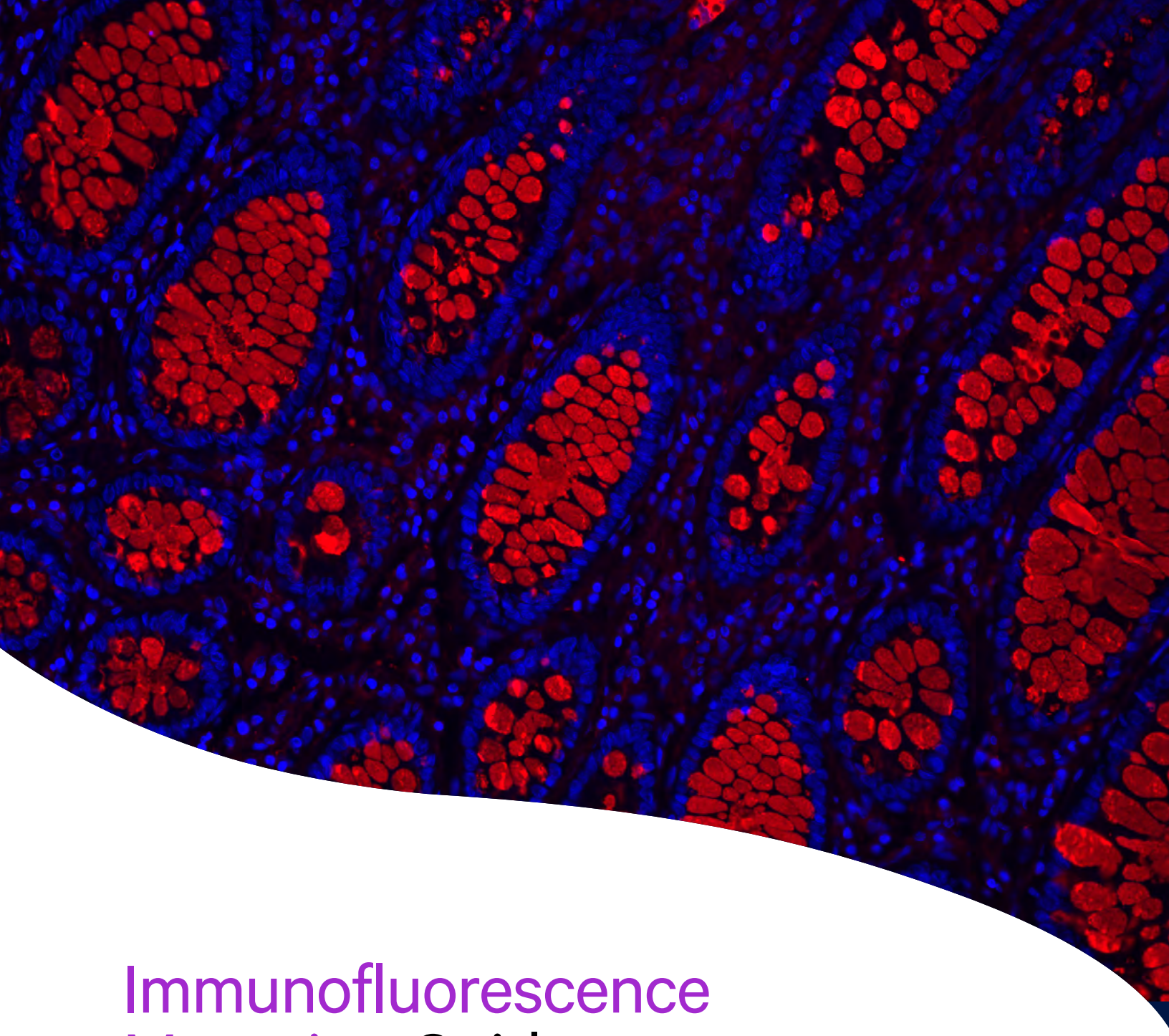

Immunofluorescence Mounting Guide

Tips & Tricks to Improve Your Immunofluorescence Staining

Available in Canada from...

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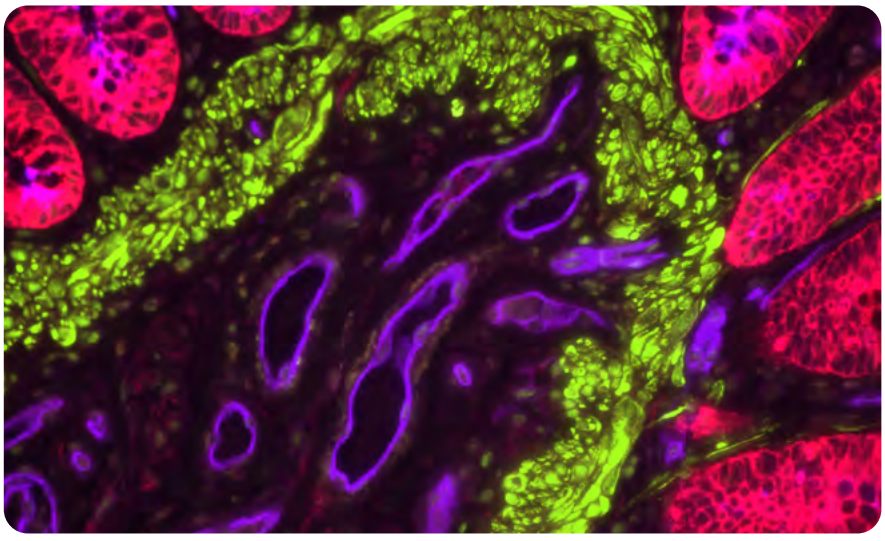
FFPE human colon carcinoma stained with Glysite™ Scout Glycan Screening Kit, Immunofluorescence 594, showing B-Jacalin staining (red). Nuclear detail was visualized with VECTASHIELD Vibrance® Antifade Mounting Medium with DAPI (blue). Tissue was treated with Vector® TrueVIEW® Autofluorescence Quenching Kit .

Introduction

Immunofluorescence (IF) staining combines versatility and high sensitivity to visualize cells and intracellular structures. It has the side benefit of yielding beautiful, vibrant, and colorful images that often make it to the cover of scientific journals, but neglecting care during the mounting step can make the fluorescent signal short-lived and turn specimen imaging into an unsuccessful endeavor.

Mounting is the last step in the IF workflow, but its importance should not be understated. Selecting the ideal mounting medium is critical for tissue integrity, optical clarity, long-term storage, signal intensity, and fluorescence preservation. The latter is a crucial factor as fluorophores lose their ability to emit fluorescence after exposure to light, a phenomenon known as photobleaching. Using the right mounting medium ensures that the stained tissue retains this capability after the first (or multiple) visualizations under the fluorescence microscope. As with every step in the immunostaining workflow, mounting medium selection requires knowledge of the different options and consideration of the unique experimental needs.

This IF mounting guide provides information to help researchers choose the best mounting medium for their project and offers tips to help reduce photobleaching during the image visualization process.



Human Colon: Rabbit Anti-Cytokeratin (AE1/AE3) and Mouse Anti-Desmin detected simultaneously with VectaFluor™ Duet Double Labeling Kit; Vasculature detected using DyLight™ 649 UEA I Lectin (purple). Mounted in VECTASHIELD® PLUS Antifade Mounting Medium.

Mechanisms of Fluorescence and Photobleaching

Fluorophore molecules are capable of absorbing light, which leads to the transition of electrons in the fluorophore from the ground to the excited state (1). Each fluorophore responds most efficiently to a specific wavelength, but excitation can also occur within a range of wavelengths known as the excitation spectrum (1). When excited electrons in the fluorophore return to their original state, they emit fluorescent light at a wavelength longer than the excitation wavelength (1). Emission occurs at maximum intensity at a specific wavelength, but similar to excitation, each fluorophore has an emission spectrum covering a range of wavelengths (1).

Photobleaching happens when a fluorophore permanently loses its ability to emit a fluorescent signal and occurs as a byproduct of the mechanisms underlying fluorescence emission (1,2). Each fluorophore can undergo a different number of excitation and emission cycles until they permanently stay in an excited state and lose the ability to emit fluorescence (1,2). Different factors such as fluorophore structure, light intensity, and time exposed to light during visualization can influence the number of excitation and emission cycles for each molecule (2). As photobleaching happens, it becomes increasingly difficult to image an IF experiment. This progressive reduction in signal intensity also increases the possibility of false-negative results and reduces the feasibility of experiments targeting quantitative analyses. Taking precautionary measures when mounting and visualizing IF experiments is essential to protect the fluorescent signal from photobleaching.

Protection from photobleaching

Different strategies can help protect the immunofluorescence signal from photobleaching. This guide organizes these strategies into 4 categories—light intensity, image exposure, fluorophore selection, and mounting media selection (Figure 1).

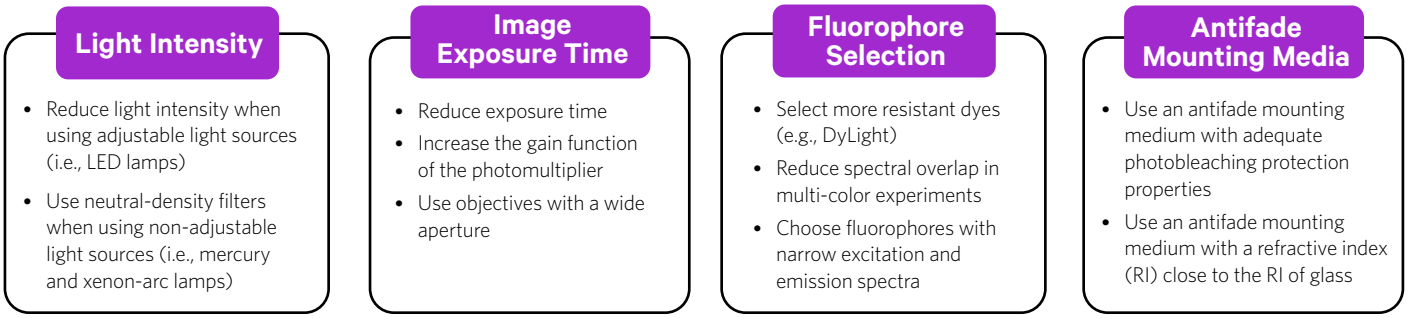


Figure 1 *Strategies to prevent and reduce photobleaching in IF experiments.*

Pro Tip

Specific experiments, such as z-stack imaging, can also contribute to photobleaching. It requires repeated excitation of the same region of interest even if individual planes were minimally exposed.

Light Intensity

The higher the intensity of the light used during specimen visualization, the quicker electrons go through multiple cycles of excitation and emission, directly contributing to photobleaching. Light intensity refers to the power of the light source, or in simple words, its brightness. Thus, one of the most straightforward strategies to reduce photobleaching and extend the life of the fluorophore is to reduce the intensity of the light during imaging (3). Traditionally, fluorescence microscopes have used mercury and xenon-arc lamps as the source of light to excite fluorophore molecules. Light intensity when using these lamps cannot be manually adjusted, but an easy solution is to use neutral-density filters. These accessories reduce light intensity by a given percentage that varies from filter to filter. For example, a neutral-density filter with 80% transmittance allows only 80% of the light to reach the sample.

Alternatively, fluorescence microscopes can use manually adjustable LED lamps as the light source. With either the addition of filters or manual light intensity reduction, fewer protons reach the sample, resulting in less excitation and a dimmer image. The researcher must empirically determine the adequate balance between photobleaching protection and proper signal strength. Reducing the light intensity might not be worth it if longer exposure is required to acquire an image with an appropriate fluorescent signal.

Image Exposure Time

Exposure is the time the camera shutter needs to remain open to properly image the specimen. Longer exposure yields brighter images but also increases the amount of light reaching the sample and accelerates photobleaching. Shorter exposures can result in dim images. Here again, the researcher needs to empirically determine the ideal balance between photobleaching protection and the risk of false-negative results.

When reduced exposure results in a dim, unsuitable image, increasing the gain function of the photomultiplier can help increase the overall signal intensity. Gain measures the amplification of the signal reaching the camera sensor. Higher gain yields a brighter image while keeping the light intensity and exposure constant. This approach increases camera sensitivity to light in a non-selective way, which means that background noise will also become brighter. In addition, higher gain can also contribute to reduced image resolution. Therefore, the perfect balance between light intensity, exposure time, and gain should be empirically determined, preferably using a test section while keeping tissue type, target protein, and fluorescent dye constant.

Using an objective with a wider aperture is one final approach to increase the amount of light reaching the sample. Objectives with a high numerical aperture allow more light to reach the sample, and as a result, the researcher can reduce light intensity, exposure, and gain and still acquire an image with adequate signal intensity. Wider apertures also result in a smaller depth of field, which can be beneficial or detrimental, depending on the objectives of the experiment.

Pro Tip

Use transmitted light to find your region of interest and focus the sample. Then switch to the fluorescent light and desired excitation filter to acquire the image. This approach reduces overall exposure to fluorescent light and can help minimize photobleaching.

In simple terms, the depth of field is the thickness of the plane in the specimen that can be in focus at the same time—a small depth of field results in sharp images of thin optical sections, and a large depth of field allows a bigger portion of 3D structures to be in focus. Despite these added benefits, adjusting the objective aperture is the least practical approach to preventing photobleaching. Researchers commonly use institutional imaging facilities that offer microscopes with a pre-defined set of objectives selected to suit the needs of a large group of people.

Fluorophore Selection

Different fluorophores have varied susceptibility to photobleaching. Newer generations of fluorophores, such as AlexaFluor® and DyLight, have more stable structures and are less susceptible to photobleaching than traditional fluorophores such as FITC, TRITC, and Texas Red™ (4). Changing the type of fluorophore is a straightforward strategy to reduce photobleaching during image visualization. When performing multi-color staining, the researcher should also select fluorophores with minimal spectral overlap. Otherwise, a single channel can excite multiple fluorophores with overlapping excitation spectra, leading to bleed-through and photobleaching (1,2). Manufacturers often report the excitation and emission spectra for each fluorophore, and this information should be carefully considered when designing an experiment. Choosing fluorophores with narrow excitation and emission spectra can also help reduce overlap. Many free online tools are available to help with fluorophore selection for multi-color staining. Fluorophores also vary in their sensitivity to light—they require different intensities of light to yield a fluorescent signal with similar strength. Thus, when considering all the factors influencing the choice of fluorophores, the best option combines good sensitivity and less photobleaching.

Antifade Mounting Media

Fading is another word commonly used to describe photobleaching. Thus, mounting media that confer protection against photobleaching are known as antifade mounting media. Adding these reagents during the mounting process is the most effective way to prevent photobleaching. Antifade mounting media prevent the reaction of excited electrons with other molecules and thus extend the number of excitation/emission cycles they can undergo (5). In addition, mounting media maximize fluorophore signal strength, requiring lower light intensity and exposure for optimal specimen visualization (5). Other benefits of mounting media include preservation of tissue morphology and improved image quality (6). As many different options for mounting media are available, researchers should be aware of the many factors influencing the final choice and their impacts on the final results.

Mounting Media Selection

Antifade mounting media are water-based reagents that have a pH suitable for sample preservation and allow mounting the specimen directly from an aqueous buffer. They are easy to apply but have a major drawback—a refractive index (RI) that does not favor high-quality images. Light travels in different media at varying speeds, and the RI for each medium captures those differences. Water has an RI of 1.33, while glass (slides and coverslips) and oil-immersion liquid have an RI of 1.51. Significant differences in RI reduce image quality and contribute to spherical aberration, chromatic aberration, reduced resolution, and reduced depth of field (7,8). Adding glycerol to water-based mounting media can help raise their RI and thus improve image quality. Higher RIs also improve axial resolution and allow better imaging of thicker sections. Although RIs for currently available mounting media do not match that of glass and oil, most products have an adequate RI for image visualization. Matching RIs for all media as close as possible is particularly important for super resolution microscopy (9).

The End of the Line: Considerations for Mounting Media Selection

Learn more ▶

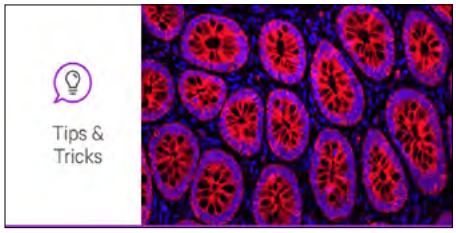
Choosing the right mounting medium for your tissue can make or break a successful tissue preservation process. From understanding the importance of refractive index to technique tips, we have insights to help you choose the right mounting medium for your applications.

Water-based mounting media come in 2 forms—hardening and non-hardening (setting and non-setting) formulations. As the name implies, in hardening formulations, the liquid cures into a hard film and does not require sealing the coverslip. In non-hardening formulations, the mounting medium remains in the liquid form. Sealing often makes handling and visualization easier, but is optional with certain non-hardening formulations. Both options have relatively similar RIs, equal photobleaching protection performance, and are compatible with frozen and formalin-fixed paraffin-embedded sections, but other factors influence the choice between them. Experiments with thick (>30 µm), whole-mount, and floating sections require non-hardening formulations as hardening media retract and might affect tissue morphology. Likewise, non-hardening formulations work best for 3D imaging, as those experiments often require thicker sections. The main advantages of hardening formulations include a slightly higher RI after curing and storage flexibility. Mounted slides are stable for extended periods without loss of signal intensity and can be stored at room temperature or under refrigeration. Curing time is another variable that might affect the choice of mounting media.

Setting media vs. non-setting mounting media: Which is right for you?

Learn more ▶

Let us help you decide whether setting or non-setting mounting media will work best for your workflow in this Tips & Tricks article.



Some products allow visualization in 1 hour after mounting while others might take up to 48 hours to fully cure. Options with a long curing time may have a higher RI upon complete curing, but that comes with the cost of delaying data acquisition. Visualizing the specimen before the recommended curing time adds the risk of displacing the coverslip, damaging the tissue, and reducing anti-fade performance, thus acquiring an image with less-than-ideal quality.

Another factor to consider when choosing a mounting media is the availability of counterstain. Most commercially available mounting media offer the choice of DAPI (4′,6-diamidino-2-phenylindole) nuclear counterstain. Using a mounting media with DAPI is applicable even when not imaging the nucleus. Having stained nuclei allows using the DAPI channel to locate the region of interest and adjust the focus before switching to the proper channel and acquiring the image. This approach helps reduce total exposure and minimizes photobleaching.

All the features listed above can add convenience when performing an IF experiment. Still, the primary purpose of antifade mounting media is to preserve the intensity of the fluorescent signal during imaging and over time upon storage. High-performance mounting media should address both needs—signal retention during both visualization and storage. In addition, mounting media should preserve the optical characteristics of the stained section. Product datasheets might contain information pertaining to mounting media performance, but each researcher should empirically determine which formulation best suits their needs.

Putting it all Together

Many formulations of antifade mounting media are commercially available, and selecting the ideal one for a particular experiment can be confusing. They all have advantages and disadvantages, and the right choice depends on the unique needs of each application. Thinking through the specific requirements of an experiment and answering a few questions can help identify and select the ideal mounting media. Use the questions outlined in Figure 2 as a guide when planning a new IF experiment.

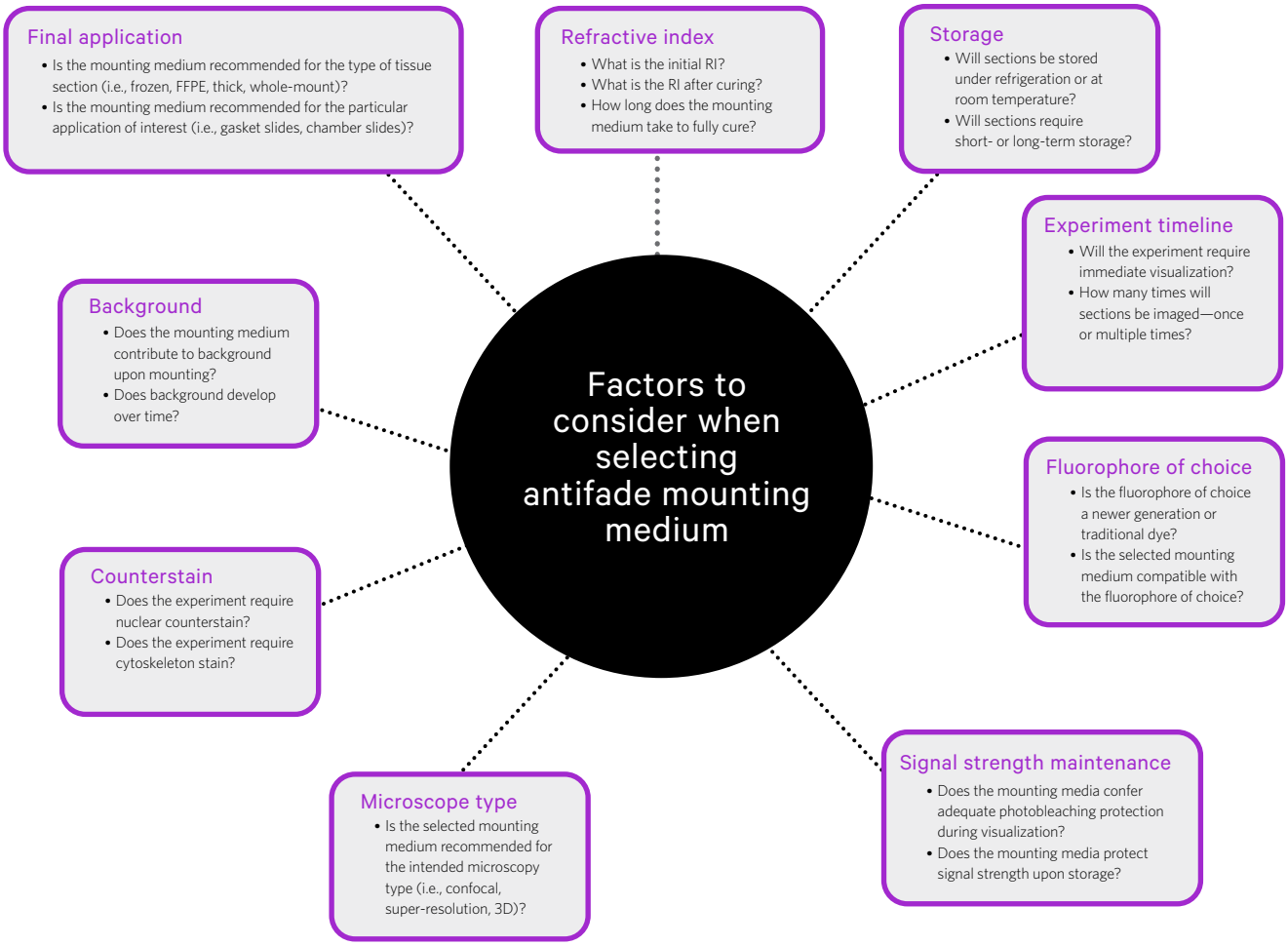
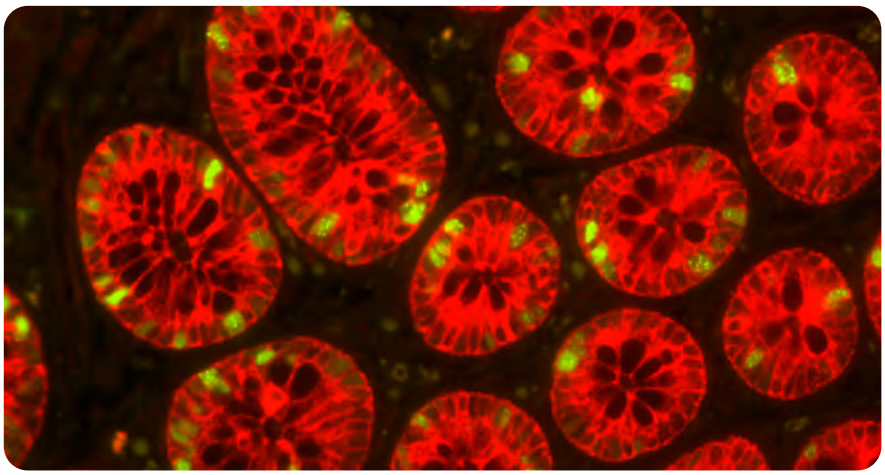


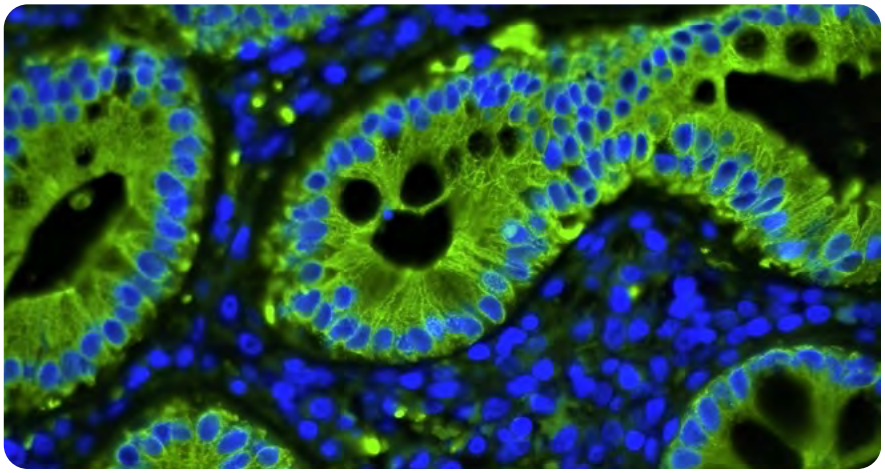
Figure 2 *Overview of the factors affecting the choice of antifade mounting medium.*

Vector Laboratories offers high-quality options for both hardening and non-hardening mounting media. VECTASHIELD PLUS is a ready-to-use, non-hardening formulation that offers superior photobleaching protection of traditional and new generation fluorophores across the spectrum from blue to far-red. Slides can be viewed immediately after mounting; however, optimal antifade performance is achieved after 1 hour. After curing, slides should be stored at 2–8°C. VECTASHIELD PLUS has an RI of 1.45 after curing and is available with and without DAPI counterstain.



Human Colon: Rabbit Anti-Cytokeratin (AE1/AE3) and Mouse Anti-Ki67 detected simultaneously with VectaFluor Duet Double Labeling Kit; DyLight 488 Anti-Mouse (green)/DyLight 594 Anti-Rabbit (red). Mounted in VECTASHIELD PLUS.

VECTASHIELD Vibrance is a ready-to-use hardening formulation which also offers superior photobleaching protection of traditional and new generation fluorophores across the spectrum, including far-red channels. In addition, VECTASHIELD Vibrance has very low background after mounting as well as after extended storage and cures to an ideal RI of 1.47. Formulations with and without DAPI counterstain are available. Slides can be viewed 30 minutes after mounting, however optimal antifade performance is achieved after 2 hours. For long-term storage, slides can be stored at room temperature or at 2–8°C. Table 1 summarizes the main characteristics and recommended applications for VECTASHIELD PLUS and VECTASHIELD Vibrance.



Human colon section (FFPE) stained for cytokeratin (green) using fluorescein anti-mouse IgG secondary antibody. Mounted in VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (blue).

Non-Hardening vs. Hardening Formulations

	VECTASHIELD PLUS	VECTASHIELD Vibrance
Compatible with most Alexa Fluor, DyLight, and CY dyes in the visible spectrum	X	X
Compatible with traditional dyes and fluorescent proteins	X	X
Refractive index after curing	1.45	1.47
Non-hardening vs. hardening	Non-hardening	Hardening
Mounted sections viewable after 1 hour	X	X
Preferred for DAPI/AMCA channels	X	X
Storage conditions	2–8°C	Room Temp or 2–8°C
Available with DAPI counterstain	X	X
Lectin imaging		X
Fixed frozen sections	X	X
Formalin-fixed paraffin sections	X	X
Whole-mount preps	X	
Gasket slides	X	
Chamber slides	X	
Microtiter plates/wells	X	
Thick sections	X	
Floating sections	X	
3D imaging	X	
Epifluorescence microscopy	X	X
Confocal microscopy	X	X
Super-resolution microscopy	X	

Other Factors to Consider with Immunofluorescence

Autofluorescence is a common cause of background in IF experiments and occurs when molecules other than the antigen-bound antibody complex emit fluorescence. Common natural sources of autofluorescence include the heme groups of red blood cells, collagen, elastin, NADH, and lipofuscin. Autofluorescence from these sources makes the interpretation of IF results challenging, particularly when using the green and red channels (10–12). The fixation process can also contribute to the autofluorescence problem as formalin-induced cross-links can emit fluorescence in a broad range of wavelengths (10).

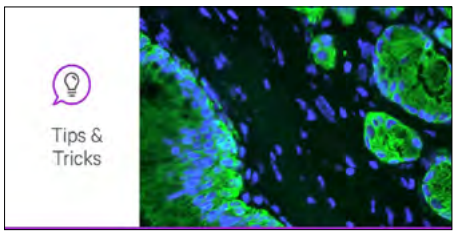
The standard solution is to use dyes, such as Sudan black, trypan blue, pontamine sky blue, crystal violet, and DAB to quench unwanted fluorescence through absorption of incident radiation (13–15). However, not all of these dyes will reduce fluorescence from all sources. For example, Sudan black reduces lipofuscin-related autofluorescence but is ineffective in quenching signals from formalin-induced cross-links, collagen, and red blood cells. A combination of different dyes might help to quench autofluorescence from lipofuscin and other sources completely (16). In addition to using fluorescence-quenching dyes, adding 1–10 mm copper sulfate in 50 mm ammonium acetate buffer to the final wash helps to reduce lipofuscin-related autofluorescence (17). One final note of caution is that these dyes will reduce both the non-specific and targeted fluorescence (16). In addition to using quenching dyes, modifying some steps in the IF workflow can help minimize autofluorescence: using a different fixative when working with blood-rich specimens; switching from formalin-fixed paraffin-embedded preservation of tissues to cryopreservation; and/or adding Tween® 20 to the wash buffer.

Vector Laboratories offers Vector TrueVIEW Autofluorescence Quenching Kits, an innovative solution to autofluorescence. Unlike the dyes previously mentioned, this quencher is a hydrophilic molecule that effectively reduces autofluorescence from non-lipofuscin sources: aldehyde fixation, red blood cells, collagen, and elastin. In addition, TrueVIEW selectively reduces unwanted fluorescence and enhances signal-to-noise, resulting in clear images with a “true view” of the target antigen.

How to improve your immunofluorescence by overcoming autofluorescence

Learn more ▶

Interfering background signal such as autofluorescence can hamper your immunofluorescence results. Check out our blog post for tips and tricks from Dr. Craig Pow to improve your IF staining.



Conclusion

Careful selection of a mounting medium is essential for the success of IF experiments, and Vector Laboratories offers state-of-the-art reagents that consistently deliver high-quality results. We also offer many educational resources to empower researchers across all levels of expertise to design, execute, and troubleshoot experiments. Vector Laboratories' team of experts is always available to provide technical support and offer additional guidance.

Product Selection Guide

Product	Counterstain	Unit Size	Cat. No.
VECTASHIELD® PLUS Antifade Mounting Medium	None	2 ml, 10 ml	H-1900
	DAPI	2 ml, 10 ml	H-2000
VECTASHIELD Vibrance® Antifade Mounting Medium	None	2 ml, 10 ml	H-1700
	DAPI	2 ml, 10 ml	H-1800
VECTASHIELD® Antifade Mounting Medium	None	10 ml	H-1000
	DAPI	10 ml	H-1200
	PI	10 ml	H-1300
VECTASHIELD® HardSet™ Antifade Mounting Medium	None	10 ml	H-1400
	DAPI	10 ml	H-1500
	TRITC-Phalloidin	10 ml	H-1600
Vector® TrueVIEW® Autofluorescence Quenching Kit	None	Kit	SP-8400
	DAPI	Kit	SP-8500

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