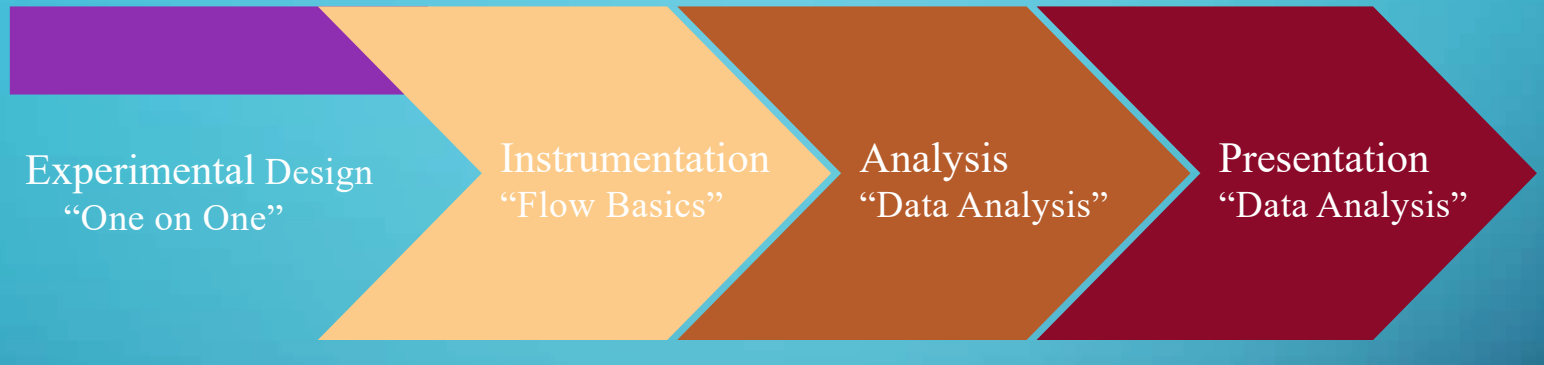
FLOW CYTOMETRY APPLICATIONS

- **Immunophenotyping**
- **DNA cell cycle/tumor ploidy**
- **Membrane potential**
- Ion flux
- Cell viability
- **Intracellular protein staining**
- pH changes
- Cell tracking and proliferation
- **Sorting**
- Redox state
- Chromatin structure

The uses of flow in research has boomed since the mid-1980s, and is now the gold standard for a variety of applications

- Total protein
- Lipids
- Surface charge
- Membrane fusion/runover
- Enzyme activity
- Oxidative metabolism
- Sulfhydryl groups/glutathione
- DNA synthesis
- **DNA degradation**
- Gene expression
- Phagocytosis
- Microparticle analysis

MANY COMPONENTS TO A SUCCESSFUL ASSAY

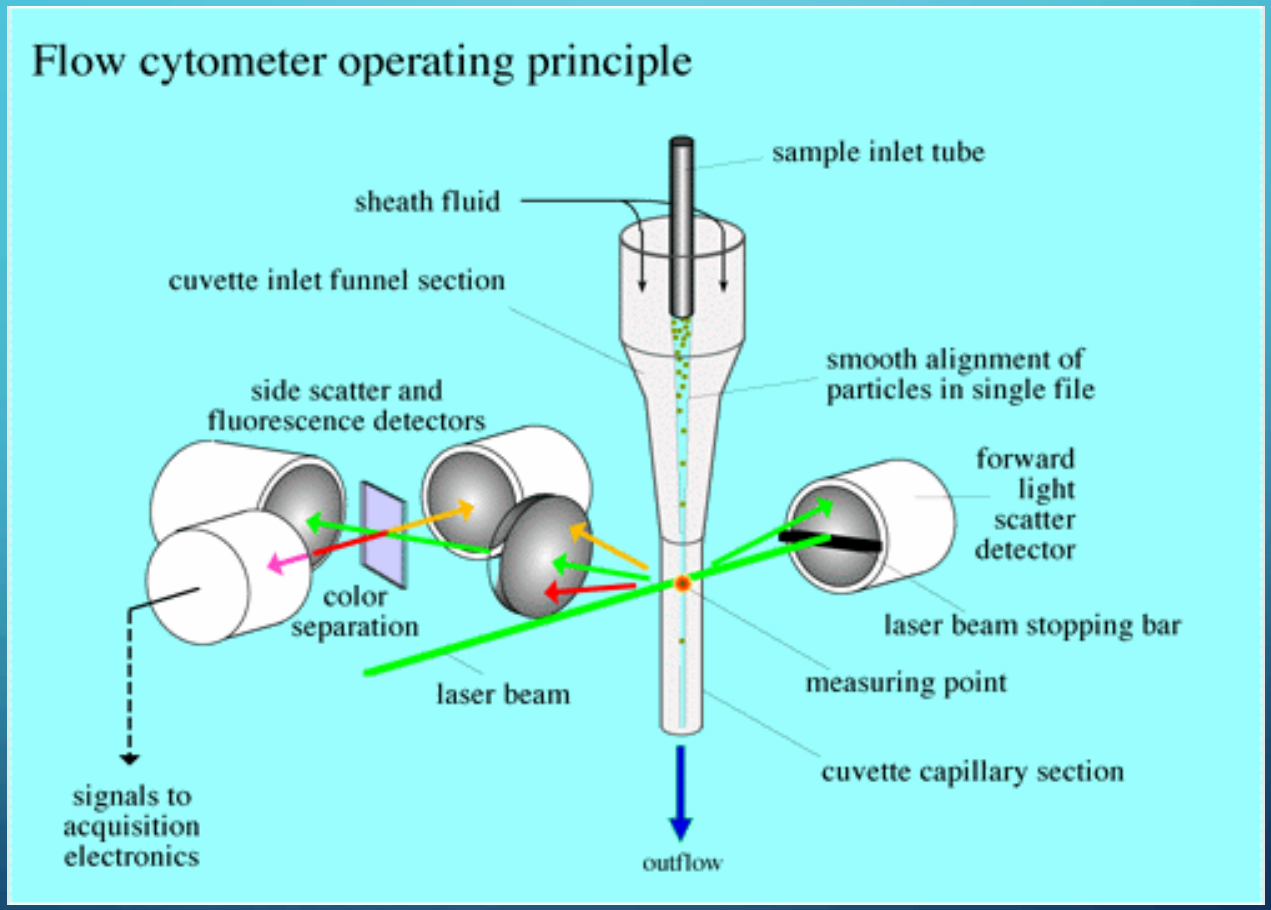


Sample Procurement
Sample preparation
Fix/Perm
Which Fluorophore
Controls
- Isotype?
- Single color
- FMO

Appropriate Lasers
Appropriate Filters
Instrument Settings
- Lin vs Log
- Time
- A, W, H
- Doublet discrimination

Interpretation
Mean, Median
% +
CV
SD
Signal/Noise
Gating

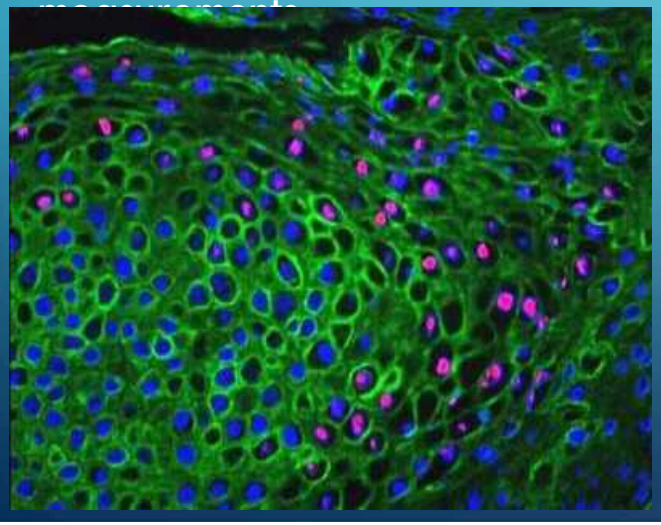
Histogram
Dot Plot
Density Plot
Overlay
Bar Graph


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

CYTOMETRY VS. FLOW CYTOMETRY

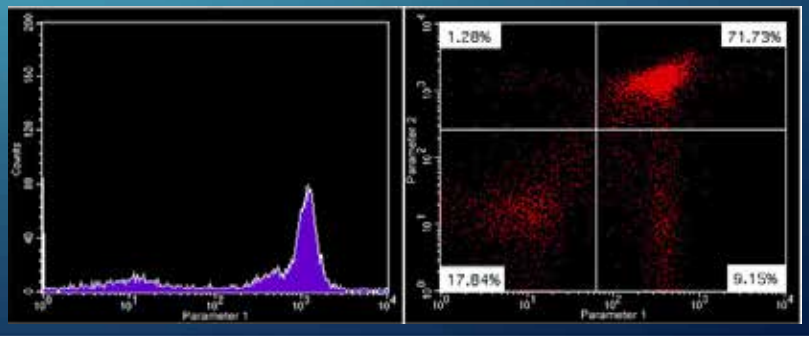
Cytometry/Microscopy

- Localization of antigen is possible
- Poor enumeration of cell subtypes
- Limiting number of simultaneous measurements



Flow Cytometry.

- No ability to determine localization (traditional flow cytometer)
- Can analyze many cells in a short time frame. (30k/sec)
- Can look at numerous parameters at once (>20 parameters)



WHAT HAPPENS IN A FLOW CYTOMETER?

- Cells in suspension flow single file
- through a focused laser where they scatter light and emit fluorescence that is filtered,
- measured, then converted to digitized values that are stored in a file
- which can then be analyzed and interpreted within specialized software.

Fluidics

Interrogation

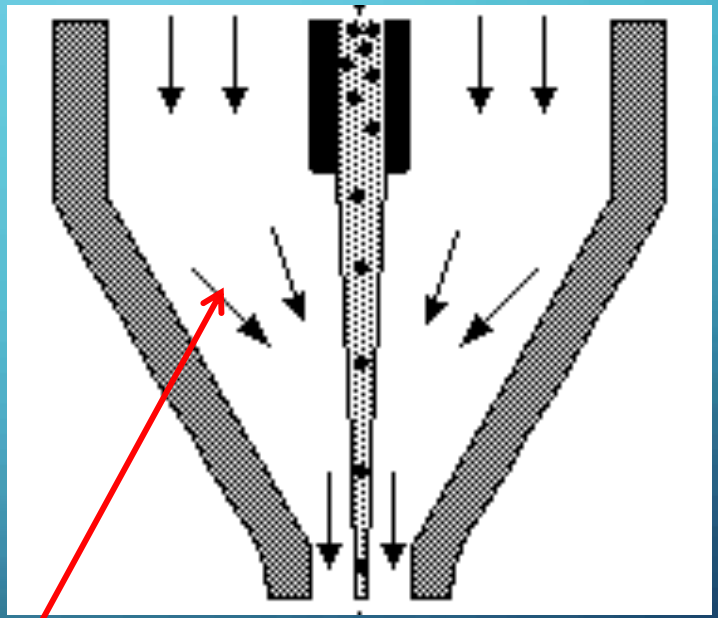
Electronics

Interpretation

THE FLUIDICS SYSTEM
"CELLS IN SUSPENSION FLOW SINGLE FILE"

- Cells must flow one-by-one into the cytometer to do single cell analysis
- Accomplished through a pressurized laminar flow system.
- The sample is injected into a sheath fluid as it passes through a small orifice (50um-300um)

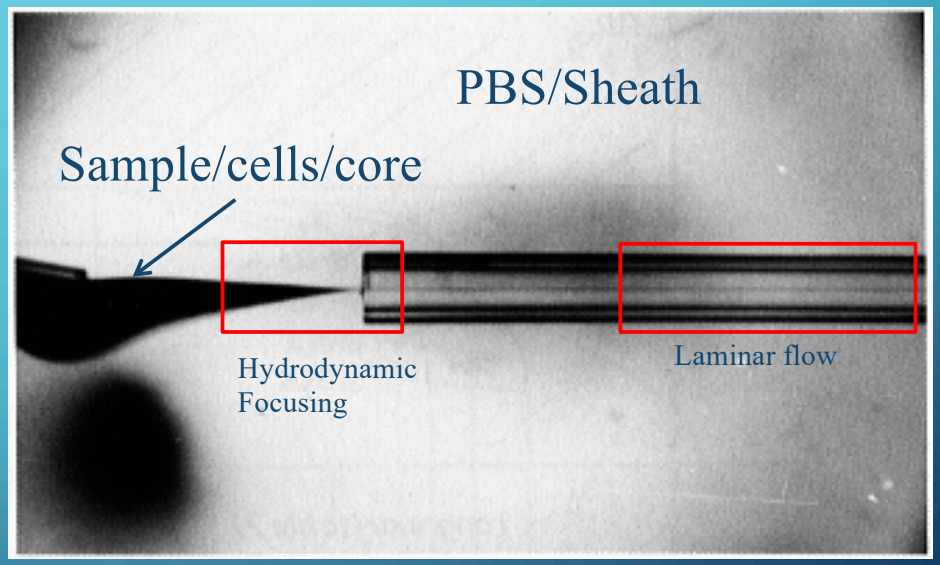
SHEATH AND CORE



Sheath

HYDRODYNAMIC FOCUSING

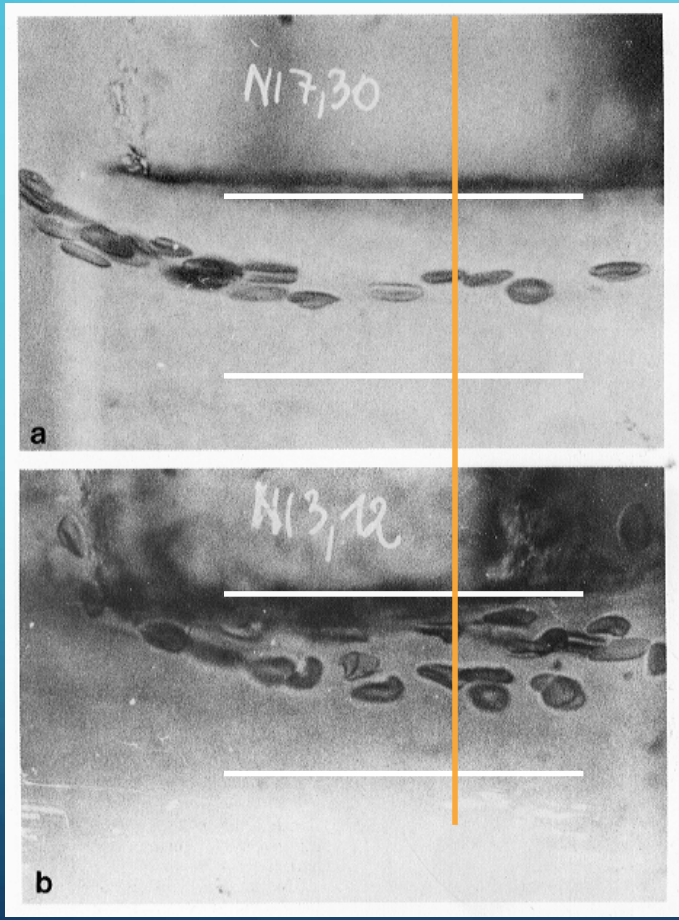
Notice how the ink is focused into a tight stream as it is drawn into the tube under laminar flow conditions.



Laminar flow occurs when a fluid flows in parallel layers, with no disruption between the layers

V. Kachel, H. Fellner-Feldegg & E. Menke - MLM Chapt. 3

PARTICLE ORIENTATION AND DEFORMATION

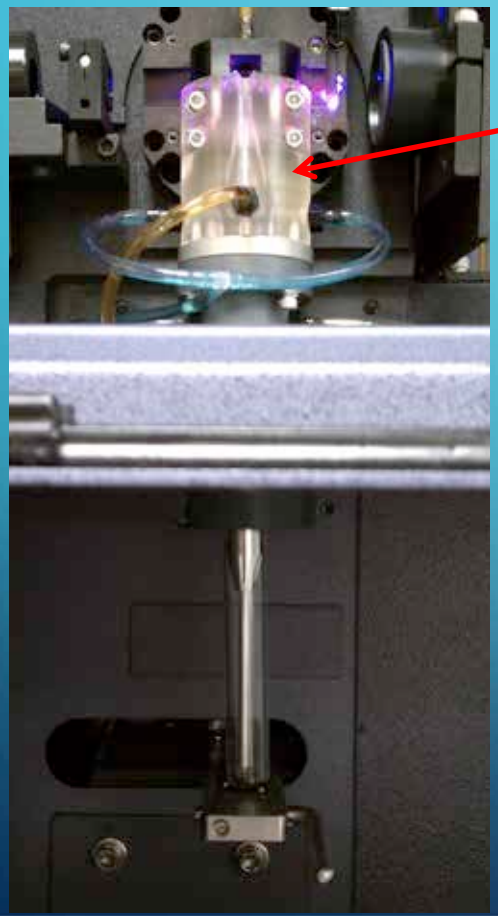


a: Native human erythrocytes near the margin of the core stream of a short tube (orifice). The cells are uniformly oriented and elongated by the hydrodynamic forces of the inlet flow.

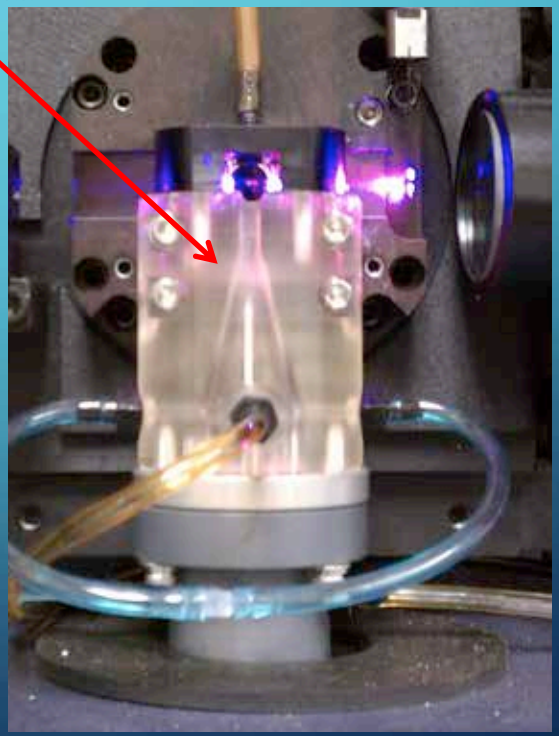
b: In the turbulent flow near the tube wall, the cells are deformed and disoriented in a very individual way. v>3 m/s.

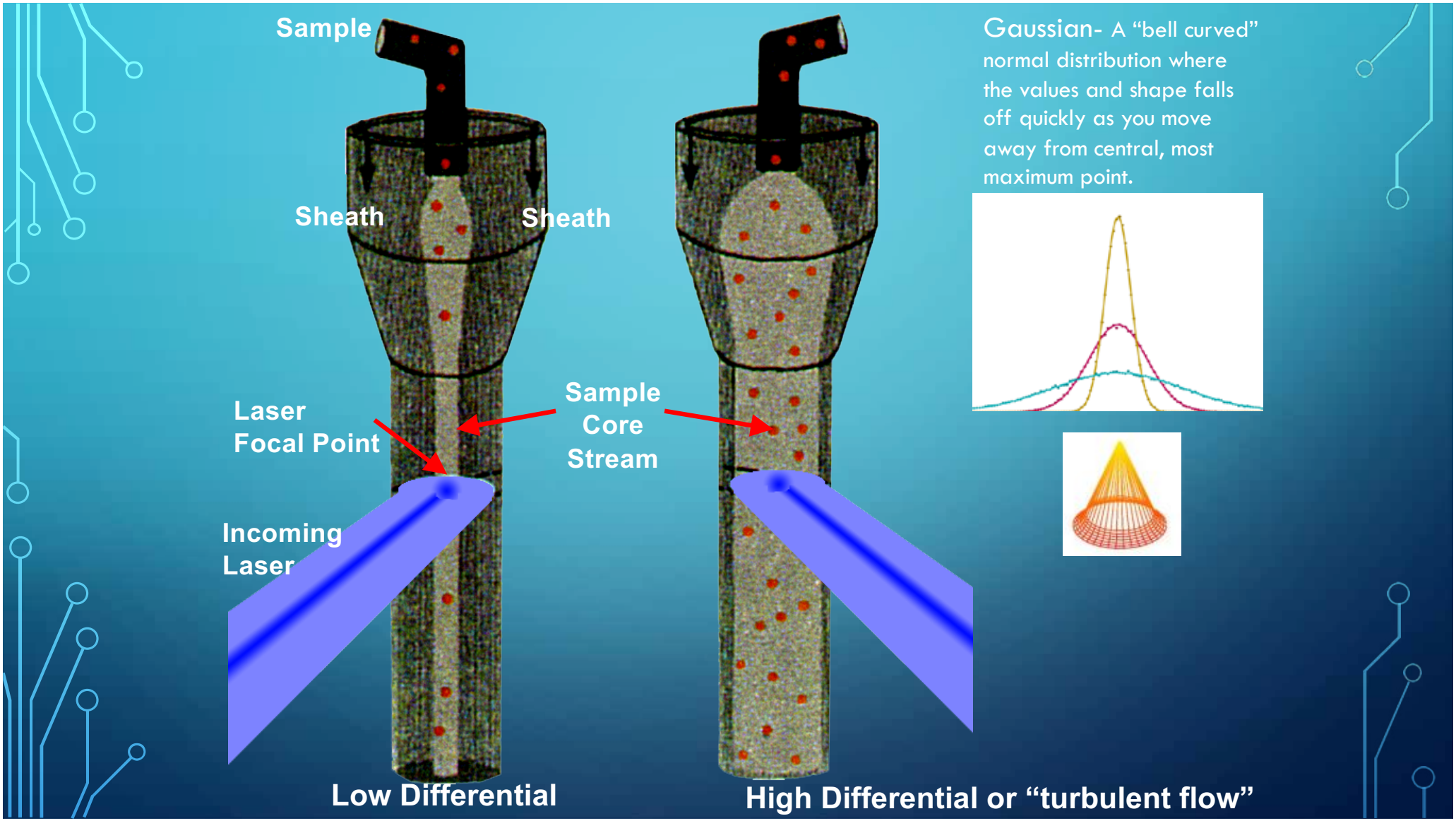
V. Kachel, et al. - MLM Chapt. 3

WHAT HAPPENS IN A FLOW CYTOMETER (SIMPLIFIED)



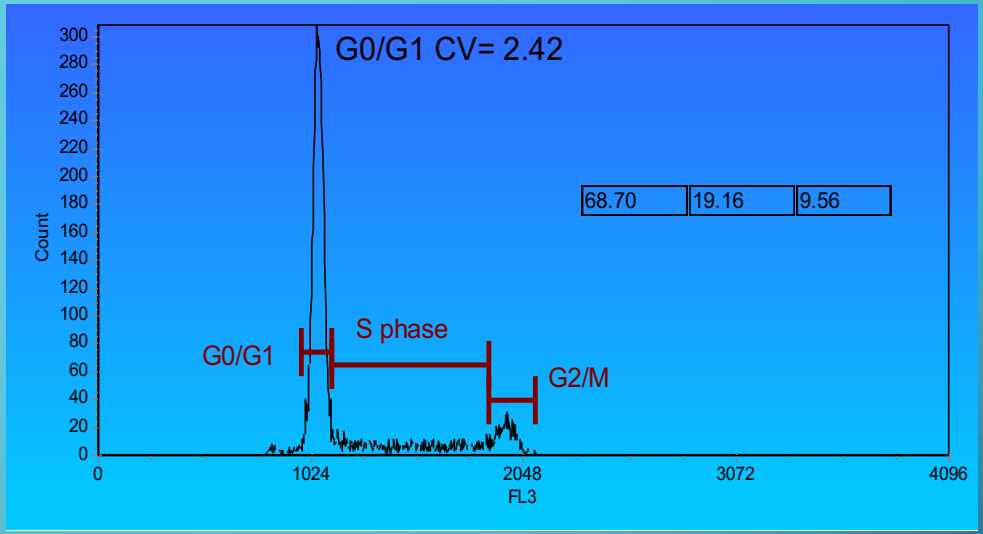
Flow Cell- the place where hydrodynamically focused cells are delivered to the focused light source



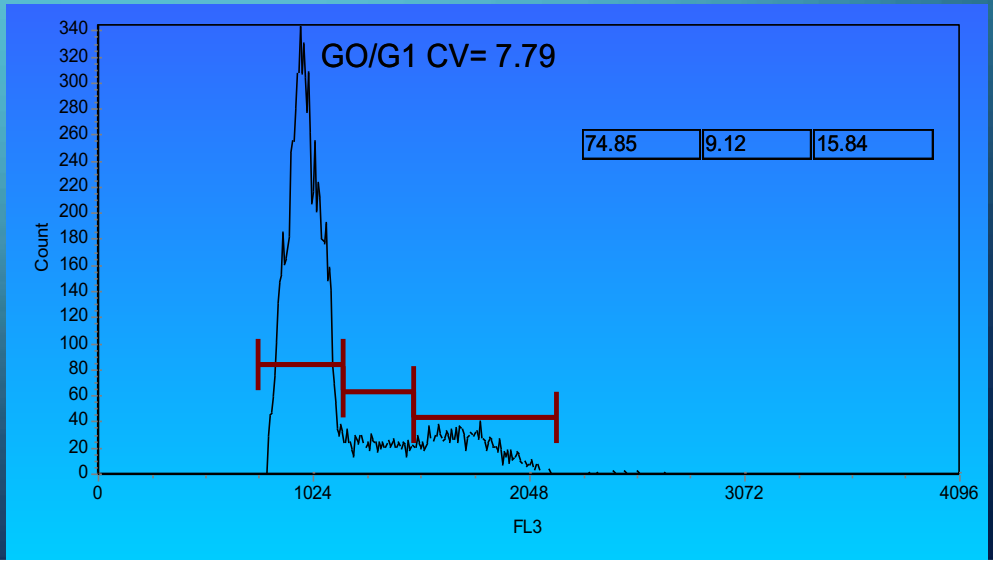


Gaussian- A "bell curved" normal distribution where the values and shape falls off quickly as you move away from central, most maximum point.

Low pressure



High pressure



FLUIDICS RECAP

- Purpose is to have cells flow one-by-one past a light source.
- Cells are “focused” due to hydrodynamic focusing and laminar flow.
- Turbulent flow, caused by clogs or fluidic instability can cause imprecise data

WHAT HAPPENS IN A FLOW CYTOMETER?

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- through a focused laser where they scatter light and emit fluorescence that is filtered,
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- Which can then be read by specialized software.

Fluidics

Interrogation

Electronics

Interpretation

LASERS

LIGHT AMPLIFICATION BY STIMULATED EMISSION OF RADIATION

- Lasers provide a single wavelength of light (monochromatic)
- They can provide milliwatts to watts of power
- Low divergence
- Provide coherent light
- Gas, dye, or solid state

Coherent: all emmiting photons have same wavelength, phase and direction as stimulation photons

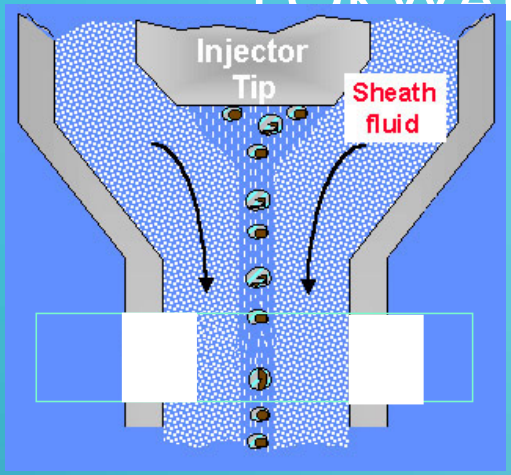
LIGHT COLLECTION

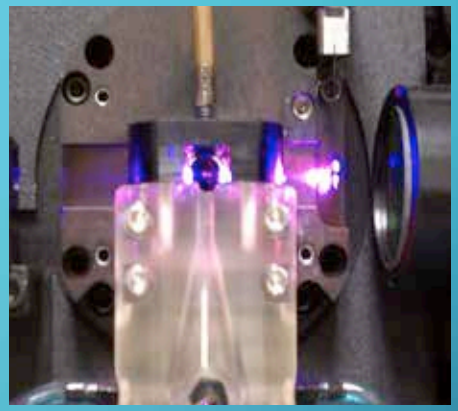
Scatter VS Fluorescence

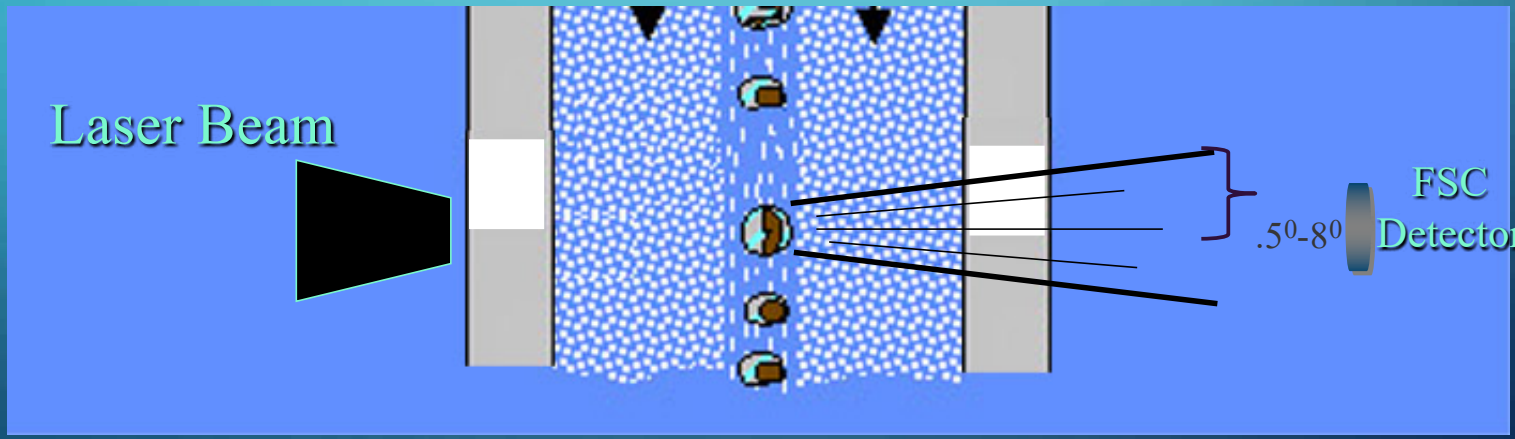
- Collected photons are the product of laser light scattering or bouncing off cells
- 488nm
- Information associated with physical attributes of cells (size, granularity, refractive index)

- Collected photons are product of excitation with subsequent emission determined by fluorophore
- 350nm-800nm
- Readout of intrinsic (autofluorescence) or extrinsic fluorescence (intentional cell labeling)

FORWARD SCATTER



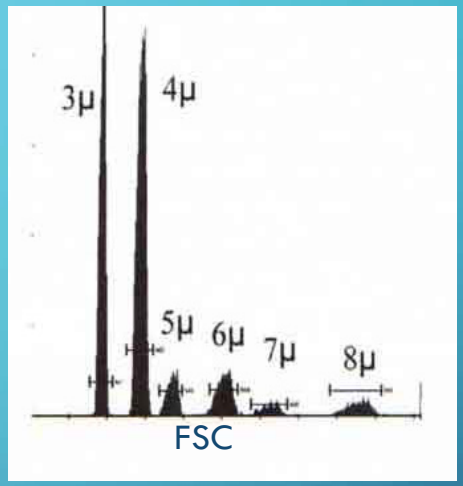


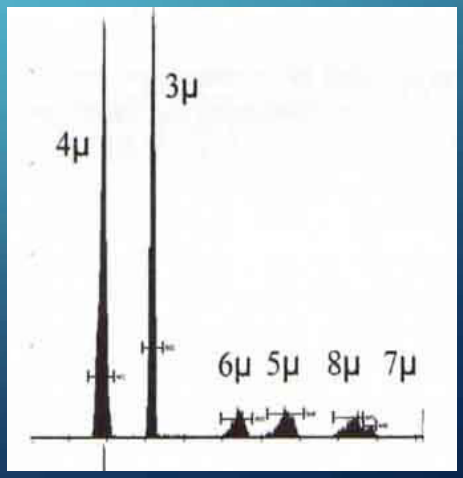


Original from Purdue University Cytometry Laboratories

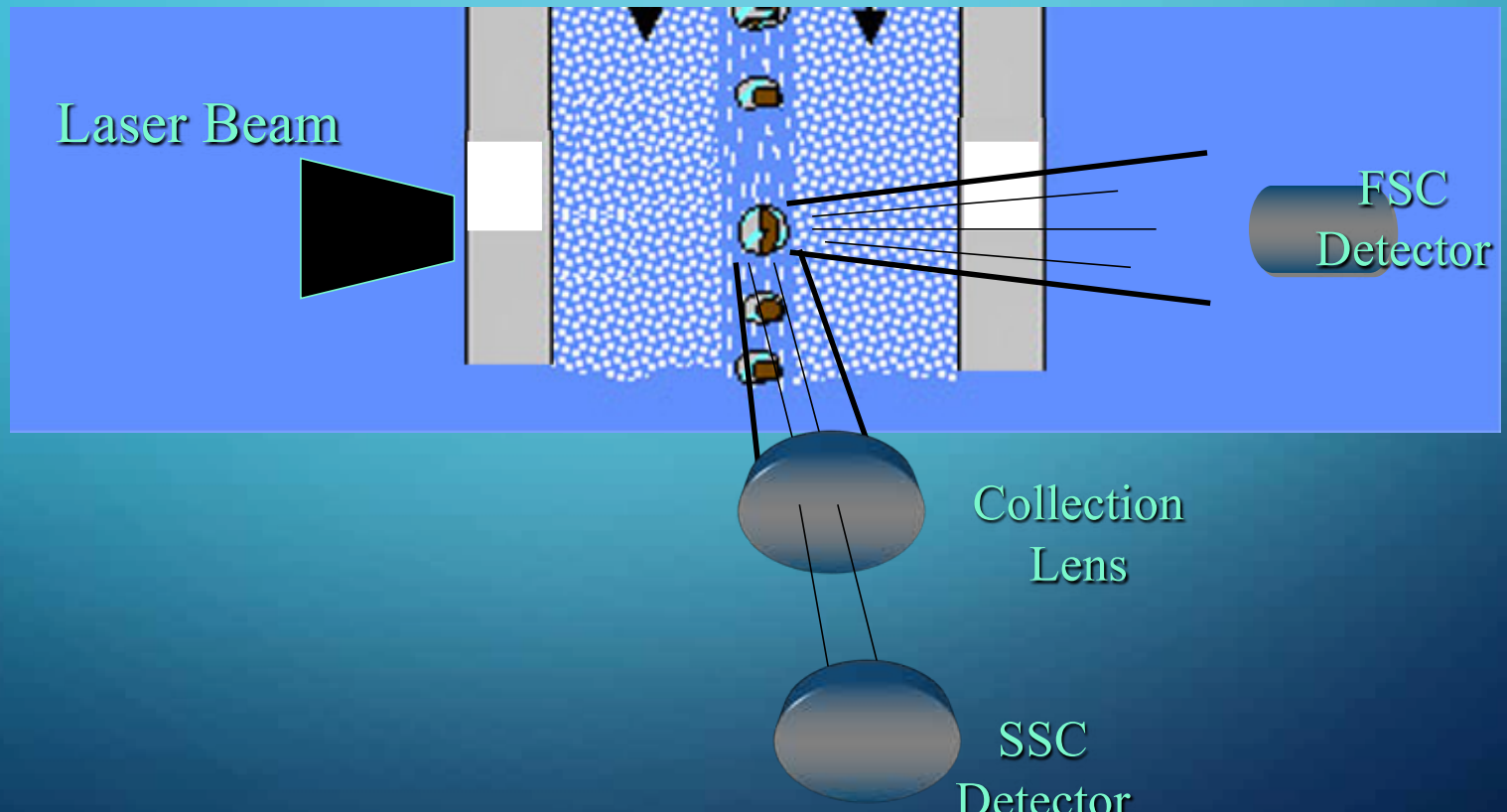
FORWARD SCATTER

- The intensity of forward scatter signal is often attributed to cell size, but is very complex and also reflects refractive index (membrane permeability), among other things
- Forward Scatter=FSC=FALS=LALS





SIDE SCATTER



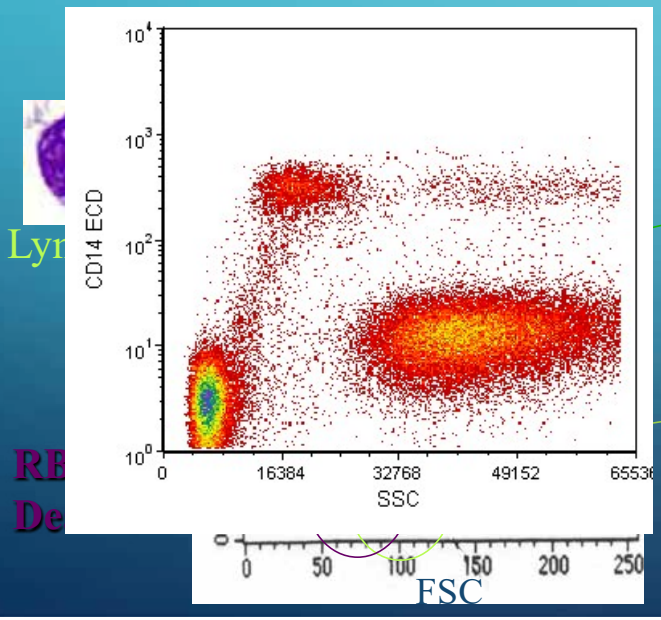
Original from Purdue University Cytometry Laboratories

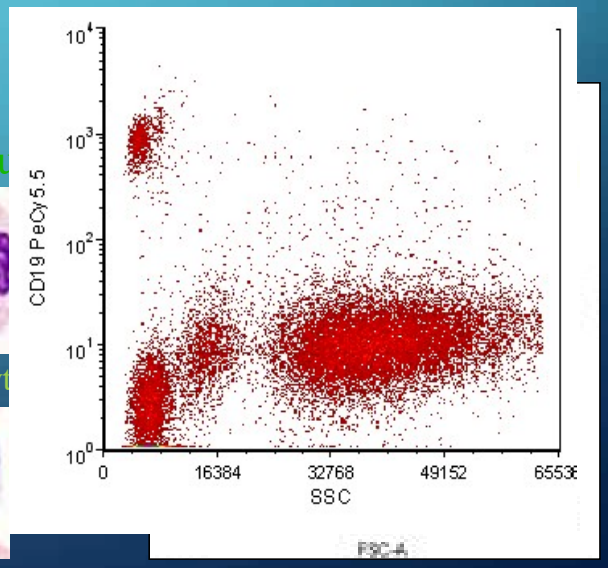
SIDE SCATTER

- 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, drug delivery nanoparticles.)
- Side Scatter=SSC=RALS=90 degree Scatter

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



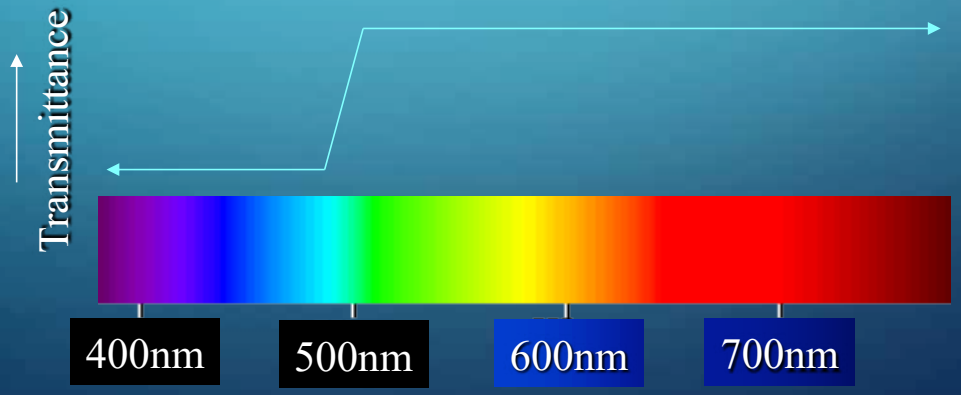


OPTICAL FILTERS

- Many wavelengths of light will be emitted 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

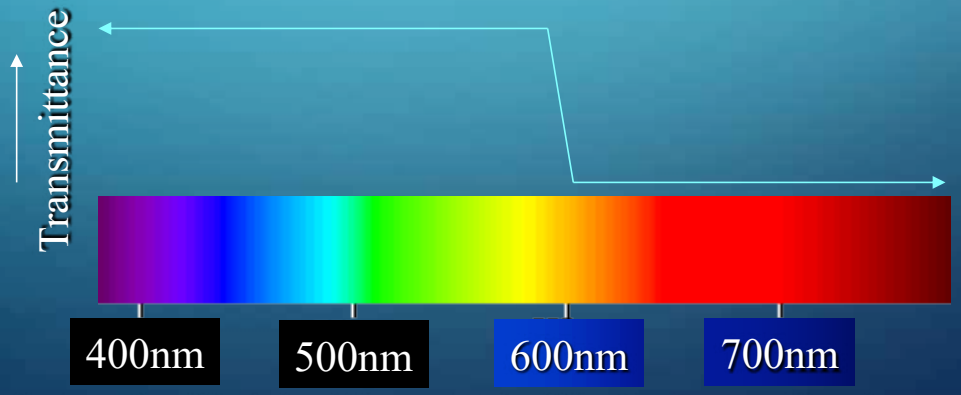
LONG PASS FILTERS

- Transmit all wavelengths greater than specified wavelength
  - Example: 500LP will transmit all wavelengths greater than 500nm



SHORT PASS FILTER

- Transmits all wavelengths less than specified wavelength
  - Example: 600SP will transmit all wavelengths less than 600nm.

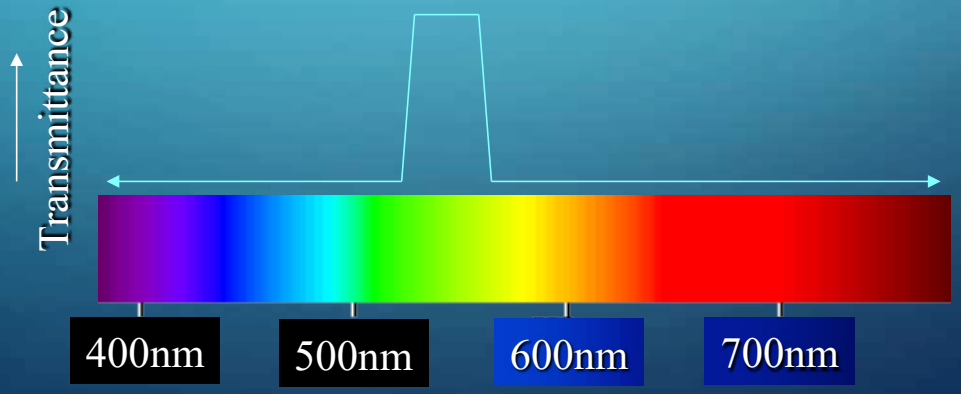


Original from Cytomation Training Manual, Modified by James Marvin

BAND PASS FILTER

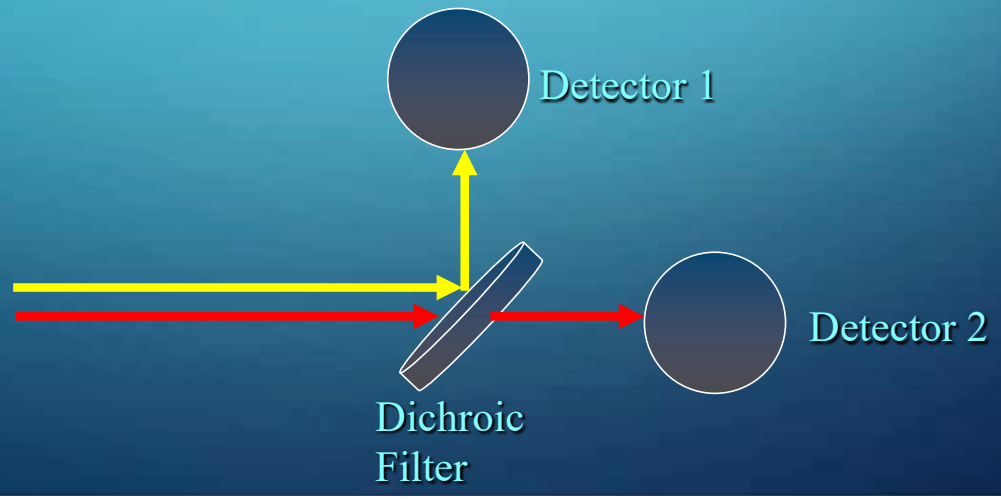
- Transmits a specific band of wavelengths
  - Example: 550/20BP Filter will transmit wavelengths of light between 540nm and 560nm

    (550/20 = 550+/-10, not 550+/-20)

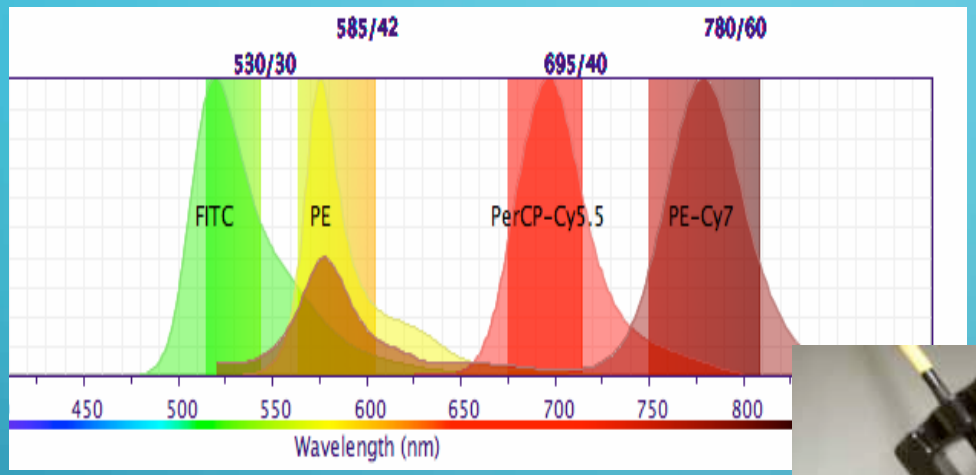


DICHROIC FILTERS

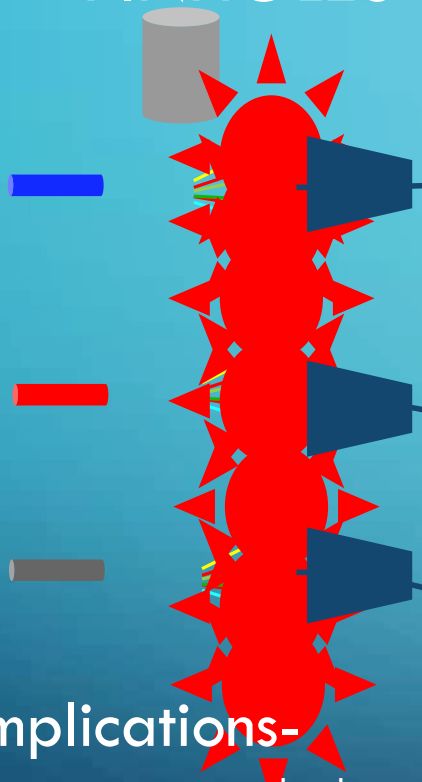
- Can be a long pass or short pass filter
- Depending on the specs of the filter, some of the light is reflected and part of the light is transmitted and continues on.

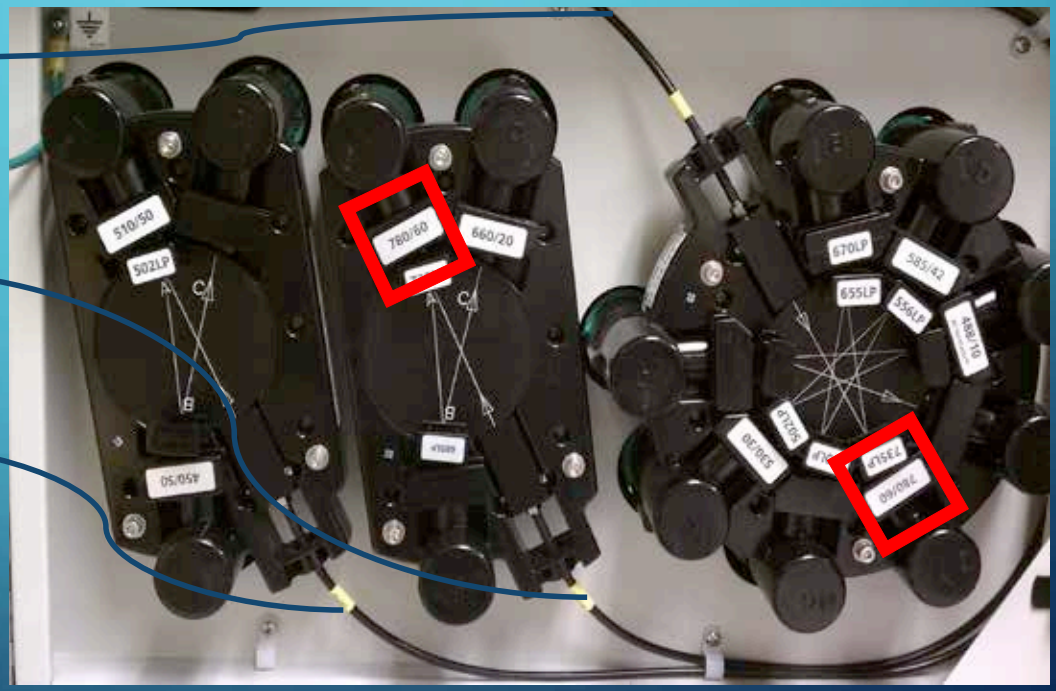


SPECTRA OF COMMON FLUOROCHROMES WITH TYPICAL FILTERS



MULTI-LASER INSTRUMENTS AND PINHOLES



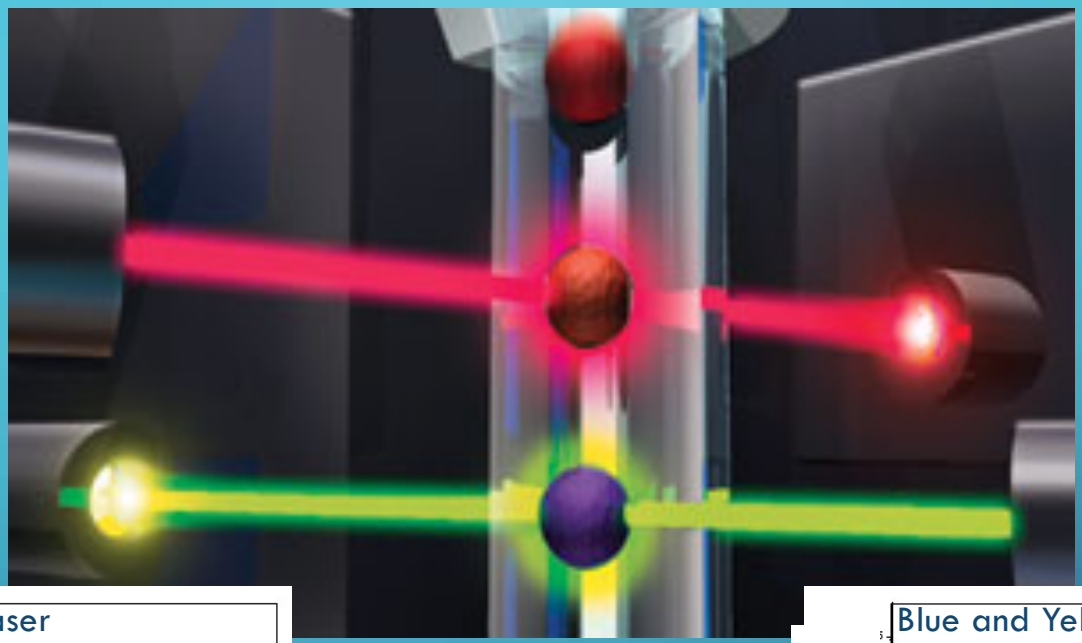


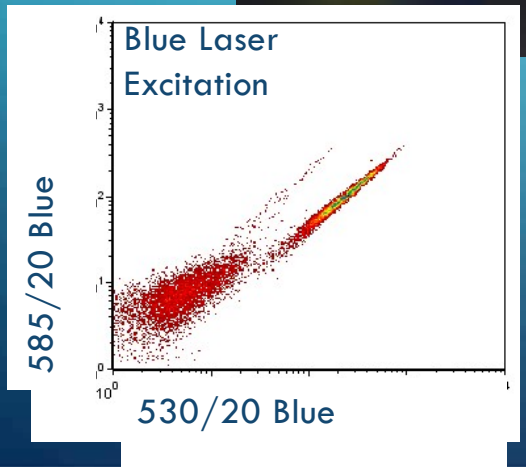
Implications-

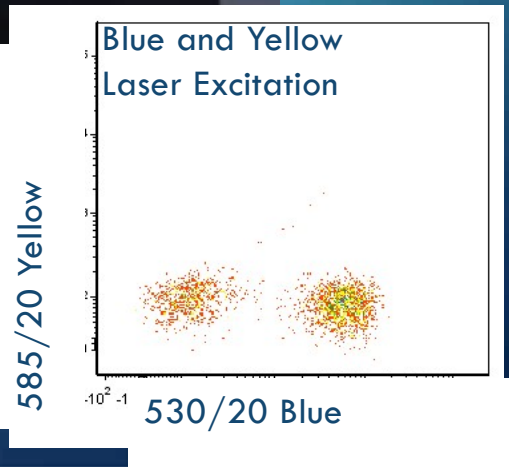
-Can separate completely overlapping emission profiles if originating off different lasers

-Significantly reduces compensation

SPATIAL SEPARATION







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 integrity
- 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

WHAT HAPPENS IN A FLOW CYTOMETER?

- Cells in suspension flow single file
- Through a focused laser where they scatter light and emit fluorescence that is filtered, measured
- and converted to digitized values that are stored in a file
- Which can then be read by specialized software.

Fluidics

Interrogation

Electronics

Interpretation

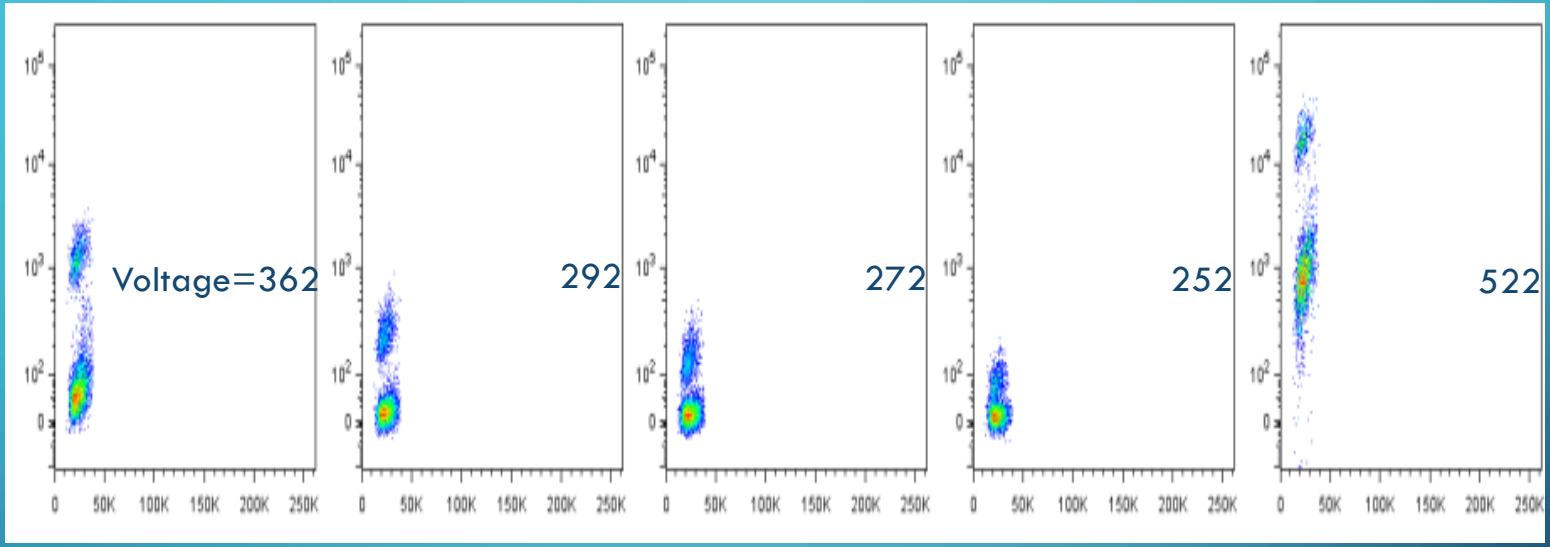
ELECTRONICS

- Detectors basically collect photons of light and convert them to an electrical current
- The “electronics” must process that light signal and convert the current to a digitized value/# that the computer can graph

DETECTORS

- There are two main types of photo detectors used in flow cytometry
  - Photodiodes
    - Used for strong signals, when saturation is a potential problem (eg. FSC detector)
  - Photomultiplier tubes (PMT)
    - Used for detecting small amounts of fluorescence emitted from fluorochromes.
    - Incredible Gain (amplification-up to 10million times)
    - Low noise

DOES VOLTAGE SETTING MATTER?



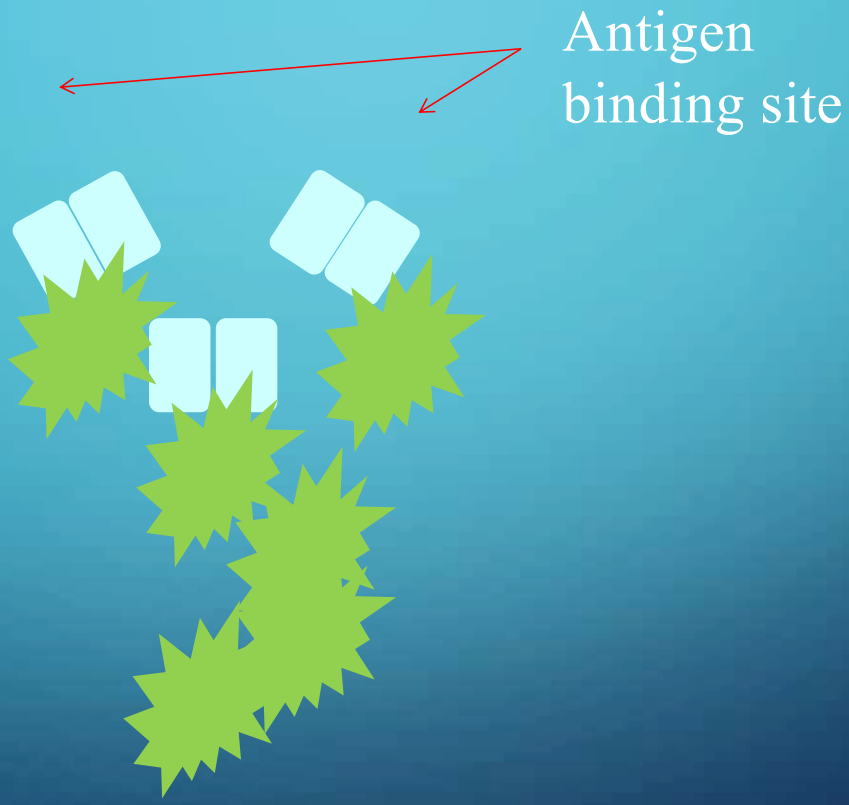
-Voltage doesn't change sensitivity or laser power
-All your doing is changing the amplification of the signal

-Caveat- there is large "sweet spot" of PMT voltage, outside of this range you may run the risk of non linear amplification

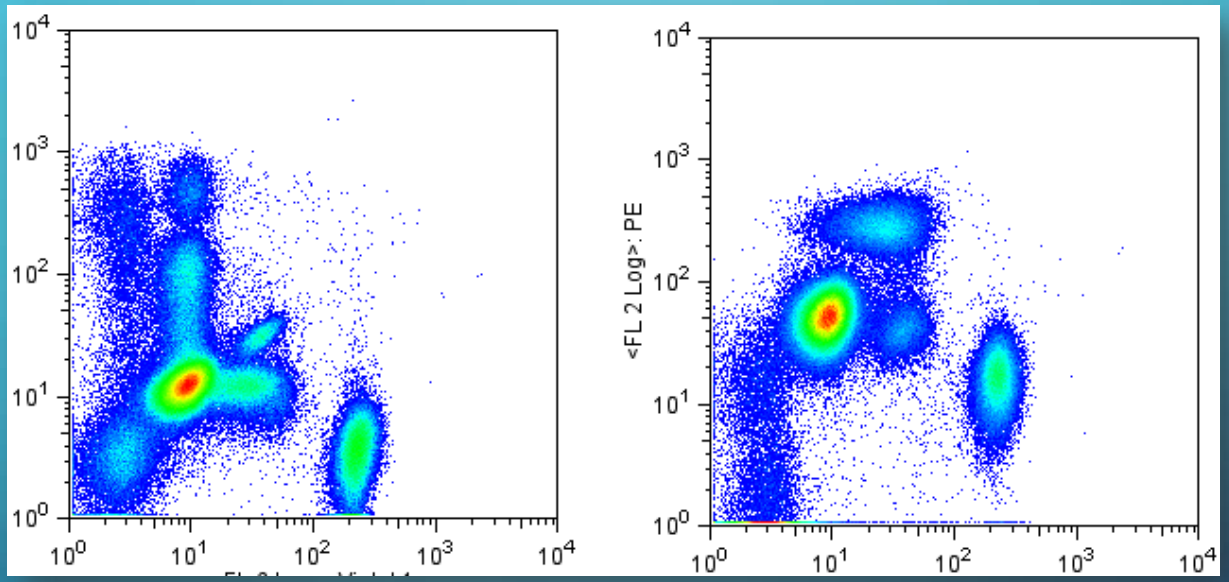
WHAT HAPPENS IN A FLOW CYTOMETER?

- Cells in suspension flow single file past — Fluidics
- a focused laser where they scatter light and emit fluorescence that is collected, filtered — Interrogation
- and converted to digitized values that are stored in a file — Electronics
- Which can then be read by specialized software. — Interpretation

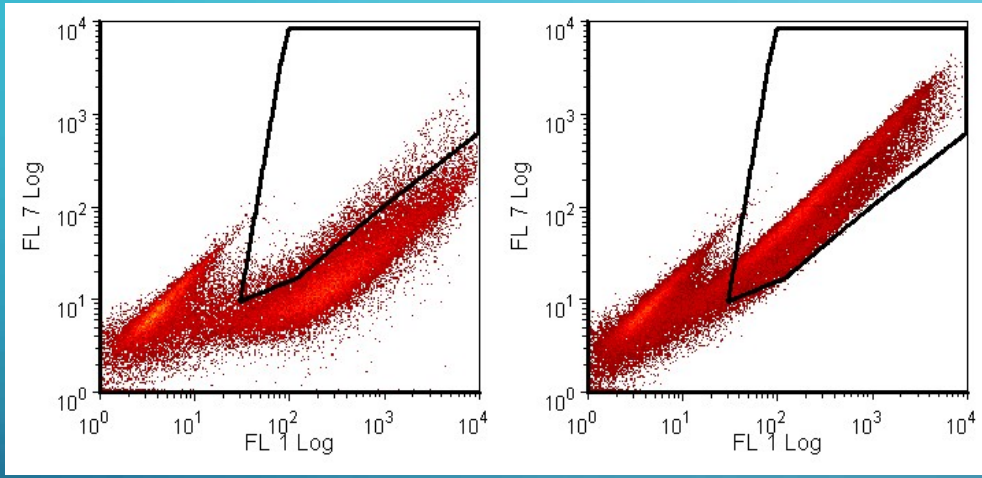
ANTIBODY

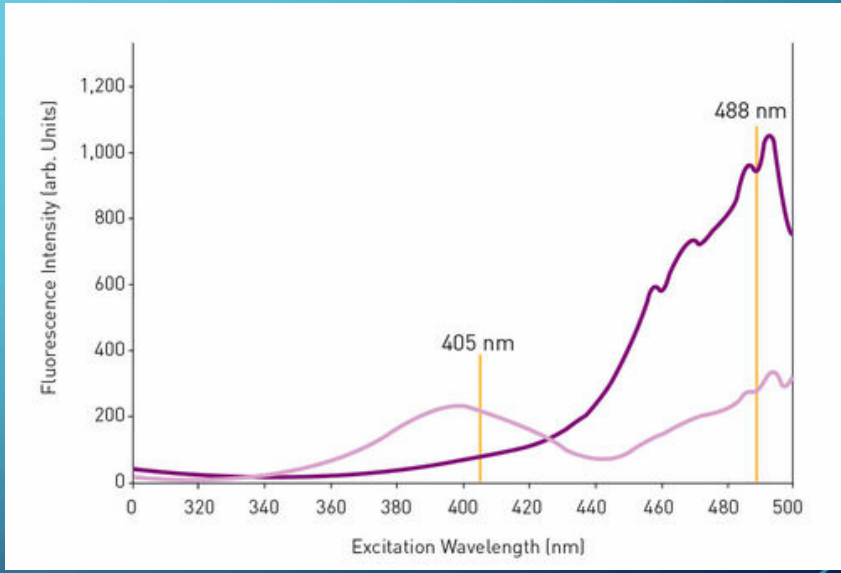


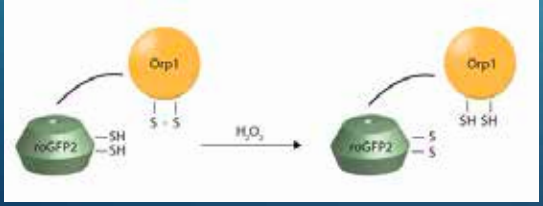
IMMUNOPHENOTYPING



ROGFP
REDOX SENSTITIVE BIOSENSOR





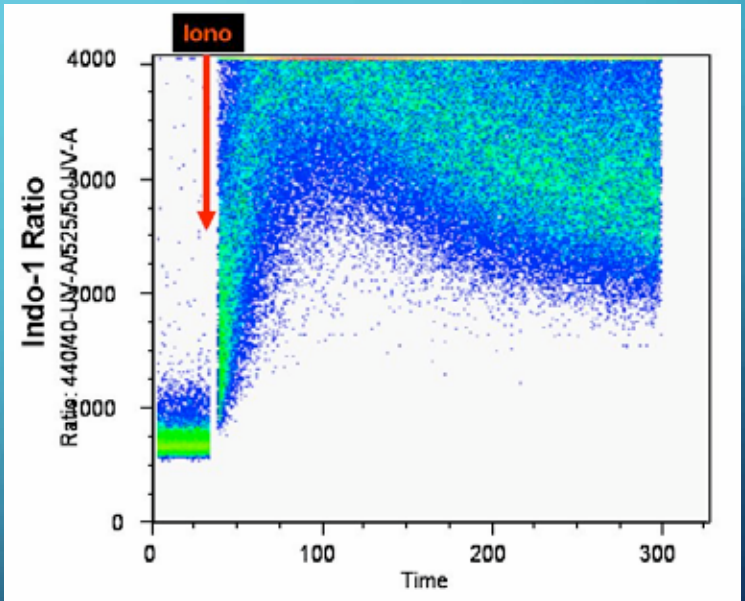


Schematic of the mechanism of action of roGFP2-Orp1. The formation of the redox-sensitive disulfide bridge between cysteines on the surface of roGFP is mediated by the presence of oxidants (e.g.: H2O2)

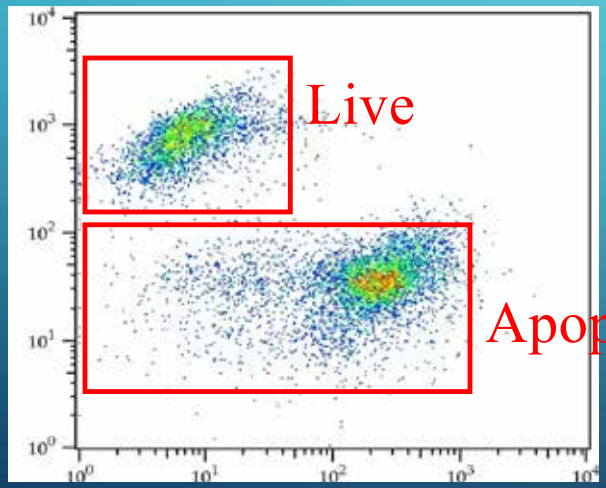
CA^{2+} FLUX

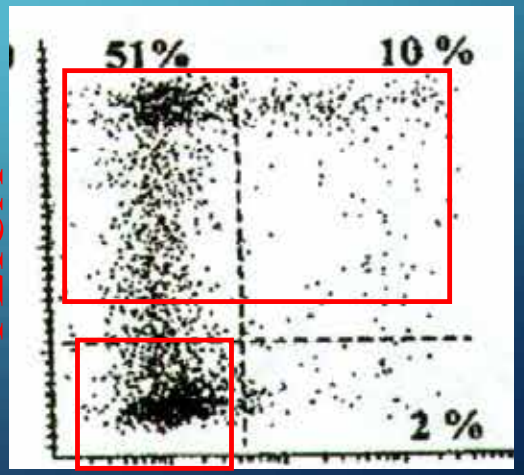
Indo-1 Ca2+ free
Emission=500nm

Indo-1 Ca2+ bound
Emission=395nm

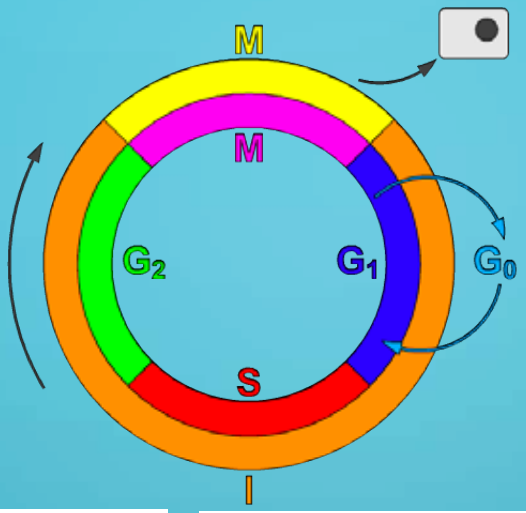


APOPTOSIS

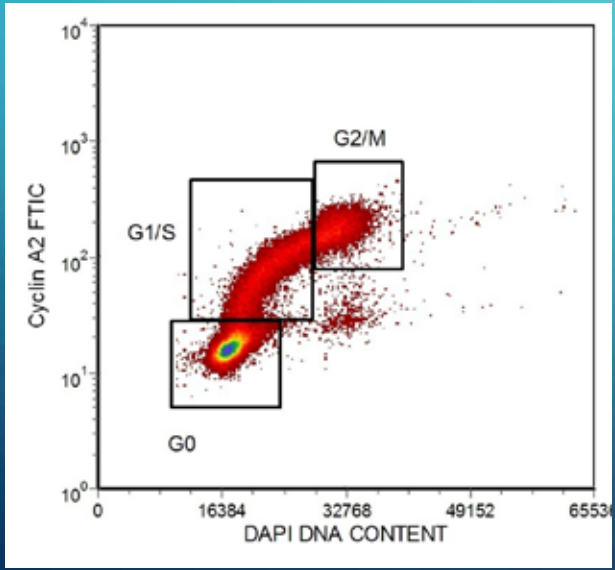


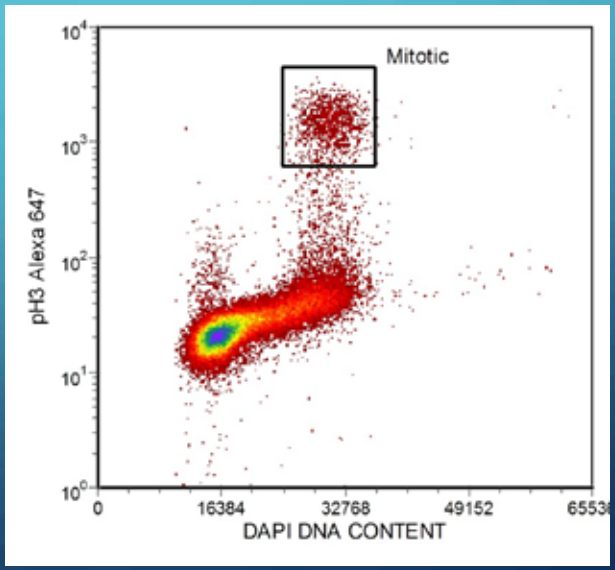


CELL CYCLE

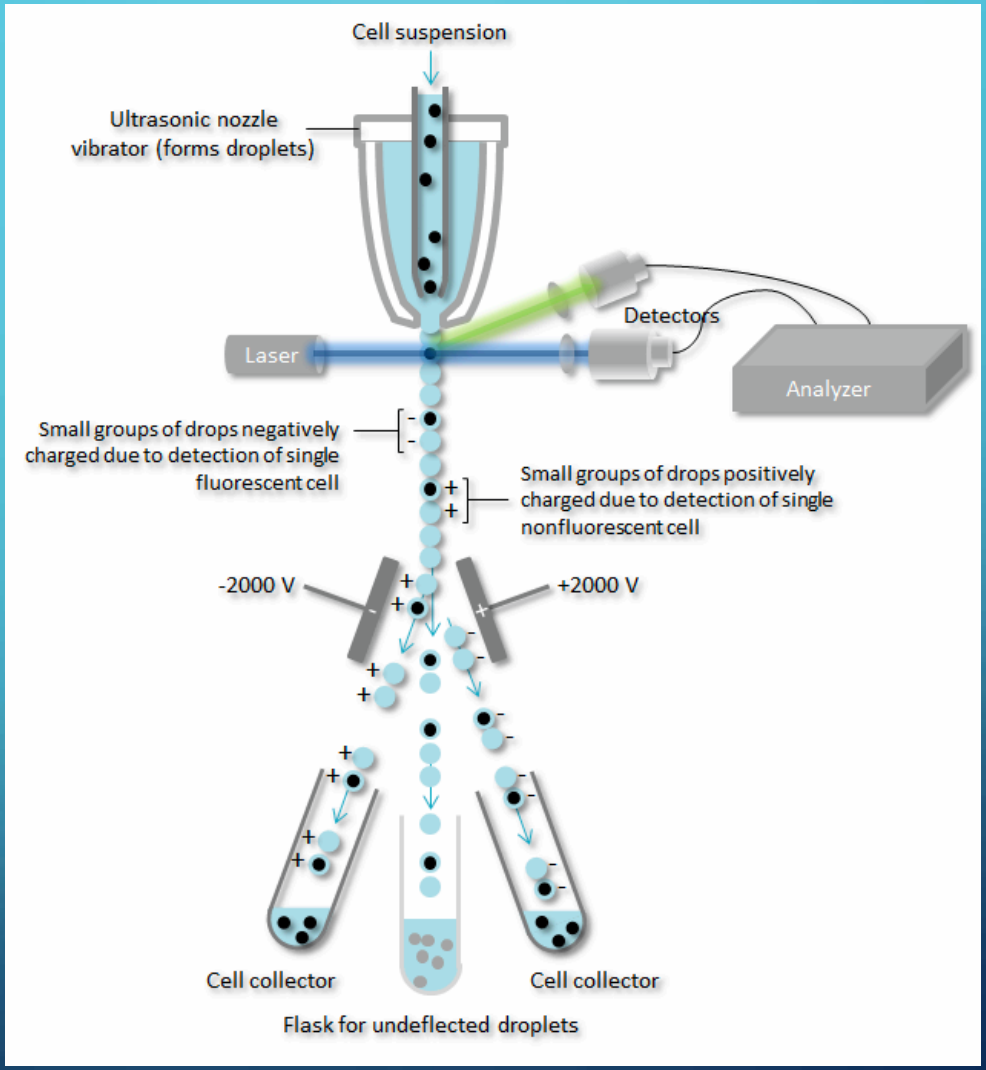


Schematic of the cell cycle. outer ring: I=Interphase, M=Mitosis; inner ring: M=Mitosis; G1=Gap phase 1; S=Synthesis; G2=Gap phase 2. The duration of mitosis in relation to the other phases has been exaggerated in this diagram





SORTING


Cell suspension
Ultrasonic nozzle vibrator (forms droplets)
Detectors
Laser
Analyzer
Small groups of drops negatively charged due to detection of single fluorescent cell
Small groups of drops positively charged due to detection of single nonfluorescent cell
-2000 V
+2000 V
Cell collector
Cell collector
Flask for undeflected droplets