Principles of the flow cytometer

1. Overview

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a **particle's relative size**, **relative granularity** or **internal complexity**, and **relative fluorescence intensity**.

These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

- **The fluidics system** transports particles in a stream to the laser beam for interrogation.
- **The optics system** consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.
- **The electronics system** converts the detected light signals into electronic signals that can be processed by the computer. For some instruments equipped with a sorting feature, the electronics system is also capable of initiating sorting decisions to charge and deflect particles.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. **Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis**. Cells from solid tissue must be disaggregated before analysis. The portion of the fluid stream where particles are located is called the sample core. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present *o*n the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer. This data can be analyzed to provide information about subpopulations within the sample.

2. Fluidics system

One of the fundamentals of flow cytometry is the ability to measure **the properties of individual particles.** When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. **The sample must therefore be ordered into a stream of single particles** that can be interrogated by the machine's detection system. This process is managed by the fluidics system.

Essentially, the fluidics system consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its center and zero velocity at the wall (see Figure 1). The effect creates a single file of particles and is called **hydrodynamic focusing**. Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid.

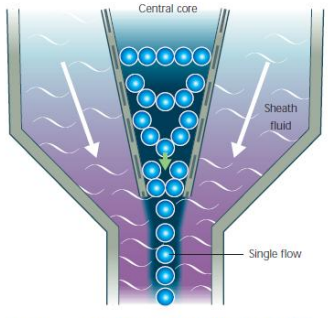


FIGURE 1 Hydrodynamic focusing produces a single stream of particles

3. Optics and detection

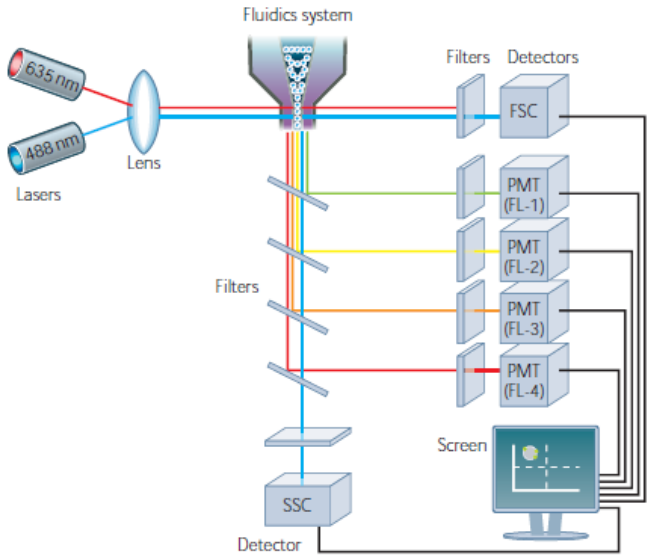
After hydrodynamic focusing, each particle passes through **one or more beams of light**. Light scattering or fluorescence emission (if the particle is labeled with a fluorochrome) provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources in modern flow cytometry. Lasers produce a single wavelength of light (a laser line) at one or more discreet frequencies (coherent light).

- Light that is scattered in the **forward direction**, typically up to 20° offset from the laser beam's axis, is collected by a lens known as the **forward scatter channel (FSC).** The **FSC intensity roughly equates to the particle's size** and can also be used to distinguish between cellular debris and living cells.
- Light measured approximately at **a 90o angle** to the excitation line is called **side scatter**. The **side scatter channel (SSC)** provides **information about the granular content within a particle**.

Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell types in a heterogeneous sample.

- **Fluorescence measurements** taken at different wavelengths can provide **quantitative and qualitative data about fluorochrome-labeled cell surface receptors or intracellular molecules** such as DNA and cytokines. Flow cytometers use separate fluorescence (FL-) channels to detect light emitted. The number of detectors will vary according to the machine and its manufacturer. Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). Silicon photodiodes are usually used to measure forward scatter when the signal is strong. PMTs are more sensitive instruments and are ideal for scatter and fluorescence readings.

The specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting (passing) others. There are three major filter types. 'Long pass' filters allow through light above a cut-off wavelength, 'short pass' permit light below a cut-off wavelength and 'band pass' transmit light within a specified narrow range of wavelengths (termed a band width). All these filters block light by absorption (Figure 2).



Schematic overview of a typical flow cytometer setup

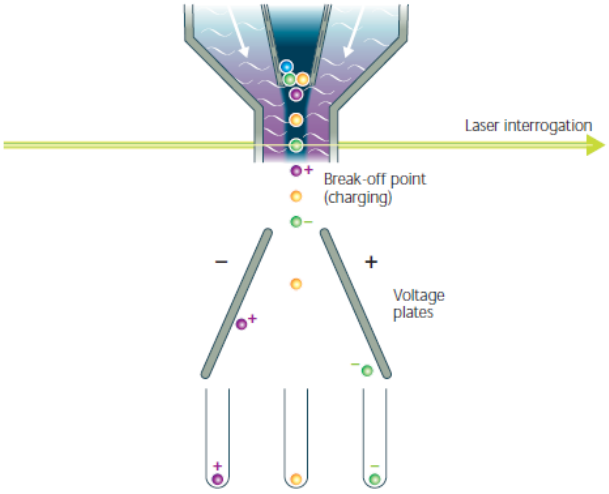
To detect multiple signals simultaneously, the precise choice and order of optical filters will be an important consideration.

4. Signal processing

When light hits a photodetector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector.

This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically. Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is preferable where there is not such a broad range of signals e.g. in DNA analysis.
The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence. The data acquired in each parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

5. Electrostatic cell sorting
A major application of flow cytometry is **to separate cells according to subtype** or epitope expression for further biological studies. This process is **called cell sorting** or **FACS analysis**. After the sample is hydrodynamically focused, each particle is probed with a beam of light. The scatter and fluorescence signal is compared to the sort criteria set on the instrument. If the particle matches the selection criteria, the fluid stream is charged as it exits the nozzle of the fluidics system. Electrostatic charging actually occurs at a precise moment called the 'break-off point', which describes the instant the droplet containing the particle of interest separates from the stream.
To prevent the break-off point happening at random distances from the nozzle and to maintain consistent droplet sizes, the nozzle is vibrated at high frequency. The droplets eventually pass through a strong electrostatic field, and are deflected left or right based on their charge (Figure 3). The speed of flow sorting depends on several factors including particle size and the rate of droplet formation. A typical nozzle is between 50–70 μM in diameter and, depending on the jet velocity from it, can produce **30,000–100,000 droplets per second**, which is ideal for accurate sorting. Higher jet velocities risk the nozzle becoming blocked and will also decrease the purity of the preparation.



Electrostatic flow sorting

6. Gates and regions
An important principle of flow cytometry data analysis is to selectively visualize the cells of interest while eliminating results from unwanted particles e.g. dead cells and debris. This procedure is called **gating.** Cells have traditionally been gated according to physical characteristics. For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Also, dead cells have lower forward scatter and higher side scatter than living cells. Lysed whole blood cell analysis is the most common application of gating, and Figure 4 depicts typical graphs for SSC versus FSC when using large cell numbers. The different physical properties of granulocytes, monocytes and lymphocytes allow them to be distinguished from each other and from cellular contaminants. On the density plot, each dot or point represents an individual cell that has passed

through the instrument. Yellow/green hotspots indicate large numbers of events resulting from discreet populations of cells. The colors give the graph a three-dimensional feel. After a little experience, discerning the various subtypes of blood cells is relatively straightforward. Contour diagrams are an alternative way to demonstrate the same data. Joined lines represent similar numbers of cells. The graph takes on the appearance of a geographical survey map, which, in principle, closely resembles the density plot. It is a matter of preference but sometimes discreet populations of cells are easier to visualize on contour diagrams.

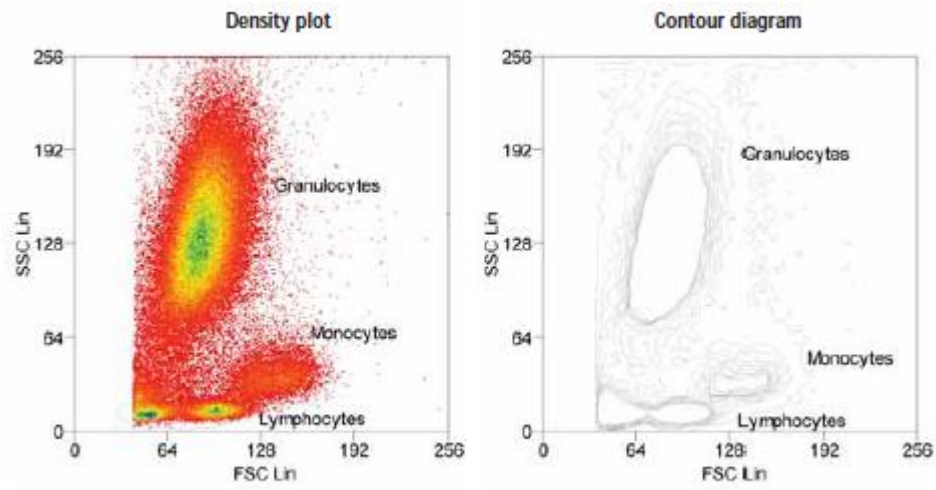


FIGURE 9 Analysis of lysed whole blood using FSC/SSC