FLOW CYTOMETRY

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1) This tech. was used for counting, examining and sorting microscopic objects / organisms or cells suspended in a stream of fluid.

2) Invented by M.I. Fulwyler & L.A. Herzenberg.

3) Principle → It involves multiparametric analysis of the physical and or chemical characteristics of a single cell flowing through an optical or electronic detection apparatus. A beam of laser light of single wavelength is aimed at the stream of fluid. Detectors are placed at placed either in line with the light beam (Forward detector). ~~or on side of light beam (Side detector). One or more fluorescent detectors are placed~~

Several Side detectors (SSC) and fluorescent detectors are placed perpendicular to the light beam.
When suspended particles pass through the beam they scatter light and fluorescent chemicals found in the particle or attached to it are excited into emitting light at a lower frequency than the source light. Both the scattered and fluorescent light are picked up by the ~~sca~~ detectors.
Analysis of fluctuations in brightness at each detector provides information about physical and chemical structure of each individual particle.

FSC detector measures cell volume
SSC detector " nuclear shape, cytoplasmic granules, membrane texture etc.

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Component of flow cytometer

i) Flow cell → liquid stream that carries and aligns the cell in a single file or row before passing through the beam of light for sensing.
ii) A light source → Mercury or xenon lamps.
iii) high power water-cooled Argon or Krypton dye lasers.
iv) low power air cooled lasers – blue, green, violet-diode laser.
v) A detector that converts analog to digital signal.
vi) A computer for analysis.

PROCEDURE

Cell suspension is passed through a small hole into a channel of flow assembly. The flow is arranged in such a way that the larger cells are separated on the basis of their diameter
Sheath fluid is pumped in the same channel through another opening and its function is help the cell suspension to move towards a third opening. from where cells emerge out.
The emerging cells are coated with fluorescent material.
A beam of light produced by lasers are directed towards the fluorescent coated cells which excites the fluorescent material and fluorescent light emitted is detected by photo multiplier tubes which relay the electric signal to the analysing system
Each fluorescent pulse detected is equivalent to a cell passing through the measuring orifice and magnitude of each pulse varies to proportion in the quantity of fluorescent material coated with each cell.

* FACS is a modified or specialized flow cytometry (Fluorescence Activated Cell Sorting). This method makes ~~it~~ separation of different cells present in a heterogenous mixture based upon specific light scattering and fluorescent characteristics of each cell.

Application of cytometry

1) Measurement of volume and morphology of cells, cell pigments
2) DNA, RNA, chromosome analysis and sorting
3) Protein expression and localization
4) To study expression of ~~in vitro~~ in vivo transgenic products, cell surface antigens, nuclear antigens, monitoring electropermeabilization of cells.
5) ~~They~~ It is useful in the field of pathology, immunology, in chemotherapy, sperm sorting in IVF.
6) In marine biology, the abundance and community structure of auto-fluorescent photosynthetic plankton can be studied with the help of flow cytometry.

Chapter

Principles of the flow cytometer

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.

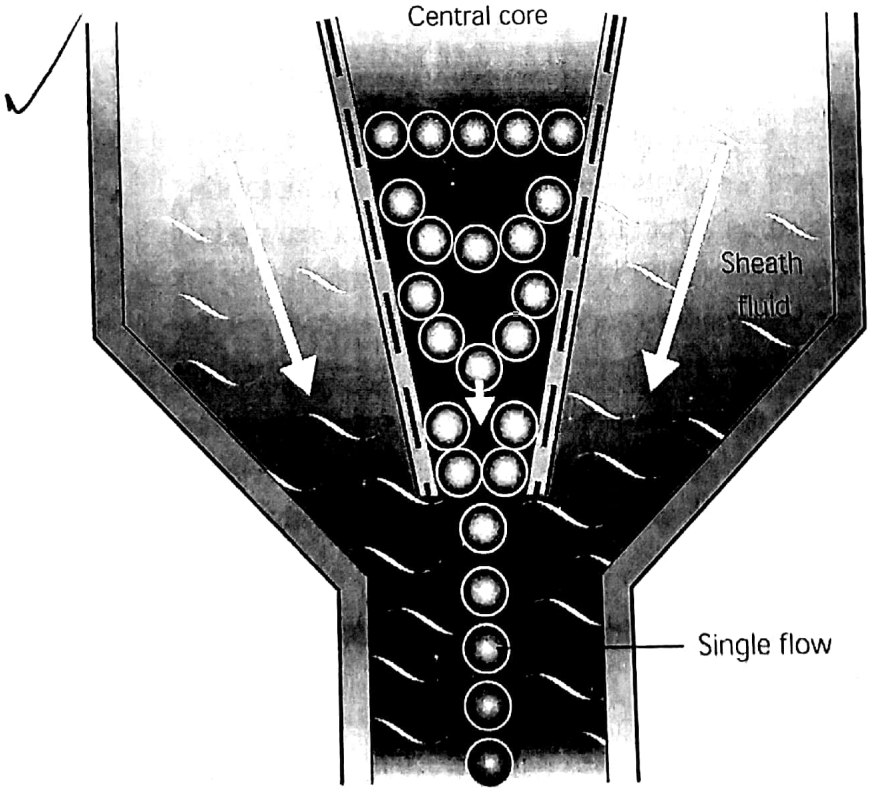

FIGURE 1 **Hydrodynamic focusing produces a single stream of particles**

The flow characteristics of the central fluid can be estimated using Reynolds Number (R_e):

$$R_e = \frac{pVD}{\mu}$$

where D = tube diameter, V = mean velocity of fluid, p = density of fluid, and μ = viscosity of fluid.

When $R_e < 2300$, flow is always laminar. When $R_e > 2300$, flow can be turbulent, which accelerates diffusion.

Without hydrodynamic focusing the nozzle of the instrument (typically 70 μM) would become blocked, and it would not be possible to analyze one cell at a time.

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). Arc lamps tend to be less expensive than lasers and exploit the color emissions of an ignited gas within a sealed tube. However, this produces unstable incoherent light of a mixture of wavelengths, which needs subsequent optical filtering.

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).

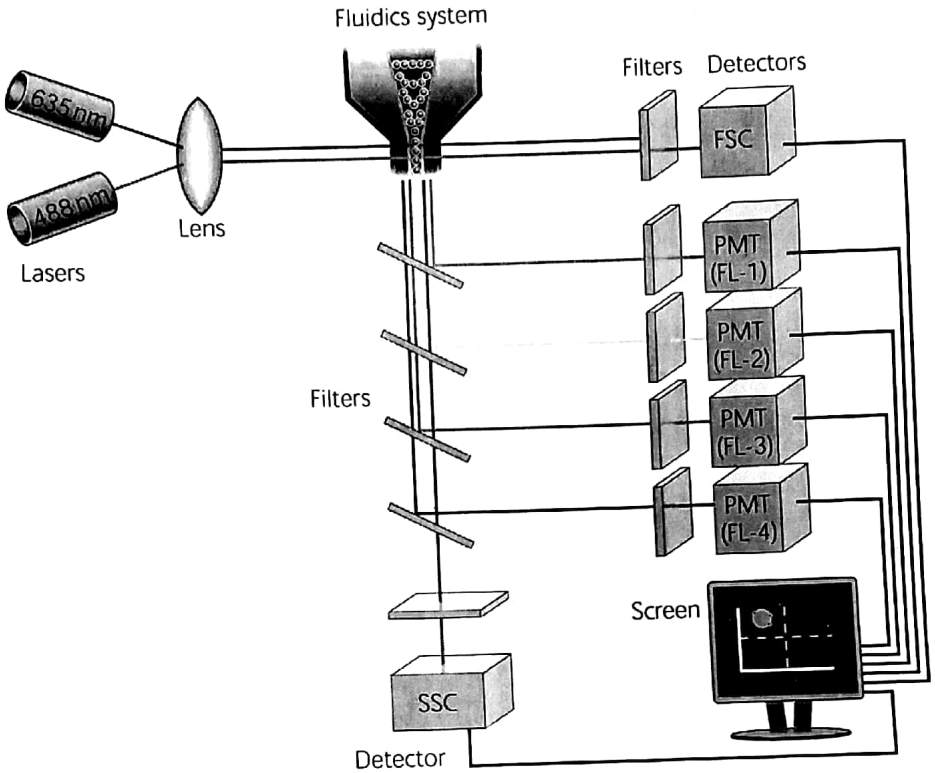

FIGURE 3 **Schematic overview of a typical flow cytometer setup**

parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

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 4).

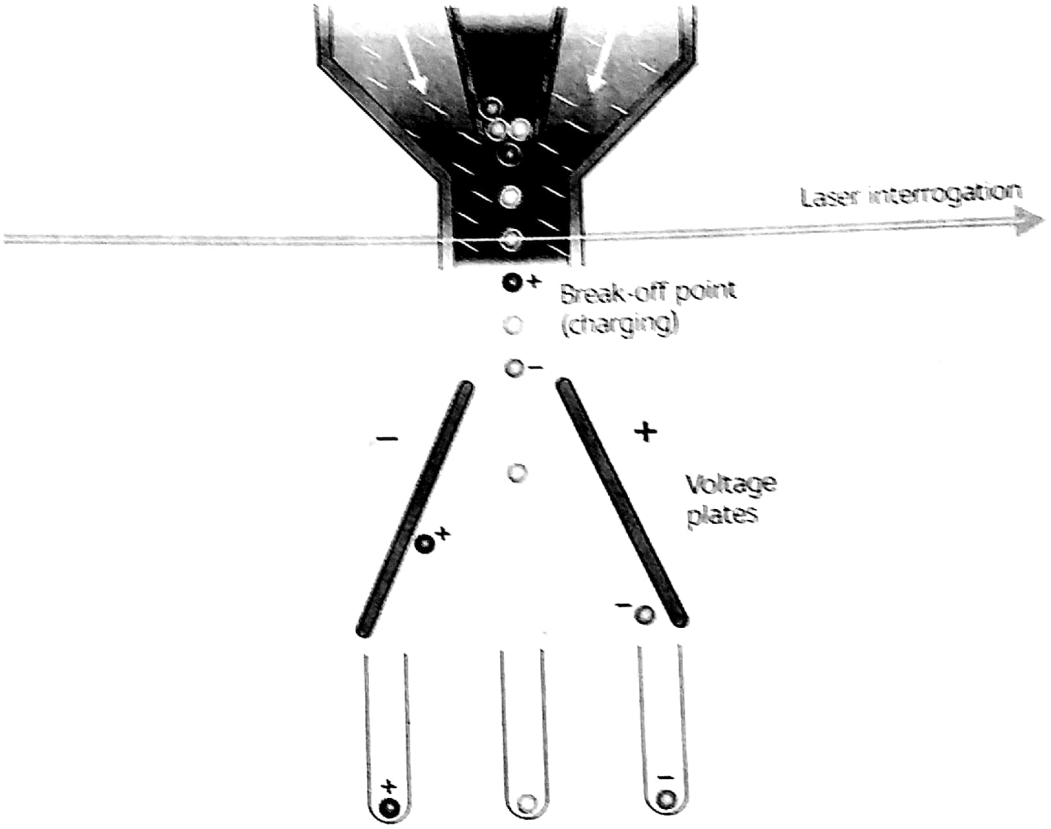

FIGURE 4 **Electrostatic flow sorting**

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.