in 1 mL of distilled water. Store at 4 °C in the dark. Dilute 2000-fold with TBS to use.
4. Fluoromount.
5. Fluorescence microscope.

3 Methods

3.1 Antigen Retrieval of FFPE Specimens

FFPE materials are widely used for histological studies because they provide good morphology and are easy to store for long periods and because FFPE specimens are commonly collected as pathological, surgical and autopsy specimens in many laboratories. The HRP-labeled antibody method is the most popular for immunohistochemistry because this technique does not require any special equipment and immunostained sections are stored almost permanently in a light proof box. This method is a convenient and quick means of studying a wide range of tissues and cell types that exhibit positive immunostaining (Fig. 1A–D).

1. Mount PFFPE section (5 µm) on a Silan-coated glass slide.
2. Place the slide glass in a metal slide holder.
3. Deparaffinize in xylene at room temperature (4 × 3 min) in a square glass staining box.
4. Immerse in 100 % ethanol (4 × 1 min).
5. Immerse in 70 % ethanol (1 min).
6. Wash with distilled water (3 × 1 min).
7. Soak in antigen retrieval solution (300–350 mL) in a 500 mM Griffin beaker.
8. Autoclave in a high-pressure sterilizer at 121 °C for 10 min.
9. Remove the beaker from the sterilizer at 70–80 °C.
10. Place the beaker in tap water in a styrol box and cool to room temperature.

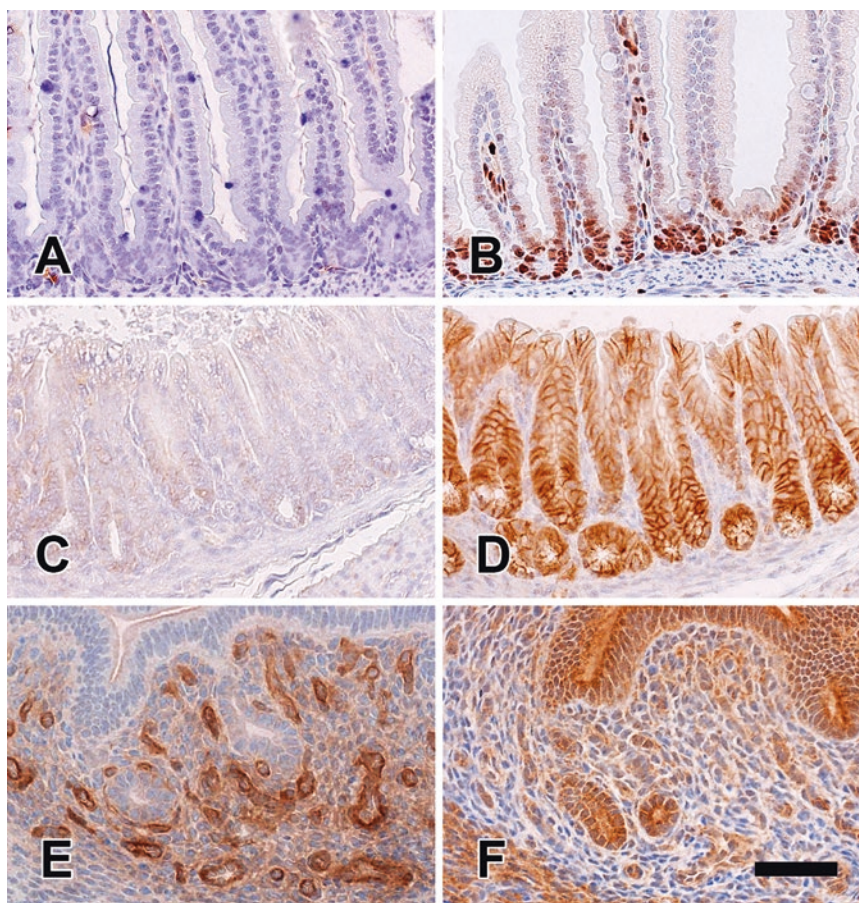


Fig. 1 Effect of HIAR and retrieval solution in FFPE and frozen sections. **(A, B)** Localization of proliferating cell nuclear antigen (PCNA) in the mouse small intestine of FFPE specimens. PCNA was immunostained without heat treatment **(A)** and after autoclaving in the 20 mM Tris–HCl, pH 9.0 at 121 °C for 10 min **(B)**. Epithelial cells locating in the basal portion of intestinal gland show the positive immunoreactions in the nuclei **(B)**. **(C, D)** Localization of β -catenin in the mouse stomach in FFPE sections. β -catenin was localized without heat treatment **(C)** and after autoclaving in the 20 mM Tris–HCl, pH 9.0 **(D)**. β -catenin immunoreactions is seen along the cell membrane of glandular and luminal epithelial cells after HIAR **(D)**. **(E)** Neuropilin-1 distribution in the frozen section in 20-day-old mouse uterus. Fresh frozen sections were fixed 10 % formalin in 0.1 M HEPES buffer, pH 7.4 containing 25 mM CaCl_2 for 5 min and heated in 20 mM Tris–HCl buffer, pH 9.0 at 95 °C for 30 min, and then immunostained. Positive reaction is seen in the endothelial cells and some vascular smooth muscle cells. **(F)** Localization of vascular endothelial cell growth factor (VEGF) in the 20-day-old mouse uterus. After frozen sections were fixed with 10 % formalin/0.1 M HEPES buffer, pH 7.4 containing 25 mM CaCl_2 , the sections were heated in 10 mM citrate buffer, pH 3.0 for 30 min. VEGF-immunoreaction is demonstrated in the cytoplasm of epithelial cells, vascular smooth muscle cells, and myometrial cells. Scale bar = 50 μm

11. Wash the sections with PBS (3×1 min), and then with TBS (1 min) in the staining box (*see Note 9*).
12. Add about 2 mL of blocking solution to the TBS and gently dunk a few times (*see Note 10*).
13. Wipe the solution with a filter paper, leaving the solution around sections, and draw a circle around the section with a PAP pen.

14. Cover the section with a blocking solution (200–300 μL) and incubate for 1 h at room temperature in a moisture chamber.
15. Blot the blocking solution using a filter paper.
16. Incubate the section with the primary antibody (about 100 μL) in a moisture chamber overnight at 4 °C. Incubate with normal IgG as a negative control (*see* **Note 11**).
17. Wash the sections with PBS (6 $\times$ 5 min).
18. Incubate with Envision Plus System-HRP labeled polymer for 1 h at room temperature in a moisture chamber.
19. Wash the section with PBS (6 $\times$ 5 min).
20. Incubate with imidazole-DAB solution at room temperature for 1–10 min while monitoring the reaction under a light microscope.
21. Wash the sections with distilled water (5 $\times$ 1 min).
22. Counterstain with Meyer's hematoxylin for 1 min.
23. Wash the sections under running tap water for 5 min.
24. Dip in 0.05 % lithium carbonate solution several times.
25. Wash with tap water for 5 min.
26. Dehydrate with 100 % ethanol (4 $\times$ 1 min).
27. Clear in xylene (4 $\times$ 1 min).
28. Mount the section with the appropriate mounting medium (e.g., Malinol).

3.2 Antigen Retrieval for Fresh Frozen Sections

Fresh frozen sections are convenient for examining the suitability of fixatives for target antigens [3, 10]. Many antigens show positive immunoreactions in FFPE sections using HIAR, whereas some antigens fixed with formalin are inactivated during paraffin embedding. Coagulating fixation (physical fixation) with organic solvents is required for the detection of few antigens that show no immunostaining in tissues fixed with formalin. HIAR is also useful for formalin or formaldehyde-fixed frozen sections (Fig. 1E, F). The application of HIAR to fresh frozen sections should provide more reproducible results than application to FFPE sections because the fixation time is precisely controlled and the fixation is uniform throughout the sections, whereas the penetration of fixative is not homogeneous and fixation at deep portions is insufficient in tissue blocks.

1. Prepare frozen sections (5 μm) with a cryostat.
2. Mount the sections on a Silan-coated glass slide.
3. Dry the sections with a hairdryer using cool air for at least 30 min (*see* **Note 12**).
4. Immerse the sections in the fixative in a glass staining box for 5 min at room temperature (*see* **Note 13**).

5. Wash the sections with distilled water (5×1 min).
6. Immerse the sections in an antigen retrieval solution and maintain about 95°C in a microwave oven for 30 min (*see Note 14*).
7. Immunostain and mount the sections according to the procedures described for FFPE sections (**steps 10–28**).

3.3 Antigen Retrieval for Highly Masked Epitopes

A few antibodies react with polypeptides in western blotting, whereas they yield negative immunostaining in tissues even after HIAR. In such cases, the epitopes might be located in a highly folded portion of the protein or they might be covered by neighboring heat-resistant polypeptides in which multiple disulfide bonds may form stable secondary and tertiary structures. The reduction of disulfide bonds using dithiothreitol or 2-mercaptoethanol prior to heat treatment can sometimes enable strong immunoreactions (Fig. 2), [10].

1. Prepare rehydrated FFPE sections or the frozen section fixed with 10 % formalin containing 25 mM CaCl_2 .
2. Draw a circle around the section using a PAP pen.
3. Place about 300 μL of 0.1 M DTT/20 mM Tris-HCl, pH 9.0 on the sections and incubate for 1 h at room temperature in a moisture chamber.
4. Wash with distilled water (3×1 min).
5. Incubate the sections with about 300 μL of 0.1 M iodoacetamide/0.1 M Tris-HCl, pH 9.0 for 15 min at room temperature in a moisture chamber.
6. Wash with distilled water.
7. Perform HIAR and immunostaining according to the procedures described for FFPE sections and frozen sections.

3.4 Double Immunostaining

Immunohistochemical double staining is powerful technique to study the topological and functional relationships of two target antigens and to identify cell types for diagnosis.

3.4.1 HRP-Labeled Antibody Method

Double immunostaining using the HRP-labeled antibodies is sequentially performed as follows. The first target antigen is visualized with imidazole-DAB reaction (brown) after HIAR or without HIAR in FFPE and frozen sections. The sections are then heated to inactivate HRP enzyme activity and antigenicity of HRP-labeled antibody, and then the second target antigen is shown by Ni-Co-DAB (dark purple), (Fig. 3A, B), (*see Note 15*).

1. Heat the FFPE or fixed frozen sections in antigen retrieval solution (*see Note 16*).
2. Treat the sections with the blocking solution, the primary antibody and HRP-labeled antibody. Visualize the antigen using the imidazole-DAB solution.

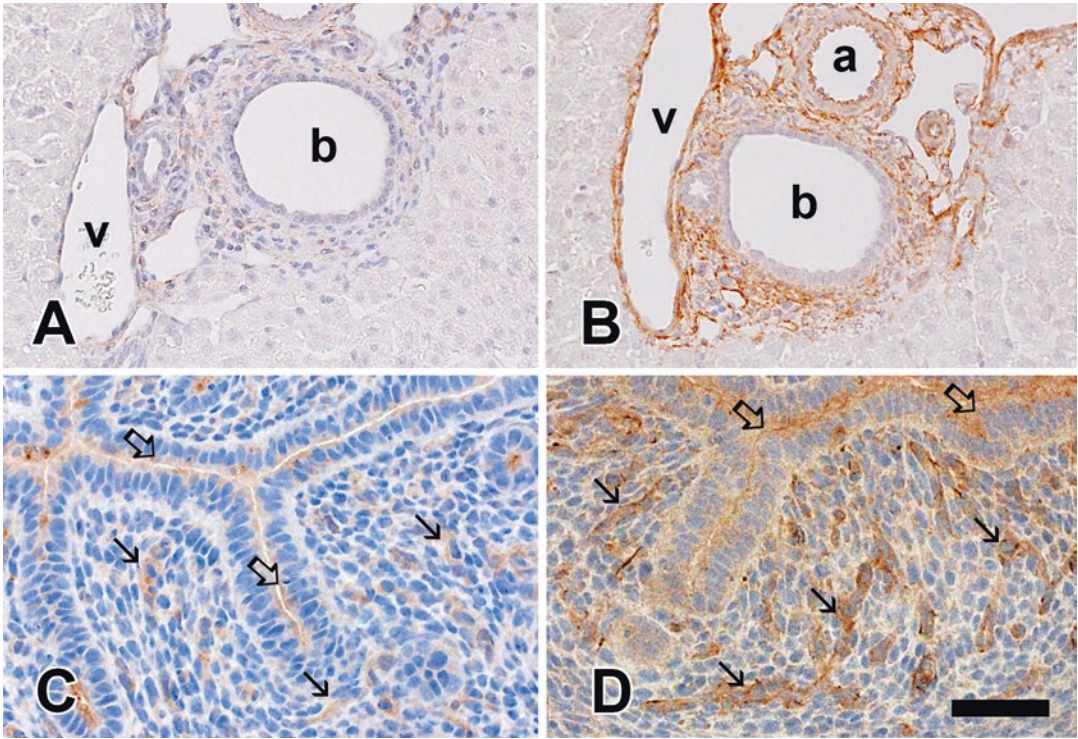


Fig. 2 HIAR of highly masked antigens in the FFPE and frozen tissues. (**A, B**) Localization of vascular endothelial cell growth factor receptor-1 (VEGFR1) in the mouse liver of FFPE sections. Sections were treated with dithiothreitol and iodoacetamide to unfold polypeptides completely and to expose epitopes and then autoclaved in 20 mM Tris-HCl, pH 9.0 (**B**) and sections without pretreatment preceding autoclaving (**A**). VEGFR1-immunoreaction is seen along the endothelial cells in the artery (*a*) and vein (*v*) and in the interlobular connective tissues (**B**), whereas it is hardly detected without pretreatment (**A**). Epithelial cells in the interlobular bile ducts (*b*) and liver cells are negative for the immunostaining. (**C, D**) Tyrosine kinase with immunoglobulin-like and EGF-like domain 2 (TIE2) localization in the frozen sections from 10-day-old mouse uterus. Fresh frozen sections were fixed 10 % formalin in 0.1 M HEPES buffer, pH 7.4 containing 25 mM CaCl_2 for 5 min. The sections were treated with dithiothreitol and iodoacetamide (**D**) or without the treatment (**C**) and then heated in 20 mM Tris-HCl buffer, pH 9.0 at 95 °C for 30 min. Strong immunoreaction for TIE2 is present in the endothelial cells (*arrows*) and apical membrane of epithelial cells (*outlined arrows*) in the uteri treated with dithiothreitol and iodoacetamide (**D**), whereas a faint immunostain is observed blood vessels (*arrows*) and luminal membrane of epithelial cells (*outlined arrows*) when HIAR is done without pretreatment (**C**). Scale bar = 50 μm

3. Heat the sections in antigen retrieval solution for 15 min at 95 °C.
4. Treat the sections with the blocking solution, antibody to the second target antigen and HRP-labeled antibody. Visualize the antigen localization with the Ni-Co-DAB solution.

3.4.2 Immunofluorescence Method

Using the immunofluorescence method, the precise distribution of an antigen in cell organelles can be obtained thanks to the high resolution and high contrast of this method (Fig. 3C, D). The immunofluorescence method is easy and suitable for the detection of colocalized antigens using double immunostaining technique, since precipitated DAB reaction products around antigens may

hinder the second antibody from interacting with the target second antigen when using the HRP-labeled antibody method (Fig. 3D). When two antibodies are generated in different species, primary antibodies and labeled antibodies are simultaneously applicable, respectively. Both FFPE sections and frozen sections can be used with the immunofluorescence method.

1. Treat the sections with the blocking solution after HIAR.
2. Incubate with a mixture of the primary antibodies diluted with the blocking solution (*see* **Note 17**) overnight at 4 °C in a moisture chamber.
3. Wash the sections with PBS (6 × 5 min).
4. Incubate with a mixture of fluorescein-labeled antibodies diluted 500-fold with the blocking solution for 1 h in a moisture chamber shielded from light.
5. Wash the section with PBS (6 × 5 min)
6. Incubate the sections with DAPI solution for 20 min at room temperature in a moisture chamber shielded from light.
7. Wash the section with PBS (6 × 5 min).
8. Blot excess PBS and mount the section with a cover glass using Fluoromount.
9. Dry the Fluoromount for 1 h in the dark (*see* **Note 18**).
10. Examine the sections under a fluorescence microscope.

4 Notes

1. The pH and ionic strength of the retrieval solution are critical factors for HIAR, as described above [11, 12]. Tris-HCl buffer, pH 9.0, yields the strongest immunoreaction for most antigens. Citrate buffer (CB), pH 6.0, is a popular solution for HIAR. CB buffer, pH 6.0, may be suitable for examining detailed nuclear structures in pathological studies, since heating in basic solutions cleaves and extracts RNAs and reduces the nuclear stainability with hematoxylin. Heating in an acidic (pH 3.0 or pH 6.0) frequently enables excellent immunostaining for the detection of basic proteins or epitopes composed of basic amino acids (Fig. 1F), [13]. Therefore, at least three antigen retrieval solutions at pH 3.0, pH 6.0 and pH 9.0 should be used to localize unknown antigens for the first time.
2. Although TBS is the standard diluent for primary antibodies and yields stronger immunoreactions than PBS for most antigens, other buffers such as 50 mM NaCl in 10 mM Tris-HCl, pH 7.4, 300 mM NaCl in 10 mM Tris-HCl, pH 7.4, or commercially available diluents for immunohistochemistry and

western blotting sometimes provide more specific and stronger reactions than TBS. Since an epitope and the corresponding paratope of the antibody have unique three dimensional structures, the most suitable binding condition may not be constant for each immunoreaction. Electrostatic forces between the antigen and antibody molecules and the net electrostatic charges around the antigens may affect the antigen-antibody association during the first step, and then they may tightly bind flexibly changing conformation of epitope and paratope. Thus, the types of buffers, the ionic strength, and the presence of detergents in the solutions are likely to exert strong influences on the antigen-antibody interaction.

3. Some primary antibodies show a high background. HIAR at pH 3.0 frequently facilitates nonspecific binding between antibodies and tissues. Blocking with 3 % skim milk in TBS is effective for reducing such nonspecific bindings.
4. At least 50 % of commercially available antibodies have no or very low specificity to antigens when used for immunohistochemical and western blot analyses. Information from published papers and investigators is important for selecting antibodies with established reputations. Polyclonal antibodies from different lot numbers frequently have different affinities and titers, whereas monoclonal antibodies prepared from the same clones may provide stable results. If a polyclonal antibody produces reliable results, antibody with the same lot number should be stocked until the completion of the study. When an antibody with the same lot number is not available, the used antibody solution may be recovered from sections and stored for reuse. Antibodies should be diluted as much as possible to conserve them and to reduce background staining.
5. Linear polymers conjugating multiple HRP and antibodies, such as Envision plus and ImmPRESS (Vector Laboratories), enable a high sensitivity and are suitable for antigen detection through the application of the HIAR technique. Although the biotin-labeled antibody-ABC method is sensitive and popular, it frequently shows nonspecific staining after heat treatment, presumably because of exposure of endogenous biotins.
6. Although imidazole-DAB solution is sensitive for the detection of HRP enzyme activity around a neutral pH, conventional DAB-H₂O₂ solution is also useful. Both solutions yield brown reaction products which are insoluble in organic solvents. Ni-Co-DAB solution shows the highest sensitivity producing dark purple reaction products and stable for organic solvents. Since the mixture is unstable, it should be prepared immediately before use.

7. The use of several heating devices, such as a high-pressure steam sterilizer, microwave oven, pressure cooker, and water bath, has been examined for HIAR. It is now recognized, however, that the total amount of heat energy applied is more important than the type of heating device that is used. The heating energy required for antigen retrieval may differ for paraffin sections and frozen sections and according to the formalin fixation time. We use a Tomy BS-235 high-pressure steam sterilizer to perform HIAR for FFPE specimens, since it is a compact unit and the temperature can be regulated relatively rapidly.
8. This fixative, 10 % formalin containing 25 mM CaCl_2 is more appropriate for the fixation of fresh frozen sections compared with 10 % formalin. Since it has a stronger fixation ability and crosslinks proteins more rapidly than buffered formalin, the extraction of antigens during fixation is minimized and excellent tissue structure is maintained after heat treatment.
9. The blocking of endogenous peroxidase activity is conventionally recommended for immunohistochemistry using the HRP-labeled antibody method before incubation with the primary antibody. Eosinophils and erythrocytes exhibit positive DAB reactions in frozen sections and FFPE sections. However, the blocking of peroxidase activity is unnecessary in both paraffin and frozen sections after heat treatment, since heating completely inactivates endogenous peroxidase. When mouse monoclonal antibody is applied to mouse tissues, endogenous mouse immunoglobulins in connective tissues and plasma cells react with the HRP-labeled second antibody. Heat treatment also inactivates the antigenicity of endogenous immunoglobulins.
10. The sections are circled with a PAP pen, creating a hydrophobic line to incubate the sections with a small amount of antibodies. However, the sections dry rapidly during the circling because of the effect of the organic solvent included in the pen. Pretreatment with BSA solution prevents the sections from the drying.
11. In addition to incubation with normal IgG, the following controls are necessary to confirm the specificity of immunostaining. (a) If possible, antibodies absorbed with purified antigens or peptides used as immunogens should be applied to the sections. (b) Incubation with the primary antibody should be omitted to examine the interaction between the labeled antibody and the tissues. (c) Incubation with the primary antibodies and the second labeled antibody should be omitted and the DAB reaction should be performed or observed under a fluorescence microscope to estimate the endogenous peroxidase activity or autofluorescence, respectively. (d) As a positive control, immunostaining in tissues or cultured cells that highly express the antigens is useful for confirming that the detection

system works well and that the antigen is expressed at a low level when the immunoreaction is negative or very faint.

12. The air-dried frozen sections are stable for at least 6 months, when they are placed in a metal slide holder and stored in a zip-lock plastic bag at -20°C . When the sections are taken out from the bag for use, they should be immediately dried using a hairdryer to remove frost on the slide glasses.
13. This fixative, formalin containing CaCl_2 should be recovered, as it can be used many times.
14. To maintain the temperature in the microwave oven, the sections are immersed in boiling antigen retrieval solution and kept in the oven without microwave irradiation; after 10 and 20 min, microwave irradiation is performed for 10–20 s each.
15. Since heating completely inactivate the HRP enzyme activity, double immunostaining for two antigens are easy when two antibodies are generated in different animal species, such as rabbit and mouse. Even when antibodies are generated in the same species, double immunostaining can be performed, since heat treatment significantly reduces the antigenicity of the primary antibody although heating may not completely dissociate the primary antibody and the HRP-labeled antibody from the tissues. It should confirm that the primary antibody to the first immunoreaction is inactive and do not bind the HRP-labeled antibody as follows. The tissues show negative staining when the sections were treated with the primary antibody and the HRP-labeled antibody for the first immunoreaction, heated, treated with the HRP-labeled antibody and then incubated with the DAB solution.
16. When one of the target antigens is sensitive for fixatives and heat treatment, frozen sections may be fixed with 10 % buffered-formalin in a short time and immunostained with the antibody to the sensitive antigen (Fig. 3A, B). After the first antigen is localized, the tissues are fixed with 10 % formalin/0.1 M HEPES buffer, pH 7.4 containing 25 mM CaCl_2 for 5 min and heated in the antigen retrieval solution at 95°C for 15 min, and then the second target antigen should be immunostained.
17. Although the concentration of primary antibody suitable for use with the immunofluorescence method depends on each antigen, $1\text{ }\mu\text{g/mL}$ may be used for the first time. An approximately 100-fold higher antibody concentration is required for the immunofluorescence method, compared with the HRP-labeled antibody method described in this protocol.
18. Specimens embedded in Fluoromount are stable for a few months at 4°C in the dark. If the specimens are stored for a long time, the edges of the cover glass should be shielded with nail polish.

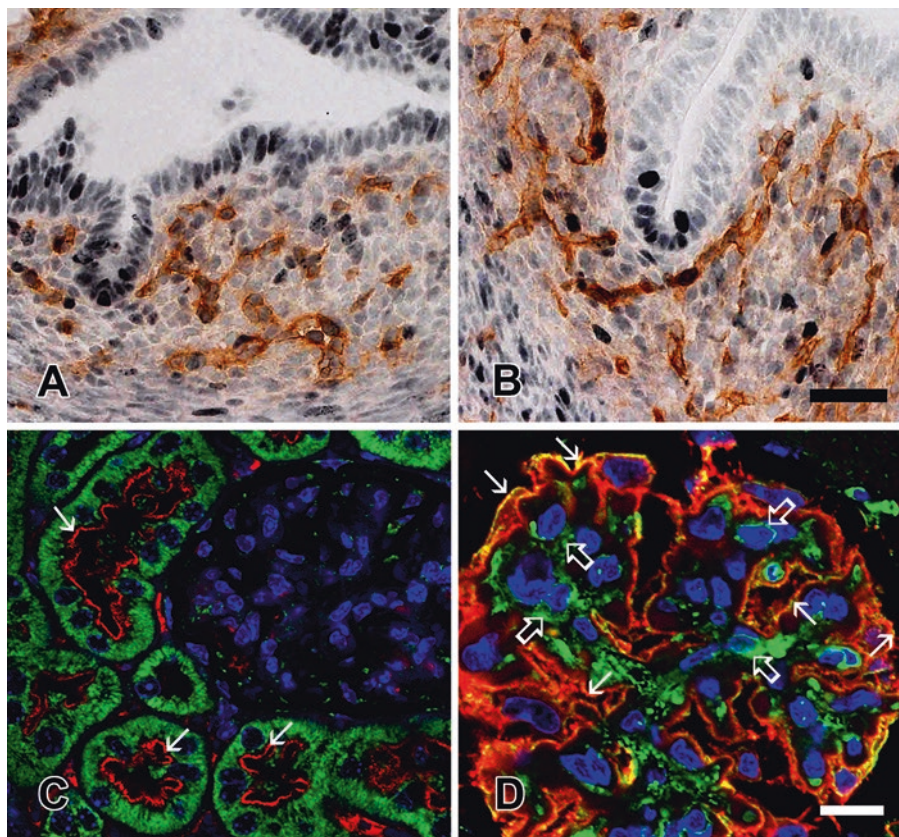


Fig. 3 Double immunostaining with the HRP-labeled method and immunofluorescence method. (**A**, **B**) Localization of platelet and endothelial cell adhesion molecule-1 (PECAM1) and PCNA in the uteri of 10-day-old (**A**) and 15-day-old (**B**) mice. Frozen sections were fixed with 10 % formalin/0.1 M phosphate buffer, pH 7.4 for 10 min and treated with anti-PECAM1 rat monoclonal antibody, successively with anti-rat IgG rabbit antibody, and then with Envision plus (rabbit). PECAM1 localization is detected by imidazole-DAB solution (*brown*). The sections were further fixed with 10 % formalin/0.1 M HEPES buffer, pH 7.4 containing 25 mM CaCl_2 for 5 min and heated in 20 mM Tris-HCl, pH 9.0 at 95 °C for 15 min, and then PCNA was localized using mouse monoclonal antibody with Ni-Co-DAB solution (*dark purple*). Both epithelial cells (*outlined arrows*) and endothelial cells (*arrows*) are positive for PCNA-immunostaining on day 10. Endothelial cells show positive PCNA-immunostaining, whereas few epithelial cells reveal PCNA-immunostaining on day 15; blood vessels are continuously formed, whereas the growth of epithelial cells is down-regulated on day 15. (**C**) Simultaneous staining of clathrin and translocase of mitochondrial outer membrane 20 (TOM20) using immunofluorescence method in the mouse kidney of FFPE specimen. After sections were autoclaved in 20 mM Tris-HCl, pH 9.0 at 121 °C for 10 min, clathrin was detected using mouse monoclonal antibody and Alexa Fluor anti-mouse IgG 549 (*red*) and TOM20 was localized with rabbit polyclonal antibody and Alexa Fluor anti-rabbit IgG 488 (*green*). Nuclei were counter-stained with DAPI (*blue*). Clathrin (*arrows*) is localized underneath the brush borders which may correspond to apical canaliculi in the proximal tubules. TOM20 is demonstrated in the mitochondria locating in basolateral cytoplasm of the proximal and distal tubules and collecting ducts. (**D**) Double immunostaining of claudin-5 and β -actin in the glomerulus of mouse kidney in the FFPE tissue. After HIAR in 20 mM Tris-HCl, pH 9.0, β -actin localization was demonstrated with the monoclonal antibody and Alexa Fluor anti-mouse IgG 549 (*green*) and claudin-5 was localized with rabbit antibody and Alexa Fluor anti-rabbit IgG 488 (*red*). β -actin and claudin-5 are colocalized along the walls of capillaries (*arrows*) showing *yellow to orange* color, which might correspond to pedicels of podocytes. β -actin is also demonstrated in the mesangial cells (*outlined arrows*). Scale bars: **A** and **B** = 50 μm ; **C** and **D** = 20 μm

References

1. Shi S-R, Key ME, Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 39:741–748
2. Yamashita S, Okada Y (2005) Mechanisms of heat-induced antigen retrieval: analyses in vitro employing SDS-PAGE and immunohistochemistry. *J Histochem Cytochem* 53:13–21
3. Yamashita S, Okada Y (2005) Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. *J Histochem Cytochem* 53:1421–1432
4. Yamashita S, Katsumata O, Okada Y (2009) Establishment of standardized post-embedding method for immunoelectron microscopy by applying heat induced antigen retrieval. *J Electron Microsc* 58:267–279
5. Yamashita S (2010) The post-embedding method for immunoelectron microscopy of mammalian tissues: a standardized procedure based on heat-induced antigen retrieval. *Methods Mol Biol* 657:237–248
6. Kiernan JA (1999) Fixation. In: *Histological and histochemical methods. Theory and practice*, 3rd edn. Butterworth-Heinemann, Woburn, MA, pp 11–35
7. Metz B, Kersten GF, Hoogerhout P et al (2004) Identification of formaldehyde-induced modifications in proteins. Reaction with model peptides. *J Biol Chem* 279:6235–6243
8. Mason JT, Fowler CB, O’Leary TJ (2010) Study of formalin fixation and heat-induced antigen retrieval. In: Shi S-R, Taylor CL (eds) *Antigen retrieval immunohistochemistry based research and diagnostics*. John Wiley & Sons, Hoboken, NJ, pp 253–285
9. Yamashita S (2007) Heat-induced antigen retrieval: mechanisms and application to histochemistry. *Prog Histochem Cytochem* 41:141–200
10. Yamashita S, Kudo A, Kawakami H et al (2013) Mechanisms of angiogenic suppression in uteri exposed to diethylstilbestrol neonatally in the mouse. *Biol Reprod* 88(116):1–13
11. Shi S-R, Imam SA, Young L et al (1995) Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J Histochem Cytochem* 43:193–201
12. Yamashita S (2010) pH or ionic strength of antigen retrieval solution: a proposal for refolding during heat treatment. In: Shi S-R, Taylor CL (eds) *Antigen retrieval immunohistochemistry based research and diagnostics*. John Wiley & Sons, Hoboken, NJ, pp 303–321
13. Kajiya H, Takekoshi S, Takei M et al (2009) Selection of buffer pH by the isoelectric point of the antigen for the efficient heat-induced epitope retrieval: re-appraisal for nuclear protein pathobiology. *Histochem Cell Biol* 132:659–667

Chapter 11

Detecting Neuronal Differentiation Markers in Newborn Cells of the Adult Brain

Sara Bonzano and Silvia De Marchis

Abstract

Adult neurogenesis is the lifelong generation of new neurons that occurs into restricted regions of the adult mammalian brain, namely the dentate gyrus of the hippocampus and the olfactory bulb. In this chapter, we describe the procedures aimed to investigate adult neurogenesis in the murine brain. Specifically, we explain how to inject into animals exogenous markers of proliferation (i.e., BrdU) and prepare brain tissues to perform immunofluorescence reactions for neuronal markers in combination with BrdU staining. As BrdU is incorporated in the DNA during the S-phase of the cell cycle of proliferating cells and is then inherited by daughter cells, by coupling BrdU-immunoreactivity together with the immunolabeling for neuronal markers, we provide the general procedures that can be applied to identify adult-born neurons and to characterize their specific phenotypes in different brain regions, under physiological condition or in pathological states.

Key words BrdU labeling, Neuron birth-dating, Adult neurogenesis, Multiple immunolabeling, Neuronal differentiation

1 Introduction

More than 50 years ago, Joseph Altman's pioneer studies based on tritiated [H^3]-thymidine autoradiography provided the first anatomical evidence for the presence of newly generated neurons in the postnatal/adult rat hippocampus and olfactory bulb [1, 2], challenging one of the central dogma in neurobiology stating that neurogenesis was confined to developmental stages. After being dismissed for decades, the field of adult neurogenesis took off following the introduction of bromodeoxyuridine (BrdU), a nucleotide analogue that can be used to label adult born neurons and can be easily revealed by immunostaining techniques [3]. BrdU, as well as other thymidine analogue exogenous compounds (e.g., IdU, CldU, and EdU), integrates into the DNA of dividing cells in the S-phase of cell cycle and it labels their progeny. By using different experimental paradigms, including single or multiple injections

and different survival times, BrdU can be used to monitor the proliferation capabilities of adult neural stem/progenitor cells, for neuronal birth-dating studies and time course analysis [4–8]. Moreover, the use of BrdU staining in combination with immunolabeling for neuronal stage/type-specific markers (e.g., immature neuronal markers such as doublecortin and PSA-NCAM; mature neuronal markers such as NeuN; markers for specific neuronal subpopulations, such as GABA, TH, and Prox1) allows the characterization of the phenotype and functional integration of newborn cells, making BrdU the “gold standard” for studying adult neurogenesis. Here, we describe the procedures that allow identification and characterization of newborn neurons in the postnatal/adult brain: from administration of BrdU to tissue fixation, sampling, multiple immunostaining, imaging, and analysis.

2 Materials

1. Tris–HCl stock solution 10× (1 M, pH 7.4): This solution can be prepared using Tris base (MW 121.14), or Tris–HCl (MW 157.60). In this protocol we use Tris–HCl to prepare 500 mL of solution. Under the chemical hood, dissolve 78.8 g of Tris–HCl in about 350 mL of ddH₂O, in a glass beaker over a stir plate (*see Note 1*). Adjust the pH to 7.4 with highly concentrated HCl (e.g., 37%) (*see Note 2*) and transfer the solution to a graduated cylinder and add dd H₂O up to 500 mL. Filter-sterilize through a 0.2 µm filter, transfer to a glass bottle and store at 4 °C until use.
2. BrdU solution 1× (10 mg/mL): Prepare a stock solution of 100 mL containing BrdU (5-bromo-2'-deoxyuridine) at 10 mg/mL. Under the chemical hood, dissolve 1 g of BrdU powder (stocked at –20 °C) in sterile 1× Tris–HCl solution [0.1 M, pH 7.4, dilute 1:10 the 10× Tris–HCl stock solution in double-distilled water (dd H₂O)] in a glass beaker. Filter-sterilize through a 0.2 µm filter to prepare injectable solution and aliquot in sterile 1.5 mL vials and store at –20 °C until use. When stored at –20 °C, the BrdU solution remains stable for months/years. Avoid multiple freeze-thaws, once thawed BrdU solution is stable at 4 °C for 2 weeks. Protect aliquots from light (*see Note 3*).
3. PB stock solution (0.2 M, pH 7.4): Phosphate buffer solution contains equimolar concentration of sodium phosphate dibasic (Na₂HPO₄, used to prepare solution A) and sodium phosphate monobasic (NaH₂PO₄, used to prepare solution B). In this protocol, we mix A and B solutions to prepare about 1 L of 0.2 M PB stock solution. To prepare solution A, dissolve 28.48 g of sodium phosphate dibasic dihydrate (Na₂HPO₄

dihydrate, MW 177.99) in a beaker containing about 700 mL ddH₂O (*see Note 1*), transfer the solution to a graduated cylinder and add ddH₂O up to 800 mL. To prepare solution B, add 7.8 g of sodium phosphate monobasic dihydrate (NaH₂PO₄•2H₂O, MW 156.01) to a graduated cylinder containing about 100 mL ddH₂O, mix to dissolve and add ddH₂O to a final volume of 250 mL. Put the solution A (800 mL) in a glass beaker and by monitoring pH add Solution B until reaching pH 7.4. Store in a glass bottle at 4 °C until use.

4. 1× Phosphate buffered saline (PBS) solution (0.01 M, pH 7.4): PBS is made up by 0.2 M PB (pH 7.4) diluted in ddH₂O and NaCl. To prepare 1 L, dissolve 8.5 g of NaCl (MW 55.44) in about 800 mL of ddH₂O and add 50 mL of PB 0.2 M pH 7.4. After adjusting to pH 7.4, add ddH₂O to bring the final volume up to 1 L in a graduated cylinder. Transfer the solution in a bottle and store at 4 °C.
5. 30% Sucrose in PB 0.1 M, pH 7.4: Dissolve sucrose in 0.1 M PB (dilute 1:10 0.2 M PB pH 7.4 solution in ddH₂O). For a final volume of 100 mL, add 30 g of sucrose to about 50 mL of 0.1 PB solution and then stir until the sucrose is properly dissolved. Add ddH₂O to bring the final volume up to 100 mL in a graduated cylinder. Transfer the solution in a bottle and store at 4 °C (*see Note 4*).
6. 1× Saline solution (NaCl 0.9%): For a volume of 100 mL, dissolve 0.9 g of NaCl in 100 mL of ddH₂O. Transfer the solution in a glass bottle and store at 4 °C (*see Note 5*).
7. 10 M NaOH: Add 40 g of NaOH to a glass beaker. Add ddH₂O to a final volume of 100 mL.
8. Paraformaldehyde (PFA) solution 1× (4%, pH 7.4): To prepare 500 mL of a solution containing 4% PFA, add 20 g of prilled PFA to 200 mL ddH₂O preheated at 60 °C on a stirrer under the chemical hood (*see Note 6*). Turn off the heater, add the PFA prills and continue to stir the solution under the chemical hood. Add 1 M NaOH drop-by-drop to clarify the PFA-containing solution (*see Note 7*). Once the PFA is completely dissolved, add 250 mL of 0.2 M PB solution (pH 7.4). Check and adjust to pH 7.4. Filter the solution by using a cotton filter paper-covered funnel in a glass cylinder. Add ddH₂O up to 500 mL. Mix the solution and transfer in a glass bottle. Store at 4 °C (*see Note 8*).
9. Triton X-100.
10. PBS-blocking solution, pH 7.4: This solution is made up by adding 0.5% Triton X-100 and 1:100 normal serum (of the same species in which the secondary antibody is raised) in 0.01 M PBS solution, pH 7.4.

11. 0.1 M Borate buffer solution, pH 8.5: For a final volume of 200 mL solution, dissolve 1.24 g of boric acid (H_3BO_3) in 150 mL of ddH₂O in a beaker. Adjust with NaOH (use first 10 M, then 1 M drop-by-drop) to reach pH 8.5. In a glass cylinder, add ddH₂O up to 200 mL. Transfer the solution in a glass bottle. Store at 4 °C.
12. 2 N HCl: To prepare 200 mL of solution, add 33.14 mL of 37% HCl (density 1.19 g/mL, MW 36.461) in 166.86 mL of ddH₂O in a glass cylinder under the chemical hood. Transfer the solution in a glass bottle. Store it at room temperature (RT) (*see* **Note 2**).
13. Glycerol.
14. Ethylen glycol.
15. 1× antifreezing solution: To make 500 mL of antifreezing solution, first prepare a solution containing 1.6825 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.385 g of NaOH dissolved in ddH₂O (final volume 50 mL; pH 7.4; *see* **Note 1**), pour it into a 500 mL glass cylinder in which you have previously added 150 mL of glycerol and 150 mL of anhydrous ethylene glycol. Transfer the obtained solution in a glass beaker over a stir plate; add 150 mL of ddH₂O and continue to mix until the solution is homogeneous. Store at 4 °C (*see* **Note 9**).
16. Mowiol mounting medium: To prepare about 100 mL of mounting medium, add 9.6 g of Mowiol to 24 mL of glycerol in a beaker over a stir plate and mix for 30 min, then add 24 mL of ddH₂O and stir for several hours (up to 6) at RT. Add to the previous solution 48 mL of 0.2 M Tris–HCl pH 8.5, and heat to 50 °C by continuous stirring overnight (o/n). After the Mowiol dissolves, transfer the solution in 50 mL falcons and clarify by centrifugation at $5000 \times g$ for 15 min. Aliquot the supernatant in 1.5 mL vials and store at –20 °C (*see* **Note 10**).
17. Gelatin-coating solution: For a final volume of 200 mL of gelatin-coating solution, prepare two different solutions as follows. Prepare solution A (1% gelatin) by dissolving 1 g of gelatin in 100 mL of preheated ddH₂O, stir and heat the solution up to max 45–50 °C. Prepare solution B (0.1% chrome alum) by dissolving 0.1 g of chromium potassium sulfate ($\text{CrK}(\text{SO}_4)_2$) in 100 mL ddH₂O. As solution A reaches RT, mix it with solution B.
18. Gelatin-coated slides: Place cleaned histological slides into racks. Dip the racks containing the slides for 7 min into the gelatin-coating solution trying to avoid the formation of air bubbles. Remove slowly the racks containing the slides and let them drain onto a filter paper to remove excess solution from

the racks. Place the racks containing the slides on the lab bench and cover them with paper towels to protect them from dust. Dry at room temperature for 24 h. Dried slides can be covered with aluminum foil and stored at 4 °C until use (*see* **Note 11**).

19. Ketamine (75 mg/kg)/xylazine (30 mg/kg) mixture, for anesthesia.
20. Syringes.
21. Peristaltic pump, for animal perfusion.
22. Surgical and dissection tools (scissors, blades, tweezers).
23. Optimal cutting temperature compound (OCT).
24. Cold isopentane.
25. Dry ice.
26. Cryostat.
27. Multi-well plastic plates.
28. Paintbrush.
29. Pasteur pipettes (glass and plastic).
30. Variable volume micropipettes (e.g., P2; P10; P200; P1000).
31. Microscope slides, coverslips.
32. Slide racks.
33. Primary antibodies.
34. Fluorescent secondary antibodies.
35. Confocal microscope.

3 Methods

All the following methods are optimized for the adult mouse brain, but can be easily adapted for other ages (i.e., developmental stages) and species as well. All experiments must be performed in accordance with relevant authorities' guidelines and instructions. All procedures should be carried out at RT unless otherwise specified.

3.1 BrdU Administration by Intraperitoneal Injection

The most common method to administer BrdU to animals is through intraperitoneal (i.p.) injection as this administration route is precise, technically simple and easily temporally controlled. A single BrdU injection labels cells during DNA replication for about 6 h [9], although this may vary depending on the initial dose given. Targeting a large population of dividing progenitors in the adult brain requires BrdU labeling over an extended period that can be obtained by repeated injections (e.g., for studying integration and survival of new neurons during adult olfactory bulb neurogenesis,

usually four injections 4 h apart at a dose of 50 mg/kg of body weight; but *see* [5, 8, 10]) (*see* **Note 12**).

1. Thaw BrdU solution (10 mg/mL) at RT.
2. Invert tube and vortex until the solution is completely clear (i.e., without any crystal). Protect BrdU solution from light (by covering the vials with aluminum foil).
3. Calculate the exact amount of BrdU solution required for each injection based on the weight of the experimental animals (*see* **Note 13**).
4. Restrain the mouse by the scruff. Put a topic anesthetic (e.g., lidocaine) over the area of the i.p. injection.
5. Intraperitoneally inject an appropriate and weight-adjusted volume of BrdU solution (at RT) using a fine dosage syringe (*see* **Note 14**).

3.2 Tissue Preparation

Proper tissue fixation and sampling processing are key passages for successful immunofluorescence analysis. In this section, we give an overview of the different steps needed to preserve a life-like tissue and consequently ensure reliable staining and consistent results.

1. Deeply anesthetize adult mice (e.g., 2-month-old C57Bl6 mice) with an i.p. injection of a mixture of ketamine (75 mg/kg) and xylazine (30 mg/kg) (*see* **Note 15**). Check the foot pinch reflex to make sure the animal is unresponsive and thus deeply anesthetized before proceed with the perfusion.
2. Transcardially perfuse each mouse with the help of a peristaltic pump. Perfuse first by passing ice-cold 0.9% saline solution (until the liver become clear, approximately 20/30 mL/animal), in order to remove blood from vessels, followed by ice-cold 4% PFA solution, pH 7.4 (about 100 mL/animal) to fix the tissue (*see* **Note 16**).
3. Dissect carefully the brain from the skull, post-fix as whole brain for about 2–4 h at 4 °C in 4% PFA solution (*see* **Note 17**).
4. Rinse three times the brain with a 0.1 M PB solution, pH 7.4.
5. Brain tissue must be cryoprotected by immersion in a 30% sucrose in 0.1 M PB, pH 7.4 at 4 °C until its precipitation (in average it takes 36–48 h) (*see* **Note 4**).
6. Embed the brain tissue in OCT by using embedding cryomolds, then freeze it by slowly submerge the cryomolds into –70/75 °C cold isopentane for a couple of minutes, and then place and store at –80 °C until cutting (*see* **Note 18**).
7. Using a cryostat, cut the brain tissue at the appropriate thickness (e.g., 25 µm of thickness for the olfactory bulb is currently

used in our laboratory) and collect the slices as free-floating coronal/parasagittal serial sections in sterile multi-well plates (*see Note 19*).

8. Free-floating sections can be stored at -20°C in antifreezing solution until analysis.

3.3 Sequential Multiple Immunofluorescence Reaction for Neuronal Markers Together with BrdU Staining

A general rule for multiple immunofluorescence is to use primary antibodies that are raised in different species. In the case they are not, perform a sequential multiple immunolabeling that has been optimized to detect multiple primary antibodies from the same host species (as described in [11]). Moreover, to minimize possible cross-reaction (i.e., nonspecific bindings), the secondary antibodies should ideally come from the same host and be appropriate for multiple immunolabeling (i.e., ELISA and/or solid-phase absorbed to ensure minimal cross-reactivity with immunoglobulins from other tested species).

HCl pretreatment to denature DNA is required for immunodetection of thymidine analogs. However, HCl treatment may compromise the detection of some antigens. To overcome this problem, we recommend sequential reactions (i.e., staining for endogenous markers first and BrdU staining thereafter) for multiple immunostaining, instead of the simultaneous multiple immunostaining reaction (i.e., HCl pretreatment followed by incubation with a cocktail containing all the antibodies together). Moreover, the sequential reactions for multiple immunostaining have the additional advantage that each staining step can be verified before continuing onto the next step. Thus, for novices, this is a good way to troubleshoot in case of any kind of problem.

1. Transfer the sections from antifreezing solution into a clean 24-multi-well plate containing 0.01 M PBS solution, pH 7.4 (*see Note 20*).
2. Rinse with PBS thoroughly to completely remove antifreezing solution (usually three times at RT for 10 min each is enough) by agitation.
3. Remove the PBS and incubate in the PBS-blocking solution, 45 min at RT on a rocker (*see Note 21*).
4. Remove the PBS-blocking solution and incubate in primary antibody cocktail (e.g., rabbit anti-TH, goat anti-DCX; *see Fig. 1*) diluted in PBS-blocking solution, o/n at 4°C on a rocker (*see Note 22*).
5. Rinse three times in PBS for 10 min each in agitation.
6. Remove the PBS and incubate in cocktail of fluorochrome-conjugated secondary antibodies (e.g., Alexa Fluor-647 anti-rabbit, Alexa Fluor-488 anti-goat; all made in donkey; *see Fig. 1*)

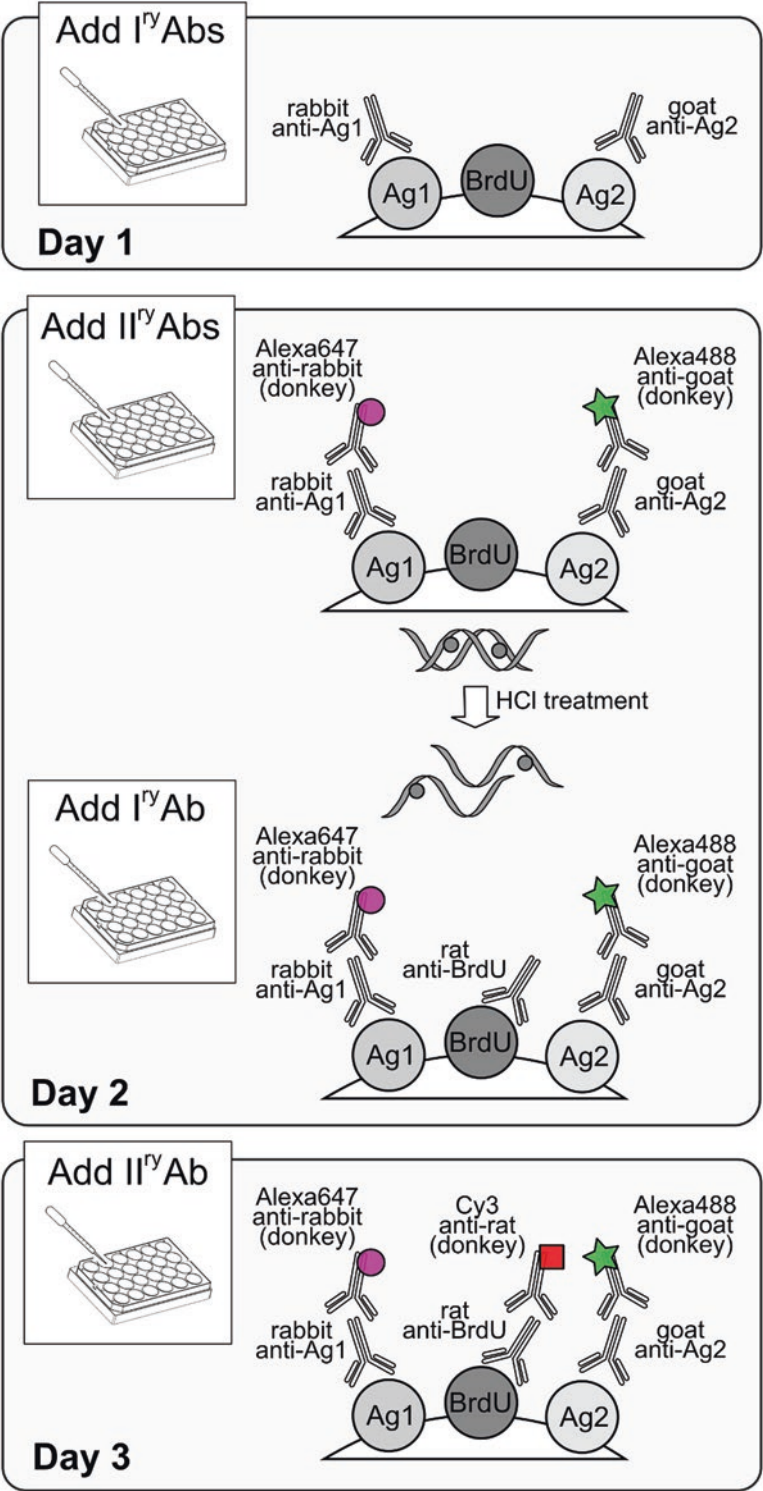


Fig. 1 Sequential multiple immunofluorescence for BrdU and cell-type specific endogenous antigens: *Ag1* antigen 1, *Ag2* antigen 2, *Iry Ab(s)* primary antibody(ies), *IIry Ab(s)* secondary antibody(ies)

diluted in PBS-blocking solution, 1 h at RT on a rocker. From now on, protect sections from light (*see* **Notes 23** and **24**).

7. Rinse three times in PBS for 10 min each, in agitation.
8. Pre-incubate sections in 2 N HCl solution at 37 °C for 30 min (*see* **Note 25**).
9. Remove HCl solution and then neutralize the pH with borate buffer solution (pH 8.5) before anti-BrdU staining for 10 min at RT on a rocker.
10. Remove rapidly and completely the borate buffer solution, and wash carefully 3 times in PBS for 10 min each wash.
11. Remove the PBS and incubate in primary anti-BrdU antibody (e.g., rat anti-BrdU; *see* Fig. 1) diluted in PBS-blocking solution, o/n at 4 °C on a rocker.
12. Rinse three times in PBS for 10 min each.
13. Remove the PBS and incubate in cocktail of fluorochrome-conjugated secondary antibodies (e.g., Cy3 anti-rat, made in donkey; *see* Fig. 1) diluted in PBS, 1 h at RT on a rocker.
14. Rinse three times in PBS for 10 min each.
15. Immerse the sections in a petri dish containing PBS and mount them onto gelatin-coated slides with the help of a fine paintbrush (*see* **Note 26**).
16. Briefly dip slides (1/2 s) in ddH₂O to remove excess salts.
17. Air-dry completely the slides.
18. Coverslip with Mowiol mounting medium. Refrigerated Mowiol-mounted sections kept in the dark will maintain their fluorescence for months, if not years.

3.4 Imaging and Analysis

To perform the phenotypic characterization of newborn cells within the adult brain at a single cell resolution, the best is to image the fluorescence labeled sections as three-dimensional multi-stacks with a confocal laser microscope equipped with appropriate lasers and filter systems, followed by analysis and quantification at ImageJ. The use of confocal microscope in a sequential scanning configuration is an advantageous tool when detecting multiple fluorophores in one tissue sample (Fig. 2). Indeed, exciting and recording in a sequential order will avoid channel cross talk (i.e., false-positive due to a possible overlaps between the emission spectrum of each excited fluorophore) that could happen when a simultaneous acquisition of all signals is done [8]. Moreover, the optical resolution of confocal microscopy and the ability to collect registered series of focal planes (i.e., multi-stacks) is ideally suited for quantitative stereology [10, 12].

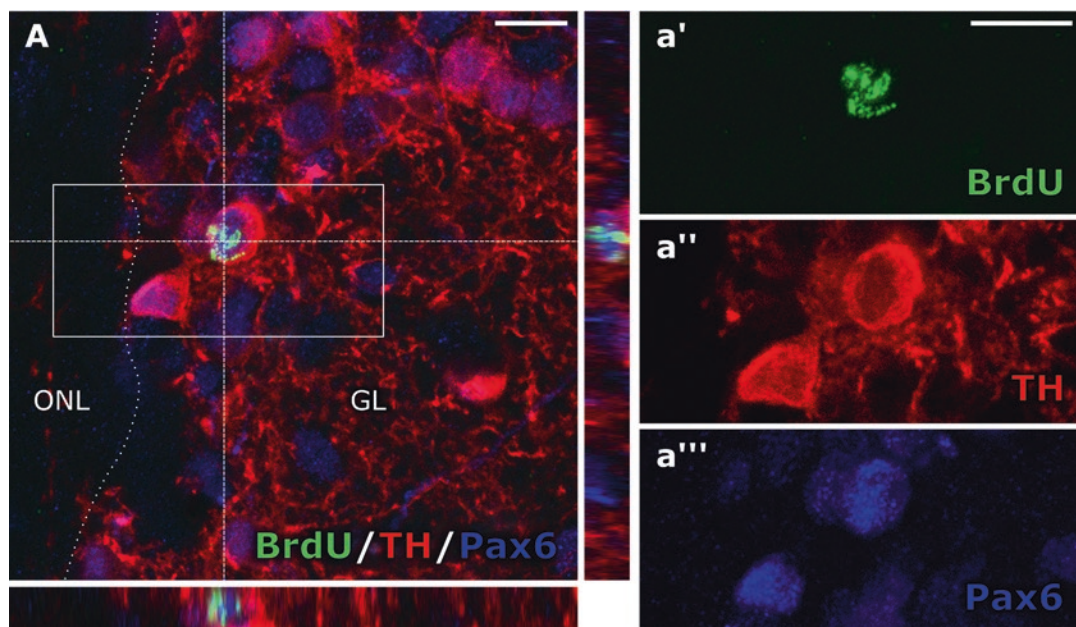


Fig. 2 Representative multiple immunostaining showing an adult-born olfactory bulb dopaminergic neuron. Mouse olfactory bulb glomerular layer (GL) stained for BrdU (in green), tyrosine hydroxylase enzyme (TH, in red), and the transcription factor Pax6 (in blue). Insets a', a'', a''' show a higher magnification of a triple labeled BrdU+TH+Pax6+ neuron. Scale bar in a = 10 μ m. Scale bar in a' = 10 μ m and refers to a'' and a'''. ONL olfactory nerve layer

4 Notes

1. To help to dissolve powders relatively easily, put a small volume of ddH₂O at the bottom of the beaker and allow the magnetic stir bar to work immediately before adding the powder, then add the remaining volume.
2. Exercise extreme caution in handling 37% HCl; please read the Material Safety Data Sheets (MSDS) before working; wear gloves and eye protection; use a glass pipette.
3. BrdU is toxic and classified as a suspected mutagen and must be handled with care. Follow the specific MSDS provided by the suppliers: wear proper protective gear, a lab coat and gloves. Room light is sufficient, do not switch on the hood light as BrdU is photosensitive. Make sure the powder is completely dissolved before aliquoting (at room temperature, with the use of a stir bar on a stir platform it takes about 15–20 min).
4. Sucrose solution acts as cryoprotectant preventing the formation of ice crystal artefact in frozen tissues by acting as a partial dehydrant. The use of cryoprotectant is critical to assure good microscopic morphology. Prepare fresh 30% sucrose solution each time and keep it at 4 °C. Consider a volume of about 15 mL per each dissected brain.

5. Saline solution is used to perfuse the animals before PFA and to flush out the fixative from the tubes of the perfusion apparatus in between sequential perfusions. To calculate the final volume, consider a volume of about 50 mL per animal.
6. Formaldehyde is highly toxic; please read the MSDS before working with this chemical. Gloves and safety glasses should be worn and solutions made inside a chemical hood. The use of prilled PFA (small white beads) can require longer time to dissolve compared to powder but it is less volatile and thus it minimize possible contamination.
7. To dissolve and clarify the PFA solution requires time (up to 1 h); do not exceed in adding NaOH in order to maintain the pH in the physiological range (start by adding 1 mL and proceed adding 200 μ L at a time).
8. In our experience the best is to prepare fresh PFA solution the day before the perfusion and keep it at 4 °C. However, if you need to use PFA solution kept at 4 °C up to 1 month you should re-filter it in order to eliminate possible crystal deposits. A volume of about 140 mL per each animal will be largely sufficient for the perfusion and post-fixation.
9. Since the glycerol and ethylene glycol are viscous, traces can remain in the cylinder; you can use 150 mL ddH₂O to rinse the cylinder before adding it to the final mix into the beaker. Before using the antifreeze solution on brain sections, you can test if it works properly by simply putting a small amount (i.e., 500 μ L) of solution in a 1.5 vial at -20 °C: if it does not freeze within a hour, you can safely use it.
10. Mowiol 4-88 is an anti-fading agent and thus aqueous Mowiol-containing mounting medium is useful to prevent rapid photo bleaching and thus maintain immunofluorescence over time. In commerce, there are different kinds of mounting media that you can use in alternative.
11. Chromium potassium sulfate will positively charge the slides allowing them to attract negatively charged tissue sections into the slides, optimizing their adhesion. In our experience, we usually stock the gelatin-coating solution at 4 °C and reuse it (after filtering it) in a week to prepare additional gelatin-coated slides. In commerce, there are different kinds of adhesive microscope slides that you can use as an alternative choice.
12. A general advice is to use the same stock of BrdU solution for the treatment of different experimental groups within the same study, in order to avoid unwanted and uncontrollable increase in variability between animals/groups. As the size of the bromine atom can alter the DNA structurally, leading possibly to impaired stability and functionality of the DNA itself (i.e., increase in the risk of sister chromatin exchange, mutations and DNA double-strand breaks; *see* [4]), the optimal concentration

of BrdU must be applied to limit possible alteration at the DNA level or the risk of artifacts. For the mouse, the BrdU dosage for each injection is normally 50–100 mg/Kg for the OB and DG, but it can rise up to 300 mg/kg depending on the experimental procedure and animal in use [5, 6, 8].

13. You can weigh animals the day before injection(s). Calculate the amount of BrdU solution required for each injection based on the concentration of the BrdU solution. For injecting at the dose of 50 mg/kg, calculate 5 μ L of BrdU (10 mg/mL) each gram of the animal (e.g., for an adult mice weighting 30 g, you should inject 150 μ L of BrdU solution); for injecting at the dose of 100 mg/kg, calculate 10 μ L of BrdU (10 mg/mL) each gram of the animal (e.g., for an adult mice weighting 30 g, you should inject 300 μ L of BrdU solution).
14. In our experience use of 0.5 or 1 mL 29 G insulin syringe with a fixed needle allow more precise and safer injections. Alternatively 1 mL syringes with 29 G needles can be used.
15. Anesthetic may vary per laboratory/institution. In our experience ketamine/xylazine mixture is very effective.
16. The goal of fixation is to rapidly and uniformly preserve tissues in a life-like state. The advantage of directly perfusing fixative through the circulatory system is that the chemical can quickly reach the brain using the vascular network. This process is critical for preserving the morphology and antigenicity of the tissue and researchers should optimize the perfusion taking into account the solution flux and velocity of the perfusion pump. Attention must be paid to avoid formation of air bubbles within the tubes. A detailed description of the perfusion procedures (although refereed to rats) can be found in [13].
17. In general, you will need to adjust the right post-fixation time for your experiment depending on the antigens you are interested in and on the outcome of the perfusion (i.e., in the case of poor perfusion longer post-fixation time is needed). Over post-fixation by leaving tissue in PFA solution for too long may cause masking of antigen-epitopes and loss of immunoreactivity and could increase autofluorescence.
18. In general, since adult neurogenesis is confined to the OB and hippocampus, by using a blade we cut out the part of the brain caudal to the cerebral hemispheres, then we cut along the longitudinal plane to obtain the two hemispheres that will be processed separately.
19. A 12-well free-floating coronal sections series is collected as follows. By using a standard 24-well cell culture plate, start at well A1 and put one slice, placing next slices into the successive wells of row A (one slice per well from A1 to A6), then go to the row B (from B1 to B6) and follow the same schema.

When reaching B6, go back to A1 and carry on as the previous series. In this way, you will obtain 12 wells containing brain slices; in each well there will be a collection of slices representative of the brain/area of interest (*see* also [11]). Taking into account the thickness of each slice (i.e., 25 μm) and the total number of wells in which they are collected (i.e., 12 wells), the gap between each consecutive slice within the same well corresponds to 300 μm (calculated by multiplying thickness by the number of wells; in our case: $25 \times 12 = 300$). This arrangement of slices allows for quantification of every n th section of an entire brain/area of interest. For quantification and phenotypic characterization of newborn neurons within the adult OB, usually it is sufficient to take every 12th brain section (i.e., just one well). Since you are using a 24-well cell culture plate, two different specimens can be collected in one plate.

20. Transfer and touch sections with the help of a fine brush that needs to be carefully rinsed when changing solutions. It is critical that the sections remain completely moist through the entire protocol; perform all the passages, as far as you can, in order to prevent sections from drying-out. Dried slices once processed for immunofluorescence will result in increased autofluorescence and background signal, making the resulting images (and thus the consequent analysis) unusable. Plates need not be sterile for antibody staining, and can be reused if well cleaned.
21. The PBS-blocking solution containing Triton X-100 (a non-ionic surfactant) and serum (of the same species in which the secondary antibody is raised from) is used to permeabilize the tissue and block unspecific secondary antibody binding sites. In general, a detergent-driven permeabilization is necessary and must be carefully calibrated to optimize staining without deterioration of the tissue. Moreover, the concentration of Triton X-100 may vary (0.1–2%) depending on the tissue thickness.
22. We usually place up to ten OB sections in 300 μL of antibody-containing solution in a single well (24-multiwell plates). Take into account that, in general, longer primary antibody incubation times (up to 2 days at 4 $^{\circ}\text{C}$) may improve staining for difficult antigen detection or when the slices are thicker (e.g., 50/60 μm). For each primary antibody, the optimal working concentration must be determined; increasing the dilution might improve the signal to noise ratio.
23. In our experience, longer secondary incubation times could increase nonspecific staining and background. In general, for multiple immunofluorescence it is advisable to use secondary antibodies that have been raised in the same species (e.g., donkey) and have minimal cross-reactivity to your tissue and to serum proteins from other species (i.e., pre-adsorbed).

Moreover, select fluorochromes with minimal spectral overlap and an excitation/emission spectral features that fit to your detection system (e.g., AMCA, Alexa Fluor 488, Cy3, Alexa Fluor 647).

24. If a nuclear counterstain is required and you have not used near-UV fluorochrome-conjugated secondary antibodies, add DAPI (10 µg/mL) to the secondary antibody cocktail or, alternatively, incubate the sections with a PBS solution containing 10 µg/mL of DAPI for 15 min at RT on the rocker. Anyway, keep in mind that BrdU has been reported to quench DNA counterstainings [14].
25. Before the incubation, check that the sections are not floating on top of the solution, but instead are all completely covered by the HCl solution. Sections will shrink and become whitish and then regain their normal appearance after washing in PBS and the neutralization with borate buffer solution.
26. There are different methodologies that can be used to mount sections. Slices can be transferred onto the slide by immersing the slide into the PBS-containing petri dish and slice moved by using the paintbrush inside the PBS. Alternatively, using a pipette place a drop of PBS in the position in which you want to mount the section on the slide, then by using the brush transfer the section into the drop, and gently unbend the tissue.

References

1. Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 124:319–336
2. Altman J (1969) Autoradiographic and histological studies of postnatal neurogenesis: IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol* 137:433–458
3. Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age related decrease of neuronal progenitor proliferation. *J Neurosci* 16:2027–2033
4. Cooper-Kuhn CM, Kuhn HG (2002) Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. *Dev Brain Res* 134:13–21. doi:[10.1016/S0165-3806\(01\)00243-7](https://doi.org/10.1016/S0165-3806(01)00243-7)
5. Taupin P (2006) BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. *Brain Res Rev* 53:198–214. doi:[10.1016/j.brainresrev.2006.08.002](https://doi.org/10.1016/j.brainresrev.2006.08.002)
6. Wojtowicz JM, Kee N (2006) BrdU assay for neurogenesis in rodents. *Nat Protoc* 1:1399–1405. doi:[10.1038/nprot.2006.224](https://doi.org/10.1038/nprot.2006.224)
7. Mamber C, Kozareva DA, Kamphuis W et al (2013) Shades of gray: the delineation of marker expression within the adult rodent subventricular zone. *Prog Neurobiol* 111:1–16. doi:[10.1016/j.pneurobio.2013.07.003](https://doi.org/10.1016/j.pneurobio.2013.07.003)
8. Kuhn HG, Eisch AJ, Spalding K et al (2016) Detection and phenotypic characterization of adult neurogenesis. *Cold Spring Harb Perspect Biol* 8(3):a025981
9. Hayes NL, Nowakowski RS (2000) Exploiting the dynamics of S-phase tracers in developing brain: interkinetic nuclear migration for cells entering versus leaving the S-phase. *Dev Neurosci* 22:44–55
10. Bonzano S, Bovetti S, Fasolo A et al (2014) Odour enrichment increases adult-born dopaminergic neurons in the mouse olfactory bulb. *Eur J Neurosci* 40:3450–3457. doi:[10.1111/ejn.12724](https://doi.org/10.1111/ejn.12724)
11. Ansorg A, Bornkessel K, Witte OW et al (2015) Immunohistochemistry and multiple labeling

- with antibodies from the same host species to study adult hippocampal neurogenesis. *J Vis Exp* 98:e52551. doi:[10.3791/52551](https://doi.org/10.3791/52551)
12. Peterson DA (1999) Quantitative histology using confocal microscopy: implementation of unbiased stereology procedures. *Methods* 18:493–507. doi:[10.1006/meth.1999.0818](https://doi.org/10.1006/meth.1999.0818)
 13. Gage GJ, Kipke DR, Shain W (2012) Whole animal perfusion fixation for rodents. *J Vis Exp* 65:e3564. doi:[10.3791/3564](https://doi.org/10.3791/3564)
 14. Latt SA, Wohlleb JC (1975) Optical studies of the interaction of 33258 Hoechst with DNA, chromatin, and metaphase chromosomes. *Chromosoma* 52:297–316

Characterizing Satellite Cells and Myogenic Progenitors During Skeletal Muscle Regeneration

Nicolas A. Dumont and Michael A. Rudnicki

Abstract

Muscle stem cells, named satellite cells, are quiescent in resting skeletal muscle. Following injury, satellite cells are activated and become proliferating myoblasts that either self-renew or differentiate. Several markers are used to identify the different myogenic populations, such as Pax7 (quiescent and activated satellite cells), MyoD (proliferating myoblasts), and myogenin (differentiating myoblasts). Immunodetection of these markers is a very useful tool to analyze myogenic cells and muscle regeneration. Here, we describe a method for immunodetection of satellite cells and their myogenic progeny in resting and regenerating skeletal muscles.

Key words Satellite cells, Myoblasts, Immunocytochemistry, Immunofluorescence, Pax7, Myogenin, Differentiation markers, Skeletal muscle, Myogenesis, Regeneration

1 Introduction

Skeletal muscles are mainly constituted of long multinucleated cells called myofibers. Adjacent to the myofibers, a population of mononuclear cells was identified and named satellite cells based on their anatomical location in between the myofiber and the extracellular matrix. Satellite cells were later characterized as muscle stem cells that have the potential to self-renew to maintain the satellite cell pool or differentiate to generate the myogenic progenitors needed for muscle regeneration [1]. Conditional deletion of satellite cells confirmed that they are absolutely required for muscle regeneration [2, 3].

First recognized by their anatomical location, many different markers were shown to specifically label satellite cells. M-cadherin was one of the first markers used to stain quiescent satellite cells in their niche [4]. Later, it was demonstrated that the paired box transcription factor Pax7 is expressed in satellite cells where it regulates myogenic specification [5]. Pax7 is expressed in both quiescent and activated satellite cells in various species, including

Table 1
Myogenic markers

Markers	Quiescent satellite cells	Proliferating myoblasts	Differentiating myoblasts
Pax7	+	+	–
Calcitonin receptor	+	–	–
CD34	+	+/–	–
Caveolin-1	+	+/–	–
Cxcr4	+	+	–
Mcad	+	+	–
c-Met	+	+	–
Itga7 and itgb1	+	+	+
Vcam1	+	+	+
Ncam1	+	+	+
Syndecan 3/4	+	+	+
MyoD	–	+	+/–
Desmin	–	+	+
MyoG	–	–	+
MyHC	–	–	+

Table showing different markers expressed by satellite cells, proliferating myoblasts, and differentiating myoblasts (myocytes). Immunostaining for these markers can be performed to analyze myogenic cells and skeletal muscle regeneration

rodent and human, and is thus a very convenient marker for immunodetection of satellite cells. In addition, different cell surface receptors are used to isolate satellite cells by FACS. In mice, FACS strategies commonly use a combination of lineage negative markers (CD11b, CD45, Sca-1, CD31) together with positive selection for one or several of these markers: alpha7-integrin (itga7), CD34, and vascular cell adhesion protein-1 (Vcam1) [6, 7]. Satellite cells can also be isolated from human muscle samples using a combination of the positive selection markers CD56 (NCAM) or EGFR, and β 1-integrin (itgb1) [8, 9].

Following muscle injury, satellite cells exit quiescence to proliferate, differentiate, and fuse to regenerate skeletal muscle [10]. The myogenic cells express specific markers during the different stages of their myogenic progression (Table 1). Activated satellite cells (myoblasts) express Pax7 together with the transcription factors Myf5 and/or MyoD, which are needed for their commitment into the myogenic lineage. Myoblasts then withdraw from the cell cycle to self-renew or differentiate [10]. Myoblasts can be characterized as

self-renewing ($\text{Pax7}^+/\text{MyoD}^-$), proliferating ($\text{Pax7}^+/\text{MyoD}^+$), or differentiating ($\text{Pax7}^-/\text{MyoD}^+$). Myoblasts progressing through the myogenic lineage (myocytes) lose Pax7 expression and start to express the differentiation factors myogenin (Myog) and/or Mrf4 (Myf6). Thereafter, myocytes fuse together to form multinucleated cells, named myotubes, which express various contractile proteins, such as myosin heavy chain (MyHC) and skeletal muscle alpha actin.

Immunodetection of satellite cells and myogenic progenitors is a very useful tool to analyze skeletal muscle regeneration. This chapter describes an immunofluorescence protocol for the detection of myogenic cells in regenerating skeletal muscle based on the markers Pax7, Myog and laminin (Figs. 1 and 2).

2 Materials

2.1 Material and Antibodies

1. Tissue-Tek OCT compound.
2. 2-methylbutane.
3. Liquid nitrogen.
4. 1× PBS.
5. Hydrophobic PAP pen.
6. Mouse on Mouse (M.O.M.) blocking reagent (Vector laboratories, MKB-2213).
7. Fluorescence mounting medium.
8. Superfrost plus microscope slides.
9. Coverslips.
10. Nail polish, for sealing coverslips.
11. Mouse monoclonal IgG1 anti-Pax7 (DHSB).
12. Rabbit polyclonal anti-Myogenin (Santa Cruz, sc-576, clone M-225).
13. Rat monoclonal IgG1 anti-laminin-2 (Sigma L0663, clone 4H8-2).
14. Goat anti-rabbit IgG (H+L) Alexa fluor 488 (ThermoFisher scientific, A-11008).
15. Goat anti-mouse IgG1 Alexa fluor 546 (ThermoFisher scientific, A-21123).
16. Goat anti-rat IgG (H+L) Alexa fluor 647 (ThermoFisher scientific, A-21247).

2.2 Solutions

1. PFA stock solution: While stirring, add 4 g of paraformaldehyde to 100 ml of 1× PBS. Add 1 drop of 2 N NaOH (the solution should become clear within a few minutes). Heating helps to dissolve the PFA (*see Note 1*). Adjust pH to 7.2. Filter the solution. Use immediately or aliquot at -20°C (avoid freeze-thaw cycles and protect from light) (*see Note 2*).

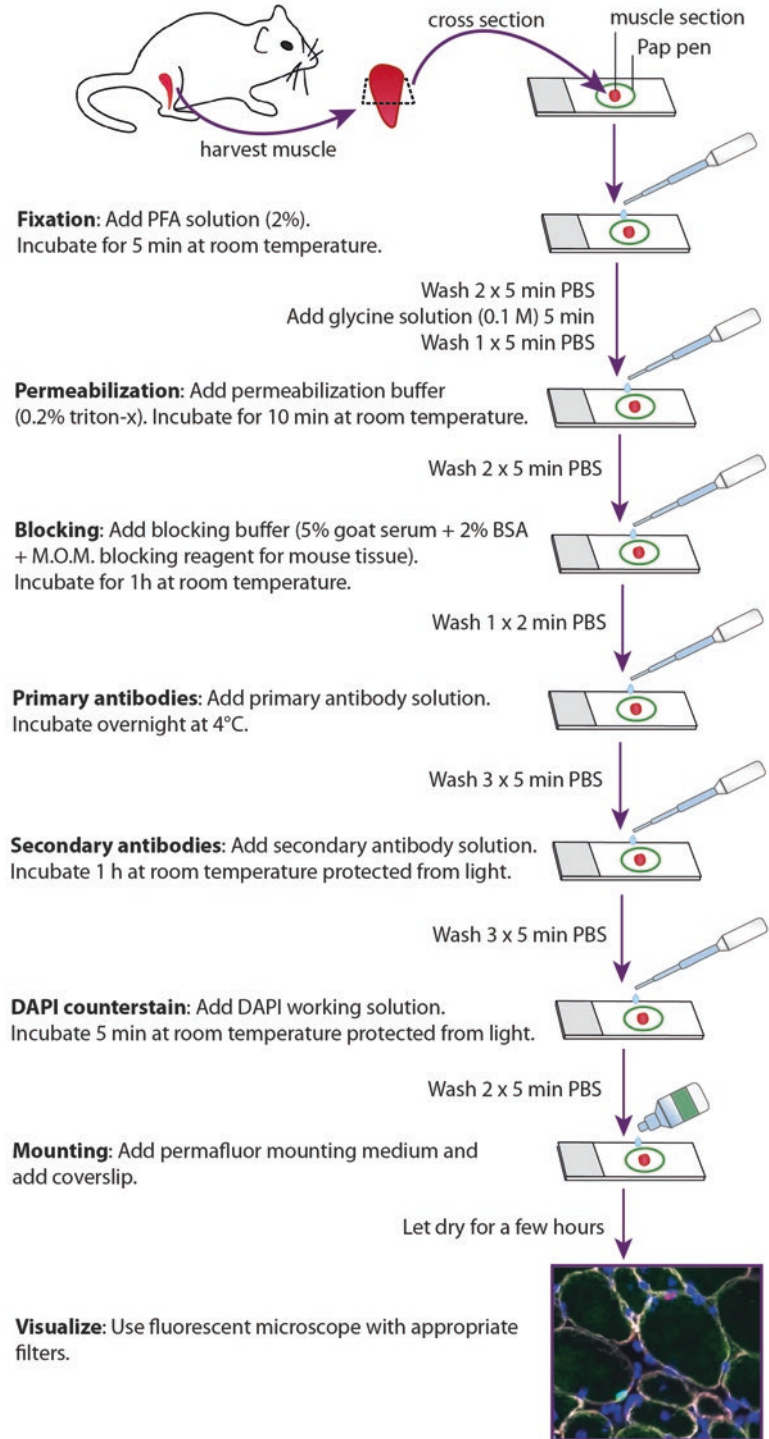


Fig. 1 Immunostaining steps. Schematic representing the steps for immunodetection of myogenic markers in healthy or regenerating skeletal muscle. Briefly, after muscle sample isolation and freezing, muscle cross sections are fixed, permeabilized, blocked, stained with primary and secondary antibodies, counterstained with DAPI, mounted, and visualized

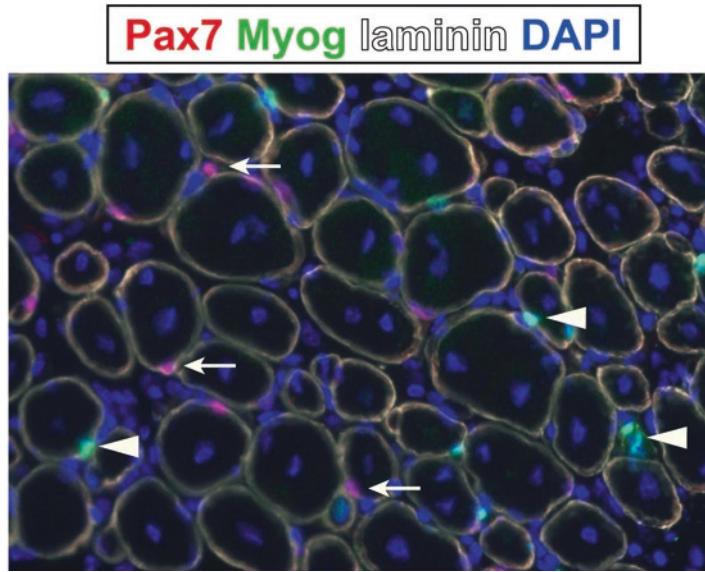


Fig. 2 Immunodetection of myogenic markers in regenerating skeletal muscle. Image of a regenerating muscle (7 days post-cardiotoxin injection) immunostained for Pax7 (red), Myog (green), laminin (white), and DAPI (for nuclei, blue). Arrows show examples of Pax7-positive satellite cells and arrowheads indicate a few of the myogenin-positive committed cells. Centronucleated myofibers (DAPI positive nuclei in the middle of a myofiber delineated by laminin staining) indicate regenerating myofibers)

2. PFA working solution (2%): dilute stock solution 1:2 in 1× PBS.
3. Glycine solution (0.1 M): Dilute 0.751 g of glycine into 100 ml of 1× PBS. Store at 4 °C.
4. Permeabilization buffer (0.2% Triton X): dilute 200 µl of Triton X into 100 ml of 1× PBS. Vortex thoroughly. Store at 4 °C.
5. Blocking buffer (5% goat serum + 2% bovine serum albumin (BSA) in 1× PBS): Add 2 g of BSA to a solution containing 5 ml of goat serum and 95 ml of 1× PBS. Store at 4 °C for up to a month or aliquot and store at −20 °C for longer period.
6. DAPI stock solution: Dilute 5 mg into 1 ml of distilled water. Store at −20 °C in the dark.
7. DAPI working solution: Dilute stock solution 1:50,000 in 1× PBS. Store at 4 °C protected from light.

3 Methods

1. Harvest muscle sample (*see Note 3*), embed in OCT (*see Note 4*) and freeze in 2-methylbutane cooled in liquid nitrogen (*see Note 5*).
2. Using cryostat, cut the muscle perpendicularly (thickness: 10 μm) and add the muscle section onto the slide (*see Note 6*).
3. Allow sections to air-dry at room temperature for 5 min.
4. Surround the muscle section with hydrophobic Pap pen.
5. Fix the muscle sections by adding a few drops of PFA working solution (*see Note 7*). Incubate for 5 min at room temperature.
6. Remove PFA solution and wash twice with 1 $\times$ PBS for 5 min (*see Note 8*).
7. Add a few drops of glycine solution to quench PFA signal. Incubate for 5 min at room temperature.
8. Aspirate glycine solution and wash once with 1 $\times$ PBS for 5 min.
9. Add a few drops of permeabilization buffer. Incubate for 10 min at room temperature.
10. Aspirate permeabilization buffer and wash twice with 1 $\times$ PBS for 5 min.
11. Add a few drops of blocking buffer. For staining with mouse primary antibody on mouse muscle section: add M.O.M. blocking reagent to the blocking buffer (1:40 dilution) (*see Note 9* and Fig. 3). Incubate for 1 h at room temperature.
12. Aspirate blocking solution and rinse once with 1 $\times$ PBS for 2 min.
13. Add a few drops of the primary antibody solution: mouse anti-Pax7 hybridoma supernatant (undiluted), rabbit anti-myogenin (1:200), rat anti-laminin (1:1000). Incubate overnight at 4 $^{\circ}\text{C}$ (*see Notes 10–13*).
14. Aspirate primary antibody solution and wash three times with 1 $\times$ PBS for 5 min.
15. Add a few drops of secondary antibody solution (diluted in blocking buffer): goat anti-rabbit Alexa Fluor 488 (1:1000), goat anti-mouse IgG1 Alexa Fluor 546 (1:1000) (*see Note 14*), goat anti-rat Alexa Fluor 647 (1:1000). Incubate for 1 h at room temperature. Protect from light from this step and for all subsequent steps.
16. Aspirate secondary antibody solution and wash three times with 1 $\times$ PBS for 5 min.
17. Add a few drops of DAPI working solution. Incubate for 5 min at room temperature (*see Note 15*).

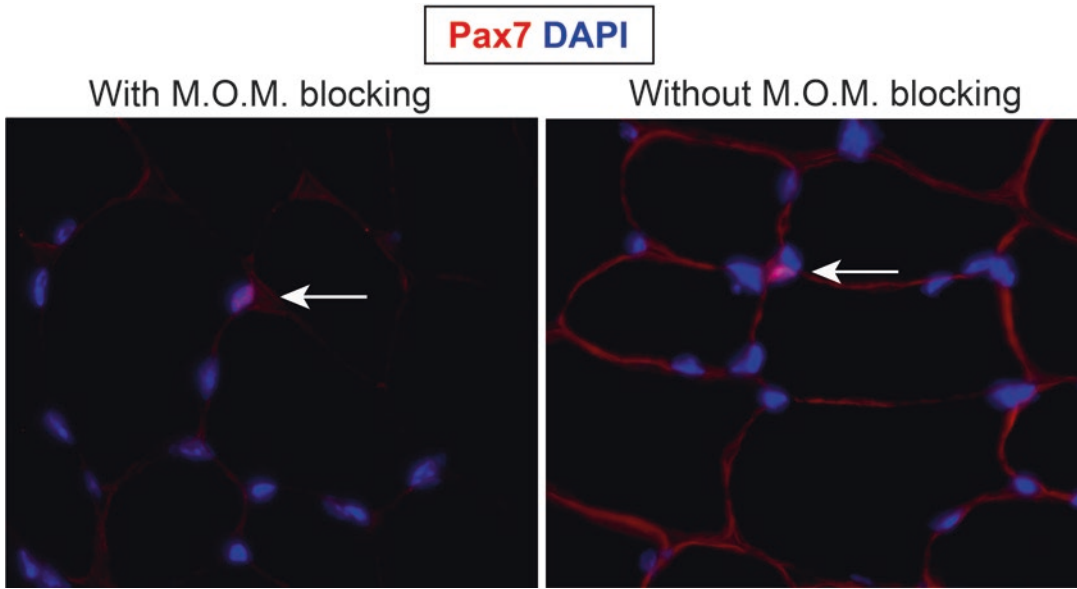


Fig. 3 Mouse on mouse (M.O.M.) blocking reagent. Schematic showing staining with mouse anti-Pax7 on mouse muscle section with (*left panel*) or without (*right panel*) M.O.M. blocking reagent. Satellite cells are distinguishable (*arrows*) in both conditions; however the background staining in the extracellular matrix is strongly decreased with M.O.M. blocking reagent

18. Aspirate DAPI solution and wash twice with 1× PBS for 5 min.
19. Aspirate PBS, add two drops of fluorescent mounting medium and carefully put the coverslip to avoid air bubbles. Let dry for a few hours. Seal the border of the coverslip with nail polish.
20. Visualize the muscle sections using a fluorescent microscope with appropriate filters (*see* **Note 16** and Fig. 2).

4 Notes

1. To avoid PFA degradation, do not heat the solution above 70 °C.
2. PFA 8% aqueous solution is also commercially available (Electron microscopy sciences). Dilute this solution 1:2 in 2× PBS to obtain the PFA 4% stock solution.
3. Immunostaining can be performed on resting or regenerating skeletal muscle. Intra-muscular injection of cardiotoxin (Latoxan, L8102; 50 µl at 10 µM) is a technique that induces severe muscle degeneration and regeneration. Myogenin-positive cells should only be observed in regenerating muscles.
4. Prefixation of the muscle in PFA before OCT embedding interferes with Pax7 staining. Prefixation is not needed for the

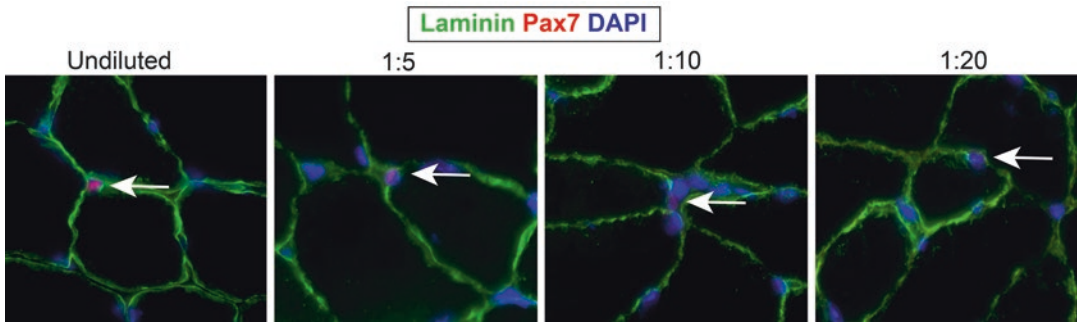


Fig. 4 Pax7 antibody concentration. Immunostaining of Pax7 (red), laminin (green), and DAPI (for nuclei, blue). Pictures show different dilution of Pax7 hybridoma culture supernatant. Satellite cells (arrows) are easily distinguishable when the culture supernatant is used undiluted or 1:5; however the staining becomes faint when it is more diluted

current protocol. However, if prefixation is needed for a specific staining (such as GFP staining), do not overfix the samples (low PFA concentration and incubation time). Use antigen retrieval if needed.

5. This protocol is optimized for immunostaining on muscle cryosection. However, it can also be used for other samples such as isolated myofibers [11] and in vitro primary myoblast culture.
6. Optimal results are obtained when immunostaining is performed immediately after sectioning. However, slides can be stored at -20°C for weeks.
7. Ensure to completely cover the muscle section. Do not let the muscle samples dry until the end of the protocol.
8. Dispose PFA in accordance with hazardous waste disposal guidelines.
9. M.O.M. blocking solution is a critical step to avoid background staining caused by the binding to endogenous mouse IgG. Background staining on mouse skeletal muscle section is classically characterized by high fluorescent staining observed in the extracellular matrix (Fig. 3).
10. Pax7 staining is brighter when the antibody is used undiluted (from hybridoma supernatant). Dilution 1:5 also gives good quality staining. Further dilution significantly decreases the staining intensity (Fig. 4).
11. Use freshly thawed aliquots of Pax7 hybridoma supernatant. Avoid freezing and thawing cycles. Do not keep at 4°C for more than a few days.
12. Other staining could be performed to characterize muscle regeneration (Table 1). For instance, Pax7 and MyoD co-staining could be done to distinguish self-renewing ($\text{Pax7}^{+}/\text{MyoD}^{-}$),

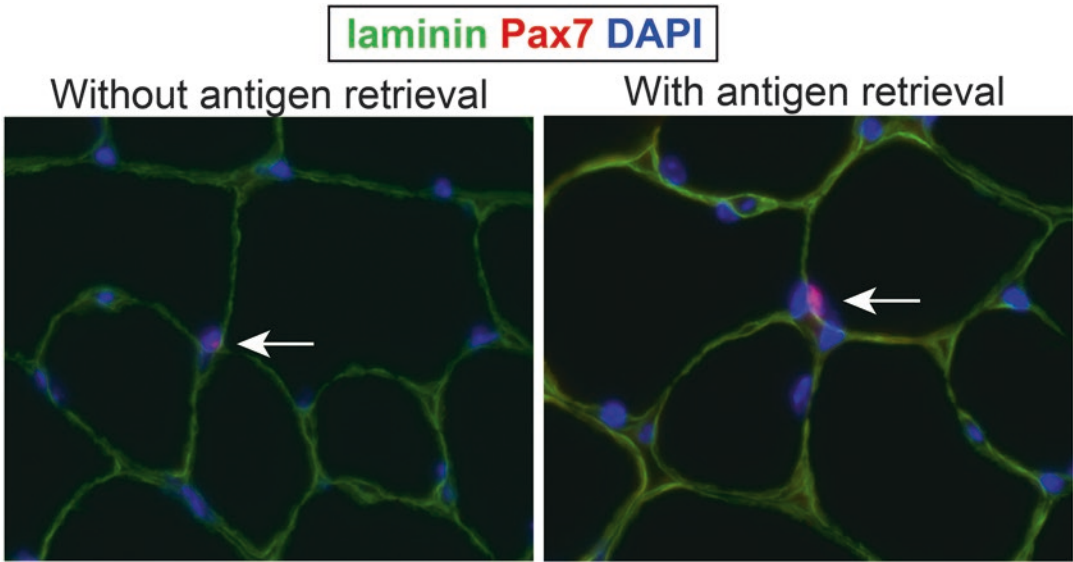


Fig. 5 Pax7 staining with and without antigen retrieval. Immunostaining of Pax7 (red), laminin (green), and DAPI (for nuclei, blue). Pictures show that antigen retrieval (heat-induced antigen retrieval) enhances staining intensity. Antigen retrieval is not needed for the current protocol, however it can be needed for other antigens. Satellite cells are indicated by an arrow

proliferating (Pax7⁺/MyoD⁺) and committed myoblasts (Pax7⁻/MyoD⁺). Pax7, M-cadherin and laminin co-staining could be done to distinguish satellite cell in their niche. Embryonic MyHC and laminin co-staining can be used to distinguish regenerating myofibers.

13. Use negative control to determine the specificity of the staining. Appropriate isotype control (such as mouse IgG) can be used instead of primary antibodies.
14. Use anti-mouse IgG1 secondary antibody to obtain more specific results. Anti-mouse IgG (H+L) secondary antibody increases background staining.
15. Alternatively, mounting medium containing DAPI can be used.
16. Antigen retrieval can be used if staining is dim or absent (Fig. 5). Different antigen retrieval methods can be used such as heat-induced epitope retrieval. To do so, after **step 8**, put your slides in a coplin jar filled with sodium citrate buffer (10 mM sodium citrate, 0.05 % Tween 20, pH 6.0), put the jar in a pressure cooker containing 500 ml of distilled H₂O and heat at high pressure for 5 min (alternatively, microwave can be used). Remove the jar and let cool down at room temperature for 20 min. Wash three times with PBS-T and proceed with **step 9**.

References

1. Dumont NA, Bentzinger CF, Sincennes M-C et al (2015) Satellite cells and skeletal muscle regeneration. *Compr Physiol* 5(3): 1027–1059
2. Sambasivan R, Yao R, Kissenpfennig A et al (2011) Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Dev Camb Engl* 138(17):3647–3656
3. Von Maltzahn J, Jones AE, Parks RJ et al (2013) Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc Natl Acad Sci U S A* 110(41):16474–16479
4. Irintchev A, Zeschnigk M, Starzinski-Powitz A et al (1994) Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev Dyn Off Publ Am Assoc Anat* 199(4):326–337
5. Seale P, Sabourin LA, Girgis-Gabardo A et al (2000) Pax7 is required for the specification of myogenic satellite cells. *Cell* 102(6):777–786
6. Pasut A, Oleynik P, Rudnicki MA (2012) Isolation of muscle stem cells by fluorescence activated cell sorting cytometry. *Methods Mol Biol Clifton NJ* 798:53–64
7. Liu L, Cheung TH, Charville GW et al (2015) Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nat Protoc* 10(10):1612–1624
8. Charville GW, Cheung TH, Yoo B et al (2015) Ex vivo expansion and in vivo self-renewal of human muscle stem cells. *Stem Cell Rep* 5(4):621–632
9. Xu X, Wilschut KJ, Kouklis G et al (2015) Human satellite cell transplantation and regeneration from diverse skeletal muscles. *Stem Cell Rep* 5(3):419–434
10. Dumont NA, Wang YX, Rudnicki MA (2015) Intrinsic and extrinsic mechanisms regulating satellite cell function. *Dev Camb Engl* 142(9): 15721–15781
11. Pasut A, Jones AE, Rudnicki MA (2013) Isolation and culture of individual myofibers and their satellite cells from adult skeletal muscle. *J Vis Exp JoVE* 73:e50074

Immunohistochemical Detection of the Autophagy Markers LC3 and p62/SQSTM1 in Formalin-Fixed and Paraffin-Embedded Tissue

Sabina Berezowska and José A. Galván

Abstract

Autophagy is a highly conserved cellular mechanism of “self digestion,” ensuring cellular homeostasis, and playing a role in many diseases including cancer. As a stress response mechanism, it may also be involved in cellular response to therapy.

LC3 and Sequestosome 1 (p62/SQSTM1) are among the most widely used markers to monitor autophagy, and can be visualized in formalin-fixed and paraffin-embedded tissue by immunohistochemistry. Here we describe a validated staining protocol using an automated staining system available in many routine pathology laboratories, enabling high-throughput staining under standardized conditions.

Key words Immunohistochemistry, LC3, Sequestosome 1, p62/SQSTM1, Autophagy

1 Introduction

Macroautophagy (hereafter called “autophagy”) is an evolutionary conserved cellular mechanism of “self-digestion” enabling homeostasis under physiological conditions by degradation of long-lived proteins [1, 2]. Disturbances of autophagy are believed to have impact in multiple diseases, including cancer. Autophagy may be also partly responsible for cancer cell resistance to therapy, due to its pro-survival role under stress conditions, such as radiation and chemotherapy [3]. Due to all this, there is increasing interest in visualizing autophagy.

A hallmark of autophagy is the autophagosome, a double-membrane, cytosolic vacuole formed around proteins and organelles that are subsequently degraded by fusion of the autophagosome with a lysosome [4, 5]. LC3 (typically LC3A, B, and C, with multiple isoforms and/or splice variants in some organisms) [5] and Sequestosome 1 (p62/SQSTM1) are among the most widely used markers to monitor autophagy [6]. LC3-I is present in the

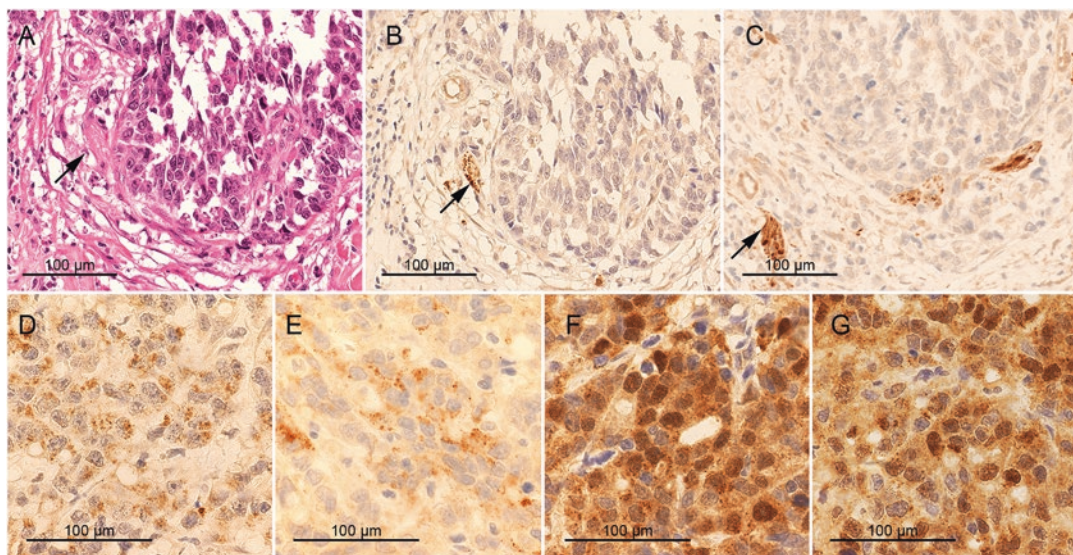


Fig. 1 Staining of LC3B and p62 in lung and gastric carcinoma. (a–c) LC3B positivity of nerve tissue (*arrow*), adjacent to a negative gastric cancer infiltrate: (a) hematoxylin and eosin; (b) LC3B/cell signaling; (c) LC3B/Novus (40×). (d–e) Dot like staining of non-small cell lung carcinoma for (d) LC3/cell signaling, (e) LC3/Novus, (f) p62/MBL and (g) p62/Sigma (100×). Images were taken on a Zeiss Axioskop microscope and corrected for brightness. Objective magnifications are reported. Scale bars: 100 µm (figure adapted from [8])

cytoplasm, and its lipidated form (LC3-II) is incorporated into the autophagosomal membrane [7] thereby allowing detection of autophagic vesicles, e.g., as dots visualized by immunohistochemistry (*see* Fig. 1b–e) [6, 8, 9]. p62 is one of the best known autophagy receptors, enabling selectivity of autophagic degradation by linking cargo to the autophagic machinery [10]. Because of its interaction with LC3, p62 is constantly degraded via autophagy and inhibition leads to accumulation of p62 positive aggregates (*see* Fig. 1f, g) [11].

As autophagy is a flux, it is best assessed in functional assays [6]. Although key markers of autophagy—like LC3 and p62—may also be detected using immunohistochemistry, there is to date uncertainty on how to validly deduce the state of autophagy from those staining patterns only [6, 9]. As an example, multiple LC3 positive dots may signify enhanced autophagic flux, or a disturbed autophagic flux due to for example blocked fusion with lysosomes. In spite of those challenges, if autophagy is to be assessed on very large patient collectives of archival tissue, and electron microscopy may thus be not feasible, immunohistochemistry may be the only method available.

Thus, we report a protocol for high volume immunohistochemical staining of the autophagy markers LC3 and p62/SQSTM1 using an automated staining system available in many routine pathology laboratories [8].

2 Materials

This protocol was developed in the automated system BOND RX[®] (Leica Biosystems, Newcastle, UK). The following reagents should be available before commencing with the staining procedures:

1. Charged tissue slides.
2. BOND Dewax Solution, Leica Biosystems.
3. BOND Wash Solution 1×, Leica Biosystems.
4. Ethanol (100%).
5. Deionized water.
6. Antigen unmasking buffers.
 - (a) ER1 (Citrate based buffer and surfactant pH 6.0), Leica Biosystems.
 - (b) ER2 (EDTA based buffer and surfactant, pH 9), Leica Biosystems.
7. Primary antibody diluent, Leica Biosystems.
8. Primary antibodies.
 - (a) LC3B antibody, rabbit monoclonal (1:3000 dilution), clone D11, Cell Signaling Technology (MA, USA) (Cat. No. 3868) (*see Note 1*).
 - (b) LC3B antibody, rabbit polyclonal (1:4000 dilution), Novus Biologicals (Cambridge, UK) (Cat. No. NB600-1384) (*see Note 2*).
 - (c) p62/SQSTM1 antibody, rabbit polyclonal (1:8000 dilution), MBL (IL, USA) (Cat. No. PM045).
 - (d) p62/SQSTM1 antibody, mouse monoclonal (1:40,000 dilution), Sigma Aldrich (Buchs, Switzerland) (Cat. No. WH0008878M1).
9. BOND polymer Refine Detection kit, Leica Biosystems which contains:
 - (a) Peroxidase Inhibitor (3% Hydrogen Peroxide).
 - (b) Post primary antibody (<10 µl g/mL IgG rabbit anti mouse).
 - (c) Polymer (<25 µl g/mL Poly HRP IgG anti rabbit).
 - (d) DAB (66 mM 3,3'-diaminobenzidine tetrahydrochloride hydrate with >90% ethylene glycol).
 - (e) DAB diluent (0.1% hydrogen peroxide).
 - (f) 0.1% Hematoxylin.
10. Mounting medium.

11. Ethanol (95 % and 100 %) and xylene (only if a xylene-soluble mounting medium is chosen).
12. Tap water.

3 Methods

All procedures are performed at room temperature, unless stated otherwise. The staining was developed on the fully automated staining system BOND RX® (Leica Biosystems).

3.1 Preparation of Samples

1. Cut the paraffin embedded tissue (tissue blocks) at 3 µm on a microtome, and float samples in a water bath at 40 °C.
2. Mount cuts onto charged slides (*see Note 3*).
3. Dry the slides in the oven at 60 °C for 45 min (*see Note 3*).
4. Finally, place the slides in the appropriate racks into the automated staining system BOND RX® (Leica Biosystems), including the cover slides and all necessary reagents according to the manufacturer's instructions.

3.2 Prestaining

The prestaining procedures take place inside the staining system BOND RX® (Leica Biosystems) (*see Note 4*).

1. Heat the slides at 60 °C for 30 min.
2. Add Dewax solution at 72 °C for 30 s followed by Ethanol 100% (*see Note 5*).
3. Antigen retrieval (*see Note 6*).
 - (a) For p62 immunostaining: ER1 buffer is applied at 100 °C for 30 min.
 - (b) For LC3B immunostainings: ER2 buffer is applied at 95 °C for 30 min.

3.3 Chromogenic Staining

The chromogenic staining procedures take place inside the staining system BOND RX® (Leica Biosystems) (*see Note 7*). The whole process is performed in the following steps:

1. Add 150 µl of 3 % hydrogen peroxide for 5 min (*see Note 8*).
2. Wash the slides three times with 150 µl of wash buffer solution.
3. Add 150 µl of diluted primary antibody to each slide, and incubate for 30 min.
4. Wash the slides three times with 150 µl of wash buffer solution.
5. For mouse primary antibody only: add 150 µl per slide of Post-primary antibody for 15 min (*see Note 9*).
6. Wash the slides three times with 150 µl of wash buffer solution.

7. Add 150 μ l of Polymer (Poly HRP IgG anti rabbit) for 15 min.
8. Wash the slides three times with 150 μ l of wash buffer solution.
9. Add 1 drop of mixed DAB Refine for 10 min (previously, the machine mixed 30 μ l of DAB Chromogen Concentrate to 1 ml of DAB diluent).
10. Wash the slides three times with 150 μ l of wash buffer solution.
11. Counterstain slides with 150 μ l hematoxylin for 5 min (*see Note 10*).
12. Wash the slides three times in deionized H₂O.
13. Finally, take out the slides from the staining system and place them in tap water for 10 min.

3.4 Dehydrating and Mounting Slides

3.4.1 Using Aqueous Medium

DAB Chromogen is compatible with either aqueous or non-aqueous mounting medium.

1. Remove slides from tap water.
2. Place a drop aqueous medium on the slide and cover with a coverslip (*see Note 11*).
3. Allow mounting medium to set and view slides on a microscope.

3.4.2 Using Non-aqueous Medium (*See Note 12*)

1. Place slides in 95 % ethanol for 10 s.
2. Place slides in 100 % ethanol for 10 s.
3. Place slides in xylene for 10 s.
4. Place a drop of non-aqueous mounting medium on the slide and cover with a coverslip (*see Note 11*).
5. Allow mounting medium to set and view slides on a microscope.

4 Notes

1. LC3B antibody from Cell Signaling is specific for LC3B [9].
2. LC3B antibody from Novus Biologicals is reported to visualize LC3B and LC3A [12].
3. Using charged cover slides and drying the slides in the oven improves adhesion of the thin tissue cuts to the slide.
4. Pre-staining procedures are necessary to prepare the tissue for staining. First, the paraffin must be removed from the sample. Then it must be rehydrated. After this, the antigen retrieval process is performed.
5. Dewax solution is a solvent-based solution applied for deparaffination and Ethanol 100% for hydration.

6. The antigen retrieval process is performed to remove the cross-links formed by formalin fixation. It is very important not to dry slides at any time during the prestaining procedure, as this can lead to inconsistent staining.
7. The chromogenic staining used is based on horseradish peroxidase (HRP). This enzyme catalyzes the oxidation of substrates in presence of hydrogen peroxide, associated to dextran polymers in the secondary antibody. Currently the most widely used HRP substrate for these applications is 3,3'-Diaminobenzidine (DAB). This reaction generates a brown precipitate.
8. This is performed to quench endogenous peroxidase activity in samples, which may lead to high background staining.
9. This step can be skipped for rabbit primary antibodies.
10. Counterstaining using hematoxylin stains the cell nuclei blue, which provides a contrast to the brown color of the DAB chromogen for better visualization of tissue morphology.
11. Be careful to avoid introducing air bubbles.
12. If choosing non-aqueous medium, the slides must be dehydrated again prior to coverslip mounting. This is achieved by placing them in ethanol and xylene as described.

Acknowledgment

This work was supported by a grant from the Bernese Cancer League to SB.

References

1. Mizushima N, Levine B, Cuervo AM et al (2008) Autophagy fights disease through cellular self-digestion. *Nature* 451(7182):1069–1075
2. Scarlatti F, Granata R, Meijer AJ et al (2009) Does autophagy have a license to kill mammalian cells? *Cell Death Differ* 16(1):12–20
3. Tschan MP, Simon HU (2010) The role of autophagy in anticancer therapy: promises and uncertainties. *J Intern Med* 268(5):410–418
4. Feng Y, He D, Yao Z et al (2014) The machinery of macroautophagy. *Cell Res* 24(1):24–41
5. Hurley JH, Schulman BA (2014) Atomistic autophagy: the structures of cellular self-digestion. *Cell* 157(2):300–311
6. Klionsky DJ, Abdelmohsen K, Abe A et al (2016) Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 12(1):1–222
7. Kabeya Y, Mizushima N, Ueno T et al (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19(21):5720–5728
8. Schlaffli AM, Berezowska S, Adams O et al (2015) Reliable LC3 and p62 autophagy marker detection in formalin fixed paraffin embedded human tissue by immunohistochemistry. *Eur J Histochem* 59(2):2481
9. Martinet W, Schrijvers DM, Timmermans JP et al (2013) Immunohistochemical analysis of macroautophagy: recommendations and limitations. *Autophagy* 9(3):386–402
10. Mijaljica D, Nazarko TY, Brumell JH et al (2012) Receptor protein complexes are in control of autophagy. *Autophagy* 8(11):1701–1705
11. Komatsu M, Ichimura Y (2010) Physiological significance of selective degradation of p62 by autophagy. *FEBS letters* 584(7):1374–1378
12. Zois CE, Giatromanolaki A, Sivridis E et al (2011) "Autophagic flux" in normal mouse tissues: focus on endogenous LC3A processing. *Autophagy* 7(11):1371–1378

Part IV

Lipid Histochemistry

Chapter 14

Tissue Fixation and Processing for the Histological Identification of Lipids

Víctor Carriel, Fernando Campos, José Aneiros-Fernández,
and John A. Kiernan

Abstract

Lipids are a heterogeneous group of substances characterized by their solubility in organic solvents and insolubility in water. Lipids can be found as normal components of different tissues and organs, and they can be affected by several pathological conditions. The histochemical identification of lipids plays an important role in histopathological diagnosis and research, but successful staining depends on adequate fixation and processing of the tissue. Here we describe methods to fix and process tissue samples for the histochemical identification of lipids in frozen or paraffin-embedded tissues.

Key words Lipids fixation, Formaldehyde, Freezing techniques, Sucrose cryoprotection, Isopentane, Liquid nitrogen, Paraffin embedding, Linoleic acid, Lecithin, Lipid histochemistry

1 Introduction

Lipids vary greatly from the structural and molecular point of view. They are soluble in organic solvents and insoluble in water [1]. Histochemically, lipids can be classified according to their hydrophobicity and hydrophilicity, or based on the presence or absence of esters and amides [1]. Lipids are essential for the normal function of the organism, playing important structural, metabolic, or endocrine roles. In this regard, significant amounts of lipids are present in adipose tissue, in sebaceous and adrenal glands [2], and in the myelin sheaths of central and peripheral nerve fibers [3, 4]. Lipids can be affected by several pathological conditions [5]. In this context, the histochemical identification of lipids plays an important role in histopathological diagnosis (e.g., atherosclerotic lesions, liposarcoma and demyelinating diseases) [2, 5] as well as in research. The aim of this chapter is to describe procedures for tissue fixation and processing for subsequent lipid histochemistry in

sections of frozen or paraffin-embedded material. The starting point of each method is chemical fixation of the tissue.

Fixation is the first and most critical step in histology [1, 6, 7], intended to immobilize the cellular and extracellular material in a condition closely resembling the structure and chemical composition of the living tissue [1, 6, 8]. Specimens can be fixed by chemical reactions, physical methods (such as heating and freezing), or by combination of both methods [1, 6, 7]. Fortunately, most of lipids are unaffected by the fixation with non-coagulant chemical agents, notably formaldehyde, which stabilizes structure by cross-linking protein molecules [1, 7]. Lipids are also well fixed (and also stained) by osmium tetroxide (for the method, *see* the Chapter 15) [9, 10]. Lipids are neither displaced nor chemically changed by freezing [1, 2, 7]. In contrast, most lipids are extracted from tissues by organic solvent-based fixatives, which immobilize proteins by coagulation. The incorporation of calcium ions into an aqueous solution of formaldehyde (Baker's formal-calcium) increases the preservation of hydrophilic phospholipids [1]. Our recommendation is for chemical fixation of specimens in 4% neutral buffered formaldehyde (10× dilution of formalin, which contains 40 g formaldehyde per 100 ml) or formal-calcium. Immersion for 24–48 h is necessary for adequate cross-linking of proteins. Once fixed, samples can be frozen and sectioned. Alternatively, the specimens may be dehydrated, cleared and embedded in paraffin. The organic solvents used for dehydration and clearing extract most lipids; only lipids that are covalently bound to protein can be detected in routinely prepared paraffin sections. A post-fixation treatment described by Tracy and Walia [11, 12], however, renders lipids insoluble in organic solvents and allows their histochemical detection in paraffin sections.

Currently, most lipid histochemistry is done on fresh (unfixed) frozen tissues, which are useful for the examination of biopsies for rapid diagnosis, and also for enzyme activity histochemistry and immunofluorescent techniques [1, 7, 13, 14]. However, it is often not feasible to obtain ideal conditions for rapid freezing. Ice crystal formation due to slow freezing produces irreversible damage to the tissue architecture (Figs. 1 and 2) [1, 7, 13, 14]. In order to improve tissue morphology and reduce damage due to ice crystal formation, biopsies must be first fixed in formaldehyde, then cryoprotected, and then frozen quickly [1, 14]. In this sense, tissues must be frozen by using cooled isopentane at -80°C (freezer) or -160°C (liquid nitrogen). This procedure minimizes ice crystal formation and allows the cutting of frozen sections of good quality, suitable for most lipid histochemical methods (Figs. 1 and 2) and other staining techniques.

Conventional paraffin embedding provides internal and external mechanical support to the tissues for microtomy [8]. In the usual method the specimens are completely dehydrated by replacing

LIPID HISTOCHEMISTRY IN ADIPOSE TISSUE

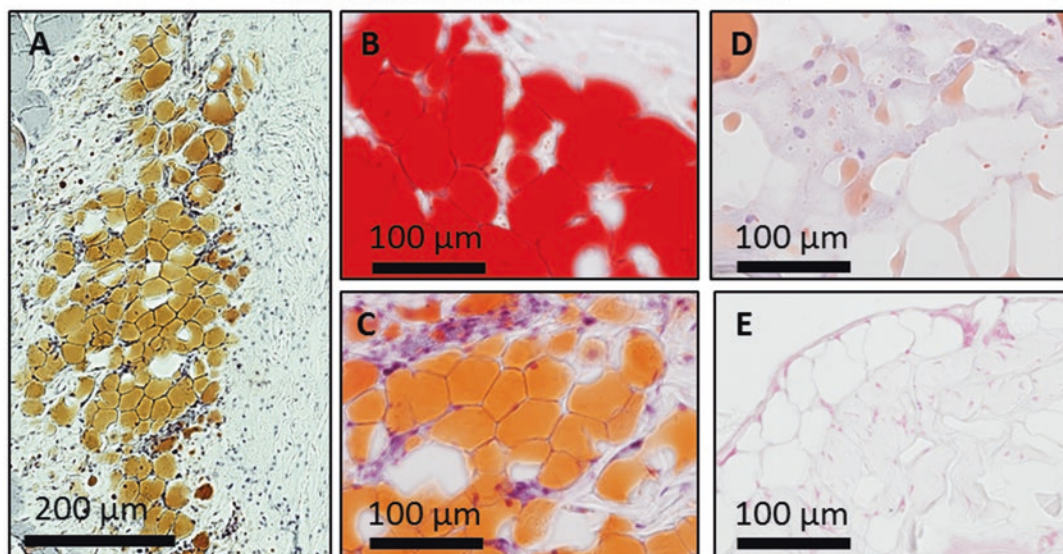


Fig. 1 Examples of lipid histochemistry of adipose tissue carried out with described methods. Positive histochemical reactions with Sudan IV (**a**, **c**, and **d**) and oil red O (**b**) in adipose-rich tissues. Images **a–c** show results obtained in chemically fixed, cryoprotected and quickly frozen tissues, whereas image **d** shows the typical artifact (holes due to ice crystal formation) caused by slow freezing (-20°C) of unfixed material. Image **e** shows complete extraction of lipids by organic solvents during the conventional paraffin embedding procedure

the tissue's water with alcohol, "cleared" in an alcohol-miscible paraffin solvent such as xylene, and finally infiltrated with molten paraffin wax [1, 6, 7]. Although lipids are not affected by formaldehyde fixation, most are extracted from the tissue by the alcohol and xylene used during tissue processing. Some lipoproteins and phospholipids resist extraction during this process, and these can be stained by some histochemical techniques. This mainly occurs with the myelin sheaths of the central and peripheral nervous systems [3, 4, 6] (Fig. 2) (*see staining methods on Chapter 15*) [9].

Tracy and Walia [11, 12] have described a method that successfully preserves lipids in paraffin sections. The formaldehyde-fixed specimens are infiltrated with an emulsion of unsaturated lipids in ethylene glycol. These lipids partition into tissue lipids. A subsequent treatment with chromic acid changes the introduced lipids into organometallic complexes that are insoluble in organic solvents and stainable in paraffin sections.

Finally, this chapter offers different procedures after formaldehyde fixation to perform general lipid histochemistry. The selection of one of these methods should be determined by the aims of the study. Tissue sections from frozen or paraffin-embedded samples can be used for other histochemical and immunohistochemical applications. The Tracy and Walia method was designed to allow

LIPID HISTOCHEMISTRY IN NERVE TISSUE

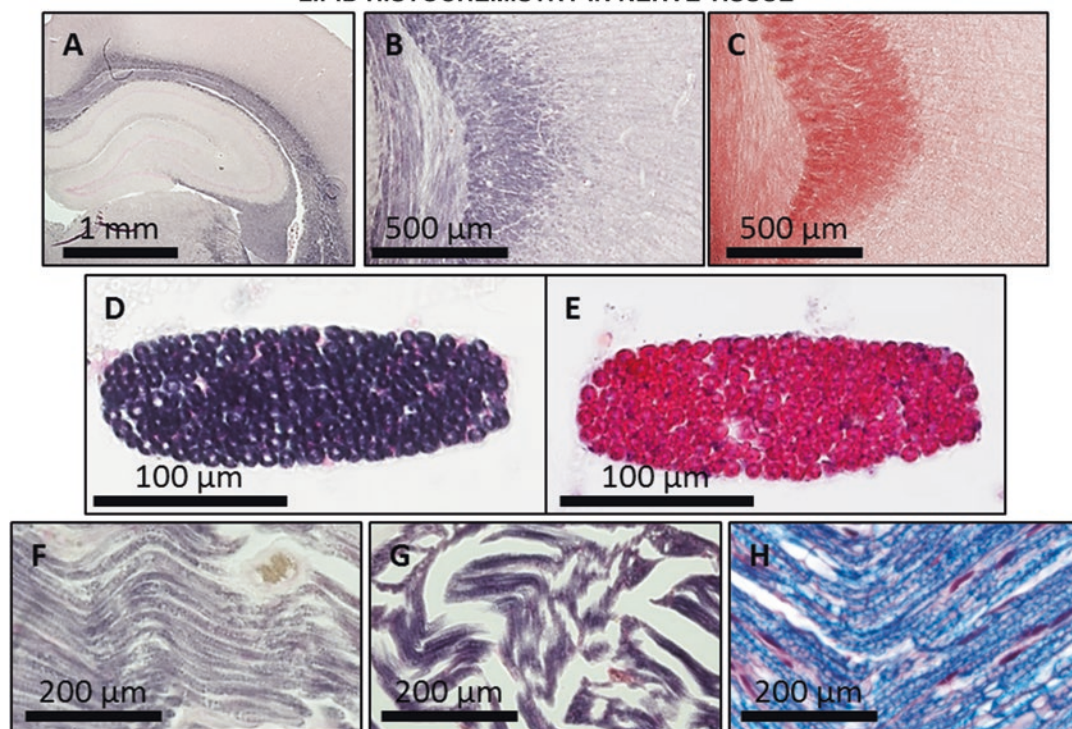


Fig. 2 Images showing positive histochemical reactions of myelin in the central and peripheral nervous system. Sudan Black B (**a, b, d, f, g**), oil red O (**c, e**) and MCOLL (**h**) histochemical methods. Images **a–f** were obtained from tissues subjected to formaldehyde fixation, cryoprotection and fast freezing, while the image **g** shows damage due to the slow freezing (-20°C) of a previously fixed, but not cryoprotected nerve. The image **h** shows myelin stained blue by the MCOLL method in a normal peripheral nerve fixed with formaldehyde and embedded in paraffin (for details of MCOLL method, see Chapter 15) [9]. Note the typical undulating course of nerve fibers (**f–h**) in longitudinal sections. If straight nerve fibers are desired, the fresh nerve can be stretched, under a dissecting microscope, until the bands of Fontana (transverse striations) just disappear. The ends of the specimen can then be pinned to a cork, or the stretched nerve can be placed on a small card for about 1 min, until it adheres, before immersing in the fixative

lipid histochemistry with paraffin sections. It is not yet known whether sections of chromated tissue are suitable for other histochemical or immunohistochemical techniques.

2 Materials

In preparing specimens for lipid histochemistry workers will be in contact with fresh biological material and they will need to use some toxic reagents. Toxic solutions must be prepared under a laminar flow hood using lab coat, gloves, and, when necessary, laboratory goggles and a mask. Tissues must be handled, fixed, and dissected in a pathology workstation (ideally) or in a chemical fume hood, while wearing

medium or heavyweight nitrile, neoprene or PVC gloves, tight-fitting safety goggles or a full face shield. Similarly, for freezing techniques, adequate gloves and eye protection are needed. Isopentane is flammable and volatile (boils at 28 °C).

2.1 Materials and Equipment for Tissue Fixation

1. Biopsy containers or similar.
2. Magnetic stirrer.
3. Dissecting instruments.
4. Electrothermal blowing drying oven or hybridization oven at 56 °C.
5. Separatory funnel.

2.2 Fixation Reagents and Solutions

1. Distilled water.
2. 0.1 M phosphate buffer solution (PB), pH 7.2–7.4.
3. 4% neutral buffered formaldehyde solution. Dilute formalin (40% HCHO, w/v) to 10× volume by adding PB. This solution can be used immediately.
4. Formal-calcium (Baker's formal-calcium) solution. Dilute formaldehyde (37–40% HCHO, w/v) to 10× volume with a 2% w/v aqueous solution of calcium acetate (monohydrate) [1].
5. 70% v/v solution of ethylene glycol (analytical reagent grade) in water.
6. 70% v/v solution of ethanol (analytical grade) in water.
7. Linoleic acid, technical grade (60%; the other major component of this liquid is oleic acid). Alternatively use pure (99%) linoleic acid.
8. Lecithin (product sold as a dietary supplement is suitable).
9. **Solution 1.** Add either 20 ml of technical grade linoleic acid [12] or 5 g of pure (99%) linoleic acid [11] and 2 g lecithin to 500 ml 70% ethylene glycol. Mix for 4 h on a magnetic stirrer and then leave it to stand for at least 4 h. Finally, draw off the lower phase in a separatory funnel. This emulsion is stable for several weeks, but the separation may need to be repeated if an oil layer forms on the surface.
10. **Solution 2.** 2% aqueous solution of chromium trioxide ("chromic acid").
11. **Solution 3.** 5% aqueous solution of sodium bicarbonate (analytical reagent grade).

2.3 Freezing Technique Reagents and Equipment

1. Laboratory freezer (−80 °C).
2. Liquid nitrogen, tank and pump.
3. Isopentane (2-methylbutane).
4. 20% aqueous solution of sucrose.

5. Falcon tubes or similar (between 15 and 50 ml).
6. Freezable plastic or metal bottle with wide cap (for isopentane).
7. Cryomolds, metallic holders or similar.
8. Cryoprotective agent OCT (optimal cutting temperature) or similar.
9. Polystyrene box with wide cap (for liquid nitrogen).
10. Large forceps (to handle samples during freezing).
11. Sealable plastic bags.

2.4 Tissue Processing, Paraffin Embedding, and Equipment

1. Histological cassettes.
2. Glass or xylene-resistant plastic vials (at least 12) with wide caps, or an automatic tissue processor.
3. Electrothermal blowing drying oven at 60 °C for paraffin or commercial embedding center.
4. Analytical grade ethanol solutions (50%, 70%, 95 %, 99 %). Ninety-nine percentage is often sold as “absolute” or “anhydrous” ethanol; it can be diluted with distilled water to make the lower percentages.
5. Xylene (or an alternative clearing agent such as d-limonene).
6. Paraffin wax for histology (*see Note 1*).
7. Paraffin embedding molds.

3 Methods

Lipid histochemistry can be performed with frozen or paraffin-embedded tissues. In all cases, adequate fixation is essential for all subsequent histological techniques. Biopsies must be fixed as soon as possible in an adequate volume of fixative. It is important to follow precautionary measures when working with formaldehyde. If fixative is not available, samples can be transported to the laboratory (as soon as possible) in a biopsy container with wet gauze (saline solution) at 4 °C in a polystyrene box. Once in the laboratory, samples must be properly labeled. We recommend that you prepare all materials, solutions, and equipment in advance.

3.1 Fixation

1. A whole organ or other large specimens must first be immersed in at least ten times its own volume of either neutral buffered formaldehyde or formal-calcium, for a few hours (1–2 h).
2. When organs or biopsies have hardened somewhat, cut them and select representative samples no more than 2 mm thick, and place them in histological cassettes or Falcon tubes (*see Note 2*).
3. Complete fixation by immersing cassettes or samples in at least ten times their volume of neutral buffered formaldehyde or formal-calcium.

4. Leave the samples in the fixative for 24–48 h at room temperature, with agitation (*see* **Note 3**).
5. Rinse fixed samples in tap water and then proceed with freezing or conventional paraffin embedding or with Tracy and Walia lipid post-fixation method.

3.2 Freezing Technique

1. Immerse fixed and rinsed samples in ten times the sample volume of 20% sucrose solution for 24 h at 4 °C.
2. Cool a sufficient volume of isopentane in a freezable bottle with wide cap at –80 °C (–80 °C freezer) or –160 °C (liquid nitrogen) (*see* **Note 4**).
3. Place cryoprotected sample (or fresh material) in a cryomold and cover it completely with OCT.
4. Hold the samples with a large forceps and freeze by immersion on isopentane at –80 °C (in a –80 °C freezer) or at –160 °C (in isopentane immersed in liquid nitrogen) (*see* **Note 5**).
5. Mount frozen samples into the cryostat specimen chuck, and cut sections 10–15 µm thick (for storage of samples, *see* **Note 6**).

3.3 Paraffin Embedding

This method is suitable for formaldehyde-fixed samples (routine method) and samples post-fixed with Tracy and Walia method. The routine method allows us to stain myelin and some lipids that resist extraction. In contrast, Tracy and Walia's method improves the preservation of all lipids, including neutral fat and cholesterol. Here we describe standard schedules for routine and rapid tissue processing, but the times can be adapted according to sizes and types of samples [1, 8]. In addition, throughout the procedure, the volume of liquid should be 10–20 times that of the specimen [1] (for general recommendations, *see* **Note 7**).

Reagent	Routine (overnight)	Rapid (small samples), min
1. 70 % ethanol	1 h	25
2. 95 % ethanol (1)	1 h	15
3. 95 % ethanol (2)	1 h	15
4. 95 % ethanol (3)	1 h	30
5. 99 % ethanol (1)	1 h	15
6. 99 % ethanol (2)	1 h	15
7. 99 % ethanol (3)	1 h	30
8. Xylene or similar (1)	30 min	10
9. Xylene or similar (2)	45 min	15
10. Xylene or similar (3)	45 min	15

(continued)

Reagent	Routine (overnight)	Rapid (small samples), min
11. Paraffin wax (1 at 60 °C)	1 h	30
12. Paraffin wax (2 at 60 °C)	1 h	30
13. Paraffin wax (3 at 60 °C)	1 h	30

**3.4 Tracy and Walia
Lipid Post-fixation
Method**

Note that this method requires 5–7 days, but only needs few minutes of active effort in this period of time. This method is suitable for freezing technique, but an important improvement of lipid histochemistry can be achieved in formaldehyde-fixed and paraffin-embedded tissues [11, 12].

1. Immerse formaldehyde-fixed tissues (no more than 2 mm thick) in solution 1 for 3 days at 56 °C (*see* **Note 8**).
2. Rinse samples in several changes of 70% ethanol for a minimum of 8 h.
3. Rinse in several changes of water for 8 h.
4. Immerse in solution 2 for 24 h at 4 °C.
5. Rinse in tap water for 24 h at room temperature.
6. Immerse in solution 3 for 24 h at room temperature.
7. Rinse in tap water for at least 8 h.
8. Proceed with the dehydration, clearing and paraffin embedding as described in Subheading 3.3 above.

4 Notes

1. Melting point of paraffin waxes range from 52 to 58 °C and as a general rule, a higher melting point gives a harder block. Waxes sold for histology commonly contain added polymers, not identified by the manufacturers but claimed to accelerate the penetration and increase the hardness. It is important to keep wax about 2 °C above its melting point. Higher temperatures are said to damage the secret additives.
2. It is important to perform a correct tissue dissection and to obtain thin representative samples that are no more than 2 mm thick. Thicker samples will increase the times needed for all steps (fixation, freezing, dehydration, clearing and paraffin embedding). In addition, for the Tracy and Walia method thin tissue blocks are necessary for uniform penetration of the post-fixative solutions.
3. The duration of fixation depend of several factors (sample size, type of tissue, temperature, static vs agitation conditions, etc.). Formaldehyde can correctly fix a small object in 8 h, but we

recommend you fix specimens for at least 24 h. Large samples and/or dense tissues will need longer times. In the case of neural tissue (brain and spinal cord) it is recommended to fix samples for at least 48 h or even longer.

4. Isopentane must be stored in an explosion-proof refrigerator at 4 °C or less, because it is an extremely volatile and flammable liquid with high vapour pressure at room temperature. It is necessary to use lab coat, gloves and mask. Isopentane can be cooled at -80 °C 1 or 2 days before using; it will not freeze at this temperature. To cool isopentane to its freezing point (-160 °C) it is necessary to deposit enough liquid nitrogen in a polystyrene box and then immerse the bottle with the isopentane for some minutes. The isopentane becomes viscous, then gelatinous, before it freezes.
5. To freeze the sample at -80 °C by using a cooled isopentane and -80 °C refrigerator, immerse the cryomold with the sample immersed in OCT directly into the isopentane for 3–5 min. To perform a quick freezing it is necessary to immerse the samples into cooled isopentane in liquid nitrogen until OCT is completely frozen. Long exposure to liquid nitrogen should be avoided because it could cause tension cracks.
6. The -80 and -160 °C frozen samples can be packed in a plastic bag, sealed and stored at either -25 or -80 °C for at least 5 months.
7. In general, tissue processing should be performed manually in a chemical fume hood or by using automatic tissue processor. For manual processing, the worker should wear lab coat, gloves, and mask, especially during fixation and clearing. Delicate tissues must start dehydration from 30 or 50% ethanol solutions (or even lower) [1]. An example of a delicate object is a peripheral nerve sample subjected to the osmium tetroxide technique (*see* Chapter 15). Times are standard for general purposes, but it is important to take into account that the size and type of tissue will have an impact on this process. In general, tissue blocks of 1 mm thickness require around 30 min in each reagent, while a tissue blocks of 5 mm thickness could require around 90 min or more in each reagent. An automatic tissue processor can be programmed to work overnight, and it is optional to start with 70% ethanol or 10% neutral buffered formaldehyde (especially if it is necessary to complete the tissue fixation), but this will leave traces of formaldehyde in the alcohols. Manual processing needs stand points, especially for long procedures. Specimens may be left overnight in 70 or 95% ethanol at 4 °C, but not in 99% ethanol or xylene, which can cause excessive hardening of tissues).

8. For the Tracy and Walia method it is recommended to process all the samples by using glass bottles. Histological cassettes are not recommended, because they affect the penetration of solutions into the tissues.

Acknowledgments

The methods described in this chapter were supported by the Spanish “Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica”, from the National Ministry of Economy and Competitiveness (*Instituto de Salud Carlos III*), Grant No FIS PI14-1343 and cofinanced by “Fondo Europeo de Desarrollo Regional” (FEDER), European Union; The Tissue Engineering Group (CTS-115) of the University of Granada, Spain; and by the “Fundación Benéfica Anticáncer San Francisco Javier y Santa Cándida, Granada, España”.

References

1. Kiernan JA (2015) Histological and histochemical methods: theory and practice, 5th edn. Scion, Banbury
2. Mills SE (2007) Histology for pathologists, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, PA
3. Carriel V, Garzón I, Alaminos M et al (2011) Evaluation of myelin sheath and collagen reorganization pattern in a model of peripheral nerve regeneration using an integrated histochemical approach. *Histochem Cell Biol* 136(6):709–717
4. Kiernan JA (2007) Histochemistry of staining methods for normal and degenerating myelin in the central and peripheral nervous systems. *J Histochem* 30(2):87–106
5. Kumar V, Abbas AK, Aster J (2013) Robbins basic pathology, 9th edn. Elsevier Saunders, Philadelphia, PA
6. Carriel V, Garzón I, Alaminos M et al (2014) Histological assessment in peripheral nerve tissue engineering. *Neural Regen Res* 9(18):1657–1660
7. Cook DJ, Warren PJ (2015) Cellular pathology: introduction to techniques and applications, 3rd edn. Scion, Bloxham
8. Crocker J, Burnett D (2005) The science of laboratory diagnosis, 2nd edn. John Wiley, Chichester
9. Carriel V, Campor A, Alaminos M, Raimondo S, Geuna S (2017) Staining methods for normal and regenerative myelin in the nervous system. *Methods Mol Biol Volumen* 1560, Histochemistry of single molecule, methods and protocols. ISBN: 978-1-4939-6787-2
10. Di Scipio F, Raimondo S, Tos P et al (2008) A simple protocol for paraffin-embedded myelin sheath staining with osmium tetroxide for light microscope observation. *Microsc Res Tech* 71(7):497–502
11. Tracy RE, Walia P (2002) A method to fix lipids for staining fat embolism in paraffin sections. *Histopathology* 41(1):75–9
12. Tracy RE, Walia P (2004) Lipid fixation for fat staining in paraffin sections applied to lesions of atherosclerosis. *Virchows Arch* 445(1):22–26
13. Meier-Ruge WA, Bruder E (2005) Pathology of chronic constipation in pediatric and adult coloproctology. *Pathobiology* 72(1-2):1–102
14. Serrato D, Nieto-Aguilar R, Garzón I et al (2009) Comparison of the effect of cryopreservation protocols on the histology of bioengineered tissues. *Histol Histopathol* 24(12):1531–1540

Staining Methods for Normal and Regenerative Myelin in the Nervous System

Víctor Carriel, Antonio Campos, Miguel Alaminos, Stefania Raimondo, and Stefano Geuna

Abstract

Histochemical techniques enable the specific identification of myelin by light microscopy. Here we describe three histochemical methods for the staining of myelin suitable for formalin-fixed and paraffin-embedded materials. The first method is conventional luxol fast blue (LFB) method which stains myelin in blue and Nissl bodies and mast cells in purple. The second method is a LFB-based method called MCOLL, which specifically stains the myelin as well the collagen fibers and cells, giving an integrated overview of the histology and myelin content of the tissue. Finally, we describe the osmium tetroxide method, which consist in the osmication of previously fixed tissues. Osmication is performed prior the embedding of tissues in paraffin giving a permanent positive reaction for myelin as well as other lipids present in the tissue.

Key words Myelin sheath, Luxol fast blue, Picrosirius staining, Osmium tetroxide method, Collagen staining, MCOLL method, Paraffin embedding

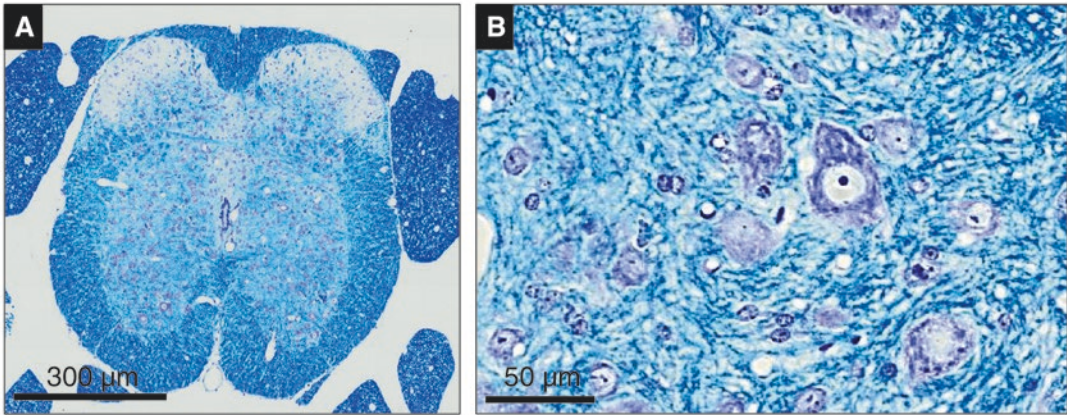
1 Introduction

Nerve fibers (NFs) are complex functional units of the nerve tissue, which are specialized in conduction of the nerve impulse through the nervous system. NFs are internally composed by the neuronal axons, which in turn are surrounded by oligodendrocytes or Schwann glial cells in the central and peripheral nervous systems respectively [1, 2]. These glial cells can form lipid-rich multilayered myelin sheaths establishing the myelinated fibers, or interact with axons without myelin sheaths, forming the unmyelinated NFs. Myelinated NFs are molecularly and structurally different in the central and peripheral nervous systems [2–4], but in both, myelin sheaths have the role to electrically insulate axons from the extracellular space, except for certain gaps between consecutive myelin sheaths, which are known as nodes of Ranvier. These nodes are essential to provide much more rapid conduction of nerve impulse as compared to unmyelinated NFs [2, 4, 5]. Histological and especially

histochemical analyses are basic tools in neurosciences [4]. These techniques allow the identification of different tissue elements including normal or degenerative myelin, which is important to establish a histopathological diagnosis (such as multiple sclerosis or peripheral neuropathy) or to confirm degenerative or regenerative processes of nerve tissue in *in vivo* nerve tissue engineering models [2, 4, 6–10].

Histochemical methods for the identification of myelin were comprehensively reviewed by Kiernan in 2007 [4]. Myelin can be microscopically evaluated on fresh material by using phase contrast microscopy, but this method has several technical limitations and applications [4]. It is well known that myelin is composed by several types of lipids, proteolipids and lipoproteins, which allow its histochemical staining [3, 4]. In general, many lipids are dissolved by organic solvents used for paraffin-embedding protocols, especially the hydrophobic ones [4]. For this reason, freezing and cryosectioning is recommended for lipid histochemistry [4, 7]. In the case of myelin, some lipoproteins resist extraction by organic solvents, and their associated phospholipids confer hydrophobic properties that allow the histochemical staining of myelin in formaldehyde-fixed and paraffin-embedded tissues [4, 7]. Here we describe three relatively simple histochemical methods for the specific staining of normal, degenerative, and regenerative myelin suitable for cryosections and especially for formaldehyde-fixed and paraffin-embedded tissues. The first method described for myelin staining is the luxol fast blue (LFB) MBS, which was introduced by Klüever and Barrera in 1953 [11]. LFB dye has a copper phthalocyanine chromogen insoluble in water, but soluble in alcohol [12] which intensely stains myelin sheaths [6]. However, the chemical bases of this reaction are still unknown. LFB staining is differentiated with lithium carbonate and then contrasted with cresyl violet. Cresyl violet is a cationic dye which stains cell nuclei, Nissl bodies and mast cells at the central and peripheral nervous systems, respectively (Fig. 1) [12]. Conventional LFB method is especially useful for the analysis of central nervous system, but its weak contrast limits its use in peripheral nerves and other tissues. The second method described on this chapter is a LFB-based histochemical staining called MCOLL (due to the simultaneous staining of myelin and collagens). This method was described in 2011 [6], and combines the specificity and sensibility of LFB, picrosirius (sirius red F3B and picric acid) staining and Harris' hematoxylin (as nuclear contrast). LFB stains myelin, while sirius red interacts with cationic groups on the surface of collagens fibers staining them in red and enhancing their natural birefringence under polarized light microscopy [6, 13]. This combination allows the simultaneous evaluation of the histological parameters within the histochemical identification of myelin and fibrillar collagens in normal, degenerative and regenerative conditions (Figs. 2 and 3). MCOLL histochemical method was successfully used to qualita-

CENTRAL NERVOUS SYSTEM



PERIPHERAL NERVES

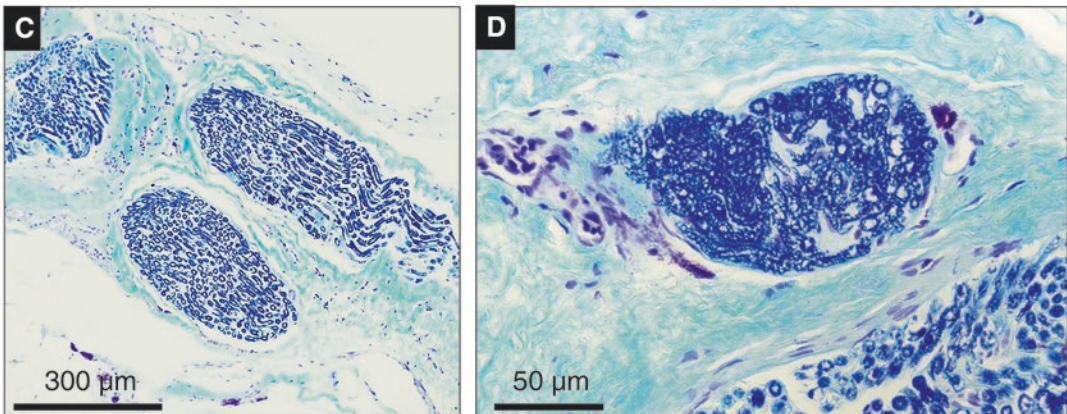
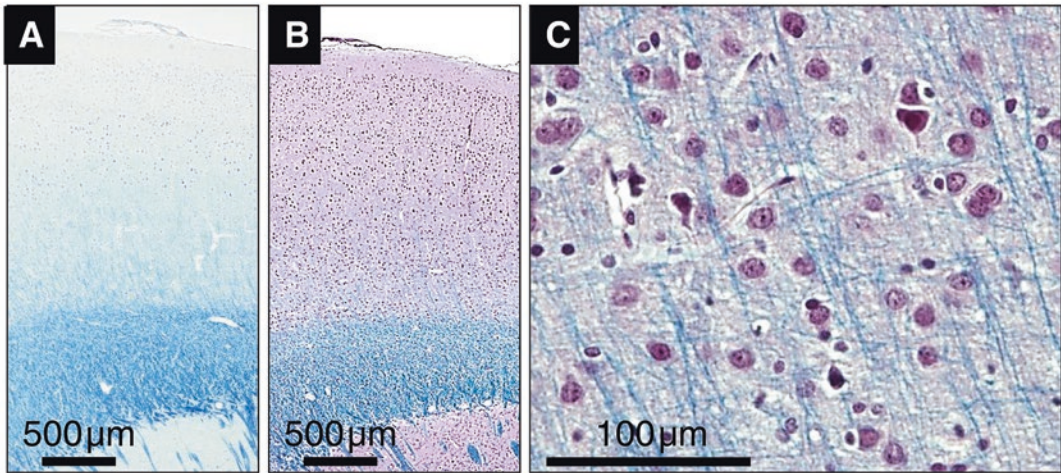


Fig. 1 Conventional luxol fast blue staining in normal rat nerve tissues. Observe the blue positive histochemical reaction for myelin in the white matter of the spinal cord at low magnification (**a**). At higher magnification, it is possible to identify myelinated nerve fibers between some α motor neurons bodies in the anterior horn. Neurons bodies were stained by cresyl violet which also stained Nissl bodies in purple (**b**). In peripheral nerves LFB method intensely stain myelinated nerve fibers in blue, gives a greenish contrast to the epineural collagens (**c**, **d**). Cresyl violet stain cells nuclei and mast cells granules (arrows in **d**)

tively and quantitatively determine the degree of peripheral nerve regeneration, myelination, and stromal remodeling in peripheral nerve tissue engineering [6, 7, 9]. Finally, the last method in this chapter is the osmium tetroxide (OsO_4) staining, which consist in the postfixation/staining (osmication) of 4% paraformaldehyde fixed tissues in 2% osmium tetroxide before the paraffin embedding [8]. OsO_4 react with proteins, but it is reduced by lipids and lipid-rich elements like myelin to a black insoluble precipitate (probably osmium dioxide) [12]. This reaction fixes lipids, and in the case of myelin sheaths prevents their swelling, and provides a permanent and stable myelin stain, which greatly facilitates the identification and evaluation of nerve fibers in normal and regenerative conditions.

CENTRAL NERVOUS SYSTEM



PERIPHERAL NERVES

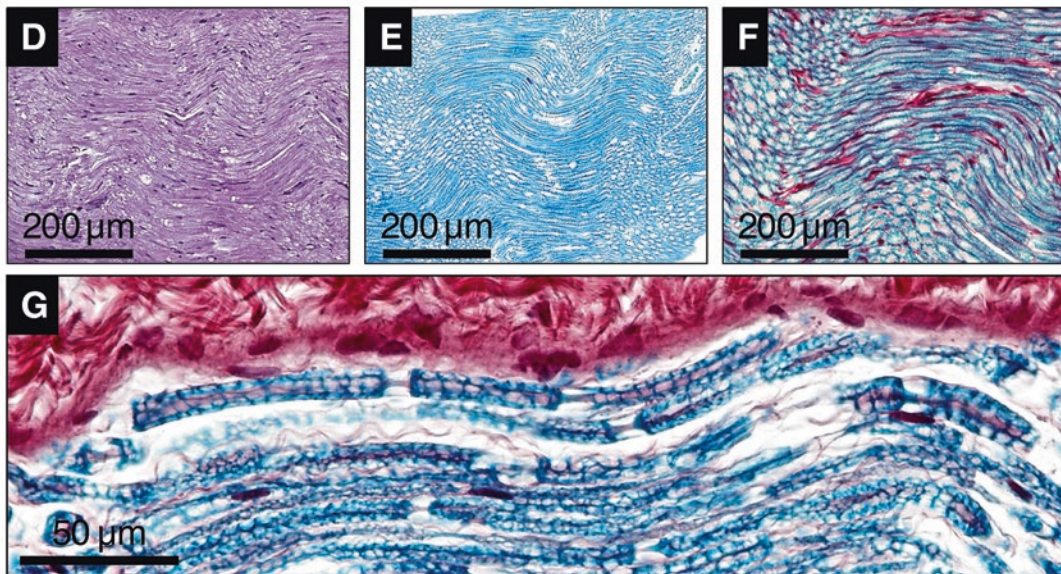
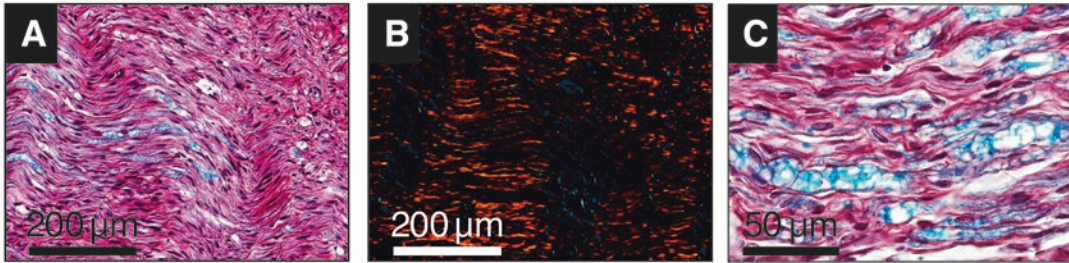


Fig. 2 MCOLL histochemical staining in normal rat nerve tissues. In the central nervous system LFB and MCOLL stains the white matter with the same intensity and specificity (**a**, **b** respectively), but MCOLL gives more contrast between both components and allows to identify the thin myelinated nerve fibers crossing between neuronal bodies of the VI cortex layer (**c**). In peripheral nerves HE staining allows to observe the morphological pattern (**d**), LFB stain the myelin (**e**) and MCOLL simultaneously stain myelin, collagen fibers and cell nuclei with accuracy (**f**). At higher magnifications MCOLL allows the identification of individual nerve fibers with their characteristic myelin sheaths (*blue*) and inner axons, Ranvier Nodes and the collagen fibers (*red*) in the perineurium and endoneurium levels (**g**). Images were reproduced from Carriel et al. 2011 [6], with permission from Springer

Fig. 3 (continued) Transversal sections (**e**, **f**) confirm the fascicular organization of the peripheral nerve regeneration and the presence of surrounded collagen fibers at light and polarized microscopy (**e** and **f** respectively). Image **g** shows a longitudinally oriented newly formed nerve fascicle composed by thin myelinated fibers, several Schwann cells and a collagen matrix in formation. Images **a–c** were reproduced from Carriel et al. 2011 [6], with permission from Springer

DEGENERATION OF PERIPHERAL NERVES



REGENERATION OF PERIPHERAL NERVES

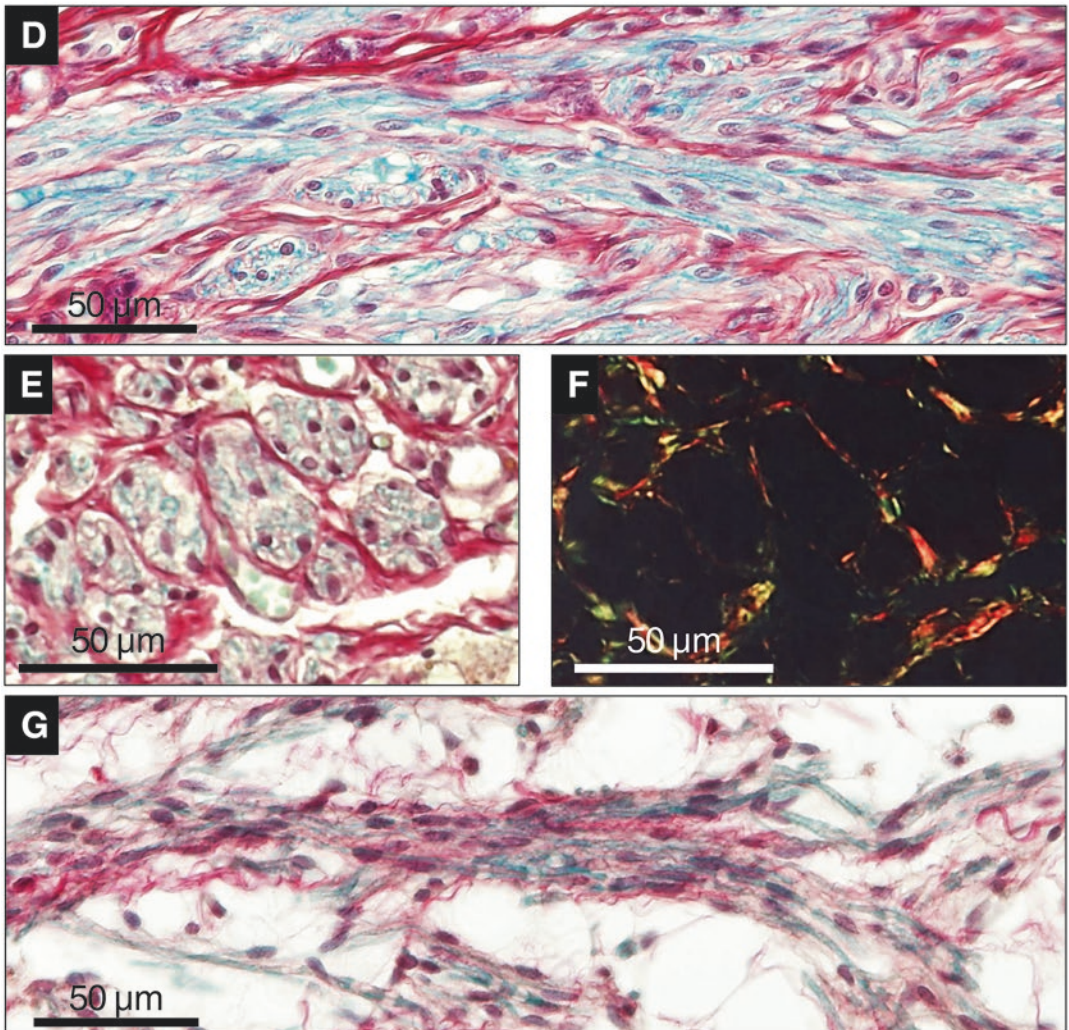


Fig. 3 Light and polarized microscopy images of MCOLL histochemical staining in rat degenerative and regenerative nerves. Unsuccessful peripheral nerve regeneration leads the Wallerian degeneration of the distal nerve stumps, in which an important decrease of myelin occurs accompanied by an increase of stromal collagens (a–c), which is confirmed by polarized light microscopy (b). Myelin sheath is progressively degraded and some remnants react with LFB as well those nerve fibers with disrupted, irregular and sometimes swollen myelin sheaths (c). Tubulization technique promotes different degrees of peripheral nerve regeneration (d–g). Collagen type I conduits filled with fibrin-agarose hydrogel and adipose-derived mesenchymal stem cells promote efficient peripheral nerve regeneration (d–f). In longitudinal sections, it is possible to observe the presence of abundant newly formed myelinated nerves fibers and Schwann cells which are surrounded by collagen fibers stained red

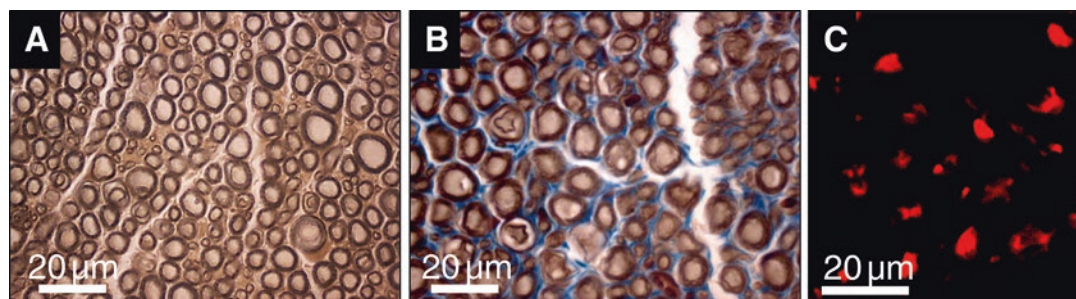


Fig. 4 Light photomicrographs of cross sections of rat median nerve from paraffin-embedded specimens that were stained with osmium tetroxide method before embedding. In image **a** the sample showed no counterstain, while in **b** the section shows Masson's trichrome counterstaining. Collagen staining obtained with Masson's trichrome counterstaining makes it easier to identify myelinated axons and also facilitates analysis of the connective tissue architecture of the nerve and especially blood vessels. Image **c** shows confocal analysis of rat median nerve cross sections immunofluorescence with PAN-antineurofilament (*red*) antibody after osmium etching by H_2O_2

Moreover, this method allows us to perform Masson's trichrome counterstaining and immunohistochemical analyses using a simple and fast H_2O_2 etching technique (Fig. 4).

On this chapter we offer three easy alternatives for the histochemical analyses of myelin on paraffin sections. Other methods such as Sudan black B or oil red O are also useful for the staining of myelin and specially lipids on frozen material (for representative images *see* Fig. 1 on Chapter 14 [14], but the morphological quality is poor and materials (samples and slides) cannot be stored for long periods. In this sense, conventional LFB, MCOLL, and osmium tetroxide method offer specificity, sensibility, good morphology, and permanent histochemical reactions. Finally, MCOLL and osmium tetroxide methods offer the advantage to allow further histological and histochemical analyses due to their integrated histochemical nature.

2 Materials

For all reagents used on these methods it is first necessary to carefully read the specification sheet and especially the safety information. We recommend preparing all solutions under chemical fume hood using lab coat, gloves and, when indicated, laboratory goggles and mask. Prepare all aqueous solution using distilled water and analytical grade reagents only.

2.1 LFB and MCOLL Histochemical Methods

1. Polylysine-coated slides or similar.
2. Glass coplin jars with cap.
3. Xylene or similar organic solvent for dewaxing tissue sections.

4. Analytic degree ethanol solutions at 99 %, 95 %, 70 %, 30 % (*see Note 1*).
5. Distilled water.
6. 10 % analytic-degree glacial acetic acid (99.5 %) in distilled water.
7. Luxol fast blue (LFB) MBS (Color Index (C.I.) 74180) staining solution. Dilute LFB at 0.1 % (w/v) in 95 % ethanol and add 2.5 mL of 10 % glacial acetic acid solution (for store and reuse *see Note 2*).
8. 0.05 % Lithium carbonate (Li_2CO_3) in distilled water.
9. Cresyl violet working solution. Dilute cresyl violet powder at 0.1 % (w/v) in distilled water and add 5 mg of oxalic acid (for store and reuse *see Note 3*).
10. Picric acid saturated solution (*see Note 4*).
11. Picrosirius staining solution. To prepare this solution dilute sirius red F3B (C.I. 35780) powder at 0.2 % (w/v) in a saturated aqueous solution of picric acid (for store and reuse *see Note 5*).
12. Ready-to-use Harris' hematoxylin solution. This solution can be prepared by using Hematoxylin (C.I. 75290) powder and following well-described procedures [15].
13. Electrothermal blowing drying oven or hybridization oven at 56 °C.
14. Commercial hydrophobic mount medium and coverslips.

2.2 Osmium Tetroxide Method

1. Phosphate buffer solution (PBS) 0.1 M, pH 7.2–7.4.
2. Magnetic stirrer with hotplate and thermometer.
3. 4 % Buffered paraformaldehyde solution. Weight 16 g paraformaldehyde (95 %) and dilute it in 350 mL of 0.1 M PBS. Heat the solution at 60 °C under continuous stirring. If it is necessary, add 1 M NaOH drop by drop to completely dissolve the paraformaldehyde precipitate. Adjust the pH (7.2–7.4) and add PBS to 400 mL (*see Note 6*).
4. 0.2 % glycine solution in 0.1 M PBS.
5. Dilute an osmium tetroxide (OsO_4) ampule at 2 % (w/v) in 0.1 M PBS by using a double glass bottle. OsO_4 is a highly reactive and toxic reagent, and it is mandatory to work following safety recommendations (*see Note 7*).

2.3 Tissue Processing, Paraffin Embedding, and Equipment

1. Histological cassettes.
2. Glass or xylene-resistant plastic vials (at least 12) with wide caps, or an automatic tissue processor.
3. Electrothermal blowing drying oven at 60 °C for paraffin or commercial embedding center.

4. Analytical grade ethanol solutions (50 %, 70 %, 95 %, 99 %). Ninety-nine percentage is often sold as “absolute” or “anhydrous” ethanol; it can be diluted with distilled water to make the lower percentages.
5. Xylene (or an alternative clearing agent such as D-limonene).
6. Paraffin wax for histology (*see* **Note 8**).
7. Paraffin embedding molds.

3 Methods

Conventional LFB and MCOLL methods are especially indicated for identification of myelin in fixed tissues (formaldehyde or paraformaldehyde) embedded in paraffin. In the case of the osmium tetroxide method, it is performed after formaldehyde fixation and prior dehydration and paraffin embedding. For these methods, it is recommended to mount histological sections using Polylysine-coated or similar slides, let them dry for 45 min at 50 °C or overnight at 37–40 °C. In addition, it is important to include normal brain or spinal cord tissues or peripheral nerve samples as technical positive controls of all these histochemical reactions.

3.1 Conventional LFB Staining

1. Dewax histological sections in three changes of xylene (10 min in each).
2. Hydrate sections in three changes of 100 % ethanol followed by three changes of 95 % ethanol (3–5 min each).
3. Stain sections overnight (16–24 h) in LFB staining solution at 56 °C (*see* **Note 9**). This solution should be preheated at 56 °C prior to staining.
4. Rinse in 95 % ethanol to remove excess of dye two or three times, and then rinse in distilled water.
5. Differentiate in 0.05 % lithium carbonate until grey and white matter can be macroscopically distinguished (for differentiation *see* **Note 10**).
6. Rinse in two changes of 70 % ethanol.
7. Stop differentiation in distilled water and control myelin staining under microscope.
8. Counterstain in cresyl violet working solution for 10 min at 56 °C. This solution should be preheated at 56 °C prior to staining.
9. Differentiate in 70 % ethanol until there is no background color (for differentiation *see* **Note 11**).
10. Dehydrate quickly in 99 % ethanol (three changes).
11. Clear using two changes of xylene and mount using a hydrophobic medium. Let slides dry at room temperature for at least 15 min.

3.2 MCOLL**Histochemical Method**

1. Perform the **steps 1–7** as described above (*see Note 10*).
2. Rinse in distilled water for 5 min.
3. Stain sections in picosirius staining solution for 30 min at room temperature.
4. Rinse in distilled water, two changes.
5. Counterstain with Harris' hematoxylin for 3 min (*see Note 12*).
6. Rinse in tap water for 3–5 min.
7. Dehydrate in increasing concentrations of ethanol starting from 95 to 99%.
8. Clear in two changes of xylene and mount using a hydrophobic medium. Let slides dry at room temperature for at least 15 min.

3.3 Osmium**Tetroxide Method**

1. Fix nerve samples in buffered 4% paraformaldehyde solution for 2–4 h at 4 °C.
2. Rinse samples with PBS and store them in a 0.2% glycine solution.
3. Rinse with two or three changes of PBS for few min.
4. Immerse tissue samples in 2% OsO₄ (fixative/staining) solution for 2 h at 4 °C.
5. Dehydrate by an increasing ethanol starting from 30 to 99% with numerous changes in each (5 × 5 min each) (*see Note 13*).
6. Clear in xylene or similar, infiltrate and embed in paraffin.
7. Cut section of 8–10 µm thickness.
8. Dewax, hydrate, and stain sections with Masson's trichrome staining or perform an immunohistochemical staining [9].

4 Notes

1. For LFB and MCOLL only 70, 95 and 99% ethanol solutions are needed, but for osmium tetroxide method, tissues should be dehydrated using 30–99% ethanol solutions. In this sense, it is recommendable to prepare these lower ethanol solutions by diluting the 70% ethanol with distilled water.
2. LFB staining solution can be reused and stored for at least 5 years [12]. It is recommended to prepare around 500 mL of this solution (stock working solution) and use aliquots of 100 or 200 mL (in use working solution) for routine use. Discard this solution when results are not optimal.
3. Cresyl violet counterstain solution can be reused with optimal results, but solution cannot be stored for longer periods and it is recommended to prepare a volume according to needs. Discard this solution when results are not optimal.

4. Picric acid is a highly reactive and explosive reagent, especially when the powder is subjected to friction and compression. It is recommended to use commercially available saturated aqueous solution with caution.
5. Picrosirius staining solution can be reused and it is stable for at least 3 years. Like LFB staining solution, it is recommended to prepare around 500 mL of this solution (stock working solution) and to use aliquots of 100 or 200 mL (working solution) for routine use. Discard this solution when results are not optimal.
6. To prepare 4% paraformaldehyde solution, it is mandatory to use the lab coat. For face and eye protection tight-fitting safety goggles or a full face shield (8-in. minimum) are recommended. To protect hands, solutions should be handled with medium or heavyweight nitrile, neoprene, natural rubber, or PVC gloves. All procedures must be performed under laminar flow hood.
7. Osmium tetroxide is a strong oxidant and has a high vapor pressure. It should be stored taking care to avoid vapor dispersion and in a refrigerator restricted to specialized personnel only. Because of its high toxicity, safety procedures for its handling should be followed carefully. Inhalation and skin contact must be avoided and it must be used only under a chemical fume hood. Handling this product and all embedding passages should be carried out by expert technician/researchers. Alcohol washes after osmium staining should be numerous (3–5 passages for each ethanol concentration). Osmium waste should be collected by specialized waste disposal personnel after being placed in properly labeled containers.
8. Melting point of paraffin waxes range from 52 to 58 °C and as a general rule, a higher melting point gives a harder block. Waxes sold for histology commonly contain added polymers, not identified by the manufacturers but claimed to accelerate the penetration and increase the hardness. It is important to keep wax about 2 °C above its melting point. Higher temperatures are said to damage the secret additives.
9. In order to avoid the evaporation of LFB solution during the staining at 56 °C it is important to cover the coplin with the slides and then seal it with Parafilm. This solution can be reused several times.
10. Differentiation should be performed controlling macroscopically and microscopically the histochemical reaction in the positive controls. We recommend not to leave the slides for longer periods in lithium carbonate and 70% ethanol solutions. If slides need more differentiation, repeat **steps 5–7**. Normally two differentiations are enough. Overfixation (more

than 48 h) affects the differentiation process and could induce background on collagen-rich structures, especially in peripheral nerves.

11. Cresyl violet is differentiated by 70 % ethanol, stop differentiation in distilled water and control under microscope until obtaining the desired positive reaction.
12. It is well known that tap water turns Harris' hematoxylin blue, but it is important not to exceed the 5 min in tap water, because it could diminish the red staining intensity of collagens.
13. The tissue processing for osmium tetroxide method differs from the conventional paraffin embedding procedure. This method needs to start dehydration from lower ethanol solutions (30 %) and in order to completely remove the excess of osmium from tissues several changes in each alcoholic must be done (at least 5).

Acknowledgments

The methods described in this chapter were supported by the Spanish “Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica”, from the National Ministry of Economy and Competitiveness (*Instituto de Salud Carlos III*), Grant No FIS PI14-1343 and cofinanced by “Fondo Europeo de Desarrollo Regional” (FEDER), European Union.

References

1. Carriel V, Alaminos M, Garzón I et al (2014) Tissue engineering of the peripheral nervous system. *Expert Rev Neurother* 14(3): 301–318
2. Geuna S, Raimondo S, Ronchi G et al (2009) Histology of the peripheral nerve and changes occurring during nerve regeneration. *Int Rev Neurobiol* 87(Chapter 3):27–46
3. Arroyo EJ, Scherer SS (2000) On the molecular architecture of myelinated fibers. *Histochem Cell Biol* 113(1):1–18
4. Kiernan JA (2007) Histochemistry of staining methods for normal and degenerating myelin in the central and peripheral nervous systems. *J Histochem* 30(2):87–106
5. Kierszenbaum AL (2002) Histology and cell biology : an introduction to pathology. Mosby inc., St Louis, MO
6. Carriel V, Garzón I, Alaminos M et al (2011) Evaluation of myelin sheath and collagen reorganization pattern in a model of peripheral nerve regeneration using an integrated histochemical approach. *Histochem Cell Biol* 136(6):709–717
7. Carriel V, Garzón I, Alaminos M et al (2014) Histological assessment in peripheral nerve tissue engineering. *Neural Regen Res* 9(18):1657–1660
8. Di Scipio F, Raimondo S, Tos P et al (2008) A simple protocol for paraffin-embedded myelin sheath staining with osmium tetroxide for light microscope observation. *Microsc Res Tech* 71(7):497–502
9. Carriel V, Garrido-Gómez J, Hernández-Cortés P et al (2013) Combination of fibrin-agarose hydrogels and adipose-derived mesenchymal stem cells for peripheral nerve regeneration. *J Neural Eng* 10(2):026022
10. Carriel V, Garzón I, Campos A et al (2014) Differential expression of GAP-43 and neurofilament during peripheral nerve regeneration through bio-artificial conduits. *J Tissue Eng Regen Med* PMID:25080900. doi:[10.1002/term.1949](https://doi.org/10.1002/term.1949)

11. Kluver H, Barrera EA (1953) A method for the combined staining of cells and fibers in the nervous system. *J Neuropathol Exp Neurol* 12(4):400–403
12. Kiernan JA (2008) *Histological and histochemical methods: theory and practice*, 5th edn. Scion, Bloxham, p 571
13. Carriel VS, Aneiros-Fernandez J, Arias-Santiago S et al (2011) A novel histochemical method for a simultaneous staining of melanin and collagen fibers. *J Histochem Cytochem* 59(3):270–277
14. Carriel V, Campos F, Aneiros-Fernández J, Kiernan JA (2017) Chapter 14: Tissue fixation and processing for the histological identification of lipids. *Histochemistry of single molecules: methods and protocols*, *Methods in Molecular Biology*, vol. 1560. doi: [10.1007/978-1-4939-6788-9_14](https://doi.org/10.1007/978-1-4939-6788-9_14), © Springer Science + Business Media LLC 2017
15. Prophet EB, Armed Forces Institute of Pathology (1992) *Laboratory methods in histotechnology*. American Registry of Pathology, Washington, DC, p 279

Nile Red Staining of Neutral Lipids in Yeast

Kerry Ann Rostron and Clare Louise Lawrence

Abstract

Determination of cellular neutral lipid levels in yeast is important for both the biotechnology industry and biomedical research. However, many of the currently available methods are labor intensive and time consuming. Here we describe a rapid and repeatable method for the detection of neutral lipids, which can be utilized in both oleaginous and non-oleaginous yeast species. The method utilizes the fluorescent dye, Nile red, which enables neutral lipid levels to either be visualized via microscopy or quantified using a 96-well plate assay.

Key words Nile red, Neutral lipids, Microscopy, Microplate, Yeast

1 Introduction

Lipids are a vital component of all eukaryotic cells and can be generally classified into polar and neutral lipid subgroups. Neutral lipids, namely sterol esters (SEs) and triacylglycerols (TAGs), are more hydrophobic molecules when compared with polar lipids, holding a pivotal role in the storage of lipids for cellular energy [1].

There is vast interest in the formation and accumulation of neutral lipid bodies, with intense investigation in a wide variety of research areas. Specifically in yeast systems, a major area of neutral lipid research focuses on the production of biofuels from oleaginous yeast species. Due to their ability to generate biomass on a large scale coupled with the ease of genetic modification, yeast are an attractive alternative to biofuel production in plant based systems [2].

Other prominent areas of investigation are the regulation of lipid metabolism as well as the accumulation of neutral lipids in human disease. Aberrant signaling leading to defects in lipid metabolism has been described for a number of pathological states including cancer, diabetes, and Alzheimer's disease [3–5]. With its extensive use as a model organism to study human disease, yeast holds an important role in furthering our understanding of

regulatory mechanisms in lipid metabolism and therefore lipid related disorders [6].

There are a number of traditional methodologies widely used to quantify fungal lipids, such as by gravimetric, gas or liquid chromatographic means. However, these methods are time consuming, laborious and often require multiple extractions along with the use of organic solvents in large quantities [7, 8]. Further to this, the lipidologist must take precautions to ensure that lipids are not oxidized either by contaminants, such as plasticisers from plastics, or enzymes and oxygen. In addition, there are inherent problems with commonly used extraction methods including inefficient cell wall breakage along with gravimetric underestimates of neutral lipids in extracts comprising more than 2% lipid [9]. As such, it is generally accepted that there is no single method capable of obtaining 100% lipid yield. Further, when utilizing non-oleaginous yeasts, gravimetric analysis of samples can prove difficult due to the low amounts of lipid accumulated.

These difficulties mean screening a large number of samples is complex and highlights the requirement for a sensitive, increased throughput methodology for determining cellular levels of neutral lipid. This would allow the identification of key strains and culture conditions, which may then be promoted for more sophisticated, in-depth analyses.

Nile red, 9-diethylamino-5H-benzo(a)phenoxazine-5-one, is an established and widely utilized lipophilic dye for assessment of cellular neutral lipid status. Previous work validating the use of Nile red has often concentrated around microplate assays to determine lipid accumulation in oleaginous species [10, 11] and phenotypic analyses in non-oleaginous yeast species via means of microscopy [12–14]. The method described in this chapter has the ability to reliably detect measurable amounts of fluorescence pertaining to cellular lipid content in low lipid accumulating species, such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Schizosaccharomyces pombe* (*S. pombe*) in all growth phases [15].

Here, we describe a simple, fast, and repeatable method for the relative quantification of neutral lipids in yeast cells. The described Nile red assay allows for rapid identification of relative neutral lipid amounts *in situ*, thus abolishing the requirement for multiple extractions of samples which may not yield an informative phenotype at the level of the general neutral lipid pool.

We have demonstrated the use of this methodology for the relative quantification of neutral lipids from the oleaginous yeast species *Lipomyces starkeyi* (*L. starkeyi*, Fig. 1). Further, we have validated the sensitivity of this method in obtaining distinguishable lipid phenotypes between wild type and mutant strains of the low lipid accumulating yeasts *S. cerevisiae*, and for the first time demonstrated the use of a Nile red assay for the fission yeast species *S. pombe* [15].

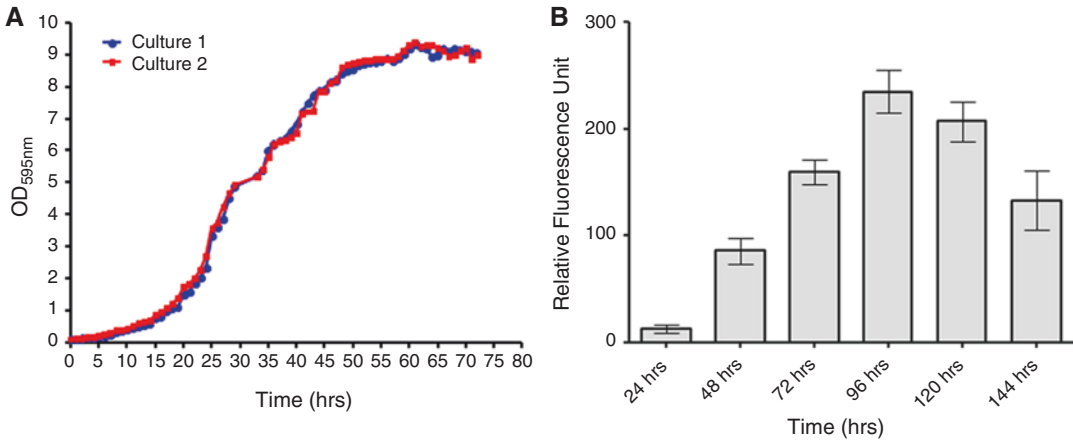


Fig. 1 (a) Growth curve of *L. starkeyi* in YNB medium. Cells from log phase YPD cultures were resuspended in yeast nitrogen base (YNB) to give a starting OD_{595nm} of 0.1. Cultures were allowed to grow, with shaking at 180 rpm 30 °C and readings until stationary phase was reached, as indicated by plateaued growth. Growth curves were conducted in duplicate with blue and red lines denoting growth pattern observed from two distinct colonies. **(b)** Neutral lipid fluorescence intensity of Nile red stained *L. starkeyi* over a range of growth phases. Cells were harvested at 24 hourly intervals until 144 h post resuspension in YNB. For Nile red analysis, cells were washed twice in PBS before adjusting to a cell density of 4×10^7 cells/mL (OD_{595nm} 1.0). 10×10^6 cells were transferred to the wells of black 96-well plates in triplicate. Twenty-five microliter PBS/DMSO (1:1 v/v) and 5 µg/mL Nile red were added to each sample. Fluorescence intensity was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence pertaining to neutral lipids increased as cells moved into stationary phase at 72 h before reaching maximal levels late stationary phase, 96 h. Post 96 h, fluorescence values decreased which is thought to be attributable to beta oxidation of accumulated lipids for the production of cellular energy. All values are normalized to a cell density of 4×10^7 cells, corresponding to OD₅₉₅ of 1.0. Data are shown as means of triplicate experiments $\pm$ SD

Although the method described here is specific to yeast cells, Nile red has been utilized in a range of organisms including *Caenorhabditis elegans* [16, 17], *Drosophila* [18], bacteria [19], zebrafish [20], and microalgae [21]. Nile red has also been utilized in staining of lipids in mammalian cells such as macrophages [22] along with MCF-7 (breast cancer) and HepG2 (hepatic cancer) cell lines [23, 24]. As such, it may be possible to further develop the described method for screening in a variety of model systems inclusive of mammalian cells.

2 Materials

2.1 Yeast Cell Culture

Growth medium should be prepared in distilled water before sterilization by autoclaving and thereafter stored at room temperature. For the purposes of microscopy, it may be preferable to sterilize growth medium by filtration in order to reduce background fluorescence.

Ensure good aseptic technique when aliquoting medium, preparing pre-cultures, taking cell density readings and during dilution or resuspension of pre-cultures into fresh medium. Sterile technique can be maintained either through the use of a Bunsen burner on a blue flame or within a microbiological safety cabinet.

For the described Nile red method, yeasts were pre-cultured in complex medium before transferring to a defined minimal medium in order to follow de novo cellular lipid accumulation. Briefly, pre-cultured yeast cells were harvested during mid-exponential growth, and the resultant cell pellet resuspended in defined minimal medium at an $OD_{595nm} \sim 0.1$ (in our hands: $\sim 2.0 \times 10^6$ cells/mL for *S. cerevisiae*/*S. pombe* or $\sim 4 \times 10^6$ cells/mL for *L. starkeyi*). Cultures were then incubated at 30 °C, with shaking at 180 rpm until desired growth phases for Nile red staining were reached (*see Note 1*).

1. *S. cerevisiae* and *L. starkeyi* were routinely cultured in Yeast Extract Peptone Dextrose (YPD) (2% glucose, 1% peptone, 1% yeast extract) complex medium and Yeast Nitrogen Base (YNB) defined synthetic medium (0.67% yeast nitrogen base without amino acids (ForMedium™ UK) 2% glucose and 0.002% appropriate amino acids except for threonine, uracil, tyrosine, lysine at 0.001% and leucine at 0.005%).
2. The fission yeast species *S. pombe* is routinely cultured in Yeast Extract Sucrose (YES) (2% glucose, 0.5% yeast extract, 0.005% amino acids leucine, lysine, adenine, histidine and uracil) and defined Edinburgh Minimal Medium (EMM) (1.2% EMM without dextrose and amino acids (ForMedium™ UK), 2% glucose and 0.0225% of the appropriate amino acids for the strain).
3. Sterile conical flasks: for pre-cultures 100 mL conical flasks are normally sufficient. If cells are to be resuspended into different media or a larger volume of culture is required for Nile red staining, ensure the use of a suitable sized conical flask. For example, 20 mL of culture in a 100 mL flask or 50 mL of culture in a 250 mL conical flask (*see Note 2*).
4. Pipettes and sterile pipette tips: 1000 µL and 200 µL for cell density readings and preparation of samples.
5. Shaking incubator 30 °C 180 rpm.
6. Cuvettes and spectrophotometer for cell density readings using absorbance at 595 nm.

2.2 Nile Red Staining and Detection: Microscopy

1. Nile red stock solution, 2 mg/mL: Nile red powder (Acros Organics) dissolved in acetone to a concentration of 2 mg/mL. The Nile red stock solution should be stored protected from light at 4 °C, or at –20 °C for long term storage.

2. 1× phosphate buffered saline (PBS: 10 mM PO_4^{3-} , 137 mM NaCl, and 2.7 mM KCl) made in distilled water to pH 7.4 and sterilized by autoclaving or filtration.
3. Molecular Biology grade DMSO.
4. Freshly prepared 10% (v/v) formalin (*see Note 3*).
5. Sterile 1.5 mL tubes for sample preparation.
6. Microcentrifuge.
7. Glass slides and coverslips for microscopy.
8. *SlowFade* antifade mountant (Invitrogen, UK).
9. Pipettes and sterile pipette tips: 1000 and 200 μL .
10. Nail varnish, for sealing coverslip.
11. Fluorescence microscope (*see Note 4*).

2.3 Nile Red Staining and Detection: 96-Well Plate Assay

1. Nile red stock solution, 2 mg/mL: Nile red powder dissolved in acetone to a concentration of 2 mg/mL. The Nile red stock solution should be stored protected from light at 4 °C, or –20 °C for long term storage.
2. 1× PBS made up in distilled water to pH 7.4 and sterilized by autoclaving or filtration.
3. Molecular biology grade DMSO.
4. 15 mL tubes. (Smaller tubes may be used dependent on volume of sample required.)
5. Centrifuge with inserts for 15 mL tubes.
6. Pipettes and sterile pipette tips: 1000 and 200 μL .
7. Black 96-well plates (*see Note 5*).
8. Microplate reader suitable for fluorescence detection.

3 Methods

All procedures, unless otherwise stated, should be carried out at room temperature.

3.1 Nile Red Staining: Fluorescence Microscopy

1. Prepare a 60 $\mu\text{g/mL}$ Nile red working solution by diluting the 2 mg/mL stock solution in acetone (60 μL of 2 mg/mL stock solution in total volume of 2 mL) (*see Note 6*).
2. Prepare DMSO:PBS (1:1) solution by mixing equal amounts of DMSO with 1× PBS and mix well.
3. At the desired growth phase, transfer a 250 μL sample of each yeast cell culture to be stained for microscopy into sterile 1.5 mL tubes.
4. Add 25 μL freshly prepared DMSO:PBS (1:1) and 5 $\mu\text{g/mL}$ Nile red in acetone (25 μL of freshly prepared 60 $\mu\text{g/mL}$

stock) to the yeast cells and mix. Incubate the samples in the dark for 5 min at room temperature.

5. Centrifuge stained cells for 2 min at $492 \times g$, carefully aspirate the supernatant and wash the resultant cell pellet twice in $1 \times$ PBS, centrifuging the sample after each wash.
6. Resuspend pelleted cells in $100 \mu\text{L}$ 10% (v/v) formalin and fix for 15 min in the dark at room temperature.
7. Centrifuge the fixed and stained cells for 2 min at $492 \times g$ before washing a further two times in $1 \times$ PBS as in **step 5**.
8. Resuspend the final cell pellet in a small volume of SlowFade ($\sim 20 \mu\text{L}$) (Invitrogen UK) to prevent photobleaching of Nile red. Pipette $5 \mu\text{L}$ of the cell sample onto a glass microscope slide, place the cover slip firmly over the sample. Seal the edges of the coverslip with nail varnish to prevent drying out and proceed to imaging using a fluorescence microscope (*see* **Note 4**).

3.2 Nile Red

Staining: 96-Well Plate Assay

1. Prepare a $60 \mu\text{g/mL}$ Nile red working solution by diluting the 2 mg/mL stock solution in acetone ($60 \mu\text{L}$ of 2 mg/mL stock solution in total volume of 2 mL) (*see* **Note 6**).
2. Prepare DMSO:PBS (1:1) solution. For a complete 96-well plate a total volume of 2.5 mL is required. Prepare by combining 1.25 mL DMSO with 1.25 mL of $1 \times$ PBS and mix well.
3. Harvest the required amount of yeast culture in the desired phase of growth by centrifugation for 2 min at $492 \times g$.
4. Wash resultant pellet twice in $1 \times$ PBS before resuspending cells in $1 \times$ PBS at an $\text{OD}_{595 \text{ nm}} \sim 1.0$ (in our hands: $\sim 2 \times 10^7$ cells/mL for *S. cerevisiae*/*S. pombe* or $\sim 4 \times 10^7$ cells for *L. starkeyi*) (*see* **Note 7**). To plate each sample in triplicate we advise that the final resuspension be in a total volume of at least 1 mL .
5. Record the optical density, 595 nm , after the final resuspension in $1 \times$ PBS. This is required for correction of the raw fluorescence data based on cell density.
6. Set up the microplate reader with the following settings: wavelengths excitation 485 nm and emission 535 nm on top 50% mirror, using the optimal gain and orbital shake for 10 s before reading (*see* **Note 8**).
7. In a black, clear bottomed 96-well plate, add $25 \mu\text{L}$ of freshly prepared DMSO:PBS (1:1 v/v) to each well required for the assay. Next, add $250 \mu\text{L}$ of sterile $1 \times$ PBS into at least 6 of the DMSO:PBS containing wells of row A (this is required so that background fluorescence may be corrected for).
8. Transfer $250 \mu\text{L}$ of each yeast sample to the relevant wells of the 96-well plate (*see* **Note 9**). Ensure that the samples are mixed prior to transferring to the wells of the 96-well plate, as

yeast cells readily sediment. Finally, using a single pipette tip, add 25 μL of the freshly prepared 60 $\mu\text{g}/\text{mL}$ Nile red stock solution to each well, to give a final Nile red concentration of 5 $\mu\text{g}/\text{mL}$ per well (*see* **Note 10**).

9. Read the plate immediately after addition of the Nile red dye to the wells for the required number of cycles (*see* **Note 11**).

3.3 Data Analysis and Normalization: 96-Well Plate Assay

1. To generate a value for background fluorescence, take the average of the relative fluorescence unit (RFU) values generated from wells containing PBS with DMSO:PBS and Nile red (row A).
2. Divide the RFU values obtained for the yeast samples by this value in order to correct the raw fluorescence data for background fluorescence.
3. To normalize the data for cell density, divide the background fluorescence corrected value by the optical density measurement recorded in **step 5** of Subheading 3.2 for each sample. If samples were plated in triplicate, determine the average RFU and use this as the final RFU value for each sample.
4. To compile data generated from repeats of Nile red assays, divide the highest average background fluorescence by the lowest average background fluorescence to give a ratio. Using this ratio adjust fluorescence values of the samples to the assay with the highest background fluorescence by multiplying fluorescence values for each sample by the calculated ratio.
5. Resultant normalized data was routinely analyzed by one way ANOVA with Tukey post hoc test, using GraphPad Prism software. Data from our studies were displayed as means of triplicates $\pm$ standard deviation. A significant result was determined to have a p value <0.05 .

4 Notes

1. It is advisable to conduct growth curves in the chosen culture volume prior to carrying out Nile red assays. This is so that appropriate growth phases for screening may be identified, for example, stationary phase cultures of oleaginous yeast species would be expected to accumulate increased levels of lipid in comparison to exponential phase cultures (Fig. 1). Contrariwise, we identified that the fission yeast *S. pombe* produced increased levels of fluorescence pertaining to neutral lipids during exponential growth [15]. This highlights that screening of cultures, both in exponential and stationary phases of growth, is advantageous when the lipid accumulation profile of a yeast species is unknown.

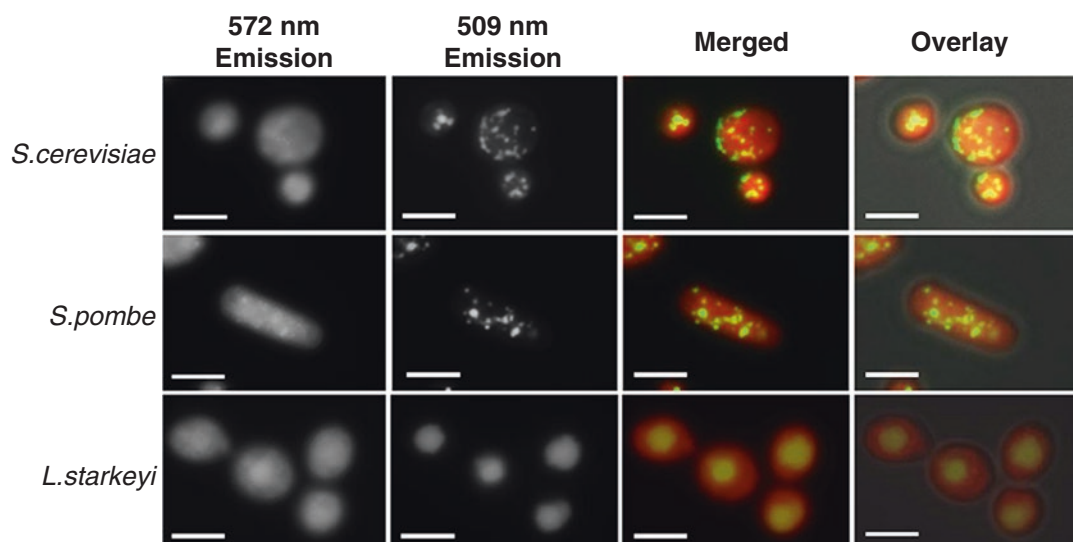


Fig. 2 Fluorescence microscopy highlighting emission properties of Nile Red stained yeast cells. *S. cerevisiae*, *S. pombe*, and *L. starkeyi* stationary phase cells were stained with Nile Red and visualized with filter sets DsRed (Ex. 545/Em 572 nm) and EGFP (Ex. 488/Em. 509 nm). Merged: Fluorescence from both DsRed and EGFP to shown co-localization of fluorescence signal. Overlay: Merged fluorescence images with bright field image of cells

2. Culture volumes should be kept consistent for samples being compared on a single plate, as well as those being used to form triplicate data sets. Differences in culture volumes can impact cell densities, time taken to reach required growth phases and therefore relative lipid amounts.
3. To prepare 10% formalin for use as a fixative, mix 1 part 37–40% formaldehyde with 9 parts distilled water (this makes a 3.7–4% formaldehyde solution). 37–40% formaldehyde is known to be 100% formalin, as formalin is formaldehyde gas dissolved in water, which reaches a saturation point at 37–40%.
4. For evaluation of Nile red staining via fluorescence microscopy, we find the widely available filter set for EGFP (Ex 488/Em 509 nm) gives good visualization of the distribution of neutral lipid droplets within cells, *see* Fig. 2.
5. We find that black plates which have individually molded wells, such as Krystal 2000 plates (Porvair Sciences), are superior for fluorescence detection. We noted that plates without individually molded wells gave more variable fluorescence readings, potentially due to lateral piping of light underneath the wells of the plates.
6. Vortex mixing of the 2 mg/mL Nile red stock in acetone before preparation of the working solution helps to ensure solution homogeneity by redissolution of any precipitated Nile red. We find that it is best to make the 60 µg/mL

working Nile red solution fresh for each experiment. This avoids variability of fluorescence readings due to evaporation of acetone.

7. When washing cells in preparation for the Nile red assay with $1\times$ PBS we find that allowing the cells to remain in $1\times$ PBS for 1–2 min between washes improves the sensitivity of the assay.
8. The described assay has been optimized and validated utilizing a filter based platereader on top 50 % mirror setting. Therefore, optimization of the assay may be required for other mirror settings or when utilizing platereaders with monochromatic technology. Additionally, as filter based plate readers have increased sensitivity for fluorescence measurements, monochromatic plate readers may not be able to detect slight differences in relative fluorescence pertaining to lipid amounts between wild type and mutant strains of non-oleaginous yeast species. The plate reader gain setting for each set of experiments was set to optimal, whereby the gain value was determined by the plate reader. This determined gain setting was recorded after the initial screen before setting manually to the same value for further screens to obtain triplicate data.

There have been a number of excitation and emission wavelengths reported in the literature for detection of neutral lipids utilizing Nile red including 485/535 nm [25] and 535/590 nm [10]. After identifying that use of the filters Ex 488/Em 509 nm in microscopy gave better visualization of cellular neutral lipid droplets, we routinely utilized the filter set Ex 485/Em 535 nm for microplate readings.

9. As a matter of course, we plated each sample in triplicate in order to ensure that relative fluorescence values obtained were similar and that pipetting was accurate between the wells of the plate.
10. Accurate pipetting is essential for ensuring consistent results within and between assays, thus it is good practice to use the same pipettes. Additionally, we find touching the pipette tip to the top of the cell suspension when delivering Nile red solution to the wells improves both signal and accuracy of the fluorescence readings. Hovering above wells while pipetting Nile red can cause variable relative fluorescence values, potentially due to evaporation of acetone containing the Nile red dye.
11. For the described assay the number of plate reader cycles was optimized for each yeast species by reading wild type samples on a kinetic reading every 60 s for 20 min. The cycle before saturation point of the Nile red dye, where fluorescence signal reached a plateau, was determined and subsequently utilized for further screens to generate triplicate data sets.

Acknowledgments

This work was supported by The University of Central Lancashire and Brain Tumour North West. The authors would like to acknowledge Dr. Gail Welsby and Dr. Vicky Jones for assistance with microscopy, Mr. Tony Dickson for technical assistance, and Dr. Christopher Smith for support with statistical analysis.

References

1. Hutchins PM, Barkley RM, Murphy RC (2008) Separation of cellular nonpolar neutral lipids by normal-phase chromatography and analysis by electrospray ionization mass spectrometry. *J Lipid Res* 49:804–813
2. Santamauro F, Whiffin FM, Scott RJ et al (2014) Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources. *Biotechnol Biofuels* 7:34
3. Di Paolo G, Kim TW (2011) Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat Rev Neurosci* 12:284–296
4. Oresic M, Simell S, Sysi-Aho M et al (2008) Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. *J Exp Med* 205:2975–2984
5. Zhang F, Du G (2012) Dysregulated lipid metabolism in cancer. *World J Biol Chem* 3:167–174
6. Nielsen J (2009) Systems biology of lipid metabolism: from yeast to human. *FEBS Lett* 583:3905–3913
7. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *C J Biochem Physiol* 37:911–917
8. Shahidi F (2001) Extraction and measurement of total lipids. In *current protocols in food analytical chemistry*. John Wiley, New York, NY
9. Iverson S, Lang SC, Cooper M (2001) Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. *Lipids* 36:1283–1287
10. Sitepu IR, Ignatia L, Franz AK et al (2012) An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *J Microbiol Methods* 91:321–328
11. Sitepu IR, Jin M, Fernandez JE et al (2014) Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated in alkaline pretreated corn stover. *Appl Microbiol Biotechnol* 98:7645–7657
12. Adeyo O, Horn PJ, Lee S et al (2011) The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. *J Cell Biol* 192:1043–1055
13. Grimard V, Massier J, Richter D et al (2008) siRNA screening reveals JNK2 as an evolutionary conserved regulator of triglyceride homeostasis. *J Lipid Res* 49:2427–2440
14. Sorger D, Athenstaedt K, Hrastnik C et al (2004) A yeast strain lacking lipid particles bears a defect in ergosterol formation. *J Biol Chem* 279:31190–31196
15. Rostron K, Rolph C, Lawrence CL (2015) Nile red fluorescence screening facilitating neutral lipid phenotype determination in budding yeast, *Saccharomyces cerevisiae*, and the fission yeast *Schizosaccharomyces pombe*. *Antonie Van Leeuwenhoek* 108:97–106
16. Lemieux GA, Liu J, Mayer N et al (2011) A whole-organism screen identifies new regulators of fat storage. *Nat Chem Biol* 7:206–213
17. Soukas AA, Carr CE, Ruvkun G (2013) Genetic regulation of *Caenorhabditis elegans* lysosome related organelle function. *PLoS Genet* 9, e1003908
18. Reis T, Van Gilst MR, Hariharan IK (2010) A buoyancy-based screen of *Drosophila* larvae for fat-storage mutants reveals a role for Sir2 in coupling fat storage to nutrient availability. *PLoS Genet* 6, e1001206
19. Zuriani R, Vigneswari S, Azizan MNM et al (2013) A high throughput Nile red fluorescence method for rapid quantification of intracellular bacterial polyhydroxyalkanoates. *Biotechnol Bioproc E* 18:472–478
20. Flynn EJ III, Trent CM, Rawls JF (2009) Ontogeny and nutritional control of adipogenesis in zebrafish (*Danio rerio*). *J Lipid Res* 50:1641–1652

21. Chen W, Zhang C, Song L, Sommerfeld M et al (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J Microbiol Meth* 77:41–47
22. Greenspan P, Mayer EP, Fowler SD (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 100: 965–973
23. Drabsch Y, Ramsay R, Gonda T (2010) MYB suppresses differentiation and apoptosis of human breast cancer cells. *Breast Cancer Res* 12:R55
24. Yao HR, Liu J, Plumeri D et al (2011) Lipotoxicity in HepG2 cells triggered by free fatty acids. *Am J Transl Res* 3:284–291
25. Dey P, Chakraborty M, Kamdar MR et al (2014) Functional characterization of two structurally novel diacylglycerol acyltransferase2 isozymes responsible for the enhanced production of stearate-rich storage lipid in *Candida tropicalis* SY005. *PLoS One* 9, e94472

Staining of Lipid Droplets with Monodansylpentane

Bo-Hua Chen, Huei-Jiun Yang, He-Yen Chou, Guang-Chao Chen,
and Wei Yuan Yang

Abstract

Monodansylpentane (MDH) is a fluorophore that displays selective labeling of lipid droplets (LDs). The dye preferentially segregates into the neutral lipid cores of LDs and emits blue fluorescence, compatible with the simultaneous use of green and red fluorescent reporters in multi-color live-cell imaging. MDH can be used for visualizing LDs not only in cell cultures, but also in fixed tissues such as the fat body and ovaries from *Drosophila*. MDH is therefore a versatile marker for LDs in fluorescence microscopy.

Key words Lipid droplets, Fluorescence microscopy, Monodansylpentane, Live-cell imaging, Tissue, *Drosophila*

1 Introduction

Lipid droplets (LDs) are dynamic organelles cells use to store neutral lipids. The known cellular roles for LDs have greatly expanded recently, ranging from stations where passive lipid storage occurs, to locations where immune responses and membrane trafficking initiate [1–4]. LDs can even act to alleviate cells from oxidative stress [5]. To image cellular LDs one can conveniently utilize hydrophobic fluorophores that preferentially partition in the LD neutral lipid core for staining; these can be performed efficiently, and are oftentimes compatible with live-cell imaging. The most commonly used LD dyes include BODIPY 493/503 and NileRed. Both fluorophores are commercially available, but their emissions spectrally overlap with green or red fluorescent proteins [6, 7]. A blue-fluorescent LD dye would therefore be useful when both green and red fluorescent reporter constructs are desired in an imaging experiment.

Monodansylpentane (MDH) is a commercially available fluorophore that displays highly selective labeling of LDs (Fig. 1). MDH achieves high contrast LD labeling through preferential segregation into LDs and through its significantly blue-shifted, highly

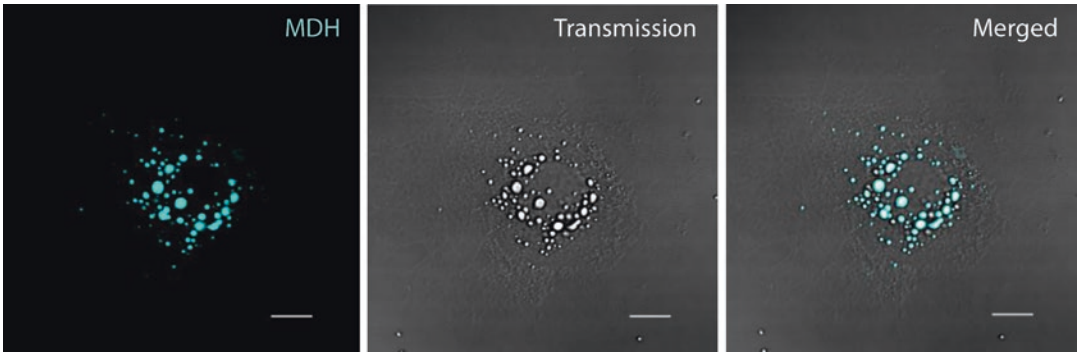


Fig. 1 MDH-stained lipid droplets in fixed HepG2 cells. Scale bars: 10 μ m

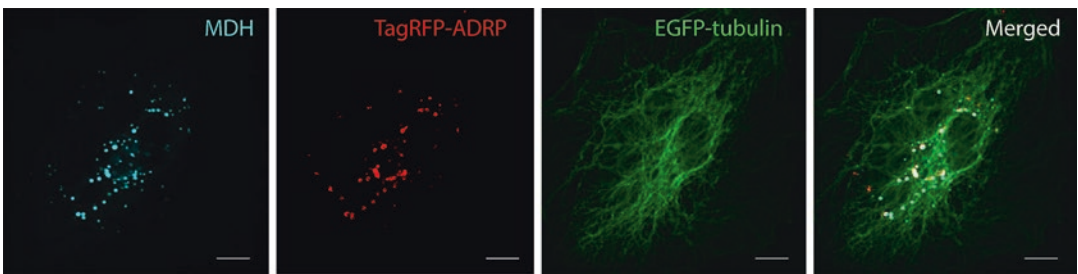


Fig. 2 Multicolor imaging with MDH. HeLa cells were transfected with TagRFP-ADRP and EGFP-tubulin plasmids. After 16 h the cells were fixed and then stained with MDH. Scale bars: 10 μ m

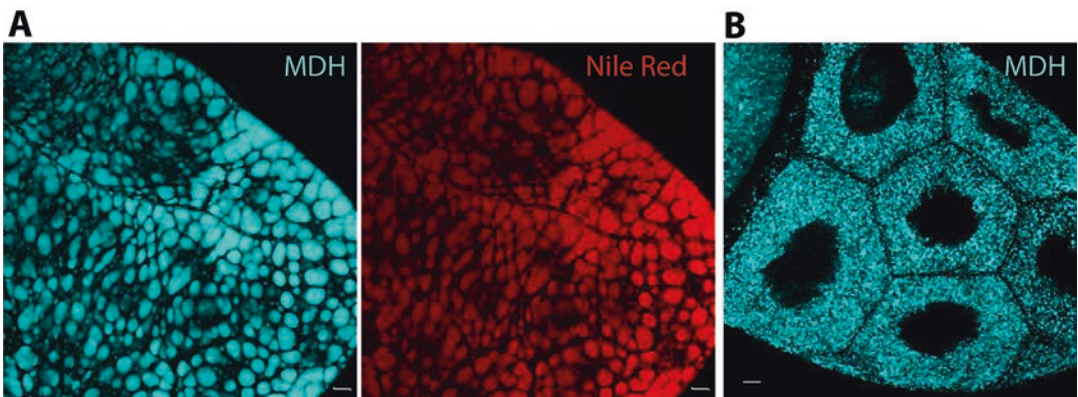


Fig. 3 MDH-stained *Drosophila* larval fat bodies and adult egg chambers (stage 10 nurse cells). (a) Wandering third instar larval fat bodies were dissected, fixed, permeabilized, and stained with both MDH (1 μ g/mL) and Nile Red (0.5 μ g/mL). (b) Ovaries of adult female flies were dissected, fixed, permeabilized, and stained with MDH. MDH displayed significantly better tissue penetration abilities when compared to Nile Red. Scale bars: 10 μ m

enhanced emission within lipophilic environments [8]. Since MDH absorbs in the violet and emits blue fluorescence when labeling LDs, it can be combined with other green and red fluorescent reporters for multi-color cell imaging (Fig. 2). The dye can also be used to label LDs in fixed tissues (Fig. 3). MDH therefore represents a versatile marker for LDs in fluorescence microscopy.

2 Materials

2.1 Staining Reagents

1. Monodansylpentane (MDH): MDH (Abgent, #SM1000a) at the concentration of 100 mM is stocked in DMSO. Dilute MDH to 100 μ M (1:1000 dilution) with PBS and mix the solution by pipetting up and down gently before use.
2. Nile Red: store Nile Red powder at room temperature. Prepare 1 mg/mL stock solution by dissolving Nile red in acetone (1 mg/mL). Aliquot in Eppendorf tubes and store them at -20°C . Dilute stock Nile Red solution to 1 μ g/mL (1:1000 dilution) with PBS and mix the solution by pipetting up and down gently before use.
3. Green and/or red florescent reporter constructs. MDH is compatible with green and red fluorescent reporter constructs. In this protocol the green EGFP-tubulin plasmid was acquired from Clontech (#6117-1). The red human TagRFP-ADRP plasmid was constructed by amplifying the ADRP gene by PCR from HeLa total cDNA and inserted into the pTagRFP-C vector obtained from Evrogen (#FP-141).
4. Lipofectamine 2000: transfect plasmids into cells using the Lipofectamine 2000 reagent (Thermo Fisher, # 11668019).
5. Phosphate-buffered saline (PBS): Use ultrapure water (18 M Ω -cm at 25°C) to prepare PBS stocks (10 $\times$) by dissolving 80 g NaCl, 2 g KCl, 14.4 g Na₂HPO₄, and 2.4 g KH₂PO₄ in 800 mL of ddH₂O. Adjust pH to 7.5 and add ddH₂O to 1 L. Sterilize by autoclaving. Dilute to 1 $\times$ with ddH₂O before use.
6. Mounting medium: prepare the mounting medium by diluting glycerol (99+%, extra pure) with PBS to 90%.

2.2 Cell/Tissue Reagents

1. Dulbecco's Modified Eagle Medium.
2. Dulbecco's Modified Eagle Medium without phenol red.
3. Fetal bovine serum.
4. Penicillin–streptomycin.
5. L-glutamine.
6. Poly-L-lysine.
7. Hemacytometer.
8. Trypan blue.
9. Cell fixation buffer: dilute the 16% paraformaldehyde stock solution with PBS (1:8 dilution) to 2% for cell fixation. Dilute the 16% paraformaldehyde stock solution with PBS (1:4 dilution) to 4% for tissue fixation.

10. Cell permeabilization buffer: 0.1 % (v/v) Triton X-100 in PBS.
11. Tissue permeabilization buffer: 0.3 % (v/v) Triton X-100 in PBS.
12. HeLa cells. The cells are cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % Fetal Bovine Serum and 1 % P/S, and maintained at 37 °C, 5 % CO₂.
13. HepG2 cells. The cells are cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % Fetal Bovine Serum and 1 % P/S, and maintained at 37 °C, 5 % CO₂.
14. Glass-bottom culture dishes: cells are cultured on 35 mm glass bottom dish for MDH staining. The thickness of the No. 1.5 glass coverglass (0.16–0.19 mm) is optimal for imaging under a confocal microscope.
15. 9-well glass spot plate (McCRONE #SKU: 131).
16. Glass slides.
17. Coverslips.
18. Forceps.
19. Nail polish for sealing coverslips.
20. Confocal microscope.
21. Medium for live-cell imaging: live-cells are cultured in Dulbecco's Modified Eagle Medium without phenol-red supplemented with 10 % Fetal Bovine Serum, 2 mM L-glutamine and 1 % P/S, and maintained at 37 °C, 5 % CO₂ for confocal imaging.
22. Microscope stage-top incubator: during image acquisition, live-cells are maintained in a microscope stage-top incubator (e.g., Tokai-Hit INUBSF-ONICS-F1 or LCI Chamliide IC) heated to 37 °C and supplied with 5 % CO₂. The confocal microscope can be placed into a heated (37 °C) enclosure for optimal stability.

3 Methods

3.1 Staining LDs with MDH in Living Cells

1. Seed 5×10^4 HeLa or HepG2 cells onto the 20 mm glass microwell of a 35 mm glass-bottom culture dish.
2. Two days after seeding the cell culture will be ~50 % confluent and ready for staining/imaging.
3. Remove culture medium thoroughly by aspiration.
4. Wash the cells twice with PBS: overlay the cells with 1 mL of PBS, and gently shake the dish for 5 s, followed by aspiration of the PBS. Repeat once.
5. Incubate the cells with diluted MDH in PBS at the concentration of 100 μ M (*see Note 1*). Maintain the cells at 37 °C, 5 % CO₂ in a culture incubator for 15 min for staining.

6. Wash the cells three times with 1 mL of PBS.
7. Add 1 mL of phenol-red free medium for imaging (*see Note 2*).
8. MDH stained LDs can then be visualized immediately on a confocal microscope. Use 405 nm laser for MDH excitation, and collect MDH emission between 430 and 470 nm.

3.2 Staining LDs with MDH in Fixed Cells

1. Seed 5×10^4 HeLa or HepG2 cells (*see Note 3*) onto the 20 mm glass microwell of a 35 mm glass bottom dish.
2. Two days after seeding the cell culture will be ~50% confluent and ready for staining/imaging.
3. (Optional) for multi-color imaging, one can further transfect the ~50% confluent HeLa cells with fluorescent reporter constructs using Lipofectamine 2000. The transfected cells will be subjected to staining 1 day following transfection.
4. Remove culture medium thoroughly by aspiration.
5. Wash the cells twice with 1 mL PBS.
6. Fix the cells with 1 mL of 2% paraformaldehyde in PBS at room temperature for 10 min.
7. Wash the cells three times with 1 mL of PBS.
8. Permeabilize the cells with 0.1% Triton X-100 in PBS at room temperature for 10 min.
9. Wash the cells three times with 1 mL PBS.
10. To stain the LDs cells are incubated with MDH (100 μ M) in PBS at 37 °C for 15 min.
11. Wash the stained cells three times with 1 mL PBS.
12. Keeps cells in PBS for imaging.
13. Image LDs as in Subheading 3.1, **step 8** (sample result: Fig. 1). MDH is compatible with the use of green/red fluorescent reporters (e.g., EGFP and TagRFP). Use 488 nm excitation and collect emission between 505 and 550 nm for acquiring the EGFP signal. Use 559 nm excitation and collect emission between 575 and 675 nm for acquiring the TagRFP signal (sample result: Fig. 2).

3.3 Staining LDs with MDH in Fixed Drosophila Tissues (See Note 4)

1. Dissect desired tissues from Drosophila. For example to dissect the fat body first rinse well fed wandering stage third instar of w^{1118} larvae with ddH₂O. Prepare a clean 9-well glass spot plate for dissection under a dissecting microscope. Dissect the fat bodies in the 9-well glass spot plate with PBS.
2. Fix the dissected tissues with 0.4 mL of 4% paraformaldehyde in PBS at room temperature for 30 min.
3. Wash the fixed tissues three times with 0.4 mL of PBS.
4. Permeabilize the fixed tissues with 0.4 mL 0.3% Triton X-100 in PBS at room temperature for 20 min.

5. Wash the fixed tissue three times with 0.4 mL of PBS.
6. Stain the fixed tissues with 0.4 mL MDH (100 μ M) in PBS at room temperature for 60 min in dark.
7. Wash the stained tissues three times with 0.4 mL of PBS.
8. Mount the stained tissues in 90% glycerol for imaging. Add a drop of mounting medium to a glass slide. Gently place the stained tissue into the drop of mounting medium with a forceps. Coverslip is placed over the mounting agent. Apply nail polish along the edges of the coverslip to seal it to the slide.
9. Image LDs within the stained tissue as in Subheading 3.1, step 8.

4 Notes

1. MDH can bind to lipoproteins in FBS, and this affects staining. MDH staining should therefore be performed in the absence of serum. Live-cells should also be washed several times to thoroughly remove serum before staining.
2. For live-cell imaging, it is best to use phenol-red free medium to reduce the fluorescence background.
3. For culturing cells that are less adherent to glass (such as HepG2), culture surfaces can be coated with 0.01% (w/v) poly-L-lysine in ddH₂O for 10 min to promote cell adhesion for best imaging results.
4. Fixed tissues from other organisms can be similarly stained.

Acknowledgments

This work was supported by the NSC-98-2311-B-001-006-MY3 grant from the National Science Council in Taiwan.

References

1. Rambold AS, Cohen S, Lippincott-Schwartz J (2015) Fatty acid trafficking in starved cells: regulation by lipid droplet lipolysis, autophagy, and mitochondrial fusion dynamics. *Dev Cell* 32(6):678–692
2. Saka HA, Valdivia R (2012) Emerging roles for lipid droplets in immunity and host-pathogen interactions. *Annu Rev Cell Dev Biol* 28:411–437
3. Shpilka T, Welter E, Borovsky N et al (2015) Lipid droplets and their component triglycerides and steryl esters regulate autophagosome biogenesis. *EMBO J* 34(16):2117–2131
4. Welte MA (2015) Expanding roles for lipid droplets. *Curr Biol* 25(11):R470–R481
5. Bailey AP, Koster G, Guillermier C et al (2015) Antioxidant role for lipid droplets in a stem cell niche of *Drosophila*. *Cell* 163(2):340–353
6. Greenspan P, Mayer EP, Fowler SD (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 100(3):965–973
7. Gocze PM, Freeman DA (1994) Factors underlying the variability of lipid droplet fluorescence in MA-10 Leydig tumor cells. *Cytometry* 17(2):151–158
8. Yang HJ, Hsu CL, Yang JY et al (2012) Monodansylpentane as a blue-fluorescent lipid-droplet marker for multi-color live-cell imaging. *PLoS One* 7(3), e32693

Part V

Nuclear Histochemistry

Fluorochromes for DNA Staining and Quantitation

Giuliano Mazzini and Marco Danova

Abstract

In these last few decades the great explosion of the molecular approaches has casted a little shadow on the DNA quantitative analysis. Nevertheless DNA cytochemistry represented a long piece of history in cell biology since the advent of the Feulgen reaction. This discovery was really the milestone of the emerging quantitative cytochemistry, and scientists from all over the world produced a very large literature on this subject. This first era of quantitation (histochemistry followed by cytochemistry) started by means of absorption measurements (histophotometry and cytophotometry). The successive introduction of fluorescence microscopy gave a great boost to quantitation, making easier and faster the determination of cell components by means of cytofluorometry. The development of flow cytometry further contributed to the importance of quantitative cytochemistry. At its beginning, the mission of flow cytometry was still DNA quantitation. For a decade the Feulgen reaction had been the reference methodology for both conventional and flow cytofluorometry; the advent of Shiff-type reagents contributed to expand the variety of possible fluorochromes excitable in the entire visible spectrum as well as in the ultraviolet region. The fluorescence scenario was progressively enriched by new probes among which are the intercalating dyes which made DNA quantitation simple and fast, thus spreading it worldwide. The final explosion of cytofluorometry was made possible by the availability of a large variety of probes directly binding DNA structure. In addition, immunofluorescence allowed to correlate the cell cycle-related DNA content to other cell markers. In the clinical application of flow cytometry, this promoted the introduction of multiparametric analyses aimed at describing the cytokinetic characteristics of a given cell subpopulation defined by a specific immunophenotype setting.

Key words DNA content, Quantitative cytochemistry, Fluorochromes, Fluorescence microscopy, Flow cytometry

1 A Bit of History (... and Local Pride): The “Pavia School” after Camillo Golgi, by MaffoVialli and Giovanni Prenna

In the evolution of cell biology studies, DNA has undoubtedly played a key role from the first discovery of the biochemist Friedrich Miescher [1] who described and chemically characterized a nuclear component (named by him *nuclein*) made of a “fragile mixture of carbohydrates and proteins.” The second milestone was the X-ray study and related images of the first DNA structure made by Rosalind Franklin [2] and finally the famous amazing discovery of

Francis Crick and James D. Watson of the “double helix” [3]. Meanwhile, other researchers focused on the chemical–physical structure of DNA, and a parallel progress was also made in the chemical methodologies aimed at demonstrating DNA in tissue and cells by histochemistry and cytochemistry [4–7]. From the mid-nineteenth century, many research groups all around Europe provided important contributions to this scientific area. Among others, the Feulgen reaction [8] must be considered the milestone in the “DNA story” and certainly it represents the first brick in the evolution of quantitative cytochemistry. Its fine chemical details have been considered in hundreds of publications by many researchers all over the world [9–14].

In Italy, namely in Pavia, Camillo Golgi made his first empirical histochemical approach based on the reduction of the silver salts leading to the demonstration of neuronal structures. The so-called “Golgi black reaction” was recognized few years later by assigning the Nobel Prize to the author.

In the Golgi stream, a “School of Histochemistry” was established at Palazzo Botta (Fig. 1): in this building of the Pavia University many famous scientists (among them, Lazzaro Spallanzani) made their discoveries. In the middle of 1900 an active member of the Pavia School, Maffo Vialli (Fig. 2) dedicated many efforts to transform the microscope from its typical application as an instrument for observation into a device for quantitative analysis starting a very prosperous matter. The time in which he operated was immediately after the Second World War so that he had the opportunity to benefit from military technology mainly in the area of optic-electronic-telecommunications providing various additional apparatuses. Among the electronic devices, the first



Fig. 1 The (A) historical “Palazzo Botta” (1700) with its Golgi round room (B) down to the *minaret* tower. In this building, around 1870, the University of Pavia transferred many of the biomedical institutions. At present, it houses the Museum of Natural History and the precious original Golgi lab



Fig. 2 Professor Maffo Vialli (1897–1983), the founder of the Pavia School of Histochemistry. In his long and very intense scientific activity at Palazzo Botta, he focused on two main topics: the enterochromaffin system with important contributions to the isolation and characterization of catecholamines (in particular, 5-hydroxytryptamine), and DNA quantitation. He pioneered the first development of histophotometry and cytophotometry

photodetectors from RCA (USA) and related power supplies became the core elements of a new generation of home-made instruments pioneering the future of quantitative histochemistry [15, 16].

These first prototypes were named microdensitometers as they were aimed at assigning density values to few histochemical reactions that Vialli was applying at that time particularly on amphibian tissues. Thanks to this peculiar application, the instrument was also termed histophotometer [17, 18]. The first original prototype was also upgraded to other models [19, 20] named according to their specific optical design such as: single/double path, multichannels and inverted (Fig. 3). Vialli's intent was to measure the "cell content" of certain histochemical reactions and to correlate these values to the evolution of physiological states as well as of pathological conditions [21]. Using optical filters, the observed color image was converted into a black-and-white picture and the "gray level" was then graded by a densitometer. Quantification could be made directly on the microscopic field, or the image-area was photographed and later quantification was performed on the corresponding photographic film. These first rudimental "home-made" approaches of microdensitometry were the base for the emerging optical industries of microscopy instruments which then produced commercial models which later widespread in many laboratories.

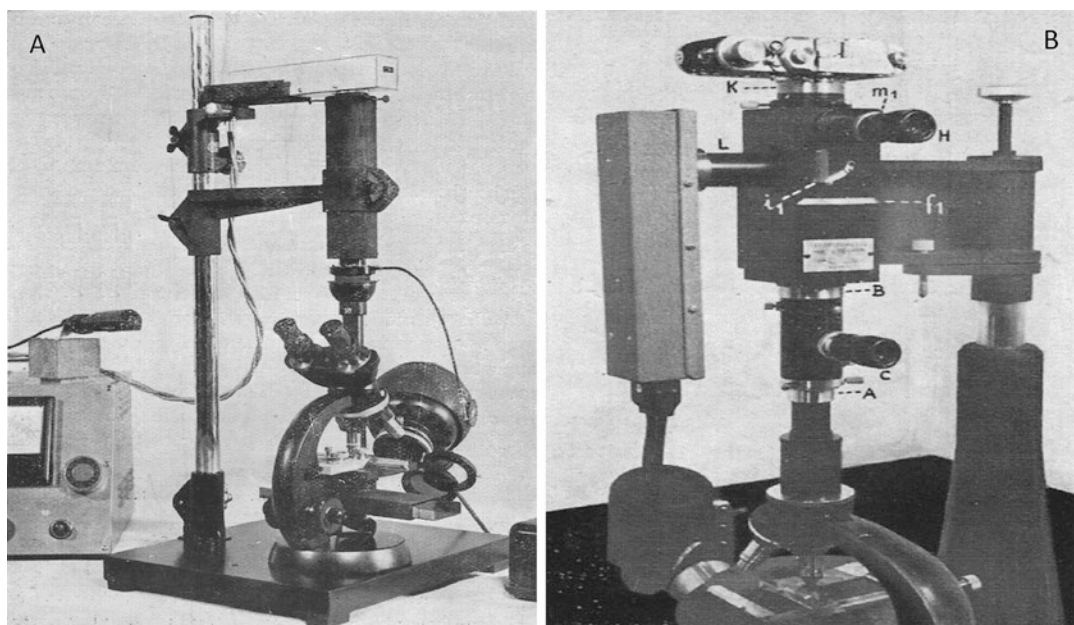


Fig. 3 First-generation microphotometers realized by the Vialli group in Pavia. Immediately after the Second World War, Maffo Vialli had access to military “surplus” electronics including the precious RCA photomultiplier 1P28, the real core element of the new generation of quantitative microscopes born in Pavia. In the model (A) a single optical pathway originated just above the conventional eyepieces, while in the model (B) a more sophisticated optical bench included measuring oculars and a second pathway for camera film [19]

The pioneering quantitative histochemistry of the 1950s then became accessible to many laboratories, further evolved to the areas of “integrated microdensitometry,” and later again to “cytophotometry.”

The measurement strategy performed at the microscope level tried to emulate the more affordable spectrophotometric techniques operated in cuvettes. The great jump from “Histo” to “Cyto” is in fact related to the so called “measurement area” that is well defined in the case of cell and quite undefined in case of biological tissue, even though a final error in the cytometric techniques still remains, due to the fact that the components (such as DNA) are not homogeneously distributed within the cell. This is the source of the so-called “distributional error” [22–24] later overcome by the advent of the integrated scanning microdensitometry [25, 26] made available by few microscope companies a decade later.

The solution came few years later and was still the Pavia School to substantially contribute to its realization. The introduction of fluorescence opened a new era of quantitative measurements in cell biology. The progress of microscopy yielded a great achievement in those years, including the development of fluorescence microscopy. The epi-fluorescence illuminator [27] gave a further improvement to cell microscopy in terms of image quality (fluorescence brilliance



Fig. 4 Professor Giovanni Prenna (1931–1977), a student of the Vialli School and later head of Centre for Studies on Histochemistry of the Italian National Research Council (established in Pavia by Maffo Vialli in 1965). In the stream of his mentor, he focused on the evolution of absorption photometry to cytofluorometry including flow cytometry. His short intense scientific life was all addressed to find advanced quantitative methodologies for fluorescence DNA analyses

and background contrast) and brought great contributions to cell biology laboratories. The Feulgen reaction, in the meanwhile described in all its physical–chemical aspects, became a test model for the new fluorescence techniques [28].

Thanks to several discoveries that contributed to the assessment of the cell cycle, the nuclear DNA became soon the model of choice to test the performances of a variety of fluorochromes candidate for being DNA quantitative probes. The original Feulgen reagent, para-rosaniline (PRA) yielding the famous *magenta*-color suitable for absorption measurements, resulted also appropriate to be excited with the green light with fluorescent emission in the red region. The original recipe was thus considered to be validated also for application in fluorescence microscopy [29].

Few groups of researchers spent many efforts in finding the optimal methodological conditions to apply the Feulgen reaction for quantitative fluorescence microscopy. Among others, Giovanni Prenna (Fig. 4) belonging to the Vialli's School was at that time very active in research dealing with cytofluorometry [30]. Around 1970, Prenna addressed the new-born “Centre for Studies on Histochemistry” of the National Research Council to a unique national laboratory focused on the methodological and instrumental aspects of fluorescence quantitative cytochemistry. The DNA quantitation was of course the core-mission, and to this goal Prenna renewed all technological equipment of the Centre from

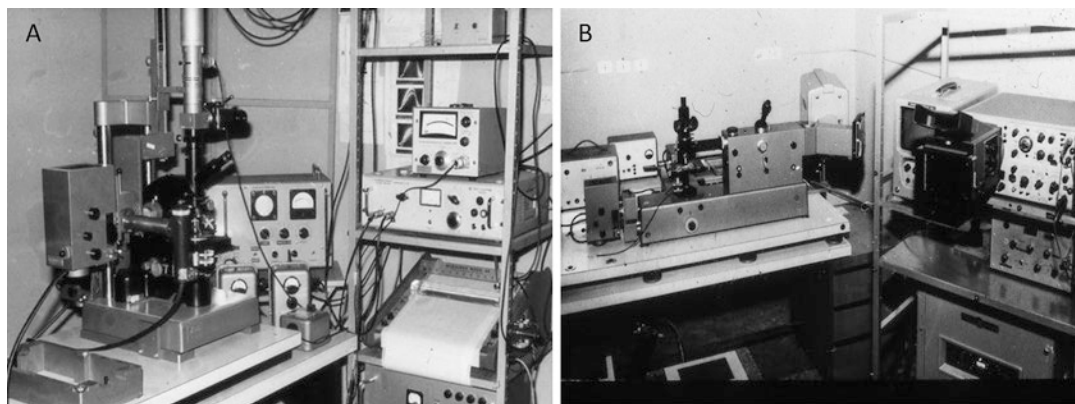


Fig. 5 Instrumentation belonging to Centre for Studies on Histochemistry in Pavia, around 1972. **(A)** The Leitz Microphotometer MPV1 previously utilized for absorption measurements was implemented by Giovanni Prenna to operate in fluorescence by means of the HBO100W mercury arc-lamp. **(B)** The advanced Leitz Microspectrograph capable to operate in both absorption and fluorescence modes, equipped with a variety of illumination/excitation arc-sources including mercury, xenon, hydrogen, sodium, and the conventional low-power tungsten. All the electronic instruments coupled to the optical ones had been conventional “analogic” for years; later, thanks to a cooperation with Professor Sergio Cova of the Polytechnic University of Milan, an electronic revolution introduced the first “photon-counting” system and the digitalization of all analyzers [32]

the original “absorption-micro-photometry” (also termed cytophotometry) to the emerging “micro-fluorometry” (or cytofluorometry). To perform advanced methodological research in the area of new fluorochromes he invested all the financial resources of the Institution to acquire the new microspectrofluorometer from Leitz (Fig. 5). This unique and advanced instrument was essential for those researchers, and very few labs in the world had at that time access to that sophisticated technology. Thanks to the availability of this advanced equipment, his group provided in few years important international contributions in the area of cytofluorometry and microspectrofluorometry [31, 32]. The paper published on 1974 [33] was worldwide recognized as a sort of guideline to DNA quantitation.

Few years later, beside the advantages introduced by the fluorescent Feulgen reaction, few methodological aspects remained unsolved. Among others, the low quantum efficiency of PRA and its low-performing spectrofluorometric properties were inadequate for the emerging multiparametric cell analyses. Few groups of researchers were thus prompted to explore the possibility to develop alternative fluorochromes more suitable to this aim. Together with others, Prenna made soon available (Fig. 6) a list of molecules able to replace PRA in the original Feulgen reaction and thus named “Shiff-type reagents” [34–42]. In the meanwhile, the development of flow cytometric techniques further promoted the introduction of new fluorescent probes. PRA was in fact incompatible with the



Fig. 6 The so-called “Shiff-type” reagents became very important in the search for new fluorochromes to replace the original para-rosaniline in the Feulgen reaction. Giovanni Prenna and few colleagues were able to test various chemical drugs with compatible chemical structures to exploit their fluorescence properties. Few of them showed to be excellent probes after excitation with UV light

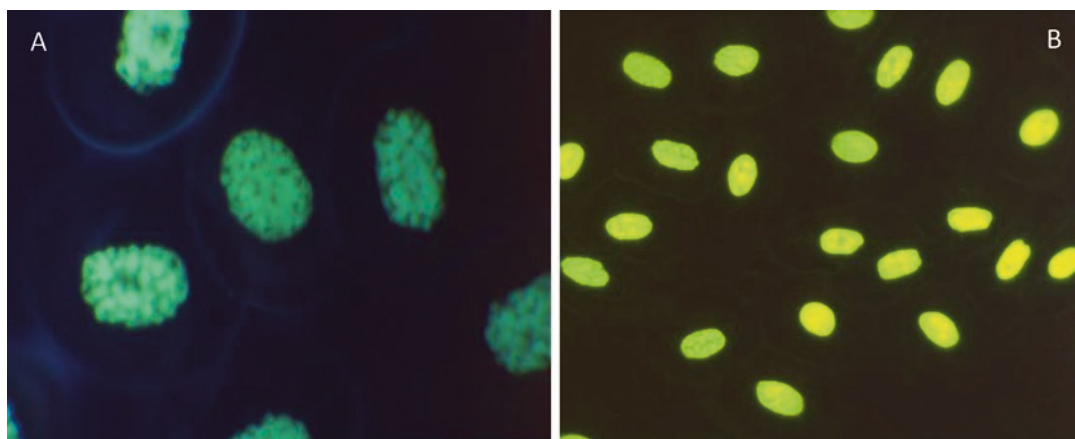


Fig. 7 Fluorescent micrographs of frog erythrocyte nuclei stained with the Feulgen reaction performed by means of Prenna’s Shiff-type reagents. The dehydrothioparalutidine (A) and BBT (B) showed their great fluorescence performances when excited by UV light. In the Vialli School, amphibian blood had been for decades the most established and simple model to test any quantitative staining oriented to DNA stoichiometry

argon-ions laser excitation systems installed in the first generation of American flow, while being inefficient for the European arc-lamp-based flow instruments.

The Pavia School did contribute to set up quantitative fluorescence methodologies (Fig. 7) [43–46] pioneering the development of flow cytometry (FC) in Italy.

2 The DNA Fluorochromes

In the years between 1960 and 1970, the implementation of the Feulgen reaction through fluorescence surely was the milestone supporting all the successive series of DNA specific probes. The Acriflavine-Feulgen method was therefore the connecting link from past to future [47]. In few years, thanks to the development of FC (see below) and the availability of a wide variety of new fluorochromes, the Acriflavine-Feulgen reaction became obsolete [48]: this time-consuming and multi-step complicated procedure was rapidly abandoned in favor of new techniques characterized by a direct labeling of DNA with specific probes.

A large family of fluorochromes prepared from the so-called “acridine derivatives” were made available by the chemical industry. The most famous and popular was Acridine Orange (AO) which is still used in few peculiar applications of cell biology [49]. At the beginning of its applications (in both fluorescence microscopy and FC), AO had been appreciated thanks to its metachromatic properties modulating the emission spectrum according to the microenvironment. In some practical applications (and under strictly controlled methodological conditions) AO is able to differentiate double-stranded (DNA) from single-stranded (RNA) configuration [50]. This fascinating property produced for years a large literature in the area of hematology and, later on, in cell kinetics as well as in cell damage and apoptosis. The peculiarity of AO was initially exploited also in oncohematology where the DNA–RNA ratio was of significant value in the classification of malignant leucosis [51].

As far as the reaction mechanism is concerned, AO was the first of the so-called DNA-intercalating dyes whose physical-chemical behavior is driven by the tridimensional conformation of the target sites. This family of fluorochromes is characterized by a complex structure including few (or several) aromatic rings producing a planar configuration responsible for the principal binding mode to the specific (stereo) target. Other secondary chemical interactions (hydrogen bonds, van der Waals bonds, etc.) may either consolidate hinder the principal binding mode. From the practical point of view, however, AO has encountered numerous obstacles mainly related to the critical methodological aspects which require highly controlled and often hardly reproducible reaction conditions.

Other DNA-binding fluorochromes with different reaction mechanisms were subsequently proposed under the name of “fluorescent antibiotics.” Among others, mithramycin (MM), chromomycin, and olivomycin react with similar chemical behavior involving the formation of a complex with guanine base (in the presence of magnesium ions) and are base-specific probes with preference for CG base pairs. They all have a maximum of excitation in the violet-blue region (410–450 nm) and emission in the

green-yellow region (500–600 nm). Therefore, they can best be excited by the mercury or xenon arc-lamps, while maintaining a sufficient level of emission even when excited by the blue line (488 nm) of the argon-ions laser. Due to their CG preferential binding they should not probe single-stranded RNA thus assuring the accurate quantitative DNA determination without the need of enzymatic RNA digestion [52]. To this aim MM has been in particular combined with ethidium bromide (EB) thanks to their complementary spectrofluorometric properties. Exploiting the energy transfer between MM and EB the proper (420 nm) excitation of the first does not allow the direct excitation of the second that can only accept “transferred energy” from MM to finally emits its typical red fluorescence: this may only happen when the distance between the couple of donor–acceptor molecules is in the order of few angstroms, as it typically occurs with double stranded DNA allowing the intercalation of the EB molecule very close to the external CG-bound MM.

These CG specific fluorochromes have also been used in combination with complementary AT specific probes (like DAPI and Hoechst, described below) to differentiate individual human chromosomes (in both fluorescence microscopy and FC) as well as for the recognition of subclasses of bacteria [53].

In more recent years, another fluorochrome belonging to this family and named 7-amino-actinomycin D (7-AD) has been proposed for the analysis of DNA in combination with a variety of immunofluorescent markers. Its physical-chemical characteristics and in particular its emission in the deep-red region allow a good separation of its fluorescence even in multi-labeling methodologies [54]. As the 7-AD cannot penetrate the intact cell membrane it has been proposed for studies on apoptotic cell death [55].

Another group of DNA probes is represented by the so-called di-amidino-phenyl-indole-derivatives among which the most popular are DIPI and DAPI which contributed to the development of DNA FC in Germany [56]: thanks to their spectrofluorometric properties they became the probes of choice to operate with the Wolfgang Göhde’s flow cytometers (see below). Their absorption spectra have in fact a maximum in the ultraviolet (UV) region where the mercury arc-lamp has an important emission band. The high quantum efficiency combined with the high power excitation yields a very intense fluorescence emission of these probes which is highly appreciated in FC.

A similar UV-excitabile group of DNA probes is those developed by the chemical brand Hoechst and named as HO followed by numbers. The most interesting fluorochromes in the area of cytofluorometry are HO33258 and its analog, HO33342 [57]. The latter is characterized by the peculiar property to be able to cross the intact cell membrane and therefore to bind DNA also of live cells (in *supravital* staining, see below).

In proliferation studies, a couple of dyes known as ethidium bromide (EB) and Propidium Iodide (PI) play a pivotal role [58, 59]. They both belong to the family of the intercalating phenanthridine derivatives characterized by very peculiar spectrofluorometric properties. EB has extensively been studied from the physical-chemical point of view, and its interaction with DNA has precisely been detailed even at the ultrastructural level. Its principal absorption band is in the blue-green region and a second one in the UV region; the emission is typically located over 550 nm with a maximum in the red region. These probes cannot penetrate the intact cell membrane thus requiring a fixation (or a permeabilization) procedure to be able to quantitatively bind nuclear DNA. PI shows very similar behavior in DNA binding, and comparable spectrofluorometric properties with the exception of an emission spectrum more restricted to the red region. For this last property it is largely preferred to EB in case of multiparametric analysis since it displays less detection problems of spectral overlapping (e.g., cross-talk with the orange emitting probes). PI definitely became soon unreplaceable for DNA quantitative analyses especially in flow cytometric clinical applications [60–66]. A unique special feature of the phenanthridine dyes is to be practically nonfluorescent as free molecules in water solution, whereas they greatly increase their quantum efficiency when intercalated into double-stranded nucleic acids. Once excited by the proper light, free molecules can disperse the accumulated energy to the surrounding polar molecules of water; on the contrary, intercalated molecules can return to the ground state only transferring the accumulated excitation energy through fluorescence emission. On a practical point of view, in a PI stained cell suspension the fluorescence of free dye molecules are irrelevant to the final fluorescence emitted by the nuclei. The little drawback of EB and PI is the peculiarity to also bind double-stranded RNA (typically the t-RNA), thus requiring an enzymatic RNA digestion when a precise DNA quantitation is mandatory for cell cycle analyses [67].

Although a large variety of new DNA probes have been proposed by many companies in these last few years [68], PI still continues to maintain its popularity as a marker of choice for DNA detection and cell cycle analyses.

3 DNA Probes for Supravital Staining

Cell biology investigation often requires staining and microscope analyses in live conditions. Unstained biological samples can be observed by means of phase-contrast as well as dark field microscopy. Cytochemistry is generally performed (at least at the intracellular level) after a proper cell fixation. This procedure chemically (or physically) modifies the status of the cell membrane thus allowing

dye molecules (and fluorochromes) to penetrate the cell and bind the target. Very few dye molecules can cross the cell membrane of live intact cells. Among them, the already mentioned HO33342 is the most established and popular *supravital* probe largely applied in fluorescence microscopy and FC [69]: as recalled above, it can best be excited by UV light, and it is therefore largely utilized in fluorescence microscopes and in flow cytometers equipped with mercury arc-lamp. Another specific property of this dye is its being meta-chromatic thus showing color variation in fluorescence emission. In fixed cells it has free access to nuclear chromatin and under condition of dye saturation (up to 5 $\mu\text{g}/\text{ml}$) the fluorescence emission is stoichiometrically related to the DNA amount; the fluorescence emission is, in this case, very brilliant and white-yellow colored. In supravital staining (at low dye concentration, i.e., 0.1 $\mu\text{g}/\text{ml}$) the HO33342 fluorescence emission in the nuclei is mainly function of two opposite events: (a) the dye “passive” entrance into the cell (modulated by the partial membrane permeability) and (b) the dye “active” exit modulated by a specific pumping system (the latter is powered by the cell metabolism and may be altered by a large variety of morpho-functional states). In supravital staining, the emission color is mainly dependent on the final dye concentration inside the nuclei. Intact viable cells are characterized by a very low nuclear dye quantity because they are able to efficiently pump it out: as a consequence, the fluorescence emission is weak and bluish colored. On the contrary damaged/dead (apoptotic or necrotic) cells are characterized by a higher concentration of HO33342 inside their nuclei being unable to pump out the entered dye: under this condition, the fluorescence emission is more intense and white-yellow colored (Fig. 8a).

A very interesting approach is represented by the combination of the couple of dyes, HO33342 and PI which show complementary characteristics [70]. As described above, the PI molecule cannot cross the intact cell membrane and therefore it can label nuclei only if the plasma membrane is seriously damaged. On the contrary, HO33342 can differentiate cells with various grades of damage simply interfering with the pumping-out system. The combination of the two probes allows a very detailed chromatic differentiation (in a sort of rainbow exhibition) of the cell status: from the viable/intact (faintly blue) to the necrotic/nude nuclei (red) including a series of white-yellow-pink palette tagging all the intermediate conditions (Fig. 8b).

4 DNA Flow Cytometry

Quantitative DNA analysis has been (and still is today) the most important cell parameter able to give comprehensive information on cell proliferation [71]. After the discovery of the Feulgen

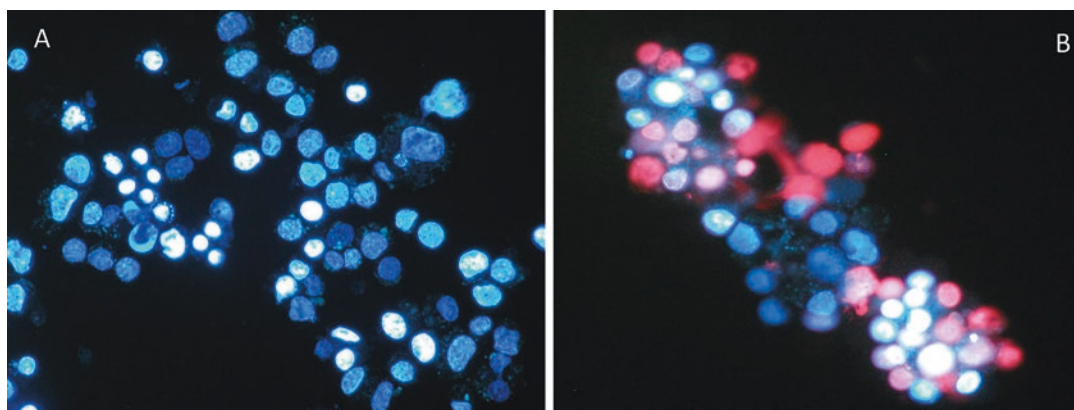


Fig. 8 Examples of the staining in supravital mode. **(A)** Epithelial cell cultures grown in presence of H033342 at low concentration prevalently show faintly labeled nuclei. These nuclei exhibit a moderate bluish fluorescence being related to the modest concentration of the dye present in the intact live cells which may indeed pump out the internalized dye molecules. Few cells (those damaged or dead) exhibit a more intense fluorescence emission and, the dye being metachromatic, they also show a color shift toward the yellow. This is the consequence of the altered membrane permeability and the low pumping-out efficiency linked to the damaged status. **(B)** The association with a second probe, such as PI, allows to stain the nuclei according to the degree of integrity/viability of the plasma membrane. Intact viable cells can only be penetrated by a modest quantity of HO and their nuclei thus appear faintly blue; the cells with seriously damaged membrane can be penetrated by both HO and PI, turning their nuclei red; in between these two conditions, a variety of rainbow colors of white/blue/pink tag each representing a peculiar physiological cell condition [70]

reaction, huge contributions have been supplied with the aim to quantify DNA focusing on cell proliferation. At that time the only alternative was the application of the autoradiographic techniques. As it is known, they are based on the incorporation of ^3H - or ^{14}C -labeled DNA precursors into the replicated DNA; the cell sample left in contact with an autoradiographic film “record” the positive cells finally turned in black spots by chemical development. The scoring under the microscope of the number of positive (black) cells over the total cell number gave the so called “labeling index,” the latter representing the fraction of actively synthesizing cells. This tedious, time-consuming, and risky procedure was, for decades, carried out in parallel with DNA Feulgen cytophotometry. The safety recommendations soon entered in the research labs actually limiting (or preventing) the use of radioisotopes. The advent of cytofluorometry definitely reduced (or even stopped) the application of the autoradiographic methodology.

The final evolution of DNA quantitative analysis occurred thanks to a new type of cell analysis made possible thanks to the advanced technological development occurred after the 1950s. In few labs (both in the USA and Germany) different prototypes of instruments capable of counting/analyzing cells in suspension were designed. Different physical strategies were experimented, starting

from the impedenziometric principle for cell counting to the electrostatic liquid droplets generation for cell displacement. A common basic analytical strategy was established to drive cells to flow in a precise interrogation point by means of the so called hydrodynamic focusing. Advanced researches were carried out by scientists such as Leonard Herzenberg, Wallace H. Coulter, Mack Fulwyle, Marvin A. Van Dilla, and Louis Kamentsky, among others, in the USA [72–75], and by Wolfgang Göhde in Germany [76, 77]. The term “flow cytometry” was established by an international consensus committee at the Fifth American Engineering Foundation Conference on Automated Cytology in Pensacola (Florida), in 1976. There was an international agreement to commonly use “flow cytometry,” a term that quickly became popular being the previous terminology different from the USA and Europe.

Around the 1970s, few models of flow instruments became commercially available and were originally characterized by their different illumination system. The American flow cytometers were equipped with the newly developed laser units while the German-made instruments used the arc-lamps. The latter were designed and made by Wolfgang Göhde who followed the idea to make the cells flowing into the conventional epi-fluorescence microscope. Lasers and lamps have both advantages and limitations, and thus, a sort of *commercial fight* between the different companies lasted years. Since at that time DNA was the unique measurable cell parameter and, for this reason, the application of FC largely oriented to ploidy analysis, the company game was focused on this subject.

As already mentioned, FC is today popular in clinical labs for large-scale routine phenotypic analyses whereas much less attention is given to DNA tests; on the contrary, during the decades between 1970 and 1990, the interest of flow cytometrists was essentially focused on the precision of DNA quantitation. Before the era of molecular genetics a lot of literature was devoted to DNA quantitation by FC for both ploidy and cell cycle analyses [78–93]. The latter is still today used in many field of cell biology as well as in the area of drug discovery. On the contrary, cytometric ploidy has today lost any significance and among the various reasons the central one is linked to the relatively limited precision of the FC approach. The first limit is the different chromatin condensation among various cell types which interferes with the staining stoichiometry, and the second one is the instrumental noise and sensitivity. The most advanced modern flow cytometers are unable to detect 1 % difference in DNA content thus FC failed in automated detection of clinical aneuploidy.

Years were spent worldwide to find the best fluorochrome and the best methodology to get very narrow differences in cell DNA content. The Pavia School contributed to this project in the stream of the experience acquired in cytofluorometry. In the 1970s, the

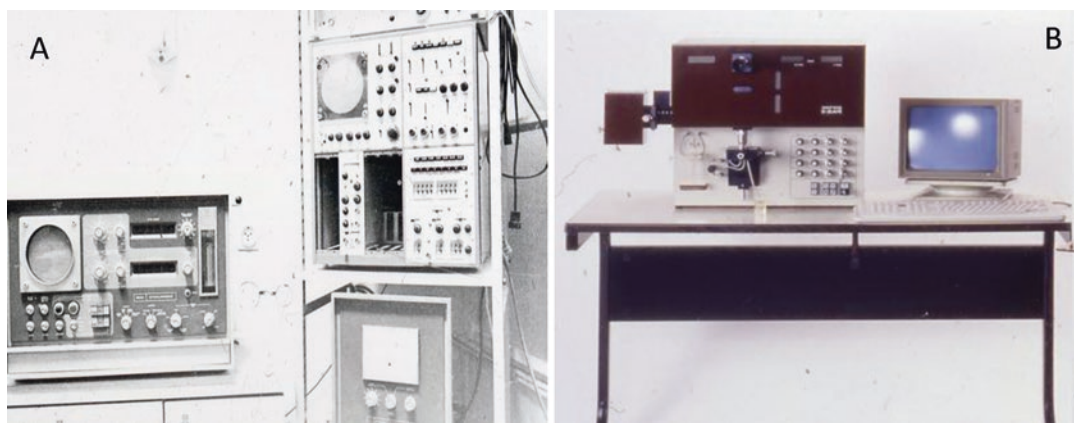


Fig. 9 Images of the first models of flow cytometers operated at the Centre for Studies on Histochemistry in the 1970s. **(A)** The Biophysics 4800A (USA) equipped with the argon ion laser and capable to detect two fluorescent bands, originally devoted to the measurement of Acridine Orange stained hematological samples. **(B)** The Partec PAS (Germany) equipped with the same excitation (HBO100W mercury arc-lamp) employed in fluorescence microscopy and more versatile for the excitation of a variety of fluorochromes. Wolfgang Göhde demonstrated for years the best performance of this instrumental configuration toward the goal of high-resolution DNA measurement

Biophysics 4800A was the first FC operated in Italy (Fig. 9a) and soon a second flow cytometer was acquired by CNR lab (Fig. 9b) who promoted the FC applications [94–97] and the foundation [98] of the Italian Society of Cytometry. The core research activity was still the methodological aspects of DNA staining and the search of new probes suitable to be applied in FC [99].

Even if FC found the new application of choice on immunofluorescence detection (clinical immunophenotyping) it is the unique technique very much appreciated today for studies on cell kinetics as well as for cell induced damages and apoptosis [100–103]. Its peculiar characteristics to allow fast measurements of thousands of cells are greatly appreciated in many biomedical applications [104–109] and remained the unbeatable analytical support for studies on cell biology [110, 111].

5 Pros and Cons of the DNA Content Data for Clinical Applications

The measurement of nuclear DNA content for the evaluation of both ploidy and cell cycle in the majority of human cancers was made for more than 25 years, using FC. Initially, this type of analysis was addressed to the hematological malignancies [112–114] but, with the development of reliable tissue disaggregation and staining techniques, coupled with more and more sophisticated programs for data acquisition and analysis, the technology was extended to almost all types of solid cancers [115–123].

Throughout this period, an impressive number of articles have been published on the potential clinical utility of the DNA cytometric data, with particular emphasis on the prognostic value of ploidy and proliferative activity in clinical oncology. In this field, early investigations have been focused on the comparison of these biological characteristics with other well-established prognostic factors such as clinical stage or histologic grade. Second-generation studies have been designed to correlate tumor ploidy and/or proliferative activity directly with the clinical course of individual patients.

Aneuploidy has clearly important biological implications in many of the common human malignancies but not always correlates with prognosis and/or with the classical clinic-pathologic parameters. Tumor proliferative activity expressed by the S-phase fraction has been studied less exhaustively, but might also be a predictor of outcome in some tumor types. Particular interest has been devoted to the study of cell proliferation performed by means of DNA synthesis precursor such as bromodeoxyuridine which was also utilized *in vivo* under particular clinical conditions [124–130].

In addition, through ploidy and proliferative activity measurements, flow cytometric studies have been focused on the possibility to provide a biological rationale to assist the oncologists in selecting the treatment for some tumors, *i.e.*, to predict the outcome of specific treatment regimens [131].

However, the unique technical capabilities of DNA FC have substantially failed in all the above mentioned possible applications and have shown a relatively little impact in clinical oncology.

It must be underlined that, in order to explore the potential clinical value of FC, both ploidy and proliferative activity should only be evaluated by examining tumor specimens from homogeneous patient series enrolled in specifically designed, large clinical trials and this has not been always accomplished. As a result, one can say that the major limitation to an even more extensive clinical application of these data is coming from the, sometimes strong, lack of concordance between the various studies. If it is true that this can be sometimes ascribed to an inadequate design of prospective studies (due to insufficient number of patients or too short follow-up), in many cases the discrepancies stem from factors directly linked to the various technical aspects of FC.

In particular, a large number of studies have been carried out using formalin fixed, paraffin-embedded tumor specimens that permitted the retrospective analysis of archival specimens. However, there are at least three significant limitations to the use of this type of material [132]. Because of fixation-induced variations in fluorochrome binding, external diploid standards cannot be used to determine which G_{0-1} peak represents the diploid subpopulation in a mixed histogram. Regarding this point, some authors stated that since clinical tumor specimens almost invariably contain a significant

non-malignant cellular component, aneuploid tumors can nonetheless be reliably identified. A second problem associated with the use of paraffin-embedded material is that enzymatic digestion, required to produce single cell suspensions, always results in a variable amount of subcellular debris, which is superimposed to the DNA histogram: if this debris fraction is not taken into account, overestimation of tumor S-phase fraction can occur. To partially overcome this drawback, in the last few years several mathematical models have been implemented with the aim to subtract the background from the histogram before cell cycle analysis. A third problem is that the “DNA resolution” from paraffin-embedded specimens is usually lower than that obtained from fresh tumor material: this obviously increases the risk that paraffin-embedded aneuploid tumors with near-diploid DNA content will be misclassified.

In summary, despite early optimism, the development and clinical applications of FC-based methods for DNA content analysis did not show a great impact on the diagnosis and classification of neoplastic disease or in the monitoring and prognosis of adult patients with solid tumors. On the other hand, flow cytometric DNA analysis will continue to be a valuable tool for in vitro analysis aimed to a better understanding of the biology of tumor growth and heterogeneity as well as to monitor the cancer resistance to chemotherapy/radiotherapy [133].

More significant clinical applications of DNA FC can be foreseen in near future through the multiparametric measurements of DNA content and proliferative activity, in association with the simultaneous analysis of additional parameters such as the presence of specific cell-cycle-related proteins, the phenotype setting, the products of oncogenes, or markers of specific cell functions (e.g., drug resistance) [134–138].

Acknowledgments

The authors acknowledge Emanuela Cova for helpful discussion and contribution, and Carlo Pellicciari for his precious assistance in finding archival documents and pictures.

References

1. Dahm R (2005) Friedrich Miescher and the discovery of DNA. *Dev Biol* 278:274–288
2. Franklin RE, Gosling RG (1953) Evidence for 2-chain helix in crystalline structure of sodium deoxyribonucleate. *Nature* 172:156–157
3. Watson JD, Crick FH (1953) Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature* 171:737–738
4. Caspersson T (1936) Über den chemischen aufbau der strukturen des zellkernes. *Scand Arch Physiol* 73:8
5. Viali M (1954) Su una possibile spiegazione di alcune presunte anomalie alla legge della costanza quantitativa dell'acido desossiribonucleico nelle cellule. *Rend Ist Lomb Sc Lett* 87:29–36

6. Vialli M (1954) Ricerche di istochimica quantitativa comparata sull'acido desossiribonucleico e sulle proteine. *Monit Zool Ital* 62:28–41
7. Vialli M (1957) Volume et contenu en ADN par noyau. *Exp Cell Res Suppl* 4:284–293
8. Kasten FH (2003) Robert Feulgen and his histochemical reaction for DNA. *Biotech Histochem* 78:45–49
9. Böhm N, Sprenger E (1968) Fluorescence cytophotometry: a valuable method for the quantitative determination of nuclear Feulgen-DNA. *Histochemie* 16:100–118
10. Duijndam WAL, Van Duijn P (1975) The influence of chromatin compactness on the stoichiometry of the Feulgen-Schiff procedure studied in model films. II. Investigations on films containing condensed or swollen chicken erythrocyte nuclei. *J Histochem Cytochem* 23:891–900
11. Gil JE, Jotz MM (1976) Further observations on the chemistry of pararosaniline-Feulgen staining. *Histochemistry* 46:147–160
12. Kjellstrand PTT (1977) Temperature and acid concentration in the search for optimum Feulgen hydrolysis conditions. *J Histochem Cytochem* 25:129–134
13. Gautier A, Schreyer M (1970) Feulgen-like electron stains for tissue sections. In: Favard P (ed) *Microscopie electronique*, vol 1. Société Française des Microscopies, Paris, pp 559–560
14. Chieco P, Derenzini M (1999) The Feulgen reaction 75 years on. *Histochem Cell Biol* 111:345–358
15. Vialli M, Reggiani M (1948) Dispositivo per lo studio colorimetrico e fotometrico di preparati microscopici. *Boll Soc Med Chirur Pavia* 62:299–301
16. Casella C, Reggiani M (1949) Istospettrografia di fluorescenza. *Arch Biol (Liège)* 60:207–234
17. Vialli M, Romanini G (1950) Dispositivi semplificati di istofotometria nel visibile. *Boll Soc Ital Biol Sperim* 26:1633
18. Vialli M, Zanotti L (1957) Due nuovi modelli di istofotometri per assunzione di curve di assorbimento. *La Ric Scient* 27:3
19. Vialli M, Perugini S (1954) Due nuovi modelli di apparecchiature istofotometriche. *Riv Istoch Norm Pat* 1(2):149–170
20. Vialli M, Zanotti L, Bianchi U (1960) Istofotometro doppio a visione diretta per visibile e UV. *Mikroskopie* 15:72–81
21. Vialli M, Gerzeli G (1955) Primo contributo alle ricerche di microscopia interferenziale. *Riv Istoch Norm Pat* 1:503–518
22. Ornstein L (1952) The distributional error in microspectrophotometry. *Lab Invest* 1:250–265
23. Adams LR (1968) A photographic cytophotometric method which avoids distributional error. *Acta Cytol* 12:3–8
24. Chieco P, Jonker A, Melchiorri C et al (1994) A user's guide for avoiding errors in absorbance image cytometry: a review with original experimental observations. *Histochem J* 26:1–19
25. Deeley EM (1955) An integrating microdensitometer for biological cells. *J Sci Instrum* 31:263–267
26. Benedetti PA, Viola-Magni MP (1966) A scanning integrating histophotometer. *J Sci Instrum* 43:141–143
27. Ploem JS (1967) The use of vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. *Z wiss Mikrosk* 68:129–142
28. Decosse JJ, Aiello N (1966) Feulgen hydrolysis: effects of acid and temperature. *J Histochem Cytochem* 14:601–604
29. Nitsch B, Murken JD, Bruck HJ (1970) Determining Feulgen-DNA of individual chromosomes by fluorescence cytophotometry with incident light. *Histochemie* 23:254–265
30. Vialli M, Prenna G (1969) Contribution to the cytospectrofluorometric measurement of 5-hydroxytryptamine in enterochromaffin cells. *J Histochem Cytochem* 15:321–330
31. Prenna G, Mazzini G, Cova S (1974) Methodological and instrumental aspects of cytofluorometry. *Histochem J* 6:259–278
32. Cova S, Prenna G, Mazzini G (1974) Digital microspectrofluorometry by multichannel scaling and single photon detection. *Histochem J* 6:279–299
33. Prenna G, Leiva S, Mazzini G (1974) Quantitation of DNA by cytofluorometry of the conventional Feulgen reaction. *Histochem J* 6:467–489
34. Vialli M (1952) Parallelo tra reazioni argentiche e reazioni di Schiff in istomorfologia e in istochimica. *Rend Ist Lomb Sc Lett* 85:323–332
35. Itikawa O, Ogura Y (1954) Simplified manufacture and histochemical use of the Schiff reagent. *Stain Technol* 29:9–11
36. Van Duijn P (1956) A histochemical specific thionine-SO₂ reagent and its use in a bi-color method for deoxyribonucleic acid and periodic acid Schiff positive substances. *J Histochem Cytochem* 4:55–63
37. Kasten FH (1958) Additional Schiff-type reagents for use in cytochemistry. *Stain Technol* 33:39–45
38. Kasten FH (1960) The chemistry of Schiff's reagent. *Int Rev Cytol* 10:1–100
39. Prenna G, De Paoli AM (1964) La deidrotiop-toluidina-SO₂, un nuovo reagente tipo Schiff altamente fluorescente. *Riv Istoch Norm Pat* 10:185–186
40. Prenna G, De Paoli AM (1964) Derivati tiazolici come reagenti tipo Schiff fluorescenti. *Rend Ist Lomb Sc Lett B* 98:267–273

41. Prenna G, Bianchi UA (1964) Reazioni di Feulgen fluorescenti e loro possibilità citofluorometriche quantitative. 5) citofotometria quantitativa in fluorescenza ed in assorbimento della reazione di Feulgen eseguita con acriflavina-SO₂. Riv Istoch Norm Pat 10:667–676
42. Prenna G, De Paoli AM (1968) Impiego del Rivanol come reagente tipo Schiff fluorescente nella reazione di Feulgen. Riv Istoch Norm Pat 14:169–170
43. Mazzini G, Giordano P (1980) Effects of some solvents on the fluorescence intensity of phenanthridinic derivatives-DNA complexes: flow cytofluorometric application. In: Laerum OD, Lindmo T, Thorud E (eds) Flow cytometry IV. Universitetsforlaget, Bergen
44. Mazzini G, Giordano P, Riccardi A et al (1980) Biological significance of flow cytometric application of phenanthridinic dyes at low concentration. Basic Appl Histochem 24:264
45. Mazzini G, Giordano PA (1981) Flow cytometry: a methodologic approach for fast quantitative cytochemical measurements and its use for the study of the chromatin structure. Basic Appl Histochem 25:303
46. Mazzini G, Bottiroli G, Prenna G (1975) An electronic device for the automatic correction of fluorescence emission spectra. Histochem J 7:291–297
47. Trujillo TT, Van Dilla MA (1972) Adaptation of the fluorescent Feulgen reaction to cells in suspension for flow microfluorometry. Acta Cytol 16:26–30
48. Ormerod MG (1990) Flow cytometry: a practical approach. IRL Press, Oxford
49. Rigler R Jr (1966) Microfluorometric characterization of intracellular nucleic acids and nucleoproteins by Acridine Orange. Acta Physiol Scand 67(267):1–122
50. Darzynkiewicz Z (1979) Acridine orange as a molecular probe in studies of nucleic acids. In: Melamed MR, Mullaney PF, Mendelsohn ML (eds) Flow cytometry and sorting. John Wiley and Sons, New York, NY
51. Laerum OD, Farsund T (1981) Clinical applications of flow cytometry: a review. Cytometry 2:1–13
52. Robinson JP (1993) Handbook of flow cytometry methods. Wiley-Liss, New York, NY
53. Shapiro HM (1998) Practical flow cytometry. Alan R Liss Inc, New York, NY
54. O'Donnell EA, Ernst DN, Hingorani R (2013) Multiparameter flow cytometry: advances in high resolution analysis. Immune Netw 13(2):43–54. doi:10.4110/in.2013.13.2.43
55. Zembruski NCL, Nadine CL, Stache V et al (2012) 7-Aminoactinomycin D for apoptosis staining in flow cytometry. Analyt Biochem 429(1):179–181. doi:10.1016/j.ab.2012.07.005
56. Göhde W (1972) Automation of cytofluorometry by use of the impulsimicrophotometer. In: Thaeer A, Sernetz M (eds) Fluorescence techniques in cell biology. Springer, New York, NY
57. Latt SA, Stetten G (1976) Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. J Histochem Cytochem 24(1):24–33. doi:10.1177/24.1.943439
58. Crissman HA, Steinkamp JA (1973) Rapid simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. J Cell Biol 59:766–771
59. Krishan A (1975) Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 66:188–193
60. Fried J, Perez AG, Clarkson BD (1976) Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. Properties of the method and mathematical analysis of the data. J Cell Biol 71:172–181
61. Costa A, Mazzini G, Del Bino G et al (1981) DNA content and kinetic characteristics of non-Hodgkin's lymphoma determined by flow cytometry and autoradiography. Cytometry 2(3):185–188. doi:10.1002/cyto.990020310
62. Zippel R, Martegani E, Vanoni M et al (1982) Cell cycle analysis in a human cell line (EUE cells). Cytometry 2(6):426–430. doi:10.1002/cyto.990020612
63. Riccardi A, Mazzini G, Montecucco CM et al (1982) Sequential vincristine, arabinosylcytosine and adriamycin in acute leukemia: cytologic and cytokinetic studies. Cytometry 3(2):104–109. doi:10.1002/cyto.990030207
64. Giraldo T, Sava G, Cherubini R et al (1984) Effects of DTIC, DM-COOK and ICRF-159 on the number of circulating Lewis lung carcinoma cells detected by flow cytometry. Clin Exp Metast 2(2):151–159
65. Giordano P, Mazzini G, Riccardi A et al (1985) Propidium iodide staining of cytoautoradiographic preparations for the simultaneous determination of DNA content and grain count. Histochem J 17(11):1259–1270
66. Riccardi A, Danova M, Montecucco CM et al (1986) Adult acute non-lymphoblastic leukaemia: reliability and prognostic significance of pretreatment bone marrow S-phase size determined by flow cytofluorometry. Scand J Haematol 36(1):11–17
67. Mazzini G, Giordano P, Montecucco CM et al (1980) A rapid cytofluorometric method

- for quantitative DNA determination on fixed smears. *Histochem J* 12:153–168
68. Haugland RP, Larison KD (1996) Handbook of fluorescent probes and research chemicals. Molecular Probes Inc, Eugene, OR
 69. Ellwart JW, Dormer P (1990) Viability measurement using spectrum shift in Hoechst 33342 stained cells. *Cytometry* 11:239–243
 70. Mazzini G, Ferrari C, Erba E (2003) Dual excitation multi-fluorescence flow cytometry for detailed analyses of viability and apoptotic cell transition. *Eur J Histochem* 47:289–298
 71. Caspersson T (1979) Quantitative tumor cytochemistry. *Cancer Res* 39:2341–2355
 72. Kametsky LA, Melamed MR, Derman H (1969) Spectrophotometer: new instrument for ultrarapid cell analysis. *Science* 150:630–631
 73. Van Dilla MA, Trujillo TT, Mullaney PF et al (1969) Cell microfluorometry: a method for rapid fluorescence measurement. *Science* 163:1213
 74. Melamed MR, Lindmo T, Mendelsohn ML (1991) Flow cytometry and sorting. Wiley-Liss, New York, NY
 75. Longobardi A (1992) Flow cytometry: first principles. Wiley-Liss, New York, NY
 76. Dittrich W, Göhde W (1969) Impulsfluorometrie bei einzelnzellen in suspension. *Z Naturforsch* 24:360–361
 77. Göhde W, Dittrich W (1970) Simultane impulsfluorimetrie des DNS und proteingehaltes von Tumorzellen. *Z Anal Chem* 352:328–330
 78. Baisch H, Göhde W, Linden WA (1975) Analysis of PCP-data to determine the fraction of cells in the various phases of the cell cycle. *Radiat Environ Biophys* 12:31–39
 79. Barlogie B, Raber MN, Schuman J et al (1983) Flow cytometry in clinical cancer research. *Cancer Res* 43:3982–3997
 80. Mares V, Giordano PA, Mazzini G et al (1987) Influence of cis-dichlorodiamineplatinum on glioma cell morphology and cell cycle kinetics in tissue culture. *Histochem J* 19(4):187–194
 81. Pellicciari C, Mazzini G, Fuhrman Conti AM et al (1989) Effect of hypertonic medium on human cell growth: III. Changes in cell kinetics of EUE cells. *Cell Biol Int Rep* 13(4):345–356
 82. Pellicciari C, Danova M, Giordano M et al (1991) Expression of cell cycle related proteins—proliferating cell nuclear antigen (PCNA) and statin—during adaptation and de-adaptation of EUE cells to a hypertonic medium. *Cell Prolif* 24(5):469–479. doi:10.1111/j.1365-2184.1991.tb01175.x
 83. Casasco A, Casasco M, Cornaglia AI et al (2001) Cell kinetics in a model of artificial skin. An immunohistochemical and flow cytometric analysis. *Eur J Histochem* 45(2):125–130
 84. Derenzini M, Montanaro L, Chillà A et al (2005) Key role of the achievement of an appropriate ribosomal RNA complement for G1-S phase transition in H4-II-E-C3 rat hepatoma cells. *J Cell Physiol* 202(2):483–491. doi:10.1002/jcp.20144
 85. Montanaro L, Mazzini G, Barbieri S et al (2007) Different effects of ribosome biogenesis inhibition on cell proliferation in retinoblastoma protein- and p53-deficient and proficient human osteosarcoma cell lines. *Cell Prolif* 40(4):532–549. doi:10.1111/j.1365-2184.2007.00448.x
 86. Rebuzzini P, Neri T, Mazzini G et al (2008) Karyotype analysis of the euploid cell population of a mouse embryonic stem cell line revealed a high incidence of chromosome abnormalities that varied during culture. *Cytogenet Genom Res* 121(1):18–24. doi:10.1159/000124377
 87. Derenzini M, Donati G, Mazzini G et al (2008) Loss of retinoblastoma tumor suppressor protein makes human breast cancer cells more sensitive to antimetabolite exposure. *Clin Canc Res* 14(7):2199–2209. doi:10.1158/1078-0432.CCR-07-2065
 88. Soza S, Leva V, Vago R et al (2009) DNA ligase I deficiency leads to replication-dependent DNA damage and impacts cell morphology without blocking cell cycle progression. *Mol Cel Biol* 29(8):2032–2041. doi:10.1128/MCB.01730-08
 89. Zonta A, Pinelli T, Prati U et al (2009) Extracorporeal liver BNCT for the treatment of diffuse metastases: what was learned and what is still to be learned. *Appl Radiat Isot* 67:67–75. doi:10.1016/j.apradiso.2009.03.087
 90. Cova E, Ghiroldi A, Guareschi S et al (2010) G93A SOD1 alters cell cycle in a cellular model of amyotrophic lateral sclerosis. *Cell Signal* 22(10):1477–1484. doi:10.1016/j.cellsig.2010.05.016
 91. Zucca E, Bertoletti F, Wimmer U et al (2013) Silencing of human DNA polymerase λ causes replication stress and is synthetically lethal with an impaired S phase checkpoint. *Nucleic Acids Res* 41(1):229–241. doi:10.1093/nar/gks1016
 92. Terranova N, Rebuzzini P, Mazzini G et al (2014) Mathematical modeling of growth and death dynamics of mouse embryonic stem cells irradiated with γ -rays. *J Theoret Biol*. doi:10.1016/j.jtbi.2014.08.042
 93. Paolini A, Curti V, Pasi F et al (2015) Gallic acid exerts a protective or an anti-proliferative effect on glioma T98G cells via dose-dependent epigenetic regulation mediated by miRNAs. *Int J Oncol*. doi:10.3892/ijo.2015.2864

94. Prenna G, Mazzini G, Bottiroli G et al (1976) Automated determination of DNA cellular content (Feulgen) by using BBT-SO₂ in flow-cytofluorometry. In: Göhde W, Schumann J, Büchner T (eds) *Flow-through cytophotometry, its application to cancer research and hematology*. European Press, Medikon, Ghent, pp 88–95
95. Del Bino G, Bruni C, Koch G et al (1985) Validation of a mathematical procedure for computer analysis of flow cytometric DNA data in human tumors. *Cytometry* 6(1):31–36. doi:[10.1002/cyto.990060107](https://doi.org/10.1002/cyto.990060107)
96. Giordano M, Danova M, Riccardi A et al (1989) Simultaneous detection of cellular ras p21 oncogene product and DNA content by two-parameter flow cytometry. *Anticancer Res* 9(3):799–803
97. Danova M, Pellicciari C, Bottone MG et al (1994) Multiparametric assessment of the cell-cycle effects of tamoxifen on MCF-7 human breast-cancer cells. *Oncol Rep* 1(4):739–745
98. Tirindelli Danesi D, Spanò M, Altavista P et al (1997) Quality control study of the Italian Group of Cytometry on flow cytometry DNA content measurements: II. Factors affecting inter- and intralaboratory variability. *Cytometry* 30(2):85–97
99. Tavecchio M, Simone M, Bernasconi S et al (2008) Multi-parametric flow cytometric cell cycle analysis using TO-PRO-3 iodide (TP3): detailed protocols. *Acta Histochem* 110(3):232–244. doi:[10.1016/j.acthis.2007.10.007](https://doi.org/10.1016/j.acthis.2007.10.007)
100. Chiozzi V, Mazzini G, Oldani A et al (2009) Relationship between Vac A toxin and ammonia in *Helicobacter pylori*-induced apoptosis in human gastric epithelial cells. *J Physiol Pharmacol* 60(3):23–30
101. Barbieri G, Palumbo S, Gabrusiewicz K et al (2011) Silencing of cellular prion protein (PrP^C) expression by DNA-antisense oligonucleotides induces autophagy-dependent cell death in glioma cells. *Autophagy* 7(8):840–853
102. Giansanti V, Tillhon M, Mazzini G et al (2011) Killing of tumor cells: a drama in two acts. *Biochem Pharmacol* 82(10):1304–1310. doi:[10.1016/j.bcp.2011.05.023](https://doi.org/10.1016/j.bcp.2011.05.023)
103. Aredia F, Giansanti V, Mazzini G et al (2013) Multiple effects of the Na(+)/H(+) antiporter inhibitor HMA on cancer cells. *Apoptosis*. doi:[10.1007/s10495-013-0898-3](https://doi.org/10.1007/s10495-013-0898-3)
104. Ferrara F, Daverio R, Mazzini G et al (1997) Automation of human sperm cell analysis by flow cytometry. *Clin Chem* 43(5):801–807
105. Cova E, Cereda C, Galli A et al (2006) Modified expression of Bcl-2 and SOD1 proteases in lymphocytes from sporadic ALS patients. *Neurosci Lett* 399(3):186–190. doi:[10.1016/j.neulet.2006.01.057](https://doi.org/10.1016/j.neulet.2006.01.057)
106. Fassina L, Saino E, Visai L et al (2008) Electromagnetic enhancement of a culture of human SAOS-2 osteoblasts seeded onto titanium fiber-mesh scaffolds. *J Biomed Mat Res* 87(3):750–759. doi:[10.1002/jbm.a.31827](https://doi.org/10.1002/jbm.a.31827)
107. Donati G, Brighenti E, Vici M et al (2011) Selective inhibition of rRNA transcription downregulates E2F-1: a new p53-independent mechanism linking cell growth to cell proliferation. *J Cell Science* 124:3017–3028. doi:[10.1242/jcs.086074](https://doi.org/10.1242/jcs.086074)
108. Riva F, Omes C, Bassani R et al (2014) In-vitro culture system for mesenchymal progenitor cells derived from waste human ovarian follicular fluid. *Reprod Biomed*. doi:[10.1016/j.rbmo.2014.06.006](https://doi.org/10.1016/j.rbmo.2014.06.006)
109. Cansolino L, Clerici AM, Zonta C et al (2015) Comparative study of the radiobiological effects induced on adherent vs suspended cells by BNCT, neutrons and gamma rays treatments. *Appl Radiat Isot*. doi:[10.1016/j.apradiso.2015.07.054](https://doi.org/10.1016/j.apradiso.2015.07.054)
110. Mazzini G, Carpignano F, Surdo S et al (2015) 3D silicon microstructures: a new tool for evaluating biological aggressiveness of tumor cells. *IEEE Trans Nanobiosciences* 7:797–805. doi:[10.1109/TNB.2015.2476351](https://doi.org/10.1109/TNB.2015.2476351)
111. Aredia F, Carpignano F, Surdo S et al (2016) An innovative cell microincubator for drug discovery based on 3D silicon structures. *J Nanomat* doi:[10.1155/2016/8236539](https://doi.org/10.1155/2016/8236539)
112. Danova M, Riccardi A, Mazzini G (1990) Cell cycle-related proteins and flow cytometry. *Haematol* 75(3):252–264
113. Danova M, Riccardi A, Ucci G et al (1990) Ras oncogene expression and DNA content in plasma cell dyscrasias: a flow cytofluorimetric study. *Brit J Cancer* 62(5):781–785
114. Rosti V, Bergamaschi G, Lucotti C et al (1995) Oligodeoxynucleotides antisense to c-abl specifically inhibit entry into S-phase of CD34+ hematopoietic cells and their differentiation to granulocyte-macrophage progenitors. *Blood* 86(9):3387–3393
115. Cavalli C, Danova M, Gobbi P et al (1989) Ploidy and proliferative activity measurement by flow cytometry in non-Hodgkin's lymphomas. Do speculative aspects prevail over clinical ones? *Europ J Cancer Clin Oncol* 25(12):1755–1763
116. Giaretti W, Danova M, Geido E et al (1991) Flow cytometric DNA index in the prognosis of colorectal cancer. *Cancer* 67(7):1921–1927
117. Mazzini G, Danova M (1994) Citometria a flusso: applicazioni cliniche dell'analisi del DNA in oncologia. Collana "I Manuali delle

- Scuole". Manuale Ph.D. 08. Scuola Superiore Oncologia e Scienze Biomediche. Genova
118. Danova M, Rosti V, Mazzini G et al (1995) Cell kinetics of CD34-positive hematopoietic cells following chemotherapy plus colony-stimulating factors in advanced breast cancer. *Internat J Cancer* 63(5):646–651
119. Danova M, Mazzini G, Alberici R et al (1996) Sequential administration of interleukin-3 and granulocyte-macrophage colony-stimulating factor following intensified, accelerated CEE (cyclophosphamide, epirubicin, etoposide) chemotherapy in patients with solid tumors. *Internat J Oncol* 9(5):971–976
120. Bozzetti C, Nizzoli R, Naldi N et al (1996) Nuclear grading and flow cytometric DNA pattern in fine-needle aspirates of primary breast cancer. *Diagn Cytopath* 15(2):116–120. doi:[10.1002/\(SICI\)1097-0339\(199608\)15:2<116::AID-DC6>3.0.CO;2-G](https://doi.org/10.1002/(SICI)1097-0339(199608)15:2<116::AID-DC6>3.0.CO;2-G)
121. Casasco A, Casasco M, Calligaro A (1997) Cell proliferation in developing human dental pulp. A combined flow cytometric and immunohistochemical study. *Europ J Oral Sci* 105(6):609–613
122. Oliani C, Barana D, Cazzadori A et al (2005) Cytofluorimetric evaluation of DNA ploidy in lung cancer: a bronchoscopic study. *Internat J Biol Markers* 20(2):87–92
123. Bozzetti C, Nizzoli R, Camisa R et al (1997) Comparison between Ki-67 index and S-phase fraction on fine-needle aspiration samples from breast carcinoma. *Cancer* 81(5):287–292
124. Danova M, Riccardi A, Gaetani P et al (1988) Cell kinetics of human brain tumors: in vivo study with bromodeoxyuridine and flow cytometry. *Europ J Cancer Clin Oncol* 24(5):873–880
125. Riccardi A, Danova M, Wilson G et al (1988) Cell kinetics in human malignancies studied with in vivo administration of bromodeoxyuridine and flow cytometry. *Cancer Res* 48(21):6238–6245
126. Riccardi A, Danova M, Dionigi P et al (1989) Cell kinetics in leukaemia and solid tumours studied with in vivo bromodeoxyuridine and flow cytometry. *Brit J Cancer* 59(6):898–903
127. Giordano M, Riccardi A, Danova M et al (1991) Cell proliferation of human leukemia and solid tumors studied with in vivo bromodeoxyuridine and flow cytometry. *Cancer Detect Prev* 15(5):391–396
128. Giordano M, Danova M, Mazzini G et al (1993) Cell kinetics with in vivo: bromodeoxyuridine assay, proliferating cell nuclear antigen expression, and flow cytometric analysis. Prognostic significance in acute nonlymphoblastic leukemia. *Cancer* 71(9):2739–2745
129. Erba E, Giordano M, Danova M et al (1994) Cell kinetics of human ovarian cancer with in vivo administration of bromodeoxyuridine. *Ann Oncol* 5(7):627–634
130. Mazzini G, Danova M, Ferrari C et al (1996) Cell proliferation and ploidy of human solid tumours: methodological experience with in vivo bromodeoxyuridine and DNA flow cytometry. *Analyt Cell Path* 10(2):101–113
131. Chang Q, Hedley D (2012) Emerging applications of flow cytometry in solid tumor biology. *Methods* 57:359–367
132. Danova M, Mazzini G, Wilson G et al (1987) Ploidy and proliferative activity of human gastric carcinoma: a cytofluorometric study on fresh and on paraffin embedded material. *Bas Appl Histochem* 31(1):73–82
133. Woo J, Baumann A, Arguello V (2014) Recent advancements of flow cytometry: new applications in hematology and oncology. *Exp Rev Mol Diagn* 14:67–81
134. Danova M, Torchio M, Mazzini G (2011) Isolation of rare circulating tumor cells in cancer patients: technical aspects and clinical implications. *Exp Rev Mol Diagn* 11(5):473–485. doi:[10.1586/erm.11.33](https://doi.org/10.1586/erm.11.33)
135. Manzoni M, Comolli G, Torchio M (2014) Circulating endothelial cells and their subpopulations: role as predictive biomarkers in antiangiogenic therapy for colorectal cancer. *Clin Col Cancer*. doi:[10.1016/j.clcc.2014.12.002](https://doi.org/10.1016/j.clcc.2014.12.002)
136. Kim KH, Sederstrom JM (2015) Assaying cell cycle status using flow cytometry. *Curr Protoc Mol Biol* doi: [10.1002/0471142727](https://doi.org/10.1002/0471142727)
137. Robinson JP, Roederer M (2015) History of science. Flow cytometry strikes gold. *Science* 350(6262):739–740. doi:[10.1126/science.aad6770](https://doi.org/10.1126/science.aad6770)
138. Adan A, Alizada G, Kiraz Y et al (2016) Flow cytometry: basic principles and applications. *Crit Rev Biotechnol* 14:1–14

Osmium Ammine for Staining DNA in Electron Microscopy

Irene Masiello and Marco Biggiogera

Abstract

The osmium ammine staining allows the specific detection of DNA in the cell nucleus and represents one of the most used techniques for EM cytochemistry.

The procedure is a Feulgen-type reaction, consisting of an acid hydrolysis to obtain free aldehyde groups on DNA followed by their binding to osmium ammine, a Schiff-type reagent. Osmium ammine is polyamminic electron-dense compound commercially available.

Here, we describe the staining procedure for ultrathin sections and the different procedures for the preparation of the reagent for acrylic and epoxy sections.

Key words DNA staining, Osmium ammine, Cytochemistry, Feulgen reaction, Schiff reagent, Electron microscopy

1 Introduction

The greatest achievement of Alain Gautier, a pioneer in electron microscopy (EM), is the translation of the Feulgen reaction to EM level. Basically, the reaction described by Robert Feulgen in 1924 for light microscopy consists of two steps: first, an acid hydrolysis leading to removal of purine bases from the deoxyribose, engendering aldehyde groups; second, the reaction of free aldehyde groups with the Schiff reagent resulting in specifically stained DNA. The Schiff reagent must be activated by SO_2 [1] and have at least one amino group which can bind the aldehydic one giving a specific stain. Osmium ammine (OA) has been used as a Schiff-type reagent for the specific DNA staining in ultrathin sections since it fulfills the described requirements and, importantly, it is an electron dense compound which can be revealed at EM [2, 3]. Moreover, OA was proved to be specific and with high resolution [4, 5].

However, the preparation technique led to an almost 50% failure rate. In 1989, Olins and coworkers synthesized a different reagent, called Osmium Ammine-B, in a much simpler and reproducible way [6]. The reagent is now commercially available and the

results are reproducible. Finally, a useful modification for preparing the active reagent has been proposed by Vázquez-Nin and collaborators [7]. The purpose of the latter paper was to avoid the use of bubbling SO_2 (potentially dangerous) through the addition of SO_2 -generating chemicals, as it is done for light microscopy. The preparation method is not expensive, safe, simple and reproducible, giving the same results of the original technique.

Some controls can be performed to test the efficiency of this staining method for EM cytochemistry both on acrylic sections and epoxy ones: after the removal of DNA by DNase treatment prior to HCl, no structures are stained by OA; treatment with dimedone in 1 % acetic acid (1 h at 37 °C) after HCl and before OA suppresses DNA staining by specifically blocking aldehyde groups [8].

After OA treatment of acrylic sections in the absence of HCl hydrolysis DNA as well as RNA can be stained: this process involves the binding of OA to the negative charges of nucleic acids and it is related to the low pH of the staining solution [9]. Nevertheless, this staining cannot be carried out on Epon sections. Interestingly, a variation of the Feulgen reaction involving pyrimidines allows the staining of DNA (unpublished data): the removal of pyrimidines from DNA is obtained by incubating the samples with 1 N NaOH; the Feulgen-type reagent binds the apyrimidinic sites staining selectively DNA, as for the normal reaction.

The procedure described here provides two different protocols for the preparation of the reagent using acrylic sections or epoxy ones.

2 Materials

2.1 Sample Preparation

Any kind of cells or tissues can be processed for EM to reveal specifically DNA through OA-staining.

2.1.1 Reagents

1. 67 mM Sörensen buffer pH 7.4: dissolve 11.88 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 1 L of distilled water (Solution A); dissolve 9.08 g of KH_2PO_4 in 1 L of distilled water (Solution B); mix 81.8 mL of Solution A and 18.2 mL of Solution B.
2. 4 % paraformaldehyde in Sörensen buffer pH 7.4; store at 4 °C for 1–2 months (*see* **Note 1**).
3. 2.5 % glutaraldehyde in Sörensen buffer pH 7.4; store at 4 °C for 1–2 months (*see* **Note 1**).
4. 2 % agar (necessary for pre-embedding isolated cells): dissolve 1 g of agarose in 50 mL of warm distilled water and store at 4 °C.
5. Graded ethanol or acetone in distilled water: 30 %, 50 %, 70 %, 90 % and absolute ethanol or acetone; store at room temperature.

6. LRWhite resin: the activation of LRWhite resin is obtained by adding 9.9 g of benzoyl peroxide in 500 mL of resin under gentle agitation by magnetic stirrer; store at 4 °C.
7. EMBED-812 resin: mix under gentle agitation by magnetic stirrer 20 g of EMBED-812, 16 g of dodecenyl succinic anhydride and 8 g of nadic methyl anhydride and finally add 1.2 mL of benzyl-dimethylamine under a fume hood; let the reagent mix for at least 30 min (*see Note 2*) and store it at -20 °C (thaw the resin for at least 20 min before use).

2.2 Preparation of the Schiff-Type Reagent, Osmium Ammine-B

It is necessary to always prepare a fresh solution of OA (*see Note 3*).

1. *For acrylic sections:* dissolve 10 mg of osmium ammine-B (*see Note 4*) in 4.8 mL of double distilled water. After the reagent has completely dissolved (*see Note 3*), add 200 µL of 5 N HCl (final concentration 0.2 N) and mix the solution; add 190 mg of sodium metabisulfite (final concentration 0.2 N) and stir. The reagent is ready for use 30 min after dissolving the sodium metabisulfite.
2. *For Epoxy sections:* dissolve 10 mg of osmium ammine-B (*see Note 4*) in 2.5 mL of double distilled water. After the reagent has completely dissolved (*see Note 3*), add 2.5 mL of glacial acetic acid (final concentration 8 N) and mix; then add 38 mg of sodium metabisulfite (final concentration 40 mM) and stir. The solution is ready for use 30 min after dissolving the sodium metabisulfite. Freshly prepared 5 N HCl (*see Note 5*) and fresh distilled water are necessary.

2.3 Equipment

1. 5 N HCl: dilute 42 mL of 12 N HCl in 58 mL of distilled water under a fume hood.
2. 200 mesh gold grids or Marinosz rings to place thin sections.
3. Thin sections obtained using an ultramicrotome.
4. Embryo dishes for the staining procedure.
5. Filter paper to dry the grids during the procedure.

3 Methods

3.1 Sample Processing

1. The samples can be fixed either in 4% paraformaldehyde in Sörensen buffer for 2 h at 4 °C or in 2.5% glutaraldehyde in Sörensen buffer for 2 h at room temperature (*see Note 6*). After the removal of fixative with a pipette, the specimens are rinsed thoroughly with Sörensen buffer first and then with PBS.
2. Pre-embedding in 2% agar in H₂O is only necessary when working with cells in suspension. Dehydration in graded ethanol or acetone follows. Finally, the samples are embedded either in LRWhite or Epon resin.

3. Thin sections of 70–80 nm are collected on naked gold grids (*see Note 7*). Alternately, the sections can be floated onto the different solutions within Marinozzi rings and, after the final rinsing, put on a formvar-coated grid which in this case can be made of nickel or copper.

3.2 OA-Staining of Sections

Carry out all passages at room temperature. The procedure is the same using acrylic or epoxy sections.

1. Fill an embryo dish with 5 N HCl and float the grid, sections facing down, on the solution for 30–45 min (*see Note 8*) to allow the formation of free aldehyde groups (*see Note 9*).
2. Blot with a filter paper.
3. After filling an embryo dish with distilled water, rinse the specimen pipetting 5–10 times.
4. Rinse the sample three times for 2 min (*see Note 10*).
5. Blot with a filter paper.
6. Fill up an embryo dish with the solution of osmium ammine-B and incubate the sample in the reagent solution for 1 h to stain DNA (*see Note 11*) under the fume hood, since SO₂ fumes are released.
7. Dry quickly with a filter paper.
8. After filling an embryo dish with distilled water, rinse the specimen thoroughly by pipetting (10–15 times). Wash the sample three times for 5 min. Finally, let the specimen float on distilled water at least for 20 min: pipetting is recommended using a Pasteur (*see Note 12*).
9. Dry the sections with a filter paper.
10. Examine the stained grids with an electron microscope operating at 80 kV (Fig. 1).

The stain can penetrate through the entire section [9], thus staining all the DNA embedded in its thickness (*see Note 13*).

4 Notes

1. Check for possible presence of precipitates in the fixative solutions: if any, discard and prepare a fresh fixative.
2. Avoid the formation of bubbles in the Epon mixture since they may remain during resin polymerization not allowing the preparation of good sections.
3. The complete dissolution of osmium ammine-B avoids the formation of its precipitates, which prevents the correct visualization of the samples. For the same reason, freshly prepared reagent solution is suggested.

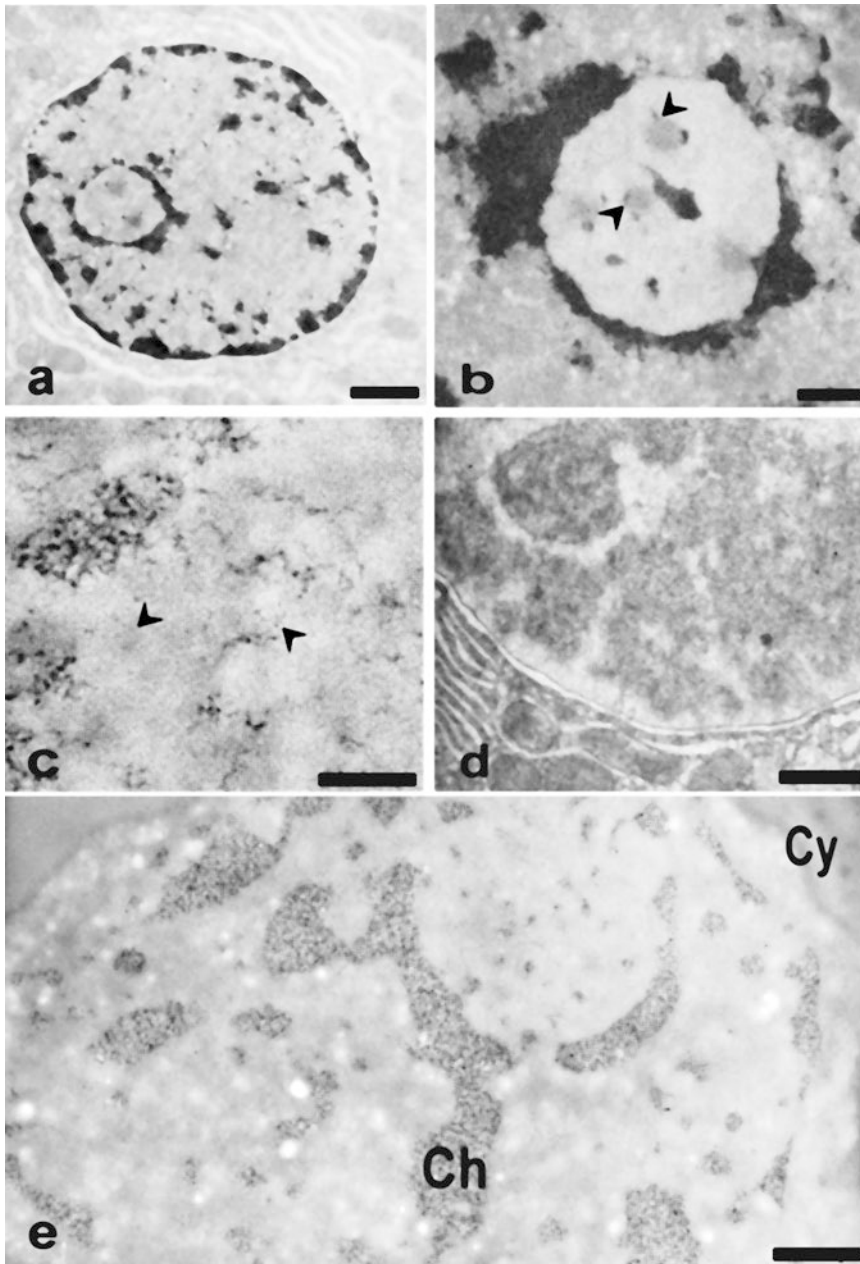


Fig. 1 DNA staining. (a) Rat liver, PFA fixation, LR White embedding. 5 N HCl for 30 min, OA for 60 min. (b) Rat liver, PFA fixation, Epon embedding. Staining as in (a). Note the thin DNA fibers inside the nucleolus which looks electron lucent due to loss of proteins after hydrolysis. (c) HeLa cell, LR White embedding. At the periphery of the condensed chromatin extremely thin fibers are visible. (d) Rat liver. Dimedone blockade of aldehyde groups after 5 N HCl. The treatment with OA does not result in staining of DNA. (e) Mouse P815 cell, high pressure freezing, cryosubstitution in Lowycryl K11M. Immunolabeling for histone H2B followed by 1N HCl for 10 min and OA for 30 min. Bar in **a**, **b**, **d**, **e** = 1 μ m. Bar in **c** = 500 nm

4. Since osmium ammine is an organometallic compound, it can interact electrostatically with the walls of recipient used for weighing. Therefore, it is recommended to use glass or paper to avoid electrostatic interactions (which are enhanced by plastic tubes).
5. Since HCl is gaseous, its leakage from the solution during the storage is possible changing the final concentration. Therefore, fresh HCl is recommended to allow the activation of osmium ammine-B and the correct removal of purine from DNA.
6. PFA fixation is suggested: in fact, glutaraldehyde should be avoided due to the high risk of free aldehyde groups. If necessary, after glutaraldehyde fixation an incubation with 0.5 M NH_4Cl in PBS for 30 min will block the free aldehyde groups which will give rise to false positive staining (several washes with H_2O are necessary). It is possible to perform the blocking reaction also after paraformaldehyde fixation.
7. The formvar-carbon coat as well as nickel grids are degraded by acid hydrolysis. Moreover, golden-plated grids increase the possibility of section detachment during the procedure.
8. The incubation time in HCl is dependent on the thickness of the sections and on the biological material involved. Usually, within the proposed range (30–45 min) there is little risk of damaging the section by excessive depolymerization of DNA.
9. When staining sections of isolated nuclei or high pressure frozen, cryosubstituted material with no chemical fixation, care must be taken for the hydrolysis step, eventually adjusting both the concentration and time: 5 N can be reduced to 1 N and 30 min can be reduced to 10–15 min.
10. Results do not change by increasing the time of rinses or their pH.
11. For acrylic sections DNA is stained also in 15–30 min. This staining method reaches saturation so the result does not change increasing the time of incubation in OA.
12. By rinsing thoroughly and pipetting, one can remove possible precipitates of OA given the tendency of this compound to precipitate after the staining.
13. While the sections stained with the classical OA [3] are stable and visible after years (at least 30), the sections treated with the commercial reagent lack stability and the stain goes to the surface of the section in form of roundish precipitates after at least 1 year. Epoxy sections appear more contrasted by osmium ammine-B than by the reagent solution prepared according to the original recipe while on acrylic sections both staining methods give the same results.

Acknowledgments

The authors would like to thank Ms. Francine Flach for excellent technical skill in preparing the ultrathin sections.

References

1. Kasten FH (1960) The chemistry of Schiff's reagent. *Int Rev Cytol* 10:1–100
2. Cogliati R, Gautier A (1973) Demonstration of DNA and polysaccharides using a new Schiff-type reagent. *C R Acad Sci* 276:3041–3044
3. Gautier A (1976) Ultrastructural localization of DNA in ultrathin tissue sections. *Int Rev Cytol* 44:113–191
4. Derenzini M, Hernandez-Verdun D, Bouteille M (1982) Visualization in situ of extended DNA filaments in nucleolar chromatin of rat hepatocytes. *Exp Cell Res* 141(2):463–469
5. Biggiogera M, Courtens JL, Derenzini M et al (1996) Osmium ammine: review of current applications to visualize DNA in electron microscopy. *Biol Cell* 87:121–132
6. Olins AL, Moyer BA, Kim SH et al (1989) Synthesis of a more stable osmium ammine electron dense stain. *J Histochem Cytochem* 37:395–398
7. Vázquez-Nin GH, Biggiogera M, Echeverría OM (1995) Activation of osmium ammine by SO₂-generating chemicals for EM Feulgen-type staining of DNA. *Eur J Histochem* 39:101–106
8. Pickett-Heaps JD (1967) Preliminary attempts at ultrastructural polysaccharide localization in root tip cells. *J Histochem Cytochem* 15:442–455
9. Derenzini M, Farabegoli F (1990) Selective staining of nucleic acids by osmium ammine complex in thin sections from Lowicryl-embedded samples. *J Histochem Cytochem* 28:1495–1501

DNA Labeling at Electron Microscopy

Nicolas Thelen and Marc Thiry

Abstract

Here, we describe a method for locating DNA on ultrathin sections. This technique is compatible with all usual fixation and embedding procedures and can be combined with cytochemical methods. Ultrathin sections are incubated in a medium containing terminal deoxynucleotidyl transferase (TdT) and various non-isotopic nucleotide analogs. The labeled nucleotides bound to the surface of ultrathin sections are then visualized by an indirect immunogold labeling technique. This high-resolution method provides a powerful tool for pinpointing the precise location of DNA within biological material, even where DNA is present in very low amounts.

Key words Terminal deoxynucleotidyl transferase, Immuno-electron microscopy, DNA labeling, Immunogold techniques, Immunocytochemistry

1 Introduction

Several cytochemical approaches are available for studying DNA distribution at the subcellular level [1, 2], including the Feulgen-like reaction with osmium ammine [3–6] and tagging DNA with DNase–gold complexes [7, 8]. Other strategies involve the use of antibodies directed against DNA [9–12] or against bromodeoxyuridine preincorporated into DNA [13, 14]. Nevertheless, these different techniques are applicable only to biological materials fixed and embedded in a special way, which does not allow excellent morphological distinction between the subcellular components.

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase found in pre-lymphocytes and at early stages of lymphoid differentiation [15]. TdT catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-OH ends of double- or single-stranded DNA during V(D)J recombination [16–19]. While playing a crucial role in the evolution and adaptation of the vertebrate immune system [19–23], this reaction is commonly used *in vitro* to label the 3' ends of double- or single-stranded DNA with labeled nucleotides and to add homopolymer

tails to DNA fragments [24–26]. By means of tritiated dATP and autoradiography, calf thymus TdT was shown to bind deoxynucleotide monophosphate to the free terminal 3'-OH group of denatured DNA [27], even after fixation and embedding of the biological material in plastic [28]. Adapting this approach for use at the ultrastructural level, Fakan and Modak [29] observed specific labeling, principally of interphase nuclei, nucleolar regions, mitotic chromosomes, and mitochondria. This autoradiographic method is compatible with all fixation and embedding procedures but do not provide high resolution.

Here we describe a method providing a very useful tool for pinpointing the precise location of DNA in situ within various biological materials at the ultrastructural level [30–32]. The technique includes two successive steps: an enzymatic reaction followed by an immuno-cytochemical approach; the labeled nucleotides added by TdT on ultrathin sections are then visualized by an indirect immunogold labeling technique involving either an anti-biotin antibody or a monoclonal anti-bromodeoxyuridine antibody and a second, colloidal-gold-coupled antibody [30, 33]. This technique is compatible with plenty of fixation and embedding procedures and can be combined with cytochemical methods [31, 32]. Moreover, this technique can be applied to a wide variety of biological materials allowing a sensitive detection of DNA-containing structures, even where these are present in very low amounts [30, 34].

2 Materials

Prepare all solutions using boiled bidistilled water and analytical grade reagents. Prepare and store all reagents at room temperature (RT), unless indicated otherwise. Diligently follow all waste disposal regulations when disposing of waste materials.

Any biological material is suitable for the TdT DNA labeling method (*see* **Note 1**).

2.1 Reagents and Tools

1. Fixative for electron microscopy (*see* **Note 2**): 2.5 % (vol/vol) glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4). Store at 4 °C, stable for 1–2 month.
2. Post-fixation solution: 1 % osmium tetroxide in 100 mM sodium cacodylate buffer (pH 7.4). Store at 4 °C, stable for up to 6 months.
3. Phosphate buffered saline (10× PBS): 1.4 M NaCl, 60 mM Na₂HPO₄, 40 mM KH₂PO₄. Stable at 4 °C for up to 1 year.
4. Graded series of ethanol solutions.
5. Embedding resin (LX 112 resin, Epon 812 replacement) (*see* **Note 3**): Mix in a 25-ml Erlenmeyer flask 5 g of dodecyl succinic anhydride (DDSA) with 5 g of methyl nadic

anhydride, 0.3 g of 2,4,6- dimethylaminomethyl phenol (DMP-30) and 10 g of LX 112; close the Erlenmeyer flask, stir until the appearance of bubbles and let the mixture rest until the complete disappearance of bubbles. Store at RT, stable for 2–5 days.

6. Graded series of ethanol 100%:LX 112 solutions.
7. 50% (wt/vol) ethanolic uranyl acetate: 0.5 g of uranyl acetate, 12.5 ml of boiled H₂O, 12.5 ml of ethanol. Store at 4 °C in a brown glass container or otherwise protected from direct light, stable at 4 °C for up to 1 year, filter (0.22 µm pore size) before use.
8. Aqueous lead citrate: 4.2% (wt/vol) sodium citrate, 2.6% (wt/vol) lead nitrate, add concentrated NaOH until clearing up of mixture. Stable at 4 °C for up to 1 year, filter (0.22 µm pore size) before use.
9. Media for Terminal transferase reaction (TdT medium a and b):
 - (a) 20 µM 5 bromo-2-deoxyuridine triphosphate, 100 mM sodium cacodylate (pH 6.5), 2 mM CoCl₂, 10 mM β-mercaptoethanol, 50 µg/ml bovine serum albumin (BSA), and 125 U/ml calf thymus TdT.
 - (b) TdT medium a, supplemented with 4 µM each of dCTP, dGTP, and dATP.
10. Saturated solution of sodium metaperiodate.
11. Mouse monoclonal anti-bromodeoxyuridine antibody (Roche).
12. Goat anti-mouse IgG coupled to gold particles 10 nm in diameter (Amersham Biosciences).
13. Platinum wire (0.1 mm in diameter).
14. 200 or 300-mesh nickel grids.
15. Anticapillary forceps style 7 or style 5.

3 Methods

Timing: Preparation for electron microscopy (Subheading 3.1, steps 1–10): 5–6 days; Terminal transferase reaction and immunogold labeling (Subheading 3.2, steps 1–12): 8–9 h; Staining of sections (Subheading 3.3, steps 1–6): 30 min.

3.1 Preparation for Electron Microscopy

1. Fix small fragments of biological material for 60 min at 4 °C (*see* Note 1).
2. Eliminate the fixative with a pipette and wash three times with sodium cacodylate buffer (pH 7.4) during 10 min at 4 °C.
3. Postfix the biological samples with post-fixation solution for 60 min at 4 °C.

4. Eliminate the post-fixative with a pipette and wash three times with distilled water during 10 min at 4 °C.
5. Replace the distilled water by a graded series of ethanol solutions for dehydrating the biological samples: ethanol 70% (vol/vol), 4 °C, 15 min; ethanol 95% (vol/vol), RT, 5 min; ethanol 100% (vol/vol), RT, 3×20 min.
6. Eliminate the solvent with a pipette and impregnate progressively the biological samples at RT in a graded series of alcoholic LX 112 solutions as follows: ethanol 100%:LX 112, 2:1, 60 min; ethanol 100%:LX 112, 1:1, 60 min; ethanol 100%:LX 112, 1:2, 60 min; pure LX 112, overnight.
7. Embed the biological samples in fresh LX 112 .
8. Polymerize resin for 3–4 days in an incubator at 42 °C (*see Note 4*).
9. Cut ultrathin sections of LX 112-embedded samples on an ultramicrotome with a diamond knife at 60–90 nm.
10. Collect ultrathin sections with a platinum ring (4 mm diameter) formed by a platinum wire and store them on distilled water (*see Note 5*).

3.2 Terminal Transferase Reaction and Immunogold Labeling

1. Incubate ultrathin sections for 30 min at 37 °C on the surface of TdT medium a (*see Note 6*).
2. Incubate the sections for 30 min at 37 °C on the surface of TdT medium b (*see Note 7*).
3. Transfer the sections for 60 min on a saturated solution of sodium metaperiodate at RT (*see Note 8*).
4. Rinse the sections two times with bidistilled water during 10 min at RT.
5. Incubate the sections for 30 min with PBS containing normal goat serum (NGS) diluted 1/30 and 1% BSA at RT.
6. Rinse the sections with PBS containing 1% BSA during 10 min at RT.
7. Incubate the sections for 4 h at RT in monoclonal anti-BUdR antibody diluted 1/50 in PBS containing 0.2% BSA and NGS diluted 1/50 (*see Note 9*).
8. Rinse the sections three times with PBS containing 1% BSA during 10 min at RT.
9. Incubate the sections for 1 h at RT in goat anti-mouse IgG coupled to gold particles diluted 1/40 with PBS (pH 8.2) containing 0.2% BSA.
10. Rinse the sections with PBS containing 1% BSA during 10 min at RT, then rinse them again two times with bidistilled water during 10 min at RT.

11. Depose ultrathin sections on 200- to 300-mesh nickel grids coated with collodion film (*see* **Note 10**).
12. Dry grids on filter paper (*see* **Note 11**).

3.3 Staining of Sections

1. Transfer grids into a petri dish with reduced CO₂ concentration (sodium hydroxide pellets in a petri dish). Incubate for 5 min at RT in darkness, on drops (20 μ l) of 50% ethanolic uranyl acetate (*see* **Note 12**).
2. Rinse grids in three 25-ml beakers filled with bidistilled water (*see* **Note 13**).
3. Dry grids on filter paper.
4. Transfer grids into a petri dish with reduced CO₂ concentration (sodium hydroxide pellets in a petri dish) for 5 min at RT, on drops (20 μ l) of aqueous lead citrate (*see* **Note 12**).
5. Rinse grids in three 25-ml beakers filled with bidistilled water (*see* **Note 13**).
6. Dry grids on filter paper (*see* **Note 11**).
7. Examine ultrathin sections in a transmission electron microscope at 60–80 kV (*see* Fig. 1).

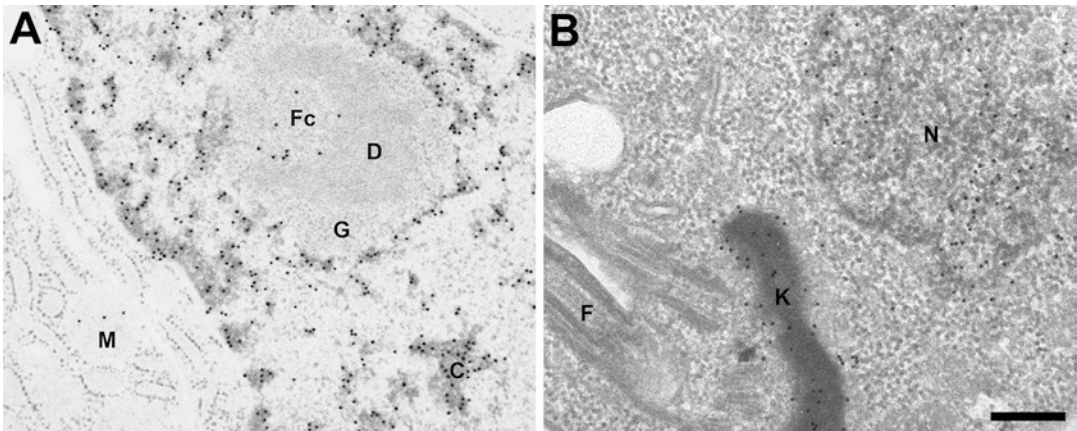


Fig. 1 Location of DNA in the lizard, *Jalapura splendida* (a), and in the symbiont-containing trypanosomatid *Blastocrithidia culicis* (b) using the TdT DNA labeling technique. An acetylation method was applied to increase the contrast of condensed chromatin blocks (c) and between various nucleolar components. *D* dense fibrillar component, *F* flagellum, *Fc* fibrillar center, *G* granular component, *K* kinetoplast, *M* mitochondria, *N* nucleus. Bar represents 0.5 μ m

4 Notes

1. The technique can be applied to a wide variety of biological materials. *See* ref. 31, 34 for examples of high resolution and sensitive detection include the DNA present in mitochondria, chloroplasts, mycoplasmas, DNA viruses and nucleoli of various animal cell lines or tissues.
2. Other fixatives and buffers can be used. For example: 4 % formaldehyde, 0.2–2.5 % glutaraldehyde, 2.5 % glutaraldehyde/2 % osmium tetroxide, 5 % acrolein, or 4 % formaldehyde, 0.1% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4) (*see* ref. 30)
3. Other plastic embedding procedure such as Lowicryl K4M (*see* ref. 35) or LR White using the technique of Newman and Jasani (*see* ref. 36) can also be used.
4. Polymerized resin can be stored at RT for several months in a closed dish.
5. **Steps 10–20** are performed on drops of medium placed on parafilm in moist petri dish; a platinum ring is used to move the ultrathin sections on a drop of medium to another. Always keep the same face of the sections in contact with the medium. Do not let the sections dry.
6. First control experiments are carried out: TdT and/or labeled nucleotides are omitted from the TdT medium.
7. This step is indispensable because it has been demonstrated that the anti-BUdR antibody weakly detects BUdR on single-stranded tails of DNA (*see* ref. 37).
8. This step is used to increase the hydrophilicity of LX 112, allowing to obtain more intense labeling.
9. Second control experiment is carried out: the monoclonal anti-BUdR antibody is omitted.
10. The coating of grids with a collodion film is important to avoid the detachment of sections.
11. The grids can be stored at RT for several months on filter paper placed in a closed petri dish.
12. **Steps 23 and 26** are performed on drops of medium placed on parafilm in moist petri dish; the face of the grid with ultrathin sections is floated or incubated on the drops of the medium. Before each incubation of grids on drops, the edge of grids and the face of grids devoid of ultrathin sections have to be blotted on filter paper to avoid dipping of grids.
13. The washing procedure is performed in beakers by agitating the grids for 10 s using anticapillary forceps.

References

1. Moyne G (1980) Methods in ultrastructural cytochemistry of the cell nucleus. *Prog Histochem Cytochem* 13:1–72
2. Gautier A (1976) Ultrastructural localization of DNA in ultrathin tissue sections. *Int Rev Cytol* 44:113–191
3. Cogliati R, Gautier A (1973) Mise en évidence de l'ADN et des polysaccharides à l'aide d'un nouveau réactif "de type Schiff". *C R Acad Sci* 276:3041–3044
4. Derenzini M, Viron A, Puvion-Dutilleul F (1982) The Feulgen-like osmium—amine reaction as a tool to investigate chromatin structure in thin sections. *J Ultrastruct Res* 80:133–147
5. Olins AL, Moyer BA, Kim SH, Allison DP (1989) Synthesis of a more stable osmium ammine electron-dense DNA stain. *J Histochem Cytochem* 37:395–398
6. Masiello I, Biggiogera M (2017) Osmium ammine for staining DNA at electron microscopy. In: Pellicciari C, Biggiogera M (eds) *Histochemistry of single molecules*. *Methods Mol Biol* 1560:261–267
7. Bendayan M (1981) Ultrastructural localization of nucleic acids by the use of enzyme-gold complexes. *J Histochem Cytochem* 29:531–541
8. Bendayan M (1982) Ultrastructural localization of nucleic acids by the use of enzyme-gold complexes: influence of fixation and embedding. *Biol Cell* 43:153
9. Raska I, Armbruster BL, Jira M et al (1984) Ultrastructural localization of DNA in tissue culture cell nuclei by means of enzyme-gold and autoimmune sera-protein A-gold techniques. *Biol Cell* 48:211–214
10. Hansmann P, Falk H, Scheer U, Sitte P (1986) Ultrastructural localization of DNA in two cryptomonas species by use of a monoclonal DNA antibody. *Eur J Cell Biol* 42:152–160
11. Scheer U, Messner K, Hazan R et al (1987) High sensitivity immunolocalization of double and single-stranded DNA by a monoclonal antibody. *Eur J Cell Biol* 43:358–371
12. Thiry M, Scheer U, Goessens G (1988) Localization of DNA within Ehrlich tumour cell nucleoli by immunoelectron microscopy. *Biol Cell* 63:27–34
13. Thiry M, Dombrowicz D (1988) Anti-bromodeoxyuridine monoclonal antibody: an alternative tool for the identification of replicated DNA at the electron microscope level. *Biol Cell* 62:99–102
14. Thiry M (1988) Immunoelectron microscope localization of bromodeoxyuridine incorporated into DNA of Ehrlich tumor cell nucleoli. *Exp Cell Res* 179:204–213
15. Chang LM, Bollum FJ (1986) Molecular biology of terminal transferase. *CRC Crit Rev Biochem* 21:27–52
16. Bollum FJFJ (1974) Terminal deoxynucleotidyl transferase enzyme. Elsevier, New York, NY, pp 145–171
17. Baltimore D (1974) Is terminal deoxynucleotidyl transferase a somatic mutagen in lymphocytes? *Nature* 248:409–411
18. Desiderio SV, Yancopoulos GD, Paskind M et al (1984) Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* 311:752–755
19. Motea EA, Berdis AJ (2010) Terminal deoxynucleotidyl transferase: the story of a misguided DNA polymerase. *Biochim Biophys Acta* 1804:1151–1166. doi:10.1016/j.bbapap.2009.06.030
20. Bertocci B, De Smet A, Weill J-C, Reynaud C-A (2006) Nonoverlapping functions of DNA polymerases mu, lambda, and terminal deoxynucleotidyltransferase during immunoglobulin V(D)J recombination in vivo. *Immunity* 25:31–41. doi:10.1016/j.immuni.2006.04.013
21. Kepler TB, Borrero M, Rugerio B et al (1996) Interdependence of N nucleotide addition and recombination site choice in V(D)J rearrangement. *J Immunol* 157:4451–4457
22. Komori T, Okada A, Stewart V, Alt FW (1993) Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. *Science* 261:1171–1175
23. Kunkel TA, Gopinathan KP, Dube DK et al (1986) Rearrangements of DNA mediated by terminal transferase. *Proc Natl Acad Sci U S A* 83:1867–1871
24. Deng G, Wu R (1983) Terminal transferase: use of the tailing of DNA and for in vitro mutagenesis. *Methods Enzymol* 100:96–116
25. Michelson AM, Orkin SH (1982) Characterization of the homopolymer tailing reaction catalyzed by terminal deoxynucleotidyl transferase. Implications for the cloning of cDNA. *J Biol Chem* 257:14773–14782
26. Roychoudhury R, Jay E, Wu R (1976) Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. *Nucleic Acids Res* 3:101–116

27. Modak SP, Bollum FJ (1970) Terminal lens cell differentiation: III. Initiator activity of DNA during nuclear degeneration. *Exp Cell Res* 62:421–432
28. Modak SP, Donnelly GM, Karasaki S et al (1973) An enzymatic assay for DNA in epoxy-tissue-sections by light microscope-autoradiography. *Exp Cell Res* 76:218–222
29. Fakan S, Modak SP (1973) Localization of DNA in ultrathin tissue sections incubated with terminal deoxynucleotidyl transferase, as visualized by electronmicroscope autoradiography. *Exp Cell Res* 77:95–104
30. Thiry M (1992) Highly sensitive immunodetection of DNA on sections with exogenous terminal deoxynucleotidyl transferase and non-isotopic nucleotide analogues. *J Histochem Cytochem* 40:411–419
31. Thiry M, Ploton D, Menager M, Goessens G (1993) Ultrastructural distribution of DNA within the nucleolus of various animal cell lines or tissues revealed by terminal deoxynucleotidyl transferase. *Cell Tissue Res* 271:33–45. doi:[10.1007/BF00297539](https://doi.org/10.1007/BF00297539)
32. Lamaye F, Galliot S, Alibardi L et al (2011) Nucleolar structure across evolution: the transition between bi- and tri-compartmentalized nucleoli lies within the class Reptilia. *J Struct Biol* 174:352–359. doi:[10.1016/j.jsb.2011.02.003](https://doi.org/10.1016/j.jsb.2011.02.003)
33. Thiry M (1995) Nucleic acid compartmentalization within the cell nucleus by in situ transferase-immunogold techniques. *Microsc Res Tech* 31: 4–21. doi:[10.1002/jemt.1070310103](https://doi.org/10.1002/jemt.1070310103)
34. Thiry M (1992) Ultrastructural detection of DNA within the nucleolus by sensitive molecular immunocytochemistry. *Exp Cell Res* 200:135–144. doi:[10.1016/S0014-4827\(05\)80081-3](https://doi.org/10.1016/S0014-4827(05)80081-3)
35. Roth J, Bendayan M, Carlemalm E et al (1981) Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J Histochem Cytochem* 29:663–671. doi:[10.1177/29.5.6166664](https://doi.org/10.1177/29.5.6166664)
36. Newman GR, Jasani B (1984) Post embedding immunoenzyme techniques. In: Polak JM, Varndell IM (eds) *Immuno-labelling for electron microscopy*. Elsevier, New York, NY, pp 53–70
37. Kitazawa S, Takenaka A, Abe N et al (1989) In situ DNA-RNA hybridization using in vivo bromodeoxyuridine-labeled DNA probe. *Histochemistry* 92:195–199

Chapter 21

Visualizing RNA at Electron Microscopy by Terbium Citrate

Marco Biggiogera and Irene Masiello

Abstract

Although the EDTA regressive technique allows the visualization of RNPs, this widely used method is not intended to be specific for RNA alone. A fine ultrastructural visualization of RNA on ultrathin sections can be obtained with terbium citrate: this method gives a weak contrast but a very fine end product which allows observations at a high resolution level.

The procedure is very simple since it consists only of a period of incubation and very short washes, which are the crucial point of this technique to avoid the Tb removal from RNA. This method does not require any special type of fixation and embedding.

Key words RNA staining, Terbium citrate, Cytochemistry, Electron microscopy, EDTA regressive stain, Lanthanides

1 Introduction

Selective RNA staining at electron microscopy (EM) has been pursued for a long time in the absence of a specific method: while for DNA a Feulgen-type reaction existed as a selective and reliable cytochemical procedure, no specific and selective procedure was present for RNA, not even at light microscopy level.

The most commonly used approach was the EDTA regressive staining for ribonucleoproteins [1] which, as clearly stated by the author, was never intended for RNA alone. Some techniques were proposed for the detection of both RNA and DNA [2–4]. By applying these methods after the enzymatic removal of DNA, it was possible to recognize only RNA on thin sections. In other papers, RNA was detected by RNase–gold complexes [5], anti-RNA antibodies or after the incorporation of labeled RNA precursors. Platinum quinolone was proposed [6] but this approach has shown several limitations.

The basic reason for this problem in revealing RNA lies in the absence of any chemical group characteristic of RNA: the hydroxyl group of the sugar in the DNA structure makes the difference for

the reactivity toward hydrolysis while for RNA no such specific markers are present.

Since data in literature described that lanthanide elements interact with guanine sites at single-stranded nucleic acids [7, 8], a series of experiments was carried out with lanthanides, a family of strictly correlated chemical elements called rare earths. In trying to find a reliable method for RNA, we have examined 134 different procedures to bind an electron dense element as a marker of RNA. Many of these elements were found to be useful in staining RNA (terbium, ytterbium, europium, lutetium) but the most useful for electron microscopy proved to be terbium (Tb): its chloride or nitrate form almost does not give rise to staining. For this reason we applied a substitution reaction [9], as in the case of lead citrate [10].

Terbium is capable of binding the RNA guanosine [7] with an efficiency of about 16,000 fold with respect to DNA guanosine [11]. The binding occurs usually within 30–60 min and reaches saturation: therefore, overnight staining does not improve contrast (unpublished results) and overstaining or unspecific staining is avoided. On the other hand, pH proved to be critical: application at pH below 7.5 does not result in staining. This staining method can be applied on acrylic and epoxy sections, although with two different procedures, and it is not dependent on the type of fixation (except for osmium); it can be used after embedding, immunocytochemistry and EM in situ hybridization (EMISH).

This technique shows a weak contrast, even if the stained structures appear more contrasted after a few minutes of eye adaptation but the staining is very fine allowing studies at high resolution. With this approach perichromatin fibrils (PFs), perichromatin granules (PGs), interchromatin granules (IGs) and the nucleolus are contrasted in the nucleus while in the cytoplasm only ribosomes are stained. Actually, it is possible to see single RNA molecules within PGs and ribosomes.

As control, enzymatic digestions are carried out: DNase or pronase do not modify the staining, confirming that DNA and proteins do not interfere with the result; RNase and Nuclease S1 digestions prevent the formation of an electron-dense product, indicating that the staining depends on single strand RNA [9].

2 Materials

2.1 Sample Preparation

All kind of cells or tissues can be processed for EM to reveal specifically RNA through Tb-staining.

2.1.1 Reagents

1. 67 mM Sørensen buffer pH 7.4: dissolve 11.88 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 1 L of distilled water (Solution A); dissolve 9.08 g of KH_2PO_4 in 1 L of distilled water (Solution B); mix 81.8 mL of Solution A and 18.2 mL of Solution B.

2. 4% paraformaldehyde in Sörensen buffer pH 7.4; store at 4 °C for 1–2 months (*see* **Note 1**).
3. 2.5% glutaraldehyde in Sörensen buffer pH 7.4; store at 4 °C for 1–2 months (*see* **Note 1**).
4. 2% agar (necessary for pre-embedding isolated cells): dissolve 1 g of agarose in 50 mL of warm distilled water and store at 4 °C.
5. Graded ethanol or acetone in distilled water: 30%, 50%, 70%, 90% and absolute ethanol or acetone; store at room temperature.
6. LRWhite resin: the activation of LRWhite resin is obtained by adding 9.9 g of benzoyl peroxide in 500 mL of resin under gentle agitation by magnetic stirrer (*see* **Note 2**); store at 4 °C.
7. EMbed-812 resin: mix under gentle agitation by magnetic stirrer (*see* **Note 2**) 20 g of EMbed-812, 16 g of dodecenyl succinic anhydride and 8 g of nadic methyl anhydride and finally add 1.2 mL of benzyl-dimethylamine under a fume hood; let the reagent mix for at least 30 min (*see* **Note 2**) and store it at –20 °C (thaw the resin for at least 20 min before use).

2.2 Preparation of Terbium Citrate

1. Freshly double-distilled or ultrapure water.
2. 1 N sodium hydroxide: dissolve 4 g of NaOH in 100 mL of distilled water.
3. 0.2 M terbium (III) nitrate: dissolve 435 mg of terbium (III) nitrate in 5 mL of double-distilled or ultrapure water.
4. 0.5 M sodium citrate buffer (for epoxy sections): dissolve 735 mg of trisodium citrate dihydrated in 5 mL of double-distilled or ultrapure water; adjust the pH to 12 with 1 N NaOH.
5. 0.2 M sodium citrate buffer: dissolve 294 mg of trisodium citrate dihydrated in 5 mL of double-distilled or ultrapure water.
6. Terbium citrate: stirring continuously, 0.2 M terbium nitrate must be added dropwise to the 0.2 M sodium citrate—a white precipitate is formed. The precipitate tends to dissolve at the beginning and afterwards forms coarse precipitates and floculates. Add then 1 N NaOH dropwise while gently stirring until the precipitates dissolve completely (around pH 6.7–7.0): the solution must become transparent. Finally, adjust the pH to 8.2–8.5 with 1 N NaOH. Control the pH after 24 h to readjust it if necessary (*see* **Note 3**). Store the solution in a syringe fitted with a filter (*see* below): it is stable for several weeks at room temperature.

3 Equipment

1. 0.22 μm pore filter.
2. Plastic syringe and pipettes.
3. Thin sections obtained using an ultramicrotome.
4. 200 mesh gold, copper or nickel grids to collect the thin sections.
5. Parafilm sheet to be used as a support for the staining procedure.
6. Filter paper to dry the grids where indicated.
7. Transmission electron microscope to observe the stained sections.

4 Methods

4.1 Sample Processing

The samples can be fixed either with 4% paraformaldehyde in Sörensen buffer for 2 h at 4 °C or in 2.5% glutaraldehyde in Sörensen buffer for 2 h at room temperature. After the removal of fixative with a pipette, the specimens are rinsed thoroughly with Sörensen buffer first and then with PBS.

Pre-embedding in 2% agar in H_2O is only necessary when working with cells in suspension. Dehydration in graded ethanol or acetone follows and the samples are respectively embedded either in LRWhite or Epon resin.

Thin sections are collected on nickel, copper or gold grids (*see* **Note 4**).

4.2 Staining Procedure

4.2.1 For Epoxy Sections

1. Place a 30 μL drop of 0.5 M sodium citrate pH 12 on the Parafilm sheet.
2. Float the grid (sections facing down) on the drop of sodium citrate for 1 h at room temperature (*see* **Note 5**).
3. Remove the excess of solution by blotting quickly the grid with a filter paper: do not allow to dry.
4. Put a 50 μL drop of terbium citrate on the support.
5. Incubate the specimen (sections facing down) for 1 h at room temperature on terbium citrate for the staining. In the meantime, prepare two large drops of distilled water (100–150 μL). Do not dry the grid with filter paper, so move the sample on the first drop of water for 30 s and then on the other for 15 s without blotting the grid between the two steps (*see* **Note 6**).
6. Dry the grid (*see* **Note 7**).
7. Observe the stained sections with an electron microscope operating at 80 kV (*see* **Note 8**).

4.2.2 For Acrylic Sections

1. Put a 50 μL drop of terbium citrate on the support.
2. Stain the specimen with terbium citrate for 30 min at room temperature (sections facing down).
3. In the meantime, prepare two drops of 100–150 μL of distilled water.
4. Without blotting the grid after terbium staining, rinse it on the first drop for 10 s and on the other for 5—do not blot the grid between two washes (*see* **Note 6**).
5. Dry the grid (*see* **Note 7**).
6. Observe the stained sections with an electron microscope operating at 80 kV (*see* Fig. 1) (*see* **Note 8**).

5 Notes

1. Check the possible presence of precipitates in the fixative solutions: in this case, discard and prepare a fresh fixative.
2. Avoid the formation of bubbles in the resin mixture since they may remain during resin polymerization not allowing the preparation of good sections.
3. At pH lower than 8.0, precipitates are formed within 2–3 days. Therefore, it is very important to check the pH after 24 h and the possible presence of precipitates before the use. The use of a syringe fitted with a filter can help to remove particles.
4. LRWhite sections have a higher inherent contrast, even if Epon sections give good staining results since chromatin is devoid of contrast. The staining can be performed on nickel, copper or gold grids, equally. It is recommended to use thinner sections (with silver-to-grey interference color) without formvar support which can decrease the contrast. An amorphous carbon-film is advisable. The idea is to ameliorate the signal-to-noise ratio in order to enhance contrast since Tb staining is a very low contrast technique. Marinozzi rings are generally not used for this cytochemical staining.
5. Treatment with alkaline sodium citrate is necessary to avoid the overnight incubation in terbium citrate using epoxy sections.
6. The critical step of this procedure is rinsing: the Tb-RNA bond is weak and a long rinsing will remove the staining. For the same reason, it is recommended not to blot the grid after terbium staining or during the short washes to avoid Tb removal from RNA. It was tested that an improvement in the retention of staining can be achieved adjusting the water pH to 9–10 using 0.1 N NaOH: in these conditions, the staining appears to be more stable and contrasted (unpublished data).

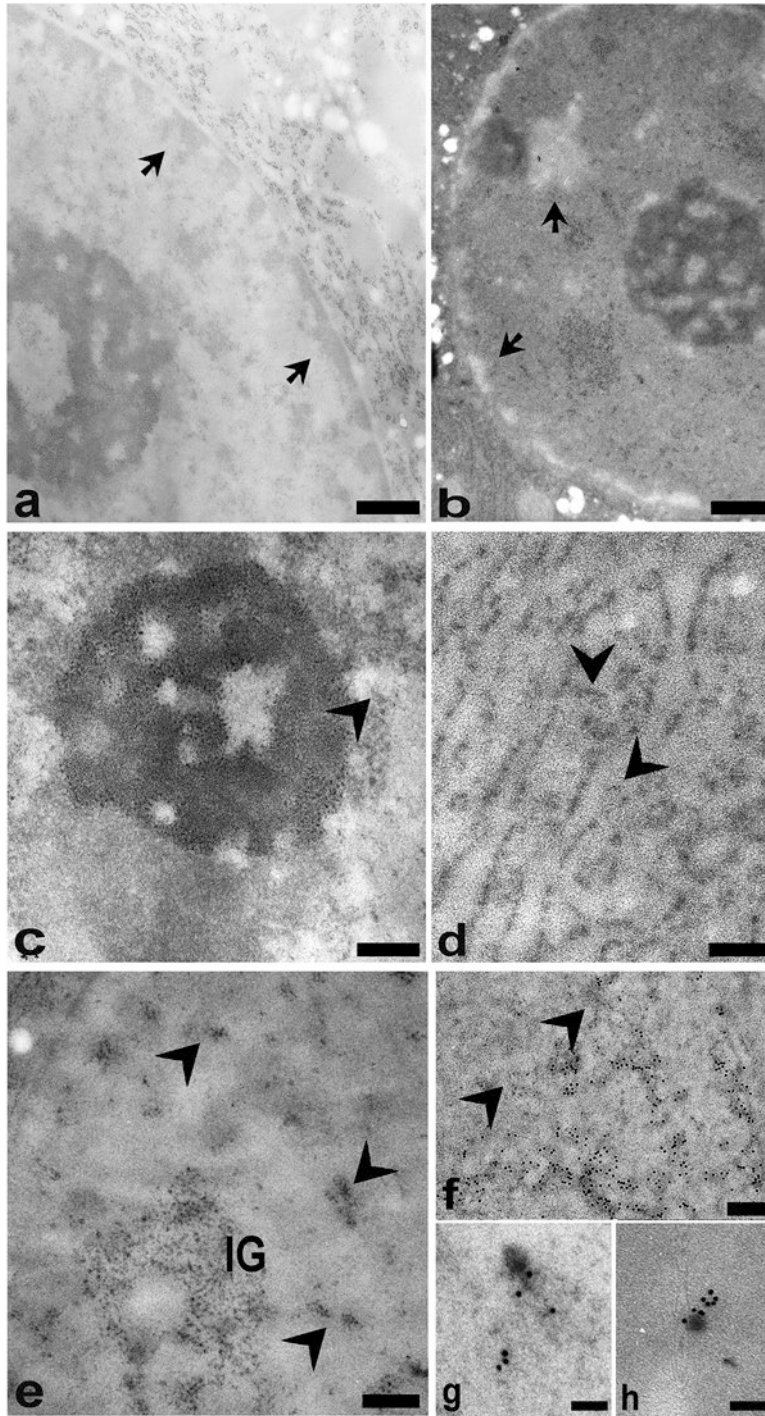


Fig. 1 RNA staining. Panel (a) rat liver, glutaraldehyde fixation, LR White embedding. The nucleolus and ribosomes are stained while the peripheral condensed chromatin (arrows) exhibits only inherent contrast. Bar = 500 nm. (b) as in (a) but Epon embedding. Note the unstained chromatin areas (arrows). Bar = 500 nm. (c) a nucleolus with an evident granular component. Bar = 250 nm. (d) ribosomes with single RNA molecules interspersed (arrowheads). Bar = 250 nm. (e) a cluster of interchromatin granules (IGs) and perichromatin fibrils (arrowheads). Bar = 250 nm. (f) anti-DNA immunolabelling followed by Tb staining. Note the perichromatin fibrils (arrowheads) emerging from labeled DNA areas. Bar = 250 nm. (g, h) *Chironomus thummi* salivary glands; Balbiani ring granules with the tail labeled by a poly d(T) probe. Bar = 100 nm

7. A post staining with lead citrate for 5 s can increase the contrast but it determines a complete loss of specificity.
8. During the direct observation, the unstained chromatin areas (which are white) help the localization of the cells. In TEM equipped with a digital image capture system the contrast enhancement will help in localizing the stained molecules. Although the contrast is weak, the negatives and printed micrographs are satisfactory. Grids stained with Tb do not fade. To our experience, sections stained under these conditions can still be observed after 10–15 years.

Acknowledgments

The authors are grateful to Ms. Francine Flach and Prof. Stan Fakan. Part of this work has been carried out at the CME, University of Lausanne, Switzerland.

This chapter is dedicated to the memory of Václav Kopečný and Jean-Luc Courtenis.

References

1. Bernhard M (1969) A new staining procedure for electron microscopical cytology. *J Ultrastruct Res* 27:250–265
2. Chan-Curtis V, Belt VD, Ladoulis CT (1970) Cytochemical localization of nucleic acids by acriflavine-phosphotungstate complex for fluorescence microscopy and electron microscopy. *J Histochem Cytochem* 18:609–627
3. Biggiogera M, Flach Biggiogera F (1989) Ethidium bromide- and propidium iodine-PTA staining of nucleic acids at electron microscopic level. *J Histochem Cytochem* 37:1161–1166
4. Derenzini M, Farabegoli F (1990) Selective staining of nucleic acids by osmium ammine complex in thin sections from Lowicryl-embedded samples. *J Histochem Cytochem* 28:1495–1501
5. Bendayan M (1982) Ultrastructural localization of nucleic acids by the use of enzyme-gold complexes: influence of fixation and embedding. *Bio Cell* 43:153–156
6. Scott JE (1973) Affinity, competition and specific interactions in the biochemistry and histochemistry of polyelectrolytes. *Biochem Soc Trans* 1:787–806
7. Ringer DP, Burchett S, Kizer DP (1978) Use of terbium fluorescence enhancement to selectively monitor DNA and RNA guanine residues and their alteration by chemical modification. *Biochemistry* 17:4818–4825
8. Hörer OL, Zaharia CN, Marcu A (1977) Terbium fluorescence in aqueous solutions of nucleic acids. *Rev Roum Biochim* 14:175–179
9. Biggiogera M, Fakan S (1998) Fine structural specific visualization of RNA on ultrathin sections. *J Histochem Cytochem* 46:389–395
10. Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* 17:208–212
11. Ringer DP, Howell BA, Kizer DE (1980) Use of terbium fluorescence enhancement as a new probe for assessing the single-strand content of DNA. *Anal Biochem* 103:337–342

Two-Tailed Comet Assay (2T-Comet): Simultaneous Detection of DNA Single and Double Strand Breaks

Elva I. Cortés-Gutiérrez, José Luis Fernández,
Martha I. Dávila-Rodríguez, Carmen López-Fernández,
and Jaime Gosálvez

Abstract

A modification of the original comet assay was developed for the simultaneous evaluation of DNA single strand breaks (SSBs) and double strand breaks (DSBs) in human spermatozoa. The two-dimensional perpendicular tail comet assay (2T-comet) combines non-denaturing and denaturant conditions to the same sperm nucleoid. In this case, the species-specific deproteinized sperm is first subjected to an electrophoretic field under non-denaturing conditions to mobilize isolated free discrete DNA fragments produced from DSBs; this is then followed by a second electrophoresis running perpendicular to the first one but under alkaline conditions to produce DNA denaturation, exposing SSBs on the same linear DNA chain or DNA fragments flanked by DSBs. This procedure results in a two dimensional comet tail emerging from the core where two types of original DNA affected molecule can be simultaneously discriminated. The 2T-comet is a fast, sensitive, and reliable procedure to distinguish between single and double strand DNA damage within the same cell. It is an innovative method for assessing sperm DNA integrity, which has important implications for human fertility and andrological pathology. This technique may be adapted to assess different DNA break types in other species and other cell types.

Key words Two-tailed comet assay (2T-Comet), DNA fragmentation, Single cell gel electrophoresis (SCGE), Single-stranded DNA breaks (SSB), Double-stranded DNA breaks (DSB), Spermatozoa

1 Introduction

The single cell gel electrophoresis (SCGE) or comet assay, developed by Ostling and Johanson [1], combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and cross linking with the single cell approach typical of cytogenetic assays.

The principle of the comet assay is based on alterations found in DNA such as strand breaks resulting in the extension of DNA loops from lysed and salt extracted nuclei, which, in turn, form a

comet-like tail after either alkaline electrophoresis, indicating global DNA damage simple (SSBs) plus double strand breaks (DSBs), or neutral electrophoresis, indicating DSBs only [2]. This technique detects both: SSB and DSB, without distinguishing between the two [3].

It is known that SSBs can be induced by a number of agents, such as ultraviolet light, reactive oxygen species, and many mutagens, and that they can be easily and rapidly repaired, but do not pose a major threat to genomic integrity. By contrast, DSBs represent the most dangerous damage inflicted by mutagens, because they are potent inducers of mutations and chromosomal abnormalities [4].

The implication of sperm DNA damage in fertilization and embryo development depends on the type of DNA lesions and the oocyte's repair capacity [5, 6]. Therefore, the differentiation of the types and levels of DNA damage that may coexist in the different sperm, provide relevant information to the study of human fertility and andrological pathology.

A modification of the original comet assay was developed for the simultaneous evaluation of DNA SSBs and DSBs in human spermatozoa. The 2T-Comet assay is a fast, sensitive, and reliable procedure for the quantification and characterization of DNA damage in spermatozoa [7, 8].

A general view of the technique is provided, emphasizing its versatility for assessing sperm DNA integrity in human [7, 8]. This technique may be adapted to assess different DNA break types in other species (eutherian and metatherian) [8–11], and other cell types as cumulus cells [12].

2 Materials

2.1 Reagents

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18.2 MΩ cm at 25 °C) and analytical grade reagents.

1. Low melting point agarose solution: A solution of 1% (w/v) low-melting point agarose is prepared by melting 1 g of low-melting point agarose in 100 mL of water and microwaving for five times 10 s. The homogeneous solution is then dispensed into 15 mL sterile conical tubes and stored at 4 °C until use.
2. Standard agarose solution: Standard agarose was prepared as described above for low-melting point agarose.
3. Lysing solution I [0.4 M Tris-HCl, 0.8 M dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS), pH 7.5].
4. Lysing solution II (0.4 M Tris-HCl, 2 M NaCl, 1% SDS, 0.05 M EDTA, pH 7.5). Stored at 4 °C. Add about 50 mL of water to a 100-mL beaker and dissolve with agitation.

5. Alkaline unwinding solution: 0.03 M NaOH, 1.0 M NaCl, pH 12.5, freshly prepared and kept cold at 4 °C. Add 50 mL of water to a 100-mL beaker and dissolve with agitation. It is generally used at room temperature.
6. Neutralizing solution: 0.4 M Tris-HCl, pH 7.5, in distilled water up to 100 mL and dissolve with agitation. Stored at 4 °C, used at room temperature.
7. Tris-Borate-EDTA buffer (TBE): 0.09 M Tris-borate, 0.002 M EDTA disodium salt, pH 7.5, stored at room temperature. Add 1000 mL of water to a 1000 mL beaker and dissolve with agitation. Store at room temperature.
8. Analytical-grade ethanol solutions in distilled water.
9. Propidium iodide (1 µg/mL) for this, a 1.0 mg/mL stock is prepared in Vectashield mounting medium and stored protected from light at 4 °C. Solution stable for at least 6 months.
10. Vectashield mounting medium (Vector Laboratories).

2.2 Equipment

1. Disposable plastic tubes (5 mL).
2. Water baths for melting agarose at 100 °C and maintaining agarose at 37 °C.
3. Hemocytometer or electronic particle counter for adjusting cell numbers.
4. Frosted-end microscope slides (*see Note 1*).
5. Diamond-tipped pen for scoring slides.
6. Covered glass containers for slide lysis, staining, and storage.
7. Microscopy glass slides (24 mm × 72 mm) and glass coverslips (24 mm × 24 mm) (*see Note 2*).
8. Large-bed horizontal gel electrophoresis chamber and power supply.
9. Epifluorescence microscope with appropriate filters (Texas red, FITC, DAPI) and high-power objectives (40, 60, 63, or 100×).
10. High-sensitivity CCD digital camera.
11. Computer and comet analysis software. Commercial systems (e.g., Visilog 5.1, Noesis France) or free software available on the Internet (*see* <http://cometassay.com>.) are appropriate.

3 Methods

3.1 Sample Preparation

Sperm cells were diluted to a concentration of 10×10^6 spermatozoa/mL in phosphate-buffered saline (PBS). Next, 25 µL of the cell suspension was mixed at 37 °C with 50 µL of freshly prepared 1 % low melting point agarose in water (*see Note 3*).

3.2 Preparation of Slides with Microgel Cell Suspensions

1. 1 % low melting point agarose in distilled water, within a glass tube, is dissolved by microwave heating and then kept in a water bath at 37 °C for 5 min at least, to achieve this temperature.
2. Single cells in suspension are gently mixed using a micropipette with the low melting point agarose solution at 37 °C, to arrive at a final concentration of 0.7 % (*see Note 4*).
3. 50 µL of the cell-agarose suspension are pipetted onto the coated surface of a pretreated slide (*Chromacell SL, Madrid, Spain*) and covered with a 24×60 mm glass coverslip, taking care to avoid trapping any bubbles. The slide is deposited on a metallic or glass surface.
4. The metallic or glass plate is placed in the refrigerator at 4 °C for 5 min, to solidify the agarose from the slide.

3.3 Lysis and Electrophoresis

1. As soon as the gel solidifies, coverslips are removed smoothly and the slides are immersed sequentially in the two lysing solutions: lysing solution I for 30 min, followed by lysing solution II for 30 min (*see Notes 5 and 6*).
2. Rinse the slides in TBE buffer for 10 min, then transfer to a first electrophoresis tank and immerse in fresh TBE electrophoresis buffer. Electrophoresis is performed at 20 V (1 V/cm), 12 mA for 12.5 min (*see Notes 7 and 8*).
3. After washing in 0.9% NaCl, nucleoids are immersed in an alkaline unwinding solution for 2.5 min and transferred to a second electrophoresis tank. The slides are oriented 90° to the first electrophoresis. This second electrophoresis is performed at 20 V (1 V/cm), 12 mA for 4 min in 0.03 M NaOH (*see Notes 9–11*).
4. The slides are rinsed once in the neutralizing solution for 5 min, briefly washed in TBE buffer, dehydrated in sequential baths of 70, 90, and 100 % ethanol (2 min each), and are then air-dried.

3.4 Slide Staining

Finally, the slides are stained 1–5 min with propidium iodide (1 µg/mL) in Vectashield (*see Notes 12 and 13*).

The type of DNA damage (ssDNA or dsDNA) can be identified by fluorescence in situ hybridization (FISH) (Fig. 1).

3.5 Analysis of Slides

The Comets could be assessed by visual scoring or digitalization, and image processing. The frequency of cells with fragmented DNA is established by measuring at least 100 cells per slide.

For visual scoring, cells are classified as undamaged (no DNA migration) or damaged (migrated DNA) cells. The type of damage could also be assessed; cells are classified as containing SSB (up down migration), DSB (right left migration) or both (Fig. 2) (*see Note 14*).

For digital image analysis, images are captured using a high-sensitivity CCD camera. Groups of 100 TIFF format images are

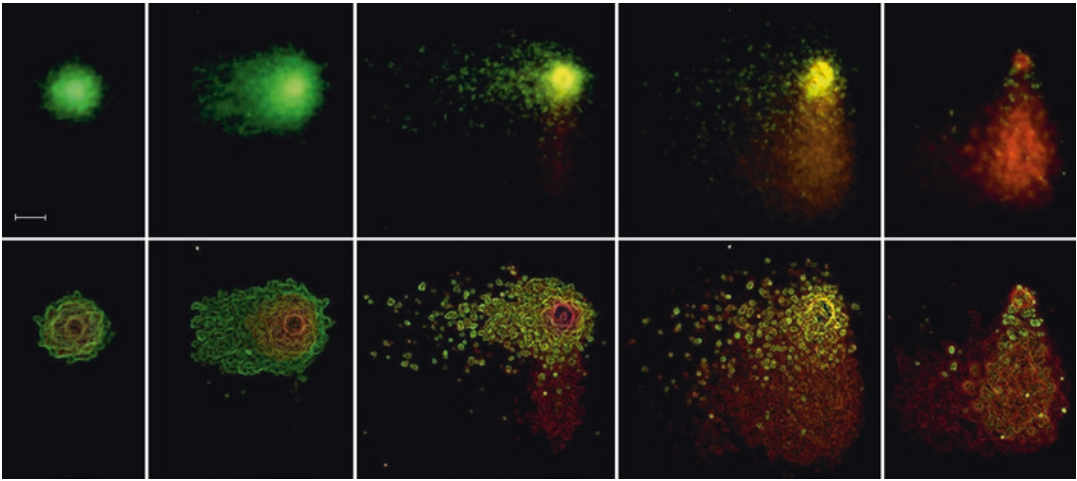


Fig. 1 Characterization of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) by fluorescence in situ hybridization (FISH). ssDNA detection by FISH with a digoxigenin-labeled whole human genome probe and fluorescein isothiocyanate-detected (*green*) on two-tailed (2T)-Comets obtained from human spermatozoa, counterstained with propidium iodide (*red*) (up images). (b) Same images after application of a common electronic filter (*down images*). Scale bar: 10 μ m (photomicrograph reproduced with permission from Enciso [7])

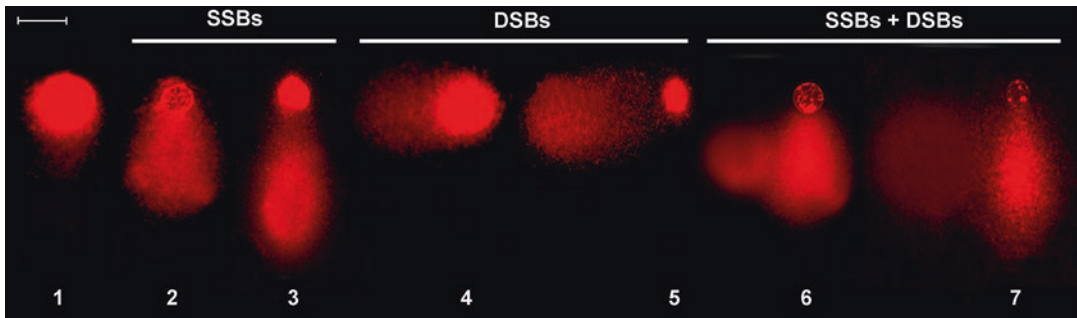


Fig. 2 Two-tailed (2T)-Comet assay protocol detects seven Comet types: 1, undamaged; 2, low level of single-stranded DNA breaks (SSB); 3, high level of SSB; 4, low level of double-stranded DNA breaks (DSB); 5, high level of DSB; 6, low level of SSB and low level of DSB; 7, high level of SSB and high level of DSB. Scale bar: 10 μ m

taken in similar conditions. An image analysis is performed using a routine designed with the comet analysis software. These data are exported as text files (*.txt) to Excel software for statistical analysis. Using image analysis software, analyze individual comet images for several features including total intensity (DNA content), tail length, percent DNA in tail, and tail moment (tail length $\times$ percent of DNA in the tail).

4 Notes

1. Better adhesion can be obtained using fully frosted slides, but slides cannot be dried and stored after staining.
2. Glass slides and coverslips are rinsed in acetic acid and methanol (3:1).
3. In each experiment include two positive controls (e.g., cells exposed with hydrogen peroxide at 20 μM during 15 min at 37 °C as DNA SSBs control and cells treated with the 50 μL of *AluI* for 10 min at room temperature as DNA DSBs control).
4. Cell concentration should be checked under phase-contrast microscopy and adjusted so that, after mixing with the agarose, cells do not overlap or are not excessively dispersed. The concentration may vary according to the type of cells.
5. Avoid moving the dish containing the slides.
6. Handle slides gently, e.g., hold slides horizontally and dip them into solutions, do not pour solutions over slides or move containers containing slides.
7. Take care not to allow DNA to renature even briefly (i.e., by lowering pH below 12.3) until after electrophoresis, as this will result in DNA tangling and reduced migration.
8. The distance in centimeters is measured between the negative and positive electrodes in the electrophoresis chamber.
9. The chamber should be filled with a consistent volume of buffer that is about 1–2 mm above the top of the agarose. Ensure that the chamber is level using a bubble leveling device.
10. The cells are first subject an electrophoretic field under non-denaturing conditions to mobilize isolated free discrete DNA fragments produced from DSBs; this is then followed by a second electrophoresis running perpendicular to first one but under alkaline conditions to produce DNA denaturation, exposing SSBs on the same linear DNA chain or DNA fragments flanked by DSBs. This procedure results in a two-dimensional comet tail emerging from the core where two types of original DNA affected molecule can be simultaneously discriminated (Fig. 3).
11. To explain the possible distribution of the actual state of the DNA in this comet, we have distributed the original image in Fig. 4a in four regions (R1–R4); the results are presented on an electronically enhanced image (Fig. 4b). **R1** includes long DNA fragments (DSBs). DNA fragments of equivalent characteristics but shorter were mobilized under neutral conditions to the end of the comet tail. **R2** includes single stranded DNA stretches originating from the constitutive comet at the original spermatozoa. **R3** includes short single

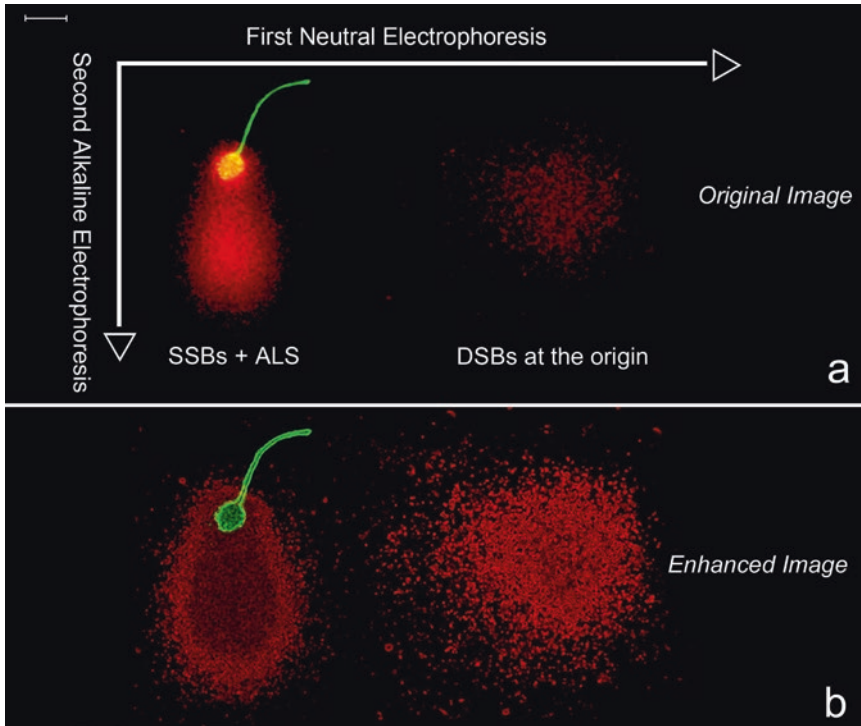


Fig. 3 Original (a) and digitally enhanced image (b) of to show whole DNA fragment distribution DNA map following a two-tailed comet assay in a human sperm cell. The first neutral electrophoresis results in a horizontal migration along the *X* axis of DNA fragments formed as a consequence of DSBs. After turning the micro gel 190°, the second electrophoresis results in the migration of DNA along the vertical *Y* axis and is conducted under alkaline conditions; the alkaline comet assay reveals both structural Alkali Labile Sites (ALSs) and “true” SSBs that have elongated comet tails. The green fluorescence is an associated with protein remnants of the sperm head and flagellum. Scale bar: 10 μm (photomicrograph reproduced with permission from Cortés-Gutiérrez et al. [9])

strand DNA stretches, which area consequence of the DNA denaturation produced on DNA fragments displaced with the neutral comet and originally positioned at equivalent areas of **R1**. **R4** includes single stranded DNA stretches, which area consequence of DNA denaturation produced from enzymatic DSBs at the origin and displaced with the neutral comet.

12. Slides can be stored in humidified, light tight boxes in the cold room (at 4 °C). If storing for more than 2–3 days, dip slides in ethanol and dry them. Dried slides can be stored for several months, but before analysis, rehydrate by adding a thin layer of agarose to reduce background fluorescence, and re-stain with propidium iodide. If the intensity of the comet image appears

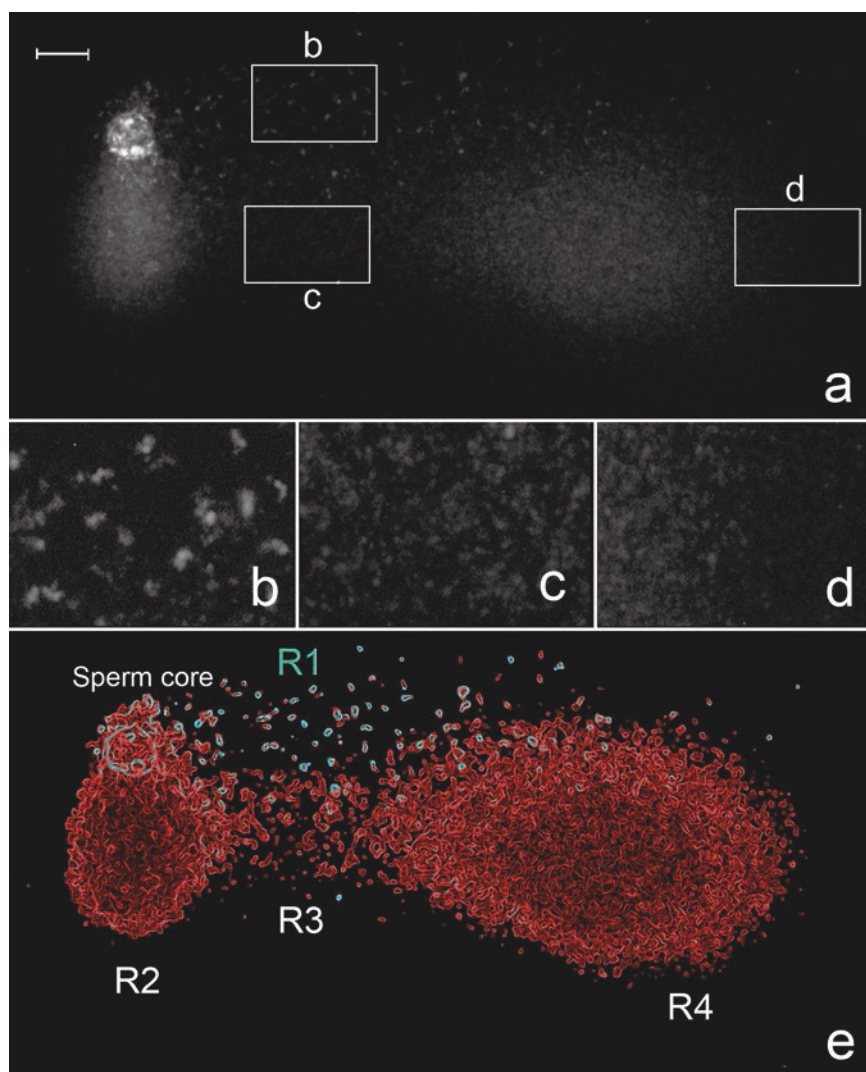


Fig. 4 2T-comet produced after controlled double strand DNA cleavage using the restriction enzyme Alu-I (AG_CT). **(a)** Original comet with selected regions to show differences in DNA texture showed in **(b, c)** and **(d)**. **(e)** Electronically enhanced. *R* different regions identified at the TT-comet. Scale bar: 10 μ m (see **Note 11**) (photomicrograph reproduced with permission from Cortés-Gutiérrez et al. [9])

to decrease while viewing with the fluorescence microscope, try using an antifade solution.

13. Slides could be immediately analyzed or stored at room temperature in the dark until needed.
14. Visual scoring can be used especially when the population is homogenous and adequate controls have been included. The use of five damage classes introduced by Collins [13] has been widely adopted. Triplicate repeat experiments are recommended.

References

1. Ostling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun* 123:291–298
2. Olive PL, Wlodek D, Banath JP (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res* 51:4671–4676
3. Singh NP, McCoy MT, Tice RR et al (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191
4. Jackson SP (2002) Sensing and repairing DNA double-strand breaks. *Carcinogenesis* 23:687–696
5. Fernández-González R, Moreira PN, Pérez-Crespo M et al (2008) Long-term effects of mouse intra cytoplasmic sperm injection with DNA-fragmented sperm on health behavior of adult off spring. *Biol Reprod* 78:761–772
6. Gunes S, Al-Sadaan M, Agarwal A (2015) Spermatogenesis DNA damage and DNA repair mechanisms in male infertility. *Reprod Biomed Online* 31:309–319
7. Enciso M, Sarasa J, Agarwal A, Fernández JL et al (2009) A two-tailed Comet assay for assessing DNA damage in spermatozoa. *Reprod Biomed Online* 18:609–616
8. Enciso M, Johnston SD, Gosálvez J (2011) Differential resistance of mammalian sperm chromatin to oxidative stress as assessed by a two-tailed comet assay. *Reprod Fertil Dev* 23:633–6337
9. Cortés-Gutiérrez EI, López-Fernández C, Fernández JL et al (2014) Interpreting sperm DNA damage in a diverse range of mammalian sperm by means of the two-tailed comet assay. *Front Genet* 5:404
10. Sánchez-Calabuig MJ, López-Fernández C, Martínez-Nevado E et al (2014) Validation of a field based chromatin dispersion assay to assess sperm DNA fragmentation in the bottlenose dolphin (*Tursiops truncatus*). *Reprod Domest Anim* 49:761–768
11. Johnston SD, López-Fernández C, Arroyo F et al (2015) The assessment of sperm DNA fragmentation in the saltwater crocodile (*Crocodylus porosus*). *Reprod Fertil Dev* 2015:PMID: 26462595. doi:[10.1071/RD15300](https://doi.org/10.1071/RD15300)
12. Barcena P, López-Fernández C, García-Ochoa C et al (2015) Detection of DNA damage in cumulus cells using a chromatin dispersion assay. *Syst Biol Reprod Med* 61:277–285
13. Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* 26:249–261

Part VI

Plant Histochemistry

Detection of Endogenous Nuclear Proteins in Plant Cells: Localizing Nuclear Matrix Constituent Proteins (NMCPs), the Plant Analogs of Lamins

Malgorzata Ciska and Susana Moreno Díaz de la Espina

Abstract

At present, two complementary approaches are used for in situ protein visualization in plant nuclei. Imaging of transformed fluorescent proteins is the election tool for the analysis of protein movement and interaction. However, this methodology presents several drawbacks for the identification/localization of endogenous nuclear factors, such as over-expression or mislocalization of transformed proteins. In contrast, immunocytochemistry with specific antibodies represents a powerful tool for the localization of endogenous nuclear proteins at their “native” nuclear sub-compartments. In plant cells, the cell wall hampers antibody accessibility during immunocytochemical analysis thereby reducing the effectivity of the technique, particularly in the case of lowly expressed proteins. To overcome this problem in nuclear protein immunodetection, we developed a method based on the in vitro incubation of isolated nuclei with specific antibodies followed by imaging by confocal fluorescence or electron microscopy. Here we describe the application of this methodology to the localization of Nuclear Matrix Constituent Proteins (NMCP), the plant analogs of lamins, of the monocot *Allium cepa*, using antibodies raised against highly conserved regions of the proteins.

Key words Immunofluorescence confocal microscopy, Immunoelectron microscopy, Nuclear isolation, Pre-embedding labeling, NMCP proteins, Plant cells, *Allium cepa*

1 Introduction

While nuclei from plants and animals are structurally and functionally similar, plants lack many important animal nuclear proteins homologs such as lamins and their associated proteins, coilin, etc. Although each year new plant genomes are sequenced, still relatively little is known about plant nuclear proteins. Since the nuclear protein sequence conservation is low between the two kingdoms, plant analogs are many times assigned taking into account subnuclear localization, biochemical properties, predicted protein structures and phenotypic characteristics of mutant cells, as is the case of the plant lamin analog Nuclear Matrix Constituent Proteins (NMCPs).

NMCPs are integral components of the plant lamina, a thin fibrillar layer lining the inner nuclear membrane and are regarded as analogs of animal lamins [1]. NMCPs are insoluble proteins thought to play an important function in maintaining nuclear shape [2, 3].

NMCPs were visualized at the nuclear envelope and, in some cases, at nucleoplasm using specific antibodies [4–6] or fluorescent protein (FP) expression [2, 3]. Their fate during mitosis was also examined by immunofluorescence [5, 7] and FP visualization [3]. Constructs of different protein regions fused to green FP were used to determine the domains responsible for the integration of carrot NMCP1 protein to the nuclear periphery [8] and to demonstrate the interaction between *Arabidopsis thaliana* NMCP1/LINC1 and SUN1/2 proteins at the nuclear envelope [9]. Thus both immunofluorescence and FP expression have been employed in the detection of these nuclear proteins but both pose important limitations. Imaging of FP tagged proteins is very useful for the analysis of movement (by FRAP: fluorescence recovery after photobleaching) and interaction (by FRET: fluorescence resonance energy transfer) of plant proteins [10]. Nevertheless, this methodology presents several disadvantages for in situ protein detection: mainly artifacts due to overexpression of the transformed proteins and the potential mislocalization owing to the presence of FPs in the chimeric construct. Addition of FPs in a region involved in protein interaction or localization (and many of these sites are not described) has been reported to result in faulty detection [9]. For this reason, immunocytochemistry with specific antibodies is still frequently chosen for the identification of endogenous plant proteins at their “native” locations [11]. This technique is based on protein detection in cells fixed either chemically by aldehyde treatment (mostly formaldehyde, FA) or cryofixed, using specific antibodies and secondary antibodies conjugated with a fluorescent dye or gold particle. However, immunocytochemistry in plant cells presents several problems. The first one is the accessibility of antibodies to the epitopes they are directed against due to the presence of a cell wall. This has been frequently resolved by treatment with cell wall-digesting enzymes, cellulase and pectinase after fixation [11]. In the case of nuclear proteins, this procedure is effective when disposing of highly reactive antibodies, proteins are highly abundant and staining clearly stands out from weaker cytoplasm background staining, as is the case of the nucleolar protein fibrillarlin [12, 13], but not for low-expressed proteins or when using antibodies with low reactivity. Another problem is the lack of availability of highly specific antibodies for the protein in question, which in most cases have to be specifically produced for a given experiment.

Over the past years we have developed a protocol for identifying and localizing nuclear proteins in plants using cell fractionation

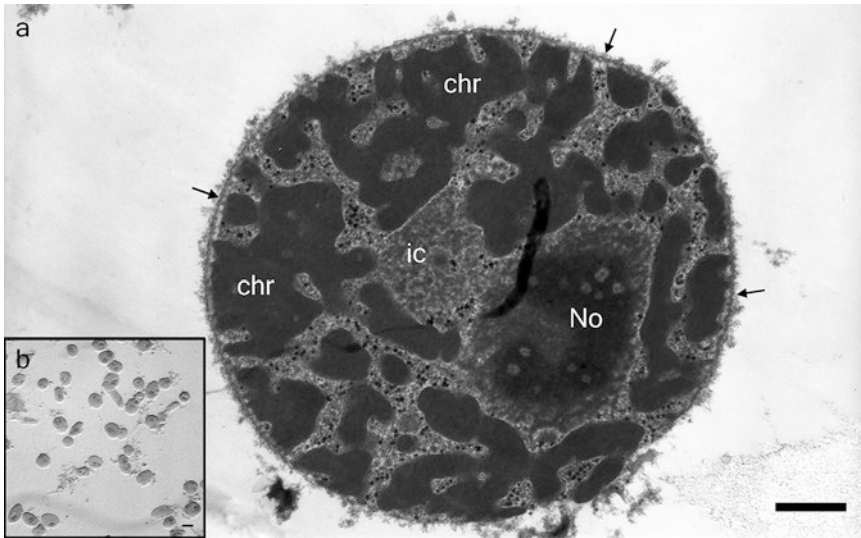


Fig. 1 Typical images of onion meristematic nuclear fractions isolated with the described procedure showing their yield, purity and the preservation of nuclear integrity and organization. **(a)** Transmission electron microscopy image of an isolated nucleus fixed in 3% glutaraldehyde and contrasted with uranyl acetate. The organization of the main nuclear structures is well preserved: nuclear envelope (*arrows*), condensed chromatin compartment (*chr*), nucleolus (*No*), and interchromatin compartment (*ic*). Scale bar = 1 μ m **(b)** DIC (differential interference contrast) image of a fresh nuclear fraction observed after methyl green staining. Scale bar = 10 μ m

to isolate well-preserved nuclei allowing a precise localization of “native” proteins, an improved antibody accessibility and avoiding excessive cytoplasmic background staining (Figs. 1a, b and 2a, c). This method can be combined with immunofluorescence (Fig. 2c, d) or pre-embedding immunoelectronmicroscopy (Fig. 3a); and western blotting as a control for primary antibody specificity. This protocol is based on mild mechanical cell disruption of root meristems, isolation of nuclei by low speed centrifugation, mild fixation with FA and incubation of nuclei with primary and secondary antibodies in suspension. After the immunological reaction, nuclei incubated with fluorescent secondary antibodies are mounted for confocal microscopy, while those incubated with gold-conjugated secondary antibodies are further fixed with FA, embedded in resin and processed for transmission electron microscopy. The procedure is simple and rendered very good results in the detection of nuclear proteins with different biochemical characteristics and nuclear localizations: MFPI (Matrix Filament Protein 1) [14], nuclear actin [15], spectrin-like protein [16] and especially in the case of NMCP1 a peripheral nuclear protein whose labeling is difficult to be discriminated from cytoplasmic background labeling [6] (Fig. 2 a, b).

Here we report the current routine procedures used in the lab for the detection of NMCP1 in isolated onion nuclei by

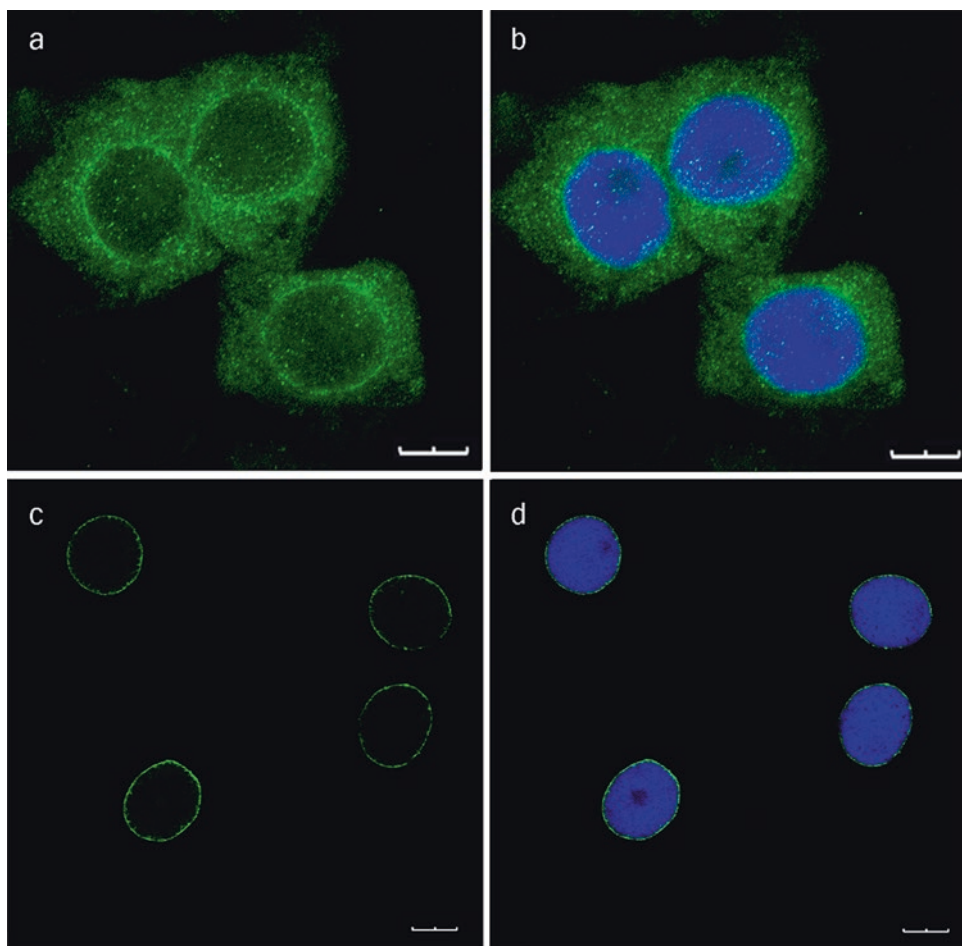


Fig. 2 Example images of immunofluorescence localization of NMCP1 in onion meristematic cells and isolated nuclei. All images correspond to z confocal sections. **(a)** Squashed cells after cell wall digestion with cellulase and pectinase. The staining of the lamina is weak and partially masked by the unspecific staining of the cytoplasm. **(c)** Incubation of isolated nuclear fractions allows a higher and sharper definition of NMCP1 staining at the nuclear periphery. **(b and d)** Overlays of NMCP1 and DAPI stainings. Scale bar = 10 μ m

immunofluorescence and immunoelectronmicroscopy imaging using an antibody produced against the highly conserved N-terminus peptide of the protein [6].

2 Materials

All solutions should be freshly prepared and protease inhibitors and detergents added shortly before use.

2.1 Isolation of Nuclei

1. Isolation Medium (IM): 2 % arabic gum (w/v) (*see Note 1*), 1.25 % (w/v) ficoll, 2.5 % (w/v) dextran (*see Note 2*), 0.01 % (w/v) BSA, 0.5 mM EDTA, 50 mM magnesium acetate,

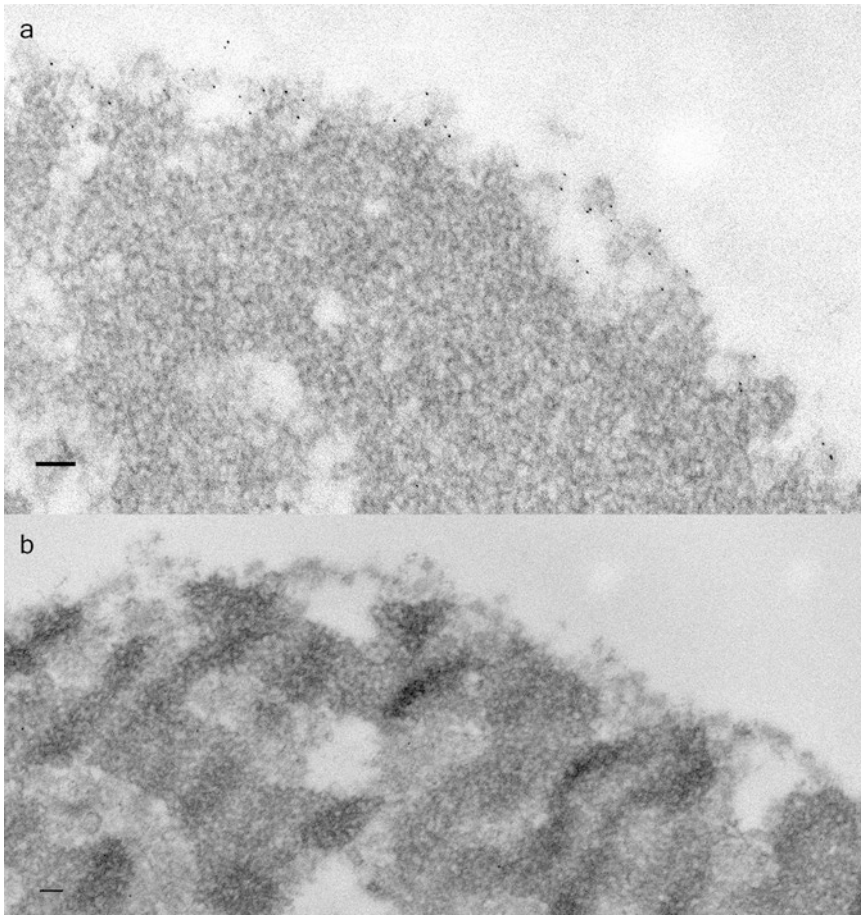


Fig. 3 High resolution localization of NMCP1 at the nuclear lamina by transmission electron microscopy immunolabeling displaying the higher efficiency of the pre-embedding procedure (a) which shows the specific accumulation of 5 nm gold particles at the peripheral nuclear lamina, in contrast with the results of the post-embedding labeling which only displays a few scattered gold particles inside the nucleus corresponding to background labeling (b). Scale bars = 100 nm

- 8 mM β -mercaptoethanol, 4 mM n-octanol, 25 mM TRIS, 7 mM diethylpyrocarbonate, 30% (v/v) glycerol, 1% (v/v) protease inhibitor cocktail (for example Sigma-Aldrich P9599).
2. Methyl Green staining solution: 1% Methyl Green in 70% ethanol.
3. Stock buffer: 10 mM Tris-HCl pH 7.4, 10 mM HEPES pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 4 mM n-octanol, 0.02% sodium azide, 0.24 mM sucrose.
4. 30, 50, and 100 μ m nylon sheets.
5. Vacuum pump.
6. Homogenizer, e.g., Ultra-Turrax IKA T25 with disperser IKA S25-10G.

2.2 Immuno-fluorescence

1. 10× PBS pH 7.4: 1.37 M sodium chloride, 27 mM potassium chloride, 80 mM sodium hydrogen phosphate, 20 mM potassium dihydrogen phosphate, adjust pH to 7.4.
2. Immunofluorescence Fixative: 2% formaldehyde (FA) freshly prepared from paraformaldehyde (PFA) powder in a ventilated hood. To dissolve FA the 1× PBS buffer pH 7.4 was first warmed up to 60 °C and then the PFA powder was added. Once dissolved, the solution was filtered through a 0.20 µm filter, e.g., Ministart (Sartorius Stedim Biotech) and cooled down. Before use, Triton X-100 was added to the solution to a final concentration of 0.5% (*see Note 3*).
3. Permeabilization buffer: 1× PBS buffer pH 7.4 containing 0.5% Triton X-100 (*see Note 3*).
4. Glycine buffer: 20 mM glycine in 1× PBS buffer pH 7.4.
5. Blocking buffer: 2% BSA in 1× PBS buffer pH 7.4 containing 0.05% Tween 20.
6. Primary antibody solution: primary antibody in blocking buffer at adequate dilution, usually 10× that estimated from Western blot (WB) essays (e.g., 1:100 for anti-AcNMCP1).
7. Secondary antibody solution: secondary antibody in blocking buffer at adequate dilution (e.g., 1:100 for Alexa 488-conjugated anti-rabbit IgG).
8. DAPI nuclear staining solution: 1 µg/ml 4',6' diamidino-2-phenylindole (DAPI) in 1× PBS buffer pH 7.4 containing 0.05% Tween 20.
9. DABCO anti-fading medium: add 200 µl of DABCO to 1 ml of 0.2 M Tris-HCl pH 8 in a 1.5 ml centrifuge tube and incubate at 70 °C for 5 min, vortex well, transfer to a 15 ml centrifuge tube containing 9 ml of glycerol, mix well, incubate again for 5 min at 70 °C, vortex and aliquot. Store at -20 °C until use.
10. Poly-lysine coated slides: multi-well slides were washed with warm tap water containing detergent, next they were washed a few times in ultrapure Milli-Q water and finally with acetone. Subsequently slides were air-dried and submerged in 0.01% poly-L-lysine solution for 15 min. The slides were air-dried at room temperature (RT) and stored at -20 °C until use.
11. Transparent nail polish to seal the coverslips.
12. Confocal microscope, in our case a Leica TCS-SP2-AOBS with the Leica-confocal software.

2.3 Electron Microscopy

2.3.1 Pre-embedding Immunolabeling

1. EM Fixative I: 0.25% FA freshly prepared in 1× PBS buffer pH 7.4 (*see Note 4*). To prepare the FA solution *see* Subheading 2.2, **item 2**. Cool down the solution and add Triton X-100 to a final concentration of 0.5%.
2. Glycine buffer: 20 mM glycine in 1× PBS buffer pH 7.4.

3. Blocking buffer: 2 % BSA in 1× PBS buffer pH 7.4.
4. Primary antibody solution: primary antibody in blocking buffer at adequate dilution, e.g., anti-AcNMCP1 at 1:50, usually two times more concentrated than for immunofluorescence.
5. Secondary antibody solution: 5 nm gold conjugated secondary antibody in blocking buffer at adequate dilution, 1:50 for 5 nm gold conjugated anti-rabbit IgG (Sigma-Aldrich G7277).
6. EM Fixative II: 2 % FA freshly prepared from PFA powder in PBS buffer pH 7.4 as indicated above (Subheading 2.2 item 2).
7. Agarose (low gelling temperature) 2 %.
8. Ethanol solutions: 30, 50, 70, 90, 100 %.
9. Ethanol: LR White resin solution 2:1.
10. Ethanol: LR White resin solution 1:2.
11. LR White resin medium grade (London Resin Company) (*see* **Note 5**).
12. Formvar-coated nickel grids (*see* **Note 6**). Commercial formvar-coated grids are available but to prepare them in laboratory conditions use a 0.5 % solution of formvar in ethylene dichloride (commercially available), dip a clean slide in it, remove and let it dry. Cut the edges with a sharp razor and let float the thin film layer on Milli-Q water by dipping the slide into the water at an angle. Place the grids on the floating film layer, collect the film with a filter paper and let to dry. Carefully remove the grids with EM tweezers.
13. EM staining solution: 5 % aqueous uranyl acetate.
14. Electron microscope, in our case Jeol 1230 EM.

2.3.2 Post-embedding Immunolabeling

1. EM fixative III: 4 % FA freshly prepared from PFA powder in 1× PBS buffer pH 7.4 as indicated above in Subheading 2.2 item 2 (*see* **Note 4**).
2. Agarose (low gelling temperature) 2 %.
3. Ethanol solutions: 30, 50, 70, 90, 100 %.
4. Ethanol: LR White resin solution 2:1.
5. Ethanol: LR White resin solution 1:2.
6. LR White resin medium grade (London Resin Company) (*see* **Note 5**).
7. Formvar-coated nickel grids (*see* Subheading 2.3.1 item 12 and **Note 6**).
8. Glycine buffer: 20 mM glycine in 1× PBS buffer pH 7.4.
9. Blocking buffer: 2 % BSA in 1× PBS buffer pH 7.4.
10. Primary antibody solution: primary antibody in blocking buffer at adequate dilution, usually two times more concentrated

than for pre-embedding immunogold labeling (1:20 for anti-AcNMCP1).

11. Secondary antibody solution: gold conjugated secondary antibody in blocking buffer at adequate dilution, e.g., 1:50 for 10 nm gold conjugated anti-rabbit IgG.
12. EM staining solution : 5 % aqueous uranyl acetate.
13. Electron microscope, in our case Jeol 1230 EM.

3 Methods

3.1 Plant Culture

Eliminate the external layers of *Allium cepa* L. francesa var. bulbs, wash them extensively in tap water for 30 min and grow them in filtered tap water at RT for at least 2 days. Root meristems are excised at appropriate lengths from the tips and used for nuclear isolation (*see Note 7*). Quiescent meristems of *A. cepa* are taken directly from unsoaked bulbs.

3.2 Isolation of Nuclei

1. Select the root segments to be isolated and submerge them in 5 ml of freshly prepared IM. Incubate the tissues in vacuum on ice for 15 min and then transfer the tissues to a centrifuge tube, add 5 ml of IM and homogenize three times 20 s at 20,000 rpm with the homogenizer.
2. Filter the homogenate through a set of 100, 50 and 30 μm nylon sheets; the 100 μm nylon sheet should be positioned on the top to facilitate the filtration and avoid stacking the pores. Repeat the homogenization and filtration steps three times and collect each batch in a separate centrifuge tube (*see Note 8*).
3. Centrifuge the homogenate at $6000 \times g$, for 15 min at 4 °C (*see Note 9*). The supernatant contains the cytoplasmic fraction.
4. Wash the pellets containing the nuclei at least four times with IM containing 0.1 % protease inhibitor cocktail and store at -20 °C in stock buffer until use. To control the purity and integrity of isolated nuclei add 20 μl of Methyl Green staining solution to a small portion of the fraction on a slide at RT, mix with a pipette tip to spread the staining solution and observe under a light microscope. If the nuclei are surrounded with a ring of cytoplasm the fraction should be washed a few times more.

3.3 Immuno-fluorescence Confocal Microscopy of Nuclear Fractions

All steps are performed at RT and in constant shaking if not stated differently. Negative controls are prepared by omitting the primary antibody.

1. Fix nuclear fractions for 1 h in 0.5–1 ml of Immunofluorescence Fixative in a 1.5 ml centrifuge tube on a tube rotator at 4 °C. Then centrifuge the samples at $6000 \times g$ for 15 min at 4 °C.

2. Wash the nuclei in permeabilization buffer for 30 min on a tube rotator at 4 °C. This step also serves to permeabilize the membranes and facilitates the antibodies reaching intranuclear epitopes. Centrifuge at $6000\times g$ for 15 min at 4 °C.
3. Resuspend the pellets in glycine buffer and incubate for 30 min on a tube rotator at 4 °C to quench aldehyde groups (*see Note 10*). Centrifuge at $6000\times g$ for 15 min at 4 °C.
4. Block in 100–200 μ l of blocking buffer for 30 min on a tube rotator.
5. Add the primary antibody to the blocking buffer to the appropriate dilution, e.g., 1:100 for anti-AcNMCP1 (*see Note 11*) and incubate for 1 h or overnight at 4 °C in constant shaking. For co-localization experiments add the two antibodies produced in different species together.
6. Wash 3 times in PBS with 0.05 % Tween 20 for 15 min. To pellet the fractions centrifuge at $6000\times g$, 15 min at 4 °C.
7. Incubate the pellets with the corresponding Alexa fluor-conjugated secondary antibody (or secondary antibodies in the case of co-localization experiments), at 1:100 dilution for 45 min, and up to 1 h at RT in the dark. From this step on, the sample should be protected from light. Use dark centrifuge tubes or to avoid the loss of pellet use common transparent centrifuge tubes covered with aluminum foil.
8. Wash twice for 15 min in PBS with 0.05 % Tween 20. Centrifuge at $6000\times g$ for 15 min at 4 °C.
9. Stain for 5 min with 200 μ l of DAPI nuclear staining solution to counterstain DNA in the nuclei. Add 800 μ l of PBS 1 $\times$ pH 7.4 with 0.05 % Tween 20 and incubate for another 10 min. Centrifuge at $6000\times g$ for 15 min.
10. Wash pellets three times for 15 min in PBS with 0.05 % Tween 20 and centrifuge at $6000\times g$ for 15 min.
11. Resuspend the labeled fractions in 1 $\times$ PBS pH 7.4, control the density of the suspension under a microscope, dilute if needed and layer onto 0.1 % poly-L-lysine coated multi-well slides (around 10 μ l/well). Air-dry and mount with mounting medium. Seal the coverslip with transparent nail polish.
12. Examine the samples in a confocal microscope.
13. Store slides at 4 °C in an opaque box to avoid the loss of fluorescent signal.

3.4 Immunoelectron Microscopy (*See Note 12*)

3.4.1 Pre-embedding Immunogold Labeling

Negative controls are performed by omitting the primary antibody.

1. Fix the isolated nuclei in 500–1000 μ l of EM Fixative I in a 1.5 ml centrifuge tube from 30 min to 1 h at 4 °C on a tube rotator. Centrifuge for 20 min at $6000\times g$ at 4 °C.

2. Wash for 30 min in 1× PBS buffer with 0.5 % Triton X-100 at 4 °C on a tube rotator. Centrifuge for 20 min at 6000×*g* at 4 °C.
3. Incubate with glycine buffer for 30 min on ice on a tube rotator. Centrifuge for 20 min at 6000×*g* at 4 °C.
4. Block in blocking buffer from 30 min up to 1 h on a tube rotator. Centrifuge for 20 min at 6000×*g* at 4 °C.
5. Add the primary antibody to the blocking buffer at the appropriate final dilution (1:50 in the case of anti-AcNMCP1), and incubate the nuclei for 1 h or overnight at 4 °C on a tube rotator.
6. Wash three times for 15 min in 1× PBS buffer pH 7.4 containing 0.05% Tween 20. Centrifuge for 15 min at 6000×*g* at 4 °C.
7. Incubate the pellets with the appropriate 5 nm gold conjugated secondary antibody, e.g., anti-rabbit secondary antibody at 1:50 from 45 min up to 1 h at 4 °C (*see Note 13*). Add 500 µl of 1× PBS buffer pH 7.4 and centrifuge for 15 min at 6000×*g* at 4 °C.
8. Wash twice for 15 min in 1× PBS buffer pH 7.4 containing 0.05% Tween 20. Centrifuge for 15 min at 6000×*g* at 4 °C.
9. Fix the pellets in EM Fixative II for 1 h at 4 °C on a tube rotator. Centrifuge for 15 min at 6000×*g* at 4 °C.
10. Wash twice for 10 min in 1× PBS buffer pH 7.4. Centrifuge for 15 min at 6000×*g* at 4 °C.
11. Embed in 2% agarose in a centrifuge tube. Heat the agarose in Milli-Q water until it dissolves, cool down to 37 °C, add to the centrifuge tubes and resuspend nuclei pellets. Once the agarose jellifies cut the tube with a razor and carefully take out the gel. Cut it into pieces and transfer to a 2 ml centrifuge tube.
12. Dehydrate the gel pieces at 4 °C in an ethanol series: 30% ethanol for 30 min; 50% ethanol for 30 min; 70% ethanol for 30 min or overnight; 90% ethanol for 45 min; three times in 100% ethanol for 45 min. To increase the contact surface of the samples with ethanol solutions and resin it is recommended to position the centrifuge tubes vertically in the fridge during the incubations.
13. Infiltrate the gel pieces in LR White resin in three steps: (a) 100% ethanol: LR White 2:1 for 2 h; (b) 100% EtOH: LR White 1:2 for 2 h; (c) LR White for 3 days changing resin every 12 h. All infiltration steps should be performed at 4 °C.
14. Close the samples in gelatin capsules with resin eliminating the air and cure them at 60 °C for 20–22 h.
15. To select the region of interest cut semi-thin sections (0.5–2 µm) of the sample and examine them by using phase

contrast light microscopy. Once the sections are selected, trim the face of the block and cut ultrathin sections (80–100 nm) on an ultramicrotome with a diamond knife. Mount the sections on nickel grids coated with a Formvar film.

16. Wash the grids three times for 10 min in Milli-Q water and then dry for 10 min on a filter paper. Contrast the samples for 30 min using the EM staining solution. The uranyl acetate solution should be centrifuged before use at $6000\times g$ for 30 min to avoid any contaminating particles in the preparations. During incubation with uranyl acetate sections should be protected from light which causes precipitation of the compound. Wash again the grids three times for 10 min in Milli-Q water and let them dry.
17. Examine in an electron microscope at 80 kV.

*3.4.2 Post-embedding
Immunogold Labeling
(See Note 12)*

Negative controls were performed by omitting the primary antibody.

1. Fix nuclear fractions for 2 h in EM fixative III at 4 °C on a tube rotator. Centrifuge at $6000\times g$ at 4 °C.
2. Wash for 10 min in $1\times$ PBS buffer pH 7.4. Centrifuge at $6000\times g$ for 15 min at 4 °C.
3. Embed the fixed nuclei in 2% agarose as described in Subheading 3.4.1 step 11.
4. Dehydrate the sample at 4 °C in an ethanol series: 30% ethanol for 30 min; 50% ethanol for 30 min; 70% ethanol for 30 min to overnight; 90% ethanol for 45 min; three times in 100% ethanol for 45 min. During incubation position the centrifuge tubes vertically to increase the contact surface of the sample with the solution.
5. Infiltrate in LR White resin in three steps at 4 °C: (a) 100% ethanol: LR White 2:1 for 2 h; (b) 100% EtOH: LR White 1:2 for 2 h; (c) LR White for 3 days changing resin every 12 h. During incubation position the centrifuge tubes vertically to increase the contact surface of the sample.
6. Close the samples in gelatin capsules with resin eliminating the air and cure them at 60 °C for 20–22 h.
7. Cut ultrathin sections on an ultramicrotome with a diamond knife and mount on nickel grids coated with a Formvar film. Post-embedding immunogold labeling was performed on ultrathin section mounted on nickel grids floating on drops of the adequate solutions in a humid chamber as follows.
8. Incubate on glycine buffer for 15 min to quench aldehyde groups.
9. Wash on $1\times$ PBS pH 7.4 with 0.05% Tween 20 for 10 min.

10. Block on the blocking buffer for 30 min.
11. Incubate the grids on drops of the primary antibody solution at appropriate dilution (e.g., 1:25 for anti-AcNMCP1) for 1 h at RT.
12. Wash 3 times for 10 min on 1× PBS pH 7.4 with 0.05 % Tween 20.
13. Incubate on the secondary antibody solution for 1 h.
14. Wash three times for 10 min on 1× PBS pH 7.4 with 0.05 % Tween 20.
15. Wash the grids three times for 10 min on Milli-Q water and then dry for 10 min on a filter paper. Contrast the samples for 30 min using the EM staining solution as previously described (Subheading 3.4.1 step 16).
16. Examine in an electron microscope at 80 kV.

4 Notes

1. After dissolving arabic gum the solution should be filtered through a 30 µm nylon sheet to avoid contamination of nuclei with undissolved particles. This step is especially important for the nuclei that will be used for microscopy.
2. If dextran does not dissolve at RT, warm up the solution shortly in a microwave oven until it dissolves and then cool down and filter through a 30 µm nylon sheet.
3. For investigation of soluble proteins the detergent should be used at much lower concentrations in the fixative and permeabilization buffers, e.g., 0.05 % Tween 20.
4. Aldehyde fixatives, like FA or glutaraldehyde, are the most common fixatives used in EM studies. They cross-link proteins as well as other macromolecules like DNA-linked proteins and lipoproteins of cellular and nuclear membranes. FA is a mono-aldehyde and its cross-linking ability is lower than that of glutaraldehyde, leaving more antigen sites that are recognized by antibodies. For this reason it is the best choice for immunolabeling. Glutaraldehyde is a dialdehyde and its cross-linking ability is higher, therefore the fixation of macromolecules is more stable but it can deform the tertiary structure of antigens and also penetrates the tissue slower than FA. Often a mixture of both, sometimes called Karnovsky fixative, is used to combine the fast penetration of FA and the stable fixation of glutaraldehyde. If the observation of membranes is a priority, the sample can be post-fixed with 1–2 % osmium tetroxide for 1 h, nevertheless since osmium also has cross-linking properties a high reduction of antigenicity should be expected. The antigen-

nicity can be partially restored by treatment with sodium metaperiodate [17].

5. In our lab, we use LR White resin which is optimal for immunogold labeling. Sections of polymerized acrylic resins such as LR White resin are hydrophilic which facilitate recognition of epitopes by antibodies by post-embedding labeling.
6. Copper and nickel grids are commercially available, but since copper ions may have inhibitory effect on antibody binding to antigen it is better to use nickel grids for post-embedding immunolabeling experiments.
7. Root meristems are a good material for extraction of nuclei since their cells contain numerous uniform, undifferentiated nuclei and little contaminating elements such as chloroplasts, fibers, etc. In contrast the upper parts of the root contain differentiated tissues with nuclei with different sizes and forms. *Allium cepa* root meristems are a very good material for microscopic analysis of nuclear proteins since their nuclei are diploid and large, which enables detection of nuclear proteins with high resolution.
8. The first batch usually is the least contaminated and the nuclei the least disturbed and it is best for the microscopy, the following batches are preferably used for WB analysis.
9. The centrifugation speed is optimized for the size of onion root meristematic nuclei, to use this protocol in different species or tissues the speed should be optimized according to nuclear size.
10. All aldehyde fixatives, glutaraldehyde more than FA, react with and cross-link protein molecules, leaving many free aldehyde groups that will combine covalently with antibodies if not quenched. Aldehyde blocking can be performed by feeding the free aldehyde groups with bland amino groups such as glycine or by reducing the $-CHO$ groups to $-OH$ with sodium borohydride [18]. The first option is much quicker and less problematic and is sufficient for samples fixed with FA.
11. The optimal concentration of the antibody is usually ten times that used for WB. The concentration and incubation time might need optimization for every antibody.
12. For the choice between pre-embedding and post-embedding immunogold labeling one should take into account several aspects like the quantity of the sample, specificity and quantity of the antibody, and availability of the antigen. Post-embedding immunolabeling is usually the first choice for its simplicity and efficiency. Ultrathin sections are incubated with antibodies, leaving the block unlabeled and different sections from the same sample can be used as controls, for different antibodies or

for multiple labeling. On the other hand, the number of epitopes available on the surface of the section is much lower than in the whole fixed nuclei, highly reducing the sensitivity of this method. If the antigen is scant, is distributed sparse throughout the nuclei or the sensitivity of the antibody is not high, the pre-embedding labeling is the best choice.

13. For double labeling it is important to choose well the antibodies. The primary antibodies should be produced in different species and the size of gold particles adsorbed to the secondary antibodies should be different for each detected protein to enable visual differentiation. The most commonly used are the secondary antibodies conjugated to 5 and 10 nm gold particles in diameter which are small enough to enter cellular structures in pre-embedding. Secondary antibodies conjugates with larger gold particles are also available and can be used in post-embedding labeling.

Acknowledgments

We would like to thank Mrs M. Carnota and past members of the lab for their contribution to the development of the methods and protocols presented here. We acknowledge the support from the Spanish Ministry of Science and Innovation [BFU2010-15,900] and the CSIC [PIE 201020E019].

References

1. Ciska M, Moreno Diaz de la Espina S (2014) The intriguing plant nuclear lamina. *Front Plant Sci* 5:166. doi:[10.3389/fpls.2014.00166](https://doi.org/10.3389/fpls.2014.00166)
2. Dittmer TA, Stacey NJ, Sugimoto-Shirasu K et al (2007) LITTLE NUCLEI genes affecting nuclear morphology in *Arabidopsis thaliana*. *Plant Cell* 19(9):2793–2803. doi:[10.1105/tpc.107.053231](https://doi.org/10.1105/tpc.107.053231)
3. Sakamoto Y, Takagi S (2013) LITTLE NUCLEI 1 and 4 regulate nuclear morphology in *Arabidopsis thaliana*. *Plant Cell Physiol* 54(4):622–633. doi:[10.1093/pcp/pct031](https://doi.org/10.1093/pcp/pct031)
4. Masuda K, Xu ZJ, Takahashi S et al (1997) Peripheral framework of carrot cell nucleus contains a novel protein predicted to exhibit a long alpha-helical domain. *Exp Cell Res* 232(1): 173–181. doi:[10.1006/excr.1997.3531](https://doi.org/10.1006/excr.1997.3531)
5. Masuda K, Haruyama S, Fujino K (1999) Assembly and disassembly of the peripheral architecture of the plant cell nucleus during mitosis. *Planta* 210(1):165–167
6. Ciska M, Masuda K, Moreno Diaz de la Espina S (2013) Lamin-like analogues in plants: the characterization of NMCP1 in *Allium cepa*. *J Exp Bot* 64(6):1553–1564. doi:[10.1093/jxb/ert020](https://doi.org/10.1093/jxb/ert020)
7. Kimura Y, Kuroda C, Masuda K (2010) Differential nuclear envelope assembly at the end of mitosis in suspension-cultured *Apium graveolens* cells. *Chromosoma* 119(2):195–204. doi:[10.1007/s00412-009-0248-y](https://doi.org/10.1007/s00412-009-0248-y)
8. Kimura Y, Fujino K, Ogawa K et al (2014) Localization of *Daucus carota* NMCP1 to the nuclear periphery: the role of the N-terminal region and an NLS-linked sequence motif, RYNLRR, in the tail domain. *Front Plant Sci* 5:62. doi:[10.3389/fpls.2014.00062](https://doi.org/10.3389/fpls.2014.00062)
9. Graumann K (2014) Evidence for LINC1-SUN associations at the plant nuclear periphery. *PLoS One* 9(3):e93406. doi:[10.1371/journal.pone.0093406](https://doi.org/10.1371/journal.pone.0093406)
10. Hanson MR, Kohler RH (2001) GFP imaging: methodology and application to investigate cellular compartmentation in plants. *J Exp Bot* 52(356):529–539

11. Brown RC, Lemmon BE (2011) Dividing without centrioles: innovative plant microtubule organizing centres organize mitotic spindles in bryophytes, the earliest extant lineages of land plants. *AoB Plants* 2011:plr028. doi:[10.1093/aobpla/plr028](https://doi.org/10.1093/aobpla/plr028)
12. Acevedo R, Cuadrado A, De la Torre C et al (2002) Behaviour of ribosomal genes and nuclear domains during activation in sugarcane (*Saccharum officinarum* L.) root primordia: from the unsoaked quiescent state to the steady state of proliferation. *Eur J Histochem* 46(2):143–158
13. Acevedo R, Samaniego R, Moreno Diaz de la Espina S (2002) Coiled bodies in nuclei from plant cells evolving from dormancy to proliferation. *Chromosoma* 110(8):559–569
14. Samaniego R, Jeong SY, de la Torre C et al (2006) CK2 phosphorylation weakens 90 kDa MFP1 association to the nuclear matrix in *Allium cepa*. *J Exp Bot* 57(1):113–124. doi:[10.1093/jxb/crj010](https://doi.org/10.1093/jxb/crj010)
15. Cruz JR, Moreno Diaz de la Espina S (2009) Subnuclear compartmentalization and function of actin and nuclear myosin I in plants. *Chromosoma* 118(2):193–207. doi:[10.1007/s00412-008-0188-y](https://doi.org/10.1007/s00412-008-0188-y)
16. Perez-Munive C, Moreno Diaz de la Espina S (2011) Nuclear spectrin-like proteins are structural actin-binding proteins in plants. *Biol Cell* 103(3):145–157. doi:[10.1042/BC20100083](https://doi.org/10.1042/BC20100083)
17. Stirling JW, Graff PS (1995) Antigen unmasking for immunoelectron microscopy: labeling is improved by treating with sodium ethoxide or sodium metaperiodate, then heating on retrieval medium. *J Histochem Cytochem* 43(2):115–123
18. Beisker W, Dolbeare F, Gray JW (1987) An improved immunocytochemical procedure for high-sensitivity detection of incorporated bromodeoxyuridine. *Cytometry* 8(2):235–239. doi:[10.1002/cyto.990080218](https://doi.org/10.1002/cyto.990080218)

Histochemical Analysis of Plant Secretory Structures

Diego Demarco

Abstract

Histochemical analysis is essential for the study of plant secretory structures whose classification is based, at least partially, on the composition of their secretion. As each gland may produce one or more types of substances, a correct analysis of its secretion should be done using various histochemical tests to detect metabolites of different chemical classes. Here I describe some of the most used methods to detect carbohydrates, proteins, lipids, phenolic compounds, and alkaloids in the secretory structures.

Key words Histochemistry, Plant secretory structures, Secondary metabolites, Secretion, Classification, Function

1 Introduction

Many of the natural products used by man since ancient times are produced by plant secretory structures [1, 2]. These substances have the most varied functions in plants and may act in defense of vegetative and/or reproductive organs against herbivores and microorganisms, in attracting pollinators, in seed dispersal, or in the capture and digestion of prey in the case of carnivorous plants [3–5].

Secretory structures occur in the vast majority of vascular plants and differ from each other in structure, topographic position and secreted material. The secretion may be released to the surface of the gland, into specific intercellular spaces within the plant, or remain stored inside the secretory cell itself, but in all such cases the product does not usually return to be metabolized by the plant [1, 3].

The plant secretory structures can be formed by a single cell or by groups of cells within the plant body or on its surface; in this last case, they are usually called glands [1–3, 6, 7]. The classification of these structures is based on their (1) morphology (e.g., trichomes (Fig. 1a), idioblasts (Fig. 1b), cavities (Fig. 1c) or ducts (Fig. 1d)), (2) secreted substances or function of the gland (e.g., nectary

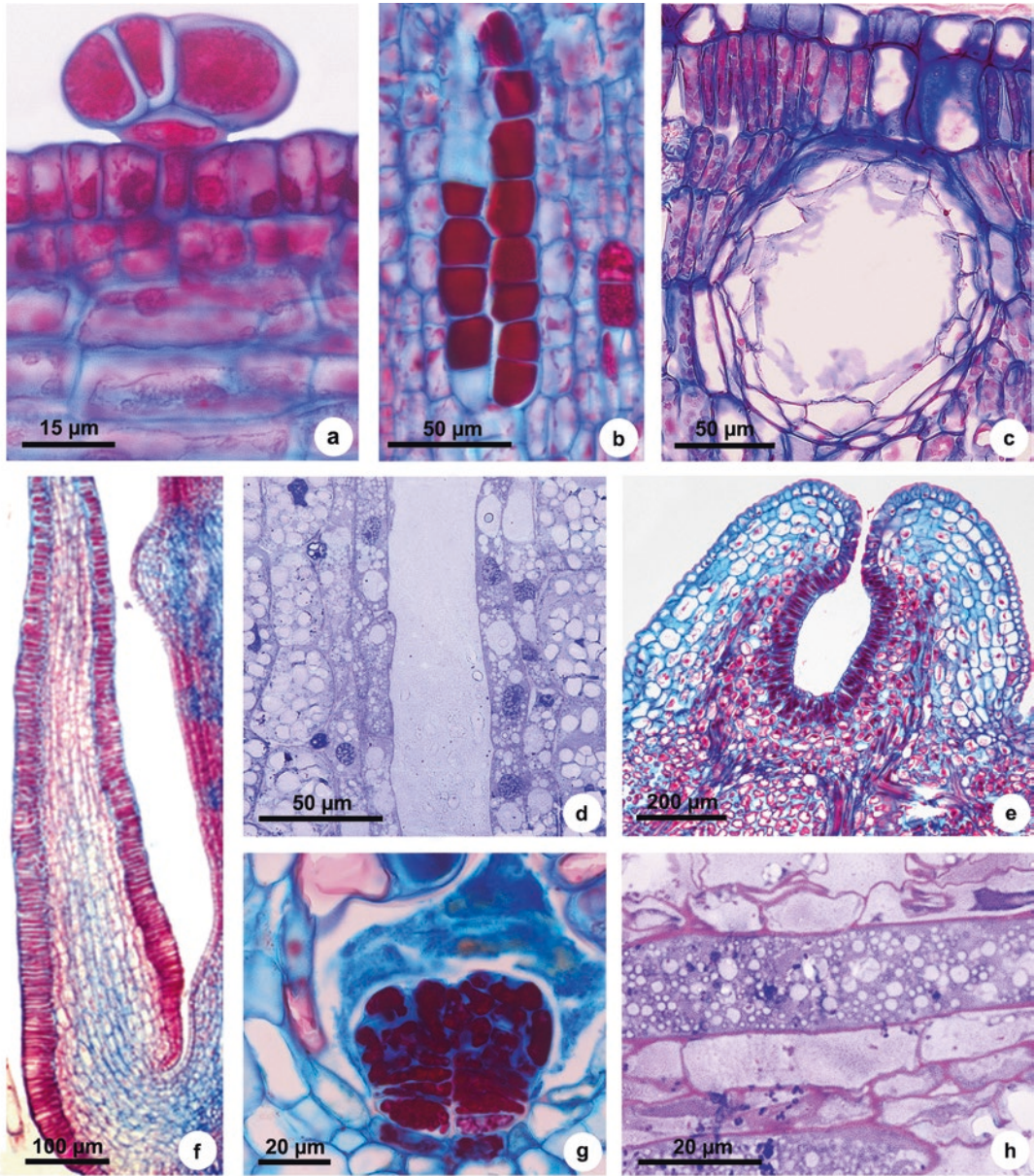


Fig. 1 Plant secretory structures. (a) Glandular trichome of *Ipomoea tricolor* Cav. (Convolvulaceae). (b) Secretory idioblasts of *Mussaenda erythrophylla* Schumach. & Thonn. (Rubiaceae). (c) Secretory cavity of *Eucalyptus* sp. (Myrtaceae). (d) Secretory duct of *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae). (e) Extrafloral nectary of *Sapium glandulatum* (Vell.) Pax (Euphorbiaceae). (f) Colleter of *Matelea denticulata* (Vahl) Fontella & E.A. Schwarz (Apocynaceae). (g) Salt gland of *Laguncularia racemosa* (L.) C.F. Gaertn. (Combretaceae). (h) Laticifer of *Ditassa gracilis* Hand.-Mazz. (Apocynaceae)

(Fig. 1e), hydathode, colleter (Fig. 1f), or salt gland (Fig. 1g)), and (3) both structure and secreted substances (e.g., laticifer (Fig. 1h)).

Even in the case of secretory structures classified based on their morphology, their subtype is based on the secretion produced,

which may be mucilaginous, oleiferous, resiniferous, etc. [2, 3, 7]. Therefore, the classification of these structures is always based on the secretion produced; to classify them correctly and verify their possible function, it is necessary to perform a histochemical analysis of their secretion.

Histochemistry is a methodological approach that allows the chemical analysis of cells and tissues in relation to their structural organization [8], but to achieve this objective for plant secretory structures, a wide histochemical analysis is necessary because the same gland and even the same glandular cell can produce several different metabolites simultaneously [1, 7, 9].

This high metabolic complexity typical of some secretory structures may be easily inferred from histochemical tests applied for several chemical classes of substances; therefore, all histochemical tests should be performed for a proper analysis and classification of the secretory structure [10].

As the histochemical analysis of plant secretory structures uses reagents and dyes that are not specific, certain precautions should be taken to correctly interpret the results: (1) the natural color of the secretion should be observed *in vivo* before applying the test (avoiding the use of reagents with the same color as the secretion), (2) attention should be paid to the color obtained in the staining since different colors can be generated in each test but the positive staining is specific, and (3) the control procedure should be carefully set up, which usually consists of removing the substance to be detected prior to the test application. The color of the test and that of the control are compared visually to interpret the result.

In general, it is necessary to section the material to analyze the plant secretory structures morphologically or histochemically. Although all the histochemical tests have been designed to be applied to fresh materials, it is often necessary to fix materials, embed them in paraffin or resin, and section them using a microtome prior to the testing due to the reduced size and fragility of some secretory structures. In this case, suitable fixatives must be used for each substance type at predetermined times, as described below. Except for a few histochemical tests, almost all tests can be applied to fixed materials without loss or alteration of the results.

The histochemical analysis of secretory structures is usually performed to detect carbohydrates, proteins, lipids, phenolic compounds and alkaloids, with some tests being more general and others more specific. Most substances produced by secretory structures may be detected by more than one histochemical method. These redundant tests are required for the histochemical study of secretory structures because the affinity of the dyes varies and the negative result of one test does not necessarily imply the absence of a given compound which may be identified by another dye or reagent. Therefore, all methods listed in this chapter and their respective controls should be performed for a conclusion about the composition of the gland secretion in histological sections.

2 Materials

All the procedures are to be performed at room temperature and all stains and reagents should be stored in amber bottles.

2.1 For Detecting Hydrophilic Substances

1. Ruthenium red solution: dissolve 0.1 g of Ruthenium red in 100 mL of distilled water. Store at 4 °C (*see Note 1*).
2. Alcian Blue solution: dissolve 1 g of Alcian Blue in 97 mL of distilled water and add 3 mL of Glacial acetic acid.
3. Tannic acid solution: dissolve 5 g of tannic acid plus 0.3 g of Phenol in 100 mL of distilled water.
4. Ferric chloride solution: dissolve 3 g of ferric chloride in 100 mL of distilled water.
5. Iodine–potassium iodide solution: dissolve 1 g of potassium iodide in 100 mL of distilled water, then add 1 g of iodine (*see Note 2*).
6. Safranin solution: dissolve 1 g of safranin O (C.I. 50240) in 100 mL of 50% ethanol.
7. Astra blue solution: dissolve 1 g of Astra blue and 2 g of Tartaric acid in 100 mL of distilled water containing 4 mL of 37% formaldehyde.
8. 1 N HCl: add carefully dropwise 8.3 mL of 37% hydrochloric acid in 91.7 mL of distilled water (*see Note 3*).
9. 1% sodium tetraborate solution: dissolve 1 g of sodium tetraborate decahydrate in 100 mL of distilled water.
10. 1% periodic acid: dissolve 1 g of periodic acid in 100 mL of distilled water.
11. Sodium metabisulfite solution: dissolve 1 g of sodium metabisulfite in 190 mL of distilled water, then add 10 mL of 1 N HCl.
12. Schiff's reagent: Dissolve 2 g pararosaniline (C.I. 42500) in 60 mL of 1 N HCl, and 2 g of sodium metabisulfite in 300 mL distilled water, mix the two solutions and let the mixture stand for 24 h in a tightly closed bottle; add 3 g of activated charcoal and stir until the solution becomes colorless; filter the solution and store at 4 °C in a bottle wrapped with aluminum foil (*see Note 4*).
13. Aniline blue solution: dissolve 0.05 g of Aniline blue (C.I. 42755) in 100 mL of 0.1 M sodium phosphate buffer, pH 8.0. Store at 4 °C (*see Note 5*).
14. Calcofluor white solution: dissolve 0.01 g calcofluor white MR2 in 100 mL of distilled water. Store at 4 °C.
15. Aniline blue black solution: dissolve 1 g of Aniline blue black (C.I. 20470) in 100 mL of 7% acetic acid.
16. Coomassie blue solution: dissolve 0.25 g of Coomassie blue in 100 mL of 7% acetic acid.

17. Solution for control of protein tests: 40 mL of acetic anhydride in 60 mL of pyridine.
18. Glycerin gelatin: dissolve 7.5 g of gelatin in 45.5 mL of distilled water (at 30–35 °C), then add 1 g of phenol and 53 mL of glycerin. Store at 4 °C (*see* **Note 6**).
19. Formalin-aceto-alcohol fixative: Mix 90 mL of 50% ethanol with 5 mL of glacial acetic acid and 5 mL of 37% formaldehyde.
20. Xylene-containing mounting medium.
21. Glass slides and coverslips.

2.2 For Detecting Lipophilic Substances

1. Buffered neutral formalin (BNF): mix 10 mL of 37% formaldehyde with 90 mL of 0.1 M sodium phosphate buffer, pH 7.0. Store at 4 °C.
2. Sudan black B solution: dissolve 0.5 g of Sudan black B (C.I. 26150) in 100 mL of 70% ethanol, filter the solution and store at 4 °C in a bottle wrapped with aluminum foil.
3. Extraction solution for lipids: mix 66 mL of methanol with 33 mL of chloroform, 4 mL of distilled water and 1 mL of 37% hydrochloric acid.
4. Sudan IV solution: dissolve 1 g of Sudan IV (C.I. 26105) in 100 mL of 80% ethanol plus 10 mL of glycerin.
5. Neutral red solution: dissolve 0.1 g of neutral red in 100 mL of 0.1 M sodium phosphate buffer, pH 6.5. Store at 4 °C.
6. Nile blue solution: dissolve 1 g of Nile blue (C.I. 51180) in 100 mL of distilled water and 5 mL of 1% sulfuric acid (*see* **Note 7**).
7. Copper acetate solution: dissolve 0.05 g of copper acetate in 100 mL of distilled water.
8. Rubeanic acid solution: dissolve 0.1 g rubeanic acid in 100 mL of 70% ethanol.
9. α -Naphthol solution: dissolve 0.01 g of α -naphthol in 10 mL of 40% ethanol. Store at 4 °C in a bottle wrapped with aluminum foil.
10. NADI reagent: mix 0.5 mL of 0.1% α -naphthol with 0.5 mL of 1% *N,N*-dimethyl-*p*-phenylenediamine and 49 mL of 0.1 M sodium phosphate buffer, pH 7.2. Store at 4 °C in a bottle wrapped with aluminum foil.
11. Ferric chloride solution: dissolve 10 g of ferric chloride in 100 mL of distilled water.
12. Potassium dichromate solution: dissolve 10 g of potassium dichromate in 100 mL of distilled water.
13. Ferrous sulfate–formalin fixative: dissolve 10 g of ferrous sulfate heptahydrate in 96 mL of distilled water plus 4.0 mL of 37% formaldehyde.

14. Vanillin solution: dissolve 0.5 g of vanillin in 100 mL of 9 % hydrochloric acid.
15. Phloroglucinol solution: dissolve 10 g of phloroglucinol in 100 mL of 95 % ethanol.
16. Acridine orange solution: dissolve 0.01 g of acridine orange in 100 mL of distilled water.
17. 12.5 % bismuth nitrate: dissolve 6.25 g of bismuth nitrate in 50 mL of 25 % acetic acid.
18. Bismuth nitrate stock solution for Dragendorff's reagent: mix 25 mL of 12.5 % bismuth nitrate with 10 mL of 40 % potassium iodide in water.
19. Dragendorff's reagent: mix 5 mL of bismuth nitrate stock solution with 10 mL of glacial acetic acid and 85 mL of distilled water.
20. Sodium nitrite solution: dissolve 5 g of sodium nitrite in 100 mL of distilled water.
21. Extraction solution for alkaloids: dissolve 5 g of tartaric acid in 100 mL of 95 % ethanol.
22. Wagner's reagent: dissolve 2 g of potassium iodide in 100 mL of distilled water, then add 1.27 g of iodine (*see* **Note 2**).

3 Methods

3.1 Detection of Hydrophilic Substances

All tests described below may be applied to fresh or fixed material. If it is necessary to use fixed material, the best fixative for hydrophilic substances is formalin-aceto-alcohol (FAA) [11]. For fixation, the material should be immersed in the FAA under vacuum for 24 h, then washed in 50 % ethanol overnight and stored in 70 % ethanol (*see* **Notes 8–10**).

3.1.1 Mucilage

This method stains acidic mucilages, pectins [11, 12], and nucleic acids magenta or red (Fig. 2a).

3.1.1.1 Ruthenium Red Staining

1. Apply 0.1 % ruthenium red to sections for 5 min.
2. Wash sections twice in distilled water to remove surplus stain.
3. Mount the sections between slide and coverslip with glycerin gelatin.

Fig. 2 (continued) (A.St.-Hil.) Ravenna (Malvaceae) by PAS reaction. **(g)** Identification of callose in the sieve tube element of the *Euphorbia milii* nectary by aniline blue under UV light. Note the blue autofluorescence of lignin in the vessel elements. **(h)** Fluorescence of cellulose in the trilamellar cell walls of *Peplonia axillaris* (Vell.) Fontella & Rapini (Apocynaceae) secretory idioblasts using calcofluor white under UV light. **(i)** Detection of protein secretion by digestive gland of *Drosera chrysolepis* Taub. (Droseraceae) by aniline blue black. **(j)** Identification of protein in the duct lumen of *Schinus terebinthifolius* Raddi (Anacardiaceae) by Coomassie blue

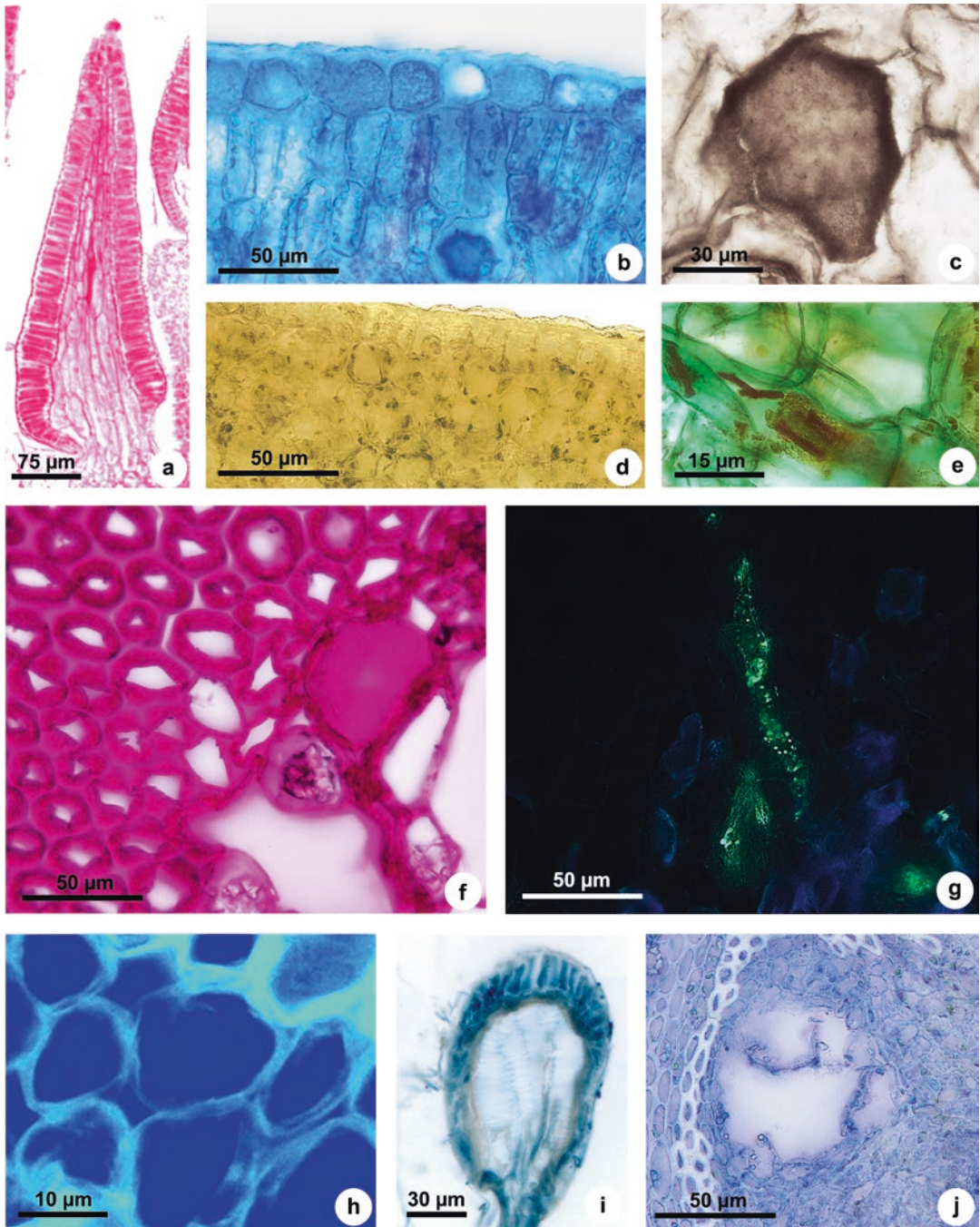


Fig. 2 Histochemical analysis of plant secretory structures. (a, c, g–i) Embedded material. (b, d–f, j) Fresh material. (a) Detection of acidic mucilage in the colleter of *Asclepias curassavica* L. (Apocynaceae) by ruthenium red. (b) Identification of acidic mucilage in the epidermis of *Rhododendron* sp. (Ericaceae) by Alcian Blue. (c) Positive result for mucilage in secretory idioblast of *Cattleya walkeriana* Gardner (Orchidaceae) using tannic acid and ferric chloride. (d) Starch grains in the nectary of *Inga edulis* Mart. (Fabaceae) detected by Lugol's reagent. (e) Observation of starch grains in laticifer of *Euphorbia millii* Des Moul. (Euphorbiaceae) and the tissue structure using the triple staining. (f) Detection of carbohydrates in the secretory idioblast of *Ceiba speciosa*

3.1.1.2 Alcian Blue
Staining

This test has a similar result as ruthenium red, staining acidic mucilages, pectins [13] and nucleic acids light blue (Fig. 2b).

1. Stain sections with 1 % Alcian Blue for 30 min.
2. Rinse sections twice with distilled water to remove surplus stain.
3. Mount the slide with glycerin gelatin.

3.1.1.3 Tannic Acid
and Ferric Chloride

This method is based on the reaction of tannic acid with mucilages [14] and pectins, substances which are further revealed by the addition of ferric chloride, producing a grey to black color (Fig. 2c).

1. Apply 5 % tannic acid for 20 min.
2. Rinse briefly with distilled water.
3. Submerge sections in 3 % ferric chloride for 5 min.
4. Wash twice in distilled water to remove surplus ferric chloride.
5. Mount the sections using glycerin gelatin.
6. Control: Compare the staining obtained in the test with that of sections treated only with tannic acid or with ferric chloride.

3.1.2 *Starch*

This reaction highlights the starch grains in dark blue to black (Fig. 2d) [11]. Almost all other structures stain yellow, but this color has no specific significance.

3.1.2.1 Lugol's Reagent

1. Submerge the sections in the Lugol's reagent for 10 min.
2. Rinse briefly with distilled water.
3. Mount the slides using distilled water or Lugol's reagent itself.

3.1.2.2 Triple Staining
for Starch Detection

This triple staining was developed to analyze structural tissue components and the starch grains concomitantly [15]. The application of safranin, astra blue and iodine–potassium iodide solution stains starch grains black, acidic substances (e.g., nucleic acids and lignin) brown, and non-lignified cell walls green (Fig. 2e).

1. Stain the sections with 1 % safranin for 1 min.
2. Rinse 3 times for few seconds in 50 % ethanol to remove surplus stain.
3. Stain with 1 % astra blue for 1 min.
4. Wash three times for few seconds in distilled water to remove surplus stain.
5. Apply the iodine–potassium iodide solution for 10 min.
6. Dip sections rapidly in distilled water.
7. Mount the slide with the smallest amount of water.

3.1.3 Carbohydrates

3.1.3.1 PAS Reaction (Periodic Acid: Schiff's reagent)

This method is based on the reaction of periodic acid with carbohydrates, forming carbonyl groups revealed by Schiff's reagent [16]. Carbohydrates stain magenta (Fig. 2f).

1. Apply 1 % sodium tetraborate (freshly prepared) for 30 min.
2. Transfer sections to 1 % periodic acid for 10 min.
3. Rinse briefly in distilled water.
4. Apply Schiff's reagent for 15 min in dark.
5. Wash the sections with sodium metabisulfite for 10 min.
6. Rinse in tap water for 10 min (*see* **Note 11**).
7. Mount the slides using glycerin gelatin.
8. Control: Repeat the test excluding **step 2** (periodic acid).

3.1.3.2 Aniline Blue Staining

This staining marks callose, which may be detected by a green fluorescence under UV light (Fig. 2g) [17].

1. Apply 0.05 % aniline blue for 10 min.
2. Rinse briefly in distilled water.
3. Mount the slide in the same buffer used for staining.

3.1.3.3 Calcofluor White Staining

This test is used to detect cellulose in cell walls, which fluoresces light blue under UV light (Fig. 2h) [18].

1. Place sections into 0.01 % calcofluor white for 10 min.
2. Rinse briefly in distilled water.
3. Mount in distilled water.

3.1.4 Proteins

3.1.4.1 Aniline Blue Black Staining

This stain reveals proteins in blue (Fig. 2i) [19], whether structural or acting in the primary or secondary metabolism.

1. Dip sections into 1 % aniline blue black for 1 min.
2. Wash twice in 0.5 % acetic acid to remove excess stain.
3. Rinse briefly in distilled water.
4. Dehydrate sections passing quickly through 90 %, 100 % ethanol, then a mixture of 100 % ethanol and xylene (1:1, v/v), and finally pure xylene.
5. Mount slides using synthetic resin.
6. Control: Put sections in a solution of acetic anhydride and pyridine (4:6, v/v) for 6 h prior to staining.

3.1.4.2 Coomassie Blue Staining

This method stains proteins blue (Fig. 2j) [19] and produces a similar result to aniline blue black.

1. Stain in 0.25 % Coomassie blue for 15 min.
2. Differentiate in 7 % acetic acid.

3. Rinse briefly in distilled water.
4. Mount in glycerin gelatin.
5. Control: Put sections in a solution of acetic anhydride and pyridine (4:6, v/v) for 6 h prior to staining.

3.2 Detection of Lipophilic Substances

The production of lipophilic compounds by plants is abundant and includes many types of substances in resin glands [20], oil cavities, and resin ducts, but other minority components may also be found. Resins may be composed of terpenes and/or phenolic compounds [21] but some resin ducts may secrete other compounds, such as proteins [22]. This heterogeneity of secretion is particularly common in the latex which may be composed of various types of lipids, carbohydrates, enzymes, phenolic compounds, and alkaloids [9].

3.2.1 Lipids (See Notes 12–15)

This is a general method which stains lipids dark blue to black (Fig. 3a) [13].

3.2.1.1 Sudan Black Staining

1. Stain with Sudan black B for 20 min.
2. Rinse briefly in 70 % ethanol.
3. Wash in distilled water.
4. Mount in glycerin gelatin.
5. Control: Sections should be kept in the extraction solution [23] for 6 h or more, depending on the composition of the secretion (determined empirically) (*see* **Note 16**). After this time, the sections should be transferred to distilled water and washed in a period of 4 h (4 × 1 h). Then, the staining proceeds as described.

3.2.1.2 Sudan IV Staining

Sudan IV also stains lipids, in general [13], which become red or red-orange (Fig. 3b).

1. Apply Sudan IV for 30 min.
2. Rinse briefly in 80 % ethanol.
3. Wash in distilled water.
4. Mount in glycerin gelatin.
5. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h (*see* **Note 16**).

3.2.1.3 Neutral Red Staining

This fluorochrome emits different colors depending on the lipid composition [24]. Under blue light, the lipids of secretion fluoresce yellow or green (Fig. 3f), cuticle fluoresces yellow and lignified cell walls fluoresce red.

1. Stain with 0.1 % neutral red for 20 min.
2. Rinse briefly in distilled water.
3. Mount in distilled water.
4. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h (*see* **Note 16**).

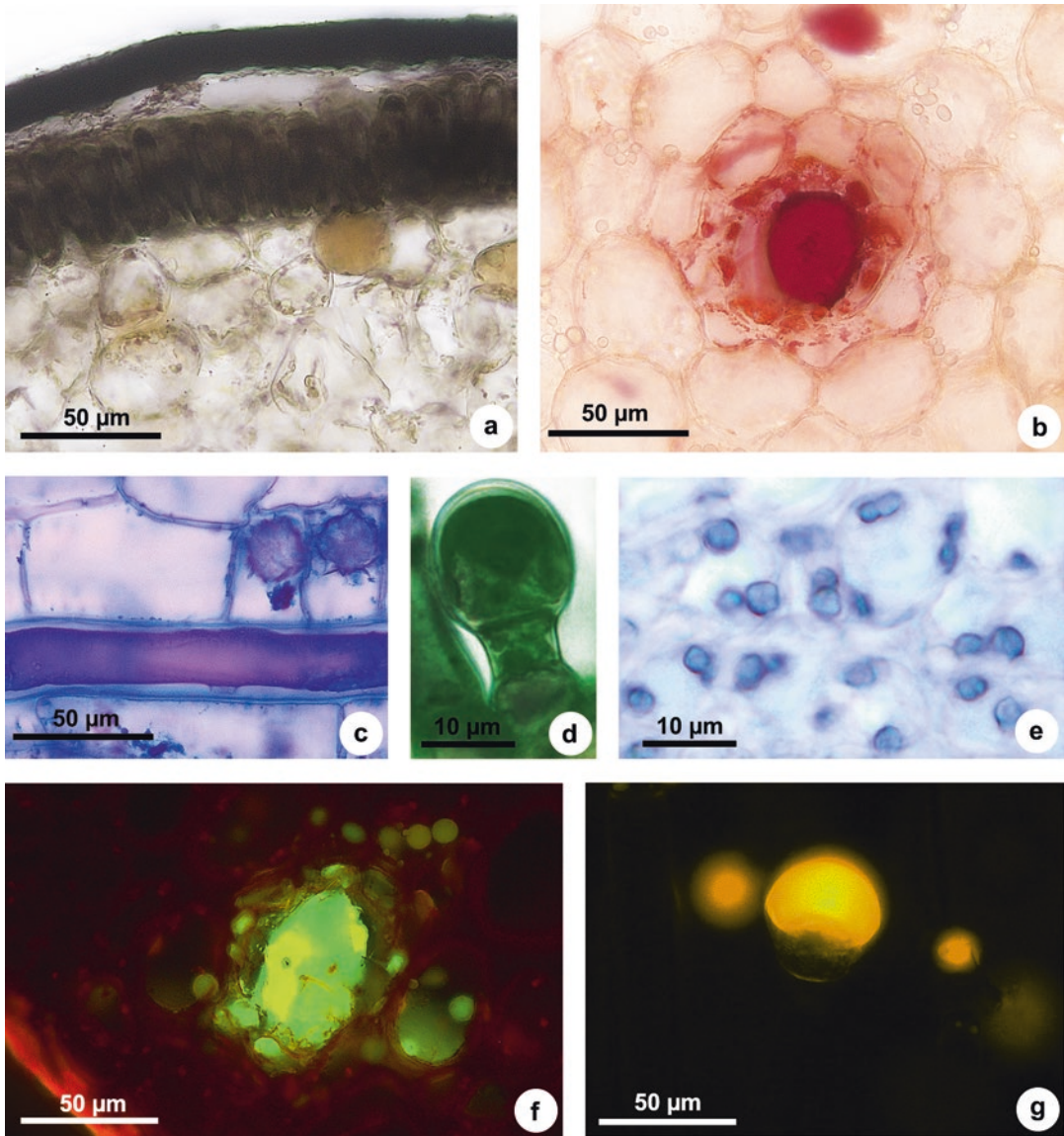


Fig. 3 Histochemical analysis of plant secretory structures. Fresh material. (a) Detection of lipids production by elaiophore of *Byrsonima intermedia* A. Juss. (Malpighiaceae) by Sudan black B. (b) Identification of lipids in the secretory duct of *Philodendron* sp. (Araceae) by Sudan IV. (c) Positive result for neutral lipids in the laticifer of *Sapium glandulatum* (Vell.) Pax (Euphorbiaceae) by Nile blue. (d) Observation of fatty acids in the glandular trichome of *Tetradenia riparia* (Hochst.) Codd (Lamiaceae) using copper acetate and rubeanic acid. (e) Detection of essential oils in the secretory idioblasts of *Peplonia axillaris* (Vell.) Fontella & Rapini (Apocynaceae) by NADI reagent. (f) Identification of lipids in secretory duct of *Kielmeyera appariciana* Saddi (Calophyllaceae) by neutral red under blue light. (g) Positive result for lipids in glandular trichome of *Tetradenia riparia* by Nile blue under blue light

3.2.2 Acidic and Neutral Lipids

3.2.2.1 Nile Blue Staining (See Note 17)

Since lipids were detected in the material, Nile blue distinguishes acidic lipids, which stain blue, from neutral lipids, which stain pink (Fig. 3c) [25].

1. Stain with Nile blue solution for 5 min at 60 °C.
2. Wash twice with 1 % acetic acid at 60 °C.

3. Rinse in distilled water.
4. Mount in glycerin gelatin.
5. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h (*see* **Note 16**).

3.2.3 Fatty Acids

3.2.3.1 Copper Acetate and Rubeanic Acid Staining

This method for lipids is slightly more specific than the Sudan tests and identifies fatty acids through the reaction of copper acetate with these acidic lipids, which subsequently turn dark green when exposed to rubeanic acid (Fig. 3d) [26, 27].

1. Treat sections with 0.05 % copper acetate for 3 h.
2. Apply 0.1 M Na₂ EDTA (EDTA acid disodium salt solution) for 5 min.
3. Wash in distilled water for 5 min.
4. Transfer sections into 0.1 % rubeanic acid (freshly prepared) for 20 min.
5. Wash in 70 % ethanol for 5 min.
6. Rinse in distilled water.
7. Mount in glycerin gelatin.
8. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h (*see* **Note 16**).

3.2.4 Terpenes

3.2.4.1 NADI Reaction

This reagent produces differential staining [28], with essential oils (Fig. 3e; monoterpenes and sesquiterpenes) staining blue and resins (diterpenes, triterpenes, tetraterpenes and derivatives) staining red. Mixtures of essential oils and resins produce varied shades of violet to purple, depending on the prevalence of each compound (*see* **Note 12**).

1. Apply NADI reagent for 1 h in the dark.
2. Wash in sodium phosphate buffer (0.1 M, pH 7.2) for 2 min.
3. Mount in the same buffer.
4. Control: As with Sudan black B, the sections should be kept in the extraction solution for at least 6 h.

3.3 Detection of Phenolic Compounds and Alkaloids

3.3.1 Phenolic Compounds

3.3.1.1 Ferric Chloride Staining

This method highlights phenolic compounds through iron precipitation, producing a dark color [11], usually black (Fig. 4a), sometimes brown.

1. Apply 10 % ferric chloride for 30 min.
2. Wash twice in distilled water to remove surplus ferric chloride.
3. Mount in glycerin gelatin.

3.3.1.2 Potassium Dichromate Staining

This method also highlights phenolic compounds, in general [29], producing a brown or red-brown color (Fig. 4b).

1. Apply 10 % potassium dichromate for 30 min.
2. Wash twice in distilled water to remove surplus reagent.
3. Mount in glycerin gelatin.

3.3.1.3 Ferrous Sulfate–Formalin Fixation

The best method to detect phenolic compounds is to introduce iron salts into the fixative since the iron compound fixes and stains the phenolic compounds (Fig. 4c) [11].

1. The samples should be fixed in the ferrous sulfate–formalin solution under vacuum for 48 h.
2. Wash 4 × 2 h (totaling 8 h) in distilled water.
3. Dehydrate the material in 30 %, 50 %, 70 % ethanol for 12 h each.
4. Embed the material according to the chosen technique (Paraplast, Historesin, or PEG) and then section in a microtome (*see* **Note 18**).

3.3.1.4 Vanillin– Hydrochloric Acid Staining for Tannins

This test is more specific for some phenolic compounds, staining tannins red (Fig. 4d) [30]. Use only sections of fresh material.

1. Treat with 0.5 % vanillin for 20 min.
2. Mount the slide using 9 % hydrochloric acid.

3.3.1.5 Phloroglucinol– Hydrochloric Acid Staining for Lignin

Phloroglucinol in an acidic medium stains lignin in cell walls pink to red (Fig. 4e) [11]. It is possible to use either fresh or embedded material.

1. Apply 10 % phloroglucinol for 15 min.
2. Mount the slides carefully with 25 % hydrochloric acid.

3.3.1.6 Acridine Orange

This fluorescent dye is useful to identify several acidic compounds under blue light, such as nucleic acids and components of the cell wall [31], distinguishing lignified cell walls (yellow-green fluorescence) from non-lignified cell walls (red fluorescence; Fig. 4g). It is possible to use fresh material as well as embedded material in this test.

1. Apply 0.01 % acridine orange for 20 min.
2. Mount the slides with distilled water.

3.3.1.7 Autofluorescence

Plant tissues have several autofluorescent components which permit their analysis under UV radiation [32] (*see* **Note 19**). In relation to secondary metabolites, many phenolic compounds (including lignin) emit a blue or blue-green fluorescence (Fig. 2g, 4f).

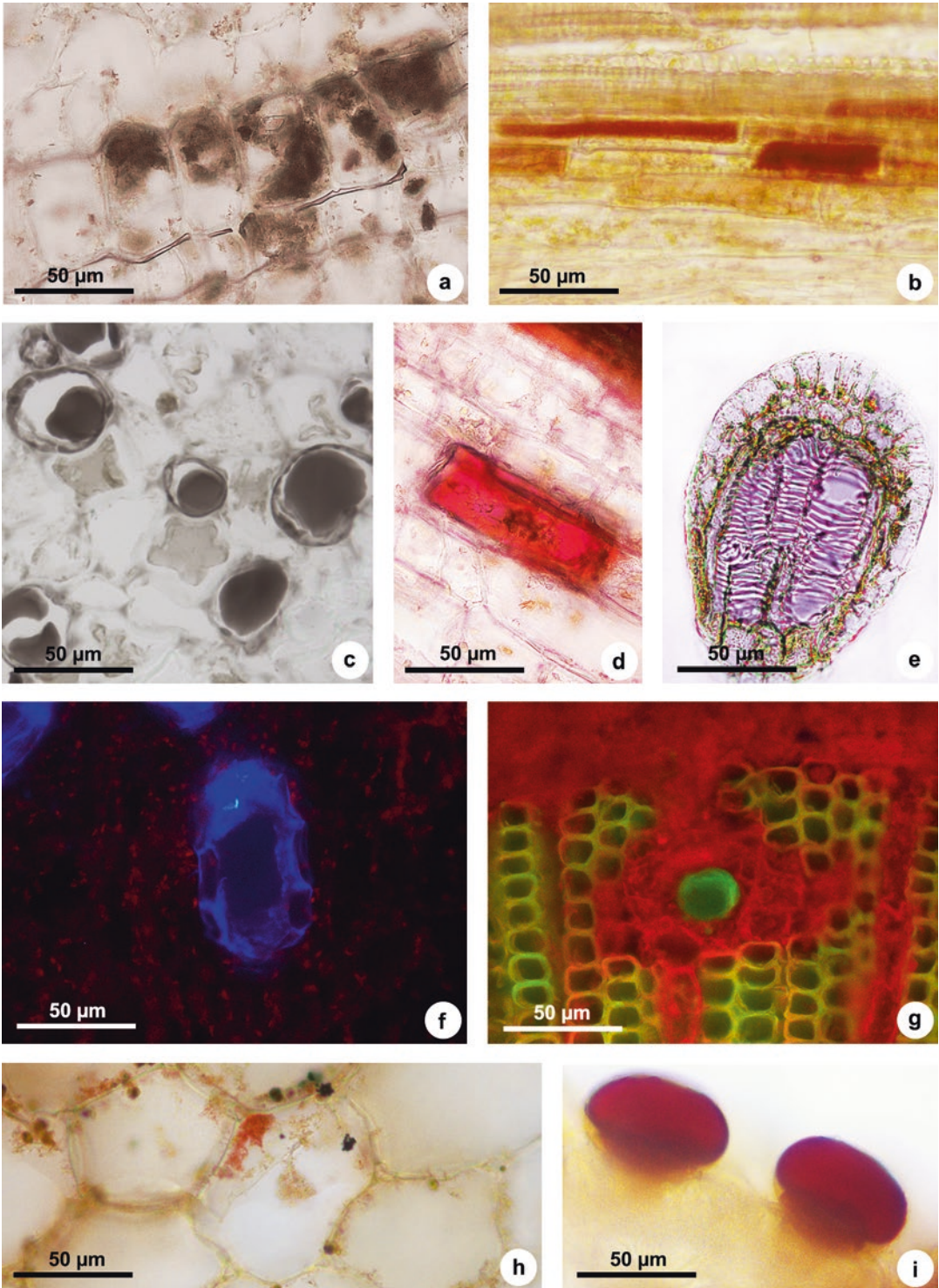


Fig. 4 Histochemical analysis of plant secretory structures. (a, b, d, f–i) Fresh material. (c, e) Embedded material. (a–c) Detection of phenolic compounds in secretory idioblasts. (a) Ferric chloride. *Acalypha amentacea* Roxb. (Euphorbiaceae). (b) Potassium dichromate. *Calliandra tweediei* Benth. (Fabaceae). (c) Ferrous sulfate in formalin.

However, it is necessary to be cautious in identifying compounds through autofluorescence because some alkaloids and terpenoids may also emit fluorescence in the blue band [32].

3.3.2 Alkaloids

3.3.2.1 Dragendorff's Reagent

This reagent marks alkaloids in red-brown (Fig. 4h) [33]. Fresh and fixed material may be used in this method, but fixed material shows a considerably loss of the alkaloids and the staining color when compared to fresh material (*see* **Note 20**).

1. Treat with Dragendorff's reagent for 20 min.
2. Rinse briefly in 5 % sodium nitrite.
3. Mount in distilled water.
4. Control: Treat sections with 5 % tartaric acid in 95 % ethanol for 72 h and repeat the staining procedure.

3.3.2.2 Wagner's Reagent

This method also stains alkaloids red or red-brown (Fig. 4i) [34]. It is recommended that fresh material be used for this test.

1. Apply Wagner's reagent for 20 min.
2. Rinse briefly in distilled water.
3. Mount in distilled water.
4. Control: Treat sections with 5 % tartaric acid in 95 % ethanol for 72 h and repeat the staining procedure.

4 Notes

1. Check the pH of the distilled water before preparing the stain. The pH should be neutral. The stain may be stored at 4 °C for a few months.
2. Iodine does not dissolve immediately. Therefore, stir the solution and let it stand from some hours to some days in dark until iodine dissolves completely.
3. Hydrochloric acid must be added drop by drop to the water under agitation. After the addition of first drops, the solution

Fig. 4 (continued) *Sapium glandulatum* (Vell.) Pax (Euphorbiaceae). **(d)** Identification of tannin in the secretory idioblast of *Caesalpinia echinata* Lam. (Fabaceae) by vanillin—hydrochloric acid. **(e)** Positive result for lignin in the vessel elements of the digestive gland of *Drosera chrysolepis* Taub. (Droseraceae) by phloroglucinol—hydrochloric acid. **(f)** Blue autofluorescence of phenolic compounds produced by secretory cavities of *Eucalyptus* sp. (Myrtaceae) under UV light. **(g)** Red fluorescence of non-lignified cell walls of the secretory duct of *Pinus palustris* Mill. (Pinaceae) and yellow-green fluorescence of lignified cell walls of the tracheids using acridine orange under blue light. **(h)** Detection of alkaloids in parenchyma cell of *Datura suaveolens* Humb. & Bonpl. ex Willd. (Solanaceae) by Dragendorff's reagent. **(i)** Identification of alkaloids in secretory trichome of *Minthostachys mollis* (Kunth) Griseb. (Lamiaceae) by Wagner's reagent

releases heat, warming the flask. Wait until the flask cools down to continue.

4. With time and use, the Schiff's reagent starts to become pink, loses its effectiveness and must be discarded.
5. Use the stain solution freshly prepared or stored for a few weeks. The solution should be light blue; if dark blue, the solution is no longer suitable for testing.
6. To mount the slides, melt the glycerin gelatin in a hot water bath.
7. Boil the solution for 4 h before filtering and storing.
8. The material may be embedded in Paraplast, Historesin, or polyethylene glycol (PEG) according the usual techniques.
9. Due to the thinner thickness of the embedded material sectioned in a microtome in relation to the freehand sections, the times of each histochemical test described here should be doubled, except in the case of ruthenium red because excessive time in this staining causes loss of the sections color.
10. Hydrophilic compounds are produced as the predominant components of the secretion of several secretory structures, such as colleter, digestive glands, and nectaries. However, the exclusive exudation of carbohydrates usually causes proliferation of microorganisms and some lipophilic compounds and/or phenolics are commonly released by these glands [5]. Therefore, the histochemical analysis should not be limited to the identification of only certain types of substances.
11. The longer the time in tap water, more magenta are the sections. This time to enhance the color may be extended to some hours, if necessary.
12. A priori, the materials used for detection of lipophilic substances may be fixed; however, the fixation interferes with the methods for fluorescence microscopy as well as the test using NADI reagent. Therefore, only fresh material should be used in these tests.
13. The embedding may be performed in Paraplast, Historesin, or PEG, but it is important to keep in mind that the Sudan stains are prepared in alcohol and Historesin shrinks in the presence of alcohol.
14. The fixative buffered neutral formalin (BNF) [35] gives excellent results for the tests for lipophilic substances (except the NADI reagent), but glutaraldehyde or Karnovsky's solution may also be used.
15. The staining time for sections obtained from embedded material should be doubled only for the methods using Sudan stains.
16. When using fixed material, the extraction of lipids for performing the control must be done before the fixation.

17. Nile blue staining may be observed in bright field, distinguishing between acidic and neutral lipids, or in fluorescence microscopy [36] under blue light, producing a yellow fluorescence when in the presence of lipids (Fig. 3g). The procedure and control for this observation in fluorescence are the same as previously described for this stain.
18. In the case of Paraplast and PEG, the embedding medium should be removed prior to observation.
19. Only sections of fresh material mounted in distilled water or glycerin should be used in autofluorescence analysis.
20. Use Dragendorff's reagent freshly prepared or stored for a few weeks preferably.

Acknowledgments

I thank the Laboratory of Plant Anatomy at the Institute of Biosciences of the University of São Paulo, where the histochemical analyses were performed.

References

1. Fahn A (1988) Secretory tissues in vascular plants. *New Phytol* 108:229–257
2. Dickison WC (2000) Integrative plant anatomy. Academic, San Diego, CA
3. Fahn A (1979) Secretory tissues in plants. Academic, London
4. Demarco D, Carmello-Guerreiro SM (2011) Pericarp ontogeny and histochemistry of the exotesta and pseudocaruncle of *Euphorbia milii* (Euphorbiaceae). *Rodriguésia* 62:477–489
5. Lacchia APS, Tölke EEAD, Carmello-Guerreiro SM, Ascensão L, Demarco D (2016) Foliar colleters in Anacardiaceae: first report for the family. *Botany* 94:337–346
6. Lüttge U (1971) Structure and function of plant glands. *Ann Rev Plant Physiol* 22:23–44
7. Evert RF (2006) Esau's plant anatomy, 3rd edn. John Wiley & Sons, Hoboken, NJ
8. Coleman R (2000) The impact of histochemistry – a historical perspective. *Acta Histochem* 102:5–14
9. Demarco D (2015) Micromorfología y histoquímica de los laticíferos de órganos vegetativos de especies de Asclepiadoideae (Apocynaceae). *Acta Biol Colomb* 20:57–65
10. Demarco D (2008) Glândulas de órgãos vegetativos aéreos e florais de espécies de Asclepiadoideae (R.Br.) Duby (Asclepiadoideae, Apocynaceae) de Mata Atlântica do estado de São Paulo. Doctoral thesis, Universidade Estadual de Campinas
11. Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York, NY
12. Gregory M, Baas P (1989) A survey of mucilage cells in vegetative organs of the dicotyledons. *Israel J Bot* 38:125–174
13. Pearse AGE (1985) Histochemistry: theoretical and applied, vol 2, 4th ed., C. Livingstone, Edinburgh
14. Pizzolato TD (1977) Staining of *Tilia* mucilages with Mayer's tannic acid-ferric chloride. *Bull Torrey Bot Club* 104:277–279
15. Demarco D, Castro MM, Ascensão L (2013) Two laticifer systems in *Sapium baematospermum* – new records for Euphorbiaceae. *Botany* 91:545–554
16. McManus JFA (1948) Histological and histochemical uses of periodic acid. *Stain Technol* 23:99–108
17. Smith MM, McCully ME (1978) A critical evaluation of the specificity of aniline blue induce fluorescence. *Protoplasma* 95:229–254
18. Hughes J, McCully ME (1975) The use of an optical brightener in the study of plant structure. *Stain Technol* 50:319–329
19. Fisher DB (1968) Protein staining of ribboned epon sections for light microscopy. *Histochemie* 16:92–96

20. Gagliardi KB, Cordeiro I, Demarco D (2016) Protection and attraction: bracts and secretory structures in reduced inflorescences of Malpighiales. *Flora* 220:52–62
21. Langenheim JH (2003) Plant resins: chemistry, evolution, ecology, and ethnobotany. Timber Press, Portland, OR
22. Joel DM, Fahn A (1980) Ultrastructure of the resin ducts of *Mangifera indica* L. (Anacardiaceae). III. Secretion of the protein-polysaccharide mucilage in the fruit. *Ann Bot* 46:785–790
23. High OB (1984) Lipid histochemistry. Oxford University Press, New York, NY
24. Kirk PW Jr (1970) Neutral red as a lipid fluorochrome. *Stain Technol* 45:1–4
25. Cain AJ (1947) The use of Nile Blue in the examination of lipids. *Quart J Microsc Sci* 88:383–392
26. Ganter P, Jollés G (1969) *Histochimie normale et pathologique*, vol 1. Gauthier-Villars, Paris
27. Ganter P, Jollés G (1970) *Histochimie normale et pathologique*, vol 2. Gauthier-Villars, Paris
28. David R, Carde JP (1964) Coloration différentielle des inclusions lipidique et terpéniques des pseudophylles du Pin maritime au moyen du reactif Nadi. *C R Acad Sci Ser D* 258:1338–1340
29. Gabe M (1968) *Techniques histologiques*. Masson & Cie, Paris
30. Mace ME, Howell CR (1974) Histochemistry and identification of condensed tannin precursor in roots of cotton seedlings. *Can J Bot* 52:2423–2426
31. Ruzin SE (1999) *Plant microtechnique and microscopy*. Oxford University Press, New York, NY
32. Talamond P, Verdeil JL, Conéjéro G (2015) Secondary metabolite localization by autofluorescence in living plant cells. *Molecules* 20:5024–5037
33. Svendsen AB, Verpoorte R (1983) *Chromatography of alkaloids*. Elsevier Scientific Publishing Company, New York, NY
34. Furr M, Mahlberg PG (1981) Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. *J Nat Prod* 44:153–159
35. Lillie RD (1965) *Histopathologic technic and practical histochemistry*, 3rd edn. McGraw-Hill, New York, NY
36. Bozzo L, Vidal BC (1968) Nile blue sulphate and brilliant cresyl blue in fluorescence methods for the histochemical demonstration of lipids. *Ann Histochim* 13:177–183

A Histochemical Technique for the Detection of Annonaceous Acetogenins

Guillermo Laguna-Hernández, Alicia Enriqueta Brechú-Franco, Iván De la Cruz-Chacón, and Alma Rosa González-Esquinca

Abstract

Annonaceous acetogenins (ACGs) are molecules with carbon numbers C35–C37, usually with tetrahydrofuran and tetrahydropyran rings and one terminal γ -lactone (usually α,β -unsaturated), in a large aliphatic chain that is varyingly hydroxylated, acetoxylation or ketonized. ACGs have ecological functions as insecticides and are pharmacologically promising due to their cytotoxic and antitumoral properties. They are found in the seeds, leaves, roots, flowers and fruits of annonaceous plants and can be detected during isolation via thin-layer chromatography using Kedde's reagent, which reacts with the unsaturated lactone. This chapter describes the location in situ of ACGs in fresh sections of annonaceous seeds using Kedde's reagent.

The acetogenins are located in the idioblasts, in the endosperm and in the embryonic axis during differentiation. This method can aid in the detection of ACGs with a terminal unsaturated γ -lactone in organs and tissues.

Key words Acetogenins, Annonaceae, Kedde's reagent, Idioblasts, Germinating seeds, Histochemistry

1 Introduction

The plants in the Annonaceae family are characterized by their ability to biosynthesize certain secondary metabolites, including alkaloids, essential oils and phenols that are common to different botanical clades, as well as “annonaceous acetogenins” (ACGs), which are exclusive to this family. ACGs were first discovered by Jolad et al. [1] with the identification of the polyketide Uvaricin, which was isolated from the roots of *Uvaria acuminata* and was the first of the ACGs to be described. The ACGs are molecules with 35- to 37-carbon atom chains. They typically consist of a long aliphatic chain with 1–3 tetrahydrofuran (THF) rings in their central region, and sometimes, tetrahydropyran rings. The chain also presents several oxygenated functional groups and invariably one terminal α,β -unsaturated or saturated γ -lactone, which sometimes is rearranged as ketolactone. The biosynthetic origin of the ACGs

is unknown, but a similarity to fatty acids has been suggested, as well as the possibility that they could originate from large chain fatty acids (32–34 carbons) combined with a molecule of 2-propanol [2–5].

ACGs have been isolated from several plant parts, but principally from the seeds, which are rich in oils. To isolate and purify the ACGs, classic extraction and chromatography techniques have been used. They can be visualized by thin-layer chromatography by inducing a reaction with Kedde's reagent [2, 3].

This reagent was originally developed for the detection of specific types of steroids, including cardenolides and bufadienolides, both of which are cardiac glycosides, and for 17-ketosteroids [6, 7]. All of these substances have a basic steroid structure, as well as a fifth ring with a lactone functional group with five (γ -lactone in cardenolides) or six atoms (δ -lactone in bufadienolides), or a simple ketone functional group (17-ketosteroids). The cardenolides with a γ -lactone group react with greater intensity. Cardiac glycosides are present in many plant families, such as Asclepiadaceae (*Gongronema gazense* (S. Moore) Bullock) [8], Scrophulariaceae (*Digitalis* species), and Apocynaceae (*Strophanthus* species) [9]. These digitaloids exhibit a five-membered lactone ring such as that in the acetogenins. Rupprecht et al. [2] also reported that all linear acetogenins have a characteristic γ -lactone subunit.

Kedde's reagent, a color-forming agent, has been used to detect unsaturated lactones with a reaction mechanism similar to the Zimmermann reaction, in which the C-methylene from the γ -lactone forms an intermediate anion in an alkaline medium, which is nucleophilically added to the 3,5-dinitrobenzoic acid in ortho position relative to the two other nitro groups. This results in a C–C bond, which produces a mesomerically stabilized pink to violet-colored anion known as a cyclohexadiene-type Meisenheimer complex [10, 11]. Although they are unstable, these colorful complexes can be photometrically measured at 485 nm for cardenolides and at 550 nm for 17-ketosteroids. Several reaction mechanisms have been suggested [12, 13].

Since their discovery, ACGs have been detected using Kedde's reagent, which produces a pink to violet color when there is a positive reaction [2, 3]. According to Alali et al. [4] Kedde's reagent detects conjugated unsaturated lactones but not translactonised ketolactones. It can be argued that this reagent was used initially for the detection and quantification of cardiac glycosides, cardenolides, digitaloids, and the first molecules that later formed the group acetogenins, as were: goniotalamin, and altholactone a novel tetrahydrofuro-pyran-5-one, biogenetically related to a number of α -pyrones of the Lauraceae, Annonaceae, and Piperaceae [14] (see Note 1).

In the review we performed about the use in chromatography of Kedde's reagent, at least six different formulations have been

reported: the first original report, in 1947 [7]; Kirchner, in 1967 [6]; Zweig and Sherma, in 1972 [15]; Jork et al., in 1990 [11]; Wagner and Bladt, in 1996 [16]; Houghton and Raman, in 1998 [17]; Parellada, in 2013 [18].

The plant species used in the present work have an active secondary metabolism. In particular, several ACGs have been isolated from their seeds, leaves, stems, and roots, and the production of secondary metabolites in the first stages of plant development, just a few days after seed germination, has been demonstrated [3, 5, 19–23].

The acetogenins laherradurine, cherimoline-2, rolliniastatin-2, and isorolliniastatin-2 were isolated from hexanic extracts of seeds of *Annona macrophyllata*; together, these acetogenins correspond to 0.1 % of the endosperm weight [20]. The stereochemical structure of all ACGs, among other characteristics, was identified and described by Rupprecht et al. [2] in a review of ACGs.

This research will expand the technique reported in Laguna-Hernández et al. [24] and Brechu-Franco et al. [25], and might help to discover other possibilities to apply histochemically Kedde's reagent which has first been used for chromatography.

2 Materials

2.1 Plant Materials

A. macrophyllata Donn. Sm, *A. purpurea* Moc. & Sessé ex Dunal, and *A. lutescens* Saff seeds were used, collected in San Lucas, Chiapas, Mexico. The herborized samples were collected in 2013 and were deposited in the Eizi Matuda Herbarium at Universidad de Ciencias y Artes de Chiapas, Tuxtla Gutiérrez, Chiapas, Mexico under reference voucher #354, #345 and #352, respectively.

The seeds were germinated in Conviron environmental chambers that were maintained at 24–28 °C with 12 h light/dark cycles and a relative humidity of approximately 65–75 %. Light was delivered by cool white ultra-high-output fluorescent lamps at an intensity of 500 mM/m²/s [26].

Seeds of *A. macrophyllata*, *A. purpurea*, and *A. lutescens* were used. The seeds were germinated, had their seed coat removed, and were imbibed for 12 days until the radicle reached approximately 2 cm in length and embryonic metabolism could be completely established [24]. The germinated seeds were dissected, with the embryonic axis being separated from the endosperm.

2.2 Reagents and Equipment

1. AGF fixative: 1.5 % acrolein/3 % glutaraldehyde/1.5 % formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4.
2. Kedde's reagent: 3,5-dinitrobenzoic acid (3,5-DNBA is prepared at 2% in ethanol, and the solution of NaOH is 1 N with a mixing ratio of 1:1 [7].

3. Tissue-Tek adhesive gel.
4. Cryostat.
5. Glass slides and coverslips.

3 Methods

3.1 Procedure

Tests were conducted at room temperature, approximately 23 °C.

1. Cut in half the endosperms transversally, or separate the embryonic axes from the radicle and hypocotyl and cut them longitudinally. The endosperm halves or the longitudinally cut embryonic axes can either be placed in water until sectioning, or immediately fixed in AGF for 12 h at room temperature.
2. After fixation, wash the pieces with several changes of distilled water for 2 h, and leave them in the last-wash water until sectioning.
3. Mount the fixed and non-fixed pieces on Tissue-Tek adhesive gel and then cut into 20 µm thick using a cryostat between −14 and −18 °C. The sections are placed on slides and may be kept dry as long as 1 week before exposing them to Kedde's reagent.
4. Once placed on the slides, the sections must be washed three times with distilled water for 5 min in each wash or until the gel is completely removed (*see* **Notes 2** and **3**).
5. Remove excess water from the section with filter paper. Depending on the size of the section, add two or three drops of Kedde's reagent, and coverslip the sections.
6. Immediately observe the samples under a microscope for 10 min, with the reaction being timed and documented with photography (*see* **Note 4**).

3.2 Observation

A positive reaction of ACGs with Kedde's reagent appears as pink color droplets in the non-fixed and fixed sections, located in large cells known as idioblasts, which are at the periphery of the endosperm, next to the tegmen of the seed coat. A positive reaction occurs in the endosperm of the seeds of all three species, in the same manner and location (Fig. 1a–c). ACGs are located in the radicle and hypocotyl sections, in the same cells—idioblasts—that will differentiate during development of the embryonic axis, forming lines in the cortical parenchyma (Fig. 1d–f). The positive reaction of the acetogenins in the idioblasts appears more clearly defined in the material fixed with AGF.

The presence of cardiac glycosides (cardenolides and bufadienolides) has not been reported in Annonaceae species. Thus, the staining observed in the histological preparations can be exclusively attributed to the presence of the unsaturated γ -lactone ACGs of the species studied here (Table 1) [3, 5, 18, 23, 27–30].

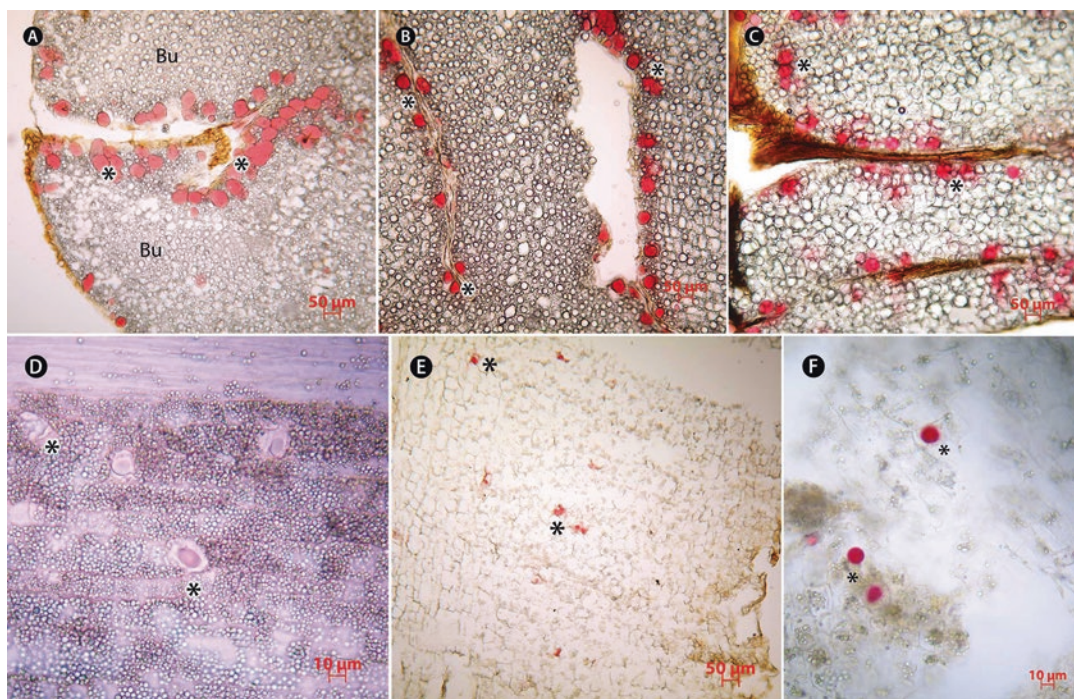


Fig. 1 Parts **a–c**: Kedde's reagent with 2% 3,5-DNBA reagent prepared in ethanol and mixed in equal parts with 1 N NaOH in water, positively reacts with the acetogenins in the idioblasts of the endosperm of fresh, unfixed sections of the three species here studied, yielding a *pink color*. 1A *A. macrophyllata*, 1B *A. purpurea*, 1C *A. lutescens*. Parts **d–f**: Kedde's reagent positively reacts with the acetogenins in the differentiating idioblasts of the embryonic axis of fresh, AGF fixed sections, yielding a *pink color*. Bubbles (Bu), Kedde's positive reaction (*asterisk*)

4 Notes

1. Even though there are secondary compounds isolated from Lauraceae and red algae that are also known as acetogenins, these acetogenins are polyketide in nature, with different structures. None of these compounds presented unsaturated or saturated lactones; therefore, they would not show a positive reaction with Kedde's reagent [31, 32].
2. Washing the slices to remove the "Tissue-Tek" adhesive gel is important to prevent the cells from being blurred or obscured by bubbles (Fig. 1a).
3. Free-hand fresh sections may also be obtained from the seeds' endosperm and must be placed directly on slides with some drops of water. These sections are immediately processed with

Table 1**Acetogenins isolated from *Annona* species of this study [3, 5, 18, 23, 27–30]**

Species	ACG	Organ	ACG type	Lactone type
<i>Annona purpurea</i>	Purpuracenin	Seed	Adjacent bis-THF	Unsaturated; A
	Purpurenin	Seed	Adjacent bis-THF	Unsaturated; A
	Purpurediolin	Seed	Adjacent bis-THF	Unsaturated; A
	Bullatalicin or Cherimolin-1	Leaf	No adjacent bis-THF	Unsaturated; A
	Purpureacin-2	Leaf	Adjacent bis-THF	Unsaturated; A
	Rolliniastatin-1	Leaf	Adjacent bis-THF	Unsaturated; A
	Rolliniastatin-2 or Bullatacin	Leaf	Adjacent bis-THF	Unsaturated; A
	Rollitacin	Seed	Adjacent bis-THF	Unsaturated; A
	Sylvaticin	Leaf	No adjacent bis-THF	Unsaturated; A
<i>Annona macrophyllata</i>	Laherradurin	Seed	Adjacent bis-THF	Saturated; C
	Rolliniastatin-2	Seed	Adjacent bis-THF	Unsaturated; A
	Cherimolin-2	Seed	No adjacent bis-THF	Unsaturated; A
<i>Annona lutescens</i>	Isoannoreticuin	Seed	Mono-THF	2-acetonil saturated; B1
	Laherradurin	Seed	Adjacent bis-THF	Saturated; C
	Rolliniastatin-2	Seed	Adjacent bis-THF	Unsaturated; A

the Kedde's reagent. Free-hand sections are thicker than those obtained by cryostat, so ACGs content was higher and the reaction more intense (Fig. 2a, b)

4. The color intensity and permanency in the timed reaction with Kedde's reagent were up to 8 min (Fig. 2e, f).

Acknowledgments

The authors thank Dr. Patricia Rivas Manzano and Biol. Carlos Tonatuih Chavira Ramírez for technical assistance with the cryostat sectioning in the Laboratory of Tissue and Reproductive Biology, Faculty of Sciences, National Autonomous University of Mexico.

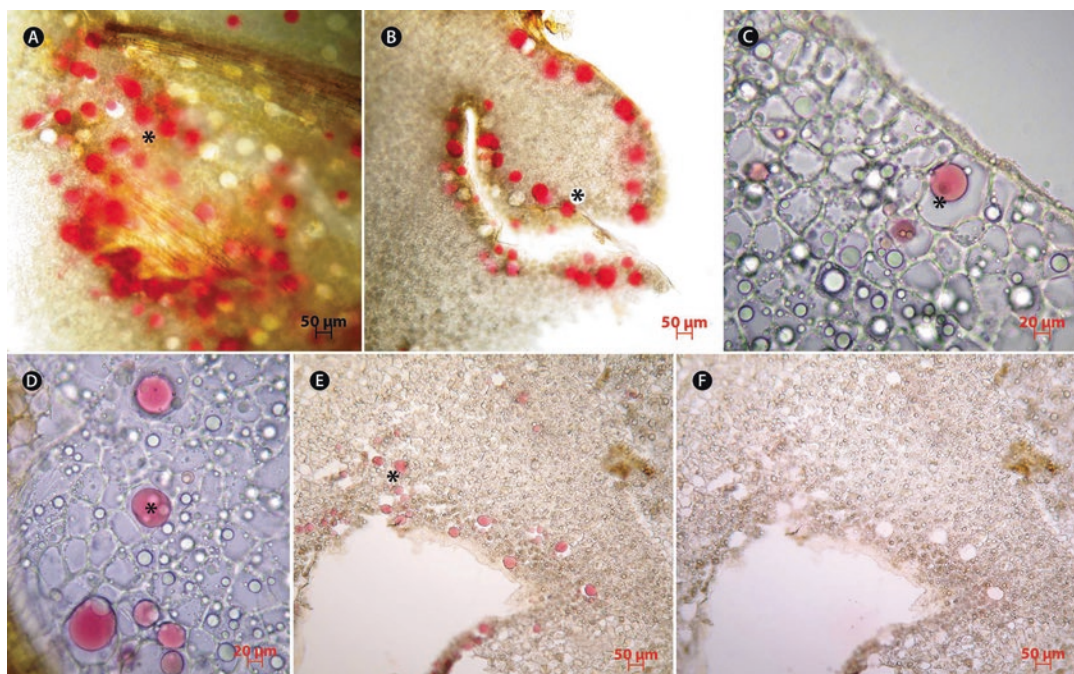


Fig. 2 Parts a–b: *A. macrophyllata* endosperm. Fresh and free-hand sections. Parts c–f: Fresh sections with Cryostat. (c, d) timed 4.00 min, (e) timed 3.30 min, (f) timed 7.30 min. Acetogenins produced a positive characteristic reaction with intense pink color that remains up to 8 min

References

1. Jolad SD, Hoffmann JJ, Schram KH et al (1982) Uvaricin, a new antitumor agent from *Uvaria accuminata* (Annonaceae). *J Org Chem* 47(16):3151–3153. doi:10.1021/jo00137a024
2. Ruprecht JK, Hui YH, McLaughlin JL (1990) Annonaceous acetogenins: a review. *J Nat Prod* 53(2):237–278
3. Cavé A, Figadère B, Laurens A et al (1997) Acetogenins from Annonaceae. In: Herz W, Kirby GW, Moore RE et al (eds) *Progress in the chemistry of organic natural products*. Springer, New York, NY, pp 81–287
4. Alali FQ, Liu XX, McLaughlin JL (1999) Annonaceous acetogenins: recent progress. *J Nat Prod* 62:504–540
5. Bermejo A, Figadère B, Zafra-Polo MC et al (2005) Acetogenins from Annonaceae: recent progress in isolation, synthesis and mechanisms of action. *Nat Prod Rep* 22:269–303
6. Kirchner JG (1967) Thin layer chromatography. In: Perry ES, Weissberger A (eds) *Technique of organic chemistry*, vol 12. Interscience, New York, NY, pp 147–186
7. Kedde DL (1947) Bijdrage tot het chemisch onderzoek van Digitalispreparaten. *Pharm Weekbl* 82:741–757
8. Lewbart ML, Wehrli W, Reichstein T (1963) Die Cardenolide von *Gongronema gazense* (S. Moore) Bullock Glykoside und Aglykone. *Helv Chim Acta* 46(2):505–517
9. Luckner M (1984) *Secondary metabolism in microorganisms, plants, and animals*, 2nd edn. Springer, New York, NY, p 247. doi:10.1007/978-3-662-02384-6
10. Kovar KA, Francas G, Seidel R (1977) Zum mechanismus der Reaktionen nach Raymond, Kedde und Baljet. *Archiv Pharmazie (Weinheim)* 310(1):40–47
11. Jork H, Funk W, Fischer W et al (1990) Thin-layer chromatography ‘reagents and detection methods’. Vol 1b physical and chemical detection methods: activation reactions, reagent sequences, reagents II. English edition: Frank and Jennifer A. Hampson. VCH Weinheim, Basel, pp 263–267
12. Flasch H, Diembeck W (1981) Chemical and chromatographic method (methods for determination

- of cardiac glycosides). In: Greeff K (ed) Cardiac glycosides, part I, handbook of experimental pharmacology, vol 56. Springer, Berlin, pp 27–42
13. Görög S (1983) Cardiac glycosides. Quantitative analysis of steroids. *Stud Anal Chem* 5:372–407
 14. Leboeuf M, Cavé A, Bhaumik PK et al (1982) The phytochemistry of the Annonaceae. *Phytochemistry* 21(12):2783–2813
 15. Zweig G, Sherma J (eds) (1972) Handbook of chromatography, vol II. Section 2.1. Detection reagents for paper and/or thin-layer chromatography. CRC Press The Chemical Rubber Co, Boca Raton, FL, p 170
 16. Wagner H, Blatt S (1996) Plant drug analysis: a thin layer chromatography atlas. Springer, New York, NY, p 362, Appendix A: spray reagents
 17. Houghton JP, Raman A (1998) Handbook for the fractionation of natural extracts. Chapman and Hall, London, p 185, Appendix A. Recipes for chemical test reagents
 18. Parellada EA, Ferrero M, Cartagena E et al (2013) Laherradurin, a natural stressor, stimulates QS mechanism involved in biofilm formation of a PAHs degrading bacterium. *Int Biodeter Biodegrad* 2013(85):78–84
 19. González-Esquinca AR (2001) Contribución al estudio del género *Annona* (Annonaceae). Análisis fitoquímico de tres especies del estado de Chiapas. Tesis doctoral. Universidad Nacional Autónoma de México, Mexico City
 20. De la Cruz-Chacón I (2001) Acetogeninas bioactivas de *Annona diversifolia* Safford. Tesis de Licenciatura. Instituto Tecnológico de Tuxtla Gutiérrez, Tuxtla Gutiérrez, Chiapas
 21. Abraján Hernández P (2002) Acetogeninas y compuestos de *Annona lutescens* Safford. Tesis (Licenciatura en Ingeniería Química). Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas
 22. De la Cruz-Chacón I, González-Esquinca AR (2013) Activities of enzymes catalyzing benzylisoquinoline alkaloid biosynthesis in *Annona diversifolia* Saff. during early development. *Russ J Plant Phys* 60(6):791–799
 23. González-Esquinca AR, De la Cruz-Chacón I, Castro-Moreno M et al (2014) Alkaloids and acetogenins in Annonaceae development: biological considerations. *Rev Bras de Frutic* 36:01–16
 24. Laguna-Hernández G, Brechú-Franco AE, De la Cruz-Chacón I et al (2015) The histochemical detection of acetogenins and storage molecules in the endosperm of *Annona macrophyllata* Donn Sm. seeds. *Eur J Histochem* 59:2502. doi:10.4081/ejh.2015.2502
 25. Brechú-Franco AE, Laguna-Hernández G, De la Cruz-Chacón I et al (2016) In situ histochemical localisation of alkaloids and acetogenins in the endosperm and embryonic axis of *Annona macrophyllata* Donn. Sm. seeds during germination. *Eur J Histochem* 60: 2568. doi:10.4081/ejh.2016.2568
 26. Ministerio de Agricultura (1976) Reglas internacionales para ensayos de semillas. Instituto Nacional de Semillas y plantas de Vivero. Dirección General de la Producción Agraria. Reimpreso por la SARH, Mexico City
 27. Mata R, Rivero-Cruz JF, Chávez D (2001) Bioactive secondary metabolites from selected mexican medicinal plants: Recent progress. In Tringali C (2001) Bioactive compounds from natural sources Isolation, characterization and biological properties. Taylor & Francis, London
 28. Cepleanu F, Ohtani K, Hamburger M et al (1993) Novel acetogenins from the leaves of *Annona purpurea*. *Helv Chim Acta* 76:1379–1388
 29. Chávez D, Mata R (1998) Purpurediolin and purpurenin, two new cytotoxic adjacent bis-tetrahydrofuran Annonaceous acetogenins from the seeds of *Annona purpurea*. *J Nat Prod* 61:580–584
 30. Chávez D, Mata R (1999) Purpuracenin: a new cytotoxic adjacent bis-tetrahydrofuran annonaceous acetogenin from the seeds of *Annona purpurea*. *Phytochemistry* 50:823–828
 31. Rodríguez-López CE, Hernández-Brenes C, De la Garza RID (2015) A targeted metabolomics approach to characterize acetogenin profiles in avocado fruit (*Persea americana* Mill). *RSC Adv* 5(128):106019–106029
 32. Ayyad SEN, Al-Footy KO, Alarif WM et al (2011) Bioactive C15 acetogenins from the red alga *Laurencia obtusa*. *Chem Pharm Bull* 59(10):1294–1298

INDEX

A

- Adult neurogenesis 163, 164, 167, 174
- Agar (pre-embedding).....73, 76–78, 88, 262, 263, 279, 280
- Alkaloids
 - Dragendorff's reagent..... 318, 326–327, 329
 - Wagner's reagent..... 318, 326–327
- Anaesthetic solutions.....71, 77
- Annonaceous acetogenins
 - Kedde's reagent.....332–336
- Antibodies
 - monoclonal antibodies.....56, 157, 158, 160, 181, 191, 270–272, 274
 - polyclonal antibodies 56, 157, 160, 181, 191
- Anticoagulant solution for hamolymph..... 71, 72, 78, 79, 87
- Aquatic arthropods..... 71, 76, 77, 79
- Autofluorescence (AF) 16–22, 24–34, 36, 37, 318–319, 325–327, 329
- Autophagosome.....189
- Autophagy markers
 - LC3 189–194
 - Sequestosome 1 (p62/SQSTM1) 189–194
- Azo-coupling method 49, 50, 70, 79, 81–83, 87

B

- Biliary tree 45–47, 49, 50, 52
- Bivalves.....70, 79
- BrdU labeling 163, 167–172, 174, 176

C

- Carbohydrate/glycoprotein retrieval
 - by microwave treatment..... 125, 127, 129, 130
 - by trypsinisation/trypsinization 125, 127, 130
- Carbohydrates.....93–98, 100, 101, 103–104, 109–111, 118, 121–125, 127, 130, 239, 315, 318–319, 321, 322, 328
- Cell separation..... 111–114, 119, 133
- Chromogenic substrates 56, 57, 60, 62, 66, 194
- Crustaceans 71, 77
- Cryostatic sections..... 36, 49, 124–127
- Curve fitting analysis.....21, 29–36

D

- Decalcification solution..... 70, 72, 77, 88
- Dechitinization solution 70, 72, 77
- Desilicification solution.....72, 77
- Differentiation markers
 - for muscle satellite cells 179–181, 183–187
 - for myoblasts..... 180, 186, 187
 - for myogenic progenitors 179, 181, 184, 186, 187
 - for neuronal cells..... 163–169, 171, 173–176
- DNA (electron microscopy)
 - immunogold labeling..... 270–273, 304–309
 - lead citrate 87, 271, 273, 278, 283
 - Osmium ammine..... 261–264, 266, 269
 - Schiff reagent.....261, 263, 316, 321, 328
 - terminal deoxynucleotidyl transferase 134, 137, 142, 269–274
 - uranyl acetate 87, 271, 273, 299, 303, 304, 307
- DNA (light microscopy)
 - DNA content..... 251–254, 289
 - Feulgen reaction 240, 243–246, 249, 261, 262, 269, 277
 - flow cytometry.....3, 134, 243–245, 248–254
 - fluorochromes.....16, 169, 171, 176, 239, 240, 242, 244–254, 322
 - microdensitometry.....241, 242
 - micro-fluorometry 244
 - supravital staining 248–250
- DNA strand breaks..... 173, 285–290, 292

E

- Earthworms.....72, 79
- Electron microscopy (EM).....4, 61, 69, 73, 76, 77, 80–87, 97, 133, 148, 185, 190, 261–264, 266, 269–274, 277–281, 283, 299, 301–304, 307, 308
- Endogenous fluorophores (EFs)..... 15–17, 19–37
- Energy metabolism.....21, 23, 24, 27, 28, 34
- Enzyme cytochemistry 4
- Enzyme histochemistry 45–47, 49, 50, 52, 69–88
 - polyvinil alcohol.....47–51
- Enzymes
 - acid phosphatase 73, 79–80
 - alkaline phosphatase 75, 84–86, 101
 - aminopeptidase.....45, 55–57, 59–67, 74, 81

Enzymes (*cont.*)

Ca ²⁺ -ATPase.....	75, 83–84
cytochrome-c oxidase	76, 87
D-amino acid oxidase	75–76, 86
dipeptidyl-peptidase IV (DPP-IV)	45–47, 49–52
β-glucuronidase	74–75, 83
lipase.....	74, 82–83, 88, 98
Mg ²⁺ -ATPase.....	75, 84, 85
β-N-acetylglucosaminidase.....	75, 83
non-specific esterase	74, 82
5'-nucleotidase.....	73–74, 80
peroxidase	75, 85, 100, 123, 125, 127, 137, 149, 158, 191, 194
phenoloxidase	76, 86–87
protease.....	74, 81–82, 99, 300, 301, 304
serine protease	74, 81–82
tripeptidyl aminopeptidase I.....	55–67

F

Fatty liver.....	46
Flavins	17, 18, 20–24, 27, 29, 30, 34
Fluorescence microscopy	4, 16, 18, 19, 21, 26, 27, 59, 64, 151, 156, 158, 223–224, 226, 232, 242, 243, 246, 247, 249, 251, 252, 292, 328, 329
Fluorogenic substrates.....	56, 58, 60, 62, 66
Formaldehyde	60, 72, 88, 123, 148, 153, 173, 198–205, 208, 214, 226, 274, 298, 302, 316, 317, 333
Formalin	72, 97, 124, 128, 130, 147, 148, 150, 152–155, 158–160, 189–194, 198, 201, 223, 224, 226, 253, 317, 318, 325–328

G

Gastropods	70, 79
Glucose-6-phosphate dehydrogenase (G6PD) deficiency	3–12
Glutaraldehyde	72, 73, 262, 263, 266, 270, 274, 279, 280, 282, 299, 308, 309, 328, 333
Glycobiology	100
Glycosylation cancer cells.....	99, 103, 110, 121
glycome.....	100, 101, 103, 104, 109, 111
sugar code	100, 104
Glycyl-L-prolyl-L-metionyl-4-hydrazido-N-hexyl-1,8- naphthalimide (GPM-HHNI)	56, 60, 64
Glycyl-L-prolyl-L-metionyl-5-chloro-1-anthraquinonylhydrazide (GPM-CAH)	56, 57, 60

H

Hemolymph	71–73, 76–88
Heat-induced antigen retrieval formalin-fixed, paraffin-embedded (FFPE) sections	123, 124, 126, 128–130, 147–156, 158, 160
for fresh frozen sections.....	124, 150–155, 158
for highly masked epitopes	149, 154

I

Immunofluorescence	149, 151, 155–156, 159, 160, 168–171, 173, 175, 181, 212, 252, 298–300, 302–305
Invertebrates	70–74, 76–88, 93–95, 102

L

Lectin-coated magnetic beads	109–111, 113–119
Lectins apoptotic cells	133–142, 247
Concanavalin A (Con A).....	96, 98, 104, 136–139
<i>Helix pomatia</i> lectin (HPA)	96, 99, 101, 102, 110, 113, 118, 122–124, 126
<i>Limax flavus</i> lectin.....	96
<i>Maakia amurensis</i> lectin	97
malignancy.....	46, 98–99, 252, 253
metastasizing cancer cells	122
non-metastasizing cancer cells.....	121–130
peanut agglutinin (PNA).....	136–140
<i>Phaseolus vulgaris</i> leukoagglutinin (PHA-L)	99, 122–124
<i>Polyporus squamosus</i> lectin	97
<i>Sambucus nigra</i> lectin	95, 97
<i>Vicia villosa</i> isolectin B4.....	99
Lignin phloroglucinol-hydrochloric acid staining	325
Lipid histochemistry formalin-fixed, paraffin-embedded (FFPE) sections	123, 124, 126, 129, 130, 155, 160
with frozen tissues	49, 59, 124, 148–156, 158–160, 172, 198, 199
post-fixation method (according to Tracy and Walia)	60, 63, 173, 174, 198, 203, 204, 270, 271
Lipid staining luxol fast blue (LFB).....	208–216
monodansylpentane (MDH)	231–236
Nile red staining	219–227, 232, 233
osmium tetroxide.....	77, 198, 205, 209, 212–217, 274
Lipofuscins	16–18, 24–30, 32–36
Liver	16–18, 21, 23–28, 30, 32, 34–36, 45–47, 49–52, 57, 58, 95, 155, 168, 265, 282

M

Metabolic mapping	4
Metal salt substitution	70, 80, 82–87
Microspectrofluorometry.....	19, 32, 244
Myelin	197, 199, 200, 203, 207–217
Myogenin	181, 183–185

N

NAD(P)H	17, 18, 20–24, 27–30, 34, 35
Neutral lipids.....	219–227, 231, 323–324, 329
Nile blue staining.....	9, 323–324, 329

O

Oxidative coupling polymerization 85–87

P

Pax7 179–181, 183–187

Phenolic compounds

 ferric chloride staining 316–320, 324–327

 ferrous sulfate-formalin fixation 317, 325

 potassium dichromate staining 317, 325–327

Photosensitivity 16

Picrosirius staining 208, 213, 215, 216

Plants..... 9, 246, 252, 301–303, 305–307,
309, 310, 317, 318, 323–327, 329

Allium cepa..... 304, 309

 cell wall

 acridine orange staining 246, 252, 318, 325–327

 nuclear isolation.....304

 Nuclear Matrix Constituent Proteins

 (NMCPs).....297, 298, 300, 302–310

 pre-embedding labelling 301–303, 305–307,
309, 310

 secretory structures

 copper acetate staining 317, 323, 324

 Nile blue staining 9, 317, 323–324, 329

 rubeanic acid staining 317, 323, 324

Porphyrins 17, 19, 24–28, 30, 34

Q

Quenching of endogenous peroxidase 125, 127, 137, 194

R

RNA (electron microscopy)

 EDTA regressive stain 277

 lanthanides.....278

 terbium citrate 277–281, 283

S

Schiff reagent.....261

Seminiferous epithelium..... 133–139, 141, 142

Single cell cytochemistry 3–12

Single cell gel electrophoresis (SCGE).....285

Skeletal muscle

 muscle regeneration 179–181, 184, 186, 187

 myogenic progenitors 179–187

Spermatozoa..... 286, 287, 289, 290

T

Tannins

 vanillin–hydrochloric acid staining 318, 325–327

Terpenes

 NADI reaction 317, 323, 324, 328

Terrestrial arthropods 71, 76, 77

Time resolved fluorescence.....20

TUNEL assay.....134–142

Tunicates 70, 79–81, 85, 86

Two-tailed comet assay (2T-Comet) 285–290, 292

V

Vitamin A 24–28, 30–32, 34