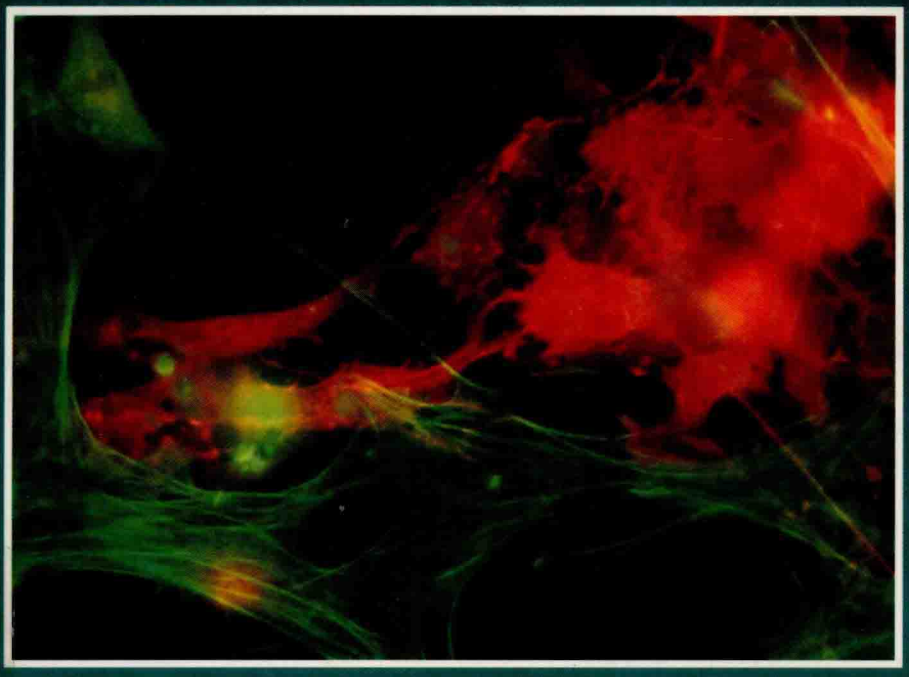
Introduction to Immunocytochemistry

Second Edition



J.M. Polak and S. Van Noorden

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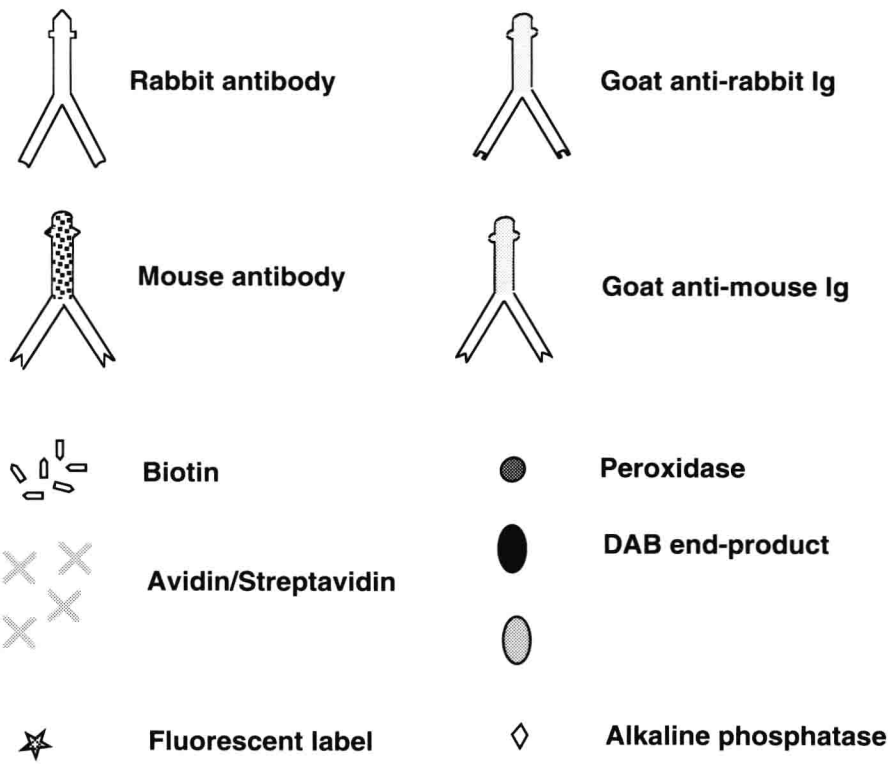
Front cover: Co-cultured muscle and nerve cells stained by double-immunofluorescence. See Section 7.2.

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

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*.

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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 diferent 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-