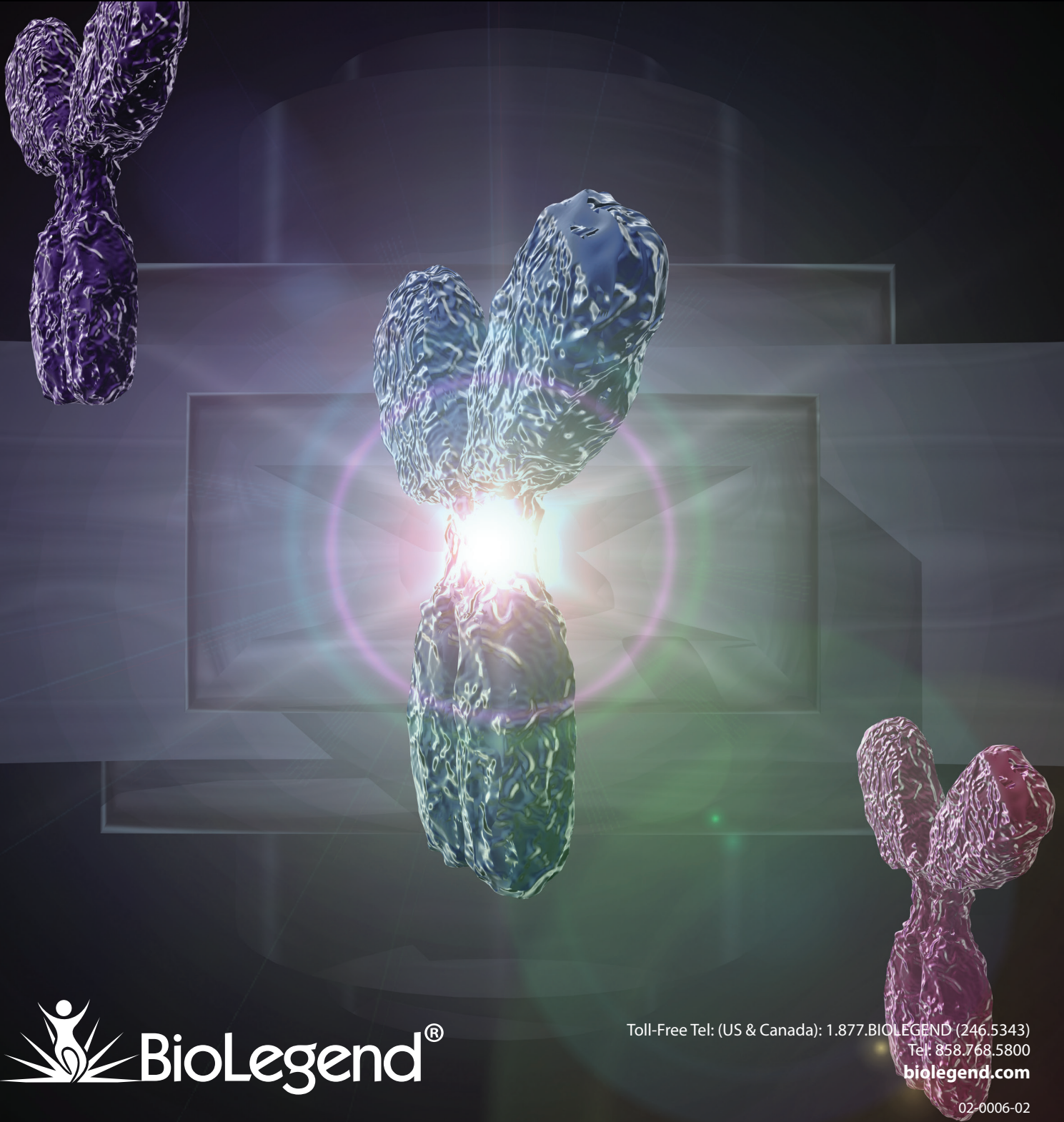# Build a Better
Multicolor Flow Cytometry Assay

![BioLegend®]

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Build a Better
Multicolor Flow Cytometry Assay:

Flow cytometric assays of four to six colors are actually not difficult to construct or analyze. At this number, choose only the brightest fluorophores and ensure that the chosen fluorophores have minimal spectral overlap. Many researchers want greater than six colors, maximizing the number of markers due to limited sample volume, or to see the inter-connectivity 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 increases, so do the complexities of experimentation 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.

Sensitivity = Signal Brightness − Background (Noise)

Event Brightness = Fluorophore Brightness ×
Degree of labeling of the Antibody ×
Expression level of the Antigen per Cell

Multicolor Panel Balance:

To build a multicolor flow cytometry assay that results in well-resolved populations, and consistent, reproducible statistical results, the following variables need to be understood and balanced:

Instrument Specifications:
Understand your instrument specifications and how to use it effectively

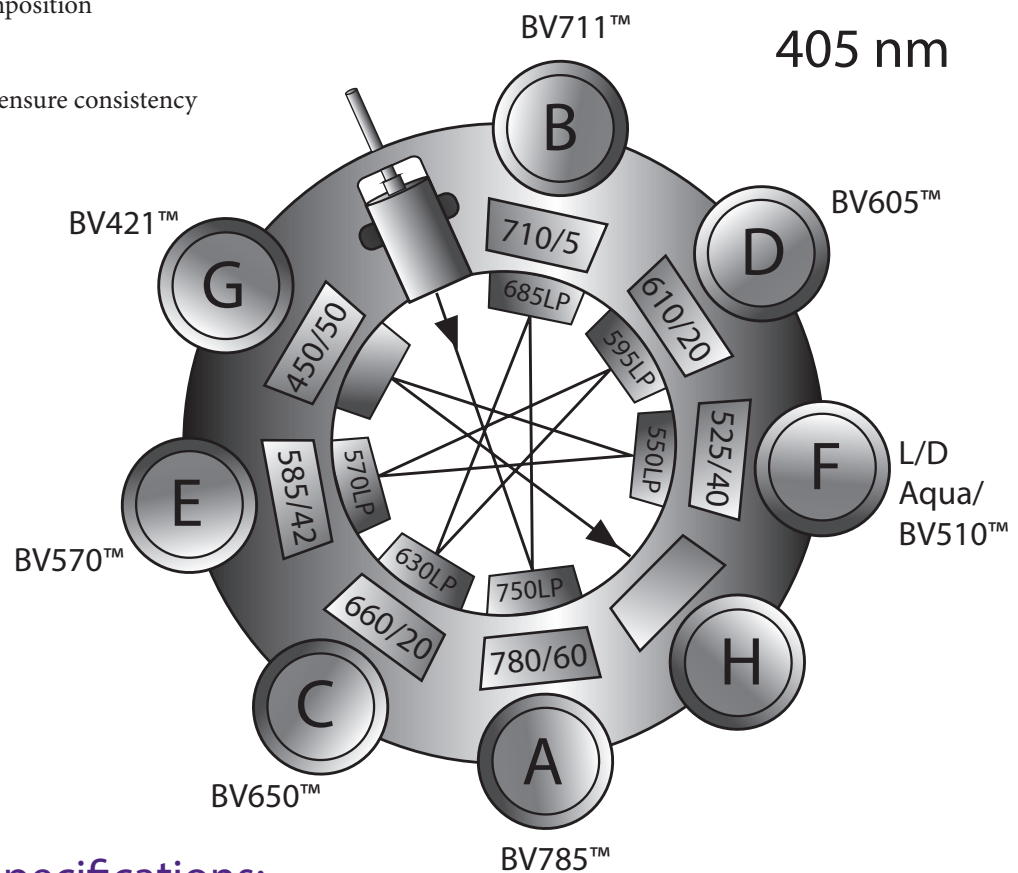### Balance Antigen Expression vs. Fluor Brightness:
Balance fluorophore brightness with level of antigen expression, consider innate biological or assay concerns

Organize a Balanced Panel:
Organize a balanced panel composition

Optimize and Control:
Utilize appropriate controls to ensure consistency

BV711™

405 nm

BV421™

BV605™

L/D Aqua/ BV510™

BV570™

BV650™

BV785™

710/5
685LP
610/20
595LP
450/50
525/40
585/42
570LP
550LP
630LP
750LP
660/20
780/60

B
D
G
F
E
C
A
H

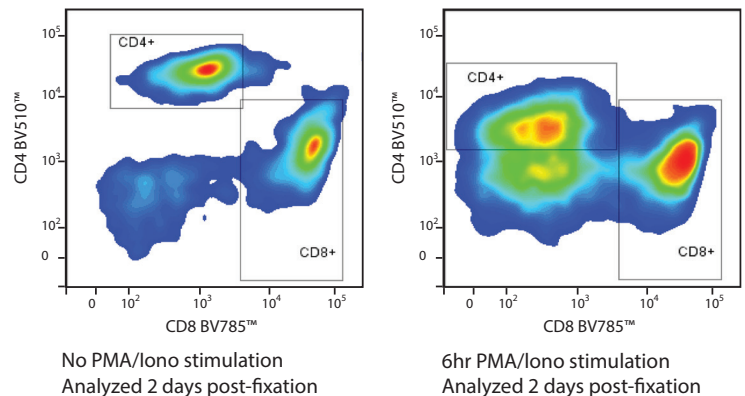## Instrument Specifications:

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. Above is an example of a common filter configuration for the violet laser used to detect the Brilliant Violet™ family of fluorophores. It is a good idea to reference the instrument's specifications while constructing a multicolor panel.

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 antibody to ensure the best chance of adequate detection.

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

In addition to dramatically affecting the signal, disease state and exogenous treatment can cause an increase in autofluorescence in certain channels, which may affect sensitivity.

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.

No PMA/Iono stimulation
Analyzed 2 days post-fixation

6hr PMA/Iono stimulation
Analyzed 2 days post-fixation

3. Refer to the following chart to match the potential abundance of the antigen in your assay with an appropriately bright fluorophore relevant to the application. Here, every fluorophore was conjugated to CD8 to construct an accurate ranking based on staining index $(MFI^+ - MFI^-)/(2 \times SD\ MFI^-)$.

- Brighter fluorophores are not always better fluorophores. For example, PE-Cy5 is very bright, but its significant cross-beam excitation by the red laser causes unwanted background in the Alexa Fluor® 647/APC channel. FITC is not a very bright fluorophore, but it emits into a channel with little background and has been conjugated to most commercially available antibodies. Staining index is just a tool to help you achieve balance in panel composition.

- A tandem fluorophore is the union of two fluorescent molecules in a FRET (fluorescence resonance energy transfer) relationship. A donor fluorophore, such as PE, APC or Brilliant Violet 421™ (BV421™) is excited by the excitation source, and upon resonating that energy, transfers it to an acceptor fluorophore, such as Cy7 in the PE-Cy7 tandem. Tandems are not 100% efficient at transferring energy, so there is always some emission of the donor detected in its own PMT.
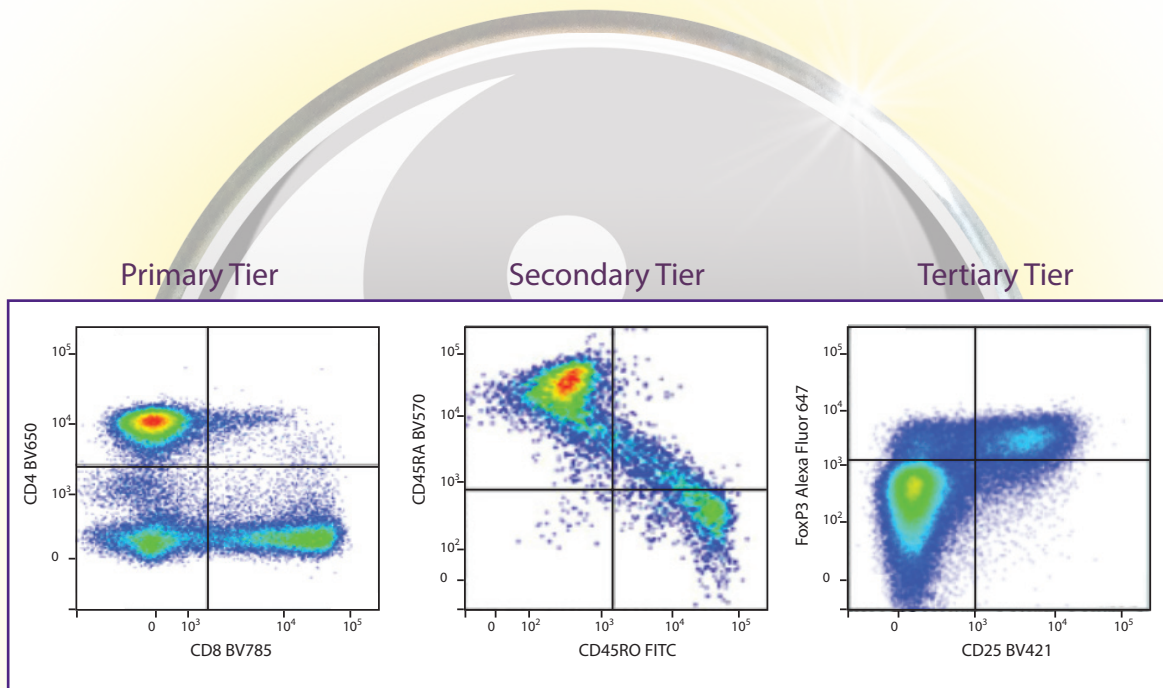
Learn more at: **biolegend.com/tandem_dyes**

CD8-Formats	405/488/561/640/ + filters	Staining Index	Brightness ranking (1-5)
PE	582/15	953	5
PE-Cy5	660/20	741	5
BV421™	450/50	718	5
PE-Cy7	780/60	567	5
APC	670/30	453	4
BV605™	610/20	355	4
BV650™	660/20	145	3
BV711™	710/50	144	3
BV510™	510/50	138	3
Alexa Fluor®647	670/30	127	3
APC-Cy7	780/60	123	3
BV785™	780/60	119	3
BV570™	585/30	112	3
PerCP-Cy5.5	710/50	90	2
PerCP	670/30	89	2
FITC	530/30	56	2
Pacific Blue™	450/50	54	2
Alexa Fluor®700	730/45	36	1

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, BV570™, BV785™ or APC-Cy7.

  Examples: basic cell surface phenotypic markers like CD4, CD8, CD56, CD11c, CD16.

- **Secondary tier antigens:** These markers are necessary for further phenotyping, but may change somewhat between parallel panels. Expression levels tend to be much more variable and are typically appropriate for moderately bright fluors like BV510™, BV650™, FITC or PerCP-Cy5.5.

  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. Often, markers in this category are commercially available only as purified antibody or in limited fluorophore selection. So for detection of these antigens, we want to save our brightest fluorophores, like BV421™, PE, APC or PE-Cy7.

  Examples: transcription factors, phospho-specific antibodies, and home-made hybridomas.
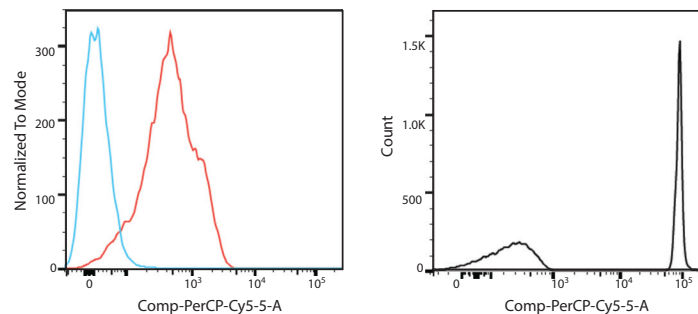
Primary Tier   Secondary Tier   Tertiary Tier

Optimize and Control:

Controls must be appropriate for the question or problem you are addressing.

1. **_Compensation Controls:_** Compensation controls need to be used every time you run an assay to ensure accuracy. Ideally, antibody capture beads will ensure that there are sufficient events in both the positive and negative peaks to populate the compensation algorithm accurately. It is very important to use the same antibody that you are using in the assay on the compensation beads. This is because tandem fluorophores, for example APC-Cy7, are sensitive to the light exposure they endure while you are staining your samples. In this example, some acceptor fluors like Cy7 will photobleach, changing the degree of spectral spillover of the APC-Cy7 into APC. However, since labeled compensation beads are often not treated the same way that cells are treated over the time course of staining, often not enduring the same fix/perm steps or light exposure as the stained samples, it is typically found that single stained biological controls can be more accurate in application.

2. **_Single Color Biological Controls:_** Single stained cells are useful to have on hand for many reasons. First, they can be used as back-up compensation controls if you realize that the compensation beads used are not entirely accurate. You can also use them to tune the voltage of the PMT at the start of the acquisition to ensure that your events are on scale, ideal for the fully stained sample, and within the linear range of the PMT. Calibration beads like CST are very important for ensuring consistent performance of the cytometer. However, the voltages that they recommend, especially for the violet laser, are often grossly inaccurate for the abundance of signal in the assay or the brightness of the fluorophore. You must optimize the PMT voltages to be relevant for the assay. Single stained biological controls also identify any non-specific staining, either due to insufficient titration of the reagent or denaturation of the antibody over time that may be causing a shift in the MFI^-.

Anti-hamster compensation beads labeled with anti-ms CD69 PerCP-Cy5.5

Blue = Unstained mouse splenocytes
Red = CD69 single stained mouse splenocytes from activation model

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.

View our webinar on Brilliant Violet™ Fluorophores in Multicolor Flow Cytometry:
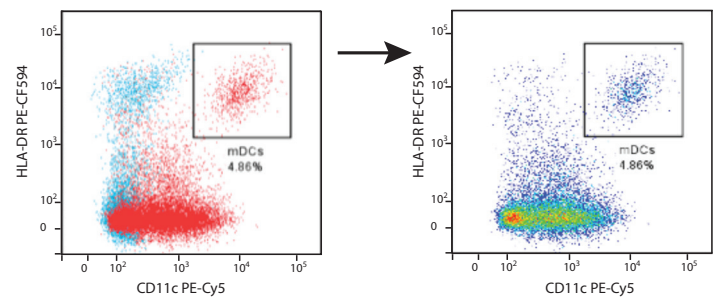

biolegend.com/brilliantviolet_webinar

3. *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 and are only truly necessary to aid in gating on a marker that lacks clear population resolution. FMOs are helpful to increase the reproducibility and decrease the statistical variance associated with these hard-to-gate markers by identifying the edge of the true negative population. In a multicolor assay of 10 colors or more, the additive increase in background signal may affect how you would place a gate excluding the negative population, since it may have shifted relative to the single stained control. FMOs also help you keep an eye on the consistent performance of your antibodies, especially tandem conjugates, to help identify problems associated with fluorophore degradation before it negatively impacts your results or creates statistical artifacts.

In this example, FMOs for both CD14 BV605™ **(Yellow)** 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.

4. *Isotype Controls:* Isotype controls are important if you suspect or are unsure 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 not an artifact with this 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.

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

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.

Multicolor Staining Guide

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

Multicolor Panel Selector

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

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