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Flow cytometry and FACS

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February 12th, 2020

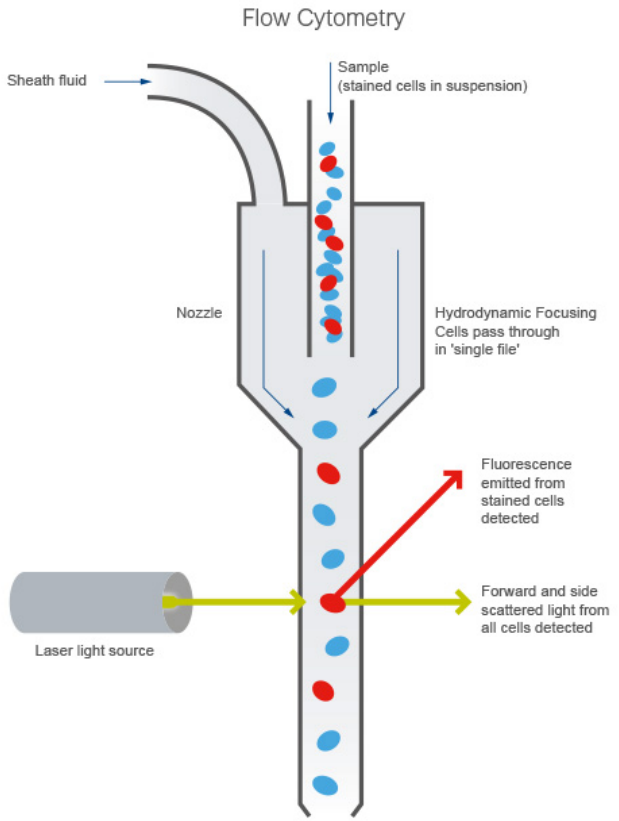
Flow cytometry

Flow cytometry

- Flow cytometry and FACS are widely used techniques to capture features of cell phenotype
- Flow cytometry allows to understand size and granularity of individual cells
- FACS allows to capture protein expression for several (pre-defined) surface proteins in individual cells

Flow cytometry

- Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam one cell at a time
- Forward and side scattered light is detected

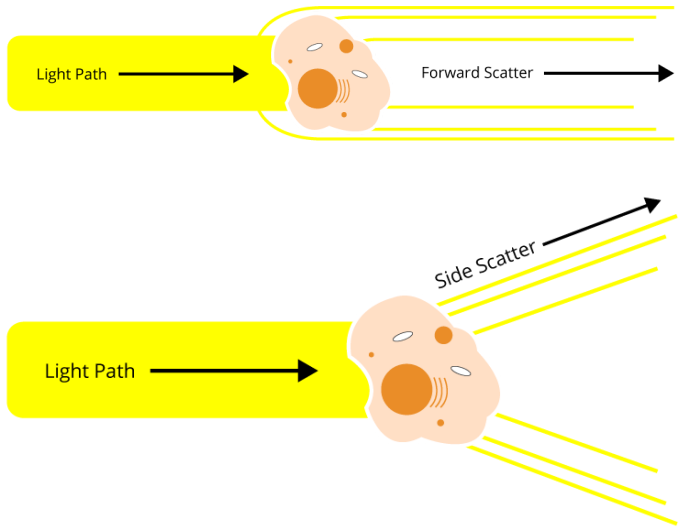


taken from https://www.abcam.com/protocols/introduction-to-flow-cytometry

Flow cytometry

Cells or particles passing through the beam scatter light, which is detected as FS and SS

- FS correlates with cell size and
- SS is proportional to the granularity of the cells

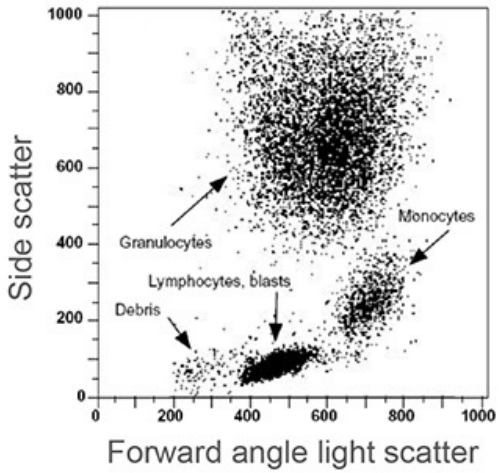


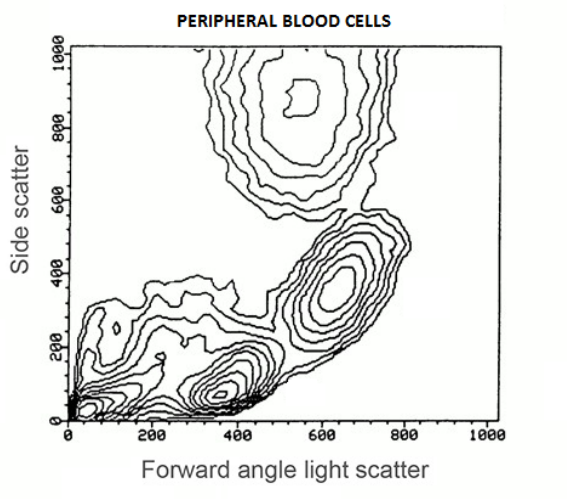
In this manner, cell populations can often be distinguished based on differences in their size and granularity alone.

taken from https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle

Flow cytometry: example

Below are dot plot and contour plot of blood cells flow cytometry.





Such plot (and density plots) are common for flow cytometry and FACS and allow cell subset identification.

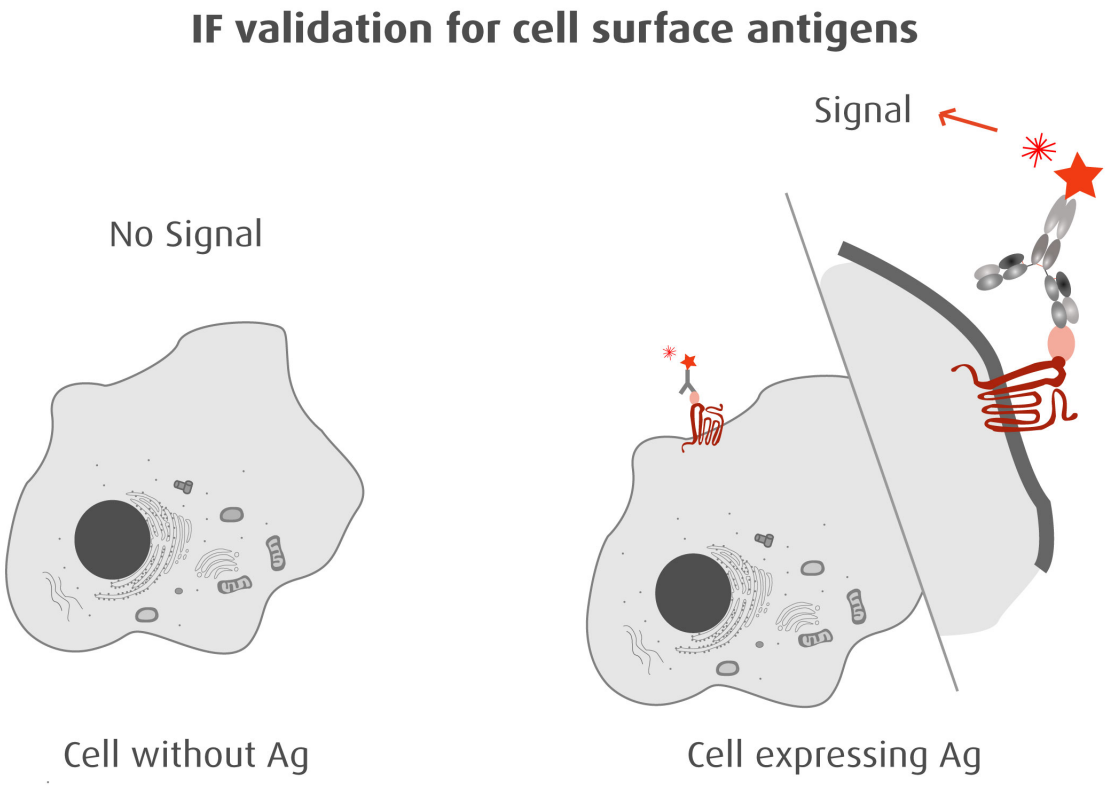
taken from https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle

FACS (fluorescence-activated cell sorting)

FACS (fluorescence-activated cell sorting)

- FACS adds another functionality to flow cytometry
- FACS detects expression of cell surface molecules
- For FACS we need to design panel of antibodies with specific fluorophores

FACS


IF validation for cell surface antigens
Signal
No Signal
Cell without Ag
Cell expressing Ag

Cell surface molecules

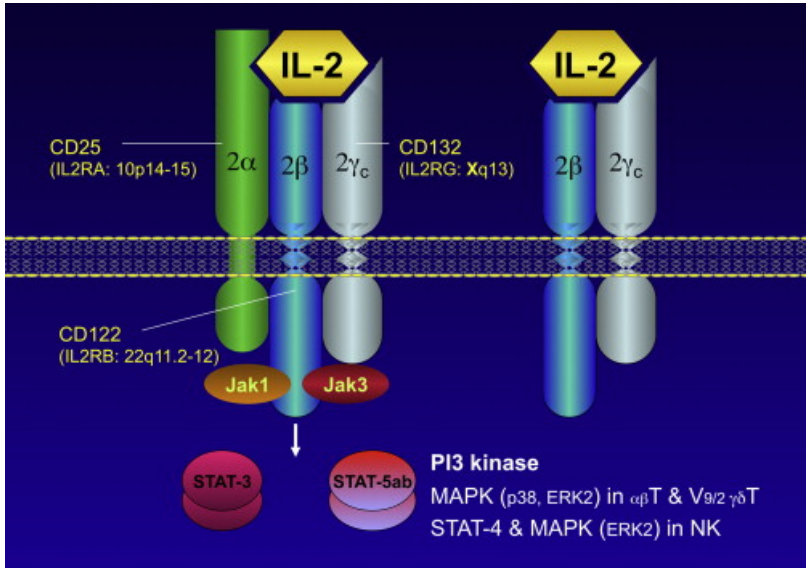
There are many cell surface molecules and ost of them have at least two names:

1. **Functional**:
   PTPRC (Protein tyrosine phosphatase, receptor type, C)
   ITGAM (Integrin alpha M)
2. **Cluster of differentiation**:
   CD45, CD11b

Sometimes these genes have just a name of CD nomenclature like CD4 and CD34.

Cell surface molecules

Cell surface molecules may be protein complexes: i.e. composed of products of several genes.



taken from https://www.sciencedirect.com/topics/immunology-and-microbiology/il-2-receptor

Cell surface molecules

- Luckily, we don't need to learn them
- Takeaway message: there are **many** cell surface molecules, and scientists very often use them to **characterize cell types**

List of human clusters of differentiation

From Wikipedia, the free encyclopedia

The following is a list of human clusters of differentiation (or *CD*) molecules.

This list is incomplete; you can help by expanding it.

* = group; ** = not listed on hcdm

CD1*	MHC-like molecule that presents lipid molecules
CD1a	CD1a (Cluster of Differentiation 1a), or T-cell surface glycoprotein CD1a, is a human protein encoded by the CD1A gene. An antigen-presenting protein that binds self and non-self lipid and glycolipid antigens and presents them to T-cell receptors on natural killer T-cells.
CD1b [de]	T-cell surface glycoprotein CD1b. Expressed on cortical thymocytes, certain T-cell leukemias and other tissues.
CD1c	T-cell surface glycoprotein CD1c.
CD1d	T-cell surface glycoprotein CD1d encoded by the CD1D gene. CD1d-presented lipid antigens activate a special class of T cells, known as natural killer T (NKT) cells, through the interaction with the T-cell receptor present on NKT membranes
CD1e	T-cell surface glycoprotein CD1e is a protein in humans encoded by the CD1E gene.
CD2	a type I transmembrane protein found on thymocytes, T cells, and some natural killer cells that acts as a ligand for CD58 and CD59 and is involved in signal transduction and cell adhesion; expressed in T-cell acute lymphoblastic leukemia and T-cell lymphoma.
CD3*	the signaling component of the T cell receptor (TCR) complex
CD3d	T-cell surface glycoprotein CD3 delta chain
CD3e	T-cell surface glycoprotein CD3 epsilon chain
CD3g	T-cell surface glycoprotein CD3 gamma chain
CD4	a co-receptor for MHC Class II (with TCR, T-cell receptor); found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. Used by HIV to enter T cells: in HIV infection. $CD8^+$ cytotoxic T cells recognize and kill infected cells hence they are predominantly for protection against intracellular pathogens, e.g. viruses, and some bacteria, i.e. Rickettsiae

taken from https://en.wikipedia.org/wiki/Cluster_of_differentiation

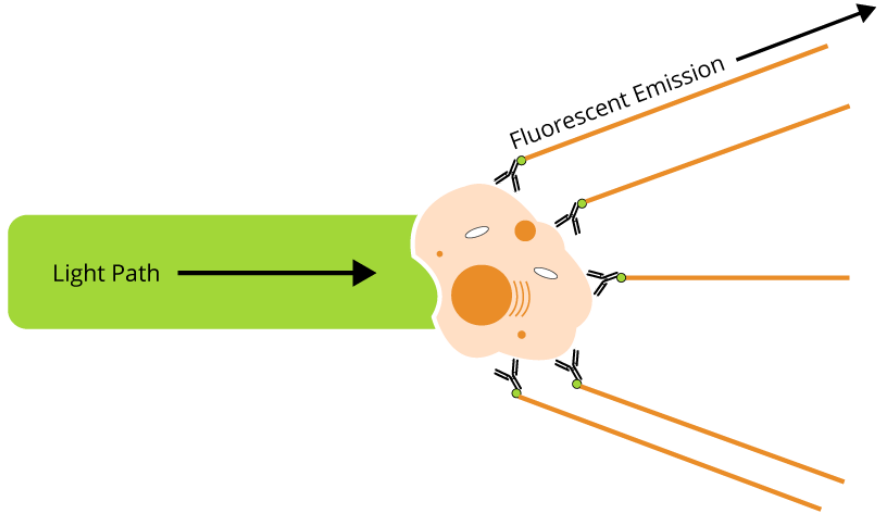
Antibodies

- An **antibody** (**Ab**), also known as an **immunoglobulin** (**Ig**), is a large, Y-shaped protein produced mainly by plasma cells that is used by the immune system to neutralize pathogens such as pathogenic bacteria and viruses
- The antibody **recognizes a unique molecule**, called an antigen, and **binds** to it
- We can engineer antibodies for cell surface molecules

Definition is taken from https://en.wikipedia.org/wiki/Antibody

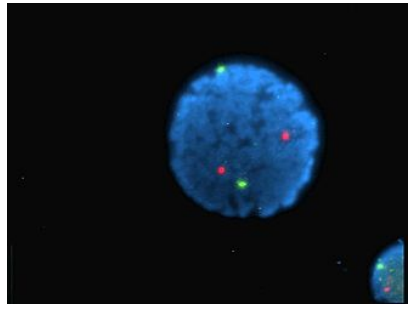
Antibodies

- We can attach fluorophores of different colors to antibodies to figure measure the expression of surface markers in individual cells



Fluorophores

- There are many fluoroscent molecules (fluorophores)
- These fluorescent molecules are excited by laser light at specific wavelengths and then emit light (fluoresce) at another wavelength
- By conjugating (pre-attaching) them to primary antibodies, we can create conjugated antibodies that allow for flow cytometry analysis



Fluorophores

Examples of frequently encountered fluorophores [edit]

Reactive and conjugated dyes [edit]

Dye	Ex (nm)	Em (nm)	MW	Notes
Hydroxycoumarin	325	386	331	Succinimidyl ester
Aminocoumarin	350	445	330	Succinimidyl ester
Methoxycoumarin	360	410	317	Succinimidyl ester
Cascade Blue	(375);401	423	596	Hydrazide
Pacific Blue	403	455	406	Maleimide
Pacific Orange	403	551		
Lucifer yellow	425	528		
NBD	466	539	294	NBD-X
R-Phycoerythrin (PE)	480;565	578	240 k	
PE-Cy5 conjugates	480;565;650	670		aka Cychrome, R670, Tri-Color, Quantum Red
PE-Cy7 conjugates	480;565;743	767		
Red 613	480;565	613		PE-Texas Red
PerCP	490	675	35kDa	Peridinin chlorophyll protein
TruRed	490,675	695		PerCP-Cy5.5 conjugate

taken from https://en.wikipedia.org/wiki/Fluorophore

Important properties of fluorophores

- **Maximum Excitation Wavelength (λex)** – The peak wavelength in the excitation (absorption) spectra, measured in nanometers (nm)
- **Maximum Emission Wavelength (λem)** – The peak wavelength in the emission spectra, measured in nanometers (nm)
- **Extinction Coefficient (ε max)** – (also called molar absorptivity) The capacity for the fluorochrome to absorb light at a given wavelength, usually measured at the maximum excitation wavelength with the units M −1 cm −1
- **Fluorescence Quantum Yield** (Φf) – The number of photons emitted per absorbed photon. A high quantum yield is important, and this number ranges between 0 and 1
- **Brightness** – The fluorescence output per fluorophore measured. Fluorophores with high brightness values can be used to detect lower-abundance targets. Calculated as the product of the extinction coefficient (at the relevant excitation wavelength) and the fluorescence quantum yield divided by 1000, with the units M −1 cm −1

Quick reminder about photon energy

$$E = \frac{hc}{\lambda}$$

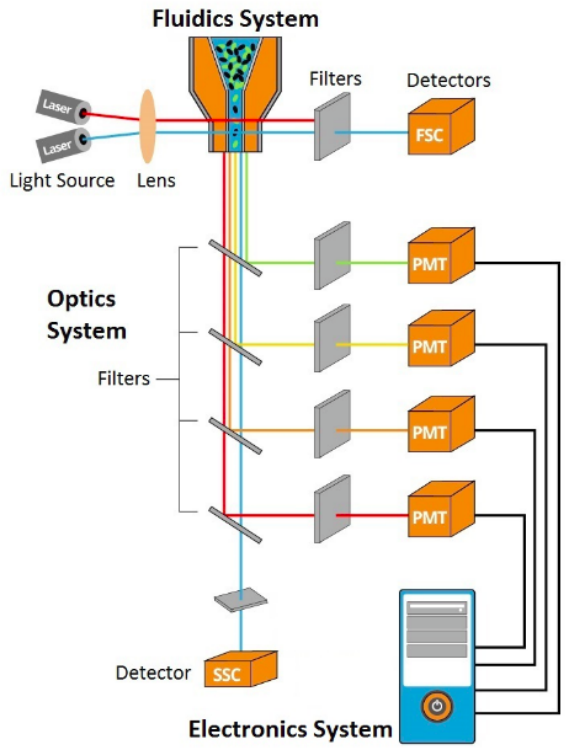
Where

- h is Planck constant
- c is the speed of light (in vacuum)
- λ is the photon wavelength

Energy of emitted photon **is always lower** than the energy of absorbed photon, so **wavelength** of emitted photon **is greater** than the wavelaength of absorbed photon.

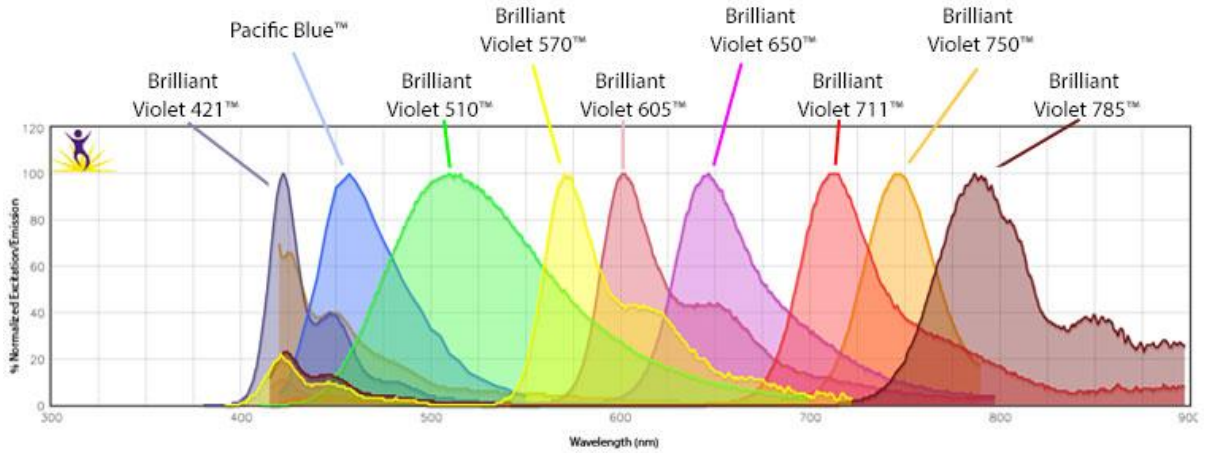
Flow cytometry with fluorescence

- So we have cell labeled with antibodies
- Antibodies for different surface molecules have different fluorophores
- **For each cell** we detect **forward scatter**, **side scatter** and **fluorescence**



FACS

- Unfortunately, emission light spectra overlap between similar colors
- We are limited to **7-10** antibodies per FACS experiment

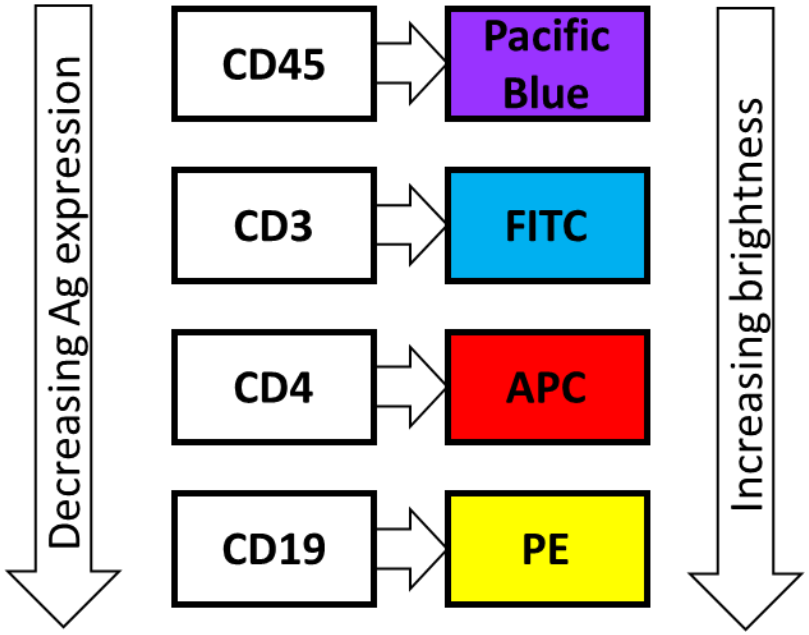


taken from https://www.biolegend.com/en-us/multicolor-staining

FACS

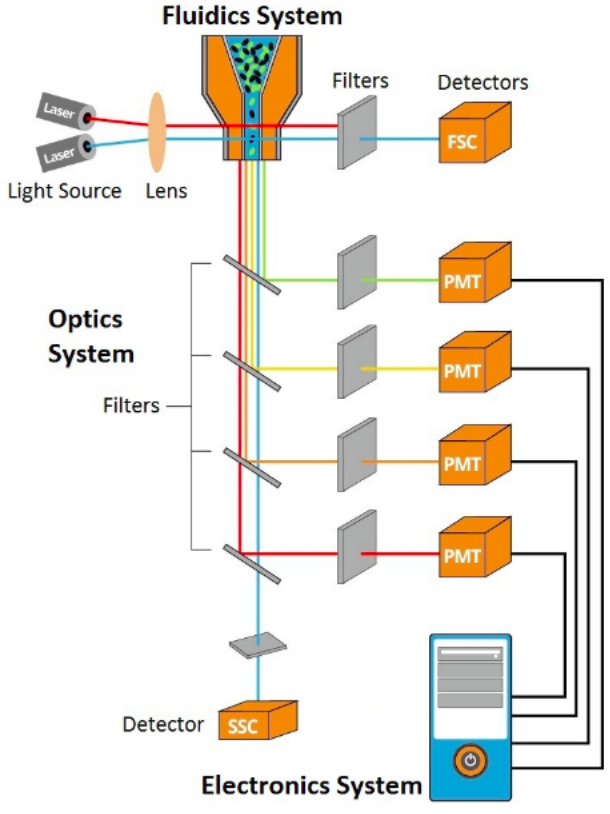
When you are only given 4 flurophores, how to chose to which antibodies to conjugate?

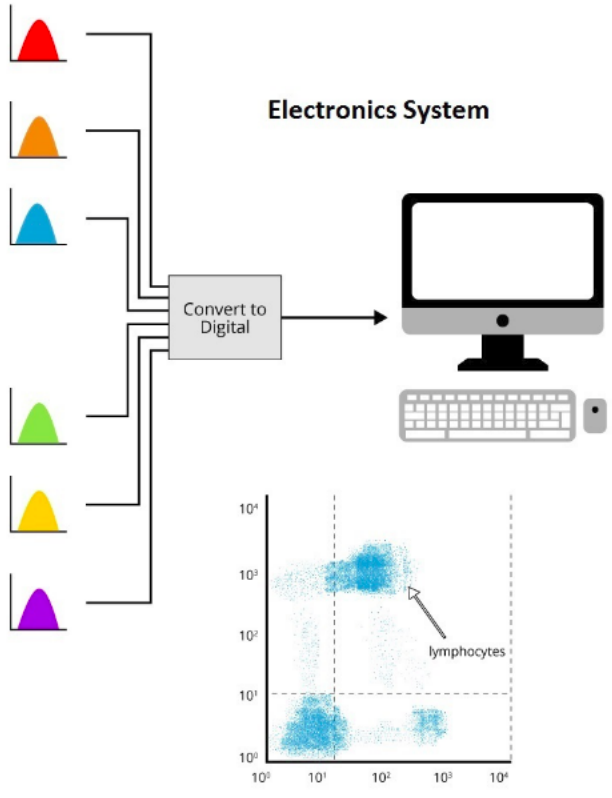
- Basic rule: lower average expression -> greater fluorohpore brightness



taken from https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-optimization/fluorescent-conjugates

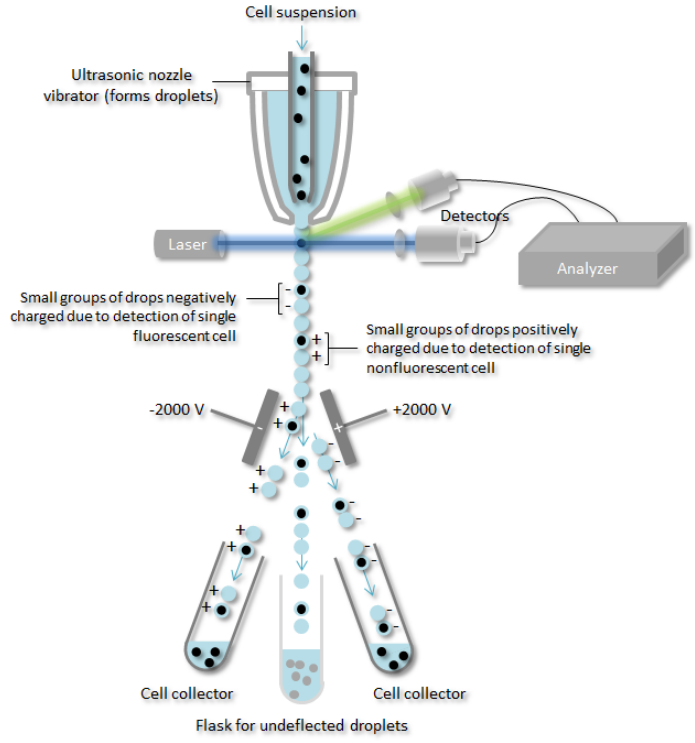
FACS


Fluidics System
Filters
Detectors
Laser
Laser
FSC
Light Source
Lens
PMT
Optics System
PMT
Filters
PMT
PMT
Detector
SSC
Electronics System


Electronics System
Convert to Digital
lymphocytes

FACS: sorting cells

- We can charge droplets with cells based on their fluorescence
- We can put magnets that will pull differently droplets with different fluorescence into different flasks
- That's called sorting



Lets interpret some FACS plots

- With this knowledge about FACS and how FACS plots look like it should be much easier for you to read and look at different biological papers (especially immunological)
- https://konsolerr.github.io/masters_2020/intro_week/facs/nature_microbiol

Any questions ?