# Immunobiology
## Handbook

BioLegend is ISO 13485:2003 Certified

# BioLegend®

World-Class Quality | Superior Customer Support | Outstanding Value

# Table of Contents

# Introduction

Immunobiology can be defined as the study of immune cells and the immune system, understanding their roles in health and disease. BioLegend is proud to provide this handbook to serve as a quick reference and guide regarding techniques and applications relevant to Immunobiology.

From flow cytometry to magnetic cell separation to cell imaging, BioLegend is a global leader in providing research reagents and support for the bioresearch community. This handbook covers a number of critical topics for immunobiology research, including flow cytometry panel design, cell health and proliferation, biofunctional antibodies, imaging, and much more. We also present commonly used protocols for immune research, as well as images of our popular immune pathway posters.

**Visit our Immunobiology page at: biolegend.com/immunobiology.**

**Our technical team is always willing find the answers you need, so don't hesitate to contact us at: tech@biolegend.com.**

3

# Multicolor Flow Cytometry

# Build a Better Multicolor Flow Cytometry Assay

The foundation of a successful multicolor flow assay is good planning and step-by-step optimization. Assays involving four to six fluorescent colors are not very complicated to construct or analyze, since the reagents chosen will be the brightest available with the least spectral spillover. Many researchers want greater than six colors, maximizing the number of markers due to limited sample volume or to see the interconnectivity of many parameters simultaneously. The task of increasing the number of markers in a multicolor panel requires that a set of guiding principles be followed, to ensure that sensitivity and statistical consistency are maintained between sample and day. As the number of markers increases, and thus the number of colors with overlapping spectra, so do the complexities of experiment and analysis. You can minimize aberrations associated with poor assay planning, lack of prior antibody titration optimization, and inadequate use of appropriate controls to ensure confidence in the resulting data set.

**In addition to the information provided here, please refer to:**
**biolegend.com/multicolor_staining**

## Multicolor Panel Balance

1. Know your instrument specifications, including which excitation lasers, laser wattages, filter widths and wavelengths are set-up on your machine.

2. Consider the expression level of the antigens you'd like to detect in the experiment and how they might change with donor, sample type or stimulation condition.

3. Understand any endogenous biological considerations that may influence your results.

4. Understand the spectra, brightness, stability and all other pros/cons associated with different fluorophores and reagents employed.

5. Optimize the assay and include appropriate controls to help with analysis.

## Instrument Specifications

**Know Your Instrument!**

Consult your flow cytometry core facility on which laser lines are available on each instrument and the filter specifications for each active photomultiplier tube (PMT) on the instrument you choose. Most instruments suitable for >10 color flow cytometry do not have a single fixed configuration. Figure 1 is just one example of a common filter configuration for the violet 405 nm laser used to detect the Brilliant Violet™ family of fluorophores.
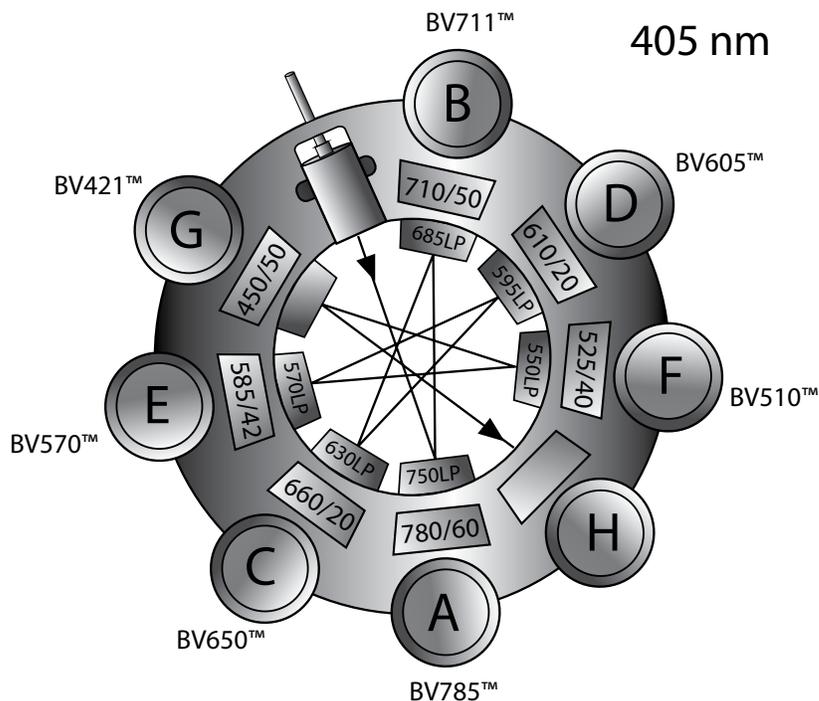


Figure 1. A typical octagon configuration off the 405nm violet laser suitable to detect the Brilliant Violet™ fluorophores.

Brilliant Violet™, Brilliant Violet 421™, BV421™, Brilliant Violet 510™, BV510™, Brilliant Violet 570™, BV570™, Brilliant Violet 605™, BV605™, Brilliant Violet 650™, BV650™, Brilliant Violet 711™, BV711™, Brilliant Violet 785™, and BV785™ are trademarks of Sirigen Group Ltd.

5

## Balance Antigen Expression vs. Fluorophore Brightness

1. Prior to choosing a particular fluorophore/antibody combination, determine the expression level of the antigen you wish to detect. If the expression level is entirely unknown, reserve the brightest fluorophores for this antibody to ensure the best chance of lower limit detection and dynamic range of expression.

**For expression of common markers on human immune cell types, go to:**
**biolegend.com/protein_expression**

2. Just as important as the common abundance of antigen is an understanding of how a disease state, exogenous treatment or stimulation may affect antigen expression. A good example is CD4: although it is abundant in both mouse and human peripheral blood, CD4 is downregulated with PMA/Ionomycin treatment.
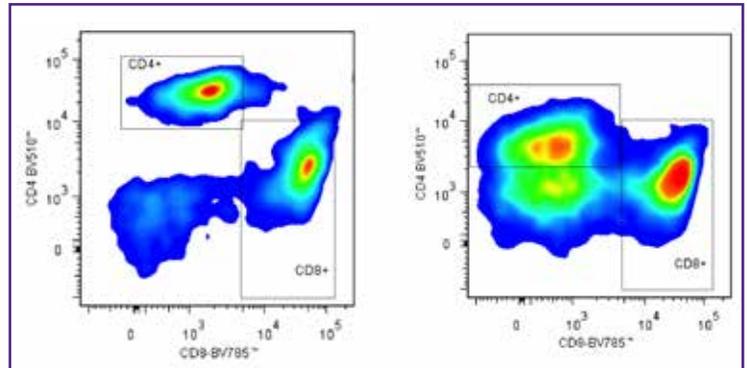


Figure 2. Left plot shows cells without stimulation and right plot shows cells stimulated for six hours with PMA and Ionomycin. Cells were analyzed 2 days post fixation.

## Overview of the Different Fluorophore Families Useful as Antibody Conjugates

There are three families of fluorescent molecules routinely employed in antibody-based cellular assays: simple organic molecules, proteins and fluorescent polymers. Simple organic fluorophores like the Alexa Fluor® dyes, Pacific Blue™, and FITC are small molecular weight chemical structures between 350D-1200 kD. Due to their small size, there are many of them conjugated to a single antibody to increase the total brightness potential of that antibody. How many fluorophores are conjugated to a single antibody is called the degree of labeling or Fluorophore to Protein (F:P) ratio. Because of their size, there is a limitation as to how much excitation energy each fluorophore can absorb. This value is called the extinction coefficient (EC). Depending on its environment and solvent, the fluorophore will exhibit a certain efficiency with which it transfers energy between the excited and emitted state called the quantum efficiency (QE) or quantum yield (QY).

Based on these three major factors, when we consider the total brightness the researcher will be able to derive from a single antibody, it follows this sort of equation:

**Brightness of Antibody= Degree of Labeling (DOL) × (EC of fluorophore × QY of fluorophore)**

The sensitivity with which you are able to detect that fluorescently labeled antibody (Ab) *in situ* then also becomes dependent on how many primary antibodies will detect the antigen (Ag), the abundance of those antigens on a cell or within a pixel minus the autofluorescence and background from the reagents associated with labeling cells and tissues from many origins. So, in a practical sense, the useful signal and lower limit sensitivity that a researcher will detect to image or quantify is more accurately depicted as:

**Sensitivity *in situ*=[Brightness of Antibody × (# Abs per Ag × # Ags)] - (autofluorescence + reagent non-specific staining)**

Refer to Table 1, the fluorophore staining index chart, to match the potential abundance of the antigen in your assay with an appropriately bright fluorophore relevant to the application and instrument to be used. Here, every fluorophore was conjugated to an anti-mouse CD19 antibody (clone 2D1) to construct an accurate ranking based on staining index calculated by $(MFI^+ - MFI^-)/(2xSD\ MFI^-)$. Staining index in this instance can be influenced by laser wattage and filter widths which we've listed in this table; thus, rankings will vary slightly between manufacturers. Rankings like this should only be used for reference purposes.

6

Table 1. Staining Index and Fluorophore Brightness

| Fluorophore | Ex (nm) Max | Em (nm) Max | Filter Used | Brightness | Histogram |
|---|---|---|---|---|---|
| PE | 565 | 575 | 585/20 | 5 | |
| PE/Cy5 | 565 | 670 | 660/20 | 5 | |
| APC | 650 | 660 | 660/20 | 5 | |
| PE/Dazzle™ 594 | 565 | 610 | 610/20 | 5 | |
| Brilliant Violet 421™ | 405 | 421 | 450/50 | 5 | |
| PE/Cy7 | 565 | 774 | 780/60 | 4 | |
| Brilliant Violet 605™ | 405 | 603 | 610/20 | 4 | |
| Brilliant Violet 711™ | 405 | 711 | 710/50 | 4 | |
| Alexa Fluor® 647 | 650 | 668 | 660/20 | 4 | |
| Brilliant Violet 650™ | 405 | 645 | 660/20 | 4 | |
| Brilliant Violet 785™ | 405 | 785 | 780/60 | 3 | |
| Brilliant Violet 510™ | 405 | 510 | 510/50 | 3 | |
| Brilliant Violet 570™ | 405 | 570 | 585/42 | 3 | |
| Alexa Fluor® 488 | 495 | 519 | 530/30 | 3 | |
| PerCP/Cy5.5 | 482 | 690 | 695/40 | 2 | |
| APC/Cy7 | 650 | 774 | 780/60 | 2 | |
| Pacific Blue™ | 410 | 455 | 450/50 | 2 | |
| FITC | 493 | 525 | 530/30 | 2 | |
| APC/Fire™ 750 | 650 | 787 | 780/60 | 2 | |
| Alexa Fluor® 700 | 696 | 719 | 720/45 | 1 | |
| PerCP | 482 | 675 | 695/40 | 1 | |
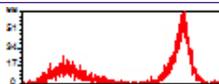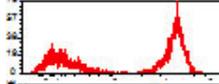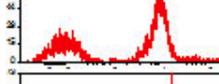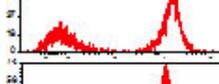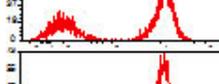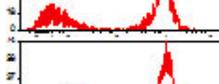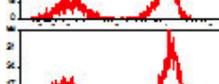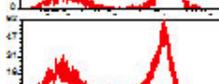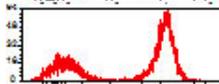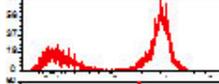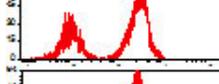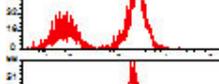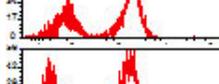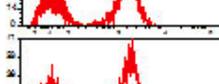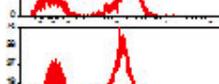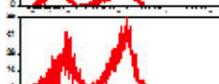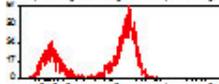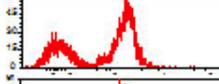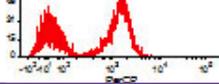
Brighter fluorophores are not always better fluorophores. For example, PE/Cy5 is very bright, but has significant cross-beam excitation by the 633nm/red laser and thus emits strongly into the APC or Alexa Fluor® 647 channel. FITC, on the other hand, is not a very bright fluorophore, however it emits into a channel that experiences little spillover from other fluorophores and is available commercially conjugated to an abundance of antibodies. Staining index is just a tool to help guide you in balancing a panel.

**For more information on fluorophore brightness, visit:**
**biolegend.com/brightness_index**

# Protein-Based Fluorophores and their Tandems

Due to their large size, protein-based fluorophores like phycoerythrin (PE: ~240 kD) and allophycocyanin (APC: 105 kD) are conjugated to antibodies at a 1:1 or 1:2 ratio respectively. In order to increase the multiplexing ability of fluorescent assays like flow cytometry, tandem fluorophores were created to diversify the spectral options available. Tandem fluorophores are two fluorescent molecules in a FRET (Förster or fluorescence resonance energy transfer) relationship, where the fluorophore capable of absorbing and transmitting the most energy at the shorter wavelength can donate that energy to an array of different kinds of acceptor molecules. A donor fluorophore, such as APC, PE, PerCP or BV421™, is excited by its particular optimal excitation source, and upon resonating that energy, transfers it to an acceptor such as Cy7 in the APC/Cy7 tandem. In this instance, the APC/Cy7 tandem is excited at the excitation peak for APC, which is 650 nm. However, while in its excited state, a single phycobilin, which is the actual fluorescent molecular subunit embedded within the APC protein, will donate its electronic energy directly to the Cy7 within a proximity of 10-80 Å (angstroms), causing an emission at the peak of Cy7, which is 775 nm. FRET is rarely 100% efficient in a tandem and a certain amount of energy will still be populated in the emission channel of the donor fluorophore and thus require some compensation be applied. For this reason and also due to the harsher and longer lasting exposure to light and photobleaching that fluorophores experience in microscopy applications, tandem fluorophores are also not recommended for imaging.

The stability of these tandems is of paramount importance for consistency in brightness and spectral spillover/compensation. For example, APC/Cy7 is known to have issues in its stability to light exposure, temperature and different fixative conditions. This is why alternatives like the APC/Fire™ 750 have effectively replaced APC/Cy7 in multicolor assays.

**Learn more about APC/Fire™ 750 at:**
**biolegend.com/apc-fire750**



| | |
|---|---|
| No Antibody | ■ (black) |
| APC/Fire™ 750 | ■ (red) |
| APC/Cy7 | ■ (blue) |
| APC-H7 | ■ (purple) |
| APC-eFluor® 780 | ■ (green) |
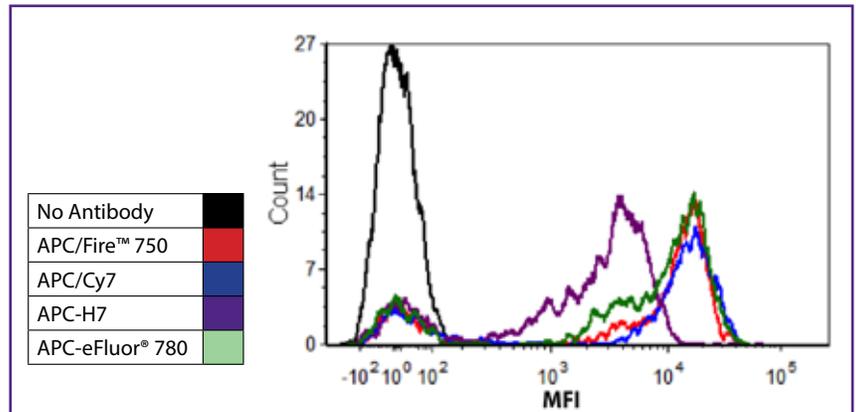
Figure 3. Human whole blood was stained for 20 min with CD3 (SK7) conjugates of APC/Fire™ 750, APC/Cy7, APC-H7, or APC-eFluor® 780 at each manufacturer's recommended optimal dilution, followed by RBC lysis and wash steps. Histograms were gated on lymphocyte populations based on forward and side scatter.
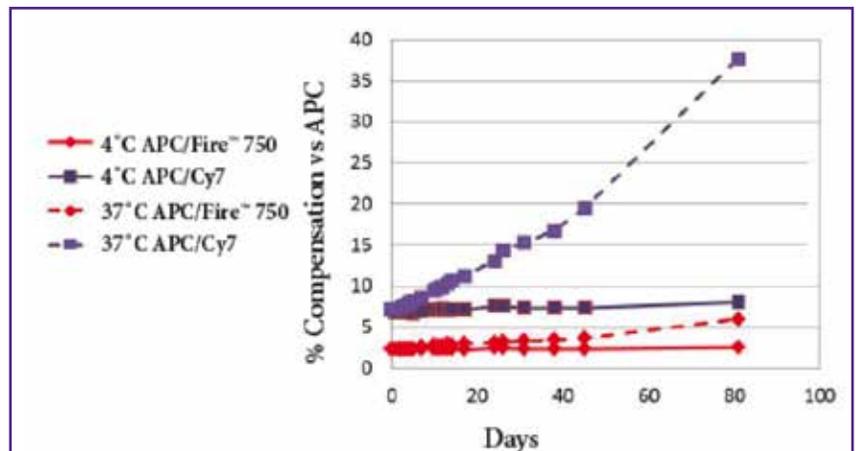


Figure 4. APC/Fire™ 750 is significantly more temperature stable versus APC/Cy7. Within 20 days of the vial of antibody being left at room temperature in the dark, there begins a significant increase in compensation with APC/Cy7. The temperature stability results were generated using the Veri-Cells™ PBMC, a lyophilized human PBMC product that effectively removes donor-dependent variation in staining for comparisons like this. Cells were stained for 15 minutes at room temperature in cell staining buffer.



| | |
|---|---|
| Unstained cells | ■ (black) |
| APC/Fire™ 750 | ■ (red) |
| APC/Cy7 | ■ (blue) |
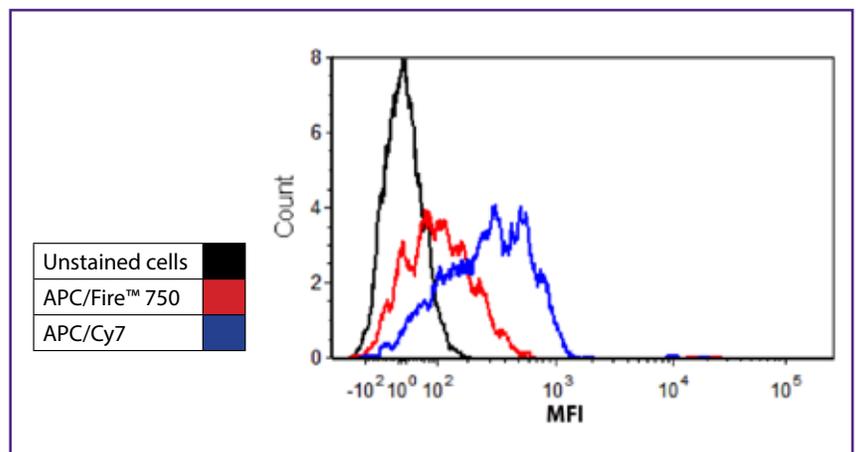
Figure 5. APC/Fire™ 750 also exhibits less unwanted non-specific binding to monocytes than APC/Cy7. Here monocytes are gated and the MFI⁻ population in the APC/Cy7 emission channel is monitored for any change in non-specific binding. More data is shown on this phenomenon and how to block it in the section on buffers for flow cytometry, specifically the True-Stain Monocyte Blocker™.

# Brilliant Violet™ Fluorophores

A relatively new family of fluorescent polymers, called the Brilliant Violet™ fluorophores, has enabled multicolor fluorescent applications in both flow cytometry and microscopy. These polymers have a very high extinction coefficient, around $2.5 \times 10^6$ M$^{-1}$cm$^{-1}$ for Brilliant Violet 421™ compared to PE which is around $2 \times 10^6$ M$^{-1}$cm$^{-1}$. The Brilliant Violet™ fluorophores provide very bright options with the widest diversity of emissions off of a single laser compared to any other reagent. Tandem fluorophores of Brilliant Violet 421™ give rise to Brilliant Violet 570™, Brilliant Violet 605™, Brilliant Violet 650™, Brilliant Violet 711™ and Brilliant Violet 785™. Each of their specific excitation and emission characteristics are outlined in the Staining Index table as well as the Fluorophores for Flow Cytometry poster.

Brilliant Violet 510™ is also a non-tandem organic polymer, similar to BV421™. However, BV510™ has unique spectral characteristics similar to AmCyan and Horizon V500™ and is also a brighter alternative to either of these fluorophores. BV421™ and BV510™ now enable the application of 5 color microscopy applications using directly conjugated antibodies. On a standard widefield microscope equipped with excitation and emission filter sets optimally matched to each of these fluorophores, simultaneous 5 color labeling with BV421™, BV510™, Alexa Fluor® 488, Alexa Fluor® 594 and Alexa Fluor® 647 is possible.
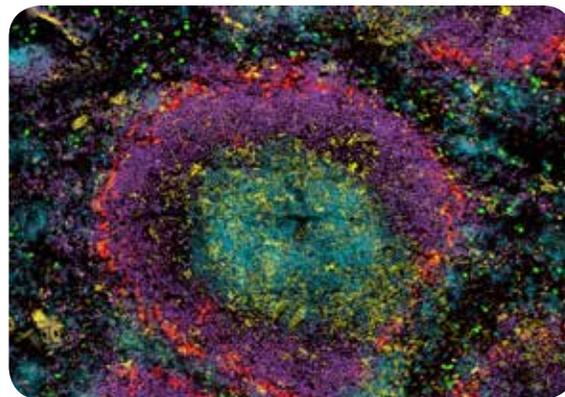


Figure 6. Frozen C57BL/6 mouse spleen was fixed, blocked, and stained with anti-mouse CD169 (clone 3D6.112) Alexa Fluor® 594 (red), anti-mouse B220 (clone RA3-6B2) BV510™ (purple), anti-mouse Ly6G (clone 1A8) Alexa Fluor® 647 (green), anti-mouse CD4 (clone GK1.5) Alexa Fluor® 488 (yellow), and anti-mouse CD8a (clone 53-6.7) BV421™ (aqua).

**For more information on Brilliant Violet™ fluorophores and their applications in imaging, visit: biolegend.com/brilliantviolet and biolegend.com/microscopy**
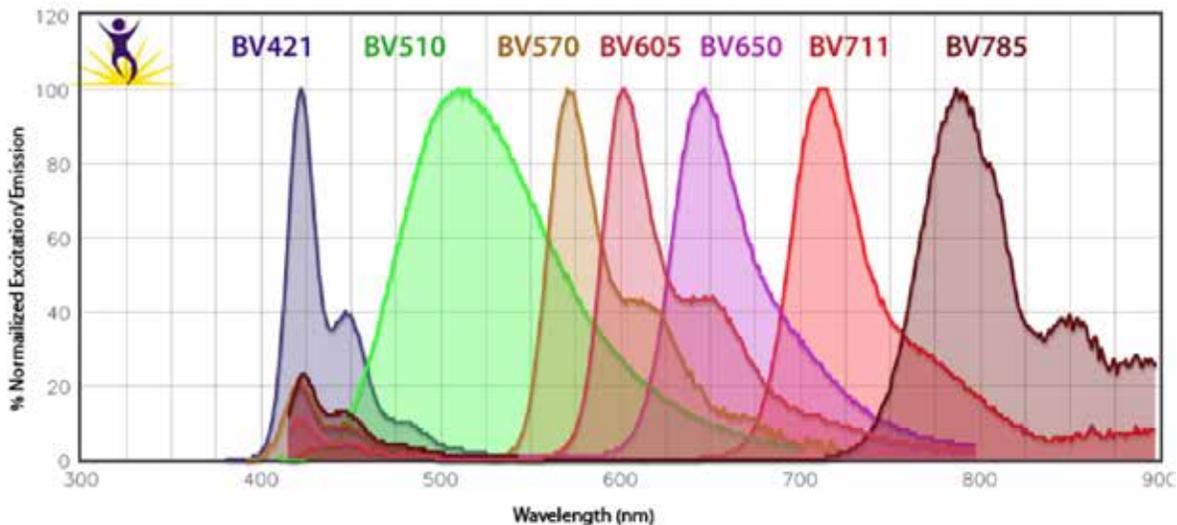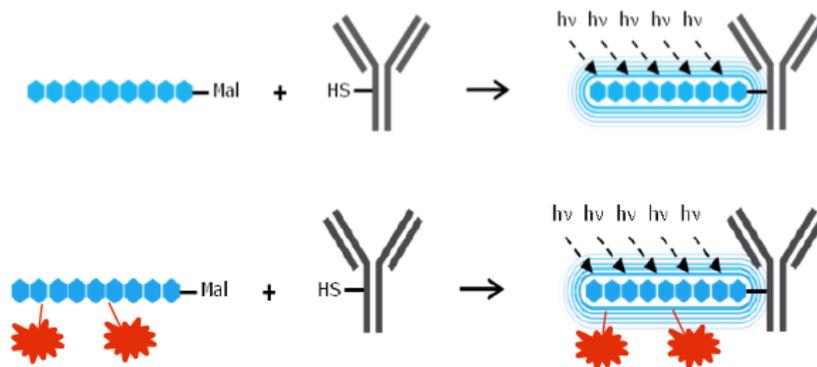
# How to Organize a Balanced Panel

Identify and organize your targets into three tiers:

- Primary tier antigens: These markers do not change between parallel panels and are often, but not always, expressed at high levels. When they are expressed abundantly, they should be dedicated to dimmer fluorophores, such as Alexa Fluor® 700, BV510™, BV570™, APC/Cy7 and APC/Fire™ 750.

Examples: basic cell surface phenotypic markers like CD4, CD8, CD14, CD56, CD11c, etc.

- Secondary tier antigens: These markers are necessary for further subset phenotyping, but may change somewhat between parallel panels. Expression levels tend to be much more variable and are typically appropriate for moderately bright fluorophores like BV650™, BV711™, BV785™, FITC, Alexa Fluor® 488 and PerCP/Cy5.5.

Examples: activation or exhaustion markers, cytokines and chemokine receptors

- Tertiary tier antigens or "wish list" markers: These markers are often the most important question you are asking in the assay and may have widely variable or completely unknown expression levels or patterns. So, for the detection of these antigens, we want to save the brightest fluorophores like BV421™, BV605™, PE, PE/Dazzle™ 594, APC, Alexa Fluor® 647 and PE/Cy7.

Examples: transcription factors, phospho-specific antibodies and homemade hybridomas.



Figure 7. An example of typical fluorophore/antibody combinations organized into tiers of the panel construction.

## Optimize and Control

Interpreting experimental results is as important as obtaining the data to support solid conclusions. To make accurate observations, every experiment should contain the proper types of controls. For flow cytometry, at least three basic types of controls should be included: instrument controls, specificity controls, and biological controls. In many situations, one control can serve multiple purposes. Instrument controls are those that will help set up or confirm the instrument parameters, or even calibrate the machine. Specificity controls are those designed to help distinguish a true positive signal from the negative, even when its distribution is affected by spreading error after compensation is applied, or false positive signals due to non-specific binding. In other words, these are the controls that help determine the specific signal detected with a given reagent. They are used to properly set gates to define positive or negative populations for specific markers. Biological controls are used when a stimulation, knockout or disease state requires a normal state control to guide gating strategies in determining true positive signal from artifact, *i.e.*, stimulated or treated cells, knockout cells and other relevant samples.

10

## Instrument Controls

Requirements to set up a balanced experiment vary from instrument to instrument, but regardless of the specific condition, two parameters must be properly qualified: the photomultiplier tube (PMT) voltage and compensation needed between the multiple fluorophores used.

1.  PMT Voltage Controls: While the most common strategy to set up PMT voltages is the use of an unstained sample, this approach doesn't fit all situations. For example, when analyzing markers that dramatically upregulate their expression after certain treatments, the positive signal may be so high that the entire population could be off scale if the voltage is relatively high. In such cases, it will be useful to have single color stained cells to verify the signal is measurable and on-scale. Calibration beads are very important for ensuring consistent performance of the cytometer. However, the recommended voltages, especially for the violet laser, often do not correlate with the abundance of signal in the assay or the brightness of the fluorophore.

2.  Compensation Controls: Compensation controls need to be used every time you run an assay to ensure accuracy. Although either compensation beads or single stained biological controls can be used to apply compensation, beads have the advantage of ease in staining, total abundance of events, and sufficient representation of both the negative and positive populations necessary to populate the compensation algorithm accurately. It is very important to use the same antibodies as were used in the assay, especially if the antibodies are conjugated to tandem fluorophores. This is because tandem fluorophores, for example APC/Cy7, are sensitive to the light, temperature and fixatives they endure while the sample is being stained. For example, in the section regarding buffers useful for flow cytometry, there is an example of an increase in compensation of APC/Cy7 into the APC channel on fresh cells versus those that have been fixed with transcription factor staining buffers. Thus, whether single stained beads or cells are used as the compensation control, for greatest accuracy, they should endure the same light, temperature and solvent exposure as was applied to the fully stained sample.
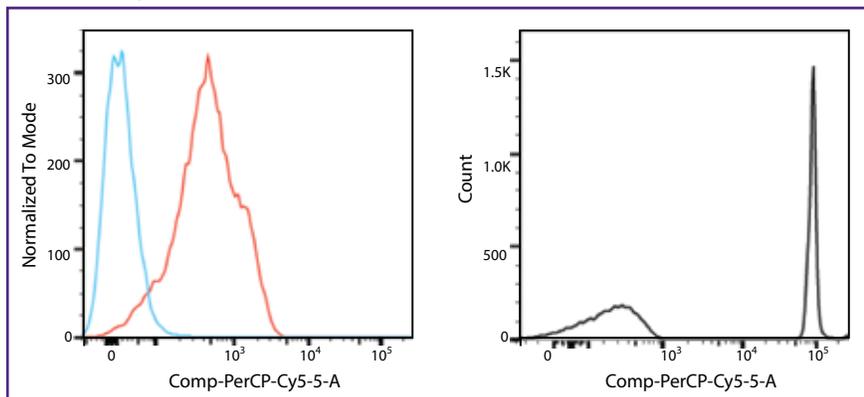


Blue = Unstained mouse splenocytes

Red = CD69 single stained mouse splenocytes from activation model

Black=compensation beads labeled with CD69 PerCP/Cy5.5

Figure 8. The plot on the left demonstrates an instance where the single stained biological control is inadequate for use as a compensation control, since there are not sufficient negative (non-activated) events to accurately populate an MFI⁻ peak. The plot on the right is the same experiment with the same antibody labeled to compensation beads that can capture an antibody raised in armenian hamster. The caution with compensation beads is that it is easy to accidentally cause the MFI⁺ peak to become off-scale due to over-labeling the compensation bead at the voltage determined to be ideal for the experimental assay. The voltage should be tuned to suit the assay, not to accommodate the overly bright compensation bead.

## Specificity Controls

After selecting PMT voltages and properly compensating all the channels used, samples can be acquired. Depending on the requirements of the experiment, it may be necessary to define one or more gates around the population or cell subsets of interest.

1. Fluorescence Minus One (FMO) Controls: FMO controls are a gating control that involves the addition of all the antibody conjugates in a panel except one marker. When overlaid with a fully stained panel, it provides a guide to where to gate the population relevant to the marker omitted from the FMO. So, simply, FMO controls are gating controls that become important in multicolor staining scenarios where there is an additive degree of spectral spillover widening the distribution of some populations that are not reflective of any change in actual antigen expression or distribution. FMOs also help you keep an eye on the consistent performance of your antibodies, especially tandem conjugates, to help identify the culprit should any problems arise that might create artifacts or confusion about the results.

2. Isotype Controls: Isotype controls are important if you suspect or are unsure of a source of non-specific binding. A good example of appropriate isotype control usage is on cell types that patrol and scavenge the biological environment, like monocytes and macrophages. It's important to provide evidence that a signal you deem positive is indeed not an artifact with any propensity to non-specifically bind. In a multicolor assay of 10 colors or more, the isotype control can be incorporated into what would have been the FMO control for that marker.



Figure 9. In this example, FMOs for both CD14 BV605™ (Orange) and CD16 APC/Cy7 (Blue) are overlaid on top of the fully stained sample (Red). By using the edges of the FMOs as a guide (for example, where the edge of the CD16 APC/Cy7 staining ends on the APC/Cy7 axis), it is much easier to accurately elicit the CD16 $^{lo}$ from the CD16$^-$ and CD16$^{hi}$ populations, identifying classical, intermediate and non-classical monocyte populations.
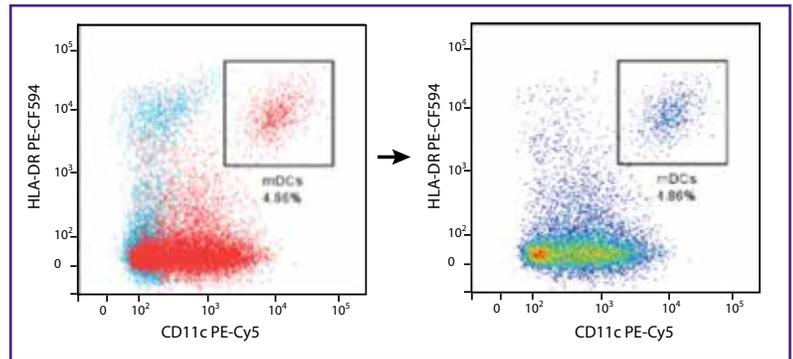


Figure 10. In this example, instead of entirely negating the CD11c PE/Cy5 in the FMO, the isotype control (Blue) replaced the antibody, ensuring that the cells that fall into the gate for myeloid dendritic cells (Red) are not the result of non-specific binding. Isotype controls should be purchased from the same manufacturer as the primary antibody and used at exactly the same concentration as the primary antibody.

## Biological Controls

Certain scenarios require controls that go beyond qualifying the instrument and the assay performance. For example, as mentioned above, certain treatments may cause a dramatic change in the signal detected with certain antibodies or reagents. Stimulated cells or cells from infected tissues may have different properties than unstimulated or non-infected cells. These samples may have higher background fluorescence, or may induce a higher non-specific antibody binding. In such cases, the best control could be the single or fully stained, or even unstained treated sample. Likewise, it may be important to have a reference value when quantifying certain cells, to be sure the results are accurate. In this regard, BioLegend Veri-Cells™ provide an excellent tool to verify assay performance.

**Learn more about Flow Cytometry controls at: biolegend.com/flow_controls**

12

1. Autofluorescence controls: Different cell types and tissues have varying levels of inherent fluorescence. Major sources of autofluorescence include NADH, riboflavin, metabolic cofactors, the crosslinking of primary amines by paraformaldehyde, and certain biological structures (*e.g.,* mitochondria, lysosomes). These proteins and molecules are more easily excited at lower wavelengths (*i.e.,* from the UV, violet, and blue lasers) and will emit at a wide range of 300-600 nm, which overlaps with several common fluorophores like BV421™, Pacific Blue™, and FITC. Myeloid cell lineages tend to be particularly autofluorescent. Furthermore, stimulating cells can cause them to become more metabolically active, producing more autofluorescent proteins, vitamins, and cofactors. An unstained control sample is helpful in delineating how much autofluorescence populates your channel of interest.

2. Stimulation Controls: Background signal can change when you stimulate samples, potentially affecting population resolution. For example, PMA stimulation can cause a decrease in surface CD4 expression in T cells while also increasing the level of autofluorescence in short wavelength emission channels with the production of autofluorescent molecules. Alternative gating strategies may have to be used (*i.e.,* CD3$^+$, CD8$^-$, CD56$^-$ events) or the intracellular detection of CD4.

3. Cell number controls/positive staining controls: In some cases, it will be necessary to include a biological control to verify that the percentage or absolute number of cells in a defined gate is accurate. It may also be needed to verify that the experimental set up is correct and working properly by verifying the detection of certain cell markers. In these cases, BioLegend Veri-Cells™ are extremely convenient and useful. Veri-Cells™ are lyophilized cells validated for multiple cell markers that have exceptional stability and exhibit a scatter profile similar to freshly isolated cells.
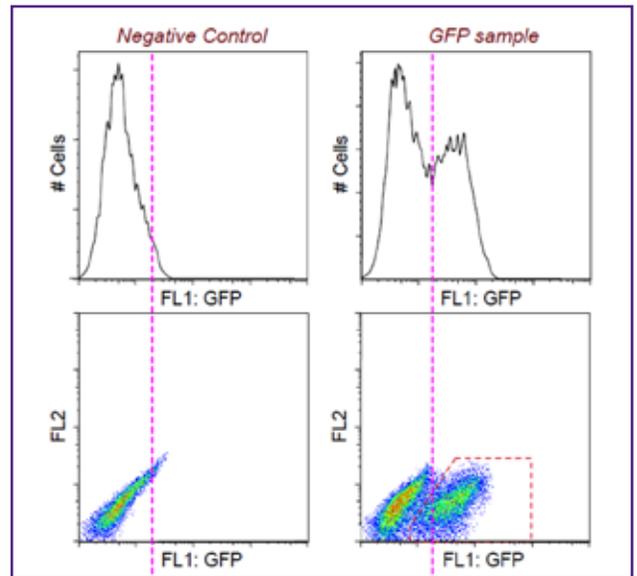


Figure 11. Histograms do not fully reflect the origin of the fluorescence and the distribution of the populations. Drawing a diagonal gate around the GFP positive population in the dot plot (rather than the histogram) more accurately defines the GFP positive population. *Figure provided by Jennifer Wilshire, Ph.D.*
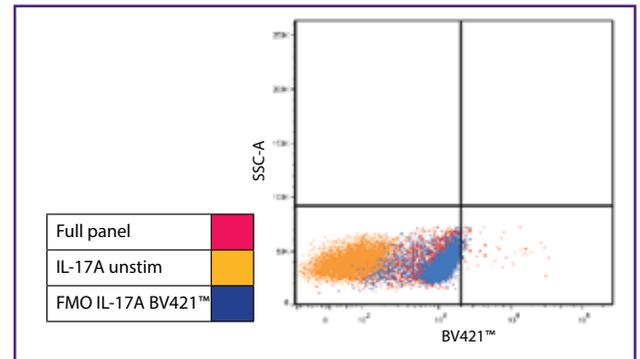


Figure 12. When cells are stimulated, they can produce vitamins and metabolic cofactors that often exhibit autofluorescence in channels excited off the violet and blue lasers.
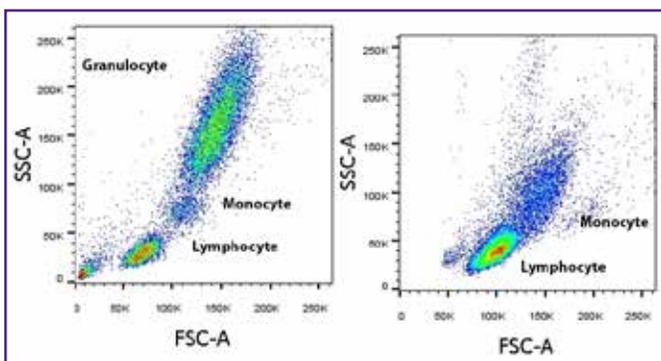


Figure 13. Unlike Veri-Cells™ PBMC and CD4-Low PBMC, Veri-Cells™ Leukocytes contain neutrophil and eosinophil populations. These cells can be used as controls or reference material to monitor expression of granulocyte markers such as CD15 and CD16. The preserved scatter profile demonstrates excellent performance.
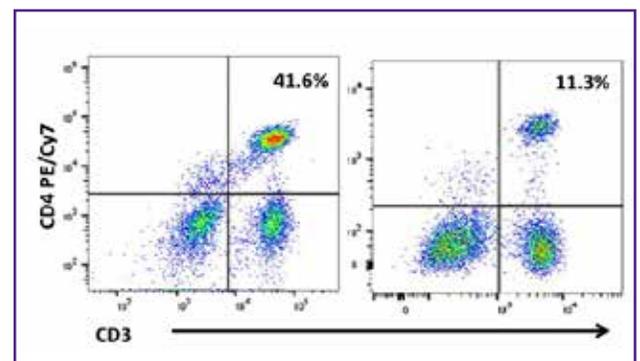


Figure 14. Veri-Cells™ CD4-Low PBMC are specially formulated to contain a lower frequency of CD4$^+$ cells, similar to that observed in patients with CD4 immunodeficiency. Left plot shows normal values of CD4, right plot shows Veri-Cells™ CD4-Low PBMC.

13

Veri-Cells™ PBMC and Veri-Cells™ Leukocytes have been verified with BioLegend's LEGENDScreen™ Human Cell Screening (PE) kit. The tables below summarize the markers that render positive signal.

Table 2. Veri-Cells™ PBMC verified markers.

| Marker | Clone | Marker | Clone | Marker | Clone | Marker | Clone |
|---|---|---|---|---|---|---|---|
| CD1c | L161 | CD45 | HI30 | CD99 | HCD99 | CD279 | EH12.2H7 |
| CD1d | 51.1 | CD45RA | HI100 | CD100 | A8 | CD284 | HTA125 |
| CD2 | RPA-2.10 | CD45RB | MEM-55 | CD101 | BB27 | CD290 | 3C10C5 |
| CD3 | HIT3a | CD45RO | UCHL1 | CD102 | CBR-IC2/2 | CD298 | LNH-94 |
| CD4 | RPA-T4 | CD46 | TRA-2-10 | CD116 | 4H1 | CD300e | UP-H2 |
| CD5 | UCHT2 | CD47 | CC2C6 | CD122 | TU27 | CD300F | UP-D2 |
| CD6 | BL-CD6 | CD48 | BJ40 | CD123 | 6H6 | CD314 | 1D11 |
| CD7 | CD7-6B7 | CD49d | 9F10 | CD124 | G077F6 | CD319 | 162.1 |
| CD8a | HIT8a | CD49e | NKI-SAM-1 | CD126 | UV4 | CD328 | 6-434 |
| CD9 | HI9a | CD49f | GoH3 | CD127 | A019D5 | CD335 | 9E2 |
| CD11a | HI111 | CD50 | CBR-IC3/1 | CD132 | TUGh4 | CD337 | P30-15 |
| CD11b | ICRF44 | CD52 | HI186 | CD134 | Ber-ACT35 (ACT35) | CD352 | NT-7 |
| CD11b | CBRM1/5 | CD53 | HI29 | CD148 | A3 | CD354 | TREM-26 |
| CD11c | 3.9 | CD54 | HA58 | CD154 | 24-31 | β2-microglobulin | 2M2 |
| CD13 | WM15 | CD55 | JS11 | CD156c | SHM14 | C3aR | hC3aRZ8 |
| CD14 | M5E2 | CD56 | HCD56 | CD158a/h | HP-MA4 | CLEC12A | 50C1 |
| CD16 | 3G8 | CD57 | HCD57 | CD158b | DX27 | CX3CR1 | 2A9-1 |
| CD18 | TS1/18 | CD58 | TS2/9 | CD158e1 | DX9 | FcRL6 | 2H3 |
| CD19 | HIB19 | CD59 | p282 (H19) | CD161 | HP-3G10 | HLA-A,B,C | W6/32 |
| CD20 | 2H7 | CD61 | VI-PL2 | CD162 | KPL-1 | HLA-A2 | BB7.2 |
| CD21 | Bu32 | CD63 | H5C6 | CD163 | GHI/61 | HLA-DQ | HLADQ1 |
| CD22 | HIB22 | CD64 | 10.1 | CD164 | 67D2 | HLA-DR | L243 |
| CD23 | EBVCS-5 | CD69 | FN50 | CD165 | SN2 (N6-D11) | HLA-E | 3D12 |
| CD24 | ML5 | CD73 | AD2 | CD172a | SE5A5 | HVEM | 122 |
| CD26 | BA5b | CD74 | LN2 | CD172b | B4B6 | IgD | IA6-2 |
| CD27 | O323 | CD79b | CB3-1 | CD172g | LSB2.20 | IgM | MHM-88 |
| CD28 | CD28.2 | CD81 | 5A6 | CD180 | MHR73-11 | Integrin β7 | FIB504 |
| CD29 | TS2/16 | CD82 | ASL-24 | CD182 | 5E8/CXCR2 | NKp80 | 5D12 |
| CD31 | WM59 | CD84 | CD84.1.21 | CD196 | G034E3 | Siglec-9 | K8 |
| CD32 | FUN-2 | CD85 | MKT5.1 | CD197 | G043H7 | TCR gamma/delta | B1 |
| CD33 | WM53 | CD85d | 42D1 | CD200 | OX-104 | TCR Vβ13.2 | H132 |
| CD35 | E11 | CD85 | GHI/75 | CD200R | OX-108 | TCR Vβ23 | αHUT7 |
| CD36 | 5-271 | CD86 | IT2.2 | CD226 | 11A8 | TCR α/β | IP26 |
| CD38 | HIT2 | CD87 | VIM5 | CD229 | Hly-9.1.25 | TCR Vβ8 | JR2 (JR.2) |
| CD39 | A1 | CD88 | S5/1 | CD244 | C1.7 | TCR Vβ9 | MKB1 |
| CD40 | HB14 | CD89 | A59 | CD268 | 11C1 | TCR Vδ2 | B6 |
| CD41 | HIP8 | CD93 | VIMD2 | CD271 | ME20.4 | TCR Vγ9 | B3 |
| CD42b | HIP1 | CD94 | DX22 | CD272 | MIH26 | TCR Vα7.2 | 3C10 |
| CD43 | CD43-10G7 | CD95 | DX2 | CD277 | BT3.1 | | |
| CD44 | BJ18 | CD97 | VIM3b | CD278 | C398.4A | | |

Table 3. Veri-Cells™ Leukocytes verified markers.

| Marker | Clone | Marker | Clone | Marker | Clone | Marker | Clone |
|---|---|---|---|---|---|---|---|
| β2-microglobulin | 2M2 | CD35 | E11 | CD85j | GHI/75 | CD245 | DY12 |
| CD1c | L161 | CD38 | HIT2 | CD86 | IT2.2 | CD263 | DJR3 |
| CD1d | 51.1 | CD39 | A1 | CD87 | VIM5 | CD268 | 11C1 |
| CD2 | RPA-2.10 | CD40 | HB14 | CD88 | S5/1 | CD278 | C398.4A |
| CD3 | HIT3a | CD43 | CD43-10G7 | CD89 | A59 | CD279 (PD-1) | EH12.2H7 |
| CD4 | RPA-T4 | CD44 | BJ18 | CD8a | HIT8a | CD284 | HTA125 |
| CD5 | UCHT2 | CD45 | HI30 | CD94 | DX22 | CD298 | LNH-94 |
| CD6 | BL-CD6 | CD45RA | HI100 | CD95 | DX2 | CD300e | UP-H2 |
| CD10 | HI10a | CD45RB | MEM-55 | CD97 | VIM3b | CD300F | UP-D2 |
| CD11a | HI111 | CD45RO | UCHL1 | CD99 | HCD99 | CD314 | 1D11 |
| CD11b | ICRF44 | CD47 | CC2C6 | CD101 | BB27 | CD319 | 162.1 |
| CD13 | WM15 | CD48 | BJ40 | CD102 | CBR-IC2/2 | CD328 | 6-434 |
| CD14 | M5E2 | CD49d | 9F10 | CD116 | 4H1 | CD335 | 9.00E+02 |
| CD15 | W6D3 | CD49e | NKI-SAM-1 | CD122 | TU27 | CD337 | P30-15 |
| CD15 (SSEA-1) | MC-480 | CD50 | CBR-IC3/1 | CD127 | A019D5 | CD354 | TREM-26 |
| CD16 | 3G8 | CD52 | HI186 | CD132 | TUGh4 | CLEC12A | 50C1 |
| CD18 | TS1/18 | CD53 | HI29 | CD141 | M80 | CX3CR1 | 2A9-1 |
| CD19 | HIB19 | CD55 | JS11 | CD155 | SKII.4 | DcR1 (TRAIL-R3) | DJR3 |
| CD20 | 2H7 | CD56 | HCD56 | CD156c | SHM14 | Galectin-3 (Mac-2) | Gal397 |
| CD21 | Bu32 | CD57 | HCD57 | CD161 | HP-3G10 | HLA-A,B,C | W6/32 |
| CD22 | HIB22 | CD58 | TS2/9 | CD163 | GHI/61 | HLA-DQ | HLADQ1 |
| CD23 | EBVCS-5 | CD59 | p282 (H19) | CD164 | 67D2 | HLA-DR | L243 |
| CD24 | ML5 | CD64 | 10.1 | CD172a/b | SE5A5 | IgD | IA6-2 |
| CD26 | BA5b | CD66a/c/e | ASL-32 | CD172b | B4B6 | LTβR | 31G4D8 |
| CD27 | O323 | CD66b | G10F5 | CD172g | LSB2.20 | NKp80 | 5D12 |
| CD28 | CD28.2 | CD7 | CD7-6B7 | CD180 | MHR73-11 | Siglec-9 | K8 |
| CD29 | TS2/16 | CD74 | LN2 | CD197 | G043H7 | TCR α/β | IP26 |
| CD31 | WM59 | CD81 | 5A6 | CD200 | OX-104 | TCR gamma/delta | B1 |
| CD32 | Fun-2 | CD82 | ASL-24 | CD226 | 11A8 | TCR Vδ2 | B6 |
| CD33 | WM53 | CD84 | CD84.1.21 | CD244 | C1.7 | Vγ9 | B3 |

**Learn more at: biolegend.com/veri-cells**

## Fluorophores and Spectra Poster

As the number of parameters able to be discriminated from one another increases in flow cytometry and microscopy platforms, there is a driving need to diversify the spectral characteristics of fluorophores. This is best achieved when we can draw from a diverse array of different fluorescent chemistries and molecules like semiconductors, fluorescent polymers, proteins and even the simple organic fluorophores we have been using for decades. Together, they create a repertoire of tools that we can use to mix and match in the optimization of a solid multicolor panel.



16

# ow Cytometry

### BioLegend®

Fluor® 488 ③

FITC ③
(Ex 495 nm/Em 520 nm)

PE ⑤
(Ex 565 nm/Em 575 nm)

PE/Dazzle™ 594 ⑤

PE/Cy5 ⑤
(Ex 565 nm/Em 670 nm)

PerCP ①
(Ex 482 nm/Em 675 nm)

PerCP/Cy5.5 ②
(Ex 482 nm/Em 690 nm)

PE/Cy7 ④
(Ex 565 nm/Em 774 nm)

600

700

800

## Zombie Fixable Viability Dyes

Zombie Aqua™
(Ex 382 nm/Em 510 nm)

Zombie Yellow™
(Ex 396 nm/Em 572 nm)

Zombie UV™
(Ex 362 nm/Em 459 nm)

Zombie Green™
(Ex 491 nm/Em 515 nm)

Zombie Red™
(Ex 600 nm/Em 624 nm)

Zombie NIR™
(Ex 719 nm/Em 746 nm)

Zombie Violet™
(Ex 400 nm/Em 423 nm)

400    500    600    700    800    900
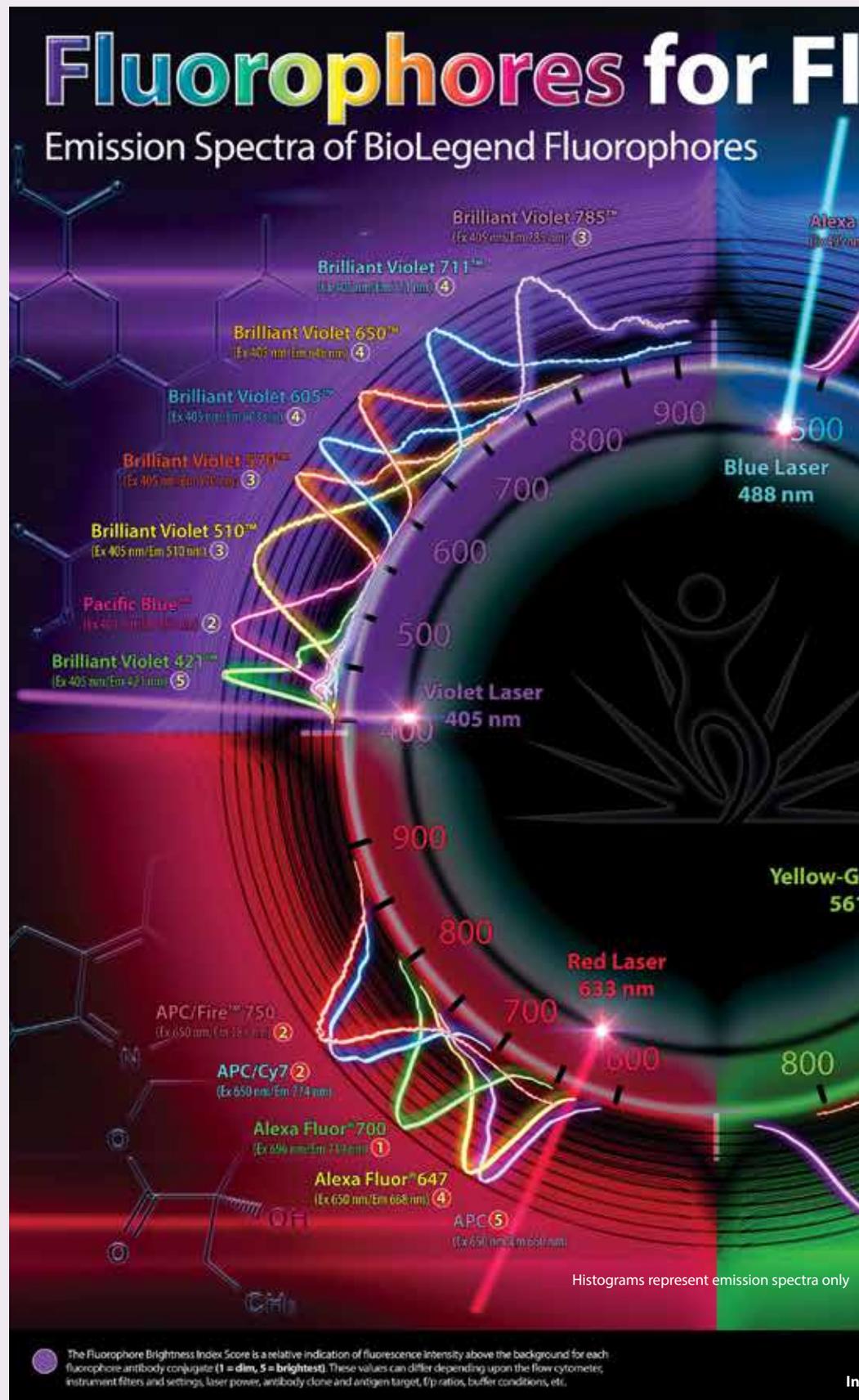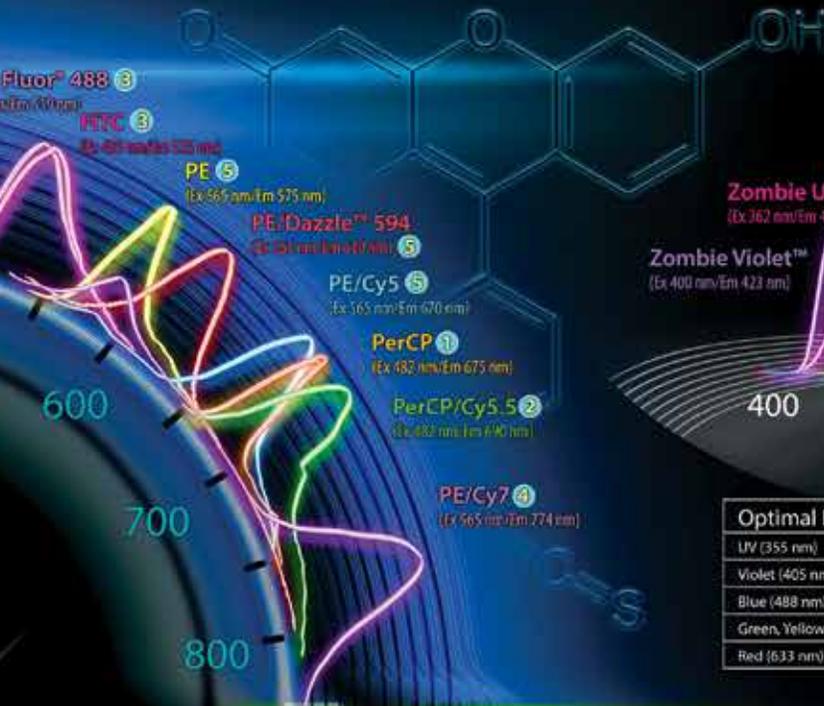
| Optimal Excitation Laser | Reagent |
|---|---|
| UV (355 nm) | Zombie UV™ |
| Violet (405 nm) | Zombie Violet™, Zombie Aqua™, Zombie Yellow™ |
| Blue (488 nm) | Zombie Green™, Zombie Red™ |
| Green, Yellow/Green (532/561 nm) | Zombie Red™ |
| Red (633 nm) | Zombie NIR™ |

## DNA Dyes

Helix NP™ NIR
(Ex 648 nm/Em 660 nm)

Helix NP™ Green
(Ex 504 nm/Em 524 nm)

7-AAD
(Ex 546 nm/Em 647 nm)

DAPI
(Ex 358 nm/Em 461 nm)

Helix NP™ Blue
(Ex 445 nm/Em 480 nm)

Propidium Iodide
(Ex 533 nm/Em 617 nm)

DRAQ7™
(Ex 598 nm/Em 678 nm)

CytoPhase™ Violet
(Ex 405 nm/Em 440 nm)

DRAQ5™
(Ex 598 nm/Em 678 nm)

400    500    600    700    800    900
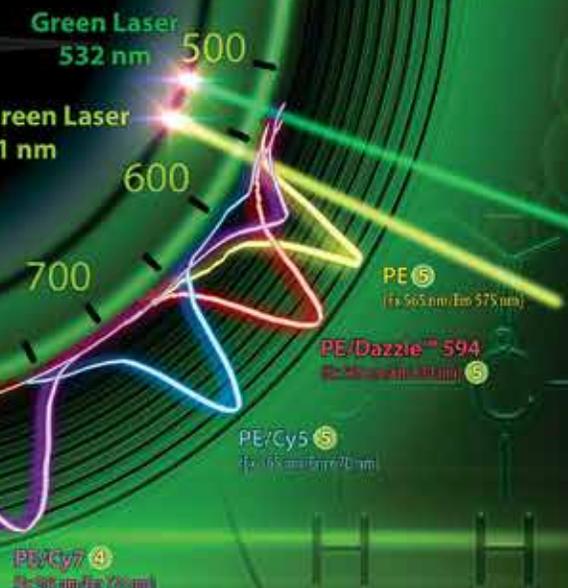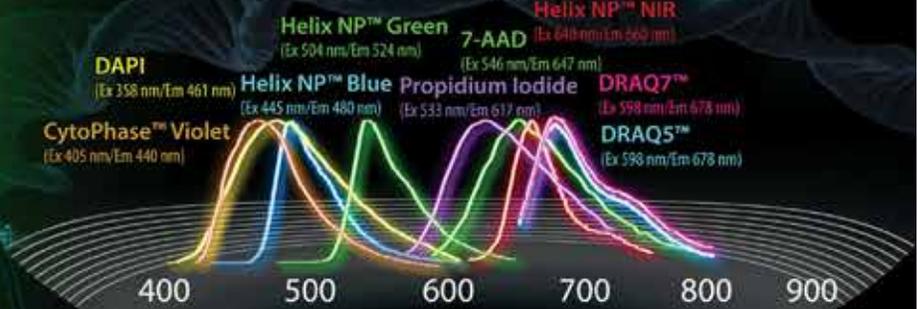
| Optimal Excitation Laser | Reagent |
|---|---|
| UV (355 nm) | DAPI |
| Violet (405 nm) | CytoPhase™ Violet, Helix NP™ Blue |
| Blue (488 nm) | 7-AAD, Helix NP™ Green, Propidium Iodide |
| Green, Yellow/Green (532/561 nm) | 7-AAD, Propidium Iodide |
| Red (633 nm) | DRAQ5™, DRAQ7™, Helix NP™ NIR |

Green Laser
532 nm

Green Laser
1 nm

500

600

700

PE ⑤
(Ex 565 nm/Em 575 nm)

PE/Dazzle™ 594 ⑤

PE/Cy5 ⑤
(Ex 565 nm/Em 670 nm)

PE/Cy7 ④
(Ex 565 nm/Em 774 nm)

# biolegend.com

Interactive Spectra Analyzer: biolegend.com/spectraanalyzer

**Contact BioLegend**
**US & Canada Toll-Free: 1.877.246.5343 (877-BIOLEGEND)**
**International: 1.858.768.5800**
Fax: 1.877.455.9587
email: cs@biolegend.com, tech@biolegend.com

US Headquarters:
San Diego, CA 92121

04-0008-01

# Buffers and Blocking Solutions

# Buffers for Flow Cytometry

The quality and optimization of sample preparation is integral to clear and consistent, internally reproducible results. In flow cytometry, this includes blocking buffers to reduce the effects caused by different sources of non-specific binding, as well as fixation and permeabilization buffers, each specially formulated for either intracellular soluble cytokine staining or intranuclear targets like transcription factors.

## Cytokine and Chemokine Buffers

Cytokines and Chemokines can be proteins, peptides or glycoproteins that are critical signaling molecules. The cytokine profile of a cell indicates a potentially wide variety of functions, whether it be a proinflammatory immune response, chemotaxis, or developmental cues in embryogenesis. Retaining soluble proteins and cytokines in order to be able to identify that function can be a challenge. A strong cross-linking fixative solution will ensure the best chance of retention, while a lighter permeabilization method will allow antibodies access to the cytoplasm, but not to other membrane-bound compartments. Cytokine buffers are not formulated to be optimal for transcription factors or any intranuclear proteins, though, since these targets are optimally detected with an opposite balance of fixation and permeabilization agents.

| Cell Activation Cocktail (With Brefeldin A) | 423303 | 423304 |
| Cell Activation Cocktail (Without Brefeldin A) | 423301 | 423302 |
| Cell Staining Buffer | 420201 |
| Fixation Buffer | 420801 |
| Fluorofix™ Buffer | 422101 |
| Cyto-Last™ Buffer | 422501 |
| Intracellular Staining Permeabilization Buffer (10x) | 421002 |

## Intranuclear and Transcription Factor Staining

Staining intranuclear and intracellular molecules presents challenges on several levels. Such challenges include 1) obtaining optimal penetration of the antibody conjugate into the nucleus or organelle while retaining integrity of cell surface markers, 2) the stability of tandem fluorophores conjugated to the antibodies, and 3) the retention of soluble cytokines. Strong cross-linking fixatives like high concentrations of paraformaldehyde (PFA) are useful in ensuring the best cytokine retention. However, due to the more extensive amine-amine protein cross-linking, the epitope may be affected, causing the antibody to exhibit lower affinity for intracellular proteins. Also, there is a challenge to have a strong enough permeabilization of the cell and nuclear membranes in order for the most efficient labeling of nuclear proteins. Thus, for the staining of intra-nuclear proteins like transcription factors, often a proprietary combination of fixative and permeabilization buffers are optimized for use together to ensure best results.
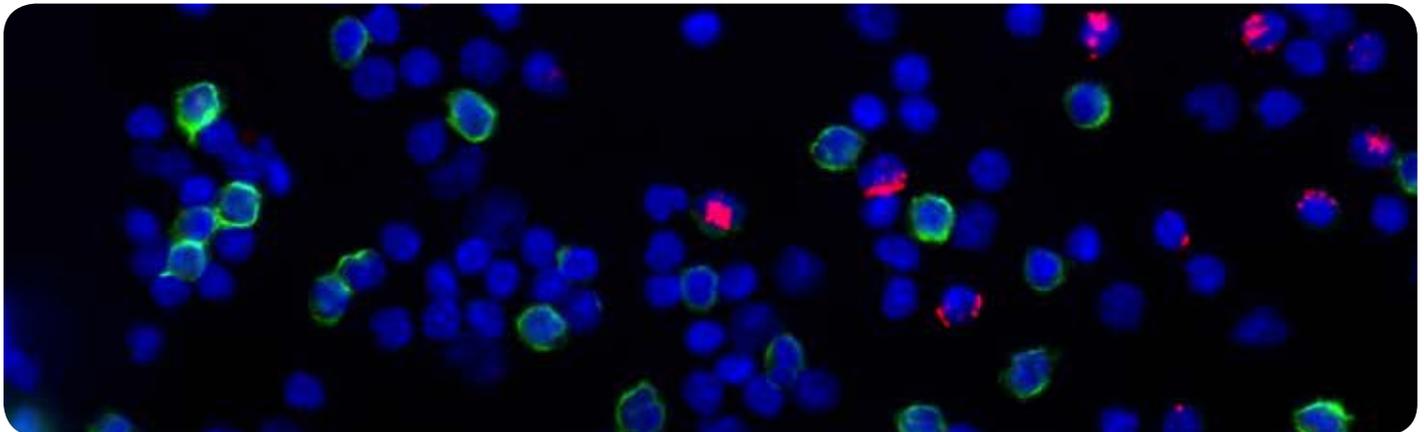


Figure 1. Human PBMCs were stained with Perforin (clone dG9) Alexa Fluor® 594 (red) and CD8 (clone HIT8a) Alexa Fluor® 488 (green). The nuclei were counterstained with DAPI (blue).

# True-Nuclear™ Transcription Factor Staining Buffer Set

We formulated our True-Nuclear™ Transcription Factor Buffer Set based on the best possible combination of buffer components that produced optimal staining results. Dramatic performance improvements are observed in two distinct instances. When comparing T-bet BV421™ conjugates using either the True-Nuclear™ Buffer or another nuclear staining buffer (Figure 2), there is a significantly improved separation between positive values compared to background.

Secondly, the True-Nuclear™ Buffer also enabled signal detection with clones that previously produced undetectable signal due to poor penetration, like anti-mouse Blimp-1 (Figure 3) and anti-FoxP3 BV421™.
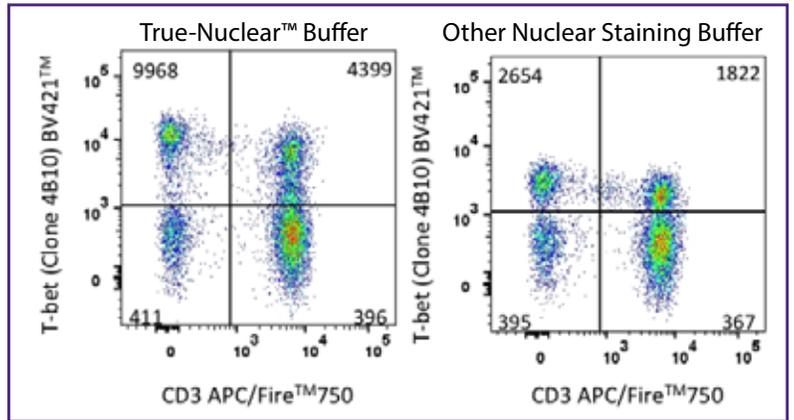


Figure 2. Intracellular staining of T-bet in human PBMCs using either the True-Nuclear™ Buffer or a previous formulation of nuclear staining buffer.

Figure 3. BALB/c spleen cells were cultured for four days in presence of LPS. Previously, Blimp-1 Alexa Fluor® 647 wasn't able to positively stain cells when used with a previous nuclear staining buffer. With True-Nuclear™ Transcription Factor Buffer Set, cells were first stained with CD45R/B220 PE, followed by fixation, permeabilization, and staining with rat IgG2a, κ Alexa Fluor® 647 isotype control (Left) or Blimp-1 (clone 5E7) Alexa Fluor® 647 (Right). Data shown was gated on live cells using Zombie Aqua™ fixable viability dye.
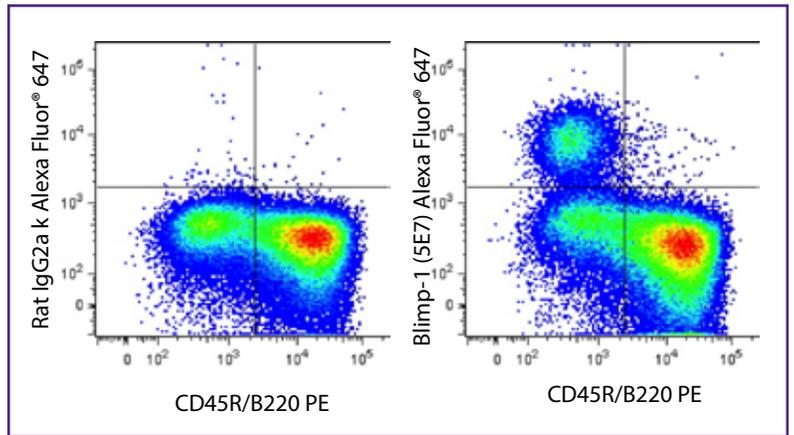


Another significant advantage of this buffer is its reduced effect on scatter properties of the cells and on the degradation of tandem fluorophores like APC/Cy7, which are particularly susceptible to fixatives when compared post-fixation with live, unstained cells. Both the use of the True-Nuclear™ Buffer and the use of APC/Fire™ 750 in lieu of APC/Cy7 can help minimize damage done to the integrity of the tandem fluorophore when first doing cell surface staining with an antibody prior to the fix/perm protocol for transcription factor staining.
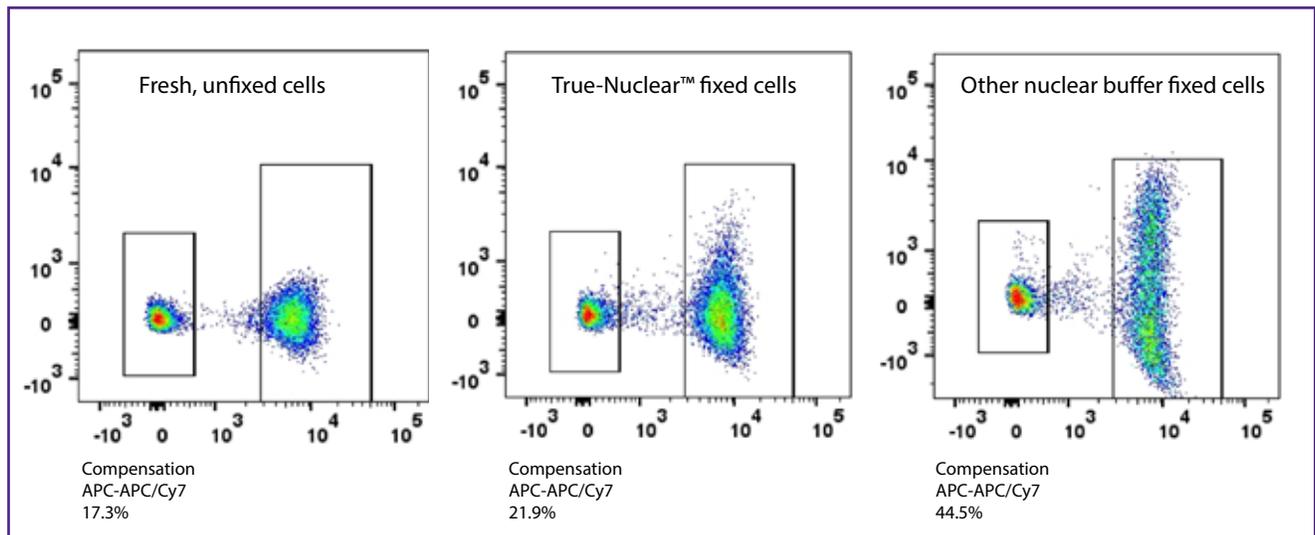


Figure 4. Cells were labeled with an APC/Cy7 antibody conjugate and changes in compensation of APC/Cy7 into the APC channel were assessed after exposure to True-Nuclear™, a previous formulation of nuclear staining buffer, or cells unfixed as a control.

# Phospho-site Specific Staining

Site-specific post-translational modification is one of the mechanisms for modifying the function of proteins. These protein modifications allow for the regulation of an array of signal transduction pathways and are involved in a myriad of cell processes, from cell health and cell cycle to synaptogenesis and modulation of neurotransmitter release. Phosphorylation generally activates, deactivates or modifies the function of a protein. For research applications, the detection of specific phosphorylation sites can be challenging, since phosphorylation can be a very transient state, whereby dephosphorylation can occur quickly. Methanol and ethanol are preferred to amine-crosslinking fixatives like PFA or formalin in order to quickly capture the phosphorylated state, while also permeabilizing the cell by solubilizing the plasma membrane, thus allowing for detection with antibodies. This can be problematic for other proteins, especially cell surface antigens and also protein-based fluorophores like PE, APC and PerCP that become denatured upon exposure to alcohol-based fixatives. Optimization of this assay is required to best understand which antigens and which fluorophores are appropriate to use before or after fixation.

**View more phospho reagents at: biolegend.com/phospho**
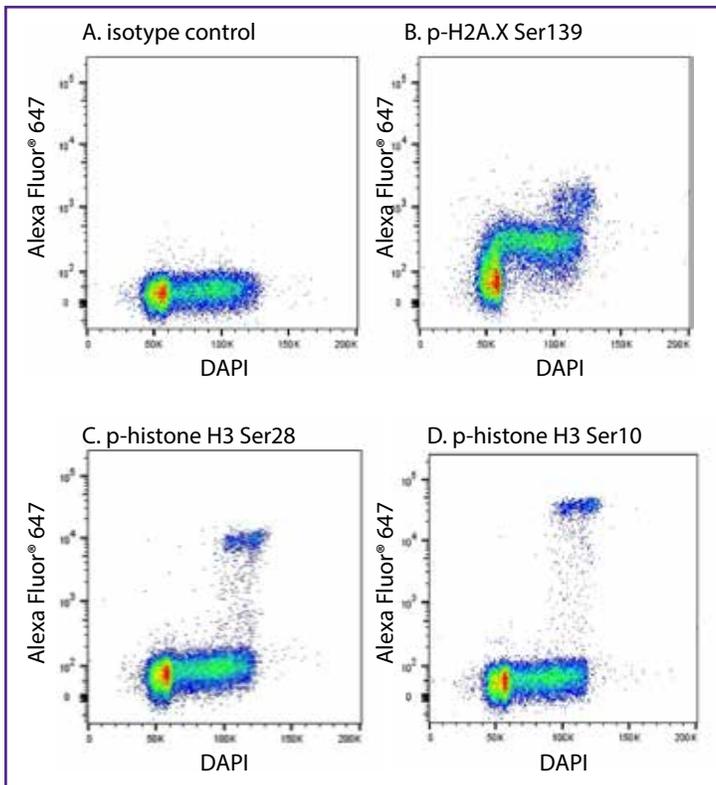


Figure 5. HeLa cells were fixed with Fixation Buffer (Cat No. 420801), permeabilized with cold True-Phos™ Perm Buffer (Cat No. 425401), then Figure A is intracellularly stained with DAPI (Cat No. 422801) and mouse IgG1,κ Alexa Fluor® 647 isotype control . Figure B is intracellularly stained with DAPI and anti-p-H2A.X Ser139 (clone 2F3) Alexa Fluor® 647. Figure C is intracellularly stained with DAPI and anti-p-histone H3 Ser28 (clone HTA28) Alexa Fluor® 647. Figure D is intracellularly stained with DAPI and anti-p-histone H3 Ser10 (clone 11D8) Alexa Fluor® 647.



21
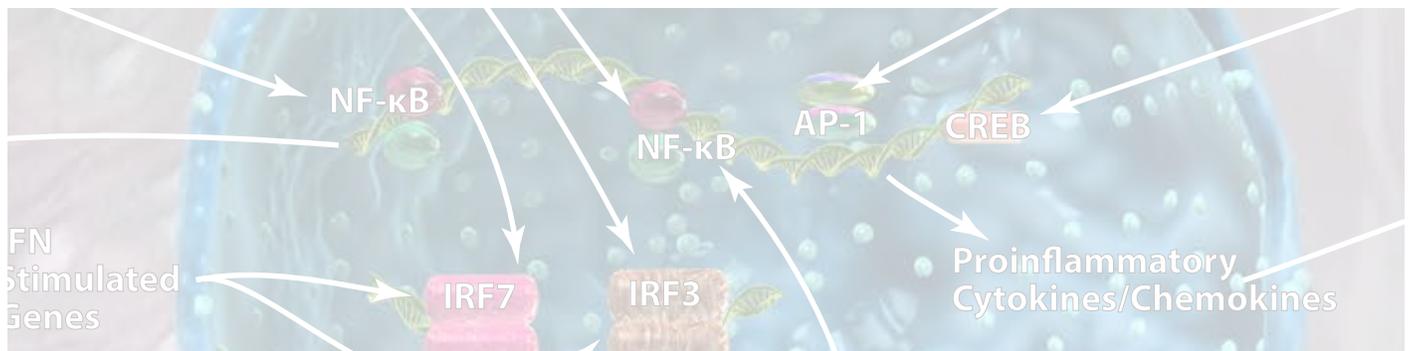
# Non-specific Monocyte Staining

Monocytes and macrophages have a scavenging function in the immune system, looking for fragments of viruses and bacteria to phagocytose, digest and present on their surface for activation of an immune response. Sometimes this function can dispose these cell subsets to non-specific binding when performing an immunoassay. Specifically, an area of concern in flow cytometry is the non-specific binding of particular cyanine-derived fluorophores like the acceptor dyes Cy5 in the PE/Cy5 tandem, Cy7 in the PE/Cy7 and APC/Cy7 tandem fluorophores, and the acceptor fluorophore in the tandem PE/Dazzle™ 594.

True-Stain Monocyte Blocker™ is a proprietary formulation that effectively blocks this fluorophore-dependent non-specific binding without activating or affecting cell health and function. It will, however, significantly improve the stringency and accuracy of multicolor staining applications in flow cytometry.
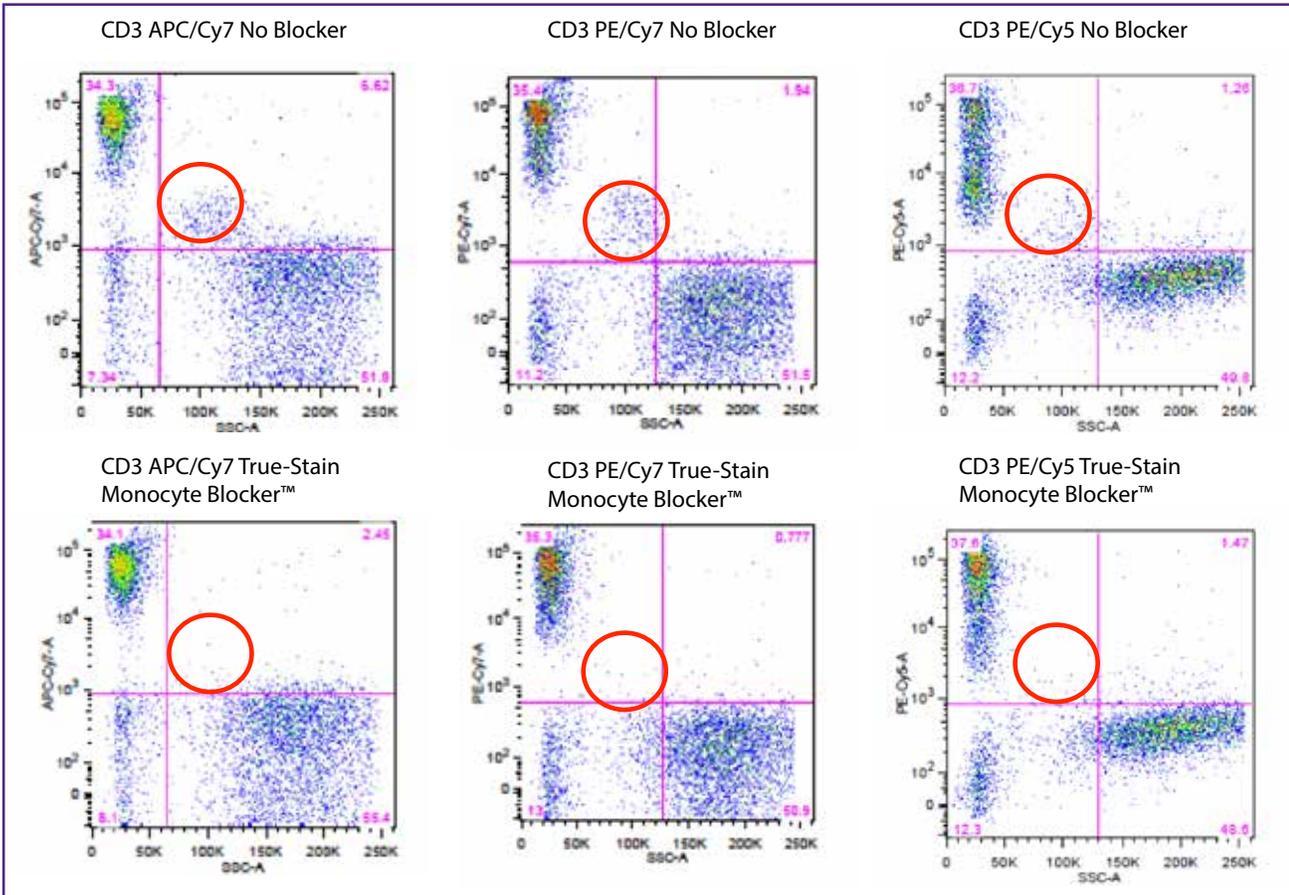


Figure 6. Plots along the top were stained without the True-Stain Monocyte Blocker™. The plots along the bottom were stained in the presence of True-Stain Monocyte Blocker™. The red gate indicates the cells involved in the non-specific binding of monocytes.

# Fc Receptor Non-specific Binding

Human Fc receptors (FcR) are expressed on a variety of cells, including monocytes, granulocytes, B cells and dendritic cells. The purpose of the Fc receptor is to bind to antibodies that might have attached to antigens and help internalize this complex. Cells with FcR expression can sometimes give false positive results in immunofluorescent staining due to binding of the Fc region of the detection antibody for the assay. Human TruStain FcX™ is specially formulated for blocking the FcR-involved unwanted staining without interfering with antibody-mediated specific staining of human cells. It is compatible with downstream flow cytometric staining of anti-human CD16 (clone 3G8), CD32 (clone FUN-2), and CD64 (clone 10.1) antibodies.

Mouse FcR-related non-specific staining caused by B cells, monocytes/macrophages, NK cells, granulocytes, mast cells, and dendritic cells can be blocked with BioLegend TruStain fcX™ (anti-mouse CD16/32) antibody.

**Learn more at: biolegend.com/fixation**

**Choose your buffers at: biolegend.com/flow_buffers**



Figure 7. Human TruStain FcX™ treated (filled histograms) or non-treated (open histograms) THP-1 cells stained with anti-human HLA-DR PE (red) or an isotype control (IgG2a PE, blue). Note: non-treated cells show false-negative HLA-DR staining due to high binding of mouse IgG2a isotype.

| Buffers for Flow Cytometry | Cat. No. |
|---|---|
| Cell Staining Buffer | 420201 |
| RBC Lysis Buffer (10x) | 420301 |
| RBC Lysis/Fixation Solution (10X) | 422401 |
| Fixation Buffer | 420801 |
| True-Nuclear™ Transcription Factor Buffer Set | 424401 |
| True-Phos™ Perm Buffer | 425401 |
| Human TruStain fcX™ (Fc Receptor Blocking Solution) | 422301 \| 422302 |
| TruStain fcX™ (anti-mouse CD16/32) antibody | 101319 \| 101320 |
| True-Stain Monocyte Blocker™ | 426101 \| 426102 \| 426103 |



Patrolling

...ytes:
...y6C⁻ (Mouse)
...man)

Step 1
Tethering
and Rolling:
P-Selectin
E-Selectin
L-Selectin
MadCAM-1
VLA-4

Step 2
Activation:
CXCL1
CCL5
CCL21
CXCL12

Step 3
Arrest and
Flattening:
LFA-1
VLA-4
α4β7

Step 4
Locomotion to
Interendothelial
Junctions:
Mac-1
LFA-1
VLA-4

Step 5
Extension of
Protrusions
Through Interendothelial
Junctions:
PECAM-1
CD99
CX3CL1
CCL3
CCL14

Step 6
Transmigration
ICAM-1

## Immunologic Networks Poster

The immune system is a complicated network, interconnected at several different junctions. The left-hand side of the poster focuses on innate immunity. Innate lymphoid cells share similarities with lymphoid cells (*i.e.* the production of cytokines associated with T helper cell classes), but lack markers that commonly define T or B cells. They also do not express common myeloid markers, making them particularly hard to phenotype. With three ILC groups, they have broad immunological activities, from cytotoxic ability to allergy and parasite defense. More traditional members of innate immunity include antigen presenting cells (APCs) such as dendritic cells and macrophages. These cells recognize pathogens with evolutionarily conserved elements like flagella or single-stranded RNA. APCs can phagocytose invading pathogens (or internal antigens like tumor cells) and digest them into small peptides for presentation. APCs can then bridge innate and adaptive immunity (the right-hand side of the poster) by presenting the captured antigen to CD4$^+$ or CD8$^+$ T cells. CD8$^+$ T cells can aid in the destruction of tumor cells and infected cells. However, constant or prolonged exposure of antigens to T cells can cause the cell to enter an exhausted state and lead to cell death. CD4$^+$ T cells can differentiate to a number of different T helper classes depending on the cytokine stimuli they receive, allowing them to influence a number of other adaptive immune responses, including antibody generation and class switching.
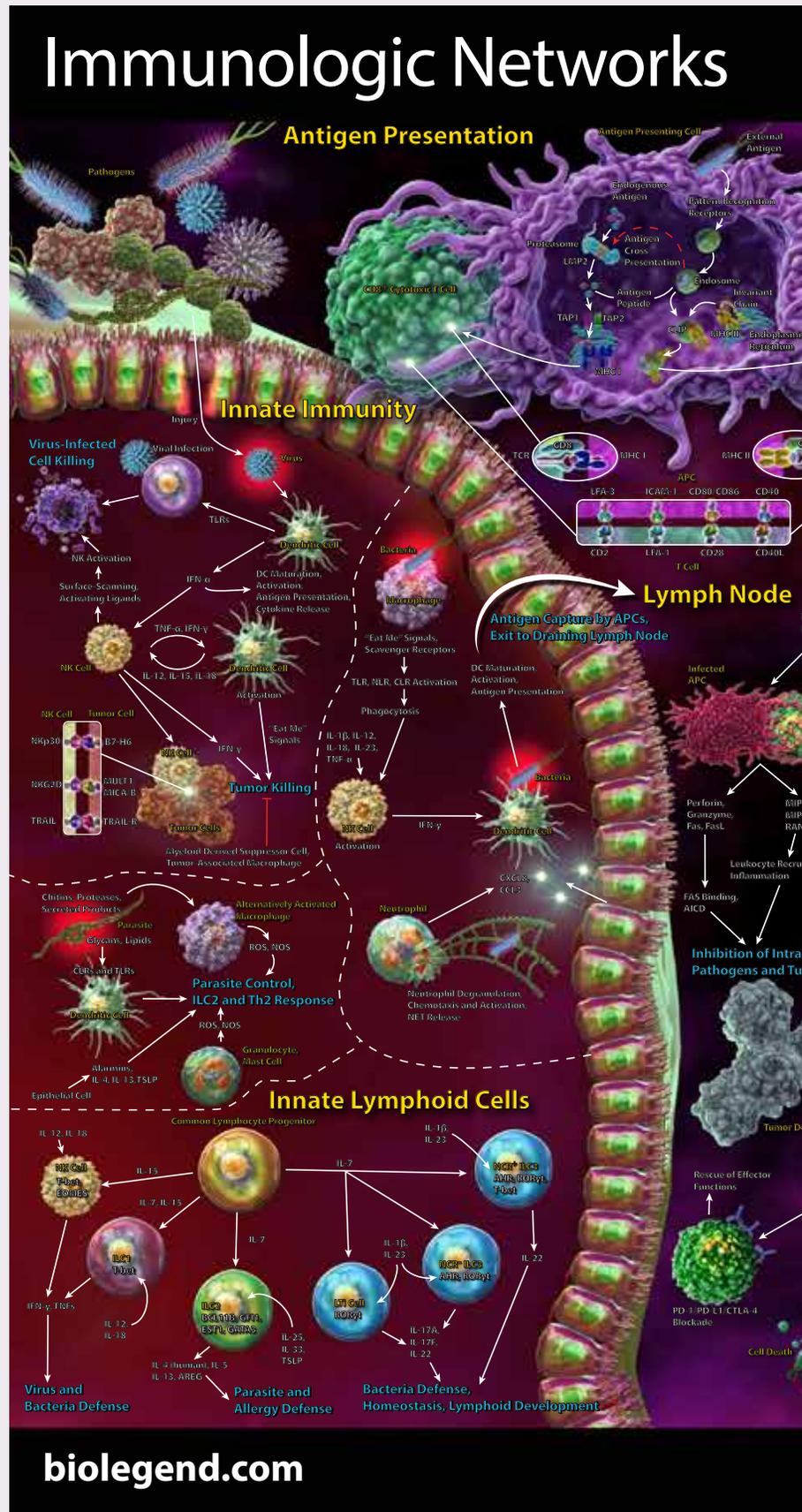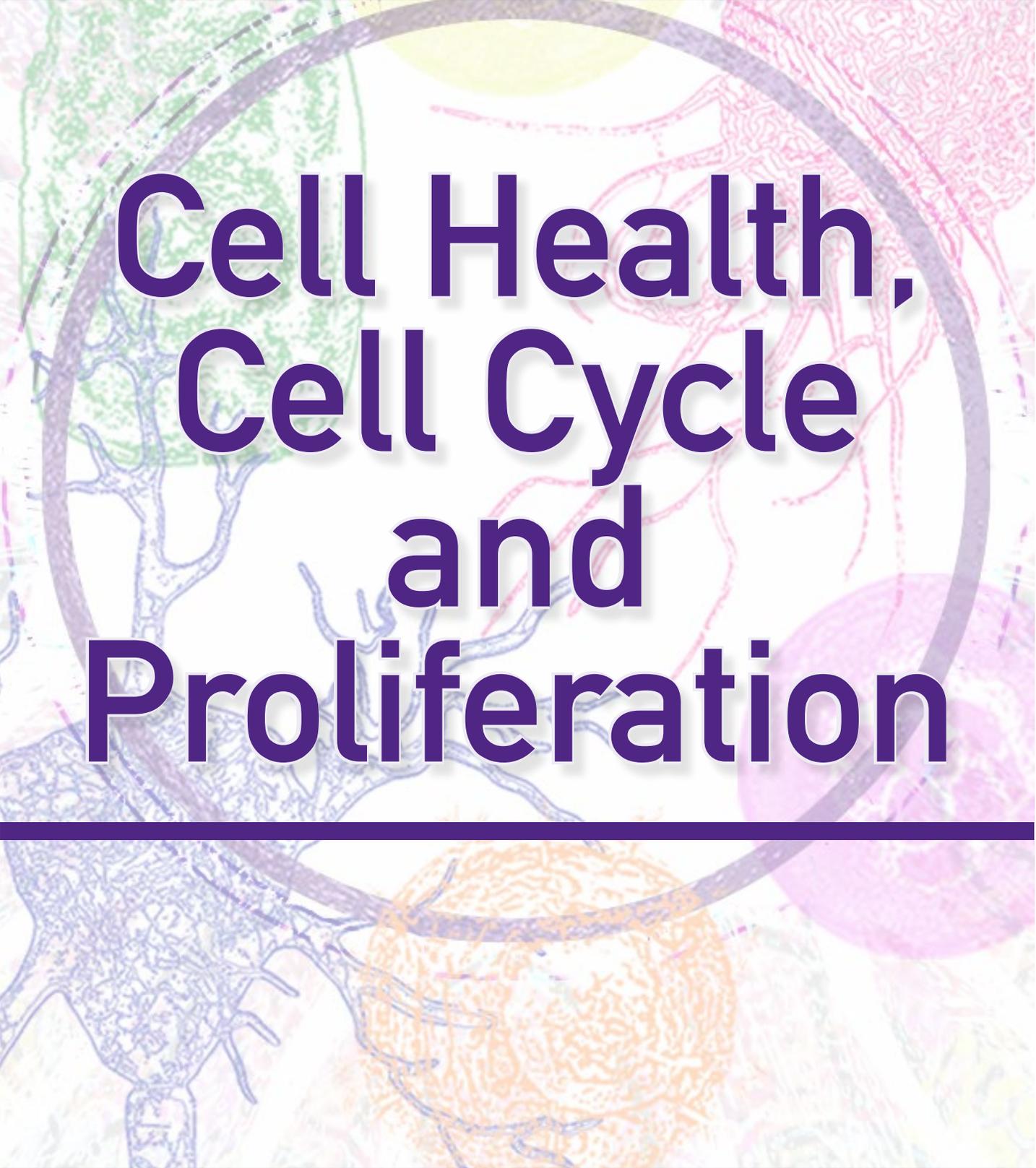


24

Adaptive Immunity

Interactive Poster: biolegend.com/immunologicnetworks
We would like to thank Dr. Vijay K. Kuchroo of Harvard Medical School for his contributions to this poster.

# Cell Health, Cell Cycle and Proliferation

# Cell Health, Cell Cycle and Proliferation

Cell health, proliferation and cell cycle are ubiquitous applications in every specialty within cell biology, from immunology to cancer biology to neuroscience. In every tissue, there are responses to cellular injury that lead to inflammation and necrosis or apoptosis, cellular trafficking and proliferation to tissue regeneration. Probes to assess these different facets of cell health can be based on antibody detection of specific proteins expressed in cell cycle or apoptosis, for example. There are also non-antibody based chemical probes available that may demonstrate cell permeability or mitochondrial polarization as indicators of cell health using a chemical mechanism inherent to the probe itself.

## Live/Dead Status Assessed through Membrane Permeability

The simplest and most commonly used method of determining live/dead status of unfixed cells is an impermeant nucleic acid stain. Impermeant nucleic acid stains only gain entrance to the cell if membrane integrity is compromised. Dead cells will stain brightly and live cells will not stain at all. Since some nucleic acid stains can start to stain late stage apoptotic cells, the best impermeant nucleic acid stains for dead cells are Propidium Iodide, Helix NP™ Blue, Helix NP™ Green, and Helix NP™ NIR.
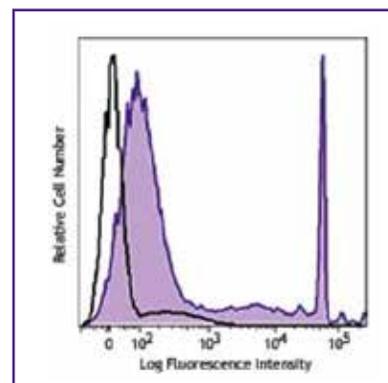


Figure 1. One day old C57 mouse splenocytes were stained with Helix NP™ NIR (filled histogram). Cells alone, without Helix NP™ NIR staining, are also shown (open histogram).

| Chemical Probe | Cat. No | Imaging Equivalent Channel | | Cell Type Suitability | | Sample Suitability | | Application | | Fixation |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | Live | Dead/ Fixed | Tissue | Cell Culture/ Single Cells | Flow Cytometry | Microscopy | Retention with PFA Treatment |
| Helix NP™ Blue | 425305 | Coumarin, BV421™ | Nucleus | | • | • | • | • | • | |
| Helix NP™ Green | 425303 | FITC | Nucleus | | • | • | • | • | • | |
| Helix NP™ NIR | 425301 | Alexa Fluor® 647, APC | Nucleus | | • | • | • | • | • | |
| DRAQ7™ | 424001 | Alexa Fluor® 700, APC/Cy7 | Nucleus | | • | • | • | • | • | |
| Propidium Iodide | 421301 | PE/Cy5, PE/Dazzle™ 594 | Nucleus | | • | • | • | • | • | |
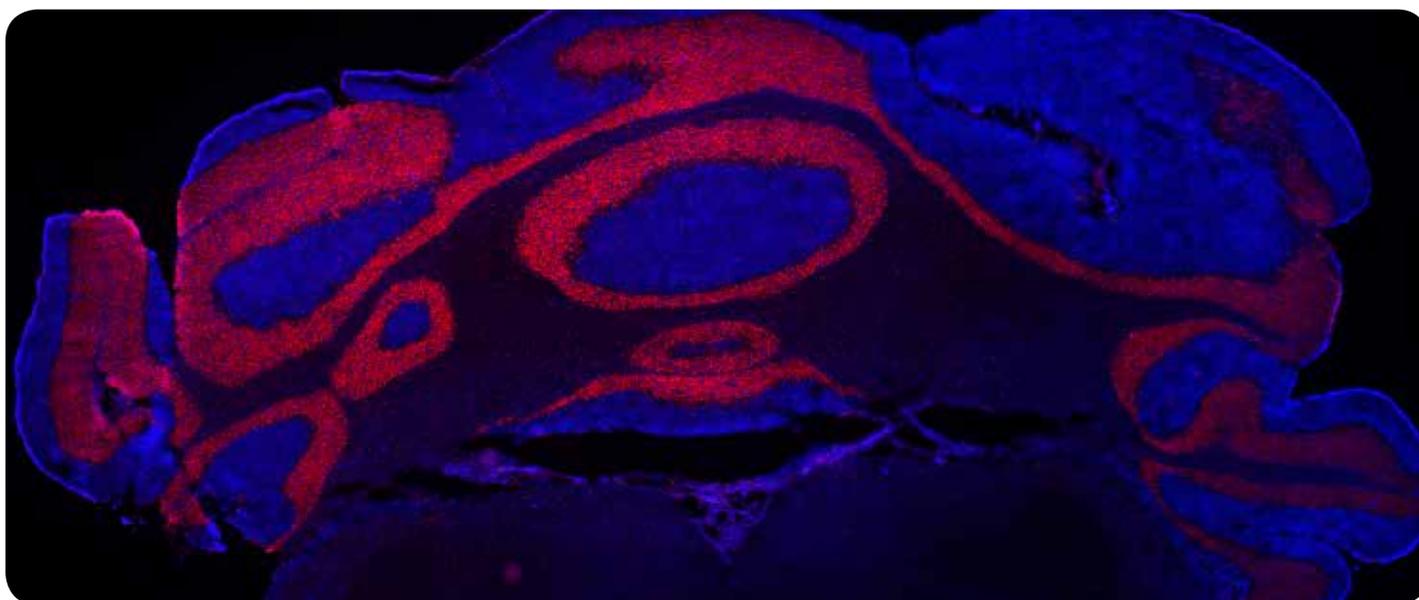


Figure 2. Whole frozen mouse brain slice, stained with Helix NP™ NIR (blue) and Flash Phalloidin™ Red (red).

27

Zombie dyes are a tool specifically suited towards applications where the live/dead status of the cells will be assessed while the cells are still alive and unfixed, but will become fixed prior to imaging or flow cytometric analysis. They exploit the permeability of the cell membrane to indicate live versus dead, but do not bind to DNA. Zombie dyes are a family of amine-reactive dyes that do not passively cross the cell membrane due to their valency. Only if the cell is compromised can these small molecules passively cross. Inside the cell, they covalently conjugate an abundance of intracellular proteins, and the signal is retained with fixation. Titration of this process is critical, since the goal is to use the lowest possible concentration of the Zombie dye to achieve a bright intracellular staining (dead cell status) with low residual cell surface staining (live cell status/background staining, Figure 4).
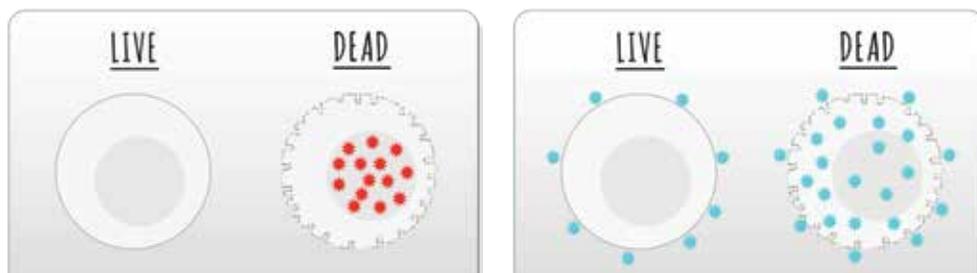


Figure 3. (Left) Impermeant nucleic acid stains can only stain DNA when the membrane is compromised. (Right) Zombie dyes bind to primary amines on proteins. Live cells receive low level surface staining, while dead cells stain at a much brighter fluorescence level due to the exposure of internal proteins.
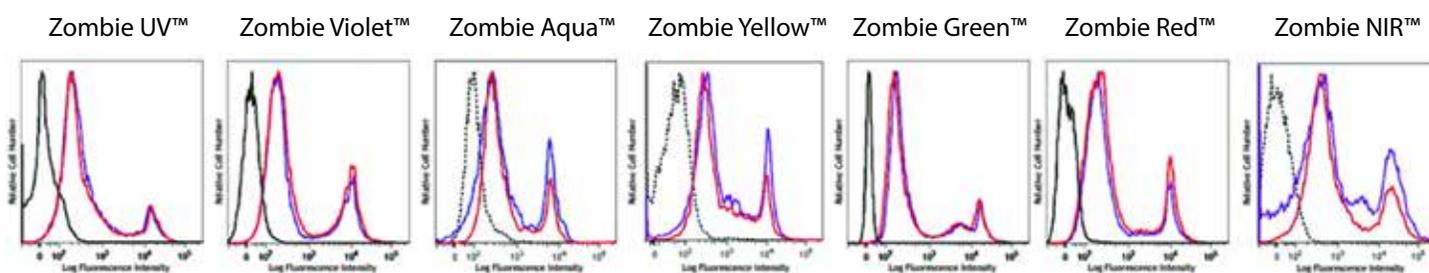


Figure 4. One day old C57BL/6 mouse splenocytes were stained with Zombie dyes as indicated and analyzed before fixation (blue/purple) or after fixation and permeabilization (red). Cells alone, without Zombie staining, are indicated in black.

| Reagent | Cat No. | Equivalent Channel | Optimal Excitation Laser | Excitation/Emission Max |
|---|---|---|---|---|
| Zombie UV™ | 423107 \| 426108 | DAPI | UV 355 nm | 362 nm/459 nm |
| Zombie Violet™ | 423113 \| 423114 | BV421™, Pacific Blue™ | Violet 405 nm | 400 nm/423 nm |
| Zombie Aqua™ | 423101 \| 423102 | BV510™ | Violet 405 nm | 382 nm/510 nm |
| Zombie Yellow™ | 423103 \| 423104 | BV570™ | Violet 405 nm | 396 nm/572 nm |
| Zombie Green™ | 423111 \| 423112 | FITC | Blue 488 nm | 491 nm/515 nm |
| Zombie Red™ | 423109 \| 423110 | PE/Texas Red® | Blue 488 nm or Yellow/Green 532/561 nm | 600 nm/624 nm |
| Zombie NIR™ | 423105 \| 423106 | APC/Cy7 | Red 633 nm | 719 nm/746 nm |
| Zombie Fixable Viability™ Sampler Kit | 423117 | DAPI, BV421™, BV510™, BV570™ and APC/Cy7 | UV 355 nm, Violet 405 nm and Red 633 nm | Varied |

**Learn more at: biolegend.com/live_dead**

# Cell Vitality Indication through Esterase-dependent Probes

Cell vitality can be assessed with probes that enter cells passively (such as CFDA-SE, Tag-it Violet™, Calcein-AM or Calcein Violet-AM). Inside the cell, esterase activity cleaves acetoxymethyl ester (AM) or diacetate (DA) groups from the probe, resulting in a charged fluorescent molecule that is retained by the intact membrane of the cell. Fluorescence intensity indicates the amount of esterase activity, which is dependent on the cell and its health status. Healthy cells will have a variable but strong conversion of the esterase substrate (Figure 6, left). Apoptotic and necrotic cells will exhibit lower esterase activity than healthy cells (Figure 6, right) and dead cells will have even less, since only a small residual amount of leftover esterase may have been retained.
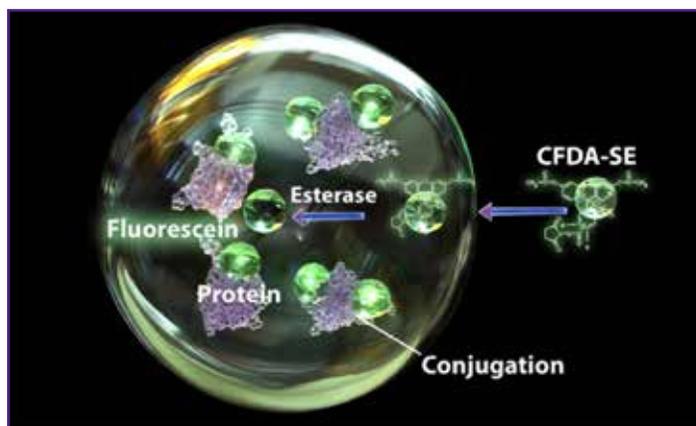


Figure 5. Acetoxymethyl ester side chains mask the charge of the small organic fluorophore, allowing it to passively cross the cell membrane. Intracellular esterases will cleave the side chains when cells are metabolically healthy. Once the charge of the fluorophore is released, it will be passively retained by the cell. For a longer term retention, some probes also contain chloromethyl or succinimidyl ester side chains to covalently cross-link either cysteine or lysine residues respectively on intracellular proteins.
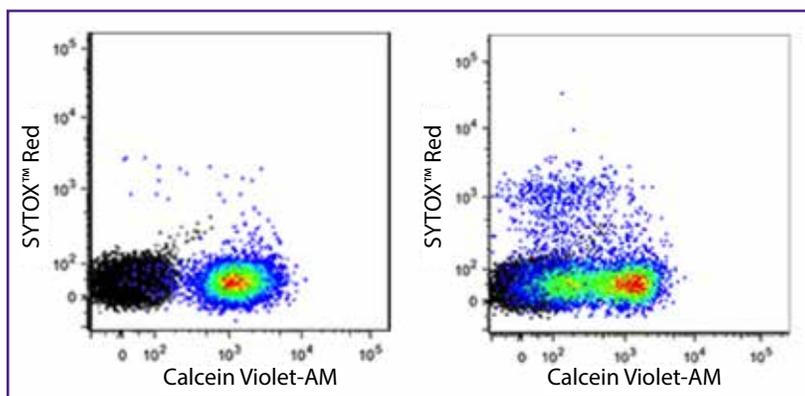


Figure 6. Fresh (left) or day-old C57BL/6 splenocytes (right) were stained with 0.01 µM Calcein Violet-AM and a cell-impermeant nucleic acid dye, SYTOX™ Red (colored). Black figure represents unstained splenocytes.

# Apoptosis Indicators

The most commonly used indicator of apoptosis, Annexin V, stains phosphatidylserine (PS) residues that have been translocated to the cell surface during the early to middle stages of apoptosis. However, it will also stain the intracellular-facing PS due to the loss of membrane integrity in death. Therefore, an impermeant nucleic acid stain or other marker of cell death must be employed to distinguish cells dying from apoptosis from those dead from necrosis. Apoptotic cells can also allow entrance of certain otherwise impermeant nucleic acid stain at late stages, namely DAPI and 7-AAD, so choosing the right dead cell indicator and also assessing multiple markers for different characteristics is important.

| | Biotin | Pacific Blue™ | BV421™ | BV510™ | BV570™ | BV605™ | BV650™ | BV711™ | BV785™ | FITC | Alexa Flour® 488 | Alexa Flour® 594 | PerCP/Cy5.5 | PE | PE/Dazzle™ | PE/Cy5 | PE/Cy7 | Alexa Flour® 647 | APC | Alexa Flour® 700 | APC/Cy7 | APC/Fire™ 750 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Annexin V | | • | • | • | | | | | | • | | | • | • | • | | • | • | • | | | • |

| Annexin V Kits and Buffer Reagents | Cat. No |
|---|---|
| Annexin V Binding Buffer | 422201 |
| APC Annexin V Apoptosis Detection kit with 7-AAD | 640930 |
| APC Annexin V Apoptosis Detection kit with PI | 640932 |
| FITC Annexin V Apoptosis Detection Kit with 7-AAD | 640922 |
| FITC Annexin V Apoptosis Detection Kit with PI | 640914 |
| Pacific Blue™ Annexin V Apoptosis Detection kit with 7-AAD | 640926 |
| Pacific Blue™ Annexin V Apoptosis Detection kit with PI | 640928 |
| PE Annexin V Apoptosis Detection Kit with 7-AAD | 640934 |

29

# Proliferation

As a cell divides, the resultant single cell will have half the fluorescent intensity of its parent cell (Figure 8). How long the signal persists depends on the length of the experiment or how fast the cells are dividing. Higher concentrations of these probes can be toxic as they interfere with protein function. As such, titration of the dye is very important. CFDA-SE and Tag-it Violet™ are retained in cells they have labeled upon fixation with paraformaldehyde to enable downstream antibody labeling for flow cytometry and imaging applications.
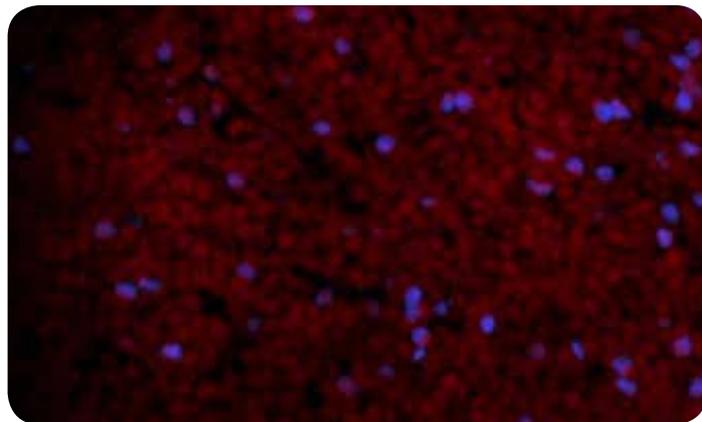


Figure 7. Mouse spleen 72 hours after adoptive transfer of Tag-it Violet™-labeled splenocytes (purple). Nucleated cells are stained with DRAQ5™ (red).
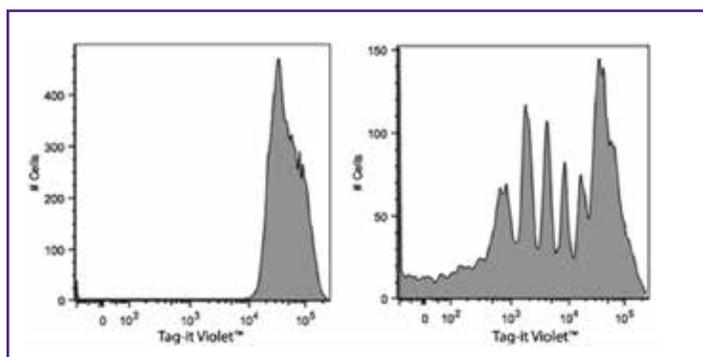


Figure 8. Mouse splenocytes stained with Tag-it Violet™ without stimulation (left) or subsequently stimulated with ConA and IL-2 for 4 days (right).

| Chemical Probe | Cat. No | Imaging Equivalent Channel | | Cell Type Suitability | | Sample Suitability | | Application | | Fixation |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Live | Dead/Fixed | Tissue | Cell Culture/ Single Cells | Flow Cytometry | Microscopy | Retention with PFA Treatment |
| Calcein-AM | 425201 | FITC | Cytoplasm | • | | | • | • | • | |
| Calcein Violet-AM | 425203 | BV421™ | Cytoplasm | • | | | • | • | • | |
| CFDA-SE | 423801 | FITC | Cytoplasm | • | | • | • | • | • | • |
| Tag-it Violet™ | 425101 | BV421™ | Cytoplasm | • | | • | • | • | • | • |

# BrdU Nucleotide Analogs and Ki-67 as Additional Indicators of Apoptosis

BrdU (bromodeoxyuridine) assays are another method of labeling proliferating cells. In culture or *in vivo*, BrdU is a nucleotide analog that can be fed to cells in culture media or via direct injection into an animal to be incorporated into the newly replicating DNA of dividing cells. The BrdU pulse time depends on the mitogen or the rate of cell division. This reveals which of the cells achieved division during the time of the BrdU pulse. While it will not give information about how many times a cell divided or the fate of all of the resultant daughter cells, it is a single cell flow cytometry application. Thus, additional parameters can be assessed on each cell, such as DNA content, phenotype of each cell or transcriptional factors, and regulators that might have differentially responded to the mitogen. As this application requires an anti-BrdU antibody, samples must be fixed and permeabilized for the antibody to gain access to the DNA for imaging.



Figure 9. C57BL/6 mouse frozen intestine section was fixed, permeabilized, and blocked. Then the section was stained with anti-Ki-67 (clone 11F6) Alexa Fluor® 647 (red) and anti-E-cadherin (clone DECMA-1) Alexa Fluor® 594 (green) overnight at 4°C. Nuclei were counterstained with DAPI (blue). The image was captured with a 20X objective.

An antibody-based method of detecting proliferating cells post-fixation is Ki-67, a nuclear protein involved in ribosomal RNA transcription. Expression occurs diffusely in the nucleus during the active phases of the cell cycle, G1, S and G2 phases, whereas in M phase or mitosis, the protein becomes localized to the surface of chromosomes. Ki-67 is not detectable in G0 or resting phase.

30

Figure 10. HEL cell line was pulsed with (left) or without (right) BrdU for 1 hour and then stained with anti-BrdU (clone 3D4) Alexa Fluor® 647 antibody. Cells were subsequently stained with 1 µg of DAPI for DNA analysis.

| Antibody | Clone | Purified | Biotin | Pacific Blue™ | BV421™ | BV510™ | BV570™ | BV605™ | BV650™ | BV711™ | BV785™ | FITC | Alexa Fluor® 488 | Alexa Fluor® 594 | PerCP/Cy5.5 | PE | PE/Dazzle™ 594 | PE/Cy5 | PE/Cy7 | Alexa Fluor® 647 | APC | Alexa Fluor® 700 | APC/Cy7 | APC/Fire™ 750 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BrdU | 3D4 | • | | | | | | | | | | • | • | | • | | | | | • | | | | |
| BrdU | Bu20a | • | • | | | | | | | | | | | | | | • | | | | • | | | |
| BrdU | MoBU-1 | • | • | | | | | | | | | | | | | | | | | | | | | |
| Ki-67 mouse/human | 11F6 | • | | | • | | | | | | | | • | • | • | | • | | | | • | | | | |
| Ki-67 mouse | 16A8 | • | | • | • | | | • | | | | • | • | | • | • | • | | • | • | • | • | • | |
| Bromodeoxyuridine (BrdU) | | | | | | | | | | | Cat. No. 423401 |
| Phase-Flow™ FITC BrdU Kit | | | | | | | | | | | 370704 |
| Phase-Flow™ Alexa Fluor® 647 BrdU Kit | | | | | | | | | | | 370706 |

# Microplate Assays for Proliferation

For time point assays, a microplate assay can be performed to give a broader view of the proliferation of cultured cells. Resazurin (Deep Blue Cell Viability™) and tetrazolium salt (TetraZ™ Cell Counting Kit) are probes that become fluorescent upon metabolic activation. The relative fluorescence units of the microplate reader are plotted on a standard curve against number of live cells to give an overall view of rate of proliferation. Ideally, they should be paired with an impermeant nucleic acid stain to indicate the number of dead cells. Permeant nucleic acid stains can render a total cell count.
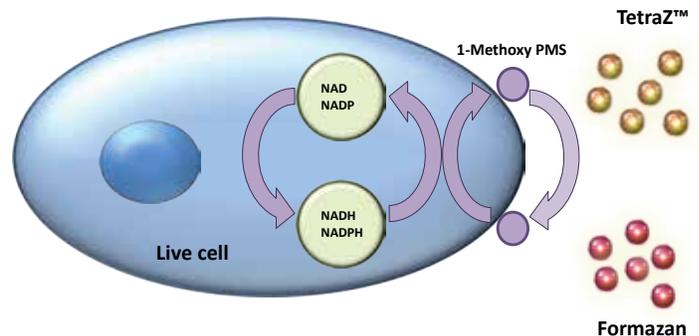


Figure 11. TetraZ™ produces a water soluble dye upon reduction in the presence of an electron mediator. Electrons are provided by cellular dehydrogenases, which transform tetrazolium salt into a colored product, formazan. The amount of formazan produced is directly proportional to the number of live cells.
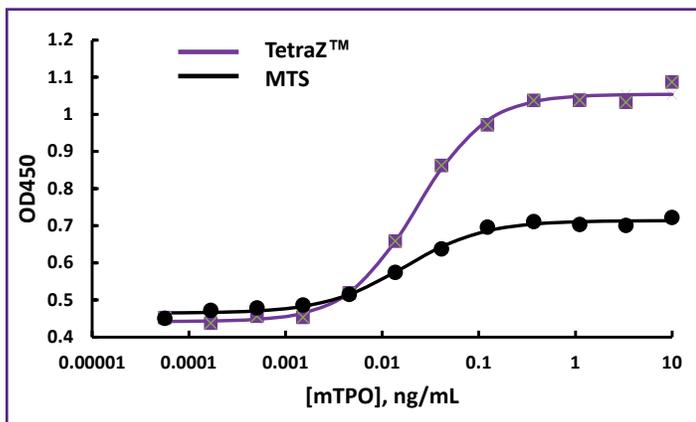


Figure 12. Recombinant mouse thrombopoietin (mTPO) induces proliferation of M0-7e cells in a dose-dependent manner. TetraZ™ was added for the last 16 hours of a 72 hour stimulation. The plate was read at 450 nm in a plate reader.

31

# Mitochondrial Health and Respiration

Mitochondrial respiration and the respiration/polarization of the mitochondrial membrane are also indicators of cell health. Probes like MitoSpy™ Orange CMTMRos and MitoSpy™ Red CMXRos are live cell-permeant fluorogenic probes that localize to the mitochondrial membrane based on their strong polarization in healthy, respiring cells. The most common application of MitoSpy™ probes is simple mitochondrial localization in live cells for imaging applications. However, when used in conjunction with other probes for cell health, like Annexin V, Calcein-AM, or impermeant nucleic acid stains, the neutralization of potential across the mitochondrial membrane can be one of the first indications that a cell is entering apoptosis.
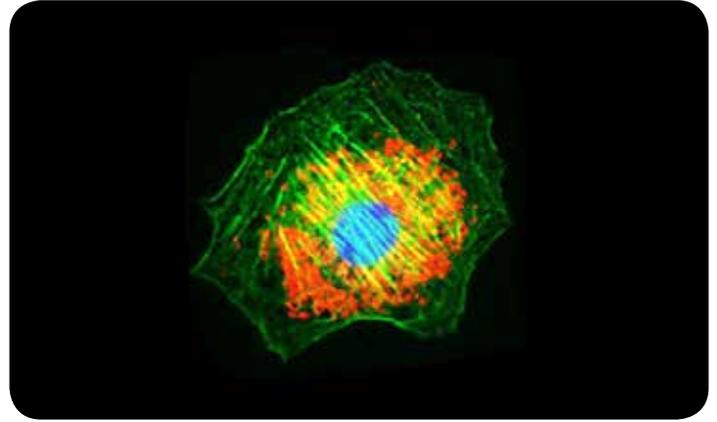


Figure 13. NIH3T3 cells were stained with MitoSpy™ Red CMXRos (red) then fixed with 1% PFA and permeabilized with 1X True Nuclear™ Perm Buffer. Then the cells were stained with Flash Phalloidin™ NIR 647 (green) and counterstained with DAPI (blue).
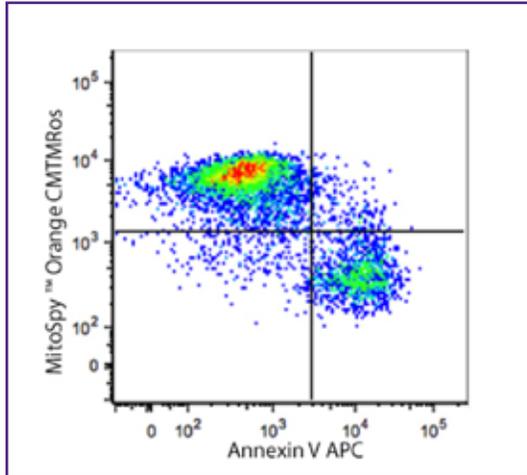


Figure 14. Human T-cell leukemia cell line, Jurkat, was treated for five hours with LEAF™ purified anti-CD95 (clone EOS9.1), then stained with an impermeant nucleic acid stain, Annexin V APC, and MitoSpy™ Orange CMTMRos. Data shown was gated on live cells.

| Chemical Probe | Cat. No. | Imaging Equivalent Channel | | Cell Type Suitability — Live | Cell Type Suitability — Dead/ Fixed | Sample Suitability — Tissue | Sample Suitability — Cell Culture/ Single Cells | Application — Flow Cytometry | Application — Microscopy | Fixation — Retention with PFA Treatment |
|---|---|---|---|---|---|---|---|---|---|---|
| MitoSpy™ Green FM | 424805 \| 424806 | FITC | Mitochondria | • | | | • | • | • | |
| MitoSpy™ NIR DilC1(5) | 424807 | Alexa Fluor® 647 | Mitochondria | • | | | • | • | • | |
| MitoSpy™ Orange CMTMRos | 424803 \| 424804 | Alexa Fluor® 555, PE | Mitochondria | • | | | • | • | • | • |
| MitoSpy™ Red CMXRos | 424801 \| 424802 | Alexa Fluor® 594 | Mitochondria | • | | | • | • | • | • |

**Learn more at: biolegend.com/mitospy**
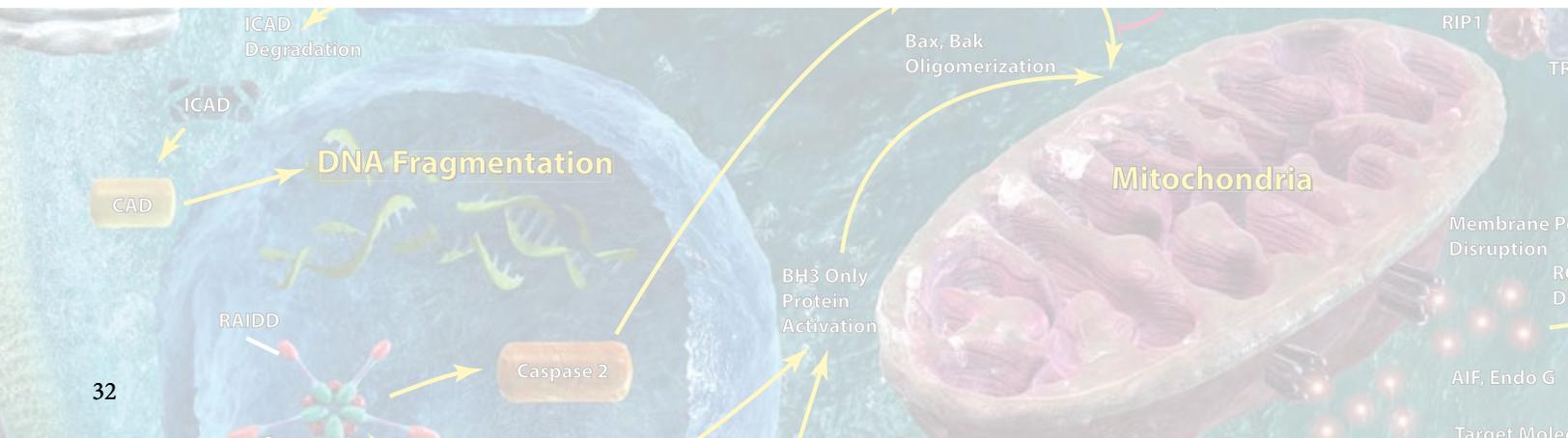
# Cell Permeant Nucleic Acid Stains and Cell Cycle Analysis

Cellular division for any cell type is dependent on the inherent function, location, and response of cells to repair, apoptosis, or death. To divide, cells must duplicate a copy of their DNA, increase mitochondrial density, and assemble/synthesize microtubules during interphase. G2 is a checkpoint stage of interphase where the cell has two sets of dsDNA and must commit to mitosis. Mitosis is the actual division stage where two daughter cells are created. There can be symmetrical or asymmetrical division, depending on the cell and tissue type. Mitosis can be further subdivided into prophase, metaphase, anaphase, and telophase as the nucleus undergoes division and chromatids are pulled away from one another. After mitosis, the cell will undergo the G0/G1 phase, when the cells rest to become ready for the next round of replication.

**Learn more at: biolegend.com/cell_health_proliferation**
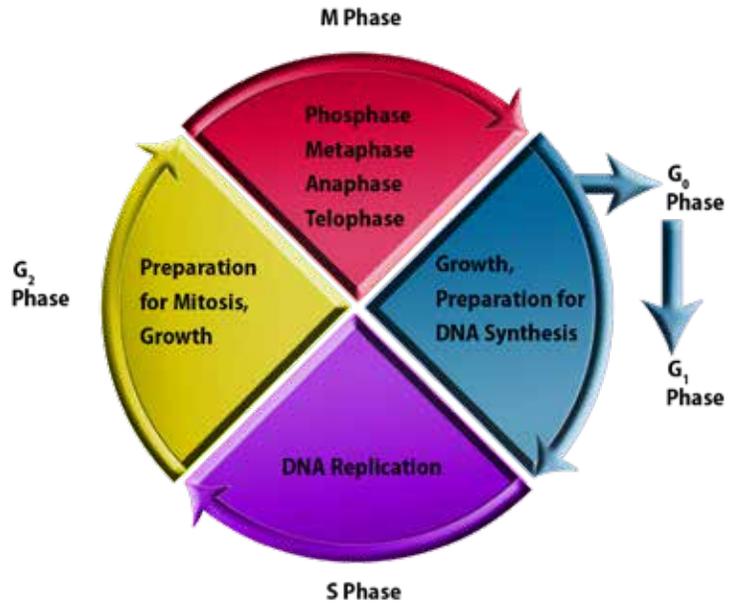
Figure 15. During G0/G1, S, and G2 phases, some fluorogenic nucleic acid stains selectively bind dsDNA stoichiometrically to give measurements of DNA mass based on fluorescence intensity. Cell-permeant nucleic acid stains can be used in instances where cells will be analyzed live. Propidium Iodide, DAPI, DRAQ5™, DRAQ7™, CytoPhase™ Violet, Helix NP™ NIR and Helix NP™ Green can all be used to stain fixed cells for cell cycle analysis. However, only CytoPhase™ Violet and DRAQ5™ can be used to assess DNA content of live cells.
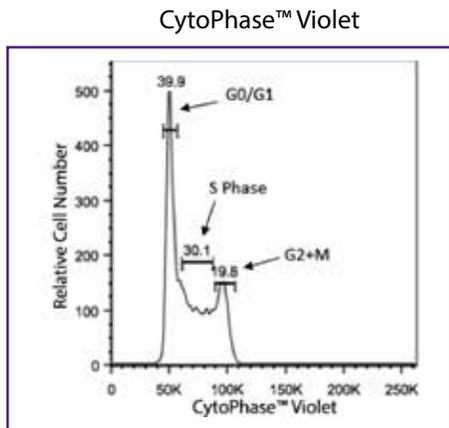
## CytoPhase™ Violet

Figure 16. Live Ramos cells treated with CytoPhase™ Violet dye for 90 minutes at 37°C.
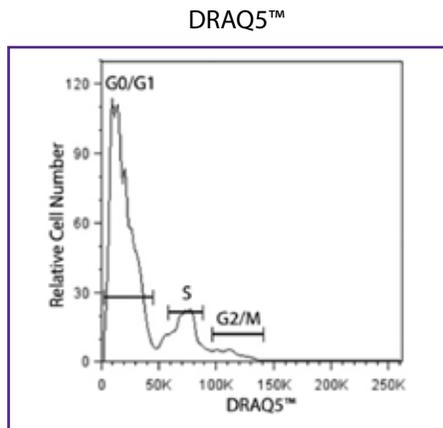
## DRAQ5™

Figure 17. Live C57BL/6 mouse bone marrow cells were stained with DRAQ5™.
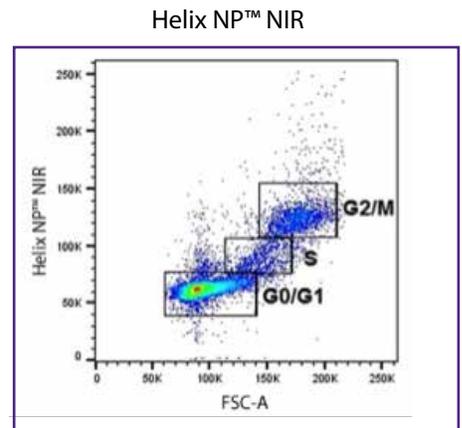
## Helix NP™ NIR

Figure 18. C57BL/6 mouse thymus cells were fixed using 70% chilled ethanol. The cells were incubated for one hour at -20°C, washed, then stained with Helix NP™ NIR at 5 µM.

33
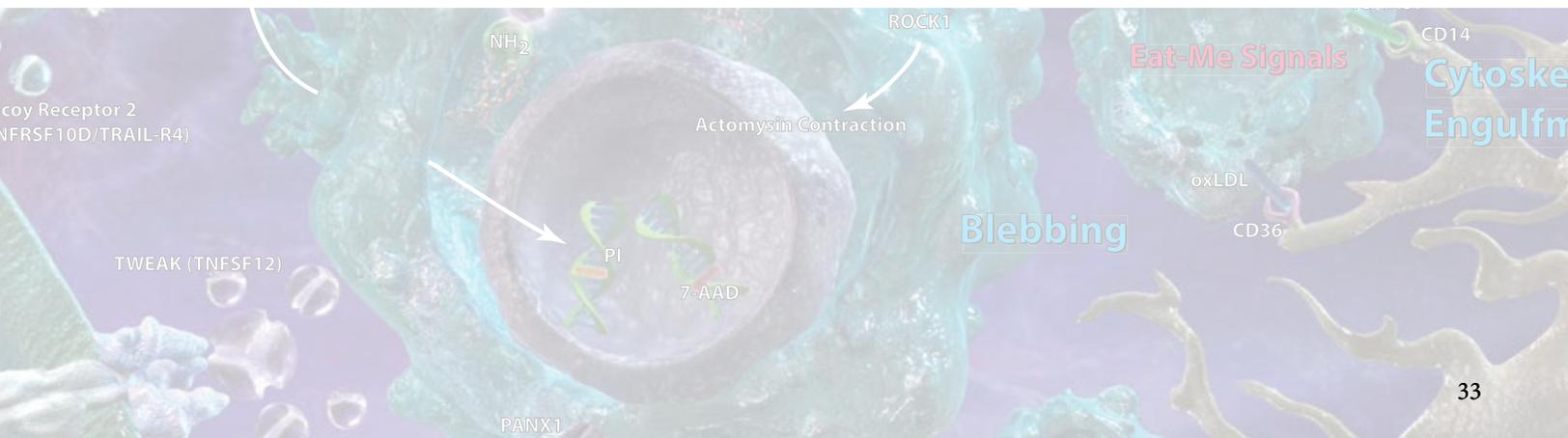
# Mechanisms of Cell Death Poster

Cell proliferation and activation are necessary; properly controlling how a cell dies is also important. This is because a cell's contents, if spilled into the environment, can cause an inflammatory response. As such, the process of apoptosis helps to package off and partition parts of a dying cell. This can be sparked by the engagement of certain TNF receptors and caspase activation. Intracellular infection can also trigger pyroptosis, which is a highly inflammatory form of programmed cell death that typically results in cell lysis, spilling the cell's contents. Necroptosis is a programmed form of cell death usually activated in response to viral threats, as it operates independently of any caspase inhibitors a virus might employ. This process, however, can also result in membrane rupture.

34

# Mechanisms of Cell

## Apoptosis

TRAIL (TNFSF10)
Death Receptor 4 (TNFRSF10A TRAIL R1)
Death Receptor 5 (TNFRSF10B TRAIL R2)
TNF-α
TNF RI (TNFRSF1A)
TNF RII (TNFRSF1B)
FAS-L (TNFSF6 CD178)
FAS (TNFRSF6 CD95)
Death Domain
K63 Ubiquitin Removal
TRADD TRADD
RIP1 RIP1
Death Inducing Signaling Complex (DISC)
FADD
CIAP1,2
K63 Ubiquitination
TWEAK (TNFSF12)
FADD
FADD
Procaspase 8,10
TRAF1
TRAF3
Death Receptor 3 (TNFRSF25, APO-3)
Procaspase 8,10
FLIP
NF-κB
TRAF5 TRAF
Caspase 8,10
Cell Survival Proliferation
RIP1 TRAIL
Decoy TRAIL Receptor R1 (TNFRSF8)
Decoy TRAIL Receptor R2 (TNFRSF2)
Decoy Receptor 1 (TNFRSF10C/TRAIL-R3)
Procaspase 3
Bid
Bcl-2, Bcl-2-xL, MCL-1
RIP1 TRA
t-Bid
Caspase 3
ICAD Degradation
Bax, Bak Oligomerization
TRAIL (TNFSF10)
ICAD
Mitochondria
Membrane Per Disruption
ROS DNA
DNA Fragmentation
CAD
RHD Only Protein Activation
FAS-L (TNFSF6 CD178)
AIF, Endo G
RAIDD
Caspase 2
Target Molecu (Actin, DNA, N
Caspase-2 PIDDosome
APAF1
smac/diablo Htra2/Omi
Caspase 2
Procaspase 2
p53
Cytochrome C
Bax, Bak
XIAP
PIDD-CC
Apoptosome
Caspase 6
Decoy Receptor 3 (TNFRSF6B)
Severe DNA Damage
Procaspase 3
Caspase 7
TRAIL (TNFSF10)
Procaspase 9
Caspase 9
Procaspase 6
Osteoprotegerin (TNFRSF11B)
Procaspase 9
XIAP
Caspase 7
Procaspase 7
XIAP
Endoplasmic Reticulum Stress
DNA Damage
Survival Factor Loss
Lactoferrin
Keep-Out Signal
Intracellular
Caspase 3,7
CD31
CD46
CD47
Phosphatidylserine (PS)
CDC50
Don't Eat Me Signals
Neutrophil Recruitment
Phosphatidylserine
Ca²⁺
ATP11C Flippase
Xkr8 Scram
Annexin V
Extracellular

### Bcl-2 Superfamily

| Pro-apoptotic | | Anti-apoptotic | |
|---|---|---|---|
| Bax | Bid | BCL-2 | A1 |
| Bok | Bik | BCL-XL | BLFL-1 |
| Bak | Noxa | BCL-W | BCL-B |
| Bim | Puma | MCL-1 | |
| Bad | | | |

### Caspases

| Initiator | Effector | Inflammatory |
|---|---|---|
| Caspase 2 | Caspase 3 | Caspase 1 |
| Caspase 8 | Caspase 6 | Caspase 4 |
| Caspase 9 | Caspase 7 | Caspase 5 |
| Caspase 10 | | |

We would like to t

BioLegend®

## Apoptotic Cell Clearance

Zombie Dyes
Propidium Iodide (PI)
7-Amino-Actinomycin (7-AAD)

TRAIL (TNFSF10)

Decoy Receptor 2
(TNFRSF10D/TRAIL-R4)

TWEAK (TNFSF12)

TWEAK-R (TNFRSF12A/Fn14)

Death Receptor 6
(TNFRSF21)

**Apoptotic Cell**

Zombie Dyes
NH₂
NH₂
ROCK1

Actomyosin Contraction

PI
7-AAD

PANX1

Nucleotides
ATP, UTP

?

MER
GAS6

BAT1, TIM-4

Phosphatidylserine

ICAM3?
CD11

**Eat-Me Signals**

oxLDL
CD36

**Blebbing**

**Cytoskeletal Rearrangement, Engulfment**

**Find-Me Signals**
Lysophosphocholine

Sphingosine-1-Phosphate

G2A?

Sphingosine-1
Phosphate Receptor

P2y₂

**Chemotaxis, Engulfment**

**Phagocyte**
Macrophage    Monocyte    Neutrophil
Dendritic Cell    Fibroblast    Mast Cell

Permeability

Generation
Fragmentation

Cleavage
Nuclear Lamins

Phospholipids

**Outer Leaflet**
**PS Exposure**
**Eat-Me Signal**

Extracellular ATP
Bacterial pore-forming toxins
Monosodium urate crystals
Cholesterol crystals

Cholera toxin B,
Gram negative bacteria

Flagellin
Type 3 Secretion
System Rods/Needles

Bacterial DNA

Cytosolic Bacteria

Intracellular LPS

NLRP3

NLRC4

Caspase 4, 5, 11

Procaspase 1

Caspase Oligomerization

AIM2

ASC Focus
Formation

ASC

?

Pyroptosis

Inflammasome

Pore formation
Cell swelling
Lysis

Caspase 1

HMGB1
ATP
IL-1α
Lactose dehydrogenase

IL-1β, IL-18

## Pyroptosis

Death Receptor

TLR

TRIF

FLIP

Necrosulfonamide

DAI

Virus

IFN

JAK-STAT

FADD

FADD    TRADD

RIPK1    Necrostatin 1

Caspase 8    Necrosome

RIPK3

MLKL
P

RKR

RIPK1

De novo
transcription

**Membrane Rupture**

## Necroptosis

©BioLegend, Inc. 2014

35

# Non-Human Primate (NHP) Research

# Antibodies for Non-Human Primate (NHP) Research

LEGENDScreen™ is a high-throughput assay that contains 371 PE conjugated anti-human monoclonal antibodies and isotype controls packaged as one lyophilized antibody per well in a 96-well plate. This presentation makes it much easier to stain single cell suspensions from any source, be it cell lines or dissociated whole tissues. As non-human primates have more than 90% genetic homology with humans, it would be reasonable to project that many anti-human antibodies will have cross-reactivity with different primates, including species such as cynomolgus, macaque, rhesus, baboon and African green monkey. By co-staining the cells with CD66, CD3, CD11b, CD20, and CD7 in complementary fluorophore conjugates to the PE conjugated antibodies in the LEGENDScreen™, researchers from Garry Nolan's lab were able to deduce not just that cells are staining positively for the marker in PE, but which cell population expressed it and to what degree. This was the backbone gating scheme applied to phenotype each population: T-cells (SSC$^{-lo}$/CD66$^-$, CD3$^+$), B cells (SSC$^{-lo}$/CD66$^-$, CD20$^+$), NK cells (SSC$^{-lo}$/CD66$^-$, CD3$^-$/CD20$^-$/CD7$^+$), monocytes (CD7$^-$/ CD11b$^+$), and granulocytes (SSC$^{-hi}$/CD66$^+$, CD7$^-$). Using the expression pattern of human cells as a reference, they found many differences in both reactivity and expression pattern.

For example, Figure 1 shows CD2 staining on gated T-cells. Human T-cells are 100% positive for CD2. All of the non-human primates tested also show a strong positive staining, but there remains a small subset of CD2 negative cells. Both baboon and African green monkey show different staining patterns with greater variability of percentage of positive T-cells, and in the case of African green monkey, a larger population of CD2 negative cells. Whether these differences are a reflection of the affinity of this clone to the epitopes of CD2 expressed in these primates or an indication of the actual expression pattern of CD2 in these primates would need to be further assessed. LEGENDScreen™ is an excellent screening tool, giving direction as to which antigens might be interesting for further "deep dive" analysis. Table 1 lists the subsets of cells the antibody stains positively versus the appropriate isotype control and in which primate. To explore the level of expression on each cell type, please visit our webpage for the most comprehensive results and interactive plots for each antibody: **biolegend.com/NHP**.

*We thank Zachary B Bjornson, Gabriella K Fragiadakis, Matthew H Spitzer, Deepthi Madhireddy and Garry P Nolan from the Stanford University School of Medicine for sharing their data.*



Figure 1.
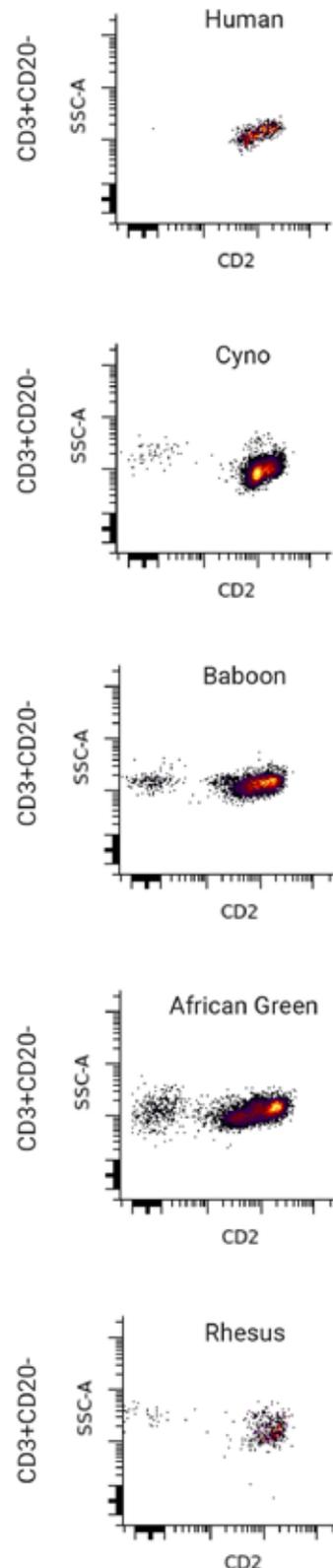
**Table 1. Cell Subsets of Primates that Stain Positively by Antibody in LEGENDScreen™ Assay**

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| CD1a | HI149 | T G | | | | |
| CD1b | SN13 (K5-1B8) | T G | M | | | T G |
| CD1c | L161 | B M | B M | B M | B M | B |
| CD1d | 51.1 | B MG | G | | | |
| CD2 | RPA-2.10 | TN | BTN | BTN | BTNM | BTN |
| CD3 | HIT3a | T | | | | |
| CD4 | RPA-T4 | T M | T | | | |
| CD5 | UCHT2 | BT G | | | | |
| CD6 | BL-CD6 | BTN G | | | | |
| CD7 | CD7-6B7 | TN | | | | |
| CD8a | HIT8a | TN | | | | |
| CD9 | HI9a | TMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD10 | HI10a | BTN G | MG | G | MG | G |
| CD11a | HI111 | BTNMG | M | | TN | NM |
| CD11b | CBRM1/5 | BT G | | | | |
| CD11b | ICRF44 | BTNMG | B NMG | BTNMG | B NMG | BTNMG |
| CD11c | 3.9 | B MG | B M | B | B M | |
| CD13 | WM15 | T MG | | | | T MG |
| CD14 | M5E2 | BT MG | MG | MG | | G |
| CD15 | W6D3 | MG | | | | |
| CD16 | 3G8 | BTNMG | TNM | TNM | TNM | N |
| CD18 | TS1/18 | BTNMG | | | | |
| CD19 | HIB19 | B | | B | | |
| CD20 | 2H7 | B | B | B | B N | B |
| CD21 | Bu32 | BT | B | | B | B |
| CD22 | HIB22 | B | | | | |
| CD23 | EBVCS-5 | | | | | |
| CD24 | ML5 | BTNMG | | | | |
| CD25 | BC96 | BTN | | | T | |
| CD26 | BA5b | TN | | | | |
| CD27 | O323 | BTN | BTN | BTN | BTNM | BTN |
| CD28 | CD28.2 | TN | T | T | T | T |
| CD29 | TS2/16 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD30 | BY88 | | | | | |
| CD31 | WM59 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD32 | FUN-2 | BTNMG | BT MG | BTNMG | BTNMG | B MG |
| CD33 | WM53 | NMG | | | | |
| CD34 | 581 | | | | | |
| CD35 | E11 | | BT MG | BT MG | BT MG | B G |
| CD36 | 5-271 | BTNMG | | | | |
| CD38 | HIT2 | BTNMG | | | | |
| CD39 | A1 | BTNMG | BTNMG | MG | BTNMG | B MG |
| CD40 | HB14 | B NM | B M | B | B | B M |
| CD41 | HIP8 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD42b | HIP1 | BTNMG | | | | |
| CD43 | CD43-10G7 | BT MG | T G | | T | T MG |
| CD44 | BJ18 | BTNMG | BTNMG | BTNMG | BTNMG | BTN G |

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| CD45 | HI30 | BT MG | | | | |
| CD45RA | HI100 | BT M | | | | |
| CD45RB | MEM-55 | BTNM | BTNMG | BTNM | BTNM | BTN |
| CD45RO | UCHL1 | | T | | | |
| CD46 | TRA-2-10 | BTNMG | | | | |
| CD47 | CC2C6 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD48 | BJ40 | BTNMG | B M | B MG | B M | BTNMG |
| CD49a | TS2/7 | TNMG | TN | T | T G | TN |
| CD49c | ASC-1 | BTN | | | | |
| CD49d | 9F10 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD49e | NKI-SAM-1 | TNMG | TNMG | TNMG | BTNMG | BTNMG |
| CD49f | GoH3 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD50 | CBR-IC3/1 | BTNMG | TNM | | TNMG | |
| CD51 | NKI-M9 | | | | | |
| CD51,CD61 | 23C6 | B M | T | | | M |
| CD52 | HI186 | BTNMG | M | M | | |
| CD53 | HI29 | BTNMG | | | | |
| CD54 | HA58 | BTNMG | | | | |
| CD55 | JS11 | BTNMG | G | | G | G |
| CD56 | HCD56 | N | NM | NM | NM | N |
| CD57 | HCD57 | N | | | | |
| CD58 | TS2/9 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD59 | p282 (H19) | BTNMG | BTNMG | BTNMG | BTNMG | |
| CD61 | VI-PL2 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD62E | HAE-1f | | | | | |
| CD62L | DREG-56 | BTNMG | | | | |
| CD62P | AK4 | | | | | |
| CD63 | H5C6 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD64 | 10.1 | MG | MG | NMG | MG | MG |
| CD66a/c/e | ASL-32 | G | | | | |
| CD66b | G10F5 | G | | | | |
| CD69 | FN50 | TNM | TN | TN | G | B N |
| CD70 | 113-16 | T | | | | |
| CD71 | CY1G4 | BTNM | | | | |
| CD73 | AD2 | BT G | | | TN | TN G |
| CD74 | LN2 | BT MG | B M | B MG | B M | BT MG |
| CD79b | CB3-1 | BTNMG | T G | G | T G | T M |
| CD80 | 2D10 | BTN | | | | |
| CD81 | 5A6 | BTNMG | T M | BT M | BT MG | BTNMG |
| CD82 | ASL-24 | BTNMG | T G | | T G | T G |
| CD83 | HB15e | T G | | T | BT M | BT |
| CD84 | CD84.1.21 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD85 | 17G10.2 | | | | | |
| CD85 | GHI/75 | BTNMG | | | | |
| CD85 | MKT5.1 | T MG | MG | MG | G | G |
| CD85d | 42D1 | T MG | | | | |
| CD85h | 24 | MG | | G | | |
| CD85k | ZM4.1 | M | | | | |

B=B cells     M= monocytes
T=T-cells     G= granulocytes
N=NK cells

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| CD86 | IT2.2 | BT MG | B M | B MG | BT MG | BT MG |
| CD87 | VIM5 | T MG | MG | BTNMG | MG | NMG |
| CD88 | S5/1 | BTNMG | | | G | T |
| CD89 | A59 | NMG | NMG | NMG | MG | T G |
| CD90 | 5E10 | T | BTNMG | BTNMG | BTNMG | BTNMG |
| CD93 | VIMD2 | M | MG | MG | TNMG | TNMG |
| CD94 | DX22 | TN | T G | | T | T |
| CD95 | DX2 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD96 | NK92.39 | TN G | | | | |
| CD97 | VIM3b | BTNMG | | B | | |
| CD99 | HCD99 | BT MG | TN | | T | T MG |
| CD100 | A8 | BTNMG | BTNM | BTNM | BTNMG | BTNM |
| CD101 | BB27 | BT MG | T MG | BTNMG | T MG | MG |
| CD102 | CBR-IC2/2 | BTNMG | BTNMG | BTNM | BTNM | BTNMG |
| CD103 | Ber-ACT8 | | | | | |
| CD104 | 58XB4 | | | | | |
| CD105 | 43A3 | NM | | | | |
| CD106 | STA | | | | | |
| CD107a | H4A3 | BTNMG | T G | BTN | BT MG | BTNMG |
| CD108 | MEM-150 | B NM | B | B | B | B |
| CD109 | W7C5 | TNM | NM | | | TN |
| CD111 | R1.302 | N | | | | |
| CD112 | TX31 | T MG | | | | |
| CD114 | LMM741 | NMG | G | | | |
| CD115 | 9-4D2-1E4 | G | G | TN G | M | T G |
| CD116 | 4H1 | T MG | | | | T |
| CD117 | 104D2 | N | | | | |
| CD119 | GIR-208 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD122 | TU27 | TN | N | N | N | N |
| CD123 | 6H6 | B NM | | | | |
| CD124 | G077F6 | B | | | | |
| CD126 | UV4 | TNMG | T M | T | T | T |
| CD127 | A019D5 | TN | T | T | TN | T |
| CD129 | AH9R7 | TN | | TN | | T G |
| CD131 | 1C1 | B MG | MG | MG | MG | G |
| CD132 | TUGh4 | BTNMG | MG | G | | T MG |
| CD134 | Ber-ACT35 (ACT35) | T | | | | |
| CD135 | BV10A4H2 | | | | | |
| CD137 | 4B4-1 | | | | | |
| CD138 | DL-101 | TNMG | T G | | T MG | T G |
| CD140a | 16A1 | | | | | |
| CD140b | 18A2 | MG | G | N | T G | T MG |
| CD141 | M80 | MG | | | M | MG |
| CD143 | 5-369 | T G | | | | |
| CD144 | BV9 | | | | | |
| CD146 | SHM-57 | BTNMG | MG | | BTNMG | T MG |
| CD148 | A3 | BTNMG | | | | |
| CD150 | A12 (7D4) | BT | BT | BT | BTN | BT |
| CD152 | L3D10 | | | | | |

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| CD154 | 24-31 | BTNMG | | | | |
| CD155 | SKII.4 | NMG | | | | |
| CD156c | SHM14 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD158a/h | HP-MA4 | N | N | | N G | |
| CD158b | DX27 | N | | | | |
| CD158d | mAb 33 (33) | TN G | | | | |
| CD158e1 | DX9 | N | | | | |
| CD158f | UP-R1 | T MG | | | | |
| CD161 | HP-3G10 | TNMG | TN | TNM | TNMG | N |
| CD162 | KPL-1 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD163 | GHI/61 | M | MG | MG | | G |
| CD164 | 67D2 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD165 | SN2 (N6-D11) | BTNMG | BTNMG | B | BTNMG | BTNMG |
| CD166 | 3A6 | BTNMG | BTNMG | BT MG | BT MG | BTNMG |
| CD167a | 51D6 | | | | | |
| CD169 | 7-239 | | | | | |
| CD170 | 1A5 | NMG | | | | |
| CD172a | SE5A5 | T MG | T MG | TNMG | MG | MG |
| CD172b | B4B6 | MG | | | N | |
| CD172g | LSB2.20 | T G | MG | TNMG | TNMG | MG |
| CD178 | NOK-1 | G | | | MG | |
| CD179a | HSL96 | M | | | | T |
| CD179b | HSL11 | | | | | BT M |
| CD180 | MHR73-11 | B M | B M | B M | B M | B M |
| CD181 | 8F1/CXCR1 | TNMG | | | | |
| CD182 | 5E8/CXCR2 | NMG | | | | |
| CD183 | G025H7 | BTNMG | BTNMG | BTNM | BTNMG | BTNMG |
| CD184 | 12G5 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD193 | 5E8 | T M | | | | |
| CD195 | T21/8 | BTNMG | T MG | MG | T M | T MG |
| CD196 | G034E3 | BTNM | BTN G | BTN | BTN | BTNMG |
| CD197 | G043H7 | BT G | BT | BT | BTN | BT |
| CD200 | OX-104 | BT G | | | BT | B |
| CD200R | OX-108 | BTNMG | | | BT MG | |
| CD201 | RCR-401 | | | | | |
| CD202b | 33.1 (Ab33) | | | | | |
| CD203c | NP4D6 | M | | | | |
| CD205 | HD30 | G | BT MG | T MG | T MG | TNMG |
| CD206 | 15-2 | | | | | |
| CD207 | 10E2 | | | | | |
| CD209 | 9E9A8 | | | | | |
| CD210 | 3F9 | BTNMG | BTNM | M | BTN | BTNMG |
| CD213a2 | SHM38 | T G | | T | | |
| CD215 | JM7A4 | T G | T G | TN G | T G | T G |
| CD218a | H44 | TNMG | N | TN | TN | TN G |
| CD220 | B6.220 | M | MG | M | M | MG |
| CD221 | 1H7/CD221 | TNMG | T MG | T | BTN G | N G |
| CD226 | 11A8 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |

**Table 1. Cell Subsets of Primates that Stain Positively by Antibody in LEGENDScreen™ Assay**

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| CD229 | HLy-9.1.25 | BTN | | | | |
| CD231 | SN1a (M3-3D9) | T G | G | | T | T |
| CD235ab | HIR2 | | | | T | B |
| CD243 | UIC2 | BTNMG | | | T | T |
| CD244 | C1.7 | TNM | | | | |
| CD245 | DY12 | TNMG | M | | TN | M |
| CD252 | 11C3.1 | G | | | | T G |
| CD253 | RIK-2 | TN G | | | | N |
| CD254 | MIH24 | G | | | | |
| CD255 | CARL-1 | | | | T | T |
| CD257 | T7-241 | BT MG | T G | TN G | T MG | BT MG |
| CD258 | T5-39 | | | TN | | |
| CD261 | DJR1 | G | T | | | T |
| CD262 | DJR2-4 (7-8) | T MG | | | | |
| CD263 | DJR3 | G | | | | |
| CD266 | ITEM-1 | | | | | T |
| CD267 | 1A1 | B | B | B | B | B |
| CD268 | 11C1 | BT | BT M | B | BT MG | BT MG |
| CD271 | ME20.4 | N | TNM | TNM | BTNM | TNM |
| CD272 | MIH26 | BT | | | | G |
| CD273 | 24F.10C12 | T G | TNM | TNM | T M | M |
| CD274 | 29E.2A3 | BTNMG | T G | N G | T MG | BTNMG |
| CD275 | 9F.8A4 | B | | | | |
| CD276 | MIH42 | TN G | N | N | | TN |
| CD277 | BT3.1 | BTNMG | B | B | BTNM | BTN |
| CD278 | C398.4A | TN | T | T | T | T |
| CD279 | EH12.2H7 | T | T | T | T | T |
| CD282 | TL2.1 | B MG | | | | |
| CD284 | HTA125 | T M | | | | |
| CD286 | TLR6.127 | MG | | | | |
| CD290 | 3C10C5 | B | | | B | |
| CD294 | BM16 | MG | | | | |
| CD298 | LNH-94 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CD300e | UP-H2 | M | | | | |
| CD300F | UP-D2 | M | | | | |
| CD301 | H037G3 | BT MG | T MG | | BT MG | BT MG |
| CD303 | 201A | | | | | |
| CD304 | 12C2 | | | | | |
| CD307e | 509f6 | B | | | | |
| CD314 | 1D11 | TN G | TN | TN | TN | N |
| CD317 | RS38E | BTNMG | BTNMG | BTNM | BTNMG | BTNMG |
| CD318 | CUB1 | | | | | |
| CD319 | 162.1 | BTNM | G | | | |
| CD324 | 67A4 | | | | | |
| CD325 | 8C11 | G | | | | |
| CD326 | 9C4 | | | | | |

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| CD328 | 6-434 | NMG | | | | |
| CD334 | 4FR6D3 | | | | | |
| CD335 | 9E2 | N | | | | |
| CD336 | P44-8 | | | | G | |
| CD337 | P30-15 | N | N | N | N | N |
| CD338 | 5D3 | | | | | |
| CD340 | 24D2 | | | | | |
| CD344 | CH3A4A7 | TNMG | G | | | TN G |
| CD351 | TX61 | N G | | | | |
| CD352 | NT-7 | BTNMG | T MG | | T G | T |
| CD354 | TREM-26 | TNMG | MG | T G | MG | G |
| CD355 | Cr24.1 | | | | | |
| CD357 | 621 | TNMG | T G | TN G | T G | T MG |
| CD360 | 2G1-K12 | BTNM | BTNM | BTNM | BTNM | B NM |
| 4-1BB Ligand | 5F4 | TNMG | T G | TN | T G | TNMG |
| C3aR | hC3aRZ8 | TNMG | | | | |
| C5L2 | 1D9-M12 | TNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| CCR10 | 6588-5 | BTNMG | B MG | BTNMG | BT MG | BT MG |
| CLEC12A | 50C1 | MG | | | | |
| CLEC9A | 8F9 | | | | | |
| CX3CR1 | 2A9-1 | TNMG | MG | G | | M |
| CXCR7 | 8F11-M16 | | | | | |
| Delta Opioid Receptor | DOR7D2A4 | | | | | |
| DLL1 | MHD1-314 | | | | | |
| DLL4 | MHD4-46 | | | | | |
| DR3 | JD3 | | | | | |
| EGFR | AY13 | | | | | |
| erbB3 | 1B4C3 | | | | | |
| FcRL4 | 413D12 | | | | | |
| FcεRIα | AER-37 (CRA-1) | M | M | M | M | |
| Galectin-9 | 9M1-3 | T G | BT G | | BT G | T |
| GARP | 7B11 | | | | | |
| HLA-A2 | BB7.2 | | | | | |
| HLA-A,B,C | W6/32 | BTNMG | BTNMG | BTNMG | BTNMG | BTNMG |
| HLA-DQ | HLADQ1 | B | | | | |
| HLA-DR | L243 | BTNM | B MG | BTNMG | BTNMG | BTNMG |
| HLA-E | 3D12 | BTNMG | | | | |
| HLA-G | 87G | B | MG | T MG | | M |
| HVEM | 122 | BTNMG | BTNMG | BTNM | BTNMG | BTNMG |
| IFN-γ R b chain | 2HUB-159 | T MG | G | T | T | T MG |
| IgD | IA6-2 | B | | | | |
| IgG1, κ Isotype Ctrl | MOPC-21 | | | | | |
| IgG1, κ Isotype Ctrl | RTK2071 | | | | | |

B=B cells  
T=T-cells  
N=NK cells  
M= monocytes  
G= granulocytes

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| IgG2a, κ Isotype Ctrl | MOPC-173 | | | | | |
| IgG2a, κ Isotype Ctrl | RTK2758 | | | | | |
| IgG2b, κ Isotype Ctrl | MPC-11 | | | | | |
| IgG2b, κ Isotype Ctrl | RTK4530 | | | | | |
| IgG3,k Isotype Ctrl | MG3-35 | | | | | |
| IgG Isotype Ctrl | HTK888 | | | | | |
| Ig light chain κ | MHK-49 | B MG | G | | | |
| Ig light chain λ | MHL-38 | B M | B MG | B MG | B MG | |
| IgM | MHM-88 | B NMG | B MG | B NMG | BTNMG | BTNMG |
| IgM, κ Isotype Ctrl | MM-30 | | | | | |
| IgM, κ Isotype Ctrl | RTK2118 | | | | | |
| IL-28RA | MHLICR2a | | | | | |
| Integrin α9β1 | Y9A2 | MG | G | N | N | N G |
| integrin β5 | AST-3T | | G | | | |
| Integrin β7 | FIB504 | BTNMG | BTNM | BTNMG | BTNMG | BTNMG |
| Jagged 2 | MHJ2-523 | N | | | | |
| LAP | TW4-6H10 | M | MG | | M | T M |
| Lymphotoxin β Receptor | 31G4D8 | MG | M | M | | BT MG |
| Mac-2 | Gal397 | T G | T G | | T G | T G |
| MAIR-II | TX45 | | T G | T | T | T G |
| MICA/MICB | 6D4 | | | | | |
| MSC | W7C6 | T | T | T G | T M | T |
| MSC,NPC | W4A5 | BT MG | T G | BTNMG | BT MG | BT MG |
| NKp80 | 5D12 | N | TN | TNM | TNM | TN |
| Notch 1 | MHN1-519 | N | | | | |
| Notch 2 | MHN2-25 | M | | | | M |
| Notch3 | MHN3-21 | | | | | |
| Notch 4 | MHN4-2 | | | | | |
| NPC | 57D2 | | | | | T |
| Please Inquire | 2H3 | N | | | | B |
| Podoplanin | NC-08 | | | | | |
| Pre-BCR | HSL2 | MG | M | M | | |
| PSMA | LNI-17 | | | | | |
| Siglec-10 | 5G6 | B M | | | | MG |
| Siglec-8 | 7C9 | N | | | | |
| Siglec-9 | K8 | NMG | | | | |
| SSEA-1 | MC-480 | G | | | | |
| SSEA-3 | MC-631 | | | | | B |
| SSEA-4 | MC-813-70 | | MG | G | G | |
| SSEA-5 | 8E11 | T | | | | T |
| SUSD2 | W3D5 | B | | TN | | BT MG |
| SUSD2 | W5C5 | N | TNM | | G | BTNMG |

| Antibody | Clone | Human | Cyno | Rhesus | Baboon | AGM |
|---|---|---|---|---|---|---|
| TCR gamma/delta | B1 | TNMG | T | TN | TN | T |
| TCR Vα24-Jα18 | 6B11 | | | | | |
| TCR Vα7.2 | 3C10 | T | | | | |
| TCR Vβ13.2 | H132 | | | | | |
| TCR Vβ23 | αHUT7 | | | | | |
| TCR Vβ8 | JR2 (JR.2) | | | | | |
| TCR Vβ9 | MKB1 | | | | | |
| TCR Vδ2 | B6 | | | | | |
| TCR α/β | IP26 | T | | | | |
| Tim-1 | 1D12 | N G | | | | |
| Tim-3 | F38-2E2 | N | | | | |
| Tim-4 | 9F4 | | | | | |
| TLT-2 | MIH61 | | | | | |
| TNAP | W8B2 | G | G | | B MG | B G |
| TRA-1-60-R | TRA-1-60-R | | | | | |
| TRA-1-81 | TRA-1-81 | N | | | | |
| TSLPR | 1B4 | | | | M | |
| Vγ9 | B3 | | | | | |
| β2-microglobulin | 2M2 | BT MG | B N | B | B | B |

**Learn more about LEGENDScreen™ at:**
 **biolegend.com/legendscreen**



IL-1β, IL-12, IL-18, TNF-α

Dendritic Cell Maturation

Macrophage

Apoptosis

TLRs, NLRs, CLRs, Other Intracellular Receptors

Phagosome

Differentiation

CCL2, 3, 5, 7

Activation

Monocyte

41
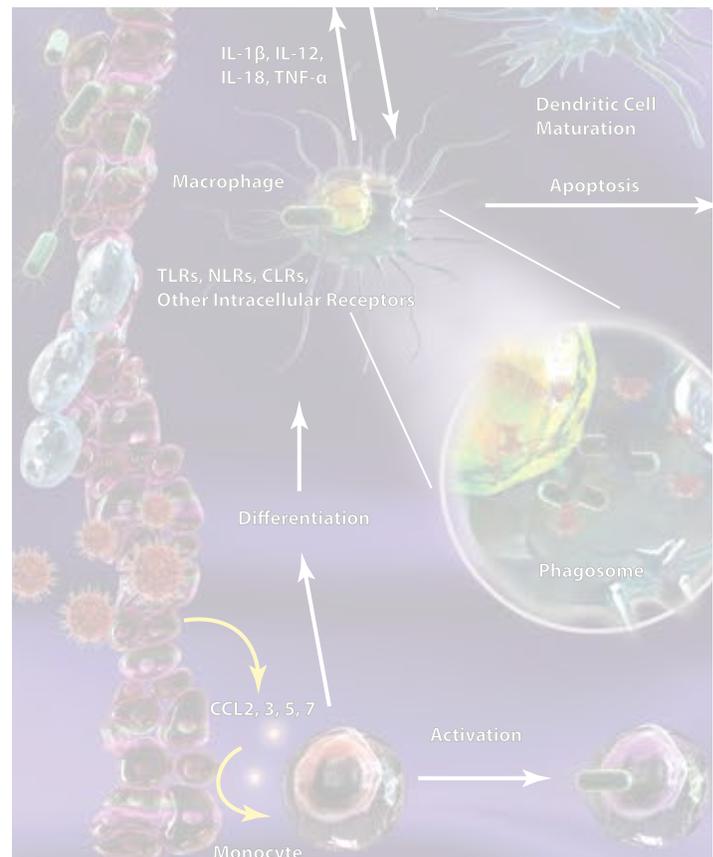
## Innate Immunity Poster

The innate immune system serves as your first line of security when pathogens attempt to breach your defenses. This involves anatomical barriers such as your skin and stomach acid. Cells like natural killer cells, dendritic cells, macrophages, and neutrophils can recognize pathogens and eliminate them by phagocytosis or induce the adaptive arm of immunity. In certain situations, even tumor cells can be destroyed if natural killer cells are able to detect the downregulation of common surface markers.



# Innate Immunity

# Pathogen control

Bacteria

Parasite

Parasite

Th1 and Th17 Response

Th2 Response

Cytotoxic T Lymphocytes

Cancer

Pathogen control

© BioLegend, Inc. 2013
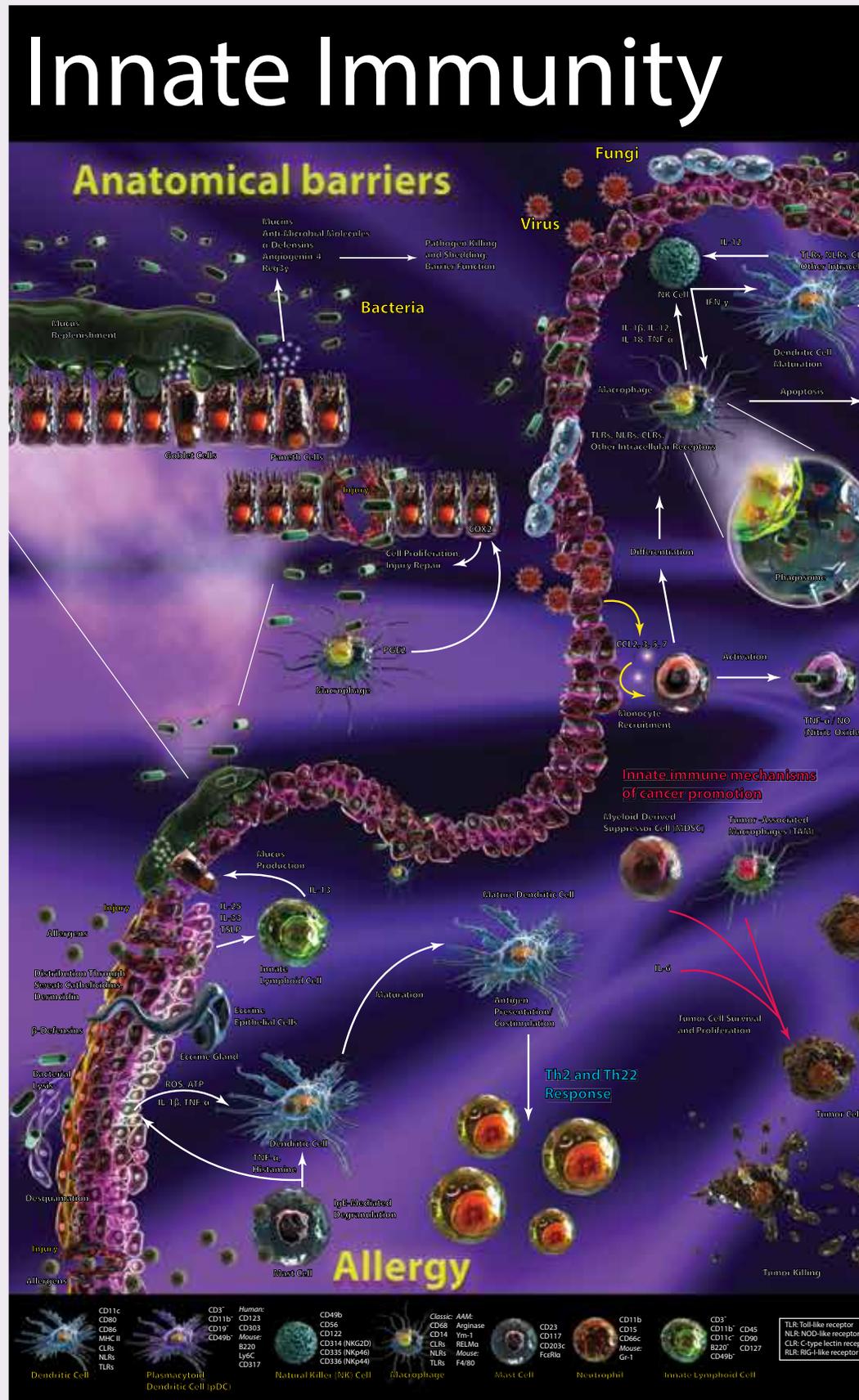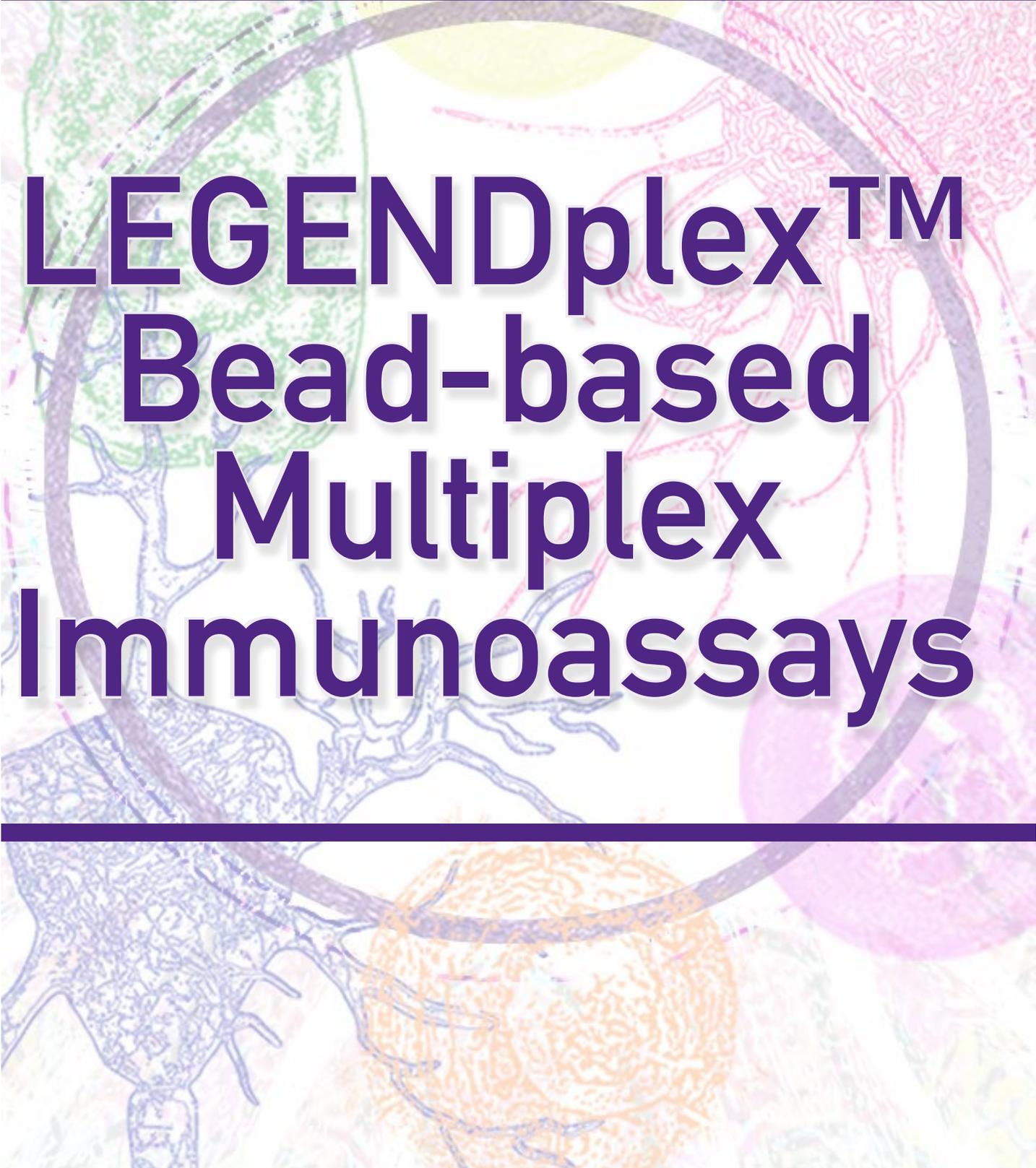
# LEGENDplex™ Bead-based Multiplex Immunoassays

# LEGENDplex™ Bead-based Multiplex Immunoassays

## Biomarkers & Multiplex Immunoassays

**Bi·o·mark·er (noun)**

A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [1].

In their role as soluble signaling molecules, biomarkers help to coordinate the multifactorial processes that govern immune-related biological responses. These molecules regulate intricate networks of synergistic and antagonistic interactions that instruct everything from the lineage choices of hematopoietic differentiation to pathogenic inflammation, cancer, and the host response to infection. Ongoing biomedical research is currently seeking to define the totality of these biomarker interactions in and around the cells they modulate [2].

Multiplex immunoassays, which simultaneously quantify the concentrations of multiple biomarker targets, are currently helping researchers to elucidate how these key molecules orchestrate the immune responses in multiple disease states ranging from inflammation, cancer, cardiovascular disease, neurodegenerative conditions, and beyond [3-7]. These assays typically provide higher detection sensitivity and broader dynamic ranges, while at the same time requiring far less sample volumes than traditional ELISA methods.

## LEGENDplex™: BioLegend's Bead-based Multiplex Assays

LEGENDplex™ bead-based immunoassays quantitate up to 13 targets simultaneously from one small sample volume. These assays are based on the same scientific principle as sandwich ELISAs:

Capture beads, which are differentiated by size and internal APC fluorescence intensity, are conjugated to antibodies specific to a given biomarker target.

Panels of defined capture bead sets are then incubated with biological samples, next a biotinylated detection antibody cocktail is added to form bead-analyte-detection antibody sandwiches.

A streptavidin-PE conjugate is added to bind to the biotinylated detection antibodies, providing a fluorescent signal intensity proportional to the amount of bound analyte.

The PE fluorescence of analyte specific bead regions are quantified using flow cytometry and the concentrations of biomarker targets are determined using the LEGENDplex™ data analysis software.

Figure 1.



45
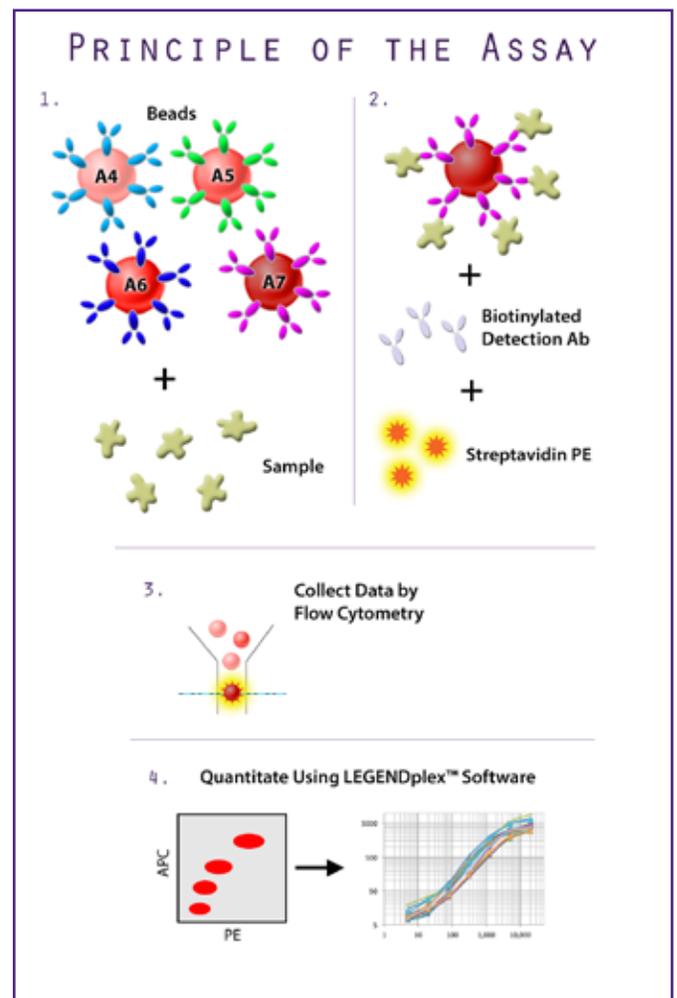
# LEGENDplex™ Features & Specifications

LEGENDplex™ assays are compatible with all flow cytometers capable of detecting PE and APC fluorescence, or PE and PerCP in the case of instruments without a red laser.

In addition, free data analysis software is available with the purchase of any assay.

| Features | |
|---|---|
| No. Analytes per assay | 1 to 13 |
| Min. volume | 12.5 µL serum/plasma \| 25 µL TC supernatants |
| Cytometer Requirements | PE & APC detection (PerCP/FL3 without red laser) |
| Analysis Software | Free PC & Mac versions with assay purchase |
| Tests/Assay | 100 tests |

# LEGENDplex™ Applications

BioLegend has developed LEGENDplex™ panels for use in a wide variety of research fields in the scientific community.

Each LEGENDplex™ panel is specifically designed to quantitate biomarker targets that are central to a given biological process or disease state. Additionally, we are constantly expanding our catalog with new assay content.

Assays are available in human, non-human primate (NHP), mouse, and rat specificities.

Figure 2.
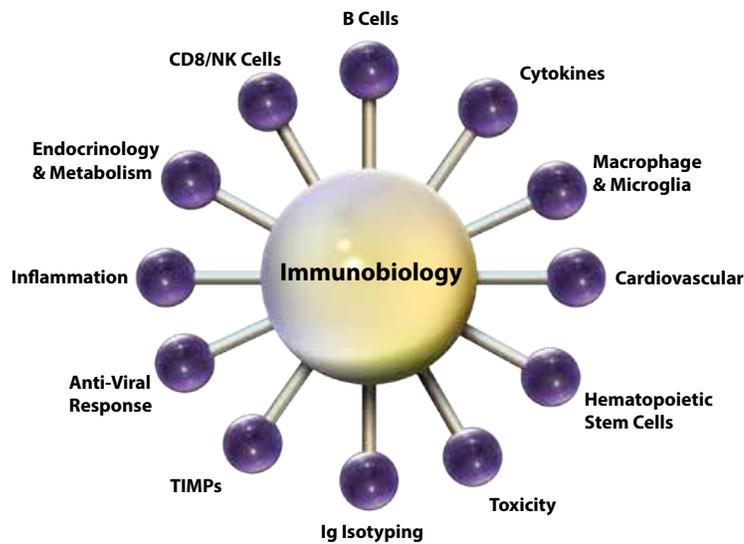


| Immunology | Species Availability | | | |
|---|---|---|---|---|
| | Human | NHP | Mouse | Rat |
| Anti-virus Response Panel | • | | • | |
| B cell Panel | • | | | |
| CD8/NK Panel | • | | | |
| T helper Cytokine Panel | • | • | • | • |
| Macrophage/Microglia Panel | • | | | |
| Inflammation Panel | • | • | • | • |
| Proinflammatory Chemokine Panel | • | | • | |
| TIMP Panel | • | | | |
| Cytokine 2 Panel | • | | • | |
| Immunoglobulin Isotyping Panel | • | | • | |
| Free Active/Total TGF-β1 Panel | • | • | • | • |
| Chemokine/Cytokine Panel | | • | | |

| Cardiovascular | | | | |
|---|---|---|---|---|
| Apolipoprotein Panel | • | | | |
| Vascular Inflammation Panel | • | | | |

| Metabolism & Endocrinology | | | | |
|---|---|---|---|---|
| Adipokine Panel | • | • | | |
| Metabolic Panel 1 | • | | | |
| Growth Factor Panel | • | • | | |
| IGFBP Panel | • | | | |
| Bone Metabolism Panel 1 | • | | | |

| Toxicity | | | | |
|---|---|---|---|---|
| Kidney Function Panel | • | | | |

| Stem Cells | | | | |
|---|---|---|---|---|
| Hematopoietic Stem Cell Panel | • | | • | |

Figure 3.

**Assay Procedure Summary for V-bottom Plate**

Capture beads

Analytes

For cell culture supernatant samples,
Add to the plate:
25 µL Assay Buffer to all wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL mixed beads to all wells

For serum and plasma samples,
Add to the plate:
25 µL Matrix A to standard wells
25 µL Assay Buffer to sample wells
25 µL diluted standard to standard wells
25 µL sample to sample wells
25 µL mixed beads to all wells

Incubate 2 hours, RT, shaking

Spin down beads
Wash 1 time
Add 25 µL Detection Antibodies
Incubate 1 hr, RT, shaking

Biotinylated Detection Antibody

Without washing, add 25 µL SA-PE
Incubate 30 min, RT, shaking

Spin down beads, remove supernatant
Wash 1 time (optional)
Add 150 µL of 1x Wash Buffer
Read on a flow cytometer

## LEGENDplex™ Assay Formats:

### Pre-defined Panels

These assays are comprised of combinations of up to 13 key targets that are central to a given biological process or disease state.

### Mix & Match

Want to assay some but not all of the pre-defined targets?

Use this option to choose a sub-group of targets from a single pre-defined panel.

### Custom Solutions

Have a project that needs to combine targets from multiple pre-defined panels?

Our custom solutions team can help you to create an assay that suits your project.

47

# Featured LEGENDplex™ Assay: Mouse Hematopoietic Stem Cell Panel

Hematopoietic stem cells (HSCs) are self-renewing multipotent cells that give rise to blood cells through the process of hematopoiesis. Found in the red bone marrow, HSCs are derived from mesodermal tissue and can differentiate into myeloid, erythroid, and lymphoid lineages of cells, which then further differentiate into specialized blood cell types (Figure 4). The process of hematopoiesis itself is the cumulative result of tightly coordinated signaling cascades which are activated by local signals in the stem cell niche and from hematopoietic cytokines [8].

For decades the scientific community has debated whether hematopoietic cytokines actively instruct lineage choice or only promote the survival and proliferation of cells already committed to a lineage fate [9-11]. Recent results have established that cytokines do in fact instruct lineage choice; however, the precise role each cytokine plays in this process remains largely unknown due to the highly overlapping signaling pathways activated by multiple cytokine receptors [11].

The **LEGENDplex™ Mouse Hematopoietic Stem Cell Panel** has been specifically developed to quantify 13 biomarker targets key to hematopoietic lineage determination. The assay has been validated for use with both serum and plasma samples as well as tissue culture supernatants. The full 13-plex panel is designed to allow flexible customization of targets and BioLegend also offers lymphoid, erythroid, and myeloid-specific 7-plex sub-panels.

**Learn more at: biolegend.com/legendplex**

Figure 4.



**Key Markers**

Lymphoid: IL-3, IL-6, IL-15, TGF-β1, SCF, LIF, CXCL12

Myeloid: IL-3, IL-6, IL-34, GM-CSF, M-CSF, SCF, CXCL12

Erythroid: IL-3, IL-6, EPO, TPO, SCF, GM-CSF, CXCL12
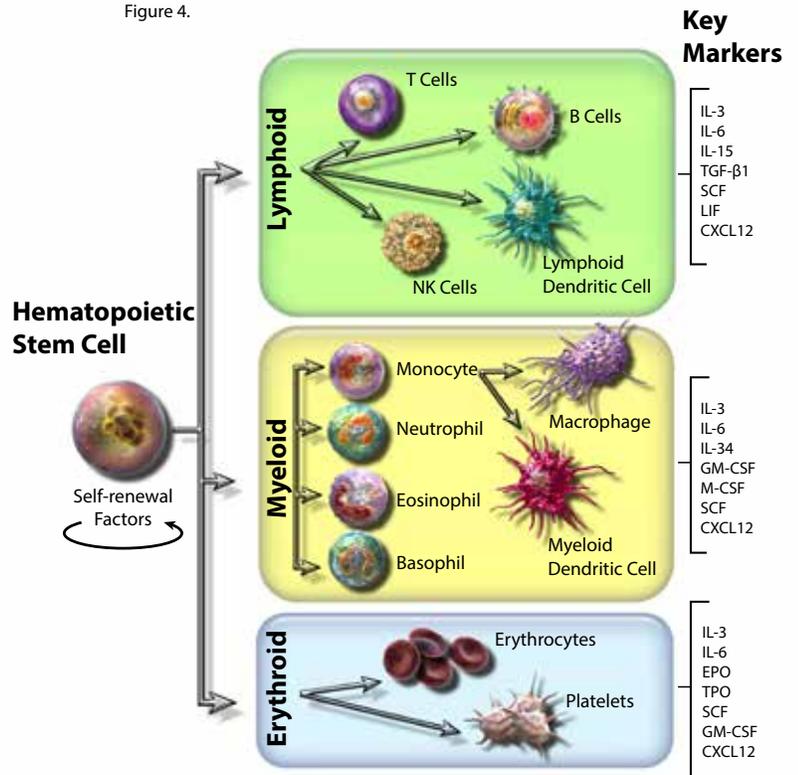
**Hematopoietic Differentiation**

Hematopoietic stem cells give rise to multiple blood cell types that are derived from Lymphoid, Myeloid, and Erythroid Lineage Precursors. Key differentiation biomarkers that are included in the LEGENDplex™ Mouse Hematopoietic Stem Cell Panel are listed.

| Target | Bead ID | HSC Full Panel | Lymphoid Sub-panel | Erythroid Sub-panel | Myeloid Sub-panel | Assay Sensitivity (pg/mL)* | | | |
| | | | | | | TC Supernatant | | Serum/Plasma | |
| | | | | | | Mean | SD | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| IL-34 | A4 | • | | | • | 7.53 | 3.86 | 10.70 | 3.15 |
| IL-5 | A5 | • | | | • | 0.25 | 0.11 | 0.21 | 0.13 |
| M-CSF | A6 | • | | | • | 1.00 | 0.52 | 0.86 | 0.28 |
| TPO | A7 | • | | • | | 1.61 | 0.39 | 0.99 | 0.23 |
| IL-6 | A8 | • | • | • | | 1.56 | 0.43 | 1.46 | 0.59 |
| GM-CSF | A10 | • | | • | • | 1.24 | 0.37 | 1.04 | 0.31 |
| IL-15 | B2 | • | • | | | 5.79 | 2.35 | 9.03 | 2.27 |
| TGF-β1 | B3 | • | • | • | • | 1.56 | 0.48 | 2.01 | 2.12 |
| IL-3 | B4 | • | • | | | 1.24 | 0.44 | 1.35 | 0.47 |
| LIF | B5 | • | • | | | 1.31 | 0.78 | 1.19 | 0.49 |
| SCF | B6 | • | • | • | • | 6.02 | 2.11 | 6.00 | 3.27 |
| EPO | B7 | • | | • | | 4.98 | 0.92 | 4.67 | 1.53 |
| CXCL12 | B9 | • | • | • | • | 43.75 | 12.22 | 40.42 | 12.43 |
| Cat. No. | Filter Plate | 740676 | 740678 | 740680 | 740682 | *Sensitivity is the theoretical limit of detection calculated using the LEGENDplex™ Data Analysis Software by applying a 5-parameter curve fitting algorithm. | | | |
| | V-bottom Plate | 740677 | 740679 | 740681 | 740683 | | | | |

# Featured LEGENDplex™ Assay: Mouse Hematopoietic Stem Cell Panel

Figure 5.



**EC+OP9-DL1**   **Microglia+OP9-DL1**   **EC+Microglia+OP9-DL1**
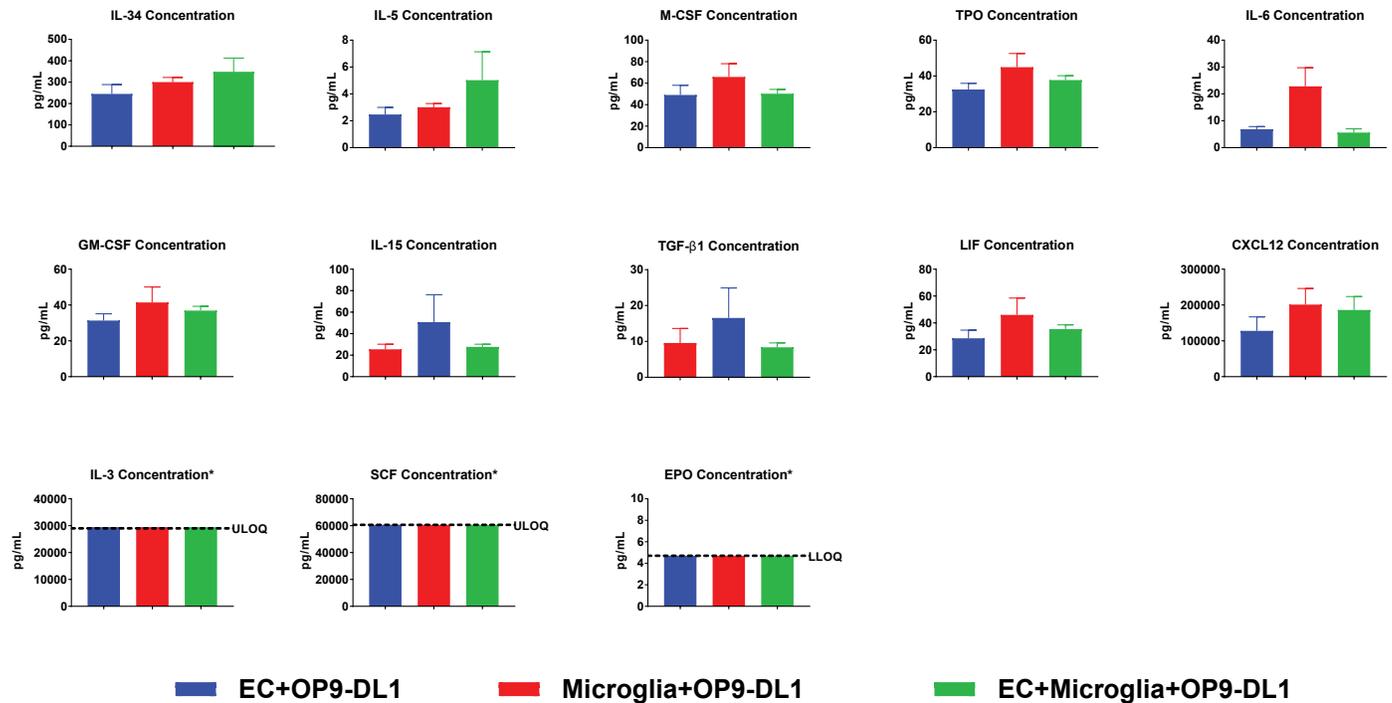
## Biological Detectability of Analyte Targets

Head tissue from E10.5 mouse embryos was digested and then sorted using FACS to isolate endothelial cells (CD31+ CD41- CD45-) and microglial cells (CD45+ CD11b+ F4/80+). Cells were then cultured with OP9-DL1 cells, a bone-marrow derived stromal cell line that ectopically expresses the Notch ligand, Delta-like 1 (DL1)[12]. This *in vitro* culture system allows for the commitment, differentiation, and proliferation of T cells from hemogenic endothelial cells. Cells were co-cultured for 7 days, and then supernatants were assayed using the LEGENDplex™ Mouse hematopoietic stem cell panel.

Concentration calculations for all assay panel targets are presented as the mean (n = 6) ± SEM. All targets were detected in the biological samples except for EPO, which was below the limit of detection.

*Note: The concentration of both SCF and IL-3 were above the upper limit of quantification for the assay as the media used in the co-culture system (alpha MEM, 10% FBS, 100 ng/ml each of SCF, FLT3L, IL-3) contained high amounts of both analyte targets.*

We thank Dr. Zhuan Li's group from the MRC Centre for Inflammation Research at the University of Edinburgh for sharing their data.

### References

1. NIH Biomarkers Definitions Working Group. 2001. *Clin Pharmacol Ther*. 69(3):89-95.
2. Constantini S, *et al*. 2010. *Bioinformation*. 6;5(4):166-7.
3. Capone F, *et al*. 2016. *Mediators Inflamm*. 2016:3064643.
4. Schett G, *et al*. 2013. *Nat Med*. 19(7):822-4.
5. Tse E, Kwong YL. 2015. *Semin Cancer Biol*. 34:46-51.
6. Reale M, *et al*. 2009. *Mini Rev Med Chem*. 9(10):1229-41.
7. Vistnes M, *et al*. 2010. *Expert Rev Mol Diagn*. 10(2):147-57.
8. Rieger MA, Schroeder T. 2012. *Cold Spring Harb Perspect Biol*. Dec 1;4(12).
9. Enver T, *et al*. 1998. *Blood* 92(2):348-51.
10. Metcalf D. 1998. *Blood* 92(2): 345-7.
11. Endele M, *et al*. 2014. *Exp Cell Res*. 10;329(2):207-13.
12. Holmes R, *et al*. 2009. *Cold Spring Harb Protoc*. 2009(2):pdb.prot5156.

# Dendritic Cell, Monocyte, and Macrophage Biology Poster

Monocytes originate in the bone marrow and can extravasate into tissues and differentiate into dendritic cells or macrophages, depending on the stimuli. These cells have key roles in pathogen killing and antigen presentation to bridge the gap between innate and adaptive immunity. Macrophages can become M1 or M2-polarized, which are generally considered proinflammatory and anti-inflammatory states respectively. They can also aid in wound repair. Dendritic cells are regarded as the better antigen presenting cell of the two, and can be classified as either classical or plasmacytoid dendritic cells. Phenotyping these myeloid cells can often prove to be very difficult, as they share several markers, and marker expression may change depending on tissue location.



50

## Distribution

**Lymph Node**

Plasmacytoid Dendritic Cells:
B220⁺ Ly6C⁺ CD317⁺ CD11c^int (Mouse)
CD123⁺ CD303⁺ CD304⁺ (Human)

Follicular Dendritic Cells:
CD21⁺ CD35⁺CD157⁺ICAM-1⁺

MHC II⁺ CD11c⁺
CD8α CD11b⁻ (Mouse)
CD11c⁺ HLA-DR⁺
CD16⁻ CD11b⁺ (Human)

Mouse Dermal Dendritic Cells:
MHC II⁺ CD45⁺ CD11b⁺
CD207⁺ and CD207⁻

Epithelium

CD11c⁺ MHC II⁺ CD103⁺ (Mouse)
CD11c⁺ HLA-DR⁺ CD103⁺ (Human)

**Cancer**

M1 Phenotype Tumor-
Associated Macrophage

Tumor-Associated
Dendritic Cells:
FOXO3a⁺ CD34⁺
CD86
CD31

VEGF, IL-10
PGE2, TGF-β

M2 Phenotype Tumor-
Associated Macrophage

**Patrolling**

Patrolling Monocytes:
CD115⁺ CD11b⁺Ly6C⁺ (Mouse)
CD14^dim CD16⁺ (Human)

M1 Polarized Macrophages:
MHC II⁺ CD86⁺
CXCL9 CXCL10⁺
IL-12 iNOS⁺

| Step 1 Tethering and Rolling: | Step 2 Activation: | Step 3 Arrest and Flattening: | Step 4 Locomotion to Interendothelial Junctions: | Step 5 Extension of Protrusions Through Interendothelial | Step 6 Transmigration: |
|---|---|---|---|---|---|
| P-Selectin E-Selectin L-Selectin MadCAM-1 VLA-4 | CXCL1 CCL5 CCL21 CXCL12 | LFA-1 VLA-4 α4β7 | Mac-1 LFA-1 VLA-4 | Junctions: PECAM-1 CD99 CX3CL1 CCL3 CCL4 | ICAM-1 |

Inflammatory Monocytes:
CD115⁺ CD11b⁺ Ly6C⁺ (Mouse)
CD14⁺ CD16⁻ (Human)
CD14⁺ CD16⁺ (Human)

Conversion

M1 Polarized Macrophages:
MHC II⁺ CD86⁺
CXCL9 CXCL10⁺
IL-12 iNOS⁺

## Principal Functions

**Pathogen Killing**

**Antigen Presentation**

MHC I
MHC II

**Tissue Repair and Wound Healing**

1. Early Inflammatory Phase

Tissue Injury

Pro-Inflammatory
M1-like Macrophages
TNF-α
ROS
MMPs
IL-12

2. Late Inflammatory Phase

Apoptotic Cells

Anti-Inflammatory
M2-like Macrophages
TGF-β
IL-10
VEGF

3. Granulation Tissue Wound Contraction

Growth Factors

Pro-Fibrotic
M2a-like Macrophages
TGF-β
VEGF
FGF-2

4. Matrix Deposition Remodeling

Fibrosis

Fibrolytic M2c-
like Macrophages
MMPs
IL-10

M1 Polarized Macrophages:
MHC II⁺ CD86⁺
CXCL9⁺ CXCL10⁺
IL-12⁺ iNOS⁺

M2 Polarized Macrophages:
CD206⁺ CD163⁺
IL-4Rα⁺ Arg1⁺ IL-10⁺

©BioLegend, Inc. 2014

51

# Biofunctional Antibodies

# Biofunctional Antibodies

Antibodies are essential components of the immune system, playing a critical role in the immune response against pathogens. They are glycoproteins that belong to the immunoglobulin superfamily. Produced by B cells, they can be expressed in the cell membrane, constituting the B cell receptor, or secreted by plasma cells to reach sites of infection.

Antibodies are complex molecules. Classical mammalian antibodies consist of two heavy chains and two light chains. There are also several types of both chains, which play a role in the recognition of the antibody by several surface cell receptors (Fc receptors) and other immune molecules. The amino terminal end of both chains defines the region that recognizes and binds other molecules, referred to as antigens.

The ultimate goal of antibodies is to help in the elimination of these antigens, and the pathogens from which they originate. Thus, the function of antibodies can be generally divided into:

1) Cross-linking (agglutination)

2) Complement activation

3) Opsonization

4) Antibody-dependent cell-mediated cytotoxicity

5) Neutralization

Figure 1. Summary of antibody functions. Specific types of antibodies may be more effective at mediating some of these effector functions.



**Protective Mechanism of Binding Antibodies to Antigens**

**Cross Linking**

Bacteria

**Enhances phagocytosis and reduces number of infectious units to be dealt with**

**Complement Activation**

**Lysis of Microbes**

**Opsonization**

Phagocyte

**Coating antigen with antibody enhances phagocytosis**

**Antibody-dependent cell-mediated cytotoxicity**

**Antibodies attached to target cell cause destruction by non-specific immune system cells**

**Neutralization**

Virus

Bacterium

Toxin

**Blocks adhesion of Microbes and Toxins to cell surface**

53

Given the biological properties of antibodies, they have been widely used both in research and clinical applications. Their high specificity allows for accurate recognition of single targets and their effector functions facilitate unique applications such as intracellular signaling activation or cell depletion. Thus, when referring to technical application, a biofunctional antibody can be defined as an antibody that can either mimic or interrupt the natural biological effects associated with ligand-receptor interaction, or have a biological effect on a target cell or molecule. Biofunctional antibodies are thus preferred in functional *in vitro* or *in vivo* studies. Antibodies without a



Figure 2. The different effects of biofunctional antibodies.

functional effect are instead preferred for phenotypic characterization or experiments where cells should not respond to the antibody treatment.

## LEAF™, Ultra-LEAF™, and GoInVivo™, BioLegend's Biofunctional Antibody Brands

When designing experiments where the use of antibodies for *in vitro* and *in vivo* treatment is required, there are factors that need to be tightly controlled. Variables such as endotoxin content, stabilizers, preservatives and any other additives can be confounding factors that are undesirable in the experimental outcome or readout. Thus, antibodies intended for functional assays should be formulated accordingly.

LEAF™ stands for Low Endotoxin, Azide-Free, and as the name indicates, these antibodies do not contain azide in the formulation, which is commonly used as an anti-microbial agent. They also do not contain any carrier protein or other additives; they are provided in PBS. Ultra-LEAF™ antibodies follow a similar concept; the difference is in the content of endotoxin guaranteed, which is ten times lower.

GoInVivo™ antibodies go several steps further, guaranteeing endotoxin less than 1.0 EU/mg of protein, very low aggregation, bulk sizes up to 1 gram, and testing for the presence of pathogens. The following table summarizes the features of these three brands.

**Table 1. Summary of key features of LEAF™, Ultra-LEAF™, and GoInVivo™ antibodies.**

| Feature | LEAF™ | Ultra-LEAF™ | GoInVivo™ |
|---|---|---|---|
| Purity | Assessed by SDS-PAGE. Guarantee purity >95% | Assessed by SDS-PAGE. Guarantee purity >95% | Assessed by SDS-PAGE. Guarantee purity >95% |
| Protein Integrity | Yes, by SDS-PAGE and IEF | Yes, by SDS-PAGE and IEF | Yes, by SDS-PAGE and IEF |
| Quality Control | Assessed by SDS-PAGE. Guarantee purity >95% | Each lot is quality-tested by flow cytometry | Each lot is quality-tested by flow cytometry |
| Azide Free | Yes | Yes | Yes |
| *In vitro* and *in vivo* applications | Yes | Yes | Yes |
| Validation | LEAF™ antibodies are functionally tested for limited targets | Ultra-LEAF™ antibodies are functionally tested for selected targets only | Most GoInVivo™ antibodies are validated by functional assays. |
| Endotoxin | <100 EU/mg | <10 EU/mg | <1EU/mg |
| Aggregation | Not Tested | Not Tested | Low aggregation, < 3% |
| Pathogen test | Not Tested | Not Tested | All GoInVivo™ antibodies are pathogen free as tested by qPCR for up to 20 pathogens, and several Mycoplasma species. |

**Learn more at: biolegend.com/ULEAF and biolegend.com/goinvivo**

# Applications in Immunotherapy Research

We have an extensive portfolio of biofunctional antibodies, and they have been widely used by the scientific community. Validation of the GoInVivo™ product line is particularly rigorous due to the scale of production and additional controls required. An important group of GoInVivo™ antibodies are clones with verified activity against important molecules of the immune system called immune checkpoints. Immune checkpoints are heavily involved in cancer development.



Figure 3. Illustration of some immune checkpoint molecules.

The progression of cancer is a complex process in which the physiological balance of the organism tilts towards dysregulation. Indeed, the term cancer encompasses a collection of related diseases that can start almost anywhere in the body. As a natural process, the cells in an organism grow and divide, giving rise to new cells as needed to replace compromised cells or cells that have died. When abnormal cells that should be eliminated survive, or divide without control, a localized tumor (in a mass of tissue for example) or cancer condition starts to evolve (in blood for example).

As our understanding of the disease improves, better and more efficient tools to combat it are developed. That is the case with immunotherapy. As its name indicates, immunotherapy is focused in manipulating the immune system to combat the disease using the body's natural immune response. The process of eliciting a T-cell mediated immune response, either to eliminate a foreign antigen or an abnormally proliferating cell, is also associated with mechanisms of self-regulation that ensure this immune response doesn't grow out of control. These regulatory mechanisms are largely controlled by the immune checkpoint molecules. Tumor cells engage these receptors to skew the immune response towards suppression, facilitating tumor cell proliferation without control by relevant cells or factors from the immune system. By targeting these molecules with monoclonal antibodies, it is possible to promote an activated state in T cells, inducing a sustained response against the cancer cells. Several drugs have been developed and approved by the FDA(1).

### *In Vitro* Data

BioLegend also has a unique set of biofunctional antibodies, developed by our R&D department, such as anti-mouse CX3CR1, clone SA011F11. CX3CR1 is expressed by several cell types, including monocytes, NK cells, a subset of T cells, and glial cells (within the brain). It is involved in cell recruitment during inflammation and participates in cell adhesion and extravasation from blood vessels. Its ligand is CX3CL1, also known as fractalkine or neurotactin, and it has been described as coreceptor for HIV1, as well as playing a role in mucosal immunity (2), atherosclerosis (3), and neurological disorders (4), among others. Figure 4 shows *in vitro* blocking activity of clone SA011F11.

In addition, we routinely validate bioactivity of GoInVivo™ antibodies *in vitro*. Antibodies that have been validated include anti-human and anti-mouse CD152 (clones L3D10, 9H10).
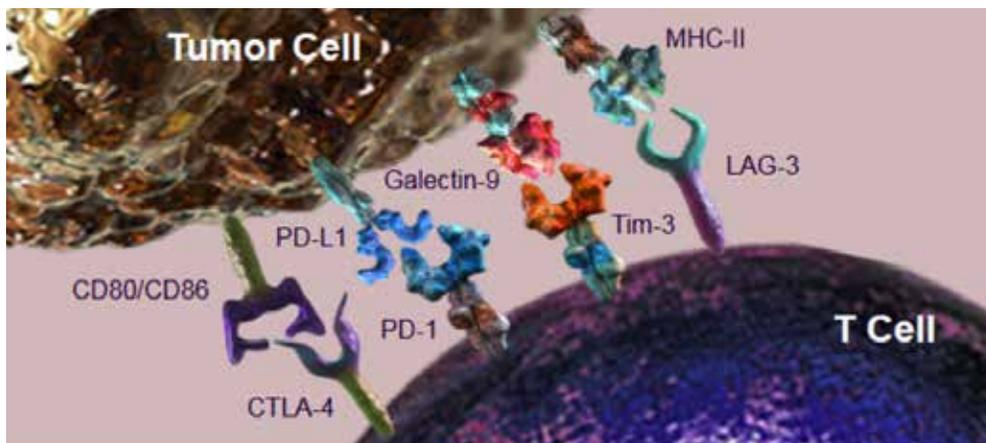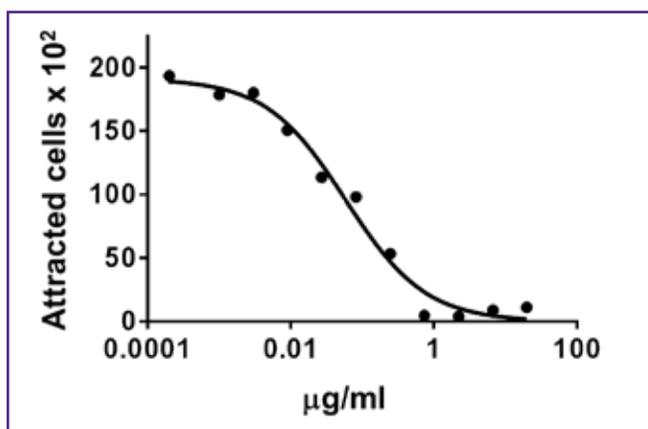


Figure 4. Ultra-LEAF™ Purified anti-mouse CX3CR1 antibody inhibits the chemotaxis induced by mouse CX3CL1 (50 ng/mL, BioLegend Cat. No. 583402) on BA/F3 mouse CX3CR1 transfected cells in a dose dependent manner with an ED50 = 0.05 – 0.15 µg/mL.

55

Figure 5. Anti-human (left panel) or anti-mouse (right panel) CD152 inhibits production of IL-2 by Jurkat cells. When Jurkat cells are stimulated with PHA (2.5 μg/ml) and soluble CD80 (B7.1, 500 ng/ml), the addition of soluble human or mouse CTLA-4 competes with CD80 and inhibits the production of IL-2 in a dose dependent manner (diamonds). When a fixed concentration of CTLA-4 is selected and pre-incubated with increasing concentrations of anti-CTLA-4 (clone L3D10 or 9H10, anti-human or anti-mouse respectively), the production of IL-2 is restored as the antibody blocks the activity of CTLA-4 (triangles), but not the isotype control (clone MOPC-21 or SHG-1, circles).
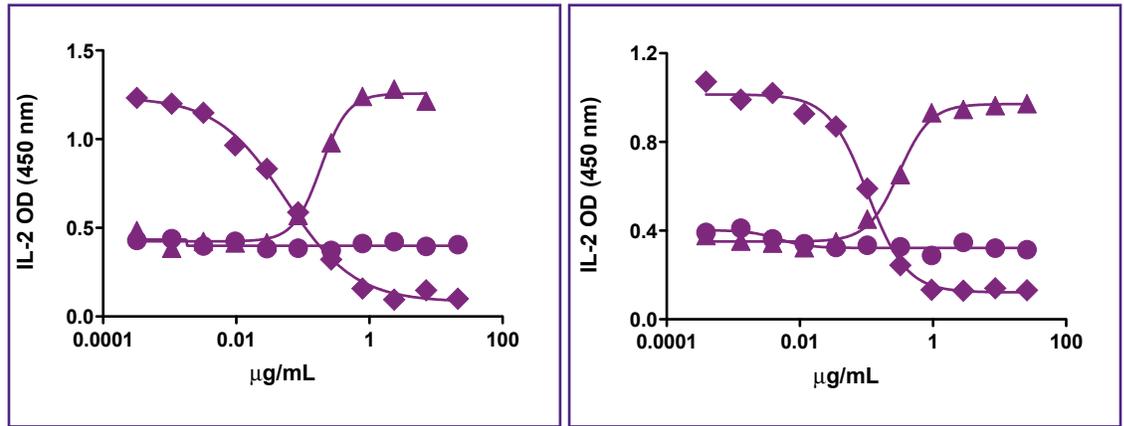


## *In Vivo* Data

Another unique clone to BioLegend is our anti-mouse CD20, clone SA271G2. This antibody is extremely efficient in depleting B cells when injected in mice. It is an excellent reagent to study autoimmunity or other conditions involving B cell deficiency, or even as an equivalent mouse model to Rituximab. Rituximab is an FDA approved drug, which is a monoclonal antibody against human CD20. The antibody destroys B cells expressing surface CD20, and is a successful treatment against leukemias and rheumatoid arthritis for example.

**Learn more at: biolegend.com/immune_checkpoints**
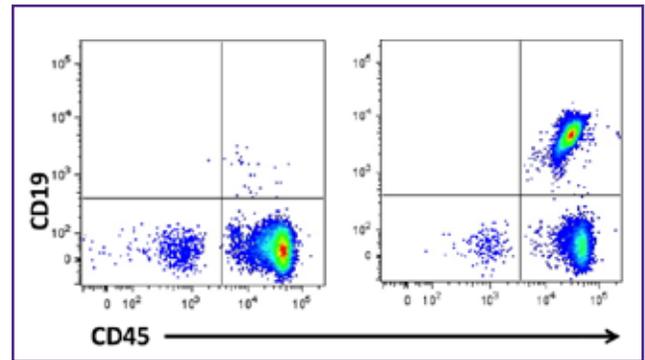


Figure 6. C57BL/6 mice were injected intravenously with 250 μg of Ultra-LEAF™ purified anti-mouse CD20 (clone SA271G2) (left panel) or Ultra-LEAF™ purified rat IgG2b, κ isotype control (clone RTK4530) (right panel). At day 7 the mice were bled and the samples were stained with anti-mouse CD19 (clone 6D5) APC and anti-mouse CD45 (clone 30-F11) Brilliant Violet 421™.
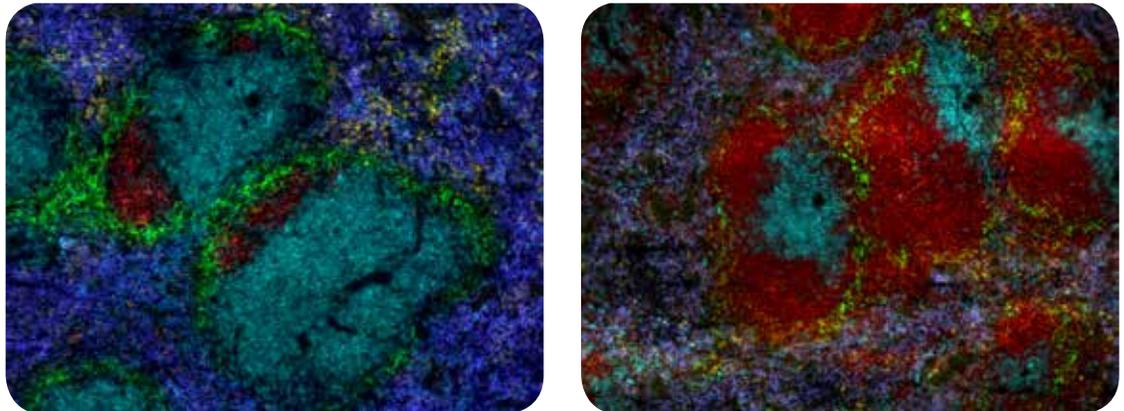


Figure 7. C57BL/6 mice were injected intravenously with 250 μg of Ultra-LEAF™ purified anti-mouse CD20 (clone SA271G2) (left panel) or Ultra-LEAF™ purified rat IgG2b, κ isotype control (clone RTK4530) (right panel). At day 7, the mice were sacrificed, spleens were collected, sectioned and stained for microscopy analysis. CD19 is shown in red, CD3 in cyan, CD169 in green, CD11b in yellow, and F4/80 in blue.

# GoInVivo™ in Melanoma Cancer Mouse Models

GoInVivo™ antibodies are an excellent tool for *in vivo* research as well, and they are specially formulated and tested to minimize variability and side effects when added to cell cultures or injected into animal models. To study the effect of anti-mouse PD-L1 (clone 10F.9G2) in a melanoma cancer mouse model, B16F10 melanoma cells were injected into the flanks of BALB/c mice, and the animals received 200 µg intraperitoneally every 3 days of either isotype control or anti-mouse PD-L1 antibodies. After 14 days, the animals were sacrificed, lymphoid organs and serum were collected, and the remaining tumor was excised. Lymphocytes were phenotyped by flow cytometry, serum was screened for cytokines and chemokines using LEGENDplex™.
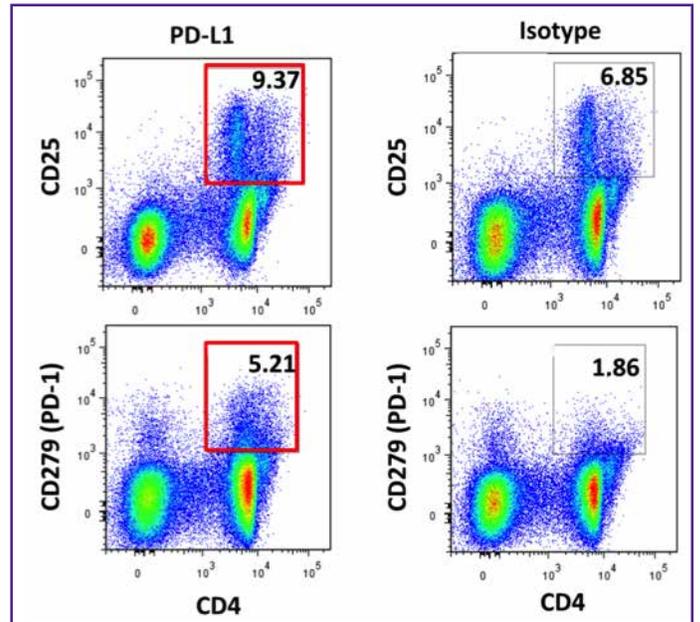


Figure 8. Animals treated with anti-PD-L1(clone 10F.9G2) (left panel) show an activated phenotype as compared to animals treated with isotype control antibody (clone RTK4530) (right panel). Dot plots show live splenocytes (7-AAD-).
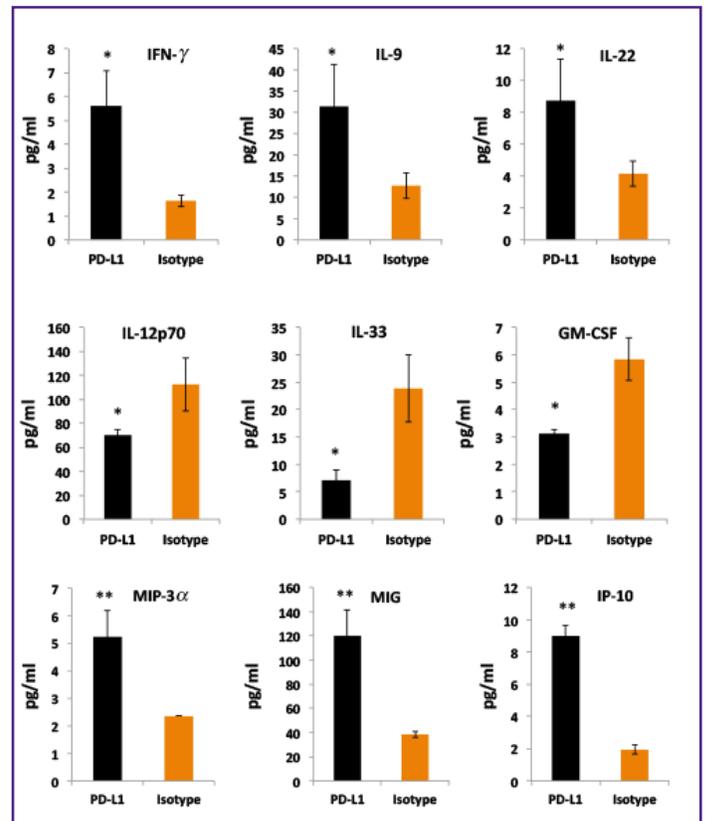


Figure 9. Bar graphs show the cytokine and chemokine profile of animals treated with anti-PD-L1 (black bars) or isotype control (orange bars). Interestingly, PD-L1 injection promotes T helper differentiation cytokine production (top panel, IFN-γ and TNF-α, Th1 profile, IL-9, Th9 profile, IL-22, Th22 profile) at the same time that downregulates systemic production of potent pro-inflammatory cytokines (middle panel). Likewise, PD-L1 treated animals are also able to produce important chemokines that help recruit relevant cells to fight the tumor cells. Bars represent 5 animals per group. Cytokines and chemokines in serum samples were measured using BioLegend's multiplex assay LEGENDplex™. * p ≤ 0.05, ** p ≤ 0.01.

Tumors and draining lymph nodes were excised and processed for microscopy imaging. Tissue slides from anti-PD-L1 and isotype control antibody treated animals were incubated with antibodies against CD4, CD8a, CD11c, and B220 (top panels), or F4/80, CD3, CD11c, and DAPI (lower panels), as indicated. All the experiments were designed and performed at BioLegend, San Diego, CA.

PD-L1

Isotype Control
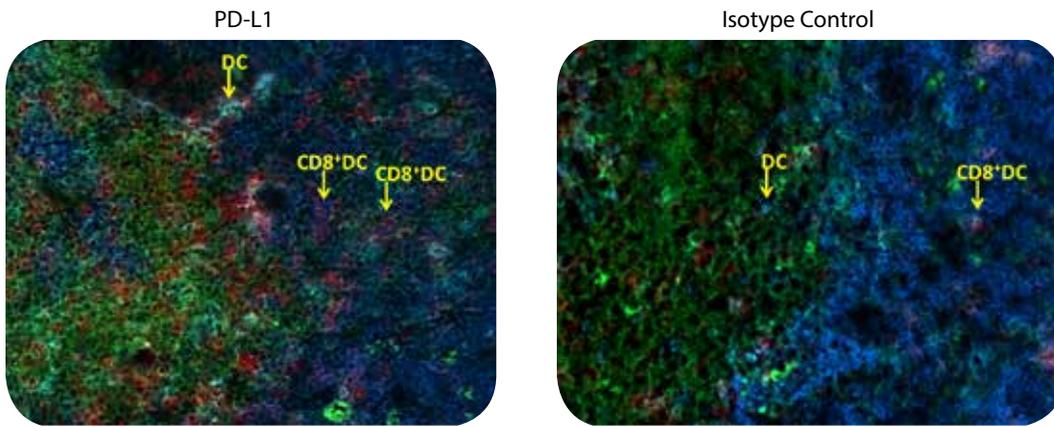


Figure 10. Animals treated with anti-PD-L1 (clone 10F.9G2) show a higher number of CD8α+ dendritic cells in the draining lymph nodes. CD8α+ dendritic cells have the capacity to cross-present antigens to T cells. In the slide, anti-CD4 in green, anti-CD8α in red, anti-CD11c in cyan, and anti-B220 in blue.

PD-L1

Isotype Control
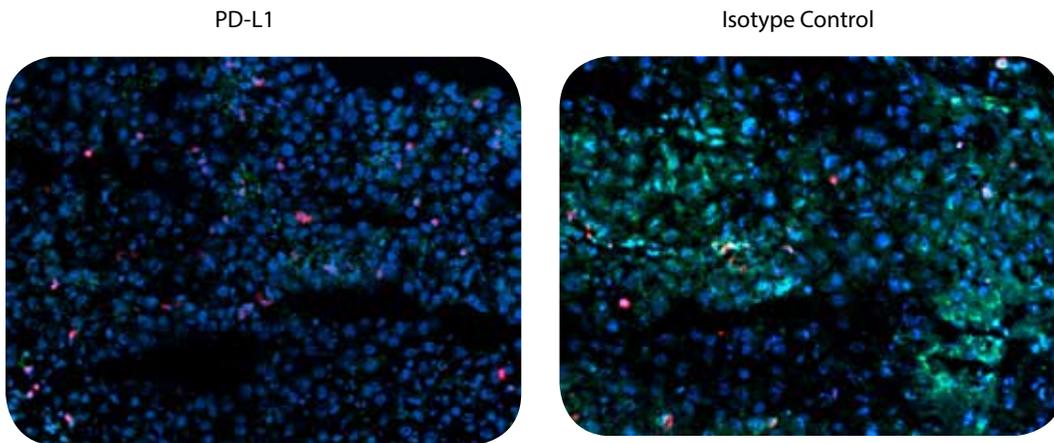


Figure 11. Animals treated with anti-PD-L1 (clone 10F.9G2) show a significantly reduced number of F4/80+ cells infiltrating the tumor. F4/80 is a typical marker for macrophages, and tumor associated macrophages (TAM) have been shown to play an important role in the development of the tumor microenvironment. In the slide, anti-F4/80 in green, anti-CD3 in red, anti-CD11c in cyan, and DAPI in blue.

In a similar series of experiments, B16F10 melanoma cells that express the SIYRYYGL model peptide (VSV Nucleoprotein 498–505) were injected into the flanks of C57BL/6 mice, and three groups of animals received 100 µg of anti-mouse PD-L1 (clone 10F.9G2) intraperitoneally. The first group received one injection at day 7 after implant. The second group received injections at days 7, 10, 13. The third group received four doses at days 7, 10, 13, and 16 after tumor implant.

At day 10 after implant (one antibody treatment at day 7), splenocytes were harvested and IFN-γ-producing cells were quantified (Figure 12). At day 14 after implant (three antibody doses administered), T cells from the tumor (TIL) and spleen were FACS sorted, stimulated, and the cytokine profile was measured (not shown). At day 24 after implant (four antibody treatments), tumor growth was evaluated, along with phenotyping of immune cells (Figure 13). Experiments were performed in collaboration with Thomas Gajewski's lab (The University of Chicago).
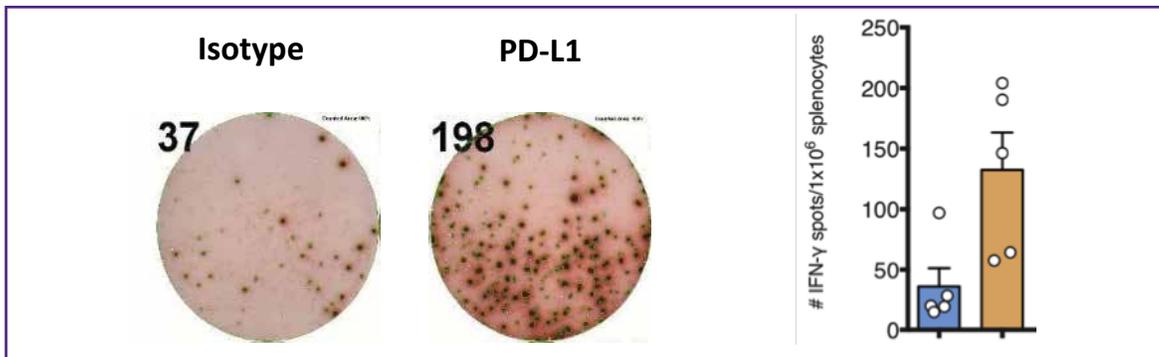


Figure 12. Splenocytes are harvested at day 10 post-implant and stimulated with SIYRYYGL peptide at 160 nM (12h ON, 37°C). Left panel shows an ELISPOT picture, right panel shows a bar graph summarizing the number of positive cells. Blue bar indicates isotype control treatment and yellow bar indicates anti-PD-L1 treatment.
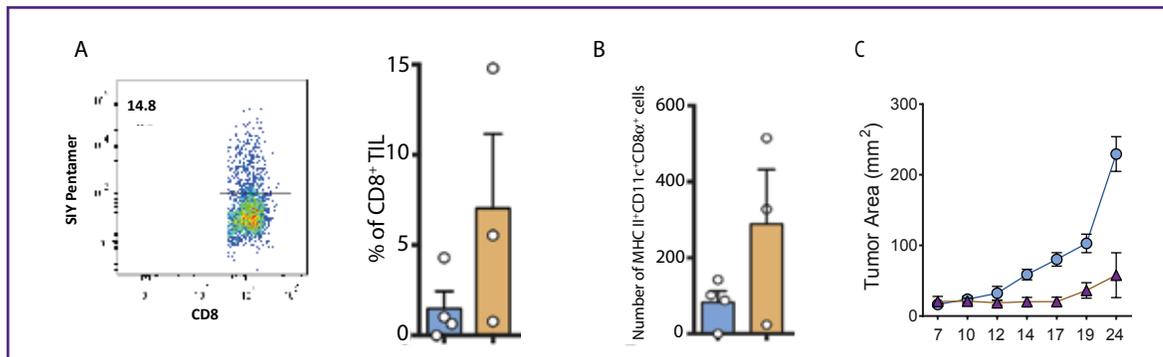


Figure 13. Panel A shows percentage of SIYRYYGL-specific TIL, as detected by MHC pentamers. Panel B shows number of CD8α+ dendritic cells in the tumor. Panel C shows tumor growth. Blue bars indicate isotype control treatment and yellow bars indicate anti-PD-L1 treatment.

In conclusion, biofunctional antibodies do not only show their potential as immunotherapeutic tools as already well documented (5, 6), they are also essential tools to dissect the molecular and cellular changes that lead to antibody-mediated tumor control.

**Learn more about GoInVivo™ at: biolegend.com/goinvivo**

**References**
1. Ito A, *et al*. 2015. *Biomed Res Int*. 2015:605478
2. Niess JH. 2010. *Gut Microbes*. 1(6):396
3. Gautier EL, *et al*. 2009. *Arterioscler Thromb Vasc Biol*. 29(10):1412
4. Chen P, *et al*. 2016. *Biomed Res Int*. 2016:8090918
5. Shin DS and Ribas A. 2015. *Curr Opin Immunol*. 33:23
6. Cook AM, *et al*. 2016. *Curr Opin Immunol*. 39:23

## Cancer Immunoediting Poster

Cancer is one of the most daunting and challenging areas of study, but our understanding develops as research progresses. Recently, Cancer Immunoediting has gained credence as the theory that the immune system is capable of both protecting the body from tumors and promoting tumor growth by selecting for tumors of low immunogenicity. This process consists of the 3 E's: Elimination, Equilibrium, and Escape. Elimination is the first phase and is initiated by the innate arm of immunity, which can then call on its adaptive counterpart. If a tumor is eliminated, the process ends without progressing to the other steps. Equilibrium occurs when tumors persist and experience immunological pressure from the body that is able to contain, but not destroy it. While some tumor cells may be destroyed in this step, others with advantageous mutations may survive. Having found ways to circumvent or elude the immune system, tumor cells will then enter the Escape phase. Allowed to grow unchecked, these tumor cells may then be detectable as clinical malignancies.



# Cancer Immunoedit

**BioLegend®**

**The 3 E's of Cancer Immunoediting**

Shedding of Tumor Recognition Antigens

Immune Editors
IFN-α/β
IFN-γ
IL-12
TNF-α
Perforin
TRAIL
Chemokines

Carcinogens
Inherited Defects
Radiation
Viruses
Chronic Inflammation

Normal Cell

Danger Signals
Uric Acid
ECM Products
Heat Shock Proteins
Type I IFNs

Tumor Cell

Normal Cell

MHC Class I Molecule
IFN-γ Response
CCL27
(Cutaneous Tumor)
CD80
PDL1

**Immunotherapy**

Cancer Therapies
Adoptive T Cell Transfer
Monoclonal Antibodies
Antibody Drug Conjugates
DCs/Peptide Vaccines

IL-12

IFN-γ

Dendritic Cell

CD8+ T Cell

CD4+ T Cell

**Immunoediting**

Necrotic Tumor

Dormant Tumor Cell

GM-CSF
CSF-1
IL-6
VEGF

COX2
PGE2

VISTA

MDSC

MDSC

Tumor Cell

M2 Macrophage

Arg1
NOS2
NO
RNOS

Arg1
IL-4
IL-10
IL-13
TGF-β

T Cell Deletion
or Anergy/Apoptosis

IL-4
IL-13

CD8+ T Cell

PDL1 (Molecular Shield)

PD-1

PDL1

B7-1

B7-1

CTLA-4

T Effector Apoptosis

IFN-γ

CD8+ T Cell

Immunosuppression

**Equilibrium**
(Cancer Persistence)

PDL1

B7-1

Immune Exhaustion or Inhibition
Tumor Cell Variants
Genetic and Epigenetic Changes
Resistance to Immune Detection
Tumor Maintained Chronically

Tumor Derived Factors
IL-6
VEGF
M-CSF
IL-1β
PGE2

**MDSC-Induced Immunosuppression**

Tumor Microenvironment

CCL2
CCL12
CXCL5
(Tumor Derived)

MDSC

Recruitment

IL-4
IL-13
GM-CSF
TGF-β

MDSC
NOS2
ROS
RNOS
Arg1

Release of MDSC into Peripheral Blood

HPC

CGP

MDSC

Bone Marrow

MDSC Maturation

iDC (immature DC)

Differentiation

pDC (Plasmacytoid DC)

IL-6
IL-13
GM-CSF

**Escape**
(Cancer Progression:
Poorly Immunogenic Tumors)

MDSC

Galectin

IDO

CD8+ T Cell

Treg

Tumor-Derived Soluble Factors

NK Cell

CD8+ T Cell

Fas Ligand

Soluble CD83

Apoptosis of Tumor-Specific Effectors

Soluble PDL1

Impair T Cell Effectors

HLA-G,E

ATP

CD39

CD73

AMP

Adenosine Receptor

Adenosine

Impair NK, CD8 T Cell Response

Immunosuppression

Epigenetic Silencing
Loss of IFN-γR Signaling

MICA/B

VEGF, M-CSF, IL-1β, IL-6

Impair NK, CD8+ T Cell, γ δ T Cell Response

Reduce T Cell Proliferation

Tumor Sculpting

Angiogenesis

**Tregs in Tumor Escape Checkpoints**

Enhanced Treg Activity

IDO
CCL22

Adenosine Receptor

FOXP3

Gangliosides
Galectin-1/9
PGE2
IL-10

TGF-β

Treg

CD4+
CD25+
CTLA-4+
GITR+

IL-13
TGF-β

VEGF
LXR-L

APC

pSTAT3

pDC

IDO

IDO
SOCS1
Arginase

CD80
CD86

APC

Tryptophan

CD80/CD86

CTLA4

Metabolized Tryptophan

IDO

Treg

Antigen Uptake

Macrophage/
Dendritic Cell
(Tolerogenic)

IL-12
MHC
CD80
CD86
IL-10
TGF-β

Effector T Cell

Effector T Cell

APC Dysfunction/
T Cell Anergy

IL-10
TGF-β
IDO
COX2
PGE2
Immunosuppressive Molecule

CD4+
CD25
T Cell

Th3/Tr1

T Cell Deletion or Anergy/Apoptosis

NOS2
NO
RNOS

Arg1
R-NOS
L-Arginine

Recruitment

MDSC

Lymph Nodes

Dendritic Cell

TGF-β
IL-10

IL-2
IL-10
IFN-α

pSTAT3
FOXP3

Treg

T Cell Apoptosis and Anergy

T Effector

CD8
Treg

Immunosuppression

Treg

Tumor Escape

**Immunosuppression**

© BioLegend, Inc. 2014

**biolegend.com**
Interactive Poster: biolegend.com/cancerimmunoediting
...ke to thank Dr. Robert Schreiber of Washington University - School of Medicine for his contributions to this poster.

**Contact BioLegend**
US & Canada Toll-Free: 1.877.246.5343 (877-BIOLEGEND)
International: 1.858.768.5800
Fax: 1.877.455.9587
email: cs@biolegend.com, techserv@biolegend.com

US Headquarters:
San Diego, CA 92121

04-0017-01

61

**biolegend.com**

# Recombinant Proteins for Immunobiology Research

# Recombinant Proteins

BioLegend offers an expansive catalog of bioactive and functional recombinant proteins that can be widely used for *in vivo* and *in vitro* research applications.

These proteins are guaranteed to be over 95% pure, and have endotoxin levels less than 1 EU per µg of protein. Our proteins are validated in-house through bioassays to ensure activity and reproducibility and tested against internal controls and competitors' equivalent protein when applicable.

Expressed with mammalian, insect, or *E.coli* expression system, our proteins can be in carrier-free formats for functional assays, or animal-free formats to avoid animal pathogens and experimental variability.

## Applications of Our Recombinant Proteins Include:

- Standard Cell Culture
- Cell Activation
- Cell Expansion
- Cell Differentiation

- Polarization
- Cytokine Production
- Growth and Proliferation
- Cell Inhibition

- Cytotoxicity
- Chemotaxis
- Adhesion

## Recombinant Protein Services:

**Catalog Proteins**

Choose from our catalog of over 700 recombinant human, mouse, and rat proteins

**Custom Solutions**

Have a project with special needs? We provide:

- Custom recombinant protein production
- Custom bioassay development

**Sample Testing**

We are happy to provide a sample for testing your system and experimental set-up before you purchase

**Bulk Orders**

Get large quantities of the same high quality proteins at an exceptional price

Email **sales@biolegend.com (for US and Canada), or**

**distributorsales@biolegend.com (for international orders).**

## Th17 Polarization with BioLegend Recombinant Proteins
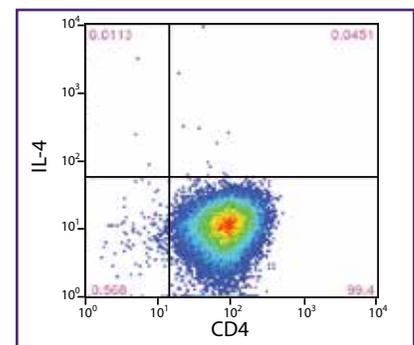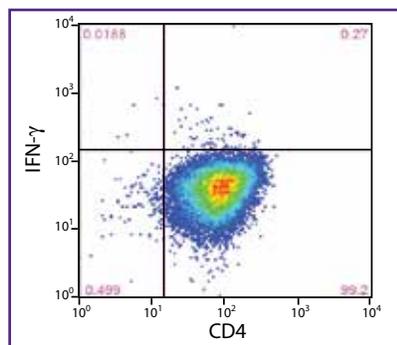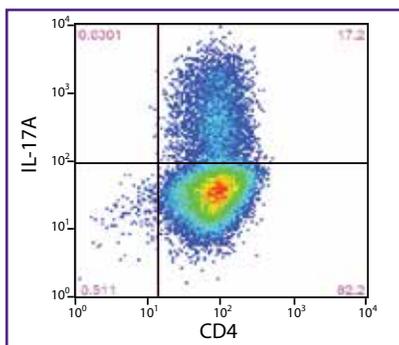


Mouse CD4+ T cells were polarized with plate-bound anti-mouse CD3, soluble anti-mouse CD28, recombinant mouse IL-6, IL-23, and TGF-β, anti-mouse IL-4, and anti-mouse IFN-γ for 4 days. After re-stimulation with PMA/ionomycin in the presence of BFA or Monensin, the cells were harvested and surface stained with CD4-FITC, and intracellularly stained with IL-17A-PE, IFN-γ-APC, or IL-4-APC.

# Commonly Used Recombinant Proteins for Immunobiology Research:

## Cytokines:

| | | |
|---|---|---|
| IFN-γ | IL-6 | IL-21 |
| IL-1β | IL-13 | IL-23 |
| IL-2 | IL-17A | IL-27 |
| IL-3 | IL-17F | IL-33 |
| IL-4 | IL-18 | IL-34 |

## Growth Factors:

| | | |
|---|---|---|
| BAFF | GM-CSF | RANK (TNFRSF11A) |
| BMP-9 | IGF-I | SHH |
| CD27L | IGF-II | TGF-β |
| CD40L | M-CSF | TNF-α |
| FLT3L | OX-40L | VEGF-165 |

## Chemokines:

| | | |
|---|---|---|
| CCL2 | CCL17 | CXCL10 |
| CCL3 | CCL19 | CXCL11 |
| CCL5 | CXCL5 | CXCL12 |
| CCL14 | CXCL8 | CXCL16 |
| CCL16 | CXCL9 | MCP-1 (CCL2) |

## Adhesion Molecules:

| | | |
|---|---|---|
| ALCAM/CD166 | EphA2 | Galectin-9 |
| CD6 | Ephrin-A1 | HVEM |
| CD200 | Galectin-1 | ICAM-1 |
| CD200 Biotinylated | Galectin-2 | ICAM-2 |
| E-Selectin | Galectin-3 | Siglec-5 |

## Soluble Receptors:

| | | |
|---|---|---|
| B7-H1 (PD-L1, CD274) | CTLA-4 | PDGFRβ Fc Chimera |
| B7-H2 | FAS (TNFRSF6) | sTNF-RI (TNFRSF1A) |
| BMPR1B/ALK-6-Fc Chimera | G-CSF Receptor/ CD114 | TLR3 |
| CD28 | IL-1R | TNFRSF10A (TRAIL R1)-Fc Chimera |
| CD200R1 | IL-1R2-Fc Chimera | TNFRSF13B |

## Enzymes and Regulators:

| | | |
|---|---|---|
| Arginase I | Granzyme A | MMP-9 |
| Cathepsin B | Granzyme B | Serpin E2 |
| Cystatin C | KLK7 | TIMP-1 |
| DPP4 | MMP-10 | TIMP-2 |
| Furin | MMP-12 | TIMP-4 |

**■ BioLegend • Competitor A**
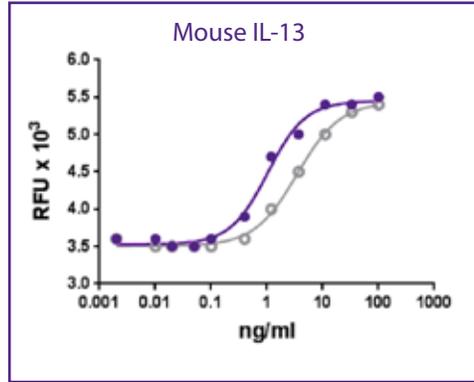
### Mouse IL-13



Recombinant mouse IL-13 induces the proliferation of TF-1 human erythroleukemic cells in a dose dependent manner.

**■ BioLegend • Competitor A**
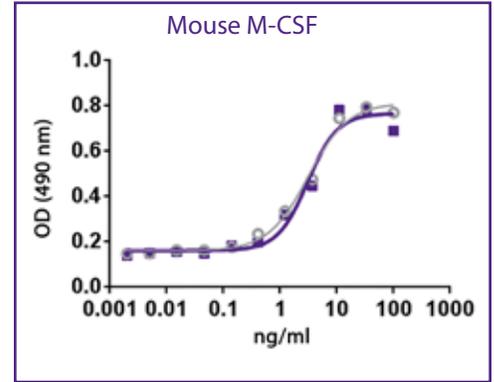
### Mouse M-CSF



M-NFS60 cell proliferation induced by mouse M-CSF.

**■ BioLegend • Competitor A**
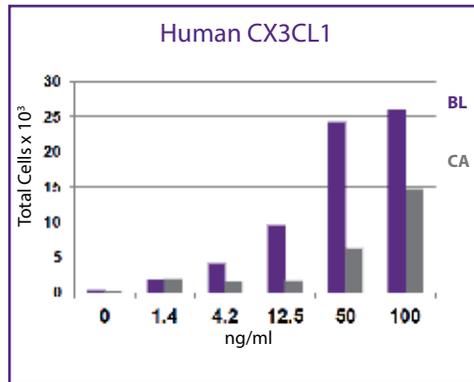
### Human CX3CL1



BaF3-mCX3CR1 transfectants attracted by human CX3CL1.

**■ BioLegend • Competitor A**
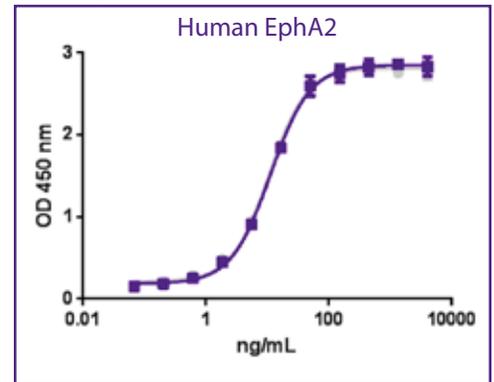
### Human EphA2



When human EphA2 is immobilized, human Ephrin-A1 binds with $EC_{50}$ of 3-12 ng/mL in a functional ELISA.

**■ BioLegend • Competitor A**
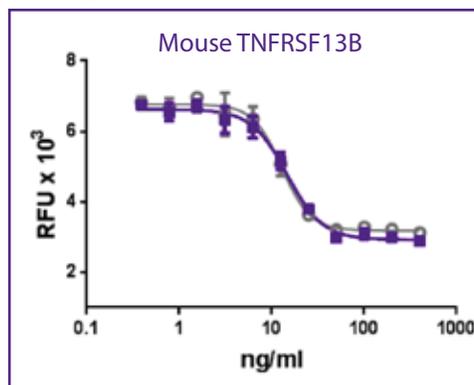
### Mouse TNFRSF13B



Mouse TNFRSF13B (TACI) inhibits mouse B cell proliferation induced by mouse BAFF (2.5 ng/mL). BioLegend's protein was compared side-by-side to the leading competitor's equivalent product.
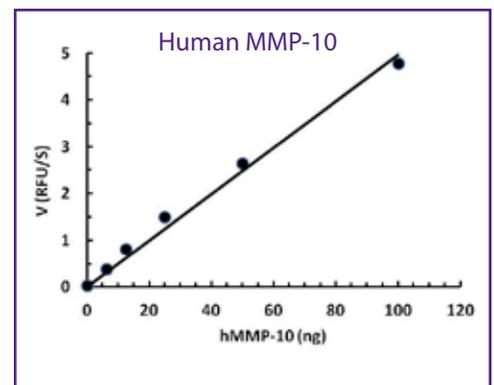
### Human MMP-10



The activity of recombinant human MMP-10 was measured with a fluorogenic MMP substrate (Mca-RPKPVE-Nval-WRK(Dnp)-NH2).

# Featured Recombinant Proteins for Cell Differentiation

Macrophages are derived from hematopoietic stem cells (HSCs) and are important because they play critical roles in processes such as cytokine production, antigen presentation and processing, phagocytosis and pathogen killing. Furthermore, macrophages can be differentiated into other cell types that share the macrophage-monocyte lineage, such as osteoclasts (bone cells), in the presence of a microenvironment rich with the right cytokines and growth factors.

To study macrophage biology, there is a big demand for obtaining a homogeneous macrophage population. In mice for example, use of immortalized macrophage-like myeloid cell lines, such as RAW264.7 cells, although popular, have several limitations including loss of genes critical for macrophage function when they are continuously sub-cultured. Hence the need developed for primary cells isolated from mice for experimental purposes.

Currently, there are three major options for obtaining primary mouse macrophages: bone marrow-derived macrophages (BMDMs), peritoneal macrophages (PMs), and alveolar macrophages (AMs). BMDMs are cultured from bone marrow cells *in vitro* in the presence of growth factors. As BMDMs are fully differentiated and high numbers can be obtained from a single mouse, they tend to be the preferred choice for obtaining primary macrophages.

BioLegend offers several recombinant growth and differentiation factors that have been successfully tested for BMDM development and differentiation.
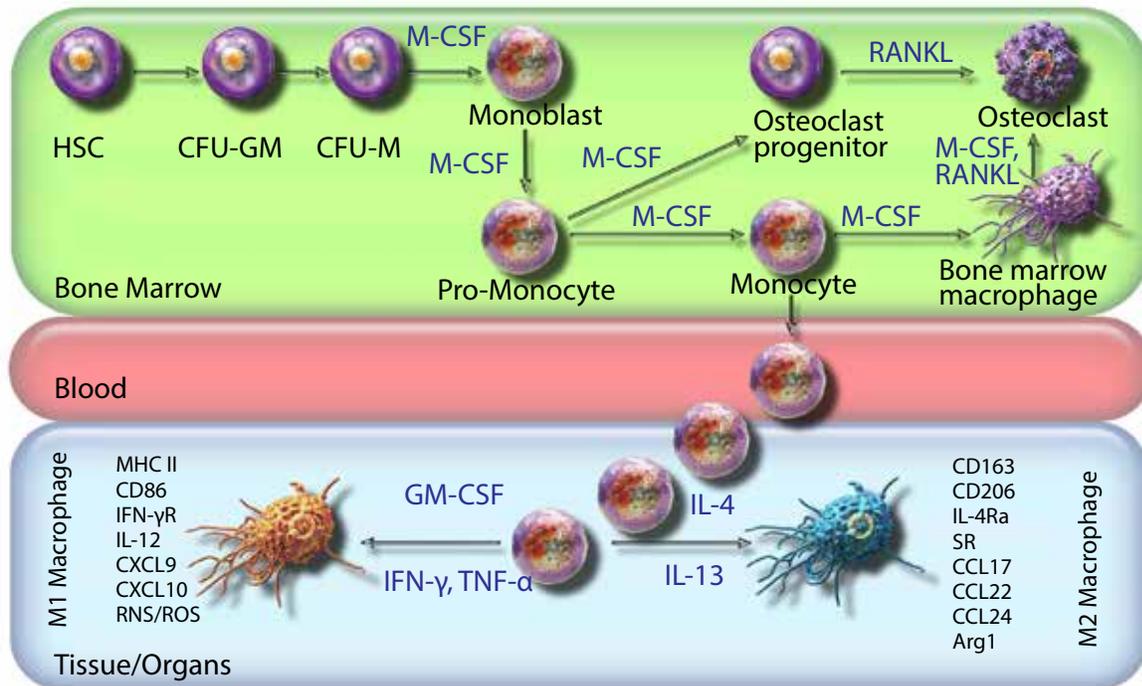


**HSC:** Hematopoietic stem cell

**CFU-GM:** Colony forming unit-granulocyte/macrophage

**IL- :** Interleukin-

**CFU-M:** Colony forming unit-macrophage

**M-CSF:** Macrophage colony-stimulating factor

**TNF-α:** Tumor necrosis factor alpha

**RANKL:** Receptor activator of NF-κB ligand

**IFN-γ:** Interferon gamma
**SR:** Scavenger Receptors
**GM-CSF:** Granulocyte macrophage-colony stimulating factor

**RNS:** Reactive nitrogen species
**ROS:** Reactive oxygen species
**CXCL and CCL:** Chemokines
**Arg1:** Arginase-1 enzyme

The following are some related experiments performed at BioLegend. After extracting and plating primary mouse bone marrow (BM) cells obtained from the mouse femur, recombinant mouse GM-CSF was used to differentiate the BM cells to BMDM. Treated cells from day 0, 7, and 14 were then surface stained with several commonly used surface markers to confirm successful macrophage development over time (figure 1). BMDMs at day 14 were confirmed to be functional by a phagocytosis assay using latex beads coated with fluorophore conjugated rabbit IgG (figure 2). And finally, BMDMs were further differentiated to osteoclasts with recombinant mouse M-CSF and mouse RANKL protein (also known as TNFSF11 or TRANCE, a member of the tumor necrosis factor (TNF) superfamily) (figures 3A and B).
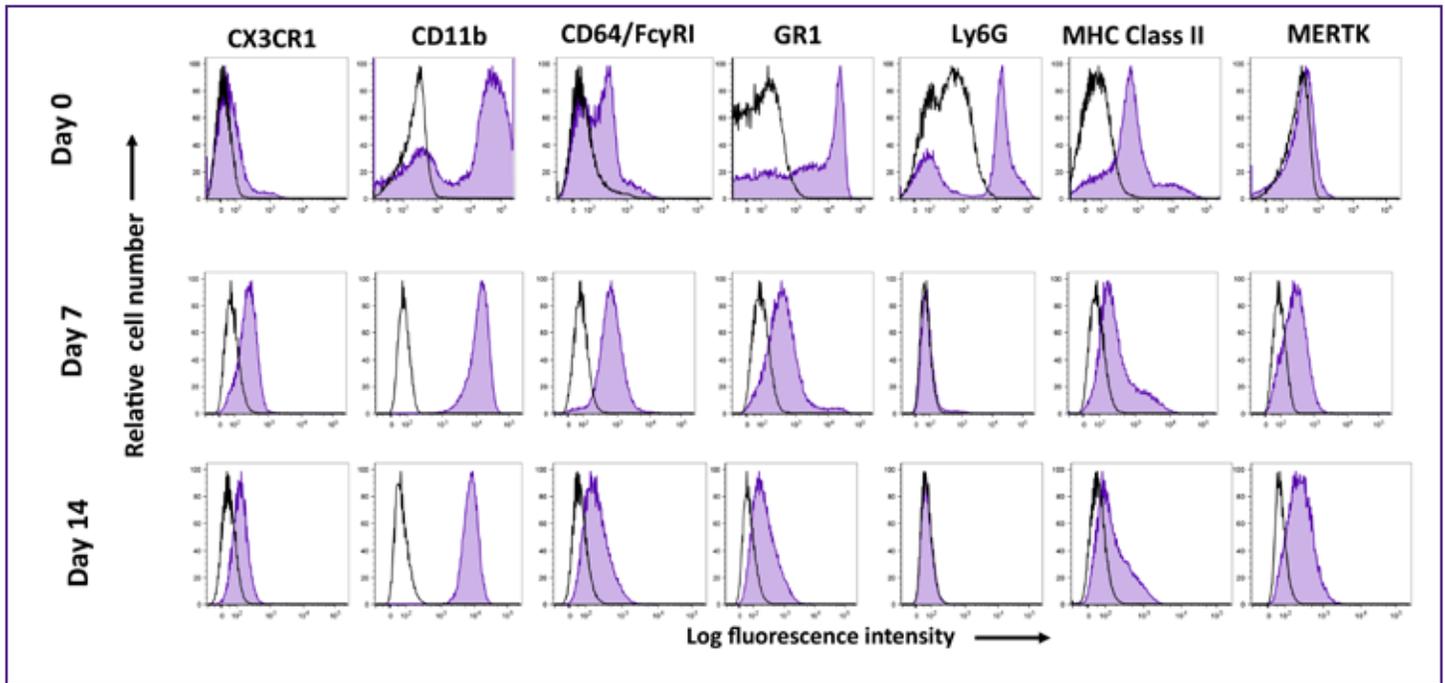


Figure 1. BMDM Staining Profile. BM cells were treated with 20 ng/mL recombinant mouse GM-CSF (Cat. No. 576302) in RPMI +10 % FBS medium for 3 days. After which, the medium was changed every 3 days with 5 ng/mL recombinant mouse GM-CSF (Cat. No. 576302) in RPMI +10 % FBS, till day 14. Cells from day 0, 7, and 14 were dissociated from the plate with cold PBS (without $Ca^{2+}$ and $Mg2^+$), and stained with antibodies: CX3CR1 (clone SA011F11), CD11b (clone M1/70), CD64/FcγRI (clone X54-5/7.1), GR1 (Ly6G/Ly6C) (clone RB6-8C5 ), Ly6G (clone HK1.4), MHC Class II (clone 39-10-8), MERTK (clone 2B10C42). All reagents used were from BioLegend.

Figure 2. Phagocytosis Assay. BMDMs at day 14 were dissociated from the plate with cold PBS (without $Ca^{2+}$ and $Mg^{2+}$) and incubated with latex beads-rabbit IgG-FITC for 3.5 hours at 37°C in RPMI + 10% FBS. After which, cells were washed in cell staining buffer, blocked with LEAF™ Purified anti-mouse CD16/32 Antibody, and stained with APC anti-mouse F4/80 Antibody.



66

**A.**

control | 120 ng/ml | 240 ng/ml | 480 ng/ml

**B.**
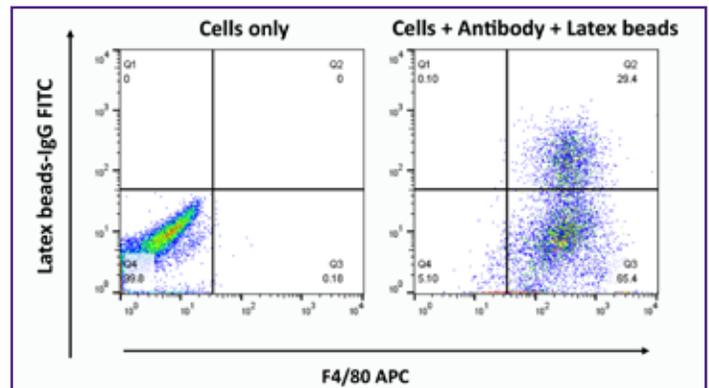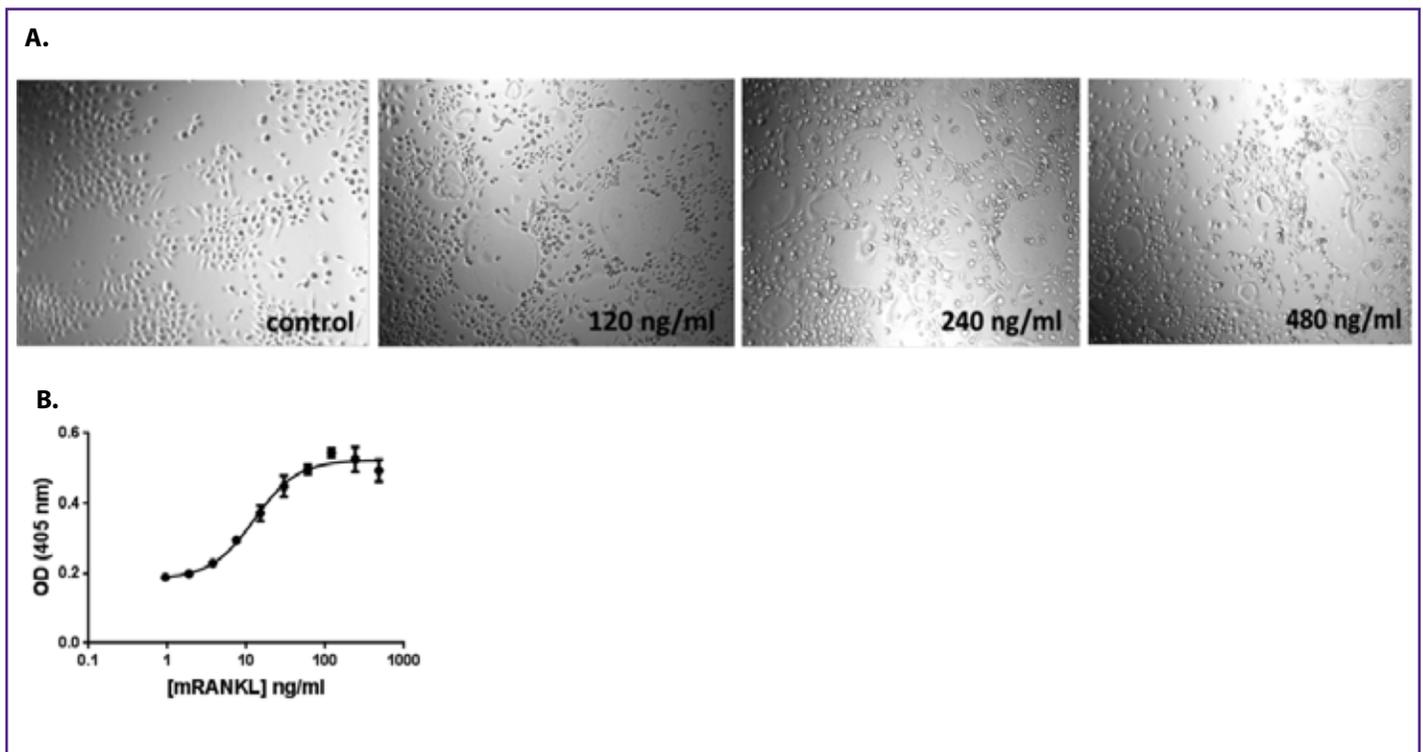
Figure 3. BMDM to Osteoclast Differentiation. BMDMs at day 14 were treated with 25 ng/mL recombinant mouse M-CSF (Cat. No. 576402) in MEM-Alpha medium + 10% FBS for 3 days. After which, the cells continued to be incubated in the same medium, but with increasing concentrations (0-480 ng/mL) of recombinant mouse RANKL (Cat. No. 769402) up to 8 days. (A) Change in morphology of the cells to resemble osteoclasts was observed at 240 ng/mL and 480 ng/mL under the microscope. (B) High levels of TRAP (Tartrate-Resistant Acid Phosphatase) activity, a signature marker of osteoclasts, was observed with increasing concentrations of recombinant mouse RANKL (Cat. No. 769402). TRAP activity was measured at OD 405 nm.

In conclusion, these experiments show that BioLegend's recombinant proteins are functional and can be used as important tools for immunology research applications.

## Selected Recent Publications Using BioLegend Recombinant Proteins:

**Mouse BAFF**

1.  Hennenberg E, *et al*. 2017. *Plos One*. 12(7):e0180834
2.  Han S, *et al*. 2016. *Arthritis Res Ther*. 17:384

**Mouse GM-CSF**

1.  Ayala M, *et al*. 2017. *Breast Cancer Res Treat*. 166(2):393-405
2.  Ayala M, *et al*. 2017. *J Cancer Res Clin Oncol*. 10.1007/s00432-017-2421-7

**Mouse IFN-γ**

1.  Davis B, *et al*. 2017. *J Virol. Aug* 10;91(17)
2.  Greer R, *et al*. 2016. *Nat Commun*. 7:13329

**Human IL-8**

1.  Popova T, *et al*. 2016. *PLoS One*. 11(9):e0163163.
2.  DuMont A, *et al*. 2015. *Infect. Immun*. 83: 3825 - 3837.

**Human TGF-β**

1.  Lin C, *et al*. 2016. *J. Exp. Med*. 213: 251 – 271
2.  Alcaide, P., *et al*. 2012. *J. Immunol*. 188:1421.

**Human CXCL10 (IP-10)**

1.  Li Y, Kaneda T. 2016. *Sci Rep*. 6:25077
2.  Sek A, *et al*. 2015. *Plos One*. 10(7):e0133266

**Learn more at: biolegend.com/recombinant_proteins**
**and**
**biolegend.com/media_assets/literature/images/02-0007-02_recombinant_brochure.pdf**

67

# Innate Immune Signaling Poster

The innate immune response is vital as the first layer of defense against potential pathogens. While the Innate Immunity Poster gives an overview of the cells involved in these pathways, this poster examines what happens when a cell's pattern recognition receptors are triggered. Toll-like receptors and C-type lectin receptors recognize highly-conserved molecules and carbohydrates on pathogens respectively.  Fc receptors are designed to pick up the Fc (fragment, crystallizable) portions of antibodies causing effects like phagocytosis and cytokine, histamine, or enzyme release. Newly synthesized viral RNA can be recognized by dsRNA helicase enzymes like Rig-I and MDA5, leading to type I interferon production to help clear viral infections. STING proteins can recognize dsDNA and cyclic dinucleotides. NOD-like receptors function similarly to TLRs in that they recognize certain pathogen-associated antigens in the cytosol. Other members of this family, like NLRP3, can form inflammasomes and lead to caspase-1 activation. Once activated, caspase-1 can induce pyroptosis, a highly inflammatory form of cell death, and activate IL-1β and IL-18 for additional proinflammatory reactions.



# Innate Immune Signaling

# NOD Signaling

# STING Signaling

# RIG-I Signaling

# NLRP/A1M2 Signaling

# Inflammasome Formation

## Nucleus

## Mitochondria

## Endoplasmic Reticulum

**Bacteria**

**Virus**

Toll - Like Receptor Ligands signaling pathway labels include:

IL-1, IL-10, IL-1α, TNF-α, GM-CSF, MIP-1α, MIP-1β, MCP-1, RANTES

TAK1, TAB2/3, TAB1, p38/MAPK, JNK, CREB, c-Jun, c-Fos, AP-1, TRAF6, MEMO, IKKα, IKKβ, p50, p65, NF-κB, RelA, Phosphorylation, Ubiquitin

Proinflammatory Cytokines/Chemokines, TNF-α, IL-1β, IL-6, iNOS

STING, Cyclic Dinucleotides, IBK1, Viral DNA, Intracellular Bacteria

IRF3, IRF7, TRAF3, IRF9, STAT1, STAT2, ISGF3, Type I IFN, IFN Stimulated Genes, IL-15, RANTES, CXCL10

pol III, CARD, MAVS Aggregation, Heterotetramer Formation, Tripolyphosphorylated ds RNA, TRIM25, RIPlet/RNF185, K63 Polyubiquitination, Dimerization, Ubiquitin, DECH Helicase, RD CID, RIG-I

ASC, Procaspase-1, CARD, PYD, HIN-200, Foreign ds DNA, Caspase-1, Pro IL-1β, Pro IL-18, IL-1β Pyroptosis, IL-18 Inflammation

NLRP3, NACHT, PYD, LRR, ASC, CARD, Procaspase-1

NOD1, NOD2, RICK/RIP2, NACHT, CARD, LRR, Muramyl Dipeptide, γ-D-glutamyl-meso diaminopimelic acid

TIRAP, Profilin, TRAF3, IKKε, TBK1, NF-κB Activation, IKKα, IKKβ, NEMO, MAPK Activation, Inflammasome Formation

JAK, TyK, Type 1 IFN Receptor, Type 1 IFNs

biolegend.com

## Contact BioLegend

US & Canada Toll-Free: 1.877.246.5343 (877-BIOLEGEND)
International: 1.858.768.5800
Fax: 1.877.455.9587
email: cs@biolegend.com, tech@biolegend.com
US Headquarters:
San Diego, CA 92121
04-0069-00

**Interactive Poster: biolegend.com/innate_signaling_poster**
We would like to thank
Dr. Glen Barber of the University of Miami Miller School of Medicine
for his contributions to this poster.

# BioLegend®

| Toll - Like Receptor Ligands | |
|---|---|
| **TLR1** - Triacetylated Lipopeptides | **TLR9** - Unmethylated CpG DNA, haemozoin |
| **TLR2** - Glycolipids, lipopeptides lipoproteins | **TLR10** - ? |
| **TLR3** - Double-stranded RNA | **TLR11*** - Flagellin, profilin |
| **TLR4** - Lipopolysaccharide, heat shock proteins, fibrinogen | **TLR12*** - Profilin |
| **TLR5** - Flagellin | |
| **TLR6** - Diacyl lipopeptides | |
| **TLR7** - Single-stranded RNA | |
| **TLR8** - Single-stranded RNA (Human) Imidazoquinoline, poly T oligonucleotides (Mouse) | |

*Mouse only

# Cell Separation

# Cell Separation

The isolation of a specific cell population from a complex sample has become a common lab procedure. Cell separation is indeed a very powerful tool that has greatly contributed to the expansion of biomedical research and clinical applications, and continues to help push the boundaries of scientific discoveries.

Thus far, several techniques have been developed to separate cells. They are based either on the physicochemical properties of the cells, or on their phenotype as defined by specific molecules that they express. These techniques can be classified thus as adherence, density, or antibody binding based. This chapter reviews these three methodologies, emphasizing BioLegend's system, MojoSort™. The information is also focused on research reagents.

## Separation Based on Adherence

This is probably the simplest way to separate cells. It is based on the capacity of the cells of interest to adhere to a specific material, and utilize that to decant unwanted cells (or strip the solution of the cells that adhere). Two classical examples of this method are the isolation of dental pulp stromal cells from digested dental pulp (1), and isolation of human monocytes from whole blood (2).

The main benefit of this approach is its simplicity, and it is also probably the most economical method to separate cells. The main disadvantage is a complete lack of specificity, as it relies on the capacity of the cells of interest to adhere, and, to some extent, their capacity to proliferate relatively quickly.
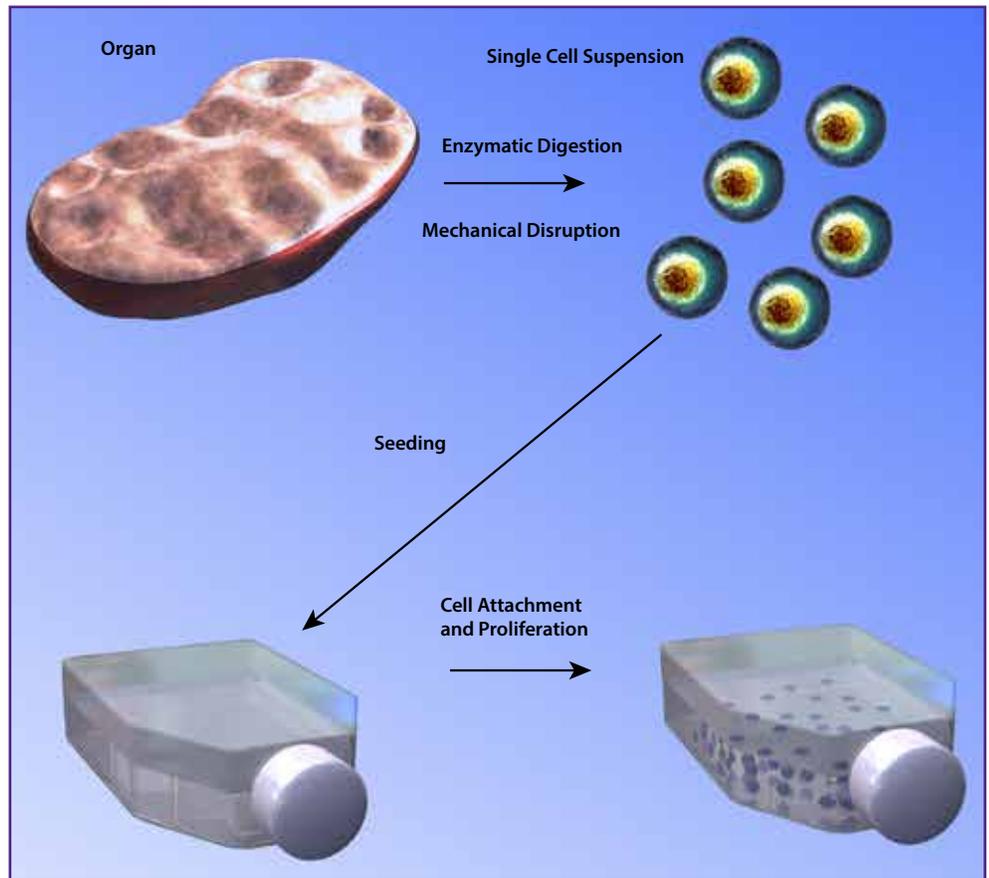


Figure 1. Diagram showing cell separation by adherence to plastic. The tissue is disrupted to yield a single cell suspension followed by seeding onto the adherent surface. The cells are cultured and adhere to the surface.

71

# Separation Based on Density

This method is based on the use of different separation media with specific density, followed by centrifugation. By modifying the density of media and the angular speed, it is possible to sediment some cells while others remain in the interface between the separation media and the cell suspension. This method is particularly useful to separate blood cells for example, although it can also be applied to cells obtained from solid organs (3).

This is also a relatively simple method, and doesn't require technically challenging equipment. However, specificity is also the main issue as the cells are separated based on physical properties, and different cell types may have similar density.

Figure 2. Diagram showing whole blood cell separation using a density gradient centrifugation protocol. Blood is diluted with saline buffer and carefully layered over the medium in a conical tube, avoiding disturbing the interphase. Following centrifugation, at the appropriate speed and no braking, well defined phases can be observed; 1 – plasma, 2 – interphase with mononuclear cells, 3 – centrifugation medium and 4 – erythrocytes and granulocytes. The cells of interest can be aspirated from the interphase.
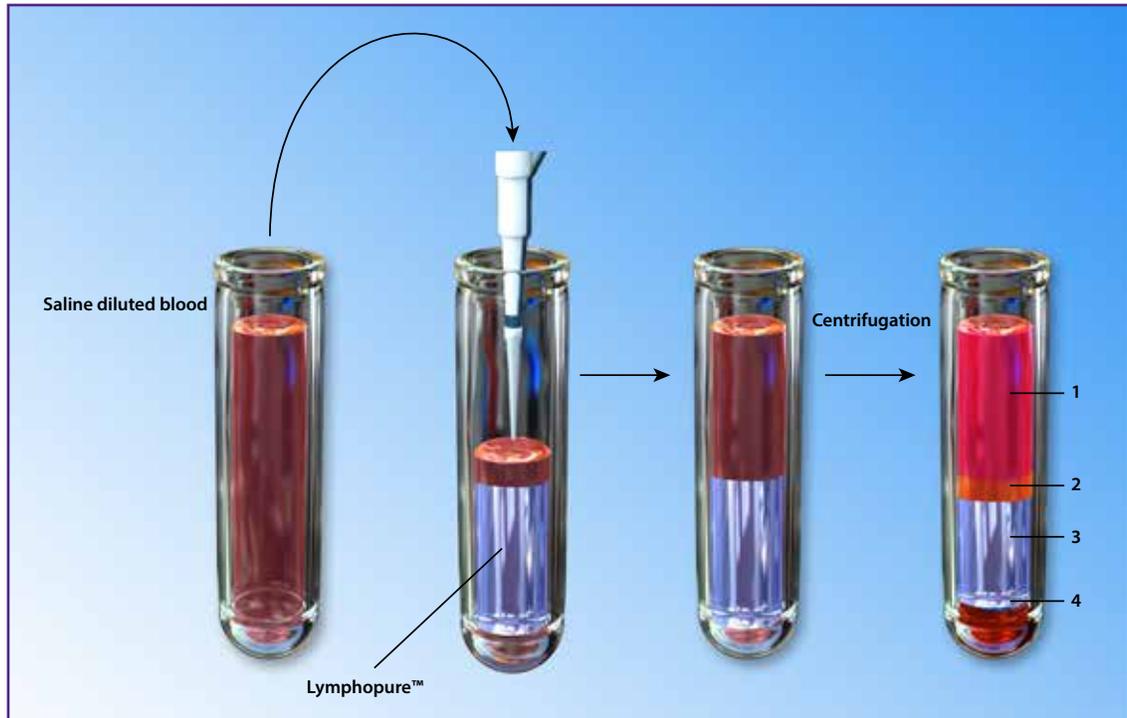
# Antibody Binding Strategies

Methods that take advantage of the specificity of the antibodies are based on their capacity to selectively bind molecules expressed by the cells of interest. The two most commonly used antibody-based techniques are Fluorescence-Activated Cell Sorting (FACS) and magnetic cell sorting.

## Fluorescence-Activated Cell Sorting

This technique is a specialized form of flow cytometry that uses fluorophore-conjugated antibodies to separate cells. A cell suspension runs through an instrument that detects the signal emitted by the fluorophores attached to the cells. After the cells are properly identified, they are separated using several mechanisms. For example, the flow stream can be broken into droplets small enough that they contain only one cell. The droplets are then electrically charged, and they can be deflected to collection tubes based on their electrical charge. Alternatively, the cells may be separated by the use of micro channels and valves (4).

## Magnetic Cell Separation

Separation of cells based on magnetic fields utilizes the same antibody-based principle. However, the antibody (or binding) molecules in this case are conjugated to magnetic particles, not fluorophores. After the cells are labeled with the antibody-magnetic particle conjugate, the sample is applied to the magnetic field which will attract the labeled cells. Depending on whether the labeled or unlabeled fraction is kept for downstream analysis, these two strategies are commonly known as positive or negative selection (5).
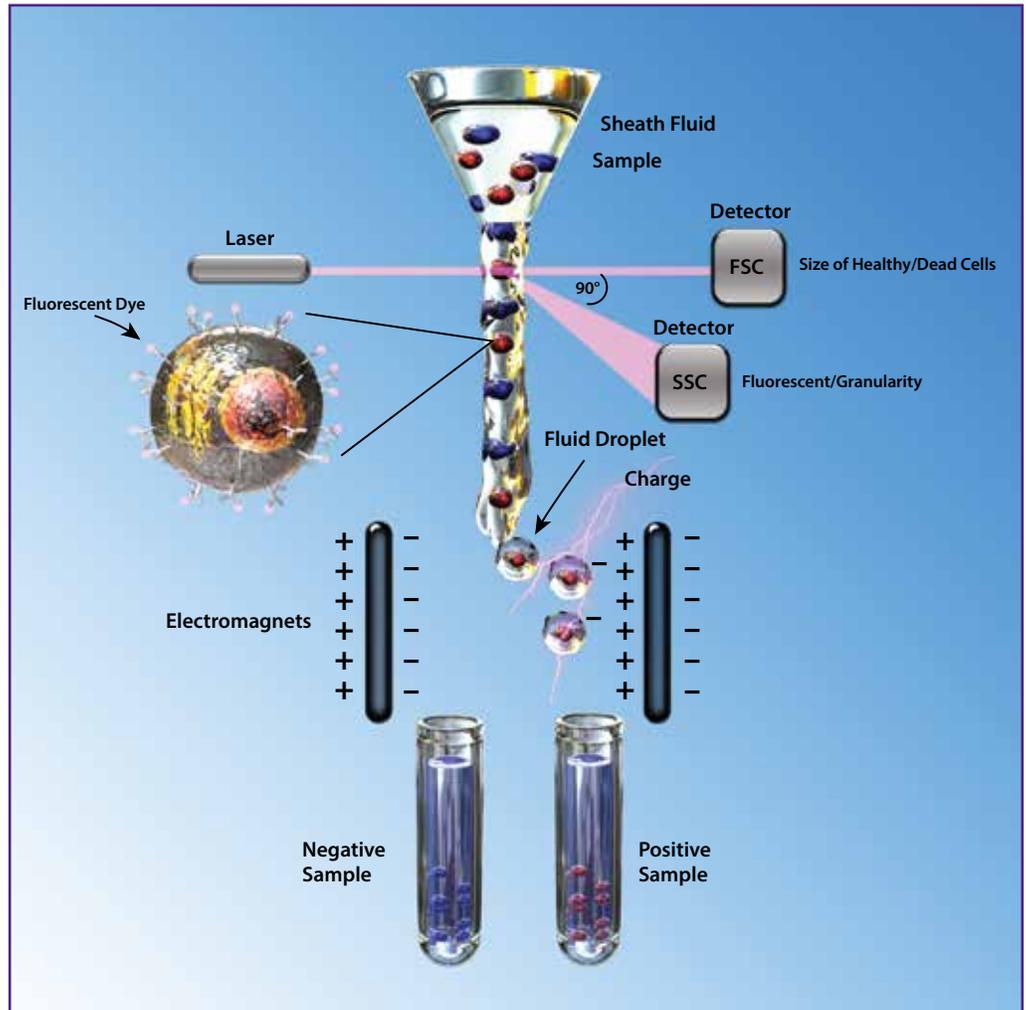


Figure 3. Schematics representing a classical droplet FACS system.
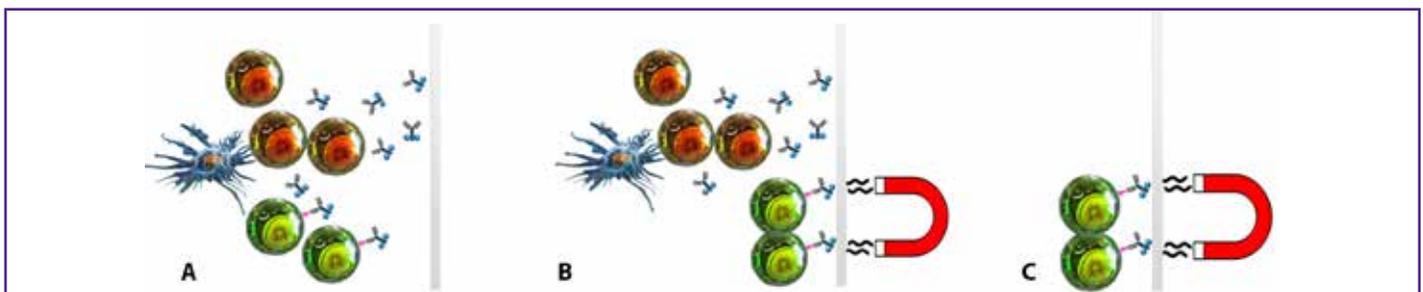


Figure 4. Illustration representing a classical magnetic separation principle. A) The cells are incubated with antibodies conjugated to magnetic particles. B) A magnetic field attracts the cells that have the conjugates bound. C) After washing or decanting the liquid, the cells of interest remain in the container. They can be retrieved by removing them from the magnetic field.

73

Both techniques have advantages and disadvantages if compared side by side. Below is a summary of key features.

|  | FACS | Magnetic separation |
|---|---|---|
| Complexity | Higher | Lower |
| Cost | Higher | Lower |
| Specificity | High | High |
| Need of specialized staff | Yes | No |
| Multi marker sorting | Yes | Limited |
| Simultaneous sorting | Yes | Limited |

Table 1. Side by side comparison of key futures of FACS and magnetic cell separation

Both methods offer high specificity, ensured by the antibodies. However, FACS is an expensive approach as it needs a complex instrument, and often dedicated personnel. Although there are some automatic systems developed for magnetic separation, these machines are less costly than flow cytometry sorters, and in general do not required specialized staff to operate them.

## MojoSort™, BioLegend's Unique Magnetic Separation Reagents

BioLegend has recently developed a unique separation platform to separate cells based on magnetic particles. Our reagents have the unique property of being able to separate cells using both stand-alone magnets and high gradient magnetic columns. Our magnetic particles can be used either with our magnets (figure 5) or with separation columns that are commercially available.



Figure 5. MojoSort™ magnets. Left panel shows a magnet that can accommodate a 12 x 75 mm tube. Right panel shows a magnet that can accommodate a 17 x 100 mm tube. MojoSort™ Nanobeads and Kits can also be used with separation columns.

## Applications

Both positive and negative selected cells using MojoSort™ can be used for *in vitro* and *in vivo* applications after isolation. Using our MojoSort™ Mouse CX3CR1 Selection Kit, we enriched CX3CR1$^+$ cells from mouse bone marrow, and differentiated them *in vitro* into Dendritic Cell and Macrophage-like cells. Following differentiation, the cells were stimulated with TLR agonists, and their surface phenotype was analyzed, as well as the cytokine profile. We also injected them in recipient C57BL/6 mice, and tracked the CFSE labeled CX3CR1$^+$ cells, detecting them back in the bone marrow 72 hours after injection.

### *In Vitro* Data

A single cell suspension from C57BL/6 bone marrow was depleted of Ly6G$^+$ cells using anti-Ly6G magnetic nanobeads, followed by positive selection of CX3CR1 cells using anti-mouse CX3CR1 biotin antibody and Streptavidin conjugated MojoSort™ Nanobeads.
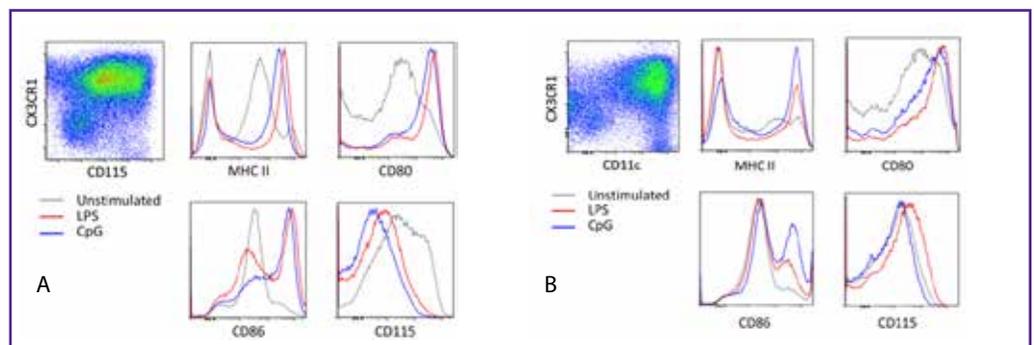


Figure 6. Surface phenotype of CX3CR1$^+$ isolated cells cultured with GM-CSF (A) or GM-CSF and IL-4 (B). The cells develop a macrophage- or dendritic cell-like phenotype and respond after LPS and CpG stimulation.

74

Figure 7. Chemokine expression of CX3CR1^{+} isolated cells cultured with GM-CSF alone (left panel) or GM-CSF and IL-4 (right panel) after stimulation with either LPS (black bars) or CpG (orange bars). The cells show a well-defined chemokine expression pattern, distinct to each cell type. The bars indicate fold increase over unstimulated control cells. Cytokine and chemokine levels in cell culture supernatants were measured using BioLegend's multiplex assay LEGENDplex™.
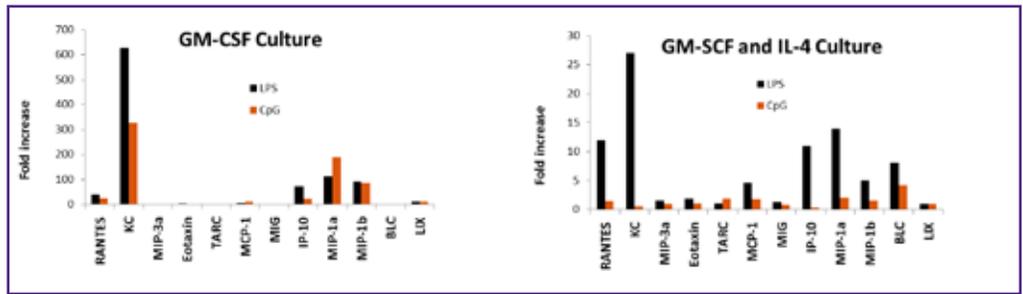
Figure 8. Cytokine expression of CX3CR1^{+} isolated cells cultured with GM-CSF alone (left panel) or GM-CSF and IL-4 (right panel) after stimulation with either LPS (black bars) or CpG (orange bars). The cells show a well-defined cytokine expression pattern, distinct to each cell type. The bars indicate fold increase over unstimulated control cells. Cytokine and chemokine levels in cell culture supernatants were measured using BioLegend's multiplex assay LEGENDplex™.
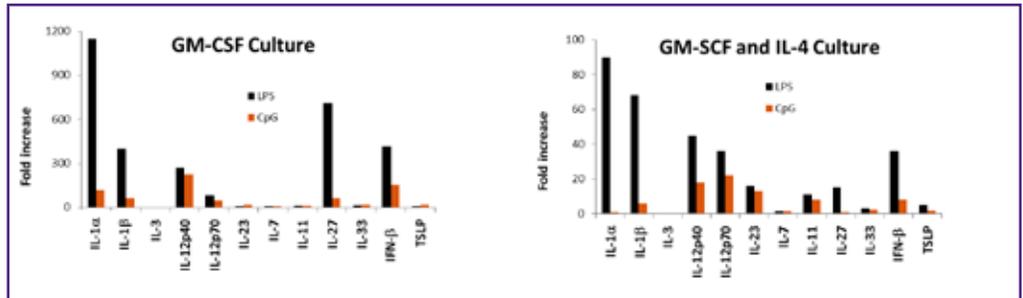
### *In Vivo* Data

A single cell suspension from C57BL/6 bone marrow was depleted of Ly6G^{+} cells using anti-Ly6G magnetic nanobeads, followed by positive selection of CX3CR1 cells using anti-mouse CX3CR1 biotin antibody and Streptavidin conjugated MojoSort™ Nanobeads. CX3CR1^{+} cells were then labeled with CFSE and injected back into a C57BL/6 recipient mouse. CFSE positive cells were tracked in the bone marrow of recipient animals.
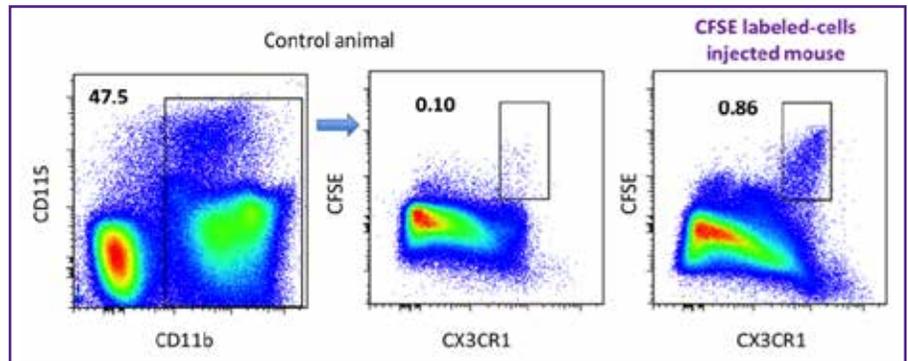
Figure 9. CFSE^{+} cells are detected in the bone marrow of recipient animals 72 hours after cells transfer. CFSE^{+}CX3CR^{+} cells were gated of CD11b^{+} cells.

In conclusion, cells isolated using MojoSort™, including positive selection, can be used for multiple applications. The isolated cells have preserved functionality and intact physiological activities.

**References**
1.  Raoof M, *et al*. 2014. *Dent Res J*. 11(2): 244
2.  Wahl LM, *et al*. 2006. *Curr Protoc Immunol*. Chapter 7:Unit 7.6A
3.  Liu W *et al*. 2011. *Proteomics*. 11(17):3556
4.  Shapiro HM. 2005. *Practical Flow Cytometry*
5.  Battye FL and Shortman K. 1991. *Curr Opin Immunol*. 3(2):238

**Learn more at: biolegend.com/mojosort**

## Chemokine Receptor Biology Poster

Chemokines are specialized cytokines that induce cell movement, better known as chemotaxis. Chemokines can serve a variety of functions, drawing cells in to investigate an injury site, and maintaining homeostasis by recruiting cells to areas of development (*e.g.,* T cell progenitors moving to the thymus for growth and development). Certain viruses, like HIV, are capable of using chemokine receptors as points of entry in T cells or dendritic cells. Tumor cells can also take advantage of chemokines by recruiting regulatory T cells to dampen inflammatory responses. They may also produce oxysterols to prevent the recruitment of immune cells and create chemokines like CXCL12 to promote angiogenesis and metastasis.

# Chemokine Receptor Bio

04-0021-00

## INFLAMMATION

Rolling Neutrophil

CXCR1, 2
CXCL8

LFA-1
ICAM-1

Blood Vessel

E-Selectin

Endothelium

Pro-inflammatory Cytokines
IFN-γ
CXCL8
TNF-α
IL-1

Tissue

Chemokine Gradient

CXCL8

Injury, Infection

Epithelium

## VIROLOGY

### HIV Entry

HIV
CXCR4
CD4
T Cell

CCR5
HIV
CD4
Macrophage

CCR5
HIV
CD4
Dendritic Cell

Other Reported HIV Co-Receptors

| | | |
|---|---|---|
| CCR2 | CCR8 | CXCR7 |
| CCR3 | CXCR6 | CX3CR1 |

## Chemokine Sink

Macrophage

CCR2

Reduced Recruitment

Chemokine Sequestration
CCL2
US28
CCL5

Cytomegalovirus

Endothelial Cell

Immunosuppression

## Immune Tolerance

B cell

Viral CCL1, 2, 3

CCR4
CCR8

Treg, Th2 Recruitment

Kaposi Sarcoma Virus

Immunosuppression

## Periphery

T Progenitor

PSGL-1
P-Selectin

Endothe

### Thymus

Cortex

CXCR4+, CCR7+, CCR9+
CD4−CD8−
Thymocyte

Positive Selection

CCR7+, CCR9+
CD4+CD8+ Thymocyte

CCL20

CXCL6, 8 — CXCR1

CXCL1, 2, 3, 5, 6, 7, 8 — CXCR2

CXCL9, 10, 11 — CXCR3

CCL19, 21 — CCR7

CCL3, 4, 5, 8, 14 — CCR5

CCR4

CCL2, 3, 5 17, 22

CCR3

CCL5, 7, 11, 13, 14, 15, 24, 26

CCL2, 7, 12, 13

We would lik

US

### Chemokine Nomenclature

CXCL1: GRO1, GROα, MGSA, NAP3, SCYB1, MGSA-α
CXCL2: CINC-2α, GRO2, GROβ, GROB, MGSA-β, MIP2
CXCL3: GRO3, GRO-γ, MGSA-γ, MIP-2β, SCYB3
CXCL4: PF-4
CXCL5: ENA-78, LIX
CXCL6: SCYB6, GCP 2, CKA-3
CXCL7: NAP-2
CXCL8: IL-8
CXCL9: CMK, MIG, SCYB9
CXCL10: IP-10
CXCL11: I-TAC
CXCL12: SDF-1
CXCL13: BCA-1, BLC
CXCL14: BRAK, Bokeline
CXCL15: LUNGKINE, SCYB15
CXCL16: SCYB16, SR-PSOX
CXCL17: DMC, VEGF Co-regulated Chemokine 1
CX3CL1: Fractalkine, Neurotactin
XCL1: Lymphotactin, ATAC, SCM-1α
XCL2: SCM-1β
CCL1: I-309, TCA-3, SCYA1
CCL2: MCP-1, MCAF, JE
CCL3: MIP-1α
CCL4: MIP-1β, ACT-2, H400
CCL5: RANTES

BioLegend®

## HOMEOSTASIS

### Bone Marrow
Specialized Stromal Cells
CXCR4
T Progenitor
CCR7
CCR9
Bone Marrow Niche
Endothelium
Blood
CXCL12
CD34+ HSC
CXCR4
G-CSF

### Secondary Lymphoid Tissues
Follicle
T cell Zone
CXCL13
CXCR5
T cell
CXCL13
CCL21
CXCR5
B cell
CCR7
CCL19
HEV
T cell

CXCL1, 8
CCL2, 5
|
DUFFY

Atypical

D6
|
CCL2, 3, 5, 7

Brain: CXCL12, CXCL14
Lungs: CXCL12, CXCL17
Bone Marrow: CXCL12
Lymph Nodes: CCL19, CCL21, CXCL13
Spleen: CCL19, CCL21, CXCL13
Liver: CXCL13
Small Intestine: CCL25, CXCL17
Skin: CCL27, CXCL14

CXCL17
CCL25
?
CCR9

### Small Intestine
α4β7+ T cell

CXCR4
CCR7
CCR9
CCL19
CCL21
CCL25
Sphingosine-1-Phosphate Gradient

Medulla
Egress
Sphingosine-1-Phosphate Receptor

Cortico-medullary Junction
CD4+ or CD8+ Single Positive Thymocyte
Mature CD4+ or CD8+ Naïve T Cell

**Negative Selection**

### Skin
Dermal Cells
CCL27
CCR10
CLA+ T cell

CCL1, 4
CCL25
CCL27, 28
CCL18
19, 21
CCR8
CCR9
CCR10
?
CCR7
CXCL17
R6
?
CXCL14
CXCL11, 12

## Chemokine Receptor Signaling
Chemokine
Chemokine Receptor
Extracellular
Membrane
Intracellular
G Proteins
PLCβ2, PLD, PKC
Arrestin, PLCβ2
spiratory Burst
anule Release
STK's, Ras
Cytoskeletal Remodeling
Shape Change
Firm Adhesion
CCR2
CCR1
CCL3, 4, 5, 7 14, 15, 16
KSHV
E1
CXCL1, 8, 10, 12
CCL11
UL12
Receptor Internalization/ Desensitization
ECRF3
US28
CXCL1, 5, 7
CCL2, 3, 4, 5, 7
CCL2, 3, 4, 5

CXCR7
CXCR6
CXCL16
CXCR5
CXCL13
CXCR4
CXCL12
XCR1
XCL 1,2
CX3CR1
CCL26, CX3CL1

## CANCER

### Tumor Defense
Tumor Cells
CXCL1, CXCL8
Tumor Cell Proliferation
Oxysterols
↓CCL27, CXCL14
CCL28
CCR7, 10
Treg Recruitment
↓CCR7
Dendritic Cell
Block Recruitment of Dendritic Cells, Lymphocytes
Immunosuppression

### Metastasis/Angiogenesis
Hypothetical Primary Tumor Site
VEGF
CXCL12
CXCR4
CXCR4
Tumor Cells
Angiogenesis
Blood Vessel
Upregulation of Chemokine Receptors
Recruitment
Hypothetical Secondary Tumor Site

**Tumor Metastasis**
CCR7: Breast, Melanoma, Head and Neck, Colorectal, Chronic Lymphocytic Leukemia, Stomach, Non-Hodgkin's Lymphoma, T Cell Leukemia
CCR9: Melanoma
CCR10: Melanoma
CXCR3: Acute Lymphoblastic Leukemia
CXCR4: Breast, Ovarian, Prostate, Pancreatic, Melanoma, Oesophageal, Non-small Cell Lung, Head and Neck, Bladder, Colorectal, Osteosarcoma, Neuroblastoma, Acute Lymphoblastic Leukemia, Chronic Lymphocytic Leukemia, Stomach
CXCR5: Head and Neck Cancer

HOMEOSTASIS
VIROLOGY

## biolegend.com

CCL6: C10, MRP1
CCL7: MCP-3, MARC
CCL8: MCP-2, HC14
CCL9: CCL10, MRP-2, MIP-1γ, CCF18
CCL11: Eotaxin, Eotaxin 1

CCL12: MCP-5
CCL13: MCP-4
CCL14: HCC-1, HCC-3, MCIF, NCC2
CCL15: MIP-1δ, MIP 5, MRP-2B, NCC3, HCC-2
CCL16: HCC-4, LEC, LCC-1

CCL17: TARC, ABCD 2
CCL18: DC-CKA1, PARC, MIP-4, AMAC-1
CCL19: ELC, MIP-3β, Exodus-3
CCL20: LARC, MIP-3α, Exodus-1
CCL21: SLC, 6Ckine, Exodus-2

CCL22: MDC, ABCD 1
CCL23: MIP-3, CKb-8, MPIF-1
CCL24: MPIF-2, Eotaxin-2, CKβ-6
CCL25: TECK, CKβ-15
CCL26: Eotaxin-3

CCL27: CTACK, ESKine, ILC, Skinine
CCL28: MEC, CCK1
**T Helper Cell Expression**
Th1: CCR5, CXCR3
Th2: CCR3, 4, 8; CXCR5

Th17: CCR4, 6
Th22: CCR4, 6, 10
Tfh: CXCR5
Treg: CCR2, 4, 5, 6, 8-10; CXCR4-6

**Cell Expression**
Hematopoietic Stem Cells: CXCR4
Naïve T Cells: CCR7, CXCR4
B Cells: CCR7, CXCR4, 5; CX3CR1
Macrophages: CCR1, 2, 5; CXCR1, 4

Dendritic Cells: CCR1, 2, 5, 6, 7; CXCR1, 2, 4
Natural Killer Cells: CCR2, 4, 5, 8; CXCR1, 3, 4; CX3CR1
Neutrophils: CCR1; CXCR1, 2, 4
Basophils: CCR1-4, CXCR4

Eosinophils: CCR3, CXCR4
Mast Cells: CCR1, 3, 4, 5; CXCR1-4
Endothelial Cells: CCR1-4; CXCR1-4
Epithelial Cells: CCR1-8; CXCR1, 4

# MHC Multimers

# MHC Multimers

The immune system is tasked with an extremely important function, the protection of the entire organism and elimination of unwanted entities, whether that is a pathogen, a cell that has become cancerous, or another insult. In most cases, an effective immune reaction involves the development of a strong T-cell mediated response. This is defined by the interaction between antigen presenting cells (APCs) and T cells, through the Major Histocompatibility Complex (MHC) and the T cell receptor (TCR) (1).

## What is an MHC Multimer, and How Do You Use It?

MHC molecules in APCs present a peptide to a T cell that recognizes this peptide. The interaction between the MHC molecules and the TCR is weak. However, multimerization of soluble MHC significantly extends the interaction, by effectively increasing the avidity of the MHC for the TCR. By simultaneously incorporating a fluorescently labeled backbone, the MHCs will maintain a more stable binding by interacting with several TCRs, thus making the multimer useful for flow cytometry detection of antigen-specific T cells. The first paper describing multimerization of peptide/ MHC molecules to identify antigen specific T cells was published in 1996, by Altman *et al* (2). The publication opened an entire new field in immunology research, and used streptavidin as the primary backbone to construct an MHC tetramer conjugated to a fluorophore.

In general, antigen-specific T cell responses can be characterized by functional assays. This includes proliferation assays, chromium-based cytotoxicity assays, $Ca^{2+}$ flux assays, and more commonly, cytokine detection assays such as ELISPOT and intracellular cytokine flow cytometry staining. However, most of these functional assays do not combine the specificity and quantification capabilities of flow cytometry MHC tetramer staining; nor can they be used to characterize the response at a single cell level (3).

## MHC Restriction, Allele Frequency and Distribution

There are several classes of MHC molecules. They are paired with the type of T cell that will recognize them. Class I molecules will stimulate CD8+ T cells and class II molecules will stimulate CD4+ T cells. Thus, to be able to effectively stimulate T cells, APCs must present an appropriate peptide in its matching MHC molecule to the corresponding T cell. Figure 1 summarizes this matching system.
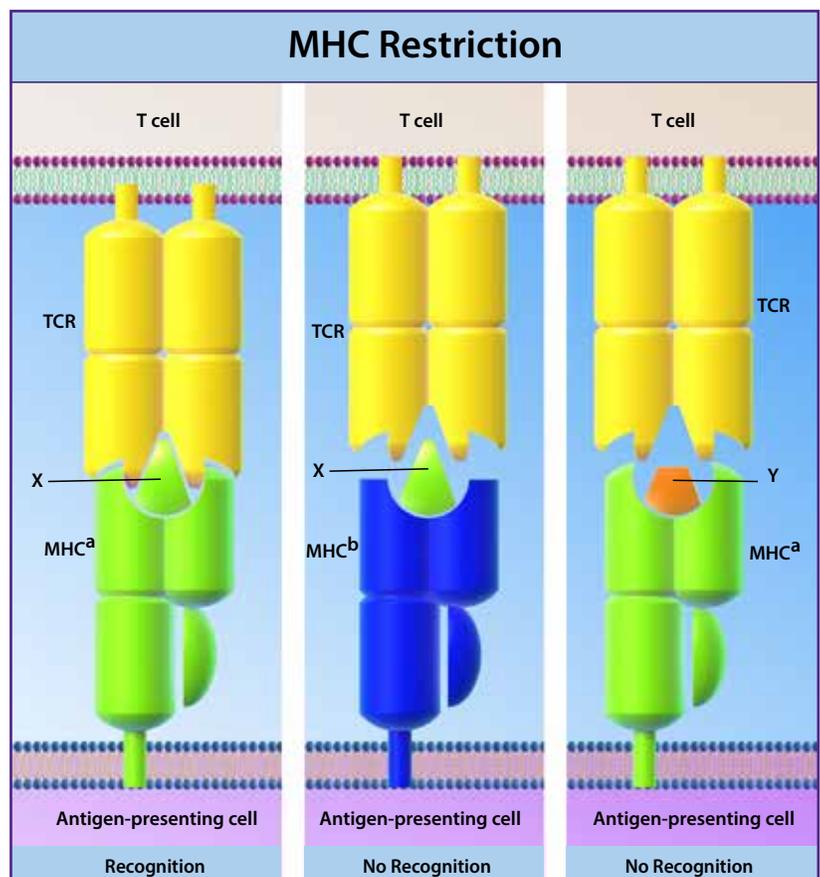


Figure 1. MHC restriction is driven by the fact that TCRs will only engage in a productive interaction if both the peptide and the MHC molecule are suited to match. The illustration shows class I molecules as an example. X and Y represent peptides.

79

The MHC molecules are extremely polymorphic, even in inbred laboratory animals. When staining samples to identify antigen-specific T cells with MHC multimers, it is then important to know which alleles are recognized by the T cells. Table 1 shows the worldwide frequency of the 25 most frequent Class I HLA- A, B, and C alleles.

| Allele | North America | Australia | Europe | North-East Asia | South-East Asia | South America | Oceania | Sub-Saharan Africa | North Africa |
|--------|--------------|-----------|--------|-----------------|-----------------|---------------|---------|--------------------|--------------|
| A*24:02 | 31.17 | 25.93 | 6.65 | 8.79 | 22.01 | 1.46 | 36.72 | 1.17 | |
| C*03:04 | 19.75 | 0.92 | 5.77 | 4.55 | 3.89 | 17.90 | 0.00 | 2.91 | |
| A*02:01 | 17.34 | 12.72 | 27.22 | 11.34 | 6.38 | 22.08 | 6.14 | 9.84 | |
| B*40:02 | 15.94 | 16.87 | 0.80 | 7.24 | 3.55 | 7.59 | 6.43 | 0.17 | 1.10 |
| C*04:01 | 14.95 | 26.05 | 10.10 | 8.84 | 5.87 | 15.94 | 10.25 | 12.93 | |
| B*35:01 | 13.47 | 0.12 | 5.93 | 5.92 | 2.11 | 1.30 | 0.63 | 4.46 | 3.31 |
| C*02:02 | 11.64 | 0.13 | 2.18 | 0.00 | 0.08 | | | 7.77 | |
| C*07:02 | 10.16 | 5.63 | 15.89 | 7.76 | 18.92 | 32.75 | 21.11 | 4.40 | |
| A*02:06 | 10.09 | 0.00 | 0.08 | 7.18 | 5.22 | 0.00 | 2.82 | 0.09 | |
| B*48:01:01 | 9.39 | 0.12 | 0.05 | 3.55 | 4.43 | 0.22 | 3.92 | | |
| B*27:05 | 9.08 | 0.12 | 2.80 | 3.16 | 0.22 | | | 0.31 | |
| A*31:01:02:01 | 6.80 | 1.60 | 2.65 | 6.99 | 2.15 | 35.83 | 1.81 | 0.77 | 2.16 |
| A*24:02:01:01 | 6.50 | 0.00 | 1.56 | 7.56 | 6.28 | 11.04 | 1.61 | 0.37 | 2.16 |
| C*01:02 | 5.94 | 24.61 | 2.77 | 13.52 | 13.31 | 3.06 | 7.79 | 0.40 | |
| B*15:01 | 5.47 | 0.37 | 3.59 | 0.00 | 1.35 | 0.22 | 0.16 | 0.45 | |
| C*15:02 | 5.25 | 16.49 | 0.87 | 4.82 | 2.32 | 3.71 | 5.94 | 0.30 | |
| A*68:01:02:01 | 4.93 | 0.00 | 1.37 | 0.66 | 0.03 | 8.13 | 0.20 | 0.14 | 1.44 |
| C*07:01 | 4.68 | 1.18 | 5.30 | 2.28 | 1.49 | 0.00 | 1.43 | 13.07 | 0.00 |
| B*51:01:01:01 | 4.46 | 0.00 | 1.54 | 9.47 | 1.15 | 0.22 | 0.94 | 0.43 | 2.94 |
| B*51:01 | 4.39 | 0.12 | 3.59 | | 2.30 | 1.74 | 0.16 | 1.73 | 1.47 |
| B*27:03 | 4.23 | | | | | | | 0.77 | 0.37 |
| A*68:01 | 3.81 | 0.12 | 1.09 | | 0.09 | | | 0.85 | |
| C*06:02:01:01 | 3.77 | 0.92 | 9.13 | 8.57 | 1.39 | 0.22 | 1.43 | 14.56 | |
| C*08:01 | 3.77 | 0.13 | 0.06 | 7.63 | 12.82 | 0.00 | 17.83 | 0.76 | |
| C*03:03 | 3.54 | 7.20 | 5.64 | 9.77 | 6.26 | 10.26 | 2.05 | 1.06 | |
| A*01:01 | 3.51 | 2.22 | 16.41 | 2.84 | 0.70 | 0.21 | 1.01 | 5.26 | 12.59 |

Table 1. Frequency of the most common class I HLA alleles worldwide. Alleles are organized according to their frequency in North America. Empty cells represent unavailable data. Data collected from the NCBI dbMHC database (https://www.ncbi.nlm.nih.gov/projects/gv/mhc/ihwg. cgi?cmd=PRJOV&ID=9).

These maps provide an approximate graphical representation of the distribution of the most common A, B, and C alleles (A*24:02, B*40:02, C*03:04).

After the seminal work done by Altman *et al.*, a number of commercially available MHC tetramers have helped advance research in this field. The NIH also funded and maintains a core facility to make sure the reagents are available to the scientific community (http://tetramer.yerkes.emory.edu/). However, most of these multimers are available with a fixed "specificity." In other words, MHC monomers or tetramers are offered pre-loaded with a specific peptide that has most likely been previously characterized by a research group.

To overcome this issue, and facilitate the development of high-throughput assay systems in which T cell responses against a multitude of epitopes can be analyzed, a collaborative effort between Sanquin (http://www.sanquin.nl/) and The Netherlands Cancer Institute (https://www.nki.nl/) resulted in the development of a technology that allows for "specificity" exchange within the same allele. In other words, a reagent was developed in which an irrelevant peptide loaded onto the MHC molecule can be swapped for another peptide of interest after being exposed to ultraviolet (UV) light. This new peptide is the new "specificity," if we interpret it as the part of the reagent that determines the specificity of the T cell receptor to which the tetramer will bind. To compare this with antibodies as an example, it would be equivalent to having a monoclonal antibody that could switch the binding region to recognize a different target by simply exposing it to UV light. This revolutionary technology has added incredible flexibility to the research of antigen-specific T cell response, and BioLegend is offering it under the brand name Flex-T™.
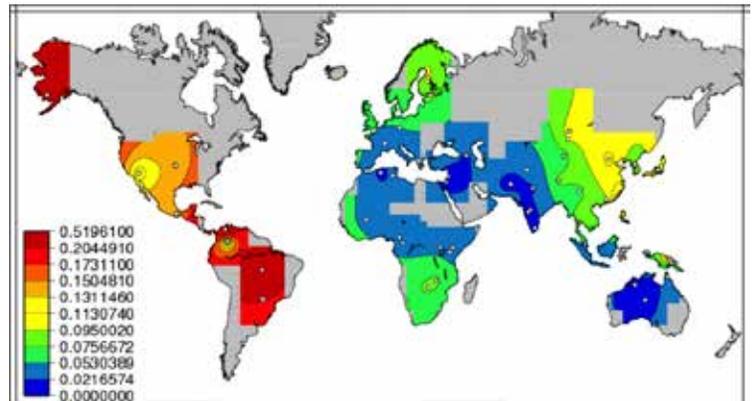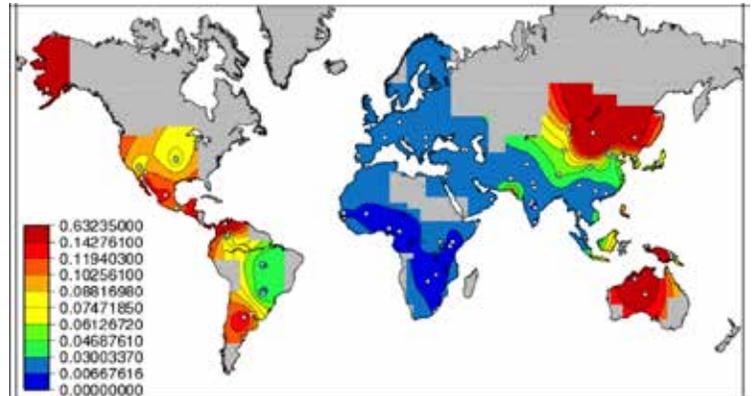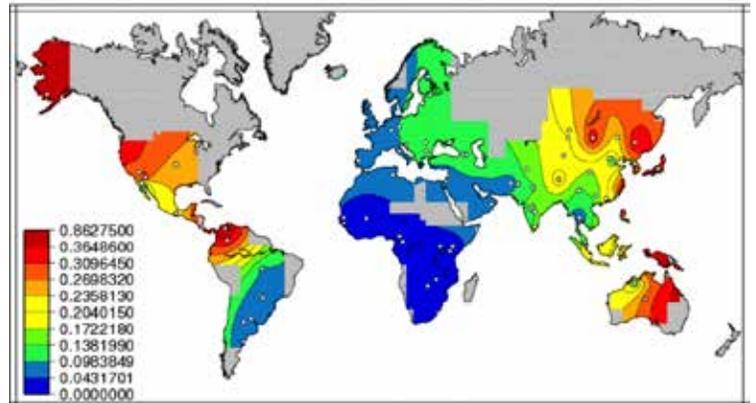


Figure 2. Geographical distribution of A*24:02, B*40:02, and C*03:04 alleles. The data is based on Lancaster A.K. *et al.* (4), and accessible here: http://www.pypop.org/popdata/2008/byfreq-A.php.

81

## How Does It Work?

Flex-T™ utilizes a UV-exchangeable system. MHC monomers are loaded with a peptide that can be degraded by the use of a UV light source. This allows for a peptide exchange when the UV irradiation is done in the presence of the peptide of interest (which is not UV-labile). This flexibility facilitates the screening of virtually any peptide of interest with enough affinity for the MHC allele that it is loaded onto.
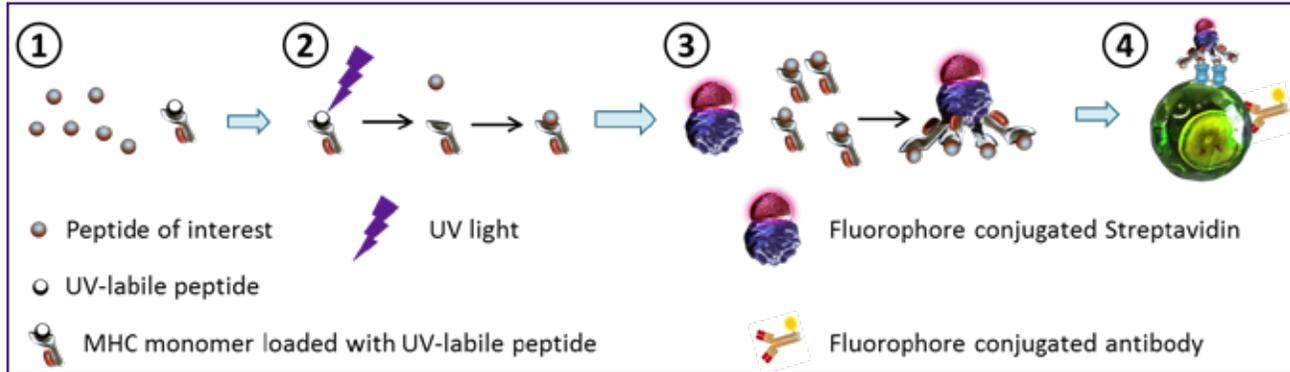


Figure 3. Diagram depicting peptide exchange and tetramerization of Flex-T™ MHC monomers. 1) UV-labile peptide loaded MHC monomer is mixed with the peptide of interest. 2) The UV light will degrade the labile peptide and the peptide of interest, which is not UV sensitive, replaces it. 3) Tetramerization occurs by incubating the MHC monomers loaded with the exchanged peptide with fluorophore-labeled streptavidin. 4) Identification of antigen-specific T cells can be achieved with the newly assembled tetramer and co-stained with conjugated antibodies.

## Applications

MHC tetramers are among the best tools available to characterize a specific T cell response. Applications include any research field that benefits from monitoring antigen-specific T cells, including, but not limited to vaccine research, identification of neoepitopes in cancer research, and immunotherapy.

To increase the resolution when detecting antigen specific T cells with MHC tetramers and simultaneously improve the specificity of the assay, it is recommended that a two color staining strategy with the same allele/peptide combination be used.

Based on the unique properties of our systems, an ELISA to evaluate the peptide exchange has been developed. This can further be used to screen multiple peptides and have an idea about the affinity by which the peptide binds the MHC molecules.
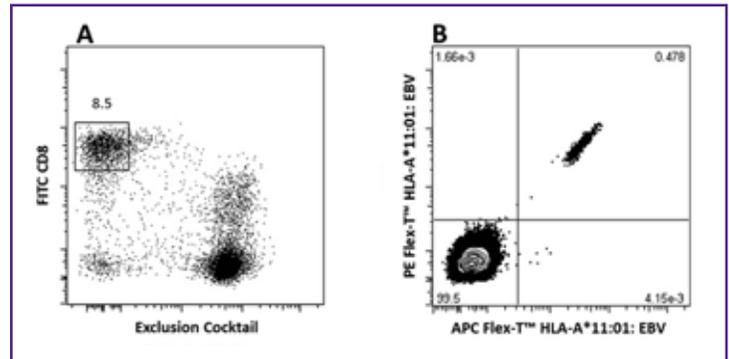


Figure 4. A) CD8^{+} T Cells previously gated on lymphocytes (FSC vs SSC) and 7-AAD negative events, the exclusion cocktail contains Alexa Fluor® 700 anti- CD4, CD19, CD14, and CD16. B) Antigen-specific CD8^{+} T Cells, gated as described, detected with Flex-T™ tagged with PE and APC. HLA-A*11:01 Flex-T™ is loaded with EBV peptide (IVTDFSVIK).
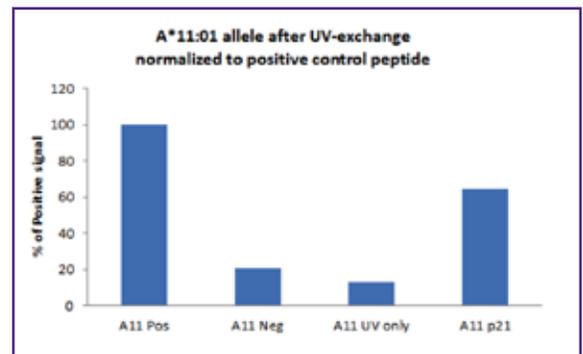


Figure 5. The bar graph shows peptide exchange of HLA-A*11:01 allele. Monomers were irradiated with UV light in the presence of positive (Pos), negative (Neg), and p21 (EBV RVRAYTYSK) peptides, or no peptide (UV only). ELISA signal was normalized using the Pos control well.

In addition, when using MHC multimers to study antigen-specific T cells, each MHC/peptide detects only a small fraction of T cells that are specific for that peptide. Thus, for a large screening of several specificities, a proportionally large volume of blood or sample will be needed. To address this issue, an excellent strategy is to use a "combinatorial color coding" system (5). The method consists of assigning a unique two-color code to each tetramer/peptide of interest. Two tetramers tagged with different colors will bind to the TCRs, yielding a specific T cell that can be



Figure 6. Diagram illustrating the use of combinatorial color coding (5).

detected in two channels in a flow cytometer. By analyzing the double positive cells, it is possible to identify the peptide that those cells are recognizing.

The number of tetramer/peptide combinations that can be used simultaneously depends on how many fluorophores are used to assemble the tetramers. For example, using 5 different streptavidin-fluorophore conjugates allows for 10 unique two-color combinations (light blue region, table 2). Using 6 conjugates, the codes expand to 15 (light red region, table 2). The codes increase as the number of conjugates increase.

Thus, as the number of colors used increase, more specificities can be covered and less sample is required. The cost of the reagents is much lower using this strategy.

## How to Boost the Signal when Working with Low Affinity TCRs

The TCR-peptide/MHC affinity required for tetramer binding exceeds that required for T cell activation. This affinity threshold difference means that tetramer staining may underestimate the frequency of functional T cells (6). This issue may be more relevant when the technique is used to study self-reactive T cells, such as those occurring in autoimmunity or cancer, which tend to express low affinity TCRs. For example, it has been documented that viral antigens have significantly higher affinity than cancer-related antigens, in the context of HLA-A2 (7). Likewise, conventional tetramer staining seemed to be insufficient to detect all functional T cells, compared to an autologous tumor killing assays (8).

| Fluorophore | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 | | ● | ● | ● | ● | ● | ● | ● |
| 2 | | | ● | ● | ● | ● | ● | ● |
| 3 | | | | ● | ● | ● | ● | ● |
| 4 | | | | | ● | ● | ● | ● |
| 5 | | | | | | ● | ● | ● |
| 6 | | | | | | | ● | ● |
| 7 | | | | | | | | ● |
| 8 | | | | | | | | |

Table 2. Possible combinations of two-color codes depend on the number of Streptavidin-fluorophore conjugates used to generate the tetramer/peptide complex (5).

## How to Improve the Signal to Detect Difficult T Cell Clones?

1) Use bright fluorophores. Three great choices to construct bright tetramers include the use of streptavidin conjugated to PE, APC and BV421™. As with any other marker that is poorly expressed, detection by flow cytometry benefits greatly from the use of bright fluorophores.

2) Using protein kinase inhibitors (PKI). Successful use of MHC multimers relies on the capacity of the reagent to bind multiple TCRs, simultaneously. Density of TCRs and availability on the cell surface are key factors to a bright signal. After engaging the cognate antigen, TCRs may be internalized (9), an event that could affect the detection using MHC multimers. TCR internalization can be inhibited using PKI such as desatanib, resulting in a significantly enhanced staining (10).

3) Signal amplification. Another approach to bring up those low affinity T cell clones is the use of conjugated antibodies with the same fluorophore as the one conjugated to the tetramer. For example, if using a PE-tetramer, it is possible to use an anti-PE antibody conjugated to PE as well to boost the signal. This approach has been successfully used to detect ILA1 T cells that recognize the peptide ILAKFLHEL with very low affinity. ILA1 T cells can be easily distinguished from regular PBMCs (which contain T cell clones with higher affinity for the same peptide) using ILAKFLHEL tetramers conjugated to PE, followed by PE conjugated anti-PE antibody staining (11).
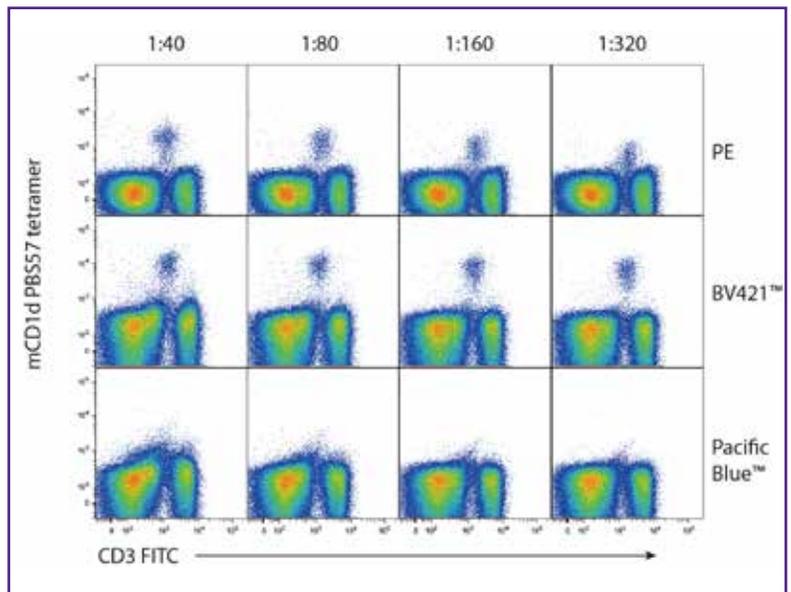


Figure 7. Mouse spleen cells were labeled with PBS57-loaded mouse CD1d tetramer bound to Streptavidin- BV421™, -PE, or -Pacific Blue™. The data shows optimal staining with BV421™ when comparing equivalent tetramer concentrations. Data provided by Dr. Rick Willis, Emory/Yerkes.

In conclusion, MHC multimers are emerging as an essential tool to characterize specific T cell responses. MHC monomers with the capacity to swap the cognate peptide are bringing a lot more flexibility to this research field. Making the technology widely available will definitely help spread the applications and utility of antigen-specific T cell identification.

**References:**
1. Rossjohn J *et al*. 2015. *Annu Rev Immunol*. 33:169
2. Altman JD, *et al*. 1996. *Science*. 274(5284):94
3. Phetsouphanh C *et al*. 2015. *Int J Mol Sci*. 16(8):18878
4. Lancaster AK *et al*. 2007. *Tissue Antigens*. 1:192
5. Hadrup SR *et al*. 2009. *Nat Methods*. 6(7):520
6. Laugel B *et al*. 2007. *J Biol Chem*. 282(33):23799
7. Aleksic M *et al*. 2012. *Eur J Immunol*. 42(12):3174
8. Dolton G *et al*. 2014. *Clin Exp Immunol*. 177(1):47
9. Valitutti S *et al*. 1995. *Nature*. 375(6527):148
10. Lissina A *et al*. 2009. *J Immunol Methods*. 340(1):11
11. Tungatt K *et al*. 2015. *J Immunol*. 194(1):463

**Learn more about MHC antigen peptides: biolegend.com/antigen_peptides**

**Discover Flex-T™ at: biolegend.com/flex-t**

# Applications of Mass Cytometry
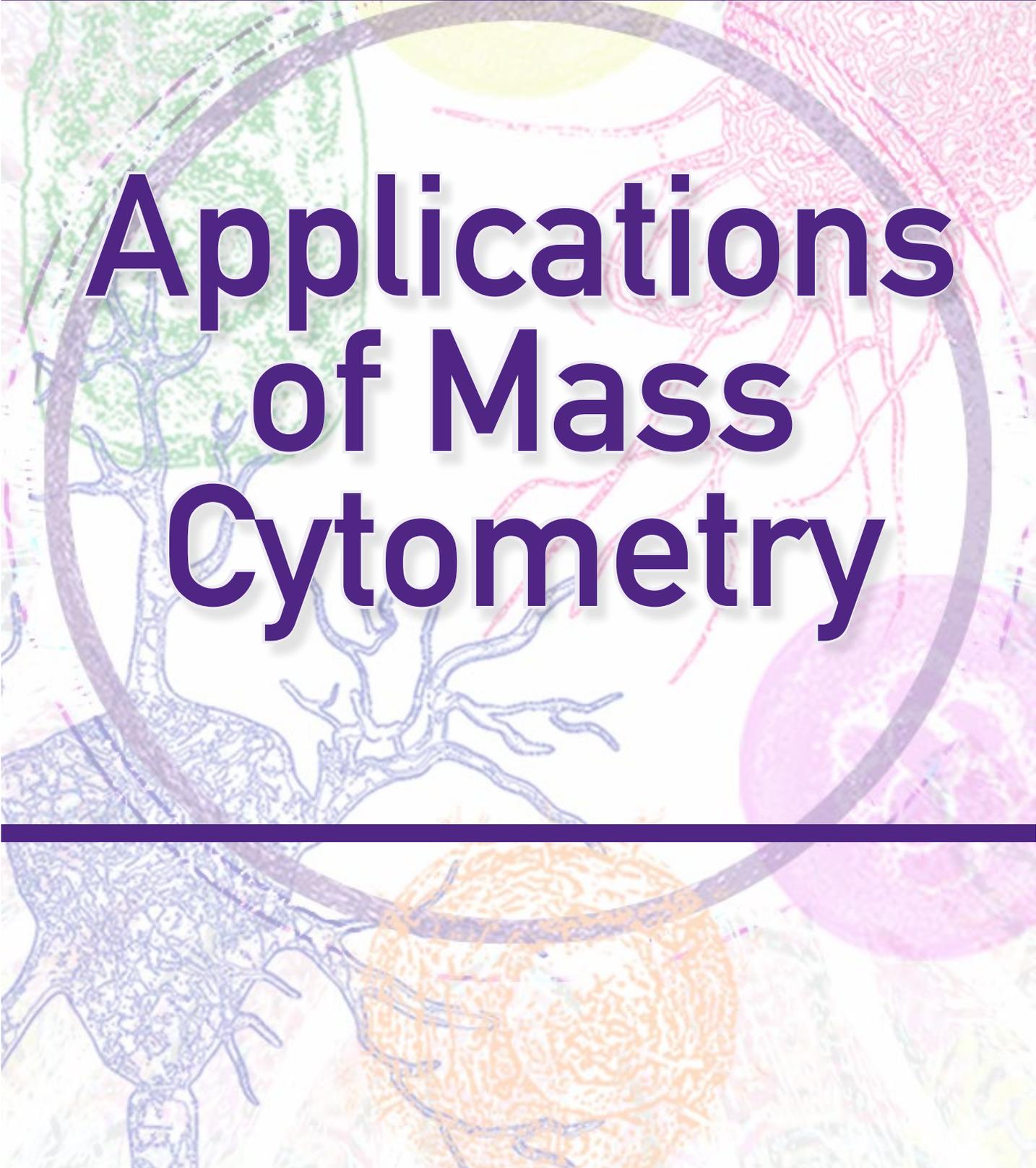
# Applications of Mass Cytometry

Mass Cytometry, also known as CyTOF®, is a unique single cell analysis platform that can enable the quantitation of 100 antigens on the same set of cells, using antibodies labeled with heavy metal ion tags like lanthenides. Although the applications of the technology are similar to flow cytometry, the platform itself is not. The cells labeled with metal ion tagged antibodies are delivered to the instrument in a single cell suspension. Each cell interrogates a laser that atomizes the cells, releasing each heavy metal ion held by each antibody into a detection array. Similar to how a mass spectrometer is able to capture and quantitate each element comprising a protein, the CyTOF® instrument detects the number of each heavy metal ion, relating a value of total abundance. The strong advantage of CyTOF® is that most elements only have a very small range of isotopic mass. For example, when a Terbium ion is detected, a mass deviance of +/- 4 might be detected from oxidized/reduced states around an atomic mass of 65. This is much more limited than the range of distribution around the entire spectrum of a fluorophore like FITC, where the peak emission may be greatest at 520 nm, but the emission spectrum begins at 480 nm and finishes around 620 nm. It's theoretically possible to detect 100 different metal ions simultaneously because of this tight detection range of atomic mass in CyTOF®.

BioLegend offers antibodies ready to conjugate with the MaxPar® isotope labeling kits. They are at the right concentration and purity to make conjugating your own antibodies as easy as possible in your panel optimization. One application recently using our antibodies and Veri-Cells™ PBMC products is from the lab of Dr. Adeeb Rahman at Mt. Sinai School of Medicine. He labeled the lyophilized Veri-Cells™ PBMC product with a combination of 19 antibodies, each conjugated to different metal isotopes on three different days over a 6 week period, to test the reproducibility of the reagents and the instrument as a control.
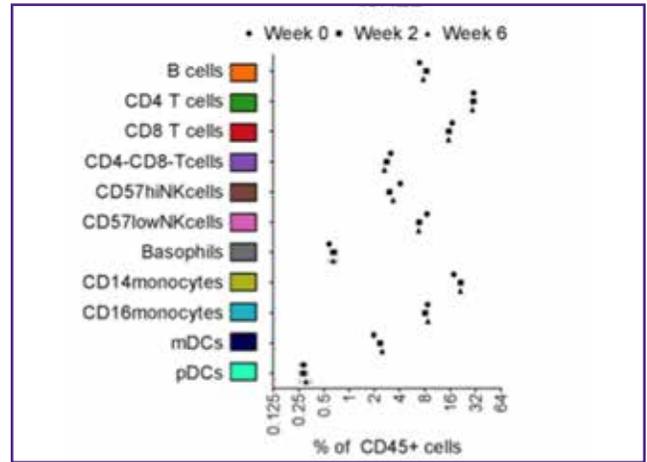


Figure 1. indicates the population frequencies of 4-5 replicate samples on each day (Week 0, Week 2 or Week 6) with a coefficient of variance across all populations and time points to be <9%.
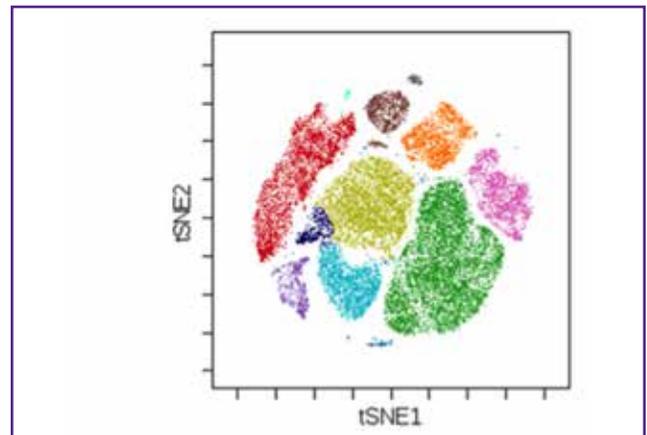


Figure 2. Using a clustering algorithm called t-SNE, the graph shows a map of the different major cell subset populations defined by the markers indicated in figure 1.
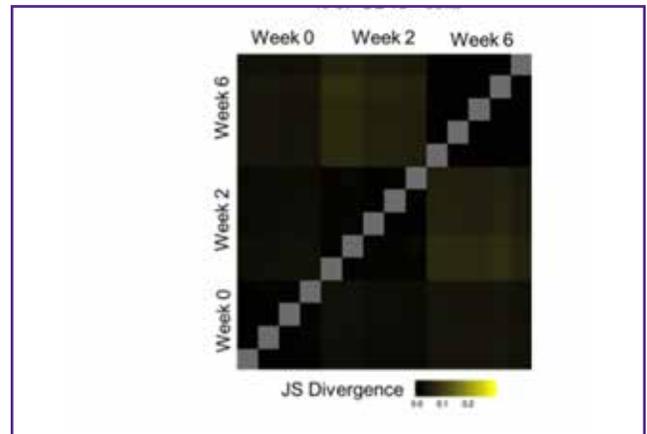


Figure 3. Applying the Jensen-Shannon (JS) divergence method between paired t-SNE plots for each replicate sample, the divergence was found to be an average of 0.02.

## Table 1. Purified MaxPar® Ready Antibodies

| Specificity | Reactivity | Clone | Cat. No. | Specificity | Reactivity | Clone | Cat. No. |
|---|---|---|---|---|---|---|---|
| Allophycocyanin (APC) | | APC003 | 408005 | CD41 | ms | MWReg30 | 133919 |
| Biotin | | 1D4-C5 | 409007 | CD41 | hu | HIP8 | 303721 |
| CD2 | hu | TS1/8 | 309219 | CD44 | ms, hu | IM7 | 103051 |
| CD3 | hu | UCHT1 | 300443 | CD44 | hu | BJ18 | 338811 |
| CD3ε | ms | 145-2C11 | 100345 | CD45 | ms | 30-F11 | 103141 |
| CD4 | ms | RM4-5 | 100561 | CD45 | hu | HI30 | 304045 |
| CD4 | hu | RPA-T4 | 300541 | CD45.1 | ms | A20 | 110745 |
| CD4 | hu | SK3 | 344625 | CD45.2 | ms | 104 | 109843 |
| CD5 | ms | 53-7.3 | 100619 | CD45R | ms, hu | RA3-6B2 | 103249 |
| CD5 | hu | UCHT2 | 300627 | CD45RA | hu | HI100 | 304143 |
| CD7 | hu | CD7-6B7 | 343111 | CD45RO | hu | UCHL1 | 304239 |
| CD8 | hu | SK1 | 344727 | CD48 | ms | HM48-1 | 103433 |
| CD8a | ms | 53-6.7 | 100755 | CD49b | ms | HMα2 | 103513 |
| CD8a | hu | RPA-T8 | 301053 | CD49d | hu | 9F10 | 304319 |
| CD10 | hu | HI10a | 312223 | CD56 (NCAM) | hu | HCD56 | 318345 |
| CD11a | hu | HI111 | 301223 | CD57 | hu | HCD57 | 322325 |
| CD11b | ms, hu | M1/70 | 101249 | CD61 | hu | VI-PL2 | 336413 |
| CD11b | hu | ICRF44 | 301337 | CD62L | ms | MEL-14 | 104443 |
| CD11c | ms | N418 | 117341 | CD62L | hu | DREG-56 | 304835 |
| CD11c | hu | 3.9 | 301639 | CD64 | hu | 10.1 | 305029 |
| CD11c | hu | Bu15 | 337221 | CD69 | ms | H1.2F3 | 104533 |
| CD13 | hu | WM15 | 301717 | CD69 | hu | FN50 | 310939 |
| CD14 | ms | Sa14-2 | 123321 | CD80 | ms | 16-10A1 | 104735 |
| CD14 | hu | M5E2 | 301843 | CD86 | hu | IT2.2 | 305435 |
| CD15 | hu | W6D3 | 323035 | CD90 (Thy1) | hu | 5E10 | 328129 |
| CD16 | hu | 3G8 | 302051 | CD90.2 | ms | 30-H12 | 105333 |
| CD16/32 | ms | 93 | 101335 | CD95 | hu | DX2 | 305631 |
| CD19 | ms | 6D5 | 115547 | CD107a (LAMP-1) | hu | H4A3 | 328635 |
| CD19 | hu | HIB19 | 302247 | CD115 | ms | AFS98 | 135521 |
| CD20 | hu | 2H7 | 302343 | CD117 (c-kit) | ms | 2B8 | 105829 |
| CD22 | hu | HIB22 | 302511 | CD117 (c-kit) | hu | 104D2 | 313223 |
| CD23 | ms | B3B4 | 101625 | CD122 | hu | TU27 | 339015 |
| CD235ab | hu | HIR2 | 306615 | CD123 | hu | 6H6 | 306027 |
| CD24 | ms | M1/69 | 101829 | CD127 | ms | A7R34 | 135029 |
| CD24 | hu | ML5 | 311127 | CD127 (IL-7Ra) | hu | A019D5 | 351337 |
| CD25 | ms | 3C7 | 101913 | CD134 | hu | Ber-ACT35 (ACT35) | 350015 |
| CD27 | hu | O323 | 302839 | CD138 | hu | DL-101 | 352311 |
| CD28 | ms | 37.51 | 102119 | CD150 (SLAM) | ms | TC15-12F12.2 | 115933 |
| CD28 | hu | CD28.2 | 302937 | CD154 | hu | 24-31 | 310835 |
| CD29 | hu | TS2/16 | 303021 | CD161 | hu | HP-3G10 | 339919 |
| CD31 | ms | 390 | 102425 | CD183 | hu | G025H7 | 353733 |
| CD31 | hu | WM59 | 303127 | CD184 (CXCR4) | hu | 12G5 | 306523 |
| CD33 | hu | WM53 | 303419 | CD196 | hu | G034E3 | 353427 |
| CD34 | hu | 581 | 343531 | CD197 (CCR7) | hu | G043H7 | 353237 |
| CD36 | hu | 5-271 | 336215 | CD200 | hu | OX-104 | 329219 |
| CD38 | ms | 90 | 102723 | CD206 (MMR) | hu | 15-2 | 321127 |
| CD38 | hu | HIT2 | 303535 | CD273 (PD-L2) | hu | 24F.10C12 | 329613 |
| CD39 | hu | A1 | 328221 | CD274 (PD-L1) | hu | 29E.2A3 | 329719 |
| CD40 | hu | 5C3 | 334325 | CD279 (PD-1) | hu | EH12.2H7 | 329941 |

**Purified MaxPar® Ready Antibodies** *(Continued)*

| Specificity | Reactivity | Clone | Cat. No. |
|---|---|---|---|
| CD303 | hu | 201A | 354215 |
| CD326 (Ep-CAM) | ms | G8.8 | 118223 |
| CD326 (Ep-CAM) | hu | 9C4 | 324229 |
| CD335 | ms | 29A1.4 | 137625 |
| CD366 (Tim-3) | hu | F38-2E2 | 345019 |
| F4/80 | ms | BM8 | 123143 |
| FcεRIα | ms | MAR-1 | 134321 |
| Fluorescein (FITC) | | FIT-22 | 408305 |
| GM-CSF | hu | BVD2-21C11 | 502315 |
| Histone H3 pSer28 | hu | HTA28 | 641007 |
| HLA-DR | hu | L243 | 307651 |
| I-A/I-E | ms | M5/114.15.2 | 107637 |
| IFN-γ | ms | XMG1.2 | 505843 |
| IFN-γ | hu | B27 | 506521 |
| Ig light chain k | hu | MHK-49 | 316527 |
| Ig light chain λ | hu | MHL-38 | 316619 |
| IgD | ms | 11-26c.2a | 405737 |
| IgD | hu | IA6-2 | 348235 |
| IgM | ms | RMM-1 | 406527 |
| IgM | hu | MHM-88 | 314527 |
| IL-2 | ms | JES6-5H4 | 503835 |
| IL-2 | hu | MQ1-17H12 | 500339 |
| IL-4 | ms | 11B11 | 504129 |

| Specificity | Reactivity | Clone | Cat. No. |
|---|---|---|---|
| IL-4 | hu | MP4-25D2 | 500829 |
| IL-5 | ms, hu | TRFK5 | 504309 |
| IL-6 | ms | MP5-20F3 | 504509 |
| IL-6 | hu | MQ2-13A5 | 501115 |
| IL-10 | ms | JES5-16E3 | 505029 |
| IL-10 | hu | JES3-9D7 | 501423 |
| IL-17A | ms | TC11-18H10.1 | 506935 |
| IL-17A | hu | BL168 | 512331 |
| IL-21 | hu | 3A3-N2 | 513009 |
| Ki-67 | hu | Ki-67 | 350523 |
| Ly-6A/E (Sca-1) | ms | D7 | 108135 |
| Ly-6C | ms | HK1.4 | 128039 |
| Ly-6G | ms | 1A8 | 127637 |
| Ly-6G/Ly-6C (Gr-1) | ms | RB6-8C5 | 108449 |
| NK-1.1 | ms | PK136 | 108743 |
| Notch 3 | hu | MHN3-21 | 345407 |
| PD-1 (CD279) | ms | RMP1-30 | 109113 |
| Phycoerythrin (PE) | | PE001 | 408105 |
| T-bet | ms, hu | 4B10 | 644825 |
| TCR-β chain | ms | H57-597 | 109235 |
| TER-119 | ms | TER-119 | 116241 |
| TNF-α | ms | MP6-XT22 | 506335 |
| TNF-α | hu | MAb11 | 502941 |
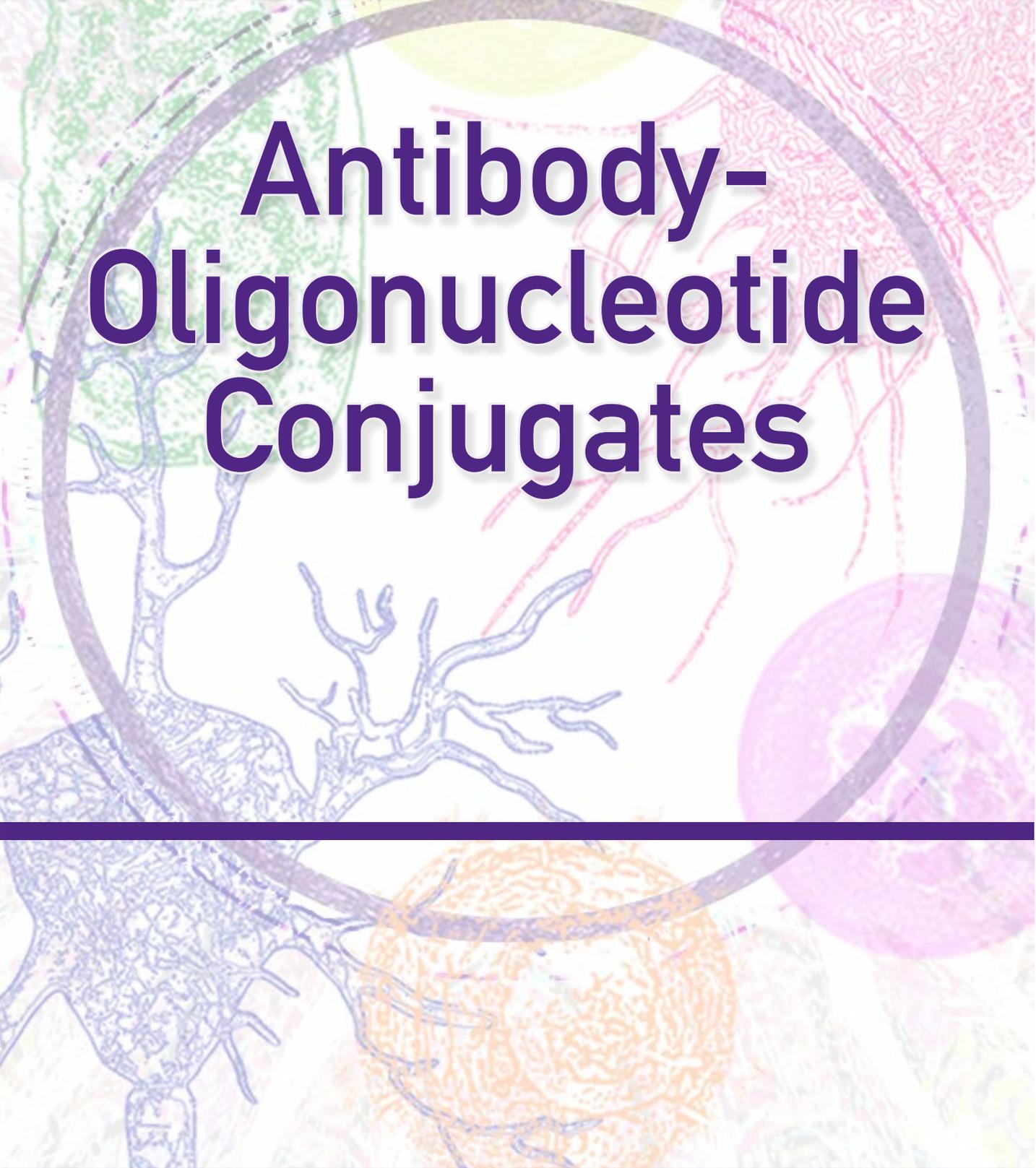
# Antibody–Oligonucleotide Conjugates

# Antibody-Oligonucleotide Conjugates

**Beyond the Crowd: Proteogenomics and The Future of Single Cell Analysis**

Recent advances in multiparametric cell analysis have greatly expanded the number of parameters that can be measured in a cell. The discovery of new fluorophores, in combination with new instruments such as spectral cytometers, as well as new technologies such as mass cytometry, has exponentially increased the amount of information that is possible to acquire from a sample. However, these methods still remain restricted to about 50 to 100 molecules that can be simultaneously analyzed.

In contrast, other techniques allow for more large-scale study of proteins, DNA, and RNA. These methods, commonly referred to as proteomics, genomics, and transcriptomics, have been traditionally limited to the analysis of entire populations of cells.

Now, imagine we could analyze not just hundreds, but thousands of targets, proteins and nucleic acids together, and have the capacity to do so not in a mixed population of cells, but at the single cell level. That's the beauty of Single Cell Multiomics Analysis. Recent workflows, powered by antibody-oligonucleotide conjugates, single cell barcoding instruments, and next generation sequencing, have enabled the capacity to measure thousands of targets in a single cell.

Conjugation of antibodies to oligonucleotides has many applications, such as their potential utility as adjuvants, targeted drug delivery systems, or sample/target barcoding. A publication by Stoeckius *et al*. is one of the first published reports to merge proteomics and transcriptomics at the single cell level using antibodies coupled with oligonucleotides. The method, termed cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), combines highly multiplexed protein marker detection with unbiased transcriptome profiling for thousands of single cells [1]. The authors also report that the method is compatible with two high-throughput single cell RNA-seq platforms, Drop-seq [2] and the Chromium™ system from 10X Genomics (10xgenomics.com). A similar approach was used by Peterson *et al*. They termed their workflow RNA expression and protein sequencing assay (REAP-seq).
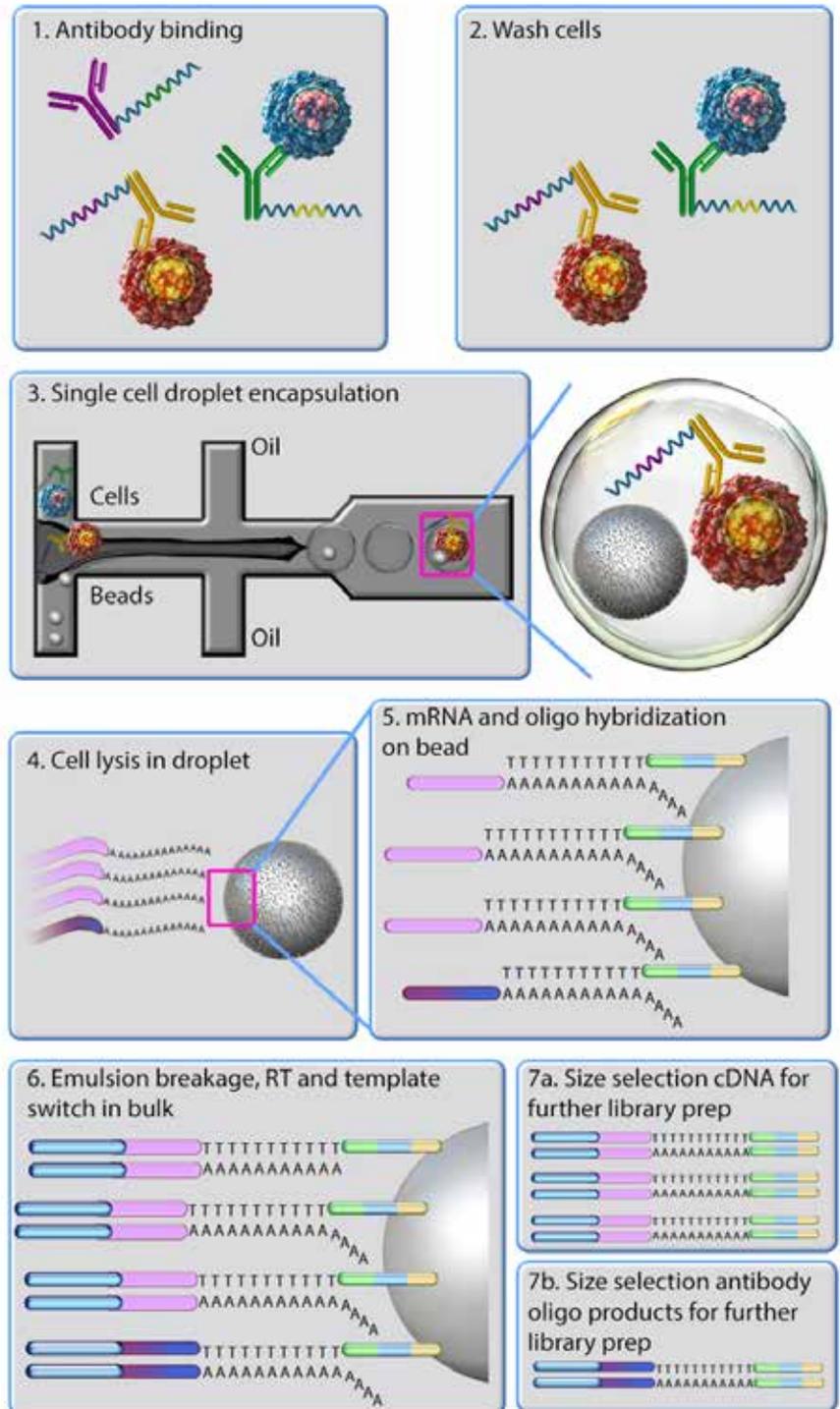


Figure 1. Diagram illustrating CITE-seq workflow. Step 3 can be performed by Drop-Seq (depicted in the figure), or another compatible method such as the Chromium™ platform from 10X Genomics.

## TotalSeq™ Antibodies and CITE-Seq Workflow

Originally, Stoeckius *et al*. used antibodies conjugated to streptavidin and biotinylated oligonucleotides to link the antibody and the oligonucleotide together. BioLegend has worked closely with the authors of the study, based at the New York Genome Center (nygenome.org), and offers antibodies directly conjugated to oligonucleotides to help accelerate single cell genomics and other applications. A similar study used directly conjugated antibodies[3].
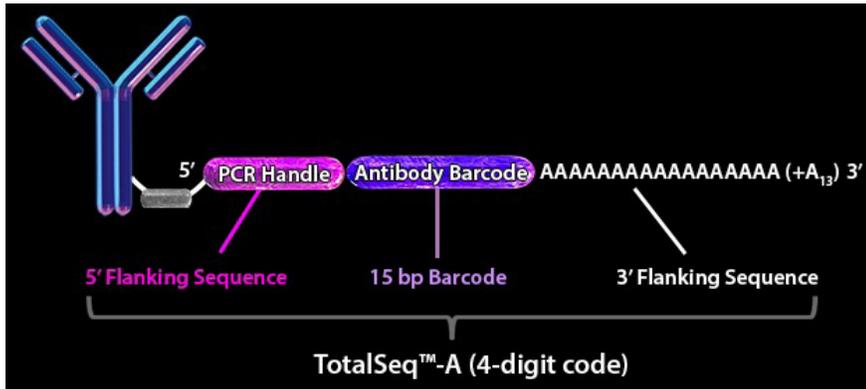


Figure 2. Schematic representation of antibody-oligonucleotide conjugates designed for CITE-seq. The full sequence of the oligonucleotide includes a PCR handle (CCTTGGCACCCGAGAATTCCA), the 15 bp barcode in the table below, and a poly A tail (BAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAA*A*A).

Each oligonucleotide barcode identifies one specific clone. Barcodes can also be used to identify samples, as they have been used before, or even true single cell events. This approach, named cell hashing, or hashtag barcodes, contributes to increase the multiplexing capability of the method, by combining multiple samples in the same sequencing run. TotalSeq™, BioLegend's brand identifying these reagents, covers conjugates developed specifically to work for CITE-seq on Illumina® sequencers and the Chromium™ 10X Genomics platform. Multiple formats of TotalSeq™ may be available, depending on the platform used for the conjugate, the specific application, and other factors.

## General Applications

TotalSeq™ antibodies used in the CITE-seq workflow facilitate experiments in many different research fields. The analysis of complex, high-dimensional datasets, may be facilitated by t-Distributed Stochastic Neighbor Embedding (t-SNE), an approach already used by researchers using CyTOF®. Using appropriate bioinformatics tools and data visualization software, researchers are providing the scientific community with an extremely useful and quickly growing amount of information. For example, analysis from several samples indicates a strong correlation between flow cytometry and CITE-seq data (1). Likewise, transcriptome analysis reveals well-defined populations as expected, based on the expression of well-characterized surface markers (Figure 3).

This technique, powered by reagents such as TotalSeq™ and related instruments, is destined to radically change how researchers analyze biological processes. From general immune response research to cancer, autoimmunity, neuroscience, and more, the future of single cell identity has already started.



Figure 3. Clustering of approximately 5,000 CITE-seq single-cell expression profiles of PBMCs reveals distinct cell populations based on transcriptome analysis. The left panel shows a two-dimensional representation (tSNE) of global gene expression relationships among all cells. Major cell types in peripheral blood can be discerned based on marker gene expression as indicated. The right panels show mRNA (blue) and corresponding Antibody-Derived Tag (ADT, green) signal for the CITE-seq antibody panel projected on the tSNE plot. Darker shading corresponds to higher levels measured.

**Learn more at: biolegend.com/totalseq**

**References:**
1. Stoeckius M, *et al*. *Nat Methods*. 2017 Sep;14(9):865-868
2. Macosko EZ, *et al*. *Cell*. 2015 May 21;161(5):1202-1214
3. Peterson VM, *et al*. *Nat Biotechnol*. 2017 Oct;35(10):936-939

91

## Regulatory T Cells Poster

Regulatory T cells (Tregs) are important members of the immune system known for their immunosuppressive abilities. While some develop in the thymus (typically recognized as natural Tregs), others can be differentiated in the periphery (also known as inducible Tregs). They are often denoted by the expression of the FOXP3 transcription factor and surface markers like CD4, CD25, GARP, CTLA-4, and LAG-3. The ability of Tregs to limit inflammation is carried out by several mechanisms, including the release of immunosuppressive cytokines (i.e., IL-10 and TGF-β) and cyto-lysis inducing cytokines like Granzyme A and B and Perforin. Tregs can also serve as sinks for valuable resources effector cells require to survive, such as IL-2. Through LAG-3, CTLA-4, and MHC II interaction, they can also influence dendritic cells to produce indoleamine 2,3-dioxygenase (IDO), an immunosuppressive molecule. These mechanisms are employed to limit inflammation in autoimmunity, intestinal homeostasis, and other situations. However, the ability to limit inflammation can also be detrimental in the proper clearance of an antigen, potentially leading to chronic inflammation and the propagation of tumor cells.
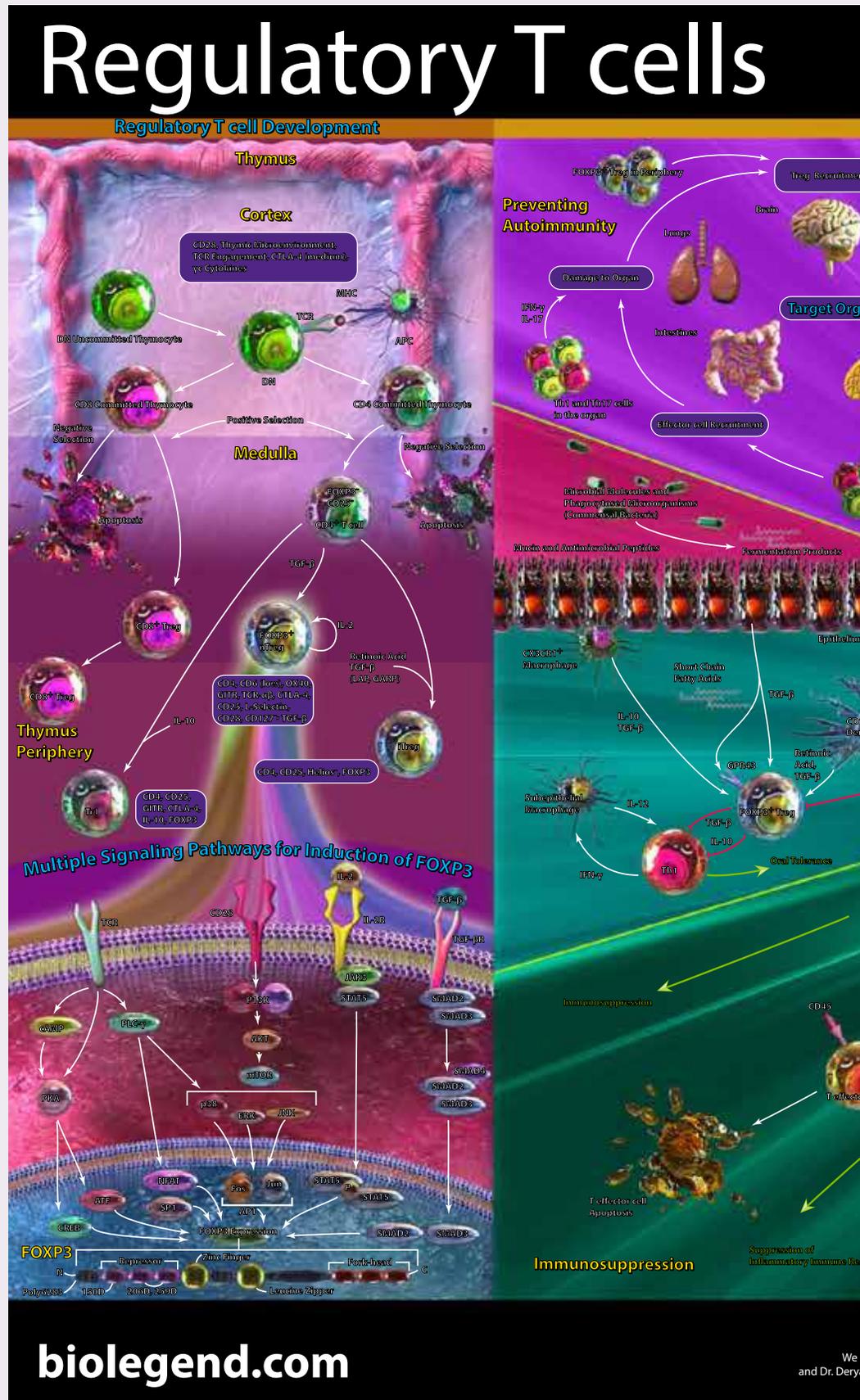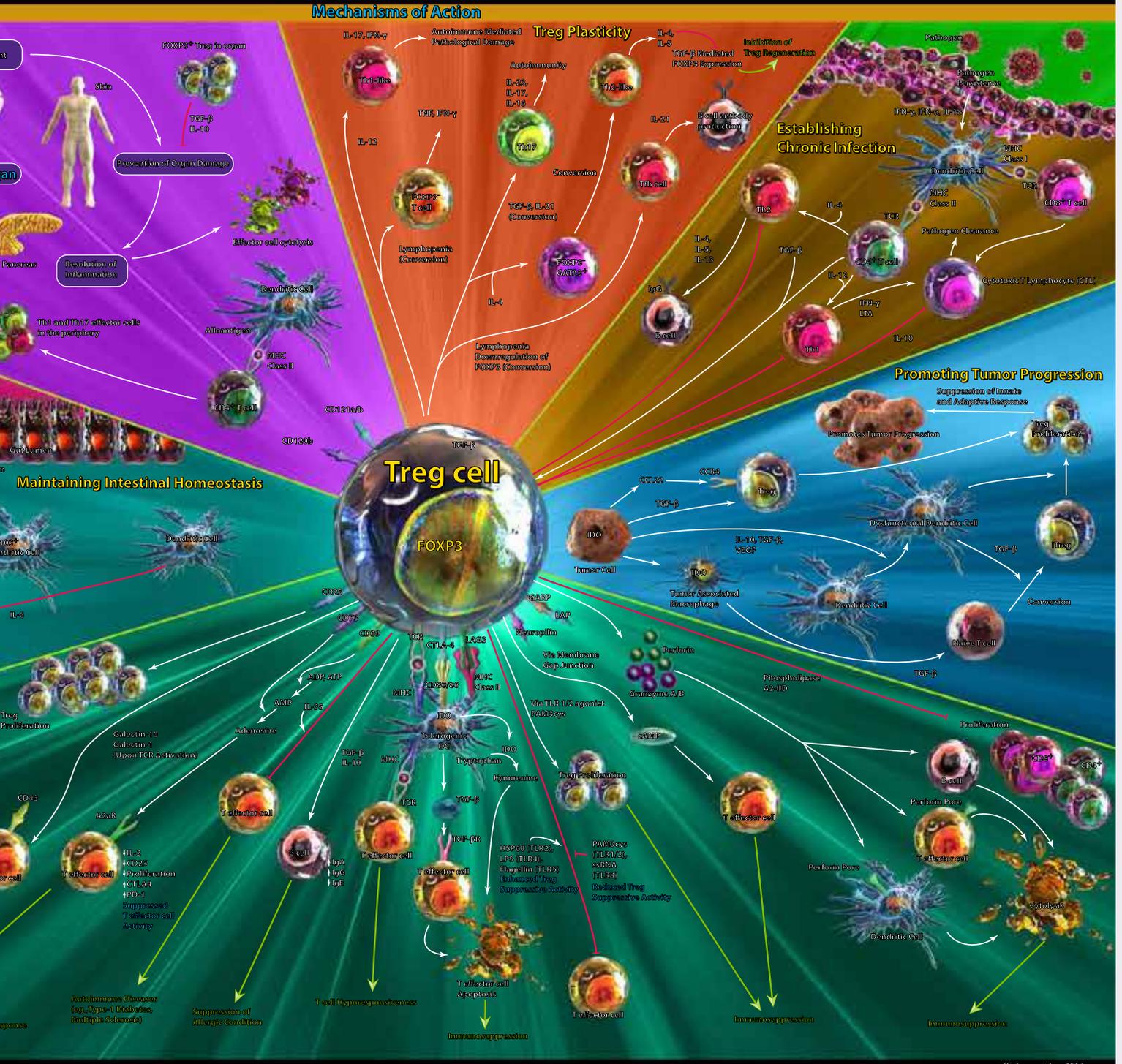
Mechanisms of Action

Treg Plasticity

Establishing Chronic Infection

Promoting Tumor Progression

Maintaining Intestinal Homeostasis

Treg cell

FOXP3

**Interactive Poster: biolegend.com/tregposter**

**Contact BioLegend**
**US & Canada Toll-Free: 1.877.246.5343 (877-BIOLEGEND)**
**International: 1.858.768.5800**
Fax: 1.877.455.9587
email: cs@biolegend.com, techserv@biolegend.com

US Headquarters:
San Diego, CA 92121

04-0006-01

# Imaging the Immune System

# Imaging the Immune System

Imaging applications in immunology give spatial information, from receptors clustered at the cell surface to proteins localized within subcellular compartments, each with a particular function or indication of cellular health in a single cell culture or an intricately connected network of cells comprising a tissue. BioLegend has both antibodies and chemical non-antibody probes to suit many interesting biological questions that are well suited for the unique microscopes available to researchers.

## 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 usable 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.



Figure 1. *Excitation source spectra courtesy of Zeiss.*

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 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. Horseradish peroxidase (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.

**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.



Figure 2. Staining of CX3CR1 (8E10.D9) on formalin fixed paraffin embedded human brain.

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.

95

# 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 Alkaline phosphatase, 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**

# Multicolor Microscopy

### Olympus IX83 (widefield)
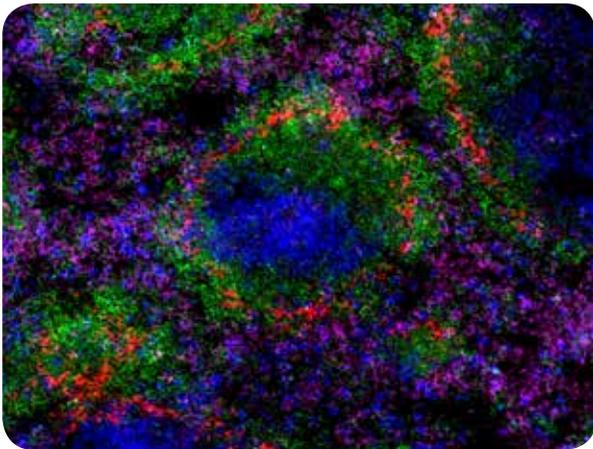


Figure 3. Mouse spleen section stained with anti-B220 BV510™ (green), anti-CD3 BV421™ (blue), anti-F4/80 Alexa Fluor® 488 (purple), and anti-CD169 Alexa Fluor® 647 (red) antibodies. Image obtained with an Olympus IX73 inverted microscope and optimized filter sets.

### Zeiss LSM 780 (confocal)
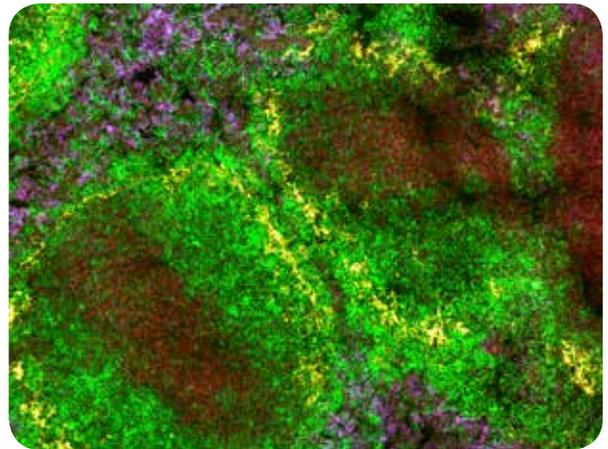


Figure 4. Mouse spleen section stained with anti-B220 BV510™ (green), anti-CD3 BV421™ (red), anti-F4/80 Alexa Fluor® 488 (purple), and anti-CD169 Alexa Fluor® 647 (yellow) antibodies. Image obtained with a Zeiss LSM 780 confocal microscope with spectral unmixing applied.

# 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**
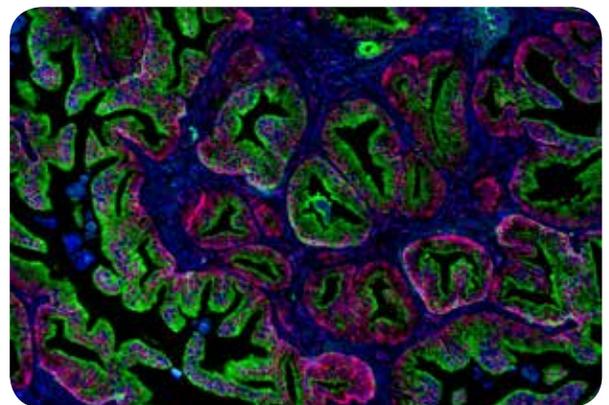


Figure 5. Paraffin embedded human prostate tissue was stained with anti-CD44 Alexa Fluor® 594 (red), purified anti-human CD107b (HB4) antibody followed by anti-mouse IgG Alexa Fluor® 488 secondary antibody (green) and DAPI (blue).

## 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)

**Learn more at: biolegend.com/cell_health_proliferation**



Figure 6. HeLa cells were stained with anti-cytokeratin (pan reactive) Alexa Fluor® 647 (red), Helix NP™ Green (green) and Flash Phalloidin™ Red 594 (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™ NIR DiIC1(5) (Cat. No. 424807)
- 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**



Figure 7. 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).

## 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)



Figure 8. HeLa cells were stained with CD171 Alexa Fluor® 594 (red), Flash Phalloidin NIR™ 647 (green) and DAPI (blue).

97

# 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.
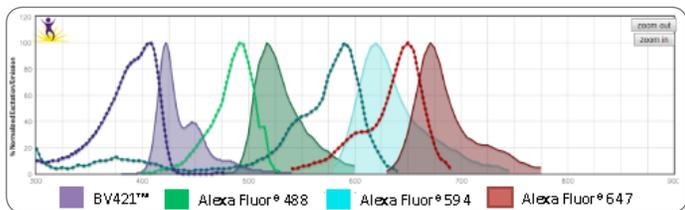


## 2. Fluorophore Combinations with Overlapping Spectra

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.

# Imaging Competition

*Cell Life*

Our imaging competition showcased the capabilities of BioLegend microscopy reagents in the hands of our customers. Many beautiful images were submitted, but in the end, we had to select a few well-deserved winners. If you didn't win this year, keep building your portfolio as new imaging competitions will be here soon. Until then, browse through our winners and other submissions at:

**biolegend.com/cell_life**

*Note: While customers may have used the linked products for their submission, the reagents may not have been validated in-house for microscopy applications.

### Hugues Lelouard –
### Centre d'Immunologie de Marseille-Luminy
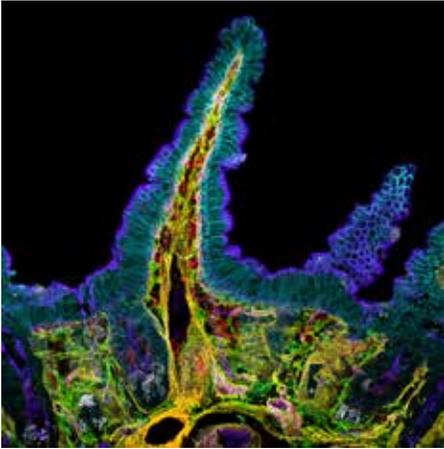


Frozen mouse intestinal villi are stained with anti-EpCAM eFluor™ 450 (cyan), purified anti-Meca 32 (magenta), anti-Lyve 1 eFluor™ 570 (orange), purified anti-CD11c (red), anti-CX3CR1 GFP (green), anti-UEA-I biotin (grey), purified anti-collagen IV (yellow) and Alexa Fluor® 660 phalloidin (purple). The purified antibodies were detected with goat anti-rabbit Alexa Fluor® 514, donkey anti-armenian hamster Alexa Fluor® 594, donkey anti-rat Alexa Fluor® 647 and Streptavidin Alexa Fluor® 405.

### Ankita Patil –
### Drexel University
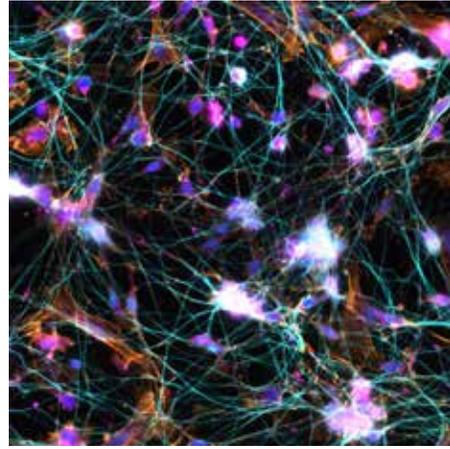


Superior cervical ganglion neurons were stained with anti-tubulin β3 (pink) and anti-TRIM46 (aqua), then stained with secondary antibodies, phalloidin (orange) and DAPI (blue).

### Sebastien Mailfert –
### Centre d'Immunologie de Marseille-Luminy
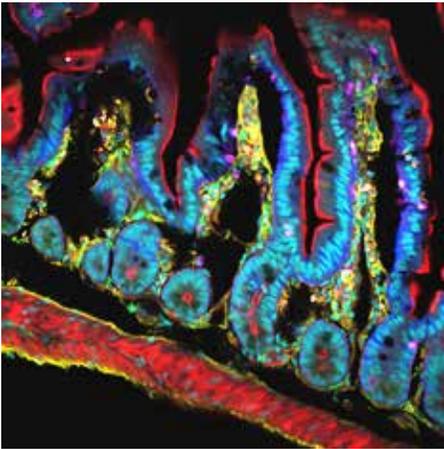


A ten-color spectral imaging strategy to reveal localization of gut immune cell subsets in mouse frozen intestine with anti-EpCam (blue), anti-GFP (light green), anti-CD45R (orange), anti-CD11c (brown), anti-CD3 (magenta), anti-UEA-1 lectin(dark green), anti-collagen IV (yellow), Alexa Fluor® 660 Phalloidin (red), Sytox Blue (cyan) and autofluorescence (light gray).

### Kif Liakathali –
### Kings College London
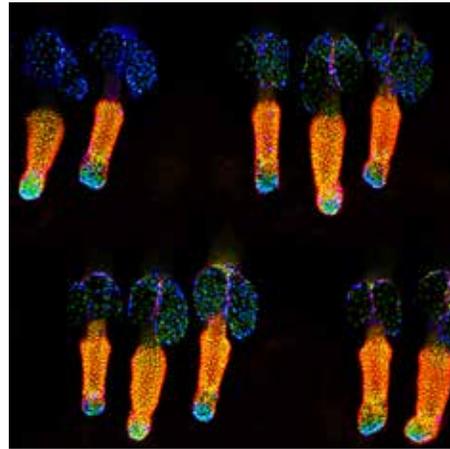


Whole mount mouse tail epidermis was stained with anti-Keratin 14 (green) and anti-Keratin 15 (orange) and DAPI (blue).

99

## Autophagy Poster

The term 'autophagy' is derived from Greek meaning 'eating of self' and was coined almost half a century ago. Autophagy is a natural process and a part of a cell's regular maintenance as it helps to dispose of dysfunctional or damaged organelles. These parts are typically gathered in an autophagosomes (and later a lysosome) and broken down so that the parts can be recycled for other purposes. If the cells sense stress, autophagy may be engaged in order to conserve energy or remove potential sources of danger (like reactive oxygen species from dysfunctional mitochondria). Macroautophagy is the main pathway of autophagy in which organelles or proteins are encapsulated in an autophagosomes and later combined with a lysosome for destruction or recycling. Microautophagy occurs when lysosomes directly engulf cytosolic cargo. Chaperone-mediated autophagy is found only in mammalian cells. Chaperones, as their name suggests, help bring targets into the lysosome. Defects in autophagy pathways have been associated with several disease states, including Alzheimer's and Parkinson's.

# Autophagy

## Chaperone-mediated Autophagy (ATG Independent)

α-Synuclein Aggregate

**Parkinson's Disease**

Mutant α-Synuclein

Mutant LRRK2

α-Synuclein Oligomers at Lysosome

**Tauopathies**

Mutant Tau

**Healthy Cell**

α-Synuclein

hsc70

Co-chaperones

Inhibition of LAMP-2A Multimerization & CMA Substrate Degradation

**Lysosome**

**Dendrites**

Site of:
• Macroautophagy-m
  Neurotransmitter R

Inhibition of CMA Substrate Degradation

Oligomers at Lysosome

LAMP-2A

**Astrocyt**
Involved in
• Clearance
• Synaptic P

Destabilization of Lysosome Membrane

LRRK2    hsc70

Co-chaperones

Degradation of LRRK2 and α-Synuclein

Tau Aggregation in the Cytosol

## Macroautophagy Process (ATG Dependent)

**Stress Signal**
Aging
Protein Aggregate
etc.

**Initiation**

Phagophore

**Nucleation**

PI3P

WIPI1/2

ULK1/2 Kinase Complex Activation

P

ULK1/2

VPS34 Complex Activation

p   VPS15

Beclin1

Protein

FIP200   ATG13    →    ATG14L   VPS34

PI3P

DFCP1

PI3P Generation

ATG5 Complex Recruitment

PI3P, WIPIs, and DFCP1 Recruitment

PI3P

**Expansion**

PI3P
WIPI1/2

Phagophore Elongation

Liptidation with LC3-PE

**Endoplasmic Reticulum**

ATG9

ATG9

Precursor Vesicles

DFCP1
PI3P

ATG12
ATG5

ATG16L
E3

ATG3
E2

ATG7
E1

Auto

ATG9

LC3-II

PE

Membrane Fusion

LC3

ATG4

LC3-I

Cleavage

Binding to Cytosolic Cargo

**Golgi Network**

Lysosome

Autolysosome

Fusion with the Lysosome

Autophagosome

**Maturation**

**biolegend.com**

**Autophagy Events in the Brain**

**Microglia**
Involved in:
• Autophagy-induced Cell Death
• Synaptic Pruning

**Soma**
Site of:
• Autophagosome Formation
• Predominant Localization of Lysosomes
• CMA-mediated Protein Degradation

**Oligodendrocytes**
Site of Macroautophagy-mediated:
• Clearance of Protein Aggregates
• Increased Myelination

mediated
Receptor Degradation

**Axon**
Site of:
• Autophagosome Formation and Maturation during Retrograde Axonal Transport
• Clearance of Synaptic Vesicles, Proteins, and Receptors

tes
Macroautophagy-mediated:
of Protein Aggregates
runing

**Autophagy in Axonal Transport**

Fusion with the lysosome in the soma
Lysosome
Autophagosome
Cargo

Retrograde Axonal Transport
Dynein
htt
HAP1 Kinesin
Dynactin

Autophagosome

**Healthy Neuron**

Cargo

-Impairment of Retrograde Transport
-Defects in Cargo Delivery
Dynein
polyQ htt
HAP1
Dynactin
Kinesin

**Damaged Neuron**

**Aggrephagy (Selective Macroautophagy)**

n Aggregate
Ubiquitin
Ubiquitinated Protein Aggregate

Misfolded Protein

**Autophagy Receptors**
(e.g. p62, ALFY, Optineurin, NBR1)

Phagophore Formation

Lysosome

PE
LC3-II

phagosome

ATG5 Complex

Autolysosome

**Mitophagy (Selective Macroautophagy)**

Parkin Recruitment, Phosphorylation & Activation

Cytosol
PINK1

Proteasome Degradation

PINK1 Accumulation
PINK1

Dimerization & Autophosphorylation

Mutant PINK1

Ubiquitin Phosphorylation

Damaged Mitochondria Loss of Membrane Potential

TOM
TIM PARL
OMM
IMM
MPP

P P

TOM TOM
TIM PARL
OMM
Substrate IMM

Mutant Parkin

**Healthy Mitochondria**

**Damaged Mitochondria**

Ubiquitination of Mitochondrial Proteins by Parkin (e.g. VDAC, Mitofusin 1/2)

Autophagosome

Autophagosome Formation and Fusion with the Lysosome, Acidification & Degradation

LC3 Phagophore
LC3 LC3 P LC3
P NDP52 Optineurin
P TBK1 TAX1BP1 P TBK1 P
NBR1 P p62 LC3 Rab7
TBC1D15
Fis1 TBC1D17

Lysosome

**Damaged Mitochondria**

Poly-Ub Chains

LC3
LC3
LC3

Phagophore

Activation and Recruitment of Macroautophagy Machinery

©BioLegend, Inc. 2017

101

# Neuroinflammation

# Neuroinflammation

Neuroinflammation refers to the inflammation of the nervous tissue and is often initiated as a response to a variety of insults, such as traumatic brain injury or infections. Neuroinflammation is a common component of many neurodegenerative disorders, including Alzheimer's (AD) and Parkinson's (PD) diseases. Production of toxic protein aggregates and/or metabolites in these disorders is often associated with chronic inflammation and sustained activation of resident brain immune cells. This inflammatory response ultimately leads to the production of inflammatory molecules, recruitment of peripheral immune cells, disruption of the blood-brain barrier (BBB) and amplification of the immune response. Glial cells, which include microglia, oligodendrocytes, and astrocytes, are essential mediators of neuroinflammation in the central nervous system (CNS).



Figure 1. Schematic representation of neurons and glial cell in the brain.

**Discover more at: biolegend.com/neuroinflammation**

## Microglia

Microglia cells are known as the resident macrophages in the CNS. They account for 10-15% of all cells found within the brain. Microglia actively survey their domain to perform housekeeping functions such as cleaning up cellular debris. These cells are very plastic and can undergo structural and morphological changes to meet the needs of their environment. Under normal conditions, microglia are found in a ramified state where they have long branching processes and a small cell body. These branches are highly sensitive to physiological alterations in their surroundings such as foreign material and damaged or dying cells. Therefore, using their processes, microglia are able to search and identify immune threats and maintain homeostasis in the CNS. Microglia can rapidly transform into a reactive form under unhealthy conditions and respond to the injury or threat by undergoing key morphological changes. In this state, they thicken and retract their processes, take on an amoeboid shape and become phagocytic. These morphological changes are also accompanied by secretion of inflammatory molecules such as cytokines and chemokines which help microglia communicate with astrocytes and peripheral immune cells.



Figure 2. Immunofluorescent staining of purified anti-P2RY12 antibody (clone S16007D) on PFA-fixed frozen mouse brain tissue.

Microglia can play a dual role in neuroinflammation, depending on the nature and duration of the insult. Acute neurotoxic insults trigger the production of anti-inflammatory factors that will eventually lead to the removal of the insult, cellular repair and regeneration, and resolution of inflammation. On the other hand, chronic neurotoxic insults, often associated with neurodegenerative disorders, lead to persistent inflammatory responses that would culminate in a variety of responses, such as aberrant synaptic pruning, axonal demyelination and degeneration, and eventual loss of neurons.

**Learn more at: biolegend.com/microglia**

## Astrocytes

Astrocytes are the most abundant glial cell type in the brain, accounting for 20 to 40% of all glial cells. Astrocytes are commonly known as possessing a star-shaped morphology with fine, elaborate processes. These cells perform many functions, such as providing metabolic support for neurons (*e.g.,* produce and secrete glutamine and lactate), as well as rapidly removing excess neurotransmitter (*e.g.,* glutamate) released into the synaptic cleft to protect neurons against neurotoxicity. In addition, astrocytes help in the maintenance of the BBB integrity and permeability by projecting astrocytic endfeet to encircle and cover endothelial cells of the blood vessel.

Similar to microglia, depending on the context and duration of the insult, astrocytes can exacerbate the inflammatory reactions and mediate tissue damage or dampen the immune reaction and promote tissue repair. They do so by secreting a variety of bioactive molecules, such as cytokines and chemokines. Furthermore, the function of astrocytes in the maintenance of the BBB has significant implications during inflammation, as dysfunction of astrocytes may lead to BBB disruption and favor the infiltration of peripheral immune cells and molecules into the CNS.

**Discover more at: biolegend.com/astrocytes**

Figure 3. IHC staining of anti-GFAP antibody (clone SMI 24) on formalin fixed, paraffin embedded (FFPE) rat brain tissue.

## Oligodendrocytes

Oligodendrocytes (ODs) are a type of glial cell with many functions in the CNS. These cells contribute to neuroplasticity and provide trophic support to neurons by producing factors such as glial cell-line derived neurotrophic factor (GDNF). One of the predominant functions of ODs is to produce myelin sheaths to insulate segments of neuronal axons. The myelin sheath enables high velocity signal transduction and is essential for the propagation of action potentials along the axon. Each OD can extend its processes to multiple axons and has a great capacity to rapidly renew its myelin sheaths. As a result, ODs have a high metabolic rate and are highly vulnerable to oxidative stress. The latter is also partly due to relatively low levels of anti-oxidative enzymes in ODs.

As mentioned earlier, microglia produce various pro-inflammatory mediators. These molecules can induce bystander damage to their neighboring glial cells and neurons. As a consequence of their high metabolic activity, oligodendrocytes are more susceptible to these factors and respond by producing poor quality myelin, which may ultimately lead to the loss of OD-neuron connections and axon degeneration. Damaged ODs can also initiate pathways of cell death, which can further activate microglia and amplify the inflammatory cascade.

**Learn more at: biolegend.com/oligodendrocytes**

Figure 4. IHC staining of anti-Myelin Basic Protein antibody (clone SMI 94) on FFPE human cerebellum.

# Complement System (CS) Activation in Alzheimer's Disease

Synapse loss is a common feature of Alzheimer's disease (AD) and often correlates with cognitive dysfunction and decline. Recent studies suggest deregulation of the complement cascade, which plays an essential role in innate and adaptive immune response, as a contributing factor leading to chronic inflammation and neurodegeneration observed in AD. Supporting evidence demonstrates localization of a full range of complement proteins, from C1q to C5b-9 (membrane attack complex), with amyloid deposits as a prominent feature of AD lesions (1). Notably, mRNA levels of complement components are upregulated in the affected brain regions of AD patients (2). Research has shown that amyloid-beta (Aβ) aggregates can initiate antibody-independent complement activation by binding to C1q and C3b (1). Furthermore, Aβ deposits are frequently surrounded by reactive microglia and astrocytes, which are capable of producing complement proteins as well as phagocytosing and degrading Aβ (3). These inflammatory responses appear to initially play a protective role by removing Aβ deposits. However, the continuous generation of Aβ aggregates leads to persistent complement activation and sustained inflammation that is detrimental to neuron health in the brain.
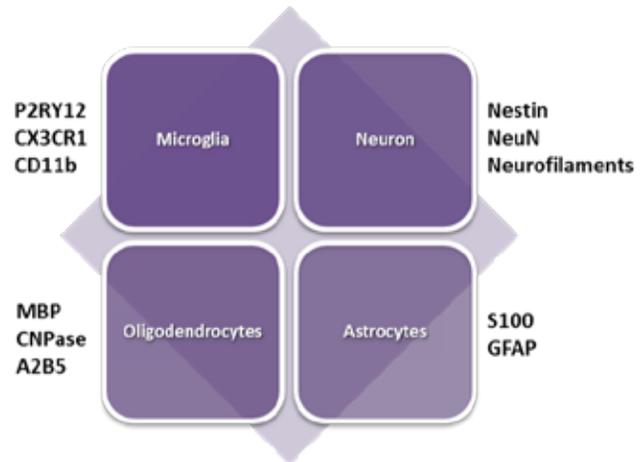


Figure 5. Common markers used to identify glial cells and neurons.
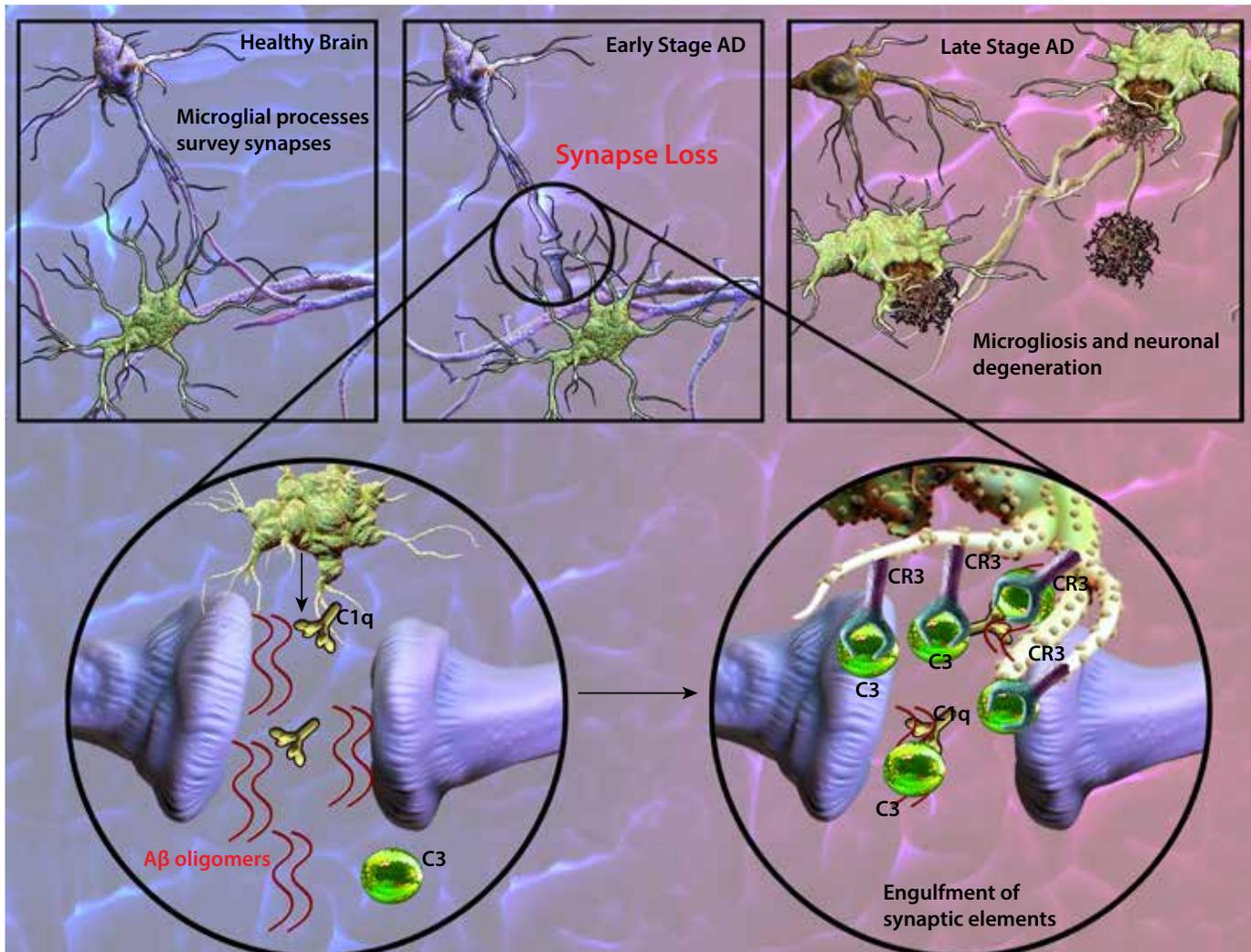


Figure 6. Schematic representation of synapse loss and neuron degeneration in AD caused by interactions between Aβ, components of the complement system and microglia. In early stages of AD, continuous production of Aβ oligomers leads to complement activation (C1q and C3), engulfment and elimination of synapses by microglia. Aberrant activation of complement and microglia coupled to excessive synapse loss are contributing factors that ultimately lead to neuron degeneration in later stages of AD.

105

# Demyelination in Multiple Sclerosis (MS)

Oligodendrocytes play a central role in the pathogenesis of a variety of disorders, including multiple sclerosis. MS is an inflammatory disease that is caused by the destruction of myelin sheaths and demyelination of axons. Evidence suggests that infiltration of myelin-specific CD4^{+} T cells into the CNS initiates a cascade that leads to degeneration and death of myelin-producing ODs, resulting in demyelination, neuronal axon damage, and degeneration (4). Secretion of a variety of cytokines and chemokines by astrocytes may modulate the recruitment and infiltration of peripheral immune cells, including CD4^{+} T cells promoting degeneration of ODs. Furthermore, loss of astrocytic endfeet encircling blood vessels contributes to lesion development and BBB disruption, a hallmark of MS progression (4). Studies have also described a role for astrocytes as inhibitors of myelination. Oligodendrocytes are derived from OD progenitor cells (OPCs), which have the capacity to continually proliferate, migrate and differentiate into myelin-producing ODs in the healthy brain. Under disease conditions such as in MS, OPCs lose their ability to respond to myelin damage and fail to re-myelinate axons. Astrocyte-derived factors such as endothelin-1 have been shown to play a crucial role in the inhibition of OPC recruitment and differentiation after OD injury (4).
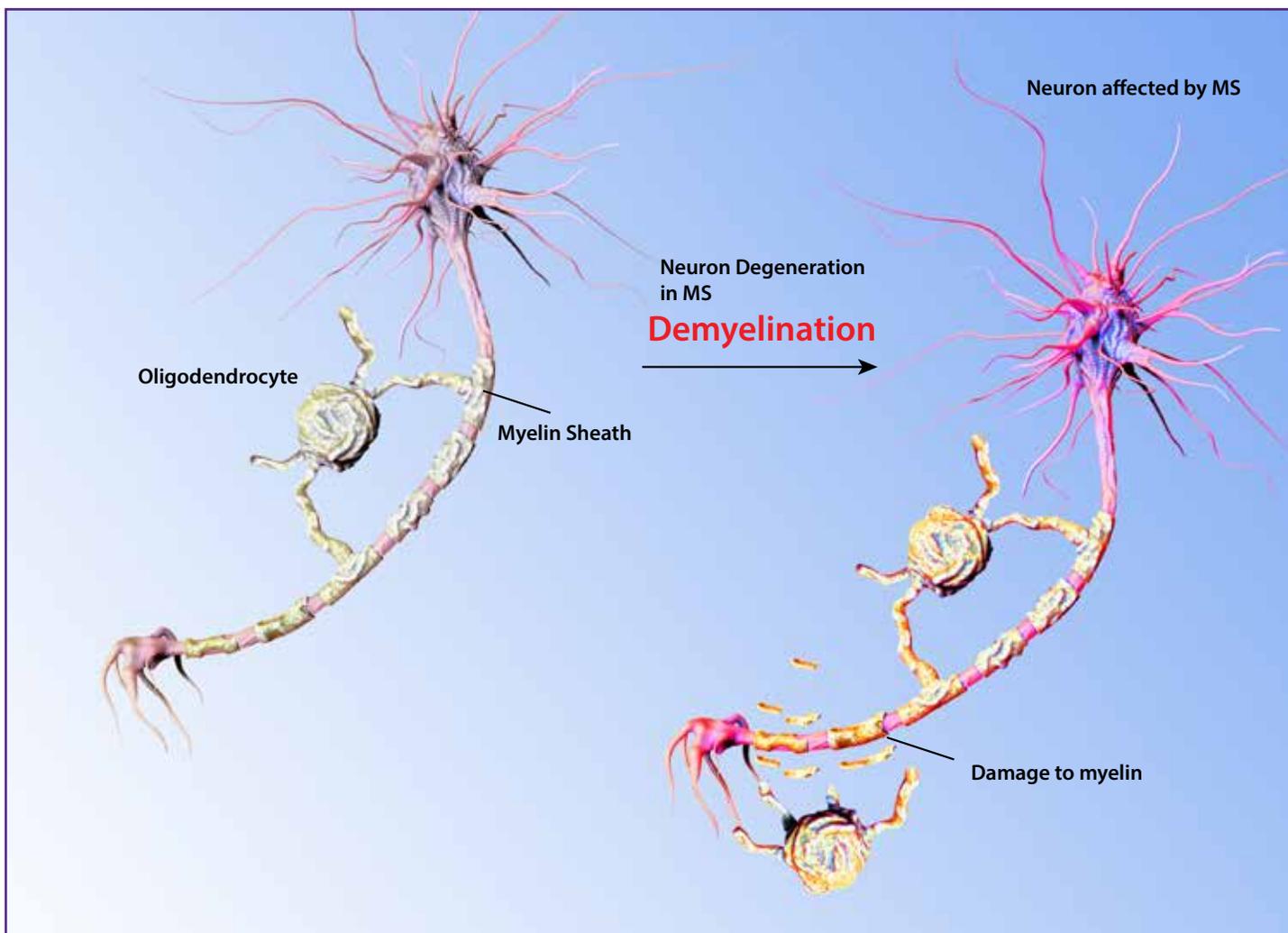


Figure 7. Neuron demyelination and axon degeneration are events associated with multiple sclerosis.

# Microglia-mediated Neuroinflammation in Parkinson's Disease

Neuroinflammation is a prominent feature of Parkinson's disease (PD) and can contribute to disease progression through loss of dopaminergic (DA) neurons in the substantia nigra (SN) of the brain. Elevated levels of pro-inflammatory cytokines and extensive proliferation of reactive microglia around dopaminergic neurons have been detected in the SN area of post-mortem brains from PD patients (5). Notably, microglia are enriched in SN compared to other regions of the brain. These findings, coupled to reduced antioxidant capacity and enhanced sensitivity of neurons to pro-inflammatory molecules, support a role for microglia-mediated DA degeneration in PD.

The leucine-rich repeat kinase 2 (LRRK2) protein may act as a mediator of inflammation through cytokine signaling and microglia-mediated pro-inflammatory responses. Mutations in the LRRK2 gene are the most common cause of familial PD. LRRK2 is a multi-functional protein with ubiquitous expression; its mRNA and protein levels are highly expressed in immune cells, as well as glial cells and neurons (5). However, recent findings indicate the presence of LRRK2 splice variants in the brain, with neurons and astrocytes expressing a different variant than microglia, suggesting a differential function for this protein in various cell types (6). In line with these observations, lipopolysaccharide (LPS)-induced inflammation in mice was associated with upregulation of LRRK2 protein expression and activity in microglia (7). Furthermore, down-regulation of microglial LRRK2 protein expression attenuated LPS-induced inflammatory responses through pro-inflammatory cytokine signaling. Interestingly, gene expression profiling of microglia from mice that over-express LRRK2 R1441G, a disease-associated mutation, revealed an expression profile corresponding to reactive and pro-inflammatory phenotype under normal, unstimulated condition (5). These findings combined indicate a role for LRRK2 in cellular pathways that induce inflammation, and that LRRK2 dysfunction may exacerbate microglial pro-inflammatory responses that would ultimately lead to neurodegeneration.

### References

1. Shen Y, *et al.* 2013. *Transl Neurodegener.* 2: 21.
2. Ysojima K, *et al.* 1999. *Am J Pathol.* 154(3): 927.
3. Serrano-Pozo A, *et al.* 2011. *Cold Spring Harb Perspect Med.* 1(1): a006189.
4. Domingues SH, *et al.* 2016. *Front Cell Dev Biol.* 4:71.
5. Russo I, *et al.* 2014. *J. Neuroinflammation.* 11:52.
6. Giesert F, *et al.* 2013. *PLosD One.* 8(5): e63778.
7. Moehle MS, *et al.* 2012. *J. Neurosci.* 32(5):1602.

**Learn more at: biolegend.com/neuroscience**



**Ramified Microglia**

Abnormal LLRK2 activity or LLRK2 mutations

**Reactive Microglia**
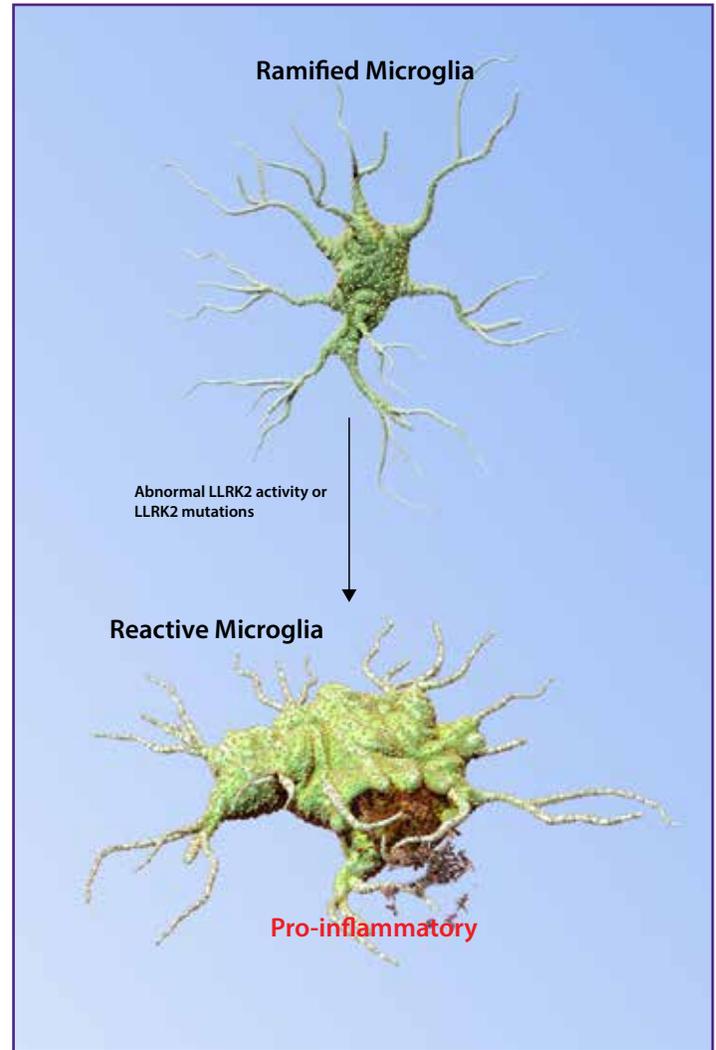
**Pro-inflammatory**

Figure 8. Abnormal LRRK2 activity affects microglia function by inducing pro-inflammatory phenotype in these cells, exacerbating the inflammatory responses, and ultimately leading to neuronal loss observed in PD.

## Neuroinflammation Poster

This poster covers different aspects of brain inflammation in response to neurotoxic insults. Neuroinflammation is a common feature of many diseases of the central nervous system (CNS), including neurodegenerative disorders. A combination of responses from neurons, microglia, astrocytes and peripheral immune cells, along with cytokines, chemokines and complement system constitute the basis for neuroinflammation. Chronic neurotoxic insults often lead to persistent pro-inflammatory responses such as over-activation of the complement system and result in aberrant synaptic pruning, axonal demyelination and degeneration. Acute neurotoxic insults, on the other hand, can trigger the production of anti-inflammatory factors such as cytokines and chemokines by the microglia, astrocytes and peripheral immune cells. These factors lead to the removal of neurotoxic insults, cellular repair and regeneration, and resolution of inflammation. A balance between pro- and anti-inflammatory responses determines whether the integrity of the blood brain barrier is maintained or disrupted, hence limiting the infiltration of peripheral immune cells into the brain, which in turn can dampen or amplify the inflammatory signals, leading to cell death or resolution of inflammation.

Immune-Mediated Aggregate Removal

Ramified State

Astrocyte

Microglia

Inflammatory Stimuli

Protein Aggregates
Acute Brain Injury
Genetic Risk Factors
Aging
Pathogens

DAMPs
(Damage-associated Molecular Patterns)
PAMPs
(Pathogen-associated Molecular Patterns)

ammatory

Anti-inflammatory

ANTES, IL-6,
17F, IFN-α

Reactive
Microglia

Reactive
Astrocyte

Reactive
Microglia

MCP-1, IL-6,
TNF-α, IL-1β,
L-17A, IL-17F

IL-10, TGF-β,
IL-4, IL-13

Reactive
Microglia

T Cell
Responses

ion of BBB Permeability
Cell Recruitment

Reactive
Microglia

Reactive
Astrocyte

Reactive
Astrocyte

Reactive
Microglia

Astrocyte
Proliferation

Misfolded/Aggregated
Proteins, Cell Debris

Reactive
Microglia

Axonal Remodeling
Neural Repair
Neurotrophic
Factor Release

Debris Clearance
Via Phagocytosis

Protein
Aggregate

Complement
Binding

Complement
Binding

Autoantibody
Binding

Diseased
Neuron

Microglia & Astrocyte
Activation

Reactive
Astrocyte

Reactive
Microglia

Circulating
Autoantibody

Complement
Expression
& Secretion

Neuron Survival

Cellular Repair
& Regeneration

Migration towards lesion
Neurogenesis
Neuronal Replacement

Neural Stem Cells

Neurotrophic Support

T Cell

Cytokines

Anti-inflammatory
Cytokines

SDF1, VEGF,
MCP-1, IGF1,
FGF2

Reactive
Microglia

Reactive
Astrocyte

Reactive
Astrocyte

Reactive
Microglia

Angiogenesis
Neovascularization
ECM Reconstruction
SDF1, VEGF,
MCP-1, IGF1,
FGF2

Astrocyte/Glial Scar

Cytokines,
Chemokines

Arg1: ECM
Reconstruction
& Tissue Repair

Endothelial Cells

Invading Pathogens

Monocyte

T Cell

B Cell

Monocyte

Circulating
Autoantibody

Injury
Site

Blood-Brain Barrier

©BioLegend, Inc. 2016

109

# Web Tools

# Web Tools

BioLegend is dedicated to providing useful web resources to make your research easier, more efficient, and more reproducible. Our Web Tools page provides an extensive set of useful tools covering a selection of applications such as flow cytometry, western blotting, ELISA, and microscopy. We also have an array of searchable libraries that aggregate useful information, such as our Publication Library, Product Reviews Library, Video Library, and much more. For those who need mobile information, we provide over 15 apps for iPhone and Android phones.

**Find all of our web tools at: biolegend.com/webtoolstab.**

**Also find our extensive selection of Technical Protocols at: biolegend.com/technical_protocols.**

**Our technical team is always willing find the answers you need, so don't hesitate to contact us at: tech@biolegend.com.**

## Featured Web Tools

**Fluorescence Spectra Analyzer, biolegend.com/spectraanalyzer**

BioLegend's Fluorescence Spectra Analyzer is useful for the analysis of excitation and emission spectra of commonly used fluorophores for flow cytometry. View data for over 90 unique fluorophores and dyes. Custom create your filters and customize the plot colors to your liking.
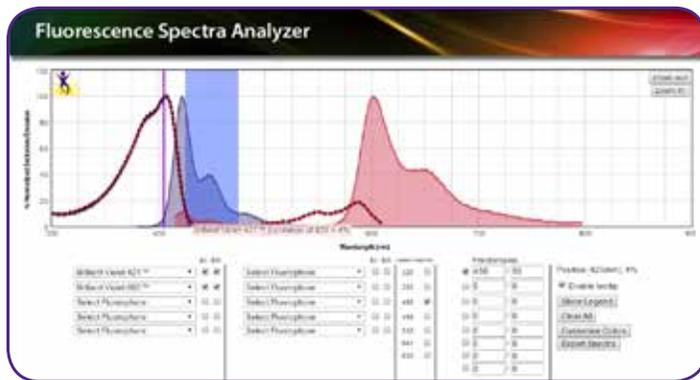


**Multicolor Panel Selector, biolegend.com/panelselector**

BioLegend's Multicolor Panel Selector tool is designed to help you find the right products for your multicolor flow cytometry experiments. View the detailed instructions for using the Panel Selector and guidelines for constructing an optimized panel below the Panel Selector.

**Cell Markers, biolegend.com/cell_markers**

The Interactive Cell Markers page shows a wide selection of over 30 cell types and the cell surface markers associated with each cell. You can learn more about the cell markers, including other names, structure, distribution, function, and ligand receptors.
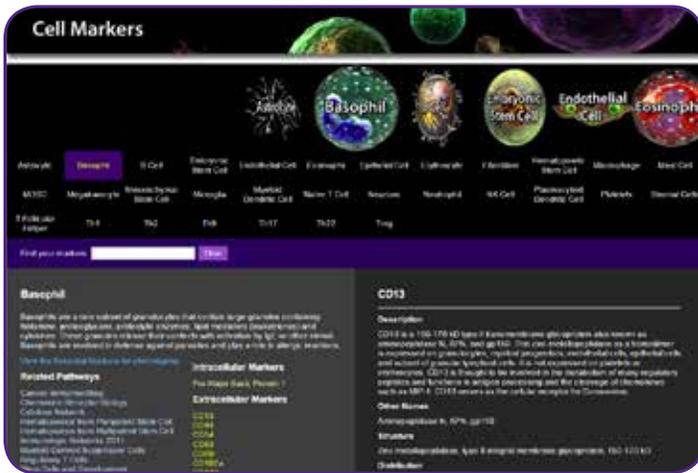


**Lab Tools, biolegend.com/labtools**

This page is full of calculators and converters that should come in very handy in the lab or in the classroom. Includes a timer, a calculator, a molarity calculator, a moles to grams converter, and other conversion tools.



**Maturation Markers, biolegend.com/maturation_markers**

BioLegend's Maturation Markers page describes the stage-specific markers during differentiation for various cell types, including T cells, Tregs, B cells, monocytes, macrophages, and dendritic cells. This page provides a unique view to compare and contrast expression of marker proteins present or absent on each cell type as the cell matures.



113
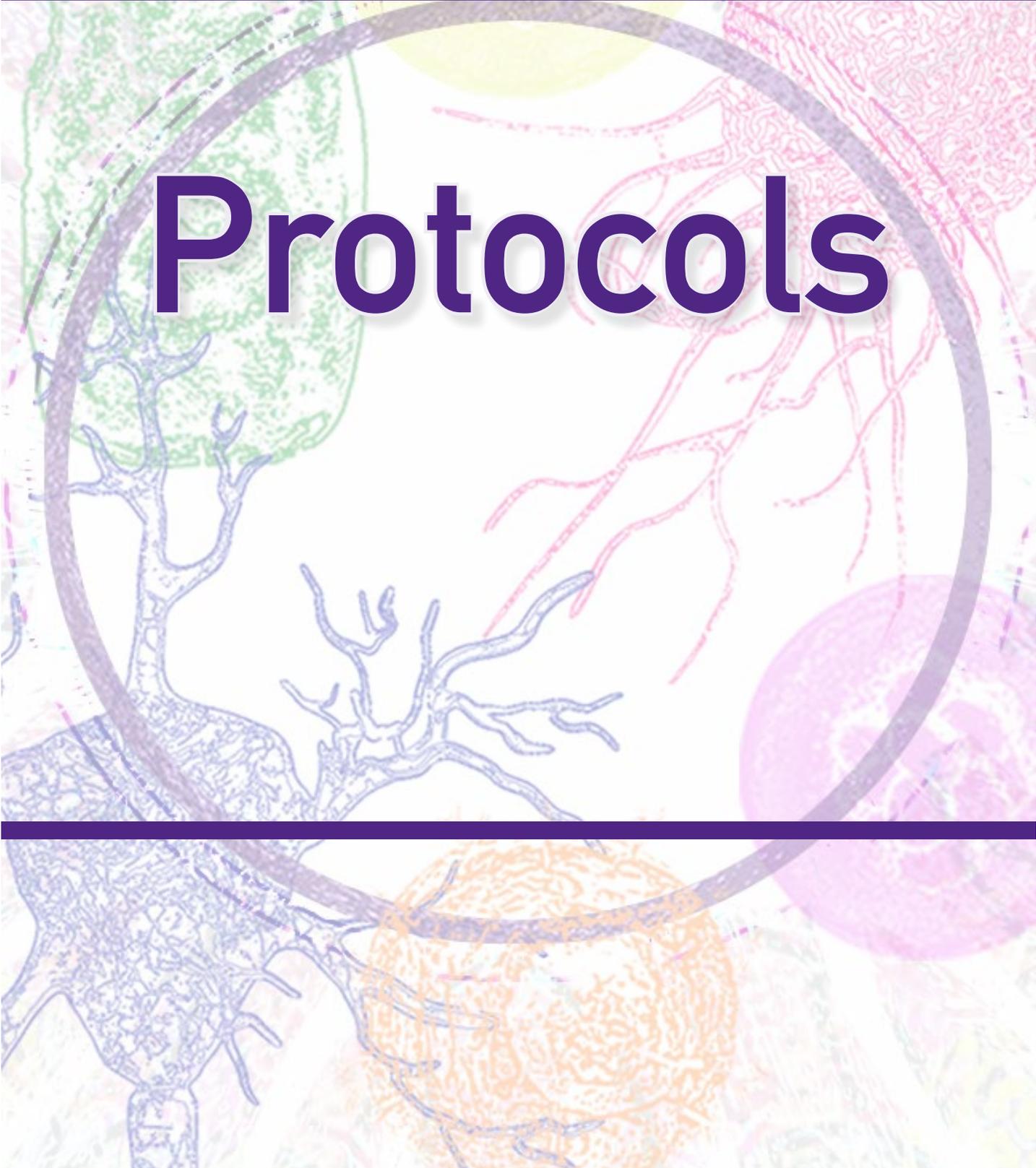
# Protocols

## Cell Surface Immunofluorescence Staining Protocol

**Reagent List**

Cell Staining Buffer (BioLegend Cat. No. 420201)

Red Cell Lysis Buffer (BioLegend Cat. No. 420301)

7-AAD Viability Staining Solution (BioLegend Cat. No. 420403)

TruStain FcX™ (anti-CD16/32, BioLegend Cat. No. 101319)

Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend Cat. No. 422301)

**Protocol Steps**

Harvest Tissue or Cells:

1. Obtain desired tissue (*e.g.,* spleen, lymph node, thymus, bone marrow) and prepare a single cell suspension in Cell Staining Buffer (BioLegend Cat. No. 420201). If using *in vitro* stimulated cells, simply resuspend previously activated cultures in Cell Staining Buffer and proceed to Step 2.

2. Add Cell Staining Buffer up to ~15 mL and centrifuge at 350 x *g* for 5 minutes, discard supernatant.

Lyse Red Cells:

3. If necessary (*e.g.,* spleen), dilute 10X Red Blood Cell (RBC) Lysis Buffer (BioLegend Cat. No. 420301) to 1X working concentration with DI water and resuspend pellet in 3 mL 1X RBC Lysis Buffer. Incubate on ice for 5 minutes.

4. Stop cell lysis by adding 10 mL Cell Staining Buffer to the tube. Centrifuge for 5 minutes at 350 *x g* and discard supernatant.

5. Repeat wash as in step 2.

6. Count viable cells and resuspend in Cell Staining Buffer at 5-10 x $10^6$ cells/mL and distribute 100 µL/tube of cell suspension (5-10 x $10^5$ cells/tube) into 12 x 75 mm plastic tubes.

Block Fc-Receptors:

7. Reagents that block Fc receptors may be useful for reducing nonspecific immunofluorescent staining. In the mouse, TruStain fcX™ (anti-mouse CD16/32) Antibody specific for FcγR III/II (BioLegend Cat. No. 101319, clone 93) can be used to block nonspecific staining of antibodies. In this case, block Fc receptors by pre-incubating cells with 1.0 µg of TruStain fcX™ (anti-mouse CD16/32) Antibody per $10^6$ cells in a 100 µL volume for 5-10 minutes on ice.

8. In humans, cells can be pre-incubated with 5 µL of Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend Cat. No. 422301) per 100 µL of cell suspension for 5-10 minutes at room temperature. In the absence of an effective/available blocking antibody, an alternative approach is to pre-block cells with excess irrelevant purified Ig from the same species and same isotype as the antibodies used for immunofluorescent staining.

*Note: Mouse TruStain fcX™ contains antibodies directed against CD16/32 (via the Fab portion of the antibody), while Human TruStain FcX™ contains specialized human IgG that bind to Fc receptors via the Fc portion of the antibodies. Human TruStain FcX™ is compatible with flow cytometric staining with anti-human CD16 (clone 3G8), CD32 (clone FUN-2), and CD64 (clone 10.1) antibodies.*

Cell-Surface Staining with Antibody:

9. Add appropriately conjugated fluorescent, biotinylated, or purified primary antibodies at predetermined optimum concentrations (*e.g.,* anti-CD3-FITC, anti-CD4-Biotin, and anti-CD8-APC) and incubate on ice for 15-20 minutes in the dark.

10. Wash 2X with at least 2 mL of Cell Staining Buffer by centrifugation at 350 x *g* for 5 minutes.

11. If using a purified primary antibody, resuspend pellet in residual buffer and add previously determined optimum concentrations of anti-species immunoglobulin fluorochrome conjugated secondary antibody (*e.g.,* FITC anti-mouse Ig) and incubate on ice in the dark for 15-20 minutes.

12. Repeat step 9.

13. Resuspend cell pellet in 0.5 mL of Cell Staining Buffer and add 5 µL (0.25 µg)/million cells of 7-AAD Viability Staining Solution (BioLegend Cat. No. 420403) to exclude dead cells.

*Note: BioLegend recommends using the Spectra Analyzer to decide compatibility with other fluors.*

14. Incubate on ice for 3-5 minutes in the dark.

15. Analyze with a Flow Cytometer.

*Note: If you are unable to immediately read your samples on a cytometer, keep them shielded from light and in a refrigerator set at 4-8°C. The samples should be resuspended in Cell Staining Buffer. Note that samples should not remain in a fixation buffer for extended periods of time as this can affect fluor conformation and fluorescence.*

# Cell Surface Immunofluorescent Staining of Whole Blood

**Reagent List**

Cell Staining Buffer (BioLegend Cat. No. 420201)

Red Cell Lysis Buffer (BioLegend Cat. No. 420301)

7-AAD Viability Staining Solution (BioLegend Cat. No. 420403)

TruStain FcX™ (anti-CD16/32, BioLegend Cat. No. 101319)

Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend Cat. No. 422301)

**Protocol Steps**

Harvest Tissue or Cells:

1. Add predetermined optimum concentrations of desired fluorochrome conjugated, biotinylated, or purified primary antibodies to 100 μL of anti-coagulated whole blood.

2. Incubate at room temperature for 15-20 minutes in the dark.

3. Dilute 10X Red Blood Cell (RBC) Lysis Buffer (BioLegend Cat. No. 420301) to 1X working concentration with DI water. Warm to room temperature prior to use. Add 2 mL of 1X RBC lysis solution to whole blood/antibody mixture. Incubate at room temperature for 10 minutes.

4. Centrifuge at 350 x $g$ for 5 minutes, discard the supernatant.

5. Wash 1X with at least 2 mL of Cell Staining Buffer by centrifugation at 350 x $g$ for 5 minutes.

6. If using a purified primary antibody, resuspend pellet in residual buffer and add a previously determined optimum concentration of anti-species immunoglobulin fluorochrome conjugated secondary antibody (*e.g.,* FITC anti-mouse Ig) and incubate in the dark for 15-20 minutes.

7. If using a biotinylated primary antibody, resuspend cell pellet in residual buffer and add a previously determined optimum concentration of fluorochrome conjugated Streptavidin (SAv) reagent (*e.g.,* SAv-PE, BioLegend Cat. No. 405204) and incubate for 15-20 minutes in the dark.

8. Repeat step 5.

9. Resuspend cells in 0.5 mL Cell Staining Buffer or 0.5 mL 2% paraformaldehyde-PBS fixation buffer.

*Tip:* *For gentler fixation (particularly with tandem fluors), FluoroFix™ Buffer (Cat. No. 422101) may be used.*

10. Analyze with a Flow Cytometer.

*Note:* *If you are unable to immediately read your samples on a cytometer, keep them shielded from light and in a refrigerator set at 4-8°C. The samples should be resuspended in Cell Staining Buffer. Note that samples should not remain in a fixation buffer for extended periods of time as this can affect fluor conformation and fluorescence.*

# Ki-67 Staining Protocol

**Protocol Steps**

1. Prepare 70% Ethanol and chill to -20°C

*Tip:* *Do not freeze ethanol for long-term storage.*

2. Prepare target cells of interest and wash 2X with PBS, centrifuging at 350 x $g$ for 5 minutes.

3. Discard supernatant and loosen the cell pellet by vortexing.

4. Add 3 mL cold 70% ethanol drop by drop to the cell pellet while vortexing.

5. Continue vortexing for 30 seconds and then incubate at -20°C for 1 hour.

6. Wash 3X with BioLegend's Cell Staining Buffer (Cat. No. 420201) and then resuspend the cells at the concentration of 0.5-10 x $10^6$/mL.

7. Mix 100 μL cell suspension with proper fluorochrome-conjugated Ki-67 antibody and incubate at room temperature in the dark for 30 minutes.

8. Wash 2X with BioLegend's Cell Staining Buffer and then resuspend in 0.5 mL cell staining buffer for flow cytometric analysis.

*Tip:* *Based on customer testing, Ki-67 staining is not recommended with our True-Nuclear™ Transcription Factor Buffer Set.*

# Intracellular Flow Cytometry Staining Protocol

**Application Notes**

1. Activated cell populations can be prepared from *in vivo*-stimulated tissues or from *in vitro*-stimulated cultures (*e.g.,* antigen-specific activation or mitogen-induced). For cytokine and chemokine detection, it is critical to include a protein transport inhibitor such as brefeldin A (BioLegend Cat. No. 420601) or monensin (BioLegend Cat. No. 420701) in the last 4-6 hours of cell culture activation. The cells can be suspended and distributed to 12 x 75 mm plastic tubes or microwell plates for immunofluorescent staining. For details on stimulation methods, please see our stimulation guide for cytokines/chemokines: (www.biolegend.com/media_assets/support_protocol/BioLegend_StimulationGuide_101711.pdf)

2. Different cytokines/chemokines have different production peaks. In order to obtain optimal staining signals, the stimulation conditions for each stimulant need to be optimized.

3. Some antibodies recognizing native cell surface markers may not bind to fixed/denatured antigens. For this reason, it is recommended that staining of cell surface antigens be done with live, unfixed cells PRIOR to fixation/permeabilization and staining of intracellular targets. Altering the procedure such that cells are fixed prior to staining of cell surface antigens requires that paraformaldehyde-denatured antigen reactive antibody clones be empirically identified. You can also take a look at our Fixation Webpage (biolegend.com/fixation) to get an idea of how epitopes stain after fixation with 4% PFA. Please note that the ability to stain post-fixation depends on the fluor, antigen expression, and several other factors.

**Reagents**

Cell Staining Buffer (BioLegend Cat. No. 420201)

Monensin (BioLegend Cat. No. 420701)

RBC Lysis Buffer (BioLegend Cat. No. 420301)

Brefeldin A (BioLegend Cat. No. 420601)

Fixation Buffer (BioLegend Cat. No. 420801)

Intracellular Staining Perm Wash Buffer (BioLegend Cat. No. 421002)

Cyto-Last™ Buffer (BioLegend Cat. No. 422501)

**Procedure**

Fixation:

1. If staining intracellular antigens (*e.g.,* IFN-γ or IL-4), first perform cell surface antigen staining as described in BioLegend's Cell Surface Immunofluorescence Staining Protocol, then fix cells in 0.5 mL/tube Fixation Buffer in the dark for 20 minutes at room temperature.

*Tip:* *For gentler fixation (particularly with tandem fluors), FluoroFix™ Buffer (Cat. No. 422101) can be used*

2. Centrifuge at 350 *x g* for 5 minutes, discard supernatant.

3. To put the experiment "on hold" at this point for future staining and analysis, wash cells 1x with Cell Staining Buffer. Resuspend cells in Cell Staining Buffer and store cells at 4°C (short term) or in 90% FCS/10% DMSO forstorage at -80°C (long term, for fixed cells without surface antigen staining). Alternatively, cells can be kept in Cyto-Last™ Buffer for the storage of cytokineproducing cells for up to two weeks. The frequencies of cytokine-producing cells present in activated human PBMC cultures can vary widely due to donor variability. Therefore, cryopreserved cells from a single donor are useful for longitudinal studies.

Permeabilization:

4. Dilute 10X Intracellular Staining Perm Wash Buffer to 1X in DI water.

5. Resuspend fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 350 *x g* for 5-10 minutes.

6. Repeat step 5 twice.

Intracellular Staining:

7. Resuspend fixed/permeabilized cells in residual Intracellular Staining Perm Wash Buffer and add a predetermined optimum concentration of fluorophore-conjugated antibody of interest (*e.g.,* PE anti-IFN-γ) or an appropriate negative control for 20 minutes in the dark at room temperature.

8. Wash 2x with 2 mL of Intracellular Staining Perm Wash Buffer and centrifuge at 350 *x g* for 5 minutes.

9. If primary intracellular antibody is biotinylated, it will be necessary to perform fluorophore conjugated Streptavidin incubations and subsequent washes in Intracellular Staining Perm Wash Buffer.

10. Resuspend fixed and intracellularly labeled cells in 0.5 mL Cell Staining Buffer and analyze with appropriate controls.

*Tip:* *To confirm specific anti-cytokine staining, a blocking experiment is recommended in which cells are fixed/permeabilized then preincubated with an excess amount of unlabeled anti-cytokine antibody and/or the recombinant cytokine of interest is preincubated with fluorophore-conjugated anti-cytokine antibody before its addition to the cells.*

Activation and Intracellular Staining of Whole Blood

11. Dilute heparinized whole blood 1:1 with sterile appropriate tissue culture medium.

*Tip:* *For details on stimulation methods, please see our stimulation guide for cytokines/chemokines: (www.biolegend.com/media_assets/support_protocol/BioLegend_StimulationGuide_101711.pdf)*

12. At this stage, *in vitro* cellular stimulation by either antigen or mitogen can be performed. If intending to stain intracellular cytokines or chemokines (*e.g.,* IFN-γ or IL-4), addition of an efficient protein transport inhibitor such

as brefeldin A or monensin is critical. After addition of a suitable cellular activator, aliquot 200 µL of the whole blood cell suspension into 12 x 75 mm plastic tubes and incubate for 4-6 hours in 5% $CO_2$ at 37°C.

13. Add 2 mL of 1X Red Blood Cell Lysis Buffer and incubate for 5-10 minutes at room temperature

14. Centrifuge at 350 $x\,g$ for 5 minutes and discard the supernatant

15. Wash cells 1X with Cell Staining Buffer and perform cell surface immunofluorescent staining as described above.

16. Fix, permeabilize, and stain intracellular antigens as described above.

Flow Cytometric Analysis:

Set PMT voltage and compensation using cell surface staining controls. Set quadrant markers based on blocking controls, isotype controls, or unstained cells. For proper flow cytometric analysis, cells stained by this method should be inspected by light microscopy and/or flow light scatter pattern to confirm that they are well dispersed. Bivariate dot plots or probability contour plots can be generated upon data analysis to display the frequencies of and patterns by which individual cells coexpress certain levels of cell surface antigen and intracellular cytokine proteins.

Related Information:

Assenmacher, M., *et al.* 1994. *Eur. J. Immunol.* 24:1097.
Elson, L.H., *et al.* 1995. *J. Immunol.* 1995.154:4294.
Jung T, *et al.* 1993. *J. Immunol. Methods* 159:197.
Prussin C., *et al.* 1995. *J. Immunol. Methods* 188:117.
Vikingsson A., *et al.* 1994. *J. Immunol. Methods* 173:219.

## Anti-BrdU Staining Using DNAse with Surface and Fluorescent Proteins

*Note: We offer two protocols here, depending on what your experiment requires. Ethanol treatment is usually harsher toward any other fluors or fluorescent proteins that may be present in your sample. As such, the DNAse method may be gentler under those conditions.*

**Protocol Steps**

Anti-BrdU Staining Using DNAse with Surface and Fluorescent Proteins:

1. Pulse actively dividing cells with BrdU (*in vitro*, cell culture media can be pulsed by adding 10-40 µM of BrdU for 1-2 hours).

2. Harvest cells and centrifuge for 5 minutes at 1200-1500 rpm (200-300 $x\,g$).

3. Wash cells in Cell Staining Buffer (Cat. No. 420201) and centrifuge for 5 minutes at 1200-1500 rpm (200-300 $x\,g$). Discard supernatant.

4. Aliquot $5 \times 10^5$ -$1 \times 10^6$ cells per 12 x 75mm tube

5. Optional: Stain cells for surface antigens if required, utilizing the Cell Surface Immunofluorescence Staining Protocol.

6. Wash cells by adding 1 mL of Cell Staining Buffer to each tube and centrifuging for 5 minutes at 1200-1500 rpm (200-300 $x\,g$). Discard supernatant.

7. Fix cells by adding 100 µL of 4% paraformaldehyde at room temperature for 20-30 minutes.

8. Wash cells by repeating step 6 twice.

*Optional: Cells can be stored in FACS buffer at 4°C for up to 72 hrs)*

9. Permeabilize cells by adding 500 µL of 0.5% Triton-X 100 in PBS for 15 minutes at room temperature.

10. Wash cells by repeating step 6 twice.

11. Treat cells with 20 µg of DNAse (Cat. No. D4513, Sigma-Aldrich) diluted in DPBS with calcium and magnesium to each tube and incubate at 37°C for 1 hour.

12. Wash cells by repeating step 6 twice.

13. Add 50 µL of Cell Staining Buffer to each tube then add the recommended concentration of antiBrdU antibody to each tube. Incubate for 20 minutes at room temperature in the dark.

14. Repeat step 6.

15. Stain DNA by adding 1 µg of either 7-AAD (Cat. No. 420403) or DAPI (Cat. No. 422801). Wait for 5 minutes prior to acquiring samples on flow cytometer.

*Note: Adding a 7-AAD or DAPI stain allows you to analyze total DNA content and provides the characteristic horseshoe flow cytometric staining pattern when compared against BrdU. This helps identify the different phases of the cell cycle.*

## Intracellular Staining with True-Phos™ Perm Buffer in Cell Suspensions Protocol

**Protocol Steps**

Buffer Preparation:

1. Warm Fixation Buffer (BioLegend Cat 420801). For each $1 \times 10^6$ cells, aliquot 0.5 mL of buffer and warm to 37°C.

2. Chill True-Phos™ Perm Buffer to -20°C. For each $1 \times 10^6$ cells, aliquot 1.0 mL of True-Phos™ Perm Buffer and chill to -20°C.

Sample Preparation:

3. Prepare a single cell suspension with the sample of interest (Human PBMC, splenocytes, cell lines, etc).

*Tips:   Prepare two aliquots, Negative control: untreated, Positive control: treated with stimuli.*

*Incubate the cells with the appropriate stimuli, at the suitable temperature and time.*

4. Fix the cells immediately after treatment by adding an equal volume of pre-warmed Fixation Buffer. Gently pipette to ensure thorough mixing.

5. Incubate at 37°C for 15 minutes to ensure cells are properly fixed.

6. Centrifuge cells at 350 $x g$ at room temperature for 5 minutes, decant supernatant, vortex to resuspend cell pellet.

Staining with Specific Antibodies:

7. Add sufficient Cell Staining Buffer to wash the cells (approximately 2 mL for each $1 \times 10^6$ cells, BioLegend Cell Staining Buffer recommended, Cat. No. 420201), centrifuge at 350 $x g$ at room temperature for 5 minutes and decant supernatant. Repeat, for a total of two washes.

8. Gently pipette cells using residual volume to resuspend cell pellet.

*Note:  If cells are not fully resuspended, True-Phos™ Perm Buffer addition will cause significant cell loss.*

9. While vortexing, permeabilize cells by adding pre-chilled True-Phos™ Perm Buffer. Example: $10 \times 10^6$ cells should be permeabilized with 10 mL of pre-chilled True-Phos™ Perm Buffer.

10. Incubate at -20°C for 60 minutes to ensure cells are properly permeabilized.

11. Centrifuge cells at 1000 $x g$ at room temperature for 5 minutes, decant supernatant, vortex to resuspend cell pellet.

12. Add sufficient Cell Staining Buffer to wash the cells, centrifuge cells at 1000 $x g$ at room temperature for 5 minutes, decant supernatant. Repeat, for a total of two washes.

13. Resuspend the cells in Cell Staining Buffer at a concentration of $10 \times 10^6$ cells/mL.

14. Transfer 100 μL (or $1 \times 10^6$ cells) to a 12 x 75mm tube.

15. Add antibody cocktail(s) to appropriate tubes, vortex to mix, and incubate for 30 minutes at room temperature in the dark.

16. Add 2 mL of Cell Staining Buffer, centrifuge cells at 1000 $x g$ at room temperature for 5 minutes, decant supernatant. Repeat, for a total of two washes.

17. Resuspend cells in approximately 500 μL of Cell Staining Buffer and analyze with a flow cytometer.

# Th17 Polarization of Mouse CD4$^+$ Cells

**Reagent List**

Sterile PBS

Cell culture medium (IMDM supplemented with 10% FBS)

Sterile plastic petri dishes

RBC Lysis Buffer (Cat. No. 420301)

Anti-mouse CD3ε, clone 145-2C11 (LEAF™ format, Cat. No. 100314)

Anti-mouse CD28, clone 37.51, (LEAF™ format, Cat. No. 102112)

Anti-mouse IFN-γ, clone XMG1.2, (LEAF™ format, Cat. No. 505812)

Mouse MojoSort™ CD4 T-cell Isolation Kit (Cat. No. 480005)

Anti-mouse IL-4, clone 11B11, (LEAF™ format, Cat. No. 504108)

Recombinant mouse IL-6 (carrier-free) (Cat. No. 575704)

Recombinant mouse IL-23 (carrier-free) (Cat. No. 589002)

Recombinant human TGF-β1 (carrier-free) (Cat. No. 580702)

Brefeldin A (Cat. No. 420601)

Monensin Solution (Cat. No. 420701)

PMA (Phorbol 12-myristate 13-acetate) (*e.g.,* Cat. No. P8139 from Sigma)

Ionomycin (*e.g.,* Cat. No. I0634 from Sigma)

**Protocol Steps**

Isolation of CD4$^+$ Cells From Lymph Nodes:

1. Harvest lymph nodes (superficial cervical, mandibular, axillary, inguinal, and mesenteric) from mice.

2. Tease lymph nodes through a sterile 70 µm nylon cell strainer to obtain single-cell suspensions in complete IMDM containing 10% FCS (complete medium).

3. Resuspend cells in complete medium and use your favorite method to isolate CD4$^+$ cells. Consider using our MojoSort™ Mouse CD4 T Cell Isolation Kit.

Th17 Polarization of CD4$^+$ Cells:

4. On day 0, coat 60 x 15mm of plastic petri dishes with anti-mouse CD3ε, clone 145-2C11 (5 µg/mL). Incubate at 37°C for 2 hours or 4°C overnight. Aseptically decant antibody solution from the plate. Wash plate 3 times with sterile PBS. Discard liquid.

5. Plate CD4$^+$ cells at 10 x 10$^6$ at 5 mL/dish. Culture cells for 4 days in the presence of anti-mouse CD28, clone 37. 51 (5 µg/mL), recombinant mouse IL-6 (50 ng/mL), recombinant human TGF-β1 (1ng/mL), recombinant mouse IL-23 (5 ng/mL), anti-mouse IL-4 (10 µg/mL), and anti-mouse IFN-γ (10 µg/mL).

6. On day 3, slowly add 5 mL of fresh media, along with the same concentration of antibodies/cytokines as used on day 0.

7. On day 4, wash cells once and then restimulate in complete medium with 500 ng/mL PMA and 500 ng/mL ionomycin, in the presence of Brefeldin A (If you are looking for IL-21 production, use monensin) for 4-5 hours.

8. After harvesting, the cells are ready for staining.

*Tip: Recombinant human TGF-β is effective for stimulating mouse cells.*

# Treg Polarization of Mouse CD4$^+$ Cells

**Reagent List**

Sterile PBS

Cell culture medium (RPMI 1640 supplemented with 10% FBS)

Sterile 12-well plate

RBC Lysis Buffer (Cat. No. 420301)

Anti-mouse CD3ε, clone 145-2C11 (LEAF™ format, Cat. No. 100314)

Anti-mouse CD28, clone 37.51, (LEAF™ format, Cat. No. 102112)

Recombinant human TGF-β1 (carrier-free) (Cat. No. 580702)

Recombinant mouse IL-2 (carrier-free) (Cat. No. 575402)

Mouse MojoSort™ CD4 T-cell Isolation Kit (Cat. No. 480005)

**Protocol Steps**

Isolation of CD4$^+$ Cells From Lymph Nodes:

1. Harvest lymph nodes (superficial cervical, mandibular, axillary, inguinal, and mesenteric) from mice.

2. Tease lymph nodes through a sterile 70 µm nylon cell strainer to obtain single-cell suspensions in complete RPMI containing 10% FCS (complete medium).

3. Resuspend cells in complete medium and use your favorite method to isolate CD4$^+$ cells. Consider using our MojoSort™ Mouse CD4 T Cell Isolation Kit.

Treg Polarization of CD4$^+$ Cells:

4. On day 0, coat 12-well plate with anti-mouse CD3ε, clone 145-2C11 (3 µg/mL). Incubate at 37°C for 2 hours or 4°C overnight. Aseptically decant antibody solution from the plate. Wash plate 3 times with sterile PBS. Discard liquid.

5. Plate CD4$^+$ cells at 1.0 x 10$^6$ at 1 mL/well. Culture cells for 5 days at 37°C, 5% CO2, in the presence of anti-mouse CD28, clone 37.51 (3 µg/mL), recombinant mouse IL-2 (5ng/mL), and recombinant human TGF-β1 (5ng/mL).

6. On day 3, if media is yellow, add 2 mL/well of fresh media.

7. On day 5, after harvesting, the cells are ready for staining.

*Note: Recombinant human TGF-β is effective for stimulating mouse cells.*

# Th1 Polarization of Mouse CD4+ Cells Protocol

**Reagent List**

Sterile PBS

Cell culture medium (RPMI 1640 supplemented with 10% FBS)

Sterile 12-well plate

Sterile 6-well plate

RBC Lysis Buffer (Cat. No. 420301)

Anti-mouse CD3ε, clone 145-2C11 (LEAF™ format, Cat. No. 100314)

Ionomycin (*e.g.,* Cat. No. I0634 from Sigma)

Mouse MojoSort™ CD4 T-cell Isolation Kit (Cat. No. 480005)

Anti-mouse CD28, clone 37.51, (LEAF™ format, Cat. No. 102112)

Anti-mouse IL-4, clone 11B11, (LEAF™ format, Cat. No. 504108)

Recombinant mouse IL-2 (carrier-free) (Cat. No. 575402)

Recombinant mouse IL-12 (p70) (carrier-free) (Cat. No. 577002)

Monensin Solution (Cat. No. 420701)

PMA (Phorbol 12-myristate 13-acetate) (*e.g.,* Cat. No. P8139 from Sigma)

**Protocol Steps**

Isolation of CD4+ Cells From Lymph Nodes:

1. Harvest lymph nodes (superficial cervical, mandibular, axillary, inguinal, and mesenteric) from mice.

2. Tease lymph nodes through a sterile 70 µm nylon cell strainer to obtain single-cell suspensions in complete RPMI containing 10% FCS (complete medium).

3. Resuspend cells in complete medium and use your favorite method to isolate CD4+ cells. Consider using our Mojosort™ Mouse CD4 T Cell Isolation Kit.

Th1 Polarization of CD4+ Cells:

4. On day 0, coat 12-well plate with anti-mouse CD3ε, clone 145-2C11 (3 µg/mL). Incubate at 37°C for 2 hours or 4°C overnight. Aseptically decant antibody solution from the plate. Wash plate 3 times with sterile PBS. Discard liquid.

5. Plate CD4+ cells at $1.0 \times 10^6$ at 1 mL/well. Culture cells for 5 days at 37°C, 5% $CO_2$, in the presence of anti-mouse CD28, clone 37.51 (3 µg/mL), anti-mouse IL-4, clone 11B11 (10 µg/mL), recombinant mouse IL-2 (5 ng/mL), and recombinant mouse IL-12 (10 ng/mL).

6. On day 3, if media is yellow, add 2 mL/well of fresh media.

7. On day 5, wash cells once and then restimulate in complete media with 50 ng/mL PMA, 1 µg/mL ionomycin and 10 µL monensin (1000x), in a 6-well plate in incubator at 37°C for 5 hours.

8. After harvesting, the cells are ready for staining.

# T Cell Activation with Anti-CD3 Antibodies Protocol - Human

**Reagent List**

Sterile PBS

Anti-human CD3 Antibody: Clone UCHT1 (LEAF™ format, Cat. No. 300413; Ultra-LEAF™ format, Cat. No. 300437)

Anti-human CD3 Antibody: Clone OKT3 (LEAF™ format, Cat. No. 317303; Ultra-LEAF™ format, Cat. No. 317325)

*Note:  Soluble forms of LEAF™ purified UCHT1 (1 µg/mL) or LEAF™ purified HIT3a (0.01 - 0.1 µg/mL) may be used to activate T cells from PBMC cell populations.*

Anti-human CD3 Antibody: Clone HIT3a (LEAF™ format, Cat. No. 300313; Ultra-LEAF™ format, Cat. No. 300331)

Cell culture medium (*e.g.,* RPMI-1640 or IMDM supplemented with 10% FBS and 2 mM L-glutamine)

Sterile single-cell suspension of Ficoll-Hypaque-purified peripheral blood mononuclear cells, isolated T cells, or T cell subsets

96-well flat-bottom tissue culture plates with lids (*e.g.,* Costar® Cat. No. 3596)

**Protocol Steps**

Method:

1. Prepare a 10 µg/mL solution of anti-CD3 (clone UCHT1, OKT3, or HIT3a) in sterile PBS.

2. Dispense 50 µL of the antibody solution to each microwell of the 96-well assay plate. For the unstimulated control wells, add 50 µL of sterile PBS.

3. Seal plate. Incubate at 37°C for 2 hours or 4°C overnight.

4. Aseptically decant antibody solution from the microwell plate.

5. Wash plate microwells 3 times with sterile PBS. Discard liquid.

6. Prepare single cell suspension of cells of interest in supplemented cell culture medium to $1\text{-}2 \times 10^6$/mL.

7. Aliquot 200 µL cell suspension into plate microwells. Cover with lid. Incubate at 37°C in 5% $CO_2$ and 100% humidity for 3 days.

# Immunohistochemistry Protocol for Frozen Sections

## Introduction

The following is a general procedure guide for preparation and staining of acetone-fixed frozen tissues using a purified, unconjugated primary antibody, biotinylated secondary antibody and streptavidin-horseradish peroxidase (Sav-HRP) and DAB detection system. Because each antigen differs in terms of requirement for fixation, amplification step, etc., it is not possible to write an inclusive protocol that will work for all antigens. The user must determine optimal conditions for each antigen of interest. Many protocols for staining individual antigens, as well as useful tips and troubleshooting guides for immunohistochemistry, can be found at the IHC World web site (ihcworld.com).

## Protocol Steps

Prepare frozen tissue sections (steps 1-8):

1. Place a freshly dissected tissue block (< 5 mm thick) on to a pre-labeled tissue base mold.

2. Cover the entire tissue block with cryo-embedding media (*e.g.,* OCT).

3. Slowly place the base mold containing the tissue block into liquid nitrogen till the entire tissue block is submerged into liquid nitrogen to ensure tissue is frozen completely.

4. Store the frozen tissue block at -80°C until ready for sectioning.

5. Transfer the frozen tissue block to a cryotome cryostat (*e.g.,* -20°C) prior to sectioning and allow the temperature of the frozen tissue block to equilibrate to the temperature of the cryotome cryostat.

6. Section the frozen tissue block into a desired thickness (typically 5-10 μm) using the cryotome.

7. Place the tissue sections onto glass slides suitable for immunohistochemistry (*e.g.,* Superfrost).

8. Dry the tissue sections overnight at room temperature. Sections can be stored in a sealed slide box at -80°C for later use.

Immunostain frozen tissue sections (steps 9-28):

9. Fix the tissue sections with a suitable fixative. One of the commonly used fixation methods for frozen tissue sections is to immerse the slides in pre-cooled acetone (-20°C) for 10 min.

10. Pour off the fixative and allow acetone to evaporate from the tissue sections for < 20 min at room temperature.

11. Rinse the slides in 300 mL of 10mM phosphate buffered saline (PBS) at a neutral pH for 2 changes, 5 min each.

12. Incubate the slides in 0.3% $H_2O_2$ solution in PBS at room temperature for 10 min to block endogenous peroxidase activity.

13. Rinse the slides in 300 mL PBS for 2 changes, 5 min each.

14. Optional: Add 100 μL blocking buffer (*e.g.,* 10% fetal bovine serum in PBS) onto the sections of the slides and incubate in a humidified chamber at room temperature for 1 hour.

15. Drain off the blocking buffer from the slides.

16. Apply 100 μL an appropriately diluted primary antibody (in antibody dilution buffer, *e.g.,* 0.5% bovine serum albumin in PBS) to the sections on the slides and incubate in a humidified chamber for 1 hour at room temperature or overnight at 4°C.

17. Rinse the slides in 300 mL PBS for 2 changes, 5 min each.

18. Apply 100 μL an appropriately diluted biotinylated secondary antibody (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min.

19. Rinse the slides in 300 mL PBS for 2 changes, 5 min each.

20. Add 100 μL pre-diluted Sav-HRP conjugates (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min (keep protected from light).

21. Rinse the slides in 300 mL PBS for 2 changes, 5 min each.

22. Apply 100 μL DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% $H_2O_2$ in PBS) to the sections on the slides to reveal the color of the antibody staining. Allow the color development for < 5 min until the desired color intensity is reached.

*Caution: DAB is a suspected carcinogen. Handle with care. Wear gloves, lab coat and eye protection.*

23. Wash slides in 300 mL PBS for 2 changes 5 min each.

24. Optional: Counterstain slides by immersing sides in Hematoxylin (*e.g.,* Gill's Hematoxylin) for 1-2 min.

25. Rinse the slides in running tap water for > 15 min.

26. Dehydrate the tissue slides through 4 changes of alcohol (95%, 95%, 100% and 100%), 5 min each.

27. Clear the tissue slides in 3 changes of xylene and coverslip using mounting solution (*e.g.,* Permount). The mounted slides can be stored at room temperature permanently.

28. Observe the color of the antibody staining in the tissue sections under microscopy.

# Immunofluorescence Microscopy Protocol

**Reagent List**

Chamber slides or cover slips

Fixation solution: 4% Paraformaldehyde, in PBS

Permeablization solution: 0.5% Triton X-100 in PBS

Antibody dilution solution: 5% FBS in PBS

**Protocol Steps**

Sample Preparation:

1.  Grow cultured cells on cover slips or chamber slides overnight, or add appropriate amount of cells to poly-L-lysine coated chamber slides and incubate at least 30 minutes at 37°C, at the time of fixation cells should be ~50% confluent.

2.  Rinse cells briefly in PBS.

3.  Fix cells by incubation with 4% Paraformaldehyde, in PBS for 15 minutes at room temperature.

4.  Rinse three times in PBS, 5 minutes each time.

5.  Add 0.5% Triton X-100 in PBS and incubate at room temperature for three to five minutes.

6.  Rinse three times in PBS, 5 minutes each time.

Sample Blocking:

7.  Block samples in 5% FBS in PBS at room temperature for one hour.

Sample Staining:

8.  Dilute the primary antibody to the recommended concentration/dilution in 5% FBS/PBS.

9.  Add 200 µL per well (8 wells) to the chamber slides and incubate two to three hours at room temperature or overnight at 4°C.

10. Rinse three times in PBS, 5 minutes each time.

*Note: If using primary antibodies directly conjugated with fluorochrome, then skip to step 14.*

11. Prepare fluorochrome-conjugated secondary antibody in 5% FBS/PBS according to the recommended manufacturer specification data sheet, and add 200 µL per well (8 wells) to the chamber slides.

12. Incubate the samples for one hour, at room temperature, in the dark.

13. Rinse three times in PBS, 5 minutes each time.

14. Coverslip with anti-fade mounting medium.

15. Seal slides with nail polish.

# Immunohistochemistry Protocol for Paraffin-Embedded Sections

**Protocol Steps**

Prepare formalin-fixed, paraffin-embedded tissue sections (steps 1-8):

1.  Fix freshly dissected tissue (less than 3 mm thick) with 10% formalin or other fixatives for 24-48 hour at room temperature. Caution: Formalin is a suspect carcinogen. It can cause eye, skin, and respiratory tract irritation. It should be handled in a hood.

2.  Rinse the tissue with running tap water for 1 hour.

3.  Dehydrate the tissue through 70%, 80%, 95% alcohol, 45 min each, followed by 3 changes of 100% alcohol, 1 hour each.

4.  Clear the tissue through 2 changes of xylene, 1 hour each.

5.  Immerse the tissue in 3 changes of paraffin, 1 hour each.

6.  Embed the tissue in a paraffin block. The paraffin tissue block can be stored at room temperature for years.

7.  Section the paraffin-embedded tissue block at 5-8 µm thickness on a microtome and float in a 40°C water bath containing distilled water.

8.  Transfer the sections onto glass slides suitable for immunohistochemistry (*e.g.,* Superfrost Plus). Allow the slides to dry overnight and store slides at room temperature until ready for use.

Immunostain formalin-fixed, paraffin-embedded tissue sections (steps 9-29):

9.  Deparaffinize slides in 2 changes of xylene, 5 min each.

10. Transfer slides to 100% alcohol, for 2 changes, 3 min each, and then transfer once through 95%, 70% and 50% alcohols respectively for 3 min each.

11. Block endogenous peroxidase activity by incubating sections in 3% $H_2O_2$ solution in methanol at room temperature for 10 min to block endogenous peroxidase activity.

12. Rinse in 300 mL of PBS for 2 changes, 5 min each.

13. Optional: Perform antigen retrieval to unmask the antigenic epitope. The most commonly used antigen retrieval is a citrate buffer method. Arrange the slides in a staining container. Pour 300 mL of 10mM citrate buffer, pH 6.0 into the staining container and incubate it at 95-100°C for 10 min (optimal incubation time should be determined by user). Remove the staining container to room temperature and allow the slides to cool for 20 min.

14. Rinse slides in 300 mL PBS for 2 changes, 5 min each.

15. Optional: Add 100 µL blocking buffer (*e.g.,* 10% fetal bovine serum in PBS) onto the sections of the slides and incubate in a humidified chamber at room temperature for 1 hour.

16. Drain off the blocking buffer from the slides.

17. Apply 100 µL appropriately diluted primary antibody (in antibody dilution buffer, *e.g.,* 0.5% bovine serum albumin in PBS) to the sections on the slides and incubate in a humidified chamber at room temperature for 1 hour.

18. Wash the slides in 300 mL PBS for 2 changes 5 min each.

19. Apply 100 µL appropriately diluted biotinylated secondary antibody (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min.

20. Wash slides in 300 mL PBS for 2 changes, 5 min each.

21. Apply 100 µL appropriately diluted Sav-HRP conjugates (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min (keep protected from light).

22. Wash slides in 300 mL PBS for 2 changes, 5 min each.

23. Apply 100 µL DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% $H_2O_2$ in PBS) to the sections on the slides to reveal the color of antibody staining. Allow the color development for less than 5 min until the desired color intensity is reached. *Caution: DAB is a suspect carcinogen. Handle with care. Wear gloves, lab coat and eye protection.*

24. Wash slides in 300 mL PBS for 3 changes 2 min each.

25. Optional: Counterstain slides by immersing sides in Hematoxylin (*e.g.,* Gill's Hematoxylin) for 1-2 min.

26. Rinse the slides in running tap water for more than 15 min.

27. Dehydrate the tissue slides through 4 changes of alcohol (95%, 95%, 100% and 100%), 5 min each.

28. Clear the tissue slides in 3 changes of xylene and coverslip using mounting solution (*e.g.,* Permount). The mounted slides can be stored at room temperature permanently.

29. Observe the color of the antibody staining in the tissue sections under microscopy.

## General Tips & FAQ

**FAQ:**

**Do I need to perform antigen retrieval on my formalin-fixed, paraffin-embedded samples prior to staining?**

In most cases, this is true. Antigen retrieval helps both the accessibility of the antibody to the tissue and also counteracts the fixation effects on the recognized epitopes. Not all antibodies indicated for use in IHC-P were validated at BioLegend, therefore be sure to check the application reference for the method of antigen retrieval utilized by the lab that validated that application.

**Can antibody X be used for immunohistochemistry? What concentration do I use?**

Typical concentrations of monoclonal antibodies for use in IHC are from 5-25 µg/mL. Polyclonal antibodies can be used at a range of 1-10 µg/mL. Some products are quality tested in-house for IHC applications, while others will indicate on the datasheet if an antibody has been published for use in this application. In addition, you can do a lit search with the clone name and immunohistochemistry/paraffin/frozen to see what the protocol details are.

*Tips:  For initial experiments, the user must titrate primary and secondary reagents so that staining with the secondary antibody alone yields no background while staining with primary and secondary antibodies yields strong, specific staining.*

*Take care to ensure that slides do not dry out by incubating with sufficient volumes and/or in a humidified chamber (such as 926301).*

125

# MojoSort™ Nanobeads Regular Protocol

**Reagent List**

MojoSort™ Buffer (5X) (Cat. No. 480017)

MojoSort™ Magnet (Cat. No. 480019) or compatible magnetic separation system

Adjustable pipettes

70 µm filters (one per sample)

5 mL (12 x 75 mm) polypropylene tubes

Reagents for sample preparation

Reagents and instruments (Flow cytometer) to determine yield and purity

Important Note:

*MojoSort™ magnetic particles can be used with other commercially available magnetic separators, both free standing magnets and column-based systems. Because MojoSort™ protocols are optimized for the MojoSort™ separator; the protocols may need to be adjusted for other systems. Please contact BioLegend Technical Service (tech@ biolegend.com) for more information and guidance. We do not recommend using MojoSort™ particles for BD's IMag™ or Life Technologies' DynaMag™.*

Product Description and Procedure Summary:

The cells targeted by the Nanobeads are either selected or depleted by incubating your sample with the directly conjugated magnetic particles. The magnetically labeled fraction is retained by the use of a magnetic separator. After collection of the targeted cells, downstream applications include functional assays, gene expression, phenotypic characterization, etc.

**Protocol Steps:**

This procedure is optimized for the isolation of $10^7$ to 2 x $10^8$ cells per tube. If working with fewer than $10^7$ cells, keep volumes as indicated for $10^7$ cells. For best results, optimize the conditions to your specific cell number and tissue. Prepare fresh MojoSort™ Buffer solution by diluting the 5X concentrate with sterile distilled water. Scale up volumes if using 14 mL tubes and Magnet, and place the tube in the magnet for 10 minutes.

1.  Prepare cells from your tissue of interest without lysing erythrocytes.

2.  In the final wash of your sample preparation, resuspend the cells in MojoSort™ Buffer by adding up to 4 mL in a 5 mL (12 x 75 mm) polypropylene tube.

*Note:  Keep MojoSort™ Buffer on ice throughout the procedure.*

3.  Filter the cells with a 70 µm cell strainer, centrifuge at 300 x *g* for 5 minutes, and resuspend in an appropriate volume of MojoSort™ Buffer. Count and adjust the cell concentration to 1 x $10^8$ cells/mL.

4.  Aliquot 100 µL of cell suspension ($10^7$ cells) into a new tube.

5.  Resuspend the beads by vortexing, maximum speed, 5 touches. Add 10 µL of Nanobeads, mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 µL for 1 x $10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

6.  Add MojoSort™ Buffer up to 4 mL and centrifuge the cells at 300 x *g* for 5 minutes.

7.  Resuspend the cells in 3 mL of MojoSort™ Buffer. Optional: Take an aliquot before placing the tube in the magnet to monitor purity and yield.

8.  Place the tube in the magnet for 5 minutes.

9.  Pour out the liquid. Resuspend the labeled cells in appropriate amount of buffer.

10. Repeat steps 7-9 on the labeled fraction 2 more times, for a total of 3 magnetic separations.

*Optional: Take a small aliquot to monitor purity and yield. If desired, pool the unlabeled fractions and process simultaneously with the positive labeled cells when assessing purity and yield.*

**Chart Protocol**
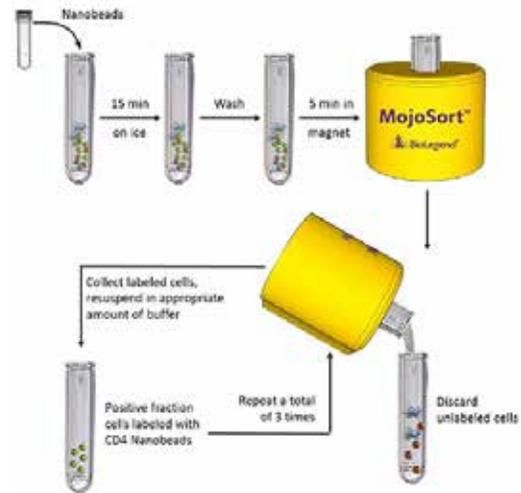


Application Notes: To use this product in magnetic separation columns, a titration of the Nanobeads should be performed. Optimal concentration for magnetic separation columns is lot-specific. Please contact BioLegend Technical Service (tech@ biolegend.com) for further assistance on how to use MojoSort™ Nanobeads in magnetic separation columns.

# MojoSort™ Nanobeads No-wash Protocol

**Reagent List**

MojoSort™ Buffer (5X) (Cat. No. 480017)

MojoSort™ Magnet (Cat. No. 480019 | 480020) or compatible magnetic separation system

Adjustable pipettes

70 µm filters (one per sample)

5 mL (12 x 75 mm) or 14 mL (17 x 100 mm) polypropylene tubes

Reagents for sample preparation

Reagents and instruments (Flow cytometer) to determine yield and purity

Important Note:

*MojoSort™ magnetic particles can be used with other commercially available magnetic separators, both free standing magnets and column-based systems. Because MojoSort™ protocols are optimized for the MojoSort™ separator; the protocols may need to be adjusted for other systems. Please contact BioLegend Technical Service (tech@biolegend.com) for more information and guidance. We do not recommend using MojoSort™ particles for BD's IMag™ or Life Technologies' DynaMag™.*

Product Description and Procedure Summary:

The cells targeted by the Nanobeads are either selected or depleted by incubating the sample with the directly conjugated magnetic particles. The magnetically labeled fraction is retained by the use of a magnetic separator. After collection of the targeted cells, downstream applications include functional assays, gene expression, phenotypic characterization, etc.

**Protocol Steps**

*Note:  This protocol has been optimized to remove washing steps after nanobeads incubation, resulting in a shorter and more convenient protocol. This procedure is optimized for the isolation of $10^7$ to $2 \times 10^8$ cells per tube. If working with fewer than $10^7$ cells, keep volumes as indicated for $10^7$ cells. For best results, optimize the conditions to your specific cell number and tissue. Prepare fresh MojoSort™ Buffer solution by diluting the 5X concentrate with sterile distilled water. Scale up volumes if using 14 mL tubes and Magnet, and place the tube in the magnet for 10 minutes.*

1. Prepare cells from your tissue of interest without lysing erythrocytes.

2. In the final wash of your sample preparation, resuspend the cells in MojoSort™ Buffer by adding up to 4 mL in a 5 mL (12 x 75 mm) polystyrene tube.

*Note:  Keep MojoSort™ Buffer on ice throughout the procedure.*

3. Filter the cells with a 70µm cell strainer, centrifuge at 300 x *g* for 5 minutes, and resuspend in an appropriate volume of MojoSort™ Buffer. Count and adjust the cell concentration to $1 \times 10^8$ cells/mL.

4. Aliquot 100 µL of cell suspension ($10^7$ cells) into a new tube.

5. Resuspend the beads by vortexing, maximum speed, 5 touches. Add 10 µL of Nanobeads, mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 µL for $1 \times 10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

6. Resuspend the cells in 3 mL of MojoSort™ Buffer. Optional: Take an aliquot before placing the tube in the magnet to monitor purity and yield.

7. Place the tube in the magnet for 5 minutes.

8. Pour out the liquid. Resuspend labeled cells in appropriate buffer.

9. Repeat steps 6-8 on the labeled fraction 2 more times, for a total of 3 magnetic separations.

*Optional: Take a small aliquot to monitor purity and yield. If desired, pool the unlabeled fractions and process simultaneously with the positive labeled cells when assessing purity and yield.*

**Chart Protocol**



Application Notes: To use this product in magnetic separation columns, a titration of the Nanobeads should be performed. Optimal concentration for magnetic separation columns is lot-specific. Please contact BioLegend Technical Service (tech@biolegend.com) for further assistance on how to use MojoSort™ Nanobeads in magnetic separation columns.

127
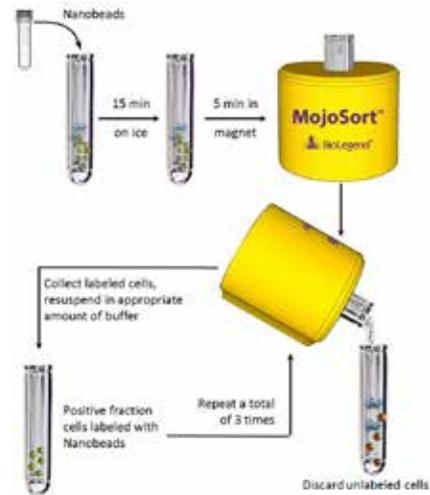
# MojoSort™ Isolation Kits Regular Protocol

**Reagent List**

MojoSort™ Buffer (5X) (Cat. No. 480017)

MojoSort™ Magnet (Cat. No. 480019 | 480020) or compatible magnetic separation system

Adjustable pipettes

70 µm filters (one per sample)

5 mL (12 x 75 mm) or 14 mL (17 x 100 mm) polypropylene tubes

Reagents for sample preparation

Reagents and instruments (Flow cytometer) to determine yield and purity

Important Note:

*MojoSort™ magnetic particles can be used with other commercially available magnetic separators, both free standing magnets and column-based systems. Because MojoSort™ protocols are optimized for the MojoSort™ separator; the protocols may need to be adjusted for other systems. Please contact BioLegend Technical Service (tech@ biolegend.com) for more information and guidance. We do not recommend using MojoSort™ particles for BD's IMag™ or Life Technologies' DynaMag™.*

**Product Description and Procedure Summary:**

Target cells are depleted by incubating your sample with the biotin antibody cocktail followed by incubation with magnetic Streptavidin Nanobeads (Cat. No. 480015 | 480016). The magnetically labeled fraction is retained by the use of a magnetic separator. The untouched cells are collected. These are the cells of interest; do not discard the liquid. Some of the downstream applications include functional assays, gene expression, phenotypic characterization, etc.

**Protocol Steps:**

*Note:  This procedure is optimized for the isolation of $10^7$ to 2 x $10^8$ cells per tube. If working with fewer than $10^7$ cells, keep volumes as indicated for $10^7$ cells. For best results, optimize the conditions to your specific cell number and tissue. Prepare fresh MojoSort™ Buffer solution by diluting the 5X concentrate with sterile distilled water. Scale up volumes if using 14 mL tubes and Magnet, and place the tube in the magnet for 10 minutes.*

1.  Prepare cells from your tissue of interest without lysing erythrocytes.

2.  In the final wash of your sample preparation, resuspend the cells in MojoSort™ Buffer by adding up to 4 mL in a 5 mL (12 x 75 mm) polypropylene tube.

*Note:  Keep MojoSort™ Buffer on ice throughout the procedure.*

3.  Filter the cells with a 70 µm cell strainer, centrifuge at 300 x *g* for 5 minutes, and resuspend in an appropriate volume of MojoSort™ Buffer. Count and adjust the cell concentration to 1 x $10^8$ cells/mL.

4.  Aliquot 100 µL of cell suspension ($10^7$ cells) into a new tube. Add 10 µL of the Biotin-Antibody Cocktail, mix well and incubate on ice for 15 minutes. Scale up the volume

accordingly if separating more cells. For example, add 100 µL for 1 x $10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

*Optional: Keep unused cells, or take an aliquot before adding the cocktail to monitor purity and yield.*

5.  Wash the cells by adding MojoSort™ Buffer up to 4 mL; centrifuge the cells at 300 x *g* for 5 minutes.

6.  Discard supernatant and resuspend in 100 µL of MojoSort™ Buffer.

7.  Resuspend the beads by vortexing, maximum speed, 5 touches. Add 10 µL of Streptavidin Nanobeads. Mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 µL for 1 x $10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

8.  Wash the cells by adding 3 mL of MojoSort™ Buffer; centrifuge at 300 x *g* for 5 minutes, discard supernatant. Optional: Take an aliquot before placing the tube in the magnet to monitor purity and yield.

9.  Resuspend the cells in 3 mL of MojoSort™ Buffer.

*Note:  If you observe aggregates, filter the suspension. To maximize yield, you can disrupt the aggregates by pipetting the solution up and down.*

10.  Place the tube in the magnet for 5 minutes.

11.  Pour out and collect the liquid. These are your cells of interest; DO NOT DISCARD.

12.  If needed, add 3 mL of MojoSort™ Buffer and repeat steps 10 and 11 with the magnetically labeled fraction up to two times, and then pool the unlabeled fractions.

*Note:  Repeating the magnetic separation increases the yield, without a strong impact on the purity. The yield will typically increase about 8-10% with a second separation, and about 2-5% with a third separation. The purity may decrease 1-2% with each separation. Optional: Take a small aliquot before placing the tube in the magnet to monitor purity and yield.*

**Chart Protocol**
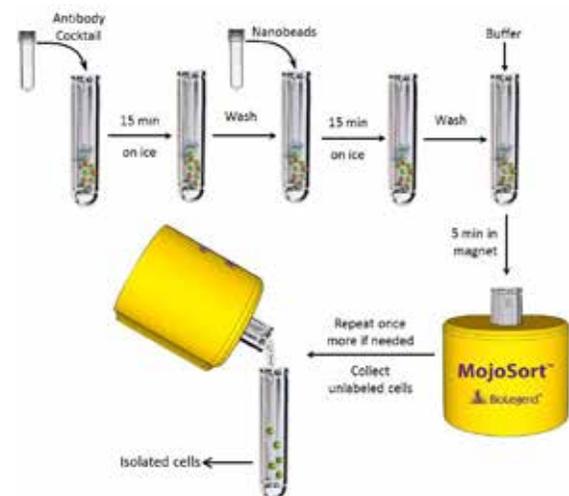
**Application Notes:**

*To use this product in magnetic separation columns, a titration of the Nanobeads should be performed. Optimal concentration for magnetic separation columns is lot-specific. Please contact BioLegend Technical Service (tech@biolegend.com) for further assistance on how to use MojoSort™ Nanobeads in magnetic separation columns.*

# MojoSort™ Isolation Kits No-wash Protocol

### Reagent List

MojoSort™ Buffer (5X) (Cat. No. 480017)

MojoSort™ Magnet (Cat. No. 480019 | 480020) or compatible magnetic separation system

Adjustable pipettes

70 µm filters (one per sample)

5 mL (12 x 75 mm) or 14 mL (17 x 100 mm) polypropylene tubes

Reagents for sample preparation

Reagents and instruments (Flow cytometer) to determine yield and purity

**Important Note:**

*MojoSort™ magnetic particles can be used with other commercially available magnetic separators, both free standing magnets and column-based systems. Because MojoSort™ protocols are optimized for the MojoSort™ separator; the protocols may need to be adjusted for other systems. Please contact BioLegend Technical Service (tech@biolegend.com) for more information and guidance. We do not recommend using MojoSort™ particles for BD's IMag™ or Life Technologies' DynaMag™.*

**Product Description and Procedure Summary:**

Target cells are depleted by incubating the sample with the biotin antibody cocktail followed by incubation with magnetic Streptavidin Nanobeads (Cat. No. 480015 | 480016). The magnetically labeled fraction is retained by the use of a magnetic separator. The untouched cells are collected. These are the cells of interest; do not discard the liquid. Some of the downstream applications include functional assays, gene expression, phenotypic characterization, etc.

**Protocol Steps:**

*Note: This protocol has been optimized to remove washing steps after antibody cocktail and nanobeads incubations, resulting in a shorter and more convenient protocol. This procedure is optimized for the isolation of $10^7$ to $2 \times 10^8$ cells per tube. If working with fewer than $10^7$ cells, keep volumes as indicated for $10^7$ cells. For best results, optimize the conditions to your specific cell number and tissue. Prepare fresh MojoSort™ Buffer solution by diluting the 5X concentrate with sterile distilled water. Scale up volumes if using 14 mL tubes and Magnet, and place the tube in the magnet for 10 minutes.*

1. Prepare cells from your tissue of interest without lysing erythrocytes.

2. In the final wash of your sample preparation, resuspend the cells in MojoSort™ Buffer by adding up to 4 mL in a 5 mL (12 x 75 mm) polypropylene tube.

*Note: Keep MojoSort™ Buffer on ice throughout the procedure.*

3. Filter the cells with a 70µm cell strainer, centrifuge at 300 x *g* for 5 minutes, and resuspend in an appropriate volume of MojoSort™ Buffer. Count and adjust the cell concentration to $1 \times 10^8$ cells/mL.

4. Aliquot 100 µL of cell suspension ($10^7$ cells) into a new tube. Add 10 µL of the Biotin-Antibody Cocktail, mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 µL for $1 \times 10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

*Optional: Take an aliquot before adding the cocktail to monitor purity and yield.*

5. Resuspend the beads by vortexing, maximum speed, 5 touches. Without washing, add 10 µL of Streptavidin Nanobeads. Mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 µL for $1 \times 10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

6. Add 3 mL of MojoSort™ Buffer.

*Note: If you observe aggregates, filter the suspension. To maximize yield, you can disrupt the aggregates by pipetting the solution up and down.*

7. Place the tube in the magnet for 5 minutes.

8. Pour out and collect the liquid. These are your cells of interest; DO NOT DISCARD.

9. If needed, add 3 mL of MojoSort™ Buffer and repeat steps 7 and 8 with the magnetically labeled fraction up to two times, and then pool the unlabeled fractions.

*Note: Repeating the magnetic separation increases the yield, without a strong impact on the purity. The yield will typically increase about 8-10% with a second separation, and about 2-5% with a third separation. The purity may decrease 1-2% with each separation.*

*Optional: Take a small aliquot before placing the tube in the magnet to monitor purity and yield. Keep unused cells to be used as control or other applications if needed.*

**Chart Protocol**
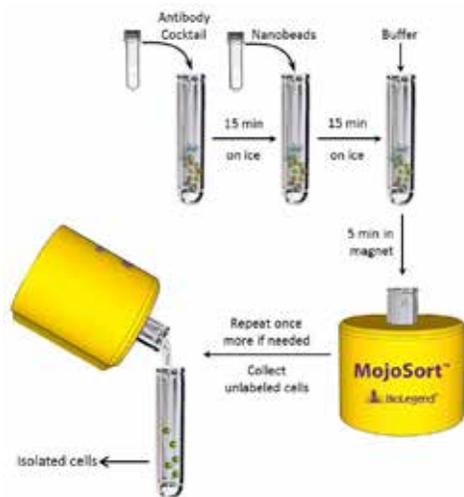


**Application Notes**

*To use this product in magnetic separation columns, a titration of the Nanobeads should be performed. Optimal concentration for magnetic separation columns is lot-specific. Please contact BioLegend Technical Service (tech@biolegend.com) for further assistance on how to use MojoSort™ Nanobeads in magnetic separation columns.*

# MojoSort™ Negative Selection Columns Protocol

## Introduction

BioLegend MojoSort™ nanobeads work in commonly used separation columns, based on our internal research as well as validation by external testing by academic labs. This simple protocol consists of following the MojoSort™ protocol to label the cells with pre-diluted MojoSort™ reagents and using the columns as indicated by the manufacturer.

*Note: Due to the properties of our beads, it may be possible to use far fewer beads and less antibody cocktail that with other commercial suppliers. We recommend a titration to find the best dilution factor. However, as a general rule, dilutions ranging from 1:2 to 1:10 for the antibody cocktail can be used. Dilutions ranging from 1:5 to 1:20 for the Streptavidin Nanobeads can be used. Please contact BioLegend Technical Service (tech@biolegend.com) if further assistance is needed.*

## Important Note:

*MojoSort™ magnetic particles can be used with other commercially available magnetic separators, both free standing magnets and column-based systems. Because MojoSort™ protocols are optimized for the MojoSort™ separator; the protocols may need to be adjusted for other systems. Please contact BioLegend Technical Service (tech@biolegend.com) for more information and guidance. We do not recommend using MojoSort™ particles for BD's IMag™ or Life Technologies' DynaMag™.*

**Protocol Steps**

1. Prepare a single cell suspension and resuspend the cells with ice cold cell separation buffer (MojoSort™ buffer recommended).

2. Pass the cells through a 70 μm filter, centrifuge (300 x $g$ for 5 minutes), discard the supernatant andresuspend the cells in cell separation buffer. Adjust the cell concentration to 1 x $10^8$ cells/mL.

3. Aliquot 100 μL ($10^7$ cells) into a new tube. Add 10 μL of the pre-diluted Biotin-Antibody Cocktail, mixwell and incubate on ice for 15 minutes. Scale up the volume if separating more cells. For example, add 100 μL of pre-diluted antibody cocktail for separating 1 x $10^8$ cells in 1 mL of buffer. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

4. Add cell separation buffer up to 4 mL; centrifuge the cells at 300 x $g$ for 5 minutes.

5. Discard supernatant and resuspend in 100 μL of buffer.

6. Vortex the Streptavidin conjugated Nanobeads (to resuspend) at max speed, 5 touches, and prepare the dilutions to test. Add 10 μL of pre-diluted Streptavidin Nanobeads. Mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 μL of pre-diluted Nanobeads for separating 1 x $10^8$ cells in 1 mL of buffer. When working with less than $10^7$ cells,use indicated volumes for $10^7$ cells.

*Note: Depending on the isolation kit you are using, a wash step may be required here. Please contact BioLegend Technical Service for details (tech@biolegend.com). If you observe aggregates, filter thesuspension. To maximize yield, you can disrupt the aggregates by pipetting the solution up and down.*

7. Resuspend the cells in appropriate amount of buffer. At least 500 μL is needed for column separation,

*Note: There are several types of commercially available columns, depending on your application,choose the one that fits best your experiment:*

Columns:

| | Max. number of labeled cells | Max. number of total cells | Cell suspension volume | Column rinse volume | Cell wash/collection volume | Elution volume |
|---|---|---|---|---|---|---|
| Medium Capacity | $10^8$ | $2 \times 10^9$ | 500 μl for up to $10^8$ cells | 3 ml | 3x3 ml | 5 ml |
| Large Capacity | $2 \times 10^8$ | $10^9$ | 500 μl for up to $10^8$ cells | 60ml | 30 ml | NA |
| Extra Large Capacity | $10^9$ | $10^{11}$ | 500 μl for up to $10^8$ cells | 500 ml | 200 ml | NA |

Example of Magnetic Separation with Medium Capacity Columns:

1. Place the column in a magnetic separator that fits the column.

2. Wash the column with 3 mL of buffer.

3. Add the labeled cell suspension to the column through a 30 μm filter and collect the fraction containingthe unlabeled cells. These are the cells of interest; do not discard.

4. Wash the column 3 times with 3 mL of buffer and collect the fraction containing the untouched cells. Combine with the collected fraction from step 3.

5. If desired, the labeled cells can be collected by taking away the column from the magnet and place it on a tube. Then add 5 mL of buffer and flush out the magnetically labeled fraction with a plunger or supplied device. The labeled cells may be useful as staining controls, to monitor purity/yield, or other purposes.

### Representative Data

Flow Cytometry. High purity and yield. "After Isolation" plots show purified population of interest using pre-diluted MojoSort™ reagents in separation columns.

Before Isolation          After Isolation
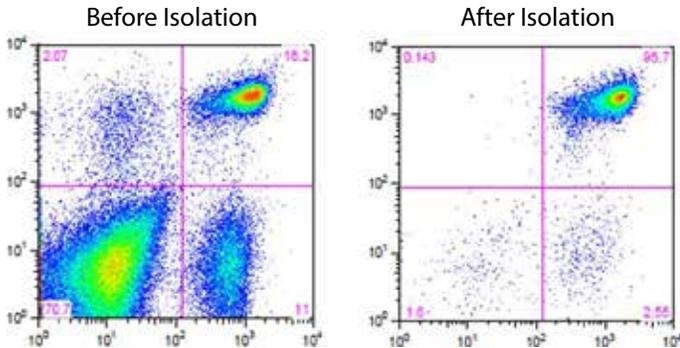


| Kit | Purity | Yield |
|---|---|---|
| Mouse CD4 T Cell Isolation Kit | 95.7% | 85% |

Electron Microscopy. CD4+ T cells isolated with MojoSort™ CD4 T Cell Isolation Kit using columns do not display particles in the cell surface. Image is representative of 36 different cells.
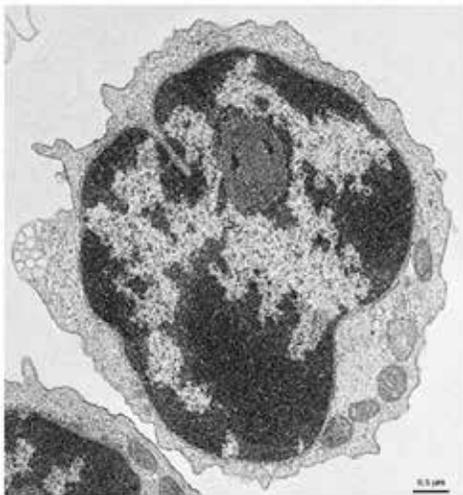


CD4+ T cells isolated with MojoSort™ CD4 T Cell Isolation Kit using separation columns.

### General Tips & FAQ

#### What dilution of the reagents shall I use?

Dilutions are lot-dependent please; contact **tech@biolegend.com** for recommended dilution.

## MojoSort™ Positive Selection Columns Protocol

### Introduction

BioLegend MojoSort™ nanobeads work in commonly used separation columns, based on our internal research as well as validation by external testing by academic labs. This simple protocol consists of following the MojoSort™ protocol to label the cells with pre-diluted MojoSort™ reagents and using the columns as indicated by the manufacturer.

*Note: Due to the properties of our beads, it may be possible to use far fewer beads than with other commercial suppliers. We recommend a titration to find the best dilution factor. However, as a general rule, dilutions ranging from 1:3 to 1:20 for the Nanobeads can be used. Please contact BioLegend Technical Service (tech@biolegend.com) if further assistance is needed.*

Important Note:

*MojoSort™ magnetic particles can be used with other commercially available magnetic separators, both free standing magnets and column-based systems. Because MojoSort™ protocols are optimized for the MojoSort™ separator; the protocols may need to be adjusted for other systems. Please contact BioLegend Technical Service (tech@biolegend.com) for more information and guidance. We do not recommend using MojoSort™ particles for BD's IMag™ or Life Technologies' DynaMag™.*

### Protocol Steps

1. Prepare a single cell suspension and resuspend the cells with ice cold cell separation buffer (MojoSort™ buffer is recommended).

2. Pass the cells through a 70 µm filter, centrifuge (300 x *g* for 5 minutes), discard the supernatant and resuspend the cells in cell separation buffer. Adjust the cell concentration to $1 \times 10^8$ cells/mL.

3. Aliquot 100 µL ($10^7$ cells) into a new tube.

4. Vortex the antibody-conjugated Nanobeads (to resuspend) at max speed, 5 touches, and prepare the dilutions to test. Add 10 µL of pre-diluted conjugated Nanobeads. Mix well and incubate on ice for 15 minutes. Scale up the volume accordingly if separating more cells. For example, add 100 µL of pre-diluted Nanobeads for $1 \times 10^8$ cells. When working with less than $10^7$ cells, use indicated volumes for $10^7$ cells.

*Note: Depending on the conjugated nanobead you are using, a wash step may be required here. Please contact BioLegend Technical Service for details (tech@biolegend.com).*

5. Resuspend the cells in appropriate amount of buffer. At least 500 µL is needed for column separation.

*Note: There are several types of commercially available columns, depending on your application, choose the one that fits best your experiment:*

131

Columns:

| | Max. number of labeled cells | Max. number of total cells | Cell suspension volume | Column rinse volume | Cell wash volume | Elution volume |
|---|---|---|---|---|---|---|
| Small Capacity | $10^7$ | $2\times10^8$ | 500 µl for up to $10^8$ cells | 500 µl | 3x1 ml | 1 ml |
| Medium Capacity | $10^8$ | $2\times10^9$ | 500 µl for up to $10^8$ cells | 3 ml | 3x3 ml | 5 ml |
| Large Capacity | $10^9$ | $2\times10^{10}$ | 5 mL for up to $10^9$ cells | 20 – 50 ml | 4x30 ml | 20 ml |

Example of magnetic separation with medium capacity columns:

1. Place the column in a magnetic separator that fits the column.

2. Wash the column with 3 mL of buffer.

3. Add the labeled cell suspension to the column through a 30 µm filter and collect the fraction containing the unlabeled cells.

4. Wash the column 3 times with 3 mL of buffer and collect the fraction containing the unlabeled cells. Combine with the collected fraction from step 3. These cells may be useful as controls, to monitor purity/yield, or other purposes.

5. Take away the column from the magnet and place it on a tube. Then add 5 mL of buffer and flush out the magnetically labeled fraction with a plunger or supplied device. These are the positively isolated cells of interest; do not discard. To increase the purity of the magnetically labeled fraction, repeat the isolation process with a new, freshly prepared column.

**Representative Data**

Flow Cytometry. High purity and yield. "After Isolation" plot shows purified population of interest using pre-diluted MojoSort™ reagents in separation columns.
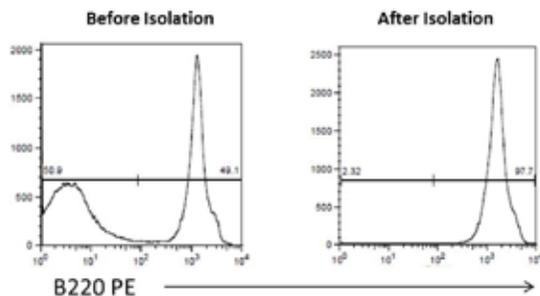


| Kit | Purity | Yield |
|---|---|---|
| Mouse CD19 Nanobeads | 97.7% | 94% |

Electron Microscopy. MojoSort™ Nanobead-isolated CD19+ cells using columns do not display more bound beads on the cell surface (A) as compared to cells isolated with a compatible commercial product using the same columns (B). Red arrows indicate where the particles are located. Numbers indicate either 2 or 3 magnetic particles adjacent to each other. Pictures were taken at the same magnification, scale shown in B. Images are representative of 41 different cells each.
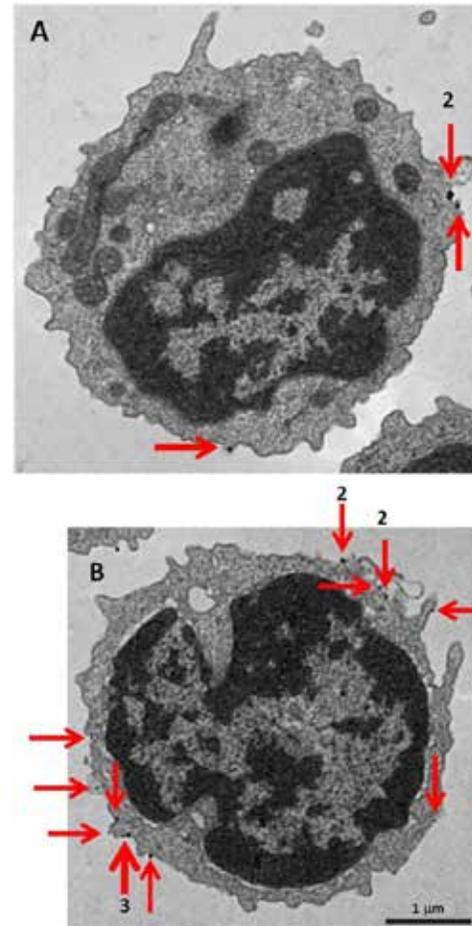
# Flex-T™ Tetramer and Cell Staining Protocol

## Background

Using UV-induced peptide exchange, MHC/peptide monomers can be generated with conditional Flex-T™ monomers that harbor peptides of interest in their binding grooves. These new MHC monomers are subsequently multimerized using streptavidin-fluorophore conjugates. The resulting Flex-T™ reagents can be used for staining antigen-specific T cells and flow cytometric analysis. In humans, the MHC molecules are called HLA (Human Leukocyte Antigen).

## Reagent List

Phosphate buffered saline pH 7.4, 10X concentrate (PBS, BioLegend Cat. 926201)

Peptide Flex-T™ monomer UVX

DMSO (*e.g.,* Sigma-Aldrich Cat. No. D5879)

50mM D-Biotin (*e.g.,* Thermo Fisher, Cat. No. B20656)

10% (w/v) NaN3 (*e.g.,* Sigma, Cat. No. S2002)

Fluorophore-conjugated Streptavidin (BioLegend Cat. No. 405203 | 405207 | 405225 or equivalent)

Cell Staining Buffer (BioLegend Cat. No. 420201 or equivalent)

96-well Polystyrene Microplate, U-shape (*e.g.,* Falcon Cat. No. 353077) or 5 mL, 12 x 75mm tubes (*e.g.,* Falcon Cat. No. 352008)

Plate sealers (BioLegend Cat. No. 423601)

1.5 mL tubes (*e.g.,* Eppendorf Cat. No. 022364111)

## Equipment

UV lamp, long-wave UV, 366 nm, 8 Watts (For example, CAMAG Cat. No. 022.9115, or Ultraviolet Crosslinker CL-1000L)

Incubator (37°C)

Centrifuge capable of accommodating microtiter plates and tubes

Single and multichannel pipettes capable of accurate delivery of variable volumes, and pipette tips

*Tips:* *DMSO can be used to dissolve the peptides. However, do not exceed an end concentration of 10% (v/v) in the exchange reaction.*

Avoid repeated freeze-thawing.

The Flex-T™/peptide solution needs to be kept on ice in the dark as much as possible. Do not work in front of a window.

The use of short-wavelength (254 nm) or broad-band UV lamps is detrimental to MHC complexes.

Centrifuge all vials before use (1 minute 3000 x $g$ at 4°C).

## Protocol Steps

Peptide Exchange:

1. Bring all reagents to 0°C by putting them on ice.

2. Dilute 10mM stock solutions of peptides of choice to 400 µM by mixing 5 µL of peptide stock solution with 120 µL PBS, and keep on ice.

3. Add 20 µL diluted peptide (400 µM) and 20 µL peptide Flex-T™ monomer UVX (200 µg/mL) into 96-well U bottom plate. Mix by pipetting up and down.

4. Seal the plate; centrifuge at 3300 x $g$ for 2 minutes at 4°C to collect the liquid down.

5. Remove the seal; put the plate on ice and illuminate with UV light for 30 minutes (the distance of the UV lamp to the samples should be 2-5 cm).

6. Seal the plate; incubate for 30 minutes at 37°C in the dark.

7. To evaluate the efficiency of the peptide exchange, follow the Protocol for HLA class I ELISA to evaluate peptide exchange.

Generation of Tetramers:

8. Transfer 30 µL of peptide-exchanged monomer into a new plate, then add 3.3 µL of conjugated streptavidin, mix by pipetting up-and-down. Incubate on ice in the dark for 30 minutes. This is enough for about 15 tests.

*Note:* *BioLegend fluorophore-conjugated streptavidin products are recommended. For 30 µL of exchanged Flex-T™ monomer, we suggest to use 3. 3 µL of BioLegend PE-streptavidin (Cat. No. 405203) or APC- streptavidin (Cat. No. 405207). For BV421™-streptavidin conjugate (Cat. No. 405225), use 1. 3 µL. For optimal reaction with other fluorophore-conjugated streptavidin products, ensure that the monomer:streptavidin conjugate has a 6:1 molar ratio.*

*(For our full list of Streptavidin conjugates, visit: biolegend. com/streptavidin_conjugates. Note that purified, biotinylated, HRP, MojoSort™, and Ultra Streptavidin (USA) kits are not recommended for this procedure.)*

9. During the incubation, prepare blocking solution by adding 1. 6 µL 50 mM D-Biotin and 6 µL 10% (w/v) NaN$_3$ to 192.4 µL PBS, mix by vortexing. After the incubation, add 2.4 µL of blocking solution and pipette up-and-down to stop the reaction.

10. Seal the plate and incubate at 2-8°C overnight (or on ice for 30 minutes in the dark).

*Tip:* *We recommend Flex-T™ to be assembled with two different streptavidin conjugates in separate reactions. This allows for two-color staining with the same tetramer allele, ensuring the highest specificity.*

133

Cell Staining and Flow Cytometric Analysis:

11. Prepare cells of interest.

12. Prior to performing staining, centrifuge the plate at 3300 x *g* for 5 minutes at 4°C. Keep on ice in the dark.

13. Add 2 x 10$^6$ cells to a 96-well U-bottom plate or 12 x 75 mm tubes. Adjust volume to 200 µL with Cell Staining Buffer. Add 2 µL per sample of Flex-T™ complex prepared in Steps 7-9, mix and incubate on ice in the dark for 30 minutes.

14. If co-staining with surface antibodies, prepare the antibody cocktail based on optimal staining concentration of each reagent. Incubate for 30 minutes on ice in the dark.

15. Wash the cells with Staining Buffer two times. Resuspend cells with Staining Buffer.

16. Acquire the samples with a flow cytometer and appropriate settings within 2 hours.

Tip: *A titration of the Flex-T™ is recommended for optimal performance.*

**Representative Data:**
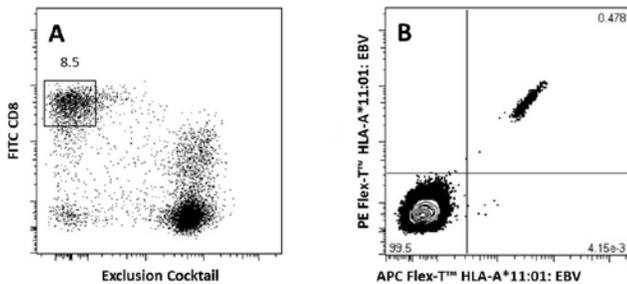


A) CD8$^+$ T Cells, previously gated on lymphocytes (FSC vs SSC) and 7-AAD negative events, were stained with FITC anti-CD8a and an exclusion cocktail containing Alexa Fluor® 700 anti-CD4, CD19, CD14, and CD16.

B) Antigen specific CD8$^+$ T Cells, gated as described, were detected with Flex-T™ tagged with PE and APC. HLA-A*11:01 Flex-T™ was loaded with an EBV peptide (IVTDFSVIK).

**Chart Protocol**

Fluorescent tetramer generation and cell staining diagram



134
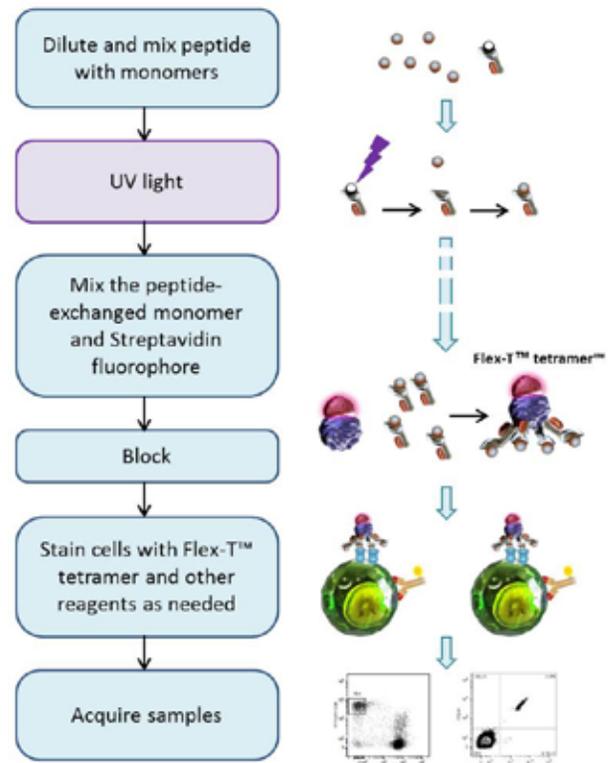
## Contact BioLegend

**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**

**BioLegend®**