

Flow cytometry is a popular laser-based technology. Discover more with our introduction to flow cytometry.

Flow cytometry is now a widely used method for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell populations, assessing the purity of isolated subpopulations and analyzing cell size and volume. It allows simultaneous multi-parameter analysis of single cells.

It is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins, 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. The cells are then incubated in tubes or microtiter plates with unlabeled or fluorochrome-labeled antibodies and analyzed on the flow cytometer.

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The flow cytometer: fluidics

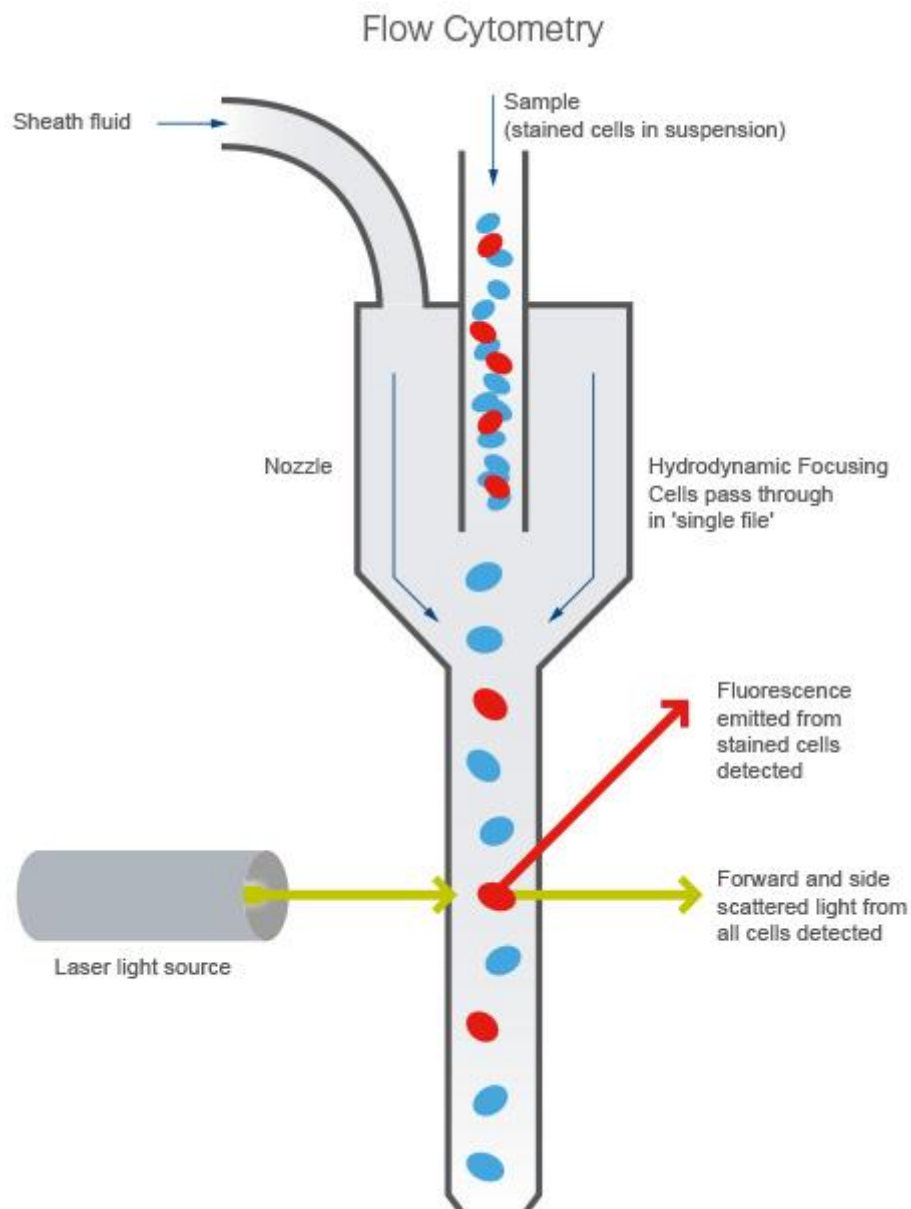


Figure 1. Overview of the flow cytometer. Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam one cell at a time. Forward and side scattered light is detected, as well as fluorescence emitted from stained cells.

When a cell suspension is run through the cytometer, sheath fluid is used to hydrodynamically focus the cell suspension through a small nozzle. The tiny stream of fluid takes the cells past the laser light one cell at a time (Figure 1).

Light scattered from the cells or particles is detected as they go through the laser beam. A detector in front of the light beam measures forward scatter (FS) and several detectors to the side measure side scatter (SS). Fluorescence detectors measure the fluorescence emitted from positively stained cells or particles.

The flow cytometer: measurement of forward and side scatter of light

Cells or particles passing through the beam scatter light, which is detected as FS and SS. FS correlates with cell size and SS is proportional to the granularity of the cells. In this manner, cell populations can often be distinguished based on differences in their size and granularity alone.

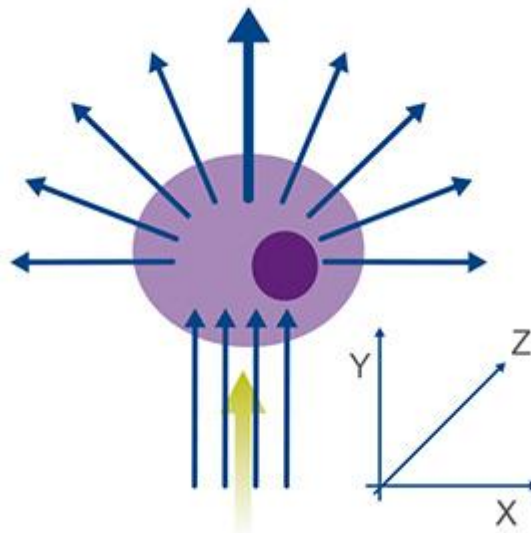


Figure 2. Light scatter as the green laser interrogates the cell. The direction of light scattered by the cell correlates to cell size and granularity.

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

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

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

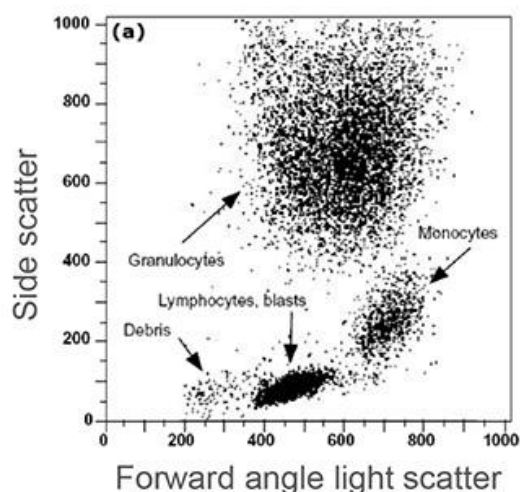


Figure 3. Dot plot of FS versus SS. Each dot represents a single cell analyzed by the flow cytometer. The characteristic position of different cell populations is determined by differences in cell size and granularity. Image reference: Riley and Idowu. *Principles and Applications of Flow Cytometry**.

*www.flowlab-childrens-harvard.com/yahoo_site_admin/assets/docs/PRINCIPLESANDAPPLICATION.29464931.pdf

The flow cytometer: measurement of scattered light and fluorescence

As well as separating cells based on FS and SS, cells can also be separated by whether they express a particular protein. In this case, a fluorochrome is often used to stain the protein of interest. Fluorochromes used for the detection of target proteins emit light when excited by a laser with the corresponding excitation wavelength. These fluorescently stained cells or particles can be detected individually.

Forward and side scattered light and fluorescence from stained cells are split into defined wavelengths and channeled by a set of filters and mirrors within the flow cytometer. The fluorescent light is filtered so that each sensor will detect fluorescence only at a specified wavelength. These sensors are called photomultiplier tubes (PMTs).

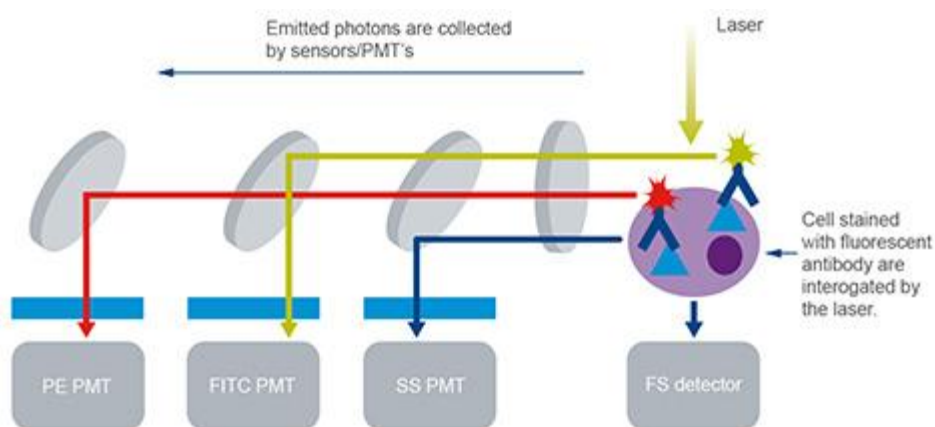


Figure 4. Fluorescent light is filtered so that each PMT detects a specific wavelength. The PMTs convert the energy of a photon into an electronic signal – a voltage.

In the example shown in Figure 4, the FITC (fluorescein isothiocyanate) channel PMT will detect light emitted from FITC at a wavelength of approximately 519 nm. The PE channel PMT will detect light emitted from PE (phycoerythrin) at 575 nm wavelength. Each PMT will also detect any other fluorochromes emitting at a similar wavelength to the fluorochrome it is detecting.

Various filters are used in the flow cytometer to direct photons of the correct wavelength to each PMT (Figure 5).

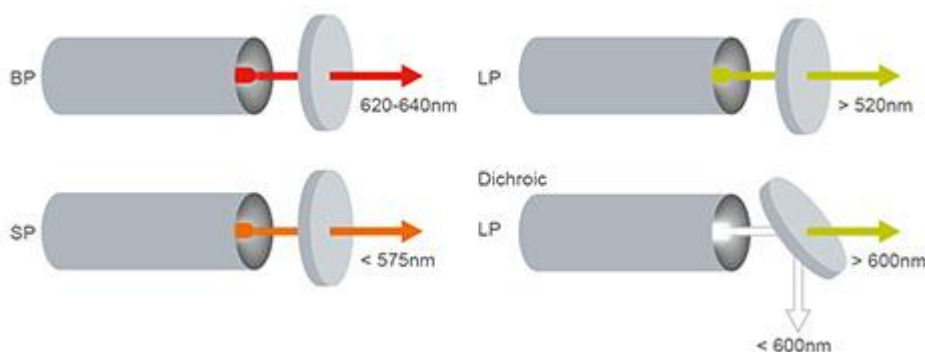


Figure 5. Filters in the flow cytometer. Band pass (BP) filters allow transmission of photons that have wavelengths within a narrow range. Short pass (SP) filters allow transmission of photons below a specified wavelength. Long pass (LP) filters allow transmission of photons above a specified wavelength. Dichroic filters/mirrors (such as dichroic LP mirrors) are positioned at a 45° angle to the light beam.

In a long pass dichroic filter, photons above a specific wavelength are transmitted straight ahead, whilst photons below the specific wavelength are reflected at a 90° angle.

Measurement of signal

As the fluorescing cell passes through the laser beam, it creates a peak or pulse of photon emission over time. These are detected by the PMT and converted to a voltage pulse, known as an event. The total pulse height and area is measured by the flow cytometer. The measured voltage pulse area will correlate directly to the intensity of fluorescence for that event.

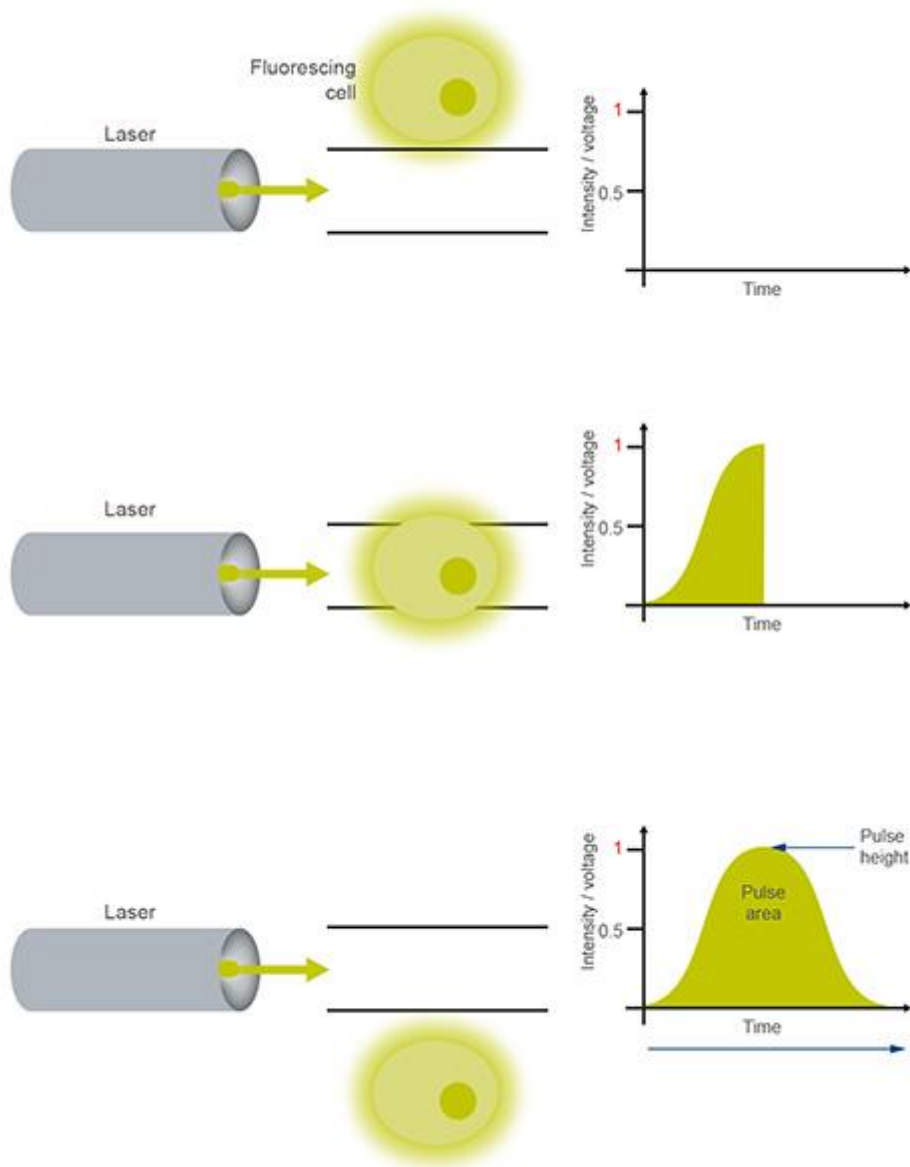


Figure 6. The PMT measures the pulse area of the voltage created each time a fluorescing cell releases photons. When no fluorescing cells pass through the optics, no photons are emitted and no signal is detected. As the fluorescent labeled cell passes through the optics and is interrogated by the laser, photons are emitted and so the intensity of the voltage measured increases. As each fluorescing cell completes its path through the laser beam, this leaves a pulse of voltage over time.

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

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

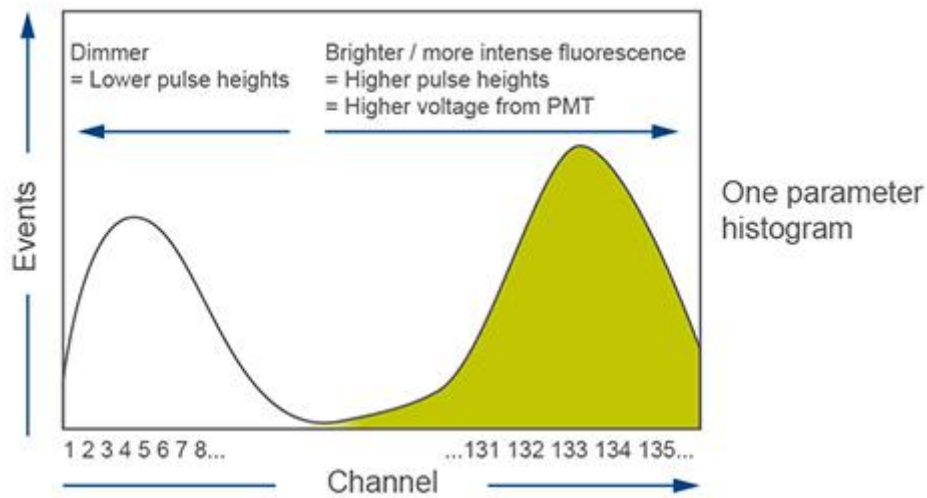


Figure 7. A one parameter histogram plotting channel number vs. number of events. The channels are usually viewed on a log scale on the x axis. Each event is given a channel number depending on its measured intensity; the more intense the fluorescence, the higher the channel number the event is assigned.

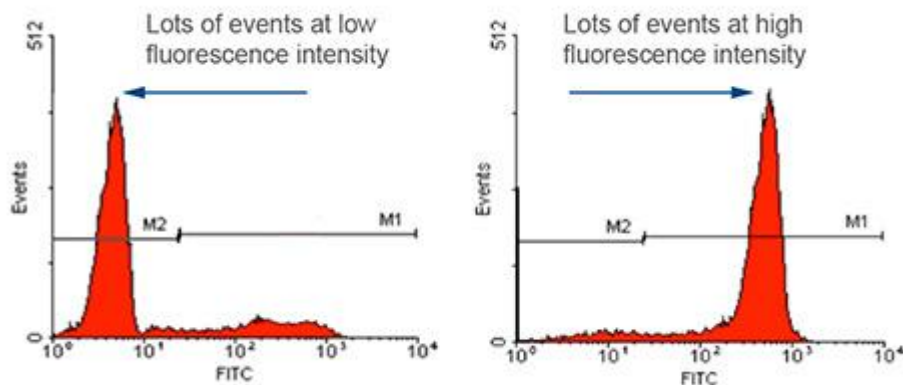


Figure 8. Fluorescence intensity measurements for a negative and positive result. The negative result shown on the left has no staining and many events at low fluorescence intensity. A positive result is shown on the right, this has a large number of events at high fluorescence intensity.

For a positive result you are looking for the shift in intensity between negative control and a positive samples (Figure 9).

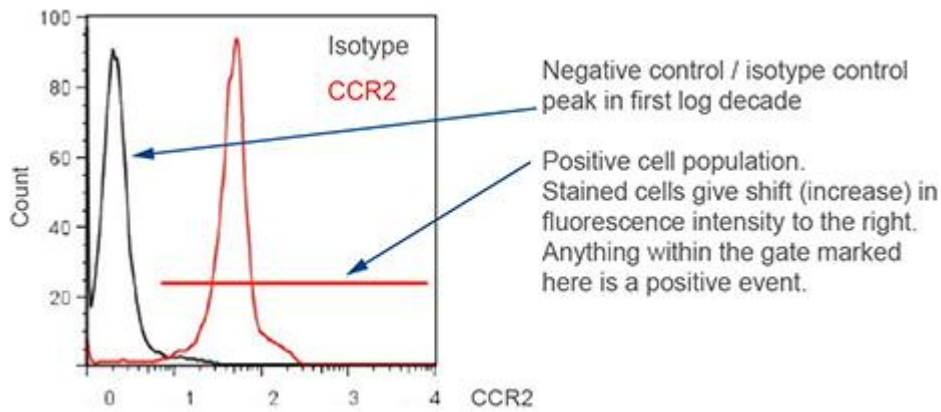


Figure 9. Anti-CCR2 antibody (ab21667) staining of human PBMC gated on monocytes. Data is from an anonymous Abreview.

Antibody staining

1. Direct staining:

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

This approach is particularly useful for intracellular staining, where large antibody-fluorochrome complexes including secondary antibodies can become trapped causing non-specific binding, or fail to enter the cell preventing primary antibody detection.

2. Indirect staining:

In indirect staining, the primary antibody is not fluorochrome-labeled, but is detected by a fluorochrome-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 an antibody is conjugated to biotin and detected with fluorochrome-labeled avidin.

With the wide range of conjugated antibodies now available, this method means that unconjugated primary antibodies raised against many different targets can be used in conjunction with a labeled secondary antibody for FACS analysis. This widens the choice of target proteins for the researcher.

3. Intracellular staining:

Staining of intracellular antigens for flow cytometry protocols depends on various fixation and permeabilization methods to allow access of antibodies to internal cellular proteins. A successful staining procedure in all cases is dependent on optimization of experimental conditions through titrating of antibodies, use of appropriate controls to set up the flow cytometer correctly and optimized fixation and permeabilization procedures.

4. Detection of secreted proteins:

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

Selecting a fluorochrome conjugate

The ability of a given antibody to resolve a positive signal from a negative signal often depends on which fluorochrome conjugate is used.

A general guideline for the relative intensities of the various fluorochromes is, from brightest to dimmest, PE, PE-Cy 7, PE-Cy5, APC, APC-Cy7, Alexa Fluor 647®, Alexa Fluor 700®, FITC, Pacific Blue, Alexa Fluor 488®. This is a general pattern; some differences in the relative intensities are seen for individual antibodies.

A highly expressed antigen will usually be detected and resolved from the negative control with almost any fluorochrome. An antigen expressed at lower density might require the higher signal to background ratio provided by a brighter PE or APC conjugate to separate the positive cells adequately from the unstained cells.

The relative fluorochrome intensity depends on the instrument. This is because of differences in the laser and filter combinations used on the different instruments. Be sure to use the appropriate FACS instrument.