

Simultaneous Detection of Multiple Antigens with Triple Immunolabeling

Zac Pujic, B.Sc., Neil W. Savage, Ph.D., P. Mark Bartold, D.D.Sc., and Laurence J. Walsh, D.D.Sc.

Immunolabeling is commonly used to localize antigens within frozen or paraffin tissue sections. We modified existing immunolabeling techniques to allow the detection of three antigens simultaneously within the one tissue section. The approach relies on the use of three monoclonal antibodies in sequential immunoperoxidase staining steps, each with colored substrates, resulting in the deposition of black, brown, and rose stains. The method is rapid and does not require novel techniques or materials. In this report, we demonstrate the colocalization of mast cell tryptase, neurofilament protein, and CD31 (platelet-endothelial cell adhesion molecule) or laminin in normal human skin and normal buccal mucosa, as an illustration of the power and simplicity of the multiple antigen localization technique.

Key Words: DAB—Immunohistochemistry—Immunolabeling—Mast cells—Skin.

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Immunoenzymatic staining uses the enzyme-catalyzed conversion of colorless chromogens into colored products to localize antigens in tissue. Although staining for different antigens on sequential sections is commonly performed, such methods provide only limited information regarding the spatial relation between the antigens of interest.

In standard immunoperoxidase techniques, the final reagent step uses horseradish peroxidase to oxidize the colorless chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) into a brown end-product in the presence of hydrogen peroxide. However, if nickel chloride is included in the reaction mixture, the final reaction product is black rather than brown. By using a sequential application of immunoperoxidase staining steps, two nonoverlapping antigens can be localized by using brown (DAB) and black (nickel-DAB) staining. This technique has been useful for exploring spatial association between immune cells and vascular and neural elements in skin and mucosal tissues (1-3). When multiple labeling is undertaken on sections, the saving in time, effort, and materials can be considerable, while yielding valuable information regarding the spatial relation between the two antigens.

With the advent of additional peroxidase substrates such as the Vector VIP Substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.), it has become feasible to achieve triple immunolabeling in tissue sections. The VIP substrate is oxidized by horseradish peroxidase to yield a rose-colored final reaction product, which provides a strong color contrast with DAB-based reaction products. For triple labeling, we found that the optimal sequence of substrates is nickel-enhanced DAB for the first label, DAB for the second label, and VIP for the third.

MATERIALS AND METHODS

Four-millimeter-diameter punch biopsies of normal non-sun-exposed human skin tissue, which had been ob-

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From the School of Dentistry, The University of Queensland, Brisbane, Australia.

Address correspondence and reprint requests to Dr. Z. Pujic, School of Dentistry, The University of Queensland, 200 Turbot Street, Brisbane, Queensland, 4000, Australia.

tained during the course of previous studies (3) were embedded in OCT, and 4- μ m cryostat sections prepared. The sections were mounted on poly-L-lysine-coated slides and stored at -80°C . Unless otherwise noted, all steps were performed at room temperature (22°C). Sections were brought to room temperature, and a grease ring drawn around the sections to limit the spread of reagents. After a 10-minute rehydration in phosphate-buffered saline (PBS; 0.1 M phosphate, 0.15 M saline, pH 7.4), sections were incubated with a blocking solution comprising 2% normal swine serum/1% bovine serum albumin (BSA) in PBS, for 15 minutes. The first staining sequence was then commenced. Sections were incubated with the primary monoclonal antibody (a 1:200 dilution of mouse anti-human mast cell tryptase antibody (DAKO, Botany, NSW, Australia) diluted in 1% BSA/PBS) for 1 hour. The sections were then washed for 10 minutes in PBS with the aid of magnetic stirring, and incubated with biotinylated anti-mouse antibody for 15 minutes, before a further washing step. The third-layer reagent, avidin-biotin-horseradish peroxidase, was then added for 15 minutes. A Vector DAB Substrate kit was used to develop the reaction by using nickel-DAB, with a 5-minute developing time, according to the manufacturer's instructions. This yielded a black reaction product at sites of mast cell tryptase.

Before the second staining sequence, the sections were washed in PBS and incubated for 15 minutes in a solution consisting of 0.3% H_2O_2 /0.1% NaN_3 , to quench residual peroxidase activity. The staining sequence for the second layer commenced with blocking solution, as described earlier. Next, the relevant primary monoclonal antibody (mouse anti-neurofilament protein antibody; DAKO, clone 2F11) diluted 1:200 in 1% BSA/PBS) was added for 1 hour. The sections were then washed and processed through steps with biotinylated secondary antibody and horseradish peroxidase, as outlined. The peroxidase was visualized by using a Vector DAB Substrate kit (Vector Laboratories), with "standard" DAB for a 5-minute reaction time, to yield a brown reaction product for neurofilaments.

Before the third staining sequence, the sections were washed in PBS and incubated in quenching solution, as described. For the third label, the primary monoclonal antibody was a 1:200 dilution of a mouse anti-human CD31 (platelet endothelial cell adhesion molecule/PECAM-1, clone JC/70A). This reagent identifies all blood vascular endothelial cells. Other incubation and washing steps were as described. The peroxidase enzyme was developed by using a Vector VIP Substrate kit for a 4-minute developing time, according to the manufacturer's instructions, to yield a rose-colored product on labeled endothelium. The sections were then washed in PBS, counterstained lightly in Mayer's acid hematoxylin (20 seconds), and rinsed in tap water before being dehy-

drated in solutions of 75, 90, and 100% ethanol (1 minute each). The sections were cleared in xylene (three changes for 3 minutes) and mounted. Normal human buccal mucosa, obtained during a previous study (4), was stained black for laminin, brown for mast-cell tryptase, and rose for neurofilament by using this procedure. Anti-mast cell tryptase, anti-neurofilament protein, anti-laminin, and anti-CD31 antibodies were obtained from DAKO. Biotinylated secondary antibody and horseradish peroxidase were from the DAKO LSAB Kit. For control purposes, an anti-immunoglobulin G (IgG) monoclonal antibody (Vector) was used in place of the first-layer monoclonal antibodies. Sections were also single labeled as positive controls. Additional sections were incubated with peroxidase substrate solutions to check for endogenous peroxidase activity in the tissues or for enzyme activity carried over from one staining sequence to the next. The concentration of primary antibody was assessed by performing staining by using 1:100, 1:200, and 1:500 dilutions on tissue sections. Although all dilutions resulted in the presence of visible staining, the 1:200 dilution gave the best overall signal-to-noise ratio, with strong staining of labeled cells and minimal background staining. The incubation time also was optimized. Overnight incubation at 4°C yielded only slightly stronger staining than a 1-hour incubation at room temperature; however, this resulted in the entire procedure becoming lengthy. Thus overnight incubation is justified only for antigens (such as neural markers) that are expressed at a low level. The concentration of anti-mouse biotinylated secondary antibody and streptavidin-horseradish peroxidase complex in the DAKO LSAB Kit has been optimized by the manufacturer, and we did not alter this during our study.

RESULTS

The triple-labeling protocol gave clear and distinct labeling of mast cells, nerves, and endothelium, with minimal background in normal human skin. Panel A and the inset in Fig. 1 show normal human skin stained for mast cell tryptase (nickel-DAB: black), neurofilament protein (DAB: brown), and CD31 (VIP: rose). Panel B in Figure 1 shows triple staining for laminin (nickel-DAB: black), mast cell tryptase (DAB: brown), and neurofilament (VIP: rose) in normal buccal mucosa. The staining for each layer was clear and distinct with minimal background. Laminin was localized to epithelial, endothelial, neuronal, and basement membranes. Spatial interactions between mast cells and cutaneous nerves could be identified as sites where the distance between the mast cell membrane and the nerve was $<20\ \mu\text{m}$ (arrowheads). Both mast cells and nerves were frequently associated with blood vascular endothelium.

Control sections in which an irrelevant antibody (anti-human IgG) was substituted for one of the first-layer

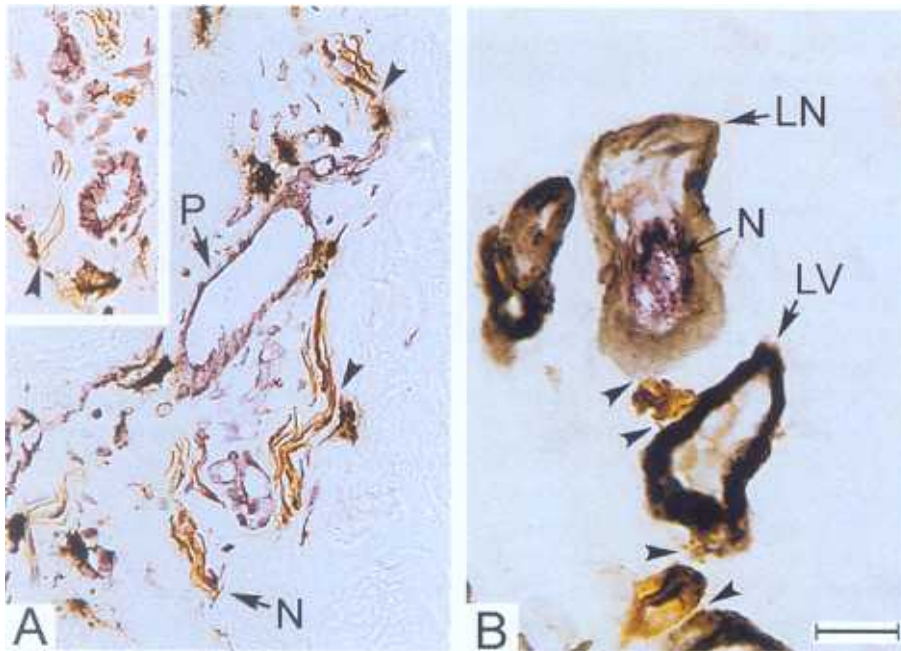


FIG. 1. Neurofilament, PE-CAM-1, and mast cell tryptase colocalization in normal human skin (**A** and insert) and neurofilament, laminin, and mast cell tryptase colocalization in normal human buccal mucosa (**B**). In skin, mast cell tryptase was stained black with nickel-DAB, neurofilament was stained brown with DAB, and PECAM-1 was stained rose with VIP. In buccal mucosa, laminin was stained with nickel-DAB, mast cell tryptase was stained with DAB, and neurofilament was stained with VIP. Arrowheads, sites of interaction between mast cells and nerves or vascular tissue. P, PECAM-1; N, neurofilament; LV, vascular laminin; LN, neuronal laminin. The scale bar indicates 40 μm for **A** and 16 μm for **B**.

monoclonal antibodies showed no immunoreactivity. All controls for endogenous and carry-over peroxidase activity were negative.

DISCUSSION

This study demonstrates the colocalization of up to three antigens within a single tissue section by using three distinctly colored products. By combining nickel-enhanced DAB staining with DAB and VIP staining, it is possible to use the simple procedure of immunohistochemistry to obtain more information than is available by using a single stain. With this technique, we demonstrated the spatial interaction between mast cells and both nerve bundles and blood vascular endothelium in normal human skin and buccal mucosa. Furthermore, we obtained an accurate measurement of the distance between mast cells and nerves or endothelia, as well as the percentage of mast cells in association with nerves in oral lichen planus (5). Studies that aim to obtain such data by using single-staining techniques of serial sections and stained consecutively for markers of these structures, are difficult to analyze because of slight distortions between tissue sections, as well as changes in the tissue architecture. However, the method developed here eliminates these problems.

In general, previous approaches to multiple labeling have typically been confined to the simultaneous visualization of two different antigens. Typically, these use immunoperoxidase followed by alkaline phosphatase (6) to achieve double labeling. Our method uses only one staining protocol (i.e., immunoperoxidase) to achieve

triple staining. There are a variety of techniques for achieving multiple staining by using histochemical techniques (7); however, such techniques can suffer from poor specificity and high background. In our method, specificity of staining is achieved through the use of antibody-antigen interactions.

In our studies of triple staining, we experimented at some length with changing the sequence of reactions to arrive at the optimal sequence of nickel-DAB, followed by DAB and VIP (i.e., black, brown, and then rose). When other sequences of staining were used, the likelihood of significant cross-contamination between the reaction products increased. If DAB was followed by nickel-DAB, there was a tendency for the nickel ions in the developing solution of the second stain to complex directly to the DAB from the first stain, darkening this and reducing contrast between the two labels. If VIP was performed before either of the DAB steps, the rose-colored VIP reaction product would acquire a subtle brownish discoloration, with some loss of color contrast. Overall, the greatest color contrast was obtained when the stain order was black, brown, and then rose.

Several additional technical issues deserve mention. First, optimization of the developing times for each enzyme substrate must be performed in each laboratory and for each tissue to ensure the greatest color difference between the three stains. This is in addition to optimizing the concentration of the primary antibody, which will be undertaken for each tissue. In our experience, incubation with lower concentrations for a longer period gives less background than high concentrations for a short period. This also provides a more economic use of expensive

first-layer reagents. Second, because it is likely that levels of background will be increased with multiple staining compared with single staining, rigorous blocking and washing is required. Third, quenching of carry-over peroxidase activity is essential to ensure that specific staining is obtained. Finally, with regard to the choice of counterstain, the color difference between hematoxylin and the rose-colored VIP reaction product will be adequate for most purposes, provided that the color of the hematoxylin is sufficiently "blue." This will vary according to the pH of the tap water (or other solution) used to wash excess hematoxylin from the sections.

Although our triple-labeling technique was developed for use on frozen tissue sections, there is no reason it cannot be applied to formalin-fixed, paraffin-embedded tissues provided that antibodies capable of binding to formalinized tissue fixed in this way are available. The method outlined for staining should be feasible for formalin-fixed, paraffin-embedded tissues; however, it will be necessary for each laboratory to optimize conditions for the tissues and antibodies. The effect of antigen retrieval, or unmasking, on signal strength in paraffin sections was not assessed in our study. Each laboratory must optimize these techniques for its own conditions of antibody use.

We found no deleterious effect on antigenicity of three stages of hydrogen peroxide/sodium azide quenching. This quenching is necessary to remove both endogenous peroxidase activity and peroxidase activity present from previous labeling procedures. In experiments using single-label immunohistochemistry with either DAB, nickel-enhanced DAB, or VIP staining, we found no difference in signal strength or alterations in the signal pattern when compared with double or triple immunohistochemistry. Because we cannot extrapolate the results of our studies to all antigens that may be studied by using this technique, it is important that each laboratory confirm that conditions of quenching are appropriate for the tissue and antigens under study.

One limitation of the triple-labeling technique described is that it is unsuitable for the colocalization of antigens expressed in or near the one site (e.g., on the membranes of the same cell, because of the visual interference between reaction products that will occur. In such cases, the options include multiple fluorescence la-

beling, combined immunohistochemistry and immunofluorescence, single labeling of serial sections, and the removal of one reaction product by chemical treatment before restaining.

In summary, by slight modification of the commonly used immunoperoxidase method, it is possible to increase greatly the amount of data obtained from one tissue section by using double or triple labeling. The technique uses materials already present in most laboratories and builds on existing techniques. The entire process as described can be undertaken in 1 day, with ~10 hours from thawing of the sections to examination under the microscope. In many laboratories, an overnight incubation step can be used, and this can be applied to simplify logistics from day to day. For instance, in our laboratory, incubations for nerve labeling (such as anti-neurofilament protein and neural adhesion molecules) are often undertaken overnight at 4°C. This gives greater penetration of the antibody into tissue sections, as well as making the procedure more manageable by splitting it over 2 days. □

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