Flow Cytometry SRL

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http://www.bidmc.org/Research/CoreFacilities/FlowCytometryCore.aspx

http://bidflow.calendarhost.com/cgi-bin/calweb/calweb.cgi

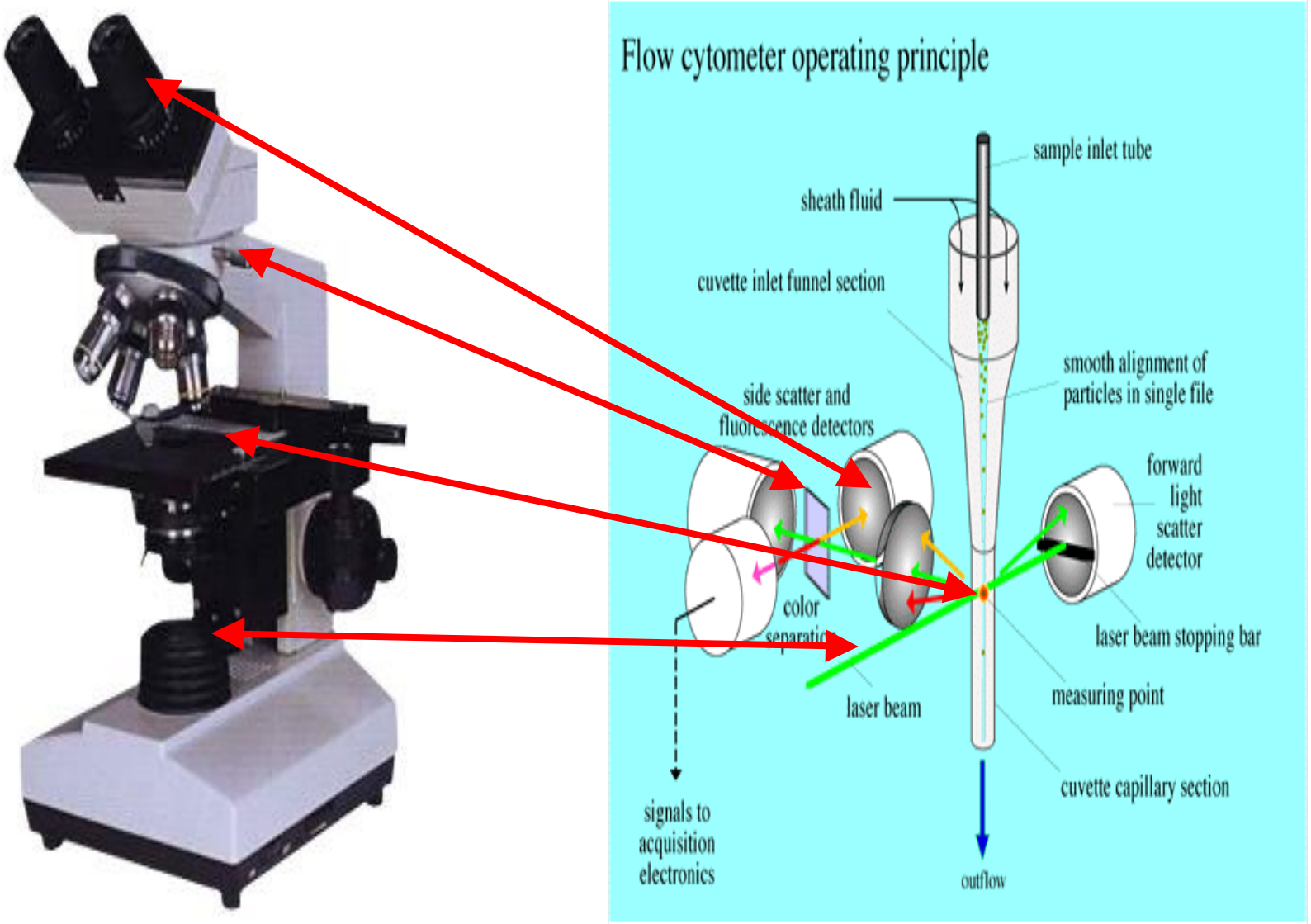
Flow Cytometry

Flow ~ fluid in motion

Cyto ~ cell

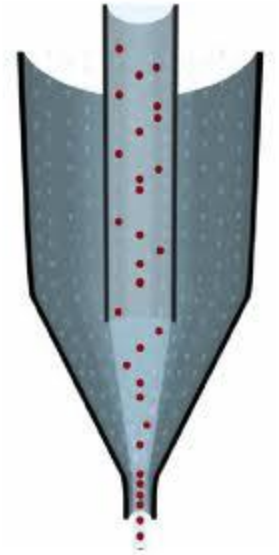
Metry ~ measure

Measuring properties of cells while in a fluid stream

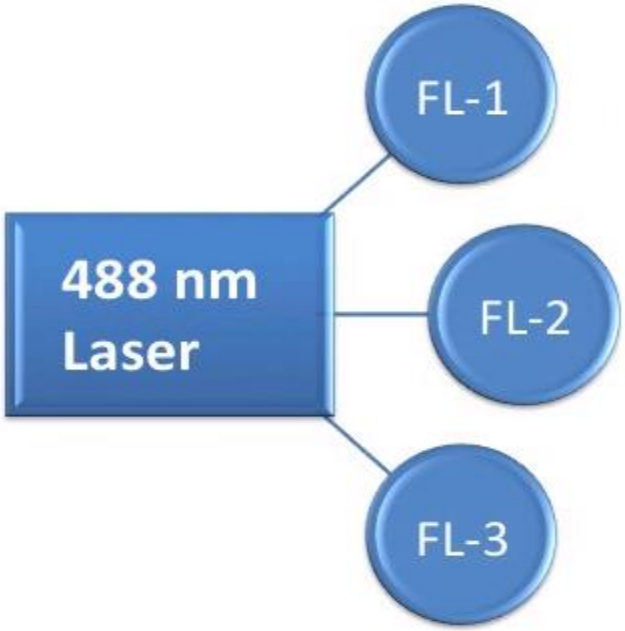

Flow cytometer operating principle
sample inlet tube
sheath fluid
cuvette inlet funnel section
smooth alignment of particles in single file
side scatter and fluorescence detectors
forward light scatter detector
color separation
laser beam stopping bar
laser beam
measuring point
cuvette capillary section
signals to acquisition electronics
outflow

Three main systems that make up a Flow Cytometer

FLUIDICS



OPTICS

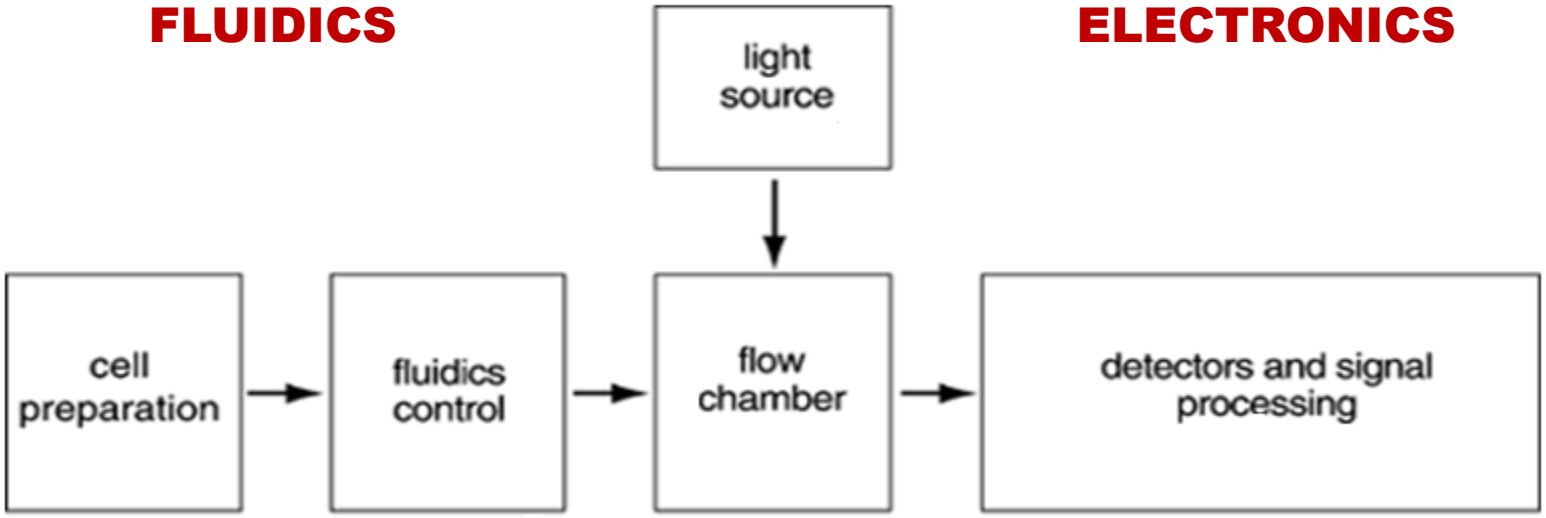


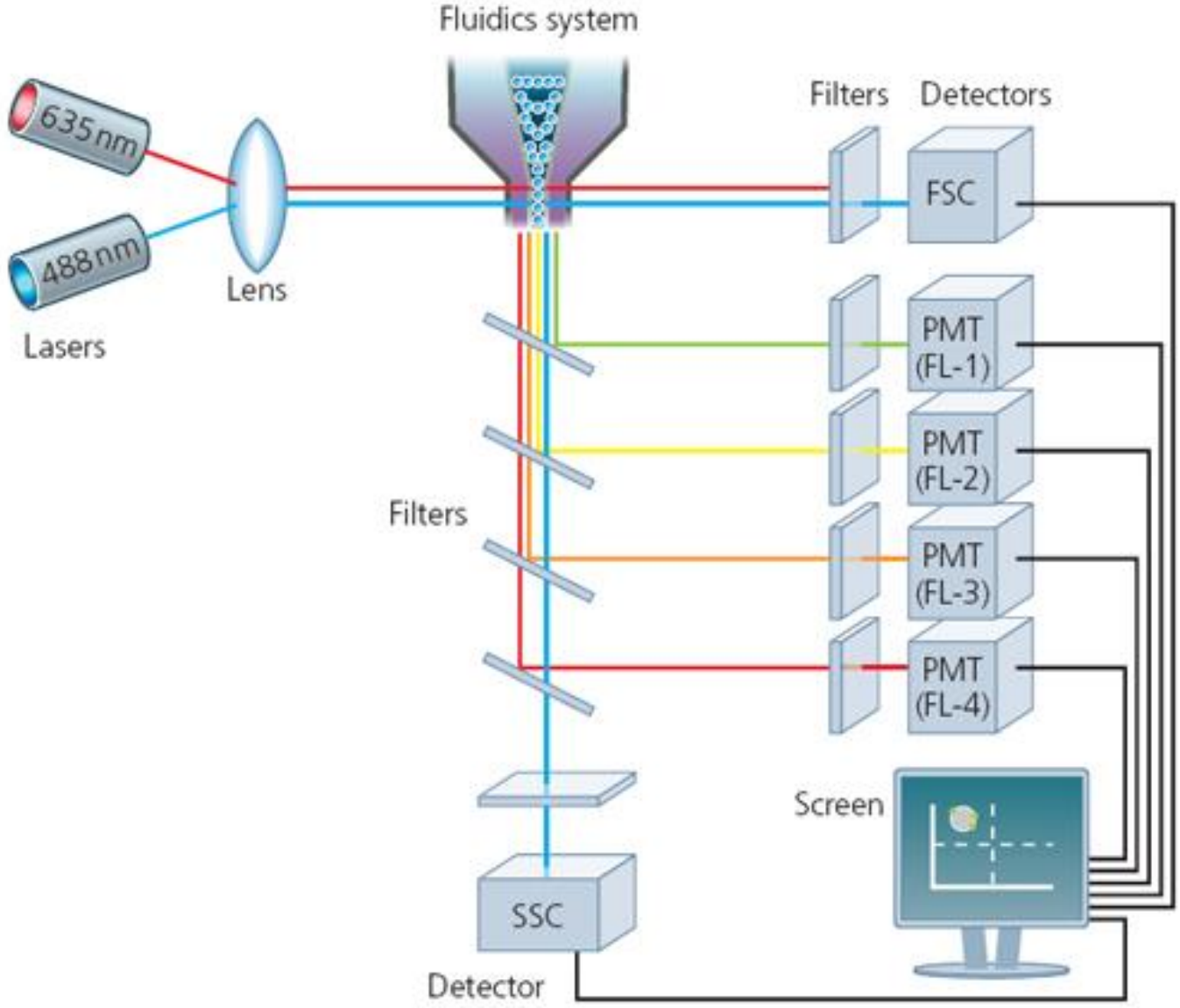
ELECTRONICS

OPTICS

FLUIDICS

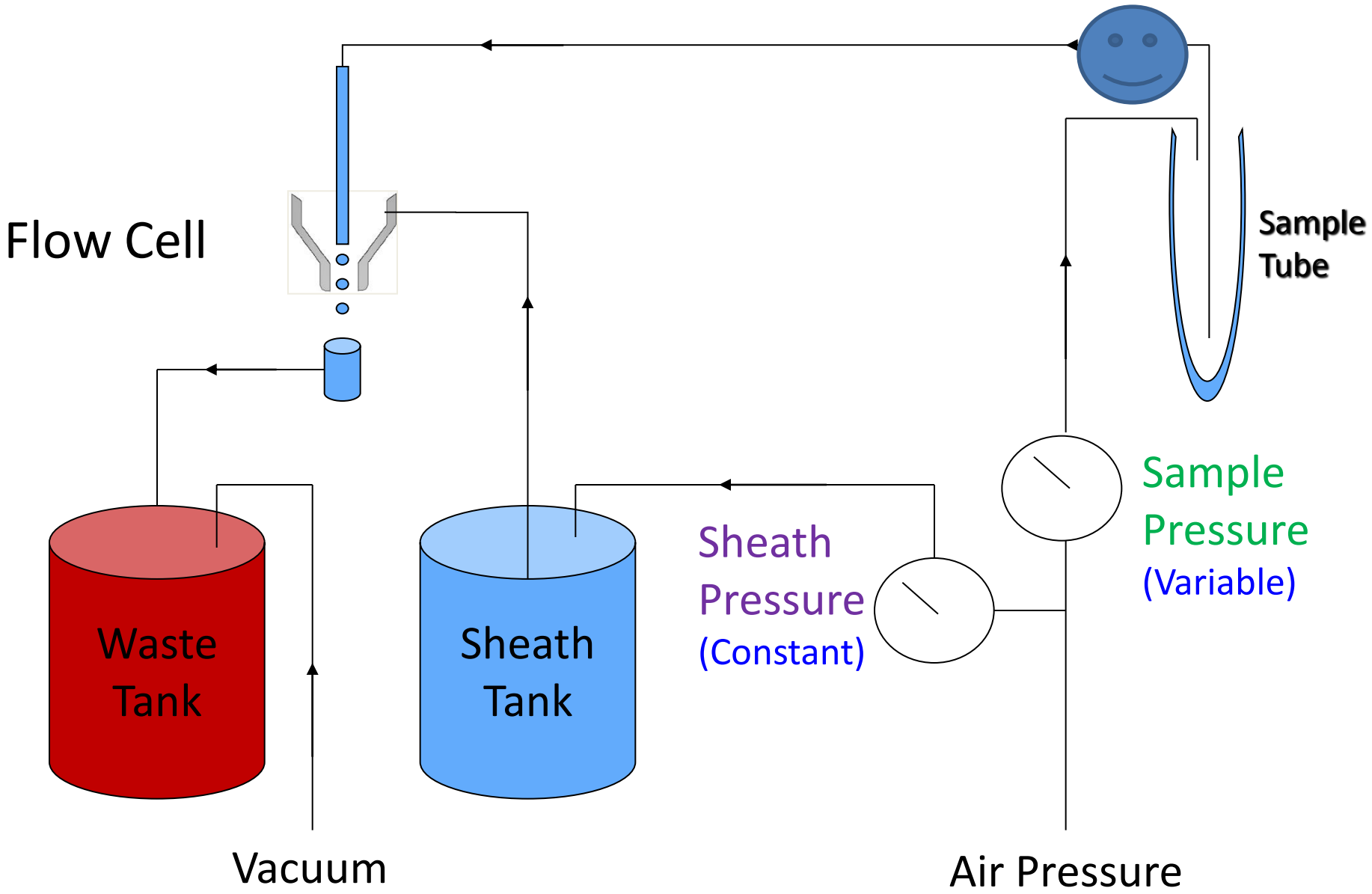
ELECTRONICS




Fluidics system
Filters
Detectors
635nm
488nm
Lens
Lasers
FSC
PMT
(FL-1)
PMT
(FL-2)
Filters
PMT
(FL-3)
PMT
(FL-4)
Screen
SSC
Detector

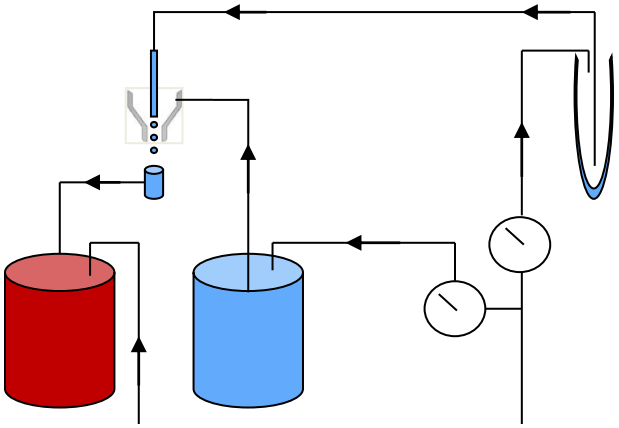
FLUIDICS

Fluidics Schematic

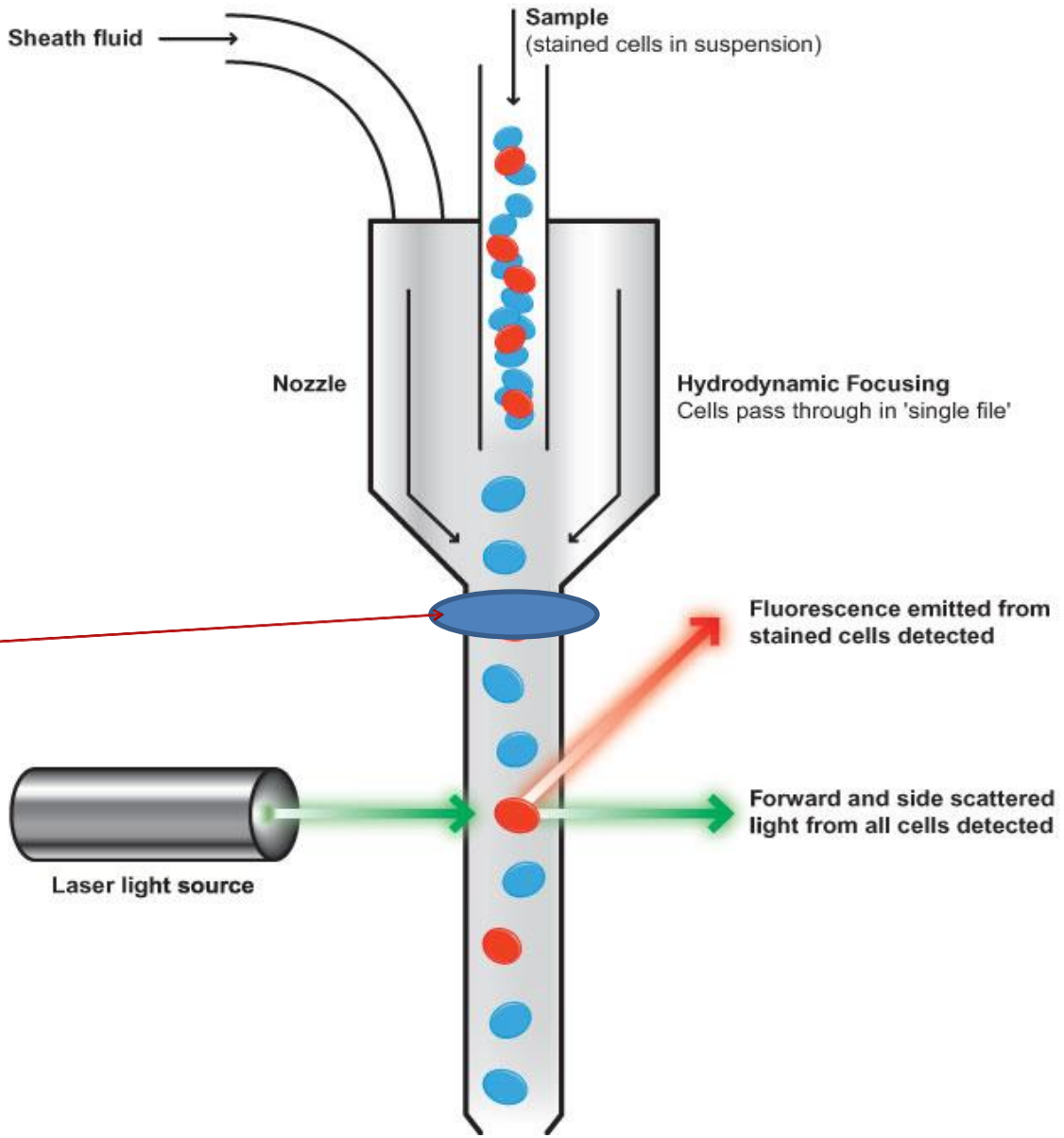


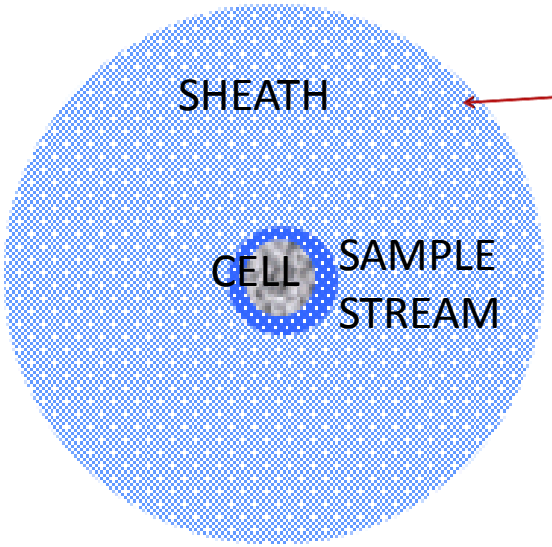
How The Flow Cell Works

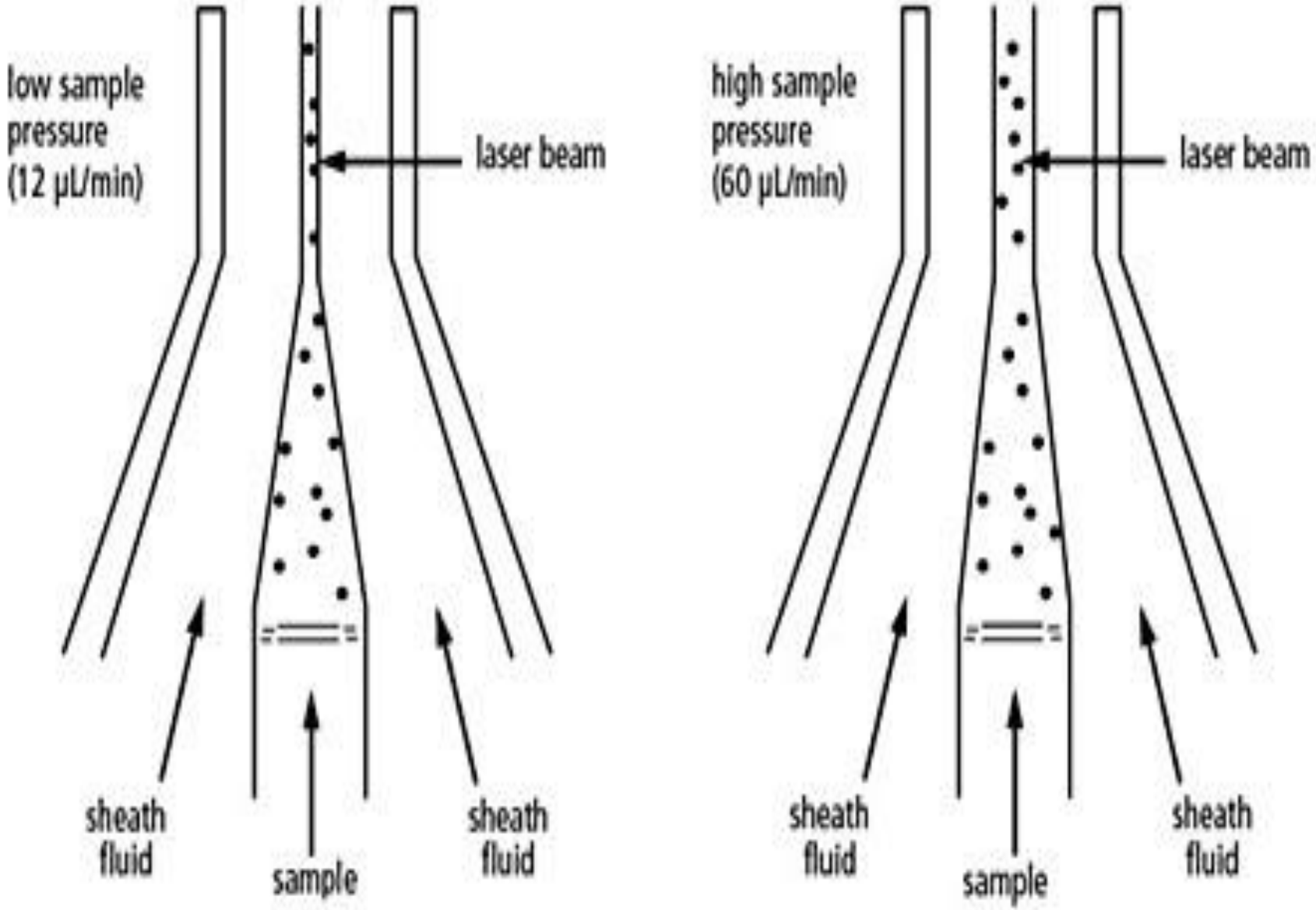
- The cells from the sample tube are injected into the sheath stream
- Flow in a flow cell is laminar.
- Hydrodynamic focusing pushes the cells to line up single file along their long axis.
- The shape of the flow cell provides the means for hydrodynamic focusing.



Flow Cytometry





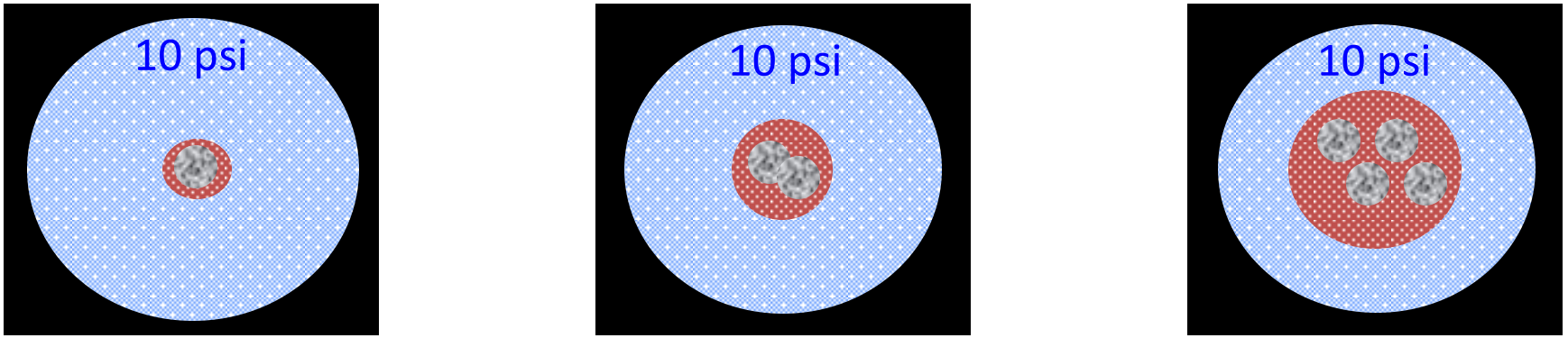

low sample
pressure
(12 µL/min)
laser beam
sheath
fluid
sample
sheath
fluid
high sample
pressure
(60 µL/min)
laser beam
sheath
fluid
sample
sheath
fluid

Sample Differential

10.2 psi

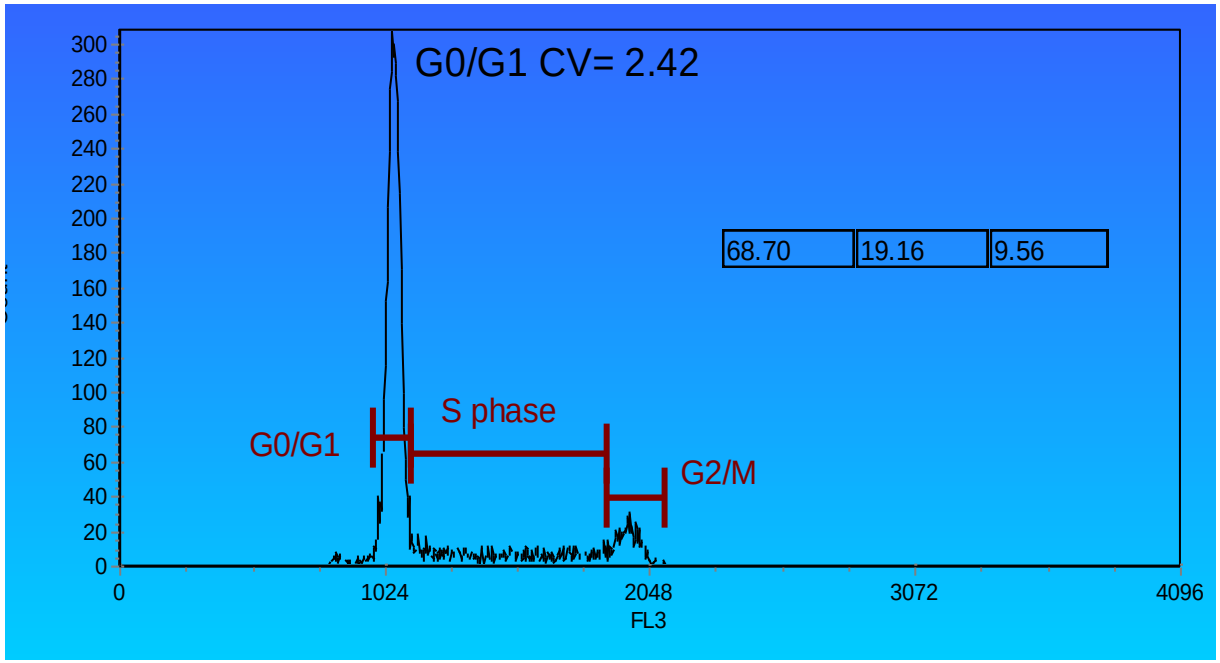
10.4 psi

10.8 psi

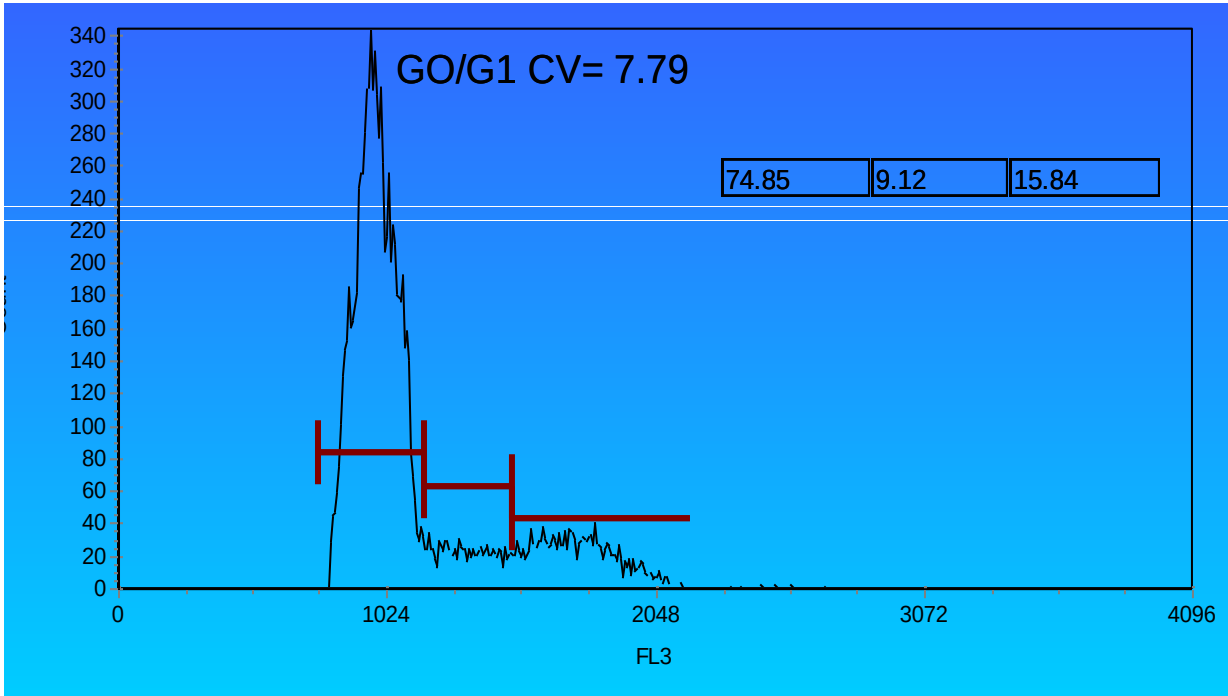


Difference in pressure between sample and sheath
This will control sample volume flow rate
The greater the differential, the wider the sample core.
If differential is too large, cells will no longer line up single file
Results in wider **CV's** and increase in multiple cells passing through the laser at once. No more single cell analysis!

Low pressure



High pressure



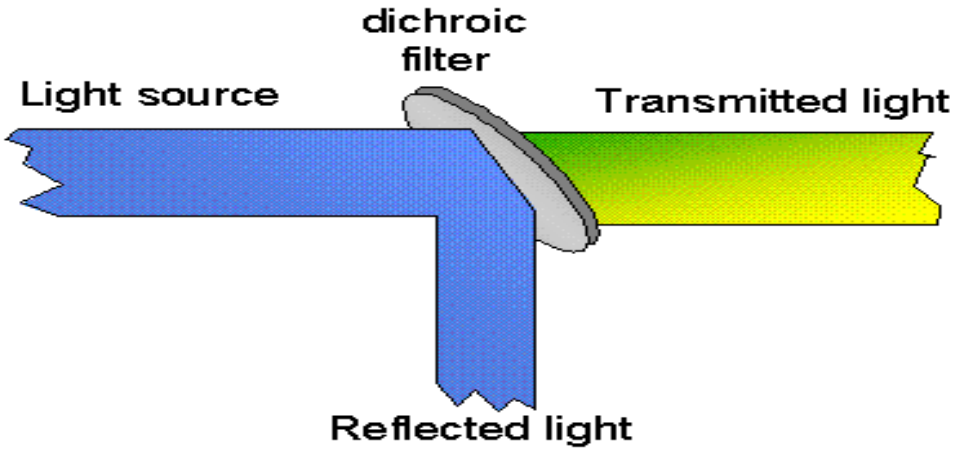
Fluidics Recap

- Purpose is to have cells flow one-by-one past a light source.
- Cells move out of tube because there is slightly greater pressure on the sample than on the sheath
- Cells are “focused” due to hydrodynamic focusing and laminar flow.

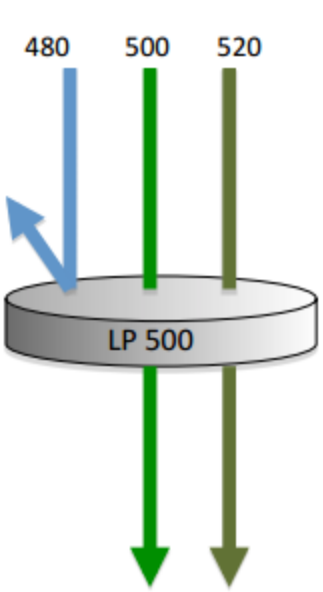
OPTICS

Filters

- Many wavelengths of light will be scattered from a cell, we need a way to split the light into its specific wavelengths in order to detect them independently. This is done with filters
- Optical filters are designed such that they absorb or reflect some wavelengths of light, while transmitting other.
- 3 types of filters
  - Long Pass filter
  - Short Pass filter
  - Band Pass filter

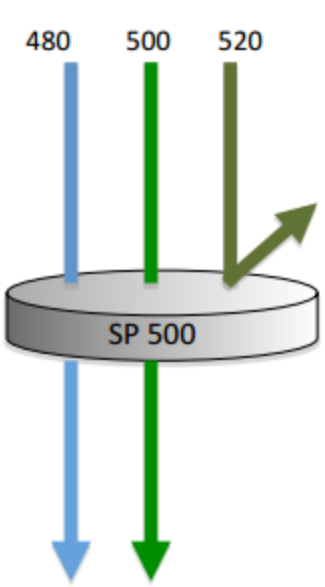


Longpass



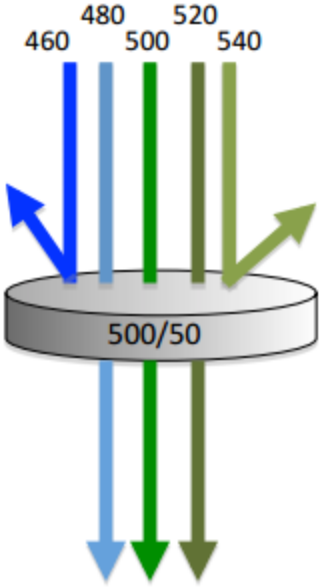
Transmits all wavelengths greater than specified wavelength

Shortpass



Transmits all wavelengths less than specified wavelength

Bandpass



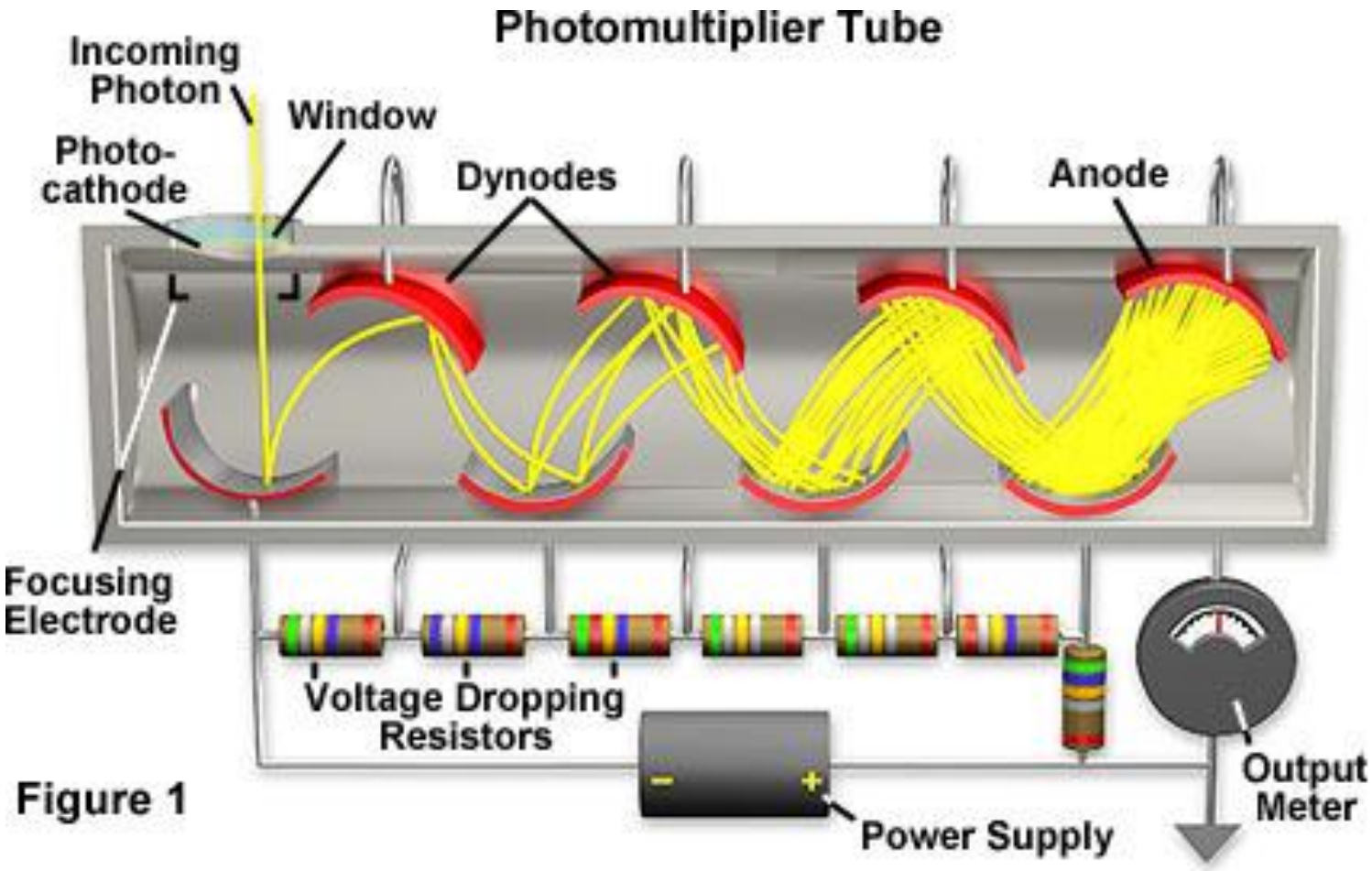
Transmits a specific band of wavelengths
i.e. 500/50 = 475-525 nm
(500 +/- 25)

PhotoMultiplier Tube

Photomultiplier tubes (**PMT**s)

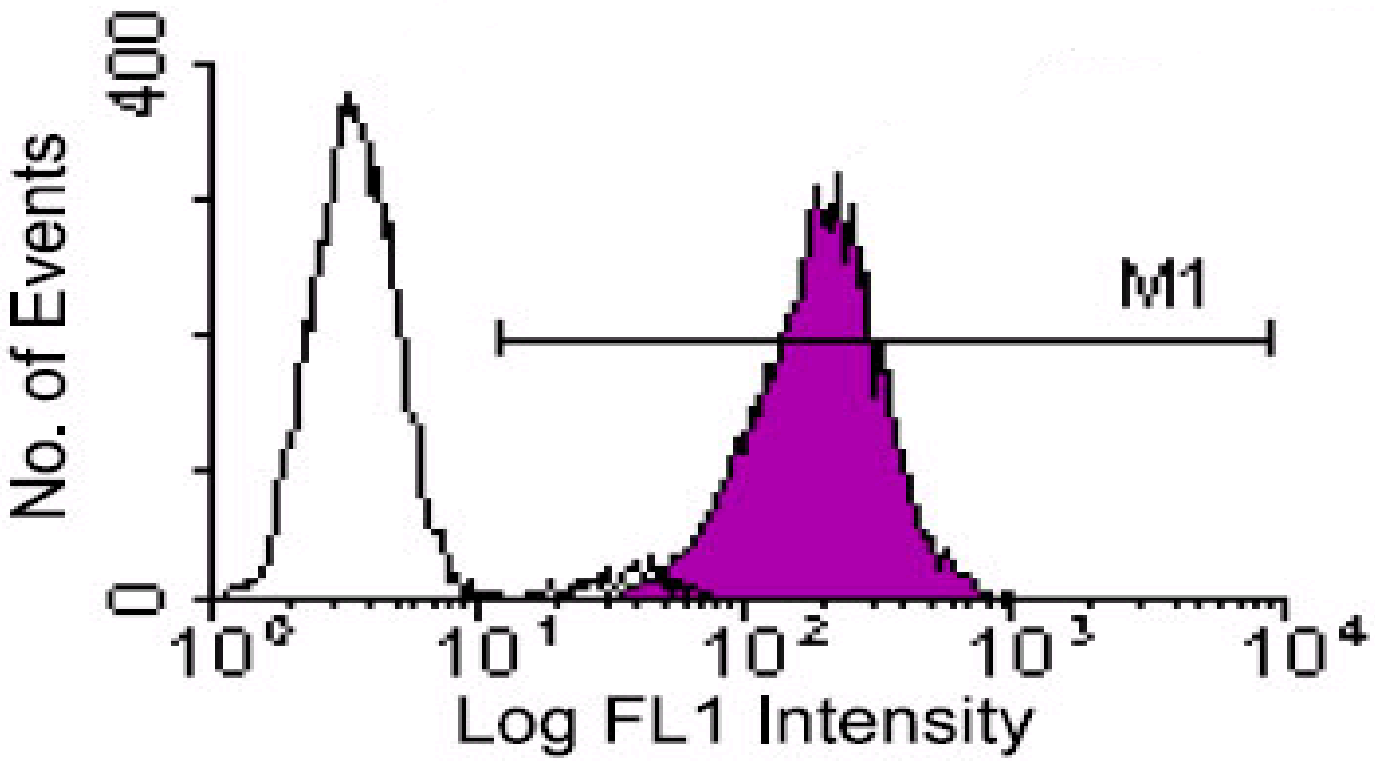
- vacuum tubes, phototubes
- detect ultraviolet, visible, and near-infrared ranges
- Multiply the current produced by as much as 100 million times enabling photons to be detected when light is very low.

PhotoMultiplier Tube



E-

PMT Adjustment for AutoFluorescence

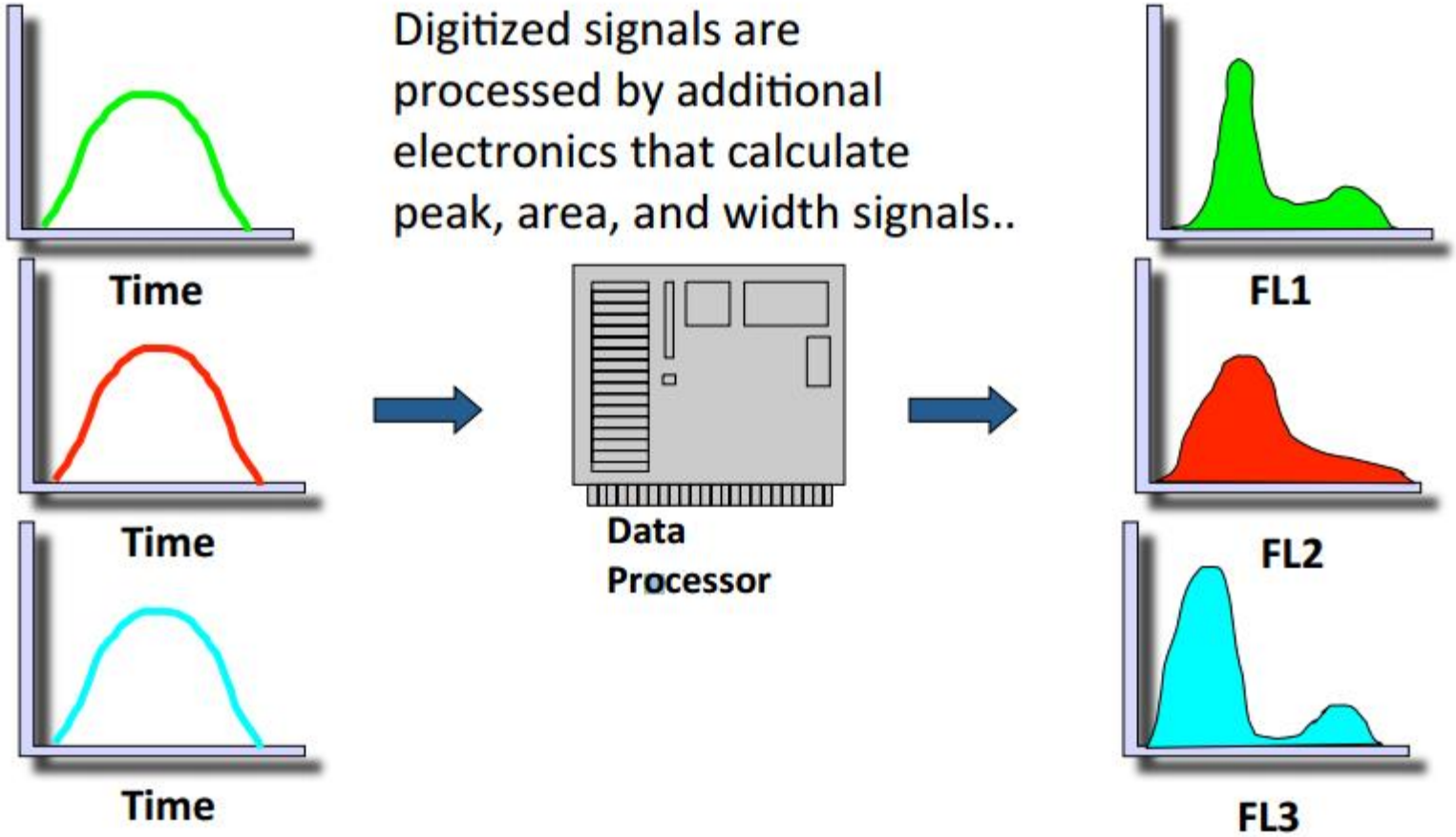


Data Collection & Display

Current produced by the PMT generates a voltage pulse; these electrical signals are digitized using Analog to Digital Converters (ADCs).

Digitized signals are processed by additional electronics that calculate peak, area, and width signals..

and generates histograms, dot plots, and FCS 3.0 files (Flow Cytometry Standard).



Amplifiers

- The current exiting the detector passes through either a linear or log amplifier converting it into a voltage pulse.

- You can adjust the intensity of the voltage by amplifying it on a linear or log scale

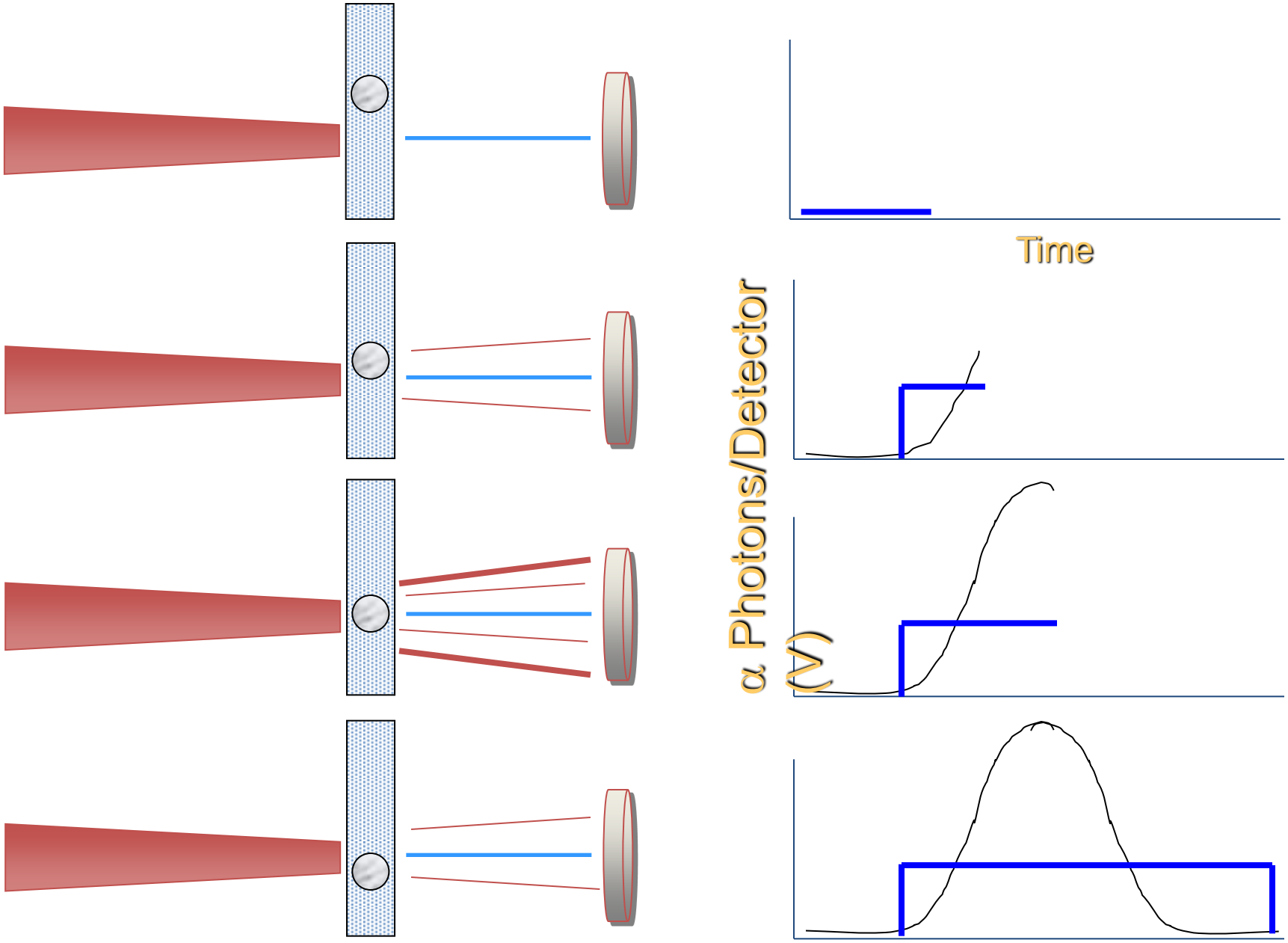
Linear Amplifiers

- Linear amplification is used when there is not such a broad range of signals e.g. in DNA analysis and calcium flux measurement.

Log Amplifiers

- The use of a log amp is beneficial when there is a broad range of fluorescence as this can then be compressed; this is generally true of most biological distributions.

The Voltage Pulse

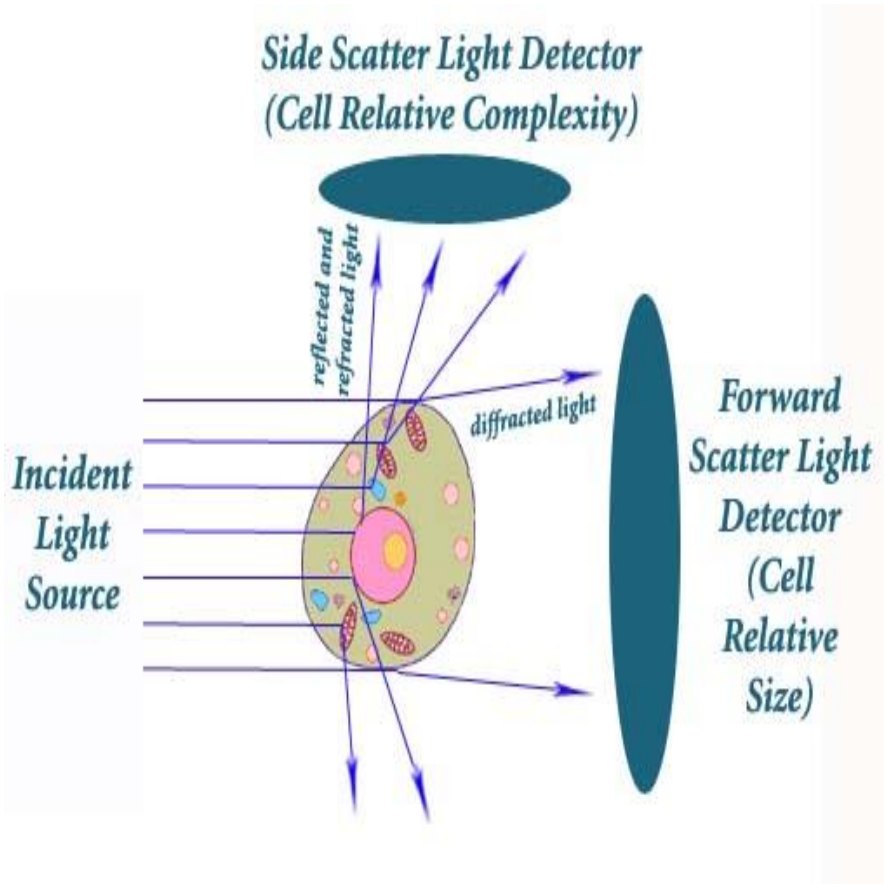


Electronics Recap

- The varying number of photons reaching the detector are converted to a proportional number of electrons
- The number of electrons exiting a PMT can be multiplied by making more electrons available to the detector (increase Voltage input)
- The current generated goes to a log or linear amplifier where it is amplified (if desired) and is converted to a voltage pulse

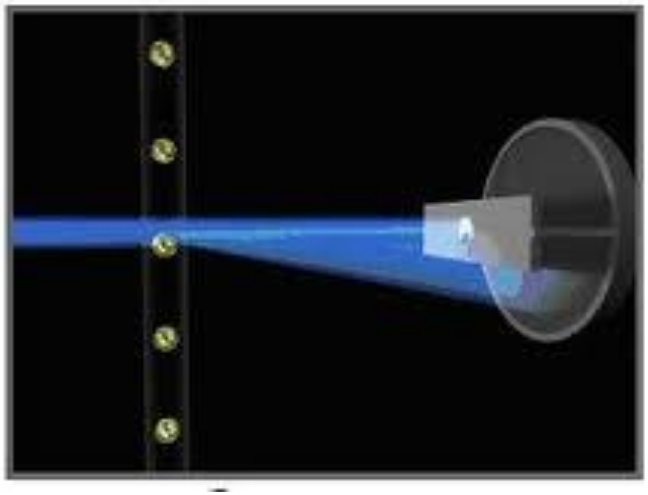
Light Scatter

- When light from a laser interrogates a cell, that cell scatters light in all directions.
- The scattered light can travel from the interrogation point down a path to a detector.



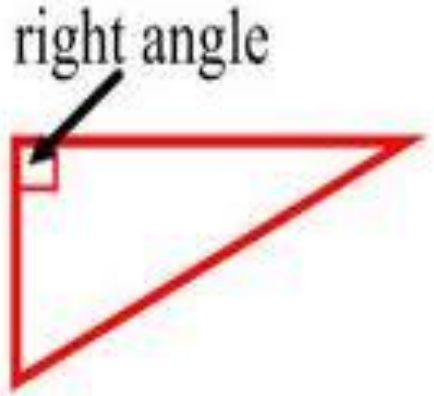
Forward Scatter

- Light that is scattered in the *forward* direction (along the same axis the laser is traveling) is detected in the Forward Scatter Channel.
- The intensity of this signal has been attributed to cell size, refractive index (membrane permeability)
- **Forward Scatter=FSC**



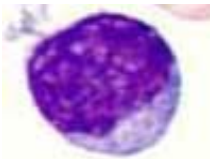
Side Scatter

- Laser light that is scattered at 90 degrees to the axis of the laser path is detected in the Side Scatter Channel
- The intensity of this signal is proportional to the amount of cytosolic structure in the cell (eg. granules, cell inclusions, etc.)
- Side Scatter=SSC

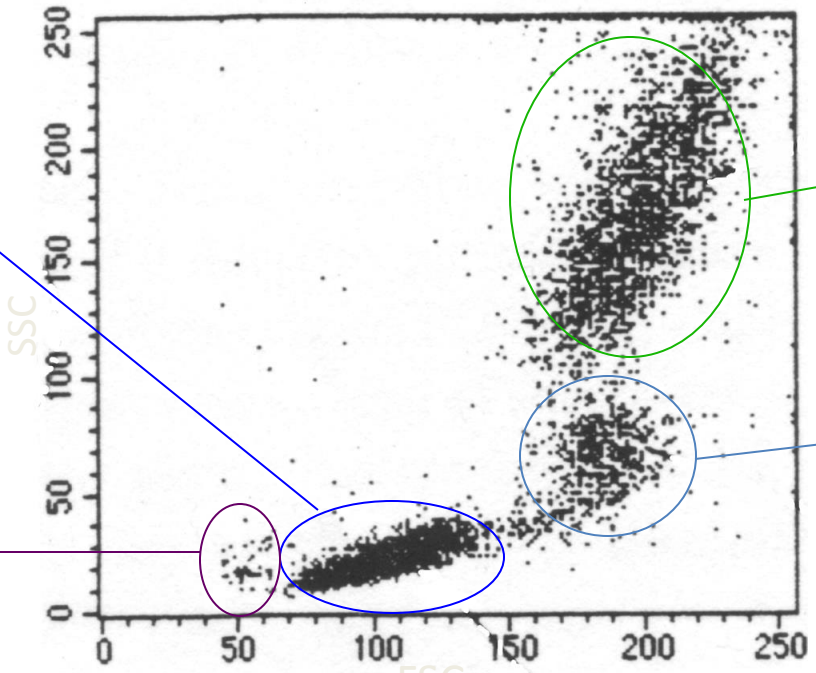


Why Look at FSC v. SSC?

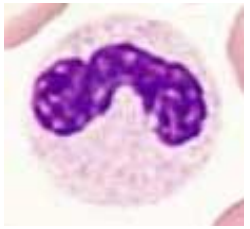
- Since FSC ~ size and SSC ~ internal structure, a correlated measurement between them can allow for differentiation of cell types in a heterogenous cell population.



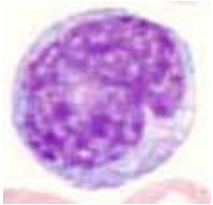
Lymphocytes



Granulocytes

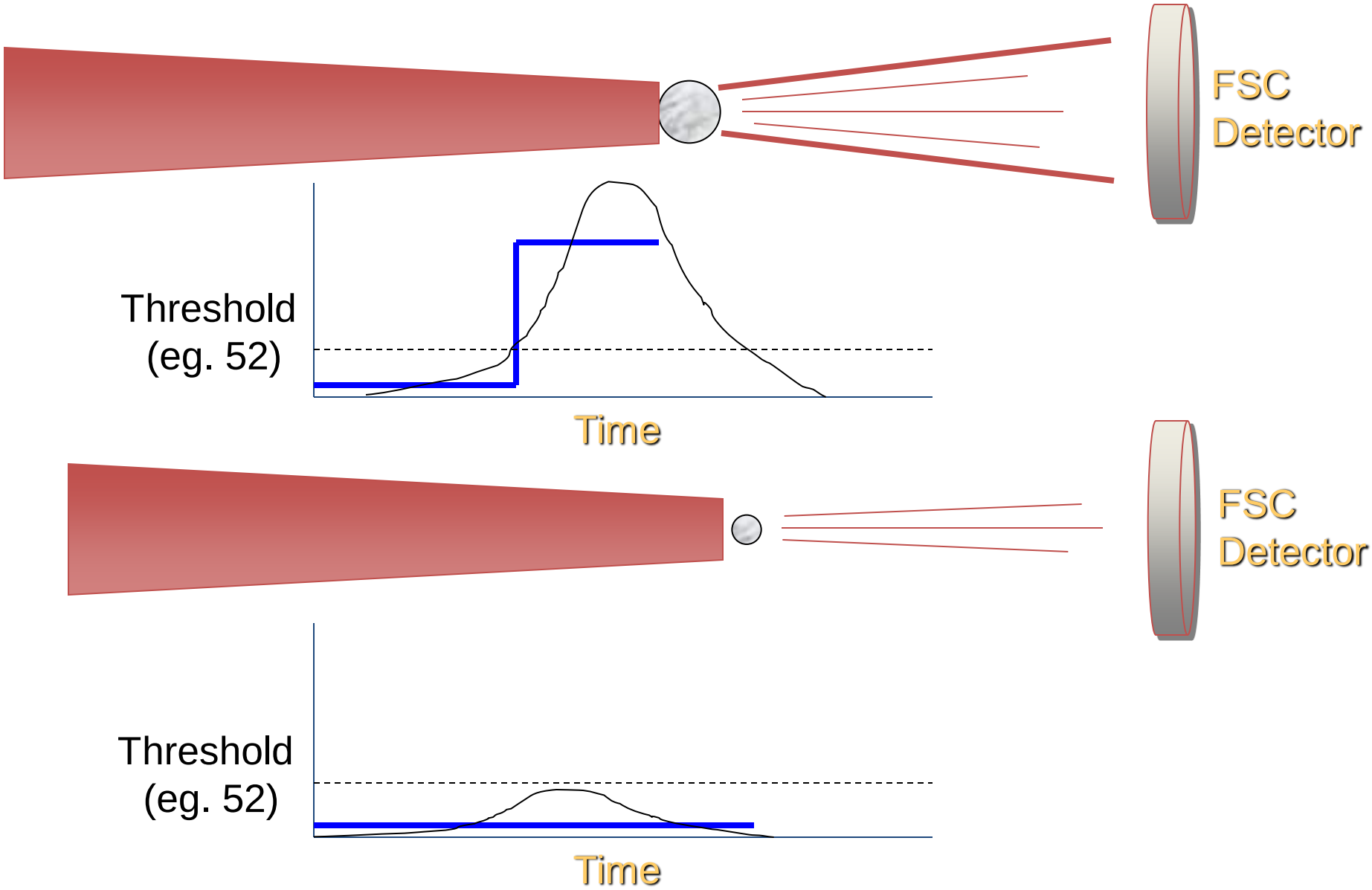


Monocytes

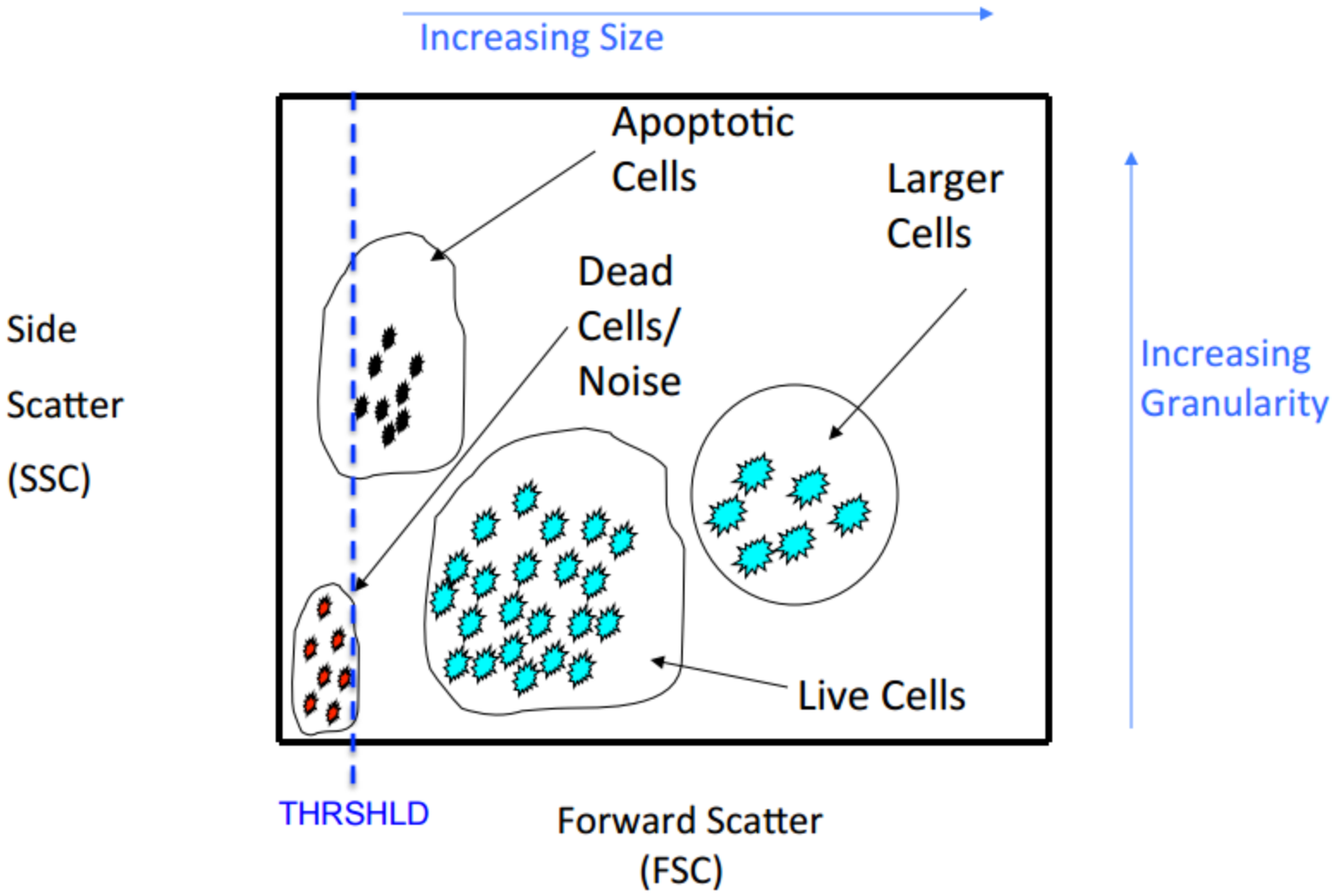


RBCs, Debris, Dead Cells

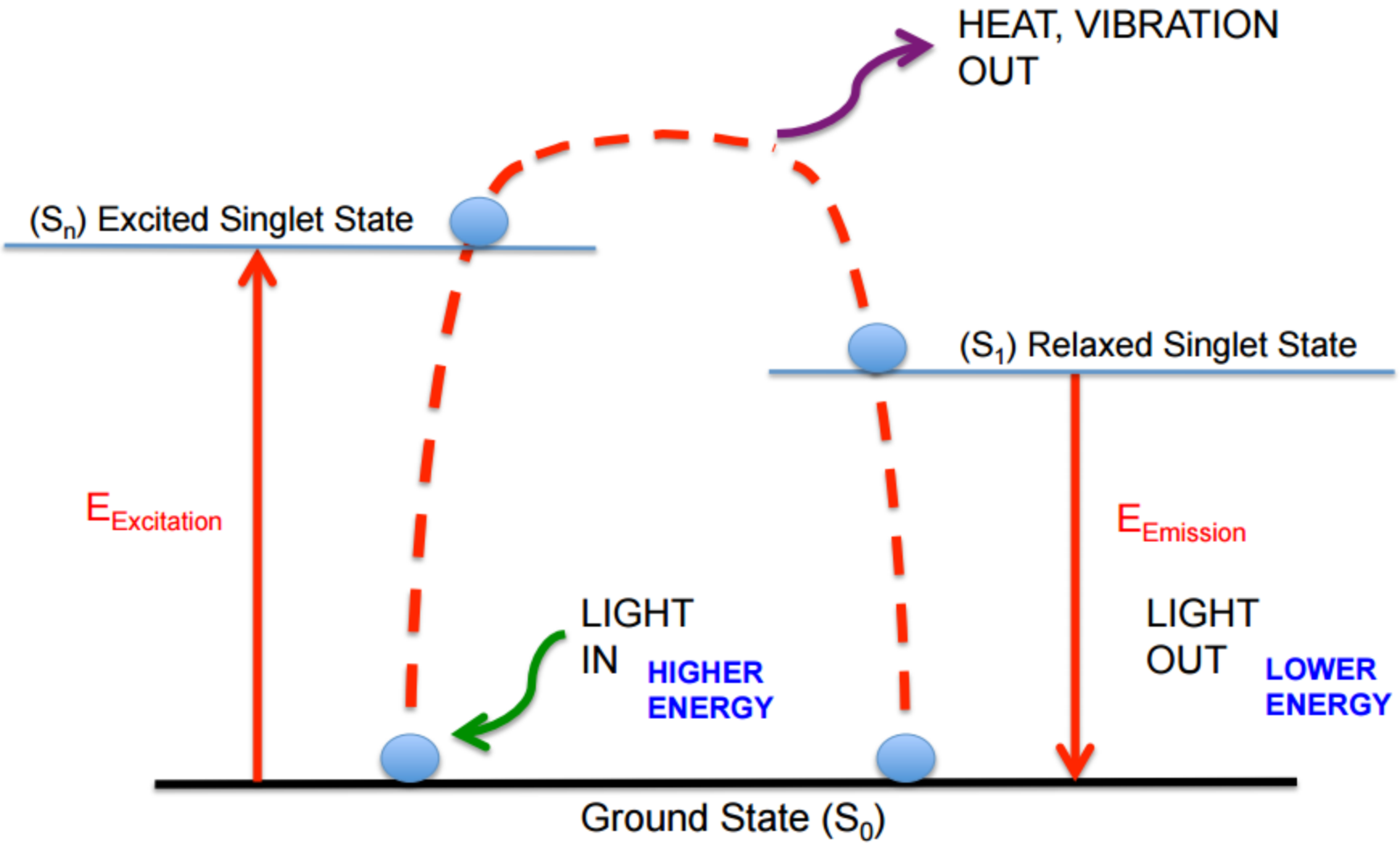
Threshold


FSC
Detector
Threshold
(eg. 52)
Time
FSC
Detector
Threshold
(eg. 52)
Time

Typical Scatter Display

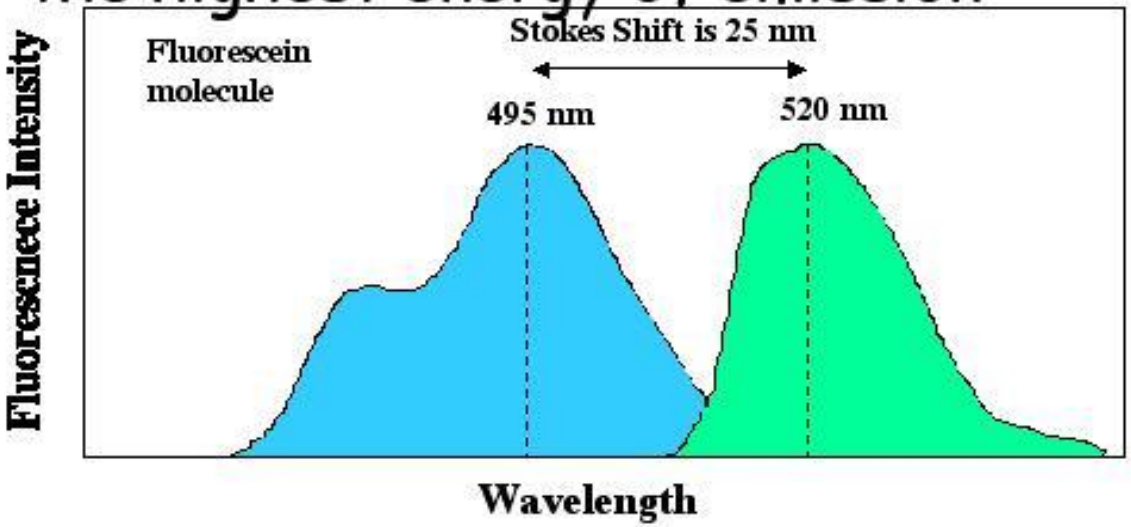


Fluorescence: Electronic Energy Transition



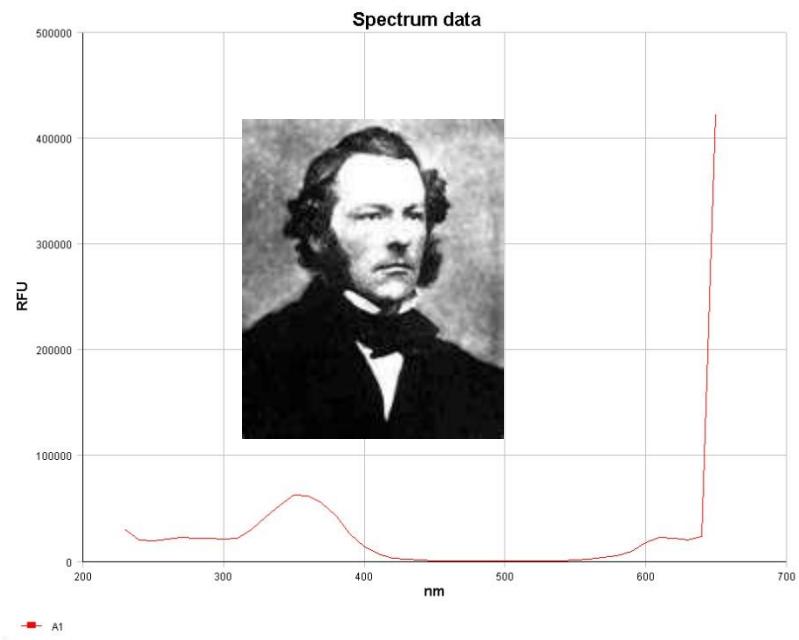
Fluorescence

- Stokes Shift
  - is the energy difference between the lowest energy peak of absorbance and the highest energy of emission

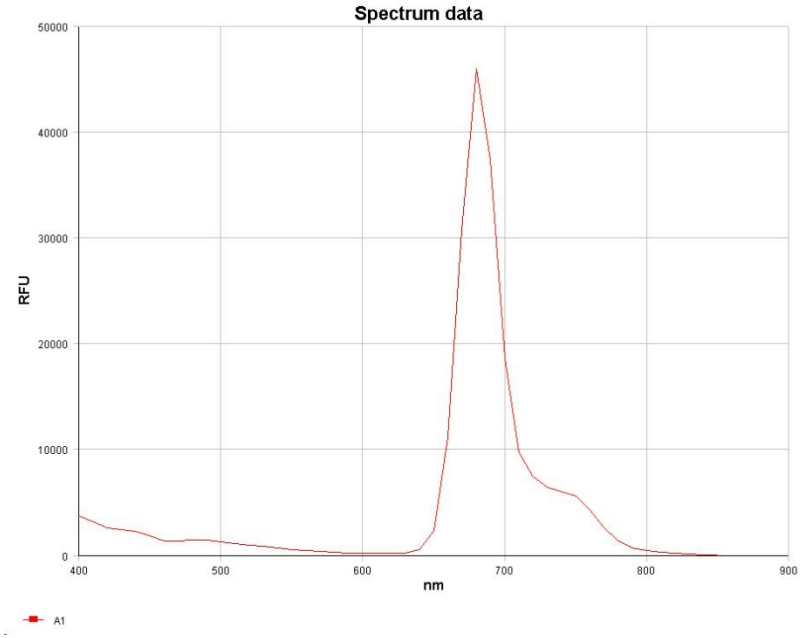


Spectral Properties

EXCITATION



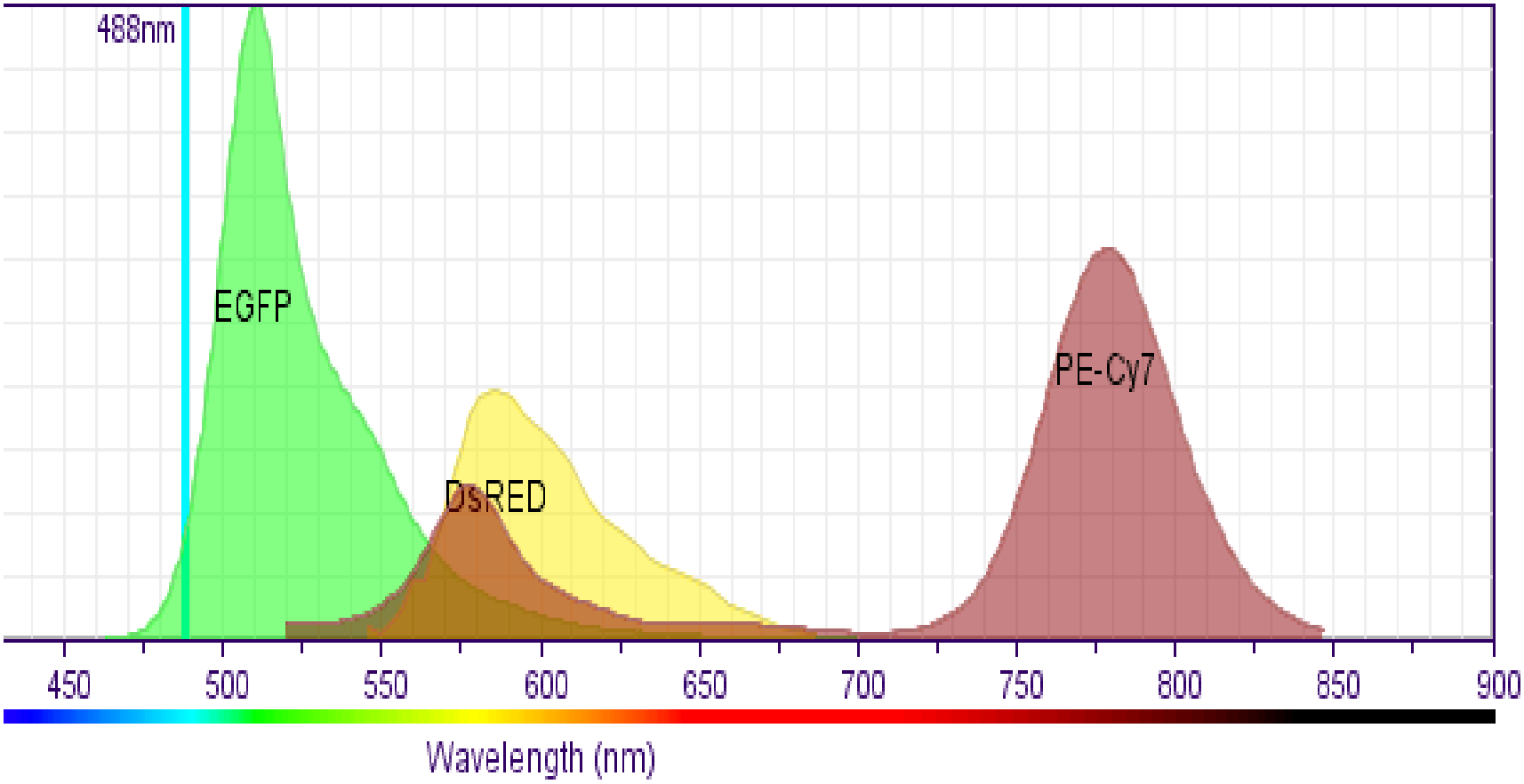
EMISSION



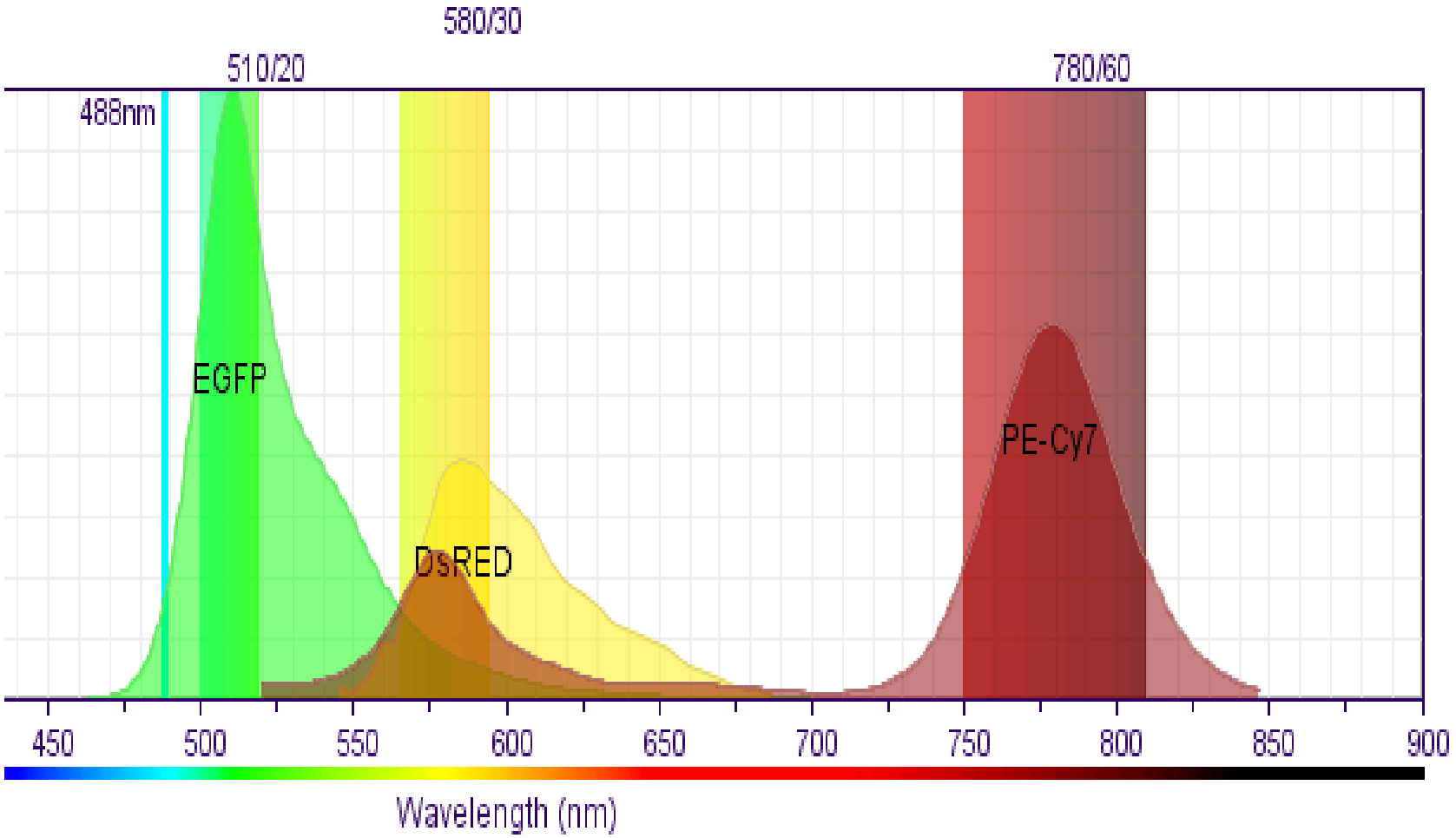
Interrogation Recap

- A focused light source (laser) interrogates a cell and scatters light
- That scattered light travels down a channel to a detector
- FSC ~ size and cell membrane shape
- SSC ~ internal cytosolic structure
- Fluorochromes on/in the cell will become excited by the laser and emit photons
- These photons travel down channels and are steered and split by dichroic (LP/SP) filters
- Specific wavelengths are then detected by PMTs that have a filter in front of them

SPECTRAL OVERLAP



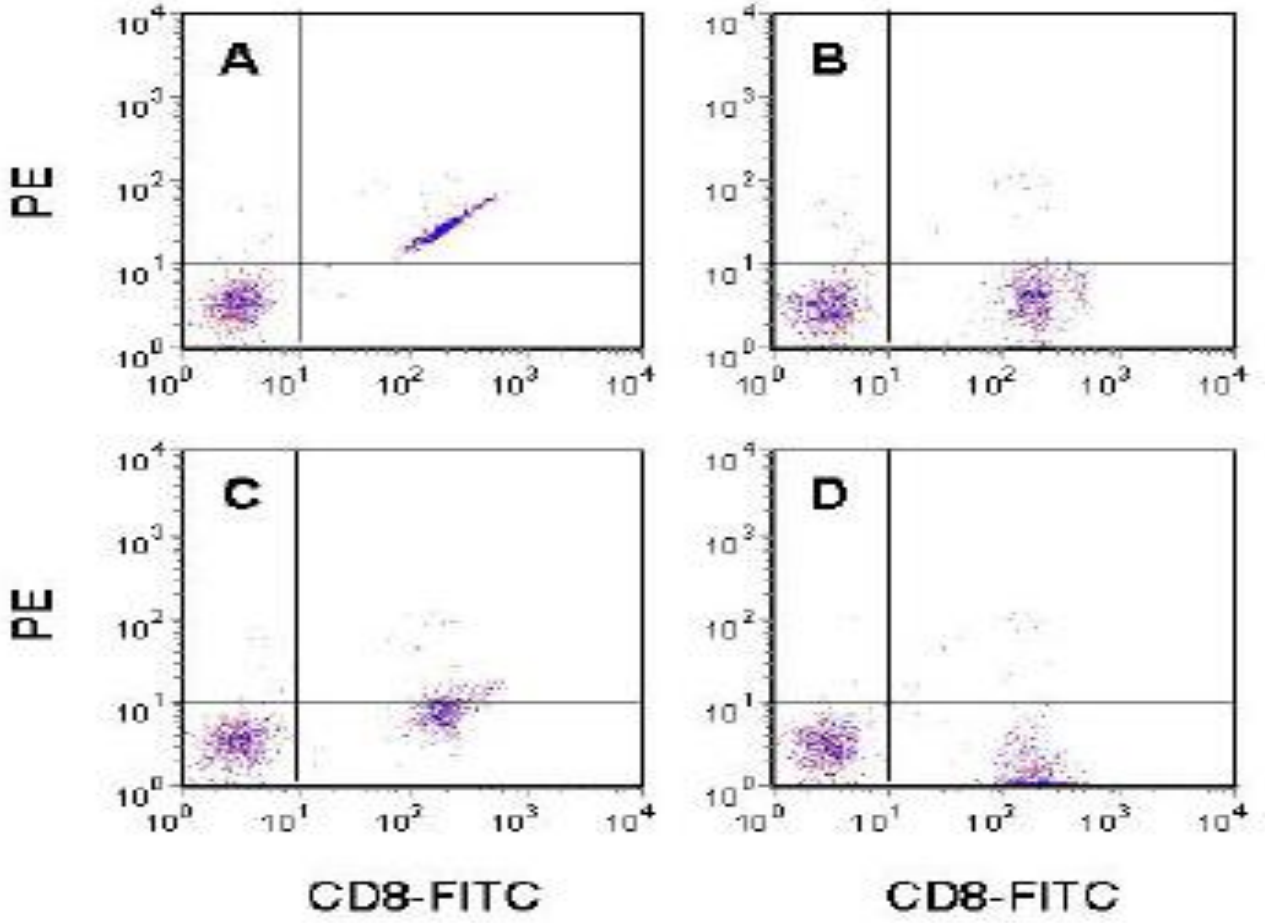
BANDPASS FILTERS *HELP*



COMPENSATION

Compensation is the process by which the fluorescence “spillover” originating from a fluorochrome other than the one specified for a particular PMT detector is subtracted as a percentage of the signal from other PMT’s.

Howard Shapiro



Human peripheral blood lymphocytes labelled with CD8-FITC showing the CD4 -ve cells only. **A**. Uncompensated data. **B**. Correctly compensated. **C**. Under compensated. **D**. Over compensated.

Michael Ormerod

Mario Roederer's COMP Recipe

(1) The compensation tube must consist of cells that are unstained as well as cells that are singly-stained with the fluorescent probes. The stained (positive) cells must have the same autofluorescence (when they are unstained) as do the unstained (negative) cells in the compensation tube (e.g., all are lymphocytes).

(2) The PMT voltages must be set high enough guarantee that the negative population is off the axis in every channel.

(3) An analysis gate is set so that only cells with identical autofluorescence characteristics are viewed (e.g., a lymphocyte gate). An analysis gate is also set to include all of the negative cells and all of the positive cells.

(4) The centers of the positive and negative cell populations are aligned by matching the median fluorescences.