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Troubleshooting tissue specificity and antibody selection: Procedures in immunohistochemical studies

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ABSTRACT

Optimal antigen detection and identification is dependent on the tissue of interest, the method of fixation, processing, and antibody specificity. We evaluated specific antigens in frozen middle cerebral artery (MCA) sections from rat brains under various conditions of fixation and differing primary and secondary antibody concentrations. Fresh MCAs were frozen, cryosectioned (8 μm), and adhered to chrom-alum coated slides. The effects of different fixation and antigen retrieval/pretreatments were tested for detection of enzymes and receptors involved in MCA tone regulation. Antigen localization was determined with specific primary antibodies and detected using fluorochrome-conjugated secondary antibodies. Spatial distribution of localized antigens was imaged using confocal microscopy. Frozen sections preserved the morphology of the endothelium and/or vessel wall within the tissue in a manner comparable to formalin-fixed sections. Fixation and tissue processing methods were modified based on the primary antibody used. Optimal antigen detection was obtained using fixatives such as 4% paraformaldehyde, 100% acetone or 100% methanol. Pretreatments, such as 1% SDS, enzymatic digestion using 0.1% trypsin, or application of heat were used to optimize antigen–antibody interaction. Stringent background and control checks were performed to ensure specificity of staining in both single and multiple labeling techniques. In a research setting where epitope detection is not used for diagnostic purposes, there is more latitude in tissue fixation. Frozen samples offer a more versatile method of linking the appropriate fixation and tissue processing to the primary antibody's unique needs. At the same time, it stabilizes the tissue in a format that allows for later analysis of multiple antigens with specific detection requirements in same tissue.

1. Introduction

Multiple techniques have been developed to assist tissue preservation in order to define changes occurring in tissues and cells during disease. Immediate tissue fixation maintains morphological integrity necessary to describe distribution and structure of specific molecules *in situ*. This has been the cornerstone in the development and use of immunohistochemistry (IHC) in biological research. IHC incorporates fixation, and processing to stabilize the microanatomy of the tissues and subsequent immunodetection allows visualization of localized antigen. (Fox, Johnson, Whiting, & Roller, 1985, Grizzle, 2009, Shi et al., 2008, Nadji, Nassiri, Vincek, Kanhoush, & Morales, 2005). The optimal visualization of the antigen within the tissue must be a compromise between proper fixation and tissue processing to allow epitope presentation to the antibody. The constraints that determine how effectively the antigen is presented and detected are a delicate balance of local antigen concentration, how the fixative modifies the antigen, access of the antibody to antigen, and antibody avidity and specificity (Harlow & Lane, 1999). The antibody–antigen interaction is optimized by ensuring that the antigen is presented in its fullest capacity after fixation and processing. Traditionally, fixatives that function by forming cross-links within and between proteins are used because of their speed of penetration into the tissue and relative uniform preservation of the macromolecules for IHC and molecular assays. Neutral buffered formalin (10% NBF) fixation followed by embedding in paraffin has been a preferred method of fixation and storage for biological tissues. However, formalin–paraffin processing is extremely harsh and may cause epitope damage and/or blockage of antibody access resulting in loss of antigen immunorecognition (Arnold et al., 1996, Otali et al., 2009). Extended time of tissue exposure to formalin results in "overfixation", or excessive cross-link formation, causing loss of epitope due to chemical modifications. During overfixation, formalin forms a tight mesh by cross-linking cellular proteins and physically blocking antibody access. Heating the tissue to 50 °C for extended periods during the paraffin embedding process, in addition to the need for organic solvents to dehydrate the tissue, all result in protein/epitope denaturation.

Various methods of antigen retrieval unmask the antigens, but overall, this means that the tissue must undergo further chemical

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modifications to reverse the fixation process constraining the antigen of interest, and further affecting the immunoreactivity of the tissue (O'Leary, Fowler, Evers, & Mason, 2009). As different antibodies will interact differently with the antigen, it is best not to restrict the method of fixation after tissue harvesting. That is why freezing a fresh unprocessed tissue cryosectioning and subsequent immunostaining of the sample is highly recommended by our laboratory. With this method you not only immediately preserve the tissue, but you are not limited in the type of fixative, and thus can tailor it to your antibody–epitope of interest. In a research based environment, a molecular friendly fixation and processing system is preferred.

Studies within our laboratory have involved the assessment of alterations in cerebrovascular function and blood flow regulation in relation to hemorrhagic stroke development in spontaneously hypertensive rat (SHRsp). These studies have required the assessment of the distribution and localization of a variety of proteins, peptides, signaling molecules, and endogenous receptors in the MCAs. Typically, the presentation of antigen and antigen recognition by antibodies differ based on the tissue being investigated, and MCAs have rarely been examined by IHC for presence of endothelial proteins. As with any methodology, epitope recognition will vary from tissue to tissue, as well as from fixative to fixative. Therefore, we tested different fixatives and tissue processing methods for each antibody to individualize the method to that antibody and epitope of interest. As well, we assessed how the fixation process affects the specificity of antibody–antigen interaction and tested the different parameters that control for specificity of both the primary and secondary antibodies in single and multiple antigen labeling methods. This paper will present potential methods and solutions related to the detection of epitopes starting with frozen tissues. In addition, we have outlined how to run proper controls when staining tissues for immunofluorescence detection. We believe that cryostat sections of unfixed frozen tissue provide the most suitable method for the selective manipulation of fixative that is needed to achieve an optimal method of antigen fixation, with a high sensitivity for antigen detection.

2. Tissue sampling, fixation and processing

In any immunodetection process, one of the primary difficulties encountered is the inability to detect the antigen of interest due to inaccessibility by the antibody. If the antigen is physically blocked due to overfixation or is oriented within an obstructing cellular structure, antibodies may not be able to penetrate to the antigen (Mason & O'Leary, 1991; O'Leary et al., 2009). This often occurs in paraffin-embedded, formalin-fixed (PEFF) sections.

The reason as to why an antibody is blocked from accessing its antigen differs with each antibody. Therefore, separate solutions are often necessary to allow each antibody optimal access to the antigen. For this reason, we believe the best method of stabilizing the tissue structure is flash freezing fresh tissue that have not undergone any previous fixations. Various studies have described immunodetection within tissues prepared from frozen sections as being gentler in maintaining antigen structure (Bergroth, 1983; Richter, Nährig, Komminoth, Kowolik, & Werner, 1999). Small blocks of tissue can be dropped directly into liquid nitrogen or into a bath of hexane cooled in liquid nitrogen. When the tissue is frozen, it is firm, with the ice acting as the embedding medium (Spencer & Bancroft, 2008). Although flash freezing creates small ice crystals, these cause minimal disruption of cellular morphology. The consistency of the frozen block can be altered by varying the temperature of the tissue during sectioning to optimize tissue consistency and the quality of cutting. For example, the majority of non-fatty unfixed tissues section well at −20 °C to −25 °C, but this temperature can be increased or decreased depending on the composition of a particular tissue. In this review, we will describe our work with MCAs embedded with surrounding brain tissue sampled from male SHRsp. To induce cardiovascular change, rats were fed a 4% NaCl Japanese-style stroke prone diet (Zeigler Bros., Gardners, PA, USA) from weaning. SHRsp were sacrificed at 9–14 weeks of age. The studies were conducted with institutional approval and in compliance with the guidelines of the Canadian Council on Animal Care outlined in The Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd Ed., revised 1993, ISBN: 0-919087-18-3) and The Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). Brain tissue blocks containing the MCA in the region of the rhinalis fissure were carefully excised and placed on a cryostat freezing disc, covered with OCT medium (Triangle Biomedical Sciences, Duhan, N.C., USA), and flash frozen directly in liquid nitrogen. Samples were then stored at −80 °C in a sealed tube. Tissues were sectioned at −20 °C using a cryostat (IEC Minotone, International Equipment Co., Needham Heights, MA, USA) and transferred onto chrom-alum–gelatin subbed glass slides. This enabled serial sections of the same MCAs to be cut, permitting accurate comparison of various fixation and processing techniques. Sections were dried at room temperature for 2 h and then stored at −20 °C until use. Slides could be stored at −20 °C for several months with no loss of quality. For processing, slices were removed from the freezer and warmed at room temperature 30 min prior to fixation. The antigens of interest were the nitric oxide synthase (NOS) isozymes [eNOS, nNOS, and iNOS], protease-activated receptor-2 (PAR2), B-2 bradykinin receptor (B_2R), angiotensin II type 1 receptor (AT_1R) and protein kinase C (PKC) alpha. Table 1 lists the most ideal fixation, pretreatment, and processing procedures used to localize the antigens. Table 2 lists specific information pertaining to the primary antibodies that targeted the antigens and includes information relating to the clone, specificity, the manufacturer, and the dilutions used. The primary goal of our IHC processes was to design the simplest method of fixation involving the least amount of chemical manipulations, to accurately determine the presence and distribution of the antigens listed. For each antibody, we identified the fixative of choice using step-wise testing of various fixatives. We started with the most common fixative, 4% paraformaldehyde, since vendor recommendations for antibody staining included procedures developed for PEFF tissues. Paraformaldehyde is the dry, highly polymerized form of formaldehyde, and is primarily used at 4% or 2% concentrations. It forms cross-links or covalent bridges between and within proteins and nucleic acids forming hydroxymethyl groups from side groups that contain an active hydrogen and/or primarily tertiary amines. Further condensation reactions lead to covalent methylene bridges between polypeptide chains (Troyer, 1980). This offers a rapid, stable fixation that maintains excellent tissue morphology, however it may mask the epitope by chemically modifying the proteins. We found this to be problematic with some of our antigens. While antigen masking was not a problem with the mouse monoclonal anti- eNOS antibody, further pretreatment, or different fixation techniques, were required to unmask the epitopes recognized by other antibodies prior to tissue staining (Table 1, Fig. 1). Coagulating fixatives were also tested. These fixatives cause protein dehydration, making them insoluble, resulting in protein precipitation (Grizzle, Fredenburgh, & Myers, 2008). Alcohols and acetone are examples of coagulants. By removing the water, the hydrophobic areas of proteins are weakened, disrupting the secondary and tertiary structures of proteins. In addition, with the water removed, the structure of the protein may become partially inverted, with the hydrophobic groups moving to the outside surface of the protein. Once the structure of a soluble protein has been modified, the rate of reversal to a more ordered, soluble state is slow and most proteins remain insoluble when returned to an aqueous environment. Fixation with acetone or alcohols also works best if the solvents are cooled to −20 °C prior to their use and kept cool during fixation (4 °C). The cold temperature halts any biological processes instantly, keeping the tissue in stasis, while the fixative precipitates the proteins.

Regardless of the fixative used, the time needed to fix an 8 µm thick section is dramatically less than that required for a tissue block