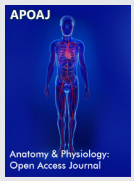

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Review Article

Immunohistochemistry and its Applications in Neuroscience

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Abstract

Immunohistochemistry (IHC) based on the specific binding between antibodies and target antigens is widely utilized to label and detect proteins of interest within tissue sections. In neuroscience, it has been instrumental in mapping the spatial distribution of neurotransmitters, receptors, ion channels, and signaling molecules across different brain regions. Specifically, IHC enables us to disclose the cellular localization and abundance of these molecules thus to advance our understanding of neural circuitry, synaptic plasticity, and neurochemical signaling pathways. Additionally, IHC allows us to identify and characterize neural cell types, aiding in the study of neurodevelopmental processes and neural pathologies such as neurodegenerative diseases and brain tumors. Recent advancements in IHC including super-resolution imaging provide enhanced spatial resolution and multiparametric analysis, thereby empowering more comprehensive investigations of complex neural networks. More importantly, integration of IHC with other molecular and imaging approaches, such as transcriptomics, electron microscopy, and functional neuroimaging, unravels the intricate molecular architecture and functional dynamics of the brain. This article provides a brief overview of the history, procedural principles and troubleshooting, and the application of immunohistochemistry to advancing our understanding of neurobiology and highlights its potential in driving discoveries to facilitate the development of novel therapeutic interventions for neurological disorders.

Keywords: Immunohistochemistry; Neurobiology; Antigen; Antibody; Cellular location; Neurodegenerative disease; Imaging; Neural circuitry

Introduction

Immunohistochemistry (IHC) is a technique used clinically and at the bench that capitalizes on the specificity of an antibody-antigen binding to identify proteins and amino acids in tissue. The antibodies are tagged with either an enzyme or a fluorescent compound that allows the binding sites to be visualized under a microscope [1]. When IHC is performed on a cellular level instead of a histological level, it is called immunocytochemistry (ICC). IHC and ICC are sometimes used interchangeably in literature [2]. Other immunostaining techniques, such as the Western Blot, are useful in the identification and quantification of proteins, but IHC has an additional advantage because of its ability to localize proteins [3]. IHC has become useful in clinical practice because of its diagnostic capabilities; pathologists frequently use the protein recognition capability of IHC to identify infections and cancerous tumors [4,5]. However, IHC is a powerful research technique that maps protein expression within tissues and has been widely used in neurobiology and other physiological fields [6].

Brief History

As the mainstay of IHC, the antibody-antigen relationship was first discovered by Emil von Behring in the 1880s [7]. Von Behring found that inoculating animals with inactivated diphtheria would produce antibodies that could be used to treat diphtheria in patients, although he did not understand the immunological mechanism that allowed it to be successful. In 1897, Rudolf Kraus demonstrated the reaction between antibodies and antigens [7]. In addition, the first immunolabeling occurred in 1942 when Albert Coons was able

to conjugate antibodies with fluorescein isocyanate and visualize them with a fluorescence microscope [2]. IHC using enzymatic labeling was developed in the 1960s, allowing immunotoxins to be visualized on an electron microscopic level [7]. Immunostaining was first applied to neurobiology in 1969 when Geffen et al. used fluorescent microscopy to visualize chromogranin, an adrenergic vesicle protein [8].

Principles of IHC

IHC consists of many steps that can be summarized as sample preparation, addition of antibodies with enzymatic or fluorescent labels, and imaging using microscopy.

Antibodies, usually of the Immunoglobin-C class that are generated from the inoculation of a mammalian host, can be monoclonal or polyclonal [9]. Monoclonal antibodies can only bind to one binding site, called the epitope, on the antigen and, therefore, have more specificity than their polyclonal counterparts. In contrast, polyclonal antibodies consist of a heterogeneous population of antibodies that can bind to multiple epitopes on one antigen. Polyclonal antibodies are more tolerable to minor changes within the antigen structure and are less expensive to produce than monoclonal antibodies [4,9].

Standard Protocol and Troubleshooting

Tissue Retrieval and Fixation

The first step in any immunohistochemical procedure is the retrieval of tissue specimens for analysis. Afterward, the tissue is fixed to preserve antigenicity. The tissue is submerged in a fixative for enough time to penetrate the sample fully. After the specimen is excised, the tissue will begin to degrade quickly, so rapid fixation is necessary to prevent the loss of antigens [10]. In neuroscience, cardiac perfusion of the whole animal with a fixative is often employed to prevent this degradation. Aldehyde fixatives, such as formaldehyde and glutaraldehyde, are used the most commonly. Paraformaldehyde, a formaldehyde polymer, can be used to create a working solution of formaldehyde [1]. During the fixation process, aldehyde groups join with proteins to form methylene cross-bridge links, creating a network that effectively preserves the in vivo state of the tissue.

It is crucial to ensure that the fixative and the antigen are compatible. If the incorrect fixative is used, test and control samples can show little to no staining. If the sample is submerged in fixative for too long or not enough time, the sample might not show staining. For this reason, it is essential to strictly follow protocol when administering fixatives. Physical damage to tissues before fixation can lead to non-specific staining patterns, so great care must be taken to protect tissue samples [10].

Tissue Sectioning

Tissue sections can take one of three formats: frozen, free-floating, or paraffin-embedded. Paraffin-embedded tissues are processed in paraffin wax and can be sliced to a single-cell thickness using a microtome. Frozen samples are best for fragile tissues that cannot withstand paraffin processing or aldehyde fixation [1] However, frozen sections tend to produce fewer clear images than their paraffin-embedded counterparts [10]. Paraffin-embedded and frozen formats are preferred when there are few samples to process, as in a clinical pathology setting [3]. The 'free-floating' method is preferred for neurological antigens. Tissue sections are about ten times thicker than paraffin-embedded tissues, but the sections are stained while floating in a buffer solution, which improves antibody penetration [1]. Additionally, the thickness of free-floating sections allows for the reconstruction of axonal and dendritic pathways, making it a powerful tool in neurobiology [3]. Free-floating tissue is cut using a vibratome [1]. The tissue samples can be damaged if the blade used in sectioning is not sharp enough. Cutting samples as thinly as possible is important to prevent reagents from getting trapped inside the sample and causing false positive staining [10].

Permeabilization, Antigen Retrieval, and Non-specific Antibody Blocking

Permeabilization and antigen retrieval are processes that improve the accessibility of the antibody to the antigen. Although fixation works to preserve the tissues, it also effectively blocks the antigen-antibody binding sites and results in weak staining [4]. Permeabilization alters the cell membrane to allow antibodies to enter the cell using a commercially available detergent, such as Triton X-100. Typically, antibodies are too large to penetrate the membrane, so this step is critical. Heat or enzymes are applied to the sections during antigen retrieval to break the methylene bridges formed during fixation, exposing the epitope to the antibody [1]. This process should not disrupt the morphology of the tissue [10].

Blocking, the final step required to prepare the tissue for the addition of antibodies, involves the addition of normal sera, protein-blocking reagent (such as bovine serum albumin, BSA), or both to prevent non-specific antibody binding. Normal serum must originate from the same species as the secondary antibody because it prevents the secondary antibody from reacting with immunoglobulins within the tissue. Horseradish peroxidase is a frequently used enzymatic label. Since endogenous peroxidase can interfere with the results of the IHC, blocking with hydrogen peroxidase is necessary to prevent background staining [1]. If the target antigen is found within DNA, HCl is added after blocking to denature the DNA and allow accessibility to the antibody [11].

Immunolabeling

Immunolabeling is considered either direct or indirect. With direct immunolabeling, the primary antibody against the antigen is labeled. This approach is best if there is an excess of primary antibody available, and the user can add a label without damaging the antibody [7]. In an indirect approach, the label is attached to a secondary antibody that binds to the primary antibody. This approach is used more commonly. Since multiple primary antibodies can bind to each antigen, and multiple secondary antibodies can bind to each primary antibody, the indirect approach has a signal-amplifying effect [1,4].

Sometimes, an additional third step is added to this process for signal amplification. In this system, the secondary antibody

is conjugated with biotin, and then a third compound, an immunolabeled avidin-biotin complex (ABC), is added. Many ABCs can bind with each secondary antibody, so they help increase the sensitivity and signal intensity of an immunoassay [1]. Endogenous biotin can interfere with results, particularly if tissue has a high biotin concentration, such as the liver tissue, so it may be necessary to add another biotin-blocking step prior to using the ABC complex [1,12].

The label may be enzymatic or fluorescent. With the addition of a substrate, an enzymatic reporter label will produce a colored or electron-dense precipitate that can be visualized with a microscope. This method is unfavorable for use in highly enzymatic tissues, such as gastric tissue [1]. Fluorescent labels, called fluorochromes, are more sensitive than enzymatic labels and allow the use of multiple labels within a single sample [13]. Fluorochromes become excited by a specific wavelength of light and then emit a longer wavelength that can be seen under a microscope [14]. However, many tissues are auto fluorescent, which can mask the signal and make interpretation difficult. Sometimes, a counterstain is added to create color contrast within the section. Many counterstains, such as DAPI (4'-6-diamidino-2-phenylindole), stain deoxyribonucleic acid (DNA), which allows the nucleus to be distinguishable from the rest of the cell.

Mounting, Imaging, and Interpretation of Results

Mounting involves the placement of tissue sections onto clear slides so that they can be visualized using microscopy [1]. Depending on which sectioning method is chosen, mounting might occur before or after the addition of antibodies. For example, paraffin-embedded sections and frozen are mounted prior to immunostaining. Free-floating sections are mounted after immunostaining. Tu et al. published a standardized protocol for the immunostaining of free-floating mouse brain samples in 2021, outlining the recommended method for mounting and imaging such sections. After slicing, the sections should be kept in a buffer solution prior to the addition of antibodies. Slices are then placed on a slide and covered with a glass coverslip. Tu et al. recommend imaging within 1-3 days to preserve fluorescence if fluorescent labeling is used. Immunotoxins are then imaged using confocal microscopy, which is preferred over other microscopy techniques because it can remove out-of-focus light to create a higher resolution image and better visualize epitopes [6,15].

Images are qualitatively evaluated based on the presence of fluorescence or colored precipitant (in the case of enzymatic reporter labels), indicating the presence of antigens at a specific location within the sample [1]. Quantitative analysis of immunotoxins is much more complicated and not yet standardized, making cross-study comparison difficult. Immunotoxins are often analyzed 'semi-quantitatively' by manually counting stains per unit area. However, this process depends on human judgment and is therefore prone to error.

Measures of Quality Control

It is essential to run control samples with every IHC assay to confirm the validity of the results [4]. A positive antigen control is a sample of tissue that is confirmed to have antigenicity and should, therefore, produce a positive staining pattern. It is used to verify protocol and reagent efficacy in detecting a specific antigen. On the contrary, a negative antigen control is a tissue sample lacking antigens that should not have any staining pattern. Staining on a negative control is indicative of non-specific antibody binding. An additional control, called a reagent control, is run to prove the relationship between the antibody and antigen. A reagent control is used to ensure that staining is produced from the primary antibody interacting with the target antigen and not with the detection system or the specimen. This can be achieved by using a detection system with diluent alone but no primary antibody [1].

The Future of IHC

IHC has been constantly evolving since its advent nearly a century ago. Each advancement in IHC technology aims to overcome its limitations. One such limitation is the inability to apply more than one antibody to a tissue sample at a time. In the near future, it will likely be possible to assess dozens of protein biomarkers simultaneously through a process known as multiplex staining (MIHC). MIHC emerged as a diagnostic tool in clinical pathology, but it can potentially be a valuable research tool for understanding the pathogenesis of complex diseases [16].

An additional drawback of IHC is its lack of a precise antigen quantification method. A new technique called mass spectrometry immunohistochemistry (MSIHC) aims to improve antigen quantification. In MSIHC, primary antibodies are labeled with an isotope with a known molecular mass, allowing the researcher to determine the amount of protein expression within a sample [17]. Another possible solution to the quantification problem is computer-automated image analysis. This approach would employ a computer algorithm to standardize the interpretation of IHC assays and circumvent human subjectivity [18].

The size of a tissue sample can limit the capabilities of IHC. Large samples may have incomplete antibody penetration, which can reduce the quality of the immunotoxin. Deep IHC is a three-dimensional histology method that aims to improve the homogeneity of staining in large samples by enhancing antibody diffusion through the tissue. Deep IHC also can be used to produce three-dimensional reconstructions of microscopic structures within tissue [19].

Applications in Neuroscience

IHC has proven invaluable in neuroscience because of the many applications of protein localization within the brain [20]. Neurons and glial cells can be identified using IHC, allowing for stereological and morphological analysis of cells in different disease states [21]. Protein markers can also be used to identify dying and progenitor cells. Markers of neurotransmitter synthesizing enzymes are used to identify transmitter specificity within neurons [22]. Antibodies can be developed for most peptides, making IHC a valuable tool [23]. In addition to characterization of cell morphologies and transmitter specificity of neurons, IHC has significantly contributed to our current understanding of neurogenesis and neurodegenerative disease [24,25].

IHC is also useful for localizing non-neuronal glial cells, such as astrocytes. Glial fibrillary acidic protein (GFAP) is one of the most commonly used markers of astrocytes. Astrocytes contribute to synaptic plasticity and respond to brain injury through a process known as reactive astrogliosis [22,26]. When undergoing reactive astrogliosis, astrocytes have elevated levels of GFAP that are detectable using IHC methods. It remains unclear whether astrocytic activation is causative or causal in neurodegeneration [26], and IHC of GFAP is a promising technique to further the understanding of the role of astrocytes in neurodegenerative disease.

IHC using bromodeoxyuridine (BrdU) is the most commonly used technique in the study of adult neurogenesis. BrdU is a thymidine analog that can be incorporated into the DNA molecule during the S-phase and functions as a marker of DNA synthesis [25]. This makes BrdU an effective way to monitor neuronal cell proliferation, particularly when combined with a secondary IHC stain to mark cell type and stage of development [25,11]. Despite this, BrdU does have certain drawbacks. For instance, BrdU can also be incorporated into DNA during other processes involving DNA synthesis, such as cell cycle reentry and DNA duplication, so it cannot technically be considered an exclusive marker of cell proliferation [25]. However, most neuronal cells will not go through these processes, so staining due to cell cycle reentry or DNA duplication would likely make up a small portion of stained cells. Still, these factors must be accounted for when monitoring neurogenesis using BrdU [27].

IHC has been instrumental in the understanding of how neurodegenerative diseases impact the brain because neurodegeneration often corresponds with an abnormal protein composition. For example, in an Alzheimer's disease state, amyloid plaques, which are peptide deposits of amyloid beta protein, aggregate within the brain and are thought to be a possible cause of the neurological degeneration associated with Alzheimer's disease [28]. IHC can be used to quantify and localize these plaques [3]. Multiple studies have reported a correlation between the number of amyloid plaques and age in mouse models through immunohistochemical techniques [28-30].

Conclusion

IHC has been evolving since the discovery of antibody-antigen specificity over a century ago. Although the process of IHC is long and precise, the current tissue preparation and analysis methods provide substantial information about protein expression. This makes IHC an indispensable tool in clinical pathology and laboratory research, particularly in neurobiology, in which great strides have been made in understanding neurogenesis and neurodegenerative disease through IHC. Although the future of IHC is still being determined, it will, without a doubt, continue to improve and become a more valuable tool in the clinic and at the bench.

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Conflict of Interest

No conflict of interest.

References

1. Renshaw S (2017) Immunohistochemistry and Immunocytochemistry. Immunohistochemistry and Immunocytochemistry: Essential Methods, Second Edition pp. 35-102.
2. Brandtzaeg P (1998) The increasing power of immunohistochemistry and immunocytochemistry. Journal of Immunological Methods 216(1-2): 49-67.
3. Potts EM, Coppotelli G, Ross JM (2020) Histological-Based Stainings using Free-Floating Tissue Sections. Journal of visualized experiments p.162: 10.3791/61622.
4. Magaki S, Hojat SA, Wei B, So A, Yong WH (2019) An Introduction to the Performance of Immunohistochemistry. Methods in molecular biology 1897: 289-298.
5. Roskams T (2002) The role of immunohistochemistry in diagnosis. Clinics in Liver Disease 6(2): 571-589.
6. Tu L, Zhang N, Conde KM, Bean JC, Wang C, et al. (2021) Free-floating Immunostaining of Mouse Brains. Journal of visualized experiments 176: 10.3791/62876
7. Childs GV (2014) History of Immunohistochemistry. Pathobiology of Human Disease: A Dynamic Encyclopedia of Disease Mechanisms pp. 3775-3796.
8. Geffen LB, Livett BG, Rush RA (1970) Immunohistochemical Localization of Chromogranins in Sheep Sympathetic Neurones and their Release by Nerve Impulses. New Aspects of Storage and Release Mechanisms of Catecholamines. Bayer-Symposium p. 2.
9. Sukswai N, Khoury JD (2019) Immunohistochemistry Innovations for Diagnosis and Tissue-Based Biomarker Detection. Current hematologic malignancy reports 14(5): 368-375.
10. Jackson S, Gandy M (2016) Quality Assurance in Immunochemistry. Immunohistochemistry and Immunocytochemistry: Essential Methods, Second Edition pp. 123-155.
11. Buenrostro-Jauregui M, Tapia-de-Jesús A, Mata J, Rodríguez-Serrano LM, Chávez-Hernández ME, et al. (2020) Immunohistochemistry Techniques to Analyze Cellular Proliferation and Neurogenesis in Rats Using the Thymidine Analog BrdU. Journal of Visualized Experiments pp. 163.
12. Cooper KM, Kennedy S, McConnell S, Kennedy DG, Frigg, M (1997) An immunohistochemical study of the distribution of biotin in tissues of pigs and chickens. Research in veterinary science 63(3): 219-225.
13. Piña R, Santos-Díaz AI, Orta-Salazar E, Aguilar-Vazquez AR, Mantellero CA, et al, (2022) Ten Approaches That Improve Immunostaining: A Review of the Latest Advances for the Optimization of Immunofluorescence. International journal of molecular sciences 23(3): 1426.
14. Cuello AC, Priestley JV, Sofroniew MV (1983) Immunocytochemistry and neurobiology. Quarterly journal of experimental physiology 68(4): 545-578.
15. Wheeler A (2016) Confocal Microscopy. Immunohistochemistry and Immunocytochemistry: Essential Methods, Second Edition 169-198.
16. Tan WCC, Nerurkar SN, Cai HY, Ng HHM, Wu D, et al. (2020) Overview of multiplex immunohistochemistry/immunofluorescence techniques in the era of cancer immunotherapy. Cancer communications 40(4): 135-153.
17. Clarke C (2022) Immunohistochemistry for Future Applications. Immunohistochemistry: A Technical Guide pp. 253-265.
18. Chlipala EA, Bendzinski CM, Dorner C, Sartan R, Copeland K, et al. (2020) An Image Analysis Solution for Quantification and Determination

of Immunohistochemistry Staining Reproducibility. Applied Immunohistochemistry & Molecular Morphology 28(6): 428-436.

19. Yau CN, Lai HM, Lee K, Kwok AJ, Huang J, et al. (2023) Principles of deep immunohistochemistry for 3D histology. Cell Report Methods 3(5): 100458.

20. Goto S, Morigaki R, Okita, S, Nagahiro S, Kaji R (2015) Development of a highly sensitive immunohistochemical method to detect neurochemical molecules in formalin-fixed and paraffin-embedded tissues from autopsied human brains. Frontiers in Neuroanatomy 9: 22.

21. Lyck L, Dalmau I, Chemnitz J, Finsen B, Schroder HD (2008) Immunohistochemical markers for quantitative studies of neurons and glia in the human neocortex. The journal of histochemistry and cytochemistry 56(3): 201-221.

22. Korzhevskii DE, Grigo'ev IP, Gusel'nikova VV, Kolos EA, Petrova ES, et al. (2019) Immunohistochemical markers for neurobiology. Medical Academic Journal 19(4): 7-24.

23. DeLaney K, Buchberger AR, Atkinson L, Gründer S, Mousley A, et al. (2018) New techniques, applications and perspectives in neuropeptide research. The Journal of experimental biology 221(3).

24. Duraiyan J, Govindarajan R, Kaliyappan K, Palanisamy M (2012) Applications of immunohistochemistry. Journal of pharmacy & bioallied sciences 4(2): 307- 309.

25. Taupin P (2007) BrdU immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations, and validation. Brain Research Reviews 53(1): 198-214.

26. Brandebura AN, Paumier A, Onur TS, Allen NJ (2023) Astrocyte contribution to dysfunction, risk and progression in neurodegenerative disorders. Neuroscience 24(1): 23-39.

27. Kuan C, Schloemer AJ, Lu A, Burns KA, Weng W, et al. (2004) Hypoxia-Ischemia Induces DNA Synthesis without Cell Proliferation in Dying Neurons in Adult Rodent Brain. Journal of Neuroscience 24(47): 10763-10772.

28. Khan S, Barve KH, Kumar MS (2020) Recent Advancements in Pathogenesis, Diagnostics and Treatment of Alzheimer's Disease. Current neuropharmacology 18(11): 1106-1125.

29. Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM (2000) High-level neuronal expression of alpha-beta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. The Journal of neuroscience 20(11): 4050-4058.

30. Shankar GM, Leissring MA, Adame A, Sun X, Spooner E, et al. (2009) Biochemical and immunohistochemical analysis of an Alzheimer's disease mouse model reveals the presence of multiple cerebral Abeta assembly forms throughout life. Neurobiology of disease 36(2): 293-302.