INSTITUTO DE MEDICINA MOLECULAR JOÃO LOBO ANTUNES

BASICS OF FLOW CYTOMETRY

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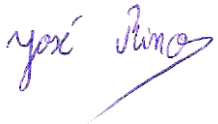


Overview

Flow: Fluid **Cyto:** Cell **Metry:** Measurement

Flow cytometry is a technology that measures and analyzes multiple physical characteristics of single particles, in an heterogenous particle suspension, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative complexity, and relative fluorescence intensity.

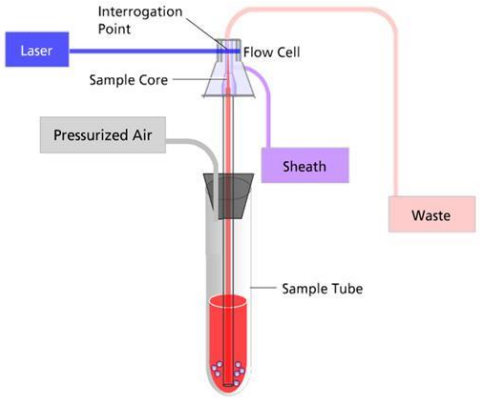
A flow cytometer is made up of three main systems: fluidics, optics, and electronics.

• 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 are processed by the computer.

In the flow cytometer, particles are carried to the laser beam in a fluid stream (usually PBS). When particles pass through the laser beam, incident laser light is scattered and fluorescence is emitted as particles pass through the interrogation point. The scattered and fluorescent light are collected by appropriately positioned lenses. Appropriate filters steer the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them.

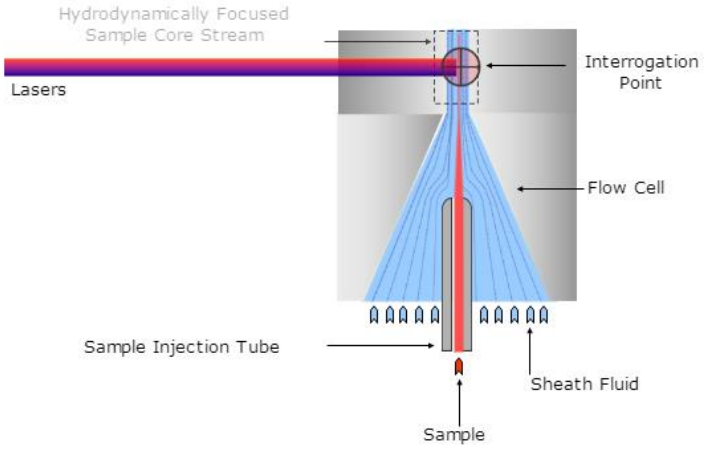
The data are collected for each particle and stored in the computer. The characteristics of each particle are based on its light scattering and fluorescent properties. This data can be analyzed to provide information about subpopulations within the sample.

Fluidics

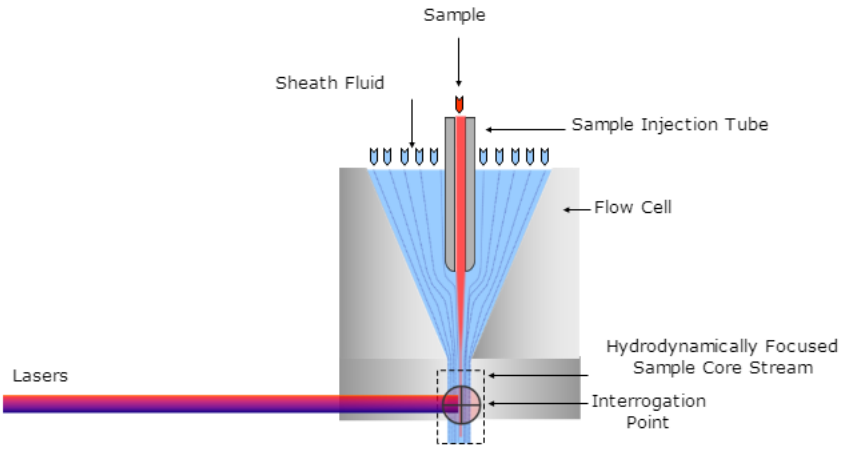


After passing through the sheath filter, sheath fluid (PBS) is introduced into the flow cell. When a sample tube is placed on the SIP, the sample is forced up and injected into the flow cell. The portion of the fluid stream where particles are located is called the sample core. Besides transporting particles in the stream to the laser beam for interrogation, the function of the fluidic system is also to align the central core with the center of the laser beam. The shape of the flow cell enables this hydrodynamic focusing.

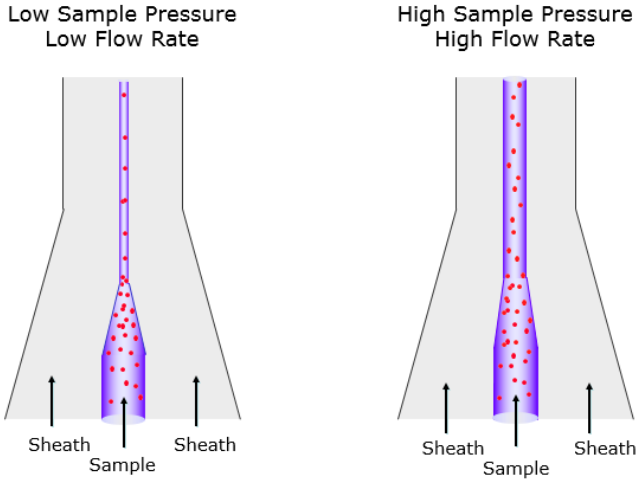
In cytometers such as BD LSRFortessa, the sample is carried upward through the center of the flow cell, where the particles are intercepted by the laser beam.



While in others such as BD FACSAria, the sample stream flows downward through the cuvette flow cell and eventually exits through the nozzle tip.

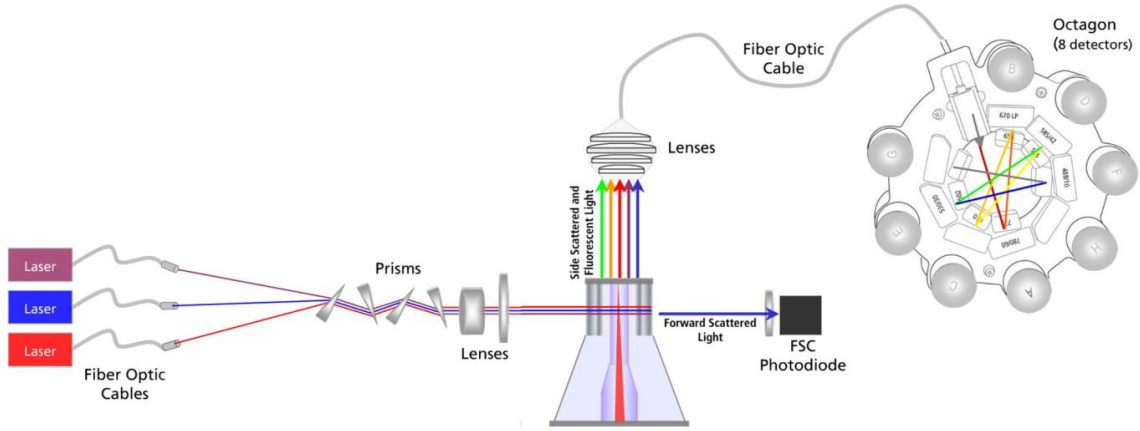


The sample pressure must always be greater than the sheath fluid pressure. The sample pressure can be adjusted through changes in the width of the sample core, which can decrease or improve the sample flow rate (events/s).

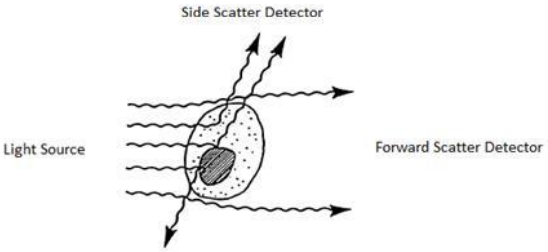


Optics

The optics subsystem provides the excitation sources and the components to collect light signals and route them to the appropriate detectors.



The Excitation Optics, through fiber optic cables, prisms and lenses, center the laser beam onto the sample core for the interrogation. The Collection Optics steer the scattered light to appropriate detectors. Scattered and emitted light are collected by appropriately positioned lenses that focus fluorescent light emitted at each of the laser interrogation points, onto the fiber optic cables. These, then steer the light into a set of filters and detectors.



Forward scattered (FSC) light indicates relative differences in the size of the cells. Side scattered (SSC) light indicates relative differences in the internal complexity or granularity of the cells.

When cells stained with fluorochrome-conjugated antibodies pass through the laser, the dyes can absorb photons, and thus are promoted to an excited electronic state. In returning to their ground state the dyes release energy, emitted as light. This light emission is known as fluorescence. The optical filters direct the light scatter and fluorescence signals to the appropriate detectors.

Visible light consists of wavelengths ranging between 400 nm and 700 nm. Wavelengths correspond to different colors. Optical filters are used to direct specific light wavelengths to each photodetector. There are 3 types of filters:

- Long Pass: Transmits light that is equal or longer than the specified wavelength.
- Band Pass: Transmits light centered on the first value, within the range specifies by the second value.
- Short Pass: Transmits light that is equal to or shorter than the specified wavelength.

Electronics

The main functions of the electronics system are to:

- Convert light signals into numerical data;
- Eliminate small signal events such as noise and debris through threshold;
- Attribute signals from multiple channels to the correct cell/particle

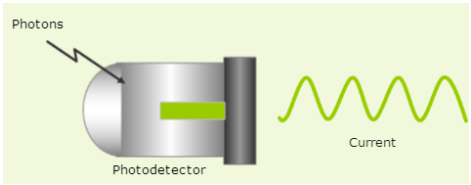
How is light converted into Data?

For light to be converted into data, 3 components are required: Photodetection, Amplification and Signal Processing.

These 3 components are crucial to convert Light into Current, Current into Voltage, and Voltage into Data for further analysis.

- Photodetection

Photodetectors are light sensors that can detect photons of light. Incoming photons cause photodetectors to produce electrical current.



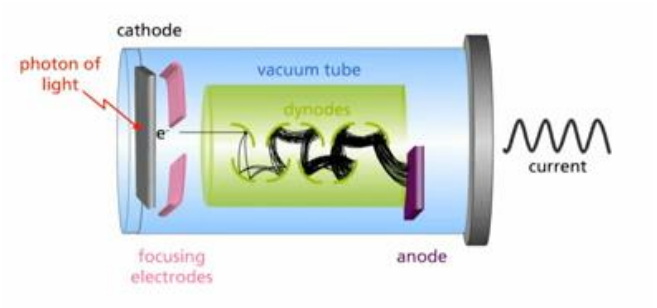
As a particle passes through the laser, it scatters light and can emit fluorescence. This scatter or fluorescence emission signal is converted into an electronic signal by the photodetectors. The electrical signal is called a pulse.

Among the photodetectors used, the **Photodiodes** are the less efficient, generating only one electron per one incoming photon. These Photodiodes are used to detect very strong signals such as FSC.

Photomultipliers (PMTs) are the most efficient photodetectors, generating billions of electrons per one incoming photon. These PMTs are used to detect very weak signals such as fluorescence and SSC.

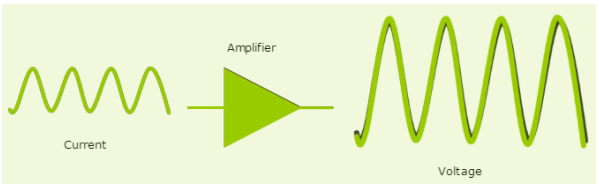
The PMT consists of a vacuum tube with a cathode, focusing electrodes, dynodes and an anode. Once the photon of light reaches the photodetector, the cathode generates one electron. Focusing electrodes guide and accelerate the electron to a series of dynodes.

Each time an electron strikes a dynode, thousands of electrons are generated until they reach the final anode. This explains how a single photon can generate billions of electrons.

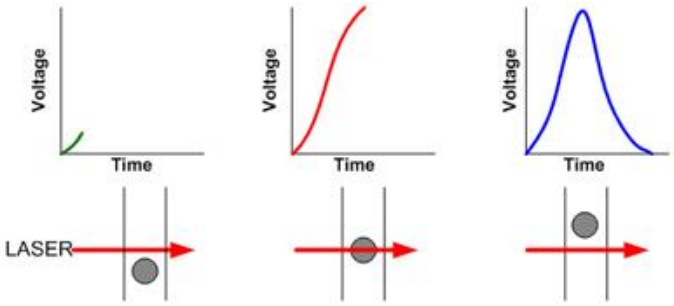


- Amplification

Amplifiers convert electrical current (pulses) from the photodetectors into a voltage. The resulting voltages are larger in magnitude than the incoming currents.



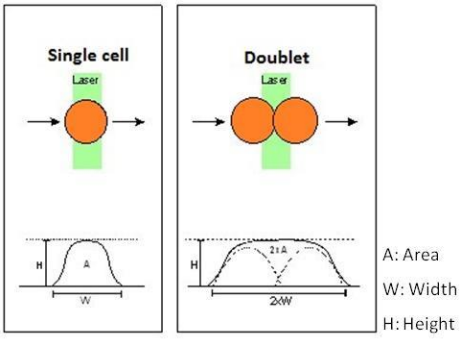
The final pulse:



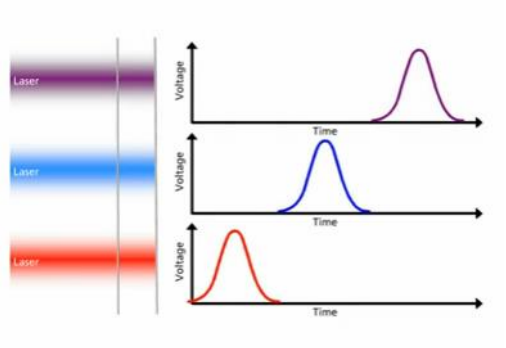
- Signal Processing

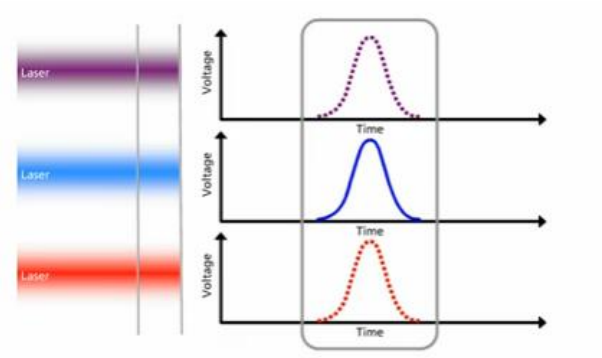
Signal processors quantify voltage pulses into numerical values for pulse height, pulse width, and pulse area.

The height and area of the fluorescence pulse will be proportional to the total amount of fluorescent material contained in the cell. The pulse width, or duration of the pulse, yields size information, and can be used to discriminate between single cells and doublets.

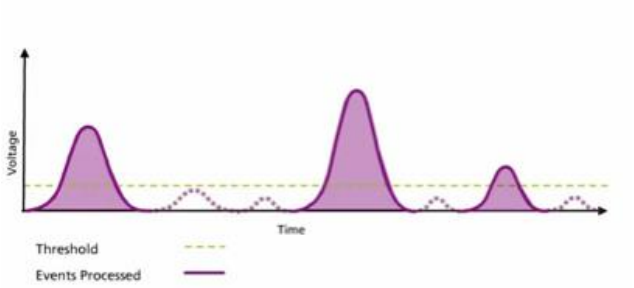


Accurate signal processing requires laser delay adjustments in cytometers with more than one laser. Laser time delay adjustments are made to ensure that signals from each laser are attributed to the correct cell/particle. In cytometers with multiple lasers, they are spatially separated so that a cell passes through only one laser at time. The spatial separation of the laser results in a single particle generating signals at different moments in time. Laser time delay ensures that all the parameters for each cell are processed together as one event. The laser time delay is calculated using specific beads during daily quality control.





Pulses are generated whenever a particle passes through the laser beam. To avoid the collection of unwanted data and debris, threshold can be used. The threshold is the minimum pulse height that a signal must have to be processed. Particles with a height pulse above the threshold are not processed and no data is recorded for those events.



What is a fluorochrome?

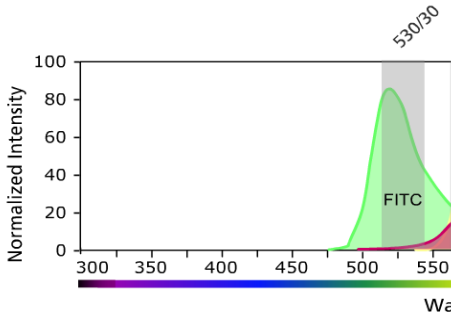
A fluorochrome is a functional molecule that fluoresces or emits light. When it is excited by an energy source (laser), the fluorochrome absorbs the energy and them emits energy of a higher wavelength. Fluorochromes are bound to monoclonal antibodies to enable cellular identification. The more binding sites on a cell for a specific antibody, the brighter the fluorescence intensity, and the higher on the fluorescence intensity scale the data appears.

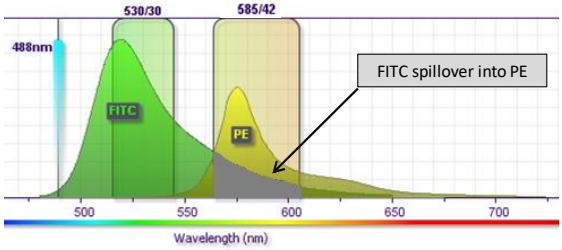
Compensation

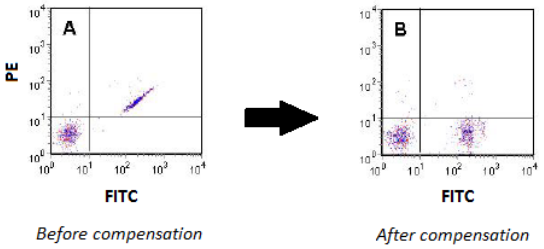
Fluorochromes emit light over a range of wavelengths. Optical filters are used to limit the range of frequencies measured by a given detector. However, when two or more fluorochromes are used, the overlap in wavelength ranges can make it impossible for optical filters to isolate light from a given fluorochromes. As a result, light emitted for one fluorochrome appears in a detector intended for another. This is referred to as spectral overlap and it can be corrected using a method called compensation.

In the following example, FITC emission appears primarily in the FITC detector, but some of its fluorescence spills over into the PE detector. Compensation allows you to distinguish cells that are truly positive from those that appear to be positive due to spillover into other detectors.

This can be a dot plof of FITC vs PE. The spillover seen in FITC detector must be corrected. The following figure shows FITC spillover correctly compensated out of the PE parameter.







Before compensation *After compensation*