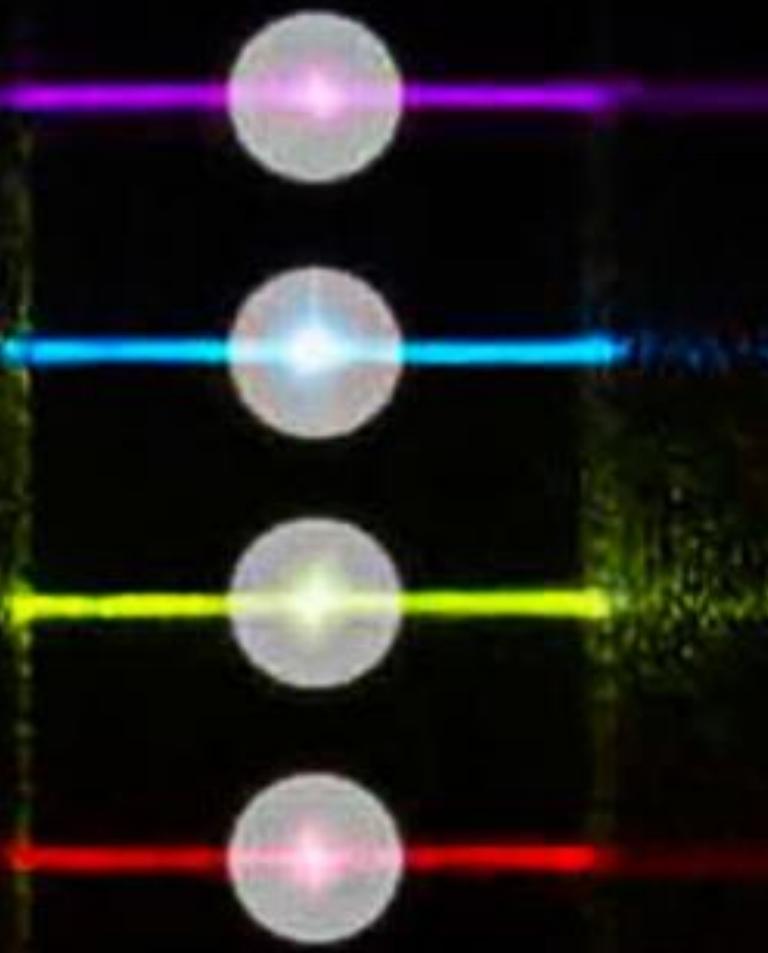
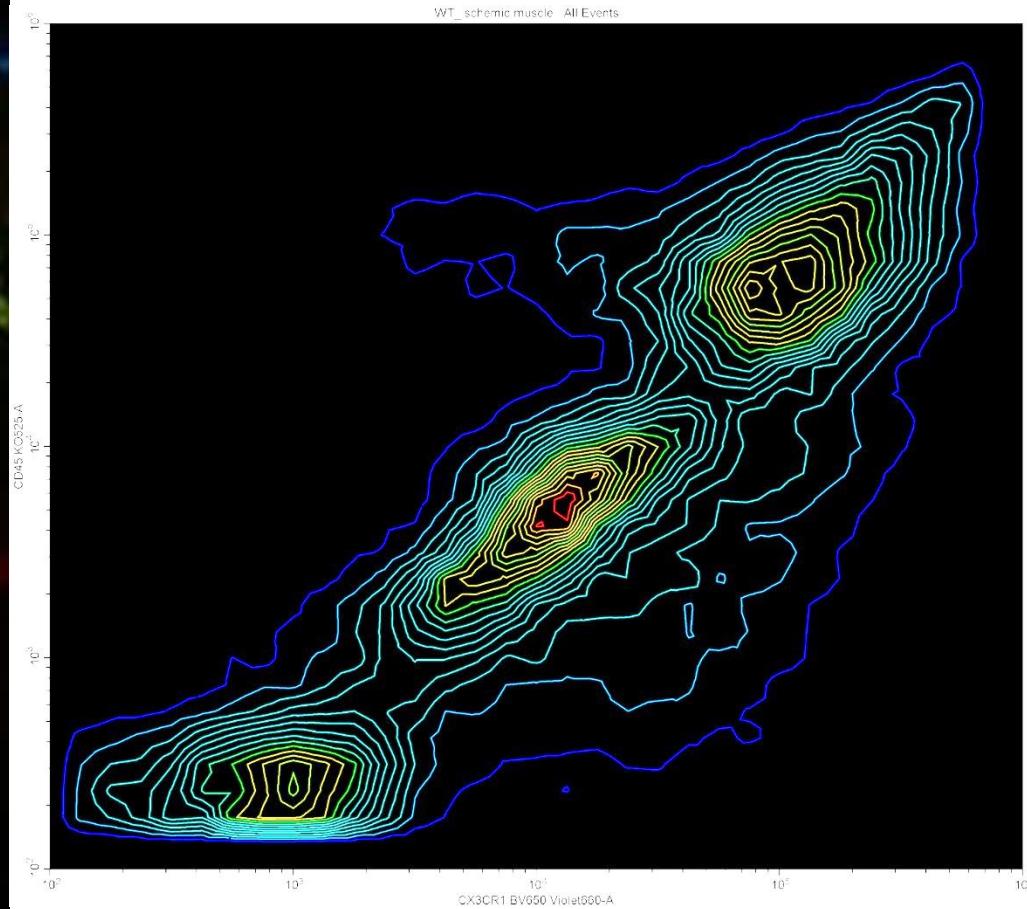




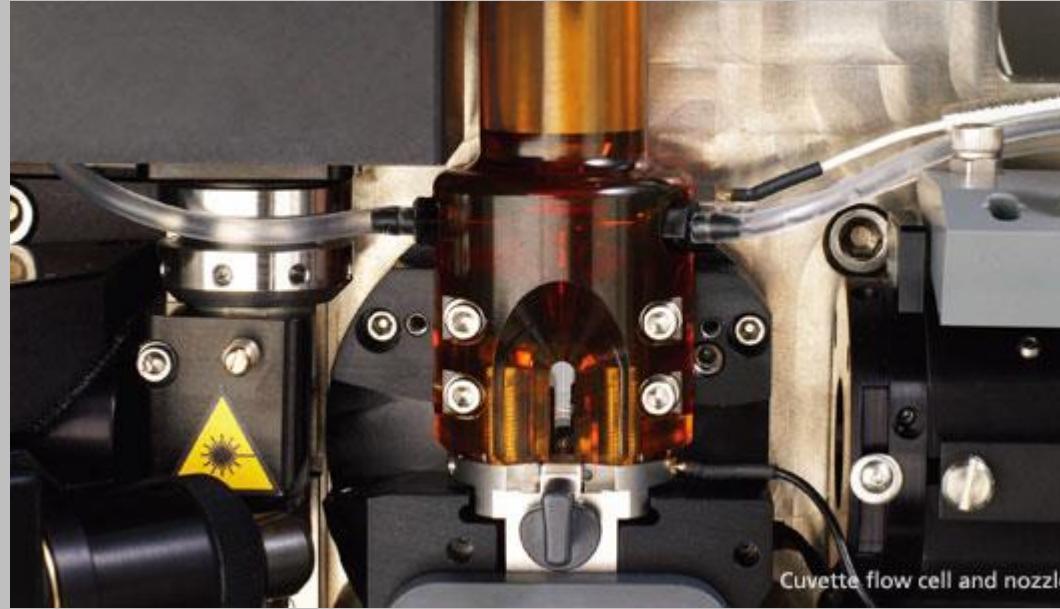
Light & Electron Microscopy : Flow Cytometry : Image Analysis

Flowcytometry

Dirk Pacholsky

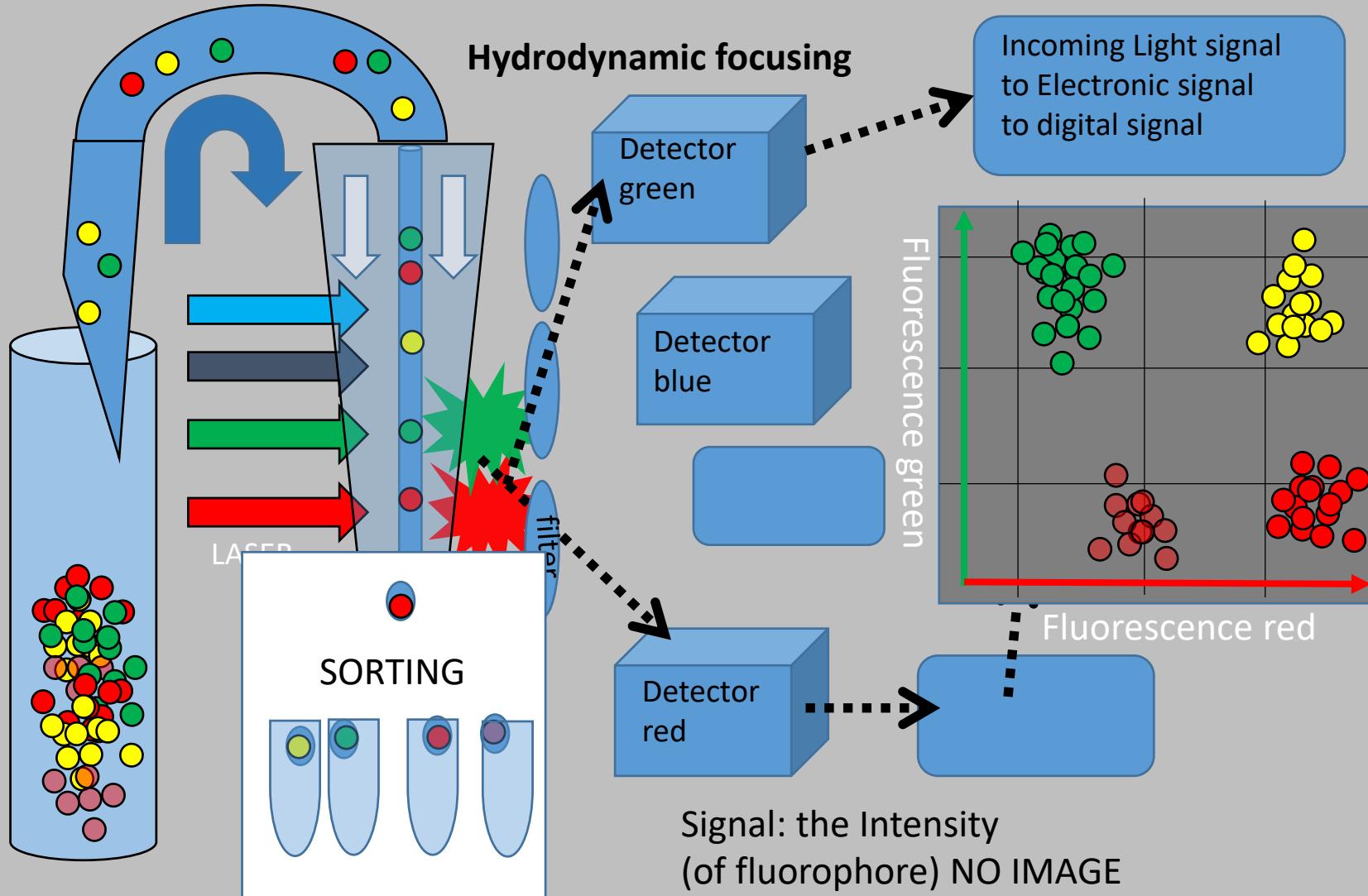


Flowcytometry: Overview

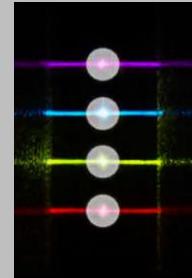


Overview

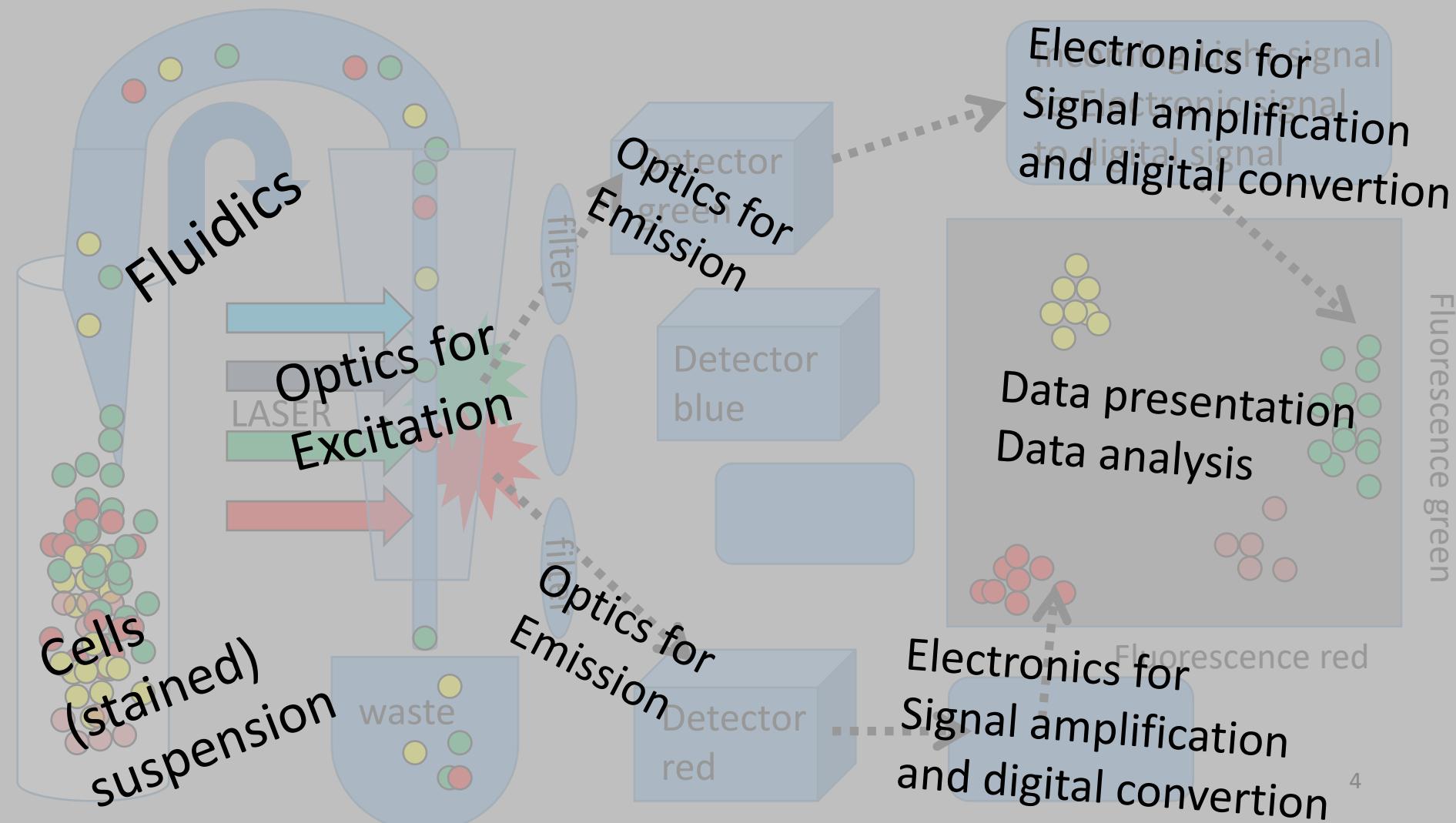
Flow -Cyto -Metry → Fluid-Cell-Measurement →
measuring cell properties of cells in suspension



Most Flow Cytometer have
Spatially separated Lasers
As seen here

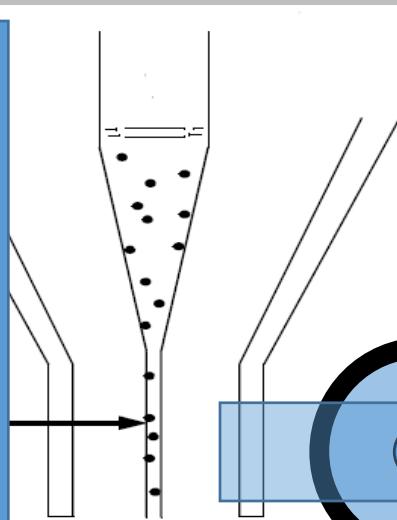


Overview



Fluidics and Signal

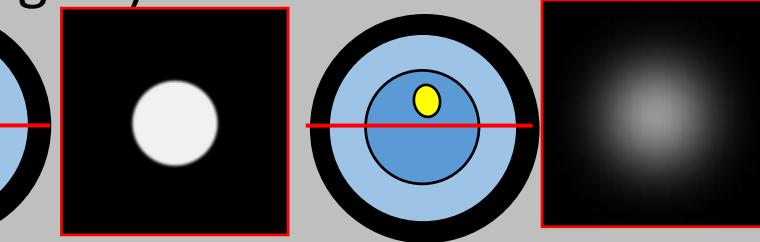
Whether Low or High : always same speed



Fluidics flow rate LOW :

Sample core with low diameter which allows
Only one cell to enter the interrogation point
Best resolution (signal)

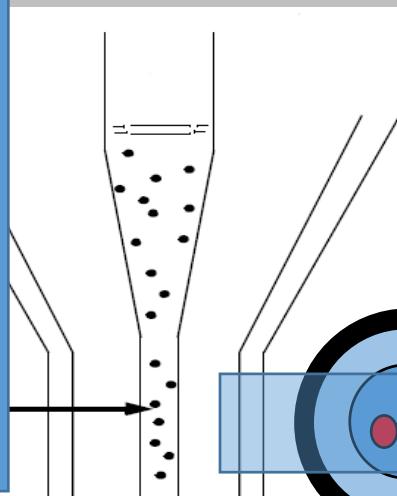
Focus



Fluidics flow rate HIGH :

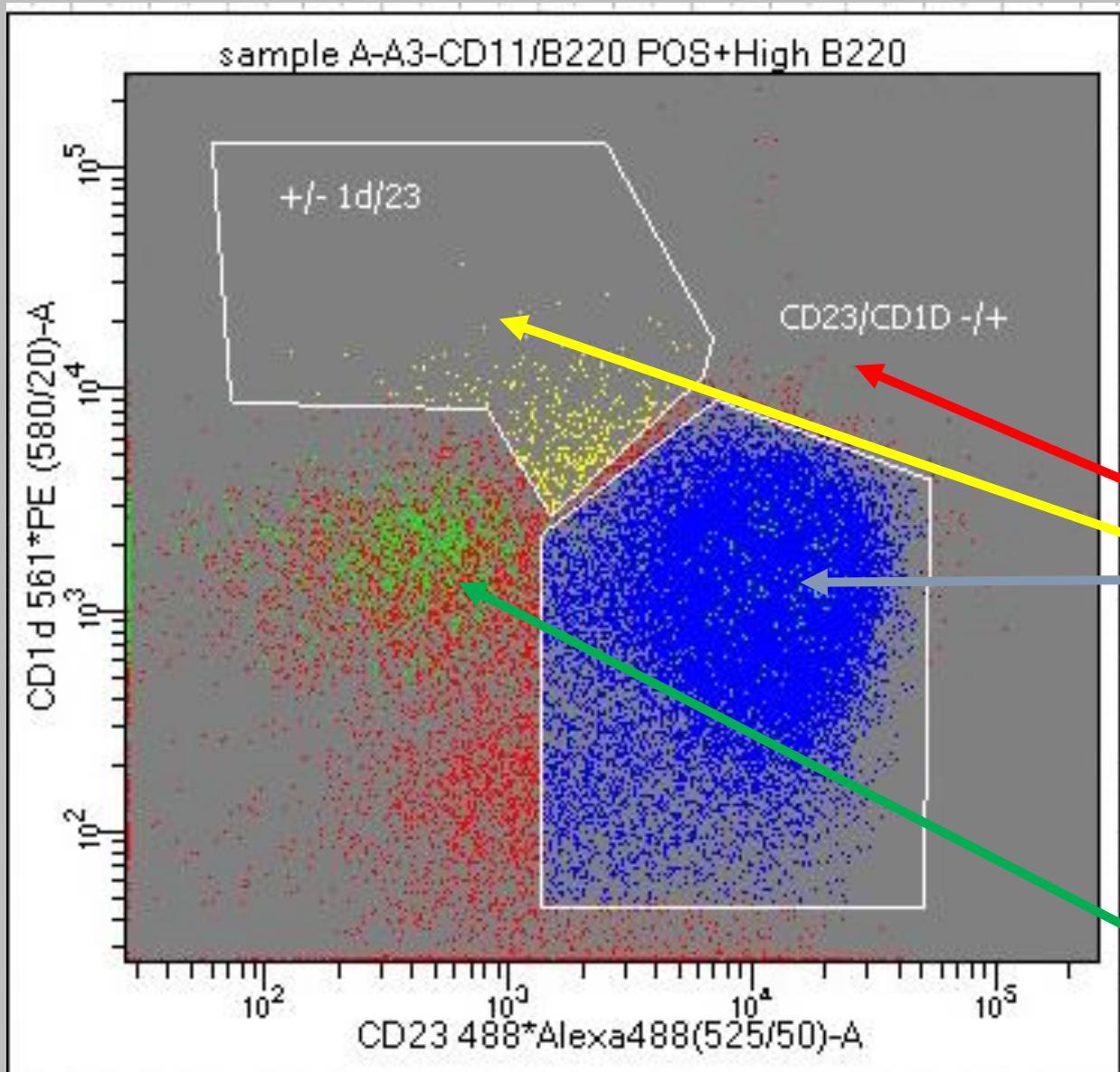
Wider sample core with more events per sec
BUT possibility of 2 or more events at
Interrogation point at same time point or
Cell off centred resulting in low signal

→ reduction in resolution



Whatever comes into the IP and results in signal is
treated as ONE event, even it comes from 2 cells.
! There is NO IMAGE in Flow Cytometry!

Overview



- each dot is one event (hopefully a cell)
- where certain parameters were measured (depending on activated detectors)
- placed in a bivariate plot (parameter 1 vs 2) in a log or linear scale
- gates can be drawn and populations identified, created and colorized
- number of events and their intensities (with certain parameters and other statistic values are available
- green events refer to a gate found in another plot

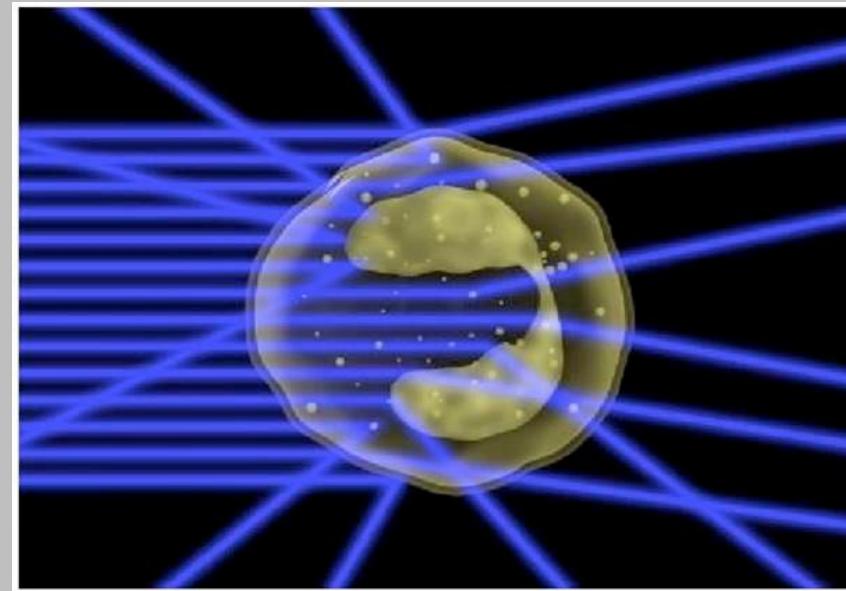
Applications of Flow Cytometry

Physical properties of cells like granularity and “size”
Fluorescent intensities

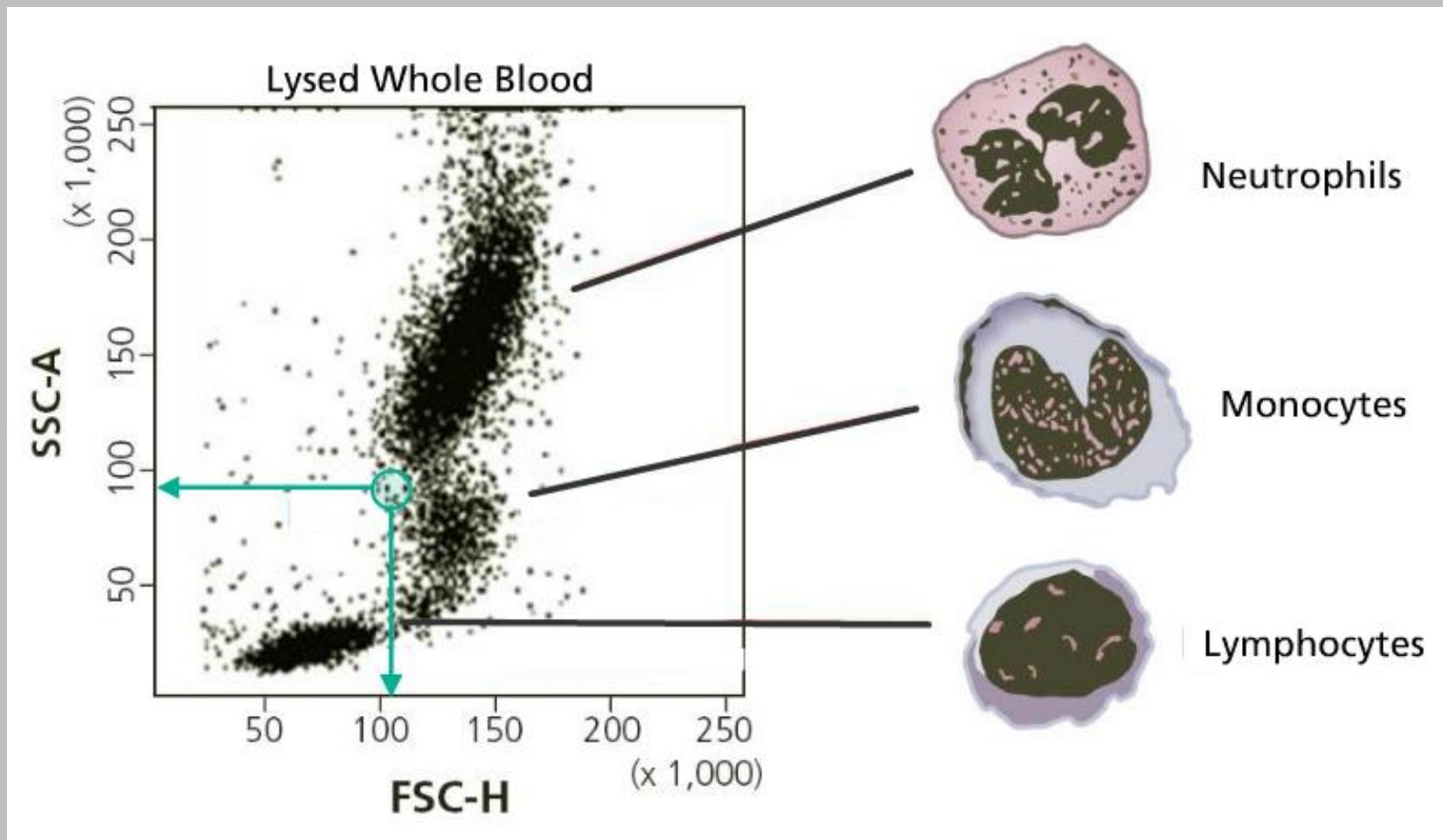
- Phenotyping (cell surface marker, intra cellular)
- xFP positives
- DNA / Cell Cycle
- Cell viability
- Cell proliferation
- Oxidative status
- ..

No image but a lot of data
Tens of thousands of events (cells) per second
Sensitive (500 molecules per cell)

Measuring physical properties of cells



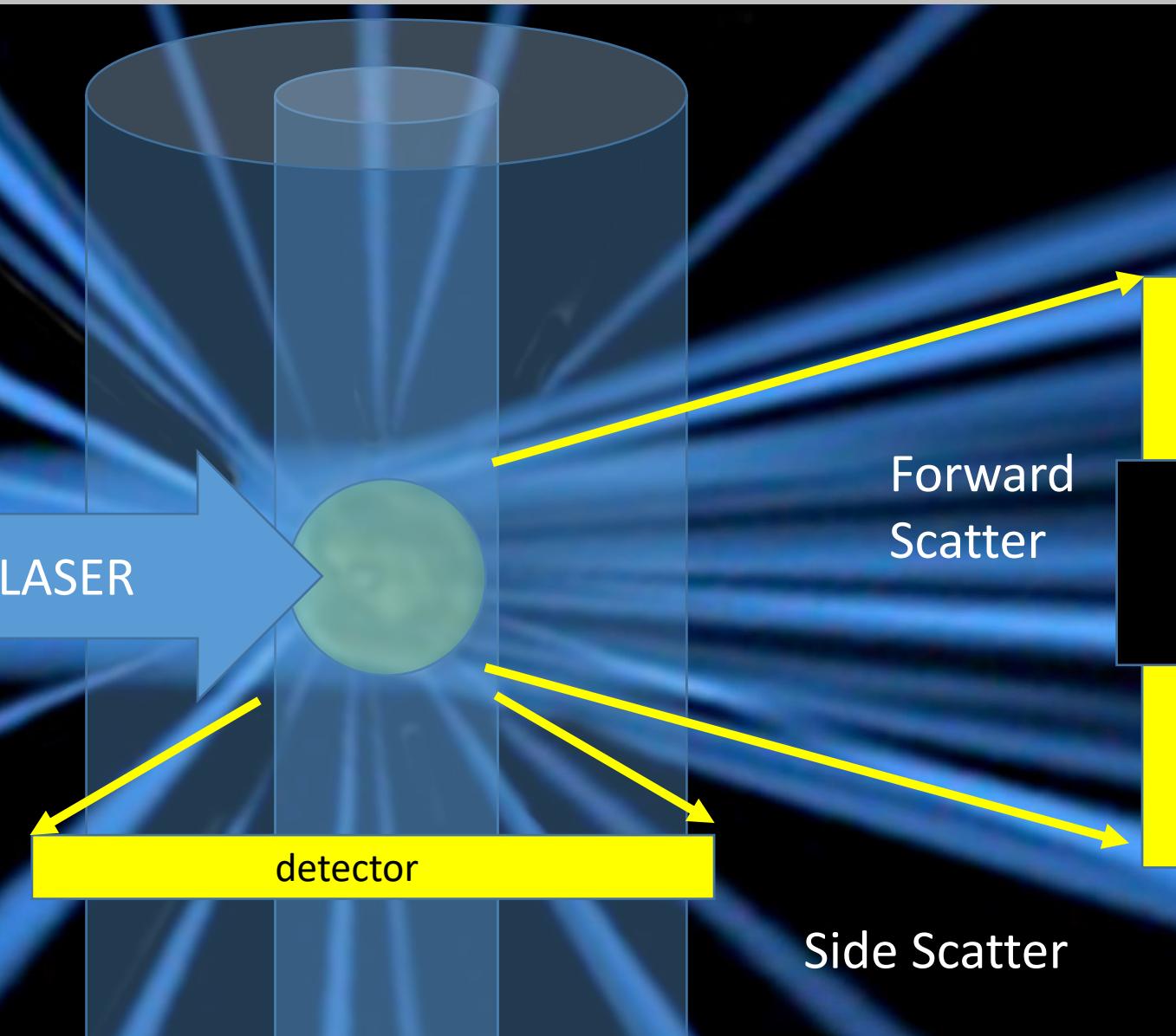
Overview: Measurements: Physical Properties



Forward Scatter FSC used to indicate size, SidewardScatter is used to indicate e.g. Granularity
Each dot in the plot indicate one cell (or better: one event)
H?, A? → see "signal to pulse to dot"

Forward & Side Scatter

Physical properties of cells define SSC & FSC values

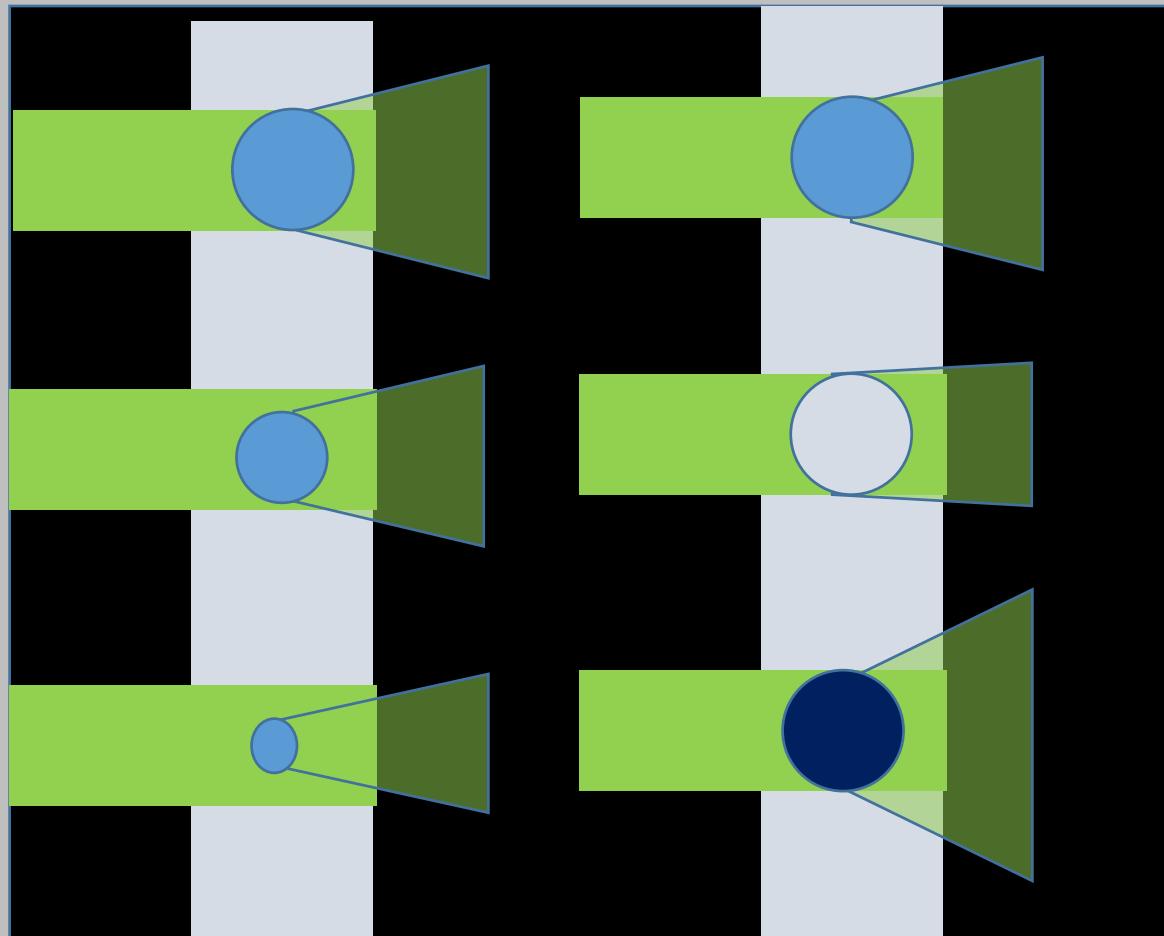


By design
488 nm Laser
is used for
Forward Scatter
Sideward Scatter
(FSC/SSC)

FSC in Laser's
direction
with blocking bar
to avoid
detection
of laser

SSC detected at
a 90° angle

Measurements FSC

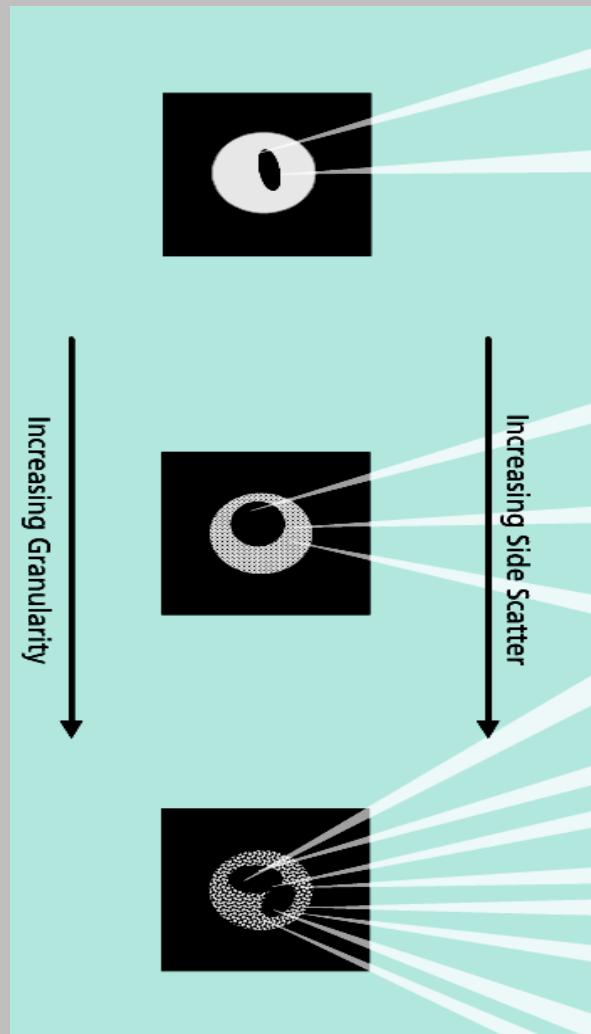


Size

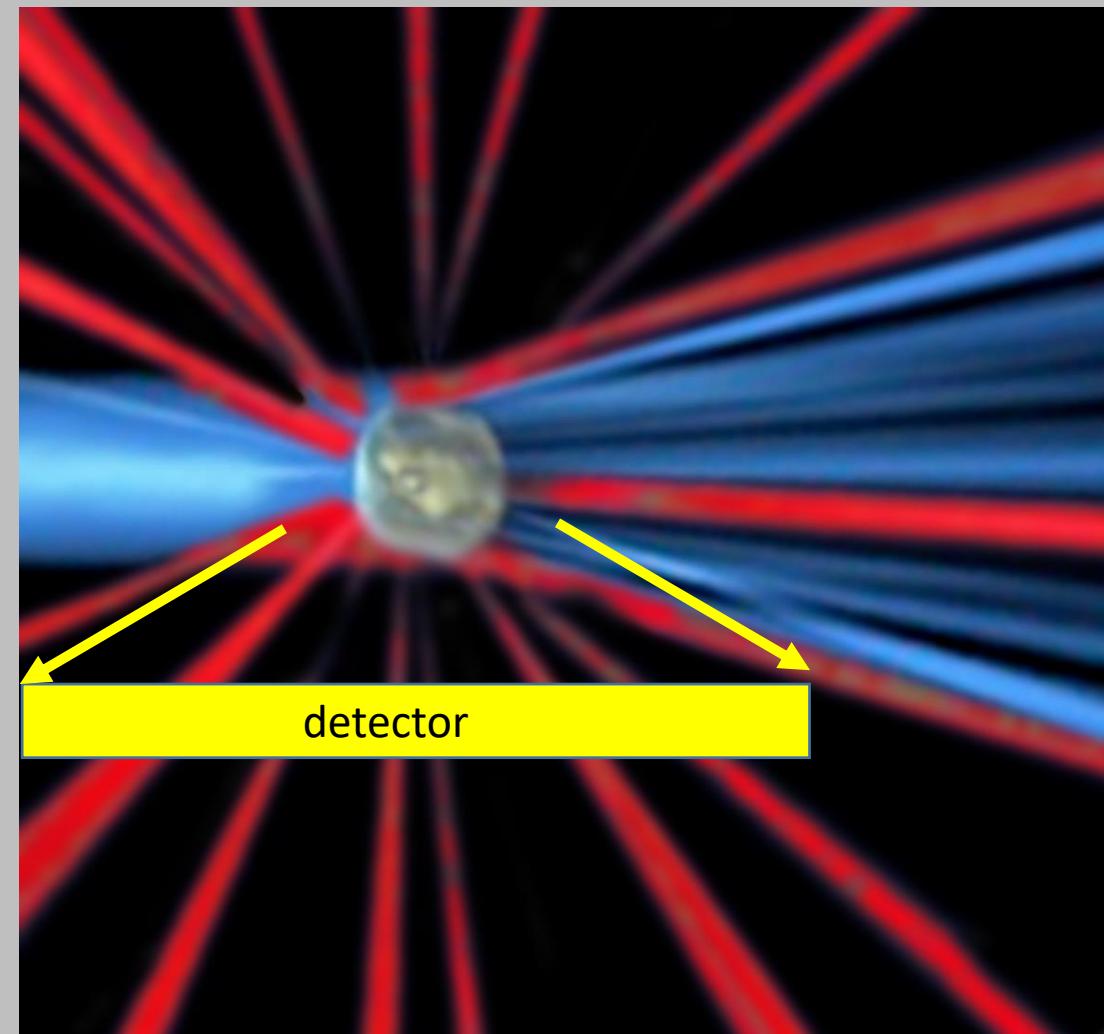
Refractive Index

FSC might be an indicator of size
BUT Not a real measure

Measurements FSC, SSC, FL

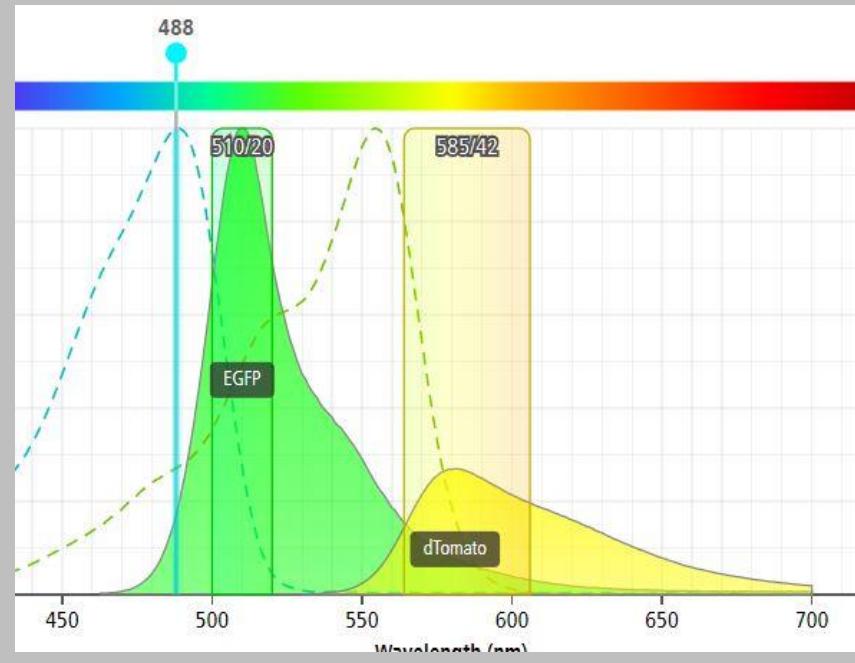


The granularity of a cell defines its SSC

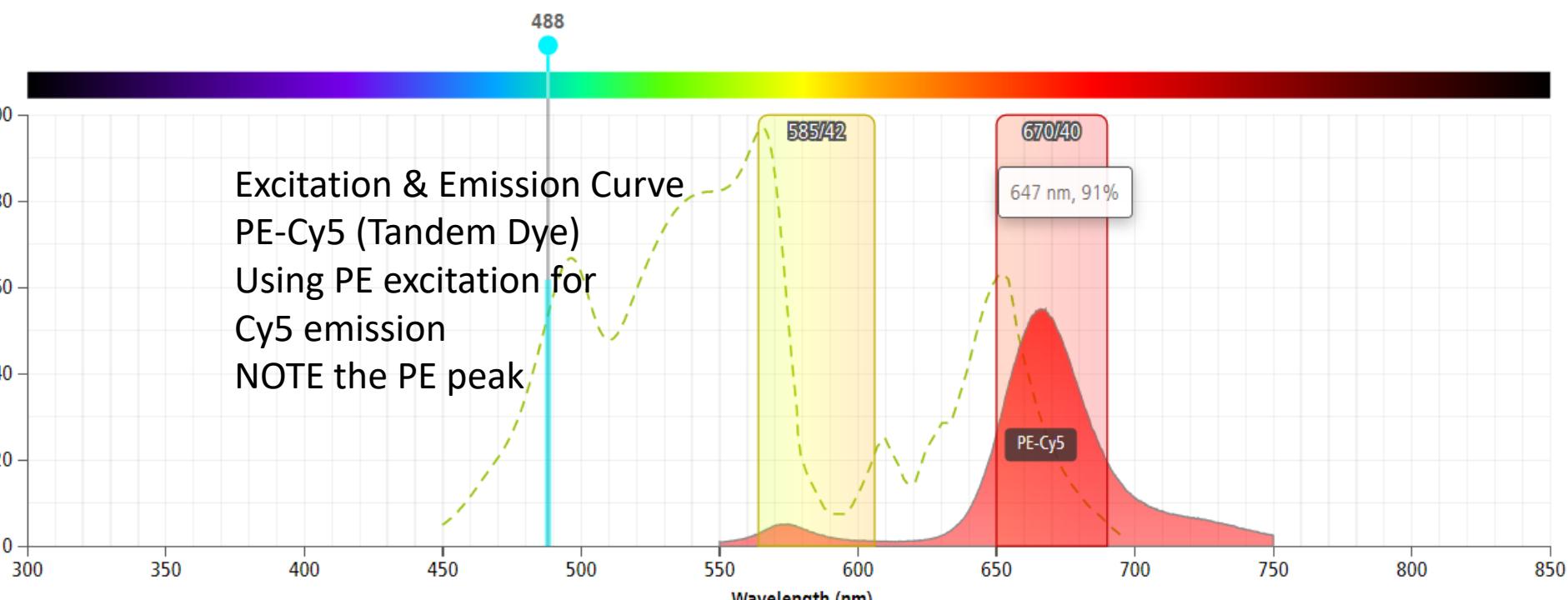
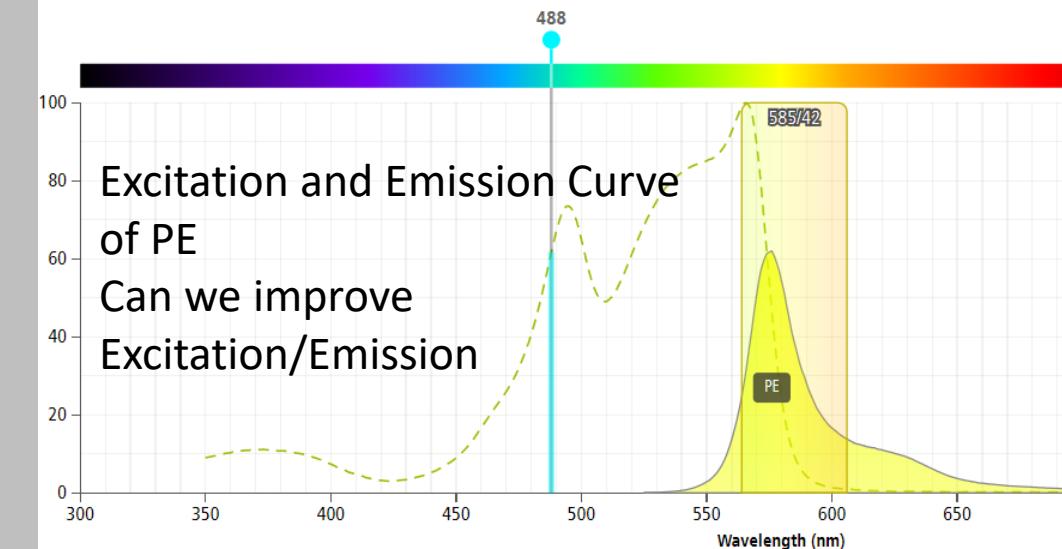
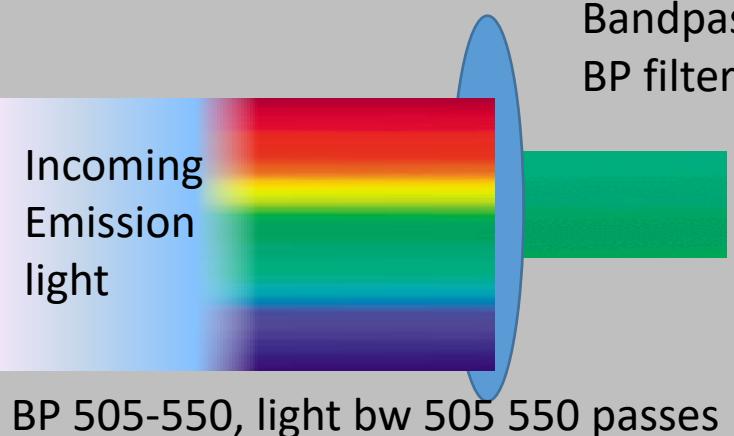


Fluorescence of a cell gets detected only at a 90 degree angle

Measuring fluorescent properties of cells

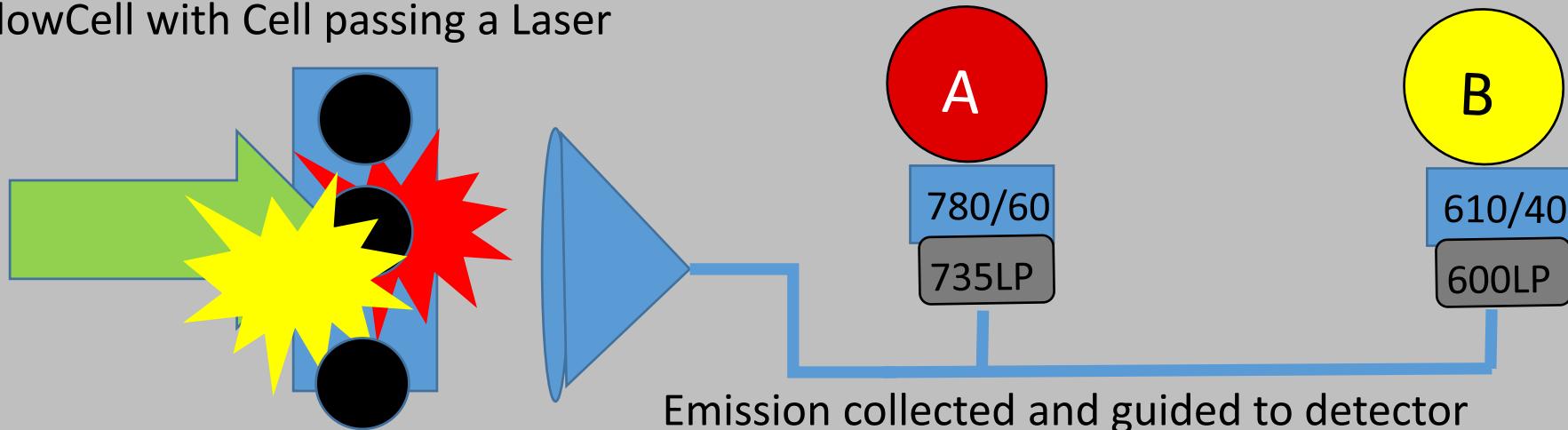


ReCap: Fluorescence



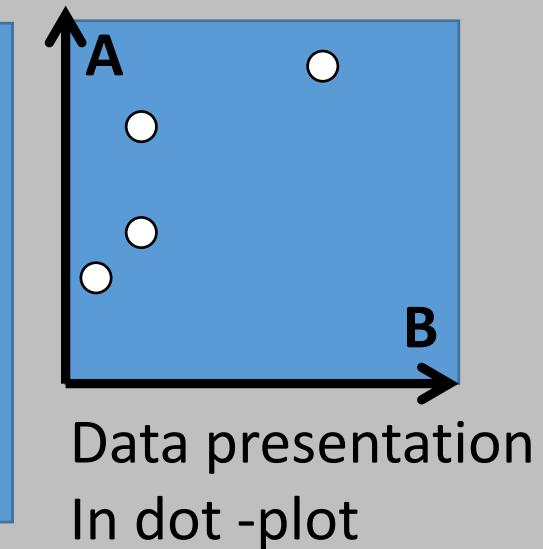
Overview: Optics:Electronics:Data

FlowCell with Cell passing a Laser



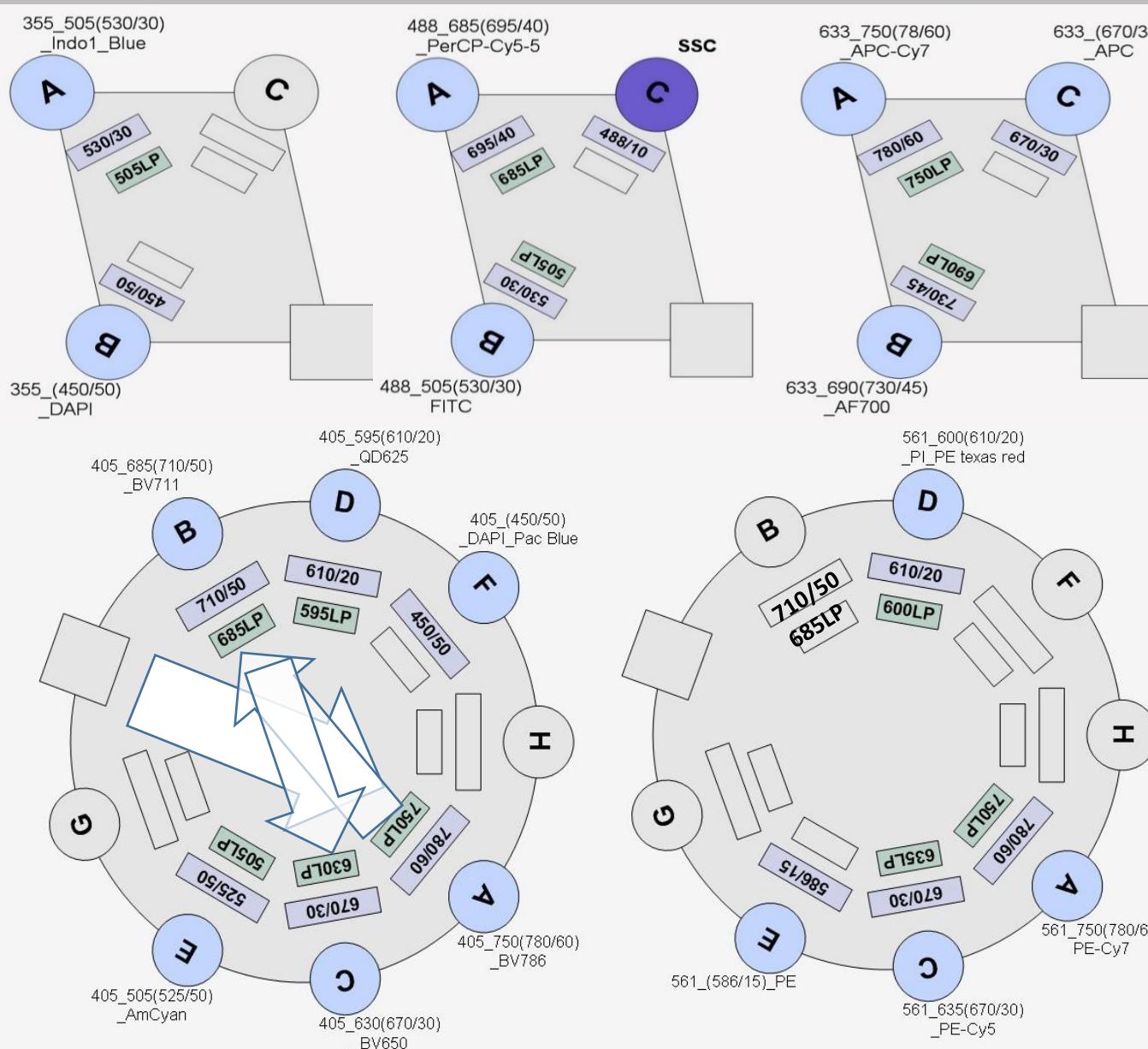
"color" A strength
Event 1: 3000
Event 2: 10000
Event 3: 10000
.....
Event 20.000: 20000

"color" B strength
Event 1: 100
Event 2: 1000
Event 3: 1000
.....
Event 20.000: 100000



Electronics convert light signals into voltages & thereafter into numerical/digital data of 18 bit depth (262.144)

Configuration of BioVis= Fortessa

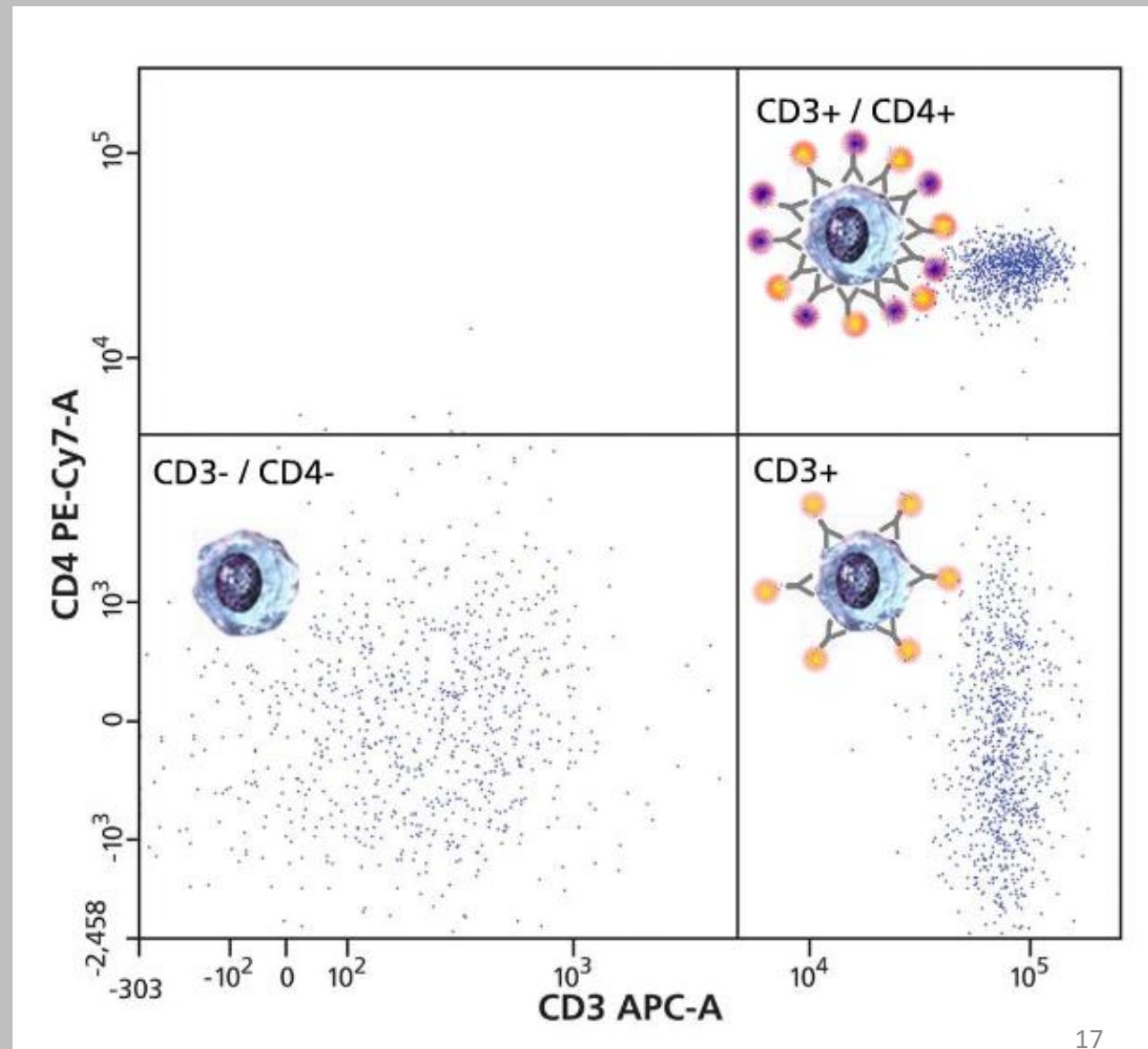


When planning the use of fluorophores :
 Check available lasers (excitation) and detector configurations (emission) on instruments you want to use

Overview: Measurements: Fluorescence

CD3, CD4
Specific proteins
(markers)

PE-Cy7, APC
Fluorophores on Abs
which detect
the marker



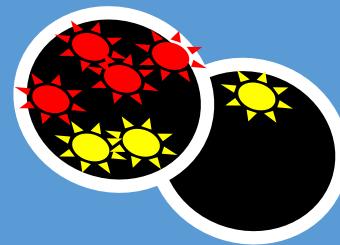
What FL information do we get?

Scenario 1
Smaller cells
clump

Doublet
Different
stains

Staining intra
Extra cellular

Scenario 20...
As scenario 3
But different



Laser

RED: 4
Yellow: 3

RED: 4
Yellow: 3

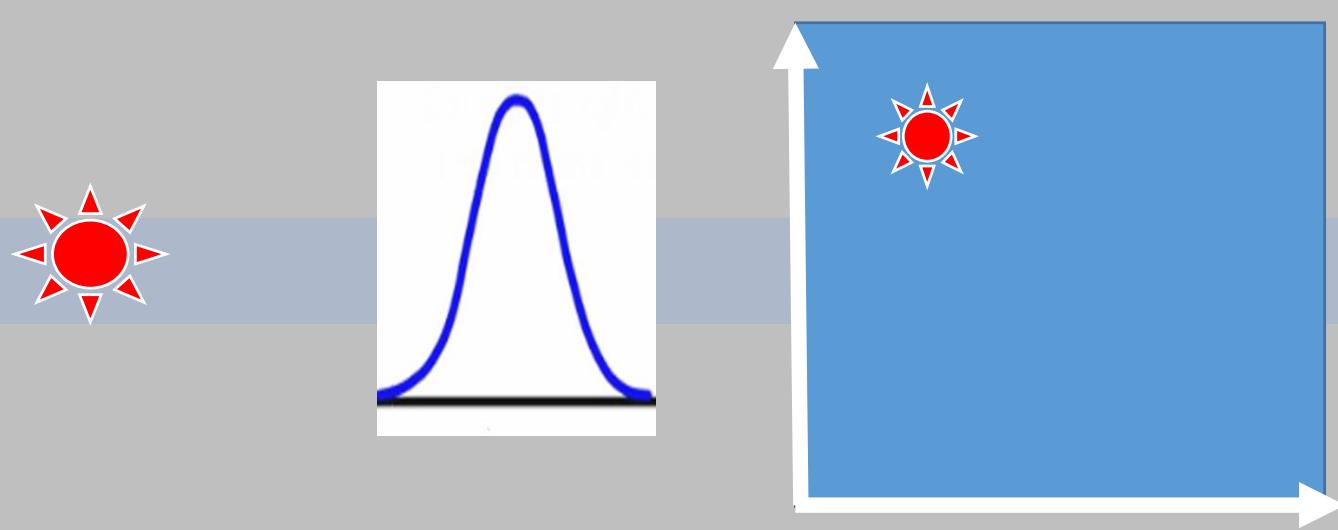
RED: 4
Yellow: 3

RED: 4
Yellow: 3

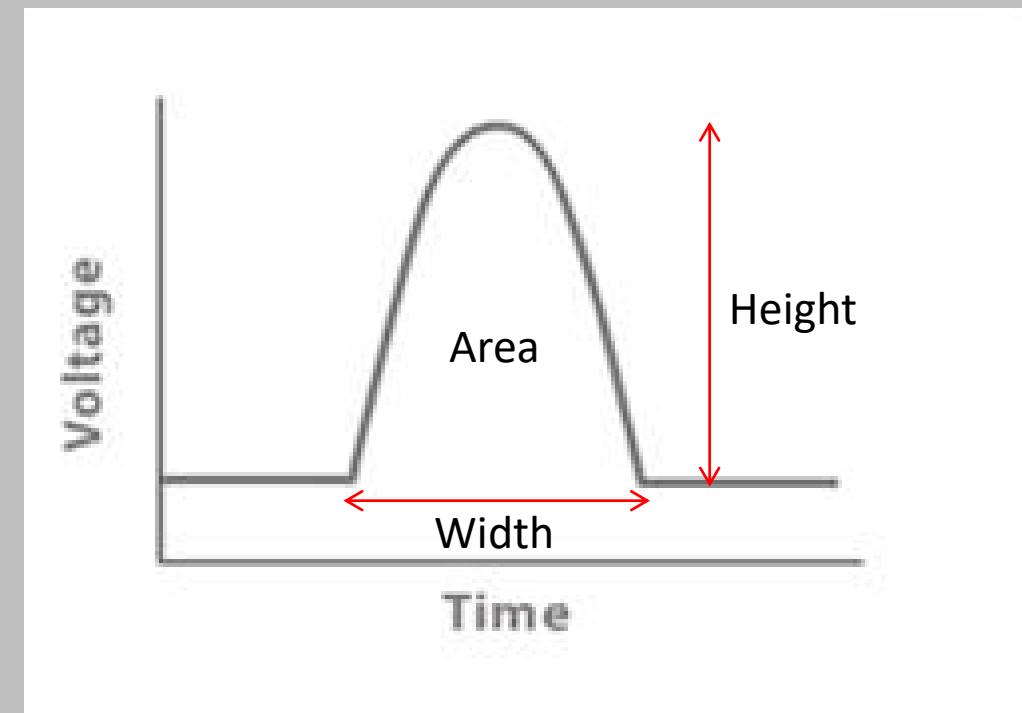
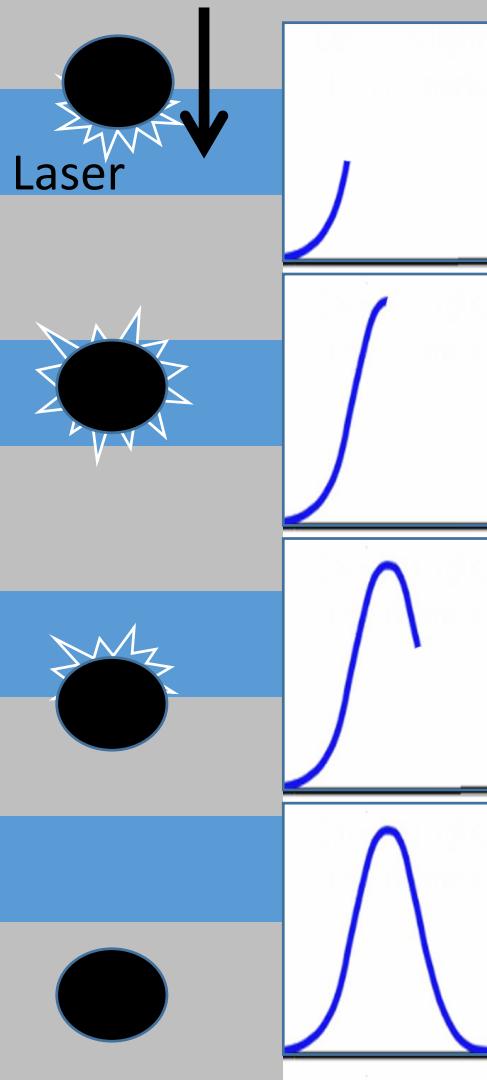
Laser

Get to know your sample (cells), (microscopy...surface/intern)
Choose the 'correct' protocol
Have controls to check for : status of cells (live/dead),
Doublets, bleeding through (compensation) etc pp

Signal to Pulse to dots on a plot

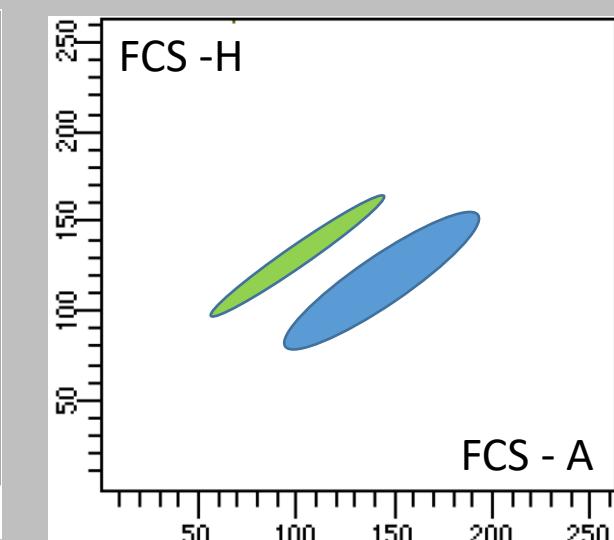
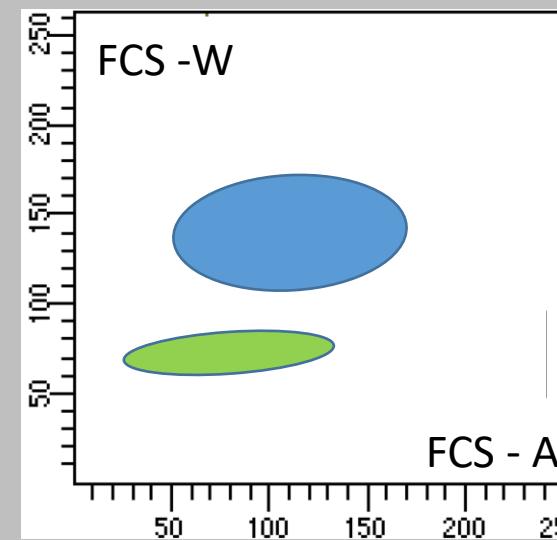
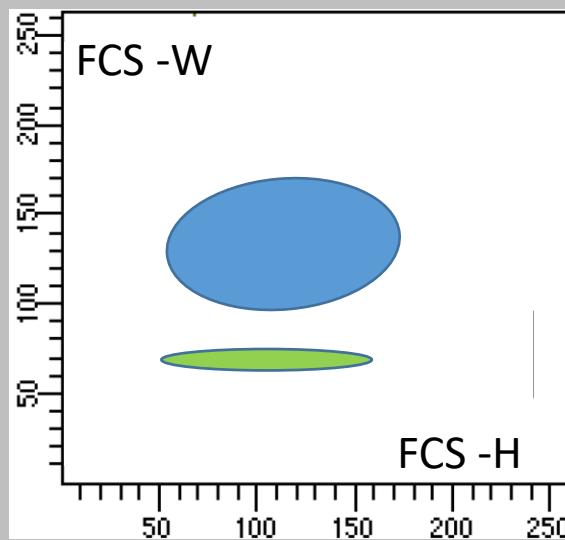
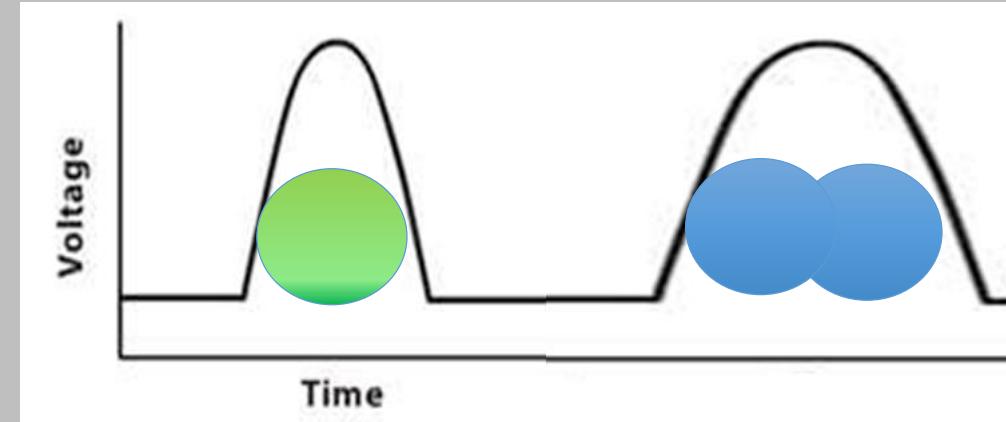
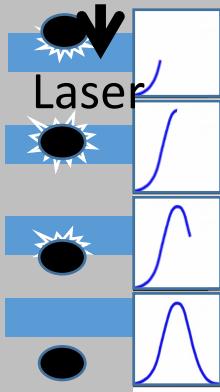


Signal to pulse to dot



FSC-H / SSC-H - Height
FSC-A / SSC-A - Area
FSC-W / SSC-W - Width

Singlet gating - why is it important?



Simplified view of appearance of singlet vs doublet(clumps) populations in plots
Comparing different parameter of the actual 'signal pulse'

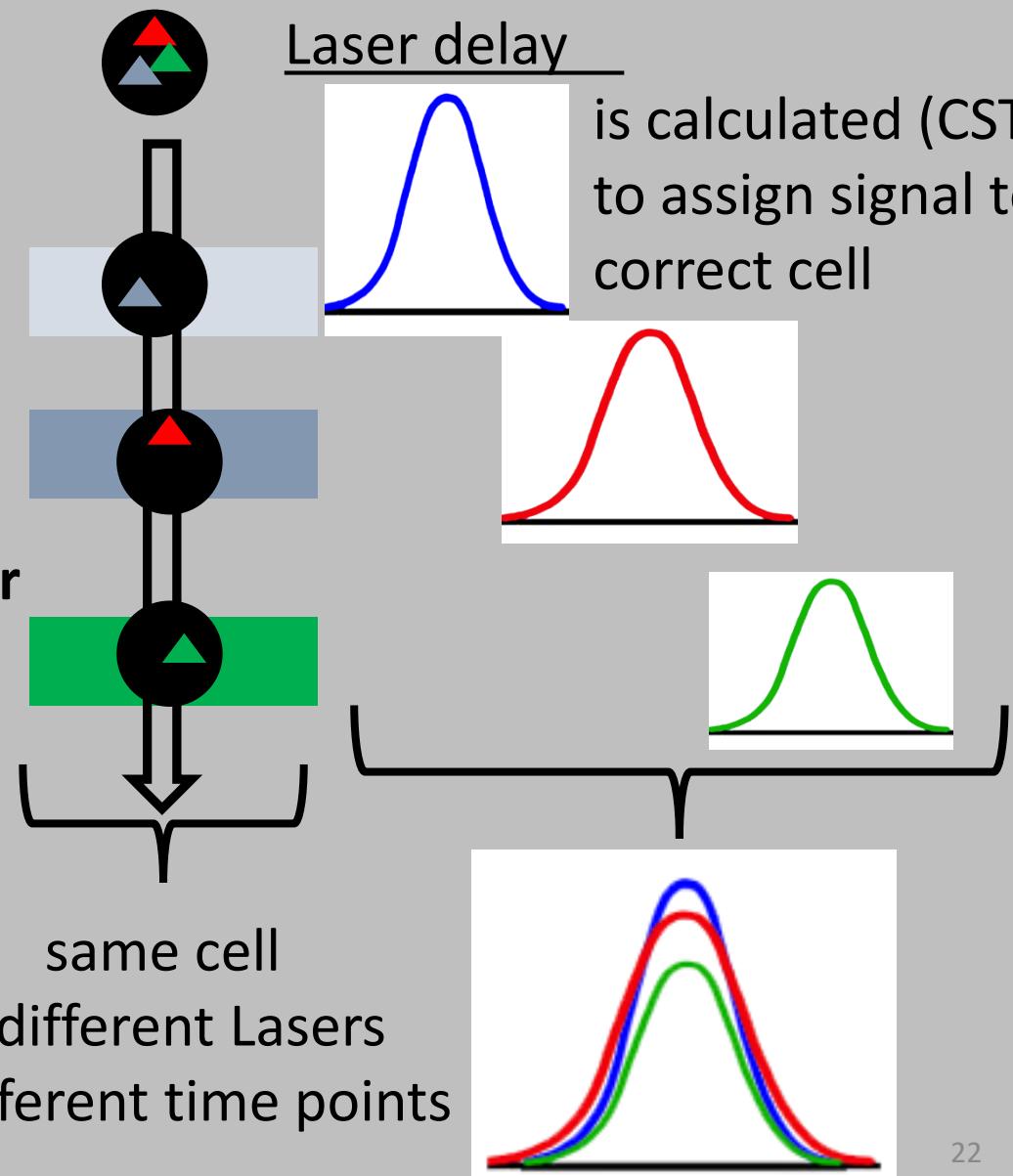
Signal to pulse to dot

Pulse formation...
Laser delay...

**! Sheath and sample core must run at same speed.
Instrument is calibrated for a certain laser delay...**

Do not have sample in higher viscosity than Sheath fluid (PBS) !

same cell
@ different Lasers
@different time points

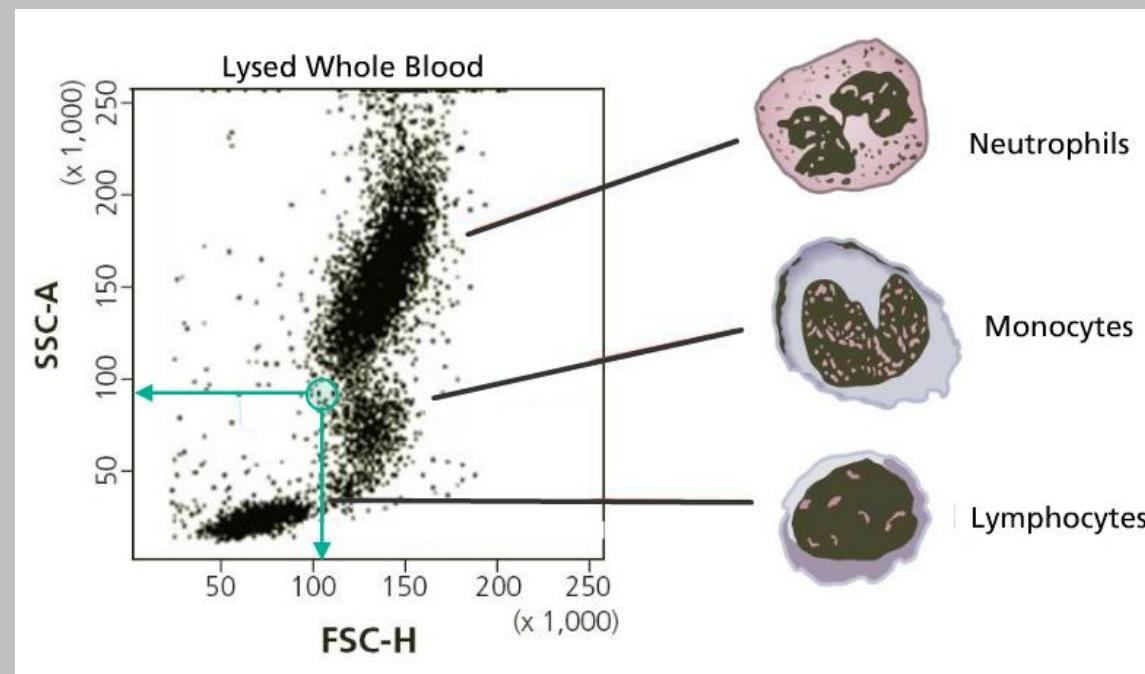


IMPORTANT

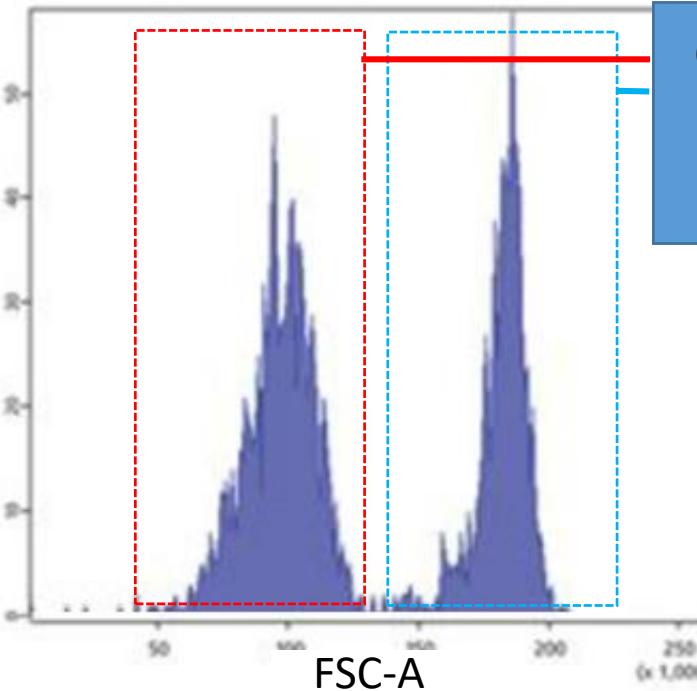
**The “performance check” procedure is needed for optimal work settings.
It sets PMT voltages to cover a wide dynamic range, to be able to record events of dim, middle and (very) bright intensities.
It takes care about Laser delay calculations**

The Cytometer set-up and Tracking procedure, or CST (performance check) should be done on daily bases

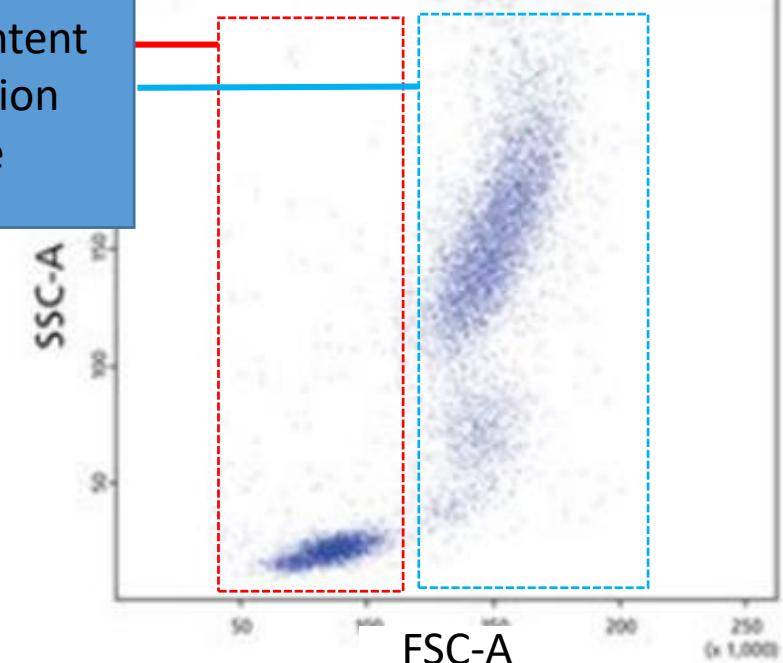
Plots : Gates : Statistics



Plot types



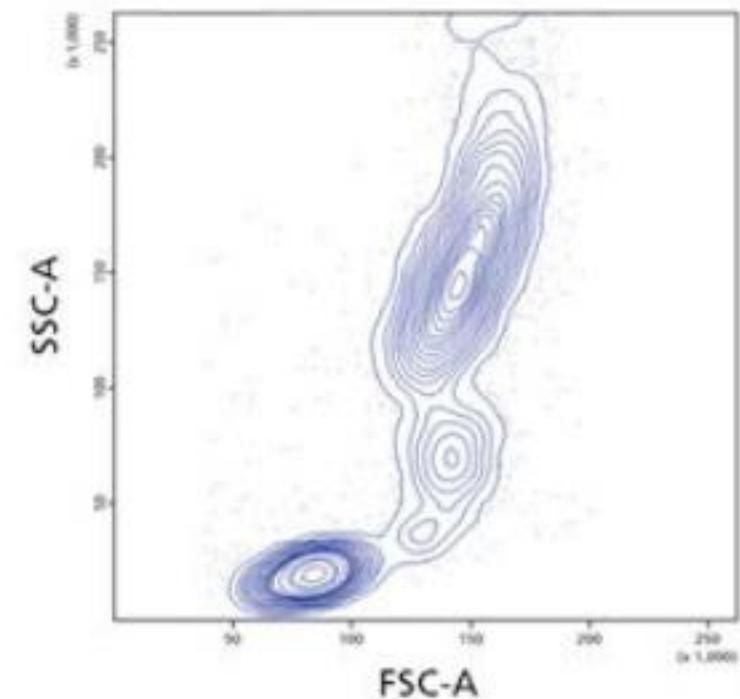
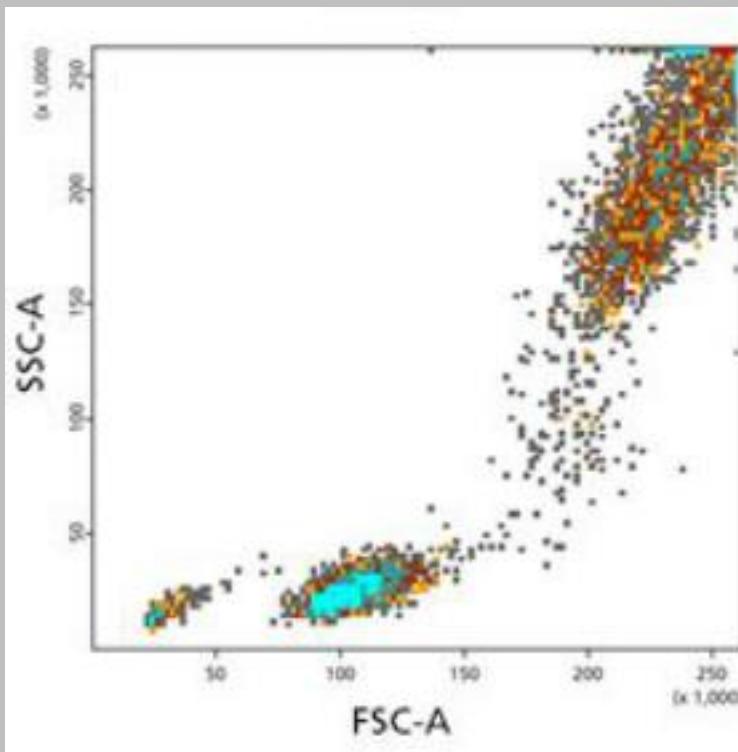
Compare content
of information
available



Histogram plot displays a single parameter against counts
The higher the parameter the more to the right

Bivariate plot displays 2 parameter (here FCS and SSC) against each other.
The higher the parameter value the higher on X, Y or/and XY

Data analysis Plot types

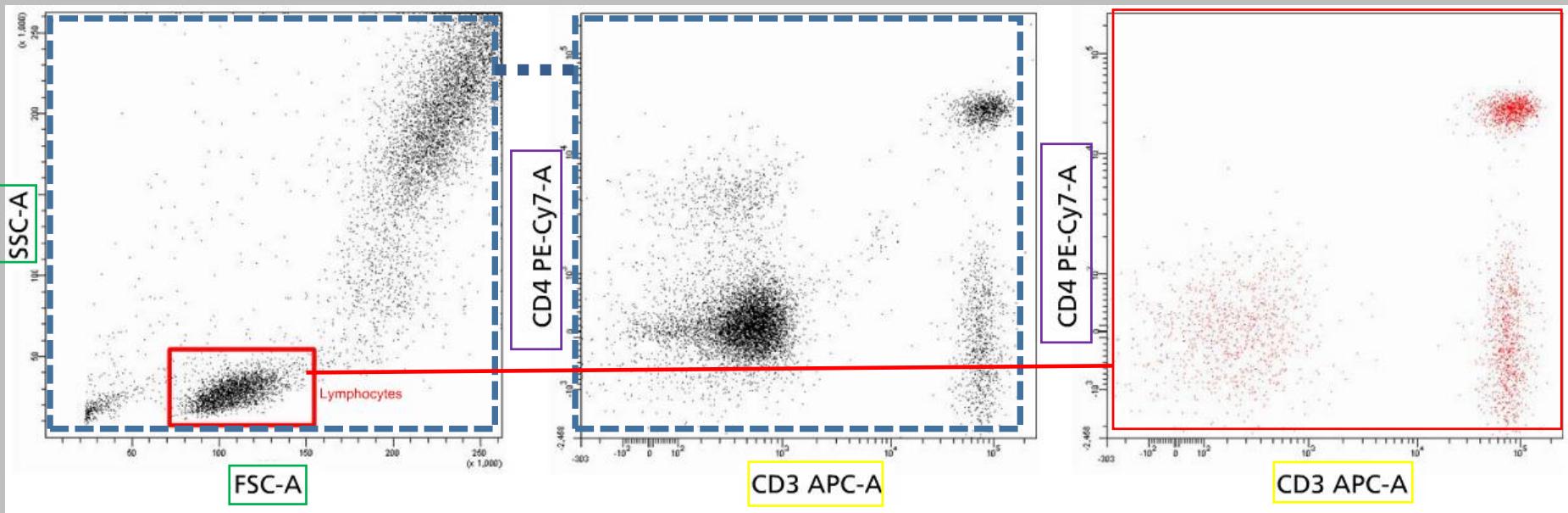


Density plot like dot plot.
Same event counts
Indicated by same color

Density plot like dot plot.
Same/similar event counts
joined by line
(like topographic maps)

Data analysis Gating

Each dot in the plots represents an event (cell) in respect to
The x and y axis of the plot



All events FSC/SSC

Gating of **Cell population** via FCS/SSC plot

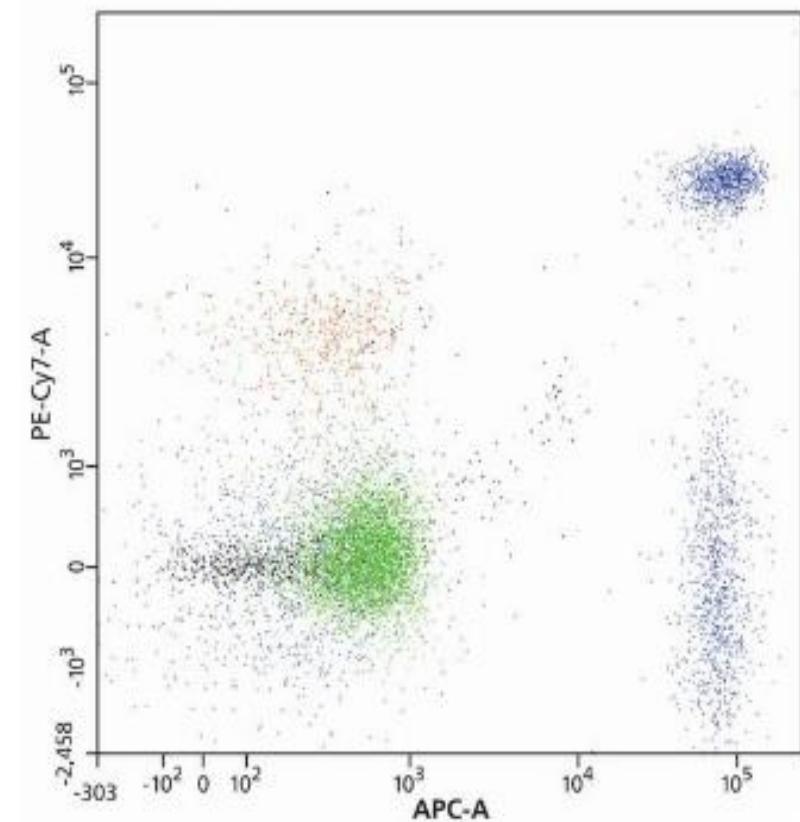
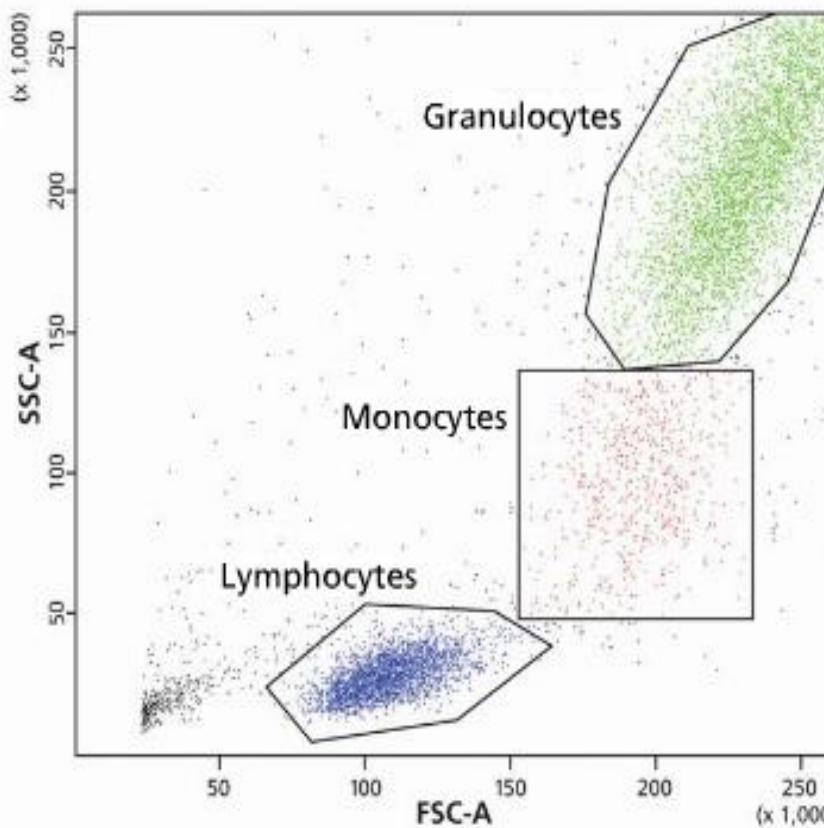
for further analysis and

Visualization on CD4 PE-Cy7 / CD3 APC plot

all events on FL plot

"Lymphocyte"
Population
separated
on FL plot

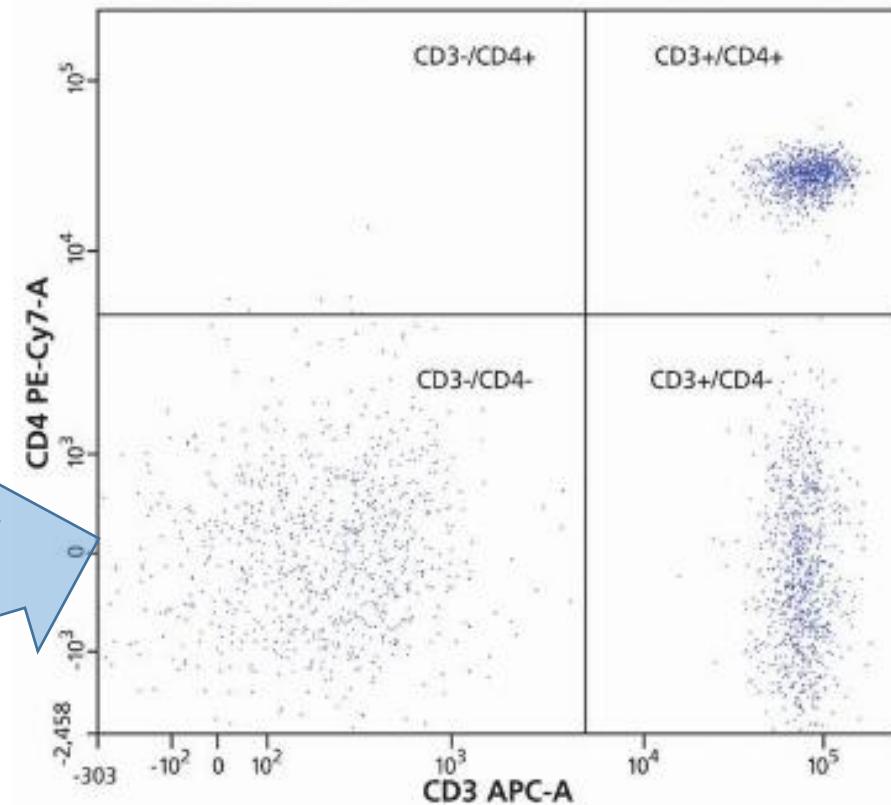
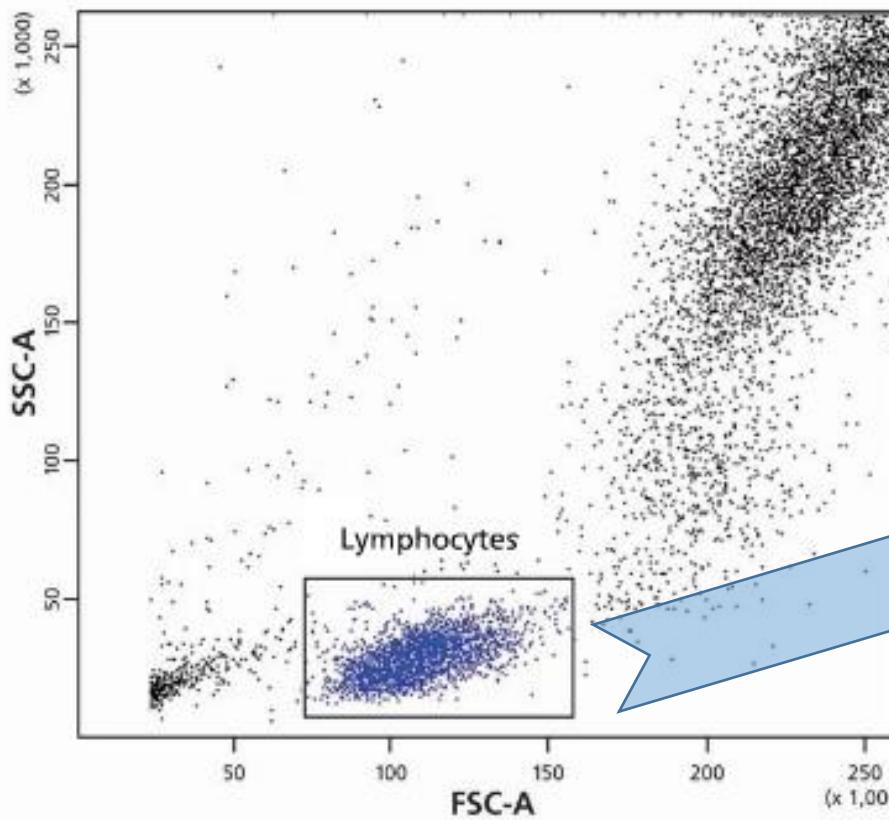
Data analysis Gating examples



Polygon SSC/FSC →
 Visualization on FL plot
 In different colors →
 Statistics

Population	#Events	% Parent	% Total
All Events	10,000	###	100.0
Lymphocytes	2,777	27.8	27.8
Monocytes	577	5.8	5.8
Granulocytes	5,792	57.9 ²⁸	57.9

Data analysis Gating example

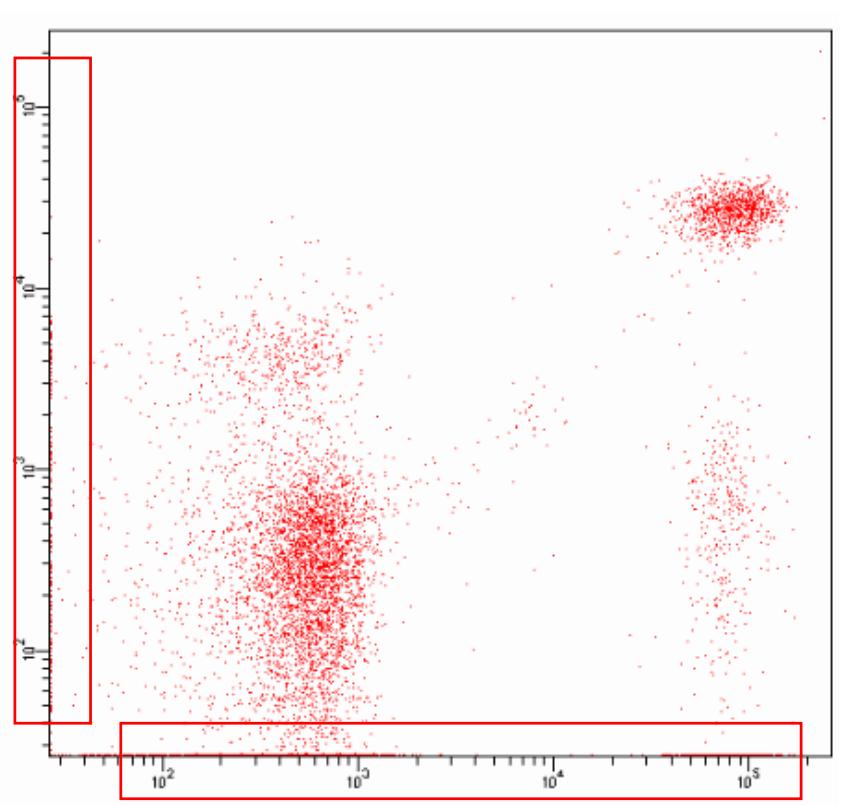


Polygon SSC/FSC →
 Quadrant FL →
 Statistics

Population	#Events	% Parent	% Total
All Events	10,000	####	100.0
Lymphocytes	2,714	27.1	27.1
CD3-/CD4+	252	9.3	2.5
CD3+/CD4+	1,067	39.3	10.7
CD3-/CD4-	851	31.4	8.5
CD3+/CD4-	544	20.0 ₂₉	5.4

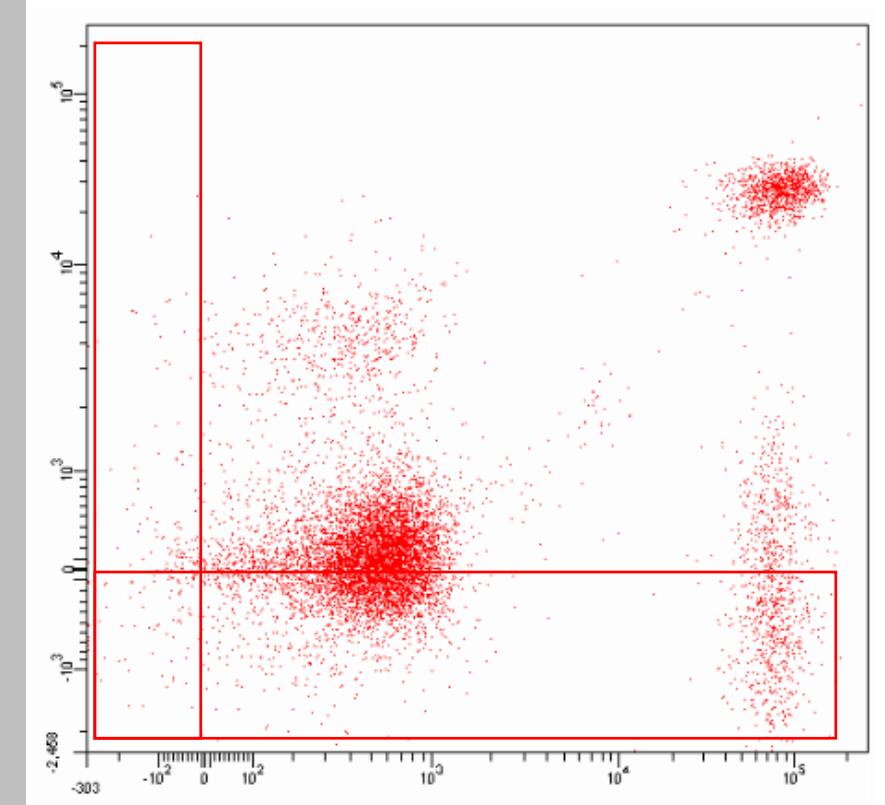
Data analysis plot scaling

Log scale



Traditionally used, but some data might end up on the scale and below the axis

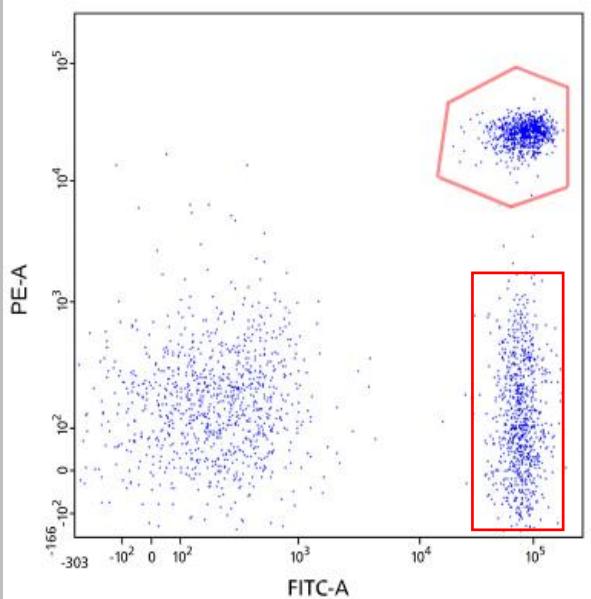
biexponential scale



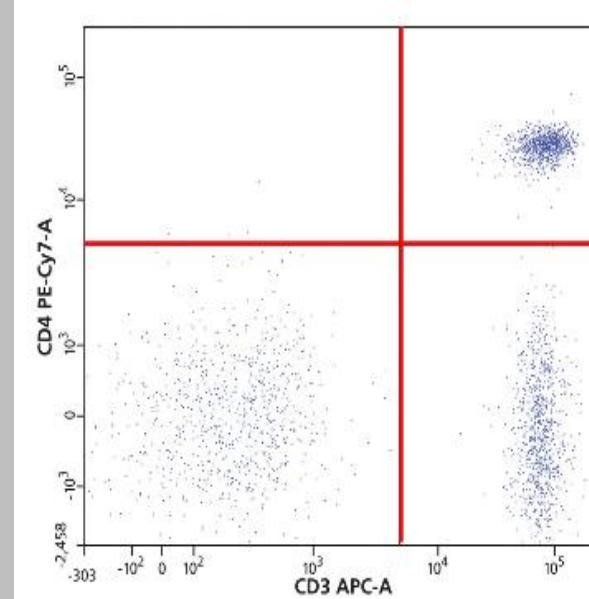
Enhances resolution,
useful for poorly resolved and/or complex population/data

Data analysis Gate types

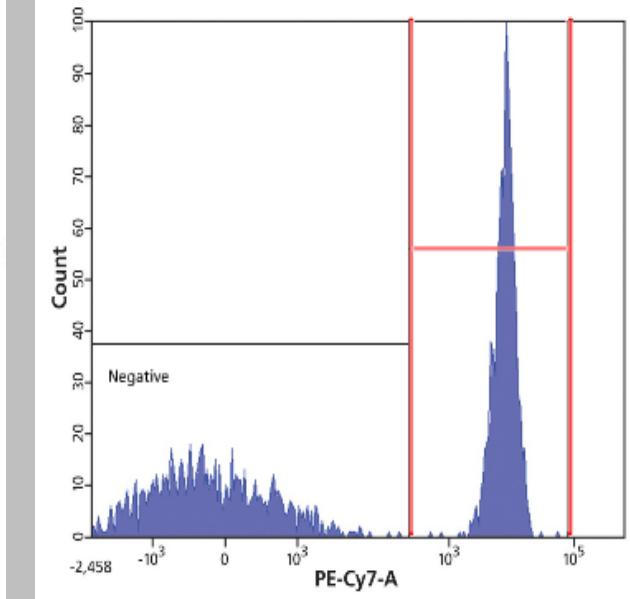
Polygonal Gate



Quadrant gate



Interval gate



boundary around a population in Dot, Density or Contour plot

Division into four separate populations in Dot, Density or Contour plot

Boundary around range of events in Histogram plot

Also available are Auto Gates And Snap-to-Gates, which are automatically drawn around cluster/range of events

Data analysis - parameter

Population

- # events (number of events recorded)
- % (grand)parent (percentage of events in relation to parental gate)
- % total (percentage in relation to total events)

Population	#Events	% Parent	% Total
All Events	10,000	####	100.0
Lymphocytes	2714	27.1	27.1
CD3-/CD4+	252	9.3	2.5
CD3+/CD4+	1067	39.3	10.7
CD3-/CD4-	851	31.4	8.5
CD3+/CD4-	544	20.0	5.4

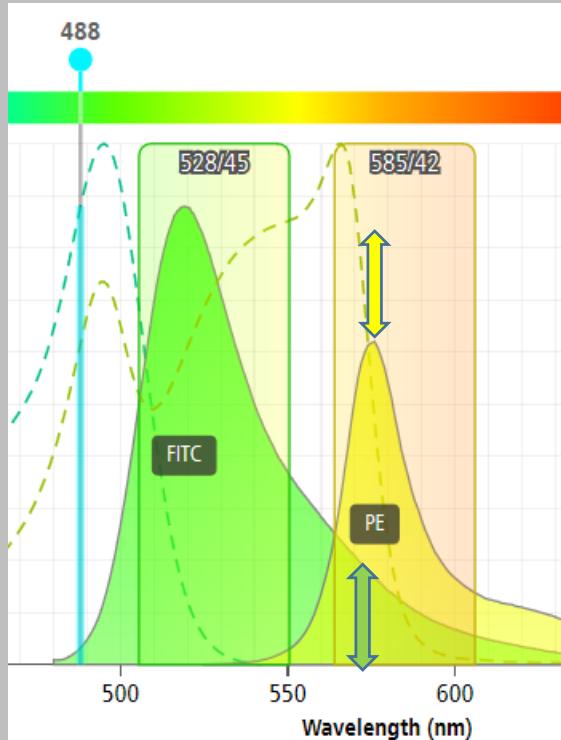
Statistics

- min / max (min/ max Intensity)
- mean/ median (average or median* Intensity)
- SD/ CV (Standard deviation / Coefficient of Variation)
- rSD/ r CV (robust , *ignores outlying events)

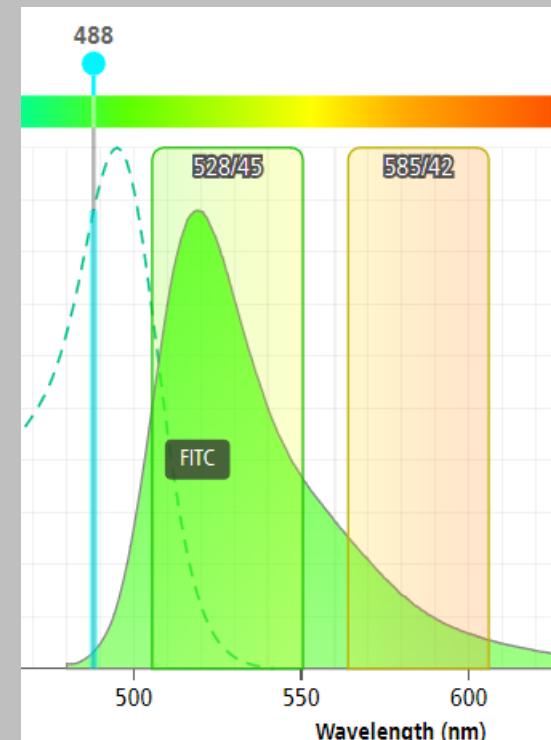
Bleeding Through : Compensation

Bleeding Through : Compensation

Staining: FITC & PE, 488 excitation, two detectors: 528/45 & 585/42



488 excitation/emission
in double stained sample
PE adds to FITC
FITC adds to PE
Highten the actual signal



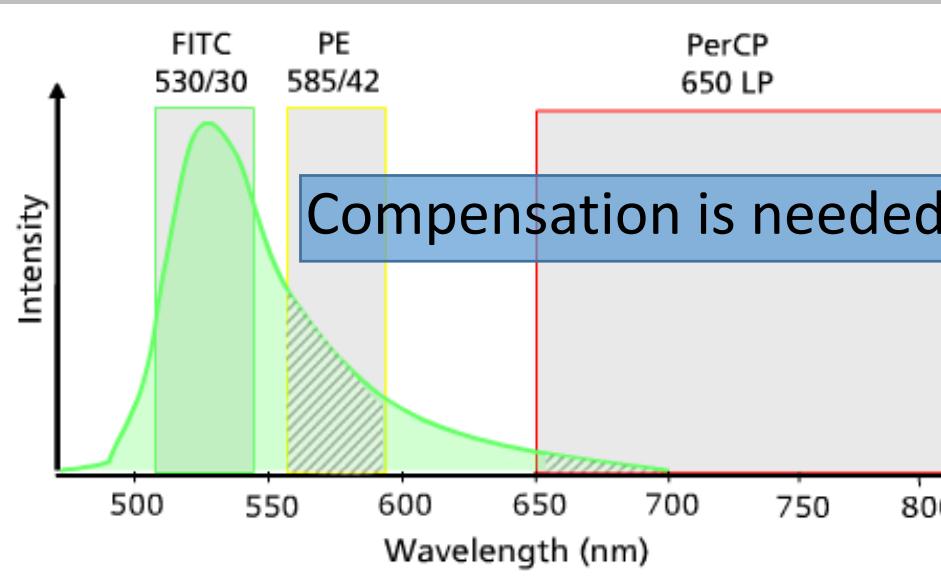
488 excitation/emission
in FITC stained sample:
Record: FITC
Record: PE



488 excitation/emission
in FITC stained sample:
Record: FITC
Record: PE

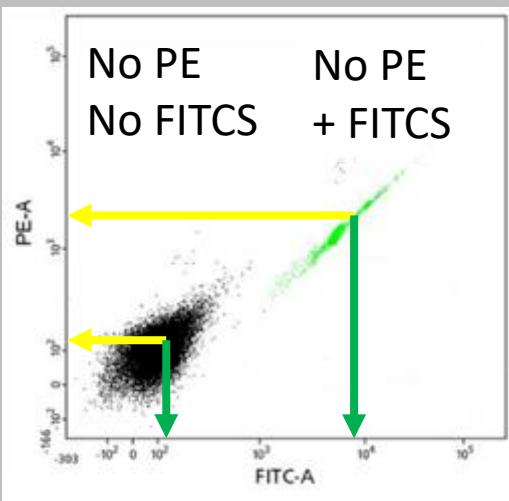
Compensation will subtract these "adds"³⁴

Bleeding Through : Compensation

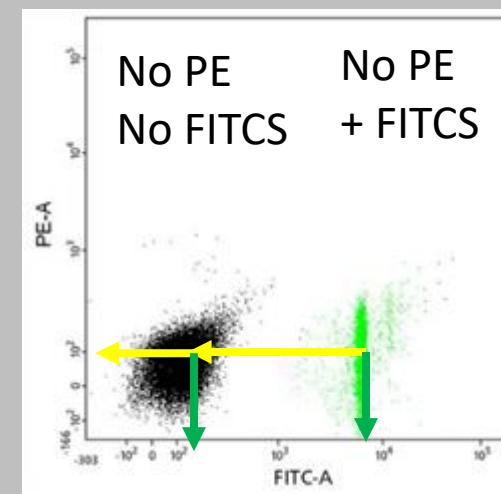


Bleeding through of FITC into PE / PerCP channels

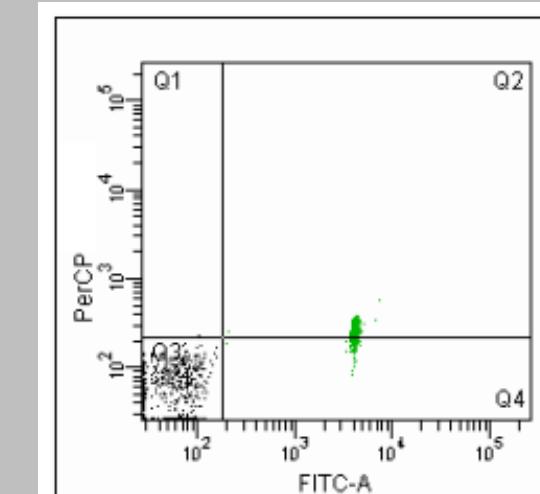
- Double negative has certain mean value of Intensity against FITC and PE
- Mean value of FITC against PE should be same like for Double negative



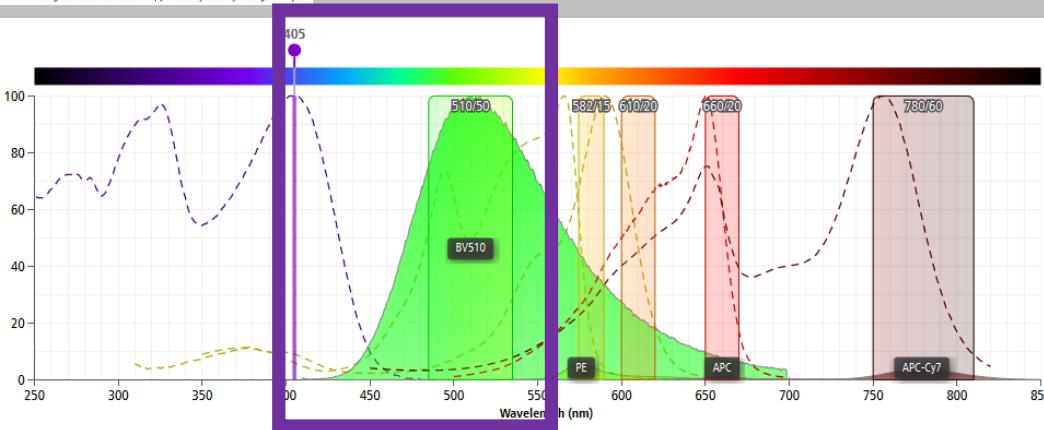
uncompensated



compensated



undercompensated



Bleeding Through : Compensation

CD3: 'AmCyan'

CD56: 'PE'

CD14: 'PI'

CD16: 'APC'

CD45: 'APC-Cy7'

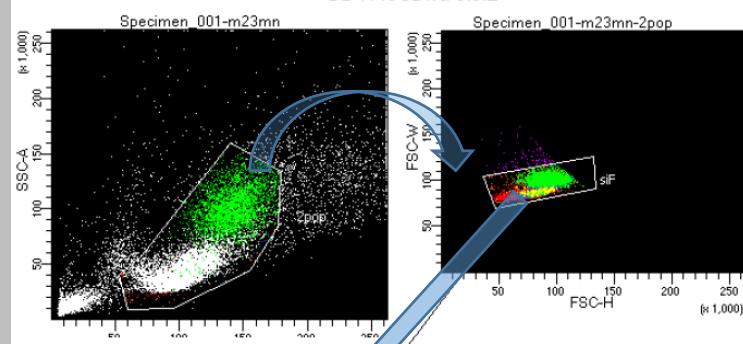
Unlying color indicates Laser wavelength

actual fluorophores for
 AmCyan = BV510 , PI = Alexa 594

Colored boxes indicate the Channels we actually observe when using a particular laser for excitation

...561 excites not only PE and A594, but also APC and hence APC-Cy7
 ... massive bleed through between PE and A594
 ...APC-

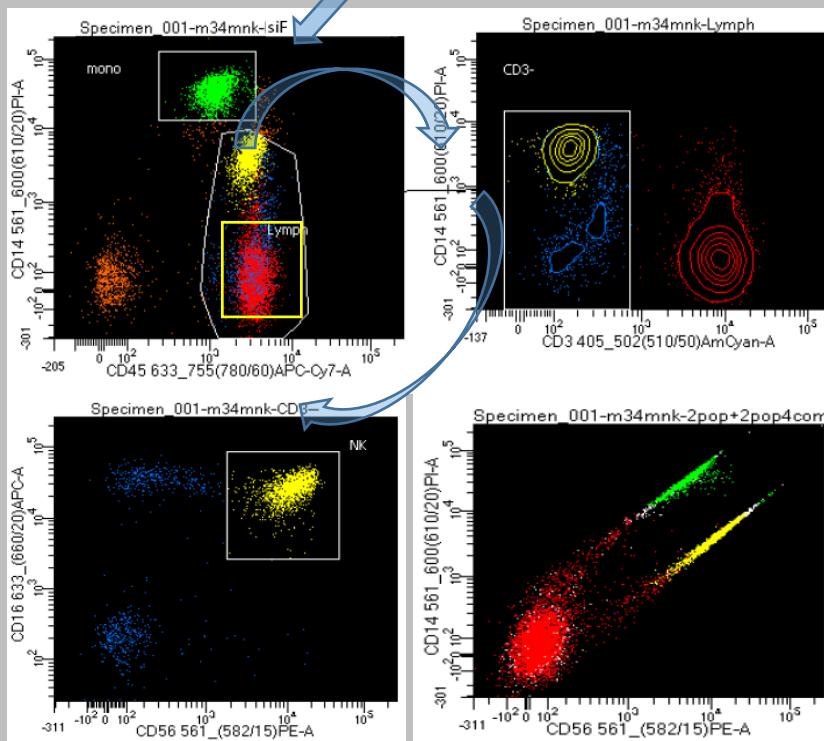
Bleeding Through : Compensation (case)



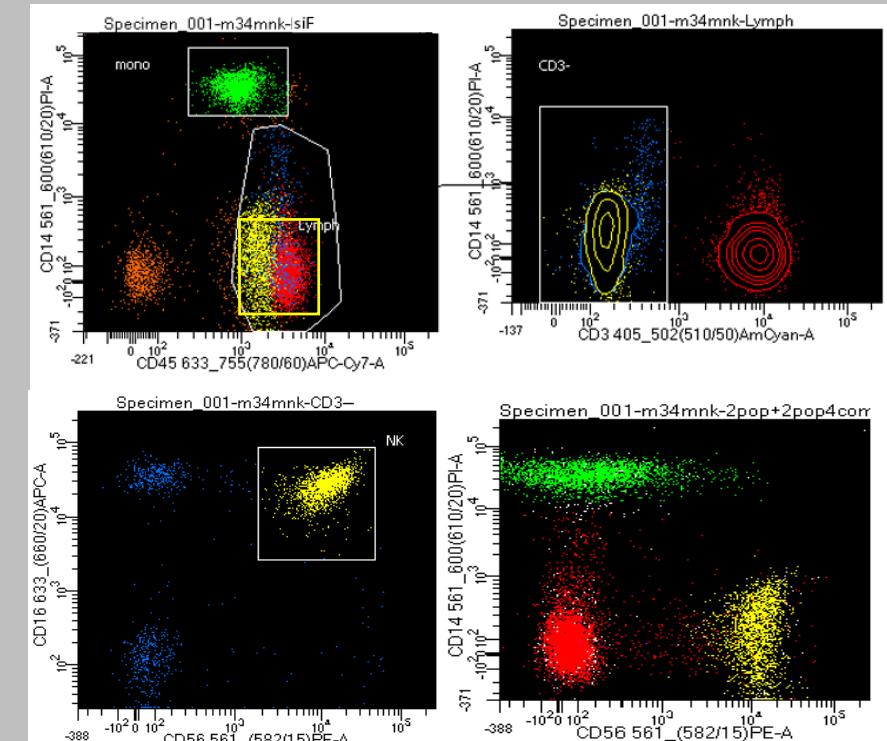
CD3: 'AmCyan'
 CD56: 'PE'
 CD14: 'PI'
 CD16: 'APC'
 CD45: 'APC-Cy7'

! Gate "*lymph*" & "*CD3*" drawn on uncompensated data to GET cells into "one" population.
 "*lymph*" and "*CD3-*" also gets cells unaffected by compensation
 Correct gate seen as yellow overlay

actual fluorophores for AmCyan = BV510 , PI = Alexa 594



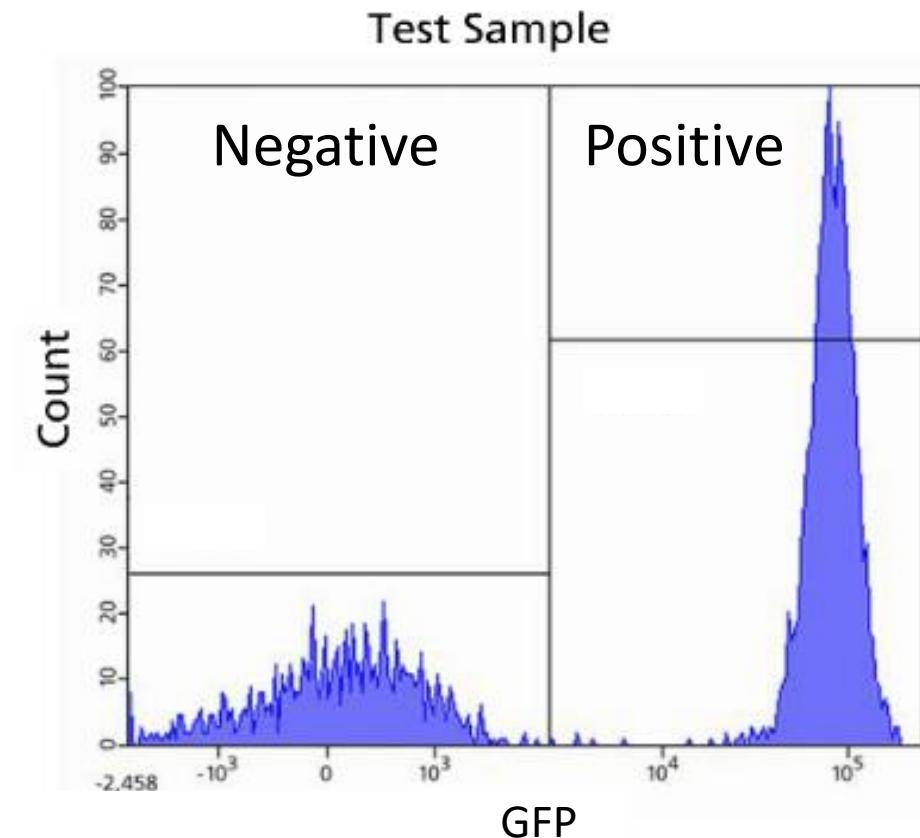
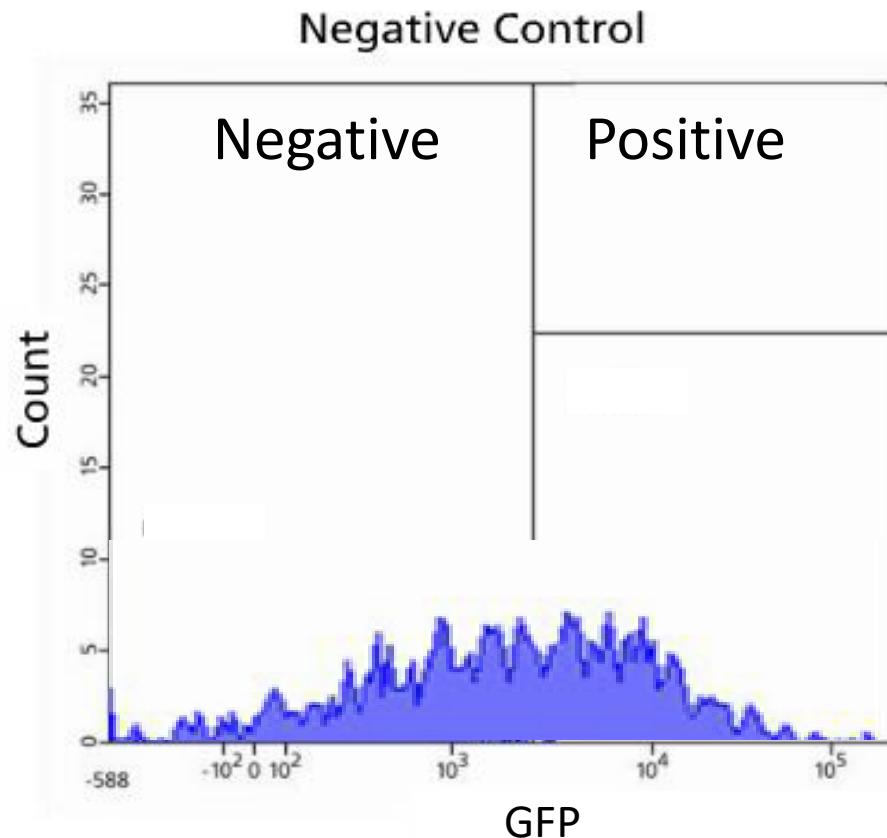
uncompensated



compensated, manually for PE and PI

Controls : Controls : Controls

A simple control : a negative



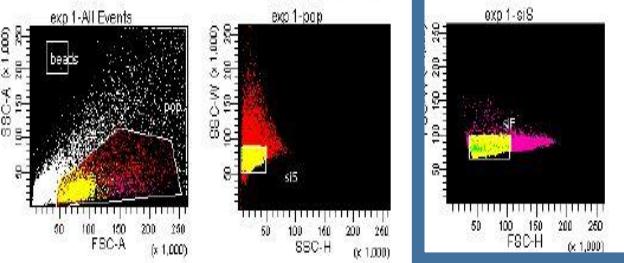
Gating on GFP negative sample
to distinguish background signal
from positive signal

Population	#Events	% Parent
Negative	921	33.3
Positive	1,834	66.7

Controls

Control	Sample type	Primary Ab	Secondary	Reason
Cells only Use treated and untreated cells	Neg control cells	No	No	Negative Control/Background Autofluorescence control
Primary Ab control	Neg control cells	Yes	No	Check for non specific binding of primary
Treated primary Ab control	Treated cells	No	Yes	Check for non specific binding of secondary Ab on treated cells
Isotype control	Neg control cells	Use Isotype control antibody. This should be the same antibody isotype as primary antibody. *	Yes	To confirm that the primary antibody binding is specific and not a results of non-specific Fc receptor binding or other protein interactions.
Compensation controls for each fluorochrome	Positive population of labeled beads or positive control cell sample	Yes	Yes	Positive control to set up cytometer alignment and to remove spectral overlap.
Cell viability control	Cell sample (identical to other samples) stained with both antibody and PI nuclear stain	Yes	Yes	Non-viable cells can be discriminated from live cells on the basis of light scatter (FSC=forward scatter). This discrimination is often lost in fixed or permeabilized cells. In these cases dead cells can be distinguished from live cells by their uptake of fluorescent DNA dyes due to loss of membrane integrity e.g. PI (propidium iodide) is used for dead-cell discrimination in unfixed and non permeabilized cells. 7-AAD (7-aminoactinomycin D, fluorescent) + AD (actinomycin D, nonfluorescent) for fixed or permeabilized cells.

Gating GFP vs Autofluorescence (case)



Typical gating strategy

Population, singlet, get the GFPs

A control (GFP+ unstained)

B Exp 1

C Exp 2

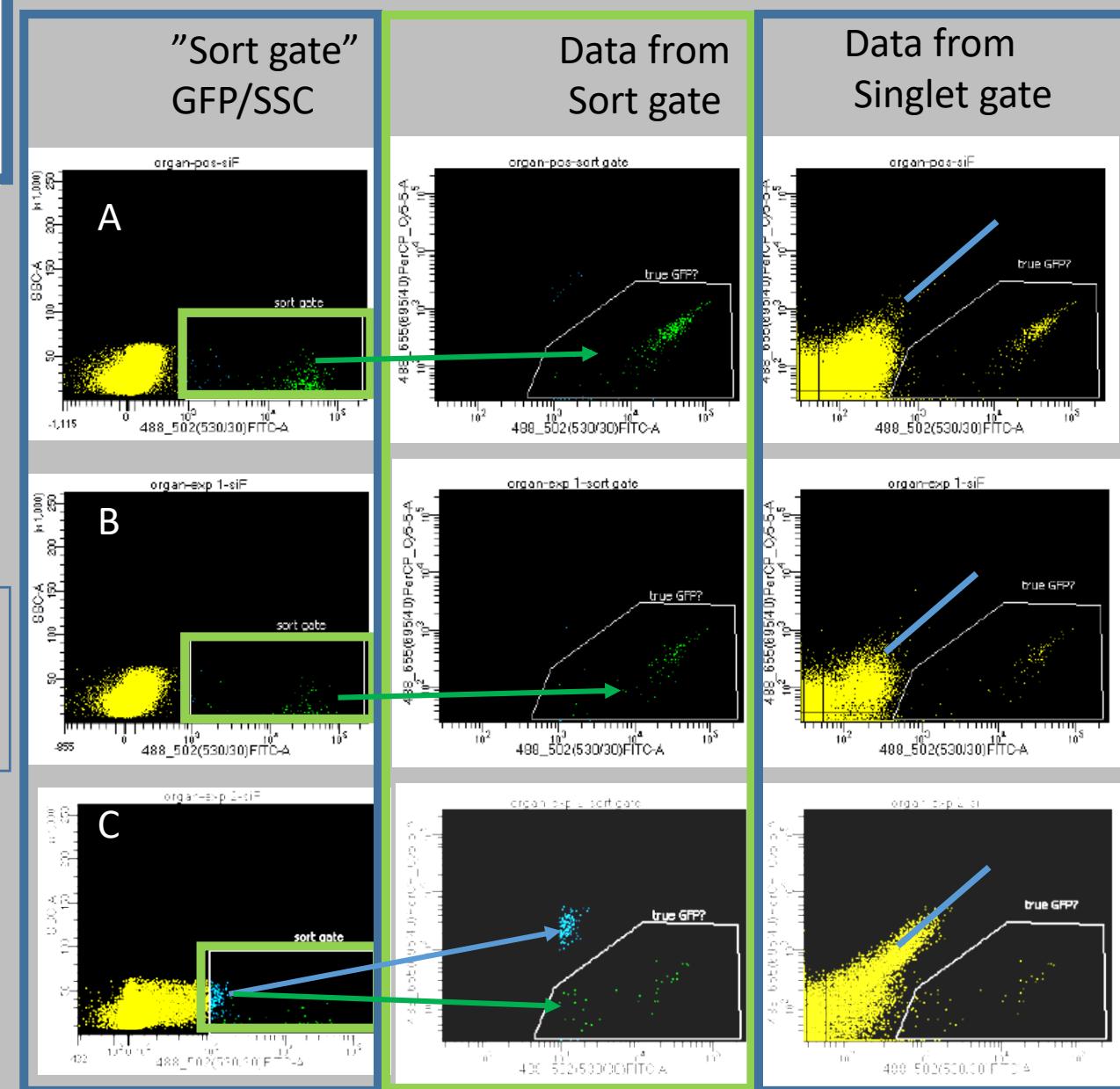
....AF is detected over many channels

- . Plot GFP against e.g. PerCP Cy5.5
- . AF cells appear as "double pos" (the diagonal)

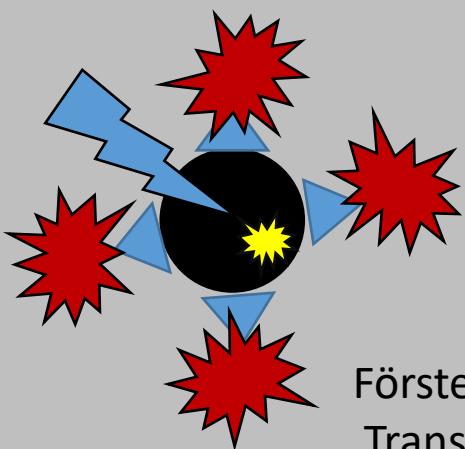
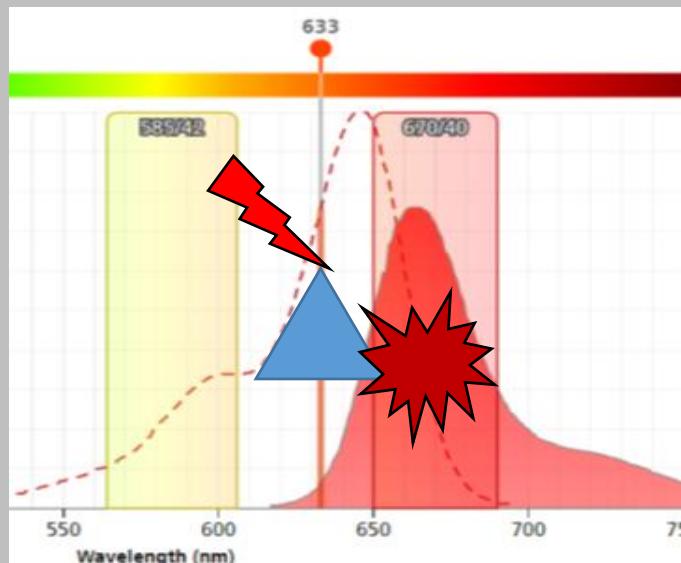
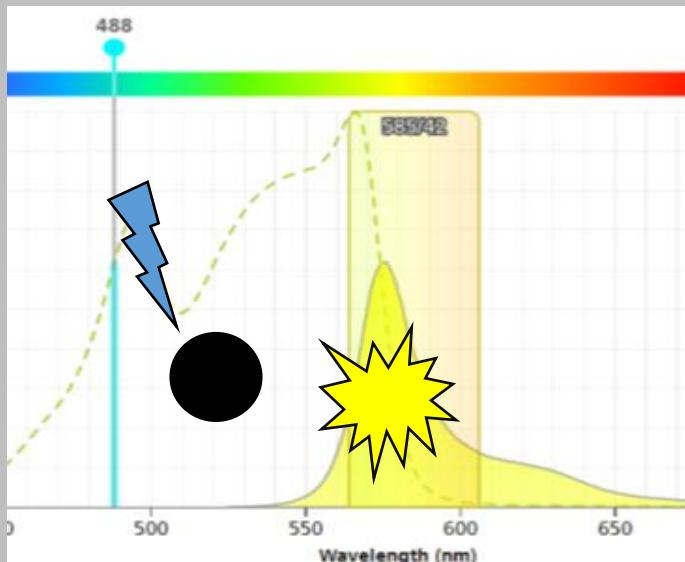
"Data from sort gate" reveals AF cells were sorted in Exp 2 (3.)

"Data from singlet" visualized the overall situation

PercPCy5.5 /GFP to estimate AF

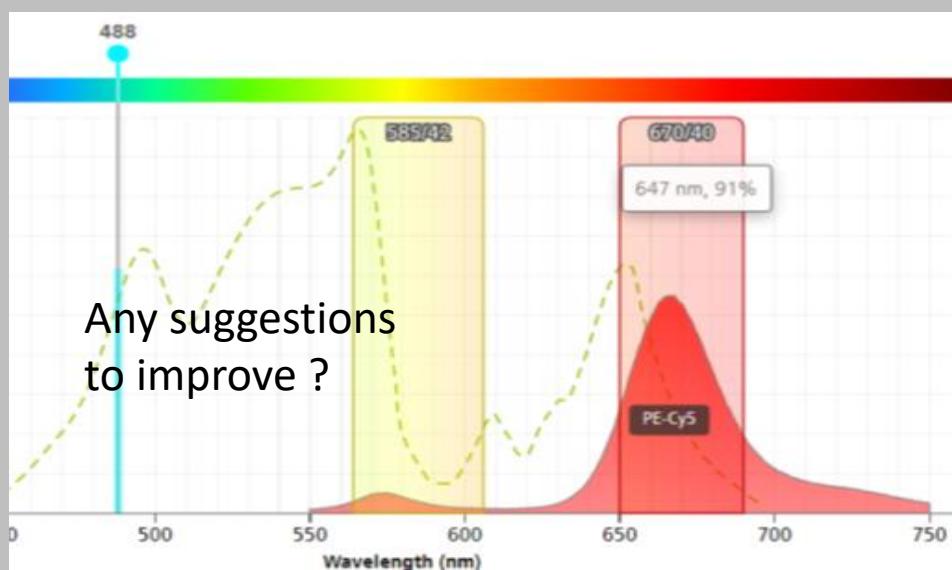


Tandem dyes (and FMOs)



Förster Resonance Transfer (FRET)

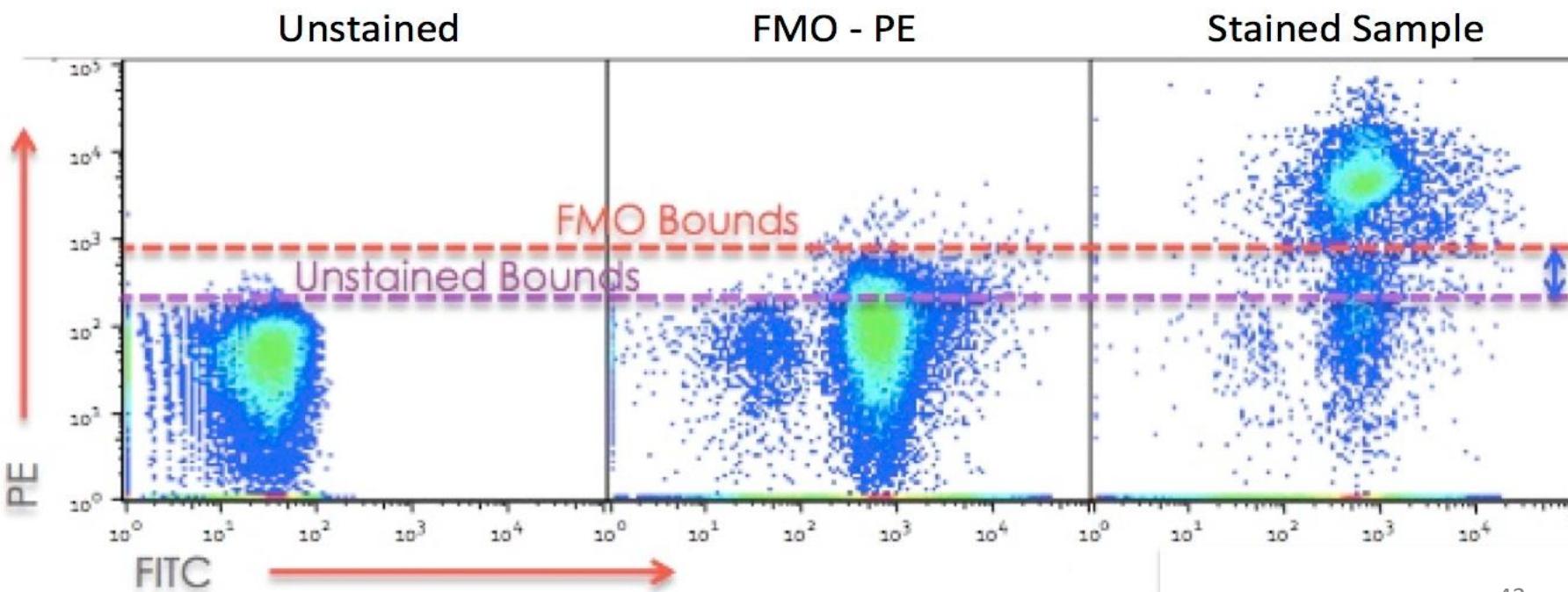
Donor's emission energy (!) gets used for excitation of acceptor who emits subsequently



Light & Temperature → Donor loses Acceptor: What's the outcome?

FMO – Fluorescence Minus One

Tube	FITC	PE	PE-Cy5	PE-Cy7
Unstained	-	-	-	-
FITC - CD3 -FMO	-	CD4	CD8	CD45RO
PE -CD4 - FMO	CD3	-	CD8	CD45RO
PE-Cy5 - CD8 - FMO	CD3	CD4	-	CD45RO
PE-CY7 CD45RO -FMO	Cd3	CD4	Cd8	-



Staining the sample

Staining the sample

1. How to detect your target cells & distinguish them from other cells

Suitable proteinmarker on your cells of interest (and non-interest)

Suitable strategy to find sub-population

2. Estimate amount of antigen you want detect on sample

Protein expressed in high numbers → gets a weak fluorophore

Protein expressed in low numbers → gets a strong fluorophore

3. Make use of the lasers and detector configuration

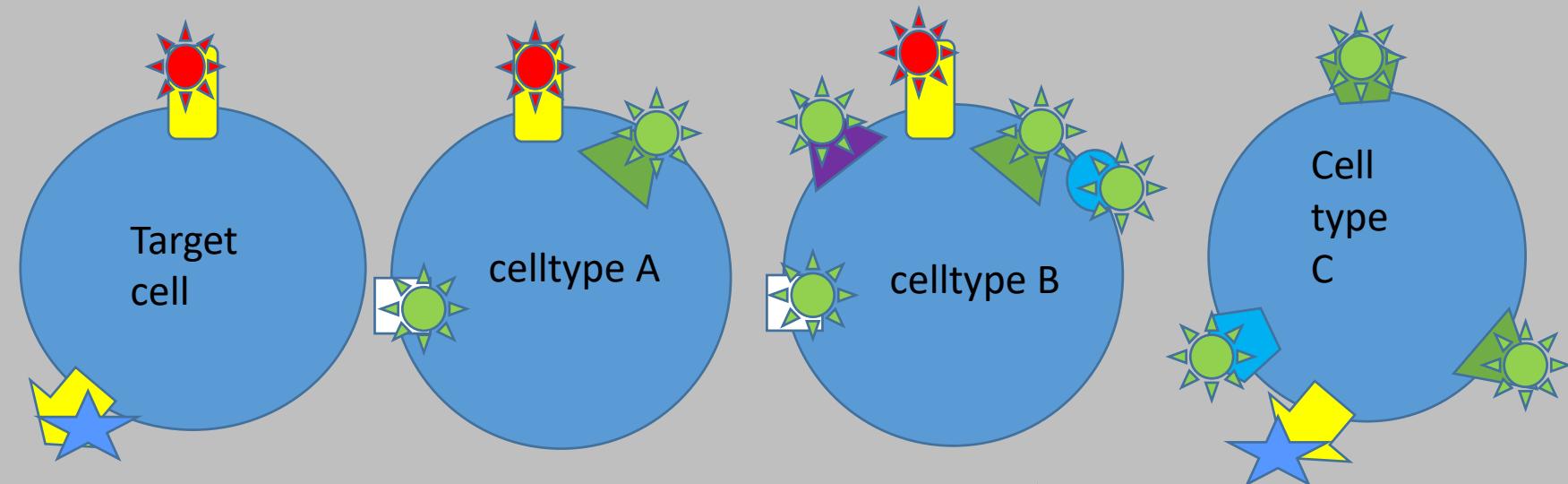
Laser 1 → marker 1, Laser 2 → marker 2 etc

Tandem dyes

4. Have controls

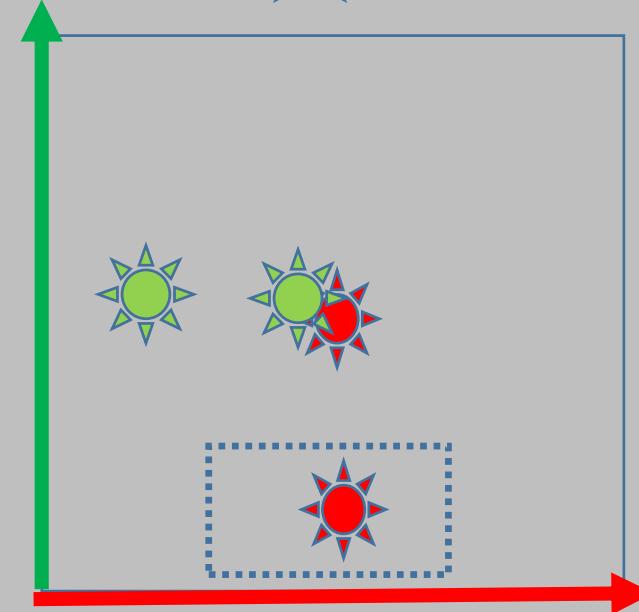
Negative, Live/dead, compensation (and FMOs)

Dump channel

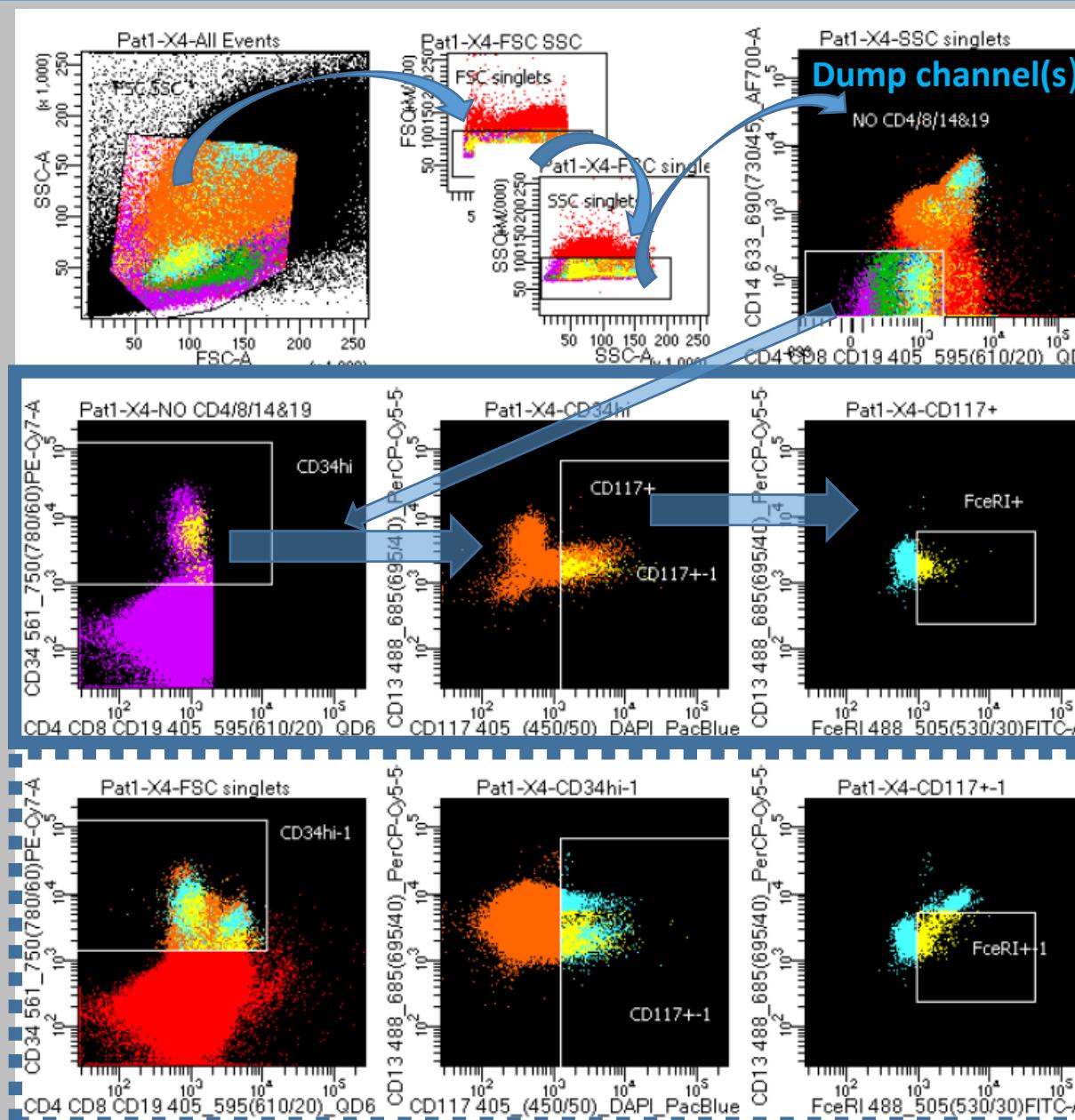


ID Marker of target cell shares that marker with non-wanted cells ?

→ Stain for other markers on 'non wanted cells' not present on target cells using same fluorophore to get rid of those unwanted cells by gating for the and working further with the single positive on other markers



Example, rare cell population / dump channel



Show in front are target population (Yellow and Green) to get rid of cells positive for CD14 & CD4,8,19

Tube: X4		#Events	%Parent	%Total
Population				
All Events		3,402,500	####	100.0
FSC SSC		3,170,212	93.2	93.2
FSC singlets		3,126,394	98.6	91.9
SSC singlets		3,118,747	99.8	91.7
NO CD4/8/14&19		1,735,426	55.6	51.0
CD34hi		10,620	0.6	0.3
CD117+		2,203	20.7	0.1
FceRI+		520	23.6	0.0
Basophils		22,373	1.3	0.7
P2		822	3.7	0.0
CD34hi-1		348,716	11.2	10.2
CD117+1		3,610	1.0	0.1
FceRI+1		978	27.1	0.0

Dump channel was used

Not having dump channel:
 False positive in final gate

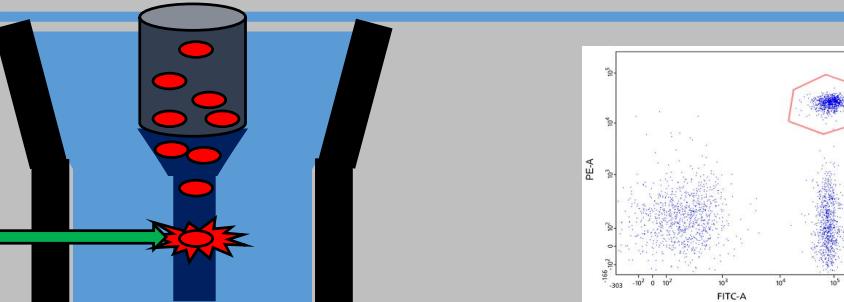
Dump channel not applied

Sorting

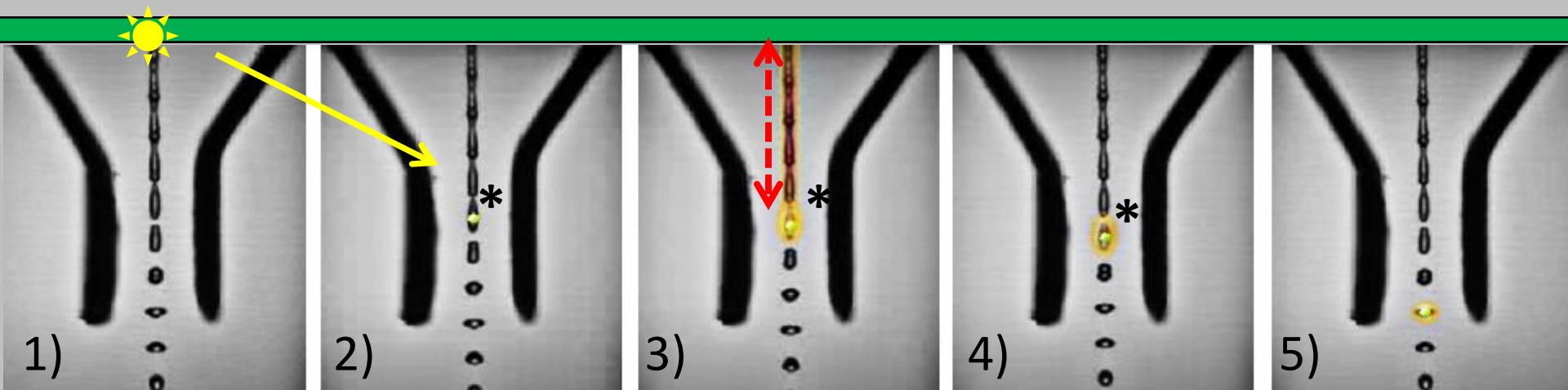


Sort collection chamber

Sorting

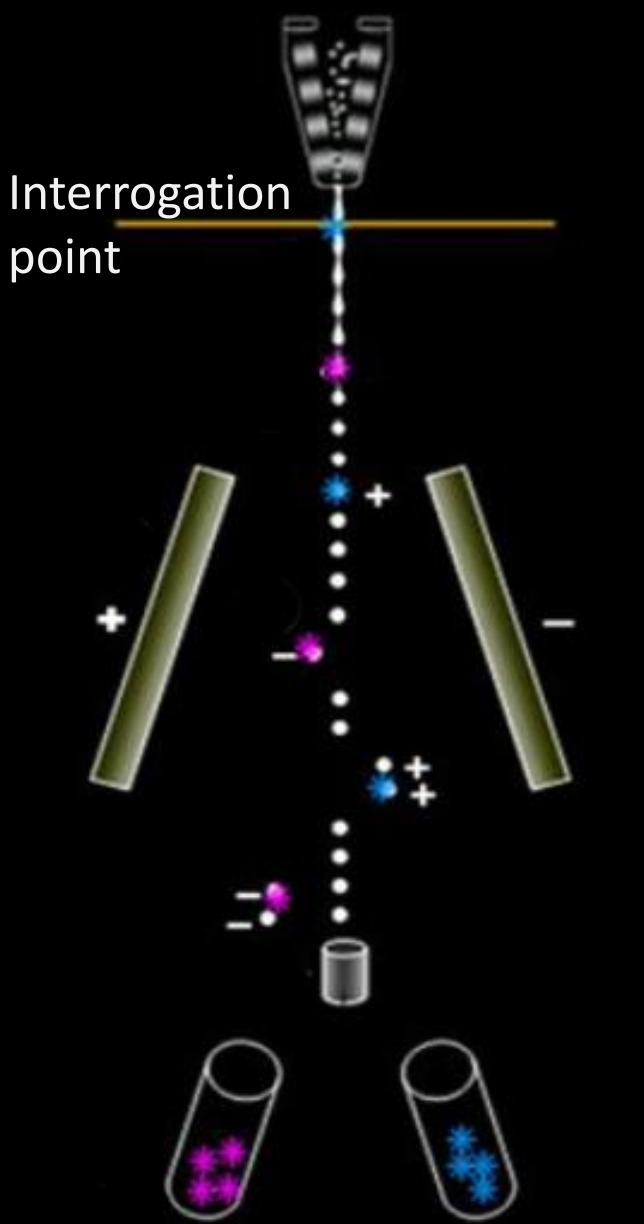


Prerequisite to sort are:
Flow cytometry data & gates
Performance check and drop delay

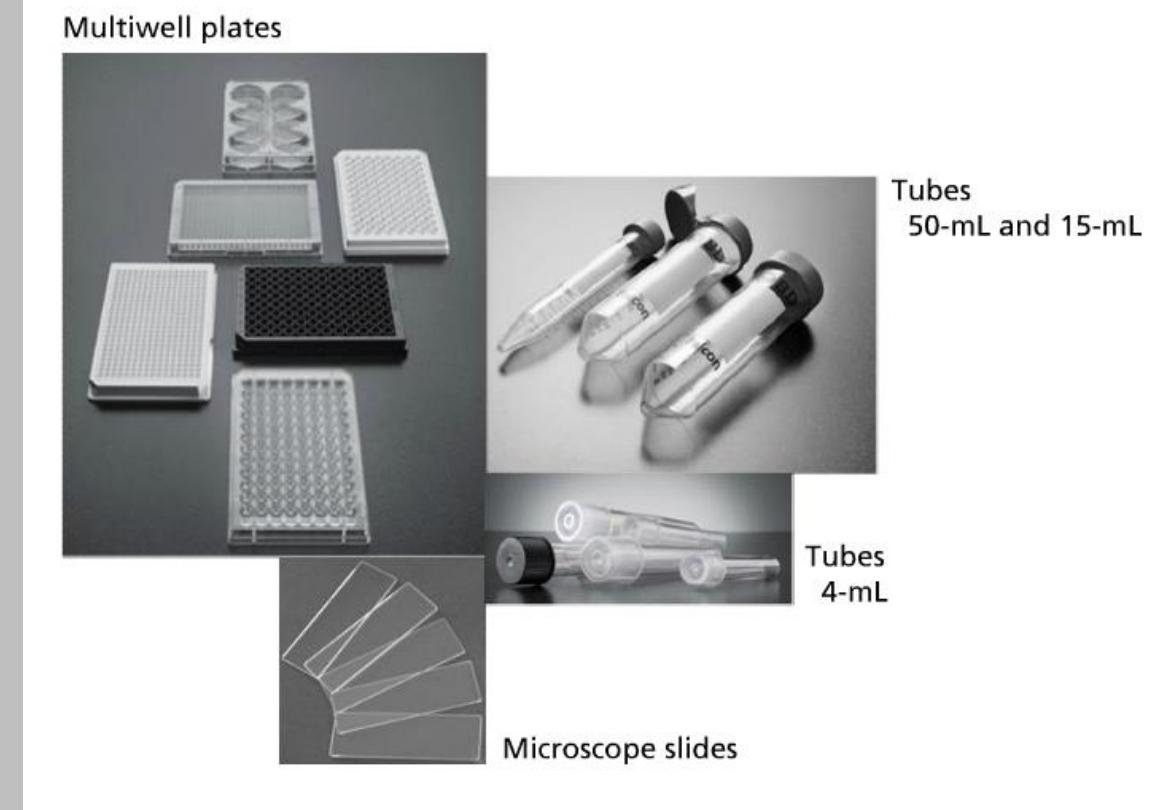


- 1) Stream vibrates, resulting in defined droplets,
- 2) cell which belongs to population to be sorted reaches break-off point*
- 3) whole stream gets charged
- 4) Cell is beyond break-off point in its charged drop and stream gets decharged rapidly, ready for another drop to be sorted
- 5) cell in its charged drop ready for sort via deflecting plates (see next slide)

Sorting



- Droplets pass through charged metal plates
- Droplets uncharged drop down directly to waste
- Droplets with charge will be deflected into sorting tube
- 2, 4, 6 sort tubes possible simultaneously



Instruments at BioVis

Melody & ArialIII – Cell Sorter

LSRII Fortessa & CytoFlex - Flow Cytometer for analysis

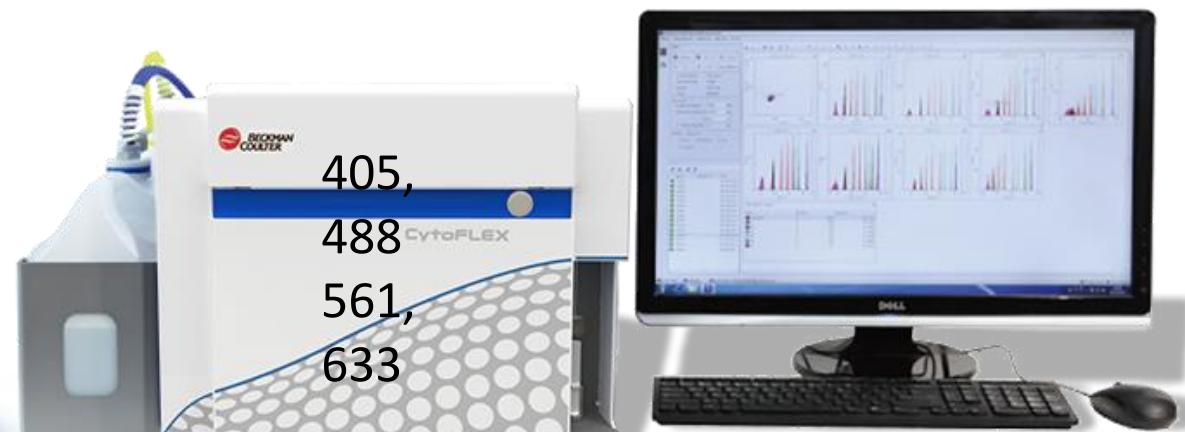
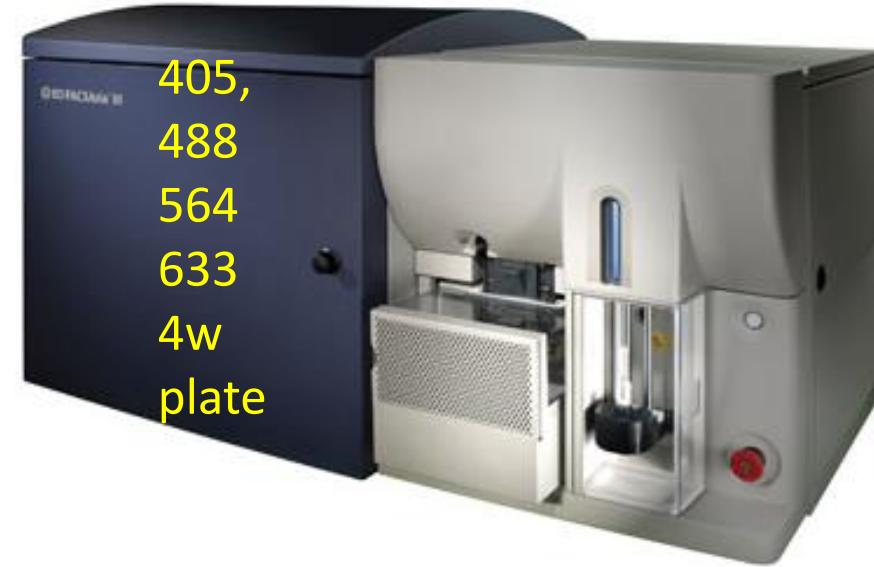


Image Flow Cytometer Merck Flowsight

