Clinical Flow Cytometry for the Perplexed

Part 2: Technical Considerations

David P. Ng, MD

Assistant Clinical Professor, University of Utah School of Medicine

ARUP LABORATORIES

HEALTH UNIVERSITY OF UTAH

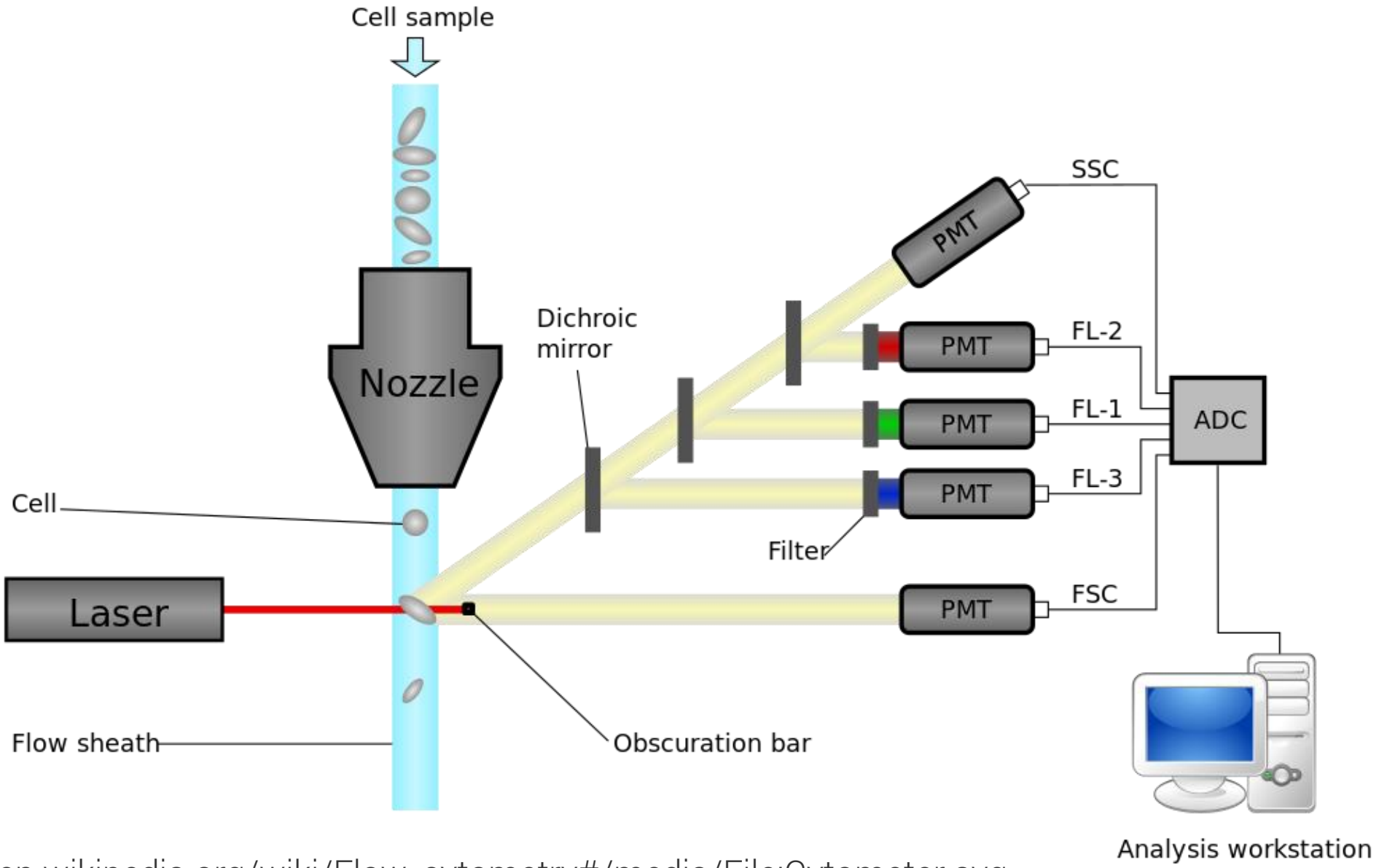
Recap from Part 1

- What is Flow Cytometry
- Gating Strategy(ies)

Goals for Part 2

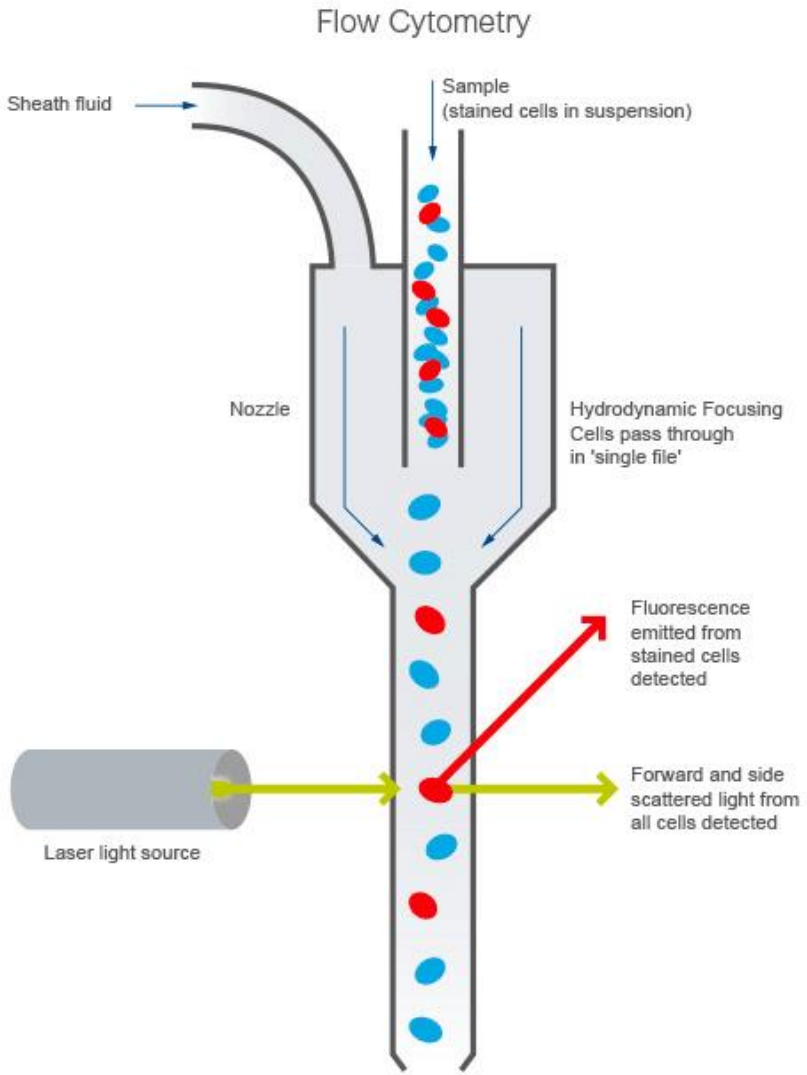
- How does a flow cytometer work?

- What are the pitfalls and artifacts that you need to know?
  - » Tandem Breakdowns
  - » Spillover/Compensation

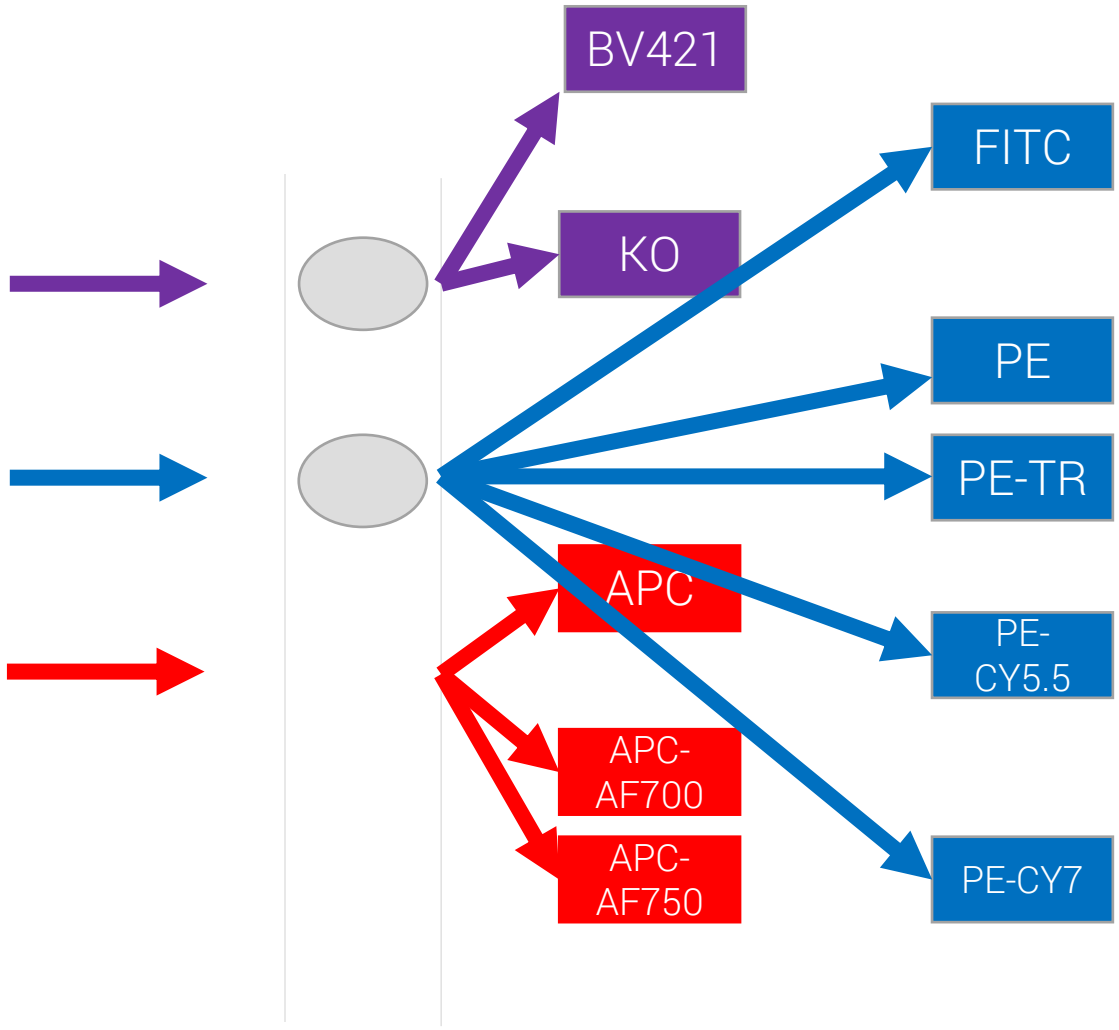


en.wikipedia.org/wiki/Flow_cytometry#/media/File:Cytometer.svg

The Flow Cell

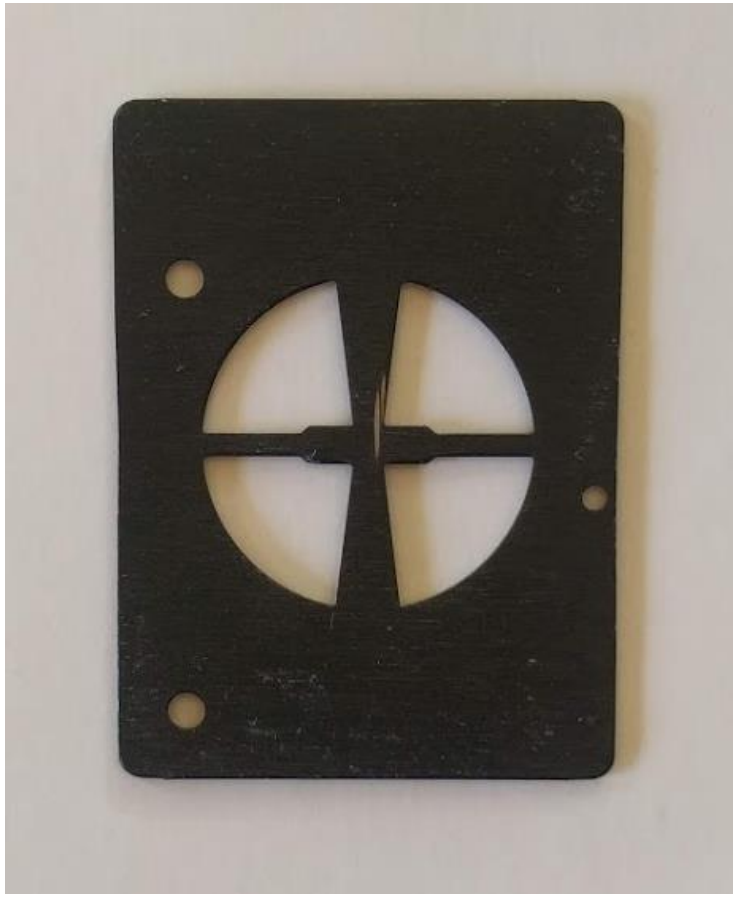


Spatial Separation

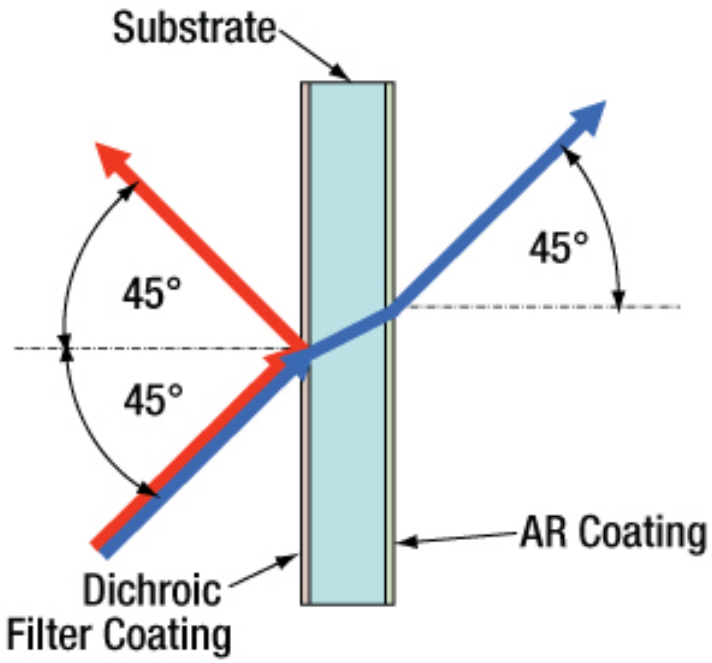


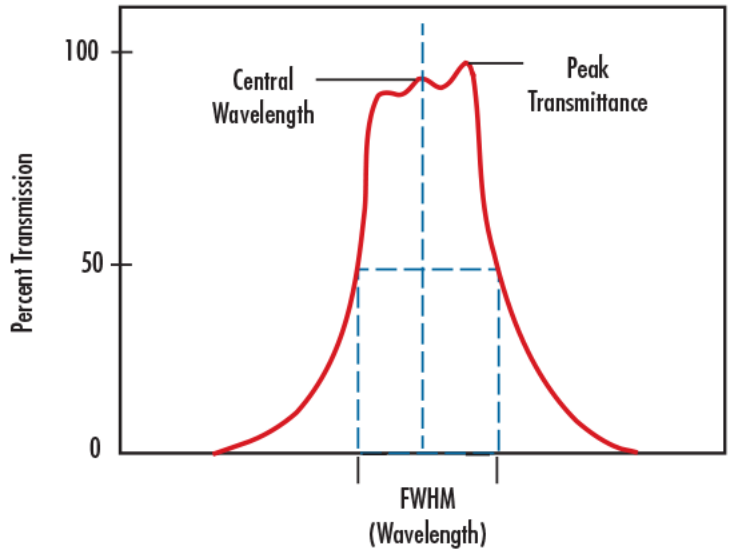
Forward Scatter

- Roughly proportional to cell size

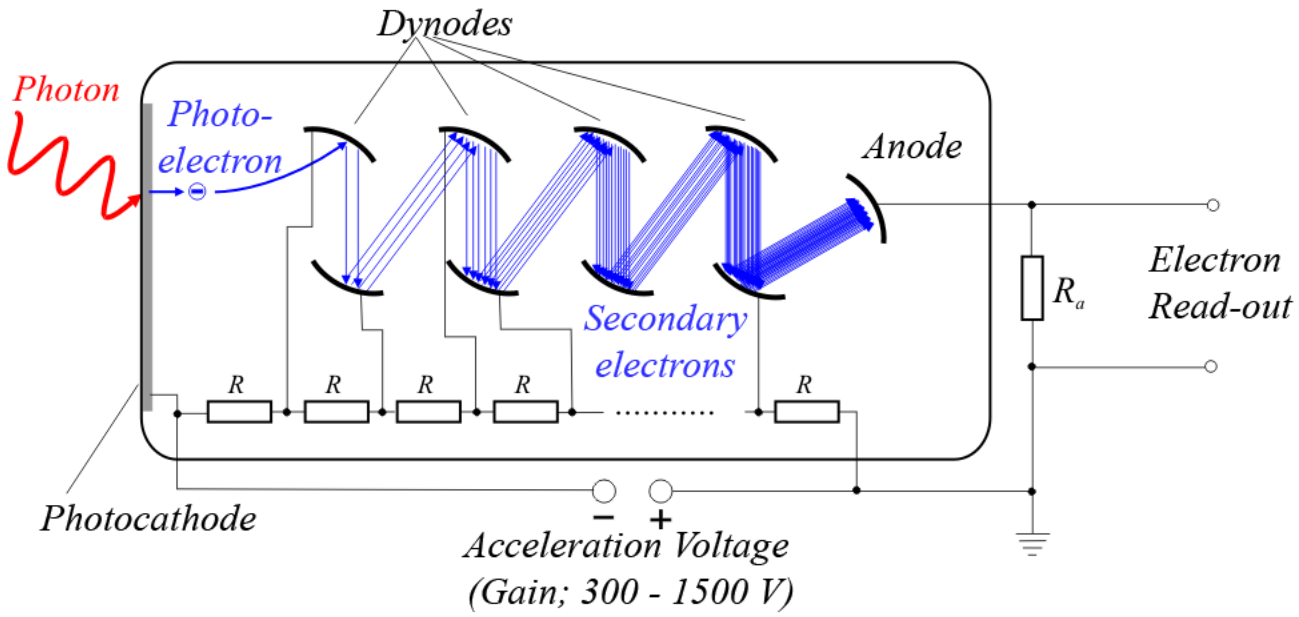


Dichroic Mirrors / Bandpass Filters

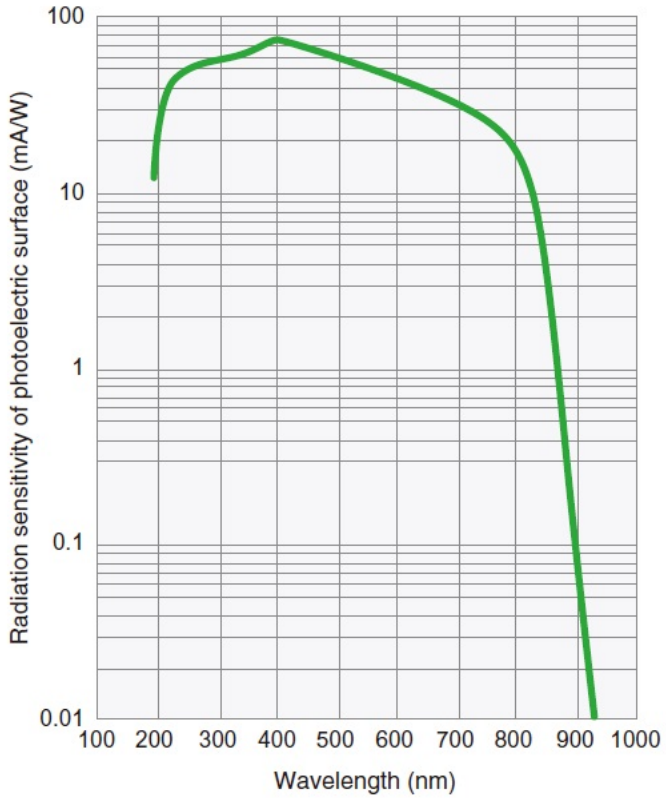




Photomultiplier Tube

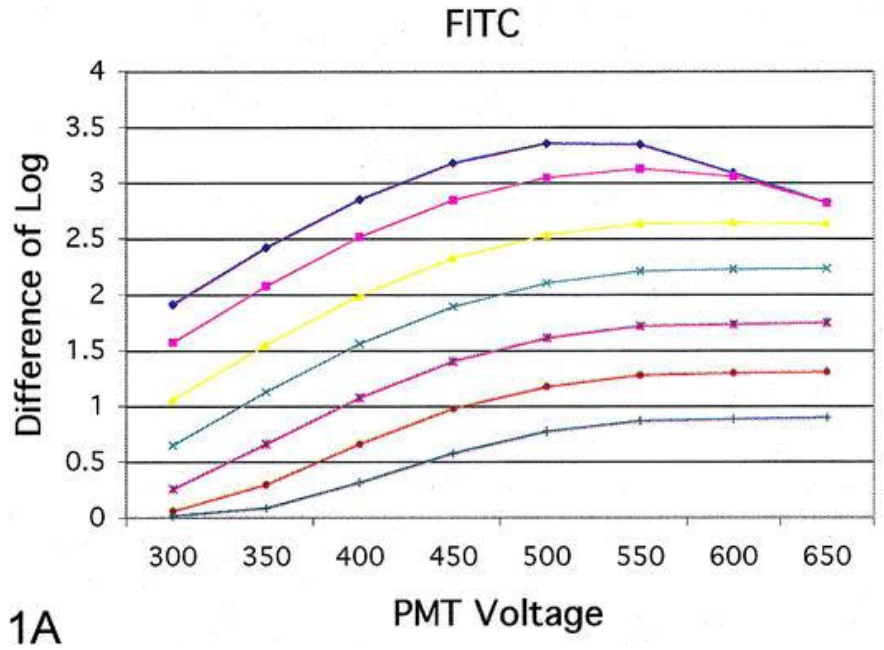


Implications (sensitivity)

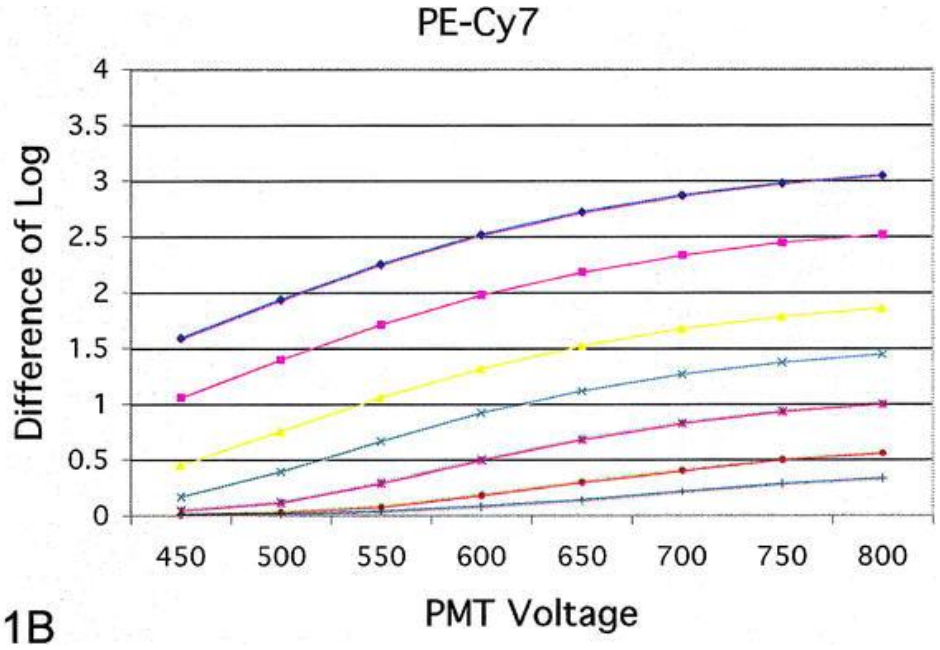


Implications (gain)

- Limited linear range of gains

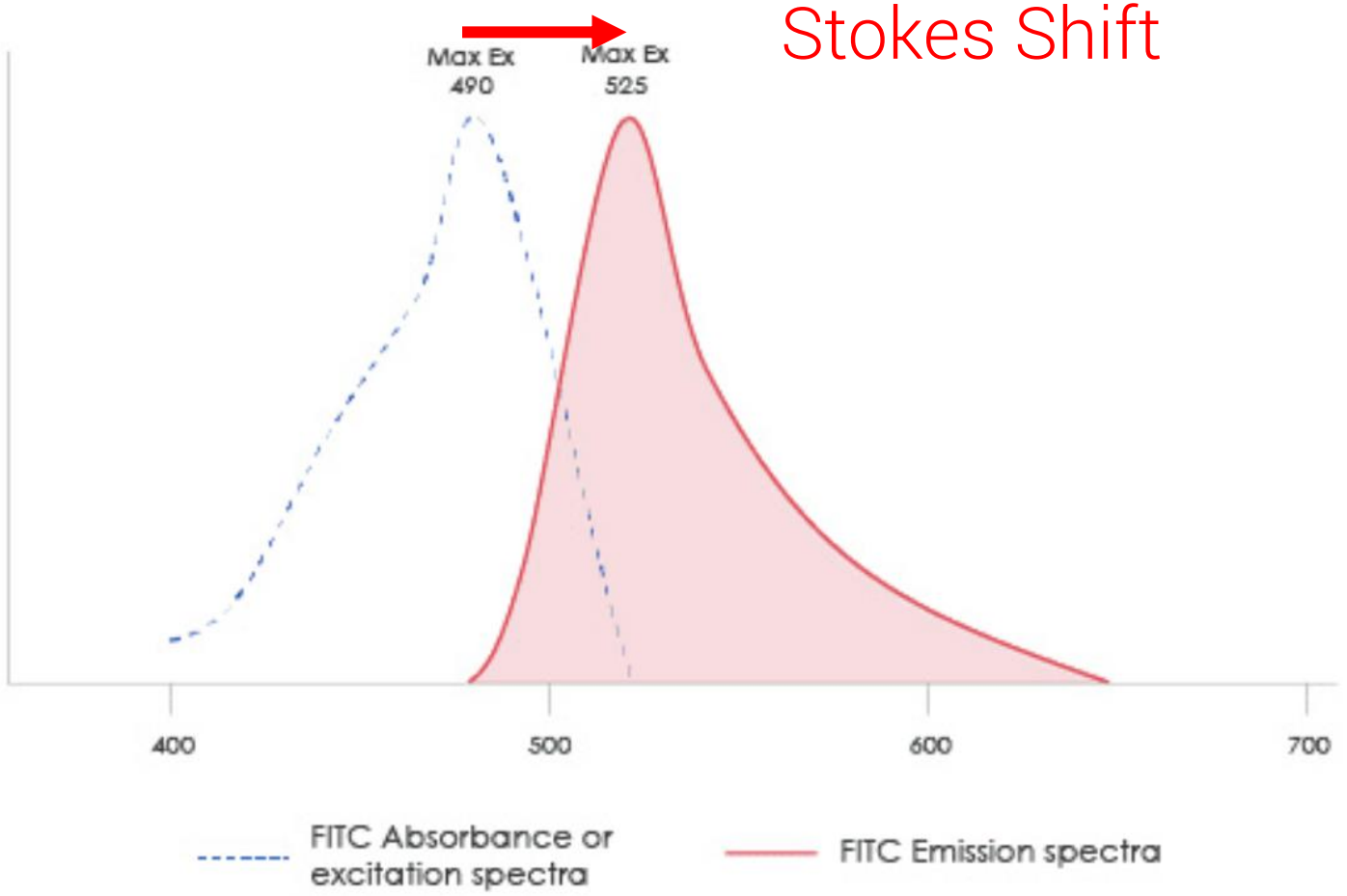


1A

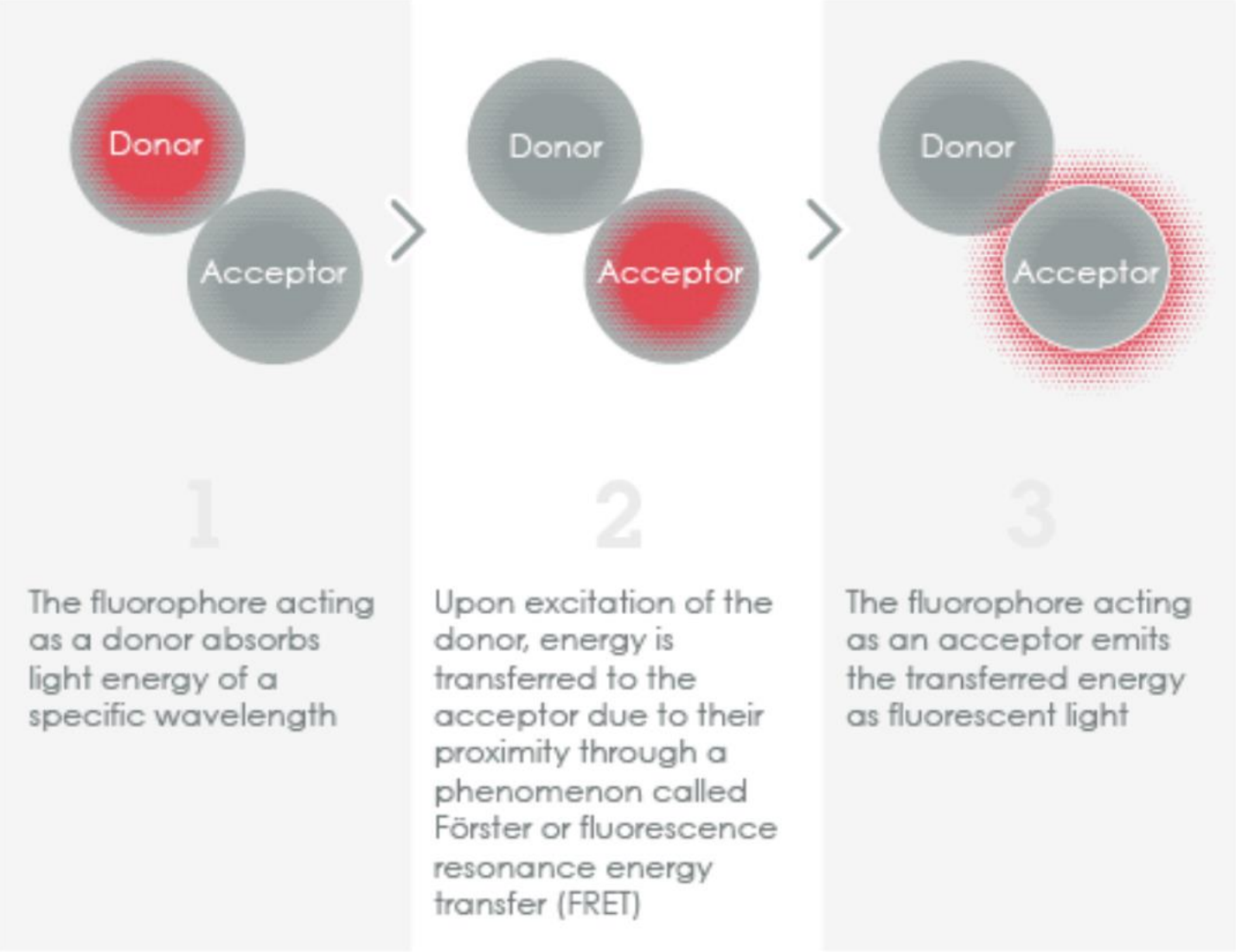


1B

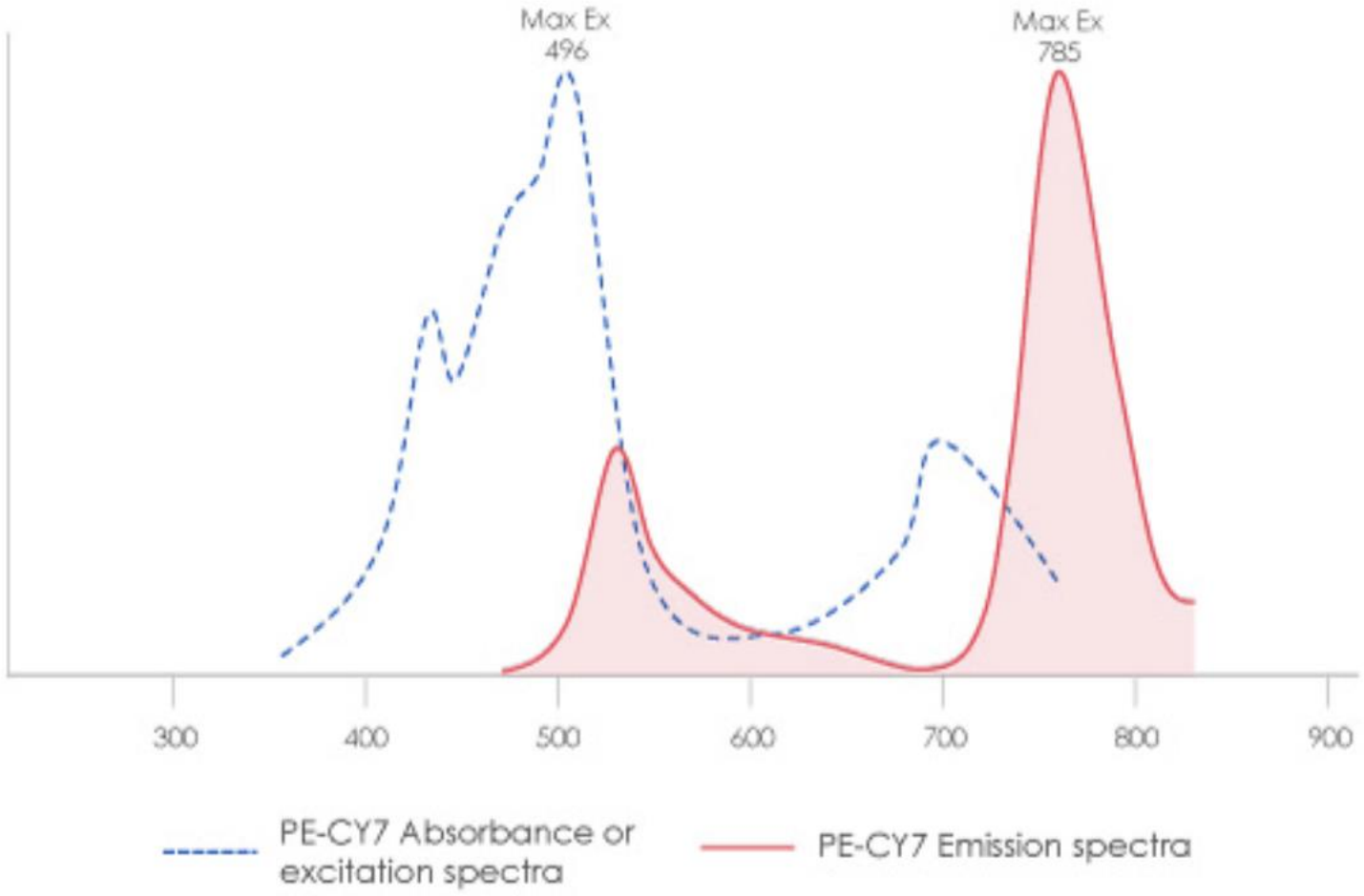
Fluorophores



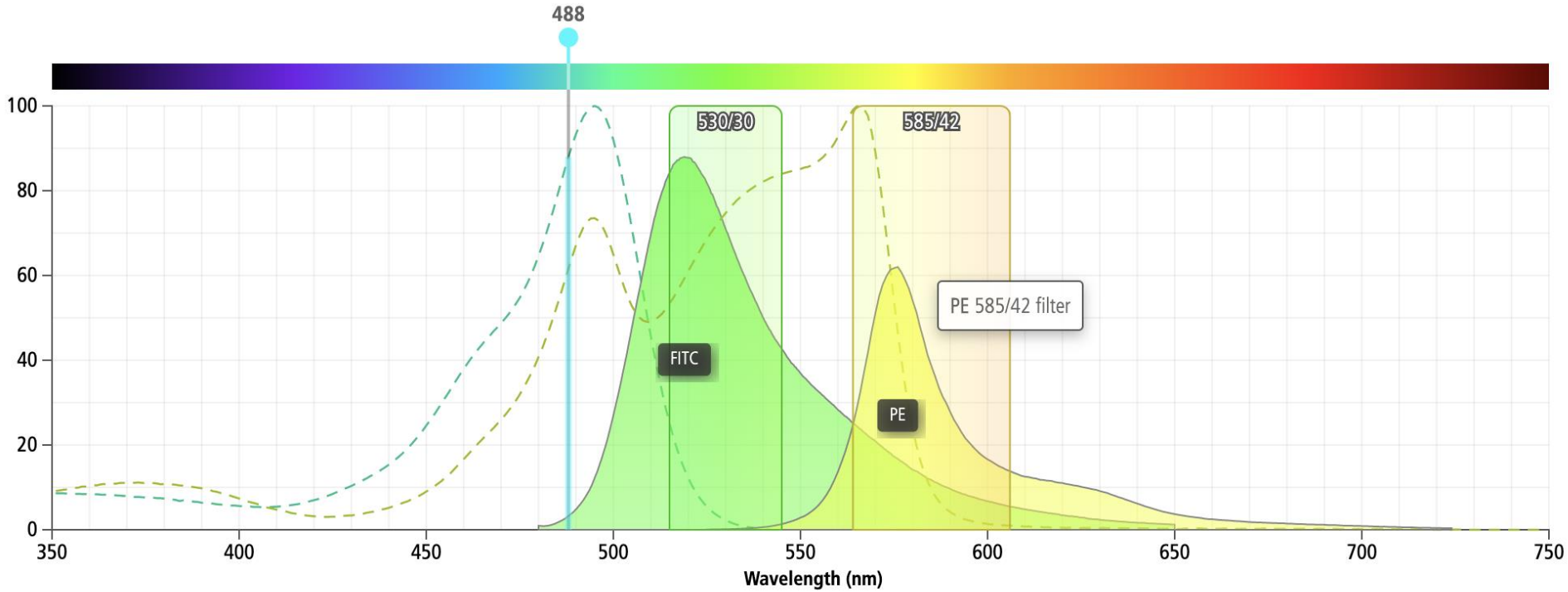
Tandem Fluorophores



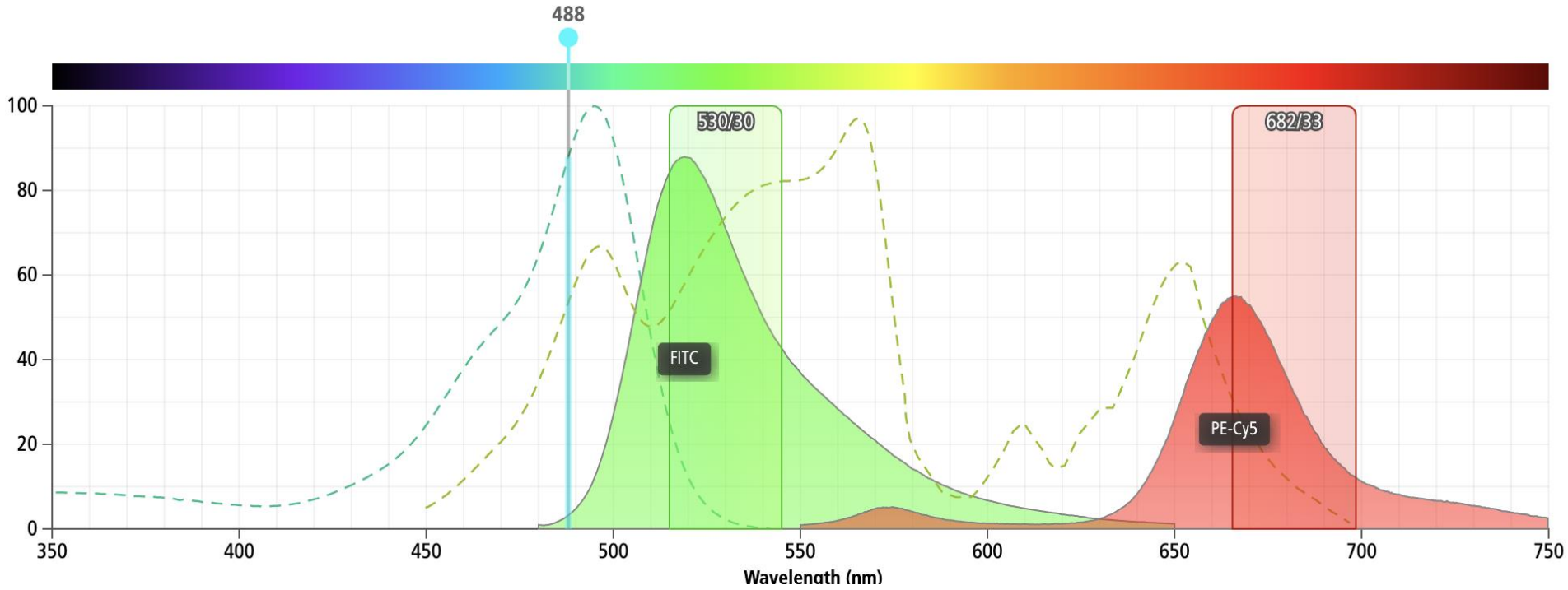
Tandem Fluorophores



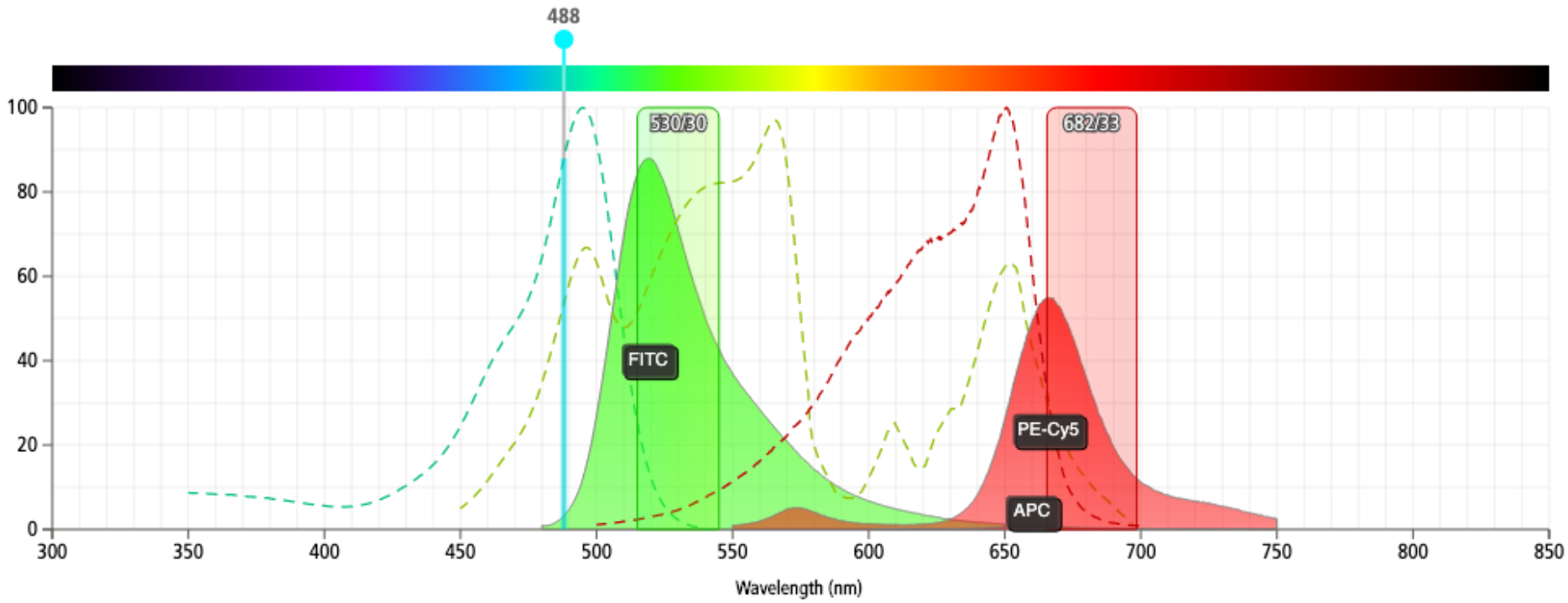
Spillover


488
100
80
60
40
20
0
530/30
585/42
PE 585/42 filter
FITC
PE
350
400
450
500
550
600
650
700
750
Wavelength (nm)

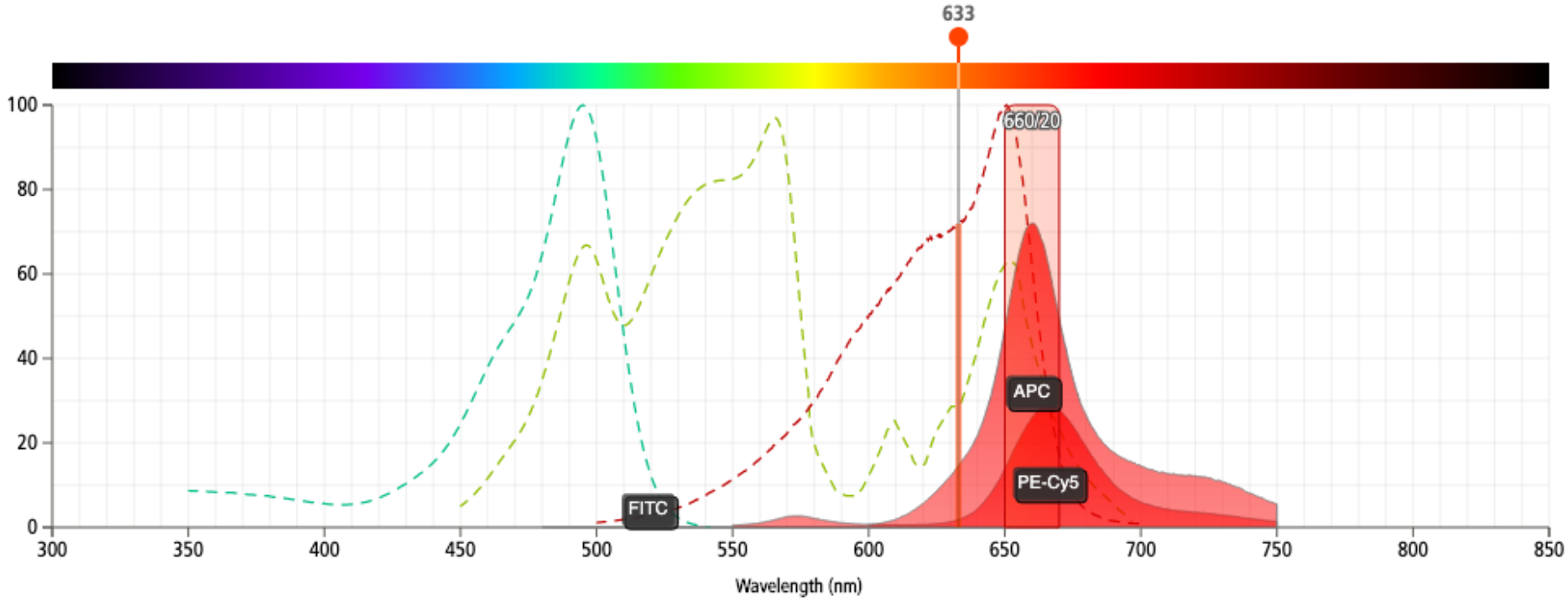
Spillover Tandems

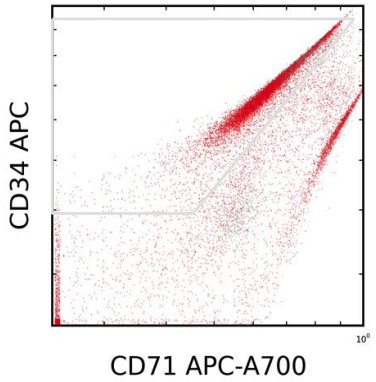


Spillover - Crosslaser

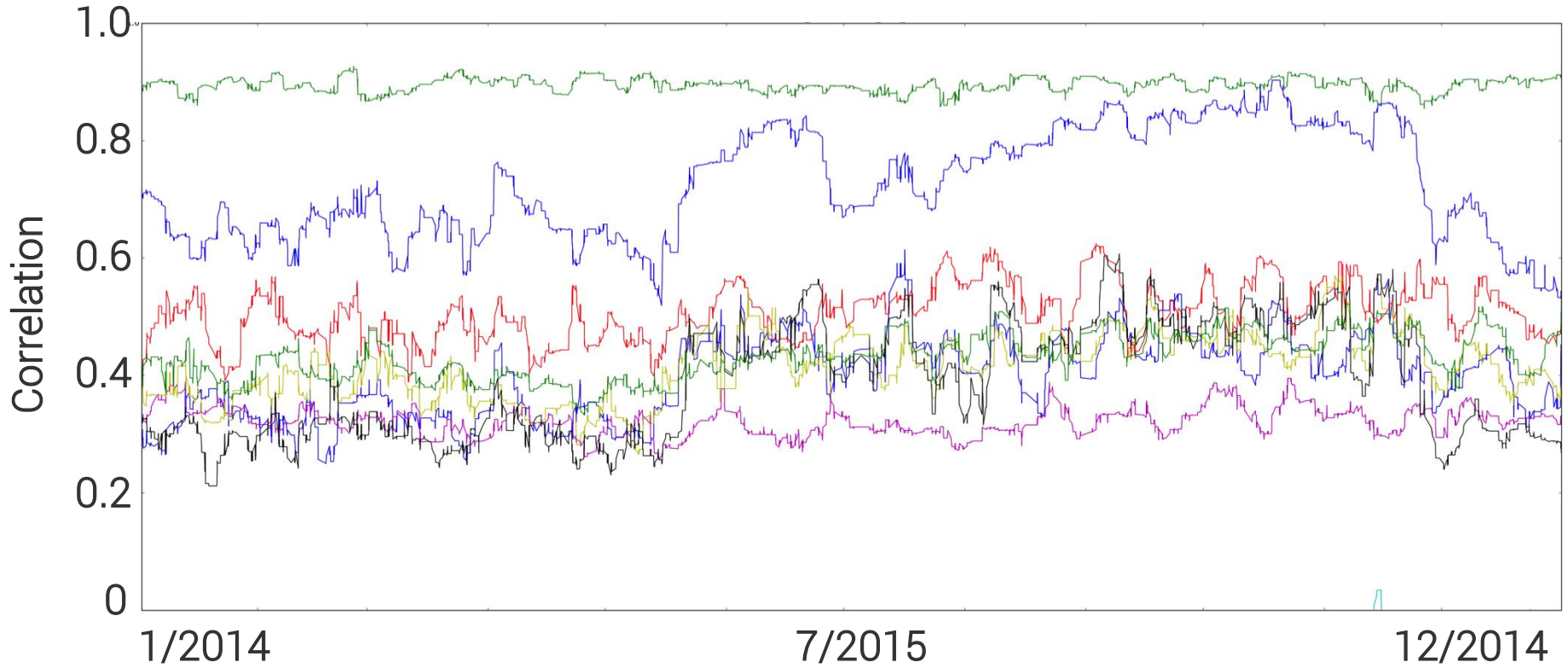


Spillover - Crosslaser





Spillover is not constant



APC, Myeloid 1, CD34

How do I avoid diagnostic errors?

- Under Calls
  - » Flow is an ancillary study…
  - » “Its not your fault” – Sampling Issues
  - » “Its entirely your fault” – Perceptual Issues

- Over Calls
  - » Is that abnormal population real?

Flow is an ancillary study…

- HG follicular lymphoma vs DLBCL
- Peripheral blood contamination vs tissue involvement

Sampling Issues

- A single follicle center can be light chain skewed…
- Plasma cell myelomas are patchy
- Poisson Statistics in paucicellular samples
  - » Population SD = $\sqrt{\#\ of\ positive\ events}$
  - » Precision drops as the number goes down!

Real vs Fake Populations

- Non specific binding of antibodies
  - » Isotype controls
  - » FMO controls

- Compensation issues

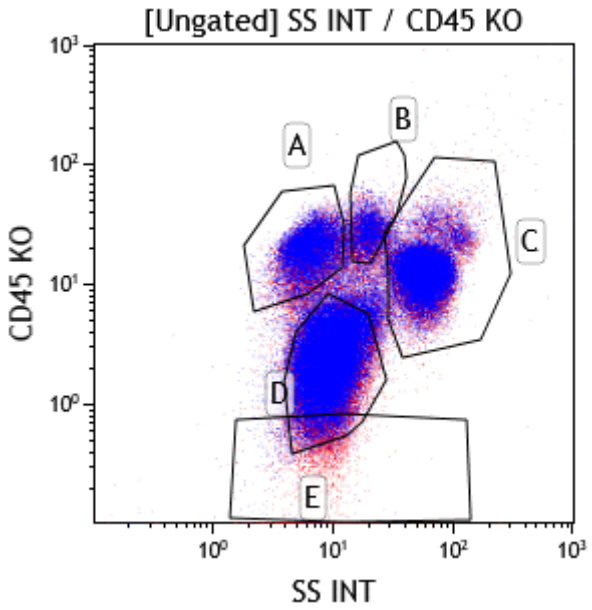
Why are isotype controls generally stupid?

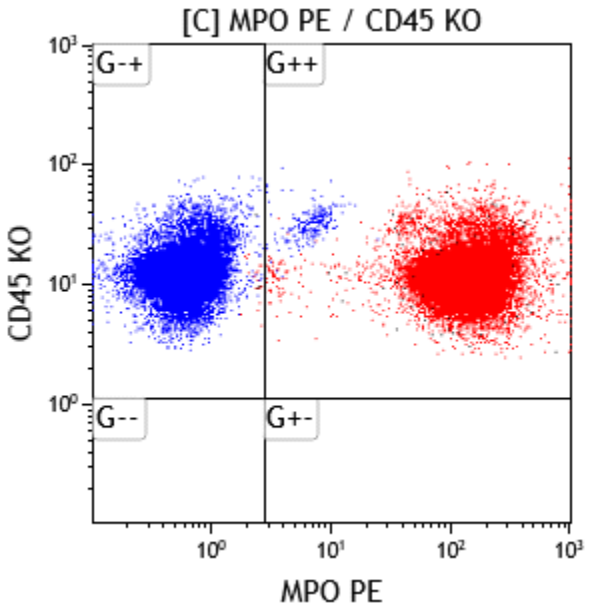
PRO

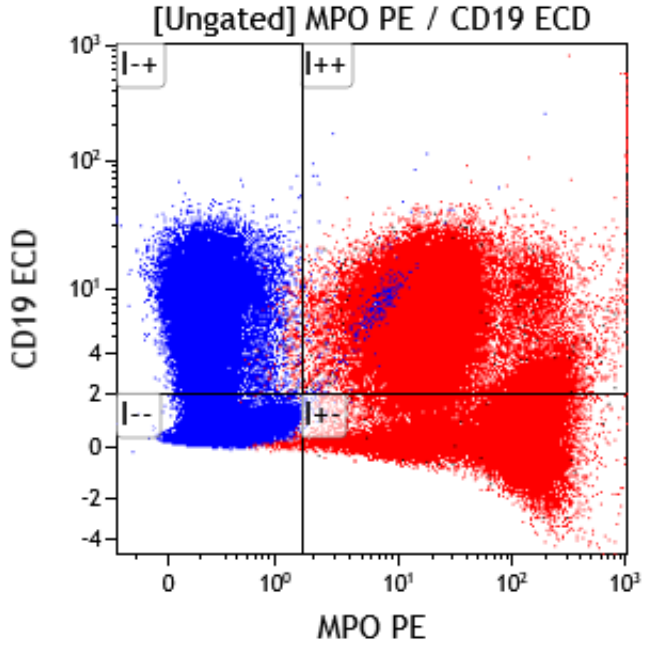
- A non-specific antibody to measure Fc interactions
- Gives you a ‘negative’ population to work with

CONS

- Its not really the same antibody
  - » There is no such thing as a true isotype
- False sense of security
- Doesn’t solve problems about autofluorescence


[Ungated] SS INT / CD45 KO
CD45 KO
SS INT
A
B
C
D
E


[C] MPO PE / CD45 KO
CD45 KO
MPO PE
G-+
G++
G--
G+-


[Ungated] MPO PE / CD19 ECD
CD19 ECD
MPO PE
I-+
I++
I--
I+-

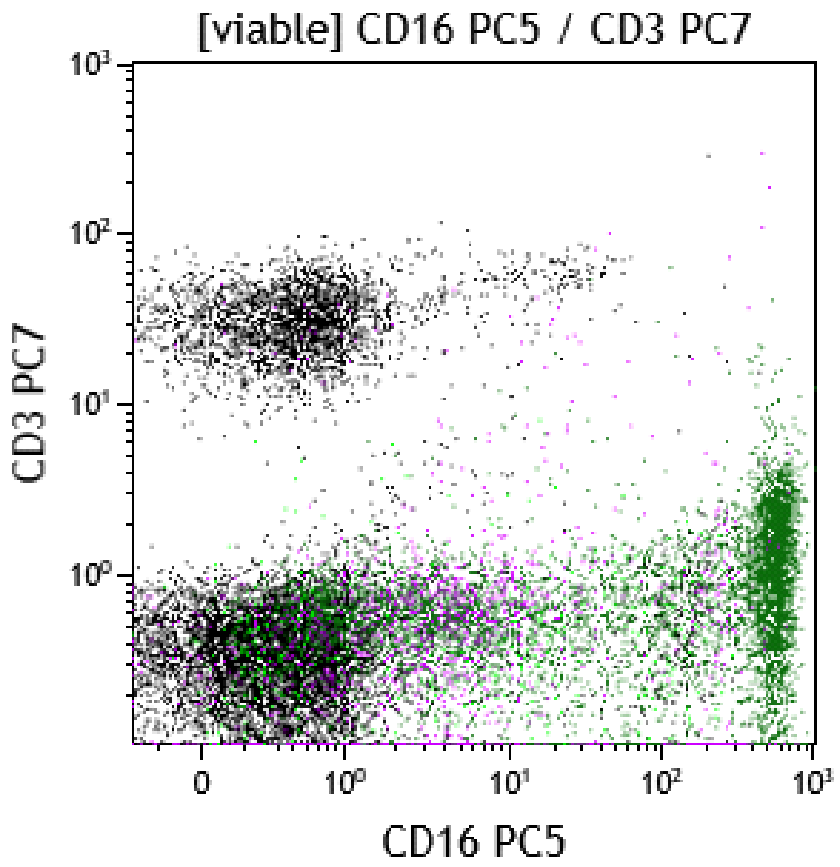
Isotype controls (con't)

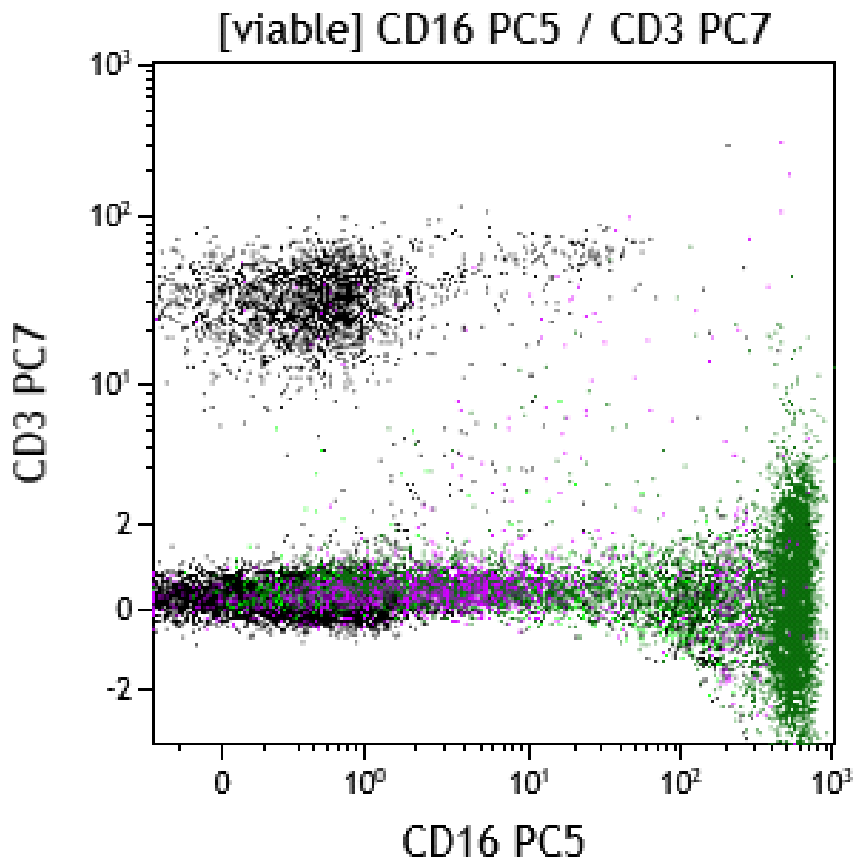
Rule: Don't use them

- » In the modern era of specific monoclonal antibodies its not needed
- » Autofluorescence should be measured by FMO control

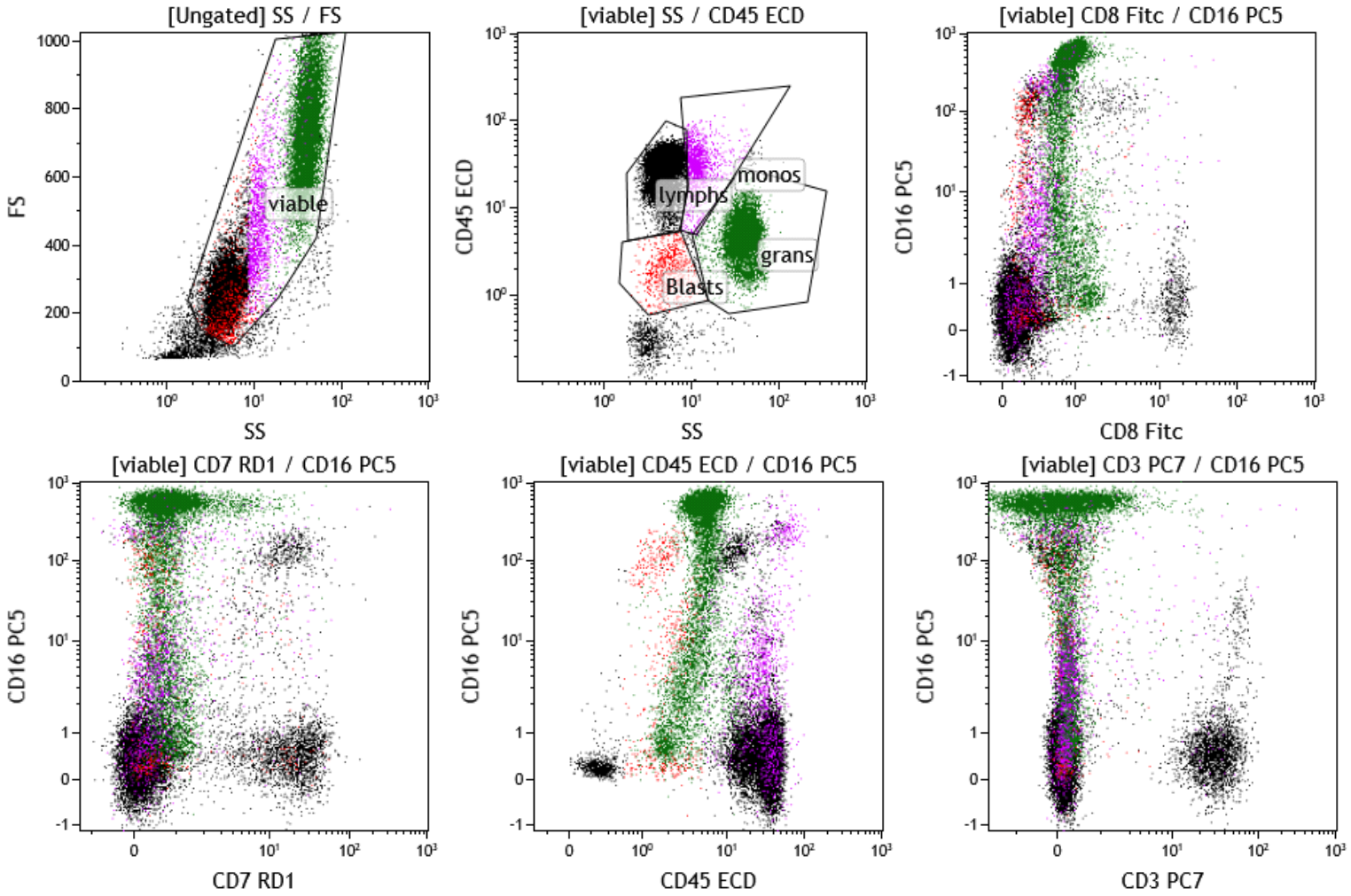
- May have applications in the monocytes, some immune subsets (Fc binding) and cytoplasmic reagents (neo-epitope formation?)

Biexponetial/Logicle Display

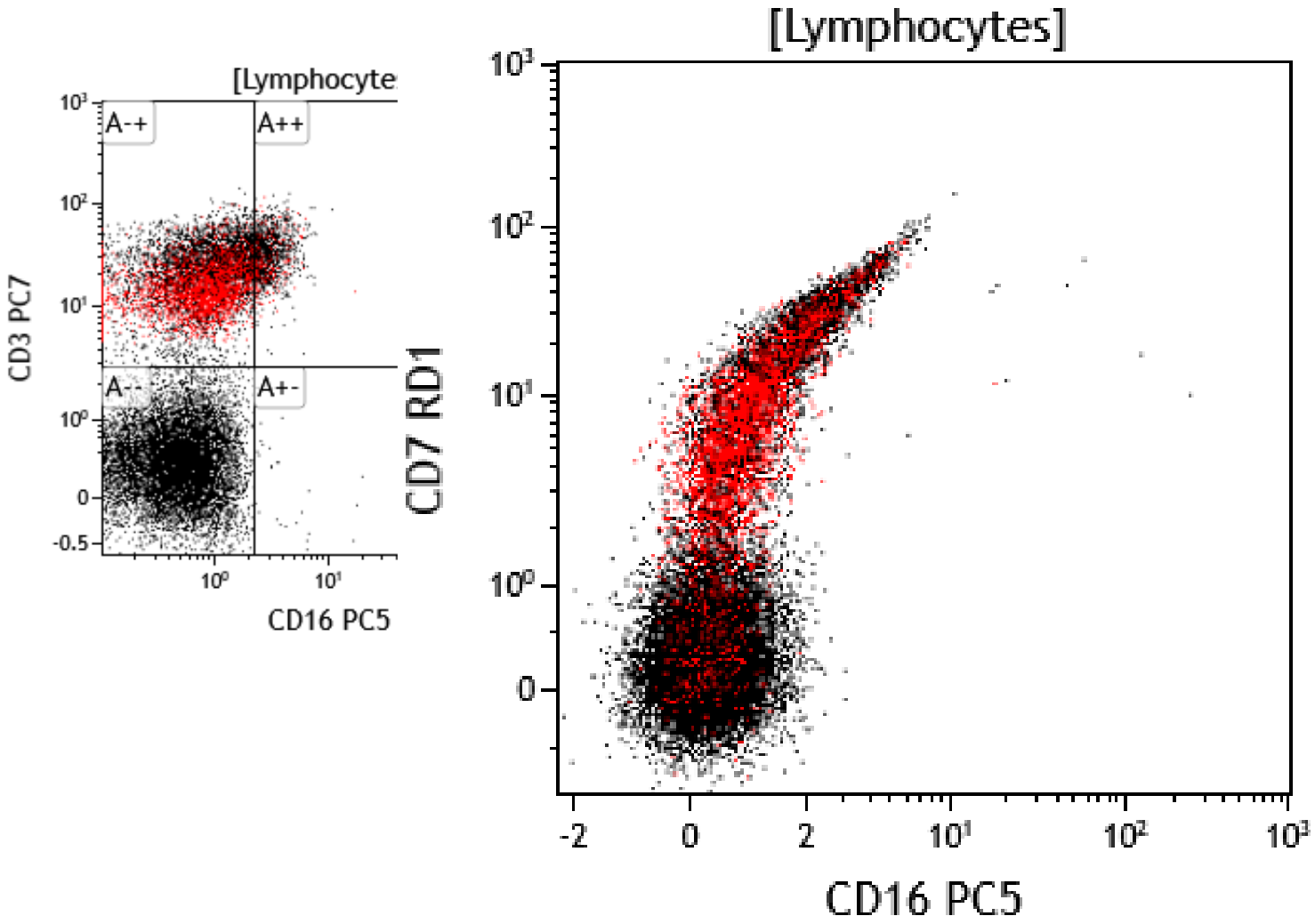


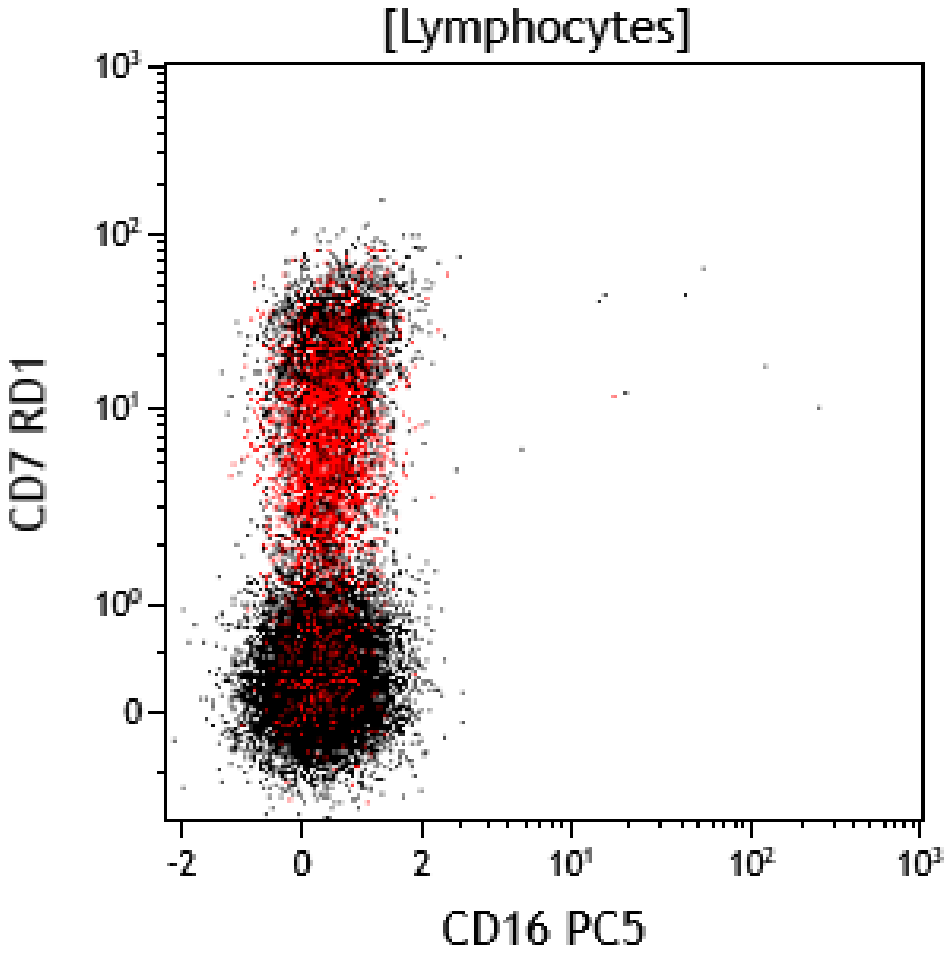


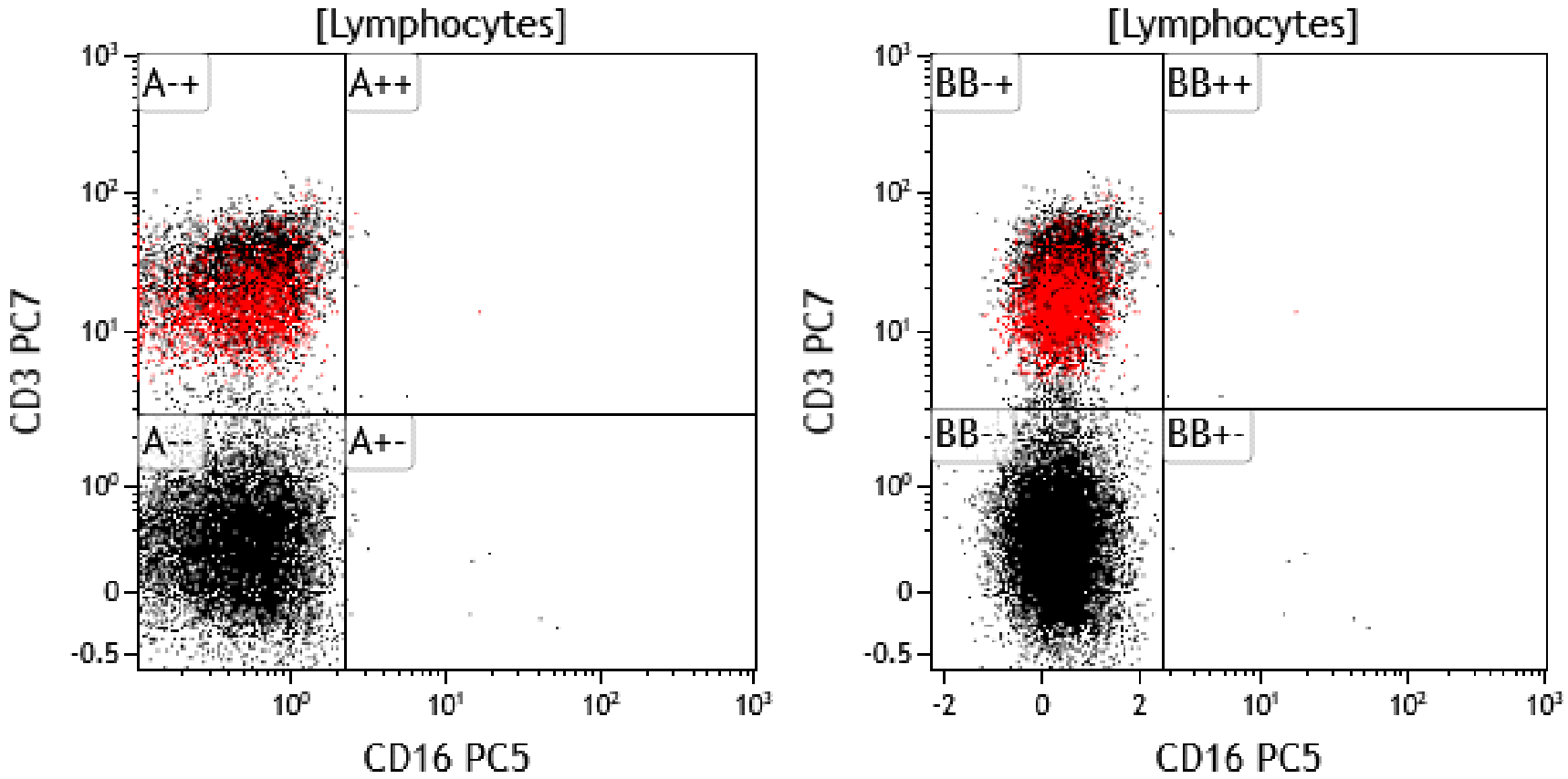
Issues with high expressors



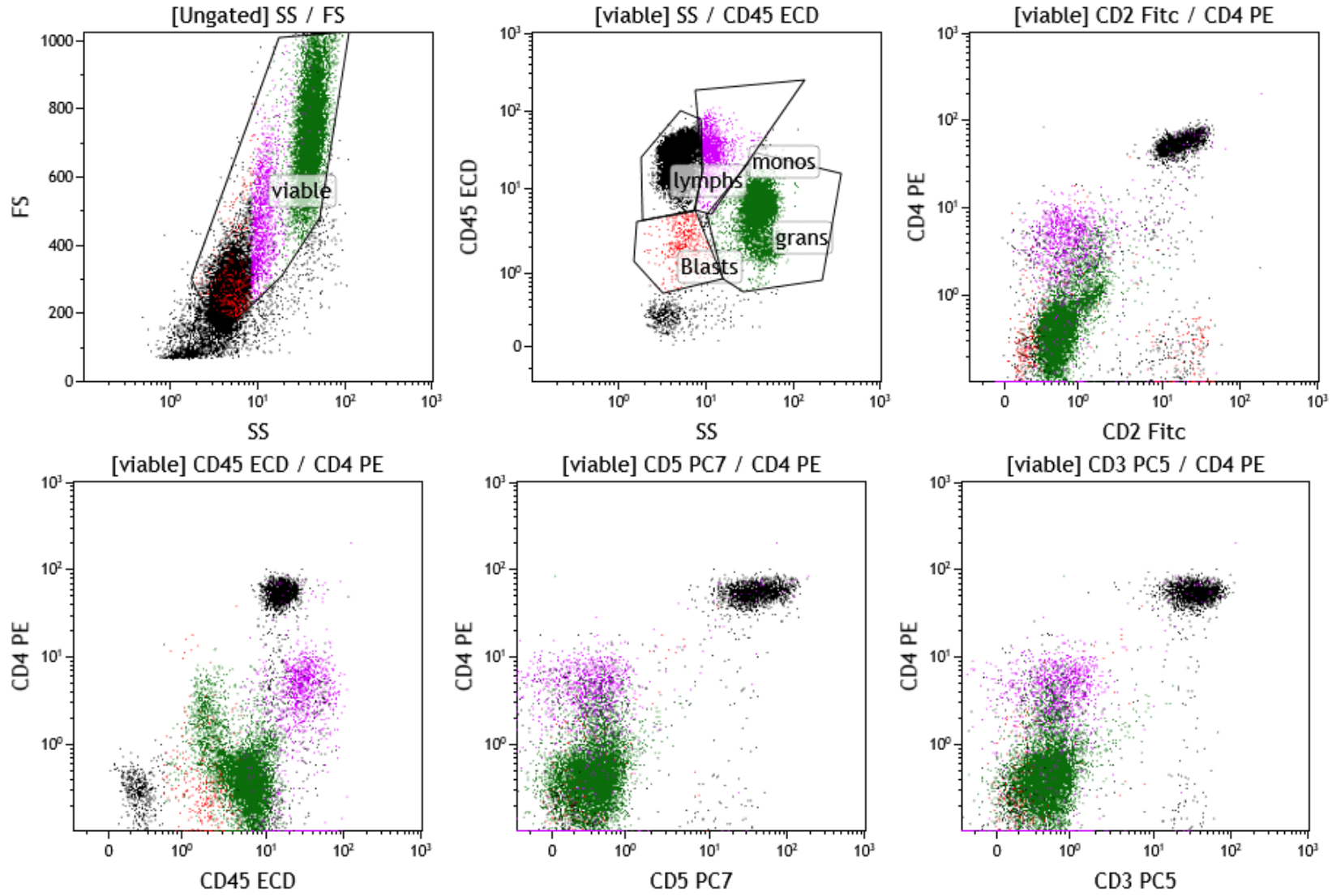
Comp issues are hard to see



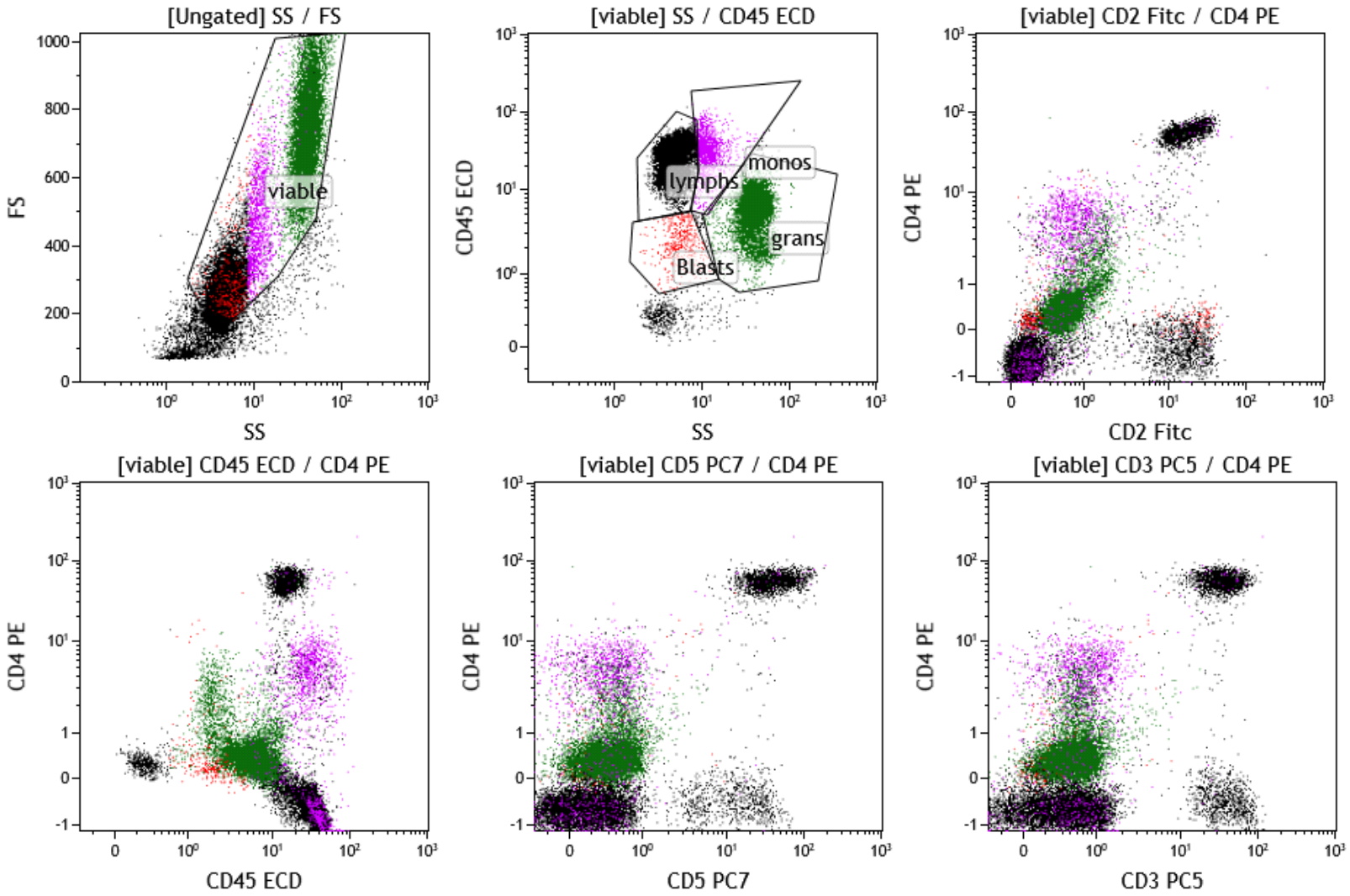

[Lymphocytes]
CD7 RD1
CD16 PC5
10^3
10^2
10^1
10^0
0
-2
0
2
10^1
10^2
10^3


[Lymphocytes]
A-+
A++
A--
A+-
CD3 PC7
CD16 PC5
[Lymphocytes]
BB-+
BB++
BB--
BB+-
CD3 PC7
CD16 PC5

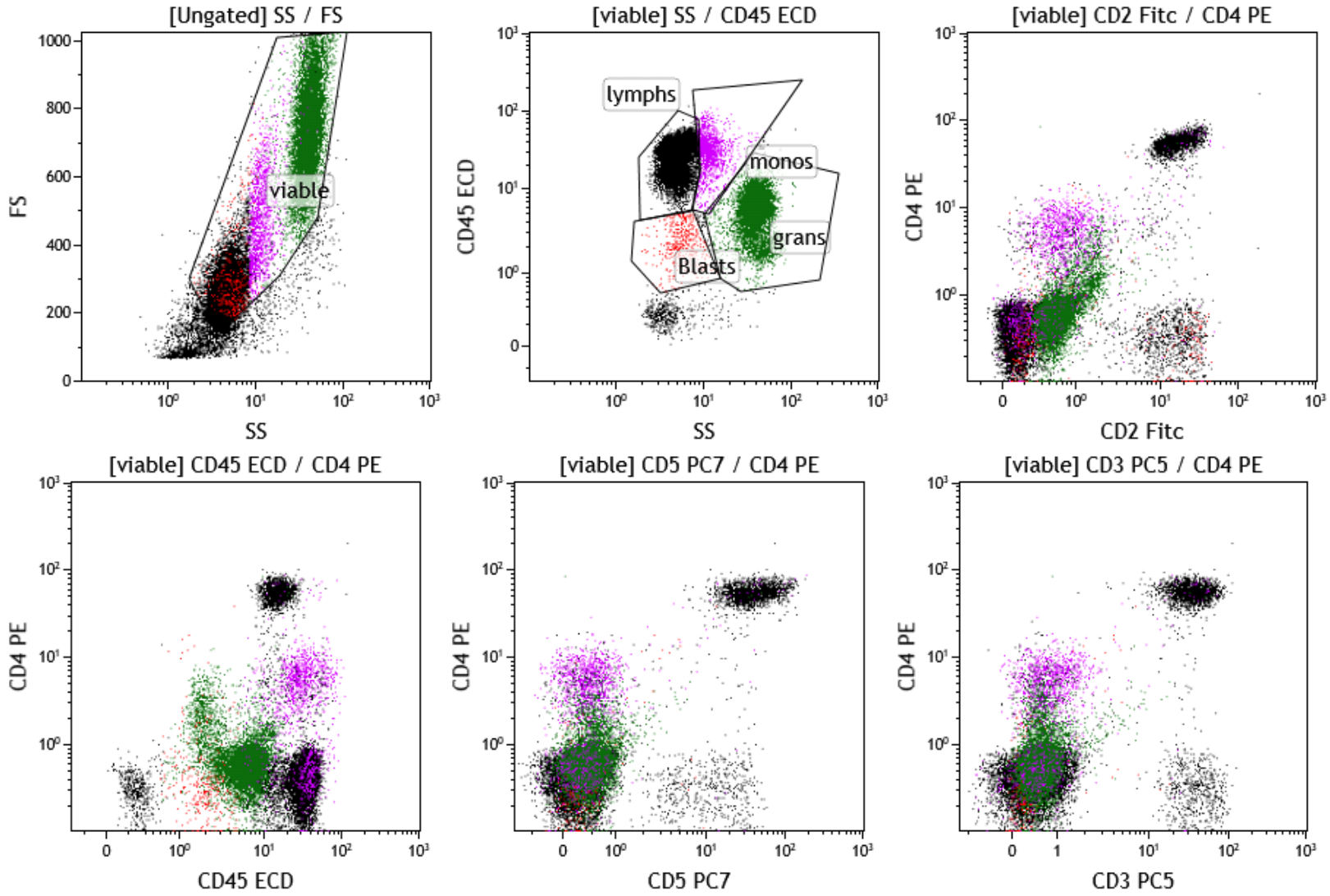
Where did my CD4- T cells go?



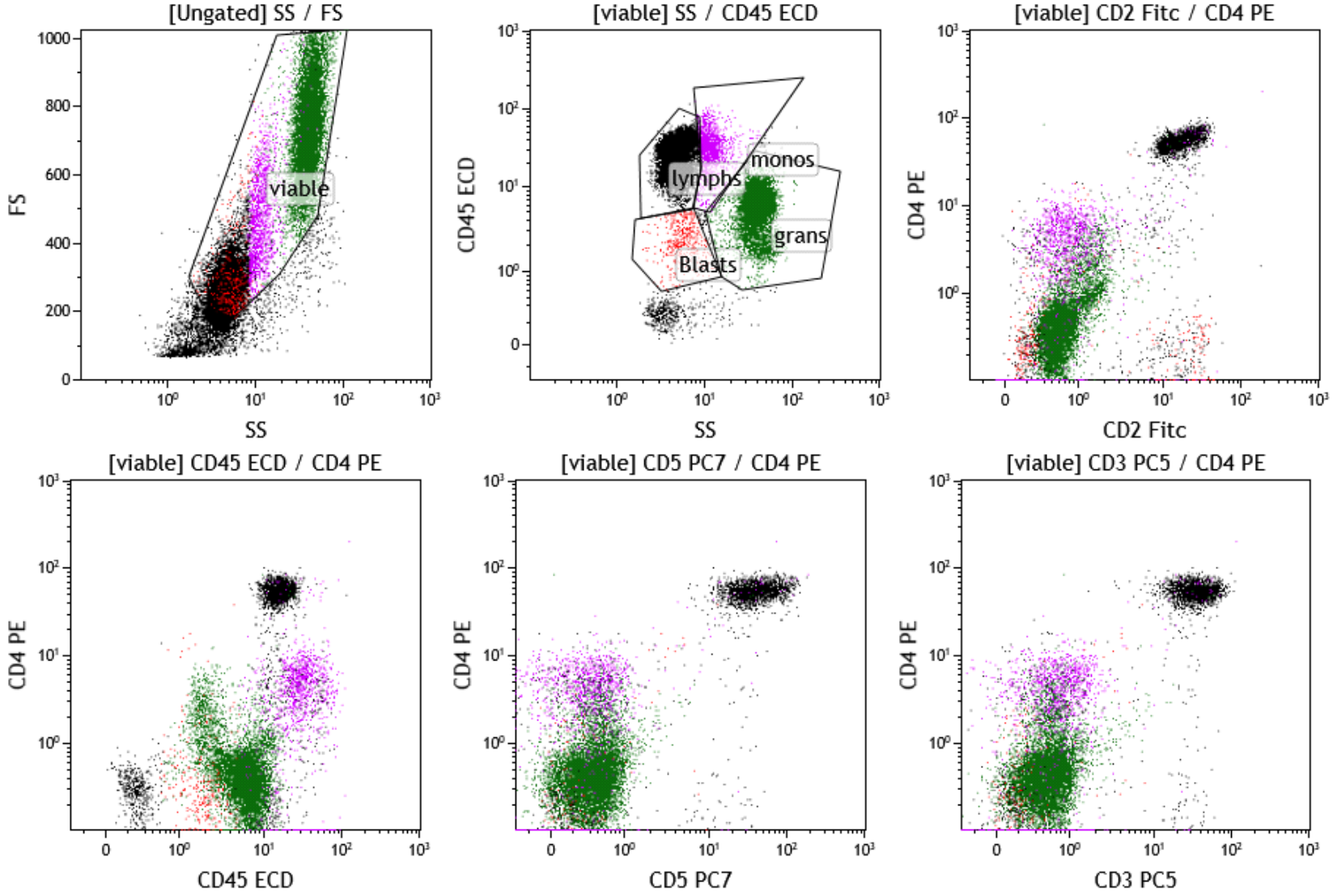
Oh there they are…



What it should look like…



Compare and Contrast…



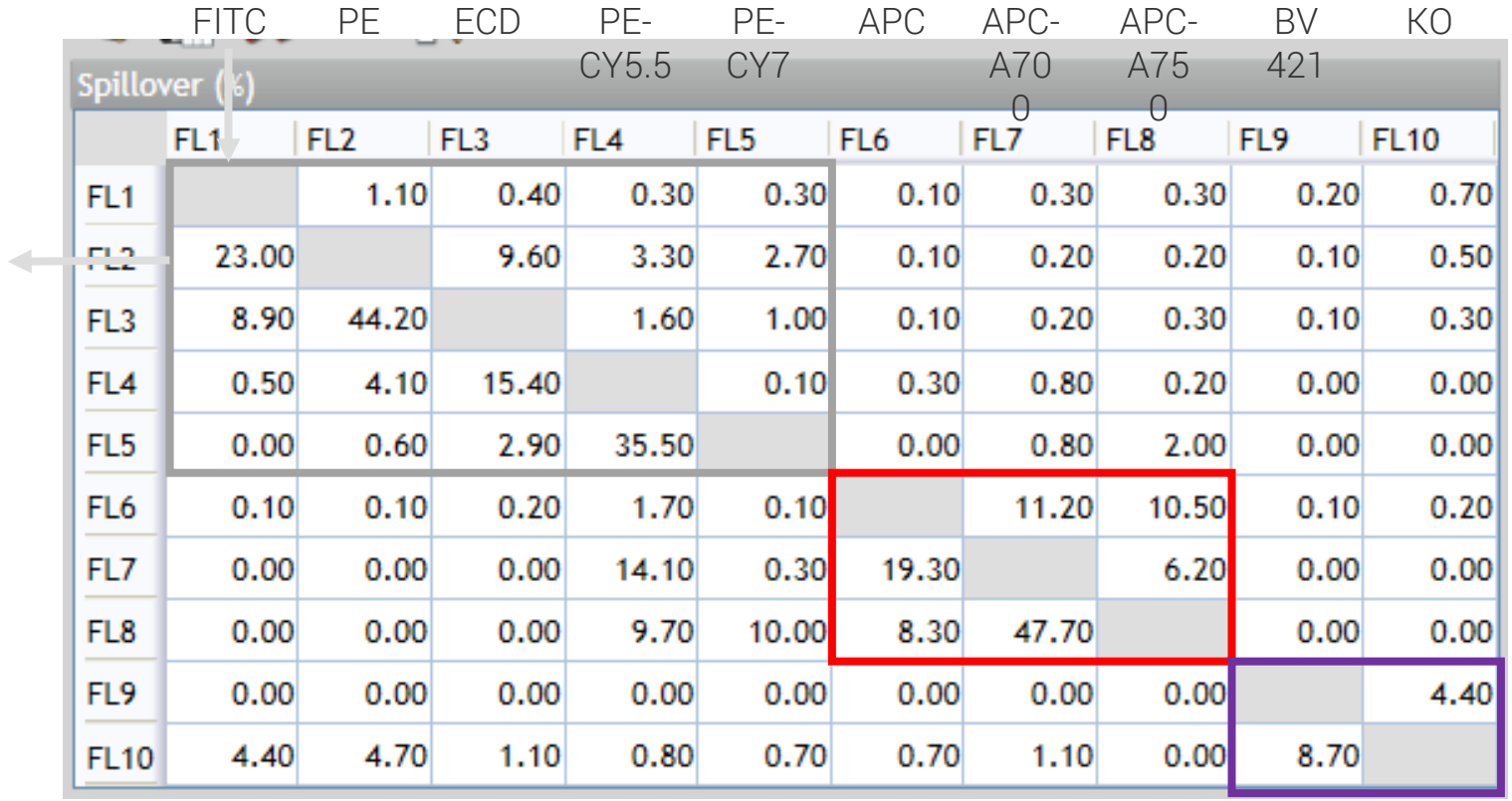
Schlemiels and Schlimazels

- Fluorophores are like people:
  - » Some fluorophores spill over into other fluorophores
  - » "The *schlemiel* spills his soup on the *schlimazel*."

- Its important to know who is who
  - » Design around the issue
  - » Recognize the artifact
  - » Adjust the spillover estimate

Spillover (%)

	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		1.10	0.40	0.30	0.30	0.10	0.30	0.30	0.20	0.70
FL2	22.00		9.60	3.30	2.70	0.10	0.20	0.00	0.10	0.50
FL3	8.90	49.10		1.60	1.00	0.10	0.20	0.30	0.10	0.30
FL4	0.80	5.30	17.70		0.10	0.30	0.80	0.30	0.10	0.00
FL5	0.00	1.00	2.90	35.50		0.00	0.80	2.00	0.00	0.00
FL6	0.00	0.10	0.20	1.70	0.10		10.20	7.50	0.10	0.20
FL7	0.00	0.00	0.00	14.10	0.30	19.30		6.60	0.00	0.00
FL8	0.00	0.00	0.00	9.70	10.00	8.30	47.70		0.00	0.00
FL9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		4.40
FL10	4.40	4.70	1.10	0.80	0.70	0.70	1.10	0.00	8.70	



Spillover (%)

	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		1.10	0.40	0.30	0.30	0.10	0.30	0.30	0.20	0.70
FL2	23.00		9.60	3.30	2.70	0.10	0.20	0.20	0.10	0.50
FL3	8.90	44.20		1.60	1.00	0.10	0.20	0.30	0.10	0.30
FL4	0.50	4.10	15.40		0.10	0.30	0.80	0.20	0.00	0.00
FL5	0.00	0.60	2.90	35.50		0.00	0.80	2.00	0.00	0.00
FL6	0.10	0.10	0.20	1.70	0.10		11.20	10.50	0.10	0.20
FL7	0.00	0.00	0.00	14.10	0.30	19.30		6.20	0.00	0.00
FL8	0.00	0.00	0.00	9.70	10.00	8.30	47.70		0.00	0.00
FL9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		4.40
FL10	4.40	4.70	1.10	0.80	0.70	0.70	1.10	0.00	8.70	

Tandem Breakdown

Tandems – the solution to and cause of all life's problems.

- Ab – Donor – Acceptor
- Antibody with a tandem looks like its donor
- CD34 APC-A700 -> acts like CD34 APC

What happens if you have CD7 on APC?

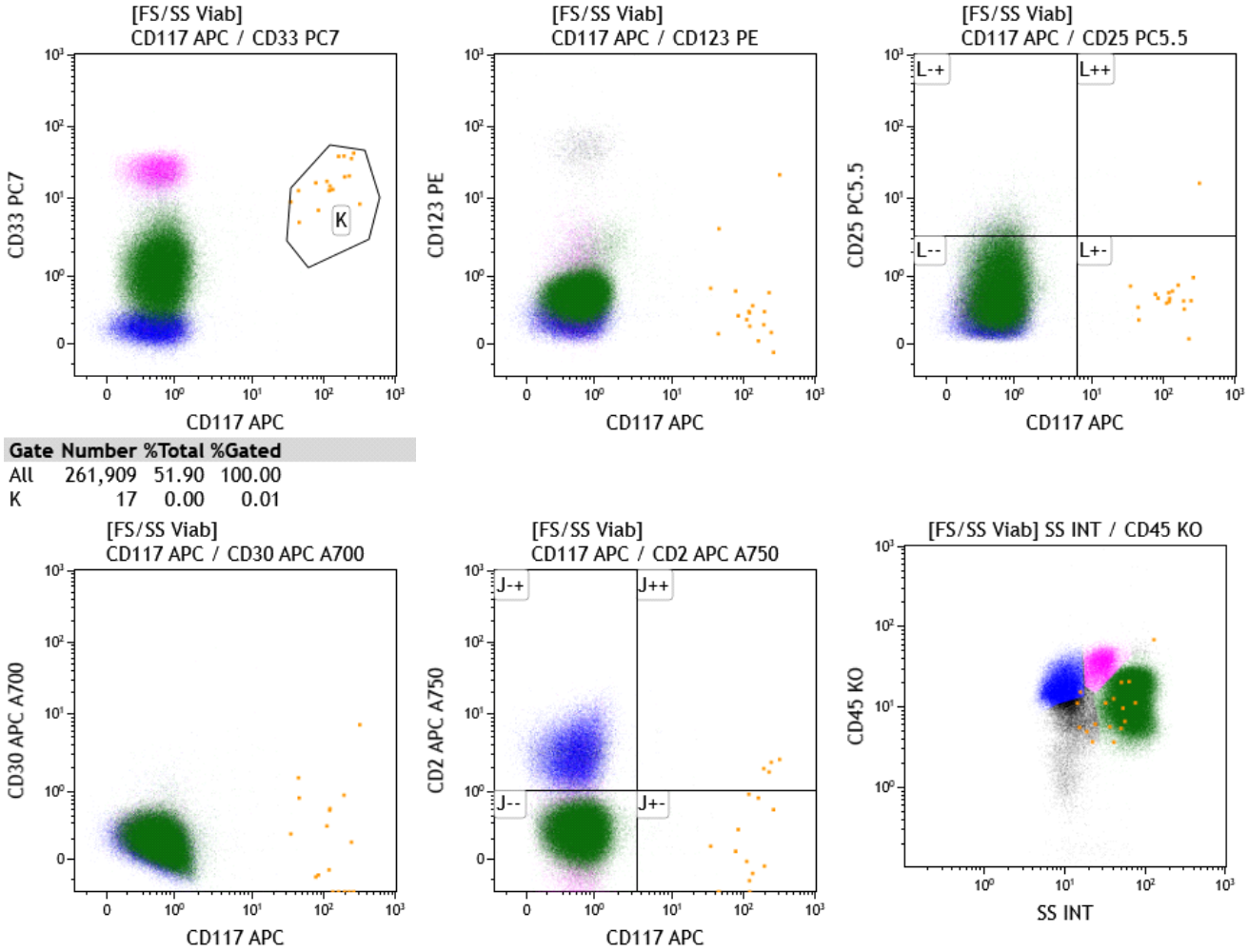
Panel Design

- What antibody and fluorophore goes where?

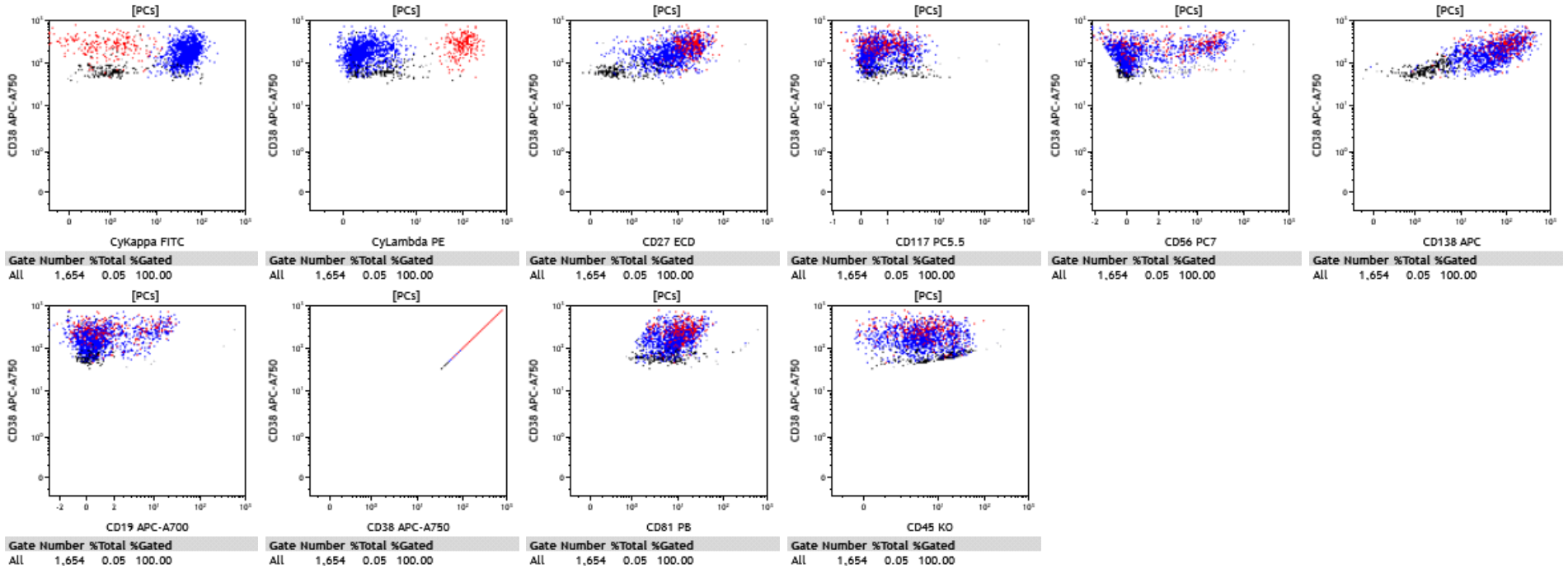
- Strong antibody and weak fluorophore (vice versa)
- Strong antibodies/fluorophore can lead to spillover issues, increased “negative” control

- Fit-for-purpose

Panel Design Example/Considerations

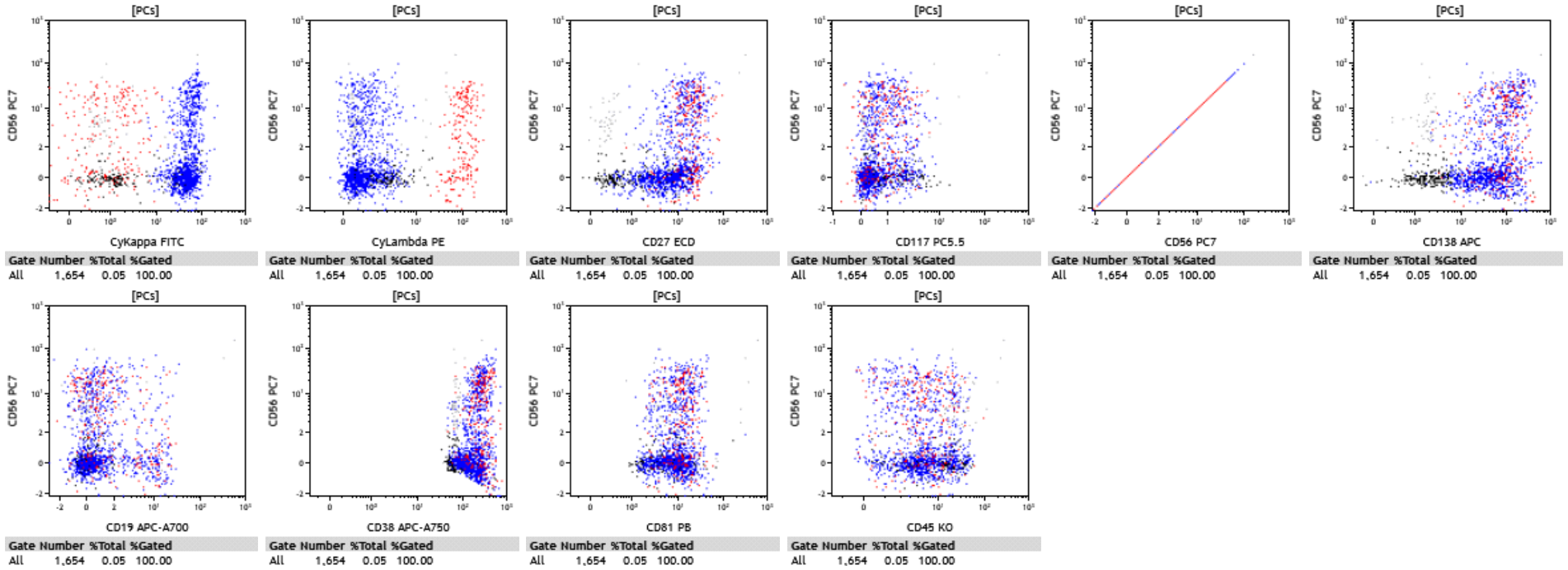


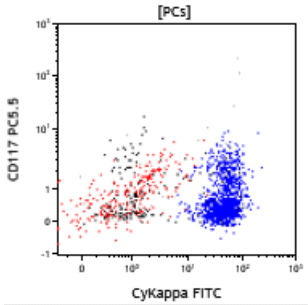
Panel Design Example/Considerations



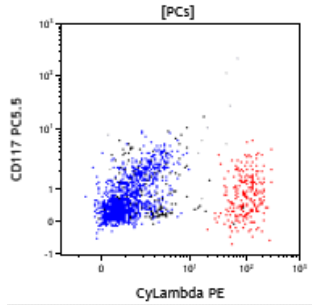
Additional things to care about

- CD56 and CD117

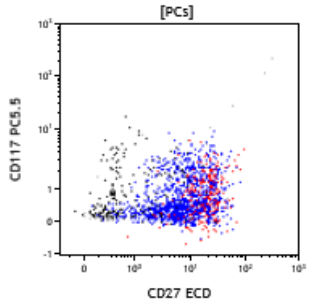




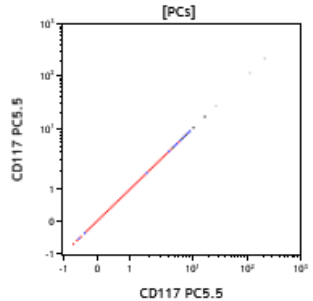
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



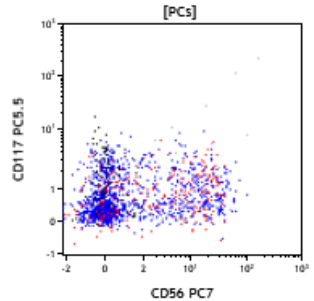
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



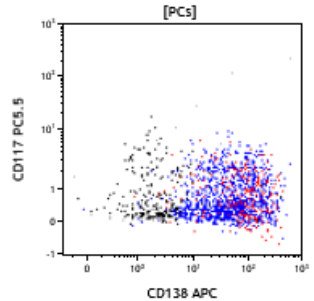
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



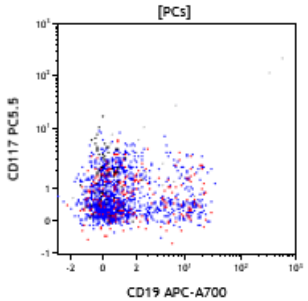
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



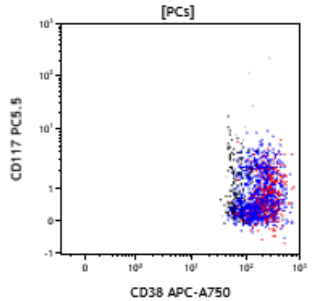
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



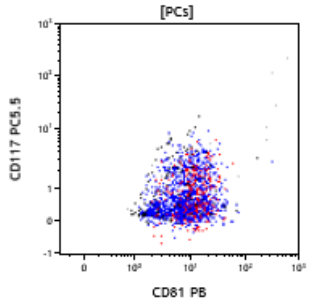
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



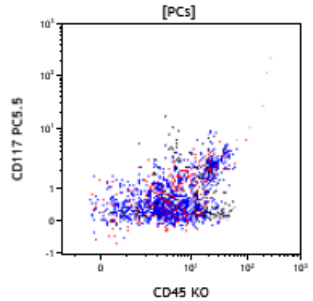
Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



Gate	Number	%Total	%Gated
All	1,654	0.05	100.00



Gate	Number	%Total	%Gated
All	1,654	0.05	100.00

Summary

- Technical artifacts need to be recognized
  - » Compensation
  - » Breakdown
  - » Positivity Discrimination

- You can decrease but never eliminate artifacts
  - » Schlemiels are always abound
  - » But bright schlimazels can’t be spilled on effectively

ARUP® LABORATORIES | HEALTH UNIVERSITY OF UTAH

ARUP is a nonprofit enterprise of the University of Utah and its Department of Pathology.