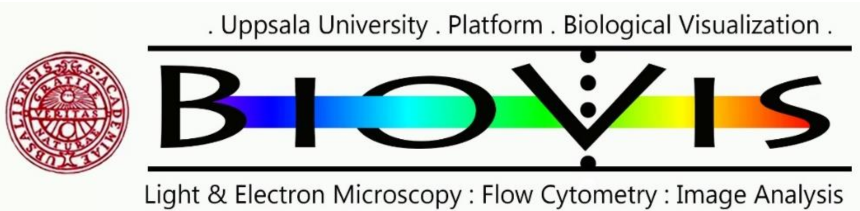
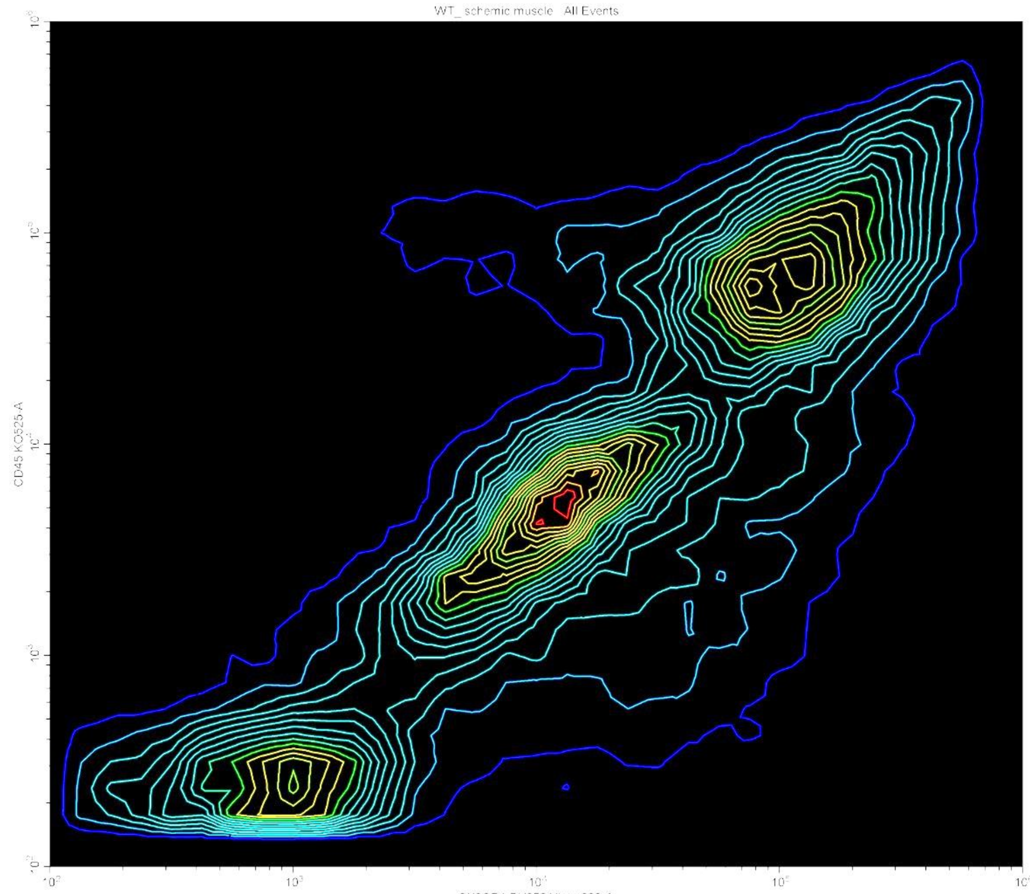
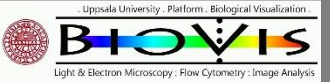


Flow Cytometry

Pt 1/2 : Basics & principle

Dirk Pacholsky





Flow Cytometry?

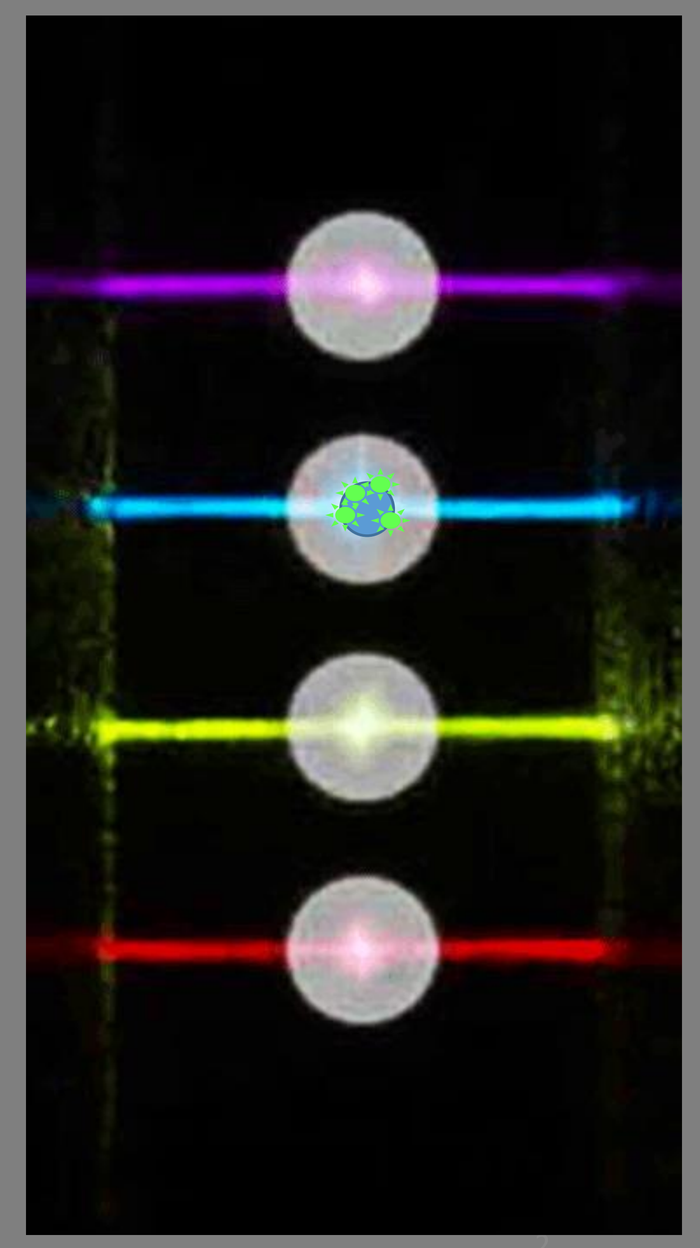
(Fluorescently labeled) cells pass through
(different) Lasers
Emission of fluorophores gets collected
This data can be later analyzed to get information about:

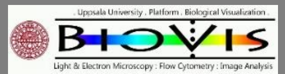
1. Is a marker present?
2. In which quantity is the marker present?

Marker: fluorescently labeled Protein or DNA etc.

This data opens up for more data

1. What kind of cells are present?
2. What is the ratio of different cells present?
3. What are the changes between experiment A and B?





Numbers and Populations

5

12

5

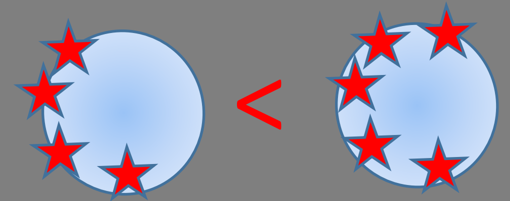
10

9

8

Number of

- Number of Cells
- Number Populations
- Combinations of staining
- 1 – 40 colors
- Strength of staining
  (expression level)

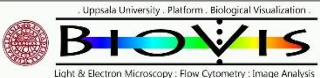


Standard Flow Cytometry

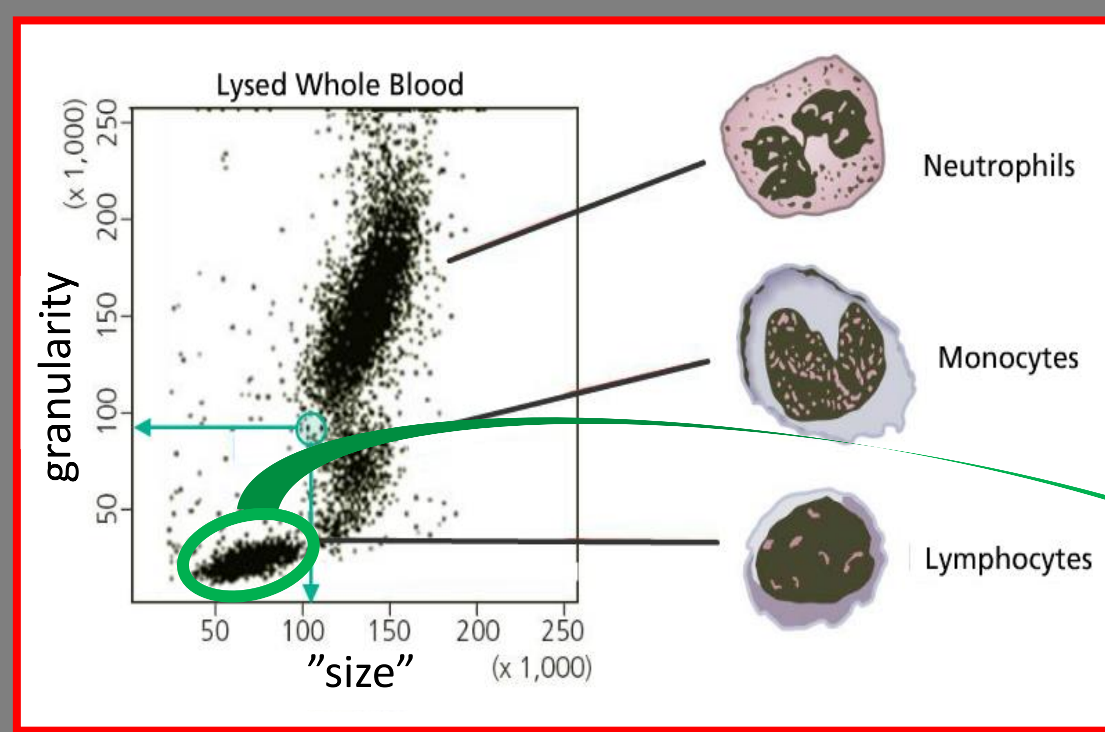
No images

But a lot of data within seconds

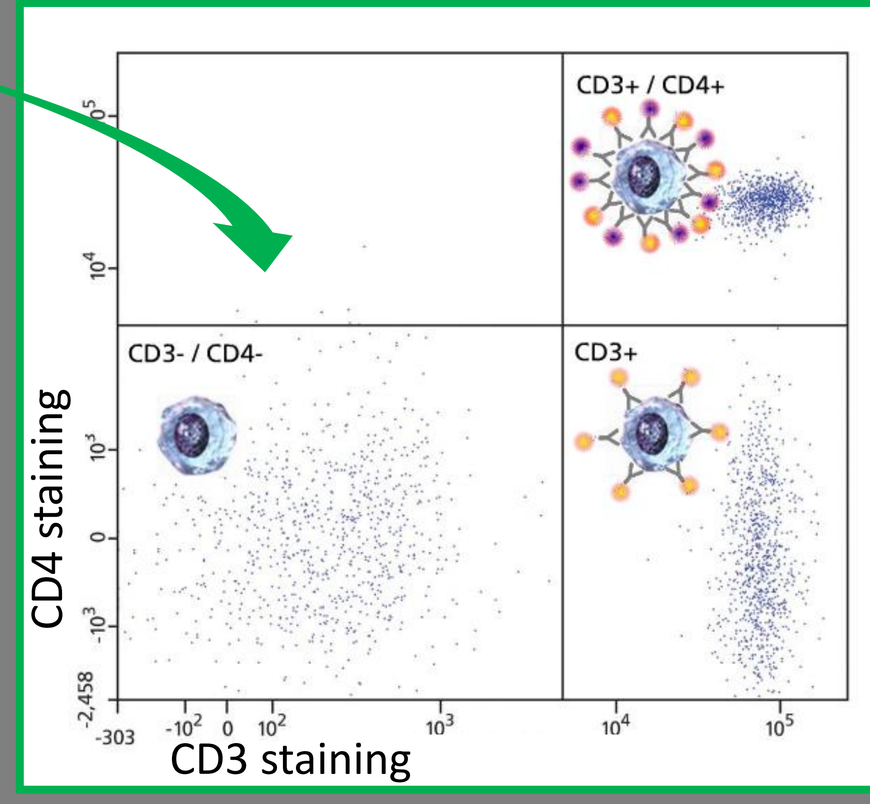
23.000 events (cells)/ second



Numbers & Populations



- Each dot equals a "cell" (event)
- Distinguish ´populations´
- in respect to different parameters
- by using "gates"

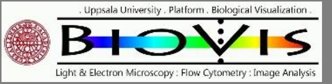


<u>"size" and granularity plot</u>

- Different populations of cells
- Cell number

<u>Staining plot</u>

- Different populations of cells
- Cell number
- Stain intensity



Applications of Flow Cytometry

Physical properties of cells like granularity and “size”

Fluorescent intensities

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

VERSATILE

If fluorescently stained, its detectable (within its limits)

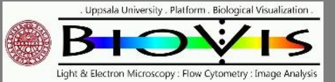
FAST

Tens of thousands of events (cells) per second

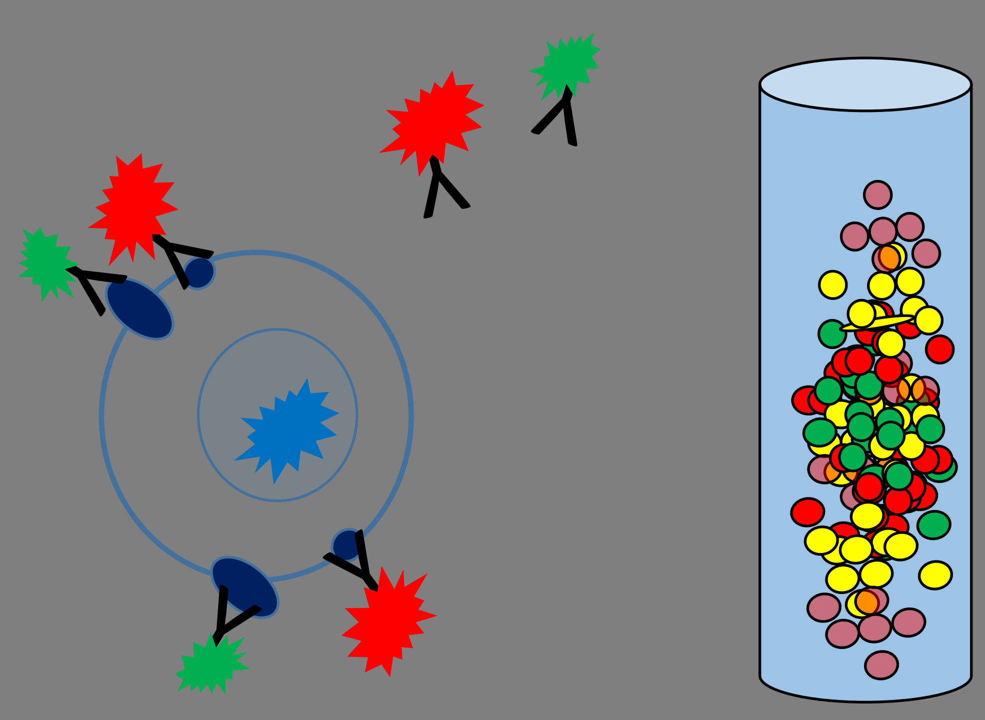
SENSITIVE

(below) 20 molecules per cell

No image



Laminar Flow and Fluorescent Cells



PBS

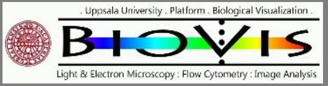
PBS

PBS

Laser

Exitation/
Emission

Laminar flow: when sheaths of molecule of a liquid
Pass by each other without mixing in lateral direction
 > All molcules move in one direction, no turbulance
Injecting cells suspended also in laminar flow into the centre of
the existing laminar flo w column, keeps the cell in the centre
of that column

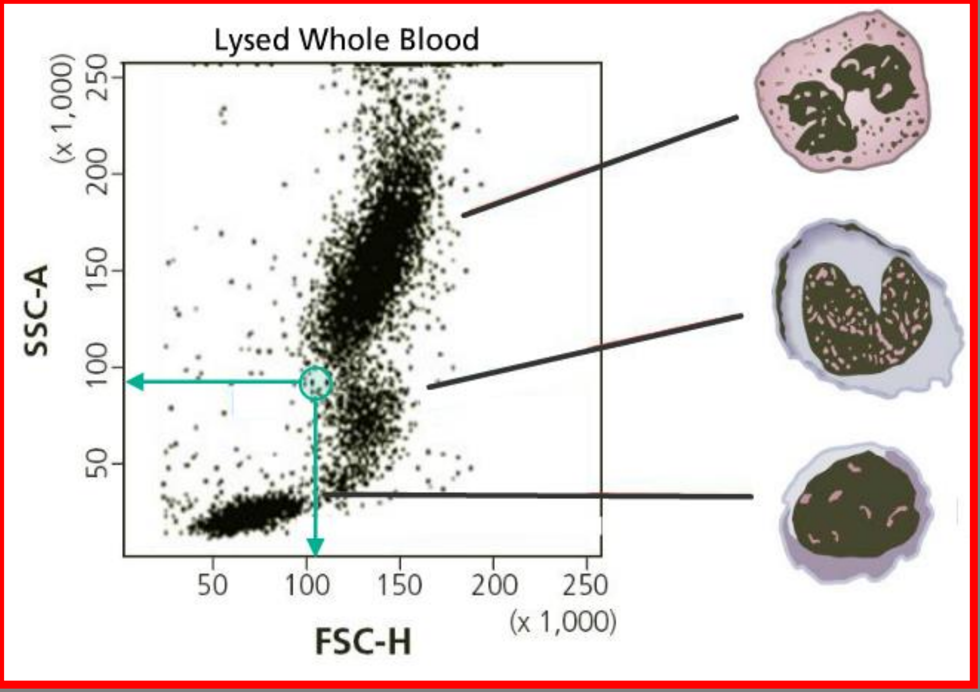


Forward & Side Scatter

LASER

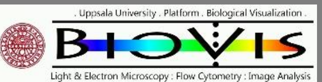
Forward Scatter

Sideward Scatter



Physical properties of cells define FSC & SSC

- By design 488 nm Laser is used for Forward Scatter & Sideward Scatter
- FSC in Laser´s direction with blocking bar to avoid detection of laser on detector
- SSC detected at a 90_o angle
- Sideward ´pathway´ also used for detection of fluorescence



ReCap: Fluorescence

488
Green is orange
Laser 488
510/20
660/20
Excitation curve
EGFP
Emission curve
APC
Wavelength (nm)

640
Laser 640
BP 510/20
=510 nm +/10 nm
BP 660/20
510/20
660/20
EGFP
APC
Wavelength (nm)

Bandpass (BP) filter

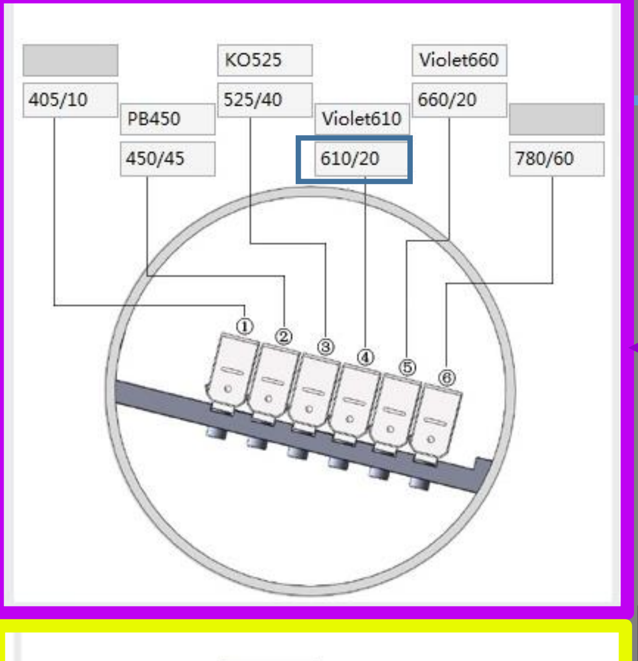
Incoming (emission) light

BP 505-550, light bw 505 550 passes

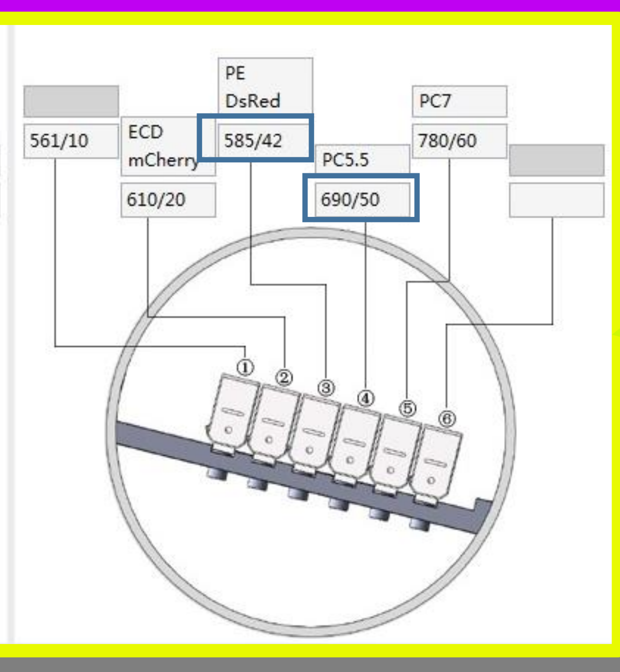
Optical Configuration*

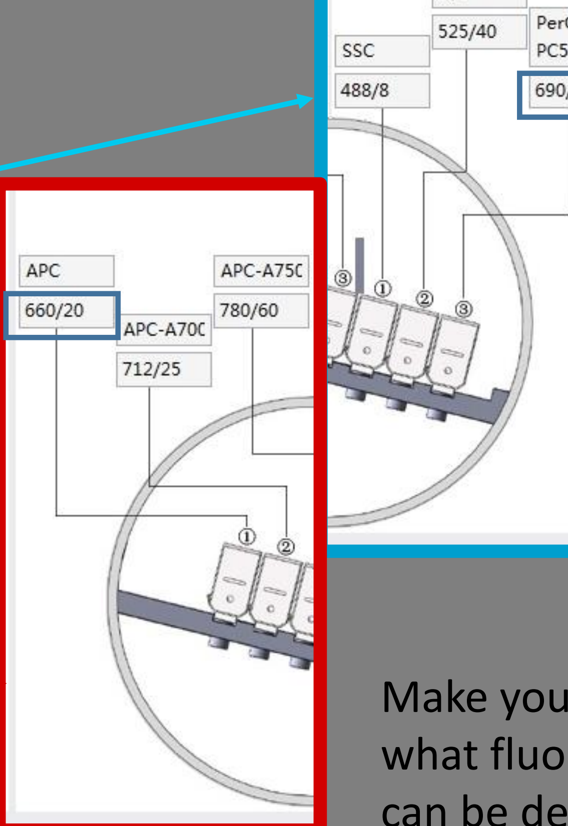
- of BioVis´Cytoflex S

Lens is collecting emission light
Light cable pass on the light to detector unit

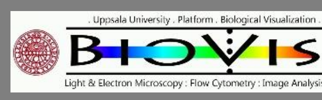


Each unit has several detectors
In front of each detector is a Bandpass filter
Ensuring that we collect a specific range of light





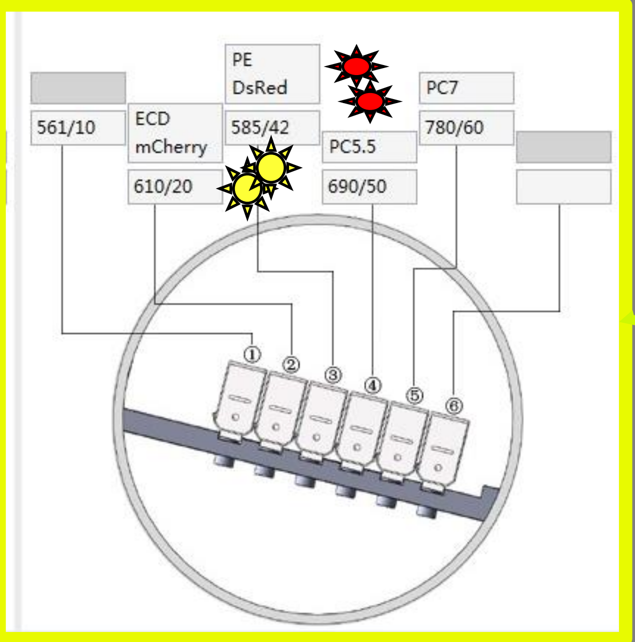
Make yourself familiar what fluorophores can be detected

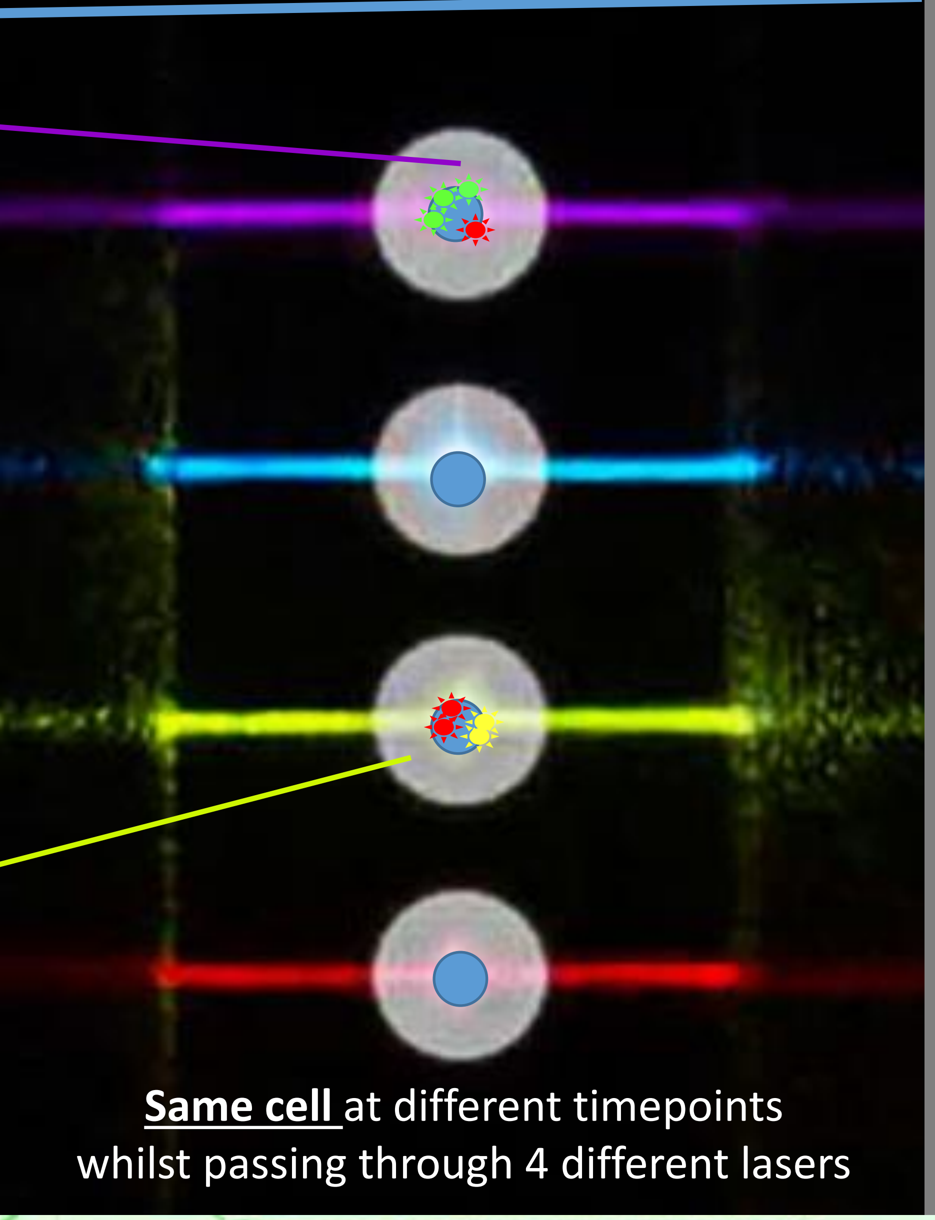


300 450 600 750

Detection of Signal

KO525 | Violet660
405/10 | 525/40 | 660/20
PB450 | Violet610
450/45 | 610/20 | 780/60





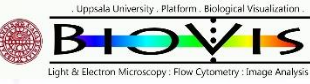
Same cell at different timepoints
whilst passing through 4 different lasers

Each cell
- is passing through each laser
- generates emission (range & strength)

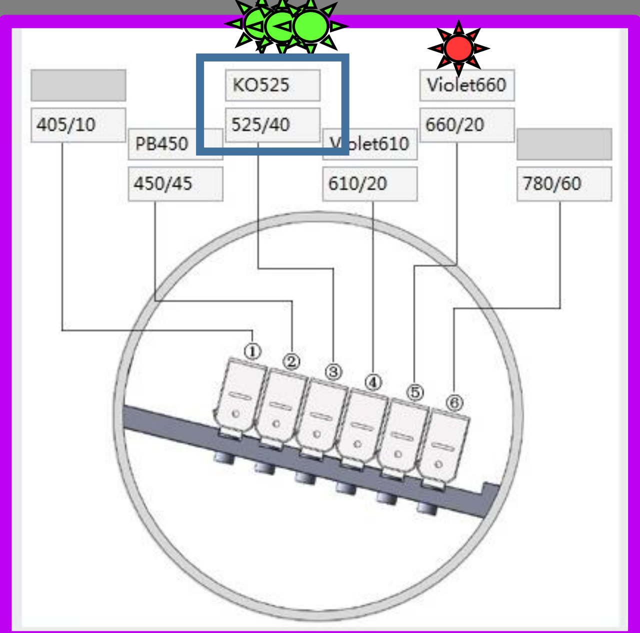
Instrument
- registers signal

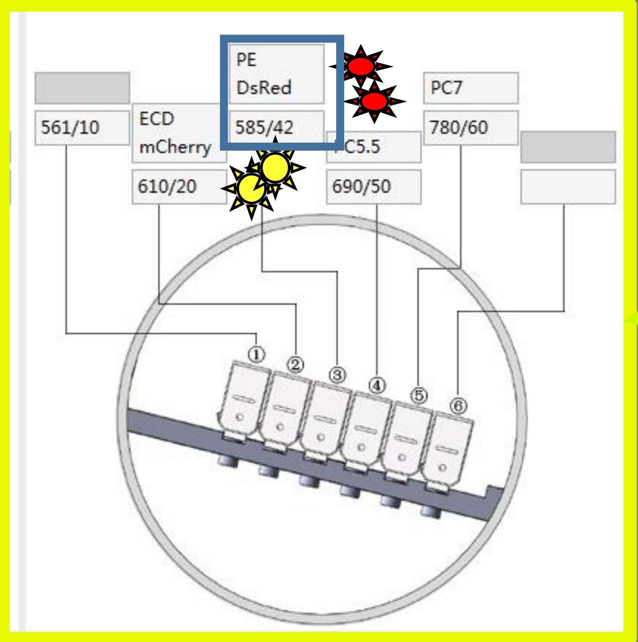
Software
- allows for analysis
- positions cells in respective plots

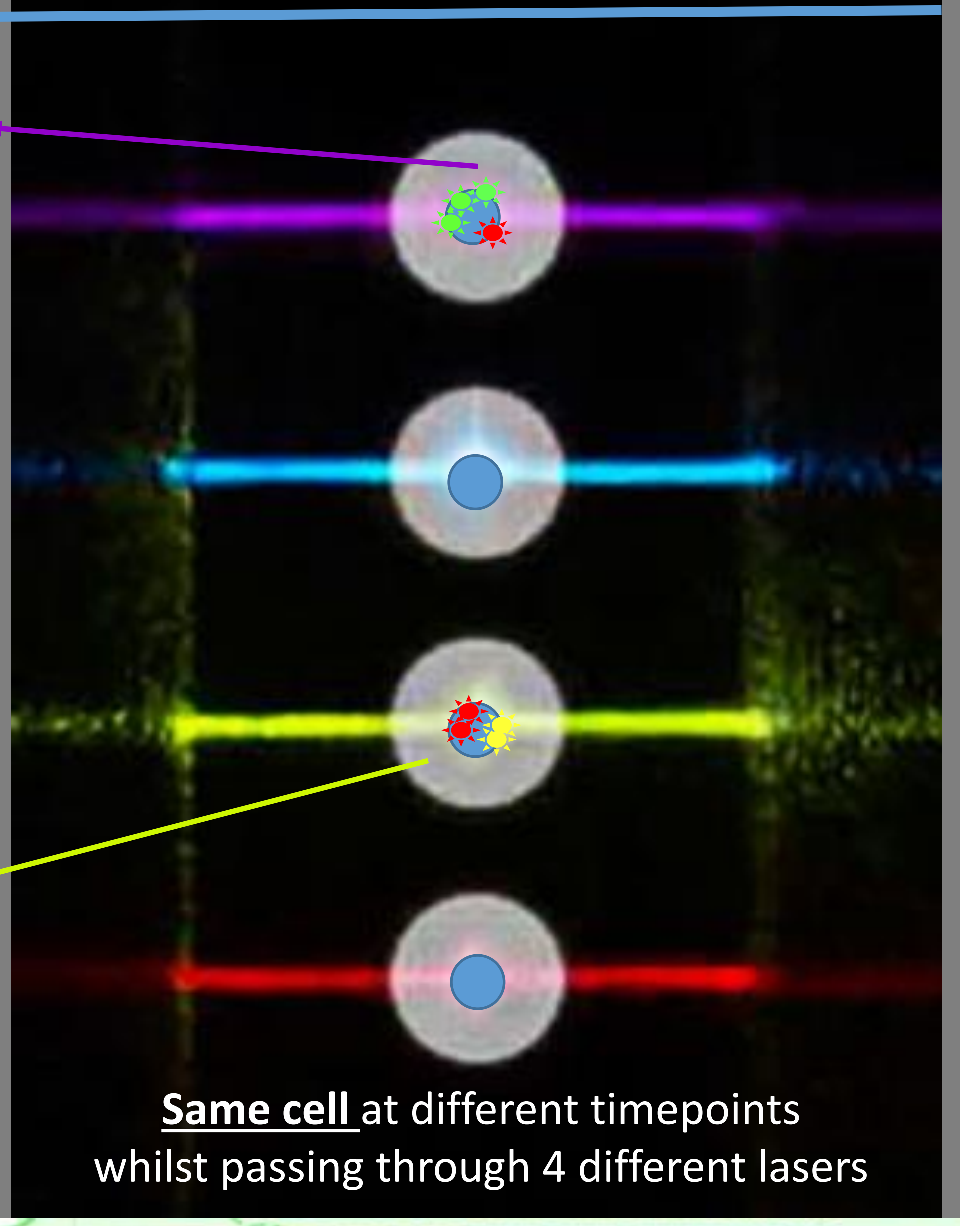
Signals caused by the specific lasers are simultaneously registered in their respective detectors. However, since the various lasers are spatially separated, the signals of the different lasers are recorded seperately in time. The electronics, together with a stable laminar flow, ensure that the signals from one and the same cell are combined. If this is not the case, the data cannot be evaluated.

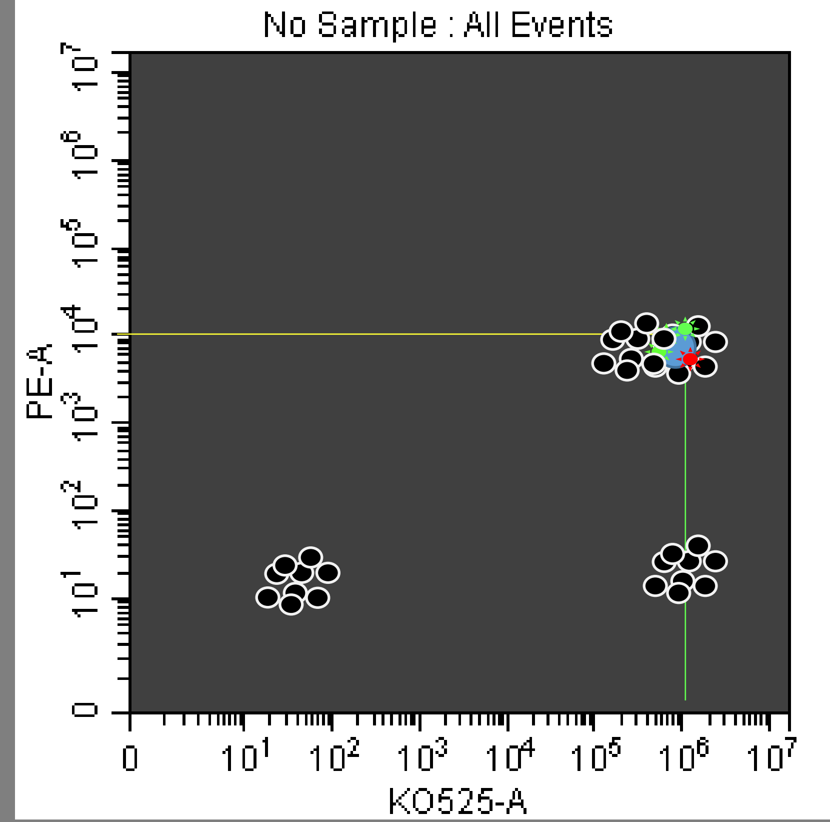


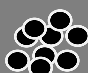
300 | 450 | 600 | 750



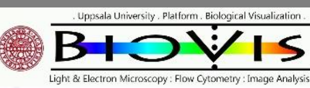




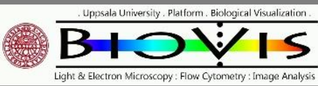




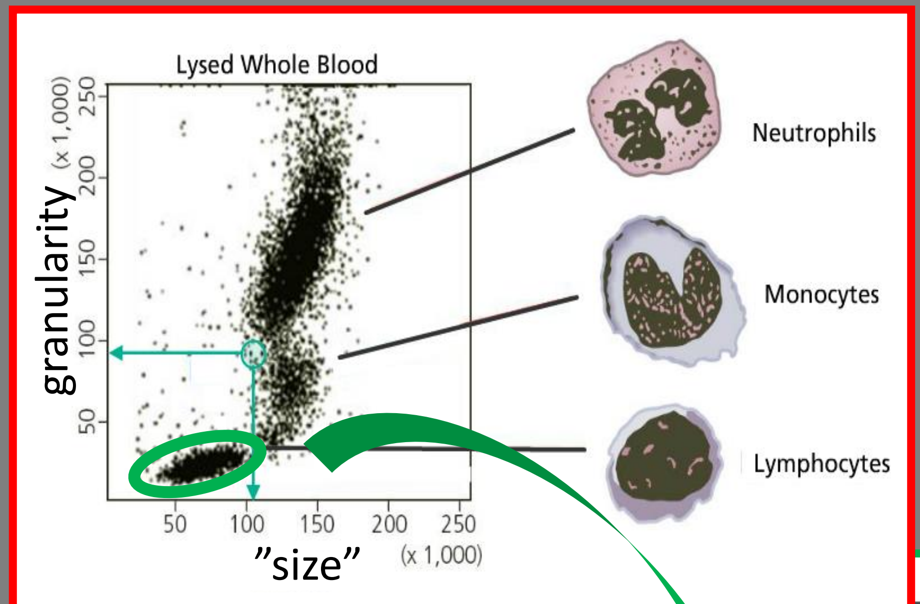
Cells of same characterization

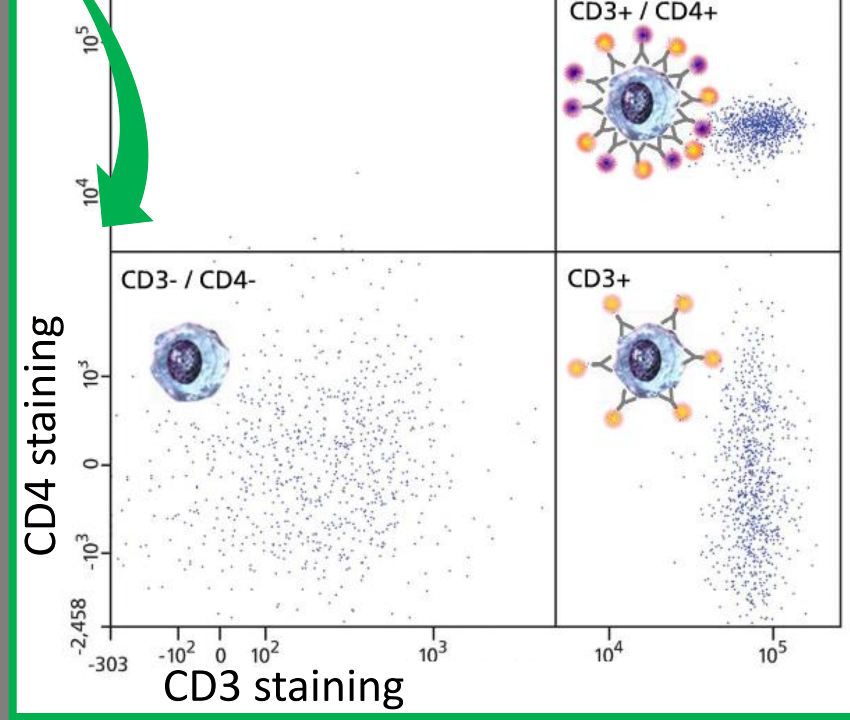


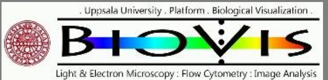
300 450 600 750



Plots and analysis in Flow Cytometry







Overview Flow Cytometry Bivariate plot

sample A-A3-CD11/B220 POS+High B220

+/- 1d/23

CD23/CD1D -/+

CD1d 561*PE (580/20)-A

10^2 10^3 10^4 10^5

CD23 488*Alexa488(525/50)-A

- each dot is one event (hopefully a cell)

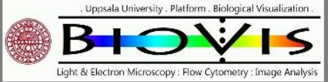
- where certain parameters were measured (depending on activated detectors)

- 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



Data analysis Plot types

Compare content of information available

FSC-A

SSC-A

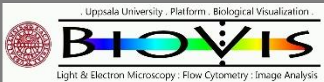
FSC-A

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

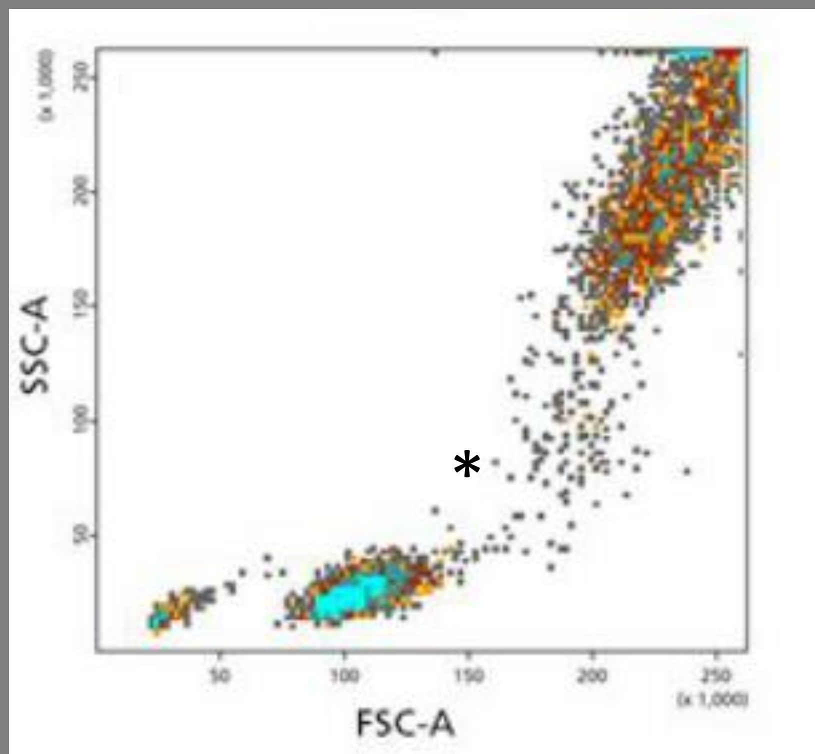
Peaks of histograms can include more than one population (see both plots in comparision

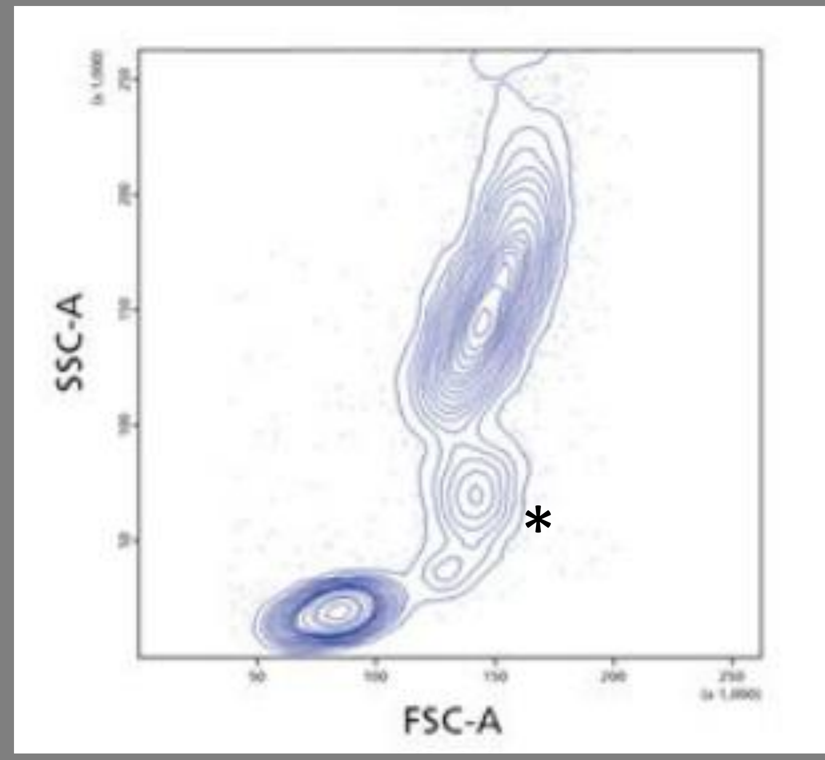
Bivariate plot displays 2 parameter (here FCS and SSC) against eachother.

Depending on data amount (massive/small) it Can be tricky to realize populations, or its centre (see next slide for solution)



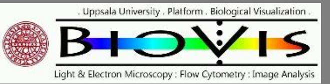
Data analysis Plot types





Density plot like dot plot.
Same event counts
Indicated by same color
Information of small amount of
Data can get lost *

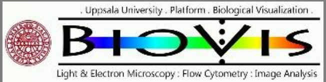
Same/similar event counts joined by line
(like topographic maps)
facilitates the representation of populations
within large amounts of data, avoiding loss of
representation of small data amount



Data analysis Gate types

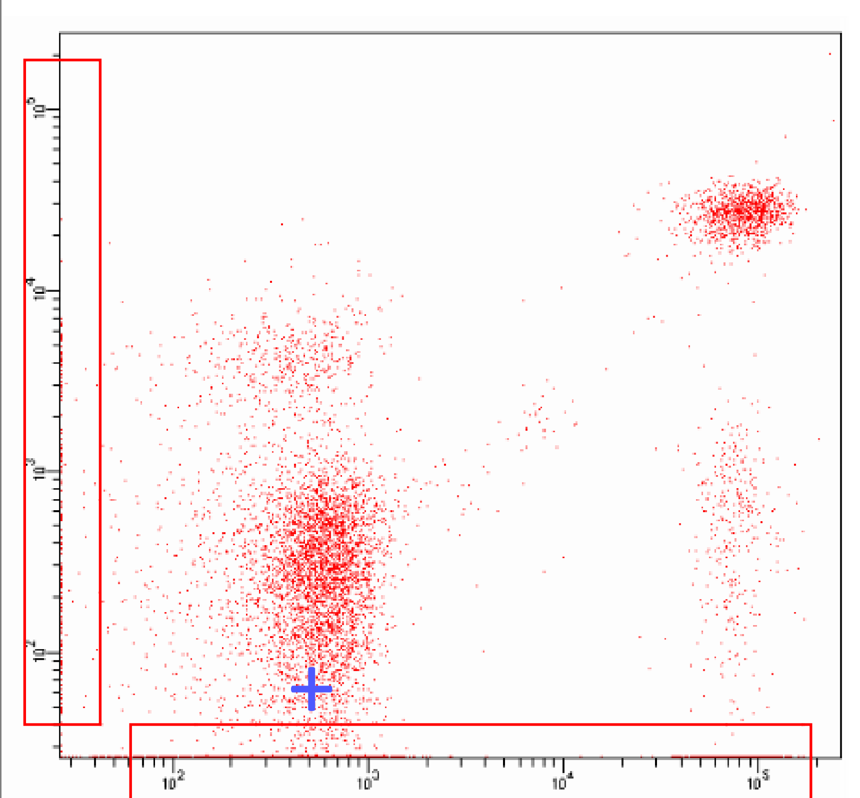
Polygonal Gate	Quadrant gate	Interval gate
boundary around a population in in Dot, Density or Contour plot	Division into four separate populations in Dot, Density or Contour plot	Boundary around range of events in Histogram plot

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



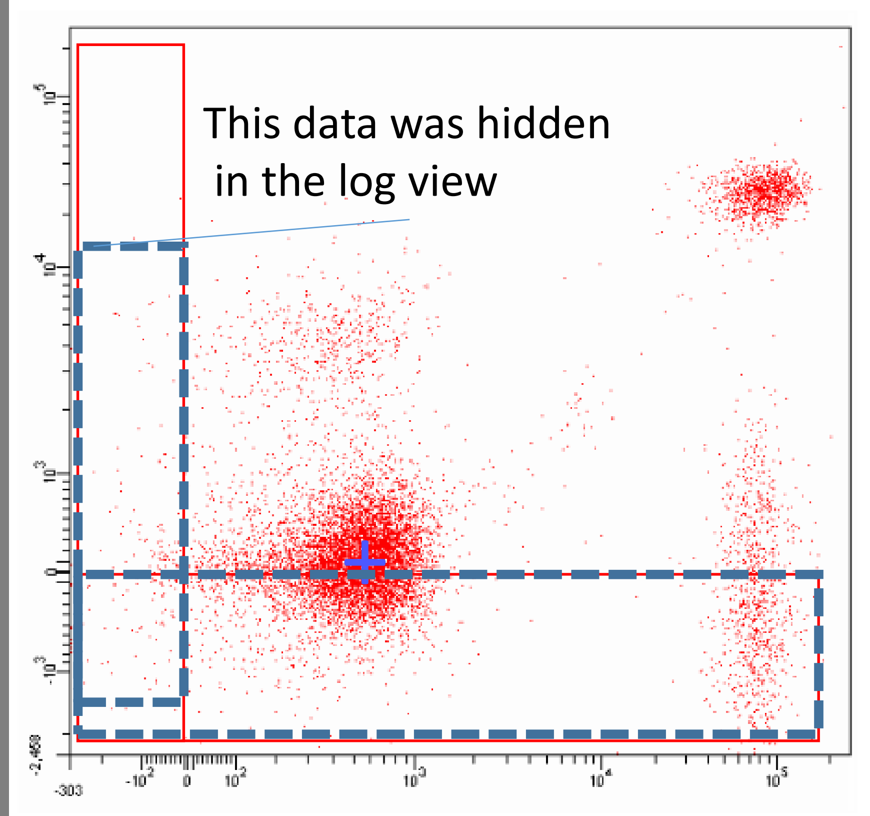
Data analysis plot scaling

Log scale



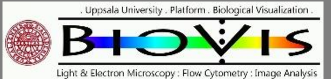
Traditionally used, but some data might end up on the scale and below the axis

biexponential scale

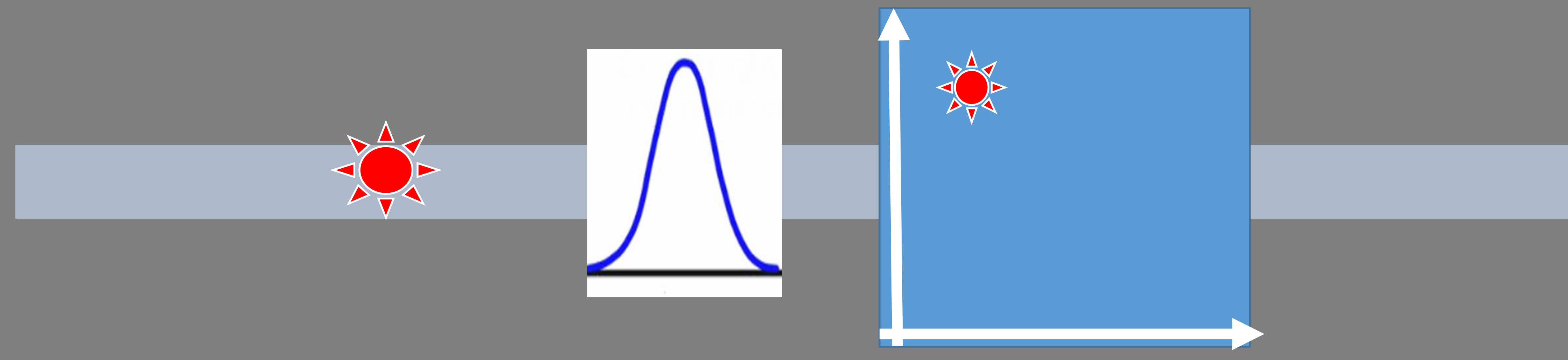


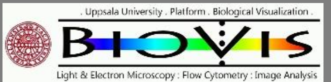
Enhances resolution,
useful for poorly resolved and/or complex population/data

+ Schematically indication of median (centre) of population

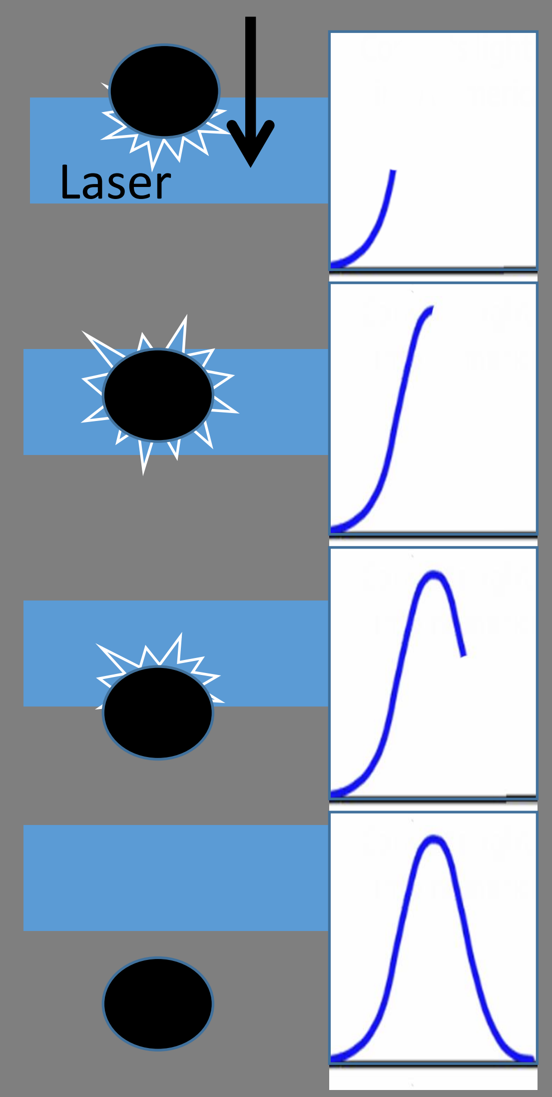


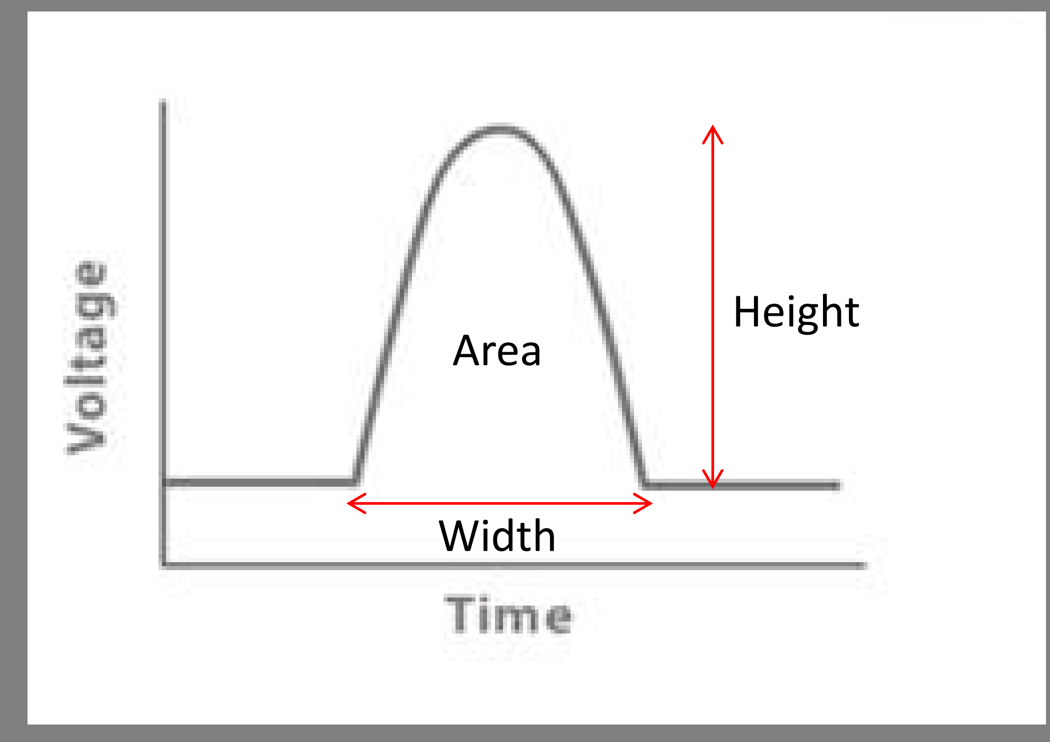
Signal to Pulse to dots on a plot
Or
How to get singlets and not doublets



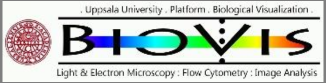


Signal to pulse to dot





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



Singlet gating

Laser

Height
Width
Area

Voltage

Time

FCS -W

FCS -H

FCS -W

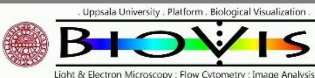
FCS - A

FCS -H

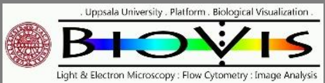
FCS - A

Whether you analyze
a single cell double stained
or a doublet of cells single stained
appearing as a double stained cell
....

Simplified view of appearance of singlet vs doublet populations (green/blue circles) in plots
Comparing different parameter of the actual ´signal pulse´. Tip: Gate for singlet, successively for FSC and SSC



Data Analysis examples



Gating – explore your data

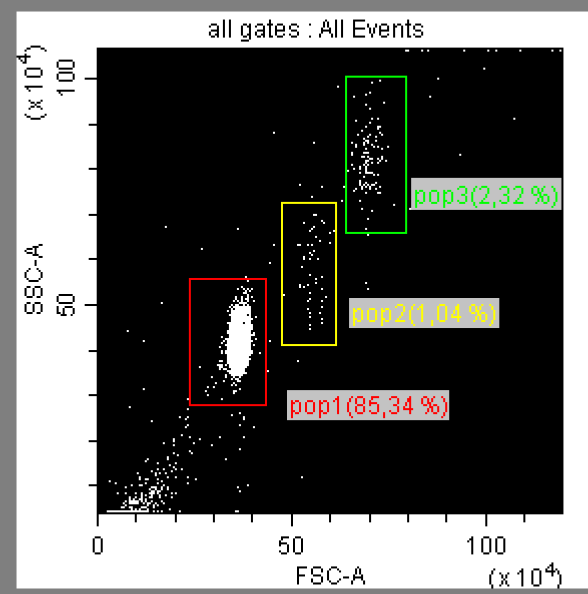
Explore your data before analysis:
→ in one or more plots make different gatings (or use one gate and move it). Events (dots) enclosed in the gate will have a certain color to be indentified by
→ Plot head line shows what data we look at - Tube name (all gates) : events (all events)

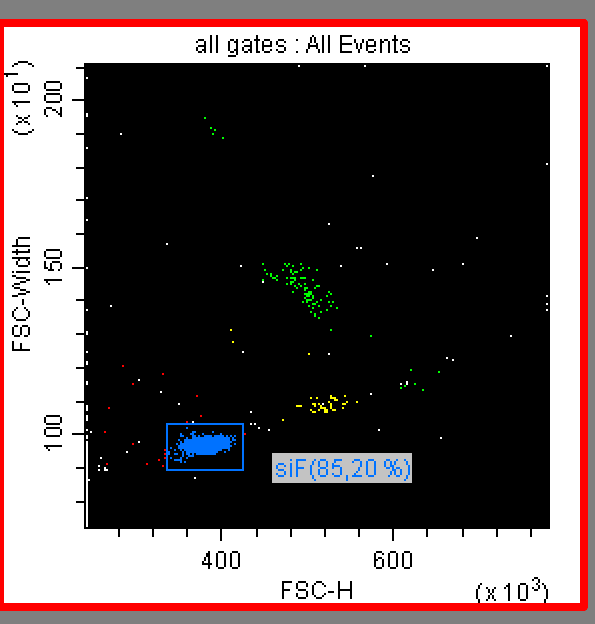
In the example above (beads of same size, and different fluorophores) only gate ”pop 1” fits into the conclusion of ”singlets” ”Pop2” and ”pop 3” are either doublets and agreggates (beads are quite sticky)

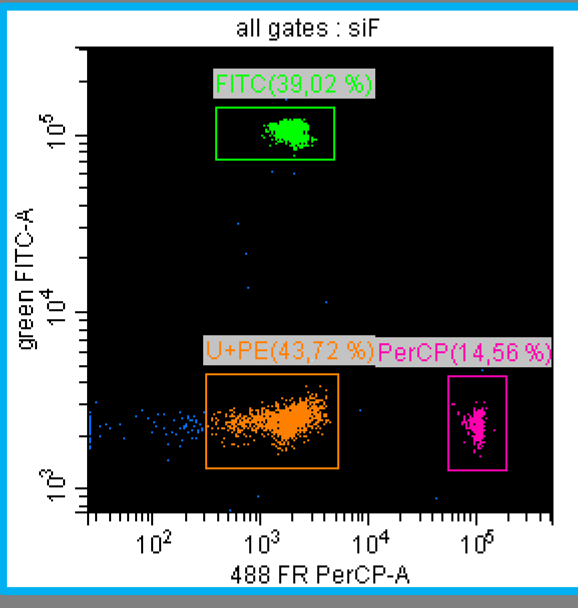
In the example above ”Pop1” is also the outstanding population, but in real experiments one is not always looking for population with the most data. The more important question is, what identifying markers there are.

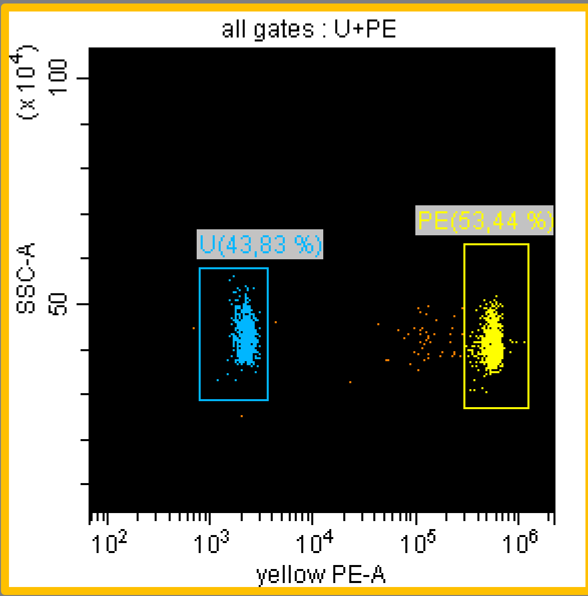
Gating – a hierarchy

Plot head line (and outline) indicates data we look/work/gate at









Task: Find the bead population of FITC-positive, PE-Positive, PerCP-posite & the unstained beads

Plot head line (and outline) indicates data we look/work/gate at

In this example other gating strategies are possible.

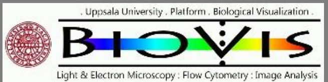
Note that the orange population contains Unstained and PE beads

To put data in relation use % parent not % total, as often debris and noise adds up to total

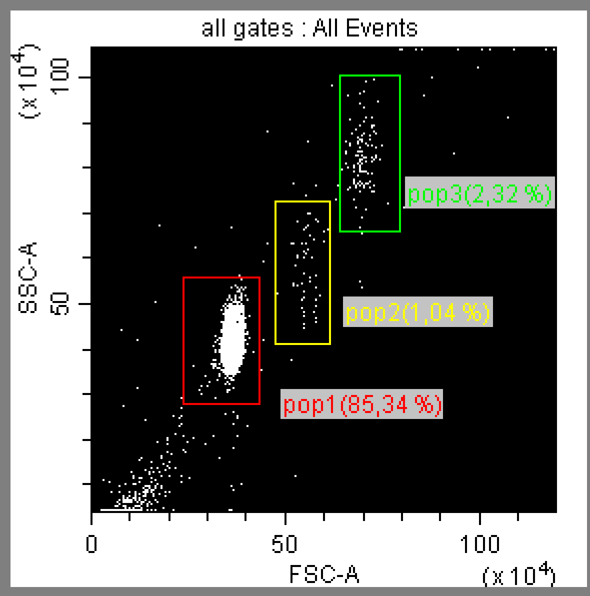
Tube Name: all gates

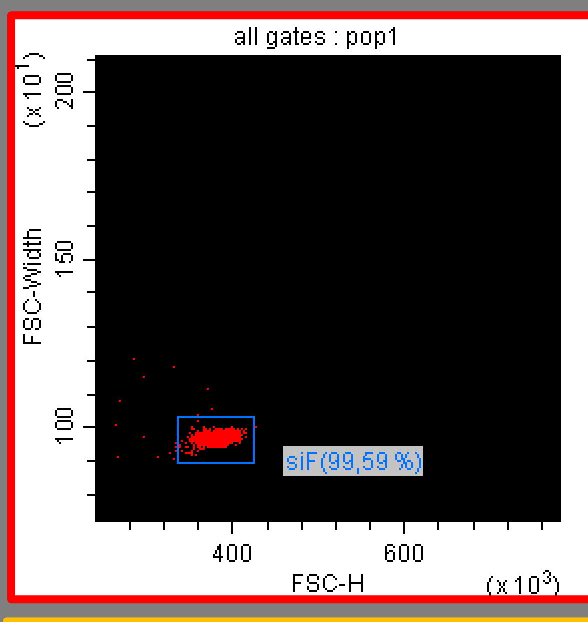
Sample ID:

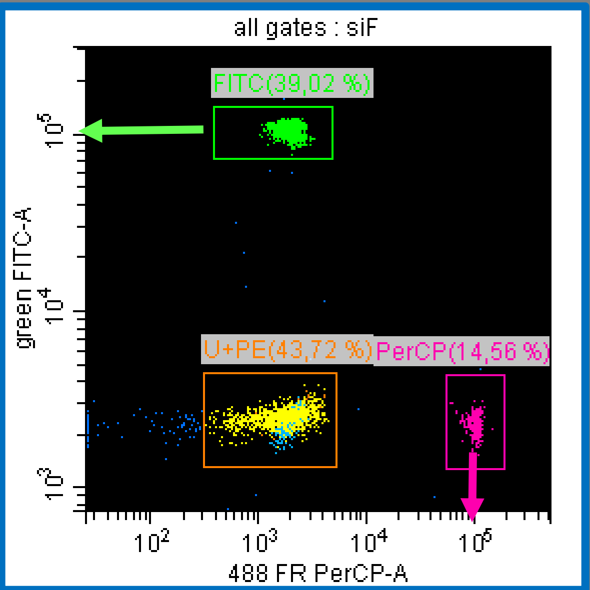
Population	Events	% Total	% Parent
All Events	4918	100,00 %	100,00 %
pop1	4197	85,34 %	85,34 %
siF	4180	84,99 %	99,59 %
FITC	1635	33,25 %	39,11 %
PerCP	610	12,40 %	14,59 %
U+PE	1830	37,21 %	43,78 %
U	802	16,31 %	43,83 %
PE	979	19,91 %	53,50 %
pop2	51	1,04 %	1,04 %
pop3	114	2,32 %	2,32 %

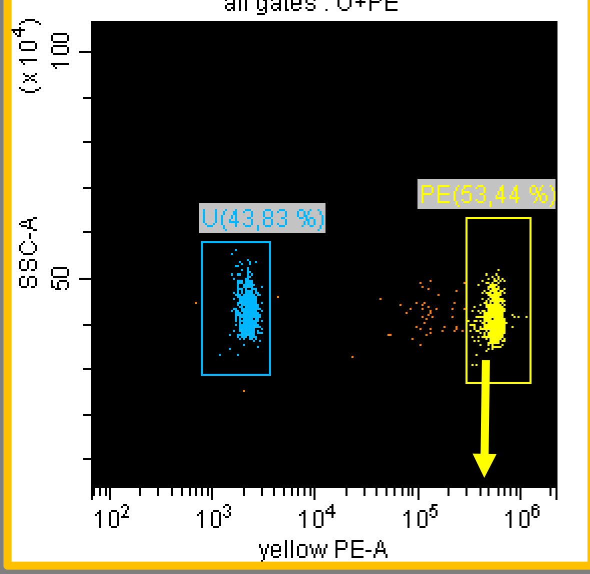


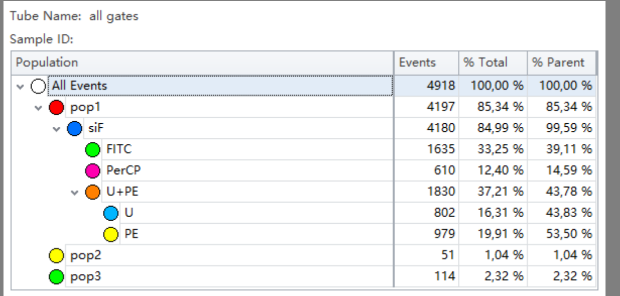
Gating: Gates, Hierarchy numbers and statistics









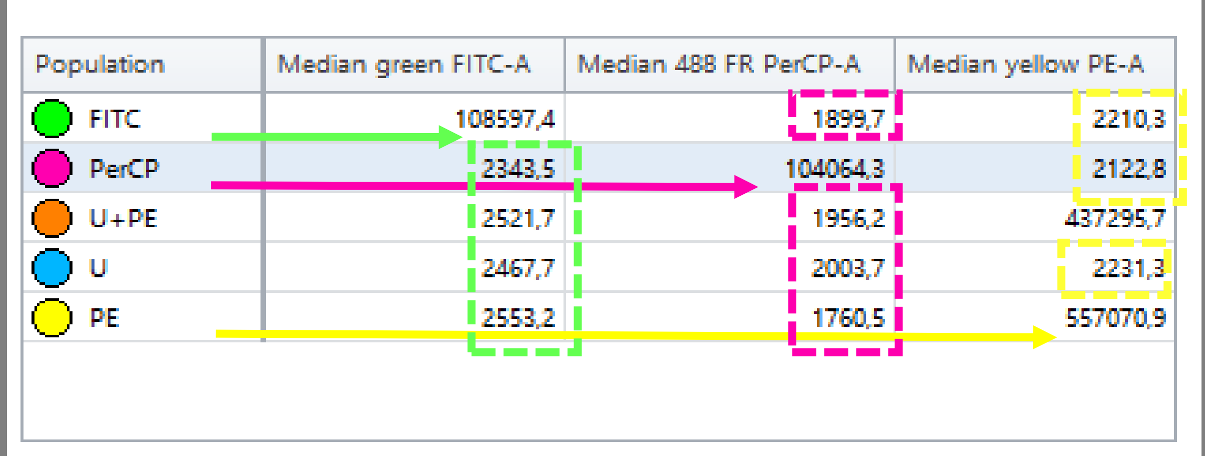


Tube Name: all gates

Sample ID:

Population	Events	% Total	% Parent
All Events	4918	100,00 %	100,00 %
pop1	4197	85,34 %	85,34 %
siF	4180	84,99 %	99,59 %
FITC	1635	33,25 %	39,11 %
PerCP	610	12,40 %	14,59 %
U+PE	1830	37,21 %	43,78 %
U	802	16,31 %	43,83 %
PE	979	19,91 %	53,50 %
pop2	51	1,04 %	1,04 %
pop3	114	2,32 %	2,32 %

Beside hierrchy and parental %-Statistical valuea available based on Intensities:
min, max, median, mean,
CV, rCV, SD, rSD ...



Population	Median green FITC-A	Median 488 FR PerCP-A	Median yellow PE-A
FITC	108597,4	1899,7	2210,3
PerCP	2343,5	104064,3	2122,8
U+PE	2521,7	1956,2	437295,7
U	2467,7	2003,7	2231,3
PE	2553,2	1760,5	557070,9

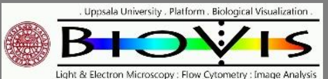
Example here shows beads which are singled stained (or not at all)

FITC pop shows high median FITC but low median in other channels

PerCP pop shows high median PerCP but low median in other channels

PE pop shows high median PE but low median in other channels

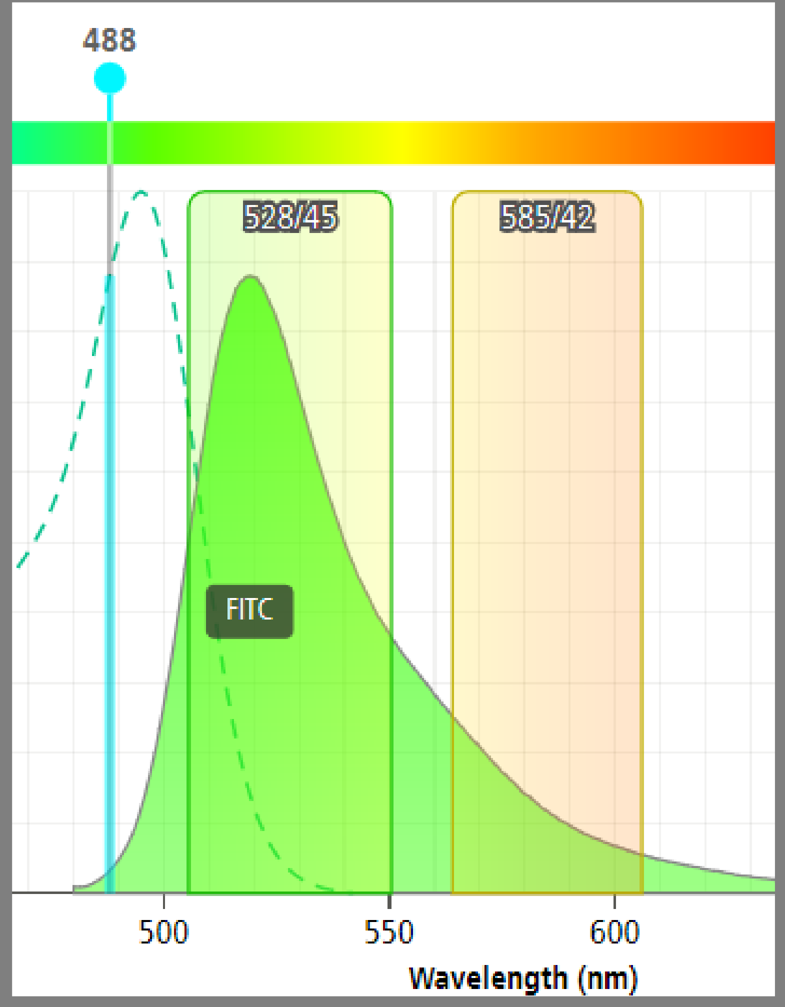
Beads not stained for the respective channel show similar median values.
(if compensated correctly)

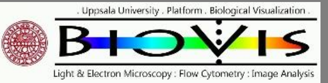


Compensation, or Green is orange

The following slides will presume that FITC and PE will be recorded Under the same laser : 488 nm. Most instruments will record PE Under 562nm Laser

The principle of compensation are easier to teach by making this assumption.





Bleeding Through : Compensation

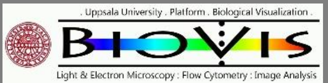
Staining: FITC & PE, 488 excitation, two detectors: 528/45 & 585/42

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

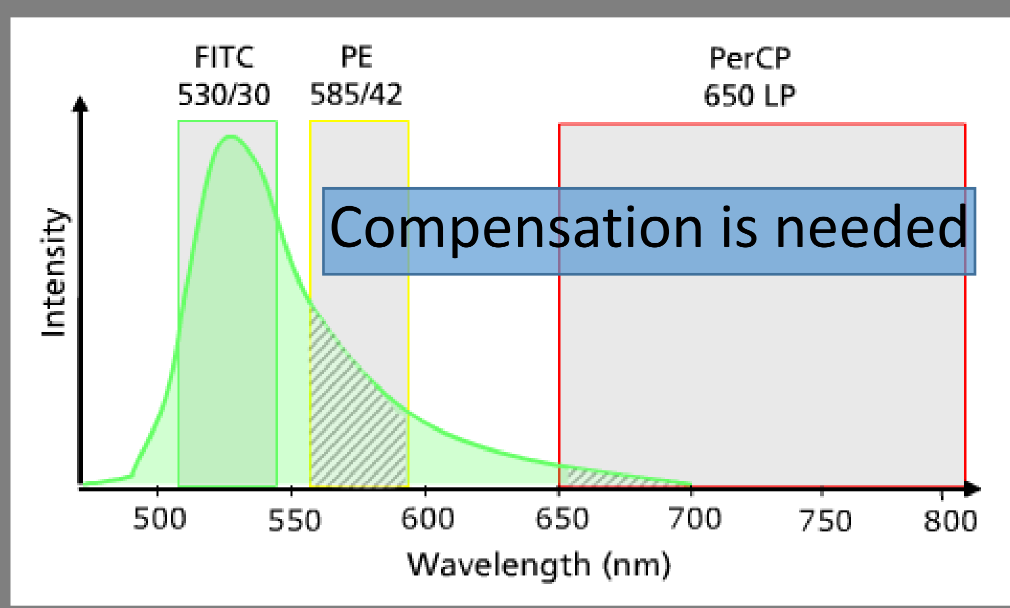
488 excitation/emission
in FITC stained sample:
Record: FITC
Record: PE

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

Compensation will substract these ”adds”



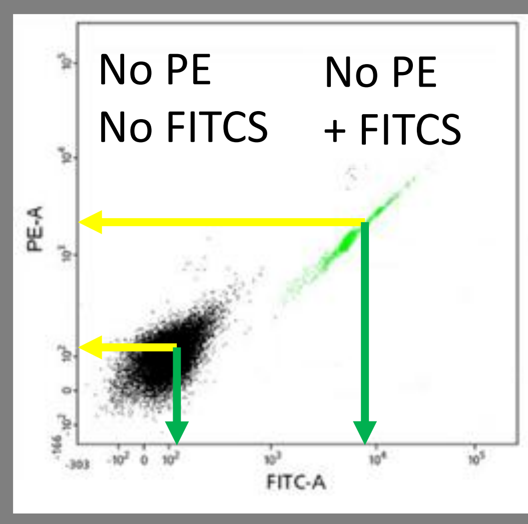
Bleeding Through : Compensation



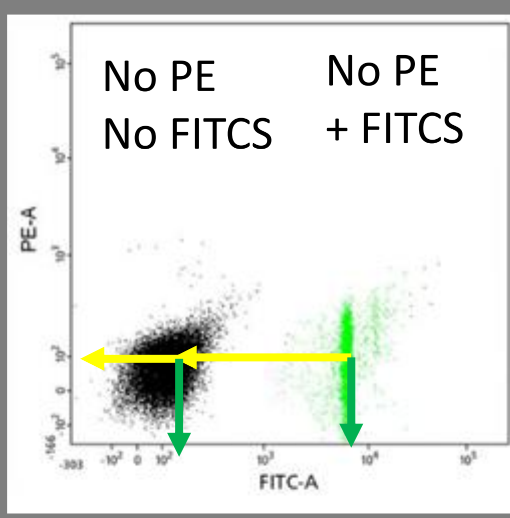
Bleeding through of FITC into PE / PerCP channels

- 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

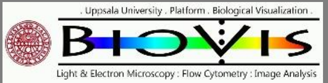


uncompensated



compensated

- Single stains can be done using compensation beads, which will bind to the antibody used



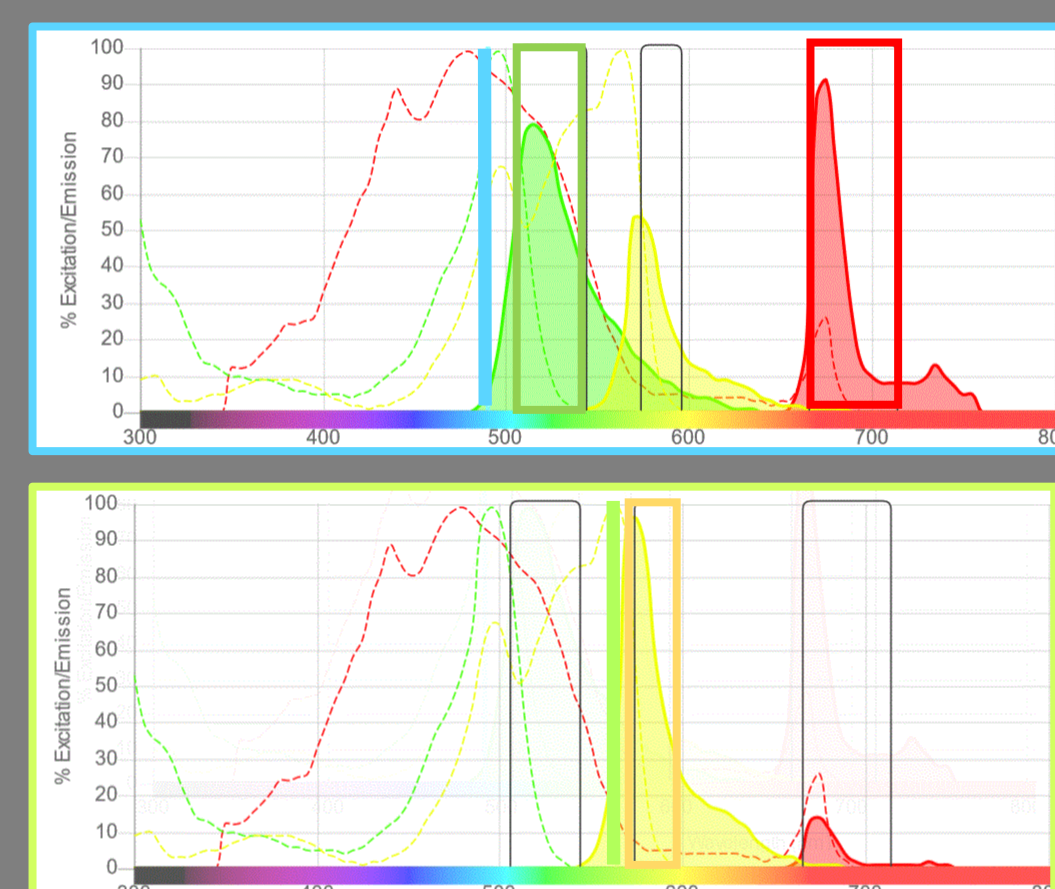
Understanding compensation

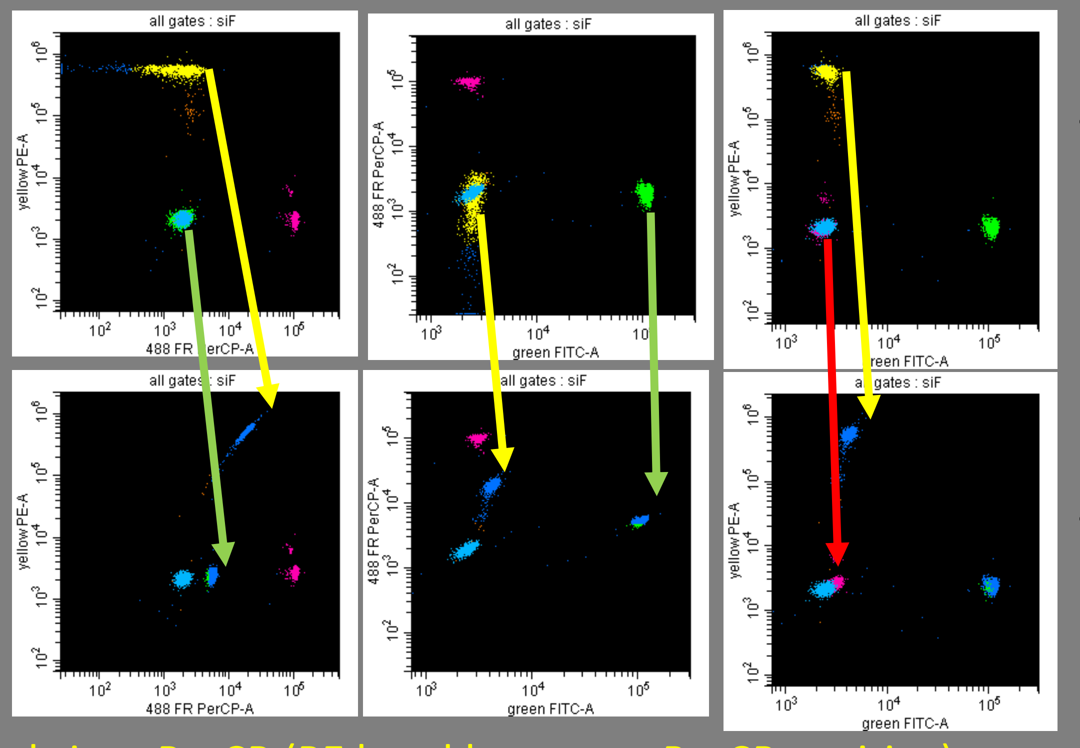
Spectra viewer with fluorophores of **FITC**, **PerCP** and **PE**
A) Instrument records FITC and PerCP under **488** (not PE)
B) Instrument records PE under **562**nm Laser (not FITC, nor PerCP)

Plots below show all color-combining plots
Compensated vs uncompensated
of single stained beads (FITC, PerCP, PE, unstained)

A)

B)

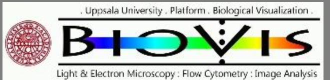




PE bleeds into PerCP (PE bead becomes PerCP positive)

FITC bleeds into PerCP (PE bead becomes PerCP positive)

PerCP, becomes slighty FITC and PE positive

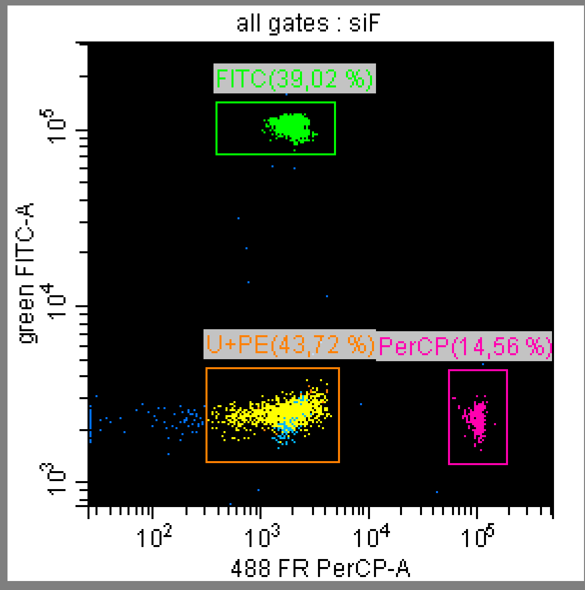


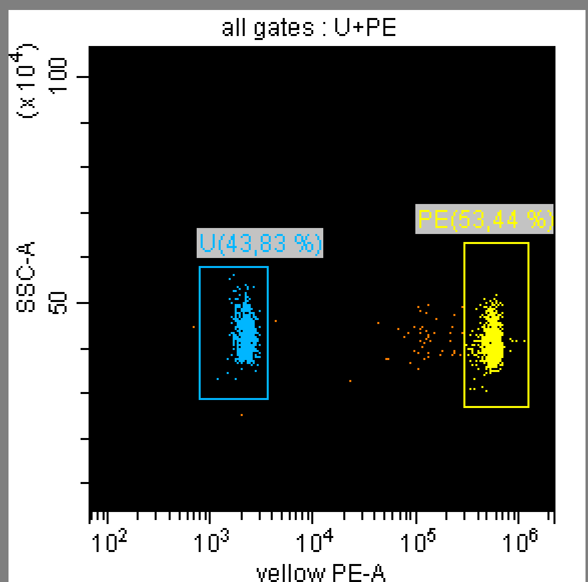
Importance of compensation

<u>Compensated data in comparision to uncompensated</u>

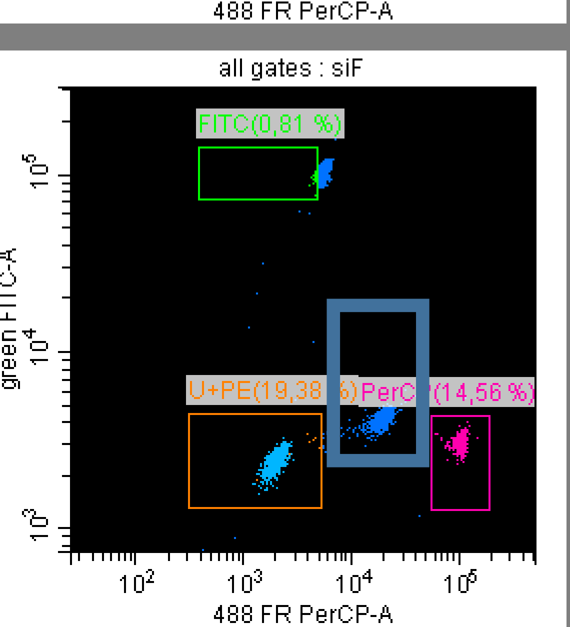
Uncompensated data

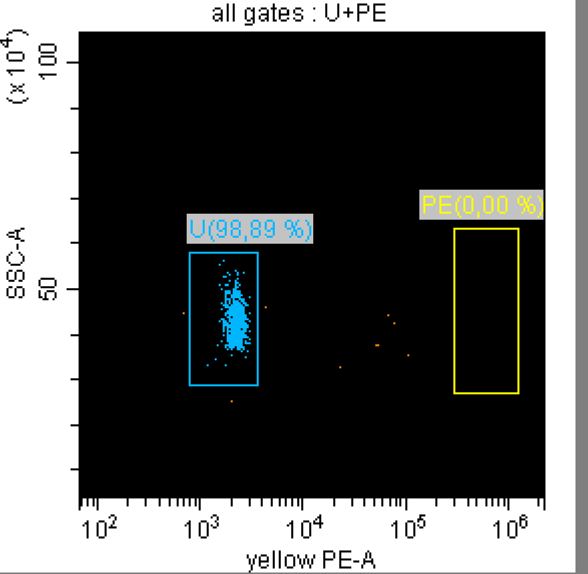
- shows a false ”mid PerCP” population
- FITC population has a false positive PerCP value
- Loss of PE in gating

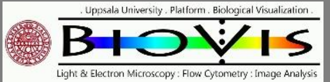




Seeing the lower plot one most likely is inclined to shift gates (the green gate) or even make a new one for the medium strong PerCP population (which in fact is the PE)







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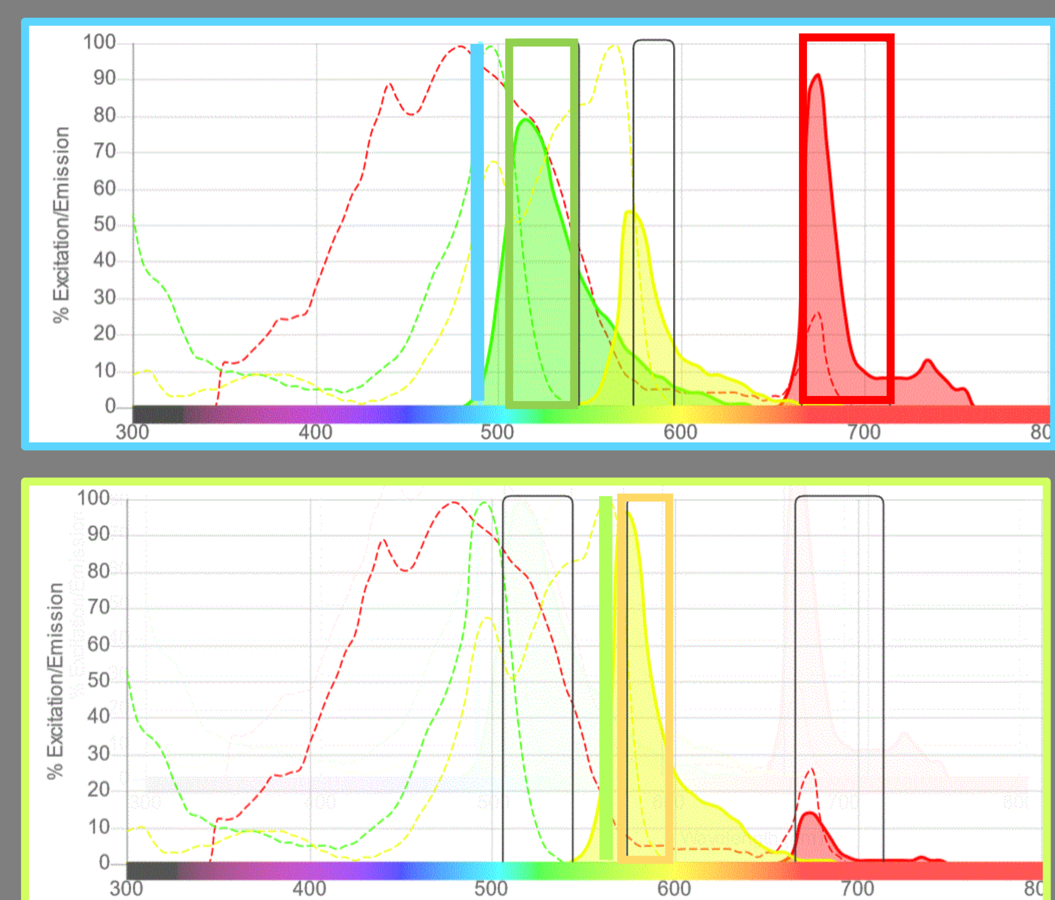
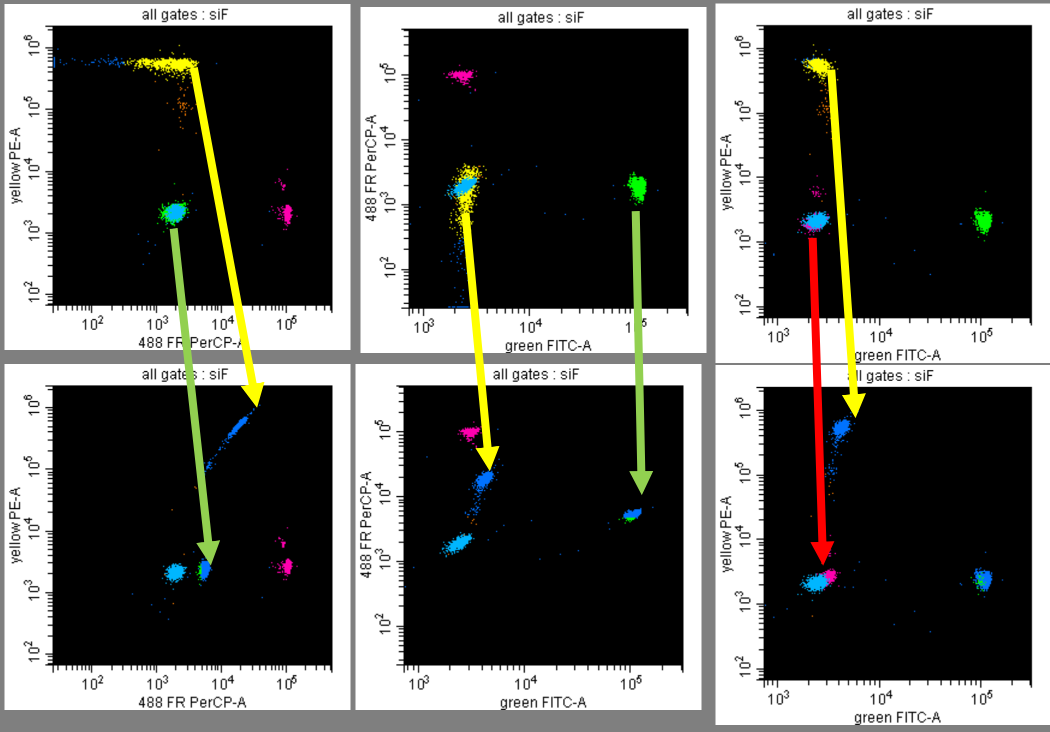
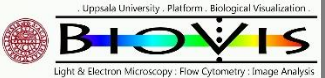






Staining the sample – some basic rules

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

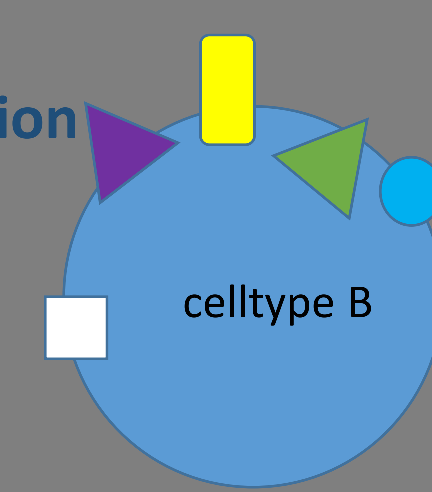
   Suitable proteinmarker on your cells of interest (and non-interest)
   Evaluate/test/ the Antibody (and Fluorophore) you want to use
   Suitable strategy to find sub-population

2. **Estimate amount of antigen you want detect on sample**

   Protein expressed in high numbers → gets a weak fluorphore
   Protein expressed in low numbers → gets a strong fluorophore

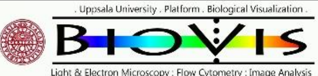
3. **Make use of the lasers and detector configuration**

   Laser 1 → marker 1, Laser 2 → marker 2 etc
   Tandem dyes,

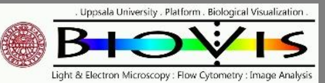


4. **Have controls**

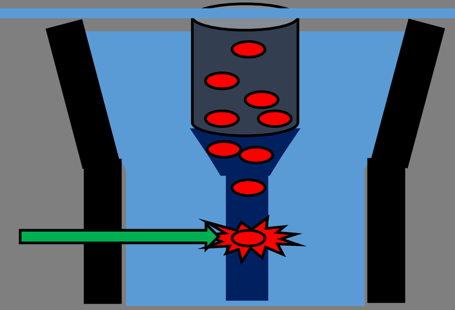
   Negative,Live/dead, compensation
   (and FMOs)

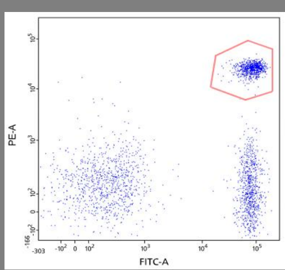


Sorting (a brief overview to avoid another hour of lecture)

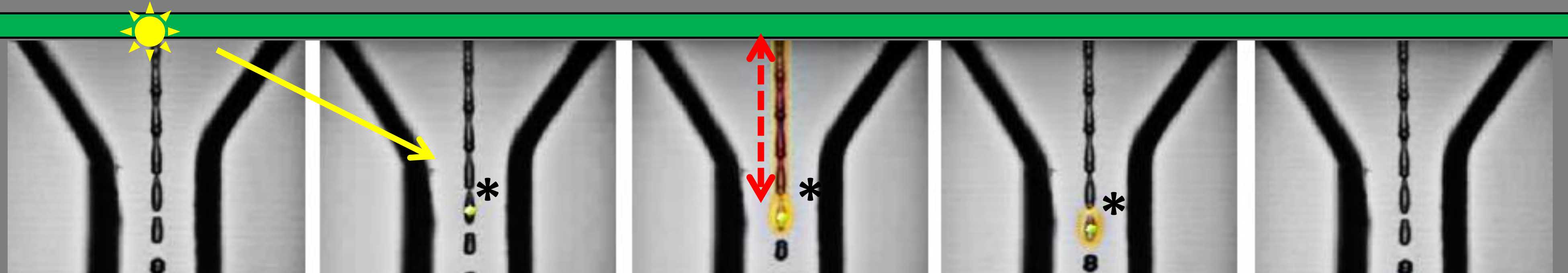


Sorting

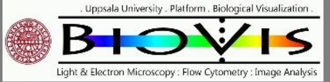




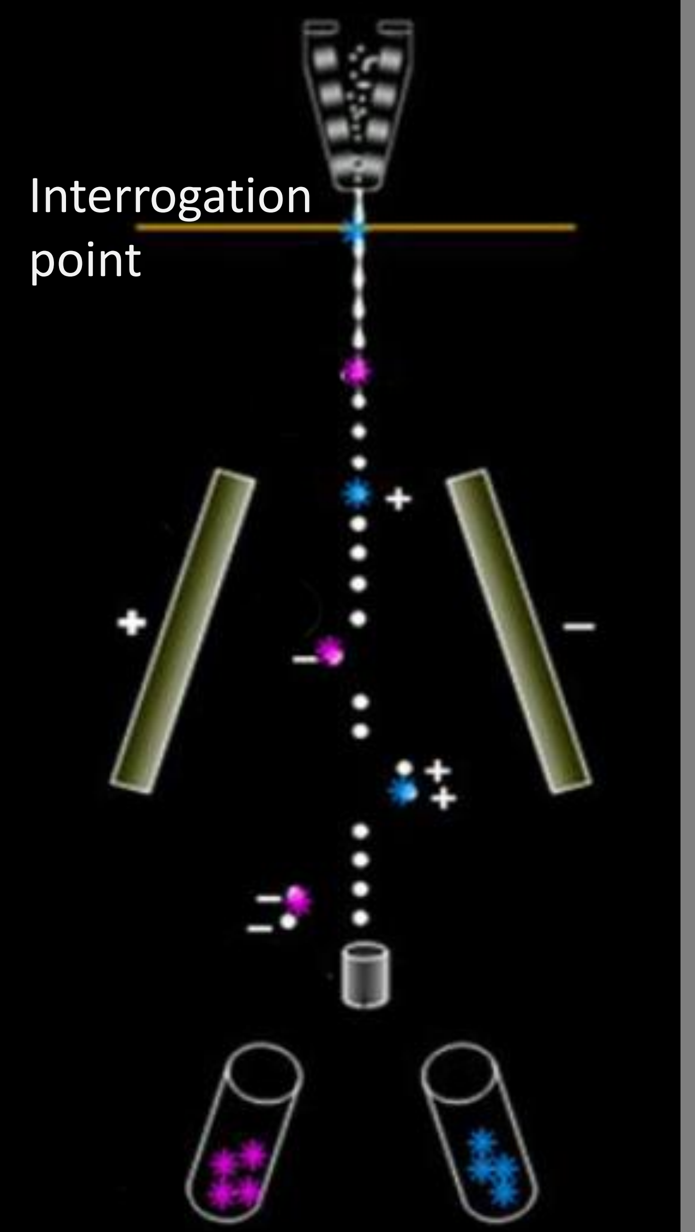
Prerequisite to sort are:
Flow cytometry data & gates
Performance check and drop delay



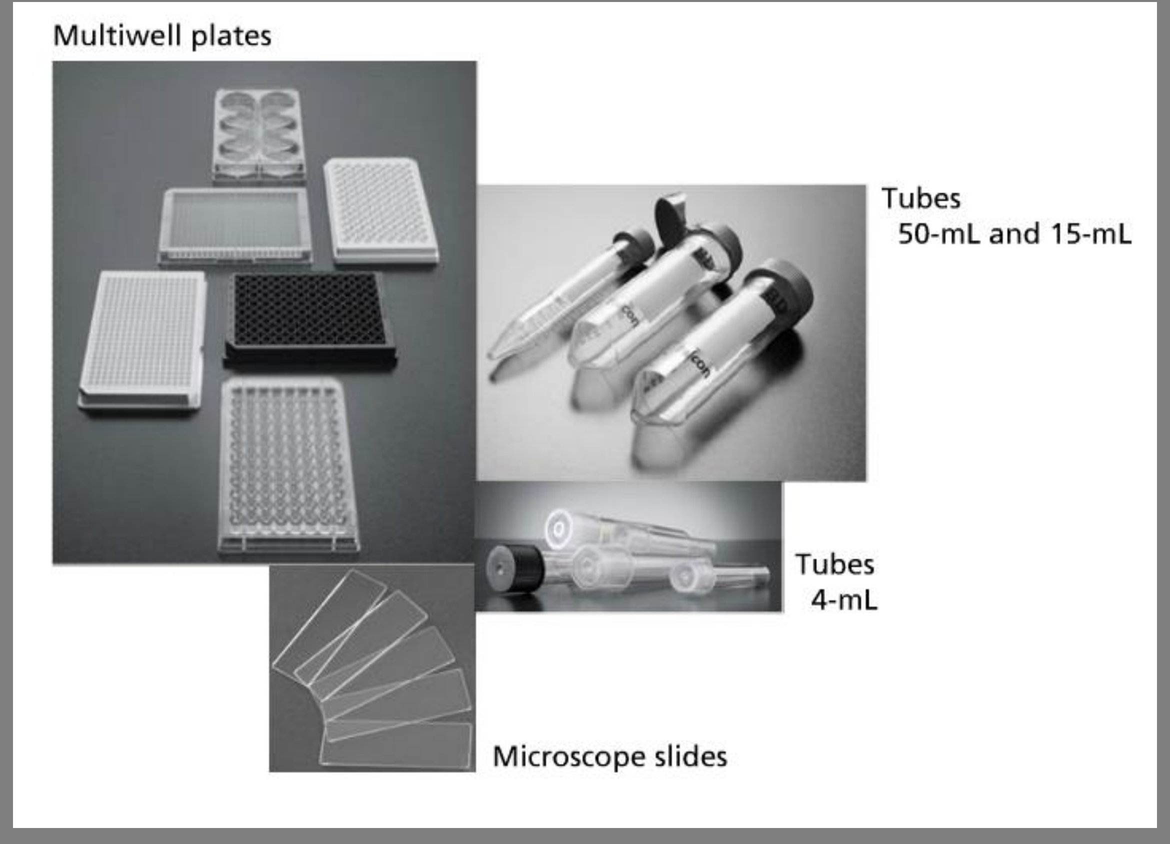
1) Stream vibrates, resulting in defined droplets,
2) cell which belongs to population to be sorted reaches break-off point*
3) whole stream gets charged
4) Cell is beyond break-off point in its charged drop and stream gets decharged rapidly, ready for another drop to be sorted
5) cell in its charged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

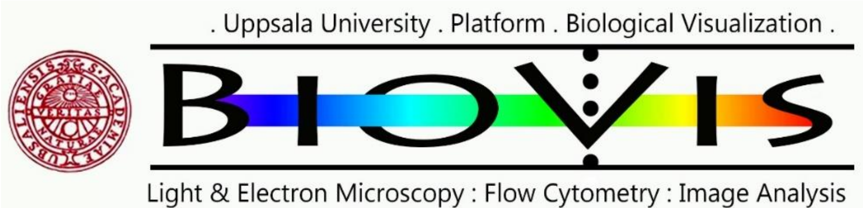


Sorting



- 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

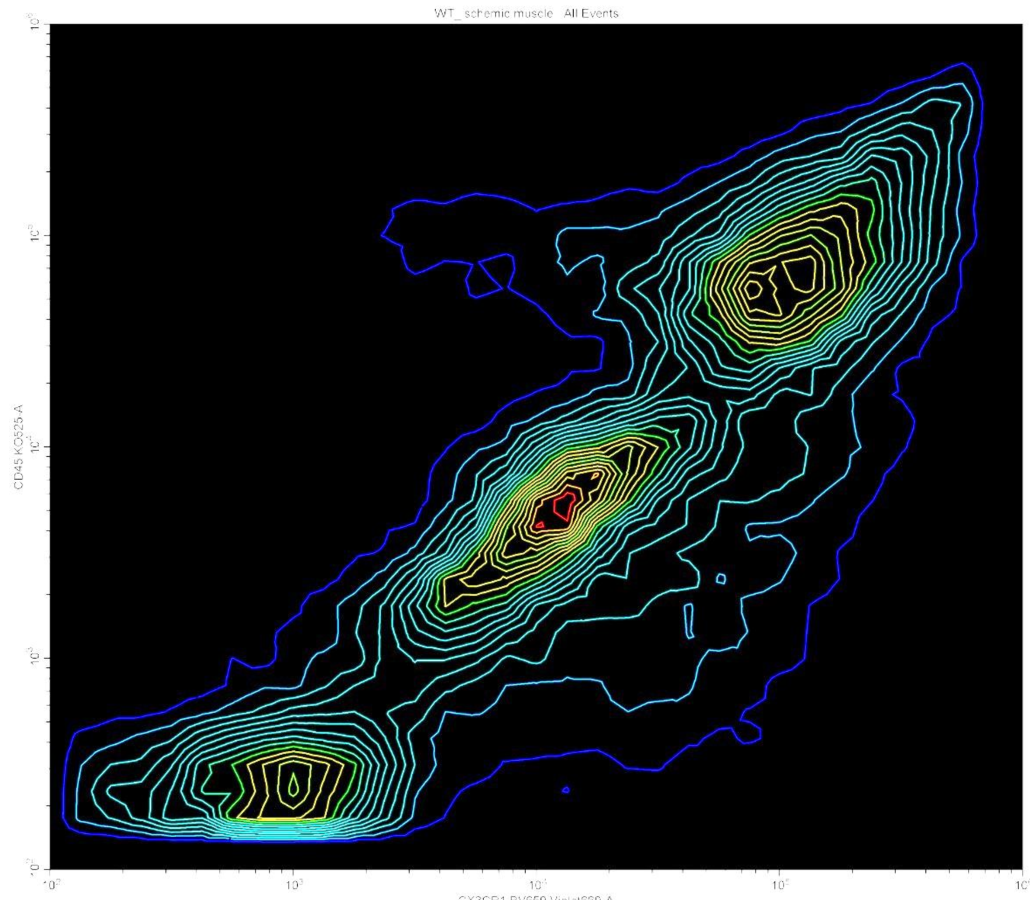


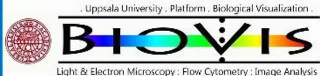


Flow Cytometry

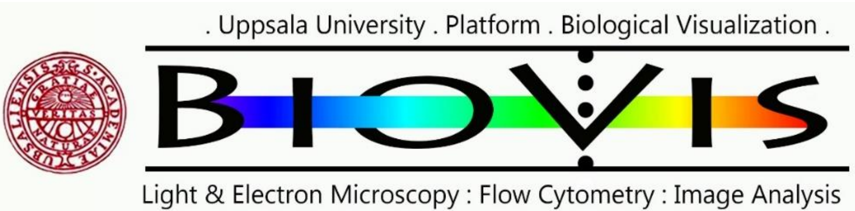
End part 1

Dirk Pacholsky





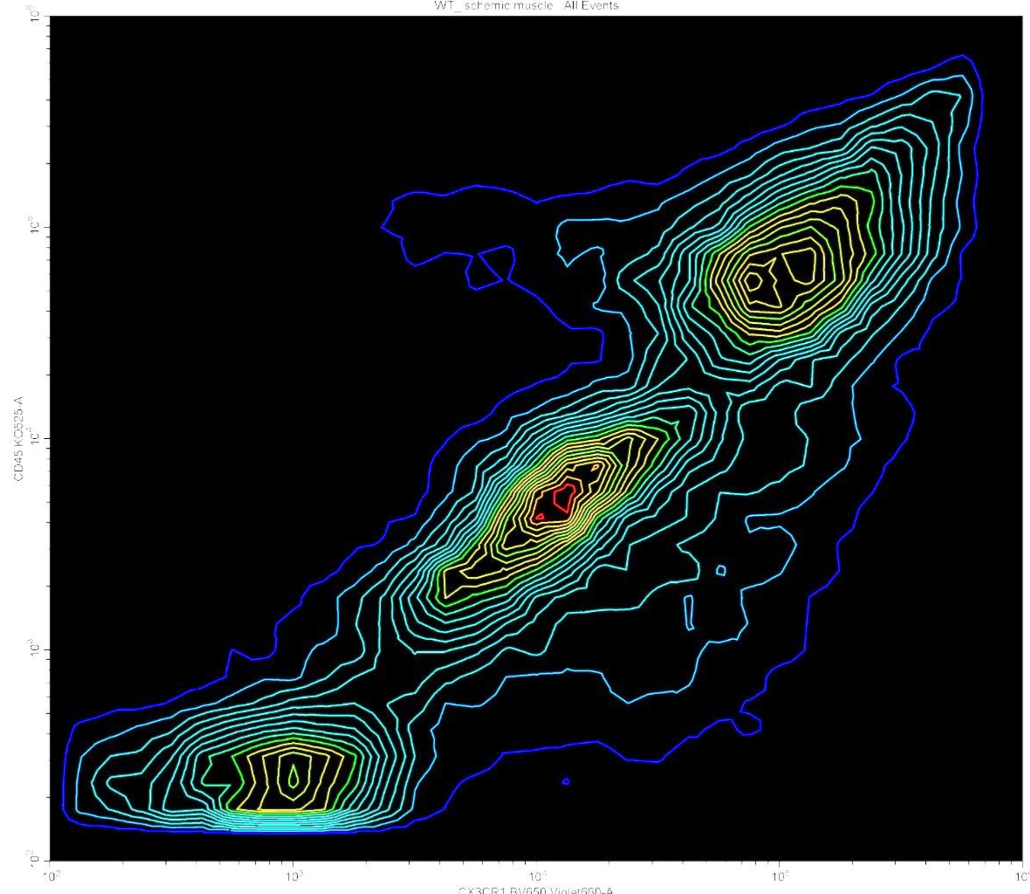
Many aspects of Flow Cytometry are not mentioned in this lecture, more more details in part 2

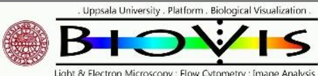


Flow Cytometry

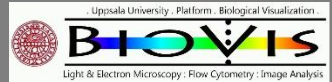
Pt 2/2 : some important details

Dirk Pacholsky





Forward Scatter (and size) and Location of the marker



FSC /RI/Size

size

RI

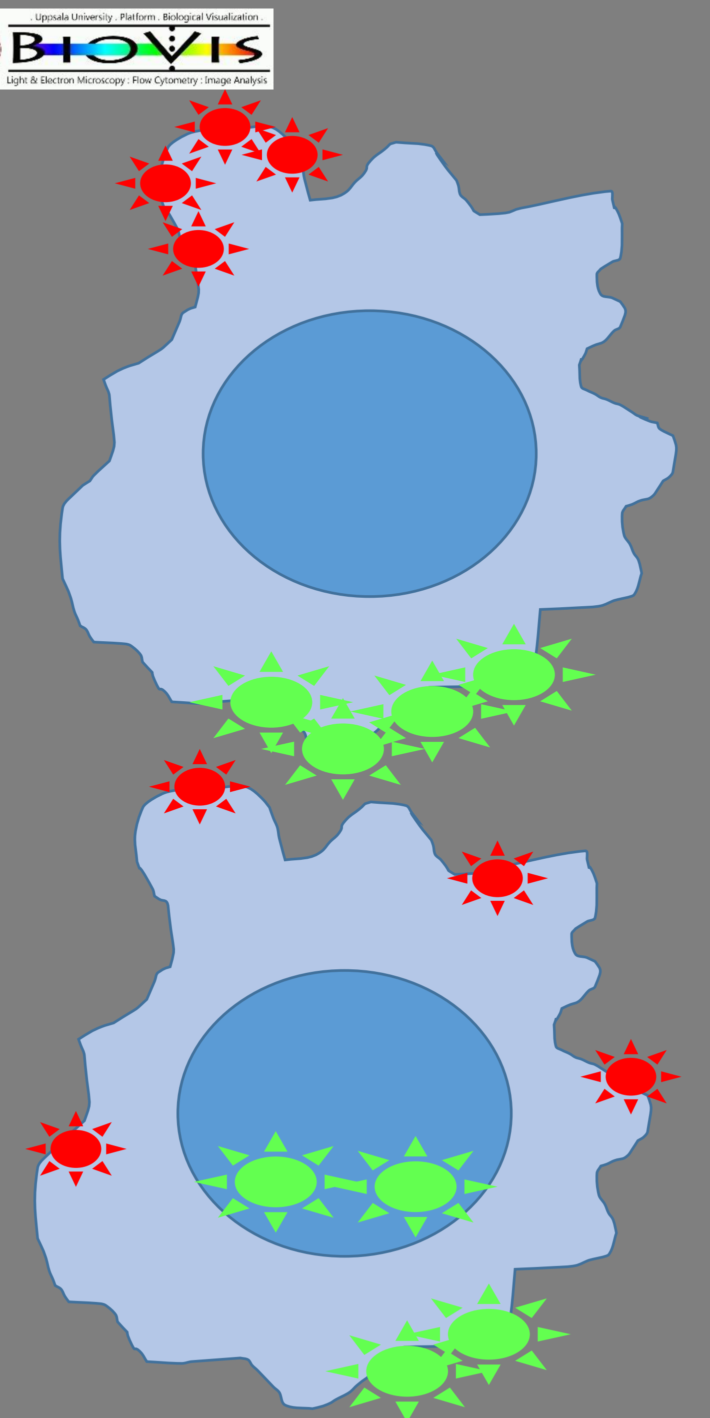
FSC

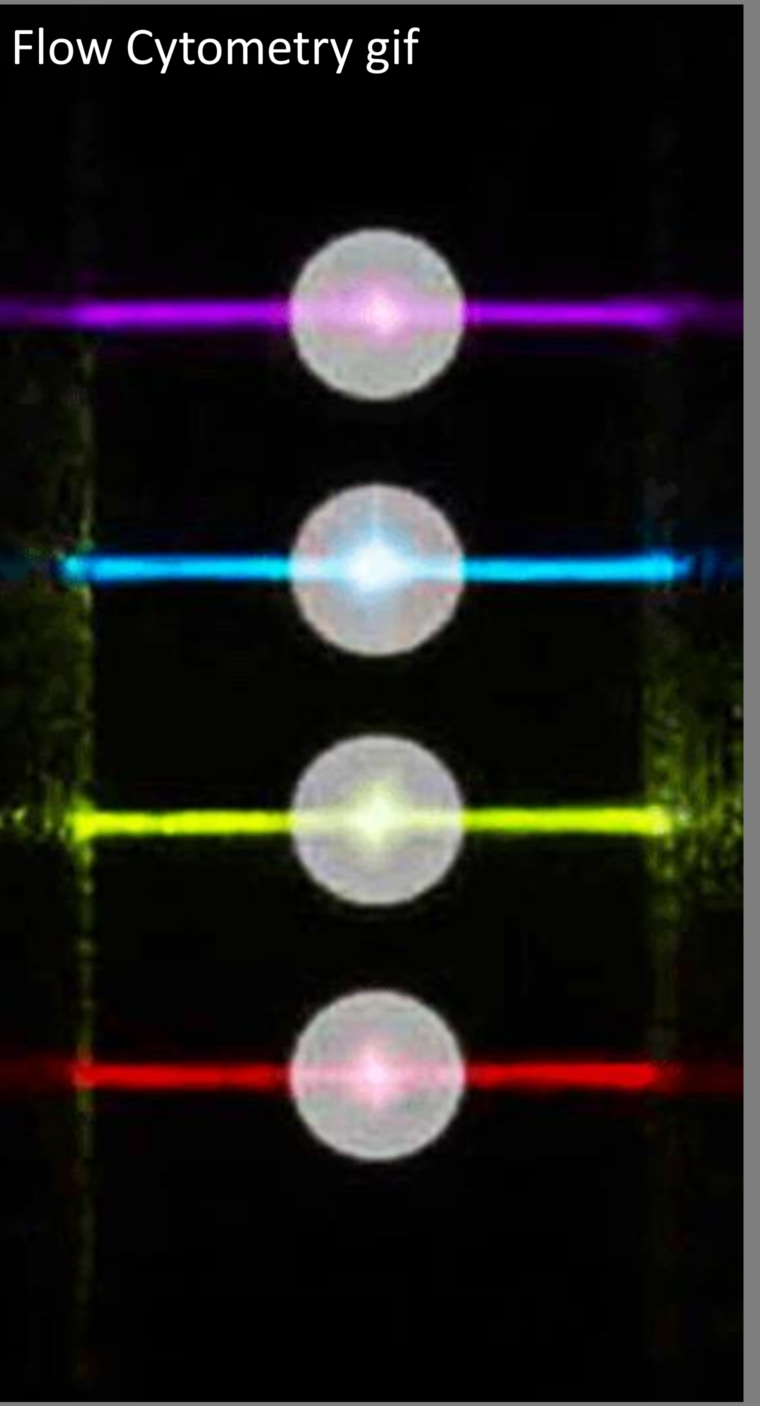
The Forward Scatter (FSC) is influenced by the size of The object AND the refractive Index

Size

Refractive Index

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



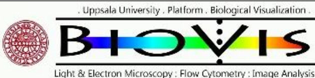


NO Images

NOTE :
The detection in a Flow Cytometer does NOT generate images
Only the detection of wavelength ranges & Intensities

There is no detecion of WHERE the fluorescence comes from within the cell

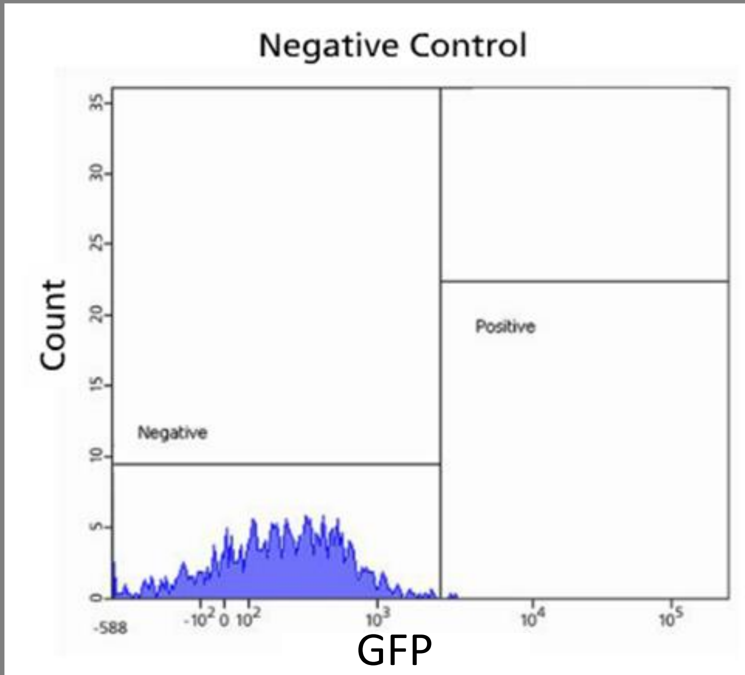
...cell membrane versus internalization...
...polarized versus even distribution...



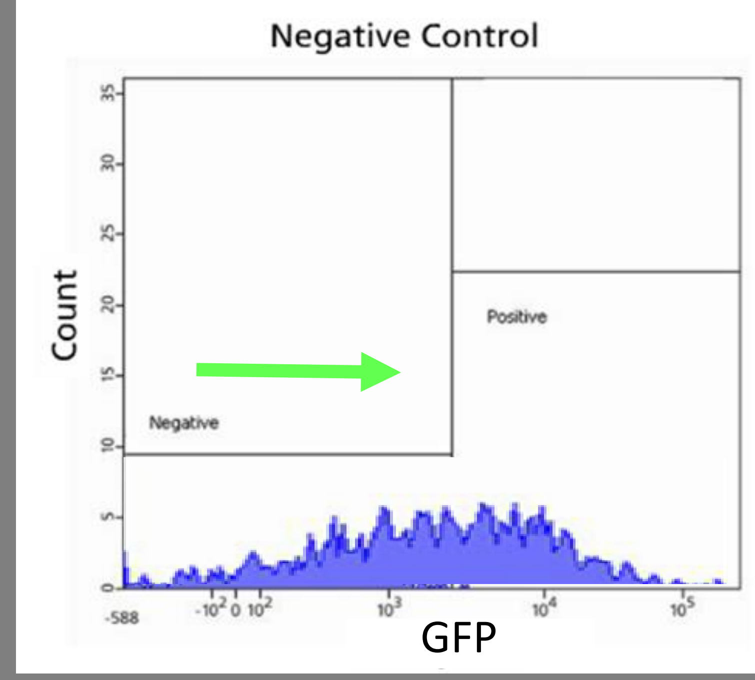
Detector values and its effects

Detector values and its effects

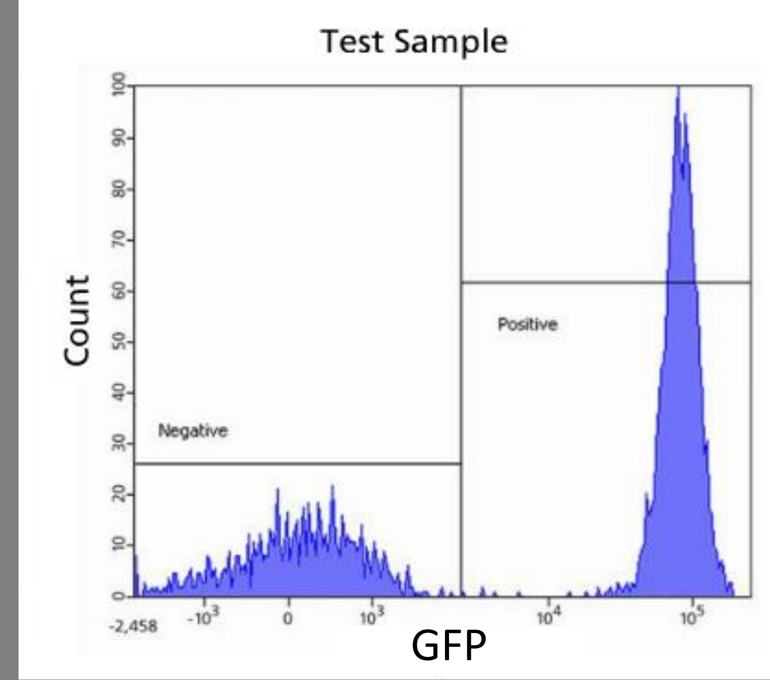
- Negative and Positive samples often needed to identify truly negative/positive populations
- Place negative population between 0 to 10^3 or 10^4 depending on instruments
- Read in data in consistent way (same settings / gates)
- Prepare sample as consistent as possible



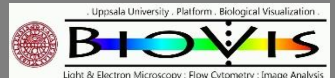
Negative sample recorded at certain detector value for GFP



Same sample as left but recorded at higher detector value for GFP



Positive sample (containing here a clear neg/pos population), recorded at same detector values as left plot

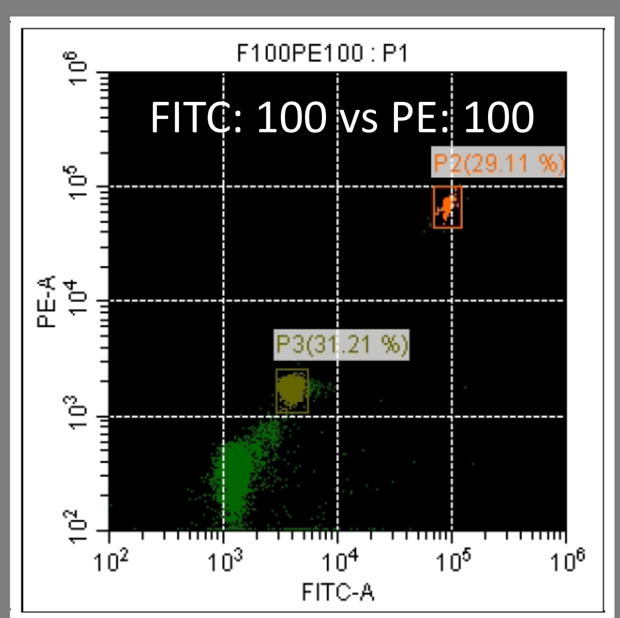


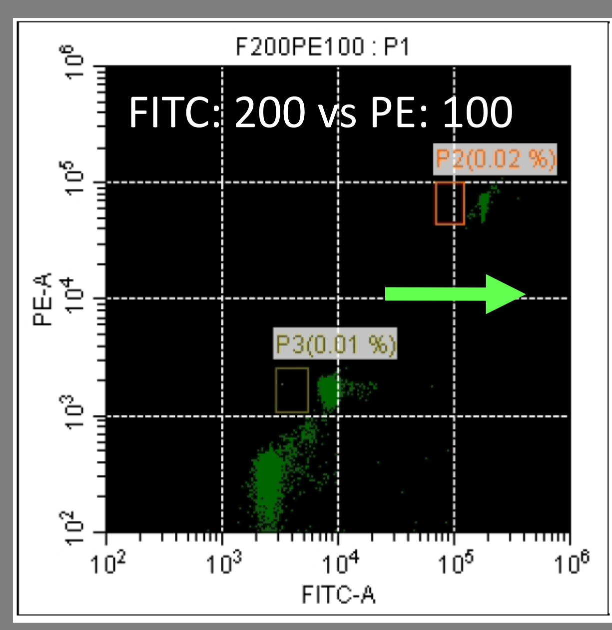
Detector values and its effects

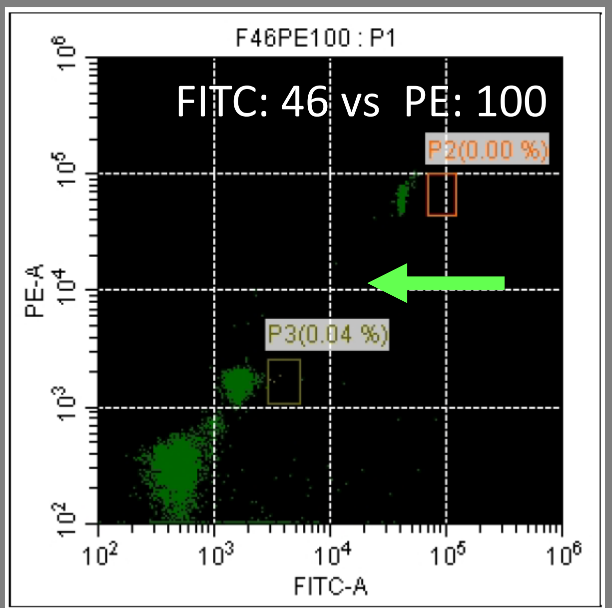
- Strenght of signal amplificiation by detector can be changed manually
- Representation of negative sample for positive or positive for negative is possible by raising /reducing detector value (gain or voltage)

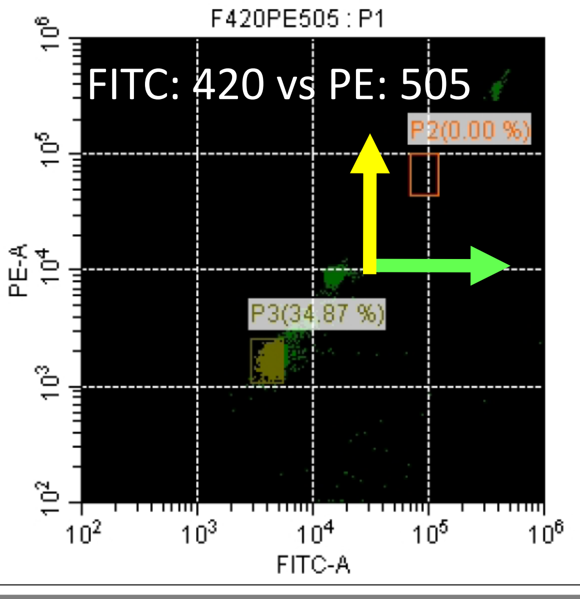
- Populations will move in relation to each other when detector values are changed

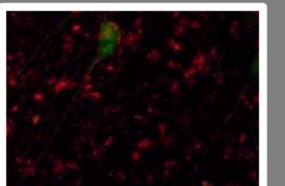
❖ Plots show beads with neg/med/high intensities in FITC and PE. Detector gains are imprinted F100 = FITC detector gain 100

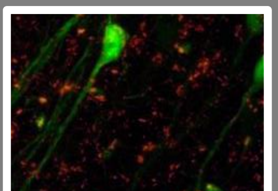




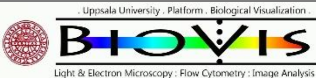




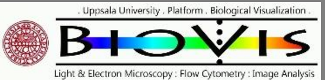




Similarity to photography: longer exposure times lead to brighter image
NOTE: in Flow this ”exposure time ” to the Laser is always the same



Where to start? : Back gating !



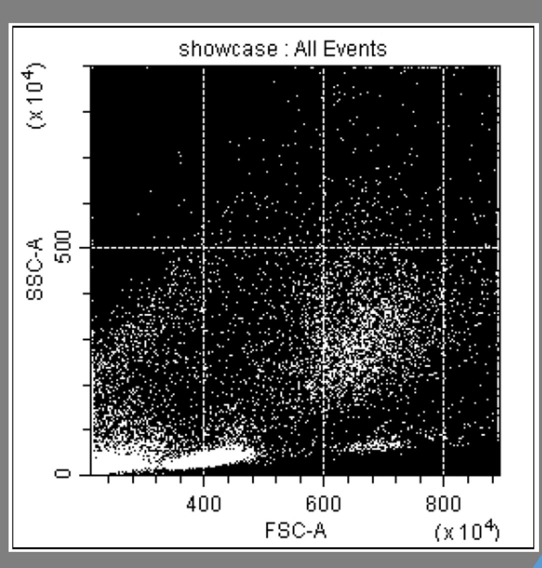
Back gating I

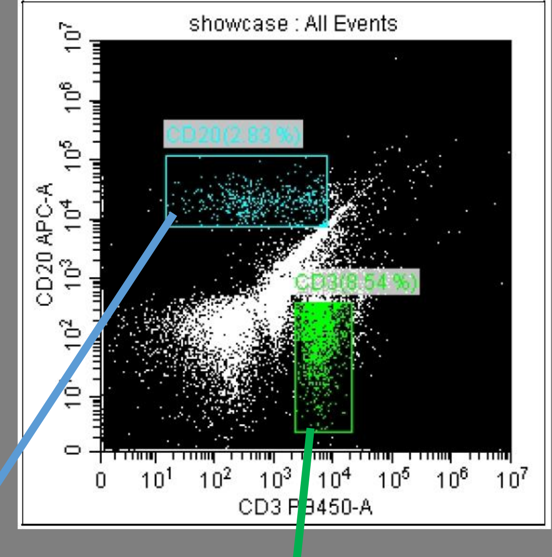
Which populationshould be gated?

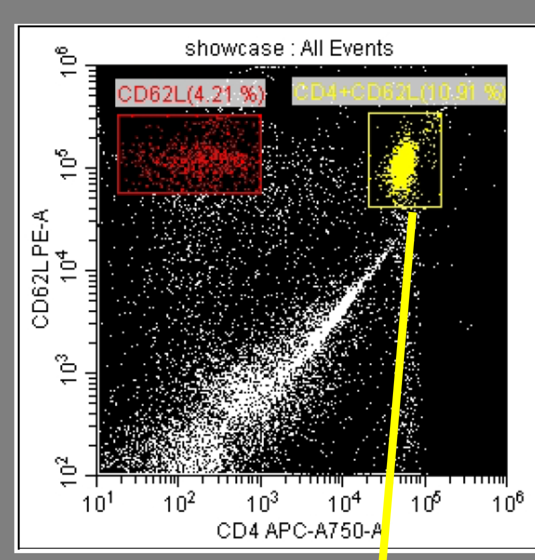
- Show ”all events” in staining plots which are important and gate the target population there

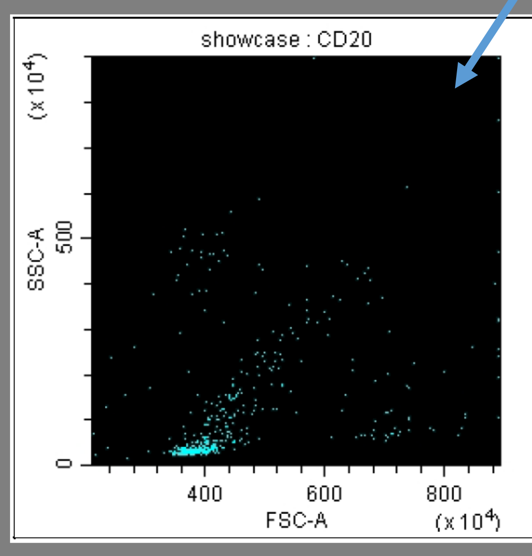
- Visualize target population in FSC/SSC plot to explore where your ”start” population lies

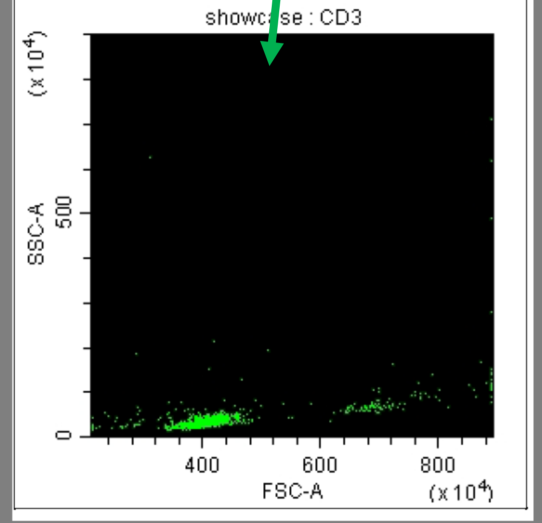
- By the way:
One does not need to start at a FSC/SSC plot either with the final gating strategy

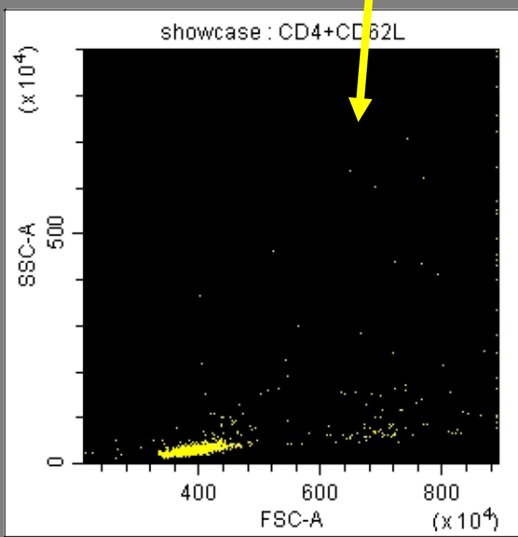


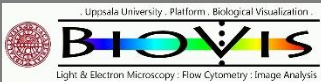






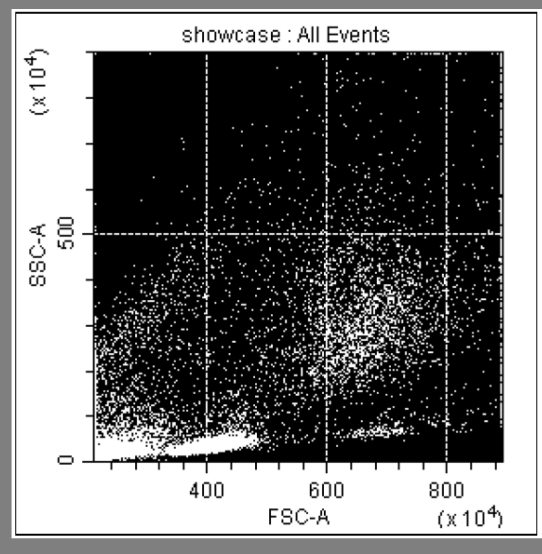


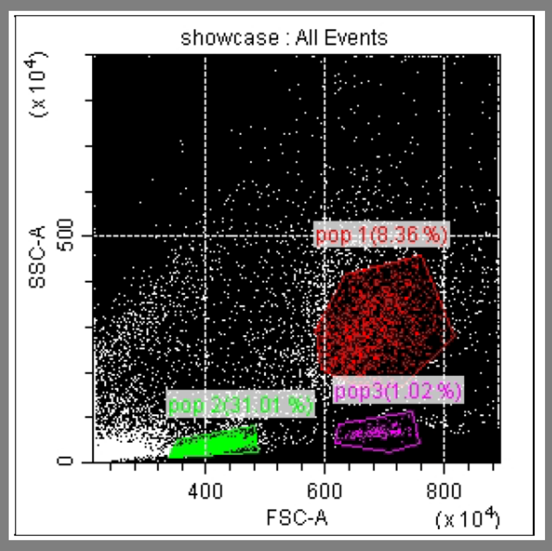




Back gating II

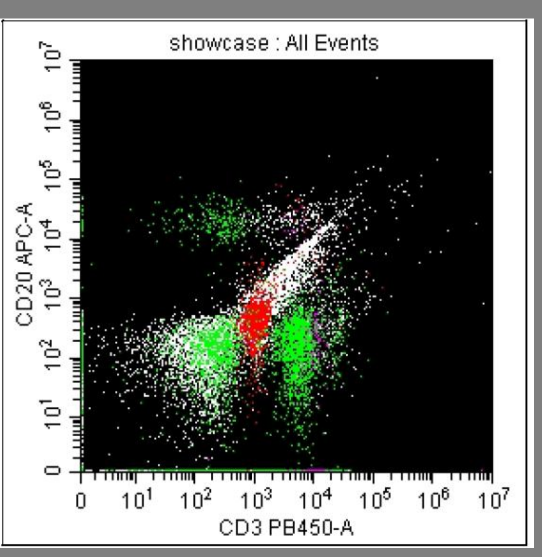
Which populationshould be gated?

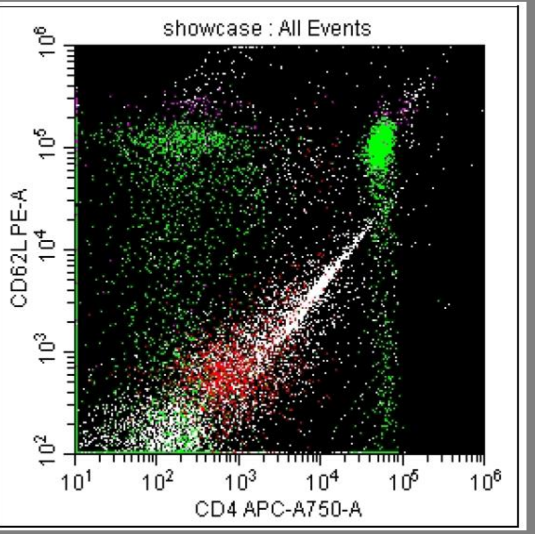


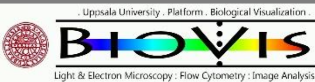


- Draw one gate into the FSC/SSC
- Show that population in the staining plots
- Move the gate around in the FSC/SSC plot and explore how the signal appear in the staining plots

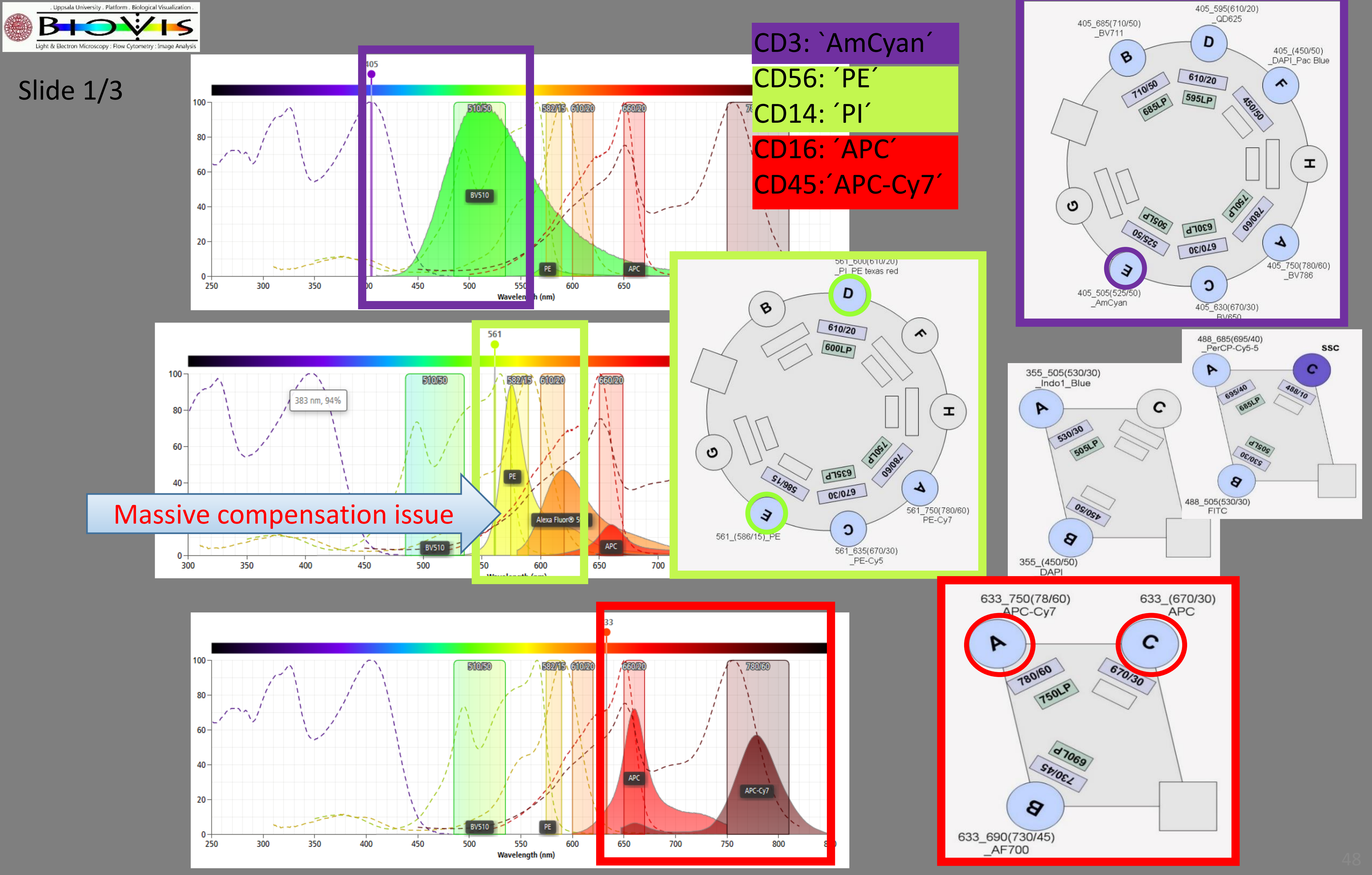
- Alternatively draw more gates in the FSC/SSC plot To visualize their respective staining in the staining plots



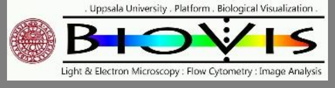




Case: compensation

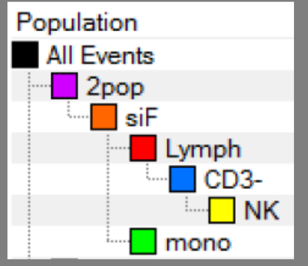


Bleeding Through : Compensation (case)



Bleeding Through : Compensation (case)

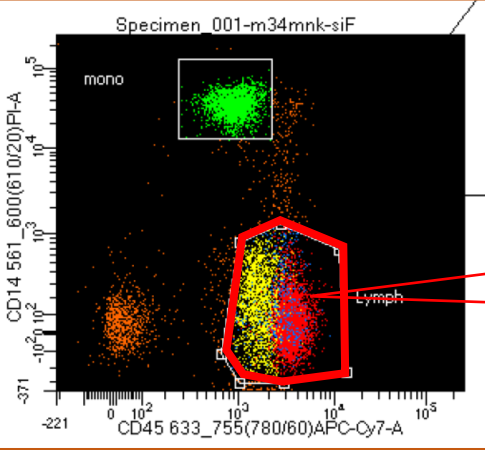
Slide 2/3

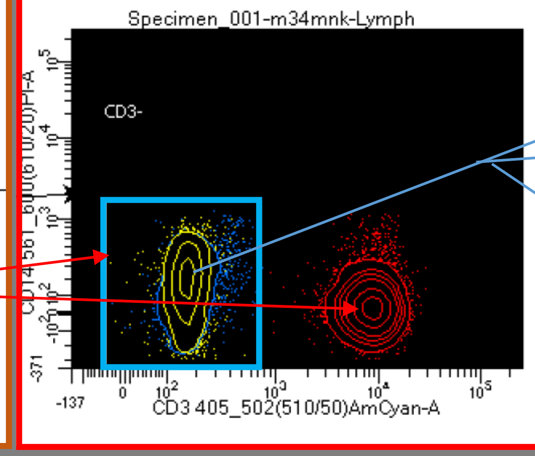


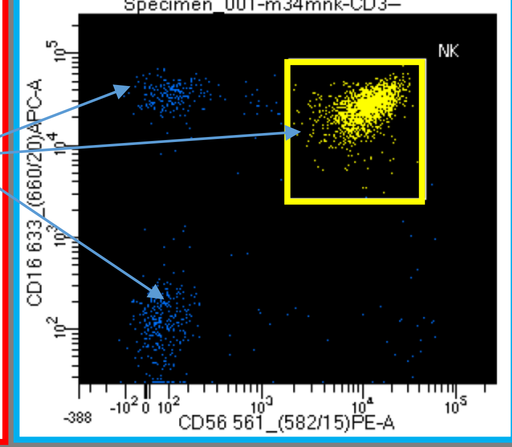
Having data uncompensated results in loss of data

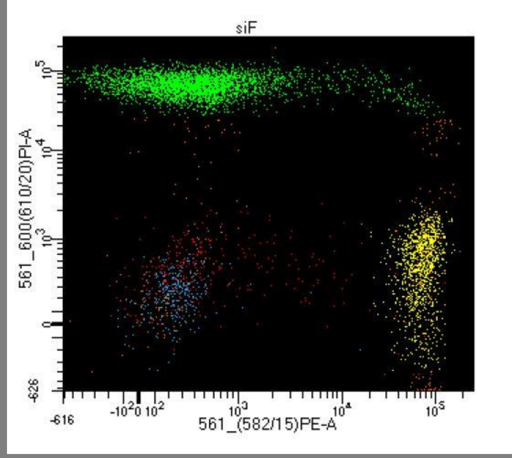
The compensation issue is not obvious as both fluorophores invollved are not plotted agains each other. In the experiment.
PE bleeding into the PI channel
Causing a false ”medium” population
In the plot APC-Cy7 vs PI.
Below are plots PE vs PI

compensated

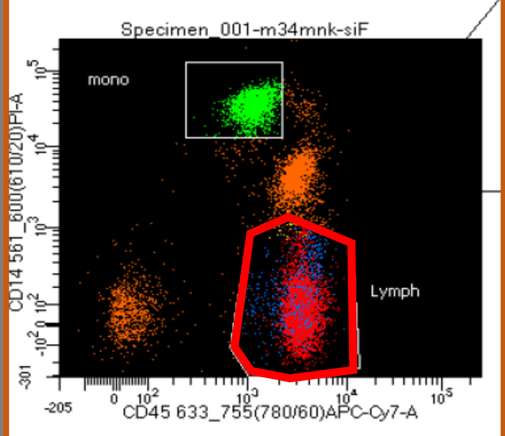


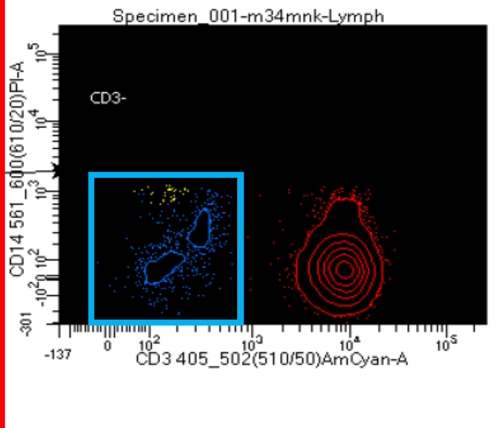


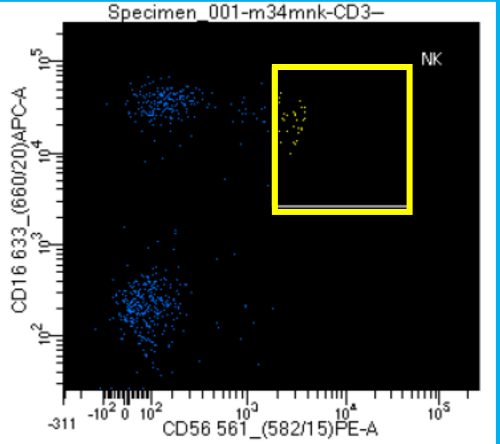


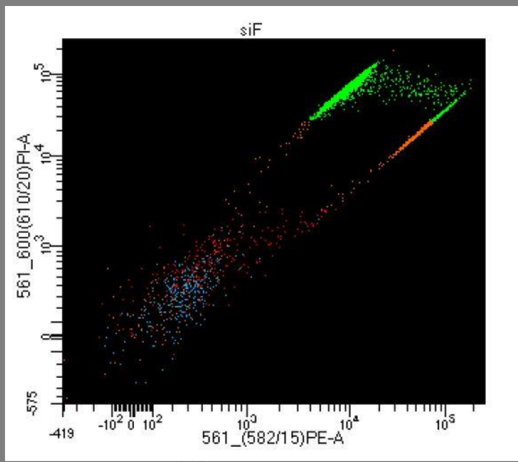


uncompensated



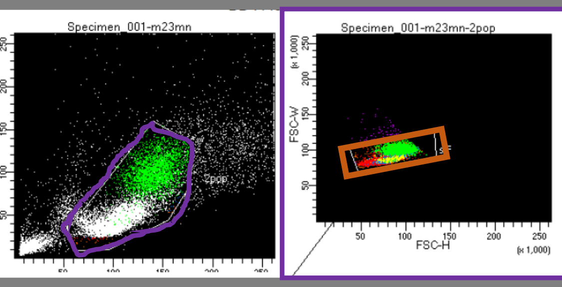


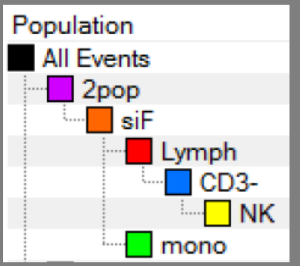




Slide 3/3

Bleeding Through : Compensation (case)

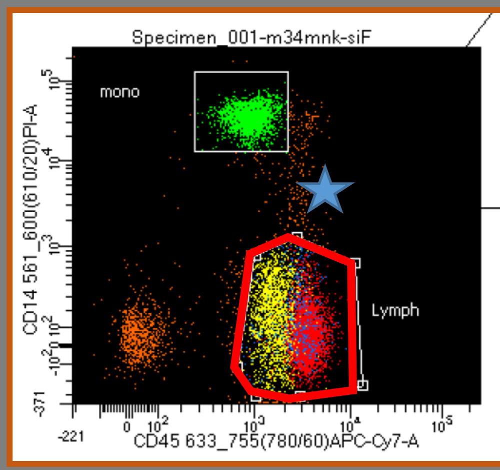


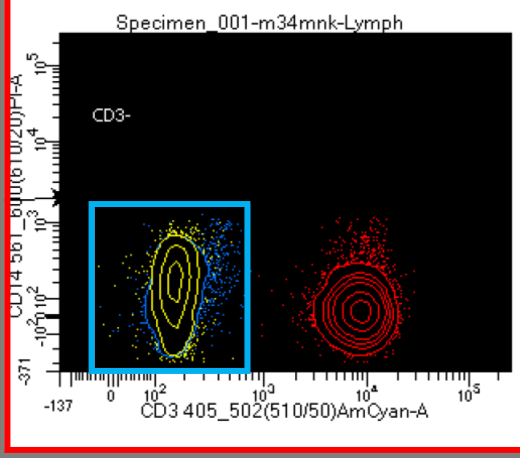


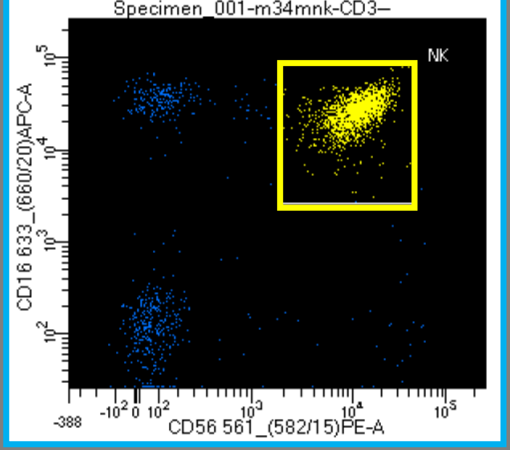
Population
- All Events
  - 2pop
    - siF
      - Lymph
        - CD3-
          - NK
      - mono

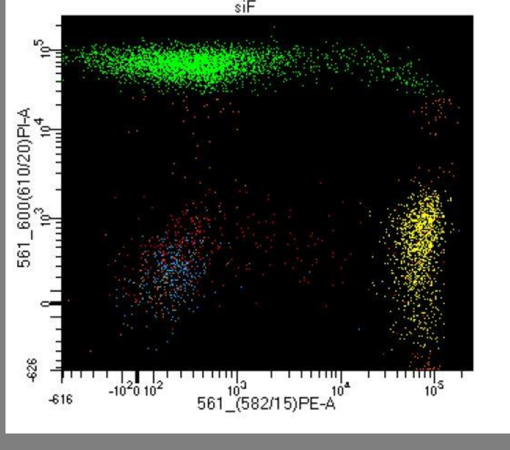
Having data uncompensated results here in loss of data (using correct gatings)
Stretching the red gate and blue gates results in recovery BUT with risk to include false positive

compensated

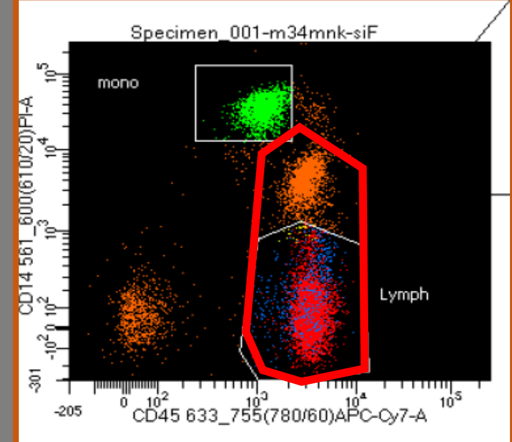


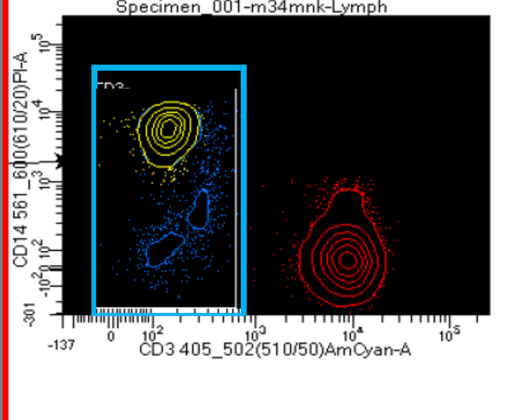


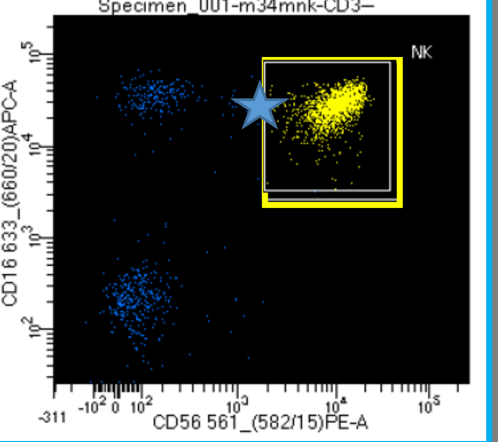


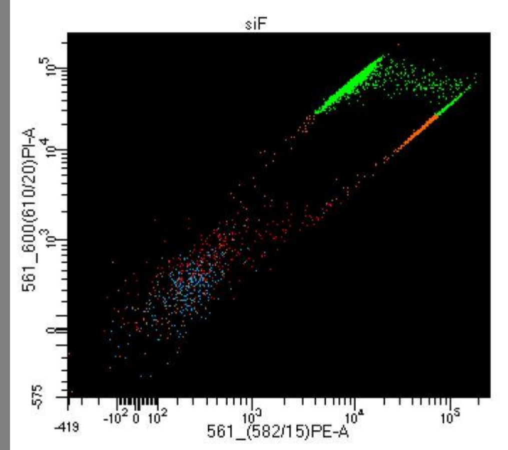


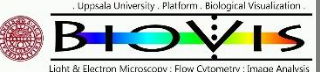
uncompensated





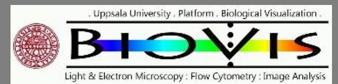




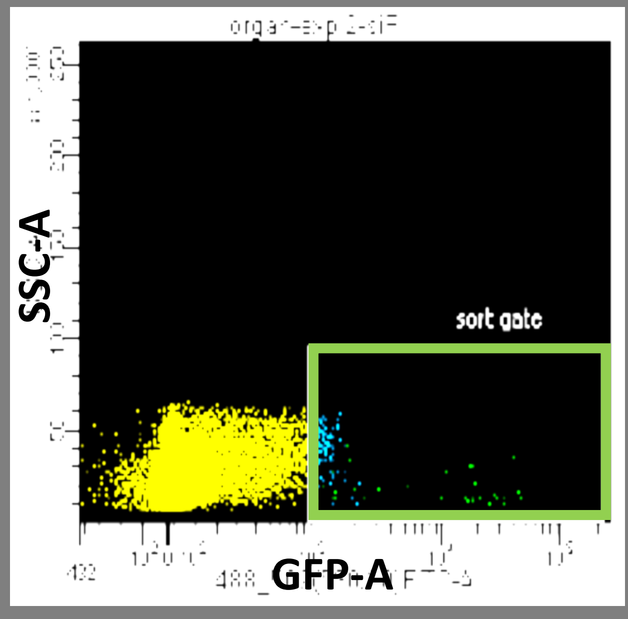


Case:
How to distinguish low GFP expression from Autofluorescence

High expression of any protein might result in stress responses
High expression of GFP might results in formation of aggregates.
Low expression might mimick the status quo of a cell

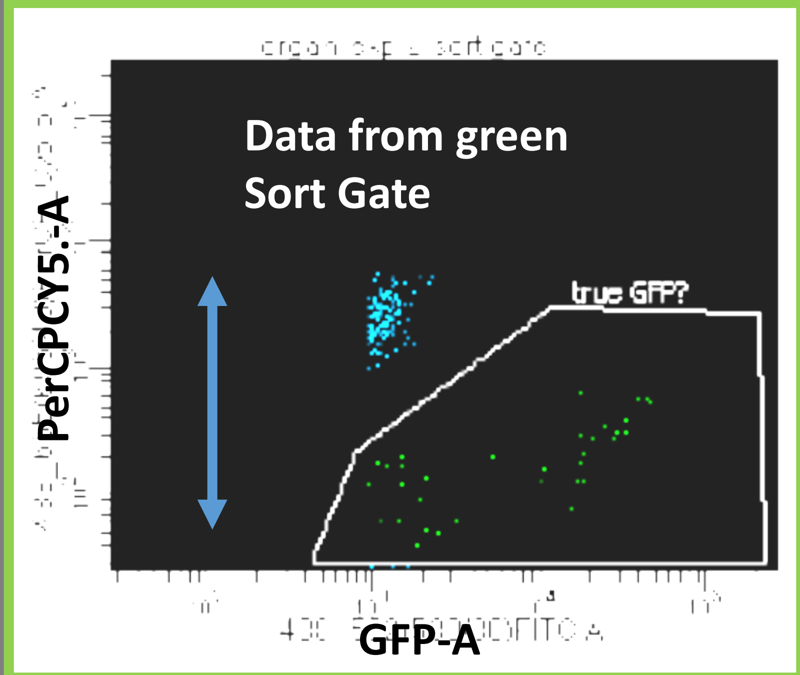


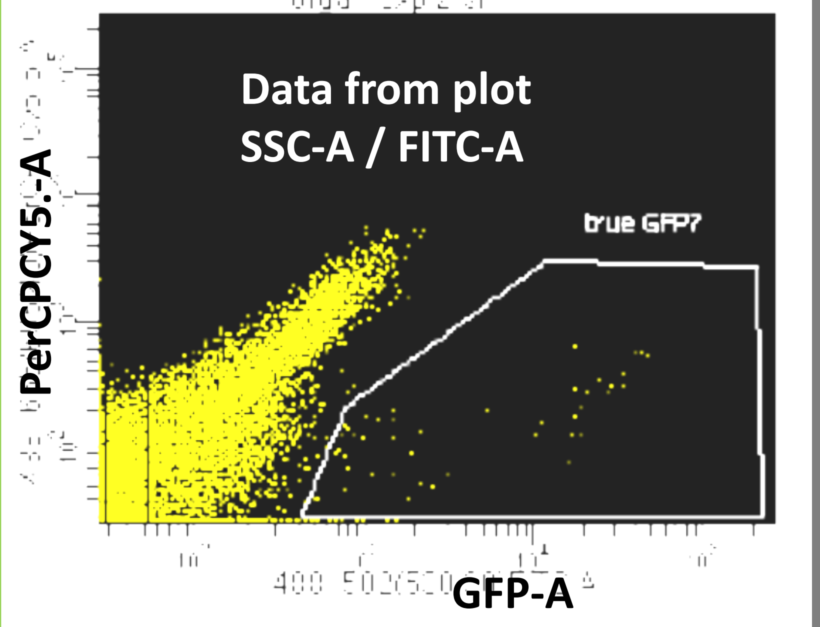
Gating GFP vs Autofluorescence (case)

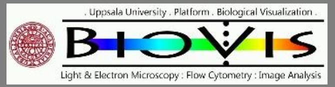


Using the PerCPCy5.5 against GFP channel separates the Background from true weak GFP

NOTE:
Background fluorescence is detected over a large range of emission and excitation.
Light of lower wavelength excite stronger
UV >>>> 405 >>> 488 >> 561 > 633







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

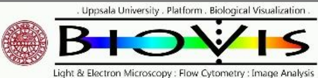
. Plot GFP against e.g. PerCPCy5.5
. AF cells appear as ”double pos” (the diagonal)

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

”Data from singlet” visualized the overall situation

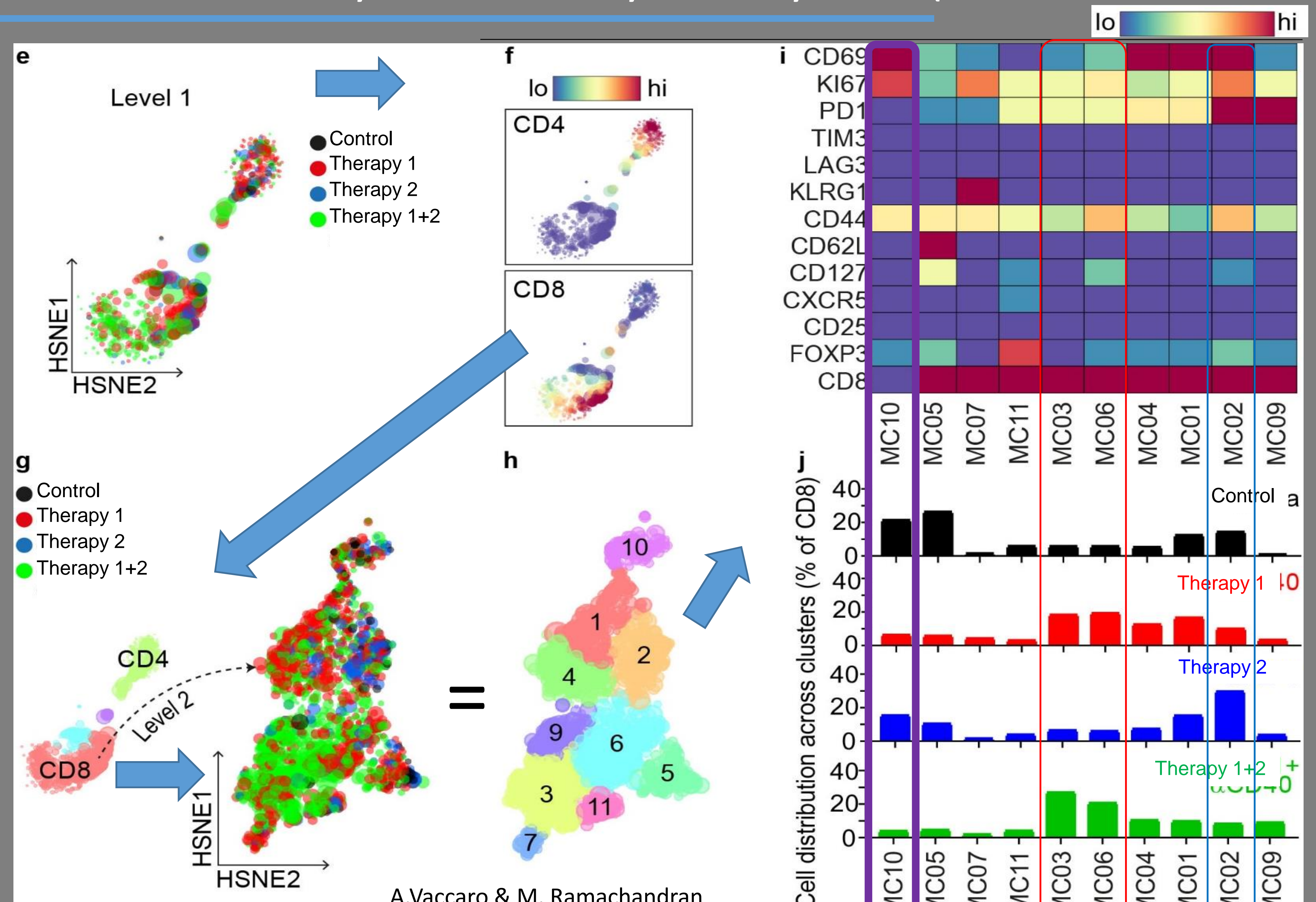
PercPCy5.5 /GFP to estimate AF

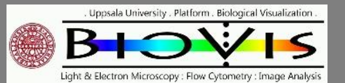
”Sort gate” GFP/SSC	Data from Sort gate	Data from Singlet gate
A		
B		
C		



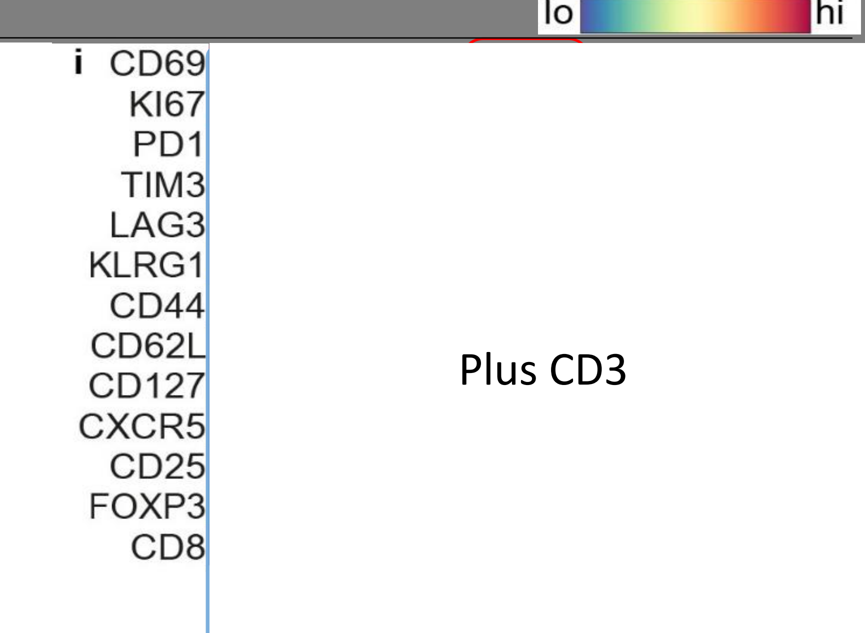
Different ways of Analysis

Multivariate Analysis of Flow Cytometry Data (17 stains and more….)



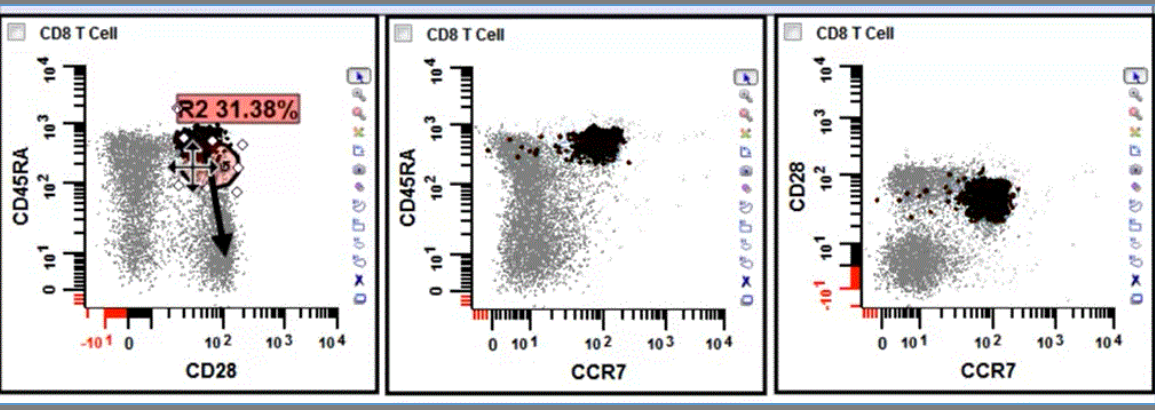


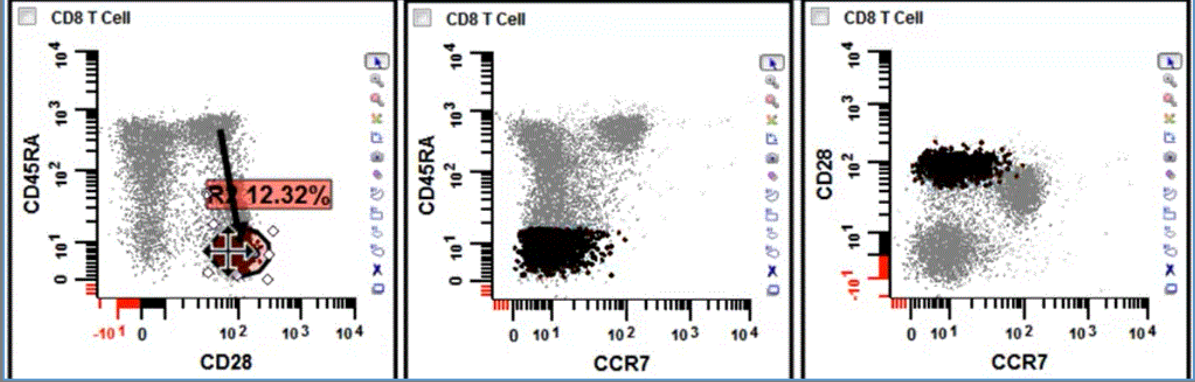
Multivariant analysis of Flow Cytometry Data



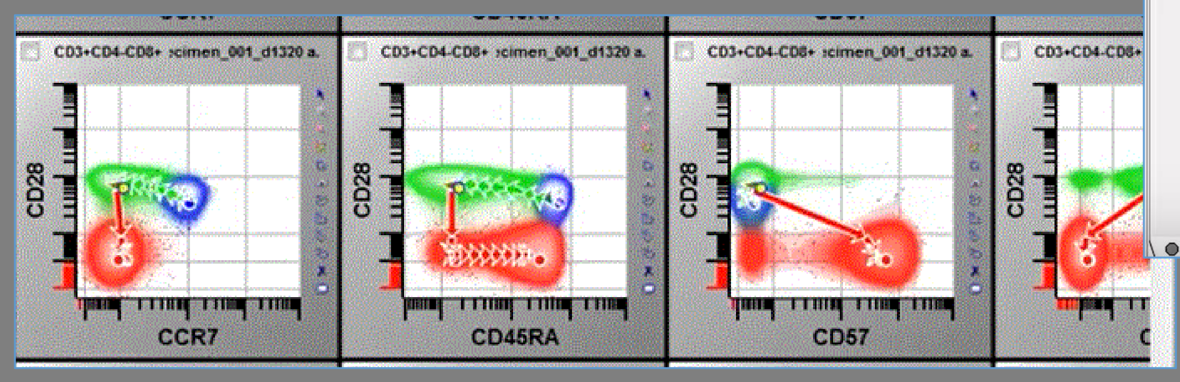
Plus CD3

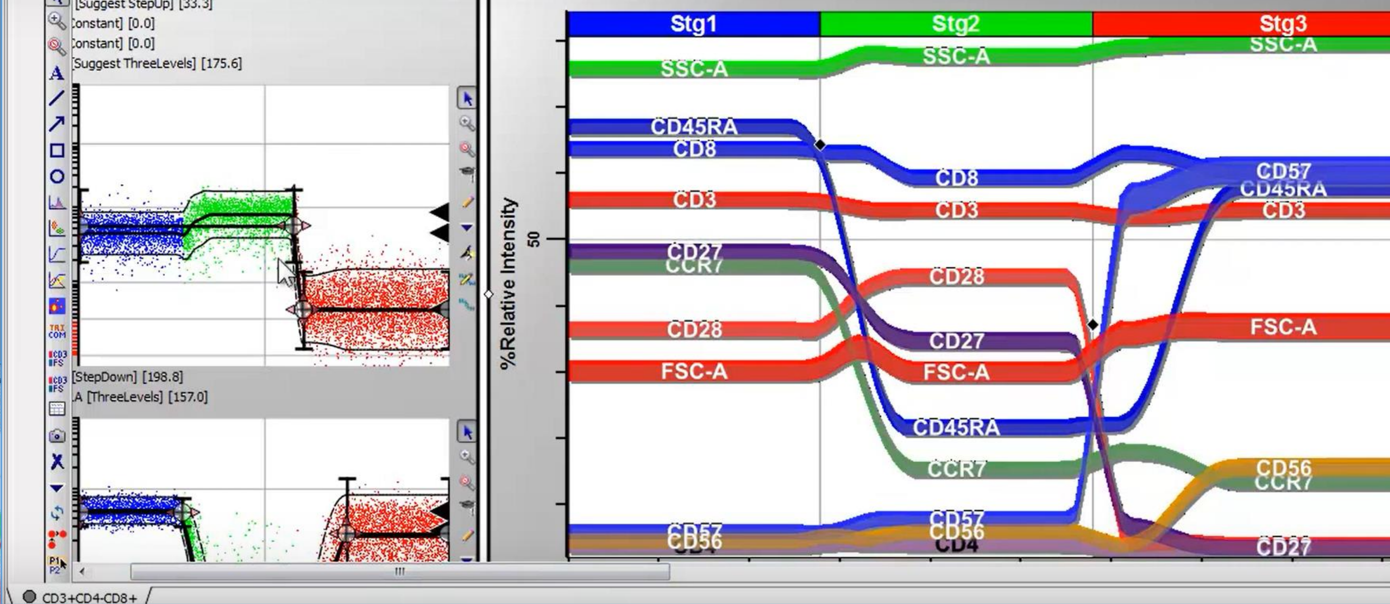
Modelling of Expression levels,





It is possible to use standard data sets to determine the relationships between the various populations. The black population marked above can be found in various other plots and thus also its relative position (expression level) to other populations. These ratios can now be used to model expression levels

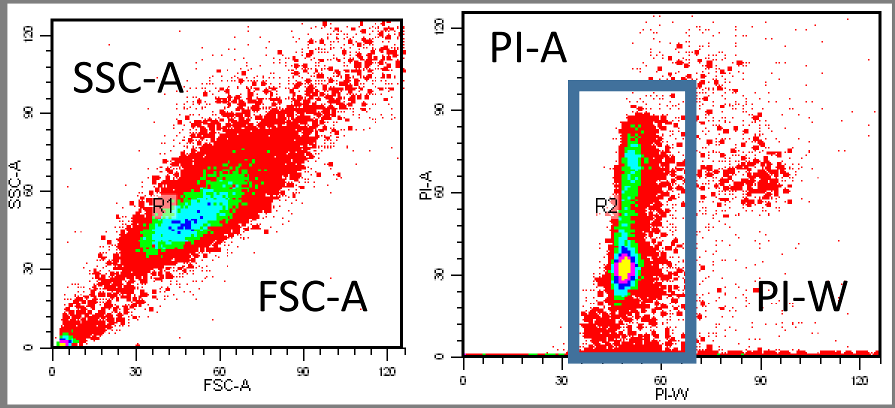




https://www.youtube.com/@veritysoftwarehouse

Cell Cycle analysis

- Staining cells DNA
- 1n Go
- 2n G2/M
  - Analysis from simpl gates to analysis by algorithms



Go/1
S
G2/M
Number

Debris
Aggregates
Dip G1
Dip G2
Dip S

File analyzed: Specimen_001_Tube_001
Date analyzed: 22-Mar-2011
Model: 1DA0n_DSF
Analysis type: Manual analysis

Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 63.94 % at 63.49
Dip G2: 11.36 % at 146.48
Dip S: 24.70 % G2/G1: 2.31
%CV: 11.61

Total S-Phase: 24.70 %
Total B.A.D.: 10.51 %

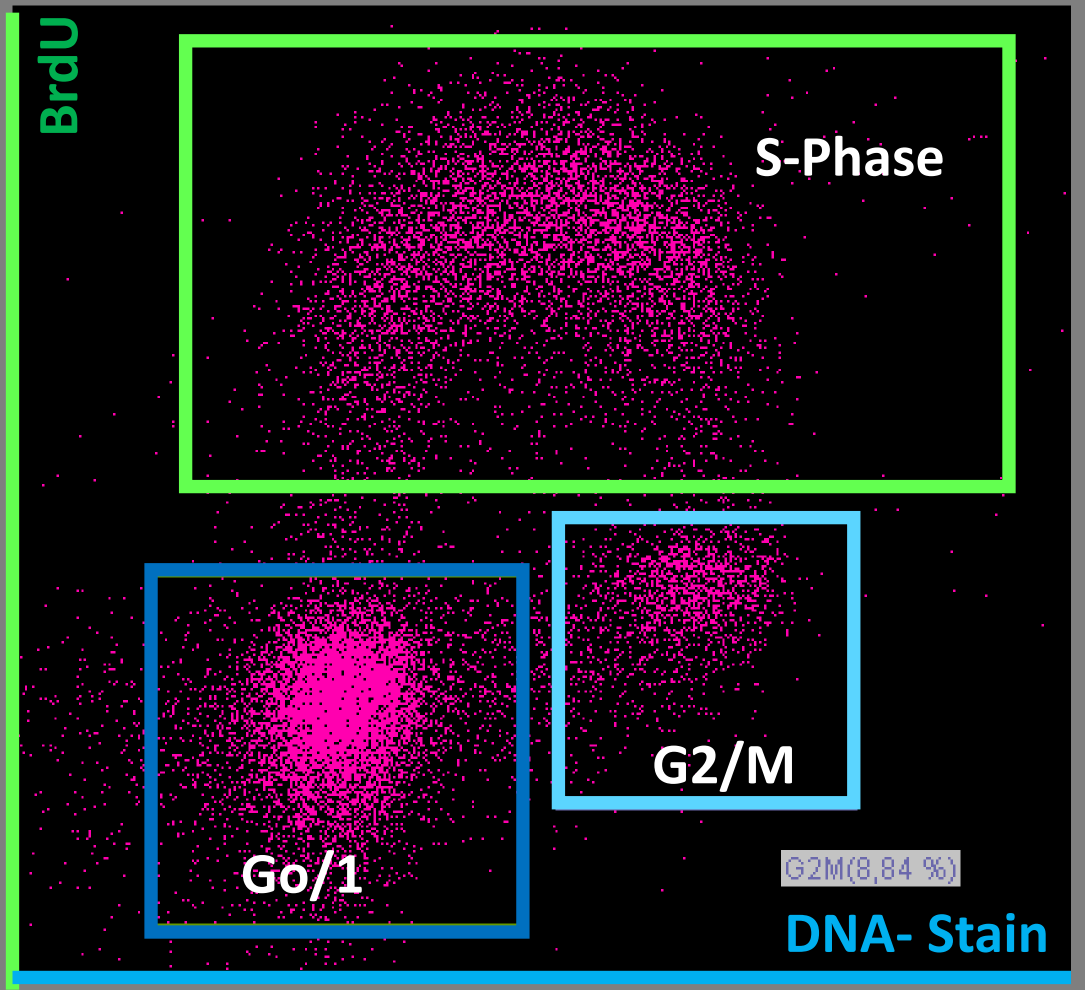
Debris: 13.32 %
Aggregates: 5.53 %
Modeled events: 23868
All cycle events: 19371
Cycle events per channel: 231
RCS: 1.958

Channels (PI-A)

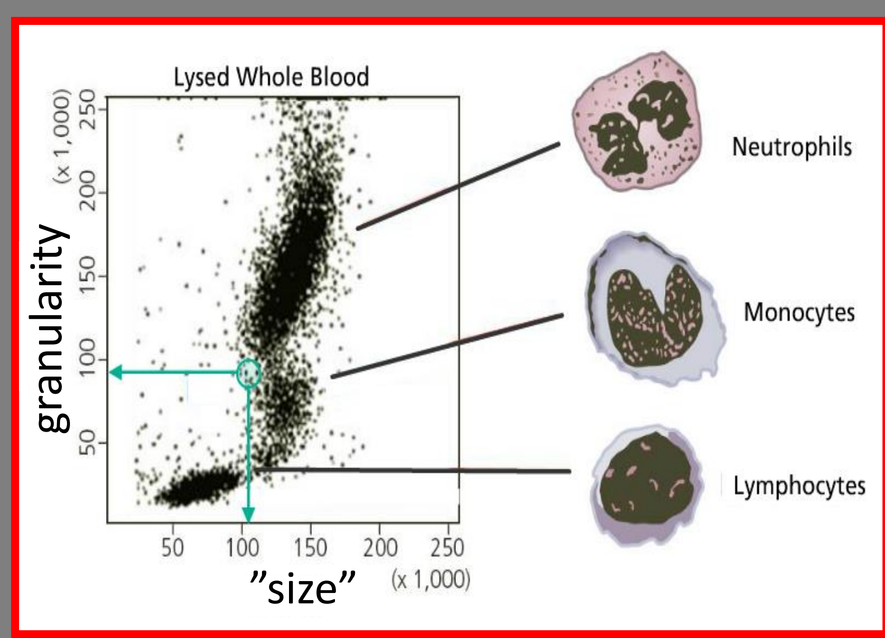
BrdU assay

To determine specifically cells undgoing S-Phase, fluorecenctly labeled thymidine analogue can be used. By adding (the non-fluorescent) Bromodeoxyuridine (BrdU) to the cell culture medium, (usually a pulse of e.g. 6 min), BrdU is taken up by cells and incorporated into the DNA which undergo DNA replication. BrdU will be fluorescently labeled whilst preparing cells for analysis, DNA is labeled ”as usal” by e.g. PI .
Analysis in a bivariate plot PI vs BrdU reveals specifically the cell undergoing S-Phase.

Note: The protocol needs refinements in respect with different celll types and their cell cycle speed.
BrdU labeled S-phase cells will not stop and undergo another round of mitosis, hence BrdU labeled cells can show up not only in S, but - at later time points of collection - also in G2 and further in G1. Signal for BrdU will be halfed by

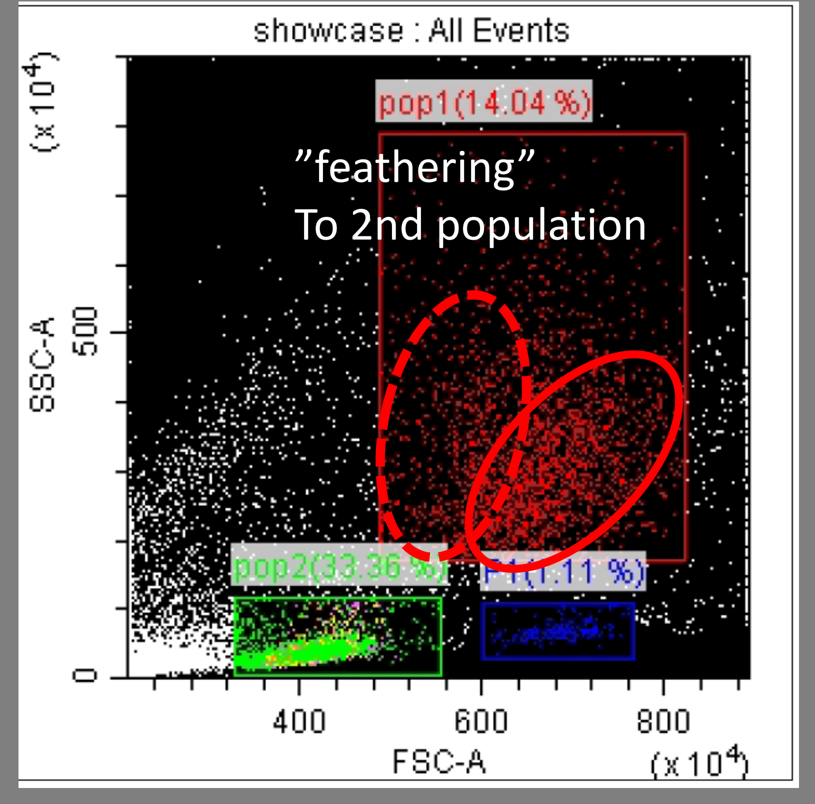


Information in the FSC/SSC plot



Cells which undergo apoptosis will have to different content

- condensed chromatin
- cell membrane blebbing
- smaller cell size

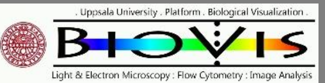


In FSC/SSC plot Apotptotic cells often seen as a "feathering" to the left or even a 2nd population

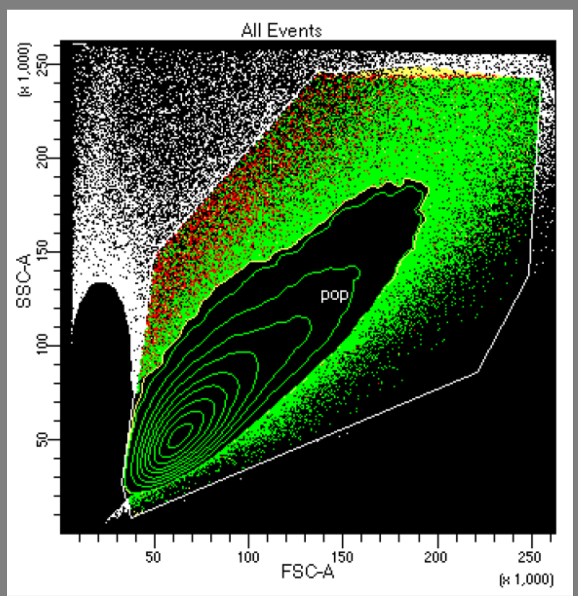
But be aware, apoptotic cells might be still
In the "normal" population depending on their combination of apoptotic feature

STAINing kits for apoptosis/living cells available.

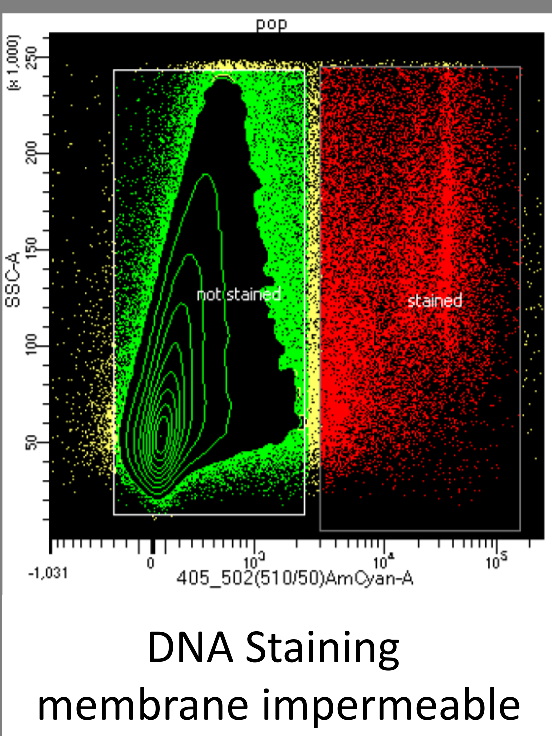
! Both plots show blood cells which appear differently in the plot due to instrument setting!



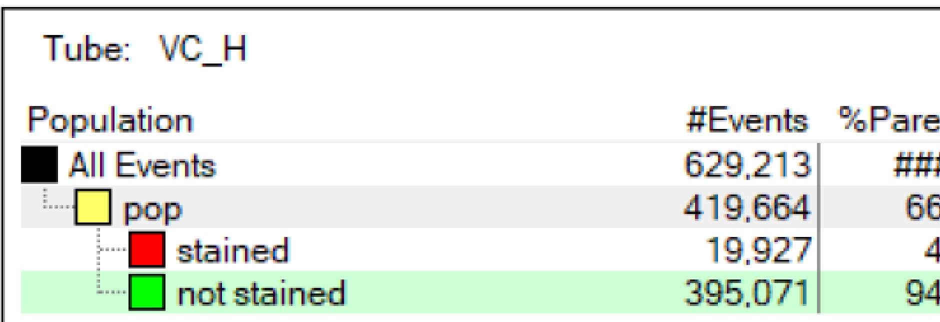
Stain Live Dead



Stain cells for compromised membrane using a dye

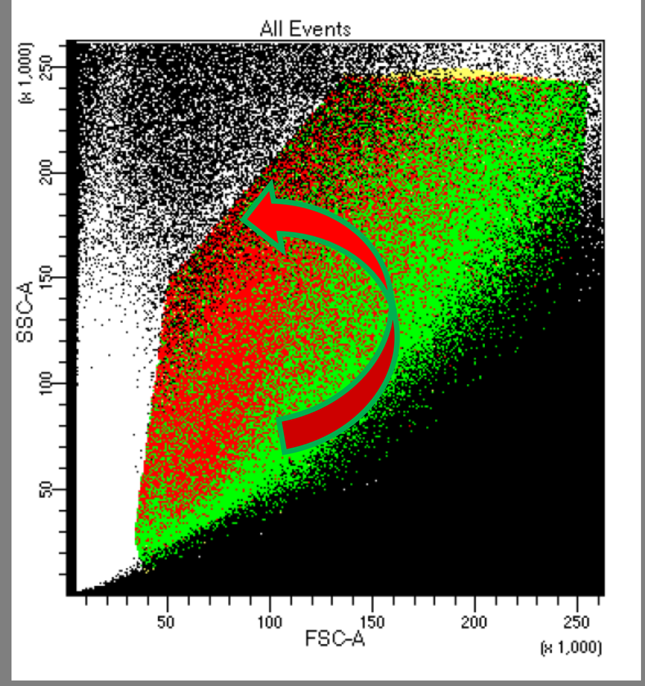


DNA Staining membrane impermeable



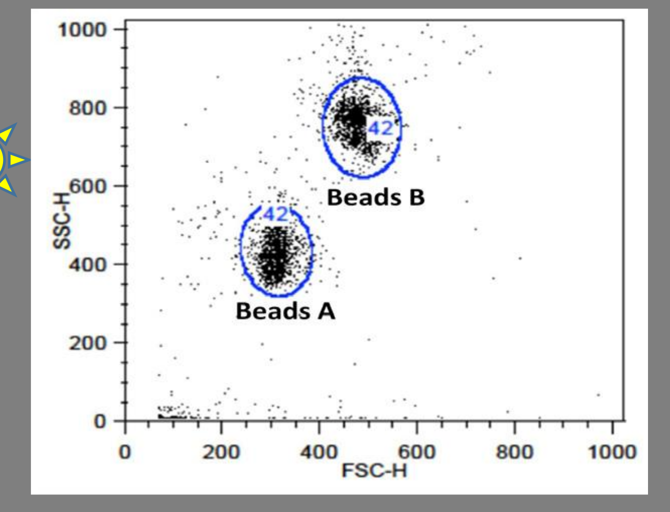
Tube: VC_H

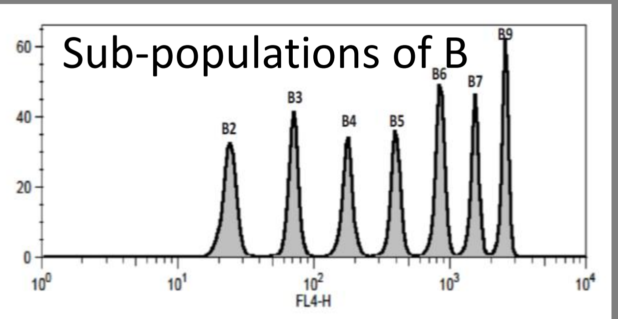
Population	#Events	%Parent
All Events	629,213	####
pop	419,664	66.
stained	19,927	4.
not stained	395,071	94.

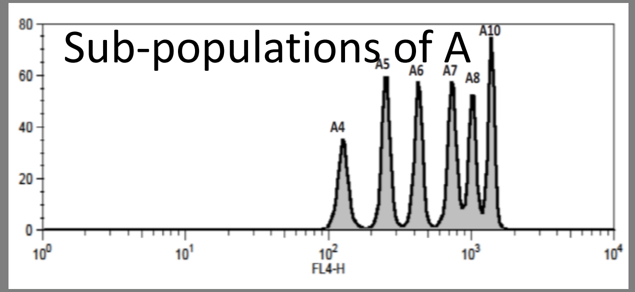


- which cannot pass intact cell membrane
- binds DNA (dye stays inside the Cell)
- Annexin V stain for early apoptosis
- Various staining kits available for different stages of cell death OR healthy cells (!)

Multiplex bead assay for Flow Cytometry





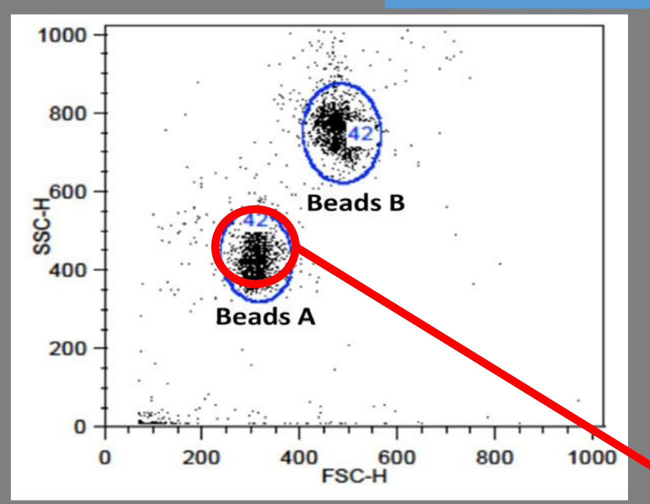


Similar to ELISA assay, but more effective
Analyze protein content of supernatants
2 bead population by size (A, B)
Each bead population contains X number
Of sub-bead population which contain the actual Abs to catch the target.
Each sub-bead poluation has the same fluorescence to be identified by.
The Antibody stains reveals the amount of targeted antigen

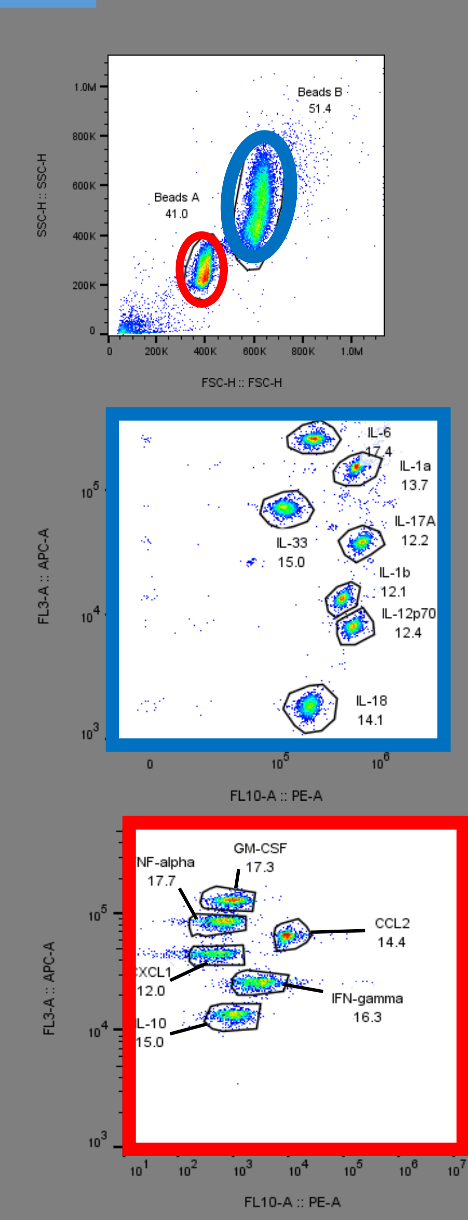
IL-18	B2
IL-12p70	B3
IL-1β	B4
IL-17A	B5
IL-33	B6
IL-1α	B7
IL-6	B9

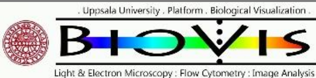
IL-10	A4
IFN-γ	A5
CXCL1/KC	A6
CCL2/MCP-1	A7
TNF-α	A8
GM-CSF	A10

Multiplex bead assay for Flow Cytometry

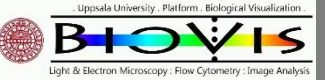


Target	Bead ID
IL-10	A4
IFN-γ	A5
CXCL1/KC	A6
CCL2/MCP-1	A7
TNF-α	A8
GM-CSF	A10
IL-18	B2
IL-12p70	B3
IL-1β	B4
IL-17A	B5
IL-33	B6
IL-1α	B7
IL-6	B9





Staining the sample



Staining the sample

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

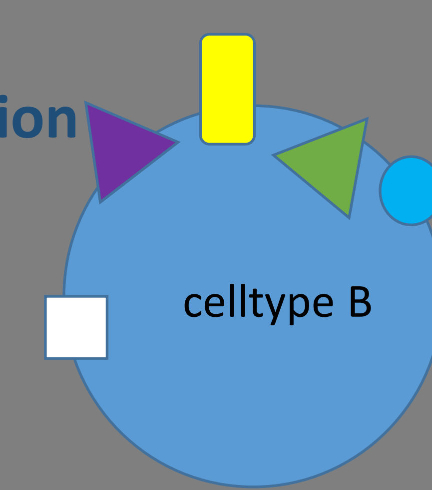
Suitable proteinmarker on your cells of interest (and non-interest)
Evaluate/test/ the Antibody you want to use
Suitable strategy to find sub-population

2. **Estimate amount of antigen you want detect on sample**

Protein expressed in high numbers → gets a weak fluorphore
Protein expressed in low numbers → gets a strong fluorophore

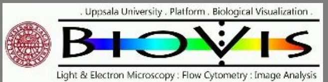
3. **Make use of the lasers and detector configuration**

Laser 1 → marker 1, Laser 2 → marker 2 etc
Tandem dyes



4. **Have controls**

Negative,Live/dead, compensation
(and FMOs)



Staining the sample – **Classification of Antigens**

Primary:

- The usual Starting point to build a pens
- well characterized, easily classified as positive or negative
- Typically defines lineage or broad subsets
- Example: CD3,CD4, CD19

Secondary:

- Well characerized,
- Typically expressed at higher density, often over continuum
- Examples CD27, CD28, CD45RA, CD45RO

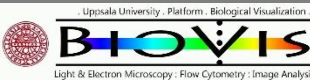
Tertiary:

- Expressed at low levels
- Variable expression depending on (unkown) activation
- Critical marker in the epxeriment
- Example CD25,STAT5, FoxP3

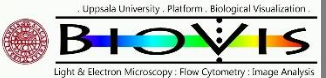
Different subpopulation can express same antigen at different densities
Antigen density assessment should be done on (sub) population of interest

WHERE TO START?
Check published panels: OMIPs

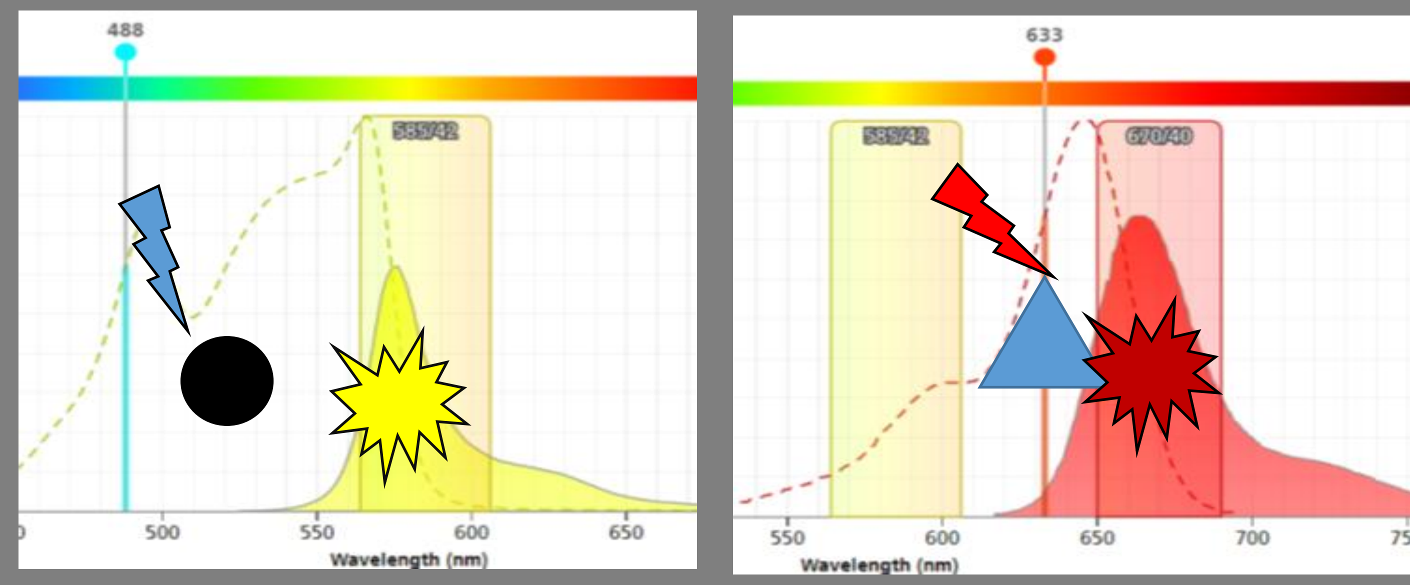
https://onlinelibrary.wiley.com/doi/full/10.1002/cyto.a.22889

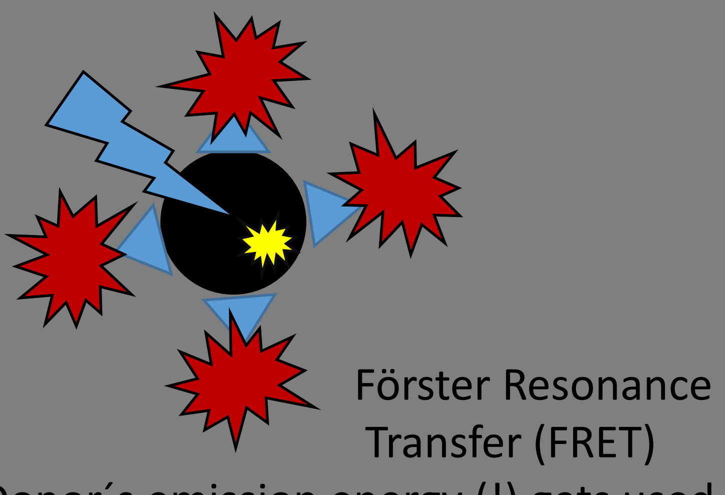


Tandem Dyes



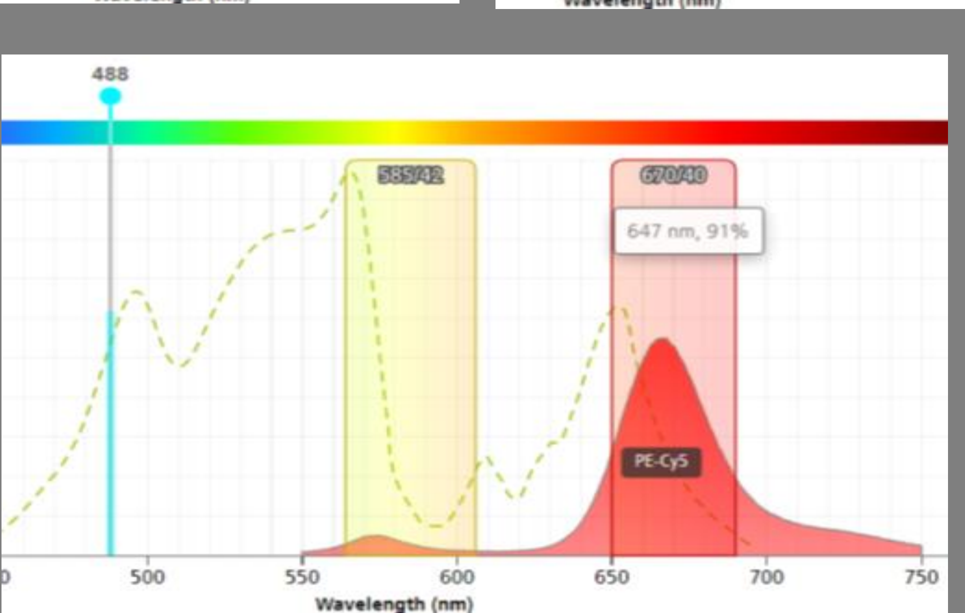
Tandem dyes (and FMOs)



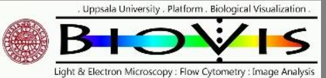


Förster Resonance Transfer (FRET)

Donor´s emission energy (!) gets used for excitation of acceptor who emits subsequentially



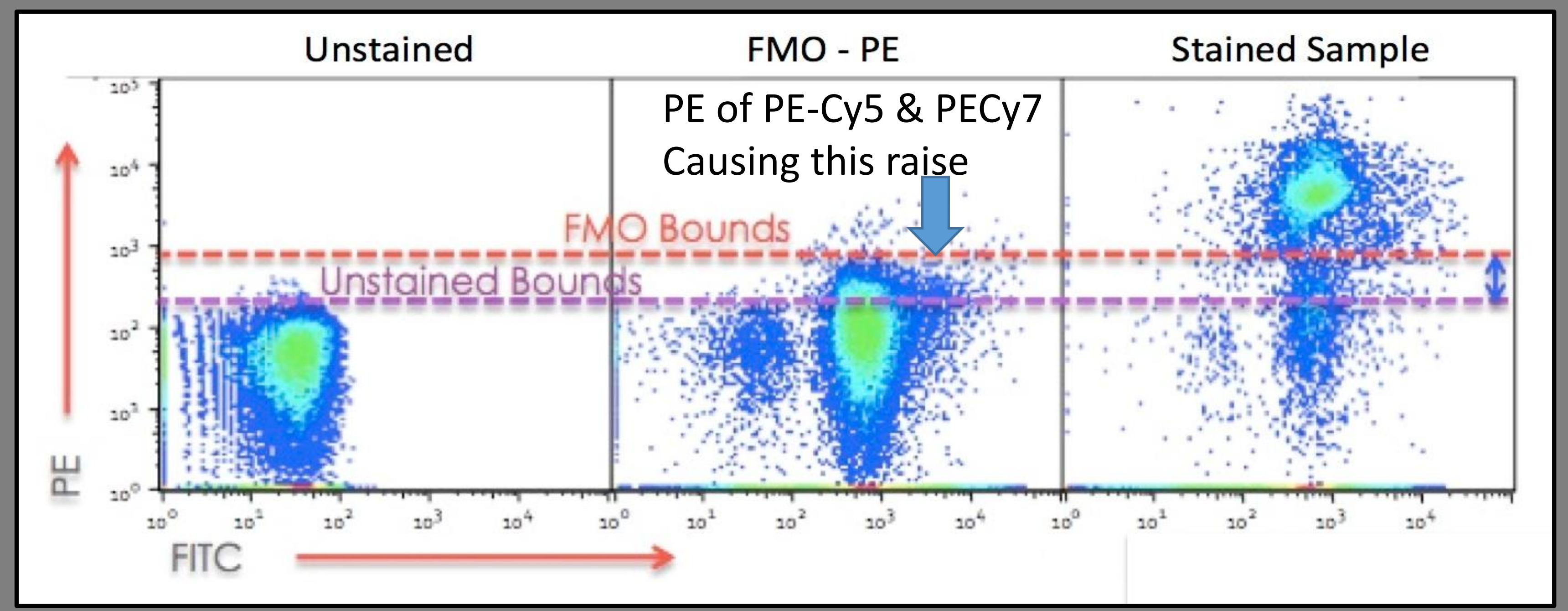
Light & Temperature → Donor looses Acceptor: Whats the outcome?

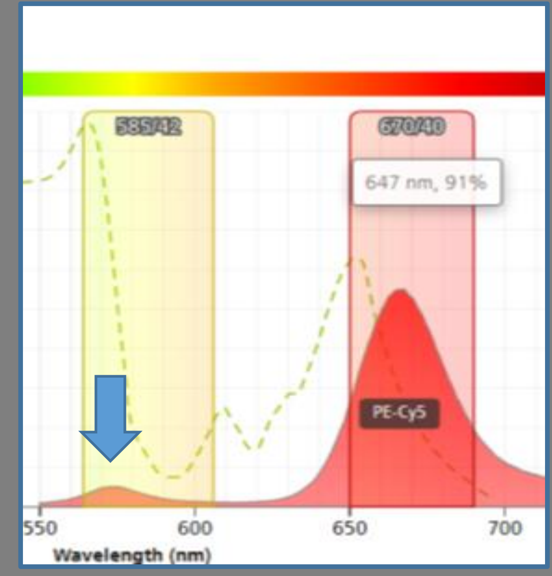


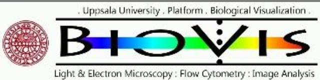
FMO – Fluorescence Minus One

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	-	CD45R0
PE-CY7 CD45RO -FMO	Cd3	CD4	Cd8	-

In multicolor experiments
Different fluorophores might interact
In a FRET manner in case they are in close proximity.
Use FMOs where resolution is critical







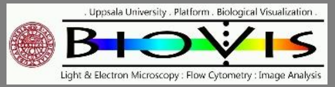
Dumb channel

What is a dump channel?
An exclusion channel to group and exclude cells
that is are NOT of interest, but have markers of interest.

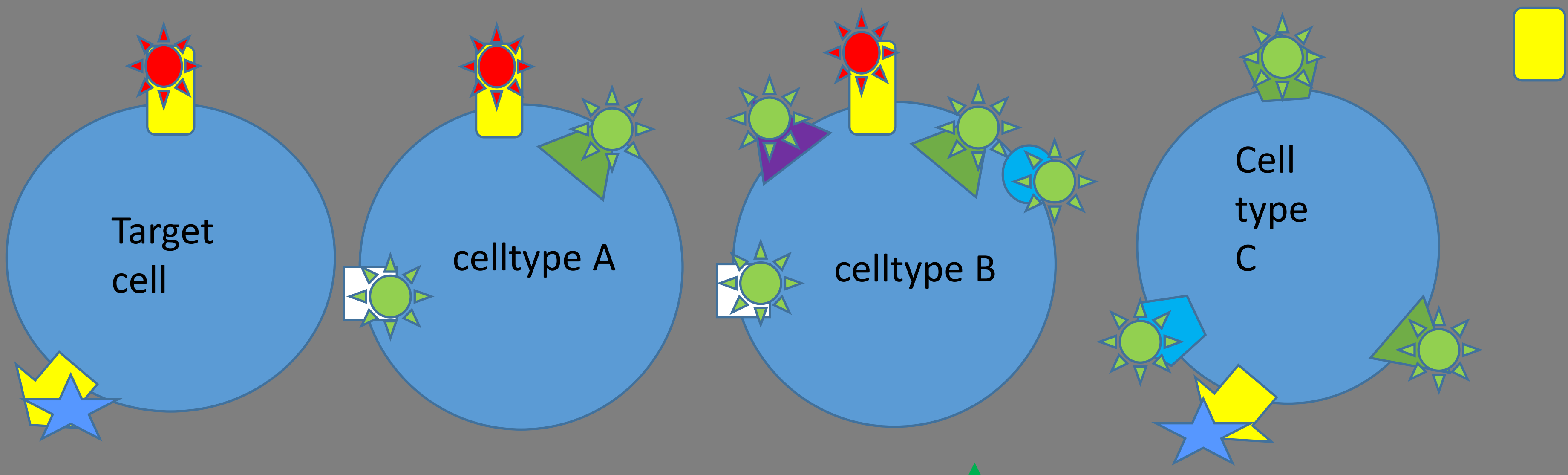
Example: of Interest Cell type 1 and Marker A
Cell type 1 expresses antigen A
Cell type 2 expresses antigens A and B
Cell type 3 expresses antigens A and C

Dump channel contains staining for B and C,
Usually labeled with same fluorochrome,
to be excluded for the actual analysis of A

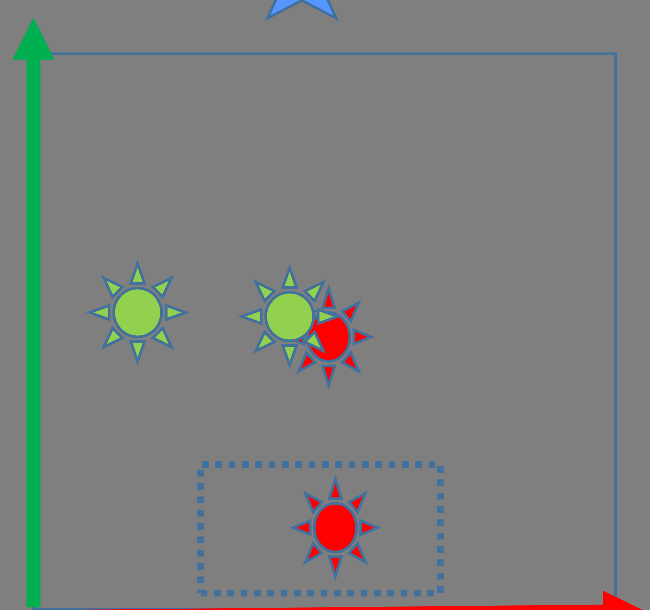
Viability dyes can be included in a dump channel
to label dead and dying cells for exclusion.
Viability dye can use the same
fluorochrome as the other dump channel.

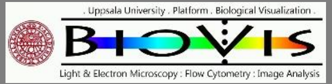


Dump channel



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 [red marker] and working further with the single positive on other markers [blue star]

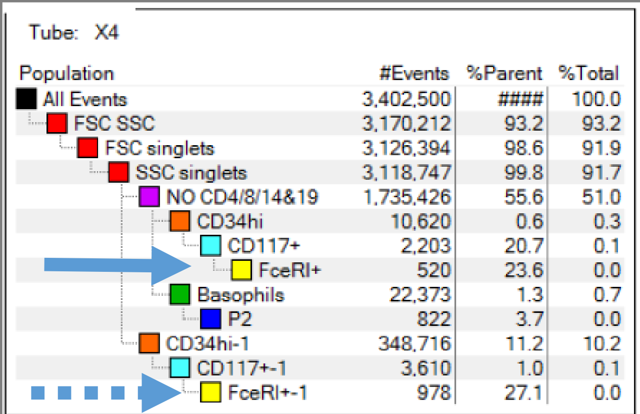




Example, rare cell population / dump channel

Dump channel(s)

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



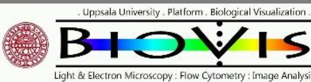
Tube: X4

Population	#Events	%Parent	%Total
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

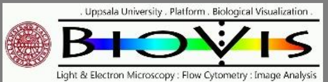
Dump channel was used

Not having dump channel:
False positive in final gate

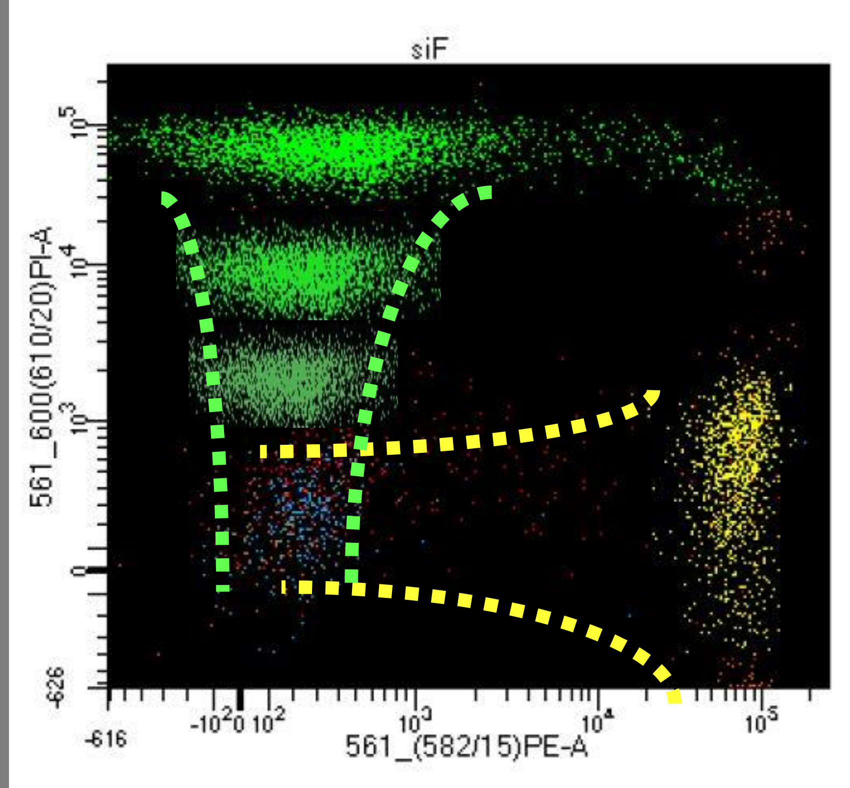
Dump channel not applied



Data spread



Data Spread– loss of resolution



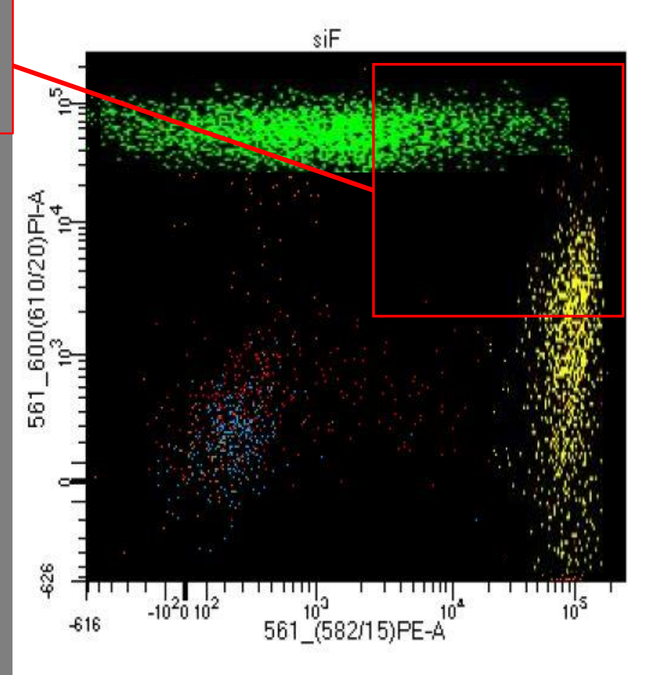
Spread of data in bivariate plots due to intrinsic ”photon counting error” of instrument. Revealed by the compensation , but not caused by it.

Spread can come from at least one other fluorophore of whole panel, not necessarily the one plotted against each other.*

More spreading error (may/can/will) occur when:

1. More fluorophores are used in panel
2. More fluorophores spill into another detector
3. Fluorescence is high (Ab stain, strong Fluorophore, high detector setting)

Data spread errors are reducing resolution to analyze co-expressed markers

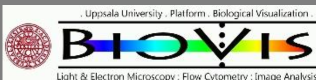


As a rule of thumb:

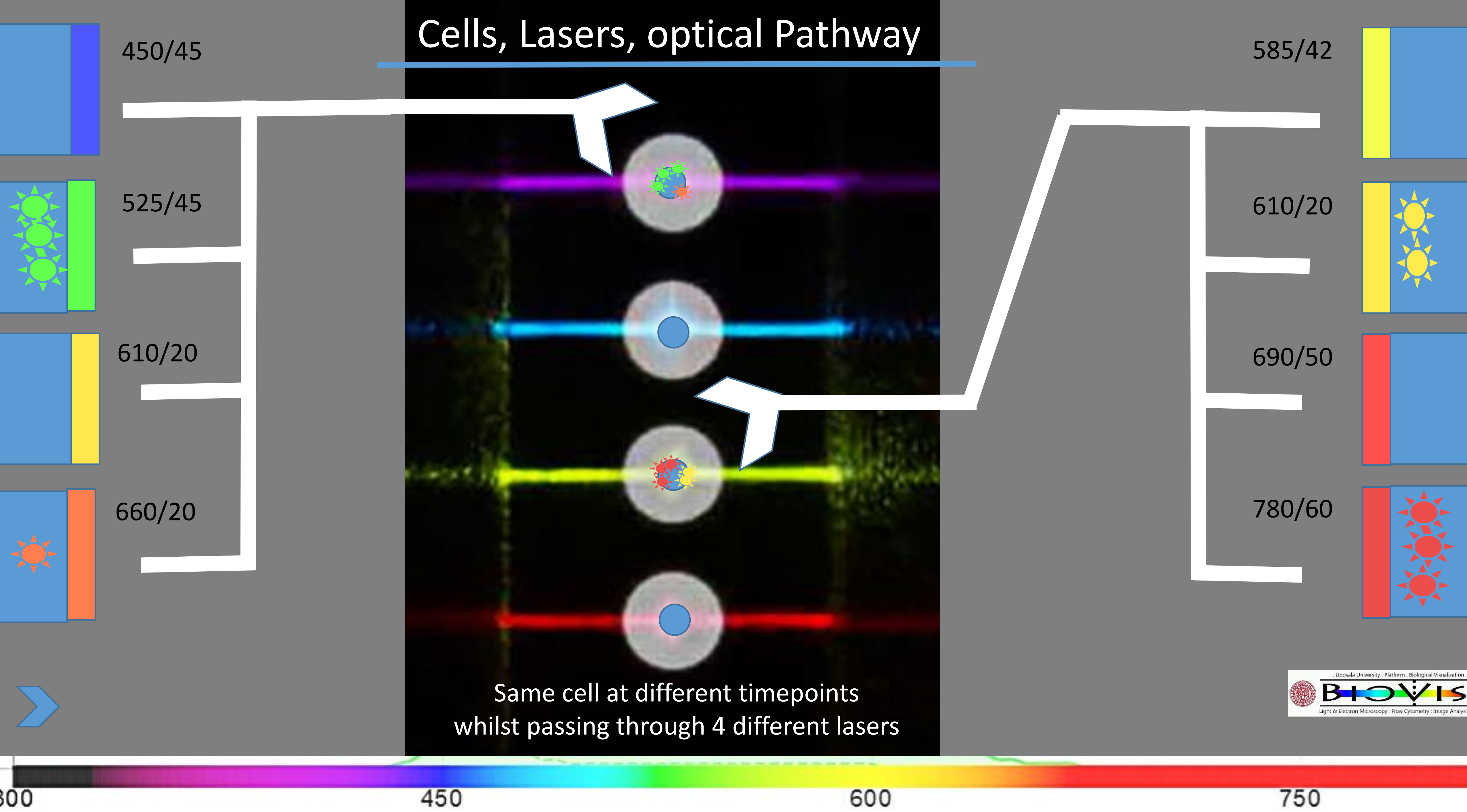
- Co-expressing markers which will be plotted in same bivariate plot should not have a similar Emission spectrum.
- Test your whole panel to reveal spread*

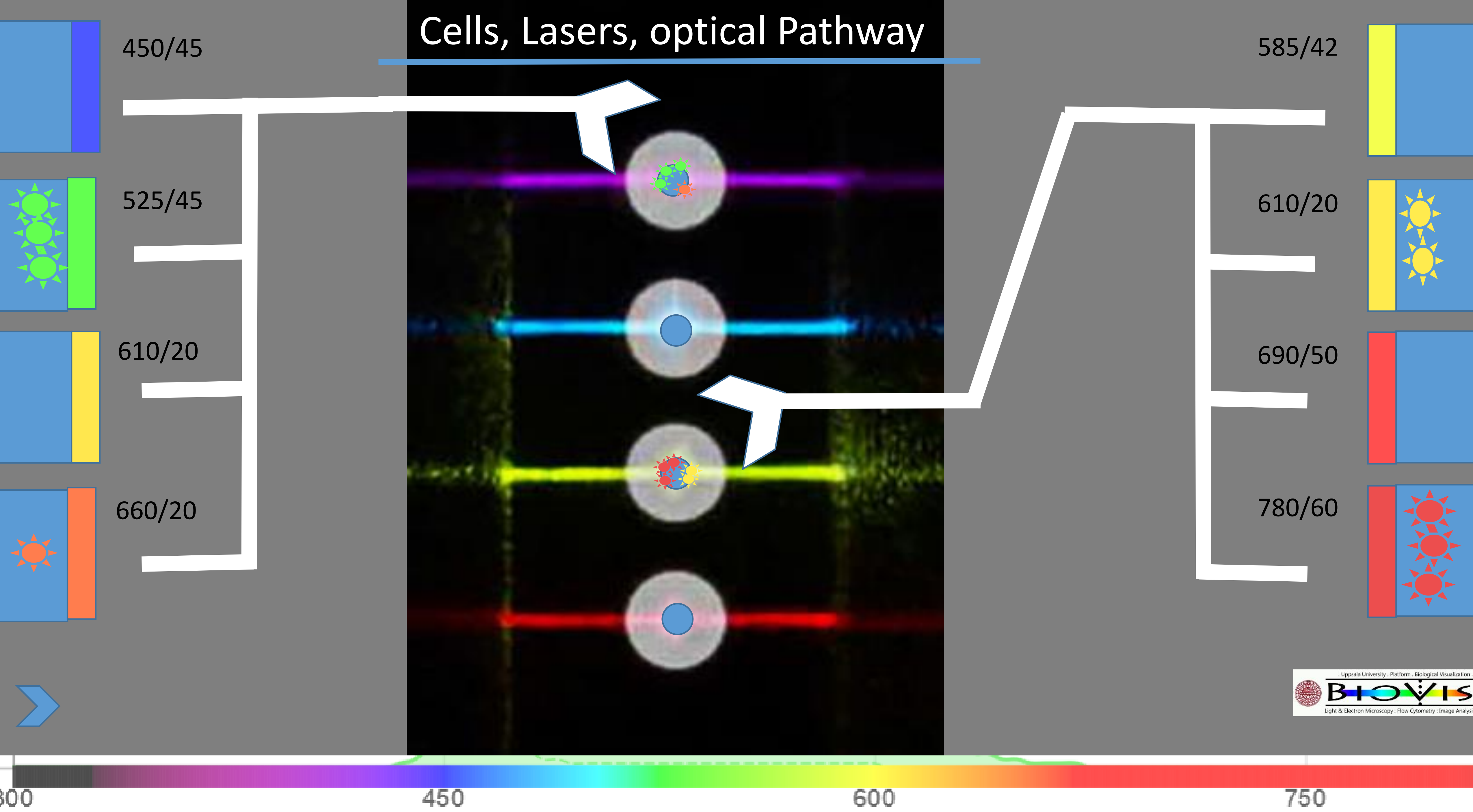
See links below for brief & conclusive overview for data spread , incl. a Spread-matrix of 30 Fluorophores

- https://voices.uchicago.edu/ucflow/2020/03/04/understanding-the-trumpet-effect-how-to-design-aurora-panels-around-spreading-error/
- https://voices.uchicago.edu/ucflow/project/cytek-aurora-panel-design/



END


Cells, Lasers, optical Pathway
450/45
525/45
610/20
660/20
585/42
610/20
690/50
780/60
Same cell at different timepoints
whilst passing through 4 different lasers
300
450
600
750



Cells, Lasers, optical Pathway
450/45
525/45
610/20
660/20
585/42
610/20
690/50
780/60
300
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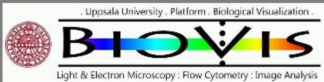
BIOVIS



The principle of a FlowCytometer (summary)

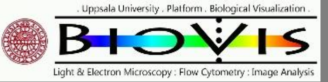
Knowledge: (slide Recap: Fluorescence)

- Different Fluorophores get excited by different Lasers
- One Laser may excite various fluorophores
- Fluorophores may get detected by not only the main-channel (bleed through, compensation)

Flow Cytometry

- Fluorecently stained cell passes through different lasers
- Cells are ”embedded” in a stream of PBS (Slide Principle of Flow Cytometry I)
- A lens, associated to a specific laser captures the emission of the cells fluorophores excited by the laser
- The different emission light passes through optical cable to a number of detectors
- The detector itself is not distinguishing different wavelength, so it has an optical bandpass filter in front of it
- Only a specific range of wavelenght (color) will pass and gets detected
- Detection of wavelenght and intensity
- Detection of events (cell sumber)

NOTE:
A Flow Cytometer detects
No image, only the wavelength
and intensity of the fluorophore



Overview

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

Hydrodynamic focusing

Detector ”green”

Incoming Light signal
to Electronic signal
to digital signal

Detector ”blue”

Fluorescence green

Optical filter

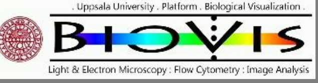
LASER

SORTING

Detector ”red”

Fluorescence red

Signal: the Intensity
(of fluorophore) NO IMAGE



Overview

Fluidics

Optical Configuration

Optics for Excitation

Optics for Emission

Optics for Emission

Cells (stained) suspension

Electronics for Signal amplification and digital convertion

Data presentation
Data analysis

Electronics for Signal amplification and digital convertion

LASER

waste

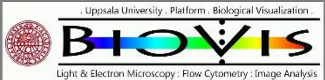
Detector green

Detector blue

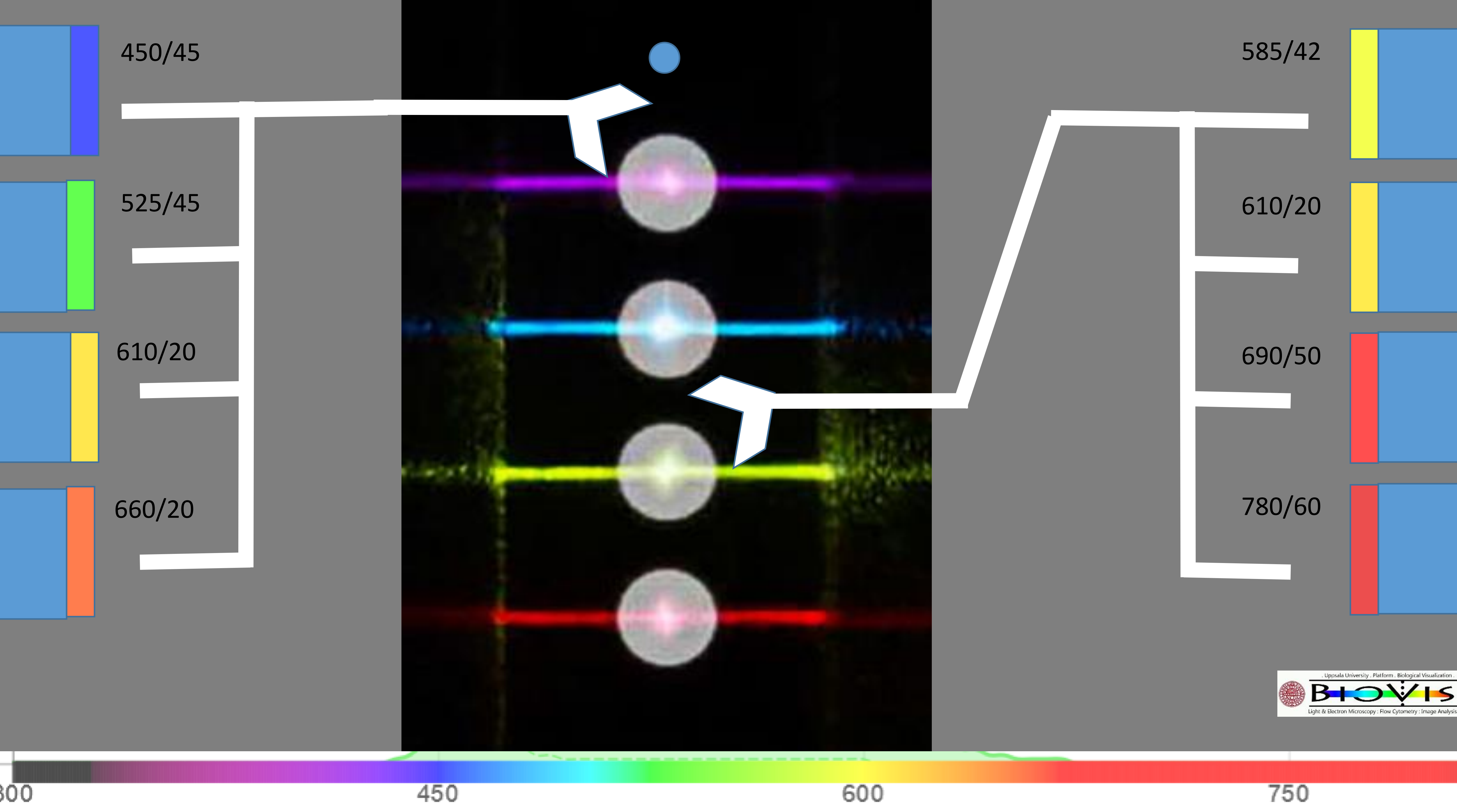
Detector red

Fluorescence green

Fluorescence red



Animation slide for part 1 part 2 needs restructer



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610/20
660/20
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610/20
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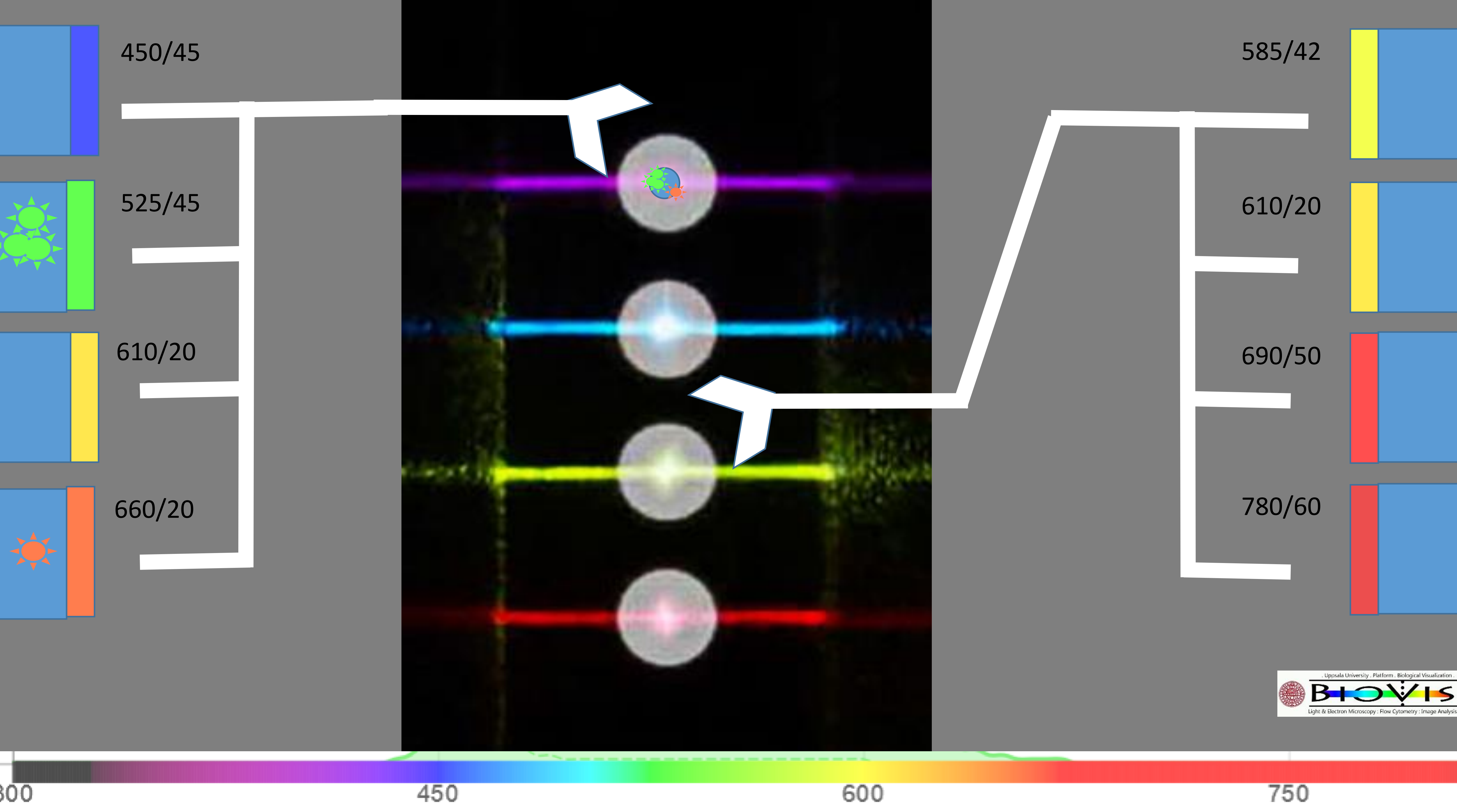
780/60
. Uppsala University . Platform . Biological Visualization .
BIOVIS
Light & Electron Microscopy : Flow Cytometry : Image Analysis
300
450
600
750



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Light & Electron Microscopy : Flow Cytometry : Image Analysis
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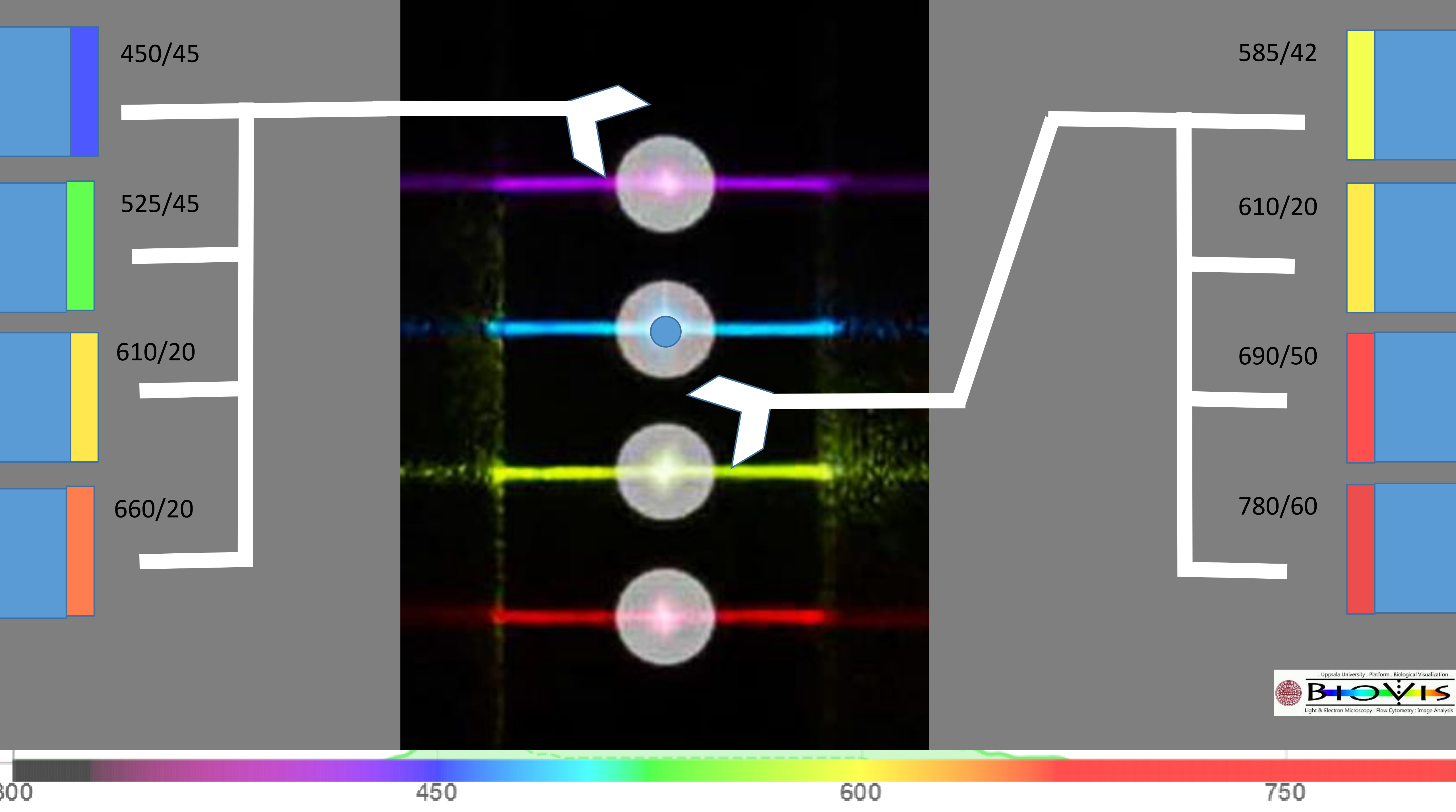
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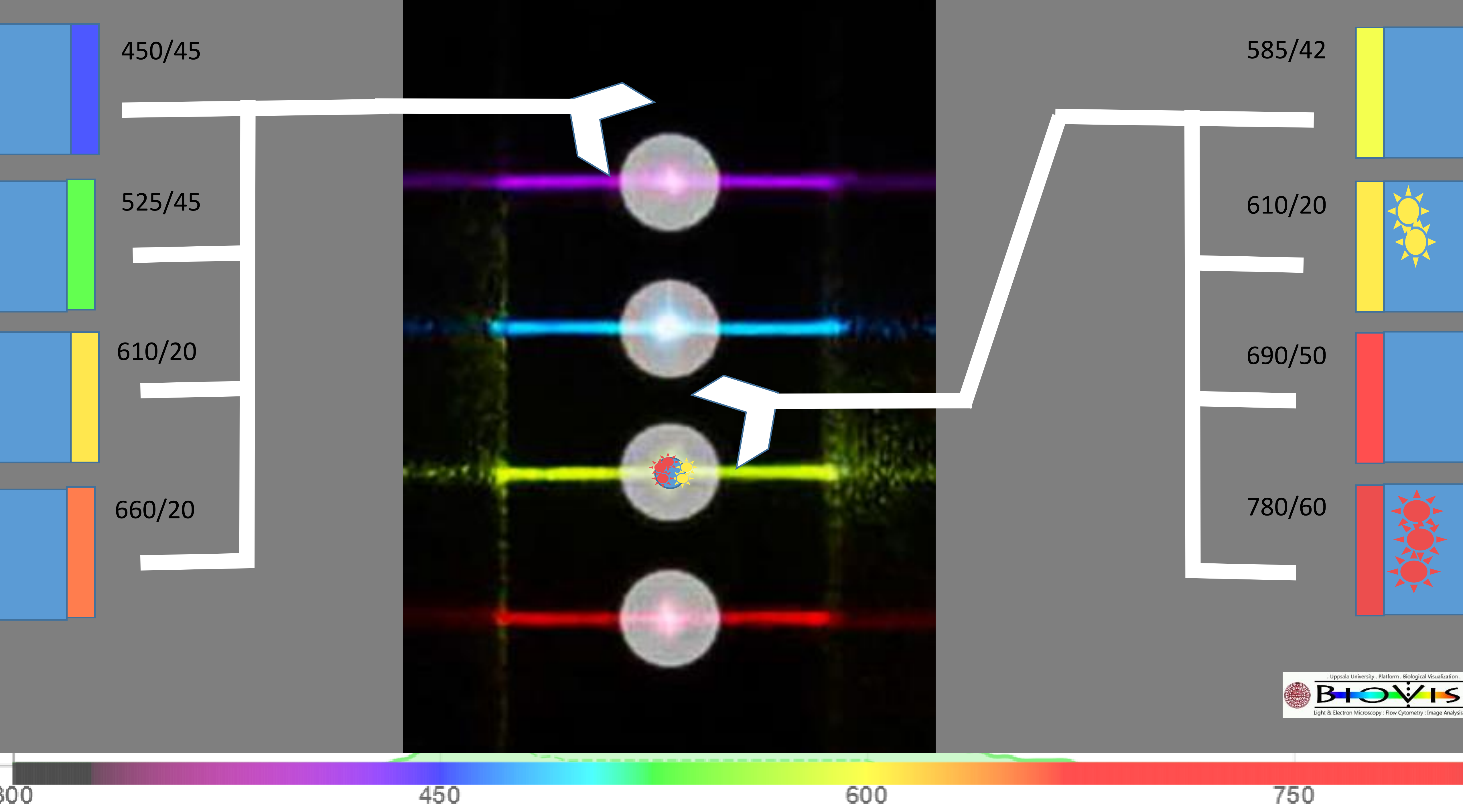
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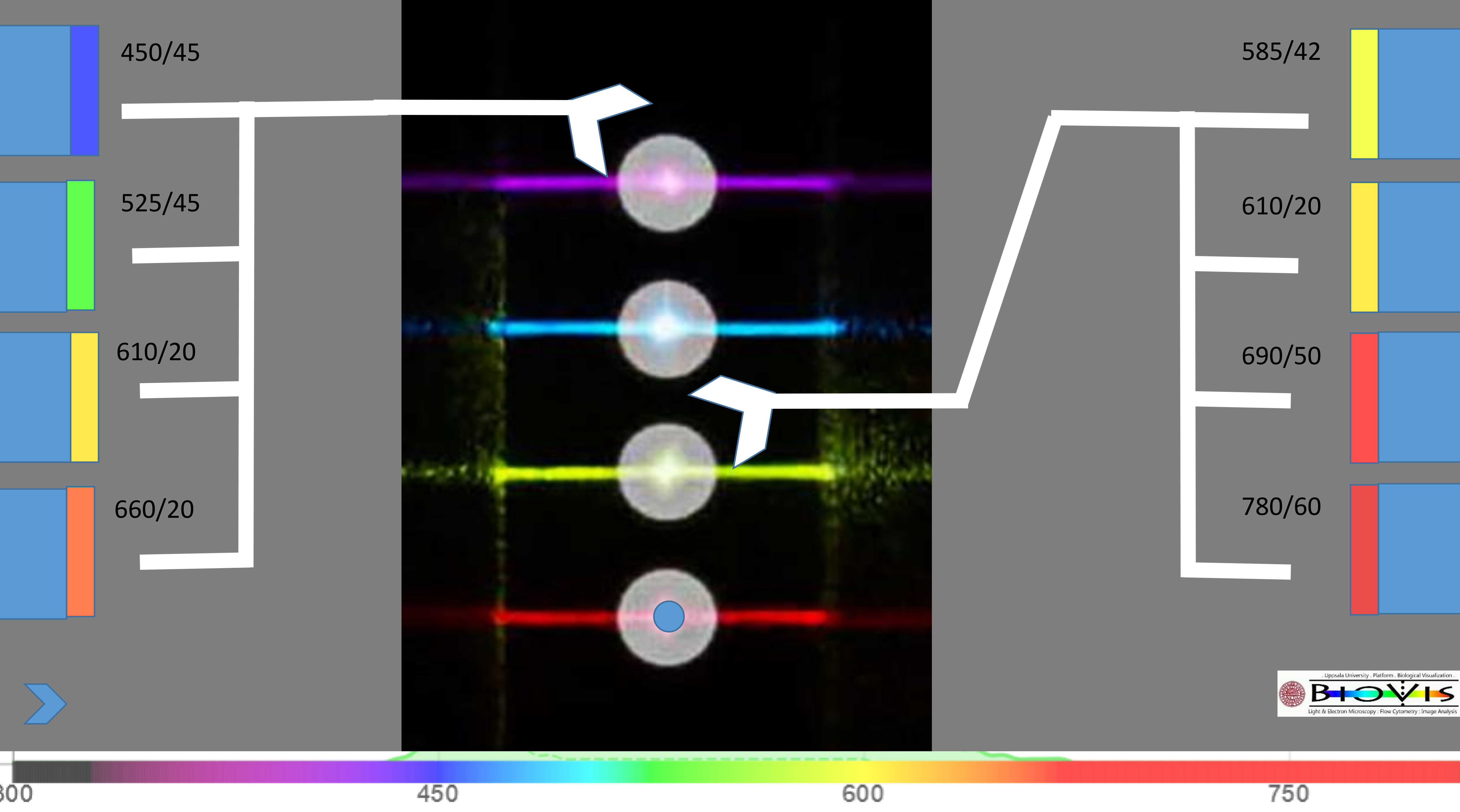

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Light & Electron Microscopy : Flow Cytometry : Image Analysis
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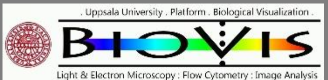
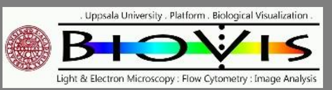
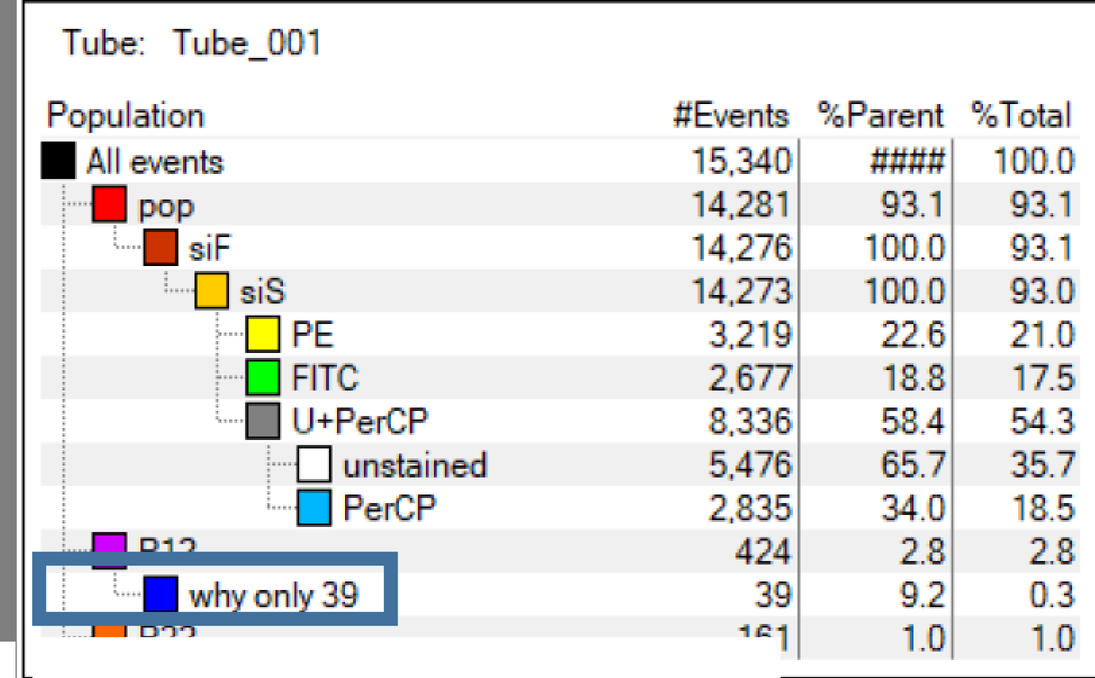


Part II/II
The small print



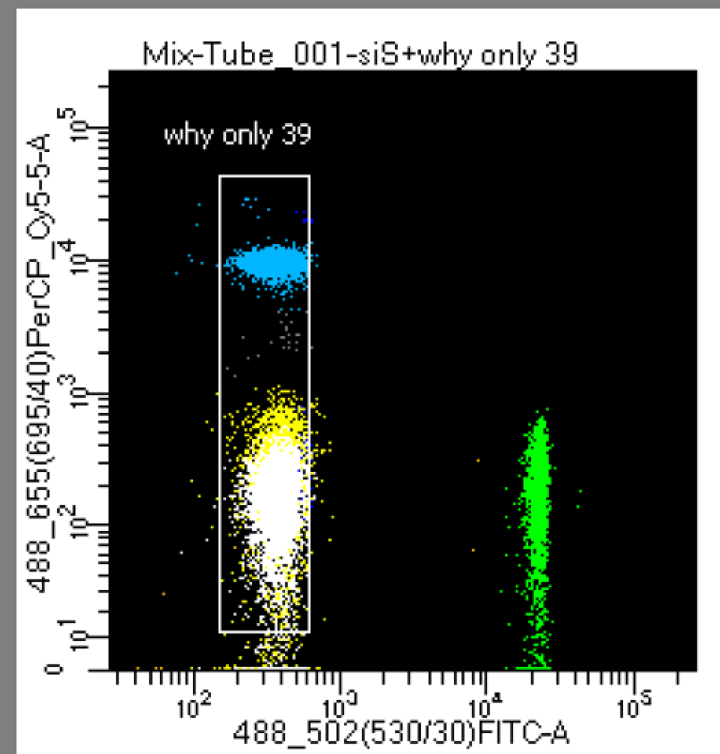
Gate ”Why only 39”

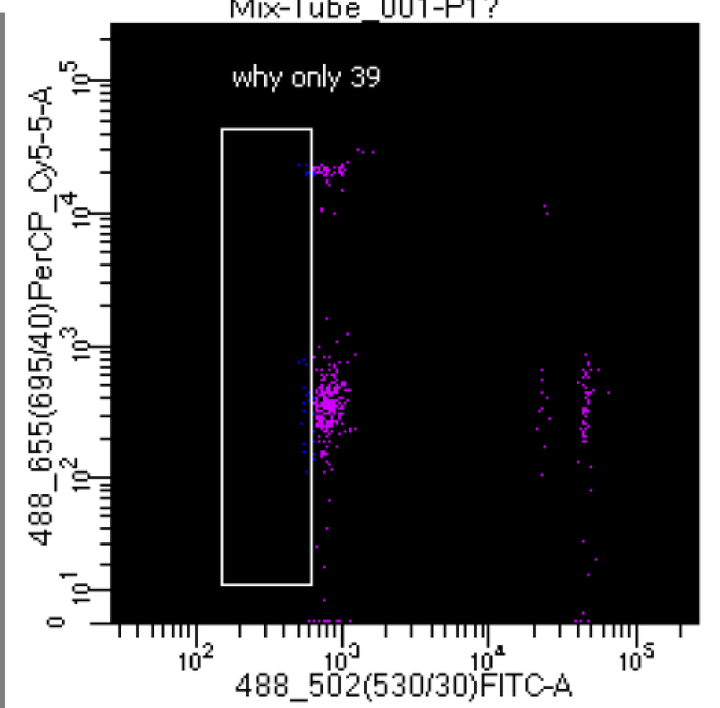
Why does this gate
Contain only 39 events
even though we clearly
see more?



Tube: Tube_001

Population	#Events	%Parent	%Total
All events	15,340	####	100.0
pop	14,281	93.1	93.1
siF	14,276	100.0	93.1
siS	14,273	100.0	93.0
PE	3,219	22.6	21.0
FITC	2,677	18.8	17.5
U+PerCP	8,336	58.4	54.3
unstained	5,476	65.7	35.7
PerCP	2,835	34.0	18.5
P1?	424	2.8	2.8
why only 39	39	9.2	0.3
P2?	161	1.0	1.0





Because it is placed
underneath
”P1?”
And this is its Data