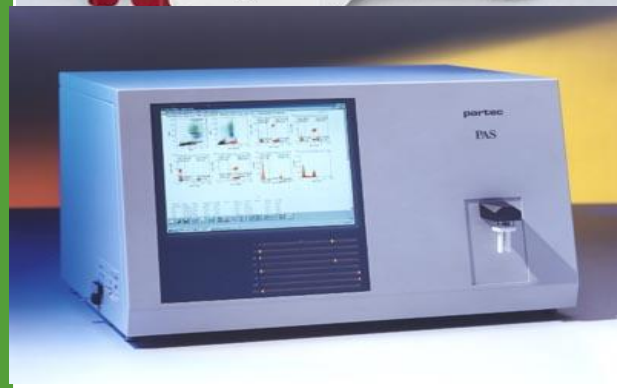
Flow cytometry



Sabzevar University of Medical Sciences

The principles and applications of flow cytometry

By

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What Is Flow Cytometry?

- ✓ An instrument for making cell-based **fluorescent** measurements.
- ✓ Method for **quantitating** cellular or structural components of a cell using fluorescent antibodies or probes.
- ✓ Allows analysis of **Tens of thousands** of cells in minutes.
- ✓ A method to **sort** and collect specific cell types.

What Is Flow Cytometry?

- ✓ Flow ~ Fluid
- ✓ Cyto ~ cell
- ✓ Metry ~ measurement
- ✓ Measuring properties of cells while in a fluid stream

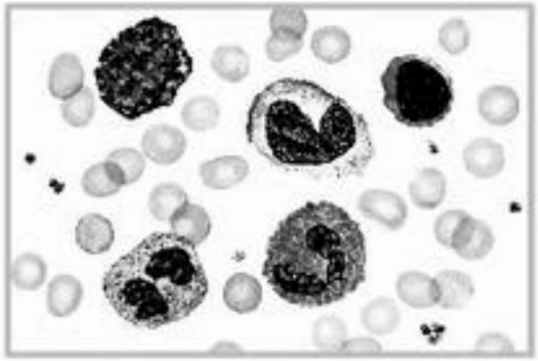
What Is Flow Cytometry?

- Flow Cytometry is a powerful technology for characterizing and analyzing particles of interest, such as cells, chromosomes, bacteria and beads.

What Is Flow Cytometry?

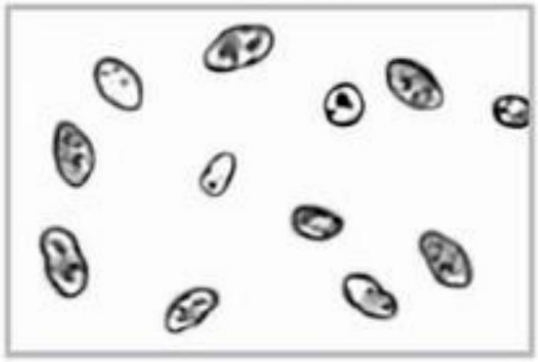
Sample Types

Cells

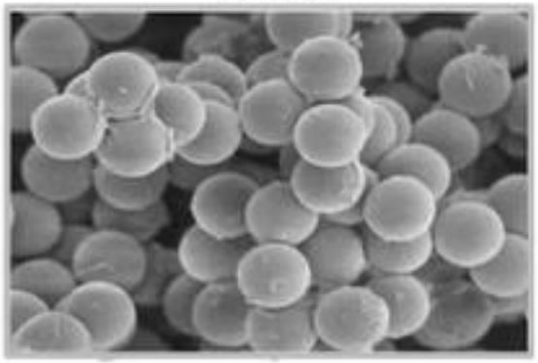


Chromosomes

Bacteria



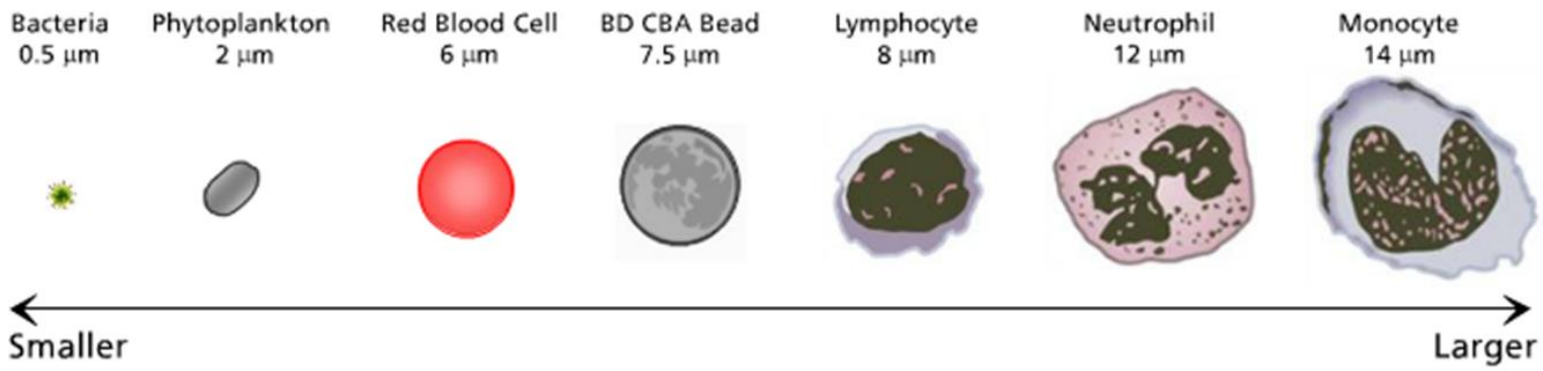
Beads

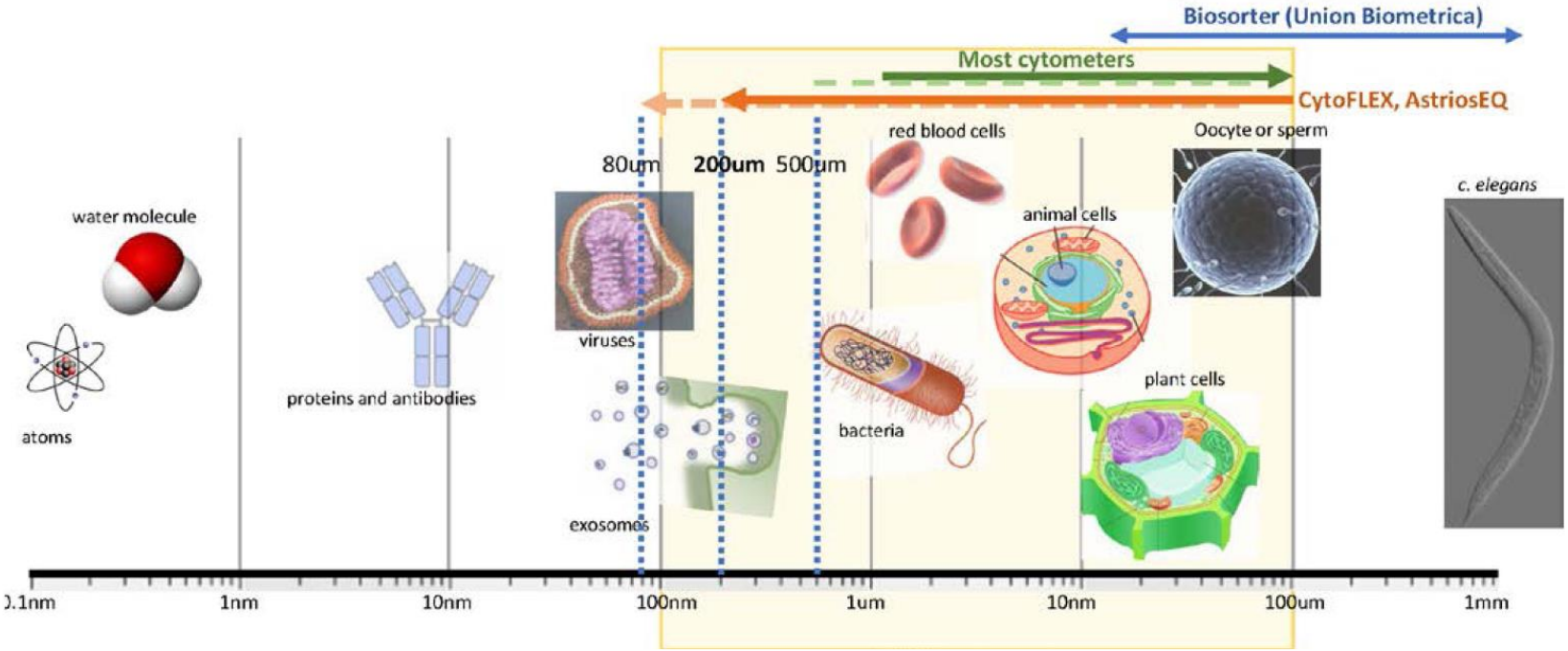


Particle Size

- All cytometer samples must be in liquid suspension.
- Cells from solid tissue must be disaggregated before analysis.
- Typically particles or cells from 0.2 to 50 micrometers in size are suitable for flow cytometric analysis.

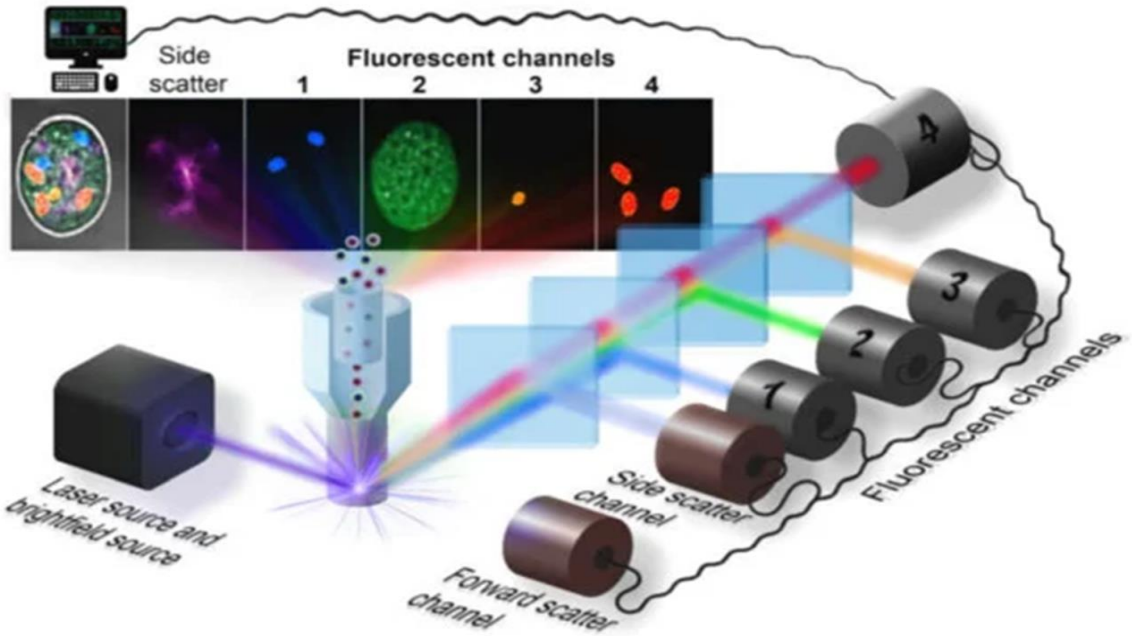
Particle Size





Flow Cytometric Measurement

- A cell's or particle's relative size, internal complexity, and fluorescence intensity can be measured by flow cytometry.

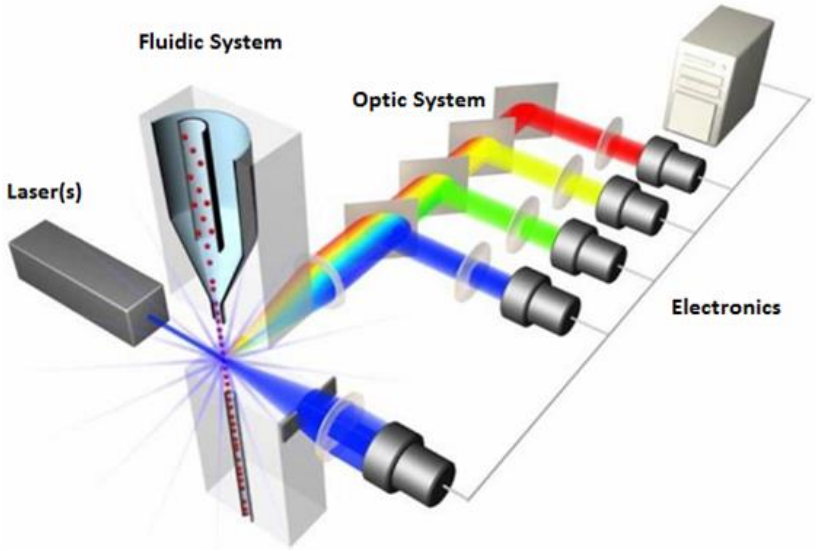


Principles of the flow cytometer

- **Fluidics system**
  - **The fluidics system transports particles in a stream to the laser beam for interrogation.**

- **Optics and detection**
  - **The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.**

- **Signal processing**
  - **The electronics system converts the detected light signals into electronic signals that can be processed by the computer.**

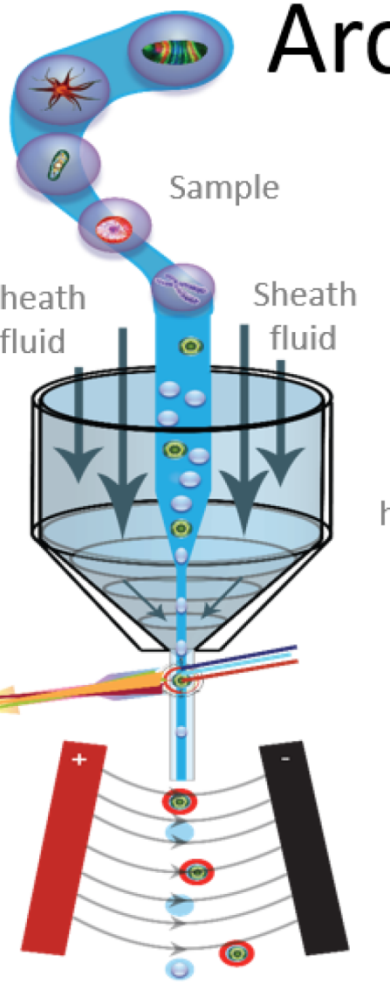


Principles of the flow cytometer

The Cytometer's Architecture

What Flow Cytometer do?
Particles in suspension flow in a single-file (I) across an illuminated volume where they scatter light and emit fluorescence that is collected, filtered (II) and converted into digital values
that are visualized and stored on a computer (III)

What is a Sorter?
Allows the isolation of particles based on the above scatter and fluorescent properties (IV)



Fluidics (I)

Flow chamber

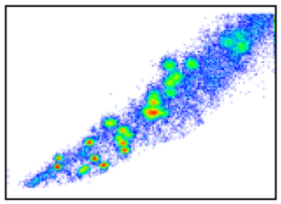
hydrodynamic focusing

Focused lasers

Optics (II)

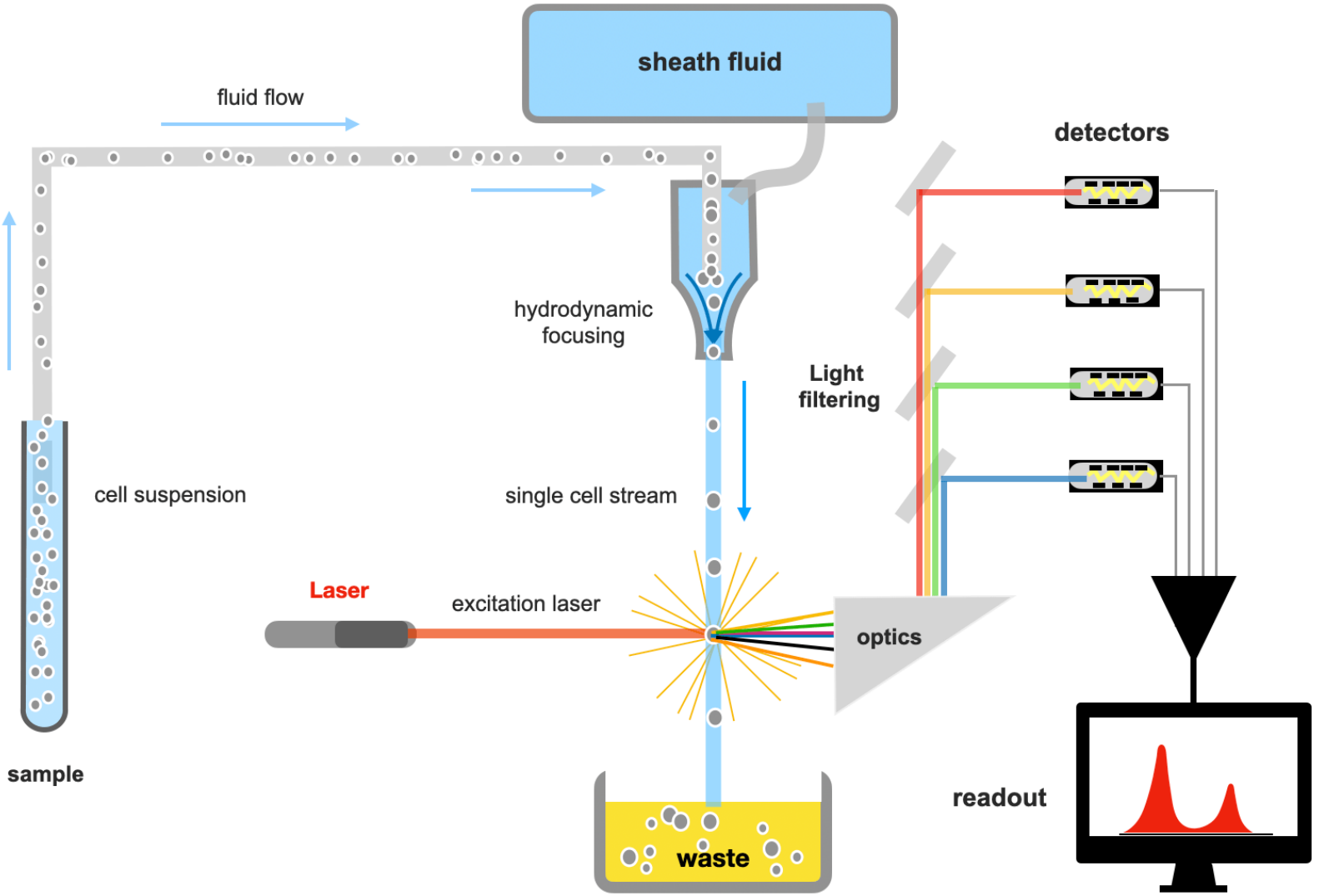
Sorting (IV optional)

Electronics and display (III)



Fluorescence + Light Scatter

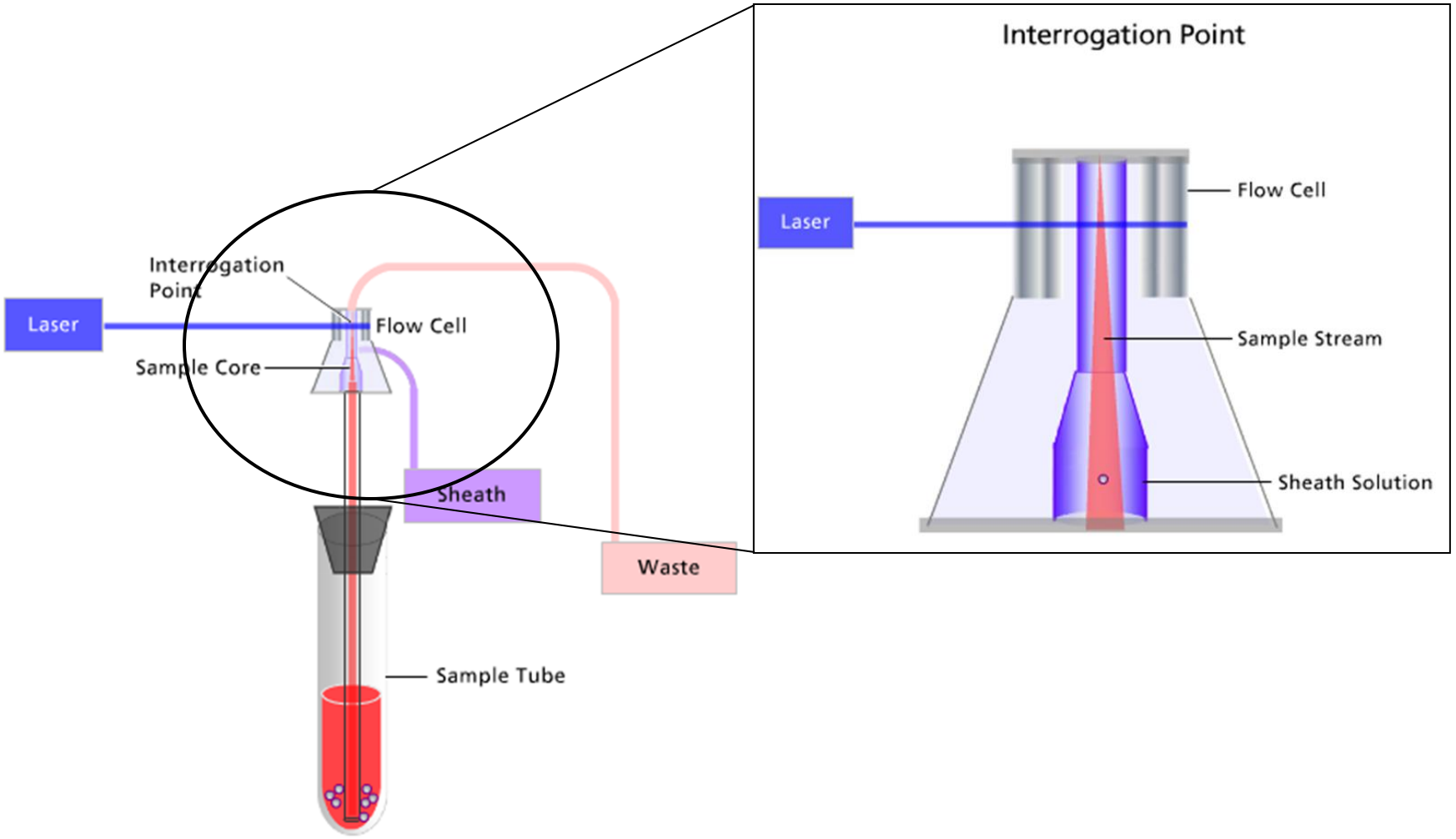
Principles of the flow cytometer



Fluidic Subsystem

- The fluidic subsystem transports particles in a fluidic stream to the laser beam for interrogation.
- The portion of the fluid stream where particles are located is called the sample core.
- The point at which particles meat the laser beam is the interrogation point. Incident laser light is scattered and fluorescence is emitted as particles pass through the interrogation point.

Fluidic Subsystem

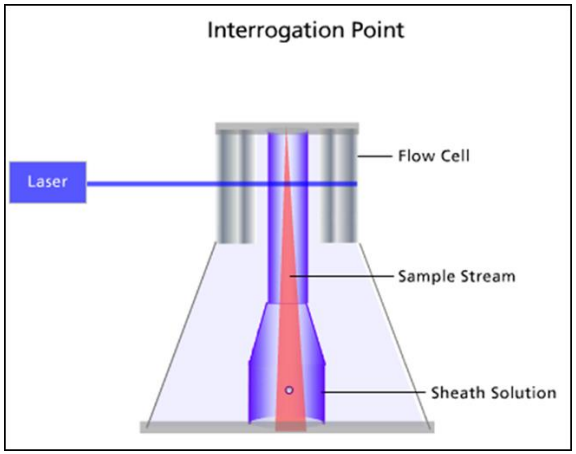


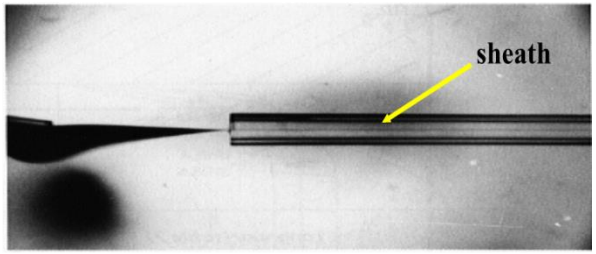
Hydrodynamic focusing

- Hydrodynamic focusing positions the sample core in the center of the sheath fluid.
- The design of the flow cell allows hydrodynamic focusing to occur and provides the means for the cells or particles to pass through the laser beam one at a time for interrogation.

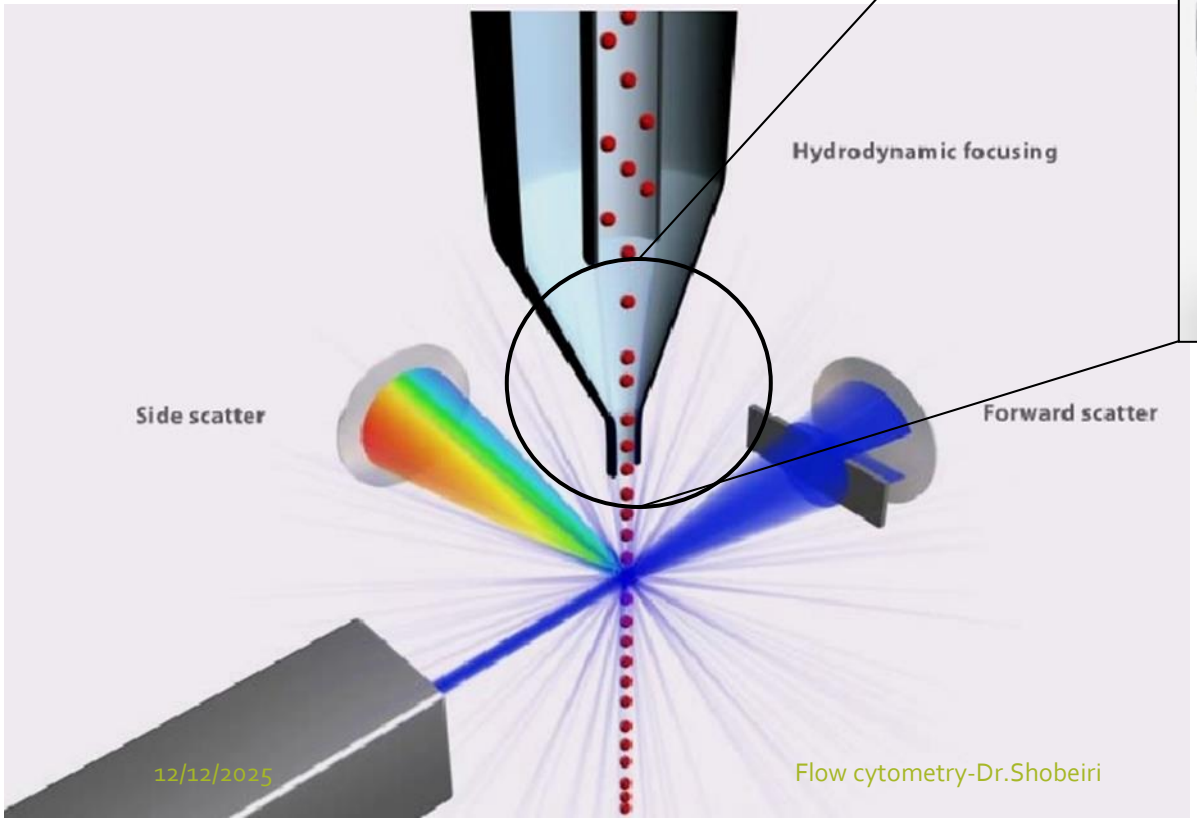
Hydrodynamic focusing

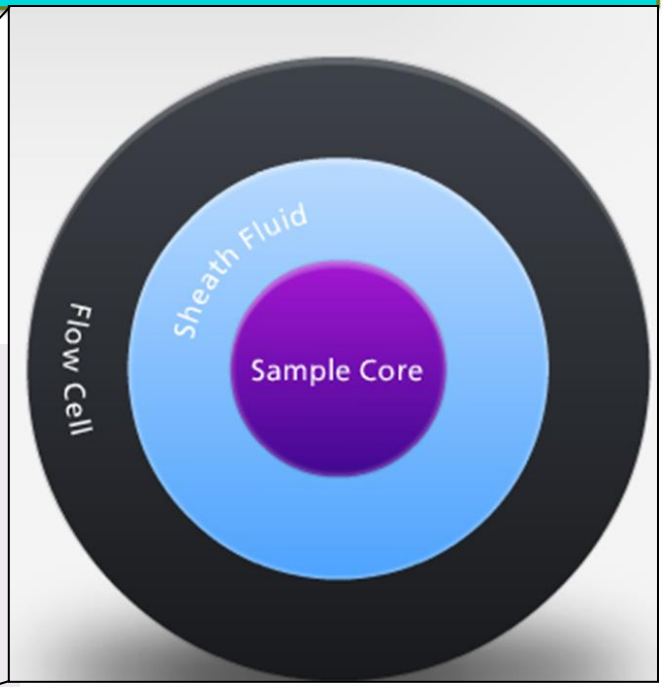
- **Flow cell:** with the flow cell, both the sheath fluid and sample fluid meet. The shape of the flow cell enables hydrodynamic focusing.
- **Sheath Fluid:** the sheath fluid surrounds the sample core. The pressure of the sample fluid can be adjusted to increase or decrease the diameter of the sample core.
- **Sample Core:** as both the sample core and sheath fluid move through a narrowing channel in the flow cell, the sample core remains in the center of the sheath fluid without mixing. This process is called hydrodynamic focusing.





Fluidic Subsystem

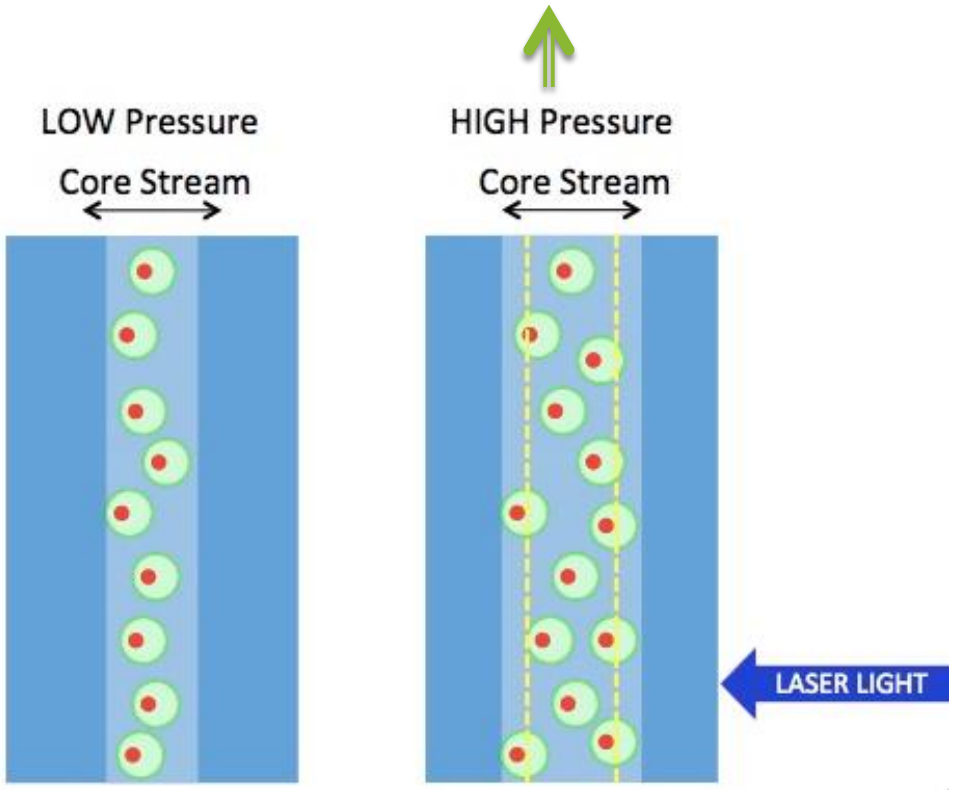




Fluidic Subsystem

Sample Pressure

- Difference in pressure between sample and sheath
  - ✓ control sample volume flow rate
  - ✓ The greater the differential, the wider the sample core.



Sample Pressure and Flow Rate

Flow rate is important from the time of sample preparation

Low sample pressure, low average cell count. This gives greater accuracy, but takes longer

Average Count

240

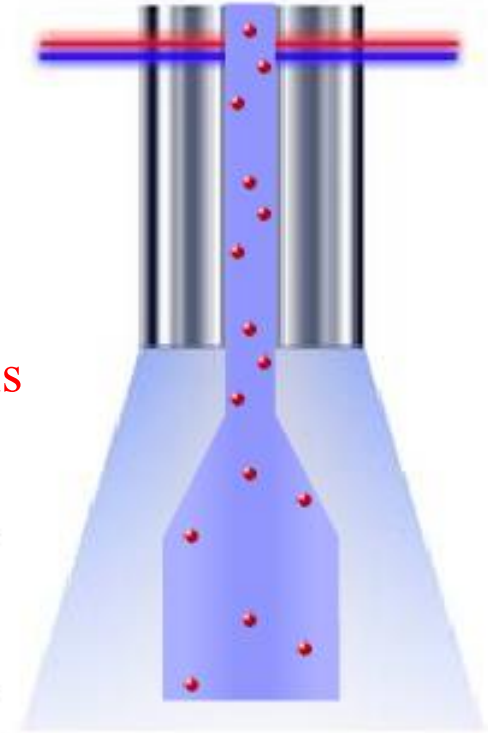
Events/sec

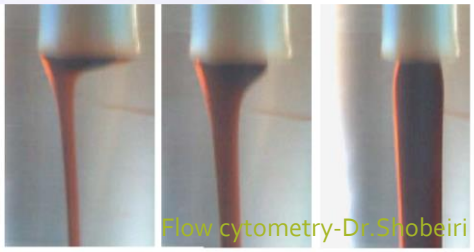
High sample pressure, high average cell count. This gives less accuracy, but is much faster

Average Count

1200

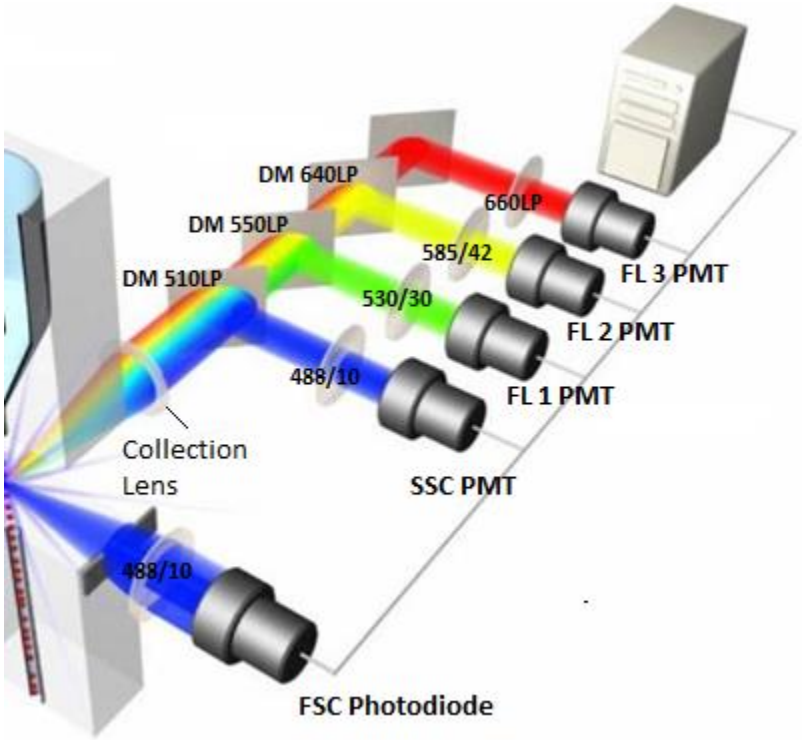
Events/sec





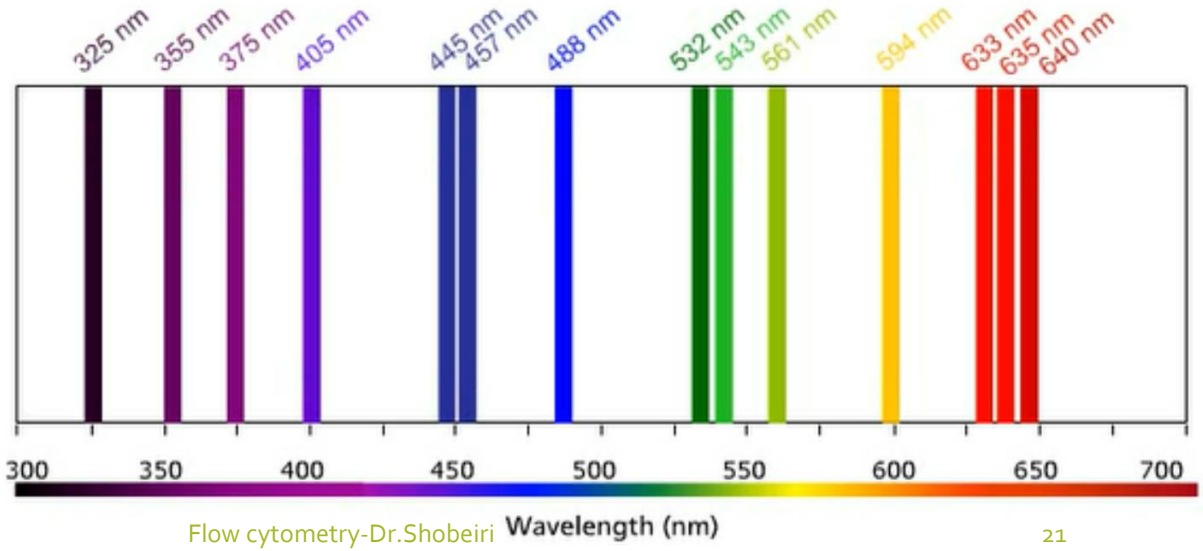
Optic Subsystem
Excitation Components

- The optic subsystem consists of excitation and collection components.
- The excitation components include lasers, beam shaping prisms, and lens to route the laser beams to the sample core.



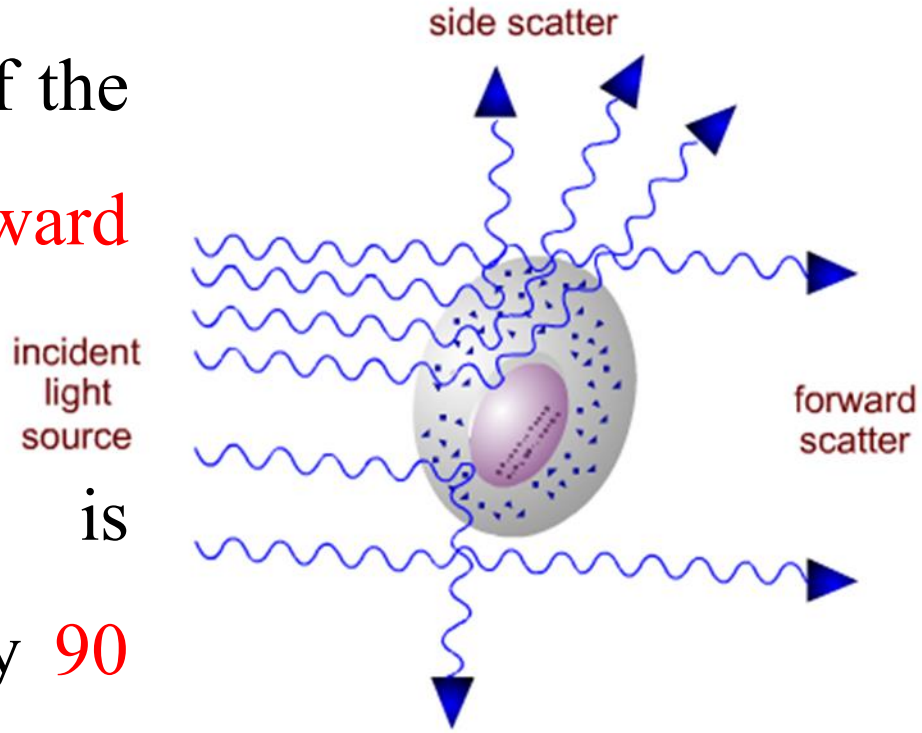
Lasers in Cytometers

- Laser is an acronym for Light Amplification by Stimulated Emission of Radiation. Lasers generate intense beams of coherent light.
- In flow cytometers, lasers serve as the excitation light source for sample interrogation.



Types of Light Scatter

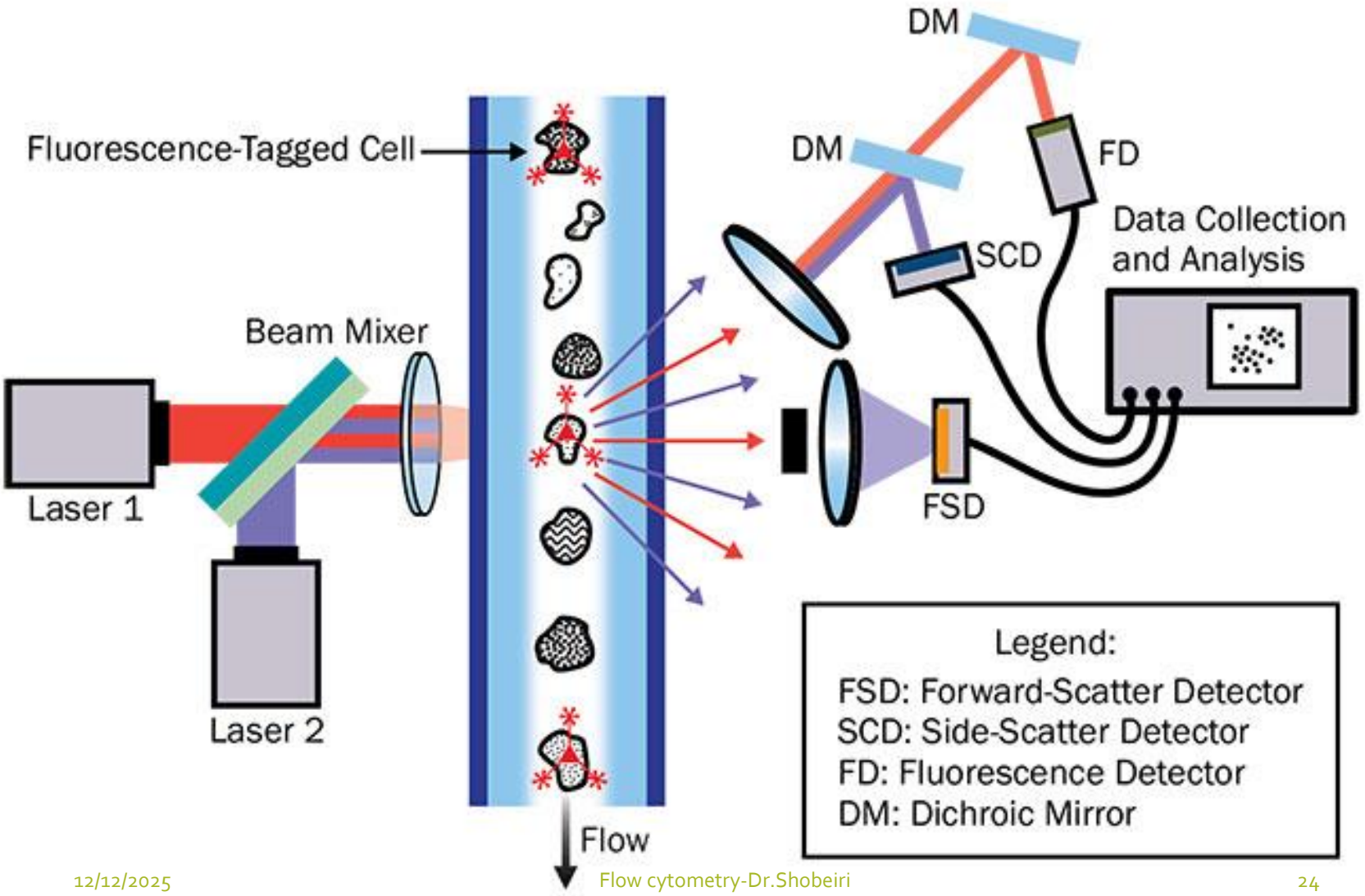
- **Forward Scatter** signal is detected just of the axis of the laser beam in the forward direction.
- **Side Scatter** signal is collected at approximately 90 degrees to the laser beam.



Forward Scatter

- Forward Scatter light is based on two properties: Size and Refractive Index (RI).
  - ✓ The larger the cell size, the more forward scatter is generated.
  - ✓ The larger the difference in RI between the cell and the surrounding sheath fluid, the more forward scatter is generated.

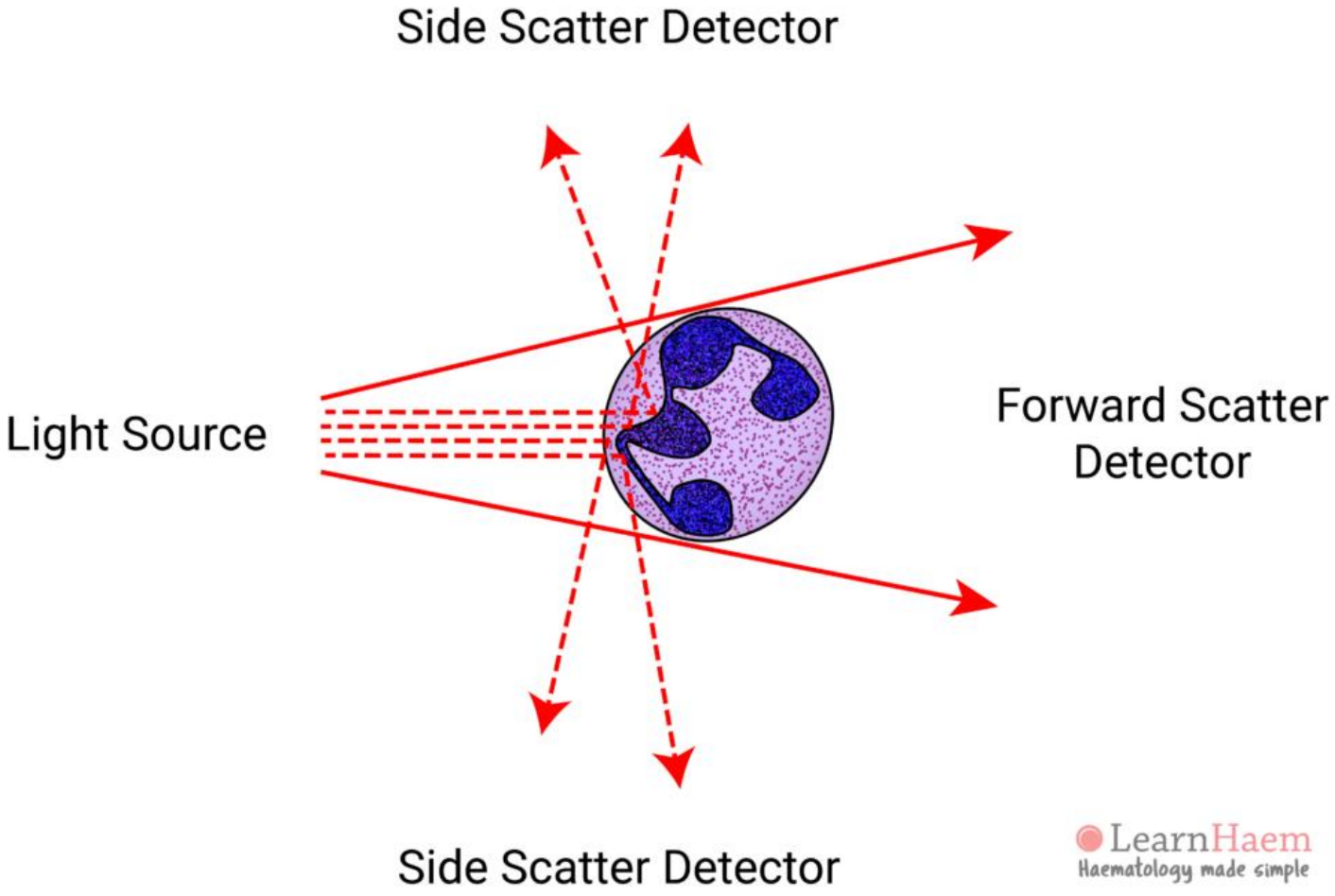
Forward Scatter

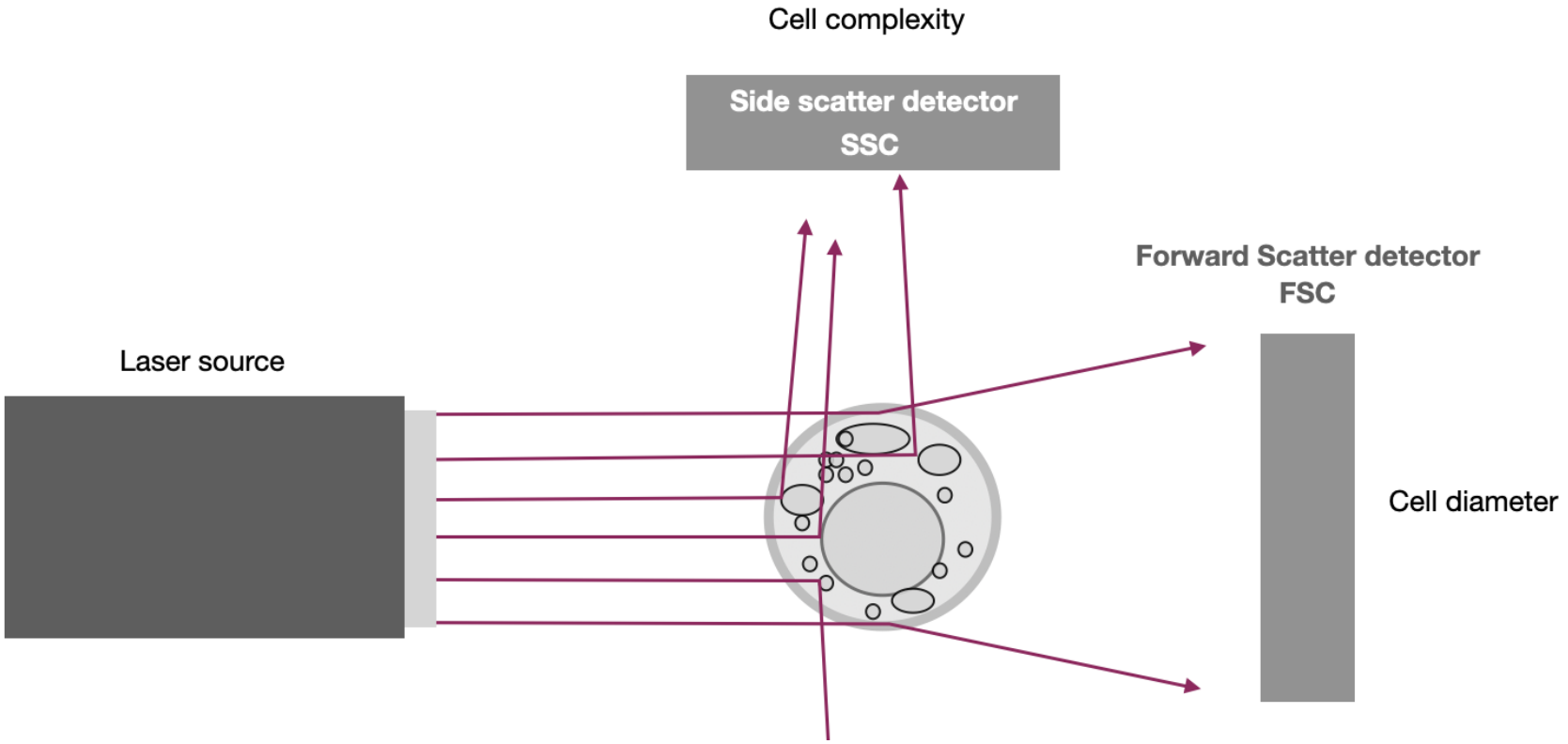


Side Scatter

- Side Scatter light is based on the granularity or internal complexity of a particle.
  - ✓ The more granular the cell, the more side scatter light is generated as a cell or particle passes through a laser.

Side Scatter



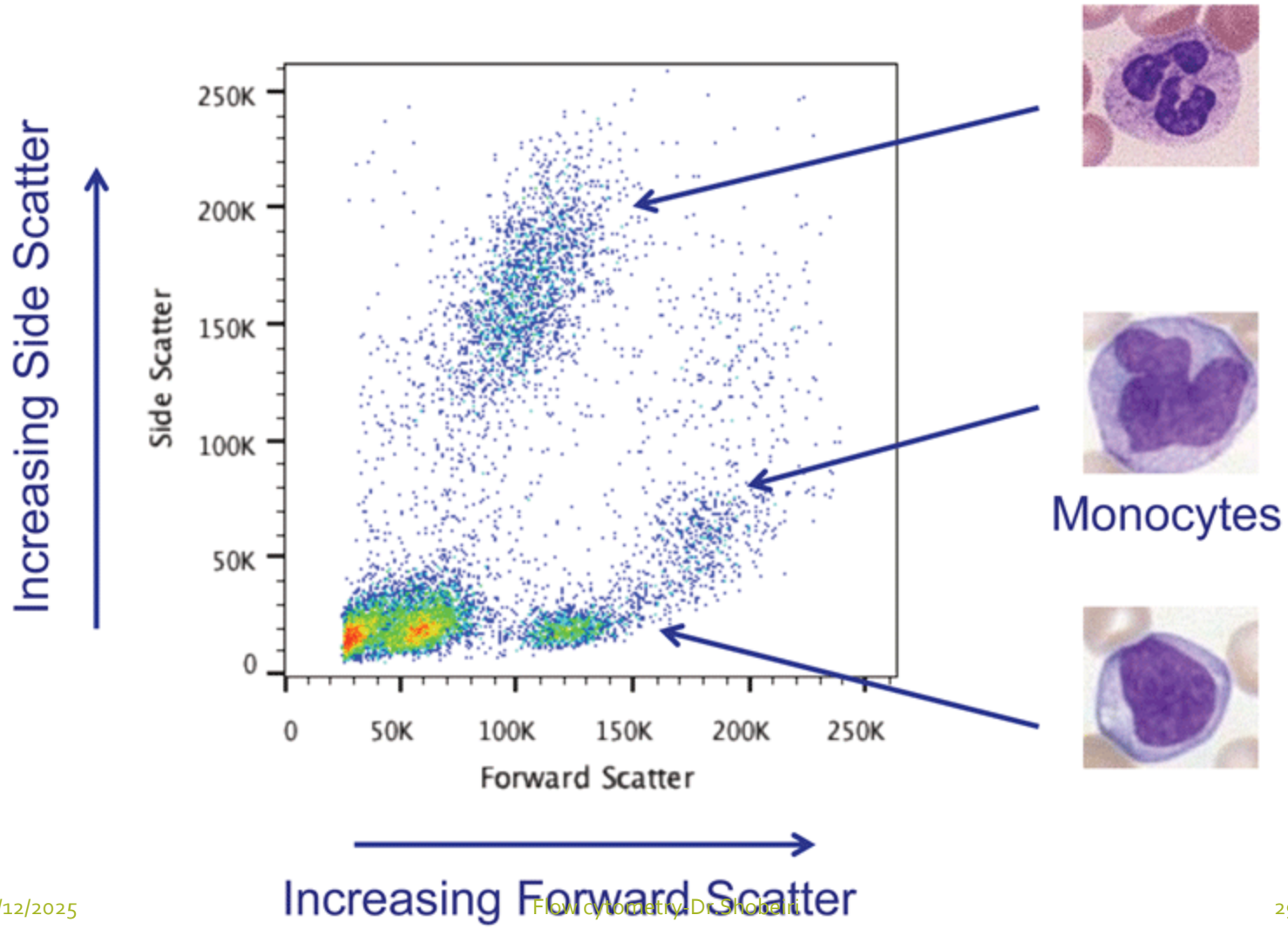

Cell complexity
Side scatter detector
SSC
Forward Scatter detector
FSC
Laser source
Cell diameter

Forward Scatter and Side Scatter of Blood Cells

- A cell's size, refractive index, and internal complexity contribute to the light that is scattered as a cell passes through a laser beam.

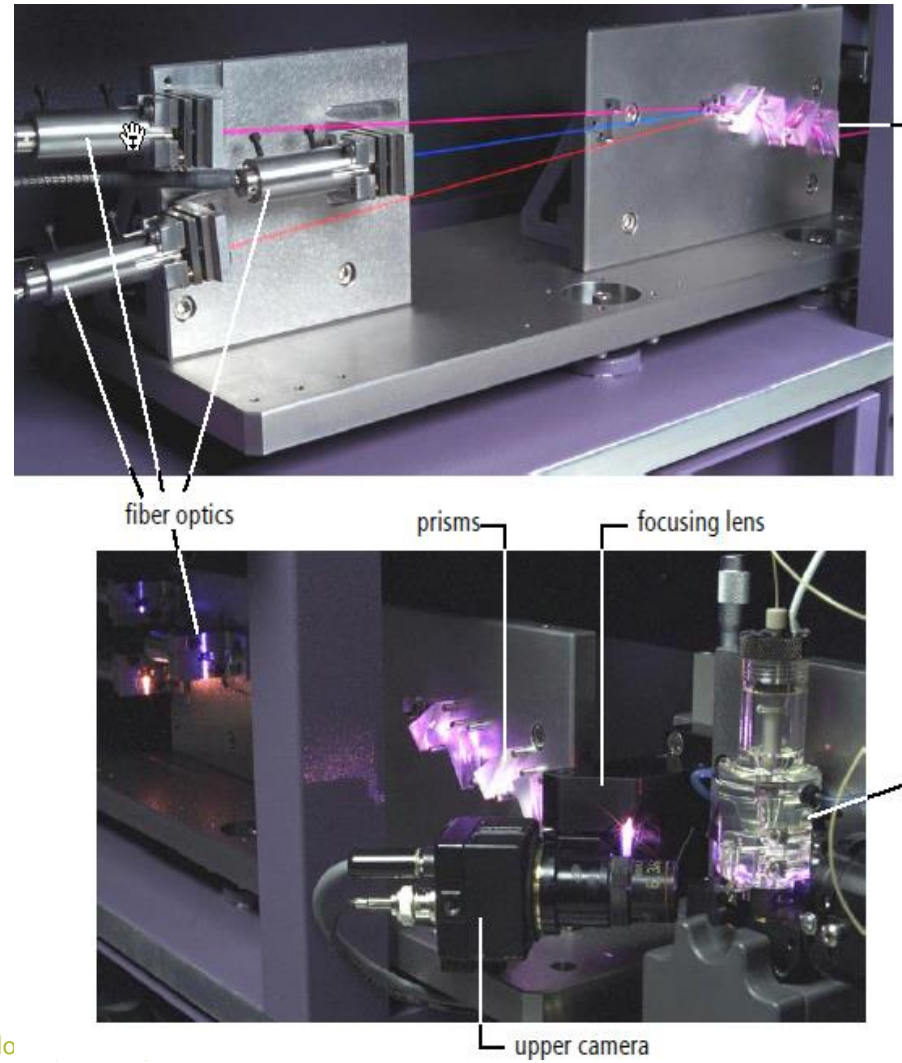
- Proteins on the cell membrane, the number of nuclei, and any granular materials or organelles inside the cell affect light scatter.

Forward Scatter and Side Scatter of Blood Cells



Optic Subsystem
Collection Components

- The positioned lenses collect scattered and fluorescent light.
- The collection components steer the scattered and fluorescent light to appropriate detectors.



Filters in Cytometers

- Optical filters are used to direct specific light wavelengths (or colors) to each photodetector. Filters are in three types:

I. **Longpass Filter:** transmits light that is equal to or longer than the specified wavelength.

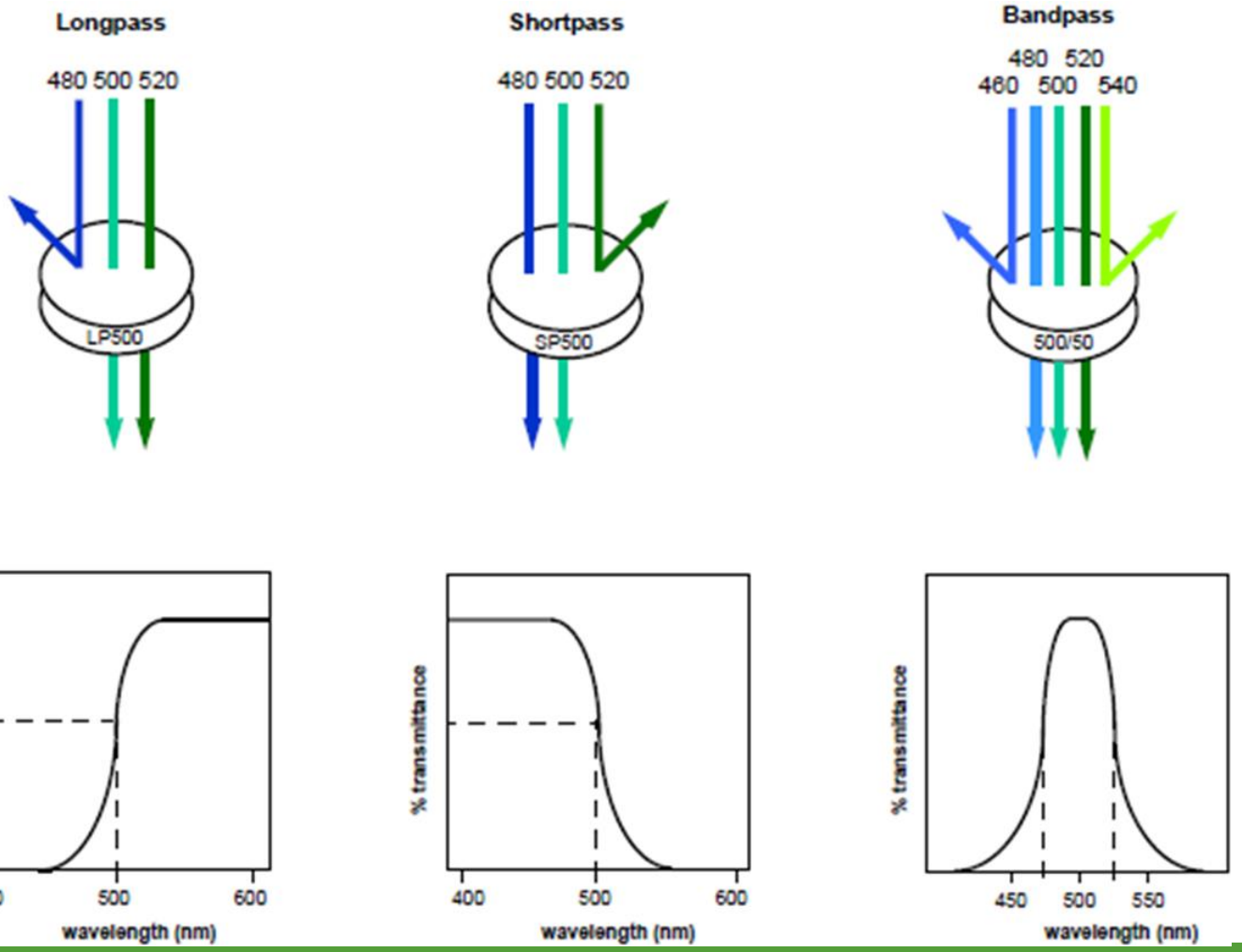
II. **Shortpass Filter:** transmits light that is equal to or shorter than the specified wavelength.

III. **Bandpass Filter:** transmits light centered around the first value, within the range specified by the second value.

Optic Subsystem
Collection Components

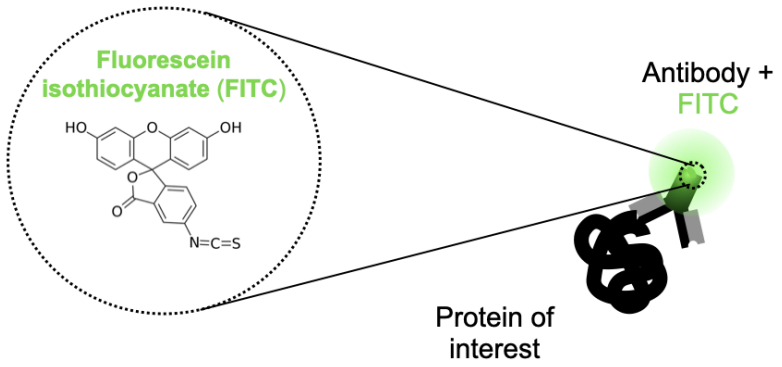
Dichroic Mirrors

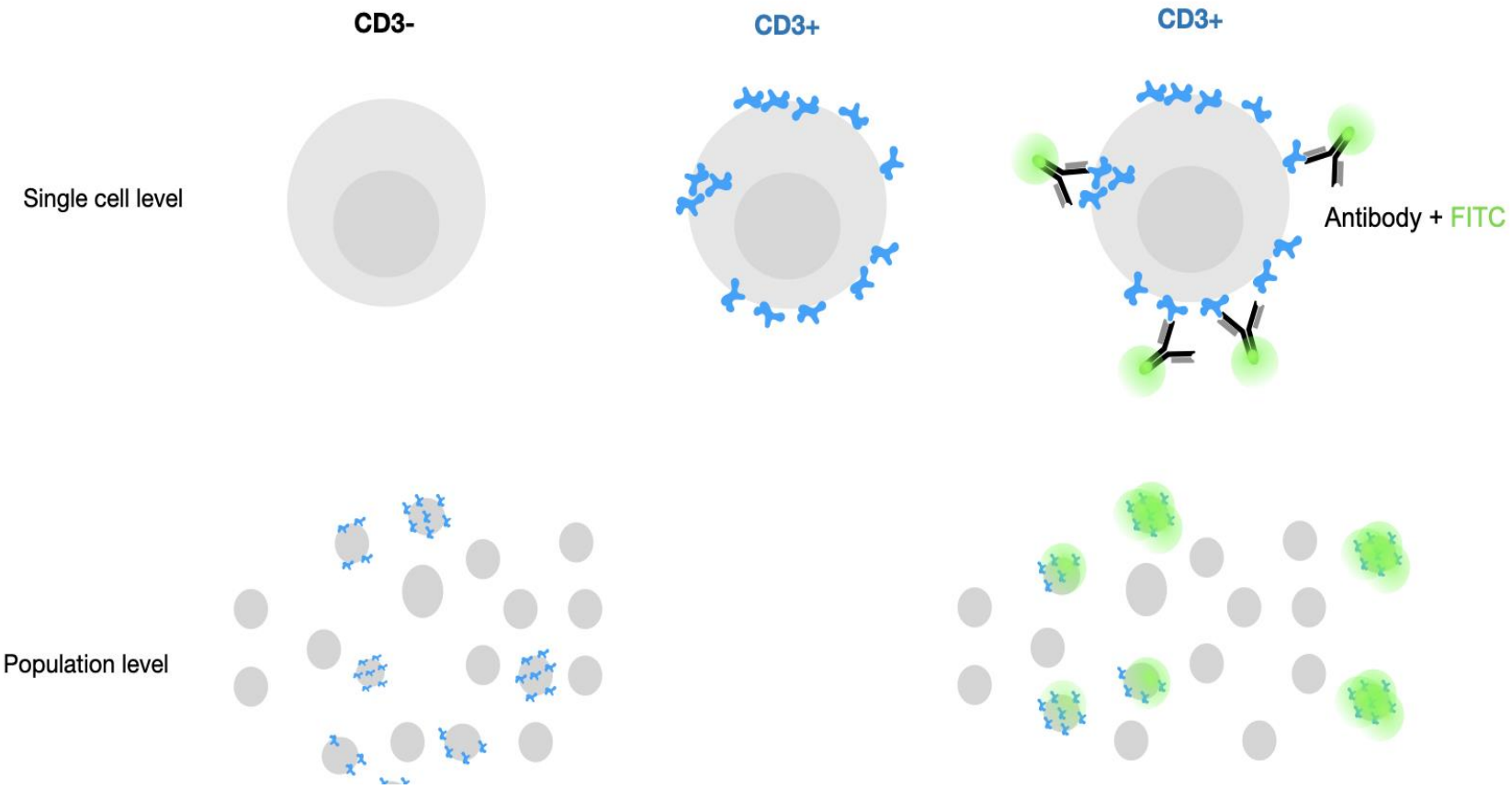
Guide Scatters To Detectors



Cellular Markers and Monoclonal Antibodies

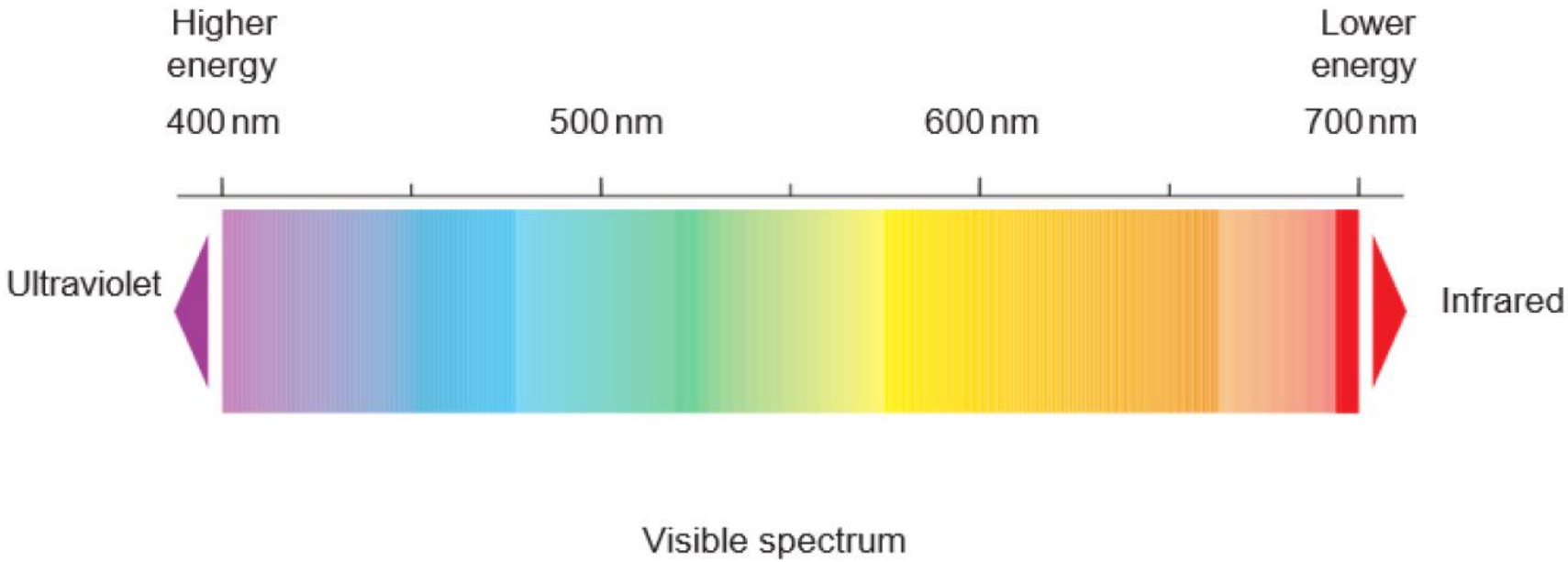
- With the use of antibodies, distinct cell population can be identified by the markers present on the cell surface.
- When a fluorescent dye is conjugated to a mAb, it can be used to identify a particular cell type based on the antigenic markers of the cell.
- A flow cytometer can detect blood cells based on the presence of mAbs with bound fluorochromes.




CD3-
CD3+
CD3+
Single cell level
Antibody + FITC
Population level

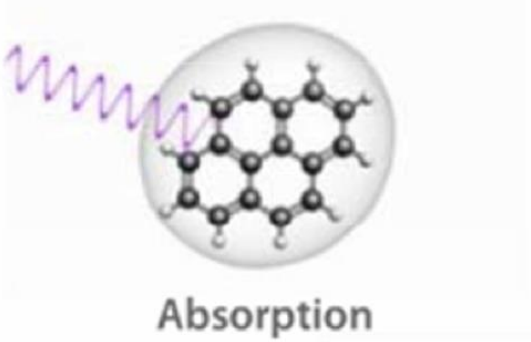
Fluorescence & Photons

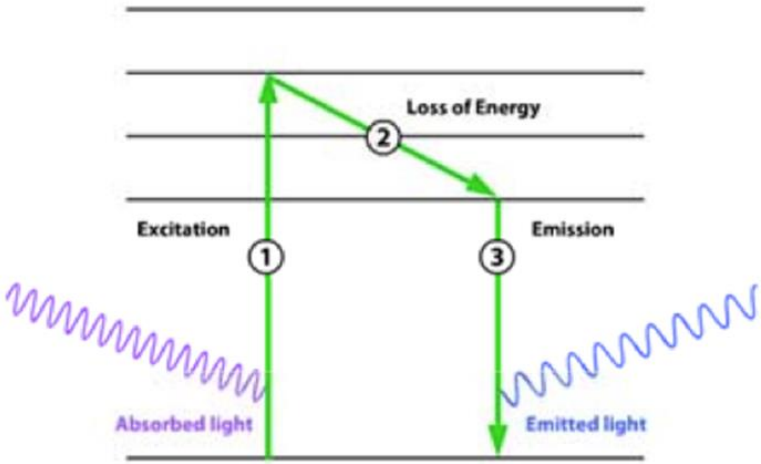
- The spectral range that is utilized in flow cytometry is between **~350 nm to ~800 nm**.
- The wavelength of visible light determines its "color".

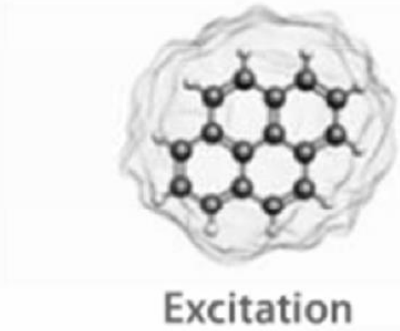


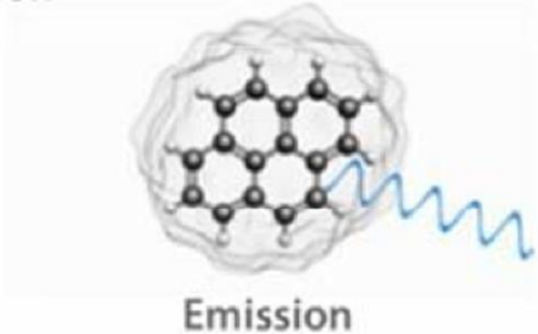
Visible spectrum

Fluorescence & Photons







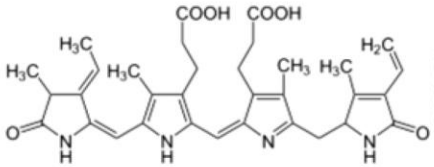


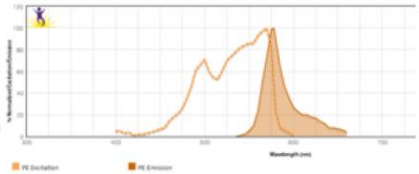
Fluorescent Molecules

Sources of common fluorescent molecules

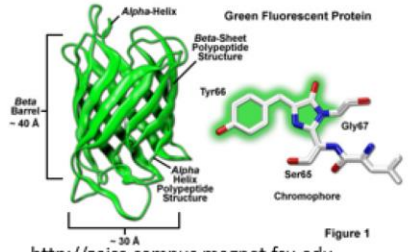
Fluorescent proteins (organic)

Phycoerythrin (PE) - pigment found in seaweed

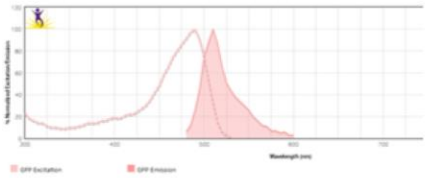




Green Fluorescent Protein (GFP) - often used as a surrogate for intracellular protein expression



http://zeiss-campus.magnet.fsu.edu

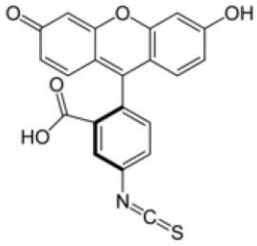


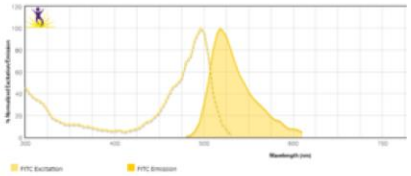
Tandem dyes

Link 2 dyes together. 1 dye is excited and it's emitted light excites the 2nd dye.
The emission of the 2nd dye is detected.

Small synthetic molecules

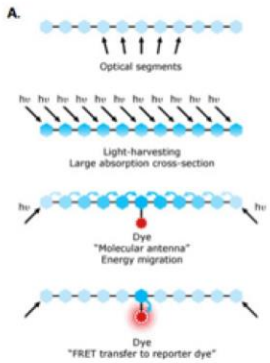
Fluorescein isothiocynate (FITC)

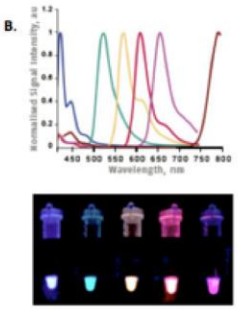




Polymer dyes (inorganic)

Brilliant violet 421



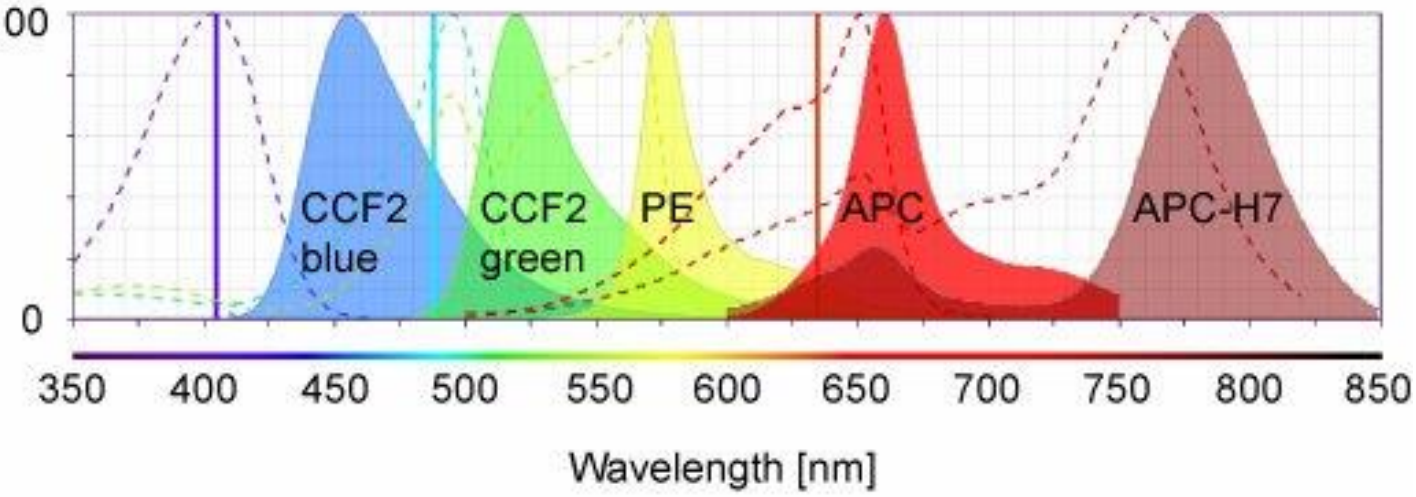


Fluorescent Molecules

FLUOROCHROME	Type of molecule	Typical excitation laser	Approximate emission peak
Fluorescein isotyocyanate (FITC)	Small organic	488 nm	518 nm
AlexaFluor 488	Small organic	488 nm	518 nm
Phycoerythrin (PE)	Protein	488 or 532 nm	574 nm
PE-Texas Red	Protein tandem	488 or 532 nm	615 nm
PE-Cy5	Protein tandem	488 or 532 nm	665 nm
Peridinin chlorophyll protein (PerCP)	Protein	488 or 532 nm	676 nm
PerCP-Cy5.5	Protein tandem	488 or 532 nm	695 nm
PE-Cy7	Protein tandem	488 or 532 nm	776 nm
Allophycocyanin (APC)	Protein	633 nm	659 nm
AlexaFluor 647	Small organic	633 nm	667 nm
AlexaFluor 700	Small organic	633 nm	718 nm
APC-Cy7	Protein tandem	633 nm	784 nm
Pacific Blue	Small organic	405 nm	454 nm
AmCyan	Protein	405 nm	487 nm

Fluorescent Molecules

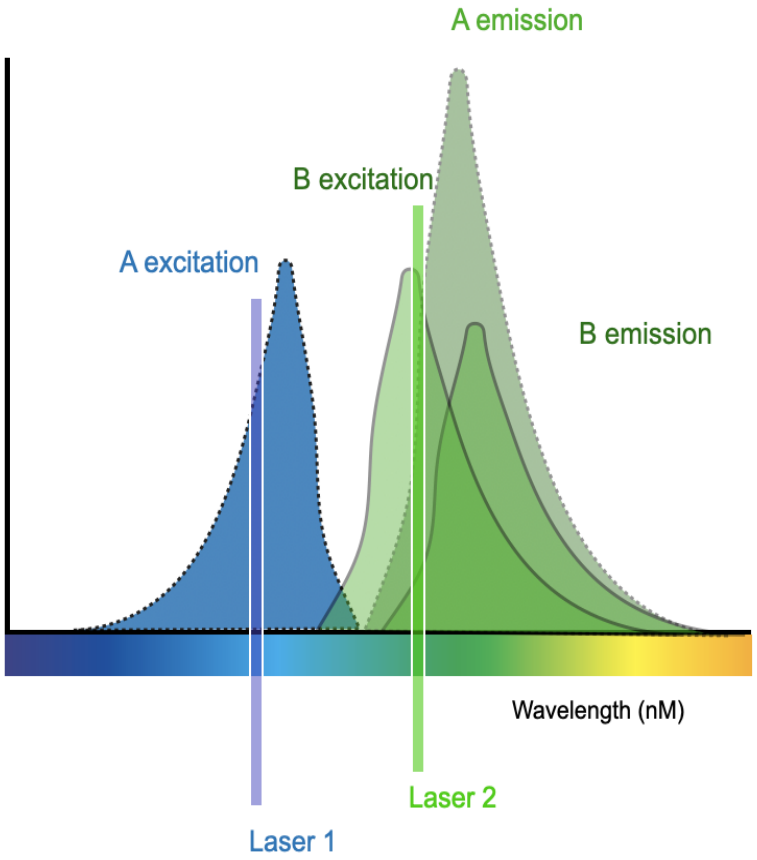
A

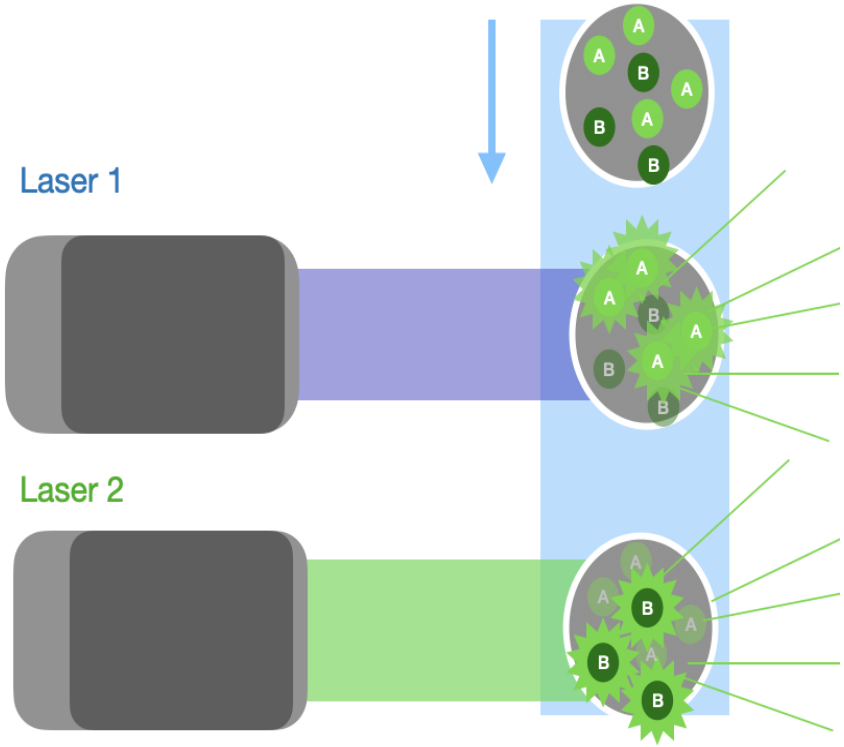


B

Fluorochrome	Ex_{max} [nm]	laser [nm]	Em_{max} [nm]
CCF2 - blue	409	405	447
CCF2 - green	409 (488)	405	520
PE	(493) 566	488	575
APC	650	635	660
APC-H7	(650) 757	635	780

- ✓ In some cases, laser are arranged co-linear and so they fire at the same time.
- ✓ An alternative setup is parallel where lasers are parallel to one another and are aimed at different points of the flow stream and fire at different times.





Electronics System

- **The main functions of the electronics system are to:**

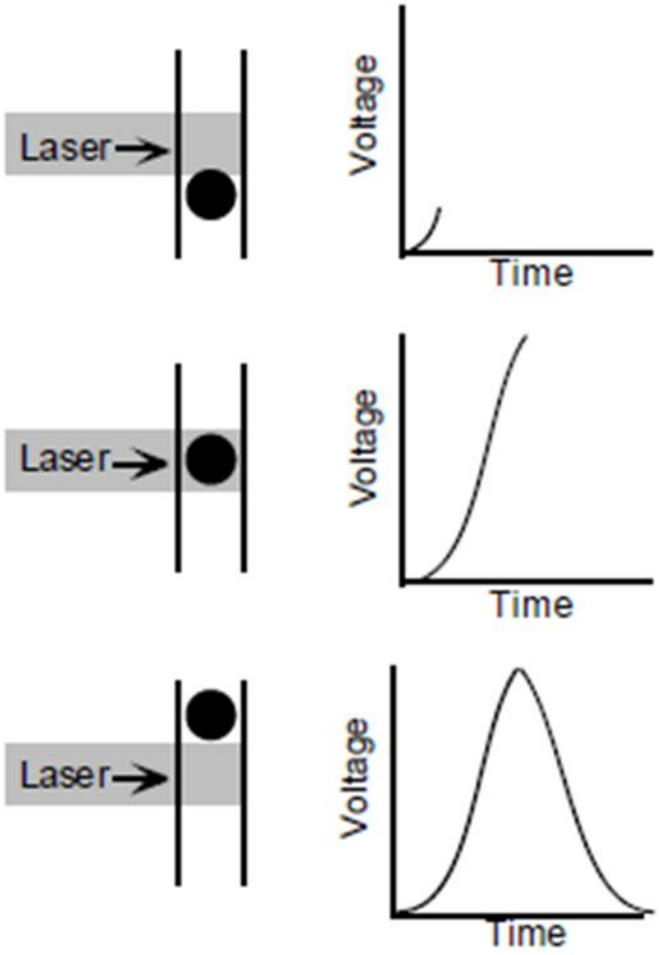
I. **Convert light signals into numerical data.**

II. **Eliminate small signal events such as noise and debris through threshold.**

III. **Attribute signals from multiple lasers to the correct cell or particle.**

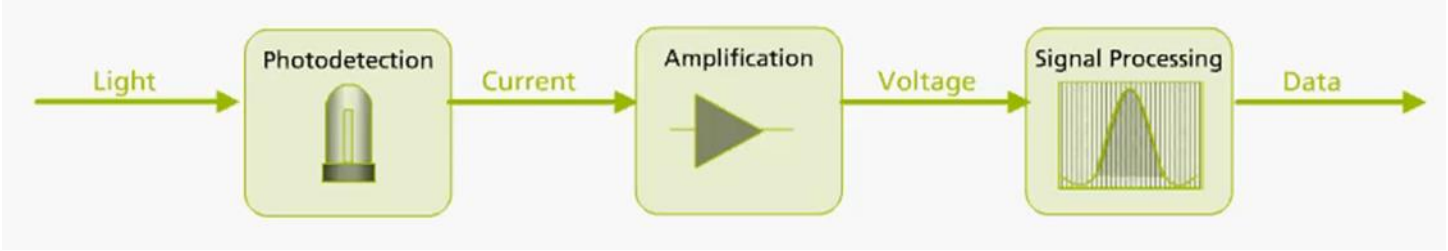
Electronics Subsystem

- The detectors in the electronics subsystem convert detected light into proportional electronic signals.
- Electronic signals are then digitized, further processed, and sent to the computer.
- Data for each cell or particle is stored in the computer and plotted in various ways for further analysis.



How Is Light Converted Into Data?

- To convert light into data, three components are needed: Photodetectors, Amplifiers, and Signal Processors.



- These electronic components work in a sequence to convert light into current, current into voltage, and voltage into data that can be further analyzed.

Photodetection

- Photodetectors are light sensors that can detect photons of light.
- Incoming photons cause photodetectors to produce electrical current.
- **Types of photodetectors:**
  1. **Photodiode**
  2. **Avalanche Photodiode**
  3. **PhotoMultiplier Tube (PMT)**

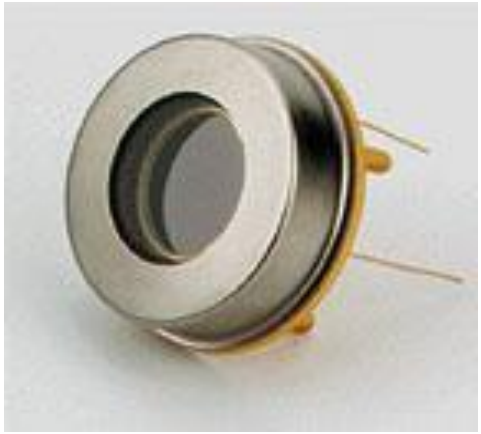
Photodetection

Photomultiplier tubes (PMT)

- ✓ "Old" but good technology
- ✓ The most common detector used in flow
- ✓ High sensitivity but poor quantum efficiencies in red (>650nm)
- ✓ Able to adjust the gain to over 100,000
- ✓ Common
- ✓ Inexpensive

Photodiode (PD)

- ✓ New[er] technology, still not common
- ✓ High quantum efficiencies for visible
- ✓ No internal gain adjustment
- ✓ Requires Cooling
- ✓ Require a high voltage bias



Amplification

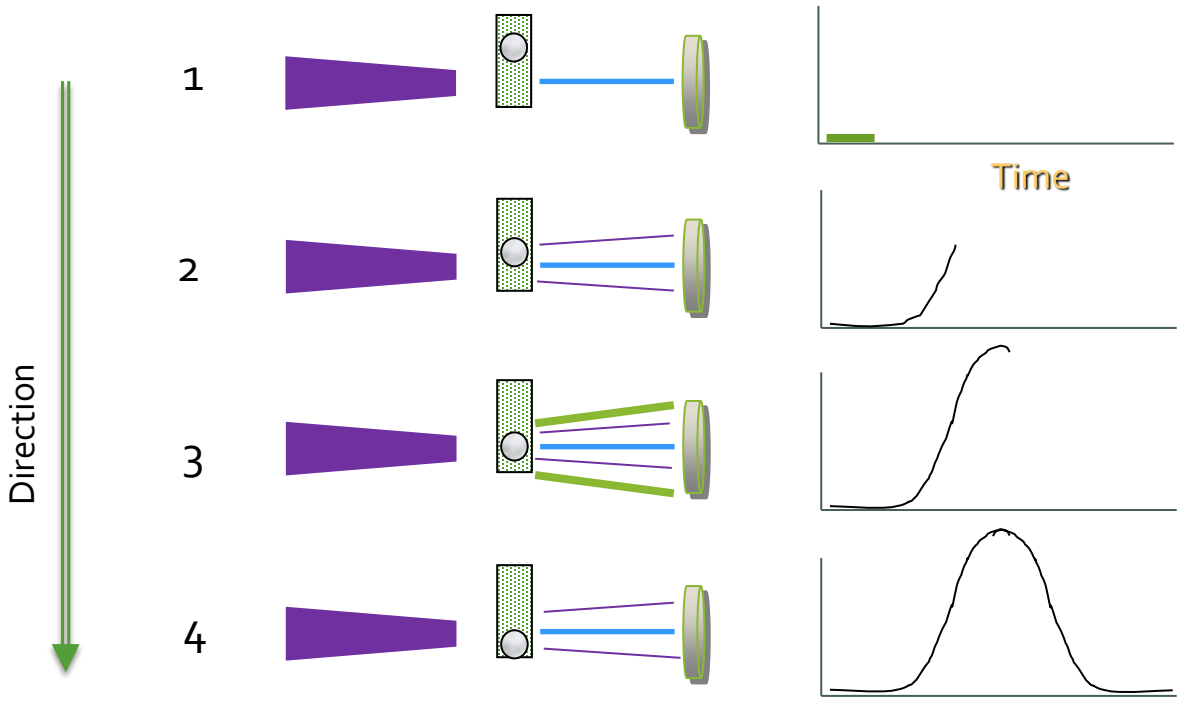
- Amplifiers convert electrical current from photodetectors into a voltage.

- The resulting voltage are larger in magnitude than the incoming currents.

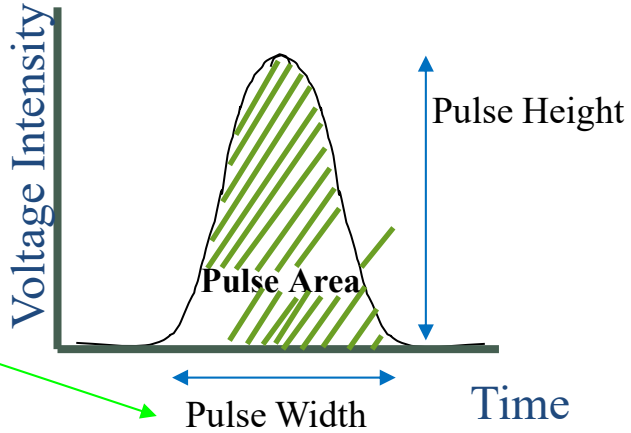
Signal Processing

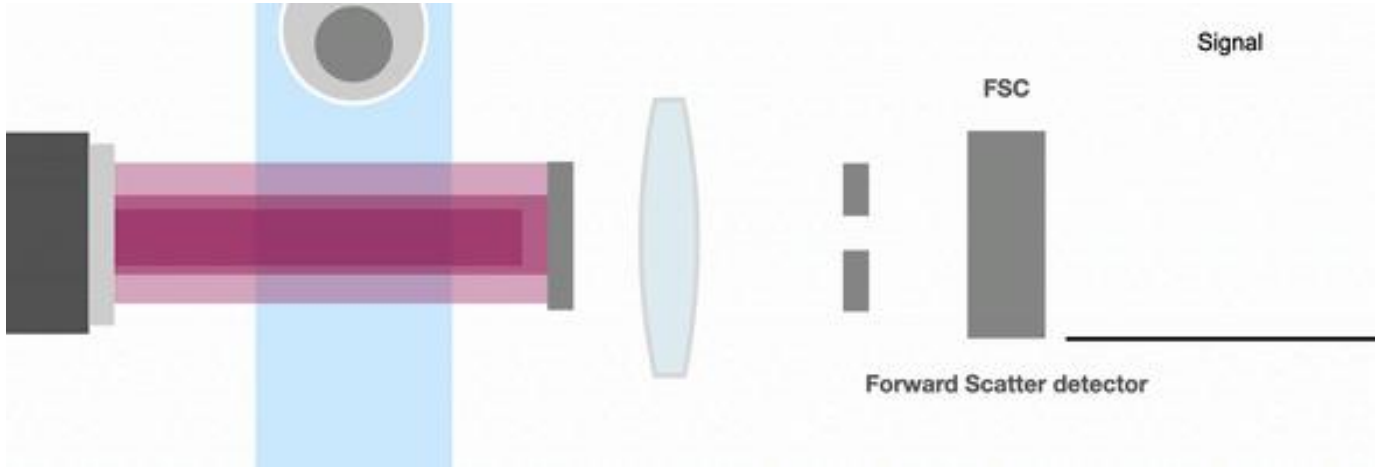
- Signal Processors quantify voltage pulses, providing numerical values for pulse height, width, and area.

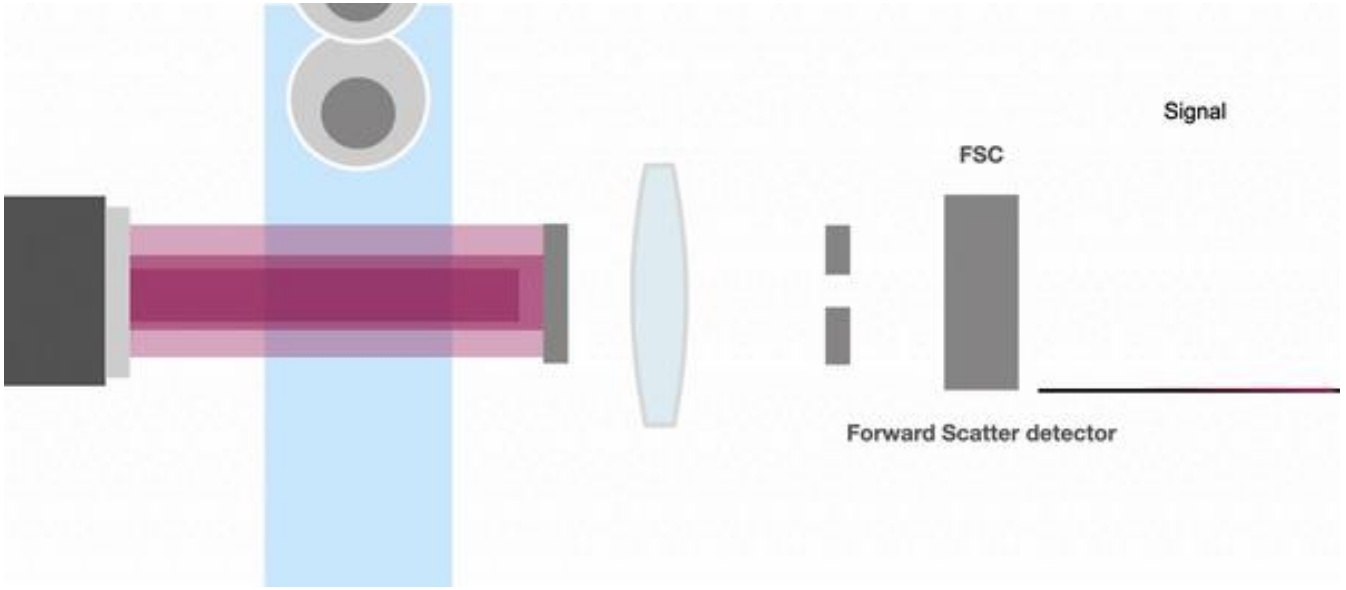
Signal Processing



Width is useful for <u>doublet</u>
Cell discrimination in analysis




Signal
FSC
Forward Scatter detector


Signal
FSC
Forward Scatter detector

Data Display in Different Plot Types

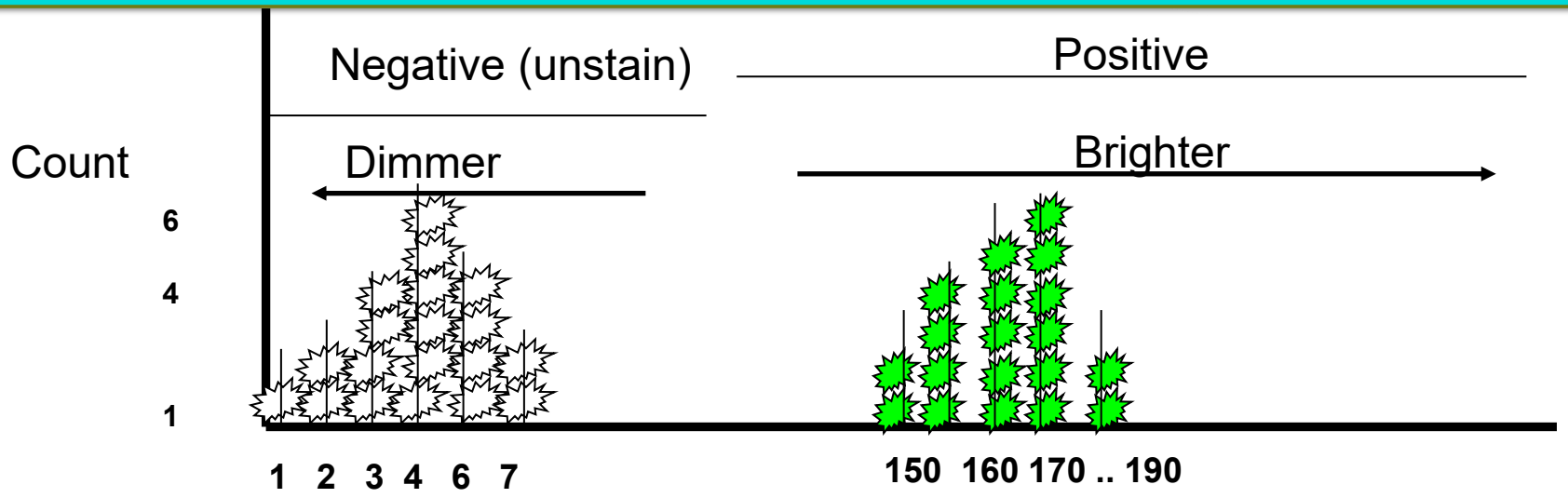
❑ Histogram Plot:

- ✓ Display a single parameter against the number of events (counts).
- ✓ Events with higher fluorescence intensity appear to the right on the X-axis.

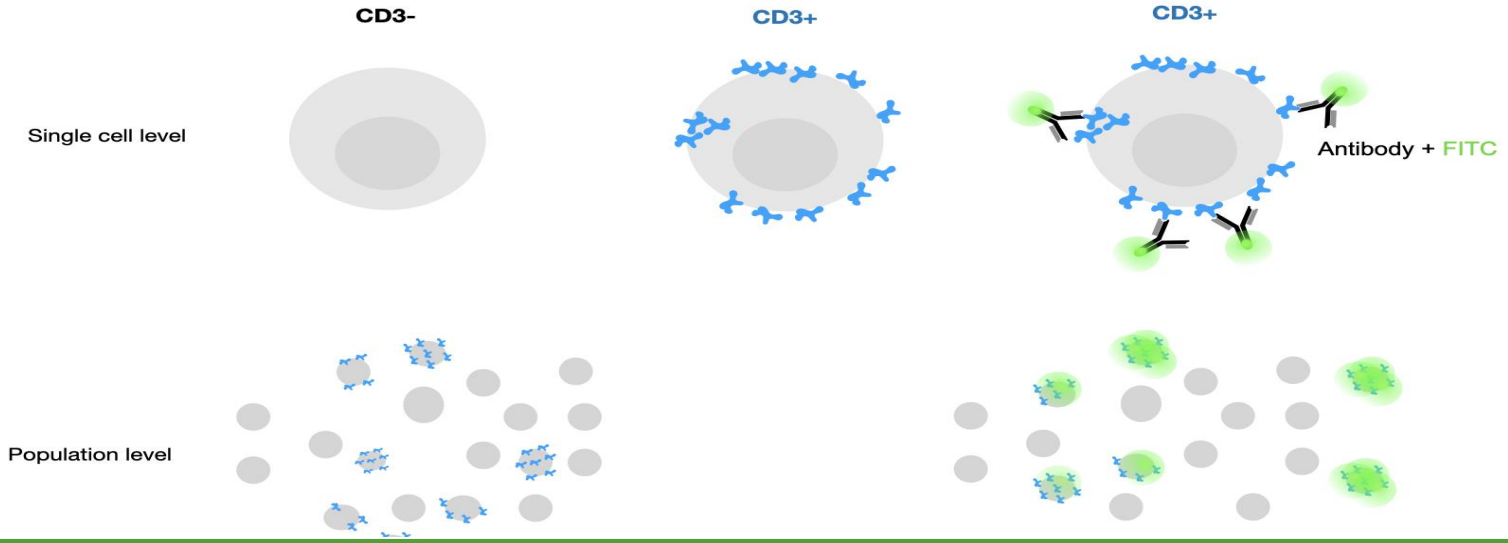
❑ Dot Plot:

- ✓ Displays two parameters simultaneously.
- ✓ Each dot represents one or more events.
- ✓ Dots appear farther to the right on the X-axis or higher on the Y-axis as signal intensity increases.

Histogram Plot



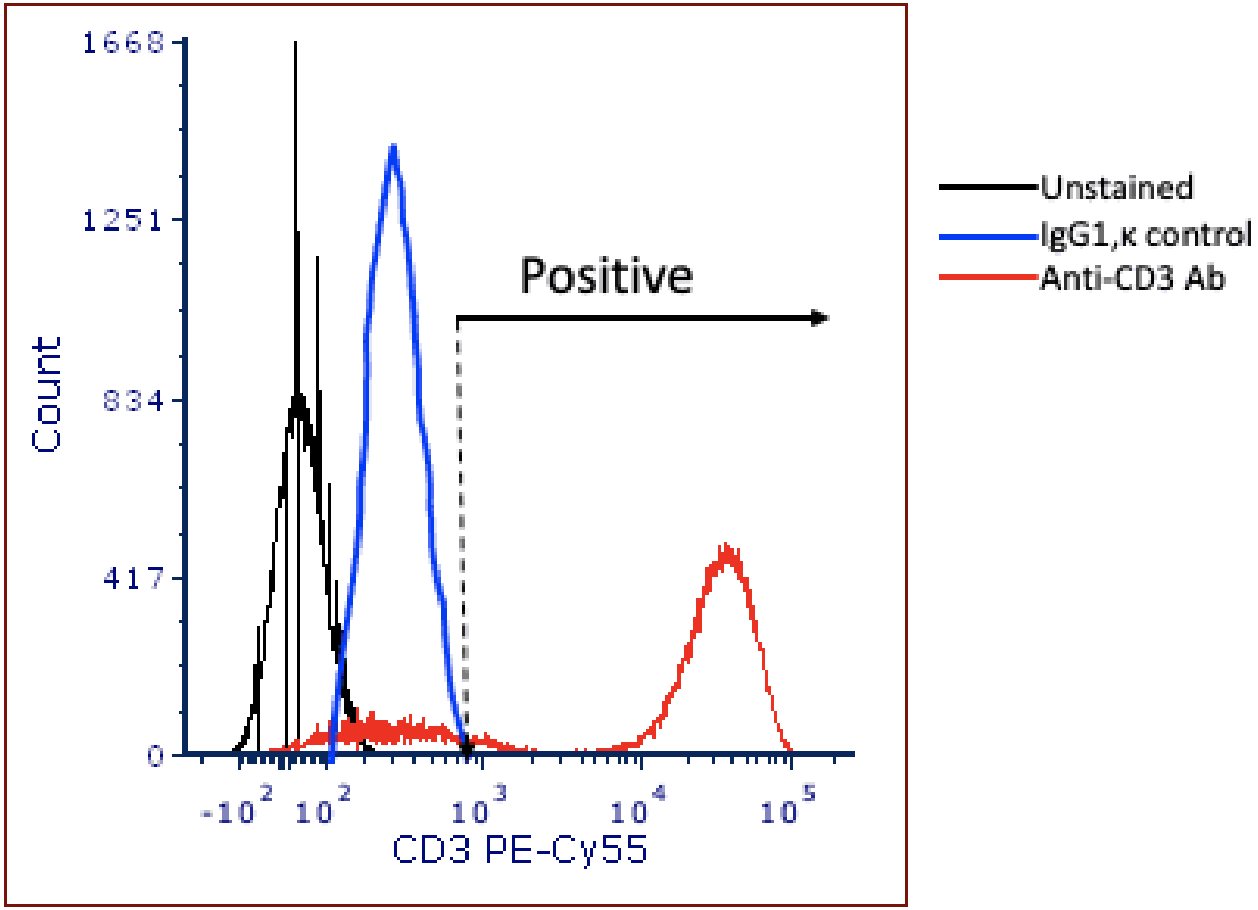
Fluorescence picked up from the FITC PMT



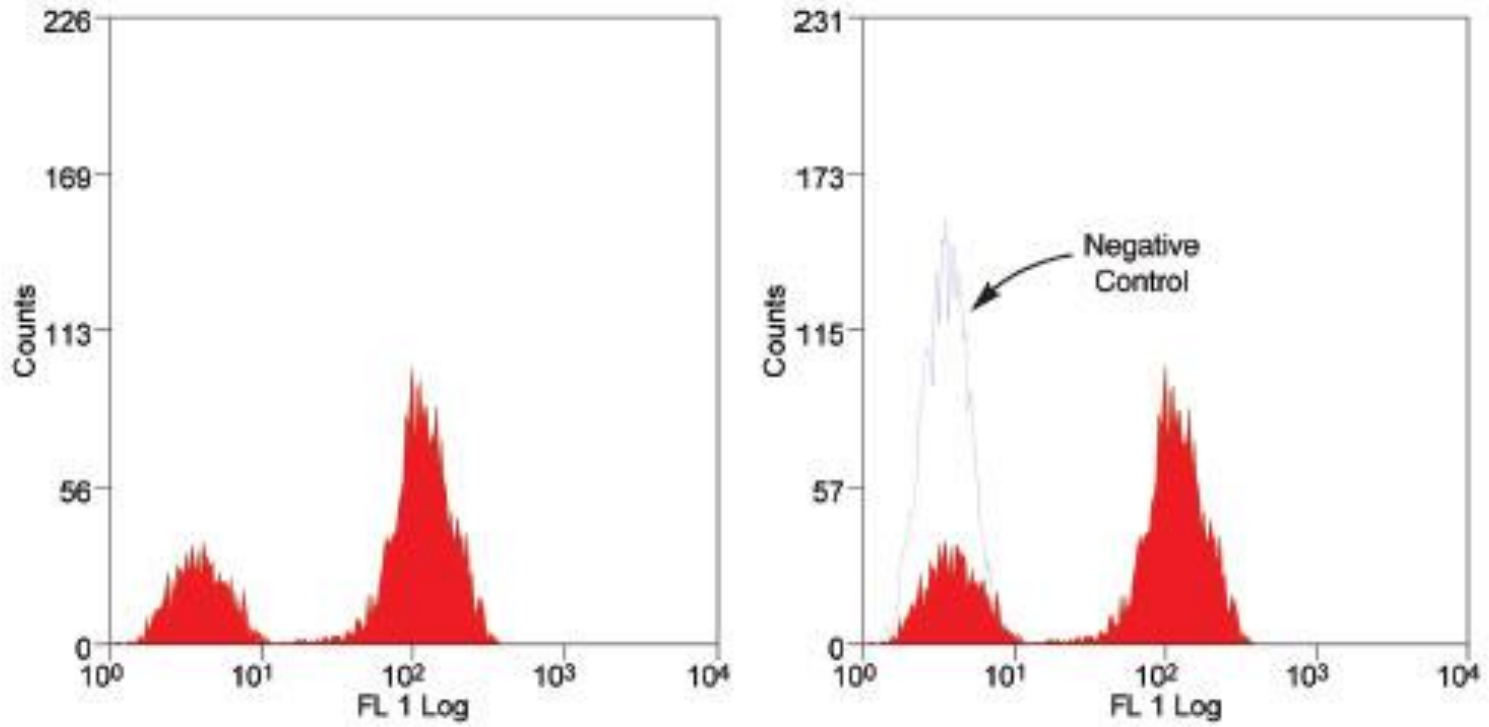
Isotype Controls

It is an antibody that has the same isotype as the experimental antibody, but has been produced against something that is not present on the cells of interest.

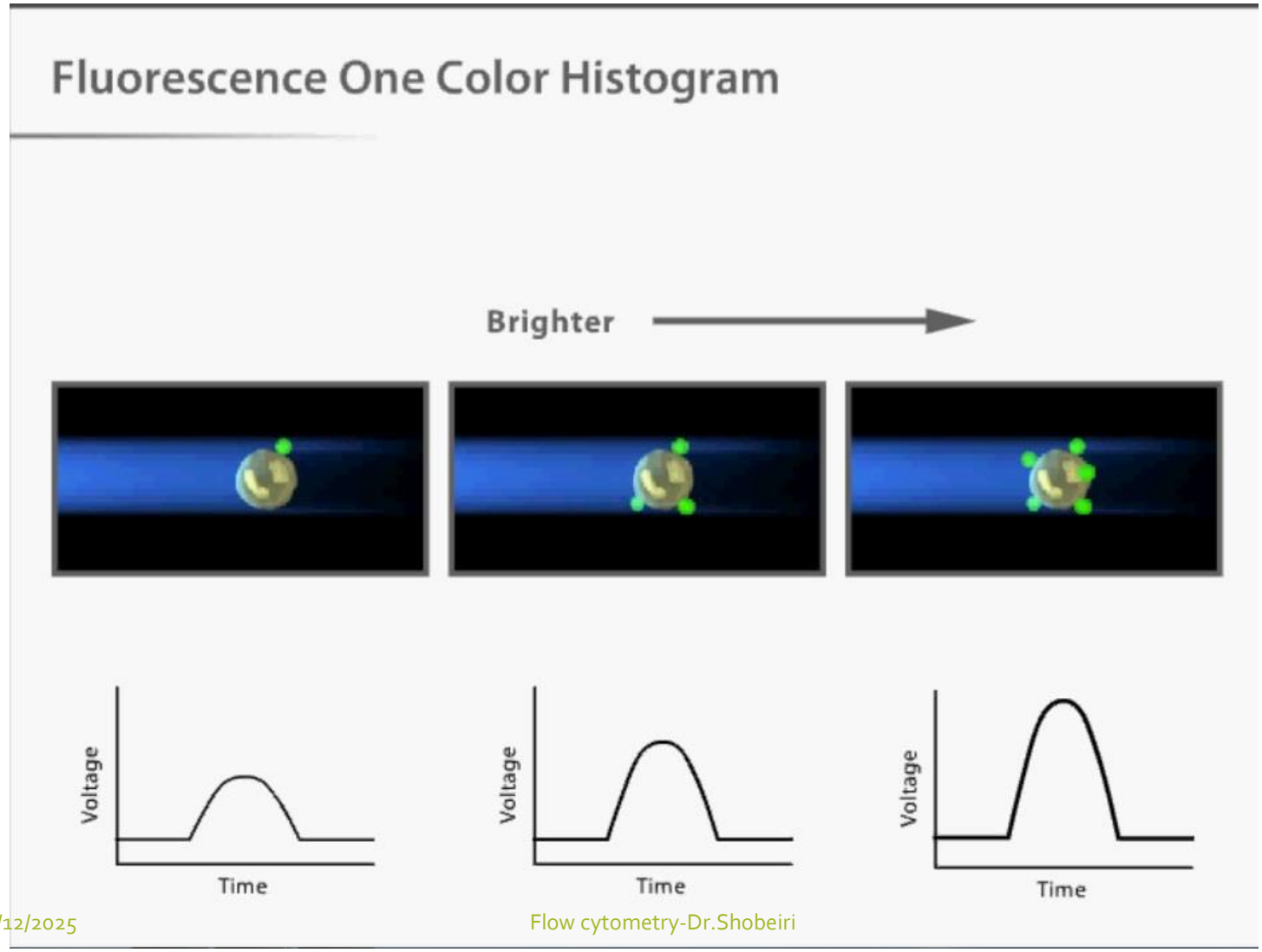
For example, clone UCHT1, (mouse anti-human CD3) has an isotype of IgG1,κ. If there were concerns over non-specific binding of this antibody on the cells of interest, one might label a different tube with an isotype control such as MOPC-21, which is also isotype of IgG1,κ. This antibody was made from mouse myeloma, and has an unknown specificity. So the theory goes that if the MOPC-21 binds to the cells, the researcher would set the positive gate above staining levels of the isotope control



Histogram Plot

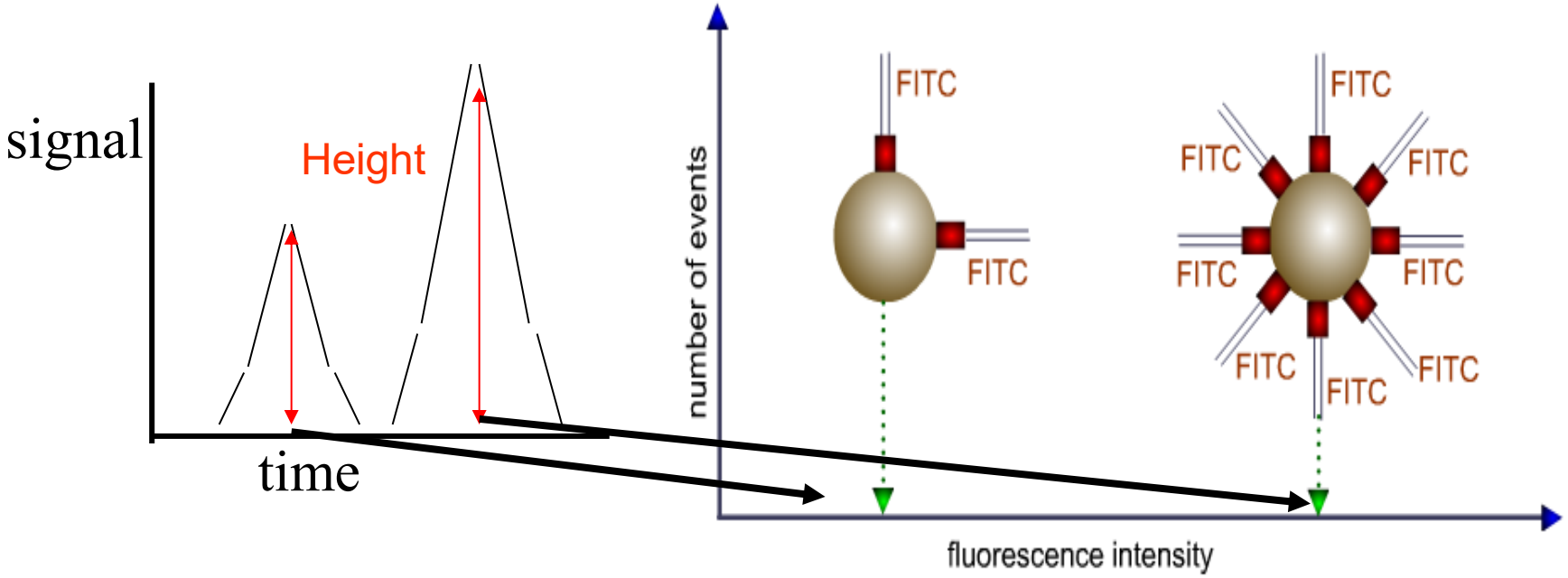


Histogram Plot



Histogram Plot

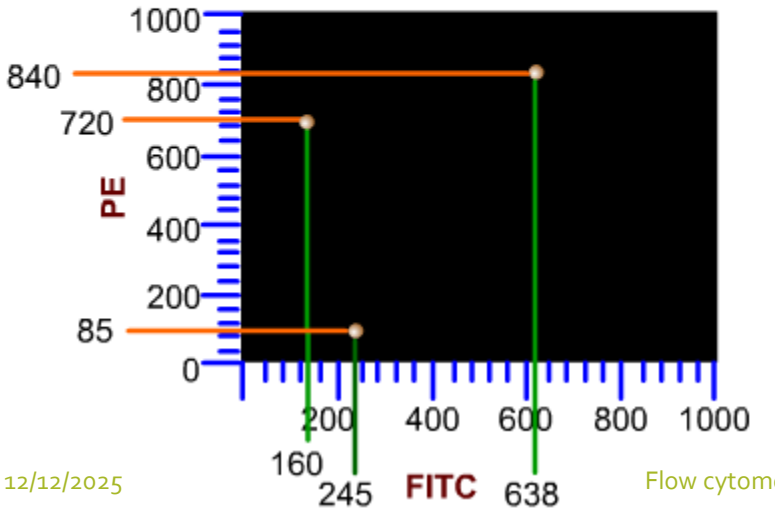
✓The place of cells in plots is depended on their fluorescence intensity or Heights of signals in detectors.

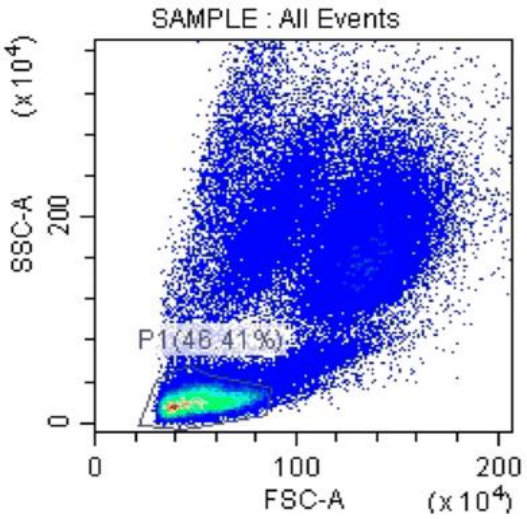


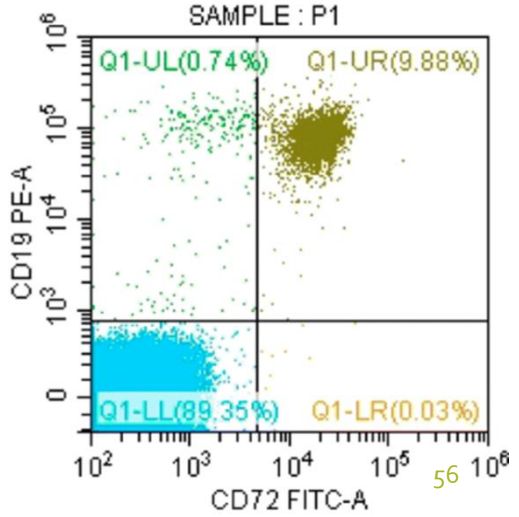
Dot Plot

- Each Dot is a cell
- Use to inspection of cell condition for two parameter, FSC vs SSC or FITC vs PE

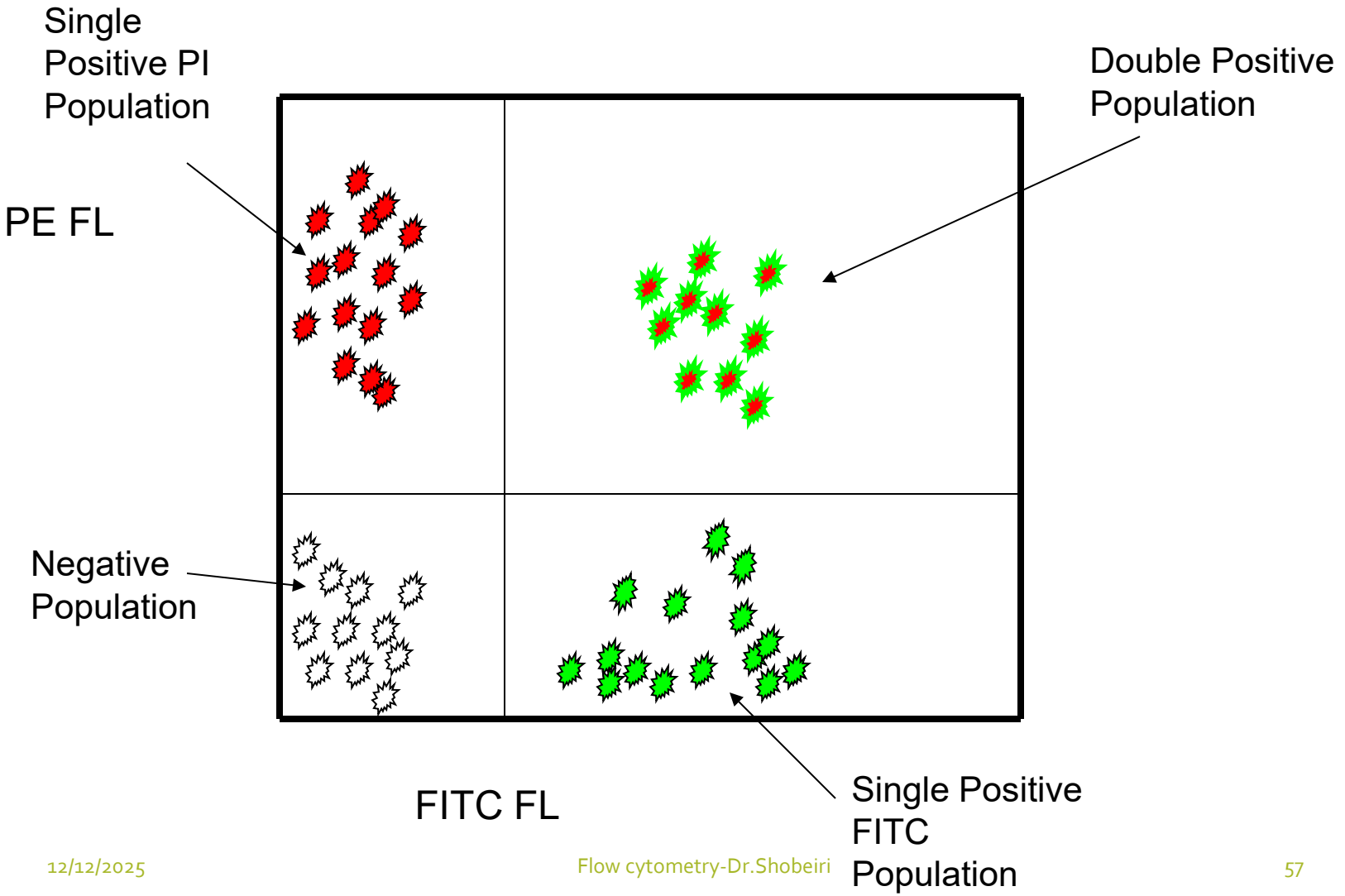
	FSC	SSC	FITC	PE
event 1	30	60	638	840
event 2	100	160	245	85
event 3	300	650	160	720







Dot Plot


Single
Positive PI
Population
Double Positive
Population
PE FL
Negative
Population
FITC FL
Single Positive
FITC
Population

Data Display in Different Plot Types

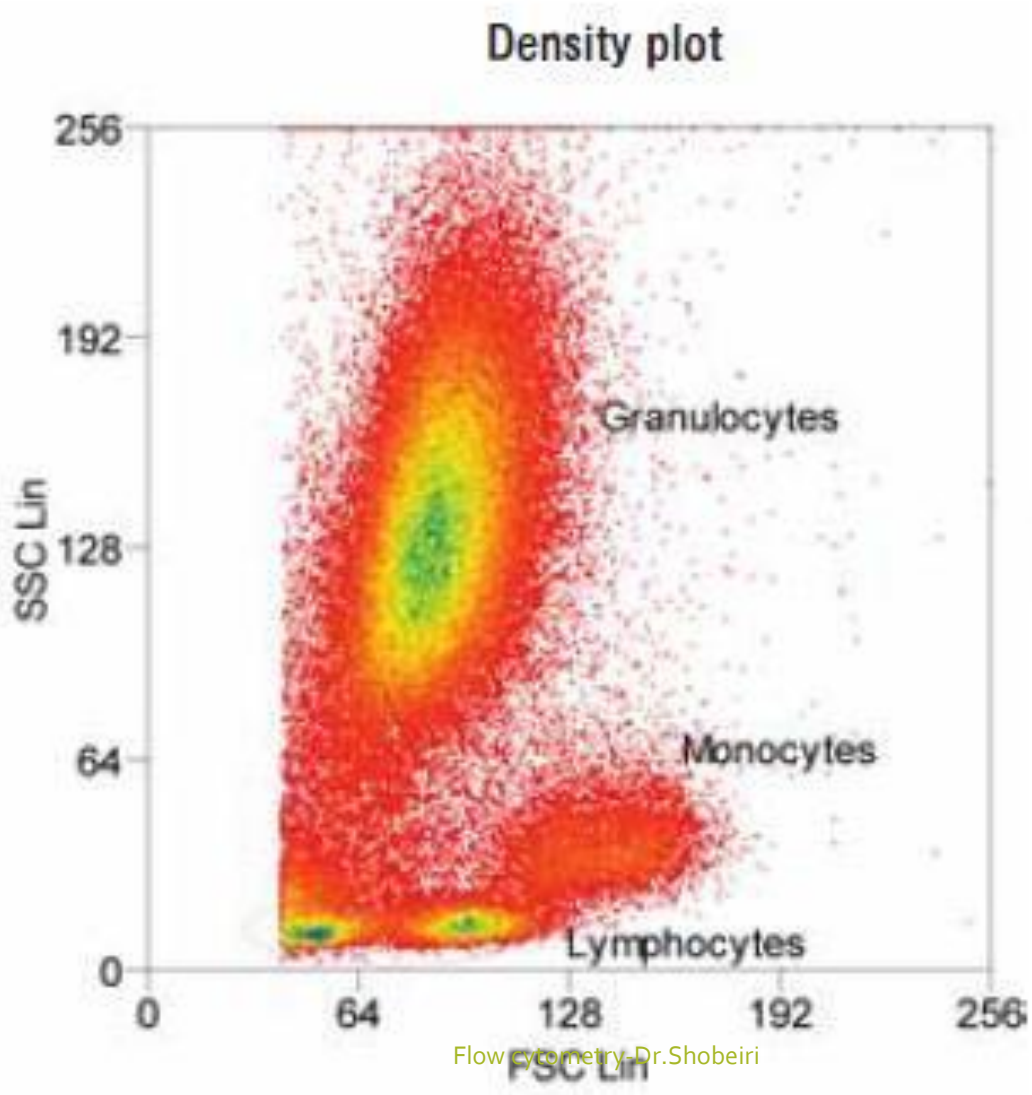
❑ Density Plot:

- ✓ Provides a third dimension using color to indicate the event count, similar to contour on a contour plot.
- ✓ Dots for coordinates with the same event count are given the same color.
- ✓ Dots appear farther to the right on the X-axis or higher on the Y-axis as signal intensity increases.

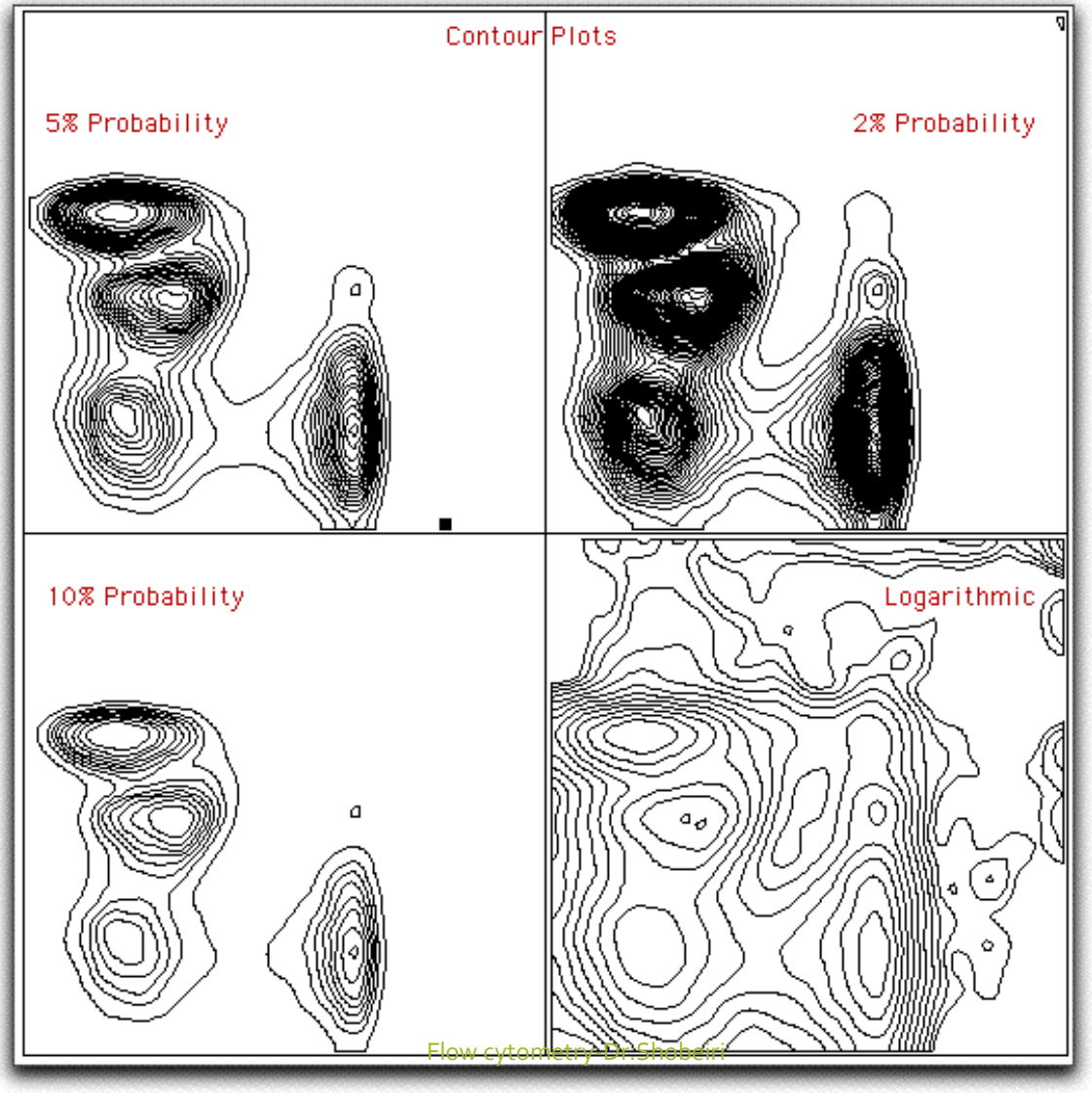
❑ Contour Plot:

- ✓ Displays two parameters simultaneously.
- ✓ Contour lines provide a third dimension by joining X- and Y-coordinates with similar event counts (similar to topographical maps).

Density Plot



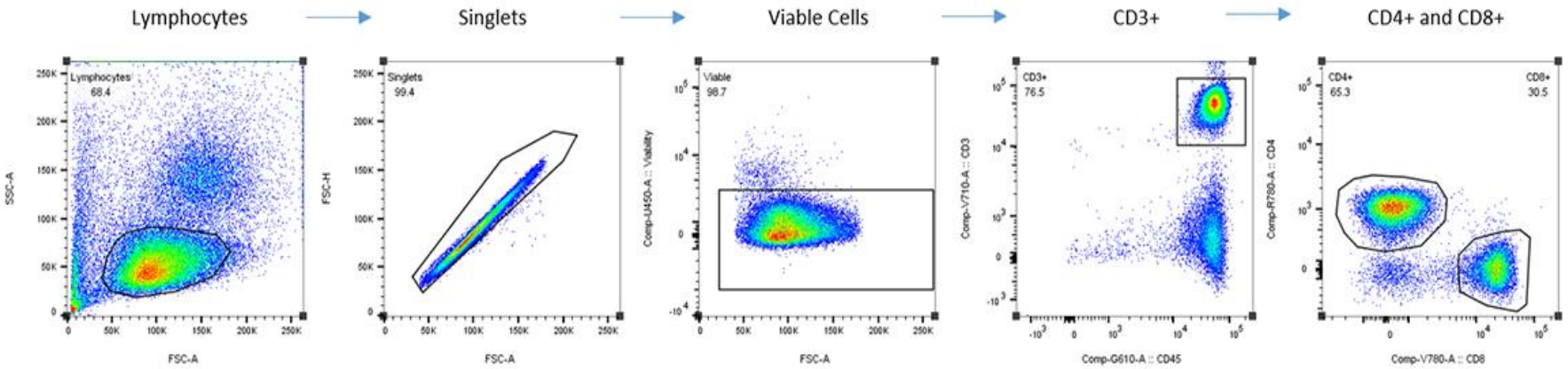
Contour Plot



Gating

- A gate is a numerical or graphical boundary that can be used to define characteristics to include for further analysis.
- Gates are used to identify subsets of data, or populations.
- Populations defined by gates can be used to generate statistics and limit the number of events collected or saved.

Gating



A representative Nested Gating Strategy illustrating lymphocyte population being subgated to the level of CD4+ and CD8+ T Cells.

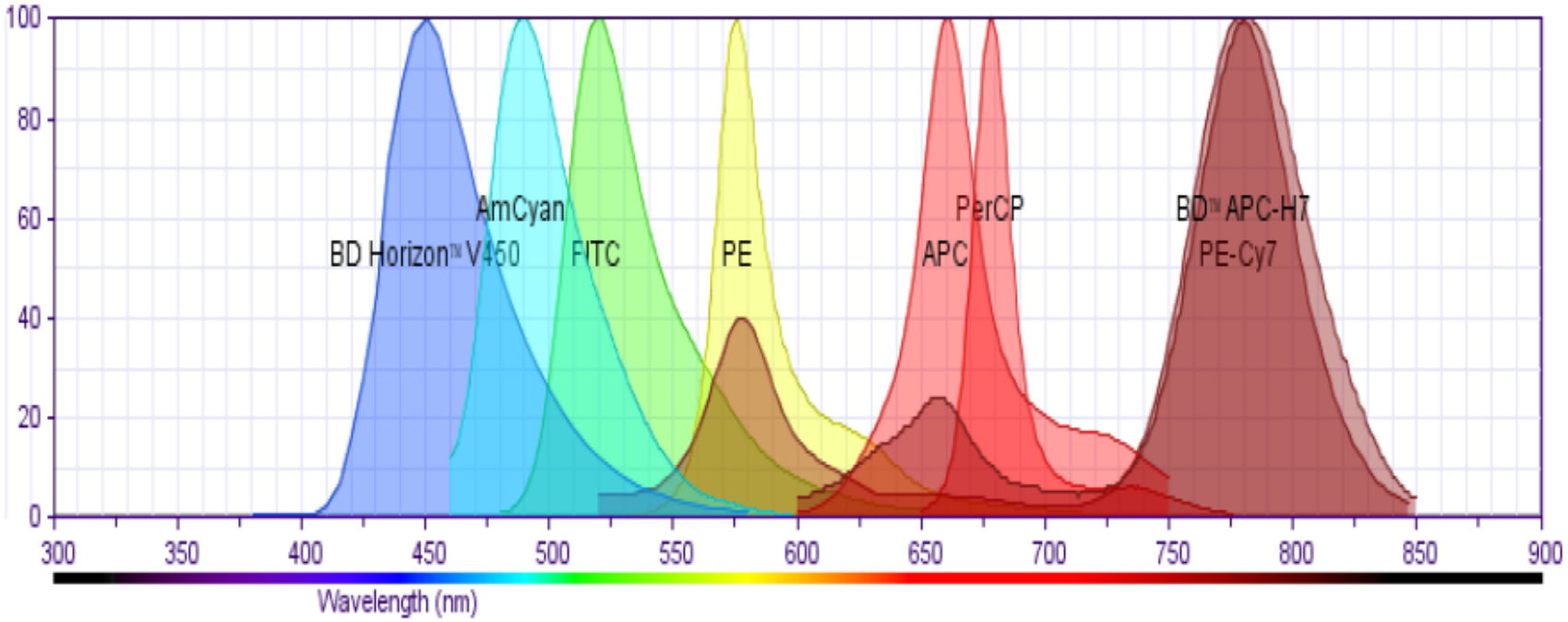
Spectral Overlap

- More than one fluorochrome can be used simultaneously to identify cells in a sample tube.

- Emission spectra of the fluorochromes may overlap each other and bleed into the detection filters for other fluorochromes. This is called spectral overlap.

How to Correct for Spectral Overlap between Fluorochromes?

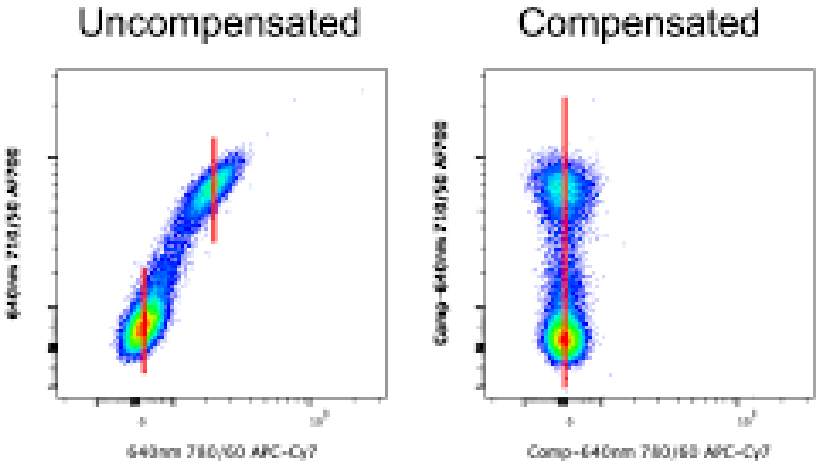
Compensation – Separation of colors



How to Correct for Spectral Overlap between Fluorochromes?

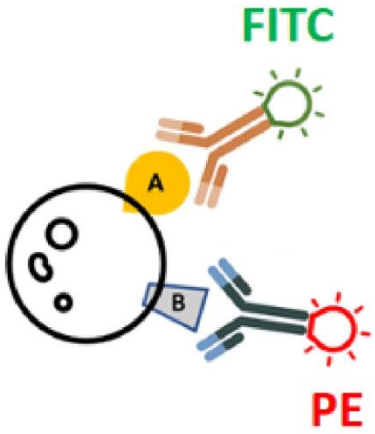
- To accurately record the fluorescence signal for a given fluorochrome, you need to correct the emission signal, and this correction is often called compensation.
- Compensation allows you to distinguish cells or particles that are truly positive for specific fluorochromes from those that appear to be positive do to spillover into other detectors.
- In order to see the amount of compensation required to correct the fluorescence, you need single-color samples such as aliquots of the cell sample stained with each fluorochromes separately.

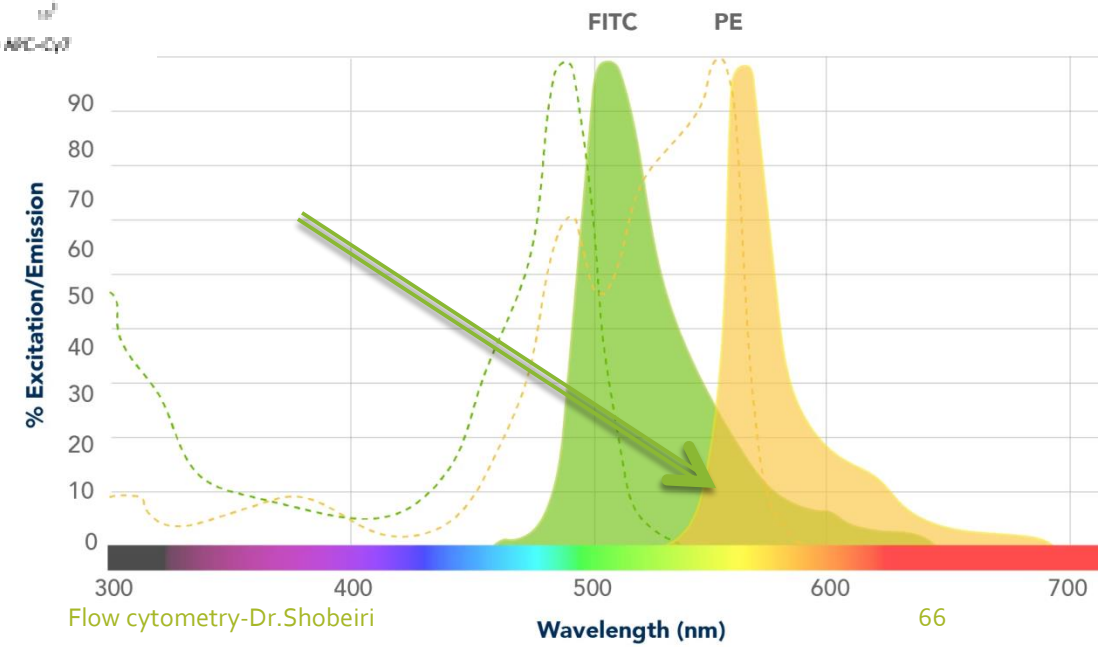
How to Correct for Spectral Overlap between Fluorochromes?



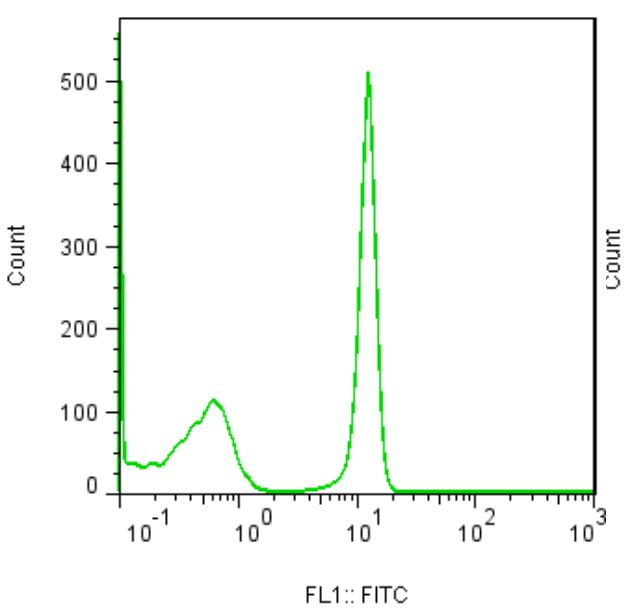
Compensation

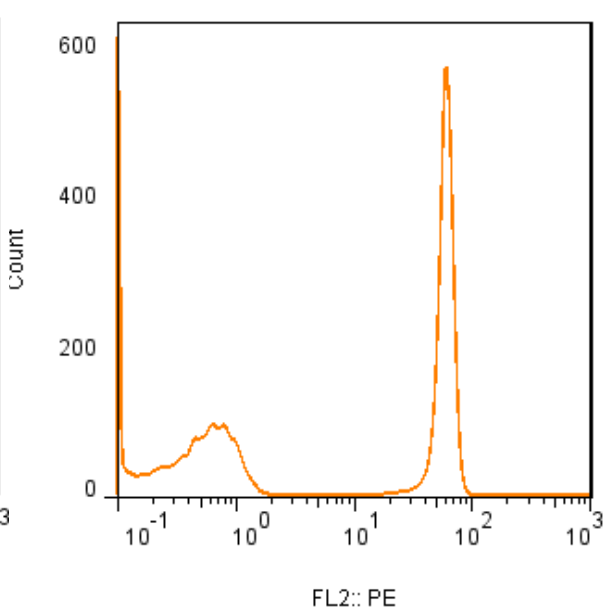
Decrease the false positive events for every parameter because of overlap in band of spectra

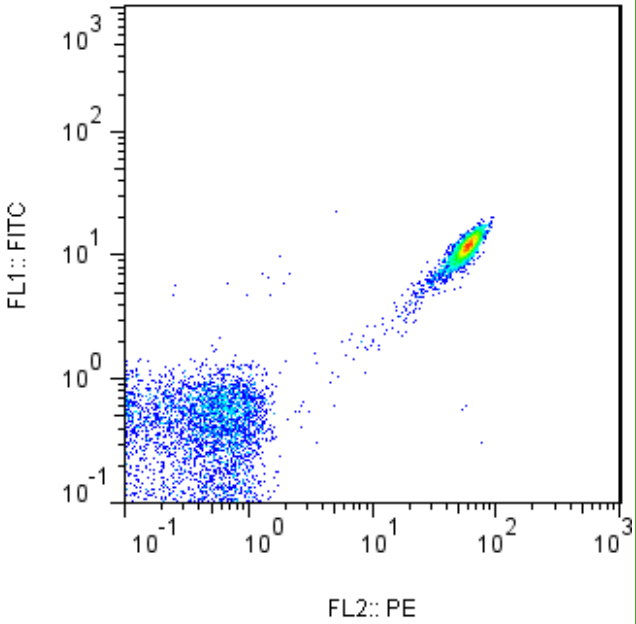




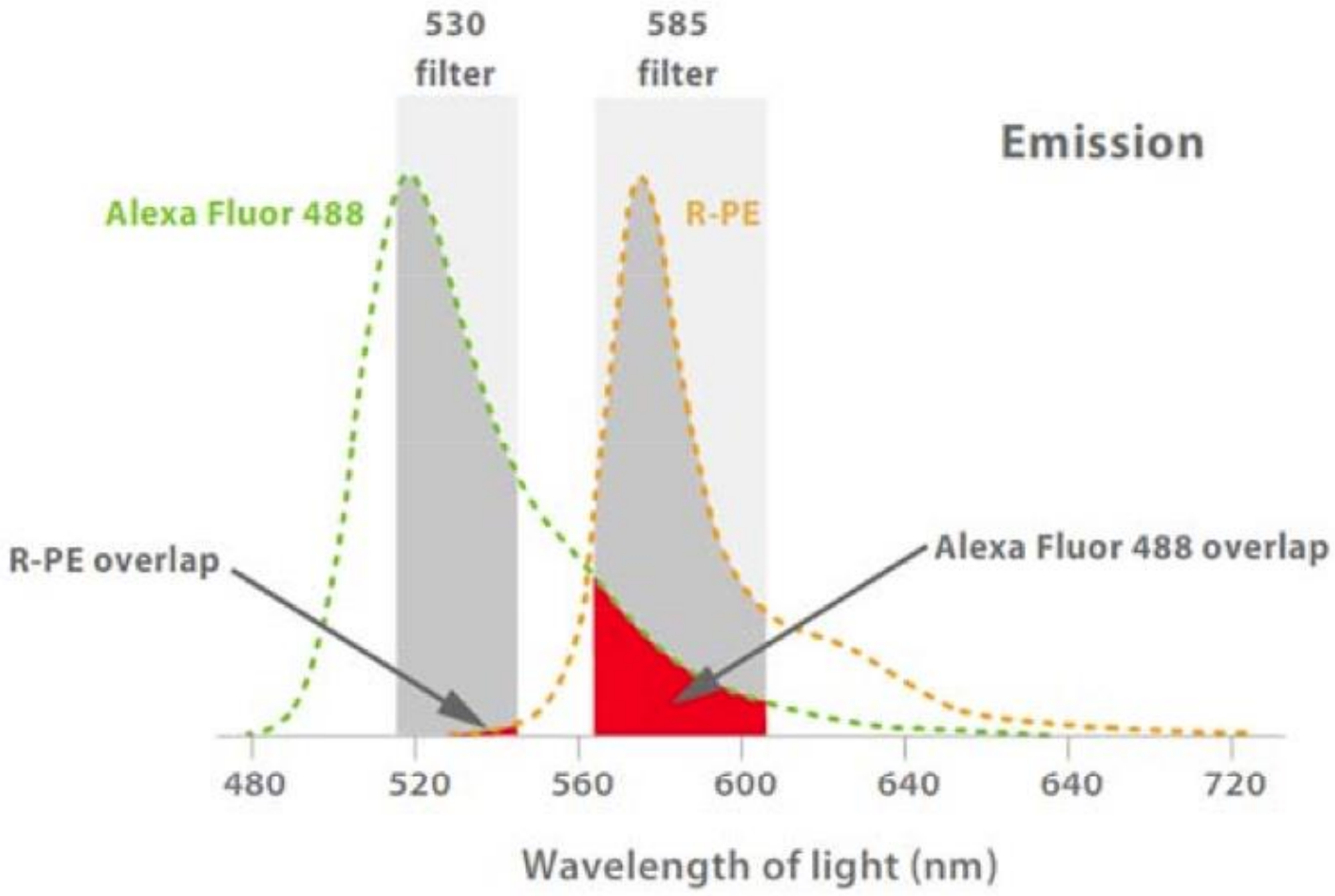
How to Correct for Spectral Overlap between Fluorochromes?



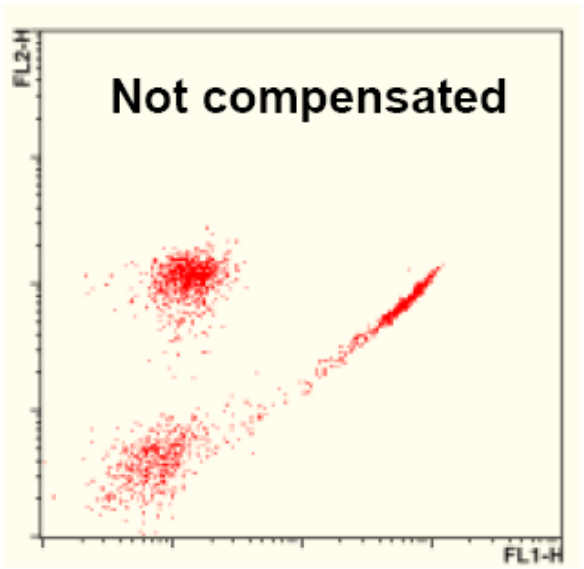


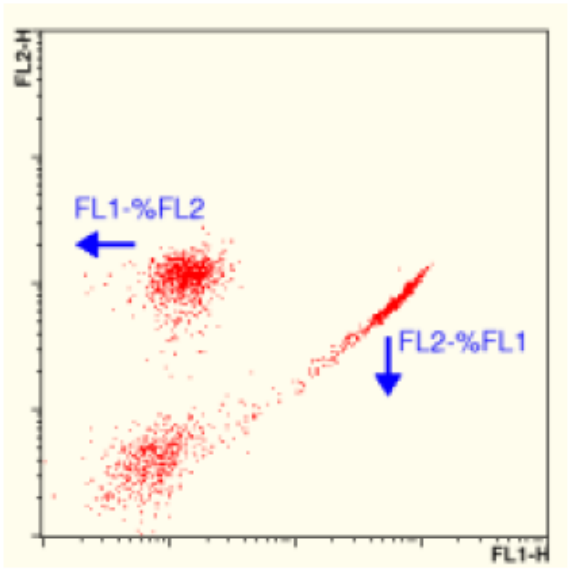


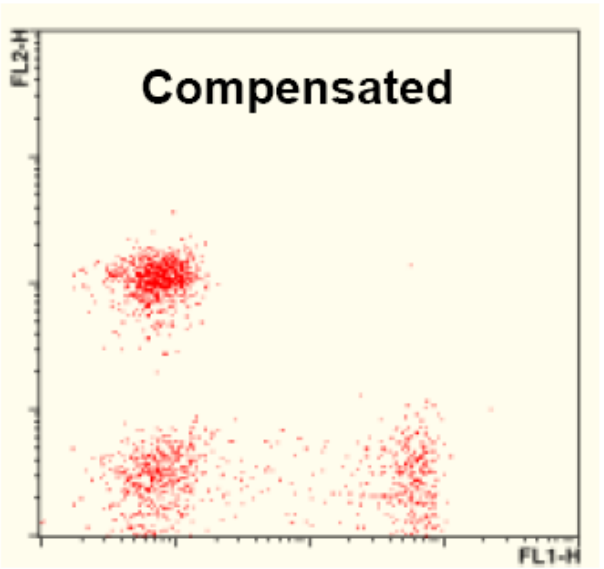
How to Correct for Spectral Overlap between Fluorochromes?

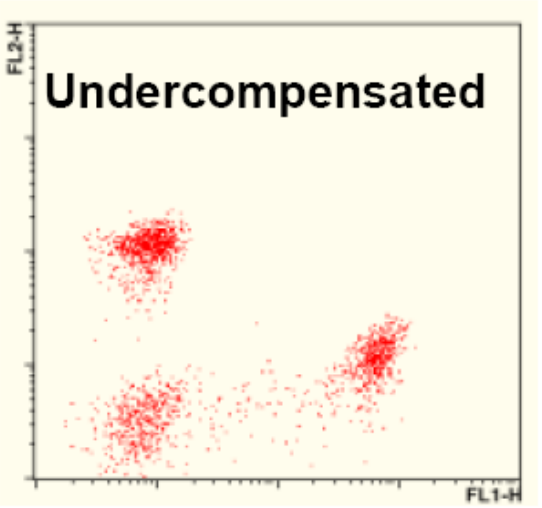


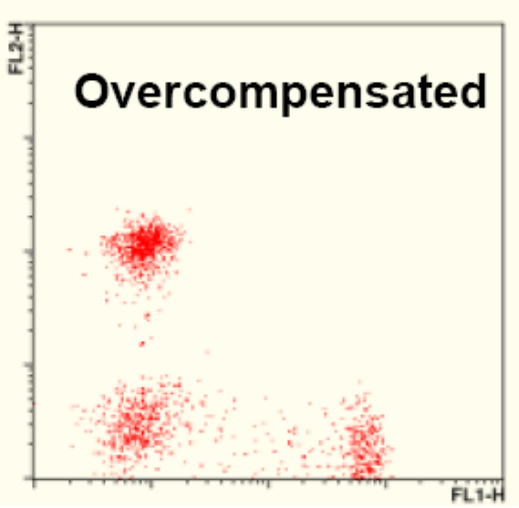
How to Correct for Spectral Overlap between Fluorochromes?

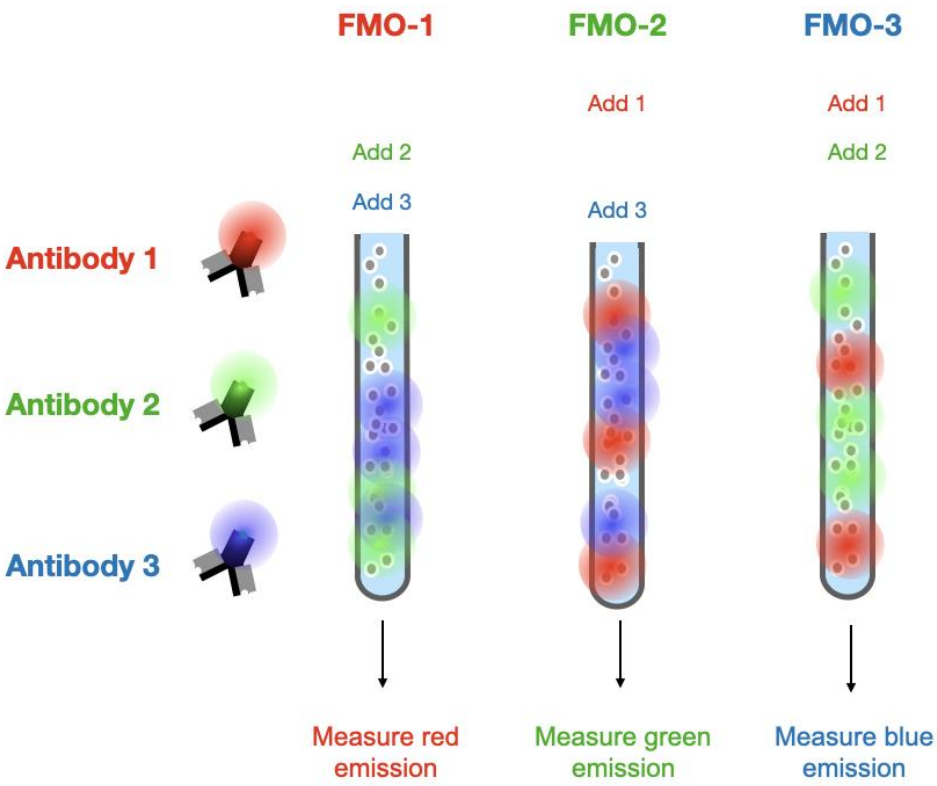


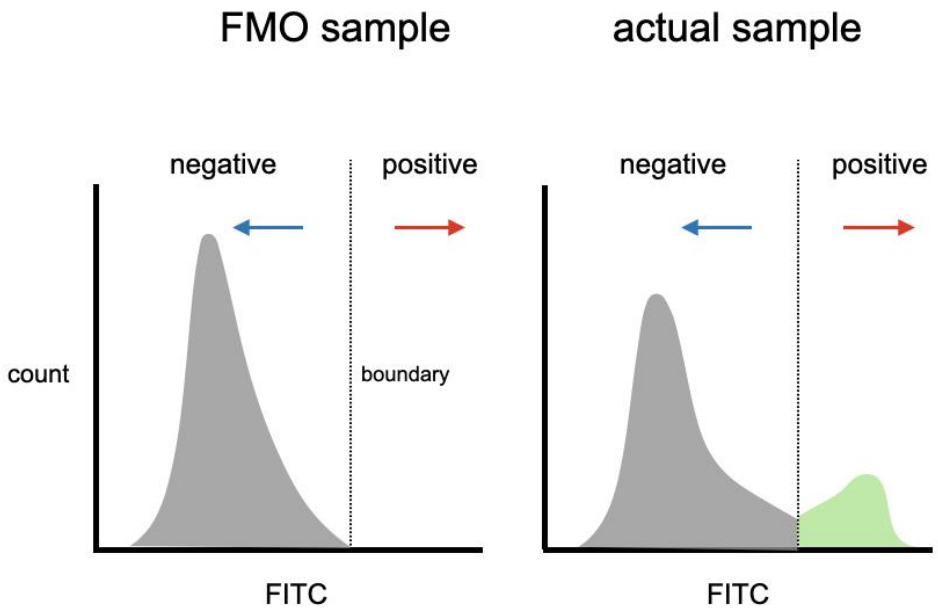






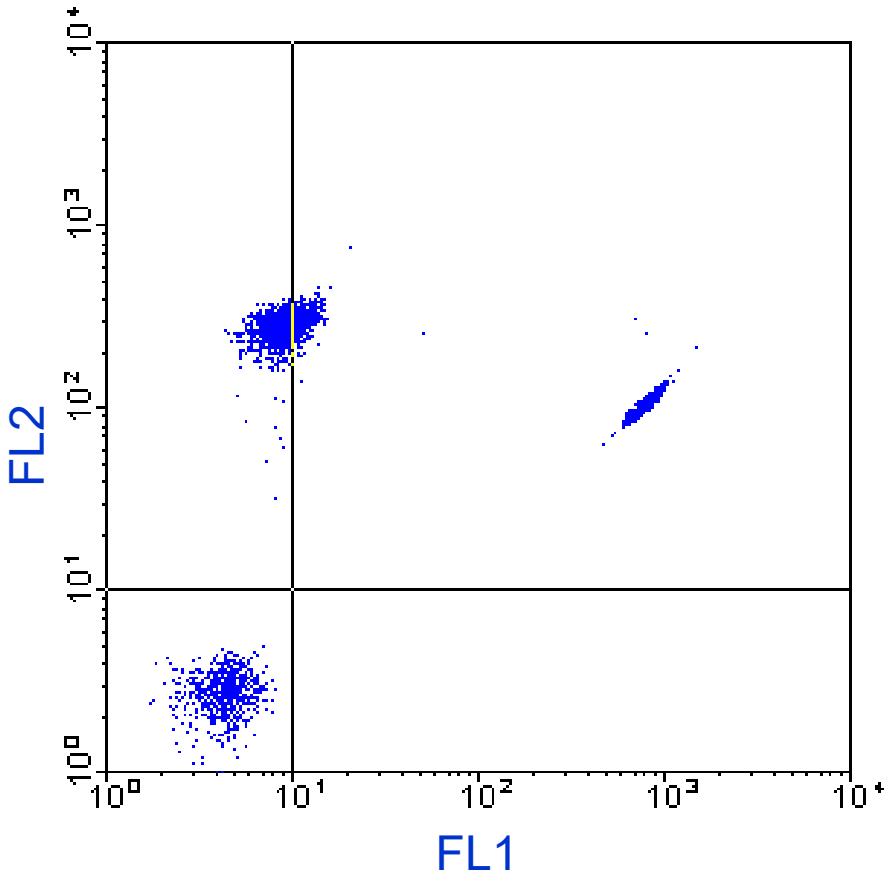



FMO-1
FMO-2
FMO-3
Add 1
Add 1
Add 2
Add 2
Add 3
Add 3
Antibody 1
Antibody 2
Antibody 3
Measure red emission
Measure green emission
Measure blue emission

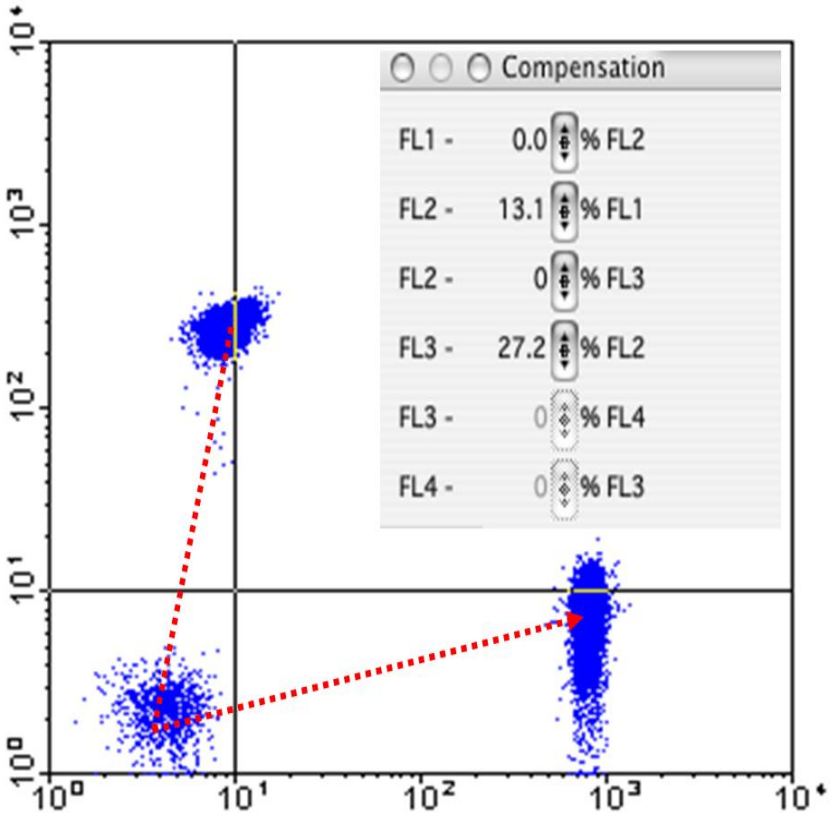

FMO sample
actual sample
negative
positive
negative
positive
count
boundary
FITC
FITC

How to Correct for Spectral Overlap between Fluorochromes?

uncompensate

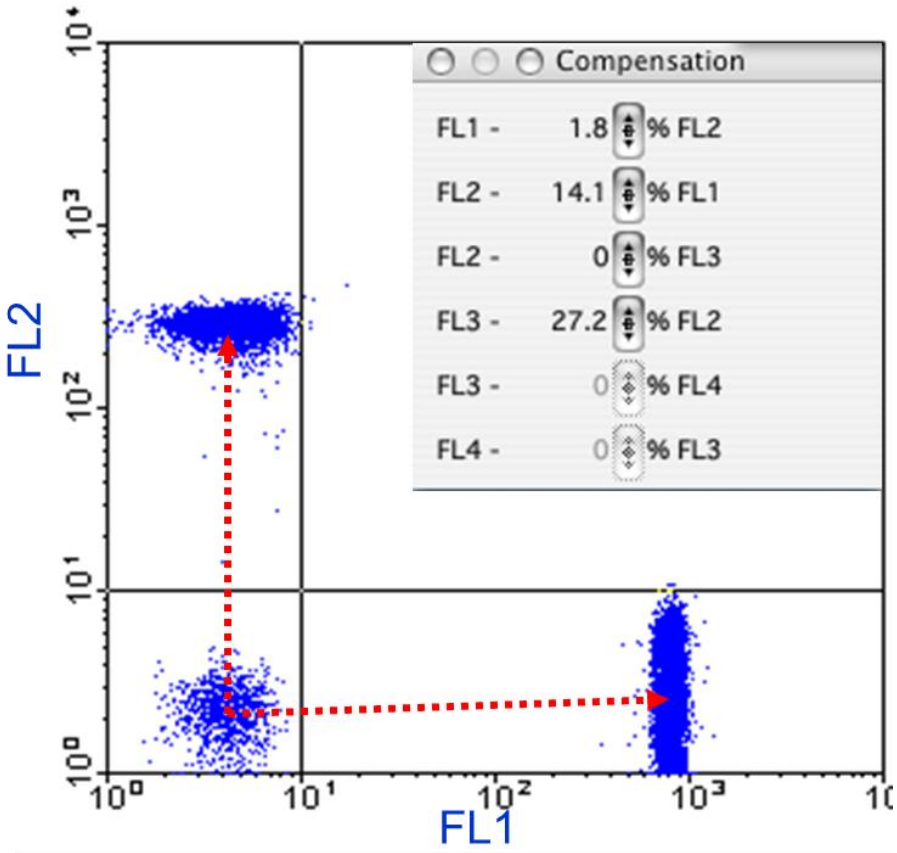


Under compensate

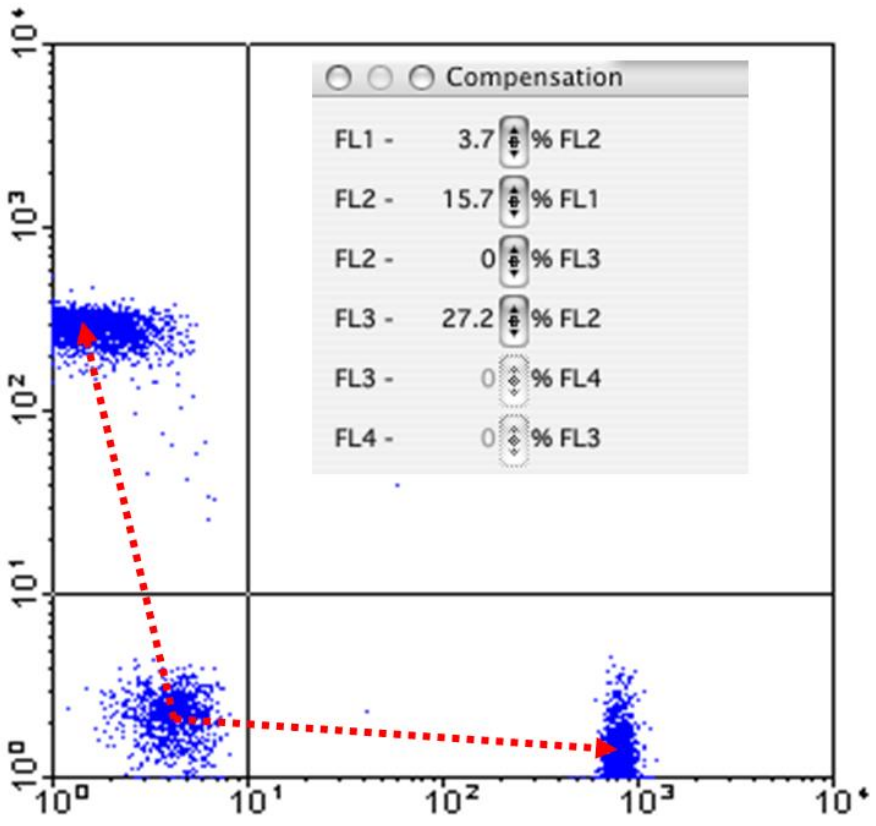


How to Correct for Spectral Overlap between Fluorochromes?

compensate



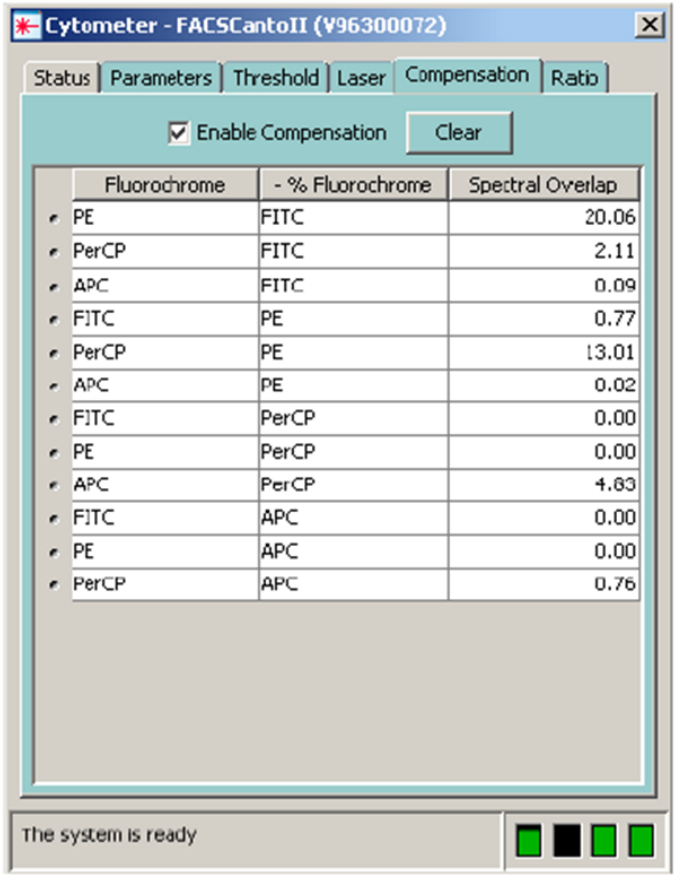
Over compensate



✓ **Compensation is specific to the fluorochrome, not the cell**

How to Correct for Spectral Overlap between Fluorochromes?

Compensation



- Correction of spectral overlap
- depend on:
  - Fluorochromes used
  - Voltage settings
- Samples:
  - Unstained cells as negative control
  - Single stained samples for the calculation of the spectral overlap

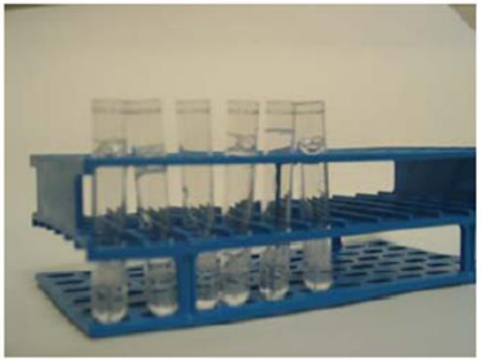
How to Correct for Spectral Overlap between Fluorochromes?

Which tool for compensation?

BD FACS™
7- Color Setup Beads

BD FACS™
CompBeads or
CompBeads Plus

Single stained cells



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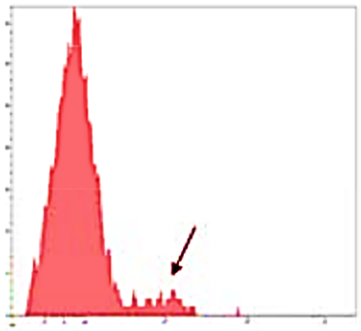
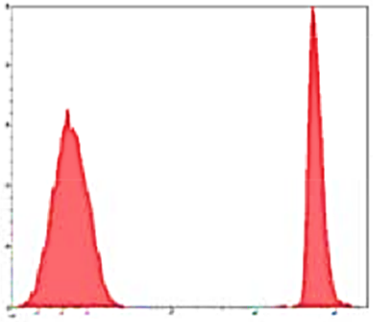
Tools for Compensation

Antibody Capture Beads for Compensation



CD56-PE

Lymphocytes

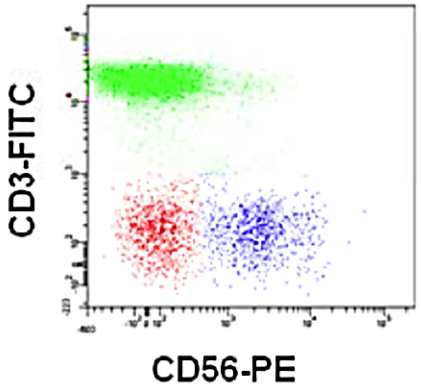


CD56-PE

AbC™ Compensation Beads

Lymphocytes

Compensation set using antibody capture beads

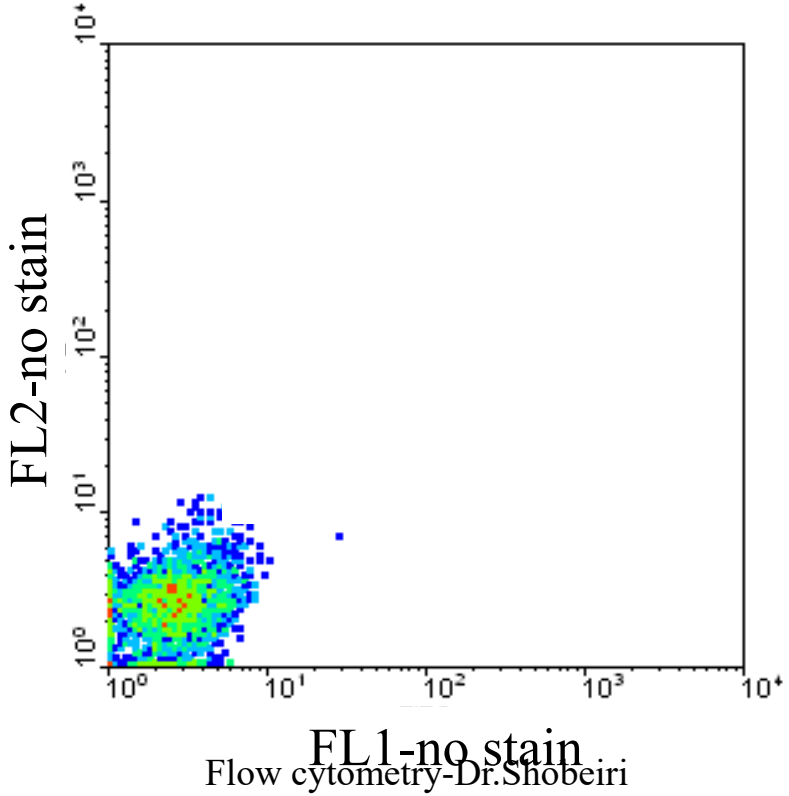


- Useful when antigen expression is dim (as in CD56 example here)
- Useful when antigen expression is normally absent
- Useful when cell number is limited
- Uses same antibody conjugates as used in experiment

Setting compensation- PMT Voltage

-Run unstained cells

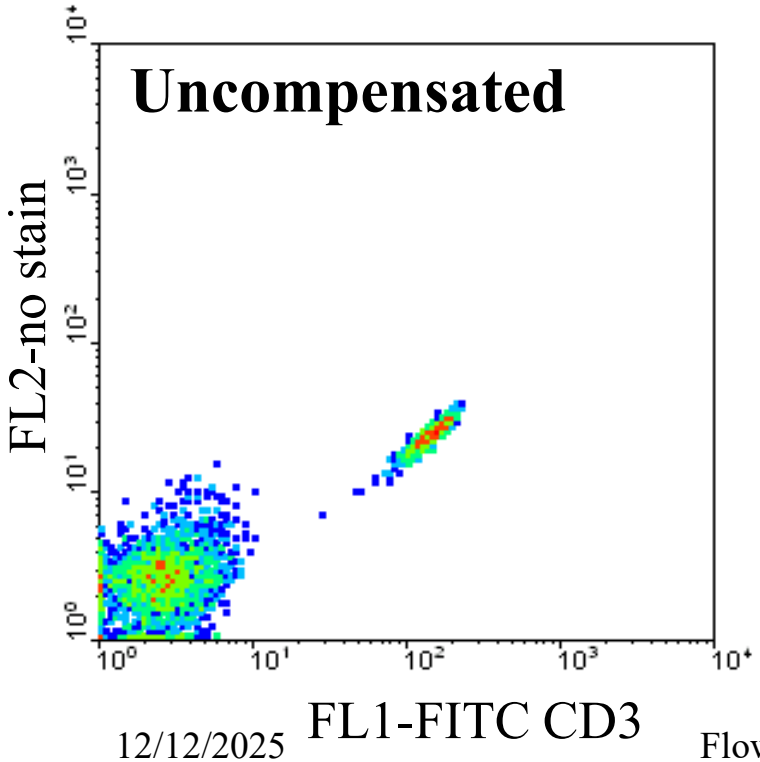
-Adjust the PMT voltages so that the negative population is off the axis in every channel.

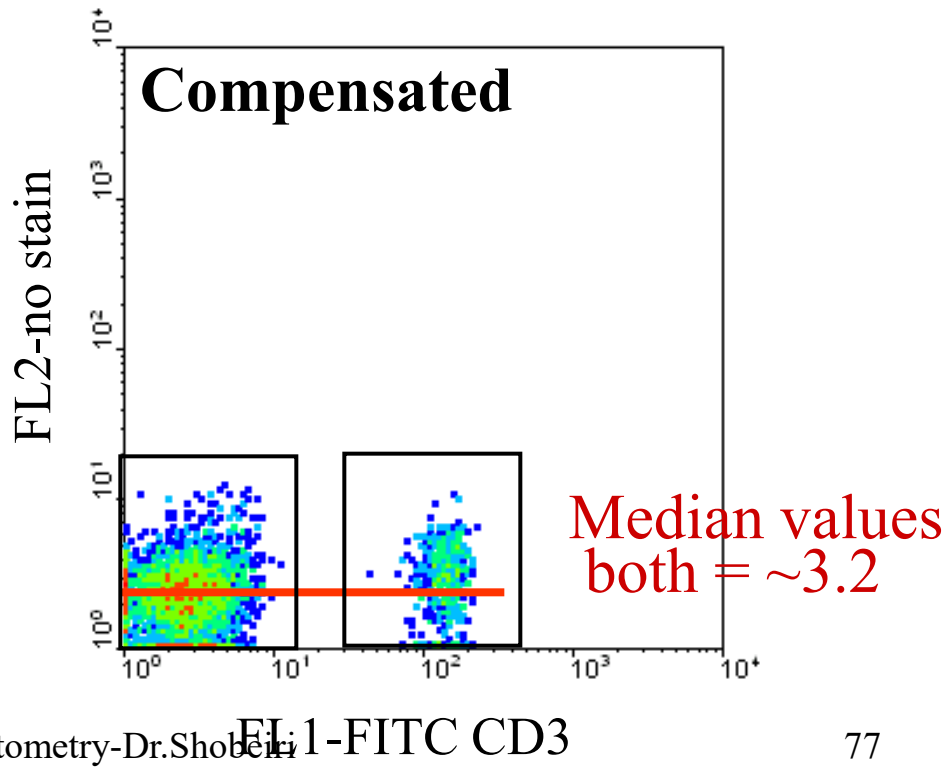


Setting compensation - FITC single stain

-Run single stained control (FITC stained only)

-Adjust the compensation value so that positive and negative population have the same FL2 median fluorescence intensity.

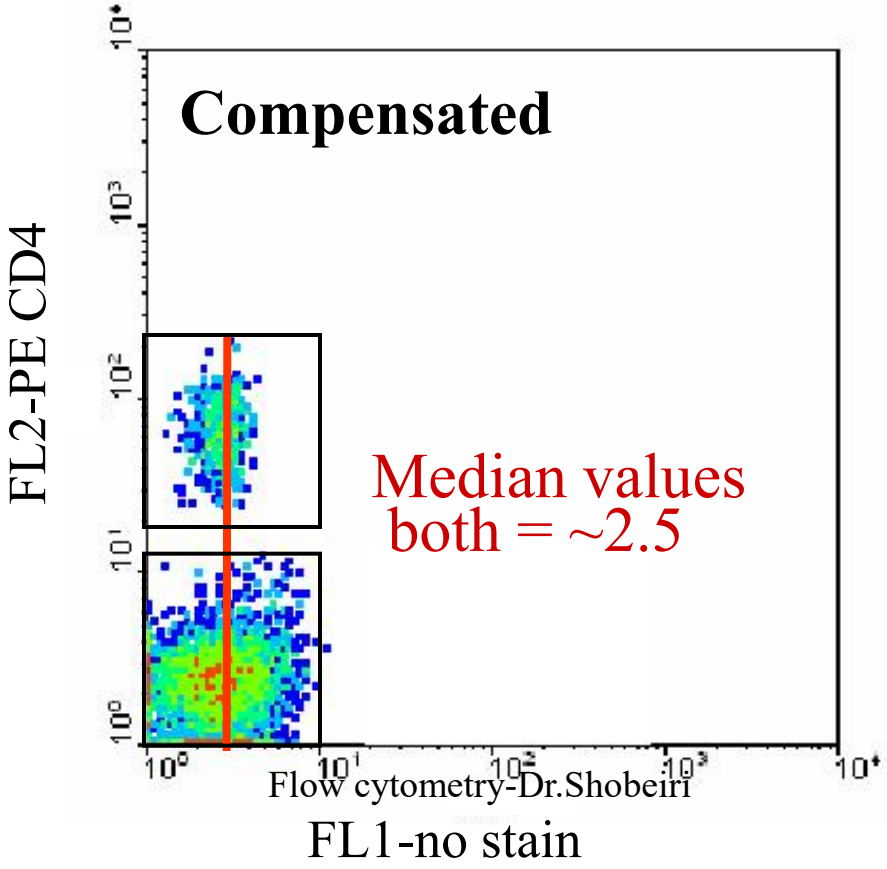




Setting compensation - PE single stain

-Run single stained control (PE stained only)

-Adjust the compensation value so that positive and negative population have the same FL1 median fluorescence intensity.



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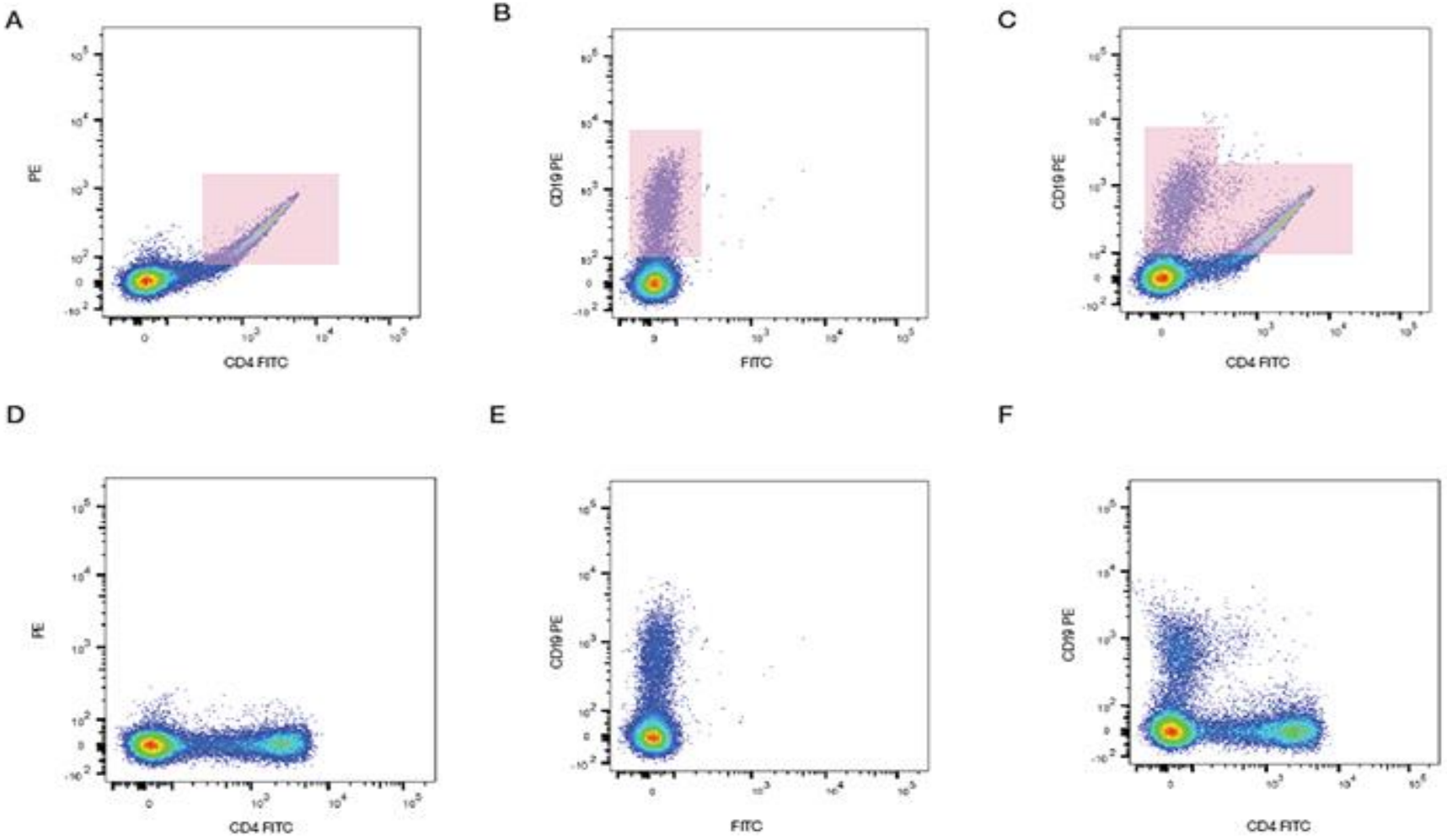
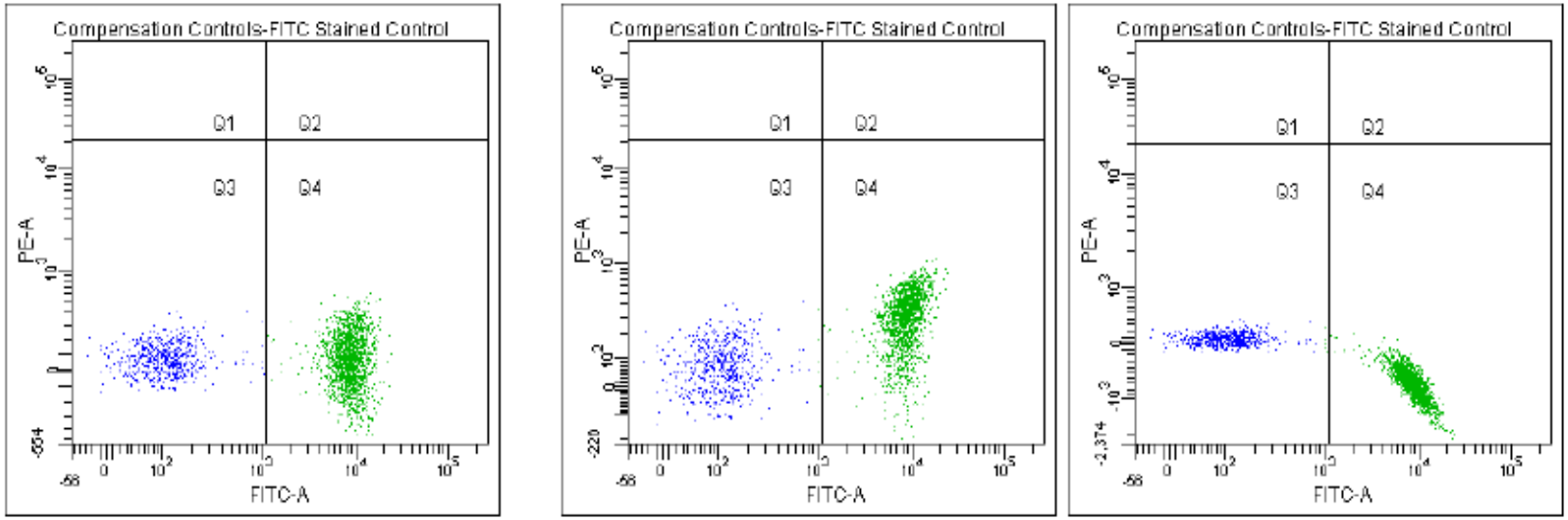


How to Correct for Spectral Overlap between Fluorochromes?

Examples

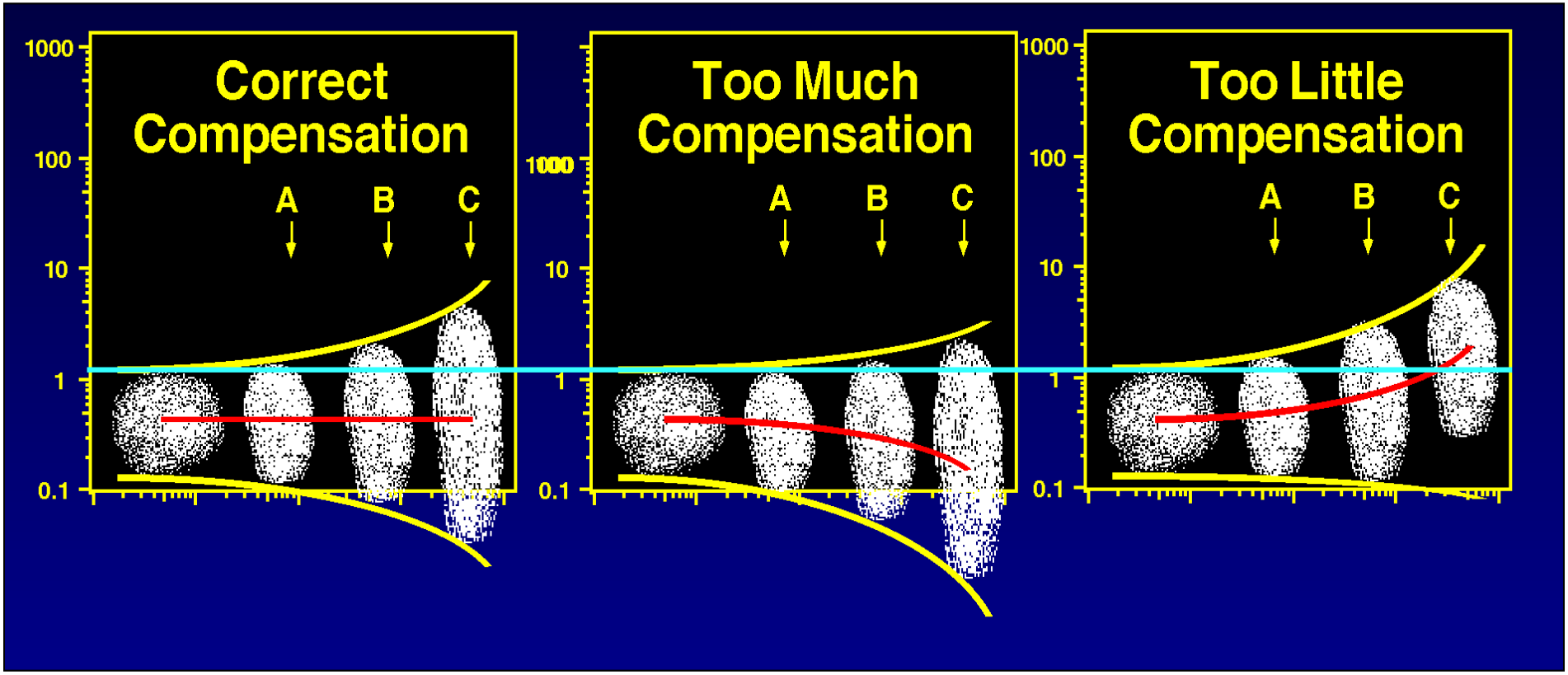
wrong compensation

ok | **less** | **Too much**



How to Correct for Spectral Overlap between Fluorochromes?

Which marker for compensation?

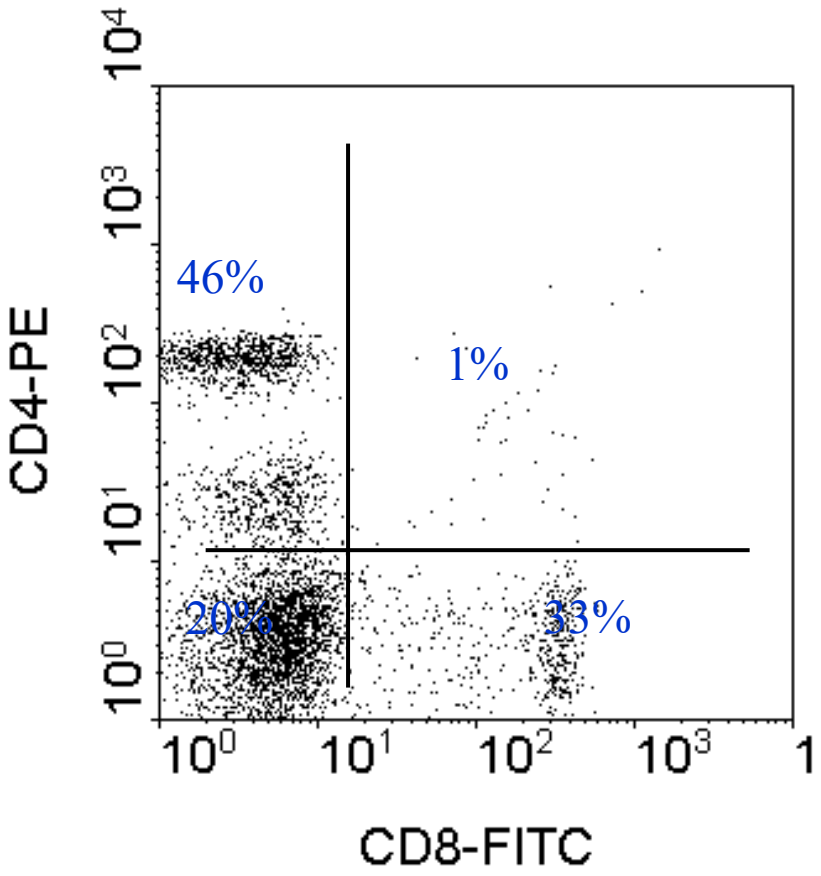


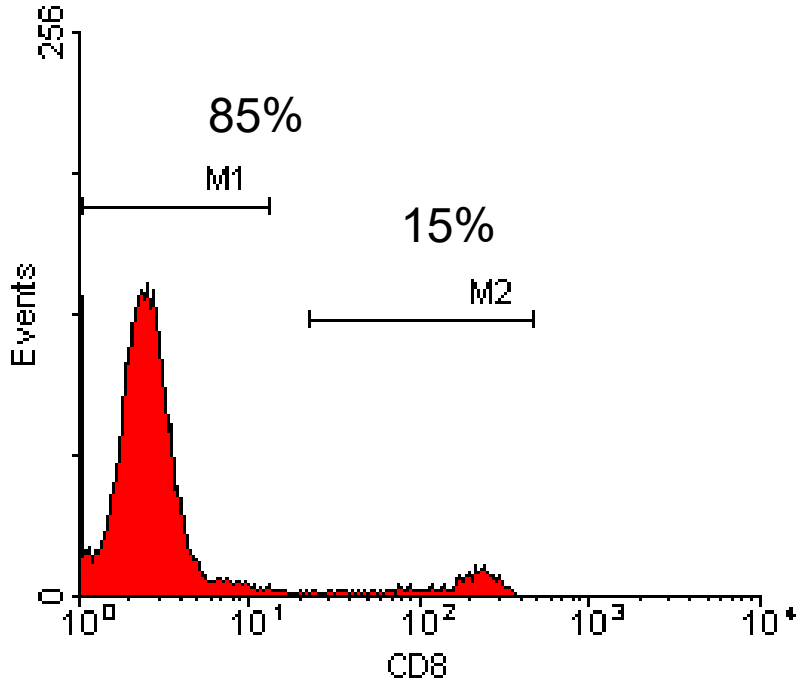
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C). Use bright markers to setup proper compensation.

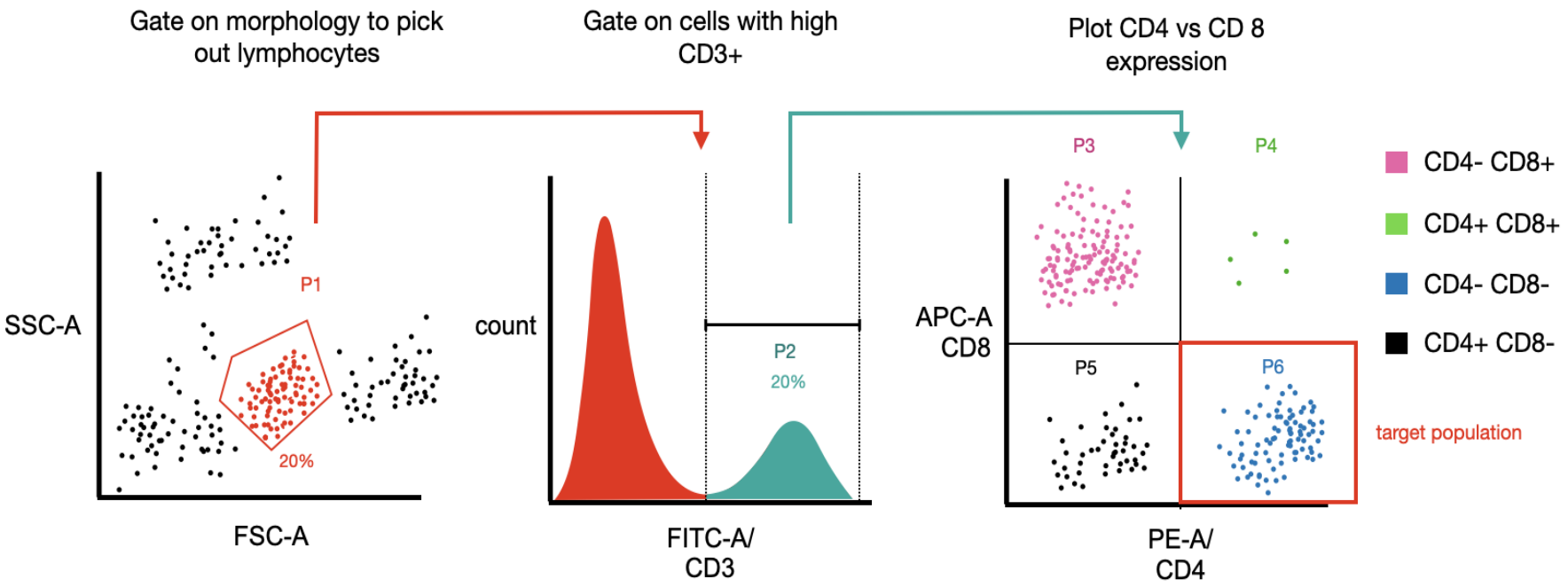
Statistical data and Data Analysis Software

- ✓**Online** Software:
  - Acquisition and Analysis (both of them)
  - Able to connect to the Cytometer
  - Cell Quest Pro(BD), Flomax(Partec), FACS Diva(BD)
- ✓**Offline** software:
  - Just Data Analysis
  - Flowjo, winMDI ,Cyflogic , Flowing software ,Cytobank

Giving Statistical data and Data Analysis






Gate on morphology to pick out lymphocytes
Gate on cells with high CD3+
Plot CD4 vs CD 8 expression
SSC-A
FSC-A
P1
20%
count
FITC-A/ CD3
P2
20%
APC-A CD8
PE-A/ CD4
P3
P4
P5
P6
CD4- CD8+
CD4+ CD8+
CD4- CD8-
CD4+ CD8-
target population


Thanks for Attention