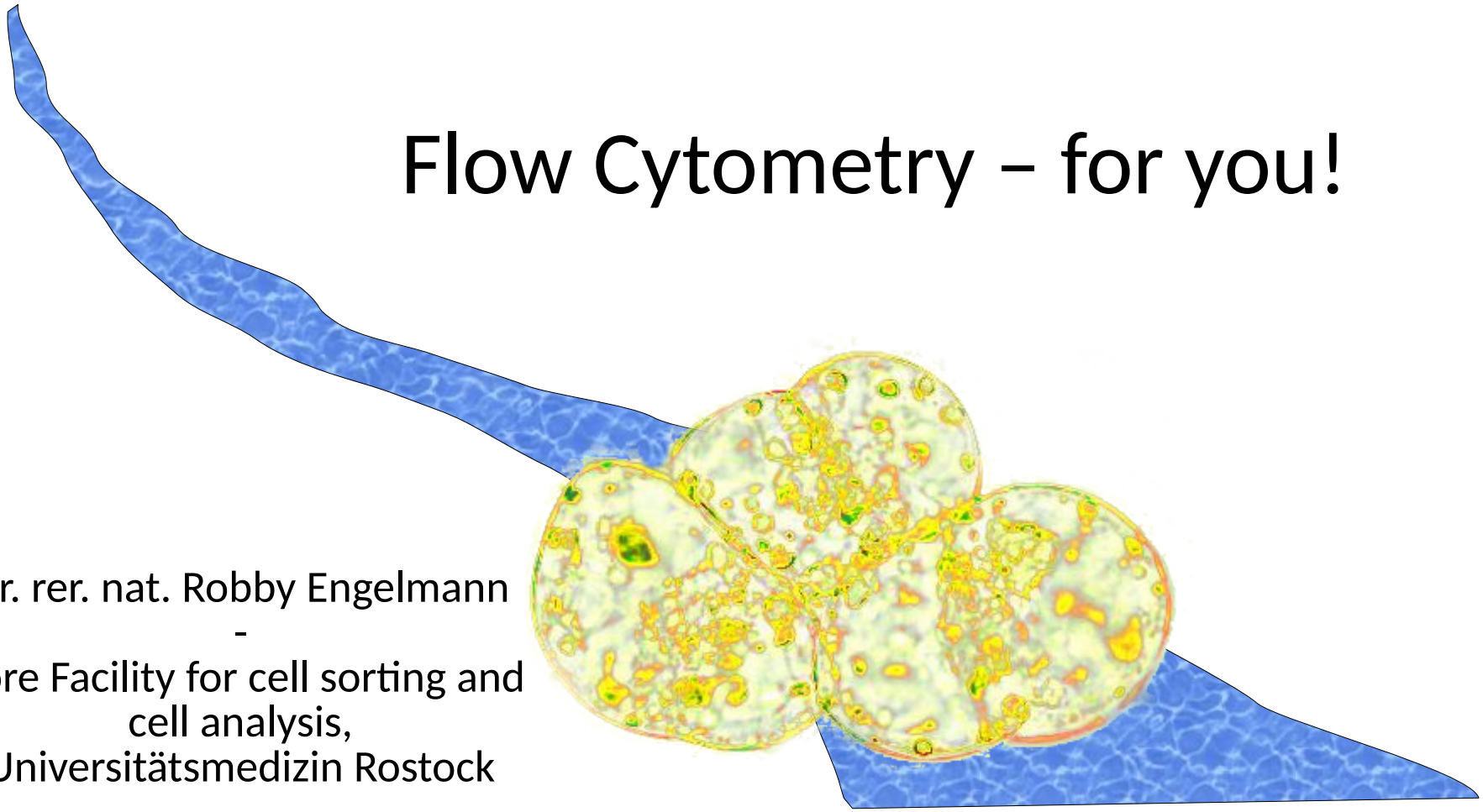
Universität Rostock Traditio et Innovatio

Universitätsmedizin Rostock

Flow Cytometry – for you!



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-
Core Facility for cell sorting and cell analysis,
Universitätsmedizin Rostock

Overview

- What is flow cytometry?
- Sample preparation
- A technical view
- Data handling
- Examples
- Questions & discussion

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What is flow cytometry?

- cyto = cell
- metry = measurement

- automated measurement of particle characteristics in a continuous sample stream

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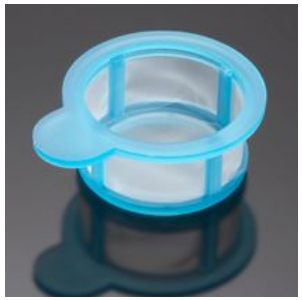
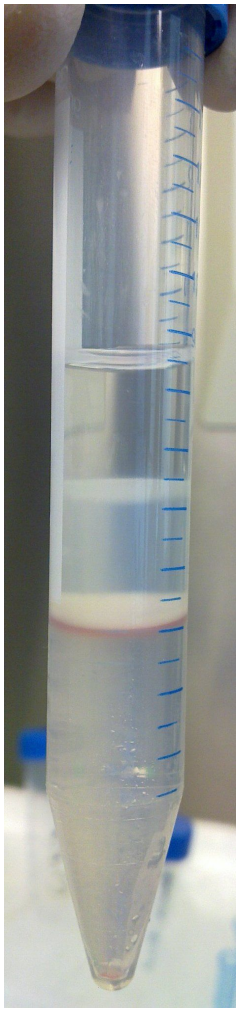
Sample preparation





- tissue – we need a single cell suspension
  - loose tissues (spleen, lymph nodes)
    → mechanic dissociation(sieve)
  - epithelial tissue → enzymatic digestion
    - trypsin, accutase, collagenases
- blood – removal of distracting particles
  - erythrozytes – lysis (hypotonic buffers)
  - density gradient centrifigation (Ficoll, Percoll)
    - peripheral blood mononuclear cells (PBMC)
- MACS

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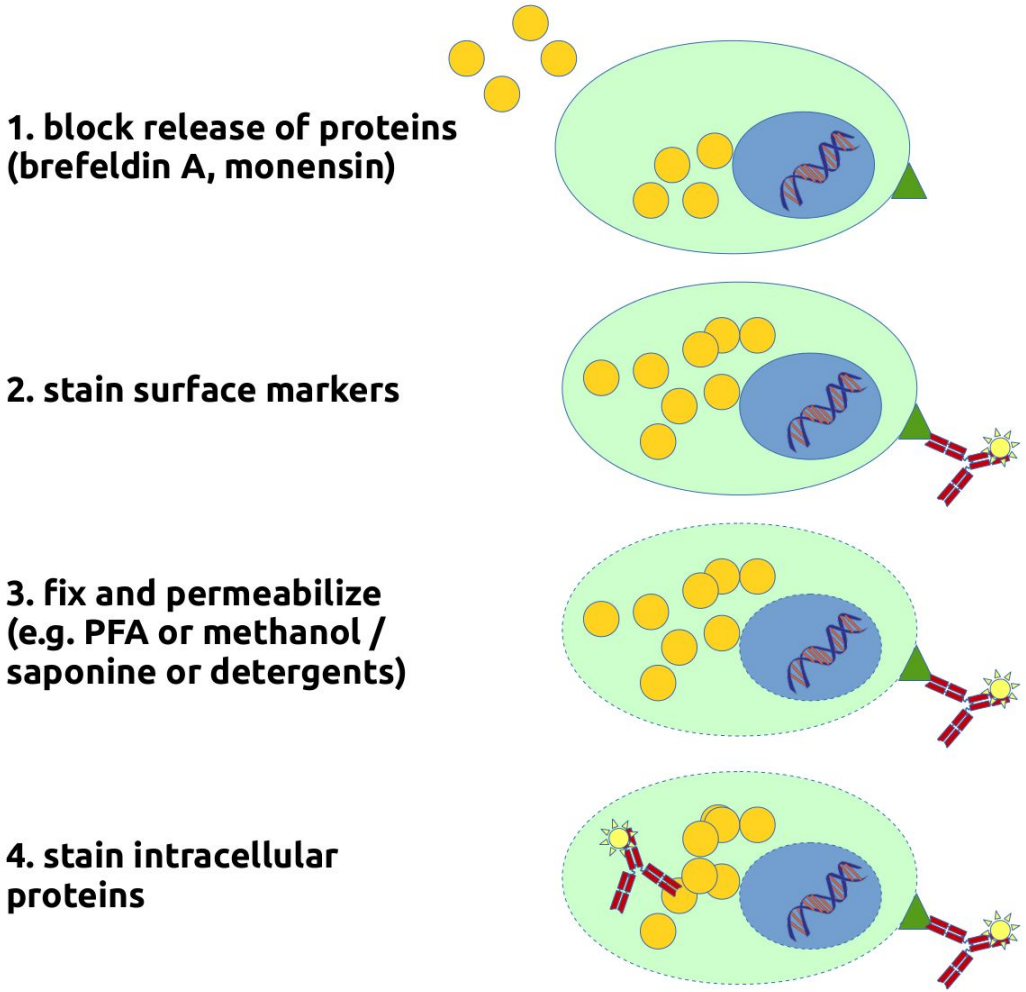
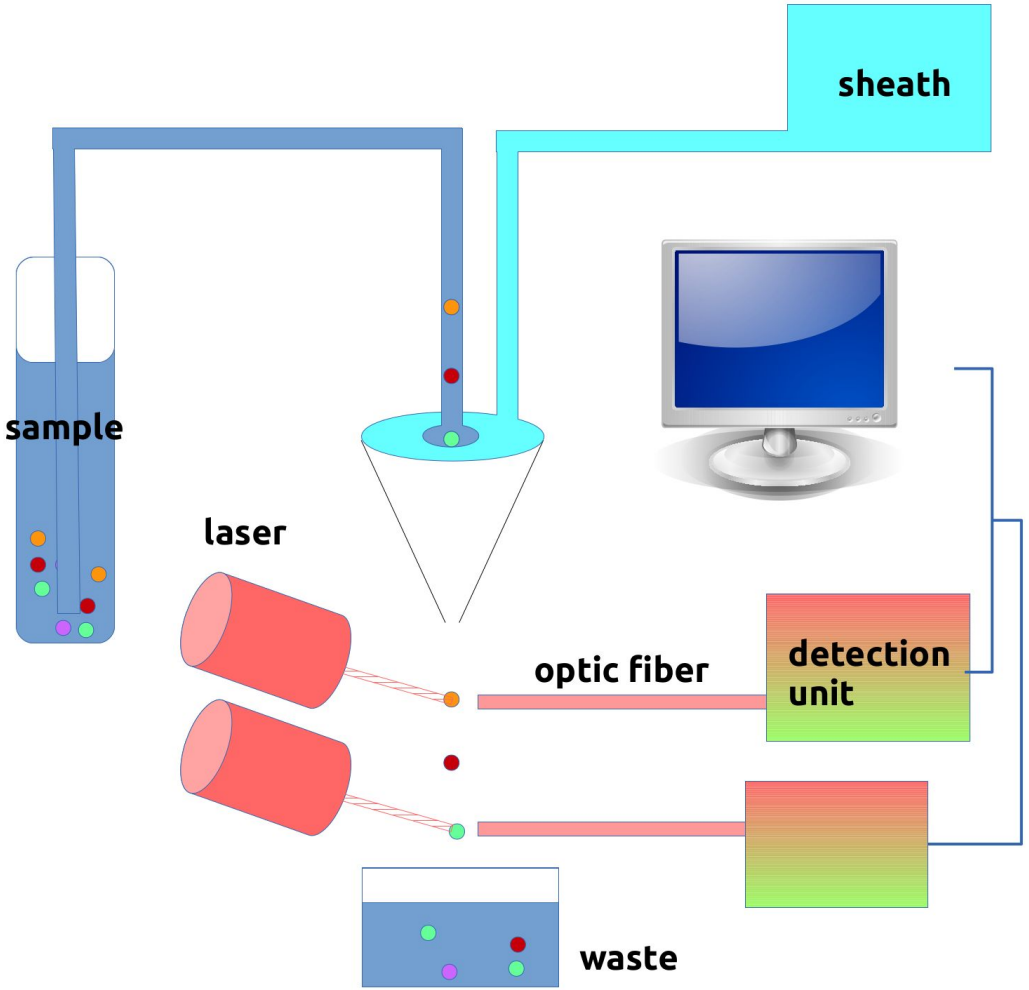
Sample preparation



- properties
  - size, granularity
  - fluorescence (specific antibodies)
- block unspecific binding (FcRs)
- staining of surface proteins
  - CD-nomenclature
- intracellular staining
  - transcription factors
  - phosphorylation
  - fixation and permeabilisation

Composition of a flow cytometer



- fluidics (blue)
  - sample stream
  - sheath fluid
- optics (red)
  - laser, lenses
  - optical fiber
  - filter, mirrors
- electronics (green)
  - photomultiplier
- computer
  - analysis

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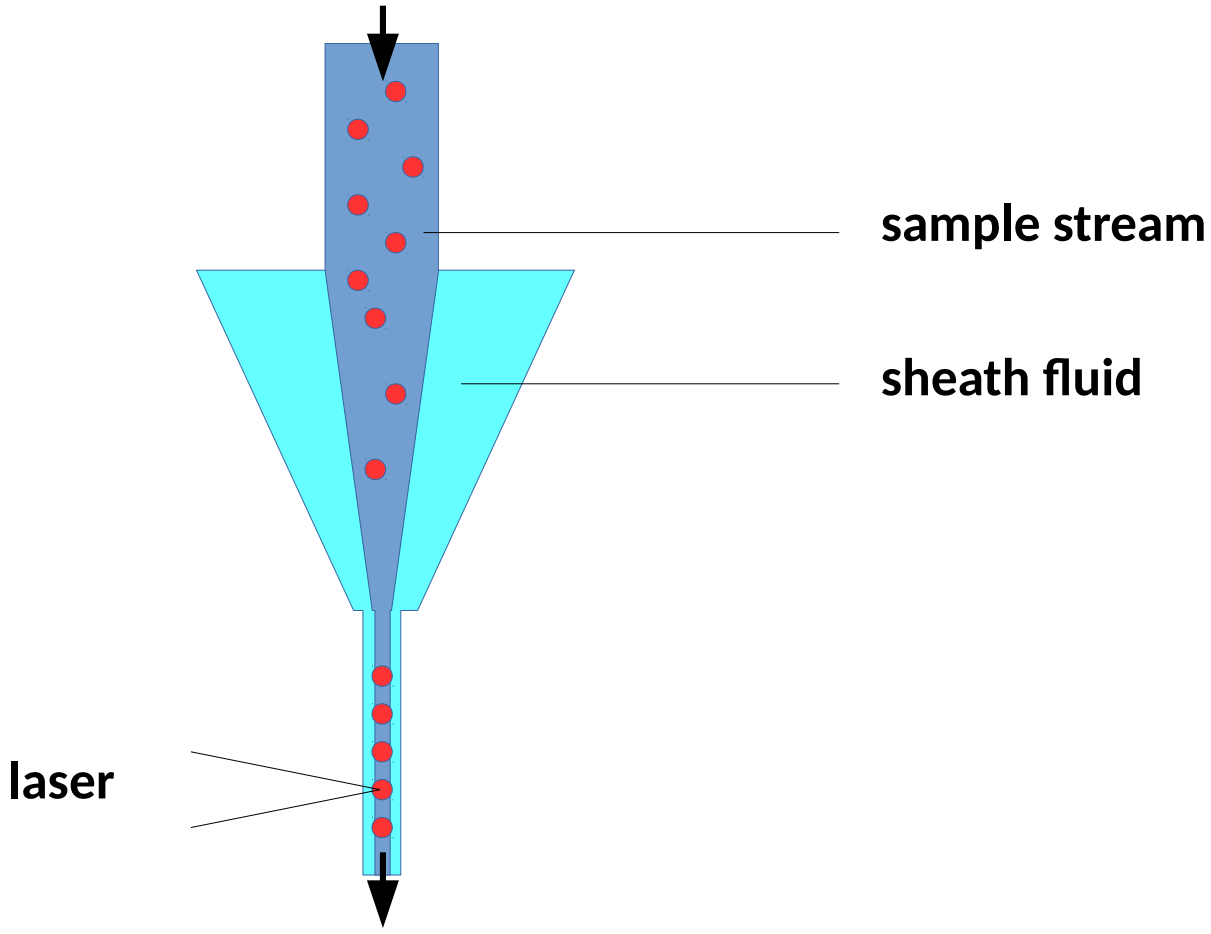
hydrodynamic focusing



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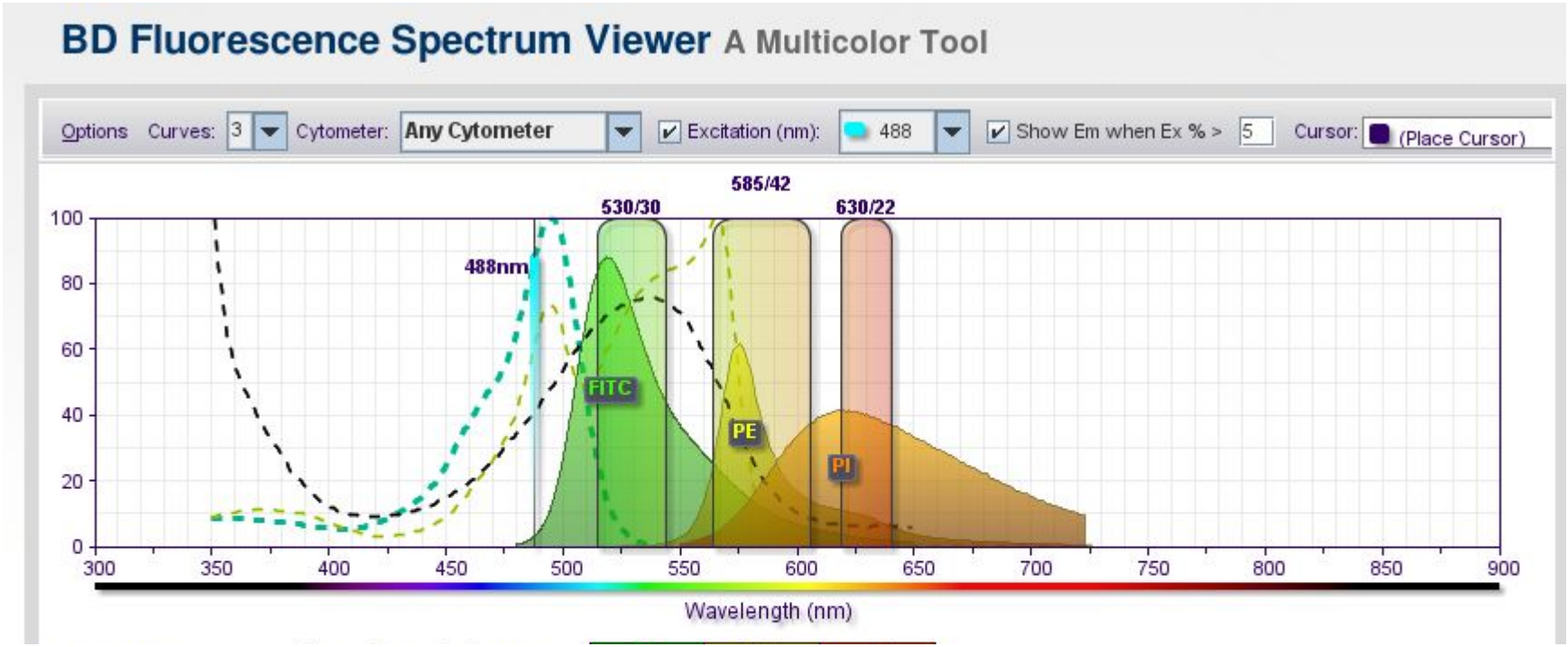
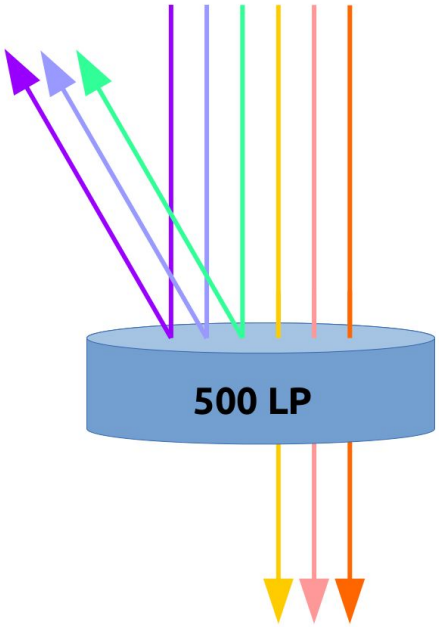
how to get the signals

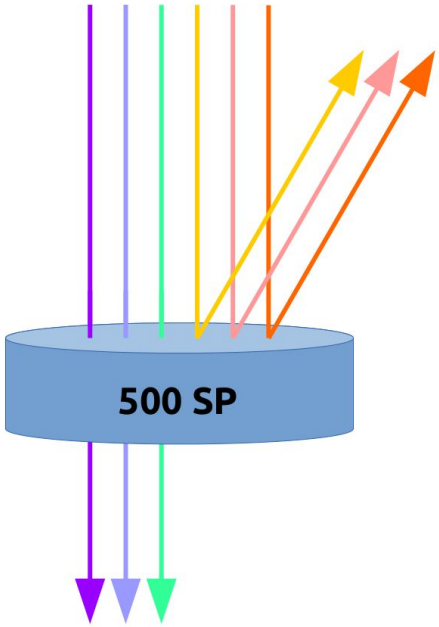


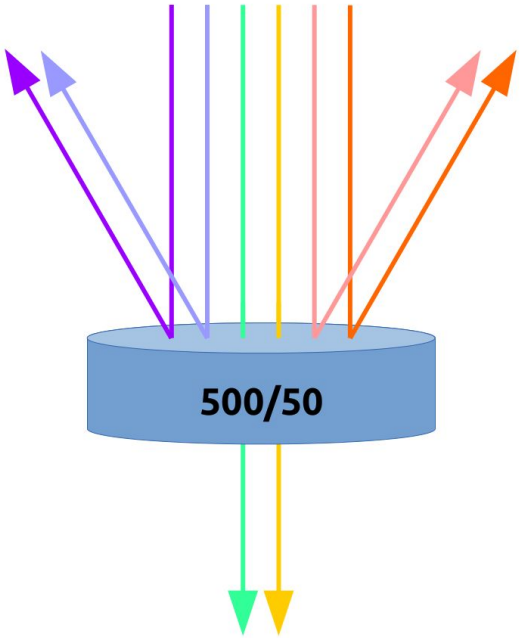
http://www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp

interference filter

Longpass **Shortpass** **Bandpass**







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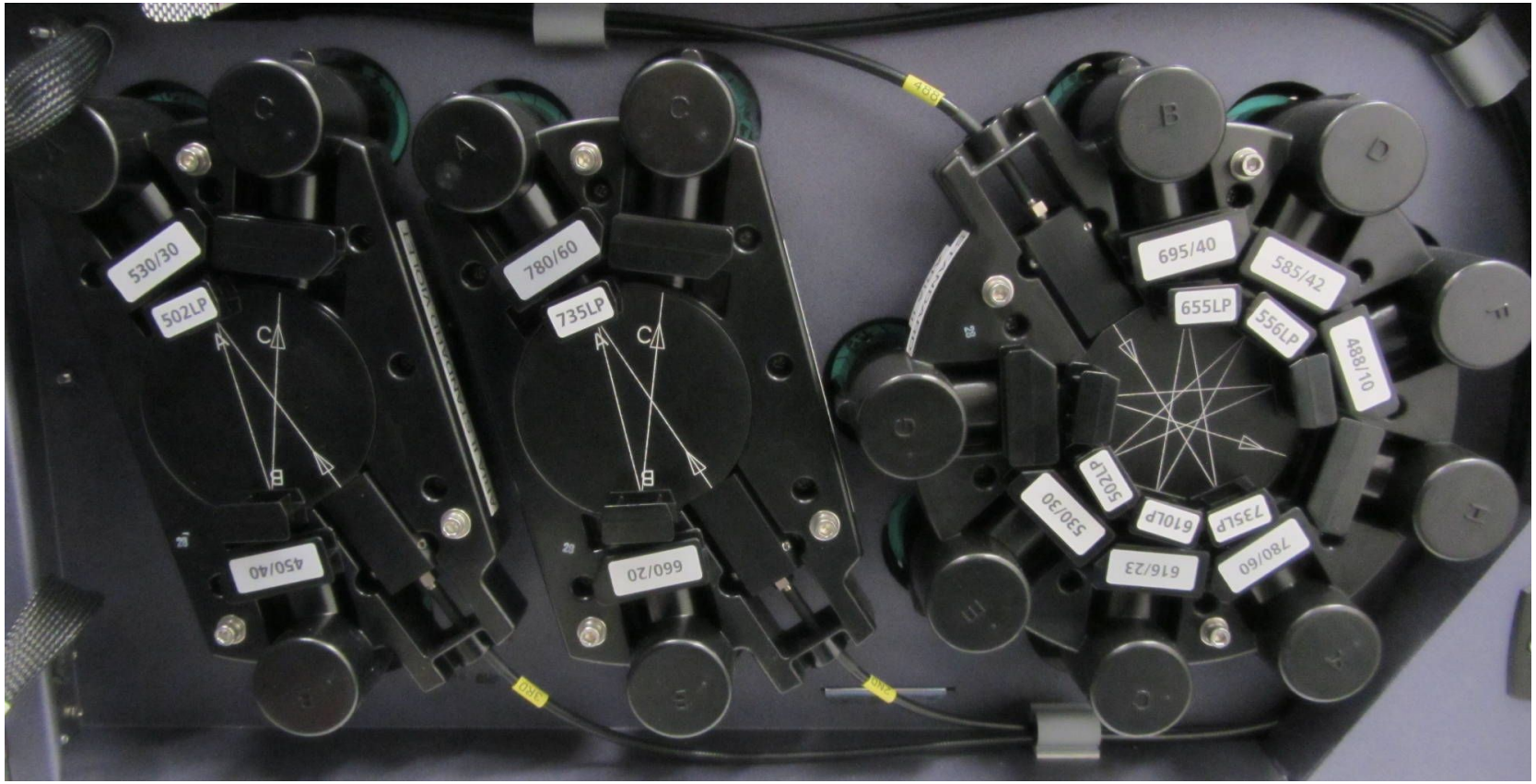
The filter system split the light

- FACS Aria II:



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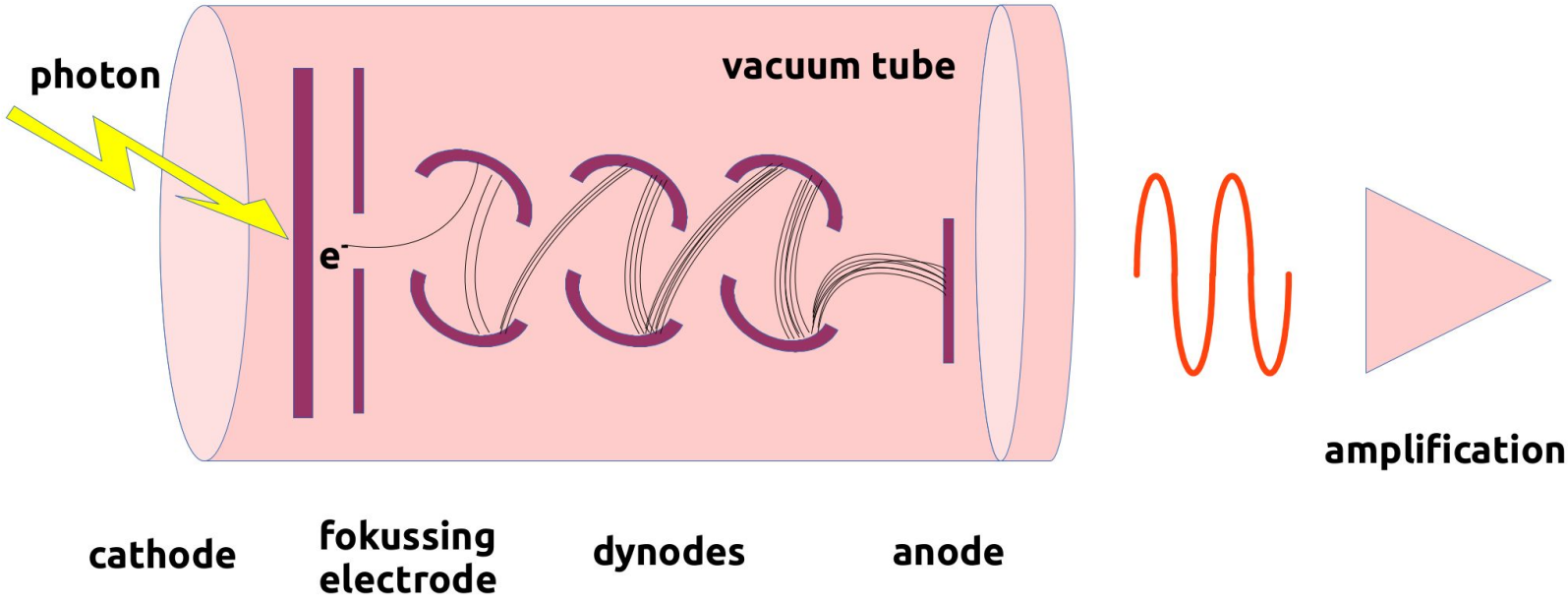
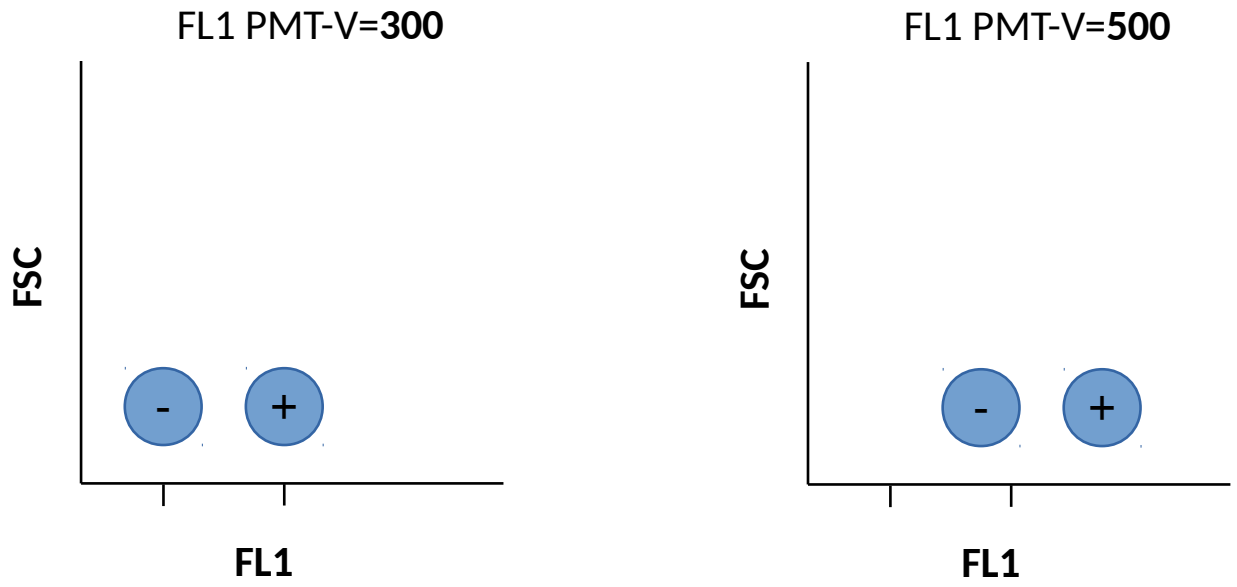
Photomultiplier (PMT)



- **all is relative in flow cytometry – you have to know where your signals are!**

Photomultiplier (PMT)

- signals are relative



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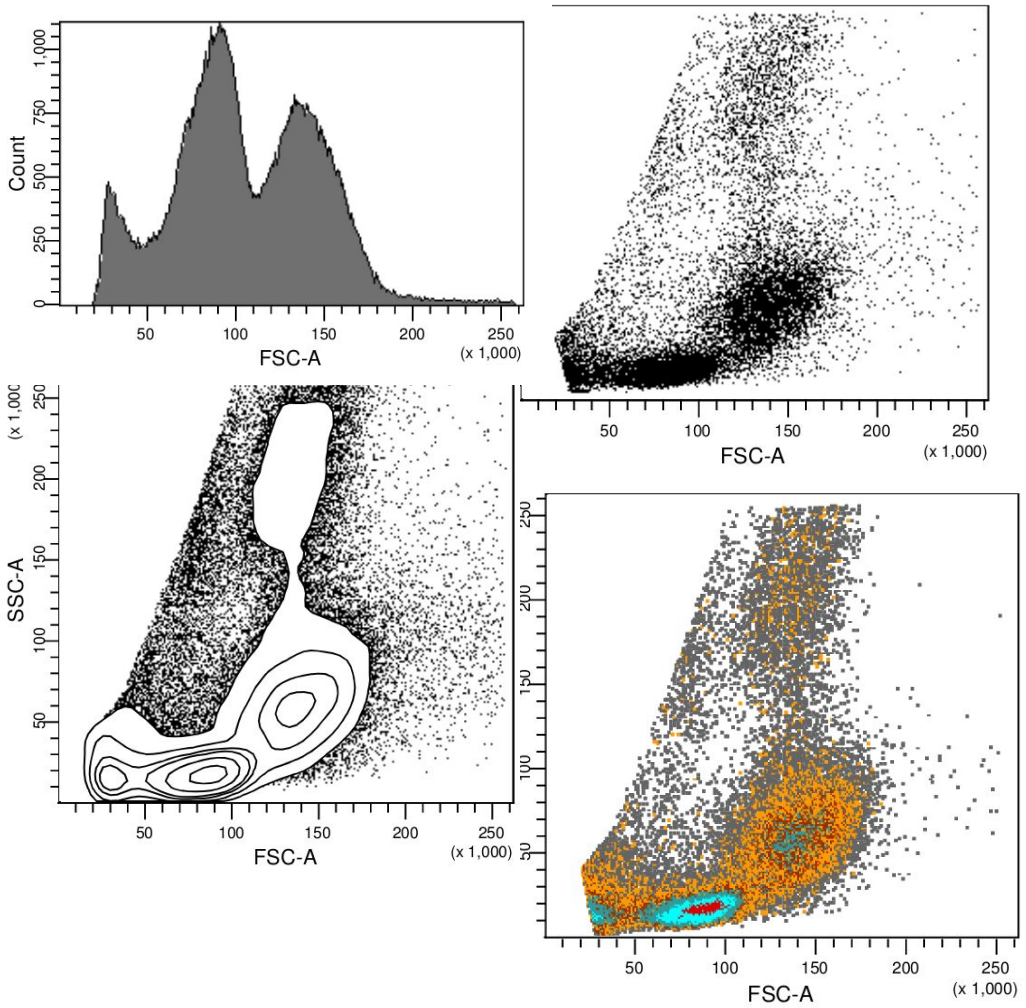
Plot types

- 1-dimensional
  - histogram
- 2-dimensional
  - dot plot
  - contour plot
  - density plot
- multidimensional
  - 3D still visualized
  - more dimensions only via algorithms



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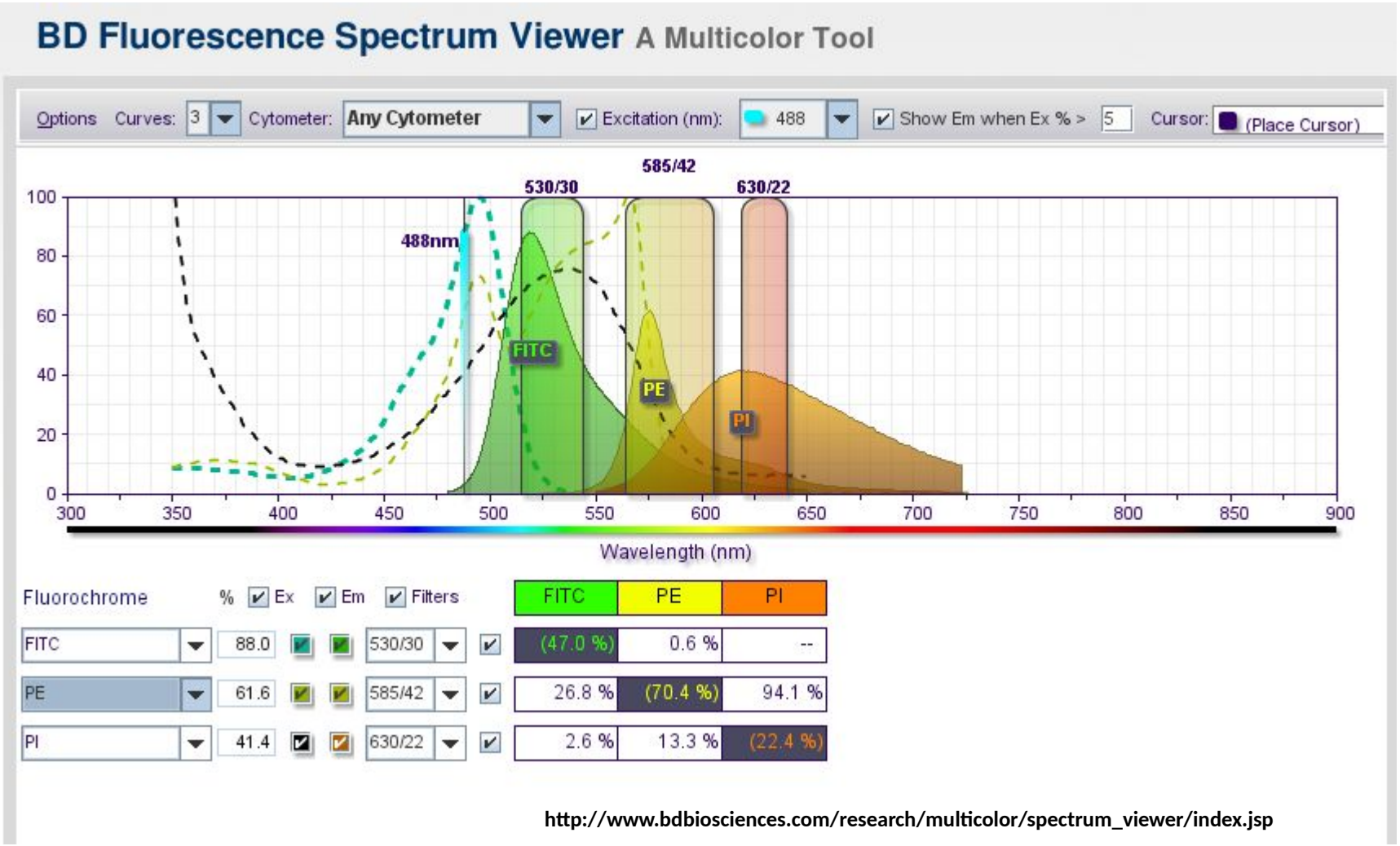
spectral overlap

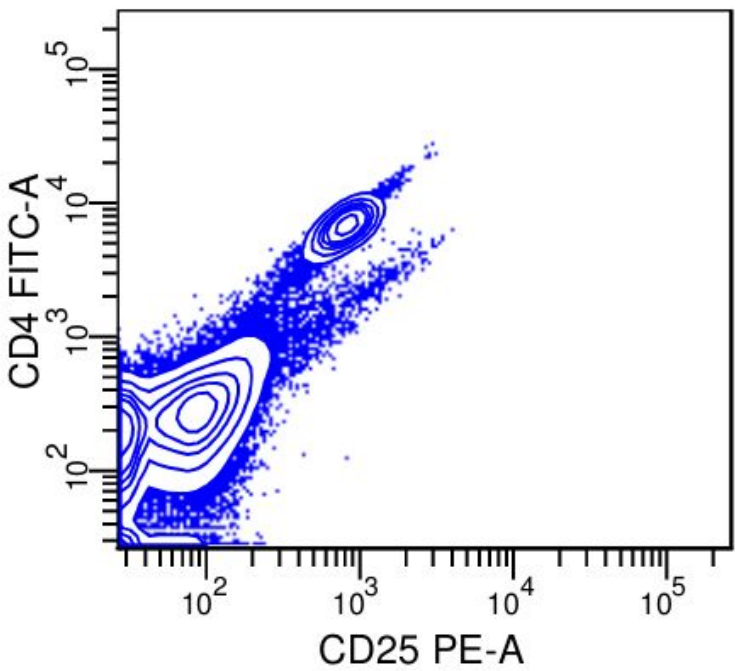


http://www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp

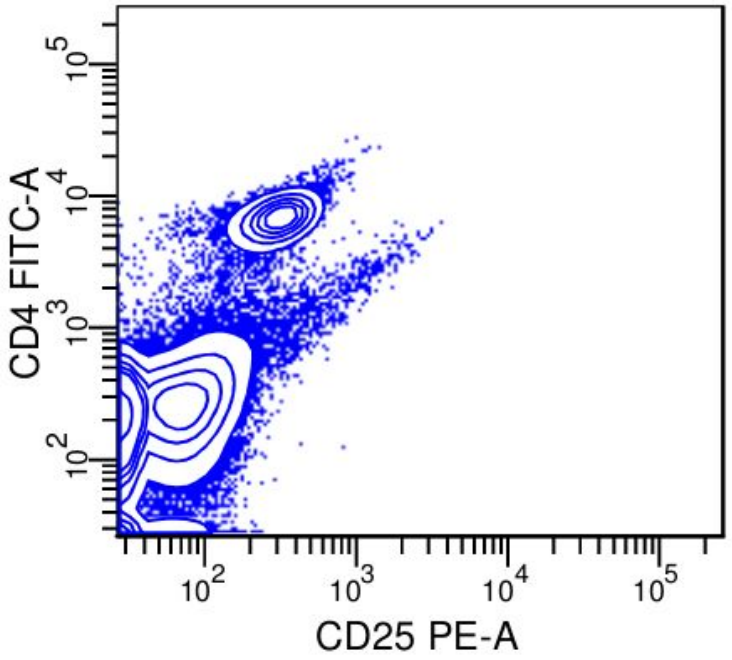
Compensation

- = removal of spectral overlap from your data

uncompensated

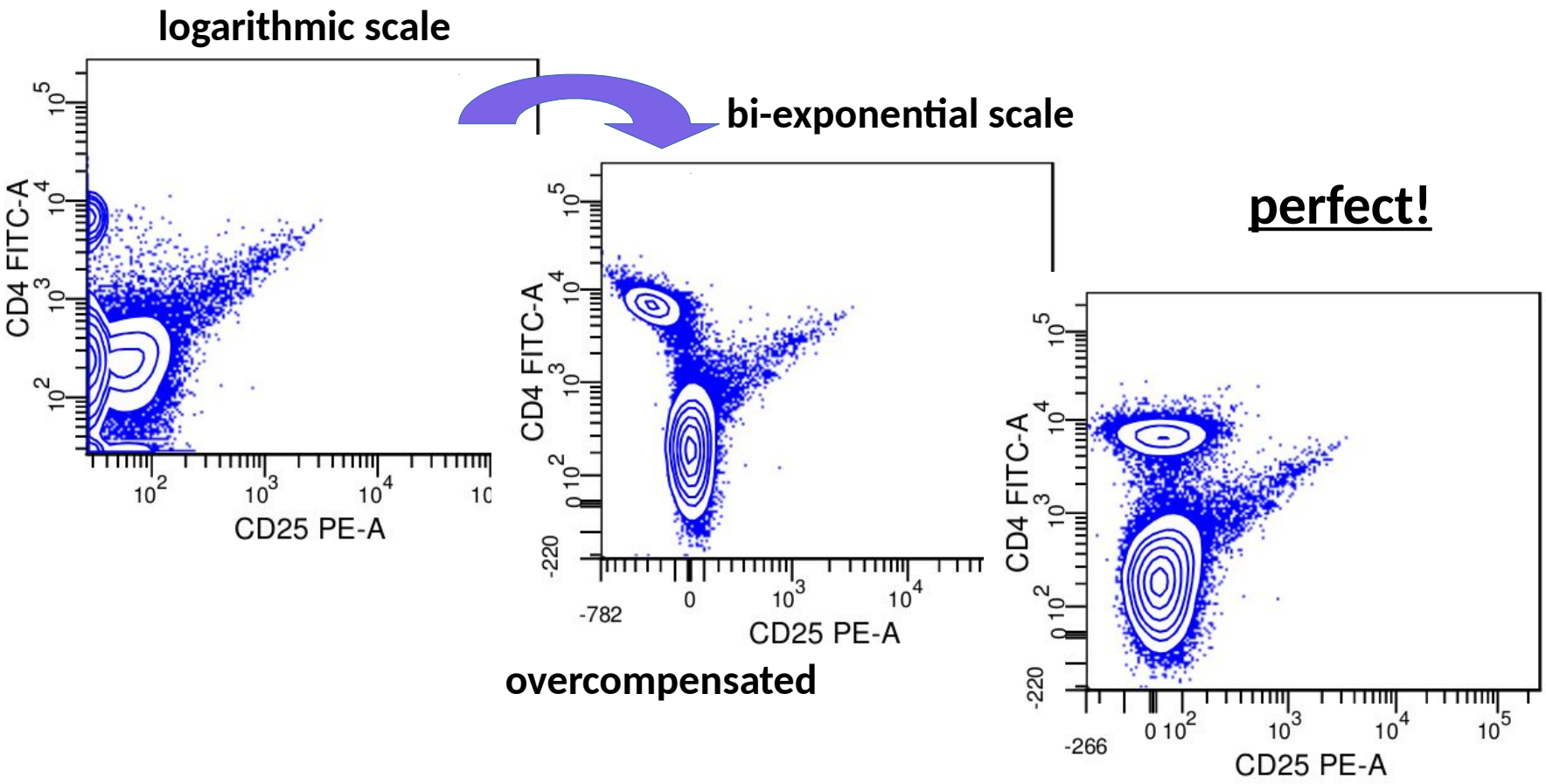


PE = PE – x% FITC



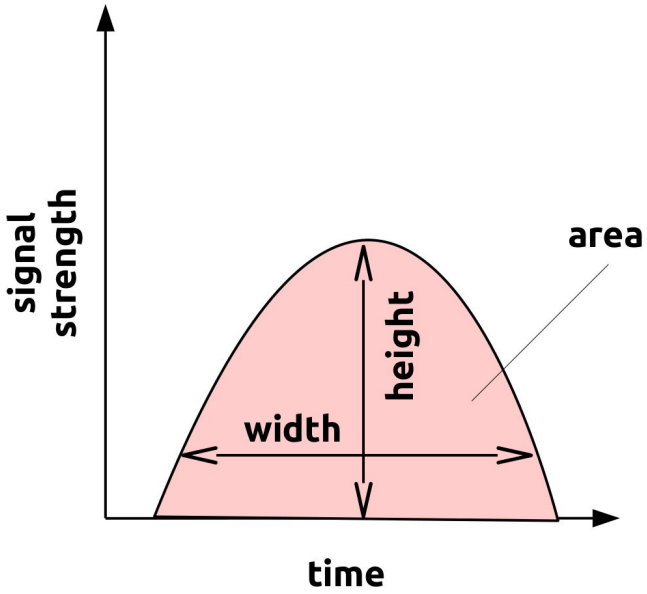
Compensation

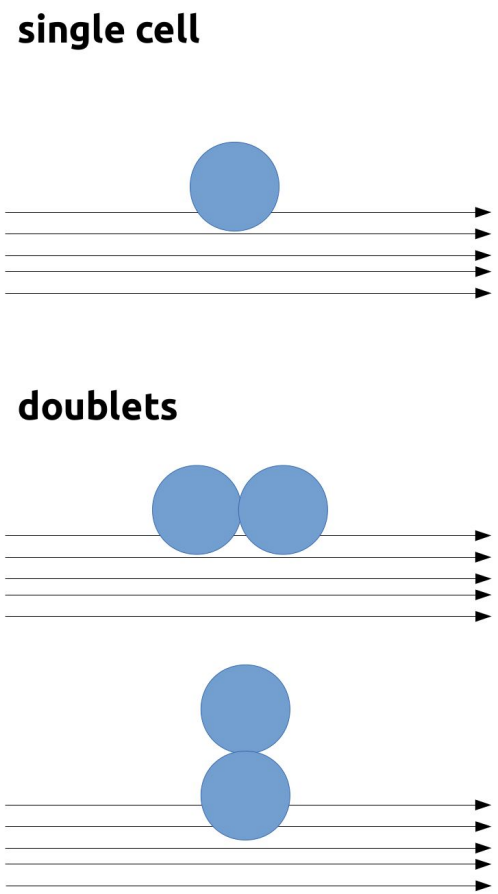
- = adjustment for the spectral overlap



Gating – single cells vs. doublets

- today's flow cytometer use three values per channel:





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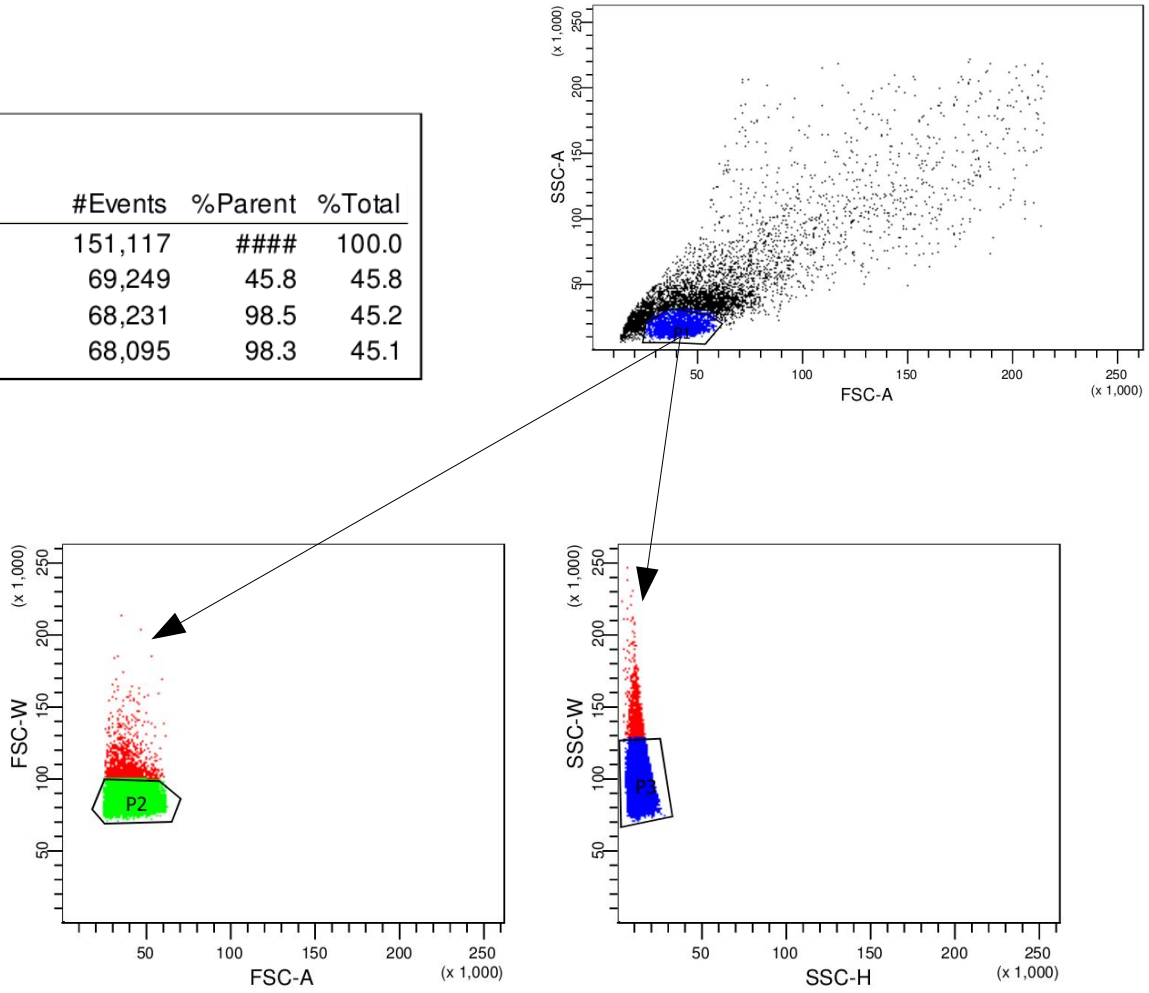
Gating – single cells vs. doublets

- example:

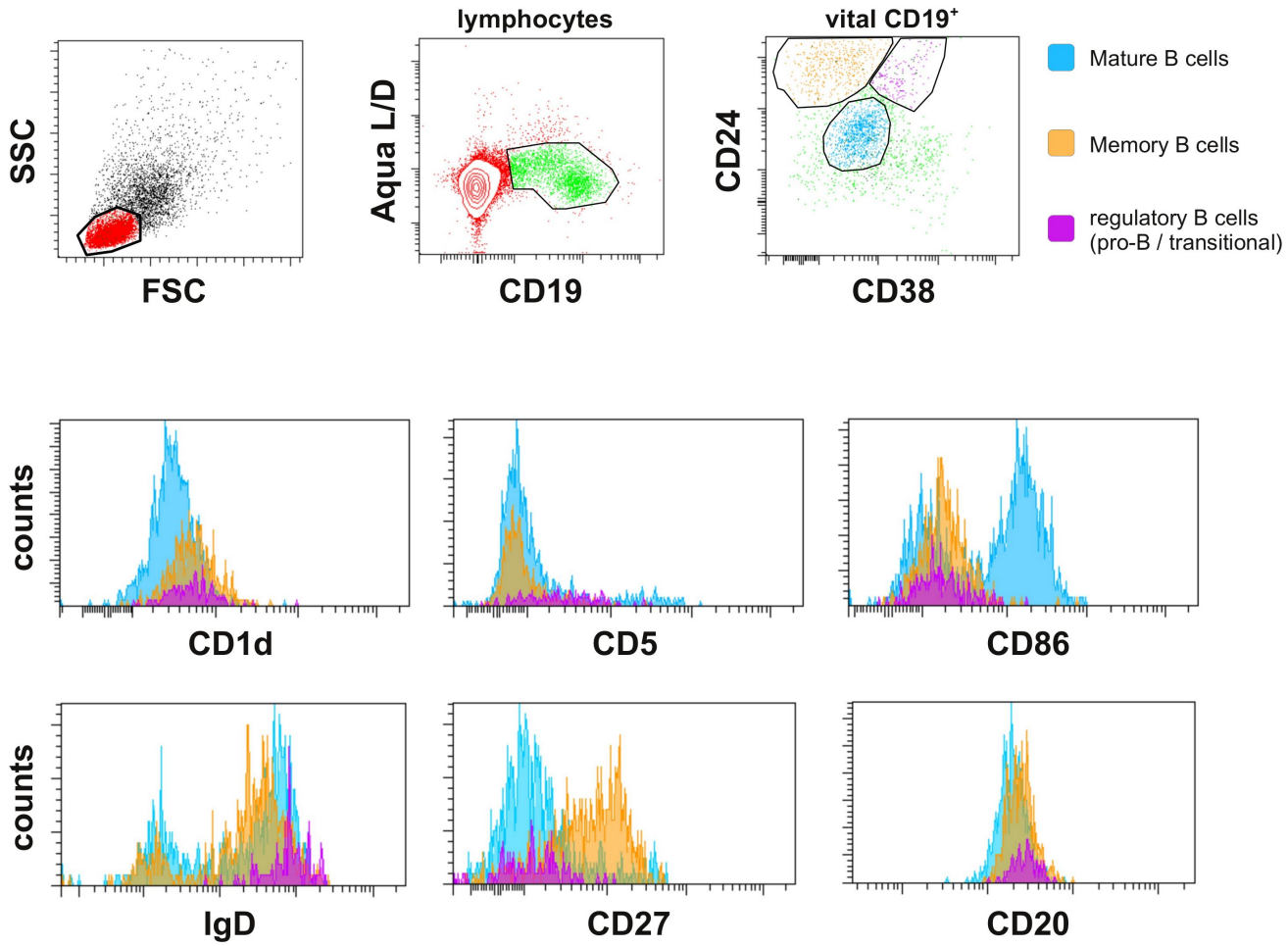
Tube: Treg_stain

Population	#Events	%Parent	%Total
all events	151,117	####	100.0
P1	69,249	45.8	45.8
P2	68,231	98.5	45.2
P3	68,095	98.3	45.1



Gating – fluorescence channels

- example:



panel design

- Basic rules:
  - low expressed marker → bright fluorochromes
  - highly expressed marker → dim fluorochromes
  - important marker → bright fluorochromes
  - „add-on“-marker → dim fluorochromes

- keep in mind:
  - spectral overlap: select fluorochromes with minimal spectral overlap for markers on the same cells

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panel design

Fluorochrome	Exzitation max (nm)	Exzitation Laser	Emission max (nm)	Brightness (1-5)
PE-Cy7	496, 565	488, 561	774	4
Brilliant Violet 570	405	405	570	3
Brilliant Violet 421	405	405, 360	421	5
Pacific Blue	410	405, 360	455	1
FITC	493	488	525	3
APC	645	595, 633, 635, 647	660	5
PE	496, 565	488, 561	575	5
PerCP-Cy5.5	482	488	690	3
PerCP	482	488	675	2
APC-Cy7	650	595, 633, 635, 647	774	2
Alexa488	495	488	519	3
Alexa647	650	595, 633, 635, 647	668	4

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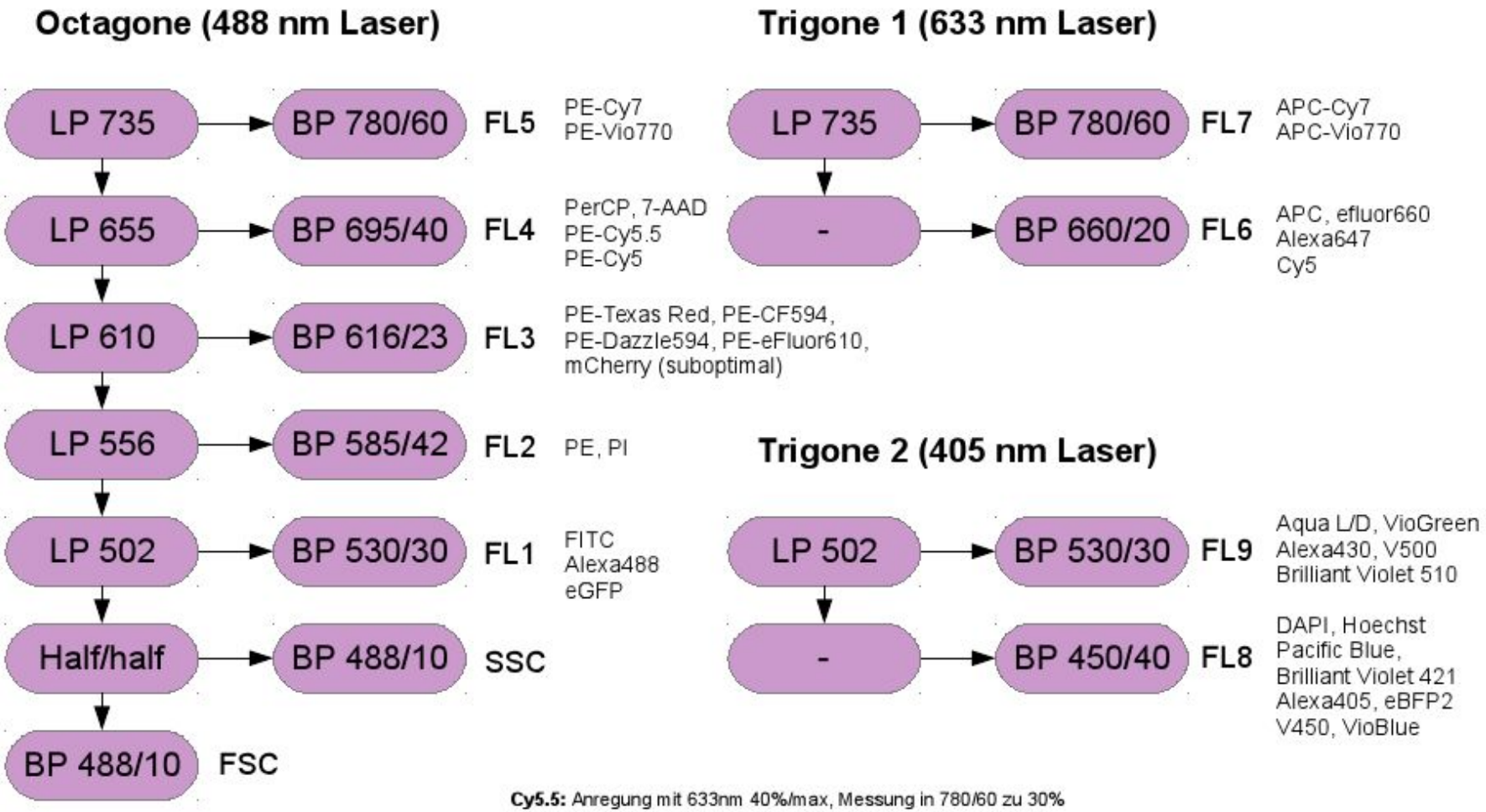
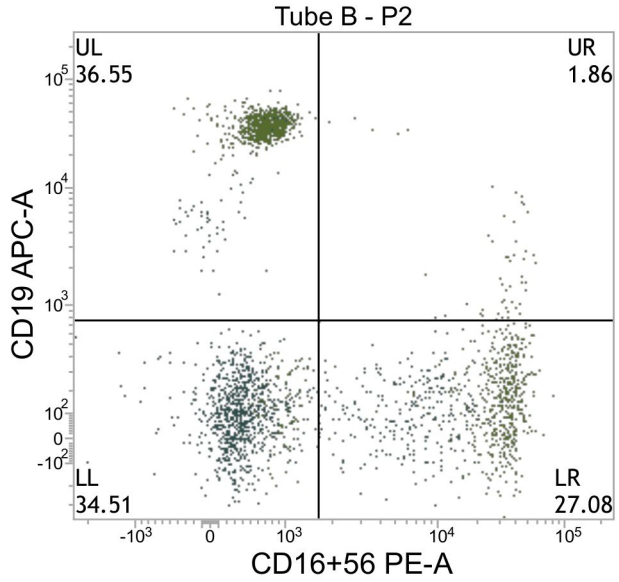
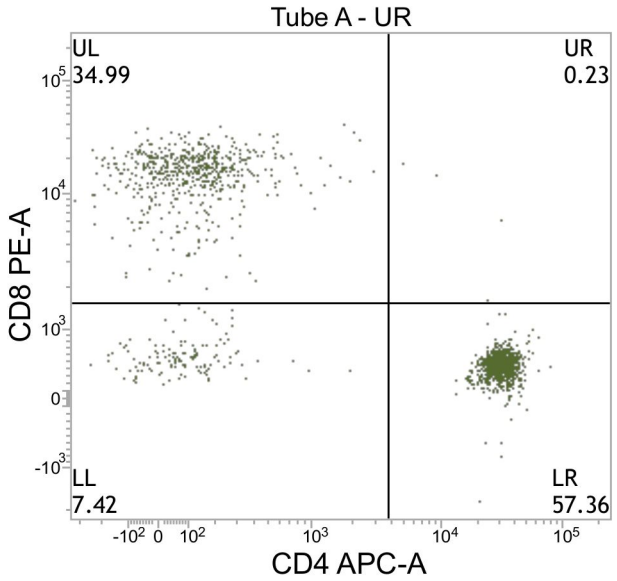
Is your fluorochrome detectable?



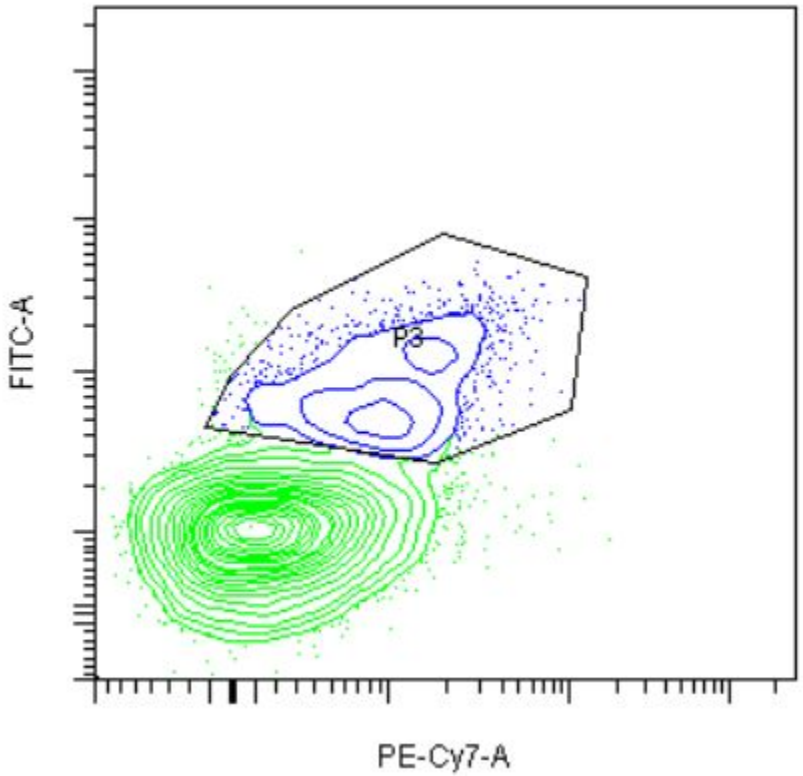
Cy5.5: Anregung mit 633nm 40%/max, Messung in 780/60 zu 30%
Cy7: Anregung mit 633nm 10%/max, Messung in 780/60 zu 80%
Cy3: Anregung mit 488nm 25%/max, Messung in 585/42 zu 80%

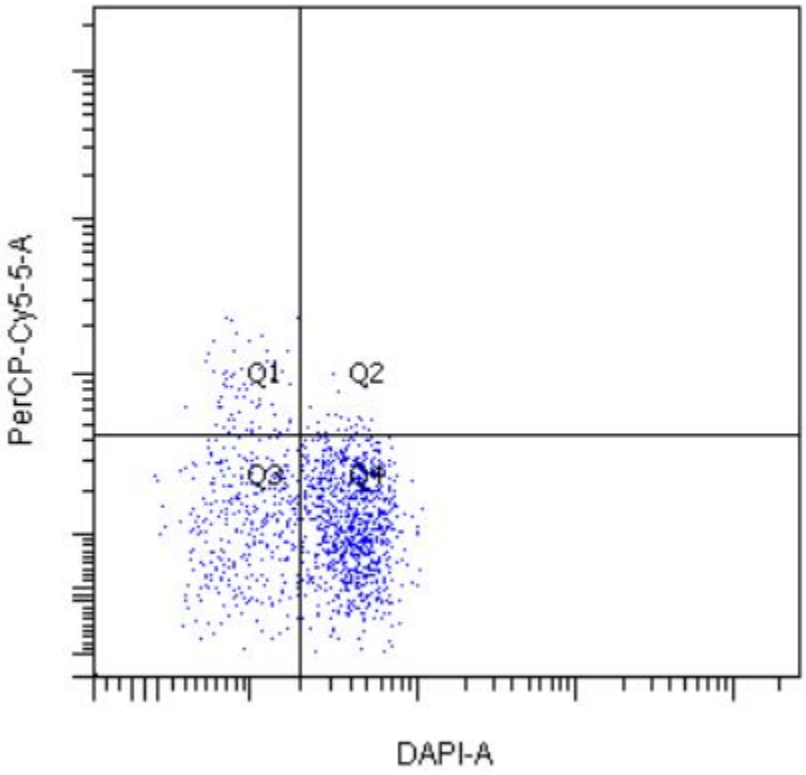
Controls – not necessary for bimodal staining





Controls – negative or positive?





Controls – isotype control

- **control for unspecific binding** via protein-protein interaction and Fc-receptor binding **IF**:
  - same species and isotype
  - same fluorochrome
  - same fluorochrome per antibody ratio
  - same concentration of the isotype control antibody
- problems
  - F:P ratio variable, tandem fluorochromes
  - degree of aggregation
  - does not control for spillover from other channels

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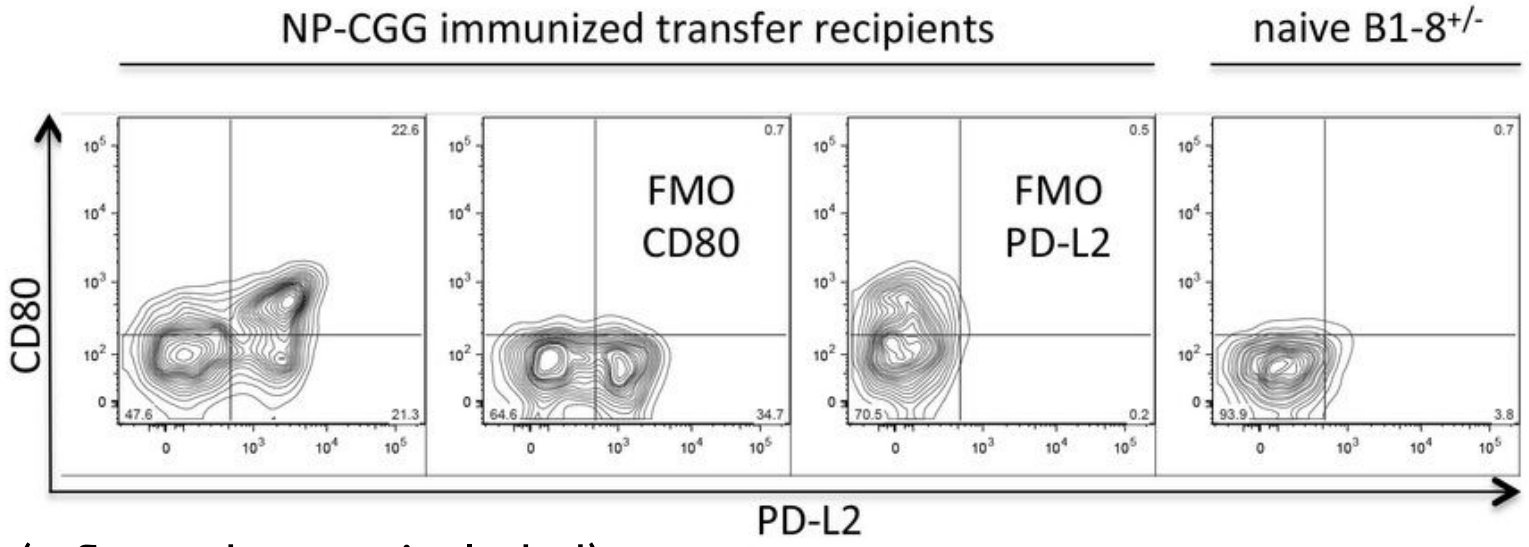
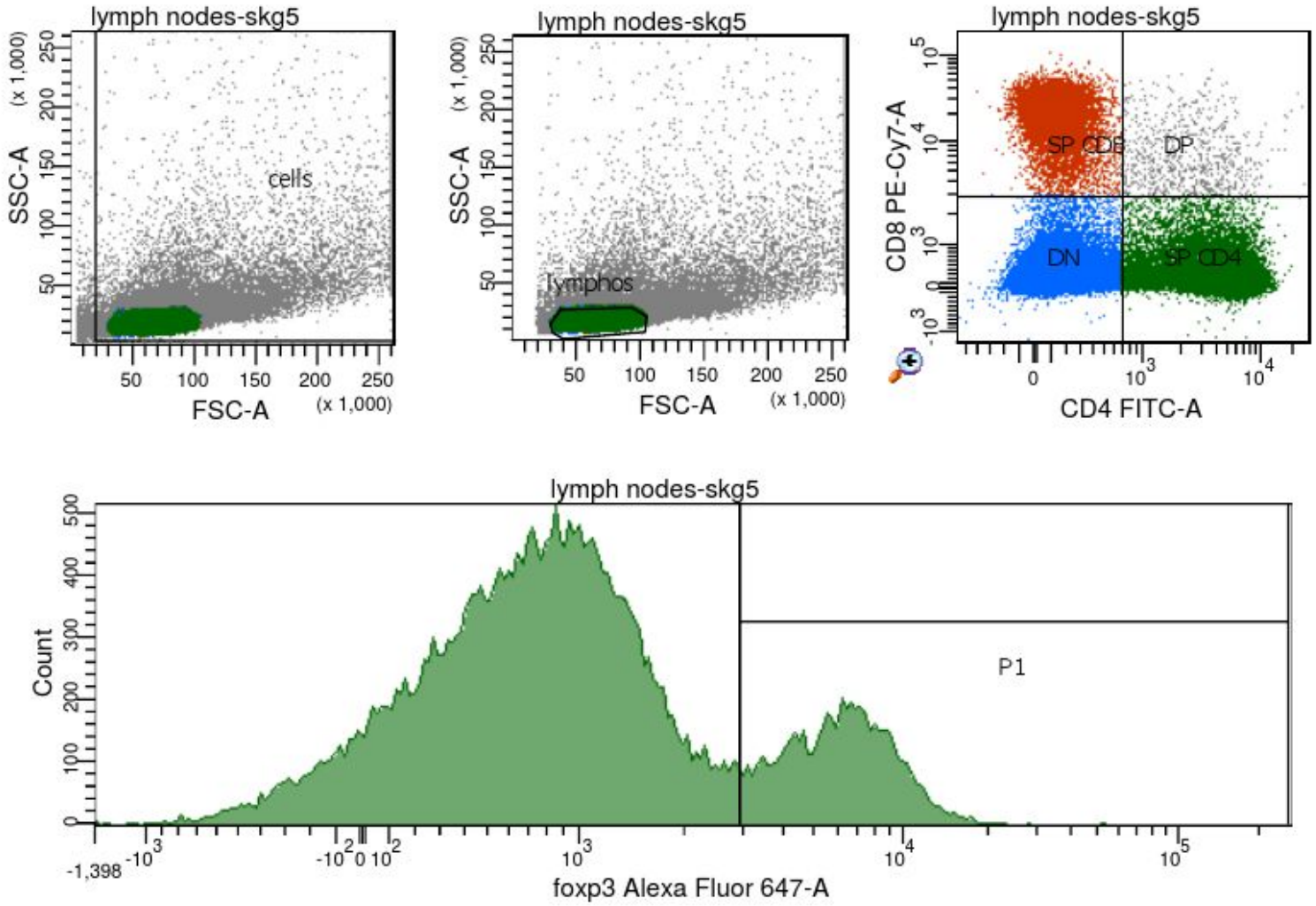
Controls – fluorescence minus one (FMO)

- **control for spillover** from other channels
  - e.g. myeloid cell gating:
  - SSC vs. CD45 → CD11b vs lineage → pDC marker vs mDC-marker, then:

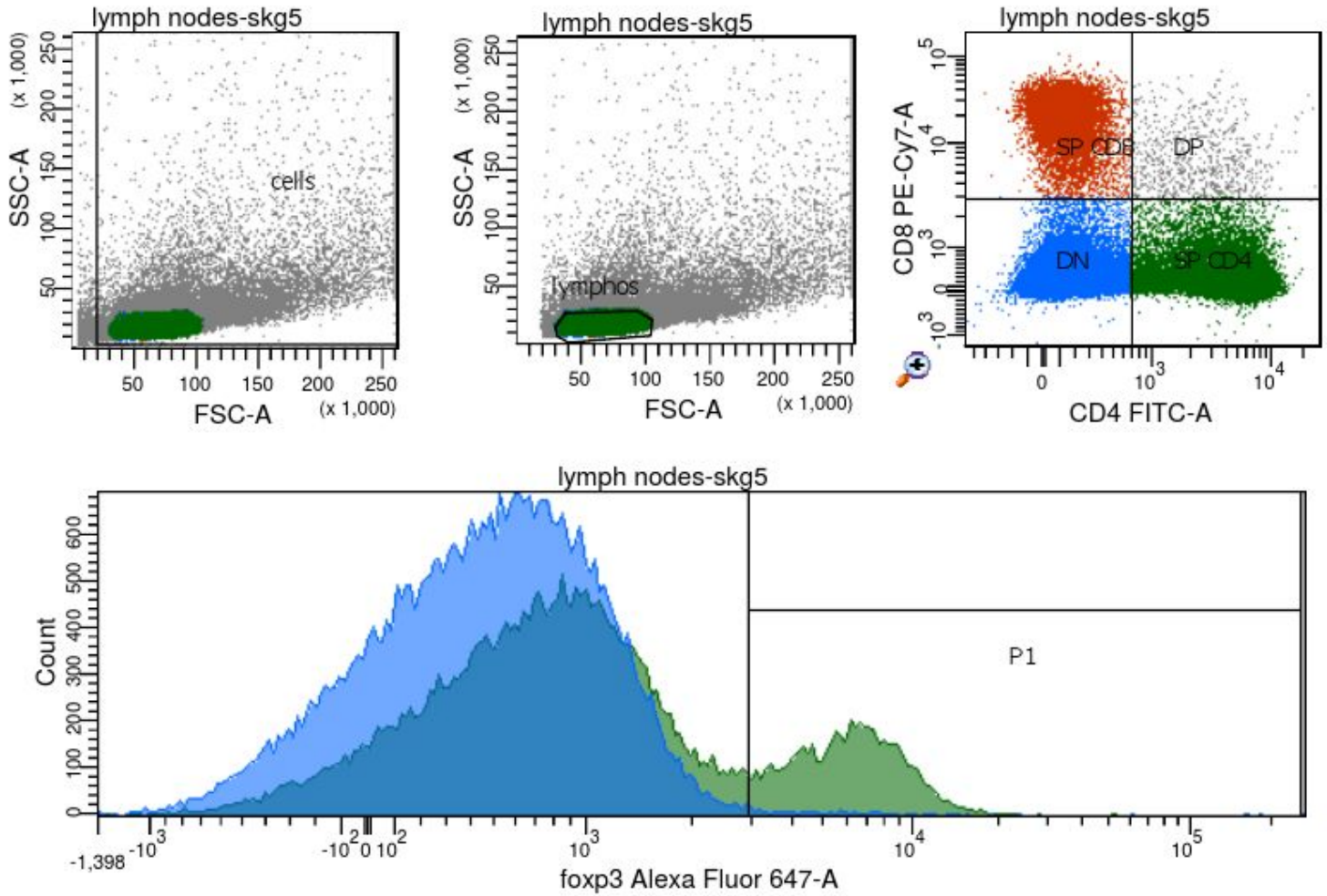


(7 fluorochromes included)

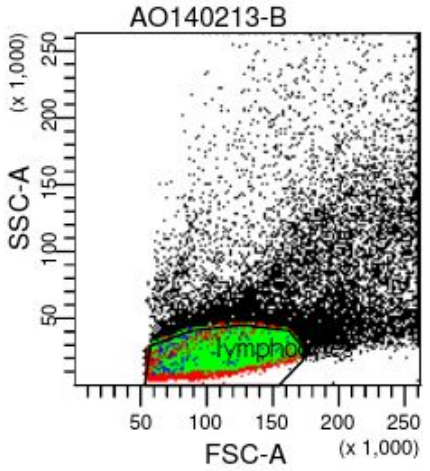
Controls – internal control

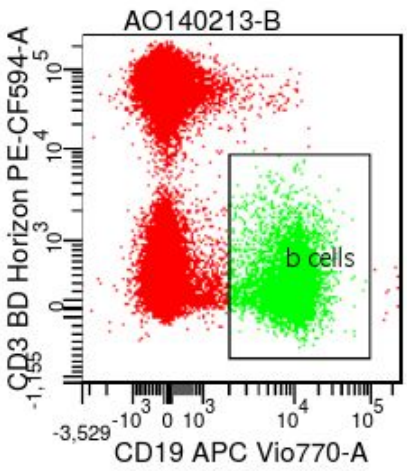


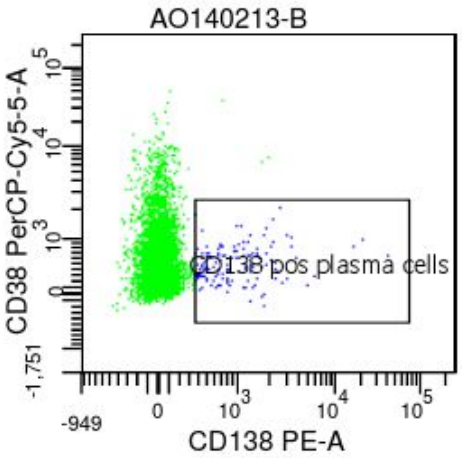
Controls – internal control



Controls – dead cells



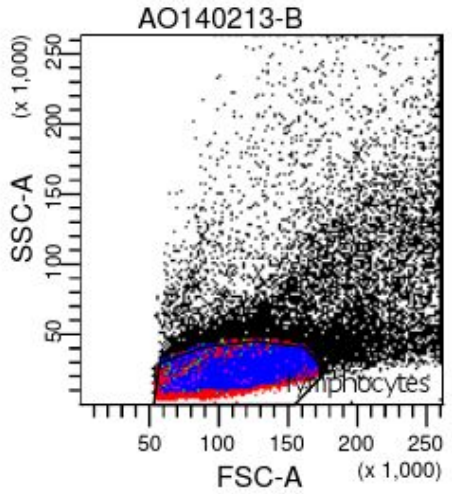


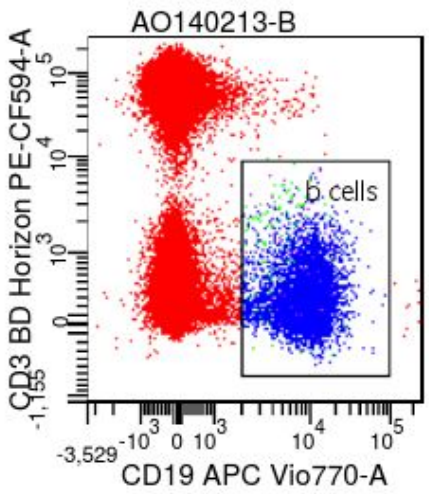


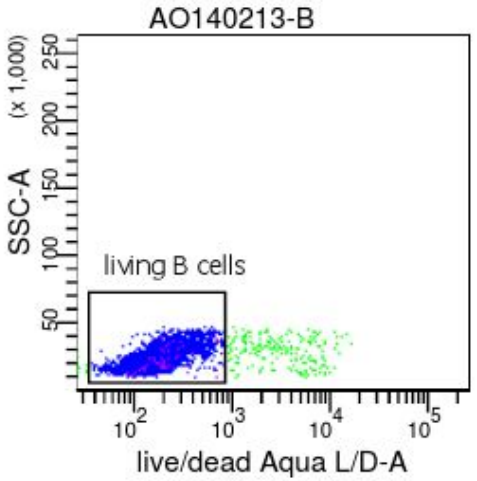
Tube: B

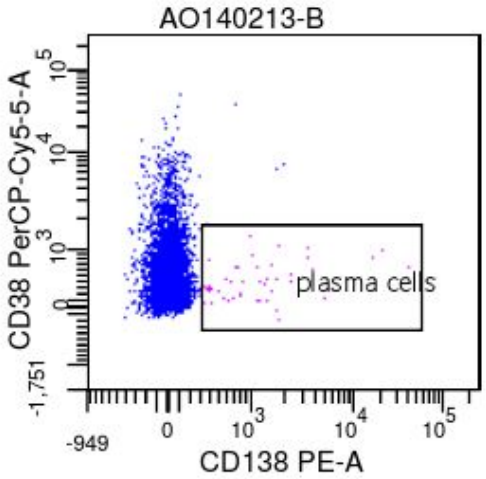
Population	#Events	%Parent	%Total
All Events	110,000	####	100.0
lymphocytes	94,689	86.1	86.1
b cells	6,201	6.5	5.6
CD138 pos plasma cell:	135	2.2	0.1

Controls – dead cells









Tube: B

Population	#Events	%Parent	%Total
All Events	110,000	####	100.0
lymphocytes	94,689	86.1	86.1
b cells	6,201	6.5	5.6
living B cells	5,980	96.4	5.4
plasma cells	50	0.8	0.0

Conclusions

- **planning phase decides upon fail or success**
- **titrate your antibodies and improve blocking step in order to minimize background – isotype controls do not help you later**
- **know your machine**
- **know your sample**

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Applications for flow cytometry

- flow cytometry is working on **single cell level** – often not all cells react the same upon treatment (e.g. impurities of <1% can cause huge effects on cytokine conc. in supernatant measured by ELISA)
- **identification** of cells (immune cell subtypes, stem cells → often >4 surface-marker standard nowadays)
- **functional** characterization of cells (cytokine expression, ligand and receptor expression, transcription factors and other effector proteins)
- analyzing signaling **pathways** (phospho-specific antibodies, Ca^{2+} flux)
- viability, proliferation, apoptosis (early up to late stages), telomere length
- multiplex **protein quantification**

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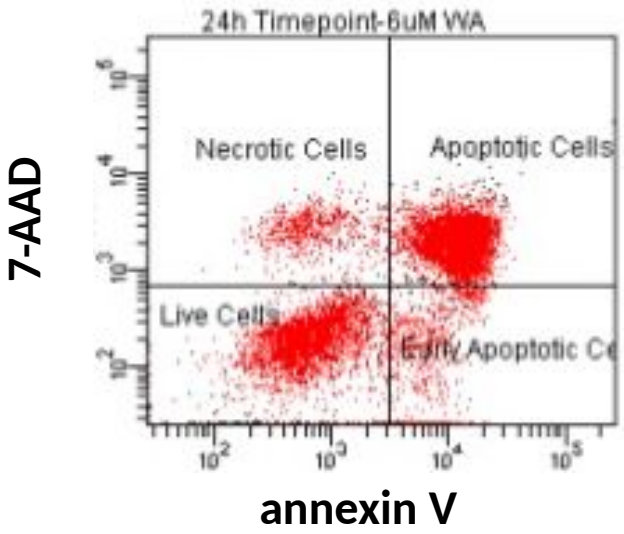
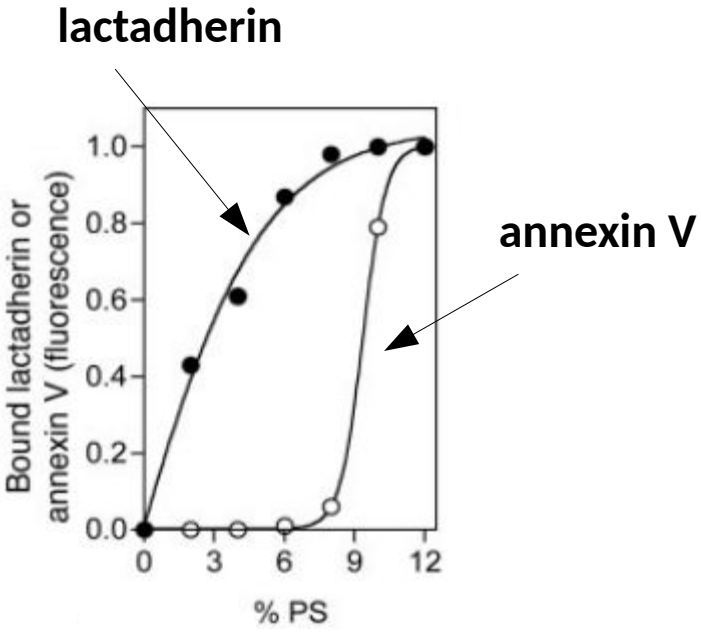
examples – apoptosis

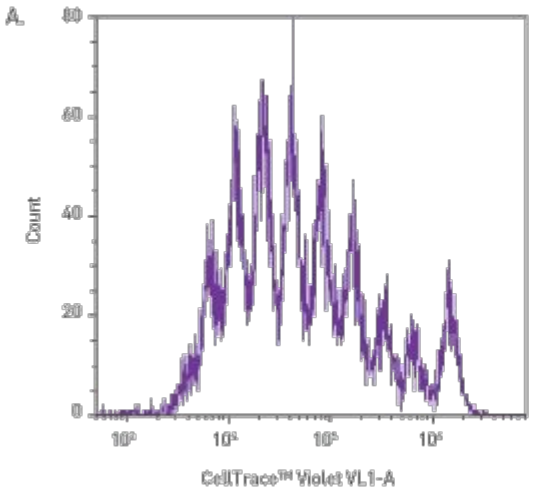


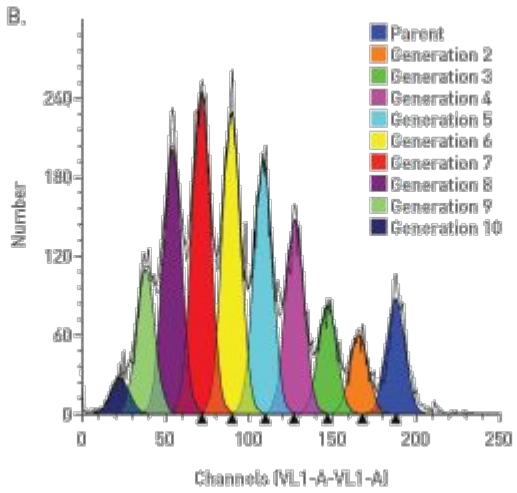


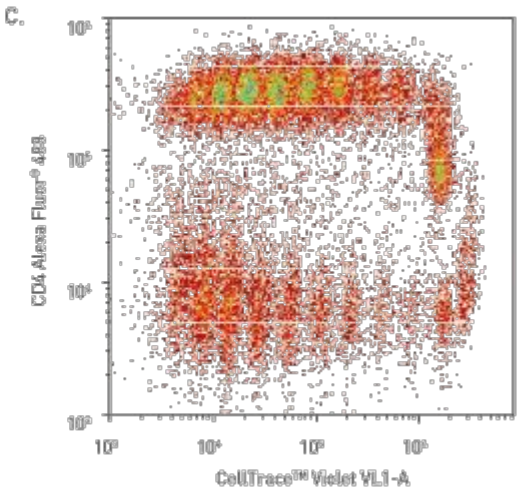
- 7-AAD has much lower spectral overlap to other channels

- no special buffers needed
- earlier and stronger staining

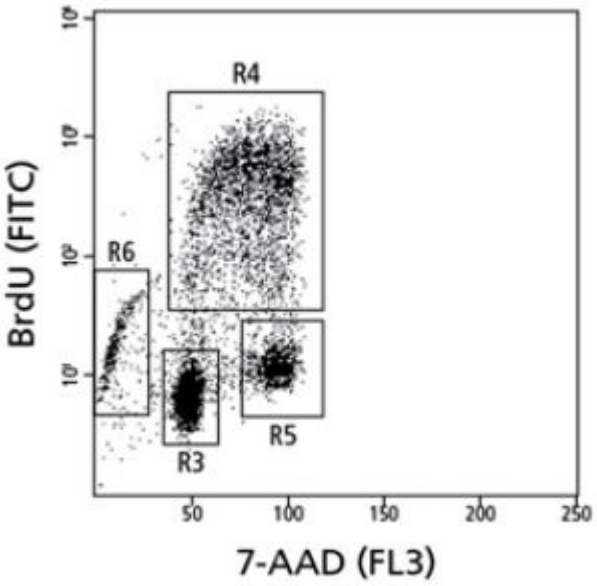
examples – proliferation



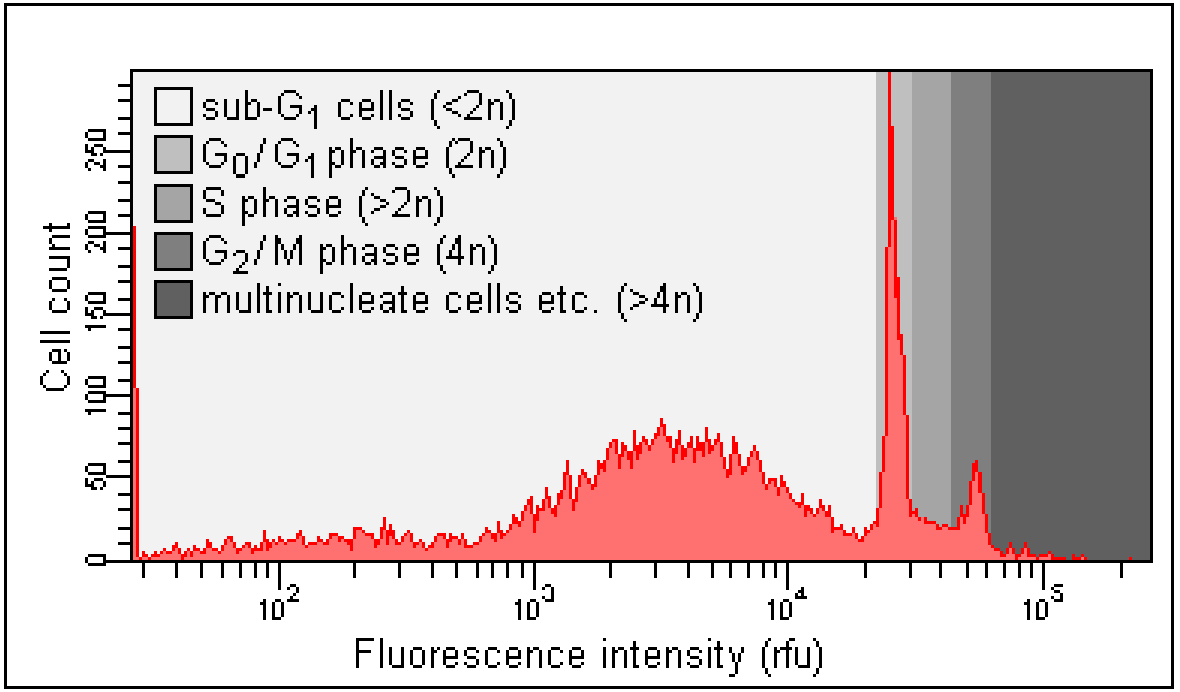




examples – cell cycle

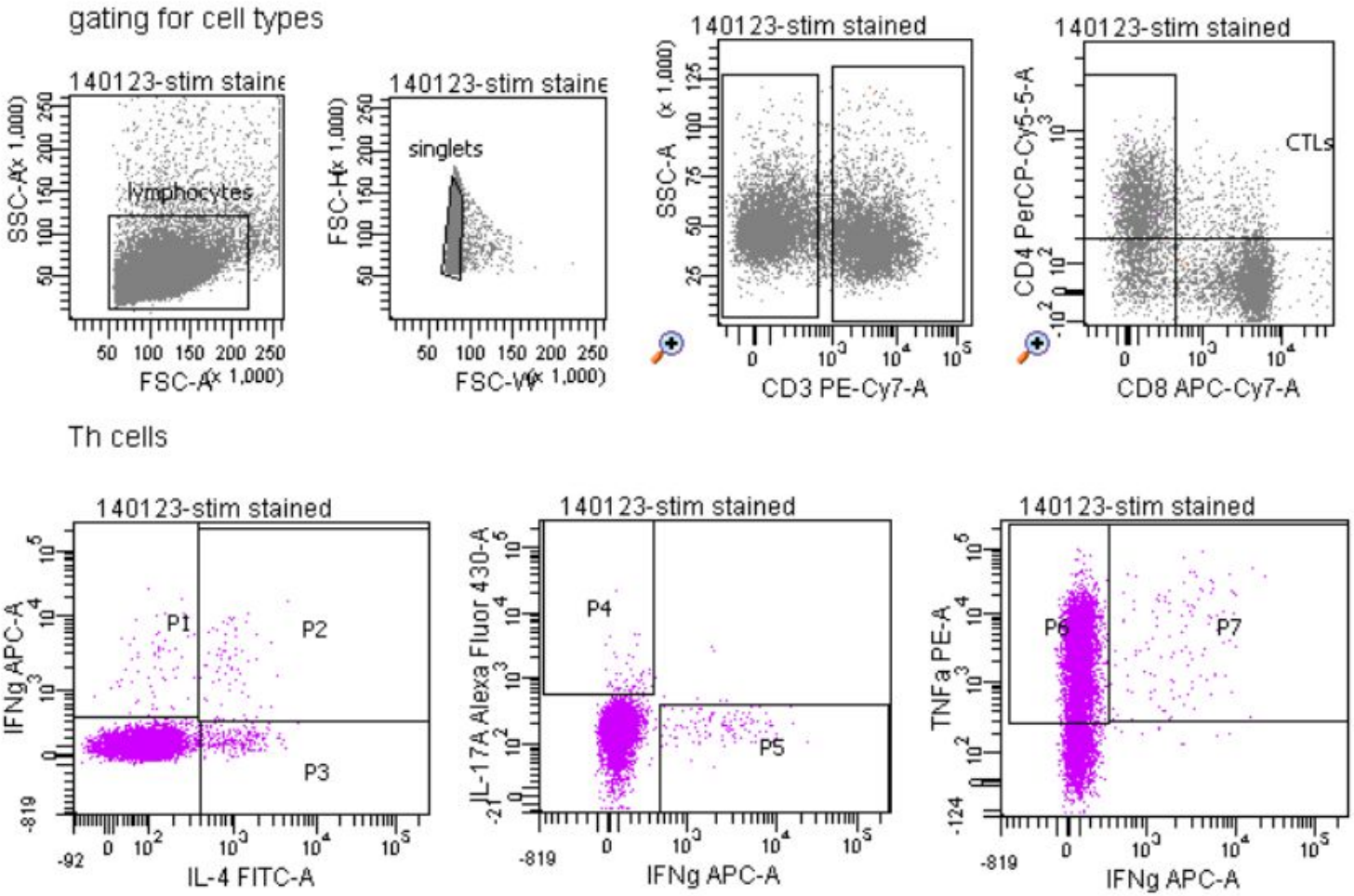


R3=G0/G1
R4=S
R5=G2/M
R6=SubG0
G1

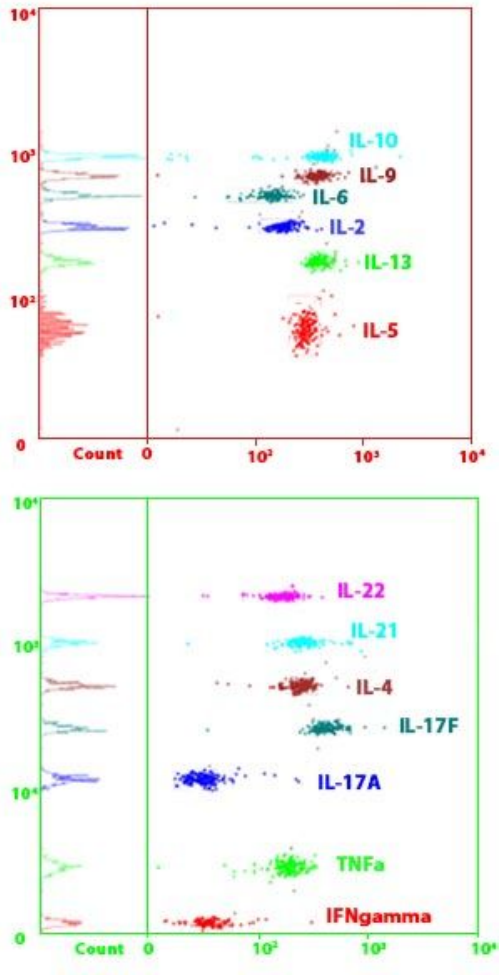


examples – intracellular proteins

- INFg
- IL-4
- IL-17A
- TNFa
- IL-10



examples – cytometric bead assay



- **e.g. from BioLegend**
  - **13 cytokine plex (min. 100 tests) for 1200€**
  - **per sample per cytokine costs:**
    - **92 cent**
- **BioPlex**
  - **8 cytokine plex (96-well) for 1900 €**
  - **costs per sample per cytokine:**
    - **2.5 €**

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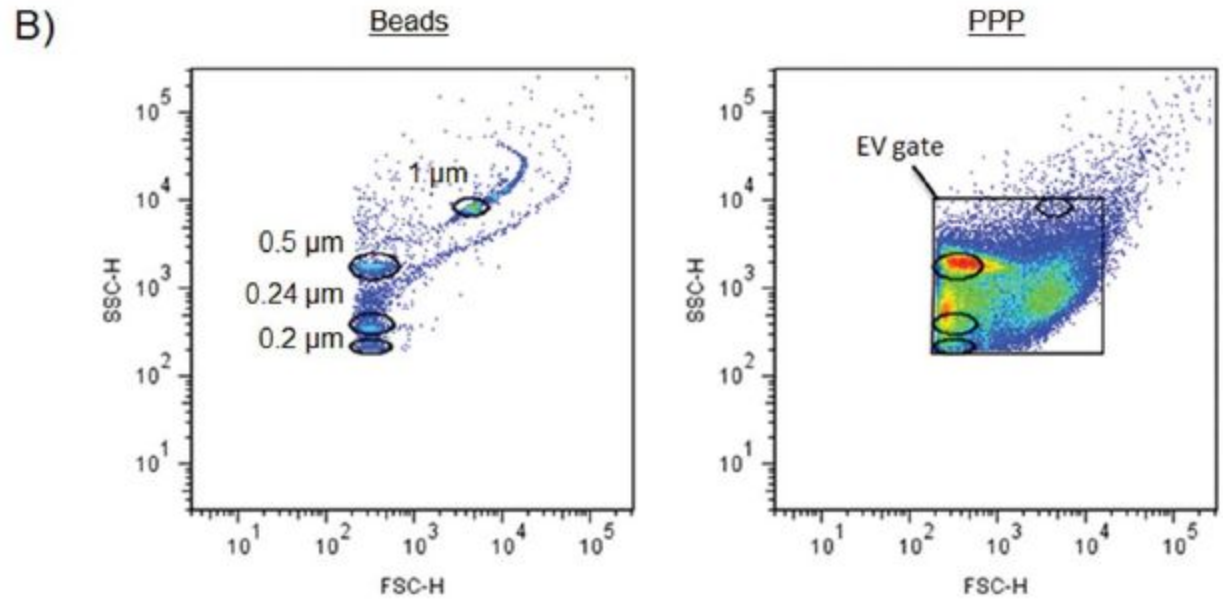
examples – microvesicle



- **bacteria, fixed thrombocytes and beads of 0.2µm size can be detected**

questions & discussion

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