BioLegend®

Microscopy Reagents

for Immunocytochemistry and Immunohistochemistry

World-Class Quality | Superior Customer Support | Outstanding Value

Microscopy Reagents

Accelerate your research with our continuously expanding portfolio of microscopy reagents. Cellular imaging empowers an understanding of subcellular localization, function, activity, and health of single cells 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 and an array of chemical and antibody-based reagents that are best designed for the application at hand. We develop reagents for microscopy-based imaging, including antibody conjugates to bright, photostable fluorophores like Brilliant Violet 421™, Brilliant Violet 510™, and the Alexa Fluor® dyes. We also provide cell tracking dyes, probes for cell health, subcellular localization, and nuclear counterstains.

Expertly-crafted by our scientists, our innovative microscopy reagents are ideally matched with the latest imaging technologies.

Learn more at: biolegend.com/en-us/microscopy

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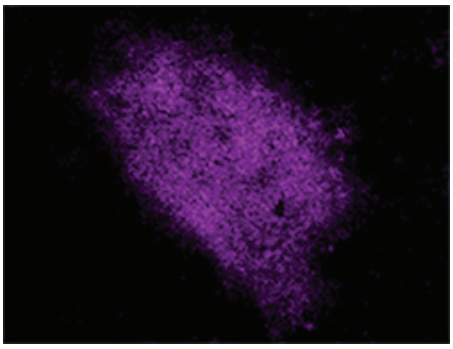
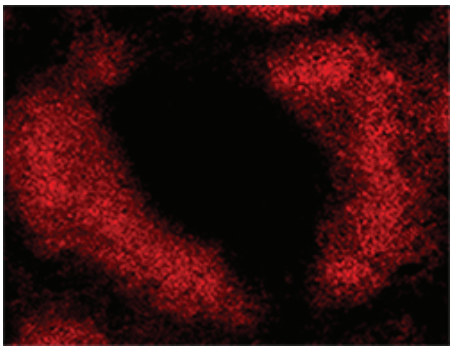
Reagents for Multicolor Fluorescence Microscopy

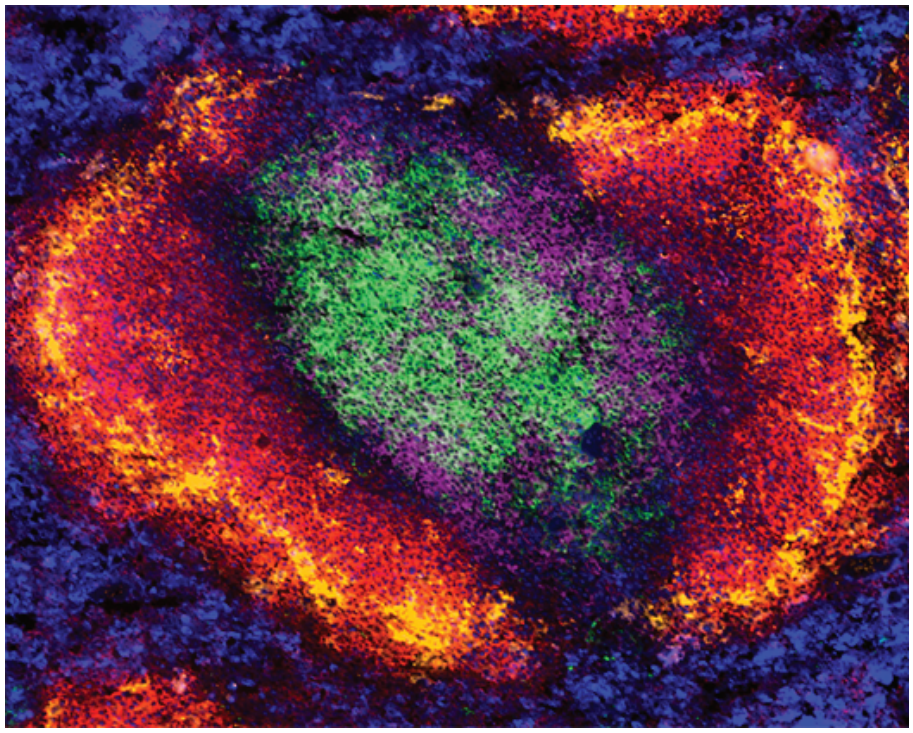
With our increased fluorophore options and the thousands of directly conjugated antibodies we provide for immunology and cell biology targets, it is possible to use fluorescence microscopy to look at multiple markers on a single sample. For larger microscopy panels, it is especially critical to know your microscope has the appropriate lasers and filters to capture the emission and excitation spectra of each of these distinct fluorophores.

Anti-CD4 Brilliant Violet 421™

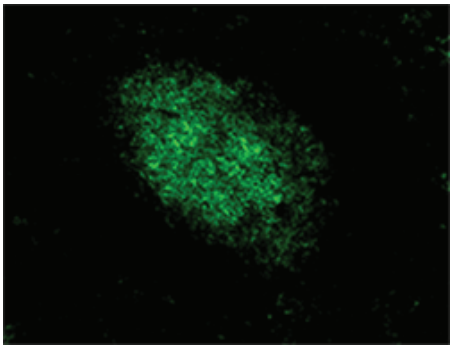


Anti-B220 Brilliant Violet 510™

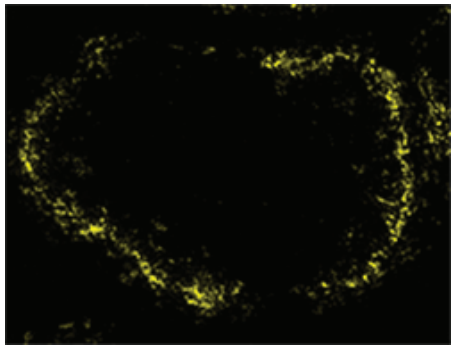




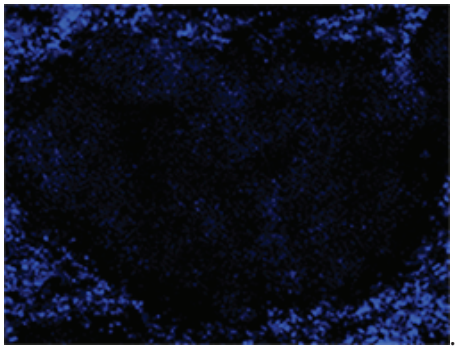
Anti-CD8a Alexa Fluor® 647



Anti-CD169 Alexa Fluor® 488



Anti-F4/80 Alexa Fluor® 594



We stained frozen mouse spleen tissue using antibodies against CD4 (violet) and CD8a (green) to detect T cells, B220 (red) to stain B cells, and CD169 (yellow) and F4/80 (blue) to detect tissue-resident macrophages. For this staining, we utilized antibodies directly-conjugated to bright photostable fluorophores including the Brilliant Violet™ and Alexa Fluor® dyes.

Fluorophores and Conjugated Antibodies

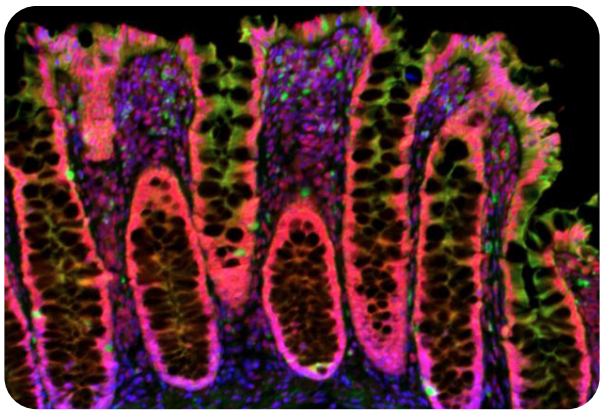
We provide a vast selection of fluorophore antibody conjugations for your microscopy research. The table below highlights some of these conjugates and emission profiles.

Direct Conjugate	Excitation max (nm)	Emission max (nm)	Emission color
DyLight™ 405	400	420	Blue
Brilliant Violet 421™	405	421	Blue
Brilliant Violet 510™	405	510	Green
DyLight™ 488	493	518	Green
Alexa Fluor® 488	495	519	Green
Alexa Fluor® 555	555	565	Orange
Spark YG™ 570	555	570	Orange
Alexa Fluor® 594	590	617	Red
DyLight™ 594	592	617	Red
Alexa Fluor® 647	650	665	Near-IR
Alexa Fluor® 700	702	723	Near-IR

Alexa Fluor® Dyes

Alexa Fluor® dyes are well-described probes with consistent output in the field of microscopy. While Alexa Fluor® 488, Alexa Fluor® 594, and Alexa Fluor® 647 are the most commonly used fluorophores in microscopy, additional family members like Alexa Fluor® 555 and Alexa Fluor® 700 can also provide strong and photostable staining for imaging. If you are looking for an Alexa Fluor® antibody conjugate not currently on our site, we provide custom fluorophore conjugations for your imaging experiments.

Learn more at: biolegend.com/en-us/custom-solutions



Paraffin-embedded human colon tissue stained with Alexa Fluor® 488 anti-Pan Cytokeratin (clone C-11, green) and Alexa Fluor® 594 anti-mouse/human CD44 (clone IM7, red) antibodies. Nuclei were counterstained with DAPI (blue).

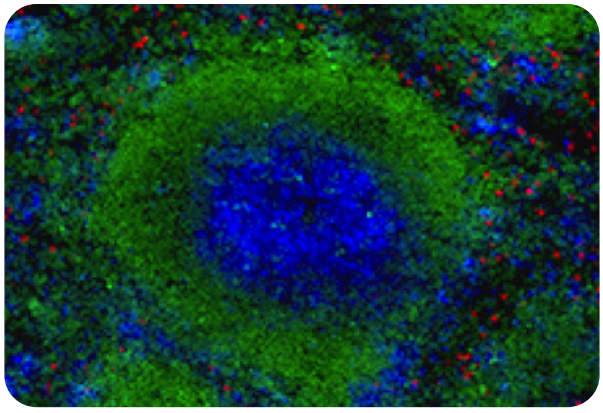
Brilliant Violet™ Dyes

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. When your filter set-up is optimized (see the filter sets below), BV421™ and BV510™ can be used simultaneously as bright, photostable options for multicolor microscopy.

Learn more about Brilliant Violet™, filter selection, and microscopy at: biolegend.com/en-us/brilliant-violet

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

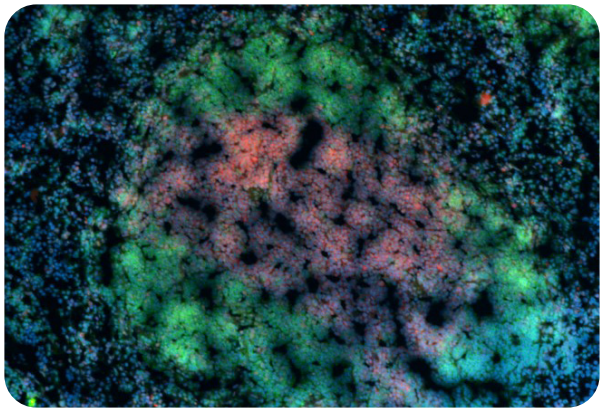
Chroma	Cat. No. 49027 | 49901
Omega	Cat No. XF403 | XF438
Semrock	Cat. No. BV421-3824A-000



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).

Spark YG™ 570

Ideal for building multicolor microscopy panels, Spark YG™ 570 stands out as a reliable addition to any multicolor microscopy panel. With an excitation max of 555 nm and an emission max of 570 nm, it can be imaged using the filter sets commonly used for Alexa Fluor® 555, Cy3, or TRITC in either widefield or confocal microscopy. Depending on the filter sets and light source of your microscope, Spark YG™ 570 can be used in combination with a variety of other fluorophores including DAPI, Alexa Fluor® 488, and Alexa Fluor® 647.

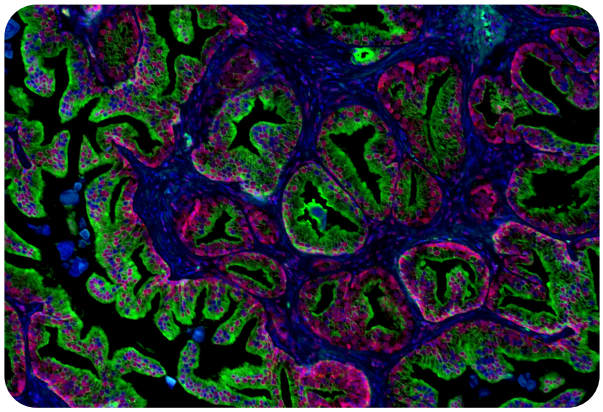


Immunohistochemistry on frozen mouse spleen tissue. Tissue was stained with anti-CD3 Spark YG™ 570 (red), anti-B220 Alexa Fluor® 647 (green), and DAPI (blue).

Secondary Reagents

Signal amplification is often required in imaging applications for lowly expressed antigens. One way to increase the likelihood of success when imaging a target is to amplify the primary 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 a selection of dye-conjugated secondary reagents.

See our secondary reagents at: biolegend.com/en-us/secondary-reagents



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

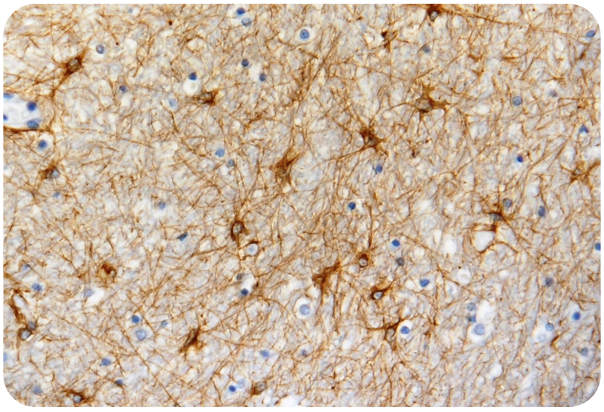
Reagents for Chromogenic Microscopy

Chromogenic detection methods have a long history in histology and pathology applications. Commonly used chromogenic immunohistochemistry (IHC) reagents include antibodies or streptavidin covalently attached to horseradish peroxidase (HRP) or alkaline phosphatase. This allows it to react to a substrate like DAB (3,3'-diaminobenzidine), AEC (3-amino-9-ethylcarbazole), or BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium). These enzymes catalyze their substrates, leaving a deposit of color where the antibody has attached to the cell or tissue. We provide a variety of accessory reagents for performing IHC including, Ultra-Streptavidin (USA) HRP Detection Kits and ACUITYAdvanced Biotin Free Polymer Detection Kits.

A chromogenic signal can also 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 due to normal cellular peroxidase activity, leading to a non-specific signal. It is only typically used to image a single marker at a time.

Our Video Library includes step-by-step protocol videos for many of our reagents, including our chromogenic tissue staining using DAB detection system.

Learn more at: biolegend.com/en-us/microscopy-protocol-videos



GFAP staining of astrocytes in human cerebellum.

Advanced Applications

IBEX Multiplex Imaging

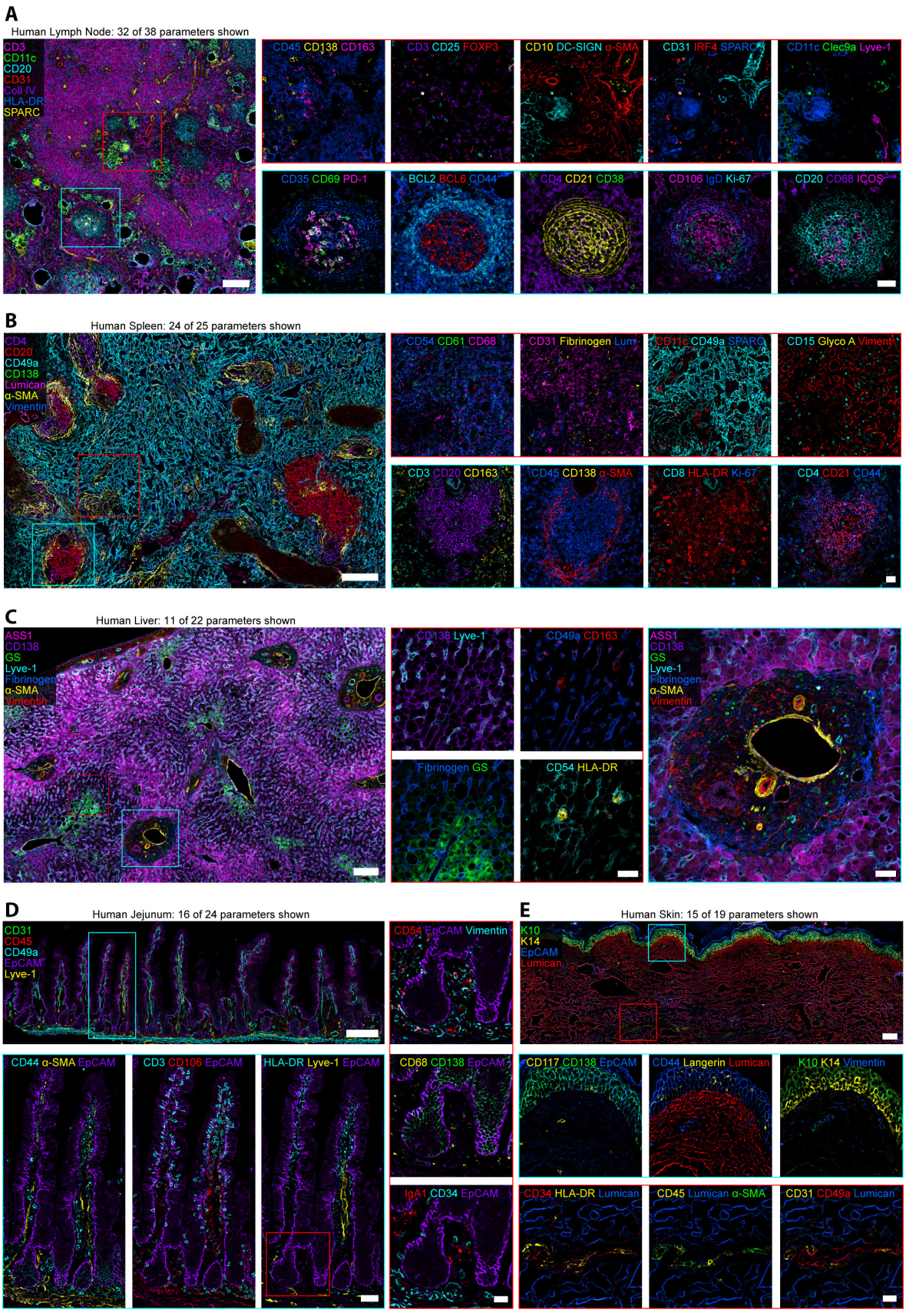
Traditional multiplexed immunofluorescence microscopy is limited by the number of spectrally unique fluorophores that can be imaged simultaneously; 2-7 parameters with common imaging systems. IBEX (Iterative Bleaching Extends multi-pleXity) increases the total number of parameters that can be imaged on a single sample using iterative cycles of antibody labeling and fluorophore inactivation. Importantly, the described method—developed by Dr. Andrea Radtke and colleagues in the laboratory of Dr. Ronald Germain (Center for Advanced Tissue Imaging, Lymphocyte Biology Section, NIAID, NIH)—requires no specialized equipment or proprietary software, making highly-multiplexed microscopy (60+ parameters) more accessible.

The next few pages contain several highly-multiplexed IBEX experiments featuring dozens of commercially available BioLegend reagents. The full panel and experimental details for human samples can be found in the IBEX *Nature Protocols* publication, and the details on the mice samples can be found in the IBEX *PNAS* publication.

Learn more about the technique and how to implement it in your research at: biolegend.com/en-us/ibex-applications

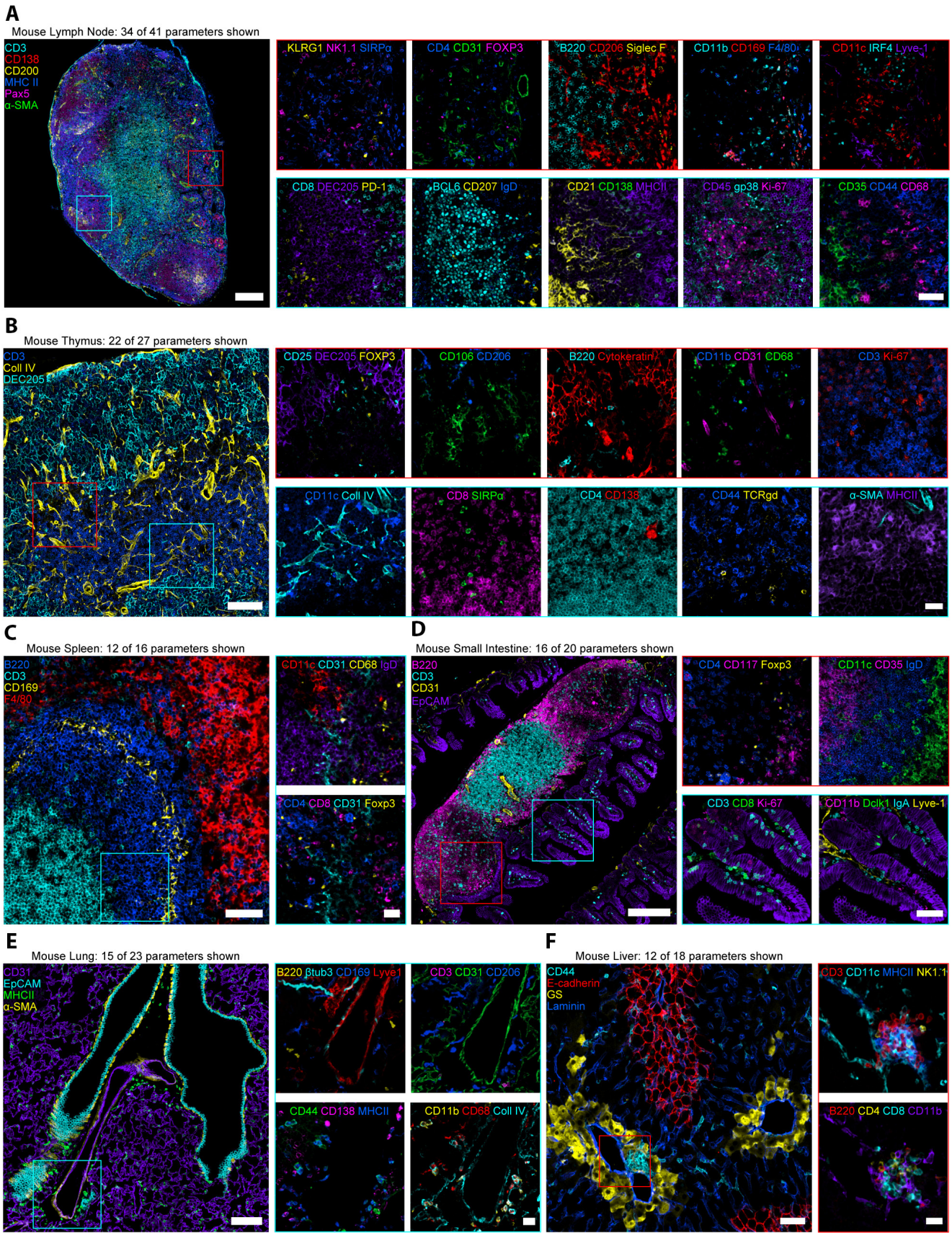
References

1. Radtke, A.J., Chu, C.J., Yaniv, Z. *et al*. (2022). IBEX: an iterative immunolabeling and chemical bleaching method for high-content imaging of diverse tissues. *Nat Protoc* 17, 378–401. DOI:10.1038/s41596-021-00644-9
2. Radtke, Andrea J *et al.* (2020) "IBEX: A versatile multiplex optical imaging approach for deep phenotyping and spatial analysis of cells in complex tissues." *Proceedings of the National Academy of Sciences of the United States of America* vol. 117,52: 33455-33465. DOI:10.1073/pnas.2018488117



A. Confocal images from a human mesenteric lymph node (LN) (nine cycles, 32 of 38 parameters shown). Scale bars: 200 µm (left), 25 µm (insets). B. Confocal images from human spleen (four cycles, 24 of 25 parameters shown). Scale bars: 200 µm (left), 25 µm (insets). Glycophorin A (Glyco A), Lumican (Lum) and Vimentin (Viment). C. Confocal images from human liver (four cycles, 11 of 22 parameters shown). Scale bars: 200 µm (left), 50 µm (insets). Glutamine synthetase (GS). D. Images from human jejunum (six cycles, 16 of 24 parameters shown). Scale bars: 200 µm (left), 50 µm (cyan box), 25 µm (red box). E. Images from human skin (five cycles, 15 of 19 parameters shown). Scale bars: 200 µm (left), 25 µm (insets). Keratin 10 (K10), Keratin. The use of information and images from Dr. Radtke and Germain lab colleagues on these informational pages does not imply any endorsement of BioLegend or its products by the US Government.

Mouse IBEX Panel

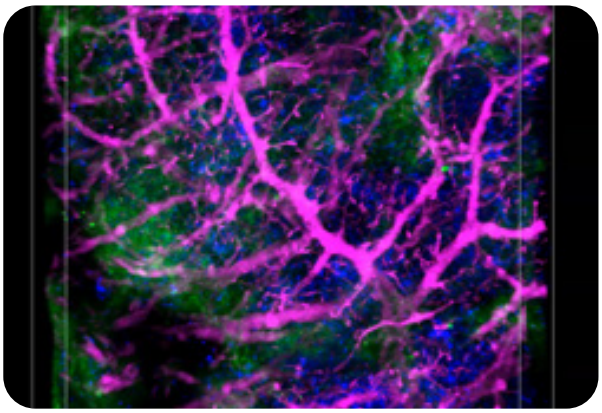


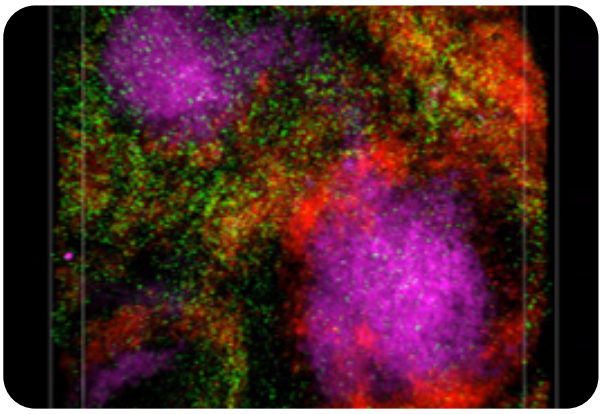
A. Confocal images from an immunized mouse LN (34 of 41 parameters shown). Scale bar is 200 µm (left), 50 µm (insets). B. Confocal images from mouse thymus (22 of 27 parameters shown). Scale bar is 200 µm (left), 20 µm (insets). Gamma Delta T cells (TCRgd). C. Confocal images from mouse spleen (12 of 16 parameters shown). Scale bar is 50 µm (left), 20 µm (insets). D. Confocal images from mouse small intestine (16 of 20 parameters shown). Scale bar is 200 µm (left), 25 µm (insets). E. Confocal images from mouse lung (15 of 23 parameters shown). Scale bar is 100 µm (left), 25 µm (insets). β-tubulin 3 (βtub3), Collagen IV (Coll IV). F. Confocal images from mouse liver (12 of 18 parameters shown). Scale bar is 50 µm (left), 20 µm (insets). Glutamine synthetase (GS). The use of information and images from Dr. Radtke and Germain lab colleagues on these informational pages does not imply any endorsement of BioLegend or its products by the US Government.

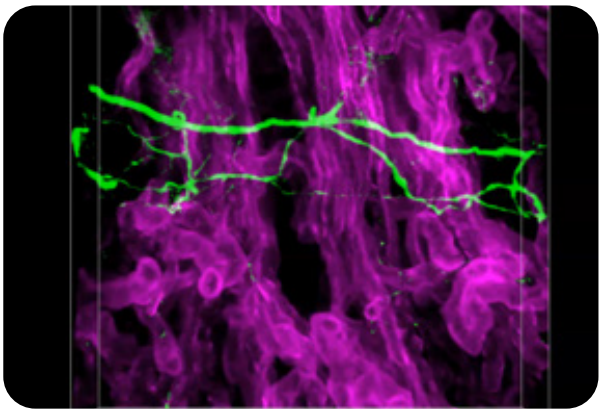
Optical Clearing Using Ce3D™

Add a dimension to your research by pairing your fluorescent antibodies with our Ce3D™ Tissue Clearing Solution. Designed by academic researchers, this buffer provides unparalleled clarity and insight as you interrogate tissues to produce mesmerizing 3D images.

Biological tissues are generally composed of proteins, lipids, and water, each of which has a different refractive index (RI). Differences in refractive indices, or RI mismatch, cause the scattering of light in the tissue and result in tissue opacity. Our Ce3D™ Tissue Clearing Solution reduces light scattering by normalizing the RI throughout the tissue, thus making the tissue transparent. This allows for rapid clearing of tissues to enable generation of 3D images in multicolor fluorescence microscopy applications.

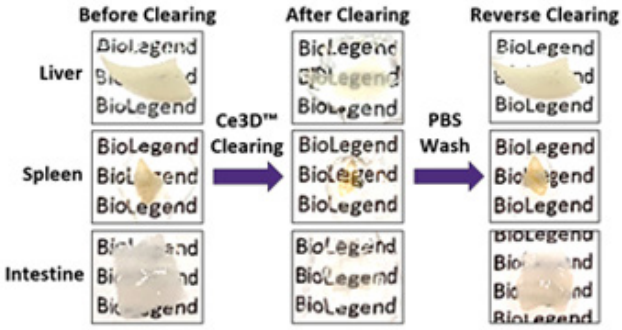






The images above are representative 3D IHC images of 500 µm-thick mouse samples. The sections were optically cleared after immunostaining, mounted in sample chambers, and imaged. Lower left: mouse spleen: CD68 (green), CD172a (red), CD8a (magenta). Upper right: mouse spleen: F4/80 (green), I-A/I-E (blue), CD146 (magenta). Lower right: TUBB3 (green), CD326 (magenta).

Ce3D™ Tissue Clearing Solution can be used in a wide variety of samples - and the process is fully reversible.



Representative 500 µm-thick mouse tissue sections before and after tissue clearing. Mice were perfused and organs were fixed with 4% PFA. Clearing was reversed by immersing tissues in PBS for one hour. For best clearing effect, 1% PFA is recommended. 4% PFA is recommended for optimal epitope preservation and immunostaining.

To learn more, visit: biolegend.com/en-us/3d-tissue-clearing

Chemical Probes for Microscopy

Structural Probes and Stains

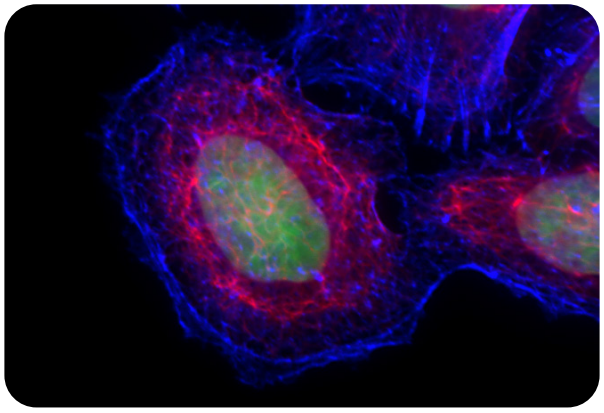
In addition to primary antibodies, a number of dyes or probes can be used to specifically stain subcellular structures. These can be used across a wide variety of microscopy applications, including counterstaining the nucleus or identifying general cytoplasmic structures, such as actin, microtubules, or organelles.

Chemical Probe	Subcellular Localization	Excitation max (nm)	Emission max (nm)	Emission color
CytoPhase™ Violet	Nucleus	355, 375, 405	440	Blue
DAPI	Nucleus	360	460	Blue
Helix NP™ Blue	Nucleus	430	470	Blue
Helix NP™ Green	Nucleus	495	519	Green
Propidium Iodide	Nucleus	488	610	Orange
7-AAD	Nucleus	488	650	Red
Helix NP™ NIR	Nucleus	640	660	Near-IR
DRAQ5™	Nucleus	568, 633, 647	695	Near-IR
DRAQ7™	Nucleus	633	695	Near-IR
MitoSpy™ Green FM	Mitochondria	490	516	Green
MitoSpy™ Orange CMTMRos	Mitochondria	551	576	Orange
MitoSpy™ Red CMXRos	Mitochondria	577	598	Red
MitoSpy™ NIR DilC1(5)	Mitochondria	638	658	Near-IR
Calcein Violet-AM	Cytoplasm	400	452	Blue
Tag-it Violet™	Cytoplasm	405	450	Blue
Calcein-AM	Cytoplasm	488	520	Green
Flash Phalloidin™ Green 488	Cytoplasm	488	520	Green
CFDA-SE	Cytoplasm	492	517	Green
Calcein Red-AM	Cytoplasm	560	574	Red
Flash Phalloidin™ Red 594	Cytoplasm	590	611	Red
Apotracker™ Green	Membrane	500	520	Green

Find out more about our chemical probes at: biolegend.com/en-us/cell-health-and-proliferation

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™ Green can be paired with a permeant nucleic acid stain like DAPI, CytoPhase™ Violet, or DRAQ5™ to assess the live to dead cell ratio.



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

Flash Phalloidin™

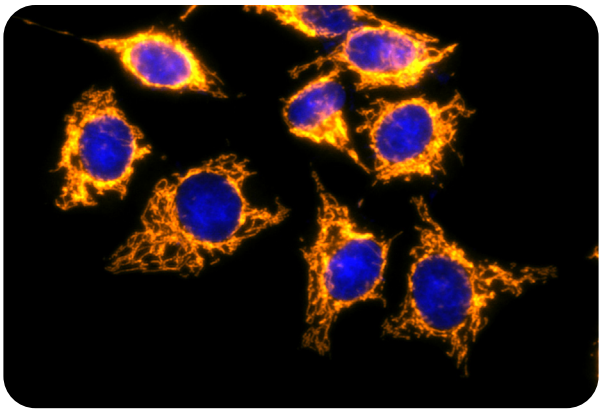
Phalloidin is a bicyclic peptide found in death cap mushrooms that binds very tightly to F-actin, preventing its depolymerization in living cells. In cellular imaging, fluorescently conjugated phalloidins are useful for imaging the fine filaments of actin, providing structural and volumetric context to the cell. We provide Flash Phalloidin™ formats that emit in the green (488 nm) and red (594 nm) regions.

Drosophila ovaries were fixed with paraformaldehyde, incubated with Flash Phalloidin™ Green 488 (green) to visualize F-actin, and mounted in VECTASHIELD® containing DAPI (red) to visualize the nucleus. Image was then collected using a Leica TCS SP5 II (Leica Microsystems) confocal microscope. Image provided courtesy of Dr. Eurico Morais-de-Sá at the Instituto de Investigação e Inovação em Saúde, Portugal.

MitoSpy™ Mitochondrial Probes

Since strong mitochondrial respiration and cellular metabolism are 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.

Learn more at: biolegend.com/en-us/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 Health and Apoptosis

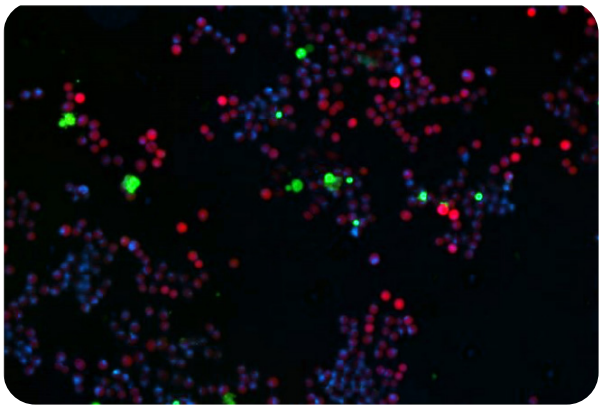
Antibodies for Apoptosis

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 can be useful in imaging early to late stages of programmed cell death. Antibodies are also available to elucidate elements of the apoptotic pathway.

See our apoptosis reagents at: biolegend.com/en-us/cell-health-tools

Apotracker™ Green (Apo-15), a Calcium-Independent Apoptosis Probe

Non-antibody probes can be particularly useful in apoptosis detection assays. Annexin V has historically been a gold standard for general apoptosis detection when coupled with another probe for necrosis like impermeant nucleic acid stains (Helix NP™ probes) and esterase substrates (Calcein-AM probes). Annexin V, however, depends on calcium in the staining media. It can also exhibit high background staining and be difficult to incorporate in microscopy applications. Detected in the FITC channel, Apotracker™ Green (Apo-15) is a fluorogenic probe that binds to apoptotic cells in a calcium independent manner, while exhibiting a linear relationship with Annexin V staining, suggesting they are both detecting externalized phosphatidylserine residues. Apotracker™ Green is useful in microscopy applications on live cells and is retained with paraformaldehyde fixation.



5-day old HeLa cells were stained with live indicator Calcein Red-AM, Helix NP™ Blue, and Apotracker™ Green (Apo-15).

Cell Vitality and Esterase Probes

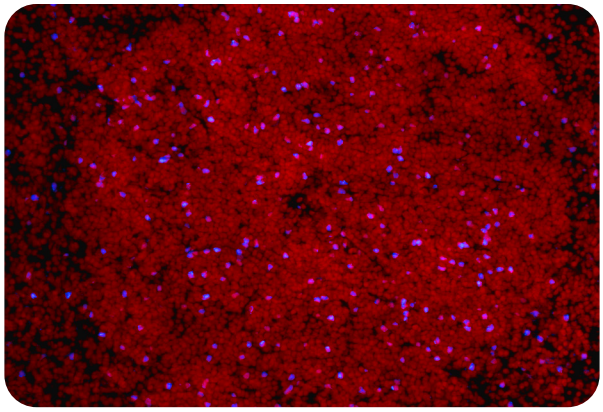
Calcein-AM is a fluorogenic esterase substrate that indicates not only that a cell is alive, but that it is also healthy, displaying an abundance of intracellular esterase activity. As cells enter and progress through apoptosis, esterase activity will diminish until only residual enzyme is left upon complete cell death. CFSE 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.

Long Term Cell Tracking and Proliferation

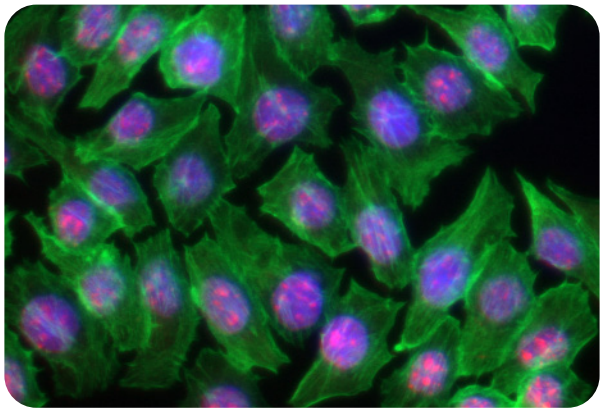
Our portfolio of microscopy products for visualizing cell dynamics and proliferation includes:

- 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. The excitation and emission wavelengths of CFSE-labeled cells are 492 nm and 517 nm, respectively.
- Tag-it Violet™ 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.
- Anti-Ki-67 antibodies are also available in several fluorescent conjugates, allowing you to identify proliferating cells post-fixation.
- BrdU is a commonly used bromine-modified uracil nucleotide analog that can be incorporated into newly synthesized DNA, highlighting dividing cells. Detection requires fixation and use of an anti-BrdU antibody available in an array of fluorescent conjugates.
- Helix NP™ NIR is a far-red emitting nucleic acid stain. It is impermeant to live cells and thus can be used for the discrimination of live and dead cells. In immunofluorescence microscopy, it can be used as a nuclear counterstain in cells and tissue. It is optimally excited at 640 nm with an emission at 660 nm, which can be detected in the Alexa Fluor® 647 channel.

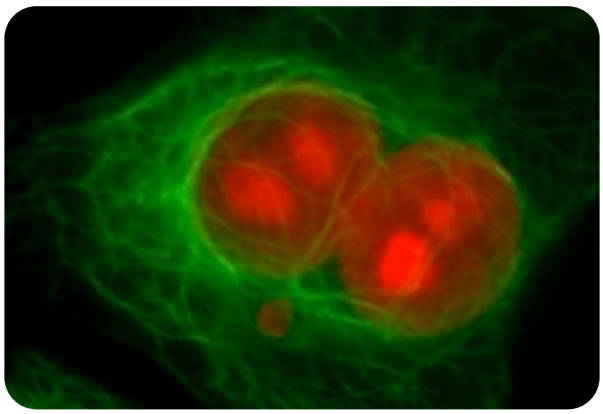
Learn more about cell tracking and proliferation at:
biolegend.com/en-us/cell-health-and-proliferation



Mouse spleen 72 hours after adoptive transfer of Tag-it Violet™- labeled splenocytes (purple). Nucleated cells are stained using 25 µM DRAQ5™ (red). Image was captured at a 40X magnification.



HeLa cells were intracellularly stained with purified anti Ki-67 (red) followed by DyLight™ 594 anti-mouse IgG and Alexa Fluor® 488 Phalloidin (green). Nuclei were counterstained with DAPI (blue).



HeLa cells were fixed, permeabilized, blocked and then intracellularly stained with Alexa Fluor® 488 anti-Cytokeratin (pan reactive; green) antibody followed by 10 µM of Helix NP™ NIR (red). Image was captured with 60X objective.

Research Areas

Our validated antibodies are used in various research areas including cell biology, immunology, and neuroscience research to label or characterize key cell types. We have highlighted some of these research areas and included several markers and the cell types of interest in the table below.

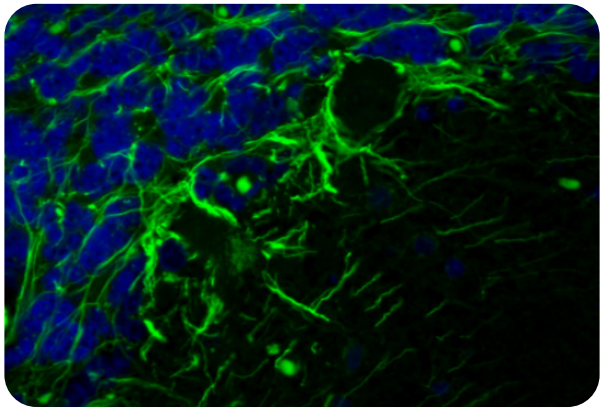
Cell Type	Markers
B cells	B220, Bcl6, CD138, GL-7, IgD, IRF4, Ki-67, Pax5, SiglecF
T cells	CD3, CD4, CD8, CD44, Foxp3, KLRG1, NK1.1, PD-1, TCRγδ
Myeloid cells	CD11b, CD11c, CD64, CD68, CD169, CD206, CD207, DEC205, F4/80, MHCII, SIRPα
Stroma/Structural	CD21, CD23, CD31, CD35, CD106, CD200, Coll IV, gp38, Lyve-1, αSMA
Neuronal stem cells	Nestin, SOX2
Neurons	MAP2, NeuN, Neurofilaments, βIII tubulin, NF-H
Microglia	CD45, CX3CR1, P2RY12
Astrocytes	GFAP, S100B
Oligodendrocytes	MBP, Olig1/2, CNP, Sox-10

Note: For cell populations like T cells and myeloid cells, subpopulations may express select markers from this listing. For example, Tregs express Foxp3, and NK T cells can express NK1.1. However, not all T cells express NK1.1 and Foxp3. Refer to the literature for more extensive phenotyping of these cells.

Cell Biology

Understanding physiological cell processes like cell signaling, proliferation, and growth is critical for life science research. In response to stimuli such as growth factors, cells undergo a series of tightly regulated steps known as the cell cycle. In addition, studying cell structures and organelles can offer important insights into the function and health of the cell. Find reagents designed by our scientists to empower your cell biology imaging.

Learn about cell biology at: biolegend.com/en-us/cell-biology

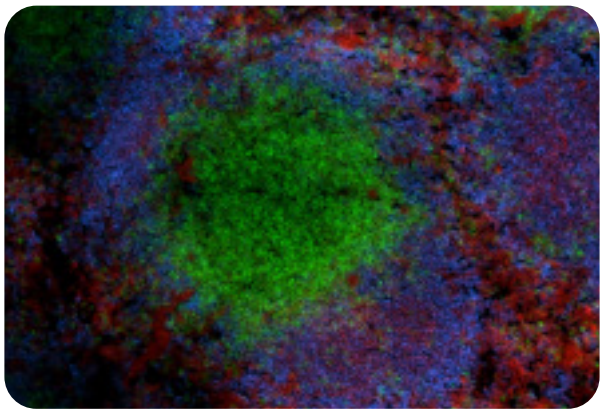


IHC staining of Alexa Fluor® 488 anti-Neurofilament H & M (NF-H/NF-M), hypophosphorylated antibody (clone SMI 35; green) on formalin-fixed paraffin-embedded human cerebellum tissue. Nuclei were counterstained with DAPI (blue).

Immunology

The immune system is comprised of various cell types and tissues involved in protecting against infection and pathological conditions. Being able to identify different immune cells and analyze cellular signaling molecules is critical to developing effective treatments for conditions such as autoimmunity, allergy, and of course, infectious diseases. Immune cells are broadly categorized according to whether they are involved in innate immunity or the adaptive immune response, with cellular characterization and analysis being based on key morphological, phenotypic, and functional differences. Microscopy is an excellent tool to complement immunology research as it provides unique contextual cell and tissue characterization.

Learn more about immunology at: biolegend.com/en-us/immunology

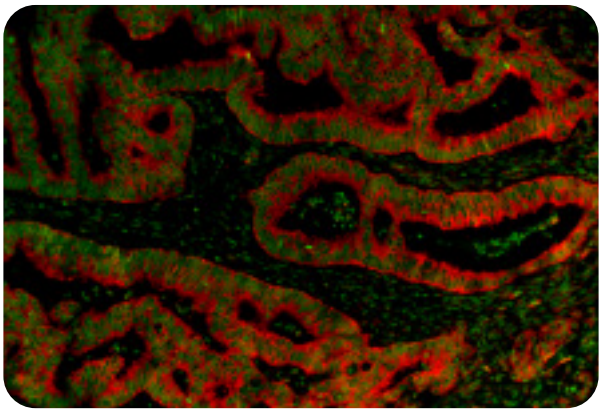


C57BL/6 mouse frozen spleen section was fixed and then stained with anti-CD24 (clone M1/69) Alexa Fluor® 594 (red), anti-CD3 (clone 17A2) Alexa Fluor® 647 (green), and anti-B220 (clone RA3-6B2) Brilliant Violet 421™ (blue).

Cancer Research

Cancer biology is broad and complex, as the causes and controls of cancer span a diverse range of biological processes, including cell cycle, epigenetics, and angiogenesis. We provide solutions for advancing all aspects of cancer research, from understanding the mechanisms and pathways associated with tumorigenesis to the development of novel immunotherapies. Microscopy has become a valuable tool for assessing various tissues and cell types for markers of interest, particularly for monitoring phenotypes of cancerous cells. We provide an extensive selection of directly conjugated antibodies as well as non-antibody chemical probes to help characterize the tumor macro and microenvironment, using techniques such as whole animal imaging and 3D microscopy.

Learn more about cancer research at: biolegend.com/en-us/cancer

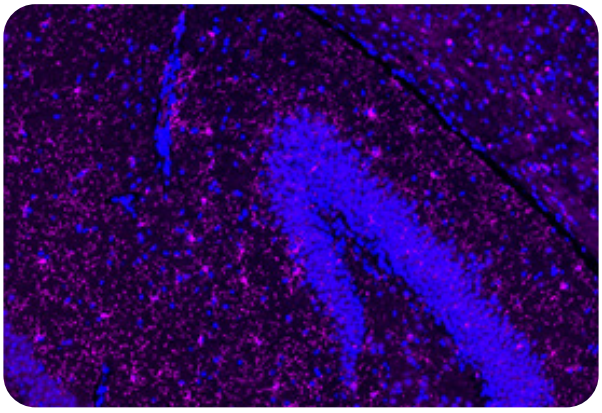


Human paraffin-embedded colon cancer tissue slices were prepared with a standard protocol of deparaffinization and rehydration. Tissue was stained with anti-CD44 (clone C44Mab-5) Alexa Fluor® 594 (red). Nuclei were counterstained with DAPI (green).

Neuroscience

The nervous system is composed of various cell types with different functions and morphological differences. Neurons have highly compartmentalized structures that are generally classified into soma, axon, dendrites, axon terminal, and the synapse. Neurodegenerative diseases result from the loss of neurons or dysregulation of their function, which can have debilitating effects like dementia, memory loss, and ataxia. Treatments for these diseases are limited, highlighting the need to gain a better understanding of their mechanisms and origins. We offer directly conjugated antibodies against markers that are expressed in each structural unit of a neuron, as well as protein- and modification-specific antibodies to visualize protein aggregation associated with neurodegeneration in applications such as IHC and ICC.

Learn more about neuroscience at: biolegend.com/en-us/neuroscience



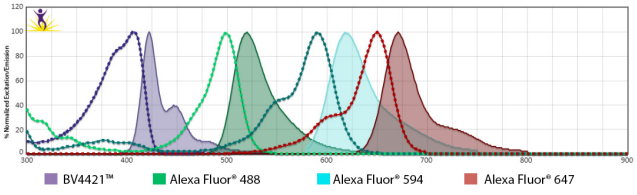
IHC staining of purified anti-P2RY12 antibody (clone S16007D; magenta) to label microglia on formalin-fixed paraffin-embedded mouse brain tissue. Nuclei were counterstained with DAPI (blue).

Best Practices

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 considerations when choosing the right reagents and instruments.

Number of Targets

It's possible to do a five-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 five 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.

Fluorophore Combinations With Overlapping Spectra

In instances where fluors are excited by other wavelengths and have some spillover into a neighboring filter, the spillover is usually suboptimal and results in a weak hazy background. To mitigate this, ensure 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. Also, make sure the fluorophore that is spilling over is on the less abundant antigen. Alexa Fluor® 555 can spill into Alexa Fluor® 594, but it is possible to resolve this by narrowing their filter bandpasses.

To view potential fluorophore or dye combinations and their excitation and emission profiles, visit: searchlight.semrock.com

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 the 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.

Instrument Choice

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

Do I want to image tissue thicker than 20 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

Sensitivity

Sensitivity is a balance between the signal strength and background noise. Biological autofluorescence is endogenous in some tissues, like brain or lung. To help with this, use an appropriately complex blocking reagent prior to the addition of antibodies, *e.g.,* serum instead of BSA. If the tissue will be fixed and permeabilized, an endogenous biotin-blocking kit can prevent the biotin found in mitochondria from binding streptavidin.

Use directly conjugated primary antibodies for abundant antigens. Use secondary antibodies, biotin/streptavidin, or hapten-based methods to amplify lowly expressed antigens. For extremely rare antigens, use enzymatic tyramide signal amplification (TSA) kits. Amplification and secondary techniques can raise the intended signal from the target, but can also increase background noise.

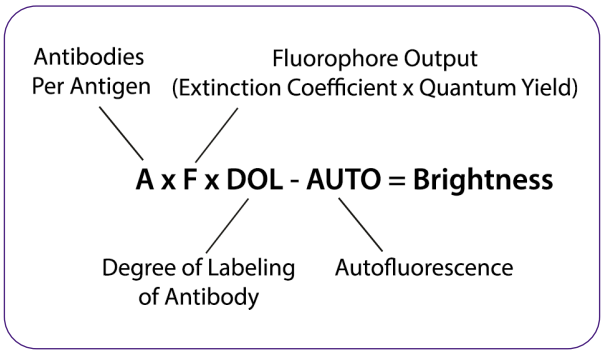
Antifade

Mounting media containing antifade is required to maintain signal strength. All organic fluors photobleach, a process where reactive oxygen species created during imaging attack the structure of the fluors and irreversibly neutralize their ability to fluoresce. Since antifade scavenges oxygen and suffocates cells, antifade is more difficult to use in live cell imaging. This is why regenerating signal, *e.g.,* GFP-proteins, are desirable for long-term, live cell imaging.

Understand "Brightness" in Microscopy

Fluor brightness is not a simple measurement of the properties of the individual fluor. Several intrinsic and extrinsic factors affect the final value of brightness, as shown in the formula to the right. The variables listed do not change the actual brightness of the antibody. Rather, they affect the brightness perceived by the end user and their ability to image the target.

If you're comparing two fluors, it is important how the pixels in the camera/PMT are binned, *i.e.,* the power of lasers exciting two fluors with different excitation wavelengths and the quantum efficiency of the cameras/PMTs at two different emission intensities. These variables are not universal and vary with the instrument and end-user.



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BioLegend products are manufactured in an ISO 13485:2016-certified facility to ensure the highest quality standards.

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