ISAC

International Society for Advancement of Cytometry

Basic Multicolor Flow Cytometry

Fluorochromes, Spillover and Compensation

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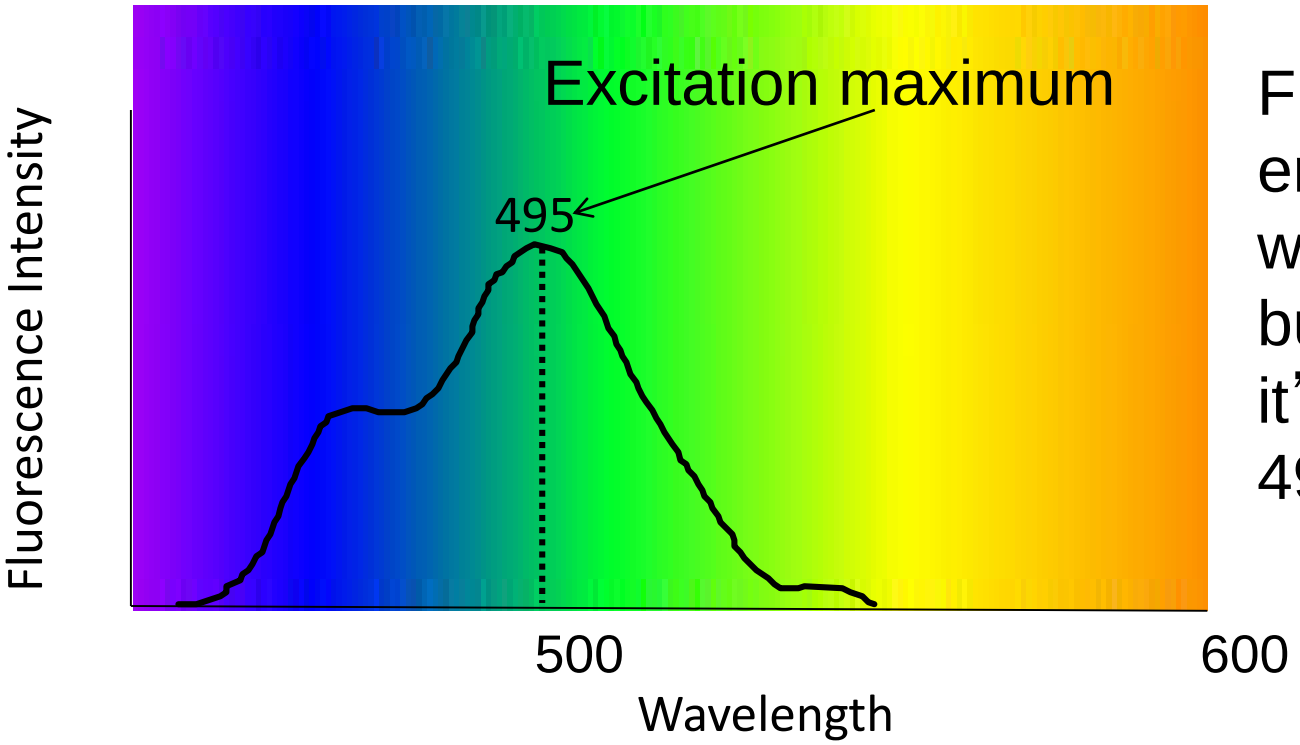
ISAC Live Education Task Force

Excitation spectrum

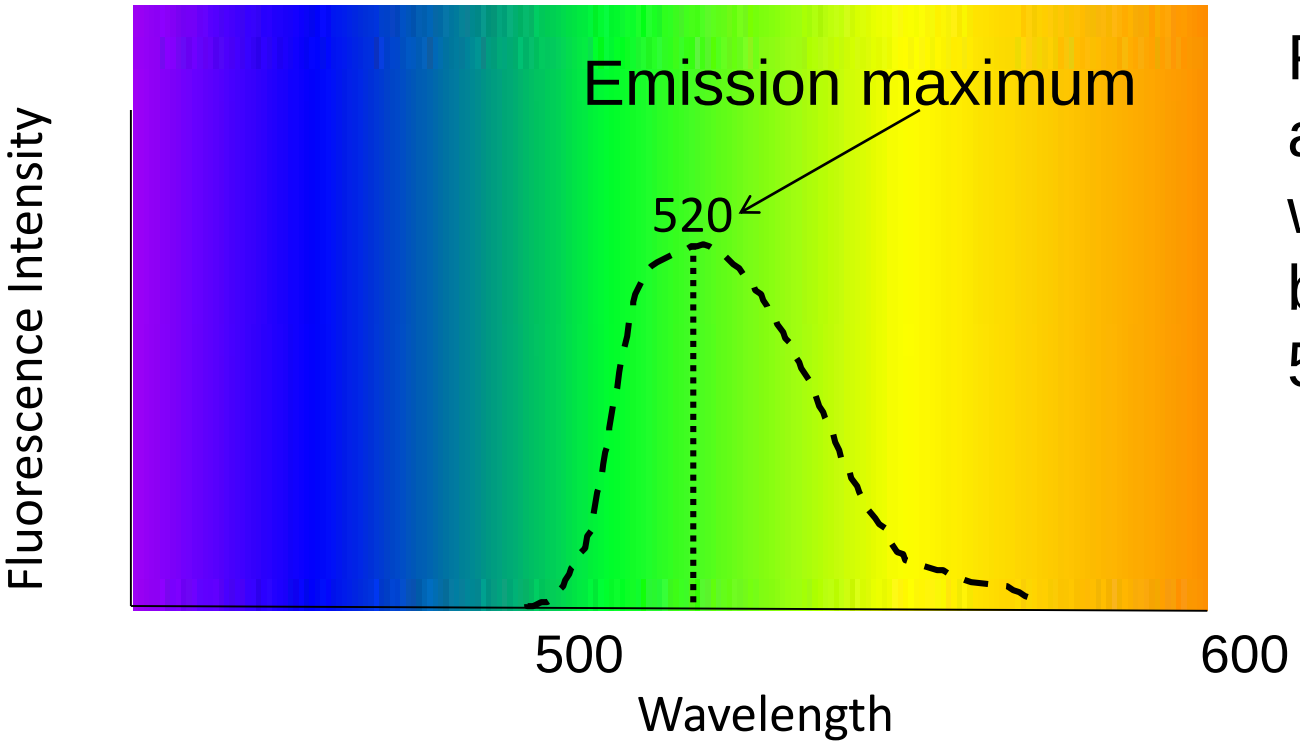
Each fluorochrome is capable of absorbing light energy over a specific range of wavelengths



FITC can absorb energy at all these wavelengths but absorbs best at it's excitation max: 495nm

Emission spectra

Each fluorochrome is also capable of emitting light energy over a specific range of wavelengths

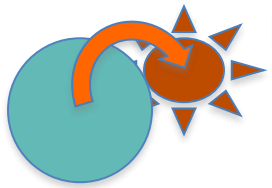


FITC will fluoresce at all these wavelengths but highest at 520nm

Tandem Dyes have 2 fluorochromes coupled together

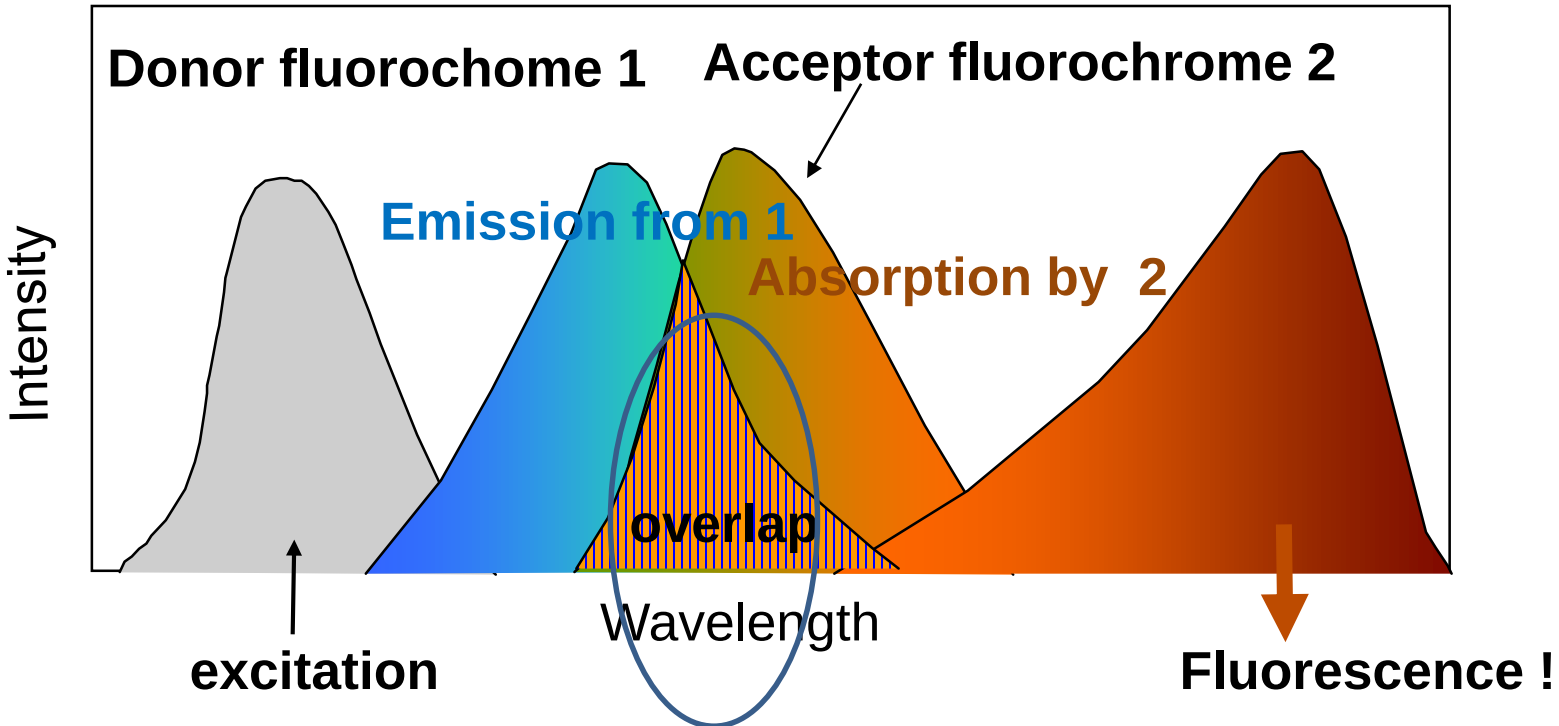
Energy Transfer

The 1st fluorochrome transfers it's absorbed energy to the 2nd fluorochrome



Fluorochrome 2

Fluorochrome 1



Tandem Dyes: conditions

Energy transfer:

- Effective between 10-100 Å only
- Emission and excitation spectrum must significantly overlap
- Donor transfers non-radiatively to the acceptor

Tandem dyes: caution

- all tandems are not the same.

- Some batches of tandems have better coupling and therefore better energy transfer than other batches.

- This means there is more or less leakage from the first fluorochrome

- More or less compensation will be necessary in that emission channel.

- ALWAYS use the same tandem in your single colors as you use in your mix!!

Commonly used tandems

PE-Cy5

PerCP-Cy5.5

PE-Texas Red

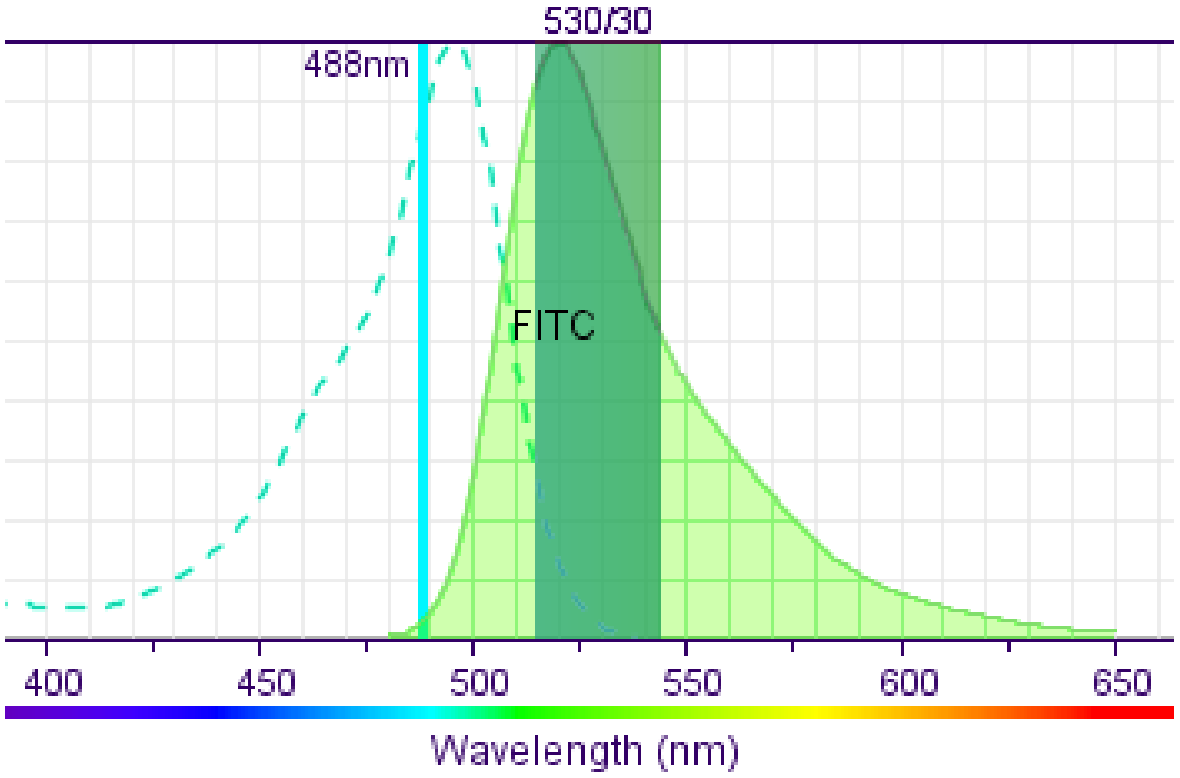
Pe-Cy7

APC-Cy7

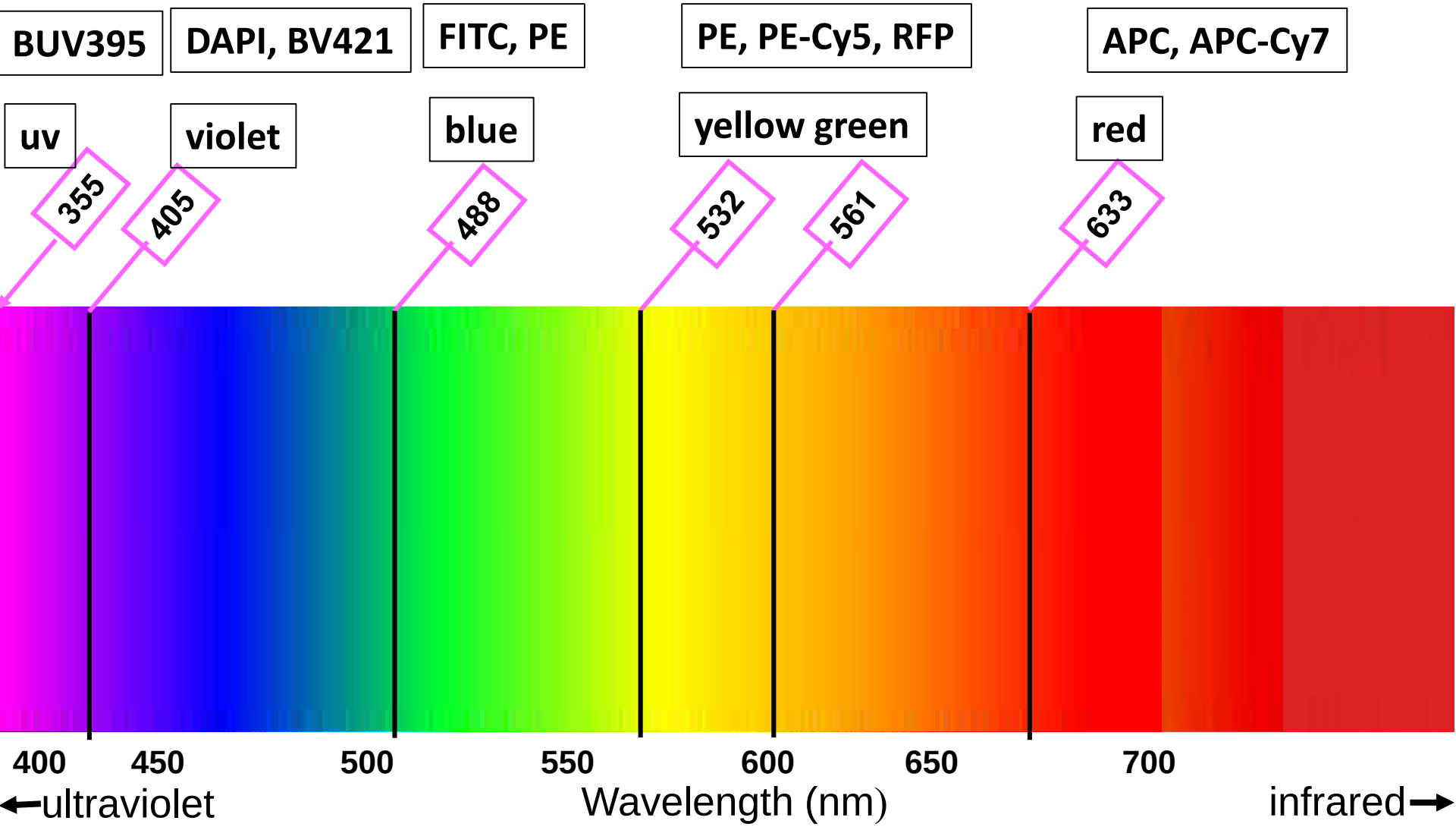
Brilliant (Sirigen) dyes (Brilliant Violet)

Choosing Fluorochromes: which lasers and filters?

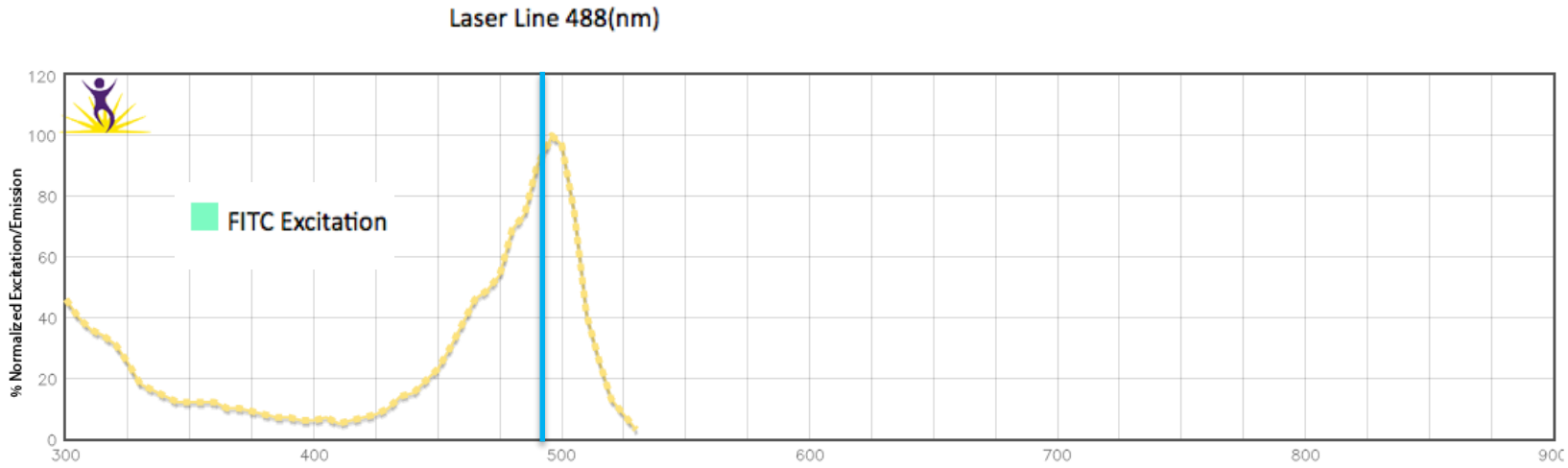
- Look at the **excitation** spectra to determine which lasers can be used to excite the fluorochrome.
- Look at the **emission** spectra to determine which filters should be used to collect the signal.



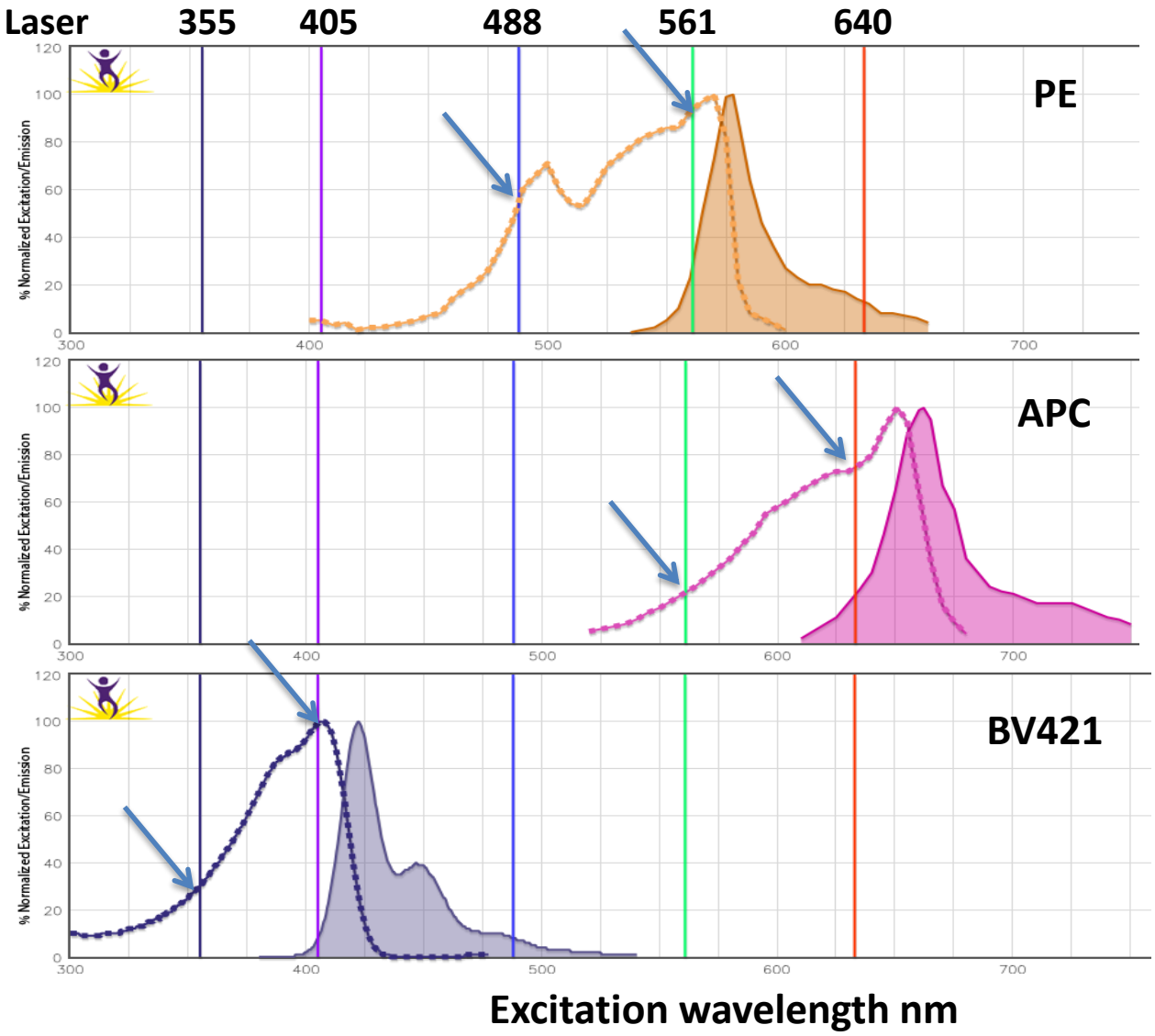
Laser wavelengths on typical cytometers



Look at the excitation spectra of your fluorochromes



Fluorochromes can be excited by different lasers.



PE has a wide excitation spectrum, excited by the 488 laser, but more efficiently with the 561

APC is excited by the 640 but also a little by the 561

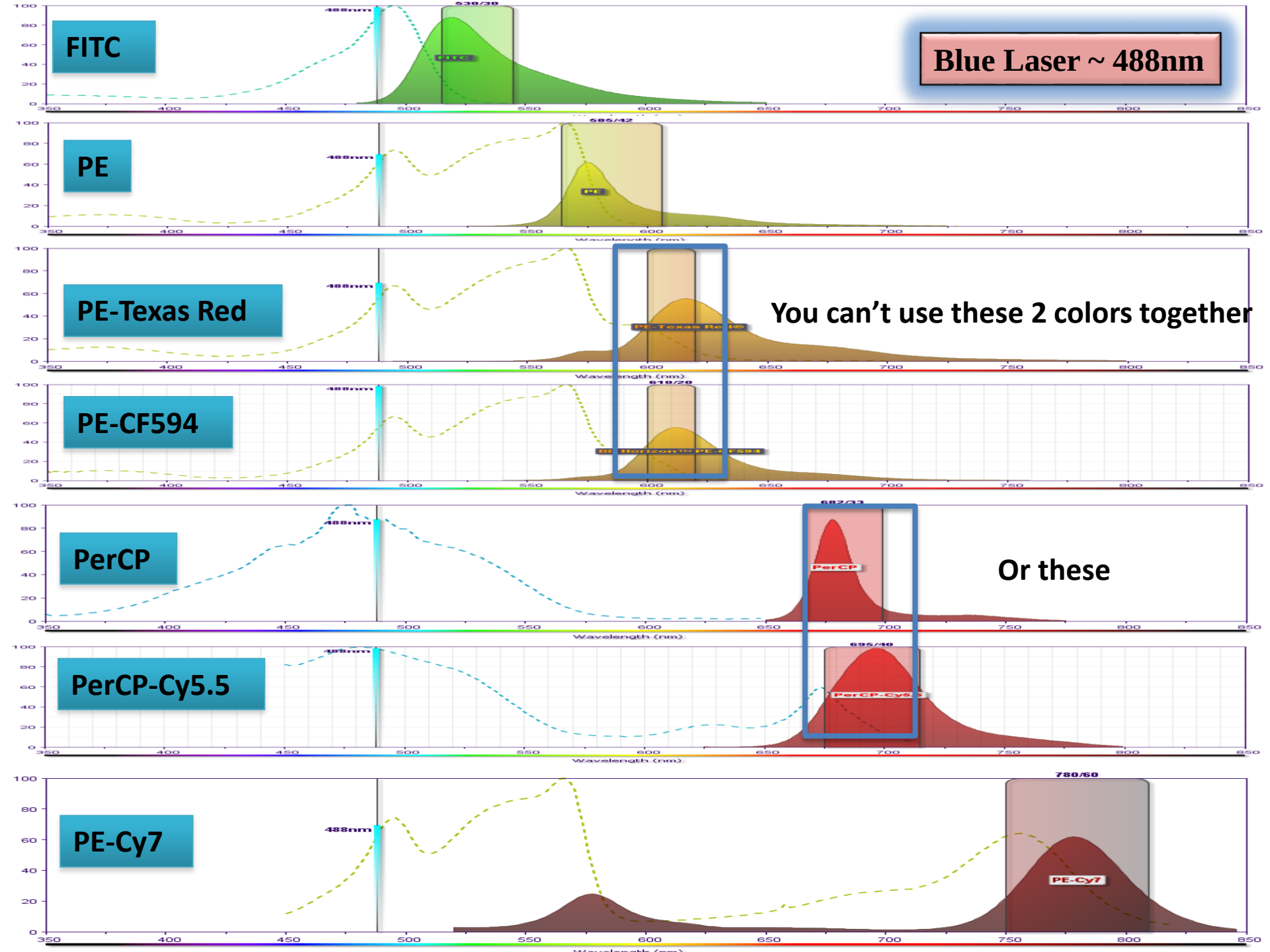
BV421 is excited best by the 405 laser but also a little by the 355.

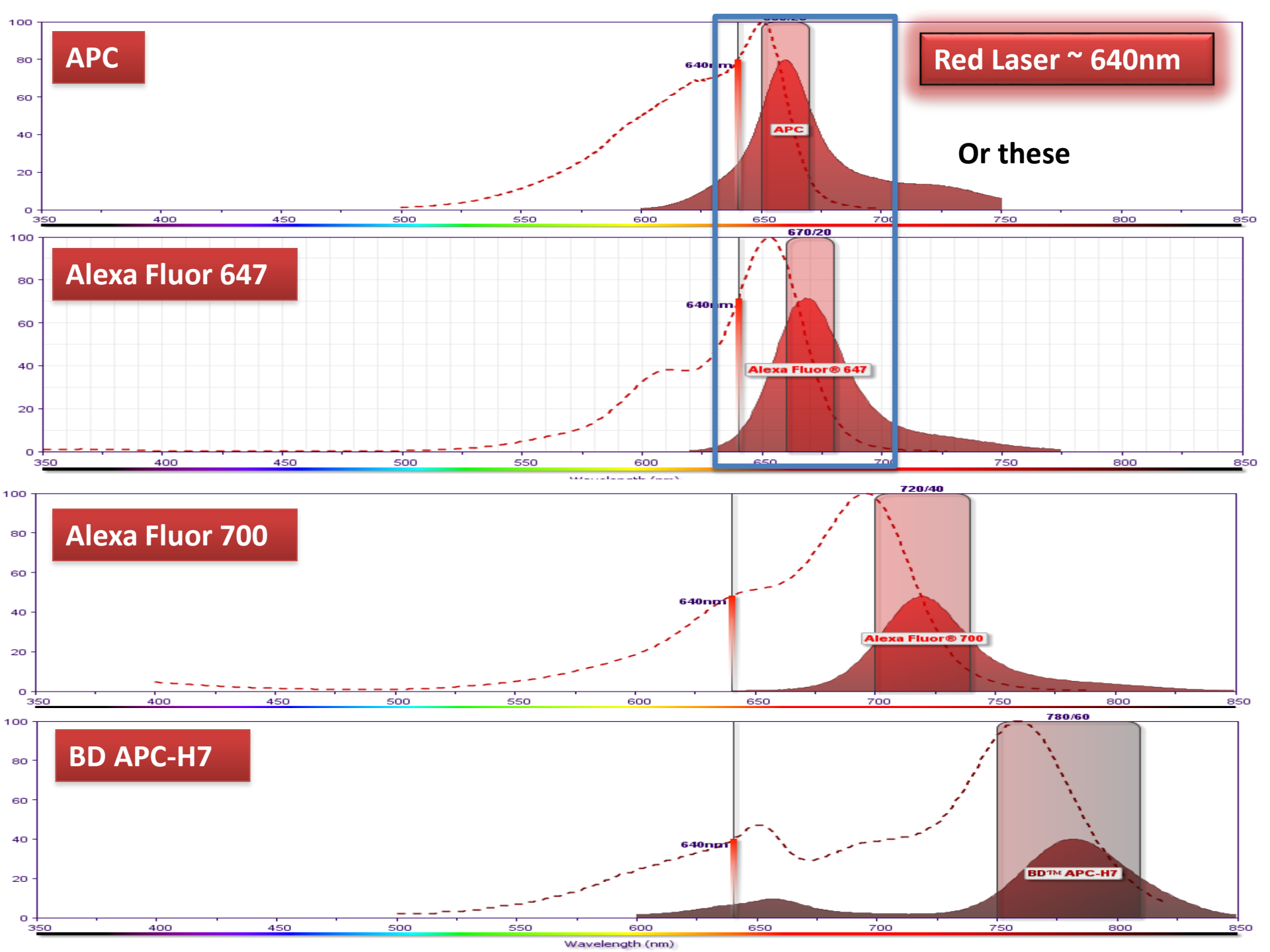
Emission spectra

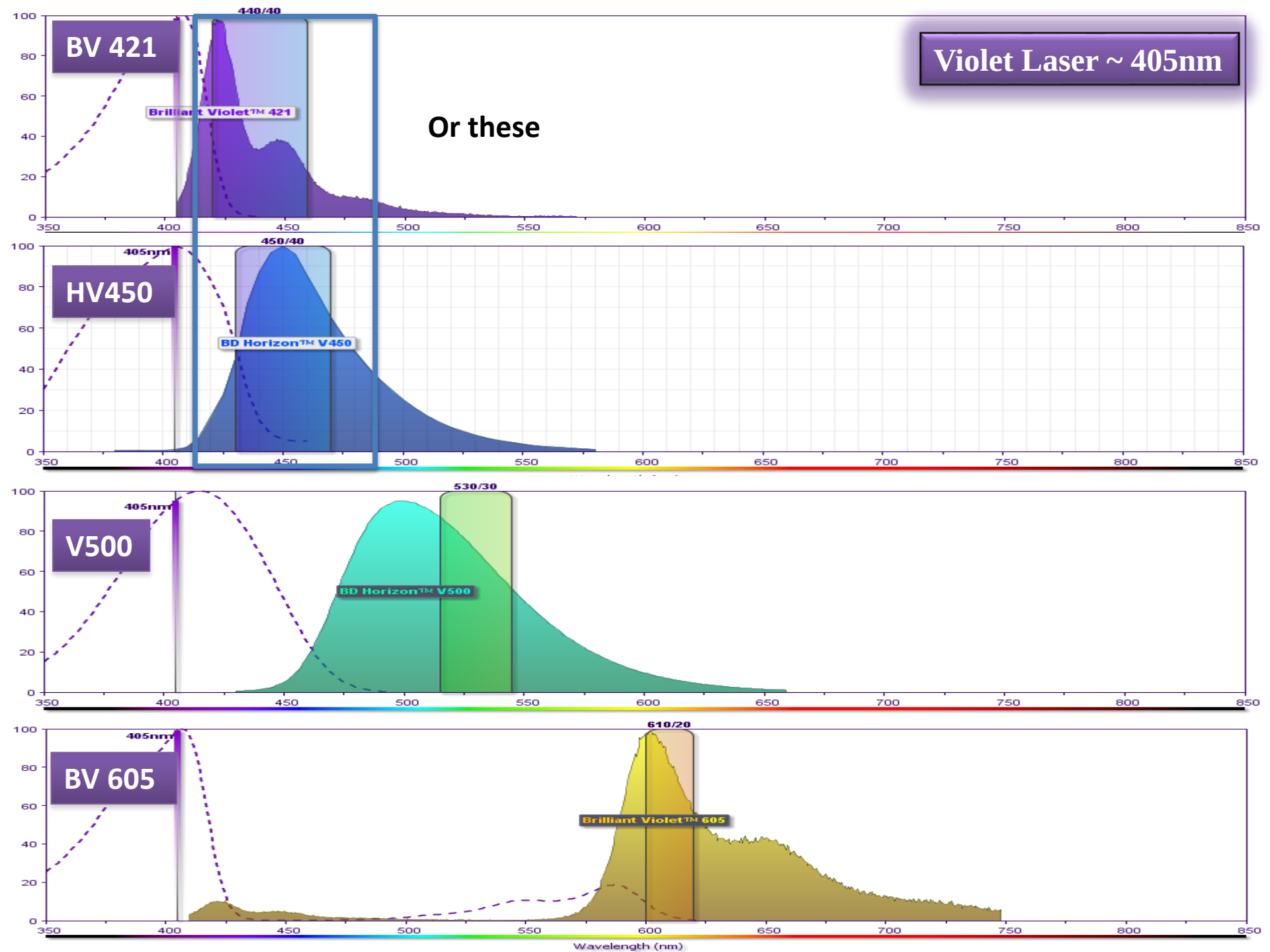
Look at the emission spectra of your fluorochromes

Make sure the filters on your cytometer correspond to the maximum emission

Fluorochromes excited off the same laser should not have overlapping emission spectra


FITC
Blue Laser ~ 488nm
PE
PE-Texas Red
You can't use these 2 colors together
PE-CF594
PerCP
Or these
PerCP-Cy5.5
PE-Cy7
488nm
Wavelength (nm)


APC
Red Laser ~ 640nm
Or these
APC
Alexa Fluor 647
670/20
Alexa Fluor® 647
Alexa Fluor 700
720/40
Alexa Fluor® 700
BD APC-H7
780/60
BD™ APC-H7
Wavelength (nm)


BV 421
440/40
Brilliant Violet™ 421
Violet Laser ~ 405nm
Or these
HV450
405nm
450/40
BD Horizon™ V450
V500
405nm
530/30
BD Horizon™ V500
BV 605
405nm
610/20
Brilliant Violet™ 605
Wavelength (nm)

Lots of “spectral viewers” online

- http://www.bdbiosciences.com/us/s/spectrumviewer
- http://www.biolegend.com/spectraanalyzer
- https://www.thermofisher.com/cn/zh/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

How bright is your fluorochrome?

Brightness is intrinsic to the fluorochrome itself

depends on: Extinction coefficient (light absorbance)

Quantum yield (photons out/photons in)

Fluorochrome	Extinction Coefficient	Quqntum Yield	Brightness x 10^5	Brightness Relative to PE	Size Daltons
PE	1,960,000	.84	16	100%	240,000
PE-Cy5	1,960,000	NA	NA	NA	241.500
APC	700,000	.68	4.7	20%	105,000
FITC	72,000	.9	.6	3%	5,000
BV421	2,500,000	.69	17	106%	69,000

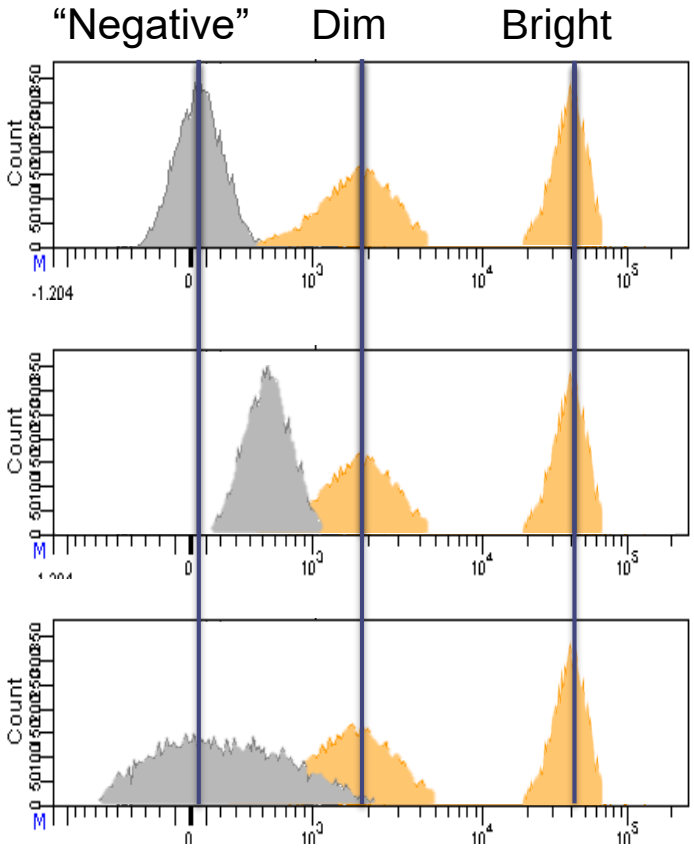
Resolution

We need to distinguish unstained from dimly stained in a mixture.

Negative population has low background
populations well resolved

Negative population has high background
Dim population not resolved

Negative population has low background but high spread (SD)
Dim population not resolved



The ability to resolve populations is a function of both background *and* spread of the negative population.

Q and B: criteria for cytometer performance

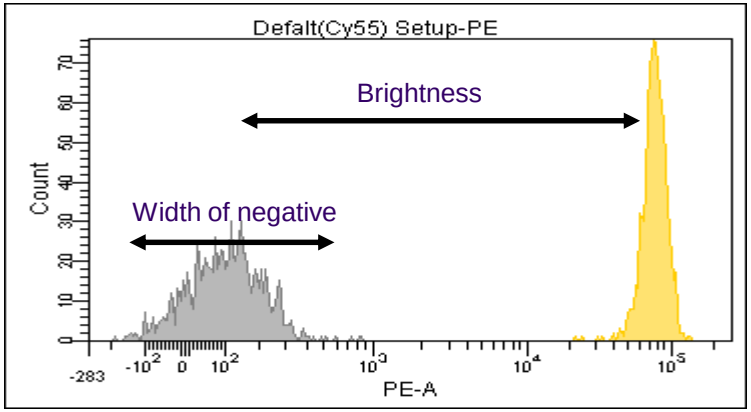
- Used to quantify detection sensitivity and background
- Q: number of photoelectrons produced per molecule of fluorochrome: sensitivity
- B: electronic and optical background when no fluorochrome is present
- Best detection when high Q and low B

Stain Index: How well separated are your populations

Stain index is a measure of reagent performance on a specific cytometer

Affected by fluorochrome brightness **AND instrument characteristics**

$$\text{Stain Index} = \frac{\text{median}_{\text{positive}} - \text{median}_{\text{negative}}}{2 \times \text{rSD}_{\text{negative}}}$$



Experiment Name: CD4 Stain Intex (081304)
Tube Name: PE

Population	#Events	%Parent	PE-A Median	PE-A rSD
PE-	827	48.9	102	97
PE+	780	46.1	75,652	12,616

$$\text{Stain Index} = \frac{75852 - 102}{2 \times 97} = 390.5$$

Stain Index Comparisons

Stain Index on Cytometer A

Reagent	CD4 Clone	Filter Set	Stain Index	Relative Brightness (compared to PE)
PE	RPA-T4	585/40	**305**	100.00%
PE-Cy5	RPA-T4	695/40	**198**	81.63%
PerCP	RPA-T4	695/40	**30**	16.66%
APC	RPA-T4	660/20	**263**	8.21%
FITC	RPA-T4	530/30	**43**	1.74%
Pacific Blue™	RPA-T4	440/40	**63**	1.5%

- **APC has a higher stain index than PE-Cy5 even though 1/10th as bright**
- **Due to less noise in red laser, thus background width is lower and stain index higher.**

Stain Index: instrument variation

Fluorochrome	Brightness x 10^5	Brightness relative to PE	Stain index	
			Cytometer 1	Cytometer 2
PE	16	100%	348	262
PeCy5	NA	NA	180	131
APC	4.7	29%	238	281
FITC	0.4	2%	132	61
BV421	16	100%	264	145

Due to cytometer differences in:

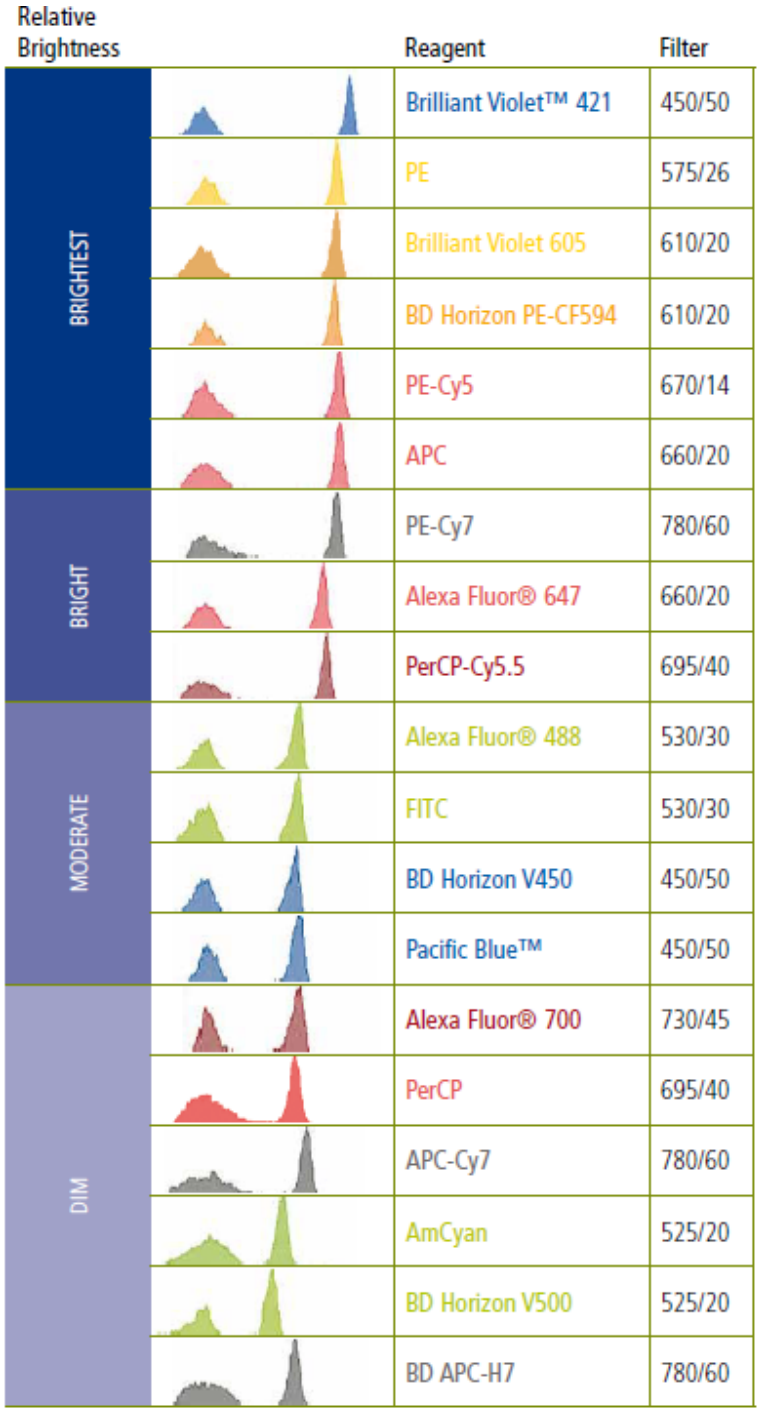
Laser configuration and power
Laser and optical alignment
Dichroic mirrors and filters
PMT sensitivity (Q)
PMT background (B)

Relative Brightness

Despite cytometer differences fluorochromes can still be grouped into

Brightest
Bright
Medium
Dim

As in this chart which is based on an average of different cytometers



Titration and PMT voltage

These can affect your stain index

Titrate your antibodies!

It is essential for all your antibodies to be correctly titrated!!

Purpose: to find the optimal concentration for each antibody to

- Maximize your stain index
- Maximize your separation of dim populations
- Minimize cost (antibodies are expensive!)

You should not depend on the manufacturer's recommendation.

They test certain cells and conditions, maybe not yours.

How to titrate

1. Serial dilution of your antibody:

    Start at 2-4x the manufacturer's recommendation

    Do 8-12 doubling dilutions

2. Add your cells of interest

    in the same conditions as your experiment

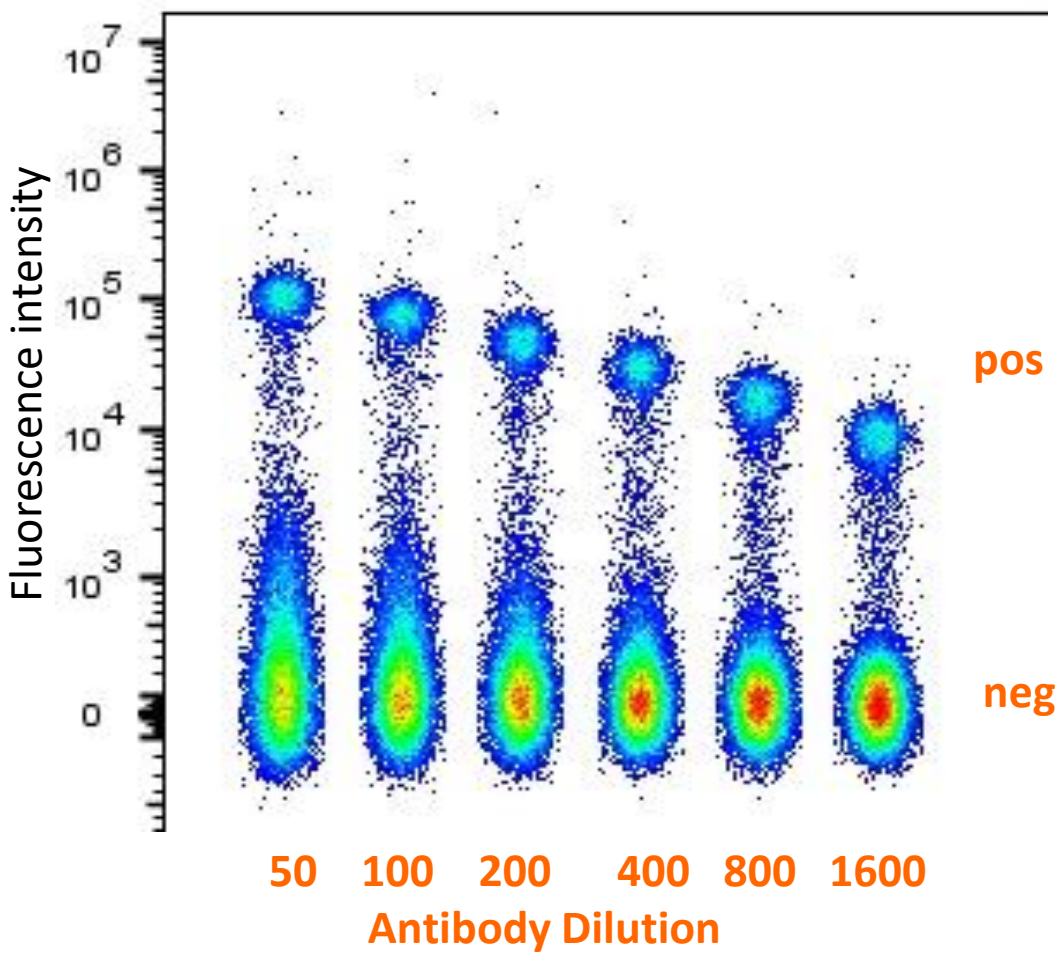
        i.e. same fixation and permeabilisation

    must have cells that are antigen positive and negative

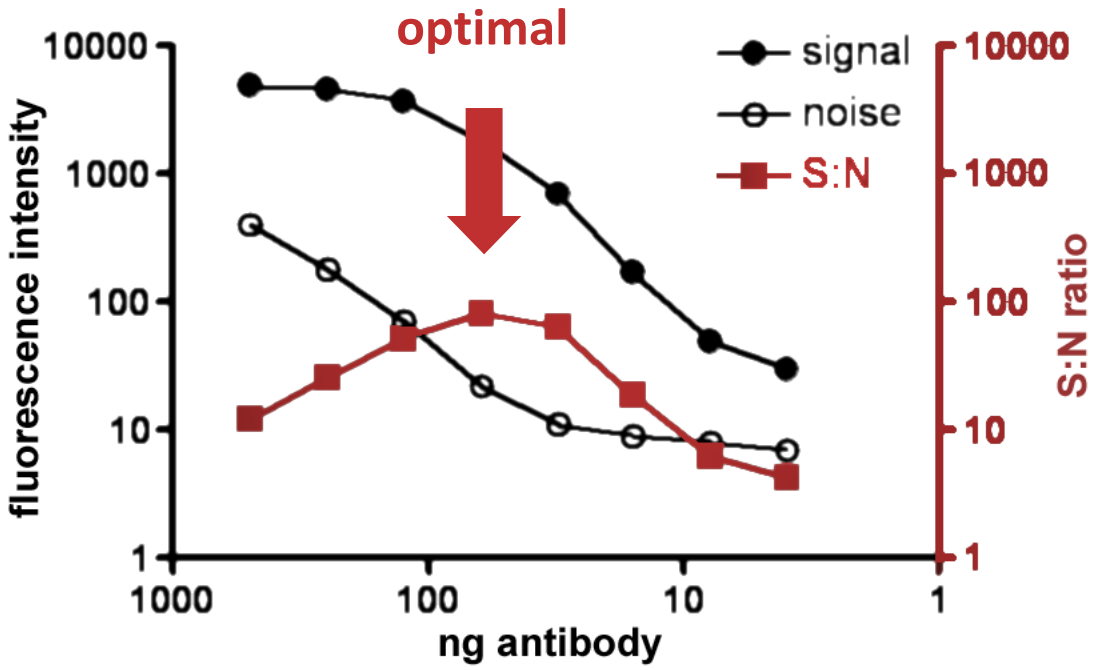
3. Wash and run on cytometer

4. Calculate stain index or signal/noise for each dilution

Titration

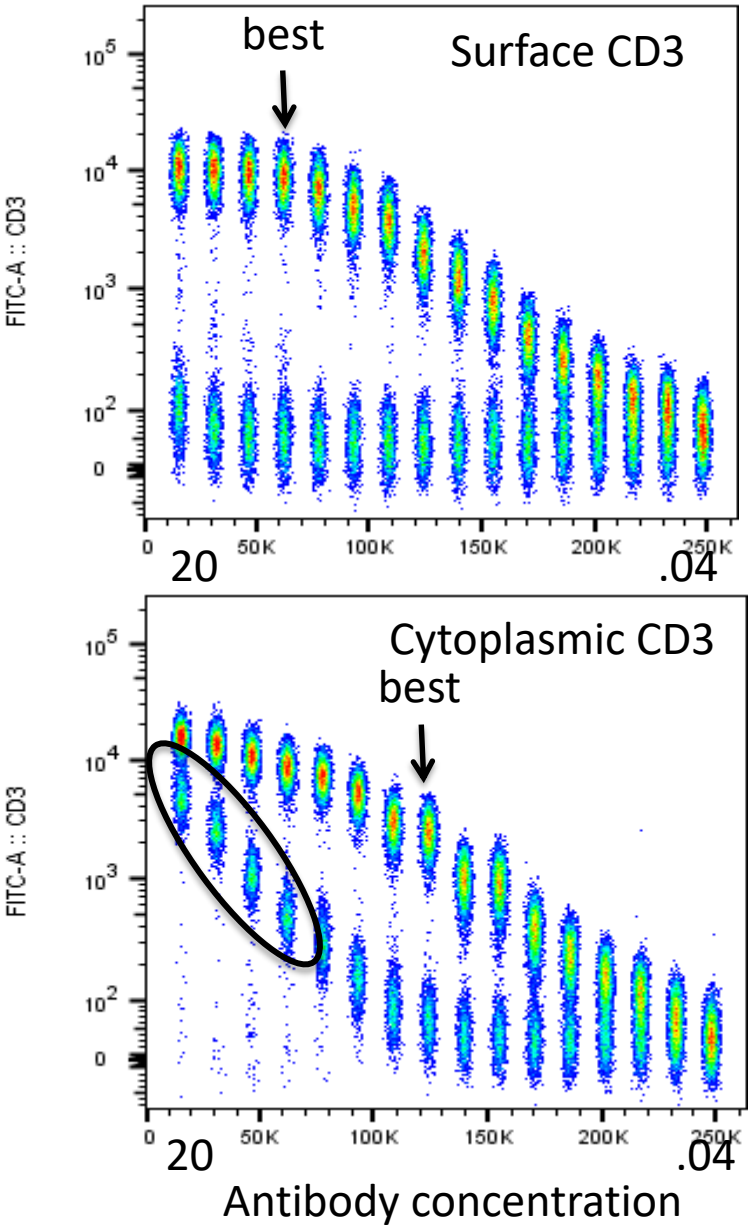

Fluorescence intensity
10^7
10^6
10^5
10^4
10^3
0
pos
neg
50
100
200
400
800
1600
Antibody Dilution

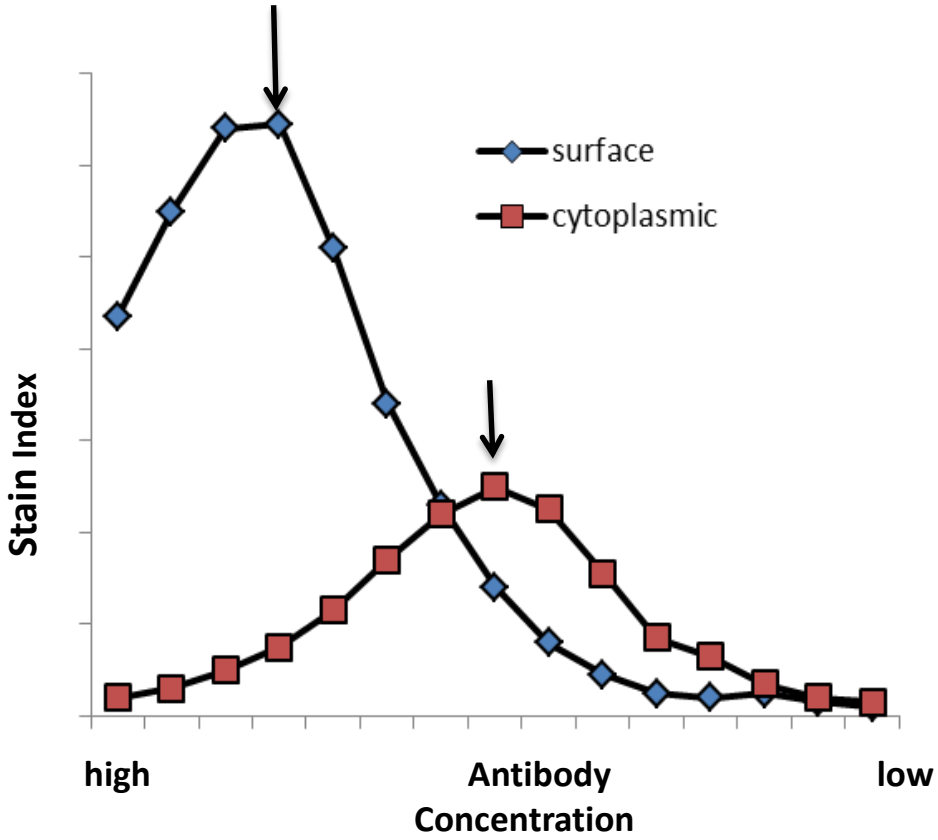
Titration



Goal is to maximize the signal (positive population) to noise (negative population) ratio.

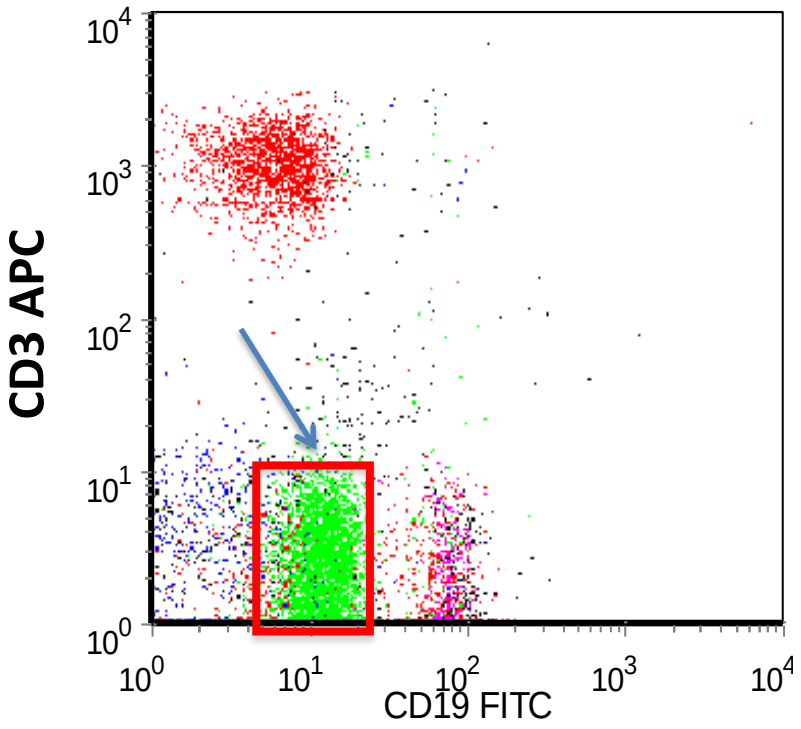
Titration CD3 surface vs CD3 cytoplasmic





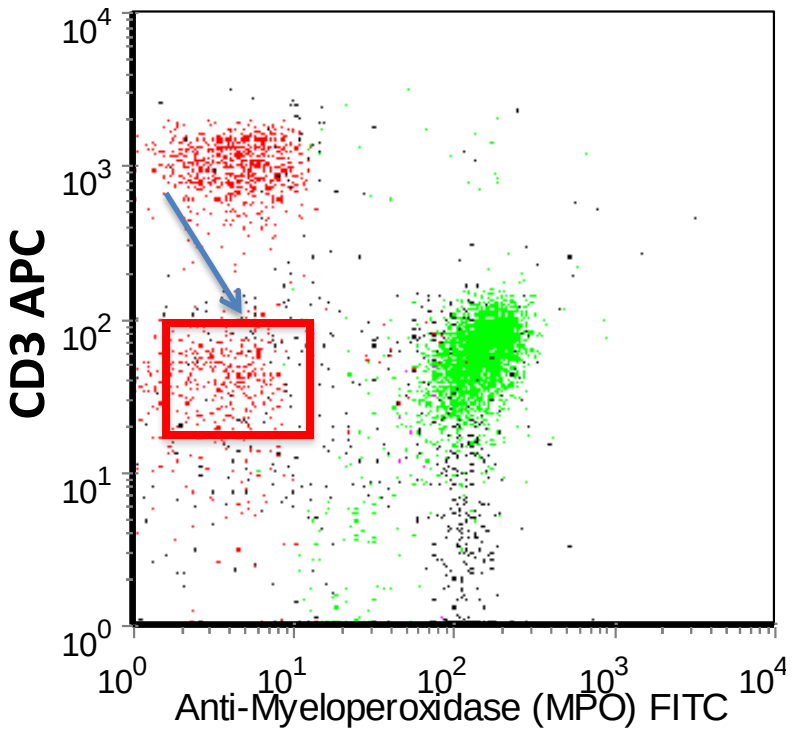
Increased Background in Fixed Cells

Surface CD3 APC (3ul):
low background



Cytoplasmic CD3 APC (3ul):
high background

due to sample processing



Titer is affected by

Staining volume (example: 100 mL)

- Number of cells (not critical up to $5x10^6$ at 100 mL volume)
- Staining time and temperature (example: 30 min RT)
- Type of sample (whole blood, PBMC, cell line)
- Scaling up to lots of cells: rough rule of thumb
    - Double the antibody concentration for every increase of every 25-50 $x10^6$ cells

PMT voltage (gain) settings

Gain or PMT voltage setting affects sensitivity

Best gain should be determined for each detector on each cytometer

Different ways to do this:

1. Voltration: Set PMT voltages to maximize Stain Index with comp beads or stained cells

2. using electronic noise and linearity criteria (CST values)

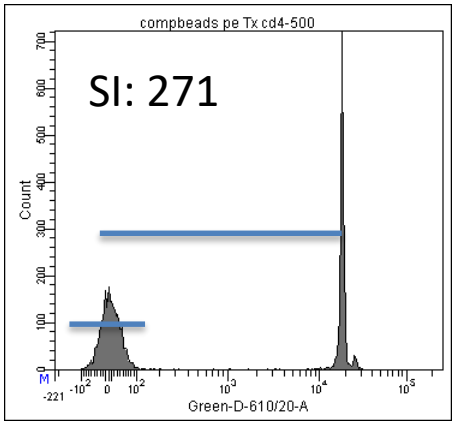
    set PMT voltage so that

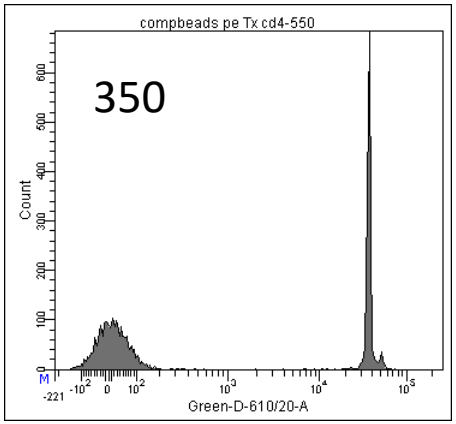
        1. unstained cells have 2.5 X SD of electronic noise

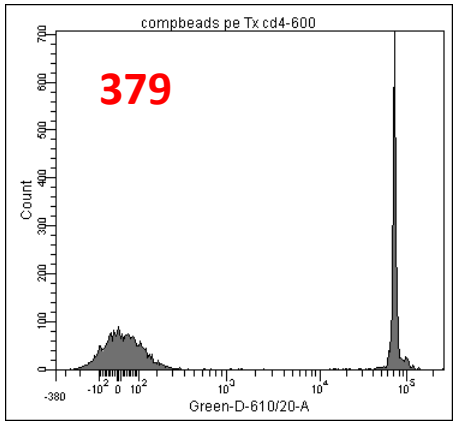
        2. positive cells not above max linearity

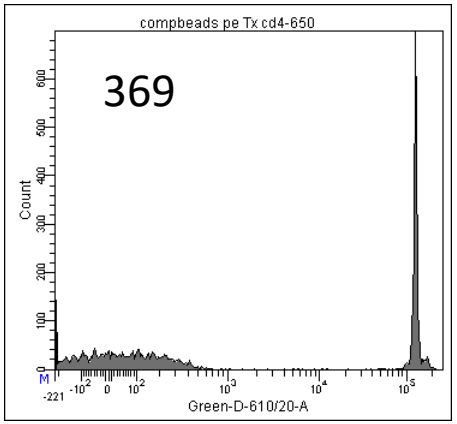
Voltration: choose the best stain index (SI)

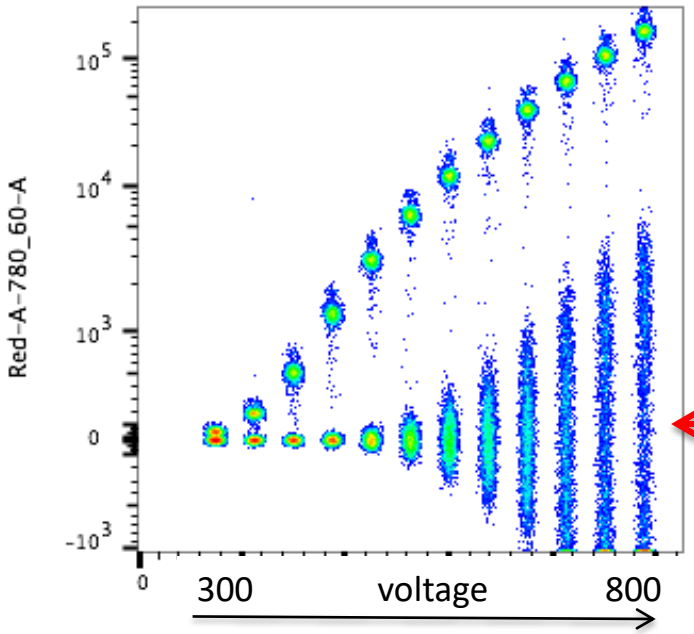
500v | 550v | **600v** | 650v







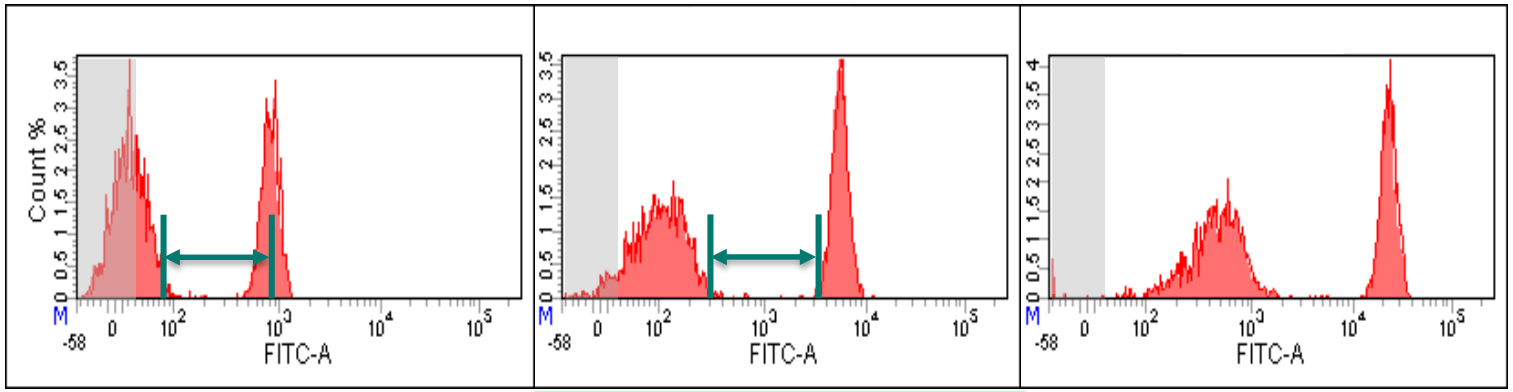




Increase of background spread at higher voltages

Voltration Example: FITC detector

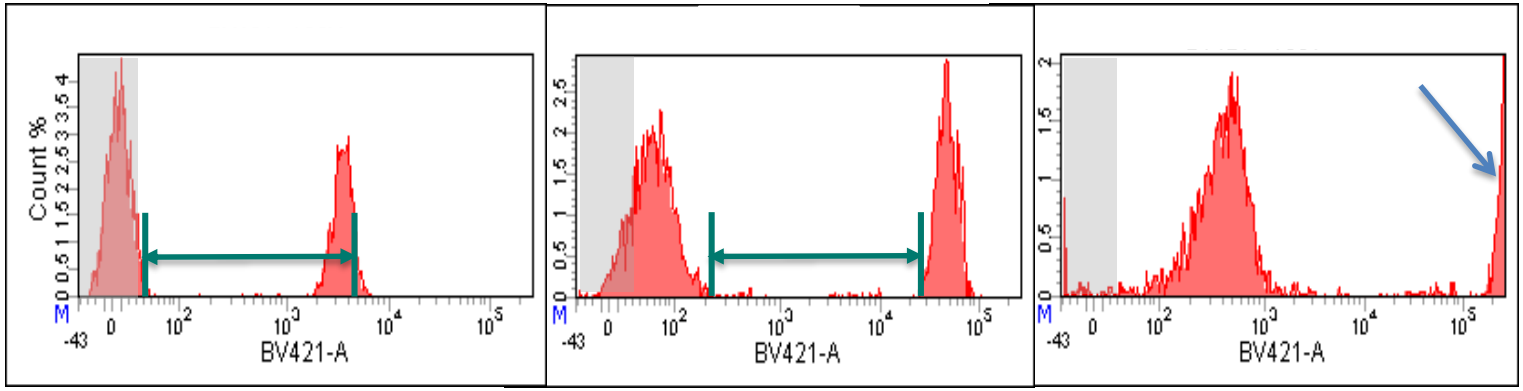
FITC Detector (SD_{EN} = 20)



PMT Voltage	370	470	570
Stain Index	15	39	42
MFI Pos Cells	750	5,072	21,183
MFI Neg Cells	15	94	415
rSD Neg Cells	24	64	245

Voltration Example: BV421 detector

BV421 Detector (SD_{EN} = 22)



PMT Voltage	350	450	550
Stain Index	125	657	>800
MFI Pos Cells	3,247	43,410	>262,143
MFI Neg Cells	4	53	368
rSD Neg Cells	13	33	207

Events should NEVER be off the top of the scale

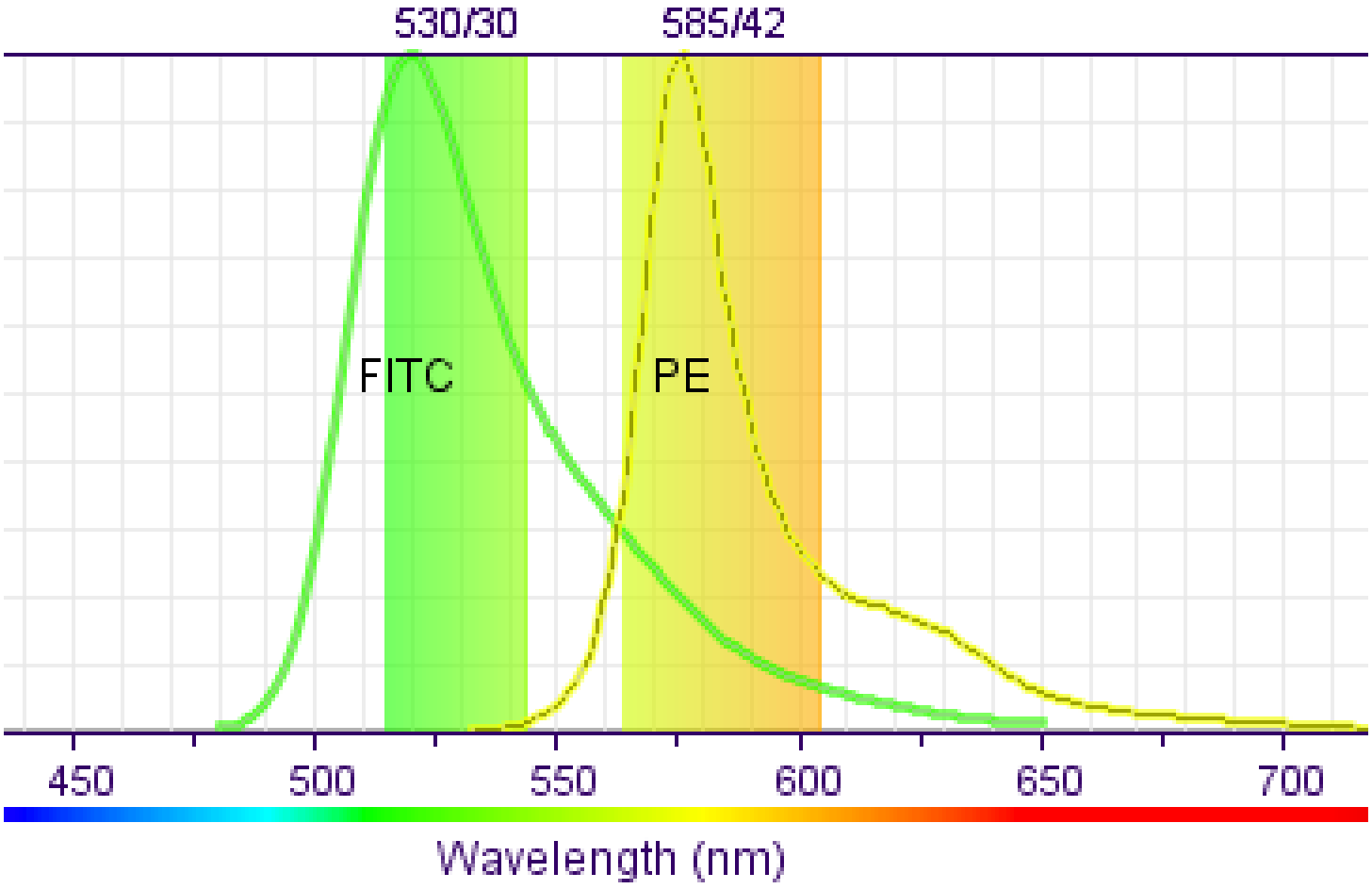
Best Gain Settings

- An issue for everyone: An entire well attended workshop was devoted to setting gains at recent CYTO meeting
- Compared several ways of best gain determination
- Most systems did not differ greatly in best gain result
- Autofluorescence affected best gain
  - Gains on lymphs or beads not the same as highly autofluorescent cells
  - may need to determine best gain separately on very autofluorescent cells

Spectral Overlap, Spillover and Compensation

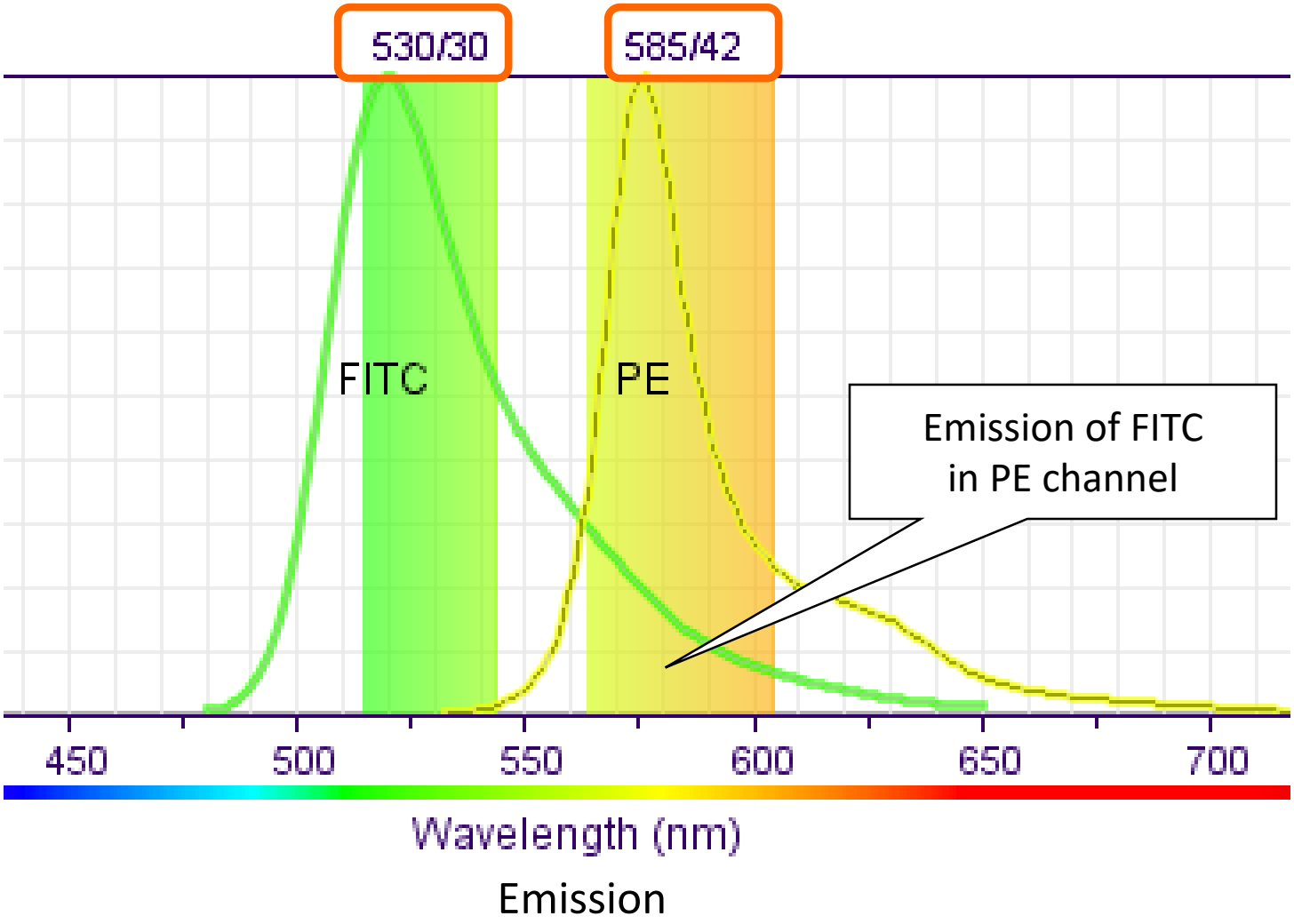
Fluorochrome Emission Range

Fluorochromes absorb and emit over a range of wavelengths specific to each fluorochrome



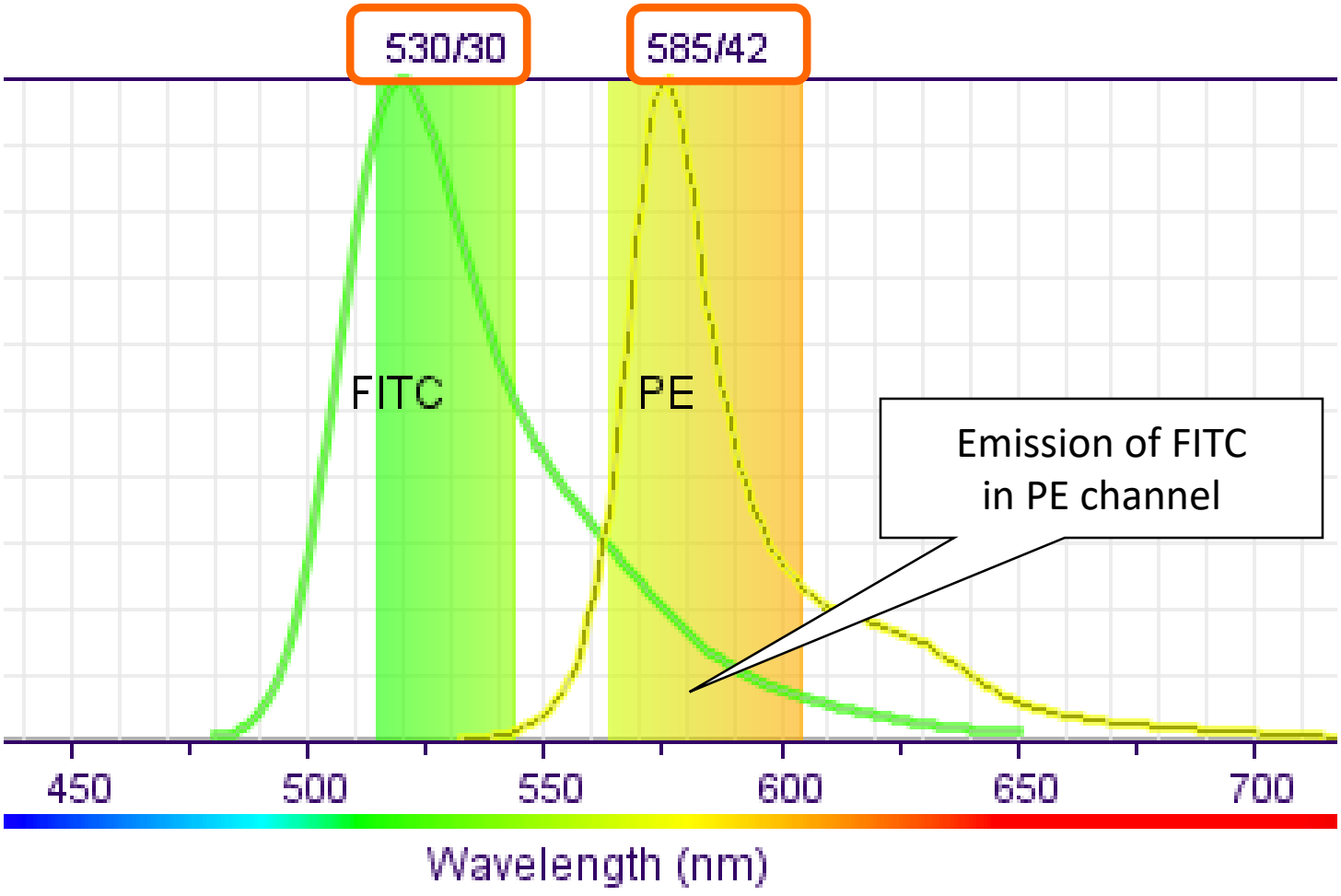
Fluorescence Spillover

These are the optical filters in which each fluorochrome is detected:
the longer wavelength emission from FITC is seen in the PE detector



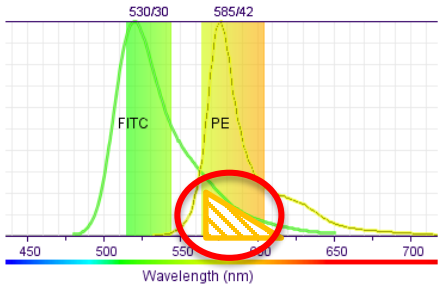
Fluorescence Spillover

Thus part of the emission measured in the PE detector is due to FITC emission
But we need to measure each fluorochrome separately



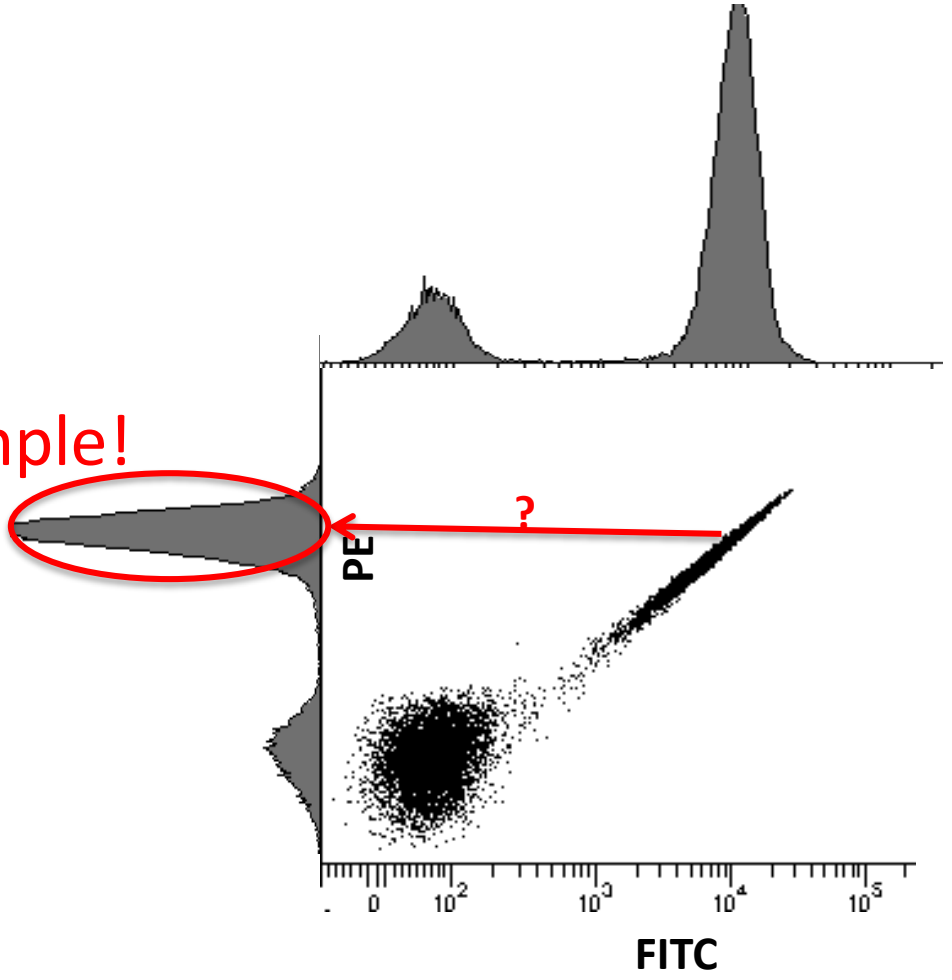
The Spillover Effect: FITC into PE

This is a single color control stained **only** with FITC.



There is no PE in this sample!

The PE signal we see is FITC fluorescence coming through into the PE detector.



So what do we do?

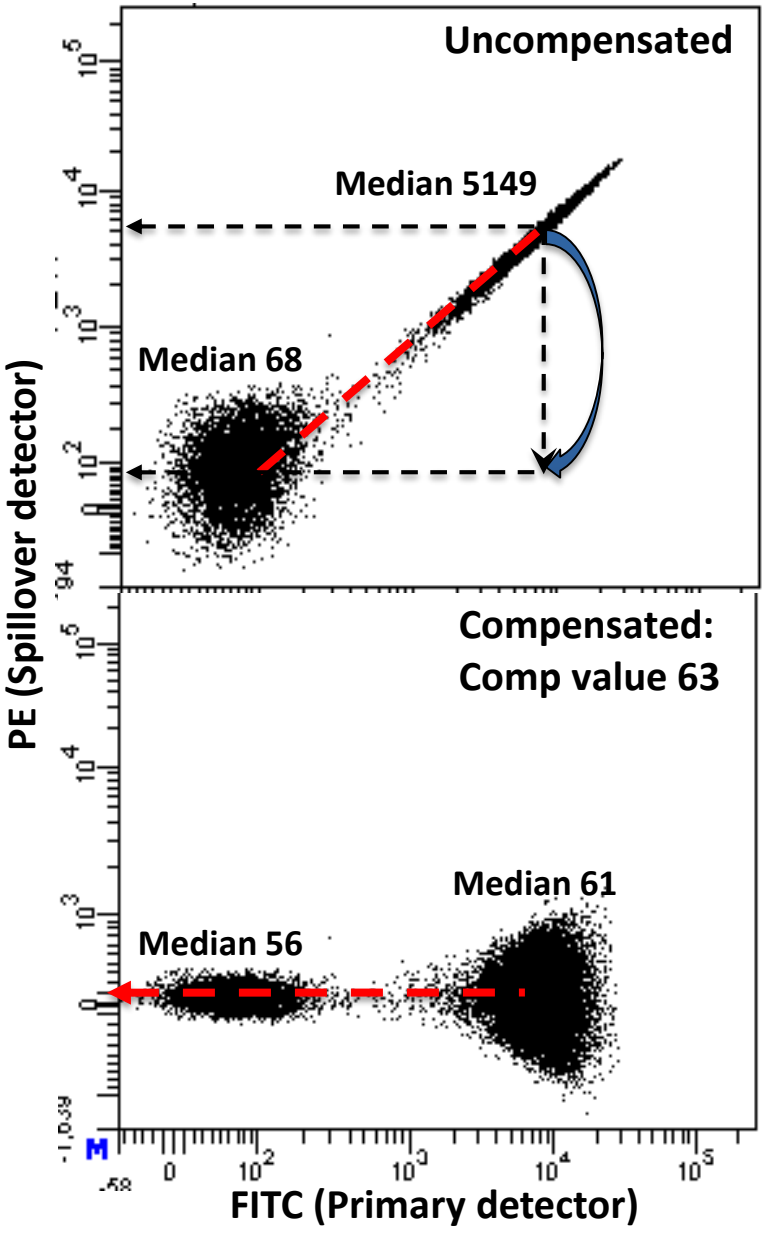
We compensate!

Compensation is a mathematical correction that is applied to correct for spillover from other fluorochromes so that:

The true PE signal =

observed PE signal – contribution (x%) of FITC signal

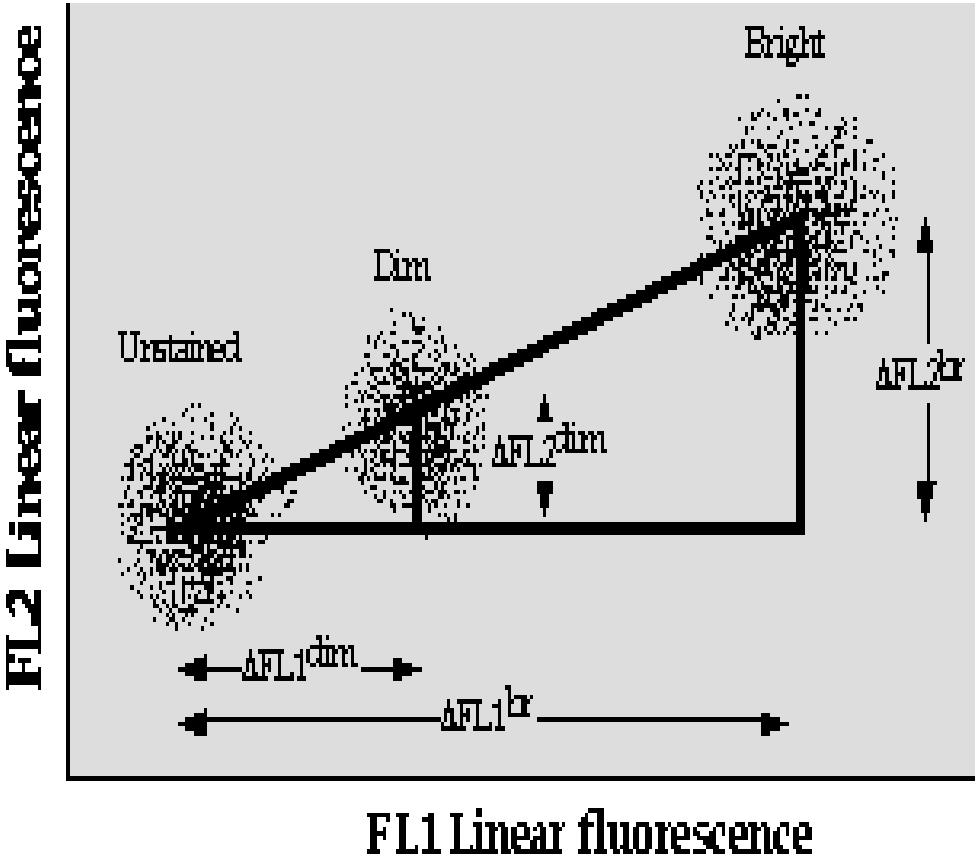
Spillover is corrected by Compensation



The median intensities
of the positive and negative populations
in the each channel are measured.

Compensation is applied so that
the PE median of the FITC positive
population
is the same as
the PE median of the FITC negative
population.

How is Compensation Calculated?



based on the slope of the line between the positive and negative medians

Actual calculation complicated:

- uses inverted spillover matrix
- matrix algebra

Compensation is not a simple percentage:

- values of over 100%
- are possible
- not necessarily a cause for concern

Compensation

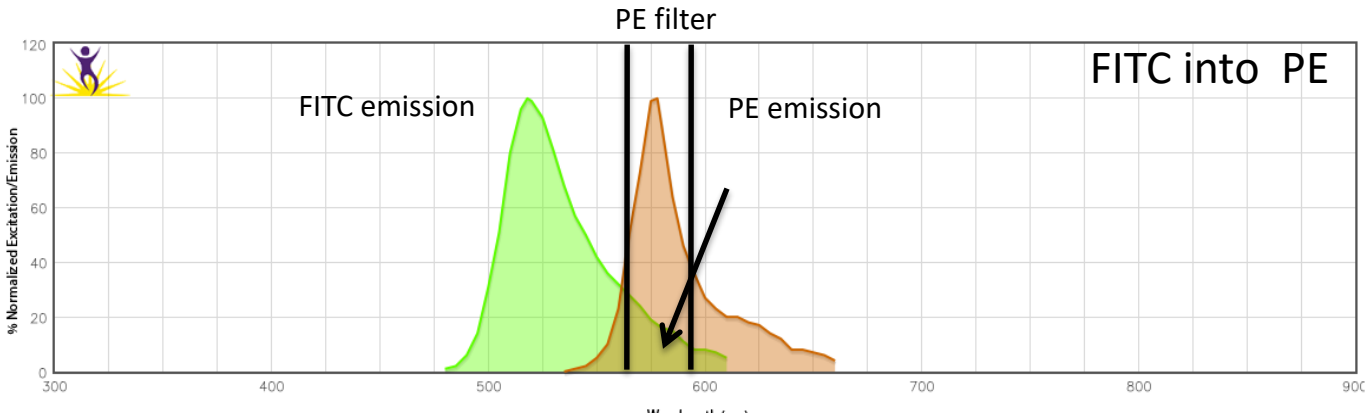
- The contribution of light signal from all other fluorochromes must be measured using single color controls for each color in the mix.

- **Compensation is more accurate using automated software than manual,** because the automated corrects for spillover of all fluorochromes into all channels simultaneously**.**

Three Sources of Spillover

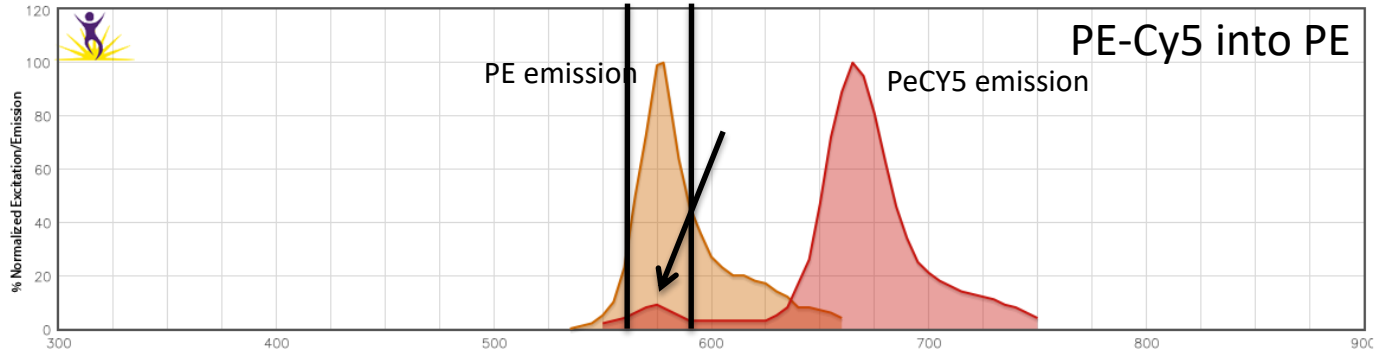
1

Adjacent spectra



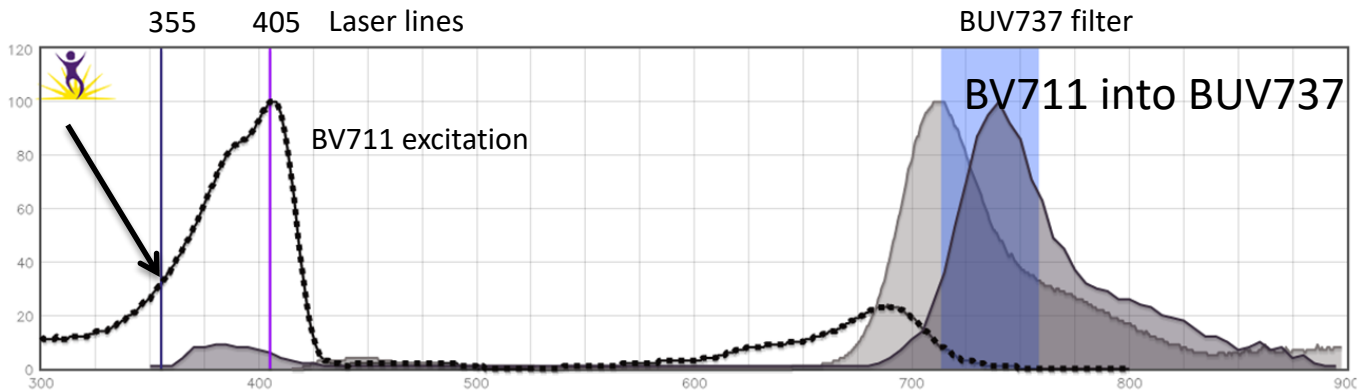
2

Tandems and their bases

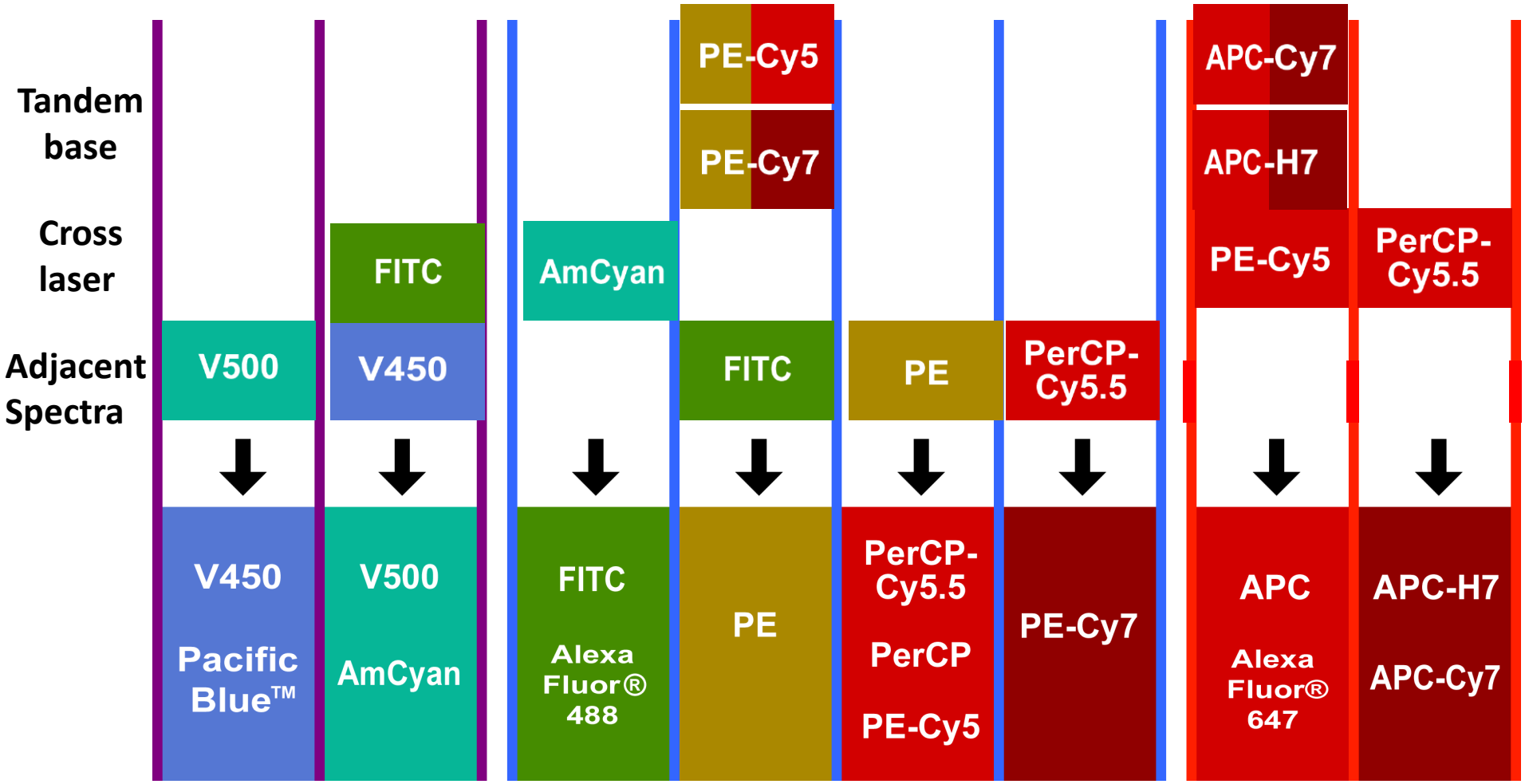


3

Cross laser excitation

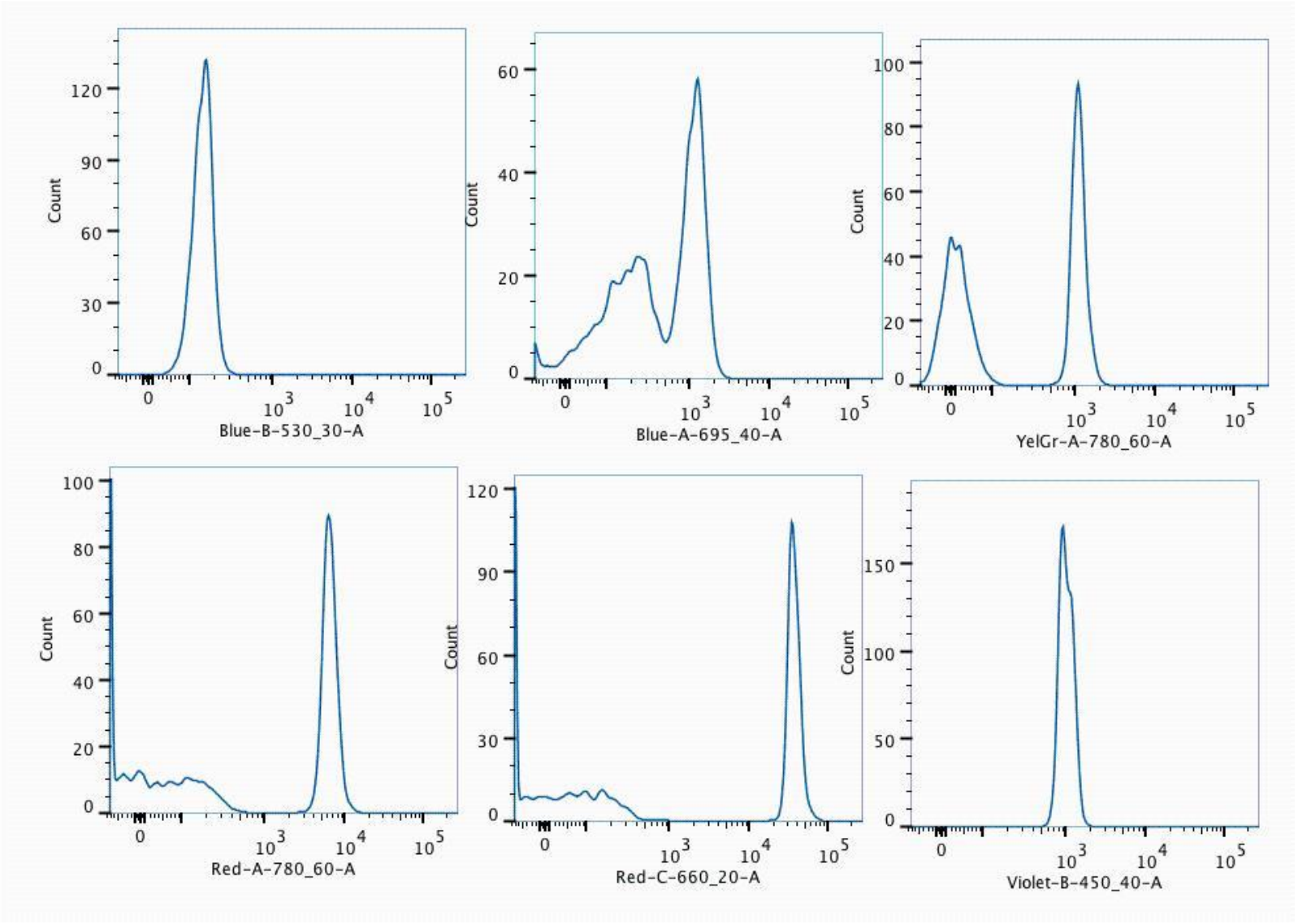


Who spills into what?



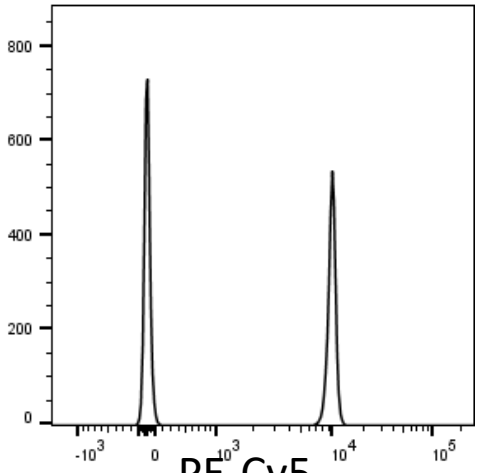
APC single color

Look where it spills!! There is only APC in this tube

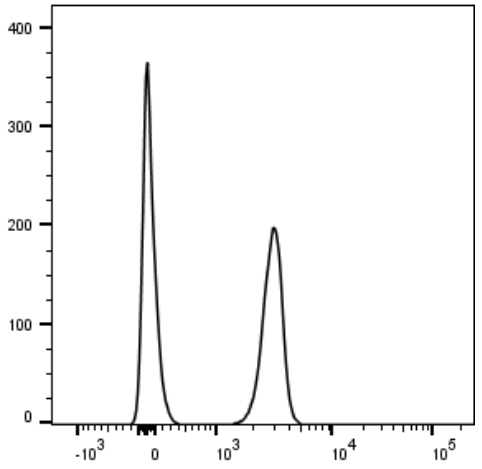


PE-Cy5 Single Color

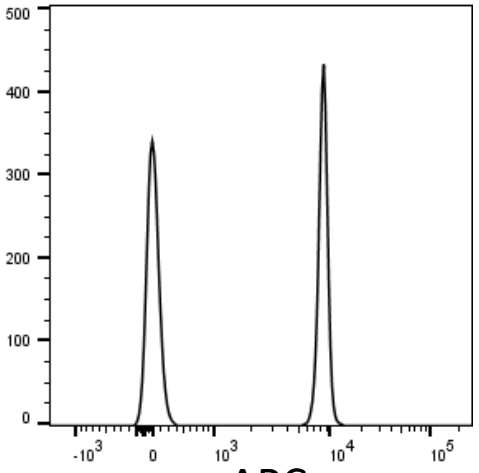
Look where it spills!! There is only Pe-Cy5 in this tube
Which categories of spillover apply here?



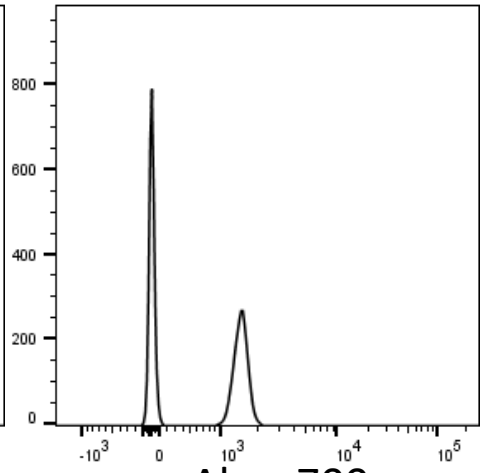
PE-Cy5



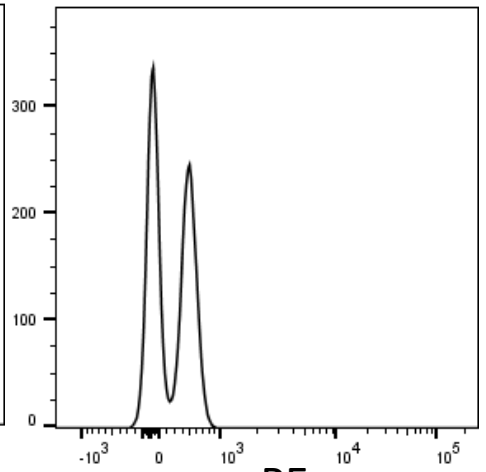
PE-Cy7



APC



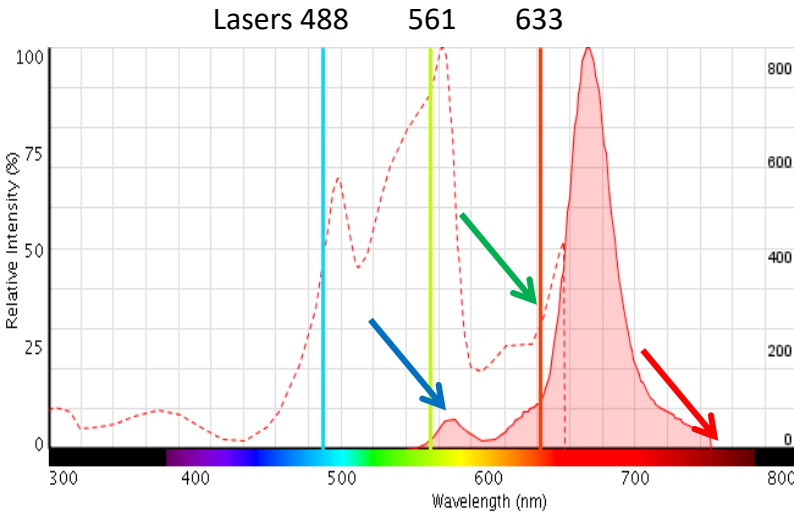
Alexa700

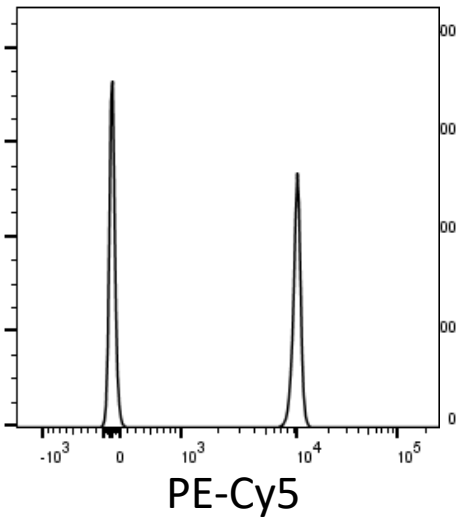


PE

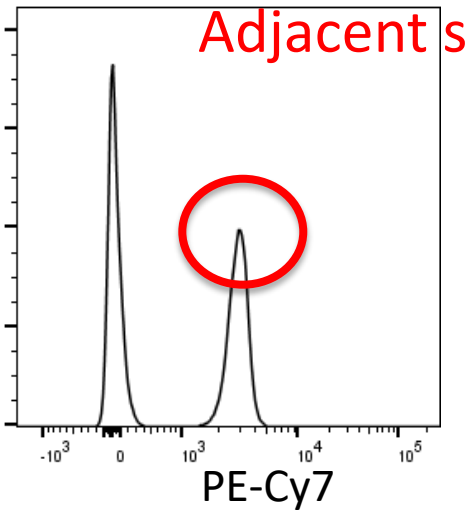
Spillover: PE-Cy5 Single Color

You can a good idea of where there will be a spillover problem by looking at the excitation and emission spectra

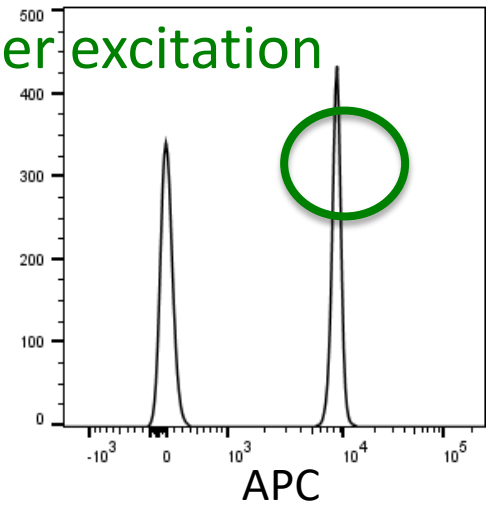


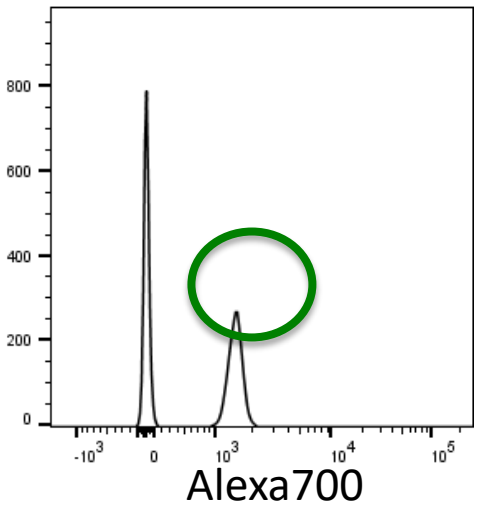


Adjacent spectra

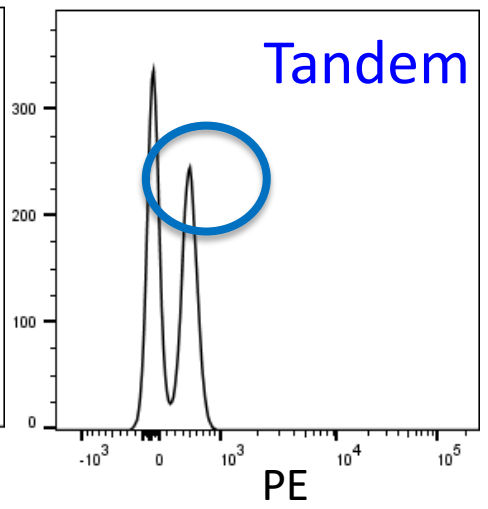


Cross laser excitation





Tandem base



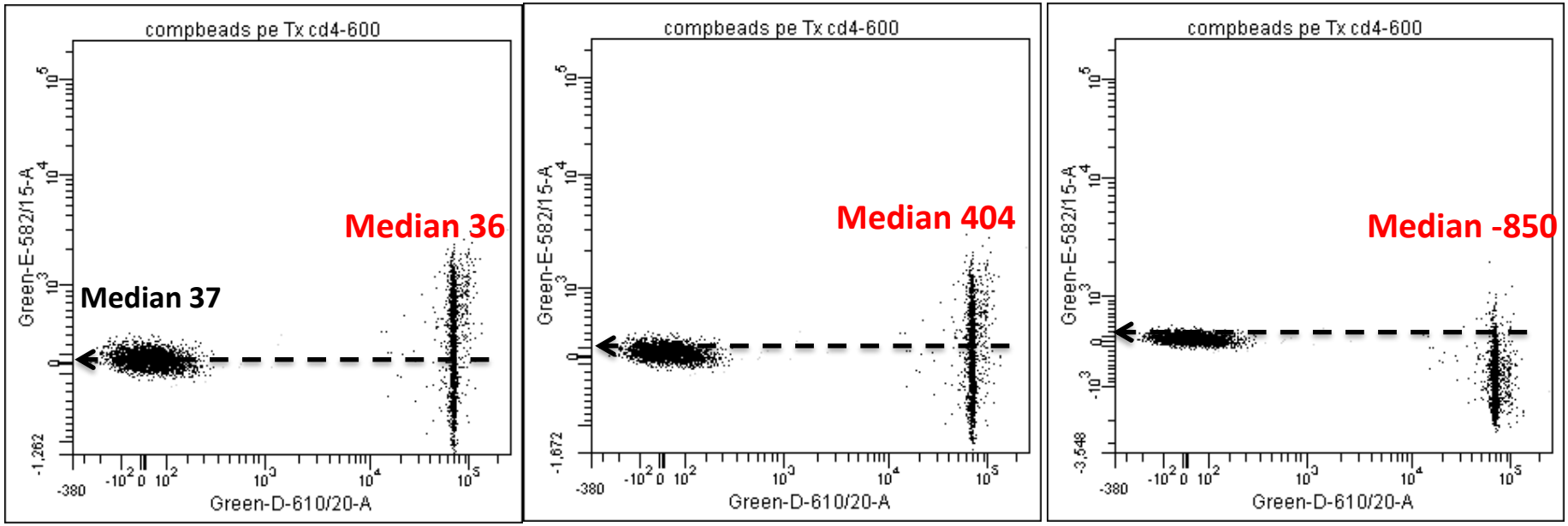
Over and under compensation

PE-Texas Red single color control

Correctly compensated
24.02%

Undercompensated
23.7%

Overcompensated
25.3%

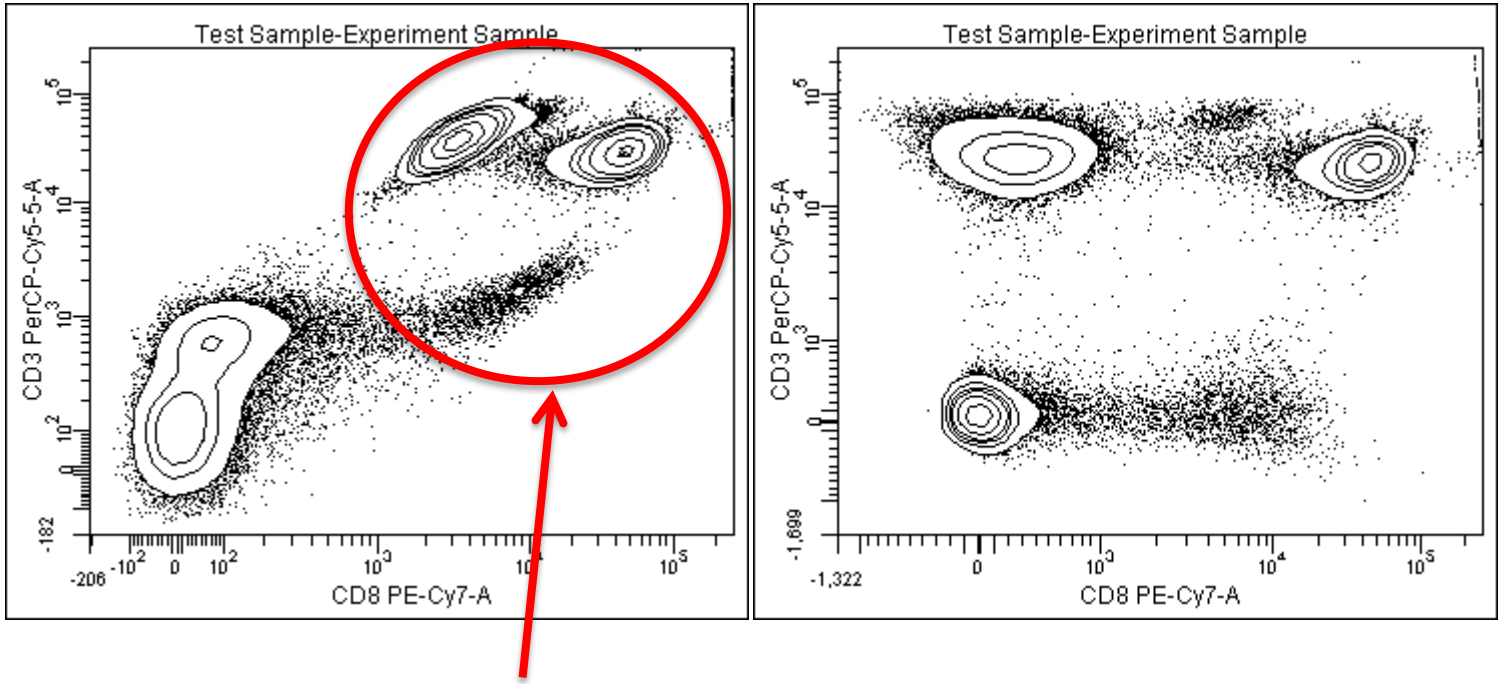


Never adjust a compensation 'by eye': Always use median statistics

Correct Compensation: the end result

uncompensated

compensated

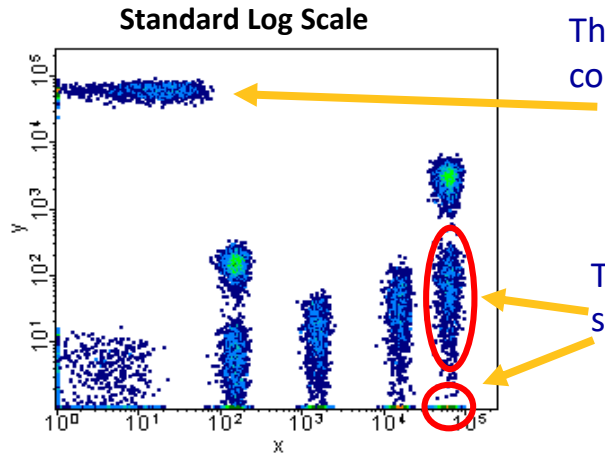


Looks like 3 double positive populations!

But it's not the case when compensated

The Bi-exponential Scale:

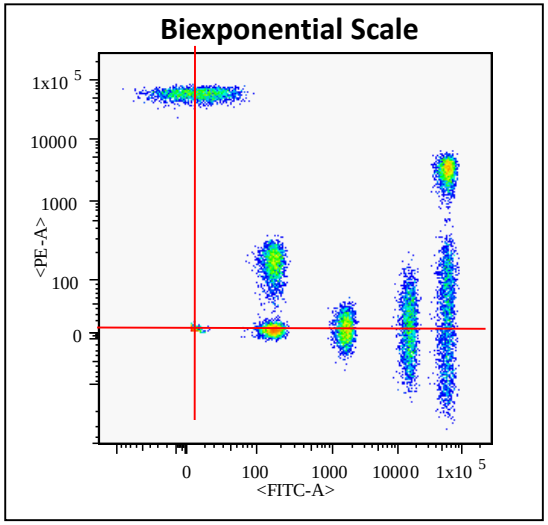
The best way to look at compensated (or uncompensated) data



This population "looks"under compensated.

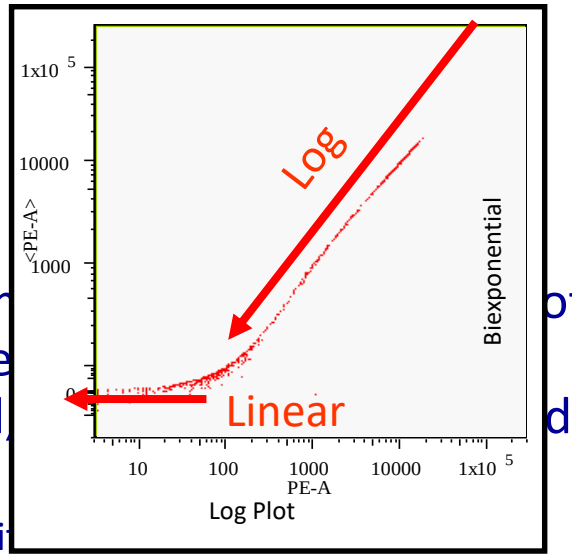
These look like two separate populations.

Visualization of compensated data is greatly improved using the biexponential scale.



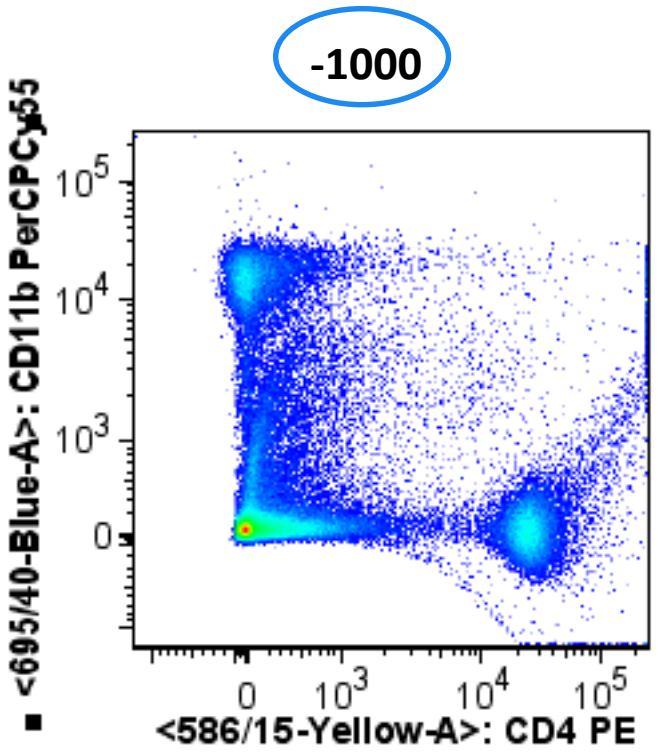
This example of the sar of the Biexponential Scale scale on the upper end, d symmetrical about the

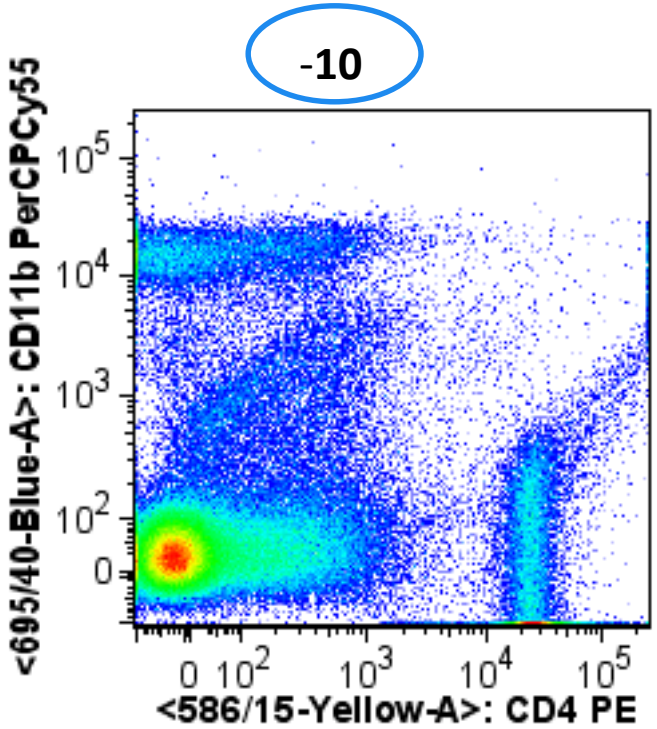
- Compensated single posi
- All populations are visible.



SCALING OPTIONS

You can choose the scaling for bi-exponential to show more or less of what is below 0

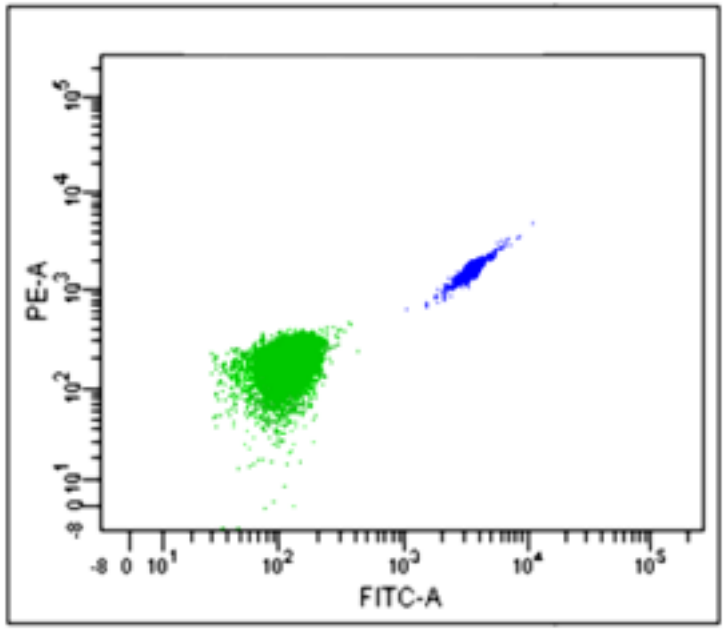


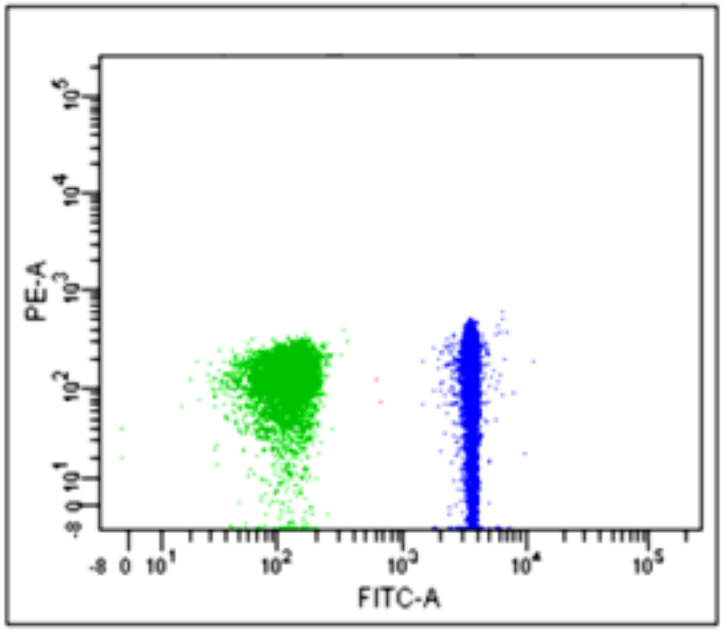


Always show axis ticks on plot, **include '0'** if you are using bi-exponential data display

How to do Compensation

You must have a good single color control for each color used!





Single color controls must have

a negative population and a population positive in one channel only

3 Rules for Compensation

1. Compensation control antibody must be the same as the one in the experiment mix

2. The positive control must be at least as bright as the experimental sample

3. The negative and positive populations for each single color control must have the same autofluorescence.

A nice discussion:
http://flowjo.typepad.com/the_daily_dongle/tips_and_tricks/

Rule #1: single color antibody must be the same as mix

Single color control antibody must have the same **spectral characteristics** as the antibody in the mix.

Similar fluorochromes have different spillover values

FITC is not Alexa 488 is not GFP!

Same antibody rule is critical for Tandem reagents:

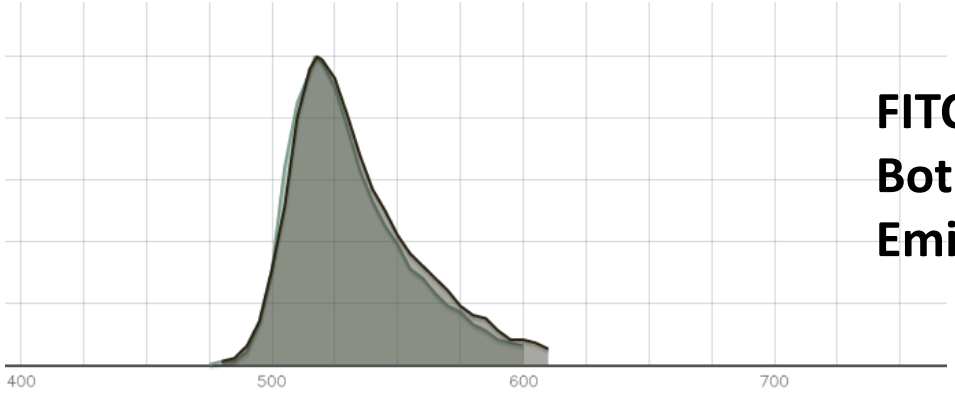
Different lots of the same antibody have different spillover

Tandems can degrade over time or in light

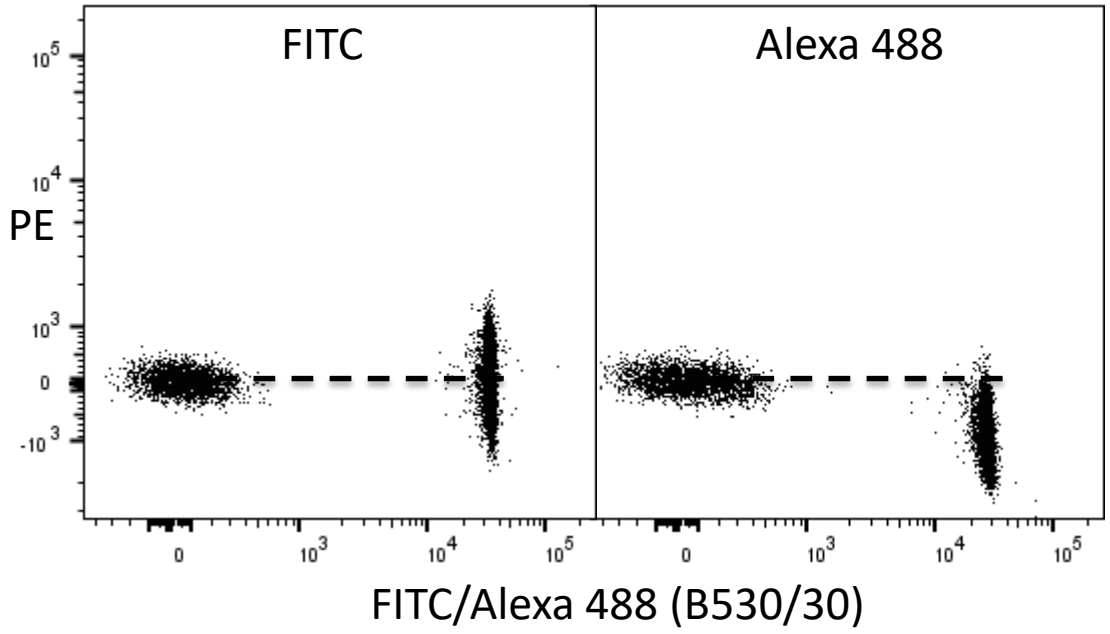
Degraded tandems will have more spillover from the base

Rule #1: FITC vs Alexa 488

When Green is not just Green



FITC and Alexa 488
Both measured in the green channel
Emission spectra look almost the same



Not in the eyes of the cytometer!

Compensation calculated using a FITC single color results in overcompensation when applied to Alexa 488

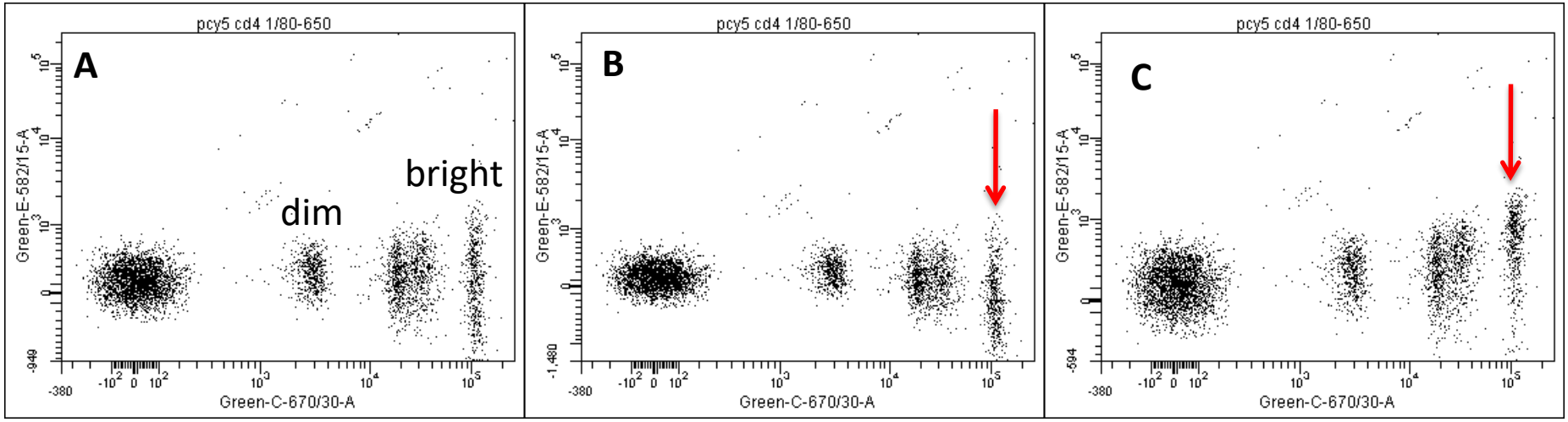
Rule #2: the single color must be as bright as the mix

Small errors in compensation using a dim single color control can result in large compensation errors with bright populations

Correctly compensated

Overcompensated

Undercompensated

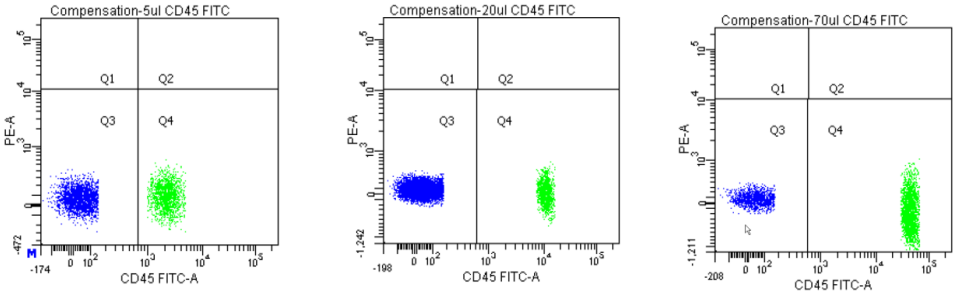


The most positive populations, whether in the single color or mix, must be within the range of linearity of the detector

Bright signals provide more accurate compensation values

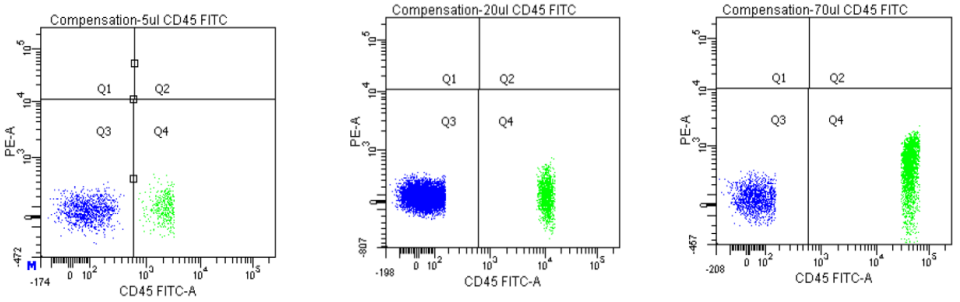
Correct compensation

Bright population used for compensation



Incorrect compensation

Dim population used for compensation



	Difference in MFI (Y-Axis) negative vs positive		
Correct compensation	**8**	**5**	**7**
Under-compensation	**4**	**85**	**245**

Rule #3: Positive and negative must have same autofluorescence

Can use antibody stained cells or beads as compensation controls

BUT the negative control must have the same autofluorescence

- beads with beads

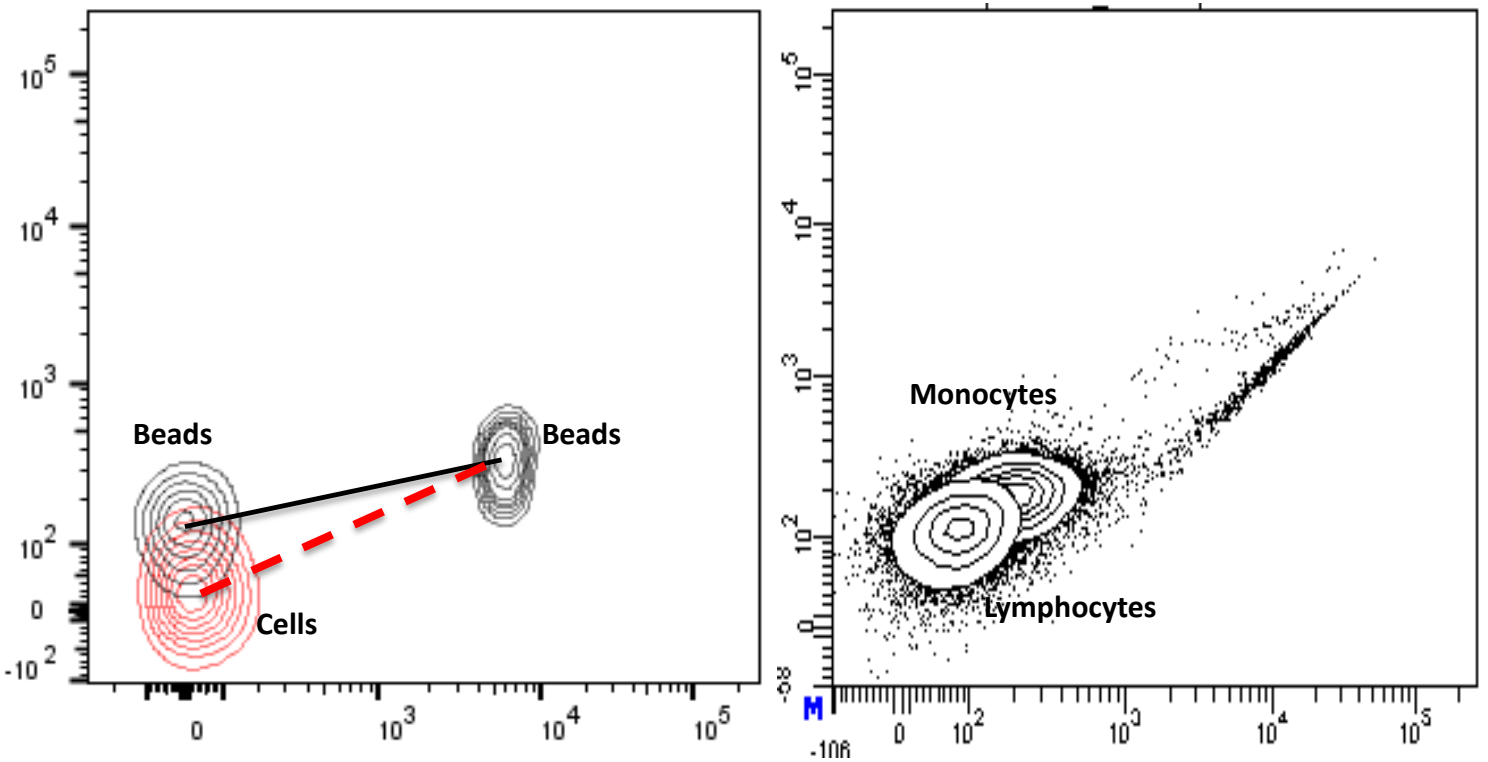
- cells with cells, same subset of cells

  use CD3+ lymphocytes to CD3- lymphocytes.

  don't use CD3+ (lymphocytes) and CD3- (monocytes, granulocytes)

Don't use beads and cells to compensate a single fluorochrome

Rule # 3: Same Autofluorescence



The slope of the line used to calculate compensation is different, depending on which negative autofluorescent population is used.

A mismatched negative will generate an erroneous compensation correction.

Compensation Controls – Cells

Single color stained cells

Pros:

Best possible match of spectra

Works with all fluorochromes

Cons: problematic when

Not enough cells available

the antigen expression is not known or dim

Few cells express antigen

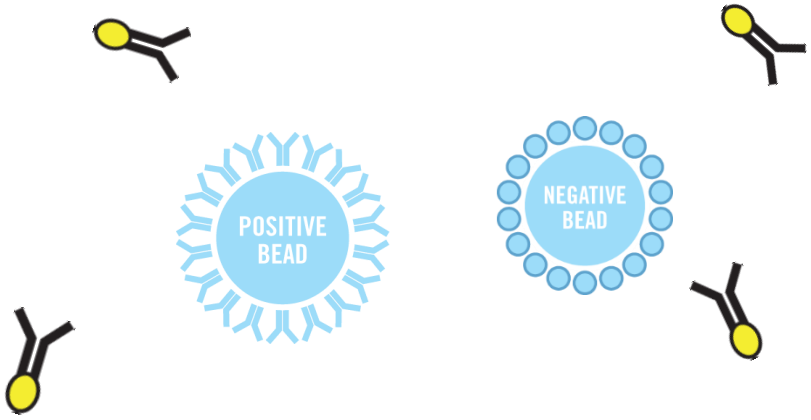
Compensation Controls – Beads

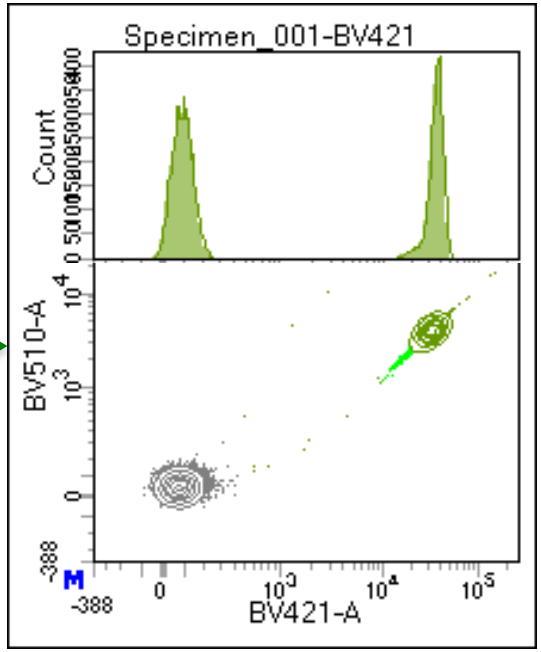
Attach to most antibodies

Give bright positive population

For most fluorochromes give the same SOV as cells

Use beads in same conditions as cells, fixation, detergent





Compensation beads

- Several kinds on the market from different companies
- Available for various situations
  - Live dead amine dyes
  - Compbeads plus: to match high autofluorescence
  - Low autofluorescence beads for violet laser
  - GFP and Cherry beads

Considerations When Using Compensation Beads

Beads are a surrogate for cells, They not a perfect match.

There can be minor differences in spillover

Compensation beads do not provide accurate spillover values when used with certain fluorochromes.

This is often, but not always stated in the antibody product sheet

For example V500 and BUV737

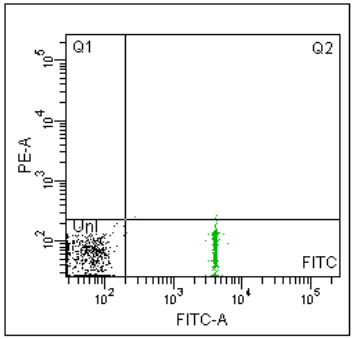
	BUV737 Spillover into					
HuCD4	**BUV395**	**BV711**	**BV786**	**APC**	**AF700**	**APC-H7**
Cells	**2%**	**4%**	**3%**	**0%**	**47%**	**12%**
Beads	**2%**	**5%**	**4%**	**1%**	**56%**	**14%**

Caution!

Compensation value is dependent on:

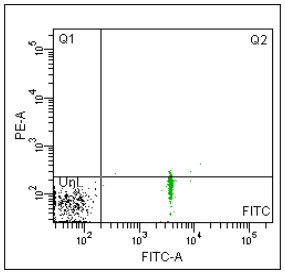
the PMT voltage of the fluorescent parameters

Correct Compensation



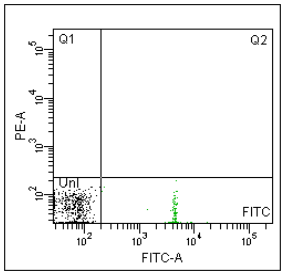
Population	PE-A Mean
Unl	69
FITC	64

PMTV = FITC Decreased by 5 V



Population	PE-A Mean
Unl	
FITC	132

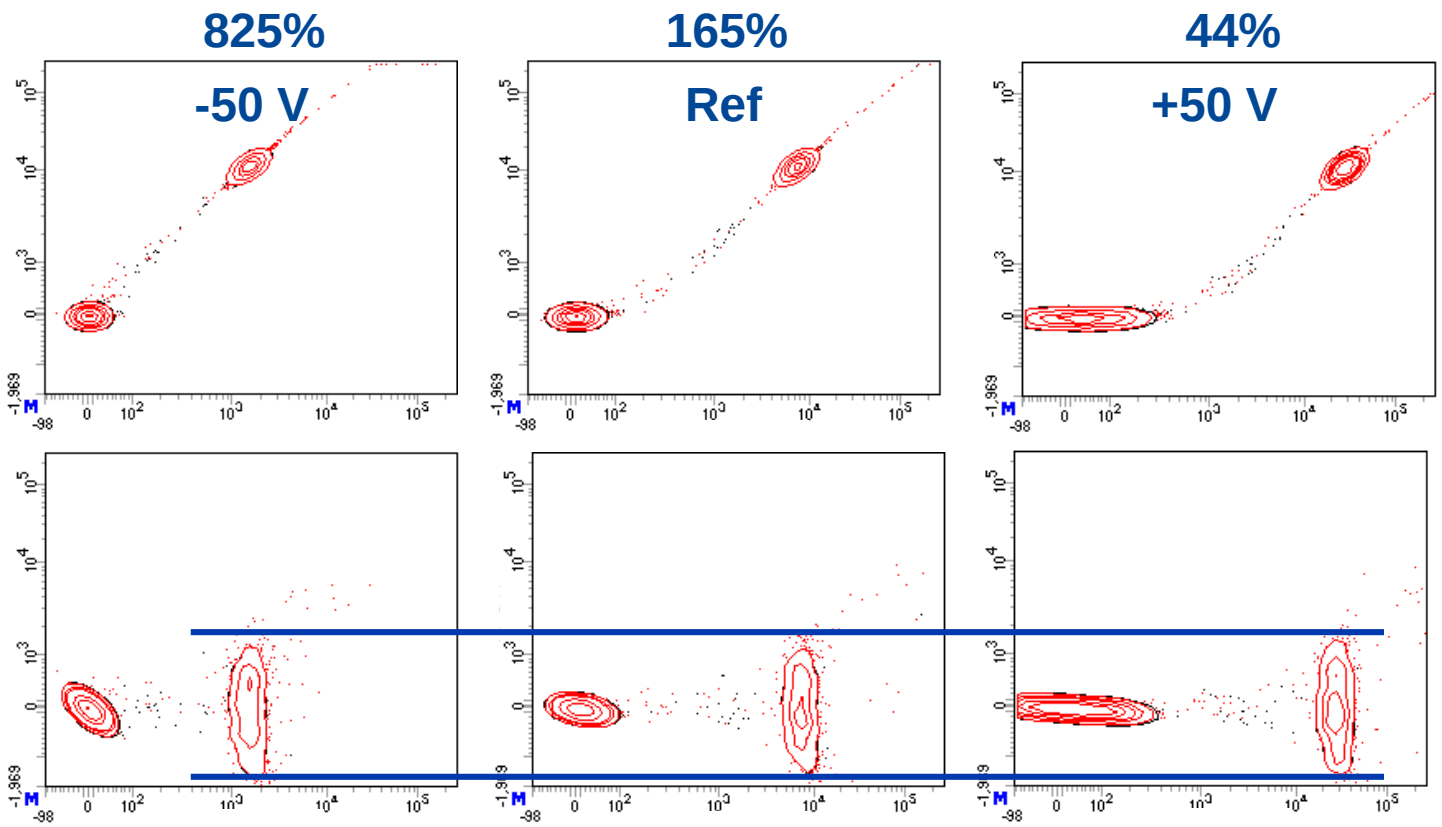
PMTV = FITC Increased by 5 V



Population	PE-A Mean
Unl	67
FITC	49

Never change PMTV for fluorescent parameters after compensation

What is an acceptable Compensation Value?



These is the same tube run at 3 different PMT Voltages

The compensation value changes greatly

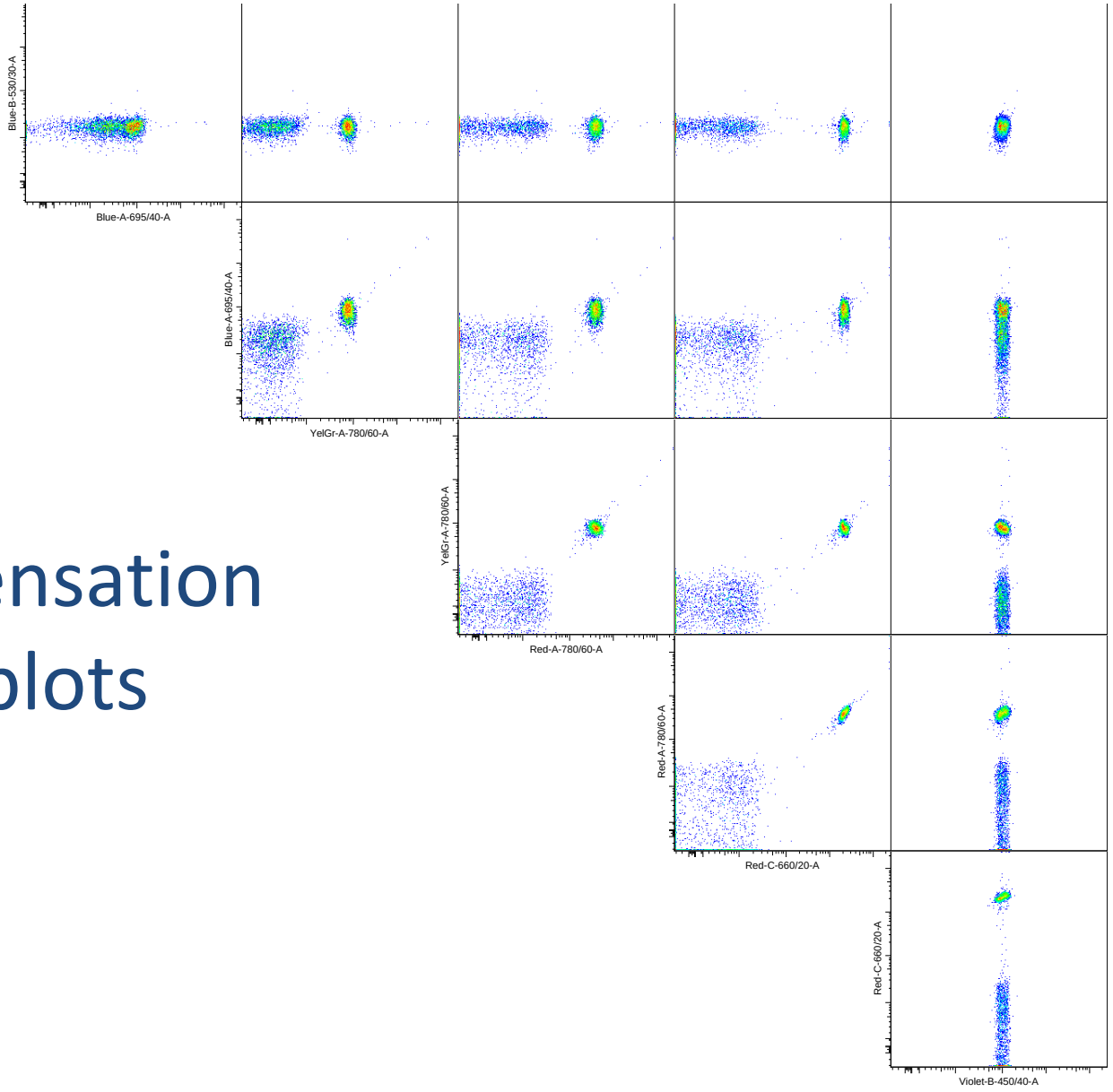
The result is the same

Check your compensations

- Good compensation dependent on good single colors!
- Look at your single colors with compensation applied
- All colors against all colors
- Make sure they look right, no leaners!
- Correct manually if necessary

  But only on the single colors!
- Then apply corrected values to experimental data

Check compensation with nxn plots

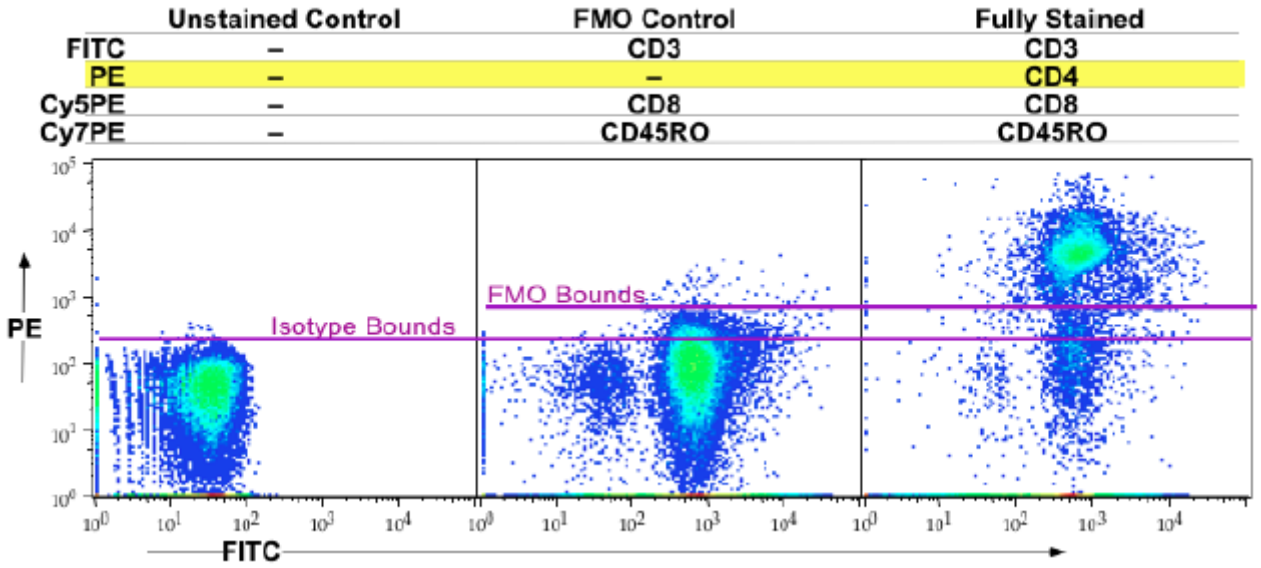


Fluorescent minus one (FMO) controls

- Fluorescence Minus One (FMO) controls contain all the fluorescent markers except one.
- Typically controls for increased background due to spectral spillover related data spread
- For low density or smeared populations (eg, activation markers), FMOs allow accurate delineation of positively vs negatively stained cells

Controls: FMO

- An FMO (fluorescence minus one) is now considered to be the best control for determining where the threshold for positive cells is.
- An FMO includes all of the fluorochromes in an experiment except for the one of interest which requires a threshold for positivity.
- A good explanation at: http://flowjo.typepad.com/the_daily_dongle/2011/09/fmo-vs-isotype-controls.html

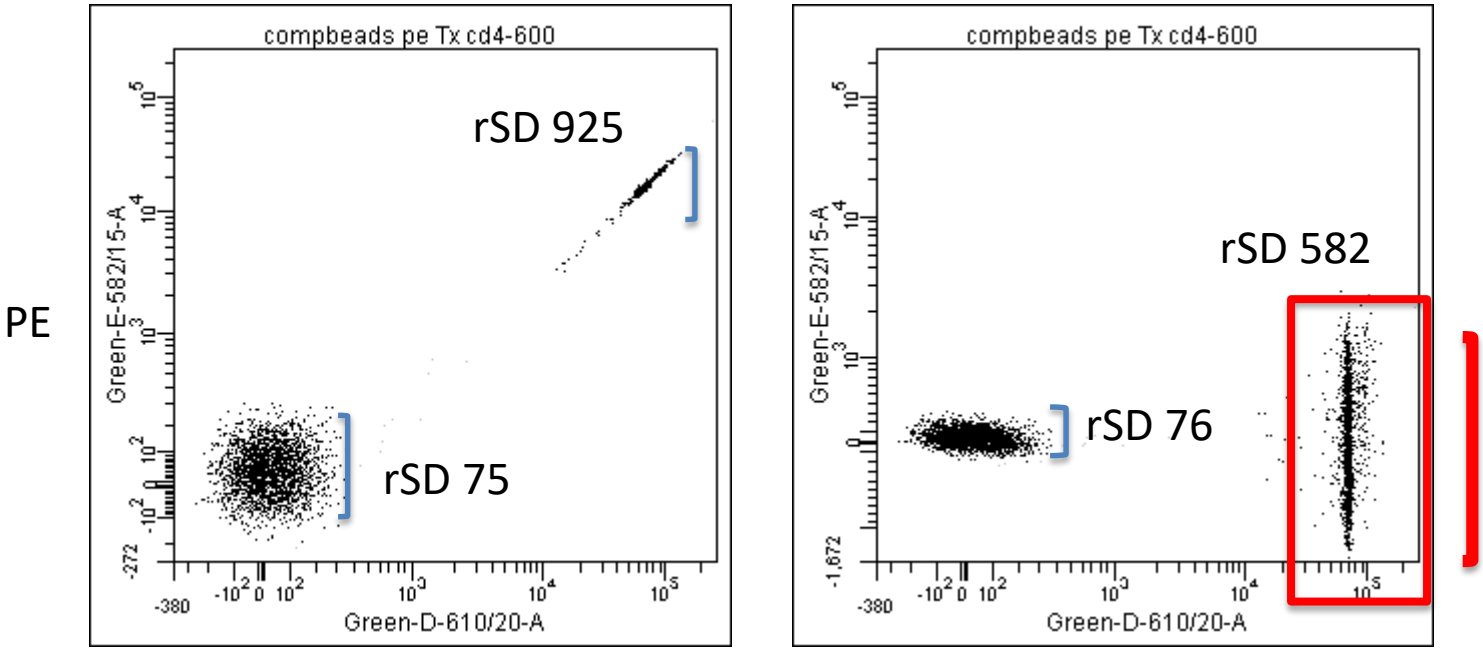


Summary

- Know your cytometer
- Choose your fluorochromes carefully for
  excitation, emission and brightness
- Look at spectra to determine possible spillover problems
- Good compensation is crucial
- **Good compensation is dependent on good quality controls**
- Gating on positive should be done on FMOs for difficult antigens

Good news:

You can correct for spectral spillover by compensation



PE

PE-TxR

Bad news:

You cannot eliminate ‘spread’ in the positive population

Reference:

Maciorowski, Z., Chattopadhyay, P.K., & Jain, P. (2017). Basic multicolor flow cytometry. Current Protocols in Immunology, 117, 5.4.1–5.4.38. doi: 10.1002/cpim.26