به نام خدا

Flow Cytometry

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عضو هیئت علمی گروه ایمونولوژی پزشکی

Section I:

Introduction and application

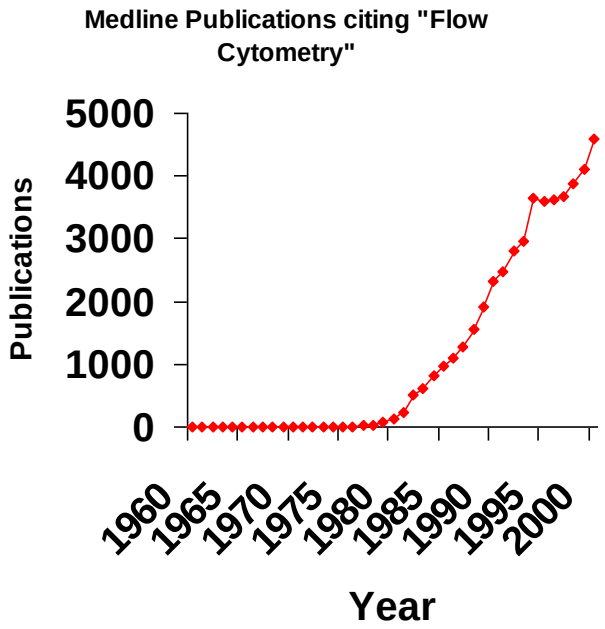
What Is Flow Cytometry?

- Flow = cells in motion
- Cyto = cell
- Metry = measure

- **Flow Sorting**
  - Sorting (separating) cells based on properties measured in flow
  - Also called :**Fluorescence-Activated Cell Sorting (FACS)**

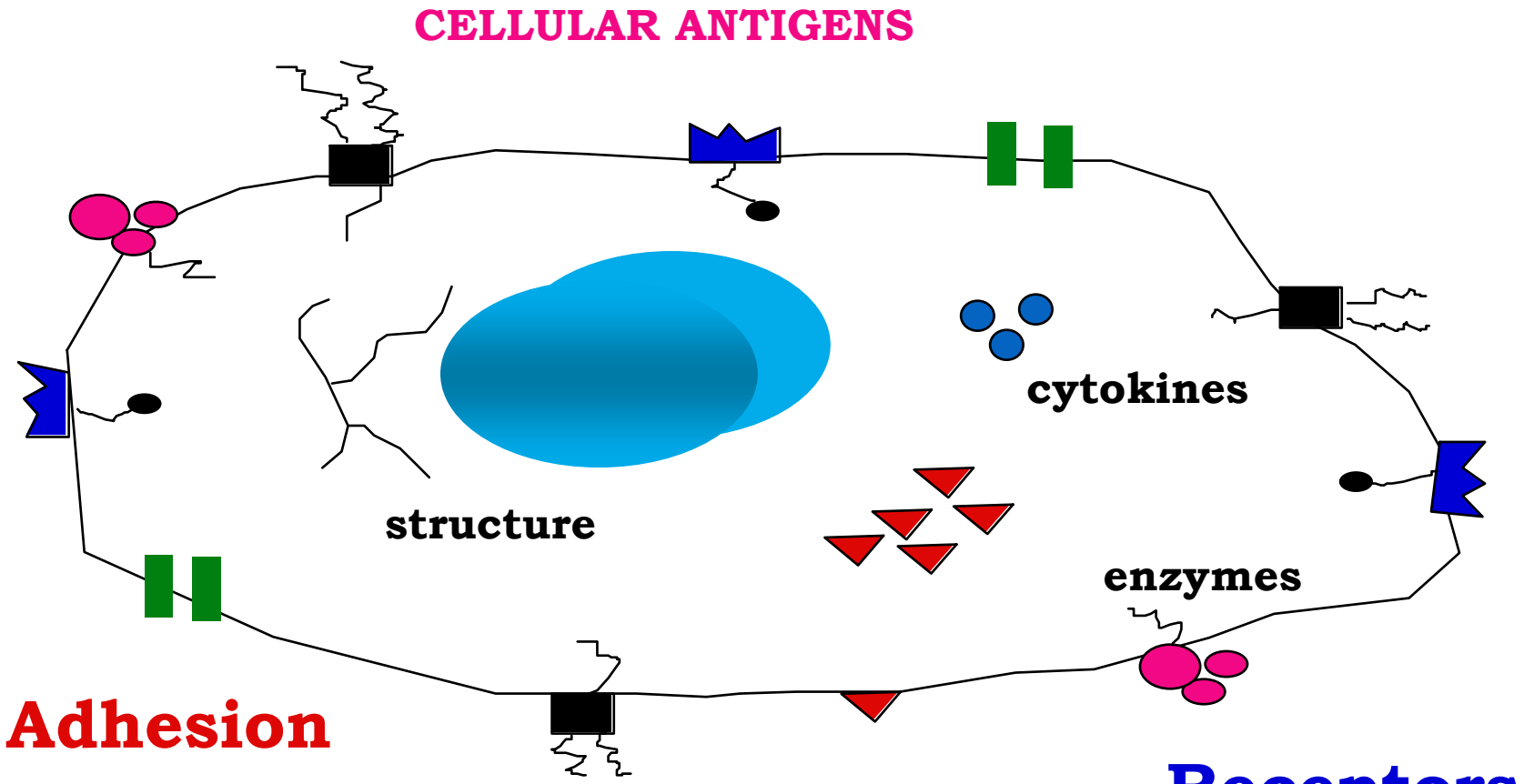
Uses of Flow Cytometry

- Immunophenoytping
- Cell viability assay
- Cell cycle Assay
- Cell surface markers identifications
- Intracellular markers identification
- DNA and chromatin studies
- Cell sorting



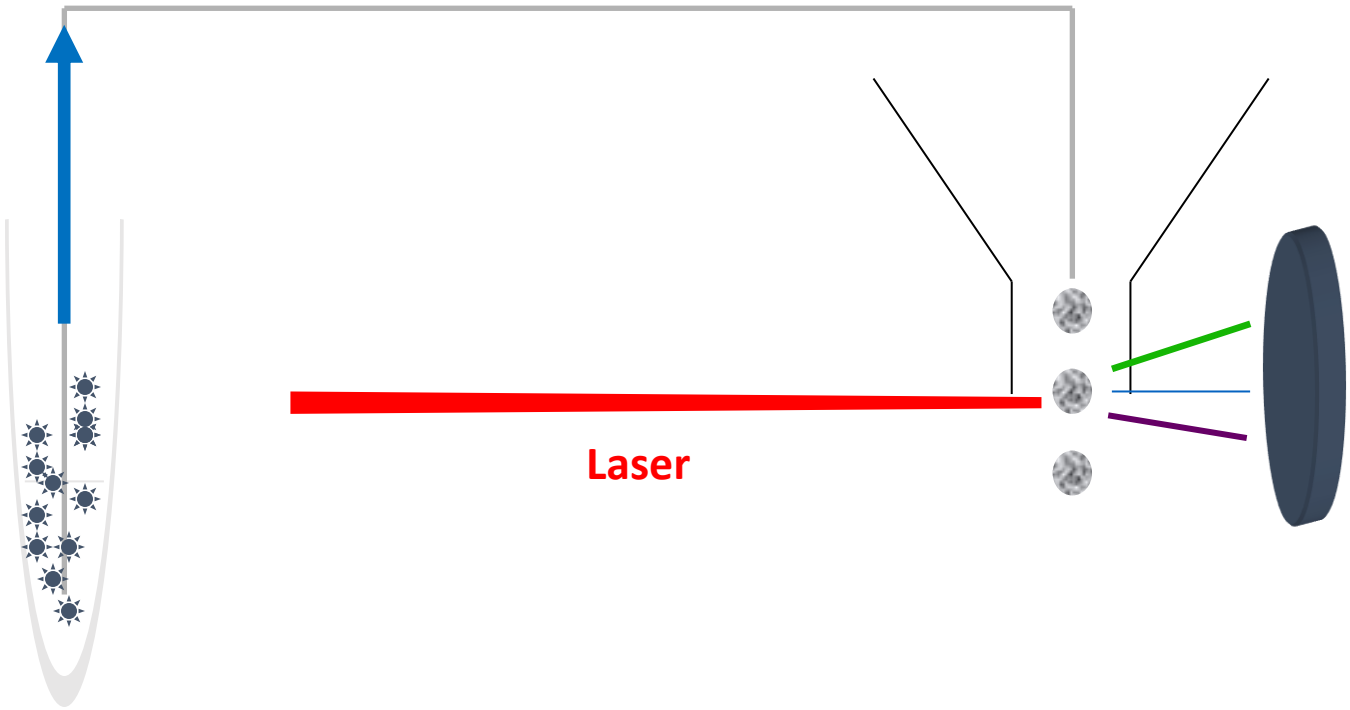
What does flow cytometry measure about cells?

- **Size**
- **Shape** (Granularity & Density)
- **Makeup** (Fluorescence Abs against markers)



T cells
B Cells

What Happens in a Flow Cytometer (Simplified)

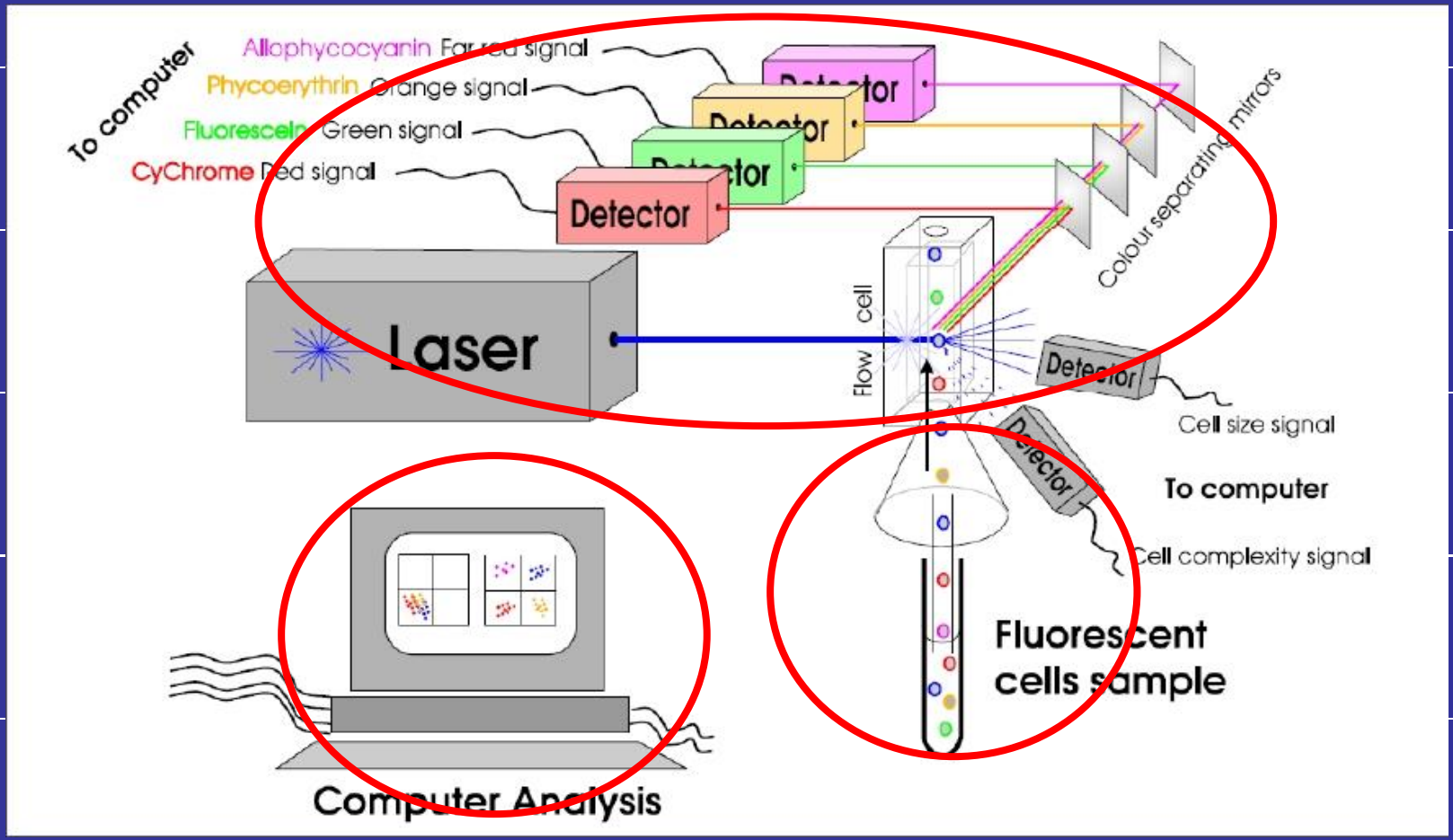


- **Size**
- **Shape**
- **Fluorescence**

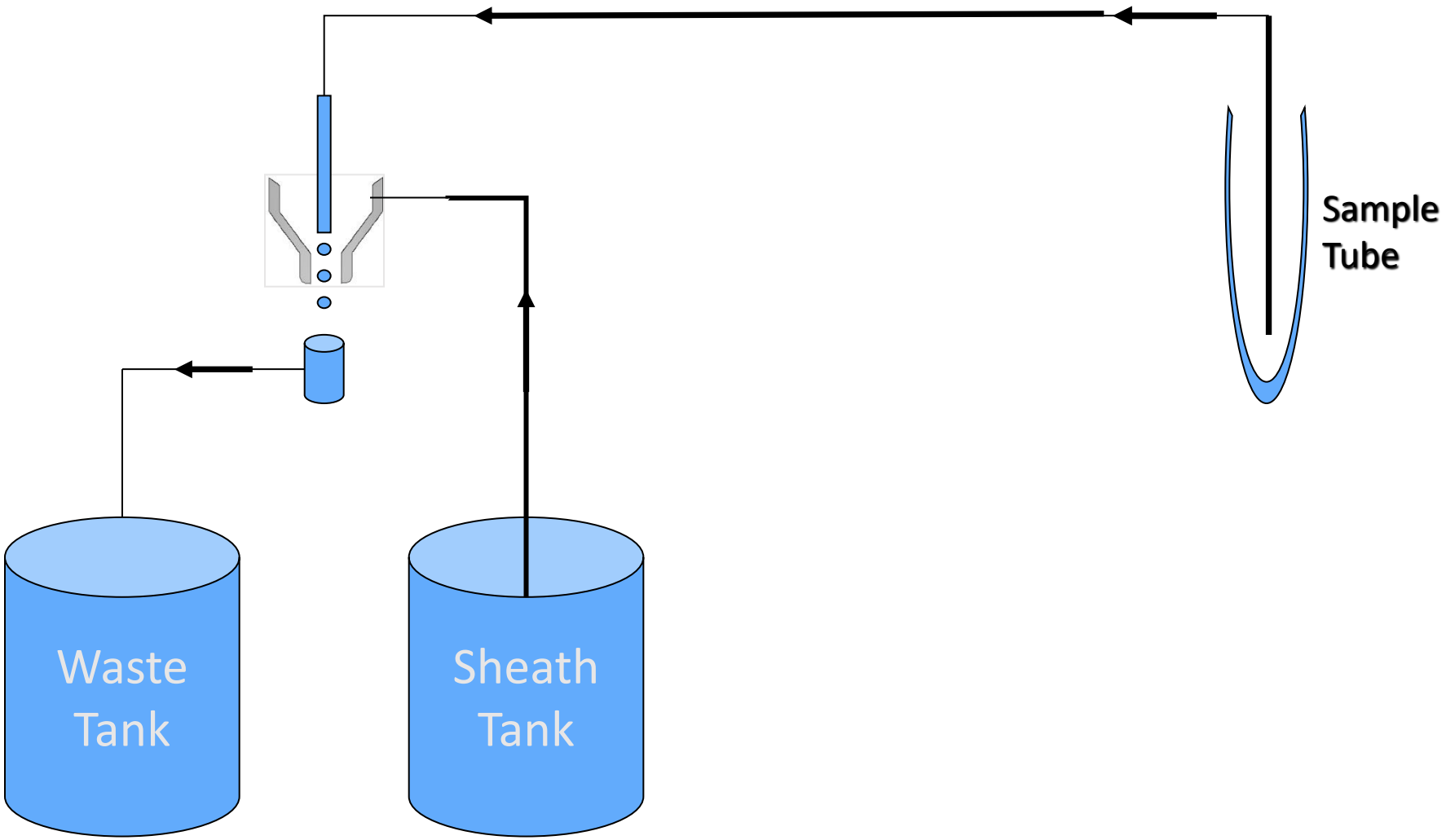
Section II:

Different parts of flow cytometry

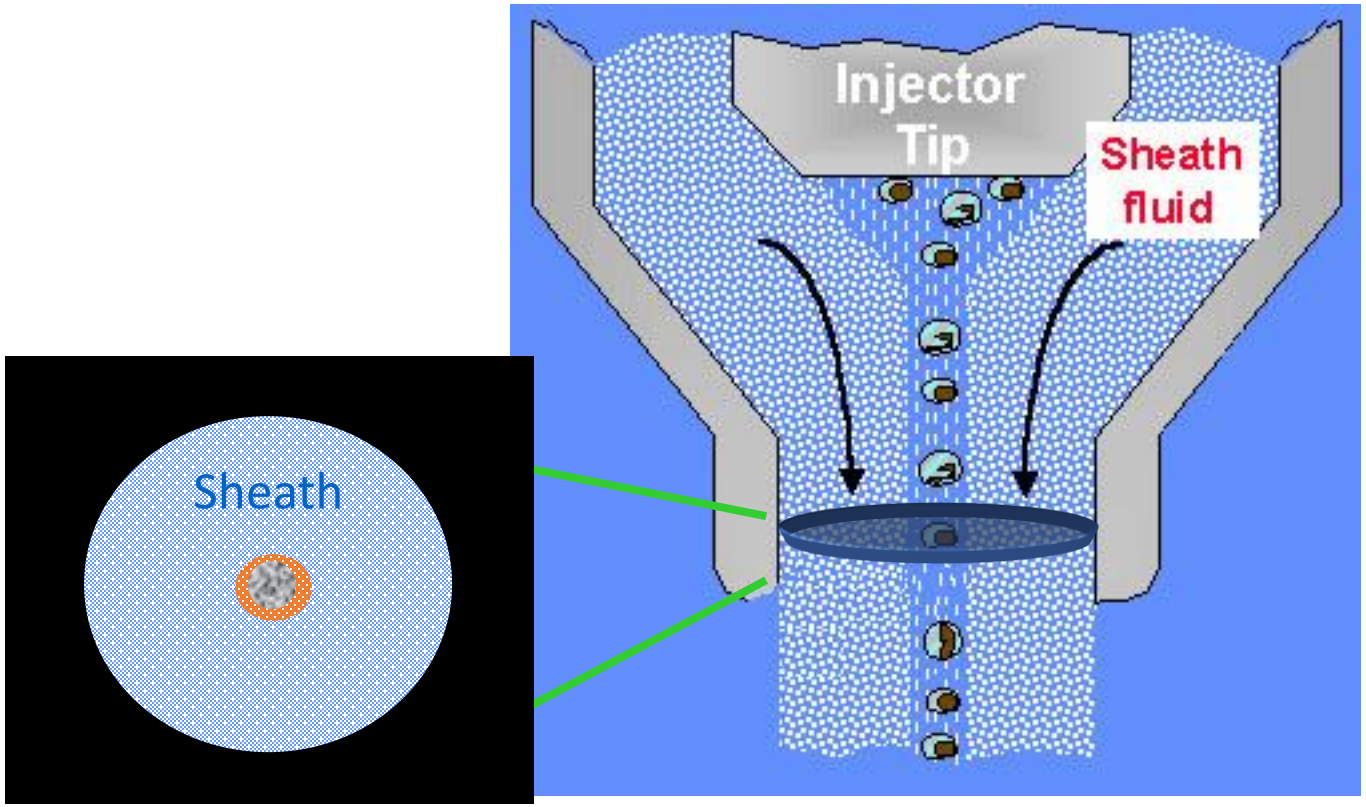
Flow Cytometer Instrumentation
Graphical Summary



Fluidics Schematic



The Flow Cell



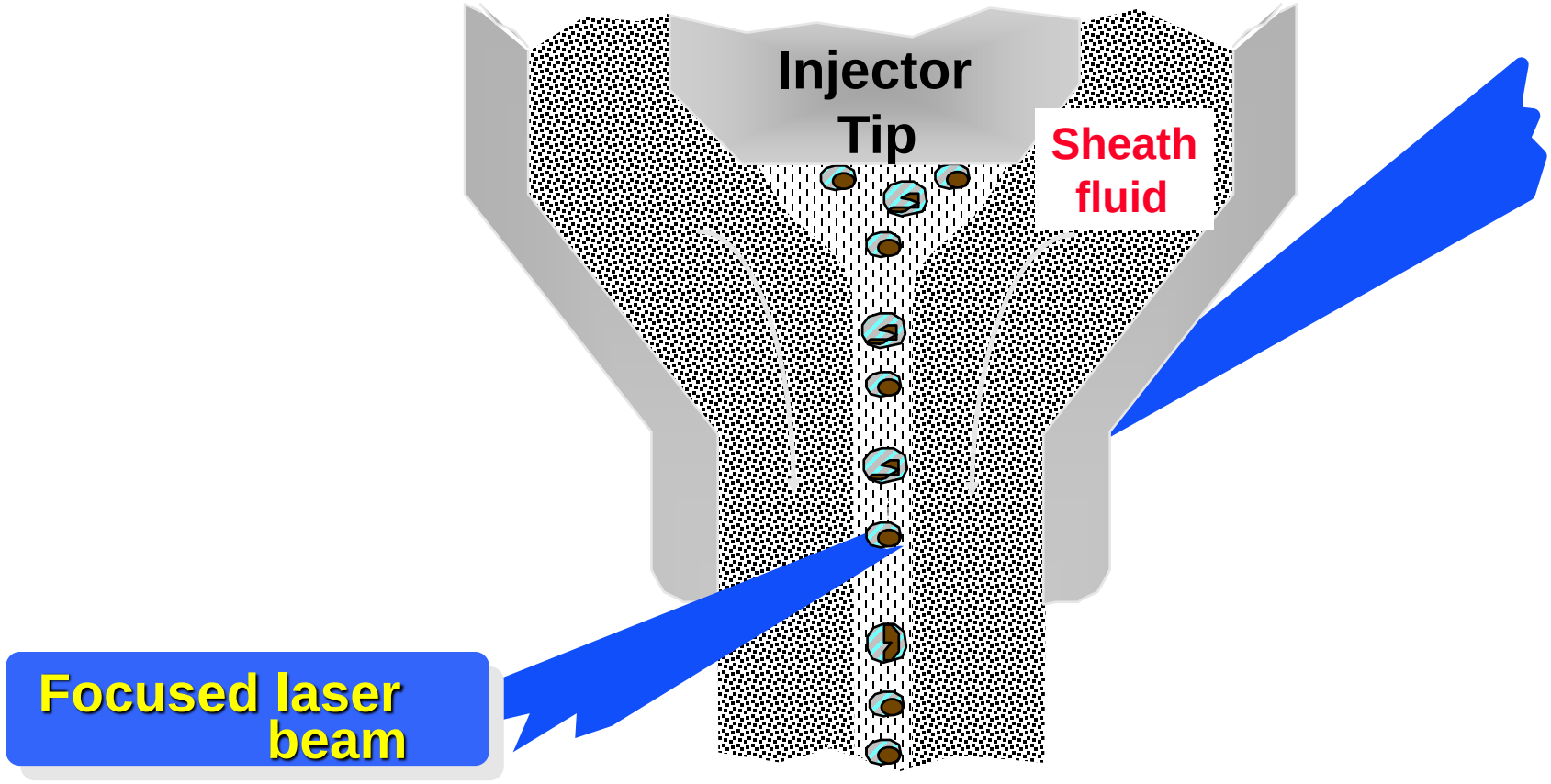
The introduction of a large volume into a small volume in such a way that it becomes "focused" along an axis is called Hydrodynamic Focusing.

Optics

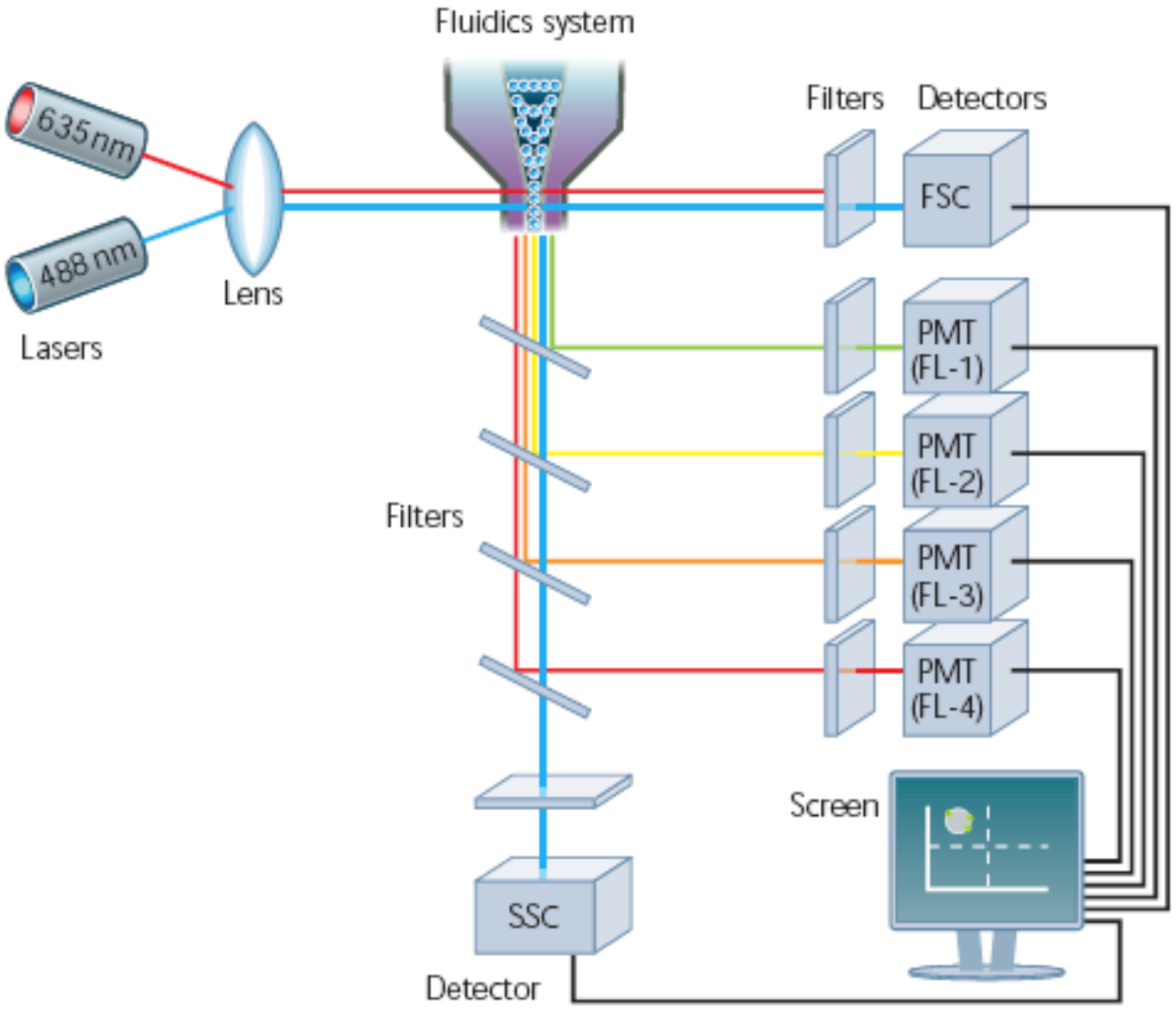
- Need to have a light source focused on the same point where cells have been focused

- light source
  - **Lasers**

Flow Cell



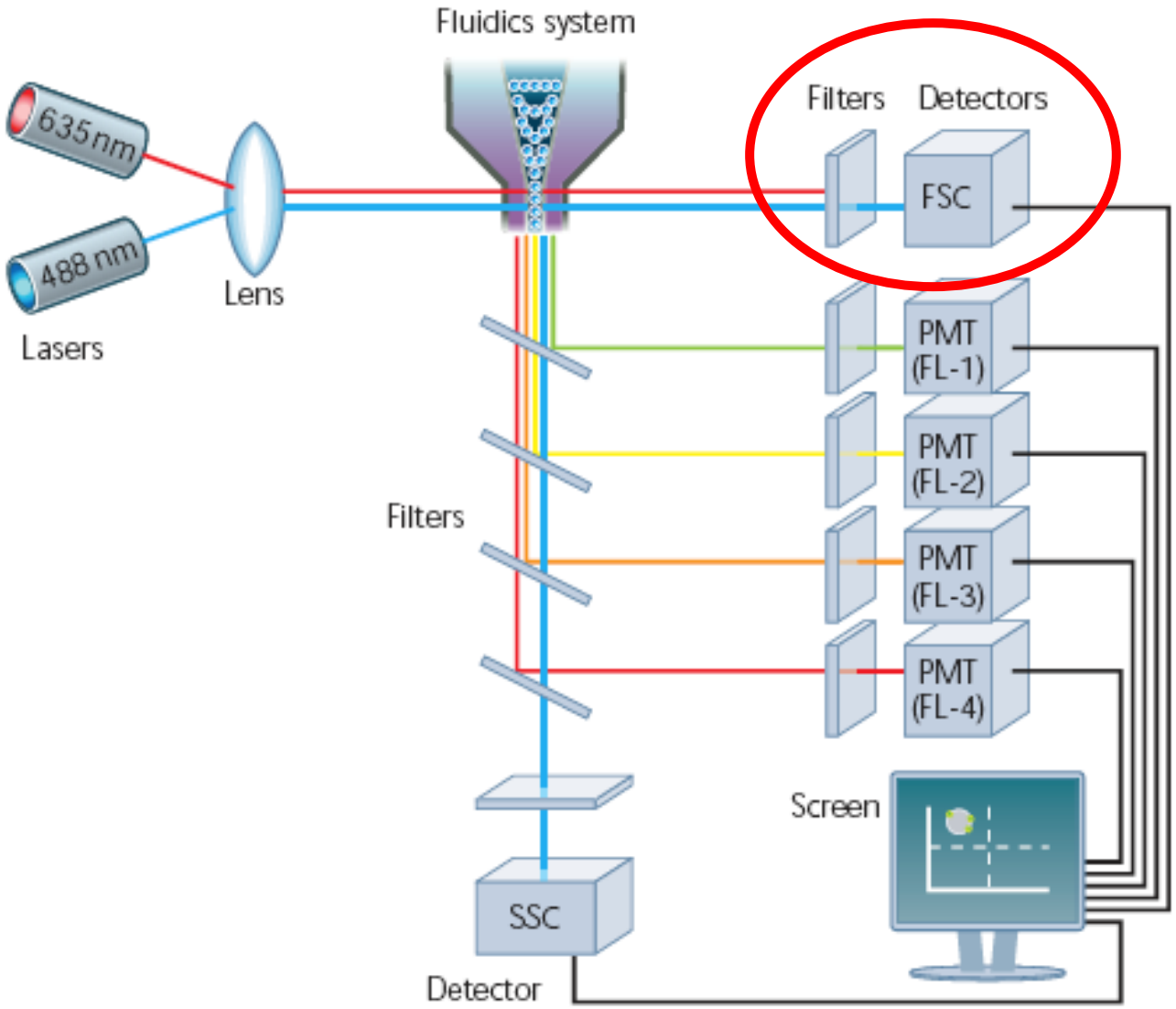
optics


Fluidics system
635 nm
488 nm
Lasers
Lens
Filters
Detectors
FSC
PMT (FL-1)
PMT (FL-2)
PMT (FL-3)
PMT (FL-4)
Filters
Screen
SSC
Detector

What does flow cytometry measure about cells?

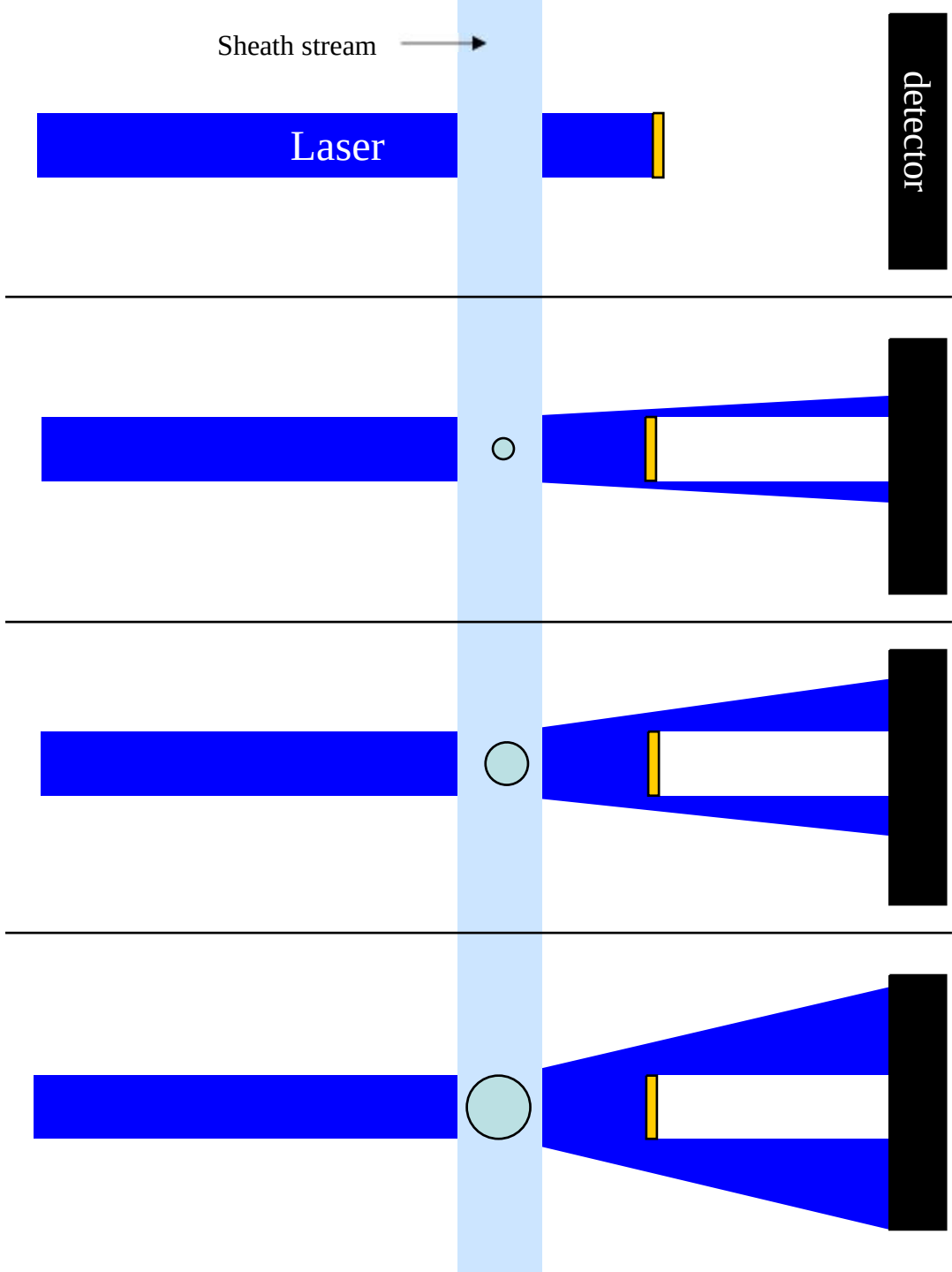
- Size
- Shape (Granularity & Density)
- Makeup (Fluorescence Abs against markers)

Forward Scatter Channel (FSC)

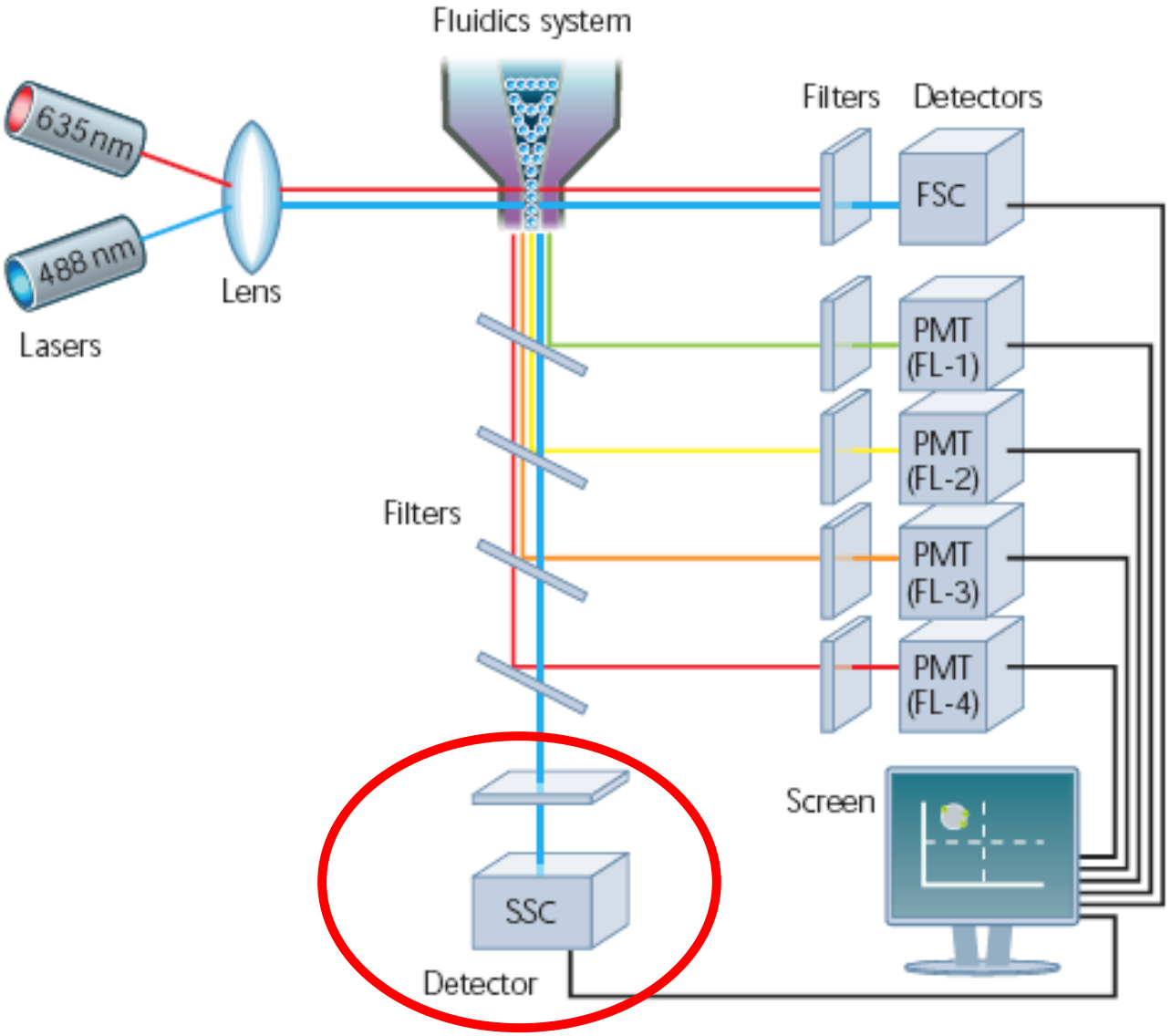


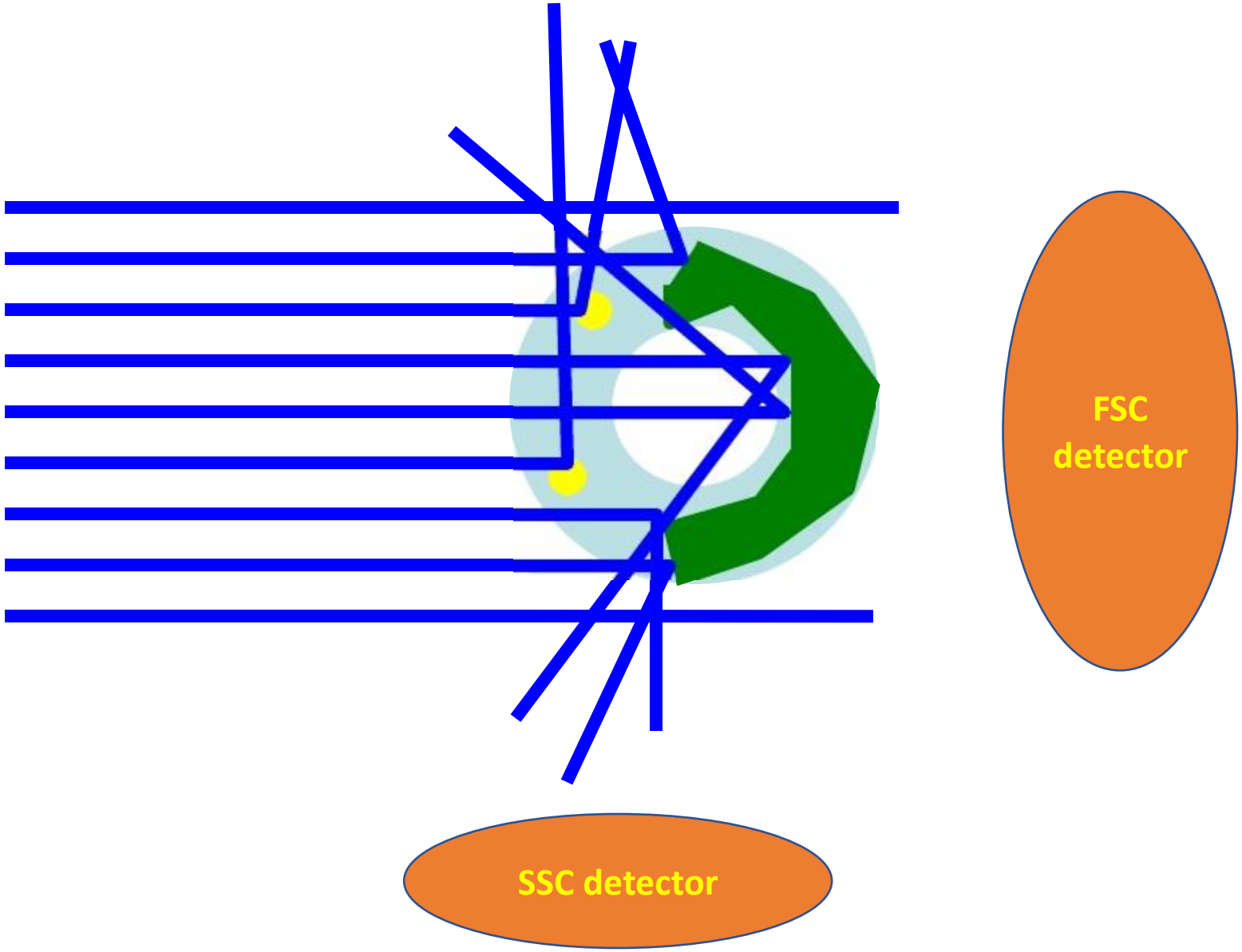
Forward scatter, small angle scatter, FSC

Diffracted light, related to particle's surface area and refractive index, detected along axis of incident light in forward direction.

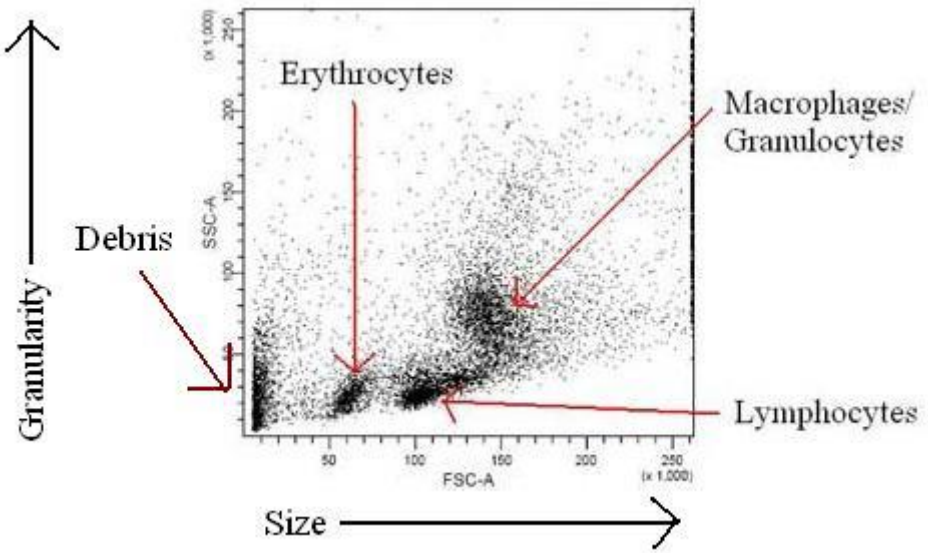


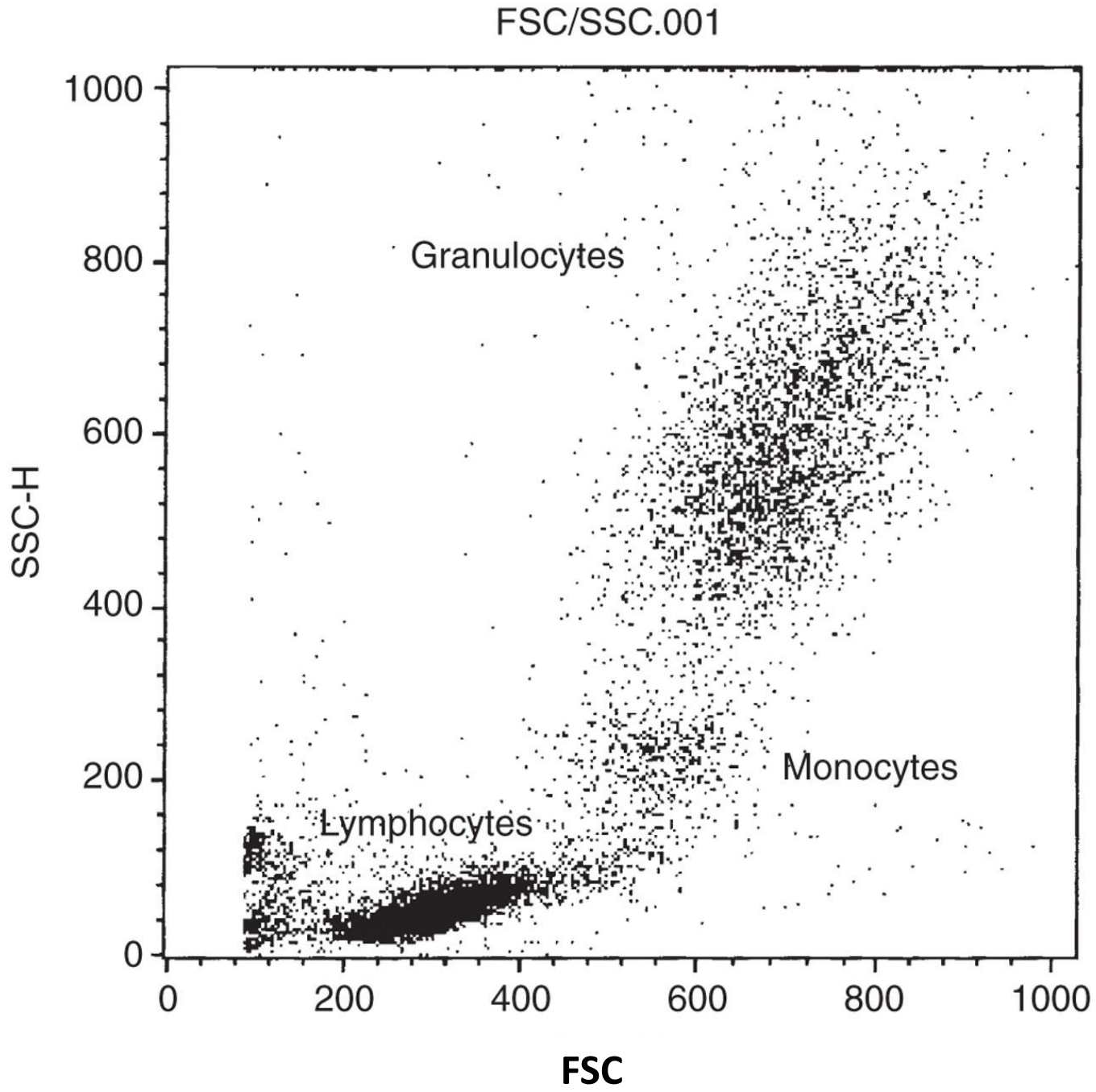
Side Scatter Channel (SSC)



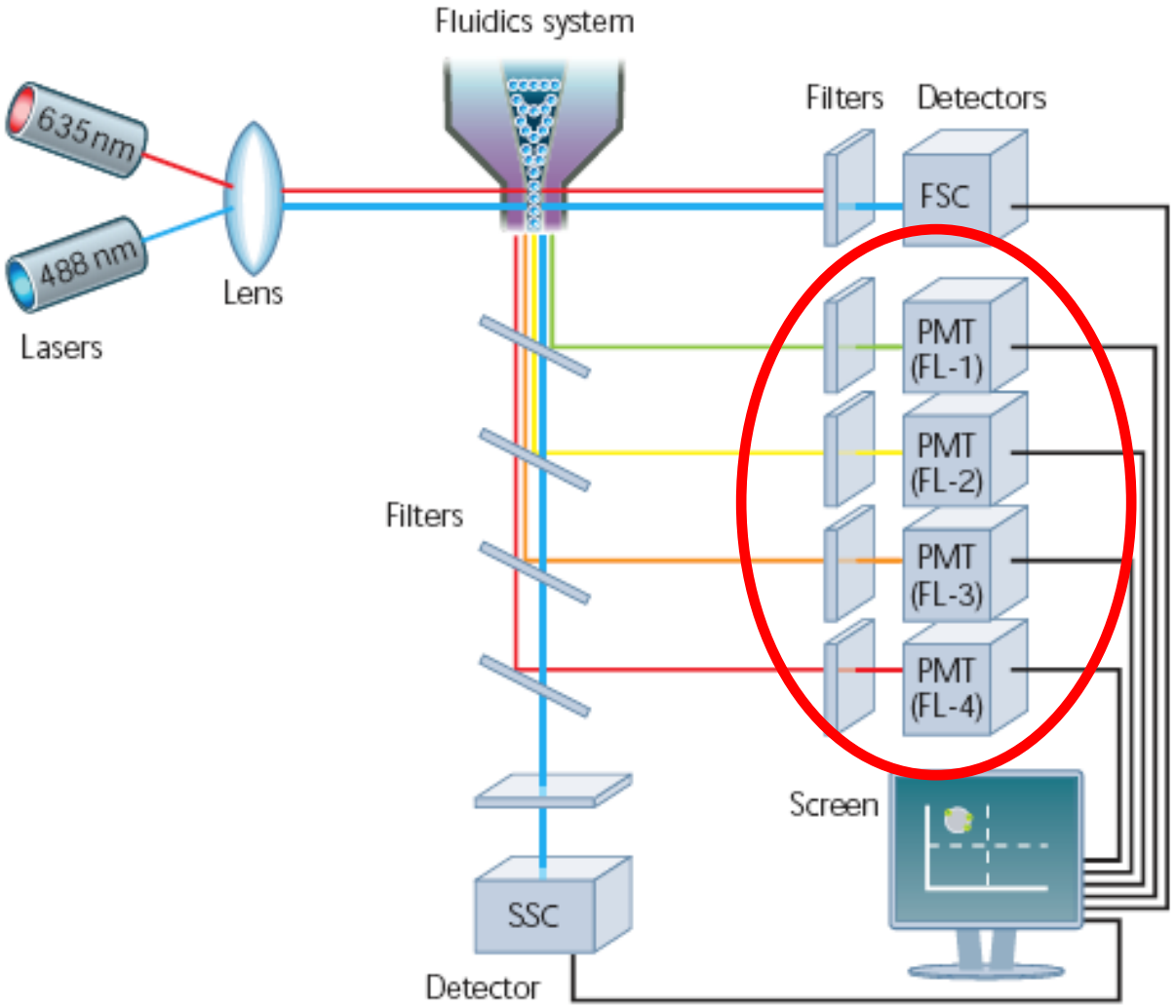

FSC
detector
SSC detector

SSC/FSC


Granularity
Debris
Erythrocytes
Macrophages/
Granulocytes
Lymphocytes
Size
SSC-A
FSC-A
(x 1.000)
50
100
150
200
250


FSC/SSC.001
1000
800
600
400
200
0
SSC-H
Granulocytes
Monocytes
Lymphocytes
0
200
400
600
800
1000
FSC

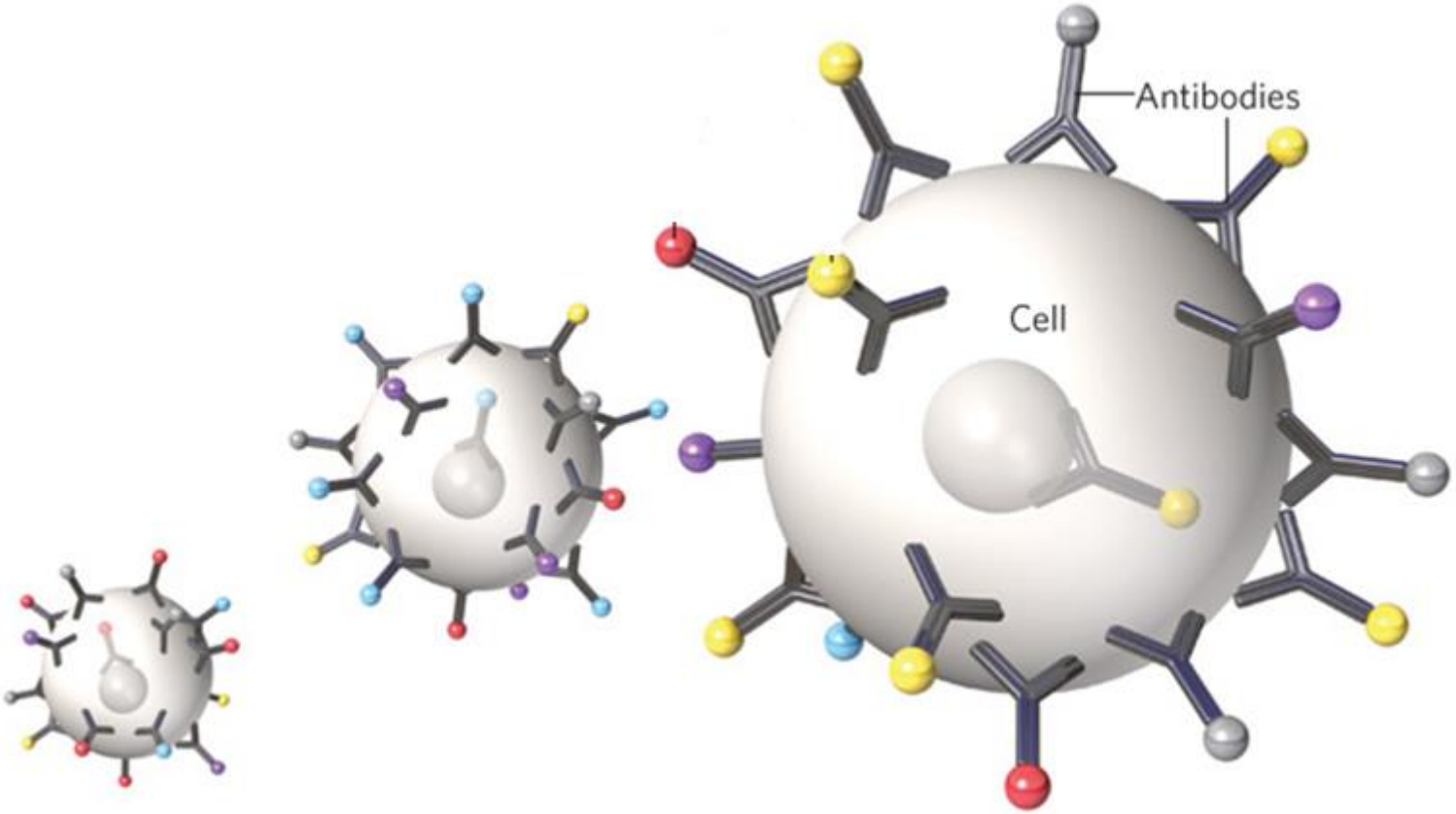
Fluorescence Channels



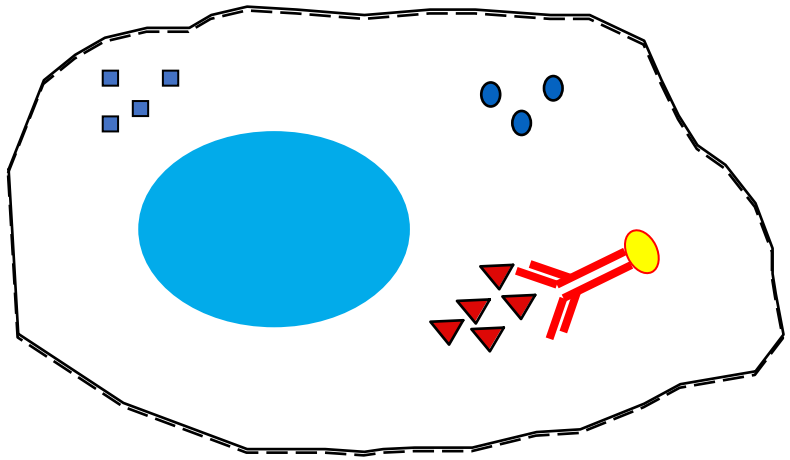
What does flow cytometry measure about cells?

- Size
- Shape (Granularity & Density)
- Makeup (Fluorescence Abs against markers)

Fluorescence Channels

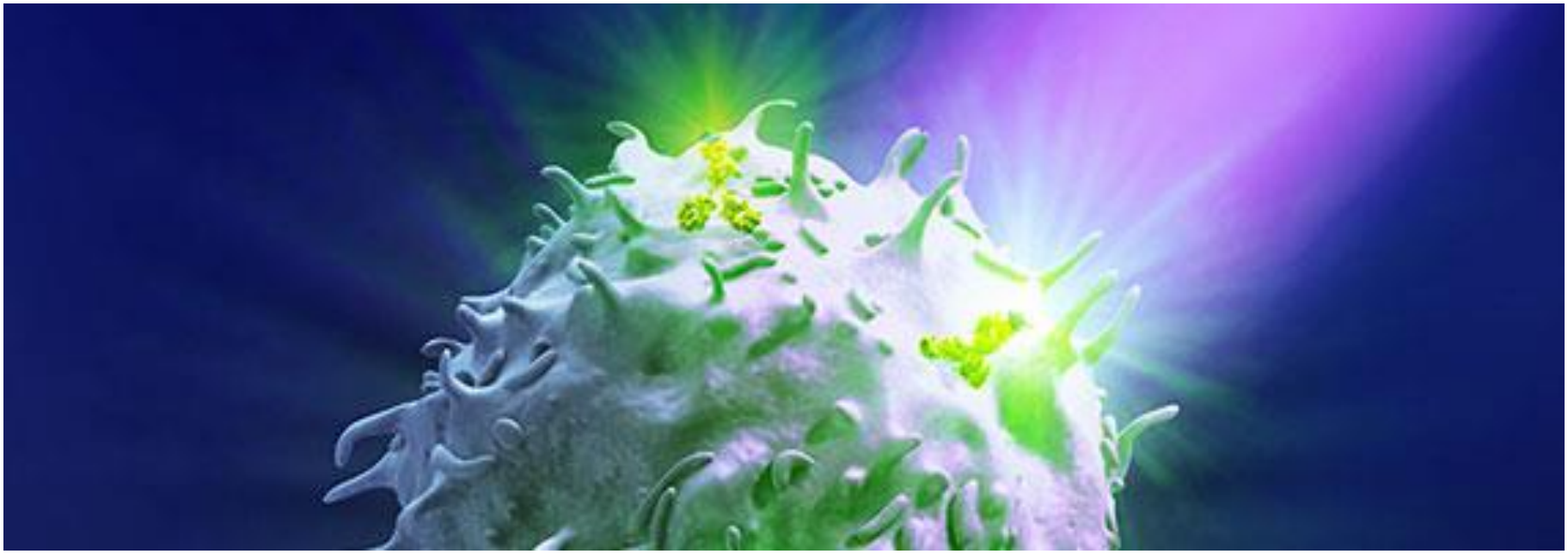


Permeabilizing solution

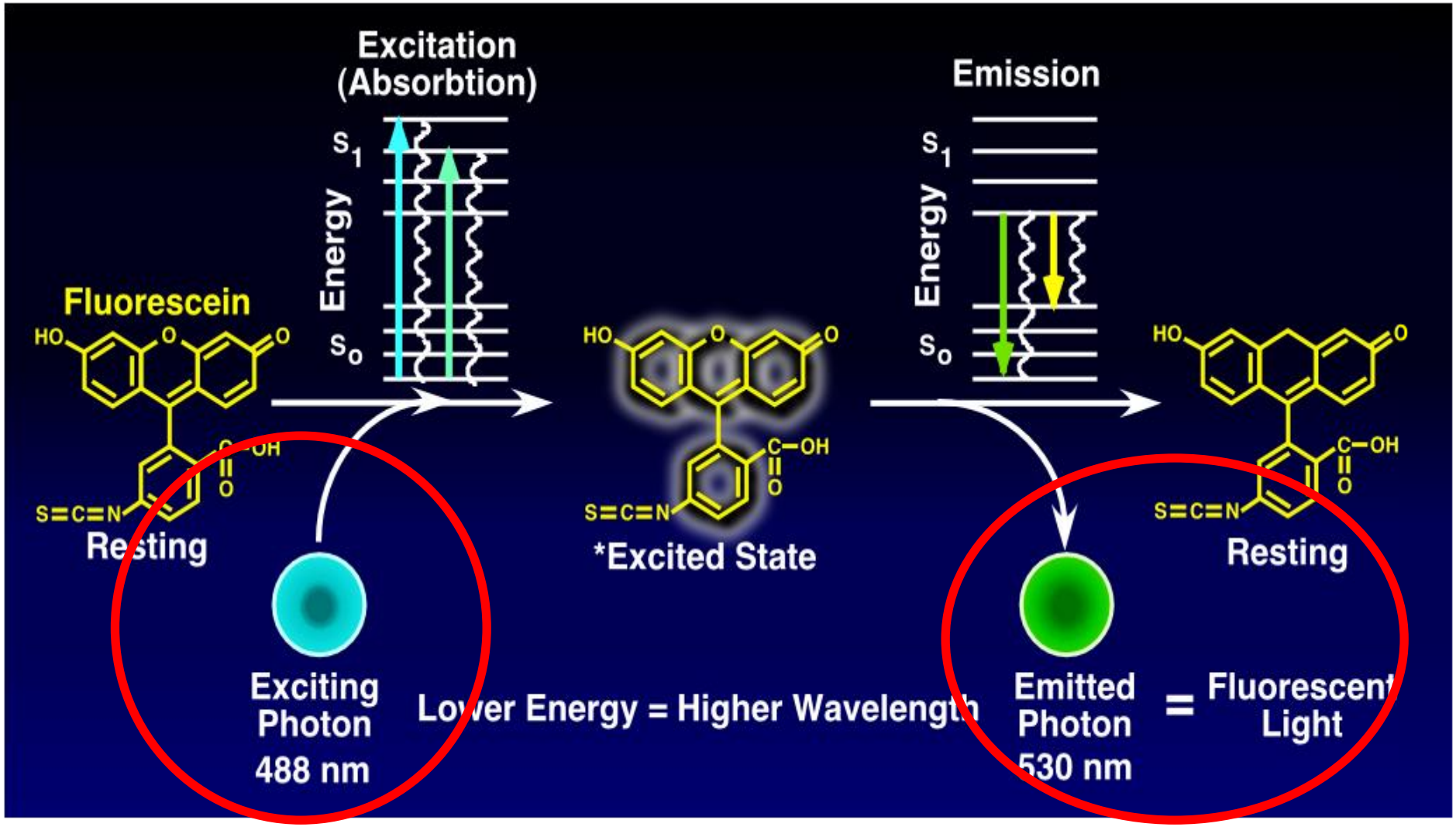


- Cytokine
- Enzyme
- signal transduction molecule
- …etc.

Fluorochromes



Fluorochromes



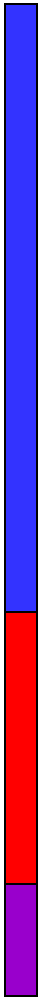
Lasers

Cytometers will have one or more lasers:

Common excitation wavelengths:

- **488 (blue)**
- **635 (red)**
- **405 (violet)**
- **532 (green)**
- **350 (UV)**
- **561 (yellow-green)**

Common Fluorochromes for Ab conjugation



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

Single dyes

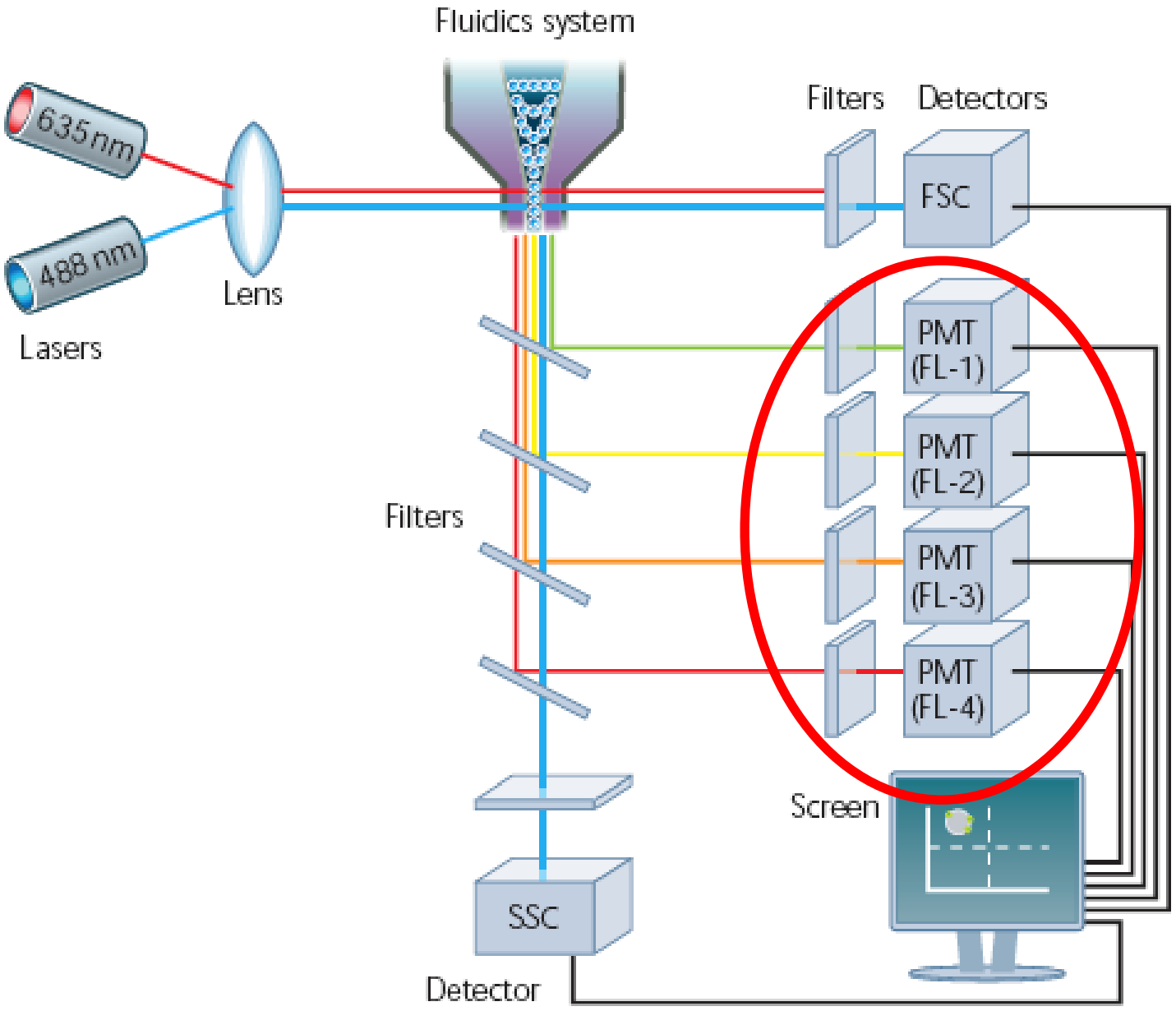
Dye	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)	Fluorescence color
Alexa Fluor® 405	405, 407	401	421	
Alexa Fluor® 430	405, 407	433	541	
Alexa Fluor® 488	488	495	519	
Alexa Fluor® 633	633, 635, 647	632	647	
Alexa Fluor® 647	633, 635, 647	650	665	
Alexa Fluor® 660	633, 635, 647	663	690	
Alexa Fluor® 680	633, 635, 647	679	702	
Alexa Fluor® 700	633, 635, 647	702	723	Infrared
APC	633, 635, 647	650	661	
FITC	488	490	525	
Pacific Blue™	405, 407	410	455	
PerCP	488	490	675	
Phycoerythrin	488	490, 565	578	

Tandem dyes

Dye	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)	Fluorescence color
APC-Alexa Fluor® 750	633, 635, 647	650	779	Infrared
APC-Cy5.5	633, 635, 647	650	695	
APC-Cy7	633, 635, 647	650	785	Infrared
PerCP-Cy5.5	488	496, 546	695	
PE-Alexa Fluor® 610	488	496, 546	627	
PE-Alexa Fluor® 647	488	496, 546	667	
PE-Alexa Fluor® 680	488	496, 546	702	
PE-Alexa Fluor® 700	488	496, 546	723	Infrared
PE-Alexa Fluor® 750	488	496, 546	779	Infrared
PE-Cy5.5	488	496, 546	695	
PE-Cy5	488	496, 546	667	
PE-Cy7	488	496, 546	785	Infrared
PE-Texas Red®	488	496, 546	615	

Abbreviations

APC Allophycocyanin
FITC Fluorescein isothiocyanate
PE Phycoerythrin
PerCP Peridinin-chlorophyll-protein complex



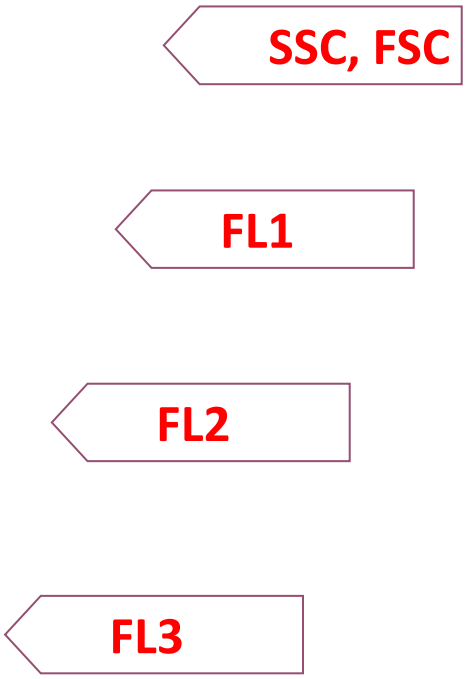
FACS Calibur™

Detector	Filter	Color	Fluorochrome
FL1	530/30nm	Green	FITC
FL2	585/42nm	Yellow/Orange	PE
FL3	670nm LP	Dark Red	PerCP, PerCP-Cy5.5
FL4	661/16nm	Red	APC

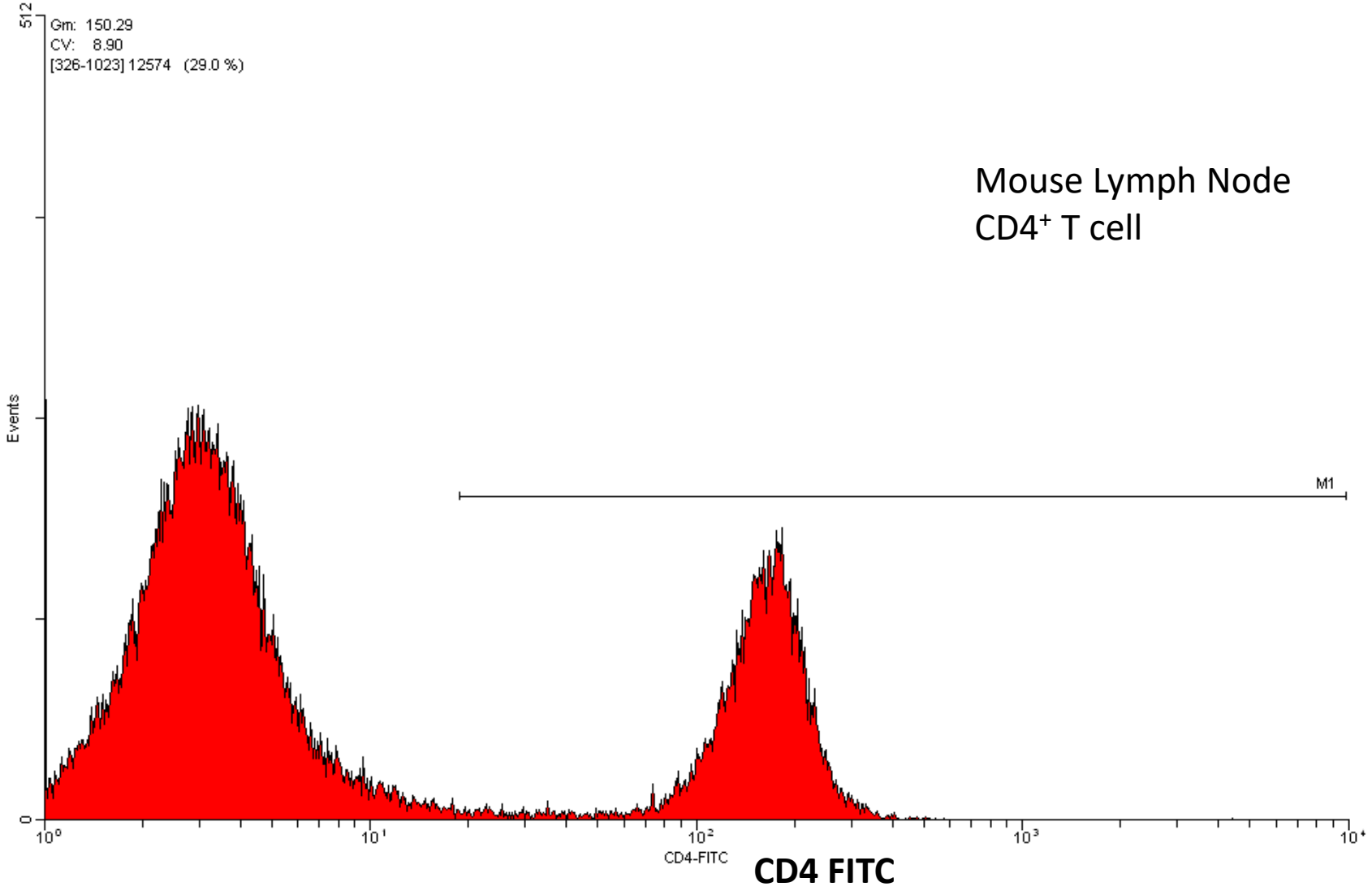

A
B
C
UV
BEACH BABES WITH BRAINS!!

example

- CD4, CD25, FoxP3 lymphocyte:
  - Lymphocyte: Size, Granulity
  - CD4: CD4 FITC antibody
  - C25: CD25 PE antibody
  - FoxP3 : Foxp3 PE-Cy5 antibody

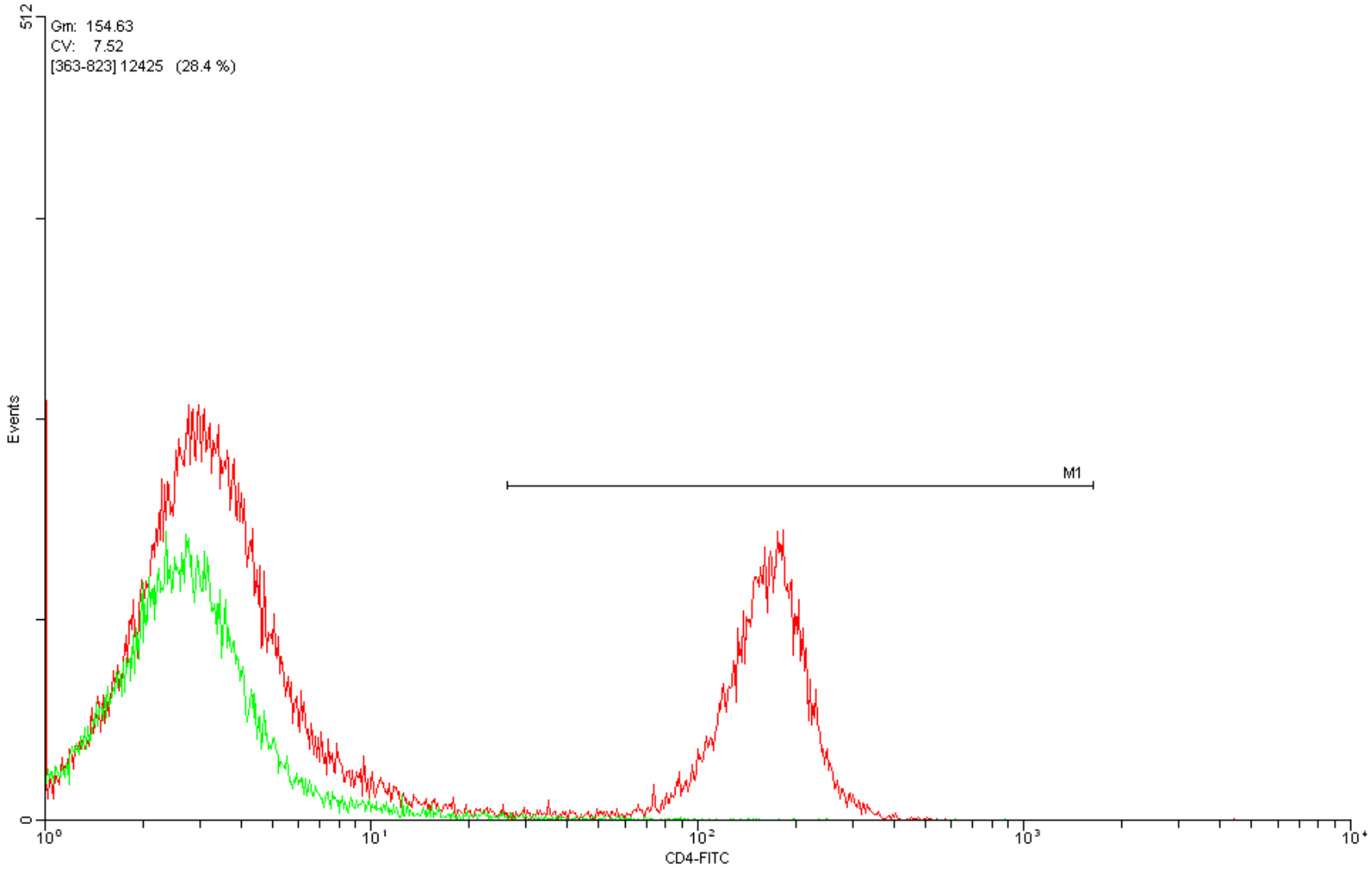


Single Color Histogram



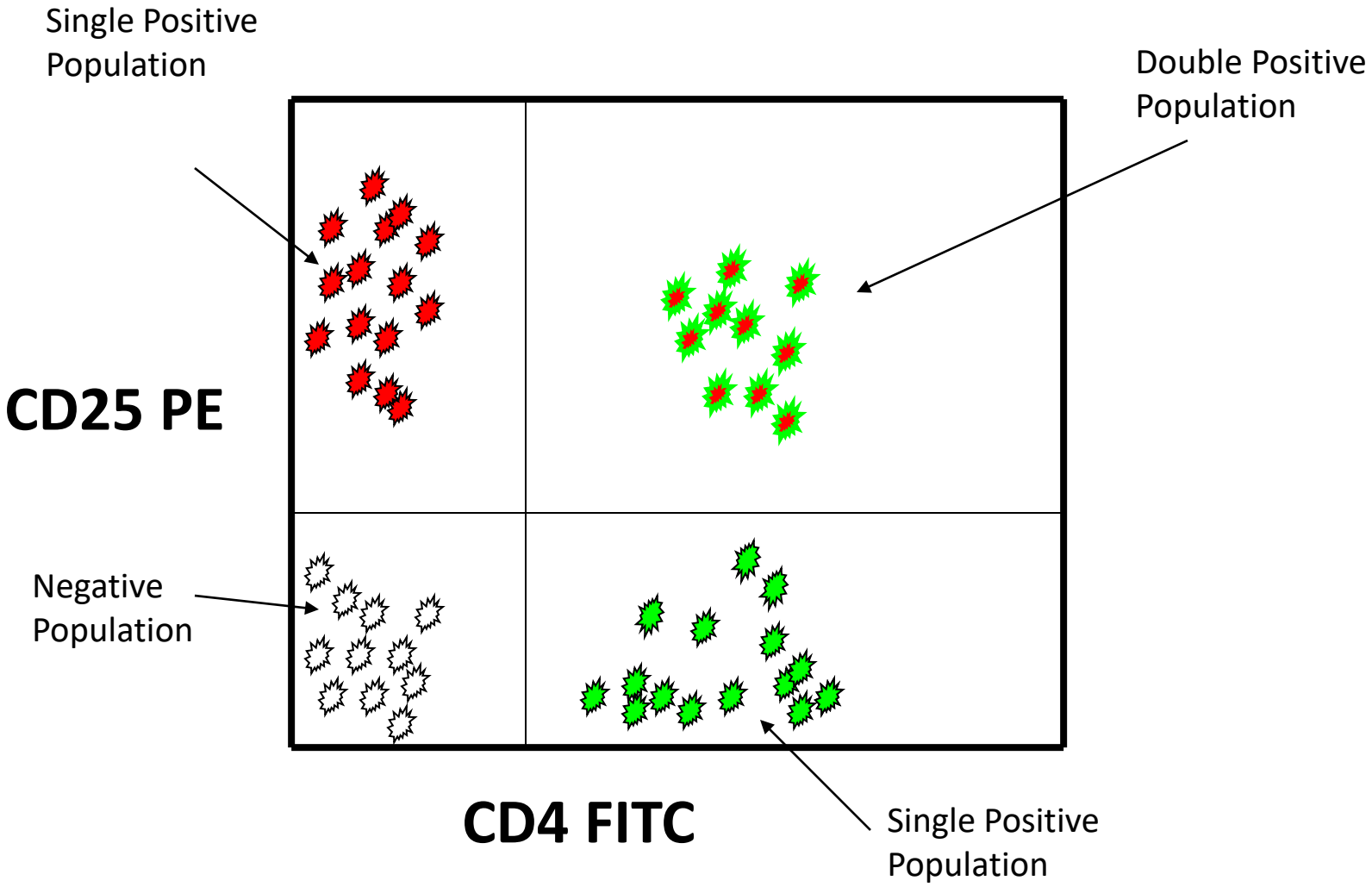
Mouse Lymph Node
$CD4^+$ T cell

CD4 FITC

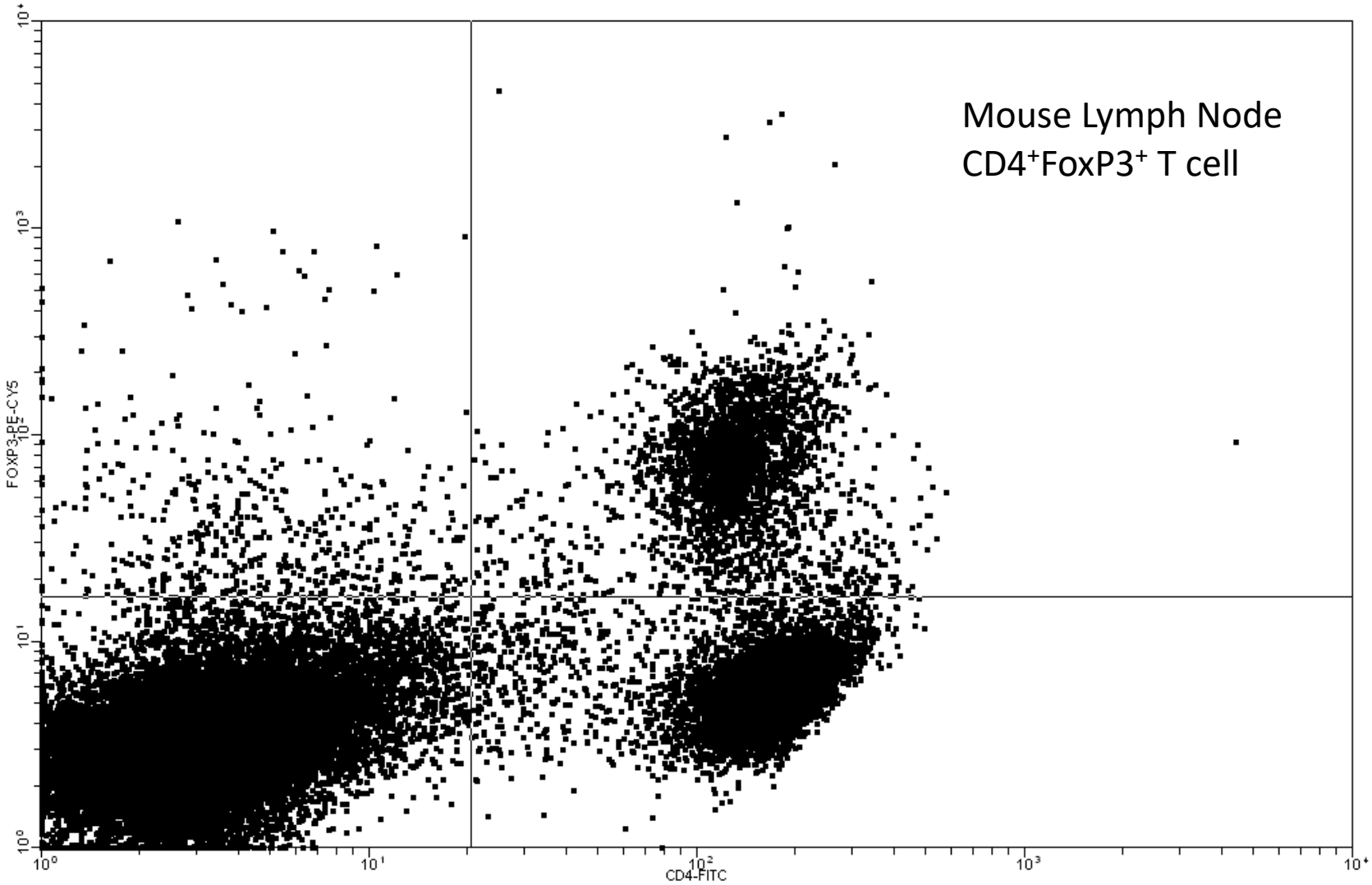

Gm: 154.63
CV: 7.52
[363-823] 12425 (28.4 %)
512
0
Events
M1
10^0
10^1
10^2
10^3
10^4
CD4-FITC

CD4 FITC

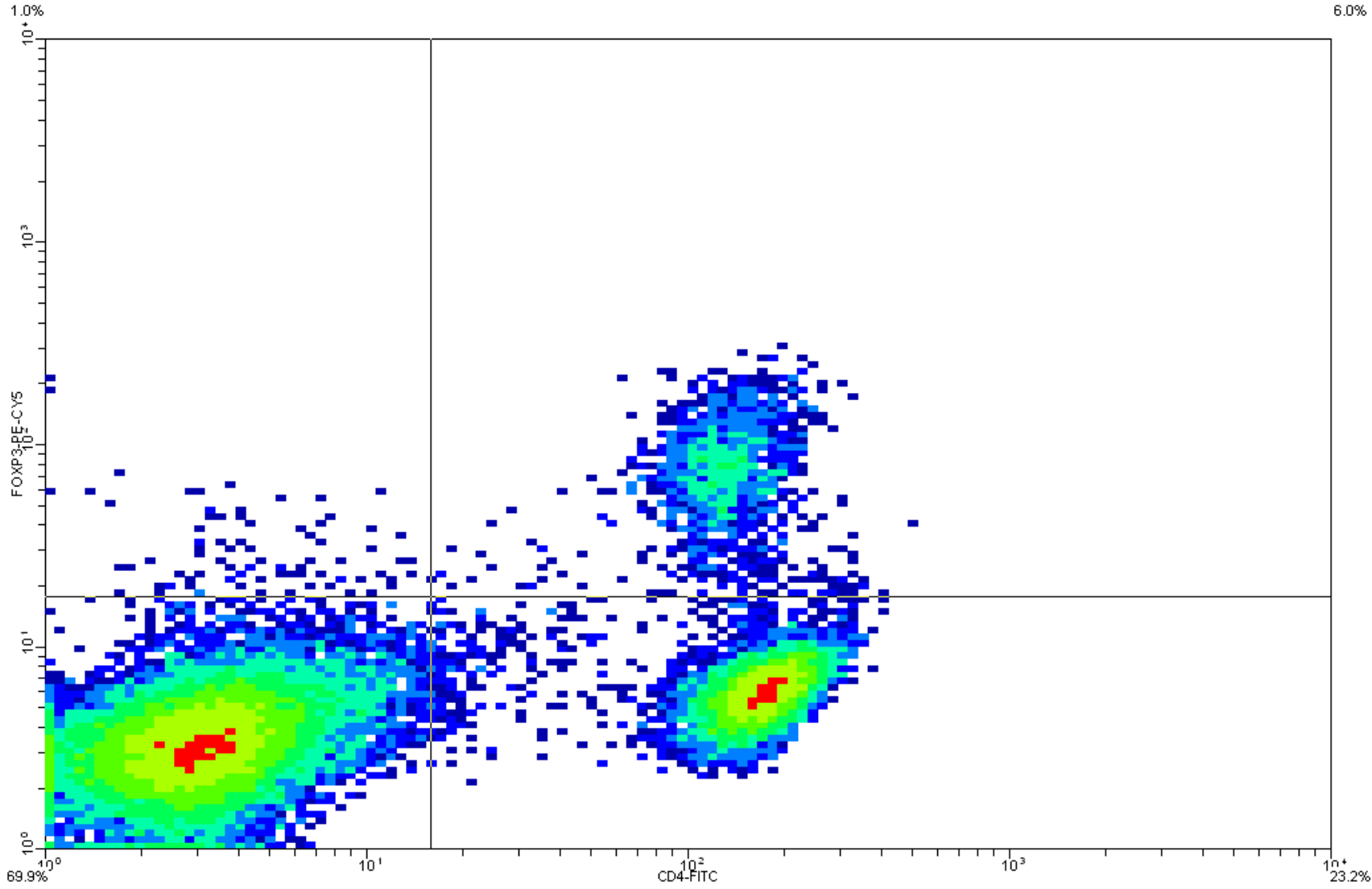
2 Parameter


Single Positive Population
Double Positive Population
CD25 PE
Negative Population
CD4 FITC
Single Positive Population

Two Color Dot Plot



Two Color Density Plot



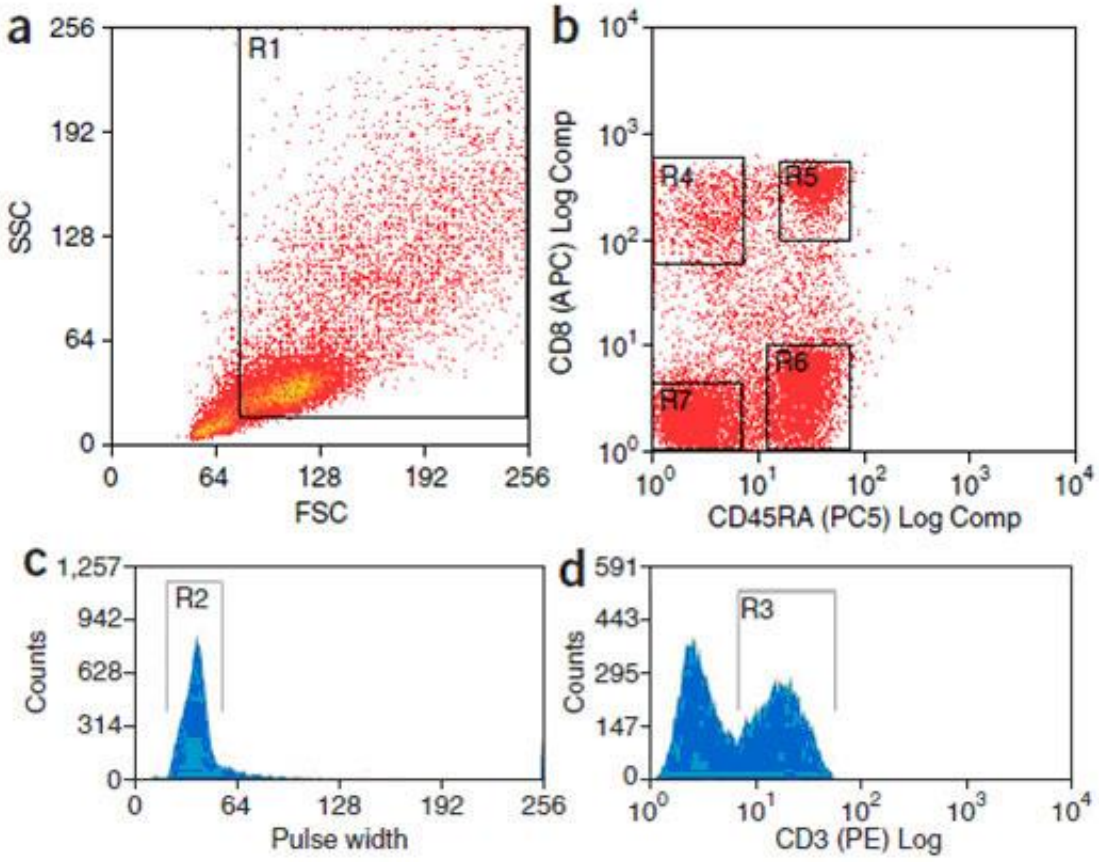
FOXP3-PE

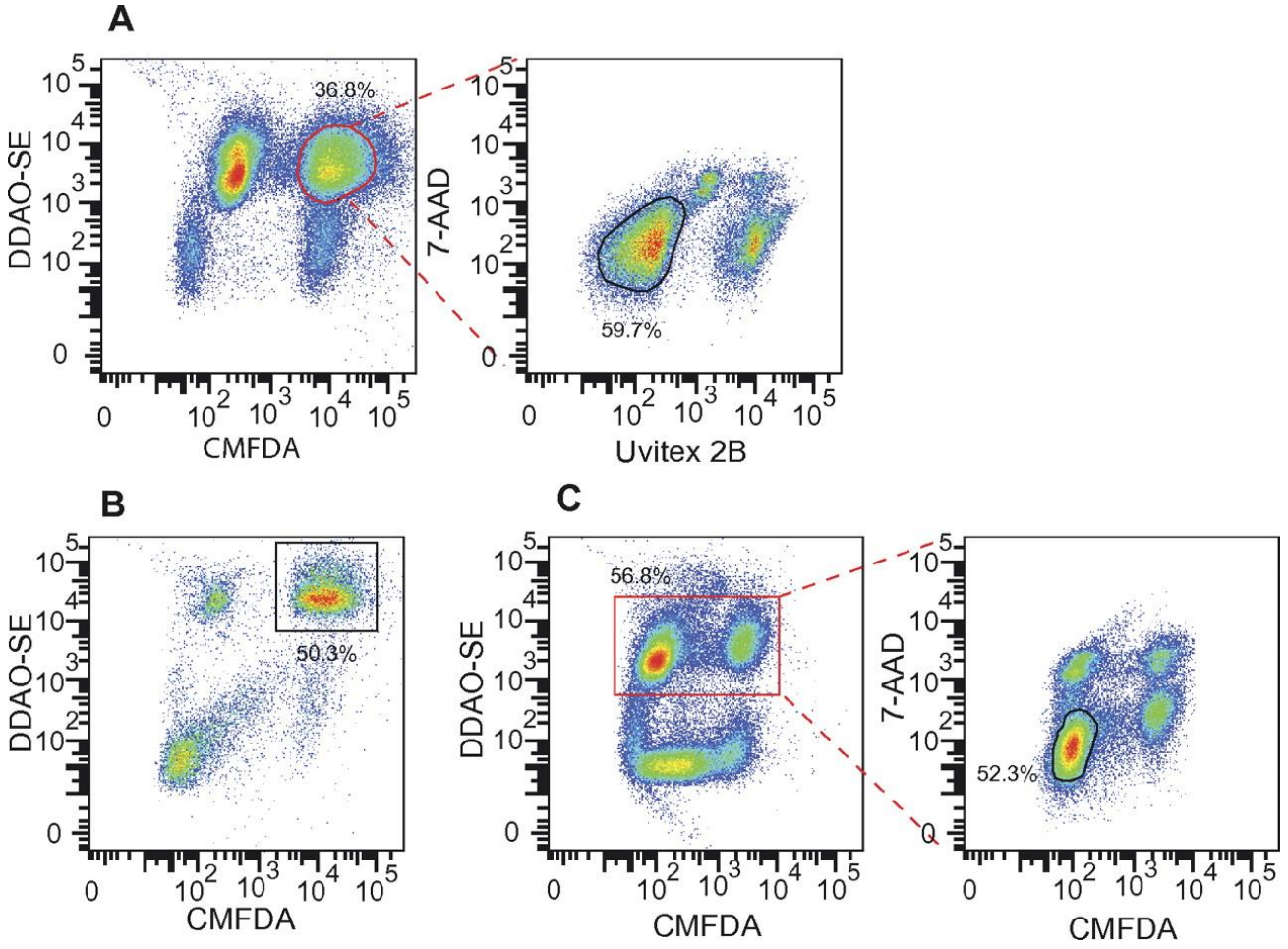
CD4-FITC

Section III:
gating

Gating

- Is used to isolate a subset of cells on a plot
- Allows the ability to look at parameters specific to only that subset




A
DDAO-SE
CMFDA
36.8%
7-AAD
Uvitex 2B
59.7%
B
DDAO-SE
CMFDA
50.3%
C
DDAO-SE
CMFDA
56.8%
7-AAD
CMFDA
52.3%

Section IV:

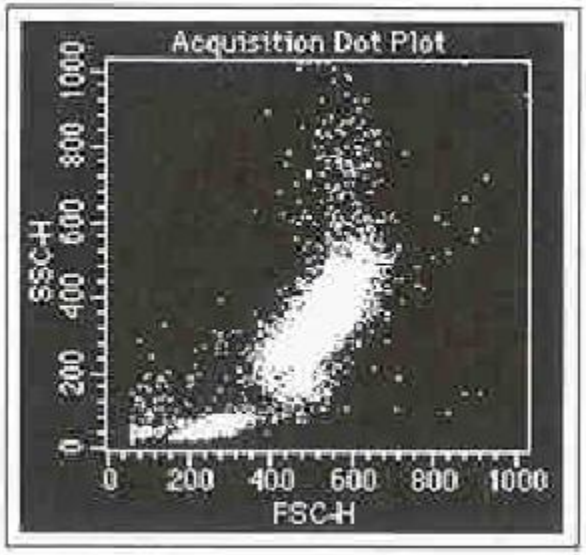
Instrument setting

Instrument Setup

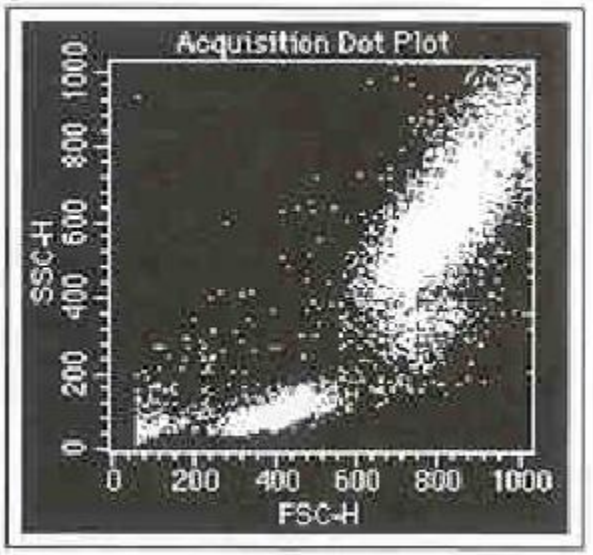
- User adjusts sensitivity of detectors so that:
  - Events of interest are on scale
  - “Negative” fluorescence on the left/bottom, providing maximum dynamic range for positive signals

1-Setting Voltages Setting FSC and SSC

Whole Blood



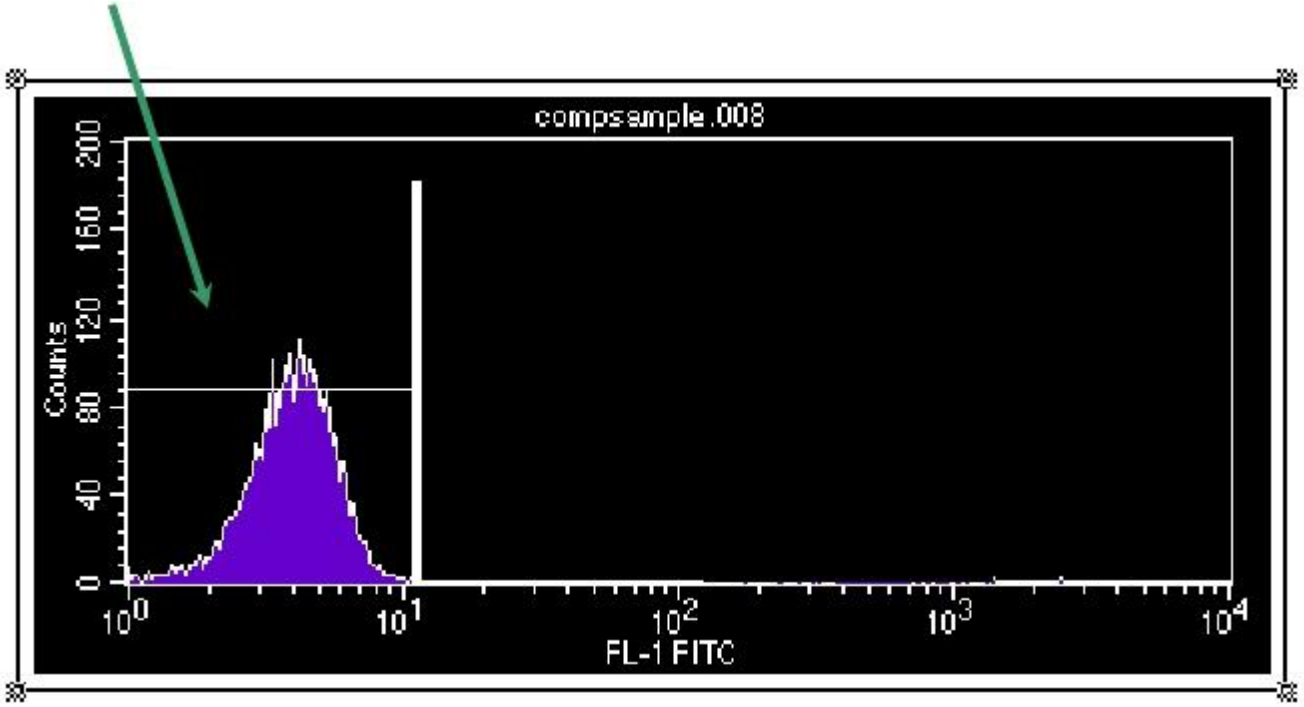
Before optimizing



Optimized

2- Setting Starting Voltages for Fluorescent Parameters

For each color, adjust voltage so that the negative population is in the first decade, off of the axis (if possible)



Auto fluorescence

- Negative" signal on cells is auto fluorescence due to flavins, porphyrins and other molecules or properties of the material (plastics fluoresce in certain excitation wavelengths).

- Different cells will have different levels of autofluorescence (e.g.lymphs vs. monos, different cell lines) affecting sensitivity in certain parameters with high base signals.

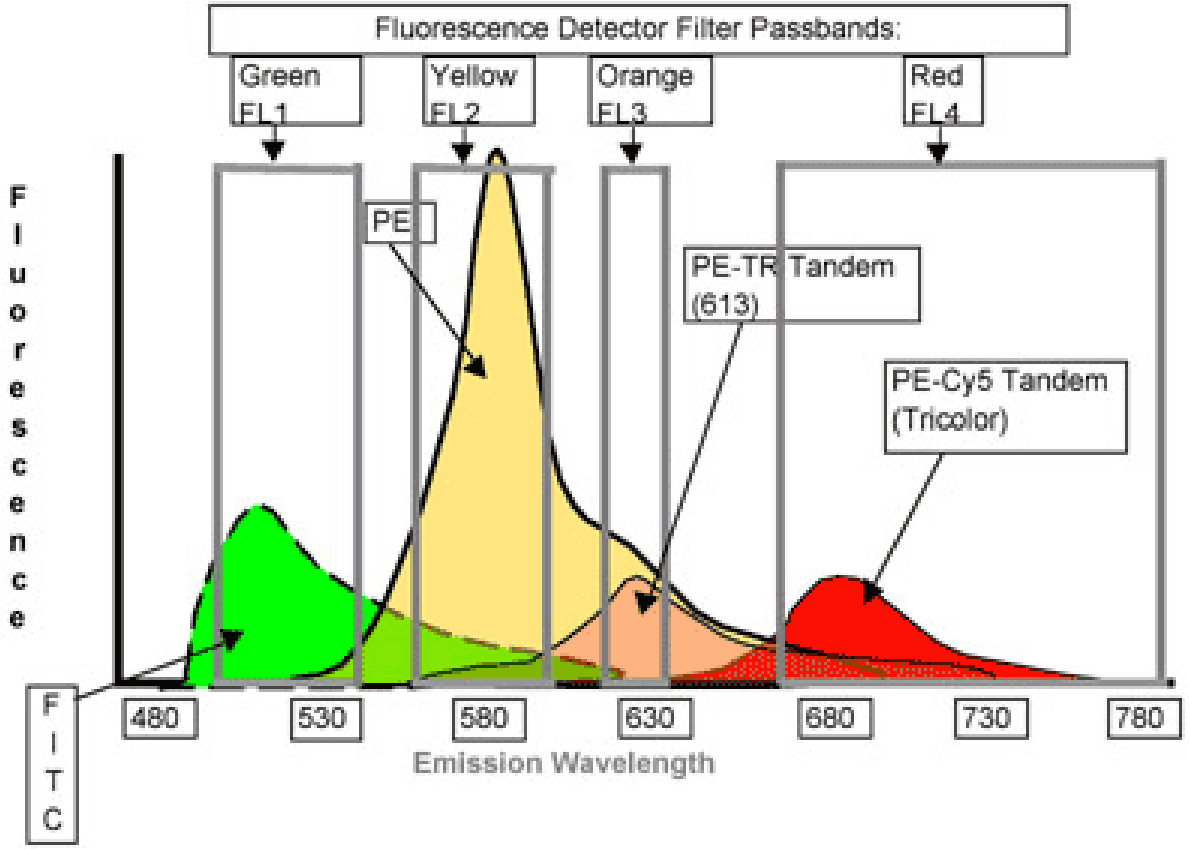
3- Compensation

- Fluorochromes typically fluoresce over a large part of the spectrum (100nm or more)

- Depending on filter arrangement, a detector may see some fluorescence from more than 1 fluorochrome

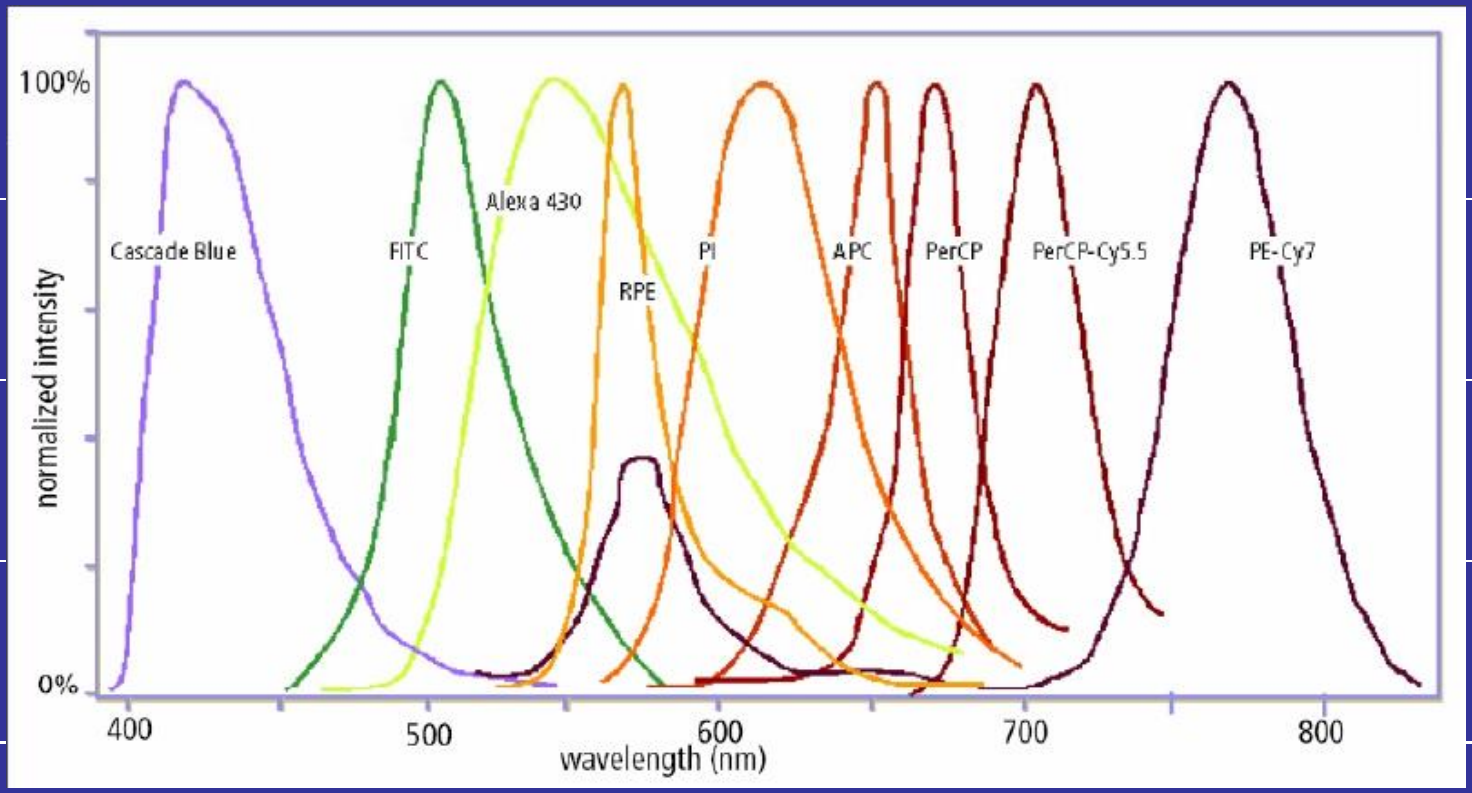
- You need to "compensate" for this bleed over so that 1 detector reports signal from only 1 fluorochrome

Compensation



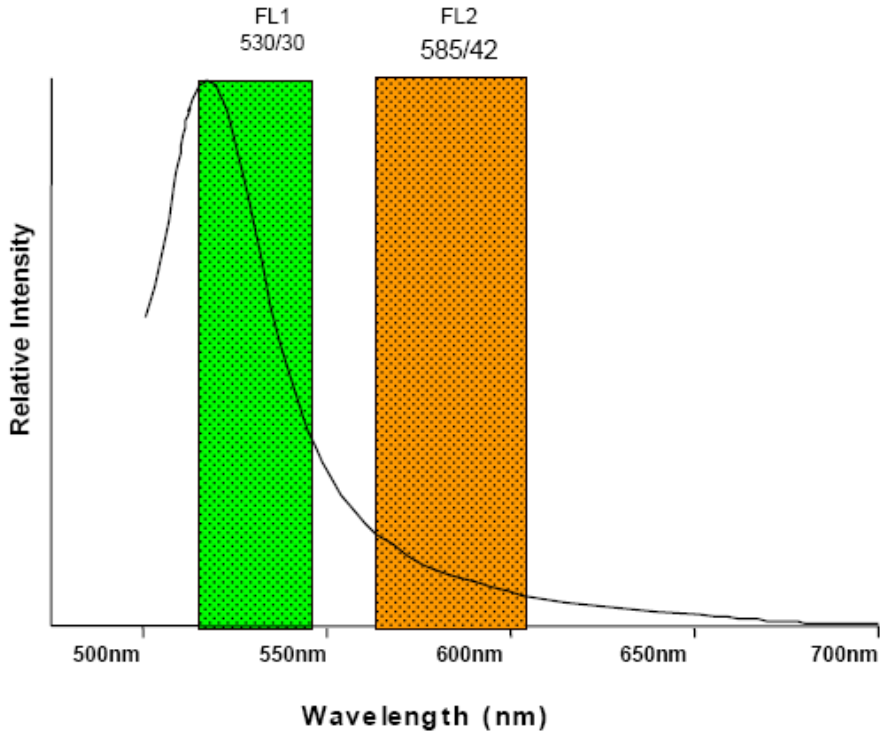
- There is some overlap between the colors emitted by different fluorescent markers, therefore mathematical compensation is used to reduce overlapping results

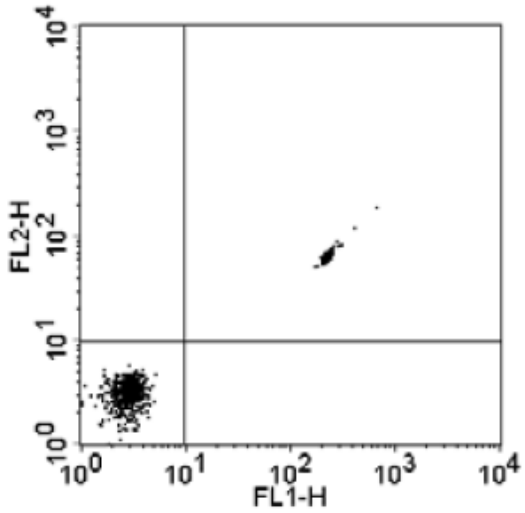
http://www.bdbiosciences.com



BD LSR II User's Guide; Becton Dickinson

FITC Fluorescence Overlap





FITC Compensation

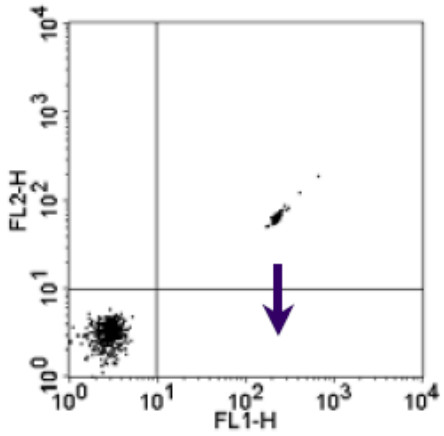


Figure A

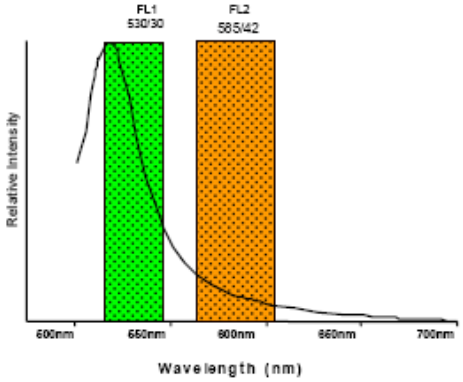


Figure B

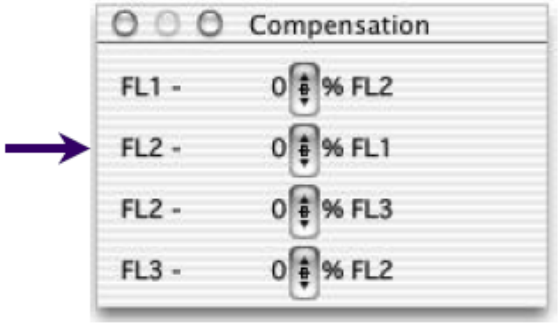


Figure C

Detector - % Signal

FITC Compensation

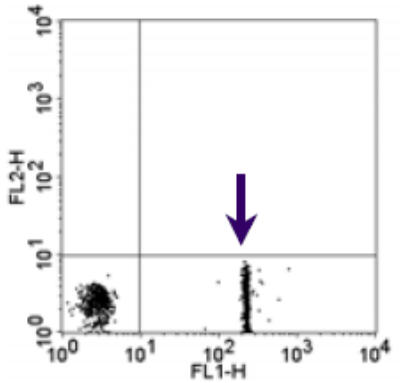


Figure A

To Lower Cluster

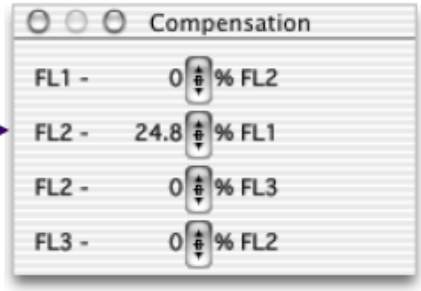


Figure B

Increase % Subtracted

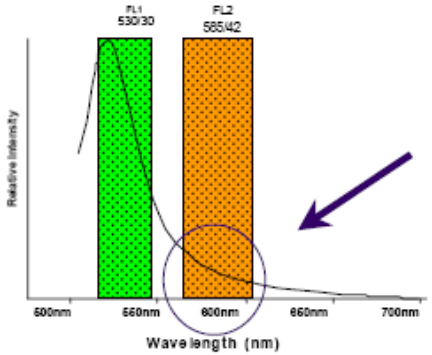
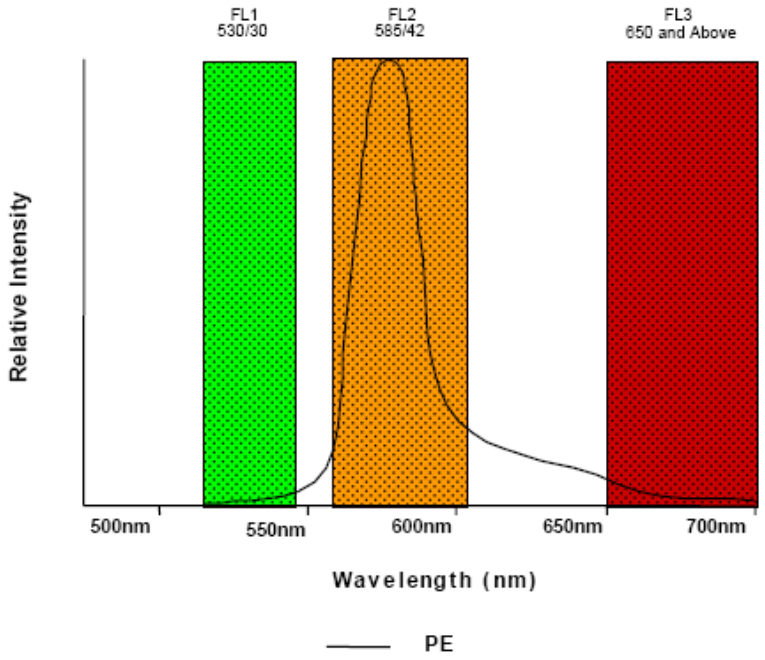
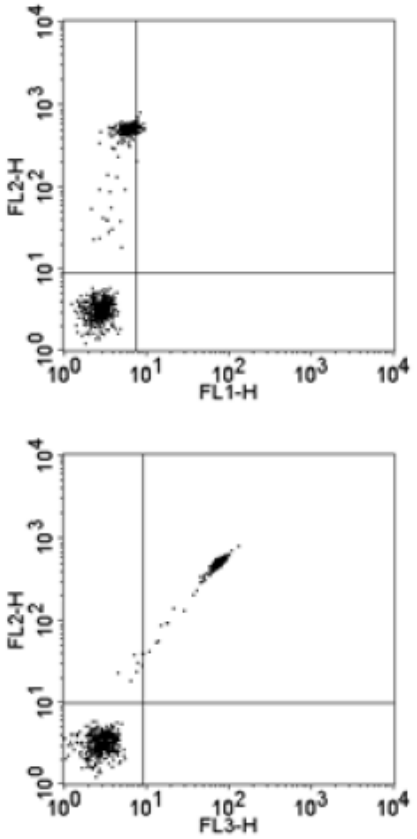


Figure C

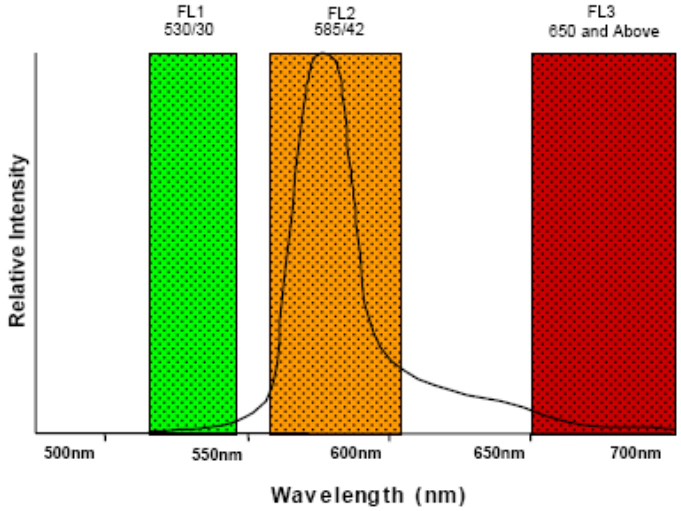
24.8% of the Signal Sensed in FL2

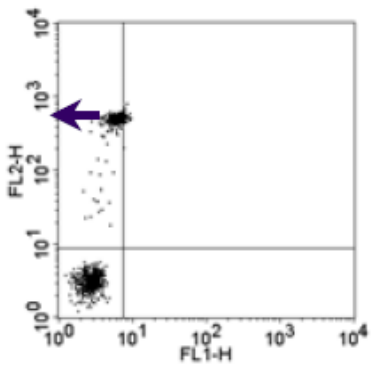
PE Fluorescence Overlap

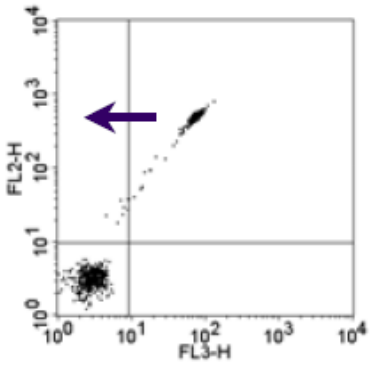


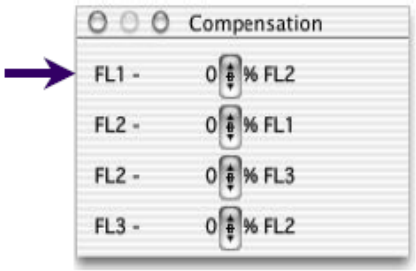


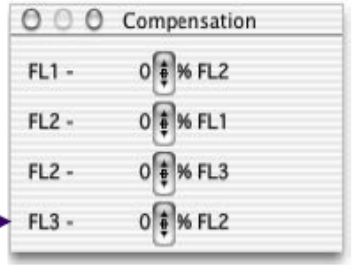
PE Compensation





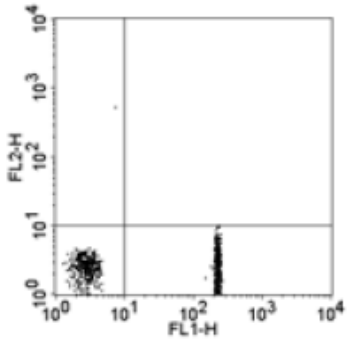


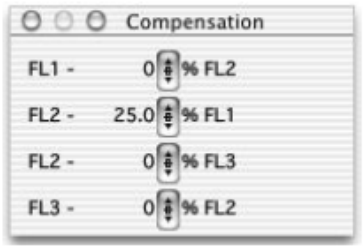




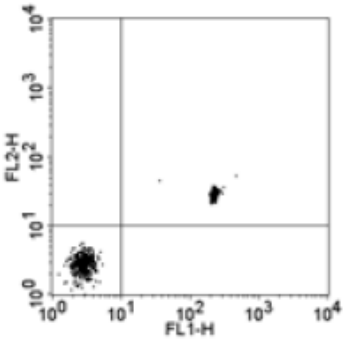
Compensation Examples

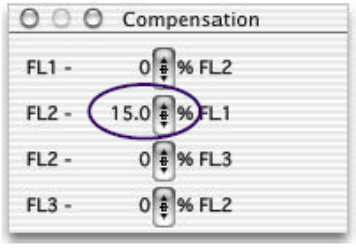
Optimal Compensation





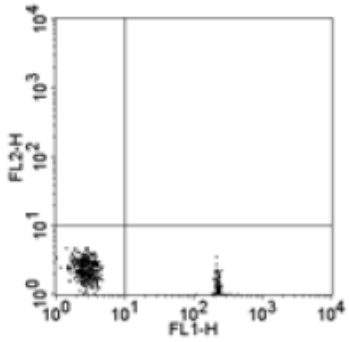
Under Compensation

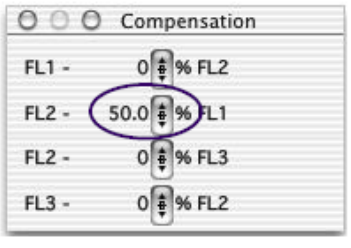




Not enough subtracted

Over Compensation



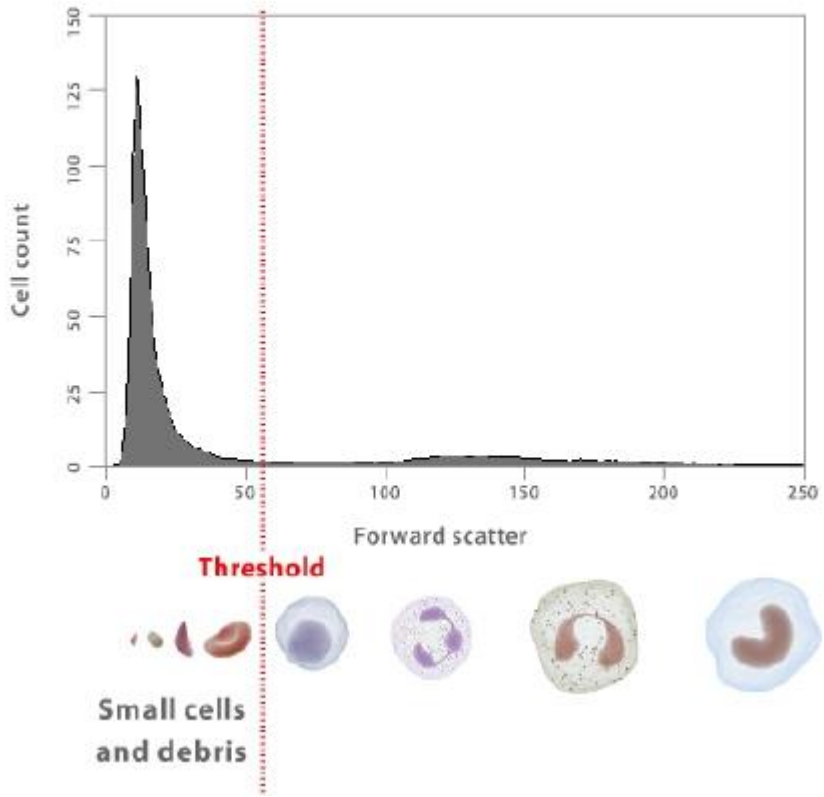


Too much subtracted

3- Threshold

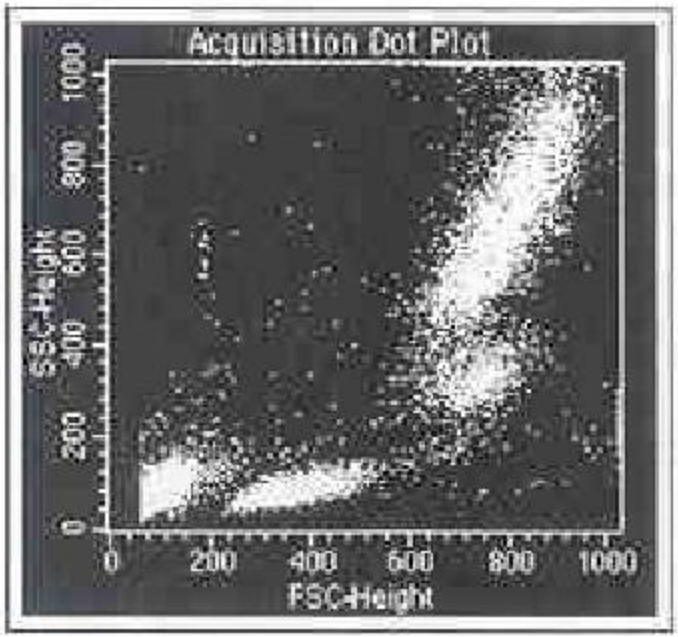
- The threshold can be set on any parameter, but is usually set on FSC

Forward Scatter Threshold

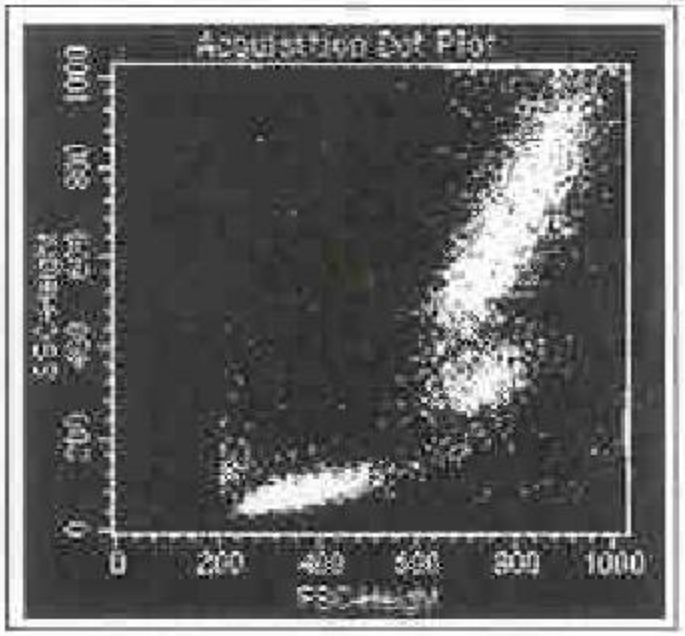


Forward scatter used as trigger signal. Events below cutoff are ignored.

Adjust FSC Threshold



Before



After

Eliminates debris, RBC's, platelets, instrument noise.

Flow Cytometers

- Beckman Coulter
  - FC500, MCL-XL, Elite, Profile, Point Care
- Becton Dickinson
  - Canto, FACSCalibur, FACSCan, FACSort, FACSCount
- Guava Technologies Inc.
  - Personal Cell Analyzer System (PCA)
- Partec - CyFlow

Interpretation

- Once the values for each parameter are in a list mode file, specialized software can graphically represent it.
- The data can be displayed in 1, 2, or 3 dimensional format
- Common programs include…
  - CellQuest
  - Flowjo
  - WinMDI
  - FCS Express

Thank you