Techniques in Immunocytochemistry

Volume 1

Edited by

GILLIAN R. BULLOCK

Research Centre, Ciba-Geigy Pharmaceutical Division, Horsham, Sussex, England

and

PETER PÈTRUSZ

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Research Centre, Ciba-Geigy Pharmaceutical Division, Horsham, Sussex, England

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PETER PETRUSZ
Department of Anatomy, University of North Carolina, Chapel Hill, USA

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Contributors

P. Brandtzaeg *Histochemical Laboratory, Institute of Pathology, The National Hospital, Rikshospitalet, Oslo, Norway*
J. Burns *University of Oxford, Nuffield Department of Pathology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, England*
J.C.W. Finley *Department of Anatomy, University of North Carolina, 111 Swing Building 217H, Chapel Hill, North Carolina 27514, USA*
R.M. Franklin *Department of Structure Biology, Biocenter, University of Basel, CH-4056 Basel, Switzerland*
R. Grzanna *Johns Hopkins University, School of Medicine, Department of Cell Biology and Anatomy, 725 N. Wolfe Street, Baltimore, Maryland 21205, USA*
R.B. Knox *Botany School, University of Melbourne, Parkville, Victoria 3052, Australia*
D.Y. Mason *University of Oxford, Nuffield Department of Pathology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, England*
P. Ordronneau *Department of Anatomy, University of North Carolina, 111 Swing Building, Chapel Hill, North Carolina 27514, USA*
P. Petrusz *Department of Anatomy, University of North Carolina, 111 Swing Building 217H, Chapel Hill, North Carolina 27514, USA*
K.B. Pryzwansky *Department of Bacteriology and Immunology, University of North Carolina, Chapel Hill, North Carolina 27514, USA*
J. Roth *Institute of Histology and Embryology, University of Geneva Medical School, CH-1211 Geneva 4, Switzerland*
L.L. Vacca *Department of Pathology, Medical College of Georgia, Augusta, Georgia 30912, USA*
R.-E. Woolston *University of Oxford, Nuffield Department of Pathology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, England*
F. van Leeuwen *Netherlands Institute for Brain Research, Ijdijk 28, Amsterdam, The Netherlands*

Foreword

Immunocytochemistry overtook Enzyme Cytochemistry some years ago to become the advancing edge of the discipline. With the current rapid increase in immunological knowledge, and through the resulting improved technology, it maintains its position without serious challenge.

It is therefore a pleasure for me to write this Foreword to the first volume of a series whose composition has employed some of the acknowledged leaders in the field and a supporting cast of experts in particular branches.

It will certainly prove an acceptable contribution to the whole field of Immunocytochemistry and I wish it, and the volumes which follow, great success.

A.G.E. Pearse
Emeritus Professor of Histochemistry

Preface

In July 1979, a meeting on immunocytochemical techniques and applications was organized by Gillian Bullock under the auspices of the Royal Microscopical Society. One of the invited speakers at that meeting was Peter Petrusz and from that beginning sprang the idea that it would be useful to put together a book on immunocytochemical techniques. The catalyst was provided by Academic Press to whose continued support and enthusiasm the two editors are indebted.

On due consideration the two editors felt that in such a fast moving field some prime considerations had to be taken into account. In the first place, a series was preferred to a single volume as the number of applications was wide—new techniques were continually being developed and it would allow a short section to be included in later volumes where proven modifications of established techniques could be included.

Secondly, with the pressures now apparent on many potential contributors, it became clear that to spread the books over a period would ensure that all the major techniques and applications could be included as well as some of the more specialist methods.

We are extremely fortunate in having a list of such illustrious contributors for our first volume, each of whom has endeavoured to provide a clear-cut contribution on their technique which will enable the newcomer to the field to understand precisely how the method should be applied, the advantages and pitfalls and examples of the applications. In some cases, the authors have seen fit to check the data in their own laboratories, an attitude we found extremely encouraging.

We would like to thank all those people who said they felt the venture worthwhile and the promptness with which we received the majority of the manuscripts whilst some were delayed to ensure the utmost accuracy. We trust this and succeeding volumes will find a good home on the library or laboratory shelf and would be delighted to receive any correspondence which would help us in the planning of successive volumes.

Horsham and Chapel Hill
February 1982

Gillian R. Bullock
Peter Petrusz

Contents of Volume 1

Tissue Preparation Methods for Immunohistochemistry

PER BRANDTZAEG

I. INTRODUCTION

However refined the immunohistochemical detection method may be, the result of antigen localization will in the end depend on the handling of the tissue specimen. Any selection of tissue preparation method must necessarily be a compromise between the limitations in obtaining morphology identical to that existing during life, and the desire to demonstrate *in situ* the antigens under study in correct proportions with the best possible signal-to-noise ratio. It is quite unrealistic to hope for a standard procedure that at the same time can immobilize all types of antigens, preserve adequately and equally their antigenicity, provide optimal access of the corresponding antibody reagents, and retain structural integrity of tissues and cells at the light and electron microscopical level.

Immobilization of antigens and preservation of morphology usually requires fixation of the tissue, although at the light microscopical level satisfactory immunohistochemical results may be obtained with cryostat sections for components that are not subject to marked diffusion. However, cryostat temperatures favor the formation of ice crystal artifacts and membrane damage, and cryostat blocks are neither permanent nor easily handled, especially when one needs to cut sections from the same specimen on repeated occasions.

The use of permanently embedded tissue (e.g. paraffin blocks) offers the advantage of relatively good preservation of morphology together with convenient handling and storage of the specimens. Although alternative methods, such as freeze-drying and freeze-substition may be used to obtain permanent blocks (Taleporos and Ornstein, 1976; Pearse, 1980), preparation of the tissue by fixation is a much simpler and usually more adequate procedure.

The purpose of fixation is, in immunohistochemical terms, to arrest enzymatic activity sufficiently rapidly to avoid structural decomposition, to hinder diffusion of peptides and proteins into and out of cells, and to fortify the tissue against deleterious effects during the various stages in the preparation of sections. Artifacts may develop when the tissue specimens are subjected to dehydration, clearing, and embedding, and when the sections are being cut, floated, stretched, dried, dewaxed, rinsed, incubated, and washed. In addition, structural decomposition and diffusion artifacts may develop before the fixation process takes place—either *in vivo* because of denaturation or necrosis of cells, or *in vitro* because of autolysis, osmotic damage, drying, or rough mechanical treatment.

The choice of tissue preparation method should be guided by the purpose of the investigation. Immunohistochemistry of immunoglobulins and complement factors, for example, poses several methodological and interpretative problems not encountered in studies of antigens that occur in serum and tissue fluids in very low concentrations. Thus, experience with the localization of peptide hormones or epithelial enzymes cannot be uncritically transferred to the field of immunobiology and immunopathology. The present account will focus on these particular problems and place emphasis on tissue preparation methods that can give reliable results at the light microscopical level in paraffin-embedded tissue specimens.

II. PRINCIPLES OF TISSUE PREPARATION

A. Fixation Methods

Although fixation is necessary to avoid artifactual diffusion of soluble tissue components and decomposition of structures, it constitutes in itself a major artifact since the living cell and its surroundings are fluid or semi-fluid in nature. A detailed description of different fixatives and their action on various tissue components can be found in several major texts on histochemistry and histopathology (Culling, 1974; Lillie and Fullmer, 1976; Nairn, 1976; Pearse, 1980).

Fixatives such as ethanol and methanol immobilize proteins and carbohydrates by precipitation. The denaturing effect of these fixatives is relatively mild and to a large extent reversible. Thus, proteins may be redissolved in a fairly native state after ethanol fixation. It follows that adequate immobilization of antigens in tissue sections is far from guaranteed. Moreover, dehydration takes place simultaneously with the fixation process so that morphological preservation may be unsatisfactory due to shrinkage.

Immobilization of peptides and proteins is best afforded by bifunctional cross-linking fixatives such as formaldehyde and glutaraldehyde, which also preserve more adequately the structure of cells and tissue. However, cross-linking necessarily leads to more severe antigen denaturation than precipitation; this is particularly so for large protein antigens whose reactivity does not depend on primary structure alone but also on conformational features (Kauzmann, 1959). Even the antigenicity of peptides is adversely affected by the aldehyde-based fixatives which react with

primary amino groups, and several alternative cross-linking agents have been suggested for the localization of peptide hormones. These fixatives include water-soluble carbodiimide (Kendall *et al.*, 1971) and the bifunctional reagent parabenzoquinone (Pearse and Polak, 1975).

Several variables will influence the effect of cross-linking fixatives on antigenicity. Thus, when formaldehyde is used the number of methylene bridges formed depends not only on the concentration of the fixative, but also on the temperature, pH and time of exposure. The deleterious effect on antigen reactivity may be partially reversed before tissue embedding by extensive washing in water or treatment with sucrose (Eidelman and Berschauer, 1969; Deng and Beutner, 1974).

The aldehyde-based fixatives induce both intermolecular and intramolecular bridges. Due to such extensive formation of cross-linkages formaldehyde, and even more so glutaraldehyde, may in addition to denaturation cause masking of antigens by steric hindrance. This phenomenon is pronounced when the actual antigen is mixed with high concentrations of other proteins (Rognum *et al.*, 1980; Hed and Enestrøm, 1981). The suggestion made by some authors (Sternberger, 1979) that the deleterious effects of aldehyde-based fixatives can be compensated for by the use of a highly sensitive immunohistochemical method is thus only partially true. It is necessary to take into account that an uneven antigen masking takes place according to location; interpretation of any observed antigen distribution can only be meaningful if this fact is kept in mind, regardless of whether immunofluorescence or immunoenzyme methods are used for detection.

B. Exposure of Hidden Antigens

Antigenic determinants of a polypeptide chain may become partially or completely hidden when it is incorporated into the quaternary structure of a protein molecule. An example is the J chain (MW $\simeq$ 15 000 daltons), which becomes part of dimeric IgA and pentameric IgM during their formation in immunocytes (Brandtzaeg, 1976a). The J chain is still more inaccessible in secretory IgA which, in addition, has incorporated the secretory component (SC) into its quaternary structure (Brandtzaeg, 1976a). SC is an epithelial glycoprotein (MW $\simeq$ 83 000 daltons) that apparently acts as a receptor for dimeric IgA and pentameric IgM and thereby determines their transport through serous-type secretory cells (Brandtzaeg, 1981a).

When attempts are made to localize epithelial J chain in sections of ethanol-fixed tissues it is almost undetectable (Fig. 1a). However, dialysis against acid urea has been shown to expose J chains of purified secretory IgA (Brandtzaeg, 1976a), and a similar treatment of tissue sections unfolds secretory IgA in the epithelium after ethanol fixation (Fig. 1b) and to some extent also after carbodiimide fixation (Section III.B). For immunohistochemical J chain studies, therefore, we routinely immerse the dewaxed sections for 1 h at 4°C in 0·1 M glycine–HCl buffer, pH 3·2, containing 6 M urea (Brandtzaeg, 1976b). Thereafter the sections are rinsed thoroughly before being incubated with the immunological reagent. This procedure has likewise been used to enhance immunofluorescence staining of other hidden antigens in columnar epithelia (Ørstavik *et al.*, 1976).

Treatment with acid urea, moreover, results in intensified staining of J chain-producing IgA (Fig. 1) and IgM immunocytes. The fact that some cytoplasmic J chain is detected in these cells without denaturation in urea (Fig. 1a) is accounted for either by partial accessibility of bound J chain or by cytoplasmic excess of free J chain (Korsrud and Brandtzaeg, 1980). Thus, J chain produced in IgG and IgD immunocytes does not complex with the immunoglobulin products, and these cells consequently show no intensification of J chain staining after urea treatment (Brandtzaeg, 1976c; Korsrud and Brandtzaeg, 1980).

Owing to their denaturing effect, some of the modified formaldehyde-based fixatives (e.g. 2% acetic acid–formol saline, Bouin's fluid, and Susa fixative) give rise to satisfactory cytoplasmic staining for J chain even in IgA immunocytes (Brandtzaeg and Rognum, 1982b). However, as discussed below (Section II.C), structural unfolding leading to exposure of the J chain is counteracted by the cross-linking properties of these fixatives so that in secretory epithelial cells most J chain determinants remain hidden.

Antigenic masking caused by aldehyde-based fixatives is virtually unaffected by treatment with acid urea except when formol-sublimate has been used (Brandtzaeg and Rognum, 1982b). However, following pronase treatment (Section II.C) acid urea may exert some unfolding effect on secretory IgA; but the result with regard to epithelial staining of J chain is, nevertheless, inadequate because of its liability to proteolytic degradation (Section III.B).

It should be noted that non-specific fluorescence staining of eosinophilic granulocytes and squamous epithelia (Brandtzaeg, 1973) is intensified by acid urea. Moreover, this treatment may affect adversely the specific staining of some antigens, such as IgM and IgD.

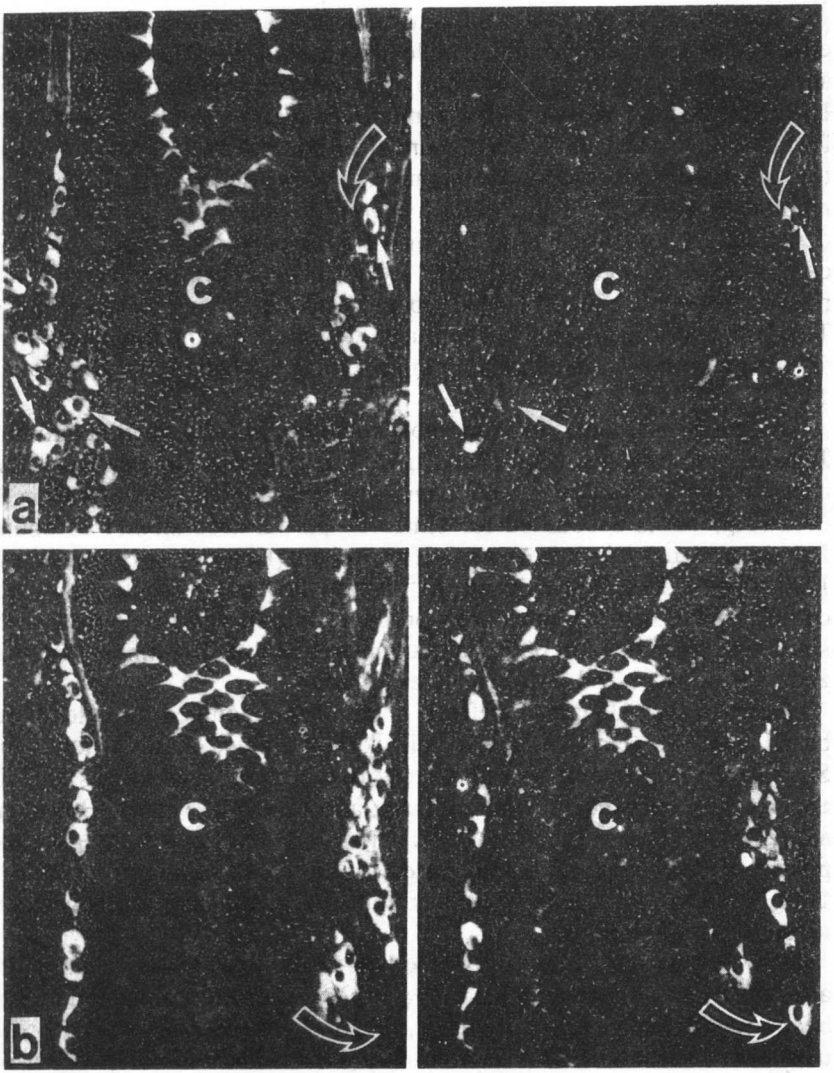

FIG. 1. Paired immunofluorescence staining for IgA (*left panel,* green fluorescence) and J chain (*right panel,* red fluorescence) in comparable fields from two serial sections of directly ethanol-fixed human colonic mucosa. (a) IgA is present in numerous immunocytes and in the crypt epithelium (C), whereas J chain is only detectable in some IgA cells (small arrows) and in one immunocyte of another isotype (open arrows). (b) This section has been denatured in acid urea; there is now bright staining for J chain in most IgA cells and in one immunocyte of another isotype (open arrows) and also in the crypt epithelium. Magnification × 296.

C. Unmasking of Antigens Concealed During Fixation

Sections of formaldehyde-fixed tissues have been subjected to proteolytic digestion in several immunofluorescence and immunoenzyme studies to improve the localization of protein antigens. The purpose has been both to reduce unwanted background staining and to restore antigenic reactivity. The latter process has been called unmasking of antigen. The various enzymes used have included trypsin, pepsin, papain and pronase (Huang, 1975; Denk *et al.*, 1976, 1977a,b; Reading, 1977; Brozman, 1978; Curran and Gregory, 1978; Radaszkiewicz *et al.*, 1979). Another recently reported advantage of proteolytic digestion is the elimination of xylene-induced antigen impairment that may be inflicted on the tissue during the clearing process (Mathews, 1981).

The mechanism of unmasking has not been definitely established; it has been suggested that proteases "etch" the surface of the tissue section sufficiently to allow contact between antibody and the corresponding antigenic determinant (Curran and Gregory, 1977). Aldehyde-based fixatives may in fact induce steric hindrance of antigenic reactivity both by directly modifying the antigen and by forming intermolecular cross-linkages. The former possibility is supported by the observation that the antigenicity of proteins can be concealed by reaction with benzyl chloroformate and subsequently unmasked by treatment with protease (Takamiya *et al.*, 1978). The latter possibility is substantiated by results showing that the degree of antigenic masking depends on the concentration of proteins surrounding the actual antigen during the fixation process (Rognum *et al.*, 1980; Hed and Enestrøm, 1981). Thus, it is more difficult to unmask immunoglobulin molecules in interstitial tissue and secretory epithelia than in immunocytes where they represent the major protein product. The drawbacks and advantages of such uneven antigen masking will be discussed below (Sections II.G and III.B.10).

Only a few studies have systematically evaluated various enzymes with regard to the best conditions of performance (concentration, incubation time, and temperature) on different types of antigens and tissues. It is recommended that such preliminary experiments are carried out when a new immunohistochemical test system is set up. The balance between under- and overdigestion is often a fine one, and adequate morphological preservation is not always compatible with satisfactory antigenic unmasking. Moreover, some antigens such as the J chain are prone to become degraded by proteolytic enzymes (Brandtzaeg and Rognum, 1982b). There have also been warnings of the possibility that new cross-reacting antigenic determinants may be created by the digestion

process (Heyderman, 1979), but there is no evidence to support this suggestion.

Theoretically, it should be easier to perform a milder and more controllable digestion with trypsin than with pronase (Huang *et al.*, 1976); but we (Rognum *et al.*, 1980) and others (Mepham *et al.*, 1979) have not detected any significant difference between the performance of the two enzymes used at optimal conditions on formalin-fixed tissues. We obtained comparable unmasking of SC and IgA in colonic epithelium when the digestion was carried out at 37°C with pronase at 1 g l^{-1} for 15 min or with trypsin at 0·5 g l^{-1} for 1–2 h. Also the effect on cytoplasmic IgG, IgA, and IgM of immunocytes was similar. However, batch differences and instability of the enzymes must be taken into account, and unpredictable variations between tissue blocks may be encountered (Mepham *et al.*, 1979).

It is important to be aware of the fact that the result of proteolytic digestion may be variable, not only for different antigens but also for the same antigen in apparently similar cells. Thus, when immunocytes in formalin-fixed tissues are subjected to pronase treatment, unmasking of cytoplasmic IgG is more consistent than that of IgA (Fig. 2). This difference is probably due to degradation of cytoplasmic IgA in some of the cells. In glutaraldehyde-fixed tissue (Section III.B) the unmasking effect may, on the whole, be more striking and the IgA immunocytes show a more homogeneous result (Fig. 3).

The heterogeneity of IgA-producing immunocytes with regard to immunoreactivity after different fixation procedures and treatment of sections with pronase is illustrated in Fig. 4. A fluorochrome conjugate with a relatively restricted specificity was used to bring out the differences more clearly than when a reagent of broader anti-α-chain activity was used. Direct fixation in cold 96% ethanol resulted in a fairly homogeneous staining of the immunocytes, but the signal-to-noise ratio was poor due to staining of IgA present in the interstitial fluid (Fig. 4a). Ethanol fixation after washing of the tissue to remove diffusible proteins (Brandtzaeg, 1974) also resulted in homogeneous staining of the cells, which were now visualized with a good signal-to-noise ratio (Fig. 4b). Fixation with 2% acetic acid–formol saline, which among the modified formaldehyde-based fixatives gives the best result for immunoglobulin-producing cells without proteolytic digestion (Curran and Gregory, 1980; Brandtzaeg and Rognum, 1982b), yielded intense staining of some cells whereas others were quite dull or contained a bright patch close to the nucleus (Fig. 4c). Formalin fixation followed by pronase treatment produced heterogeneous staining with many dull cells and some intensification close to the nuclei (Fig. 4d). Pronase treatment after glutaraldehyde fixation

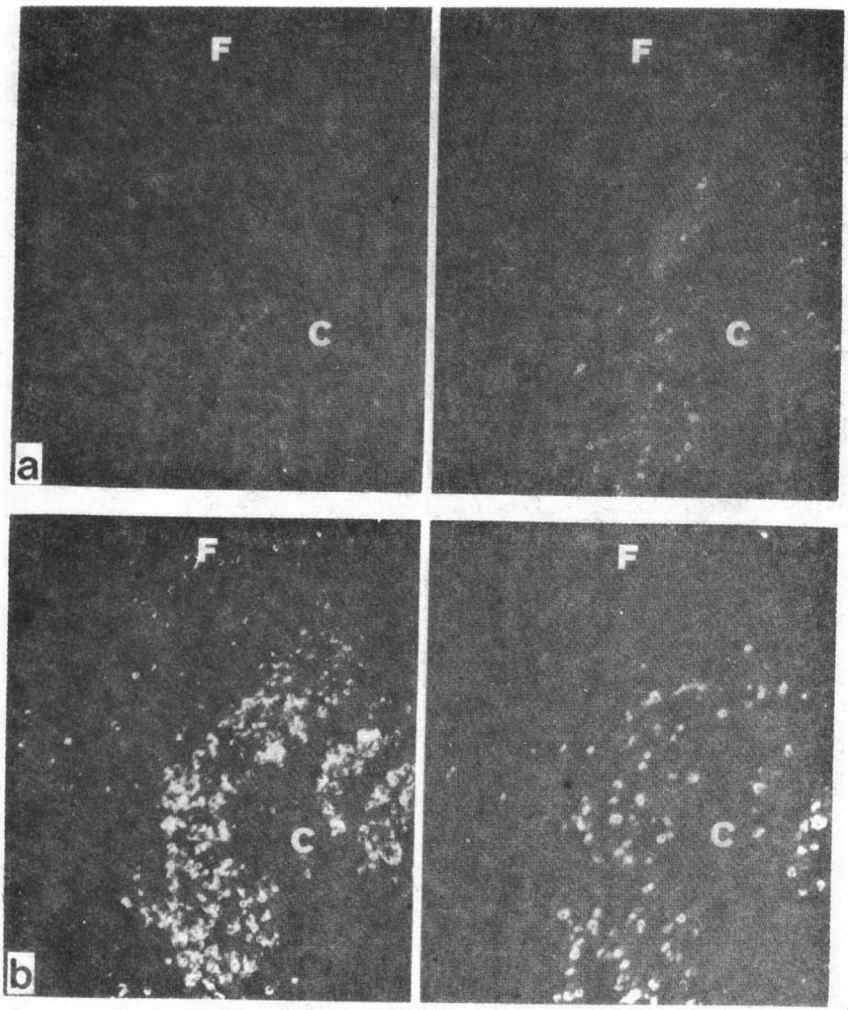

FIG. 2. Paired immunofluorescence staining for IgG (*left panel,* red fluorescence) and IgA (*right panel,* green fluorescence) in comparable fields from two serial sections of human tonsillar tissue subjected to routine formalin fixation. (a) No IgG- or IgA-producing immunocytes can be seen in the centre of a lymphoid follicle (F), and the numerous cells of both isotypes present adjacent to a crypt (C) are hardly visible. (b) In this pronase-treated section there is strikingly intensified staining of both intra- and extra-follicular IgG cells, whereas the result varies more for IgA cells. Isotype restriction of individual cells could be verified in double-exposed colour slides which thus attested to the reliability of the staining (see Plate I—between pp. 148 and 149). Exposure times were the same in (a) and (b). Magnification × 112.