Microscopy Reagents

for Immunocytochemistry and Immunohistochemistry

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

BioLegend®

Toll-Free Tel: (US & Canada): 1.877.BIOLEGEND (246.5343)
Tel: 858.768.5800
biolegend.com

02-0011-01

World-Class Quality | Superior Customer Support | Outstanding Value

Immunohistochemistry and Immunofluorescence:

What's in a Name?

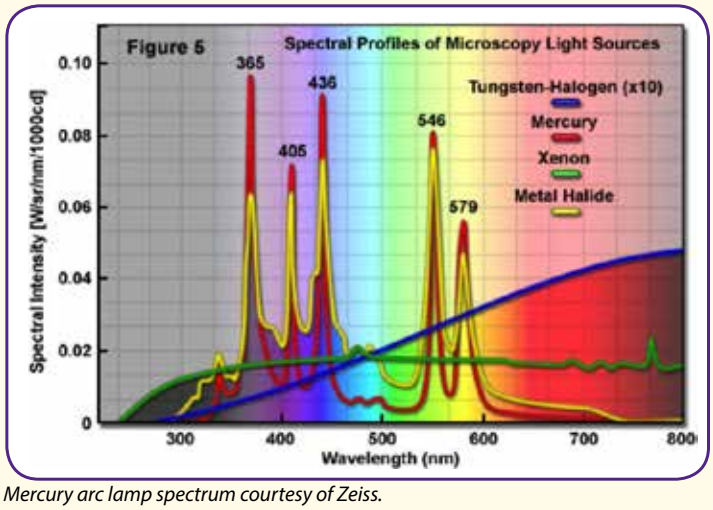
Imaging platforms enable understanding of subcellular localization, function, activity and health in a single cell culture or a network of cells comprising a tissue. For each biological question that could benefit from quantitative, structural, spatially relevant information, there is a sophisticated 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 reagent combination. It is becoming increasingly common to now also have white light excitation sources like a Tungsten-Halogen bulb that more evenly covers the entire useable spectrum rather than the principal lines of excitation common to mercury and metal halide sources. There are many modifications that can be made to a 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

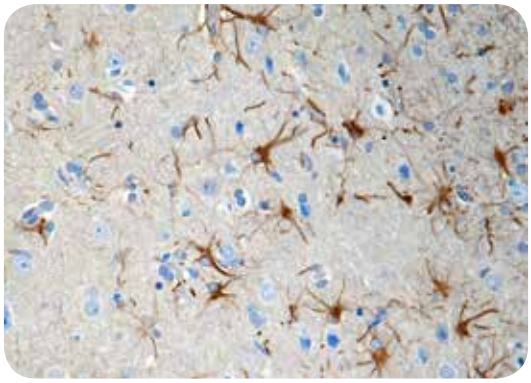


Mercury arc lamp spectrum courtesy of Zeiss.

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.

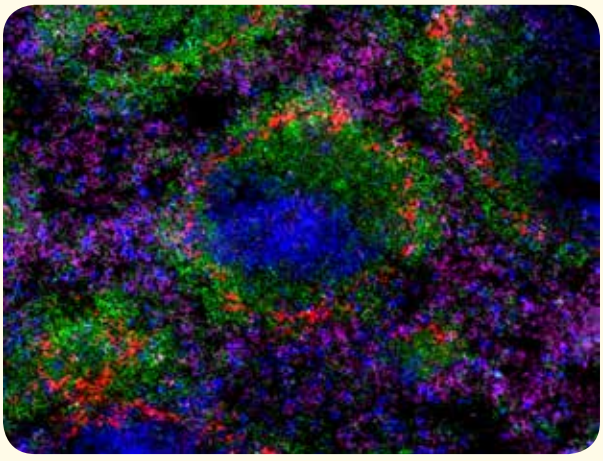


Staining of CX3CR1 (8E10.D9) on formalin fixed paraffin embedded human brain.

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 350-450 nm. 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.

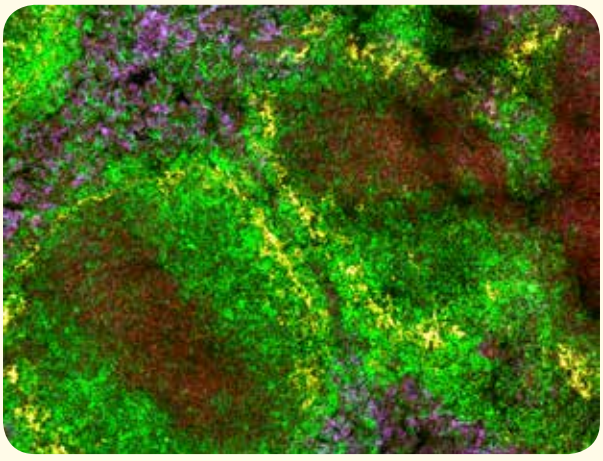
Multicolor Microscopy

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. Image obtained with a Zeiss LSM 780 confocal microscope with spectral unmixing applied.

Microscopy Reagents for Immunocytochemistry and Immunohistochemistry

BioLegend provides a variety of reagents supporting microscopy-based imaging of cells and tissue samples. Our reagents include antibody conjugates to bright, photostable fluorophores like Brilliant Violet 421™, Brilliant Violet 510™ and the Alexa Fluors®. We also provide cell tracking dyes, probes for cell health, subcellular localization and nuclear counterstains. BioLegend will continue to release new microscopy products as we discover innovative technologies.

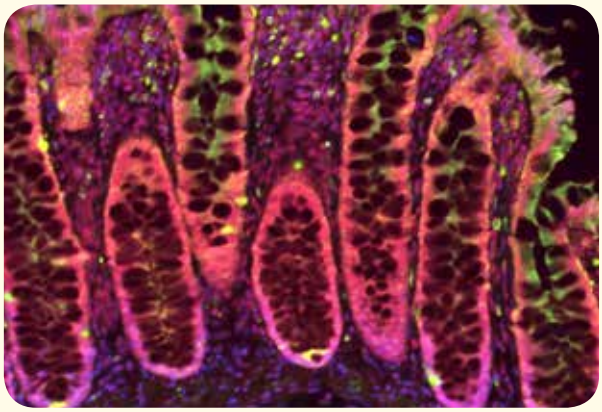
Learn more: **biolegend.com/microscopy**

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

Alexa Fluor® 488, Alexa Fluor® 594 and Alexa Fluor® 647 are popular fluorophores that provide strong signal and are photostable for imaging. They can be used in confocal or widefield microscopy as long as the microscope has the required lasers and/or excitation and emission filter sets that suit the spectra of these fluorophores. Also, the antigen being detected needs to be abundant enough that a directly conjugated primary antibody provides sufficient signal for sensitive detection.

Some of our most recent Alexa Fluor® 594 releases include:

- **Anti-human Perforin (Cat. No. 308124)**
- **Anti-Tubulin 3 (TUBB3) (Cat. No. 801208)**
- **Anti-human MMP2 (Cat. No. 679904)**
- **Anti-mouse CD169 (Siglec-1) (Cat. No. 142416)**
- **Anti-Cytokeratin (pan reactive) (Cat. No. 628606)**
- **Anti-Mouse I-A/I-E (Cat. No. 107650)**
- **Anti-Nestin (Cat. No. 656804)**
- **Anti-Cytochrome C (Cat. No. 612306)**



Human paraffin-embedded colon was prepared with standard deparaffination, rehydration, antigen retrieval and blocking protocols. Tissue stained with anti-CD44 (clone IM7) Alexa Fluor® 594 (green), anti-cytokeratin (pan reactive) (clone C-11) Alexa Fluor® 488 (red) and DAPI (blue).

Learn about Alexa Fluor® 594 and see our full line of Alexa Fluor® products at: **biolegend.com/AF594**

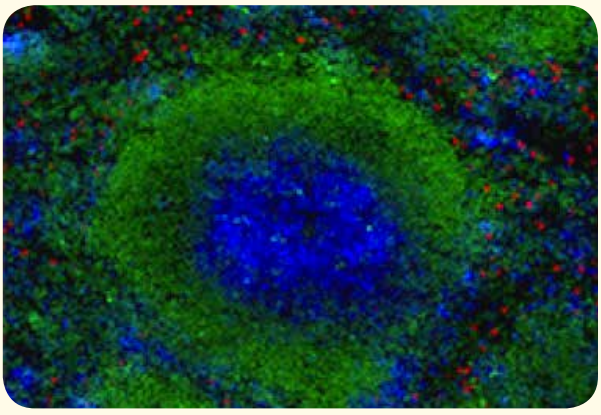
Brilliant Violet 421™ and Brilliant Violet 510™

Brilliant Violet 421™ (BV421™) and Brilliant Violet 510™ (BV510™) allow for expanded options in multicolor microscopy. BV421™ is used in the "blue" channel which is typically occupied by DAPI or Alexa Fluor® 405. BV510™ is also excited at 405 nm but emits at 520 nm. There are no other fluorophores that fit this particular spectral profile. Therefore, matching the excitation, emission and dichroic filters to this unique spectra is particularly important. However, when your filter set-up is optimized, BV421™ and BV510 ™ can be used simultaneously as bright, photostable options for multicolor microscopy. Some commercially available filters are listed below.

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

Vendors and Catalog Numbers for BV421™ and BV510™ Widefield Filters:

Chroma	Cat. No. 49027 | 49901
Omega	Cat No. XF403 | XF438
Semrock	Cat. No. BV421-3824A-000

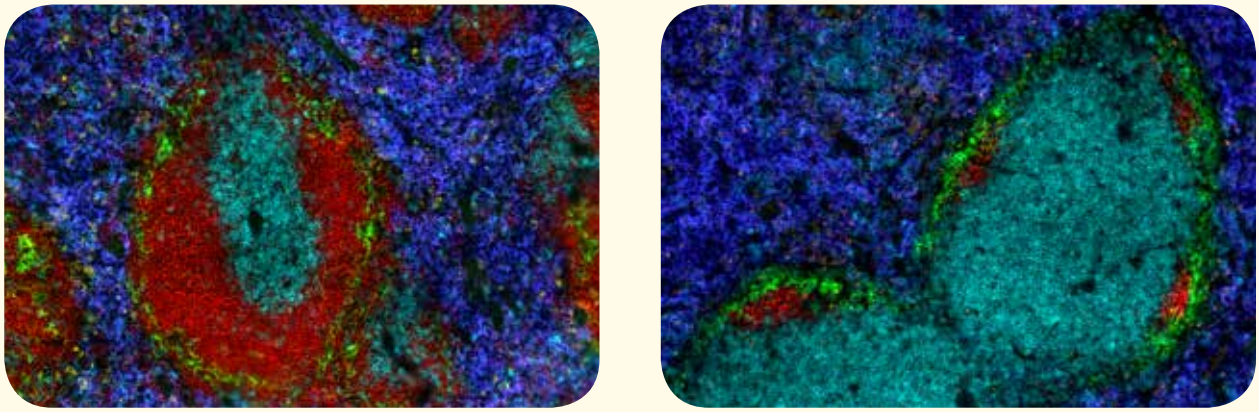


C57BL/6 mouse frozen spleen section was fixed, permeabilized and blocked using standard protocols. It was stained with anti-mouse/human CD45R/B220 (clone RA3-6B2) Brilliant Violet 510™ (green), anti-mouse CD8a (clone 53-6.7) Brilliant Violet 421™ (blue) and anti-mouse Ly-6G (clone 1A8) Alexa Fluor® 647 (red).

Application Note:

An ultra-purified version of novel anti-mouse CD20 antibody, clone SA271G2, or the equivalent isotype control was administered in a single dose of 250 µg. It depleted more than 95% of B cells from peripheral blood, spleen and lymph nodes without affecting $CD20^-$ cell types. B cell depletion was sustained for over 20 days before a gradual return over 50 days.

Learn more about applications being tested at:
biolegend.com/poster_library

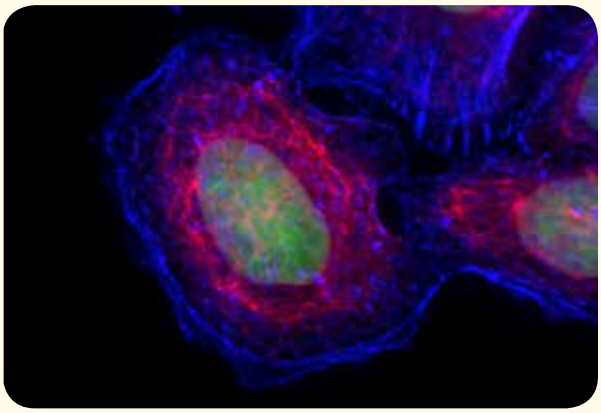


Mouse spleen was labeled *ex vivo* with CD3 (17A2) Alexa Fluor® 647 (cyan), CD11b (M170) Brilliant Violet™ 510 (yellow), CD19 (6D5) Alexa Fluor® 594 (red), CD169 (3D6112) Alexa Fluor® 488 (green), and F4/80 (BM8) Brilliant Violet™ 421 (blue).

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. In a live-cell imaging application, an impermeant nucleic acid stain like Helix NP™ Blue, Green or NIR can be paired with a permeant nucleic acid stain like DAPI, CytoPhase™ Violet or DRAQ5™ to assess the live to dead cell ratio.

- **DRAQ5™ (Cat. No. 424101)**
- **DRAQ7™ (Cat. No. 424001)**
- **DAPI (Cat. No. 422801)**
- **Helix NP™ NIR (Cat. No. 425301)**
- **Helix NP™ Green (Cat. No. 425303)**
- **Helix NP™ Blue (Cat. No. 425305)**
- **CytoPhase™ Violet (Cat. No. 425701)**
- **Propidium Iodide (Cat. No. 421301)**



HeLa cells were stained with anti-cytokeratin (pan reactive) Alexa Fluor® 647 (red), Helix NP™ Green (green) and Flash Phalloidin™ Red 594 (blue).

Find out more about our permeant and impermeant nucleic acid counterstains at:
biolegend.com/cell_health_proliferation and **biolegend.com/microscopy**

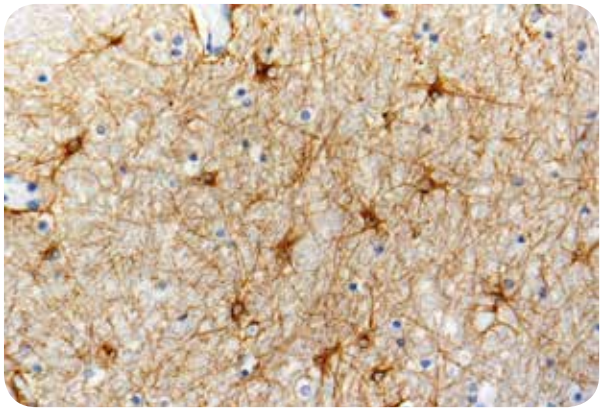
Reagents for Chromogenic IHC

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

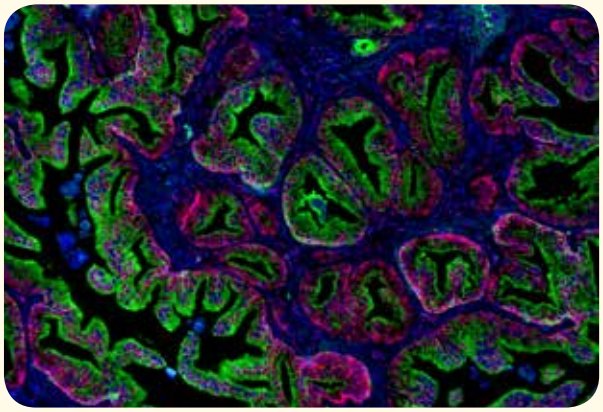


GFAP staining of human cerebellum.

Secondary Reagents

Amplification is often required in imaging applications. One way to increase the likelihood of success when imaging a target is to amplify the primary intended signal without raising 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® and DyLight™ dye conjugated secondary reagents.

See our secondary reagents:
biolegend.com/lg-second-step-1610/

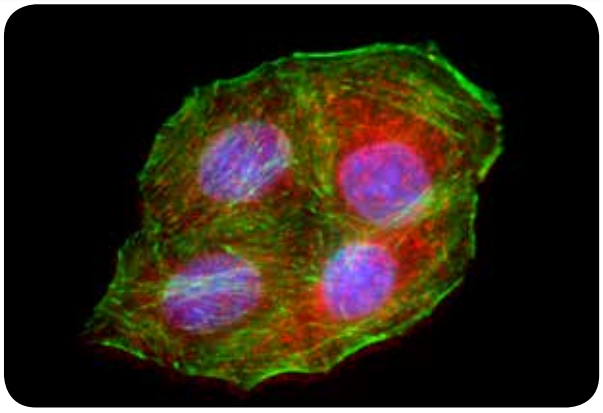


Paraffin embedded human prostate tissue was stained with anti-CD44 Alexa Fluor® 594 (red), purified anti-human CD107b (HB4) antibody followed by anti-mouse IgG Alexa Fluor® 488 secondary antibody (green) and DAPI (blue).

Flash Phalloidin™ NIR 647, Red 594 and Green 488

Phalloidin is a bicyclic peptide found in death cap mushrooms that binds very tightly to F-actin, preventing its depolymerization in living cells. In cellular imaging, fluorescently conjugated phalloidins are useful for imaging the fine filaments of actin, providing structural and volumetric context to the cell.

- **Flash Phalloidin™ Green 488 (Cat. No. 424201)**
- **Flash Phalloidin™ Red 594 (Cat. No. 424203)**
- **Flash Phalloidin™ NIR 647 (Cat. No. 424205)**



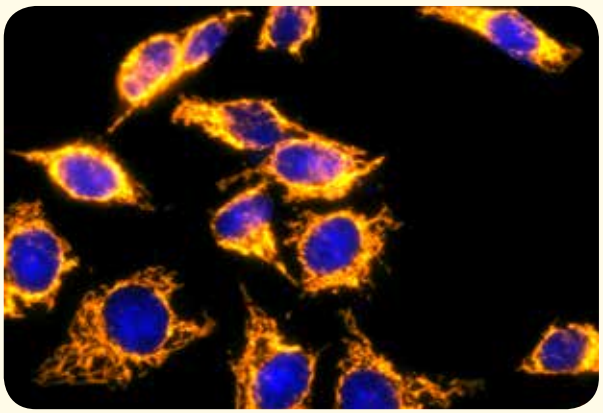
HeLa cells were stained with CD171 Alexa Fluor® 594 (red), Flash Phalloidin NIR™ 647 (green) and DAPI (blue).

Mitochondrial Localization Probes

Fluorogenic chemical reagents that are attracted to the polarization of the mitochondrial membrane make ideal probes for imaging the mitochondrial localization in microscopy and assessing cellular health based on mitochondrial respiration in flow cytometry. Initially, the cells must be live while labeling, getting excellent signal at low concentrations. However, MitoSpy™ probes containing a chloromethyl group (CM), like MitoSpy™ Orange CMTMRos and MitoSpy™ Red CMXRos, 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 is mitochondrial potential independent and is useful for mitochondrial localization in live cells and has also been indicated for use in mitochondrial mass assessment in flow cytometry. If the sample will be fixed right away, like a tissue slice, it's better to use antibodies against mitochondrial localized proteins like HSP60 and Cytochrome C.

- **MitoSpy™ Green FM (Cat. No. 424805 | 424806)**
- **MitoSpy™ Orange CMTMRos (Cat. No. 424803 | 424804)**
- **MitoSpy™ Red CMXRos (Cat. No. 424801 | 424802)**
- **Anti-Cytochrome C conjugated to Alexa Fluor® 488, Alexa Fluor® 594, Alexa Fluor® 647 and Biotin.**
- **Anti-HSP60 purified (Cat. No. 681502)**
- **Anti-VDAC1 purified (Cat. No. 820701)**

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



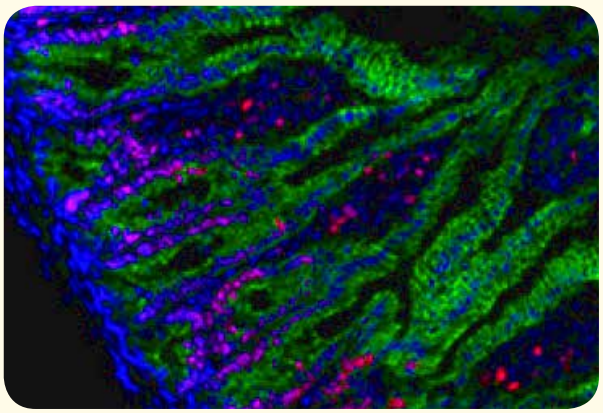
HeLa cells stained with MitoSpy™ Orange (yellow), fixed and permeabilized with 4% PFA and 0.1% Triton X-100 and stained with Cytochrome C Alexa Fluor® 647 (red) and DAPI (blue).

Long Term Cell Tracking and Proliferation

Our diverse portfolio of microscopy products includes:

- **CFDA-SE (CFSE)** is a classical, cell-permeant, long-term tracking dye that can be used for cell proliferation and tracking in microscopy and generational analysis in flow cytometry.
- **Tag-it Violet™** is an alternative to CFSE, that excites at 405 nm and emits at 450 nm. It can be used in all the same applications of tracking, proliferation and generational analysis as CFSE but provides an additional color option that helps when tracking two populations of cells.
- **Zombie Dyes** are reagents that label all cells through a covalent attachment to cell surface amine-containing proteins, offering a long-term cell tracking alternative without intracellular delivery or cytotoxicity. Cells can proliferate uninhibited until the signal intensity becomes too diluted.
- **Anti-Ki-67** antibodies are also available in several fluorescent conjugates, allowing you to identify proliferating cells post-fixation.
- **BrdU** is a bromine-modified nucleotide analog that when available in the presence of dividing cells, can become incorporated into newly replicated DNA. Detection requires fixation and permeabilization of the cells or tissue and use of an anti-BrdU antibody available in an array of fluorescent conjugates.

To learn more about cell tracking and proliferation: **biolegend.com/cell_health_proliferation**



C57BL/6 mouse frozen intestine section was stained with Ki-67 Alexa Fluor® 647 (red), E-cadherin Alexa Fluor® 594 (green) and DAPI (blue).

Neuroscience

BioLegend offers a wide range of antibodies relevant to applications in neuroscience, including neurodegeneration, neuroinflammation and neuro-related structural markers.

Neurodegeneration

Protein aggregation is a hallmark of neurodegenerative disease pathology. Post-translational modification of key target proteins plays an important role in aggregate formation, deposition and neurotoxicity. BioLegend offers a strong portfolio of high quality reagents to detect native and modified forms of these key disease targets.

Featured protein aggregation antibodies suitable for IHC and IF by disease area:

Alzheimer's disease

- **Amyloid Precursor Protein (APP)**
- **Amyloid beta (Aβ)**
- **Apolipoprotein E (ApoE)**
- **Presenilins**

Parkinson's disease

- **α-Synuclein**
- **Phosphorylated α-Synuclein (Ser129)**
- **α-Synuclein, aggregate-specific**

Tauopathies

- **Tau**
- **Phosphorylated Tau**
- **Nitrated Tau**

Neuroscience Cellular and Structural Markers

Well-characterized and highly specific neuroscience cell and structural marker antibodies allow detection of distinct cell populations in the brain for use in tissue section or cell culture applications.

Featured antibodies include:

Neurons

- **MAP2**
- **Tubulin β3**
- **Neurofilaments**

Astrocytes

- **GFAP**
- **S100B**

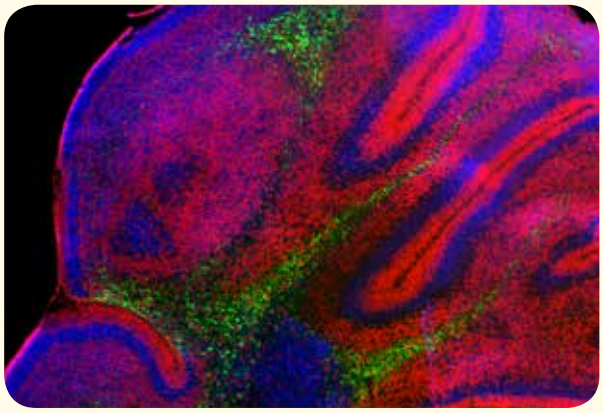
Microglia

- **CX3CR1**
- **CD11b**

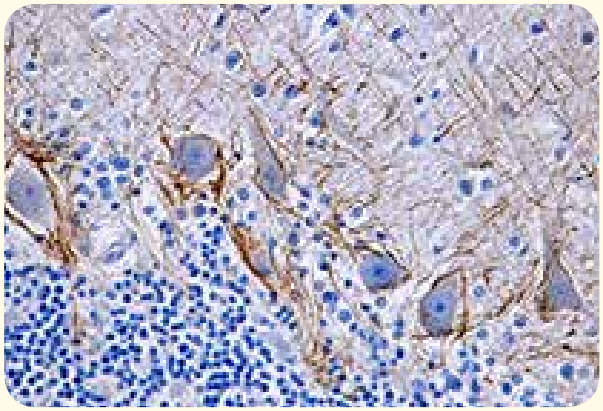
Oligodendrocytes

- **Myelin Basic Protein (MBP)**
- **Myelin CNPase**

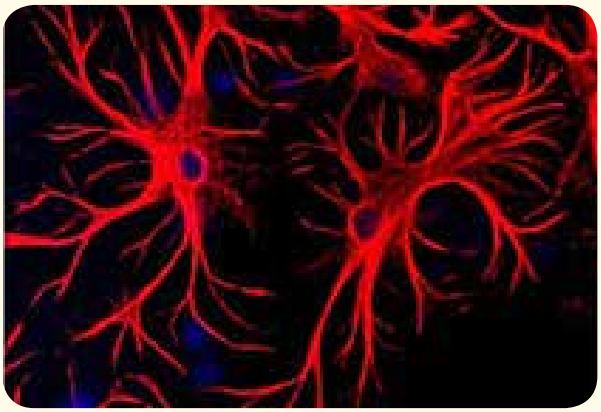
Explore our neuroscience applications and offerings:
biolegend.com/neuroscience



Mouse cerebellum stained with Brilliant Violet™ 421 anti-Tubulin Beta 3 (clone AA10) (Blue), Alexa Fluor® 488 anti-GFAP (clone 2E1.E9) (Green), and DRAQ5™ (Red).



IHC staining of human cerebellum tissue with anti-neurofilament H antibody (clone SMI 34) followed by counterstaining with hematoxylin.



Rat glial cells in mixed culture stained with anti-GFAP antibody (Poly28294, red).

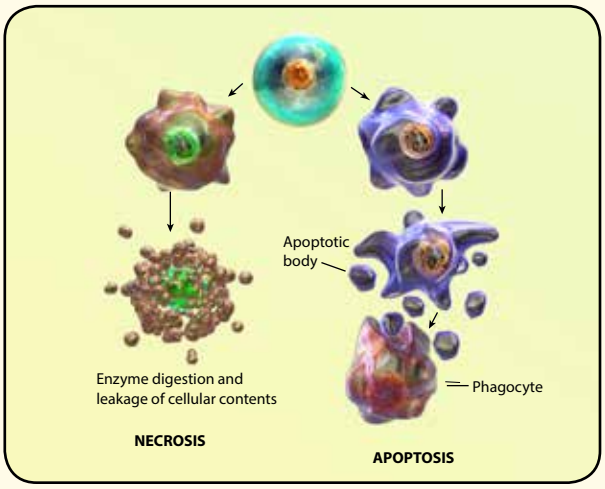
Cell Health and Apoptosis

Apoptosis is a death process defined by the internal degradation of cellular components without the instigation of a systemic inflammation response. An array of reagents are useful in imaging early to late stages of programmed cell death.

Annexin V

Annexin V is a protein that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS residues will translocate to the outer leaflet of the plasma membrane in cells undergoing membrane asymmetry associated with apoptosis. Annexin V alone will not indicate apoptosis, since it will also detect intracellularly faced PS residues in dead cells once membrane integrity is lost. Therefore, it must be used in conjunction with other probes that will assess live/dead status like the Zombie dyes or impermeant nucleic acid stains like the Helix NP™ family. Annexin V conjugates come in an array of spectral options, including BV421™, BV510™, PE, FITC and Alexa Fluor® 647.

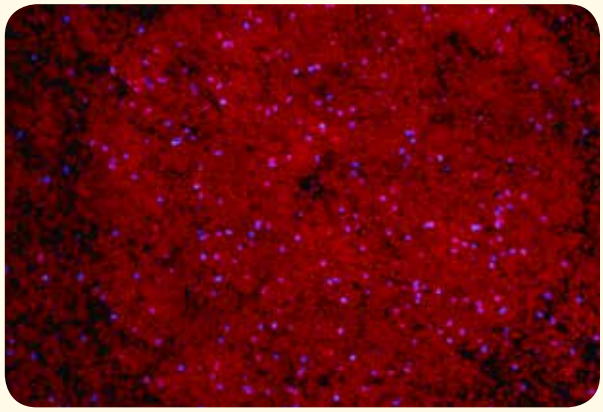
- **Annexin V conjugates in BV421™, BV510™, FITC, PE and Alexa Fluor® 647**



Cell Vitality and Esterase Probes

Calcein-AM and Calcein Violet-AM are fluorogenic esterase substrates that indicate not only that a cell is alive, but that it is also healthy, displaying an abundance of intracellular esterase activity. As cells enter and progress through apoptosis, esterase activity will diminish until only residual enzyme is left upon complete cellular death. CFDA-SE and Tag-it Violet™ can also be used to detect the vitality of esterase activity if the cell sample will need to be fixed prior to analysis or imaging.

- **Calcein-AM (Cat. No. 425201)**
- **Calcein Violet-AM (Cat. No. 425203)**
- **CFDA-SE (Cat. No. 423801)**
- **Tag-it Violet™ Proliferation and Cell Tracking Dye (Cat. No. 425101)**

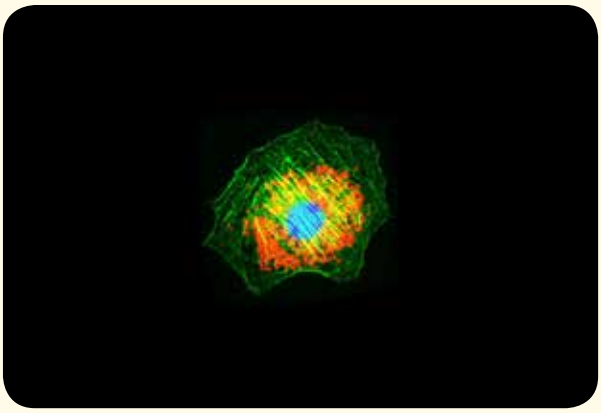


Mouse spleen 72 hours after adoptive transfer of Tag-it Violet™-labeled splenocytes (purple). Nucleated cells are stained using 25 µM DRAQ™ (red). Image was captured at a 40X magnification.

MitoSpy™ Mitochondrial Probes

Since strong mitochondrial respiration and cellular metabolism are also an indication of vitality and not just live versus dead status, the MitoSpy™ mitochondrial probes can also be helpful in this application when used in conjunction with other probes for cell health. However, since a reduction in the intensity of the staining of MitoSpy™ Orange CMTMRos and MitoSpy Red™ CMXRos indicates reduced mitochondrial polarization, it is more appropriate for flow cytometry or quantitative high-content imaging platforms.

- **MitoSpy™ Orange CMTMRos (Cat. No. 424803 | 424804)**
- **MitoSpy™ Red CMXRos (Cat. No. 424801 | 424802)**

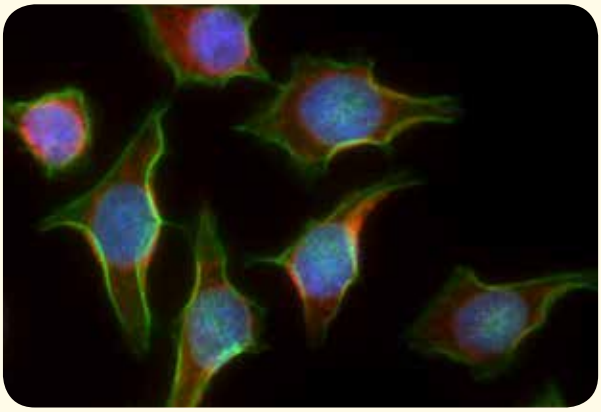


NIH3T3 cells were stained with 100 nM of MitoSpy™ Red CMXRos (red) for 20 minutes at 37°C, fixed with 1% paraformaldehyde (PFA) for ten minutes at room temperature, and permeabilized with 1X True Nuclear™ Perm Buffer for ten minutes at room temperature. Then the cells were stained with Flash Phalloidin™ NIR 647 (green) for 20 minutes at room temperature and counterstained with DAPI (blue). The image was captured with a 60x objective.

Antibodies for Apoptosis

Antibodies are also available, especially useful for applications involving fixed tissue, to elucidate elements of the apoptotic pathway. For example, there are many different caspases that are involved in degrading cellular components to dispose of the debris. They can have different functions, especially upon activation/cleavage. Cytochrome C is released from the intermembrane space of the mitochrondria upon the induction of apoptosis. PARP is involved in repairing nicked DNA and thus the cleavage of PARP can activate the apoptotic cascade in a cell. The Bcl-2 family of proteins can inhibit apoptosis by interfering with the function of pro-apoptotic proteins at the mitochondrial outer membrane, promoting cell survival. Bax, on the other hand, when assembled into homodimers, can induce apoptosis or interfere with the activity of Bcl-2 in a heterodimer.

- **Anti-Bcl-2 conjugates in BV421™, Alexa Fluor® 488, and Alexa Fluor® 647**
- **Anti-Bax (2D2) purified (Cat. No. 633601 | 633602) and Alexa Fluor® 488 (Cat. No. 633603 | 633604)**
- **Anti-PARP (5A5) purified (Cat. No. 614301 | 614302) and anti-PARP Alexa Fluor® 594 (Cat. No. 614306)**
- **Anti-Cytochrome C conjugated to Alexa Fluor® 488, Alexa Fluor® 594, Alexa Fluor® 647 and Biotin.**



HeLa cells were stained with anti-Cytochrome C Alexa Fluor® 594 (red), Alexa Fluor® 488 phalloidin (green) and DAPI (blue).

See our apoptosis reagents:
biolegend.com/ cell_health_proliferation

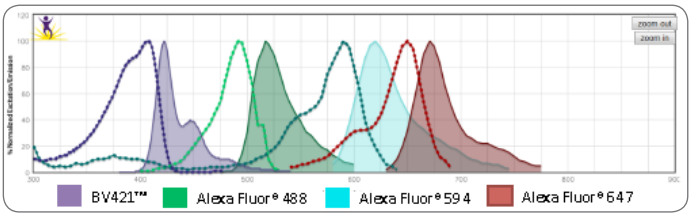
Tips and Tricks for the Best Images

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.

Contact BioLegend

Customer Service:

US & Canada Toll-Free: 1.877.246.5343 (877-BIOLEGEND)

International: 1.858.768.5800

Fax: 1.877.455.9587

email: cs@biolegend.com

Technical Service:

US & Canada Toll-Free: 1.877.273.3103

International: 1.858.768.5801

email: tech@biolegend.com

Headquarters:

BioLegend

9727 Pacific Heights Blvd.

San Diego, CA 92121

USA

International Offices

Europe:

BioLegend

4B Highgate Business Centre

33 Greenwood Place

London NW5 1LB

United Kingdom

Tel: +44 (0) 20 3475 3880

Fax: +44 (0) 20 3318 3271

email Inquiries: infoeurope@biolegend.com

email Technical Support: techeurope@biolegend.com

Japan:

BioLegend

8F, SB bldg., 1-4-6, Nezu, Bunkyo-ku, Tokyo

113-0031, Japan

Tel: +81-3-3823-9071

Fax: +81-3-3823-9072

email: supportjp@biolegend.com

biolegend.com/jp

For complete worldwide ordering details, visit: **biolegend.com**

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