Flow cytometry (and FACS)

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Flow cytometry

Flow cytometry, by its most basic definition, is the measurement of single cells in a fluid suspension: cyto= cells; meter = measurement; flow = fluid medium. Flow cytometers are capable of measuring and recording multiple characteristics of a large number of cells at rates exceeding 5000 cells per second, so that cell populations can be analyzed with a high degree of statistical accuracy.

It is a laser-based technique used for cell sorting, counting, and profiling, based on their physical and chemical characteristics, to provide information on cell characteristics such as DNA/RNA profile, gene expression, receptors, and cell size.

It allows the rapid identification and quantification of individual cells, by 'tagging' them with antibodies that are conjugated to a fluorescent molecule (a fluorochrome or a fluorescently-conjugated secondary antibody that detects the primary antibody) and passed through the flow cytometer in a stream of fluid where the

fluorochrome is excited by a laser and its emission is detected. It is predominantly used to define different cell types by analyzing the expression of cell surface or intracellular molecules (Abcam n.d.). Ideally, sample cells can pass through a laser beam one at a time, and the light scattered can be used for analyzing the cells. From there, the cells are either sorted into specific collection tubes or discarded. The light used in a flow cytometer can be divided into refracted and emitted light.

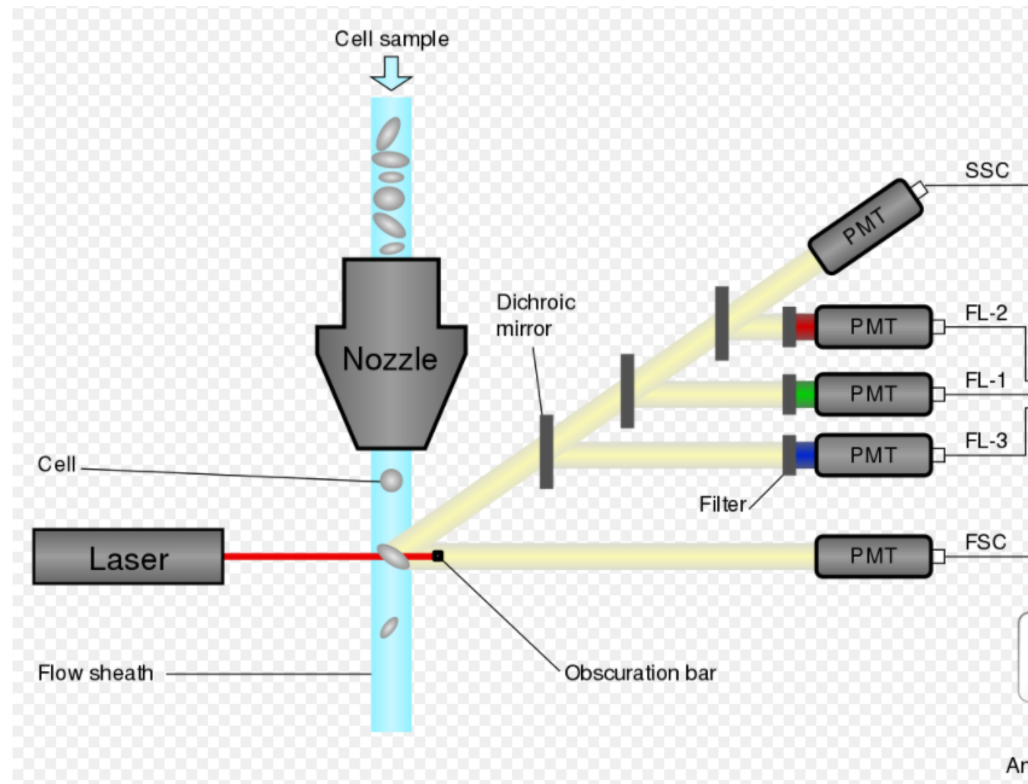
Antibodies can be used to label antigens on the surface of cells (for example, CD3 found on T cells) and/or antigens/molecules inside cells (for example, FoxP3, a marker of regulatory T cells or even soluble cytokines such as IL-10 produced by regulatory T cells). Using a number of different fluorochromes conjugated to antibodies with different specificities allows cells to be analyzed for a range of different characteristics.

The essence of the technique of flow cytometry

In essence, Laser light is passed through a stream of particles or cells in suspension, and any cell/particle intersecting the light beam disperses the light in any direction. The direction and amount of light scatter is determined by the physical and chemical characteristics (e.g. size, granularity, amount of

fluorophores present) of the cell/particle, and can, therefore, be measured by the amount of light scattering and fluorescence emission detected as the cells pass through a laser beam in single file. One detector is placed in the opposite direction of the beam (forward scatter; FSC). Another detector is located at right angles to the beam (side scatter; SSC). The FSC data collected for each particle are used for the analysis of the size and the SSC data are used for the analysis of cell/particle complexity (granularity).

Data are collected about each particle for size (**forward scatter*see below**), complexity (**side scatter*see below**), and the presence of fluorescent markers (the latter in the derivative technique of flow cytometry, called **Fluorescence-Activated Cell Sorting**). This can then be analyzed through a series of strategies for gating – i.e., applying filters to allow certain signals only to come through. You can see the basic elements of flow cytometry and FACS in the diagram below. Notice the two photomultiplier tubes(PMT labels) with the labels **FSC** and **SSC**? That's for forward and side light scatter, which we will discuss below.



A FACS machine

https://upload.wikimedia.org/wikipedia/commons/3/3f/Cytometer.svg by Kierano under CC BY 3.0 license (https://creativecommons.org/licenses/by/3.0)

Gating, usually done initially on the physical characteristics of the cells, allows the exclusion of unwanted particles (e.g. dead cells and debris) and inclusion of cells of interest. Debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Dead cells have low forward and side scatter than living cells.

The different physical characteristics of cells such as granulocytes, monocytes, and lymphocytes allow them to be distinguished from each other.

Flow cytometry allows thousands of cells per second to be analyzed, and certain machines also allow the collection of individual cells. These machines are known as fluorescence-activated cell sorters (FACS). Cells are first analyzed based on their size and their complexity and then fluorescence-labeled antibodies allow for more sophisticated analysis.

As noted in Hematopathology (Third Edition), 2018:

Flow cytometry (FC) is an immunophenotyping technique in which suspensions of living cells are stained with specific, fluorescently labeled antibodies and then analyzed with a flow cytometer.

It is used to identify cells in solution and is most commonly used for evaluating peripheral blood, bone marrow, and other body fluids.

In this technique, a suspension of single cells are labeled with a fluorescent tag, usually, something like dye a fluorescently conjugated monoclonal

antibody, and then passed through a flow cytometer, at very high rates of up to 20,000 cells per second.

The detector contains a focused beam of a laser which is set to activate the fluorescent molecule (the fluorophore) at the appropriate wavelength for excitation of the molecule. The flow cytometer contains detectors to detect the amount of fluorescent light that is emitted as well as the light-scattering properties of each cell.

The intensity of the emitted light is directly proportional to the density of the antigen which has bound to the antibody or the characteristics of the cell that is being measured.

The three elements of flow cytometry

Flow cytometry has three elements, namely the fluidics, optics, and electronics.

- The cells to be sorted are first stained using specific fluorophore-conjugated antibodies.
- They can be stained using direct, indirect or intracellular staining techniques.
- The flow cytometer consists of a fluidics system into which the

fluorophore-conjugated cells are injected. The target sample is suspended in liquid and injected into the flow cytometer apparatus with fluid and funneled at the hydrodynamic focusing region which ensures that only one cell passes through the nozzle and into the path of the laser beam at any time.

- These cells then pass through the optics system which consists of a number of filters, light detectors, and a laser source.
- The filters have different wavelengths. The conjugated cells pass through at least one filter and these filters excite the specific fluorophore-labeled cells.
- When the particles pass through the beam of laser light, light-scattering occurs which is specific to a particular cell, and scattered light determines the characteristics of the particles based on forward scatter (FS) or side scatter (SS). Forward scattered light signals (FSC) are a type of refracted light that depend on (and therefore provide information about) particle size in the forward light path. Side scattered light can be used for testing the

complexity of the cells. A detector measures the forward scatter light signals (FSC) and side scatter light signals (SSC).

- In this way, cells in the heterogeneous mixture are segregated based on the fluorophores used and also on the basis of FSC and SSC.

The cells are often fluorescently labeled so the wavelengths of the scattered light can be measured; then, photomultiplier tubes detect the specific wavelength emitted by the fluorophore. In that case, the data collected for each particle are the size (forward scatter), complexity (side scatter) **and** the presence of fluorescent markers. This technique, a derivative of flow cytometry, is called **Fluorescence-Activated Cell Sorting**, and we present some brief information below.

The cell suspension is centrifuged and stained with live/ dead fixable dye followed by incubation in fluorescently labeled antibodies for 30 min at 4°C diluted with PBS and brilliant violet buffer in 1:1.

The analysis is conducted using a computer connected to the cytometer, which can include a number of different lasers and fluorescence detectors, with more of these allowing for greater

antibody labeling and therefore, a better understanding of the cell population.

Emitted light such as fluorescent light, functions in detecting fluorescent molecules that originated from the cells or fluorescent dyes.

Forward (FSC) and side (SSC) scatter in flow cytometry

Flow cytometers have two light scatter detectors, one for side scatter (SSC) and one for forward scatter (FSC) and four fluorescence detectors.

Populations of cells can be distinguished based on their forward and side scatter properties. Forward and side scatter give an estimation of the size and granularity of the cells respectively, although this can depend on several factors such as the sample, the wavelength of the laser, the collection angle, and the refractive index of the sample and the sheath fluid. Distinguishing populations of cells can be relatively straight forward for cell lines where there is only one type of cell, but it can be more complex for samples where there are multiple cell types.

FSC and SSC allow the size and granularity of cells to be determined, can allow cell debris and dead

cells to be identified (and excluded) and can allow different cell types to be distinguished

The data from FSC and SSC are plotted in a density plot where FSC and SSC are the two axes. Clustering of data points in the 2-d space of these two axes allows distinguishing between discrete populations of cells and can be distinguished from cellular debris and dead cells, which often have a lower level of forward scatter and are found at the bottom left corner of the density plot.

The forward scatter threshold can be increased to avoid collecting these events, or they can be removed by gating on the populations of interest. This method is often used to remove dead cells which have increased autofluorescence and non-specific binding of antibodies.

The following article from the Journal of Investigative Dermatology gives a nice summary of this technique (although it's from 2012, the principles and analytic processes have not changed much, so it's still a valuable resource)

Download (PDF, 657KB)

Caveat: FSC = size and SSC = granularity (complexity) is a simplification

As noted on https://www.bio-rad-antibodies.com/blog/a-guide-to-gating-in-flow-cytometry.html:

Forward scatter does not necessarily relate to size and side scatter is not really granularity.

While these are an indication based on light refraction, it depends on the sample, the sheath fluid, and the laser wavelength.

For example, FSC vs SSC gating is most useful for blood samples but even then, granulocytes (12-17 µm) can sometimes appear larger than monocytes (20-25 µm).

Furthermore, for small samples, the level of light scatter does not always correlate with size.

Protocol for preparing single-cell suspensions for flow cytometry

Protocol

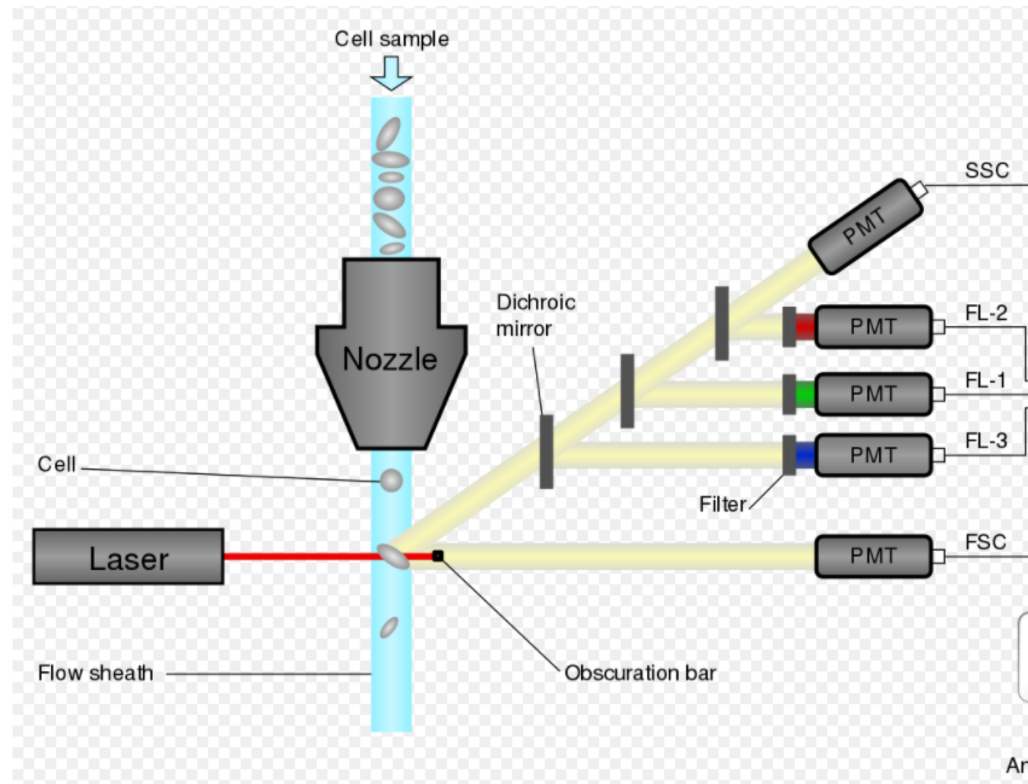
FACS – fluorescence-activated cell sorting

FACS is a derivative of flow cytometry. In this technique, as the name implies, you use fluorescence to sort cells. The fluorescence comes from fluorescent antibodies that have been used to tag cells differentially on the basis of the particular proteins (antigens) on their cell surfaces.

As noted above, photomultiplier tubes detect the specific wavelength emitted by the fluorophore. In that case, the data collected for each particle are the size (forward scatter), complexity (side scatter) **and** the presence of fluorescent markers.

You can see this in the image below, which shows a FACS machine. Notice the two photomultiplier tubes (labelled "PMT") with the labels FSC and SSC? That's for forward and side light scatter. Then there are the three photomultiplier tubes labelled FL-1, FL-2 andJ FL-3 (fluorescence 1, 2 and 3 respectively). Those are to detect fluorescence at different wavelengths.

The entire read-out goes to the ADC (analog-to-digital convertor) and then the computer.



A FACS machine

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You'll find an excellent comparison of the two techniques here: comparison of Flow cytometry and FACS.