



Take Control

Crucial Controls for Flow Cytometry





There are many components to consider when planning a flow cytometry experiment. These include familiarizing yourself with the cytometer you will use, knowing the biology of your sample, choosing the right antibody and fluorophore combinations as well as the right controls, optimizing your staining protocol, and analyzing the data correctly. Following these critical steps will ensure your experiments are successful.

Controls in Flow Cytometry

Controls are vital to any flow experiment to reliably distinguish your results from background variation and nonspecific effects. Here you will learn about the essential controls you should include in your experiment and when to use them to obtain publication quality data.

Unstained Controls

Use unstained cells to set up the instrument so that all of your cells can be easily visualized on forward scatter (FSC) and side scatter (SSC) plots. Then set photomultiplier tube (PMT) voltages so that negative cells and dim signals can be distinguished from electronic noise while keeping bright cells within the scale. This will allow you to determine the level of background fluorescence or autofluorescence in your sample (Figure 1) and set your voltages appropriately for each fluorescence channel, ensuring all signals can be detected.

An alternative method for setting PMT voltages uses beads dyed with eight different fluorescence intensities. For this method, select the voltage that gives the maximum difference between the first and second fluorescence peaks to ensure optimum PMT sensitivity.

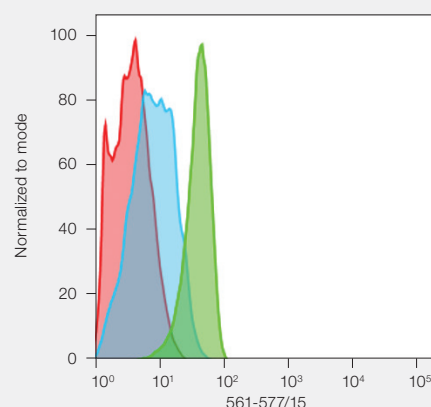
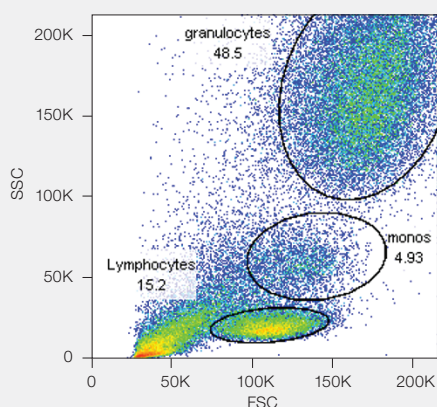


Fig. 1. Unstained peripheral blood. Unstained peripheral blood was used as a negative control to set the PMT voltage, SSC, and FSC (561 nm laser, 577/15 filter shown). The autofluorescence of different populations can be clearly observed in the histograms. Red, lymphocytes; blue, monocytes; green, granulocytes.

Fc Block Controls

Fc receptors are found on monocytes, macrophages, dendritic cells, and B cells. They bind antibodies via the constant Fc domain, rather than the antigen-specific Fab domain, leading to multiple antibodies binding to unintended targets.

This type of binding can lead to false positives, reduced resolution between the positive and negative cells, and poor data. Fc blocking reagents, such as Human Seroblock and Murine Seroblock FcR, can be added to your staining protocol to ensure that only antigen-specific binding is observed. Alternatively, diluted serum from the sample type will bind to Fc receptors (for example, mouse serum can be used for mouse cells).

Isotype Controls

Isotype controls were developed for surface staining. Their role is to ensure specificity of antibody binding. They are raised against an antigen, such as keyhole limpet hemocyanin or dinitrophenol, that is not found on the cell type or sample being analyzed.

An isotype control will:

- Determine the nonspecific binding of antibody to Fc receptors found on monocytes, macrophages, dendritic cells, and B cells

- Ensure the observed staining is due to specific binding rather than an artifact
- Reveal other nonspecific binding of the antibody or fluorophores to cellular components, such as RPE and FITC (Takizawa et al. 1993, Hulspas et al. 2009)

The role of isotype controls in determining background staining can be observed in Figure 2. The specific F4/80 staining can be clearly seen in Figure 2C.

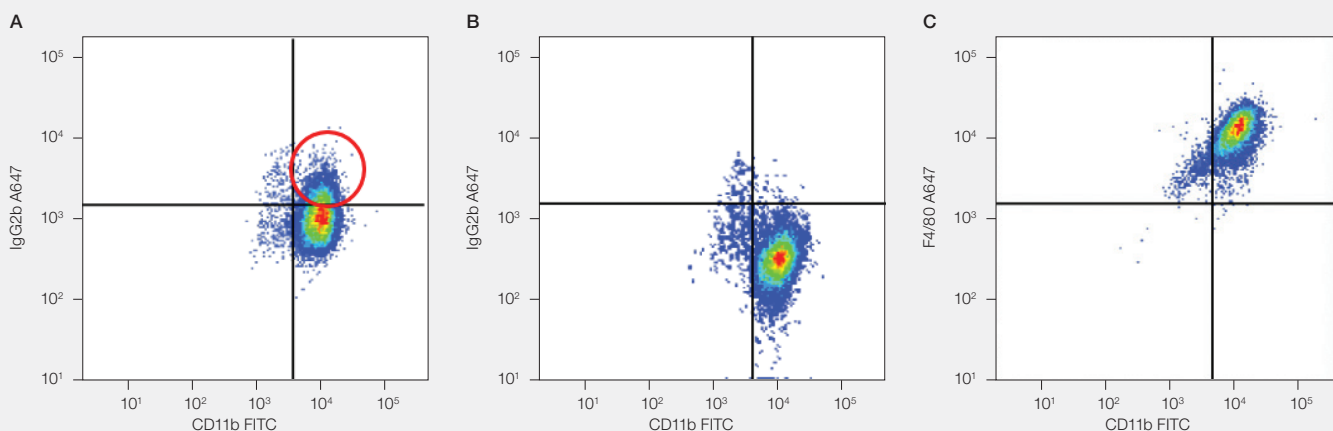


Fig. 2. J774 macrophages were stained with CD11b FITC (catalog #MCA74F) and IgG2b Alexa Fluor 647 (#MCA6006A647). **A**, in the absence of Fc block, a significant population of cells (circled in red) appears as positive, with the A647 isotype control showing the Fc binding. **B**, in the presence of Fc block (Mouse Seroblock FcR, #BUF041A), the positive cell population disappears. **C**, specific F4/80 A647 (#MCA497A647) staining.

The most appropriate isotype control matches:

- Host species
- Ig subclass
- Fluorophore of the primary antibody

As the fluorophore conjugation to the antibody (known as the F/P ratio) can vary between suppliers, we recommend purchasing the isotype control from the same supplier as the primary antibody. It is also advisable to use the isotype control at the same concentration as the primary antibody.

Nonspecific antibody binding can be reduced by:

- Blocking Fc receptors
- Adding bovine serum albumin (BSA) to your staining buffer
- Titrating your antibody
- Gating out dead cells using a live/dead marker

Viability Controls

The quality of your sample will determine the quality of your data.

Dead cells lead to:

- Greater autofluorescence
- Increased nonspecific antibody binding
- False positives
- Reduction in the detectable dynamic range
- Difficulty detecting dim and rare cells

While using a forward and side scatter gate will allow you to exclude debris and some dead cells, it will not remove them all and there will be dead cells within your gate. Therefore a viability dye should be included in your flow cytometry panel (Figure 3).

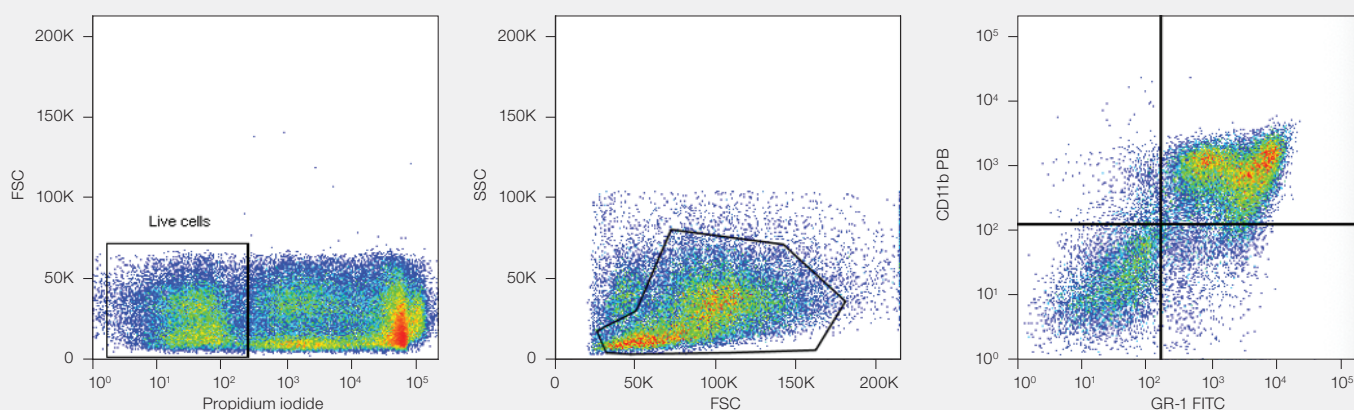


Fig. 3. Combining a viability dye and forward and side scatter gating. Using a combination of the viability dye ReadDrop Propidium Iodide (#1351101) and a forward and side scatter gate, CD11b- (#MCA711PB) and GR-1- (#MCA2387F) positive cells can be identified in murine bone marrow.

Types of Viability Dyes

DNA-binding dyes

These include propidium iodide, 7-aminoactinomycin D (7-AAD), and 4',6-diamidino-2-phenylindole (DAPI), among others, which will fluoresce upon binding to nucleic acid. They cannot pass through an intact cell membrane; therefore, only dead cells with a permeable membrane will fluoresce. When using these types of viability dyes, samples cannot be fixed, as this would make all of the cells permeable to the dyes.

Protein-binding dyes

These dyes covalently bind to free primary amines on proteins, which are present on the surface of cells. When a cell membrane is compromised, the dyes permeate the cells and react with intracellular primary amines. Greater fluorescence is observed in dead cells due to their increased content of accessible free amines, allowing them to be easily distinguished from live cells. VivaFix Cell Viability Assays are fixable viability dyes available in a wide range of excitation and emission spectra for convenient analysis and addition to multicolor flow cytometry panels.

Compensation Controls

Compensation controls are single-stained samples for each antibody in your panel and are essential for any multicolor experiment. They reveal the level of spillover of the fluorophore into the other detectors. The spillover can then be mathematically removed, ensuring only specific signals are used in the final analysis. This is known as compensation.

A compensation control must:

- Be used in every channel in which a fluorophore is used
- Be as bright as the sample

- Have a positive and a negative population in the sample
- Be the same fluorophore as the sample
- Have sufficient events collected (~5,000)

Once the compensation has been set, do not change the PMT voltages, as this will negate the compensation settings. Historically, compensation was performed manually by increasing or decreasing the compensation until the mean fluorescence intensity (MFI) of the negative and positive populations in neighboring detectors was equal. Luckily, modern software has enabled this process to be automated, thus improving accuracy.

Fluorescence Minus One (FMO) Controls

When acquiring and analyzing data, in addition to spillover, fluorescence spread can occur, which is particularly noticeable after compensation and from brighter fluorophores. Each FMO control, as the name suggests, is the addition of all fluorescently labeled antibodies in the panel minus one to see the influence of each fluorophore on the panel and its spread into neighboring channels (Figure 4). Ideally, an FMO control should be performed for all fluorophores in the panel, especially when starting a new multicolor panel.

Tube	FITC	PE	PE-A750	PE-Cy5
Unstained	–	–	–	–
FITC-FMO	–	+	+	+
PE-FMO	+	–	+	+
PE-A750-FMO	+	+	–	+
PE-Cy5-FMO	+	+	+	–

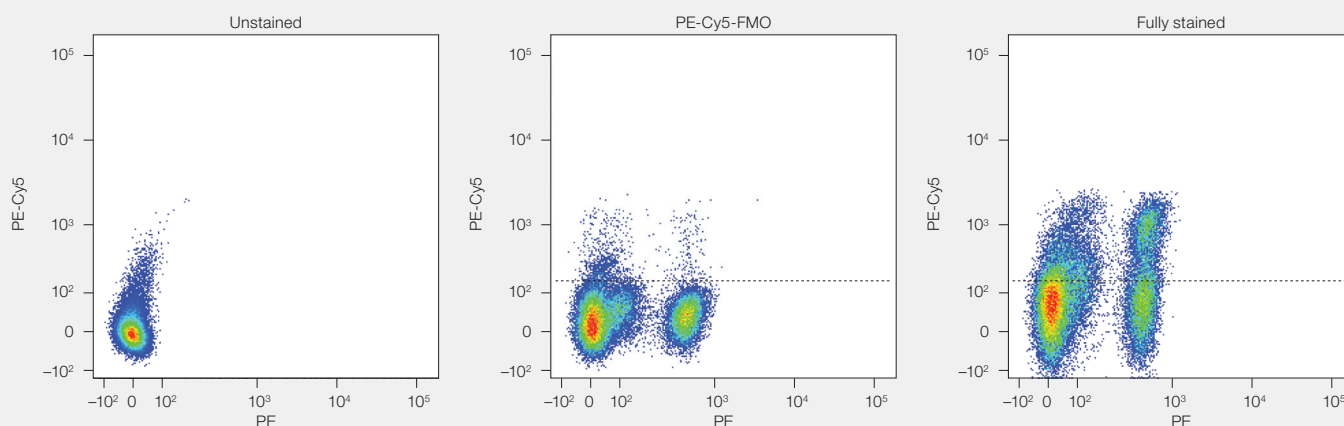


Fig. 4. Use of FMO controls to determine fluorescence spread. Dot plots showing the fluorescence spread into the PE-Cy5 channel in the PE-Cy5-FMO control compared to the unstained control, allowing correct gating (black dotted line) for the fully stained sample.

Intracellular Staining Controls

Intracellular staining can be more problematic than surface staining, often due to higher levels of background within cells caused by protein-protein interactions. Isotype controls have been optimized for cell surface staining, to control for nonspecific binding of antibody and fluorophore, so they may not always be suitable as an intracellular staining control.

Consider using:

- A negative cell line
- An antibody known to be negative on your cells
- A secondary antibody alone (if using primary and secondary antibodies)
- No permeabilization buffer
- Alternative fixation and permeabilization reagents

Biological Controls

In addition to staining and isotype controls, you should also consider biological controls that will enable you to determine staining specificity and experimental limitations. Biological controls are important for all staining, but especially for intracellular staining, which can have higher background fluorescence than cell surface staining.

Consider using:

- Known negative and positive samples
- Knockdown or knockout cells (RNAi or CRISPR-Cas9)
- Transfected cells
- Treated and untreated samples

For other experiments, such as cytokine release measurement or cellular activation, unstimulated and fully stimulated controls are important. This will determine both positive and negative results and the dynamic range of fluorescence staining in your experiment, as well as ensure the antibody is performing as expected. An example of this can be seen in Figure 5, in which human peripheral blood lymphocytes were stained for CD154 on stimulated and unstimulated cells.

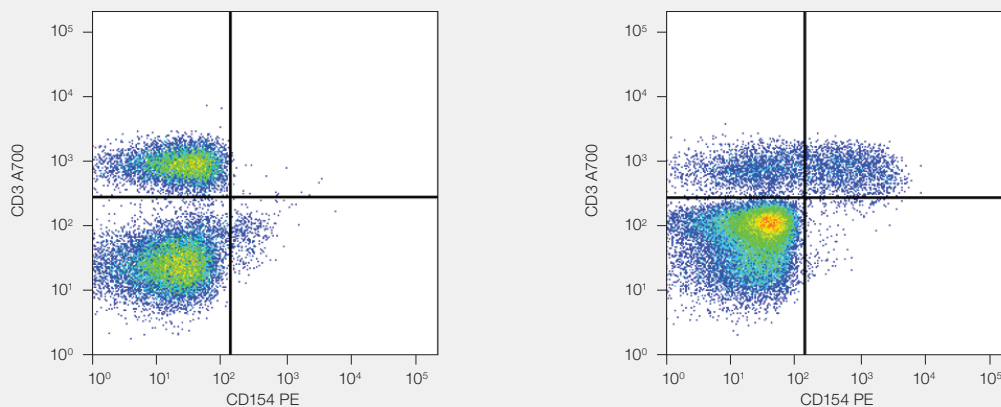
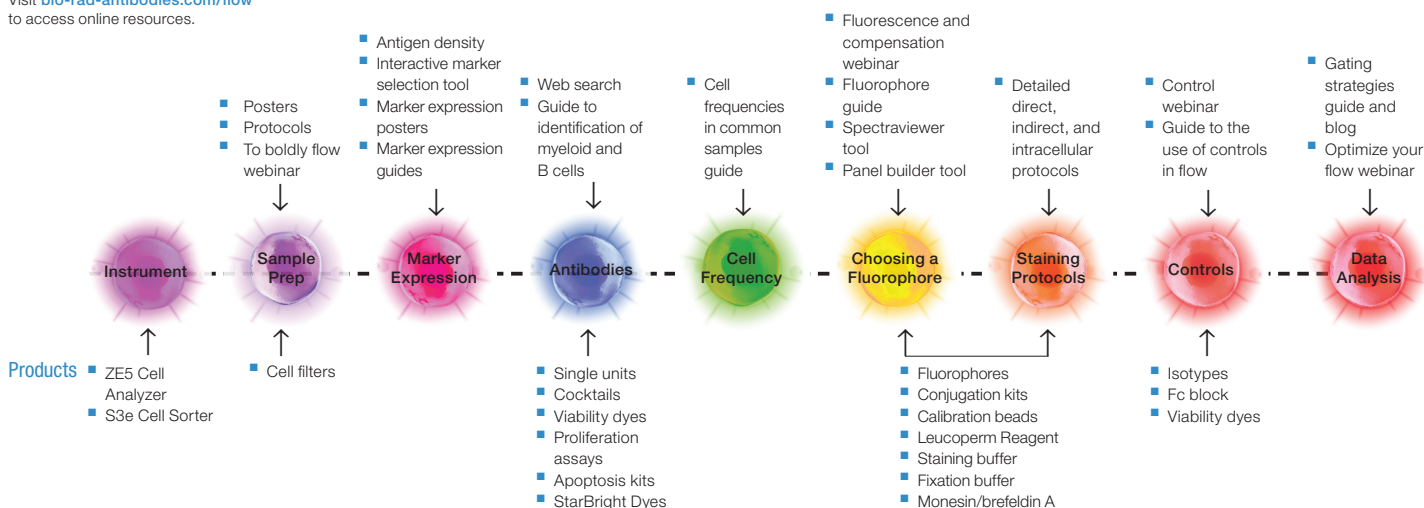


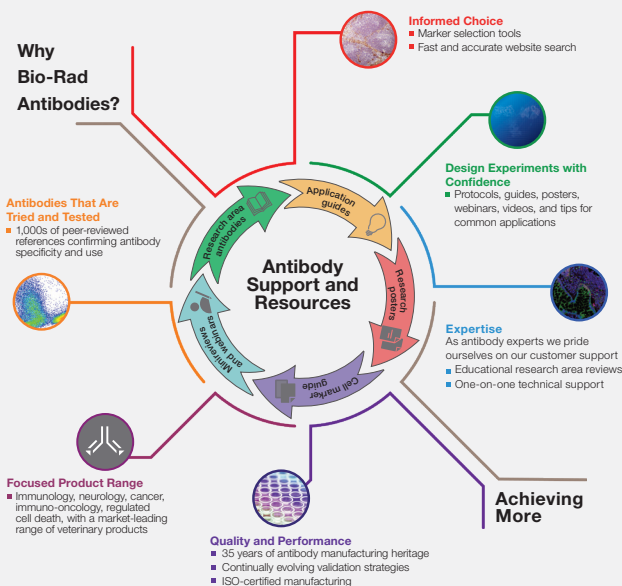
Fig. 5. Use of stimulated and unstimulated controls. Human peripheral blood was unstimulated (left) or stimulated (right) with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 hr and then stained for CD3 (#MCA463A700) and CD154 (#MCA1938PE).

Resources

Visit bio-rad-antibodies.com/flow to access online resources.



Think Antibodies. Think Bio-Rad.



References

Hulspas R et al. (2009). Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom* 76, 355–364.

Takizawa F et al. (1993). Binding of phycoerythrin and its conjugates to murine low affinity receptors for immunoglobulin G. *J Immunol Methods* 162, 269–272.

Visit bio-rad-antibodies.com/flowcontrols to find out more about controls.

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