Immunohistochemistry in Ophthalmology

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Abstract

Immunohistochemistry is a pathological technique wherein the distribution of a particular protein or lipid antigen in a tissue specimen is determined using a combination of immunological, histochemical and biochemical principles. It is based on the specificity of antigen-antibody binding and the fact that the primary antibody, being a protein itself, can also serve as an antigen for a secondary antibody. These antibodies are then detected using fluorescent probes or enzyme labels. Techniques like enzyme digestion and antigen retrieval may be used to unmask tissue antigens and improve staining. False negatives and false positives are possible. Hence, the results must be compared with histopathological and clinical characteristics. Periodic checking of antibody activity, strict procedure protocols and appropriate positive and negative controls help decrease errors. IHC is a remarkable technique that can be applied to routinely formalin fixed tissue, to frozen sections and even to cytological preparations. It can be used to detect cell surface or even intracellular antigens. IHC is commonly used for tumour diagnosis and classification, as well as detecting prognostic markers. The use of IHC in diagnosis and characterization of ophthalmologic tumours is increasing day-by-day. It helps avoid diagnostic errors and plan appropriate management. Advancements in IHC today aim to make the method faster, simpler and more reliable with better sensitivity and specificity. Proper standardization is the need of the hour to make it more widely applicable and increase accuracy and reproducibility of the results.

Keywords: immunohistochemistry, primary antibody, primary antigen, enzyme label

Introduction

Immunohistochemistry (IHC) is a technique which combines immunological, histological and biochemical techniques for the identification of specific tissue components. It is a method for localizing and seeing the distribution of specific antigens in cells or tissues, based on antigen-antibody recognition. It exploits the fact that antibodies bind to the antigen in a specific manner, thereby giving us the spatial localization of the antigen. The technique of IHC was born when the pathologists felt a need for validating their morphologic judgement. A variety of special stains were initially developed for identification, based on chemical reactions of cell and tissue components in frozen sections (histochemistry). However, these methods often merely highlighted the histological features, without being highly specific. By combining immunology with histochemistry, IHC was able to greatly increase the specificity. This method was first used by Coons in 1940, who used immunofluorescence technique to detect pneumococcal antigen in frozen section. However, it is only many years later, after several technological advancements, that IHC has found its place in routine surgical pathology. IHC doesn’t aim to replace, but instead serve as a valuable adjunct to histochemistry, greatly extending the variety of tissue components that can be demonstrated specifically within sections. As has been said, “the object of all staining is to recognize microchemically the existence and distribution of substances which we have been made aware of macrochemically.”

IHC technique exploits the fact that antibody molecules are themselves proteins, thus they can serve as both antibodies (binding specifically to tissue antigens) and as antigens (providing antigenic determinants to which secondary antibodies may be attached). The usefulness of an antibody for IHC depends on the sensitivity and specificity of the antigen-antibody reaction. The development of the hybridoma technique facilitated the manufacture of abundant, highly specific monoclonal antibodies. However cross reactions may occur even with these. Polyclonal antibodies, on the other hand, though more sensitive than monoclonal antibodies, give more non-specific background staining in slides and their affinities may vary from batch to batch. Hence, these are generally avoided. Recently, recombinant DNA techniques have been used to develop antibodies with increased specificity, using recombinant protein epitope signature tags (PreSTs). Before using any antibody type, one must ensure that the antibody is validated for that particular application, else results will not be reliable.

However, antibody molecules cannot be seen directly. These can be detected only when labelled or flagged with other substances such as fluorescent compounds, active enzymes with colorogenic substrates, or even labels that themselves are directly visible, such as gold, viruses etc. To simply state, in IHC, there is a primary antibody which is directed against the antigen to be detected. This is either directly labelled or a secondary labelled antibody may be used directed against the primary antibody. This label is then detected in tissue sections. (Figure 1) The enzymatic label (horseradish peroxidase), developed by Avrameas and by Nakane and colleagues, allowed visualization of the labeled antibody by light microscopy in the presence of a suitable colorogenic substrate system. Since then, various methods and detection systems have been developed for detecting the antigen-antibody conjugate in tissue sections.
or other cell preparations. These include:

1. **Direct Conjugate-labeled Antibody Method** (Figure 2)- A label is attached to an antibody by chemical means and then this labeled conjugate is directly applied to tissue sections. Aim is to label all the available antibody molecules, while preserving their activity. Disadvantage of this method being its exacting requirements which are hard to meet, need for a relatively larger concentration of antibody and a high risk of false positives.

2. **Indirect/ Sandwich Procedure** (Figure 3)
   Modification of the direct method. The primary antibody binds to the antigen which is to be detected. The labeled secondary antibody, is then added, which has specificity against an antigenic determinant present on the primary antibody. Hence, it serves to label the sites of tissue localization of the primary antibody, which, in turn, is bound to the antigen.
   This method has various advantages like – Increased versatility, since a single conjugated antibody can be used with several different primary antibodies. The labeling process is applied only to the secondary antibody. The primary antibody can usually be used at a higher working dilution. Increased specificity.

3. **Unlabeled Antibody methods**-
   a. Enzyme Bridge Technique: The labelled probe is linked to the antigen by immunologic binding, not chemical conjugation. Useful for research purposes.
   b. Peroxidase Antiperoxidase Methods (PAP): The PAP reagent consists of the horseradish peroxidase enzyme and the antibody against it as a stable immune complex. Two antibodies are used, one directed against the primary tissue antigen and the antiperoxidase antibody, both from the same species. A third antibody, from a different species is then used to link these two.

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**Figure 1: Basic Technique of IHC**

**Figure 2: Direct Method**

**Figure 3: Indirect/ Sandwich Procedure**
4. **Biotin- Avidin Procedure**: Biotin is linked chemically to the primary antibody that localizes the antigen within the section. Subsequently, avidin conjugated to horseradish peroxidase, is added, which binds tightly to the biotinylated antibody. False positive staining may occur if the tissues contain endogenous biotin.

5. **Avidin- Biotin Conjugate Procedure**: It is a modification of the Biotin-Avidin system with increased sensitivity.

6. **Biotin-Streptavidin Systems**: It substitutes avidin with streptavidin, which is then directly conjugated to the enzyme molecule. This method has greater specificity and the reagent is more stable.

7. **Polyvalent Detection Systems**: To demonstrate more than one antigen in a single tissue section.
   - a. Alkaline phosphatase- Antialkaline phosphatase method
   - b. Polymer-based labeling
   - c. Tyramine signal amplification

Taylor and Burns ushered in the 'Brown revolution' when they first demonstrated antigens in routinely processed formalin-fixed paraffin-embedded (FFPE) tissues. However, a critical issue in IHC was the need for greater sensitivity, i.e. to achieve a satisfactory ‘signal-to-noise’ ratio (amplifying the signal while reducing the non-specific background staining or noise) to improve results. Various methods have been developed for this, mainly aiming to expose or ‘unmask’ the antigenic sites (epitopes) that may otherwise be unexposed. These include, digestion with proteolytic enzymes, treatment with wet heat etc.

Enzyme digestion, though widely used, did not improve IHC staining of many antigens. Also, it was difficult to control and standardise the optimal “digestion” conditions for individual tissue sections when stained with different antibodies. Hence there was need for a more powerful, more widely applicable and an easier technique, which should enhance the immunohistochemical staining of routine FFPE tissue sections in a reproducible and reliable manner. This led to the birth of the antigen-retrieval (AR) technique.

This simply involves heating routinely processed paraffin sections at high temperature (e.g. in a microwave oven) before IHC staining, which dramatically increases the intensity of staining. The basis behind this may be explained by the fact that antigen-antibody recognition is based on the 3-dimensional structure of the antigen, which maybe compromised during formalin processing, and may be restored in part by AR. Also variability in composition and arrangement of antigenic epitopes may explain the variable influence of formalin fixation on antigenicity of different antigens and of the effectiveness of AR.

IHC is a revolutionary technique with many advantages including, remarkable sensitivity and specificity, applicability to routinely processed material, compatibility with most of the commonly used fixatives and fairly accurate correlation with the traditional morphologic parameters. It is feasible even in decalcified material, material stored for long or in previously stained microscopic sections. It can also be adapted to cytological preparations and to electron microscopy. A quick labelling method has been developed for using IHC intra-operatively, for instance in evaluation of sentinel lymph nodes or for frozen sections. Another recent development is that of a ‘sausage tissue block’, that allows simultaneous evaluation of over 100 tissue samples on a single slide, with one drop of antibody.

Hence due its various advantages, and constant evolution, the use of IHC in diagnostic pathology has expanded, especially for tumor diagnosis and classification. (Figure 4) Furthermore, IHC has been adapted for the identification and demonstration of prognostic and predictive markers. If properly standardized and used, IHC is a technique which not only detects the presence of an antigen, but also helps accurately determine its relative or real amount in the tissue section. However, the potential pitfalls of this technique must be kept in mind by the pathologist while interpreting the results, to prevent mistakes. Many of these can be avoided by scrupulous technique, regular checking of antibody activity and proper use of controls.

False negatives can occur when, antibody is inappropriate, denatured or used at the wrong concentration, or when antigen is lost or denatured or present at very low density so as to escape detection. False positives are possible due to, cross-reactivity of antibody with different antigens and non-specific antibody binding. This is more of a problem with polyclonal antibodies. This staining obscures the specific staining, thereby adding to the problems. Pre-incubation with normal serum may help decrease non-specific binding. Results may also be misinterpreted when there is ectopic antigen expression or when some markers presumed to be specific, are shared by other tissues or neoplasms. For instance, the S100 protein, originally thought to be specific for neuronal tissue is now known to be produced by chondrocytes also. Hence other clinical and morphological features must be taken into account while interpreting the results. Another problem is entrainment or association of normal tissue with neoplastic tissue. For instance, the S100 protein, originally thought to be specific for neuronal tissue is now known to be produced by chondrocytes also. Hence other clinical and morphological features must be taken into account while interpreting the results. Another problem is entrainment or association of normal tissue with neoplastic tissue. For instance, in pancreatic tumors, it is difficult to determine which hormone is produced by the tumor cells and which is from the residual non-neoplastic cells. Blocking endogenous enzyme activity is also important, in order to avoid false-positives, especially when using similar enzymes as labels. Eg. Peroxidase-blocking step is added while studying

### Figure 4: Uses of IHC

- Identify replicating cells
- Locate cells that are signaling
- Locate apoptotic cells
- Identify activation states
- Identify the different types of cells in a tissue
- Examine cytoskeletal structure
preparations of erythrocytes, neutrophils, eosinophils and hepatocytes which have endogenous peroxidase activity. The number of antigens that can be detected by IHC is increasing everyday. We can target cell membrane, cytoplasmic or nuclear antigens, both lipid and protein. Detergents or alcohol may be used to increase cell membrane penetration so as to access intracellular antigens. Among the different antigens, the recent ones include transcription factors, which are gaining popularity due to their greater specificity and lesser diffusion into surrounding tissue. Also, since these are nuclear antigens, their detection can be combined with another different chromogen marker to identify cytoplasmic/ cell membrane marker, thereby increasing sensitivity and specificity.

With increasing usage of IHC, search for simpler and faster methods continues, with growing focus on method reproducibility and standardization.

Just to give the readers an idea of the immense value of IHC, a short list is given below of antigens that are used in ophthalmic pathology as diagnostic aids, prognostic or predictive indicators or as histogenetic probes.

- **Melanoma**: S100, HMB45, Melan-A, Vimentin
- **Squamous cell carcinoma**: CK5/6 (cytokeratin 5/6), EMA (Epithelial Membrane Antigen)
- **Adenoid cystic carcinoma**: High molecular weight keratin
- **PNET/ Ewing’s sarcoma**: CD99
- **Lymphoma**: LCA (Leukocyte Common Antigen), CD3, CD20
- **Plasma cell tumor**: CD138, kappa light chain, gamma light chain
- **Optic nerve glioma**: GFAP (Glial Fibrillary Acid Protein), MIB-labelling index
- **Retinoblastoma**: IRBP (Interphotoreceptor Retinoid Binding Protein), S-Antigen, NSE (Neuron specific enolase)
- **Sebaceous cell carcinoma**: Fat stain on frozen section. No marker!

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