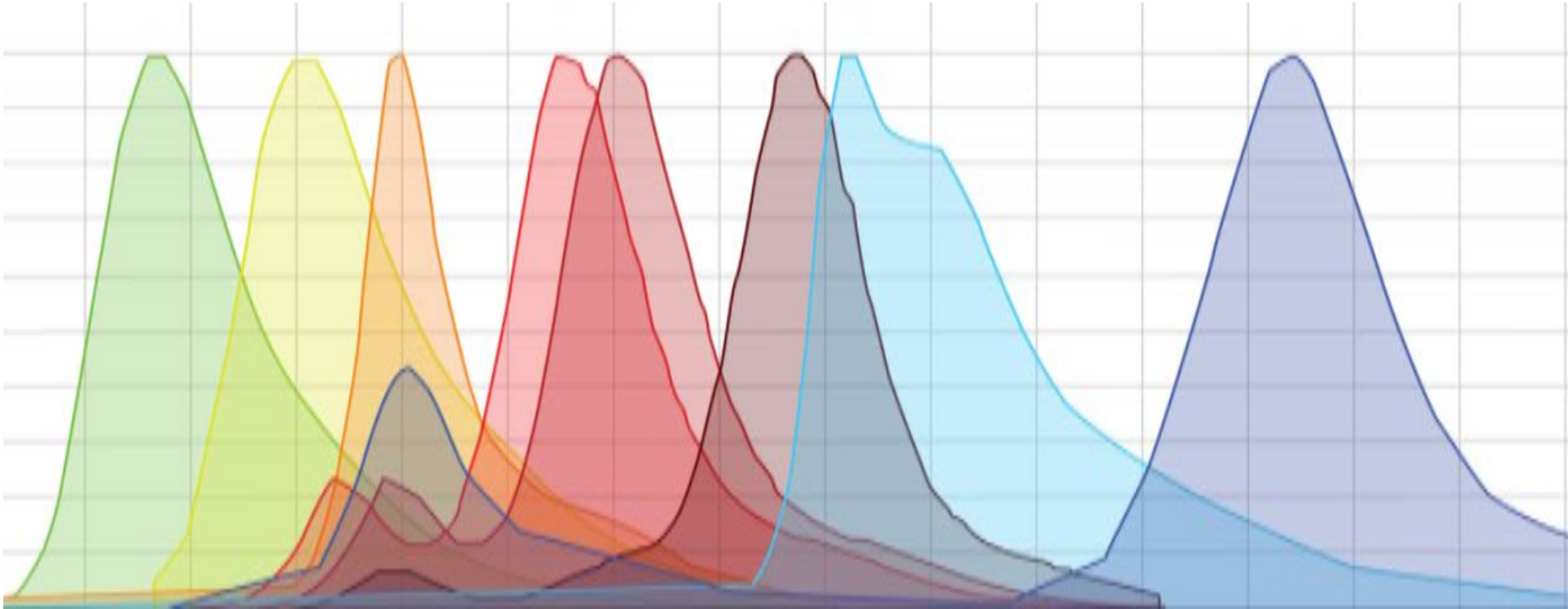
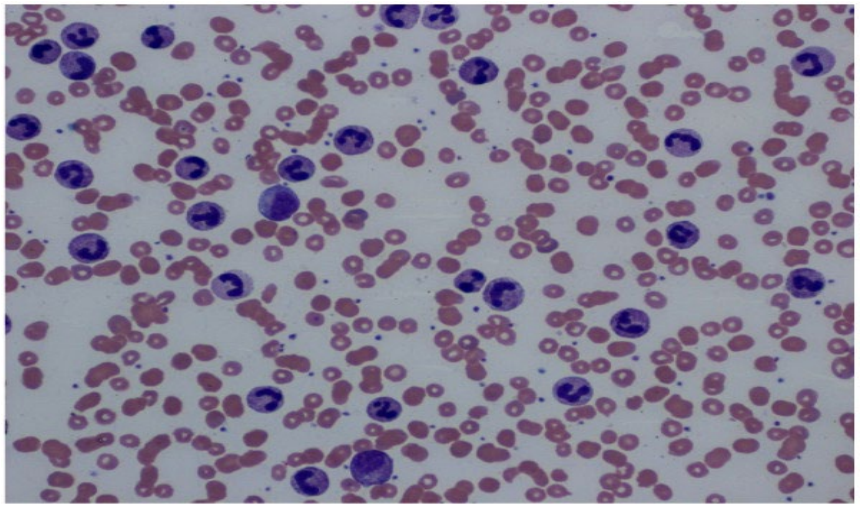
# Introduction to Flowcytometry

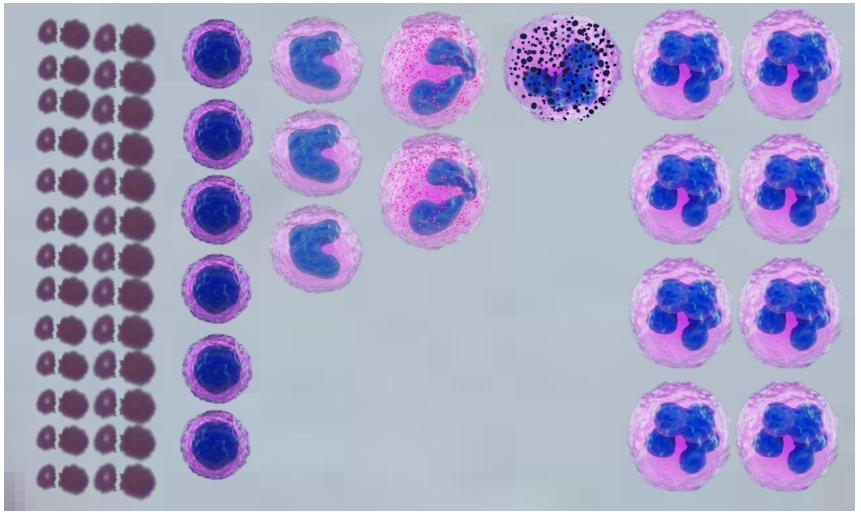


# What is flowcytometry

## Creating order in the Chaos

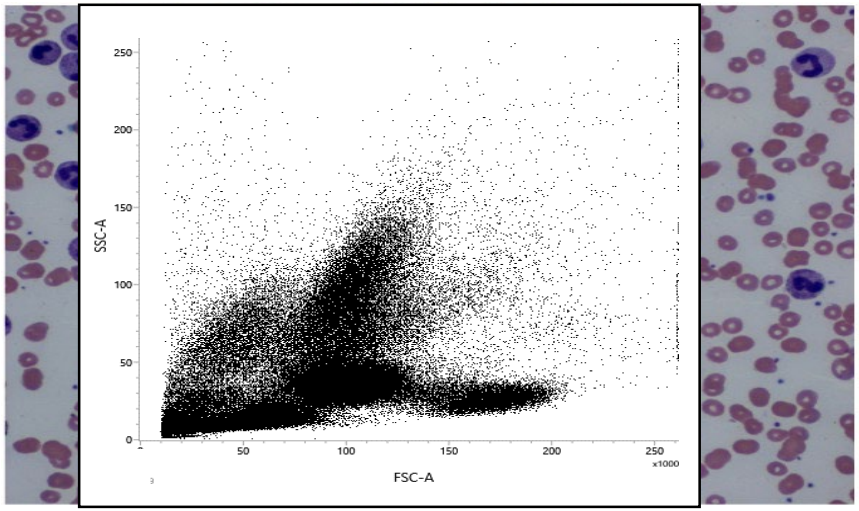
URSUS WEHRLI

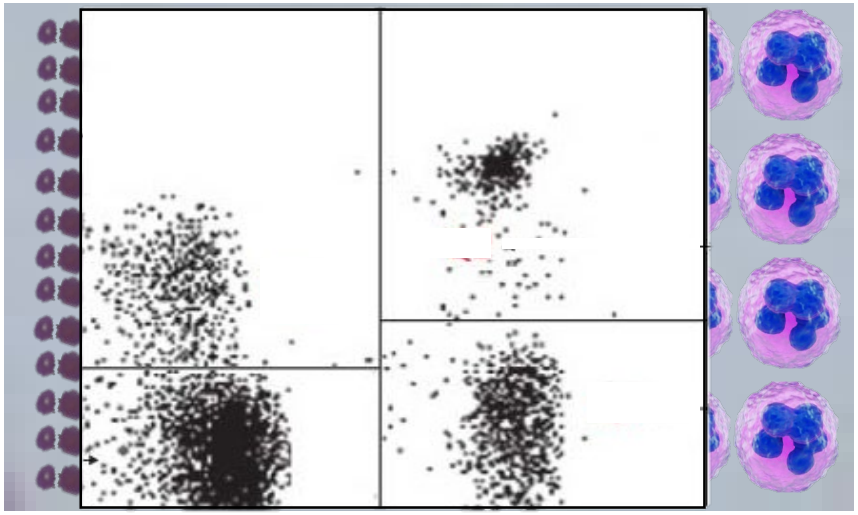




Leukocytes

# What is flowcytometry



# Some history

**1934** Photo-detection of red blood cells: **Moldavan**

**1950** Measuring cells (size) based on electrical condutivity : **Coulter**

**1953** Development of laminair flows: **Crosland – Taylor**

**1965** Electrostatic charges breaks-up a stream in droplets (inktjet printing): **Sweet**

**1967** IBM developed a rapid cell spectrometer with arc lamp and a computer: **Kamentsky**

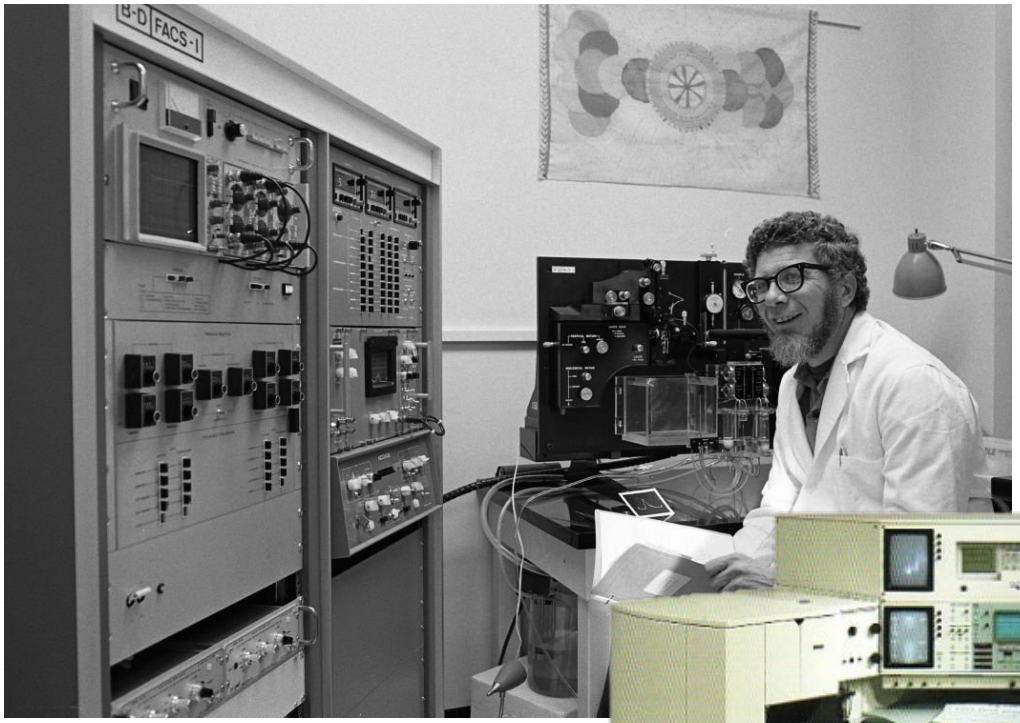
**1972 F**luorescence **A**ctivated **C**ell **S**orter: **Herzenberg**

**1981** First benchtop analyzer

**1985** 3 colors available

# Some history

## Len Herzenberg





# Some history

## What's now?







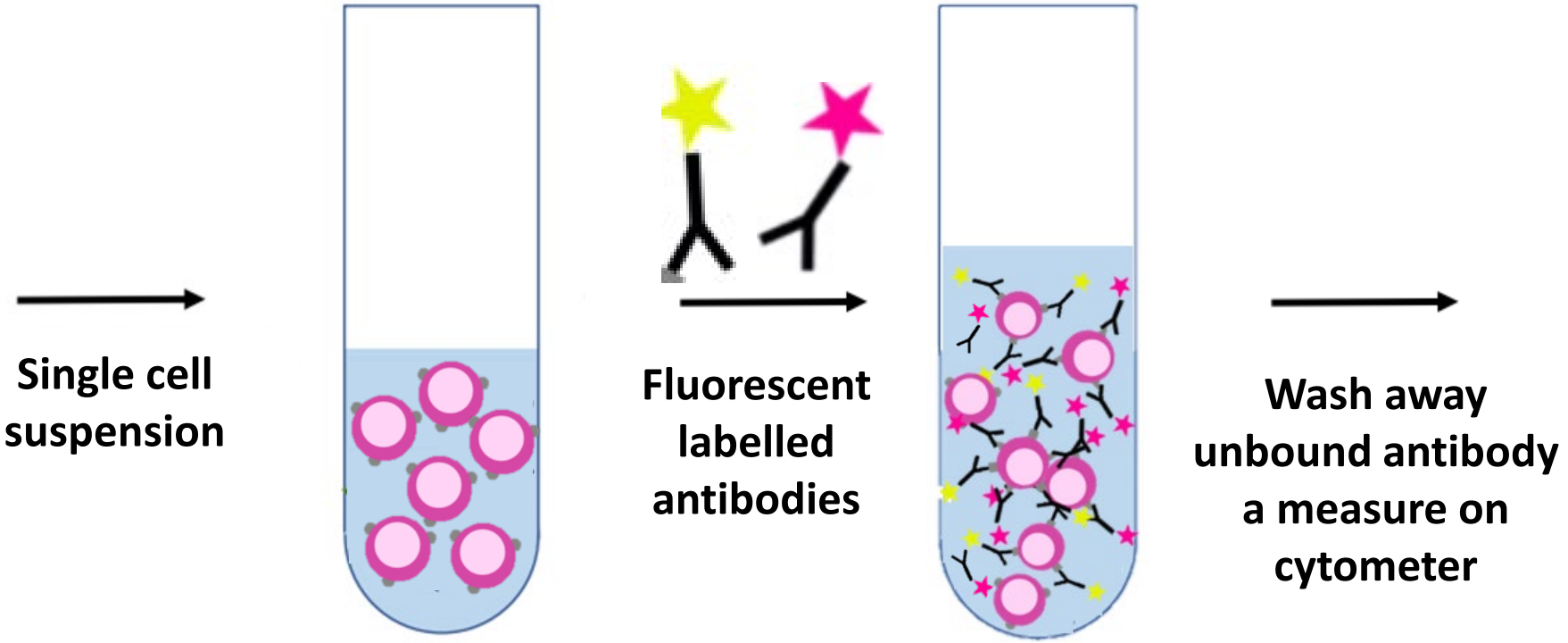


# What is flow cytometry

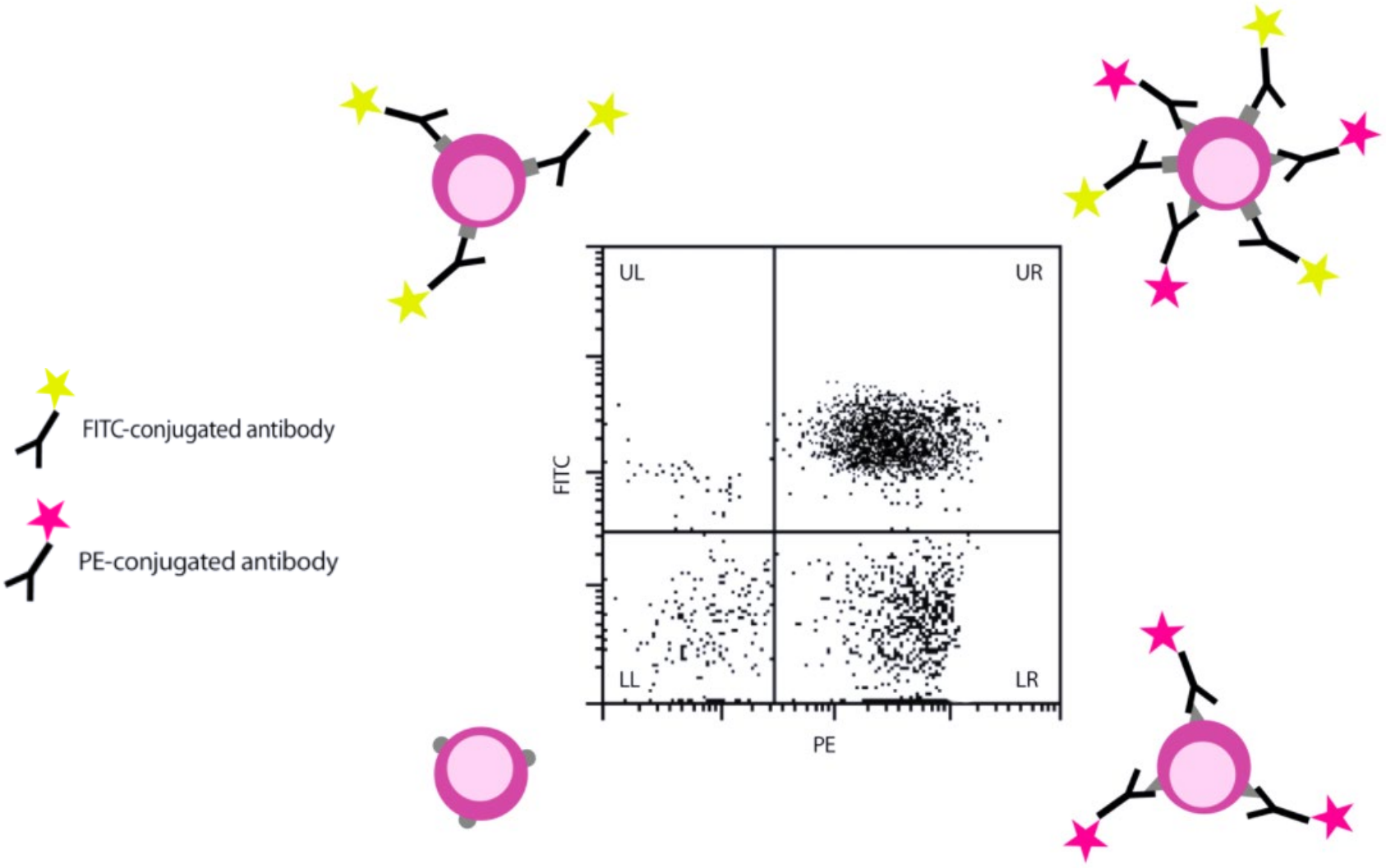
- Flow : cells in motion
- Cyto: cell
- Metry: measure

- Measuring multiple properties of single cells in a fluid stream
- Gives us the ability to analyze many properties of many cells in over 1000 cells per second
- You need single cells

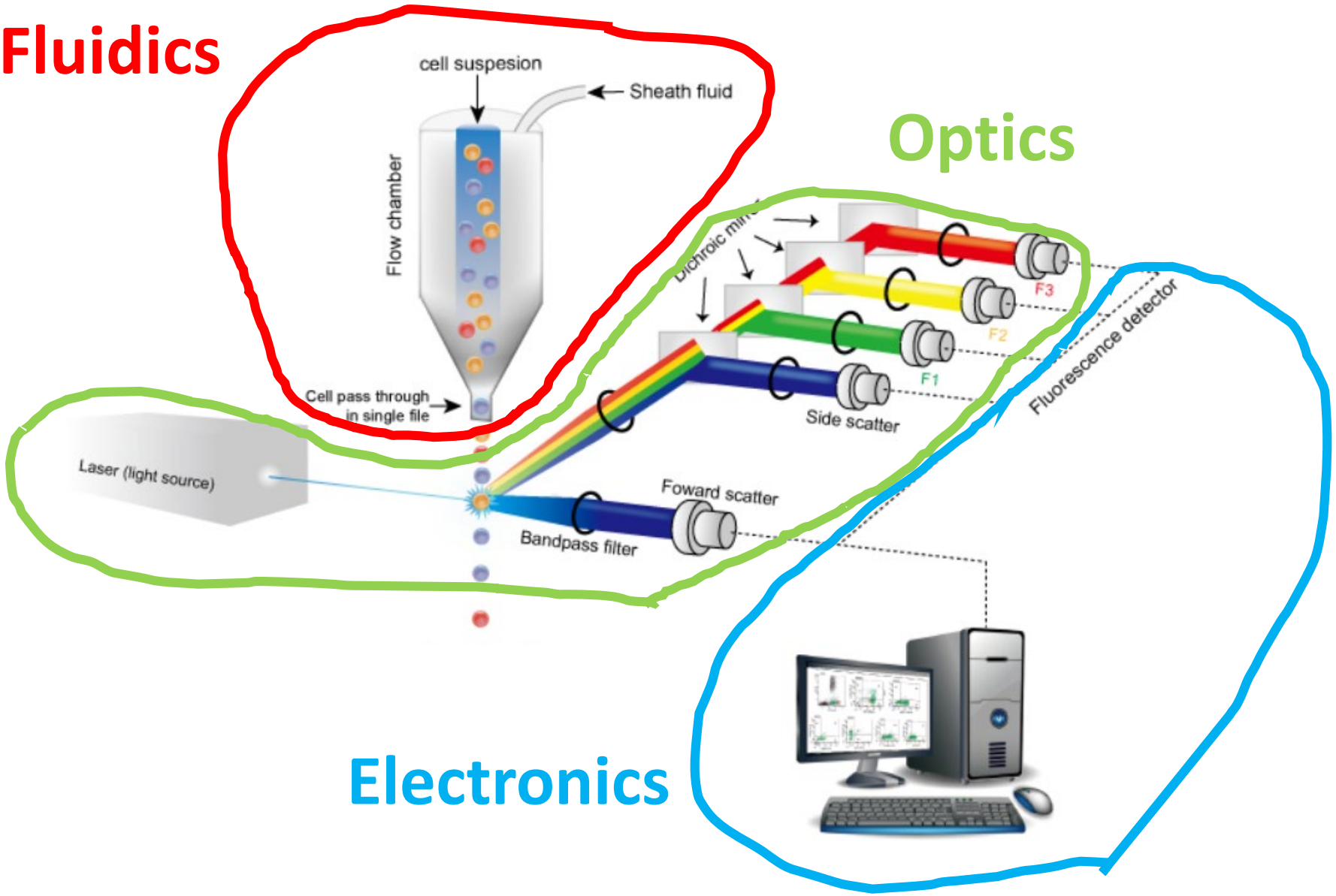
# What is flow cytometry



# What is flow cytometry

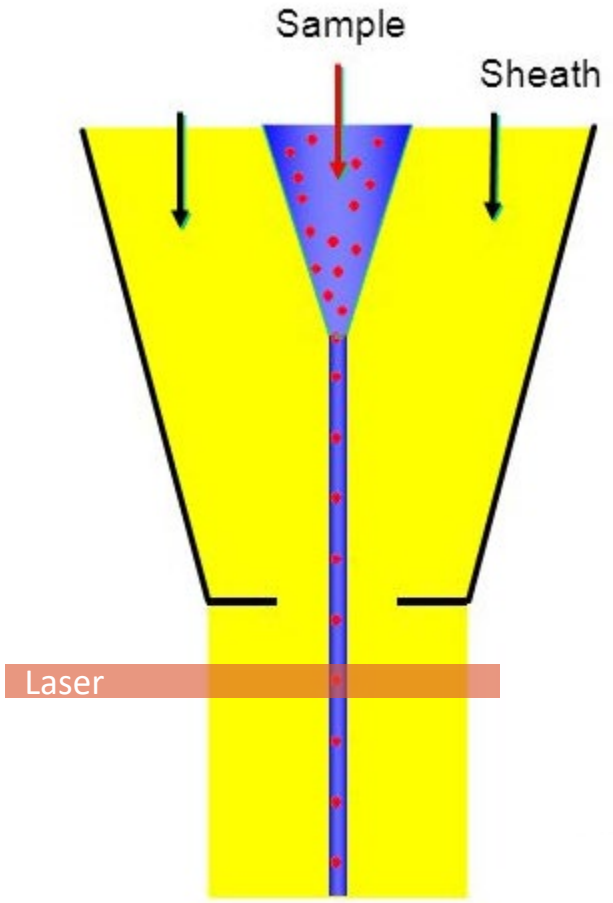


# Flowcytometric process

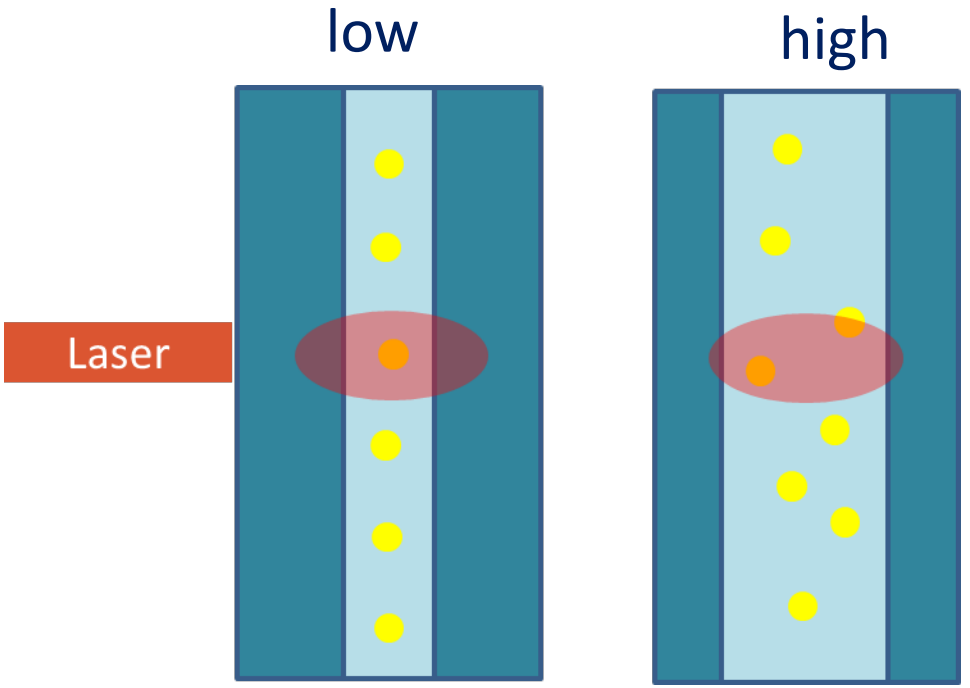


# Fluidics

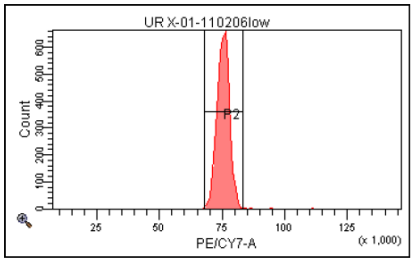
## Hydrodynamics focussing



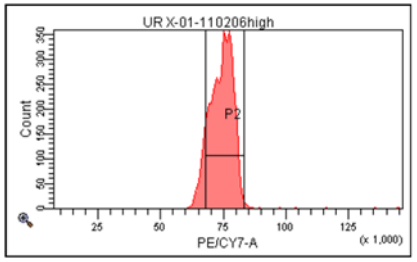

## Flowrate



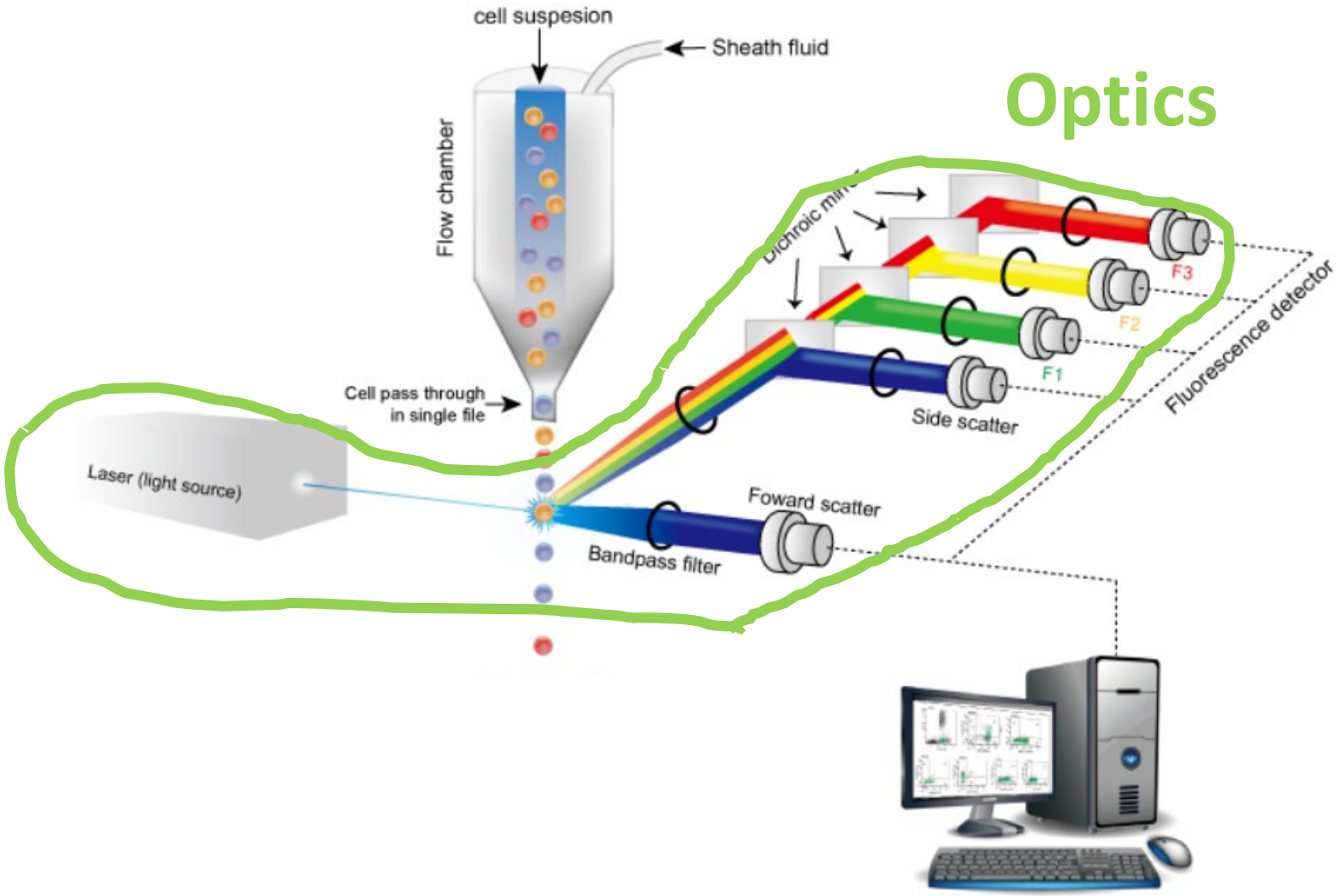

CV=3.04




CV=5.76



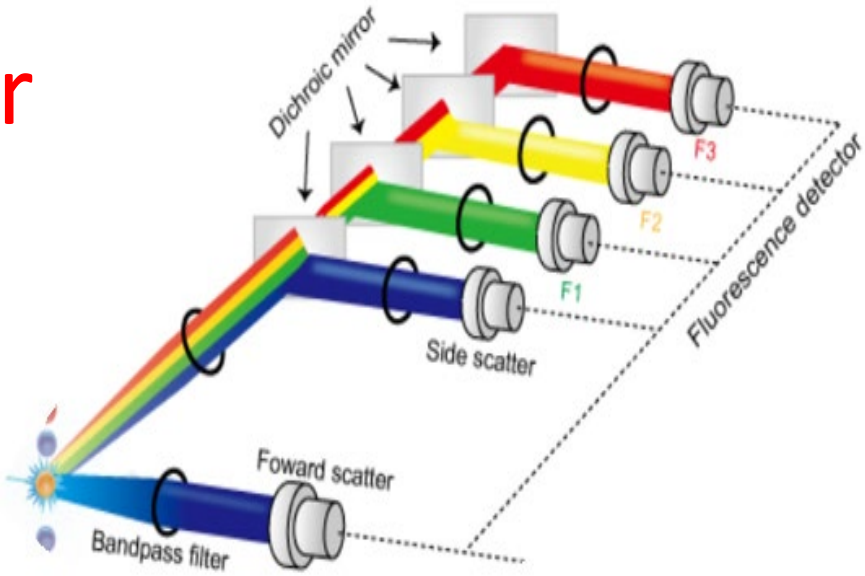
# Optics


Optics
cell suspesion
Sheath fluid
Flow chamber
Cell pass through
in single file
Laser (light source)
Bandpass filter
Foward scatter
Side scatter
F1
F2
F3
Fluorescence detector

# Optics

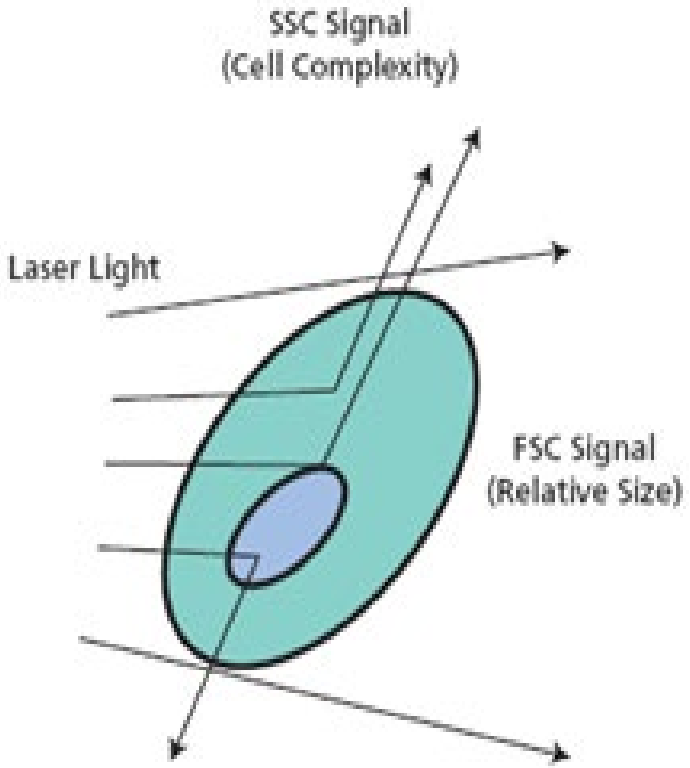
3 characteristics are measured by the optics:

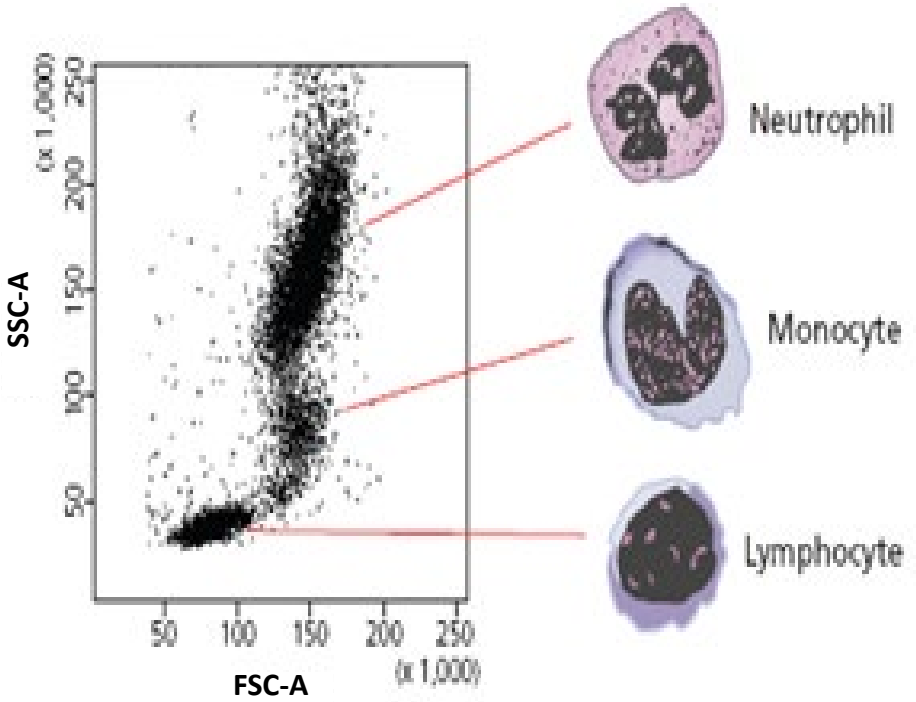
- Forward scatter
- Side scatter
- fluorescence



# Optics

## Forward / side scatter



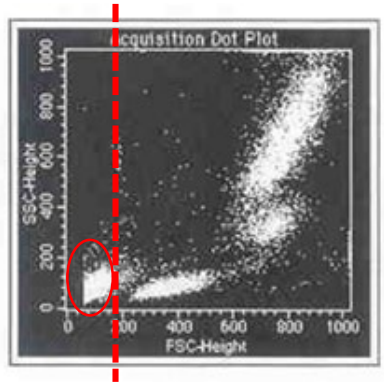
# Optics

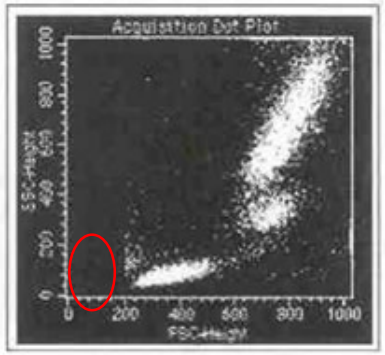
## Forward / side scatter

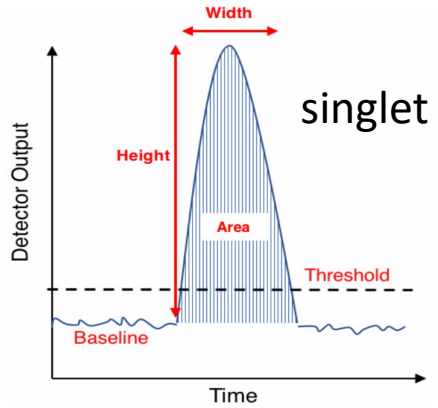
Treshold

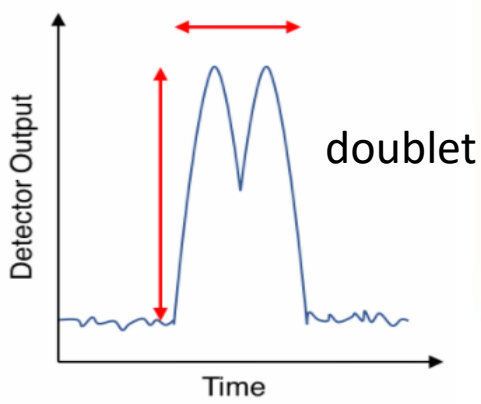
Doublet exclusion

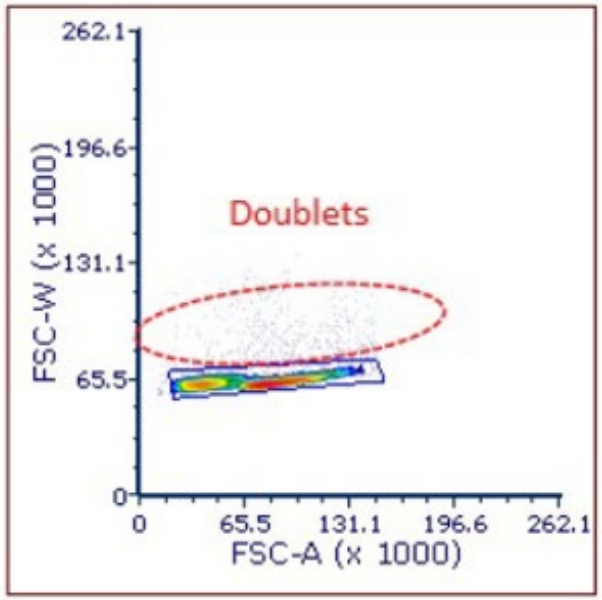
debris



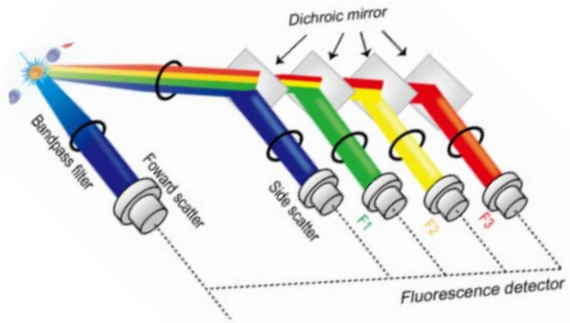



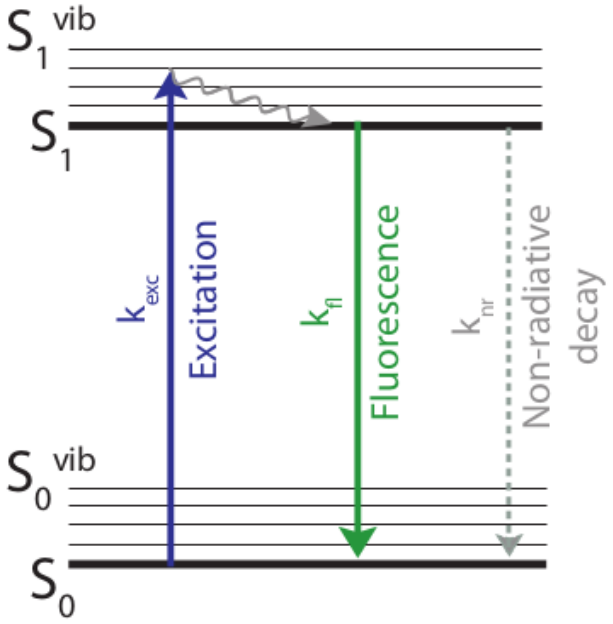

It is important to remember to turn on the W and H parameter before collecting data, otherwise it's not included in the FCS data file.

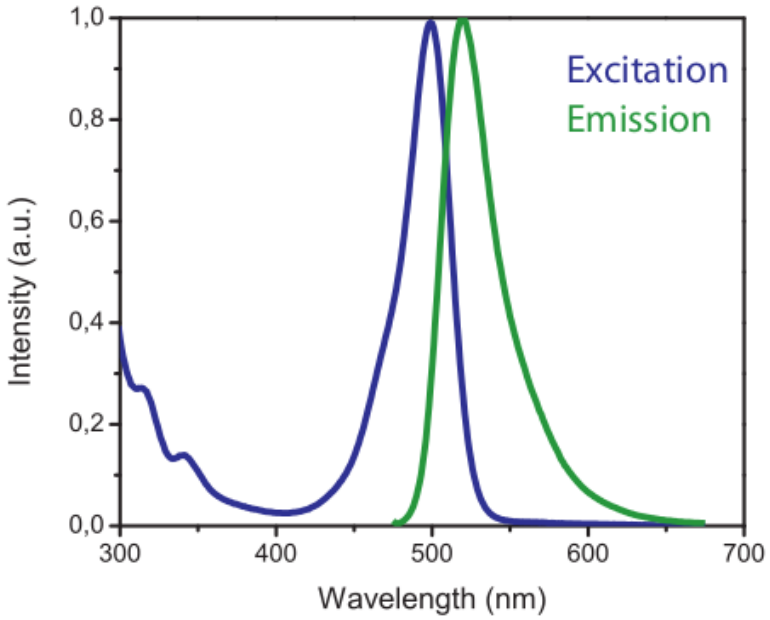



# Optics

## Fluorescence

Absorption of energy from excitated light by a photo-reactive chemical (fluorchrome) which then emmites the energy in a higher wavelenght of light



# Optics

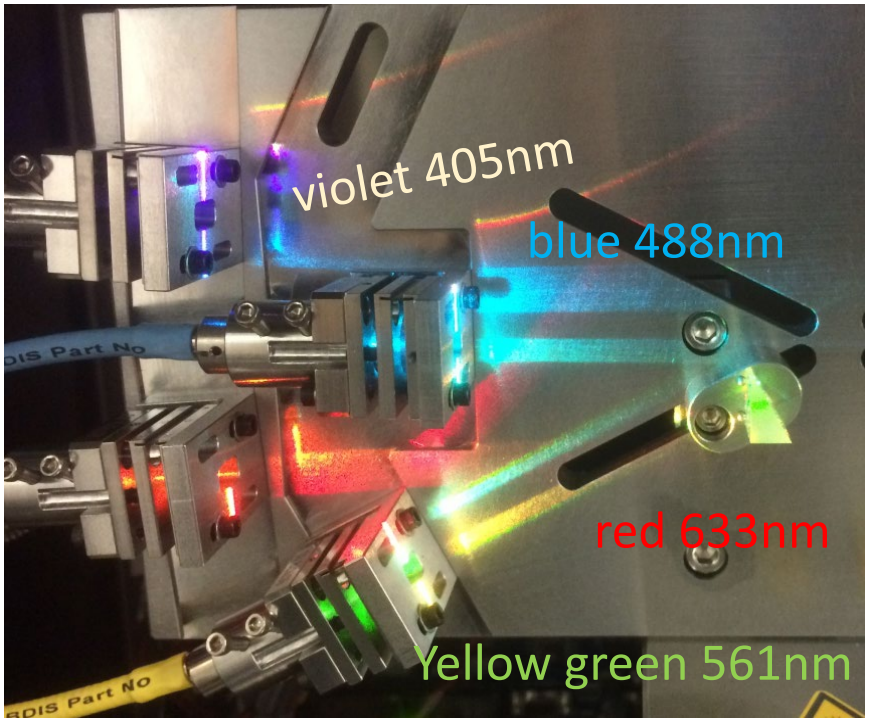
## Fluorescence in flowcytometry

### Excitation

### Emmision

LASERS

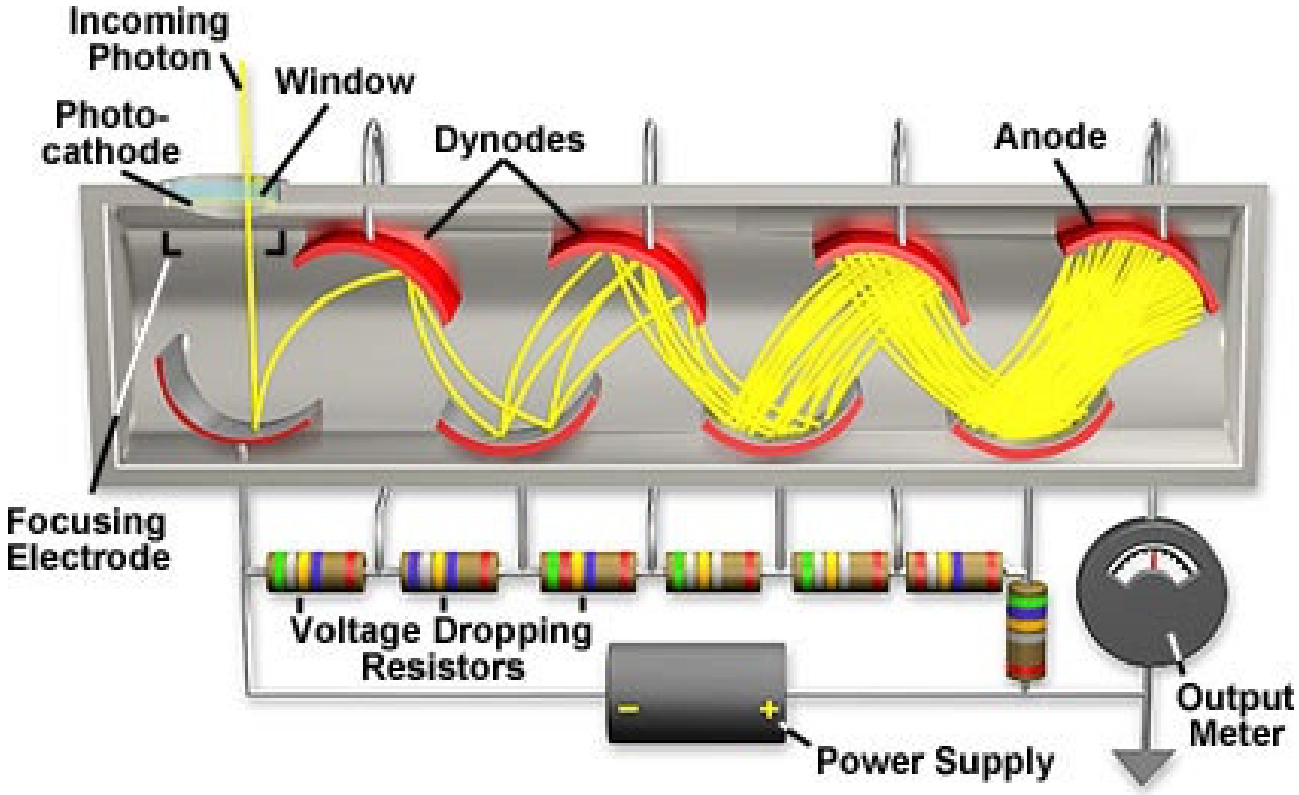
DETECTORS




# Optics

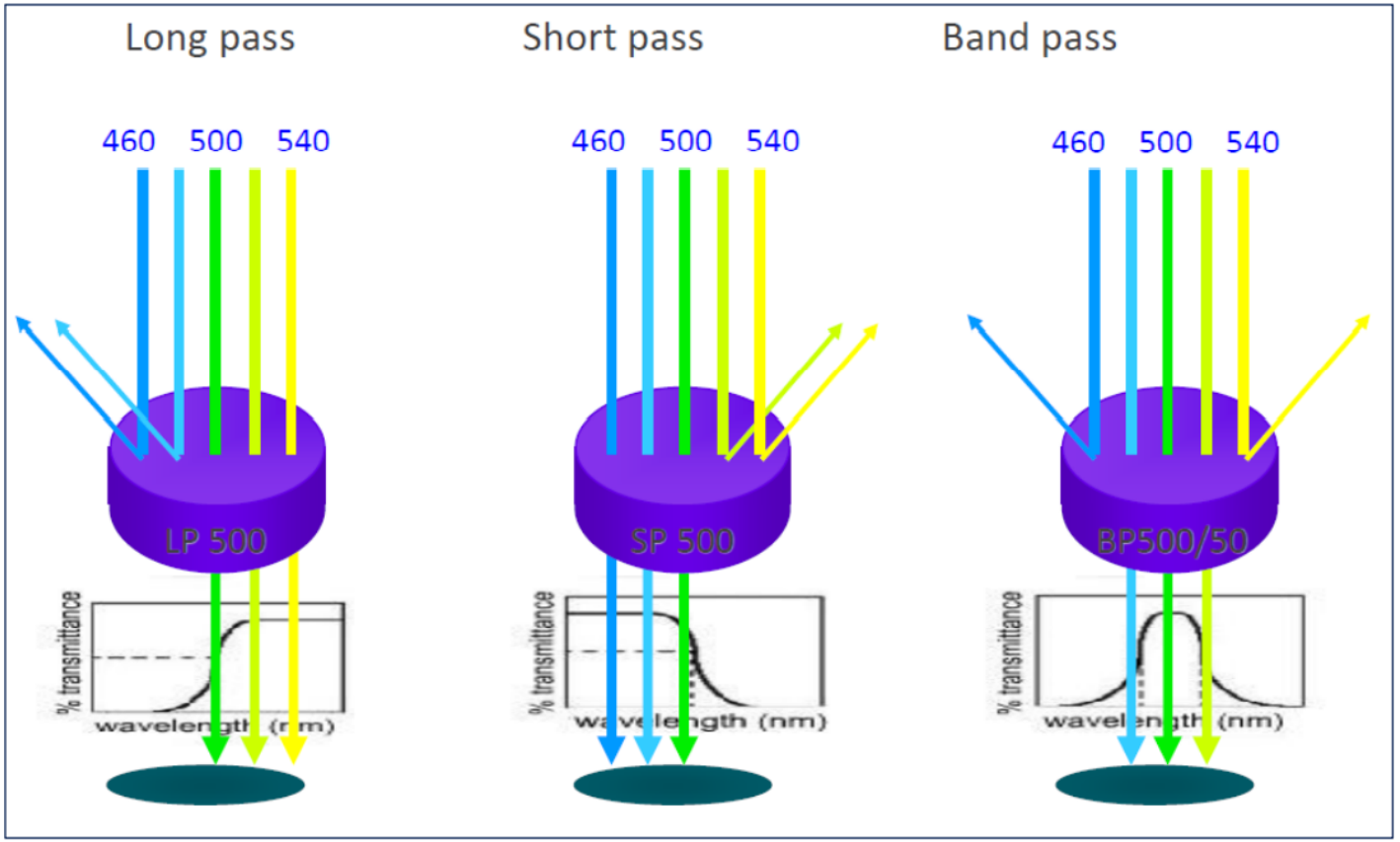
## Detectors

### PMT: Photo Multiplyer Tube

# Optics

## Filtersets in front of detectors



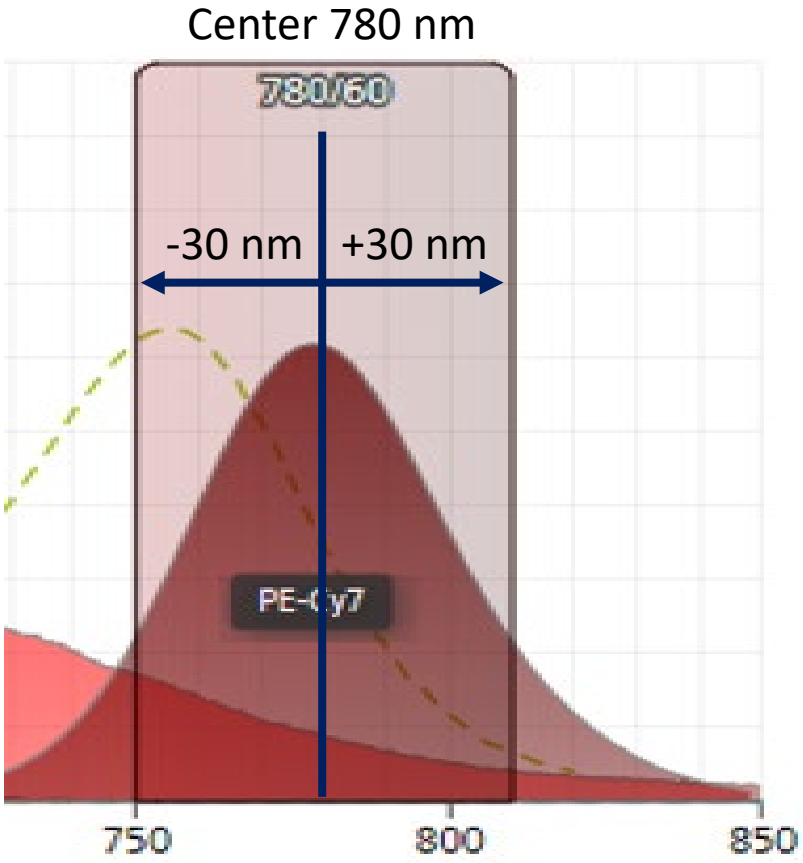

*LP: transmission of photons above a specified wavelength*

*SP: transmission of photons below a specified wavelength*

*BP: transmission of photons that have wavelengths within a narrow range*

# Optics

## Filtersets in front of detectors

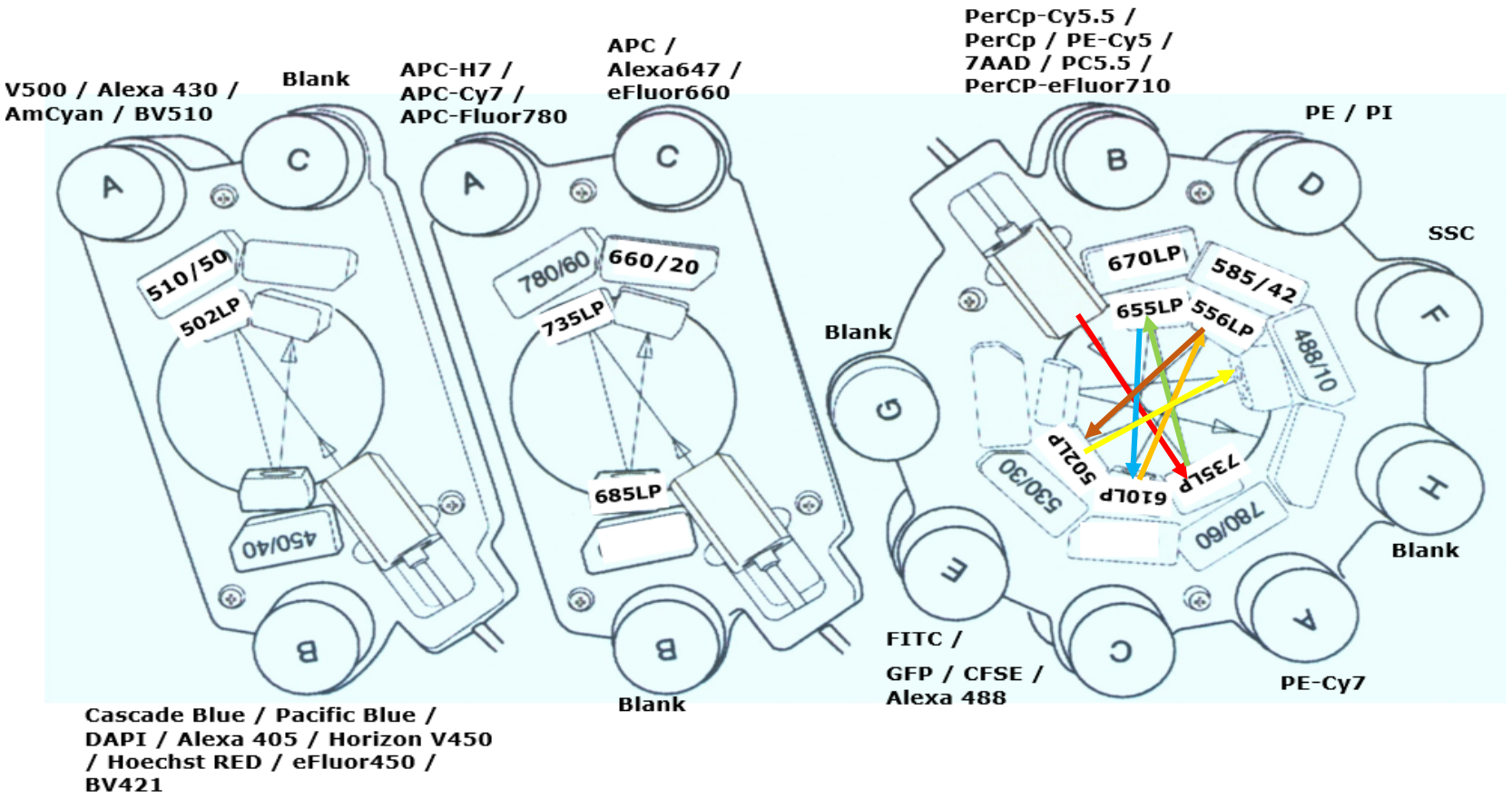



**780/60 BP filter**

Transmission of photons in the range of 750 to 810 nm

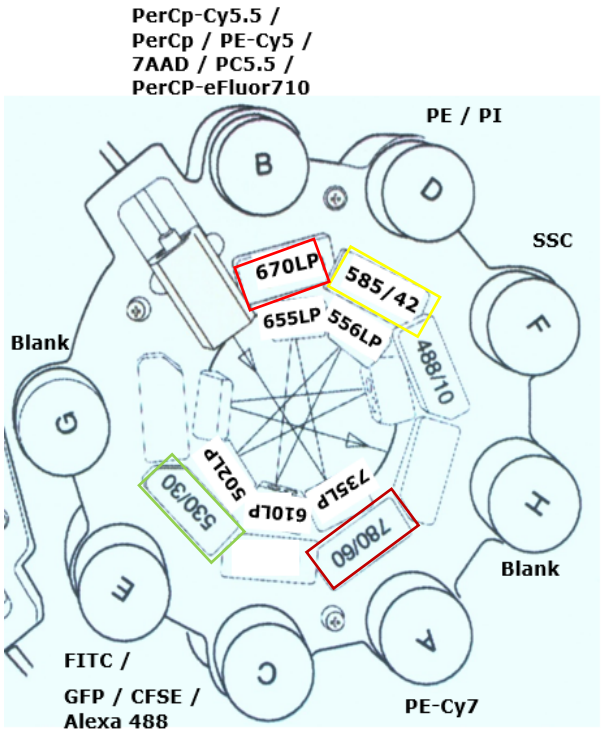
# Optics

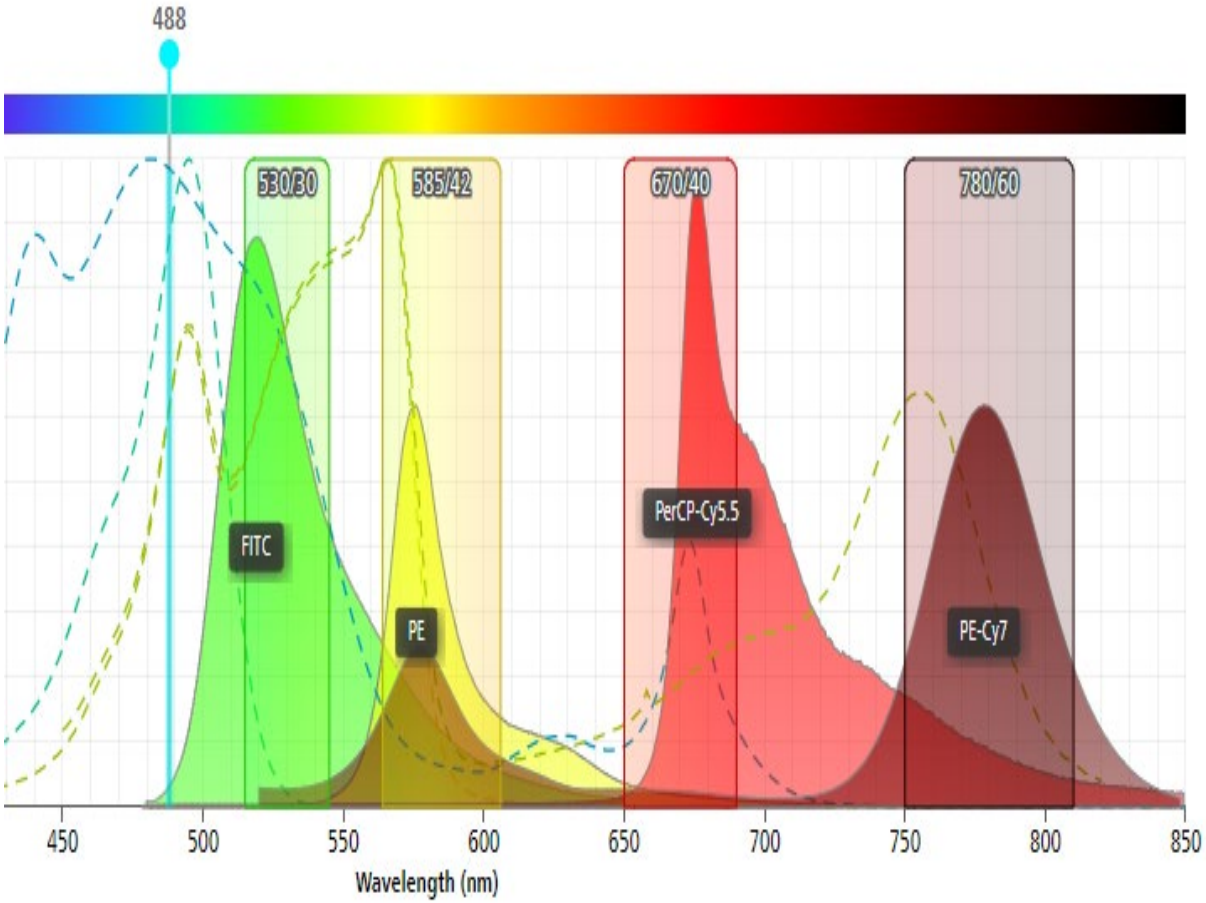
## Detectors



# Optics

## Detectors



# Optics

## Which fluorchromes can we detect at MUMC+?

## -> depends on configuration of the machine:

BD FACS Canto
- 3 laser
- 8 colours

| Laser | PMT | LP | BP | Fluorchromes |
|---|---|---|---|---|
| 488 | A | 735 | 780/60 | PE-Cy7 |
| | B | 685 | 710/50 | PE-Cy5.5<br>PerCp |
| | D | 556 | 585/42 | PE |
| | E | 520 | 530/30 | FITC<br>A488 |
| | | | | |
| 633 | A | 735 | 780/60 | APC-Cy7<br>APC-H7 |
| | B | - | 660/20 | APC<br>A647 |
| | | | | |
| 405 | A | 750 | 510/50 | V500<br>BV510 |
| | B | - | 450/40 | Pacific blue<br>Hoechst<br>Dapi<br>BV421 |

BD Fusion sorter
- 4 lasers
- 16 colours

| Laser | PMT | LP | BP | Fluorchromes |
|---|---|---|---|---|
| 488 | A | 655 | 695/40 | PerCp-Cy5.5<br>PerCp |
| | B | 502 | 530/30 | FITC |
| | C | - | 488/10 | SSC |
| | | | | |
| 561 | A | 735 | 780/60 | PE-Cy7 |
| | B | 685 | 710/50 | PE-Cy5.5 |
| | C | 630 | 670/14 | PE-Cy5 |
| | D | 600 | 610/20 | PE-Cy594<br>PI<br>mCherry<br>PE-TxRed |
| | E | - | 582/15 | PE<br>DsRed |
| | | | | |
| 640 | A | 755 | 780/60 | APC-Cy7<br>APC-H7 |
| | B | - | 670/30 | APC<br>A647 |
| | C | 690 | 730/45 | Alexa700 |
| | | | | |
| 405 | A | 750 | 780/60 | BV786 |
| | B | 690 | 710/50 | BV711 |
| | C | 630 | 660/20 | BV650 |
| | D | 595 | 610/20 | BV605 |
| | E | 505 | 525/50 | BV480<br>BV510<br>V500 |
| | F | - | 450/40 | BV421<br>V450<br>Pacific Blue<br>eFluor450 |

# Optics

## Cytek Aurora:

## full spectrum flowcytometry


UV laser (355): 7 channels

Violet laser (405): 18 channels

Red laser (635): 6 channels

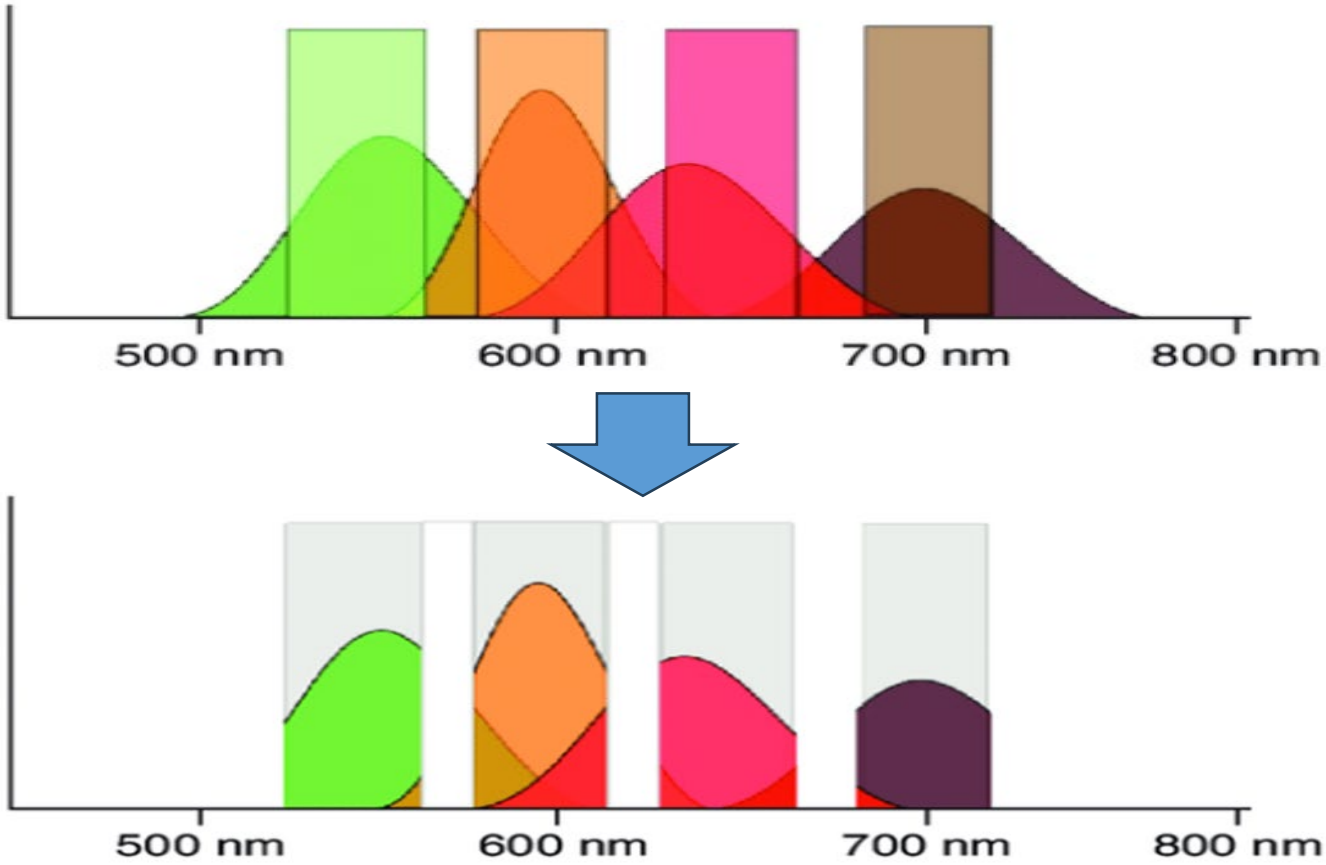
Blue laser (488): 7 channels

**38 channels in total**

# Optics

## Conventional versus full spectrum

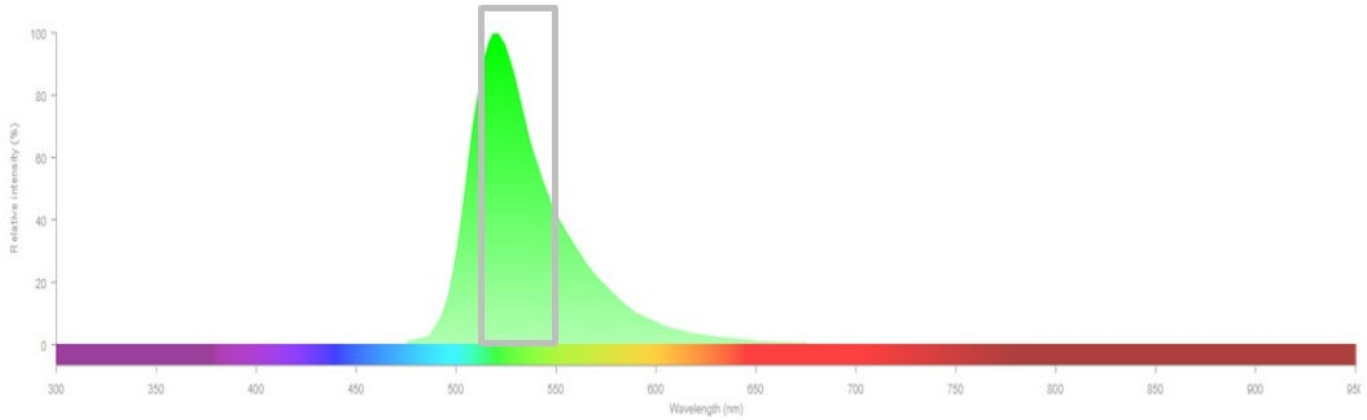
Conventional:



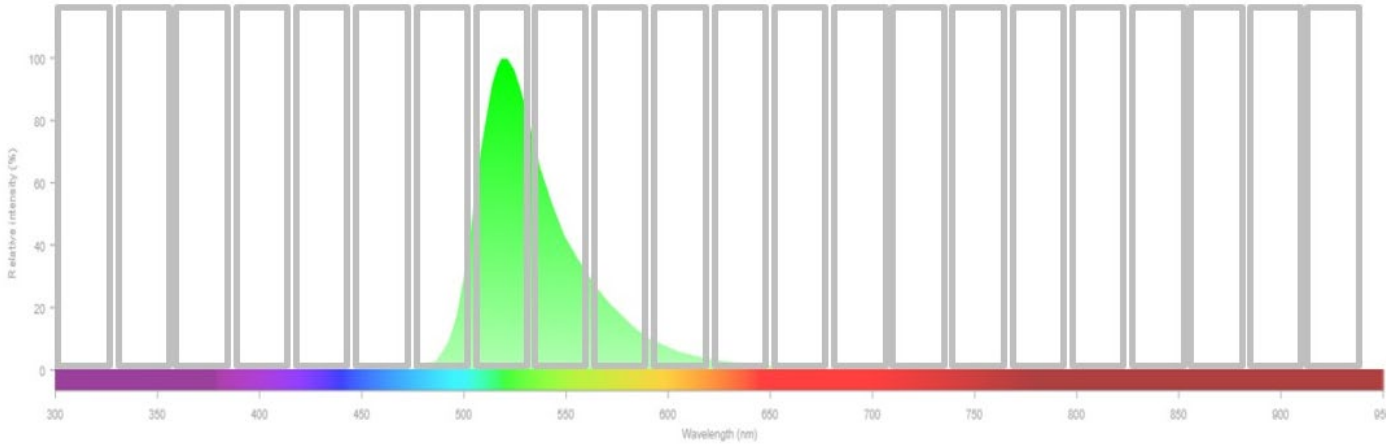
# Optics

## Conventional versus full spectrum

Conv




Full spect

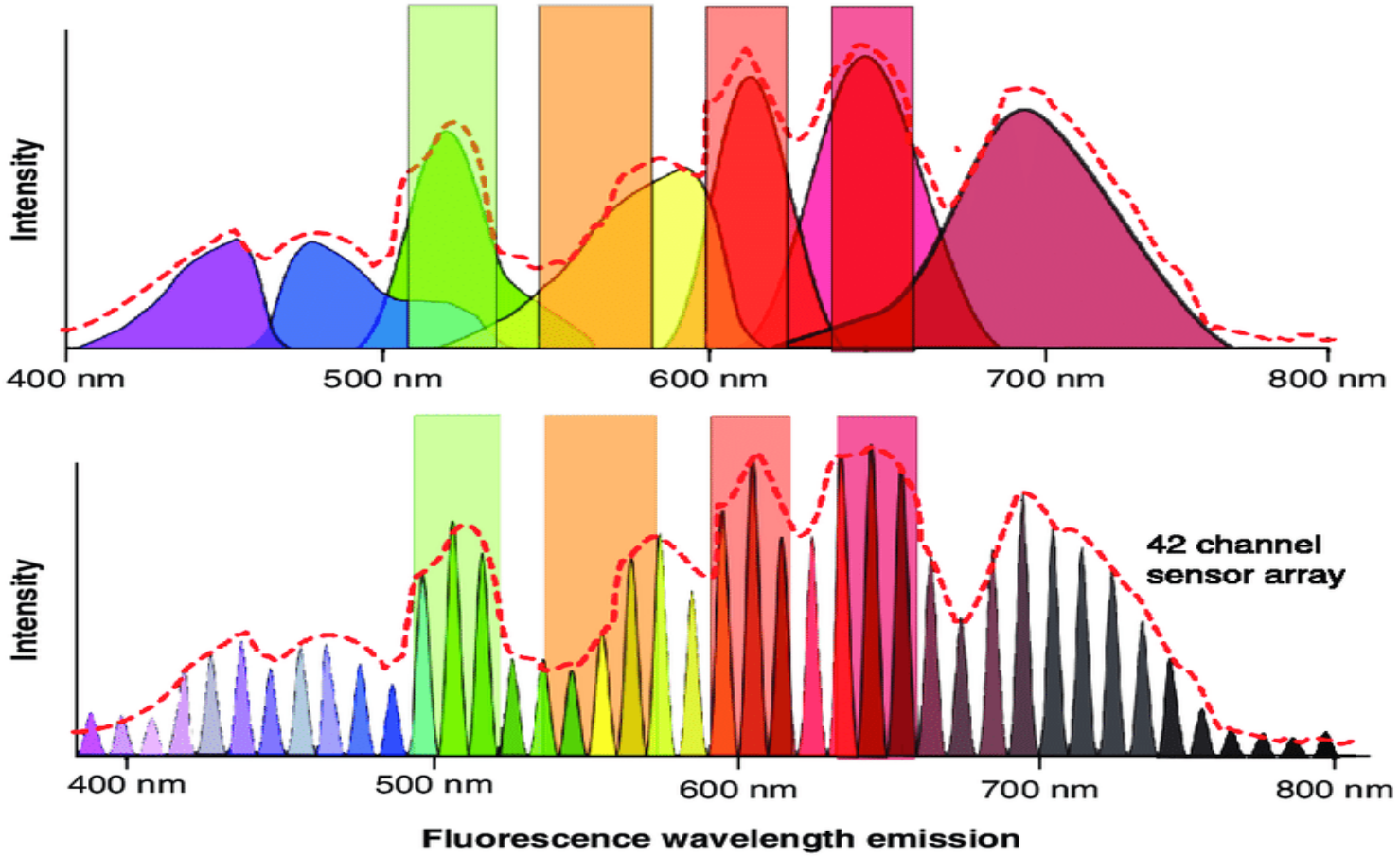


# Optics

## Conventional versus full spectrum

Conv

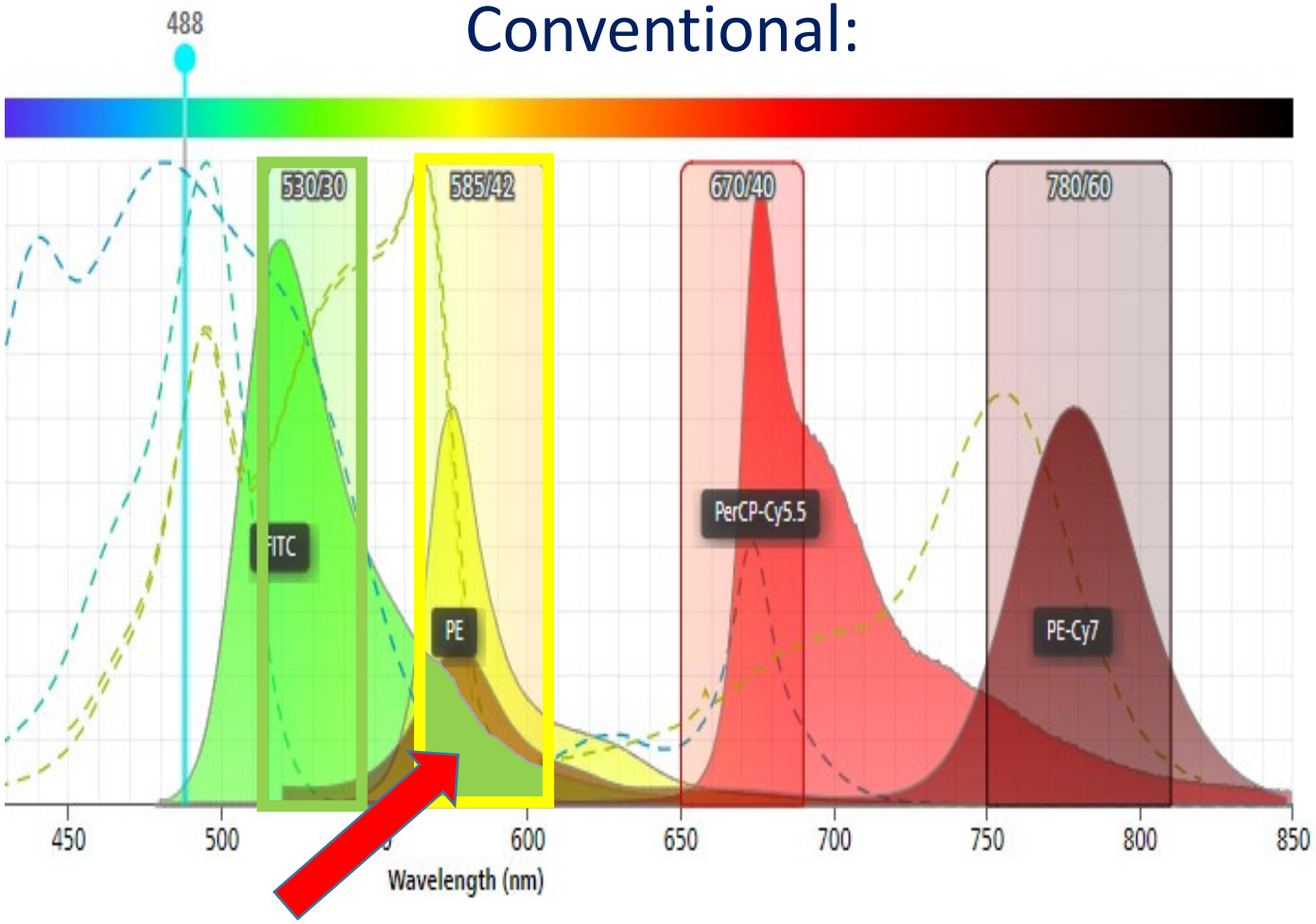
Full spect



# Optics

## Compensation

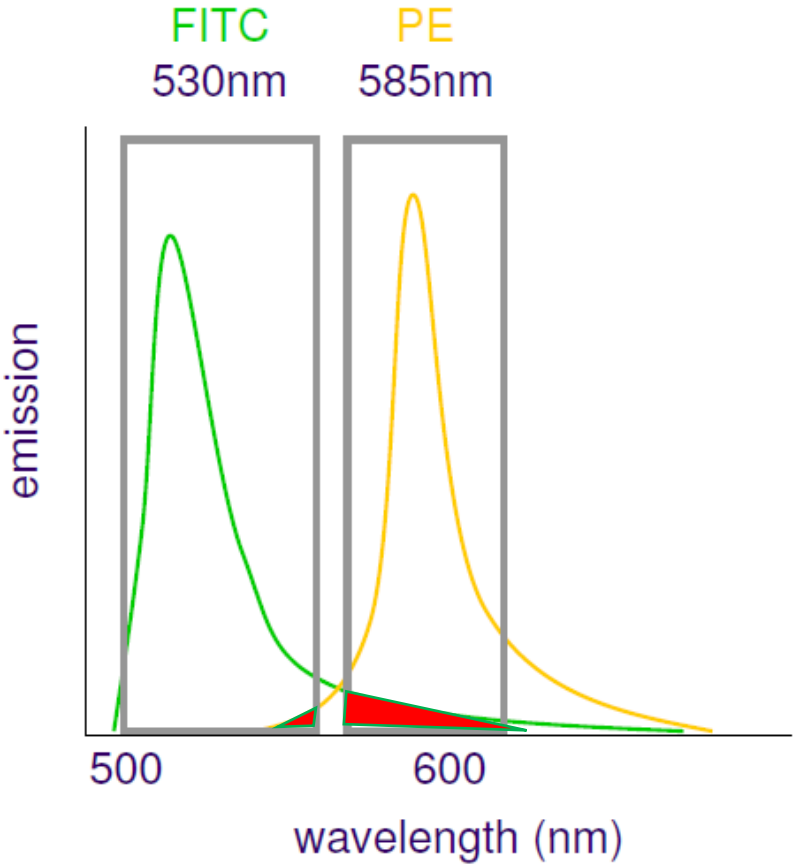
### Conventional:

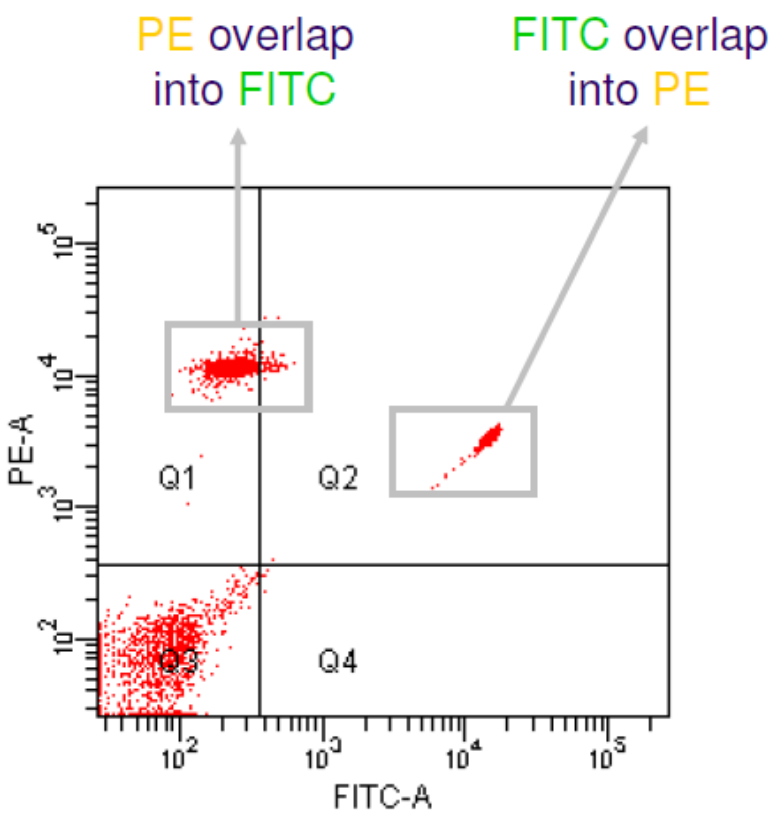


# Optics

## Compensation

### Conventional:



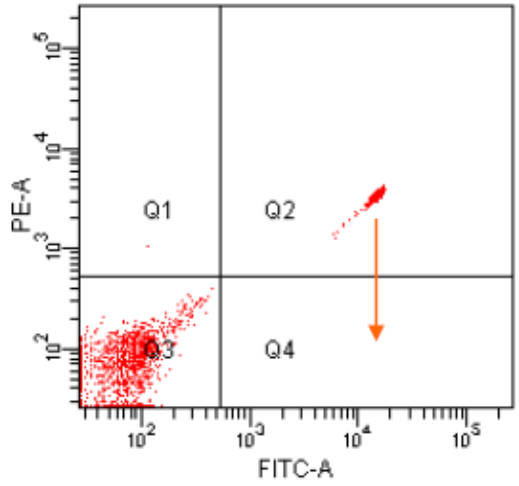
# Optics

## Compensation

### Conventional:

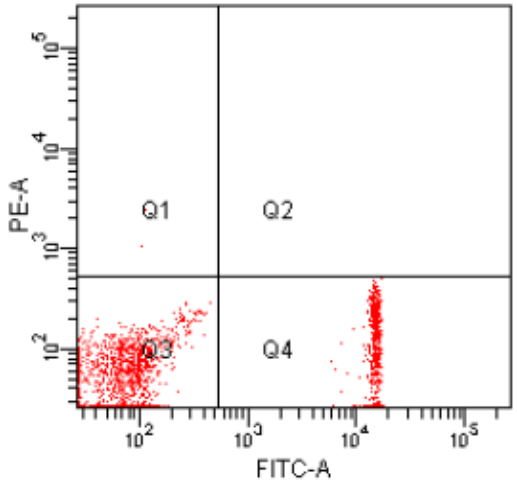
**FITC single stain**:

**No compensation**




| Population | PE-A Mean |
|---|---|
| Q1 | 11,675 |
| Q2 | 3,511 |
| Q3 | 78 |
| Q4 | #### |

**compensated**




| Population | PE-A Mean |
|---|---|
| Q1 | 11,657 |
| Q2 | #### |
| Q3 | 60 |
| Q4 | 60 |

# Optics

## Compensation

### Conventional:

**How do we do compensation:**

Applying unstained and a single stains to the machine

**Full stain:**

- ❑ CD3 FITC
- ❑ CD19 PE
- ❑ CD56 APC

**Compensation controles:**

1. Unstained
2. Only CD3 FITC stained
3. Only CD19 PE stained
4. Only CD56 APC stained

# Optics

## Compensation

### Conventional:

Spillover matrix of available fluorochromes on Canto II (4-2-2)

| | FITC | PE | PerCP | PE-Cy7 | APC | APC-H7 | BV510 | BV421 |
|---|---|---|---|---|---|---|---|---|
| **FITC** | | 18.7 | 2.1 | 0 | 0 | 0 | 0 | 0 |
| **PE** | 0.6 | | 14 | 3.5 | 0 | 0 | 0 | 0 |
| **PerCP** | 0 | 0 | | 9.8 | 11.6 | 3.6 | 0 | 0 |
| **PE-Cy7** | 0 | 4.3 | 4.1 | | 0 | 2.8 | 0 | 0 |
| **APC** | 0 | 0 | 1.1 | 0 | | 14.2 | 0 | 0 |
| **APC-H7** | 0 | 0 | 0 | 1.2 | 0.9 | | 0 | 0 |
| **BV510 / V500** | 1.8 / 2.6 | 0 | 0 | 0 | 0 | 0 | | 1.8 / 0.3 |
| **BV421** | 0 | 0 | 0 | 0 | 0 | 0 | 1.6 | |

Example of Spillover Values on BD FACSCanto II (4-2-2): PMT-V setting by CS&T

Avoid combination on the same cell (if not possible: FMO Control necessary)

Make sure that the "Troublemaker" is lower expressed than the other (FMO Control advisable)

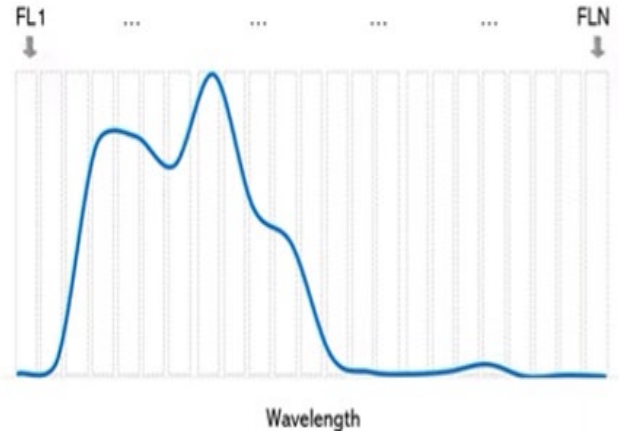
Typically no/low effects on resolution (FMO Control unnecessary)

# Optics

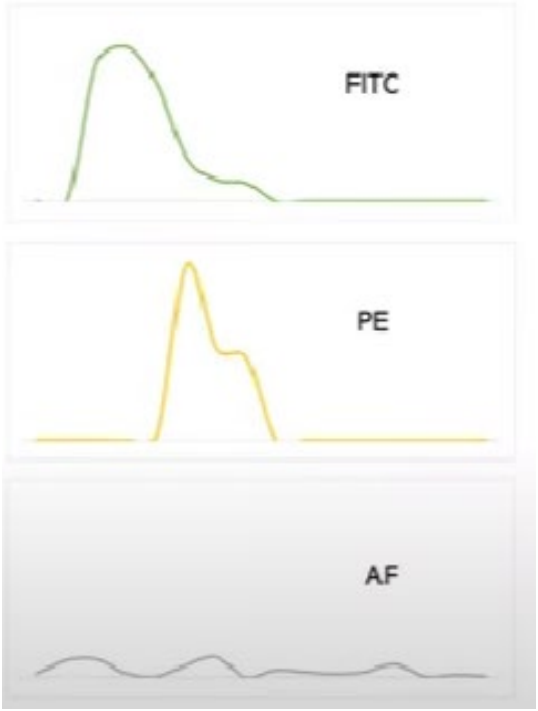
## Compensation

### Full spectrum

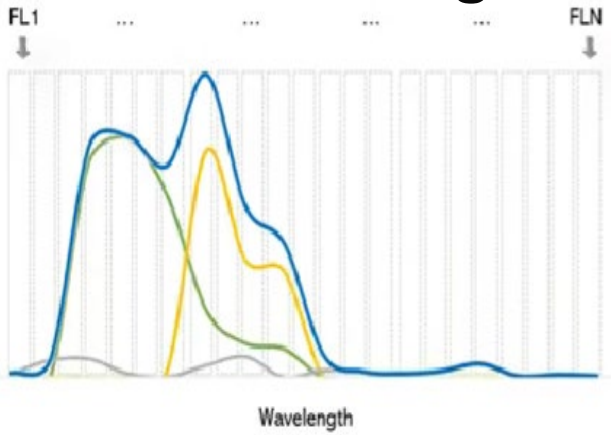
**Full stain (FITC +PE)**




**Single stain contr. / Reference spectra**




**Compensation / unmixing**



# Optics

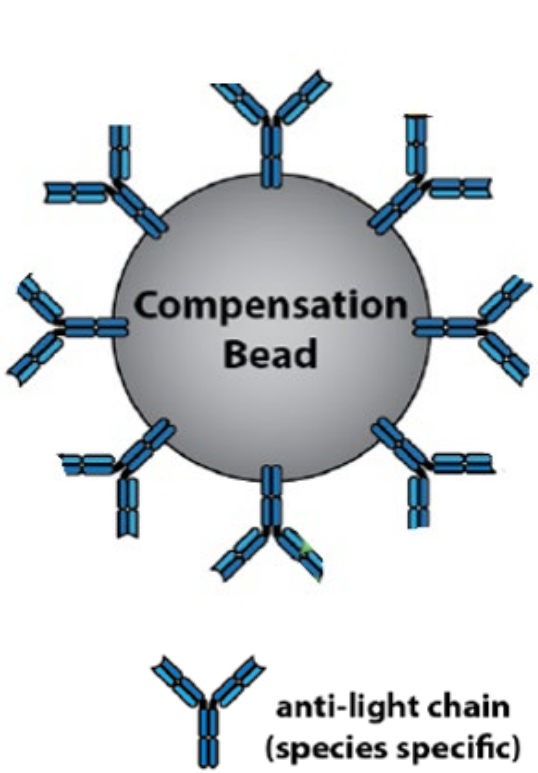
## compensation

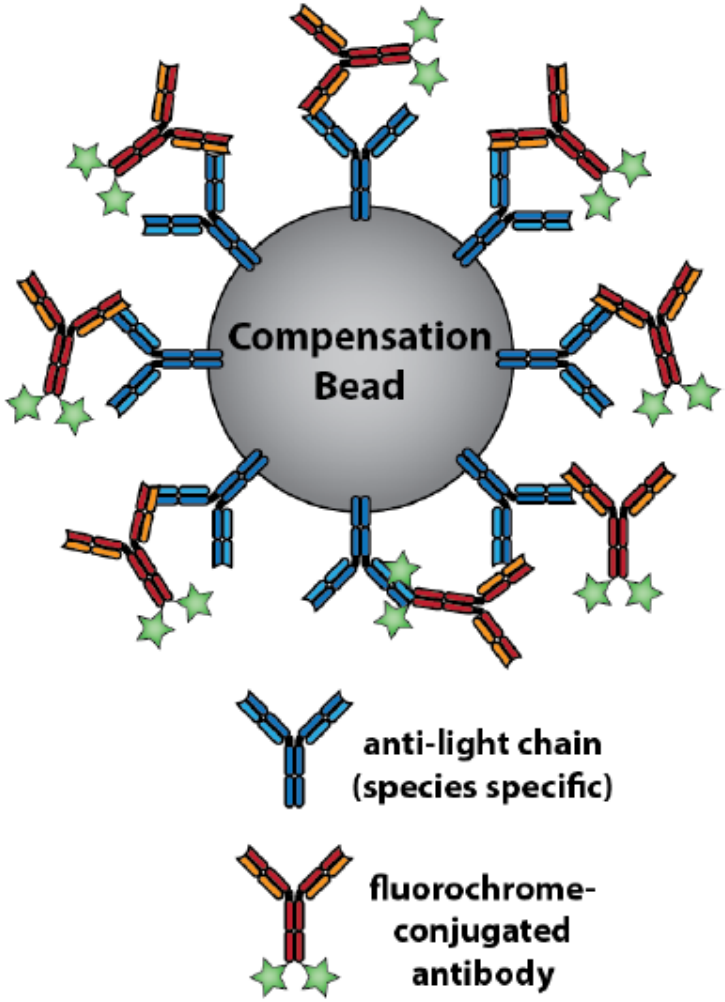
### 3 rules of compensation:

1. The control must be at least as bright as the experimental sample the compensation will be applied to.
2. The backgrounds of the positive and negative samples must be identical.
   - Use unstained cells for compensations stained on cells and negative beads for bead compo. Because the spill-over is compensation based on the mean of the negative population
3. The control must match the experimental fluorochrome. This means the tube must be acquired at the same voltage and the exact same fluorochrome has been used
   - So FITC is compensated with FITC and not Alexa488. Tandem-dyes need lot specific compensation.

# Optics

## compensation



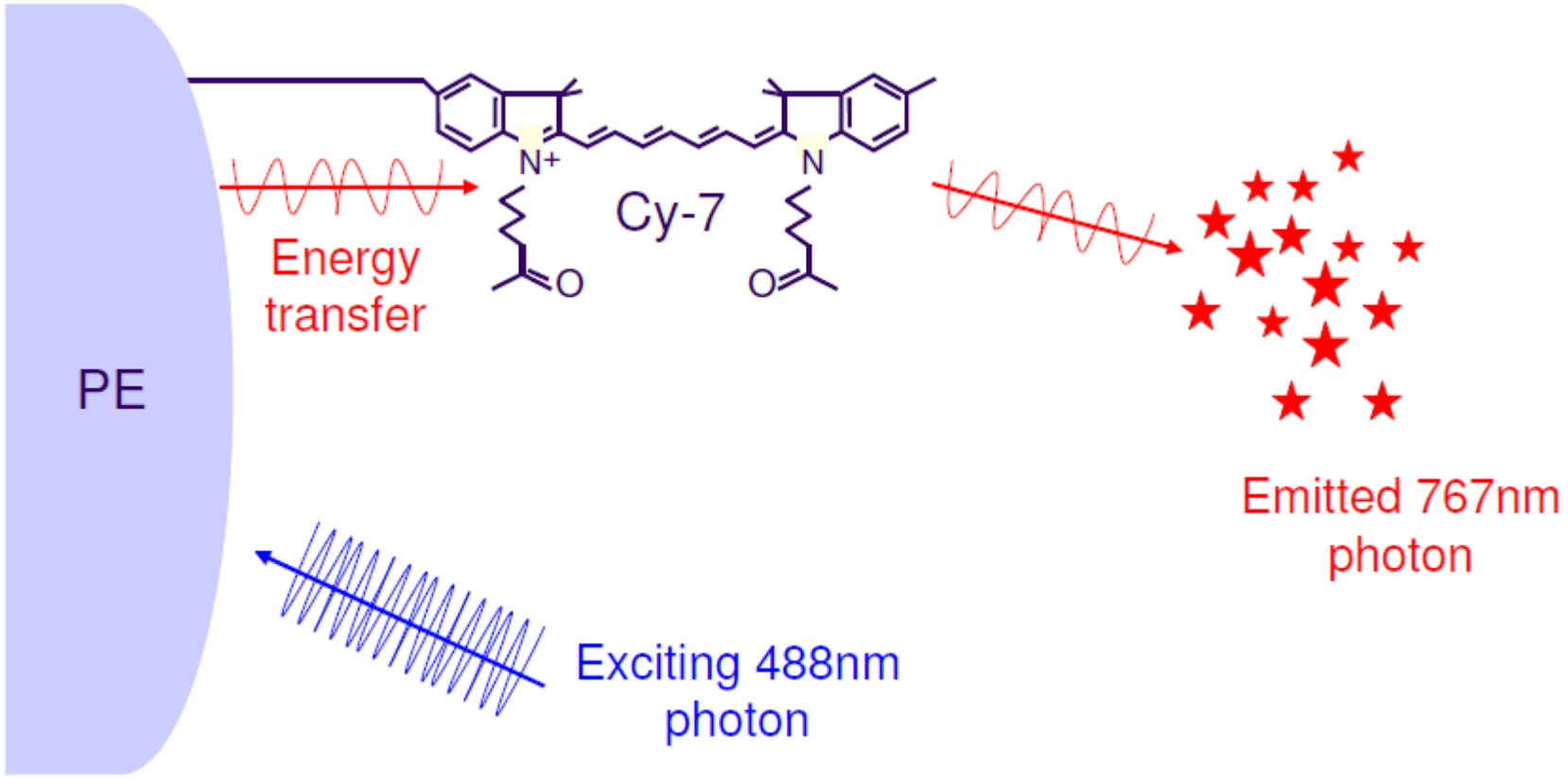
# Optics

## Fluorchromes

- Fluorescent proteins
  - Green fluorescent protein (GFP), YFP, RFP
  - PE, APC, PerCpD
- Synthetic small molecules
  - FITC / Cy5
- Polymer dyes
  - Briljant Violet dyes (BV421, BV510, etc)
- Tandem conjugates
  - PE-Cy7, APC-Cy7, Perp-Cy5.5

# Optics

## Tandem conjugates



# Optics

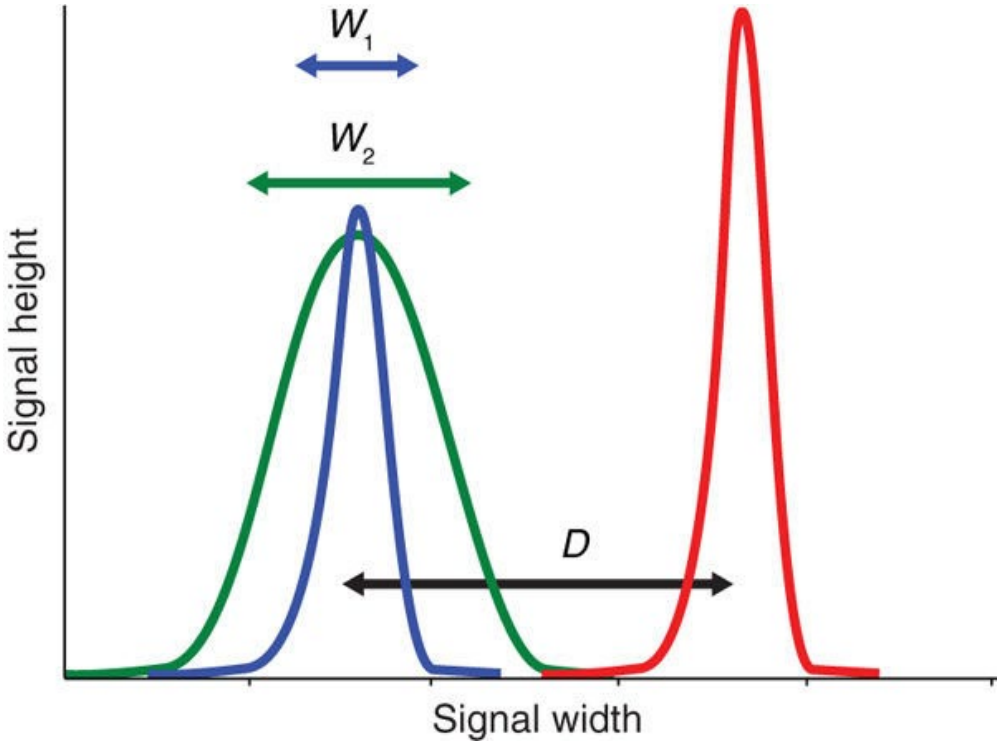
## Tandem conjugates

- Compensation for tandem dyes can vary:
  require experiment-specific compensation
- Tandem dye degradation:
  - In bottle
  - On stained cells
- Aggravated by exposure to:
  - Light
  - Elevated temperature
  - Formaldehyde based fixation

# Optics

## Fluorchromes

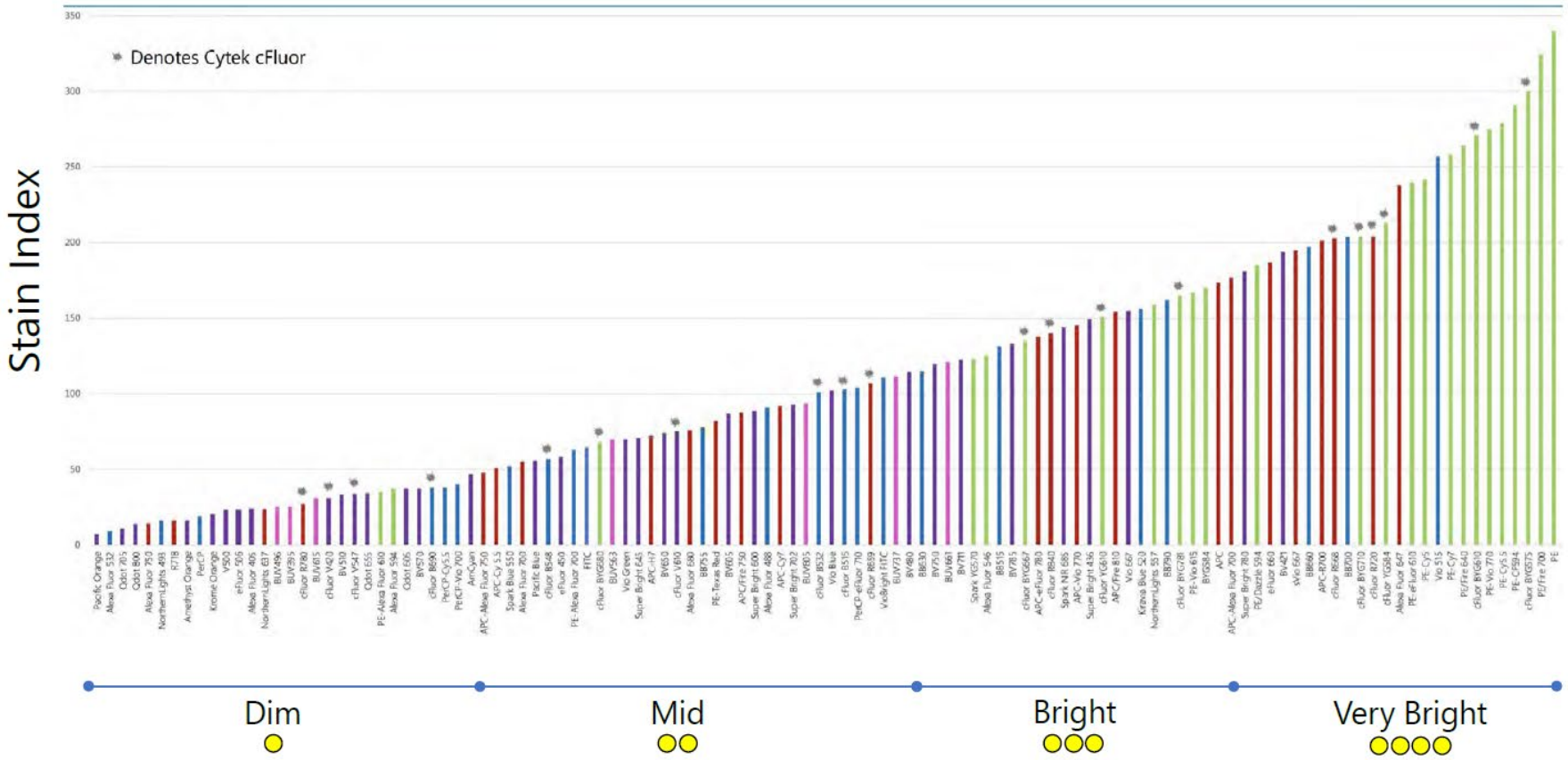
### Staining index




| | Reagent | Clone | Filter | Stain Index |
|---|---|---|---|---|
| | PE | RPA-T4 | 575/26 | 305 |
| | APC[1] | RPA-T4 | 660/20 | 263 |
| | PE-Cy™5[2] | RPA-T4 | 695/40 | 198 |
| | Alexa Fluor® 647[1] | RPA-T4 | 660/20 | 184 |
| | PE-Cy™7 | RPA-T4 | 780/60 | 122 |
| | PerCP-Cy™5.5[2] | RPA-T4 | 695/40 | 99 |
| | Alexa Fluor® 488[3] | RPA-T4 | 530/30 | 68 |
| | BD Horizon™ V450[5] | RPA-T4 | 450/50 | 65 |
| | Alexa Fluor® 700 | RPA-T4 | 720/40 | 64 |
| | Pacific Blue™.[5] | RPA-T4 | 450/50 | 63 |
| | FITC[3] | RPA-T4 | 530/30 | 43 |
| | AmCyan[6] | RPA-T4 | 525/50 | 37 |
| | APC-Cy7[4] | RPA-T4 | 780/60 | 36 |
| | PerCP[2] | RPA-T4 | 695/40 | 30 |
| | BD Horizon™ V500[6] | RPA-T4 | 525/50 | 27 |
| | BD APC-H7[4] | RPA-T4 | 780/60 | 25 |

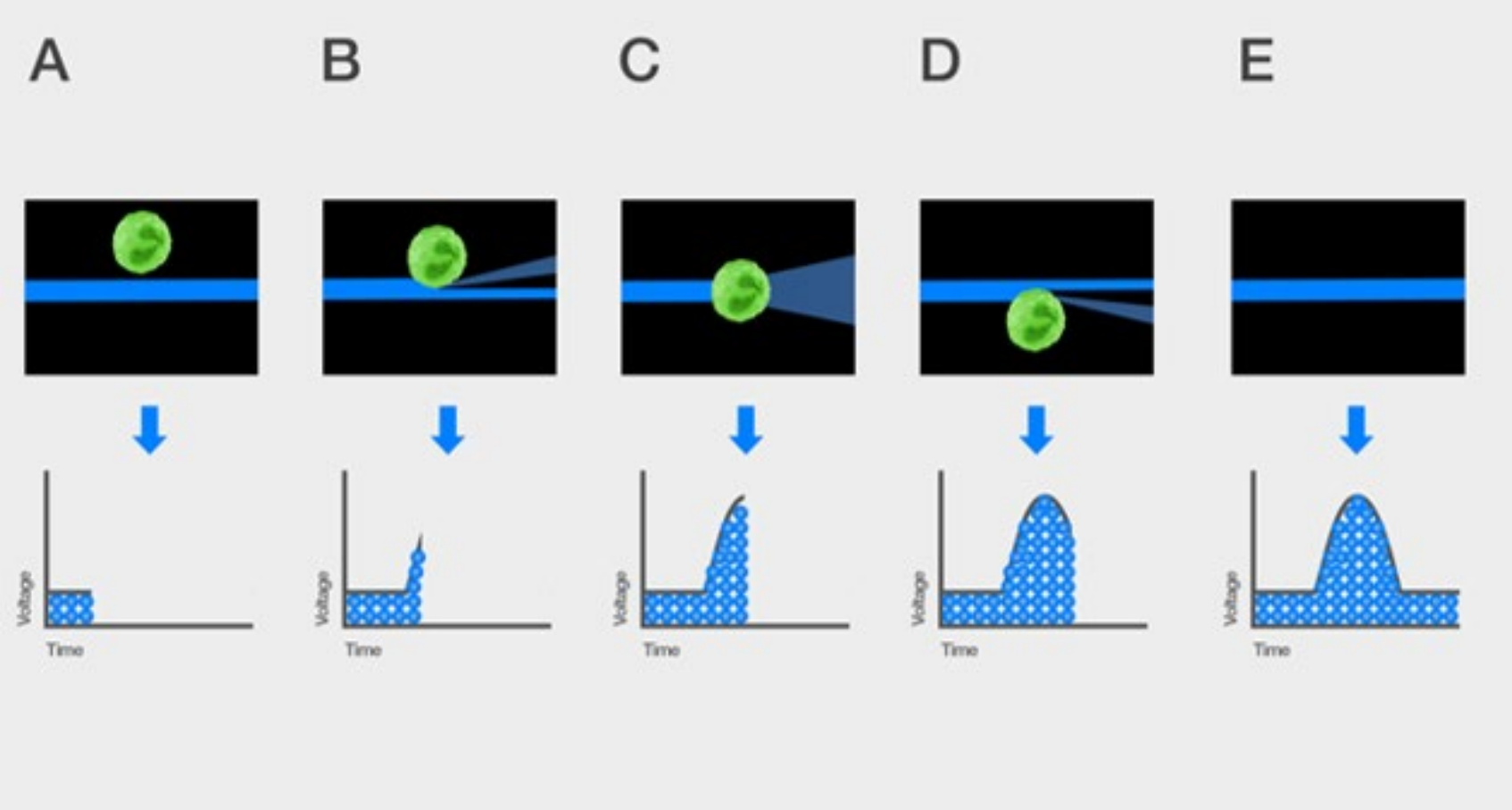
# Optics

## Fluorchromes



# Electronics

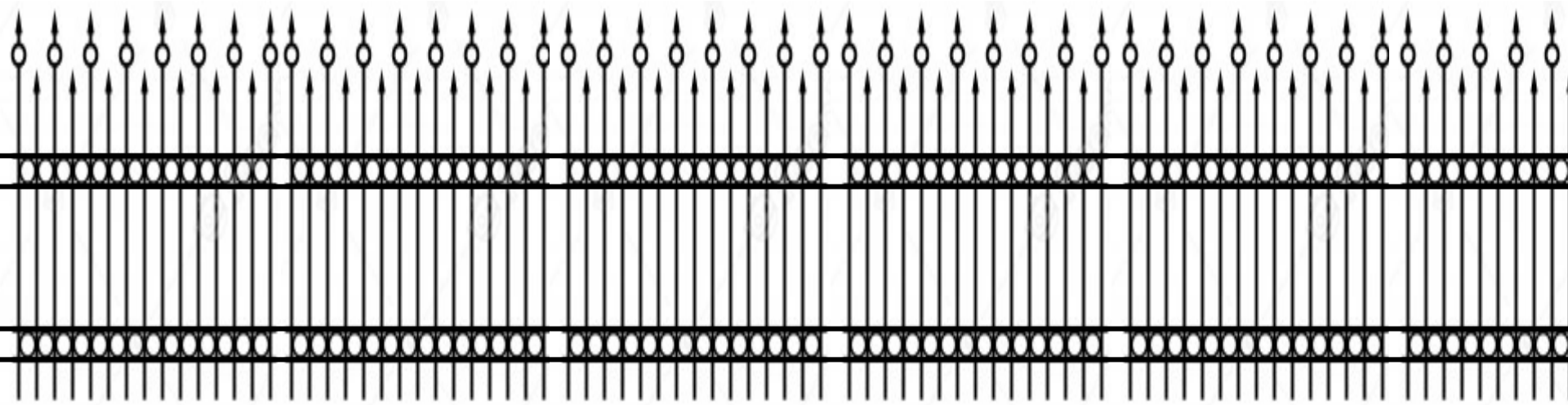
## Conversion of light into data



# Data display and gating

## Which plots do we have?

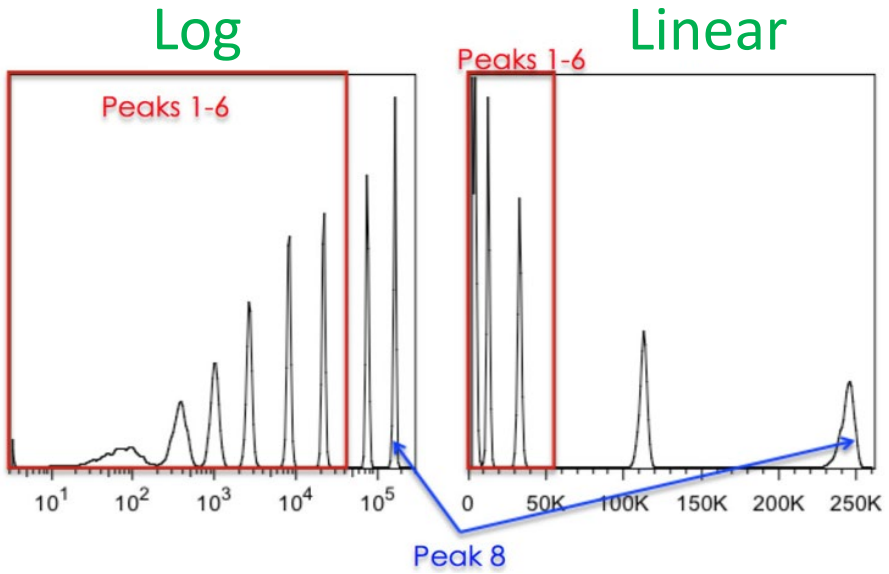
- Univariant: Histogram
- Bivariant: Dotplot
- Higher order plots: 3D-plots, SPADE trees, etc

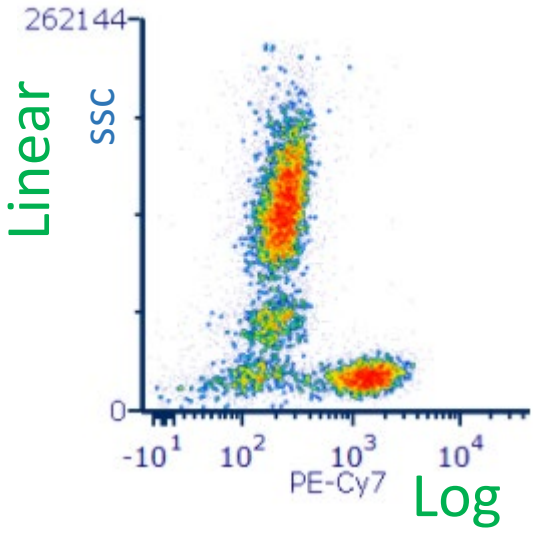


# Data display and gating

## Which plots do we have?

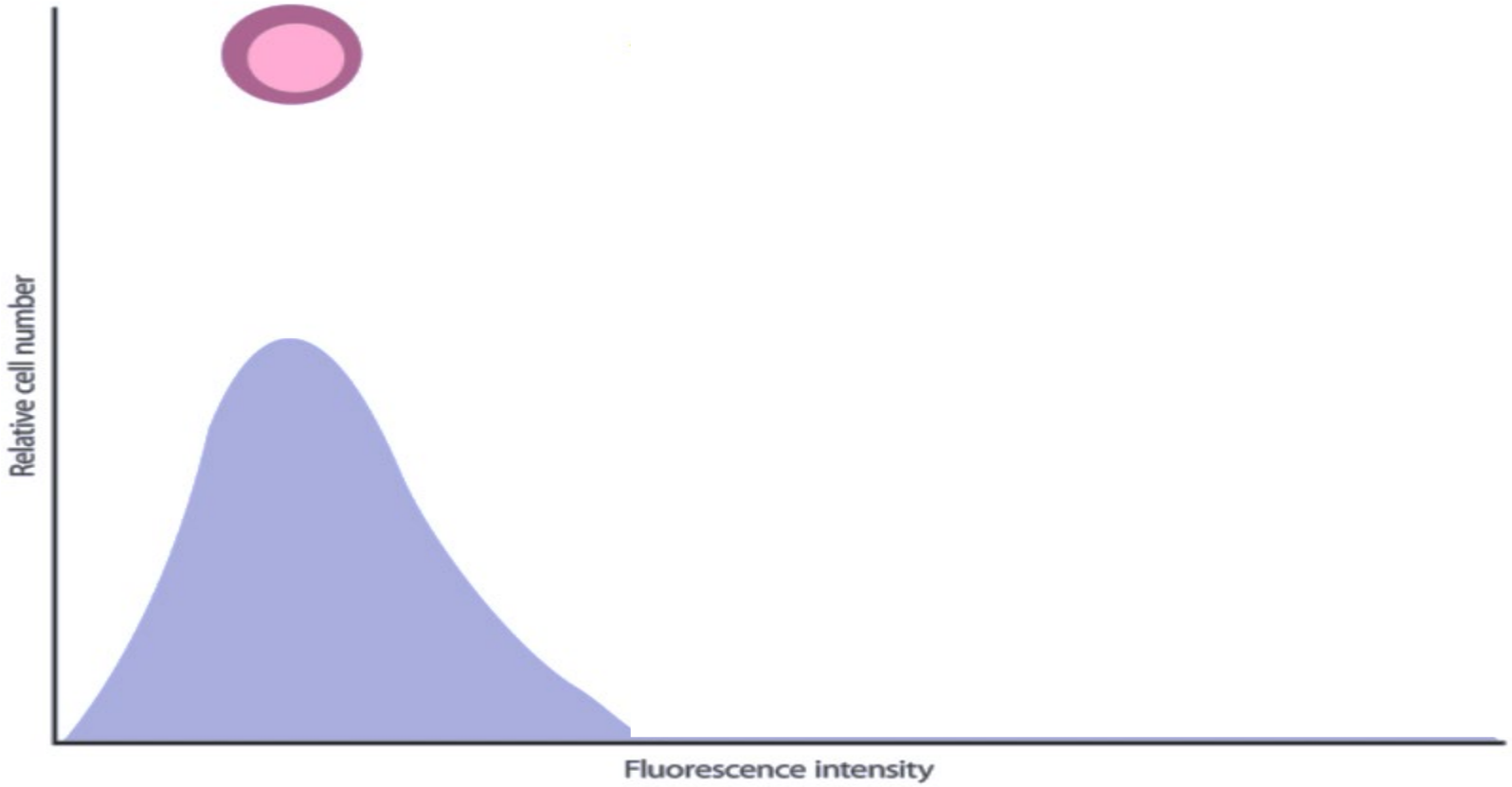
- **Linear scale:** light scatter measurement where particle differ subtly in signal intensity
- **Log scale:** fluorescence measurment where particles differ quite starkly in signal (exception: cell cycle)



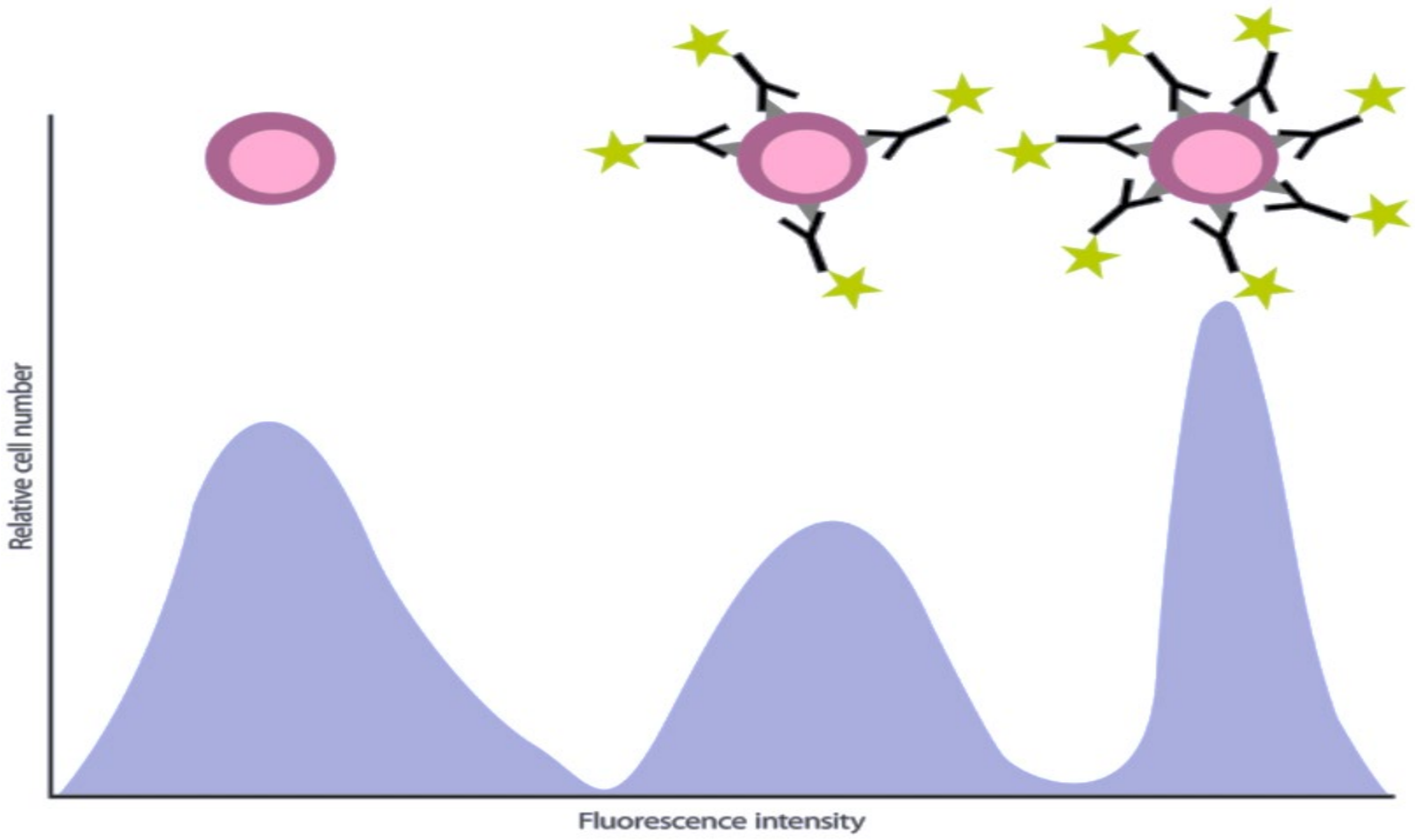
# Data display and gating

## Histogram



# Data display and gating

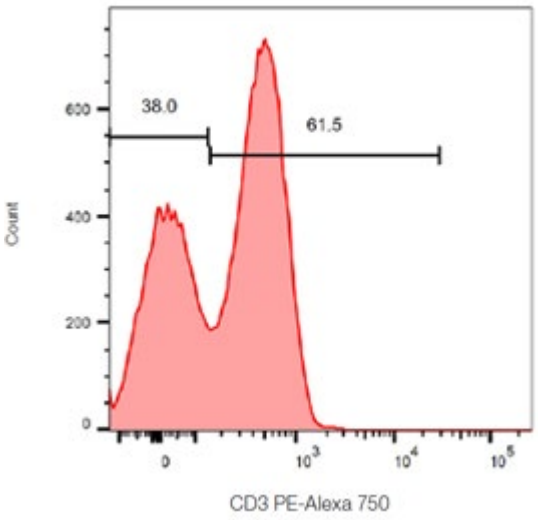
## Histogram



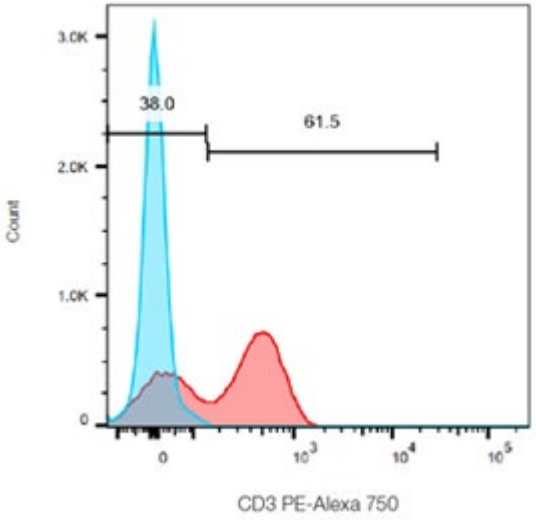
# Data display and gating

## Histogram

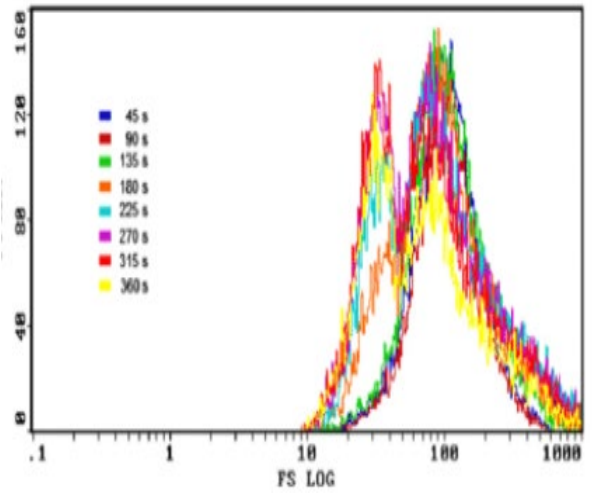
1 population




2 populations



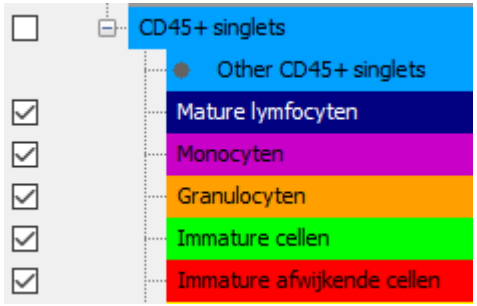

Overlay of
Several samples

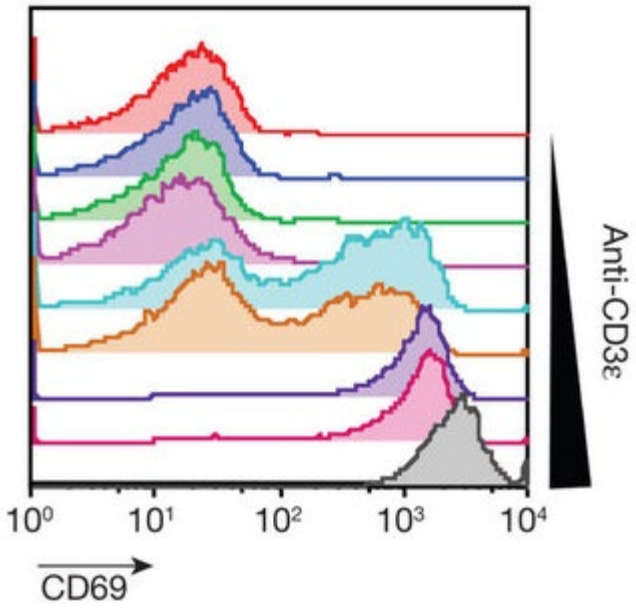


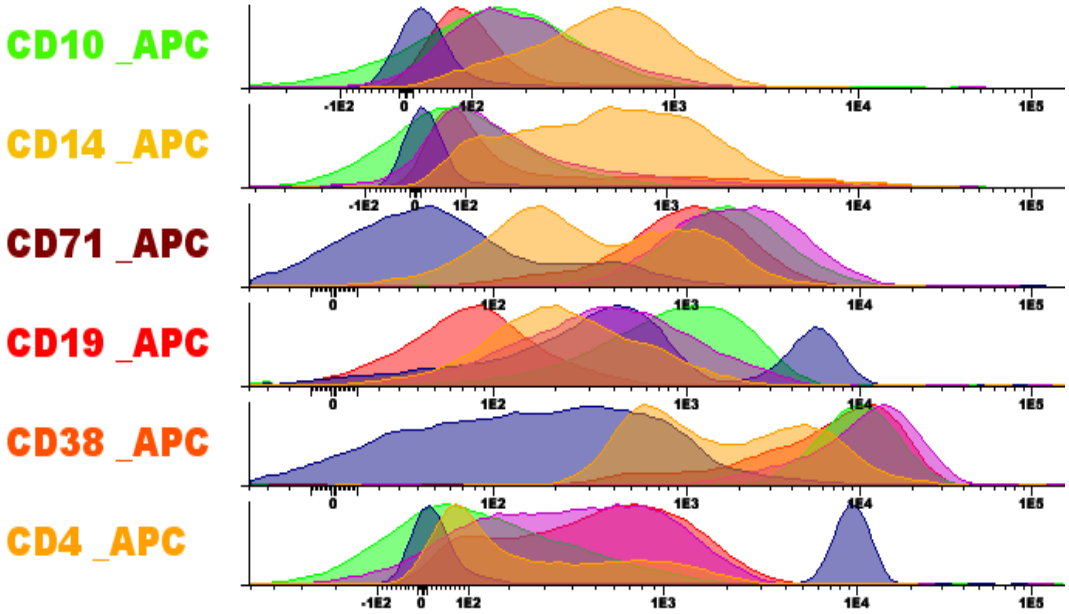
# Data display and gating

## Overlay histogram

Staged Histogram: Several sampels



# Data display and gating

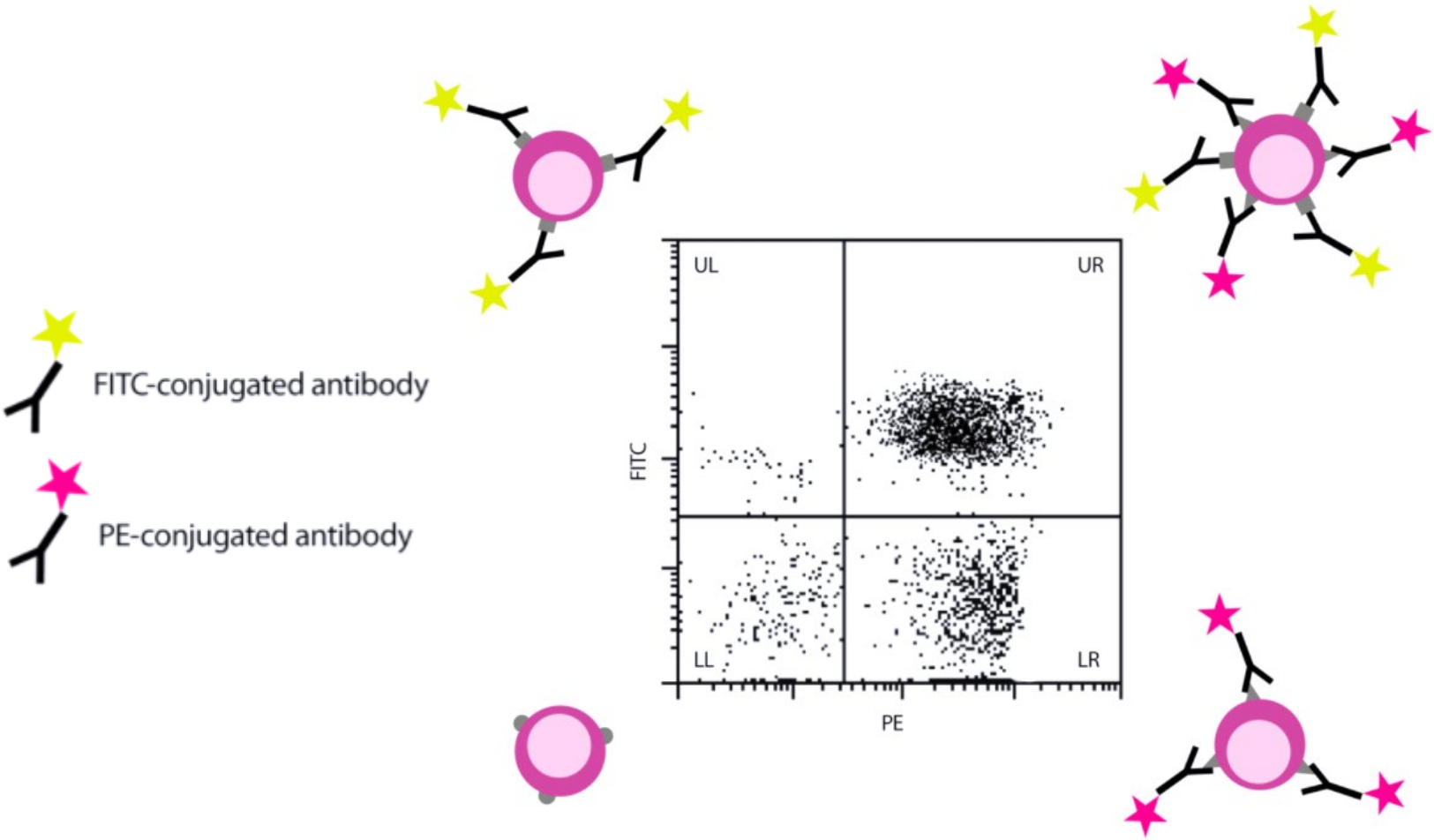
## Histogram

**Be aware:**

- You cannot see the relationship between two populations
- You can miss sub-populations that have similar values in one parameter
- You can see false positive artifacts as real signals

# Data display and gating

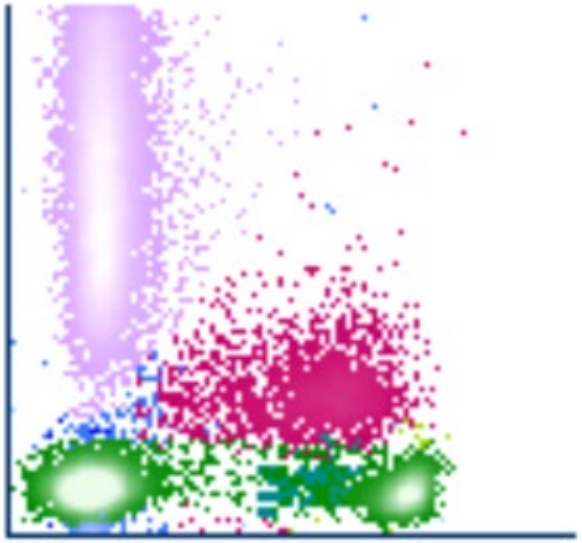
## Dot plot



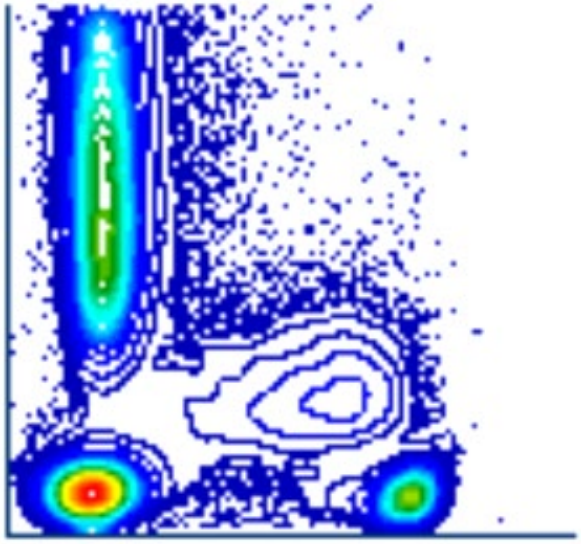
# Data display and gating

## Dot plot

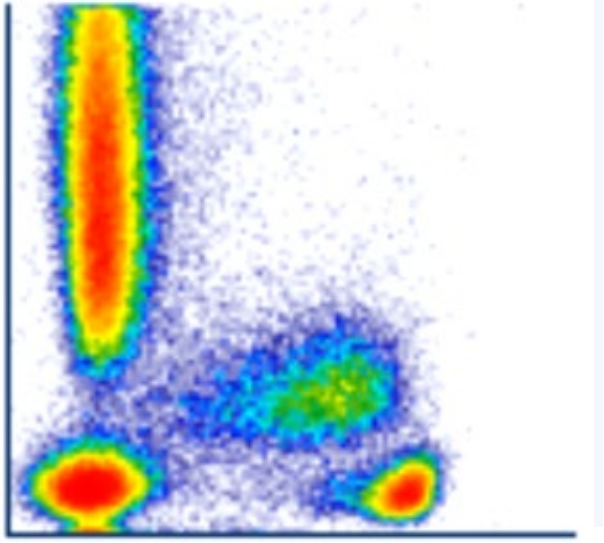
(color) Dotplot



Contourplot



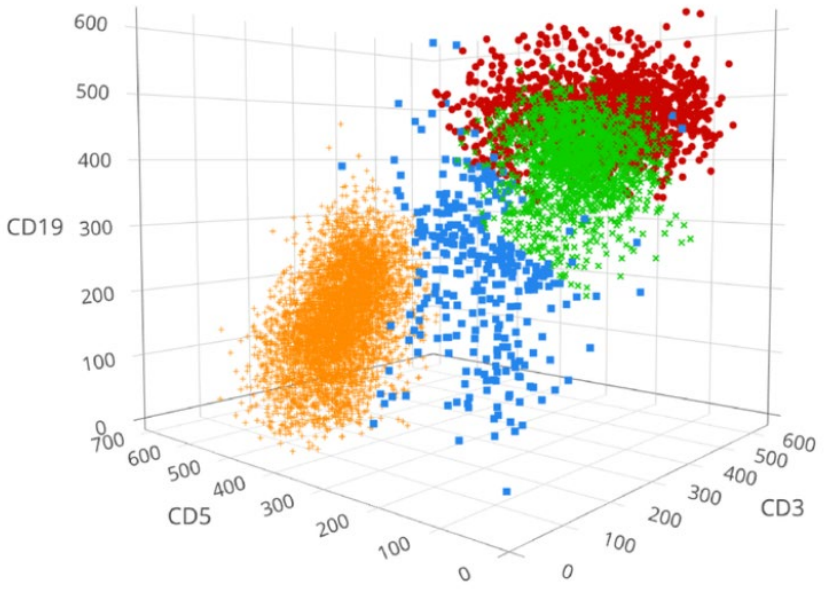
Denstity plot



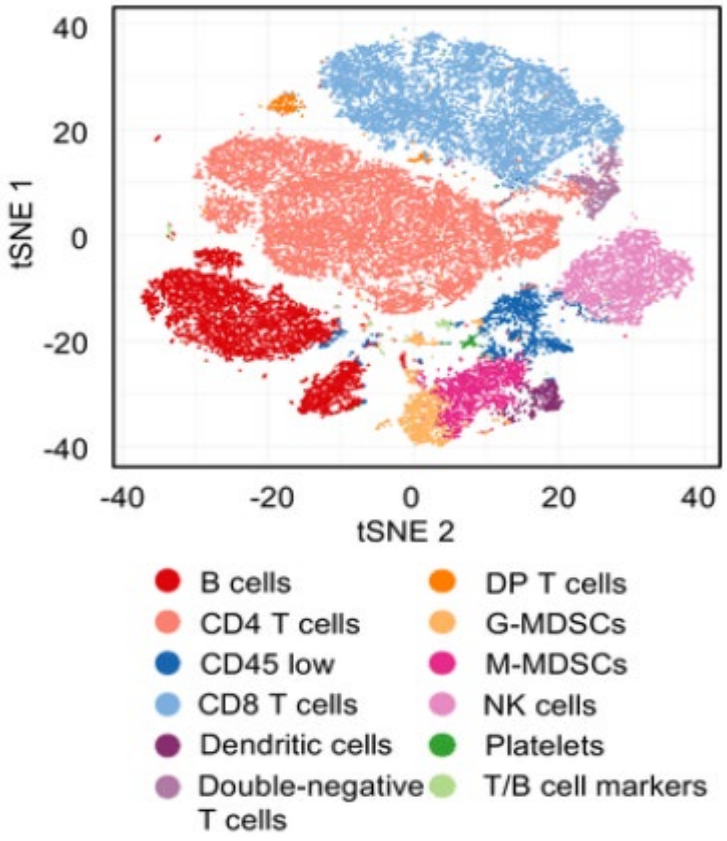
# Data display and gating

## Higher order plots

3D-plot



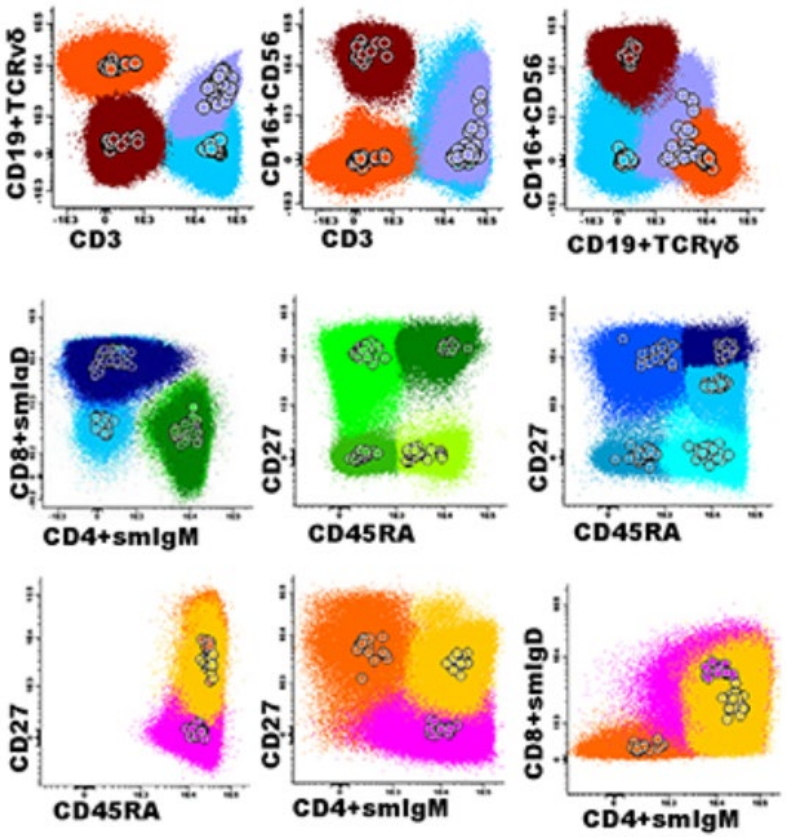

Cluster-analysis plots (high dimensional)



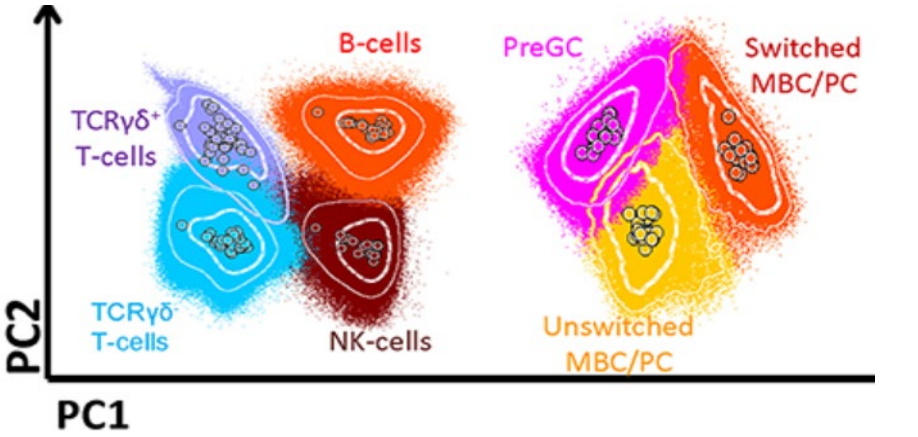
# Data display and gating

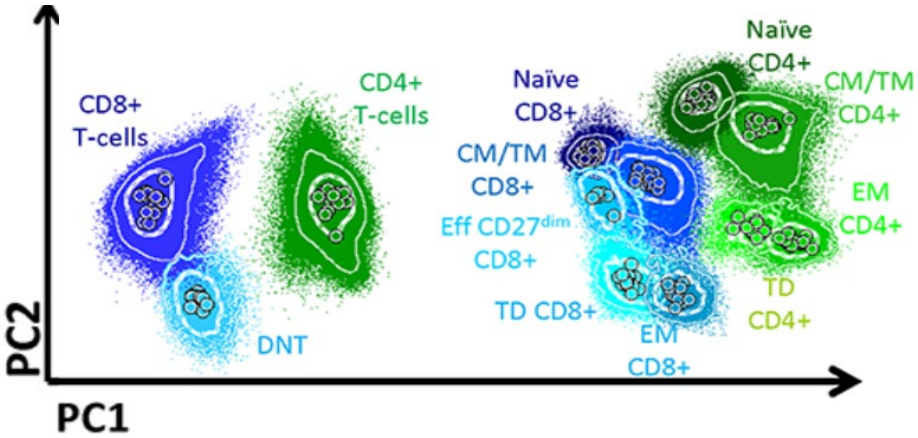
## Higher order plots

"Authentic" dotplot



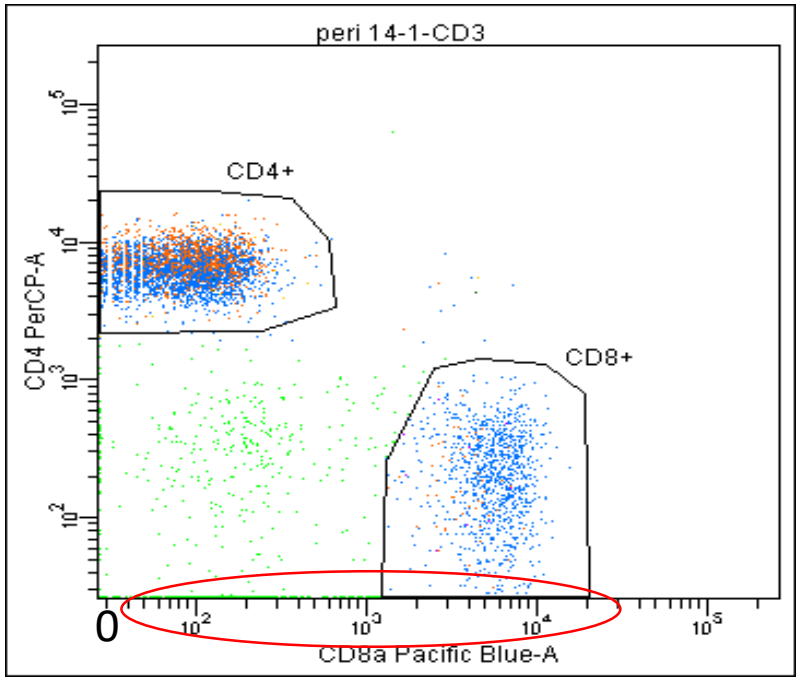

Automatic Population Selection

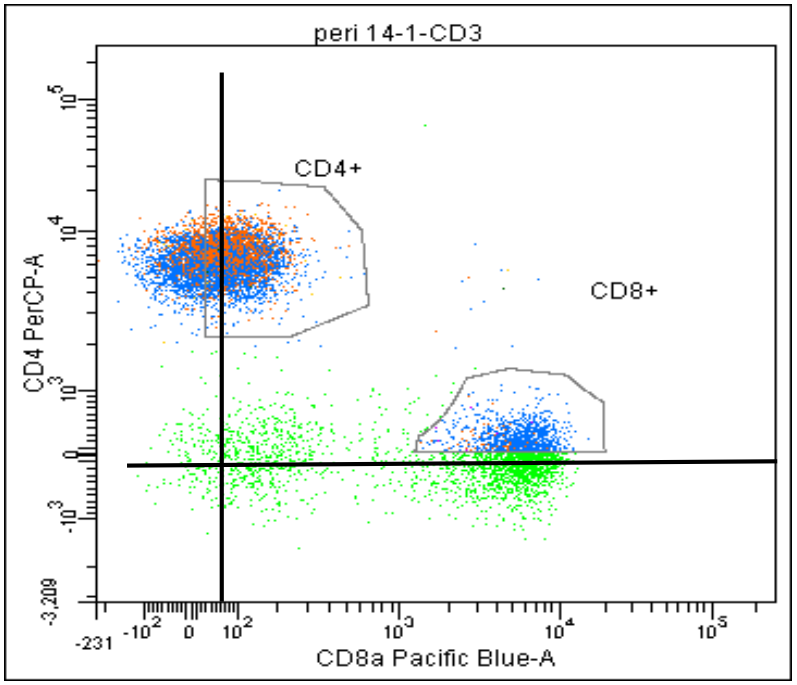


# Data display and gating

## Biexponential display



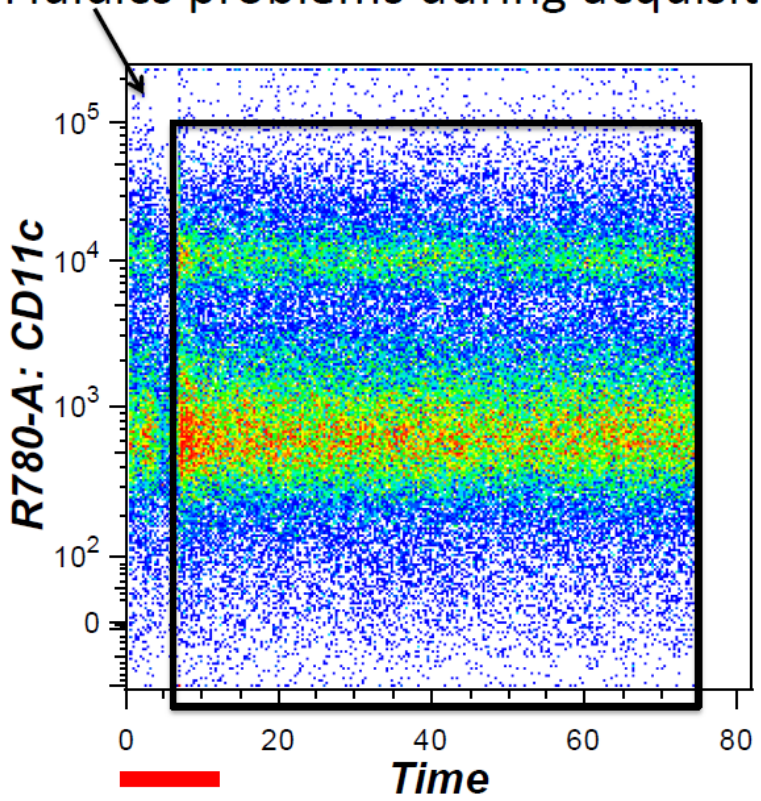




**Changing the scaling does not change the values, just the display of the data**

# Data display and gating

## Fluidics artefacts

Fluidics problems during acquisition cause artifacts in the data




To visualize:

look at data vs time

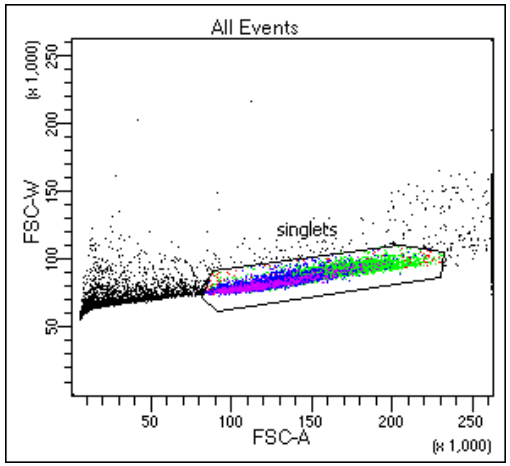
Then gate out the bad data

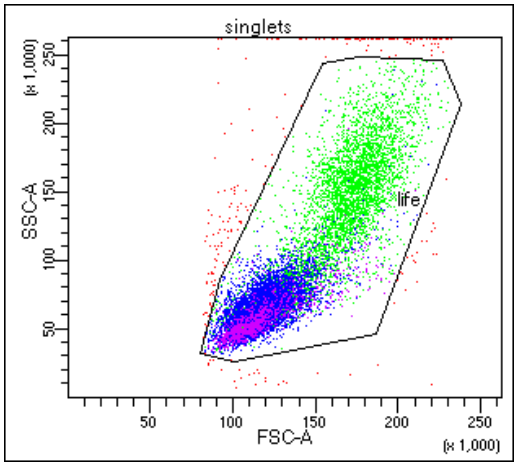
ZosiaMaciorowski

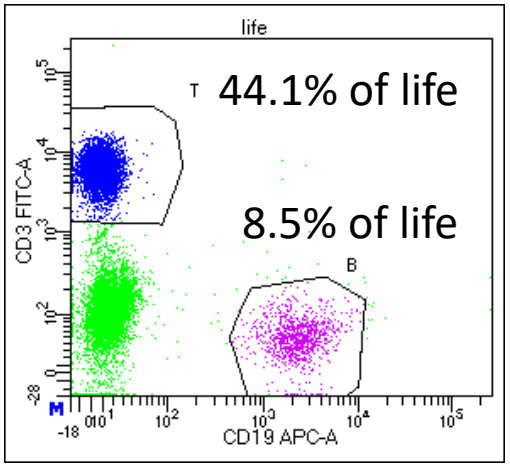
# Data display and gating

## Basic statistics

### ❑ Frequency



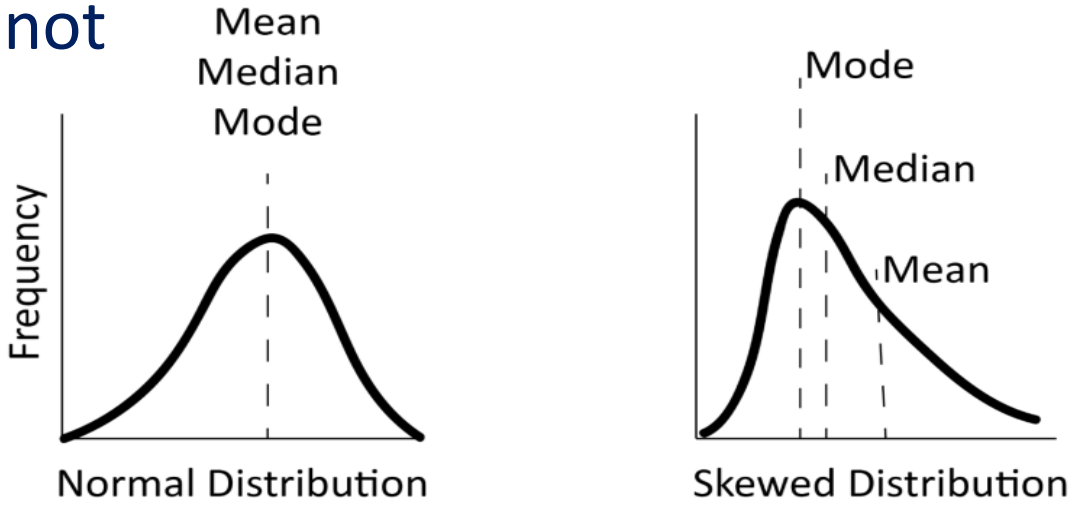

Tube: 1

| Population | #Events | %Parent | %Total |
|---|---|---|---|
| All Events | 34,160 | #### | 100.0 |
| singlets | 12,720 | 37.2 | 37.2 |
| life | 12,463 | 98.0 | 36.5 |
| T | 5,490 | 44.1 | 16.1 |
| B | 1,056 | 8.5 | 3.1 |

# Data display and gating

## Basic statistics

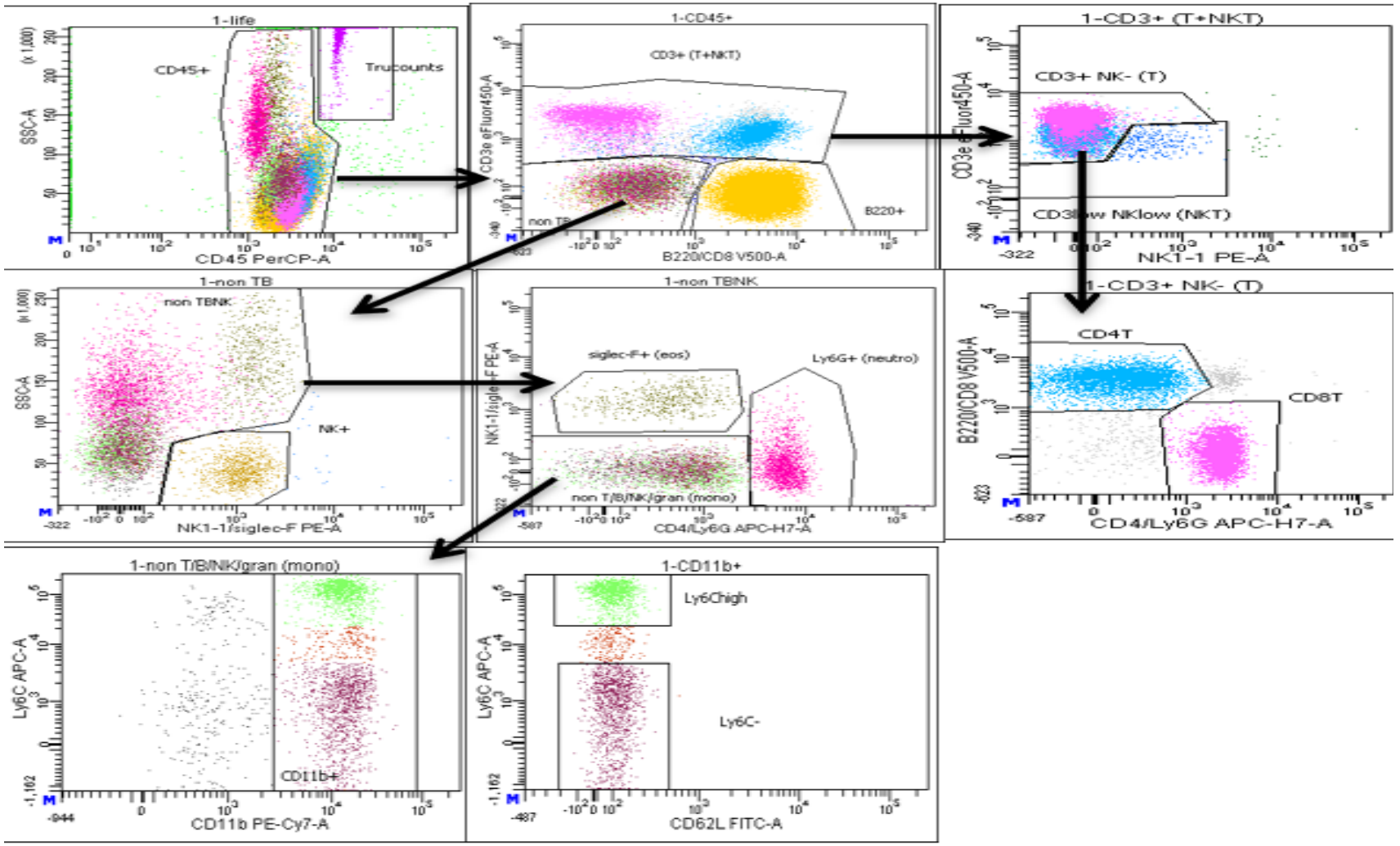
- **MFI**
  - **M**edian **F**luorescence **I**ntensity
  - Mean is sensitive for outliers and skewed data, median is not

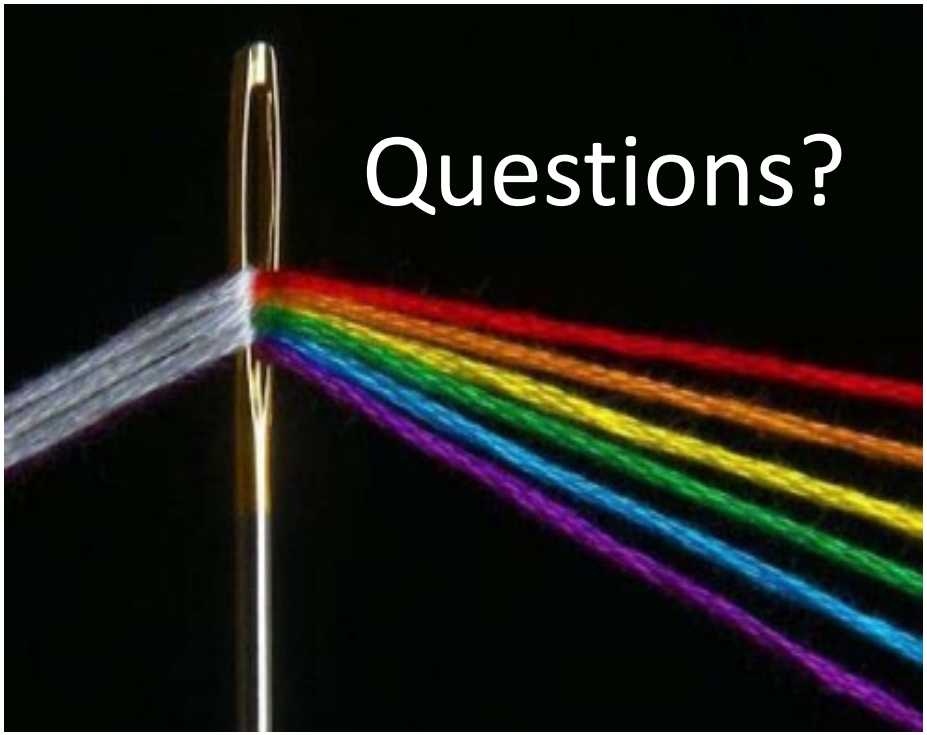



**Most flow cytometry data is displayed on a Logarithmic scale – What looks symmetrical is actually skewed!**

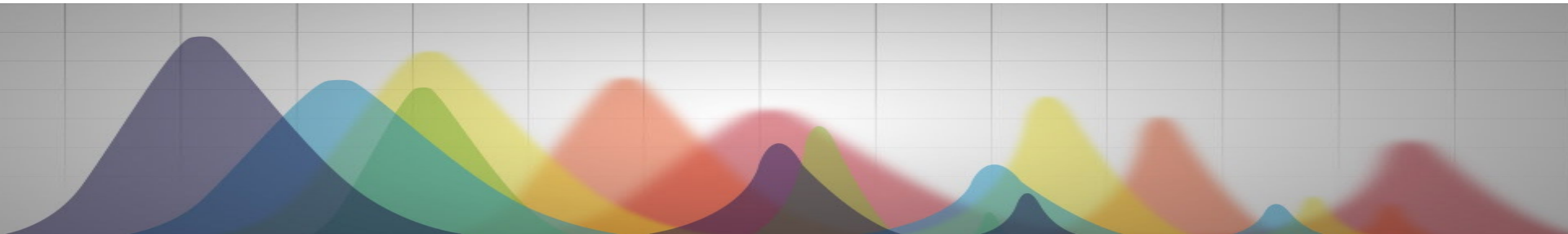
# Data display and gating

## At the end:




fccf.cdl@mumc.nl
erwin.wijnands@mumc.nl



# Experimental design

## How to perform a flowcytometrie experiment