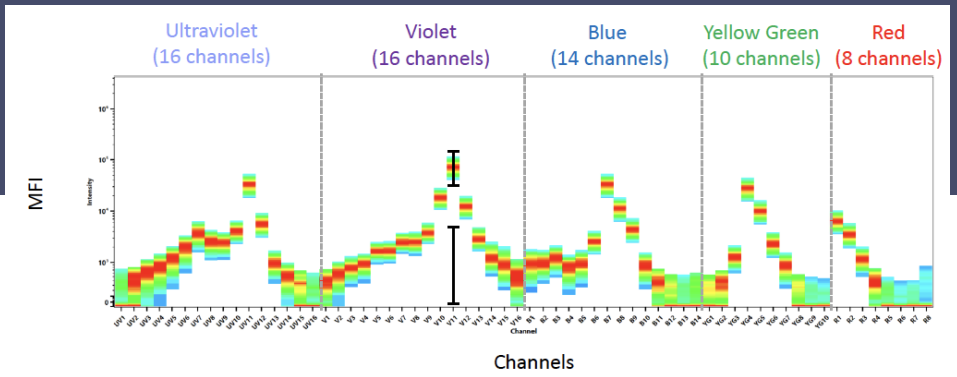
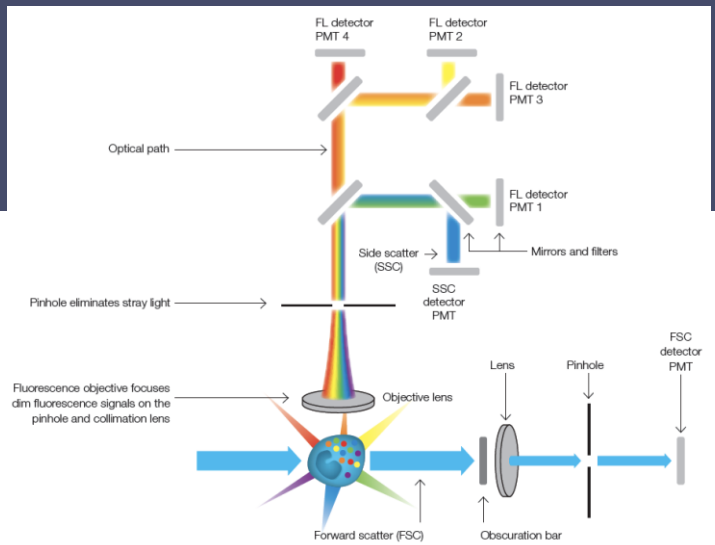
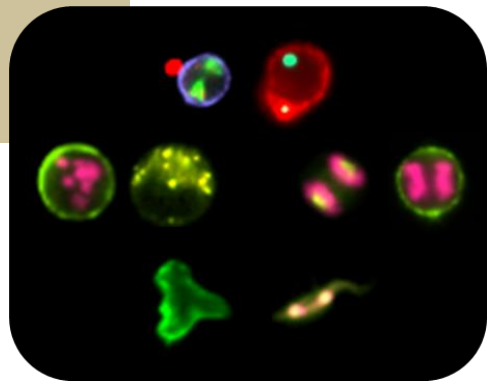




Flow Cytometry
&
Image Stream



PD Dr. Stella Autenrieth

Leiterin Core Facility Durchflusszytometrie

Universitätsklinikum Tübingen

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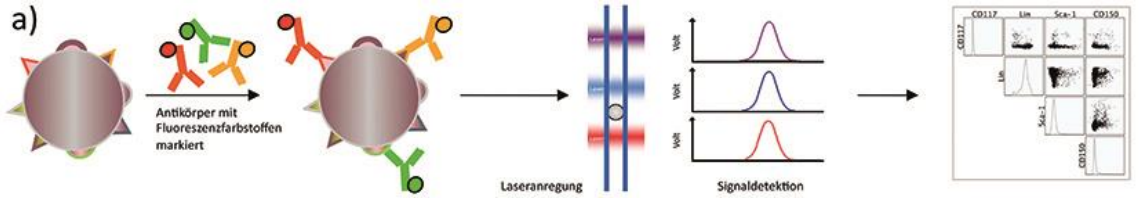
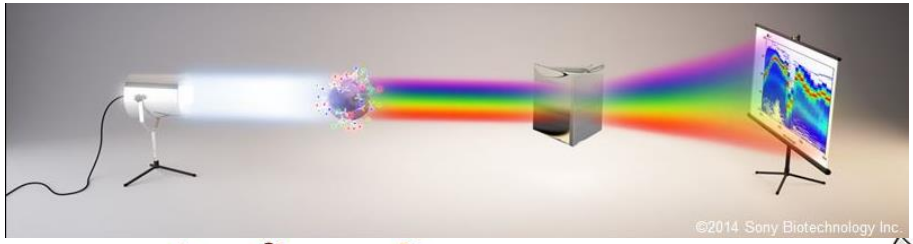
Today

- overview

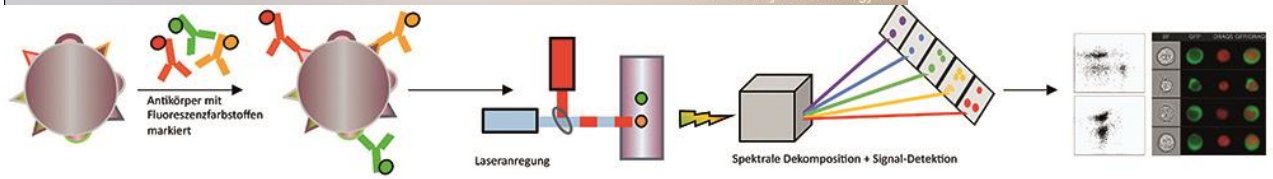
Flow cytometry

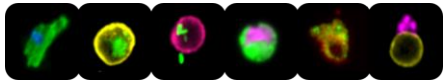


Spectral
flow cytometry



Imaging
flow cytometry

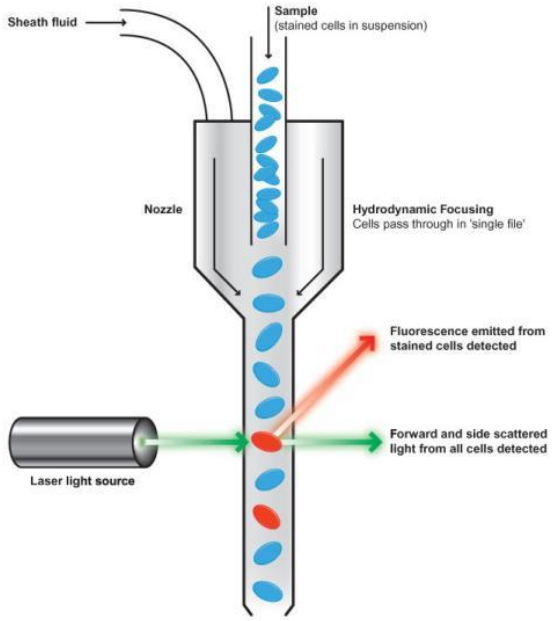




Definition of flow cytometry?

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

> Measuring properties of cells while in a fluid stream



https://www.withfriendship.com/user/mithunss/Flow-cytometry.php

What is flow cytometry?

An instrument for making cell-based fluorescent measurements.

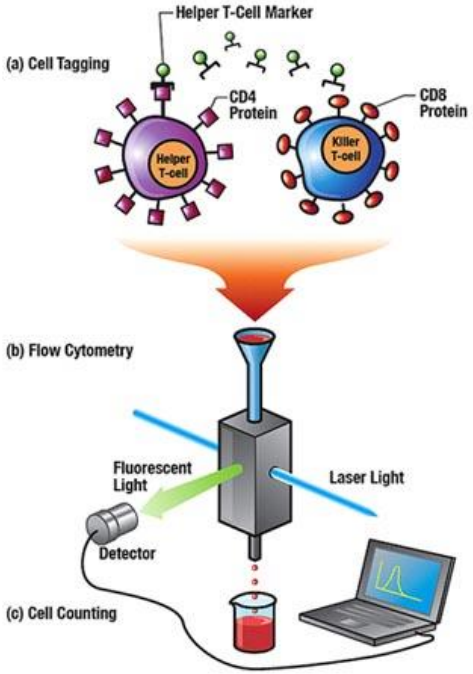
Method for quantitating cellular or structural components of a cell using fluorescent antibodies or probes.

Allows analysis of tens of thousands of cells in minutes.

A method to sort and collect specific cell types.



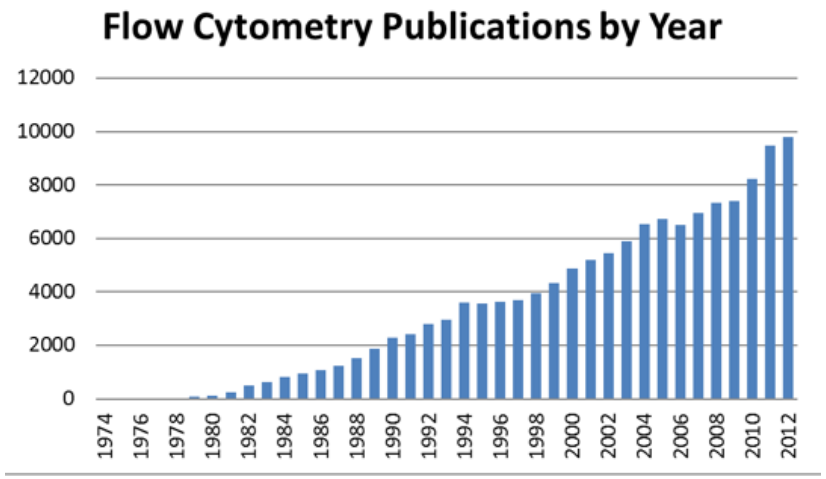
The BD FACS ARIA with sorter



https://www.withfriendship.com/user/mithunss/Flow-cytometry.php

Use of Flow Cytometry

- 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
- Enzyme activity
- Oxidative metabolism
- Signaling
- DNA synthesis / degradation
- Gene expression

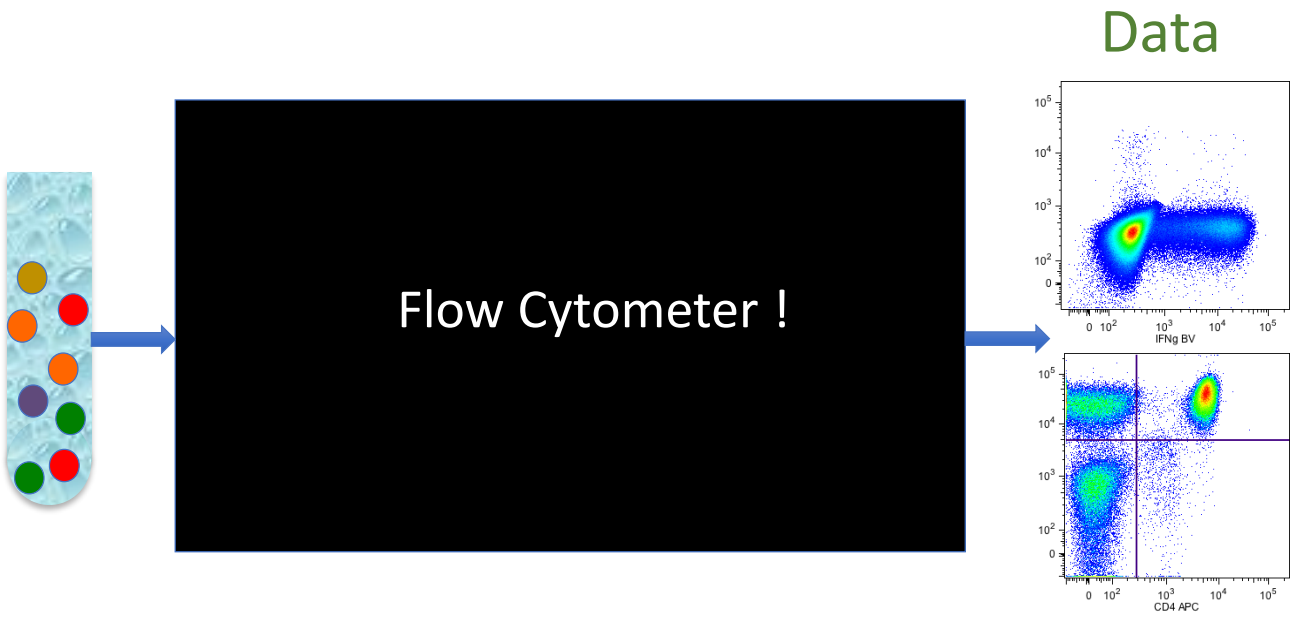


http://expertcytometry.com/flow-cytometry-resources/

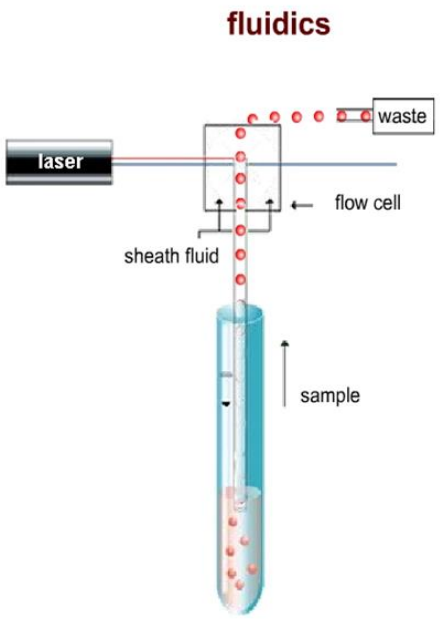
Workflow of a flow cytometry experiment



The Black box



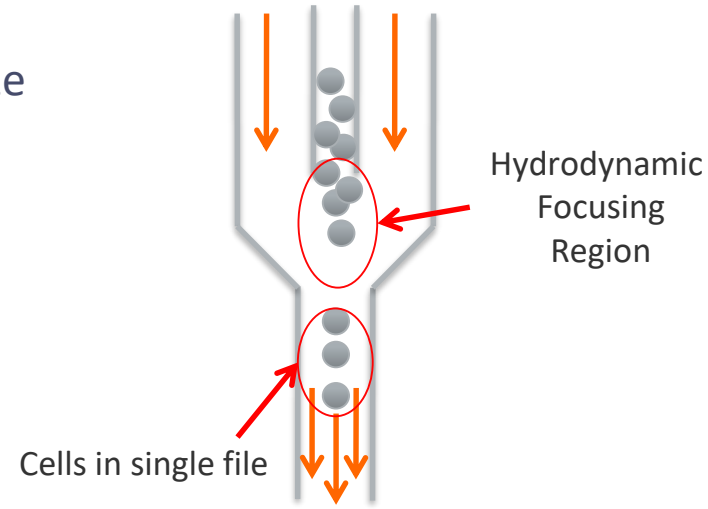
Into the black box: Flow Cytometry



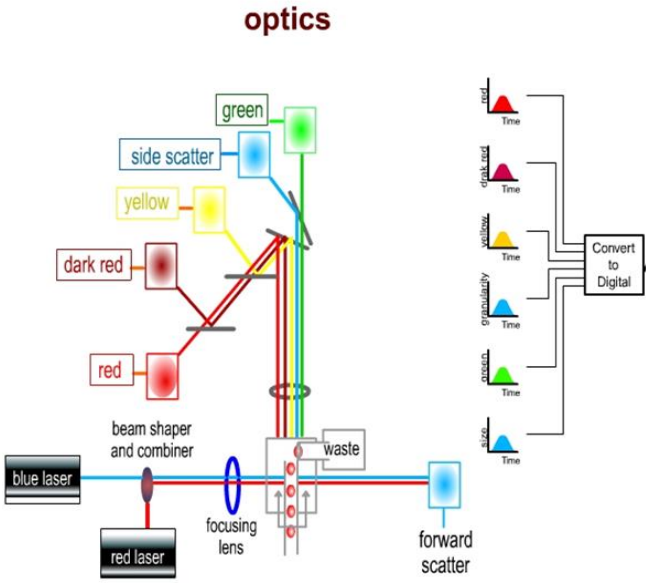
http://www.bu.edu/flow-cytometry/files/2010/09/Picture11.png

The Flow Cell

- Place where the sample is introduced to the sheath fluid
- Sample is hydrodynamically focused so the cells spread out to look like ‘pearls on a string’
- Sheath flow rate sets fluid flow rate
- Differential pressure sets core stream size

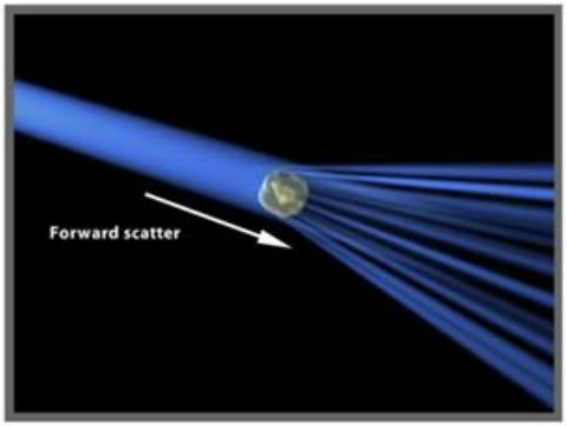


Into the black box: Flow Cytometry



http://www.bu.edu/flow-cytometry/files/2010/09/Picture11.png

FSC & SSC

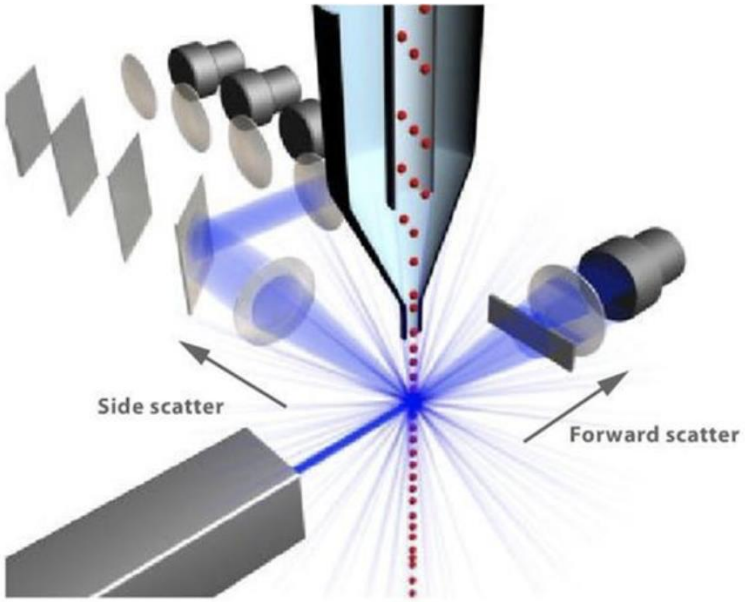


Cell surface

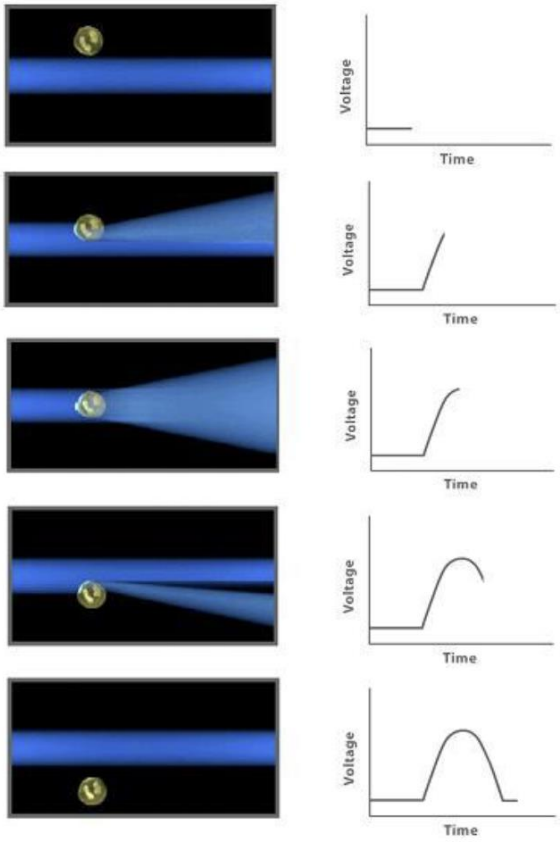
vesicles

Forward Scatter: FSC

- Light that is scattered in the *forward* direction is detected in the Forward Scatter Channel.
- The intensity of this signal has been attributed to cell size

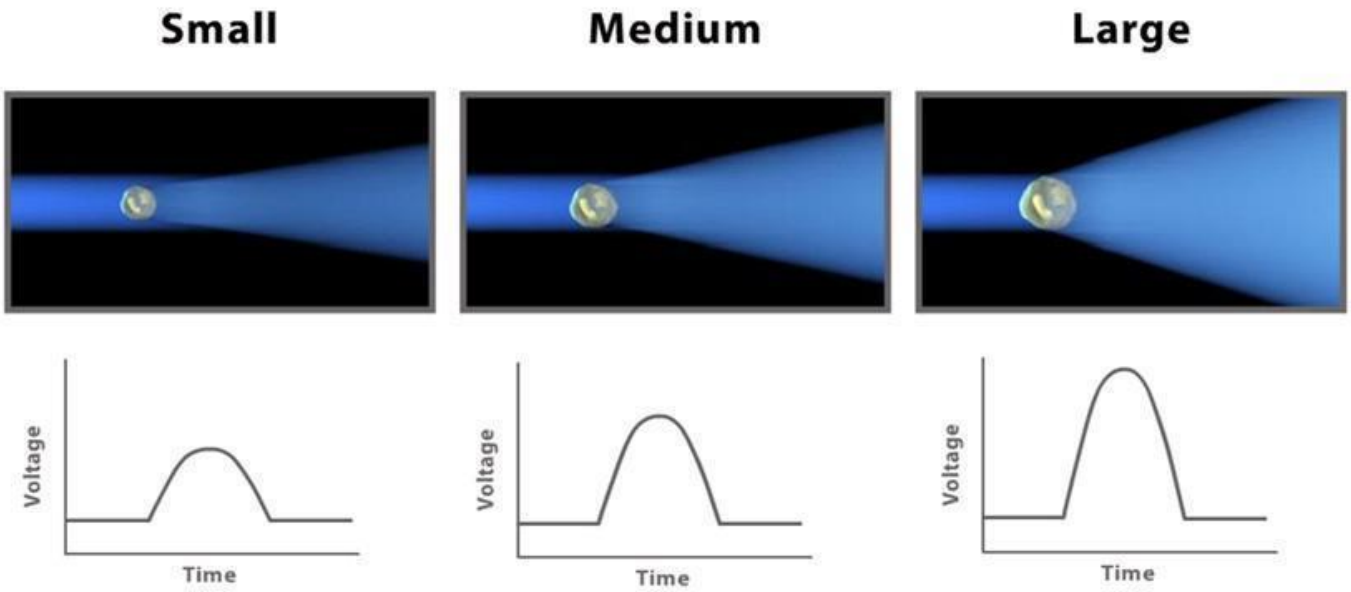


Signal formation



http://docplayer.org/4044770-Versuch-2-grundlagen-der-durchflusszytometrie.html

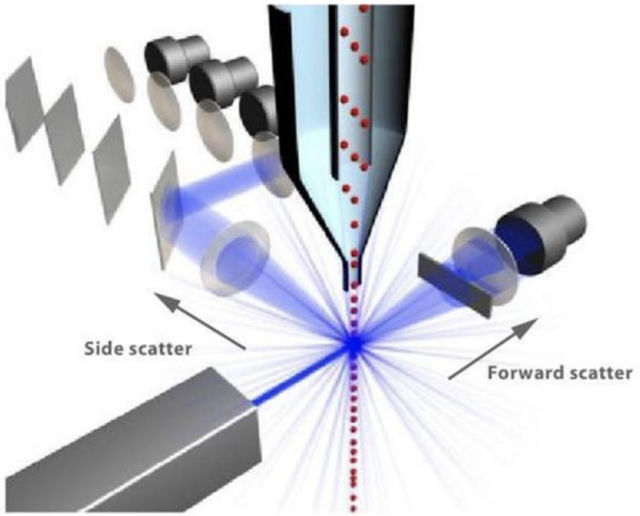
Signal - FSC



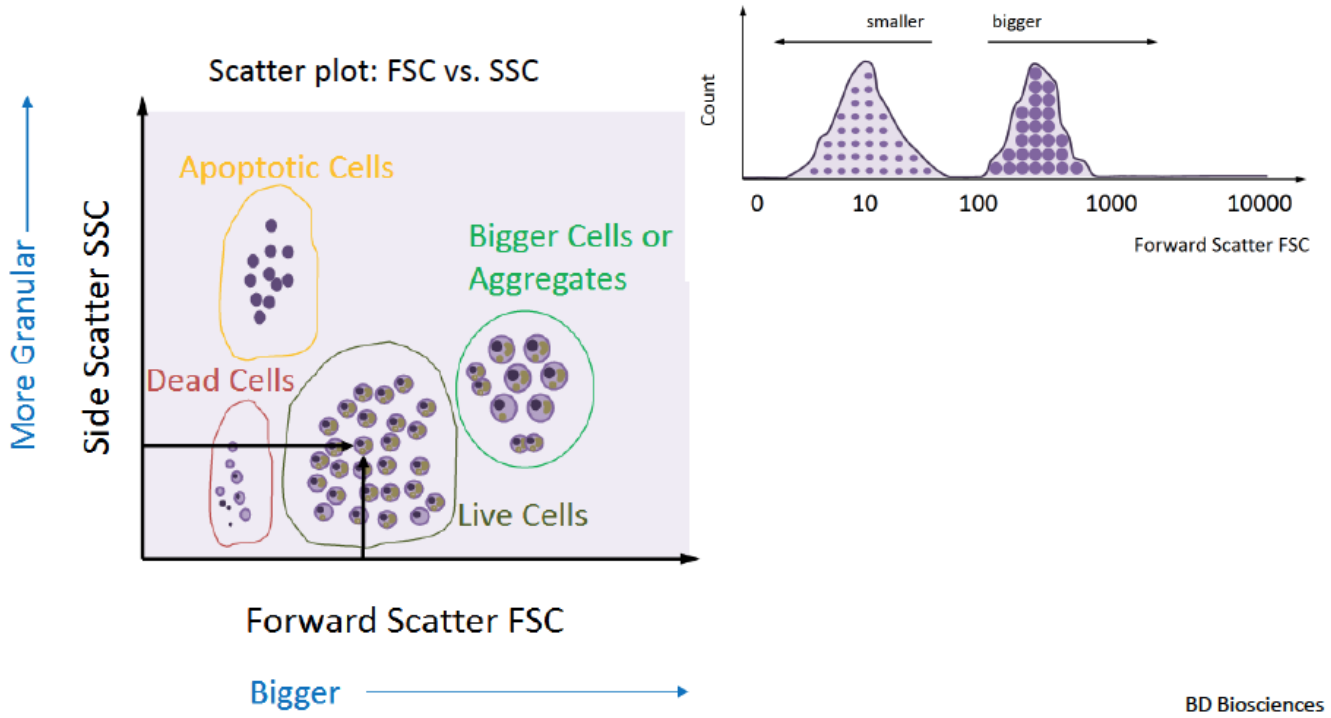
http://docplayer.org/4044770-Versuch-2-grundlagen-der-durchflusszytometrie.html

Side Scatter: SSC

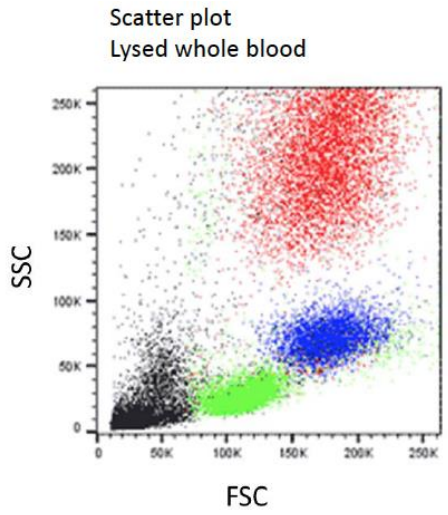
- Laser light that is scattered at 90 degrees to the axis of the laser path is detected in the Side Scatter Channel
- The intensity of this signal is proportional to the amount of cytosolic structure in the cell (eg. granules, cell inclusions, etc.)



Cell analysis based on morphology



Cell analysis based on morphology



Which population show lymphocytes, monocytes and granulocytes?

Fluorescence

- the molecular absorption of a photon triggers the emission of another photon with a longer wavelength.

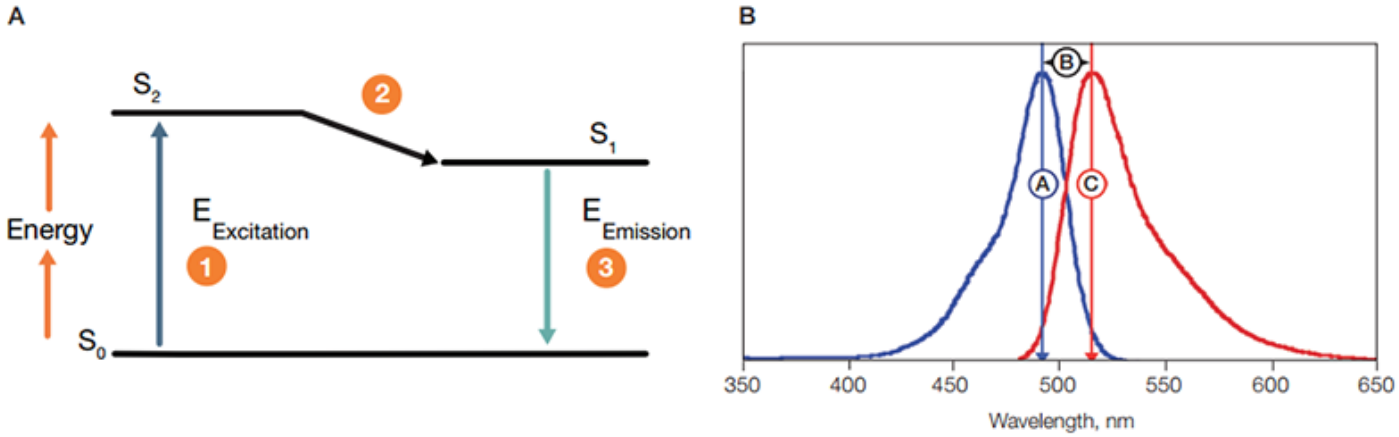


Figure 8. Stokes shift. A, upon excitation, **1,** electrons in a fluorophore move from a resting state, S_0 , to the excited electronic single state, S_2. Some energy is released as heat, **2.** The remaining energy is released as fluorescence, **3,** as the electrons return to their ground state, S_0. **B,** the difference between the excitation maxima, Ⓐ , and the emissions maxima, Ⓒ , of a fluorophore is called its Stokes shift Ⓑ .

Fluorescence

- Light = electromagentic energy that travels in wave
- Wavelength determines the colour

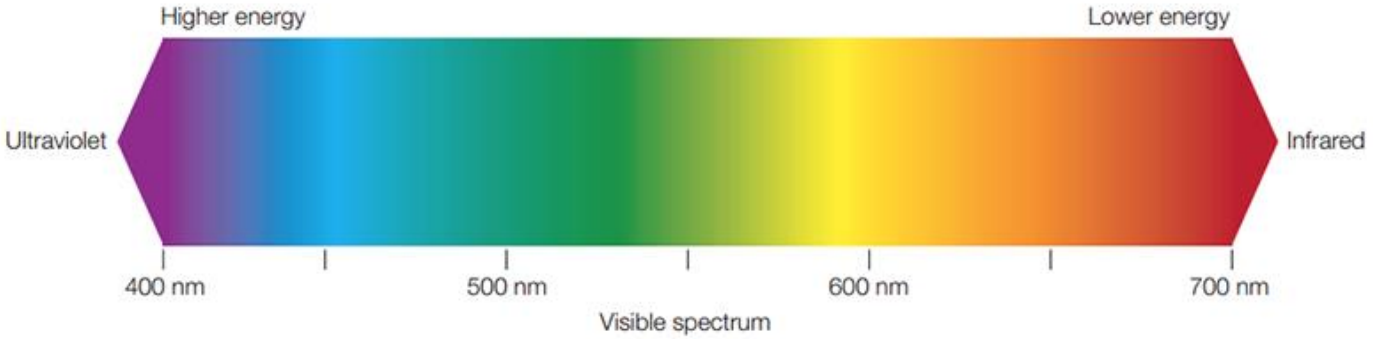


Figure 7: The electromagnetic spectrum

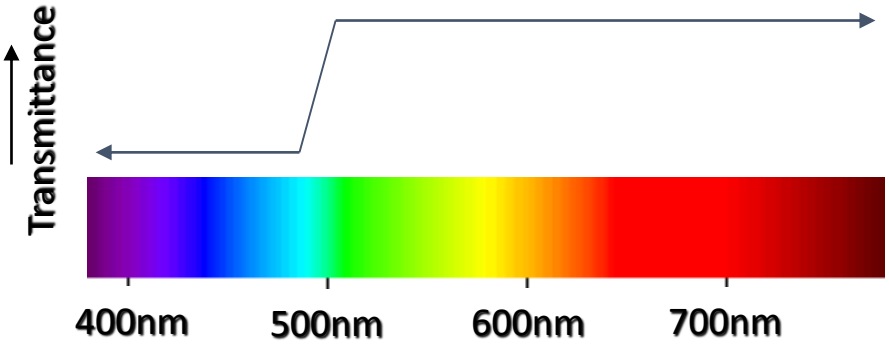
Filters

- Many wavelengths of light will be scattered from a cell, we need a way to split the light into its specific wavelengths in order to detect them independently. This is done with filters
- Optical filters are designed such that they absorb or reflect some wavelengths of light, while transmitting other.
- 3 types of filters
  - **Long Pass filter**
  - **Short Pass filter**
  - **Band Pass filter**



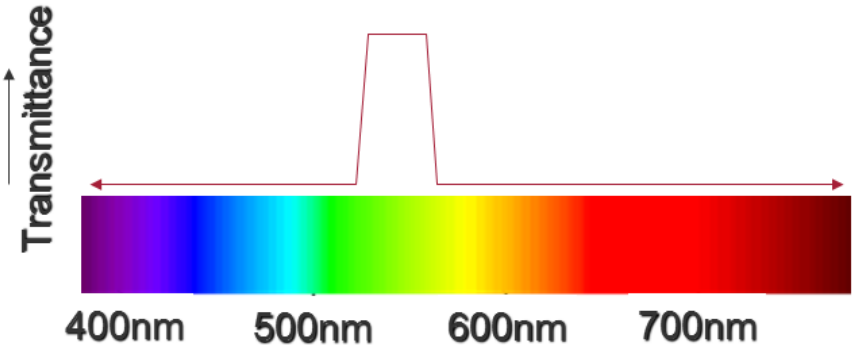
Long Pass Filters

- Transmit all wavelengths greater than specified wavelength
  - Example: 500LP will transmit all wavelengths greater than 500nm



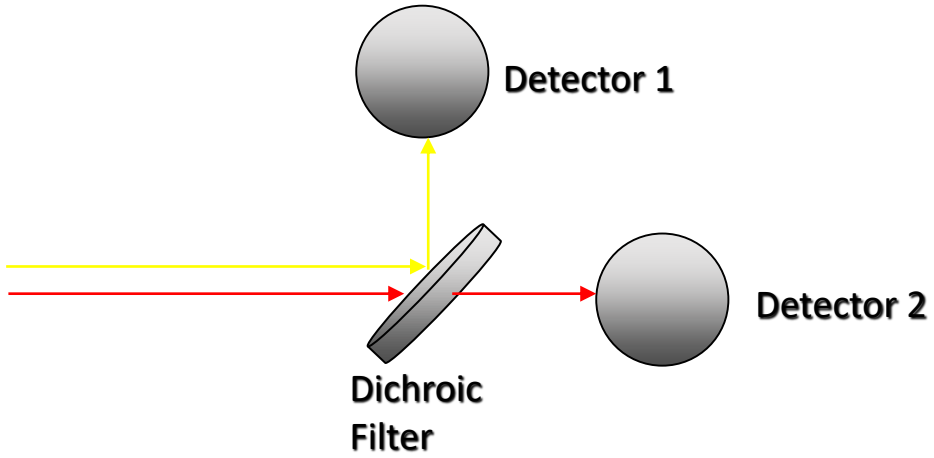
Band Pass Filters

- Transmits a specific band of wavelengths
  - Example: 550/20BP Filter will transmit wavelengths of light between 540nm and 560nm *(550/20 = 550+/-10, not 550+/-20)*

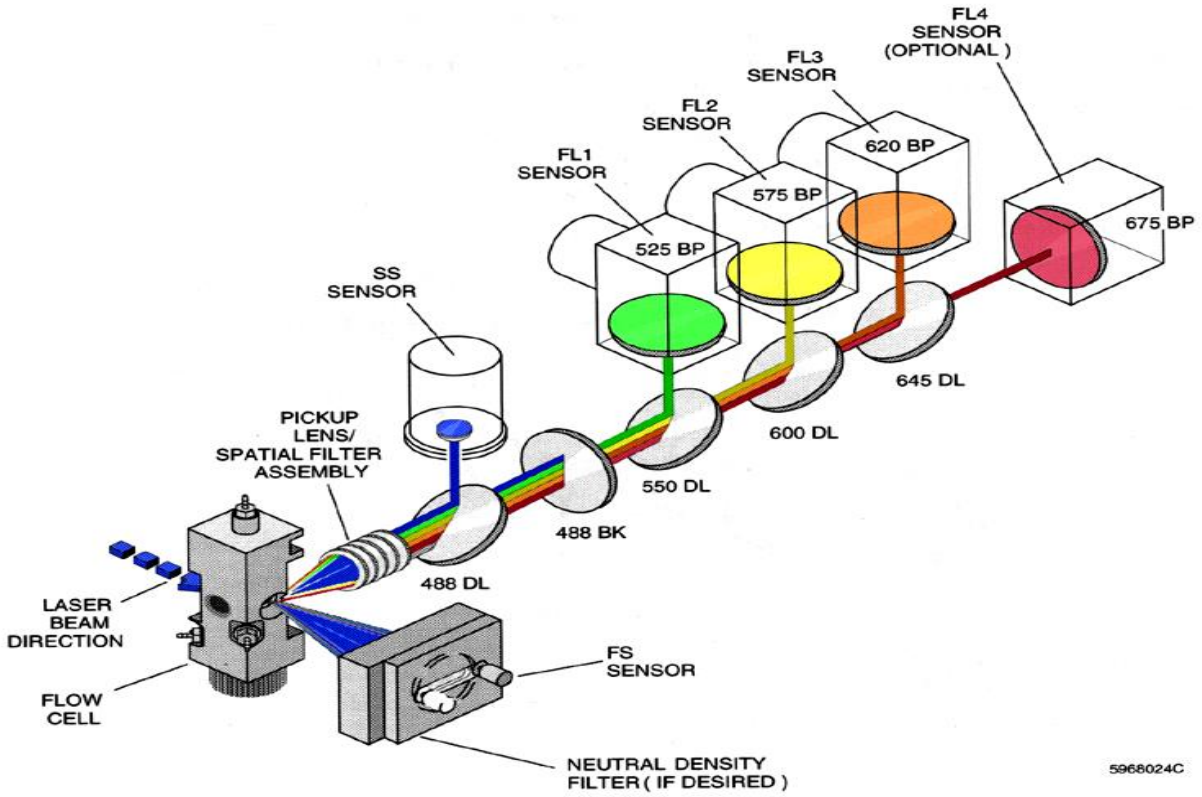


Dichroic Filters

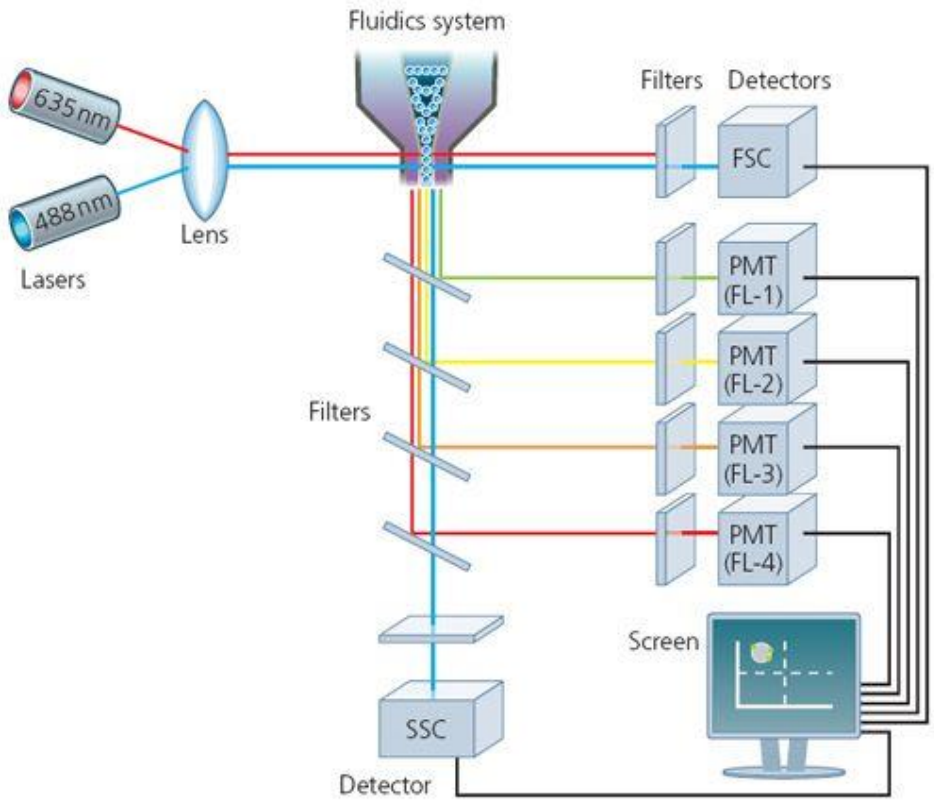
- Can be a long pass or short pass filter
- Filter is placed at a 45° angle to the incident light
- Part of the light is reflected at 90° to the incident light, and part of the light is transmitted and continues on.



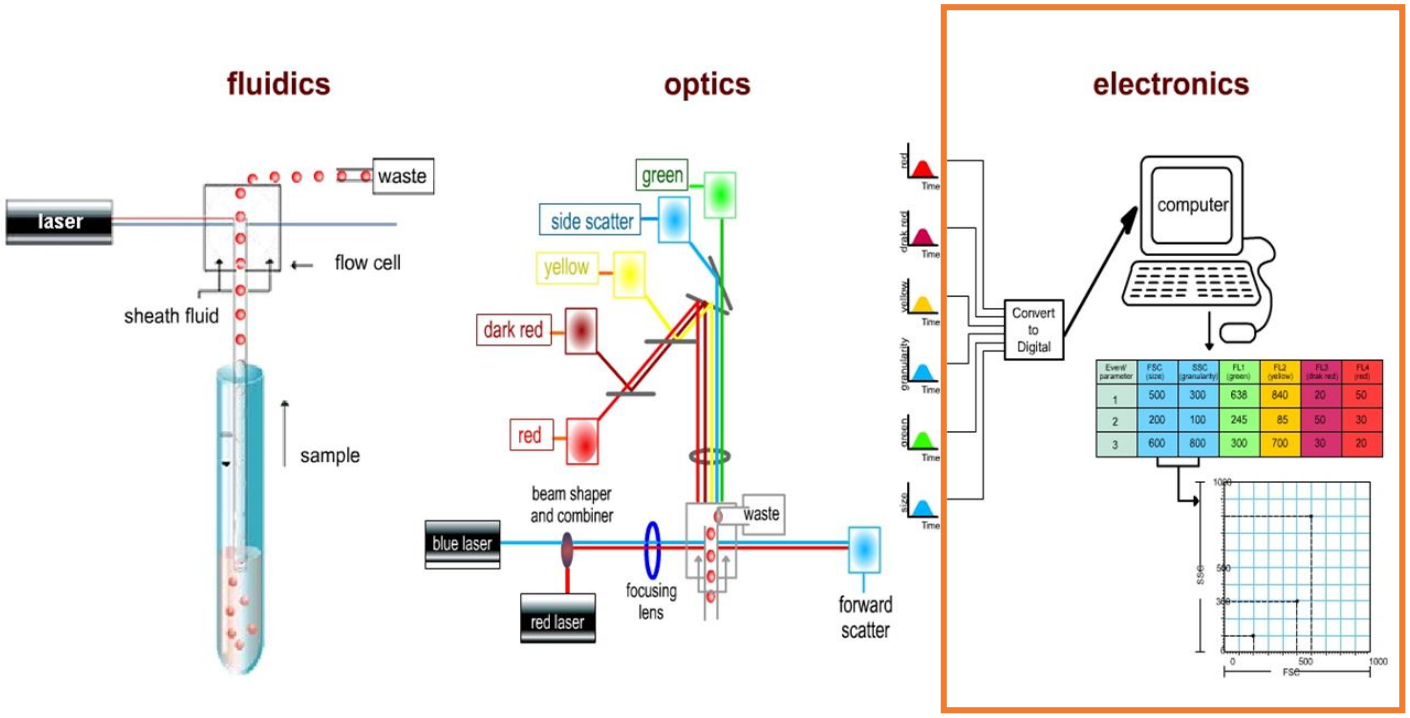
Example Channel Layout



PMT – Photomultiplier tube



Into the black box: Flow Cytometry



http://www.bu.edu/flow-cytometry/files/2010/09/Picture11.png

Today…

Flow Cytometry

- Instrumentation: Into the black box
  - Fluidics
  - Optics
- Fluorochromes & Compensation
- Examples

Spectral flow cytometry

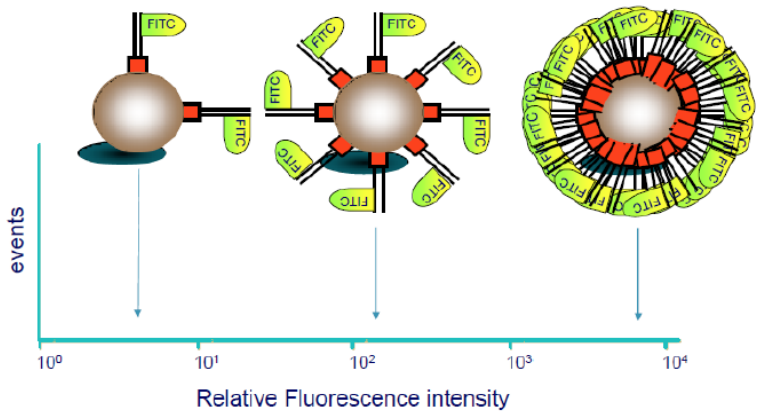
- Instrumetnation
- Examples

Image Stream

- Instrumentation
- Examples



Florescence intensity



The emitted fluorescence light is proportional to the amount of bound fluorescence molecules

BD FACSAria Operator Manual

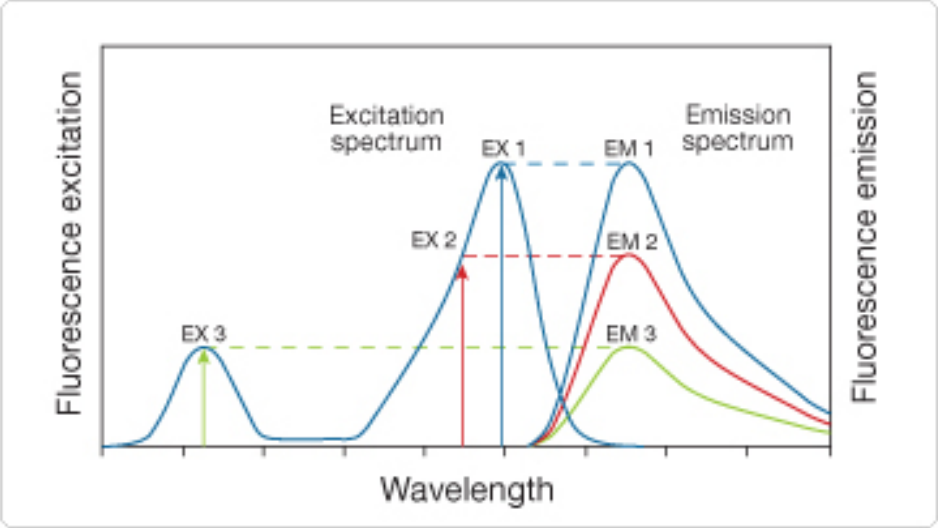
Types of fluorochromes

- Fluorescent proteins: found in nature
  - Phycoerythrin (PE), allophycocyanin (APC)
  - GFP, YFP, etc..

- Synthetic Compounds
  - Cyclic ring compounds
    - FITC, Texas Red, Alexa 488
  - Tandem dyes
    - BV570, Cy5-PE, Cy7-APC
  - Polymer and nanocrystal dyes
    - BV421, Quantum Dots



Excitation and emission spectra

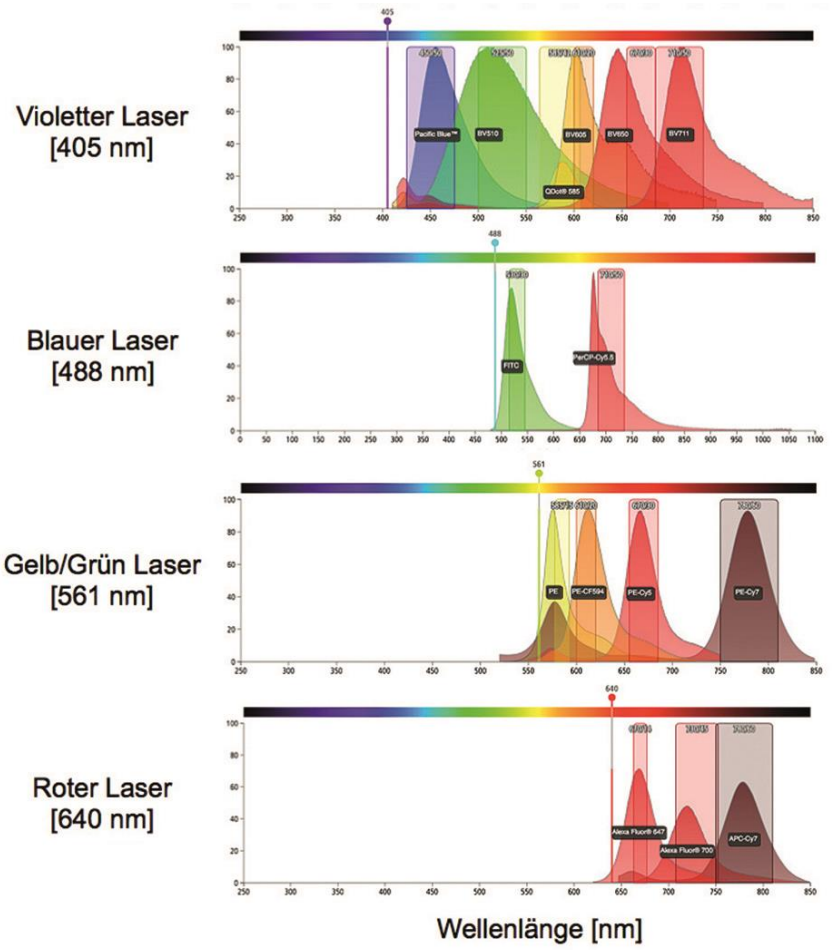


This fluorophore is excited by more than one wavelength, shown as Ex 3, Ex2, and Ex1

Peak excitation occurs at Ex1,
off peak excitation occurs at Ex2, and is measurable,
but substandard excitation occurs at Ex3.
Regardless of the strength of the excitation stimulus, the **color** range of light emitted by the dye is the same; however, the emission energy is brighter, or more intense, when the dye is optimally excited by Ex1.

http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Introduction-to-Fluorescence-Techniques.html

LSR Fortessa:
emmision spectra of fluorochromes

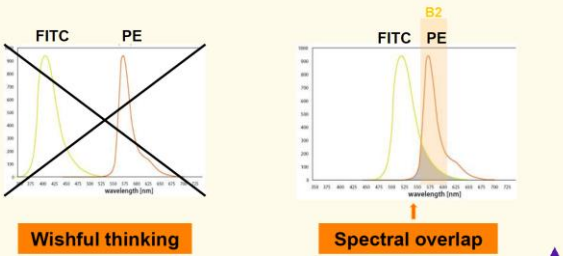


Compensation

Fluorochromes

Absorb light with a certain wavelength and emit light of longer wavelengths (low-energy)

FITC: Extinction. 493 nm, Emission. 525 nm



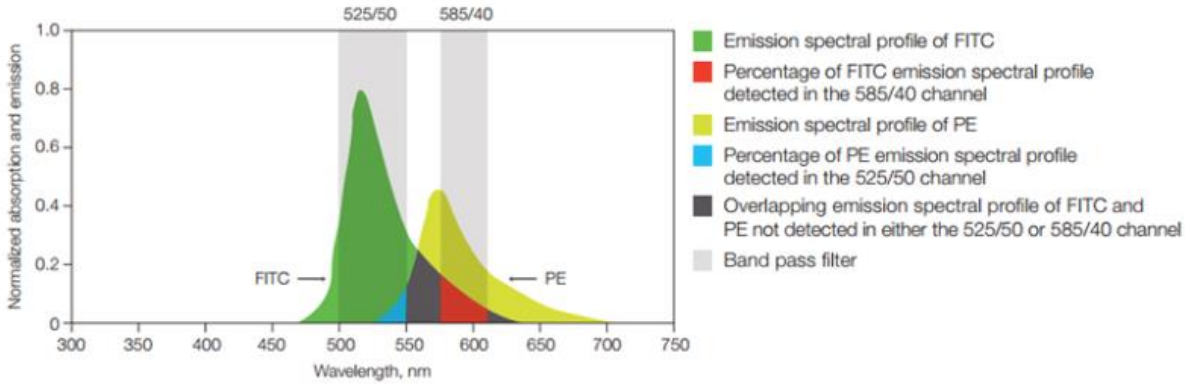
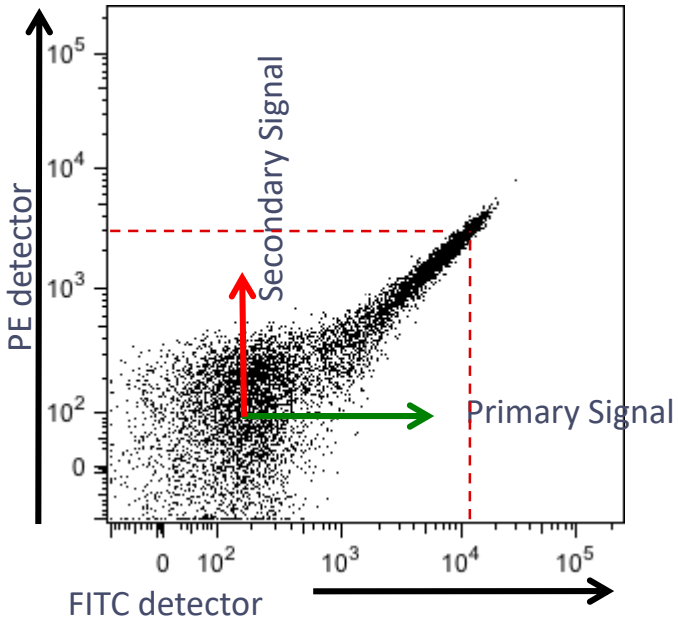


Figure 11. Fluorescence compensation. Emission spectra of two fluorophores commonly used in flow cytometry, FITC and PE are shown. Also shown is a graphical representation of two commonly used filters, 525/50 and 585/40, to detect these fluorophores. Shown in red is the portion of the FITC spectrum that will be detected in the PE detector (585/40) and that must be subtracted from the PE signal using compensation. This process becomes even more complicated when photons from multiple dyes are detected in each PMT.

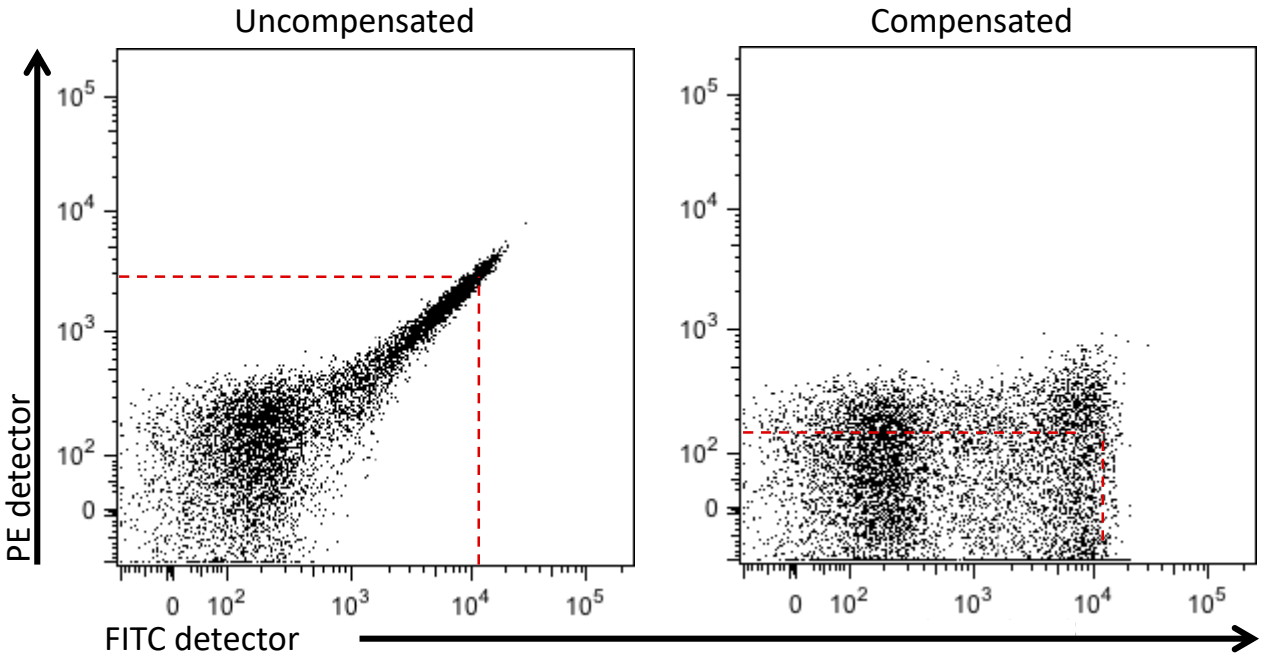
Compensation

PMBCs labeled with a FITC-conjugated antibody



Primary signal in FITC only sample is measured with a green optical filter

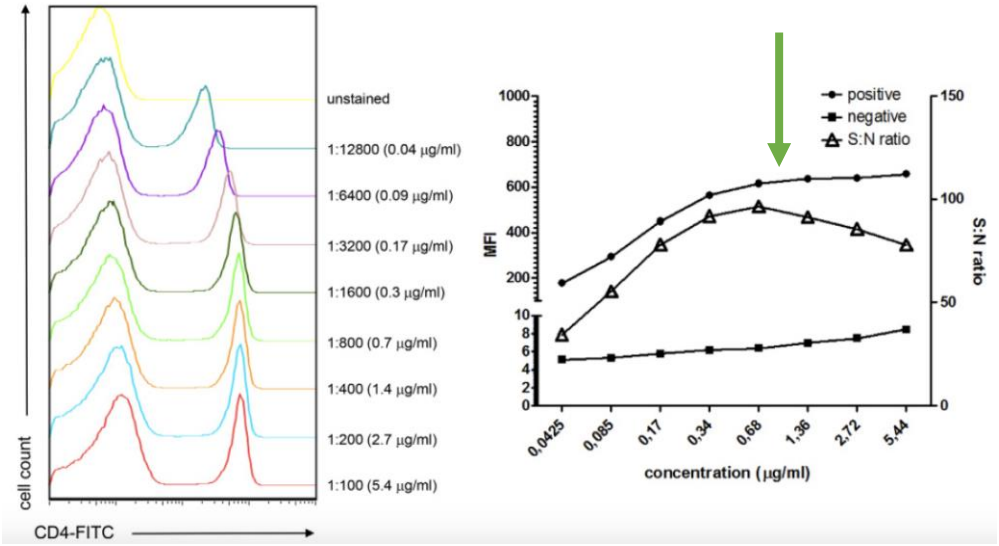
Compensation



Workflow of a flow cytometry experiment



Ab titration

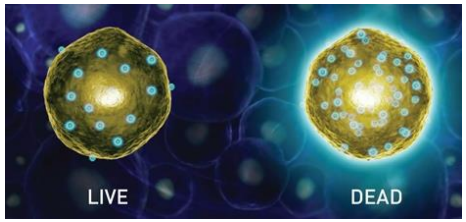


1. Improved staining accuracy
2. Saves money
3. Decreases non-specific binding

Controls - cell viability

Staining of cells with cell-viability tools

http://www.thermofisher.com

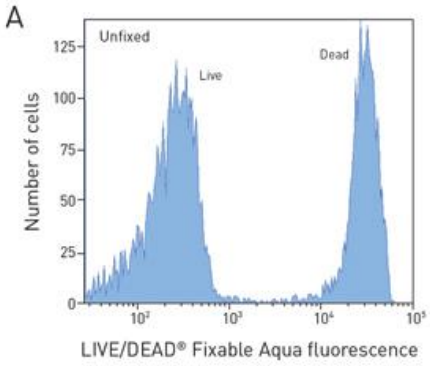


- DAPI (4',6-diamidino-2-phenylindole)
- PI (Propidium iodid)
- 7-AAD (7-amino-actinomycin D)

} Stain nuclei by binding to double stranded nucleic acids
Prerequisite:
pores in cell membrane

- Fixable Viability Dyes
  (amine reactive dyes)

} Suitable for fixation conditions



Dead cells unspecifically bind antibodies and exhibit a higher autofluorescence

Understanding the capabilities of the flow cytometer

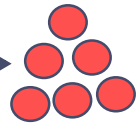
- How many lasers? What are the wavelengths?
- How many detector channels for each laser?
- Which fluorochromes can be detected?

Laser	violet 402 nm						blue 488 nm		yellow/green					red 640 nm		
PMT position	F	E	D	C	B	A	B	A	E	D	C	B	A	C	B	A
Splitter		495 LP	555 LP	635 LP	695 LP	750 LP	495 LP	635 LP		600 LP	635LP	685LP	750 LP	None	695 LP	750 LP
BP filter	450/50	525/50	605/15	661/11	711/25	785/60	525/50	690/50	586/15	615/20	670/30	700/40	785/60	670/30	725/40	785/60
fluorochromes	BV421	BV510	BV605	BV650	BV711	BV786	FITC	PerCP-Cy5.5	PE	PE-Dazzle594	PE-Cy5	PE-Cy5.5	PE-Cy7	AlexaFluor®647	AlexaFluor®700	APC-Cy7
Antibody used	CD366	lin/viability	CD11b	CD123	CD279	CD16	CD15	CD14	CD274	CD1c	-	-	CD33	-	HLA-DR	CD11c

Example 1 – Extracellular Staining - Workflow



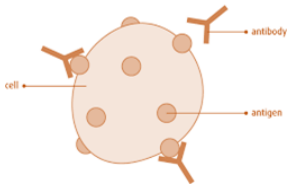
Preparation of single cell suspension



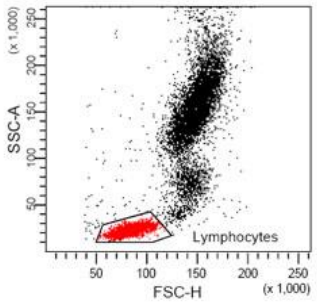
Blocking of Fc receptors

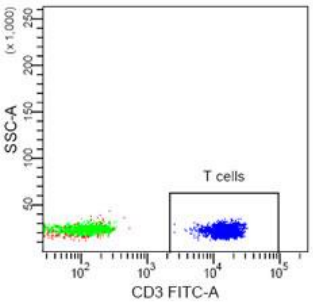


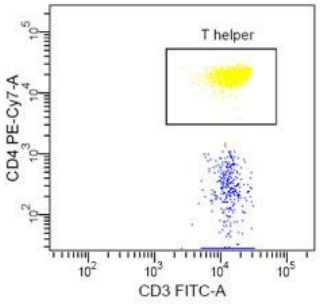
Extracellular staining with surface markers (e.g. T-cells)

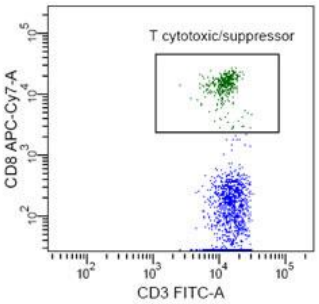


Analysis by flow cytometry







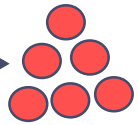




Example 2 – Intracellular Staining - Workflow



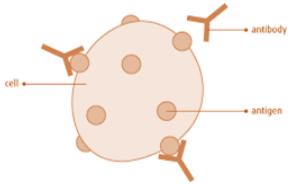
Preparation of single cell suspension



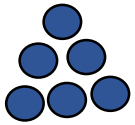
Blocking of Fc receptors



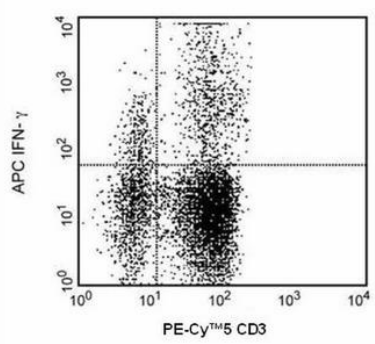
Extracellular staining with surface markers (e.g. T-cells)

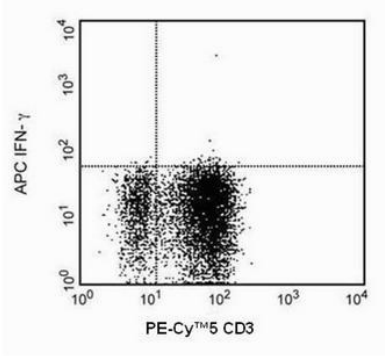


Fixation and Lysis



Intracellular staining (e.g. IFN-γ) & analysis by flow cytometry

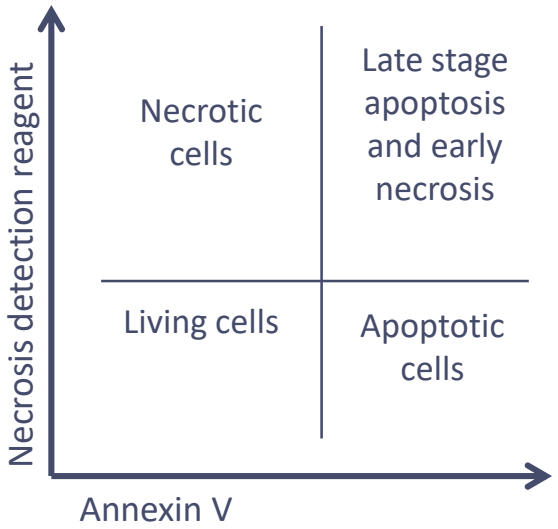


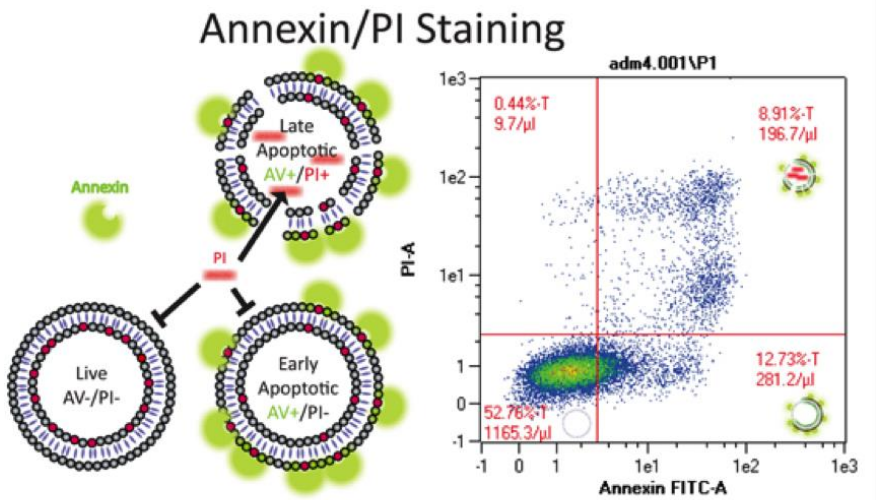


Example 3 – Apoptosis / Necrosis

Class of reagents staining apoptotic cells

- Annexin V (apoptosis) – phosphatidyl serine
- PI or 7-AAD (necrosis)





Example 4 – Cell Proliferation

Dye dilution proliferation analysis

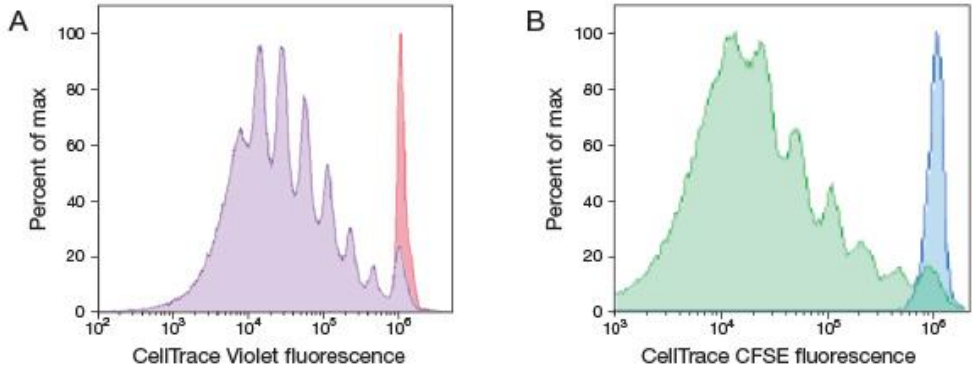
- number of cell divisions can be determined following the dilution of the label

carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)

diffuses into cells

converted to carboxyfluorescein succinimidyl ester by reaction with amines

stabilizes the fluorescent product in the cell



Why using high parameter flow cytometry?

Profile deep into a lineage of cells

CXCR4 (CD184)	CD28
CD16	CXCR6
CD45	CD127
CCR6	CD103
CD2	CD62L
CD57	Live.Dead Marker
CCR4 (CD194)	IL13
CD45RA	IL17A
CD25	IFNγ
CD3	CCR9
CXCR3 (CD294)	TNF
CD8	CD4
CRTH2 (CD294)	

Profile broadly across all types of blood cells

Subsets:

T cells

B cells

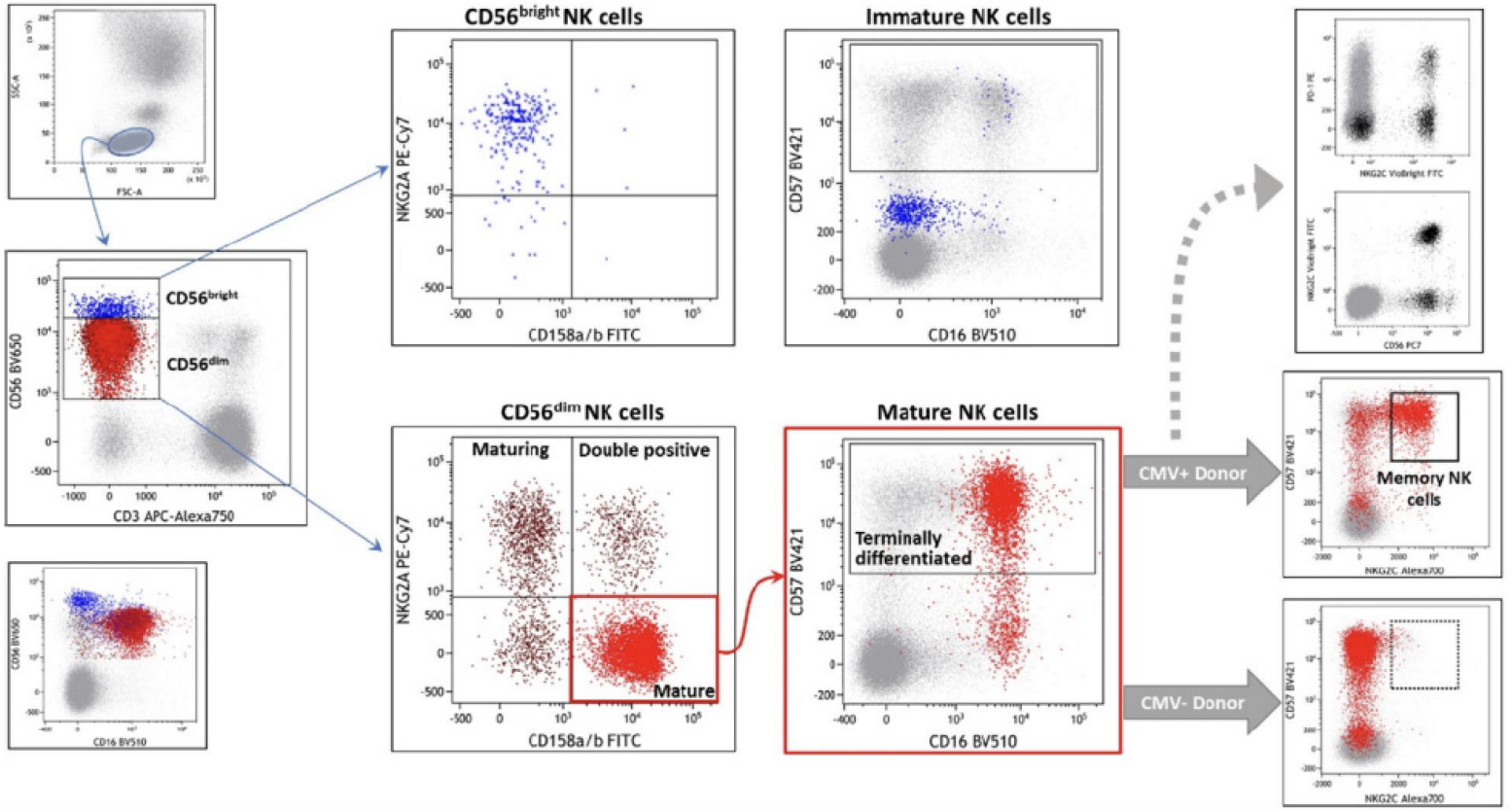
NK cells

DCs

Granulocytes

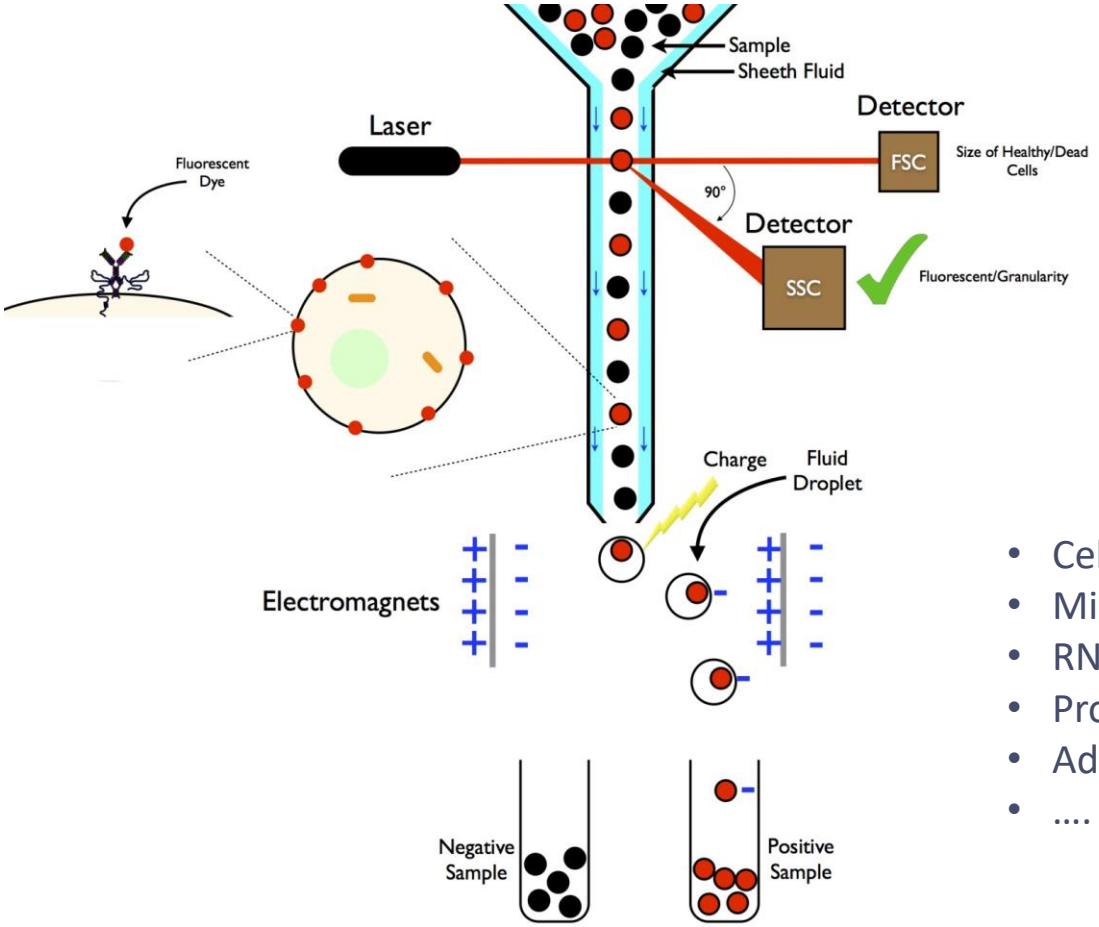
Monocytes

Immunophenotyping: NK cells from CMV patients



Cossarizza et al. Eur J Immuinol 2019

Example 5 – Cell Sorting

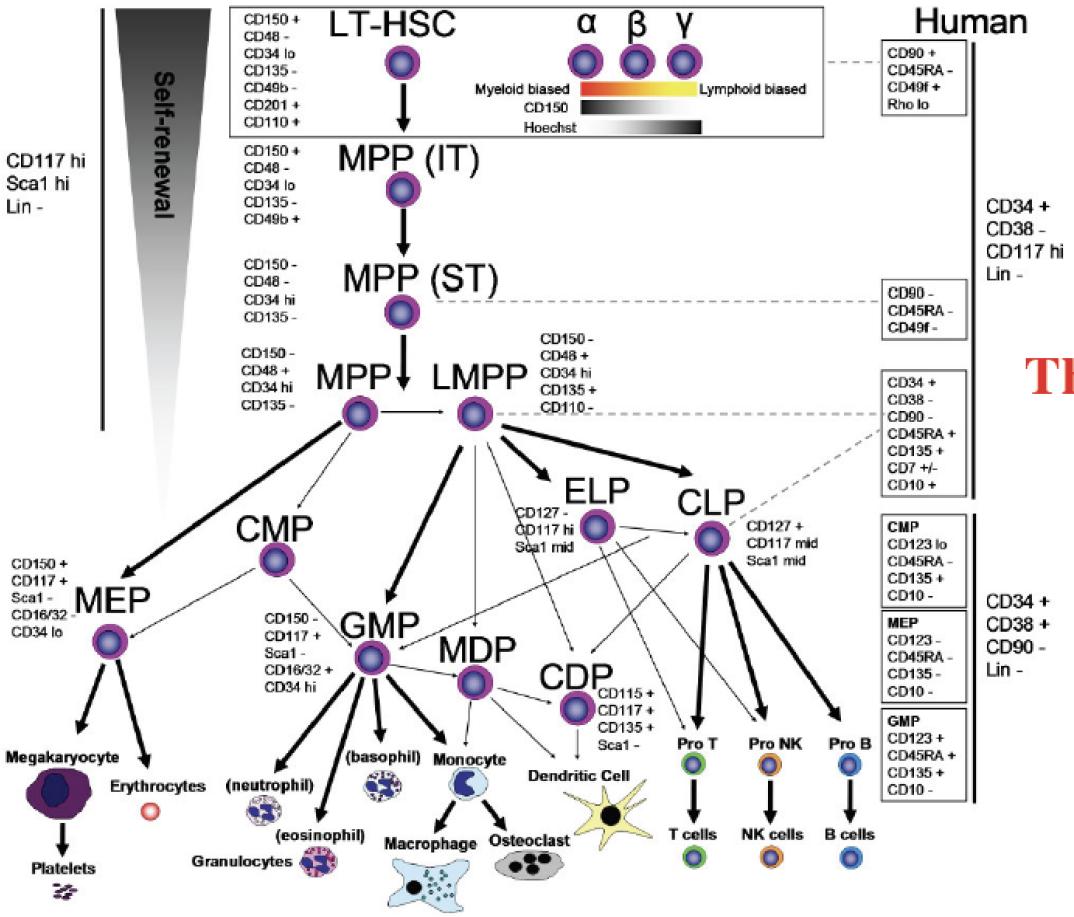


- Cell culture
- Microarray
- RNA seq
- Proteomics
- Adoptive transfer experiments
- ….

Examples of flow cytometrical measurements

Analysis parameters	Examples
Surface antigens Intracellular antigens	Phenotyping, cell type identification , protein expression (e.g. GFP), developmental stages, activation markers
DNA/RNA content	Cell cycle analysis
Cell activation	cell death, apoptosis, proliferation, membrane potential, Ca^{2+} release, phosphorylation
Intracellular distances Protein-protein interaction	FRET
Cell or particle number	Counting of cells, quantification

How many single cell measurements are enough to determine cellular identity?



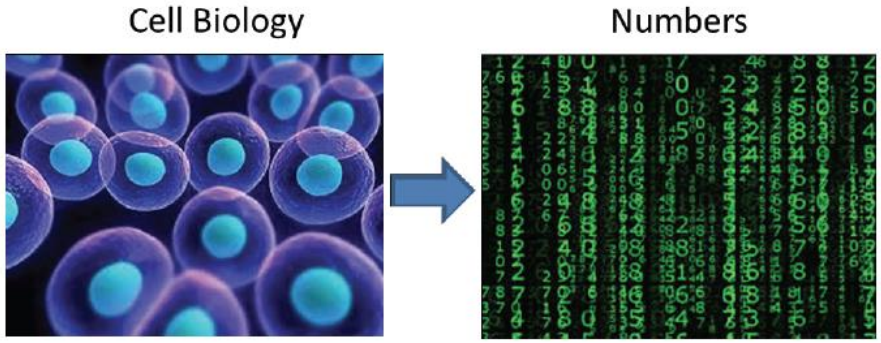
This scheme requires >20 markers!!

from Andrew Filby

Cytometry: We turn into numbers…

Questions:

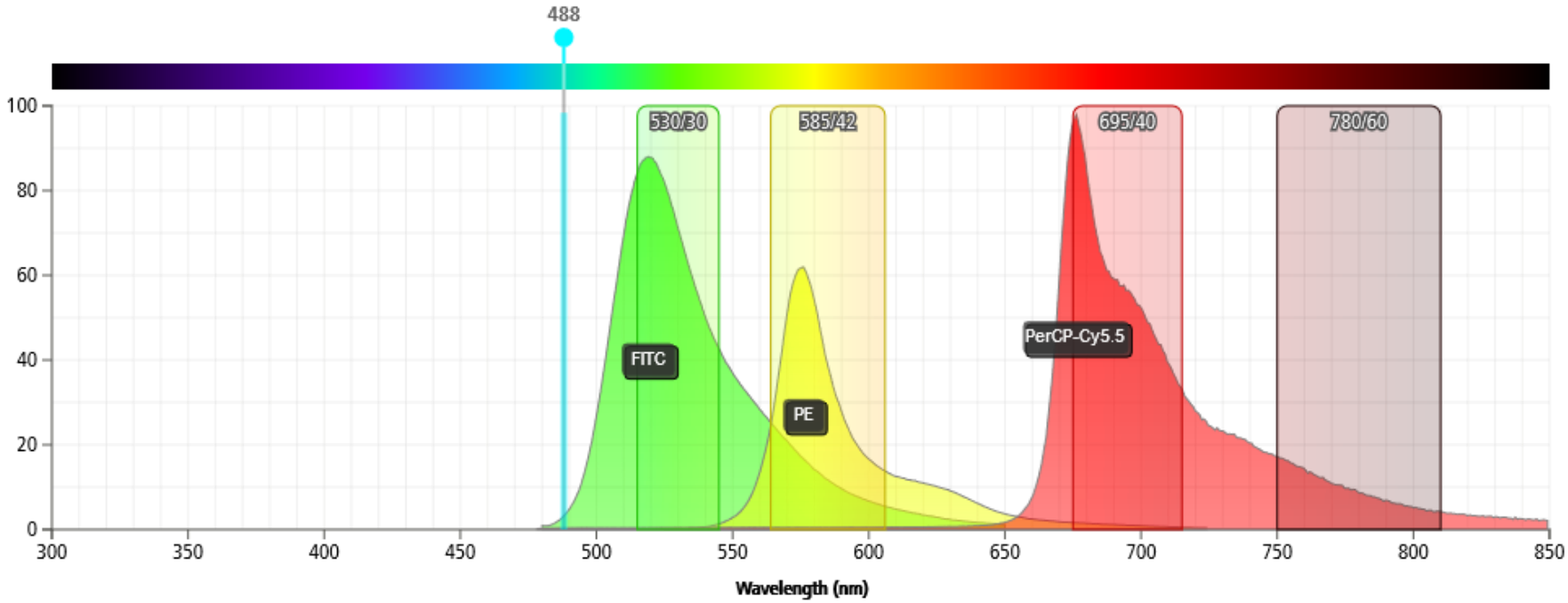
- What types of cells are there?
- How many are there?
- What do they do normally / abnormally?
- Can cell A become cell B?
- What role in development / disease?



Conventional flow cytometry

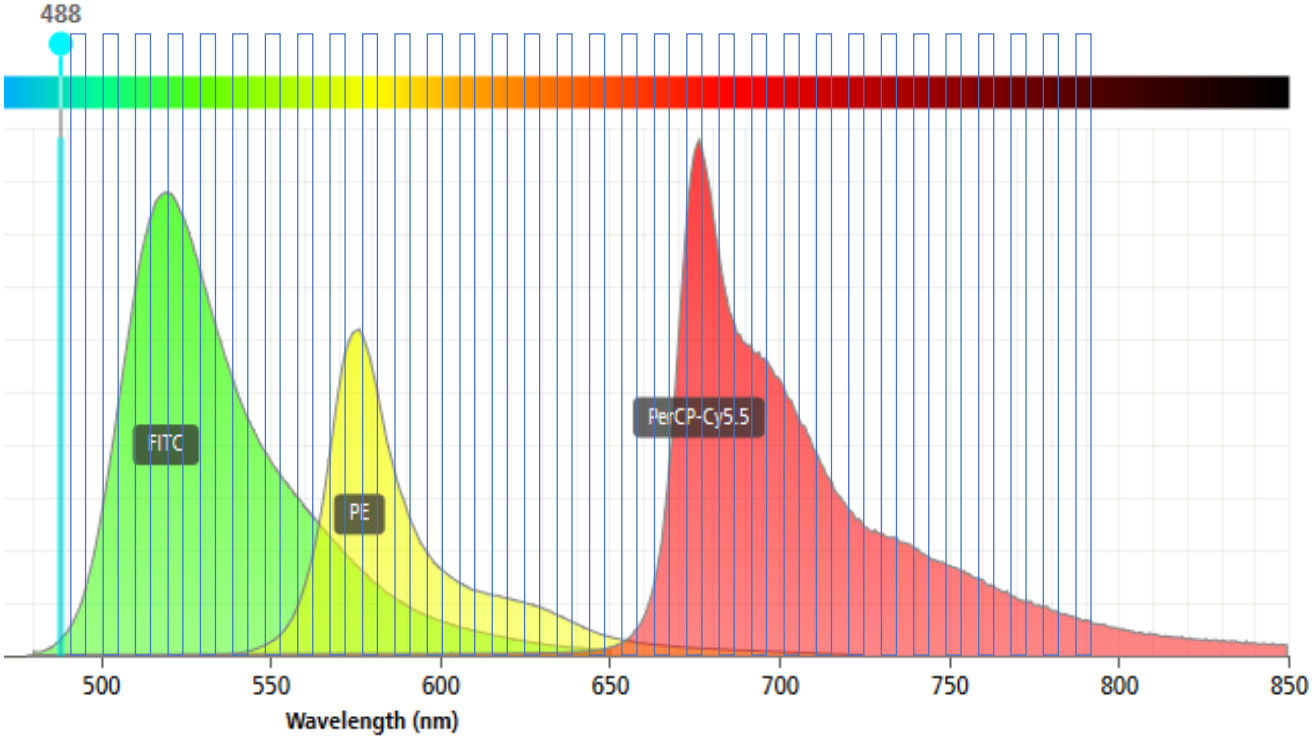
Limited number of fluorochromes detectable due to filter settings
BD Symphony with 5 lasers 27 fluorochromes

Mass cytometry – 45 parameters

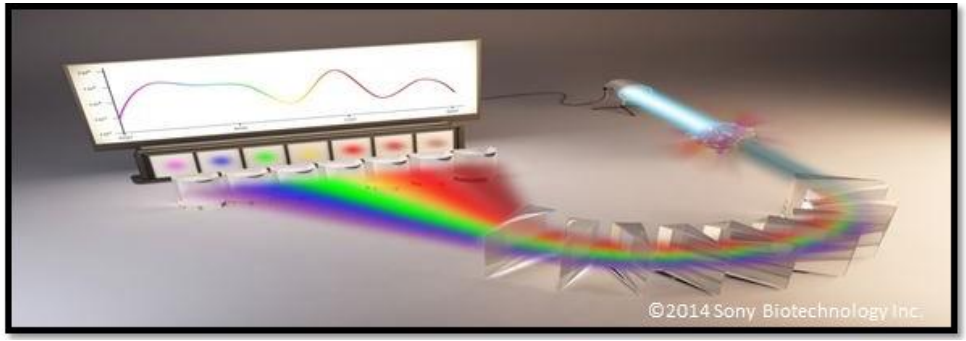


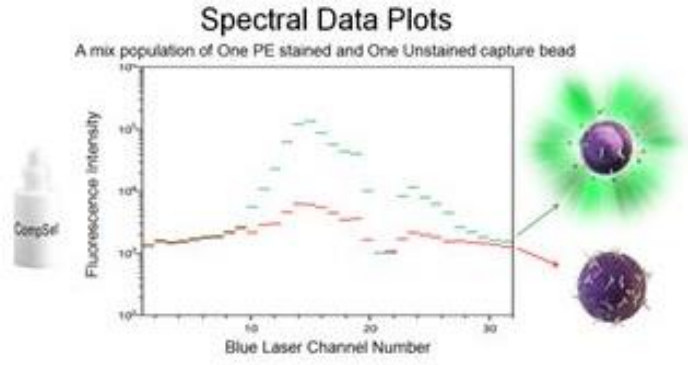
The spectral mindset

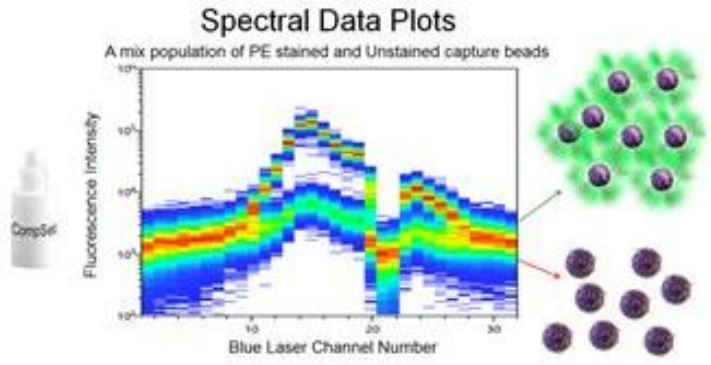
- Use the information from ALL the detectors to figure how much signal came from each dye
- Assume that each detector contains a “mixture” of photons from different fluorochromes, and our goal is to “unmix” them
- **The idea came from imaging. Satellite imaging to be precise…**



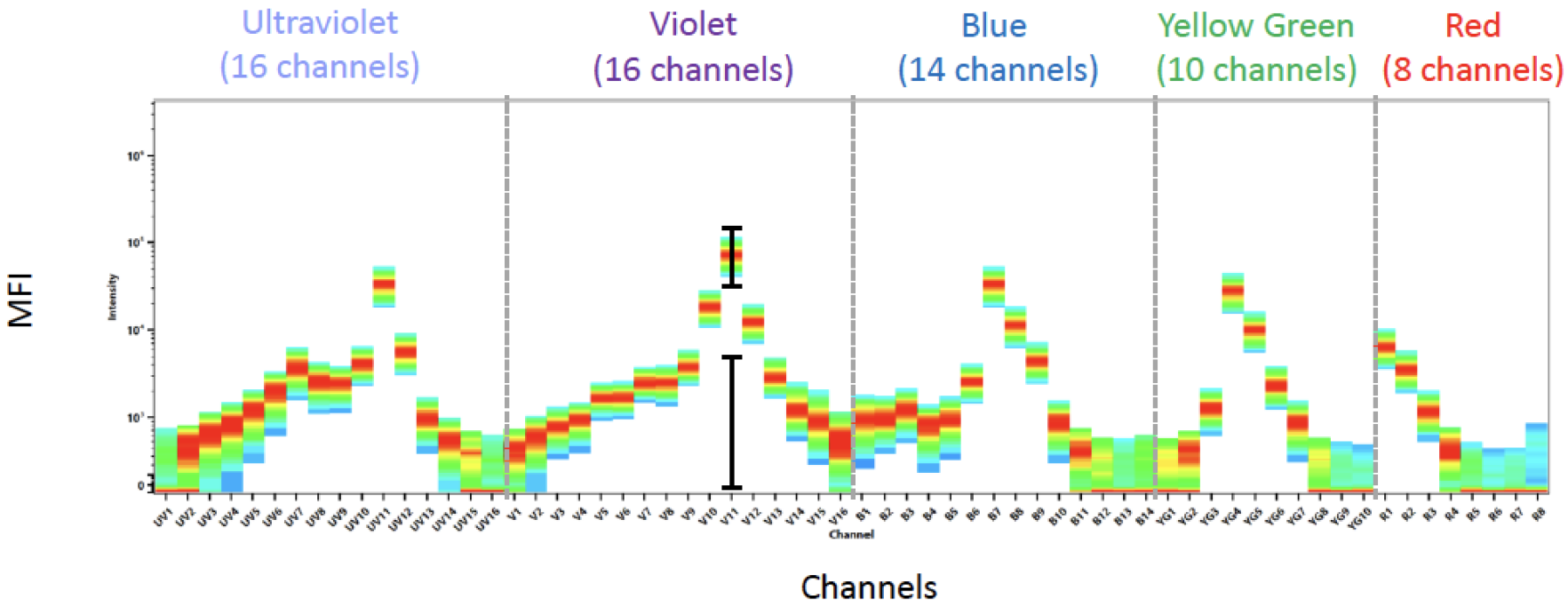
Spectral flow cytometry





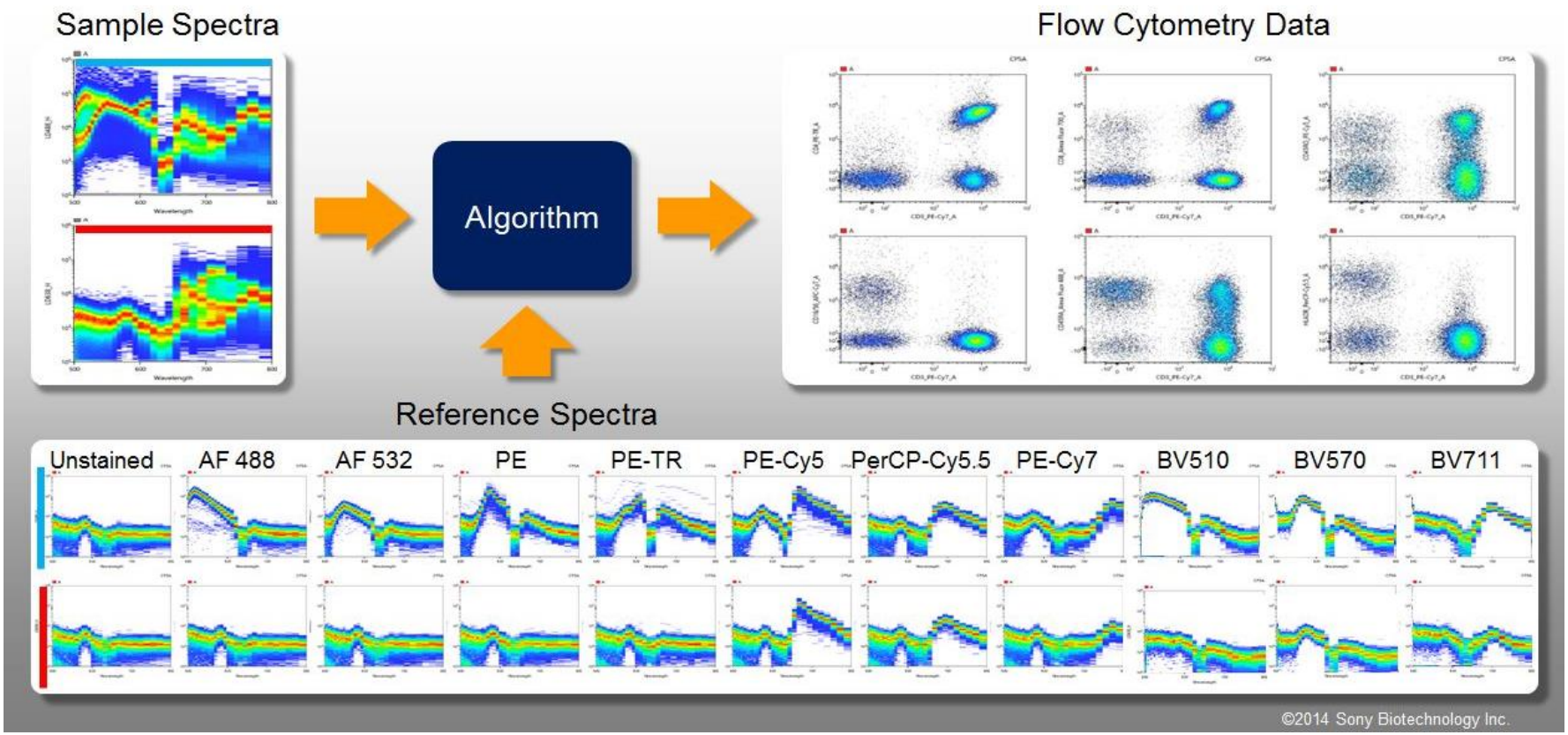


Spectral flow cytometry



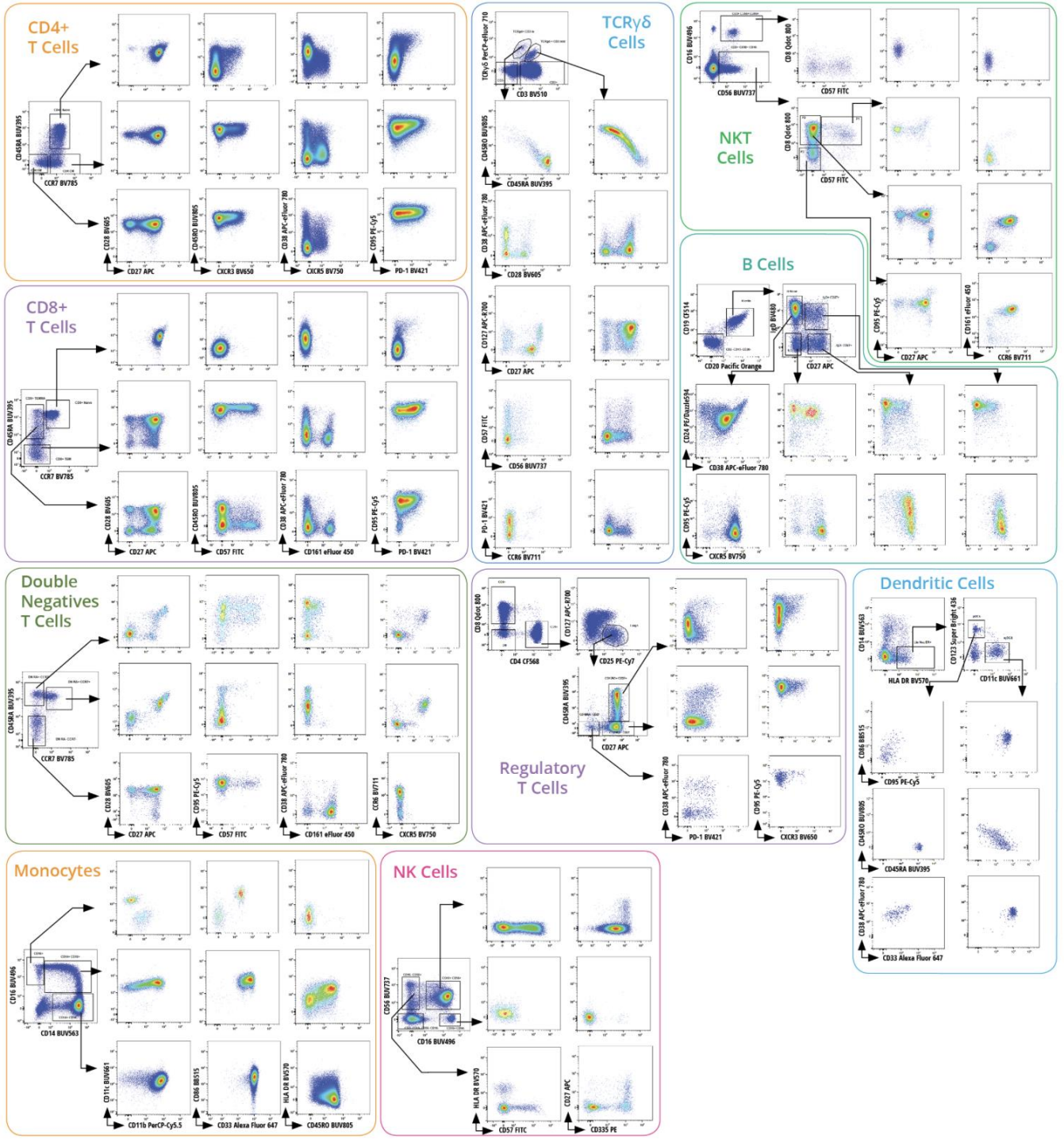
Advantage: use of every dye with unique emission spectrum

Spectral unmixing

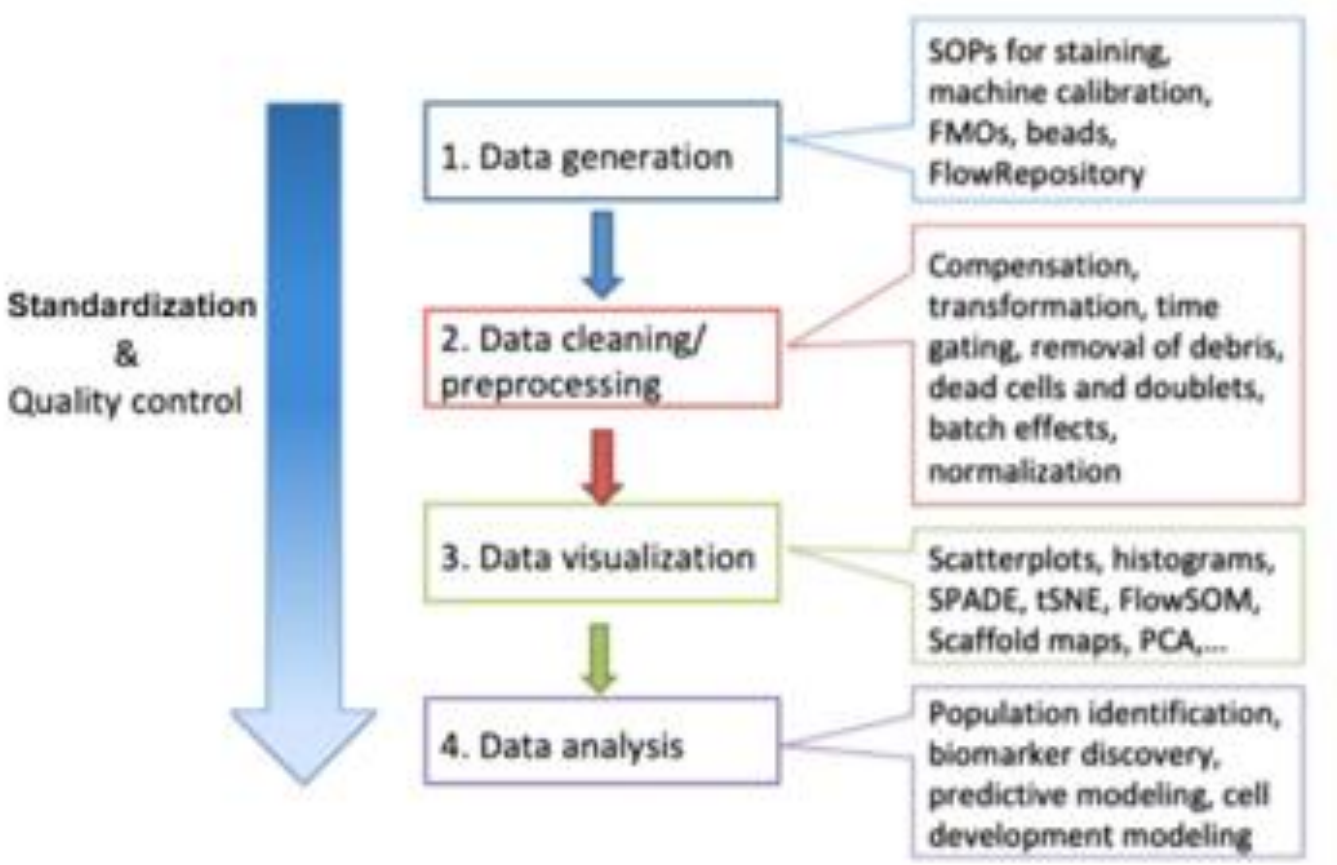


Example of 35 dyes that can be used in combination

UV		VIOLET		BLUE		YELLOW GREEN		RED	
SPECIFICITY	FLUOROCHROME	SPECIFICITY	FLUOROCHROME	SPECIFICITY	FLUOROCHROME	SPECIFICITY	FLUOROCHROME	SPECIFICITY	FLUOROCHROME
CD45RA	BD Horizon™ BUV395	PD-1	BV421	CD86	BB515	CD335	PE	CD27	APC
CD16	BD Horizon™ BUV496	CD123	Super Bright 436	CD57	FITC	CD4	CF® 568	CD33	Alexa Fluor 647
CD14	BD Horizon™ BUV563	CDC161	eFluor 450	CD19	CF® 514	CD24	PE/Dazzle 594	CD127	APC-R700
CD11c	BD Horizon™ BUV661	IgD	BV480	CD45	PerCP	CD95	PE-Cy5	CD38	APC-eFluor 780
CD56	BD Horizon™ BUV737	CD3	BV510	CD11b	PerCP-Cy5.5	CD25	PE-Cy7		
CD45RO	BD Horizon™ BUV805	CD20	Pacific Orange	TCR γδ	PerCP-eFluor 710				
Dead Cells	LIVE/DEAD™ Blue	HLA DR	BV570						
		CD28	BV605						
		CXCR3	BV650						
		CCR6	BV711						
		CXCR5	BV750						
		CCR7	BV785						
		CD8	Qdot 800						


CD4+
T Cells
CD45RA BUV395
CCR7 BV785
CD28 BV605
CD27 APC
CD45RO BUV805
CXCR3 BV650
CD38 APC-eFluor 780
CXCR5 BV750
CD95 PE-Cy5
PD-1 BV421
TCRγδ
Cells
TCRγδ PerCP-eFluor 710
CD3 BV510
CD127 APC-R700
CD57 FITC
CD56 BUV737
CCR6 BV711
NKT
Cells
CD16 BUV496
CD8 Qdot 800
B Cells
CD19 CF514
IgD BV480
CD20 Pacific Orange
CD24 PE/Dazzle594
CD161 eFluor 450
CD8+
T Cells
Double
Negatives
T Cells
Regulatory
T Cells
CD4 CF568
CD25 PE-Cy7
Dendritic
Cells
CD14 BUV563
CD123 Super Bright 436
HLA DR BV570
CD11c BUV661
CD86 BB515
CD33 Alexa Fluor 647
Monocytes
CD11b PerCP-Cy5.5
NK Cells
CD335 PE

Challenge - Data handling and analysis



Challenge:
Analysis of high-dimensional single cell data

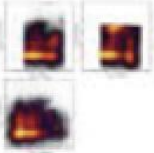
2 parameters

1 plot



3 parameters

3 plots



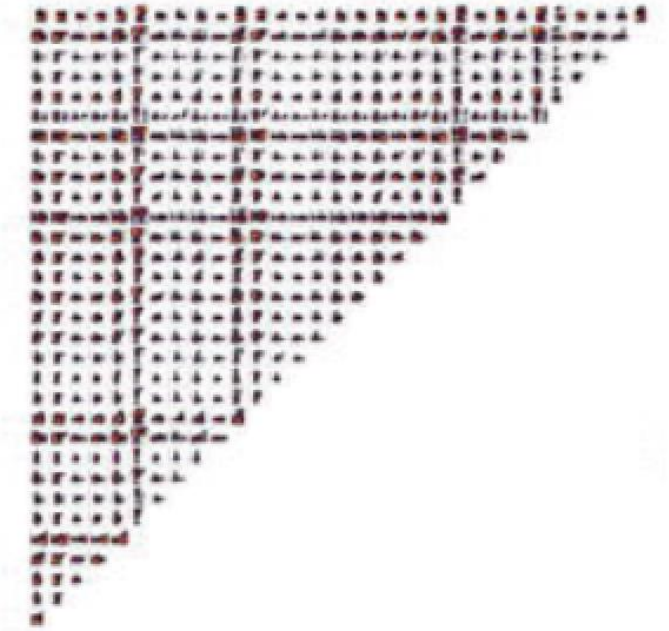
9 parameters

36 plots



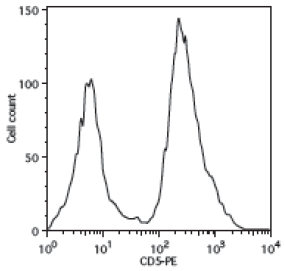
32 parameters

496 plots

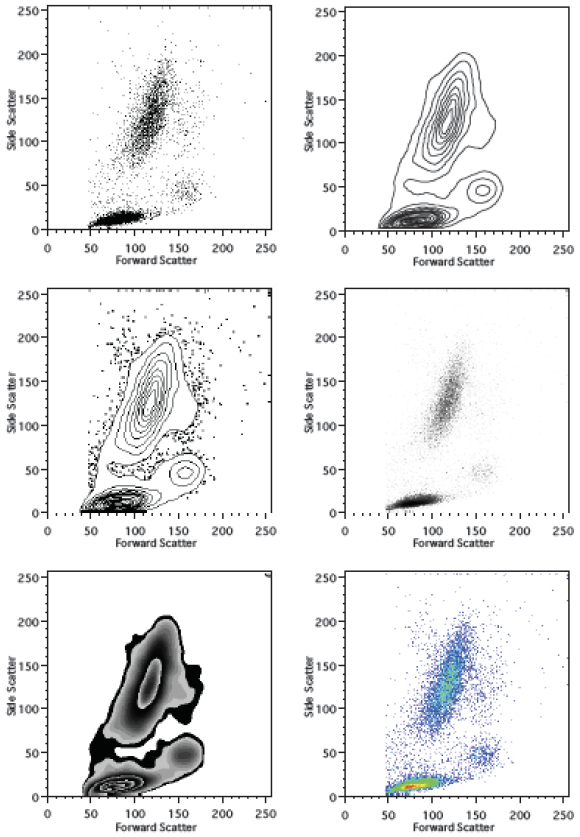


Distribution / cluster analysis

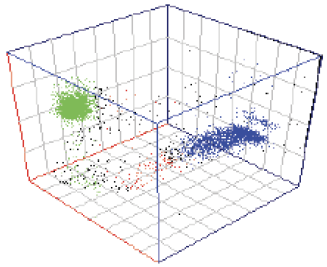
1 parameter



2 parameter



3 parameter



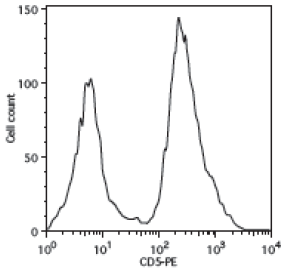
from Andrew Filby

Visualising multi-dimensional data in 2D space

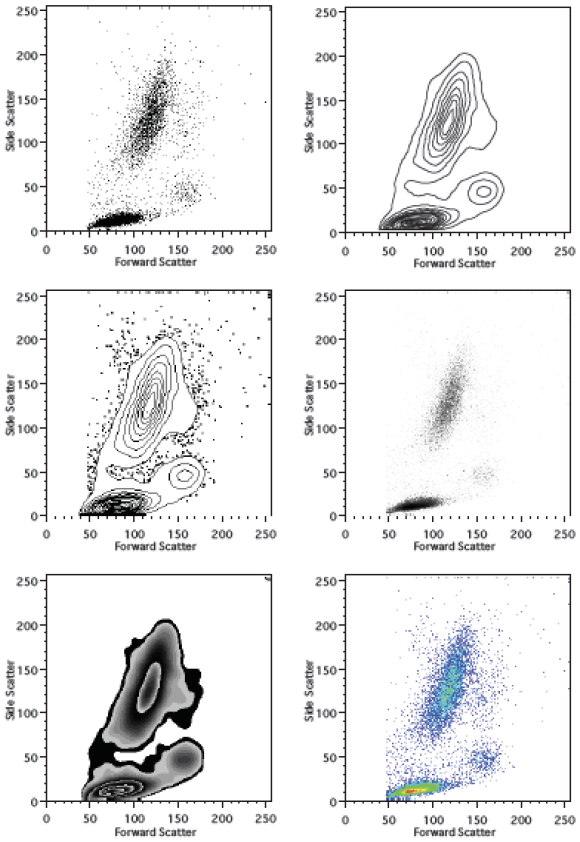


Distribution / cluster analysis

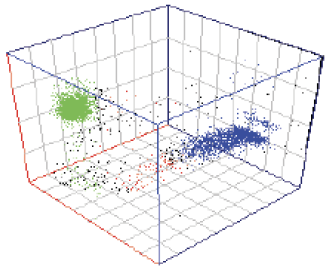
1 parameter



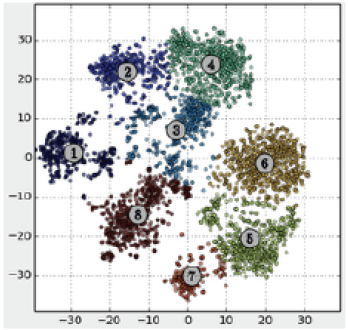
2 parameter



3 parameter



n parameter?



from Andrew Filby

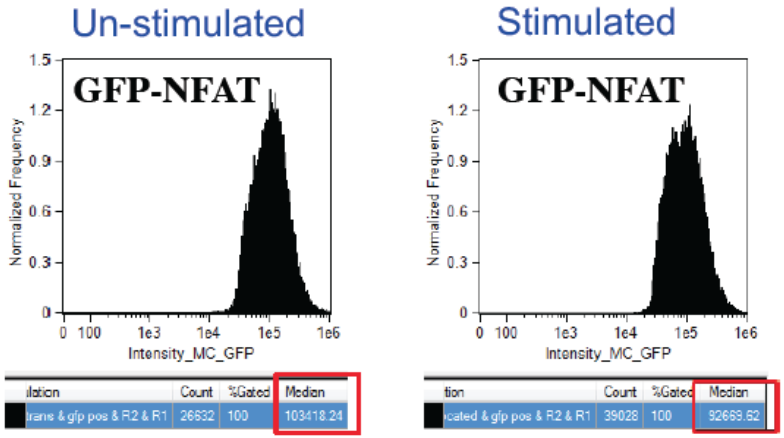
Summary - Flow cytometry

- High throughput multi-parameter analysis
- Conventional flow cytometry is limited in parameters
- Spectral flow cytometry possible with any unique fluorescence emission spectrum of a fluorochrome
- Challenge: data analysis

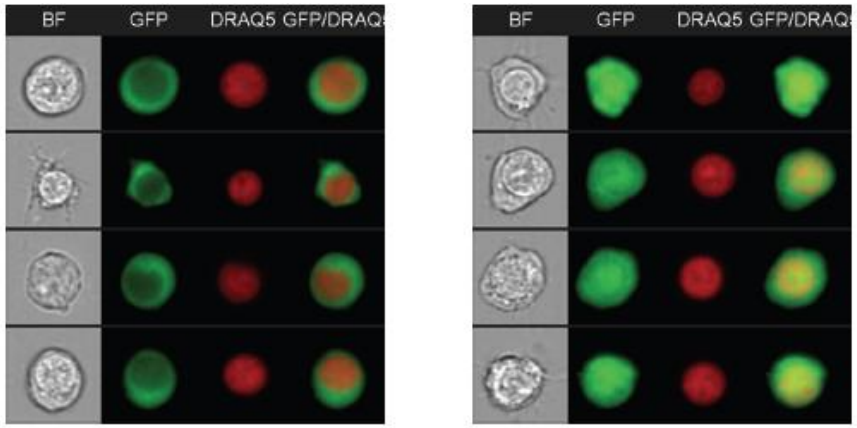
Disadvantage of Flow Cytometry – NO spatial resolution

But sometimes we need an image….

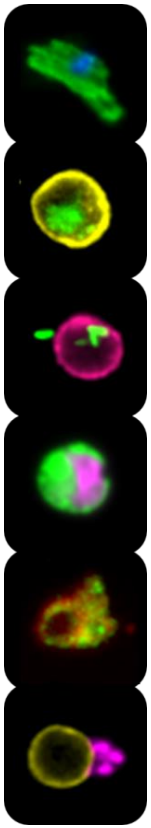
Q: nuclear translocation of GFP-labeled NFAT?



Localization and special context may be ESSENTIAL to the biological question.



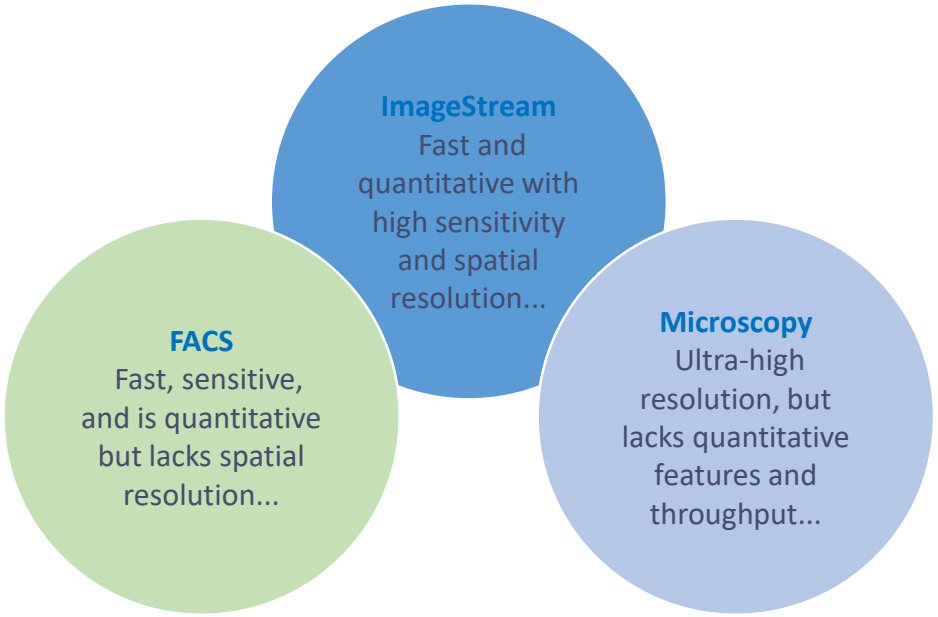
Discriminating cells based on appearance



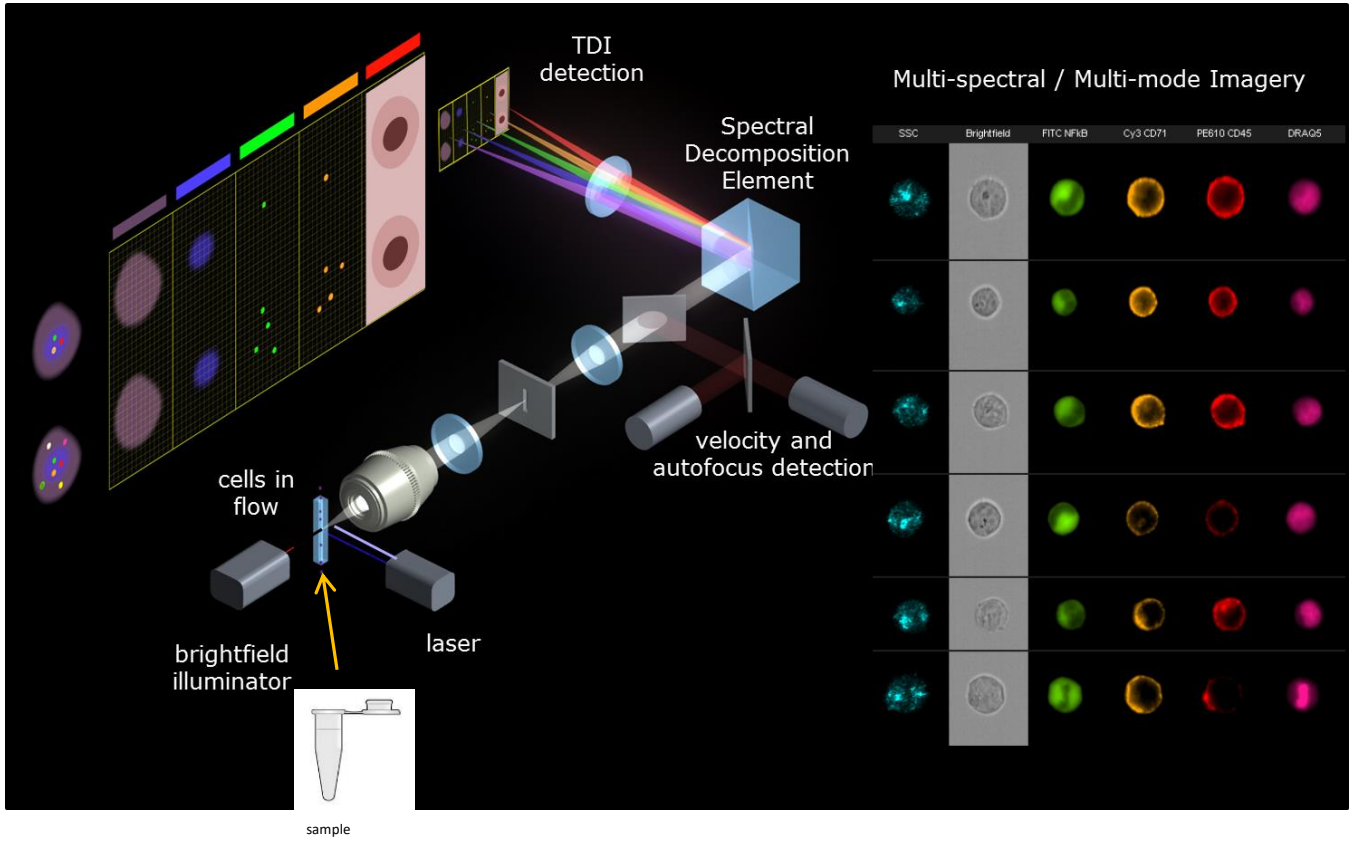


Luminex Corp.

ImageStream MkII combines FACS and Microscopy



Amnis Imaging Flow Cytometer – Principles of Operation

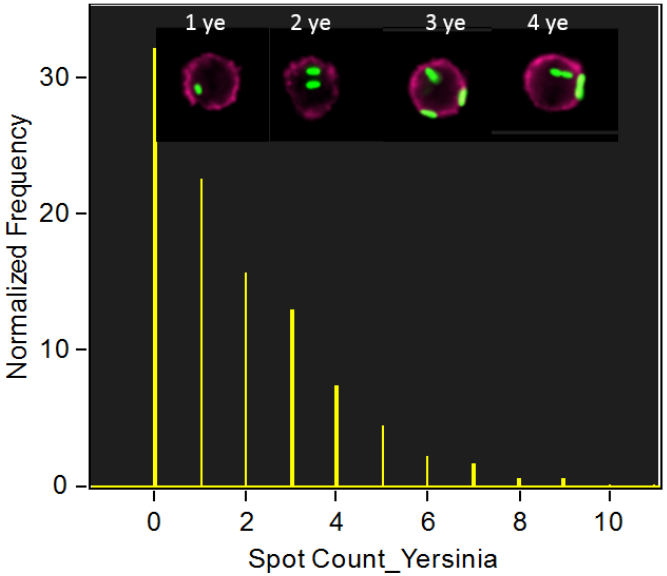


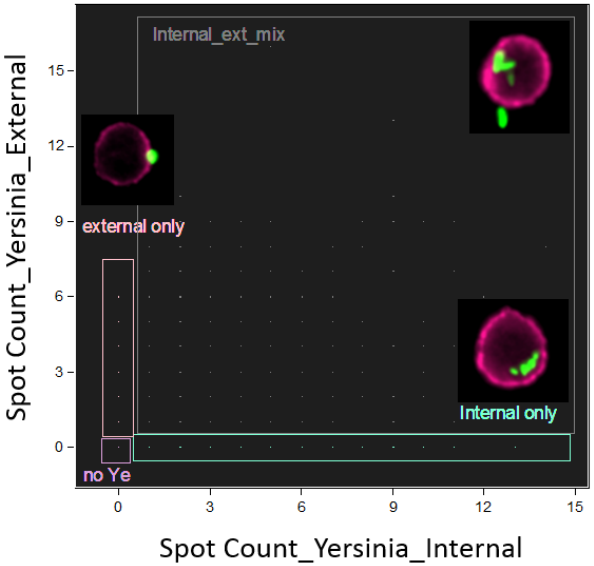
Benefits

- unique spectral decomposition element enables brightfield, side scatter, and up to 10 fluorescent images per cell
- Precise spatial registry produces detailed localization of signal from fluorescent probes

Application: Phagocytosis/Internalisation

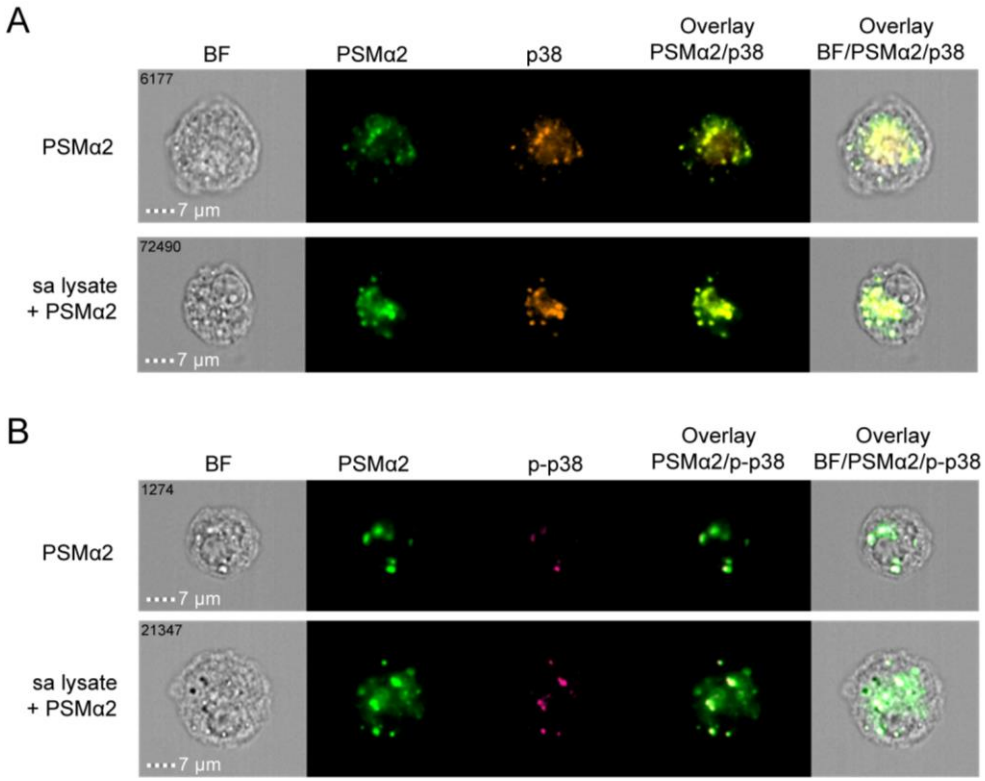
Goal: Quantification of GFP expressing bacteria in CD11b positive macrophages





Application: co-localization / signaling

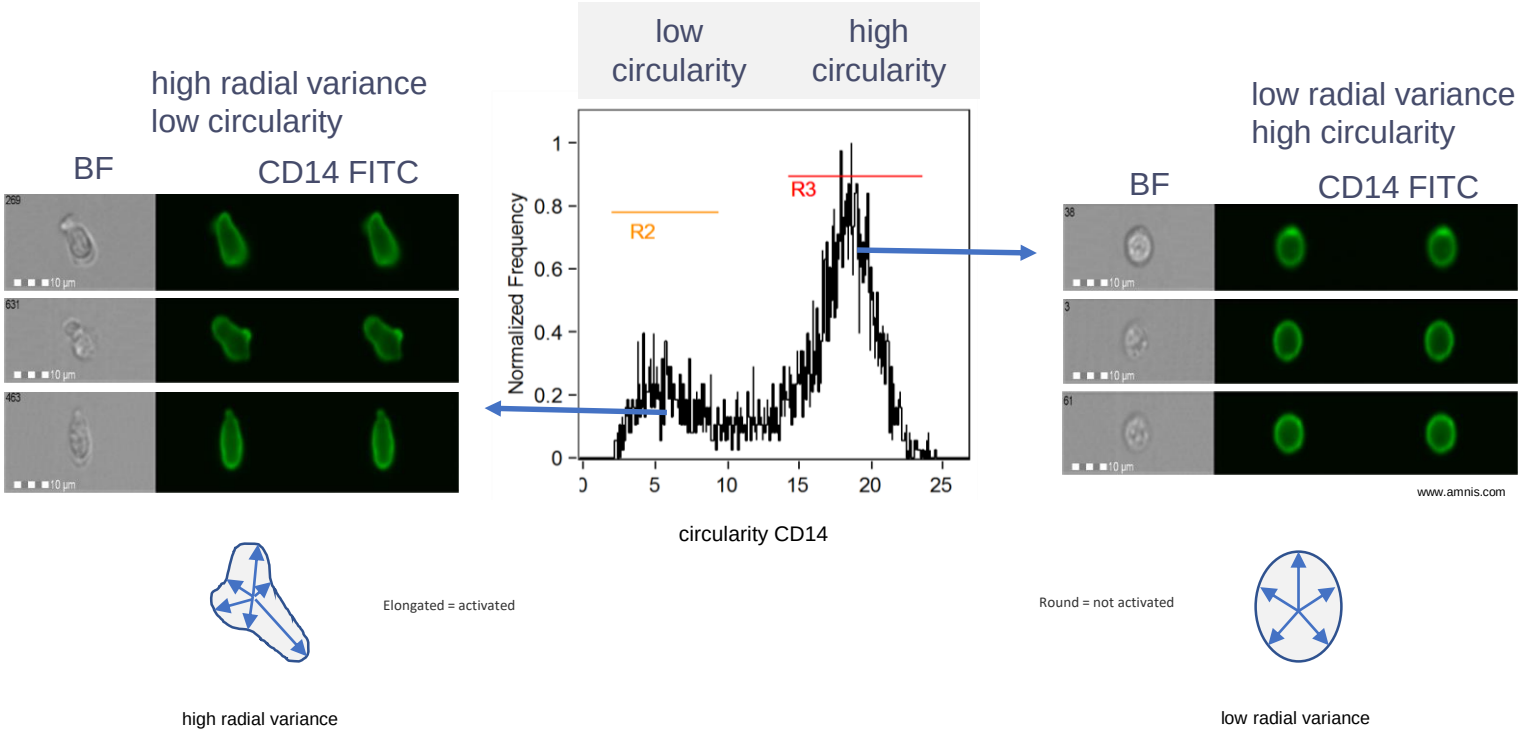
Q: Does the peptide PSMa2 from *Staphylococcus aureus* co-localizes with p38 MAPK?



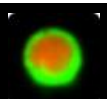
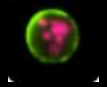
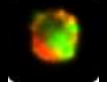
Armbruster et al. J Immunol 2016

Application: morphology / shape change

primary human monocytes after treatment with MCP-1



Applications for ImageStream

Field of study	*Example from ISx*
 Cell signaling	*NFkB translocation*
 Shape change & Chemotaxis	*MCP-1 activation of Monocytes*
 Cell-cell interaction	*Immune synapse formation, virus-induced cell fusion*
 Internalization	*Phagocytosis*
 Cell death & Autophagy	*Apoptosis, nuclear fragmentation*
 Cell cycle	*Mitosis*
 Co-localisation	*Ligand-Co-localisation*

Summary Image Stream

- Unique combination of Microscopy and Flow Cytometry
- Overcomes limitations of both techniques
- Opens door to range of novel applications

Challenges:

- niche product, routinely use rather unlikely
- High sample quality essential
- very complex software - experienced staff

The ImageStream®X is a powerful tool for Image analysis



Flow Cytometry Core Facility - FCF

Staff:

Dr. Kristin Bieber, Sabrina Grimm,

Simone Pöschel (ISX)

Medical Faculty - UKT
Internal Medicine II
Otfried-Müller Straße 10
72076 Tübingen
Bettenbau West –
Floor 2 – Room 580
07071-29 82745
fcf-berg@med.uni-tuebingen.de



Questions?