Multicolor Flow Cytometry
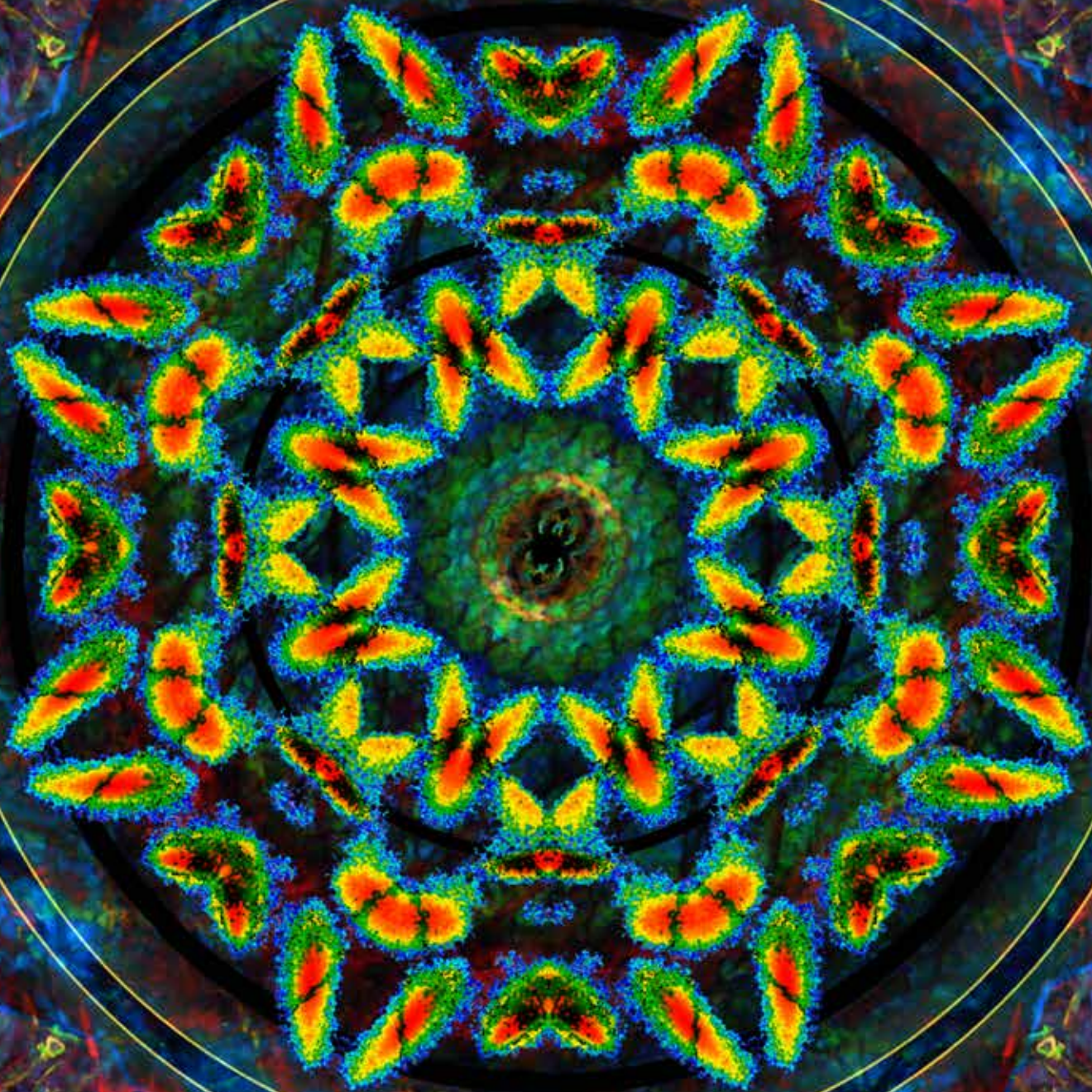## Best Practices for Optimization

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# Build a Better Multicolor Flow Cytometry Assay

Flow cytometric assays of four to six colors are relatively easy to construct and analyze. At this number, you can choose the brightest fluorophores and ensure that they have minimal spectral overlap. However, many researchers prefer to maximize the number of markers in their panel due to limited sample volume or to see the inter-connectivity of many parameters simultaneously. Increasing the number of markers in a multicolor panel requires that a set of guiding principles be followed to ensure that the sensitivity and statistical consistency are maintained between sample and day. As the number of markers in a panel increases, the number of colors with overlapping spectra increases, and so do the complexities of experimentation and analysis.

Sensitivity = Signal Brightness − Background (Noise)

$$(MFI^+) - (MFI^-)$$

Population Resolution = Fluorophore Brightness ×
Degree of Labeling of the Antibody ×
Expression Level of the Antigen per Cell
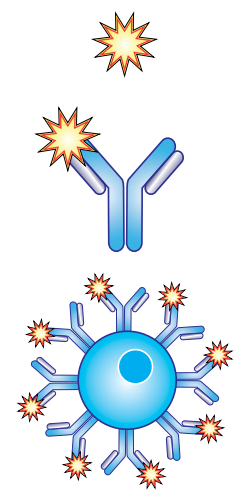
Building a balanced panel, titrating and optimizing your antibodies, and using the appropriate controls can all help to minimize aberrations and ensure confidence in your data set. In this brochure, you can learn more about how to get the most out of your multicolor flow cytometry experiment by following these guiding principles.

View all of our fluorophore conjugated antibodies at: biolegend.com/flow_cytometry_conjugates

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Instrument Specifications
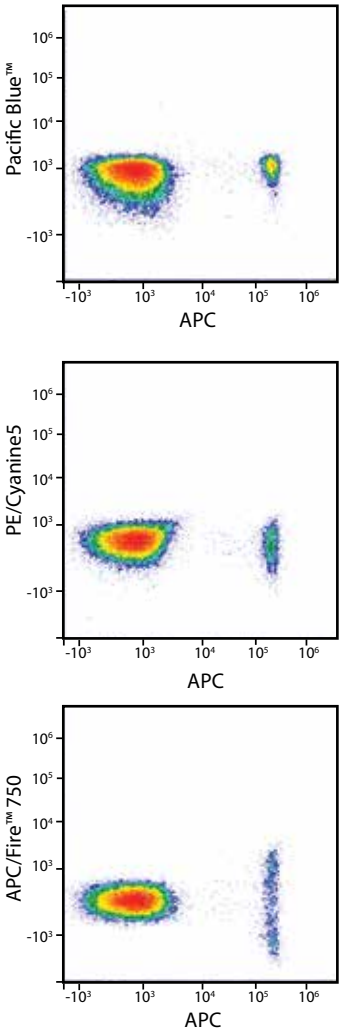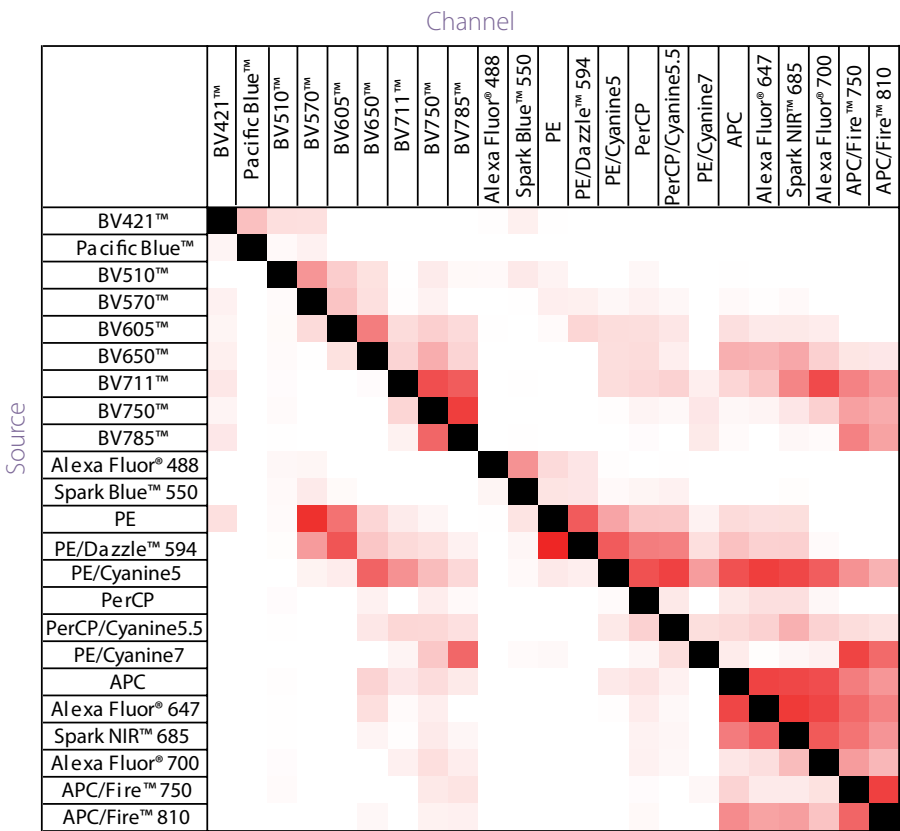
Know your Instrument!

Reference your instrument's specifications while constructing a multicolor panel to ensure that you can optimally detect the fluorophores that you choose for your experiment. If you don't have this information, contact your flow cytometry facility for the specifications. Most advanced cytometers have up to 5 lasers including ultraviolet (355 nm), violet (405 nm), blue (488 nm), green (532 nm) or yellow-green (561 nm), and red (633 nm). In addition to the lasers on your instrument, there are a limited number of detectors and filter combinations which will dictate how many and which fluorophores you can detect.

Many standard flow cytometers utilize photomultipler tubes (PMTs) and specific bandpass filters to detect specific fluorophores. Alternatively, some newer instruments utilize spectral unmixing for up to 48 fluorescent parameters in a single assay. This is done, in part, by integrating the full spectral signature of fluorophores with very similar spectra, such as Brilliant Violet 421™ and Pacific Blue™. In lieu of using PMTs, these cytometers have Avalanche Photodiode (APD) detectors, which also enables the detection of fluorophores in the near-IR and infrared wavelengths.

Spillover Matrix

When running any flow cytometry experiment, keep in mind that high compensation values are not inherently problematic as long as you are able to distinguish your positive and negative populations. Additionally, compensation values can be affected by the absolute voltage of each channel. For each experiment, use unstained and single stained biological controls to adjust the voltage for each channel.

The spread matrix below was generated using anti-CD4 antibodies (clone SK3) conjugated to each of the fluorophores and run on a Cytek™ Aurora spectral cytometer with 3 lasers (405 nm, 488 nm, 633 nm). On the right, you can see how this translates to your experiments as the amount of spillover increases.

Spillover matrix. Channel (columns) vs Source (rows) for: BV421™, Pacific Blue™, BV510™, BV570™, BV605™, BV650™, BV711™, BV750™, BV785™, Alexa Fluor® 488, Spark Blue™ 550, PE, PE/Dazzle™ 594, PE/Cyanine5, PerCP, PerCP/Cyanine5.5, PE/Cyanine7, APC, Alexa Fluor® 647, Spark NIR™ 685, Alexa Fluor® 700, APC/Fire™ 750, APC/Fire™ 810.

Dot plots: Pacific Blue™ vs APC; PE/Cyanine5 vs APC; APC/Fire™ 750 vs APC.

Balance Antigen Expression vs. Fluor 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 target to ensure the best chance of adequate detection.

2. Understand how a disease state, exogenous treatment, or stimulation may affect antigen expression. For example, while CD4 is abundant in both mouse tissue and human peripheral blood, it is downregulated upon PMA/Ionomycin treatment. In this case, you can try to use a brighter fluorophore for CD4 or gate on $CD3^+$ $CD8^-$ events. Keep in mind that antigen expression may be donor-dependent and change over the course of maturation or within different cellular subsets.
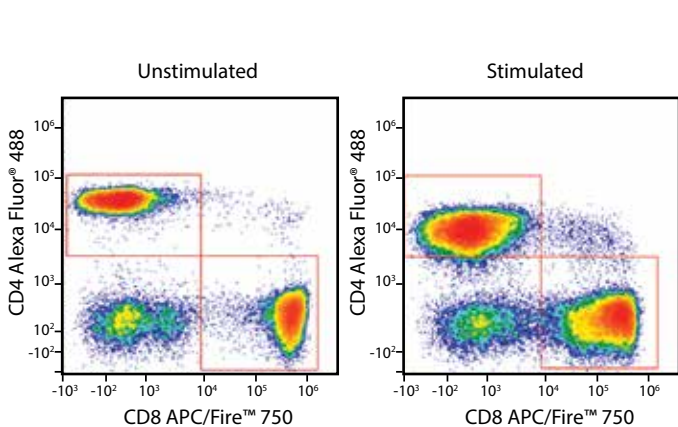
Human PBMCs were isolated and stained without stimulation (left) or stimulated (right) for 6 hours with Cell Activation Cocktail (Cat. No. 423303) and stained with surface antibodies.
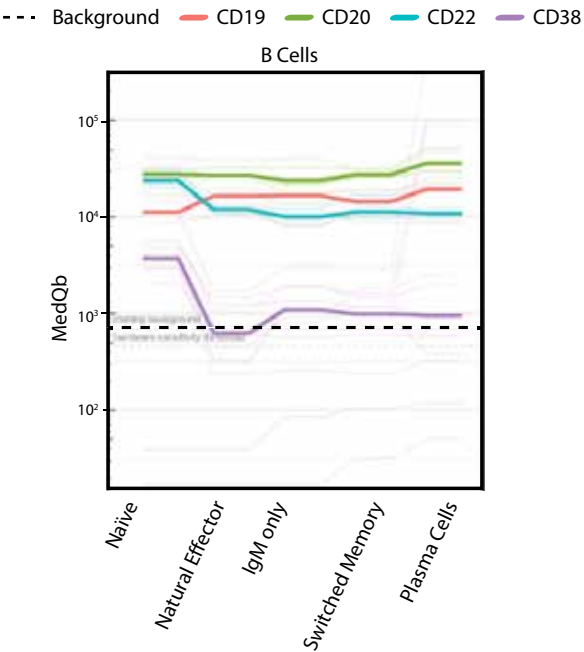
Expression of CD markers changes across different cell types and during maturation. Data provided by CDMaps.

3. Refer to our Brightness Index to help match the potential abundance of the antigen in your assay with an appropriately bright fluorophore. Keep in mind that a bright fluorophore isn't always the best fluorophore.

In this table, every fluorophore was conjugated to an anti-CD4 antibody (clones RPA-T4 and SK3) and normal human whole blood was stained. The fluorophore brightness score is a relative indication of fluorescence intensity above background and is calculated using the stain index ((MFI of the positive population - MFI of the negative population)/ 2 * Standard Deviation). These values can differ depending upon the flow cytometer, instrument filters and settings, laser power, the antibody clone, antigen target, F/P ratios, and the buffer conditions, etc.

Brightness Index

| Fluorophore	Cytek™ Aurora Channel	Excitation Max (nm)	Emission Max (nm)	Brightness Ranking (1-5)
PE/Cyanine5	B7	488/561	665	5
Brilliant Violet 421™	V1	405	420	4
Brilliant Violet 605™	V9	405	605	4
Brilliant Violet 650™	V11	405	650	4
Brilliant Violet 711™	V13	405	711	4
Brilliant Violet 785™	V15	405	785	4
PE	B4	488/561	575	4
Brilliant Violet 750™	V14	405	750	3
PE/Dazzle™ 594	B6	488/561	615	3
Alexa Fluor® 647	R1	650	675	3
APC	R1	650	665	3
Brilliant Violet 570™	V8	405	570	2
PE/Cyanine7	B13	488/561	780	2
Pacific Blue™	V3	405	450	1
Brilliant Violet 510™	V5	405	510	1
Alexa Fluor® 488	B2	488	520	1
FITC	B2	488	520	1
Spark Blue™ 550	B3	516	548	1
PerCP/Cyanine5.5	B8	488	680	1
Spark NIR™ 685	R2	660	690	1
Alexa Fluor® 700	R4	695	720	1
APC/Fire™ 750	R7	650	790	1

Fluorophore Considerations

When choosing your fluorophores, there are a number of factors to keep in mind. Ideally, you want to spread your markers across multiple lasers and choose fluorophores that have minimal spectral overlap. As your panel grows, there is a higher likelihood that you will need to use multiple fluorophores on the same laser and include tandem fluorophores.

Brighter fluorophores aren't always better fluorophores. For example, PE/Cyanine5 is very bright, but it has significant cross-beam excitation with the red laser, which causes unwanted background in the Alexa Fluor® 647 or APC channel. Alternatively, while FITC is not a very bright fluorophore, it emits into a channel with little background and is commonly conjugated to most commercially available antibodies.

Tandem fluorophores are the union of two fluorescent molecules which undergo fluorescence resonance energy transfer (FRET) upon excitation. A donor fluorophore, such as PE, APC, or Brilliant Violet 421™, is excited by the excitation source, and upon resonating that energy, transfers it to an acceptor fluorophore such as Cyanine7 (in the case of PE/Cyanine7).

Tandems are never 100% efficient at transferring energy, so there is always some emission of the donor detected in its own channel. As such, when building a panel, it is good practice to not to place a donor fluorophore and one of its tandem dyes on two markers that will be directly compared on a bivariate plot.
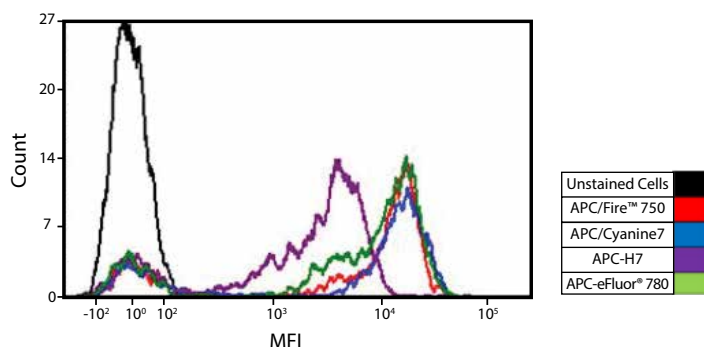
Learn more at: biolegend.com/tandem_dyes
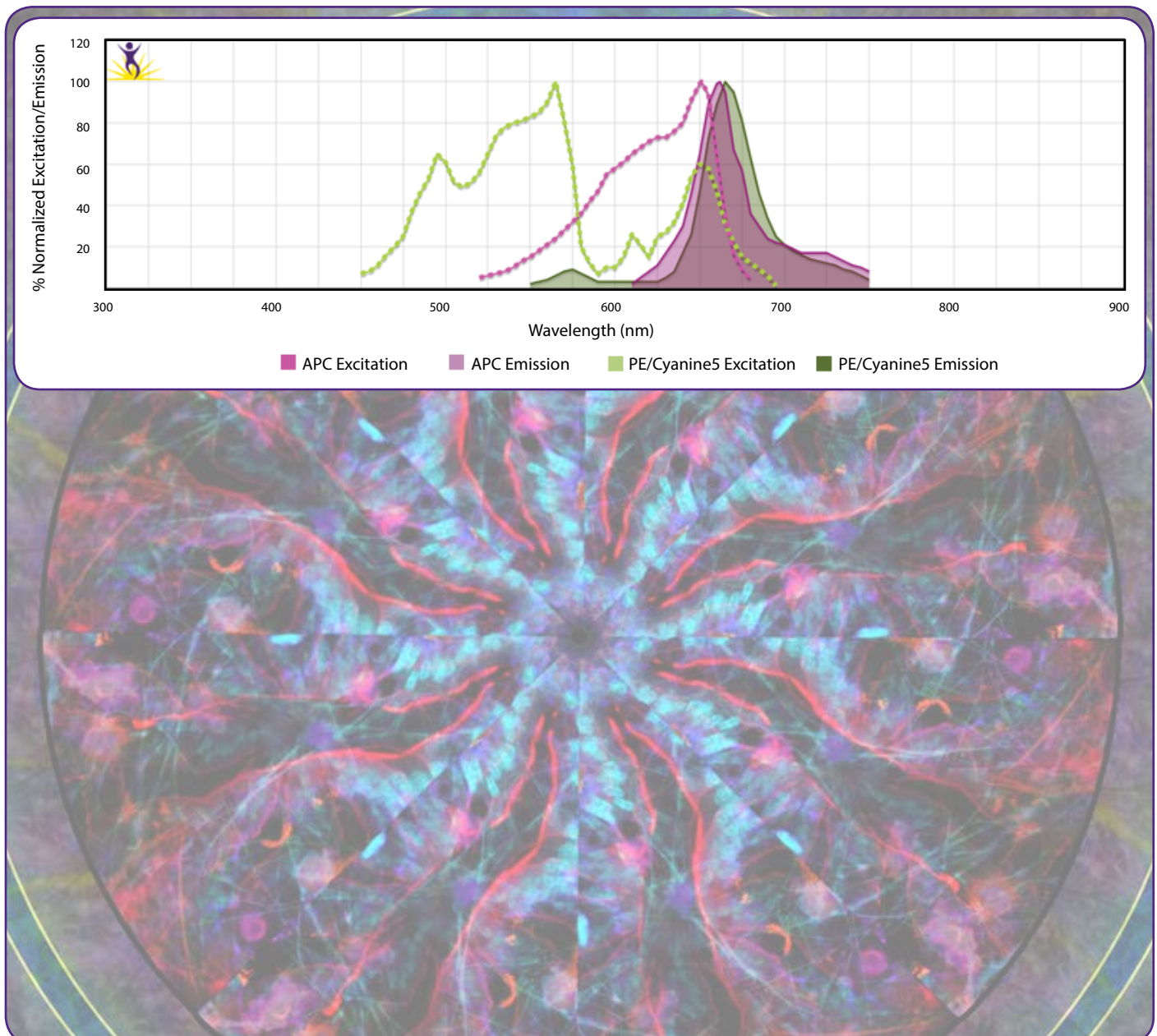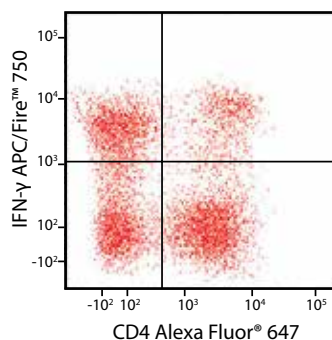
Featured Fluorophores

APC/Fire™ 750

APC/Fire™ 750 provides an alternative to APC/Cyanine7 that is more stable for long-term storage and is more photostable. It maintains the same levels of brightness, but has lower compensation requirements and is more stable to an array of fixatives as compared to APC/Cyanine7.

Learn more at: biolegend.com/fire

Human PBMCs were stimulated for 6 hours with PMA and Ionomycin in the presence of Monensin. Cells were stained with anti-CD4 Alexa Fluor® 647 and then fixed and permeabilized before staining with anti-IFN-γ APC/Fire™ 750.

| Unstained Cells |
| APC/Fire™ 750 |
| APC/Cyanine7 |
| APC-H7 |
| APC-eFluor® 780 |

Human whole blood was stained with anti-CD3 (SK7) conjugates of APC/Fire™ 750, APC/Cyanine7, APC-H7, or APC-eFluor® 780. Histograms were gated on lymphocyte populations.
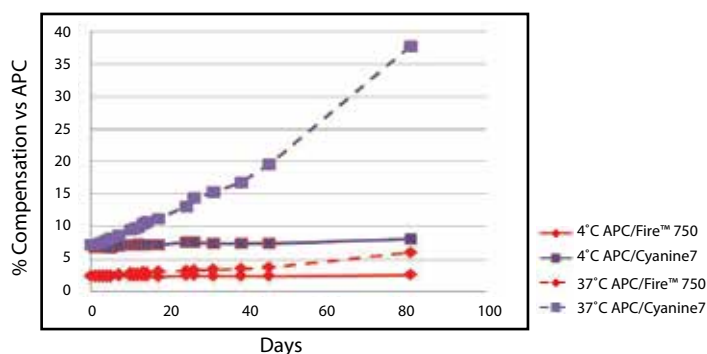

Vials of APC/Cyanine7 and APC/Fire™ 750 were stored in the dark at 4°C and 37°C for 81 days. At certain intervals, an aliquot of reagent was used to stain Veri-Cells™ PBMC, a lyophilized human PBMC product that effectively removes donor-dependent variation in staining (Cat. No. 425002). Cells were stained for 15 min at room temperature in cell staining buffer.

Brilliant Violet™ Family

The Brilliant Violet™ family of fluorescent polymers are extremely bright and stable with fixation. Adding versatility to panel selection, we offer 8 different fluorophores with discrete excitation/emission spectra with a large selection of antibody specificities. When building your panel, keep in mind that many of the Brilliant Violet™ dyes (BV570™, BV605™, BV650™, BV711™, BV750™, BV785™) are tandem dyes using BV421™ as a donor.

Learn more at: biolegend.com/brilliantviolet
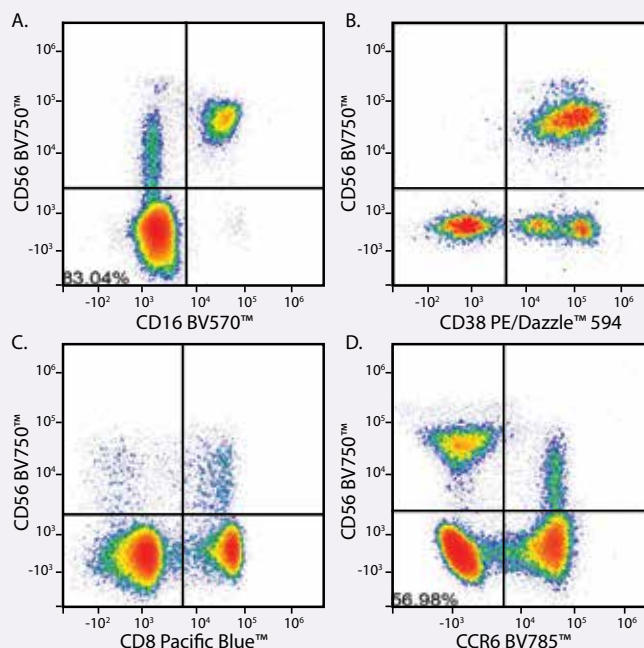
Human whole blood was stained and analyzed on the Cytek™ Aurora Spectral Flow Cytometer. Gates were applied based on the plots as follows: A/D: lymphocyte scatter profile, B. CD3⁻/CD20⁻ cells, C. CD3⁺ cells.

PE/Dazzle™ 594

PE/Dazzle™ 594 is a tandem dye that offers outstanding brightness and exceptional photostability. It is optimally excited by the 488 nm blue, 532 nm green, or 561 nm yellow-green lasers and is detected in the same channel as PE/Texas Red®, PE-CF594, PE-eFluor® 610, and ECD.
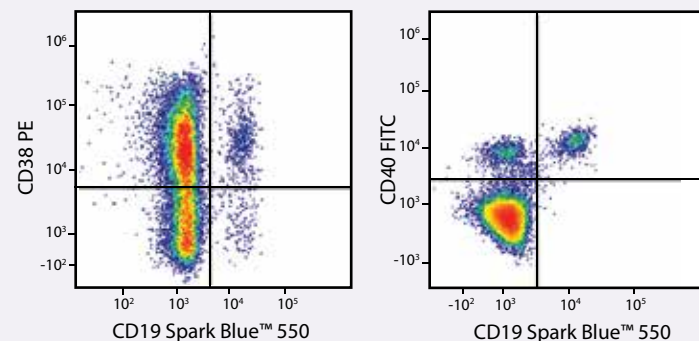
Learn more at: biolegend.com/dazzle
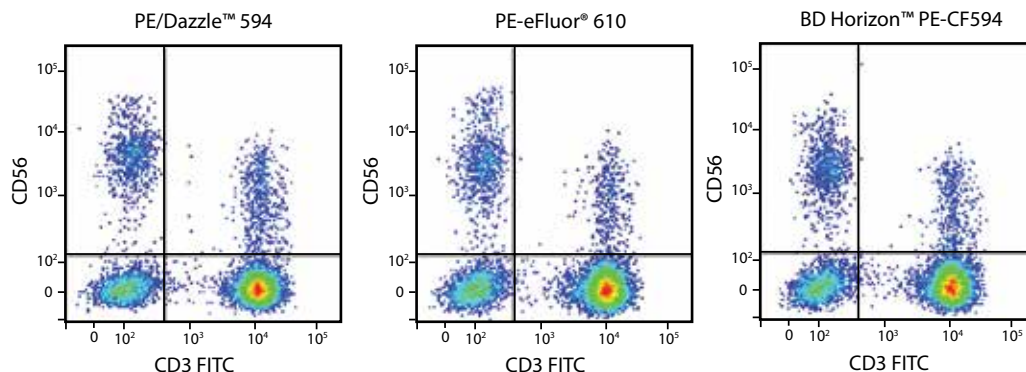
Human peripheral blood lymphocytes were stained with FITC anti-human CD3 (clone UCHT1) and anti-human CD56 conjugated to either PE/Dazzle™ 594 or other companies' equivalent fluorophore.

Spark Fluorophores

Our Spark Fluorophores offer unique spectra that can be added to multicolor panels built for a spectral unmixing cytometer like the Cytek™ Aurora or Northern Lights.

Spark Blue™ 550 is excited off the 488 nm blue laser and emits at 550 nm between the peak emissions of FITC and PE, but is spectrally distinct enough to be used in conjunction with FITC and PE antibodies.

Expanding your options off of the 633 nm red laser, **Spark NIR™ 685** fills the spectral gap between the emission peaks of APC and Alexa Fluor® 700. When using highly overlapping combinations of fluorophores, consider the spreading error from plotting these antibodies against one another on a bivariate plot.

Human whole blood was stained with antibodies for anti-CD19 conjugated to Spark Blue™ 550, CD38 PE and CD40 FITC. Samples were unmixed on a Cytek™ Aurora Cytometer using compensation beads and cells. All plots are gated on lymphocytes.

Learn more about the Spark fluorophores at:
biolegend.com/aurora
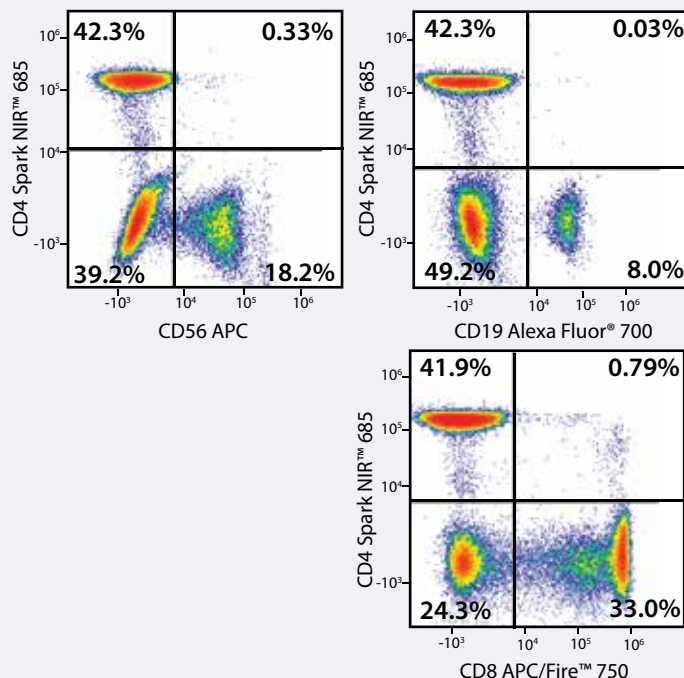
Whole blood from the same donor was stained with antibodies for anti-human CD4 Spark NIR™ 685 , CD19 Alexa Fluor® 700, CD8 APC/Fire™ 750, CD3 PE, and CD56 APC. Cells were washed and fixed with Fluorofix™ prior to analysis.

Viability Dyes

Dead or dying cells in your sample can result in false positive events due to increased autofluorescence or non-specific binding to antibodies. As not all dead cells can be distinguished using only forward and side-scatter, it is helpful to add a viability dye into your panel to exclude those cells from analysis. For this, you can use a DNA dye like Helix NP™ (Blue, Green, NIR), DRAQ7™, Propidium Iodide, or 7-AAD, which are impermeable to live cells. Alternatively, you can use one of our Zombie dyes or a vitality dye like Calcein-AM.

Learn more at: biolegend.com/cell_health_proliferation

Zombie Dyes

Zombie Dyes are fixable viability dyes that react with primary amines on proteins. Live cells exclude the reagents and only cell surface proteins will be labeled. Dead cells will allow the entry of the dyes into the cytoplasm increasing the amount of labeling. As such, dead cells will be brighter for Zombie fluorescence than live cells.

Learn more at: biolegend.com/live_dead
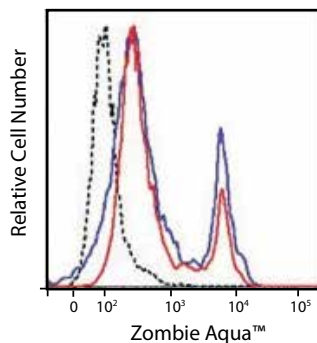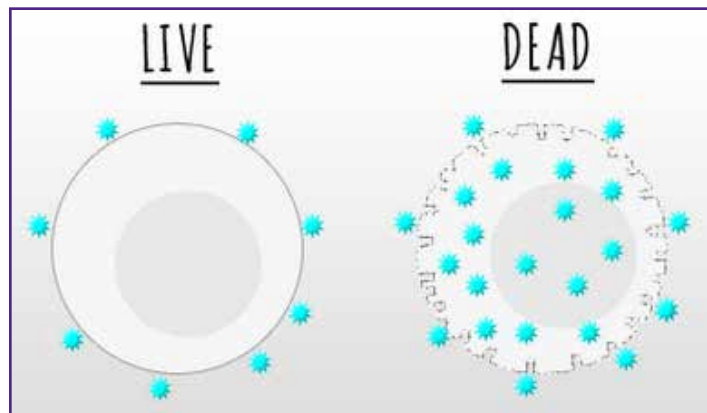
One day old splenocytes were stained with Zombie Aqua™ and analzyed without fixation (blue) or after fixation and permeabilization (red). Unstained cells are indicated in black.

Esterase-Dependent Probes

Instead of using a dye to mark your dead cells, you can consider using a vitality probe to assess cell health. Calcein-AM, Calcein Red-AM, and Calcein Violet-AM are esterase probes that are cell-permeant. Once inside the cell, non-specific esterases convert the probe, making it fluorogenic. As the signal of these dyes is proportional to cell vitality, the signal will decrease in dying cells.
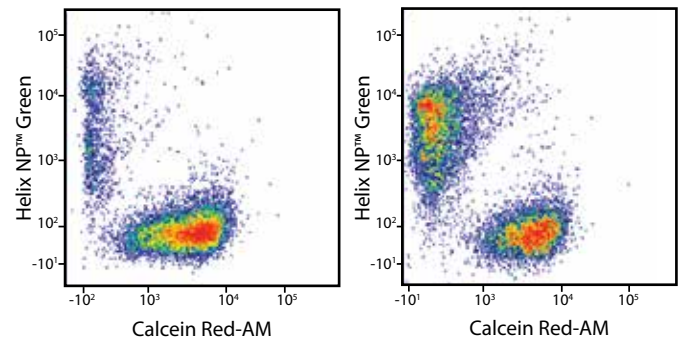
Fresh (left) or day-old mouse splenocytes (right) were stained with Calcein Red-AM and a cell-impermeant nucleic acid dye, Helix NP™ Green.

Organize a Balanced Panel
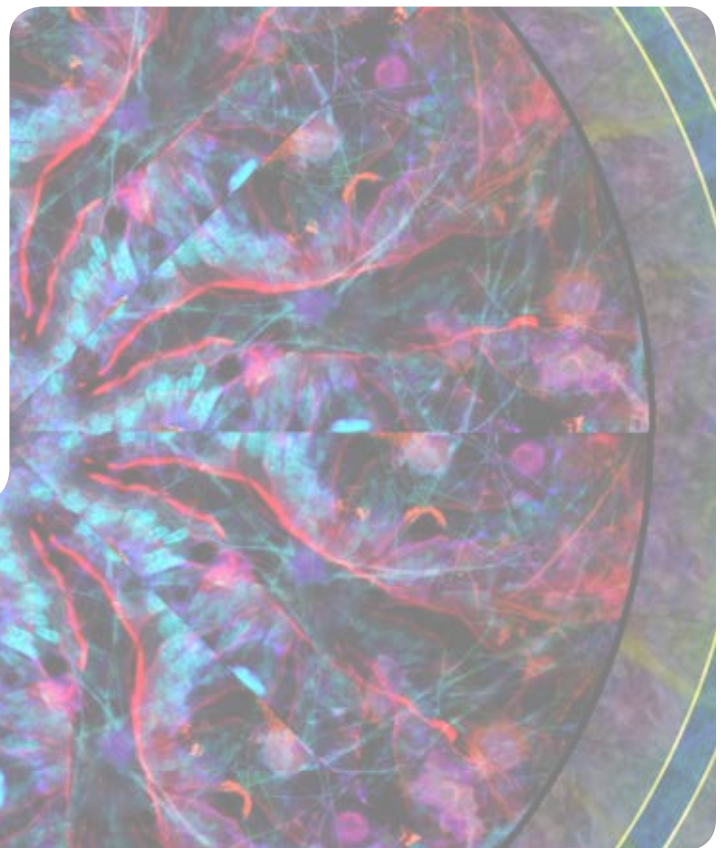
To begin panel building, identify and organize your targets into three tiers:

- **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 fluors such as Alexa Fluor® 700, APC/Fire™ 750, or BV570™.
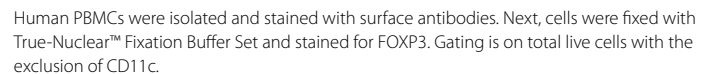
   Examples: basic cell surface phenotyping markers like CD4, CD8, CD3, B220, or CD16.

- **Secondary tier antigens:** These markers are necessary for further phenotyping but may change somewhat between parallel panels. Expression levels tend to be more variable and they are typically appropriate for moderately bright fluors like BV650™, PE/Dazzle™ 594, and PE/Cyanine7.

   Examples: activation or exhaustion markers and cytokines necessary for subtype identification.

- **Tertiary tier antigens or "wish list" markers:** These markers are often the most important question you are asking and may have widely variable or completely unknown levels of expression. Typically, markers in this category are commercially available in only limited fluorophore options. Reserve your brightest fluorophores for these antigens, such as PE, APC, Brilliant Violet 421™, PE/Cyanine7, or Alexa Fluor® 647.

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

Human PBMCs were isolated and stained with surface antibodies. Next, cells were fixed with True-Nuclear™ Fixation Buffer Set and stained for FOXP3. Gating is on total live cells with the exclusion of CD11c.

Consider your Path of Analysis

When designing your panel, think about how to you plan to analyze your experiment. Ask yourself which markers you will want to compare on a bivariate plot and which markers will be co-expressed on the same cell type? Knowing these things in advance can help you better decide which fluorophores to place on which marker.

For example, if you know that two fluorophores have highly overlapping spectra, try to put them on different cell types. Remember, all tandems will have some degree of spillover back into the original donor channel, so try not to put these fluorophores on two markers that you know will be cross-compared on a bivariate plot.

Optimize and Control

Use the appropriate controls for the question or problem you are addressing.

1. **Single Color Biological Controls:** Regardless of what you use to compensate, single stained cells are useful to have on hand for a variety of reasons. Single stained cells can be used to tune the voltage of the PMTs at the start of acquisition to ensure that your events are on scale, within the linear range of the PMT, and will work well with your fully stained samples. Calibration beads, such as CST beads, are important for ensuring consistent performance of the cytometer. However, the voltages they recommend, particularly for the violet laser, are often grossly inaccurate for the abundance of signal in the assay or the brightness of the fluorophore. PMT voltages should be optimized to be relevant to your specific assay. Before you run your samples, always check single stained biological controls to ensure that accurate compensation or spectral unmixing has been applied.

2. **Compensation Controls:** Compensation controls should be used every time you run an assay to ensure accuracy. Whether you choose to use compensation beads or single-stained cells to run your compensation, you should ensure that there are sufficient events in both the positive and negative peaks to populate the compensation algorithm accurately. This is particularly true for tandem dyes as the degree of spectral spillover may change. Ideally, you should treat your compensation controls in the same way over the course of staining including the same fix/perm steps and light exposure. As such, in these circumstances, biological controls can be more accurate for compensation. Beads may be preferred when the population is exceedingly rare in the biological sample.
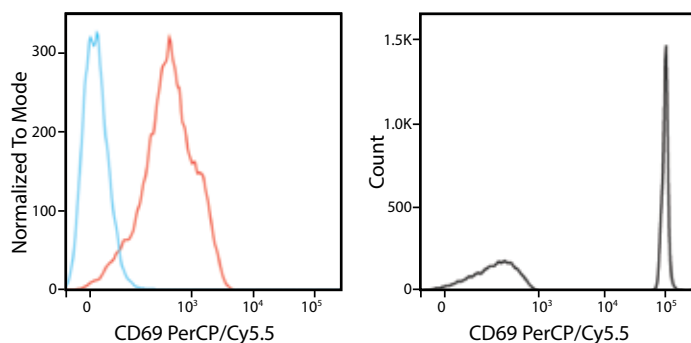

Plot on the left demonstrates an instance when a biological control is inadequate to use as a compensation control, since there are not enough negative events to populate an MFI⁻ peak. The plot on the right shows the same antibody on compensation beads. With compensation beads, the MFI⁺ peak can become off-scale, so the voltage should be tuned to suit the assay based on single color controls and not to accommodate the bead.

Blue = Unstained mouse splenocytes. Red = CD69 single stained mouse splenocytes from activation model. Black = compensation beads labeled with CD69 PerCP/Cy5.5

3. **Fluorescence Minus One (FMO) Controls:** FMO controls are used to establish accurate gating. They include the addition of all antibody conjugates in a panel except one marker. Typically, they are only truly necessary to aid in gating of markers that lack clear population resolution. FMOs are helpful to increase reproducibility and decrease statistical variance associated with hard-to-gate markers by identifying the edge of the true negative population. In larger multicolor assays (10 colors or more), the additive increase in background signal and spreading error may affect where you would place a gate to exclude the negative population, since it may have shifted relative to the single stained control. FMOs also help you keep an eye on consistent performance of your antibodies, particularly tandem conjugates, to help identify problems with FRET efficiency before it negatively impacts your results or creates statistical artifacts.

4. **Isotype Controls:** Isotype controls are important if you suspect or are unsure if there is binding of the Fc component of an antibody. It's important to use isotype controls when staining cell types that express Fc receptors, such as monocytes and macrophages. They can be used to provide evidence that a signal you deem positive is not an artifact. 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. When using isotype controls, it is important to use the same amount of antibody (by mass) for both the test antibody and isotype control.
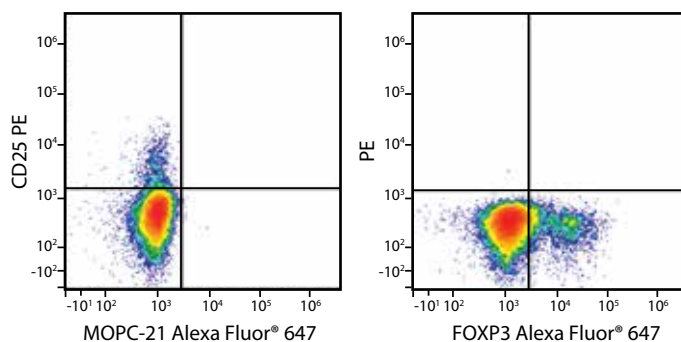

Human PBMCs were stained with surface markers before fixation and permeabilization with the True-Nuclear™ Transcription Factor Buffer Set. Next, cells were stained for intracellular markers like FOXP3. The tube on the left contained anti-CD25 PE and an isotype control for the FOXP3 antibody at the same µg concentration. The tube on the right contained the full panel of reagents but without anything in the PE channel. This served as an FMO for CD25 gating.

5. **Autofluorescence Controls:** Different cell and tissue types can have varying levels of inherent fluorescence. For example, the myeloid cell lineage tends to be particularly autofluorescent. Autofluorescence is typically caused by metabolites and certain biological structures (*e.g.* mitochondria and lysosomes). Additionally, stimulation can cause cells to become more metabolically active and produce more autofluorescent proteins. An unstained cell control is helpful to determine the level of autofluorescence of your samples in your channel of interest.

Blockers and Buffers

Fc Blocking

Fc Blocking Controls are used to block binding of the Fc component of antibodies to Fc receptors present on certain cell types. To prevent this, we recommend using Human TruStain FcX™ or Mouse TruStain FcX™ PLUS. Mouse TruStain FcX™ PLUS contains an antibody directed against CD16 and CD32 (FcγRIII and FcγRII respectively) which binds to Fc Receptors via the Fab portion of the antibody. Human TruStain FcX™ is a specialized mix of human IgG immunoglobins that bind to Fc receptors via the Fc portion of the immunoglobulin. Human TruStain FcX™ is compatible with simultaneous staining of Fc receptor antibodies.
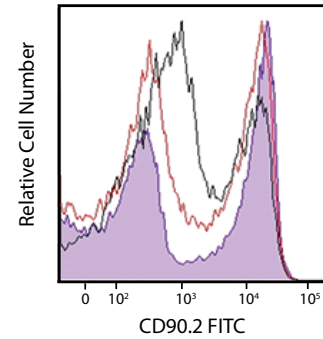

Mouse splenocytes were incubated with TruStain FcX™ PLUS (0.25 μg/test, purple filled histogram), TruStain FcX™ (clone 93, 1 μg/test, red line) or were left untreated (black line) and stained with CD90.2 FITC.

Monocyte Blocker

Monocytes and macrophages can exhibit a different form of non-specific binding by binding to certain fluorophores, particularly PE, APC, and PerCP-based tandem dyes. While the specific mechanism of this binding is not known, several factors can influence the existence and severity of this binding including sample type, stimulation of the cells, donor-dependent variation, and fixation conditions. True-Stain Monocyte Blocker™ reduces this form of non-specific binding, allowing you to stain monocytes with confidence. As True-Stain Monocyte Blocker™ blocks a different type of non-specific binding, it should be used in conjunction with Fc blocking.

Learn more at: biolegend.com/monocyte_blocker
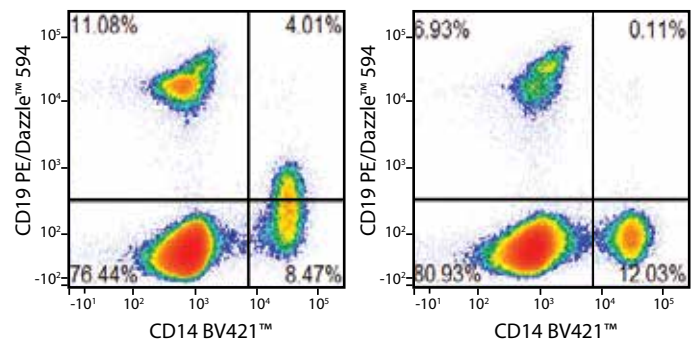
Whole blood was stained as indicated with 5 μL/test of antibody in 200 μL blood prior to RBC lysis. Cells were first treated with either TruStain FcX™ blocker or True-Stain Monocyte Blocker™ as indicated.

Fixation

Fixation is a common practice used to help lock proteins in place. Fixation is performed for a variety of reasons including timing/convenience, safety for biohazardous samples, or staining of intracellular targets. If fixation is performed prior to surface staining, it can alter the epitope in a way that interferes with antibody binding. Alternatively, if fixation is performed after surface staining, some fixatives may quench fluorescent signal, particularly signal from protein-based fluorophores such as PE or APC and their tandems. However in most cases, they should be able to withstand a standard 1-4% PFA fixation.

Learn more at: biolegend.com/fixation

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