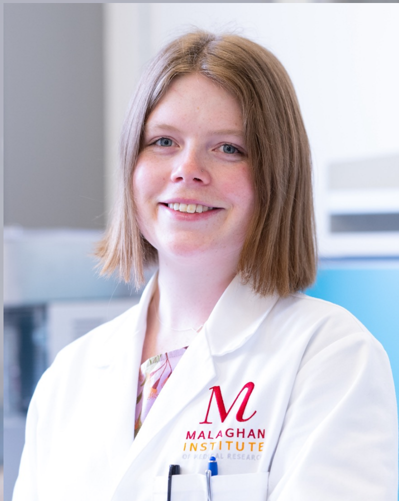
Basics of Flow Cytometry

18th July 2024



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MALAGHAN INSTITUTE OF MEDICAL RESEARCH

Hugh Green Technology Centre

Molecular → Structural → Functional

DNA → mRNA → Protein → Cell → Tissue → Organ → Organism

Genomics

Flow Cytometry

Histology & Bioimaging

Bioinformatics and High-Dimensional Data Analysis

Flow Cytometry

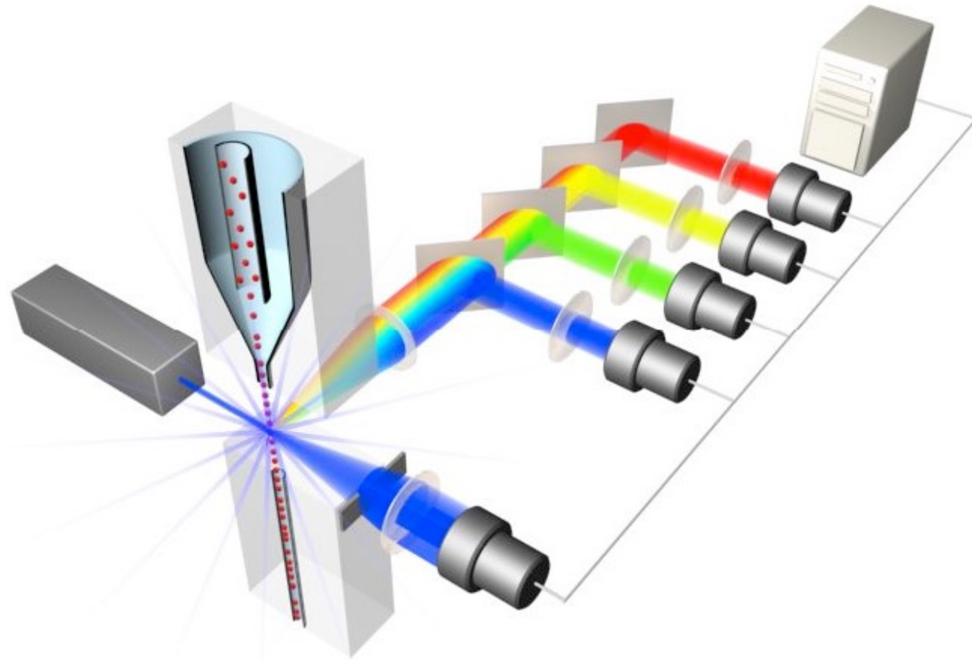
Flow cytometry is a powerful technique used to analyse single cells

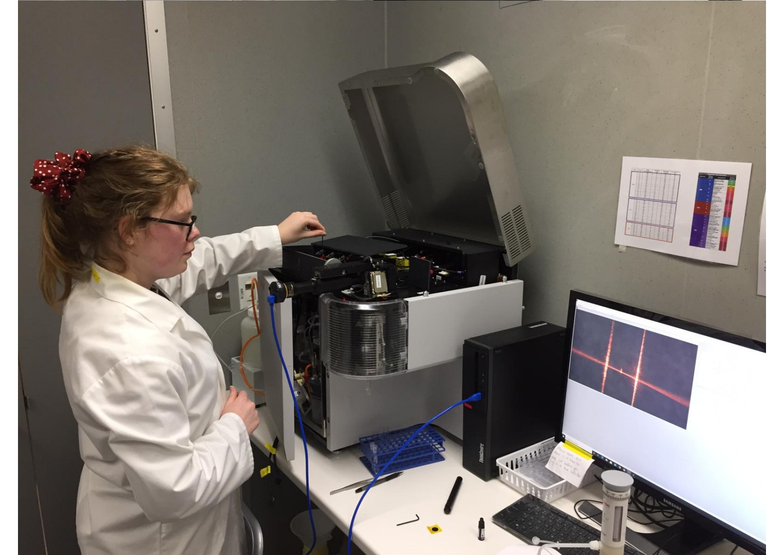
Multiparametric – can look at multiple facets of a single cell at once e.g., physical and chemical properties of individual particles based on how they scatter light and/or the amount of fluorescence emitted after passing a light source in a liquid stream

A flow cytometer is capable of rapid, quantitative, multiparametric analysis of heterogeneous cell populations on a cell-by-cell basis

5 Main Components of a Cytometer

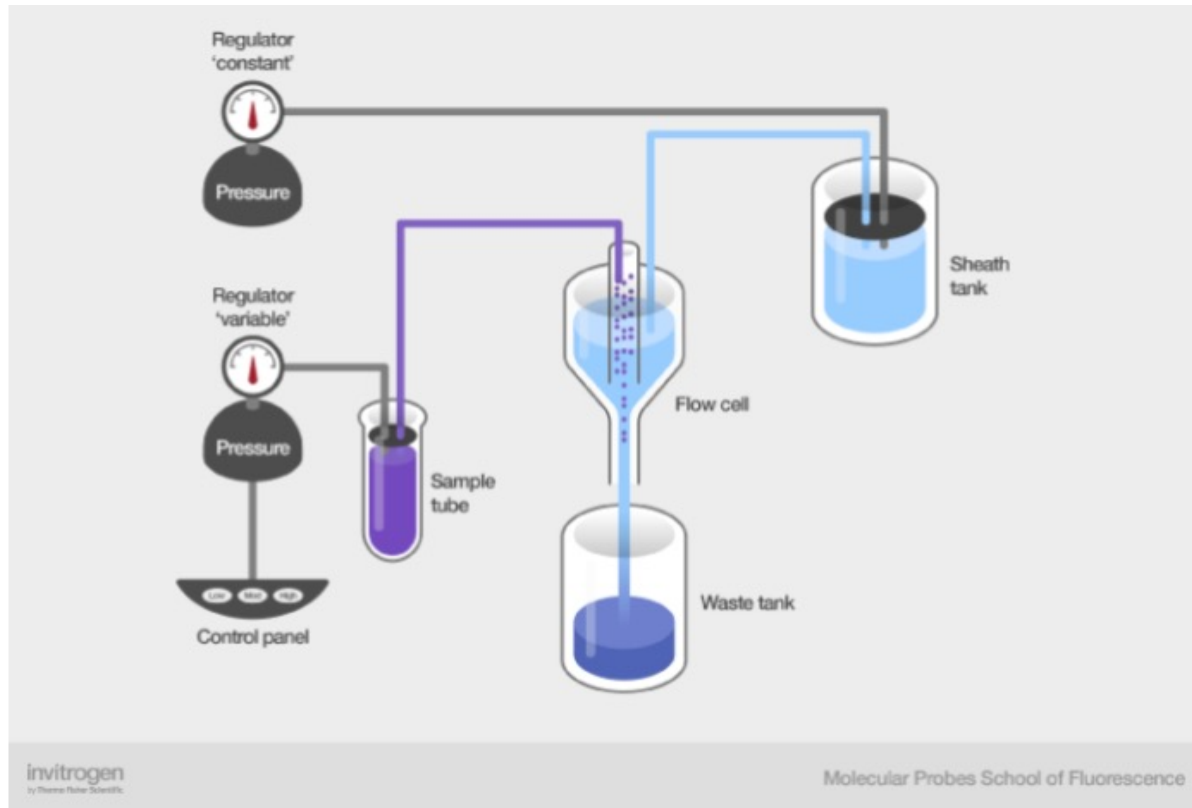
1. Fluidics
2. Lasers
3. Optics
4. Detectors
5. Electronics

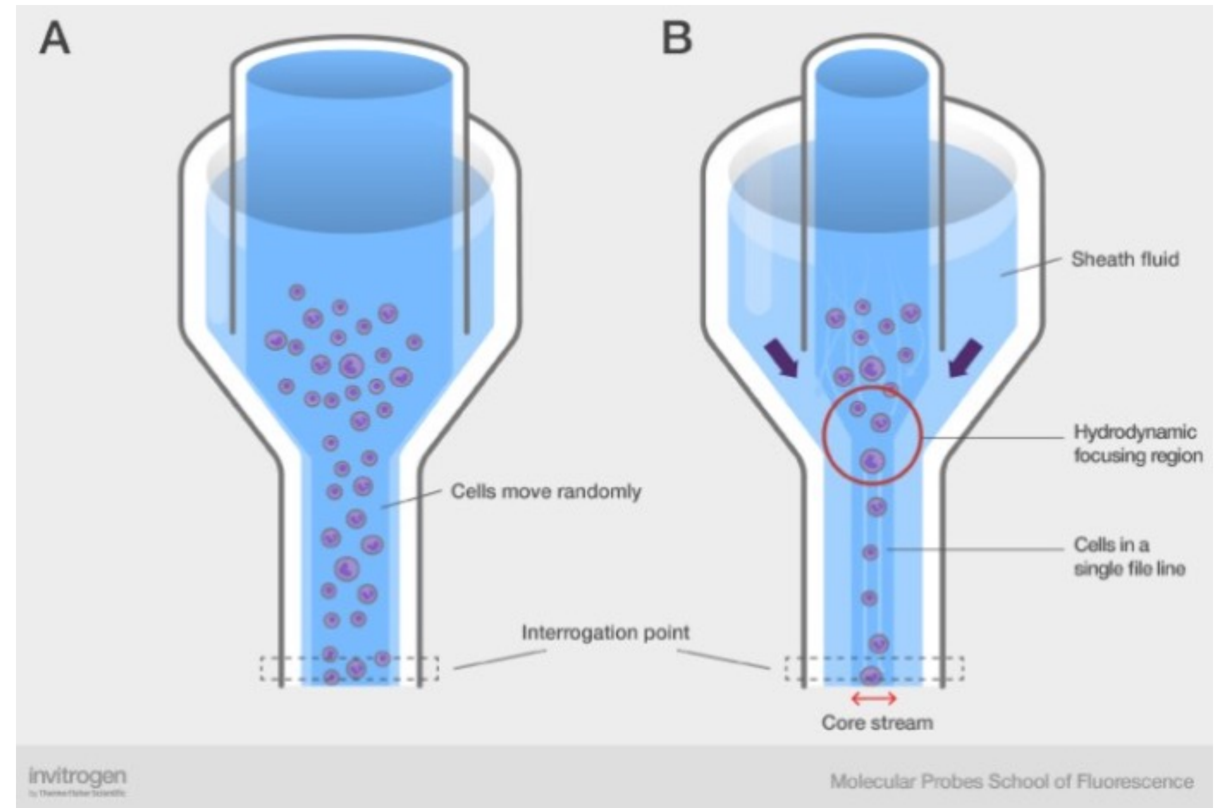




Fluidics

Position cells to flow one-by-one past the laser beam



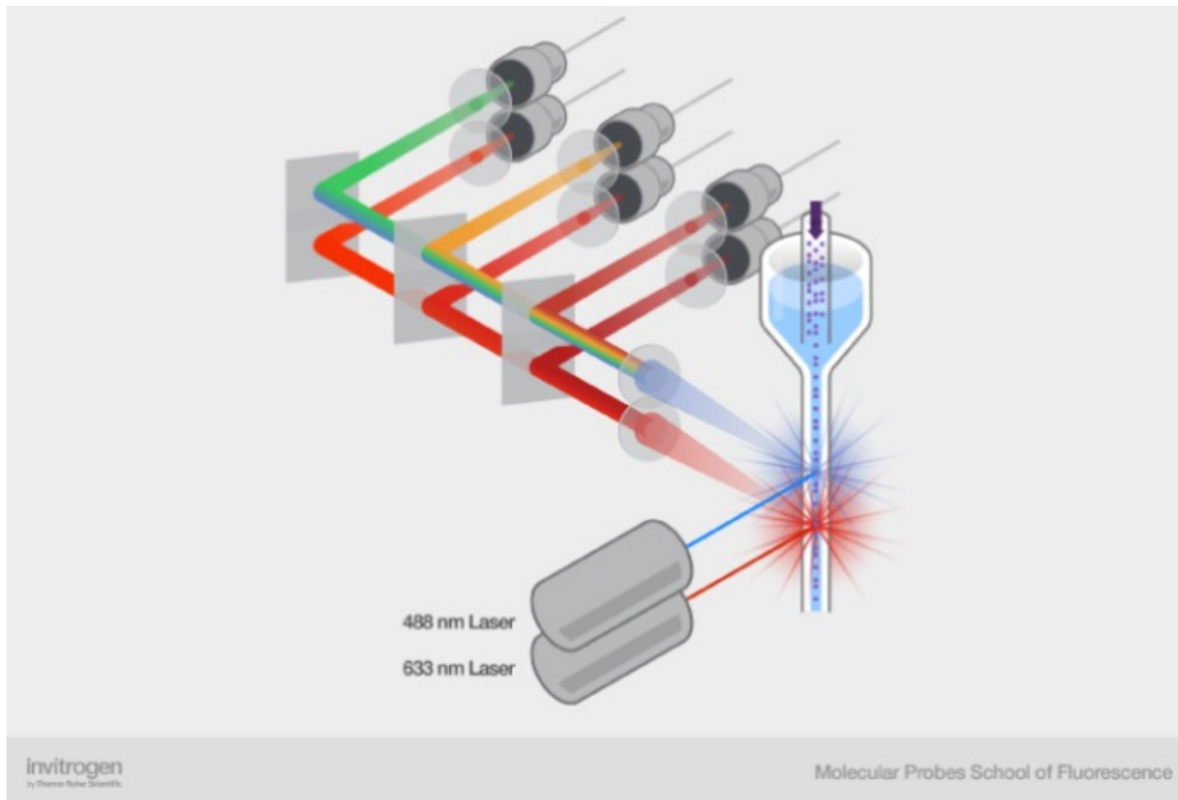


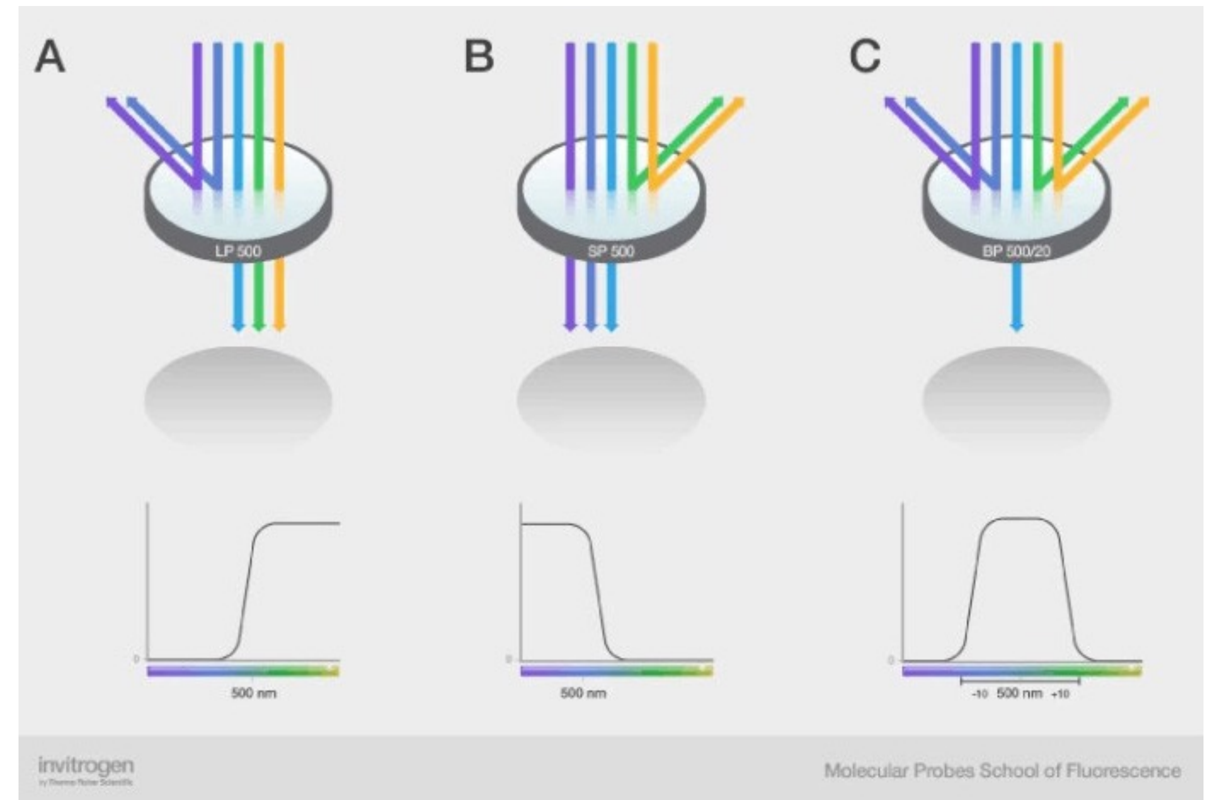
Lasers

Excitation sources used to excite fluorophores and for light scatter

Optics

Filters are used to separate the light emission from different fluorophores and direct towards detectors.





Detectors

Photodetectors transform light into electrical current

Types of photodetectors used in cytometers:

Photodiodes

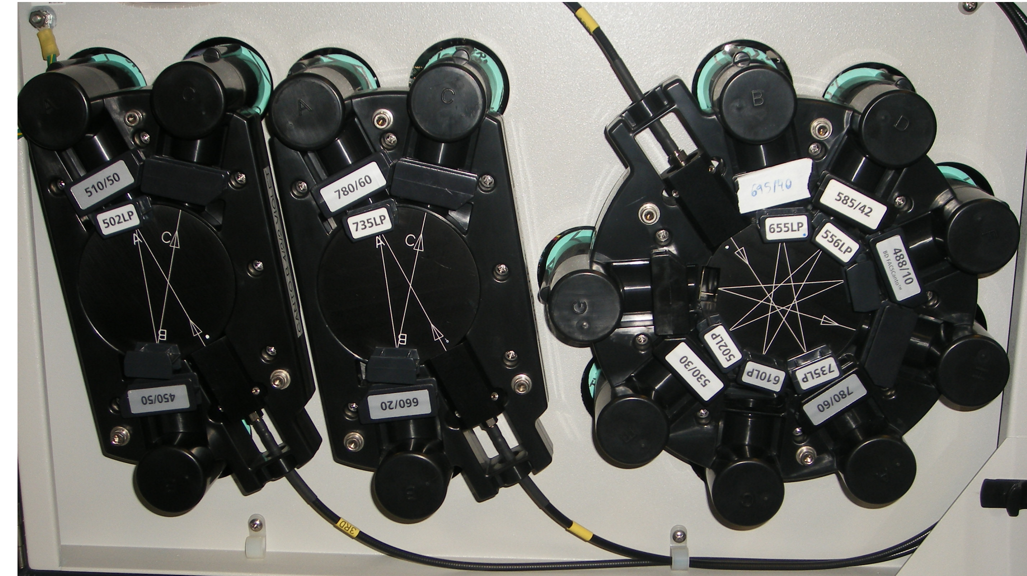
- Forward scatter (used for strong light signals)
- Avalanche Photodiodes APD (Auroras – spectral cytometers)

Photomultiplier tubes (PMT)

- Used for weak light signals
- Side scatter and all fluorescence parameters

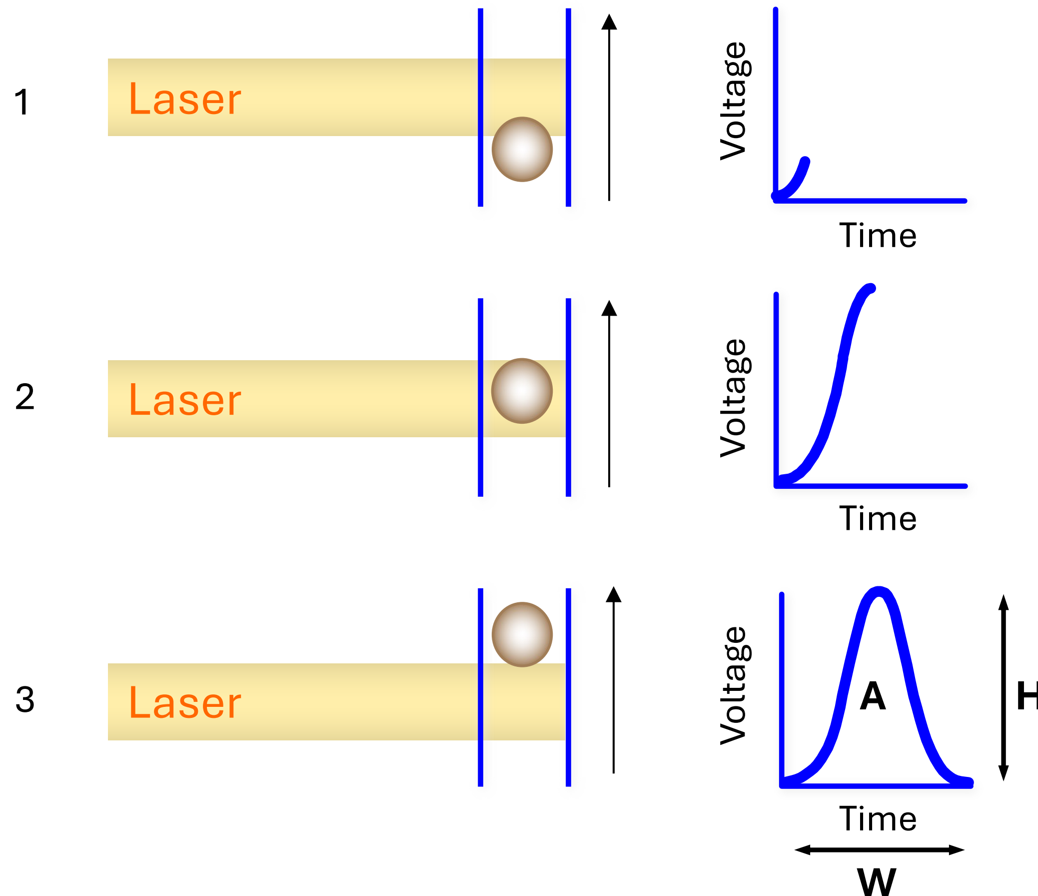
Charge Coupled Device (CCD)

- Contains many light sensitive areas
- These areas give individual signals to form an image of fluorescence output



Electronics

Signal Processing - voltage pulse (A, H, W)



- **H**eight = The intensity of the particle as it passes through the laser
- **W**idth = Time it takes for a particle to pass through laser
- **A**rea = Calculated by integration and represents the whole pulse

Light Scatter – Physical Property of Cells

Laser Beam

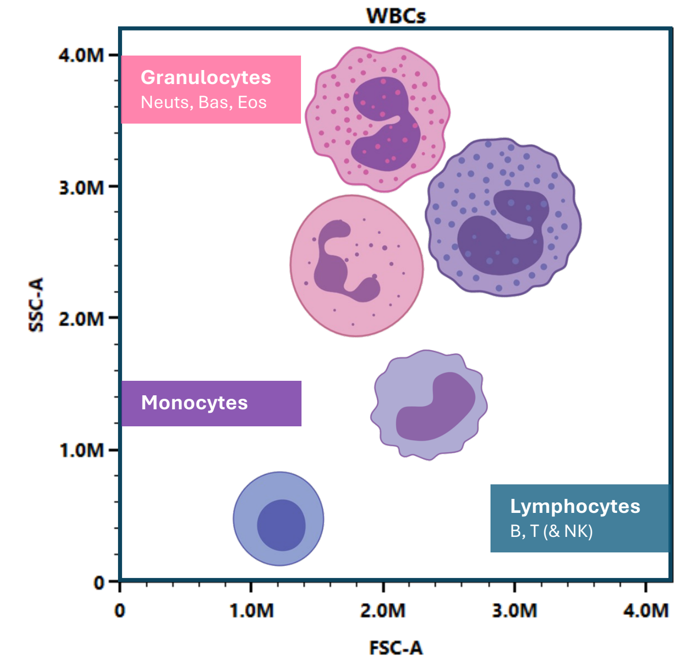
LIGHT

Cuvette

Flow Cell

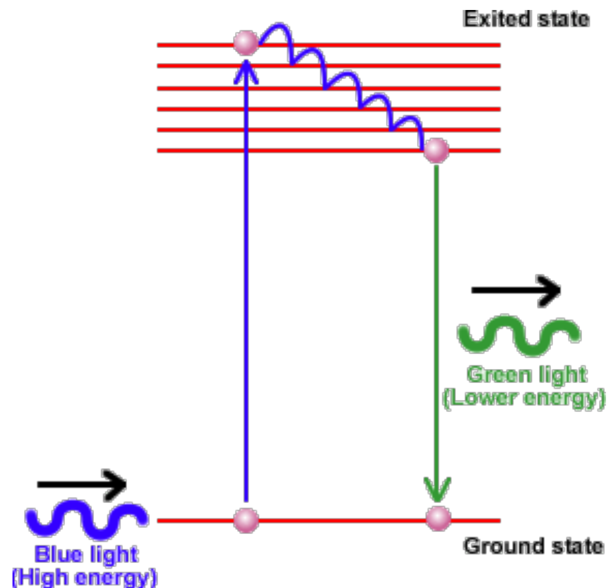
Forward Scatter (FSC)

Side Scatter (SSC)



What is Fluorescence?

- Fluorescence can be either endogenous or exogenous
- Fluorescent compounds absorb energy (photons) from an excitation source



1. Electrons enter an “excited” state – which is unstable
2. Electrons decay rapidly to ground state
3. Upon decay, photons of a longer wavelength are emitted

Fluorophores – Excitation/Emission

Every fluorophore has distinctive excitation and emission curves

Excitation and emission curves of PE

561

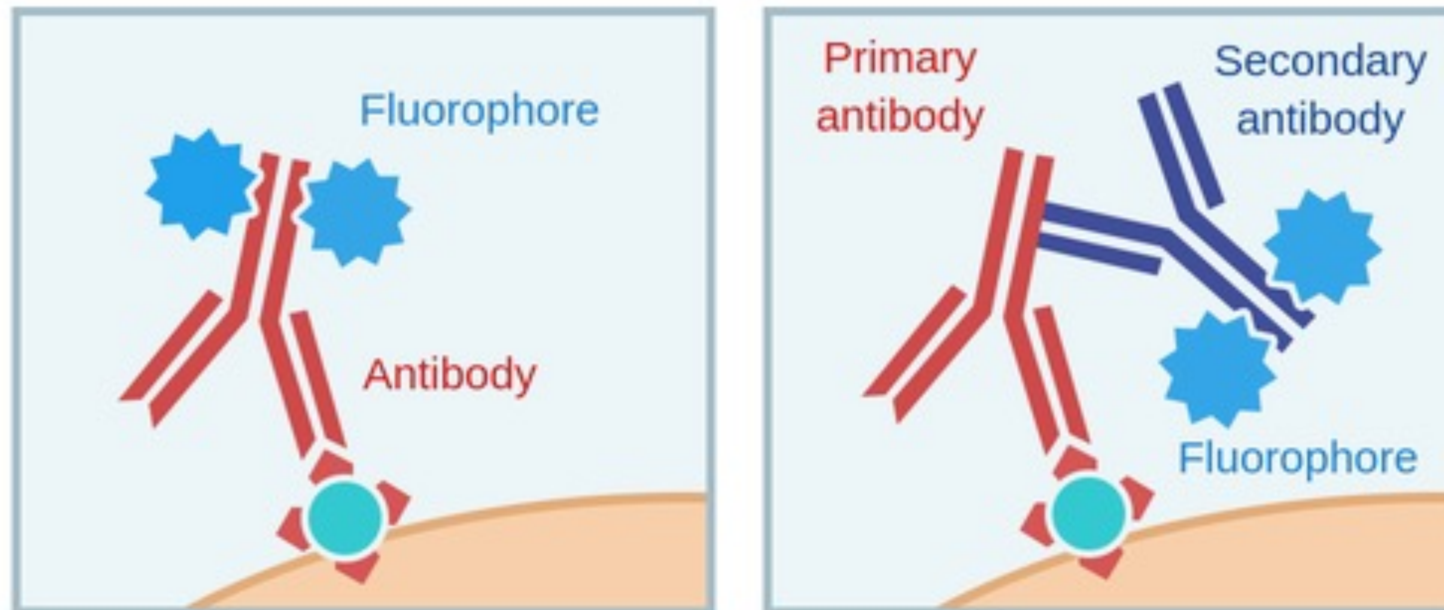
100
80
60
40
20
0

350 400 450 500 550 600 650 700 750

Wavelength (nm)

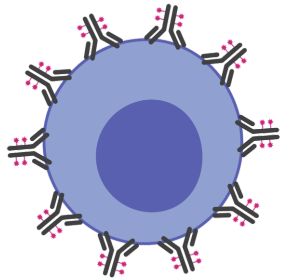
Attaching Fluorescence to Cells

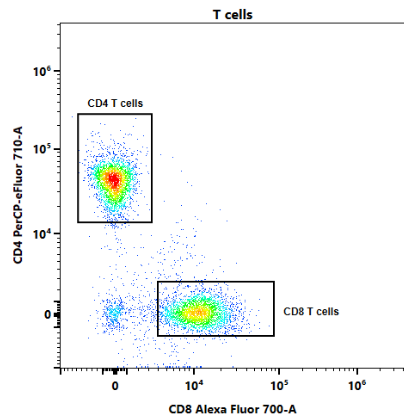
- To specifically bind fluorescent molecules to cellular targets we conjugate them to monoclonal antibodies
- We use these antibodies to “stain” cells with fluorescence
- Antibodies can target antigens on the cell surface or inside the cell or even the nucleus



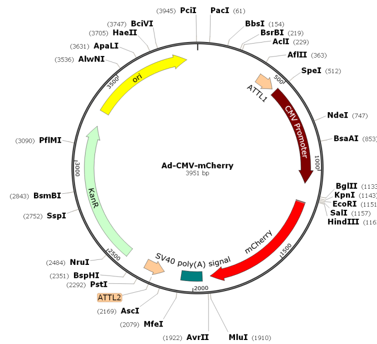
Sources of Fluorescence

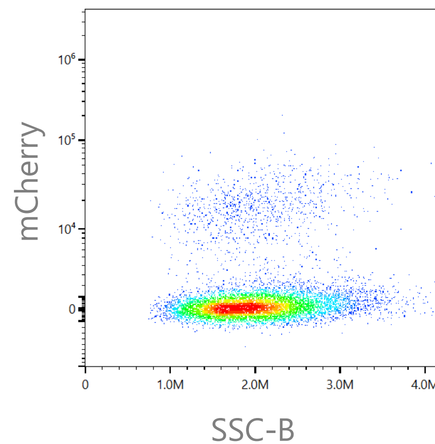
Fluorochromes



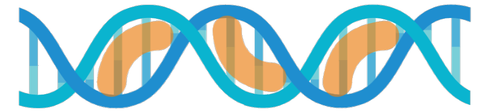


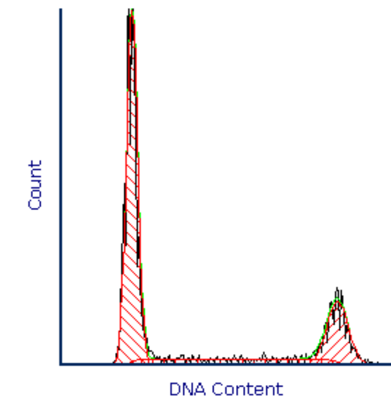
Fluorescent Proteins



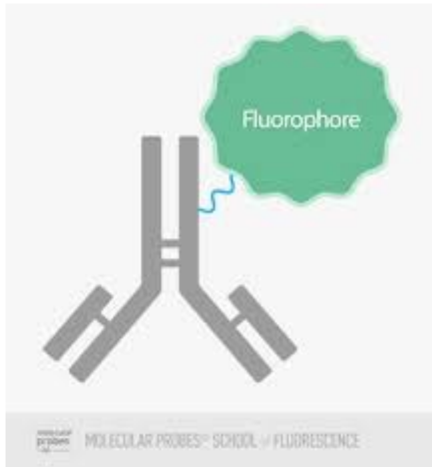


Fluorescent Dyes



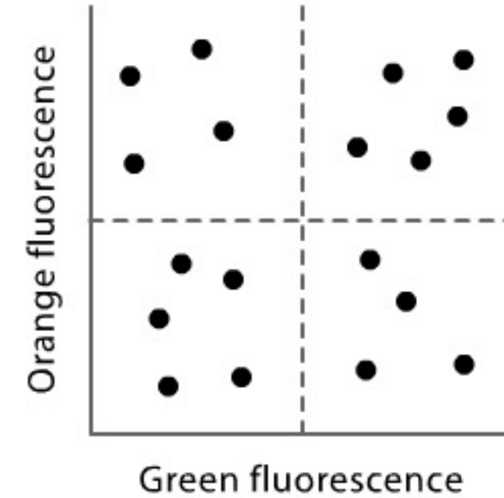


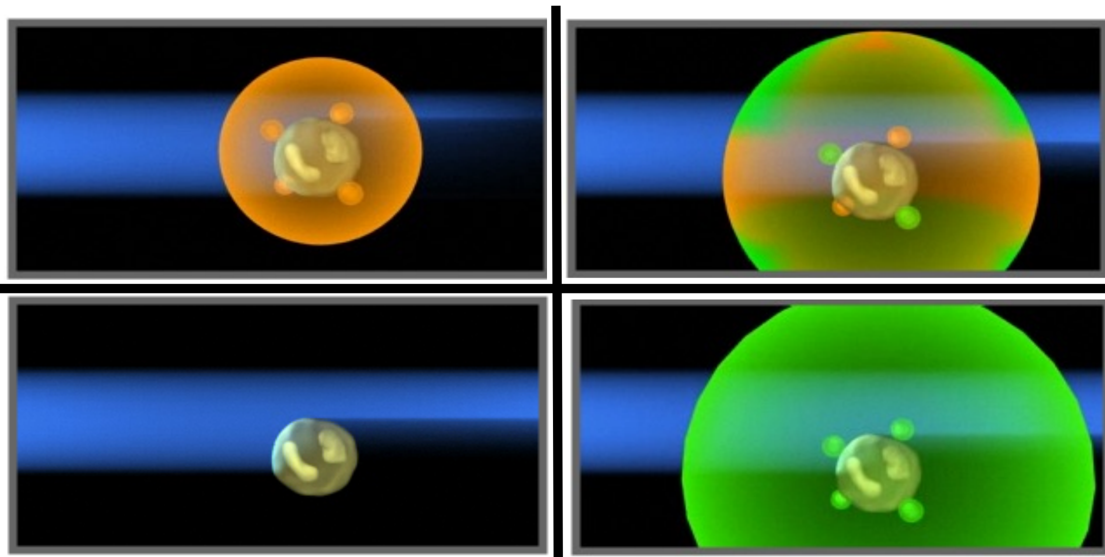
How Are Cells Visualised?

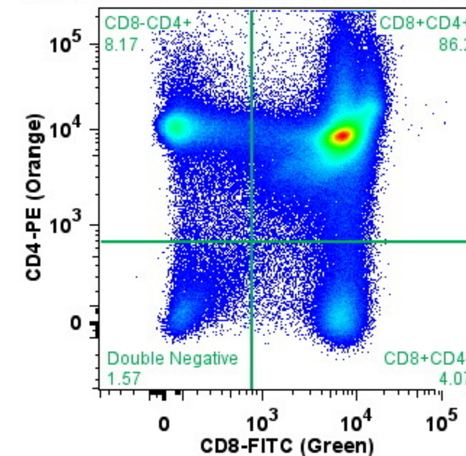


Example: In Thymus

- Run unstained sample first to set background
- CD4-AlexaFluor 488 (Green)
- CD8-PE (Orange)



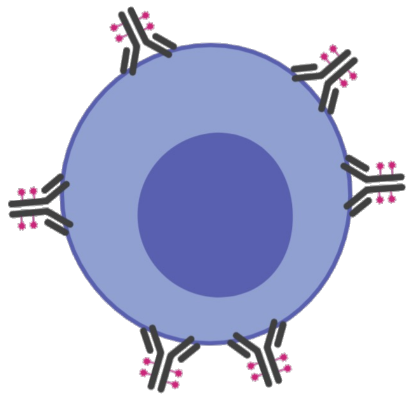


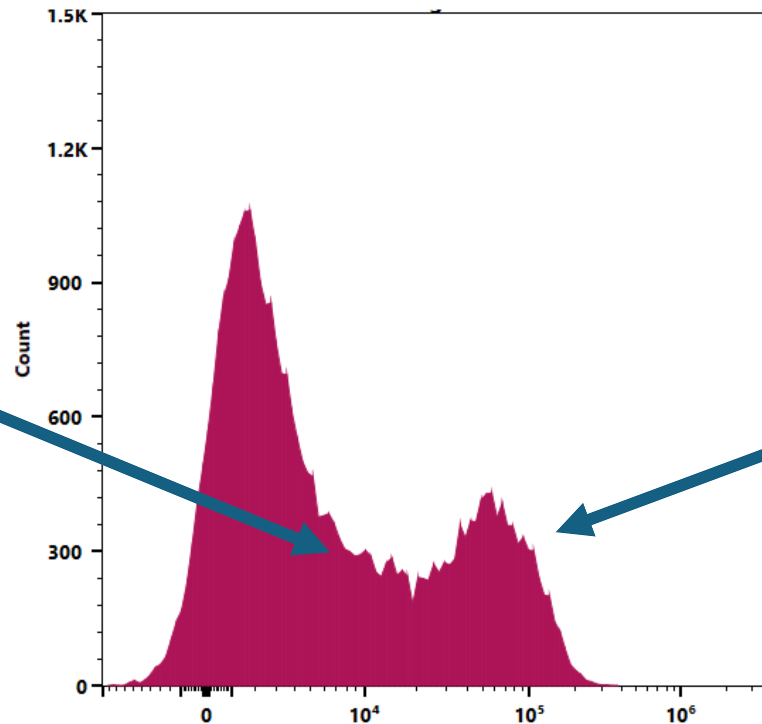


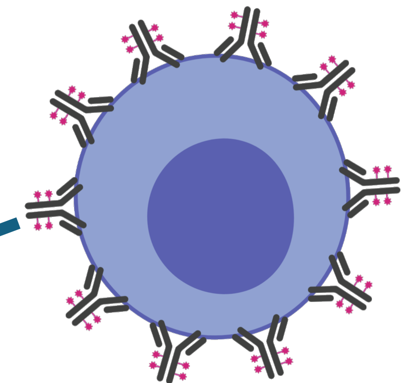
Images from Life Technologies FCM Tutorials

Fluorescence Intensity

Fluorescent dye/protein or antigen abundance in or on the cell is proportional to the fluorescence level detected



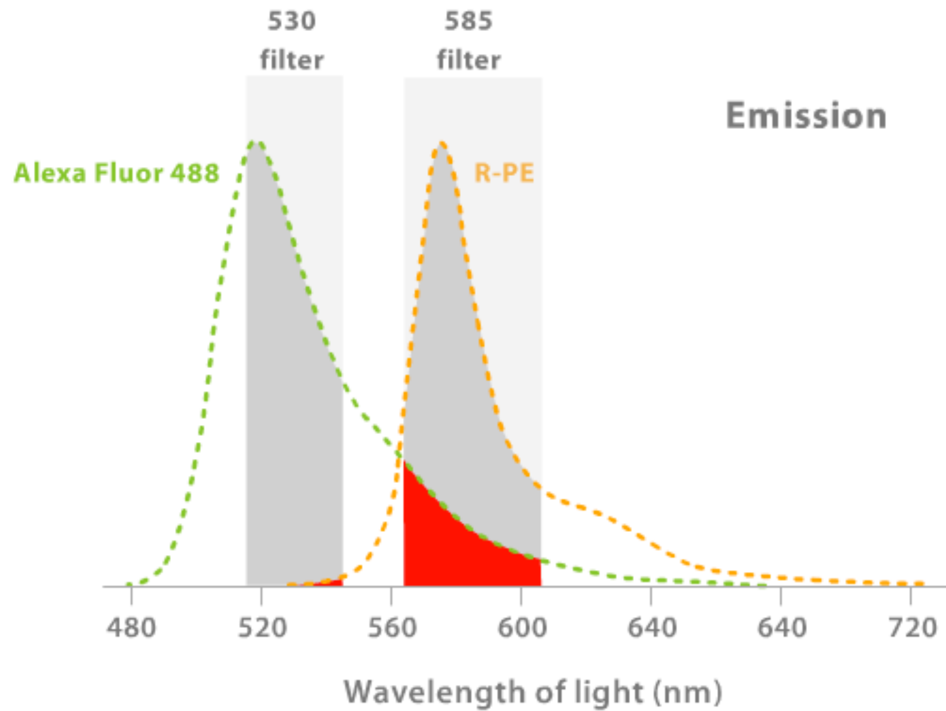


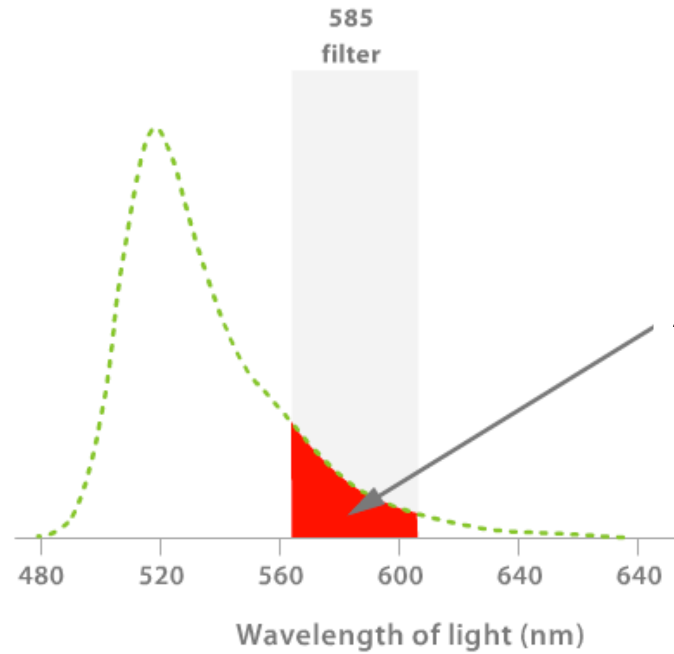


Dim Fluorescence
= lower antigen density

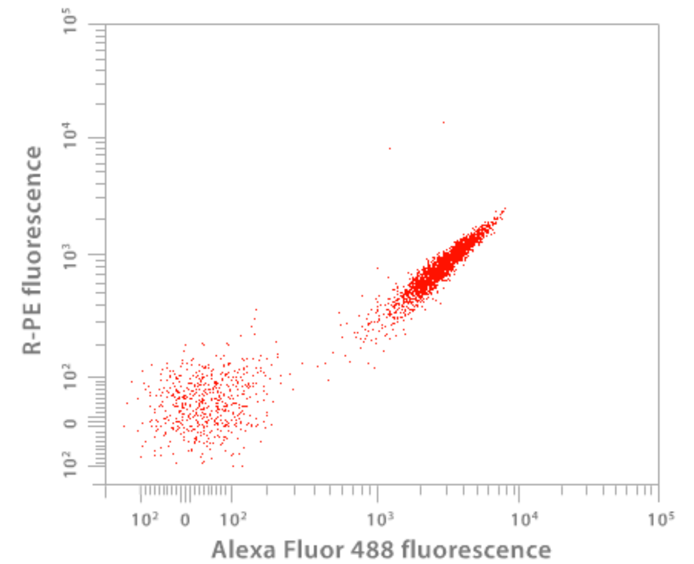
Bright Fluorescence
= higher antigen density

Compensation

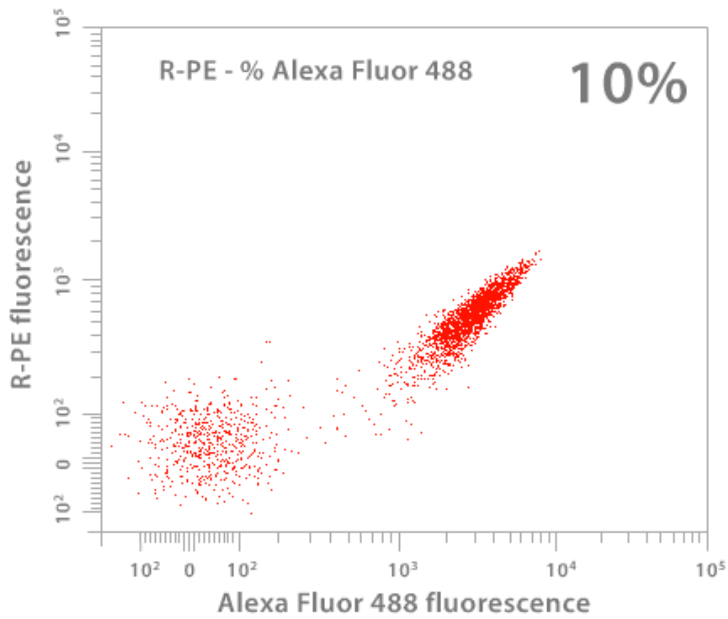


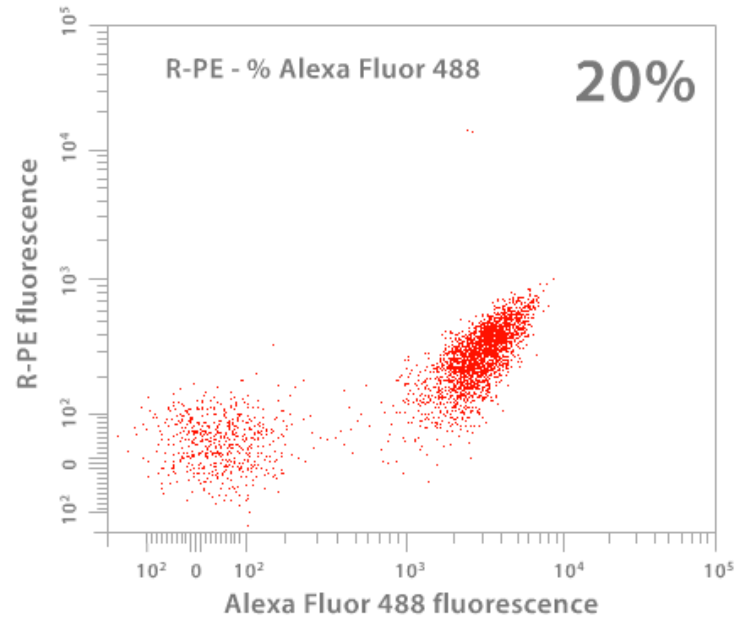


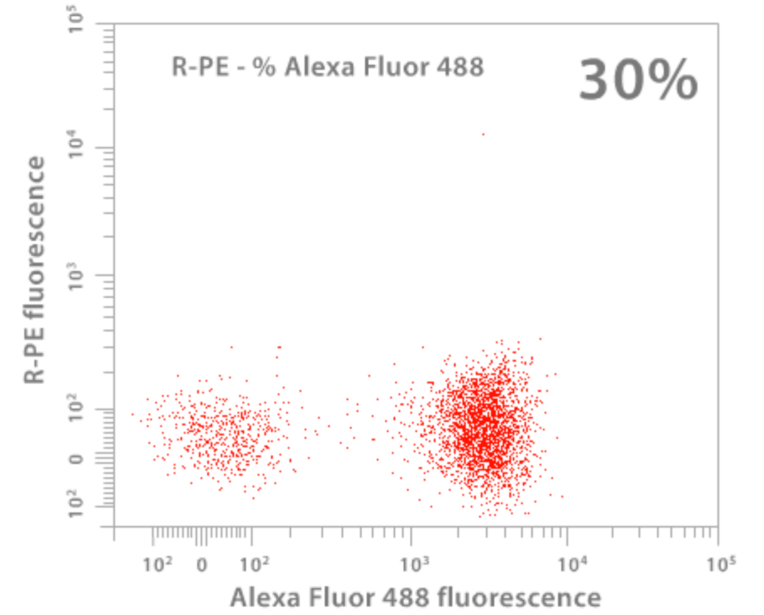
Alexa Fluor 488 overlap into PE detector



Compensation

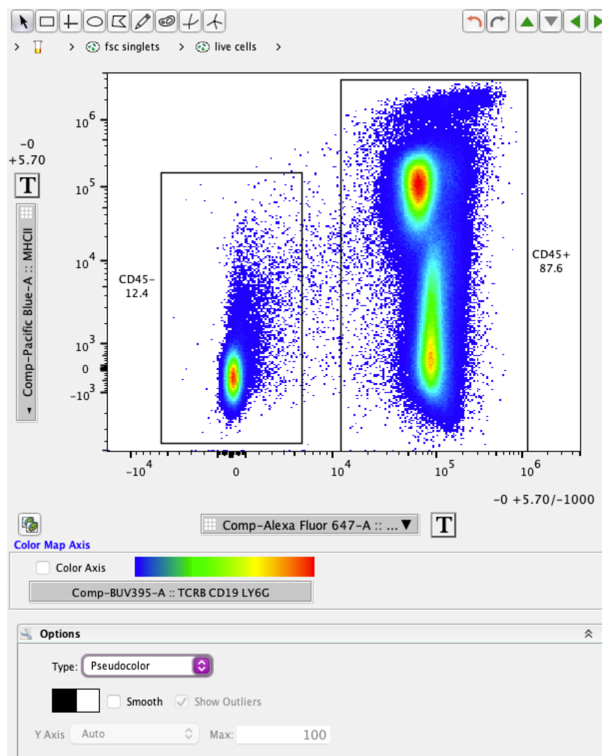


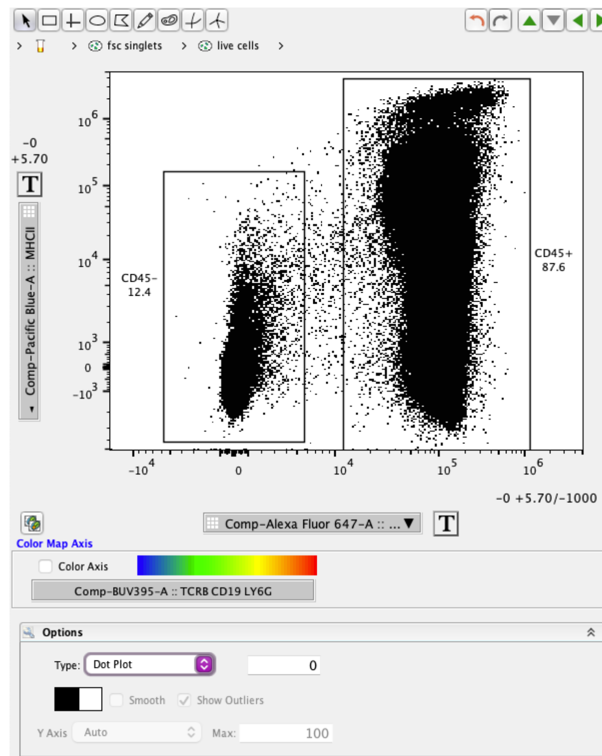


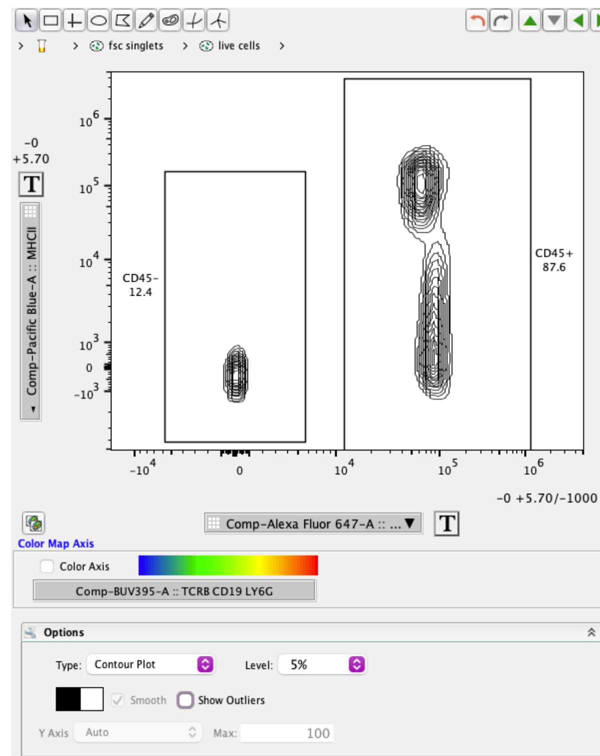


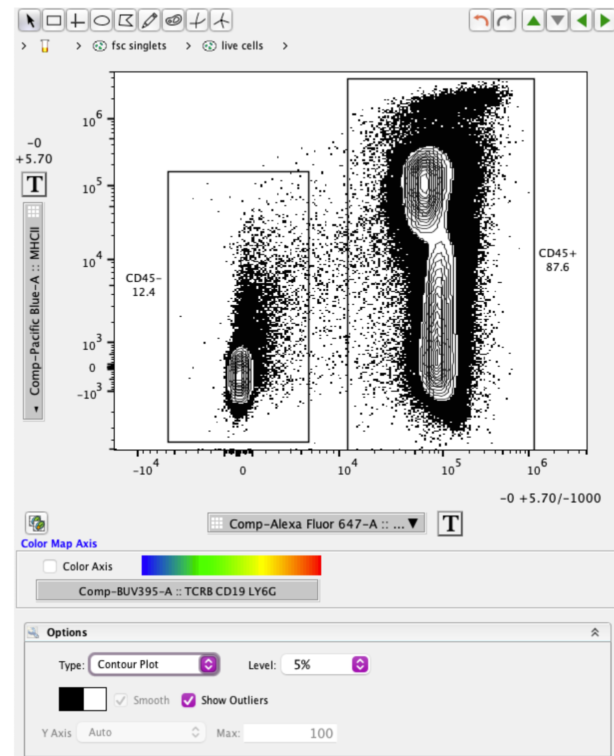
Bivariate Plots

In FCM we talk about “events” rather than cell number, because there is a chance that not everything in a flow cytometry file came from a cell, **dark current, debris etc.**
10,000 events might not actually be exactly 10,000 cells (but it’s close).









Analysing Data – Gating

- A “gate” may be defined as an electronic window encompassing a given region of a distribution delineated by upper and lower limits
- FlowJo's hierarchical gating method automatically designates AND Boolean gates by showing one subpopulation indented and underneath another in the Workspace window

Name	%	Count
Specimen_001_p1 bm2.fcs		492308
Lymphocytes	75.5	371883
Singlets	99.5	370017
Live	97.4	360316
B220+	14.4	51798
IgM+ IgD+	18.5	9578
IgM+ IgD-	22.6	11682
IgM- IgD+	2.56	1327
IgM- IgD-	21.6	11203
CD4+	1.11	4011
PD-1	9.22	370
CXCR5+	1.08	4

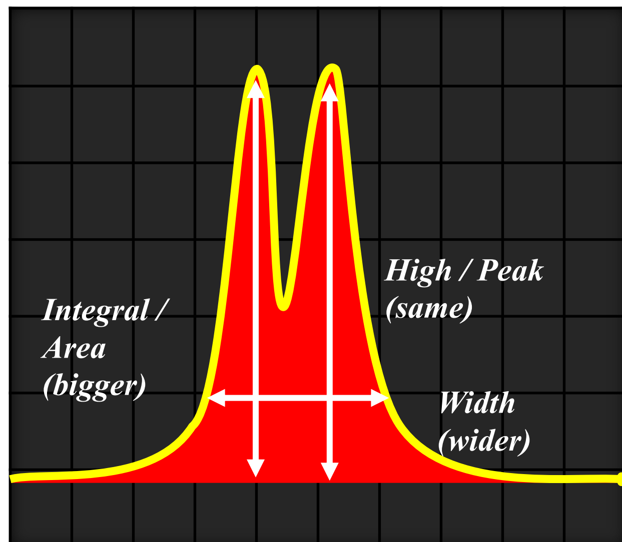
Flow Cytometry in Clinical Diagnosis, 4th Edition

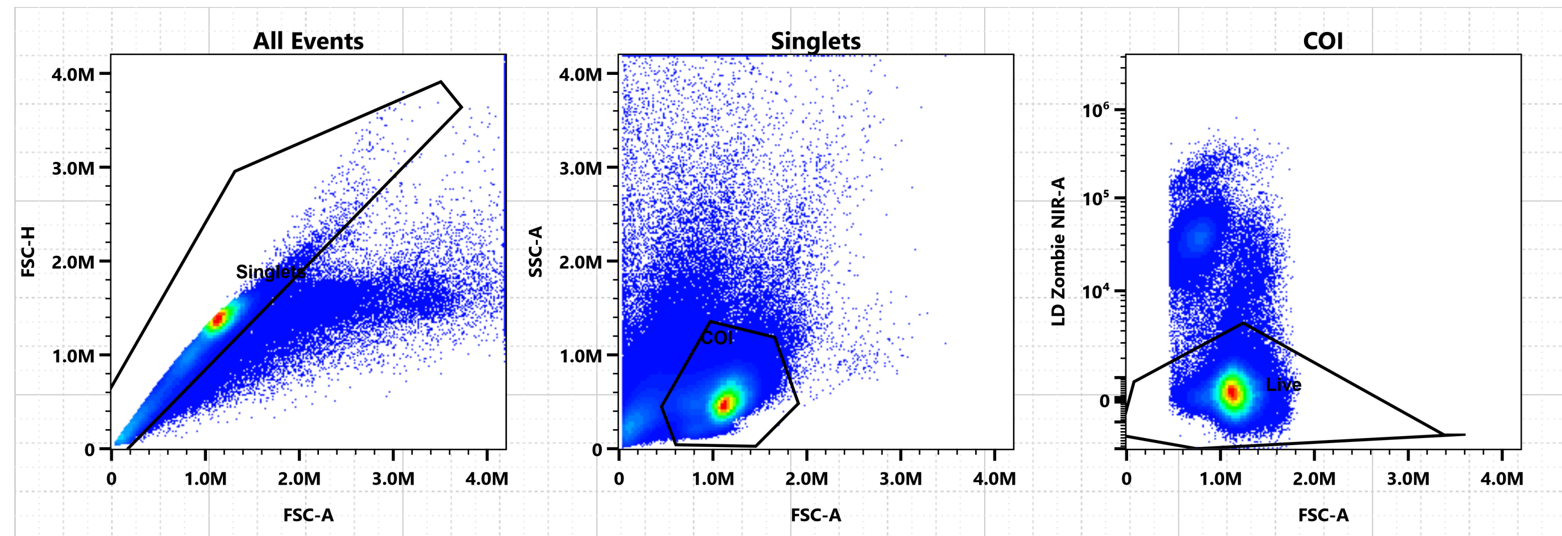
Gating Strategies

1. Use parameters Area (A), Height (H) and Width (W) to gate out doublets

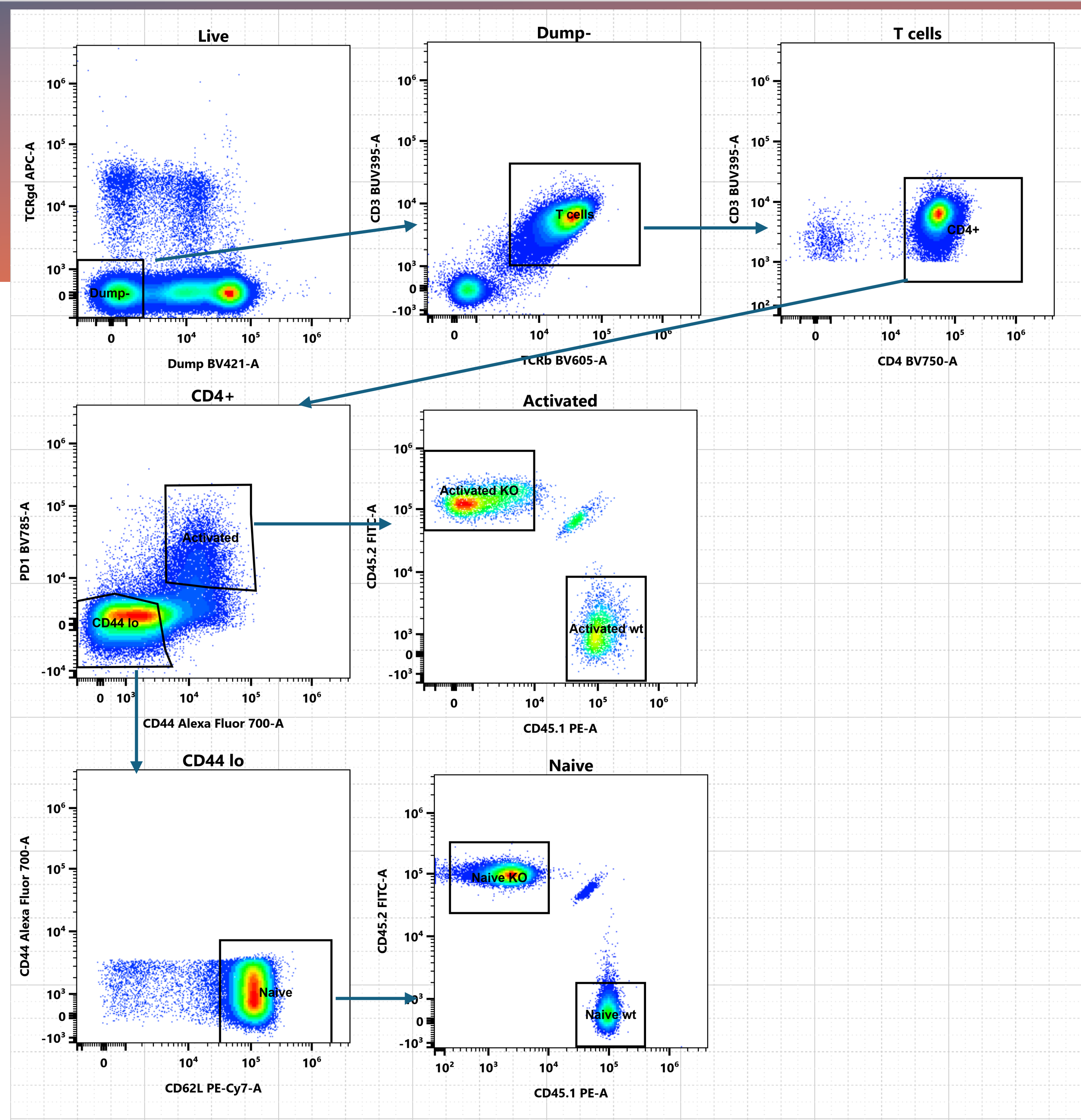
2. Cells of interest gate (typically SSC-A vs FSC-A): gate out small debris and gate in on cells of interest (e.g., lymphocytes, DCs etc..)

3. Live cells (viability dye negative events)





Gating Strategies



Gating Controls

Fluorescence Minus One (FMO)

A control containing all fluorescent antibodies in the panel except one