Overview of Immunohistochemistry

Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context. While there are multiple approaches and permutations in IHC methodology, all of the steps involved are separated into two groups: sample preparation and labeling.

History

The principle of IHC has been known since the 1930s, but it was not until 1942 that the first IHC study was reported. Coons *et al.* (1942) used FITC-labeled antibodies to identify *Pneumococcal* antigens in infected tissue. Since then, improvements have been made in protein conjugation, tissue fixation methods, detection labels and microscopy, making immunohistochemistry a routine and essential tool in diagnostic and research laboratories.

Applications

IHC is used for disease diagnosis, drug development and biological research. Using specific tumor markers, pathologist use IHC to diagnose a cancer as benign or malignant, determine the stage and grade of a tumor, and identify the cell type and origin of a metastasis to find the site of the primary tumor. IHC is also used in drug development to test drug efficacy by detecting either the activity or the up- or down-regulation of disease targets.

Samples are prepared on individual slides, or multiple samples can be arranged on a single slide for comparative analysis, such as with tissue microarrays. IHC slides can be processed and stained manually, while technological advances now provide automation for high-throughput sample preparation and staining. Samples can be viewed by either light or fluorescence microscopy, and advances in the last 15 years have improved the ability to capture images, quantitate multiparametric IHC data and increase the collection of that data through high content screening.

Sample preparation

A note about sample preparation: While using the right antibodies to target the correct antigens and amplify the signal is important for visualization, complete preparation of

the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. Patient biopsies, or tissue, must be rapidly fixed to prevent the breakdown of cellular protein and tissue architecture.

Tissue Fixation

Fixation chemically crosslinks proteins or reduces protein solubility, which can mask target antigens during prolonged or improper fixation. Therefore, the right fixation method must be optimized based on the application and the target antigen to be stained.

The most common fixative is formaldehyde, a semi-reversible, covalent crosslinking reagent that can be used for perfusion or immersion fixation for any length of time, depending on the level of fixation required. Other fixatives are available, and their use depends on the antigens that are being sought.

Formalin-fixed, paraffin-embedded tissues are sectioned into slices as thin as 4-5 µm with a microtome. These sections are then mounted onto glass slides that are coated with an adhesive. This adhesive is commonly added by surface-treating glass slides with 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine, which both leave amino groups on the surface of the glass to which the tissue directly couples. Alternatively, slides may be coated with physical adhesives, including gelatin, egg albumin or Elmer's glue. After mounting, the sections are dried in an oven or microwave in preparation for deparaffinization.

Frozen sections are cut using a pre-cooled cryostat and mounted to adhesive glass slides. These sections are often dried overnight at room temperature and fixed by immersion in pre-cooled (-20°C) acetone, although the drying step is sometimes skipped depending on the target antigens and tissue used.

Epitope (Antigen) Recovery

The paraffin from formalin-fixed, paraffin-embedded sections must be completely removed for the antibodies to reach the target antigens. Xylene, a flammable, toxic and volatile organic solvent is commonly used to remove the paraffin from IHC slides, although commercial alternatives are available.

Formaldehyde fixation generates methylene bridges that crosslink proteins in tissue samples; these bridges can mask antigen presentation and prevent antibody binding. Formalin-fixed, paraffin-embedded sections commonly require a treatment to unmask

the antibody epitopes, either by heat (heat-induced epitope retrieval; HIER) or enzymatic degradation (proteolytic-induced epitope retrieval; PIER).

Quenching/Blocking Endogenous Target Activity

For staining approaches that depend on biotin, peroxidases or phosphatases for the amplification or enzymatic detection of target antigens, quenching or masking endogenous forms of these proteins prevents false positive and high background detection. The general strategies include physically blocking or chemically inhibiting all endogenous biotin or enzyme activity, respectively.

Blocking Nonspecific Sites

Although antibodies show preferential avidity for specific epitopes, antibodies may partially or weakly bind to sites on nonspecific proteins (also called reactive sites) that are similar to the cognate binding sites on the target antigen. In the context of antibody-mediated antigen detection, nonspecific binding causes high background staining that can mask the detection of the target antigen. To reduce background staining in IHC, ICC and any other immunostaining application, the samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA or gelatin, and commercial blocking buffers with proprietary formulations are available for greater efficiency.

Immunodetection

Detecting the target antigen with antibodies is a multi-step process that requires optimization at every level to maximize the signal detection.

Both primary and secondary antibodies are diluted into a buffer to help stabilize the antibody, promote the uniform dissemination throughout the sample and discourage nonspecific binding. While one diluent may work with one antibody, the same diluent may not work with another antibody, demonstrating the need for optimization for each antibody.

Rinsing the sample in between antibody applications is critical to remove unbound antibodies and also to remove antibodies that are weakly bound to nonspecific sites. Rinse buffers are usually simple solutions of only a few components, but the right components must be considered to maximize sample washing and minimize interference with the signal detection.

Antibody-mediated antigen detection approaches are separated into direct and indirect methods. These methods both use antibodies to detect the target antigen, but the selection of the best method to use depends on the level of target antigen expression and availability and also the readout desired. Most indirect methods employ the inherent binding affinity of avidin to biotin to localize a reporter to the target antigen and amplify the signal that is detected.

IHC target antigens are detected through either chromogenic or fluorescent means, and the type of readout depends on the experimental design. For fluorescent detection, the reporter that the primary or secondary antibody is conjugated to is a fluorophore that is detected by fluorescent microscopy. Chromogenic detection is based on the activites of enzymes, most often horseradish peroxidase (HRP) or alkaline phosphatase (AP), which form colored, insoluble precipitates upon the addition of substrate, such as DAB and NBT/BCIP, respectively.

Counterstaining

Counterstains with H & E gives contrast to the primary stain and can be cell structure-specific. These single-step stains are usually added after antibody staining.

Sealing the Stained Sample

After all staining is completed, the sample should be preserved for long-term usage and storage and to prevent enzymatic product solubilization or fluorophore photobleaching. Sealing the sample by mounting a coverslip with an appropriate mountant stabilizes the tissue sample and stain. An antifade reagent should also be included if fluorescent detection will be performed to prolong fluorescence excitation. The coverslip can then be sealed with clear nail polish or a commercial sealant after the mountant has cured to prevent sample damage.

Once the sections are prepared, the samples are viewed by light or fluorescent microscopy. Depending on the antibody detection method, one can perform confocal microscopy for greater detail and enhanced imaging capabilities. Additionally, samples can be analyzed by high content screening for rapid quantitation and comparison of data from multiple samples.

References

Coons, A.A., *et al*. (1942) *J. Immunol.* **45**, 159-170.

http://www.piercenet.com/browse.cfm?fldID=F95B91A9-3DC1-4B56-8E8D-59CA044A8BA7

Simple Animations for Immunohistochemistry

http://www.sumanasinc.com/webcontent/animations/content/immunohistochemistry.html