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Review Article

Methodological Approaches to Conducting Immunohistochemical Studies

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Abstract

Immunohistochemical (IHC) approaches use immune and chemical processes to identify antigens. This approach is extremely precise and sensitive. This study describes typical IHC procedures and chemicals applied in those methods. This paper also addresses the benefits and drawbacks of each strategy.

Keywords: Immunohistochemistry; Polymer Based Detection; Methodological Approaches

Introduction

Immunohistochemistry (IHC) incorporates immunologic and biochemical approaches to scan distinct features in materials by utilizing antibodies that have been specially designated to attach to their particular targets. IHC is applied to diagnose diseases, conduct experimental studies, and manufacture drugs. IHC staining is commonly applied to diagnose atypical cells, particularly ones present in malignancies. IHC is also used in studies which investigate the availability and placement of biomarkers in various areas of tissues [1].

This may be carried out in simple steps. Initially, formaldehyde needs to be used to fixation. To enable antibody to penetrate the tissue and attach to cell surface proteins, it must then be permeate with a detergent like Triton.

Secondary antibodies containing enzymes including horseradish peroxidase (HRP) linked to their own Fc domain are used to address the primary antibodies. Some molecules, such as diaminobenzidine (DAB), can be targeted by enzymes include HRP, which catalyze an oxidation reaction that yields a colored chemical.

This colorful substance may remain concentrated in the region where the antibodies were applied, coloring the region nearest to the target protein a distinct color from the surrounding tissue. In order to establish difference in between tissue labeled with IHC and the non-colored parts for improved viewing, tissues are lastly counter-stained using a marker as a hematoxylin [2].

Selecting antibodies for IHC

Both primary and secondary proteins are applied in this approach. Although secondary antibodies attach to the primary antibody, primary antibodies specifically attach to the antigen. There are 3 different types of antibody compositions that can be selected while choosing primary antibodies: monoclonal antibodies, polyclonal antibodies and pooled monoclonal antibodies [1].

Both monoclonal and polyclonal antibodies provide benefits and drawbacks. Although polyclonal antibodies produce better signal and more coloring, they can also cause false positive findings by attaching to unintended locations. High specificity monoclonal antibodies reduce the amount of false-positive binds, but they frequently produce a considerably poorer strain. Pooled monoclonal antibodies have great selectivity and good coloring; even so there is a restricted supply of those that do not attach in a non-competitive manner [2].

Direct method

The primary antibody is selectively bound to its distinctive target. This contact can then be illustrated by using a chromogenic reaction facilitated by reporter enzymes like Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP). This is a single step immunohisto-

chemical method that uses a labeled antibody to react directly with antigen. This method employs just one antibody and is a rapid and simple procedure. Nevertheless, due to insufficient signal enhancement, it is unresponsive. The primary antibody (Ab I) on its own labeled with HRP or AP enzymes. The reporter enzyme ultimately transforms a chromogenic substance into a colored precipitate, dyeing the tissue in response to the focused antigen. For starters, this method might considerably improve epidemiologic management in rural places where rabies occurrence data is difficult to gather. Moreover, the test might increase the ability to respond to epidemics with good management decisions [4].

In conclusion, this approach has the potential to be helpful for quick diagnoses because of its quick results. The lack of signal enhancement, incredibly limited sensibility, significant prices, lack of adjustability, and requirement for marked primary antibodies for every objective antigen are some of its drawbacks.

Indirect/simple method

This technique uses an unmarked primary antibody that reacts with antigen and a marked secondary antibody that reacts with the primary antibody. This approach is particularly efficient because of the signal enhancement from many secondary antibody interactions with various antigenic regions on the original antibody. The indirect immunofluorescence approach involves labeling the second layer antibody using a fluorescent dye like FITC, rhodamine, Texas red. The indirect immunoenzyme approach involves labeling the second layer antibody by an enzyme like peroxidase, alkaline phosphatase, and glucose oxidase. Since many secondary antibodies might attach various isoforms of the main antibody, this approach is often utilized since it provides an enhanced signals and better sensibility. Furthermore, it provides for greater versatility because only some forms of secondary antibodies may be employed to identify a large variety of primary antibodies towards various antigens. C. pyfori may be identified with high likelihood utilizing immunohistochemical techniques and particular antibodies. Just like with previous coloring procedures, the goal of this immunohistochemistry approach is to locate curved bacteria in tight proximity to the epithelial cells under mucus; however single bacteria may still be observed [5].

In general, this approach has benefits over direct methods in that it is more sensitive, costs less, and is more flexible because only the secondary antibody requires to be tagged. Weak transmission intensification and decreased sensibility are drawbacks.

Peroxidase/anti-peroxidase (PAP) detection method

PAP works by forming a peroxidase/anti-peroxidase combination that is linked to the main antibody through a subsequent "bridging" antibody. This complex includes 3 peroxidase units with 2 anti-peroxidase antibodies, which take advantage of the recombinant characteristics of IgG. This approach is more sensitive than indirect screening since many enzymes are concentrated each antigenic target. Furthermore, because the enzyme is never chemically changed, there is little possibility of it losing its perceived function. This method is an extension of the indirect approach, including a third layer of rabbit anti-peroxidase antibody combined to peroxidase to form an extremely solid peroxidase anti-peroxidase interaction. The combination, which consists of rabbit gaba-globulin plus peroxidase, functions like a 3rd layer antigen and binds to the 2nd layer's uninflected goat anti-rabbit gaba-globulin. The responsiveness is around 100 to 1000 fold greater because the peroxidase component is antigenically attached rather than chemically coupled to the anti IgG and retains basic entire enzymatic function. This even enables the significantly greater primary antibody concentration, which eliminates several undesired antibodies and reduces non-specific interference coloring [6].

In summary, this approach has several benefits. Amplifying the signal, hypersensitivity in comparison to earlier techniques, less ambient stains, permits primary antibody dilutions that are greater, the absence of chemical component attachment. Limitations are, as is the requirement that the PAP complex originated from the same organism as the main antibody. A major limitation may be a large amount of intrinsic peroxidases. Greater time-consuming than earlier techniques, p potentially insufficiently responsive for the examination of FFPE tissues.

ABC (Avidin-Biotin Complex) detection method

This is a conventional IHC procedure and among the most often used techniques for immunohistochemical marking. Avidin, a big glycoprotein, has a strong attraction towards biotin that could be tagged with peroxidase or fluorescein. Biotin, a vitamin with a lower molecular weight, may be attached to a range of components, including antibodies. 3 levels are used in the process. The initial step consists of an unmarked primary antibody. The 2nd step is a secondary antibody that has been biotinylated. The last stage is an ABC. The DAB or related precursor then develops the peroxidase to yield various colorimetric final metabolites [7].

The higher enzyme: antibody proportion and the resulting significant responsiveness are the key benefits of ABC method. Still, there are a certain negatives to examine. Massive ABC complexes, for instance, may have difficulty diffusing effectively in tissues. Furthermore, the existence of intrinsic biotin might be a cause of interference. Furthermore, its carbohydrate constituent may react with various proteins like lectins. Even though the LSAB technique may help with those difficulties [7].

The above processes are highly widespread unlike most immunologic methods since they just require a biotinylated supplementary antibody for every primary antibody type employed. Biotin is a tiny chemical that can be readily attached to immunoglobulin by amino substitutions at alkaline pH without affecting immunoglobulin function. These secondary antibodies are very affordable, economically accessible, and responsive to immunoglobulins from a broad range of organisms. Because the attachment throughout this approach is not fully immunologic and is essentially irreversible according to avidin's significant sensitivity for biotin, it is lesser liable to assay-related mistakes compared to other immunologic procedures [8].

Although the sensibility given by those techniques is often higher than that achieved by PAP, the material might not seem to be acceptable using ABC approach owing to fixing or for another factor. Tissues can contain intrinsic biotin or biotin-like attaching abilities, resulting in non-specific interaction. Because the coupling is permanent, the process may be carried out within the greatest strict circumstances. The criteria for antigen adhesion remain to be met, but the intense Avidin-biotin coupling provides this method significantly more tolerant. The benefits of those same approaches stem from the simplicity with which appropriate secondary agents may be found [8].

Therefore, the benefits of this strategy are as follows: quicker approach, allowing for the diluting of the main antibody, the Avidin-biotin complex can be utilized over multiple days, propagation of a powerful signal, better sensibility than prior approaches, drawbacks include false positives: Endogenous biotin; Avidin-related non-specific interaction; and complicated structure may reduce tissue penetrating effectiveness.

Labeled Streptavidin Biotin (LSAB) Detection Method

The key variation between this procedure and the previous one is that it employs streptavidin rather than Avidin. Streptavidin seems to be a tetrameric protein derived from Streptomyces avidinii, a bacterium. Streptavidin may attach 4 biotin units, but it is not glycosylated, which eliminates non-specific connections between lectin-like compounds, and it contains a neutral pI, which limits the danger of electrostatic attachment. As a result, the LSAB approach decreases ambient coloring and is 10 fold sensitive than the ABC technique [9]. The streptavidin protein prevents electrostatic interaction. Furthermore, its lacks carbohydrate units that may attach to lectins, leading in background coloring. This is a 3-step technique, comparable to the ABC method: The first level consists of an unmarked primary antibody. The second stage is a secondary antibody that has been biotinylated. Enzyme-Streptavidin complex are used in the third step to substitute the combination of Avidin-biotin peroxidase. The enzyme is subsequently observed by using precursor chromogen mixtures to generate various colorimetric outputs. If fluorescence marking is desired, the third level may also include fluorescent dye-Streptavidin [10].

In proposition, the benefits of this technique include decreased Avidin-related non-specific marking, the ability to dilute the primary antibody, robust signal intensification, LSAB combinations being more persistent than ABC interactions. The biggest limitation is the possibility of background difficulties due to endogenous biotin. Nevertheless, there is remains a risk of mistakes due to the occurrence of intrinsic biotin when using the LSAB technique.

Polymer-based Detection Method

This approach entirely avoids biotin detection. This technique helps exceptionally specific identification and indicates a 2-step methodology, which is quicker. One potential drawback is the large molecular weight of the dextran backbone, which might make polymer entry into tissue and binding with particular antigens challenging [11].

Therefore, a different technique has been devised. This involves maximizing the steric barrier of the polymer and producing a significant density of activated reporters. While maintaining a simple two-step process, this significantly boosts the IHC's sensibility, selectivity, and signal strength.

In summary, this method's benefits include a quicker process, less background and non-specific labelling, strong signal enhancement, and significant sensibility. Dextran polymers have the disadvantage of being more costly, and steric resistance can make tissue infiltration challenging.

Fluorescein isothiocyanate (FITC)

During tissue cross-reactivity (TCR) experiments enabling the creation of pharmaceutical antibodies, fluorescein isothiocyanate (FITC) antibodies have been frequently utilized as primary antibodies. The findings demonstrated an unfavorable correlation between an antibody's FITC-labeling index and its attraction towards its targets. An antibody having a greater labeling value tended to be highly precise when used in immunohistochemistry, however it was simultaneously more inclined to produce non-specific labeling. Based on the above results, we advise that a FITC-labeled antibody utilized as a primary antibody in a TCR experiment must be chosen attentively from a variety of antibodies with various labels in order to reduce the loss of binding attraction and obtain the proper sensitivity and understanding of the immunohistochemistry [12].

Western blot

Western blotting is a method for detecting a certain protein in a specimen. To isolate the proteins in the mixture, gel electrophoresis is used. The isolated proteins are transported from the gel to the membrane. An antibody targeted to the particular protein is introduced to the membrane. A radiological or chemical label is used to identify antibody interaction. A western blot can be used to diagnose illness.The following processes are included in the western blotting technique: sample preparations, gel electrophoresis, membrane transferring, block, primary and secondary antibodies, detection, and analysis. Protein detection techniques include chemiluminescence, chemifluorescence, and fluorescence [13].

- **Chemiluminescence:** Chemiluminescence is a technique of identification that utilizes a chemical interaction among an enzyme that results in light production.
- **Chemifluorescence:** Chemifluorescence is a labeling and identification approach that merges fluorescence and chemiluminescence. Chemiluminescence produces illumination by an enzymatic process, whereas chemifluorescence binds a fluorescent compound to either the secondary or tertiary antibody, which needs activation by a light origin.
- **Fluorescence:** Fluorescence is achieved by utilizing secondary antibodies that have been coupled with a fluorophore. When a fluorophore is activated by a photo origin, photons are released when the stimulated molecule returned to its resting condition, which is then recognized as light. Fluorophores generate steady light that is precisely proportionate to the quantity of protein upon the membrane. The capacity to identify several aims on the same blot at the same time is one of the benefits of employing fluorescence, enabling the identification of the normalization/loading standard and protein of relevance on the similar blot [14].

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a method for detecting the existence of antigens. ELISA uses incredibly precise antibody-antigen reactions to identify a specific antigen. The antigen is fixed on a solid surface. This is accomplished either directly or by the application of an acquisition antibody mounted on the surface. The antigen is subsequently complexed with a recognition antibody that has been tagged with a detectable molecule, for instance an enzyme or a fluorophore [15].

Immunofluorescence assay (IFA)

IFA is a test that detects antibodies to particular antigenic substance using fluorescence microscopy. This test is frequently performed to verify positive ELISA results. This is a classic virology approach for detecting antibodies based on their unique capacity to bind to viral antigens produced in diseased cells.

During the technique virus-infected cells are cultured. Then treated with paraformaldehyde to make the protein in the cells ready for antibody attachment in order to see the antigen. A particular antibody is administered to the surface, allowing the antibody to recognize the viral antigen. The secondary antibody, which is sensitive to the Fc segment of the primary antibody's IgG molecule, is next administered. Because the secondary antibody is coupled to a fluorescence dye, the antigen may be seen using a fluorescence microscope. IFA can show the viral antigen's subcellular location. Furthermore, by utilizing two to three different antibodies, two or perhaps 3 antigens could be easily detected concurrently. IFA is now employed for scientific research rather than diagnostic reasons [16].

Conclusion

Immunohistochemistry is a technique applied to identify antigens in tissues. It's a highly delicate and specific approach. The response that results from poisons and injuries existing in the same region is significant for diagnosticians.

IHC primarily focuses on infectious disorders and malignancies. IHC has several assays that can assist identify a tumor's source, unlike many regular histological examinations that are unable to do so. IHC further provides the opportunity for predictive indicators. In conclusion, the use of IHC is essential for locating and identifying harmful antigens in clinical specimens.

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