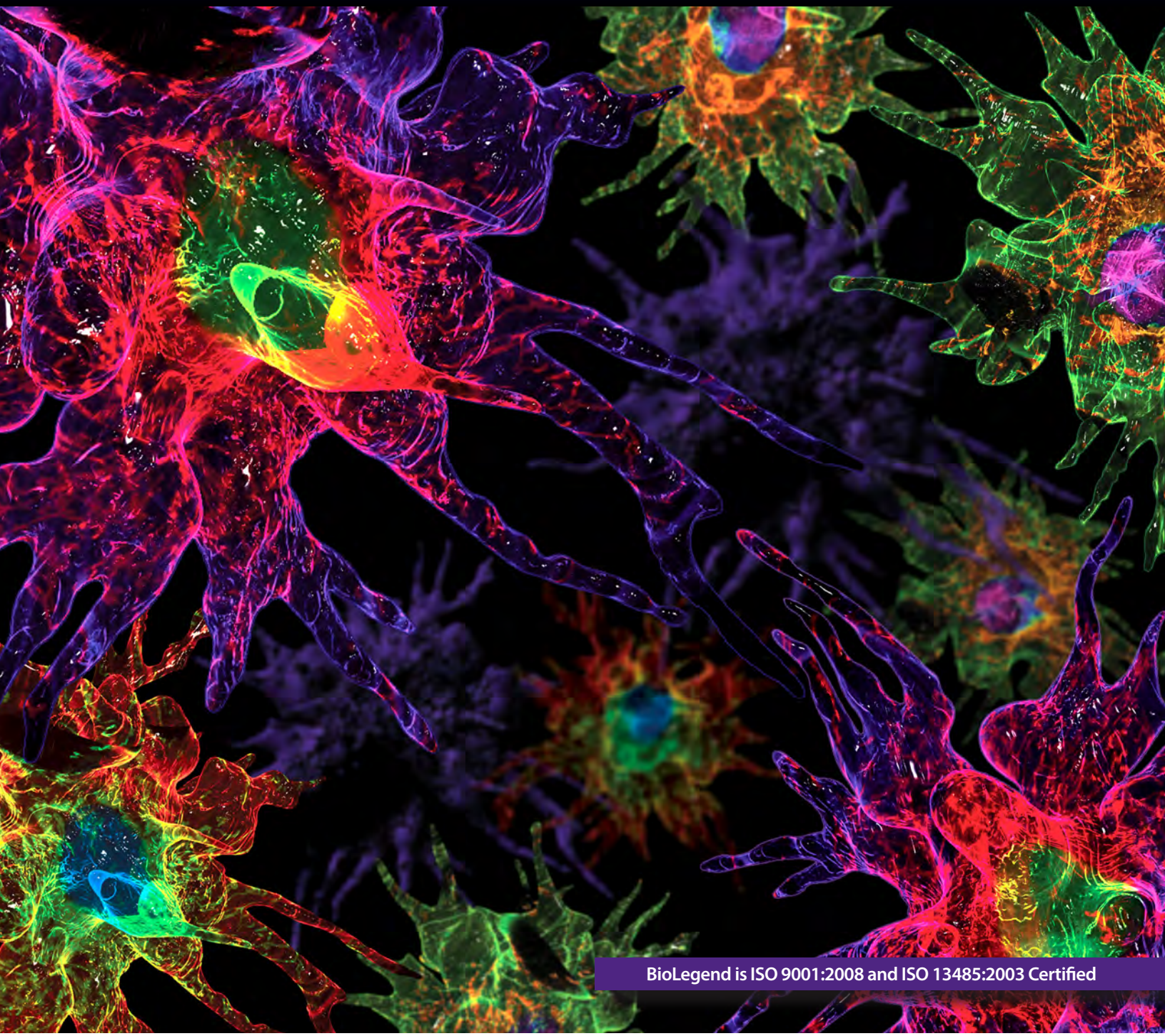
Microscopy Reagents

for Immunocytochemistry and Immunohistochemistry



BioLegend is ISO 9001:2008 and ISO 13485:2003 Certified

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02-0011-00

World-Class Quality | Superior Customer Support | Outstanding Value

Immunohistochemistry and Immunofluorescence:

What's in a Name?

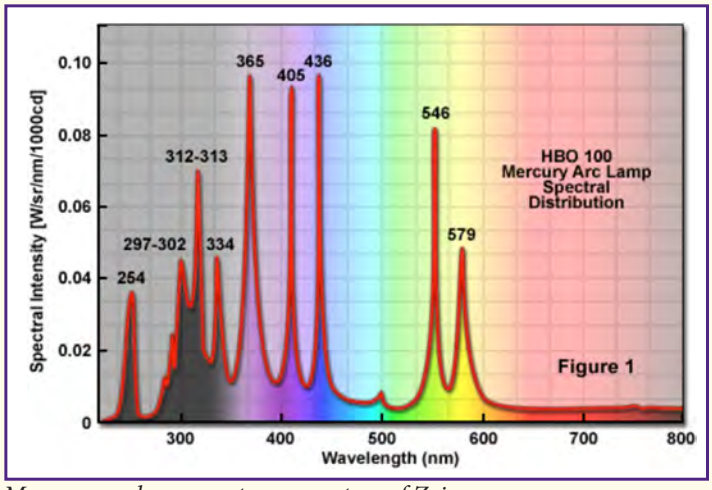
Imaging platforms enable understanding of subcellular localization, function, activity and health in a single cell culture or a network of cells comprising a tissue. For each biological question that could benefit from quantitative, structural, spatially relevant information, there is a sophisticated microscopy platform that is best designed for the application at hand. Based on the biological application and the microscope of choice, there is an array of both chemical and antibody-based reagents to enable visualization. The biological application, the instrument and the reagents must all be well matched for ultimate success.

Immunocytochemistry (ICC) is often called immunofluorescence (IF) and is characterized by imaging primary cells or cell lines in culture. Immunohistochemistry (IHC) is simply the detection of antibodies in tissue sections, whether it be by chromogenic or fluorescent realization methods. IHC-P indicates the antibody is useful in formalin-fixed paraffin-embedded (FFPE) sections and IHC-F indicates the antibody is only useful in tissue that has been fixed and frozen prior to sectioning. If there is only an IHC designation, check with the literature citations or additional information provided by the manufacturer to determine the method of tissue preparation compatible with each reagent.

Widefield vs. Confocal Microscopy

Widefield microscopy is the most common and accessible imaging platform. Historically, widefield microscopes relied on a mercury arc lamp as the primary excitation source and excitation and emission filters to choose specific wavelengths of light to be matched to the reagents combination. It is becoming increasingly common to now also have white light excitation sources that can more efficiently cover the full spectrum rather than the principle lines of excitation characteristic of a mercury arc lamp. There are many modifications that can be made to widefield scope that may increase sensitivity or resolution, or enable a particular advanced imaging modality. Widefield scopes rely on a diverse combination of excitation, emission filters and dichroic mirrors. You should optimize these for the reagents desired.

In contrast, confocal microscopy does not employ barrier filters. Confocality is a technique to focus a laser beam on a particular point of a focal plane aimed at reducing background excitation and scattered light while increasing optical resolution. Lasers are focused light at a particular wavelength and thus reagents are chosen based on this. The width of the emitted light allowed to hit the PMT (photomultiplier tube) can often be "tuned" digitally, based on what is ideal for that particular spectral fingerprint. The use of lasers for illumination is both a strength and weakness, since the strength of the laser output can photobleach

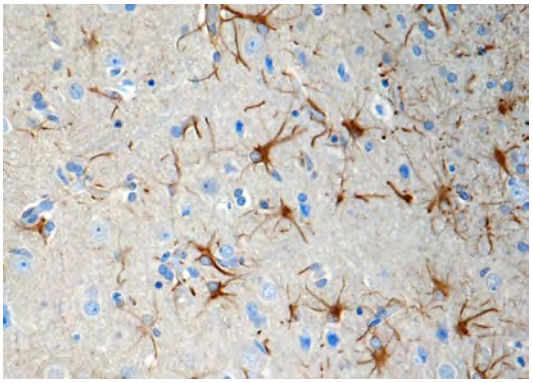


Mercury arc lamp spectrum courtesy of Zeiss.

a fluorescent molecule quite quickly. Employing antifade is imperative to retaining signal for practical imaging applications. However, the strength of confocal is the ability to focus that small beam of light to only a small area within the x, y, and z axes of the sample, thus also allowing for 3-D reconstruction of a tissue sample.

Chromogenic vs. Fluorescent Imaging Methods

Chromogenic detection methods are advantageous because a signal can be amplified simply by extending the amount of time and substrate in the reaction. Also, it does not require sophisticated instruments for detection, only a microscope with phase contrast. HRP detection can, however, be accompanied by endogenous background associated with cellular peroxidase activity, non-specific signal and is only typically used to image a single marker at a time.

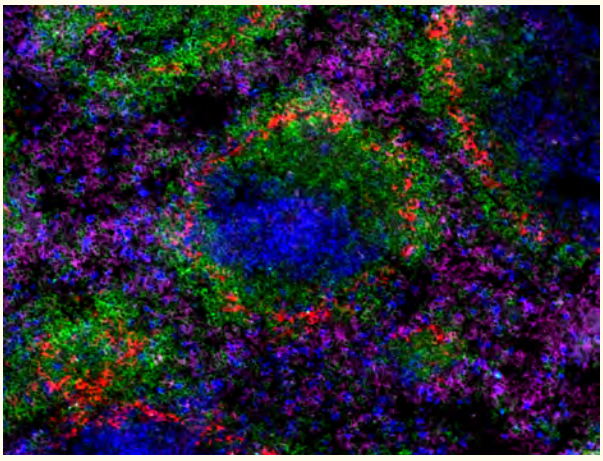


Staining of CX3CR1 (8E10.D9) on formalin fixed paraffin embedded human brain.

Fluorescent detection, on the other hand, allows visualization of multiple markers at a time, albeit most commonly through the use of discrete excitation sources optimal for each fluorophore. Fluorescent detection introduces the opportunity for advanced imaging applications as well, like live-cell imaging, multiphoton imaging, super-resolution microscopy, FLIM and FRET, just to name a few. Each of these techniques has their own additional advantages over standard widefield microscopy platforms. Sensitivity can be a limitation of fluorescence microscopy at certain wavelengths, especially reagents that emit in the range of 350-450nm. However, the intensity of emission can be modulated through varying enzymatic and immunologic amplification techniques, the use of higher sensitivity instrumentation, and near-infrared emitting fluorophores that can be used to escape the range most affected by autofluorescence.

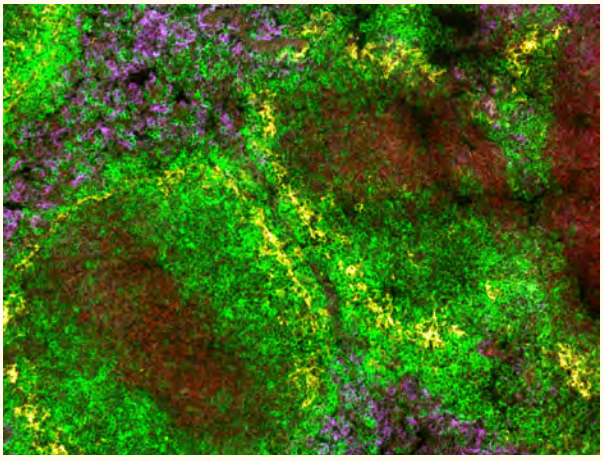
Multicolor Microscopy

Olympus IX83 (widefield)



Mouse spleen section stained with anti-B220 BV510™ (green), anti-CD3 BV421™ (blue), anti-F4/80 Alexa Fluor® 488 (purple), and anti-CD169 Alexa Fluor® 647 (red) antibodies. Image obtained with an Olympus IX73 inverted microscope and optimized filter sets.

Zeiss LSM 780 (confocal)



Mouse spleen section stained with anti-B220 BV510™ (green), anti-CD3 BV421™ (red), anti-F4/80 Alexa Fluor® 488 (purple), and anti-CD169 Alexa Fluor® 647 (yellow) antibodies. Imaged obtained with a Zeiss LSM 780 confocal microscope with spectral unmixing applied.

Microscopy Reagents

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BioLegend is proud to provide a variety of reagents supporting microscopy-based imaging of cells and tissue samples. Our reagents include antibody conjugates to bright, photostable fluors like Brilliant Violet 421™ and Alexa Fluors®. We also provide cell tracking dyes and nuclear counterstains. BioLegend will continue to release new microscopy products as we discover innovative technologies.

Learn more: **biolegend.com/microscopy**

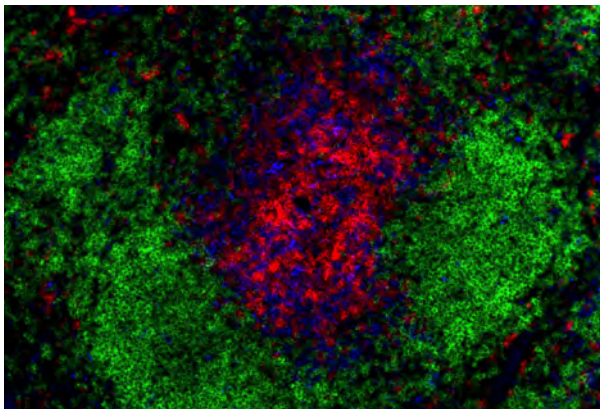
Brilliant Violet 421™

Brilliant Violet™ 421 (BV421™) allows expanded options in multicolor microscopy. BV421™ is used in the "blue" channel which is typically occupied by DAPI or Alexa Fluor® 405. Filter choice based on the wavelength of the excitation, emission and dichroic filters is particularly important. If your filter setup is suitable, BV421™ can provide you with a bright, photostable option for multicolor microscopy.

Learn more about Brilliant Violet™, filter selection, and microscopy: **biolegend.com/brilliantviolet**

Vendors and Catalog Numbers for BV421™ Widefield Filter Sets

Chroma	Cat. No. 49027
Omega	Cat No. XF403
Semrock	Cat. No. BV421-3824A-000

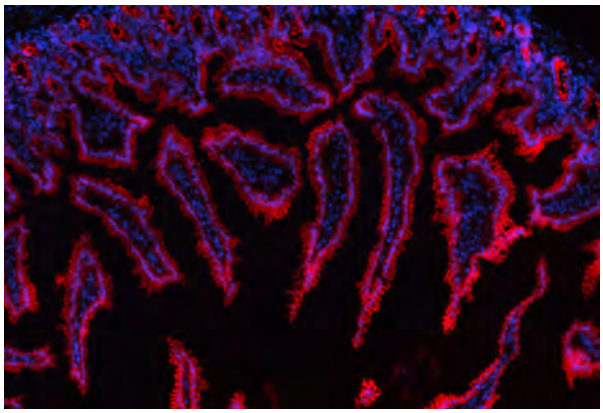


Mouse spleen, frozen, fixed and labeled with anti-B220 (clone RA3-6B2) BV421™ (green), anti-CD4 (clone GK1.5) Alexa Fluor® 488 (red), and anti-CD8a (clone 53-6.7) Alexa Fluor ®647 (blue).

Alexa Fluor® 488, Alexa Fluor® 594 and Alexa Fluor® 647

Alexa Fluor® 488, Alexa Fluor® 594 and Alexa Fluor® 647 are popular fluors that provide a strong signal and are photostable for imaging. We offer 280 Alexa Fluor® 488, 90 Alexa Fluor® 594, and 400 Alexa Fluor® 647 directly conjugated antibodies, and these numbers will grow as we find additional appropriate targets.

Learn about Alexa Fluor® 594: **biolegend.com/AF594**

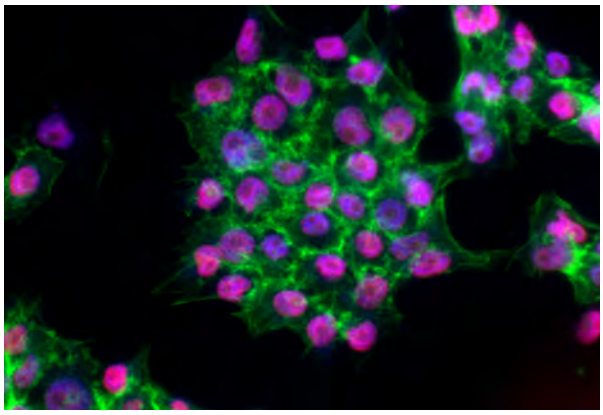


C57BL/6 mouse frozen intestine section was fixed, blocked, then stained with anti-mouse CD324 (clone DECMA-1) Alexa Fluor® 594 (red) antibody. Nuclei were counterstained with DAPI (blue).

Nucleic Acid Stains

Permeant and impermeant nucleic acid stains are an excellent tool for visualizing the location and number of cells in a sample and providing spatial context for the antigens of interest.

Description	Size	Cat. No.
DRAQ5™	50 µl	424101
DRAQ7™	1 mL	424001
DAPI	10 mg	422801
Propidium Iodide	2 mL	421301



NCCIT cells fixed and stained with DRAQ7™ (red), anti-SOX2 (clone 14A6A34) Alexa Fluor® 594 (blue) antibody and Alexa Fluor® 488 Phalloidin (green).

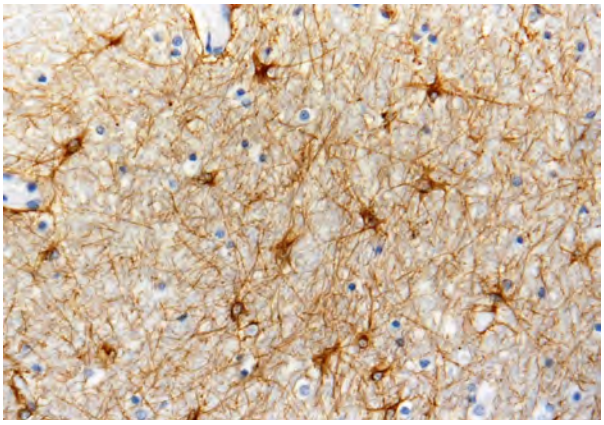
Reagents for Chromogenic IHC

Chromogenic detection methods have a long history in histology and pathology applications. There are chromogenic stains like H&E (hematoxylin and eosin), where the basophilic hemalum stains nuclei blue and the acidophilic eosin stains primarily the cytoplasm of cells or red blood cells in the tissue varying degrees of pink to red. Also commonly used in chromogenic IHC (immunohistochemistry) are antibodies or streptavidin covalently attached with HRP or AlkPhos, that convert a substrate like DAB or BCIP/NBT, respectively. These enzymes catalyze their substrates, leaving a deposit of color where the antibody has attached to the cell or tissue.

BioLegend offers reagents for HRP detection including:

- **ACUITYAdvanced Biotin Free Polymer Detection kits**
- **Ultra-Streptavidin (USA) HRP Detection Kits**
- **Retrieve-All Antigen Unmasking System**

A full list of our IHC detection reagents can be seen at:
biolegend.com/ihc_detection_reagents

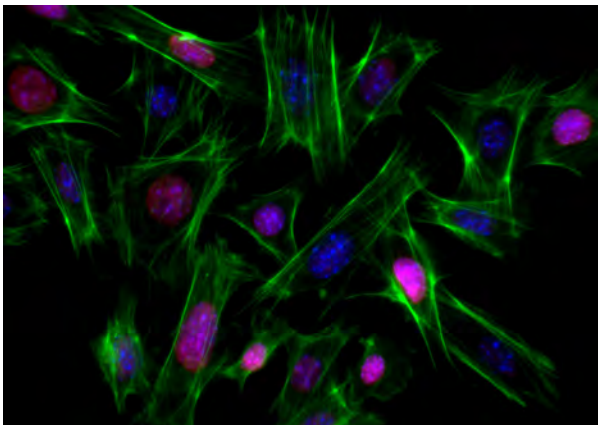


GFAP staining of human cerebellum

Secondary Reagents

Amplification is often required in imaging applications. One way to ensure the likelihood of success when imaging a target is to amplify the primary intended signal without increasing the background signal. In addition, amplifying a signal can also decrease the amount of exposure time, limiting the spillover of other fluors into your channel of interest. For these purposes, we provide many Alexa Fluor®, DyLight™, and Brilliant Violet™ conjugated secondary reagents.

See our secondary reagents:
biolegend.com/secondary_reagents



Mouse TE-71 cell line fixed, permeabilized and stained with purified anti-mouse Ki-67 and Alexa Fluor® 647 anti-Rat IgG2a antibodies, Alexa Fluor® 488 Phalloidin and DAPI.

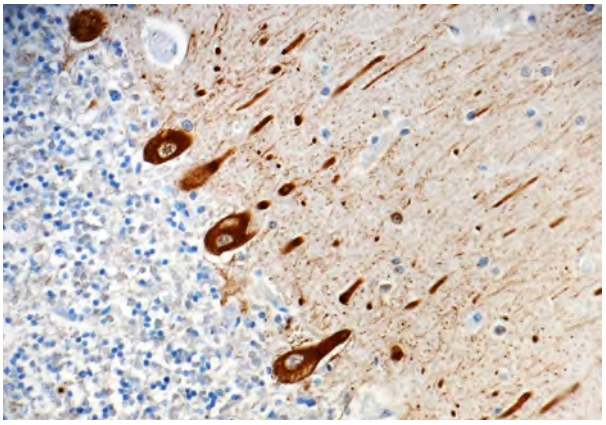
Sternberger Monoclonal Antibodies

Originally developed by Dr. Ludwig Sternberger, these antibodies have become the gold standard as neural and glial markers and have an extensive publication history in scientific literature. Many of these antibodies have been verified for use in immunohistochemical staining of formalin-fixed paraffin-embedded (FFPE) tissues as well as immunocytochemistry, ELISA and western blotting. The SMI® product line offers researchers high quality reagents to support investigations into neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease and neuroinflammation.

Featured reagents include:

- **Anti-GFAP**
- **Anti-MAP2**
- **Anti-Neurofilament**
- **Anti-Tau**

See a full list of the Sternberger monoclonal antibody portfolio: **biolegend.com/sternberger**



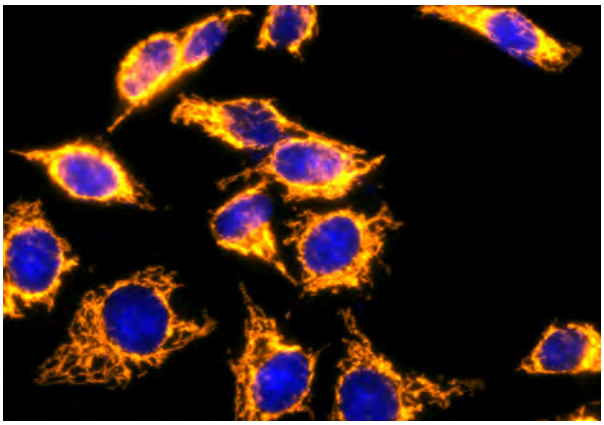
Staining of Neurofilaments H & M Non-Phosphorylated (SMI 33), on formalin fixed paraffin embedded human cerebellum tissue at 1/1000.

Mitochondrial Localization Probes

Fluorogenic chemical reagents that are attracted to the polarization of the mitochondrial membrane make ideal probes for imaging the mitochondria in both live and fixed specimens. Initially, the cells must be live while labeling, getting excellent signal at very low concentrations. However, MitoSpy™ probes containing a chloromethyl group (CM), like MitoSpy™ Orange, can be retained with an aldehyde-based fixative when incubated at higher concentrations of the probe in order to be combined with intracellular antibody detection.

- **MitoSpy™ Green FM**
- **MitoSpy™ Orange CMRos**
- **Anti-Cytochrome C conjugated to Alexa Fluor® 488, Alexa Fluor 594®, Alexa Fluor 647® and Biotin.**

To learn more about MitoSpy: **biolegend.com/mitospy**



HeLa cells stained with MitoSpy™ Orange (yellow), fixed and permeabilized with 4% PFA and 0.1% Triton X-100 and stained with Cytochrome C Alexa Fluor® 647 (red) and DAPI (blue).

Cell Tracking Dyes and Cell Proliferation Probes

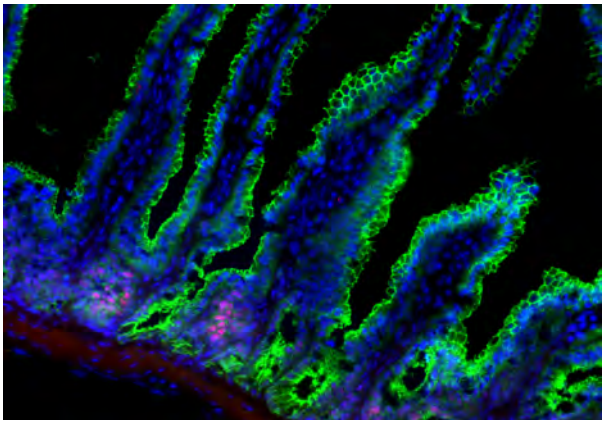
Our diverse portfolio of microscopy products includes:

CFSE (Cat. No. 423801) is a classical, cell-permeant, long-term tracking dye that can also be used for cell proliferation.

Zombie dyes are reagents that label all cells through a covalent attachment to cell surface amine-containing proteins, offering long-term cell tracking without intracellular delivery or cytotoxicity. Cells can proliferate uninhibited until the signal intensity becomes diluted with each daughter generation.

Anti-Ki-67 antibodies are also available in several conjugations, allowing you to identify proliferating cells post-fixation.

Discover Zombie dyes at: **biolegend.com/live_dead**



Mouse intestine labeled with purified anti-Ki-67 (clone 16A8) and anti-Rat IgG2a Alexa Fluor® 647 (red), and anti-E-Cadherin Alexa Fluor® 488 (green) antibodies and DAPI (blue).

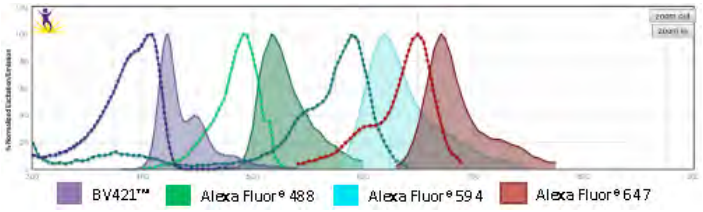
Tips and Tricks for the Best Images

Generating the best image involves many factors, all dependent on what is ideal for imaging the marker of interest in its biological context. Here are some questions to ask yourself when choosing the right reagents and instruments.

1. Number of Targets

It's possible to do a 4 color imaging experiment with relative ease in both confocal and widefield microscopy. With information about the spectrum of each fluorophore, you can make choices about optimal filter selection to minimize spectral spillover resulting from fluorophores with overlapping excitation and emission spectra. Above 5 colors, a microscope employing spectral detection becomes useful to unmix the spectral spillover.

Also, if using antibodies for detection, problems can arise with the species-dependence of the primary and secondary antibody combination. Ideally, the use of directly labeled antibodies or haptens like biotin/streptavidin can help.



2. Fluorophore Combinations with Overlapping Spectra

In instances where fluorophores are excited by other wavelengths and have some spillover of their emission into a neighboring filter, the spillover is usually suboptimal strength and results in a weak haze of background. However, one tip when using fluors where one spills into the other is to make sure the two antibodies are not imaged on markers that co-localize. For example, image one on a marker in the nucleus and the other at the cell surface, when possible. Also, make sure the fluorophore that is spilling over into the neighbor filter is on the less abundant antigen.

3. GFP or a fluorescent protein variant

Fluorescent proteins do not survive exposure to methanol or acetone. If the GFP signal was present prior to fixation but signal is lost upon fixation, check to see if the paraformaldehyde was reconstituted with the help of methanol. If the fixative can't be changed to be organic solvent-free, anti-GFP antibodies can be employed to recover the GFP signal.

4. Instrument Choice

The instrument is made to be an ideal tool for the biological question, not the reverse. The better you understand the goal of the image, the better you can match the application to the instrument.

Do I want to image tissue thicker than 10 µm?	→	Confocal or Multiphoton Microscopy
Do I want to image more than 4 colors on a cell sample?	→	Spectral Unmixing
Do I want to reconstruct the sample in 3D?	→	Confocal or ApoTome
What level of resolution is desired/required?	→	Deconvolution, Structured Illumination, STED or PALM
Do I want to demonstrate colocalization/binding or bioactivity in live cells?	→	FRET or FLIM

5. Sensitivity

Ultimately, once your biological application is matched to the right microscope and imaging condition, the reagents are the last variable in ensuring high sensitivity. Sensitivity is a balance between the signal strength and non-specific staining/autofluorescence/background. There is not much we can do about the biological autofluorescence endogenous in certain tissues, like brain, liver, lung, etc. However, employing an appropriately complex blocking step, for example serum instead of BSA or milk, prior to adding antibodies can ensure a minimal amount of non-specific binding. If streptavidin is used and the tissue will be fixed and permeabilized, an endogenous biotin-blocking kit can prevent the biotin found naturally in mitochondria from binding the streptavidin. However, these are application-specific blocking requirements.

The use of directly conjugated primary antibodies may not exhibit the same signal strength as the use of secondary antibodies, but they are associated with less background staining. Often, antigens you want to detect are not abundant enough for detection with conjugated primary antibodies. In that event, you need to employ secondary antibodies or other amplification methods. Another option is to use biotin and streptavidin or other hapten-based amplification methods. If these methods fail, the last resort is to use enzymatic amplification kits like tyramide signal amplification (TSA) kits. The more antibodies or enzymes employed in amplifying the signal, the higher the residual background will be as well. It's a fine balance between signal amplification and background reduction.

6. Antifade

Finally, mounting media containing antifade is required for the maintenance of signal strength. All organic fluorophores photobleach, a process where reactive oxygen species created in the process of imaging attack the structure of the fluorophores, irreversibly neutralizing their ability to fluoresce. Using antifade is more difficult when the cells are imaged live, since any antifade scavenges oxygen from the media, thus suffocating the cells. This is why regenerating signal, like proteins expressing GFP, are desirable for long-term, live-cell imaging.

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