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Systemic Review

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Abstract
Immunohistochemistry (IHC) is the process, which works in combination with anatomical methods, immunological, and biochemical techniques to visualize discrete components, and pathologies present in tissues by using target-specific, appropriate antibodies. These antibodies bind specifically to their target antigens in situ. With the advancement in IHC, it is possible to visualize and document the high-resolution imaging, distribution, and localization of specific cellular components within cells and within their proper histological context. Although there are many approaches and permutations in IHC methodology, all of the steps involved are separated into two groups: sample preparation and sample staining.
Keywords: Immunohistochemistry, Tissue fixation, immunostaining,
Abbreviation: Immunohistochemistry (IHC), formalin-fixed, and paraffin-embedded (FFPE).

INTRODUCTION
The methods of Immunohistochemistry (IHC) have been known since the 1930s, but it was not until 1942 when the first IHC report of FITC-labeled antibodies identified Pneumococcal antigens in infected tissue was reported by coons. Since then, major advancements have been made in tissue fixation, sectioning methods, antigen/epitope retrieval, antibody conjugation, immunostaining methods, and reagents, as well as microscopy itself. As a result of improvements, IHC has become a routine and essential tool in diagnostic and research laboratories(1).

APPLICATIONS
IHC is used for disease diagnosis, biological research, and drug development. IHC became a crucial investigation in cancer management by using specific tumor markers, it is used to diagnose if a tumor is benign or malignant, to determine its stage and grade, and to identify the cell type and origin of a metastasis to find the site of the primary tumor. Not only cancers but many other non-neoplastic diseases and conditions are diagnosed using IHC as a primary tool or as a confirmatory procedure. In the context of research, IHC can be used alone or in conjunction with other analytical techniques to study, for example, normal tissue and organ development, pathological processes,

wound healing, cell death and repair, and many other fields. IHC also plays a great role in the development of drugs, for example, testing drug efficacy or the up and down-regulation of disease markers in the target tissues and elsewhere is done by IHC applications (2). Traditional IHC is performed on the immunostaining of thin sections of tissues attached to individual glass slides. Comparative analysis on multiple tissues such as tissue microarray is also done. Still, in many setups, conventional methods of slides preparation, procession, and staining are performed. However, with the help of new technology, high-throughput sample preparation and staining are done automatically. Samples can be viewed by either light or fluorescence microscopy, and advances in the last 15 years have improved our ability to capture images, quantitate multiparametric IHC data, and increase data collection through high content screening. In (fig-1), One of the IHC examples is shown with Invitrogen antibodies and other IHC reagents. Detection of HDAC4 by IHC in human skin. Chromogenic IHC was performed on thin sections of human skin obtained from biopsies. The sections were stained with a rabbit polyclonal antibody against HDAC4 (Cat. No. PA1-863) or without this antibody (the negative control). HDAC4 detection was performed using a biotinylated anti-rabbit IgG secondary antibody and streptavidin-Horseradish peroxidase (HRP), followed by colorimetric detection using DAB. The sections were then counterstained with hematoxylin and mounted under coverslips. In the left-hand panel, above the HDAC4 antigen is stained brown by the precipitated DAB reaction product. The control section on the right is not stained brown because no anti-HDAC4 primary antibody was used. Only the blue hematoxylin counterstaining can be seen(3).

SAMPLE PREPARATION

Preparation of the sample is critical to maintaining cell morphology, tissue architecture, and the antigenicity of target epitopes, just like the use of the right antibodies to target the right antigens and amplify the signal is crucial for optimal visualization of tissues.

TISSUE COLLECTION

According to the requirement of the researcher or doctor, different specimens like Human or, animal biopsies, whole organs, or specific tissues or cells, are collected for preservation and IHC analysis. Tissue and cells must be quickly preserved to prevent tissue autolysis, cellular protein breakdown, and degradation of the normal tissue architecture. Routinely, the tissues are perfused in vivo or in vitro, or simply rinsed free of blood, before fixation/preservation(4). The aim is to make tissue free from any blood-derived antigen, which may interfere with the detection of target antigens.

TISSUE FIXATION

Most tissue fixatives chemically interact with proteins and/or reduce protein solubility, which leads to the masking of target antigens during prolonged or improper fixation. Therefore, a suitable fixation method must be chosen, based on the application and the target antigen to be stained(5). The most commonly used fixative is formaldehyde (formalin), it is a semi-reversible, covalent crosslinking reagent that can be used for tissue fixation, perfusion, or immersion fixation for any length of time, depending on the level of fixation required. Formaldehyde fixed tissues are embedded in paraffin wax before sectioning and further processing. Such tissues and the sections cut from them are often referred to as formalin-fixed and paraffin-embedded or FFPE. Other than formaldehyde, many fixatives like (e.g acetone, and methanol) are also used routinely. Generally, the use of these alternative fixatives depends on how the target antigens react to fixation in the first place. In (fig-2) representative example of IHC localization of an antigen, p21 in an FFPE section from a human colon cancer specimen is shown. Detection of p21 in human lung colon carcinoma by IHC. IHC staining for p21 in

formalin-fixed paraffin-embedded (FFPE) section of human colon carcinoma using a monoclonal antibody (Cat. No. MA1-19271) as the primary antibody and an anti-mouse IgG-HRP conjugate as the secondary antibody. The brown precipitating HRP substrate DAB was used. Before staining, (heat-induced epitope retrieval (HIER) was performed in 10 mM citrate buffer(6).

TISSUE EMBEDDING

Samples that are too sensitive for chemical fixation or the solvents (used to remove the paraffin), can be encased in a cryogenic embedding material and then frozen in liquid nitrogen. Then thin slices of these frozen tissue samples are sectioned on a freezing microtome (cryostat), transferred to slides, and then dried to preserve morphology. Such sections are referred to as frozen or cryosections.

SECTIONING AND MOUNTING

Paraffin wax is the most frequently used embedding medium for routine histological applications. FFPE sections give good and satisfactory results in detecting most of the tissue antigens using antigen retrieval techniques. However, some antigens are destroyed during routine fixation and paraffin embedding; in which case, frozen tissue sectioning becomes the method of choice. Despite having short-comes like poor morphology, decreased resolution at high magnifications, and special storage needs; frozen sectioning is not limited due to these disadvantages(7). FFPE tissues are usually cut into sections as thin as 4 to 5 μm with a microtome. These sections are then mounted onto glass slides that are coated with a tissue adhesive. This adhesive is commonly added by surface-treating glass slides with 3-aminopropyltriethoxysilane (APTS) or poly-L-lysine, both of which leave amino groups on the surface of the glass to which the tissue adheres. If necessary, slides can be coated with actual adhesives, including gelatin, egg albumin, or even Elmer's glue. After mounting, the sections are dried in an oven or microwaved in preparation for de-paraffinization. These sections can be dried overnight at room temperature and are usually post-fixed by immersion in pre-cooled (-20°C) acetone, fresh paraformaldehyde, or formaldehyde/formalin at ambient temperature(8,9). The drying step is sometimes skipped depending on the target antigens and tissue being used. In (fig-3) IHC protein, VEZF was detected in human brain tissue. Chromogenic IHC staining of a paraffin section of the human brain. Tissues were processed and probed with PA541131, a rabbit anti-VEZF polyclonal primary antibody. An anti-rabbit IgG secondary antibody labeled with HRP and the red-precipitating HRP substrate 3-amino-9-ethyl carbazole (AEC) were used for detection. The red-colored regions and fibers represent the locations of VEZF.

DE-PARAFFINIZATION AND EPITOPE (ANTIGEN) RETRIEVAL

The paraffin in FFPE sections must be completely removed before IHC staining for the target antigens to react with antibodies. If this de-paraffinization is not complete, the target antigens will be obscured and can't react with antibodies. Paraffin's hydrophobicity actually repels aqueous solutions containing the IHC staining reagents. Flammable, toxic, and volatile organic solvent xylene has traditionally been used to de-paraffinize FFPE slides, although xylene-free de-waxing alternatives are now available and used nowadays(10). Formaldehyde fixation generates methylene bridges that are covalently attached to proteins in tissue samples. These bridges can mask antigen accessibility and inhibit antibody binding. As a result, FFPE sections typically require treatment designed to unmask or retrieve the antigenic epitopes before staining(11). This is called epitope or antigen retrieval.Epitope/antigen retrieval is usually performed by heating or boiling the de-paraffinized sections in various buffers at different pH values, which is called heat-induced epitope retrieval or HIER. Antigens

can also be retrieved by digesting the tissue sections with a proteolytic enzyme like pepsin, trypsin, or proteinase K. If antigen or epitope-specific retrieval conditions are not already documented in the literature or on our antibody data sheet, an effective method must be determined empirically. It is also necessary to mention that although thorough de-paraffinization is always required before IHC staining, antigen or epitope retrieval is not. In some FFPE tissues, certain individual antigens are not obscured, so a retrieval step is not required before staining(12).

QUENCHING/BLOCKING ENDOGENOUS TARGET ACTIVITY

Many popular staining approaches depend on biotin and its binding proteins like strept(Avidin) (SA), NeutrAvidin (NA), and avidin (AV). Most detection strategies employ horseradish peroxidase (HRP) or alkaline phosphatase (AP) activity for enzyme-mediated detection of target antigens in the presence of specific substrates. Thus, inactivating (quenching) or masking endogenous forms of these proteins prevents false positive detection and high background staining. The general strategies include physically blocking or chemically inhibiting all endogenous biotin or enzyme activity, respectively(13).

BLOCKING NONSPECIFIC SITES

Sometimes, antibodies may partially or weakly bind nonspecifically to sites on non-antigen proteins that mimic the correct binding sites on the target antigen despite having antibodies showing preferential avidity and affinity for specific epitopes. In this context of antibody-mediated antigen detection, nonspecific binding causes high background staining that can mask the detection of the target antigen(14). Thus to reduce background staining in IHC, ICC, and any other immunostaining application, before staining, the samples are incubated with a buffer that blocks the non-specific sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include some percentage of normal serum, non-fat dry milk, BSA (bovine serum albumin), gelatin, and one or more gentle surfactants to aid in wetting. Many other commercial blocking buffers with proprietary formulations are available for greater blocking efficiency(15).

SAMPLE LABELING

Immunodetection

Detecting the target antigen with antibodies is a multi-step process that requires optimization at every single step to maximize signal detection. Both primary and secondary antibodies are diluted into a buffer formulated to help stabilize the antibody, promoting its complete and uniform diffusion into the sample, this also discourages nonspecific binding. Every antibody needs a different or specific diluent, as one diluent may work with one antibody, and that same diluent may not work with another antibody, so optimization for each one and every level is required(16). 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 with only a few components, but the right components must be considered to maximize washing efficiency and minimize interference with signal detection. Antibody-mediated antigen detection approaches are different for direct and indirect methods. Both of these methods use antibodies to detect the target antigen, but the selection of the best method to use depends on the level of target antigen expression, its accessibility, and the type of readout desired(17). IHC target antigens are detected directly through either chromogenic or fluorescent means and on the type of the experimental design. Chromogenic detection is based on antibodies conjugated to enzymes. Most often, the enzymes used are horseradish peroxidase (HRP) or alkaline

phosphatase (AP), which are conjugated to primary or secondary antibodies. When incubated with appropriate substrates, the enzyme activity leads to the precipitation of insoluble, colored precipitates at the antigen localization site. Such chromogenic, precipitating substrates include DAB and AEC for HRP, and Fast Red and NBT/BCIP (rarely used) for AP, respectively. For fluorescence detection, the primary or secondary antibody is conjugated to a fluorophore that is detected by fluorescent microscopy(18).

COUNTERSTAINING

Counterstains are cell-specific stains, different structures of the cell take different stains, and provide contrast to the primary stain. These single-step stains are usually added after antibody staining. Common counterstains include hematoxylin, eosin, nuclear fast red, methyl green, DAPI, and Hoechst fluorescent stain.

SEALING THE STAINED SAMPLE

After all, staining is completed, the sample should be preserved for archiving purposes and to prevent enzymatic product solubilization or fluorophore photobleaching. Sealing the sample by mounting a coverslip with an appropriate mounting solution (mountant) is done, which stabilizes the tissue section and the stain(19). An antifade reagent should also be included if fluorescent detection was used 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. Mountants with organic and aqueous formulations are commercially available.

SAMPLE VISUALIZATION

Once the sections are prepared, the samples are viewed by light or fluorescence 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.

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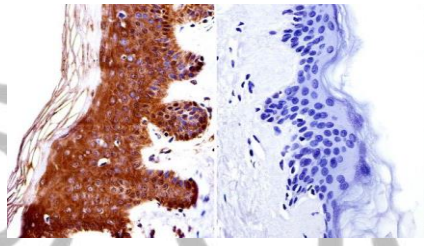
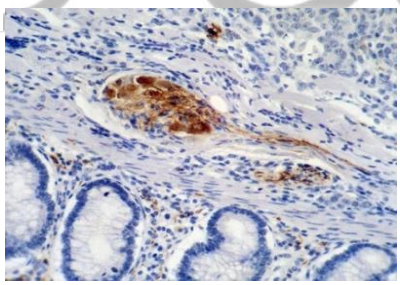
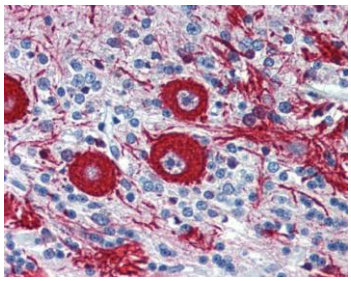
1. **Figure:**



2. **Figure**



3. **Figure**



4. **Description of images used in the manuscript:**
    1. Detection of HDAC4 in human skin by IHC
    2. p21 in an FFPE section from a human colon cancer specimen
    3. IHC protein, VEZF was detected in human brain tissue