MEDICAL UNIVERSITY OF VIENNA

The dark side of flow cytometry

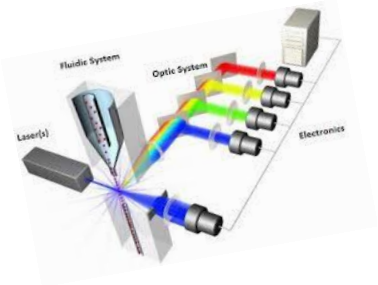
–

how to avoid mistakes

Andreas Spittler

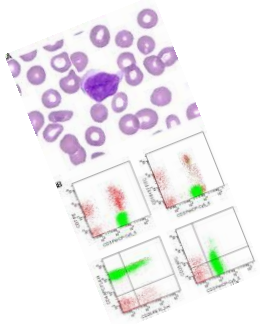
Core Facility Flow Cytometry & Surgical Research Laboratories

Flow Cytometry – Pitfalls & Troubleshooting

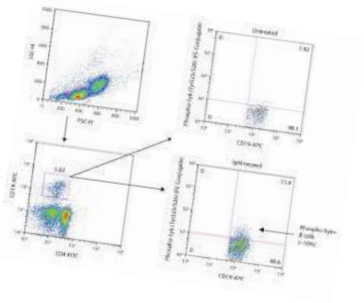


Pre-analytic

Analytic



Post-analytic



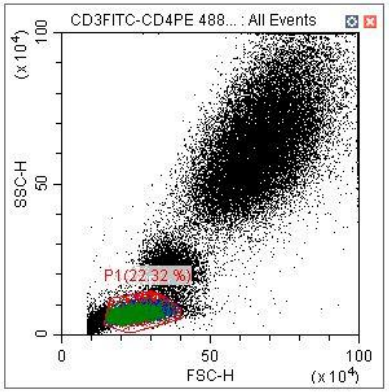
1. Pre-analytics

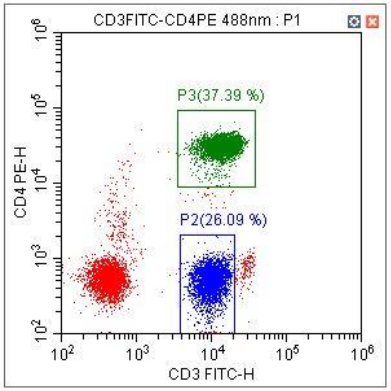
Flow cytometer strategies

- Which instrument should I use?
- Which instrument should I buy?
- How many colours – how many lasers?

… and why ??? …

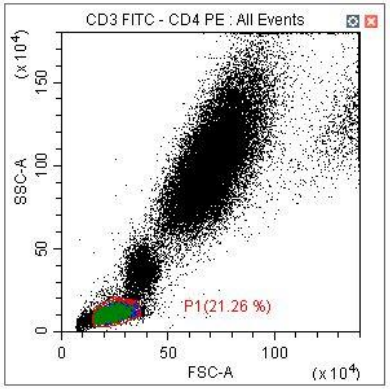
… is there a difference ???

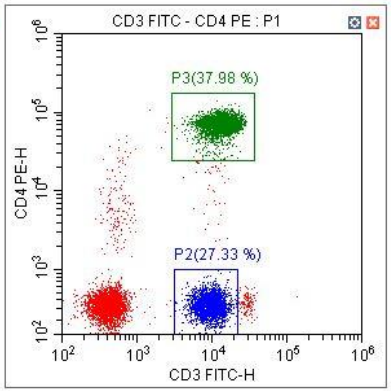




Experiment Name: Exp_20171031_3
Tube Name: CD3FITC-CD4PE 488nm
Sample ID:
Record Time: 2017-11-01 10:42:07
Volume(µL): 67.0

Population	Events	% Total	% Parent	Events/µL(V)	Median CD3 FITC-A	Mean CD4 PE-H	rSD CD4 PE-H
All Events	49172	100.00...	100.00 %	733.73	429.5	3683.2	282.7
P1	10977	22.32 %	22.32 %	163.80	10807.3	12172.4	507.9
P2	2864	5.82 %	26.09 %	42.74	11577.6	554.0	204.4
P3	4104	8.35 %	37.39 %	61.24	16356.5	31346.1	5352.5





Experiment Name: Exp_20171031_1
Tube Name: CD3 FITC - CD4 PE
Sample ID:
Record Time: 2017-11-01 10:30:22
Volume(µL): 53.7

Population	Events	% Total	% Parent	Events/µL(V)	Median CD3 FITC-A	Mean CD4 PE-H	rSD CD4 PE-H
All Events	46797	100.0...	100.00 %	871.97	428.7	7213.0	170.4
P1	9949	21.26 %	21.26 %	185.38	10388.1	27547.1	292.5
P2	2719	5.81 %	27.33 %	50.66	11040.9	351.6	97.0
P3	3779	8.08 %	37.98 %	70.41	15319.0	71358.0	12179.4

Pre-analytical human errors

Planning is everything !!!!

- **Plan your steps**
- **Pipetting errors**
- **Multi-center studies**

  antibody-precoated tubes

Optimal instrument-setup (QC & QA)

- **QC-beads:** laser delay, target channel (delta PMTV – delta GAIN), %CV, area scaling, window extension
- **Assay-specific settings** (optimized PMT voltages; $rSD_{neg.cells} > 2{,}5xSD_{EN}$) – Goal: highest sensitivity, reproducibility
- **Target voltages** for longitudinal studies and inter-laboratory studies

Sample preparation

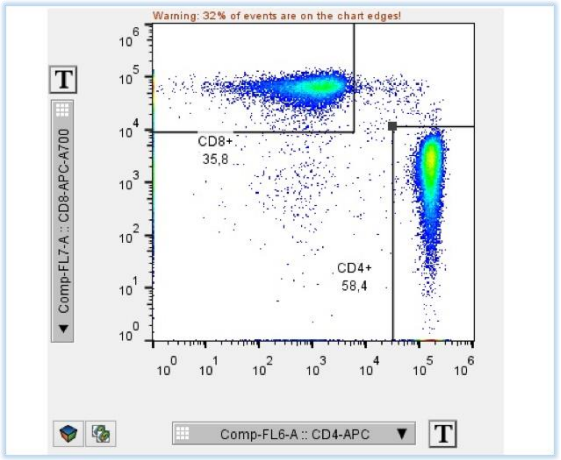
- **Single cell suspension! (10^6-10^8 cells/ml)**
  - easy for suspension cells
  - adherent cells, tissue: gentle but efficient (tissue) dissociation protocol mechanical disaggregation (chopping, grinding, passing through a needle) and/or enzymatic digestion – trypsin/accutase; collagenase, pepsin - critical factors: pH, temperature, cofactors (Ca^{2+},…)
- **Whole blood lysis:** fixing/non-fixing (ammonium chloride)
- **Count cells** (to ensure there are enough cells from the beginning and to check the preparation protocol)

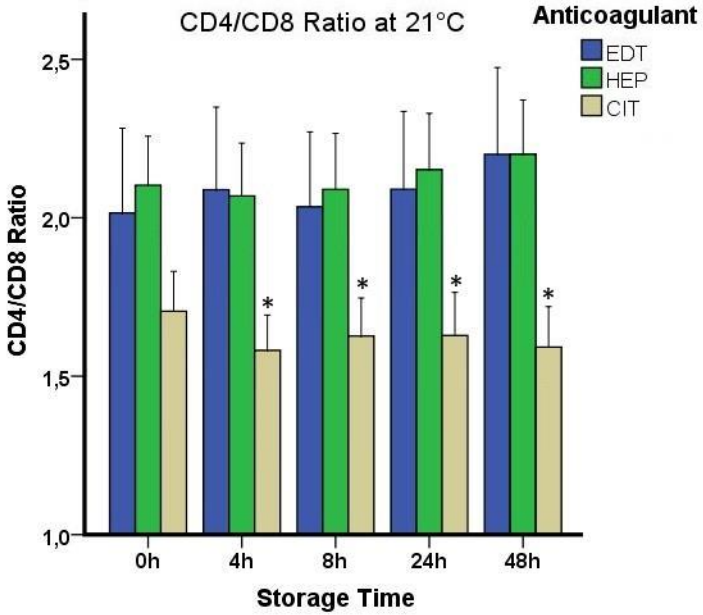
Sample preparation

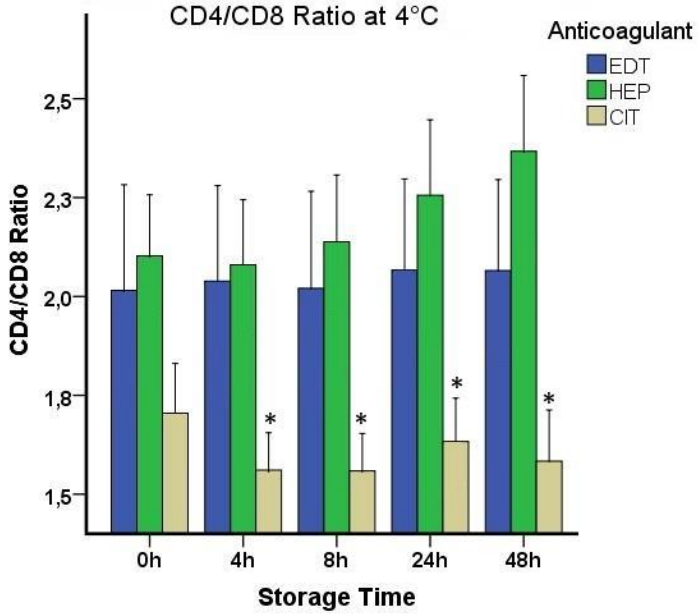
- **Intracellular staining:** specific protocols for fixation/permeabilization (formaldehyde, alcohol, detergent)
- Check correct centrifugation speed (rpm vs. xg)
- Fixation: cells may not pellet well
- Filter samples (70-200µm)

Different anticoagulants give different results

CD4:CD8 ratio

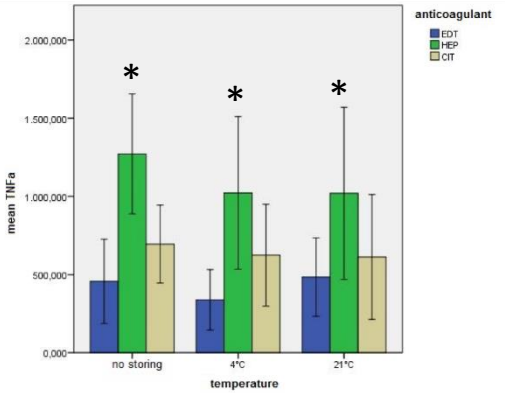


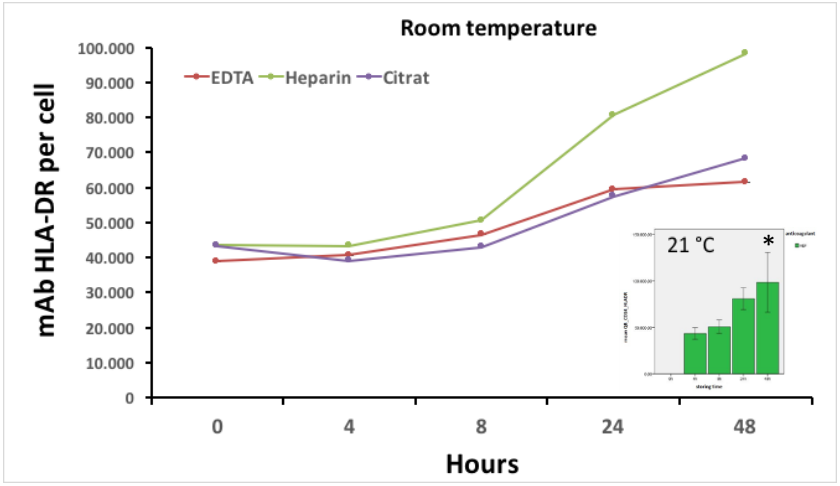


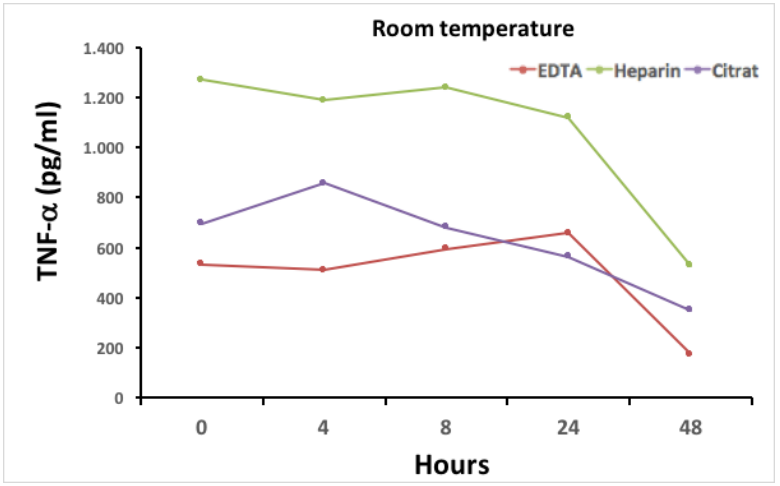


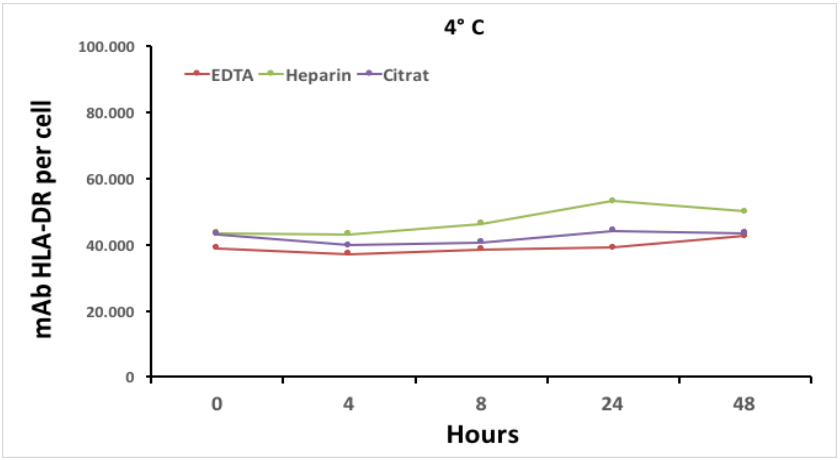
Anticoagulants, storage time and temperature

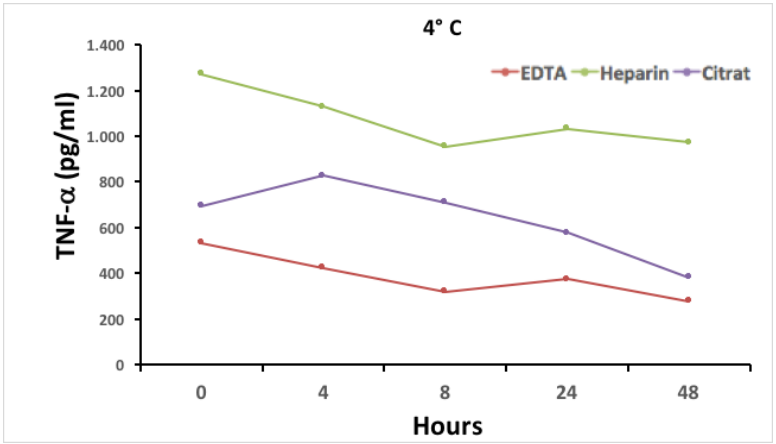
Functional assays and activation marker











Choice of fluorochromes

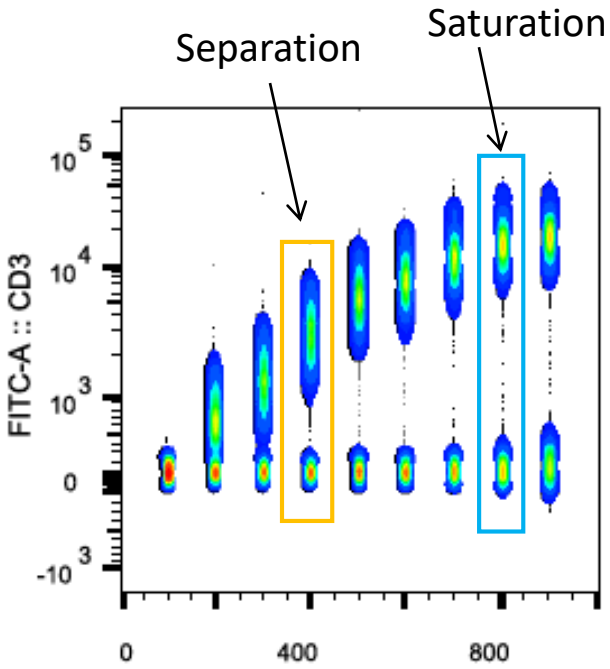
- Use bright dyes for dim markers/weak dyes for strongly expressed markers
- Minimize spillover, esp. for co-expressed markers
- Use “silent” dye for strong markers (no/little spillover into other channels)
- Use “untouched” channel for dim markers (no/little spillover from other channels)
- **Data spread** = loss of sensitivity in other channels!

Controls

- Compensation controls
- Unstained control (background fluorescence)
- Isotype control: only for detection of nonspecific binding, not for determination of positivity!
- Negative control (no expression, untreated, etc.)
- Positive control
- **FMO** (Fluorescence-minus one) control: to determine threshold for positivity
- **SWOFF** control (switch-off fluorescence): to reveal hiding of dim markers by data spreading

Titration

- **Separation titer** vs. **Saturation titer**



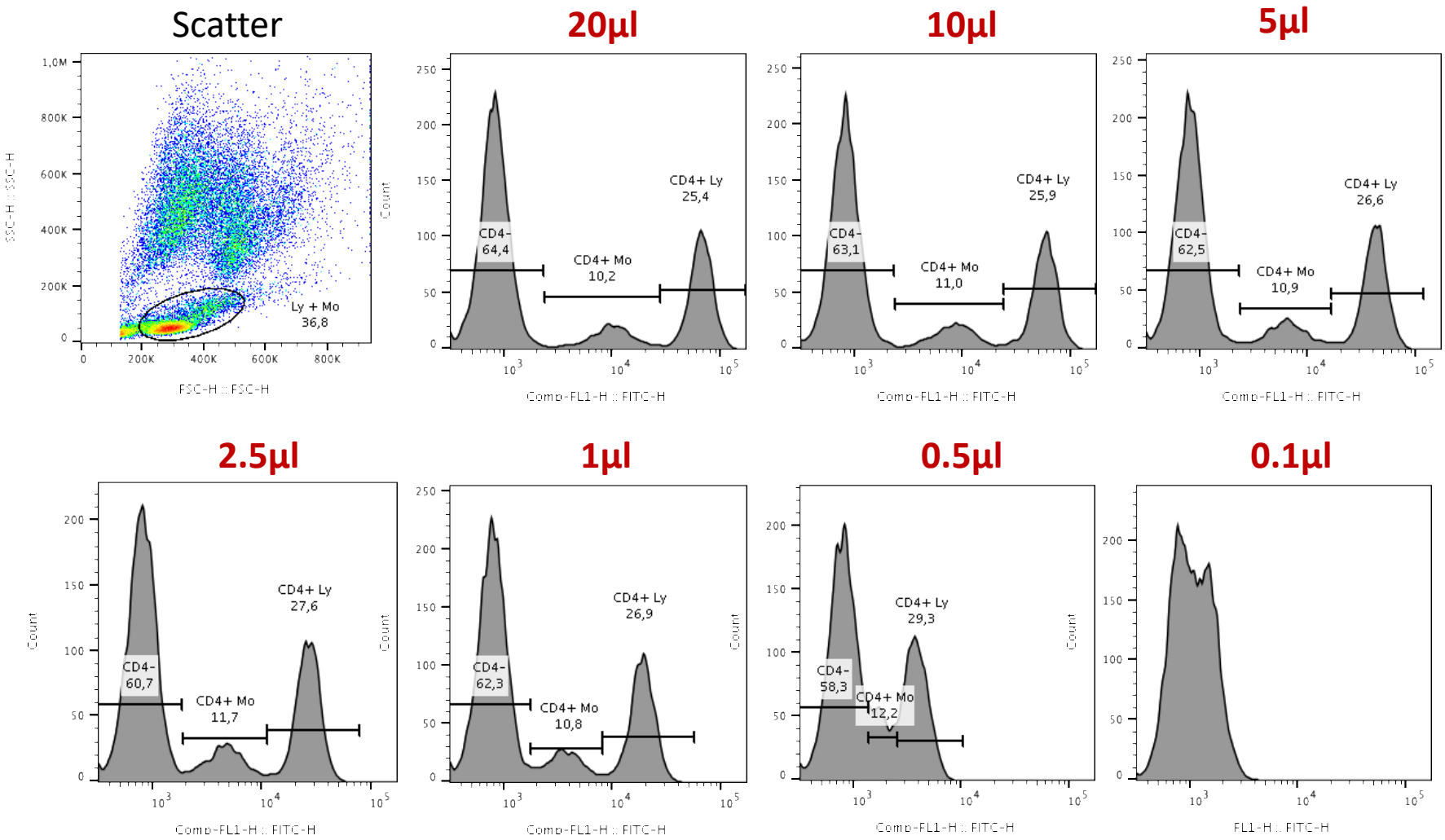
- Same conditions as in final assay
- Same cell number as in final assay
- **Stain index:**

  [MFI (pos. population) – MFI (neg. population)] / [2xSD (neg. population)]

Antibody titration

Save money, avoid unspecific staining, critical if comparing MFIs

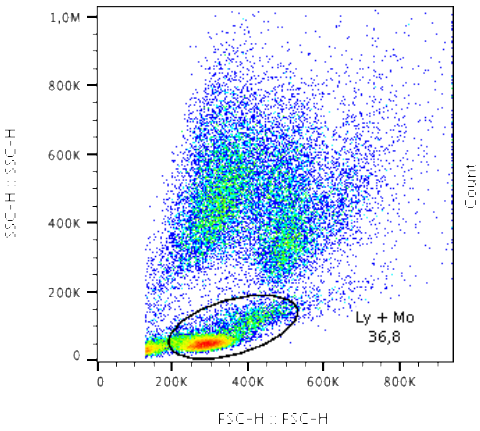
CD4 FITC – recommended concentration 20µl



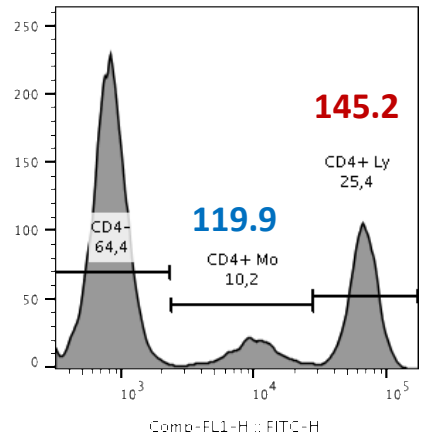
Stain index Monos - T-cells

[MFI (pos. population) – MFI (neg. population)] / [2xSD (neg. population)]

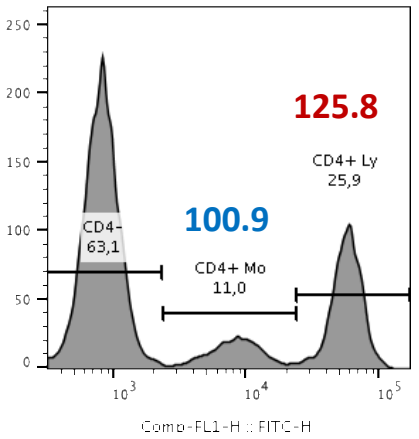
Scatter



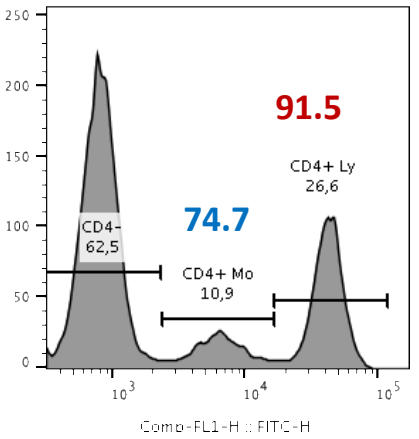
20µl



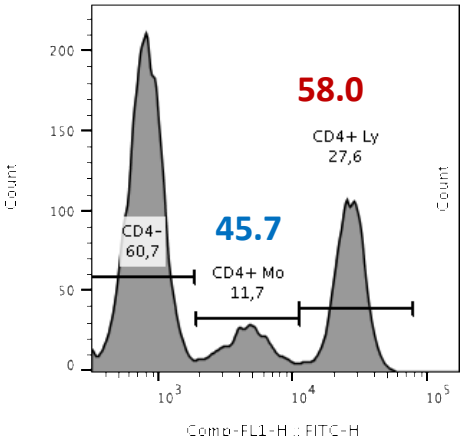
10µl



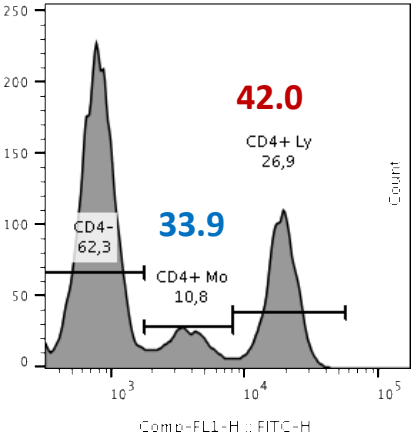
5µl



2.5µl



1µl



0.5µl

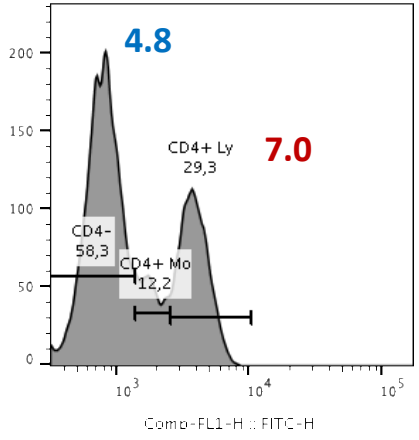
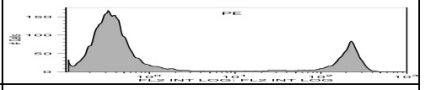
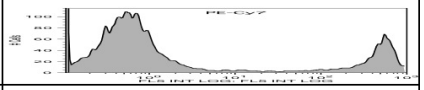
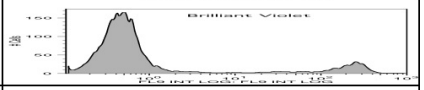
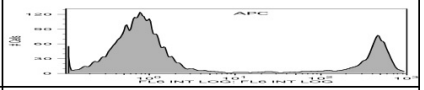
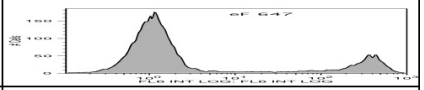
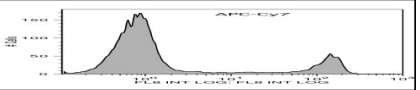
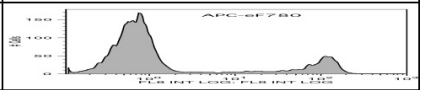
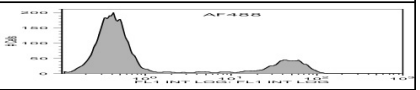
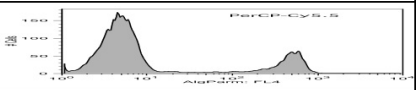
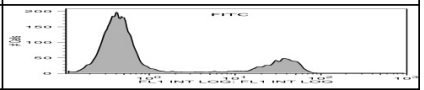
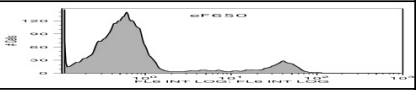
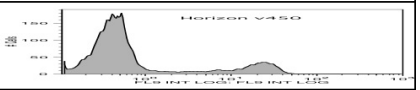
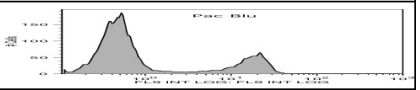
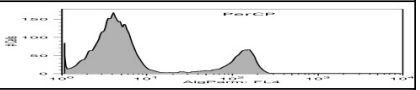
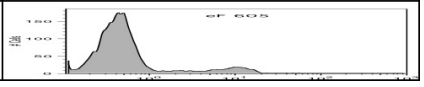


Table of stain index

Reagents	Clone	Fluorochrome	Stain Index
CD4	RPA-T4	Brilliant Violet 421	561
		Pacific Blue™	24
		BD Horizon V450	29
		PE	142
	VIT4	VioBlue®	31
CD8	RPA-T8	Brilliant Violet 421	782
		Pacific Blue™	29
		PE	198
CD56	B159	Brilliant Violet 421	45
		PE	18
CD127	hIL-7R-M21	Brilliant Violet 421	55
		PE	14

Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

Fluorochrome	Laser	Filter	PMT	Stain Index	Gallios
PE	blue	575/30 nm band pass	FL-2	617	
PE-Cy7	blue	755 nm long pass	FL-5	532	
Brilliant Violet-421	violet	450/40 nm band pass	FL-9	476	
APC	red	660/20 band pass	FL-6	440	
Alexa Fluor 647	red	660/20 band pass	FL-6	307	
APC-Cy7	red	755 nm long pass	FL-8	196	
APC eFluor-780	red	755 nm long pass	FL-8	174	
Alexa Fluor 488	blue	525/40 nm band pass	FL-1	133	
PerCP-Cy5.5	blue	675/20 nm band pass	FL-4	118	
FITC	blue	525/40 nm band pass	FL-1	97	
eFluor-650NC	red	660/20 band pass	FL-6	71	
BD Horizon-V450	violet	450/40 nm band pass	FL-9	61	
Pacific Blue	violet	450/40 nm band pass	FL-9	44	
PerCP	blue	675/20 nm band pass	FL-4	32	
eFluor-605NC	blue	620/30 nm band pass	FL-3	27	

Staining procedure

- On ice (or 4°C) or at room temperature? Mostly room temperature, on ice when measuring activation markers
- Correct volume – antibody concentration
- Intracellular staining: follow instruction in datasheet, use isotype controls to assess background (excess antibody may not be washed out of cells completely!)
- Blocking antibodies or AB serum
- Protect from light

Cell aggregation/clumping

- Buffer (PBS) w/o Mg, Ca
- Include BSA/FBS, EDTA in buffer
- DNase I
- Filter sample (40µm)
- Use hypotonic lysis buffer (0.1% sodium citrate, Triton X-100) for DNA-staining

2. Analysis

No/very few events

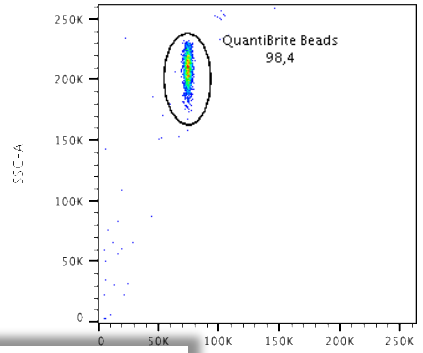
- System clog or pressure leak (see manual for instrument-specific measures), tube possibly cracked
- Threshold (FSC) too high, voltage too low
- Use log scale for small particles (linear scale for DNA analysis)

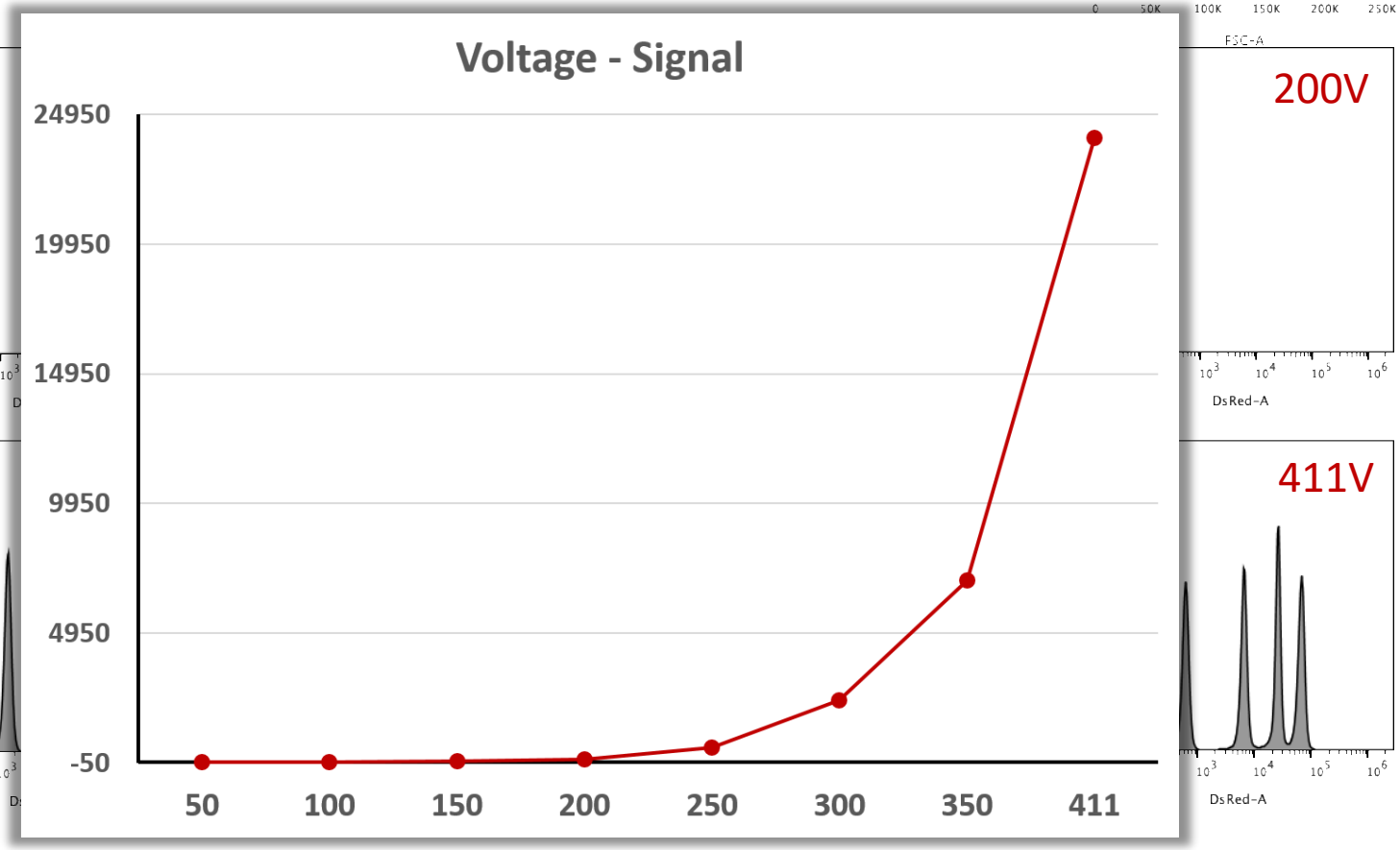
No/low positive signal

- **Fluorescence-threshold** set too high (dim-positives are lost)
- **High spill-over** obscures low signal
- **Not enough antibody** - antibody properly titrated?
- **Antibody gone bad** (expired, not stored properly – light! Do not freeze!)
- **Antigen or fluorochrome sensitive** to fixation protocol
- **Receptor internalized** (e.g. upon activation) or sensitive to trypsinization – stain at 4°C
- **Wrong secondary antibody** – use proper species

No/low positive signal

- **Voltage too low**
- **optimize PMT voltages;** $rSD_{neg.cells} > 2{,}5xSD_{EN}$





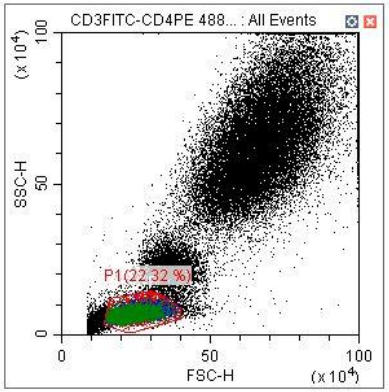
Signal goes off-scale

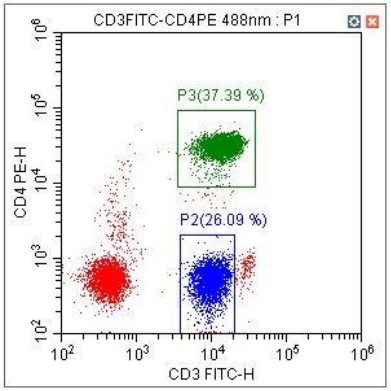
- Lower voltage
- (Re) titrate antibody
- Use less antibody
- Add non-conjugated antibody
- Use dimmer fluorochrome
- 488nm – 561nm laser (PE and PE conjugates are much brighter)

488nm – 561nm laser
(PE and PE conjugates are much brighter)

[MFI (pos. population) – MFI (neg. population)] / [2 x rSD (neg. population)]

75.3

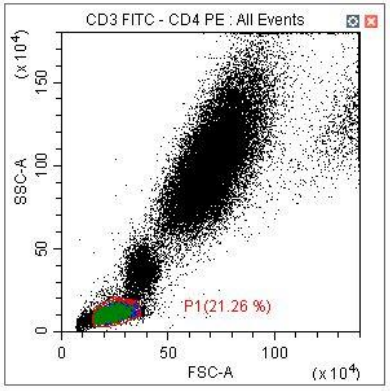


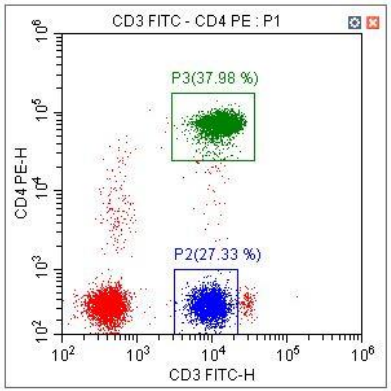


Experiment Name: Exp_20171031_3
Tube Name: CD3FITC-CD4PE 488nm
Sample ID:
Record Time: 2017-11-01 10:42:07
Volume(µL): 67.0

Population	Events	% Total	% Parent	Events/µL(V)	Median CD3 FITC-A	Mean CD4 PE-H	rSD CD4 PE-H
All Events	49172	100.00...	100.00 %	733.73	429.5	3683.2	282.7
P1	10977	22.32 %	22.32 %	163.80	10807.3	12172.4	507.9
P2	2864	5.82 %	26.09 %	42.74	11577.6	554.0	204.4
P3	4104	8.35 %	37.39 %	61.24	16356.5	31346.1	5352.5

366.0





Experiment Name: Exp_20171031_1
Tube Name: CD3 FITC - CD4 PE
Sample ID:
Record Time: 2017-11-01 10:30:22
Volume(µL): 53.7

Population	Events	% Total	% Parent	Events/µL(V)	Median CD3 FITC-A	Mean CD4 PE-H	rSD CD4 PE-H
All Events	46797	100.0...	100.00 %	871.97	428.7	7213.0	170.4
P1	9949	21.26 %	21.26 %	185.38	10388.1	27547.1	292.5
P2	2719	5.81 %	27.33 %	50.66	11040.9	351.6	97.0
P3	3779	8.08 %	37.98 %	70.41	15319.0	71358.0	12179.4

Compensation

- Use single-stained controls (cells or antibody capture beads) with same fluorochrome as used in the assay
- Unstained control must have same autofluorescence as single-stained controls (don't mix!)
- Single-stained controls must have at least same fluorescence intensity as strongest signal in assay (linearity!)
- Data spread = loss of sensitivity => no compensation = best sensitivity
- More lasers are better – reduced spillover

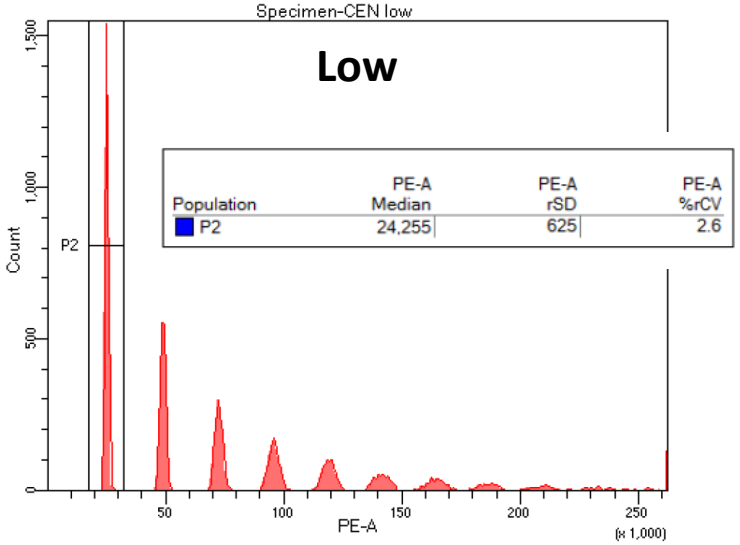
Compensation looks wrong

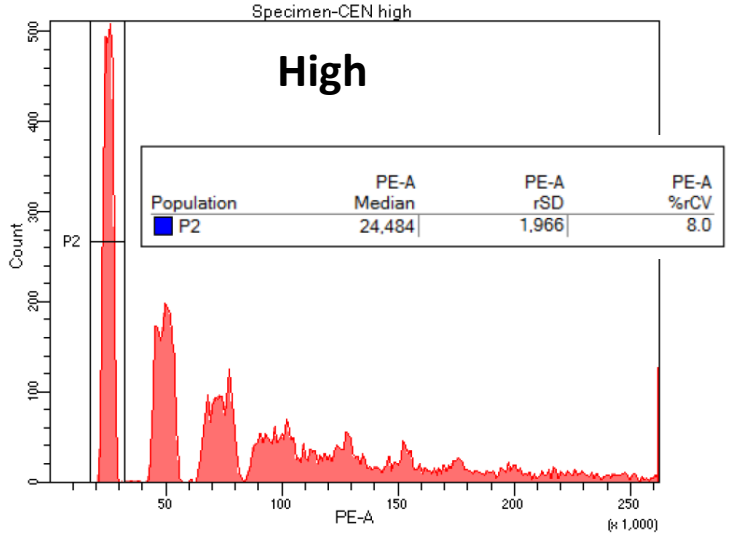
over/undercompensated

- Mixed positive and negative controls (must have same background signal/autofluorescence!)
- Positive control has lower signal intensity as population for that colour – use (antibody capture) beads for compensation
- Tandem dye - must compensate each new lot!
- BV dyes require special staining buffer (company recommendation)

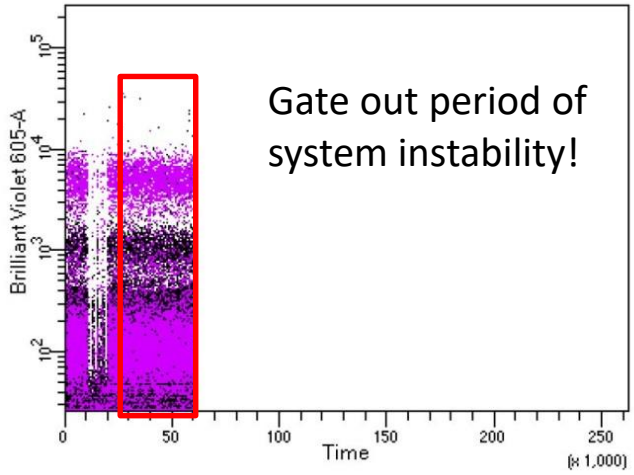
Acquisition

- Slow (optimal resolution); esp. for DNA analysis



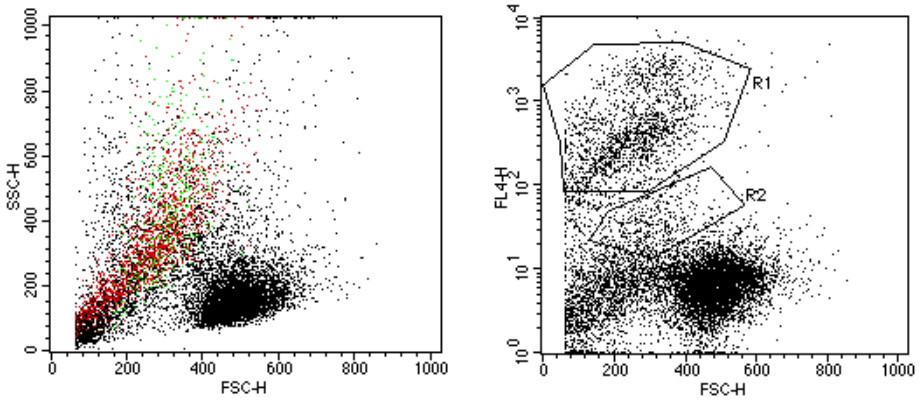


- Time parameter (to detect instrument changes, e.g. pressure/laser, during acquisition)



Acquisition

- Position of live – dead – apoptotic cells



http://www.cyto.purdue.edu/cdroms/cyto5/tech/icrfapop.htm#SCAT

- CD34+ and dead cells

Loss/shifting of signal & unusual compensation

Degradation of tandem dyes

Sensitive to light (and temperature)

Date of expiry

Sensitive to fixation

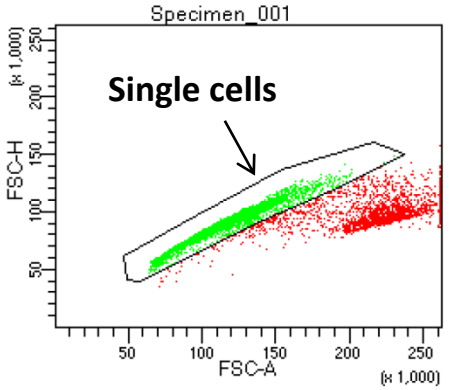
Each lot possibly requires new compensation

Controls frequently

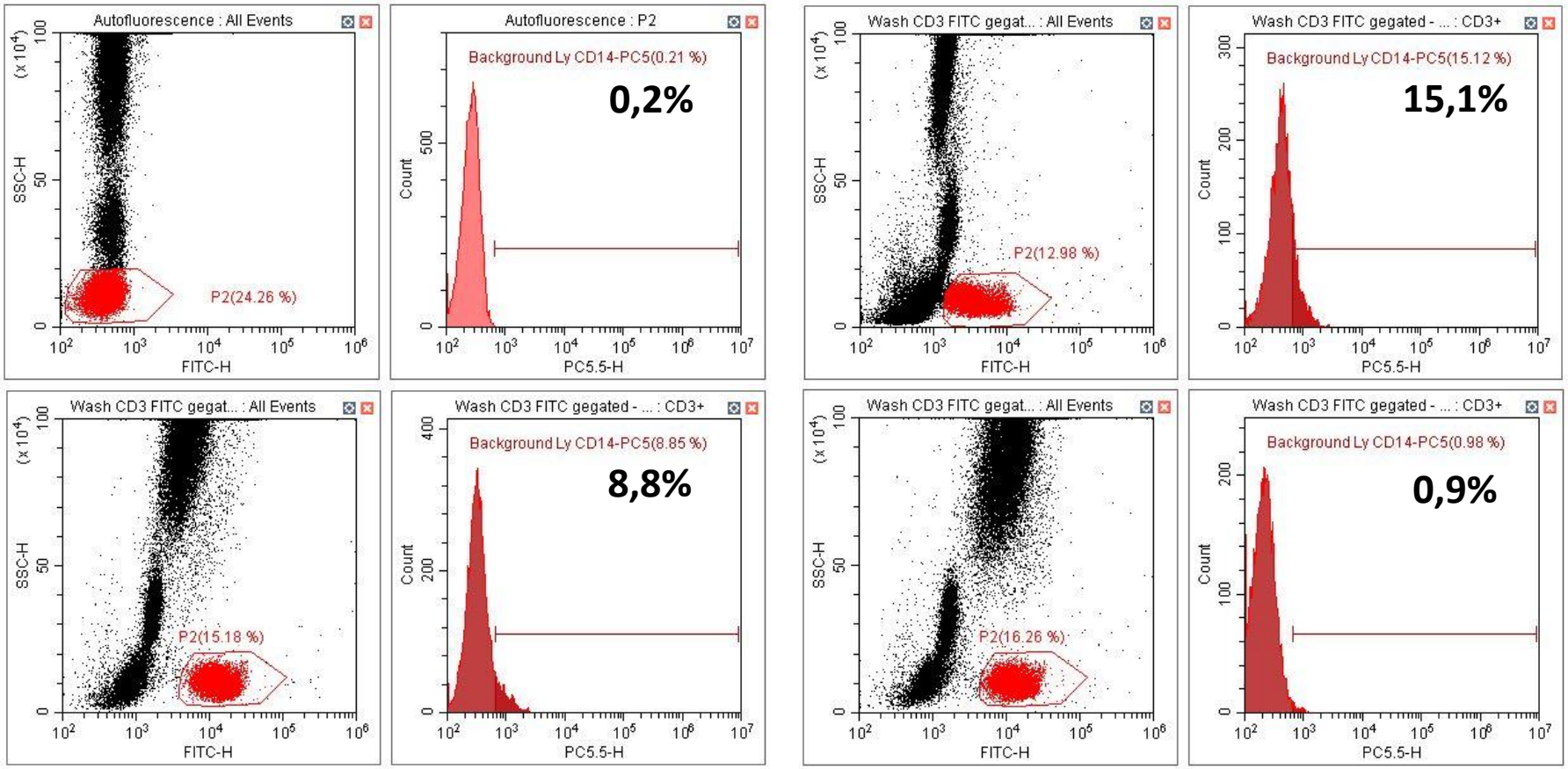
System fluctuations (laser, sheath pressure) – always record time parameter

High background (autofluorescence)/ non-specific staining - False-positives

- Antibody properly titrated? Isotype controls may be used to indicate non-specific binding.
- Gate out AF-cells (use dump channel)
- Dead cells, cell debris (high autofluorescence, bind antibodies non-specifically) - use live/dead dye, DNase I
- No doublet discrimination – exclude doublets (Dot plot: FSC-H vs. FSC-A)
- **Wash cells 3 times after staining**

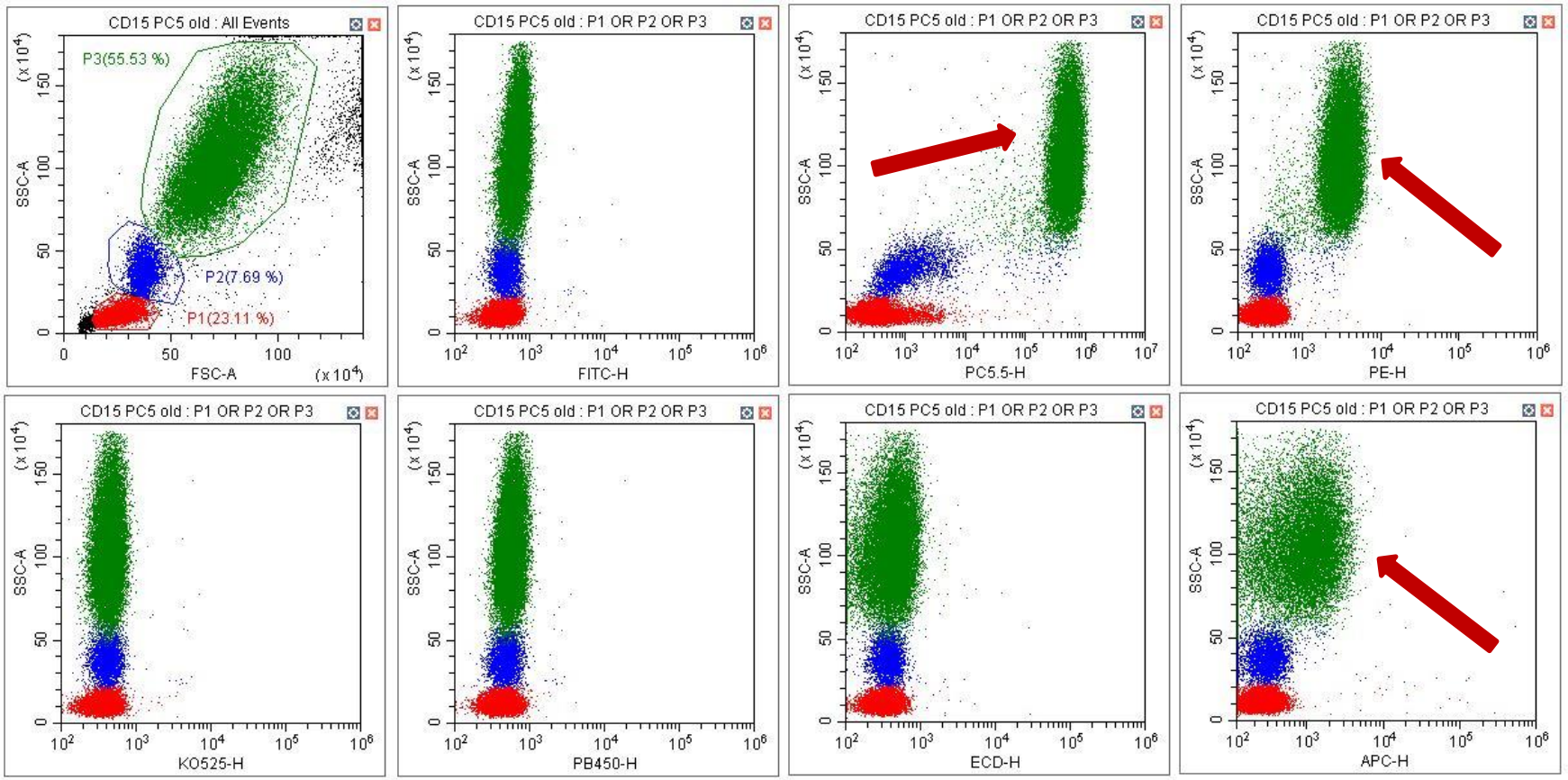


Wash your cells



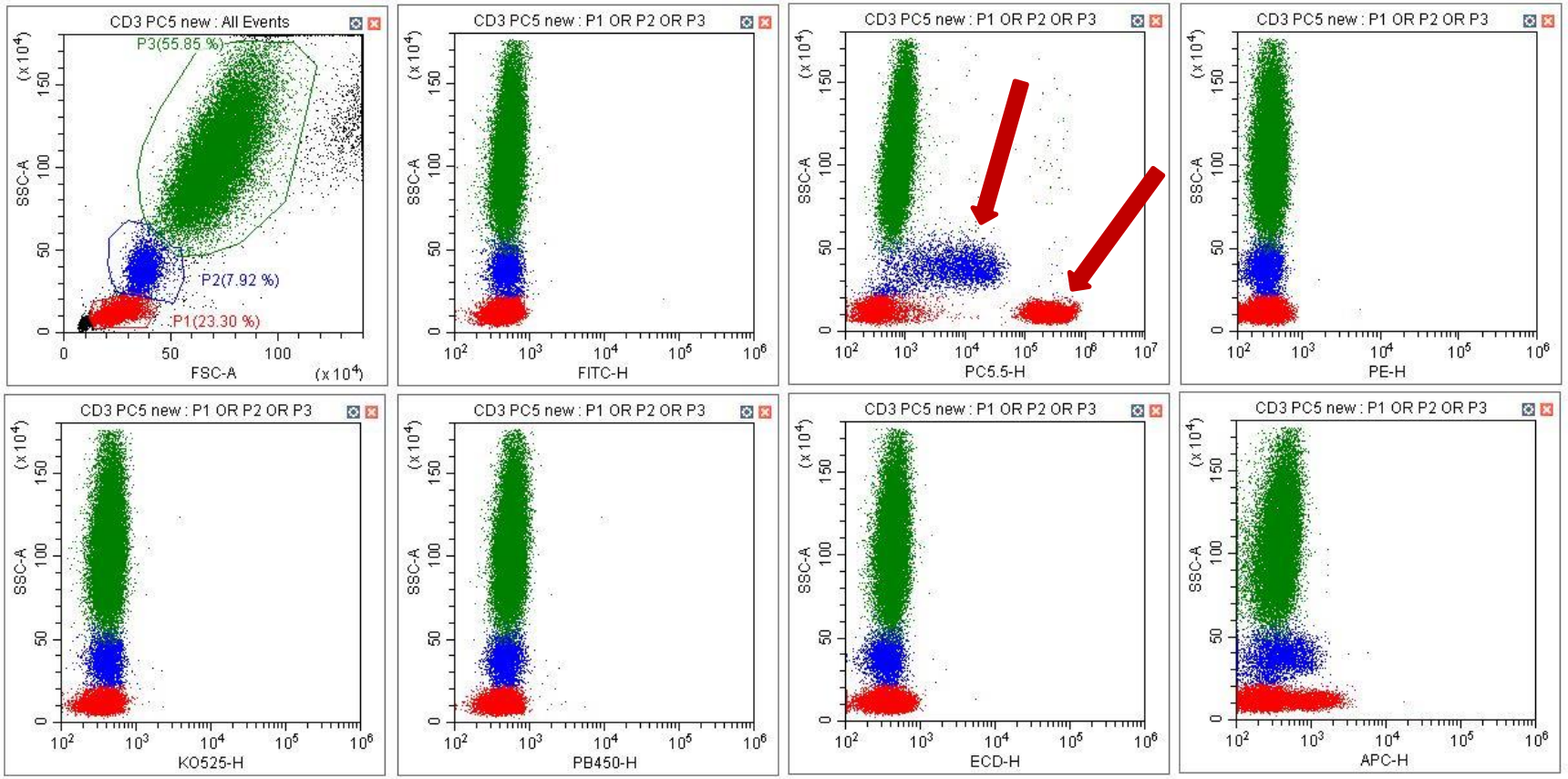
Whole blood was stained with an anti-CD3 FITC antibody and an anti-CD14 antibody. After incubation for 30 minutes at T in the dark, red blood cells were lysed and sample was washed several times. CD3+ cells were gated and non-specific binding of CD14 on CD3+ cells was detected

“Old” PE-Cy5 conjugate



High background (autofluorescence) - non-specific staining - False-positives

Blocking (Fc receptors)
avoid Cyanine dyes (e.g. PE-Cy5, PE-Cy5.5) – they stain myeloid cells (monocytes)

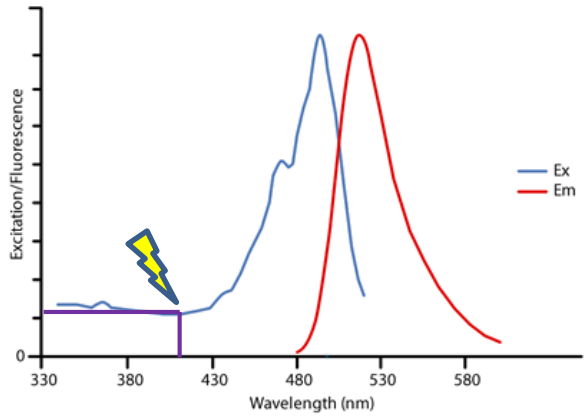


High background (autofluorescence)/ non-specific staining - False-positives

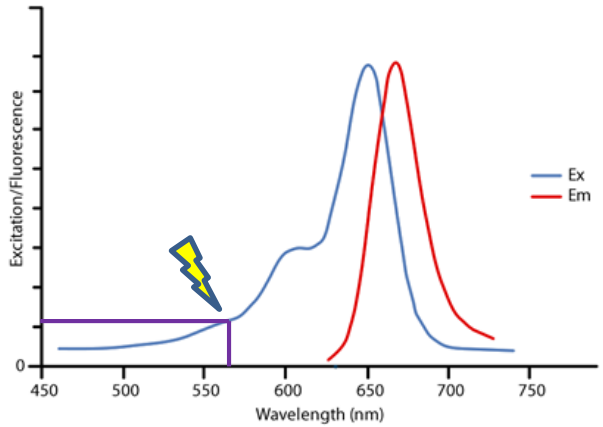
- Washing steps between samples (PI: hypochlorite, acquire unstained cells)
- Cells after culture e.g. monocytes (day 1) – monocyte derived macrophages are much bigger (day 7) – higher autofluorescence
- Autofluorescence depends on the laser and/or the wavelength measured

Some (possibly all) dyes are excited from multiple lasers

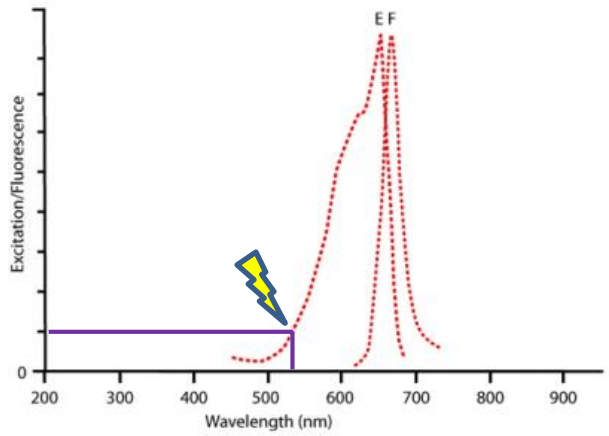
FITC



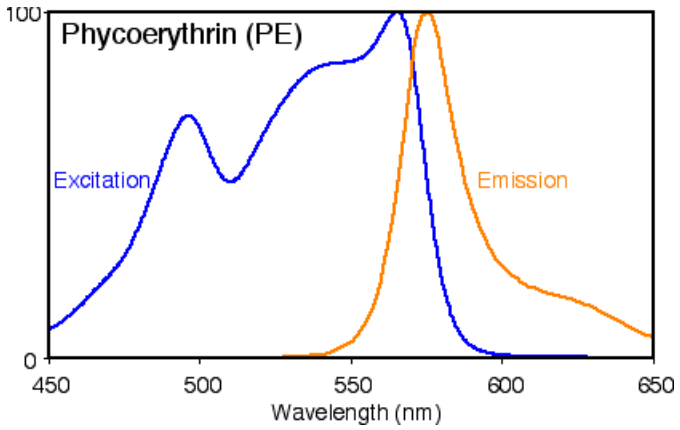
Cy5



APC



PE

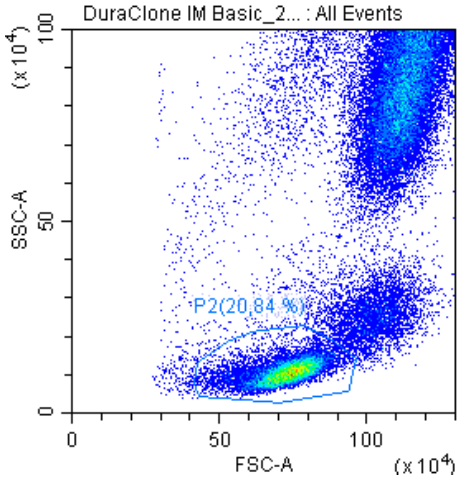


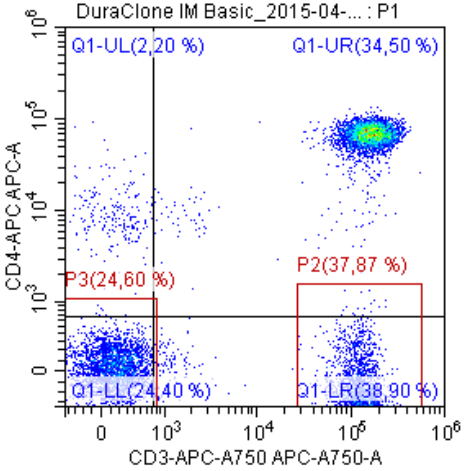
3. Post-analysis

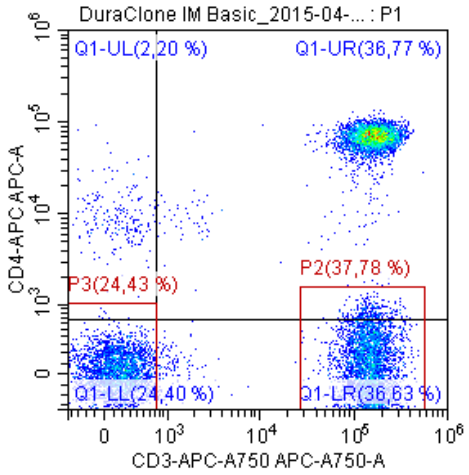
- **Morphology of the cells**
  - Live – dead – apoptotic
  - Cells after culture
  - Different settings for different cell types due to different autofluorescences? (lymphos, monos, granulos)
- **Correct gating strategy**
- **Gating strategy regarding apoptosis**

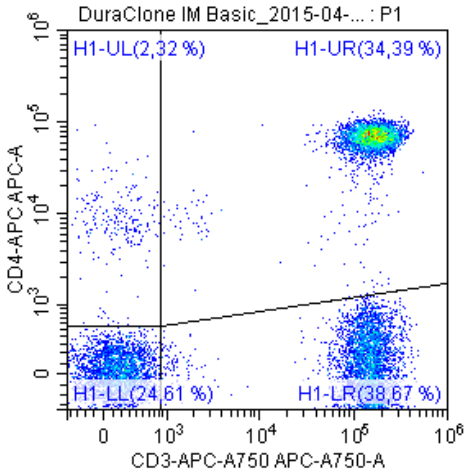
Gating Strategy

- Quadrants vs hinged regions
- Wrong vs. correct compensation









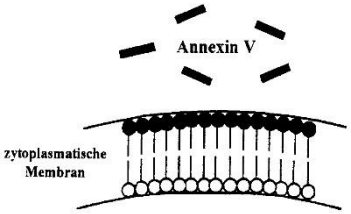
LL Y_{mean} -49.1

LR Y_{mean} -588

P3 Y_{mean} -48.7

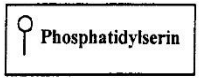
P2 Y_{mean} -50.9

Apoptotic cells change morphology

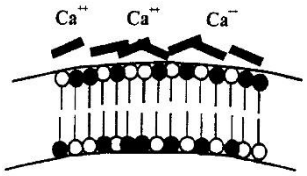


viable cell

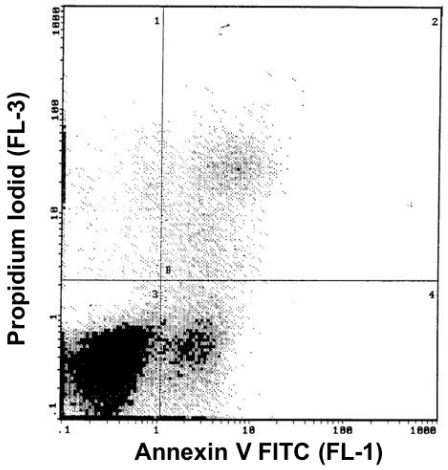
Apoptosis

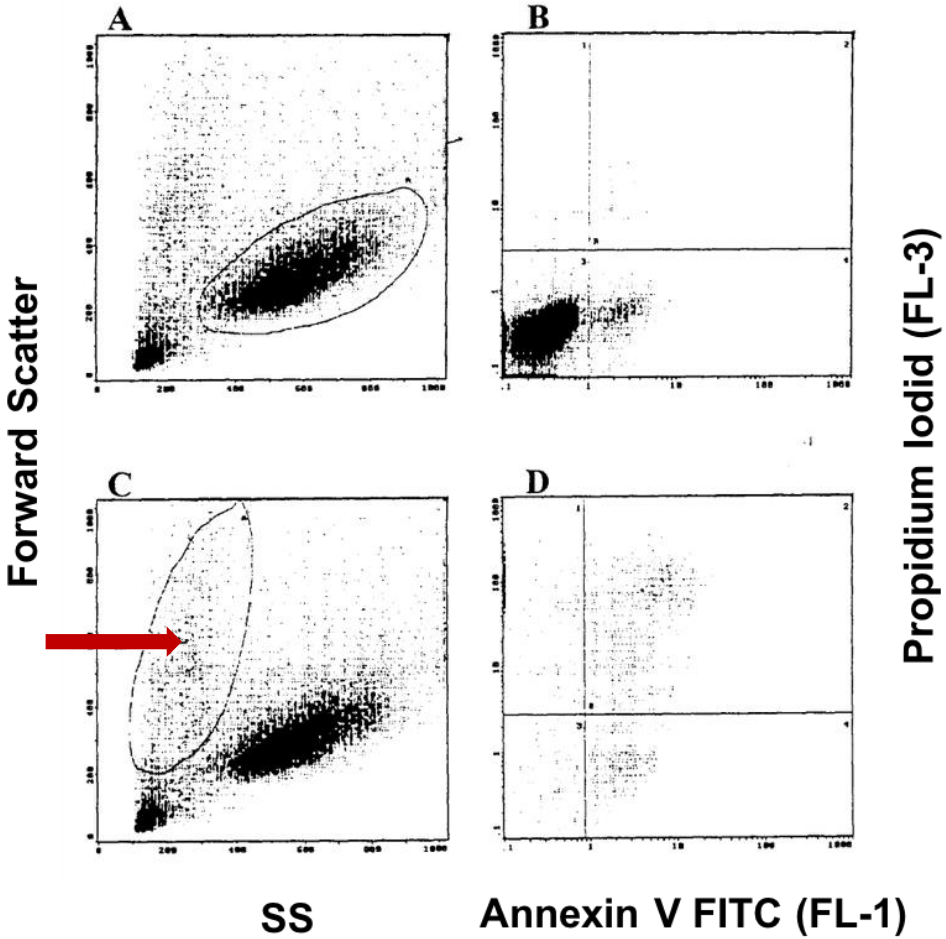


Annexin V binding



apoptotic cell





Shapiro's Zeroth Law: There is no Magic

The cytometers and reagents in your experiments use the principles of physics and chemistry to produce your results. Therefore, there is no magic involved in your experiments.