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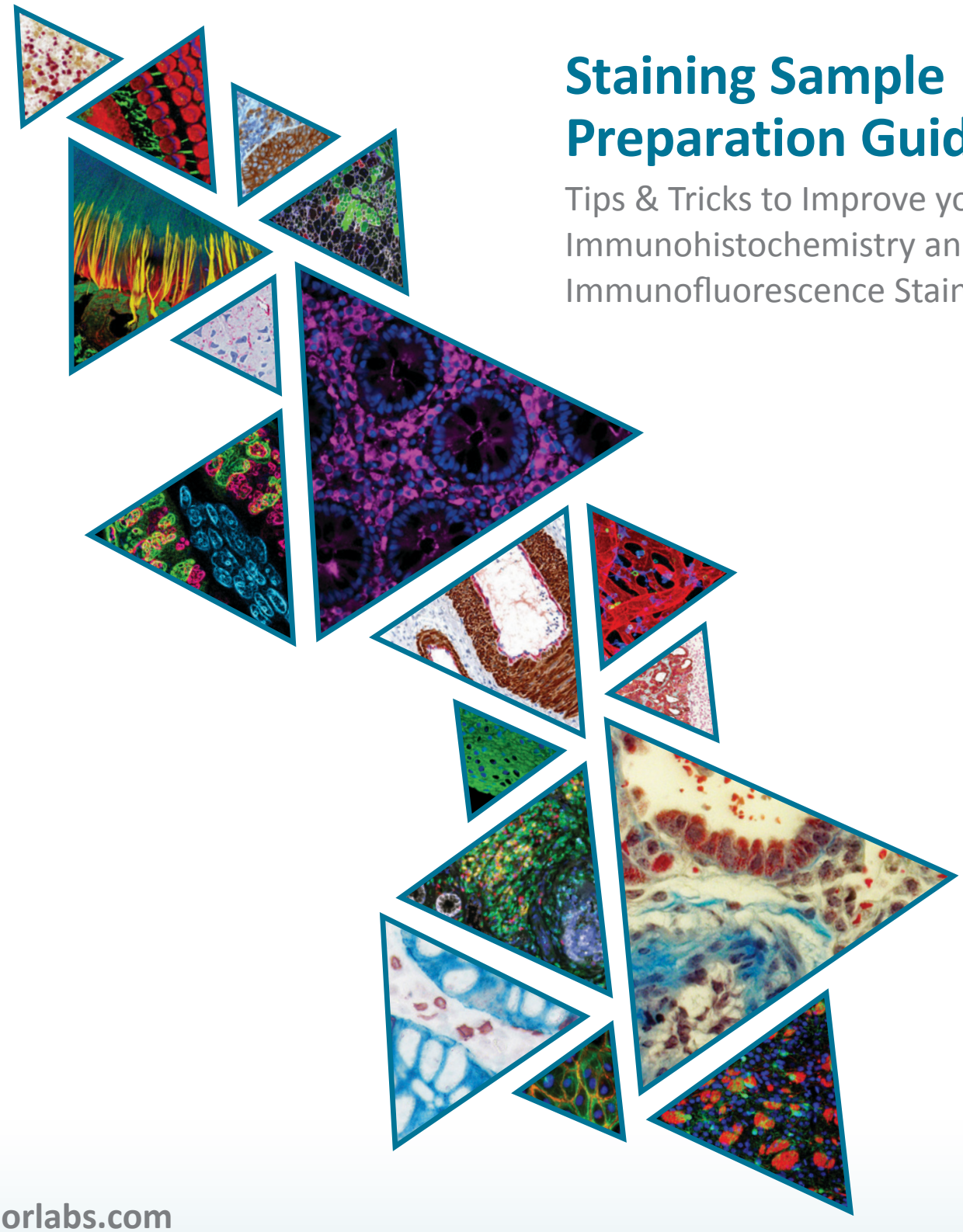

Staining Sample Preparation Guide

Tips & Tricks to Improve your Immunohistochemistry and Immunofluorescence Staining

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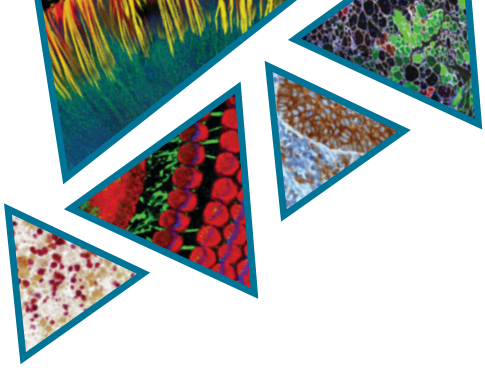

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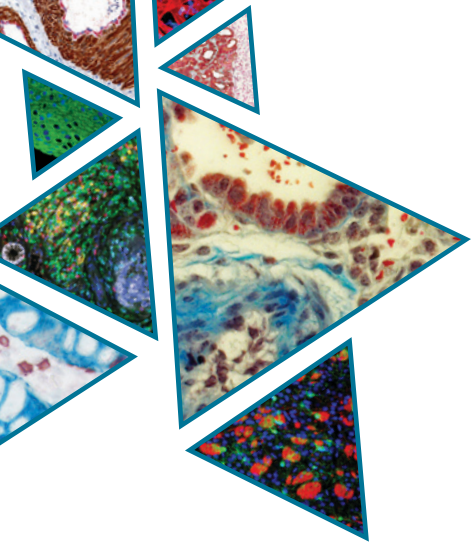


Introduction

Sitting at the microscope at the end of an immunohistochemistry (IHC) or immunofluorescence (IF) experiment can spark feelings of amazement or frustration. Successful experiments result in clearly stained specimens with appropriate tissue morphology and low background. On the other hand, frustration can stem from slides with poor morphology due to damaged specimens, high background, or staining that is inconsistent or absent.

IHC and IF are relatively simple techniques employed in various research and clinical applications. They use antibodies along with different chromogens and fluorophores to detect specific antigens in tissue sections. Because each IHC/IF step offers many choices, a successful workflow demands careful planning and execution to ensure the best results. Sample preparation alone includes multiple steps, and many of them offer different choices—paraffin-embedding vs. cryopreservation, section thickness, and antigen retrieval buffer, among others. Making informed decisions and troubleshooting issues throughout the entire process requires technical knowledge and a clear perspective of experimental needs. One of our goals is to educate and empower our end-users to ask the right questions and optimize protocols when planning experiments.

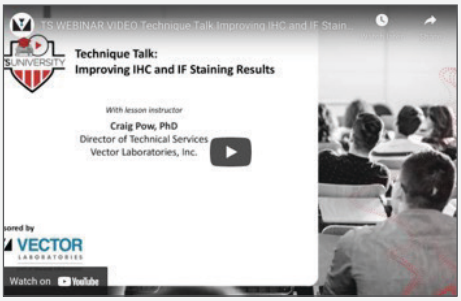
Improving IHC and IF Staining Results

This on-demand webinar, presented in partnership with *The Scientist*, focuses on optimizing IHC and IF staining workflows.

Learning objectives include:

- Selecting staining reagents
- Identifying and eliminating sources of non-specific staining
- Appropriate controls

Watch Now →



The IHC workflow comprises sample preparation, antigen retrieval, blocking, primary and secondary antibody incubation, detection, counterstaining, and mounting. This guide covers the steps included in the sample preparation process—sample fixation, sectioning, deparaffinization and rehydration, antigen retrieval, and PAP pen application (Figure 1). Adequate sample preparation ensures tissue and antigen preservation for long-term storage as well as successful staining. The information provided in this guide will help to inform experimental decisions, customize technical protocols, and ensure quality results.

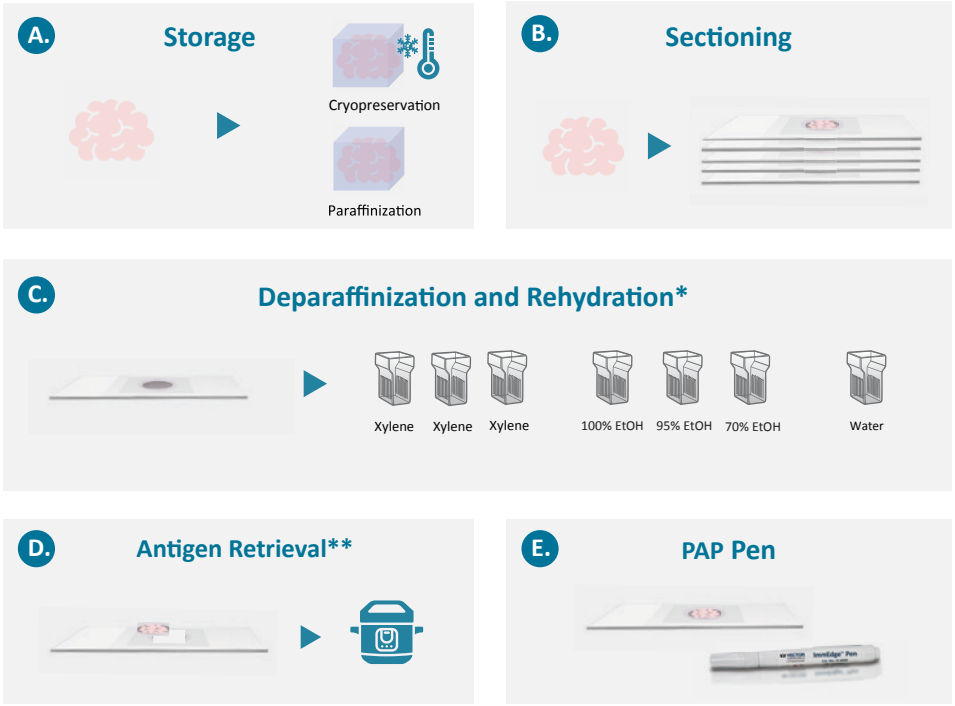
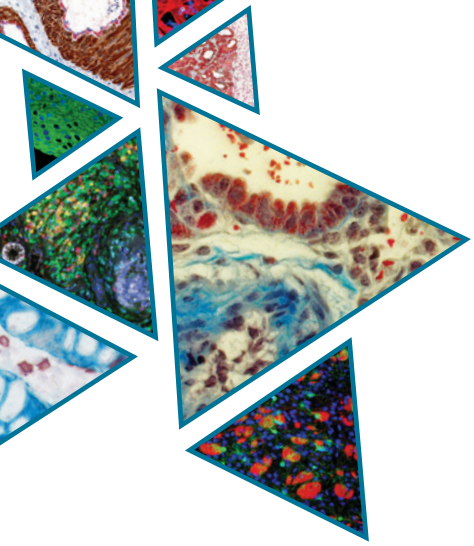


Figure 1. Sample preparation workflow. **A.** Options for tissue preservation include FFPE or frozen block. **B.** Depending on the final application, tissue blocks can be sectioned at various levels of thickness using the appropriate equipment. **C.** A series of washes in xylene ensures complete paraffin removal. Washes in graded alcohol gradually replace xylene for water.* **D.** Heat-induced epitope retrieval promotes breakage of cross-links formed during fixation and unmasking of antigen.** **E.** PAP pen applies a hydrophobic barrier around the tissue section, containing reagents within a small area.

*Deparaffinization and rehydration steps are only applicable when working with FFPE tissue sections.

**Heat-induced epitope retrieval may not be is necessary when working with tissue that was snap-frozen immediately following dissection. Antigen retrieval improves staining in specimens that underwent formalin fixation regardless of the subsequent choice of storage via paraffinization or cryopreservation.



Sample Storage

The options for long-term storage of tissue samples fall into two major categories—paraffin-embedding and cryopreservation (Figure 2). The formalin-fixation, paraffin-embedding process includes aldehyde-based fixation, dehydration with a series of alcohols, clearing with xylene, and impregnation with paraffin (1). Formalin fixation helps to inhibit biochemical reactions in the tissue and thus prevents autolysis and putrefaction. It also forms covalent bonds, or cross-linking, between protein residues, making the tissue more robust and contributing to tissue morphology preservation. Next, the formalin-fixed tissue is processed through a series of graded alcohols, moving from a lower to a higher concentration to replace the fixative with alcohol. Next, incubation with an organic solvent, such as xylene, clears the alcohol. In the final step, warm paraffin (60 °C) impregnates the tissue and then cools down to harden and create a formalin-fixed, paraffin-embedded (FFPE) block.

The FFPE block may be stored at room temperature for an extended period before continuing with the IHC/IF workflow. Uncut FFPE blocks are stable for as long as 25 years with no alteration in immunogenicity for most epitopes (Figure 2). Still, mild to moderate loss in immunoreactivity may occur for a few antigens (i.e., nuclear and membrane proteins) after 24 months of storage (2,3). Pre-cut sections may be susceptible to a quicker deterioration in immunogenicity, likely due to exposure of thin slices of tissue to the atmosphere (1). Storing pre-cut sections in plastic boxes at 4 °C helps to prevent this loss of antigenicity, but when working with FFPE tissue, best practices include keeping the paraffin block in a cool, dark place and sectioning it no more than 1 month before staining (Figure 2) (3). These guidelines ensure that immunostaining yields consistent IHC results for many years after formalin fixation and paraffin embedding.

Freezing the biospecimen at -20 °C or -80 °C is another option for storing tissue for IHC/IF applications (Figure 2). In the conventional cryopreservation workflow, the fresh tissue is snap-frozen in liquid nitrogen, isopentane, or dry ice; mounted in a block of OCT (optimal cutting temperature) compound; and then sectioned on a cryostat. Tissue fixation is usually performed after sectioning, and choices of fixatives include acetone, methanol, ethanol, and formaldehyde, among others (4,5). Uncut frozen OCT blocks are stable for many months when stored at -80 °C or -20 °C (Figure 2). Excessively long storage of cut, frozen tissue sections may result in freezer burn and loss of antigenicity and tissue architecture (5). The recommendation is to store sectioned frozen tissue for no longer than 2–3 months. Alternatively, uncut fixed tissue can undergo impregnation with a cryoprotectant (i.e., 30% sucrose solution), snap freezing, and long-term storage at -80 °C (Figure 2) (6).

Did you know?

Although formalin is a good general fixative, other solutions are available for specific applications (7, 8).

Fixature	Recommendation
Zenker's fixative	Nucleic acid, connective tissue
Bouin's solution	Delicate and soft tissue
Carnoy's solution	Nucleic Acid, glycogen
Ethanol/ methanol	Exfoliative cytology smears and blood films
Helly's fixative	Hematopoietic tissue
B-5 fixative	Hematopoietic tissue
Acetone	Enzyme preservation, large proteins
Acetic acid	Nucleic acids—combined with ethanol

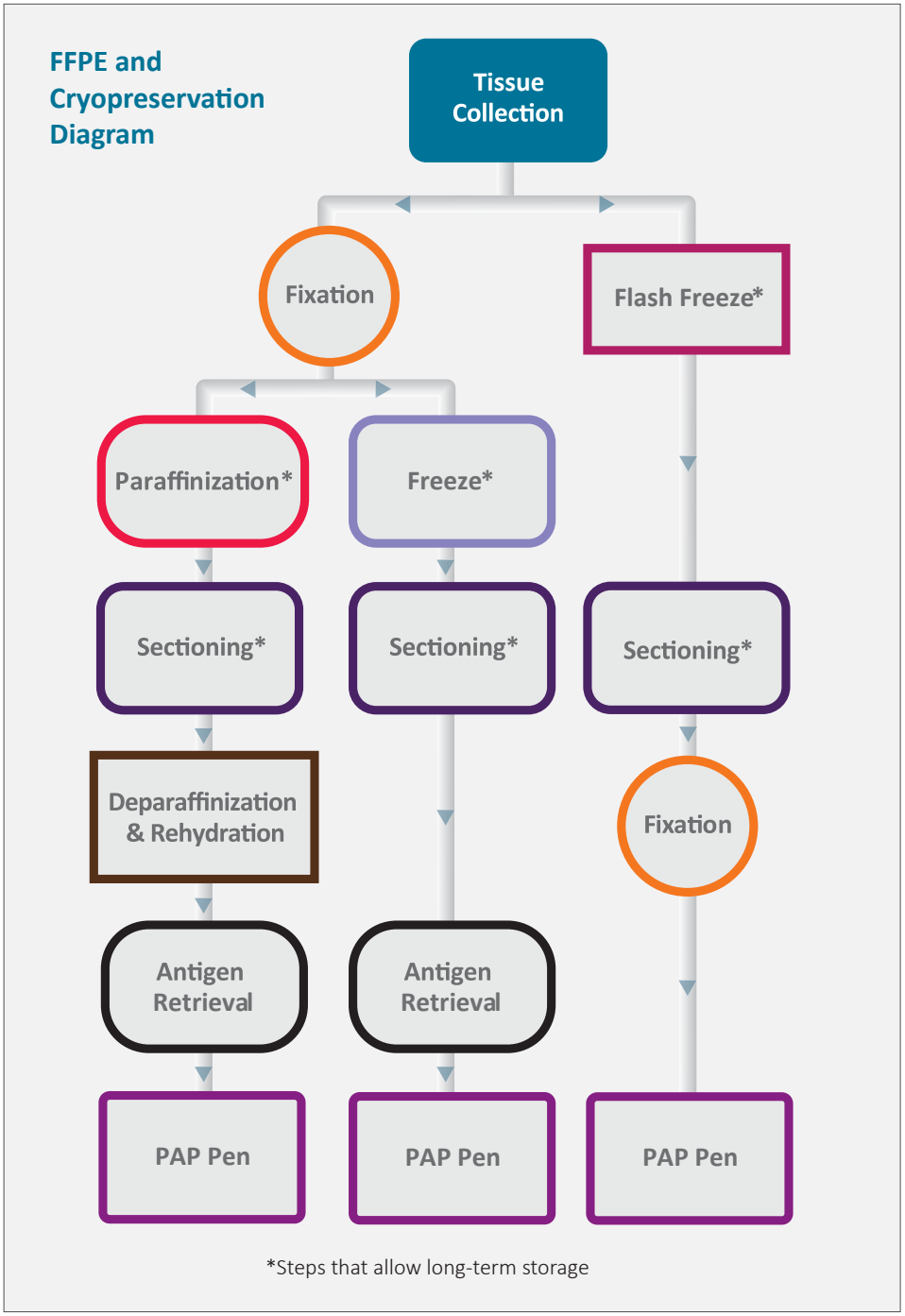


Figure 2. Diagram of FFPE and cryopreservation workflows. Immediately after dissection, tissue can be flash-frozen or fixated in formalin-based or other fixatives. Subsequent steps vary with the method of preservation. Similar shapes in the diagram represent analogous processes in the workflow..

*Steps that allow long-term storage. Refer to Table 1 for the recommended length of storage in each step.

Pro Tip

Fully consider your experimental needs and the advantages and disadvantages of each choice before beginning the experiment and collecting the tissue. Refer to Table 1, and check the literature and reagents manufacturer's recommendations to ensure best results.

Both paraffin embedding and cryopreservation have many advantages and disadvantages, and the final choice ultimately depends on the experimental needs of the study (Table 1). Therefore, it is essential that the researcher considers all options and makes a final decision before collecting the tissue. FFPE blocks offer excellent tissue stability and can be used to generate very thin (1–5 µm) sections, yielding higher microscopic resolutions and lower background. Paraffin-embedded samples generally possess well-preserved tissue morphology, which facilitates data analysis and interpretation. Another significant advantage of FFPE blocks is that they allow space-efficient long-term storage of samples at room temperature. This particular advantage enables the creation of large tissue repositories. Formalin fixation, however, brings some disadvantages as well. Though cross-linking between proteins offers tissue stability and preserved morphology, it may also mask antigen epitopes and reduce antigenicity. To address this problem, researchers must perform antigen retrieval, adding an extra step to the IHC or IF workflow to unmask the epitope.

Cryopreservation, especially when samples undergo fixation post-sectioning, is a

Table 1—Comparison of advantages and disadvantages of tissue preservation options

	Paraffin-embedded tissue	Fresh frozen tissue	Formalin-fixed frozen tissue
Fixation	Pre-embedding	Post-sectioning	Pre-freezing
Fixative	Formaldehyde	Acetone, methanol, ethanol, formaldehyde, and others	Formaldehyde
Embedding/ Cryoprotection	Paraffin	OCT	Impregnation with cryoprotectant before freezing Immersion in cryoprotectant if storing free-floating sections
Storage	FFPE block — several years at room temperature FFPE sections — up to a month at 4 °C.	Frozen OCT block (non-fixed) — up to a year at -80 °C. Frozen tissue sections — 2–3 months at -80 °C or -20 °C	Uncut frozen tissue (formalin-fixed) — several years at -80 °C. Sectioned frozen tissue (formalin-fixed) in cryoprotectant — up to 2 years at -20 °C
Sectioning	Microtome	Cryostat	Microtome, Vibratome, Cryostat
Antigenicity	May be masked by fixation-induced cross-linking	Retains antigenicity and does not require antigen retrieval	May be masked by fixation-induced cross-linking
Section Thickness	As thin as 1 µm	6–10 µm	6–20 µm
Morphology	Well-preserved	Ice crystal formation may affect tissue morphology	Well-preserved
Other Advantages	Thinner sections have higher microscopic resolution and lower background Undemanding storage conditions are ideal for the creation of large tissue repositories	Time-saving approach Lipid retention Preserves highly sensitive proteins, enzymatic activity, and nucleic acids Ideal for experiments targeting post-translational modifications	Ideal for studies looking at 3D morphology (i.e., axonal tracks) Also employed in stereology experiments

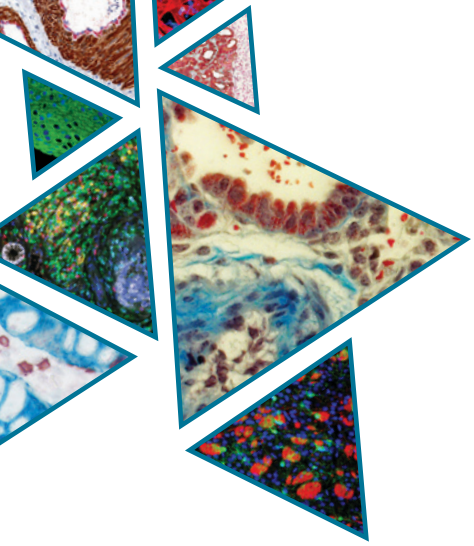
Pro tip

Freezing the specimen very quickly is key to preserving tissue and cell morphology. Slow freezing leads to the formation of large ice crystals, resulting in morphological alterations. Here are two methods for quickly freezing specimens:

- Place a metal recipient filled with isopentane inside a small dewar with liquid nitrogen. Wrap the tissue in aluminum foil and immerse it in the cold isopentane.
- Pulverize dry ice and place it in a small Styrofoam box. Place the tissue in the box and cover it with pulverized dry ice.

Always remember to label all dishes and wraps beforehand.

much quicker process. Acetone or alcohol-based fixatives are the common choice in this process and do not promote cross-linking between proteins. For this reason, cryopreservation of tissue before fixation retains antigenicity and does not require antigen retrieval. This approach preserves membranes, lipid deposits, highly sensitive proteins, enzymatic activity, and nucleic acids, and is the choice for experiments targeting post-translational modifications. The freezing process, however, may lead to the formation of ice crystals, which is problematic when tissue and cell morphology are important experiment outcomes. When samples are fixed and cryoprotected before freezing, tissues retain better morphology, but this choice introduces antigenicity loss and may require the addition of an epitope retrieval step into the workflow. In addition, frozen tissue requires thicker sectioning (>5 µm), which may lead to poorer microscopic resolution and higher background. Storage requirement is another downside of cryopreservation in general, regardless of fixation pre- or post-sectioning. It requires freezers with no temperature oscillation or liquid nitrogen tanks. Even when properly frozen, non-fixed fresh frozen tissue will not retain viability longer than one year.



Sectioning

After freezing or embedding in a paraffin block, the tissue undergoes sectioning on a microtome (FFPE blocks) or a cryostat (frozen tissue). Standard section thickness varies according to the final application, and both FFPE and frozen blocks are appropriate for thin and thick tissue slices. FFPE blocks, however, yield better results than frozen blocks on applications that require thinner (< 6 µm) sections. The investigator can experiment with different thicknesses for both applications and determine the optimal setting for a particular experimental need. For example, thinner sections are preferable when the investigator needs a high microscopic resolution and only a few cells in the visual plane. Studies examining 3D morphology or axonal tracks in brain tissue may benefit from thicker sections (> 6 µm). Even thicker sections (20–40 µm) are ideal for stereological quantification of cells and free-floating applications. When deciding on specimen thickness, it is important to remember that thicker sections may require longer incubations later in the workflow. In the case of thick frozen sections, investigators can also consider using a detergent in the blocking step of their protocol to improve tissue permeabilization.

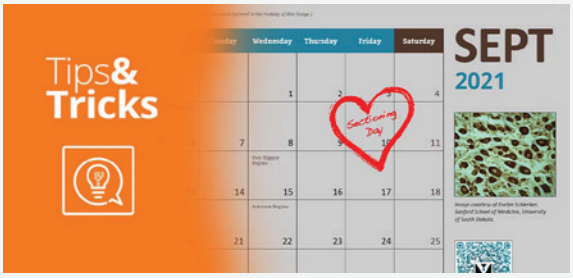
After sectioning, slides need to air dry for approximately 24 hours at room temperature or in an oven at 50–60 ˚C for 1 hour. It is essential to closely monitor tissue drying in the oven as extended slide drying at high temperatures may decrease immunogenicity (2). Some tissues can also benefit from being heated at 50 ˚C on a slide warmer for 30–60 minutes to avoid any possible detachment of specimen from the slide. Using chemical adhesive reagents, such as VECTABOND®, helps the tissue to stay attached to the slide throughout the workflow even when it involves extreme conditions (e.g., *in situ* hybridization, high-temperature antigen unmasking).

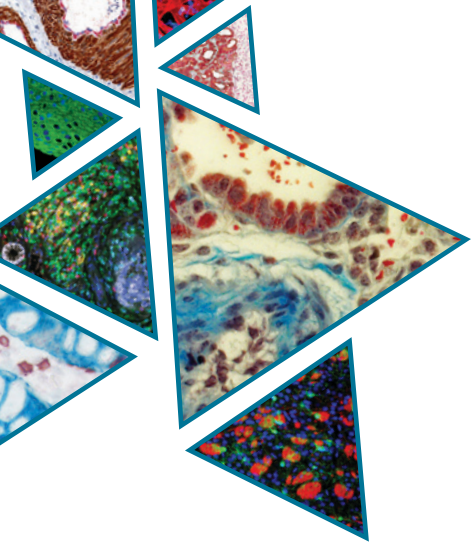
Tips and Tricks to Get the Most Out of Your Sectioning

Let's Cut to the Chase

No matter how you slice it, if your research includes immunohistochemistry or immunofluorescence, sectioning is a key part of your experimental workflow. If you aren't the type of researcher who draws a heart around "Sectioning Day" on your lab calendar, cut yourself a little slack and read on for some tips and tricks.

Read more →





Deparaffinization and Rehydration

FFPE specimens need to undergo deparaffinization and rehydration after sectioning (Figure 3). This process follows the exact reverse order of the dehydration step during paraffin impregnation. First, xylene removes paraffin, and then a series of graded dilutions of alcohol (100%, 80%, 70%, etc.) rehydrates the tissue (9). Incomplete paraffin removal leads to inconsistent results—uneven or "patchy" staining of the same antigen in the same specimen. Using fresh reagents is one measure that can be taken to avoid this issue. In addition, slides must remain in each solution for an appropriate length of time, which increases with section thickness. For example, complete deparaffinization of 4–5 µm thick sections usually requires a minimum of two changes of xylene for 5 minutes each. Options for thicker slices include extending wash time or increasing the number of washes.

Pro tip

Using a high-quality permanent marker to identify slides containing tissue sections is essential for success. Sequential immersion in alcohol and other solvents may clear the slide labeling and render the experiment useless.

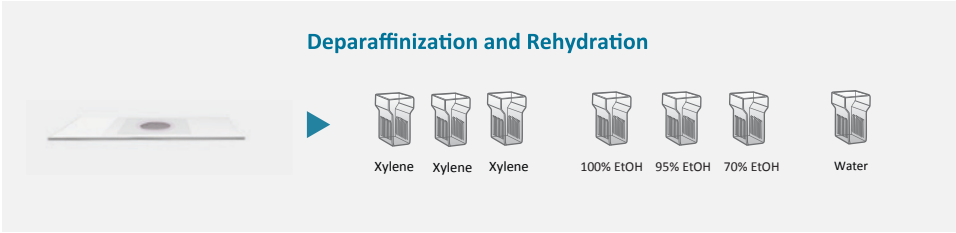
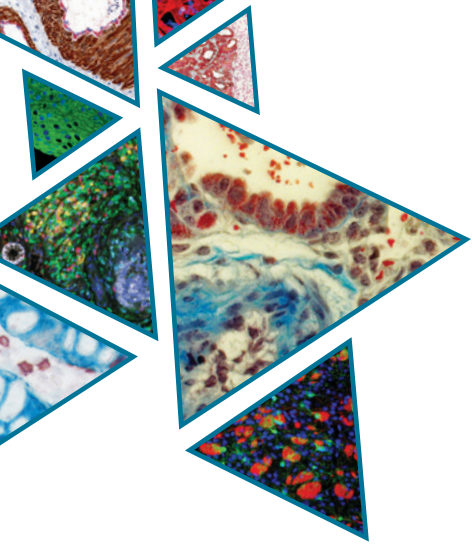


Figure 3. Overview of the deparaffinization and rehydration steps. The tissue is processed through a series of xylene washes to remove paraffin. Next, sections are immersed in a series of graded dilutions of alcohol and in a final wash in water to remove xylene and rehydrate the tissue.



Antigen Retrieval

Formalin fixation promotes the formation of cross-links between amino acids of proteins in the tissue. While this process is essential for hardening the tissue and preserving overall morphology, it can also mask the epitope and reduce antigenicity (Figure 4). The result is weak or absent staining. However, the addition of an antigen retrieval step into the IHC/IF workflow can help recover the masked epitopes for optimal staining. Heat-induced epitope retrieval (HIER), heating the tissue in a specific buffer to break the cross-links, offers advantages over the proteolytic approach, as it is easier to control and results in superior tissue preservation.

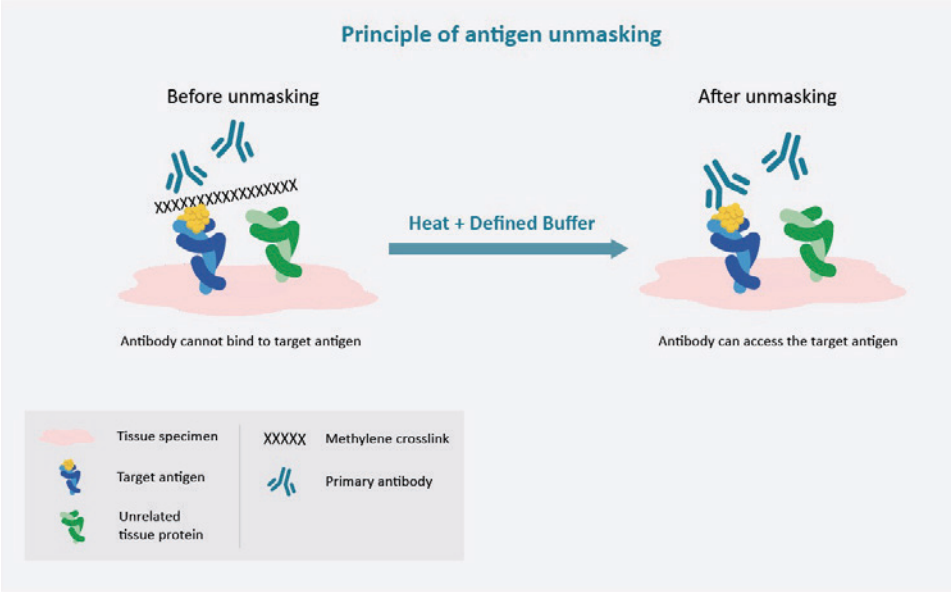


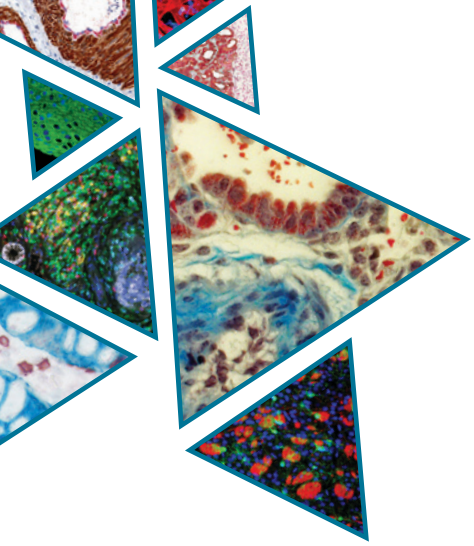
Figure 4. Heat-induced epitope retrieval process. During fixation, formalin-based fixatives promote the formation of methylene cross-links between protein residues. This process promotes tissue hardening and preservation, but also masks target antigens and prevents antibody binding. Heating the specimen in a defined buffer breaks the cross-link and exposes the epitope (13).

Pro tip

Avoiding over-fixation is essential to ensure effective antigen retrieval while preserving tissue integrity. Investigators should also take extra precautions to avoid tissue detachment during this step.

Various protocols suggesting different equipment, buffers, temperature, and incubation time are available. But, the investigator needs to determine these variables empirically for best results. Many laboratories already have at least one of the acceptable heat sources—microwave, pressure cooker, water bath, or autoclave—and each option has its own advantages. Autoclaves and pressure cookers are capable of heating the samples above 100 ˚C without heavy boiling (11, 12). Microwaves offer the same advantage along with temperature control (in the professional laboratory models), but require close monitoring and buffer replenishment as it evaporates (8). Water baths also offer temperature control and allow HIER at lower temperatures, which may be ideal for some applications (13). Regardless of equipment choice, the investigator needs to determine the ideal temperature (if the equipment allows) and incubation time. The general recommendation is to start low, increase as needed, and stop before the tissue shows signs of damage (10).

Temperature has a more significant impact on the effectiveness of the antigen retrieval than the buffer of choice, but buffer pH influences the process as well (10). Many options are available, but citrate- and Tris-based solutions are among the most popular choices. When choosing a retrieval buffer, the investigator should first check the antibody manufacturer's recommendation as it often contains specific information on which buffer to use. In the absence of guidelines from the manufacturer, the investigator must test the options available and determine the best pH to retrieve the target epitope.



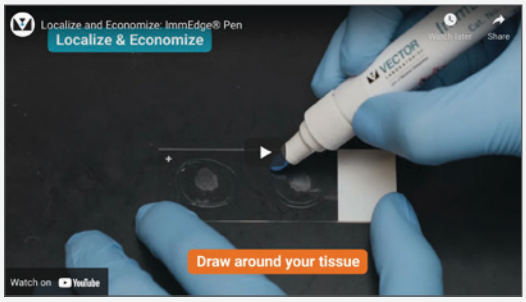
Hydrophobic Barrier Pen

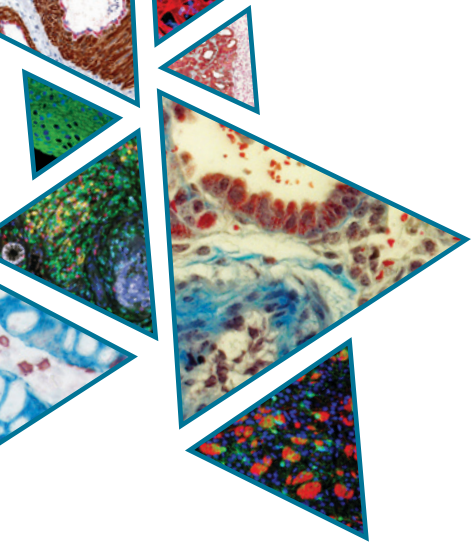
The last step in the sample preparation process is to use a Hydrophobic Barrier Pen to draw a water-repellent line around the section. This hydrophobic pen allows the creation of a quick-drying barrier to keep reagents localized in a particular area of the slide or separate two different reagents on the same slide to prevent them from mixing. PAP pens are simple and quick to use, but their most significant advantage is their cost-reduction potential. Investigators use considerably less reagent when covering a small area instead of the entire slide. Unlike traditional PAP pens, Vector Laboratories' ImmEDGE® PAP Pen solution is free of ozone-depleting hydrocarbons, making it a good choice both scientifically and environmentally.

Save precious time and resources

With a hydrophobic barrier pen, simply draw a line around your specimen to create a heat-stable, water-repellent barrier around your tissue that will keep reagents localized. See how it works in this quick video demonstration.

Watch Now →



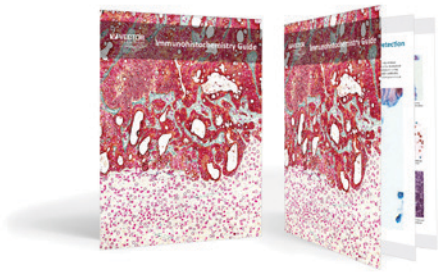


Conclusions

Prepping tissue for IHC/IF applications correctly is essential for successful staining and a frustration-free experience at the microscope. Experimental needs must inform decisions for each step of the process. Each part of the workflow contains steps that, done improperly, can negatively impact the experiment outcome. Considering all of the choices upfront, making informed decisions, and using high-quality reagents throughout the entire process are necessary factors for success. As always, reach out to the Vector Laboratories technical support team for additional help and guidance.

Essential Workflow Tools at Your Fingertips

When it comes to your staining workflow, clear and accurate results are essential. Optimize your IHC and IF experiments—explore our collection of guides. Get started quickly and move forward confidently.



Immunohistochemistry Resource Guide

The IHC Resource Guide will help you navigate the many choices of detection systems, secondary antibodies, substrates, and ancillary reagents that make up your workflow.

Download the guide ›

- Learn about IHC workflows and detection reagent selection
- Considerations for choosing enzyme substrates and species on species detection
- Troubleshooting background signal

Immunohistochemistry Multiplexing Resource Guide

The IHC Multiplexing Guide will guide you through all of the steps and considerations for creating stunning multicolor IHC images.

Download the guide ›

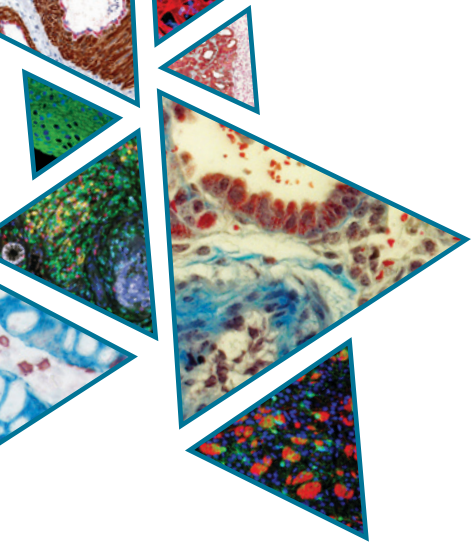
- Learn about reagent selection considerations for multiple antigen labeling
- Explore single, double, and triple staining protocols
- Useful FAQs to help prevent costly mistakes

Immunofluorescence Resource Guide

The IF Guide can help you in choosing the appropriate reagents that will enable you to produce IF results with high specificity and sensitivity.

Download the guide ›

- Learn about IF workflows and optimization
- Considerations for choosing the most appropriate fluorescent secondary conjugates
- Discover how and why quenching unwanted autofluorescence is important
- Guidance in choosing a suitable antifade mounting medium to preserve your images



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Product Selection Guide

Product	Cat. No.
VECTABOND® Reagent, Tissue Section Adhesion	SP-1800-7
Antigen Unmasking Solution, Citrate-Based	H-3300-250
Antigen Unmasking Solution, Tris-Based	H-3301-250
ImmPrint™ Permanent Marking Pen	H-6100
ImmEdge® Hydrophobic Barrier PAP Pen	H-4000