# BASICS OF FLOW CYTOMETRY

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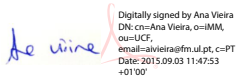
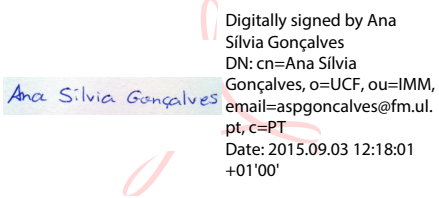



Ana Sílvia Gonçalves




**APPROVAL:** Henrique Veiga Fernandes

## Overview

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

Flow cytometry is a technology that measures and 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.

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

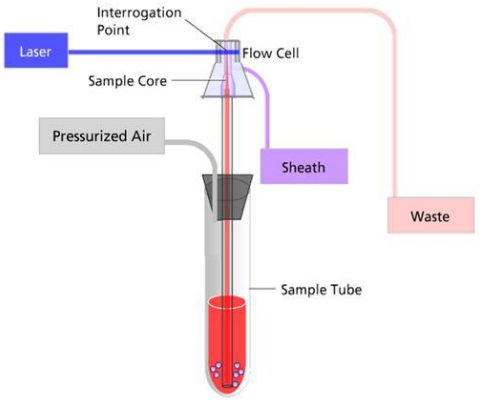
• The fluidics system transports particles in a stream to the laser beam for interrogation.
• The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.
• The electronics system converts the detected light signals into electronic signals that are processed by the computer.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream (usually PBS). 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. 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.

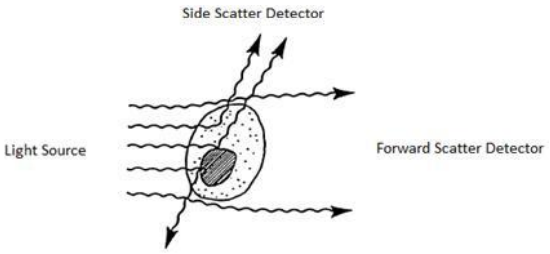
### Fluidics

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 sample is carried upward through the center of the flow cell, where the particles are intercepted by the laser beam.

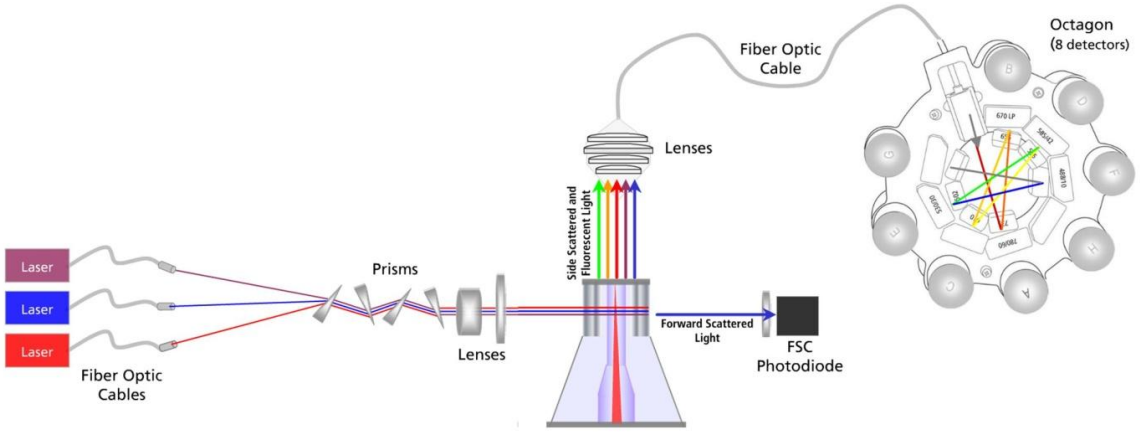


## Optics

The optics subsystem provides the excitation sources and the components to collect light signals and route them to the appropriate detectors. When a cell passes through a laser beam, laser light is scattered in all directions. 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 raging between 400nm and 700nm. Wavelengths correspond to different colors. Optical filters are used to direct specific light wavelength (or colors) 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.

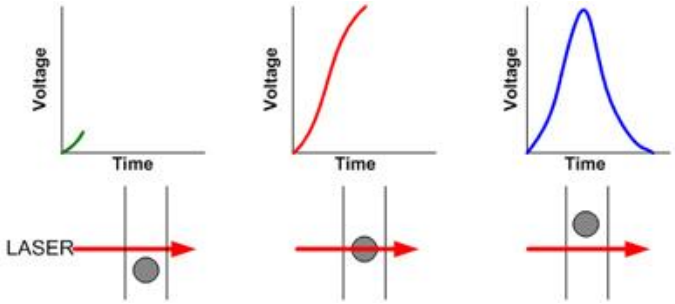
## Electronics

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 lasers to the correct cell/particle

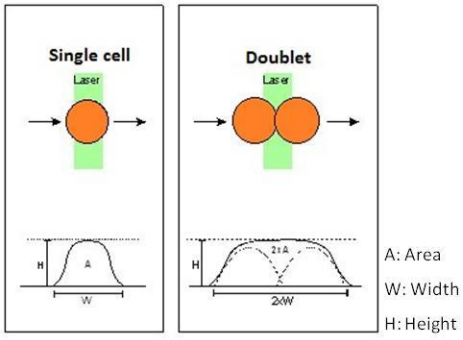
### Elements of Signal Processing:

Formation of a voltage pulse: 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 detectors. The electrical signal is called a pulse.




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.

Pulse quantification: Information about the cell is derived from the characteristics of the pulse. 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.



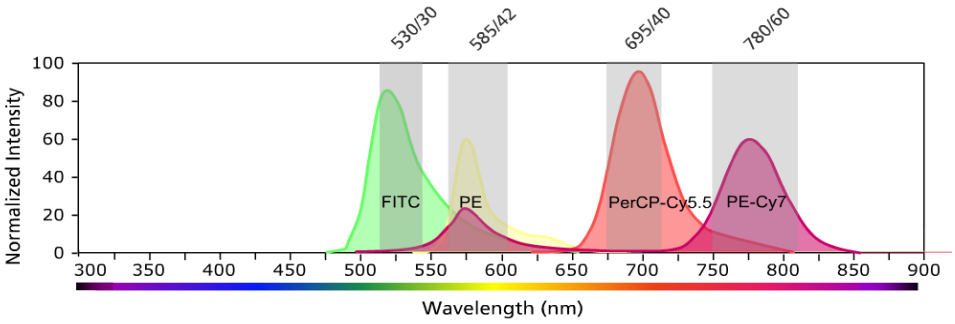
## What is a fluorochrome?

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.

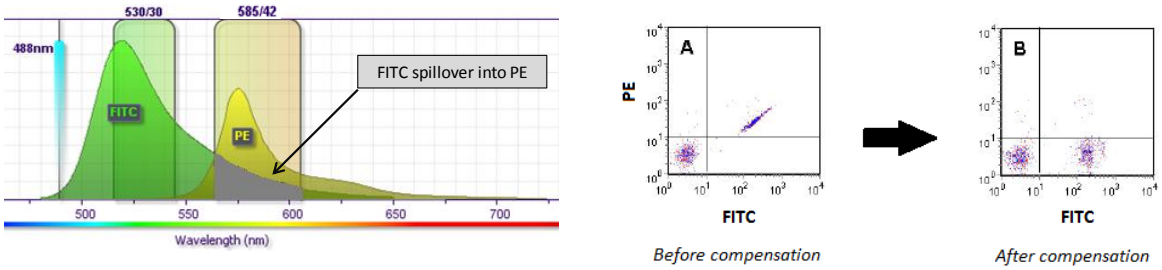
### Compensation

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.

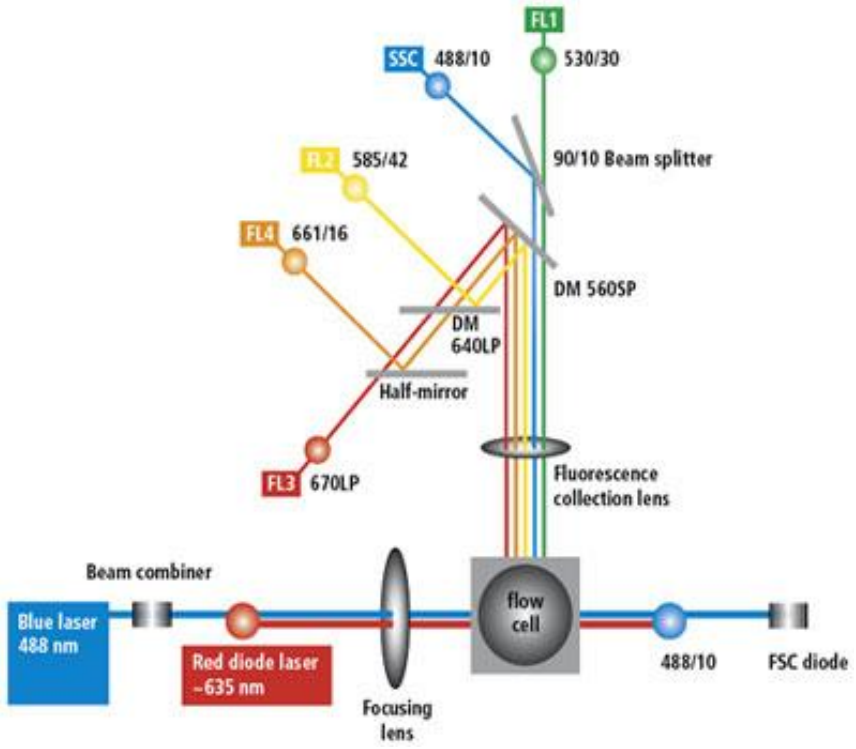
In the following example, FITC emission appears primarily in the FITC detector, but some of its fluorescence spills over into the PE detector. Compensation allows you to distinguish cells that are truly positive from those that appear to be positive due to spillover into other detectors.



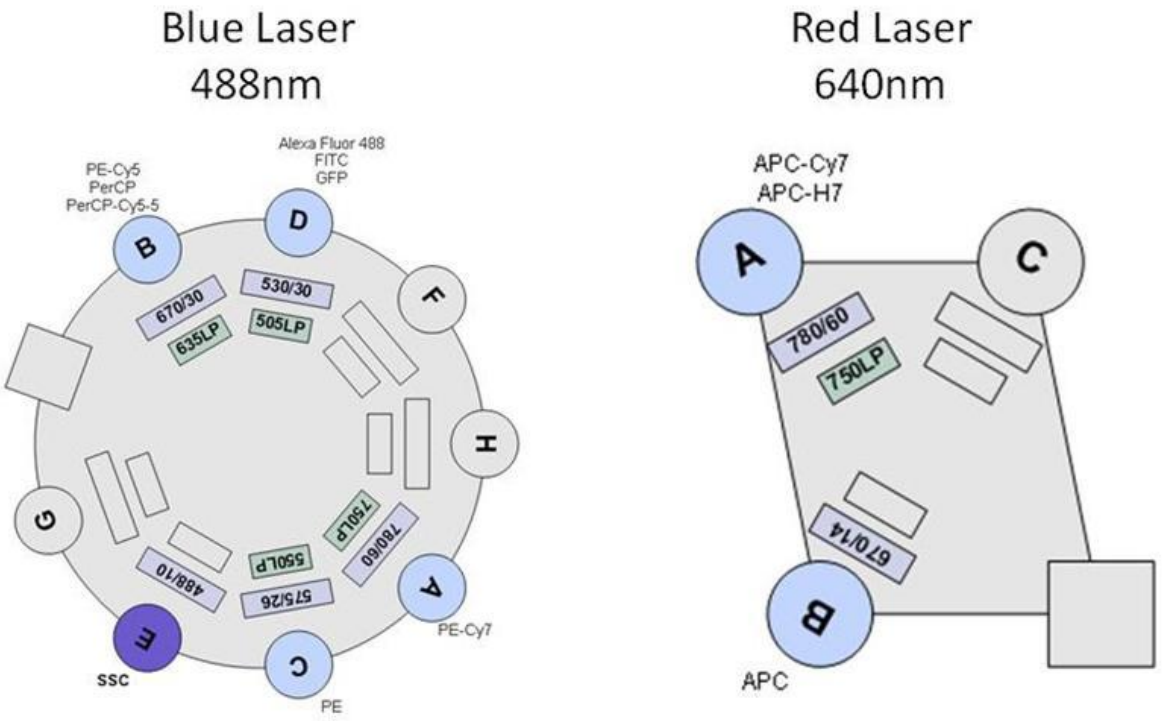

This spillover can be seen in a dot plof of FITC vs PE. The FITC spillover in the PE detector must be corrected. The following figure shows FITC spillover correctly compensated out of the PE parameter.

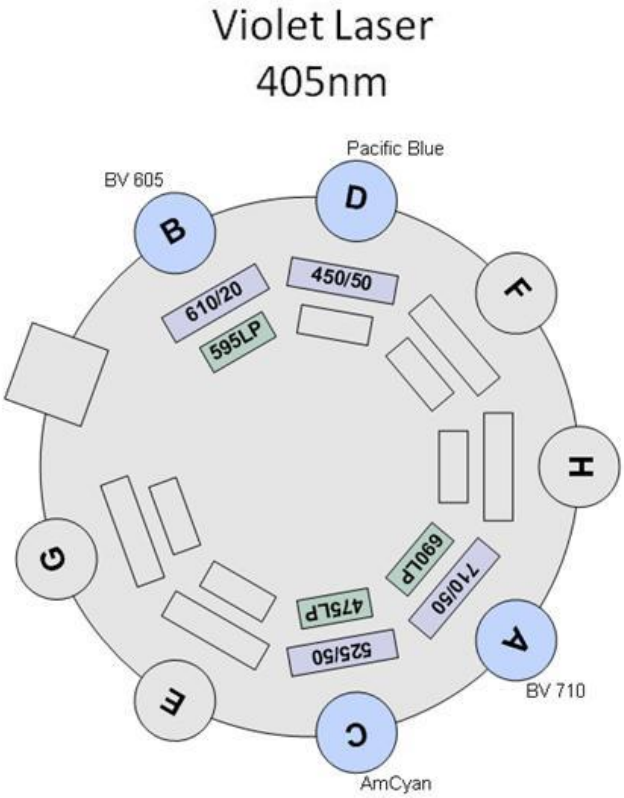


## FACSCalibur Configuration

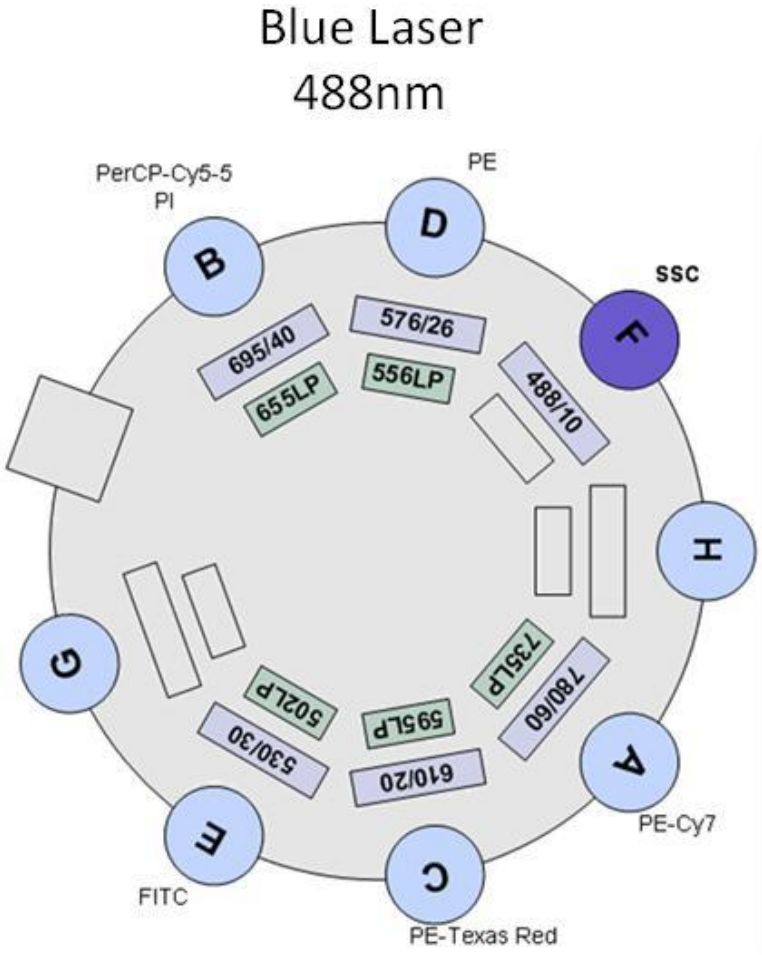


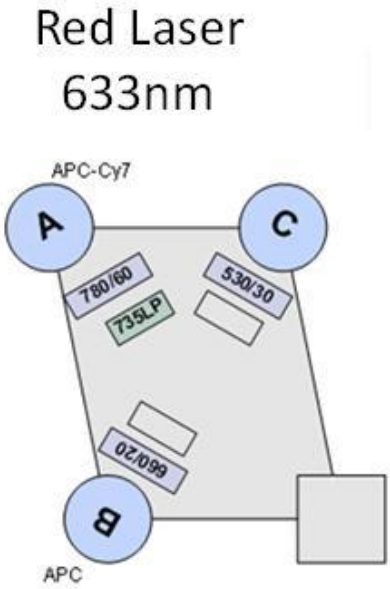
## LSR Fortessa Configuration

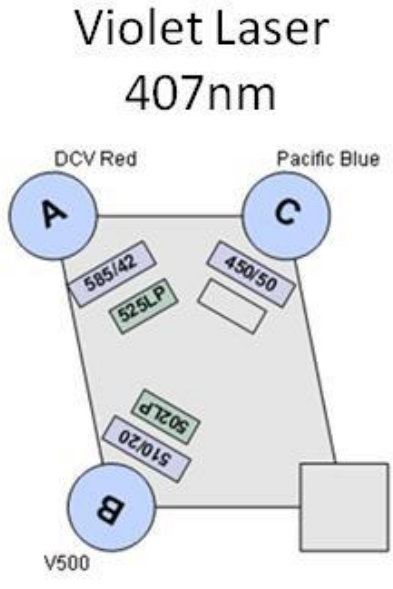


## FACSAria IIu Configuration



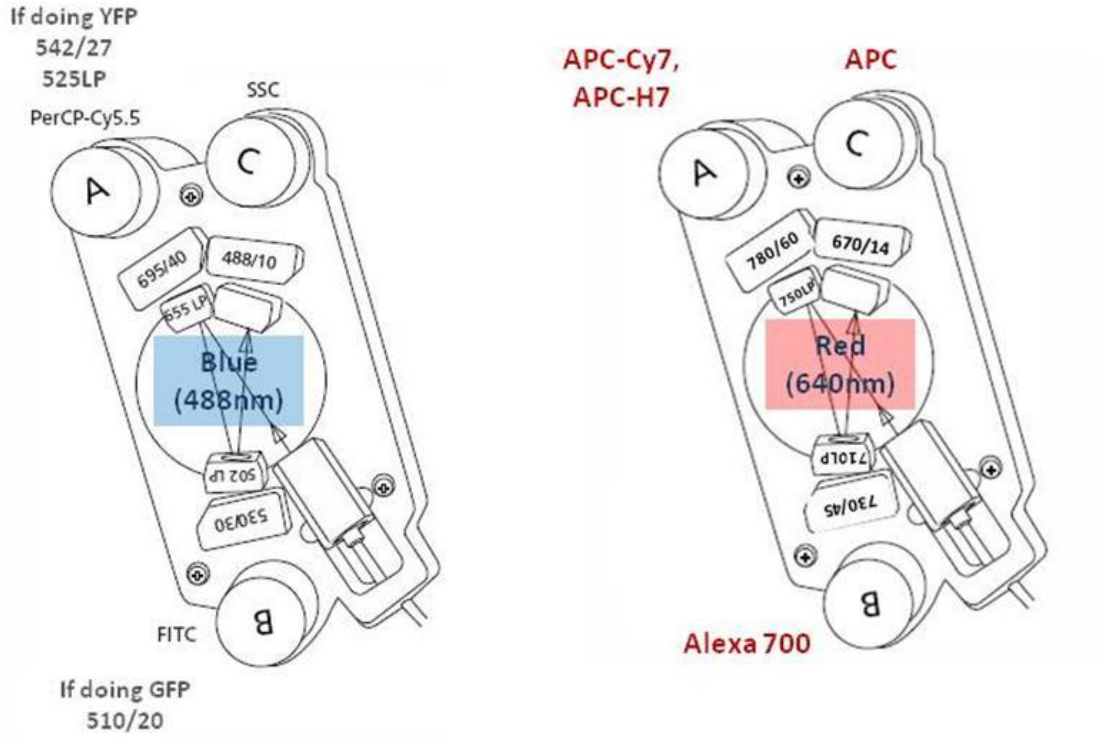



## FSCAria III Configuration

Blue Laser
488nm

Red Laser
640nm




Yellow-Green Laser
561nm