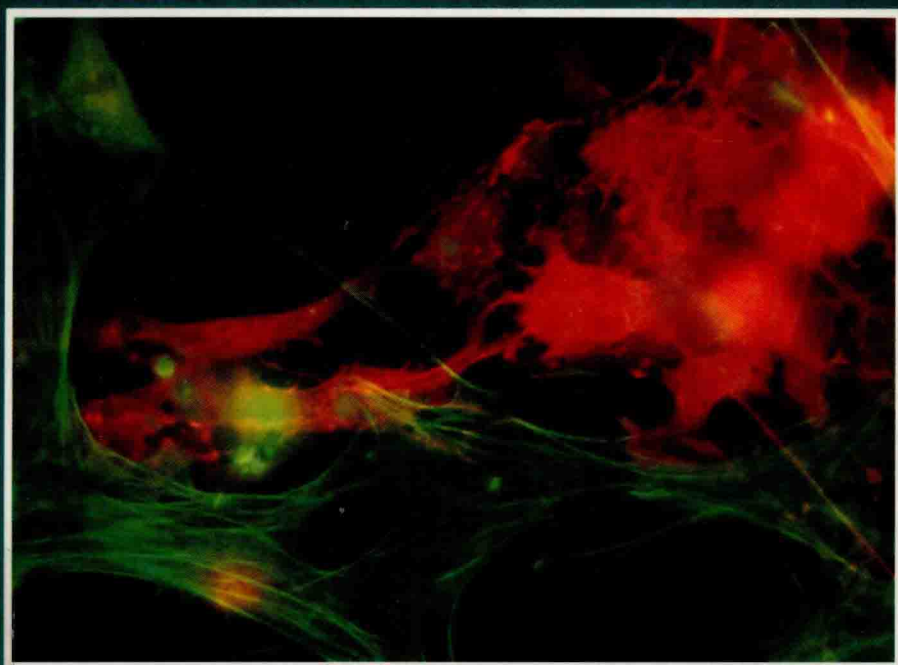




Introduction to Immunocytochemistry

Second Edition



J.M. Polak and S. Van Noorden



世界图书出版公司

Introduction to Immunocytochemistry

SECOND EDITION

J.M. Polak

Department of Histochemistry,
Royal Postgraduate Medical School,
London, UK

S. Van Noorden

Department of Histopathology,
Royal Postgraduate Medical School,
London, UK

BIOS
SCIENTIFIC
PUBLISHERS



Springer

In association with the Royal Microscopical Society

J.M. Polak and S. Van Noorden
*respectively Histochemistry Department and Histopathology Department, Royal
Postgraduate Medical School, Du Cane Road, London W12 0NN, UK*

First Edition © Royal Microscopical Society, 1984, 1987

Second Edition © BIOS Scientific Publishers, 1997

This edition has been authorized by the Publisher for sale throughout Hong Kong, Taiwan, Singapore, Thailand, Cambodia, Korea, The Philippines, Indonesia, The People's Republic of China, Brunei, Laos, Malaysia, Macau, and Vietnam only and not for export therefrom.

All rights reserved. No part of this book may be reproduced or transmitted, in any form or by any means, without permission.

The use of general descriptive names, trade names, trademarks, etc., in this publication, even if the former are not especially identified, is not to be taken as a sign that such names, as understood by the Trade Marks and Merchandise Marks Act, may accordingly be used freely by anyone.

While the advice and information in this book are believed to be true and accurate at the date of going to press, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

A CIP catalog record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data

Polak, Julia M.

Introduction to immunocytochemistry / J.M. Polak and S. Van
Noorden. -- 2nd ed.

p. cm.

Includes bibliographical references and index.

ISBN 0-367-91513-3 (softcover : alk. paper)

1. Immunocytochemistry. I. Van Noorden, Susan. II. Title.

QR187.I45P65 1997

571.9'64--dc21

97-13062

CIP

ISBN 981 3083 35 2 Springer-Verlag New York Berlin Heidelberg

Production Editor: Priscilla Goldby.

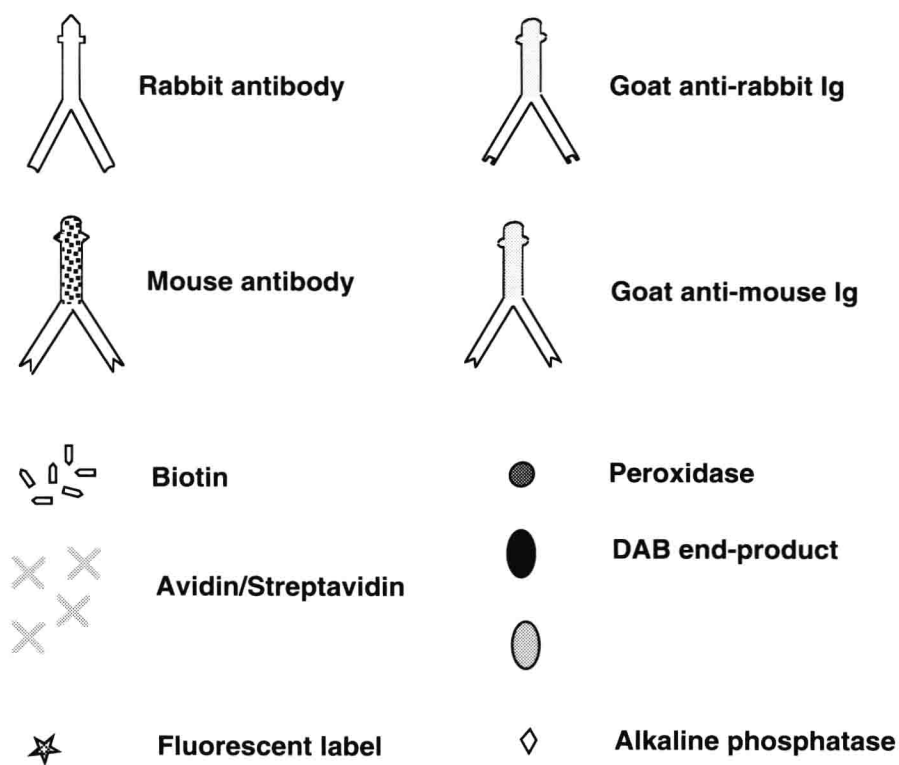
Typeset by Poole Typesetting, Bournemouth, UK.

Printed by Information Press, Eynsham, Oxon, UK.

Front cover: Co-cultured muscle and nerve cells stained by double-immunofluorescence.
See Section 7.2.

Abbreviations

ab	antibody
ABC	avidin-labelled biotin complex
AEC	3-amino-9-ethylcarbazole
ag	antigen
APAAP	alkaline phosphatase–anti-alkaline phosphatase
BSA	bovine serum albumin
DAB	diaminobenzidine
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent antibody cell sorting
FITC	fluorescein isothiocyanate
Ig	immunoglobulin
IGSS	immunogold staining with silver
LCA	leukocyte common antigen
PAP	peroxidase–anti-peroxidase
PBS	phosphate-buffered saline
PGP	protein gene product
PLP	periodate-lysine-paraformaldehyde
RIA	radioimmunoassay
TBS	Tris-buffered saline
TSA	tyramine signal amplification
UV	ultraviolet



Key to symbols used in diagrammatic figures.

Preface

Immunocytochemistry, the accurate localization of tissue constituents with labelled antibodies, was fathered by A.H. Coons in the 1940s, grew up during the sixties and seventies, and in its maturity has become an indispensable investigative technique in diagnostic histo- and cytopathology and many branches of biomedical science. Its versatility allows it to be used on whole cells or on tissue sections, whether from frozen or fixed and embedded samples, and at both light- and electron-microscopical levels. Immunocytochemistry can be combined with other localization methods such as histological or histochemical staining and *in situ* hybridization of nucleic acids. It is applicable to plant as well as animal tissue, the only requirements being a specific antibody to the antigen in question, suitable preservation of the antigen and a revelation method sensitive enough to depict even low quantities of antigen.

Despite its well-established position, the technique, like so many, has its own tricks of the trade. Newcomers need to know not only how to perform the tests, but also why the various steps are necessary, how to get the best results, what are the essential controls and what to do when things go wrong. These can all be learnt from experienced teachers, but after spending a lot of time teaching a seemingly endless stream of novices in the field, we decided in 1980 to write an introductory text to save ourselves some effort. We used the notes successfully in conjunction with practical courses, and in 1984 they were expanded into the first edition of this book (published by Oxford University Press). A revised edition followed in 1987.

This second edition has been rewritten extensively, expanded and updated to include major advances in methods of antigen retrieval and ways of increasing sensitivity. We hope that it will provide a practically directed basis for carrying out current immunocytochemical methods with enough theoretical information to allow a newcomer to understand the whys and wherefores of the technique. The text is backed up by a reference list for readers wanting further depths of knowledge.

We are grateful to the many colleagues who have helped with suggestions and by providing illustrations.

Julia M. Polak
Susan Van Noorden

Safety

Attention to safety aspects is an integral part of all laboratory procedures, and both the Health and Safety at Work Act and the COSHH regulations impose legal requirements on those persons planning or carrying out such procedures.

In this and other Handbooks every effort has been made to ensure that the recipes, formulae and practical procedures are accurate and safe. However, it remains the responsibility of the reader to ensure that the procedures which are followed are carried out in a safe manner and that all necessary COSHH requirements have been looked up and implemented. Any specific safety instructions relating to items of laboratory equipment must also be followed.

Contents

Abbreviations	xi
Key to symbols	xii
Preface	xiii
1. Introduction	1
Definition	1
History and development	1
References	3
2. Production of Antibodies	5
Immunization	5
Testing	6
Region-specific antibodies	6
Monoclonal antibodies	8
Characteristics of a 'good' antibody	9
References	10
3. Requirements for Immunocytochemistry	11
Fixation	11
Cross-linking fixatives	12
Precipitant fixatives	13
Combination fixatives	13
Fresh tissue	14
Pre-fixed, non-embedded material	14
Freeze-drying	15
Tissue storage	15
Adherence of sections and cell preparations to slides	16
Antigen retrieval in fixed tissues	16
Washing	17
Protease treatment	17
Heat-mediated antigen retrieval	19
Visualizing the end-product of reaction	20

Fluorescent labels	21
Enzyme labels	23
Colloidal gold	27
Other labels	28
Absence of non-specific staining	29
Causes and prevention of non-specific staining	30
References	31
Colour plates	36
4. Methods	41
General considerations	41
Buffer	41
Antibody diluent	42
Antibody dilution relative to reaction time, temperature and technique	42
Methods	44
Nature of antibodies (IgG)	44
Application of antibodies to preparations	45
Direct method	47
Indirect method	47
Three-layer methods	50
Avidin–biotin methods	51
References	54
5. Specificity Problems and Essential Controls	55
Testing for non-specific binding due to tissue factors	55
Testing for non-specific binding by the primary antibody	55
Testing for non-specific binding of second and third reagents	57
Non-specific or unwanted specific staining due to antibody factors	57
Unwanted specific staining of unknown antigens	57
Non-specific binding of antisera to basic proteins in the tissue	58
Unwanted specific cross-reactivity of anti-immunoglobulin	58
Cross-reactivity of the primary antibody with related antigens	58
Remedies for non-specificity due to tissue factors	60
Blocking binding sites with normal serum	60
Absorption with tissue powder	60
Remedies for non-specificity due to heterogeneity of the antibody	60
Dilution	60
Affinity purification	60

Remedies for non-specificity due to cross-reactivity	61
Controls	61
Negative controls	61
Positive control	61
Experimental controls	61
References	62
6. Enhancement of Standard Methods	63
Build-up methods	63
Intensification of the peroxidase/DAB/H ₂ O ₂ product	66
Post-reaction intensification	66
Intensification during the peroxidase reaction	67
Tyramine signal amplification (TSA)	67
References	71
7. Multiple Immunostaining	73
Primary antibodies raised in the same species	73
Separately labelled primary antibodies	73
Unlabelled primary, labelled secondary antibodies	74
Indirect double staining without elution	76
Double immunostaining with primary antibodies raised in different species	77
Triple immunostaining	79
References	79
8. Post-embedding Immunocytochemistry for the Transmission Electron Microscope	81
Principles	81
Fixation	81
Processing to resin	82
Labels	84
Sectioning	86
Immunolabelling procedure	86
Pre-treatment	87
Immunolabelling	87
Contrasting	87
Multiple labelling	87
References	89
9. In Vitro Methods for Testing Antigen–Antibody Reactions	91
Radioimmunoassay	92
Enzyme-linked immunosorbent assay (ELISA)	92

Western blotting	93
Dot blots	94
References	94
10. Applications of Immunocytochemistry	95
Histopathological diagnosis	95
Controls	96
Research	97
Quantification	97
Confocal microscopy	98
Flow cytometry and fluorescent antibody cell sorting (FACS)	98
Simpler methods of quantitation	99
Non-immunocytochemical uses of labelled probes	100
Receptor localization	100
Lectin histochemistry	101
<i>In situ</i> hybridization of nucleic acids	101
References	102
11. Microscopy	105
References	107
Appendix: Technical Notes	109
Buffers	109
Phosphatase-buffered normal saline (PBS)	109
Tris-buffered normal saline (TBS)	109
Antibody diluent and storage of antibodies	110
Adherence of preparations to slides	110
Coating slides with <i>poly</i> -L-lysine	110
Coating slides with silane	111
Blocking endogenous peroxidase reaction	112
Paraffin sections	112
Milder methods for cryostat sections and whole-cell preparations	113
Blocking endogenous biotin	113
Enzyme pre-treatment	114
Trypsin	114
Protease	114
Pepsin	115
Neuraminidase	115
Heat-mediated antigen retrieval using a microwave oven	116
Enzyme development methods	117
Peroxidase	117
Alkaline phosphatase	120

Glucose oxidase	121
β -D-Galactosidase	122
Intensifying the peroxidase/DAB reaction product	122
Following standard development	122
During development	123
Immunostaining methods	125
Initial procedure	125
Immunostaining – all preparations	127
Immunogold staining with silver enhancement	128
Silver acetate auto-metallography	130
Double immunoenzymatic staining	131
Post-embedding electron microscopical	
immunocytochemistry using epoxy resin-embedded	
tissue and an indirect immunogold method	132
Absorption specificity control (liquid phase)	133
References	134
Index	137

1 Introduction

1.1 Definition

There are several uses in biology and medicine for the strong and specific attraction between an antigen and an antibody, including the measurement of antigen in tissue extracts by radioimmunoassay (RIA) and the sorting and analysis of populations of dispersed cells after labelling with a fluorescent antibody, i.e. fluorescent antibody cell sorting (FACS). However, immunocytochemistry is the only technique which can identify an antigen in its tissue or cellular location. Thus the definition of immunocytochemistry is the use of labelled antibodies as specific reagents for localization of tissue constituents (antigens) *in situ*.

1.2 History and development

The practice of immunocytochemistry originated with Albert H. Coons and his colleagues (Coons *et al.*, 1941, 1955; Coons and Kaplan, 1950) who were the first to label an antibody with a fluorescent dye and use it to identify an antigen in tissue sections with a fluorescence microscope. As a result of this work much of the uncertainty has now been removed from some aspects of histopathology which were previously entirely dependent on special stains, with interpretation sometimes precariously based on intuition and deduction. Because an antigen–antibody reaction is absolutely specific, positive identification of tissue constituents can now be achieved, though there are still problems as will become apparent in the following pages.

The first fluorescent dye to be attached to an antibody was fluorescein isocyanate, but fluorescein isothiocyanate soon became the label of choice because the molecule was much easier to conjugate to the antibody and more stable (Riggs *et al.*, 1958). Fluorescein compounds emit a

bright apple-green fluorescence when excited at a wavelength of 490 nm.

Following the early work, and as better antibodies to more substances became available, the technique has been enormously expanded and developed. New labels have been introduced, including red, yellow, blue and green fluorophores and a variety of enzyme labels which, when developed, can give differently coloured end-products, visible in a conventional light microscope. The methods used to develop the enzyme labels are the standard ones used in histochemistry to identify native enzymes in the tissue. The first enzyme to be used was horse-radish peroxidase (Nakane and Pierce, 1966; Avrameas and Uriel, 1966). Other enzymes include alkaline phosphatase (Mason and Sammons, 1978), glucose oxidase (Suffin *et al.*, 1979) and β -D-galactosidase (Bondi *et al.*, 1982). The end-product of reaction of some of these enzyme reactions can be made electron-dense, but other intrinsically electron-dense labels such as ferritin (Singer and Schick, 1961) have been used for electron microscopical immunolabelling and colloidal gold particles, introduced by Faulk and Taylor (1971), are likely to remain the label of choice for this technique (see Beesley 1993). Antibodies have been labelled with radioactive elements and the immunoreaction visualized by autoradiography, and some other labels in addition to colloidal gold particles, for example latex particles, can be used in scanning electron microscopy.

Among the techniques, the first modification of the original, directly labelled antibody was the introduction of the two-layer indirect method (see Section 4.2). This was followed by the unlabelled antibody–enzyme methods which avoided entirely conjugation of a label to an antibody and the damage that may entail. Other methods involved the use of a second antigen (hapten) as an antibody label, visualized by a further antibody raised to the hapten (Cammisuli and Wofsy, 1976; Jasani *et al.*, 1981), the exploitation of the strong attraction between avidin and biotin (Guesdon *et al.*, 1979; Hsu *et al.*, 1981), and numerous ways of improving the specificity and intensity of the final reaction product and of carrying out multiple immunostaining.

Some of these methods are described here, and the Appendix gives details of the basic techniques. However, the subject is too vast to be covered completely in this handbook, which aims only to introduce the concept, and the reader is referred to several publications which provide more detail on selected aspects (Polak and Van Noorden, 1986; Sternberger, 1986; Bullock and Petrusz, 1982, 1983, 1986, 1989; Larsson, 1988; Beesley, 1993; Cuello, 1993; Jasani and Schmid, 1993; Leong, 1993). In addition, several scientific journals provide reviews of antibodies and papers on new methods and applications, including: *Applied Immunohistochemistry*, *Journal of Histochemistry and Cytochemistry*, *The Histochemical Journal*, *Histochemistry and Cell Biology* (formerly *Histochemistry*) and *Journal of Cellular Pathology*.

References

- Avrameas S, Uriel J.** (1966) Méthode de marquage d'antigène et d'anticorps avec des enzymes et son application en immunodiffusion. *C. R. Acad. Sci. Paris Sér. D* **262**, 2543–2545.
- Beesley JE.** (ed.) (1993) *Immunocytochemistry, A Practical Approach*. Oxford University Press, Oxford.
- Bondi A, Chieriegatti G, Eusebi V, Fulcheri E, Bussolati G.** (1982) The use of β -galactosidase as a tracer in immunohistochemistry. *Histochemistry* **76**, 153–158.
- Bullock GR, Petrusz P.** (eds) (1982) *Techniques in Immunocytochemistry*, Vol. 1. Academic Press, London.
- Bullock GR, Petrusz P.** (eds) (1983) *Techniques in Immunocytochemistry*, Vol. 2. Academic Press, London.
- Bullock GR, Petrusz P.** (eds) (1986) *Techniques in Immunocytochemistry*, Vol. 3. Academic Press, London.
- Bullock GR, Petrusz P.** (eds) (1989) *Techniques in Immunocytochemistry*, Vol. 4. Academic Press, London.
- Cammisuli S, Wofsy L.** (1976) Hapten-sandwich labelling, III. Bifunctional reagents for immunospecific labelling of cell surface antigens. *J. Immunol.* **117**, 1695–1704.
- Coons AH, Creech HJ, Jones RN.** (1941) Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol. Med.* **47**, 200–202.
- Coons AH, Kaplan MH.** (1950) Localization of antigen in tissue cells. *J. Exp. Med.* **91**, 1–13.
- 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.
- Cuello AC.** (ed.) (1993) *Immunohistochemistry II*, IBRO Handbook Series: Methods in the Neurosciences, Vol. 14. J. Wiley and Sons, Chichester.
- Faulk WR, Taylor GM.** (1971) An immunocolloid method for the electron microscope. *Immunocytochemistry* **8**, 1081–1083.
- Guesdon JL, Ternynck T, Avrameas S.** (1979) The uses of avidin–biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* **27**, 1131–1139.
- Hsu SM, Raine L, Fanger H.** (1981) Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques; a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29**, 577–580.
- Jasani B, Schmid KW.** (1993) *Immunocytochemistry in Diagnostic Histopathology*. Churchill Livingstone, Edinburgh.
- Jasani B, Wynford Thomas D, Williams ED.** (1981) Use of monoclonal anti-hapten antibodies for immunolocalisation of tissue antigens. *J. Clin. Pathol.* **34**, 1000–1002.
- Larsson L-I.** (1988) *Immunocytochemistry, Theory and Practice*. CRC Press, Boca Raton, Florida.
- Leong AS-Y.** (1993) *Applied Immunohistochemistry for the Surgical Pathologist*. Edward Arnold, Melbourne.
- Mason DY, Sammons RE.** (1978) Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. *J. Clin. Pathol.* **31**, 454–462.
- Nakane PK, Pierce Jr GB.** (1966) Enzyme-labeled antibodies: preparation and application for the localization of antigens. *J. Histochem. Cytochem.* **14**, 929–931.
- Polak JM, Van Noorden S.** (eds) (1986) *Immunocytochemistry, Modern Methods and Applications*, 2nd Edn. Butterworth Heinemann, Oxford (originally John Wright and Sons, Bristol).
- Riggs JL, Seiwald RJ, Burkhalter JH, Downs CM, Metcalf T.** (1958) Isothiocyanate compounds as fluorescent labeling agents for immune serum. *Am. J. Pathol.* **34**, 1081–1097.

4 INTRODUCTION TO IMMUNOCYTOCHEMISTRY

- Singer SJ, Schick AF.** (1961) The properties of specific stains for electron microscopy prepared by conjugation of antibody molecules with ferritin. *J. Biophys. Biochem. Cytol.* **9**, 519–537.
- Sternberger LA.** (1986) *Immunocytochemistry*, 3rd Edn. John Wiley and Sons, New York.
- Suffin SC, Muck KB, Young JC, Lewin K, Porter DD.** (1979) Improvement of the glucose oxidase immunoenzyme technique. *Am. J. Clin. Pathol.* **71**, 492–496.

2 Production of Antibodies

This book is not the place for a detailed description of antibody production, but it may be of practical use to know something about the basic procedure in order to understand why antibodies can be so variable.

2.1 Immunization

Antibodies, which are mainly γ -globulins, are raised by immunizing rabbits (or mice, guinea pigs, etc.) with antigen. The antigen must be completely pure or (preferably) synthetic to ensure as specific an antibody as possible. Despite this, the resulting antiserum will not be directed solely to the injected antigen. The antibodies produced by the donor animal will be directed to various parts of the antigen molecule and to any carrier protein (see below). The host animal serum will also contain many antibodies which may react with tissue components. A positive-appearing immunoreaction cannot, therefore, be assumed to be due to the specific, desired antigen-antibody reaction unless stringent controls are carried out. It may be necessary to immunize many animals in order to end up with even one usable antiserum, because little is known about what makes an animal react to a foreign protein, and the production of antibodies is still a matter of chance.

If the antigen is large, for example an immunoglobulin, it can be used by itself to immunize an animal. If it is as small as many bioactive peptides, or if the molecule itself is not immunogenic, it must be combined with a larger one for immunization. The small molecule (hapten) is chemically coupled (e.g. by glutaraldehyde or carbodiimide) to a larger 'carrier' protein molecule, such as bovine serum albumin, thyroglobulin or limpet haemocyanin. The larger complex is a better stimulant of antibody formation than the small molecule alone. The donor animal's serum will contain a mixture of antibodies, reactive with different amino acid sequences of the hapten and the carrier molecule, but the antibodies to the carrier molecule will either not react with the tissue to be stained (unless it were, for example, limpet tissue and the carrier pro-