Basic Principles in Flow Cytometry

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Flow Cytometry

- Flow Cytometry is the technical process that allows for the individual measurements of cell fluorescence and light scattering. This process is performed at rates of thousands of cells per second.
- This information can be used to individually sort or separate subpopulations of cells.

History

- Flow cytometry developed from microscopy. Thus Leeuwenhoek is often cited in any discussion regarding it's history.
- F.T. Gucker (1947)build the first apparatus for detecting bacteria in a **LAMINAR SHEATH** stream of air.
- L. Kamentsky (IBM Labs), and M. Fulwyler (Los Alamos Nat. Lab.) experimented with fluidic switching and electrostatic cell sorters respectively. Both described cell sorters in 1965.
- M. Fulwyler utilized Pulse Height Analyzers to accumulate distributions from a Coulter counter. This feature allowed him to apply statistical analysis to samples analyzed by flow.

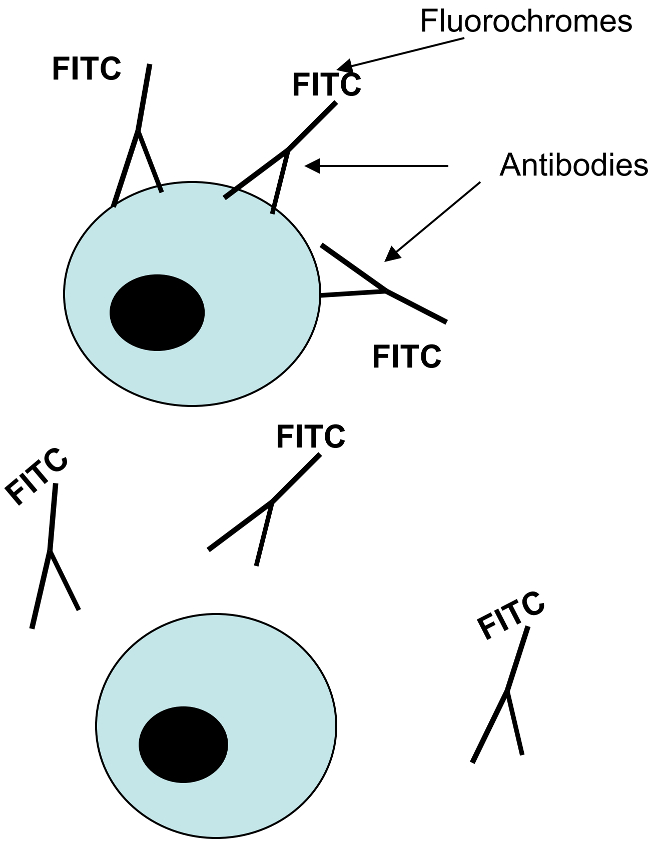
History

- In 1972 L. Herzenberg (Stanford Univ.), developed a cell sorter that separated cells stained with fluorescent antibodies.The Herzenberg group coined the term Fluorescence Activated Cell Sorter (FACS).

Fluorescence Activation Process (or Immunofluorescence)

Antibodies recognize specific molecules in the surface of some cells

Antibodies are artificially conjugated to **fluorochromes**



But not others

When the cells are analyzed by flow cytometry the cells expressing the marker for which the antibody is specific will manifest fluorescence. Cells who lack the marker will not manifest fluorescence

Cellular Parameters Measured by Flow

Intrinsic

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

Extrinsic

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

Flow Cytometry Applications

- Immunofluorescence
- Cell Cycle Kinetics
- Cell Kinetics
- Genetics
- Molecular Biology
- Animal Husbandry (and Human as well)
- Microbiology
- Biological Oceanography
- Parasitology
- Bioterrorism

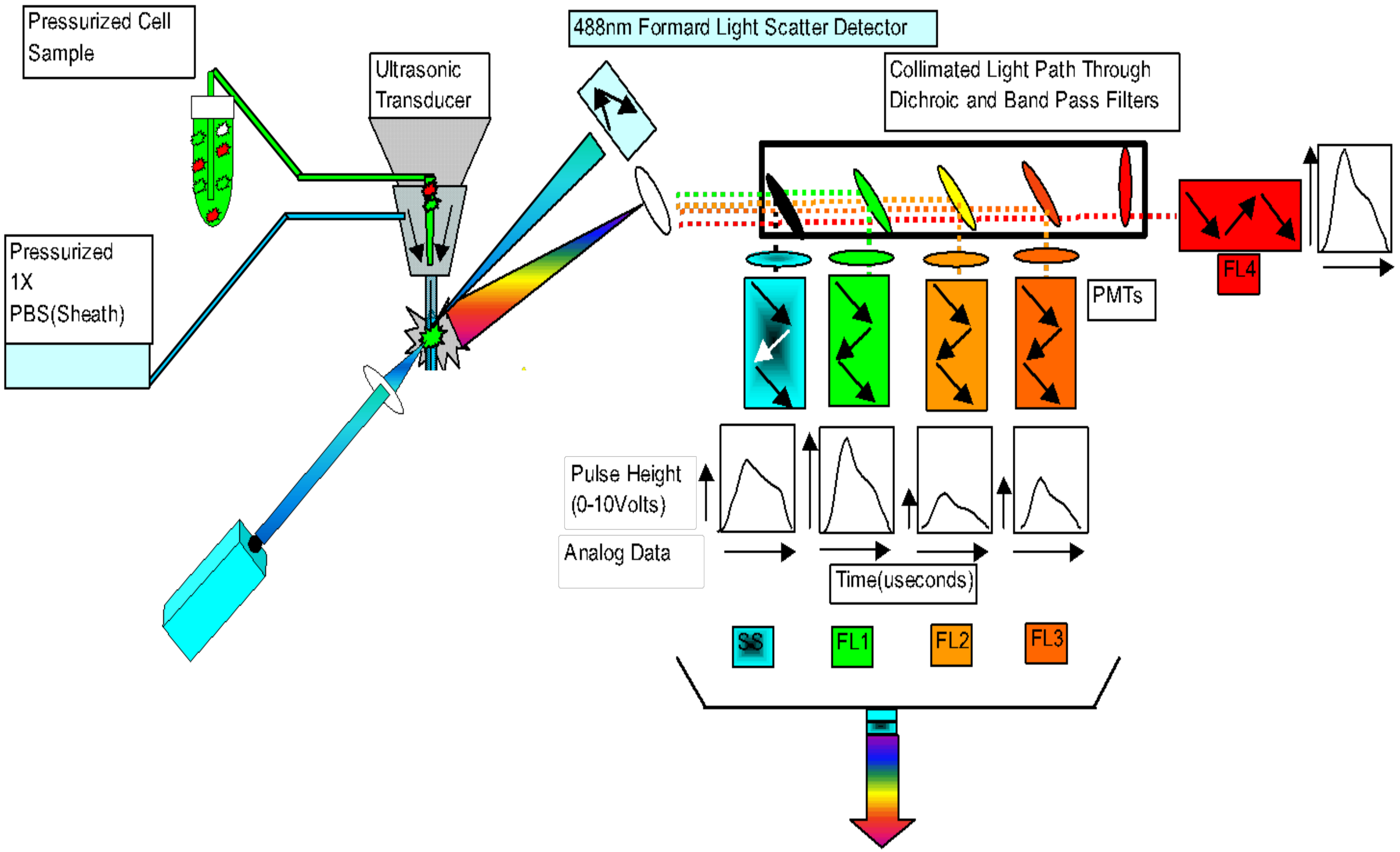
- Flow cytometry integrates electronics, fluidics, and optics.

Electronics are involved in signal processing, computer display and analysis.

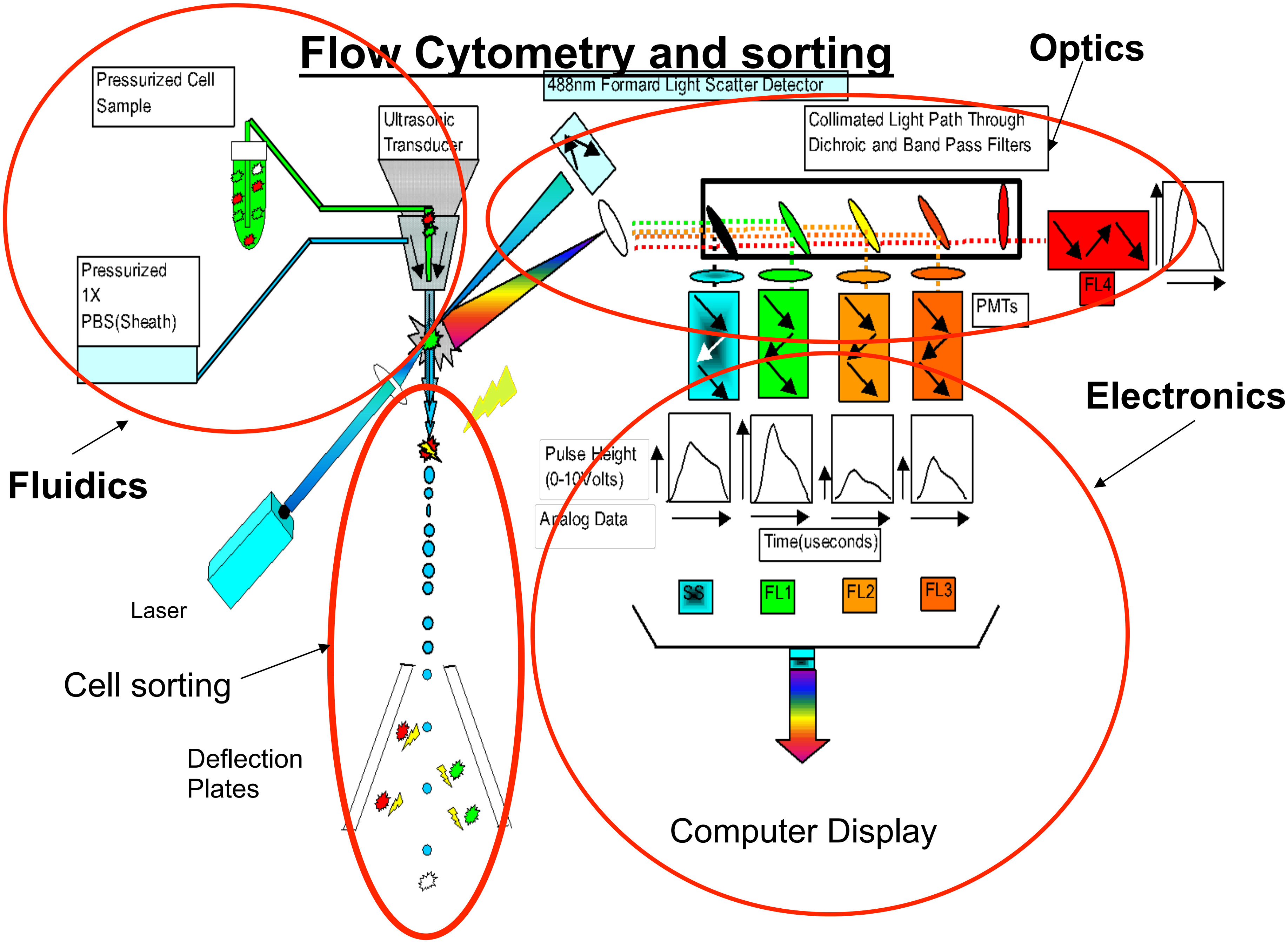
Fluidics are applied to sample processing and cell sorting.

Optics component are involved in fluorescence detection.

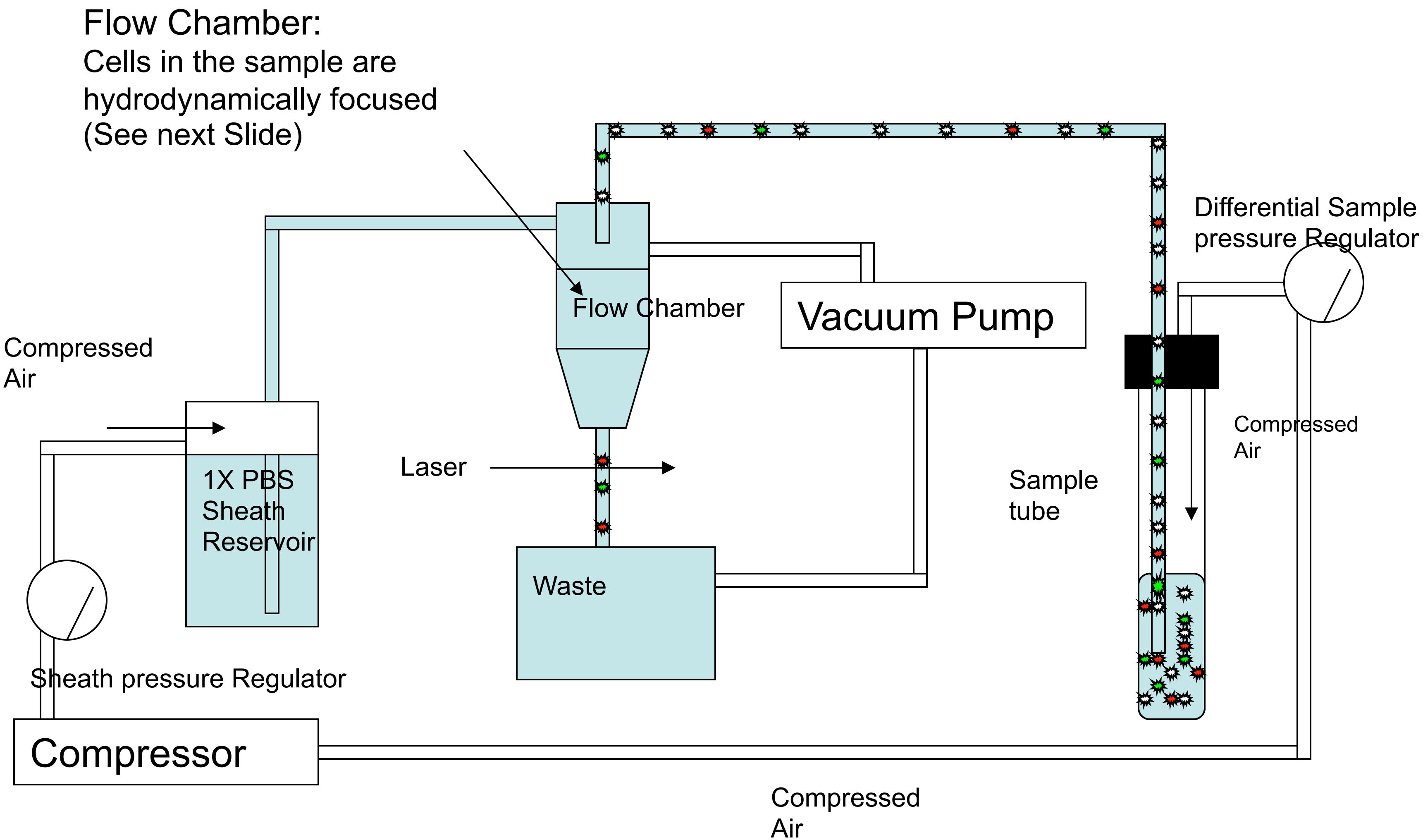
Flow Cytometry

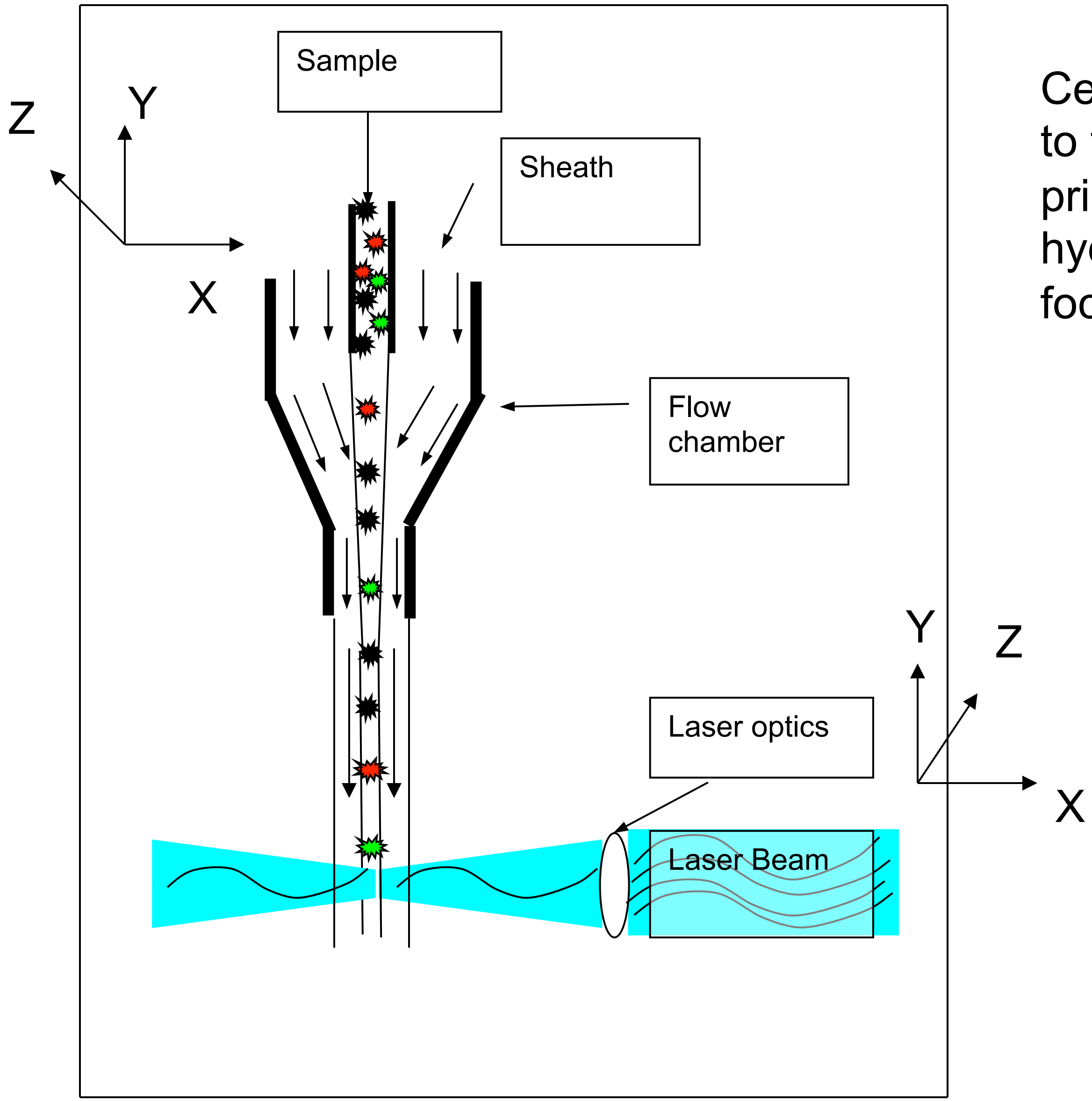


Flow Cytometry and sorting



Fluidics

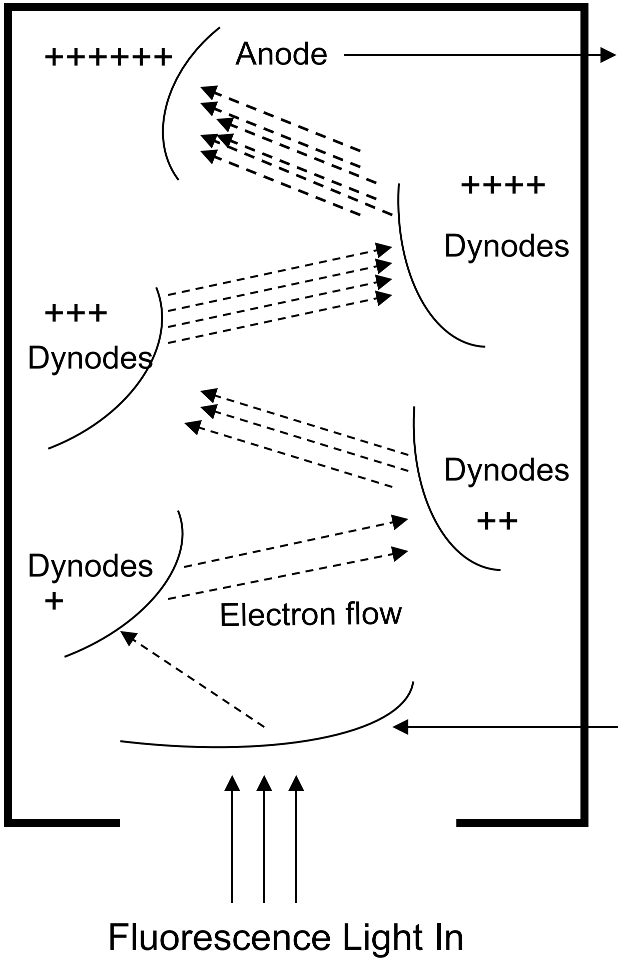

Flow Chamber:
Cells in the sample are
hydrodynamically focused
(See next Slide)
Differential Sample
pressure Regulator
Flow Chamber
Vacuum Pump
Compressed
Air
Laser
1X PBS
Sheath
Reservoir
Compressed
Air
Sample
tube
Waste
Sheath pressure Regulator
Compressor
Compressed
Air



Cells are presented to the laser using principles of hydrodynamic focusing

Electronics

Photomultiplier Tube (PMT) → PMTs convert light into electrical signals



Voltage output or signal

The higher the voltage applied to each PMT the more electrons are generated. Thus, the greater the amplification of the light coming in (gain).

***Increasing the voltage does increase sensitivity. Instrument sensitivity is determined by the optics*

Electrons flow from dynode to dynode. Each dynode generates a secondary emission of more electrons.

Each dynode has a potential voltage more positive than the preceding dynode

Photocathode
Light sensitive. When photons hit it, it generates electrons (photoelectrons).

Derived from Practical Flow Cytometry, 4th edition By Howard Shapiro

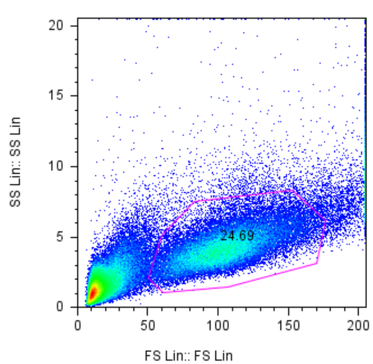
Electronics

Signal from PMTs → Pre-amplifiers → Signal Peak Detectors / Pulse Integrators / Pulse Width Circuitry → Analog to Digital Converters (ADCs)

- Signal Peak Detectors
- Pulse Integrators
- Pulse Width Circuitry

Analog to Digital Converters (ADCs)

Each Pulse (or event), for each parameter, is given a numerical value. This value is then plotted. These numerical values are proportional to the light scattering and/or fluorescence intensity of the event.



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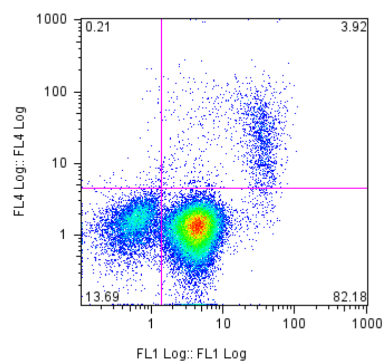
Ungated



Count: 39801
FS Lin, SS Lin subset

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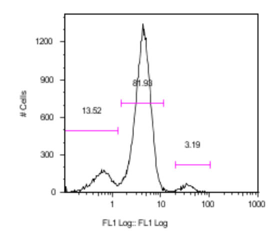
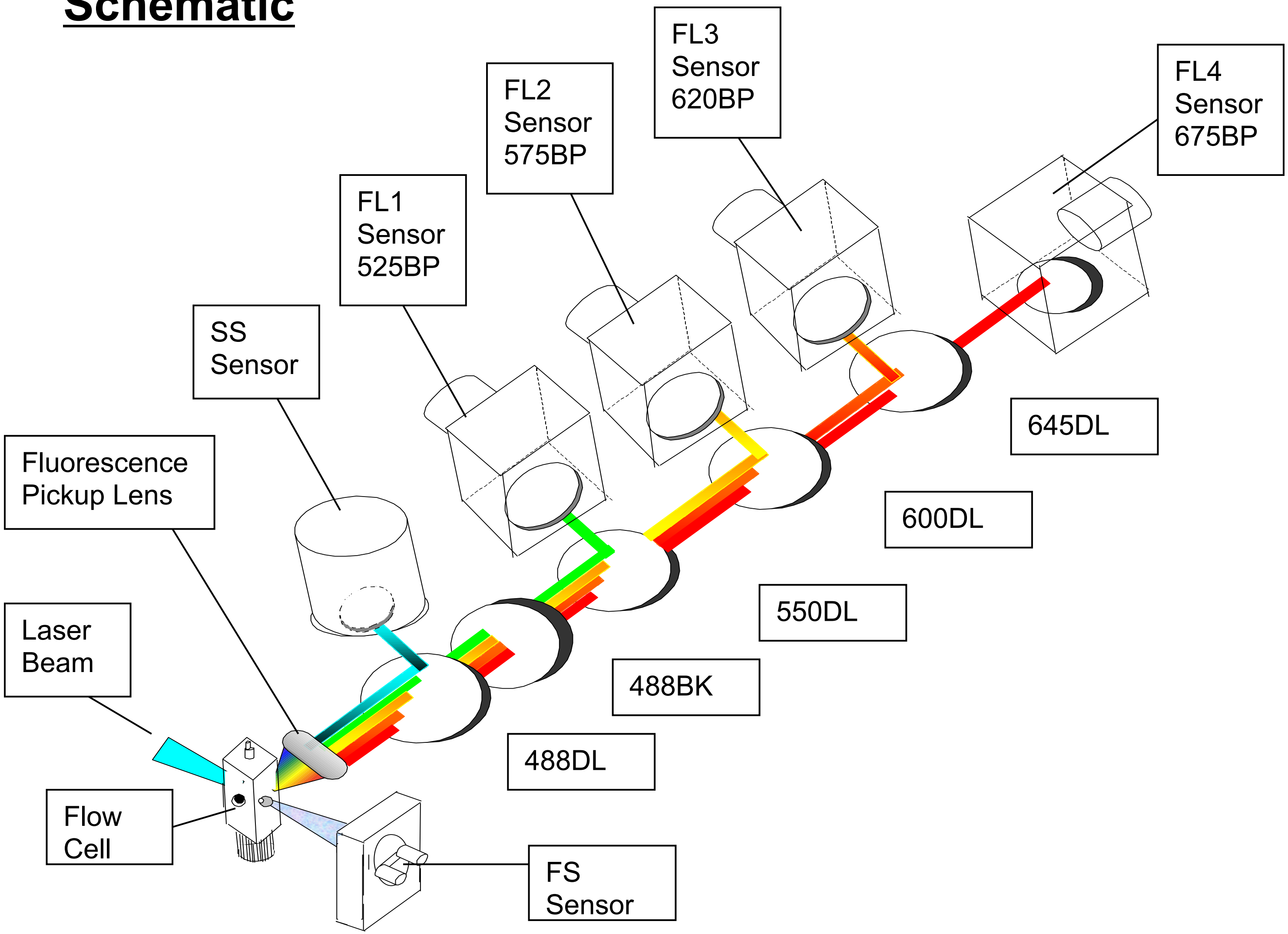
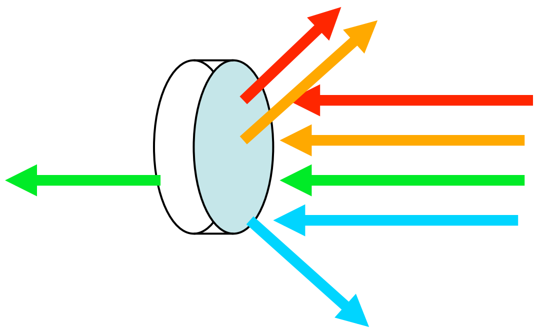


Optical Bench Schematic

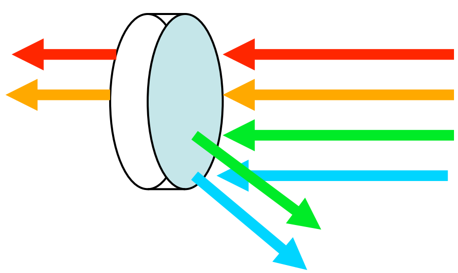


Optical Filters



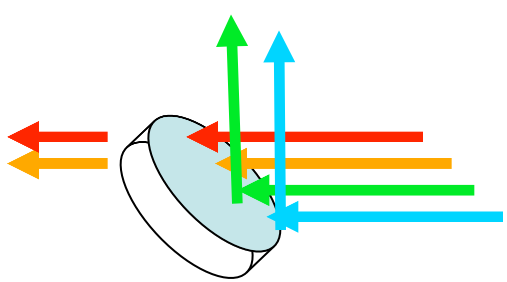
Band Pass Optical Filter

525/30nm Band Pass



Long Pass Optical Filter

550nm Long Pass



Dichroic Long Pass Optical Filter

Positioned at a 45 degree angle.

530 Dichroic Long Pass

Light Absorption

Quantum mechanics requires that molecules absorb energy as quanta (photons)

Absorption of a photon raises the energy of the molecule from a ground state to an excited state

As molecules relax to a lower energy state, light is released

From: J.Paul Robinson, Purdue University

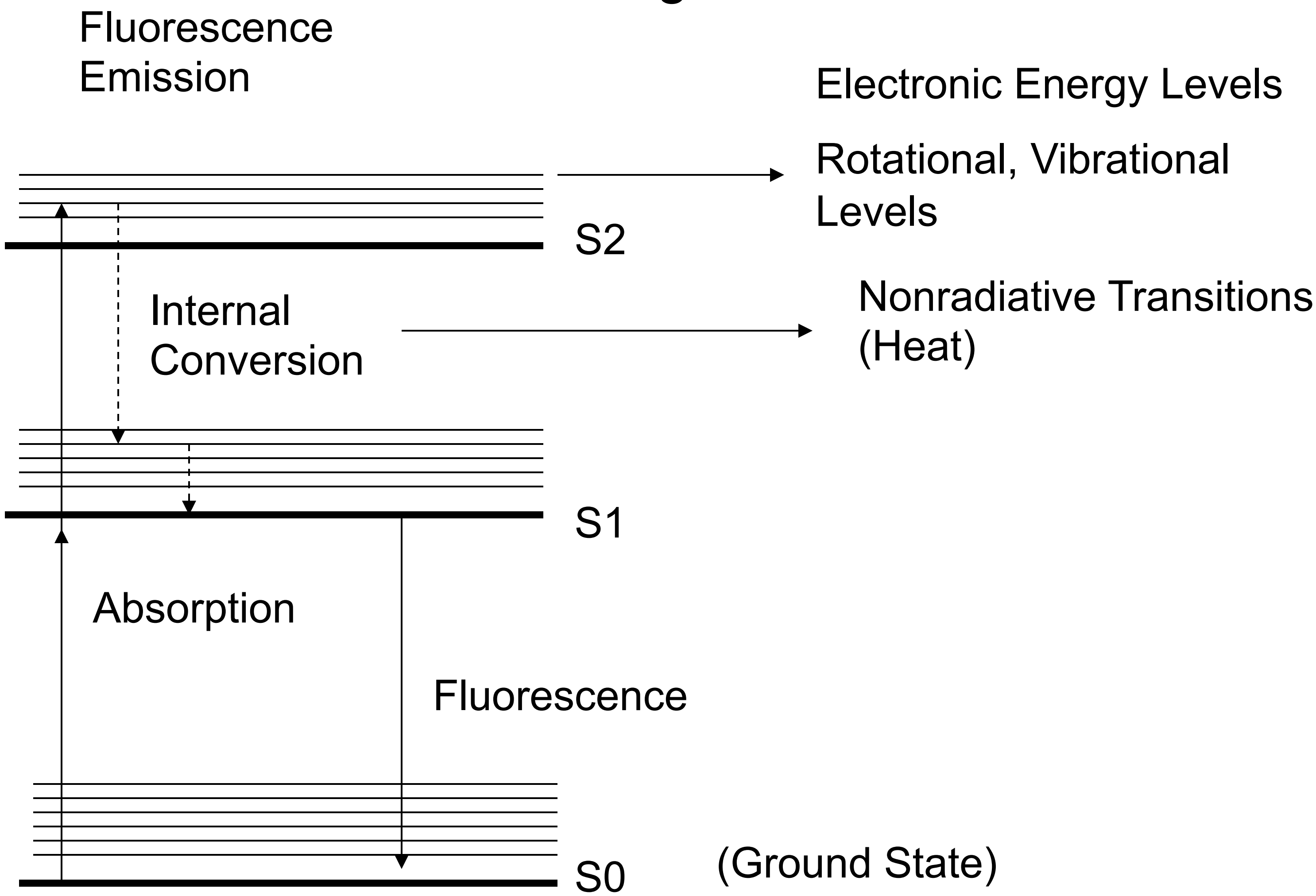
Fluorescence

Chromophores are the components of molecules which absorb light, they are generally aromatic rings.

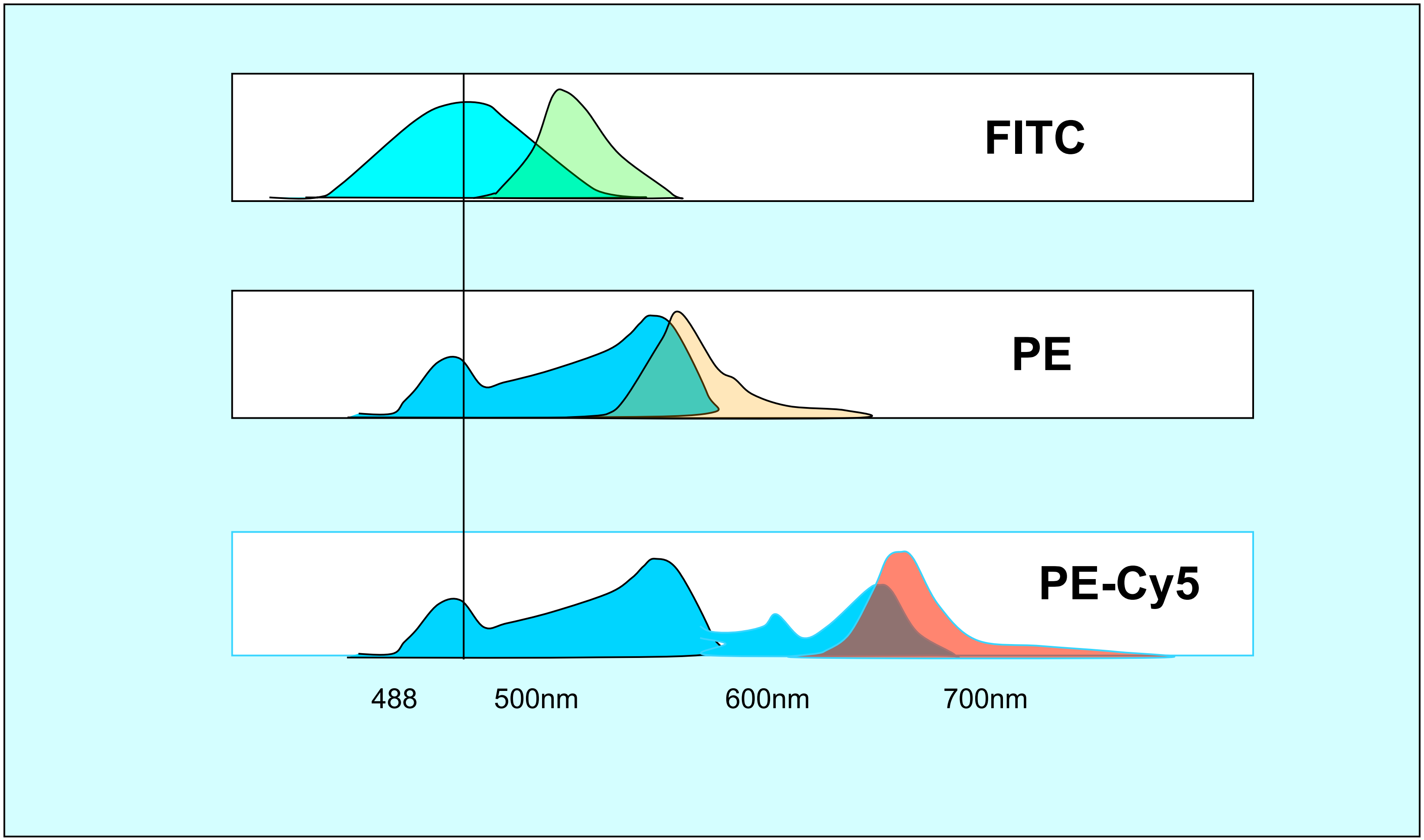
Fluorescence lifetimes can be measured in femptoseconds

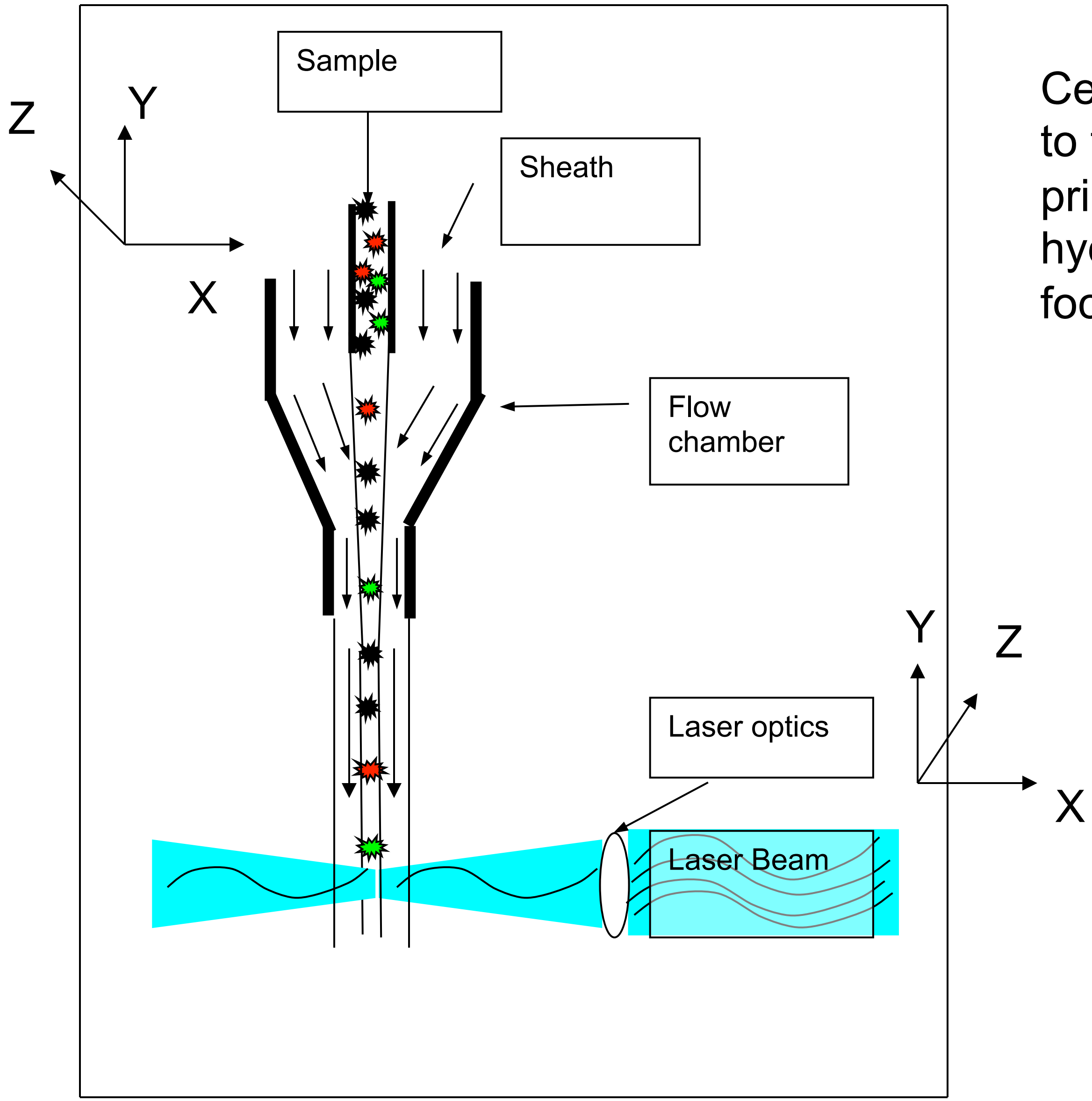
Quantum Yield measures the efficiency between photons absorbed and photons emitted

Jablonski diagram



Excitation and Emission Spectra





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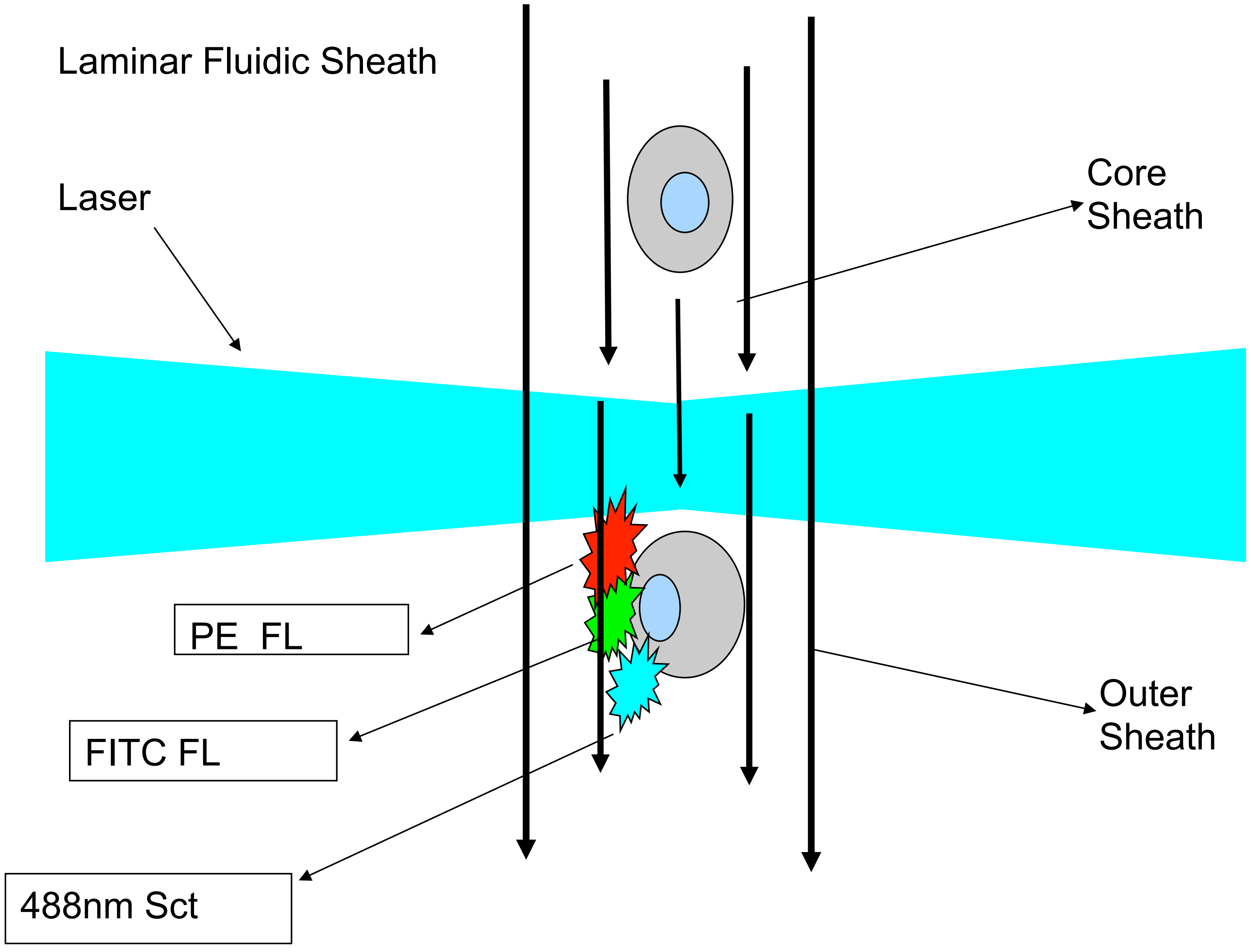
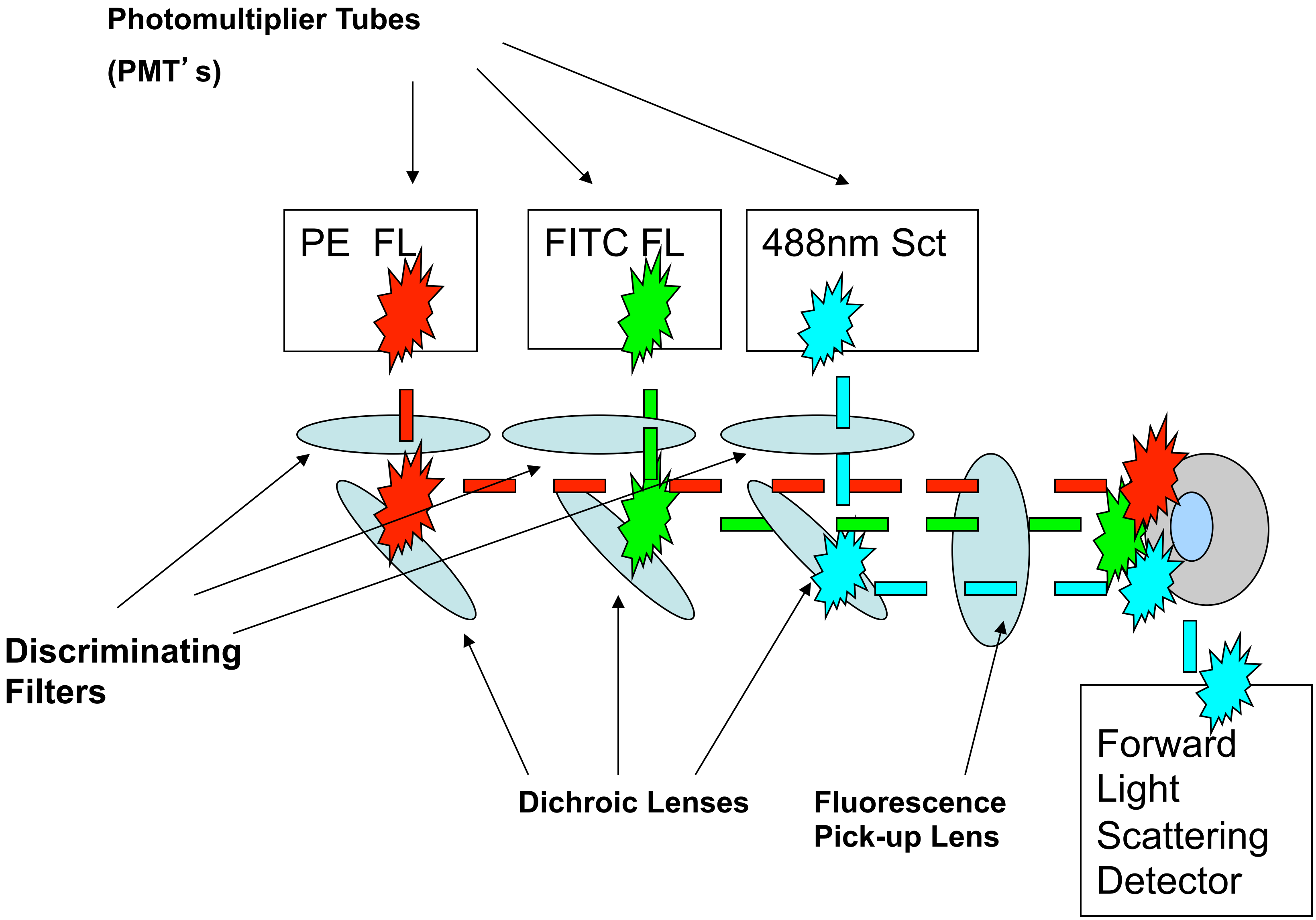
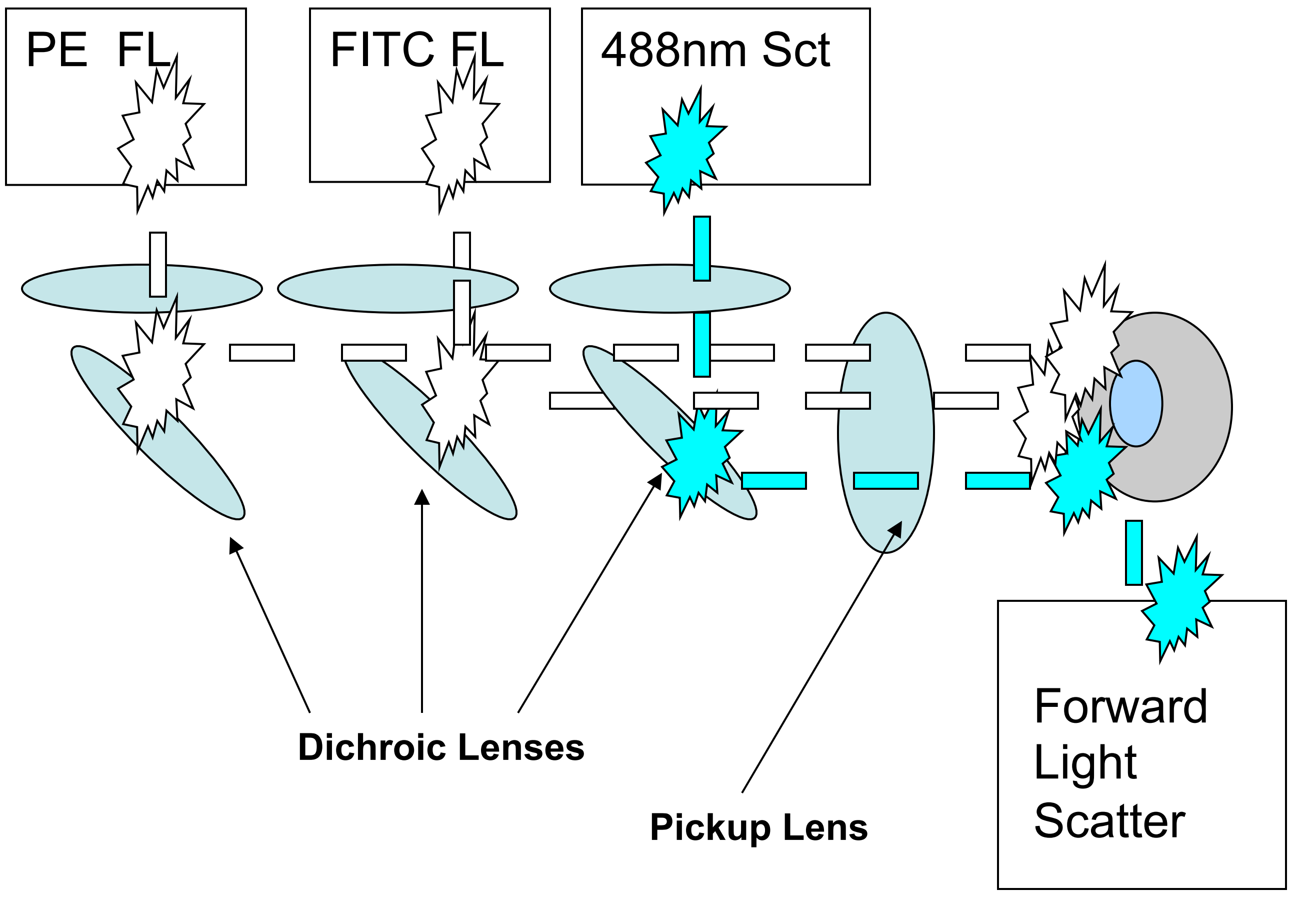


Laminar Fluidic Sheath
Laser
Core
Sheath
PE FL
FITC FL
488nm Sct
Outer
Sheath

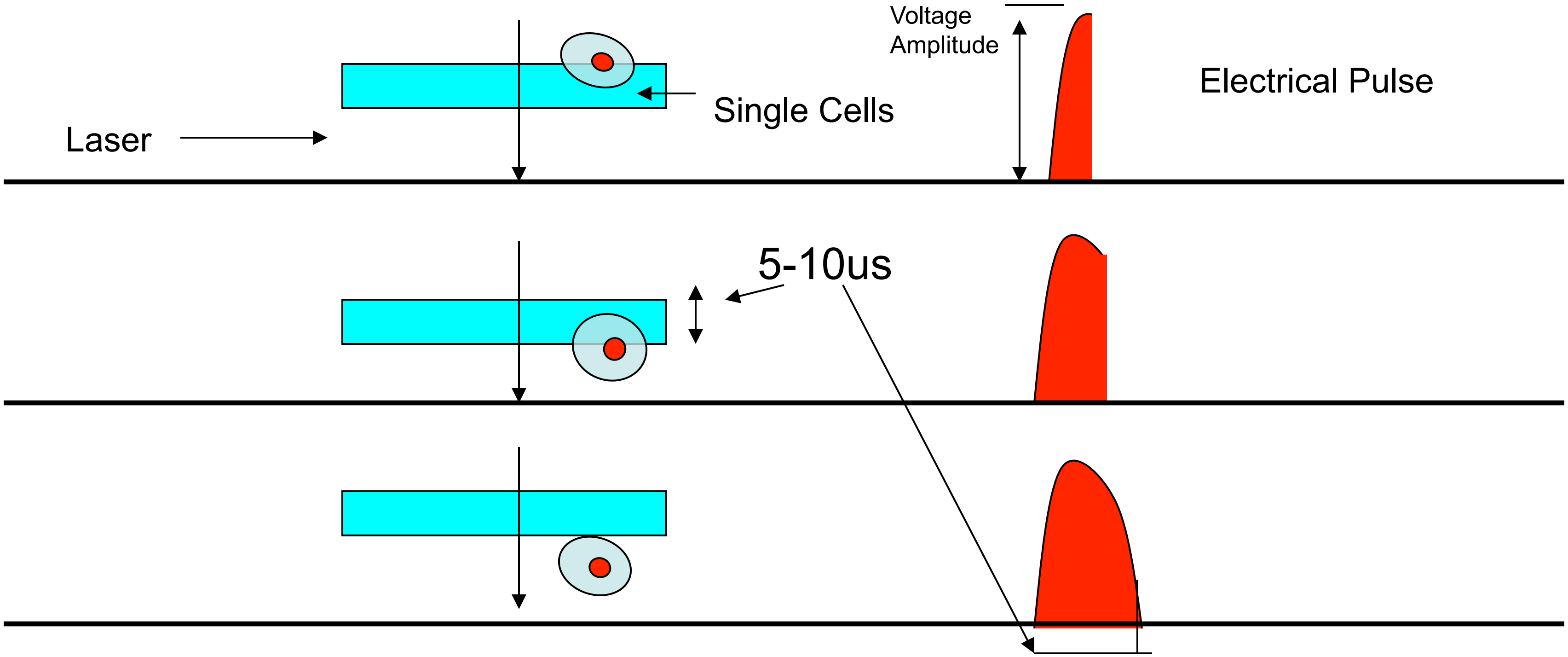
- Each cell generates a quanta of fluorescence



Negative cells are also detected



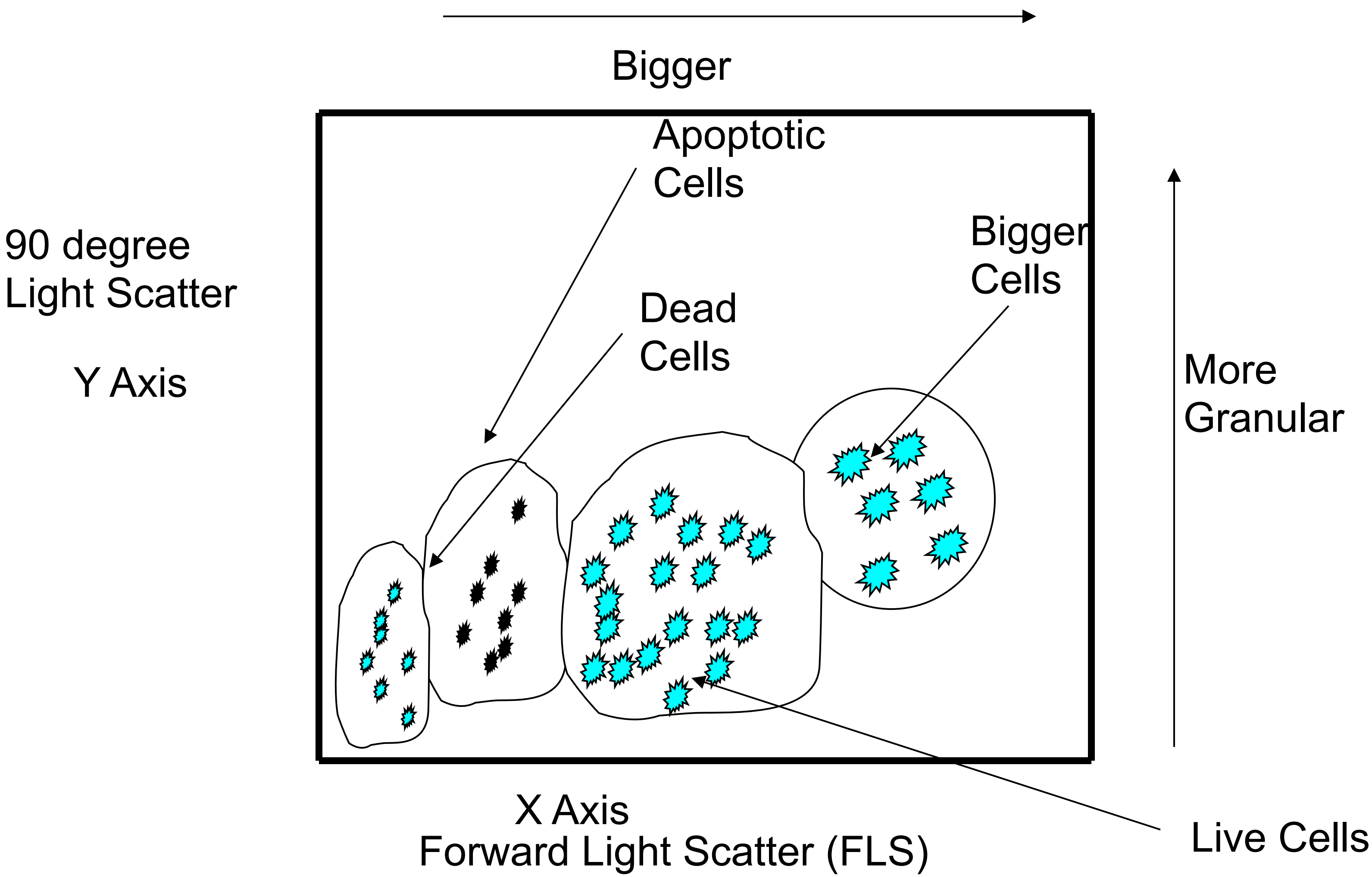
How Signals are Generated



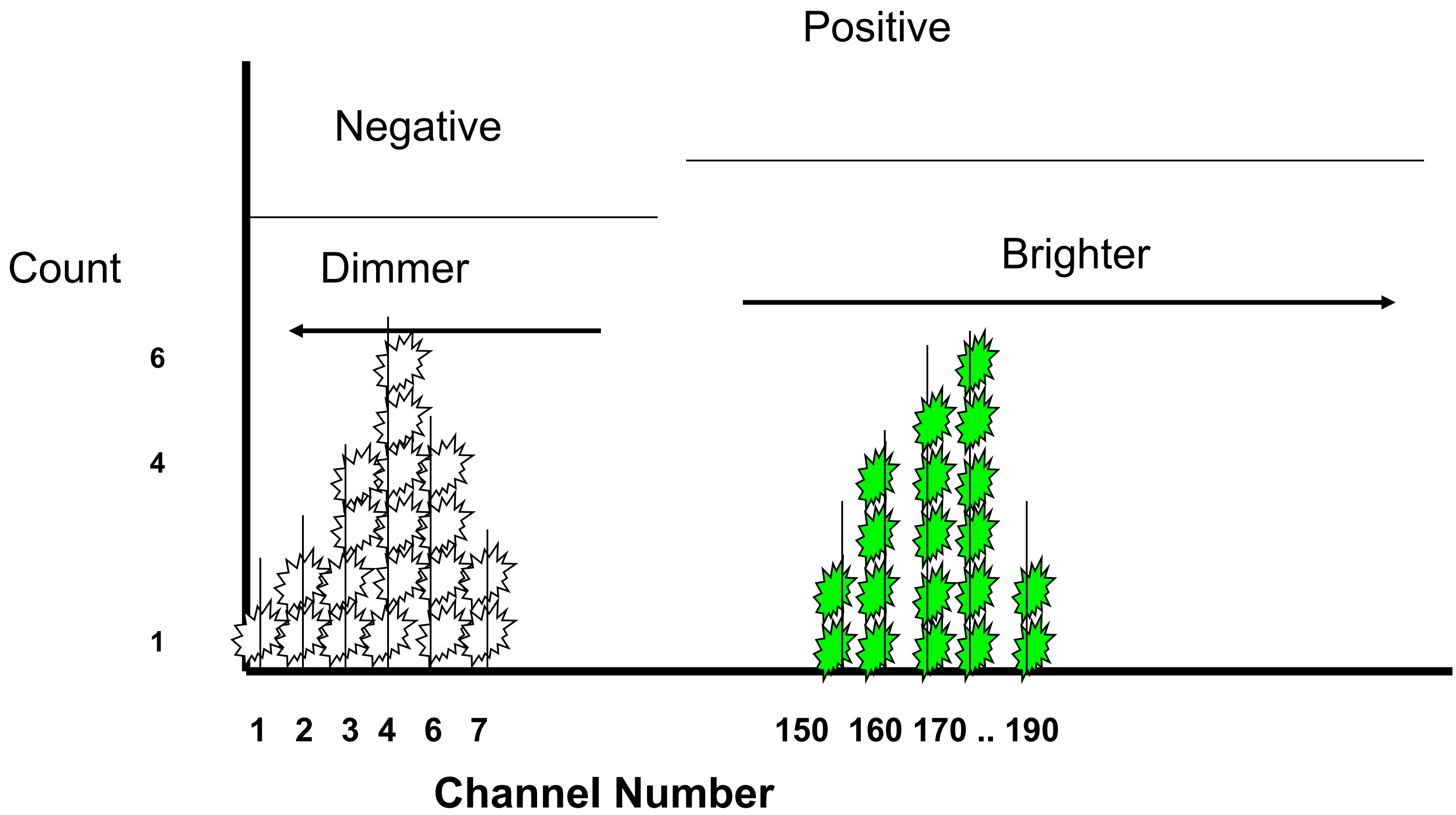
From Fluorescence to Computer Display

- Individual cell fluorescence quanta is picked up by the various detectors(PMT’s).
- PMT’s convert light into electrical pulses.
- These electrical signals are amplified and digitized using Analog to Digital Converters (ADC’s).
- Each event is designated a channel number (based on the fluorescence intensity as originally detected by the PMT’s) on a 1 Parameter Histogram or 2 Parameter Histogram.
- All events are individually correlated for all the parameters collected.

Light Scattering, 2 Parameter Histogram

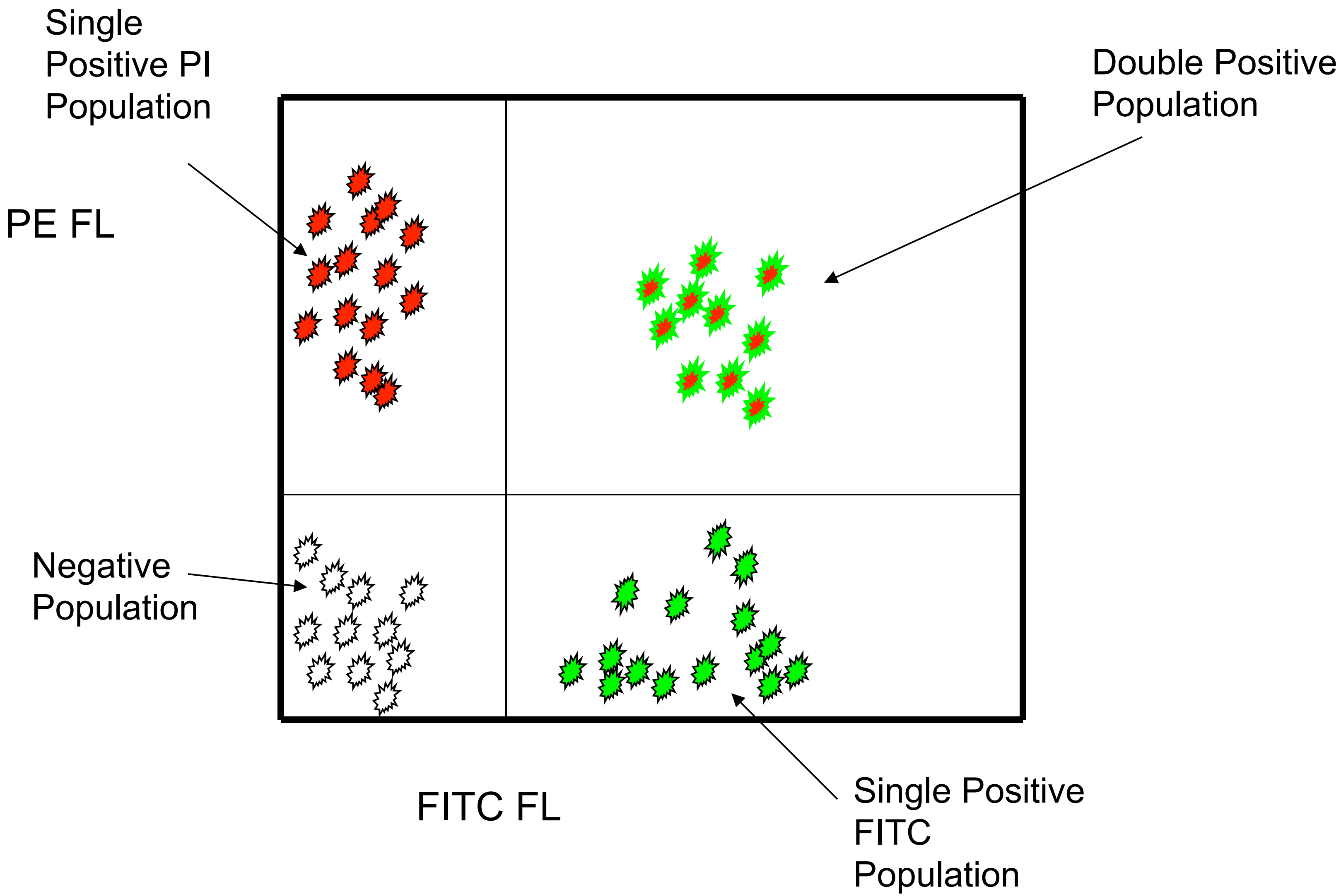


1 Parameter Histogram



Fluorescence picked up from the FITC PMT

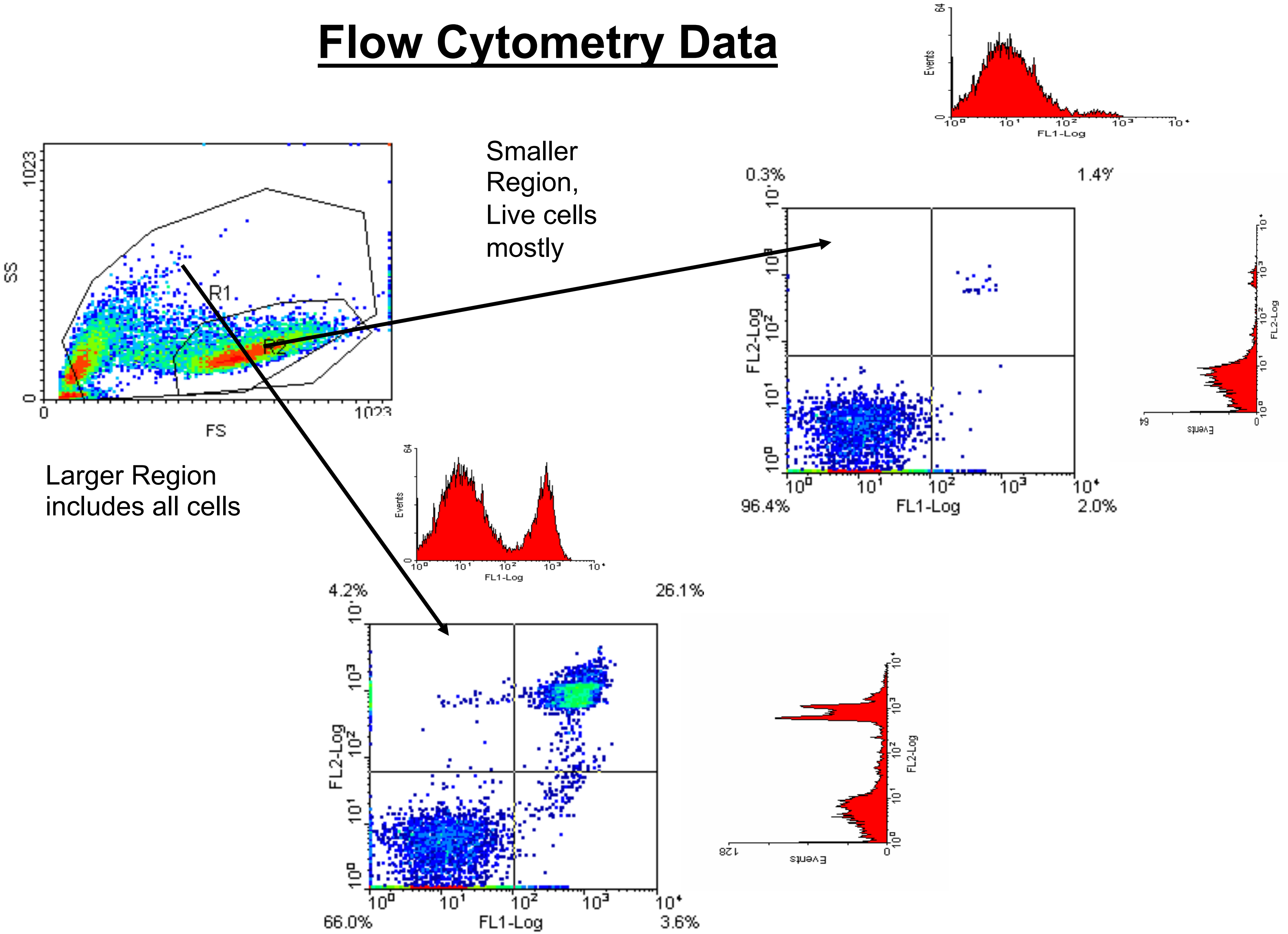
2 Parameter Histogram



Gating and Statistics

- Data generated in flow cytometry is displayed using **Multiparamater Acquisition and Display** software platforms.
- Histograms corresponding to each of the parameters of interest can be analyzed using statistical tools to calculate percentage of cells manifesting specific fluorescence, and fluorescence intensity.
- This information can be used to look at fluorescence expression within subpopulations of cells in a sample (gating).

Flow Cytometry Data



Running Samples

- Prepare samples.
- One sample should be completely negative. This sample should be analyzed first. Adjust the Forward Light Scatter and Side Scatter **amplification**.This sample is also used for adjusting the Fluorescence PMTs **amplification** voltage.
- Adjust the PMT Voltage until you can see a population peak in the first decade of your 1 parameter histogram and or your two parameter plot.
- Once the instrument settings are optimized, run samples and collect data.
- If you are analyzing 2 or more fluorescence parameters you have to prepare Single Color samples for each of your fluorochromes.

Sorting

Lord Rayleigh, liquid stream emerging from an orifice becomes unstable, and breaks up into droplets (1800’s)

If a vibration is applied to a stream (emerging from an orifice) the droplet formation becomes stable

R. Sweet develops the drop charging and deflection technique for ink-jet printing (1965)

In cell sorters, an electromagnetic tunable transducer is incorporated in the flow chamber. This causes the fluid stream to break-off into individual droplets

The stream behaves like a wavelength, drops are spaced one wavelength apart

Sorting

The resulting droplet pattern can be described using the wavelength equation :

$$v = f\lambda$$

v is the velocity of the stream

f is the vibration frequency

λ is the wavelength or droplet spacing

The droplet pattern is most stable when the break-off point is closest to the orifice

This is achieved when the wavelength is 4.5 stream diameters

Substituting λ for 4.5 stream diameter

$$v = f(4.5d)$$

From Howard Shapiro, Practical Flow Cytometry, fourth edition

Sorting

Putting it together: $v=f(4.5d)$

For a 75uM orifice and a stream velocity of 20m/s:

$f=(20m/s)/(75 \times 4.5)$

$f=(2.0e7)/(337.5)$

f=59,259cycles /s or hertz

The ideal frequency for a 75um flow chamber nozzle is 59,259Hz

From Howard Shapiro, Practical Flow Cytometry, fourth edition

Sorting

Droplet charging circuitry

When a cell meets a pre-determined criteria to be sorted, a voltage is applied to the stream

The applied charge will travel down the stream to the last attached drop

The droplet charge is delayed to coincide with the arrival of the cell to the precise position in the stream of the last attached drop

Charged droplets are physically deflected as they pass through a set of two deflecting plates with opposite polarities.

From Howard Shapiro, Practical Flow Cytometry, fourth edition

Droplet Sorting