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Flow Cytometry Facility – UNIL

Master EPFL

27th of October 2023

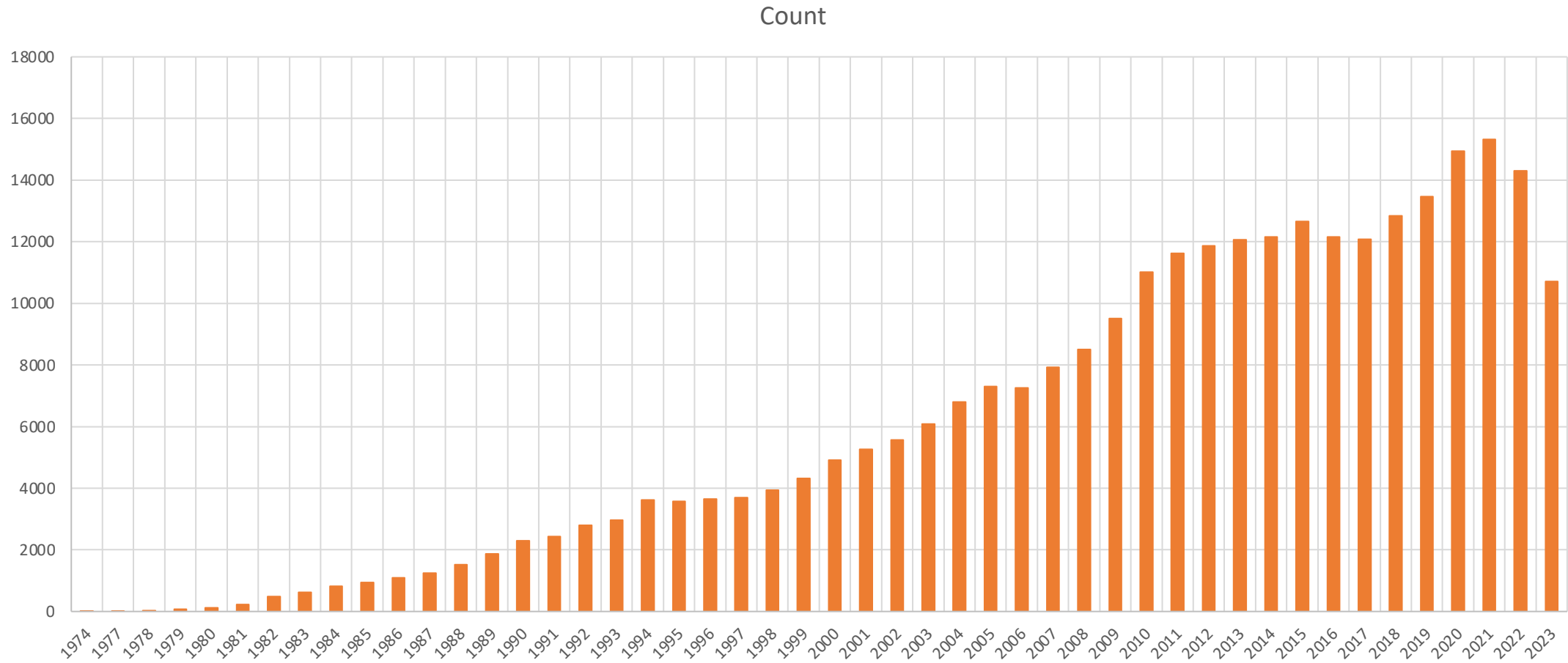
Principles of Flow Cytometry

- an invaluable tool for
research and in the clinic

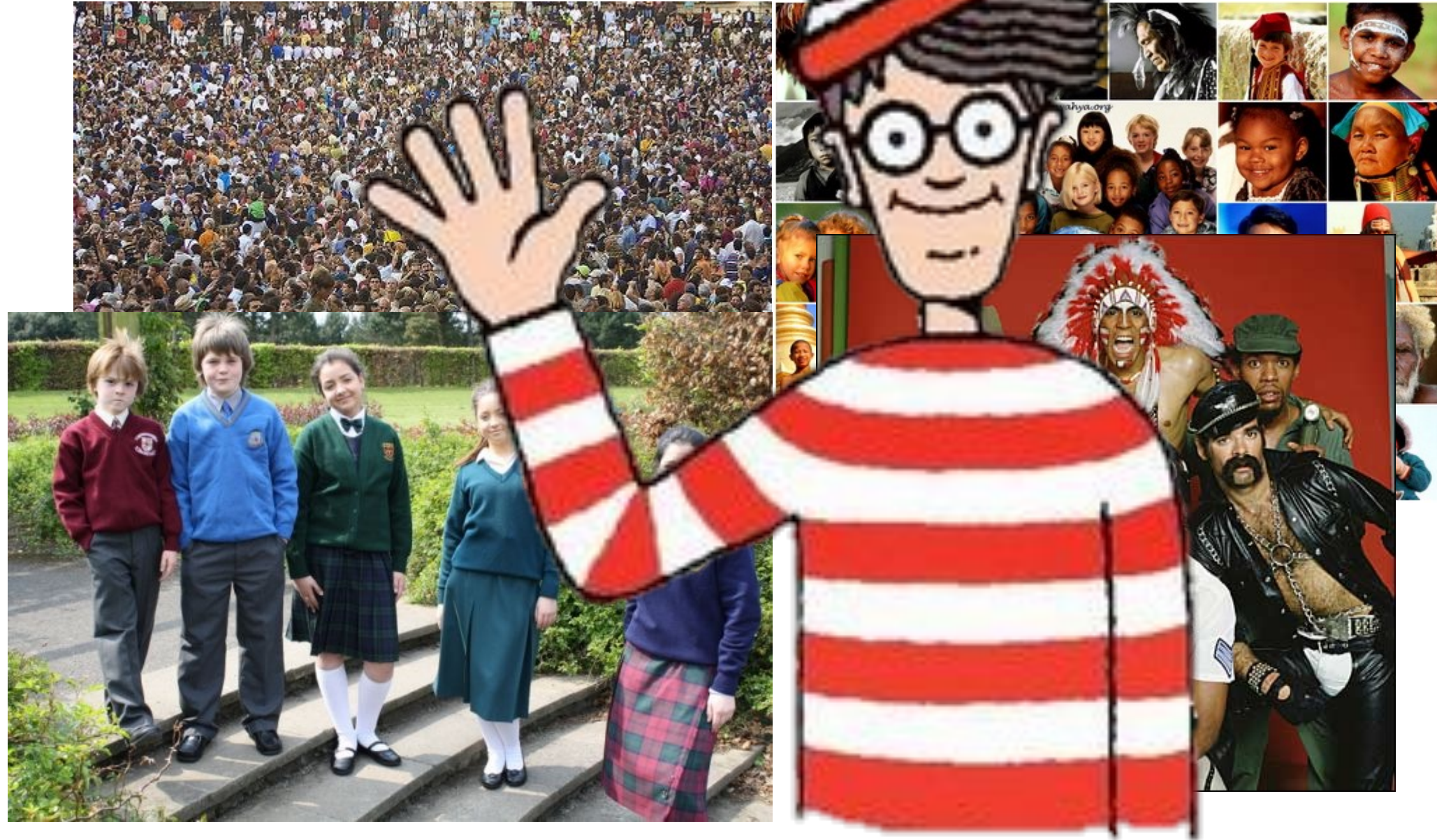


What is flow cytometry ?

Over 10,000 publications per year use 'Flow Cytometry' as a keyword!



What is flow cytometry ?



What is flow cytometry ?

Flow cytometry

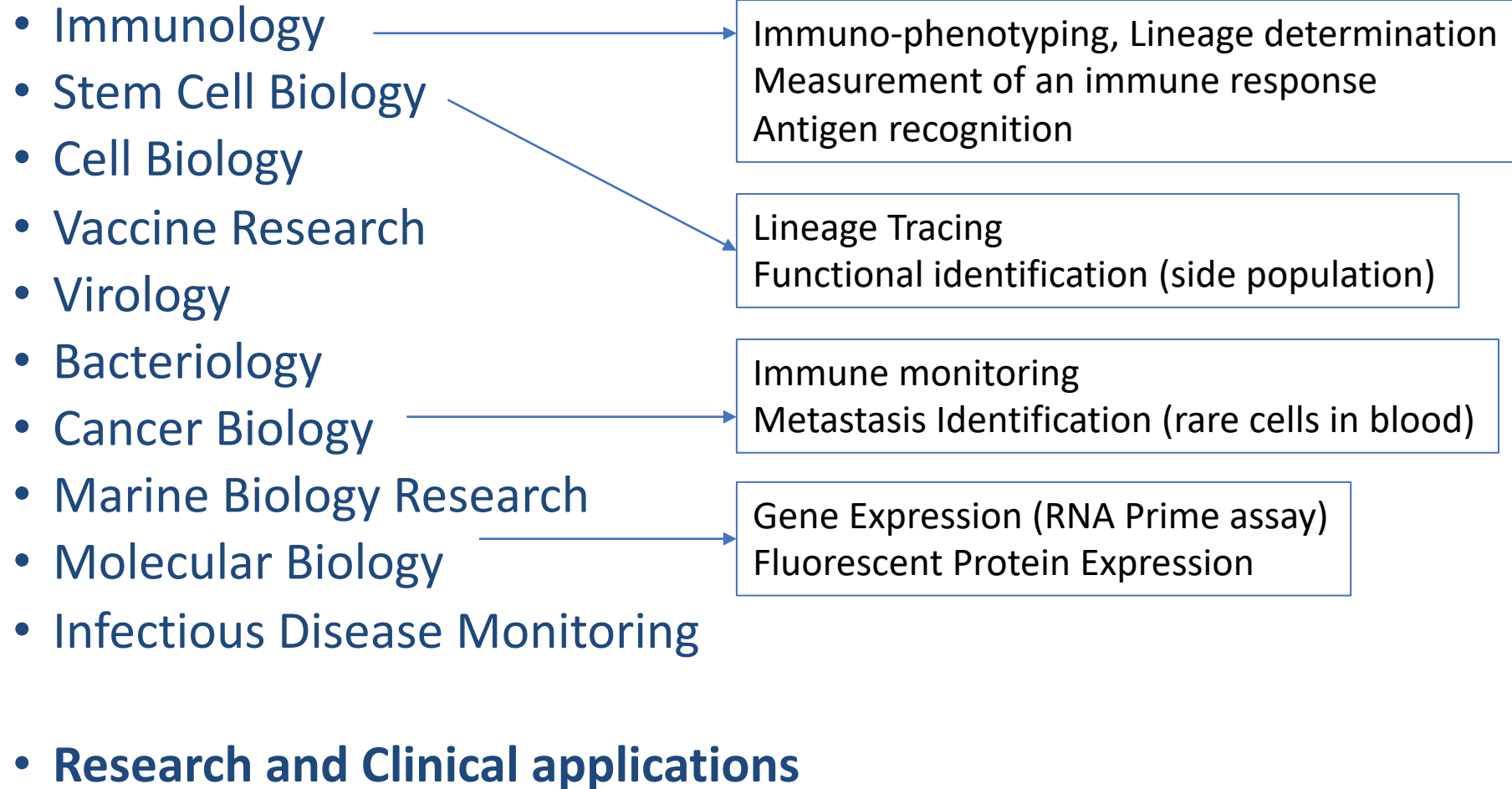
In motion
with fluidics

Prefix
denoting a cell

To Measure

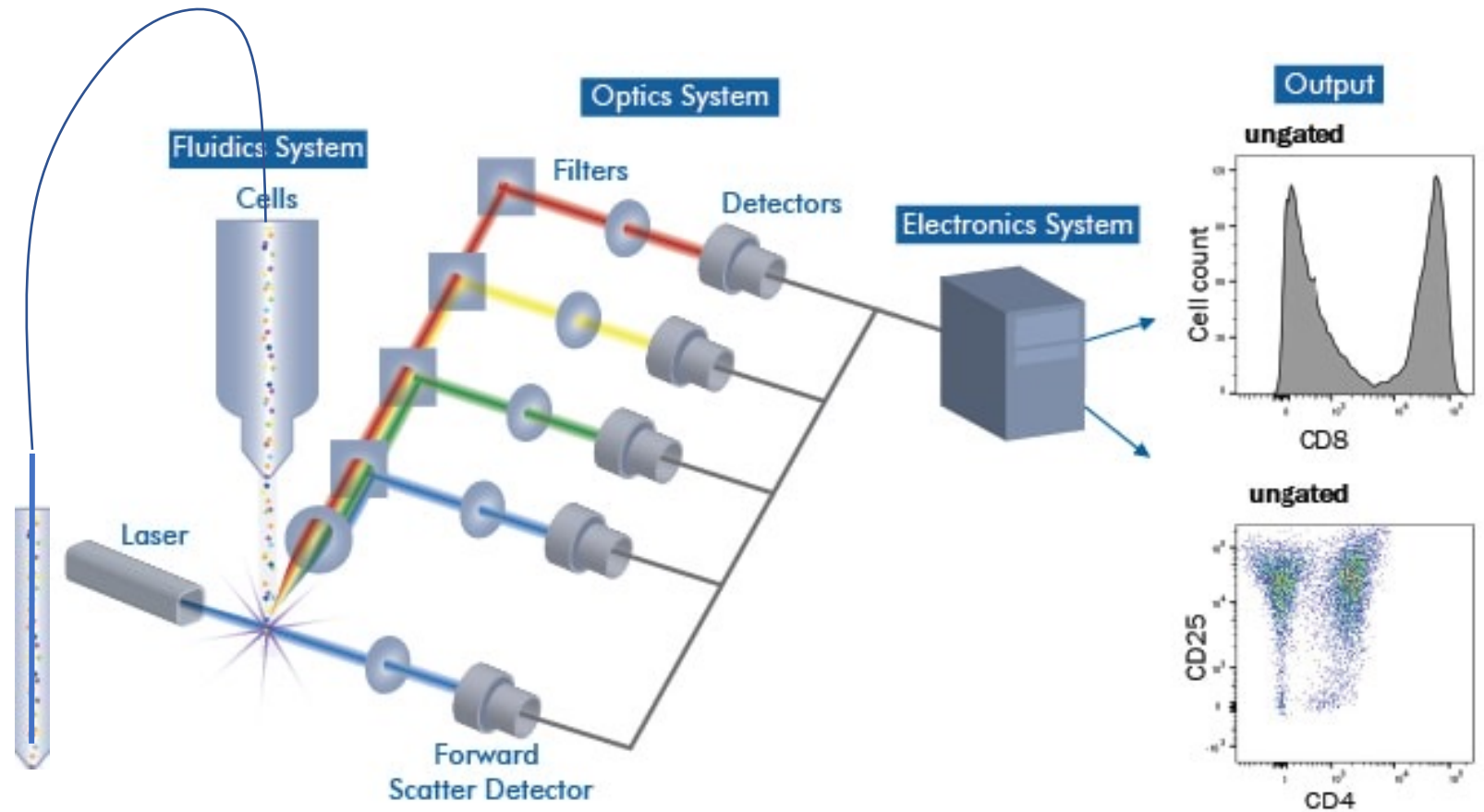
- A technology to measure multiple characteristics of single cell suspensions or particles (eg nuclei, yeast, parasites, bacteria, beads)
- First commercial flow cytometer was developed in 1974 in the Herzenberg lab in Stanford – Fluorescent Activated Cell Sorter (FACS) – 1 laser, 2 colours
- State of the art flow cytometers have up to 7 lasers, can process 100,000 events/s and collect up to 50 parameters
- Newest developments are mass spectrometry cytometry (CYTOF), Imaging Flow cytometry (IFC), Spectral Flow Cytometry (SFC)

Powerful tool with applications in:



From photons to dot plots

- Cells in a tube are passed by an illumination point
- Photons are counted by detectors and generate a current
- The current pulse is measured and digitized
- Data is displayed and analyzed



What can be analysed with flow cytometry ?

Intrinsic characteristics

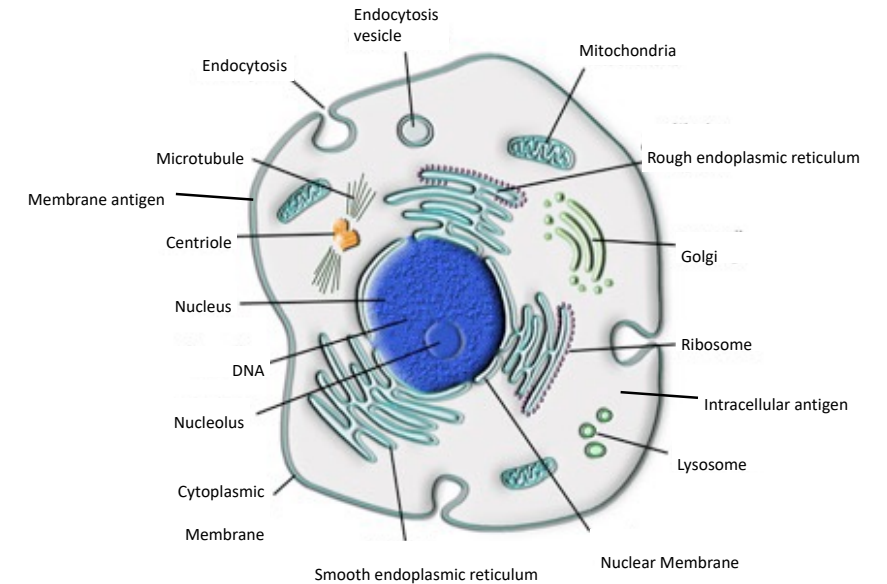
Size

Shape

Granularity

Pigments (chlorophylls, carotenoids, etc.)

Autofluorescence



Extrinsic characteristics

Internal and External Receptors probes and labelled antibodies (CD-3 FITC, CD4-PE,...)

Membrane Structure, cytoskeleton, ... probes (JC1, eosin-5-maleimide , ...)

Physiological Parameters Enzyme activity, Drug kinetics, pH, Ca^{+2} ,... (Indo 1, SNARF-1,...)

Transfection/infection efficiency Fluorescent proteins (eGFP, mCherry, dsRed, ...)

DNA Content, composition and synthesis: intercalating dyes (PI, DRAQ5, Hoechst 33342,...)

Apoptosis, Necrosis Several targets: nuclei (PI, YoPro,...), Caspases, PS (annexinV)

Time

Cell/particle concentration

What is inside the box ?



3 major elements working closely together

Fluidics

- Transport of cells to the laser interrogation point
- Hydrodynamic focusing
- Evacuation of waste

Optics

- Excitation with lasers
- Spectral separation of emitted fluorescence

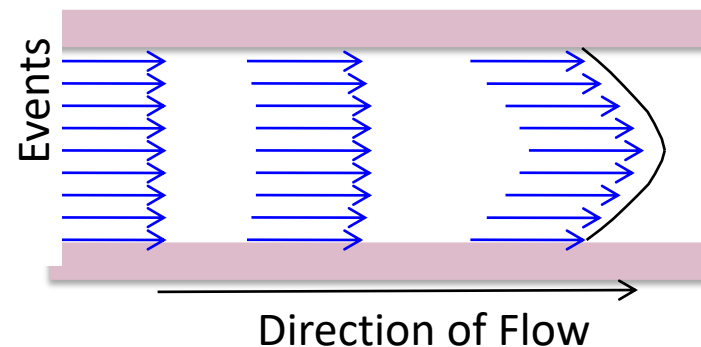
Electronics

- Conversion of photons to electrons
- Digitalization of electronic signals for computer analysis

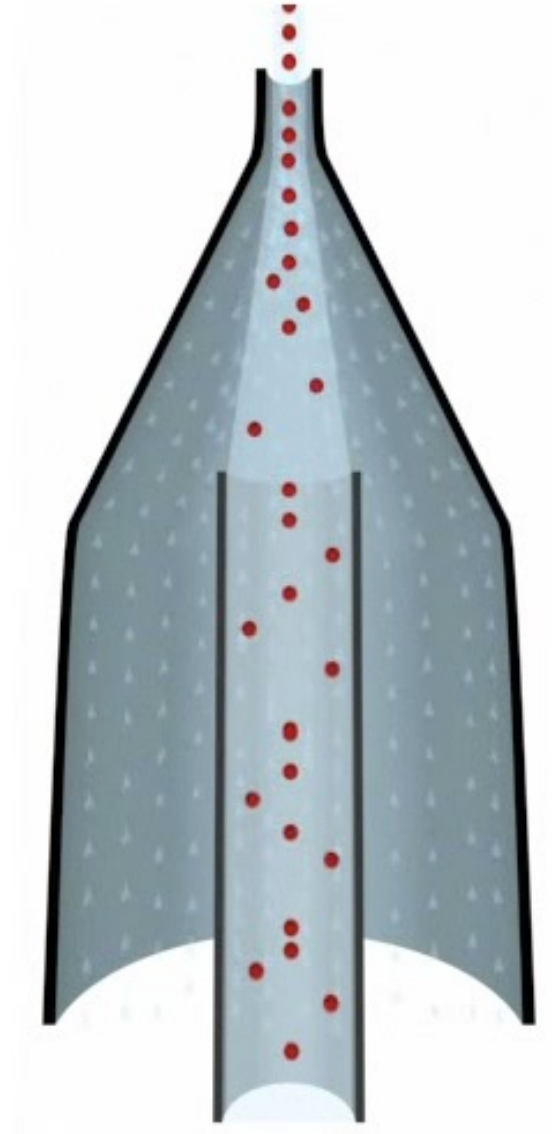
Light detecting device capable of detecting photons of different wavelengths over a wide dynamic range!

The Fluidic system

- **Moving cells from tube through interrogation point and to waste**
- **Requires Laminar Flow**
 - Uniform fluid flow in parallel layers
 - Outer 'sheath' fluid
- **Cells are Hydrodynamically focused**
 - Sample contained to core stream (center of sheath)
 - Spread out in direction of flow

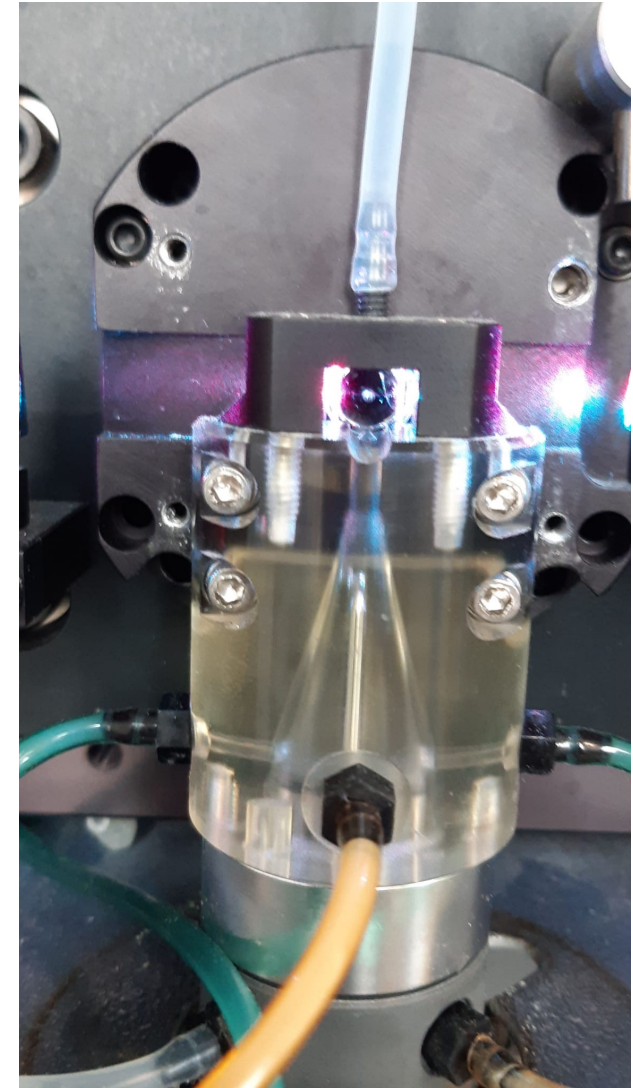
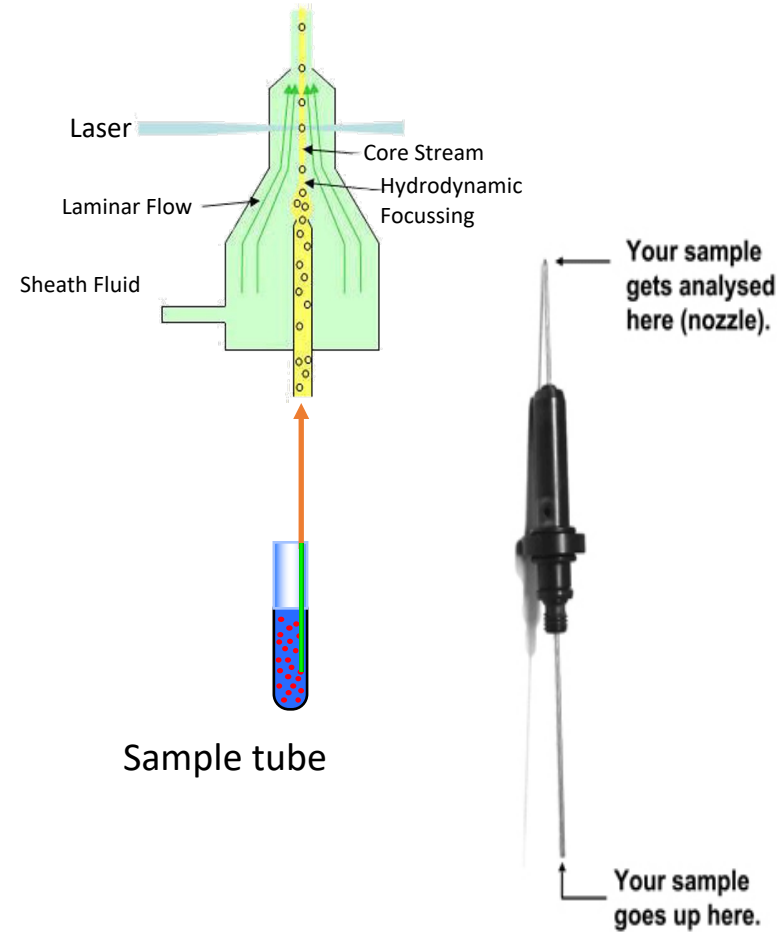


The hydrodynamic focusing



The Flow Cell

- The place where the sample is introduced to the sheath fluid
- Sample is hydrodynamically focused so the cells spread out to look like 'pearls on a string'
- Sheath and sample do not mix



Fluidic - Differential Pressure

Positive Pressure Systems

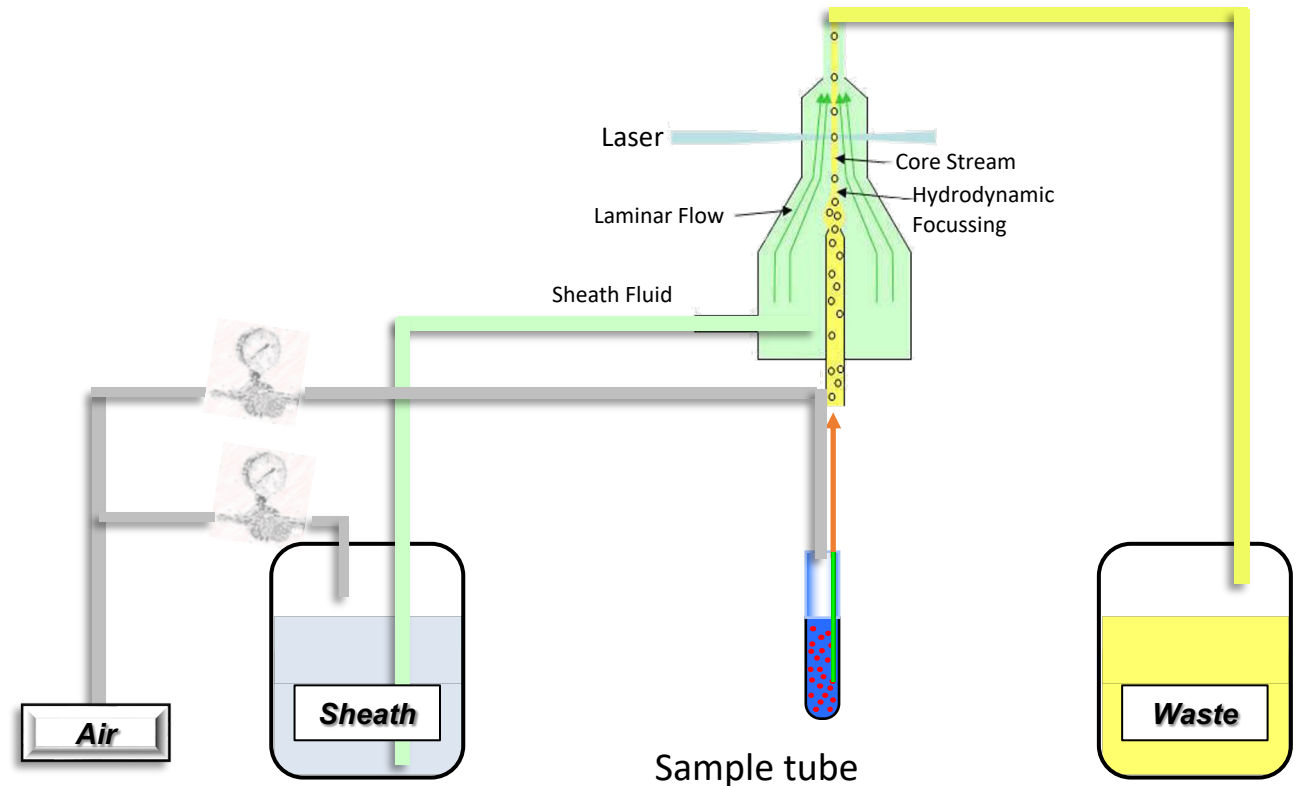
- Based on differential pressure between sample and sheath
- Require balanced positive
- Sample is pushed into injection tubing

Extremely important to use the right tube. Wrong tubes,

- Sample will not be acquired
- You can damage the instrument (!!!)

Check no cracks on the tubes

Resuspend your sample



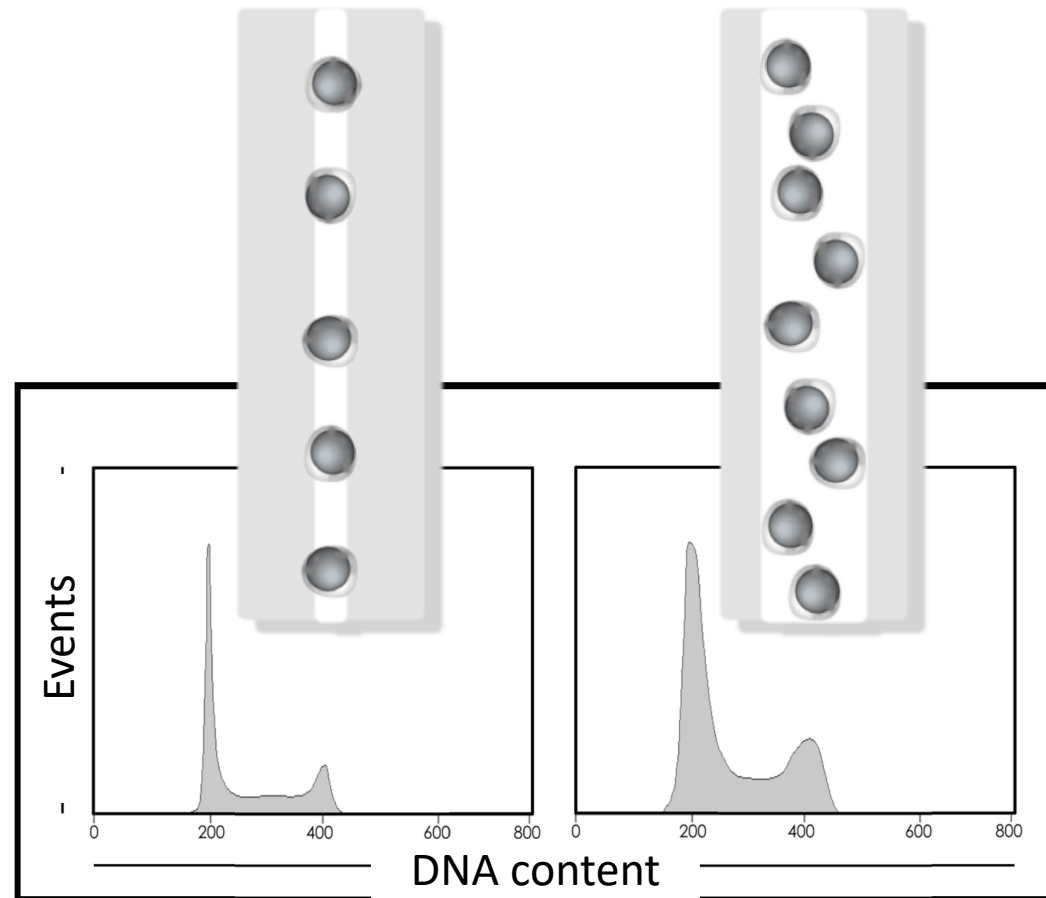
Fluidic – Differential Pressure

- Sheath flow rate sets flow rate
- Differential pressure sets core stream size

Low differential pressure

- Tight core stream
- Cells pass single file
- Lower variability

Low differential pressure High differential pressure



High differential pressure

- Wide core stream
- Faster sample rate
- More coincident events
- Increased abort rate

Light and optics

Scatter vs. Fluorescence

- Two types of light are measured as particles pass through the illumination source
- **Laser light scatter:** refraction of illuminating beam by the particle
 - Forward scatter
 - Side scatter
- **Fluorescence:** illumination source excites the fluorochromes used for the cell staining

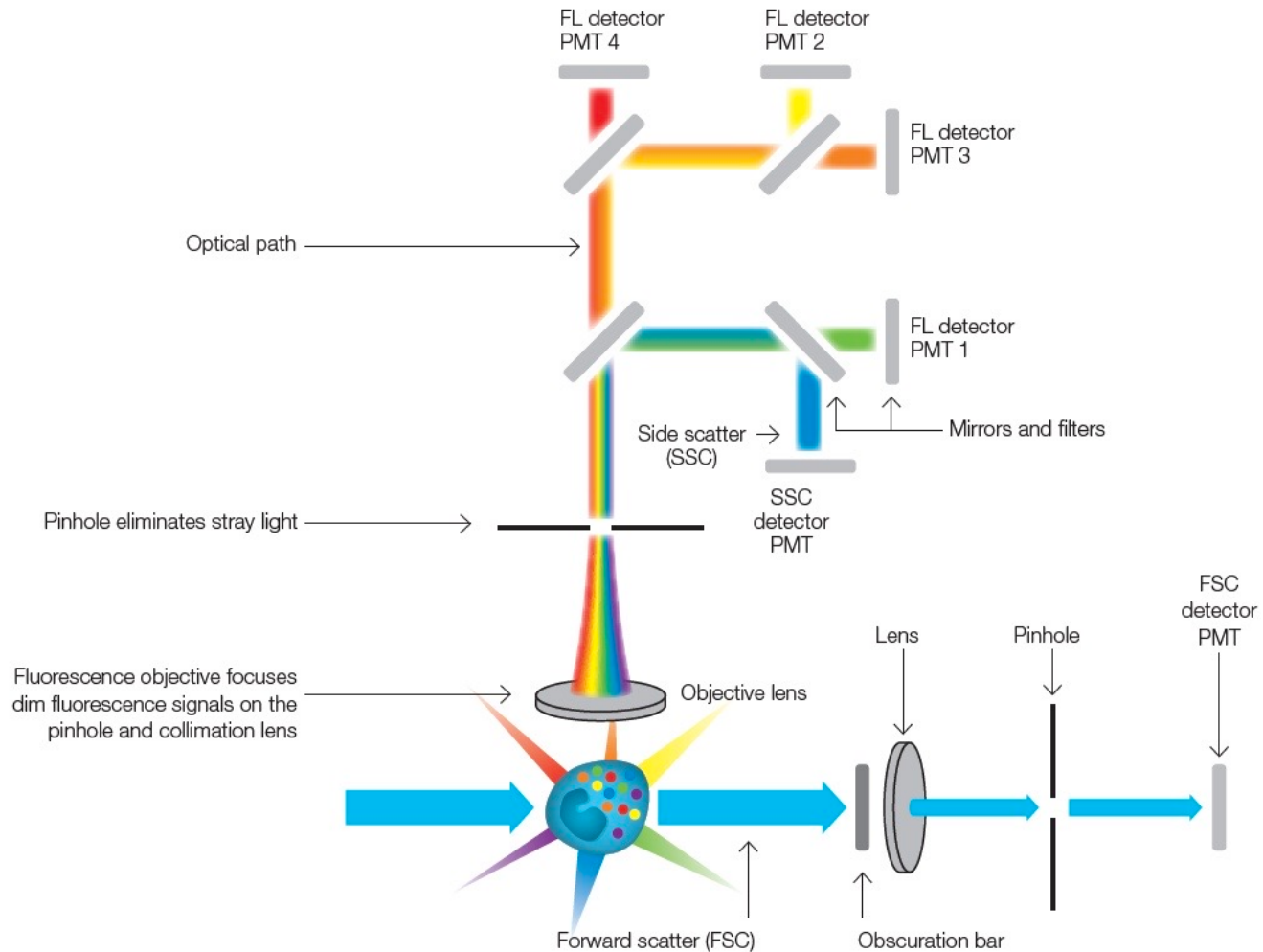
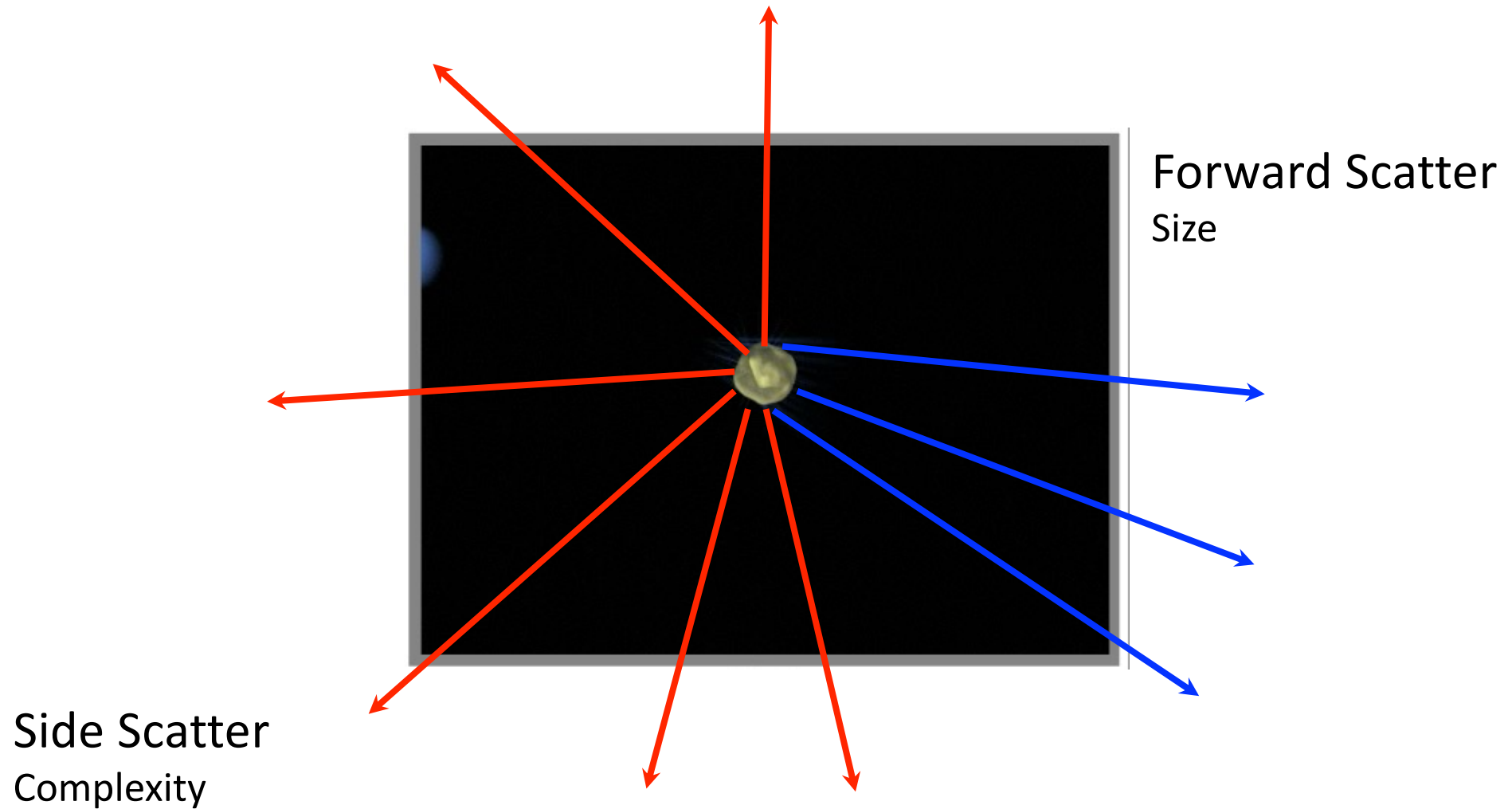
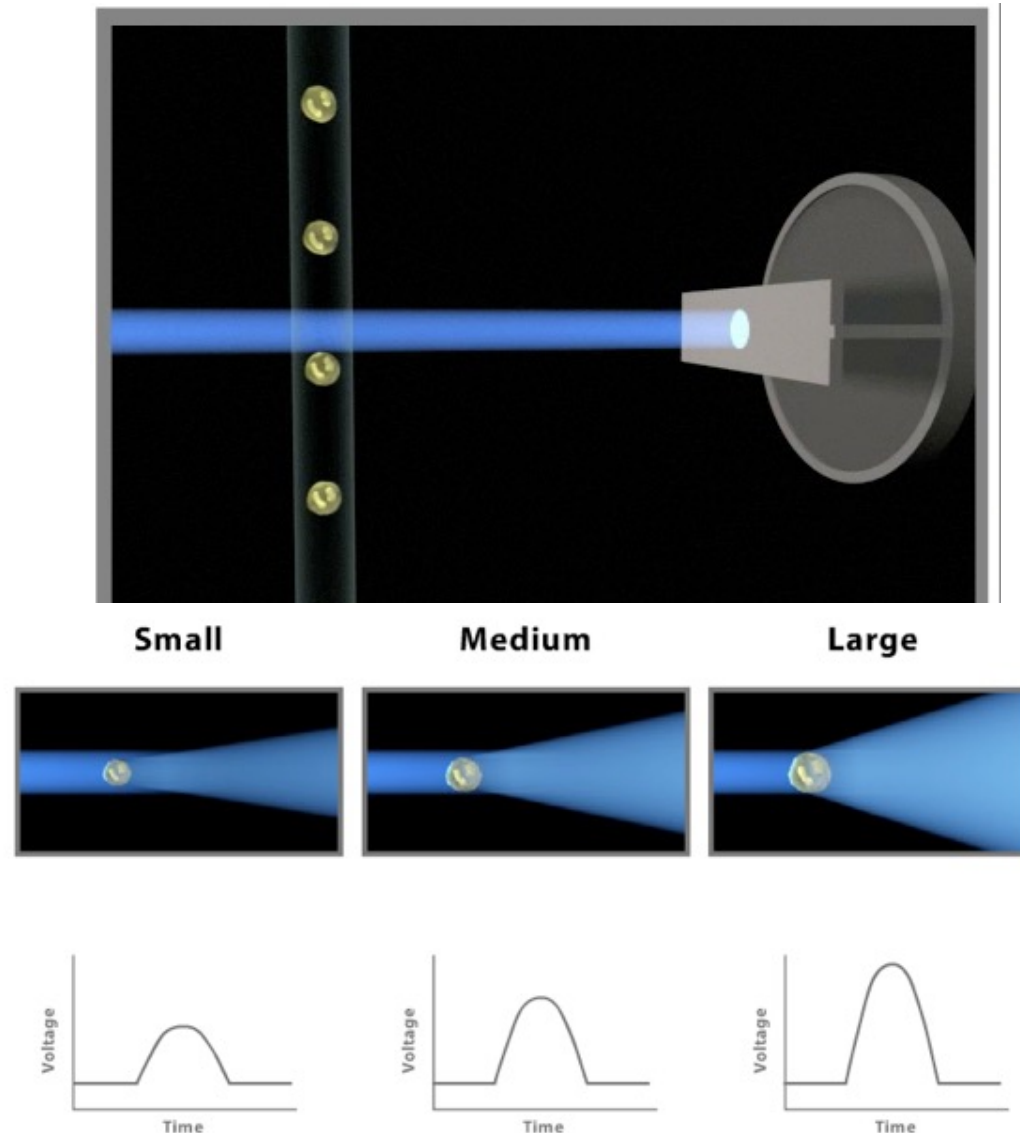


Fig. 3. Schematic overview of a typical flow cytometer setup. FL, fluorescence; PMT, photomultiplier tube; SSC, side scatter; FSC, forward scatter; blue arrow, light path.

Light and optics: the scattered light

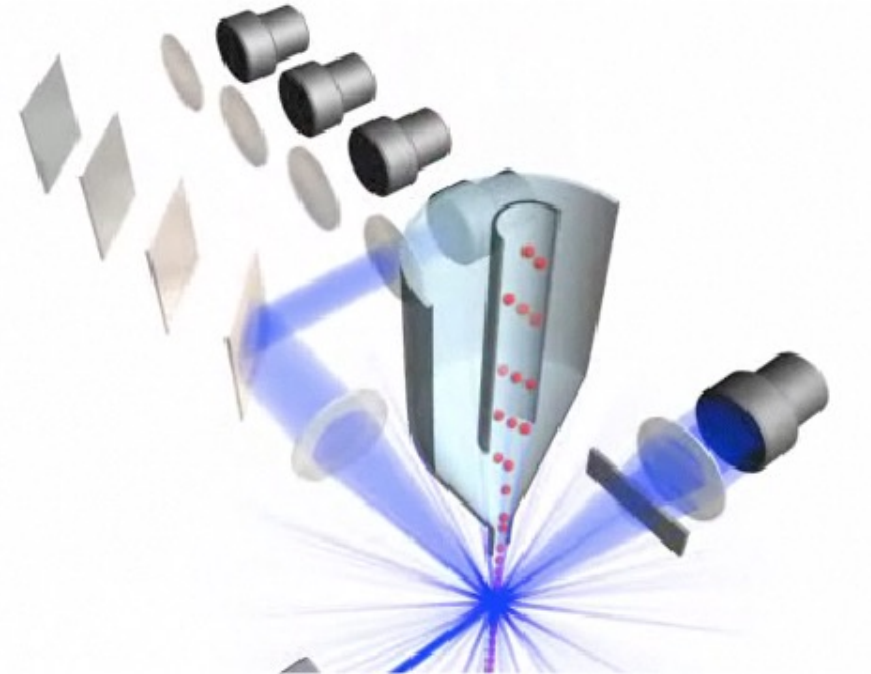
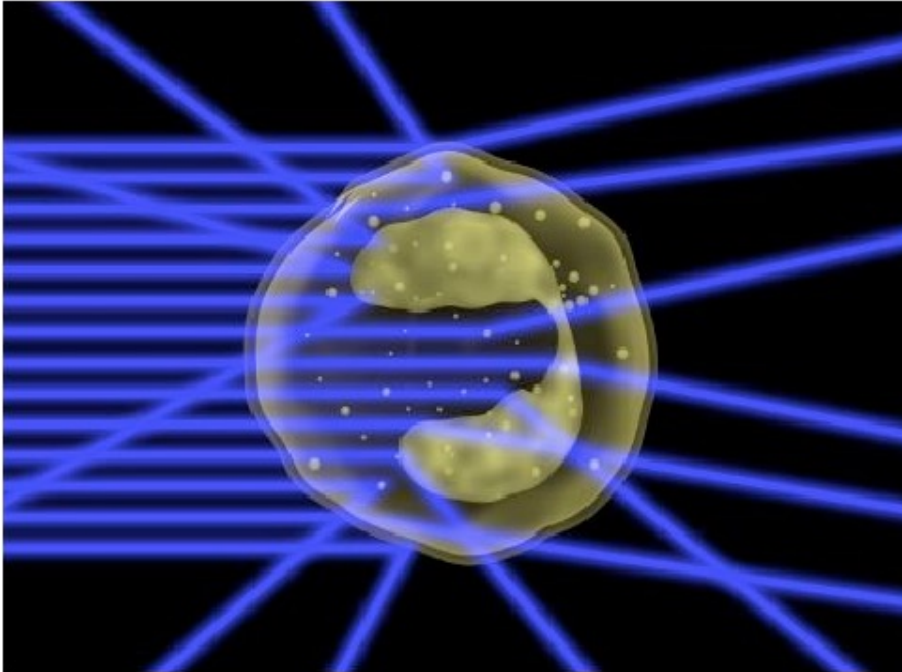


Light and optics: the Forward Scatter (FSC)



- Measured $\sim 2-20^\circ$ off the axis of the laser intercept
- Scatter is proportional to square of the diameter of the cell
- Based on 'spherical' particles
- Strong angle dependent
- Obscuration bar is critical to prevent saturation of the FS detector

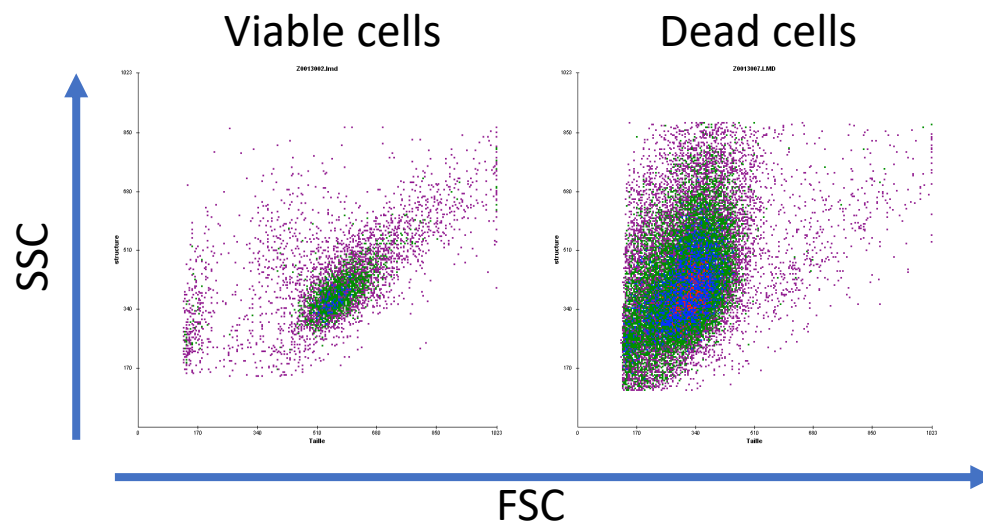
Light and optics: the Side Scatter (SSC)



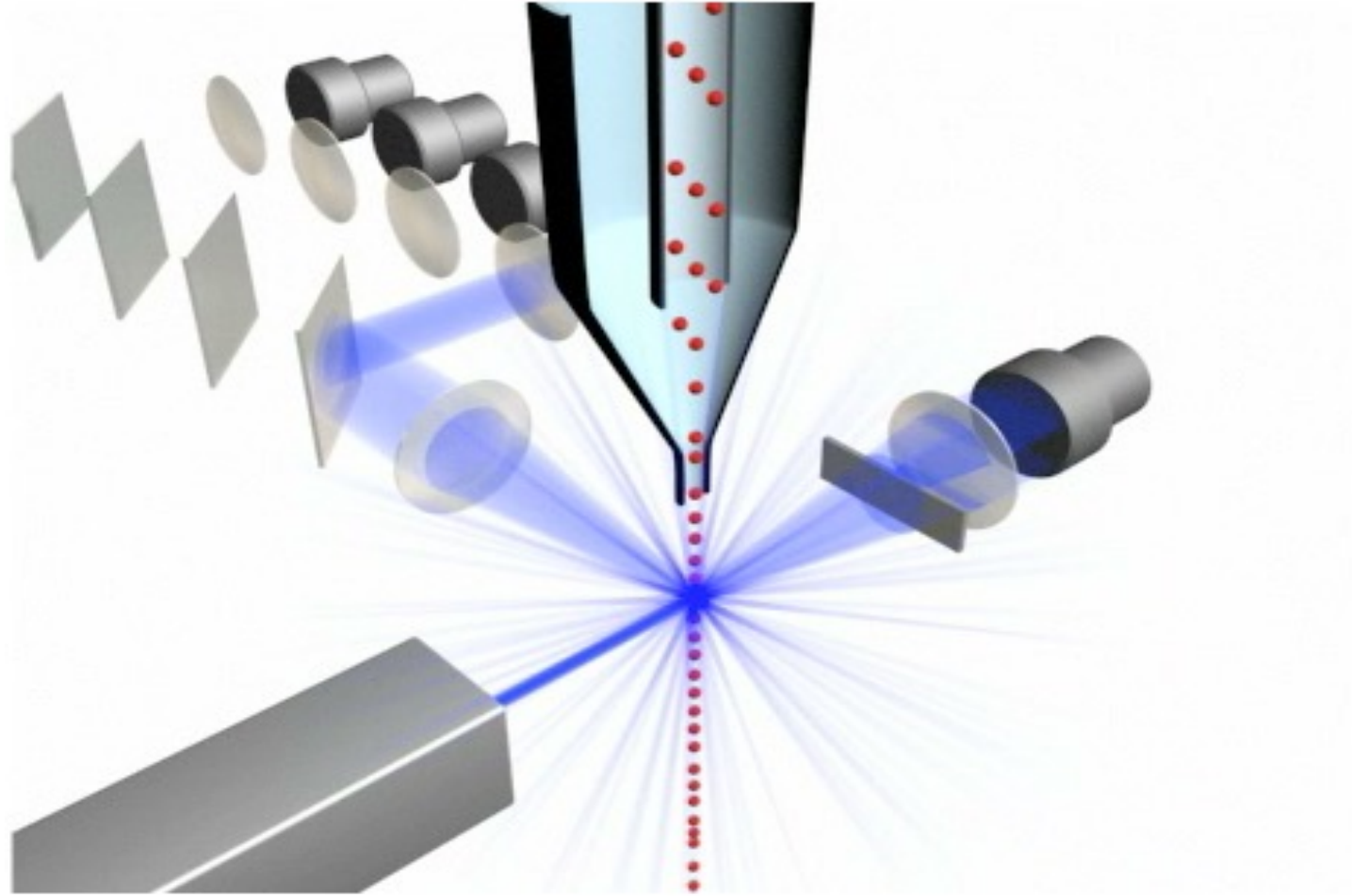
- Measured perpendicular to the plane of the FSC signal
- Is roughly proportional to complexity of the cell

Light and optics: Scatter Light - Caution

- The refractive index of beads and cells are different, therefore, they've different scatter characteristics
- The RI also change in:
 - viable and non viable cells,
 - fixed and unfixed samples,
 - some drug treatments...
- I.e. viable cells have a higher FSC and a lower SSC than cells in apoptosis



Light and optics: The fluorescence

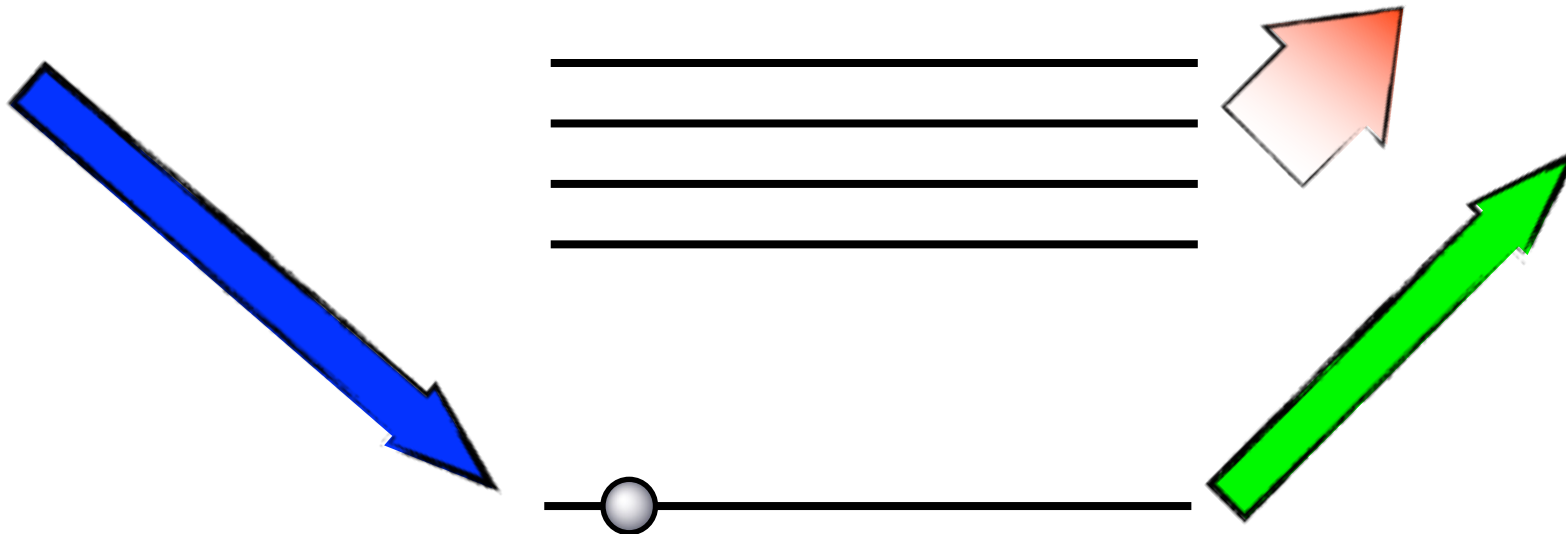
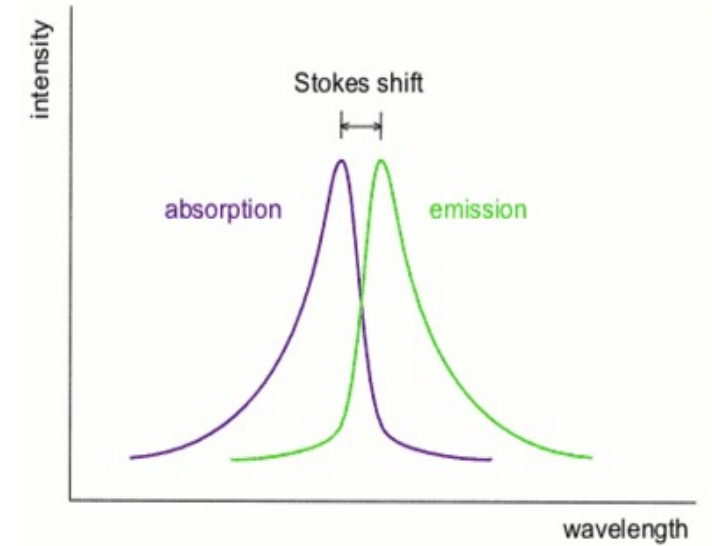
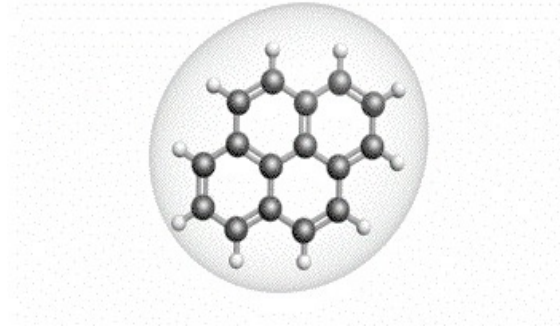


Light and optics: The fluorescence

Fluorescent molecule absorbs light of specific wavelength

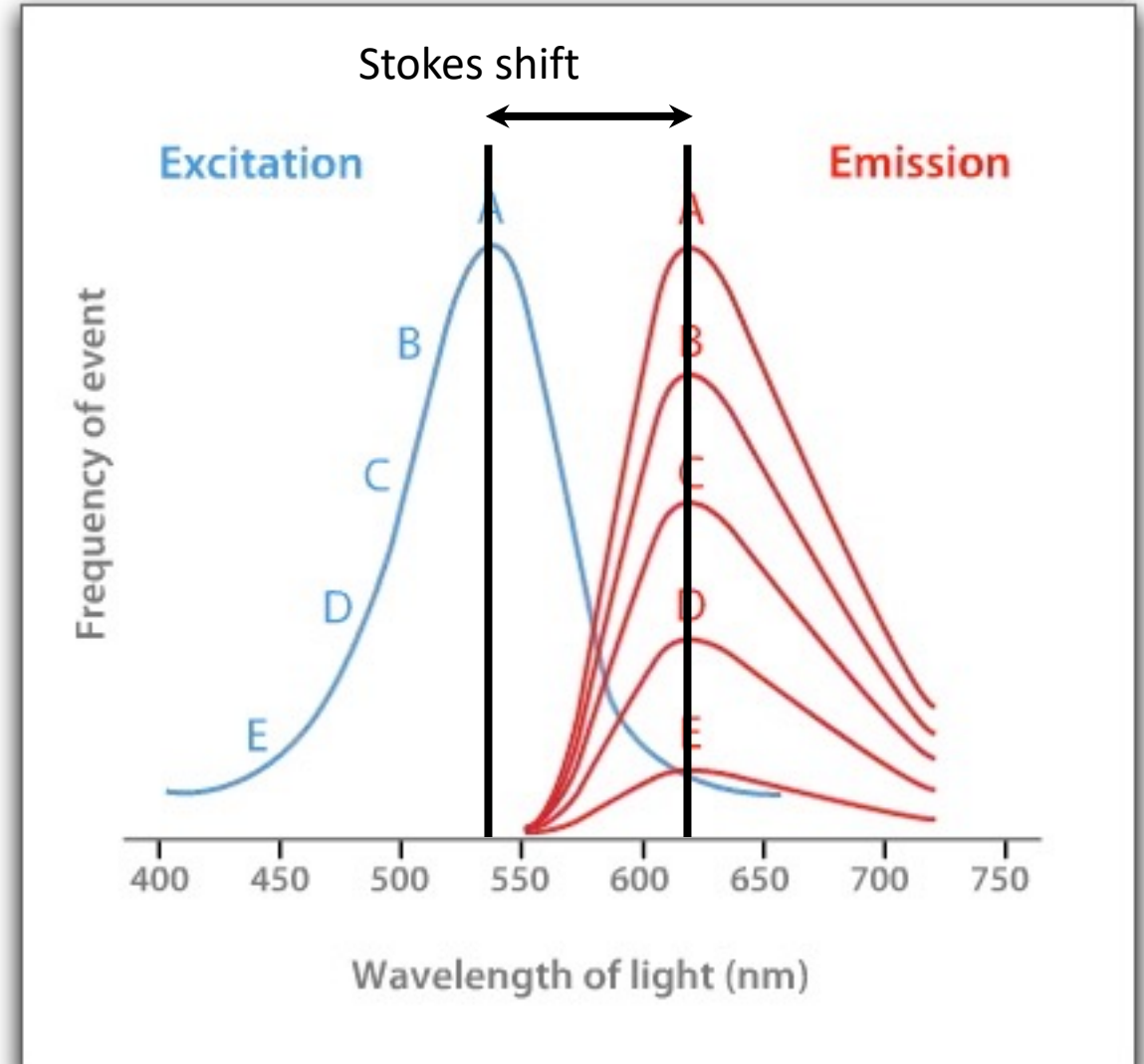
Electrons move to excited state, emit energy in form of

- Oscillation, thermal and light energy
- Emitted photon has longer wavelength than absorbed photon
- Difference is termed 'Stoke's Shift'

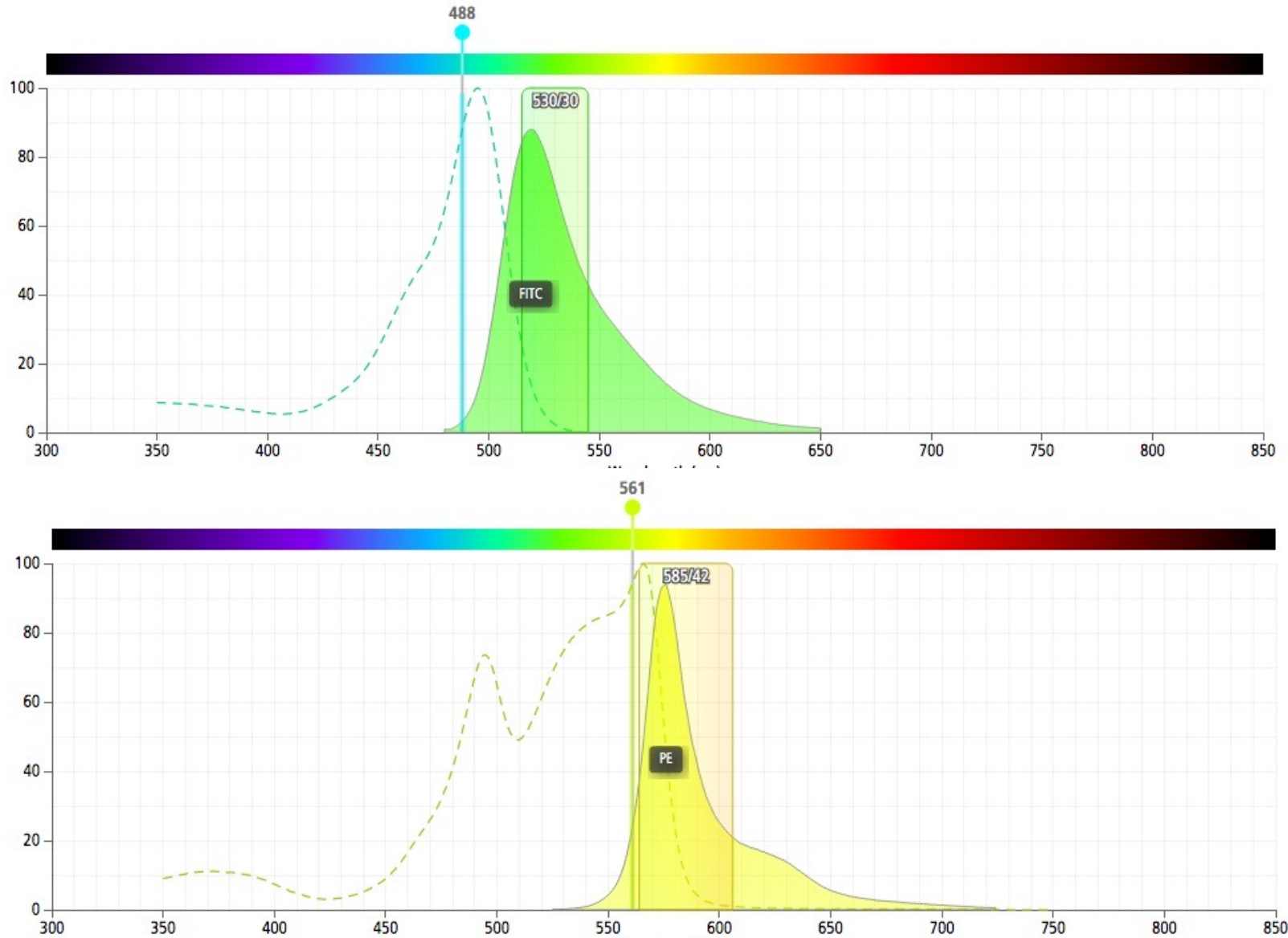


Light and optics: The fluorescence

Excitation and emission spectra



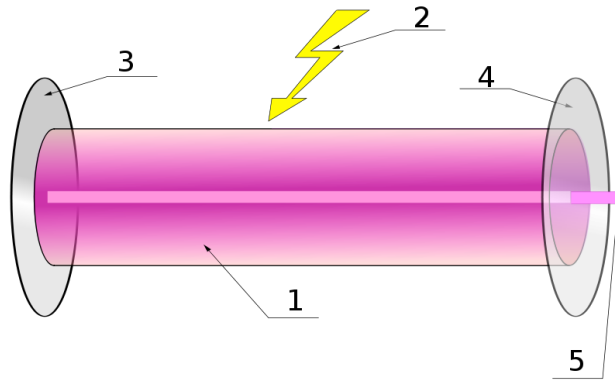
Light and optics: The fluorescence



Light and optics: LASER

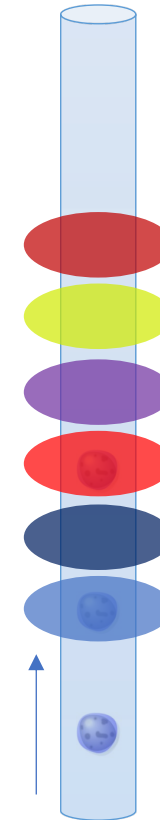
Light Amplification by Stimulated Emission of Radiation

Lasers are useful excitation sources for flow cytometry because they produce monochromatic or polychromatic coherent light.



1. Active laser medium
2. Laser pumping energy
3. High reflector
4. Output coupler
5. Laser beam

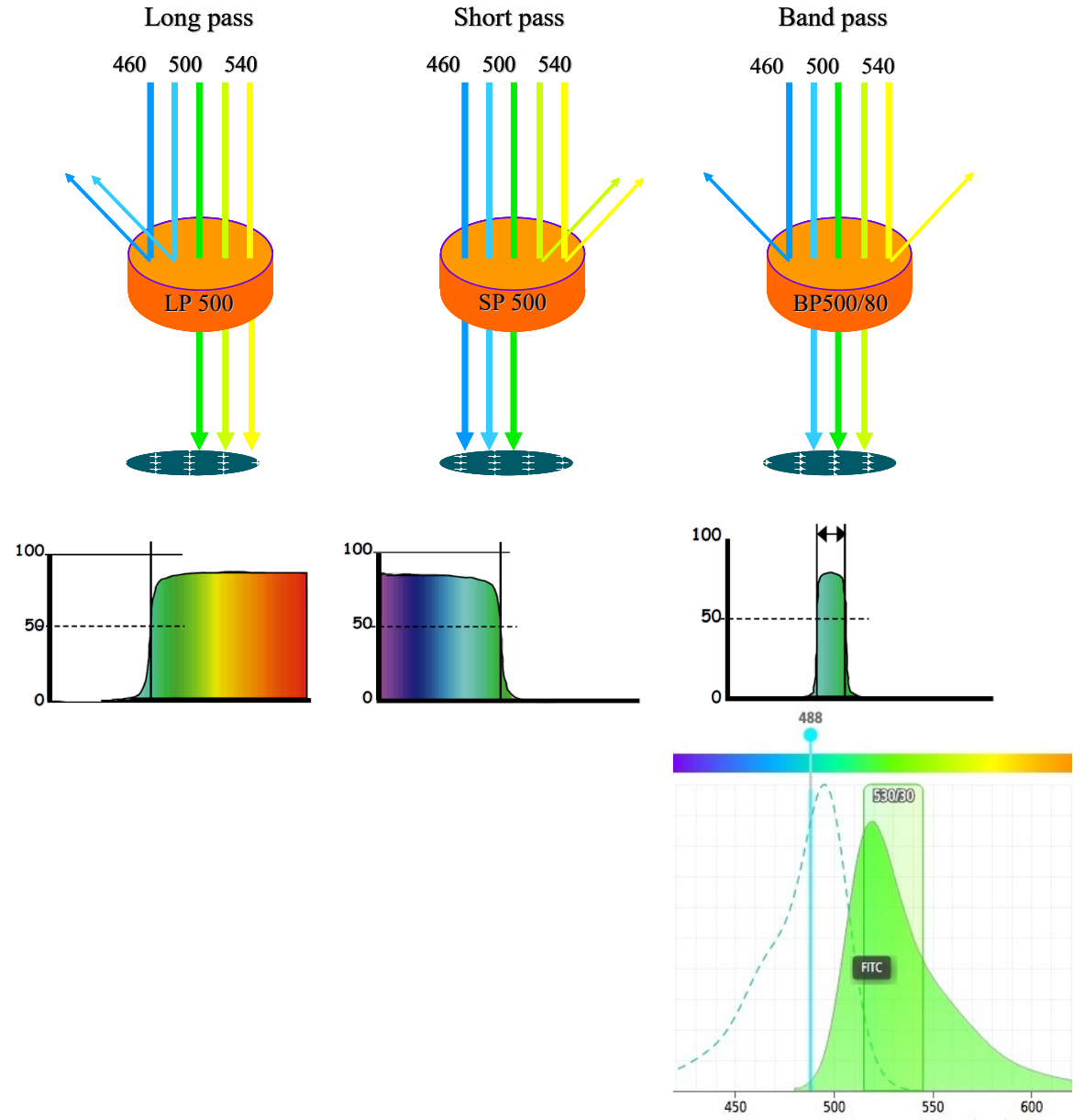
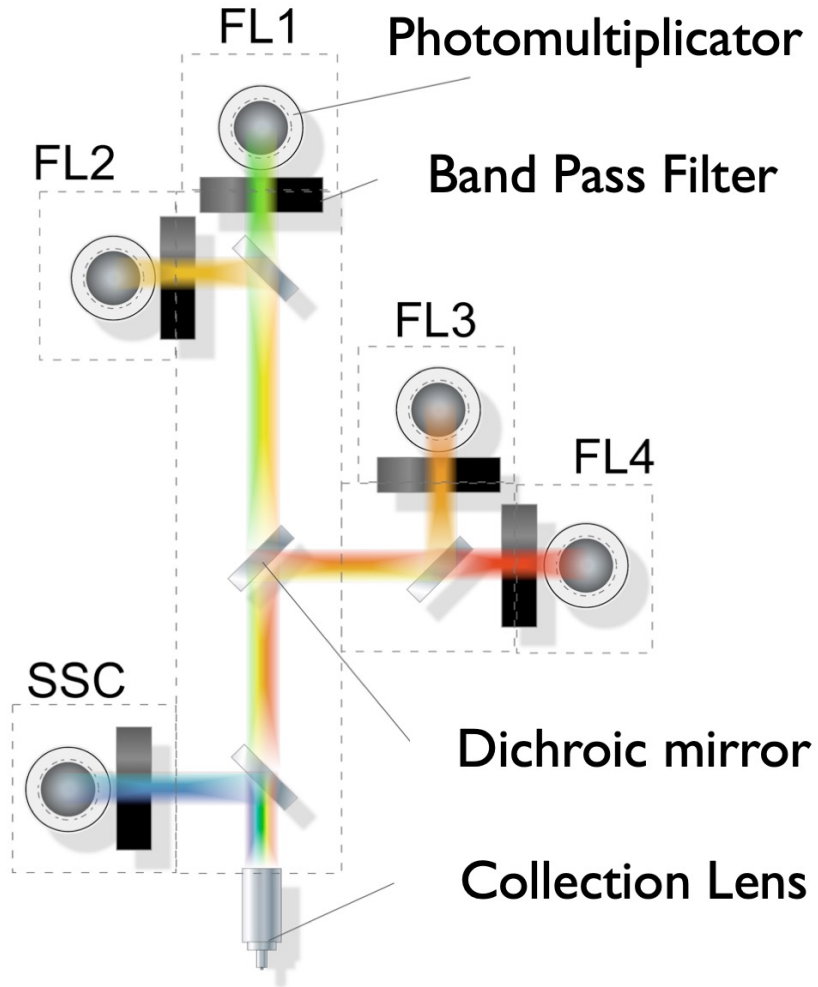
- Coherent
 - Spatial (little divergence of beam)
 - Spectral (generally a single wavelength)
- High intensity
- Polarized



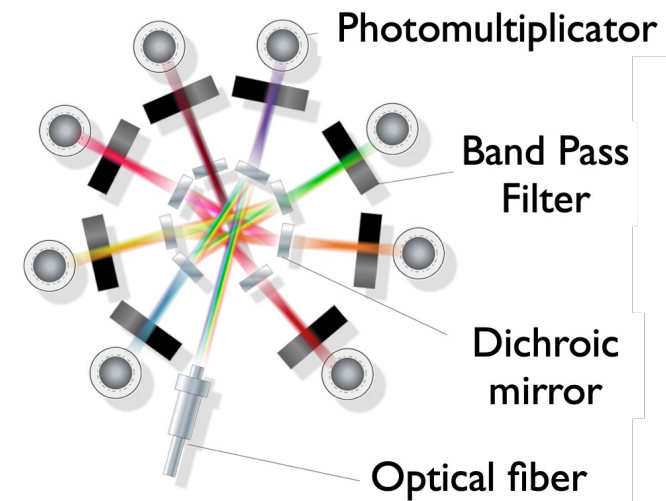
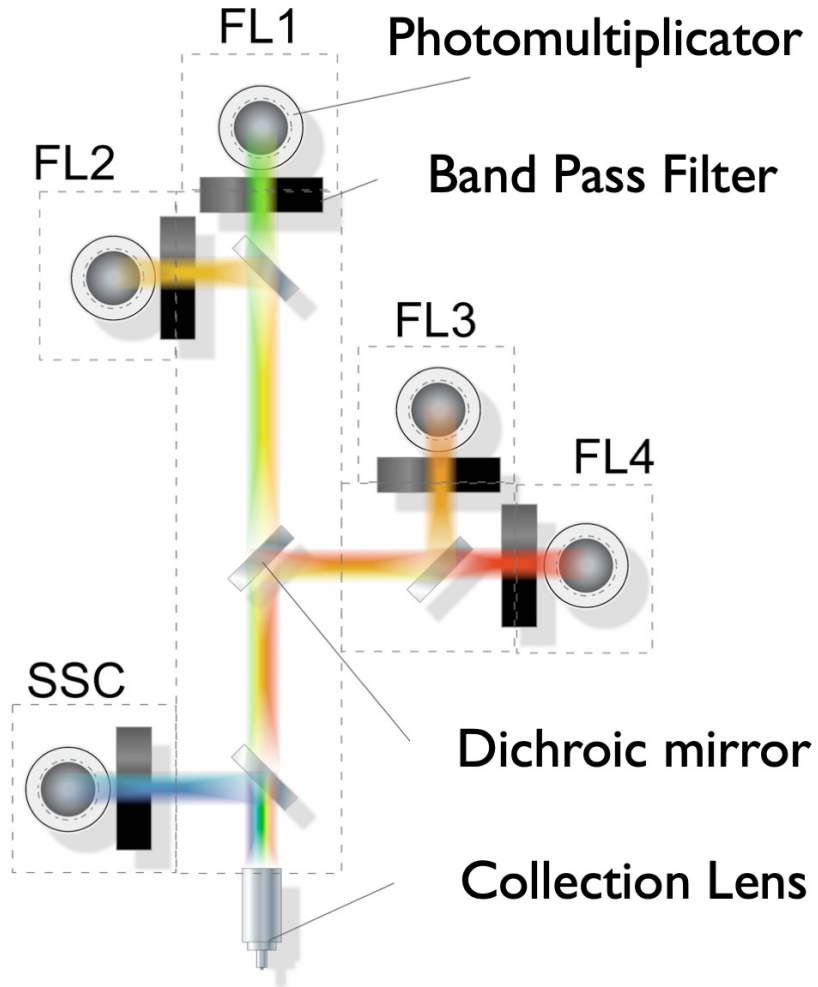
Common wavelengths include:

- UV – 355 nm
 - Near UV – 375 nm
 - Violet – 405 nm
 - Blue – 488 nm
 - Green – 532 nm
 - Yellow-Green – 561 nm
 - Red – 633 nm
 - Near Infra Red – 808nm
- Important to know where fluorochromes will be excited

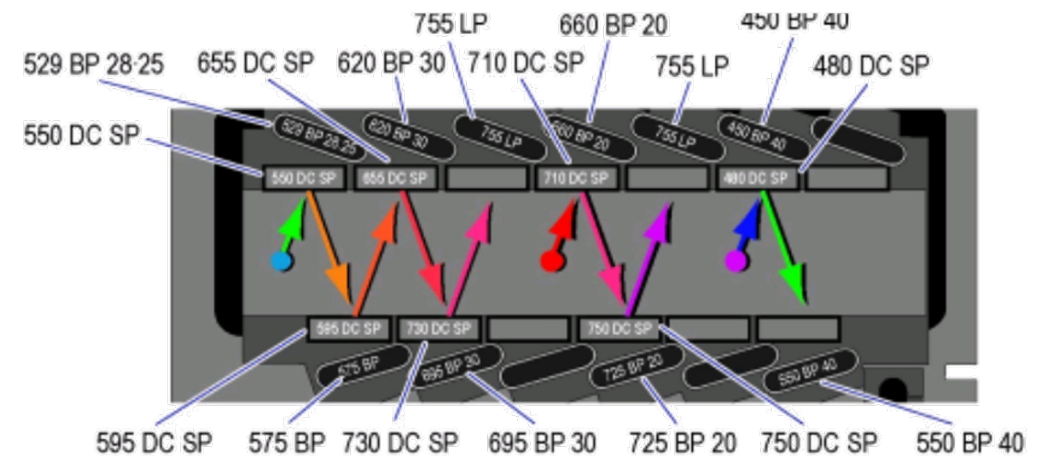
Light and optics: The Optical Bench



Light and optics: The Optical Bench

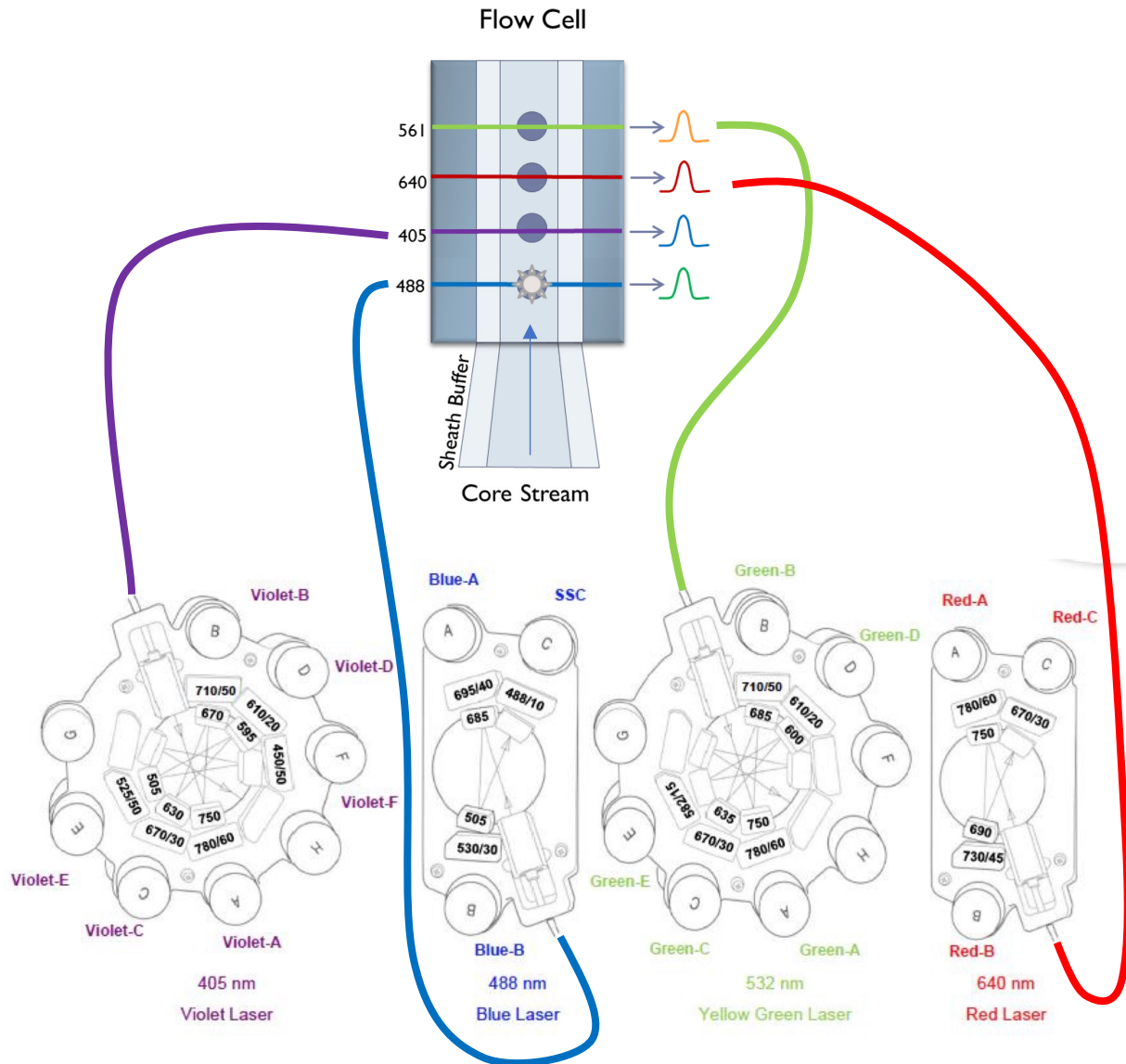


LSR II, Canto, Fortessa



Gallios

Light and optics: The Optical Bench



Typical Fluorochromes

405F	405E	405D	405C	405B	405A
BV421 Alexa405 eFluor450 Pacific Blue DAPI SYTOX Blue	BV510 Alexa430 AmCyan Pacific Orange	BV605 QD605 eFluor605	BV650 QD655	BV711	BV785

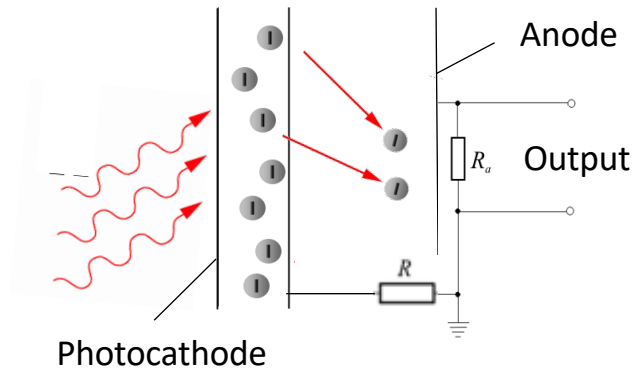
488B	488A
FITC GFP YFP Alexa488 CFSE	PerCP PerCP-Cy5.5 PerCP-eFluor710 PI

561E	561D	561C	561B	561A
PE Alexa568 PI	PE-TexasRed PE-Alexa610 PI 7-AAD mCherry	PI 7-AAD	PE-Cy5.5 PE-Alexa700 PI 7-AAD	PE-Cy7

640C	640B	640A
APC Cy5 Alexa647 Alexa660	Alexa680 Alexa700	APC-Cy7 APC-H7 APC-Alexa750

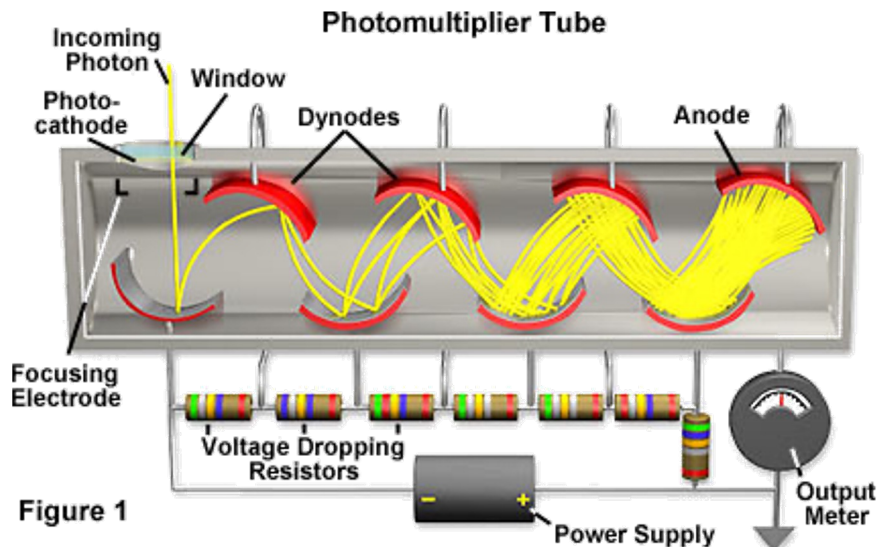
Light and optics: The Detectors

The Photodiode



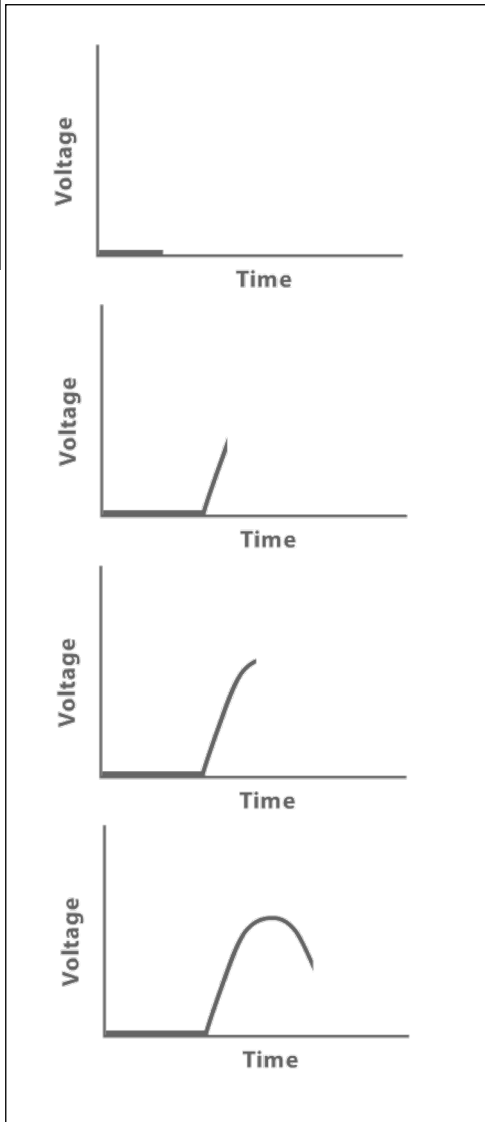
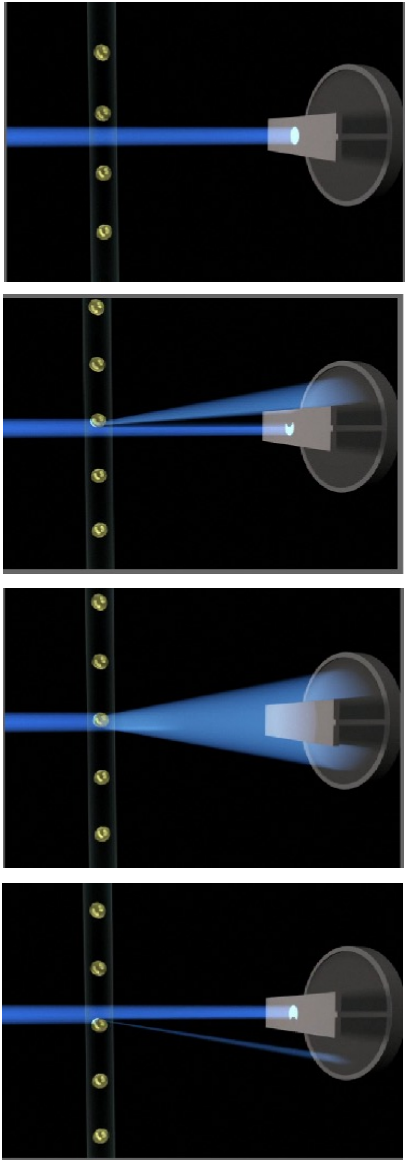
- Light sensitive semiconductor
- Not highly sensitive
- Relatively inexpensive
- Used for strong signals
 - Forward scatter

The Photomultiplier (PMT)

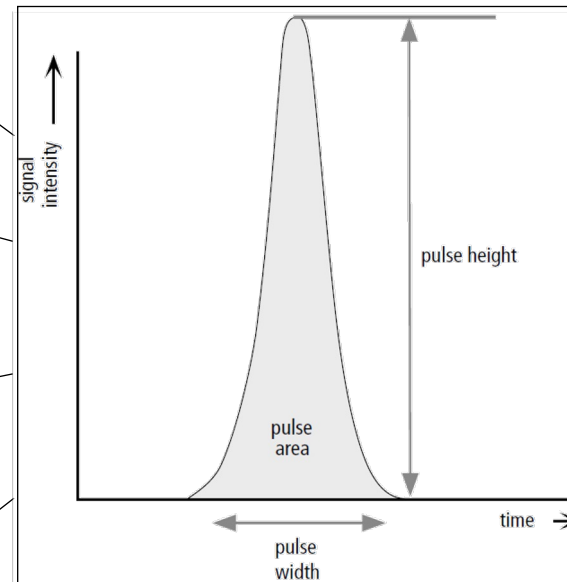


- Very sensitive to weak signals
- Sensitivity adjusted by voltage
- Expensive
 - Used for fluorescence and side scatter

The Electronic: the Voltage Pulse



Each cell creates a voltage pulse proportional to its size and the fluorescence intensity



Height: The maximum amount of current output by the PMT

Area: The integral of the pulse

Width: The time interval during which the pulse occurs

Signal intensity can be measured by either height of area

The width parameter measures the time that the cell spends in the laser

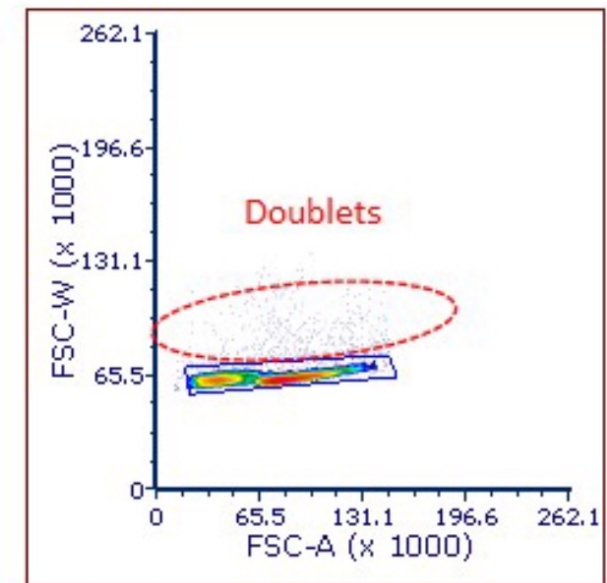
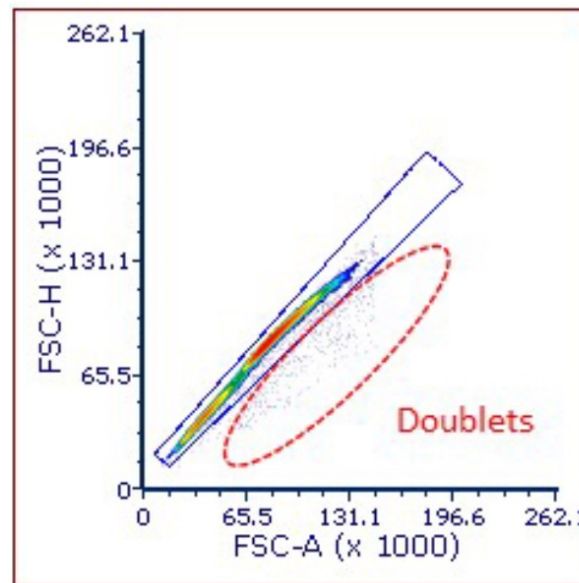
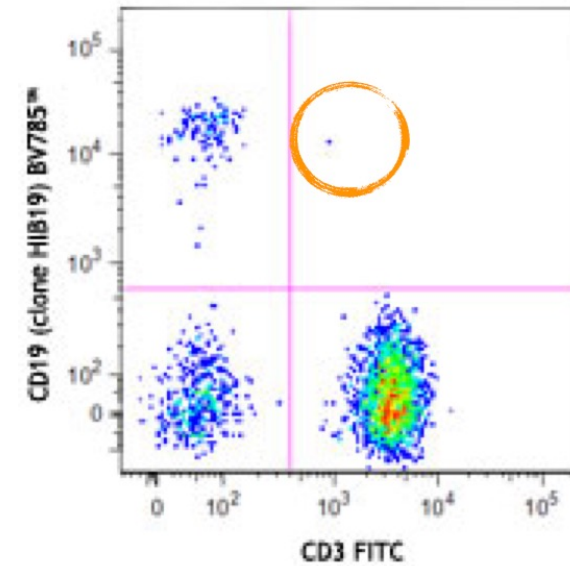
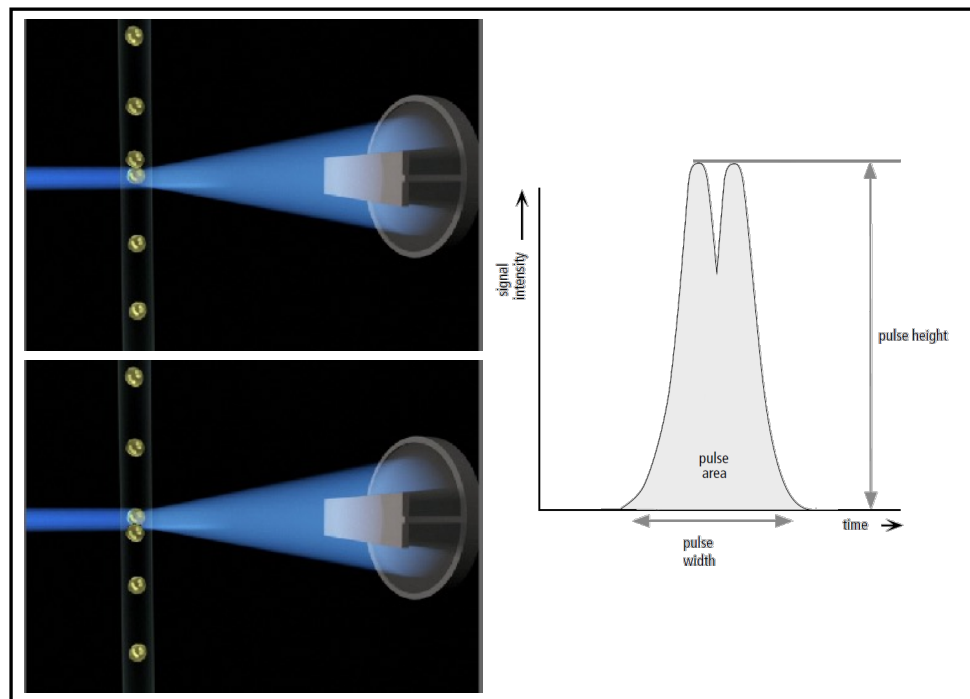
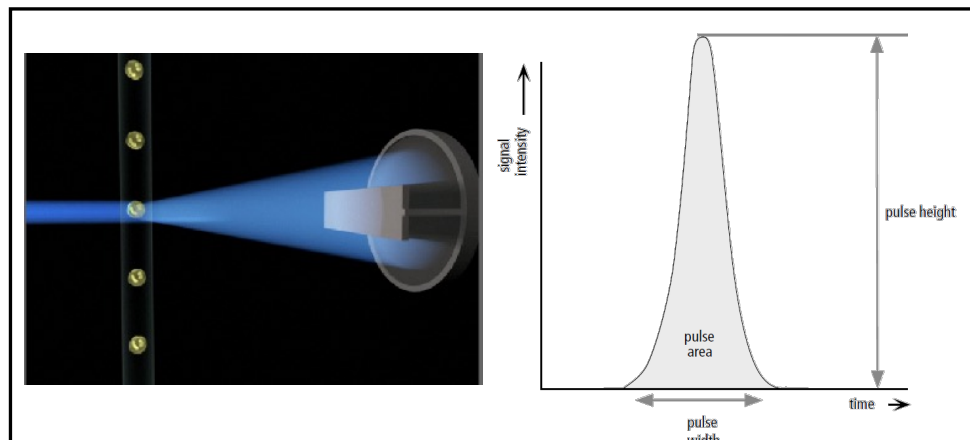
Cytometer - LSR II (1)

Parameter	Voltage	Log	A	H	W
FSC	606	☐	☒	☒	☒
SSC	315	☐	☒	☒	☒
Alexa Fluor 488	636	☒	☒	☐	☐
PerCP	778	☒	☒	☐	☐
PE	447	☒	☒	☐	☐
DsRed	434	☒	☒	☐	☐
PE-Cy5	547	☒	☒	☐	☐
PE-Cy 5-5	559	☒	☒	☐	☐
PE-Cy7	669	☒	☒	☐	☐
Alexa Fluor 405	529	☒	☒	☐	☐
Alexa Fluor 430	610	☒	☒	☐	☐
Qdot 605	805	☒	☒	☐	☐
Qdot 655	610	☒	☒	☐	☐
Brilliant Violet 711	618	☒	☒	☐	☐

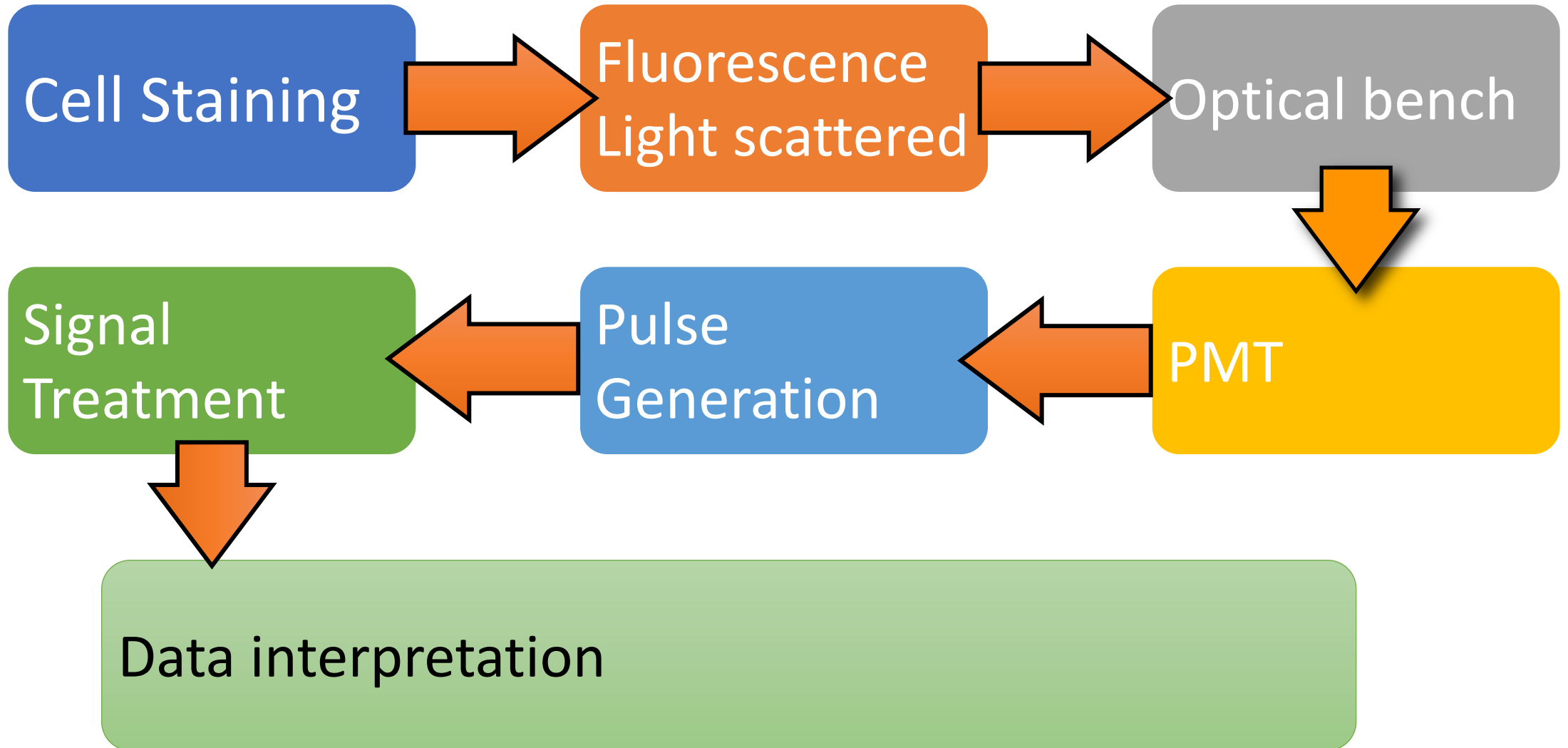
Add Delete

Cytometer Connected

The doublet discrimination



From photons to dot plots



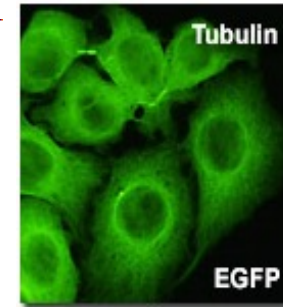
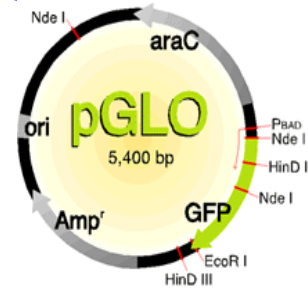
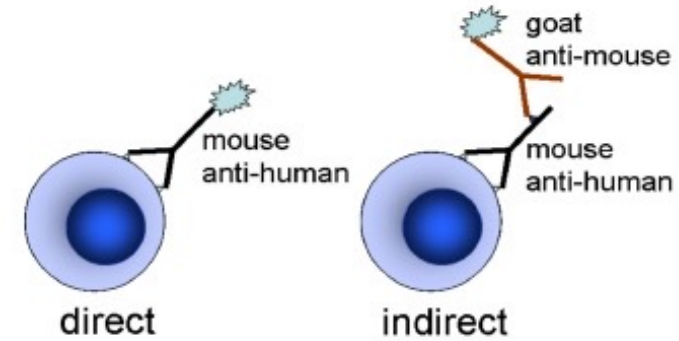
How do we stain cells ?

Monoclonal/polyclonal abs or other molecules coupled to fluorochromes

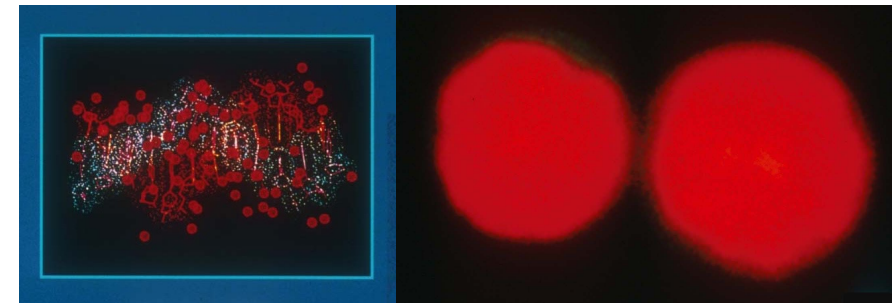
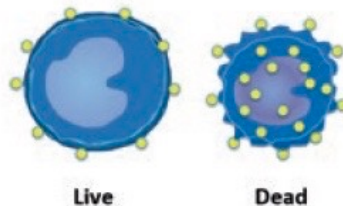
Fluorescent proteins (GFP, YFP, mCherry)

Nucleotide binding molecules
(DAPI, Hoechst, PI, 7AAD)

Amine binding dyes

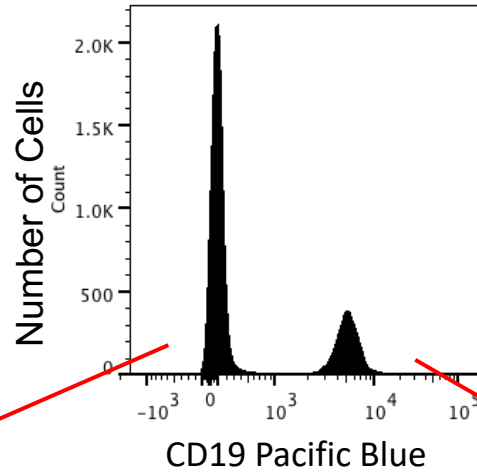


a. Amine-reactive Fixable viability stains



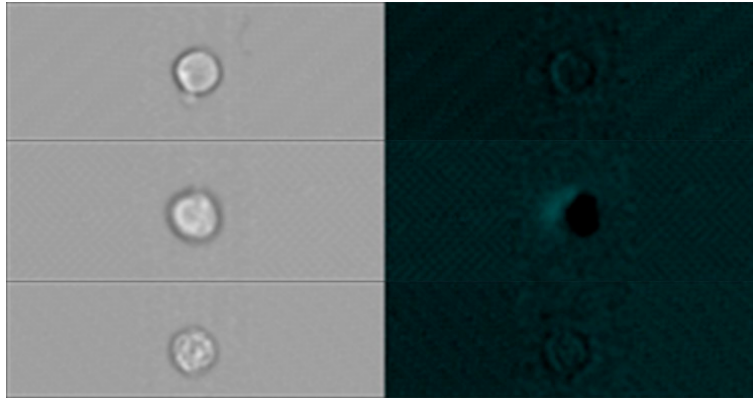
Data representation: Histograms

X: The farther to the right, the
brighter the fluorescence
Y: Cell count



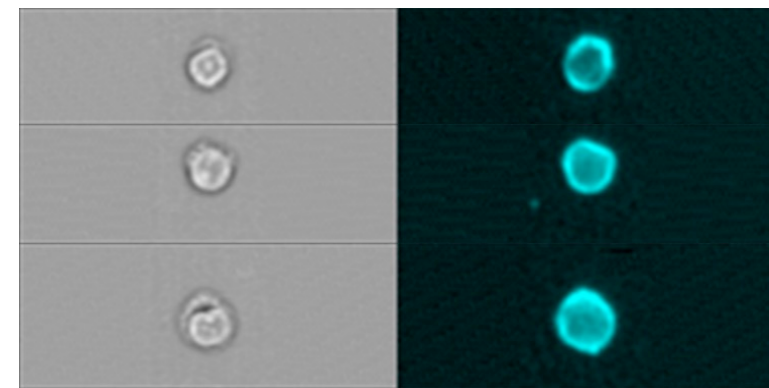
Brightfield

CD19 Pacific Blue



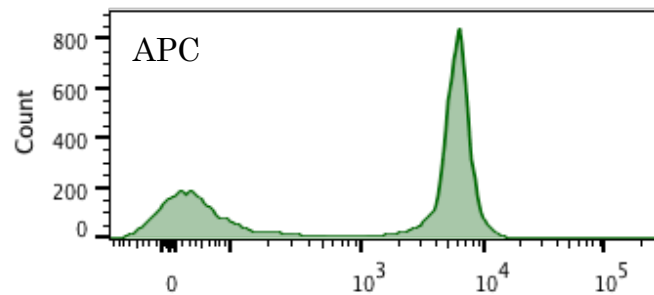
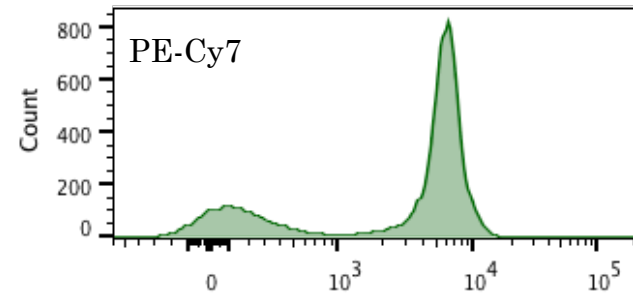
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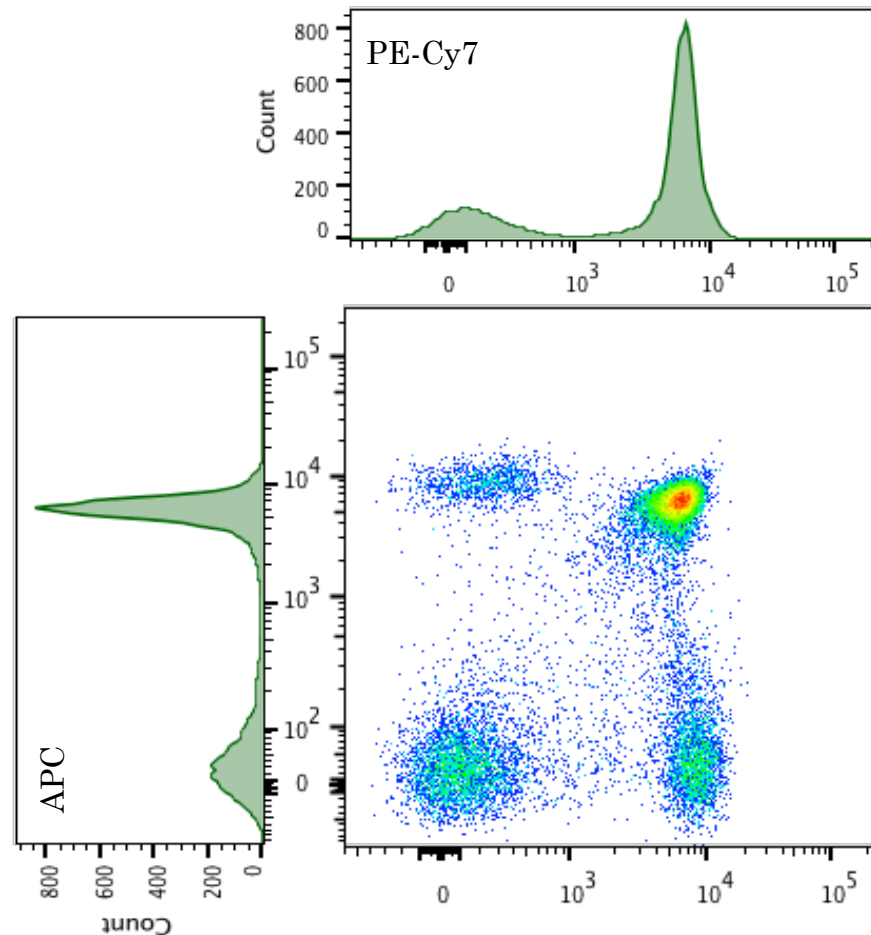
Data representation: Bivariate plots

- Univariate Plots show one dimension
- Bivariate plots provide more graphical information
- AND more data



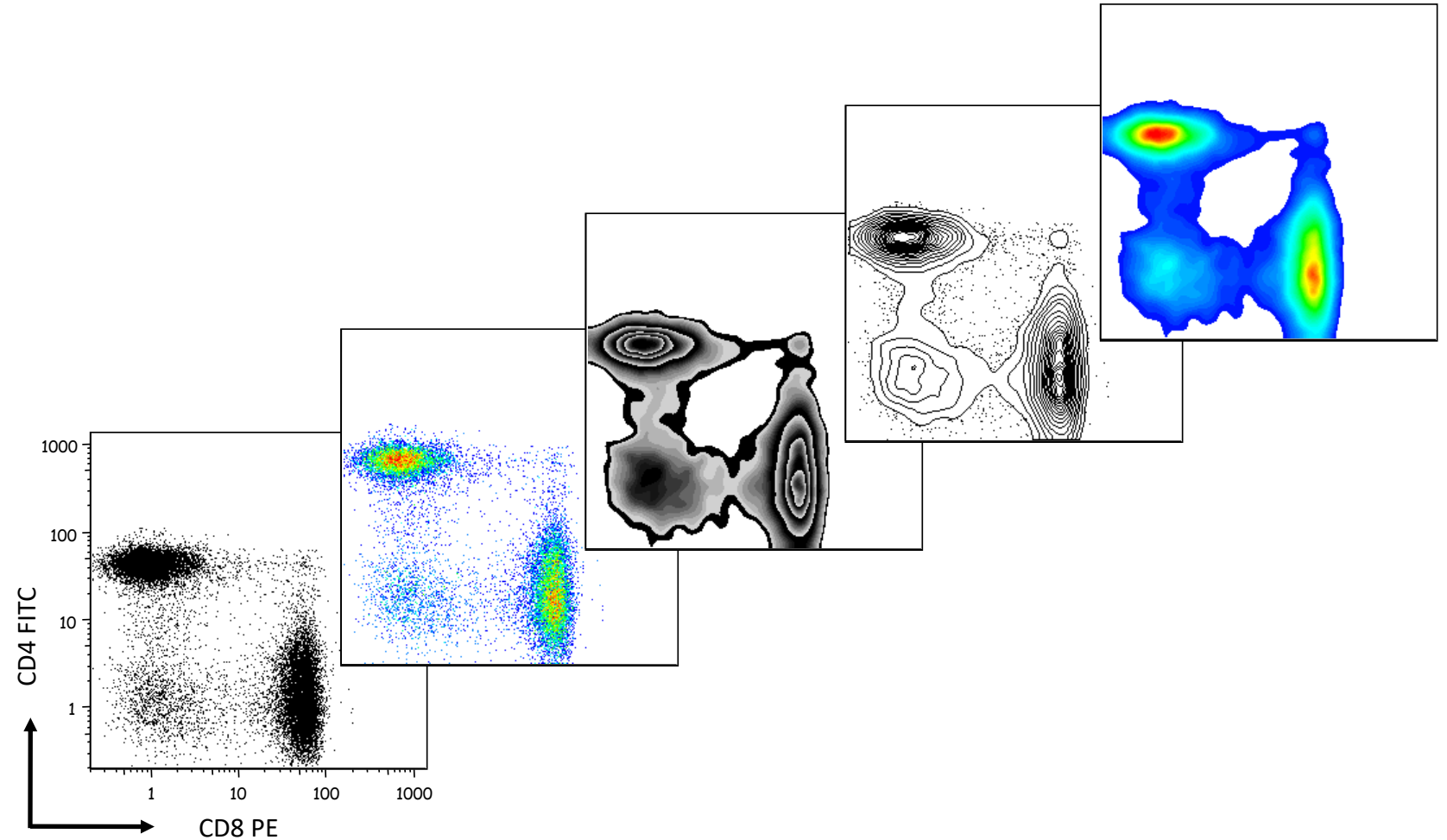
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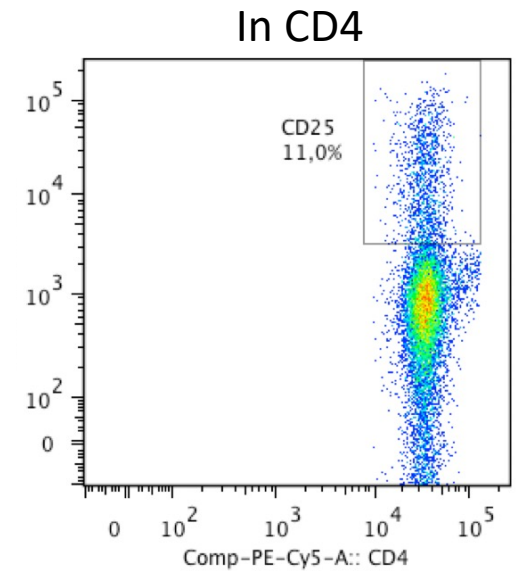
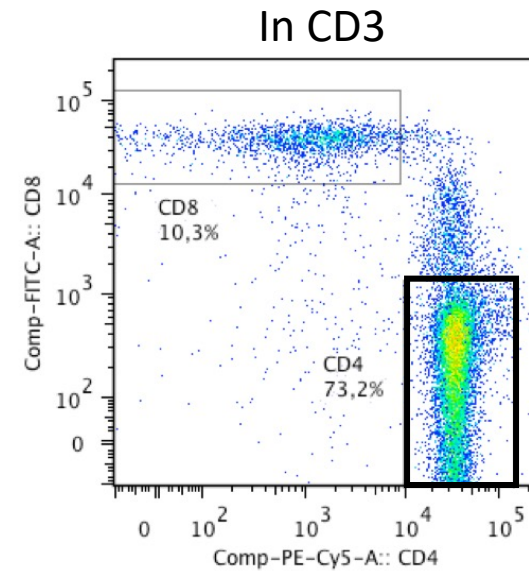
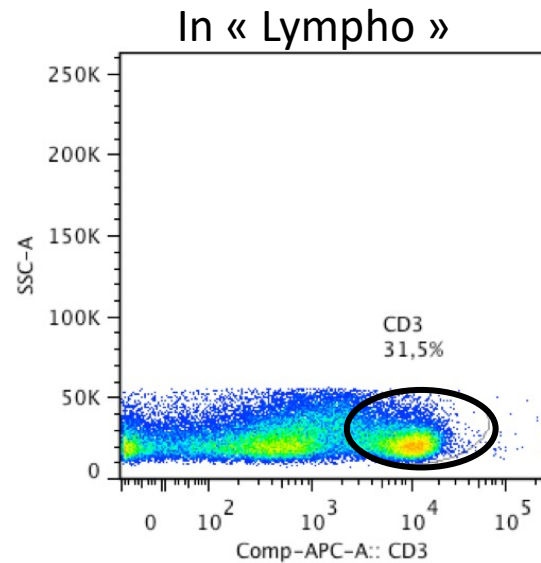
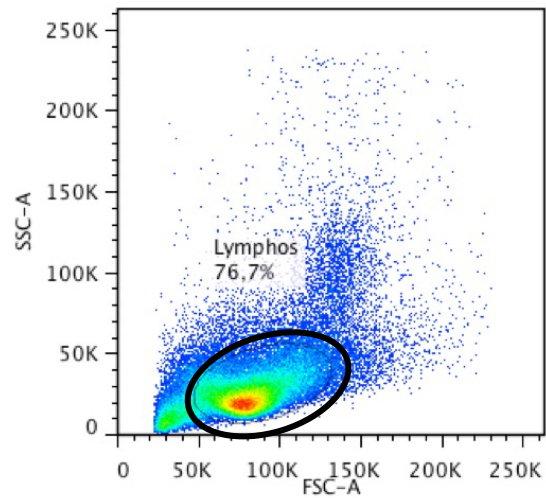
Data representation: Bivariate plots

- Several plot options
 - Dot plots
 - Contour plots
 - Density plots
 - Isometrics
 - Overlays



Data representation: The gating strategy

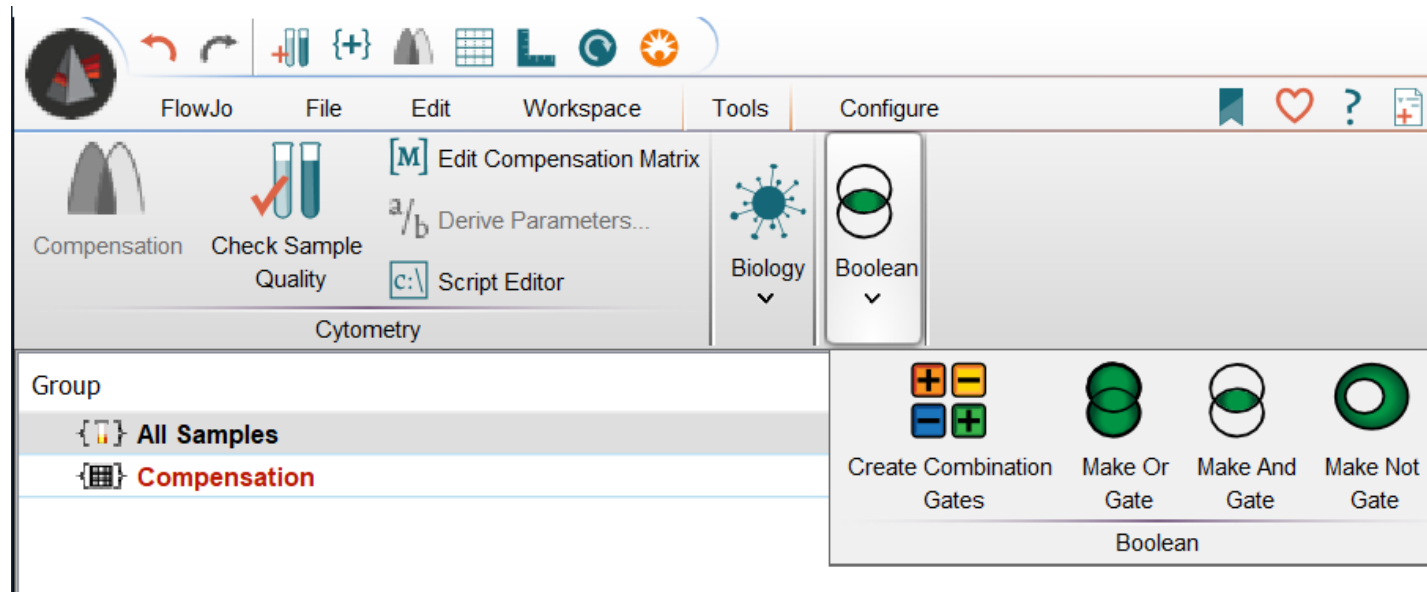
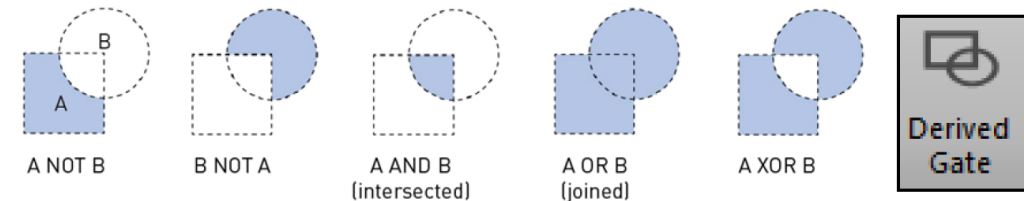
Hierarchical gating strategy



Data representation: The gating strategy

Boolean gating strategy

- Gates can be customized by using Boolean logic (OR, AND, NOT, XOR) to link multiple gates together



AND gates = all events that are shared

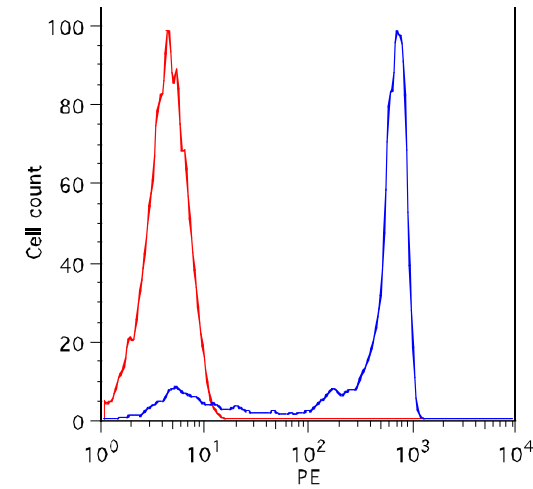
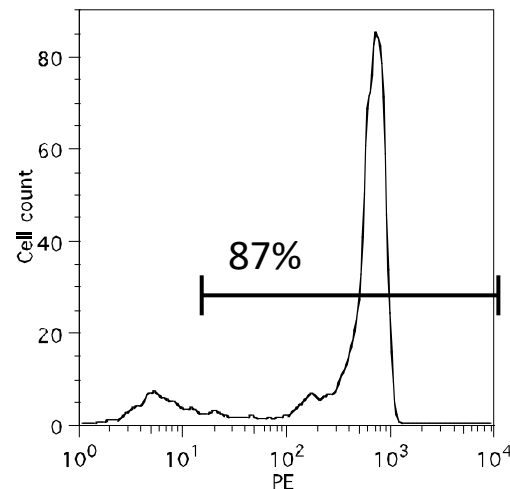
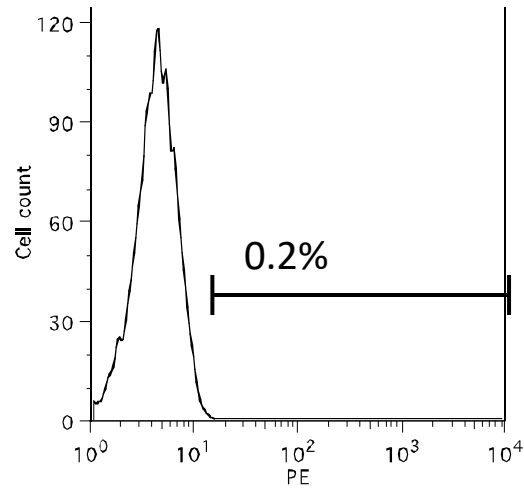
OR gates = all events found within 2 or more individual gates

NOT gates = all events found outside the gate

XOR gates = unique events found within an individual gates

Data analysis: Metrics

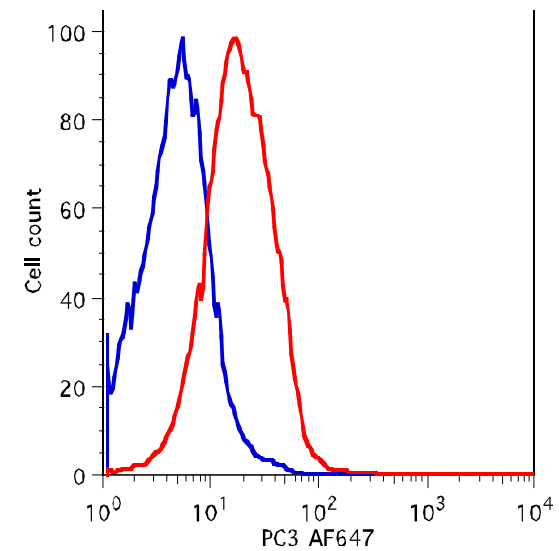
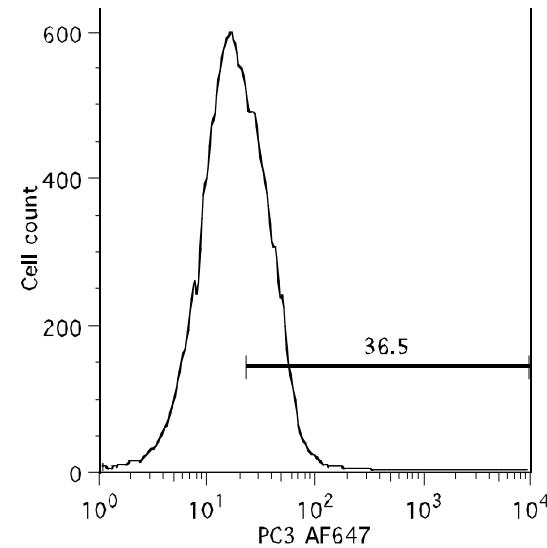
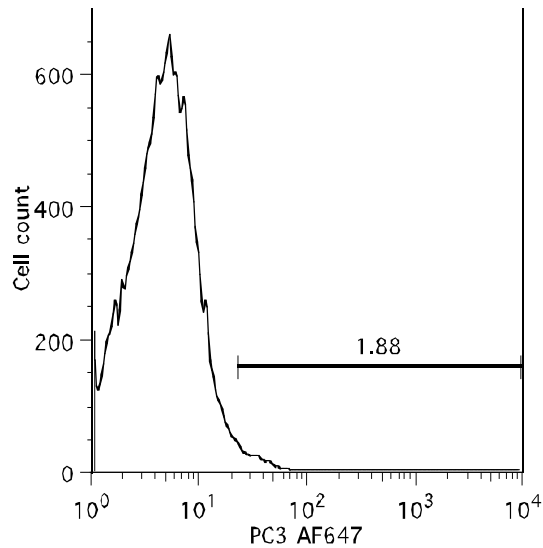
- Measuring the 'positive' fraction



- Need to define the negative population by a control

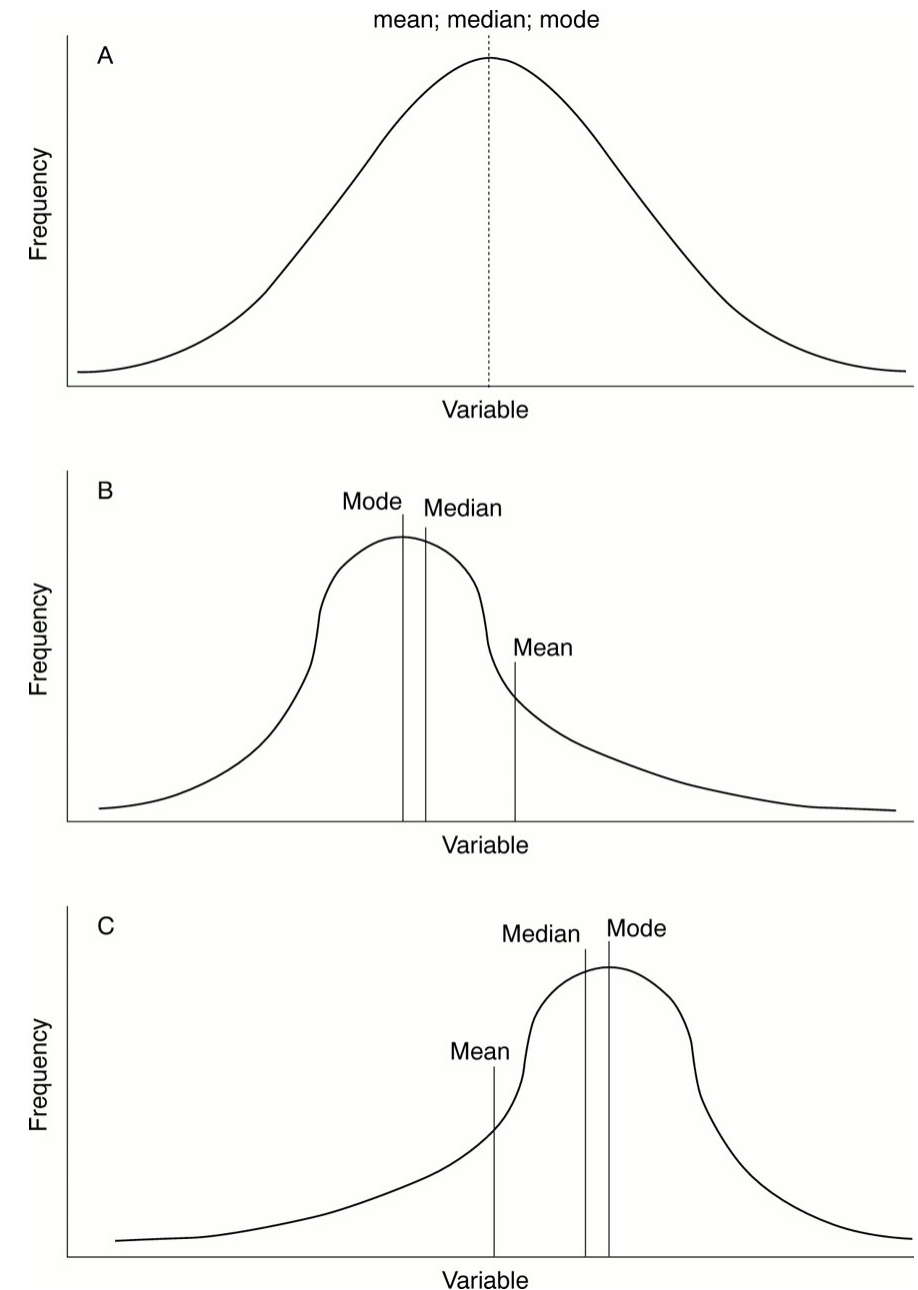
Data analysis: Metrics

- Not all distributions are as easy
 - How do we describe this?



Data analysis: Metrics

- Fluorescence Intensity measurement
 - The **median** is more robust than the **mean** for most datasets
 - The median can cause errors if more than 50% data is off-scale



Conclusion

- Flow cytometry can analyse cells in suspension emitting different signals:
 - Scattered light
 - Fluorescences
- Cells can be stained with fluorescent antibodies and/or fluorescent probes for their characterization
- Data are represented in mono or multivariates histograms for the data interpretation.