Basics of Flow Cytometry

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ICMR-NIRRCH

TCS Sponsored Flow Cytometry Workshop

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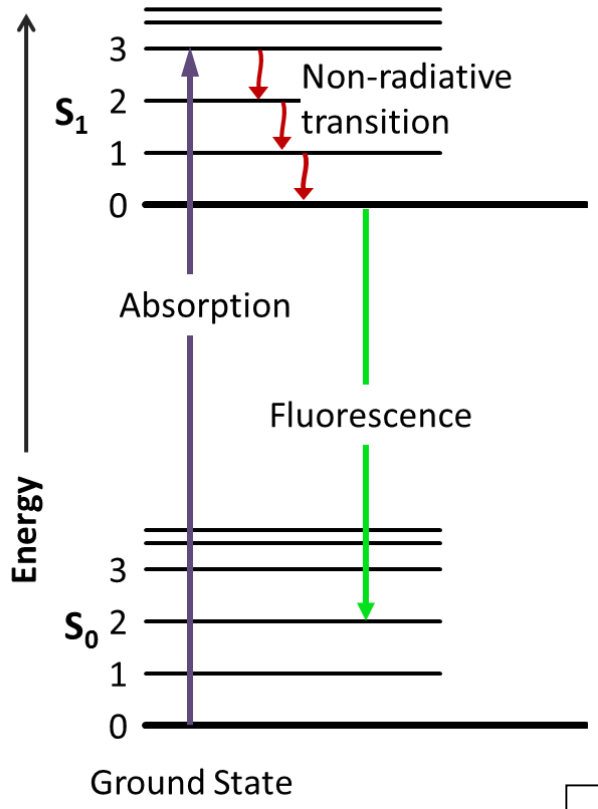
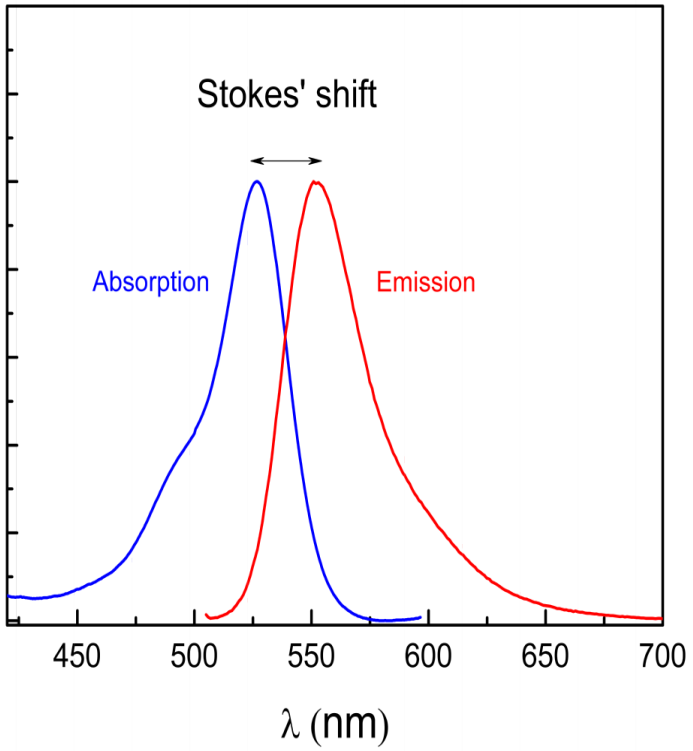
Fluorescence

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to subsequently emit light of longer wavelength

Energy Level Diagram





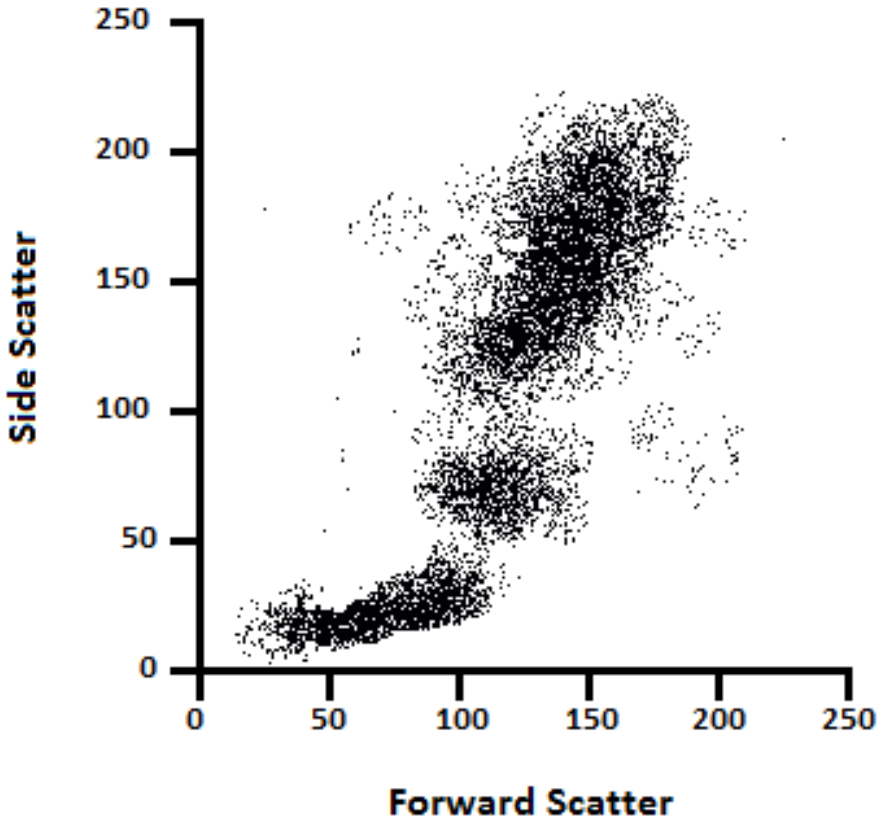
$E = h f, \upsilon = f \lambda$

Flow Cytometry

Flow cytometry is a method of measuring multiple physical and chemical characteristics of particles in a fluid stream by optical means i.e. as they pass through one or more lasers.

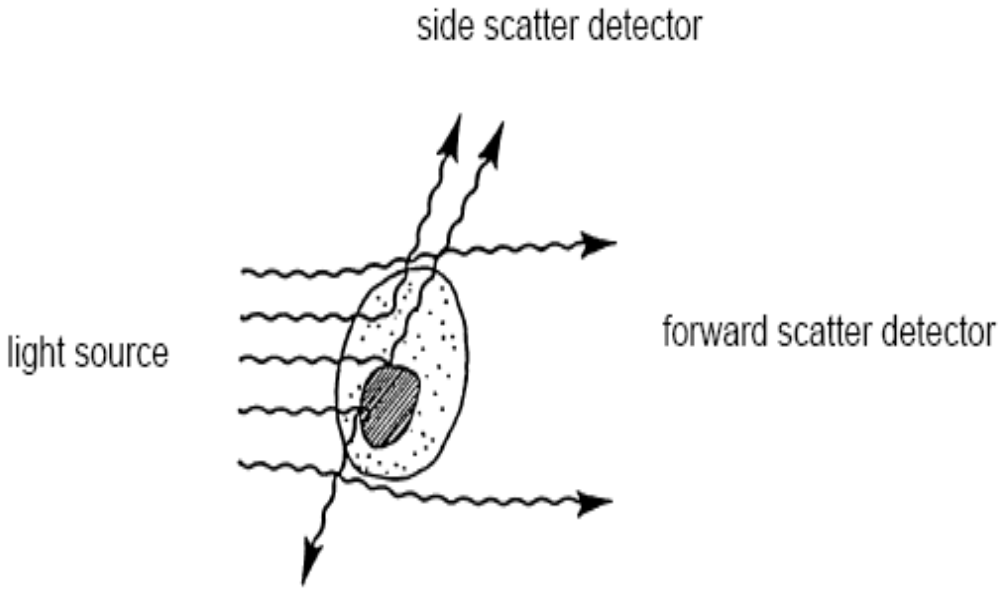
- Process for quantifying cells
- Measures different property of cells
- Able to categorize and quantify
- Even able to separate out & analyze subpopulations of cells
- Assay large numbers of cell $>10^6$ cells/tube
- Fast: assay up to 10,000 cells/second
- Identify rare populations eg. stem cells, dendritic cells

FACS: Fluorescence Activated Cell Sorting—not all flow cytometry is FACS!


250
200
150
100
50
0
Side Scatter
0
50
100
150
200
250
Forward Scatter

Light Scatter

- Light scattering occurs when a particle deflects incident laser light.
- The extent to which this occurs depends on the physical properties of a particle, namely its size and internal complexity.
- Factors that affect light scattering are the cell's membrane, nucleus, and any granular material inside the cell.
- Cell shape and surface topography also contribute to the total light scatter.



Physical properties—based on physical interaction of particle with laser light, measured in same wavelength as laser.

Relative size*

Relative granularity or internal complexity*

Often a characteristic profile for a given particle

• Chemical Properties—based on signals from reagents that interact with the laser light.

Relative Fluorescence intensity*

(FL1, FL2, etc. or FITC, PE, etc.)

"Relative" because user can adjust sensitivity of detectors to change the output

"Particle" referring to either a cell, bead, organism, or other measurement-triggering object

Cellular Parameters Measured by Flow Cytometry

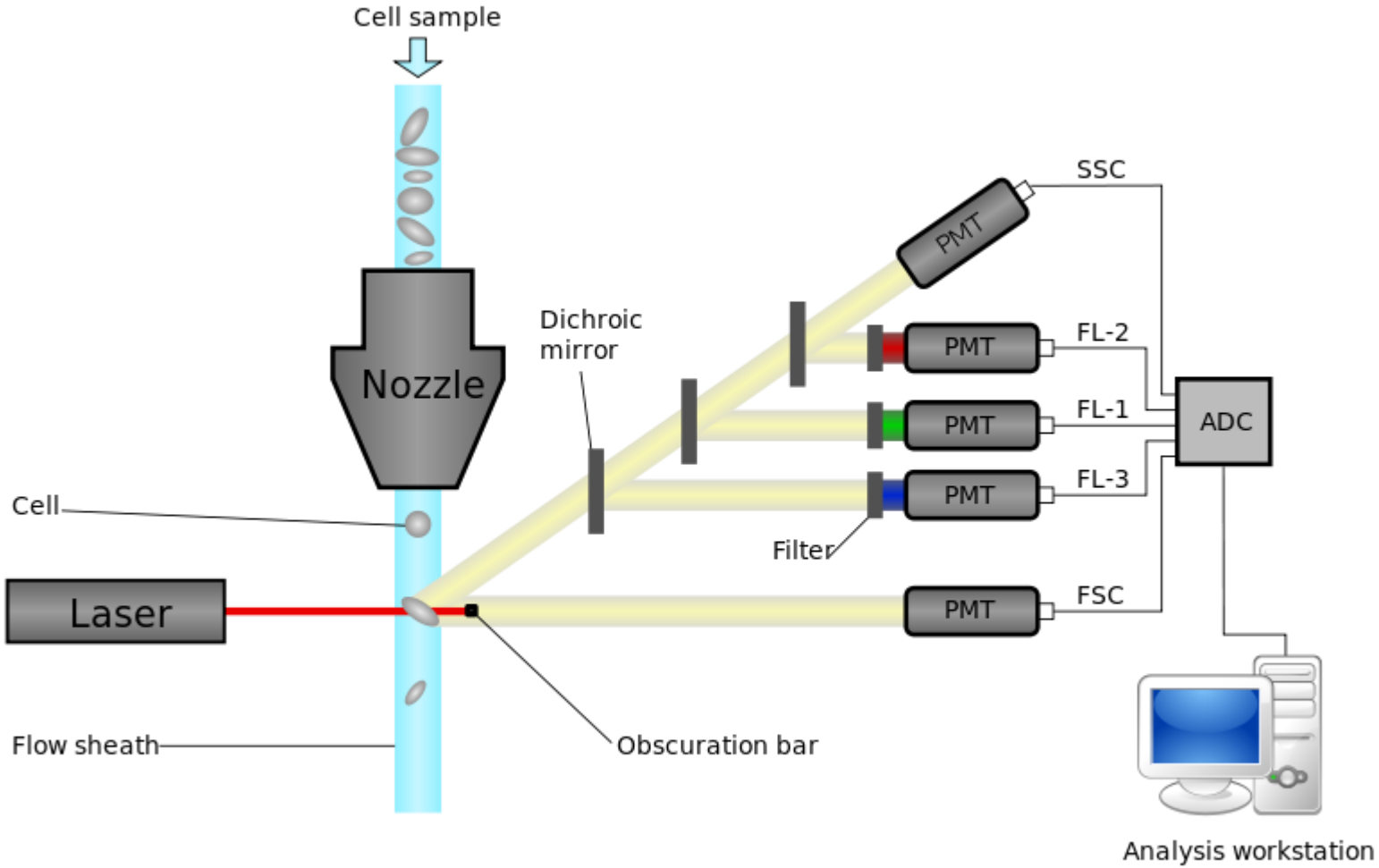
Intrinsic

- No reagents or probes required (**Structural**)
  - Cell size(Forward Light Scatter)
  - Cytoplasmic granularity (90 degree Light Scatter)
  - Photsynthetic pigments

Extrinsic

- Reagents are required.
  - **Structural**
    - DNA content
    - DNA base ratios
    - RNA content
  - **Functional**
    - Surface and intracellular receptors.
    - DNA synthesis
    - DNA degradation (apoptosis)
    - Cytoplasmic Ca++
    - Gene expression

Schematic diagram of a flow cytometer



FACS COMPONENTS

LASER – Source of light

OPTICS - Generate and collect light signals

FLUIDICS - Introduces and focuses the cells for "interrogation"

ELECTRONICS -Change optical signals to electronic signals and digitize for computer analysis

LASER

1. **ARGON**
2. **EMISSION LINE OF 488 nm**
3. **VISIBLE LIGHT**

Cytometers will have one or more lasers:

The laser color determines what fluorochromes can be used on that instrument.

Common excitation wavelengths:488 (blue)
635 (red)
405 (violet)
532 (green)
350 (UV)
561 (yellow-green)

OPTICS

1. BEAM SPLITTERS
2. DICHROIC MIRRORS
3. LONG / SHORT PASS FILTERS
4. BAND PASS FILTERS

Optics

-Excitation Optics consist of:

One or more lasers

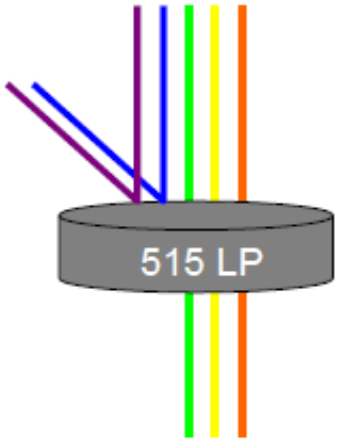
Lenses to shape and focus the beam(s)

-Collection Optics consist of:

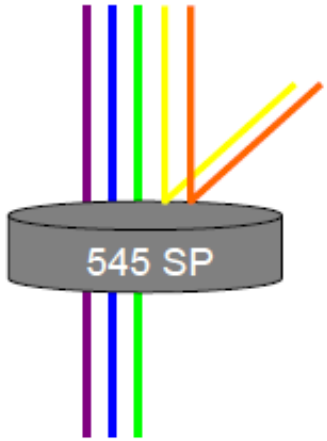
- A collection lens to collect light emitted from the particle-beam interaction
- A system of optical mirrors and filters to route specified wavelengths of the collected light to designated optical detectors

Collection Optics: Optical Filters

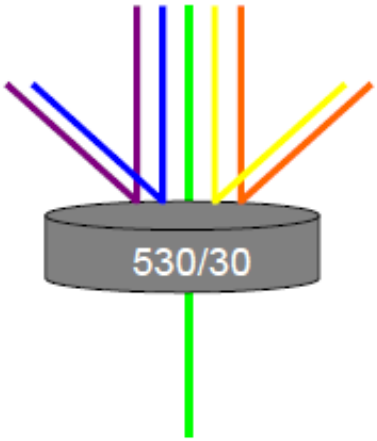
Combinations of filters determine the wavelength of light that reaches your detector



Long pass
Longer than cutoff passes through, shorter is reflected

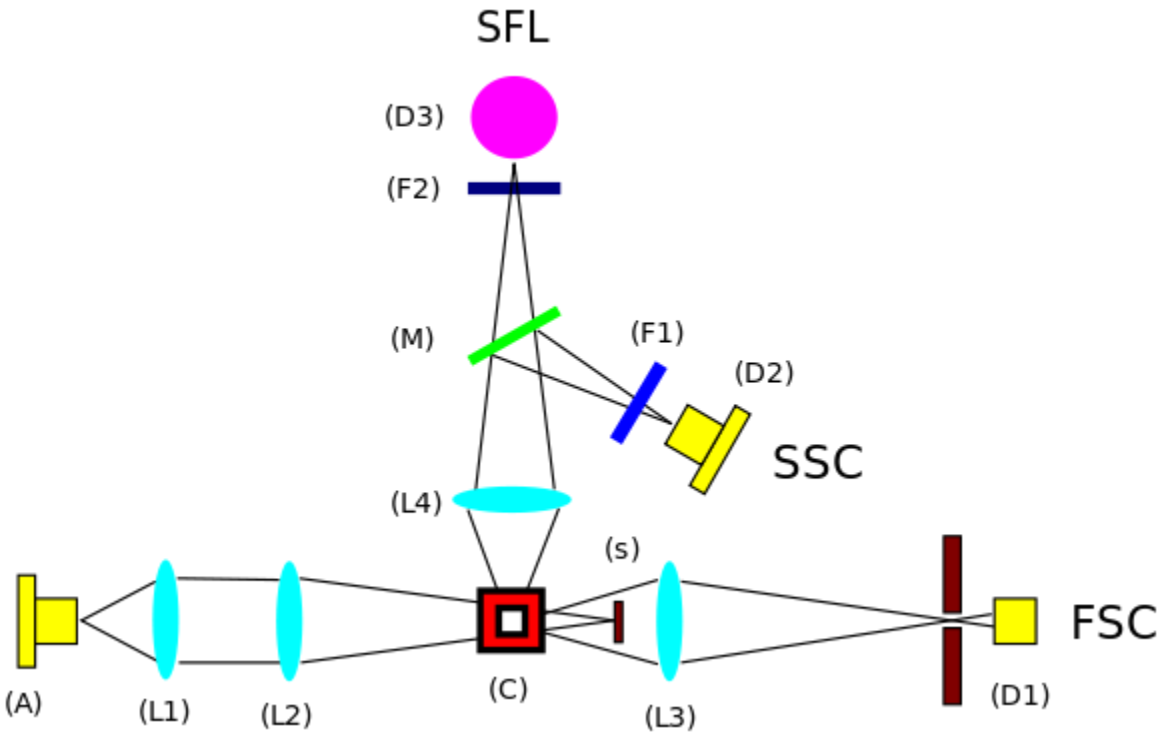


Short pass
Shorter than cutoff passes through, longer is reflected

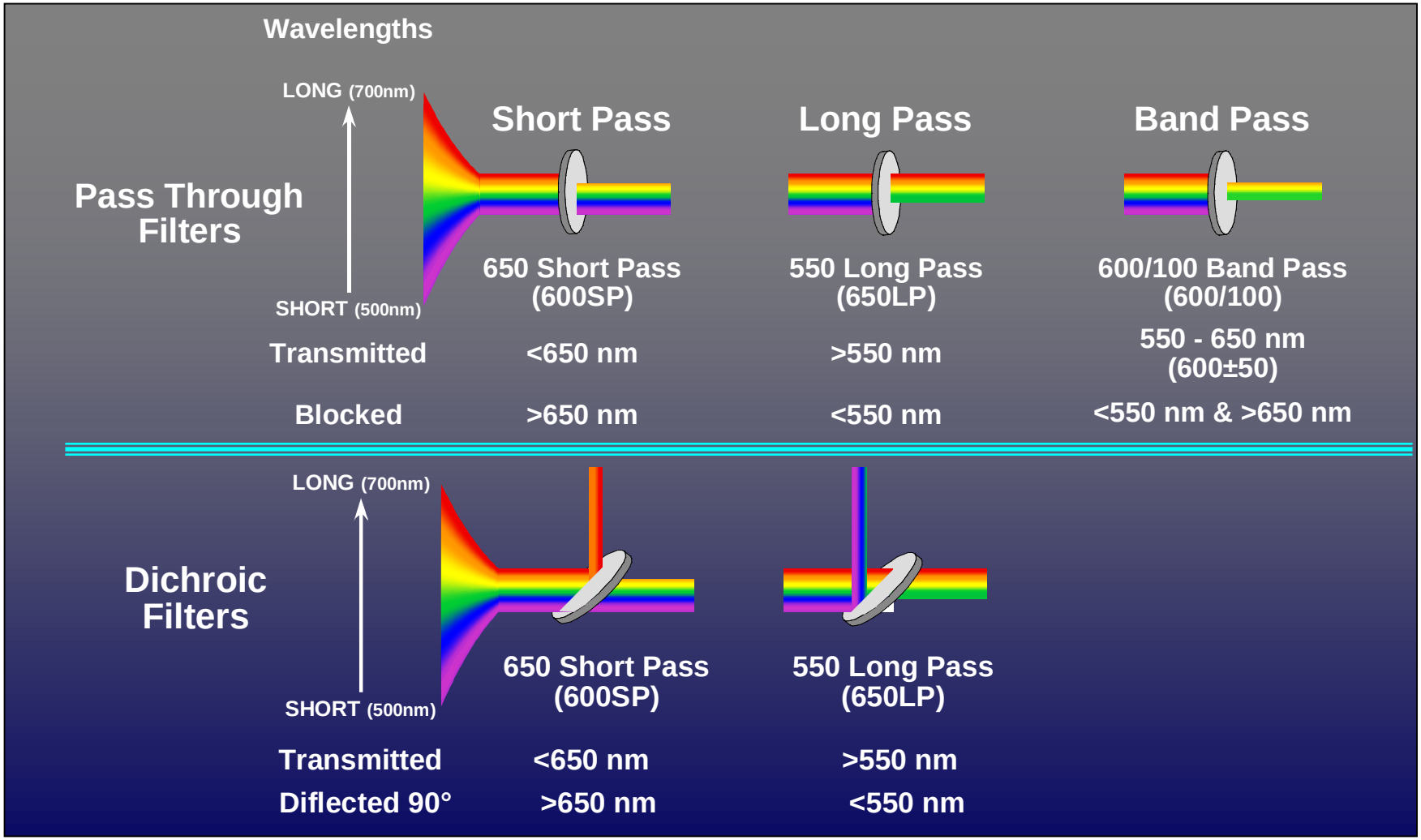


Band pass
Allows a band of light to pass through. First number indicates midpoint, second number indicates width (in this case 515-545)

Optics of Flow Cytometer



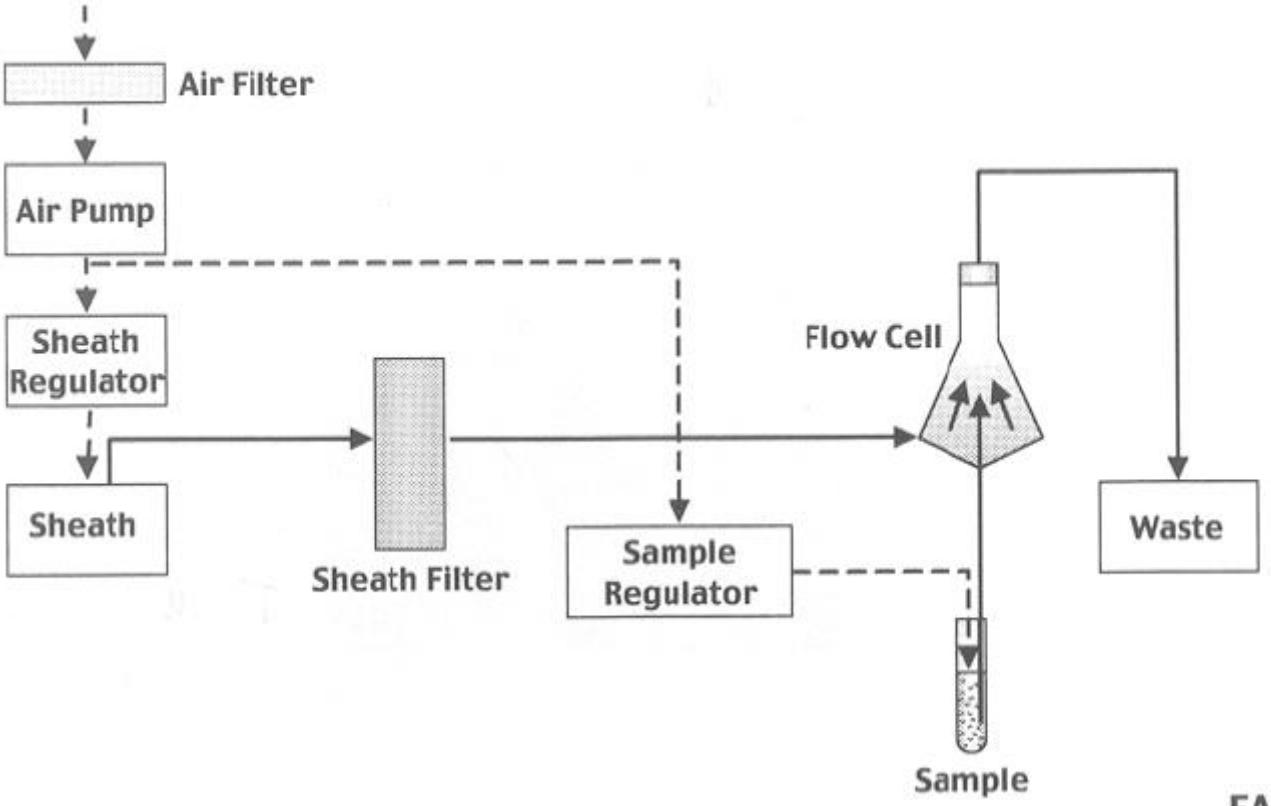
Types of Optical Filters



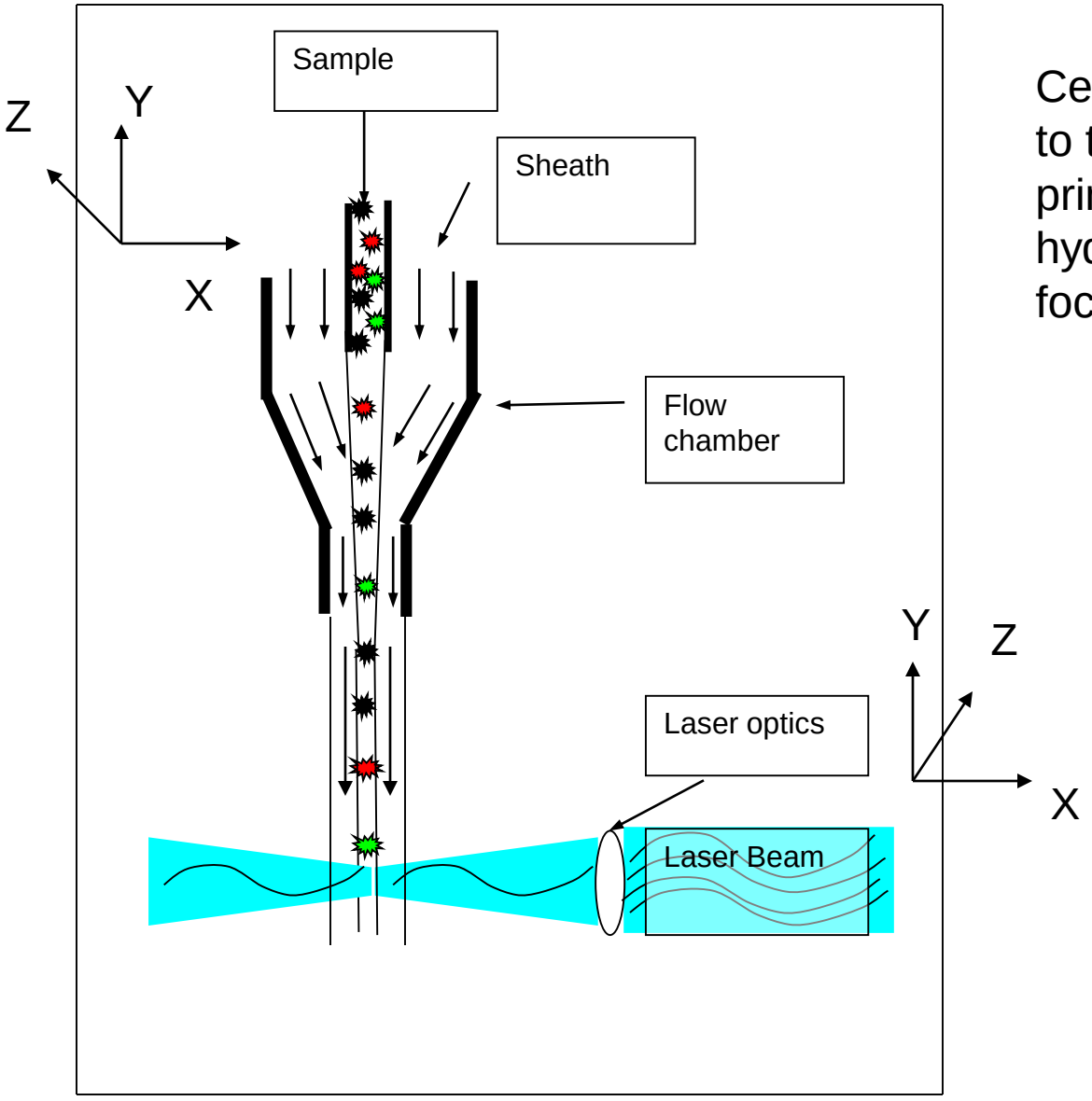
FLUIDICS

1. SAMPLE FLUID
2. SHEATH FLUID
3. FLOW CELL
4. NOZZLES, 70, 100, 400 um

Fluidics–FACSCalibur™



FACS Academy

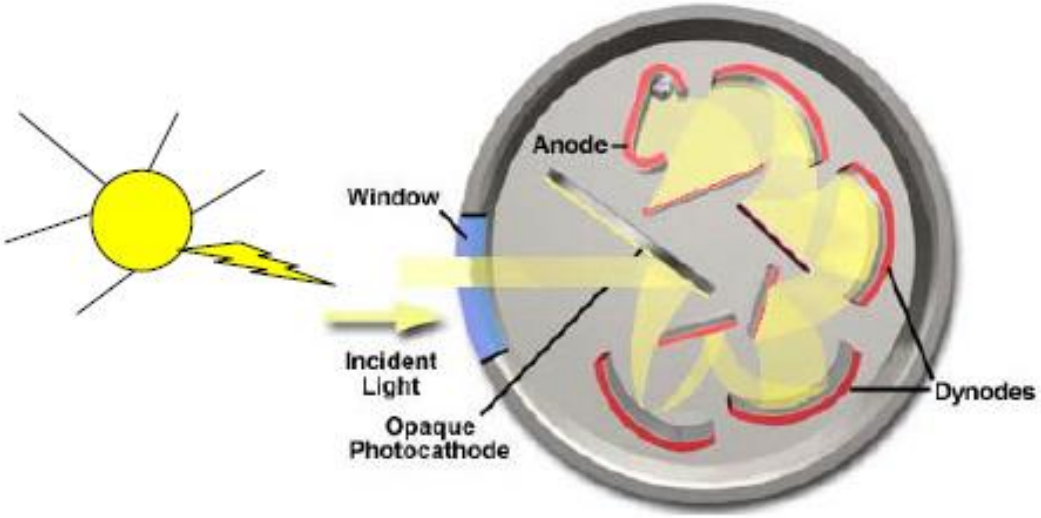


Cells are presented to the laser using principles of hydrodynamic focusing

Electronics

- Converts optical signals to proportional electronic signals (voltage pulses)
- Analyze voltage pulse height, area, or width
- Interfaces with computer for data transfer.

Conversion of Optical Signals to Proportional Electronic Signals



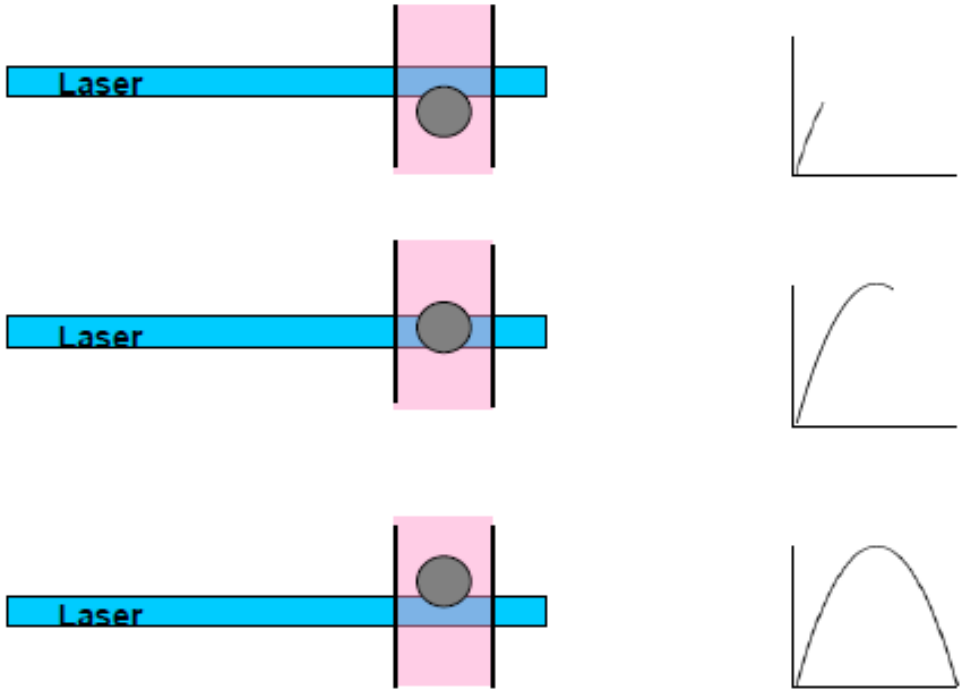
2 types of detectors—PMT and photodiode—generate voltage when struck by photons.

PD used for FSC which produces more light.

PMT amplifies the signal depending on voltage supplied.

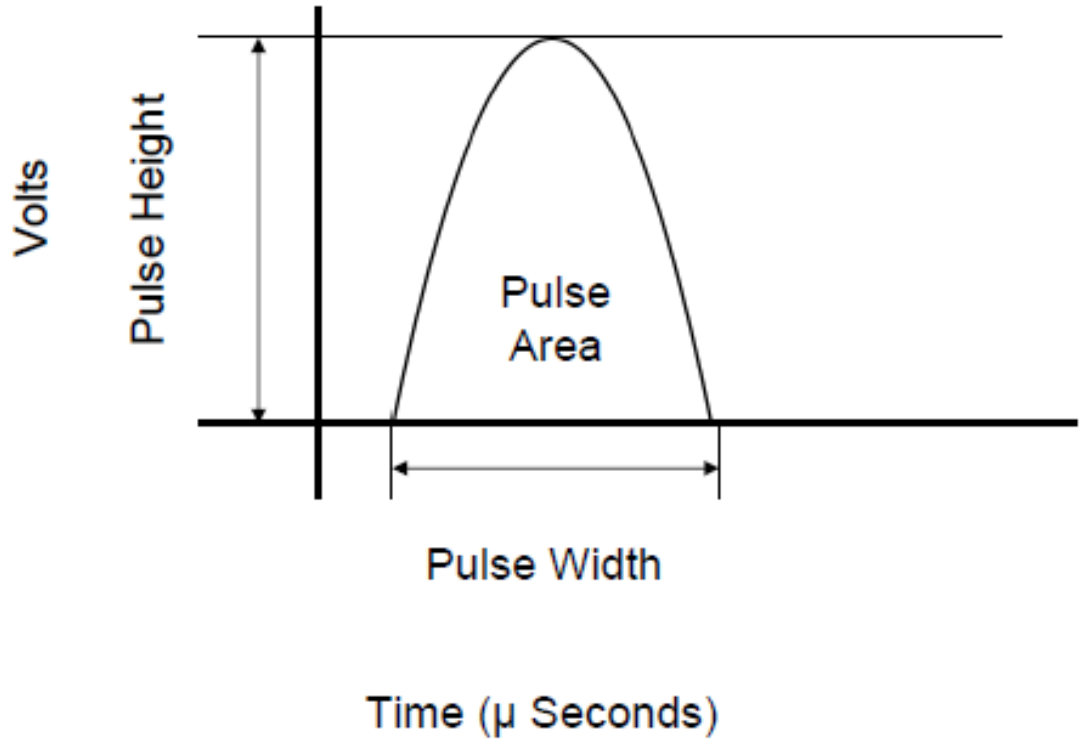
Detectors are color blind, so it is important that filters separate different colors of light.

Creation of a Voltage Pulse

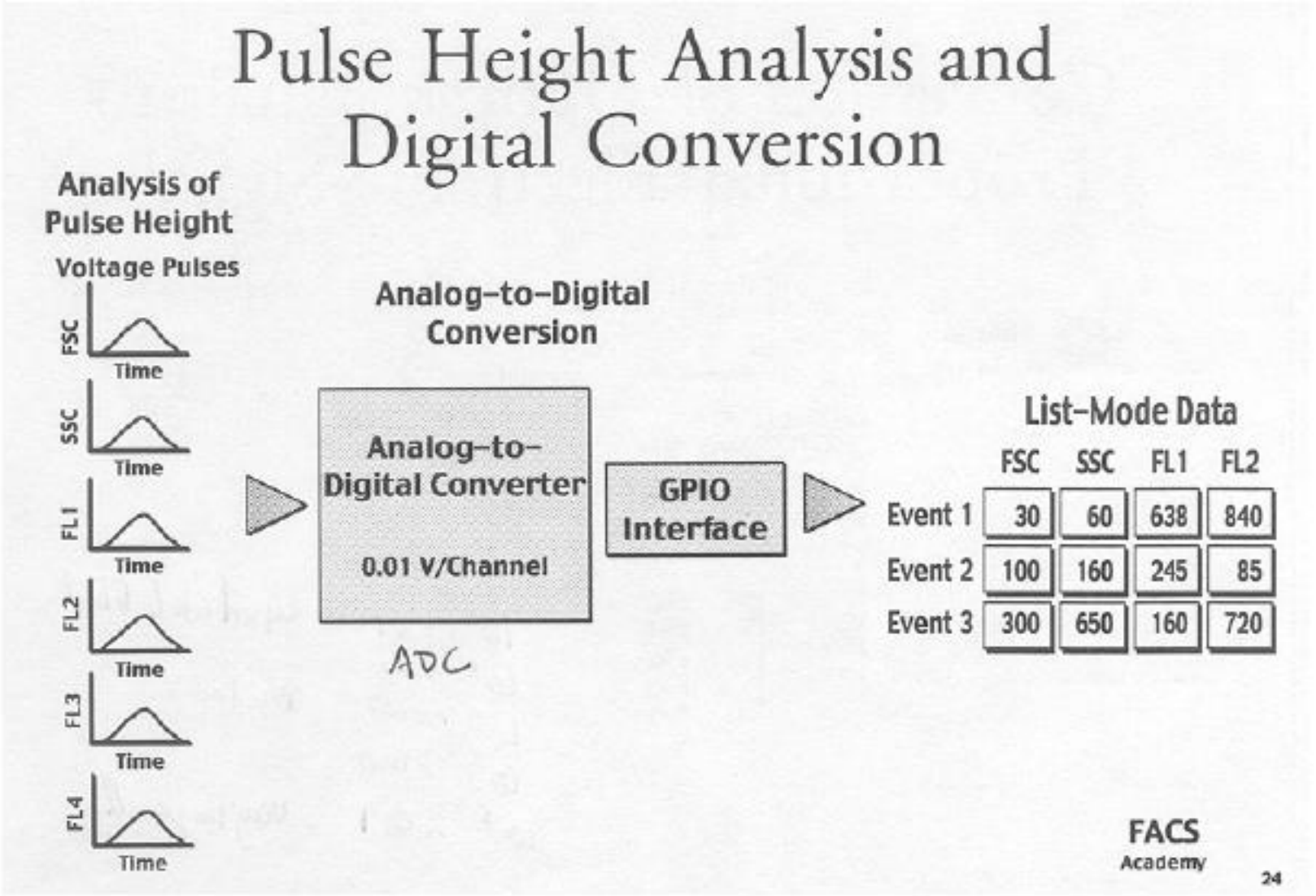


Total signal is collected, not just what is bound to particle!
(e.g. unbound dye, Phenol Red in culture media)
Maximal signal when particle is entirely within the laser beam.
Light signal turned into voltage signal, which can be quantified.

Quantification of a Voltage Pulse

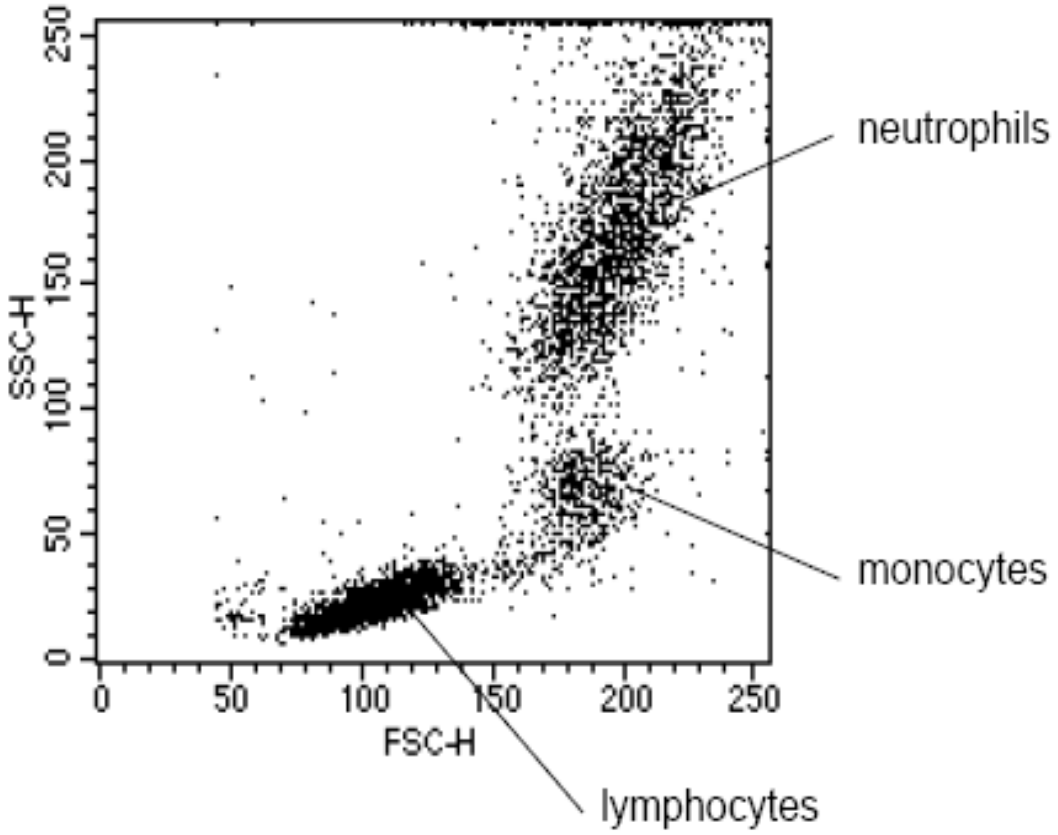


Pulse Height Analysis and Digital Conversion



Voltage signals are converted to a value and sent to a computer.
Data stored in "list-mode" format, where each event has a value for each parameter being measured. Files are saved as ".fcs" (Flow Cytometry Standard)

FSC vs SSC Dot Plot

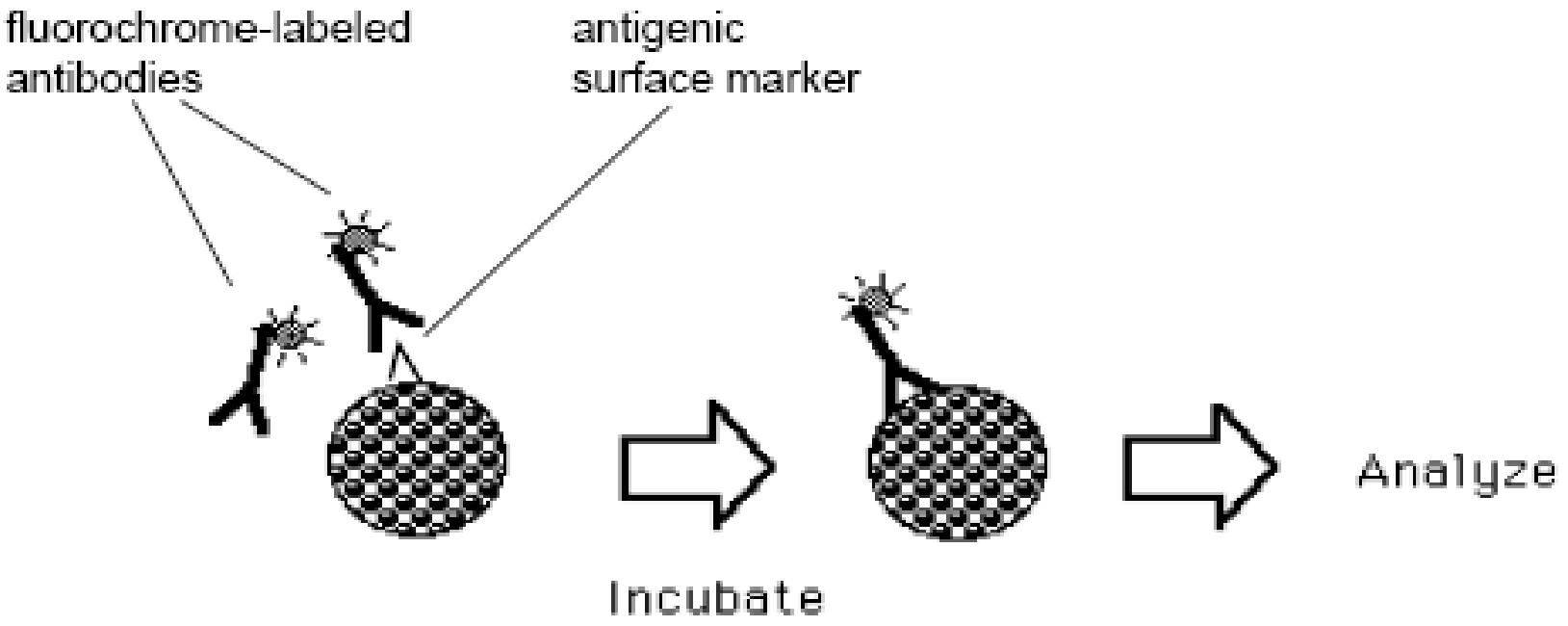


lysed whole blood

Limitations With Light Scattering

- Some Information Can Be Obtained
- FSC Correlates With Cell Size
- SSC Correlates With Internal Complexity
- To Distinguish Between 2 Cell types
  - A. Size Has To Be Different OR
  - B. Internal Complexity i.e amount of granules
- If These Two Parameters Are The Same, Then No Distinction Can Be Made

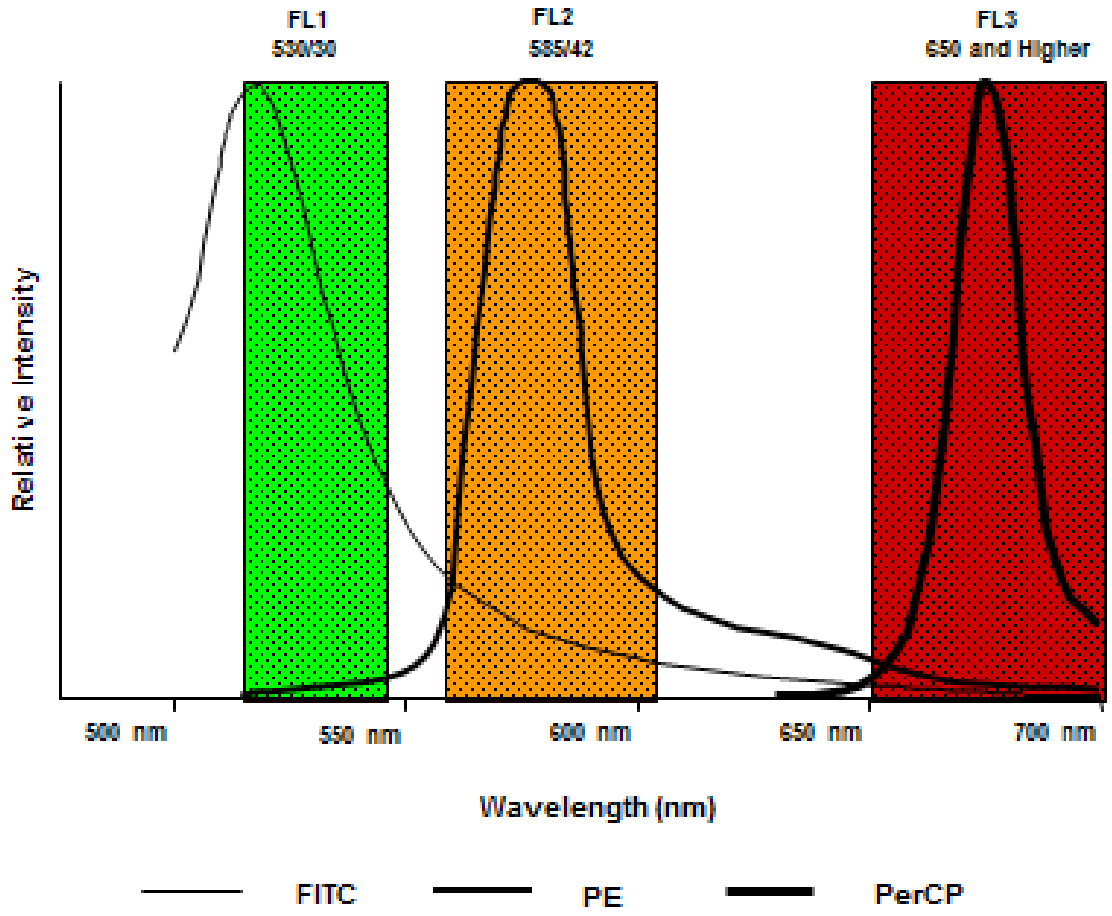
Fluorescence And Antibodies To The Rescue



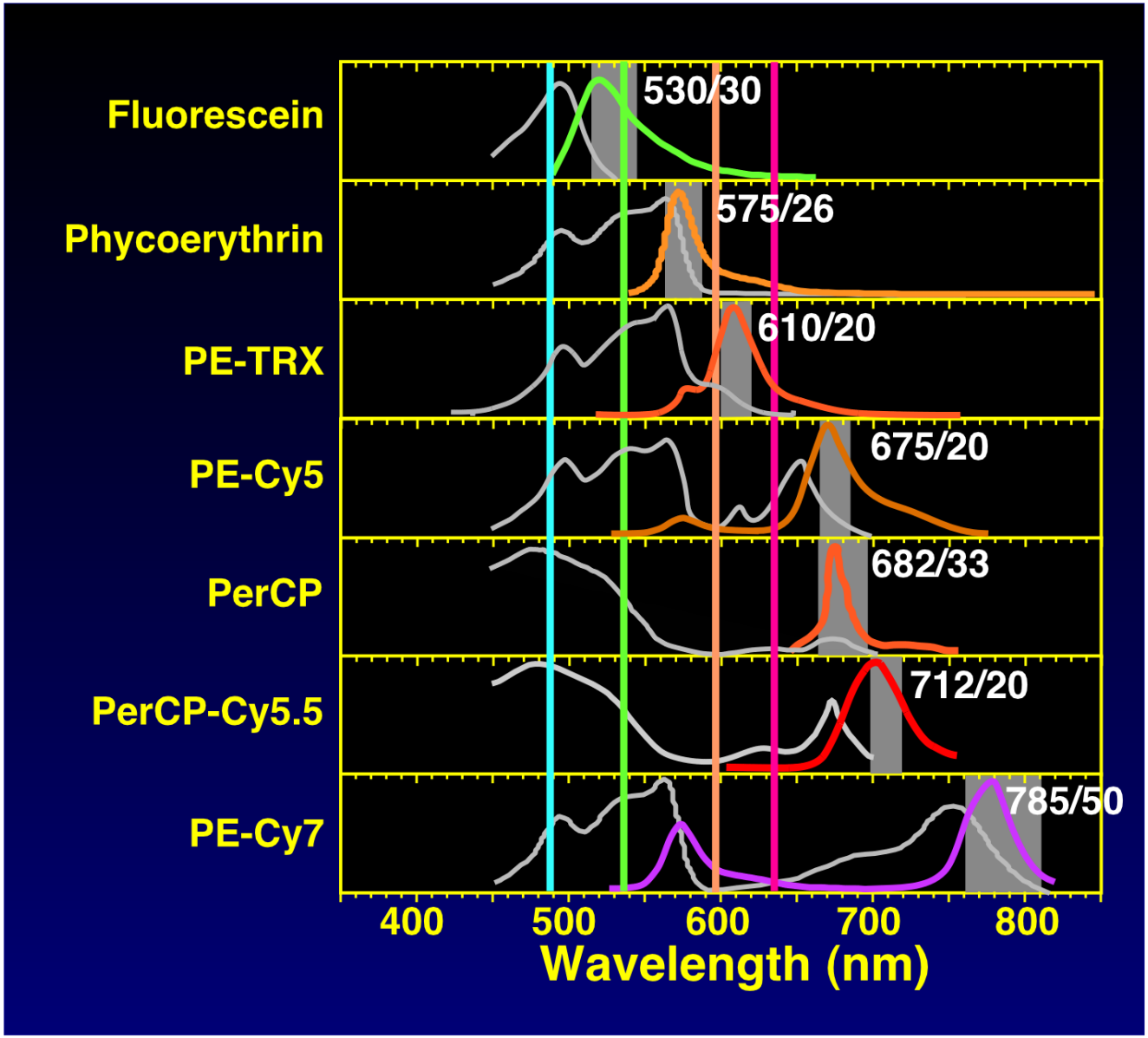
Fluorescent Dyes And Antibodies

- Fluorochromes Are Molecules That Emit Fluorescence Upon Excitation With Light
  - Ex. FITC (Fluorescein Isothiocyanate)
  - PE (Phycoerythrin)
  - PerCP (Peridinin Chlorophyll Protein)
  - APC (Allophycocyanin)
- Some Fluorochromes Are Proteins, Some Are Small Organic Compounds
  - Ex. PE (Phycoerythrin)-Protein
  - Ex. FITC (Fluorescein Isothiocyanate)

Emission Spectra



Spectra of Fluorochromes



Classical Data Analysis: Types of data displays

- Frequency distribution/Histogram

  -single parameter only, array created

  - acquisition and analysis

- Dot plot

  -bivariate, two parameters

  -acquisition and analysis

- Density plot

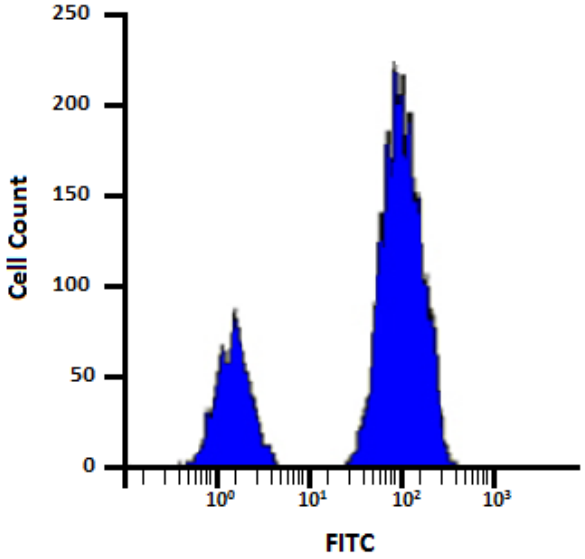
  -bivariate, 64x64, 128x128, or 256x256 2D array

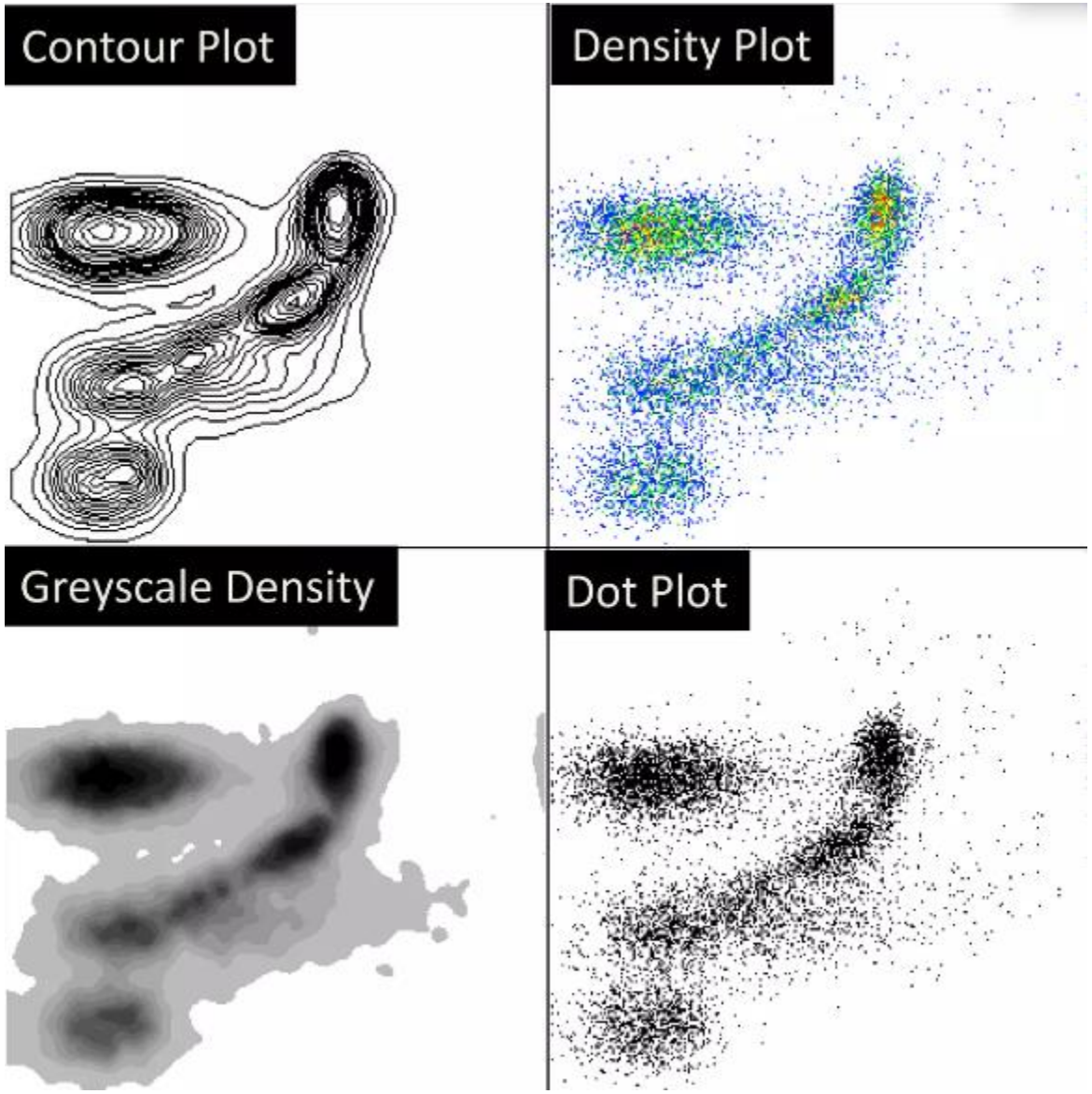
  -acquisition and analysis

- Contour plot

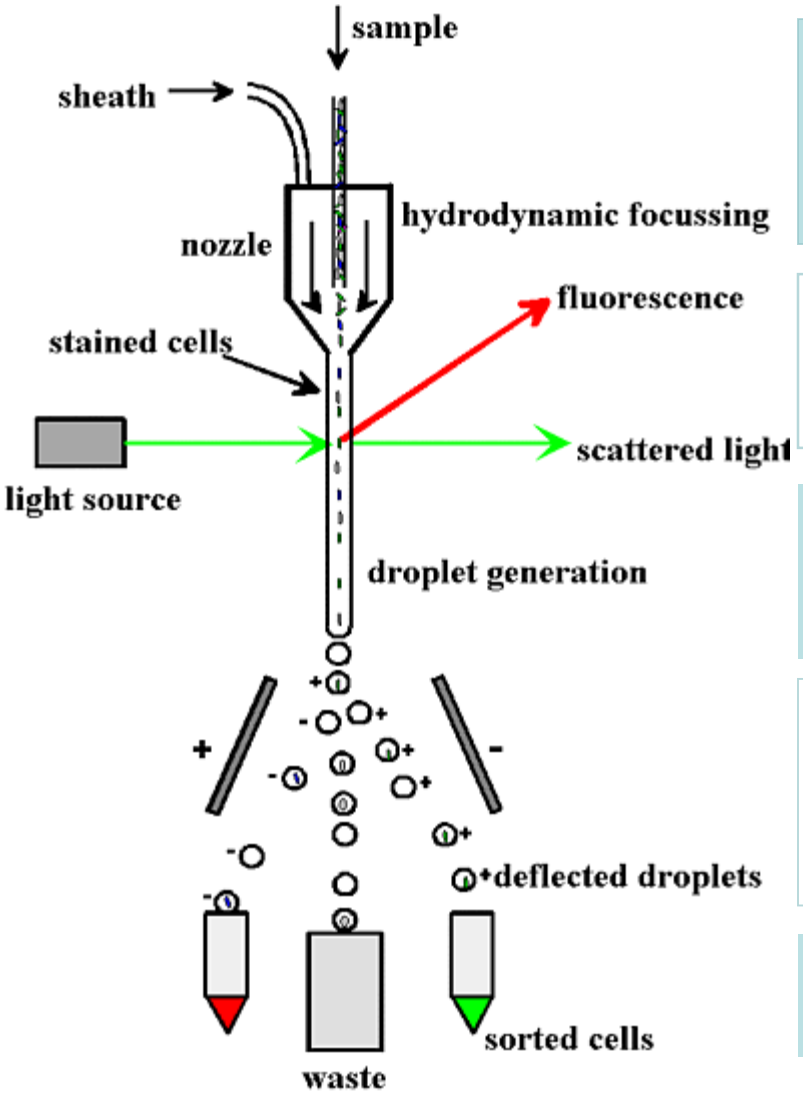
  -bivariate, 64x64, 128x128, or 256x256 2D array

  - analysis only


250
200
150
100
50
0
Cell Count
10^0
10^1
10^2
10^3
FITC


Contour Plot
Density Plot
Greyscale Density
Dot Plot

Flow Cytometry Sorting Schematic



The nozzle/flow cell is vibrated by a transducer (converts electrical energy into mechanical energy) so it produces a stream breaking into droplets.

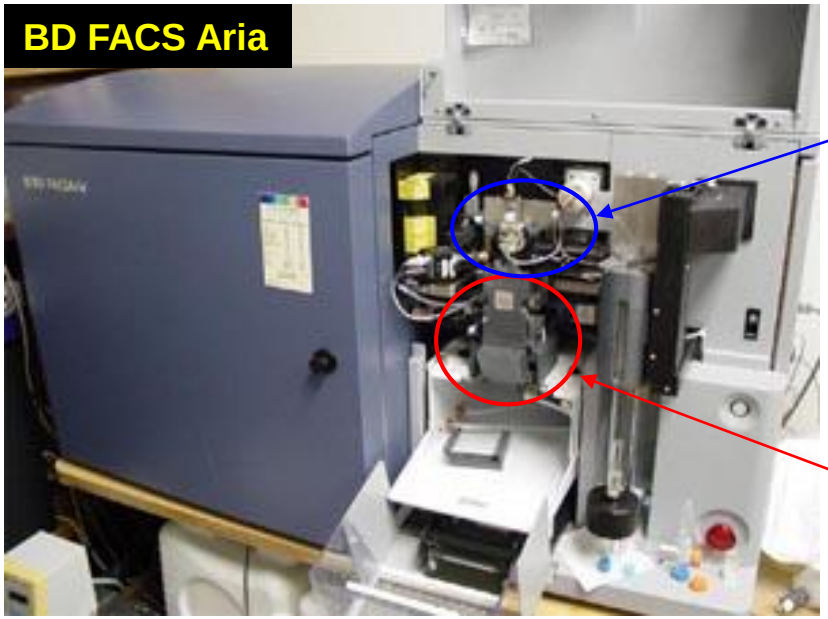
Laser interrogation and signal processing followed by sort decision: sort right, sort left, or no sort.

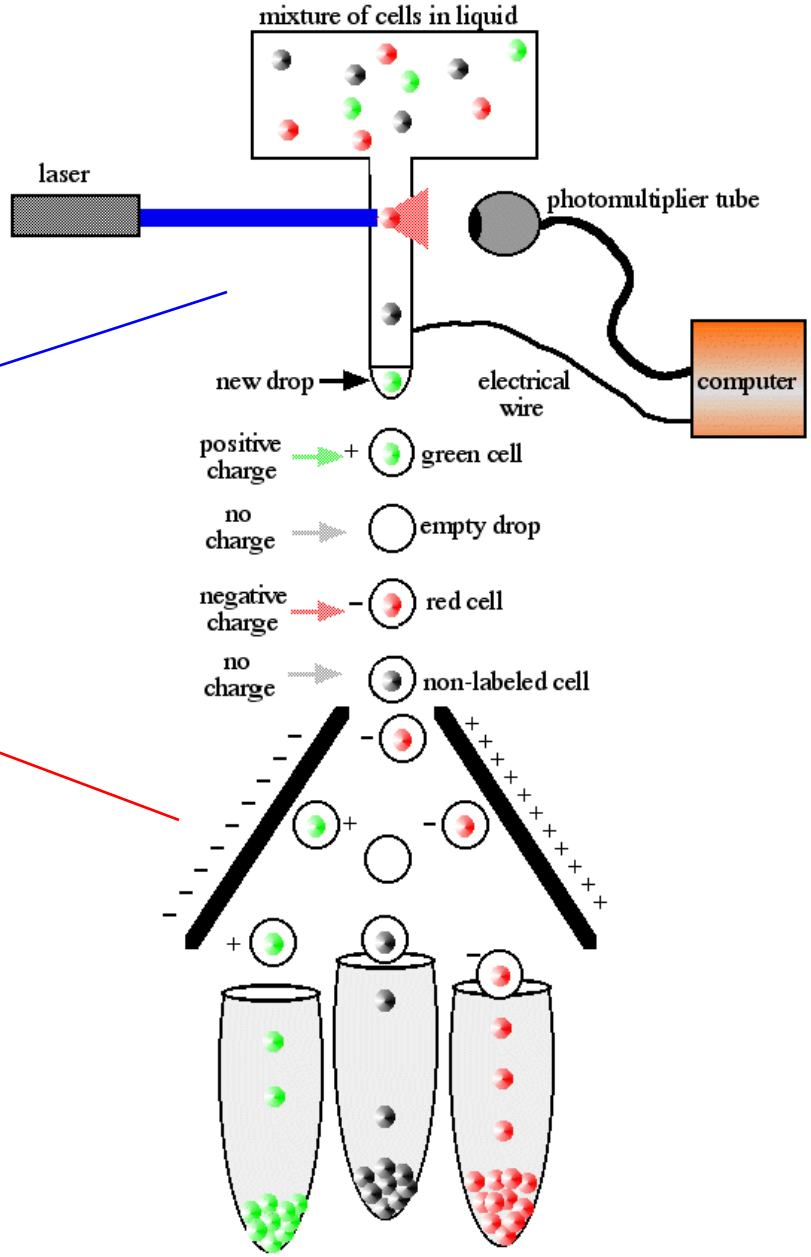
Electronic delay until cell reaches break off point. Then the stream is charged : + or -.

Charged droplets deflected by electrostatic field
from plates held at high voltage (+/-3000 volts).

Besides tubes can sort onto slides or multi-well plates.

FACS sorting





Applications of Flow Cytometry

- Cell size.
- Cytoplasmic granularity.
- Cell surface antigens (phenotyping).
- Apoptosis.
- Intracellular cytokine production.
- Intracellular signalling.
- Gene reporter (GFP).
- Cell cycle, DNA content, composition, synthesis.
- Bound and free calcium.
- Cell proliferation (BRDU and CFSE)
- Cell sorting

Shapiro's Seventh Law of Flow Cytometry:

"No data analysis technique can make good data out of bad data"

Practical Flow Cytometry (4th Eds; Wiley-Liss)