The Basics of Flow Cytometry

FACS Core Facility

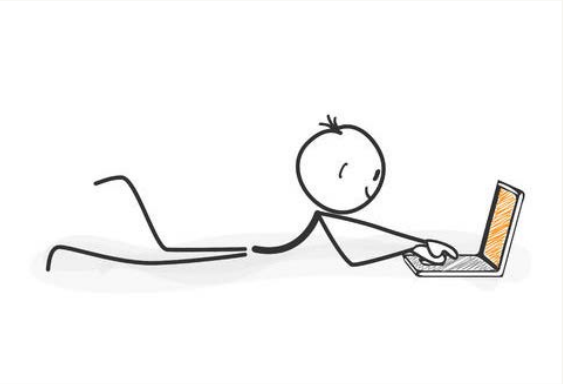
Janine Bögli, Biozentrum, 29. January 2018

The functions of the FACS Core Facility

Centralization of equipment and expertise

Train users

Sorter operation



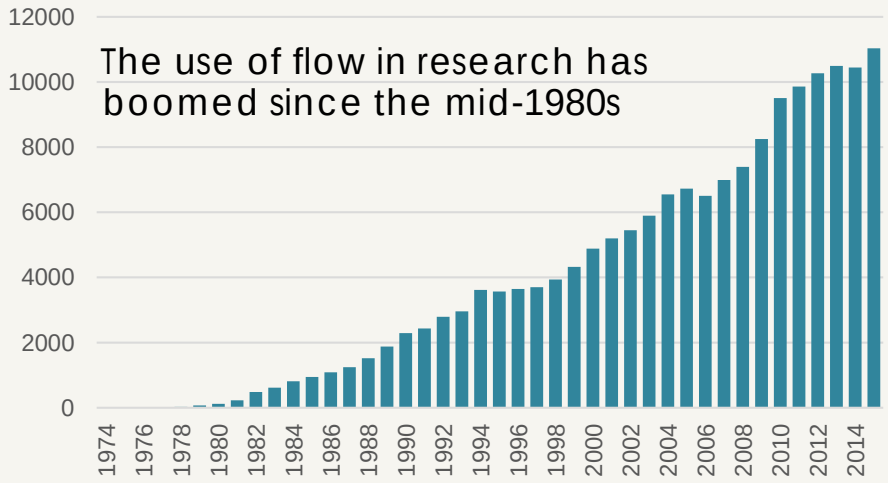
Advice and troubleshooting

Why is Flow Cytometry important?

Why we use it…

- Analyze thousands of cells in a short time
- Statistical information obtained quickly
- Flexibility of data acquisition
- Ability to re-analyze

Publications citing 'flow cytometry'



… and what it is used for

- Immunophenotyping
- DNA cell cycle/tumor ploidy
- Membrane potential
- Ion flux
- Cell viability
- Intracellular protein staining
- pH changes
- Cell tracking and proliferation
- Sorting
- Redox state
- Chromatin structure
- Total protein
- Lipids
- Surface charge
- Membrane fusion/runover
- Enzyme activity
- Oxidative metabolism
- Sulfhydryl groups/glutathione
- DNA synthesis
- DNA degradation
- Gene expression

Plus many others!

Overview

- What is Flow Cytometry
- Fluorescence
- The basics of a flow cytometer
  - Fluidics
  - Optics
  - Electronics
- Data analysis
  - How does flow cytometry data look like
  - Gating
- Applications

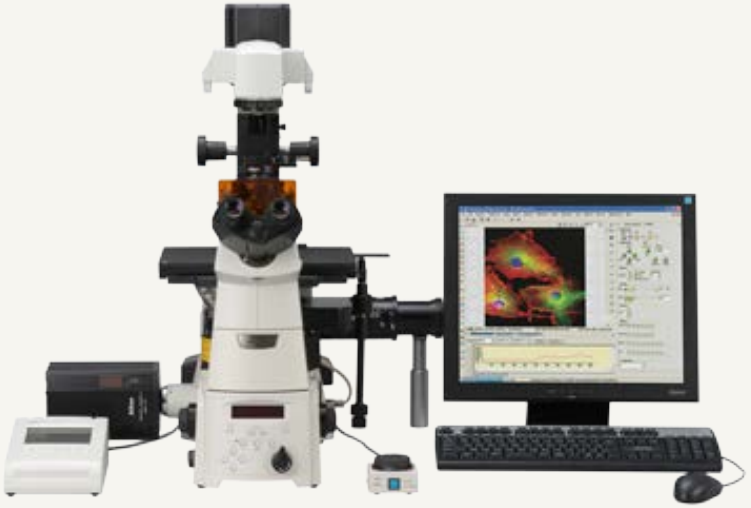
What is Flow Cytometry?

Cytometry

"kytos" Greek:
Hollow/cell

"Greek:
Measurement

The measurement can be substrate-based…



What is Flow Cytometry?

Flow Cytometry

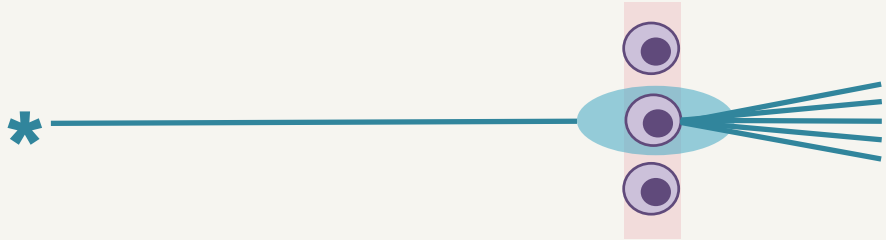
"kytos" Greek: Hollow/cell

"Greek: Measurement

...or flow-based.

What is Flow Cytometry?

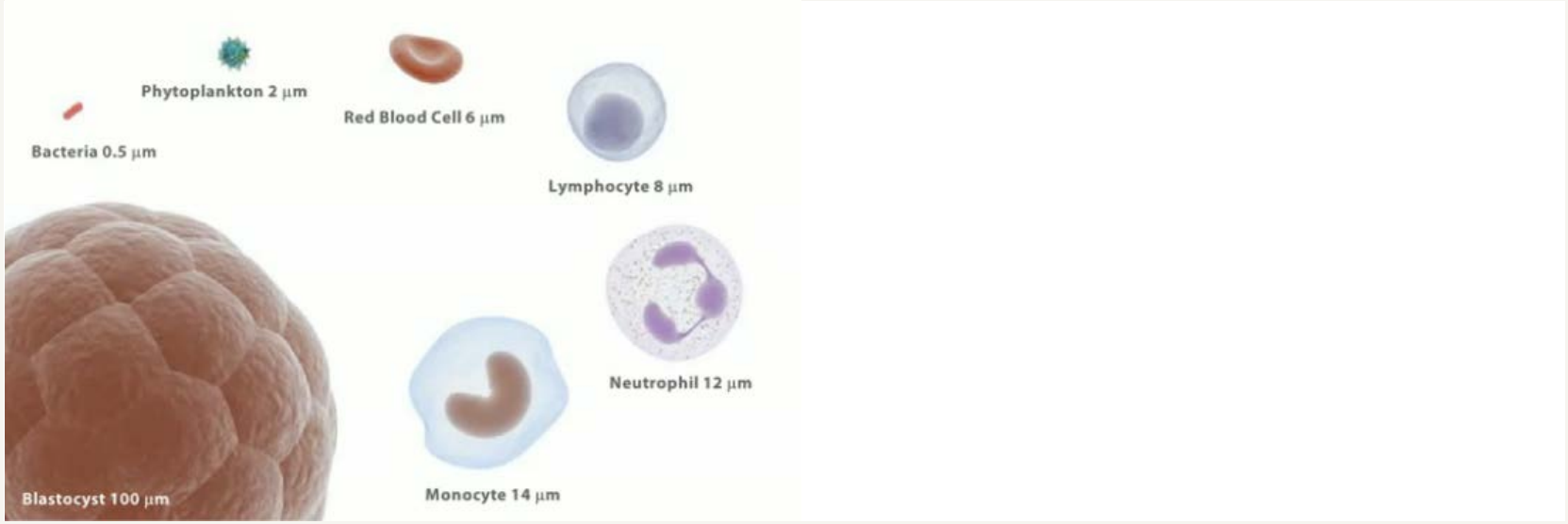
A means of measuring the physical and chemical characteristics of particles in a fluid stream as they pass one by one past a sensing point.



What can Flow Cytometry do?

Analyze light signals to:

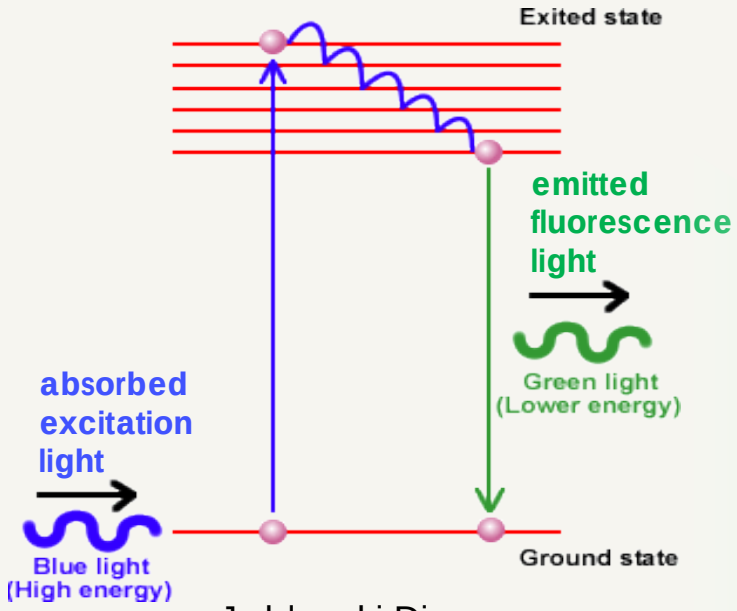
- Enumerate particles in suspension
- Evaluate 10^5 to 10^6 particles in less than 1 min
- Detection of rare cell populations
- Measure multiple parameters
- Sort single particles for subsequent analysis



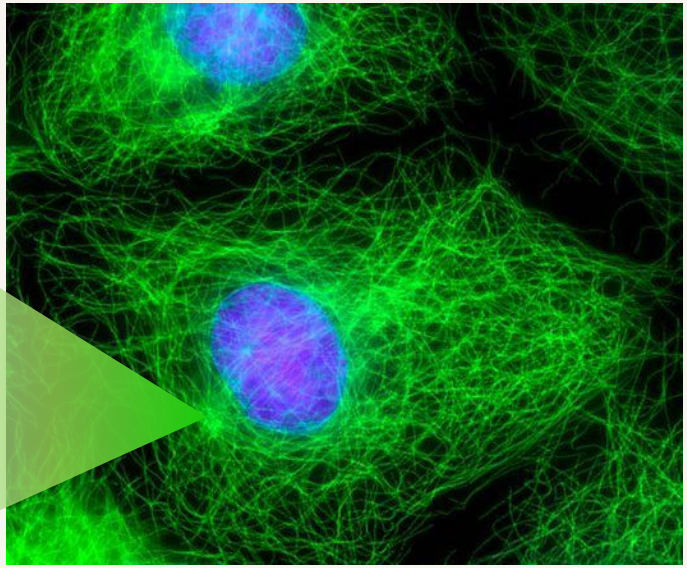
Fluorescence

- Intrinsic fluorescence
  - Inheritent molecules within the cell
  - Autofluorescence
- Extrinsic fluorescence
  - Added to the cells by investigators
  - Includes dyes and fluorescent proteins

What is Fluorescence?

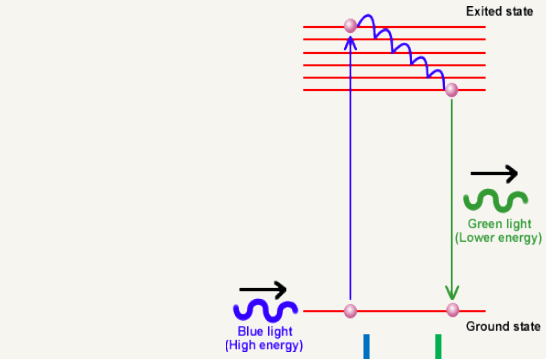


Jablonski Diagram

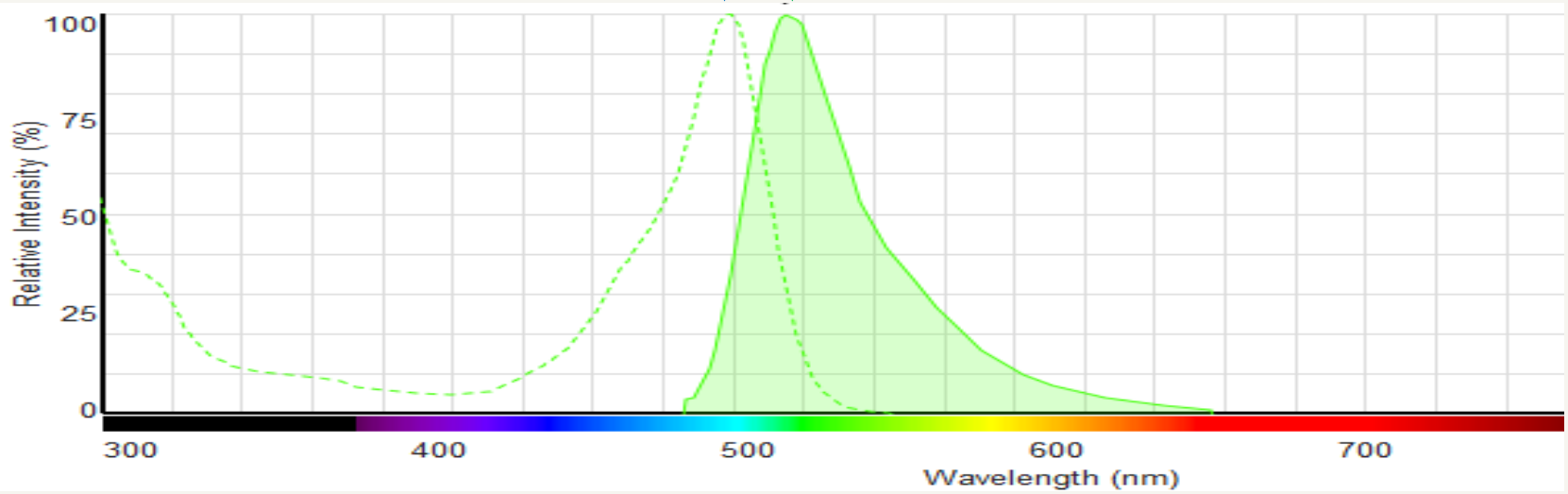


- Excitation (Absorbance) with one color
- Emission (Fluorescence) with a different color
- Fluorophore: the part of the molecule that is fluorescent
- Fluorochrome: the whole molecule (can have several fluorophores)

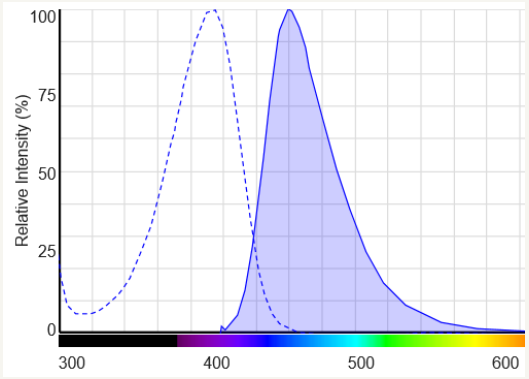
Concepts of Excitation and Emission



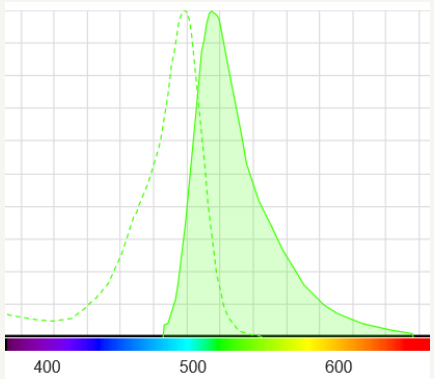
FITC Excitation FITC Emission



Fluorochromes

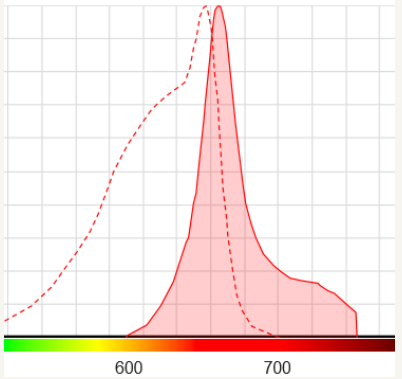


Pacific Blue



FITC

PE



APC

Make use of spectral viewers:

https://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

http://www.bdbiosciences.com/sg/research/multicolor/spectrum_viewer/index.jsp

Fluorescent Probes

Antibodies

FITC
Phycoerythrin
Allophycocyanin
PerCP
AlexaFluor dyes
PE-Cy5, PE-Cy7
BV, BUV

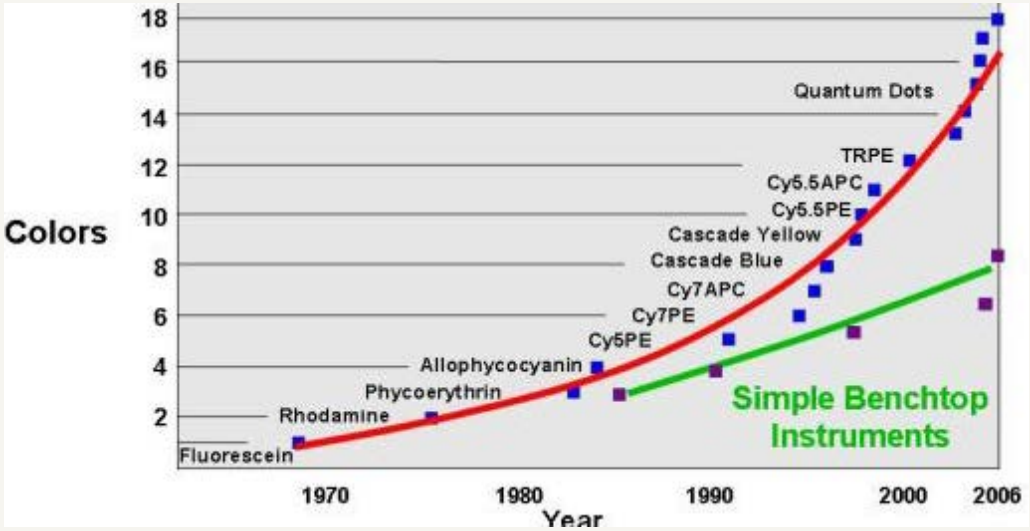
Fluorescent dyes

DAPI
Hoechst dyes
Propidium iodide
Acridine Orange
TO-PRO-3
DyeCycle dyes
SYTOX dyes

Fluorescent proteins

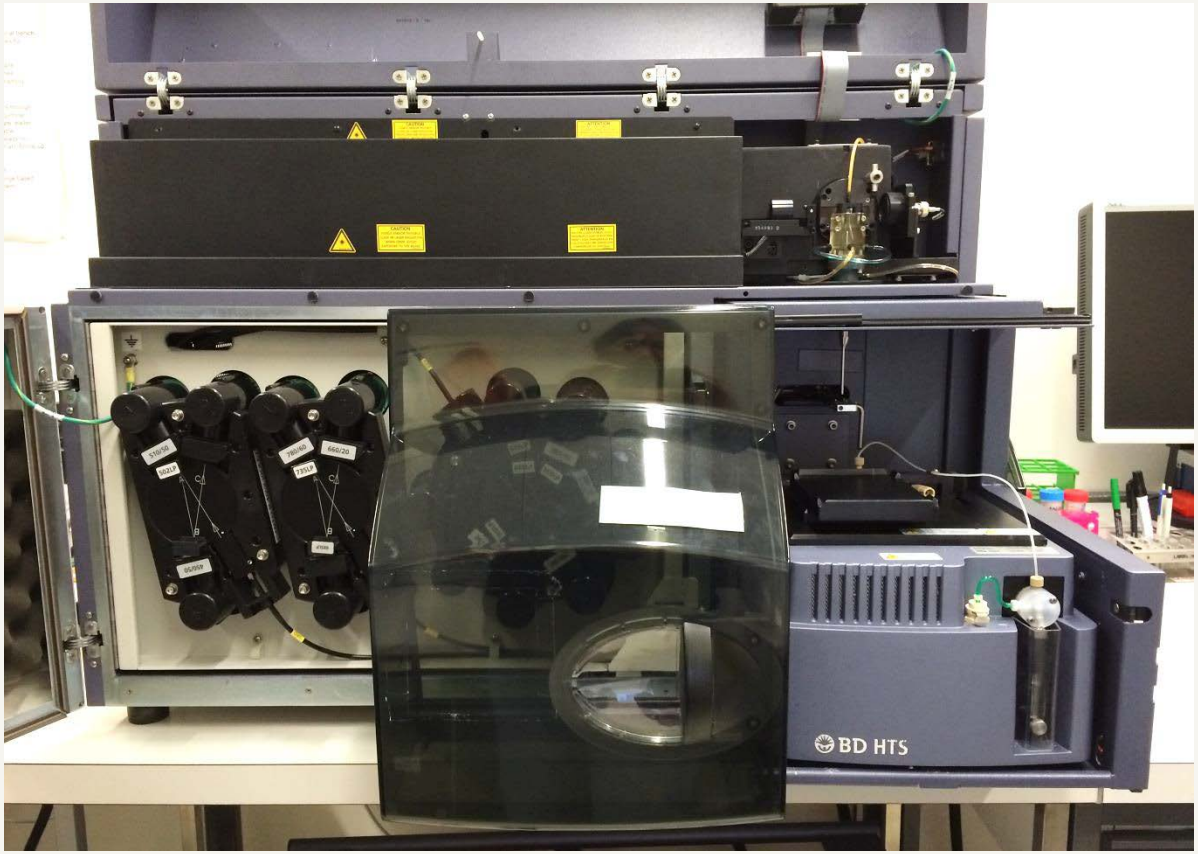
Cyan FP
Green FP
Yellow FP
Orange FP
Red FP
mCherry
mTomato

- Monoclonal antibodies
- Fluorochromes
- DNA, RNA and functional stains
- Computers and miniaturization of electronics
- Lasers



What is inside the Flow Cytometer?

The Many Parts of Flow



Basic components:

Fluidics Optics Electronics

The Basic Components

Fluidics

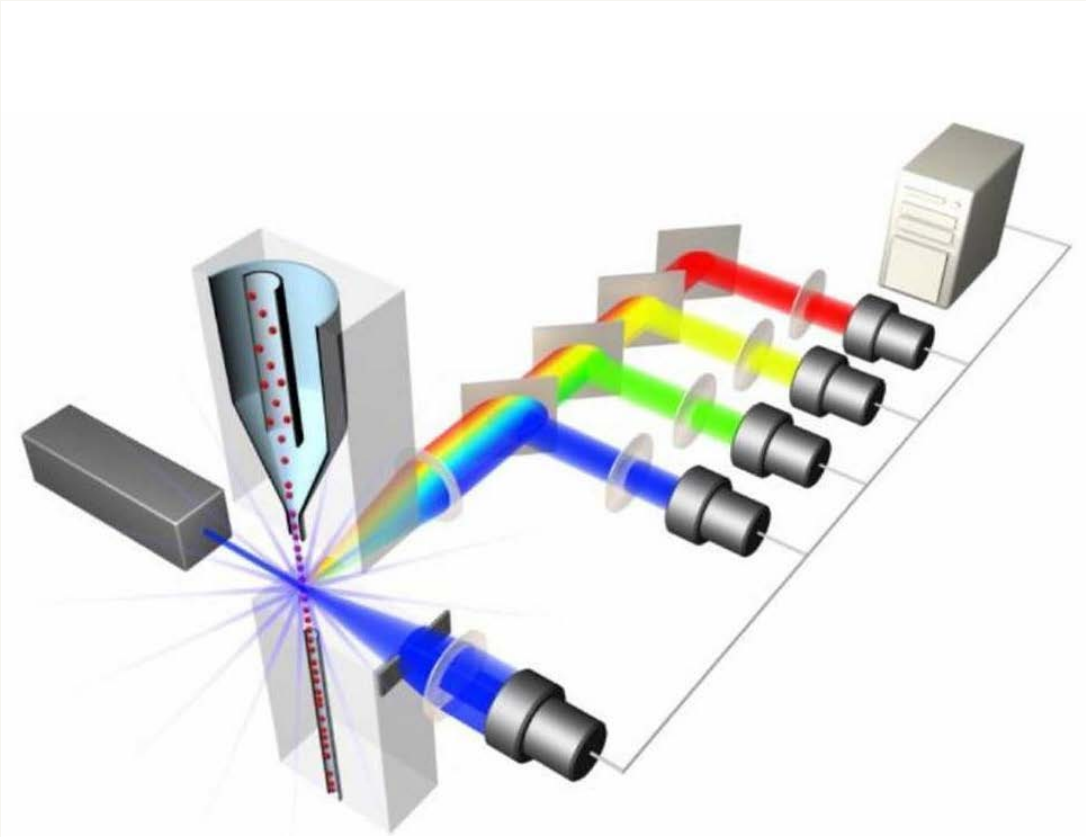
Cells in suspension flow in single-file through an illuminated volume where they scatter light and emit fluorescence…

Optics

…that is collected, filtered and converted…

Electronics

…to digital values that are stored on a computer.

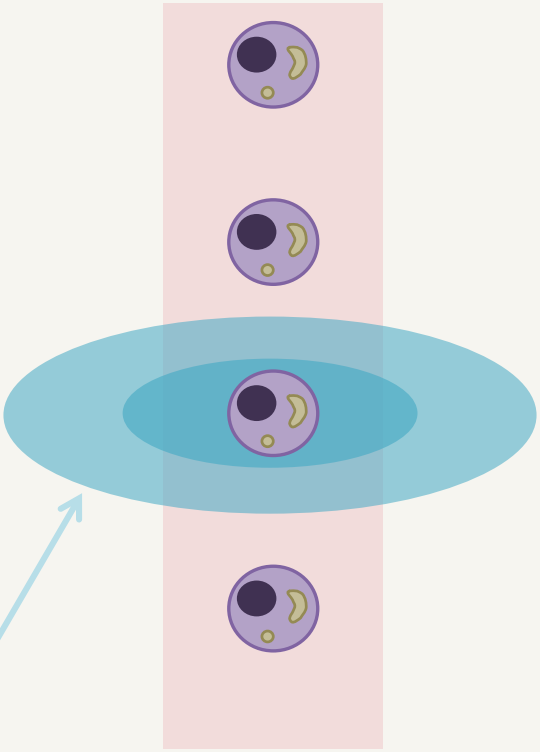


http://flow.csc.mrc.ac.uk/?page_id=5

Fluidics – Function

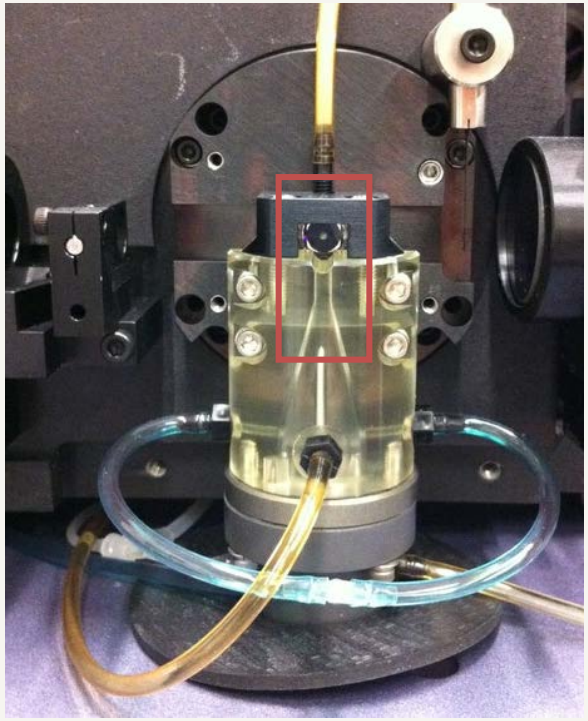
Move the cells through the cytometer and ensure that:

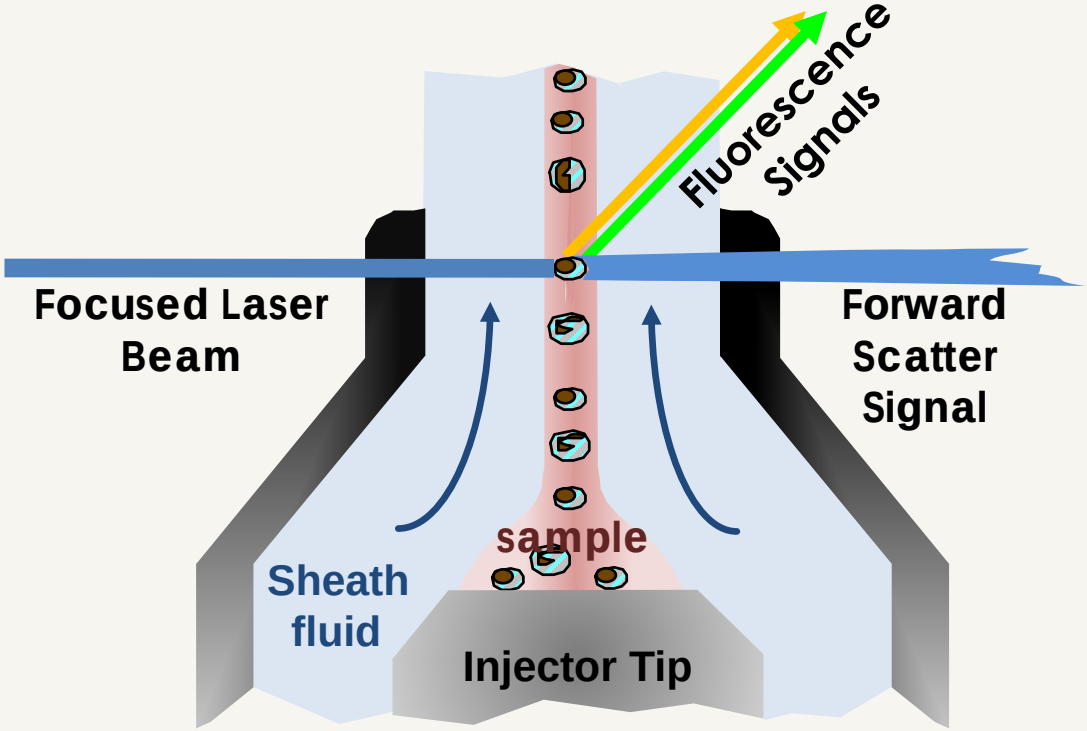
- Cell passes through the center of the light source
- Cells pass one by one
- Flow is smooth
  - no pulsing
  - no turbulence
- More than one way to do this



Interrogation point
Illumination volume
Intercept
Laser intersection point

Fluidics – Hydrodynamic Focusing

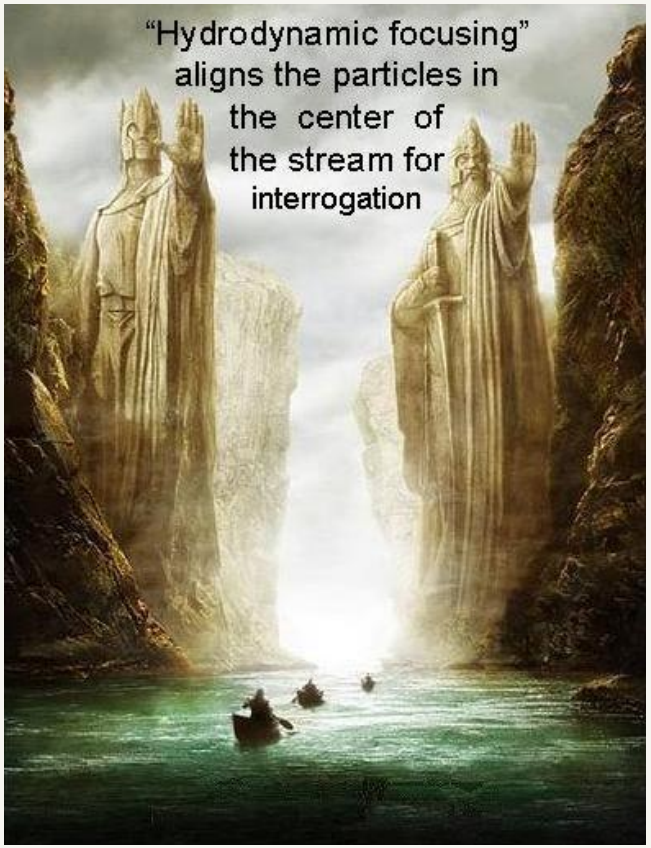




Adapted from Purdue University Cytometry Laboratories

Fluidics – Laminar Flow

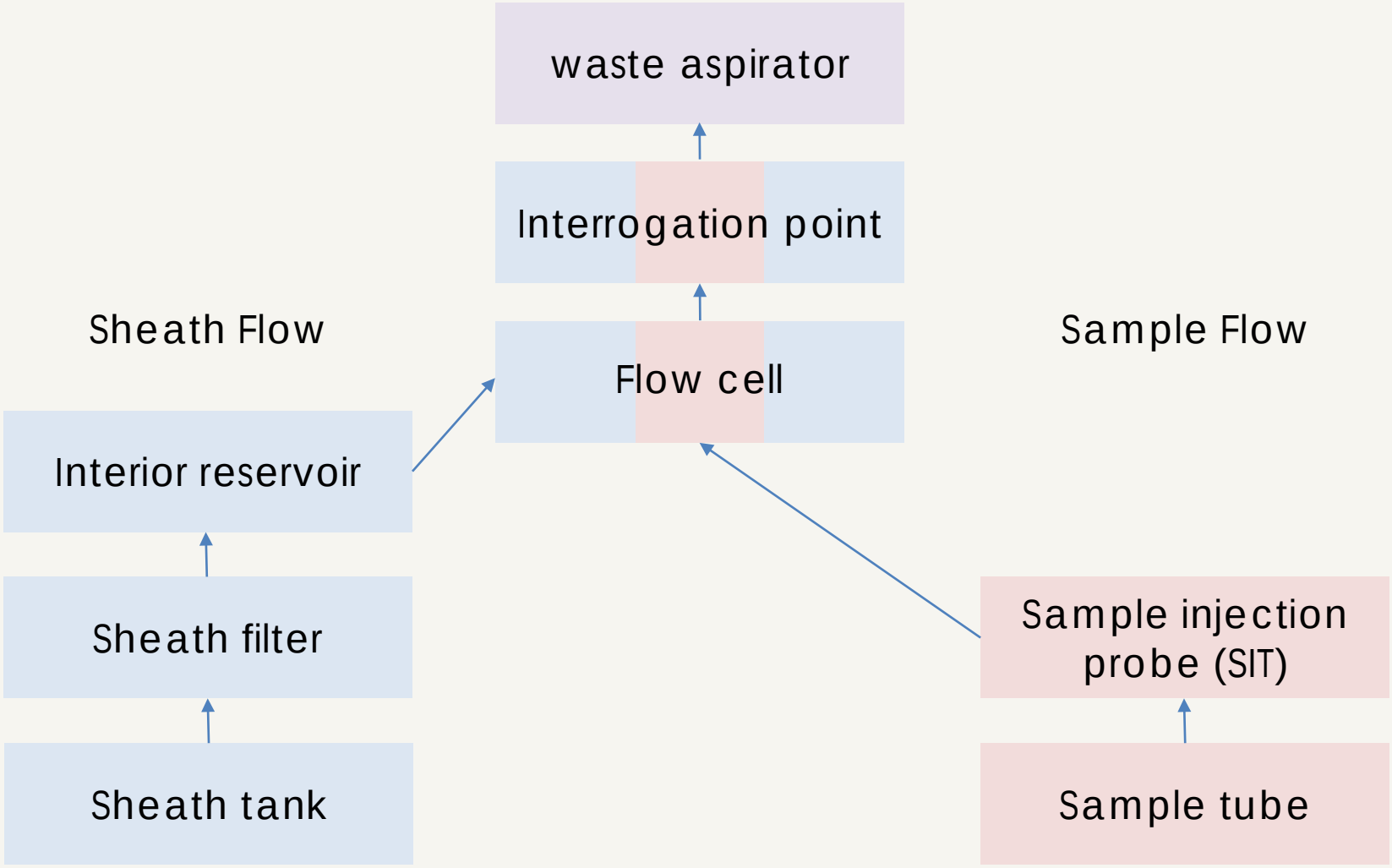
- Sheath and sample fluids stream go in parallel through the flow cell
- The sample flows in the very center of the sheath
- Sample and sheath fluids don't mix



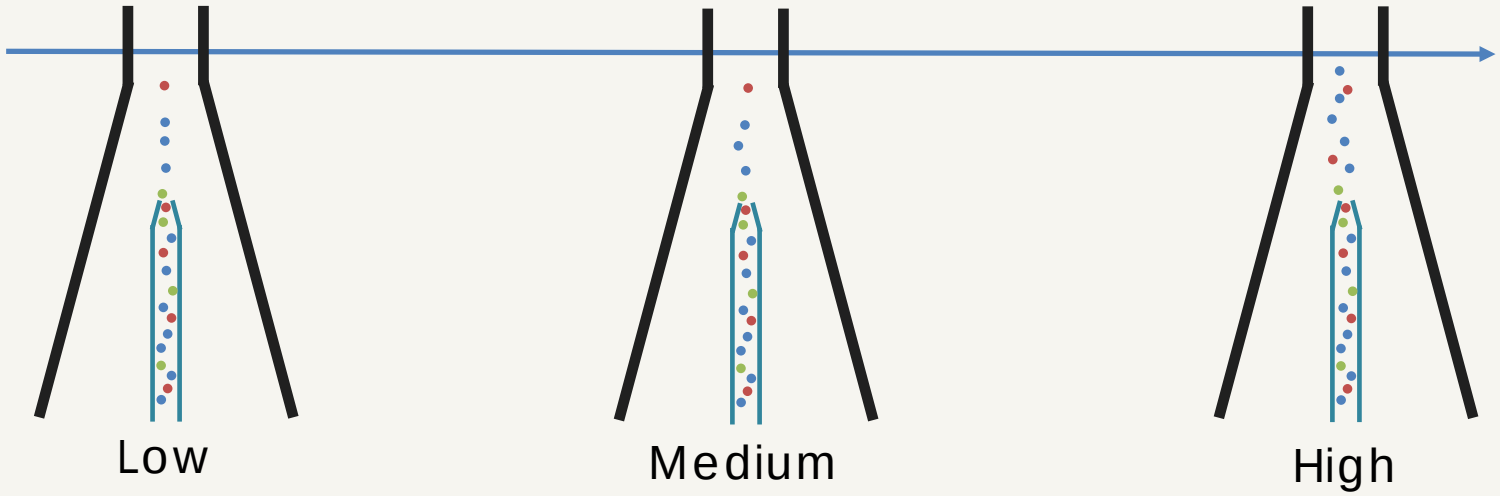
We need to keep a smooth laminar flow!

no aggregates no air

Fluidics – Schematic Overview

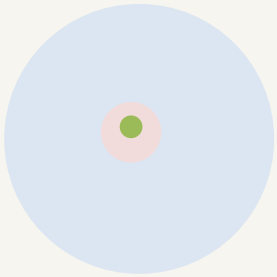


Fluidics – Sample Differential

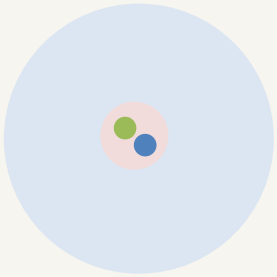


The sample pressure determines the flow rate…

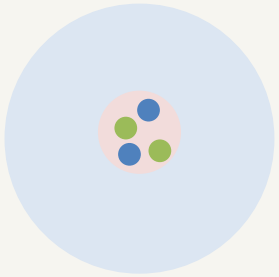
Fluidics – Sample Differential



Low



Medium



High

...and thus the core diameter.

Increased differential pressure will increase the sample flow rate, but will also increase the incidence of multiple cells passing through the laser at the same time.

Fluidics – There is a Speed Limit

With a higher flow rate we…

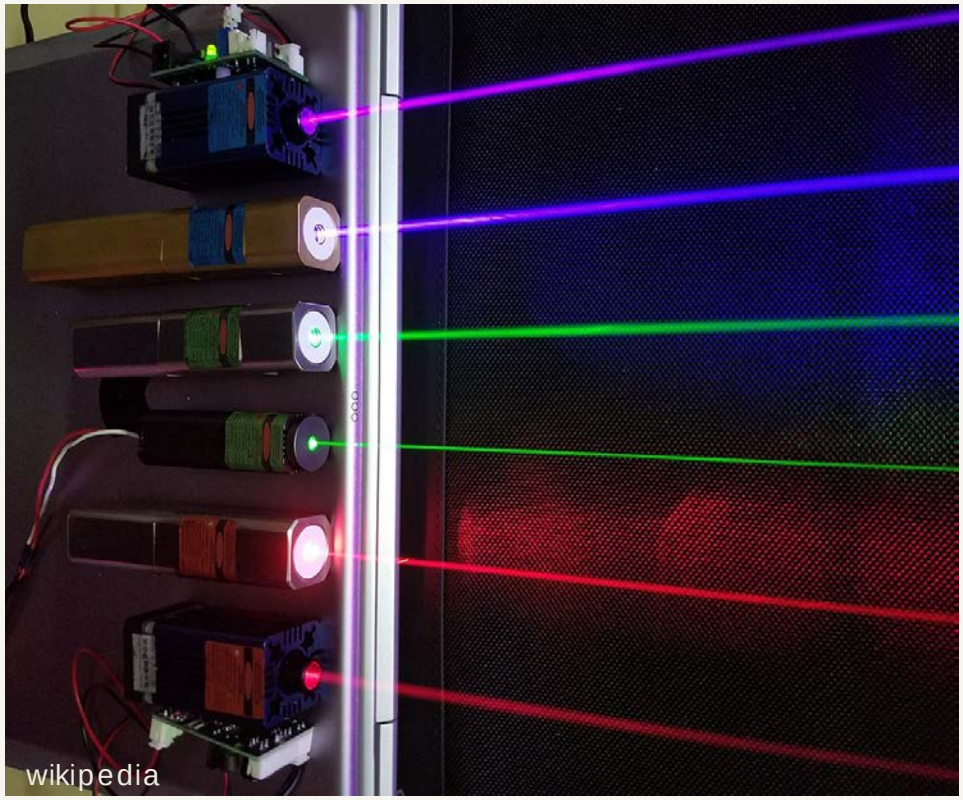
- …increase coincidences
- …get no single-cell analysis
- …get sub-optimal data
- …lose data

BAD

To increase aquisition rates you have to concentrate your sample

Optics - Lasers

- Laser light is coherent and generally of a single wavelength
- Flow cytometers can have a single or up to 7 lasers (or more)



UV (325, 355, 375nm)

Violet (405nm)

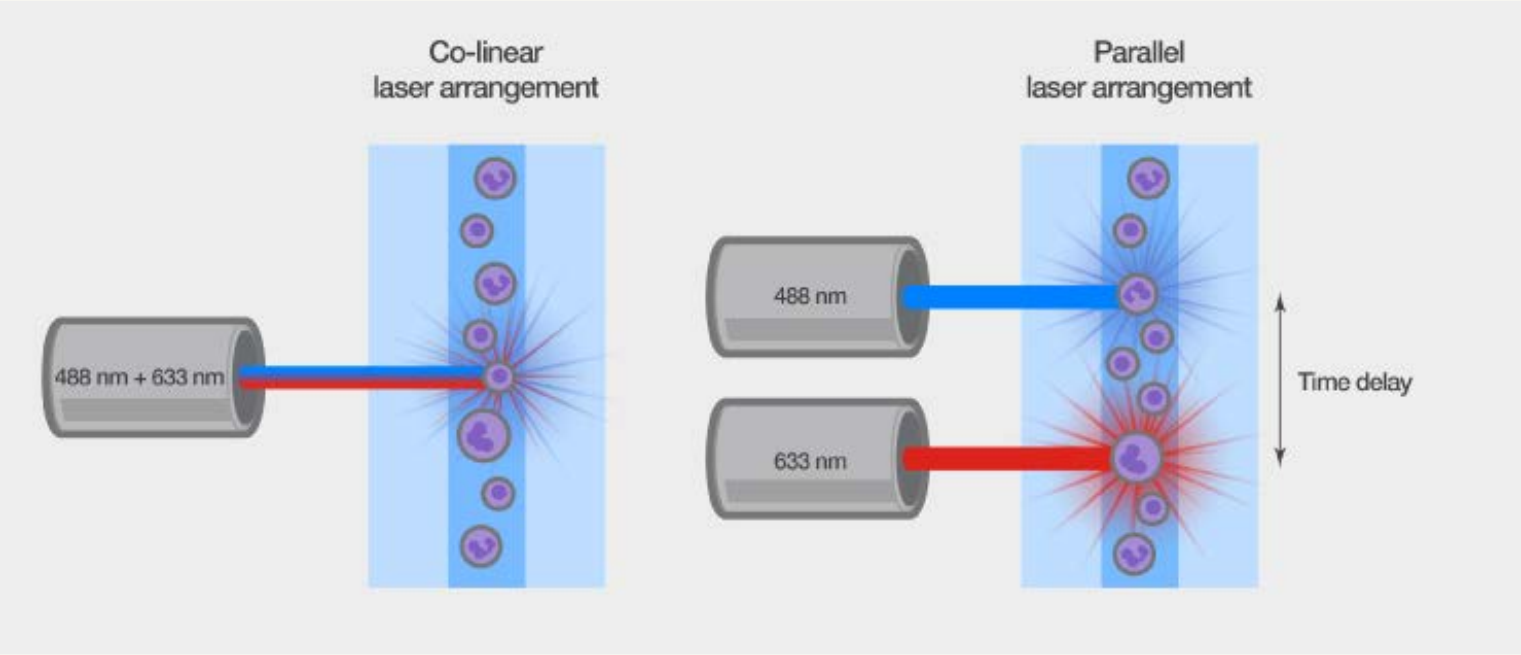
Blue (488nm)

Green (532nm)

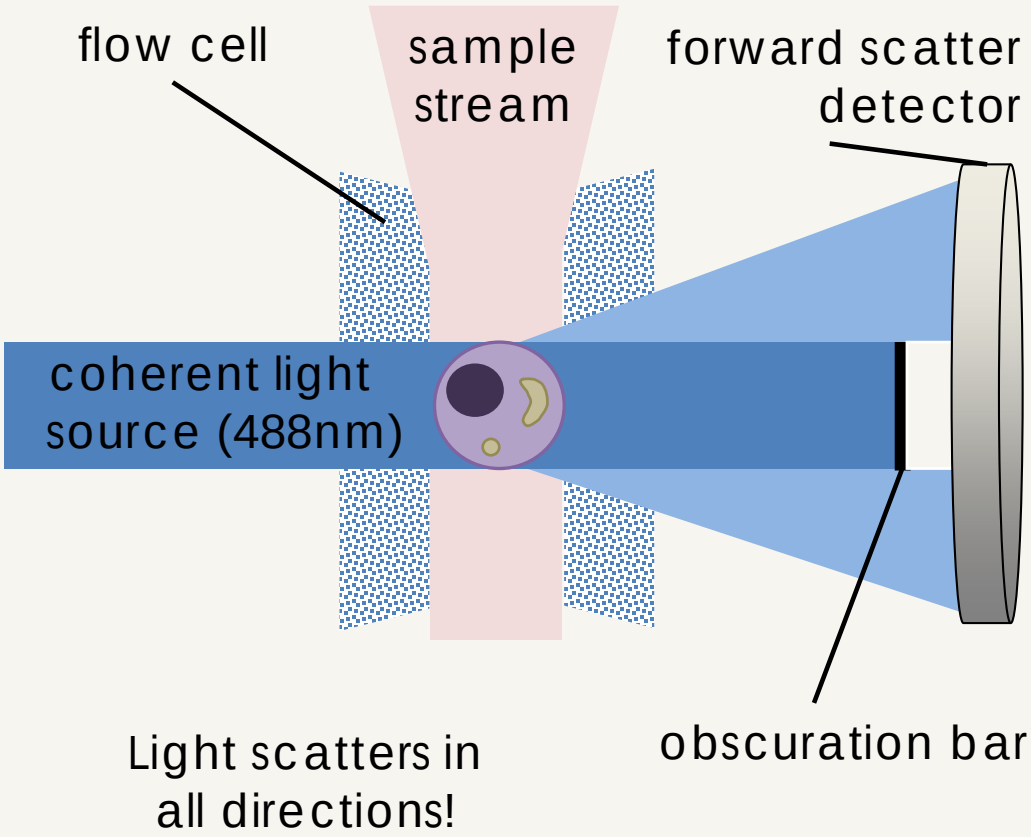
Yellow (561nm)

Red (633nm)

Optics – Laser Arrangement



Optics – Optical Arrangement



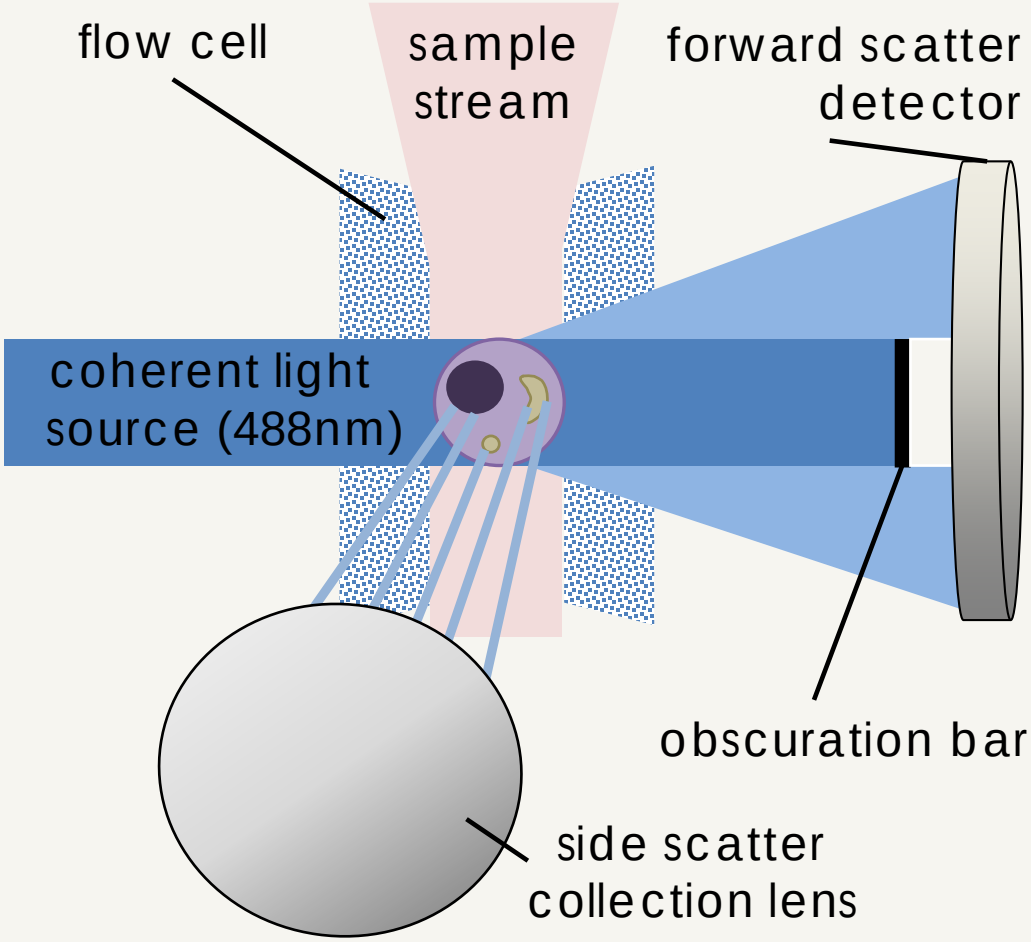
Forward Scatter (FSC,FALS,FS)

- ~2-20° of the laser intercept
- Based on **Mie Scatter**
  - Scatter proportional to the square of the diameter of the cell
  - Based on 'spherical particles

Optics – Notes on Scatter

- Using FSC to determine size only works when comparing apples to apples
- The definition fails when comparing cells and microspheres
- The refractive index of particles and cells are different
- Scatter signal is influenced by
  - Cell size
  - Refractive index
  - Nuclear to Cytoplasmic ratio
- The refractive index can change in
  - Viable and non-viable cells
  - Fixed and unfixed samples
  - Drug treatment…
  - Granularity

Optics – Optical Arrangement

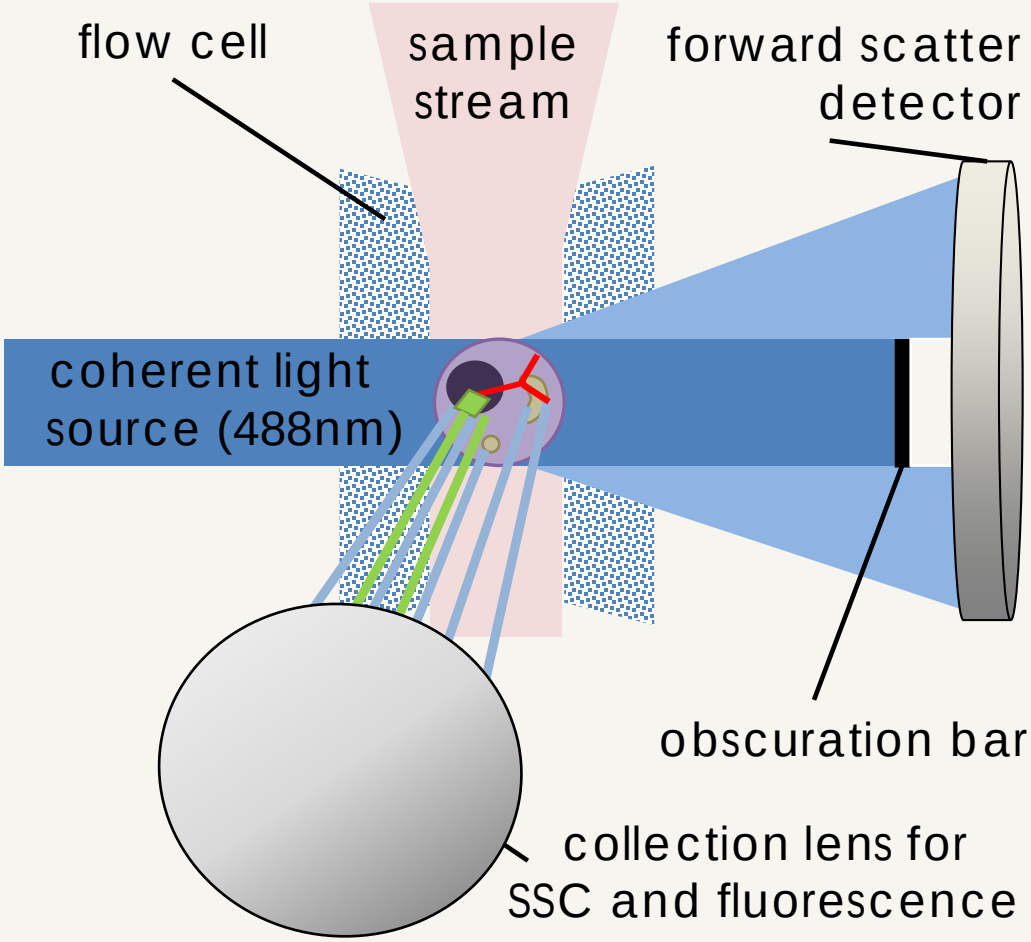


Side Scatter

(SSC, SS, orthogonal scatter)

- large angle scattering (15-150°)
- darkfield
- complexity and granularity

Optics – Optical Arrangement

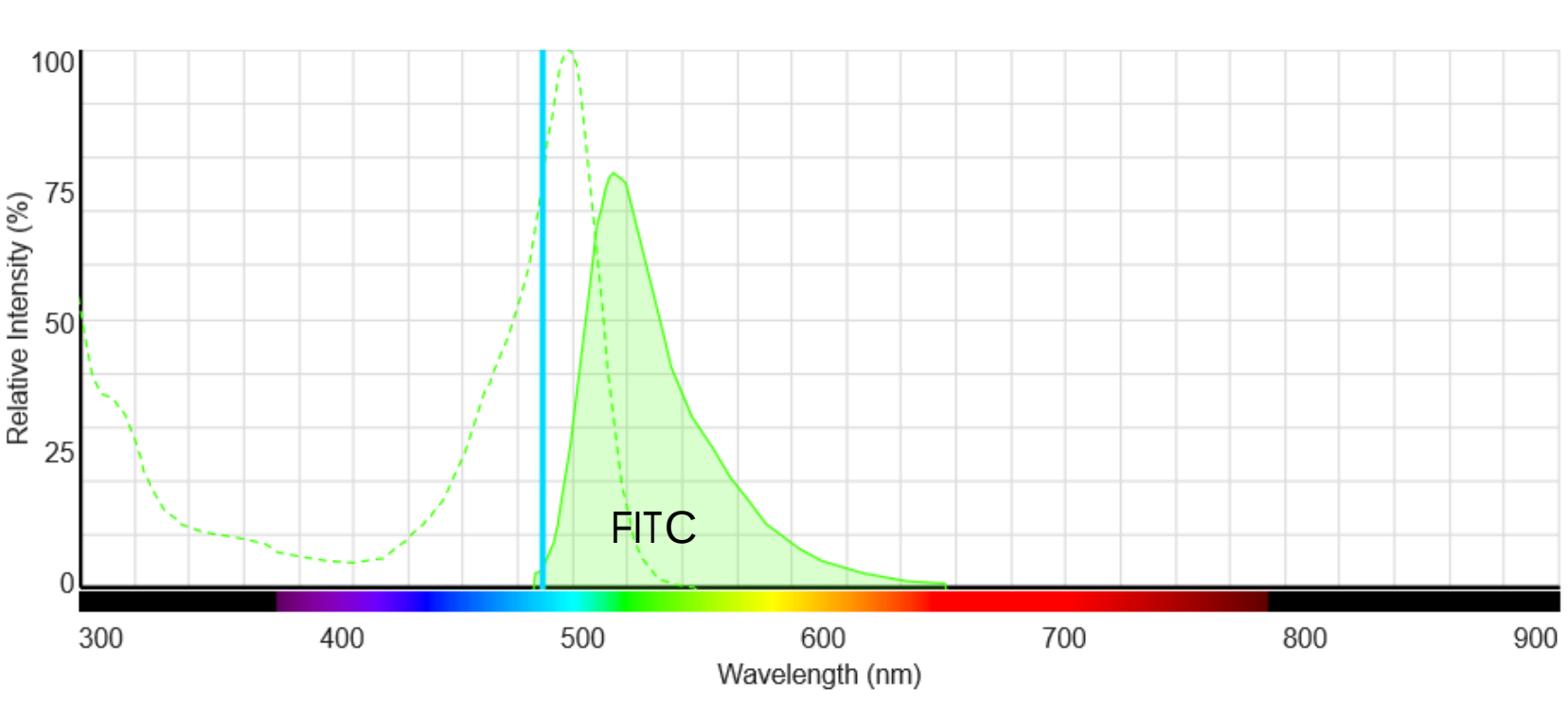


Fluorescence emission

- Collected with the same lens as the SSC (15-150°)
- Intrinsic or extrinsic fluorescence

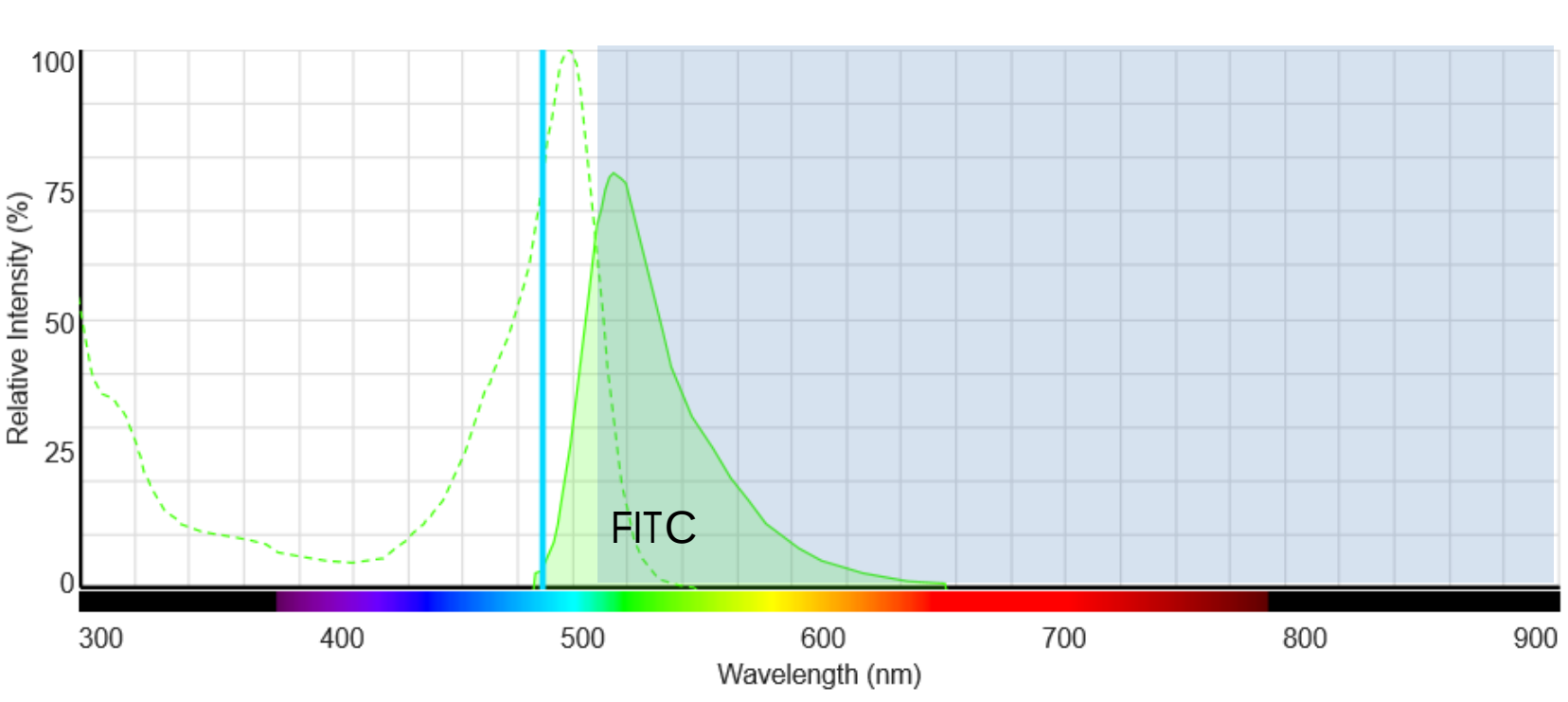
Optics – Fluorescence

488nm



Optics – Capture Fluorescence

488nm



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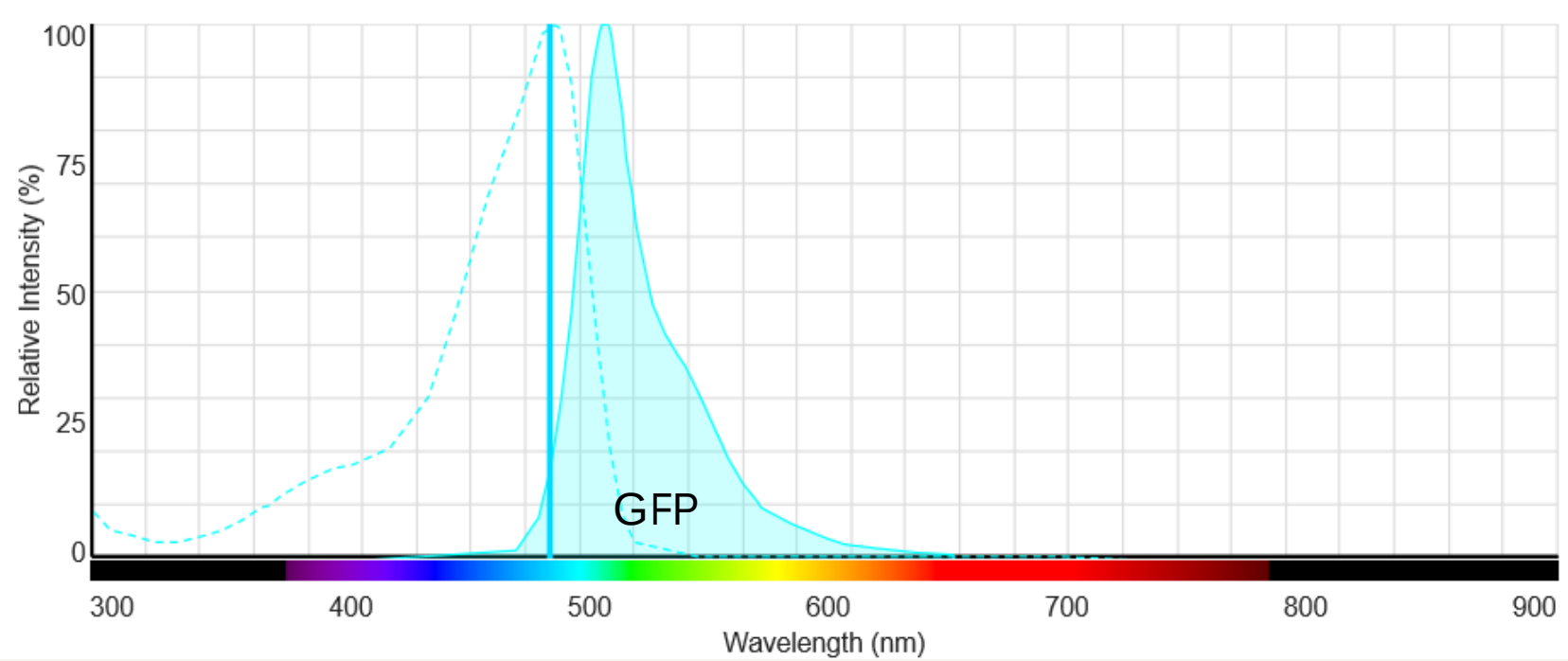
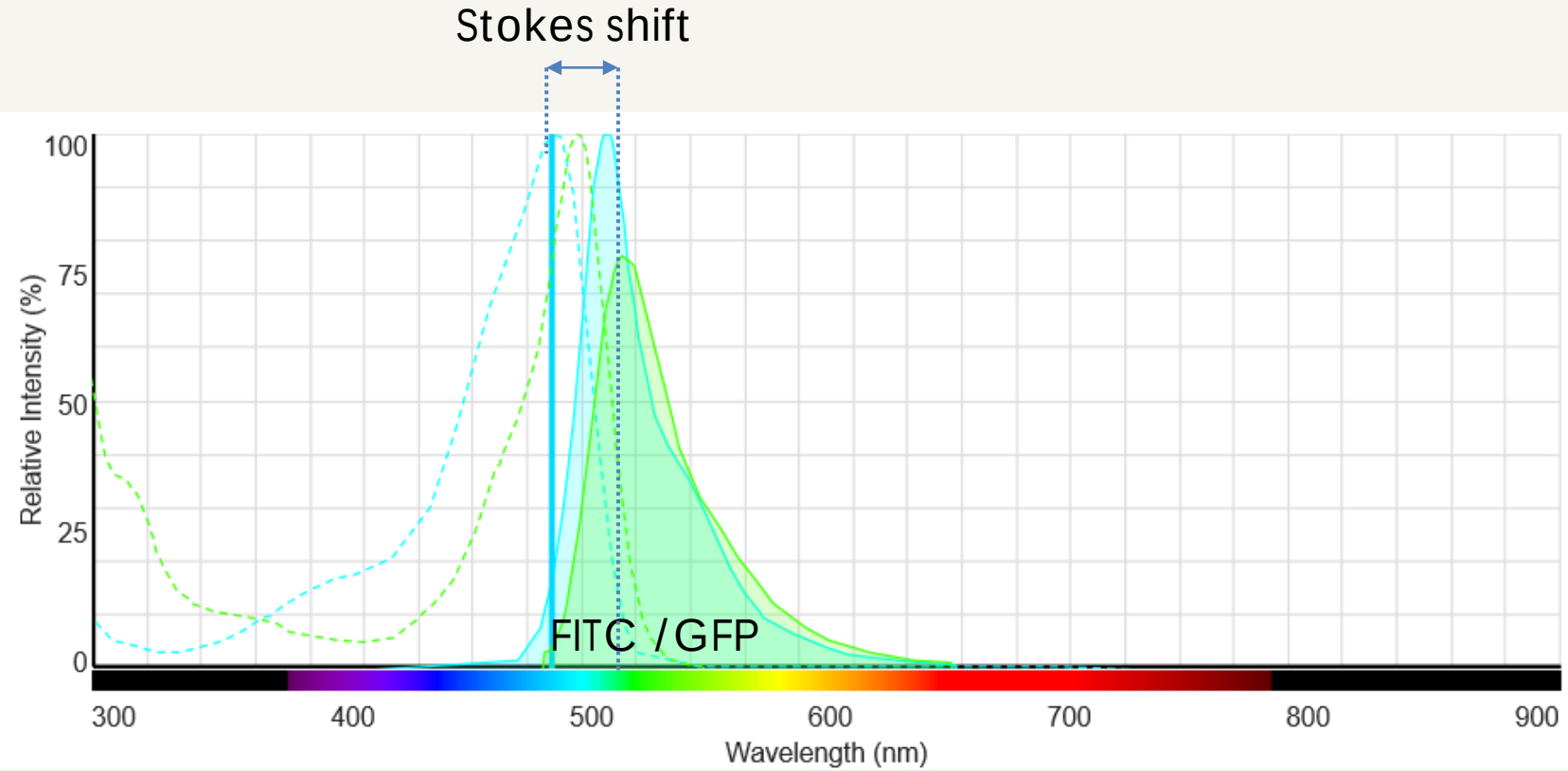


Optics – Capture Fluorescence



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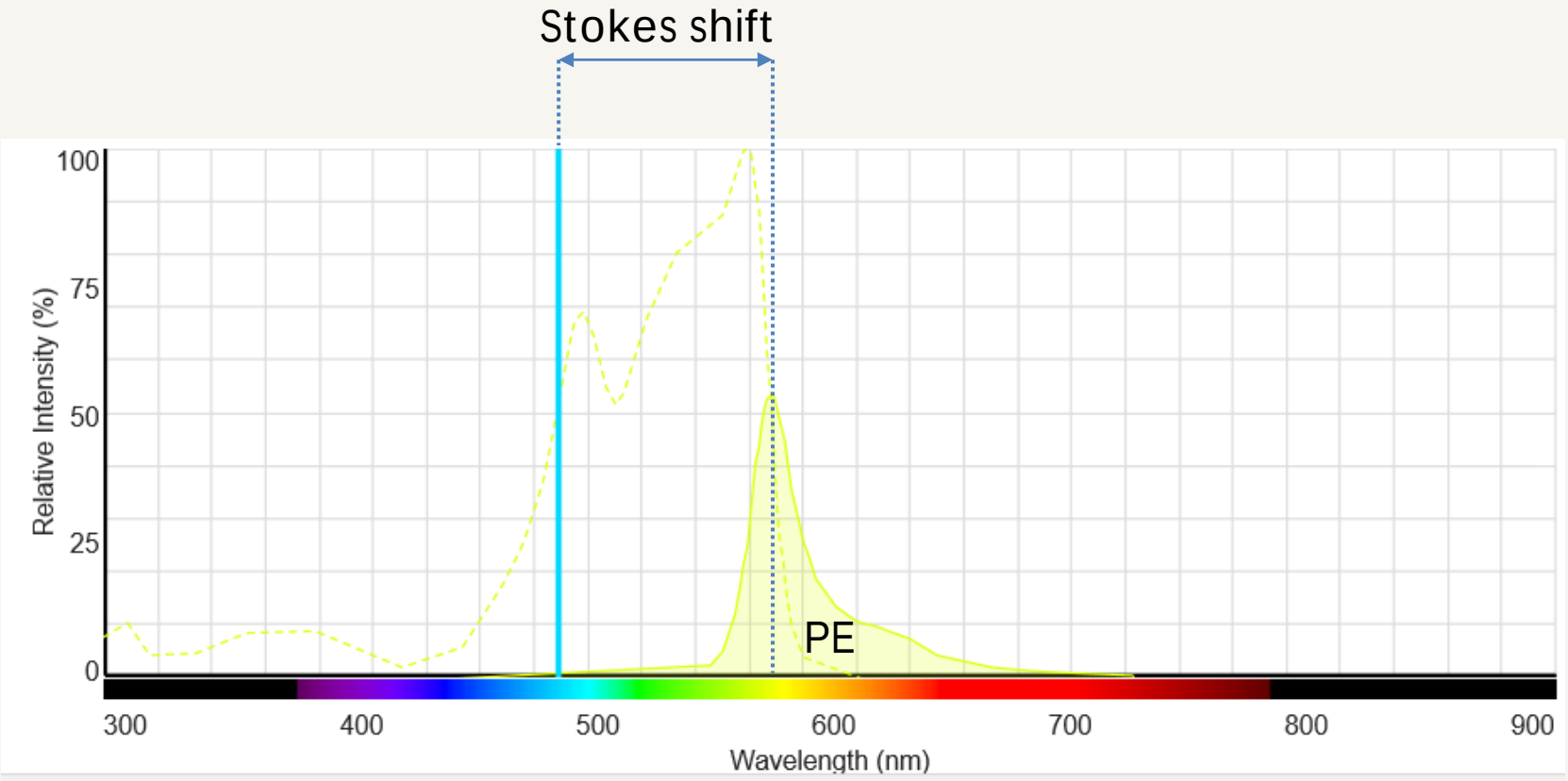
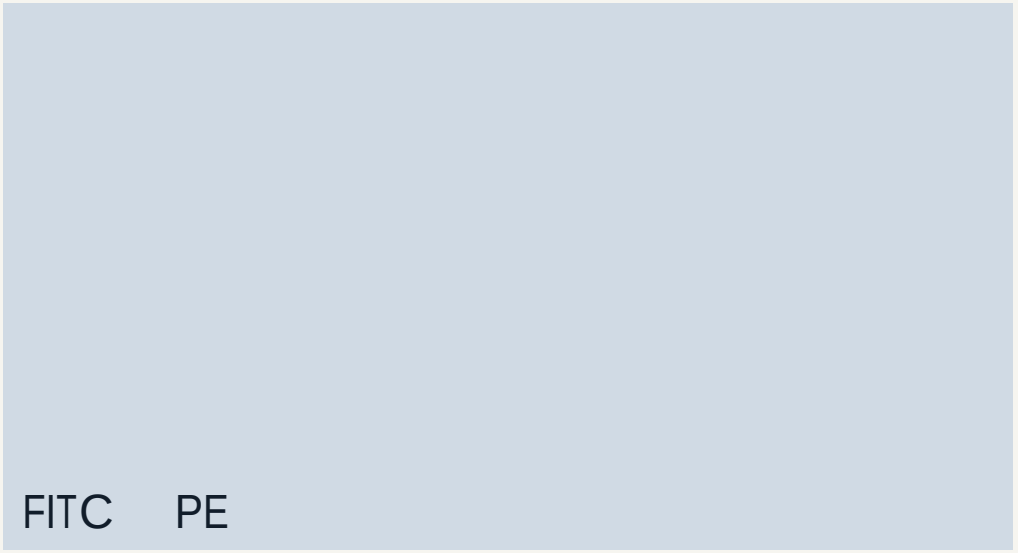
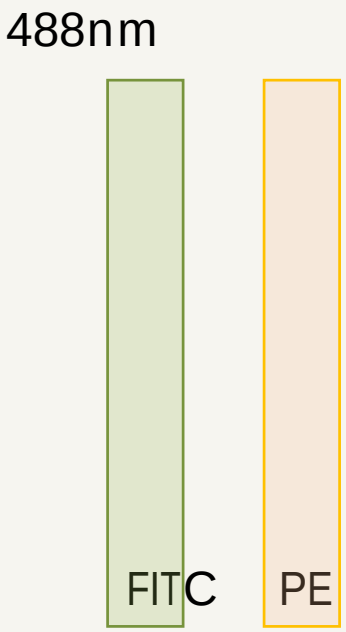


Optics – Capture Fluorescence

488nm

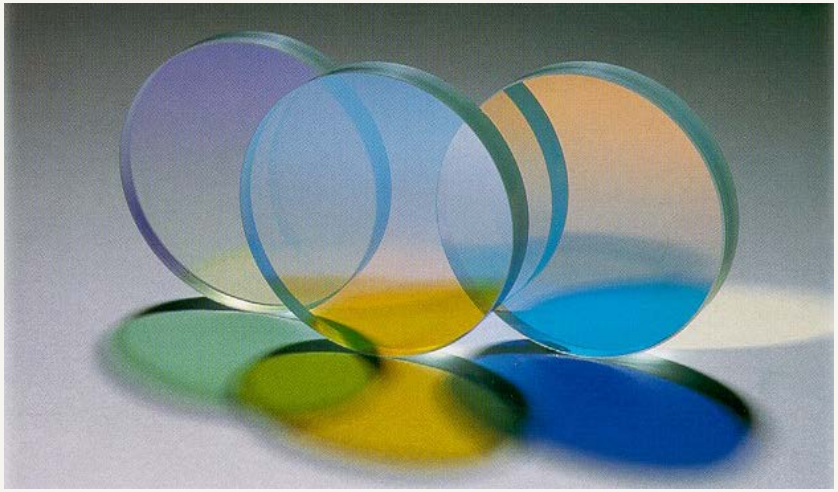


Optics – Capture Fluorescence



Optics – Optical Filters

- Filters transmit light of a specific wavelength while reflecting other wavelengths
- There are three types of dichroic filters:
  - Shortpass (SP) filters
  - Longpass (LP) filters
  - Bandpass (BP) filters

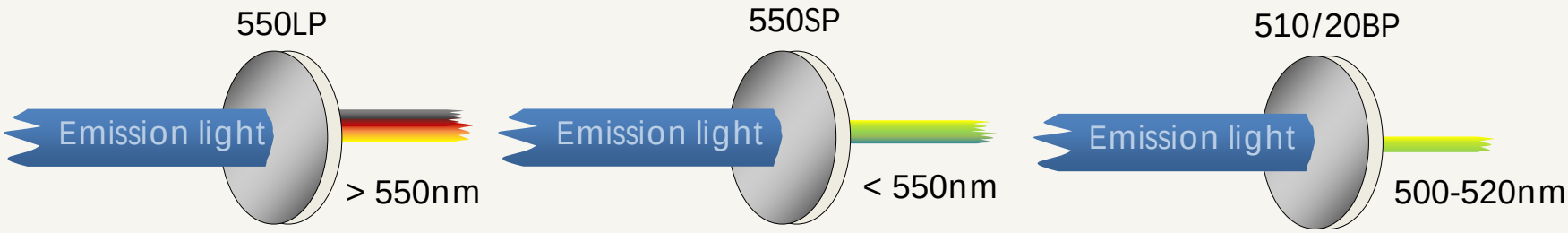


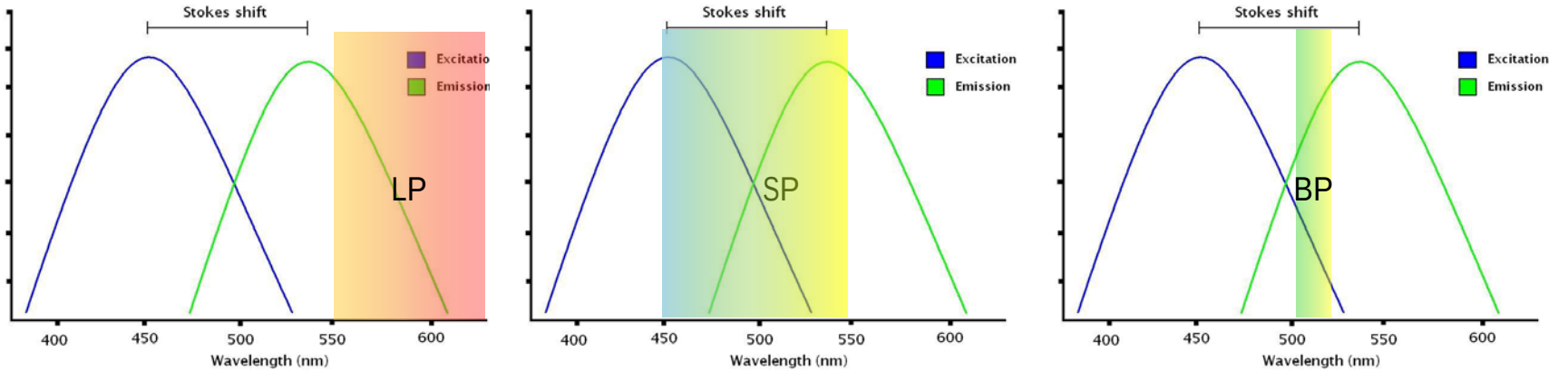
Optics - Filter Properties

Longpass filters transmit wavelengths above a cut-on wavelength

Shortpass filters transmit wavelengths below a certain wavelength

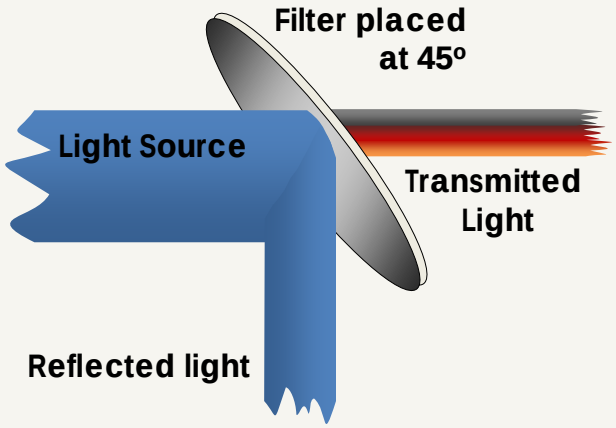
Bandpass filters transmit wavelengths in a narrow range around a specified wavelength

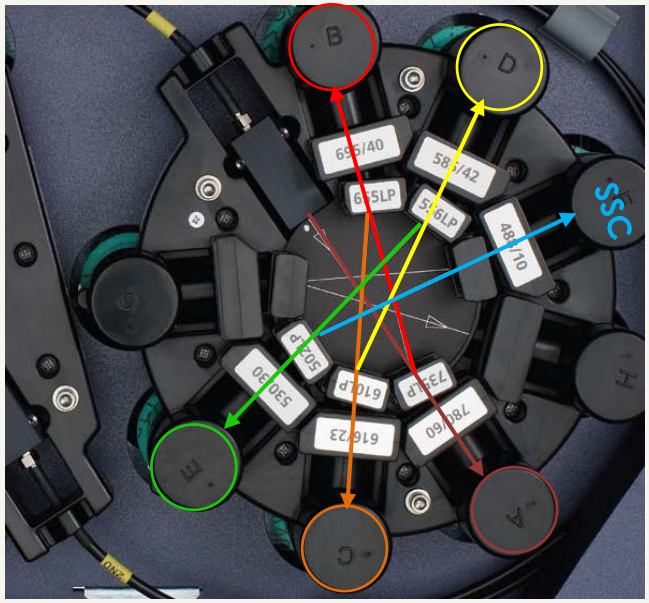


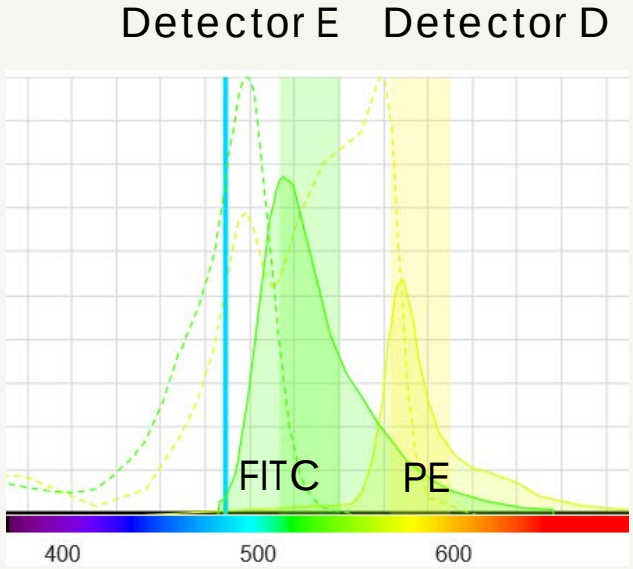


Optics - Dichroic Mirror

With a specific angle the filter can be used as a **dichroic mirror**



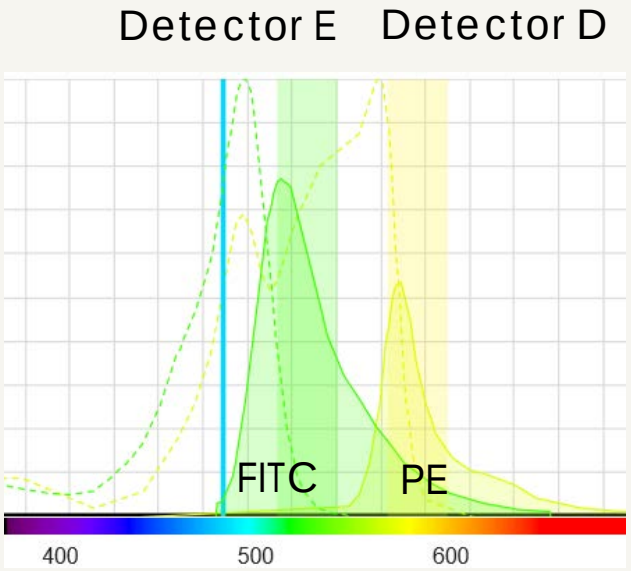




Optics –Instrument Configuration

- Excitation spectrum: will determine whether we can use that fluorochrome
- Emission spectrum: will tell us which filter to use and whether we can combine the fluorochrome with others.

Know your instrument configuration when you select for fluorescent probes.



Electronics – Light detection

- Photomultipliers (PMTs) simply detect photons
- Light needs to be optically filtered before
- Photon energy is converted into a signal that is dependent on:
  - Number of photons
  - Voltage applied to the PMT

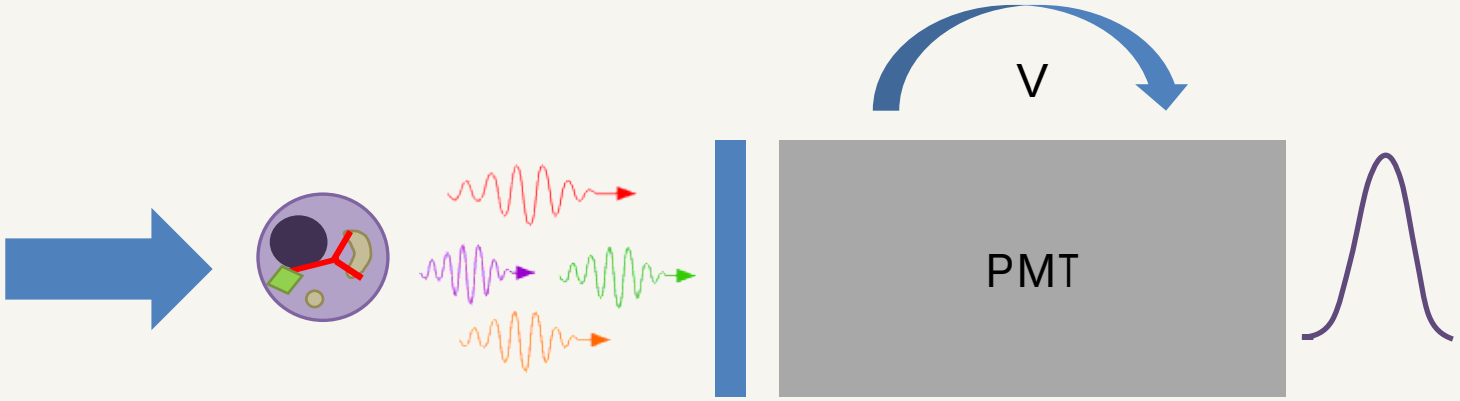
The measurement is only relative!

PMT voltage setup is important

Controls, controls and controls!

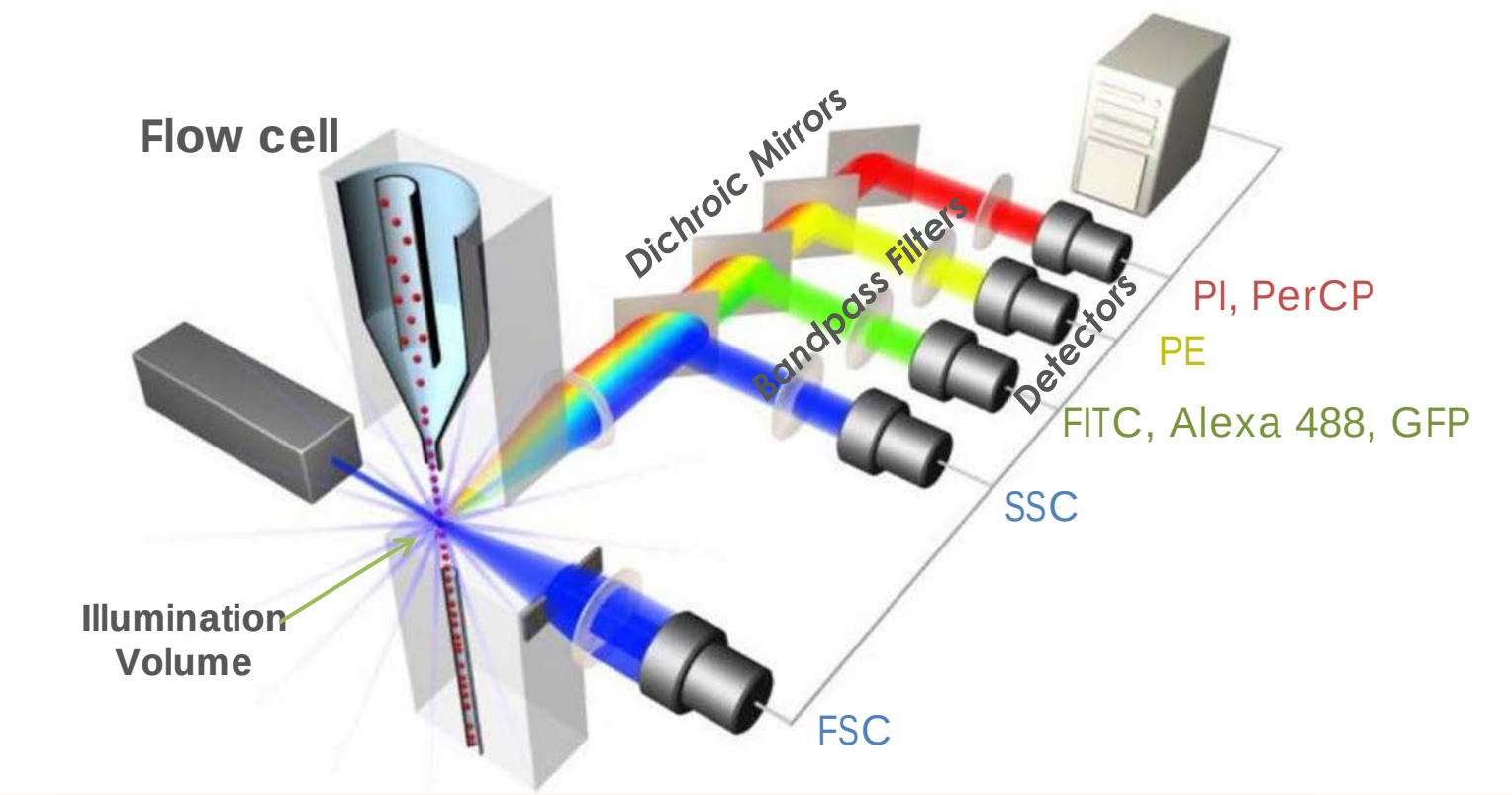
Electronics - Overview

- photons are filtered, collected, and multiplied by the PMT
- The current generated is converted to a voltage pulse
- The voltage pulse is digitized
- The values are stored into a List Mode File



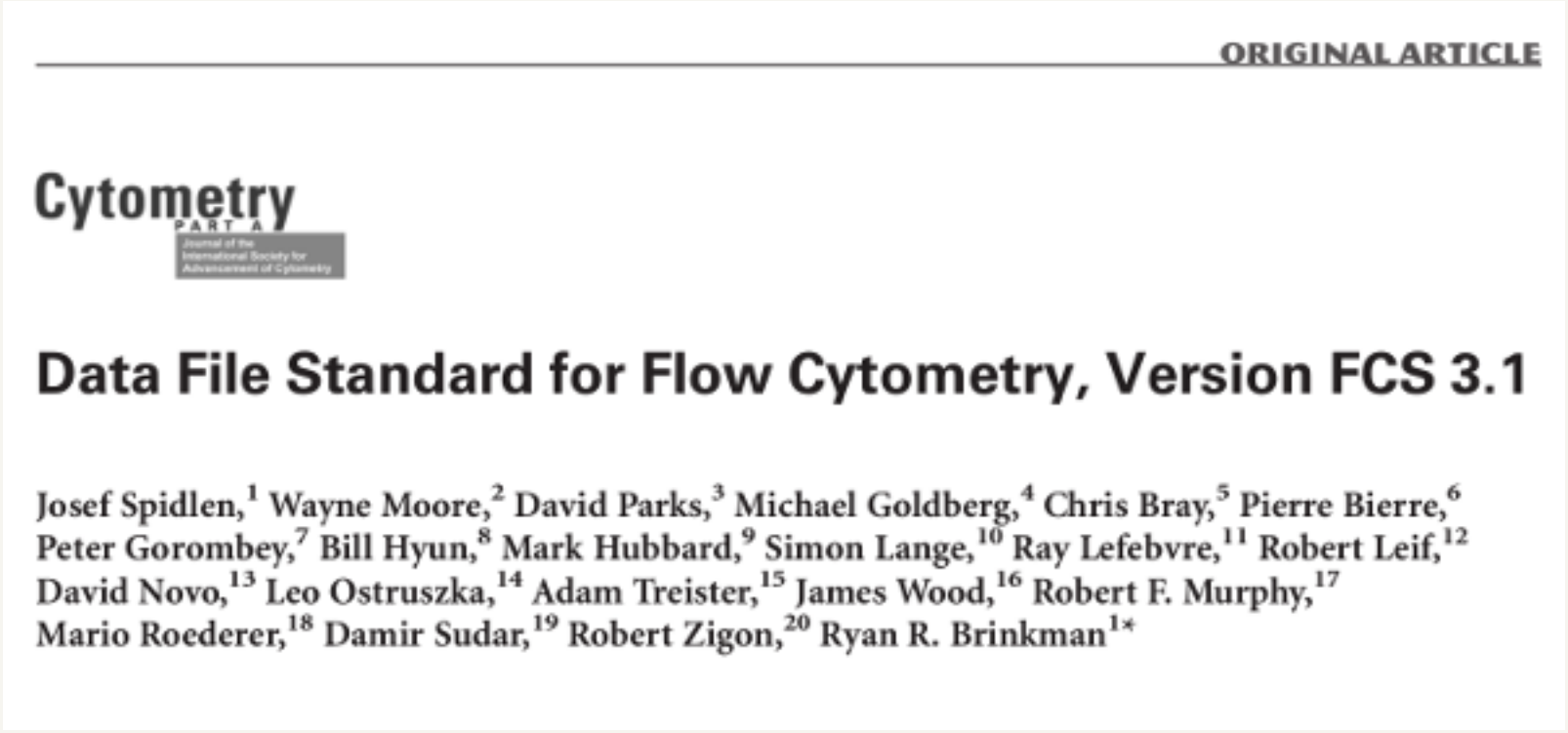
Summary I: Channel Layout

- Photon-distribution to detectors according to energy-levels (wavelengths)
- Optical elements provide separation of channels and wavelength selection



What does a cytometer give us?

- We get light scatter, fluorescence information and time
- We collect the data in a defined way
- We are in control of how the data is displayed



ORIGINAL ARTICLE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Data File Standard for Flow Cytometry, Version FCS 3.1

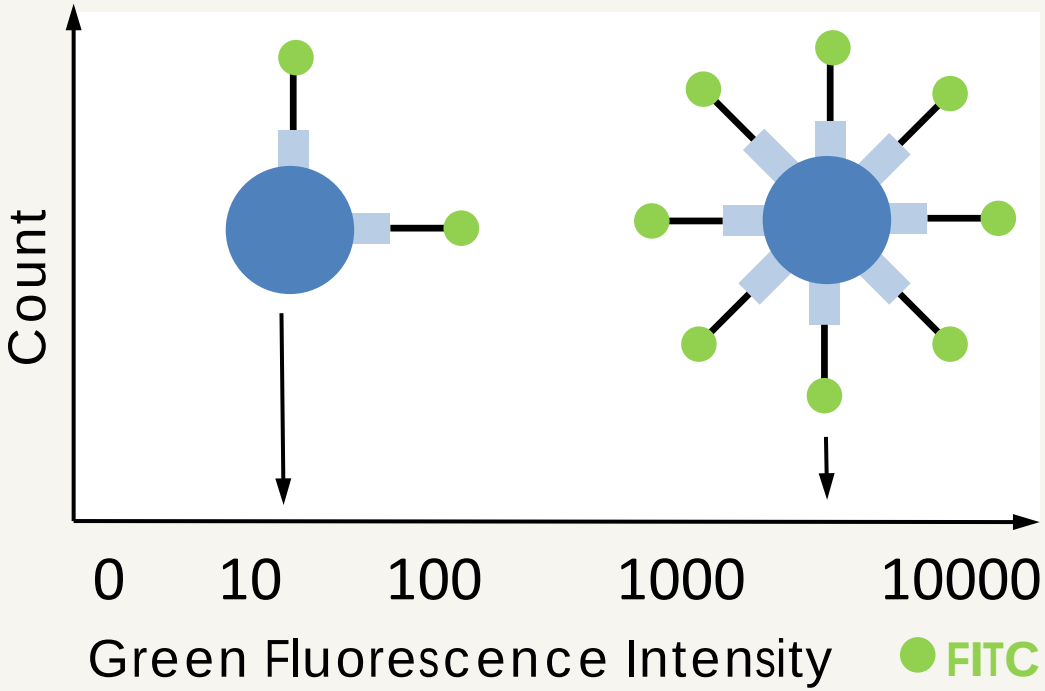
Josef Spidlen,[1] Wayne Moore,[2] David Parks,[3] Michael Goldberg,[4] Chris Bray,[5] Pierre Bierre,[6] Peter Gorombey,[7] Bill Hyun,[8] Mark Hubbard,[9] Simon Lange,[10] Ray Lefebvre,[11] Robert Leif,[12] David Novo,[13] Leo Ostruszka,[14] Adam Treister,[15] James Wood,[16] Robert F. Murphy,[17] Mario Roederer,[18] Damir Sudar,[19] Robert Zigon,[20] Ryan R. Brinkman[1*]

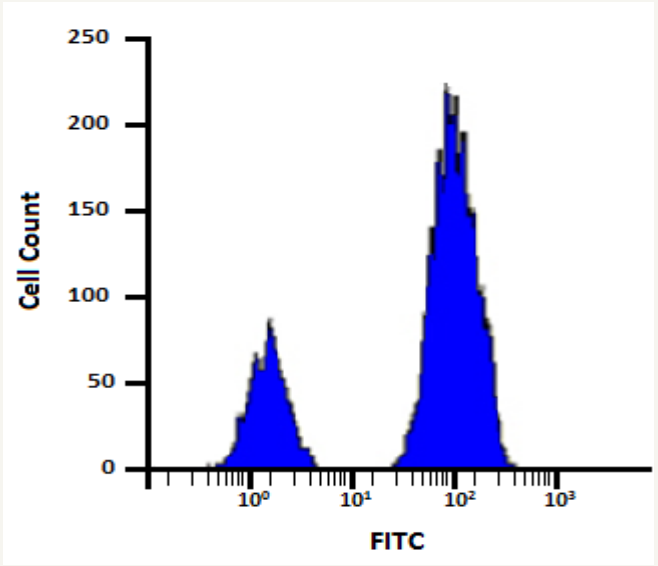
Flow Cytometry Data Files

- Listmode file: correlated data file where each event is listed sequentially, parameter by parameter
- Flow cytometry standard (FCS 3.1)
  - Allows other software programs to recognize and analyze data
  - Data and header portion

	FSC	**SSC**	**FL1**	**FL2**	**…**	**FLn**
Cell 1	50	20	45	2000		686
Cell 2	55	18	47	1867		600
…						
Cell n	67	234	86	2134		765

Display Options - Histogram

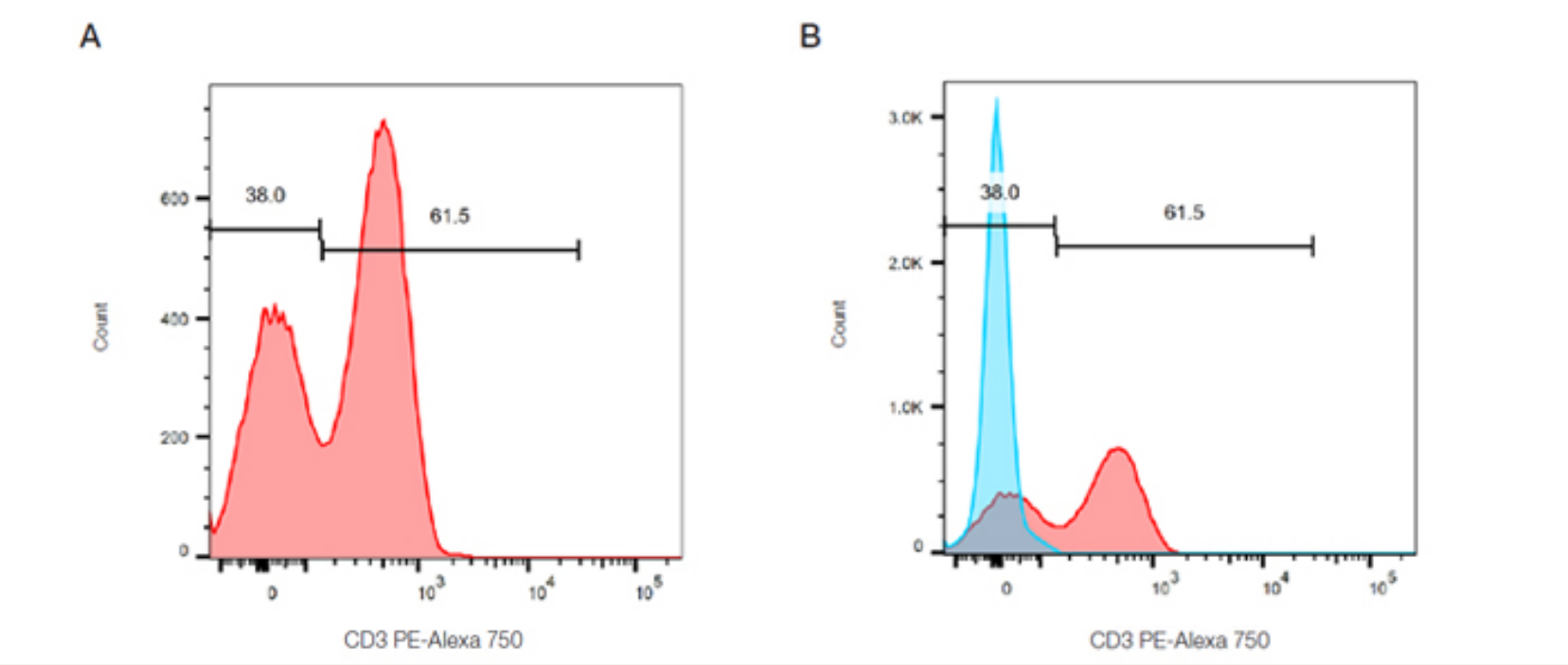




- Univariate plot
- The farther to the right, the brighter the fluorescence
- Good to display normal distributions

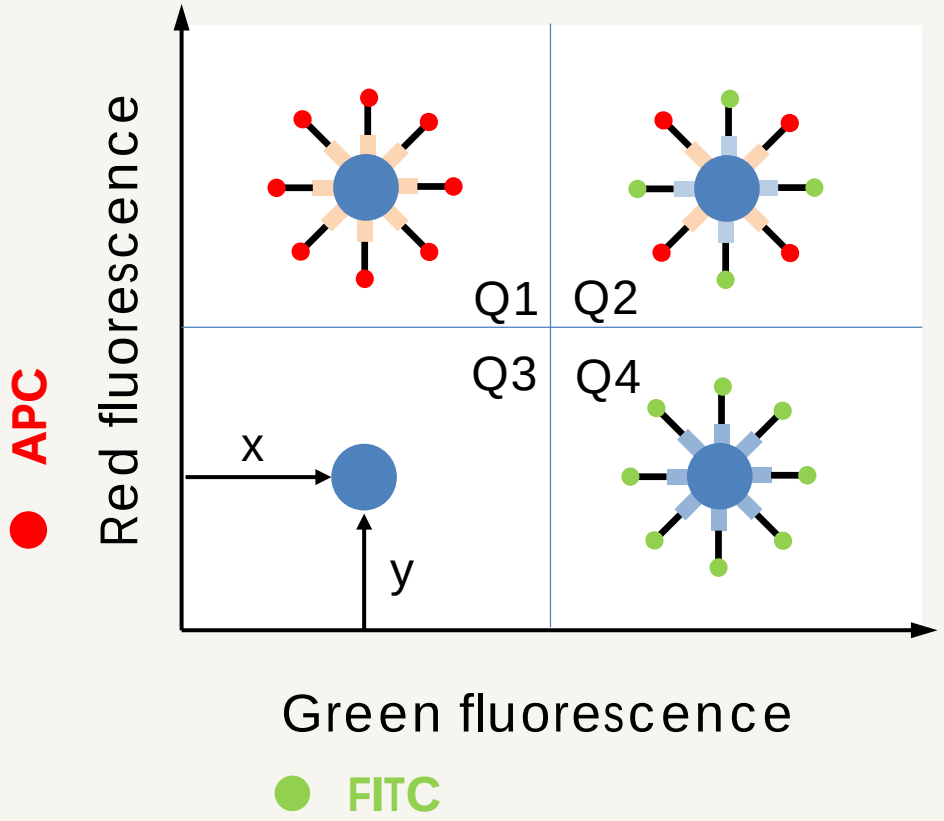
Display Options – Histogram Overlays

- Show differences between samples/populations
- Peak hight is a function of the CV (spread) and the number of events



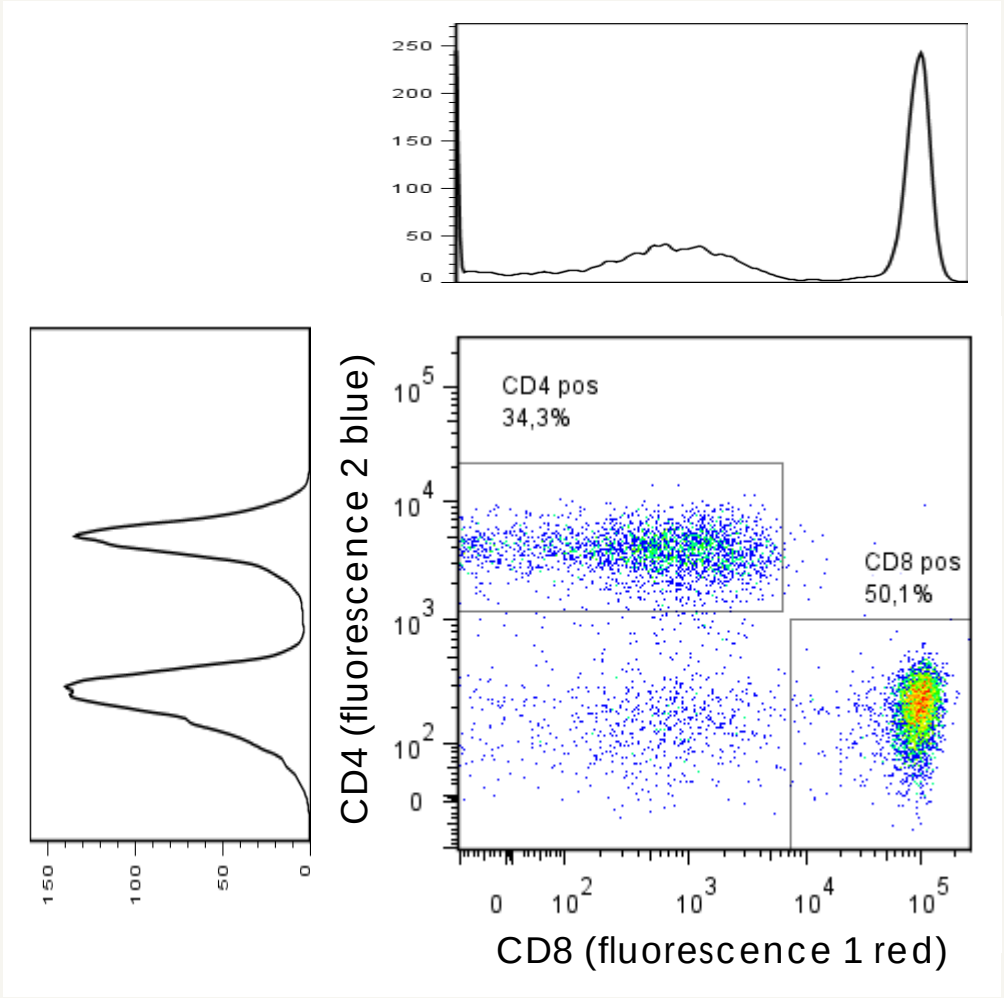
Display Options – Dot Plot

Each event is plotted according to its value in the x and y dimensions

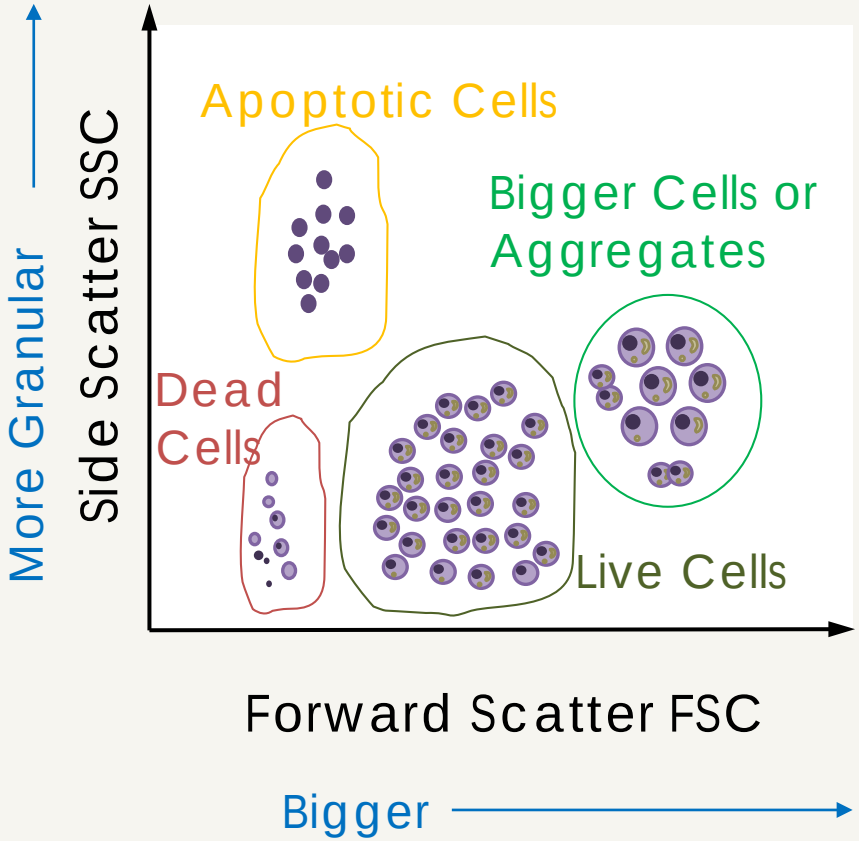


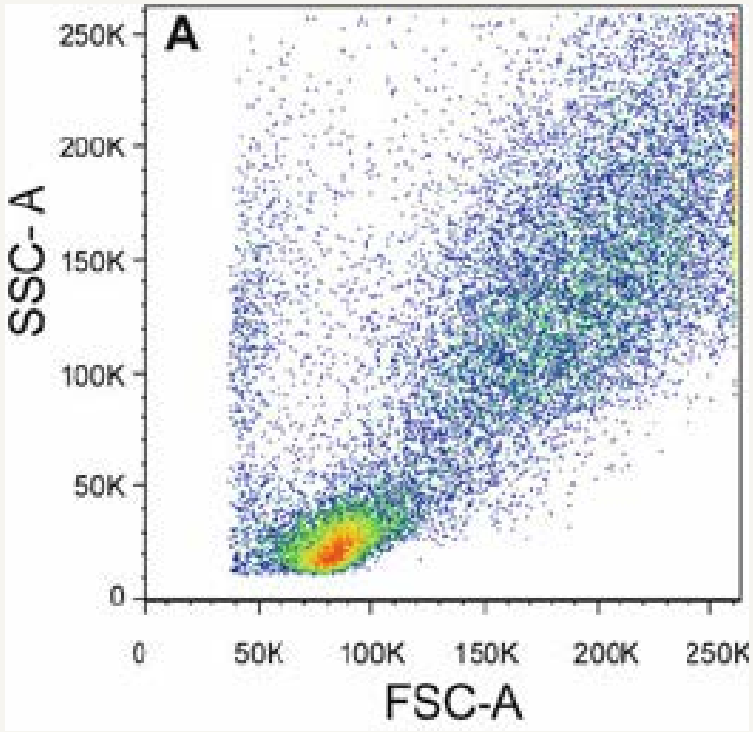
Display Options – Dot Plot

- Univariate plots show one dimension
- Bivariate plots provide more graphical information and more data

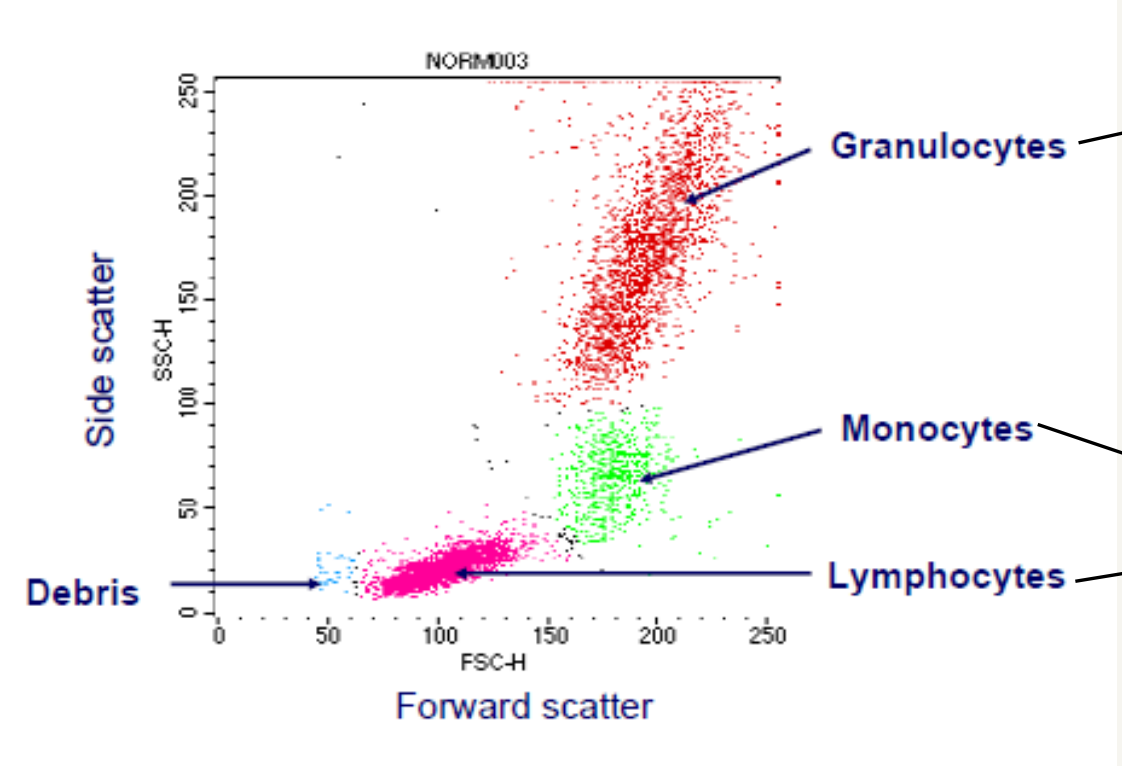


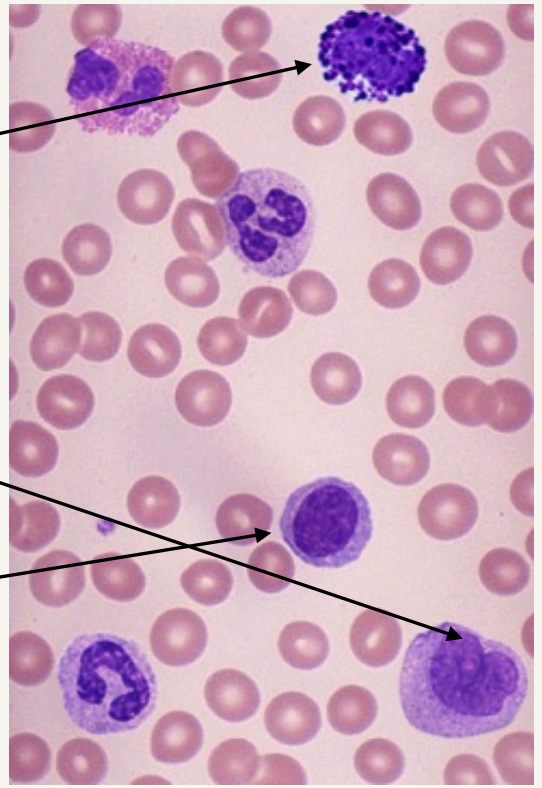
What we learn from Light Scattering





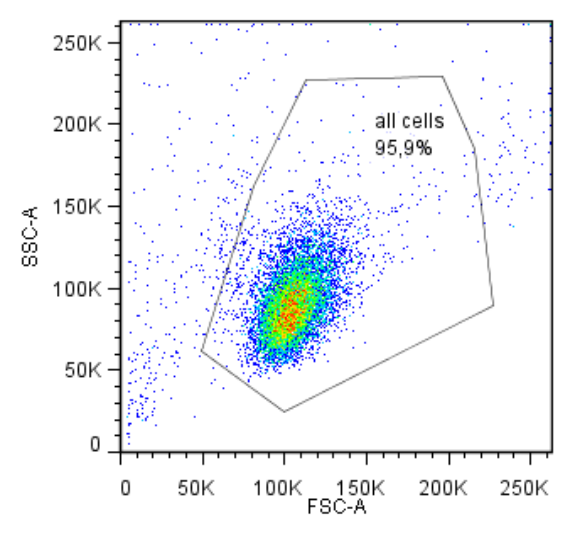
Light Scattering in Whole Blood

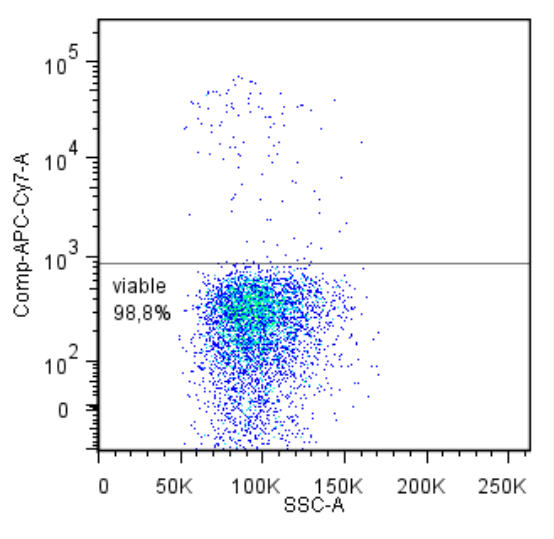


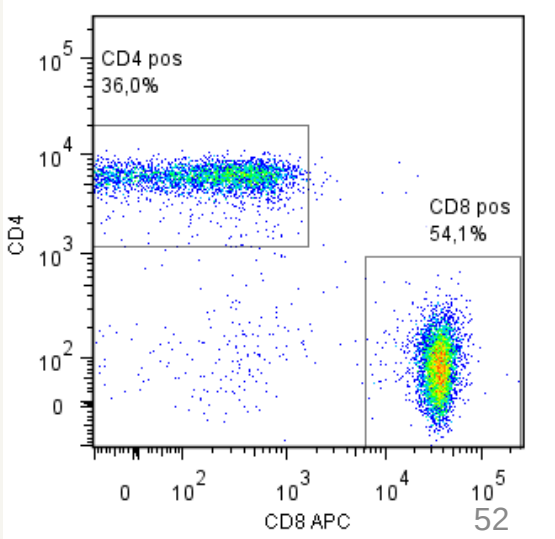


Common Modes for Dot Plots

- Forward scatter vs side scatter
  - To look at the distribution of cells based upon size and granularity
- Single color vs side scatter
  - To visualize the expression of the fluorescence of cells
- Two-color fluorescence plot
  - To differenciate between those cells that express only one of the particular fluorescent markers, those that express neither and those that express both



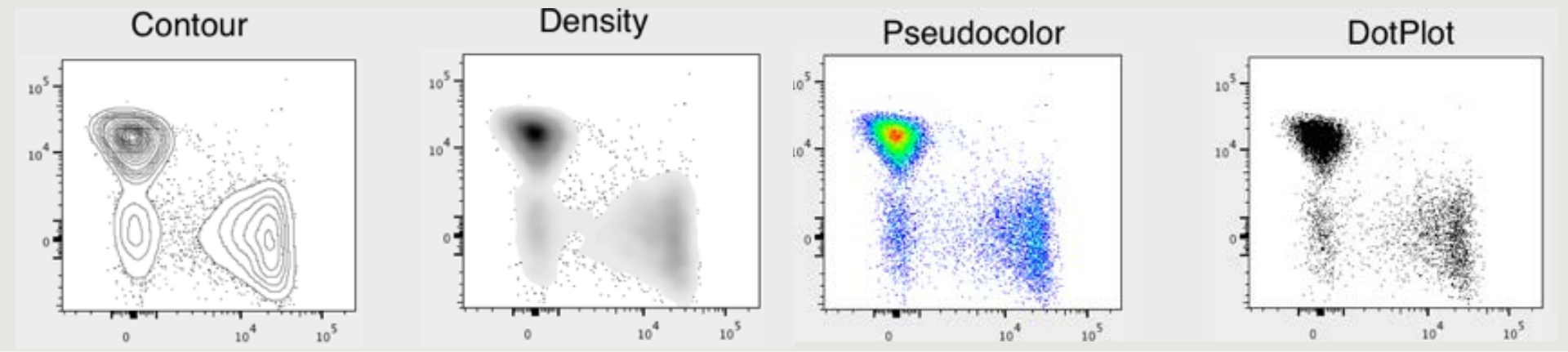




Lots of Options!

- DOT plots mask the amount of data
- Density plots and Contours show where concentrations are heaviest
- Contours do not display rare events well - use Contour showing outliers (Contour and Dot Plot combination)

Display options:



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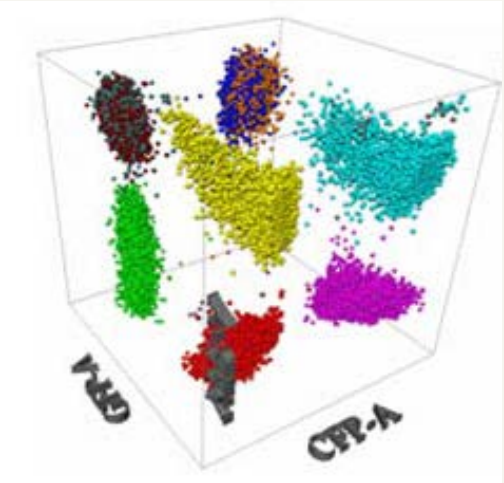
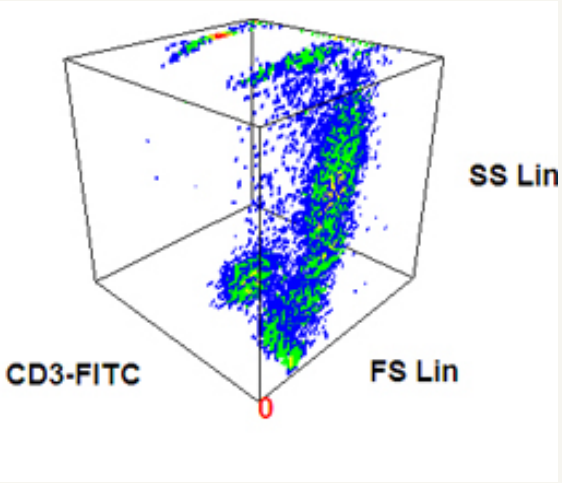
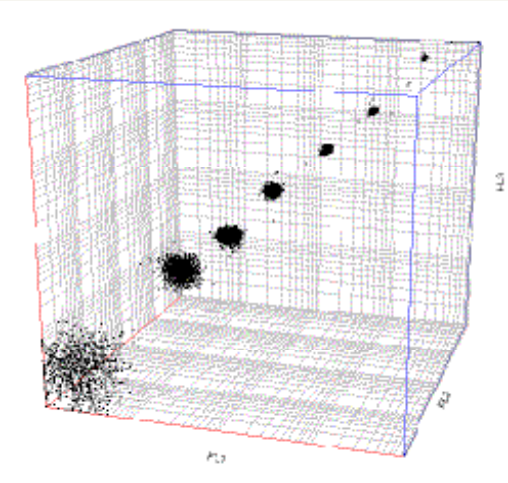
N-Dimensional Plots



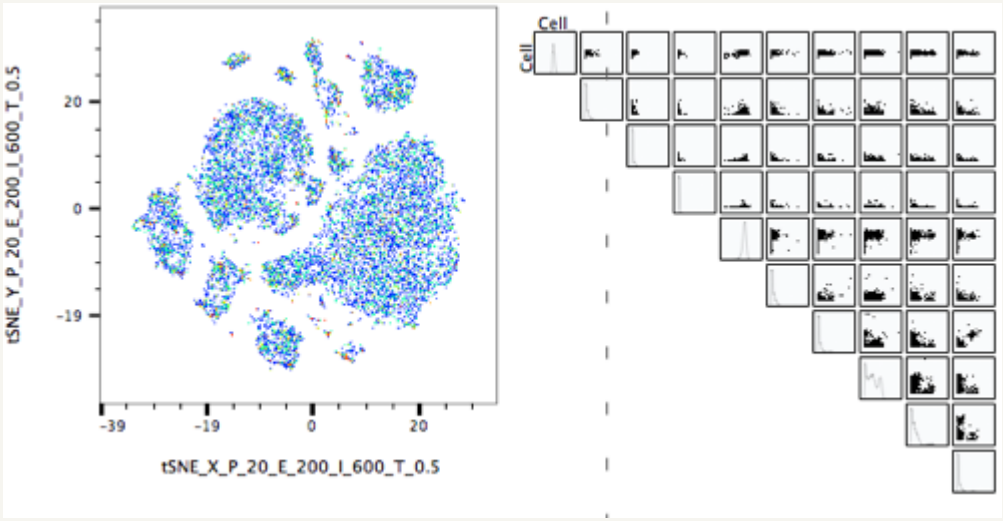


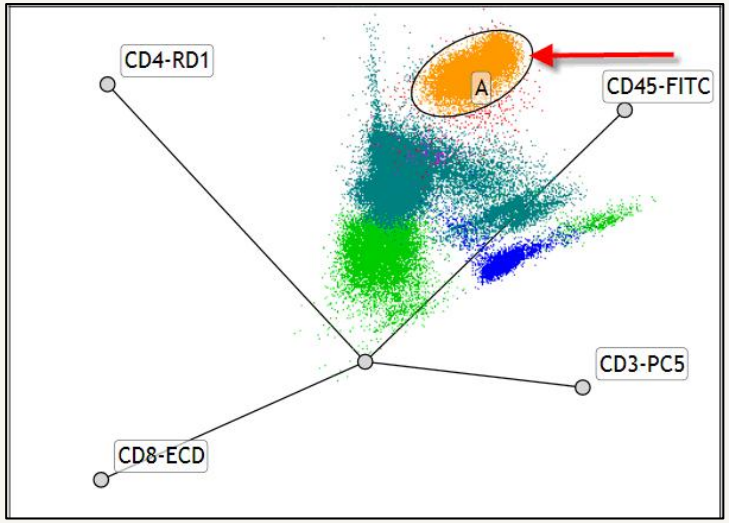


…and 4+ parameters?

- Can rotate them
- For analysis only usually
- Statistics are hard to determine on these

N-Dimensional Plots



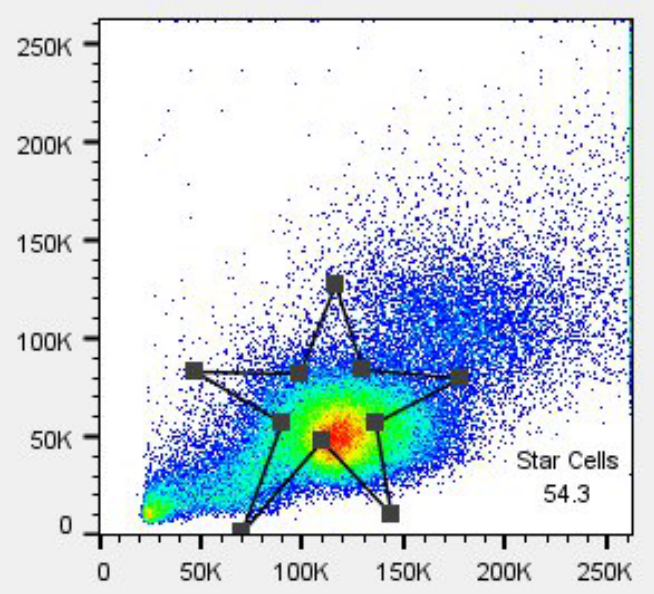


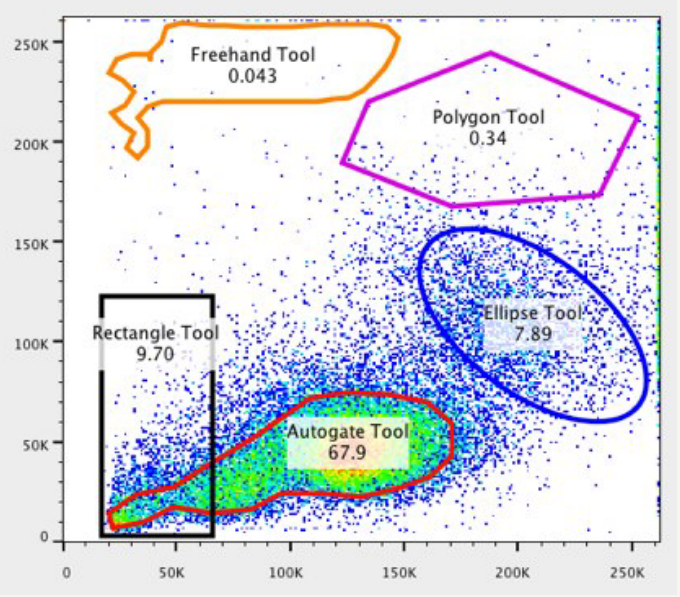
…and 4+ parameters?

- We have to reduce the data
- For this we define populations and gates

Gates

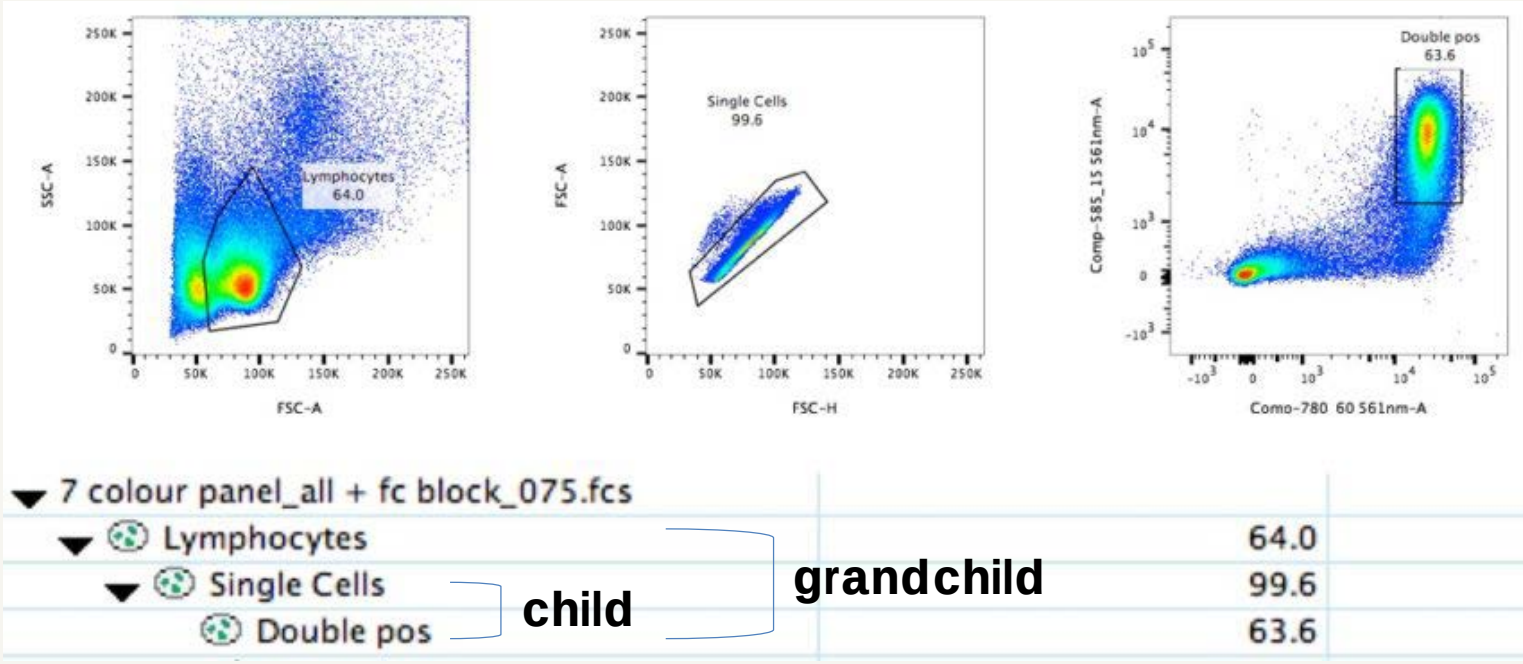
- A region (or region of interest ROI) can be drawn on
  - A histogram to define boundaries and calculate percentage of positive
  - A bivariate plot to define populations and calculate percentages
- Gating should be based on some scientific basis
- Proper controls are essential
- Follow the density of the population





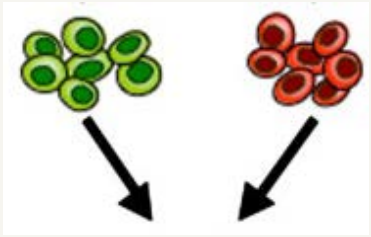
Gates

- Gates can be combined and are used to isolate subsets of data
- Gates are usually hierarchical organized, like a family tree

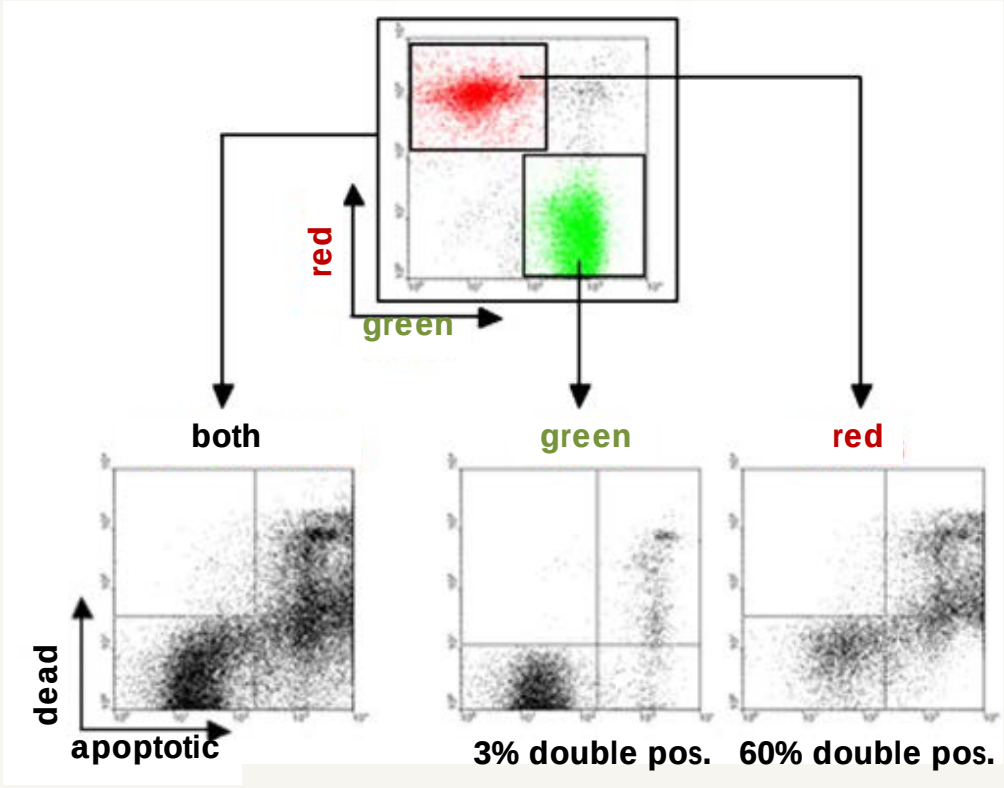


Why Gating is useful

- Is used to isolate a subset of cells on a plot
- We can use them to select a population for further study
- Allows the ability to look at parameters specific to only that subset



Mixed T cell population



Why Gating is useful (2)

- Doublet exclusion based on FSC-A vs -H vs –W gating
- Eliminates false positive events and cleans up your plots

From Annual Flow Cytometry course, University Zürich

Important Points on Analysis

- Flow data is primarily concerned with descriptive statistics
  - Enumeration of subsets
  - Level of fluorescence intensity
- Visualization
- What kind of data are you looking for?
  - How much fluorescence?
  - What percent are positive?
  - How much more positive is x than y?
  - What is the ratio between green and red fluorescence?
- What kind of statistics are available
  - MFI
  - %-ages
  - CV
  - ...

Summary II - the Main Points

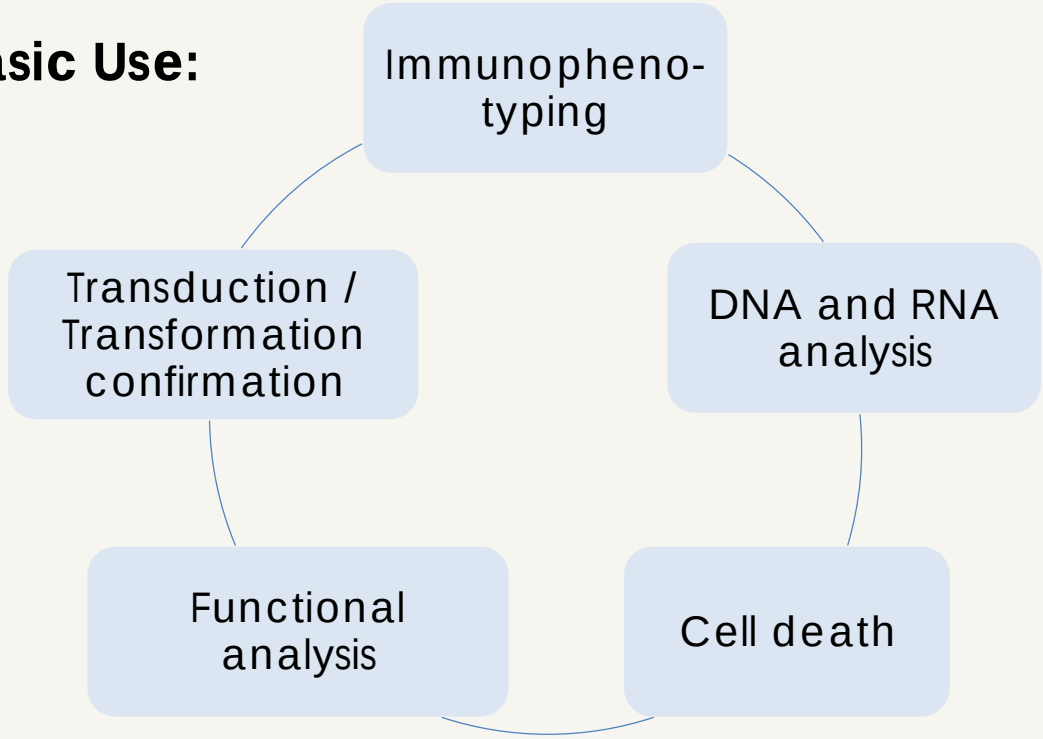
- Keep a smooth laminar flow! Prevent air and aggregates.
- To increase acquisition rates you have to concentrate your sample.
- The key to good results is good sample preparation.
- Know your instrument configuration when selecting for fluorescent probes.
- Gating helps to define your cells of interest.
- All measurements are relative, don't forget the controls.
- Include a viability dye and doublet discrimination gating to eliminate false positive events.

There is more...

Applications:

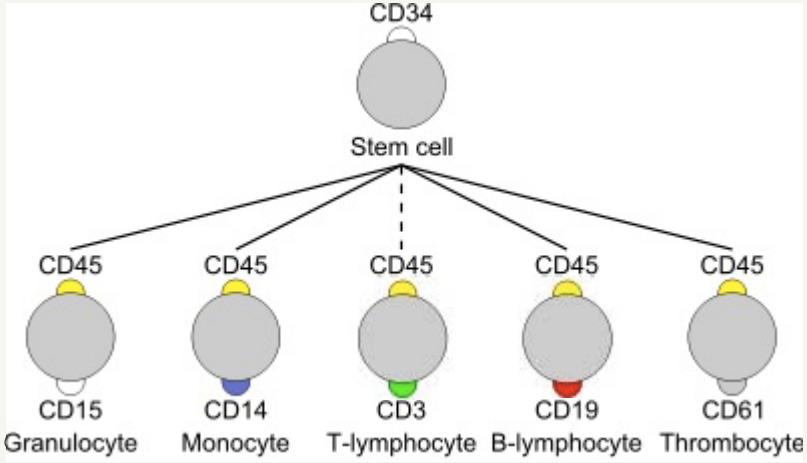
- Major applicacions phenotyping and fluorescent proteins
- Examples for special applications: RNAFlow & FRET

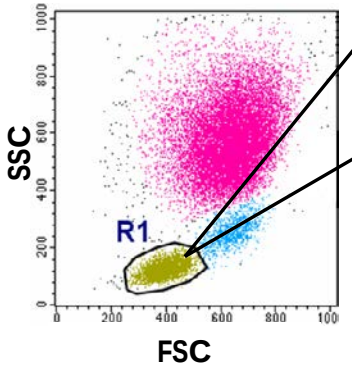
Basic Use:

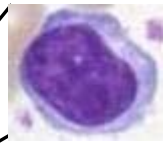


Immunophenotyping

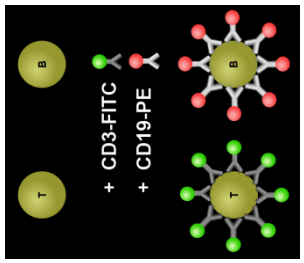
- Establish the presence or level of an antigen
- Use of labeled antibodies to identify cells of interest
- Detection of cell surface molecules as example cluster of differentiation

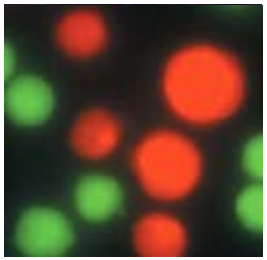


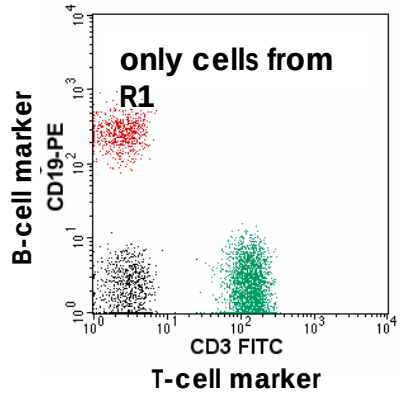




Lymphocyte
T or B????







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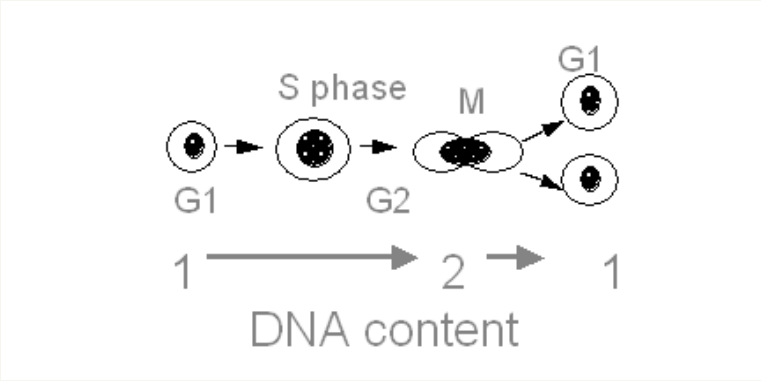
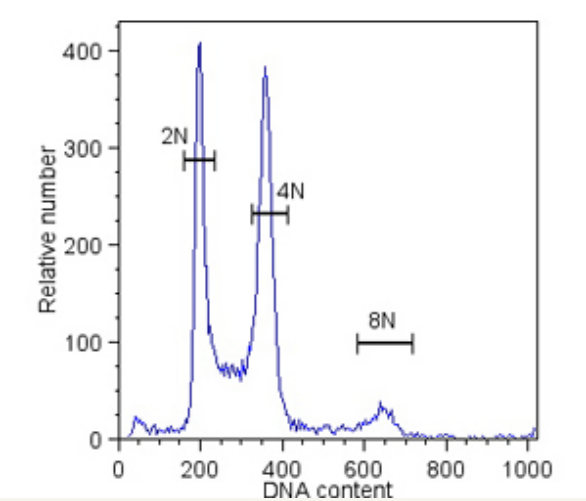
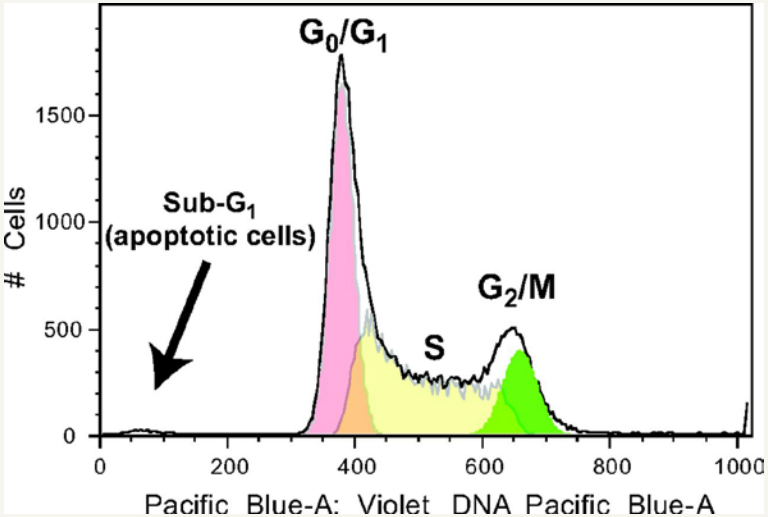
DNA Analysis

- DNA content of individual cells gives information about their ploidy
- Suitable dyes: PI, 7-AAD, DAPI
- Combination with light scatter or immunofluorescence



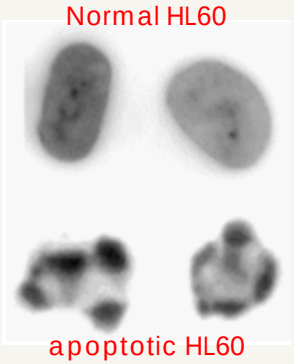


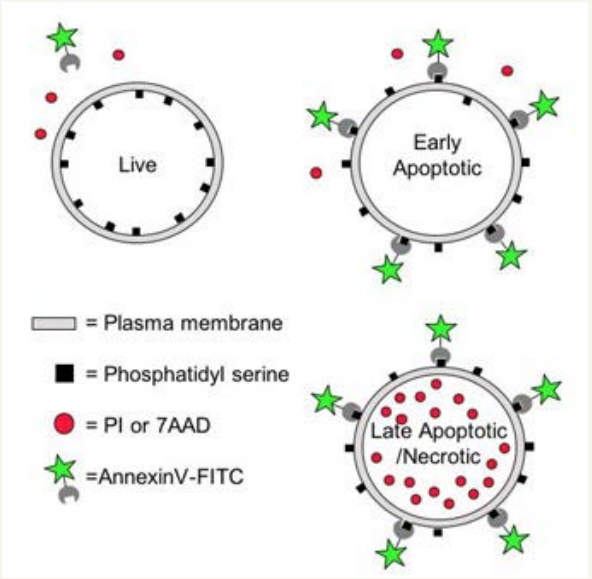


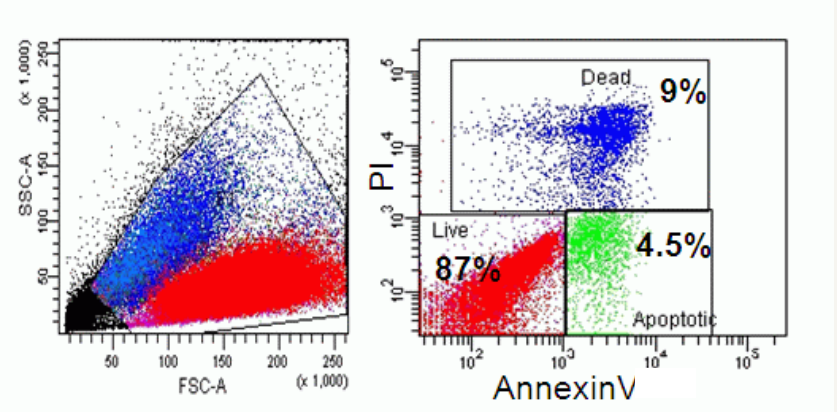
Cell Death

Measurements of cell death:

- Expression of proteins involved in apoptosis
- Activation of caspases
- Changes in the mitochondrial membrane potential
- Changes in the plasma membrane
- Cell shrinkage
- Chromatin changes
- DNA degradation

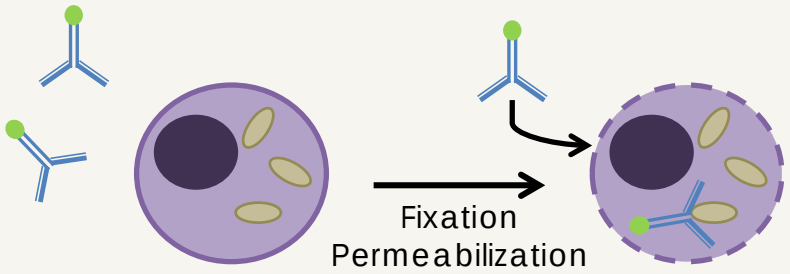


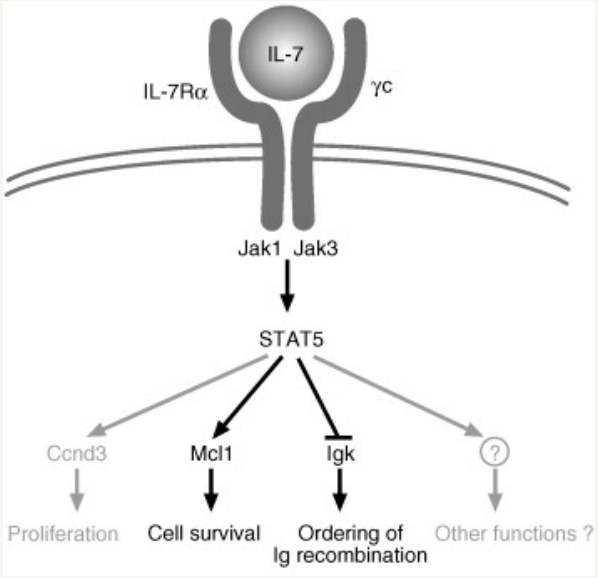


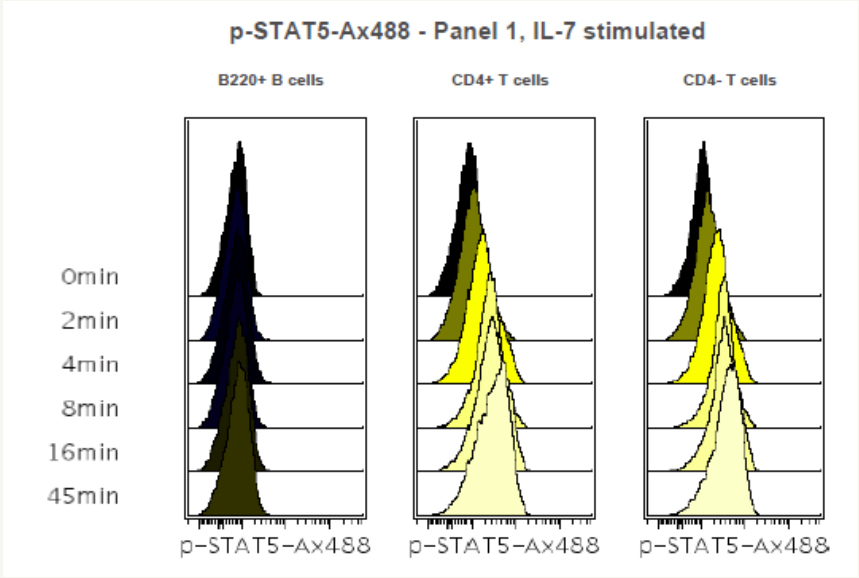


Intracellular staining

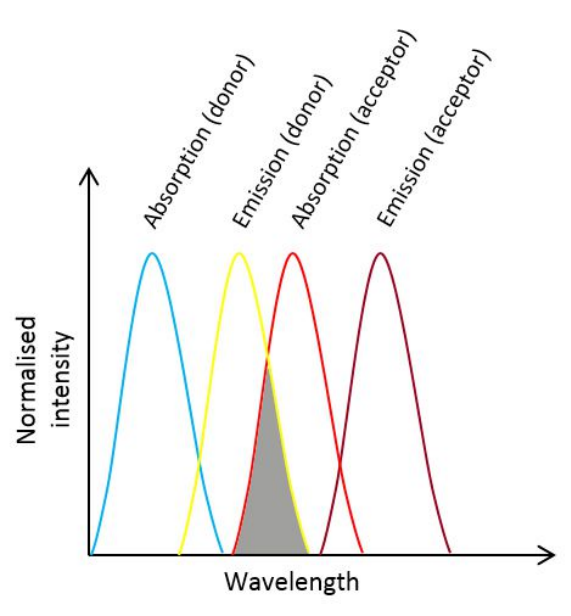
- Intracellular staining of cytokines, cytoskeleton, enzymes, transcription factors, signaling molecules
- Monitor signaling cascades



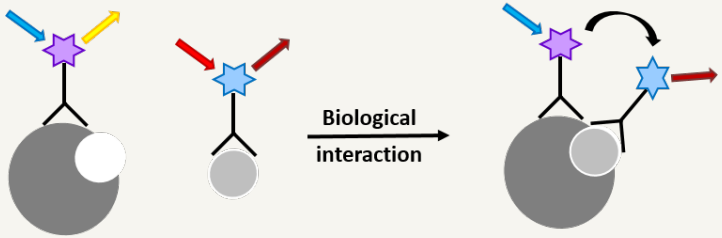


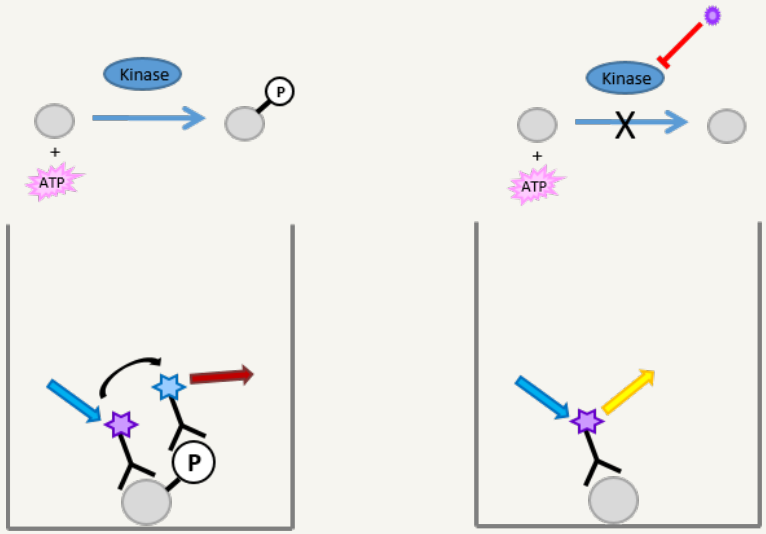


Fluorescence Resonance Energy Transfer Assays



- protein-protein interactions with the help of fluorescently labeled proteins
- distance between the two proteins must be less than 10nm
- The emission spectrum of the donor fluorophore must overlap the absorption spectrum of the acceptor fluorophore

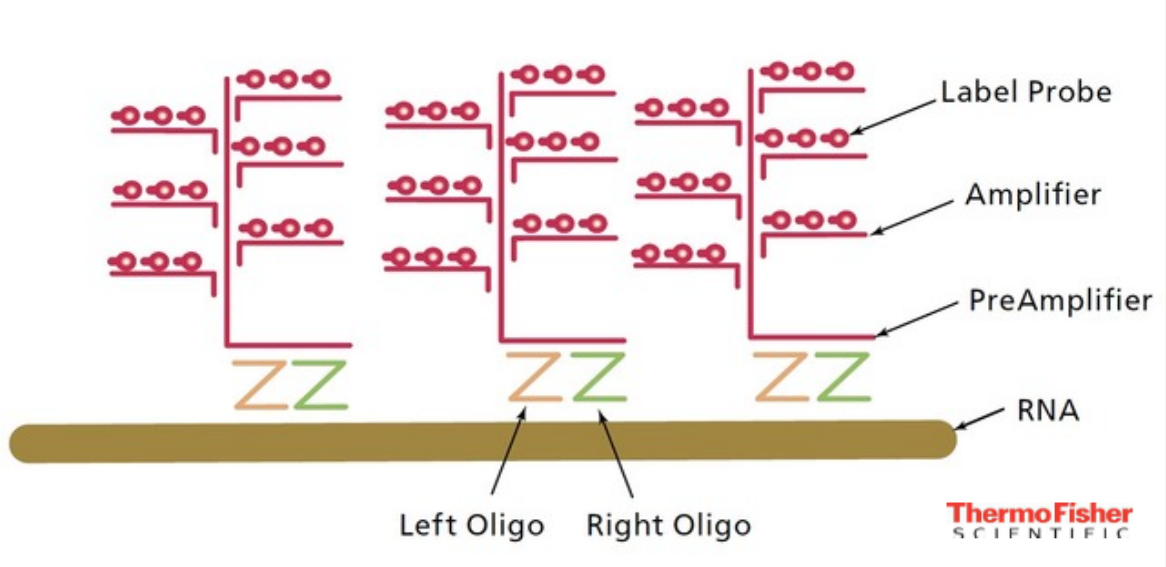




https://www.innovabiosciences.com/resources/applications/fret/

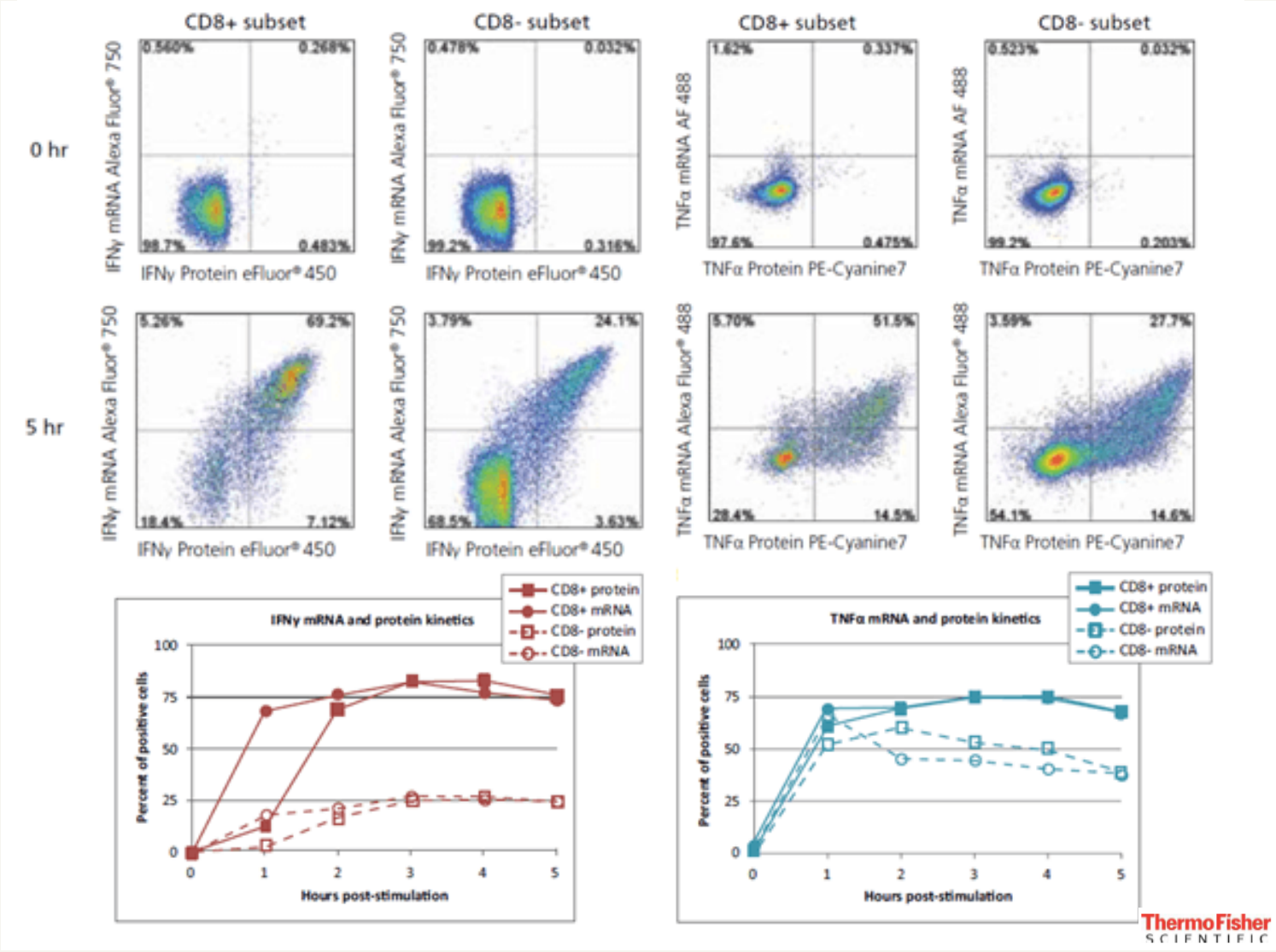
RNA Flow Assay

Gene-specific oligonucleotide probe set and branched DNA technology:



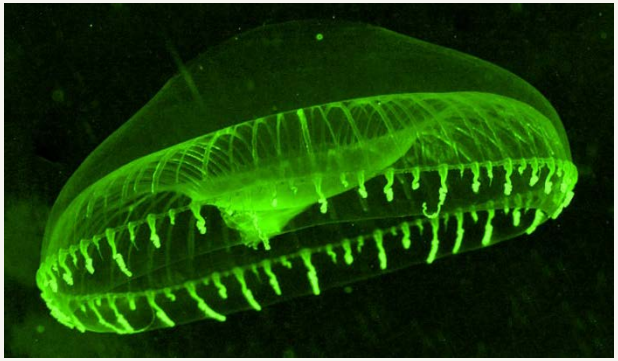
- Compare RNA and protein kinetics in the same cell
- Parallel analysis of microRNA targets in combination with antibody staining
- Detect target-specific RNA for which flow cytometry antibodies are nonexistent

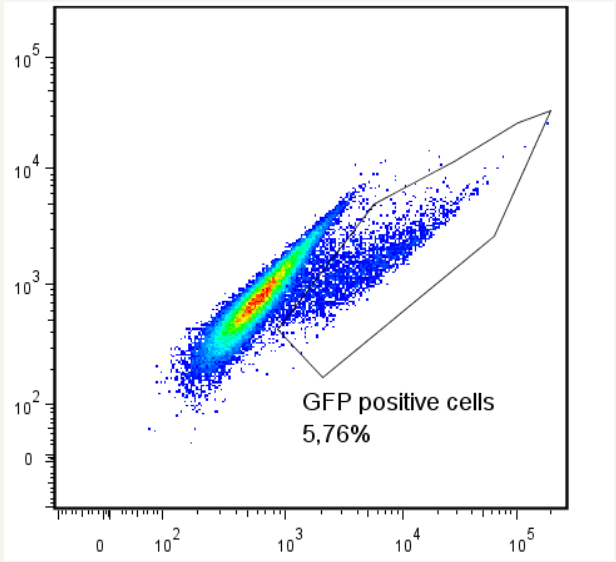
Compare RNA and Protein Kinetics



Gene Expression

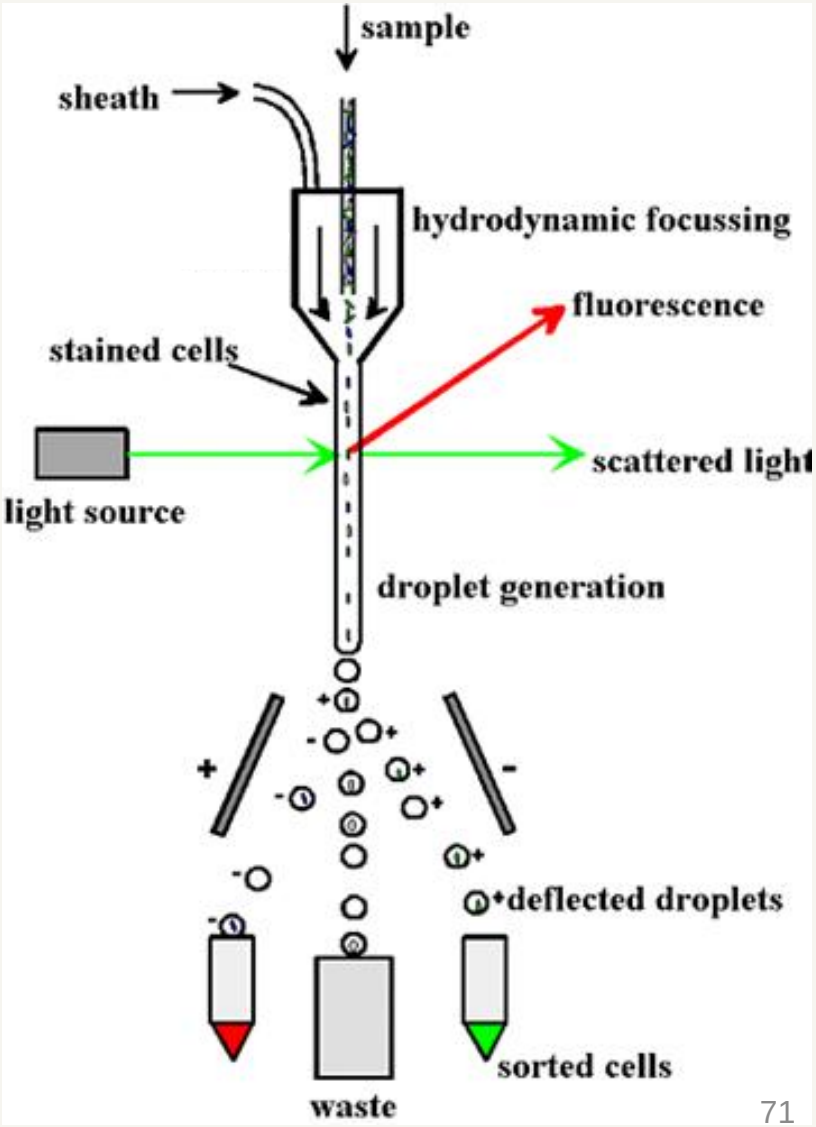
- Genes well characterized and can be cloned in frame with gene of interest
- Can be used to monitor rates of gene expression
- Commonly used as marker of transfection
- Level of intensity can be variable
- Great for cell sorting applications

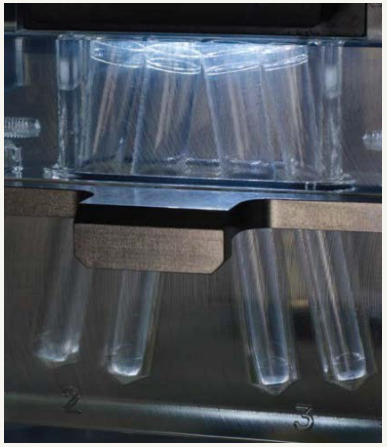




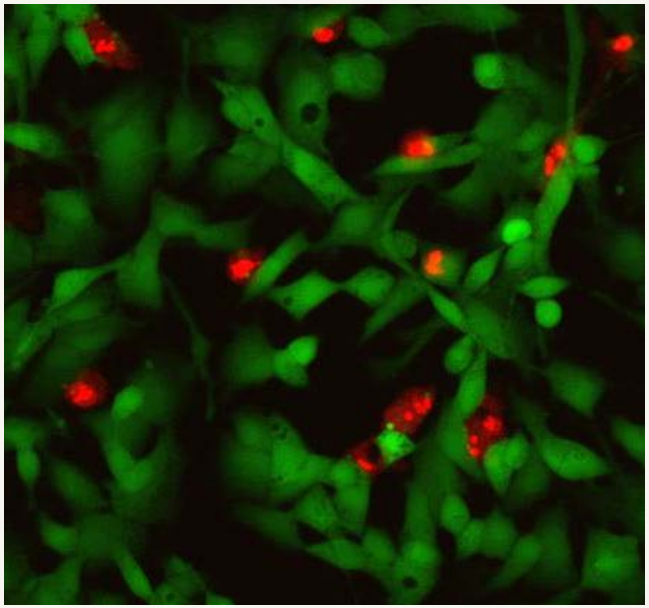
Principle of Sorting

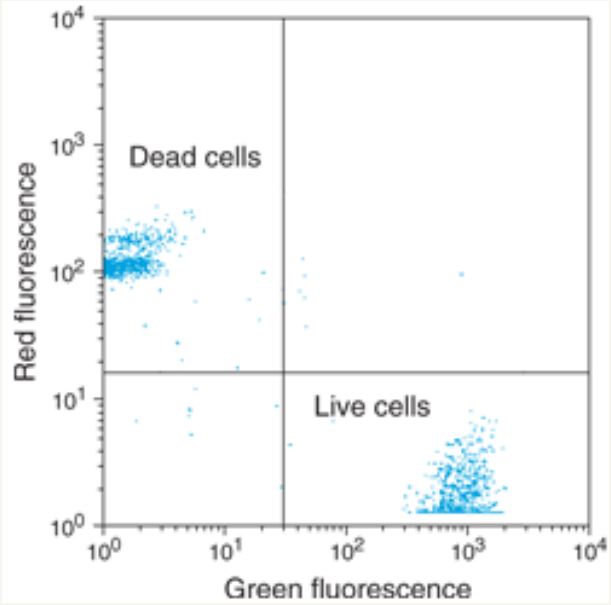
- Separating cells based on properties measured in flow is also called Fluorescence-Activated Cell Sorting (FACS)
- High-speed cell sorting is based on droplet deflection





Microscopy vs Flow Cytometry





Microscopy

- Localization of antigen is possible
- Poor enumeration of cell subtypes
- Limiting number of simultaneous measurements

Flow Cytometry

- Can not tell you where antigen is
- Fast
- Subpopulation analysis
- Multiparameter analysis
- Characterization of rare events

You are not alone!

Resources:

www.biozentrum.unibas.ch/research/groups-platforms/overview/unit/fcf/

isac-net.org

www.cyto.purdue.edu

flowbook-wiki.denovosoftware.com

www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp

www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

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