Beginner's Guide

A beginner's guide to immunohistochemistry

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Using the affinity between antibodies and antigens, immunohistochemistry (IHC) is a helpful method for analysing the cellular components within tissue sections placed on glass slides. The chemistry of antibodies was not discovered until 1941, and since then, their use has increased due to the discovery of enzymes and fluorochromes conjugated to antibodies, enabling observations through optical or fluorescence microscopy (FM). Hence, immunofluorescence (IF), or IHC with fluorescent antibodies, has been associated with the advancement of FM and fluorochromes. Specifically, the discovery of three fluorochromes that emit blue, red and green led to a rise in IF application in the context of visualizing numerous components in a single cell. The proximity ligation assay (PLA) method, so termed because the antibody is conjugated with short DNA strands called PLA probes, is the most recent development in the field of antibody conjugation. Protein–protein interactions and sub-localization of proteins can both be studied using this technique. The improvement of conjugation techniques has occurred in parallel with the development of sophisticated microscopies, including confocal and slide scanners. Confocal microscopy allows for the reconstruction of three-dimensional structures while a slide scanner can digitally convert up to 100 slides' worth of high-resolution pictures. The first step in the IHC process is the collection and fixation of tissues from animal models and humans. Paraformaldehyde is the most commonly used fixative. Fixation prevents antigen blocking and creates cross-links between proteins to preserve tissue structures. Fixation is followed by cutting tissues using microtome or cryostat, then slides are treated for antigen exposition (antigen retrieval) and then included in Triton X solution, which increases membrane permeability. Subsequently, incubations with enzyme- or fluorochrome-conjugated antibodies enable observations via optical microscopy or FM, respectively. In clinical practice and research, IHC and IF are still helpful methods for identifying the antigen-proteins linked to specific diseases.

Immunohistochemistry (IHC) is a technique useful for examining the cellular components within tissue sections placed on glass slides. The strong affinity that antibodies and antigens share is the fundamental idea behind this technique. The information produced by IHC allows us to detect both the presence/absence of the antigen and its specific cellular localization. This article provides a brief exploration of the history of IHC with a focus on immunofluorescence (IF) and fluorescence microscopy (FM). Furthermore, the procedural steps are outlined to facilitate comprehension and implementation of this valuable technique.

History of IHC

The basic tools of IHC that we use today were made available over 100 years ago when Von Behring discovered serum antibodies named 'antitoxins' in 1890 and used them to cure diphtheria and tetanus. However, little was known about their chemistry until 1941, when conjugated antibodies were first put to use. In fact, Coons and Fieser employed the IF technique for the first time, staining pneumococcal antigens using fluorescein-5-isothiocyanate (FITC)-conjugated antibodies through UV light-equipped microscopy. Due to the weak fluorescence of the first FM, researchers looked for alternative antibody conjugations. Horseradish peroxidase (HRP), an enzyme that oxidizes the substrate 3,3'-diaminobenzidine, was discovered by Nakano and Pierce in the 1960s. The peroxidase–anti-peroxidase complex was then found to increase the antibody signal. Because these enzymes produce colours visible under a standard optical light microscope (which has been around since the 16th century), their easy use made them suitable for clinical and research settings. However, in the 1960s, the fluorescence signal was amplified with the introduction of dichroic mirrors. In the same year, Shimomura identified a light-emitting molecule protein named green fluorescent protein (GFP), and Mukai, Inoue and Akune first documented in 1969 the presence of a red fluorescent protein. Later, in the 1990s, the blue fluorescent protein (BFP) was discovered by Roger Y. Tsien and his collaborators while, during the 2000s, the GFP was cloned by Shimomura, Chalfie and Tsien earning the Nobel Prize. The use of combined fluorochromes

offered benefits such as high-contrast imaging of multiple components in a single cell. Nevertheless, fluorochromes' loss of fluorescence when exposed to light is one drawback to their employment. Proximity ligation assay (PLA), which dates back to 2002, thanks to Fredriksson, is the most recent discovery in the field of antibody conjugation. It provided an advantage over traditional IF by examining the subcellular localization of individual proteins and protein–protein interactions. This technique uses two primary antibodies, each of which is capable of recognizing the same antigen-protein. Secondary antibodies directed against constant regions of the primary antibodies are connected to a short DNA strand named PLA probes. The DNA strands can engage in rolling circle DNA synthesis if the PLA probes are close together (maximum 40 nm). To bind the circle DNA, fluorescent-labelled oligonucleotide probes are then added. Concurrently, with the development of antibody conjugations, light microscopies also significantly improved. In fact, in 1977, Sheppard and Choudhury coined the term 'confocal microscope'. By taking several two-dimensional pictures at various depths, this microscopy made it possible to reconstruct three-dimensional structures while removing out-of-focus light. It has gained usefulness in the analysis of tissues, cells and intricate interactions within biological structures using antibodies conjugated with either enzymes or fluorochromes. However, limitations are the relatively slow speed when acquiring images and the small range of available excitation wavelengths. Another limitation includes the phototoxicity of the scanning process, which may not be favourable to live-cell imaging. The creation of the slide scanner, a tool that can digitally scan up to 100 slides at once and produce high-resolution images, in the 2000s represents the most recent advancement in microscopy technology. This cutting-edge technology made it possible to create and share digital images obtained from staining antibodies conjugated with enzymes or fluorochromes, enabling immediate remote examination, diagnosis and archiving. However, one drawback is that it could be difficult to focus on areas where a lot of data is recorded. Figure 1 shows the benefits and drawbacks of the various microscopies while Figure 2 describes the historical development of IHC.
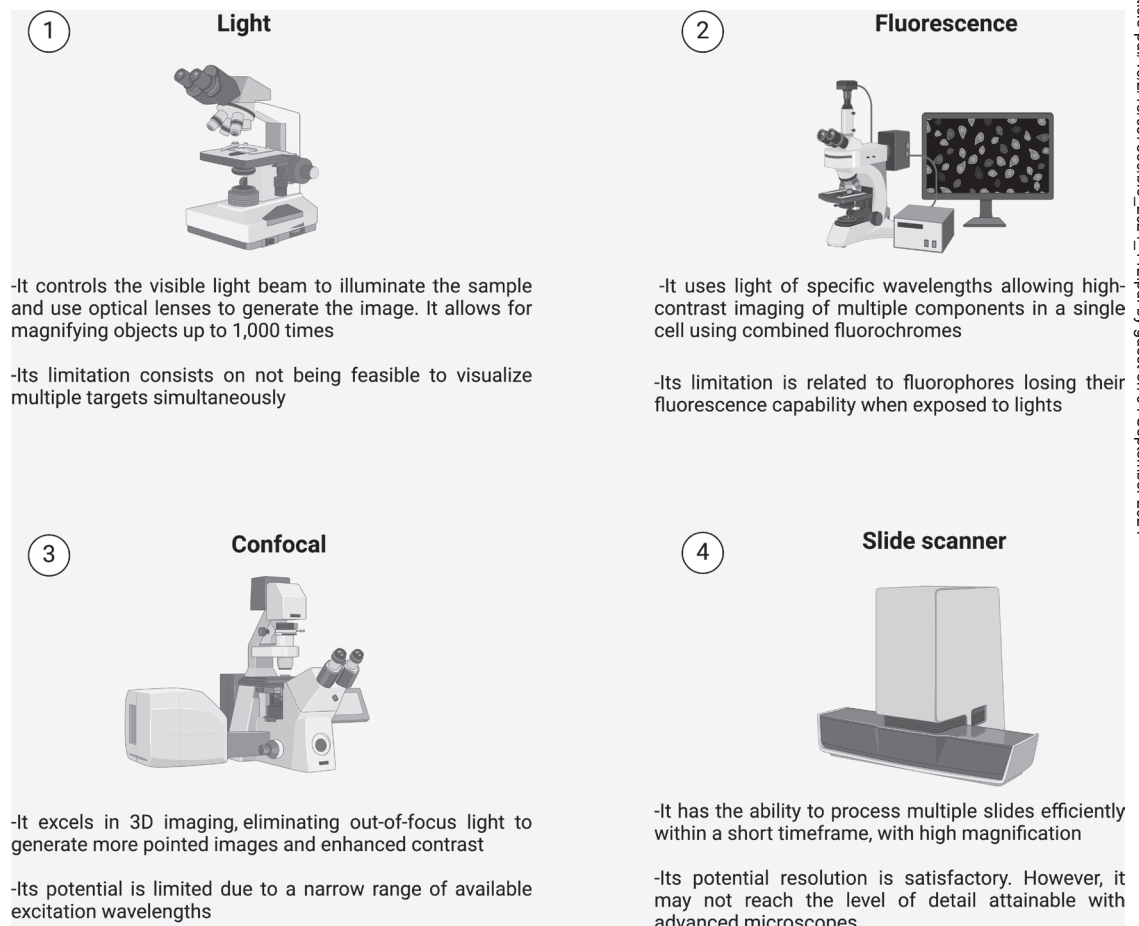
① **Light**

-It controls the visible light beam to illuminate the sample and use optical lenses to generate the image. It allows for magnifying objects up to 1,000 times

-Its limitation consists on not being feasible to visualize multiple targets simultaneously

② **Fluorescence**

-It uses light of specific wavelengths allowing high-contrast imaging of multiple components in a single cell using combined fluorochromes

-Its limitation is related to fluorophores losing their fluorescence capability when exposed to lights

③ **Confocal**

-It excels in 3D imaging, eliminating out-of-focus light to generate more pointed images and enhanced contrast

-Its potential is limited due to a narrow range of available excitation wavelengths

④ **Slide scanner**

-It has the ability to process multiple slides efficiently within a short timeframe, with high magnification

-Its potential resolution is satisfactory. However, it may not reach the level of detail attainable with advanced microscopes

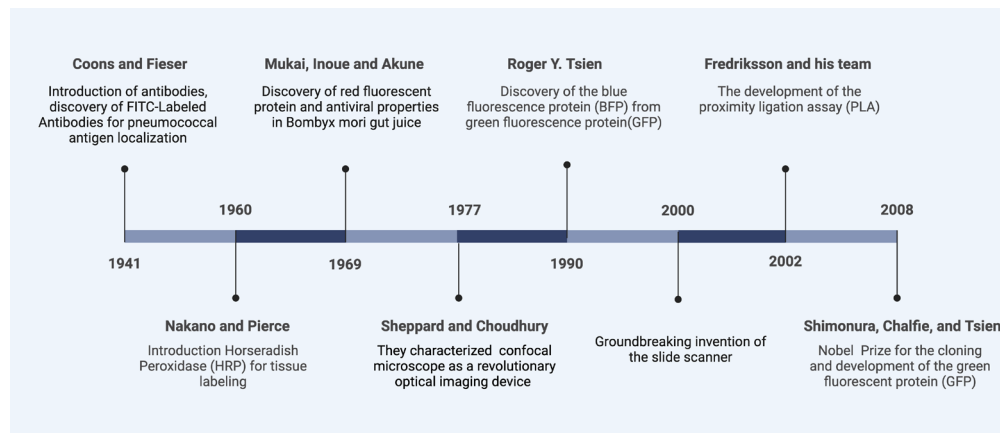**Figure 1.** Different types of light microscopy.

Figure 2. Timeline of IHC history.

Steps of IHC

Here, we have described the sample preparation and staining steps for visualization using light microscopy.

Tissue manipulation

The initial step in IHC involves collecting tissue specimens obtained from animal models or human subjects through biopsies, surgical excisions or post-mortem autopsies. Tissues are fixed after collection. Paraformaldehyde, which is formalin 4% in distilled water, is the fixative used most often. Fixation creates cross-links between proteins preserving biological tissues from autolysis. Tissue sectioning, which is accomplished with a microtome or cryo-microtome, comes after fixation. For the cryo-microtome, cut tissue samples are first immersed in the optimal cutting temperature (OCT) medium, which is a water-soluble blend of glycols and resins providing a matrix for cryostat sectioning at low temperatures. Instead, the microtome is used at room temperature with tissue samples that have been dehydrated (by the alcohol-grade scales) and embedded in paraffin to facilitate tissue cutting. Compared to a microtome, the cryostat is equipped with more advanced technology, allowing it to control thickness and temperature (up to −35°C). In 2003, Cocco C suggested a low-cost, hand-made OCT medium that can be produced in-house, as well as the development of multiple personalized OCTs depending on the type of tissue. Sections that are slide-mounted or floating in solution (also known as free-floating) can be used for IHC. Although the free-floating method allows antibodies to enter the section from any side, making labelling excellent, its main disadvantage is that it is difficult to handle and requires slides with not less than 40 um to keep them from breaking.

Staining steps

After cutting, antigen retrieval is the next step, which is important for some antibody-based procedures. Before the antigen retrieval step, in the case of paraffin-inclusion tissues, the slides should undergo deparaffinizing and rehydrating. Antigen retrieval involves breaking down cross-links to expose antigens and thus enhances antibody binding. Physical methods like heat, chemical or ultrasound treatment can achieve antigen retrieval by restoring the native structure of antigens. Following antigen retrieval, an important step is Triton X inclusion, which, by increasing membrane permeability, allows antibody penetration. Before starting the incubation steps, sections undergo the blocking step. This minimizes non-specific binding sites of antibodies by applying a blocking solution usually consisting of bovine serum albumin or serum from the same animal species used for secondary antibody production. The blocking step is followed by the incubation of conjugated primary antibodies (produced by immunizing an animal with the antigen of interest) or primary followed by secondary conjugated antibodies. In the latter case, the primary incubation is followed by secondary incubation with the secondary antibody produced against the species used to obtain the primary antibody. For instance, for a primary antibody produced in rabbits, an anti-rabbit secondary antibody produced in a different species, e.g., a goat anti-rabbit secondary, can be used. Testing the optimal dilution of primary antibodies is crucial to avoid excess or inadequate binding, both causing problems during the microscope examination. Every step is followed by washing with buffer solutions. The final step consists of slide mounting and adding the coverslip. The mounting medium protects cells against physical action and enhances the clarity of images under the microscope. There are different types of mounting mediums, including water-based or insoluble. In the latter case, samples must be completely dehydrated before mounting.

Microscopy examination

At the end of the above steps, tissue sections undergo microscopy examination. Antibodies could be conjugated
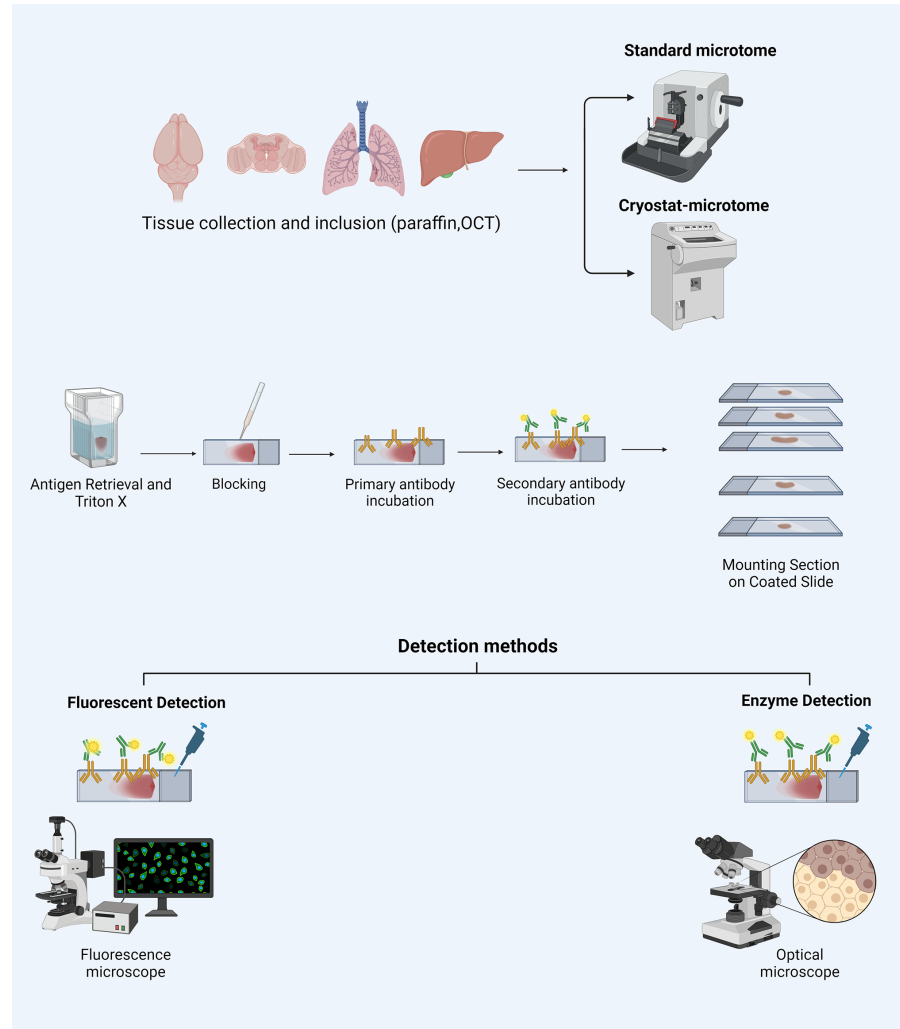
Figure 3. Fundamental steps of the IHC process.

with enzymes or fluorochromes, hence observed with optical microscopy or FM, respectively. Following the image capture process, researchers employ statistical techniques to analyse the staining. Among other software tools, ImageJ is an open-source software that is frequently used to determine the mean pixel intensity, count positively stained cells and evaluate the degree of staining localization. Figure 3 lists the fundamental IHC processes.

The application of IHC

IHC is the only technique capable of examining proteins at anatomical levels. It is a crucial technique for anatomy and pathology research, and it is also applied in clinical practice to pinpoint specific proteins linked to a particular disease state. For example, p63 and TTF-1 markers on biopsy specimens can identify non-small and small-cell lung cancer, while human epidermal growth factor receptor two can indicate cell division in breast cancer. Using antibodies

that specifically target microbial DNA or RNA, IHC has also been used to confirm the presence of a variety of infectious agents; the hepatitis B and C viruses are examples of this. IF can also be used as an initial screening test for individuals suspected of having autoimmune diseases (including lupus erythematosus and Sjögren's syndrome), by analysing the patient's serum using HEp2 cells (a human laryngeal epithelium cancer cell line) as cell substrate. The positive signal is due to the presence of autoantibodies, and different fluorescence patterns have been found related to specific autoimmune diseases (for instance, the centromere pattern is related to systemic sclerosis, while the nuclear-dense fine-speckled pattern is more prevalent in healthy individuals than in patients with systemic autoimmune rheumatic diseases). However, because IHC is believed to be observer dependent, it is mostly used for research rather than routinely in the clinic. For example, the IHC findings in animal models and post-mortem human brains have increased our understanding of neurodegenerative diseases. In studies

on Parkinson's disease (PD) and Alzheimer's disease (AD), IHC/IF was useful in examining the loss of neurons and alpha-synuclein aggregations in PD, as well as in detecting abnormal protein assembly of amyloid β-plaques in AD. Nevertheless, IHC has limitations, such as cross-reactivity wherein antibodies designed for specific proteins may bind to unrelated but similar antigens, leading to no specific staining. Another drawback includes the semi-quantitative (as opposed to quantitative) interpretation of the labelling intensity. Despite these limitations, IHC's versatility and specificity make it a valuable tool for research and clinical practice, substantially expanding our understanding of diseases and physiological processes. ■

Further reading

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