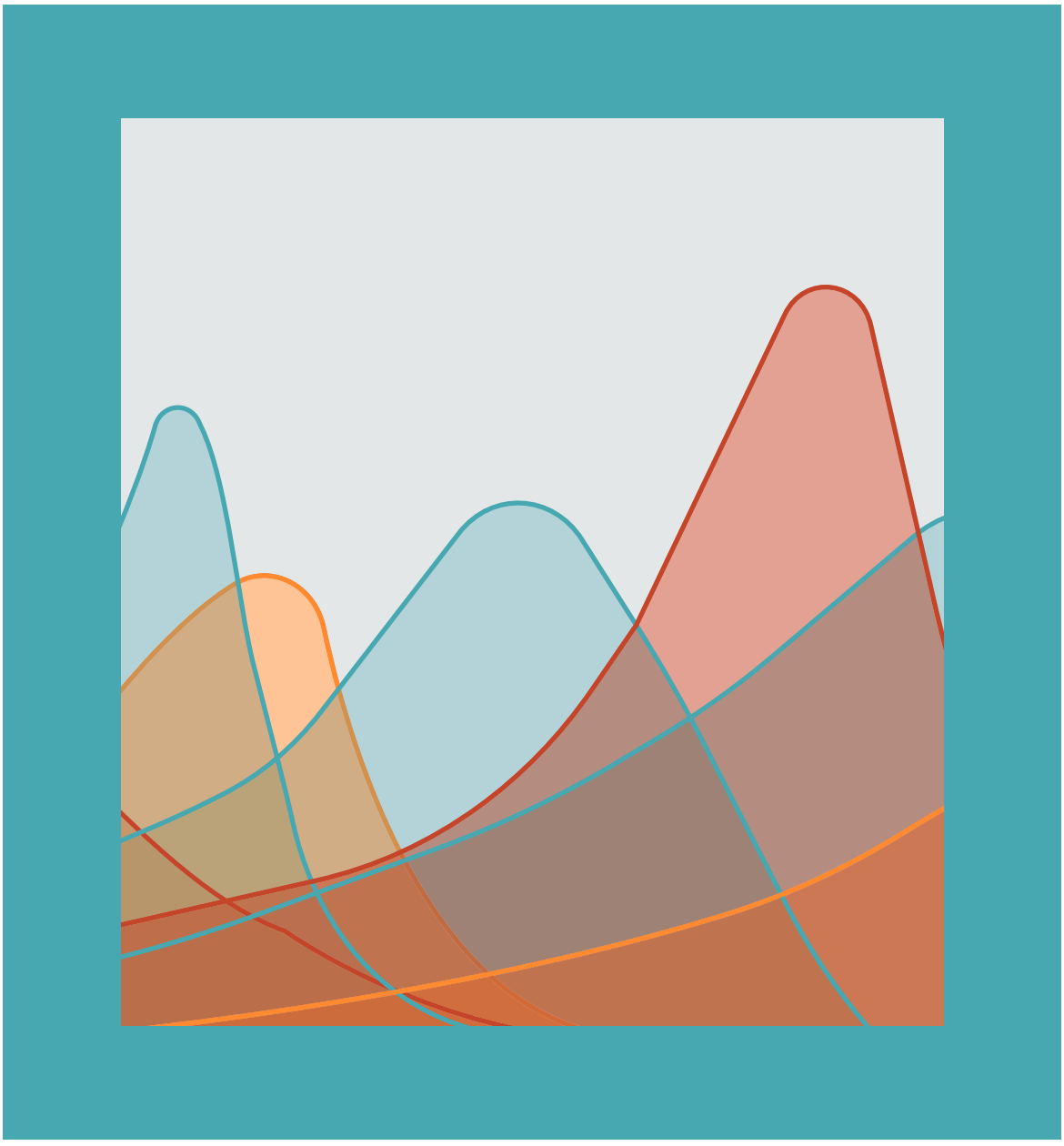
The complete flow cytometry guide

The essential guide to support your flow and multicolor flow cytometry experiments



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Introduction to flow cytometry

Flow cytometry is a widely used, laser-based method for analyzing the expression of cell surface and intracellular molecules.

There are many applications of flow cytometry in research and diagnostics, including simultaneous, multi-parameter analysis of single cells and characterizing and defining different cell types in heterogeneous cell populations. Flow cytometry assays can also assess the purity of isolated subpopulations, analyze cell size and volume, and sort different cell populations, known as fluorescence-activated cell sorting (FACS)[1].

A flow cytometry test measures fluorescence intensity produced by fluorescently labeled antibodies specific to proteins on or in cells or ligands that bind to specific cell-associated molecules, such as propidium iodide binding to DNA.

The staining procedure involves making a single-cell suspension from cell culture or tissue samples. Then, the cells are incubated in tubes or microtiter plates with unlabeled or fluorophore-labeled antibodies and analyzed on the flow cytometer.

Multicolor flow cytometry takes this further by analyzing multiple parameters on thousands of single cells or other particles in seconds[2,3]. In multicolor flow cytometry, fluorescent markers are used to characterize and define different cell types of interest in heterogeneous cell populations, assess the purity of isolated subpopulations, and analyze cell size and shape.

In this guide, we'll take you through flow cytometry basics, principles, protocols, and analysis to give you an advanced understanding of how flow cytometry works, when it is valuable, and how to do it. You will also find everything you need to quickly and easily set up your multicolor flow cytometry experiment, from instrumentation basics to recommended controls and data analysis.

Understand the instrumentation basics

The flow cytometer

When running a cell suspension through a flow cytometer, sheath fluid hydrodynamically focuses cells to get them to pass in a single file through a small nozzle. The resulting tiny stream of fluid takes cells one at a time past a laser light, as shown in Figure 1[4].

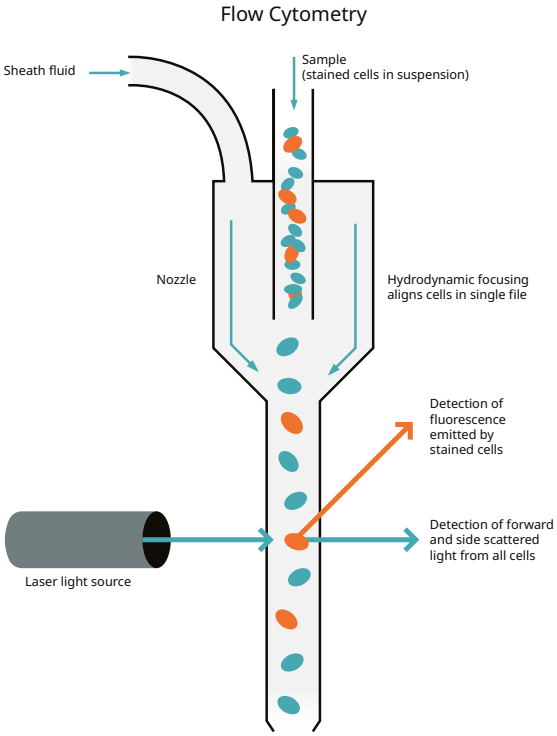


Figure 1. Diagram showing an overview of the flow cytometer. Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam one at a time. Forward and side scattered light is detected, with fluorescence emitted from stained cells.

The multicolor flow cytometer

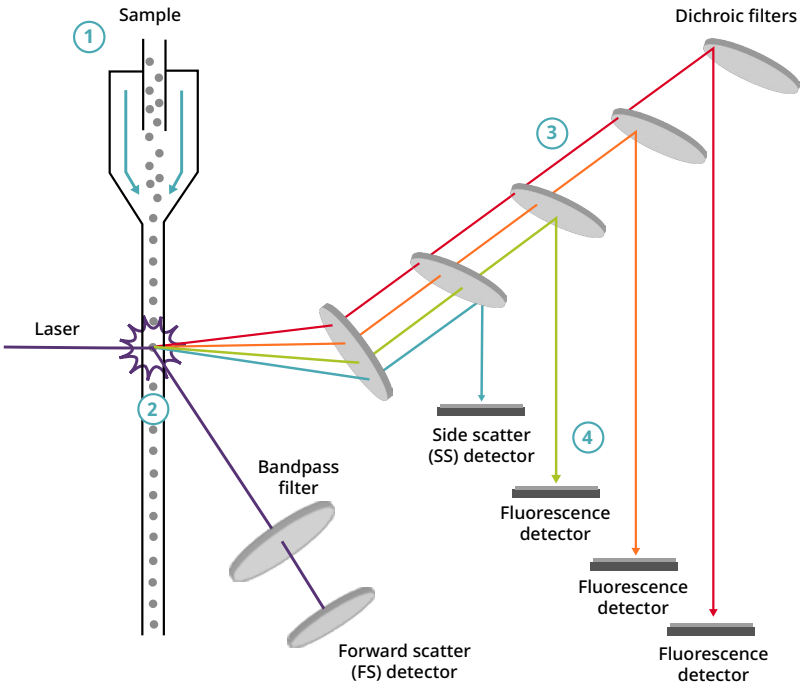


Figure 2. Overview of basic multicolor flow cytometry technology

1. When a sample is introduced into the multicolor flow cytometer flow chamber, it enters the fluidics system and separates into single cells in a process known as hydrodynamic focusing. Hydrodynamic focusing uses a controlled fluid flow to focus the sample into a narrow diameter, causing the cells to separate and align in a single file.
2. As each cell passes the laser, the instrument records it as an event. For each event, forward scatter (FS) and side scatter (SS) are subsequently recorded. If a cell is fluorescently labeled, the laser excites the fluorophore, and the emitted light collects as fluorescence intensity.
3. For the instrument to detect the specific wavelength emitted by a fluorophore, the emitted light is passed through a series of mirrors and filters until it reaches the appropriate detector. Detectors are known as photomultiplier tubes (PMTs) and will only detect fluorescence at a specific wavelength.
4. Optical filters block certain wavelengths and let others pass. When placed at an angle, a dichroic filter acts as a mirror, allowing specific wavelengths to pass through while reflecting others. The type and order of dichroic filters allow the simultaneous detection of multiple signals.

Measurement of forward and side scattered light

All cells or particles passing through the beam scatter laser light, measured as FS by detectors in front of the light beam, and SS, measured from detectors to the side of the light beam (Figure 3).

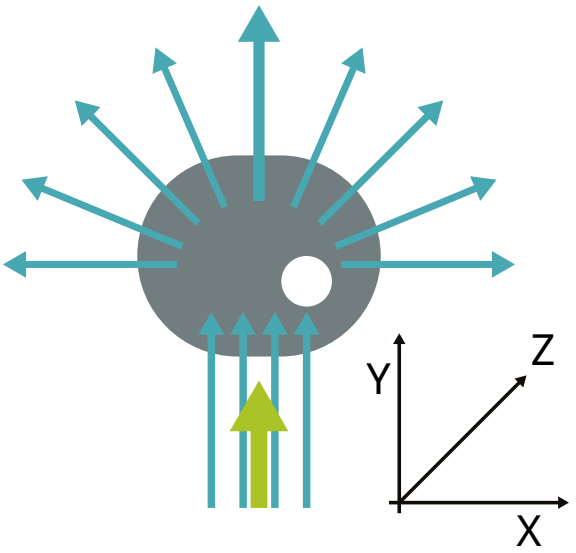


Figure 3. Light scatter as the green laser interrogates the cell. The direction of light scattered by the cell correlates to cell size and granularity.

Cell populations are often distinguishable based on differences in their size and granularity because FS correlates with cell size, and SS is proportional to the granularity of the cells (Figure 4).

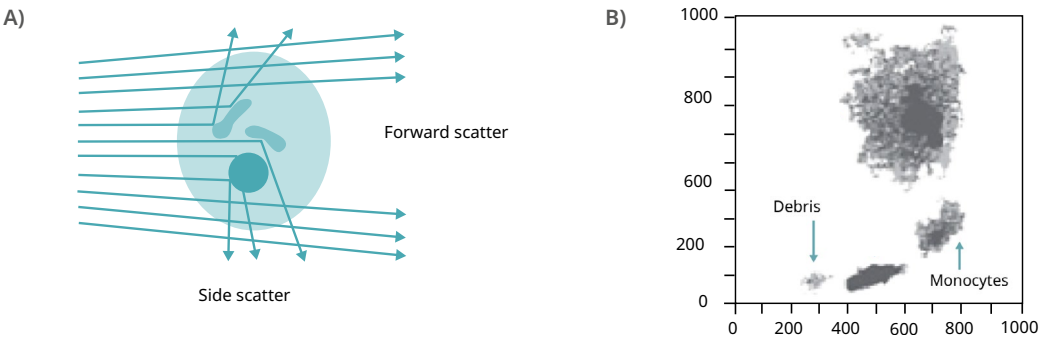


Figure 4. a) Flow cytometry measures the FS and SS of laser light, reflecting cell size and granularity. b) Typical plot distinguishing immune cell types based on FS and SS data.

A helpful example of this is running blood samples on the flow cytometer.

- Larger and more granular granulocyte cells show as a large population with a high SS and FS
- Monocytes are large cells, but not so granular, so these produce a separate population with a high FS but lower SS
- Smaller lymphocytes and lymphoblasts form a separate population with less FS, and also have a low SS as they are not granular cells

Therefore, these cells can be separated into different populations based on their FS and SS alone (Figure 5).

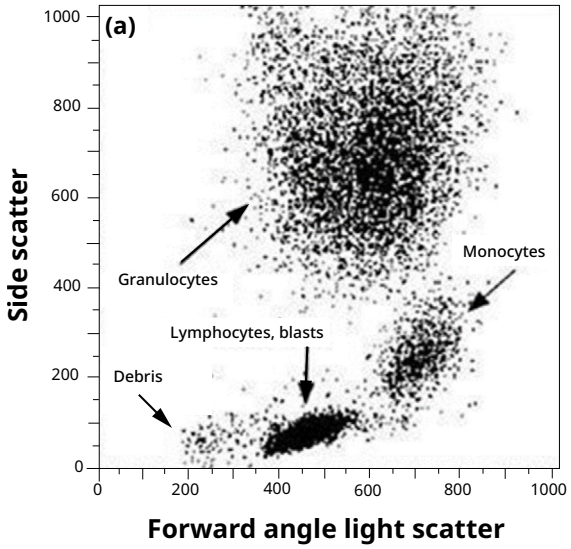


Figure 5. Flow cytometry graph: dot plot of FS versus SS. Each dot represents a single cell analyzed by the flow cytometer. Differences in cell size and granularity determine the characteristic position of different cell populations. Image reference: Riley and Idowu, Principles and Applications of Flow Cytometry.

Measurement of scattered light and fluorescence

As well as measuring forward and side scattered light from all cells or particles in a sample, fluorescence detectors within the flow cytometer measure the fluorescence emitted from positively stained cells or particles. For example, these could be stained with a fluorescently labeled antibody against a particular protein or a fluorescent ligand that binds a specific structure such as DNA. These fluorophores emit light when excited by a laser with the corresponding excitation wavelength.

FS and SS light and fluorescence from stained cells are split into defined wavelengths and channeled by a set of filters and mirrors within the flow cytometer towards sensors known as photomultiplier tubes (PMTs). The PMTs convert the energy of a photon into an electrical signal (voltage). The fluorescent light is filtered so that each sensor will only detect fluorescence at a specified wavelength.

In the example shown in Figure 6, the fluorescein isothiocyanate (FITC) channel PMT will detect light emitted from FITC at a wavelength of approximately 519 nm. The phycoerythrin (PE) channel PMT will detect light emitted from PE at 575 nm wavelength. Each PMT will also detect any other substances present in the sample emitting light at a similar wavelength to the fluorophore it is detecting.

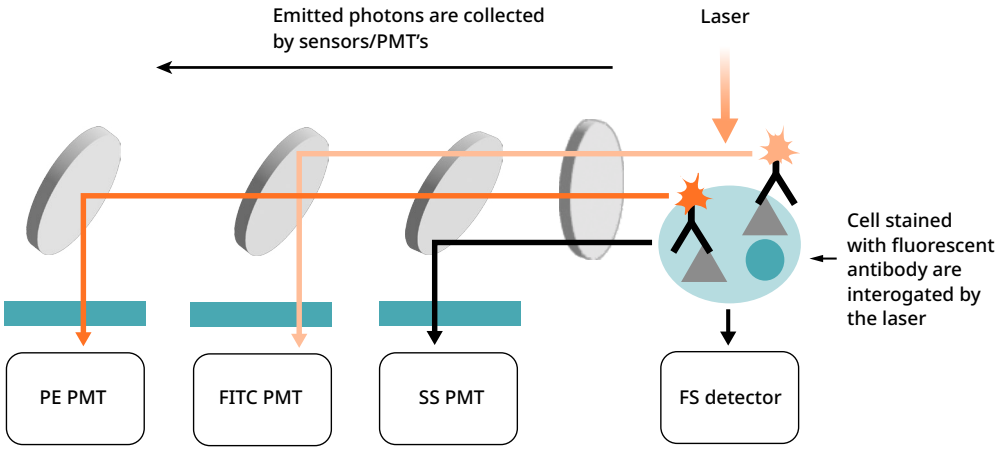


Figure 6. Cells stained with fluorescent antibodies pass by the laser.

Various filters are used in the flow cytometer to direct photons of the correct wavelength to each PMT (Figure 7). Short pass (SP) filters allow the transmission of photons below a specified wavelength, whereas long pass (LP) filters allow the transmission of photons above a specified wavelength.

Dichroic filters/mirrors (such as dichroic LP mirrors) are positioned at a 45° angle to the light beam and redirect, rather than completely blocking, light of undesired wavelengths. For example, photons above a specific wavelength are transmitted straight ahead in a long pass dichroic filter, while photons below the specific wavelength reflect at a 90° angle.

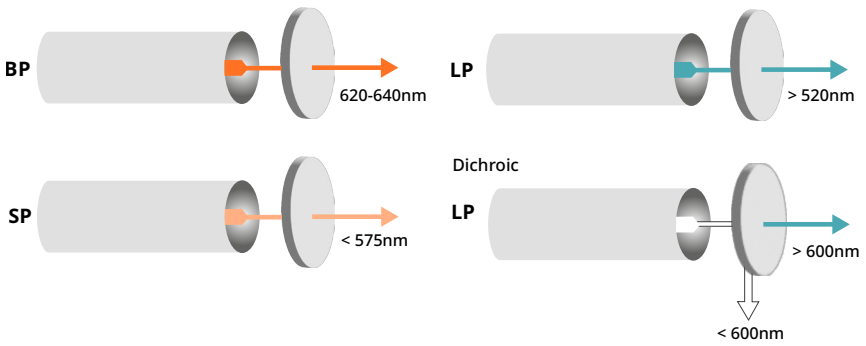


Figure 7. Band pass (BP), short pass (SP), and dichroic filters in the flow cytometer.

Antibody staining for antigen detection

To provide measurability, most immunoassays must also directly or indirectly have a label incorporated. Labels can include enzymes, fluorophores, or biotin. There are multiple methods to stain antibodies for use in flow cytometry. The choice of which method to use for your flow cytometry assay will depend on the available cells, antigens, and flow cytometry reagents.

Direct vs indirect staining

In direct immunofluorescence staining, cells are incubated with an antibody directly conjugated to a label (eg FITC). This has the advantage of requiring only one antibody incubation step and eliminates the possibility of non-specific binding from a secondary antibody.

The primary antibody is not directly labeled in indirect staining, but is detected by a labeled secondary antibody. This second reagent may be an antibody with specificity for the first antibody. Alternatively, the avidin-biotin system can be used, whereby the primary antibody is conjugated to biotin and detected with fluorochrome-labeled avidin.

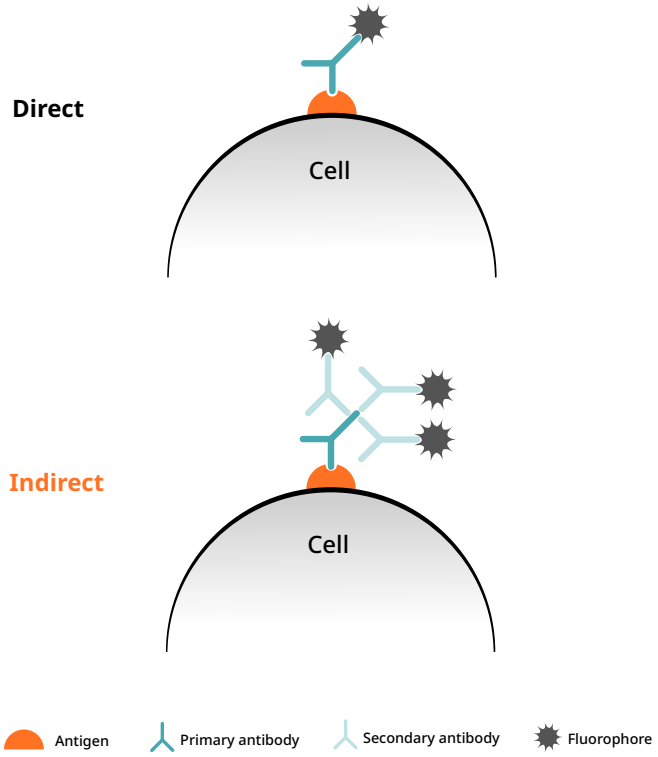


Figure 8. Direct and indirect labeling methods for flow cytometry

Benefits and limitations of direct and indirect antigen detection

Both direct and indirect methods for antigen detection each have benefits and limitations (Table 1). The direct method is typically preferred for flow cytometry experiments to save time and for ease of use. These factors are also relevant for multicolor experiments; however, using secondary antibodies can provide signal amplification[5].

Table 1. Comparison of direct and indirect antigen detection methods.

Factor	Direct	Indirect	Comments
Time	Low	High	Protocols for direct methods are usually shorter as they only require one labeling step.
Cost	High	Low	Secondary antibodies are relatively inexpensive compared with conjugated primary antibodies. Furthermore, the same secondary antibody can be used to detect many different primary antibodies.
Complexity	Low	High	Indirect methods require appropriate secondary antibody selection and additional controls.
Flexibility	Medium	Medium	Commercially available, pre-conjugated primary antibodies limit the flexibility of fluorophore and target combinations. However, indirect approaches only allow the detection of a limited number of antigens at the same time.
Sensitivity	Low	High	Several secondary antibodies bind to each primary antibody, resulting in an amplified signal.
Specis cross-reactivity	Minimized	Potential species cross-reactivity	Secondary antibodies may cross-react with species other than the target. The use of pre-adsorbed secondary antibodies can prevent cross-reactivity.
Background	Reduced	Potential background	Samples with endogenous immunoglobulins may exhibit a high background with indirect methods.

Intracellular staining

Protocols for the staining of intracellular antigens for flow cytometry utilize various fixation and permeabilization methods to allow antibodies access to internal cellular proteins. Successful staining depends on optimizing experimental conditions through titering of antibodies, appropriate controls to set up the flow cytometer correctly, and optimized fixation and permeabilization procedures[6].

Detection of secreted proteins

The detection of secreted proteins is difficult as the protein will be released from the cell before detection or may degrade rapidly. Drugs that interfere with secretion, such as Brefeldin A, can be used to inhibit the secretion of expressed proteins by retaining them in the Golgi apparatus. The intracellular staining method can then be used to detect the target protein.

Understanding fluorophores

Fluorophores, also known as fluorochromes, are critical for detection in flow cytometry. Understanding their properties is essential for correcting for fluorescence spillover in multicolor experiments.

Fluorophores are commonly used with antibodies as detection reagents in flow cytometry. They absorb and emit light within a range of wavelengths, generally referred to as the absorbance (excitation) and emission spectra (Figure 9)[7].

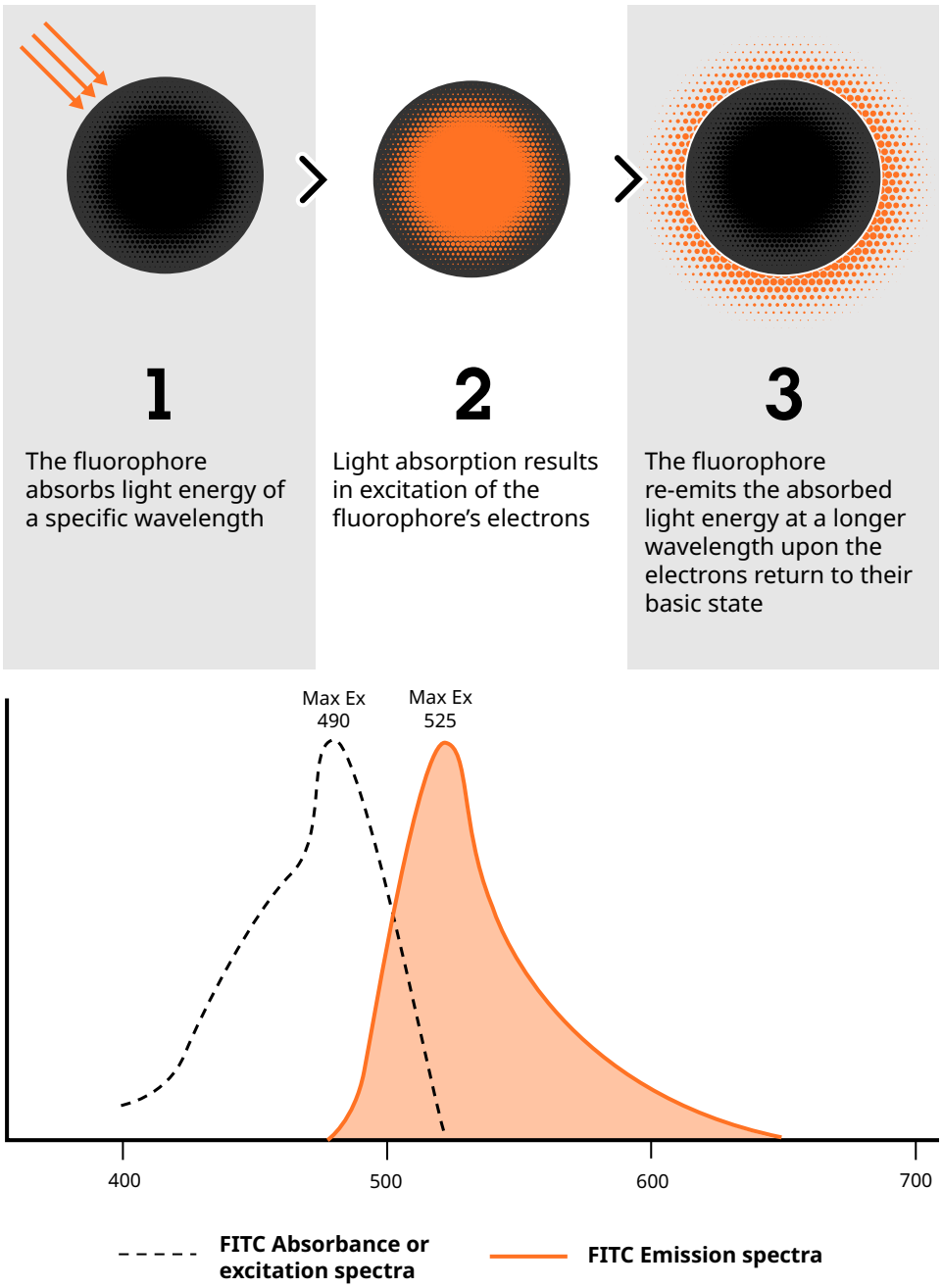


Figure 9. Fluorophore excitation and emission.

Tandem dyes for flow cytometry

The design of multicolor panels often requires tandem dyes consisting of two different, covalently attached fluorophores. One fluorophore (donor) is excited at a particular laser wavelength, while the other emits light at a different wavelength. For example, in phycoerythrin-Cy7 (PE-Cy7), PE and Cy7 act as the donor and acceptor fluorophores, respectively. Therefore, PE-Cy7 will have the excitation characteristics of PE and the emission characteristics of Cy7 (Figure 10)[8].

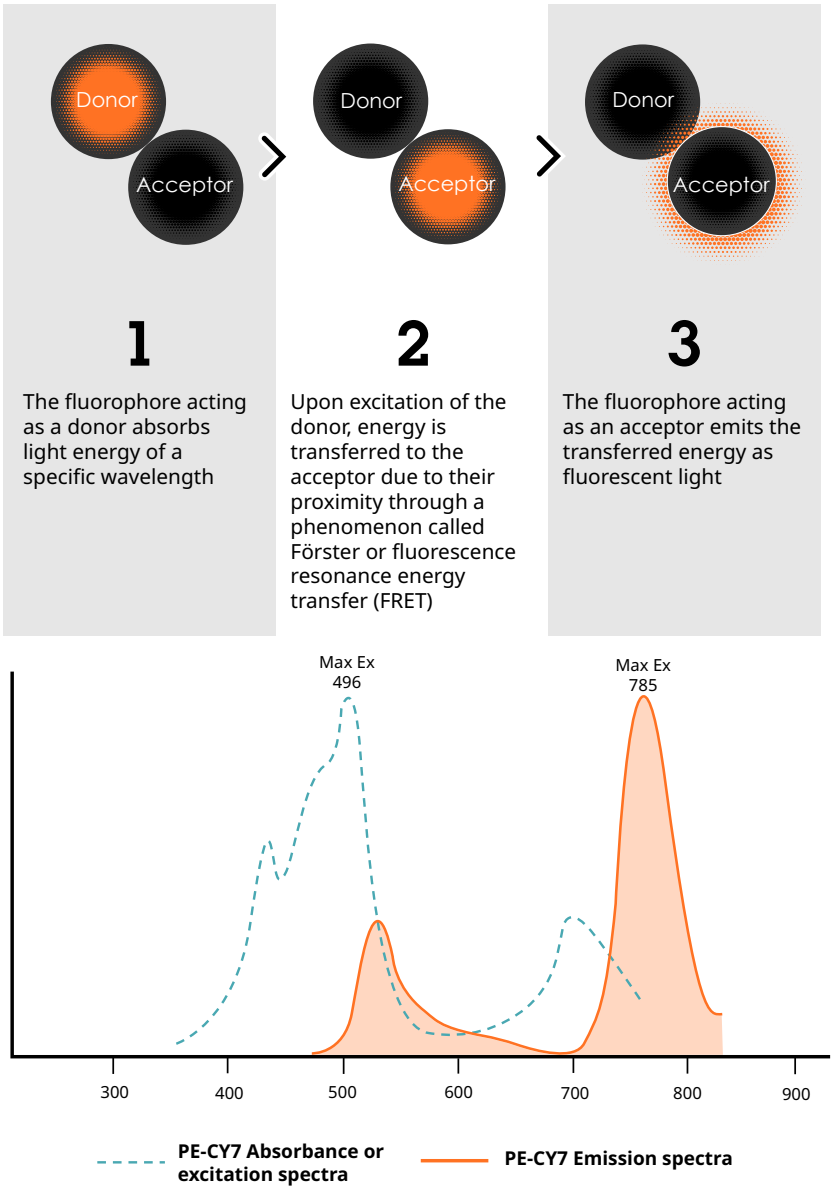


Figure 10. Tandem dye excitation and emission.

Tandem dyes increase your panel size and flexibility because several fluorophores can be excited by a single laser and measured by different detectors. For example, Alexa Fluor® 488, PE, PerCP-Cy5.5, and PE-Cy7 are excitable with a blue laser (488 nm). However, they will produce green, yellow, purple, and infrared emissions (Figure 11).

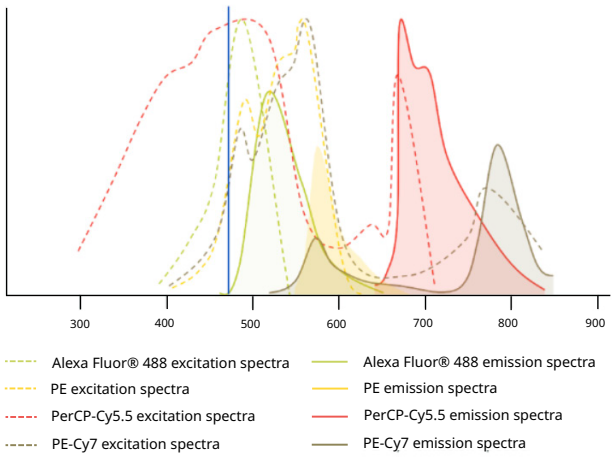


Figure 11. Excitation and emission spectra for a range of tandem dyes.

Hints and tips for handling tandem dyes

- Always protect tandem dyes from light as they are highly susceptible to photobleaching
- Do not freeze tandem dye antibody conjugates as this may result in denaturation of the donor fluorophore
- Minimize sample fixation or permeabilization as much as possible as this reduces their brightness
- Each batch requires optimization due to high batch-to-batch variation
- Some bleed-through emission from the donor might be observed since FRET efficiency is never 100%
- Label cells at 4°C to avoid degradation or decoupling of tandem dyes

Selecting a fluorophore conjugate for flow cytometry

The antibody's ability to resolve a positive from a negative signal in flow cytometry often depends on the fluorophore used.

Table 2 is a general guide to the properties of various common fluorophores .Further to this, there are some differences in the relative intensities of each fluorophore, depending on the specific flow cytometry antibodies used.

Table 2. Properties of common fluorophores used in flow cytometry.

Fluorophore	Excitation wavelength (nm)	Emission wavelength (nm)	Relative brightness	Notes
Phycoerythrin (PE)	565	575	5	Protein-based, very bright
Allophycocyanin (APC)	645	660	5	Protein-based, very bright
Alexa Fluor 647®	650	668	5	Similar to APC but small molecule more suitable for intracellular staining
PE-Cyanine 7 (PE-Cy7)	496, 565	774	4	Tandem, dye, highly sensitive to light
PE-Cyanine 5 (PE-Cy5)	496, 565	670	4	Tandem dye, not suitable for use together with APC
Alexa Fluor 488®	495	519	3	Similar to FITC but more stable and brighter
Alexa Fluor 700®	696	719	2	Useful for labelling highly abundant antigens
FITC	494	520	2	Sensitive to pH changes and photobleaching
APC-Cy7	650	774	2	Tandem dye, not suitable for use together with PE-Cy7
Pacific Blue	410	455	1	Useful for labelling highly abundant antigens

For more detailed information about fluorophores, download **our fluorophore chart** (Figure 12).

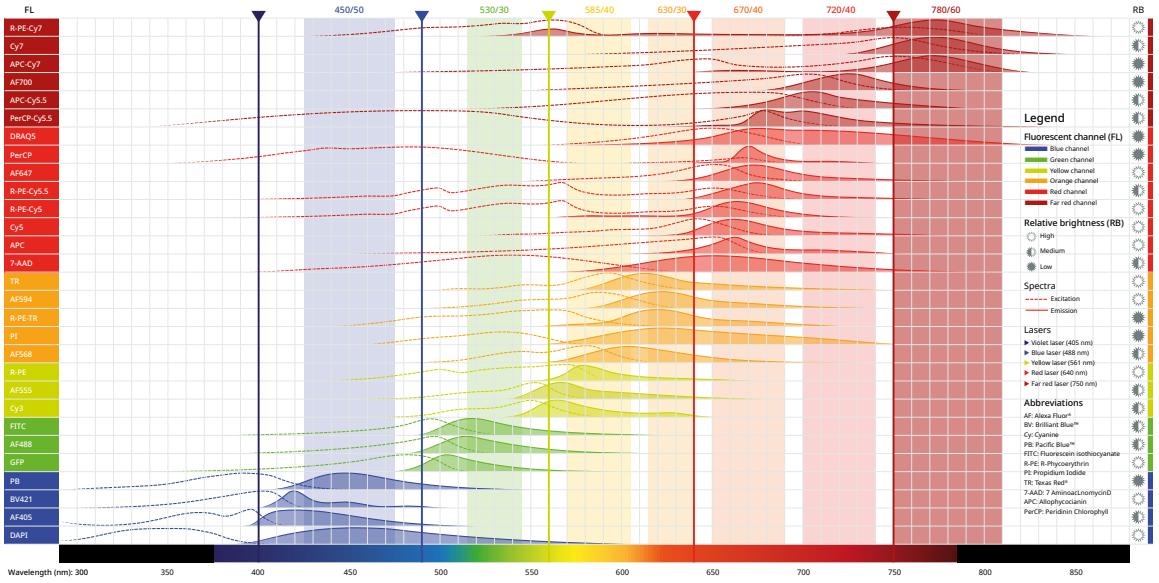


Figure 12. Fluorophores used in flow cytometry.

Designing a multicolor flow cytometry protocol

While flow cytometry is a powerful tool for identifying and analyzing multiple antigens simultaneously, increasing the number of antigens and fluorophores also increases the complexity of the experimental design. Once you know which antigens you want to study, you need to build your panel. Usually, this is the most challenging aspect of setting up a multicolor flow cytometry experiment. The steps below aim to provide a quick and easy guide to help you in the process[9].

Know your flow cytometer

Before designing your multicolor flow panel, you will need to determine the following factors:

1. The number and type of lasers
2. The number of detectors
3. The type of filters available on your flow cytometer

You will only be able to use fluorophores excited by the corresponding wavelength of light from the laser (Figure 13).

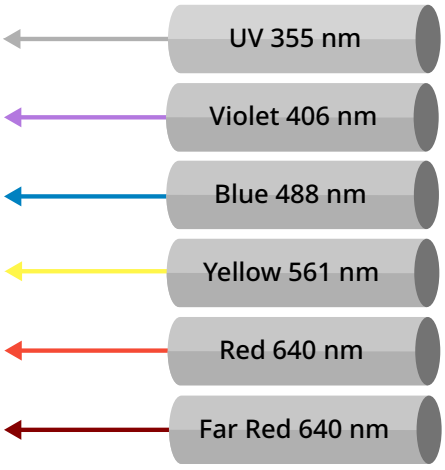


Figure 13. Common lasers used in flow cytometry.

You should match the emission wavelength of your fluorophores to the available filters and the wavelengths they allow to pass. You can check the relative brightness of fluorophores and their excitation and emission spectra using our **fluorophore chart**.

Refer to your instrument's manual or speak to your core facility manager to ensure optimal detection.

Know your cell populations, antigens, and fluorophores

Some cell populations are rare, or antigen density is low due to functional differences and cell activation levels.

A highly expressed antigen will usually be detected and resolved from the negative control with almost any fluorophore. Generally, when designing a multicolor flow cytometry panel, use the brightest fluorophores (such as PE or APC) for low or unknown antigen expression targets or rare cell populations (Figure 14) and dimmer fluorophores to detect higher abundance targets.

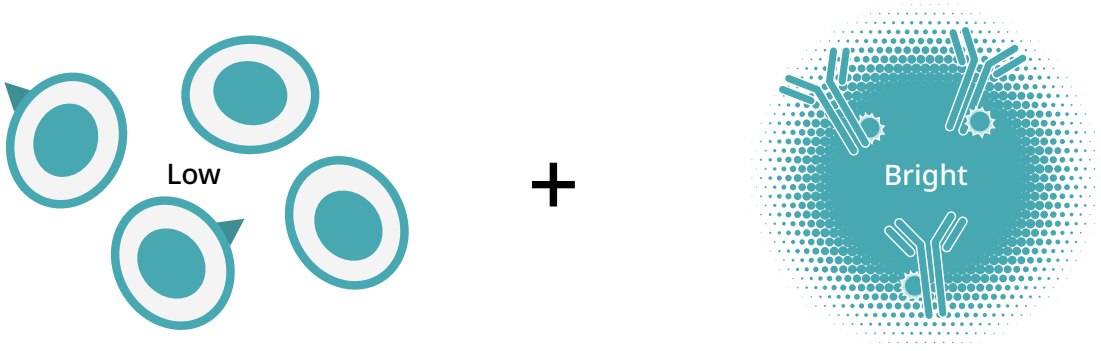


Figure 14. An antigen expressed at lower density might require a higher signal to background ratio provided by a brighter PE or APC conjugate to separate positive cells adequately from unstained cells.

Bear in mind that other factors, such as sample buffer pH, can affect fluorophore brightness. The relative fluorophore intensity also depends on the instrument due to differences in the laser and filter combinations. Be sure to use the appropriate FACS instrument for the fluorophores you wish to detect.

Minimize spectral overlap

For best results with multicolor flow cytometry, choose the brightest fluorophores with little or no overlap between their emission spectra (Figure 15). You can use compensation to control the effects of spectral overlap (see below), but it is worth sacrificing some brightness in one detector to avoid spill over (Table 3).

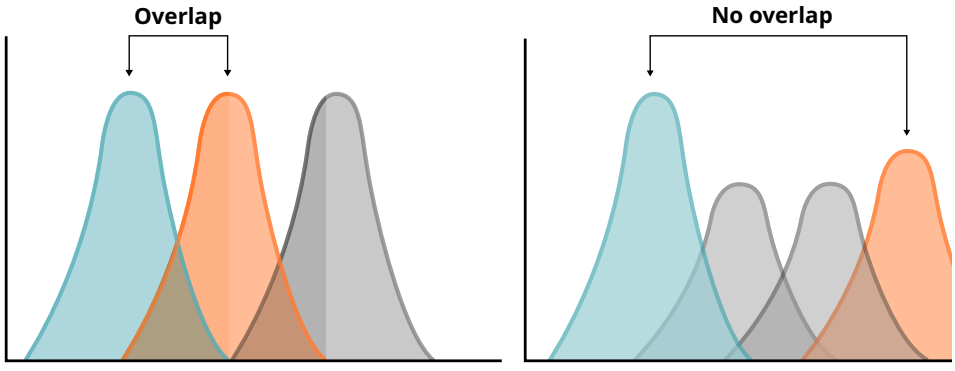


Figure 15. Where possible, choose fluorophores with little or no overlap in their emission spectra.

Table 3. Examples of good and poor fluorochrome combinations for flow cytometry.

Examples

Fluorochrome	Target Expression	Lasers	Channels	Brightness	Compensation	Combination
FITC APC	High Low	Blue Red	Green Red	Medium High	Mild	Good
FITC PE	High Low	Blue Yellow	Green Yellow	Medium High	Moderate	Medium
PerCP 7–AAD	High Low	Blue Blue	Red Red	Low Medium	Moderate	Poor

Fluorescence compensation

Fluorophores often have some overlap in their emission spectra, as demonstrated in the example below for PE and FITC. This overlap can generate a false positive signal as fluorescence from more than one fluorophore may be detected in a single channel. Spectral overlap can be problematic in multicolor experiments. Therefore, it must be corrected using compensation to ensure that the fluorescence detected is genuinely derived from the measured fluorophore[10].

Before compensation

Following excitation with the blue laser (488 nm), FITC emission is primarily detected in the channel specific for FITC, but the emission tail lies within the range of the filter used to detect PE. These are seen as false positive signals in the PE channel, meaning that cells positive for FITC will appear positive for PE (Figure 16).

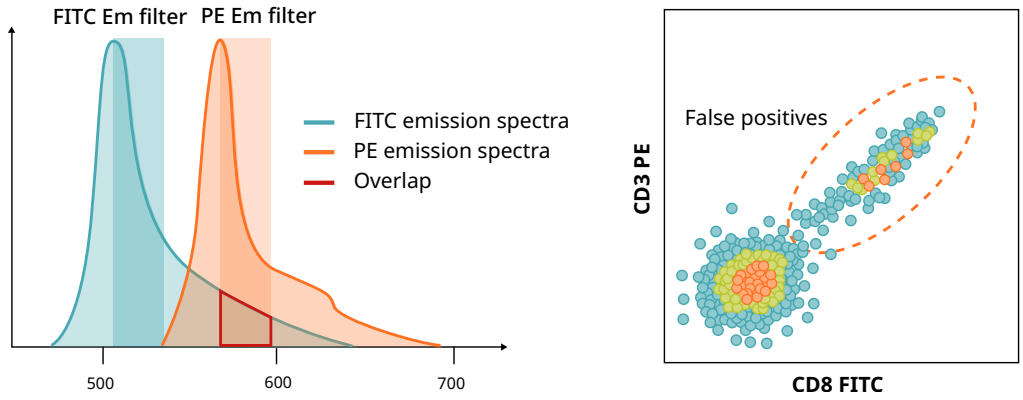


Figure 16. False-positive PE signals generated by FITC due to spectral overlap.

After compensation

To compensate, a sample stained only with a FITC-labeled antibody is required. The settings can then be adjusted until no FITC signal is seen in the PE channel. Following compensation, FITC emission is solely detected in the channel specific for FITC (Figure 17).

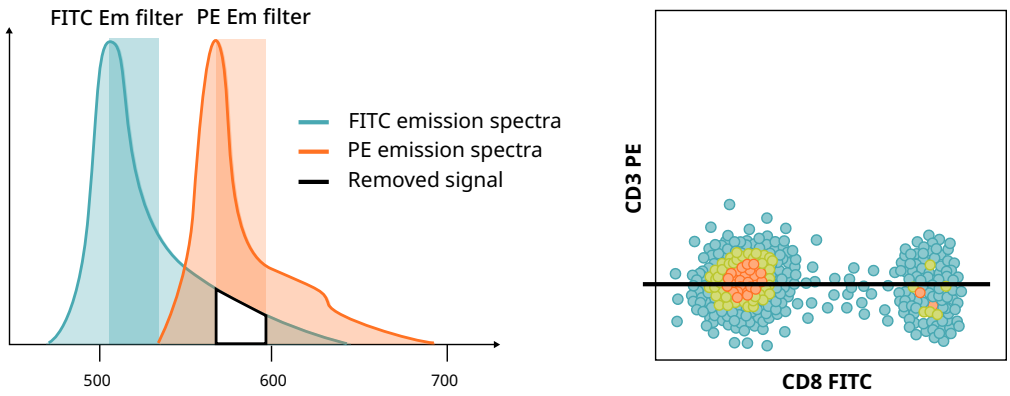


Figure 17. Compensation removes false positive signals caused by spectral overlap.

Hints and tips for applying compensation in multicolor flow cytometry

The procedure for setting the correct fluorescence compensation follows the same basic principles on any cytometer. However, due to subtle differences between instruments, we always recommend reviewing the manufacturer's instructions. These are some valuable principles to follow:

- Some fluorophore combinations should be avoided (eg APC and PE-Cy5) due to the high degree of emission overlap
- Generally, compensate with the fluorophores from the far-red end of the spectrum (higher wavelength) stepwise down to the lower end of the spectrum. Do not forget to check the compensation in all channels
- Avoid fluorophore combinations where a bright fluorophore can spill over significantly into a channel if attempting to detect a dimmer population
- Compensation is correctly set when the median of the negative population is equal to the median of the positive population in the spillover channel

Compensation controls are required for each fluorophore and should contain a positive and negative population. The following guidelines should be applied:

- These controls should only be used to set compensation
- The fluorophore used as a compensation control must match the fluorophore used in the experiment
- The positive population should be at least as bright, if not brighter, than any sample the compensation will be applied to
- The positive population should form at least 10% of the total sample population
- The background fluorescence (autofluorescence) of the positive population should be the same as the negative control
- Ideally, the positive and negative populations in the control samples should be the same type of cell. If this is not possible, consider the use of compensation beads. Alternatively, different cells for your compensation controls can be used if they express the markers of interest
- If a marker of interest is rare or possibly absent in the control cells, a different antibody directed against a more common marker can be used if it carries the same fluorophore

Recommended controls for flow cytometry

Every flow cytometry assay starts with having the appropriate controls to ensure that your data is robust and accurate (Figure 18). For more information, see our **recommended controls for flow cytometry guide.**

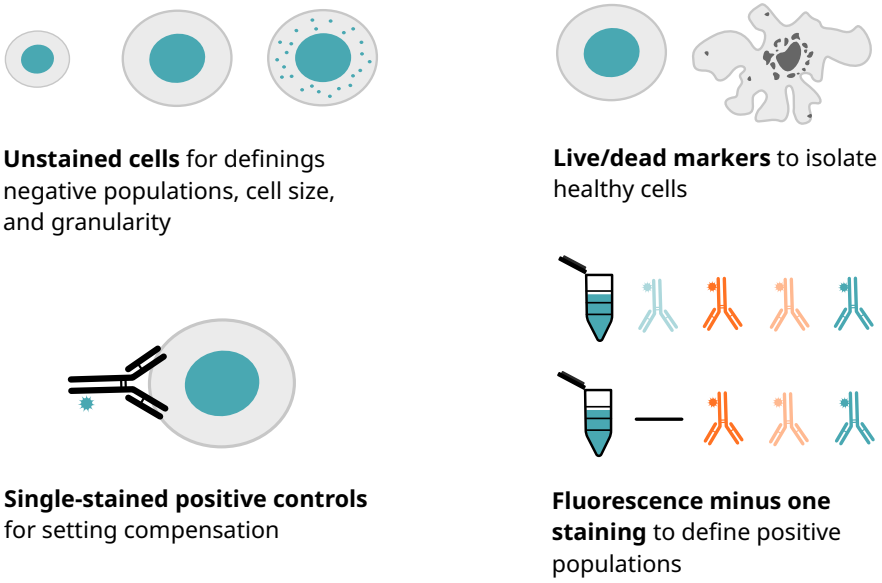


Figure 18. Some of the required controls for flow cytometry.

When setting up your flow cytometry test, make sure you include the appropriate controls for:

- **Cell viability:** Dead cells can produce artifacts due to non-specific binding and autofluorescence, potentially leading to inaccurate results
- **Autofluorescence:** Naturally occurring cell components, such as NADPH and flavins, can emit fluorescence that may mask antigen-specific signals
- **Spectral overlap:** Fluorescence emitted from one fluorophore may also be detected on a different channel, significantly affecting measurements on a given channel
- **Undesirable antibody binding:** This occurs when the antibody binds to either an off-target epitope, Fc receptor (FcR), or cellular components through its conjugated fluorophore. FcR binding can be reduced with the addition of specific blocking reagents before staining

Cell viability

It is essential to eliminate dead cells from your flow cytometry data analysis because they can give rise to false positives due to autofluorescence and increased non-specific binding.

Several markers are available that can distinguish between dead and live cells. As some of these dyes bind DNA, they may also be used for DNA content or cell cycle analysis.

Cell impermeable dyes such as 7-Aminoactinomycin D (7-AAD), propidium iodide, Nuclear Green DCS1, or DRAQ7™ are used on unfixed cells. These dyes discriminate between live and dead cells by staining only dead cells and being actively excluded from living ones.

An alternative method for determining cell viability is to use the cell-permeable fluorescent dye calcein AM. This dye is hydrolyzed to green fluorescent calcein by intracellular esterases in living cells. Cells stained with this dye can also be fixed with paraformaldehyde and then analyzed.

Autofluorescence

Cell type and physiological conditions influence autofluorescence. Naturally occurring cell components, such as NADPH and flavins, can emit fluorescence upon 488 nm wavelength laser excitation.

To check if autofluorescence presents a problem in your experiment, analyze an aliquot of unstained cells on the flow cytometer using the same cell treatment and machine settings as the experimental sample. If there is significant autofluorescence, using a different laser wavelength can resolve the problem. Refer to our fluorophore chart or multicolor selector to explore alternative lasers using your dye of choice.

Spectral overlap

When carrying out a multicolor flow cytometry experiment, the emission spectra of the various fluorophores can overlap, resulting in detection in a different channel (also called spillover).

This phenomenon can result in false positives or incorrect gating when positive or negative boundaries are ambiguous. However, this can be controlled with compensation, where spectral overlap is estimated and subtracted from the total detected signal to yield an estimate of the actual amount of each dye, or including fluorescence minus one (FMO) controls to define the positive/negative populations.

FMO controls

FMO controls, samples stained with all antibodies in a panel except for one, are essential for providing a measure of spillover in a given channel and accurately discriminating positive and negative cell populations[11]. This control provides a true negative control as it considers how the other fluorophores in your panel affect the signal observed in the channel used for the examined fluorophore. For example, in a multicolor panel of FITC, PE-Cy5, PE-Cy7, and PE, the PE FMO control would contain the FITC, Cy-PE, and Cy7-PE reagents, but not the PE (Figure 19).

Dye	Unstained control	FMO control	Sample
FITC	-	CD3	CD3
CyPE	-	CD8	CD8
Cy7PE	-	CD45RO	CD45RO
PE	-	-	CD4

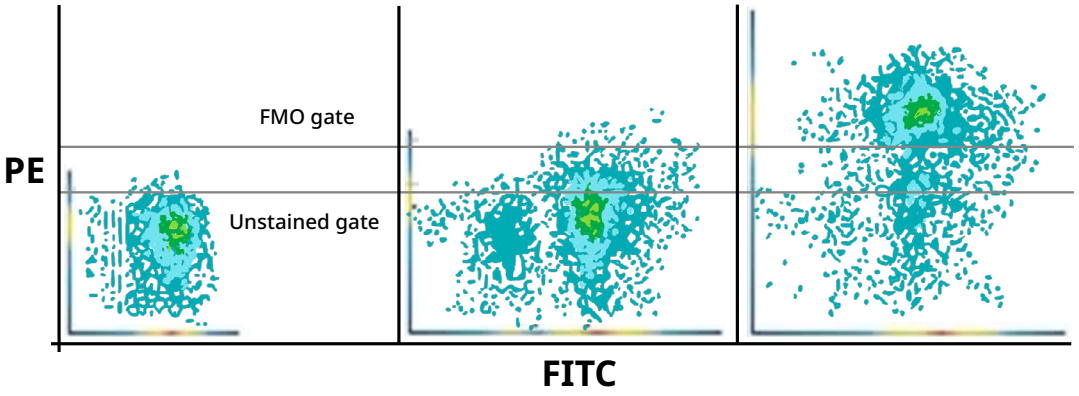


Figure 19. Example of an FMO control. If an unstained control was used to set the gate for PE positive/negative cells (lower line, unstained gate), all cells above this line in the right-hand dot plot would show as positive for PE. However, when using an FMO control to set the gate (upper line, FMO gate), it becomes clear that there is a spread of signal, and some cells are not positive for PE.

Undesirable antibody binding

This broad term includes every instance of antibody binding that prevents correct interpretation of the data. Optimizing your staining protocol and running appropriate negative controls can help to detect and alleviate these effects.

Optimizing your multicolor flow cytometry staining protocol

Antibody validation

Antibodies are key components of flow cytometry techniques, yet many have not been validated for specificity, lack of cross-reactivity, or use in flow cytometry applications [12]. To ensure reproducible, robust data, it is important to either **validate your antibodies** or purchase validated antibodies from a trusted supplier.

Antibody concentration

Non-optimal antibody concentrations can increase non-specific binding or reduce the sensitivity of the measurement. Therefore, titrate all antibodies to determine the best signal-to-noise ratio (Figure 20).

Figure 20. Titrate antibodies to improve sensitivity in flow cytometry.

Fc blocking

Phagocytic cells, such as monocytes, have Fc receptors (FcRs) on their surface that can bind non-specifically to the Fc region of antibodies—adding FcR blocking reagents before staining can block this binding (Figure 21). You should also include this blocking step in homogenized tissue samples, which may contain macrophages, as well as cell culture lines such as Daudi and THP-1.

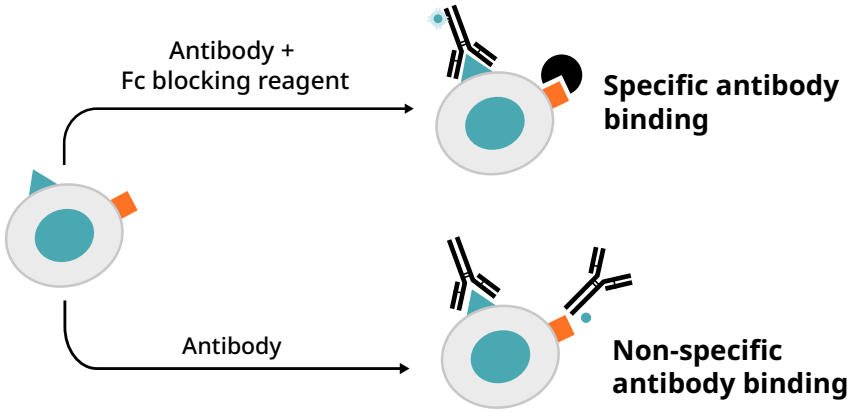


Figure 21. Fc blocking to reduce non-specific antibody binding in flow cytometry.

Negative controls

The negative control should be a population of cells that do not express the antigen of interest, ideally a knock-out cell line. This sample should be exposed to the same experimental conditions as the population in the study. Use this control to set gating regions and discern positive from negative cells.

Positive controls

The positive control is the cells known to express your target of interest. This control allows us to avoid false negatives resulting from a faulty antibody. However, positive control cells might not always be available.

Isotype controls

An isotype control is an antibody raised against an antigen not present on or in the analyzed cell type. Isotype controls determine the level of background fluorescence caused by non-specific antibody binding. They should not be used to distinguish positive from negative cells or set positive gating regions.

An ideal isotype control should:

- Match the primary antibody in host species, class and subclass of heavy and light chains, fluorophore type, and number of fluorophore molecules per immunoglobulin
- Be derived by the same manufacturing process and presented in the same formulation

Refer to our **complete guide to isotype controls** for more information.

Secondary antibody controls

This type of control is essential when using a secondary antibody, as it will allow us to assess the extent of non-specific binding associated with the secondary antibody. To set up a secondary antibody control, we can use cells that have only been treated with the secondary antibody and haven't been exposed to the primary antibody.

Isoclonic controls

The isoclonic control shows whether a fluorophore or other antibody conjugate is binding non-specifically to cellular components.

Cells are stained with the conjugated antibody in the presence of an excess of identical (isoclonic) unlabeled antibody. Specific antibody binding sites in the sample are taken up by the unconjugated antibody, while the conjugated antibody can only bind through the conjugate.

A lack of fluorescent signal suggests that the conjugate is not binding non-specifically to any components within the sample. As with any isotype control, this type of control is solely qualitative.

Considering all these factors will enable you to design and run complex multicolor flow cytometry experiments while avoiding common pitfalls. For more information on flow cytometry, visit our flow cytometry resource page.

Data analysis

Flow cytometry interpretation and data analysis are crucial to getting the correct answers from your experiments[13].

Signal measurement in flow cytometry

As the fluorescing cell passes through the laser beam during flow cytometry, it creates a peak or pulse of photon emission, which the PMT detects and converts to a voltage pulse, known as an event (Figure 22).

The flow cytometer measures the total voltage height and area, with the area correlating directly to the intensity of fluorescence for that event. When no fluorescing cells pass through the optics, no photons are emitted, and therefore no signal is detected.

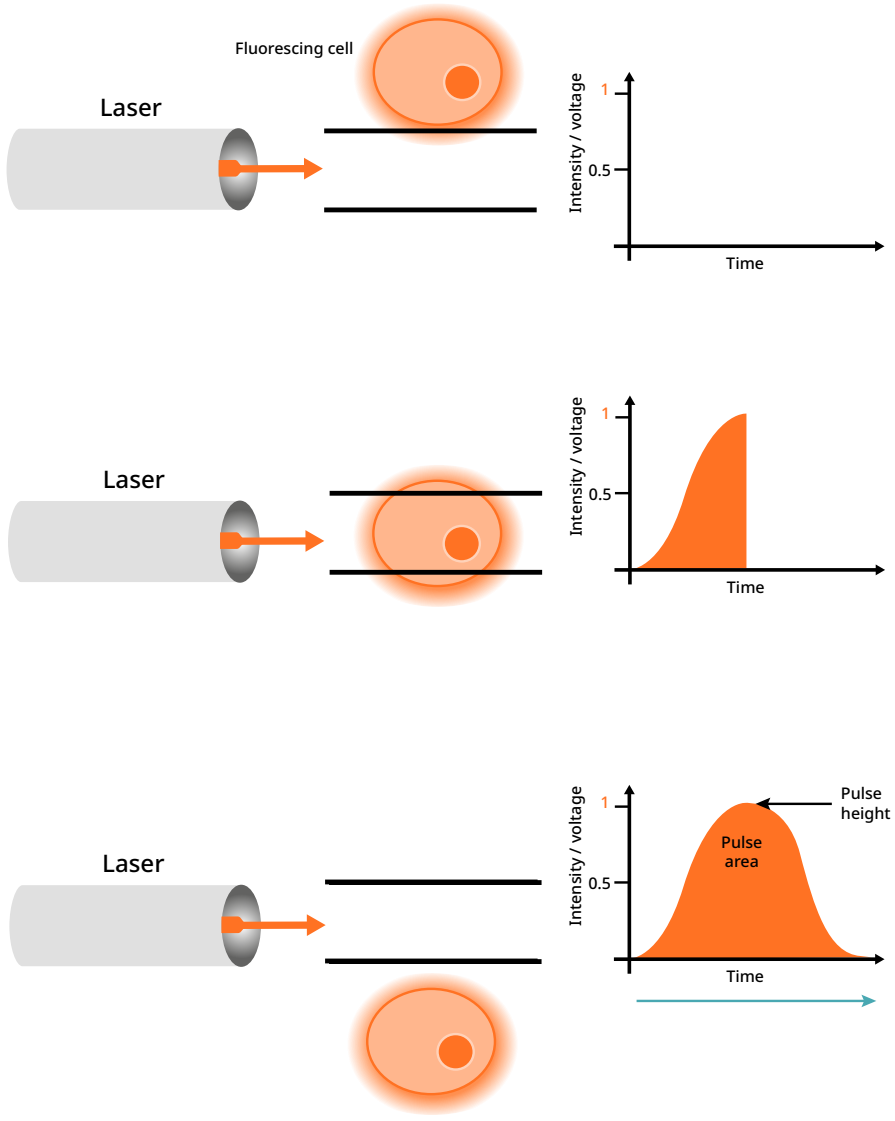


Figure 22. The PMT measures the pulse area of the voltage created each time a fluorescing cell releases photons as it passes through the detectors.

The pulse area is calculated by adding the height values for each time slice of the pulse as determined by the speed of the analog-to-digital converter (ADC), which is 10 MHz (ie 10 million per second, or 10 per microsecond).

These events are then assigned channels based on pulse intensity (pulse area). This signal can be amplified by turning up the voltage going through the PMT (Figure 23).

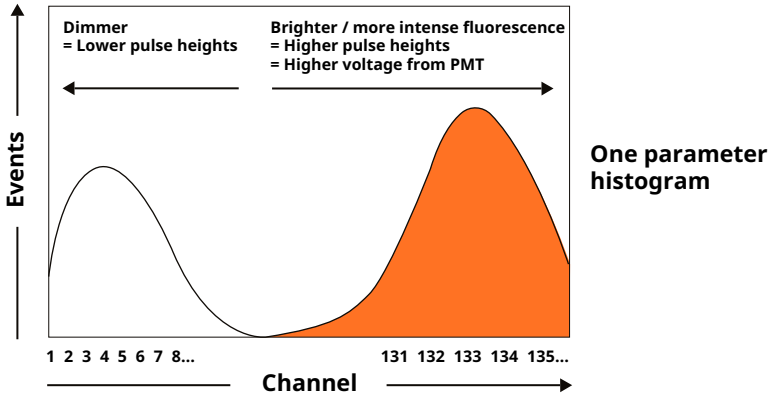


Figure 23. A one-parameter histogram plotting channel number versus the number of events. The channels are usually viewed on a log scale on the X-axis. Each event is given a channel number depending on its measured intensity; the more intense the fluorescence, the higher the channel number the event is assigned.

When plotted as a histogram, a negative result (no staining) will reveal many events at low fluorescence intensity. In contrast, a positive result gives many events at high fluorescence intensity (Figure 24).

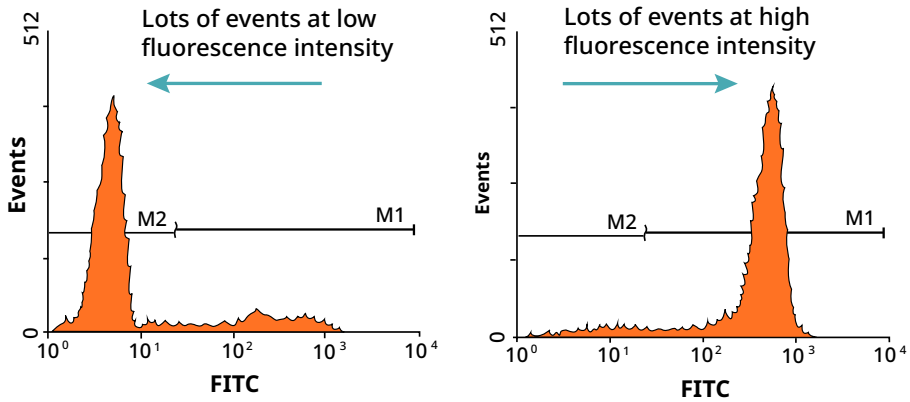


Figure 24. Fluorescence intensity measurements for a negative (left) and positive (right) result.

For a positive result, there should be a shift in intensity between the negative control and a positive sample (Figure 25).

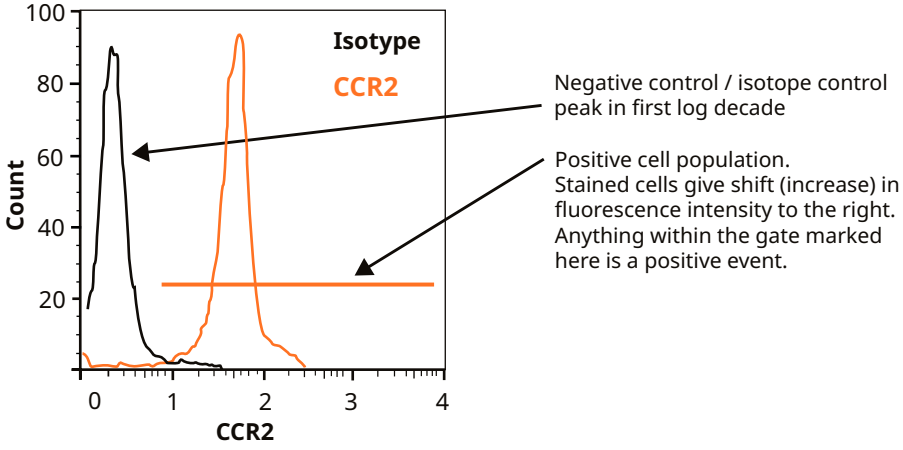


Figure 25. Anti-CCR2 antibody (ab21667) staining of human PBMC gated on monocytes. Data is from an anonymous review.

Visualizing multicolor flow cytometry data

There are several ways to visualize and analyze multicolor flow cytometry data (Figure 26). No one way is inherently right or wrong, so it's essential to determine which approach best represents your data[2].

Histograms work best when most cells express a marker of interest, and the staining is bright. The mean fluorescence intensity (MFI) measures brightness and is a relative measure of antigen abundance.

Dot plots can enable you to delineate cell populations using only FS/SS for further analysis, depending on your sample. However, using two fluorescent markers will allow you to separate your population if more than one cell population in your sample has a similar FS/SS profile or shares markers with other cell types.

Converting dot plots to pseudocolor can help highlight where there might be more than one population in regions that are close together. However, converting to contour can help highlight smaller cell populations that did not appear significant in dot plot form.

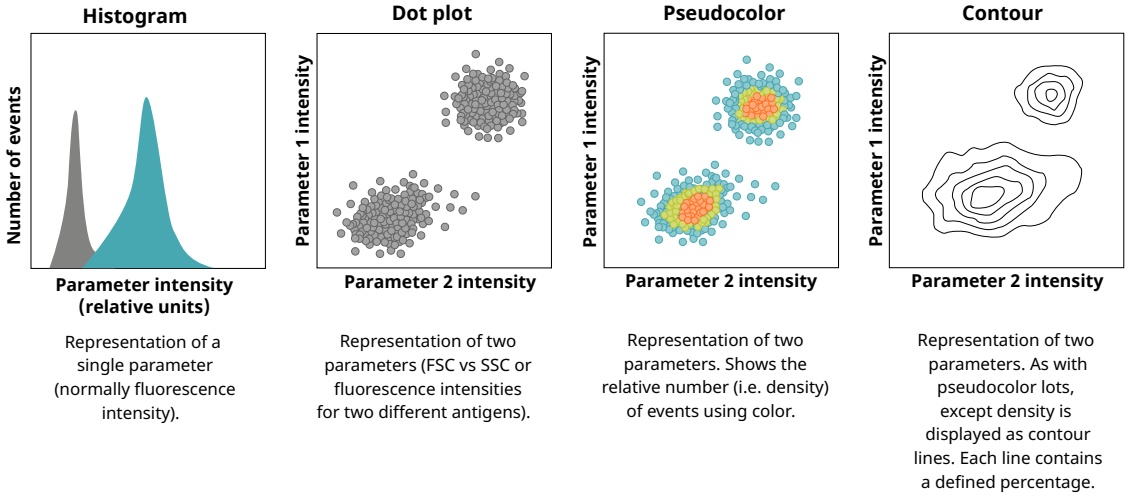


Figure 26. Examples of different ways of visualizing flow cytometry data.

Gating strategies to quantify your data

Drawing gates allow you to quantify your populations. When viewing data as a histogram, gate on a peak to identify the percentage of cells that express a particular marker (Figure 27a). Several tools are available for gating data in dot plots. The quadrant is commonly used as a gate as it easily identifies single or double-positive populations. However, other tools, such as rectangular, elliptical, and polygon gates, are also available (Figure 27b).

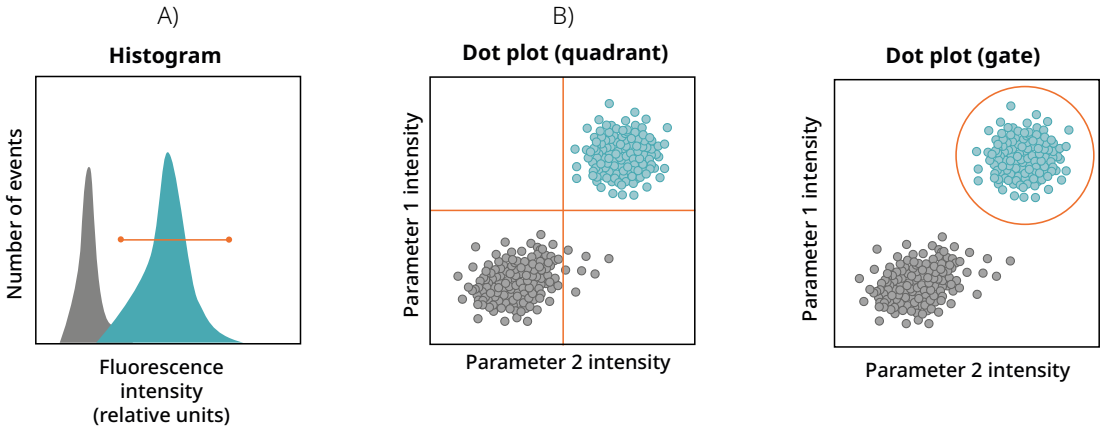
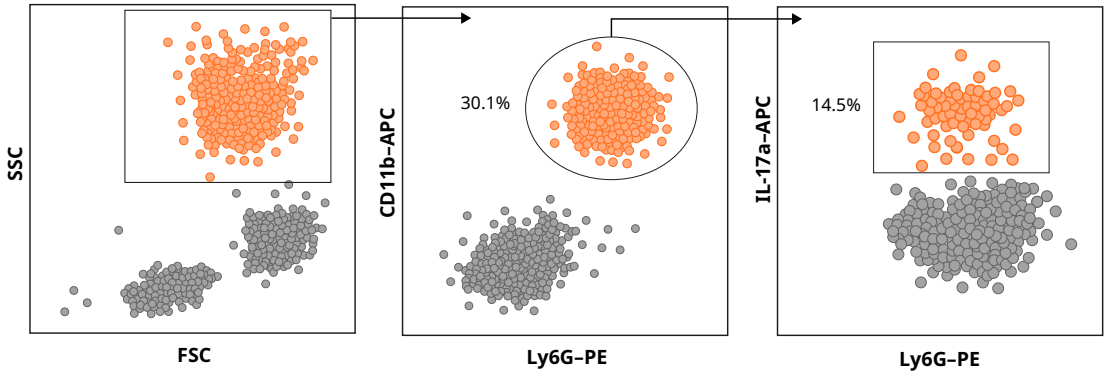


Figure 27. Examples of gating strategies. a) Gating on a peak when viewing data as a histogram. b) Gating when viewing data as a dot plot.

Calculating percentages from gated cell populations

A gate represents a subset of your total population. If you drill down on a population and gate within that, you will need to back-calculate your total population. In the example below (Figure 28), 30.1% of the total population are neutrophils, and 14.5% of neutrophils express IL-17a. However, 4.36% (30.1 x 0.145) of the total sample are IL-17a-expressing neutrophils.



A PBMC sample from a treated mouse. Granulocytes can be easily distinguished from other cell types in blood due to their FSC and SSC profile.

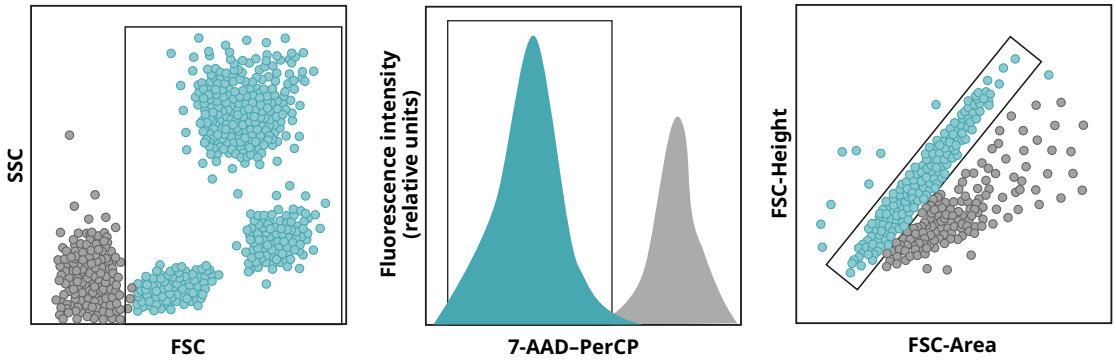
Applying a gate: 30.1% of the PBMCs in this sample are neutrophils (Ly6G+/CD11+).

Drill down: open the neutrophil gate and apply a new gate. 14.5% of neutrophils in this sample express IL-17a.

Figure 28. Example of calculating percentages of the total population after drilling down into a gated population.

Eliminating dead cells and doublets

Dead cells and doublets can be removed from flow cytometry analysis using various gating strategies (Figure 29).



Gating out dead cells using FSC/SSC. Debris and dead cells often remain in the bottom left corner of dot plots due to low FSC and SSC.

Gating out dead cells using a dead cell stain. Viability dyes often work by penetrating dead cells so they can be excluded due to their fluorescence.

Gating on single cells using area and height. Cell clumps take longer than single cells to pass through the laser. This affects the area of the signal, so they can be excluded using a simple FSC vs FSC dot plot.

Figure 29. Examples of gating strategies to eliminate dead cells and doublets from flow cytometry analysis.

Fluorescence-activated cell sorting (FACS) of live cells

Fluorescence-activated cell sorting (FACS) of live cells separates a population of cells into subpopulations based on fluorescent labeling. Sorting involves more complex mechanisms in the flow cytometer compared to a non-sorting analysis.

Cells stained using fluorophore-conjugated antibodies can be separated from one another depending on which fluorophore they have been stained with. For example, a cell expressing one cell marker may be detected using a FITC-conjugated antibody that recognizes the marker, while another cell type expressing a different marker could be detected using a PE-conjugated antibody specific to that marker. This is the fundamental principle of flow cytometry.

Live cell sorting goes one step further:

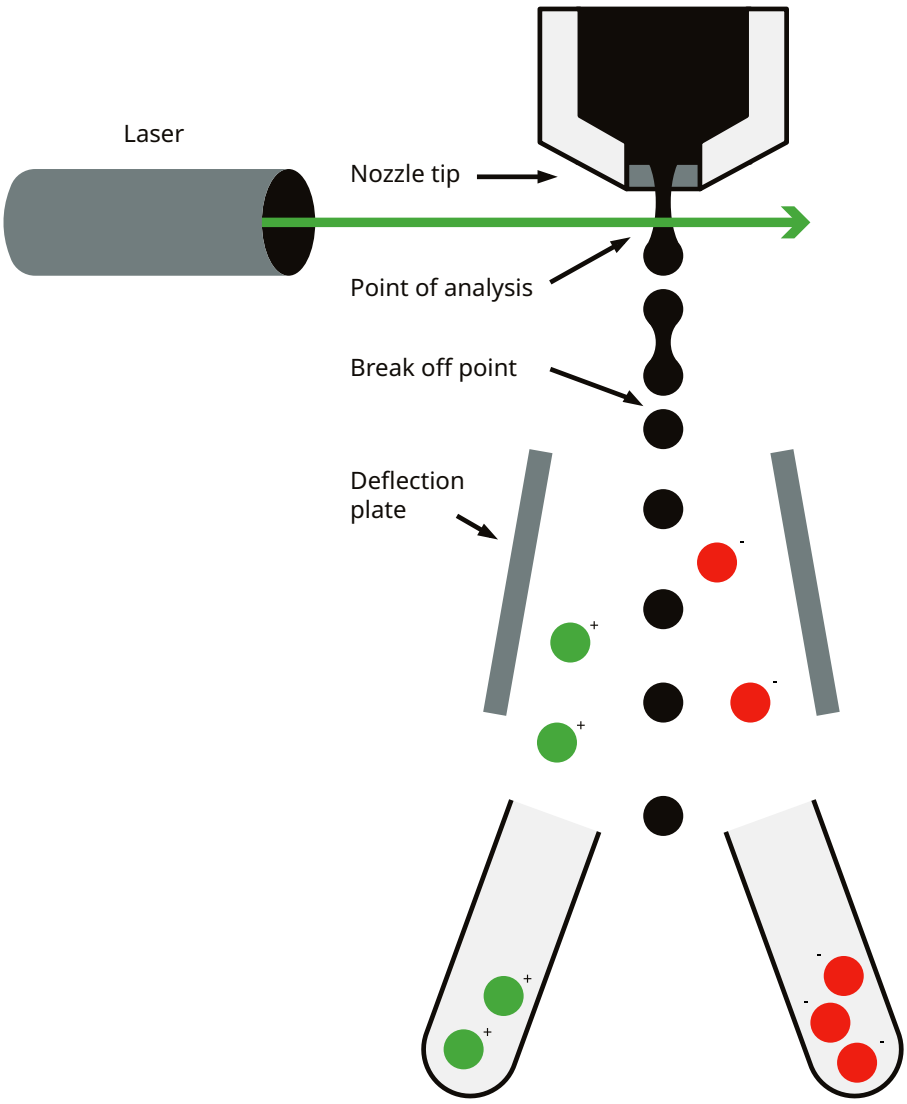


Figure 30. Schematic representation of FACS of live cells.

1. Individual cells are "interrogated" by the laser as in a standard flow cytometer.
2. The machine is set up so that each individual cell then enters a single droplet as it leaves the nozzle tip. This droplet is given an electronic charge, depending on the fluorescence of the cell inside it.
3. Deflection plates attract or repel the cells accordingly into collection tubes.

For example:

A single FITC-stained cell in a single droplet would be given a positive charge and be attracted to the left. Collection tubes to the left would collect all the positively charged FITC-stained cell droplets.

A single PE-stained cell in a single droplet would be given a negative charge and be attracted to the right. Collection tubes to the right would collect all the negatively charged PE-stained cell droplets.

4. Sorted cell populations are then analyzed to ensure successful cell sorting.
5. Sorted cells can then be cultured.

To maintain cell viability and prevent contamination for subsequent culture, consider the following tips:

- Include serum in buffers.
- Avoid using sodium azide in the buffers during staining, as it can be toxic to cells and compromise viability.
- Ensure the experiment is conducted in aseptic sterile conditions to prevent cell contamination.
- Performing intracellular staining before sorting live cells is usually not possible, as permeabilization requires damaging the cell membrane, which would compromise cell viability.

Summary

Flow cytometry provides a method to characterize, define, and quantitatively assess cell types within a mixed population. With the discovery of the increasingly helpful and advancing technology behind multicolor flow cytometry, researchers are now able to rapidly and efficiently characterize several cellular parameters simultaneously using multiple fluorescent markers.

This guide has covered vital topics to help you achieve the best possible data from your cells, from basic principles and instrumentation to challenging aspects, such as panel design and analyzing your data.

For additional flow cytometry support, try our free, on-demand **flow cytometry training course** to further build your confidence in this powerful technique.

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