Flow Cytometry

Flow Cytometry — The Essentials

Pocket Guide to Flow Cytometry

Visit bio-rad-antibodies.com/flow for more information.

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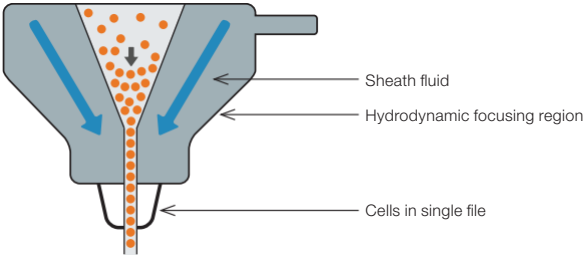
1. Principles of the Flow Cytometer

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 three essential components.

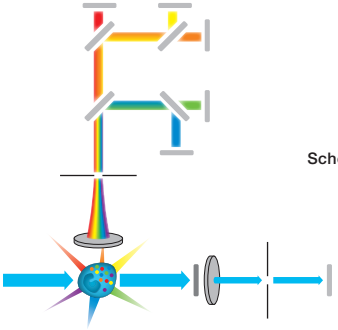
Fluidics — an outer sheath fluid running at higher pressure encloses and focuses the sample creating a single file of particles. This allows one particle at a time to be measured.



Lasers — multiple lasers of different wavelengths can be used to excite different fluorophores attached to a particle, for example 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.

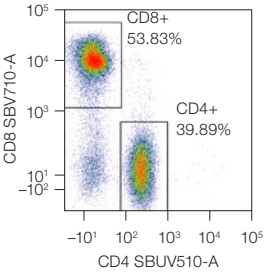
Before you start

- 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. Principles of Fluorescence

Fluorophores are fluorescent molecules that 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, respectively.



Data acquired on the ZE5 Cell Analyzer.

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.

Find out more at **bio-rad-antibodies.com/StarBright**.

Bio-Rad™ StarBright™ Dyes are a new range of superior fluorescent dyes available for all common laser lines. StarBright Dye–labeled antibodies are available conjugated to highly cited, popular immunology markers.

Ensure you have the right fluorophore

Ensure fluorophore excitation and emission spectra are compatible with your cytometer's laser and filter configuration.

Fluorophores for flow cytometry.

Fluorophores	Fluorescence Color	Max Absorbance, nm	Max Emission, nm	Relative Brightness
StarBright UltraViolet 400		335	394	
DyLight 405		400	420	
Alexa Fluor 405		401	421	
StarBright Violet 440		383	436	
StarBright UltraViolet 445		347	440	
Pacific Blue		410	455	
StarBright Violet 475		405	479	
StarBright UltraViolet 510		340	513	
StarBright Violet 515		402	516	
DyLight 488		493	518	
Alexa Fluor 488		495	519	
FITC		490	525	
StarBright UltraViolet 575		340	569	
StarBright Violet 570		402	571	
DyLight 550		562	576	
PE		496/546	578	
StarBright Yellow 575		548	579	
StarBright Blue 580		475	582	
StarBright Yellow 605		572	606	
StarBright Violet 610		403	607	
StarBright UltraViolet 605		340	609	
StarBright Blue 615		475	612	
APC		650	661	
Alexa Fluor 647		650	665	
StarBright Red 670		653	666	
StarBright Violet 670		401	667	

continued

Fluorophores for flow cytometry (cont)**.**

Fluorophores	Fluorescence Color	Max Absorbance, nm	Max Emission, nm	Relative Brightness
PE-Alexa Fluor 647		496, 546	667	
PE-Cy5		496, 546	667	
StarBright UltraViolet 665		340	669	
StarBright Yellow 665		554	670	
DyLight 650		654	673	
PerCP		490	675	
PerCP-Cy5.5		490	695	
PE-Cy5.5		496, 546	695	
StarBright Blue 700		473	703	
StarBright Red 715	Infrared	638	712	
StarBright Violet 710	Infrared	402	713	
StarBright Yellow 720	Infrared	549	719	
Alexa Fluor 700	Infrared	702	723	
StarBright UltraViolet 740	Infrared	344	743	
StarBright Violet 760	Infrared	403	754	
StarBright Blue 765	Infrared	476	764	
StarBright Red 775	Infrared	653	778	
PE-Alexa Fluor 750	Infrared	496, 546	779	
StarBright Violet 790	Infrared	402	782	
PE-Cy7	Infrared	496, 546	785	
APC-Cy7	Infrared	650	785	
StarBright Yellow 800	Infrared	549	788	
StarBright UltraViolet 795	Infrared	340	792	
StarBright Blue 810	Infrared	477	802	
StarBright Red 815	Infrared	654	811	

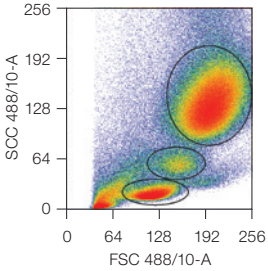
* PE is the same as R-phycoerythrin.

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

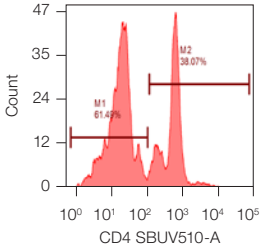
Find out more at **bio-rad-antibodies.com/fluorophores**.

3. Data Analysis

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

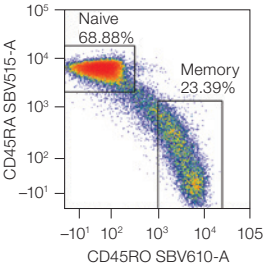


Forward and side scatter can give an indication of the size and granularity. Similar cells will group together.



Histograms are single parameter plots that give information on the percentage 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 and allowing you to identify single- or dual-positive cells.

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

Collect enough cells because the number of cells can significantly decrease as you refine your populations.

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 in Flow

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; do you have the right isotype?

- Same species
- Same subclass
- Same fluorophore

Biological controls — essential for most experiments, not just flow cytometry. Include the following controls:

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

Compensation controls — single stains to determine fluorophore spectral overlap; do you have the right stain?

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

Fc block — Fc receptors, which can increase background staining, are present on cells such as:

- Macrophages
- B cells
- NK cells
- Dendritic cells

Fluorescence minus one (FMO) 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. Optimizing Your Experiments

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. Keep cells healthy with these tips:

- Defrost cells and remove DMSO quickly
- Prepare cell suspensions as gently as possible
- If necessary, use enzymes 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 nonspecifically, 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 and 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 cell frequency can affect the number of cells collected. Knowing your cell frequency will help when planning 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 sample staining and storage. Different antigens require different permeabilization strengths. For DNA staining, alcohol fixation and permeabilization are best.

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

6. Multicolor Panel Building

Here are some simple rules to guide you through multicolor experimental design.

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

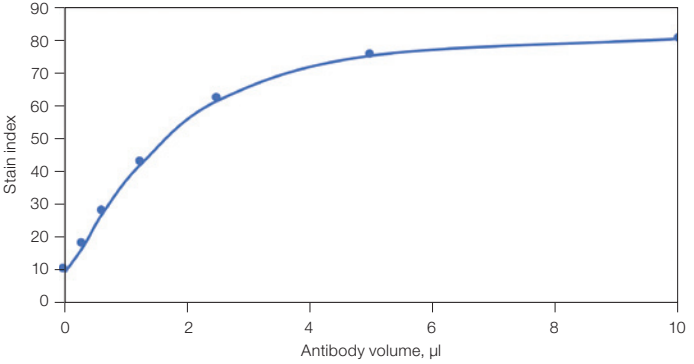
Antigen density — as a general rule it is best to match bright fluorophores (e.g. PE) with poorly expressed markers and dimmer fluorophores (e.g. Pacific Blue) with highly expressed markers.

Expression patterns — place fluorophores with spillover on mutually exclusive markers, and coexpressing 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 – these remove 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 with low affinity, creating background fluorescence that can reduce signal resolution. 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 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. Common 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 phosphate buffered saline (PBS)/bovine serum albumin (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% paraformaldehyde (PFA).
5. Acquire.

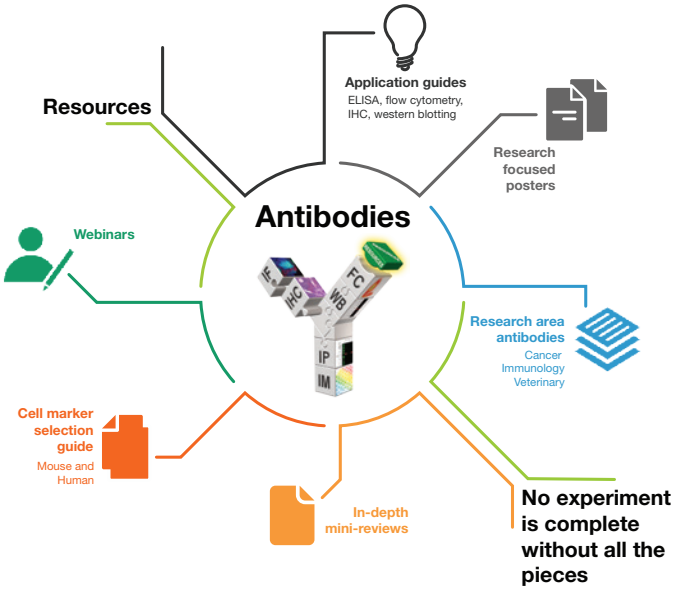
Intracellular staining using Leucoperm Reagent

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. Some optimization may be required depending on the sample and location of epitope.

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

8. Flow Cytometry Resources



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

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