

Brief guide to immunostaining

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ABSTRACT

Immunostaining is an essential biological technique that determines the localization and level of target antigen molecules using antibodies within cells or tissues. Here, we present a brief guide to immunostaining, including the principles, methods, and different types of immunostaining. This manuscript will also provide common challenges and optimization strategies. This work will be useful for researchers with basic knowledge in immunostaining.

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INTRODUCTION

Immunostaining is a widely used technique in biological research and clinical diagnostics (Kim et al., 2023; Piña et al., 2022; Ryu et al., 2023). Immunostaining utilizes the principle of antigen-antibody interaction, in which antibodies are used to specifically bind to target antigens (Magaki et al., 2019). To visualize the antigen-antibody complexes using microscopy, antibodies are usually conjugated with a detectable probe, such as fluorescent dye or enzyme (Im et al., 2019). The visualized samples provide crucial information about the presence, localization, and abundance of the target antigens in cells or tissues (Piña et al., 2022). In early days, immunostaining was mainly used to detect specific antigens in tissue sections and to gain information about cellular structures (Ortiz Hidalgo, 2022). Immunostaining is currently exploited as a critical tool for cell biological research and disease diagnosis, such as infections or cancer (Ahn et al., 2023; Duraiyan et al., 2012; Piña et al., 2022). Here, we provide a brief guide to immunostaining for researchers in basic and clinical scientific research fields who are not familiar with cell biology tools.

MAIN BODY

Principles of Immunostaining

The principle of immunostaining is based on the specific binding of antibodies to their target antigens (Fig. 1) (Im et al., 2019). The detection of antigen-antibody complexes depends on markers, which are conjugated to antibodies. When an enzyme is used, a colorimetry or chemiluminescence-based substrate for the enzyme is required. The enzyme catalyzes a reaction that produces a colored product, which is detected using a light microscope (Sukswai and Khoury, 2019). For fluorescent dyes,

fluorescent signals are measured using fluorescence microscopy without the need for additional liquid substrates.

Methods of Immunostaining

Two main methods, indirect and direct immunostaining methods, are used for immunostaining (Table 1) (Piña et al., 2022). The indirect method uses both primary and secondary antibodies. Primary antibodies specifically bind to antigens of interest. The primary antibodies are typically produced in animals such as goats, mice, or rabbits by injecting them with specific antigens. A secondary antibody is usually conjugated with a detectable marker, such as an enzyme or a fluorescent dye, and binds to the primary antibody (Ramos-Vara, 2005). Secondary antibodies produced from species different from those that produced the primary antibodies are desirable to prevent cross-reactivity (Lan et al., 1995). Multiple secondary antibodies can interact with each primary antibody, enhancing the detectable signal. In addition, one type of secondary antibody can be used with various primary antibodies (Im et al., 2019). The direct method uses a single antibody conjugated with a detectable marker that specifically binds to an antigen. This method involves 1-step antibody incubation, which makes it simpler and faster than the indirect method. However, because the direct method uses a single antibody, it has lower sensitivity compared to the indirect method, and the usage is restricted because available probe/enzyme-conjugated primary antibodies are limited (Im et al., 2019). Because both methods have their specific advantages, researchers need to choose a proper method depending on the purpose of their experiments.

Types of Immunostaining

Immunohistochemistry (IHC) is used for analyzing the level and localization of a specific antigen within the tissue structure.

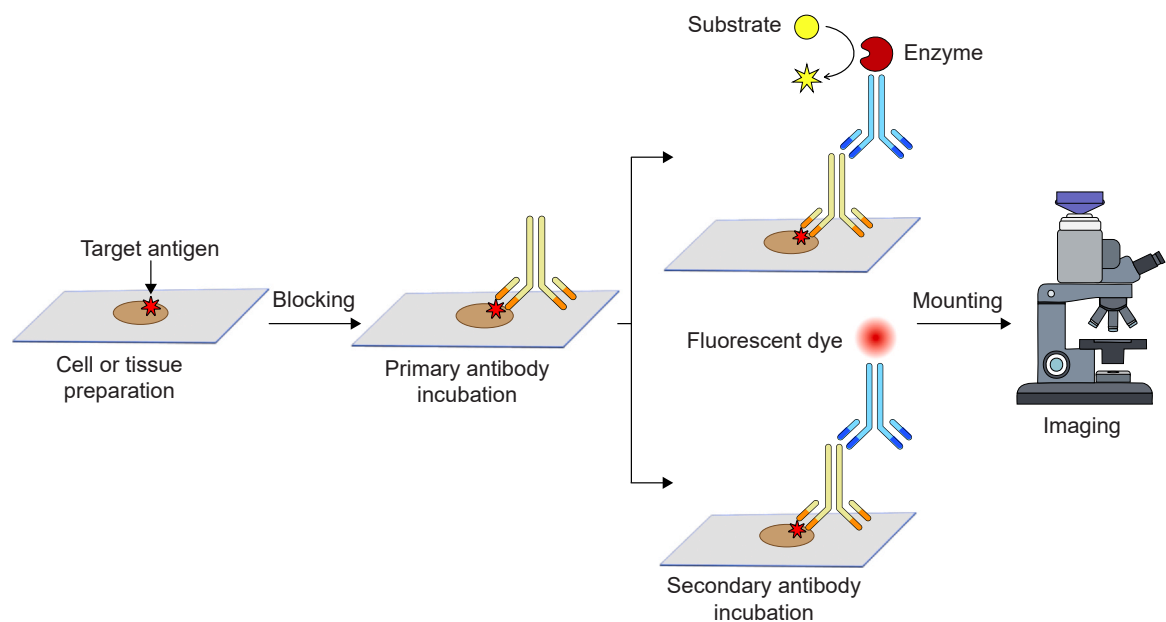


Fig. 1. Typical workflow of immunostaining. Immunostaining is initiated with cell or tissue preparation, in which specific cell or tissue samples are fixed to preserve their structures. The samples are then incubated with a blocking buffer to prevent nonspecific binding between antigen and antibody. Subsequently, the samples are incubated with a primary antibody that specifically binds to the target antigen, followed by incubation with a secondary antibody linked to a fluorescent dye or an enzyme. After washing to remove excess antibodies, the sample is mounted on a slide glass using a mounting medium. Lastly, the antigen-antibody complexes are visualized using proper microscopy.

Table 1. Features of direct and indirect immunostaining methods

	Direct	Indirect
Primary antibody	Binding specific antigens Conjugated with a detectable marker	Binding specific antigens
Secondary antibody	Unnecessary	Binding primary antibody Conjugated with a detectable marker
Processing time	One-step antibody incubation Fast	Two-step antibody incubation Slow
Sensitivity	Low	High
Signal amplification	No	Yes
Commercial products	Limited	Many
Cross-reactivity	Avoided	Need for using primary and secondary antibodies from different species

Samples for IHC are prepared by either paraffin-embedding or cryopreservation (Table 2) (Hofman and Taylor, 2013). Paraffin embedding fixes tissue in formalin before embedding in wax, requiring antigen retrieval due to epitope masking. Cryopreservation, freezing samples below -80°C , avoids this need (Hofman and Taylor, 2013; Kim et al., 2016). By using enzymes conjugated to antibodies, the target antigen, usually protein, in a tissue can be visualized under a light microscope (Ramos-Vara, 2005). Because of these features, IHC has the advantage of being permanent (Hofman and Taylor, 2013). However, it has disadvantages of low resolution for fine structures, tissue or cell damage due to enzymatic reactions, and limited colorimetric options, making it difficult for multiple labeling (Piña et al.,

2022). IHC is widely used in pathology to diagnose diseases such as cancer and research on tissue-specific protein assays (Ding et al., 2023; Magaki et al., 2019; Mikami et al., 2022; Park et al., 2022).

Immunocytochemistry (ICC) is a technique for visualizing the presence and location of specific antigens in all types of cells, including cultured and suspended cells, rather than whole tissue sections (Table 2) (Jeong et al., 2023; Park et al., 2023; Renshaw, 2017). For this technique, cells are prepared on coverslips or in multiwell plates and fixed to preserve their structure by using paraformaldehyde (Marchenko and Flanagan, 2007). By using fluorescent dyes or enzymes conjugated to antibodies, the target antigens can be visualized

Table 2. Features of different types of immunostaining

	Immunohistochemistry (IHC)	Immunocytochemistry (ICC)	Immunofluorescence (IF)
Sample type	Tissues	Cells	Tissues or cells
Sample preparation	Formalin-fixed paraffin-embedded Cryopreserved	Paraformaldehyde-fixed	Formalin-fixed paraffin-embedded or cryopreserved (tissues) Paraformaldehyde-fixed (cells)
Label	Enzyme	Enzyme Fluorescent dye	Fluorescent dye
Detection	Light microscope	Light microscope Fluorescent microscope	Fluorescent microscope
Signal duration	Permanent	Permanent with enzyme Temporary with fluorescent dye	Temporary (quenching, photobleaching, limited photostability)
Multiplex staining	Difficult	Easy with enzyme Difficult with fluorescent dye	Easy
Resolution	Low resolution (fine structure)	Low resolution with enzyme High resolution with fluorescent dye	High resolution (fine structure)

IHC, Immunohistochemistry; ICC, Immunocytochemistry; IF, Immunofluorescence.

under a fluorescence microscope and a light microscope, respectively (Piña et al., 2022). ICC is applied for the identification of the presence of specific biomarkers in cells, subcellular localization, and in situ macromolecule interactions (Kanber et al., 2021).

Immunofluorescence (IF) is used for both tissue sections and cells to detect target antigens (Table 2) (Piña et al., 2022; Song et al., 2022; Song et al., 2023). The sample preparation is the same as the IHC and ICC. IF utilizes fluorescence microscopes to detect fluorescent dyes that are conjugated with antibodies (Im et al., 2019). Such features allow high-resolution imaging for fine structures. Additionally, IF provides a large variety of colorimetric options, allowing researchers to perform multiple labeling. However, disadvantages also exist, such as quenching (reduction of fluorescence emission), limited photostability, and photobleaching (the permanent loss of fluorescence emission) (Piña et al., 2022). This technique is effective when multiplex staining is required, in particular for assessing the colocalization of macromolecules (Duplancic and Kero, 2021). For multiplex staining, researchers need to choose fluorescent dyes with nonoverlapping emission spectra to prevent spectral overlap that can lead to signal misinterpretation. For example, using 4',6-diamidino-2-phenylindole dihydrochloride (emission peak at 465 nm), fluorescein isothiocyanate (emission peak at 519 nm), tetramethylrhodamine isothiocyanate (emission peak at 576 nm), or Cy5 (emission peak at 667 nm) can prevent spectral overlap (Bolognesi et al., 2017).

Strategies to Overcome Challenges in Immunostaining

Nonspecific binding, resulting from the interaction between antibodies and off-targets, which causes false-positive signals (Buchwalow et al., 2011), is a critical issue that needs to be avoided in immunostaining. Several factors contribute to nonspecific binding, such as insufficient blocking of samples, low specificity, and high antibody concentrations. Inadequate

washing protocols, which leave residual unbound antibodies in the sample, can also cause nonspecific binding (Kim et al., 2016). To overcome this obstacle, using antibodies that are highly specific to target antigens and optimizing their concentrations are important. Utilization of compatible blocking buffers can also reduce unintended antigen-antibody interaction (Daneshtalab et al., 2010). It is also important to optimize washing steps in protocols to effectively remove unbound antibodies. Adjusting antibody concentrations and incubation times can also reduce background signals (Piña et al., 2022).

Photobleaching is another key issue in immunostaining that markedly affects the quality of fluorescence imaging. This occurs when fluorophores gradually lose their ability to emit signals from prolonged exposure to the excitation source, diminishing fluorescence over time. One effective way to reduce photobleaching is to minimize the intensity and exposure time of the excitation light (Hoebe et al., 2007). For example, after applying fluorophore-conjugated secondary antibody, samples should be incubated and stored in the dark (Im et al., 2019). Additionally, using antifade mounting media such as VECTASHIELD (Vector Laboratories) can protect fluorophores from photobleaching (Espada et al., 2005; Florijn et al., 1995).

Autofluorescence is another challenge in fluorescence-based immunostaining experiments. This phenomenon is caused by the natural light emission from tissue components, such as lipofuscin, elastin, and collagen, which can interfere with the detection of specific fluorescent signals (Surre et al., 2018). One strategy to overcome this issue is to select fluorophores with emission spectra that sufficiently differ from the autofluorescent signal (Deal et al., 2018). Utilizing an autofluorescence quenching reagent can help specifically detect the signal over autofluorescence (Baschong et al., 2001).

Bleed-through in fluorescence imaging can also pose a challenge during immunostaining. This occurs when fluorescence from a neighboring channel is detected in the channel of

interest, particularly when multiple fluorophores are simultaneously used (Kim et al., 2010). A simple solution is to select fluorophores that emit in distant spectral ranges. However, if this is not feasible, it is recommended to reduce the intensity of high fluorescence signals by lowering the concentration of secondary antibodies (Rénier et al., 2007).

CONCLUDING REMARKS

Here, we briefly describe how to perform immunostaining, discussing principles, types, and strategies to improve common issues. Although detailed methods need to be adjusted to the types of experiments, our manuscript will provide basic key information about immunostaining for biologists who do not have relevant expertise.

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DECLARATION OF COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The author is an Editor-in-Chief for *Molecules and Cells* and was not involved in the editorial review or the decision to publish this article.

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