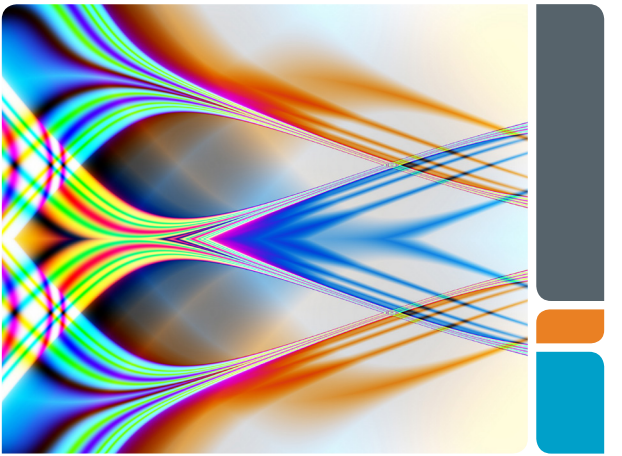
Flow Cytometry - The Essentials



Pocket Guide to Flow Cytometry

BIO-RAD

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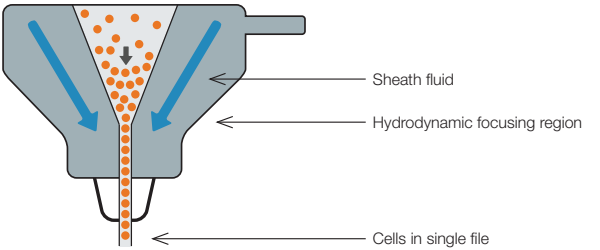
1. Know your Cytometer

The basis of flow cytometry is the measurement of how light is scattered in the forward or side direction as it passes through a particle. There are 3 essential components.

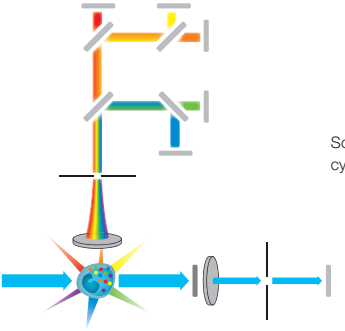
Fluidics: an outer sheath fluid running at higher pressure encloses and focuses the sample creating a single file of particles. This allows one particle at a time to be measured.



Lasers: multiple lasers of different wavelengths can be used to excite different fluorophores attached to a particle, e.g. 488 nm and FITC, 640 nm and APC. These can be focused together or be spatially separated.

Filters and mirrors: the arrangement of filters and mirrors, known as the configuration, allows separation of the light and specific wavelengths to be measured.

Short pass – light below a specific wavelength
Long pass – light above a specific wavelength
Band pass – light within a specified range



Schematic of a typical flow cytometer setup

Before you start

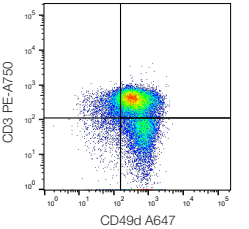
- Do you have a single cell sample at the right concentration?
- Do you know your lasers?
- Do you know what filters you have?

Find out more at
bio-rad-antibodies.com/flow-principles

2. Understanding Fluorescence and Fluorophores

Fluorophores are fluorescent molecules, which can be attached to antibodies to allow detection of cellular markers. They accept light energy (for example, from a laser) at a given wavelength and re-emit it at a longer wavelength as it has lower energy. The wavelengths of greatest absorption and emission are termed maximal absorbance and maximal emission wavelengths.

In addition to single dyes, tandem dyes comprise a small fluorophore covalently coupled to another fluorophore. When the first dye is excited its energy is transferred and activates the second fluorophore, which then produces the fluorescence emission.



Data acquired on the ZE5 Cell Analyzer.

Ensure you have the right fluorophore

Ensure the fluorophore is excited by the correct laser and the filters in the cytometer are compatible with the maximal emission to ensure optimal signal detection.

Table 1. Fluorophores for flow cytometry.

Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
DyLight 405		400	420	3
Alexa Fluor 405		401	421	3
Pacific Blue		410	455	1
Alexa Fluor 488		495	519	3
FITC		490	525	3
DyLight 550		562	576	4
PE*		496, 546	578	5
APC		650	661	4
Alexa Fluor 647		650	665	4
DyLight 650		654	673	4
PerCP		490	675	2
Alexa Fluor 700	Infrared	702	723	2

* PE is the same as R-phycoerythrin.

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Table 2. Tandem dyes for flow cytometry.

Fluorophores	Fluorescence Color	Maximal Absorbance, nm	Maximal Emission, nm	Relative Brightness
PE–Alexa Fluor 647		496, 546	667	4
PE–Cy5		496, 546	667	5
PerCP-Cy5.5		490	695	3
PE–Cy5.5		496, 546	695	4
PE–Alexa Fluor 750	Infrared	496, 546	779	4
PE–Cy7	Infrared	496, 546	785	4
APC–Cy7	Infrared	650	785	2

* PE is the same as R-phycoerythrin.

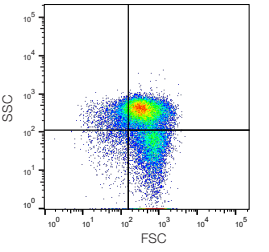
APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

Find out more at
bio-rad-antibodies.com/fluorophores

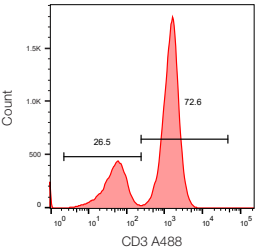
3. Gating Process

Gates and regions can be placed around populations of cells with common characteristics to investigate and quantify populations you are interested in.

Forward and side scatter can give an indication of the size and granularity. Similar cells will group together.

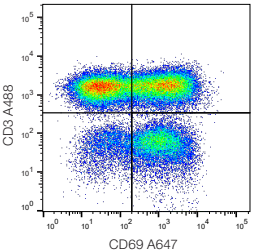


Histograms are single parameter plots that give information on the percent and number of cells positive for any given marker. The positive or negative cells can be selected to measure other parameters.



Data acquired on the ZE5 Cell Analyzer.

Two–parameter dot-plots display two parameters, giving you more information, allowing you to identify single positive or dual positive cells.



Data acquired on the ZE5 Cell Analyzer.

The simple principle of sequential gating can be applied to further characterize and identify cell populations based on an increasing number of markers.

Ensure you collect enough cells, because as you refine your populations the number of cells can significantly decrease.

Don’t be afraid to try lots of different gating combinations and strategies in your analysis, your cells may not be where you expect!

Find out more at
bio-rad-antibodies.com/gatingstrategies

4. Controls

Here are the essential controls you should include in your flow cytometry experiment:

Unstained – determine where your cells are and what is negative.

Isotype controls – determine specific surface staining, have you got the right isotype?

- Same species
- Same subclass
- Same fluorophore

Biological controls – essential for most experiments, not just flow cytometry, make sure you include these controls.

- Known positive
- Known negative
- Unstimulated
- Fully stimulated
- Fixation and permeabilization controls

Compensation controls – single stains to determine fluorophore spectral overlap, have you got the right stain?

- Positive and negative population
- Bright staining
- Same fluorophore
- Collect enough events

Fc block – some cells have Fc receptors which can increase background staining, including;

- Macrophages
- B cells
- NK cells
- Dendritic cells

Fluorescence minus one control – after compensation, fluorescence spread can be a problem. Set your gates accurately in a multicolor panel using an FMO control, where all antibodies bar one are included.

Find out more at
bio-rad-antibodies.com/fc-controls

5. Optimization

Good sample prep – in order to measure single cells and get reliable data, your sample also has to be as good as it can be. Here are some tips to healthy cells.

- Defrost cells quickly removing DMSO
- Prepare cell suspensions as gently as possible
- Use enzymes if necessary to extract cell populations
- Remove contaminating tissue
- Use the appropriate anticoagulant
- Avoid vortexing and leaving a dry pellet
- Keep cells on ice if possible

Include a viability dye – dead cells have increased autofluorescence, bind antibodies non specifically, and reduce the dynamic range. Gating on forward and side scatter may not be sufficient to remove the dead cells.

Doublets – plot the height or width against the area to exclude doublets from your analysis to avoid unwanted false positives.

Collect enough cells – the number of cells you need to collect during analysis to have significant data can vastly differ depending on the sample and frequency of your cells. The table below shows an example of how the frequency of cells can affect the number of cells collected. Knowing your cell frequency will help your experiment.

Starting Population	Frequency	Number Collected
1,000,000	10%	100,000
1,000,000	1%	10,000
1,000,000	0.1%	1,000

Intracellular staining – extra steps such as fixation and permeabilization can alter your staining. Choose your fixative carefully as it can alter your staining and how long you can store your sample. Permeabilization strength may need to be different depending on your antigen. Alcohol fixation and permeabilization is best for DNA staining.

Find out more at
bio-rad-antibodies.com/flow-optimization

6. Panel Building

Simple rules to guide you through multi color experimental design.

Fluorophores – separate fluorophore excitation across lasers, and where possible, the emission across the detectors. This will minimize the amount of spillover.

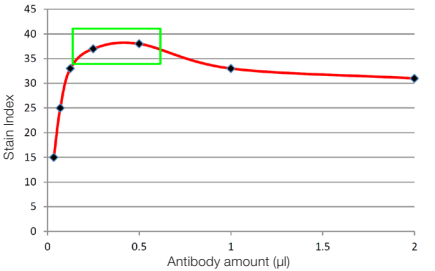
Antigen density – as a general rule it is best to match bright fluorophores (e.g. PE) with low expressing markers and dimmer fluorophores (e.g. Pacific Blue) with highly expressed markers.

Expression patterns – place fluorophores with spillover on mutually exclusive markers and co-expressing markers should be identified using fluorophores with little spillover. Unknown expression and activation markers should also be placed away from potential spillover conflicts.

Dump channels – this removes all unwanted cells by labeling them with the same fluorophore, allowing you to ignore both the cells and the fluorescence from those cells. A viability stain can be included in this channel for convenience.

Antibody titration – excess antibody can bind at low affinity creating background fluorescence that can reduce the resolution of signal. Determine the best antibody concentration by diluting the antibody to the concentration that gives the best stain index to both improve your experiment and save you money.

Find out more at
bio-rad-antibodies.com/flow-multicolor



Online Resources

Check fluorophore excitation and emission spectra compatibility using our spectraviewer, or use our online panel builder to build panels in just a few simple steps.

bio-rad-antibodies.com/spectraviewer

bio-rad-antibodies.com/panel-builder

7. Simple Staining Protocols

To have the best staining, ensure sample preparation is optimized before you start.

These short protocols are a good starting point but some optimization may be required depending on sample and antibody used.

Surface staining

1. Wash 1 x 10^6 cells and resuspend in cold PBS/BSA
2. Incubate with antibody in a small volume for 30 min at 4°C, avoiding direct sunlight
   - For indirect staining wash the cells in PBS/BSA, then add secondary reagent at vendor recommended dilution
3. Wash with cold PBS
4. Fix if necessary in 1% PFA
5. Acquire

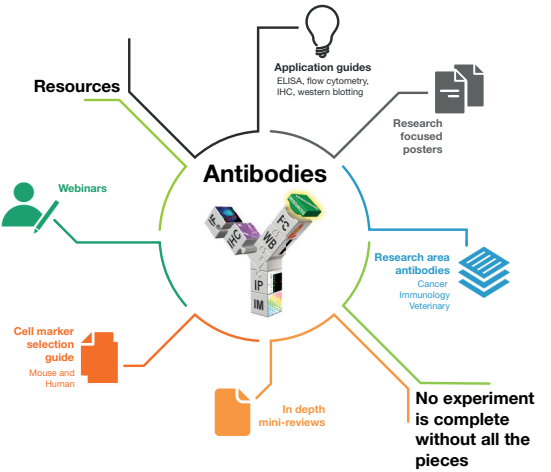
Intracellular staining using Leucoperm

1. Wash 1 x 10^6 cells and resuspend in PBS/BSA
2. If required stain surface epitopes
3. Wash cells in cold PBS/BSA
4. Resuspend in Leucoperm Reagent A (fixative)
5. Wash in PBS/BSA
6. Resuspend in Leucoperm Reagent B (permeabilization reagent)
7. Incubate with antibody in a small volume for 30 min at 4°C, avoiding direct sunlight
8. Wash with PBS
9. Acquire

Other fixatives and permeabilization reagents can be used and some optimization may be required depending on the sample and location of epitope.

Find out more at
bio-rad-antibodies.com/fc-protocols

Think Antibodies. Think Bio-Rad.



To request or download your copy of the flow cytometry guide go to **bio-rad-antibodies.com/flowguide**

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