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The Fundamentals of Immunohistochemistry

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INTRODUCTION

Immunohistochemistry, or, IHC is a valuable technique that evaluates the status of target molecules in tissues of interest by interaction between antigens and antibodies. It has evolved into a powerful method that allows for the direct visualization of tissue antigens using labelled antibodies that are specific to the target antigen, combined with microscopy and image analysis. IHC plays a crucial role in fields such as histology, pathology, cancer biology, neurology, and drug development due to its versatility, ease of use, and cost-effectiveness.

IHC with specific tumor markers is utilised to diagnose the nature of tumors, distinguishing between benign and malignant tumors, determining their stage and grade, and pinpointing the cell type and origin of metastases to locate the primary tumor site. Additionally, IHC plays a pivotal role in diagnosing various non-neoplastic diseases and conditions, either as the primary diagnostic tool or as a means of confirmation. In research, IHC is employed to investigate diverse areas such as the development of normal tissues and organs, pathological processes, wound healing, cellular responses to injury and repair, and a broad spectrum of other scientific inquiries. Furthermore, IHC contributes significantly to drug development by assessing drug effectiveness through the detection of changes in disease markers, whether it involves alterations in their activity levels or their up- or down-regulation, not only within target tissues but also in other relevant areas.

Although there exist several variations and combinations in the IHC procedure, it can be categorized into two main phases: the preparation of the sample and the subsequent staining of the sample.

Preparation of the sample

Ensuring the appropriate antibodies to target the specific antigens and enhancing the signal are essential for achieving optimal visualization. However, sample preparation is equally crucial in preserving cell structure, tissue organization, and the antigenic properties of target epitopes.

Tissue handling and fixation

Most tissue fixatives crosslink the proteins and/or reduce protein solubility. However, prolonged or inappropriate fixation can obscure the target antigens. The most commonly employed fixative is 10% Neutral buffered Formalin. It serves as a semi-reversible, covalent crosslinking agent. Typically, tissues fixed with formaldehyde are embedded in paraffin wax for subsequent sectioning and further processing. Such tissues and the resulting sections are commonly referred to as formalin-fixed and paraffin-embedded (FFPE) specimens. Various alternative fixatives available including acetone and methanol may also be used. Excessive fixation can lead to permanent damage to certain epitopes. To prevent ischemic or cold-induced damage, which can result in the deterioration of proteins or tissue enzymes, it is crucial to ensure rapid fixation.

Sectioning and mounting

The typically suggested thickness for tissue sections in IHC is 4 μm, although it can vary depending on the specific objectives of the study. These sections are subsequently transferred onto glass slides that have been treated with a tissue adhesive. This adhesive is typically applied by surface-treating the glass slides using substances like 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine, both of which introduce amino groups to the glass surface for tissue adhesion. In the past and, if necessary, even today, slides can be coated with actual adhesives, such as gelatin, egg albumin, or even products like Elmer's glue.

De-paraffinization and antigen retrieval

It is necessary to entirely remove the paraffin from FFPE sections prior to IHC staining. Incomplete de-paraffinization can lead to the concealment of target antigens, rendering them inaccessible to antibody reactions. Additionally, paraffin's hydrophobic properties act to repel aqueous solutions containing the IHC staining reagents. Organic solvent xylene has been conventionally employed for de-paraffinizing FFPE slides. However, nowadays, xylene-free alternatives for de-waxing are accessible.

Formaldehyde fixation creates methylene bridges, which covalently link proteins within tissue specimens. These bridges have the potential to obscure access to antigens and epitopes, hindering or even preventing antibody binding. Also, epitopes can be masked in formaldehyde-based fixation due to cross-linkings of amino groups on adjacent molecules. Consequently, in the case of FFPE sections, a specific treatment is usually needed to expose or retrieve the antigenic epitopes before staining. This process is commonly referred to as epitope or antigen retrieval.

Epitope or antigen retrieval can be achieved by subjecting the de-paraffinized sections to various buffer solutions with different pH levels, a process known as heat-induced epitope retrieval (HIER). Alternatively, antigens can be retrieved by treating the tissue sections with proteolytic enzymes like pepsin, trypsin, or proteinase. However, the most commonly employed technique for antigen retrieval is HIER. Heat can be applied using various methods such as microwave ovens, heating plates, pressure cookers, autoclaves, and water baths, with pH levels ranging from 6 to 10. Typically, when using an autoclave or microwave oven, the temperature is set at 120°C under full pressure and 750–800 W of power, respectively, for a duration of about 10 minutes. If a heating plate is used, the sections are incubated at 100°C for 30 minutes. It is essential to determine the

optimal retrieval conditions for each antigen-antibody pairing through empirical testing by comparing the staining results achieved with different retrieval methods. In cases, when enzymatic retrieval is utilized for specific antigens, such as some cytokeratins and immunoglobulins, tissue sections are incubated with trypsin or proteinase for approximately 10–20 minutes at 37°C. The reaction is then terminated by adding phosphate-buffered saline (PBS).

Protein blocking/Blocking nonspecific sites

A protein blocking step is essential to diminish undesired background staining. One of the primary contributors to this background signal is the nonspecific attachment of the Fc portion of either the primary or secondary antibodies. Antibodies exhibit a strong preference and affinity for specific epitopes but also exhibit partial or weak binding to sites on non-antigen proteins that resemble the actual binding sites on the target antigen. To mitigate background staining in IHC, samples are pre-treated by incubating them with a buffer designed to block the non-specific binding sites. These blocking buffers commonly contain components such as 5%–10% normal serum of same species, non-fat dry milk, bovine serum albumin (BSA), gelatin, and one or more mild surfactants. Additionally, there are numerous commercially available blocking buffers with proprietary formulations designed to enhance blocking efficiency. The duration of incubation for the blocking step can range from 30 minutes to overnight, with incubation temperatures varying from 4°C to room temperature.

Quenching/blocking endogenous target activity

Several widely used staining methods rely on biotin and its binding proteins, such as streptAvidin (SA), NeutrAvidin (NA), and avidin (AV). The majority of detection techniques utilize the enzymatic activity of horseradish peroxidase (HRP) or alkaline phosphatase (AP) for detecting target antigens when specific substrates are present. Consequently, to prevent false positive detection and minimize high background staining, it is necessary to neutralize (quench) or mask the presence of naturally occurring forms of these proteins.

When employing the peroxidase antiperoxidase system during the detection step, it is essential to block the inherent peroxidase activity. A common method involves using a 3% diluted hydrogen peroxide solution to block the endogenous peroxidase activity. Similarly, endogenous AP, which is often present in frozen tissue, should be blocked using a 10 mM levamisole solution. Another challenge is dealing with endogenous biotin in tissues. To block endogenous biotin, especially in biotin-rich tissues like the liver and kidney, tissue sections can be pre-treated by incubating them in an avidin solution (15-minute incubation in avidin solution followed by a quick rinse in PBS, and then a 15-minute incubation in biotin solution, all at room temperature). These strategies physically obstruct or chemically inhibit the activity of endogenous biotin or enzymes, respectively.

Antibody selection and validation

Before performing IHC, the selection of an appropriate antibody is paramount, and it commences with a thorough review of the available literature to understand the target. Antibodies used in research are typically categorized into three types based on their validity and reliability: well-established antibodies with strong supporting literature, well-known antibodies applied to different species or unverified tissues, and unknown antibodies with inconsistent or no literature backing.

Antibodies can generally be classified into two main categories. Polyclonal antibodies are generated by repeatedly stimulating experimental animals with antigens, and these antibodies can bind to multiple distinct epitopes within a single antigen. Conversely, monoclonal antibodies specifically target a single epitope in an

antigen and are derived from a single hybridoma clone that produces antibodies.

When validating an unknown antibody, it is crucial to carefully choose suitable positive and negative control tissues. It is advisable to validate the antibody using non-IHC methods, such as western blotting or flow cytometry. Following this, optimization becomes essential to parameters like antibody dilution, incubation durations, and blocking procedures to suit the specific laboratory conditions. Adequate validation and optimization of the IHC staining method is required for consistent results across different laboratories.

Detection system

Detecting the target antigen using antibodies is a complex process that requires optimization at various stages to maximize signal detection. Immunostaining, a method for detecting specific antigen-antibody interactions, often involves using secondary antibodies labelled with various markers like enzymes. Commonly employed detection systems include the avidin-biotin complex method, labelled streptavidin-biotin method, phosphatase anti-phosphatase method, polymer-based detection system and the tyramine amplification system. Compared to standard IHC techniques, polymeric and tyramine-based amplification methods typically offer substantially increased sensitivity (50 times or more). The use of an alkaline phosphatase based detection system is recommended for tissues rich in endogenous peroxidase, such as bone marrow or lymphoid tissue. Conversely, a peroxidase-based detection system may be suitable for tissues containing numerous endogenous APs, though the enzyme can be easily inactivated by high-temperature antigen retrieval. In cases of tissues with abundant endogenous biotin, like liver and kidney, a biotin-free synthetic polymer system is the preferred choice.

In IHC, target antigens can be directly detected through either chromogenic or fluorescent means, depending on the experimental design. Chromogenic detection relies on antibodies conjugated to enzymes, most commonly HRP or alkaline phosphatase AP, which are linked to primary or secondary antibodies. When incubated with suitable substrates, enzyme activity leads to the formation of insoluble, colored precipitates at the site of antigen localization. Examples of such chromogenic, precipitating substrates include brown coloured product giving diaminobenzidine (DAB) and red coloured product giving 3-amino-9-ethyl carbazole (AEC) for HRP, and Fast Red and nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) for AP, respectively. For fluorescence detection, the primary or secondary antibody is conjugated to a fluorophore that can be visualized through fluorescent microscopy.

Counterstaining

Counterstains are substances that enhance the contrast in stained samples. Counterstaining serves to enhance the differentiation of the target signal from the chromogens, contributing significantly to the ability to discern target elements. Some common counterstains include hematoxylin, eosin, nuclear fast red, methyl green, 4',6-diamidino-2-phenylindole (DAPI), and Hoechst fluorescent stain.

Sample visualization

After the sections have been prepared, the samples can be observed using either light or fluorescence microscopy. Depending on the method used for antibody detection, it's possible to utilize confocal microscopy to achieve more detailed and improved imaging. Furthermore, high content screening can be employed for rapid quantification and comparative analysis of data across numerous samples.

CONCLUSION

IHC has become an indispensable tool in everyday practice and fundamental research aimed at unravelling the pathophysiology of various diseases. Moreover, IHC plays a vital role in the validation of biomarkers, a pivotal

step in the journey toward personalized medicine. Despite the recent automation and standardization of IHC procedures, there remain numerous factors that require careful consideration to achieve optimal IHC results and accurate interpretation.

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