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INTERNATIONAL CLINICAL RESEARCH CENTER

# Flow cytometry

Mgr. Ondřej Vymazal

# Introduction

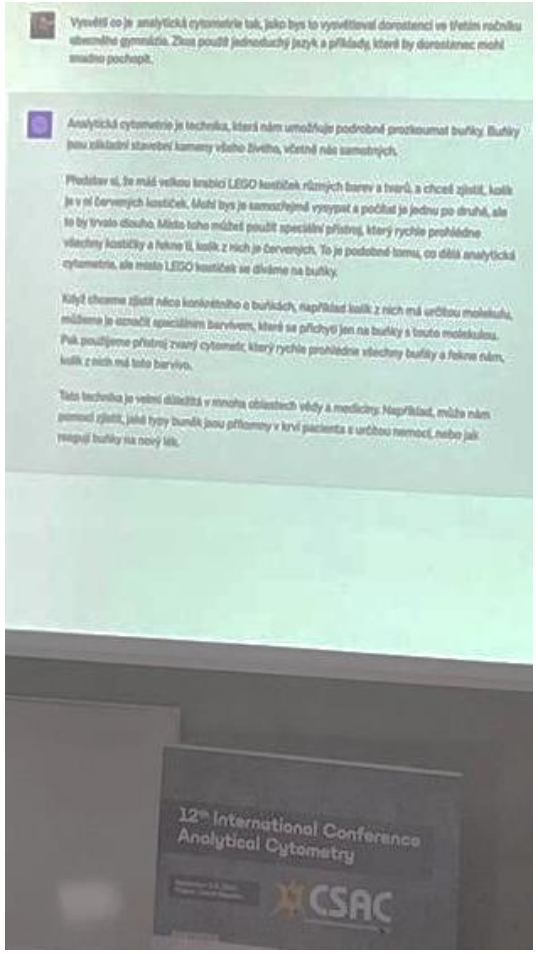
- What is the Flow cytometer?
- For which application it can be used?
- How does it work?

# Question for chatGPT: explain what analytical cytometry is as if you were explaining it to teenagers in the third year of general grammar school. Try to use simple language and examples that a teenager could easily understand.

Analytical cytometry is a technique that allows us to examine cells in detail. Cells are the basic building blocks of all living things, including ourselves.

Imagine you have a big box of LEGO bricks of different colors and shapes and you want to find out how many red bricks are in them. You could of course dump them out and count them one by one, but that would take a long time. Instead, you can use a special device that scans all the dice and tells you how many of them are red. This is similar to what analytical cytometry does, but instead of LEGO bricks, we're looking at cells.

When we want to find out something specific about cells, for example how many of them have a certain molecule, we can mark them with a special dye that will only attach to cells with this molecule. Then we use a machine called a cytometer, which quickly looks at all the cells and tells us how many of them have this dye. This technique is very important in many fields of science and medicine. For example, it can help us find out what types of cells are present in the blood of a patient with a certain disease, or how the cells respond to a new drug.



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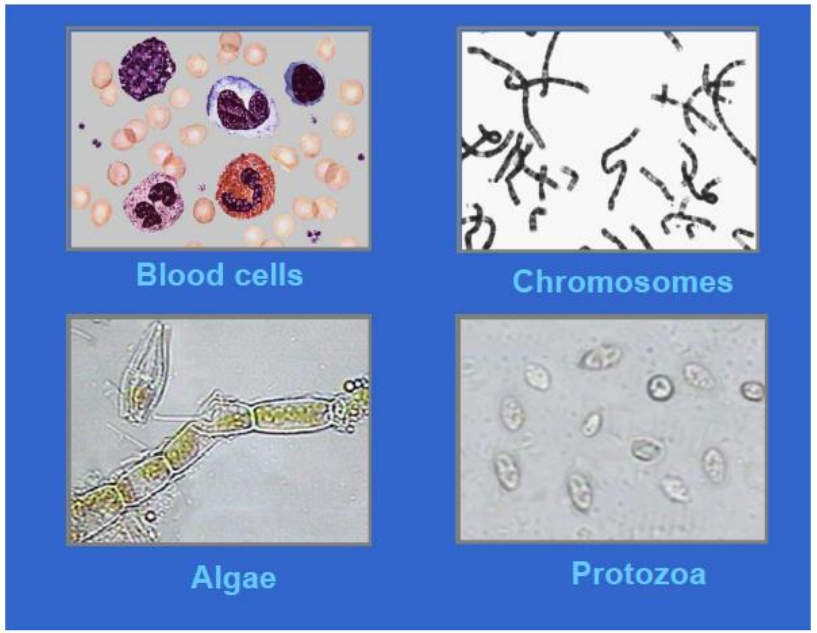
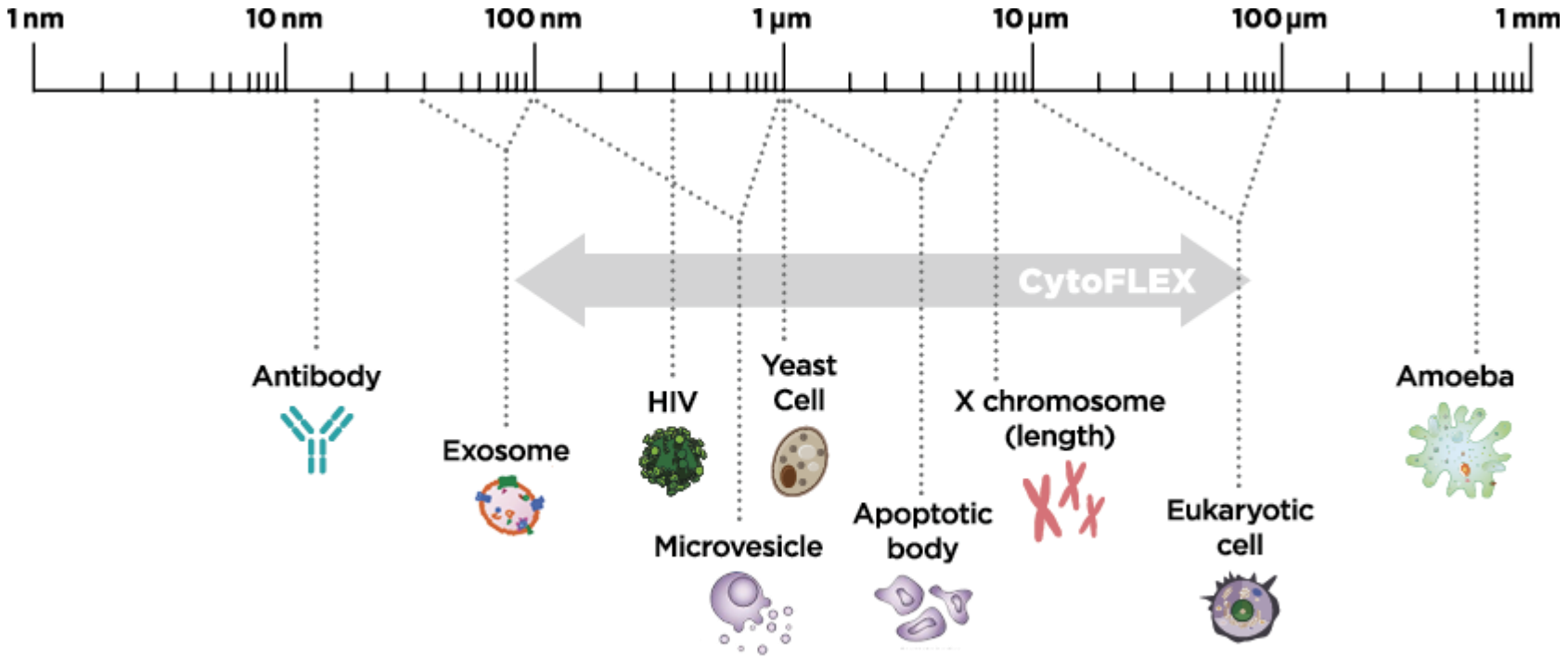
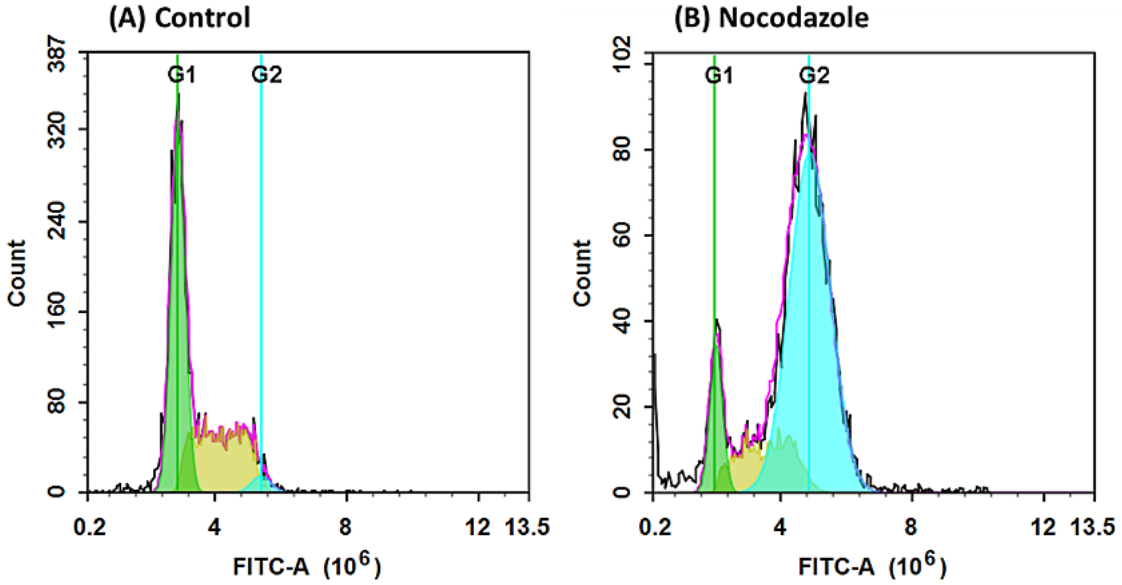
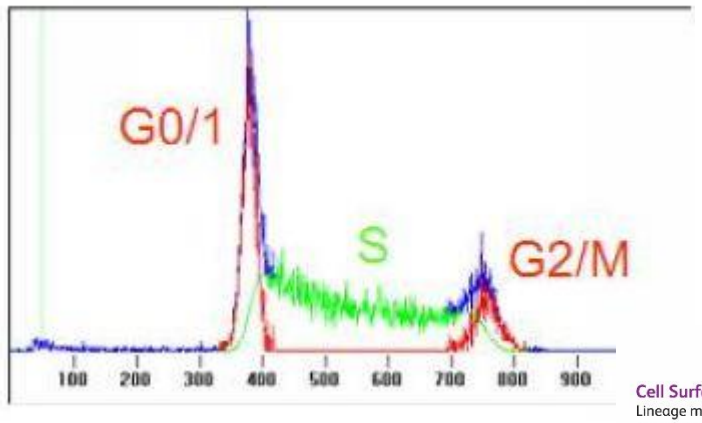

Blood cells
Chromosomes
Algae
Protozoa


1 nm
10 nm
100 nm
1 µm
10 µm
100 µm
1 mm
CytoFLEX
Antibody
Exosome
HIV
Yeast Cell
Microvesicle
Apoptotic body
X chromosome (length)
Eukaryotic cell
Amoeba

# Introduction

- What is the Flow cytometer? The machine that counts the particles/cells and measures their parameters at the single-cell level.
- For which application it can be used?
- How does it work?

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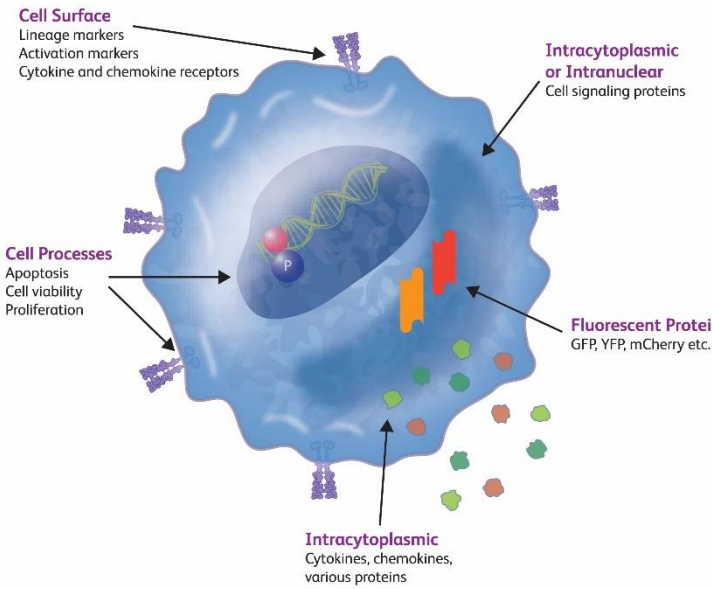


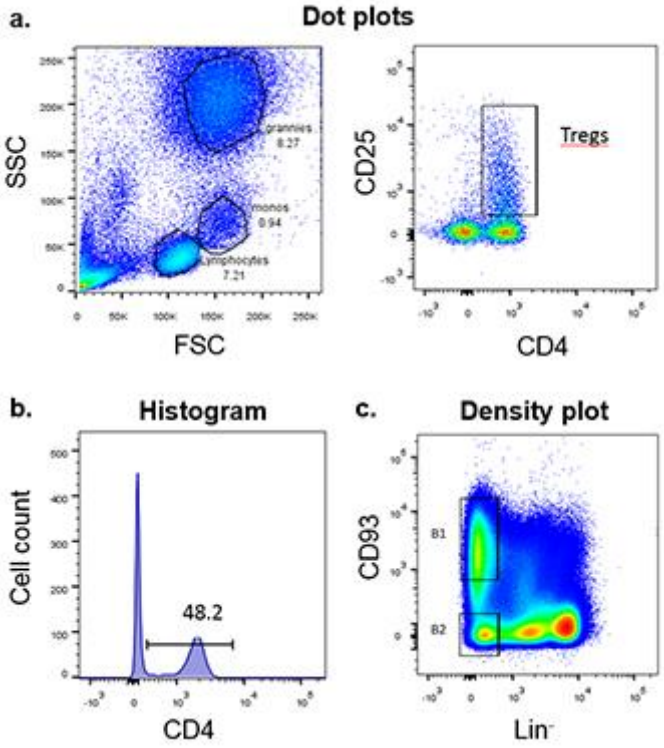


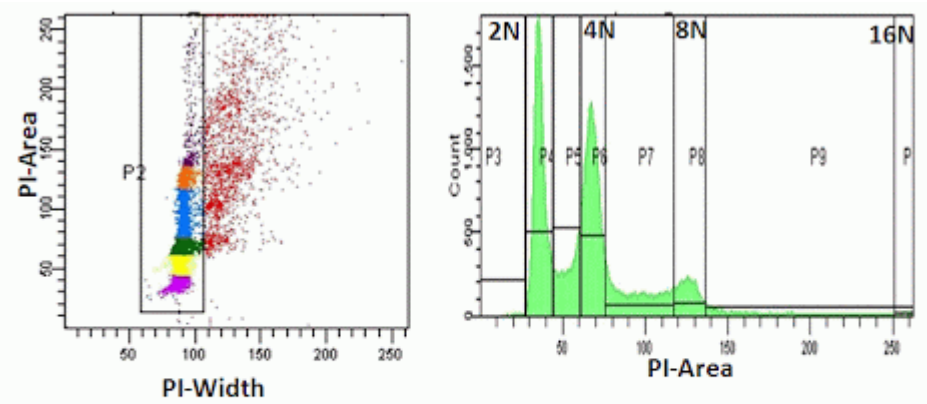


Legend. Gating on Single cells by P2 gate on PI Area v Width parameters shows the degree of Ploidy, from G1 (2N), $G_2$m (4N), 8N and 16N.
Courtesy to Eleni Pantazi Cutaneous Research, ICMS, 4 Newark Street, London E1 2AT, UK.

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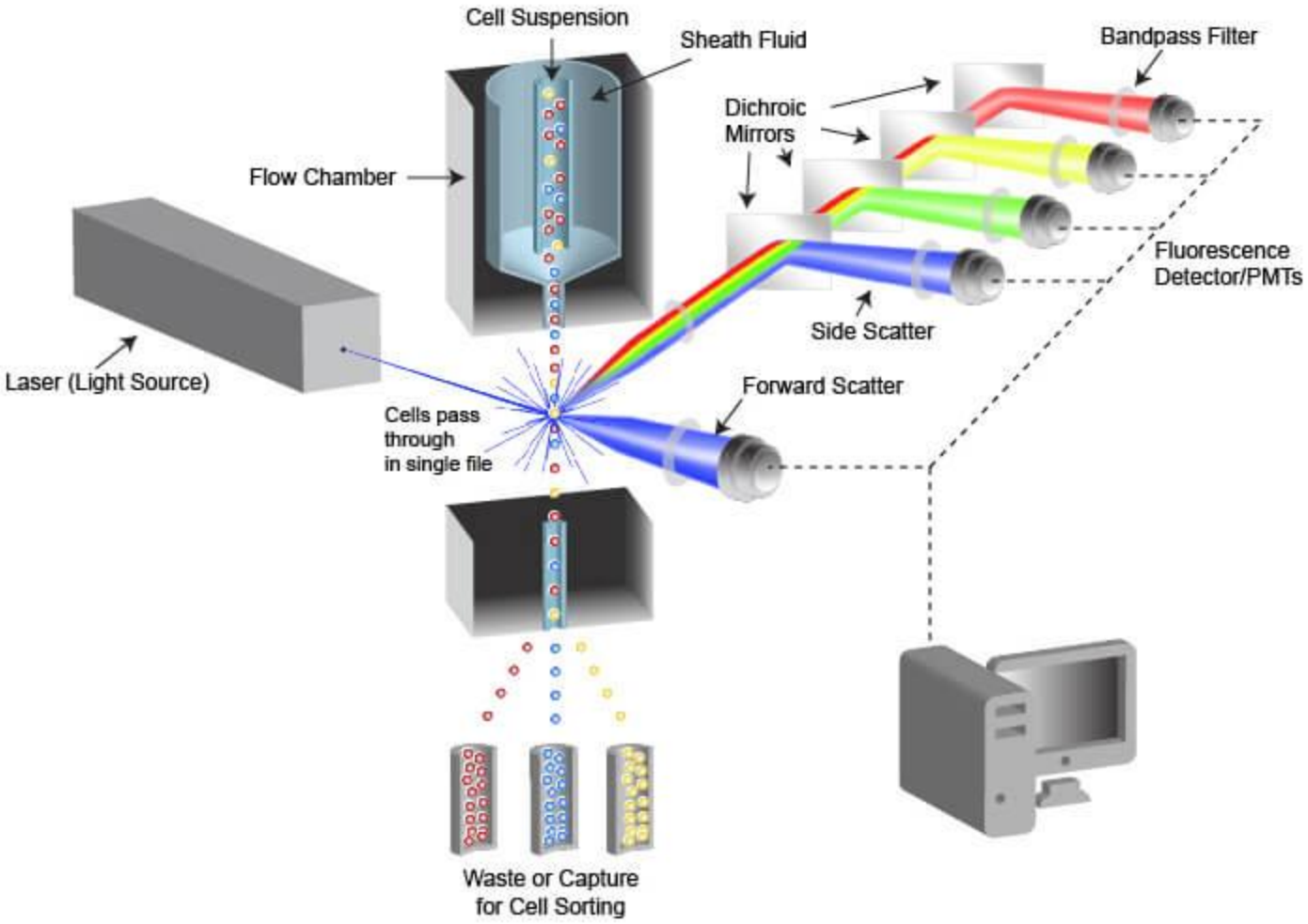
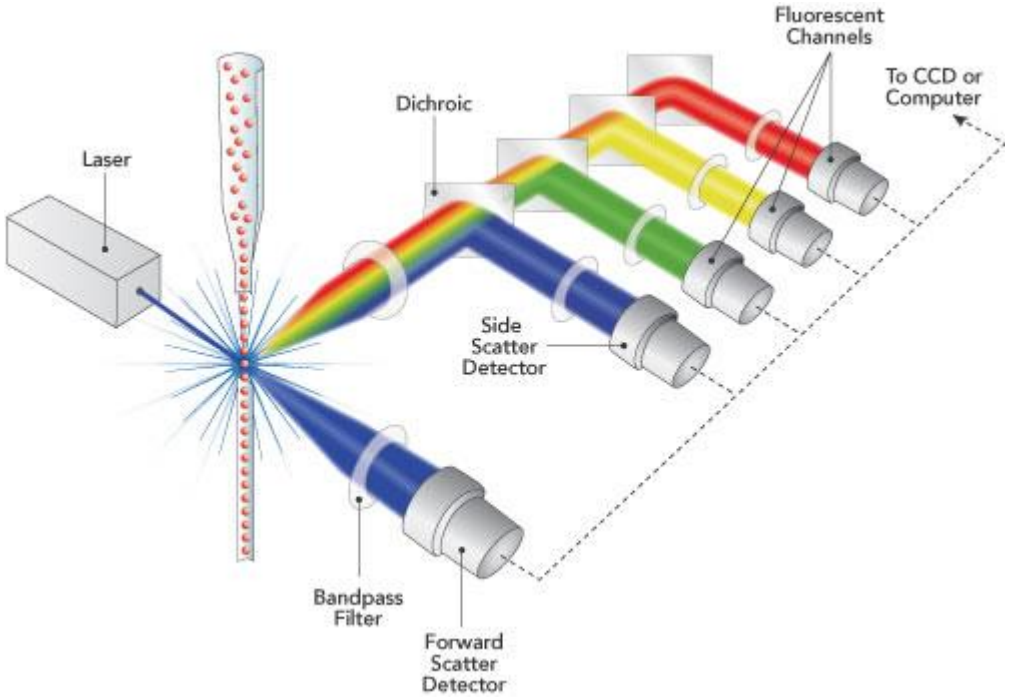
# Introduction

- What is the Flow cytometer? The machine that counts the particles/cells and measures their parameters at the single-cell level.
- For which application it can be used? Phenotyping, cell cycle analysis, cell viability, functional analysis.
- How does it work?


Cell Suspension
Sheath Fluid
Bandpass Filter
Dichroic Mirrors
Flow Chamber
Fluorescence Detector/PMTs
Side Scatter
Laser (Light Source)
Forward Scatter
Cells pass through in single file
Waste or Capture for Cell Sorting

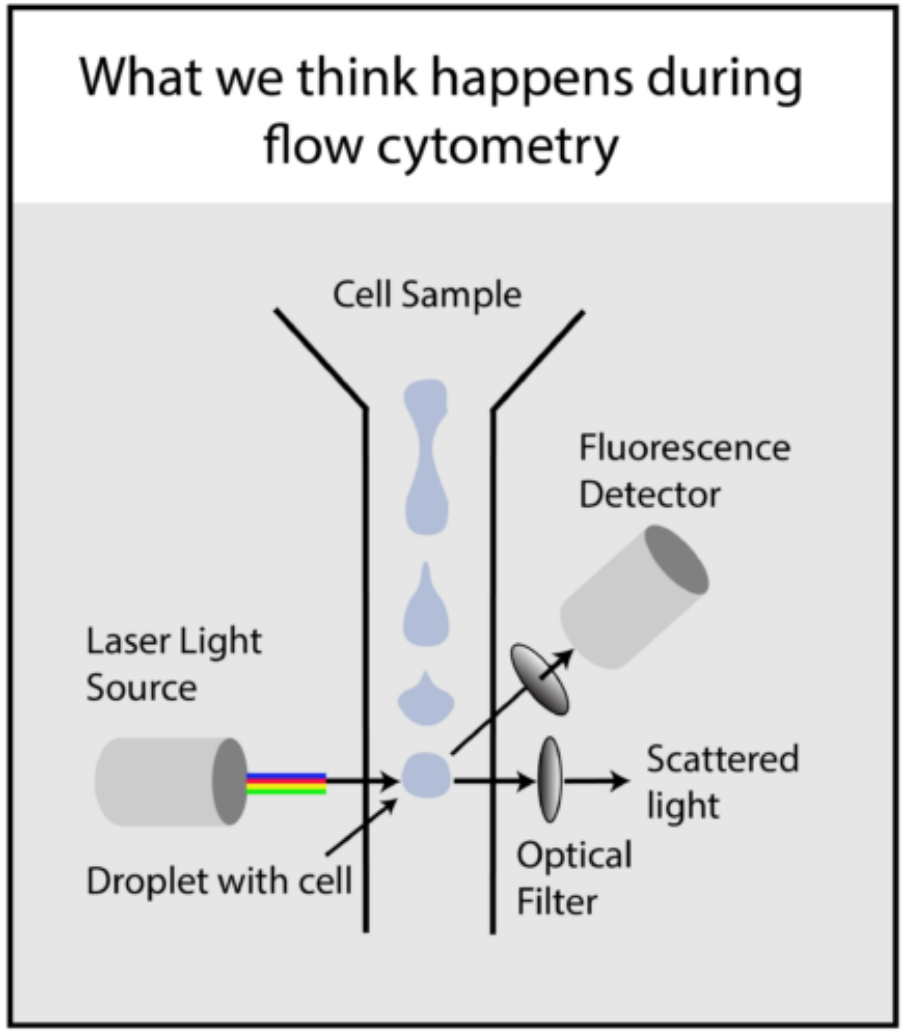

Fluorescent Channels
To CCD or Computer
Dichroic
Laser
Side Scatter Detector
Bandpass Filter
Forward Scatter Detector

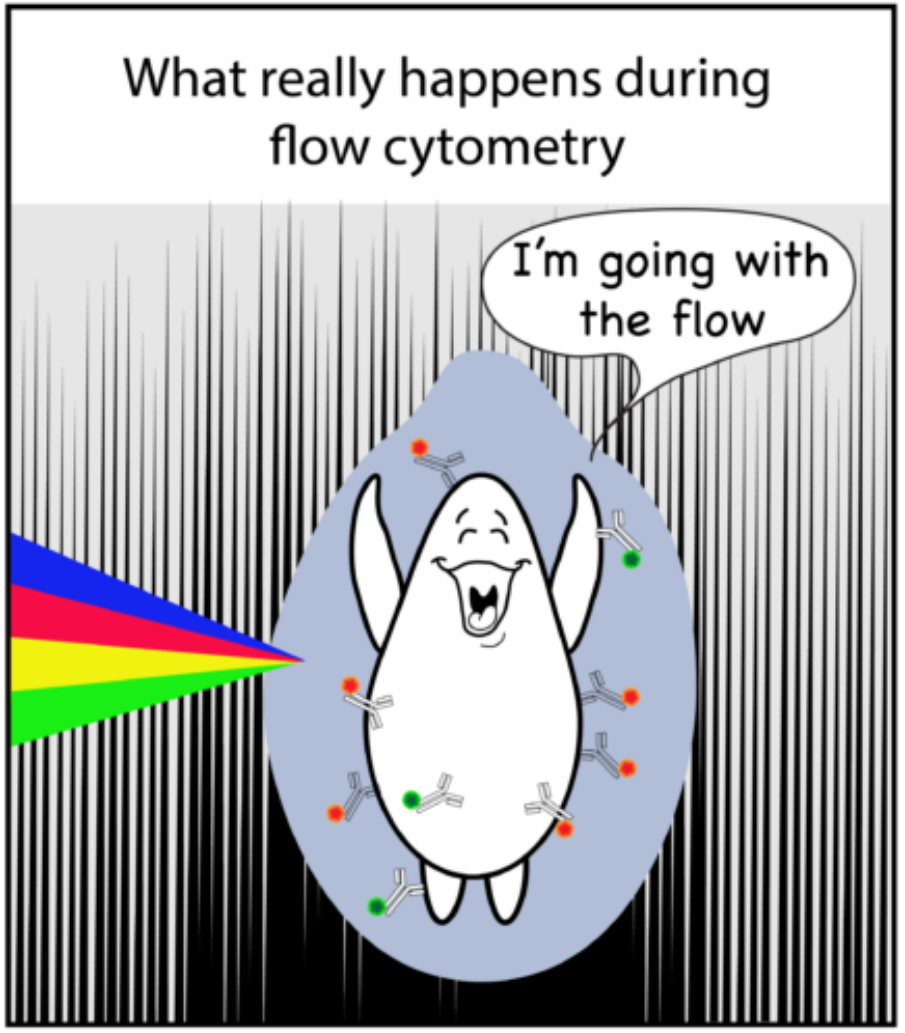
# Introduction

- What is the Flow cytometer? The machine that counts the particles/cells and measures their parameters at the single-cell level.

- For which application it can be used? Phenotyping, cell cycle analysis, cell viability, functional analysis.

- How does it work? Stream of single cells in suspension is flowing through the illuminated part, where parameters of cells are measured based on light scattering.


What we think happens during flow cytometry
Cell Sample
Fluorescence Detector
Laser Light Source
Scattered light
Droplet with cell
Optical Filter


What really happens during flow cytometry
I'm going with the flow



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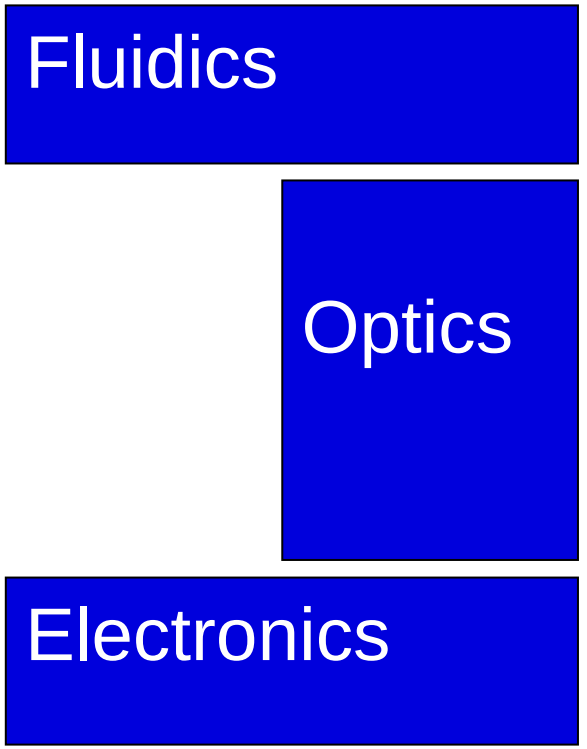
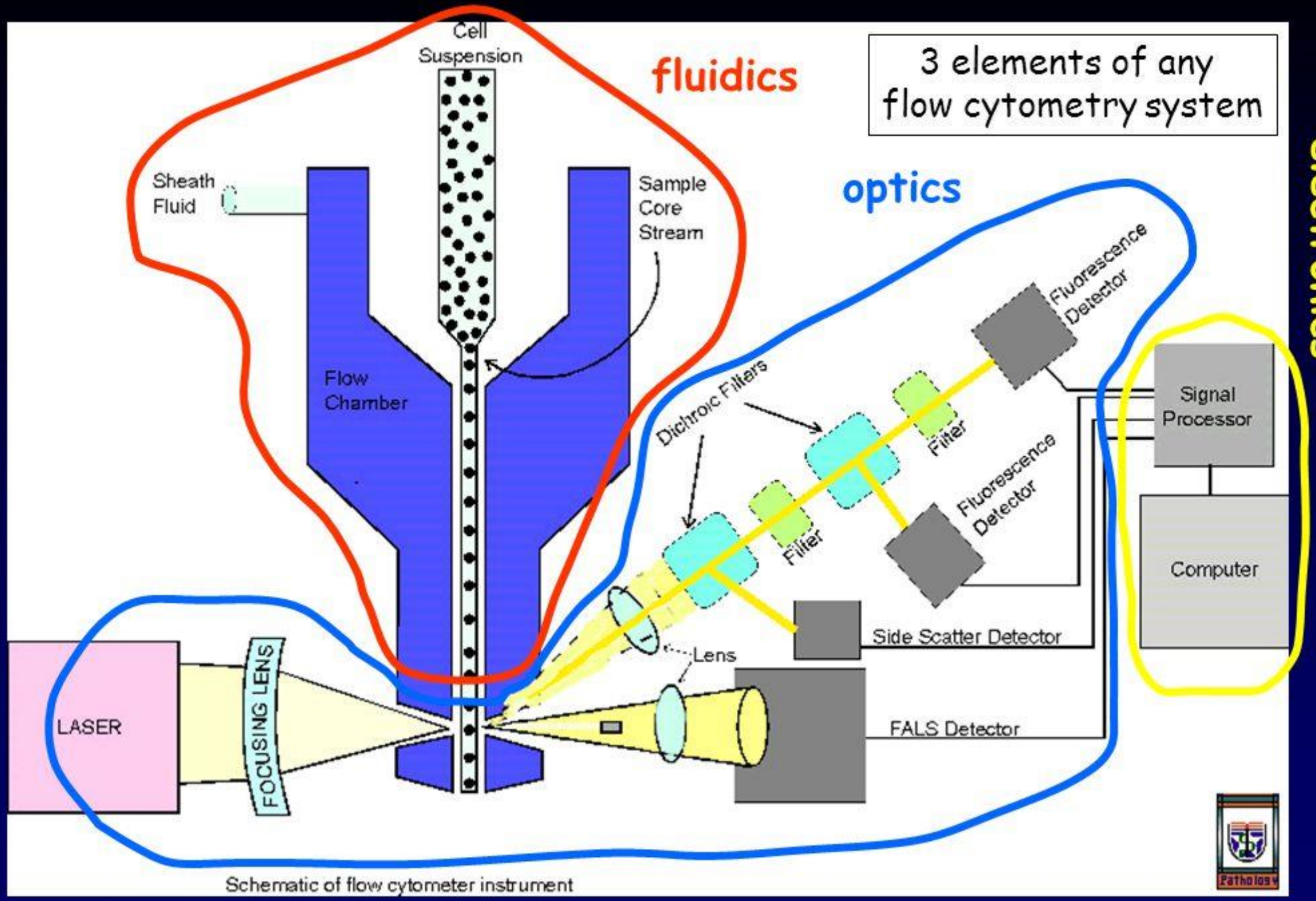
# Brief history

- **1940-1950** Development of principles used in flow cytometry, fluorescent dyes, particles and spores in air counting.
- **1953** – Sheath flow principle.
- **1956 Wallace Coulter –** Measuring the change in conductivity during the passage of cells in suspension through a small hole.
- **1960-1970** Flow cytometry development.
- Flow cytometry expansion and advancement.

# Technical components

Cells in suspension
flow individually across
illuminated part where
they scatter light and emit
fluorescence,
which is detected, filtered and
converted to digital values analyzed
and stored on your computer.




3 elements of any flow cytometry system
fluidics
optics
electronics
Cell Suspension
Sheath Fluid
Sample Core Stream
Flow Chamber
Dichroic Filters
Filter
Filter
Fluorescence Detector
Fluorescence Detector
Signal Processor
Computer
Side Scatter Detector
Lens
FALS Detector
LASER
FOCUSING LENS
Schematic of flow cytometer instrument



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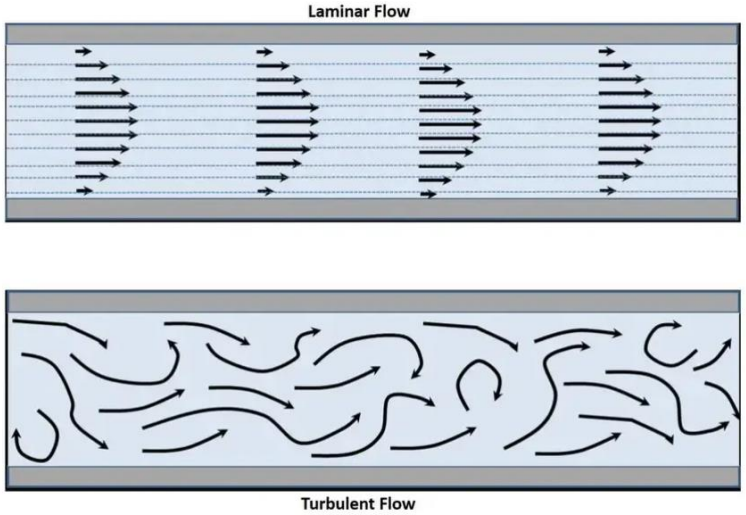
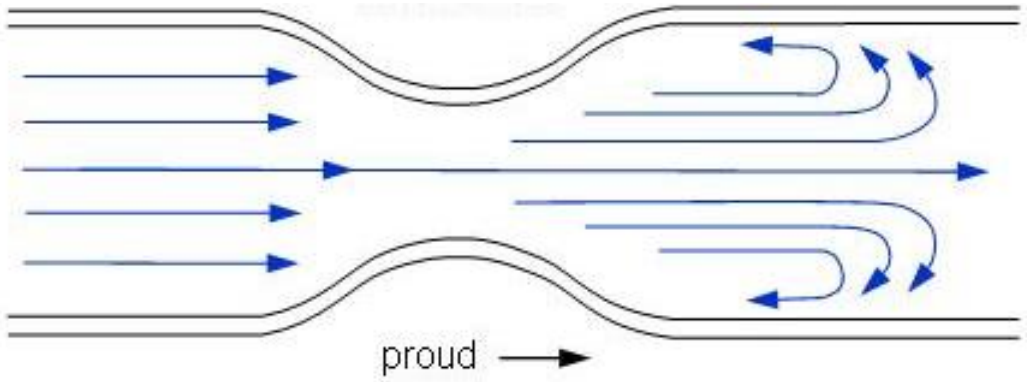
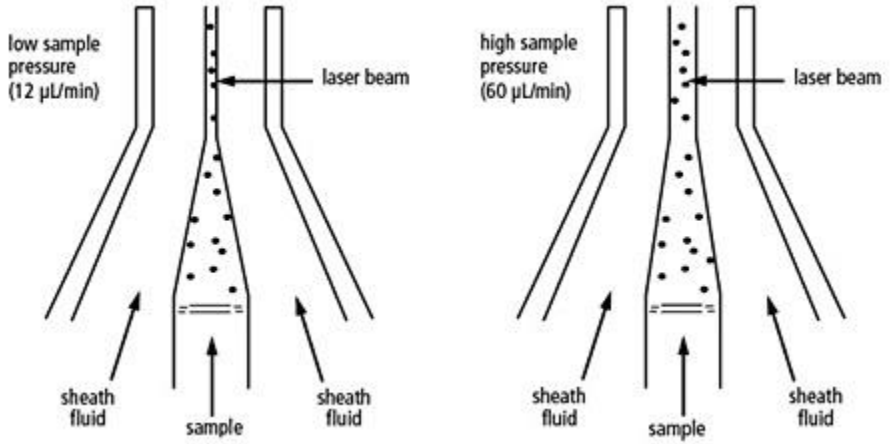
# Fluidics

– Laminar flow.

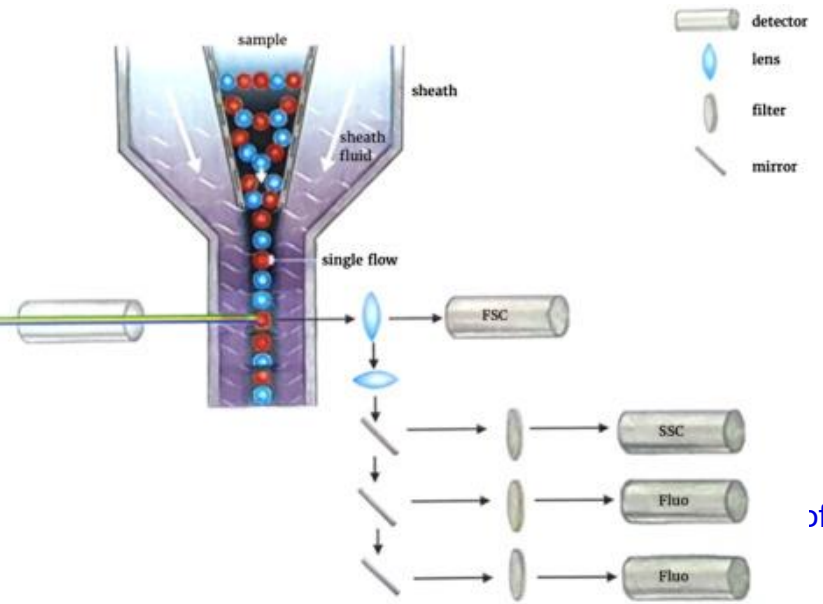


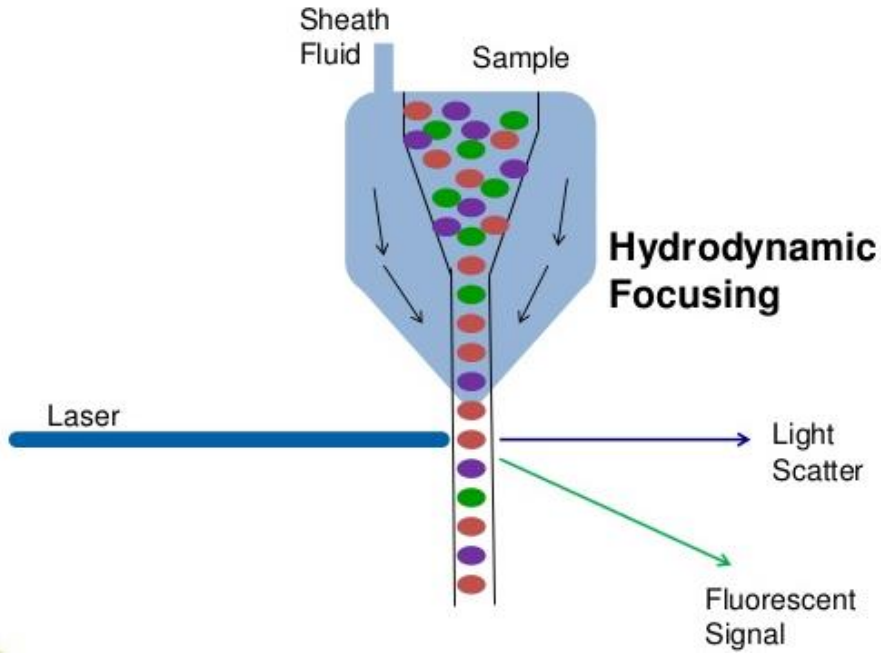



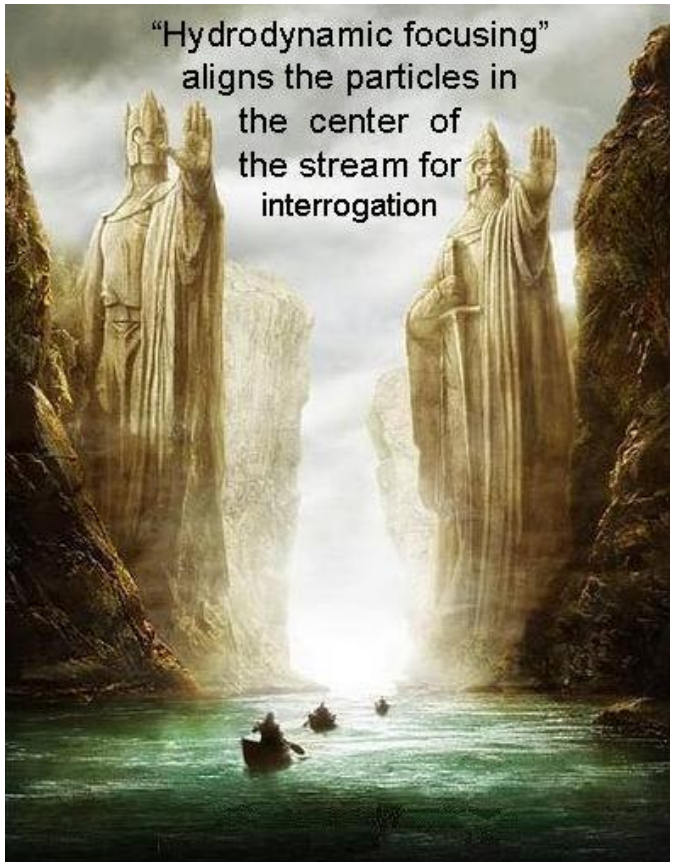

# Fluidics




- Hydrodynamically focused cells in fluid system are analyzed using light/laser rays.
- Laminar flow.



# Technical components

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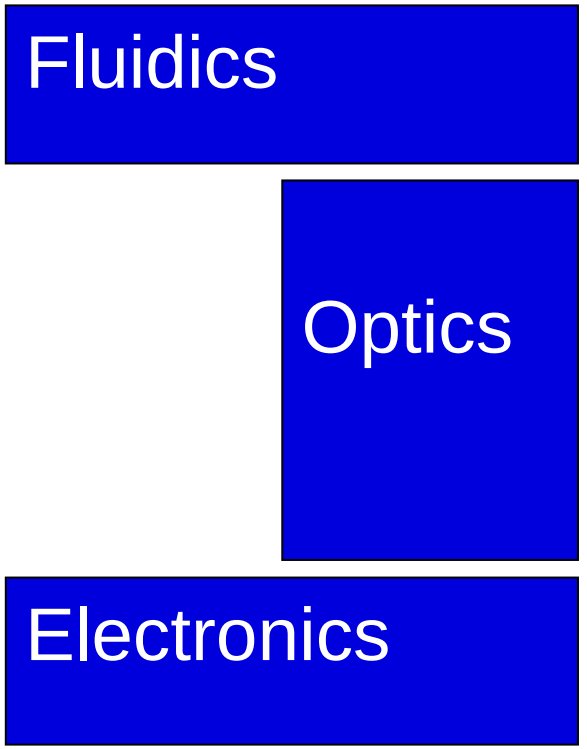
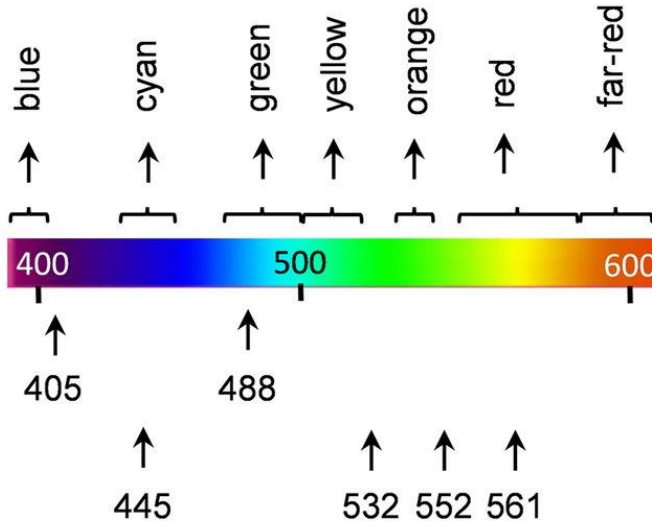
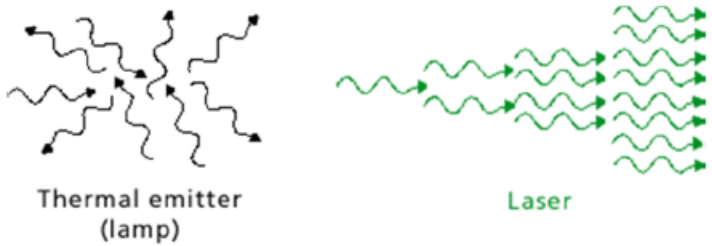


# Light/laser sources

- UV/Arc lamps
- Lasers (350-363, 420, 457, 488, 514, 532, 600, 633 nm)

  Argon ion, Krypton ion, HeNe, HeCd, Yag

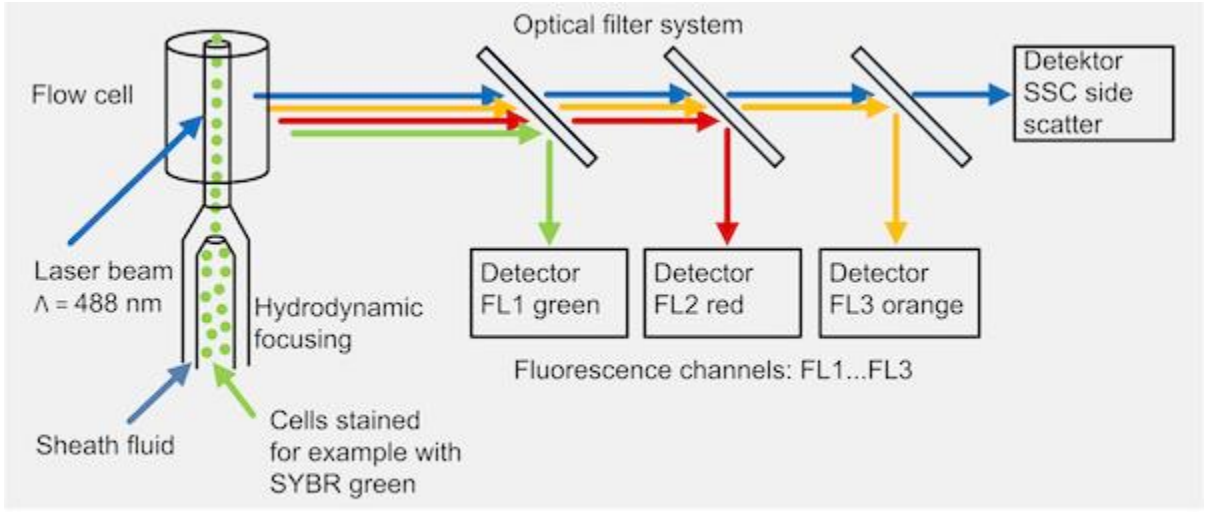


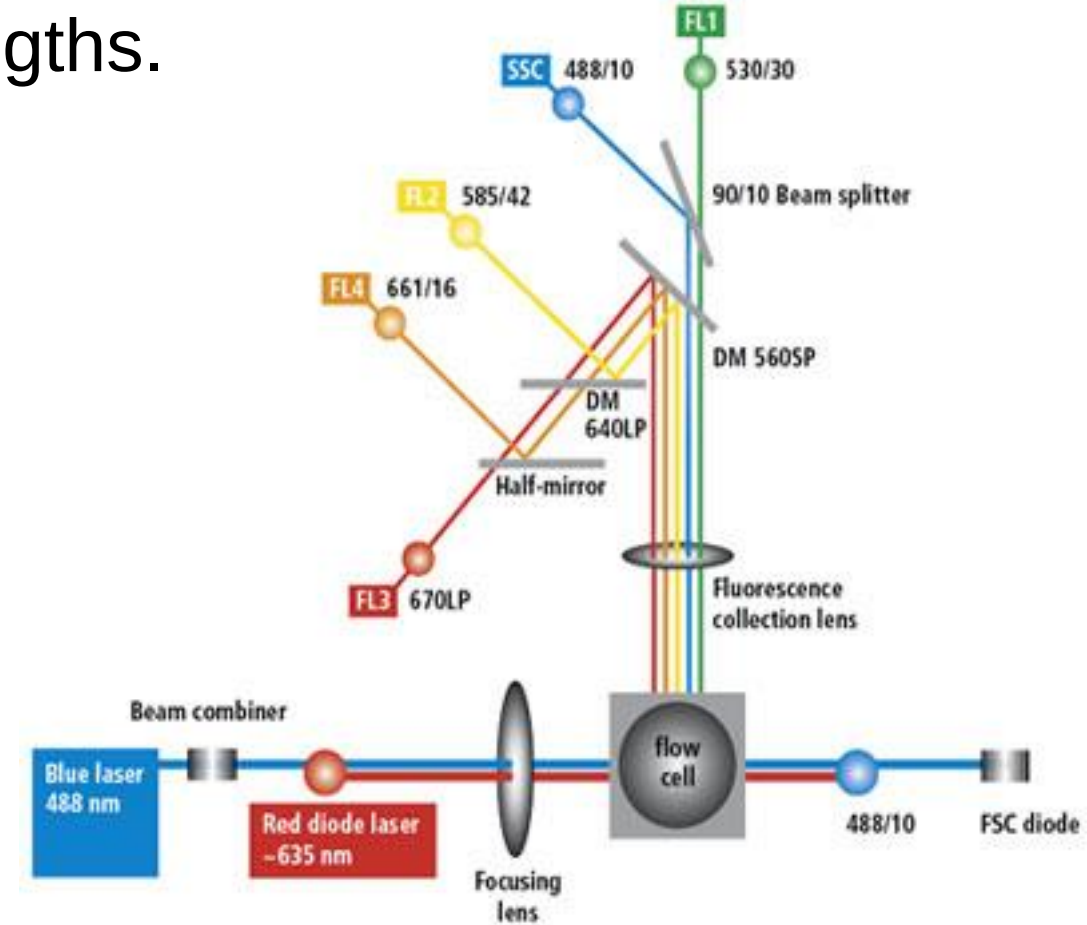



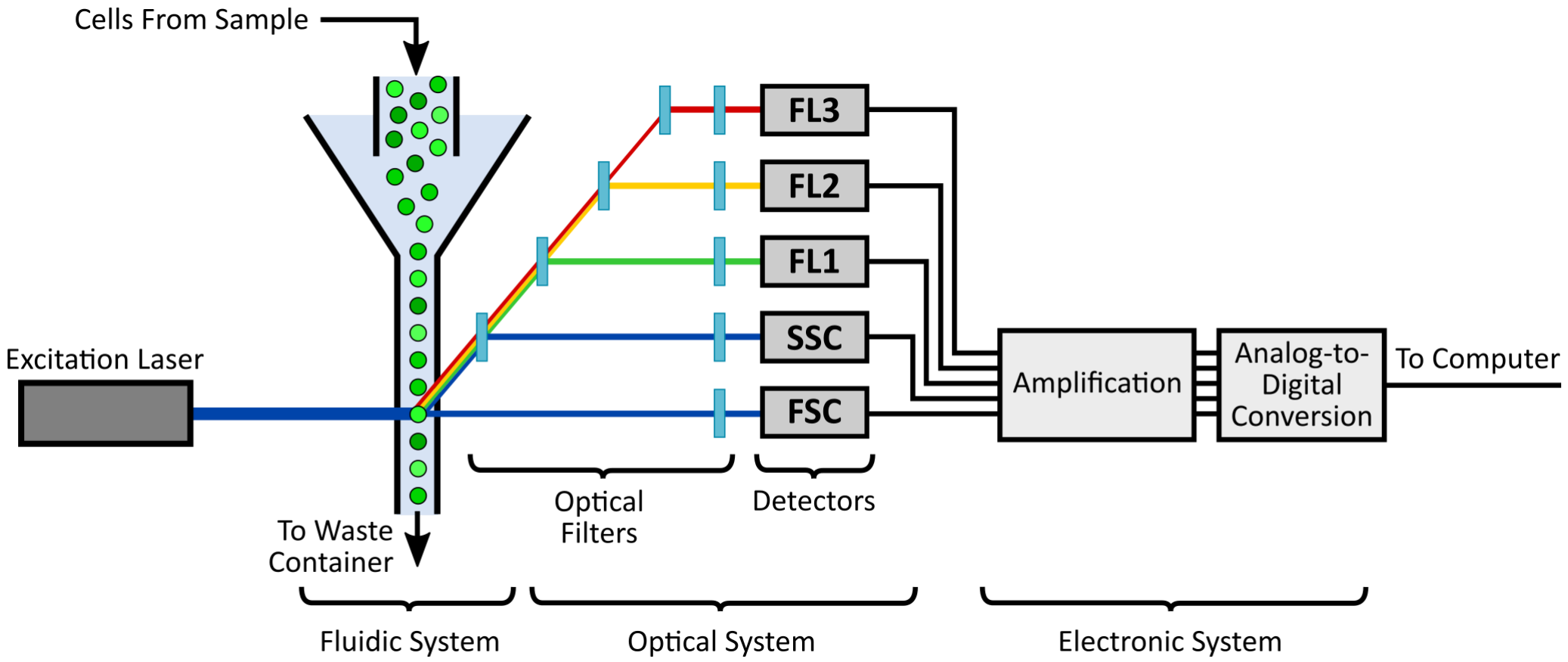

http://www.ilt.fraunhofer.de/eng/100053.html

# Optical channels

- Way of the light from the point of cell illumination to the detector.
- Optical parts separate certain wavelengths.




Cells From Sample
FL3
FL2
FL1
SSC
FSC
Excitation Laser
Amplification
Analog-to-Digital Conversion
To Computer
Optical Filters
Detectors
To Waste Container
Fluidic System
Optical System
Electronic System



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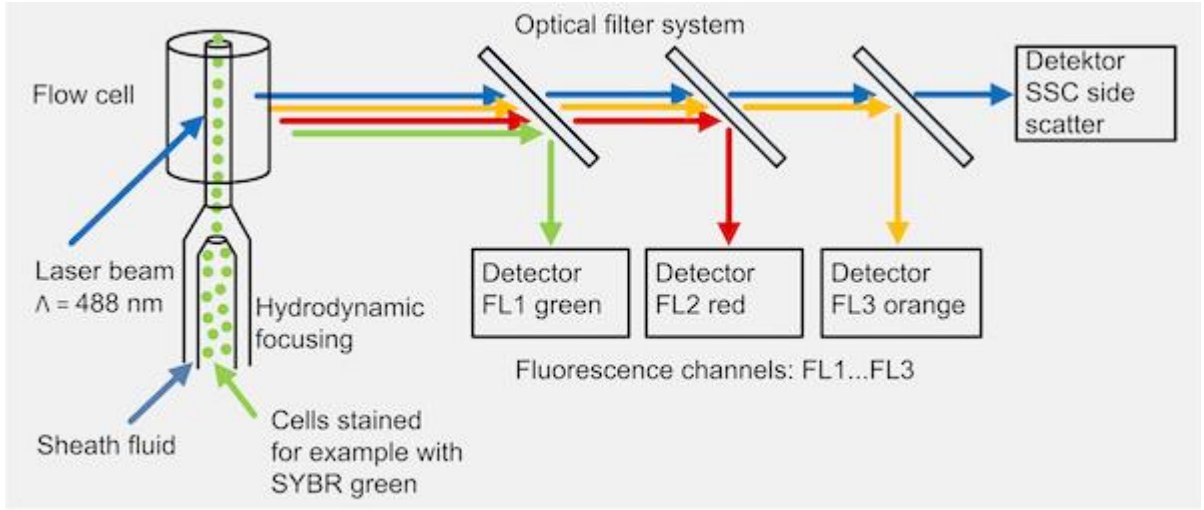
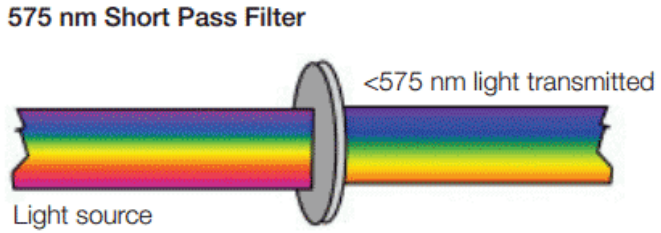
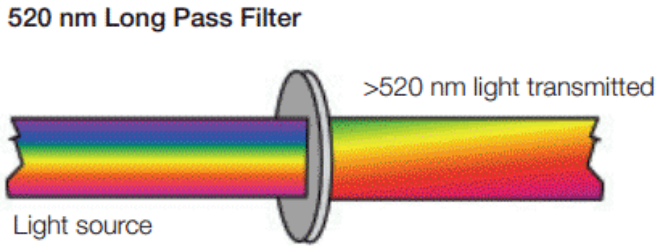
Optics

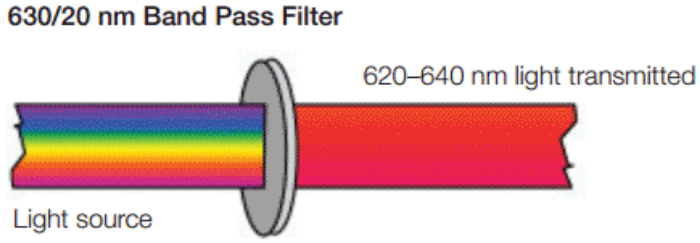
# Filters

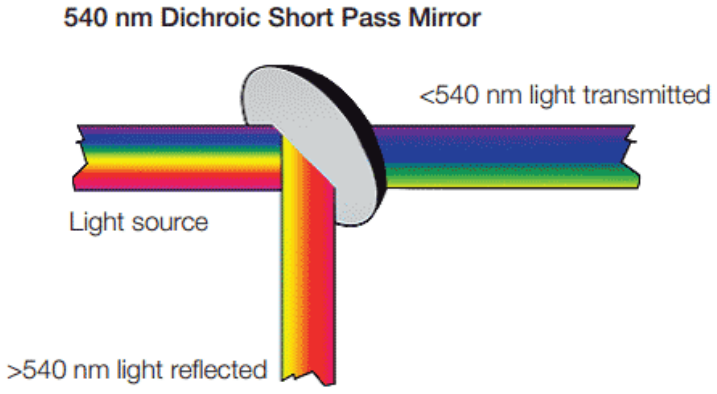
- Used for wavelength separation.
- Placed in 45° angle as a dichroic mirror.

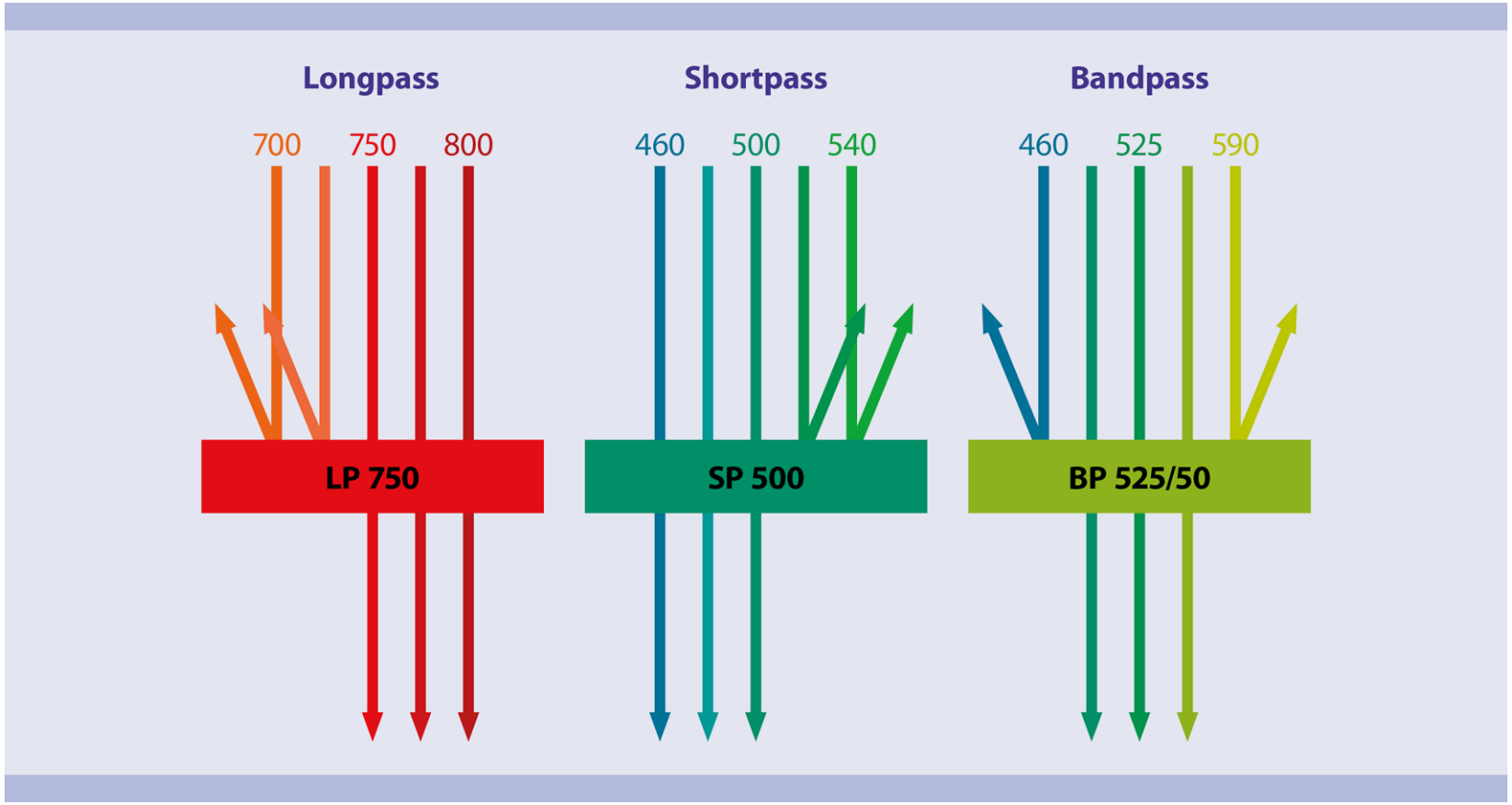



Longpass
700
750
800
LP 750
Shortpass
460
500
540
SP 500
Bandpass
460
525
590
BP 525/50



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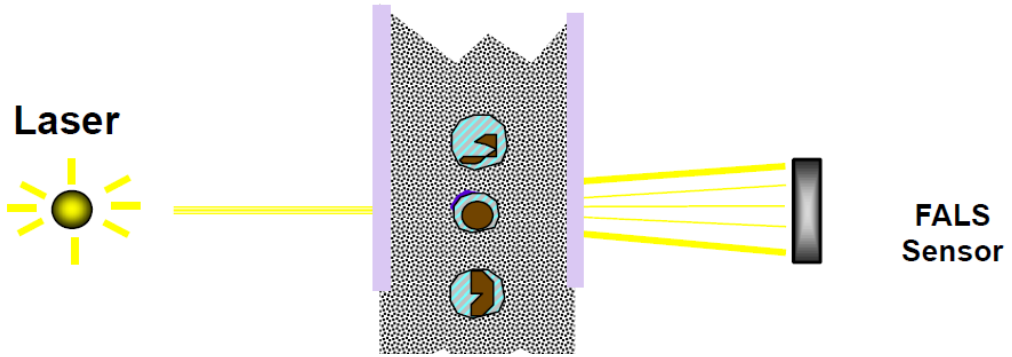
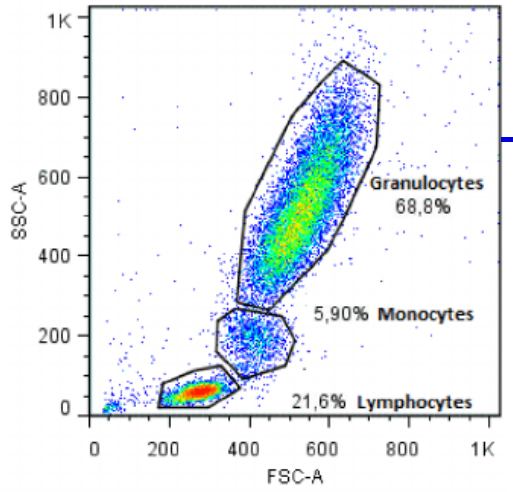
Optics

# Channels

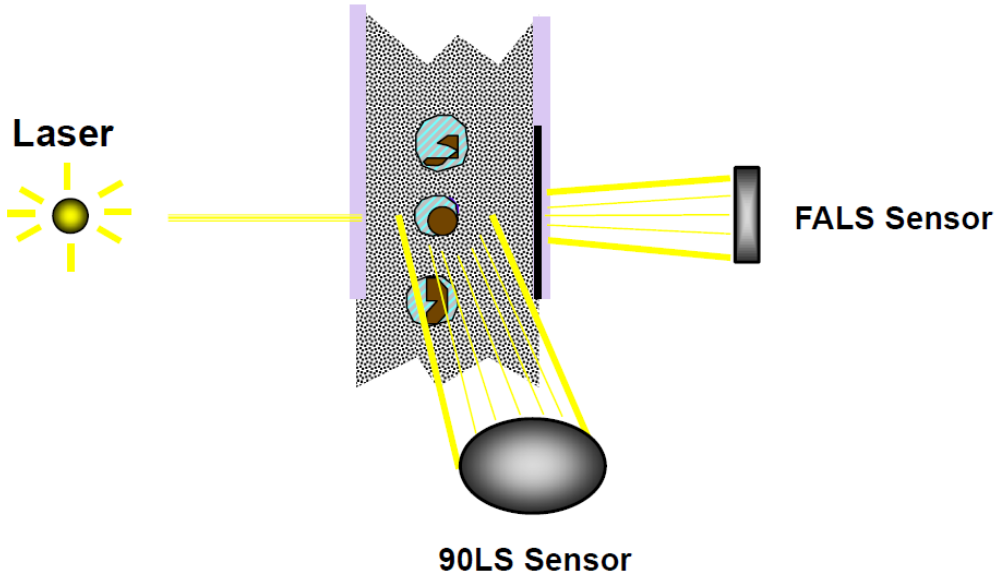
## Forward Angle Light Scatter




- Information about surface properties and cell size.
- Can distinguish live and dead cells.




## 90 Degree Light Scatter




- Information about inclusions in cells and cell density.
- Can distinguish granular cells.



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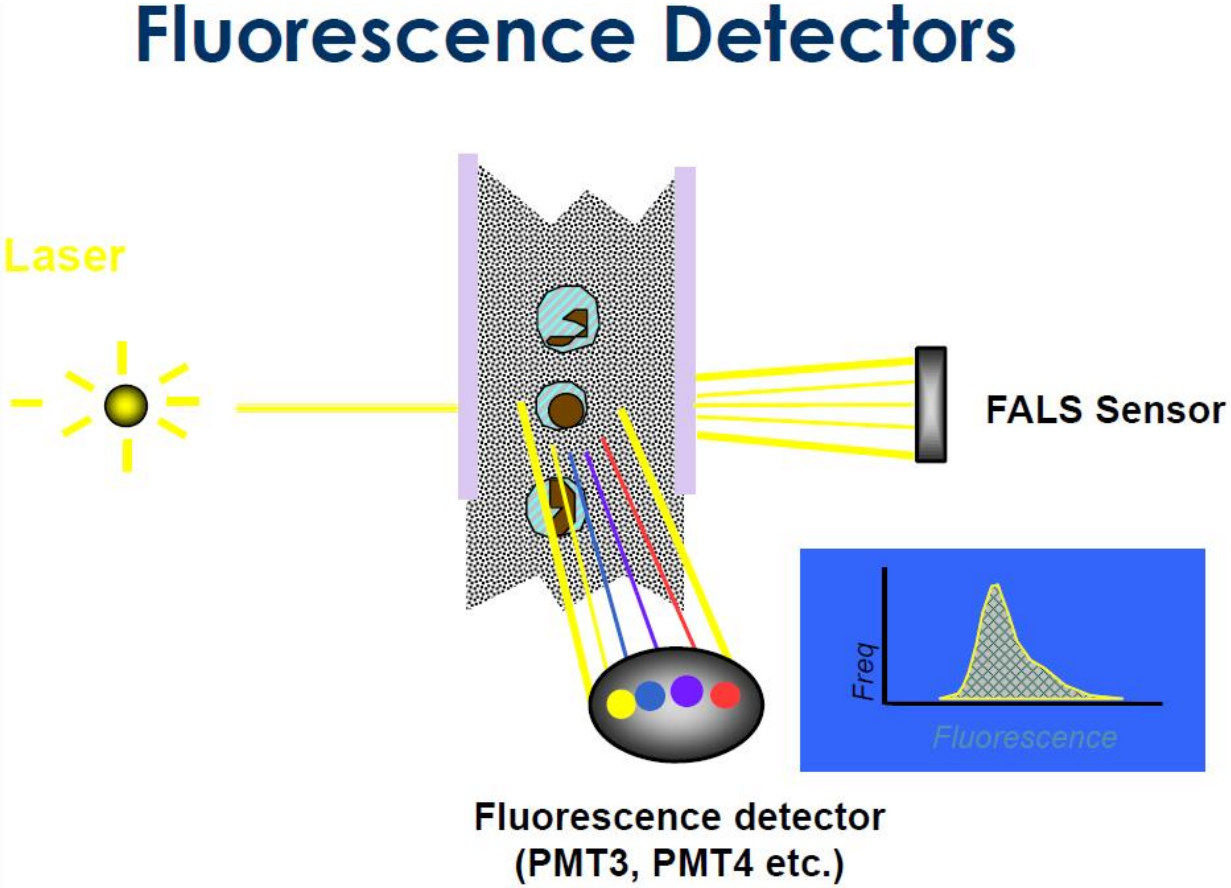
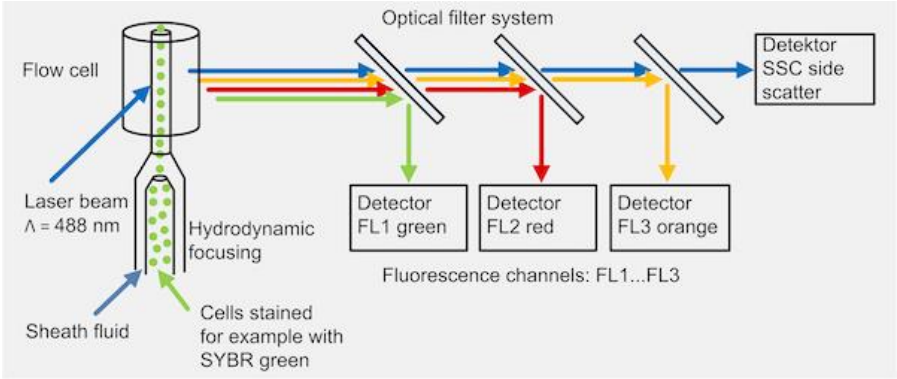
# Channels

- Fluorescence emitted from each fluorochrome is detected by specific fluorescence channel.
- The specificity of the detection is provided by selectivity of the filters and mirrors.



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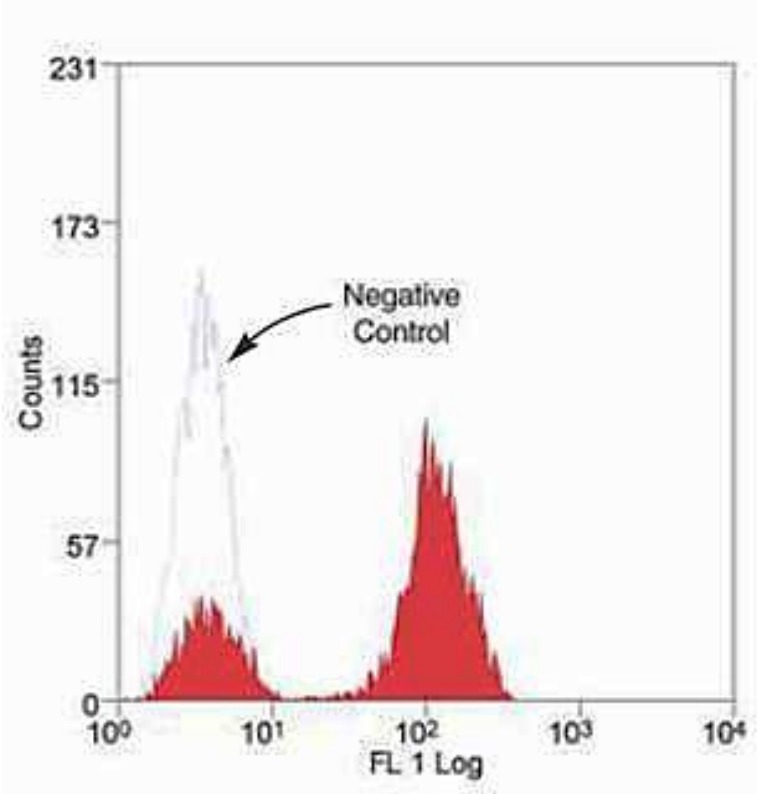
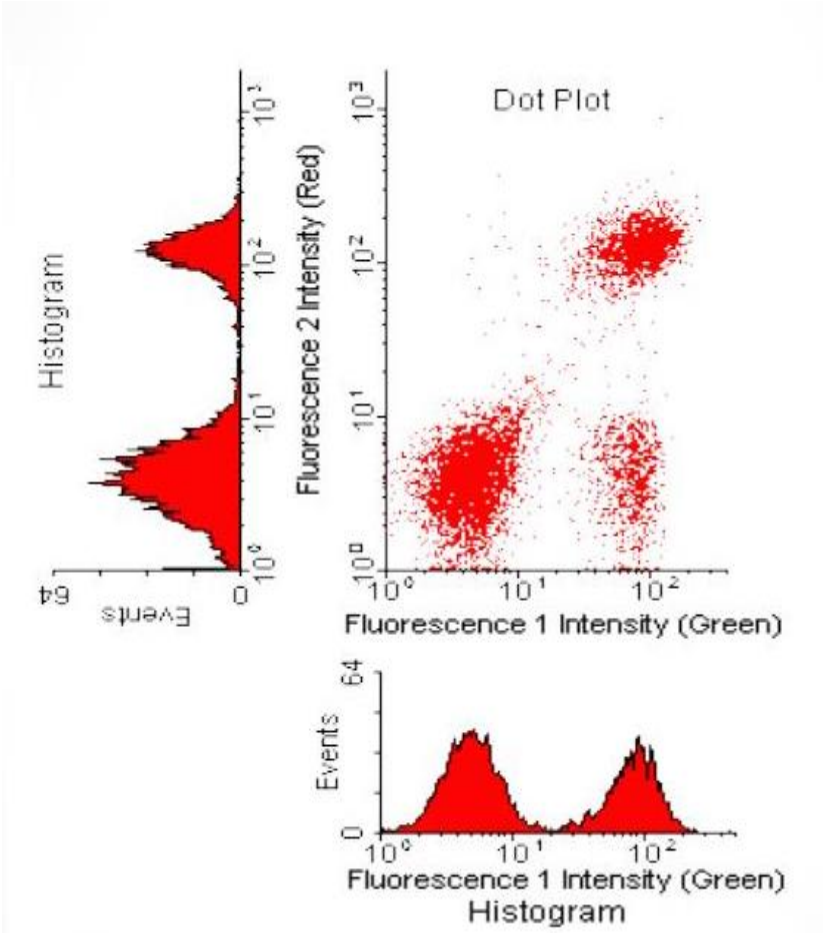
# Channels



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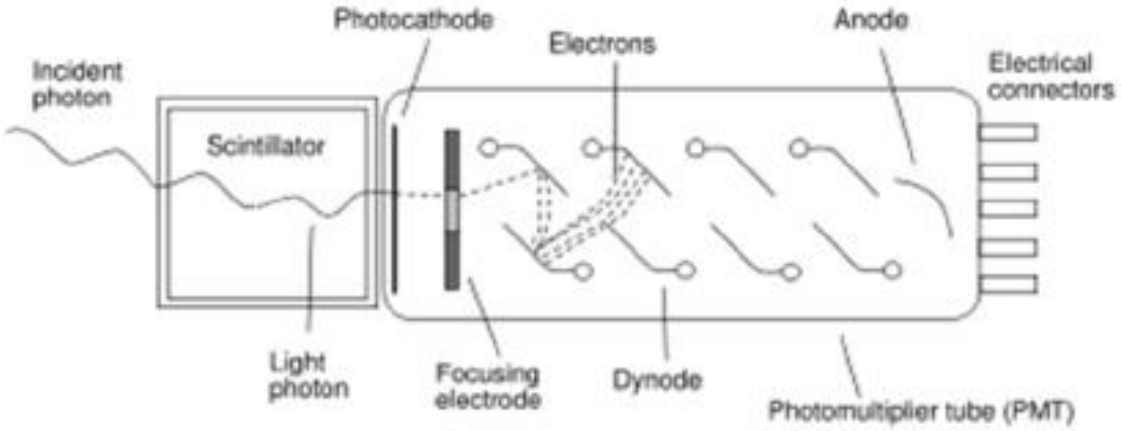
# Detectors

- Diodes (can by used in forward scatter).
  - Only for high signal, better linearity.
- Photomultipliers (PMT detectors for fluorescence).
  - Sensitive, but can be harmed by overexposure by high laser power.



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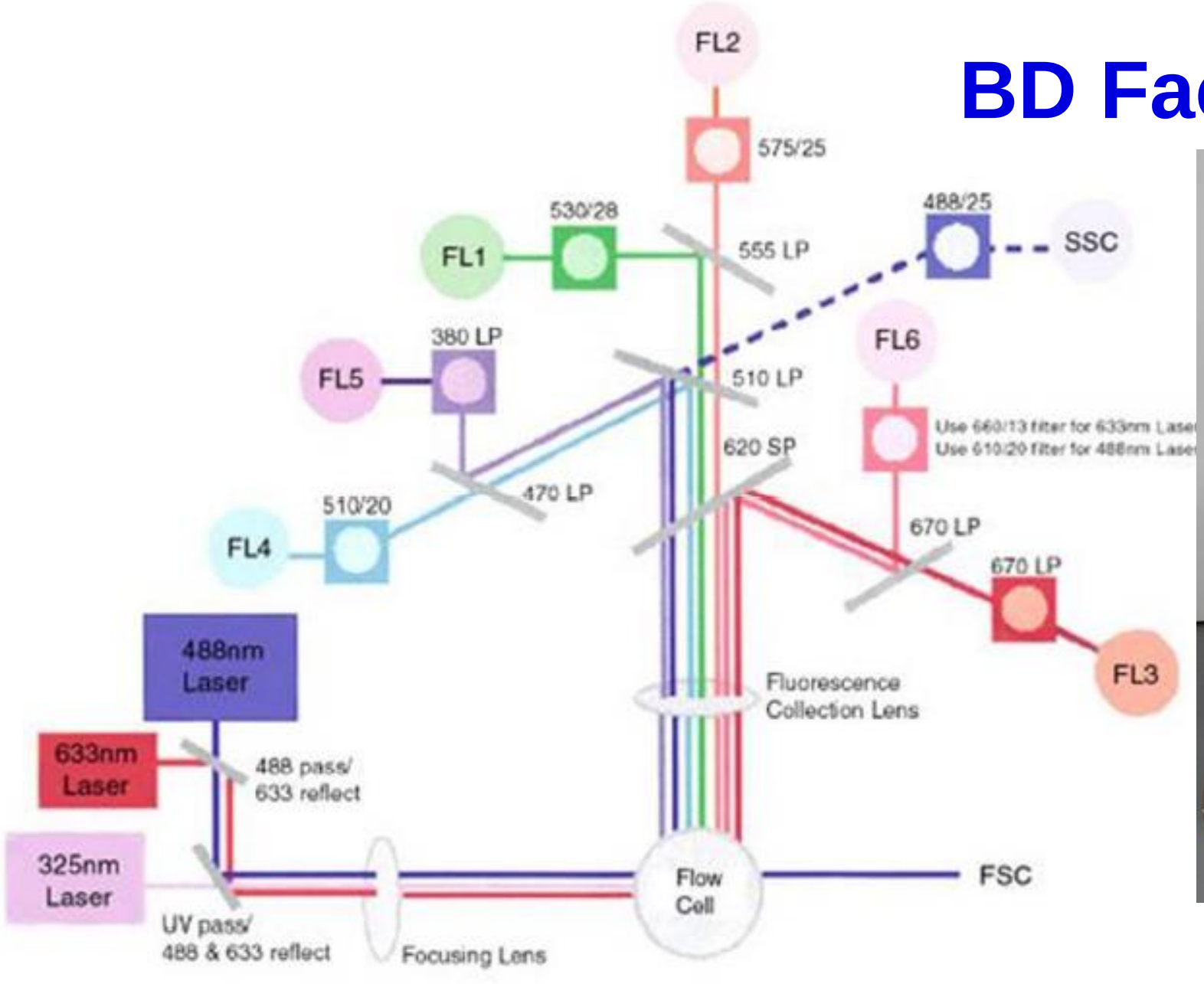
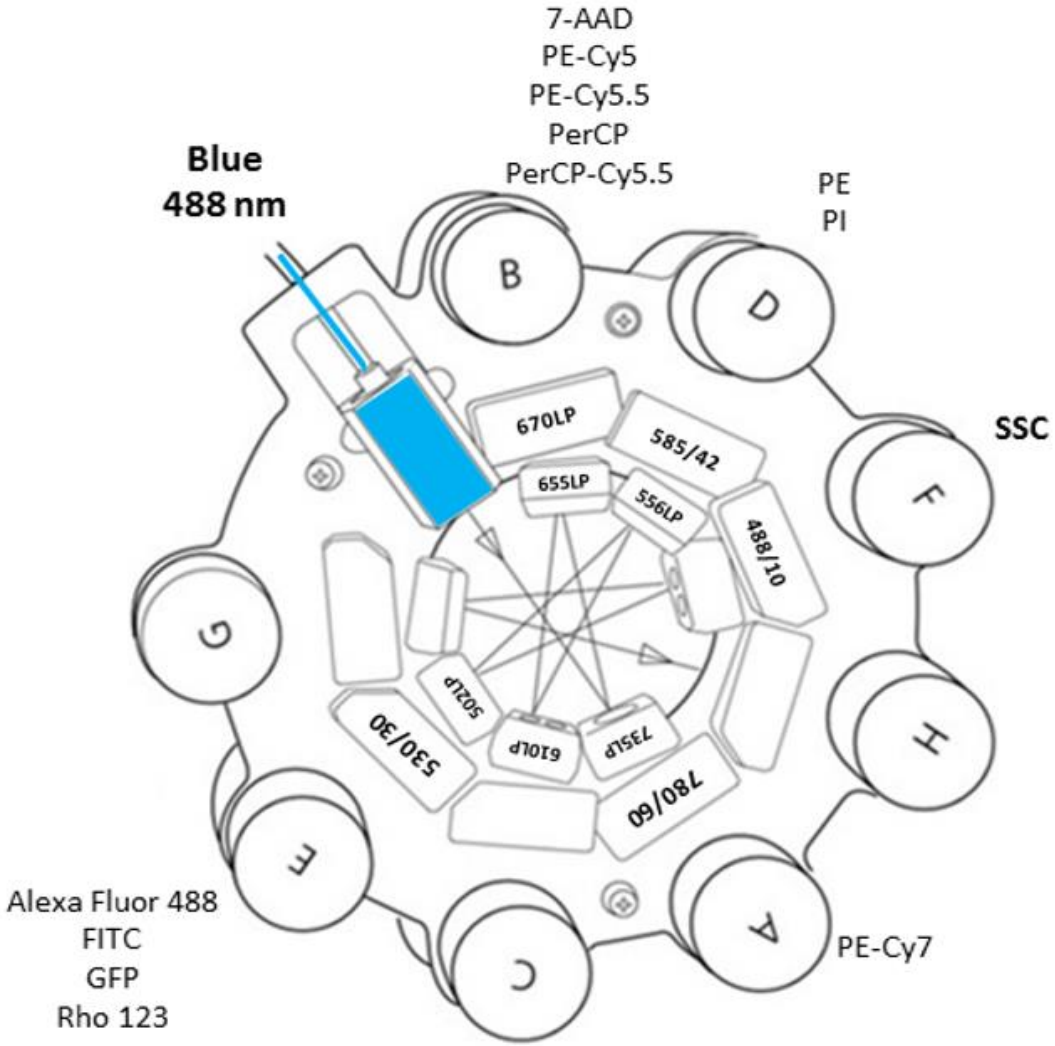
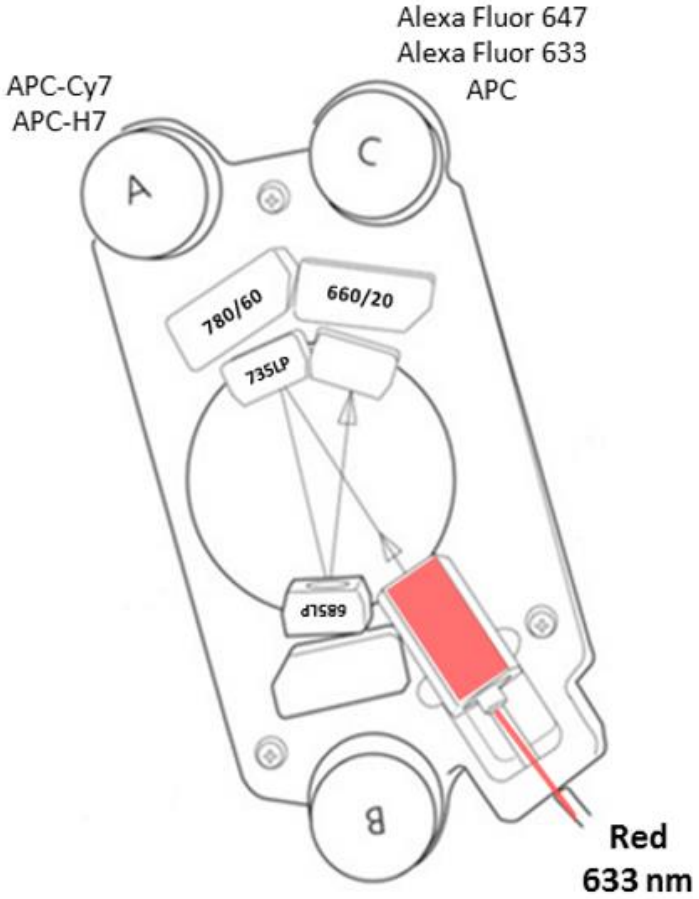
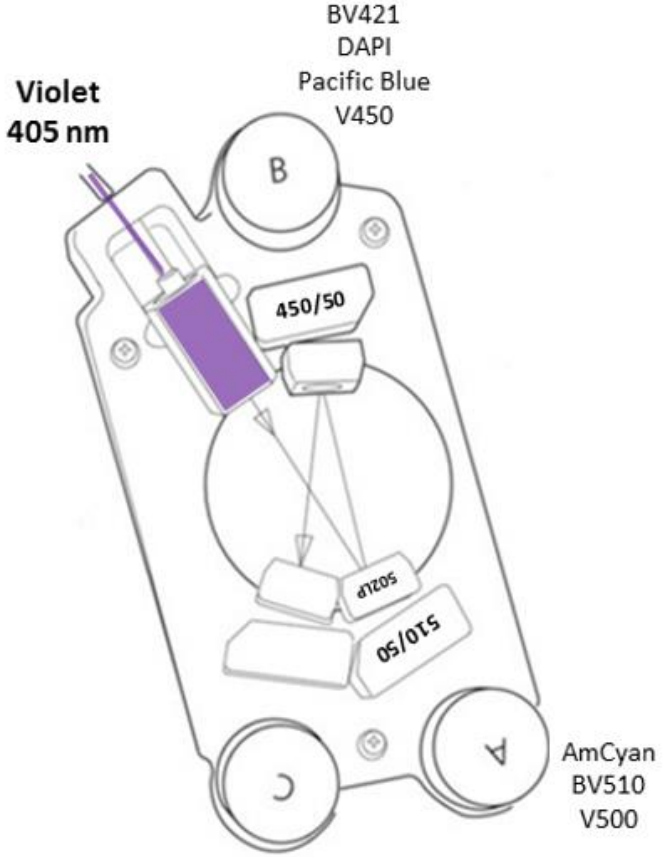
# BD Facs Canto II





7-AAD
PE-Cy5
PE-Cy5.5
PerCP
PerCP-Cy5.5
Blue
488 nm
PE
PI
SSC
Alexa Fluor 488
FITC
GFP
Rho 123
PE-Cy7
670LP
585/42
655LP
556LP
488/10
530/30
502LP
610LP
735LP
780/60
A
B
C
D
E
F
G
H


Alexa Fluor 647
Alexa Fluor 633
APC
APC-Cy7
APC-H7
Red
633 nm
780/60
660/20
735LP
685LP
A
B
C


BV421
DAPI
Pacific Blue
V450
Violet
405 nm
AmCyan
BV510
V500
450/50
502LP
510/50
A
B
C


FL1
FL2
FL3
FL4
FL5



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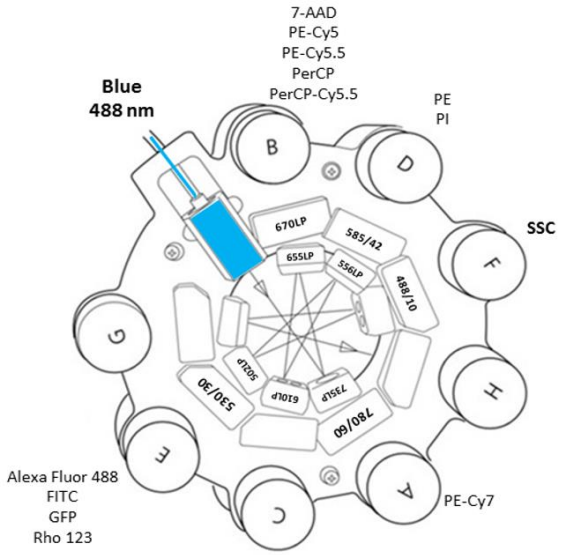
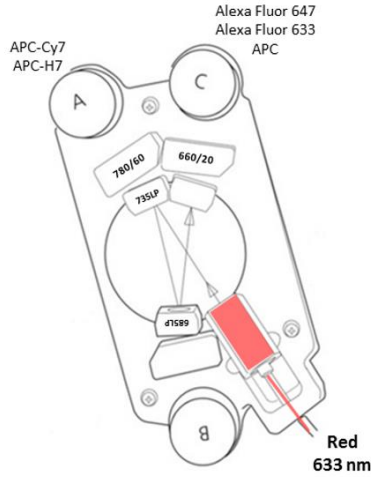
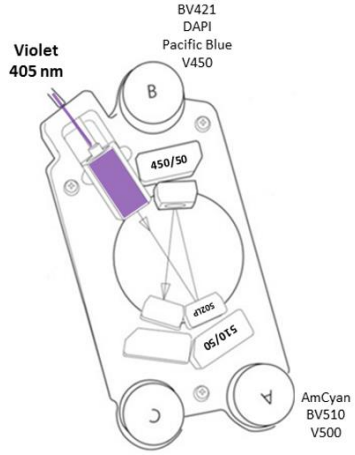
# FACSCanto

| Laser | Detector | Fluorochromes |
|---|---|---|
| 405 | | |
| | 450/50 | Pacific Blue, eFluor 450, V450, BV 421, Cascade Yellow, (DAPI) |
| | 510/50 | AmCyan, BV 510, Krome Orange, V500, Cacsade Blue |
| 488 | | |
| | 530/30 | FITC, Alexa Fluor 488, BB 515, CFSE, Fluo-3, GFP |
| | 585/42 | PE, PI |
| | 670LP | PerCP, PerCP-Cy 5.5, PC5, 7-AAD |
| | 780/60 | PC7 |
| 633 | | |
| | 660/20 | APC, Alexa Fluor 647, eFluor 660 |
| | 780/60 | APC-Cy7, APC-H7, APC - Alexa Fluor 750, APC-eFluor 780 |



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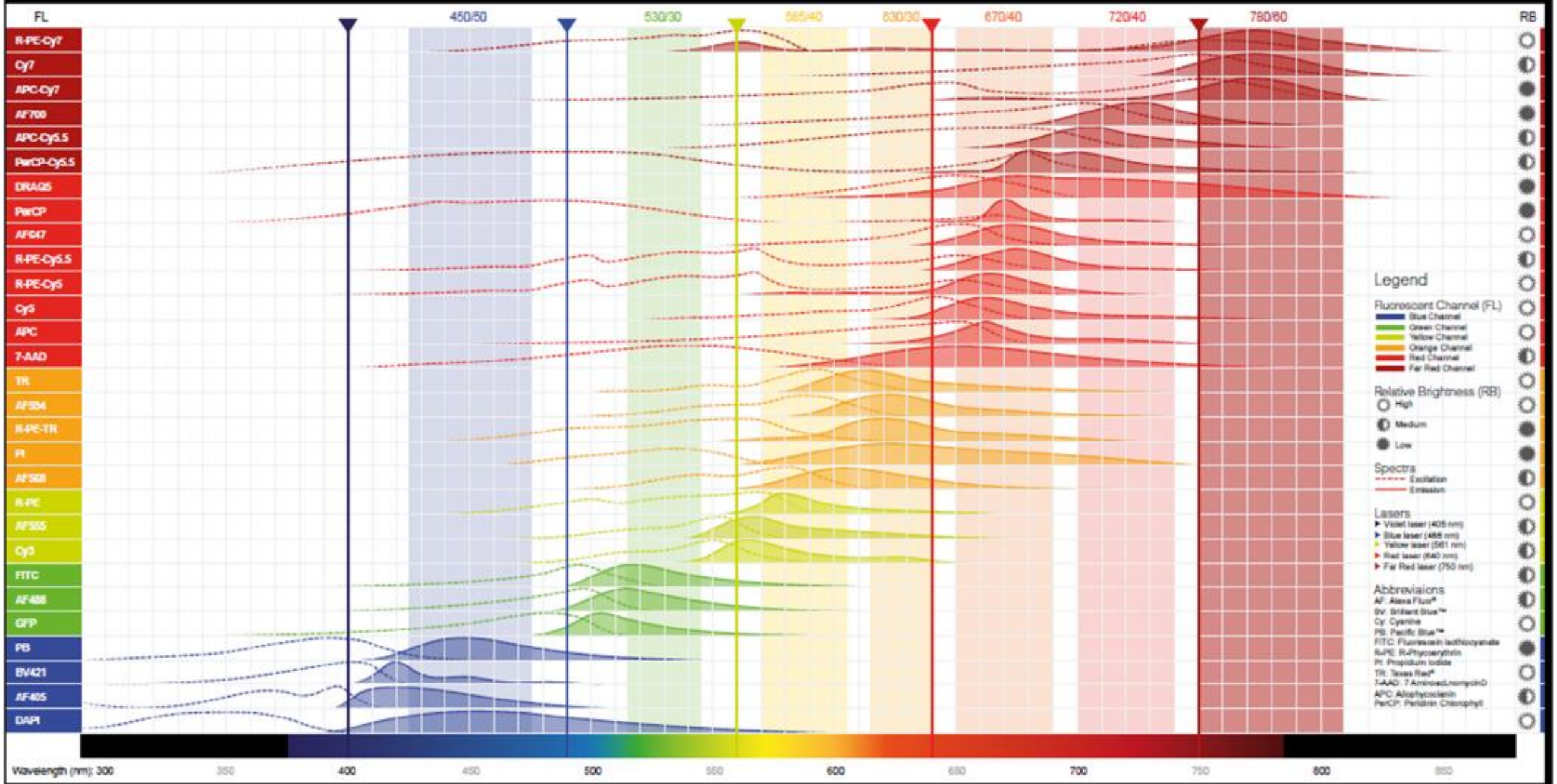
# Sources of fluorescence

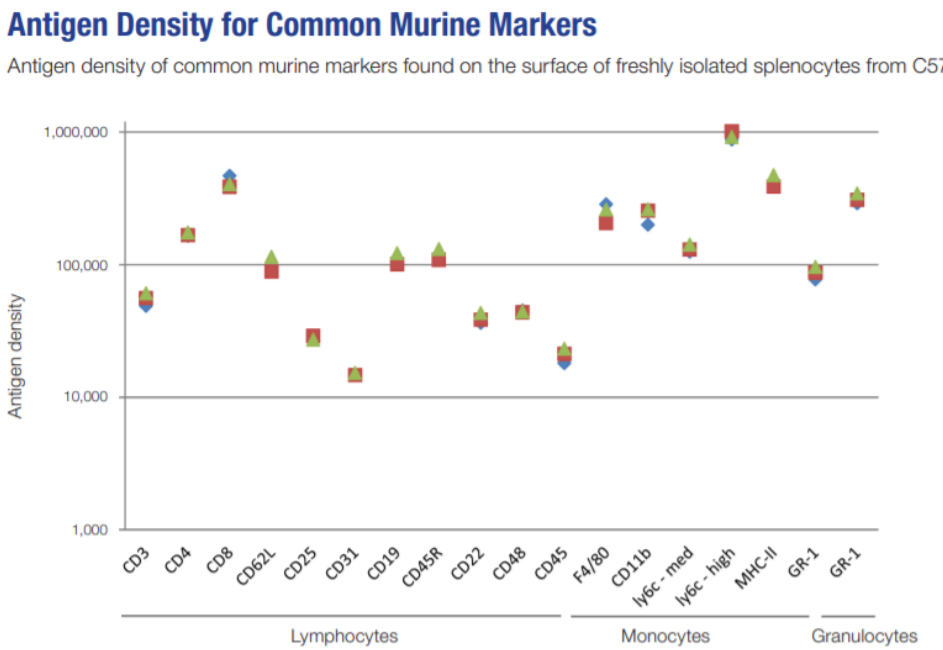
- Fluorophores conjugated antibodies.
- Autofluorescence.
- Fluorescent dyes.
- Other chemicals with fluorescence properties (drugs,…).

# Fluorochrome chart



# Multicolor panel building

- Pair bright fluorophores (e.g. PE) with low expressing markers.
- Dimmer fluorophores (e.g. Pacific Blue) with highly expressed markers.

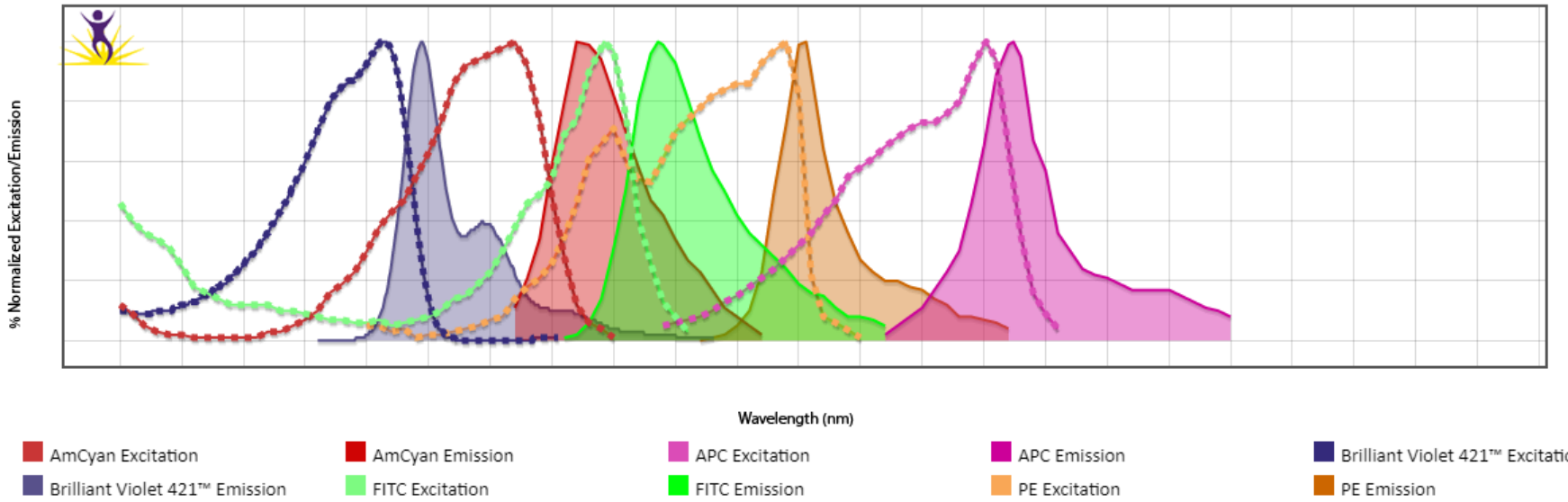



Fluorochrome

| Laser | Very Bright | Bright | Moderate | Dim |
|---|---|---|---|---|
| Ultraviolet (355 nm) | | BD Horizon™ BUV661<br>BD Horizon™ BUV737 | BD Horizon™ BUV395<br>BD Horizon™ BUV496 | BD Horizon™ BUV805 |
| Violet (405 nm) | BD Horizon™ BV421<br>BD Horizon™ BV650<br>BD Horizon™ BV711 | BD Horizon™ BV605<br>BD Horizon™ BV786 | BD Horizon™ BV510 | BD Horizon™ V450<br>BD Horizon™ V500 |
| Blue (488 nm) | BD Horizon™ BB515<br>BD Horizon™ PE-CF594<br>PE-Cy™5 | PE<br>PE-Cy™7 | FITC<br>Alexa Fluor® 488<br>PerCP-Cy™5.5 | PerCP |
| Yellow/Green (561 nm) | PE<br>BD Horizon PE-CF594<br>PE-Cy5<br>PE-Cy7 | | | |
| Red (640 nm) | | APC<br>Alexa Fluor® 647<br>BD Horizon™ APC-R700 | | Alexa Fluor® 700<br>APC-H7<br>APC-Cy7 |

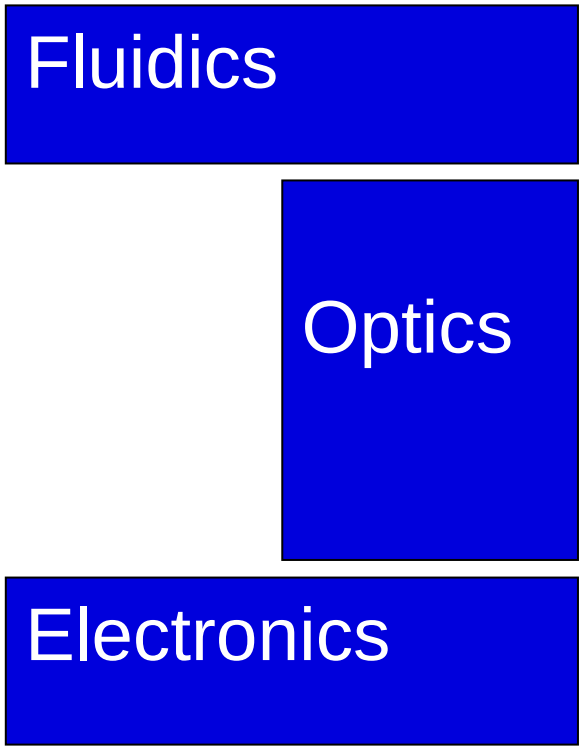
# FACSCanto

| Laser | Detector | Fluorochromes |
|---|---|---|
| 405 | | |
| | 450/50 | Pacific Blue, eFluor 450, V450, BV 421, Cascade Yellow, (DAPI) |
| | 510/50 | AmCyan, BV 510, Krome Orange, V500, Cacsade Blue |
| 488 | | |
| | 530/30 | FITC, Alexa Fluor 488, BB 515, CFSE, Fluo-3, GFP |
| | 585/42 | PE, PI |
| | 670LP | PerCP, PerCP-Cy 5.5, PC5, 7-AAD |
| | 780/60 | PC7 |
| 633 | | |
| | 660/20 | APC, Alexa Fluor 647, eFluor 660 |
| | 780/60 | APC-Cy7, APC-H7, APC - Alexa Fluor 750, APC-eFluor 780 |



# Technical components

Cells in suspension
flow individually across
illuminated part where
they scatter light and emit
fluorescence,
which is detected, filtered and
converted to digital values analyzed
and stored on your computer.

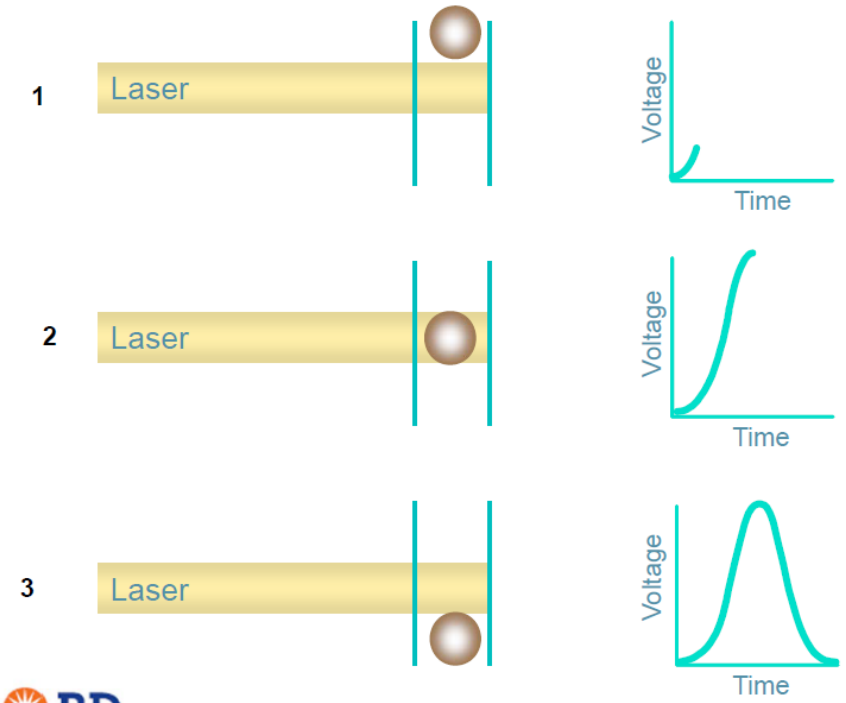


Electronics

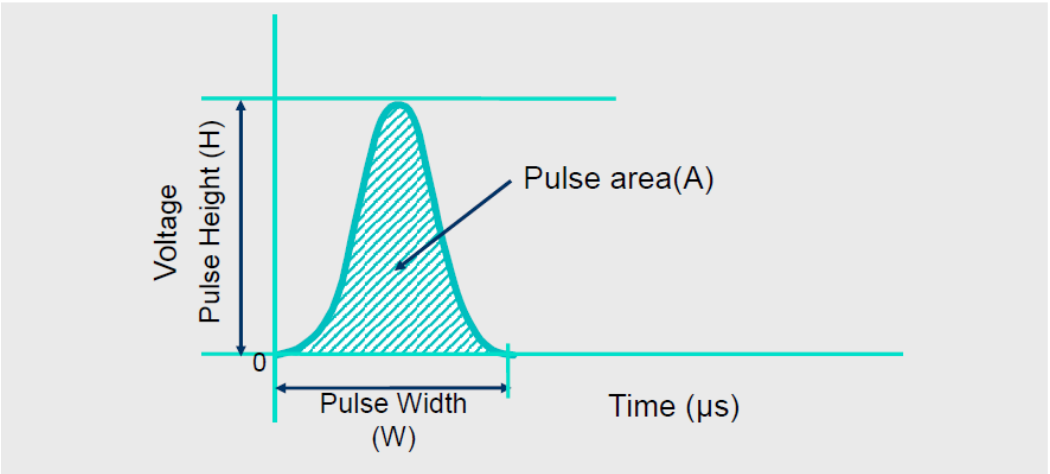
# Signal processing

– When cells go through laser scattered light makes pulse at the sensor.

## Creation of a Voltage Pulse

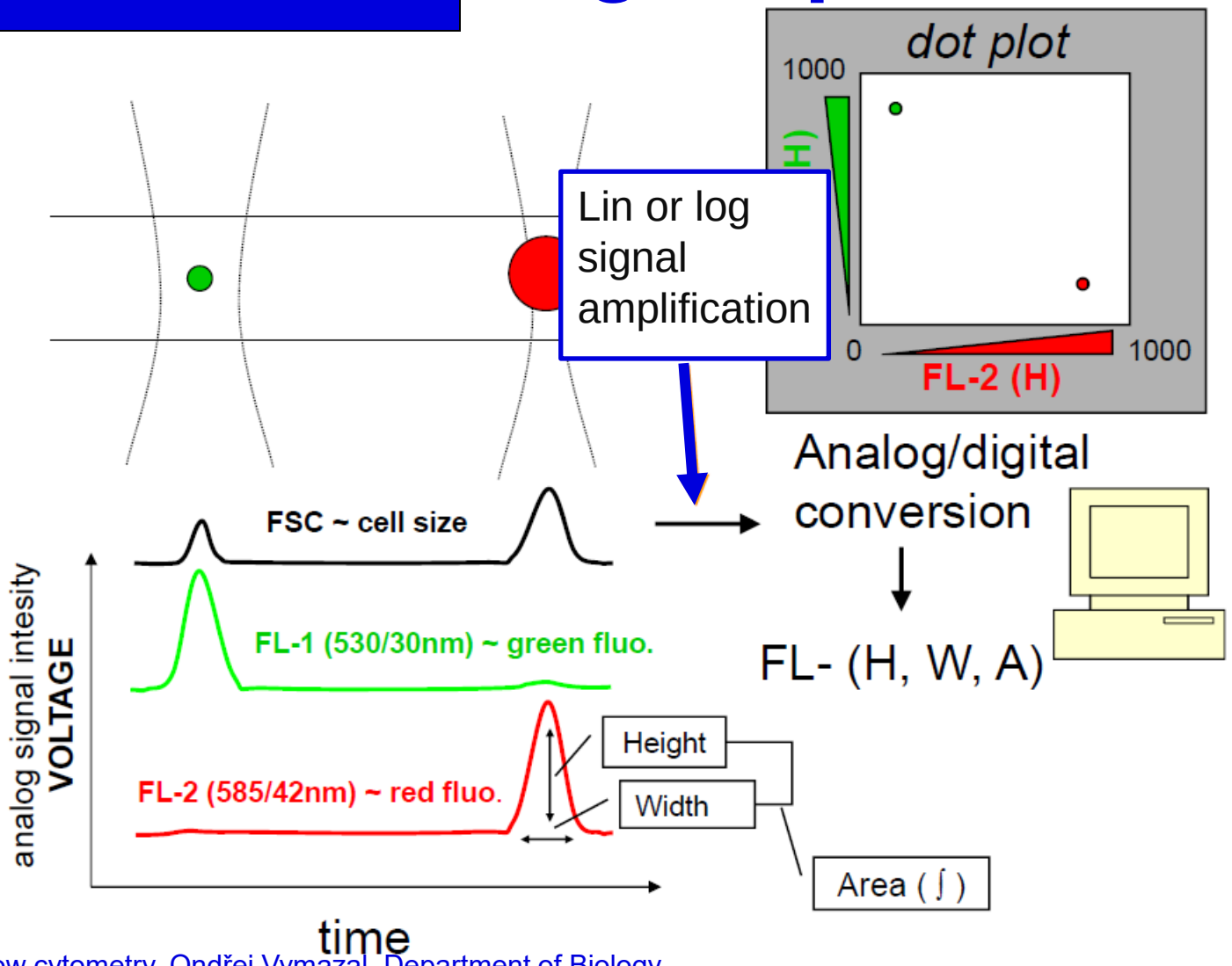



## Height, Area, and Width

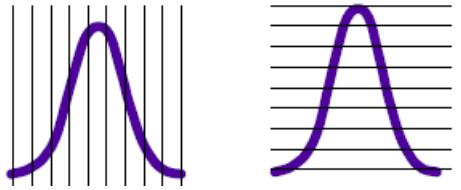


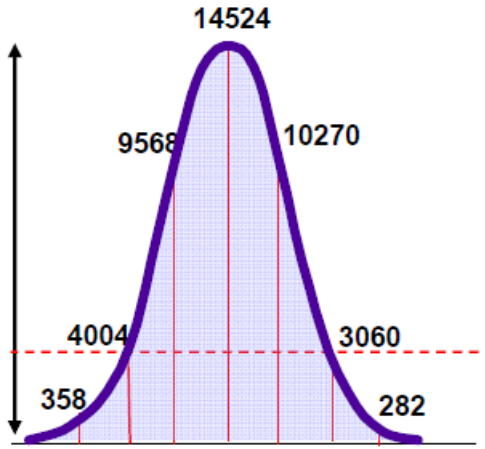
Electronics

# Signal processing




## Analog/digital conversion





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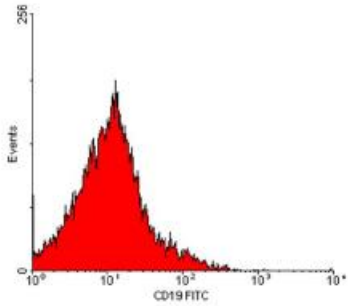
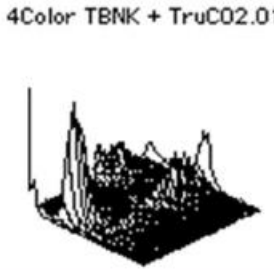
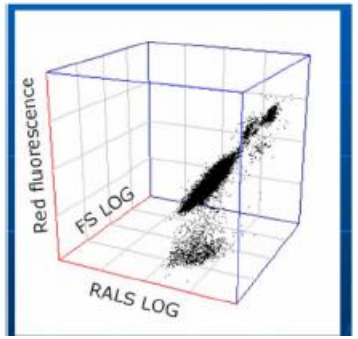
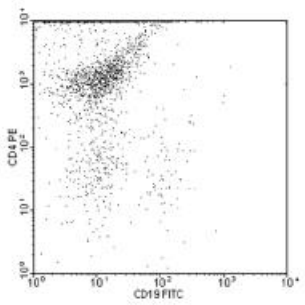
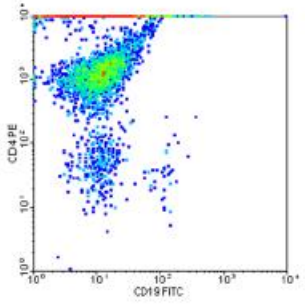
# Data analysis

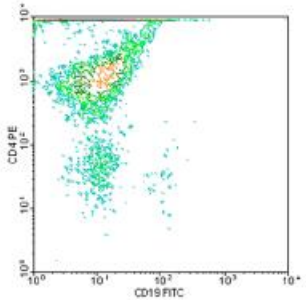


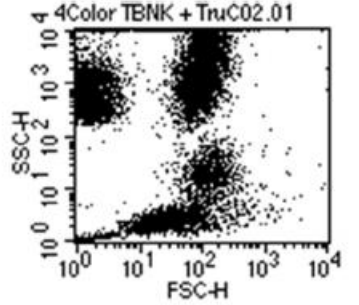



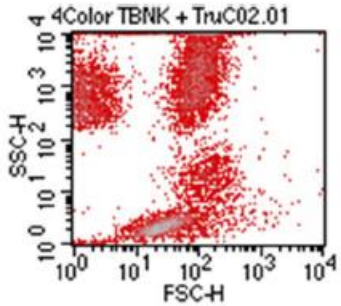



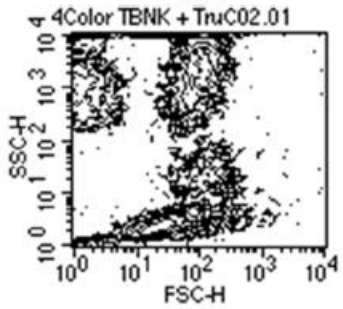




## Data visualization

- **histogram**
- **dot plot**
- isometric display
- contour plot
- chromatic (color) plots
- 3D projection



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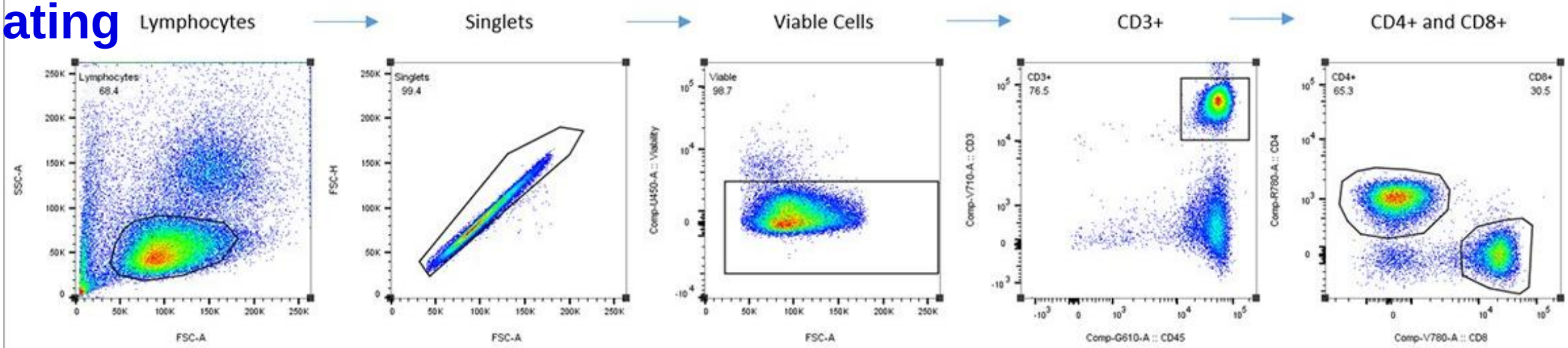
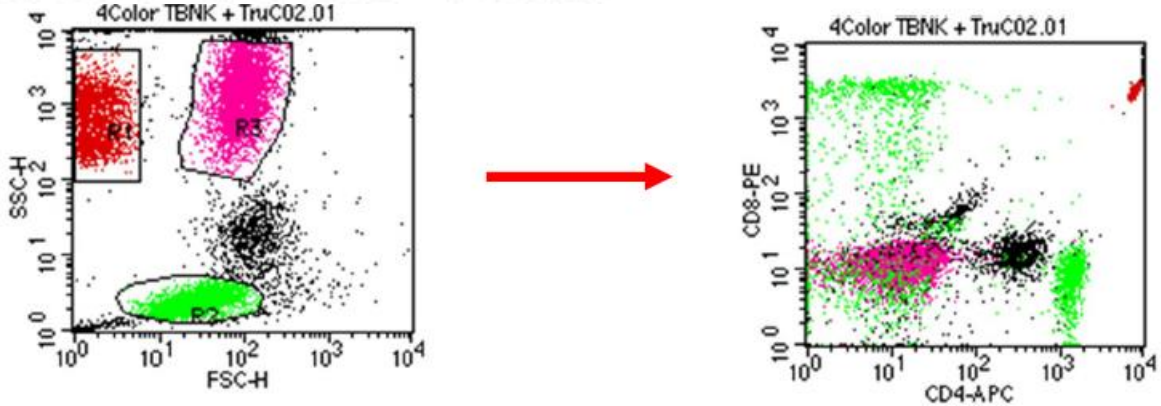
# Data analysis

## Gating

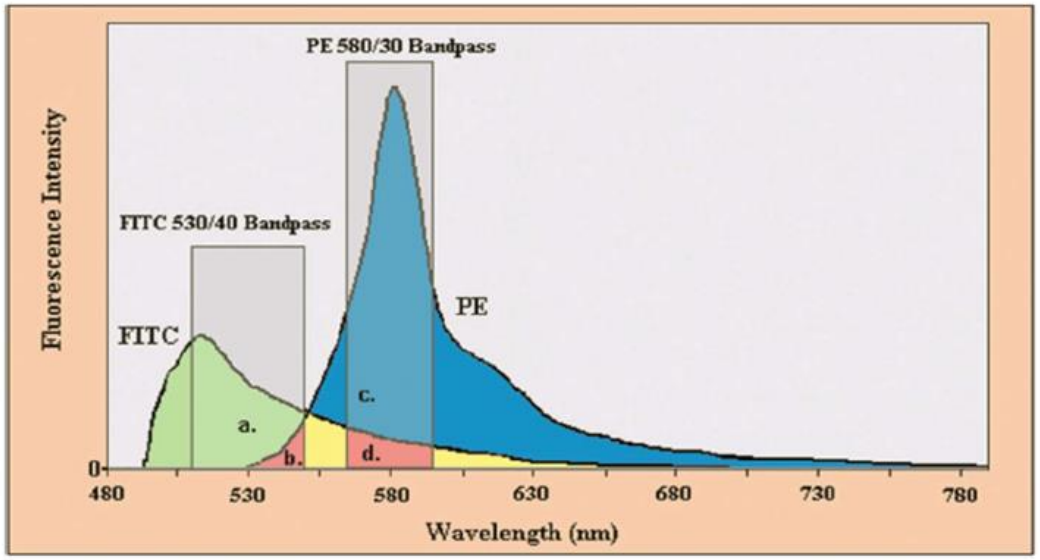


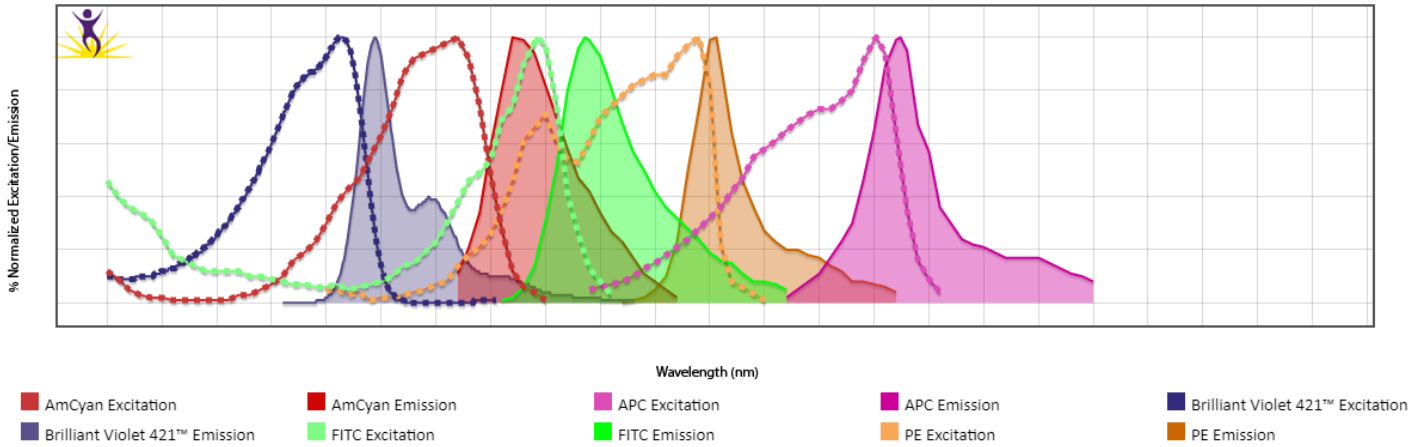

A representative Nested Gating Strategy illustrating lymphocyte population being subgated to the level of CD4+ and CD8+ T Cells.

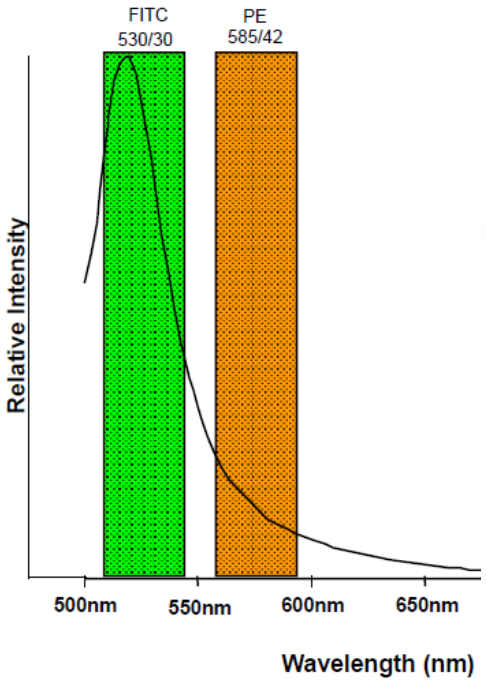
## Back gating

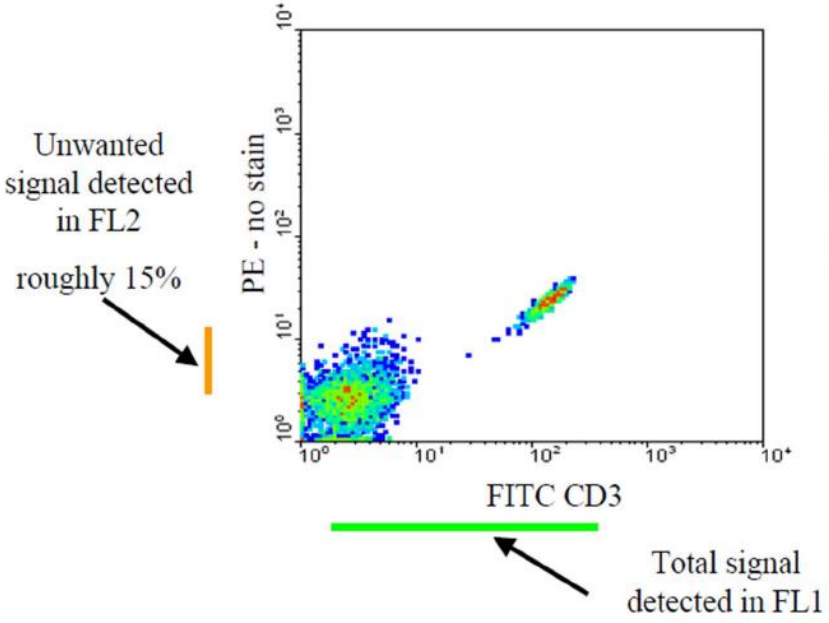


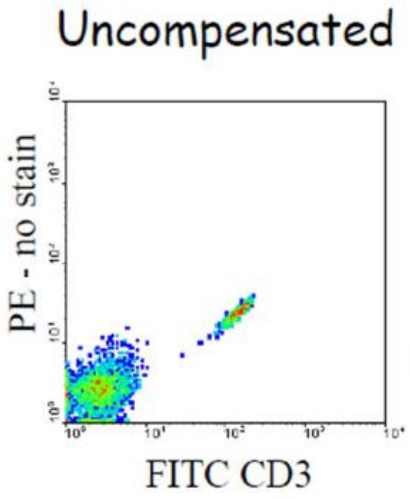
# Compensation



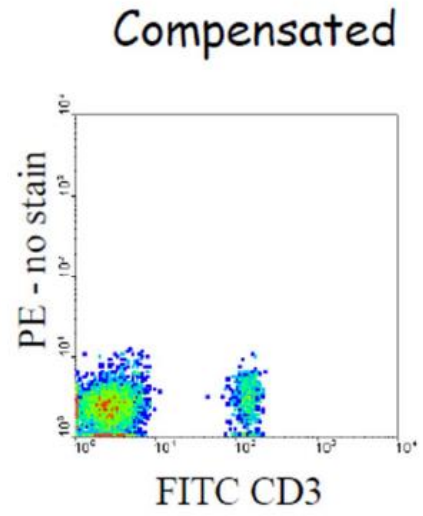





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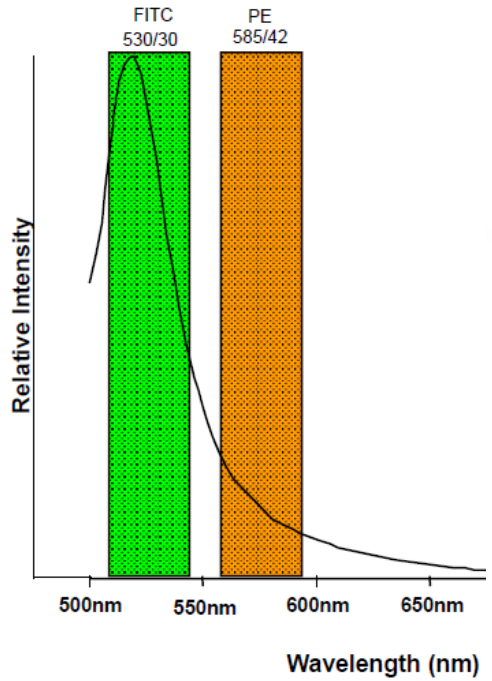
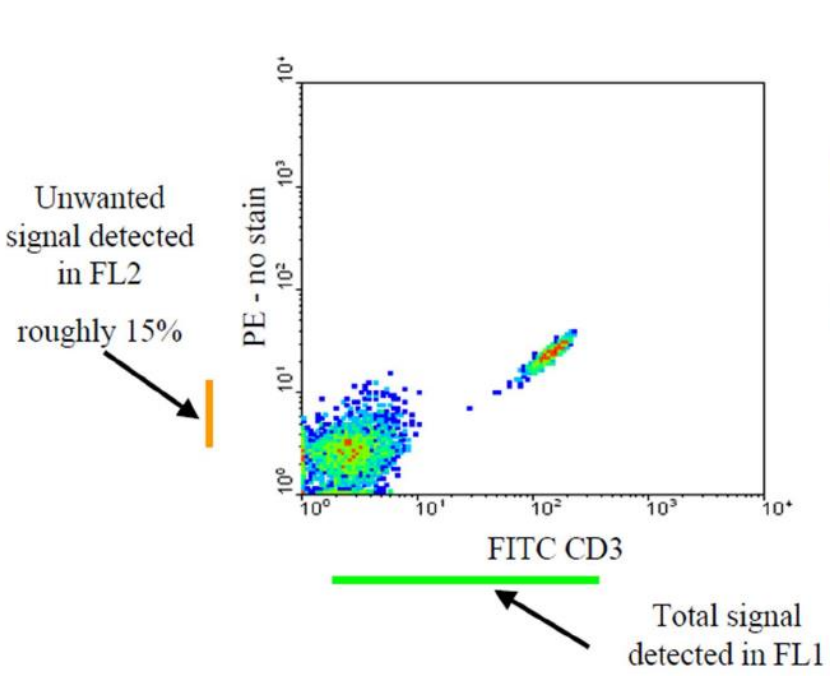
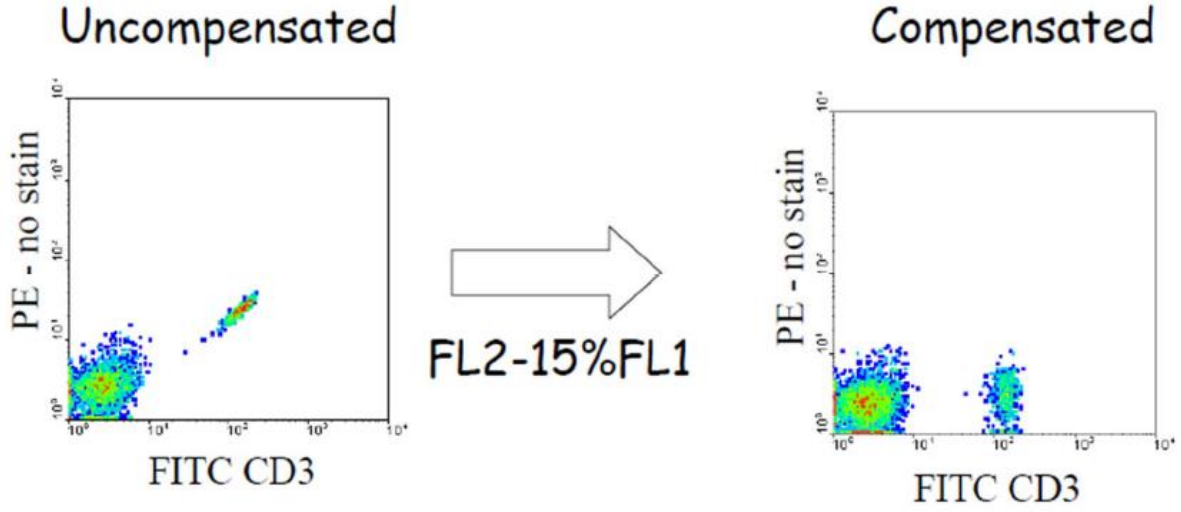
# Compensation

- Single stained control for each fluorophore in antibody cocktail.
- Cells or compensation beads.
- New compensation after any changes in cocktail, same compensation for comparable experiments.

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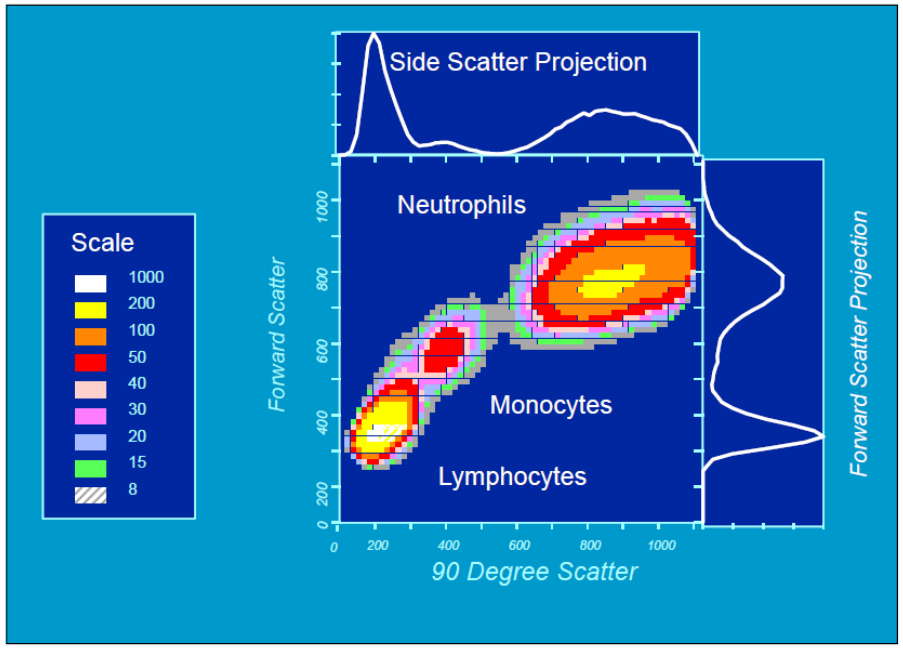
# Compensation

- Video with compensation in BD software
- Compensation of a 7 color panel on the BD LSR II:
  https://youtu.be/5UHw4DIArx4

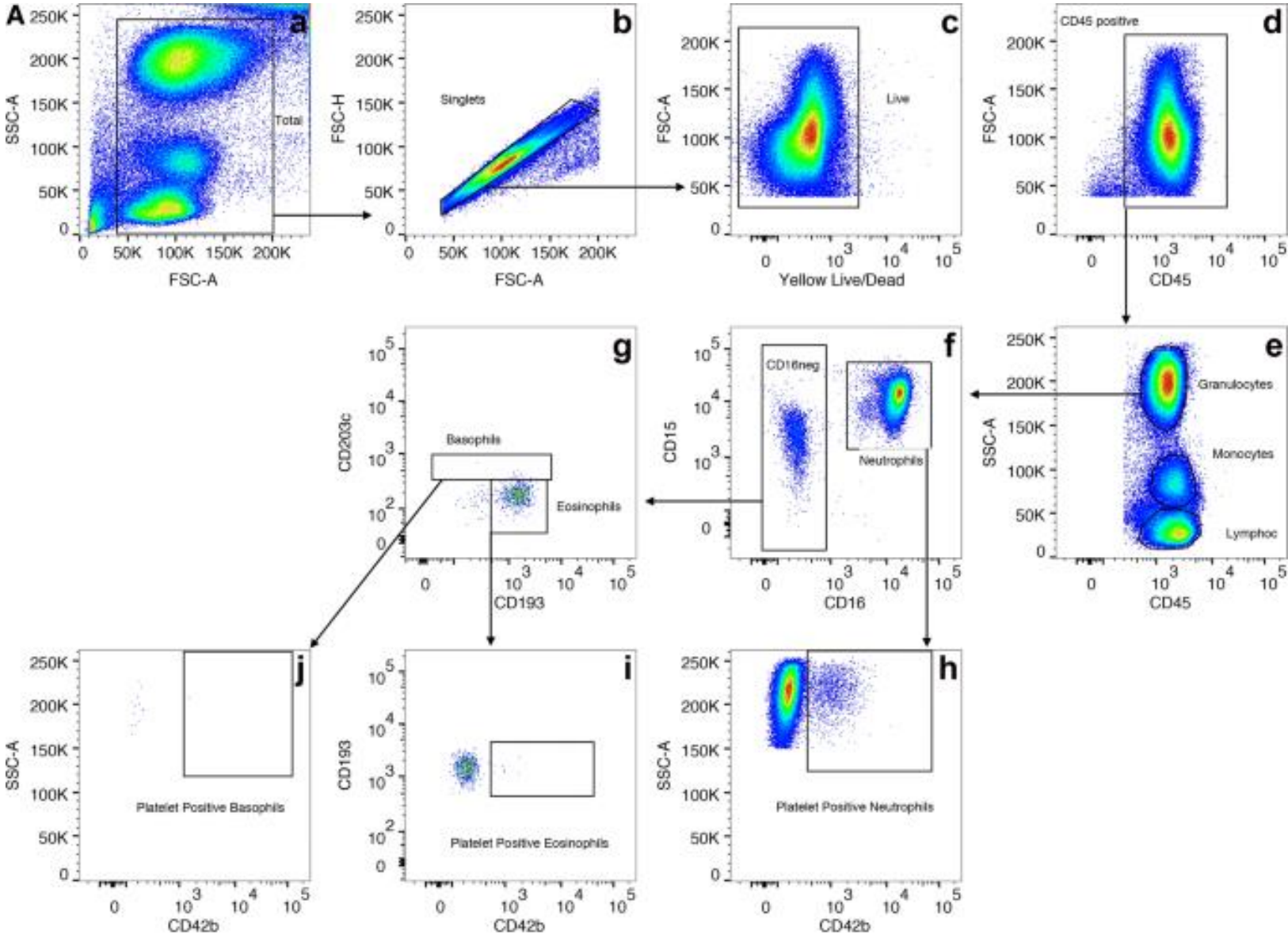
# Applications

Phenotyping

## Light Scatter Gating




upraveno podle J.P.Robinson






Baumer, Y., Gutierrez-Huerta, C.A., Saxena, A. *et al.* Immune cell phenotyping in low blood volumes for assessment of cardiovascular disease risk, development, and progression: a pilot study. *J Transl Med* **18,** 29 (2020). https://doi.org/10.1186/s12967-020-02207-0

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# Applications

Cell cycle analysis

- Fluorescent dyes binding DNA.
- Cells have to be fixed and permeabilized.

- Quaternary benzo[c]phenanthridine alkaloids (QBAs)
- **I. Slaninova**, J. Slanina and E. Taborska, "Quaternary benzo[c]phenanthridine alkaloids--novel cell permeant and red fluorescing DNA probes," Cytometry A, vol. 71, no. 9, pp. 700-708, 2007.
- Can be used to label viable cells.

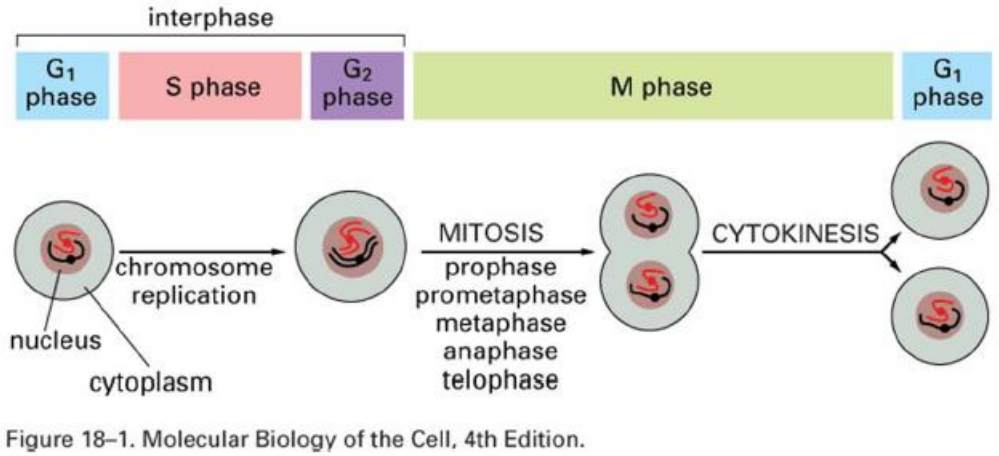
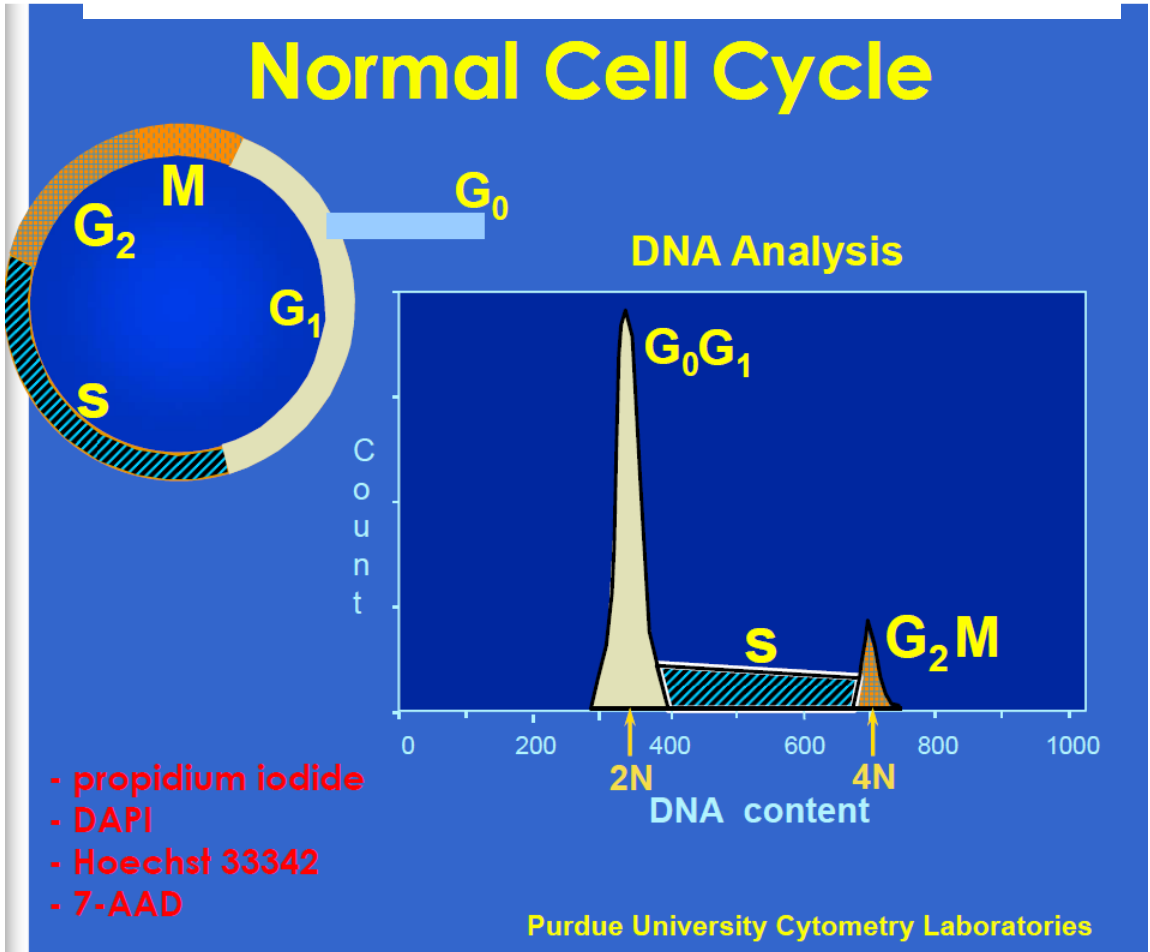


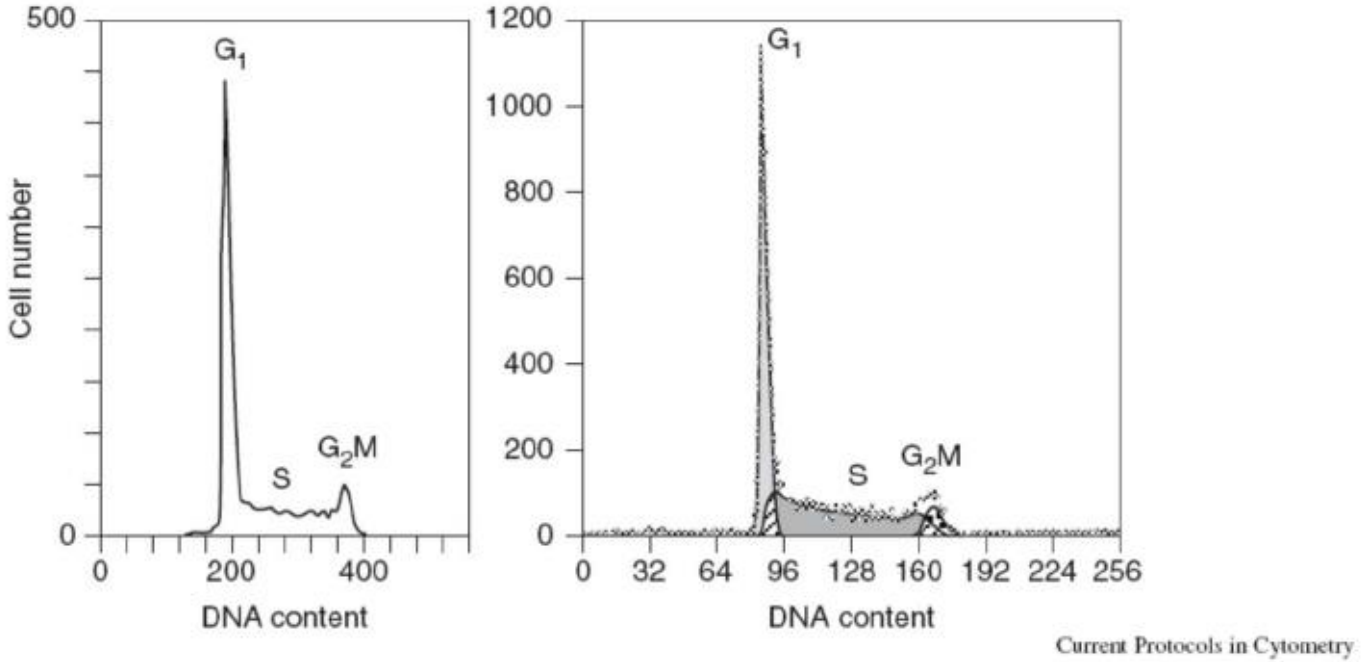

Figure 18–1. Molecular Biology of the Cell, 4th Edition.



# Applications

Cell cycle analysis

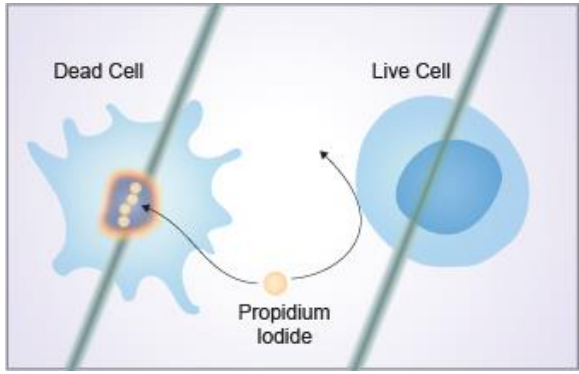
– Software for analysis of distribution of cells in individual phases.



# Applications

Viability

- Based on detection of membrane integrity.
- Some fluorescence dyes (PI,EBr) don´t leak into live cells.

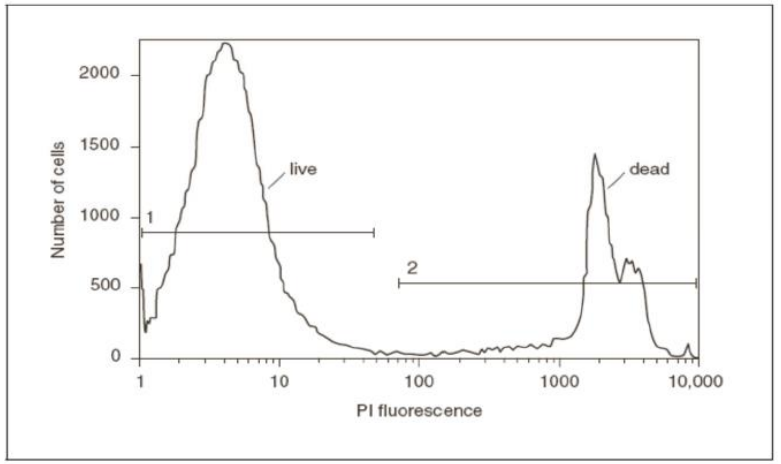



LIVE/DEAD® Fixable Dead Cell Stain Kits

invitrogen™

| Reactive dye | Excitation source | Ex* | Em* |
|---|---|---|---|
| blue fluorescent reactive dye (L23105) | UV | 350 | 450 |
| violet fluorescent reactive dye (L34955) | 405 nm | 416 | 451 |
| aqua fluorescent reactive dye (L34957) | 405 nm | 367 | 526 |
| yellow fluorescent reactive dye (L34959) | 405 nm | 400 | 575 |
| green fluorescent reactive dye (L23101) | 488 nm | 495 | 520 |
| red fluorescent reactive dye (L23102) | 488 nm | 595 | 615 |
| far red fluorescent reactive dye (L10120) | 633/635 nm | 650 | 665 |
| near-IR fluorescent reactive dye (L10119) | 633/635 nm | 750 | 775 |

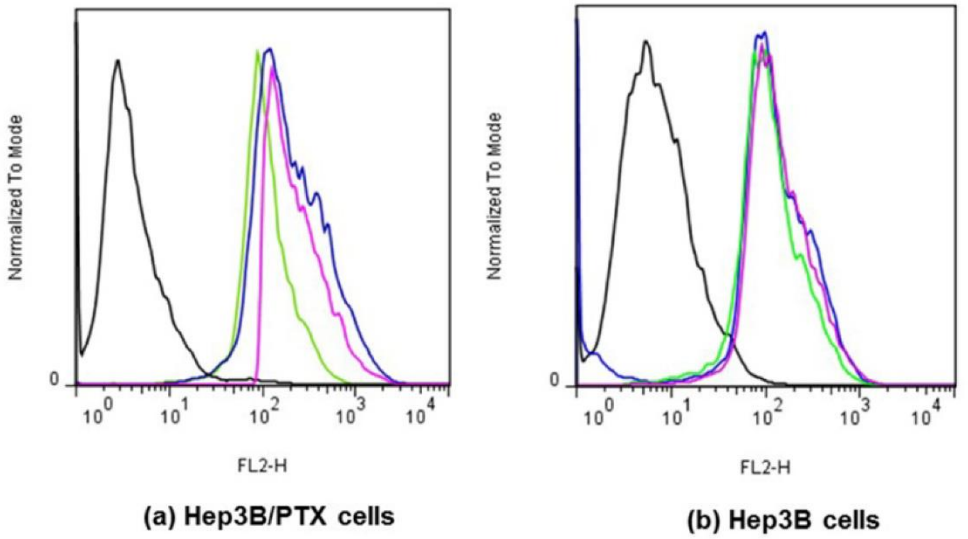
*Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.



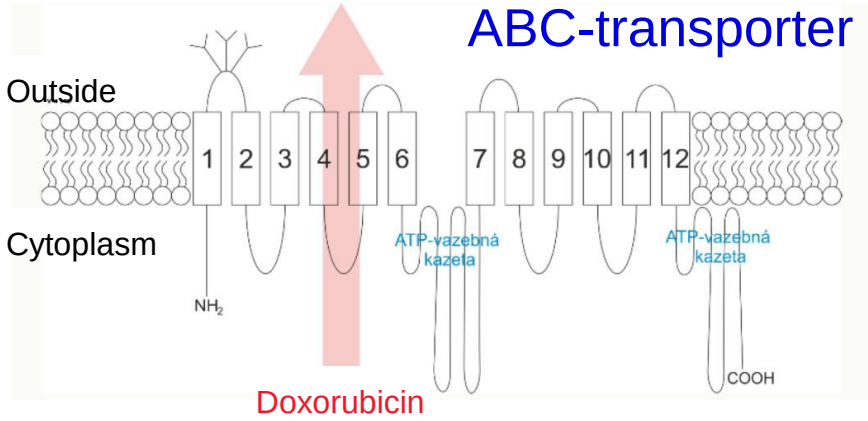
# Applications

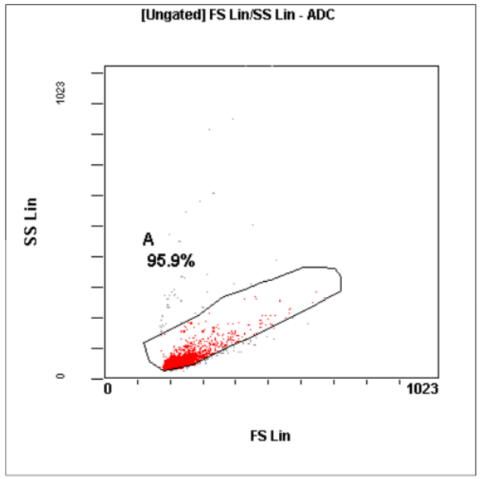
Doxorubicin accumulation

- Chemotherapeutic drug with fluorescent properties.
- Cells get rid of it from intracelular space using ABC-transporters in cytoplazmic membranes.




| | Sample Name |
|---|---|
| | Negative control |
| | Doxorubicin |
| | Achillin +Doxurubicin |
| | Verapamil+ Doxorubicin |

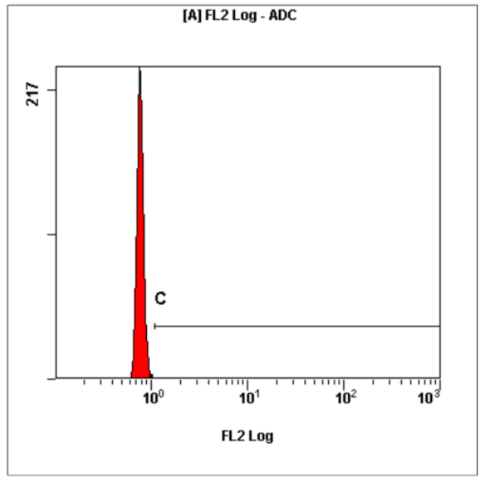





[Ungated] FS Lin/SS Lin

| Region | Number | %Total | %Gated | X-Mean | Y-Mean |
|---|---|---|---|---|---|
| ALL | 4776 | 100.00 | 100.00 | 241 | 70.7 |
| A | 4578 | 95.85 | 95.85 | 238 | 64.5 |

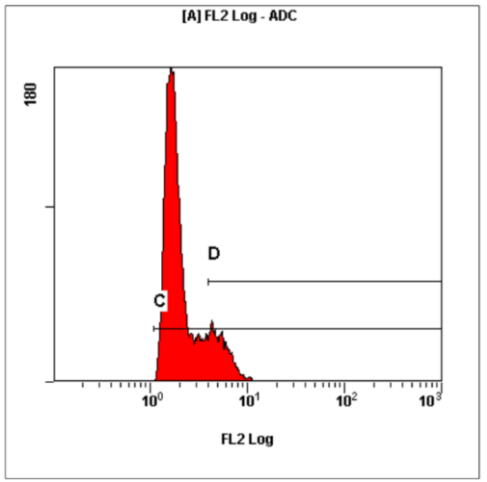
## -DOXO



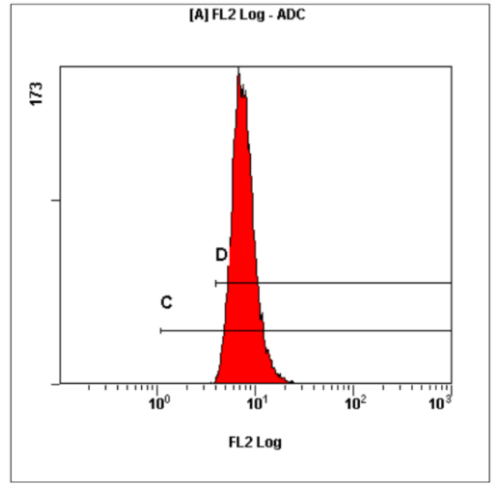

[A] FL2 Log

| Region | Number | %Total | %Gated | X-Mean | Y-Mean |
|---|---|---|---|---|---|
| ALL | 4578 | 95.85 | 100.00 | 0.772 | ### |
| C | 20 | 0.42 | 0.44 | 1.24 | ### |

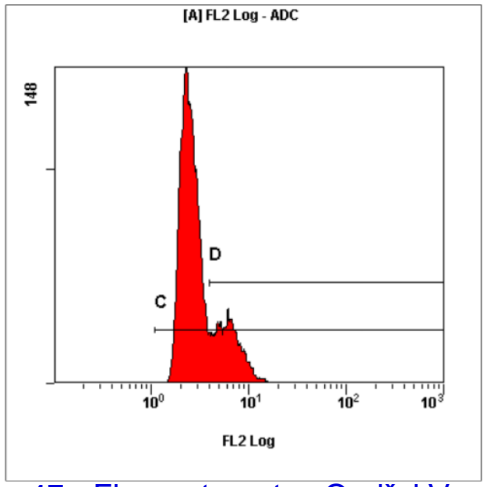
## +DOXO

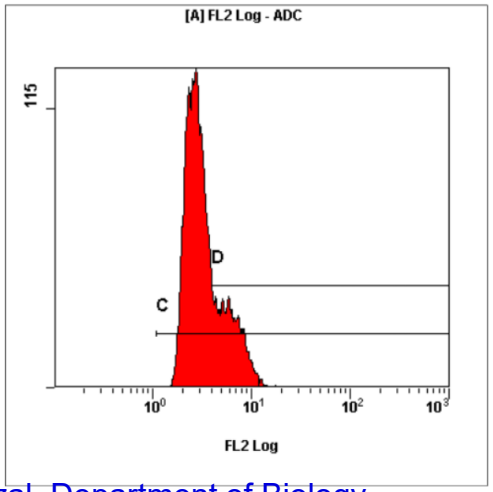


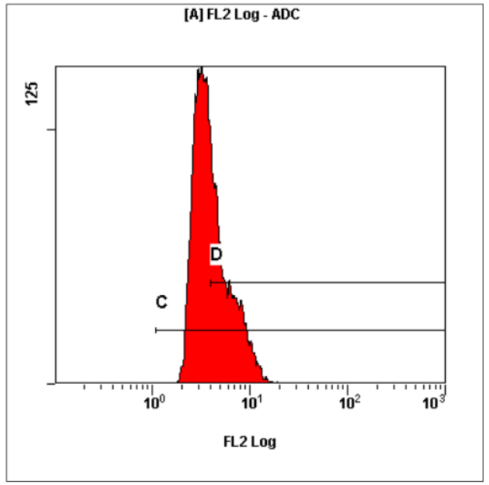

## Verapamil

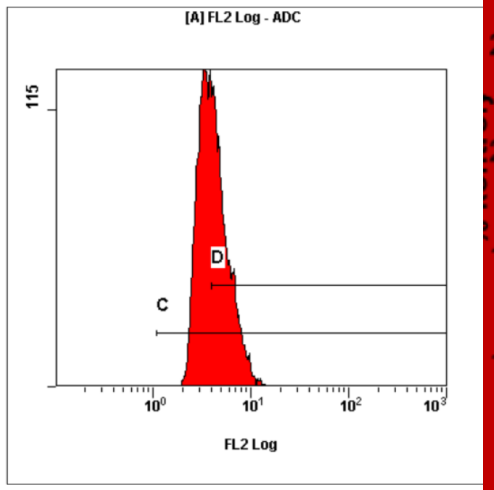


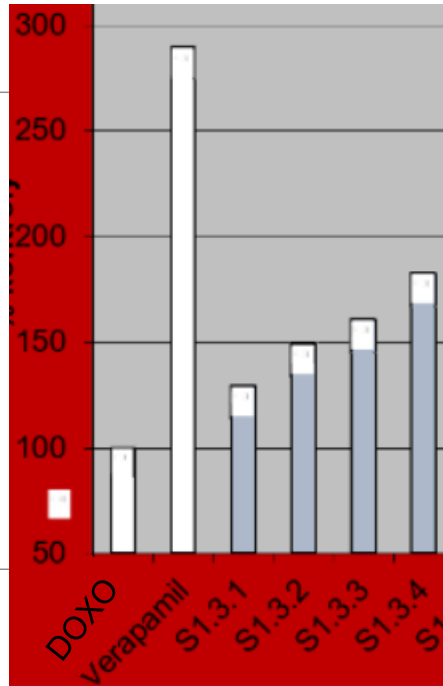

## Lignans



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# Thanks for attention!