# 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 on 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.

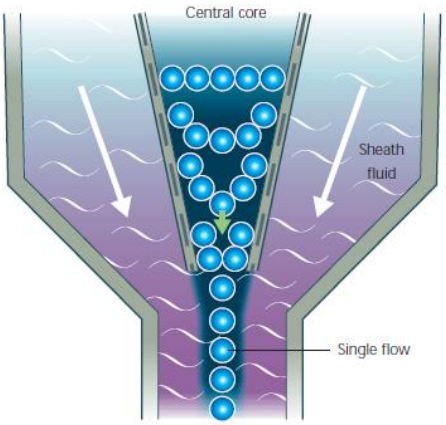



FIGURE 1 Hydrodynamic focusing produces a single stream of particles

The **acoustic technology within Attune Flow Cytometers** generate ultrasonic waves that act to transport particles to the center of the sample stream. The pre-focused stream is injected into the sheath stream, which applies hydrodynamic pressure to the sample. The combination of these two forces results in a narrow core stream and an increased probability that cells are passing single file through optical system (**Figure 2**).

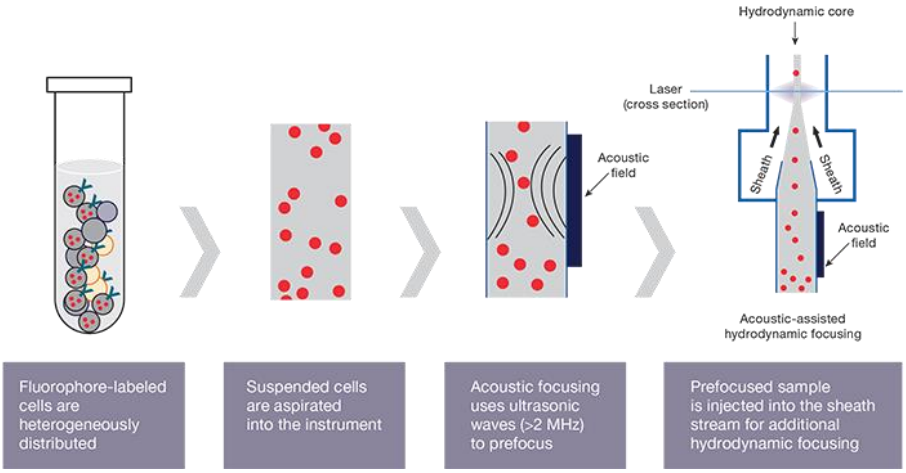



Figure 2. Overview of acoustic-assisted hydrodynamic focusing employed in the Attune Flow Cytometers.

### 3. Optics and detection

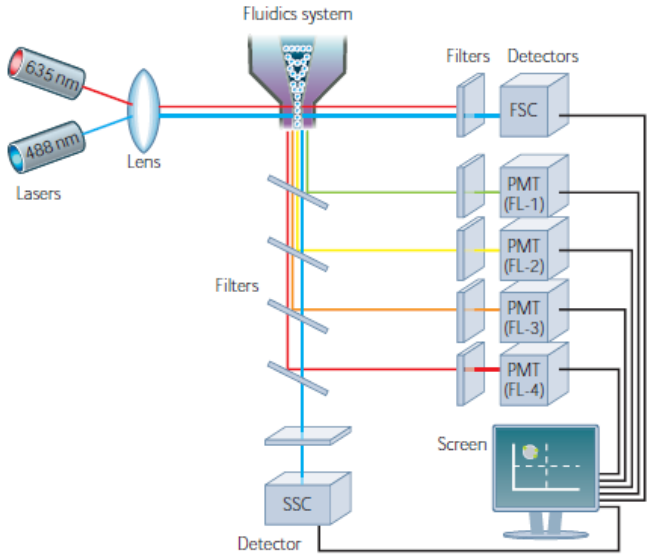
After hydrodynamic focusing, each particle passes through **one or more beams of light**. Light scattering or fluorescence emission (if the particle is labeled with a fluorochrome) provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources 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 90° 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.

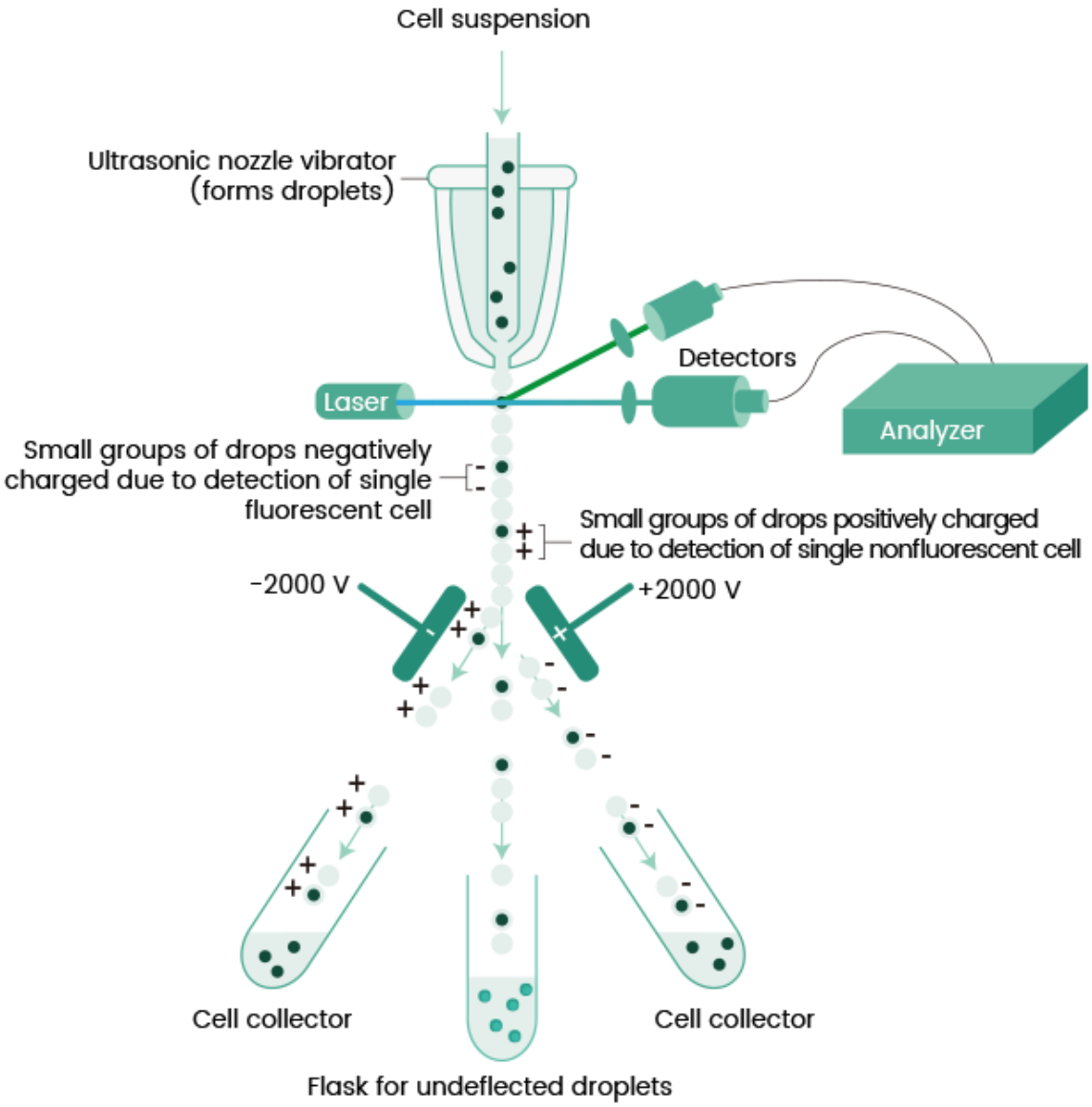
**5. Electrostatic cell sorting**

A major application of flow cytometry is **to separate cells according to subtype** or epitope expression for further biological studies.

Fluorescence-activated cell sorting (***FACS***) is a specialized type of flow cytometry. It provides a method for **sorting a heterogeneous mixture of biological cells into two or more containers**, **one cell at a time**, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. **A vibrating mechanism** causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An **electrical charging ring** is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off. ).

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.




**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.

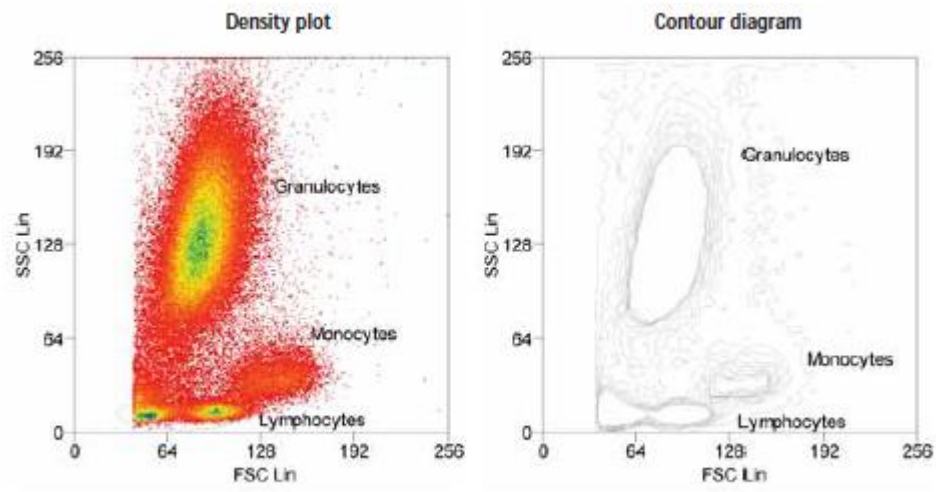



FIGURE 9 Analysis of lysed whole blood using FSC/SSC