Tribhuvan University
त्रिभुवन विश्वविद्यालय

ISAC
International Society for Advancement of Cytometry

2nd Virtual Flow Cytometry Workshop Nepal

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International Society for Advancement of Cytometry

Basic Multicolor Flow Cytometry

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Overview

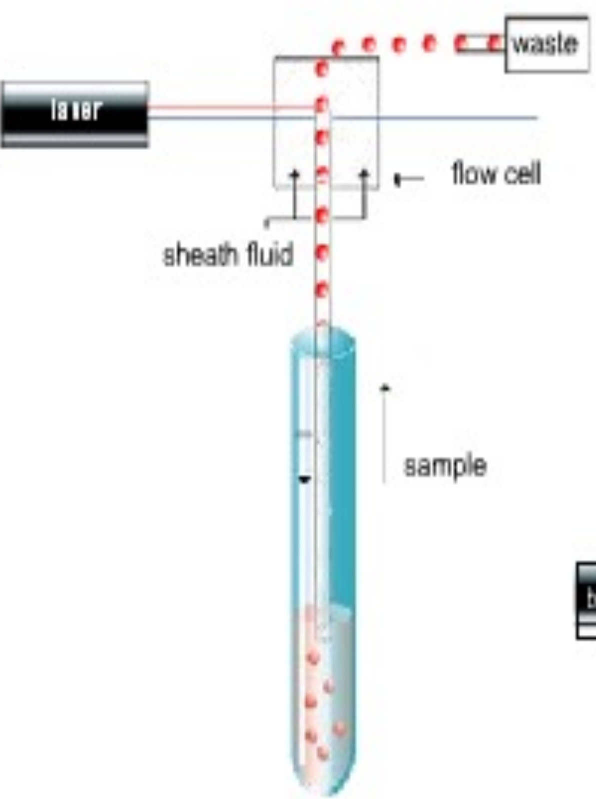
- A quick review of cytometer function
- Fluorochromes and fluorescence
- Excitation and Emission
- Fluorochrome brightness
- Stain Index to maximize resolution:
  - Characterize your instrument
  - Titrate your antibodies
- Spectral Spillover

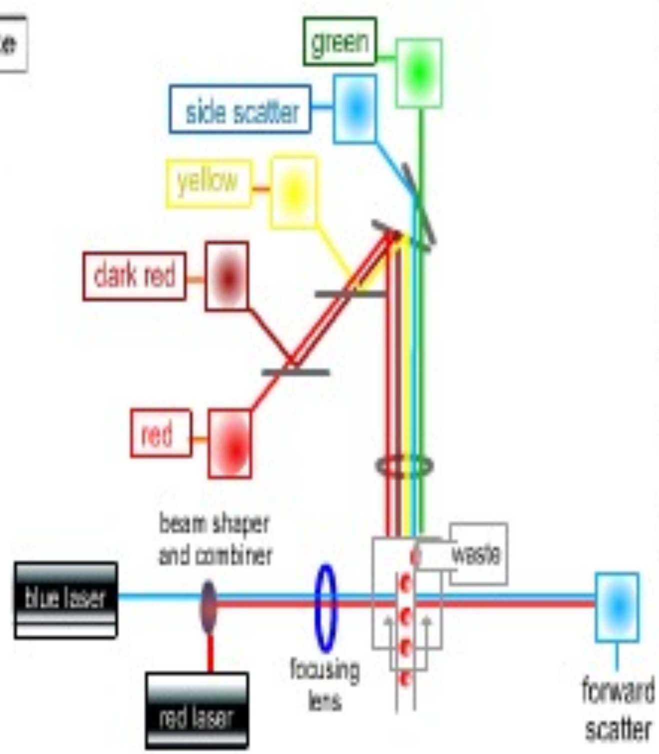
What do you find inside a Flow Cytometer?

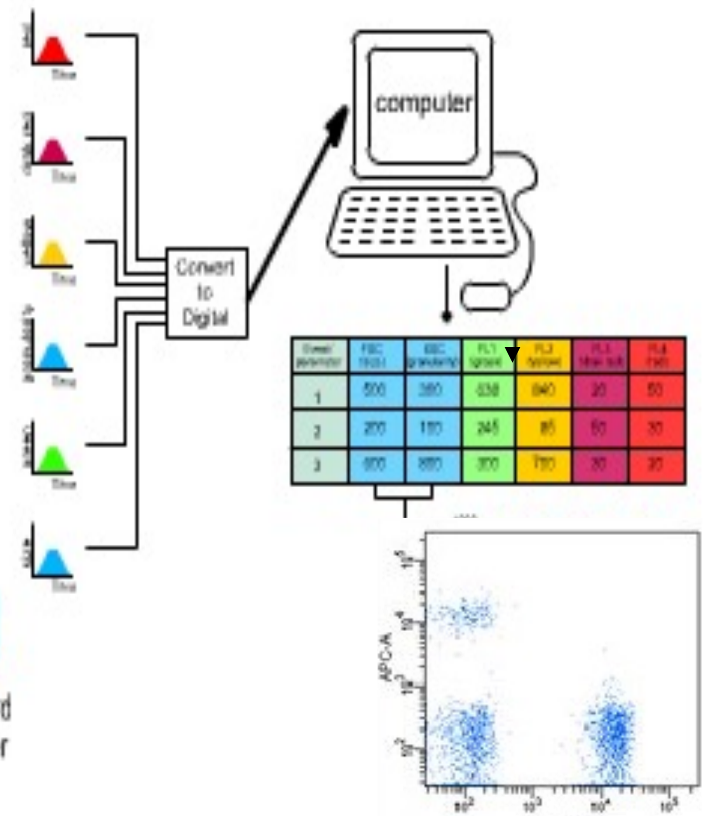
Fluidics
Position cells
to flow one by one
past the laser beam

Optics
Separate the light emission
from different fluorochromes
and direct towards detectors

Electronics
Detectors convert light
emission to voltage pulses
which are digitalized







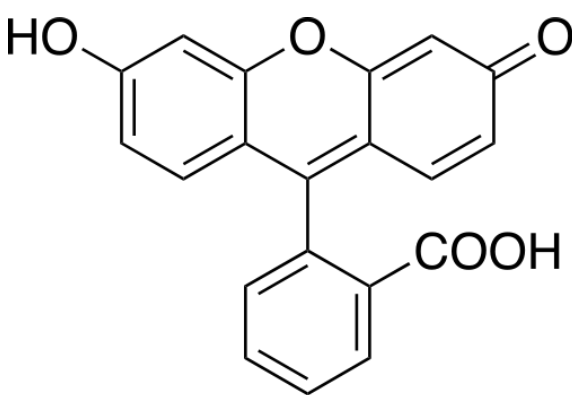
Fluorochromes and Fluorescence

Fluorochromes

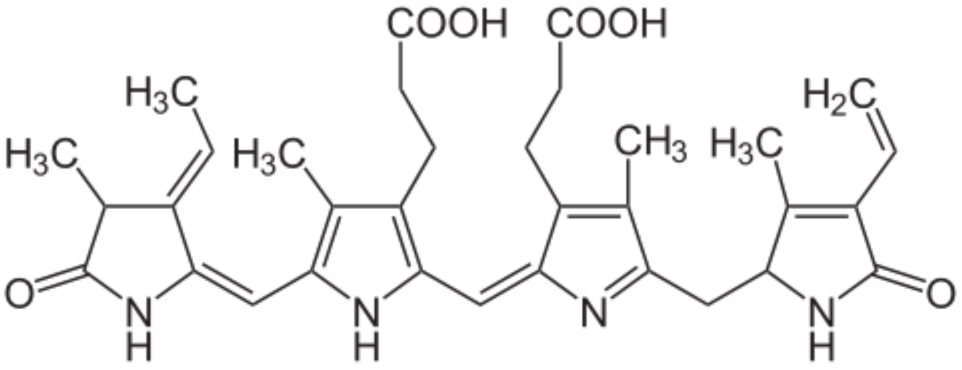
Fluorochromes are molecules
which absorb light at one wavelength
then re-emit the light energy at a longer wavelength

Structures are generally aromatic rings

Fluorescein (FITC)

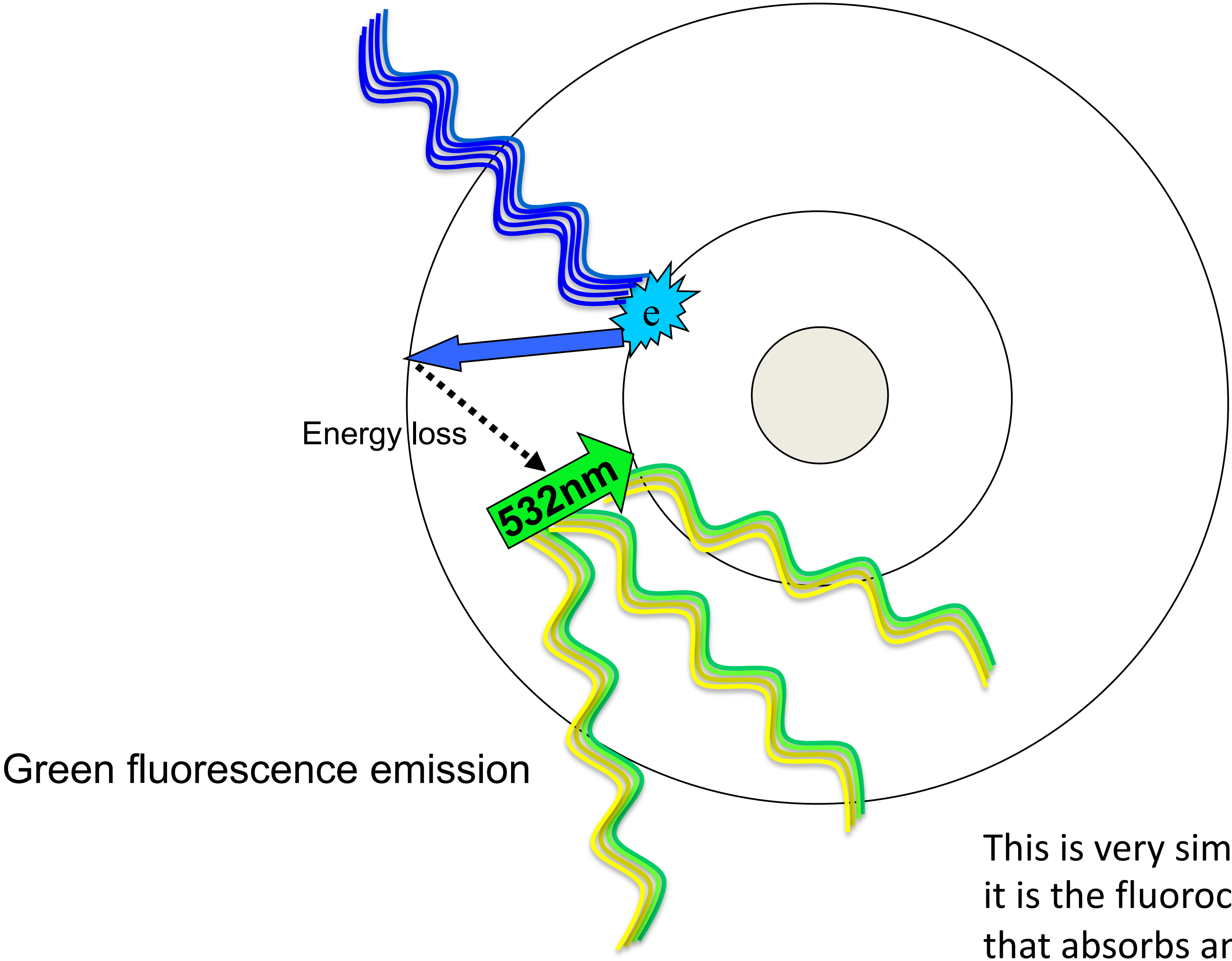


Phycoerytherin (PE)



Fluorescence

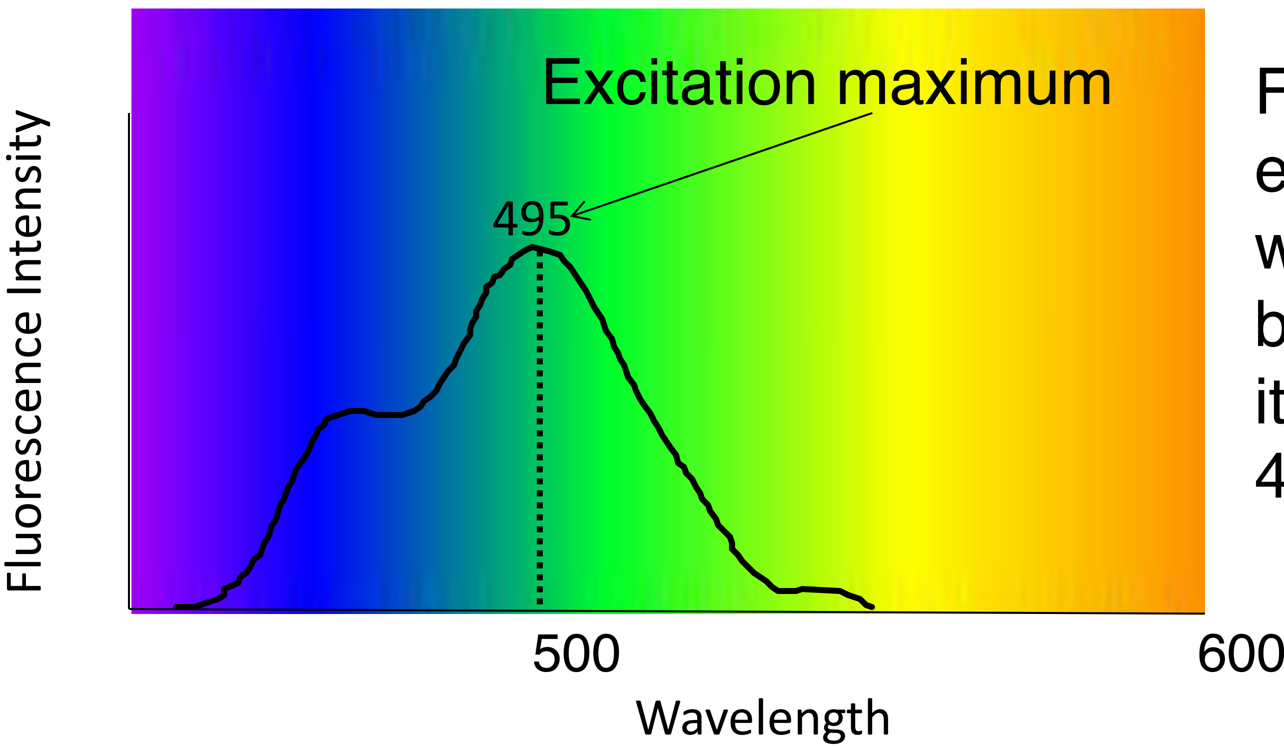
Blue 488 Laser excitation



This is very simplified:
it is the fluorochrome's electron cloud
that absorbs and emits light energy

Excitation spectrum

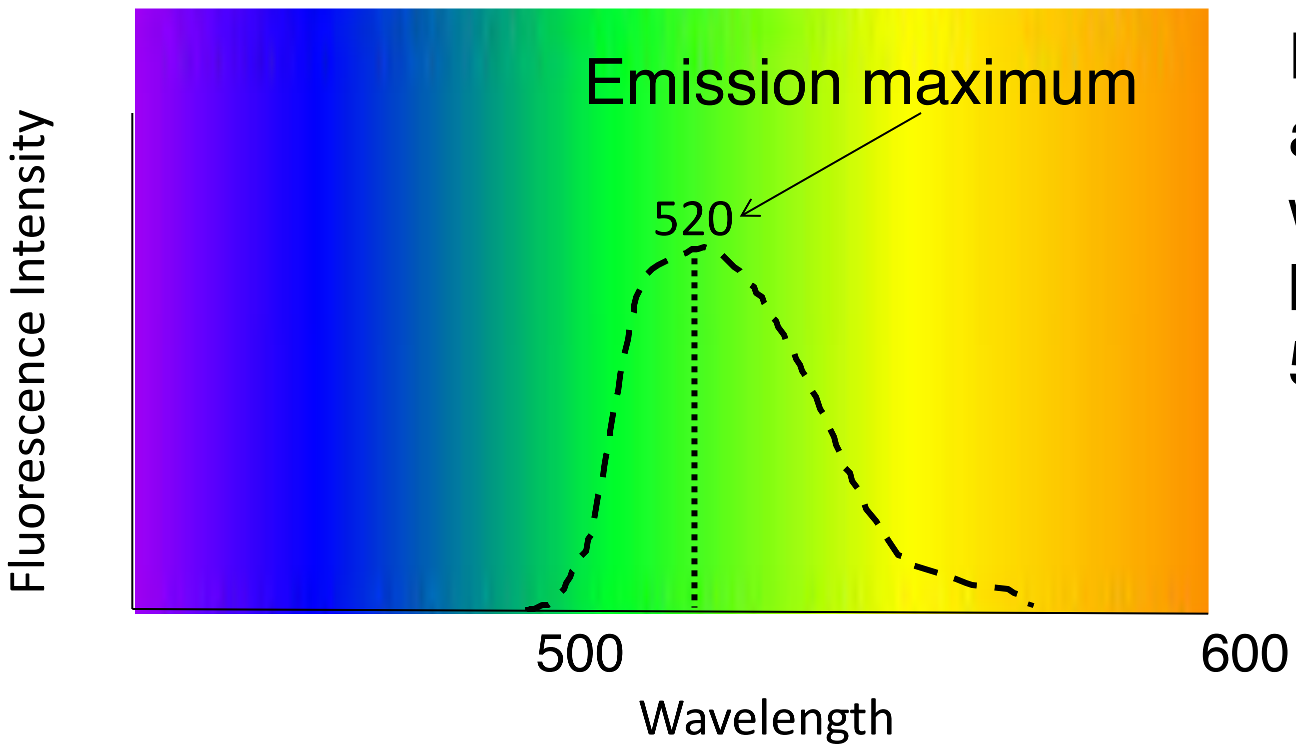
Each fluorochrome is capable of absorbing light energy over a specific range of wavelengths



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

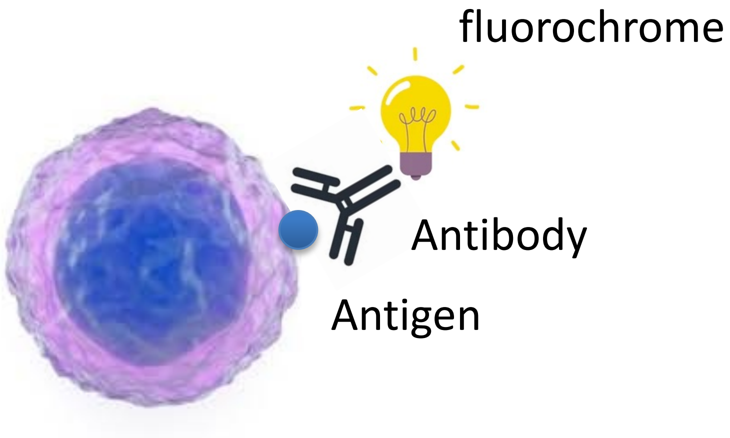
Emission spectra

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

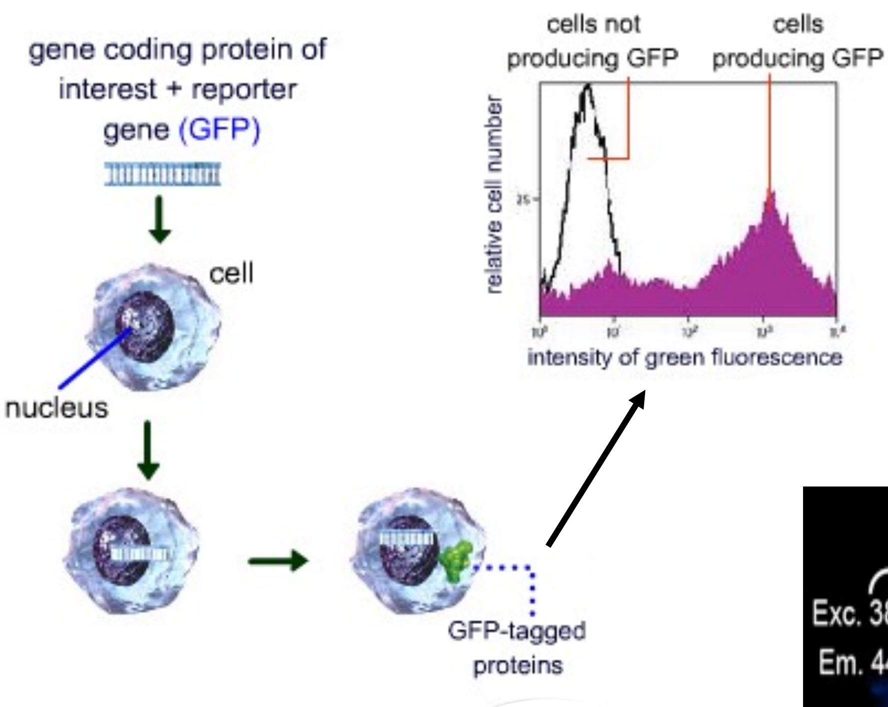


FITC will fluoresce at all these wavelengths but highest at 520nm

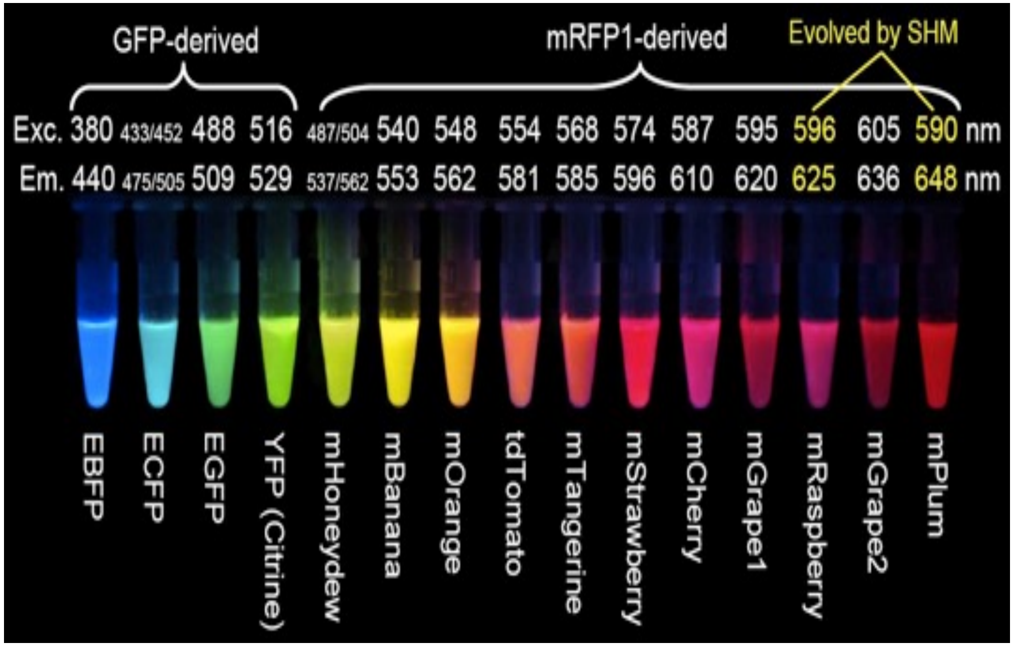
Fluorochromes are often used coupled to antibodies



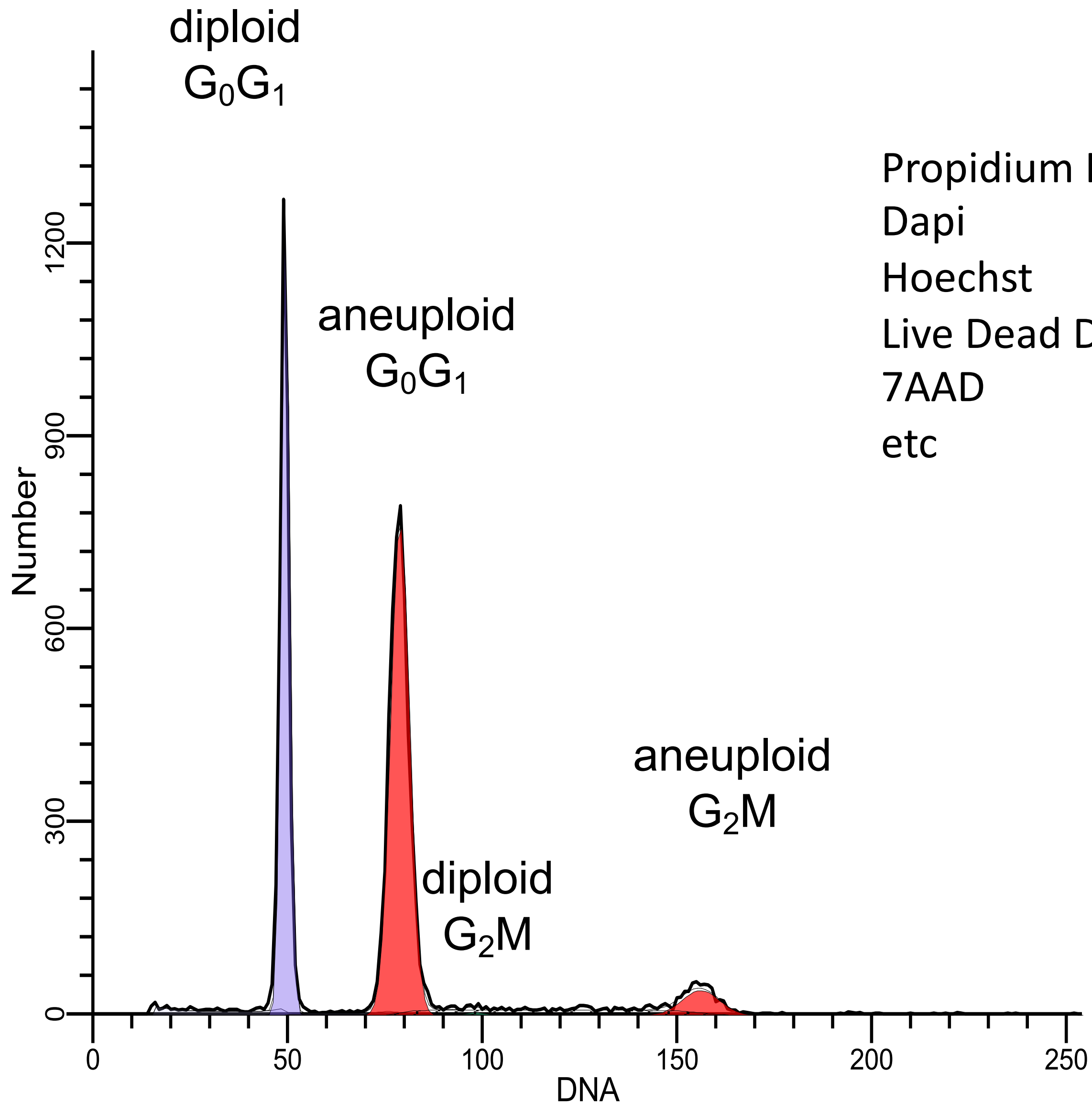
Many other applications for fluorochromes



Gene transfection with
fluorescent proteins



DNA Dyes: ploidy, cell cycle, S-Phase or viability



Propidium Iodide
Dapi
Hoechst
Live Dead DNA dyes
7AAD
etc

Fluorescent Probes for specific targets

- Apoptosis
- Organelle probes for Golgi, mitochondria, lipids
- pH sensitive probes
- Oxidative state probes
- Ion probes, e.g. bound vs unbound for Calcium flux
- Antibody capture beads for multiple proteins

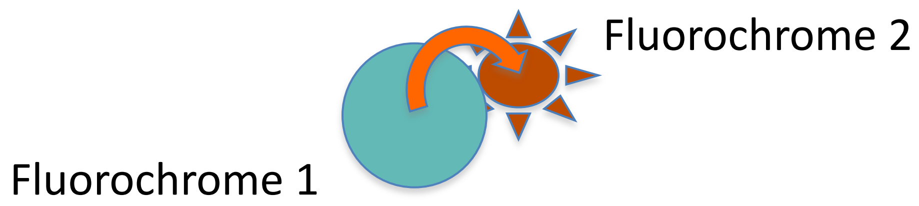
To name but a few!

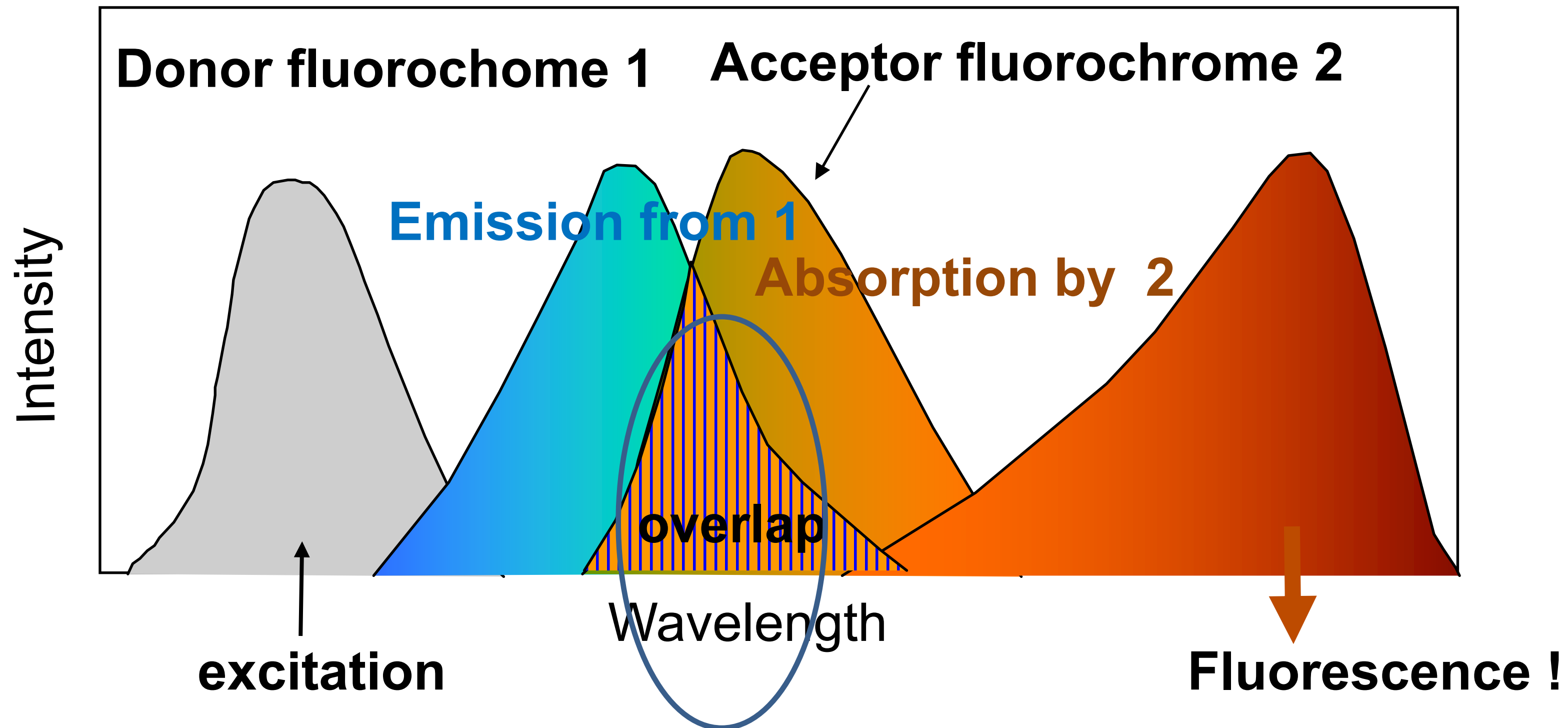
Tandem Dyes

Tandem Dyes have 2 fluorochromes coupled together

Energy Transfer

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





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

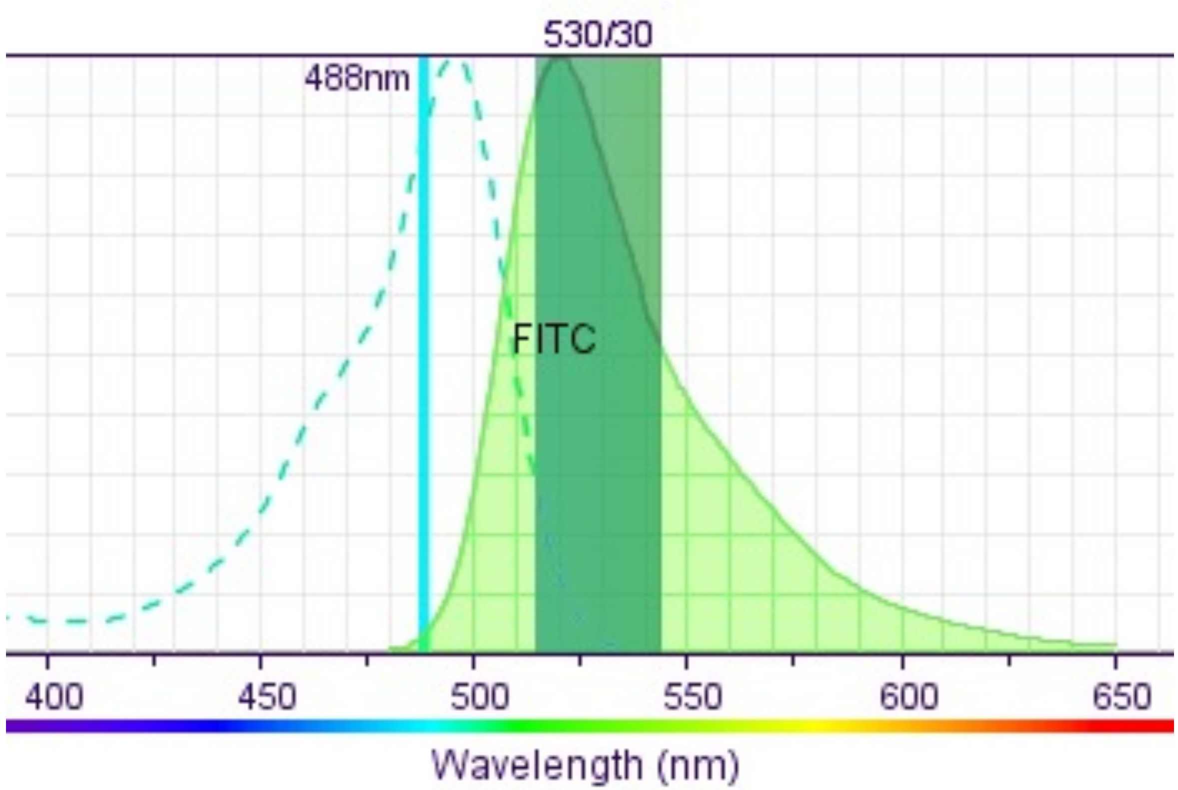
Pe-Cy7

APC-Cy7

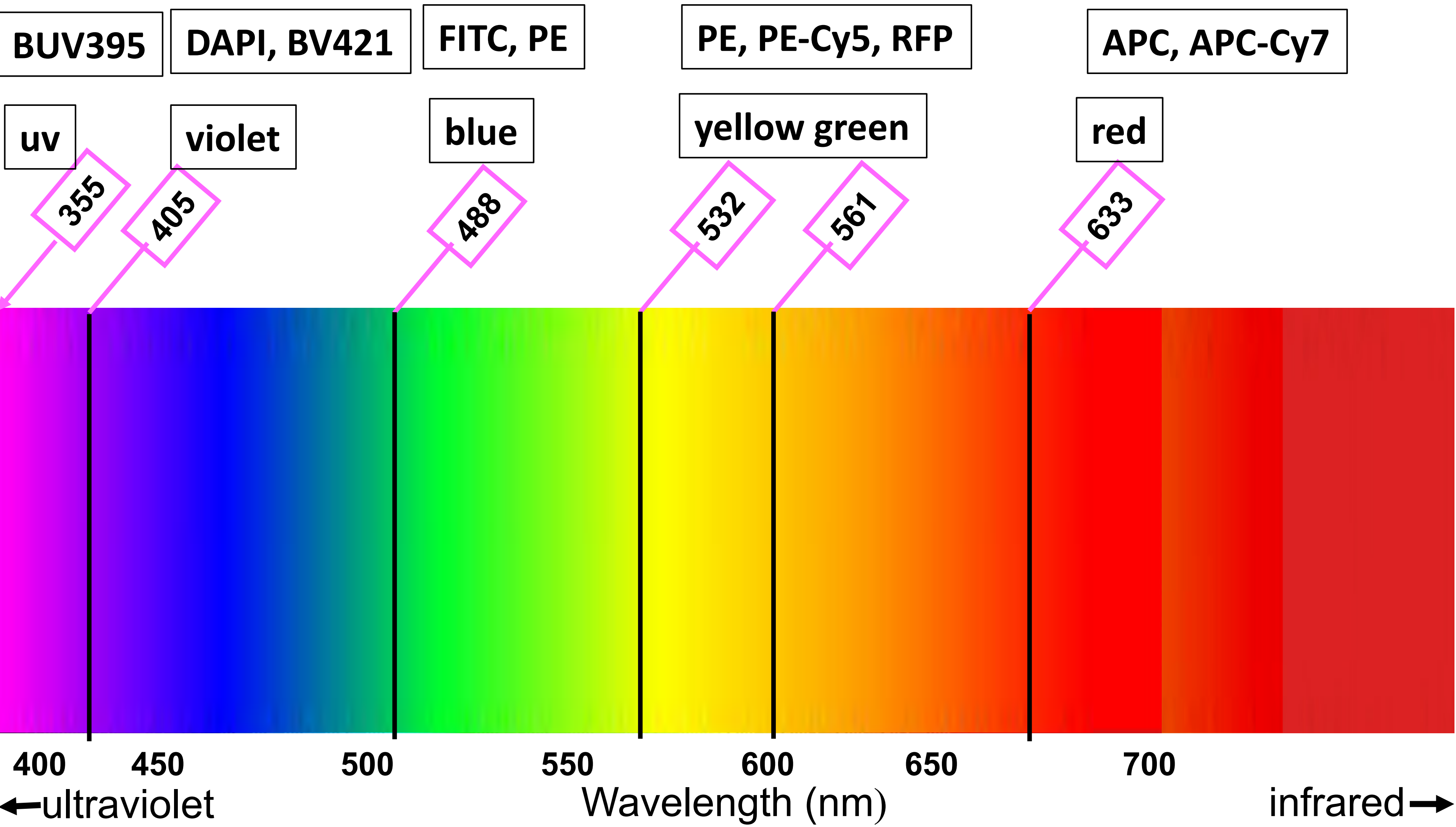
Brilliant (Sirigen) dyes (Brilliant Violet)

Choosing Fluorochromes: which lasers and filters?

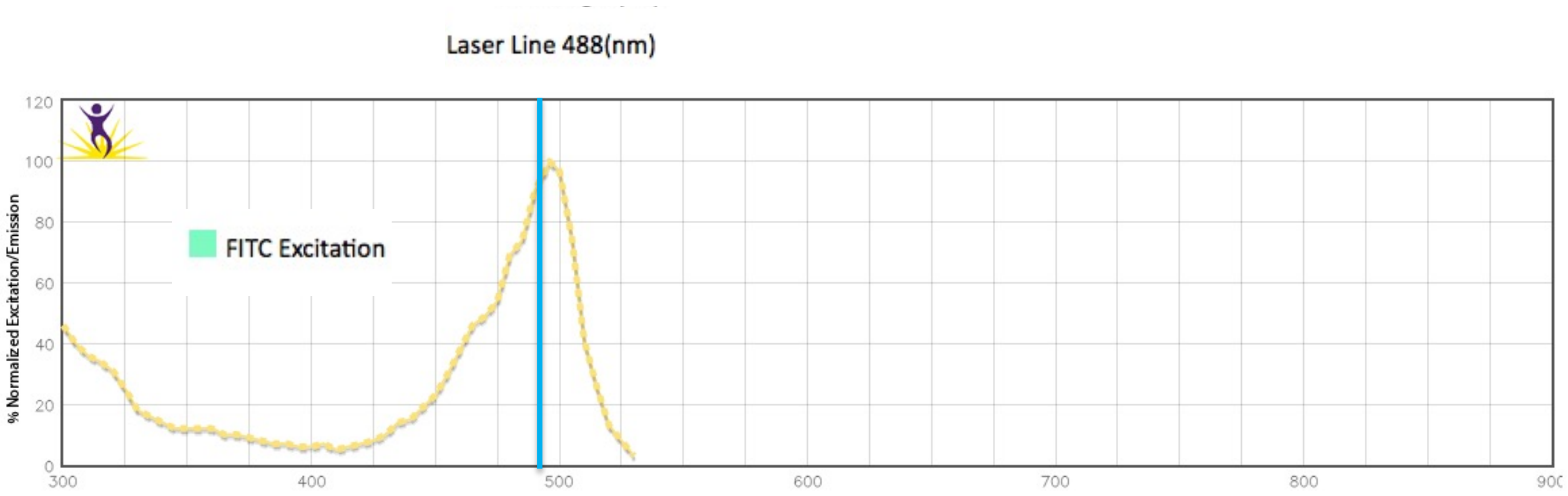
- Look at the **excitation** spectra to determine which lasers can be used to excite the fluorochrome.
- Look at the **emission** spectra to determine which filters should be used to collect the signal.



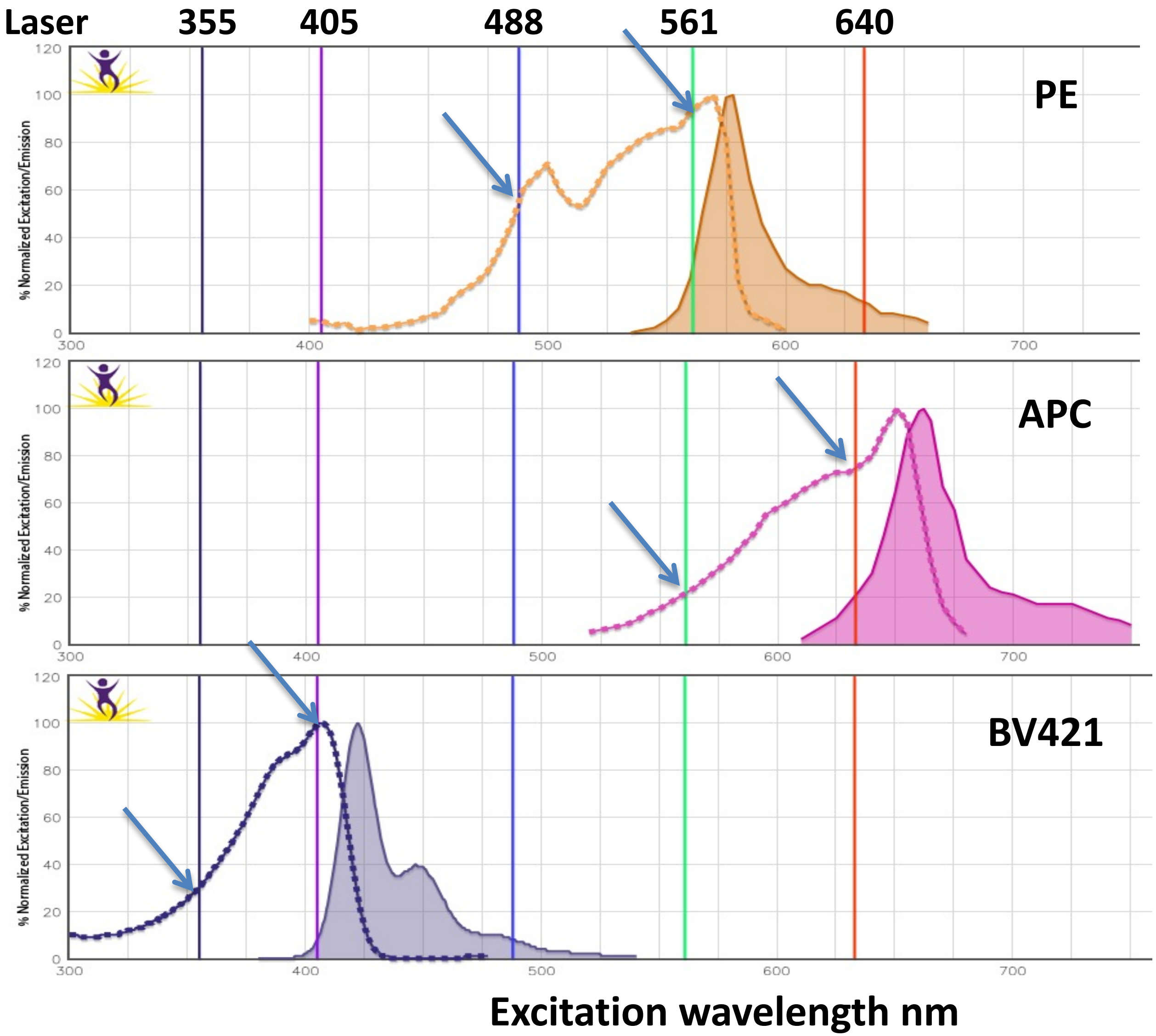
Laser wavelengths on typical cytometers



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

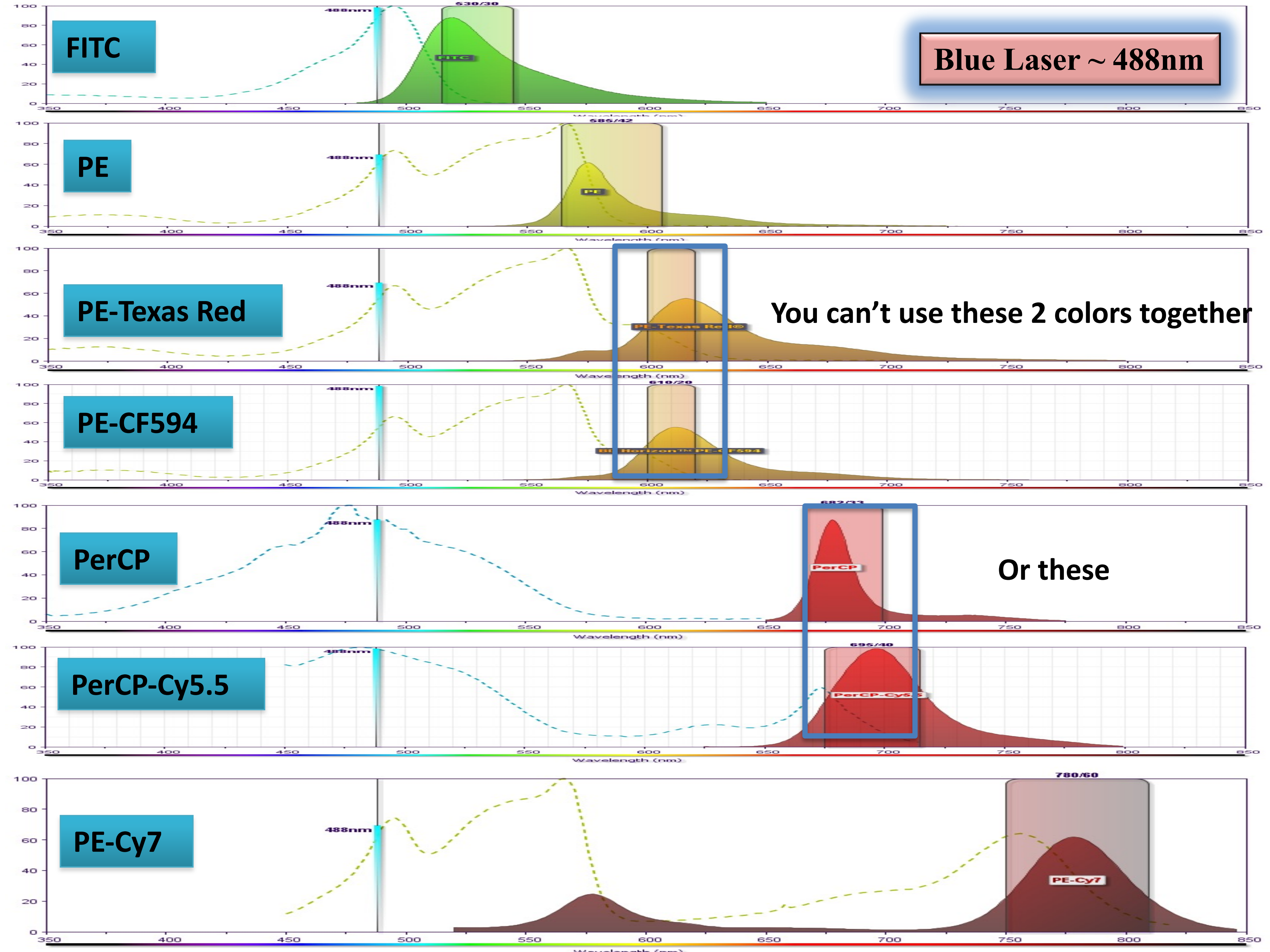
BV421 is excited best by the 405 laser but also a little by the 355.

Emission spectra

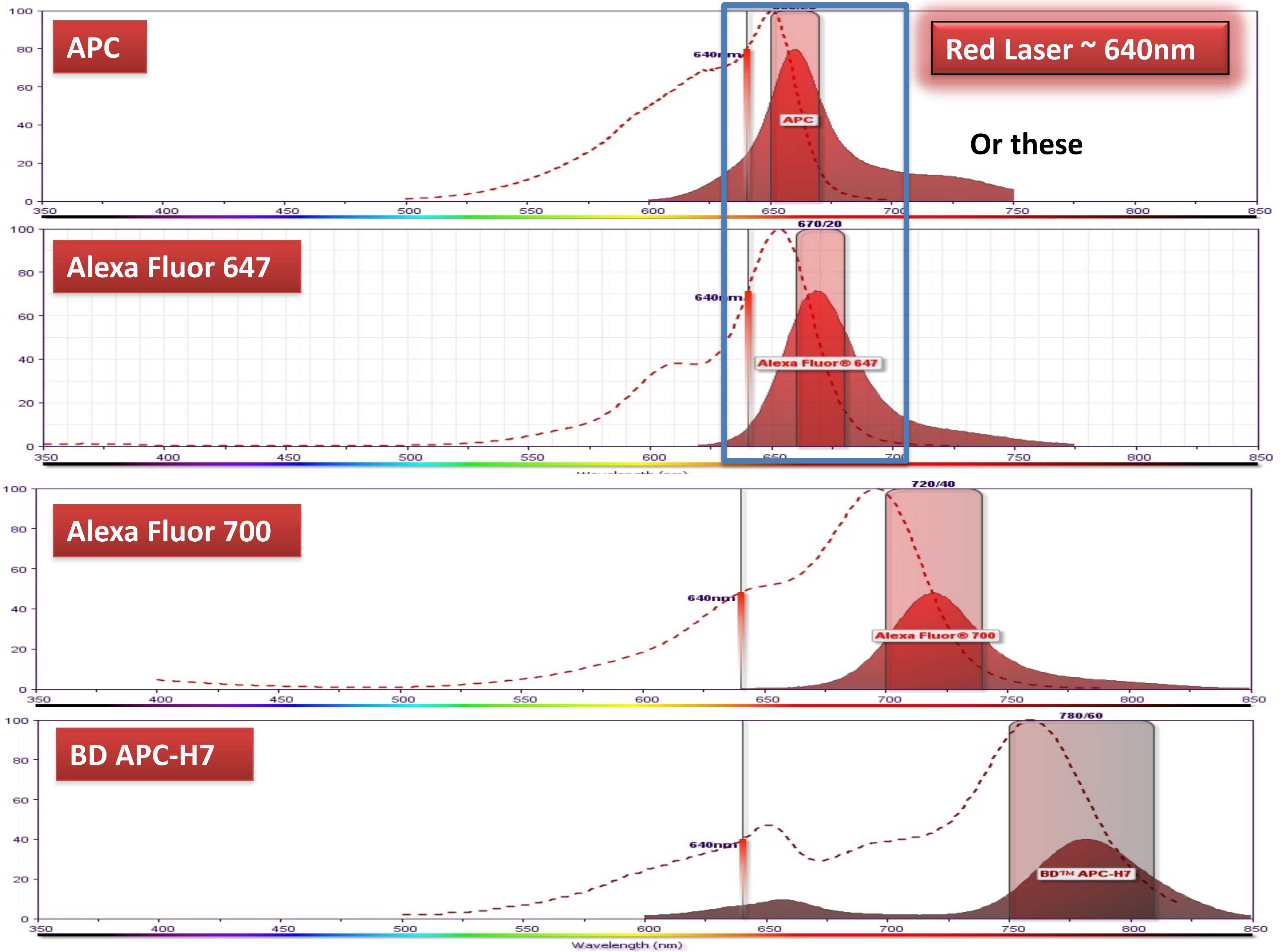
Look at the emission spectra of your fluorochromes

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

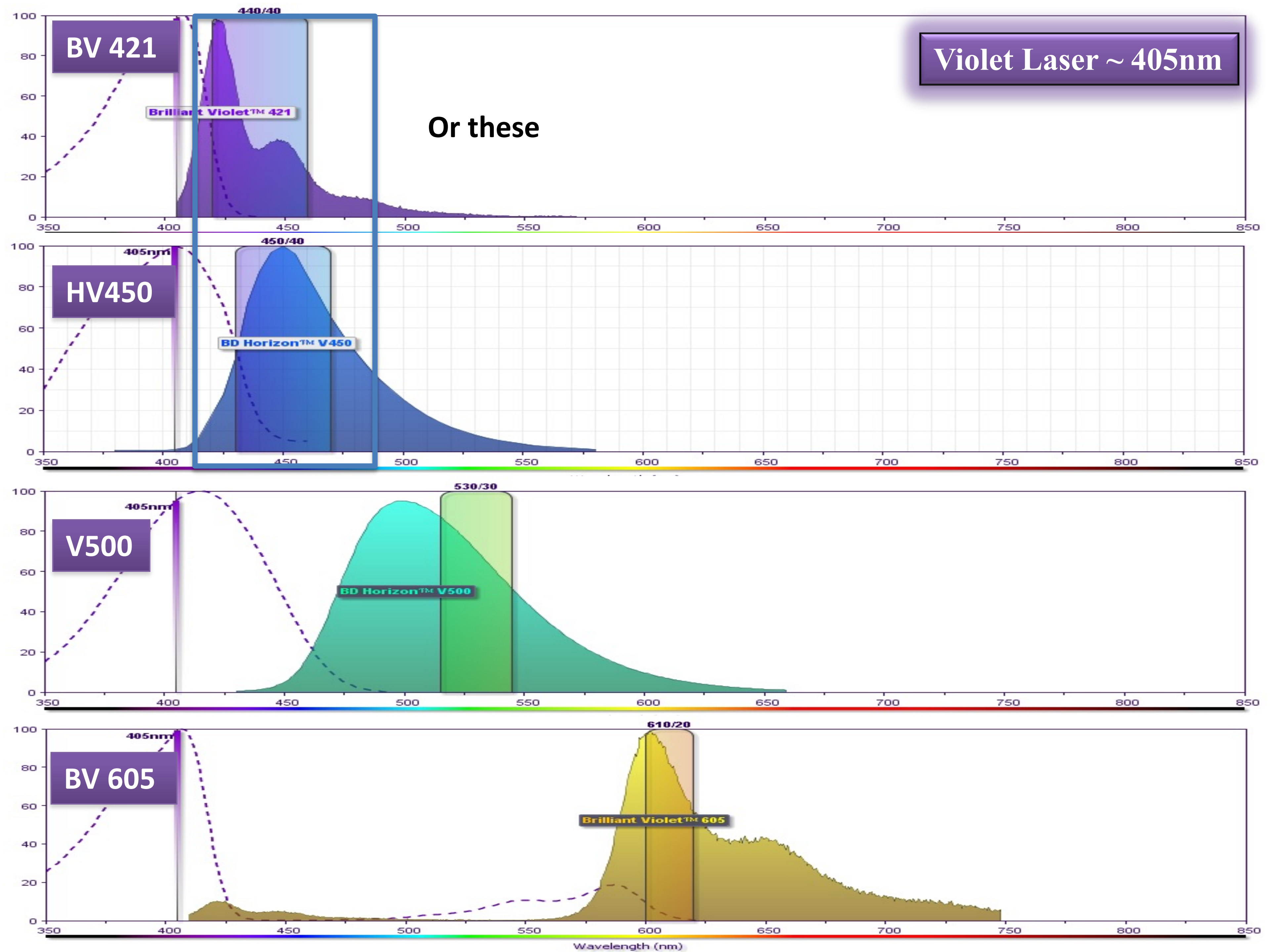
Fluorochromes excited off the same laser should not have overlapping emission spectra



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
530/30
585/42
610/20
695/40
780/60
Wavelength (nm)



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



Violet Laser ~ 405nm
Or these
BV 421
440/40
Brilliant Violet™ 421
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	29%	105,000
FITC	72,000	.9	.6	3%	5,000
BV421	2,500,000	.69	17	106%	69,000

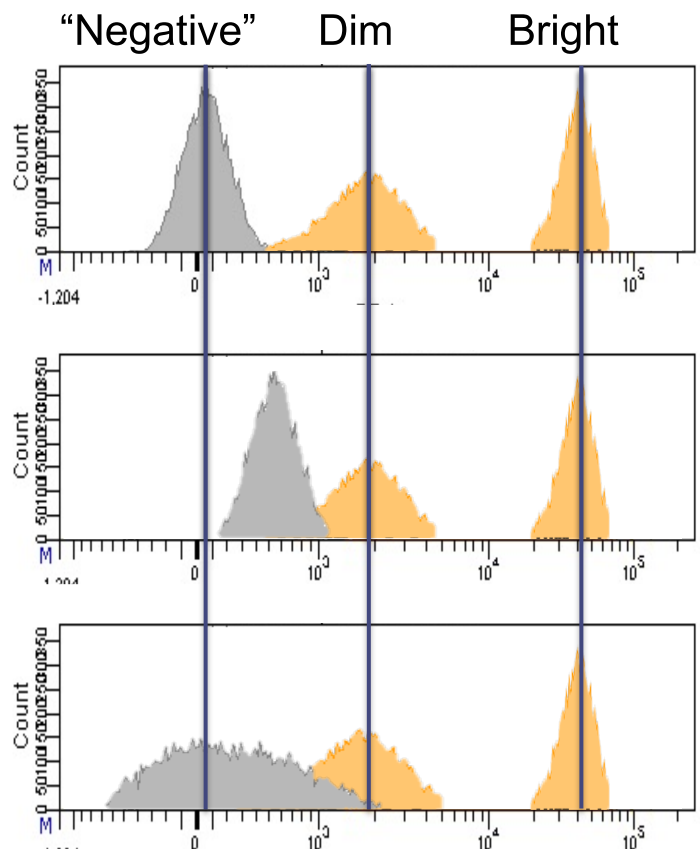
Resolution

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



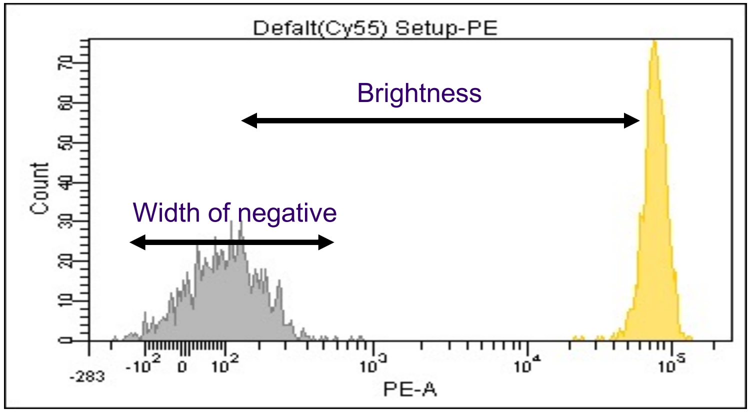
The ability to resolve populations is a function of
both background *and* spread of the negative population.

Stain Index: How well separated are your populations

Stain index is a measure of resolution 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**	82%
PerCP	RPA-T4	695/40	**30**	17%
APC	RPA-T4	660/20	**263**	29%
FITC	RPA-T4	530/30	**43**	3%
Pacific Blue™	RPA-T4	440/40	**63**	2%

- **APC has a high stain index even though less intrinsic brightness**
- **Due to less autofluorescence in red wavelengths, background 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%	$\boxed{348}$	262
PeCy5	NA	NA	180	131
APC	4.7	29%	238	$\boxed{281}$
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.

You can determine relative brightness for your own cytometer

Relative Brightness	Reagent	Filter
BRIGHTEST	Brilliant Violet™ 421	450/50
BRIGHTEST	PE	575/26
BRIGHTEST	Brilliant Violet 605	610/20
BRIGHTEST	BD Horizon PE-CF594	610/20
BRIGHTEST	PE-Cy5	670/14
BRIGHTEST	APC	660/20
BRIGHT	PE-Cy7	780/60
BRIGHT	Alexa Fluor® 647	660/20
BRIGHT	PerCP-Cy5.5	695/40
MODERATE	Alexa Fluor® 488	530/30
MODERATE	FITC	530/30
MODERATE	BD Horizon V450	450/50
MODERATE	Pacific Blue™	450/50
DIM	Alexa Fluor® 700	730/45
DIM	PerCP	695/40
DIM	APC-Cy7	780/60
DIM	AmCyan	525/20
DIM	BD Horizon V500	525/20
DIM	BD APC-H7	780/60

Instrument voltage/gain settings
How do you choose?

Settings can affect your stain index
and your sensitivity

Optimize your Instrument settings

Instrument detector gain or voltage settings affect sensitivity!

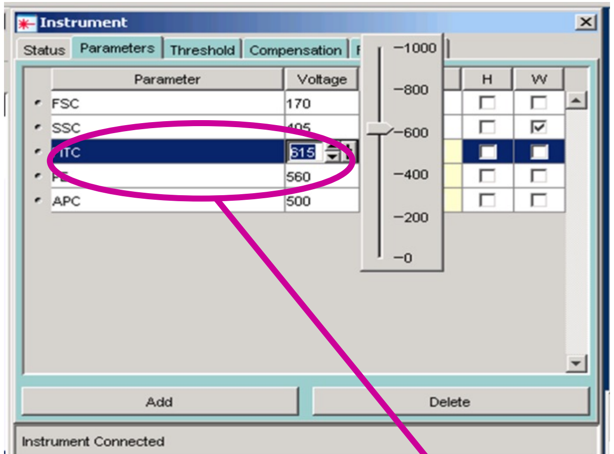
Best settings should be determined for each detector on each cytometer

This applies to photomultiplier tube (PMT) voltage settings and also to avalanche photodiode (APD) gain settings

There are different ways to do this:

1. **Voltration**: Set PMT voltages to maximize Stain Index with comp beads or stained cells
2. Use CST electronic noise and linearity values so that
    1. unstained cells have 2.5 X SD of electronic noise
    2. positive cells not above max linearity

Changing the PMT voltage



- Changing the voltage applied to the dynode chain increases or decreases output signal (current) from the PMT

- This is done using the PMT voltage control on the software

- 10^3 to 10^8 electrons may reach the anode for every electron that left the cathode, depending on the voltage applied

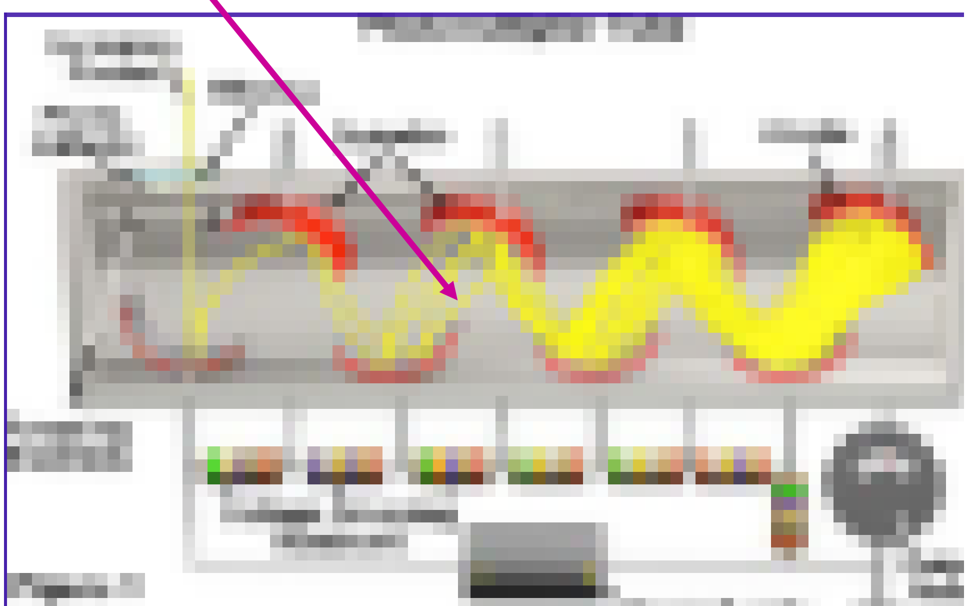
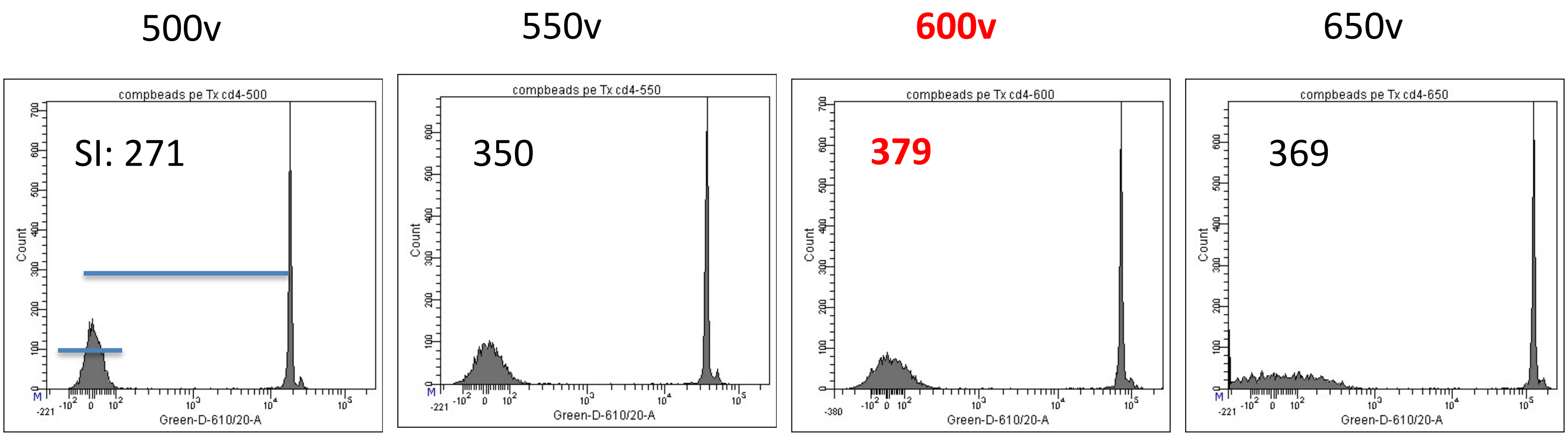
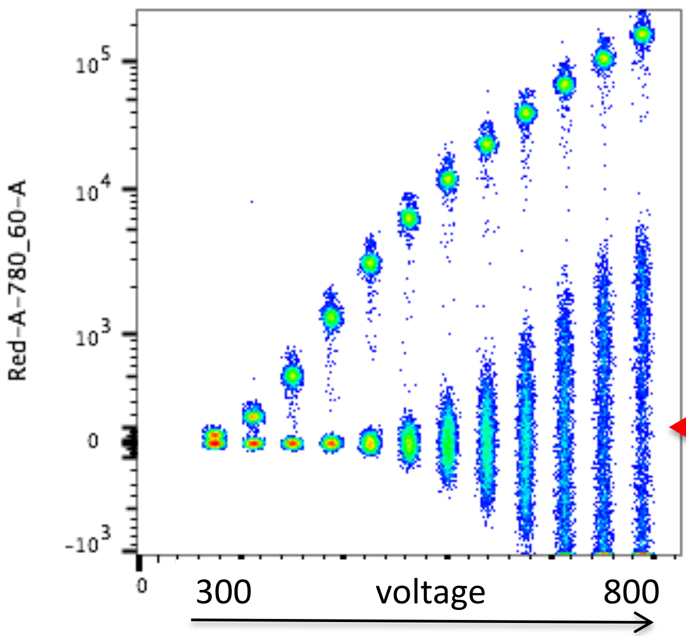


Diagram from Dakocytomation

Voltration: choose the best stain index (SI)

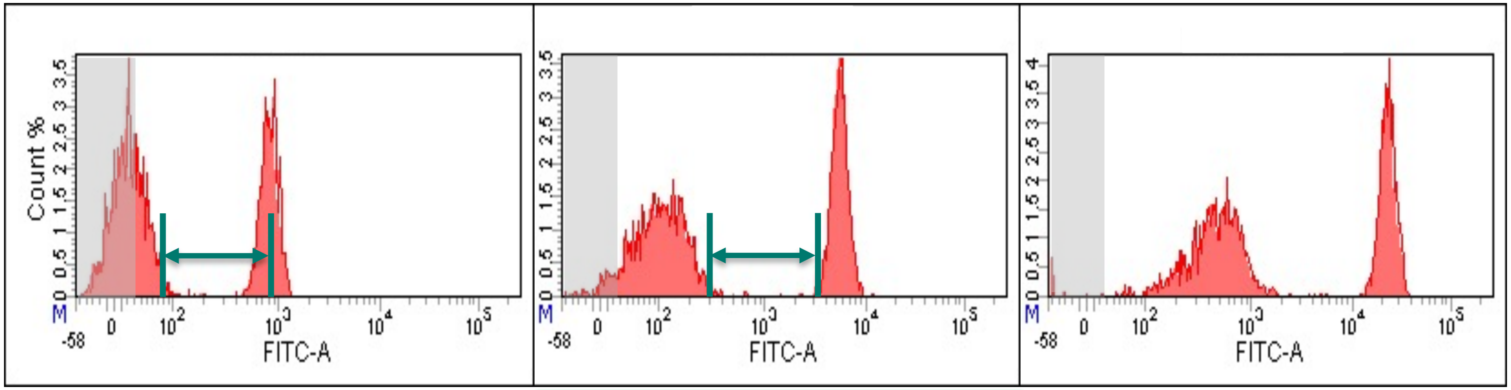




Increase of background spread at higher voltages

Voltration Example: FITC detector

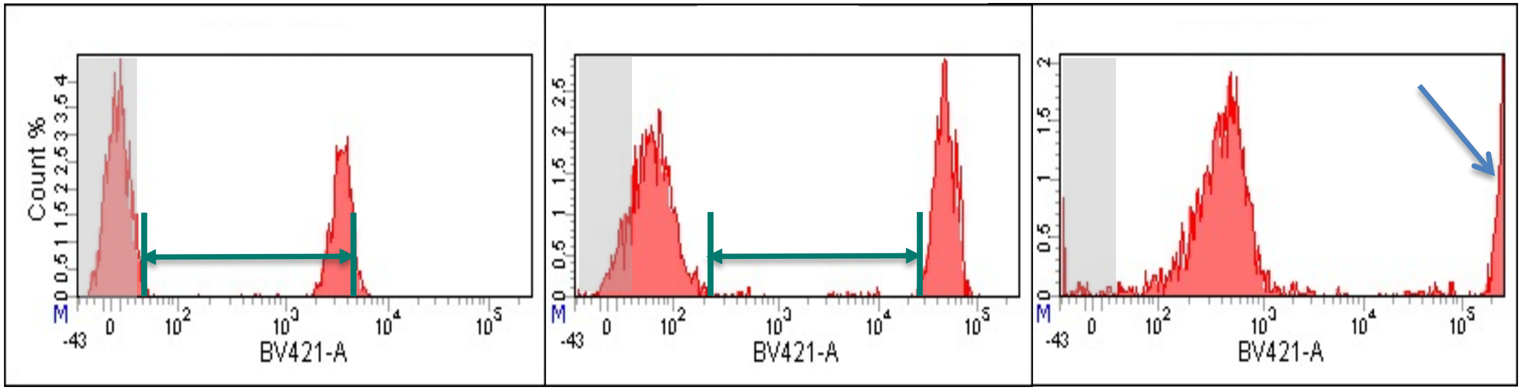
FITC Detector (SD_{EN} = 20)



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

Optimized Instrument Settings

1: Determine best voltage or gain setting for each detector

Once the best setting is determined:

- It should be good for all fluorochromes read on that detector
- It is valid until change or major maintenance to the instrument (laser change/alignment, optical filter change)

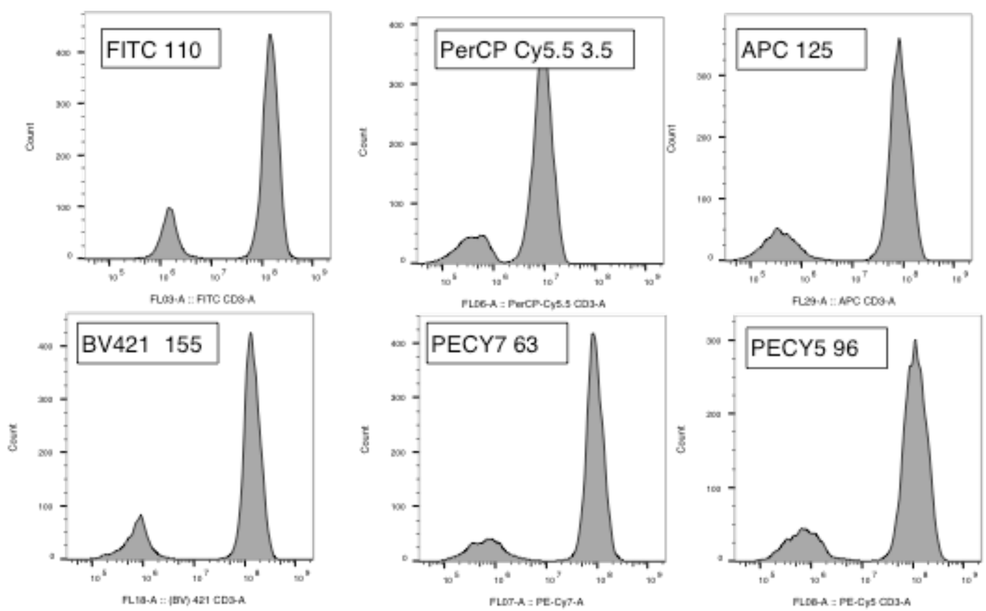
2. Highly positive cells whould NEVER be off the top of the scale.

In that case, reduce the voltage or gain so that cells are on scale and within the linearity range of the detector.

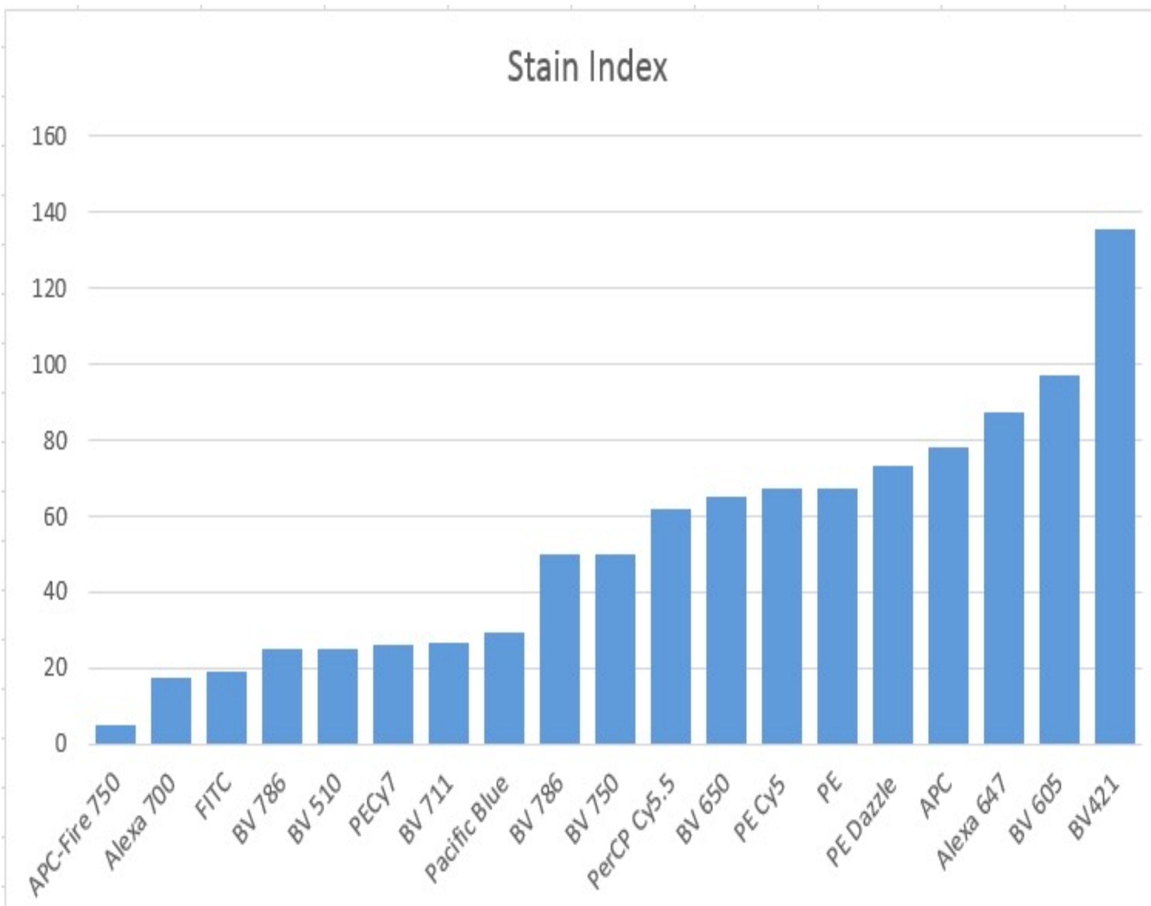
Comparative Stain index across detectors

To compare detector sensitivity for different fluorochromes on your instrument:

Run single color controls stained with the same antibody/clone
Coupled to appropriate fluorochrome for each detector
At each detector's optimized voltage/gain setting
Calculate Stain Index



Comparative Stain Index



ZE5 data courtesy of Karen Helm, University of Colorado

Titration

can affect your stain index and your sensitivity

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

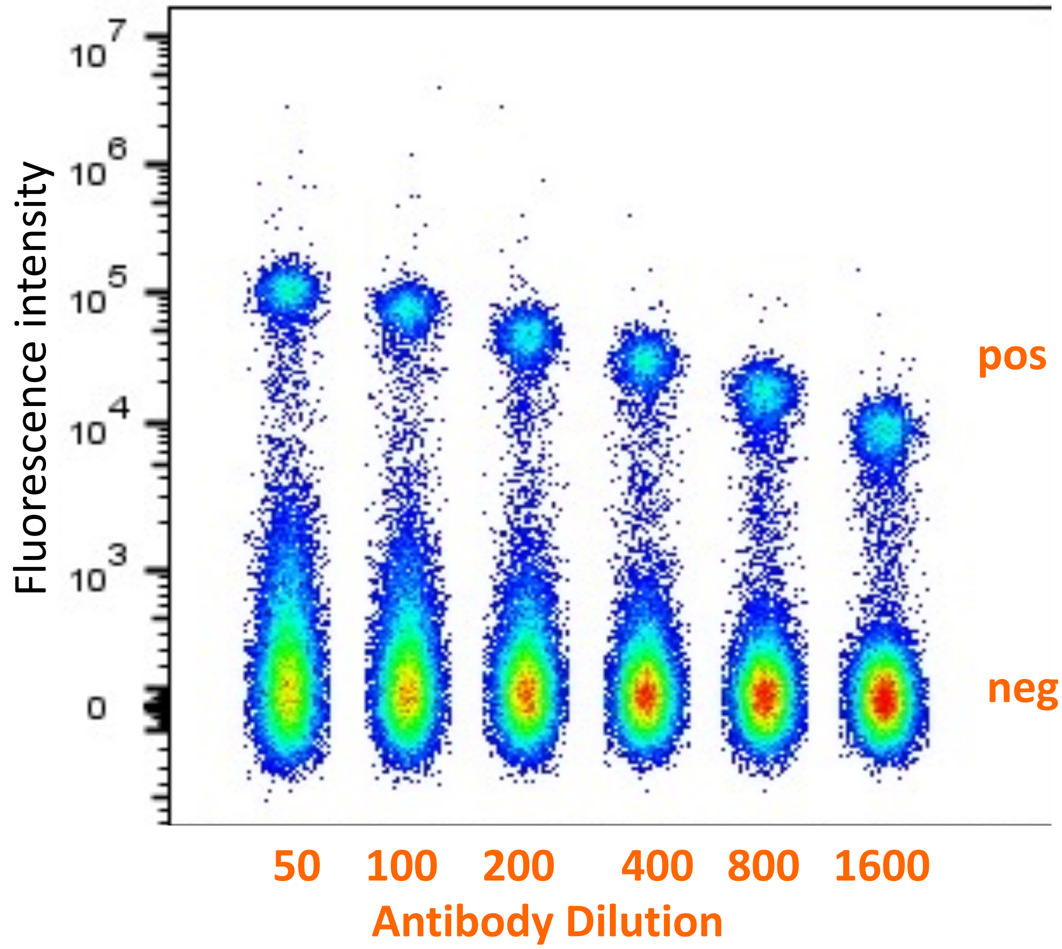
      i.e. same fixation and permeabilisation

   must have cells that are antigen positive and negative

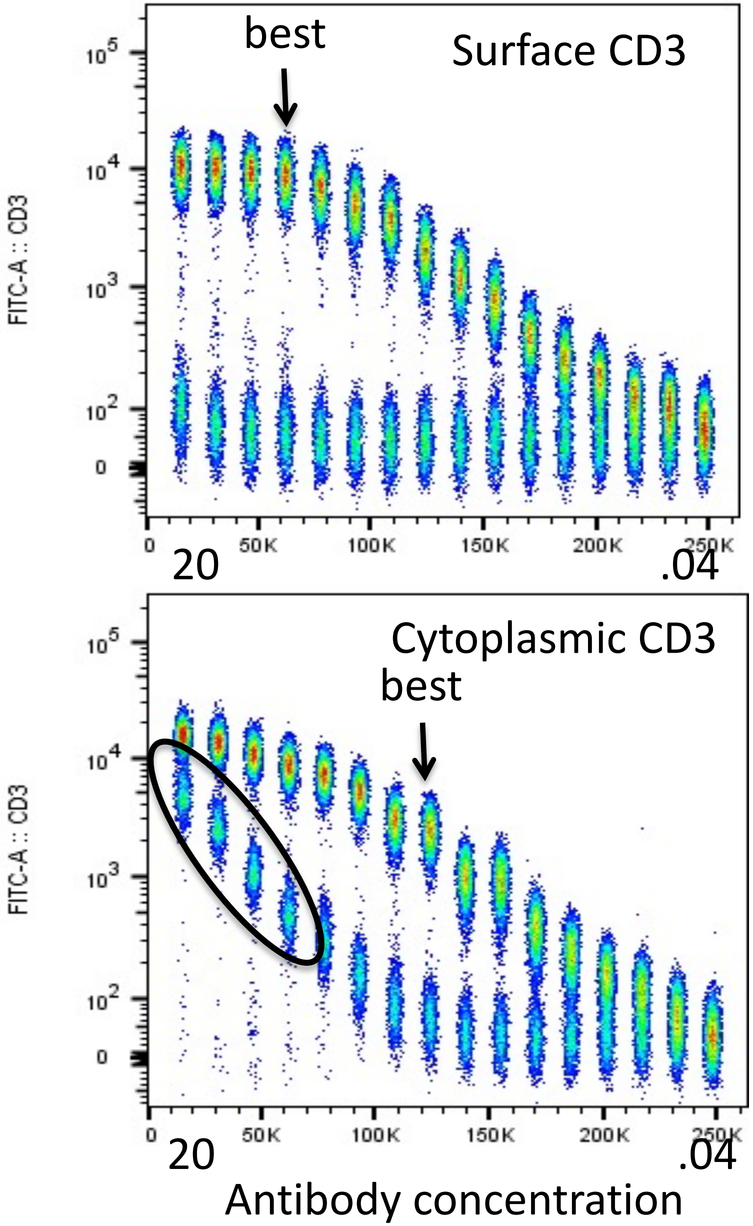
3. Wash and run on cytometer

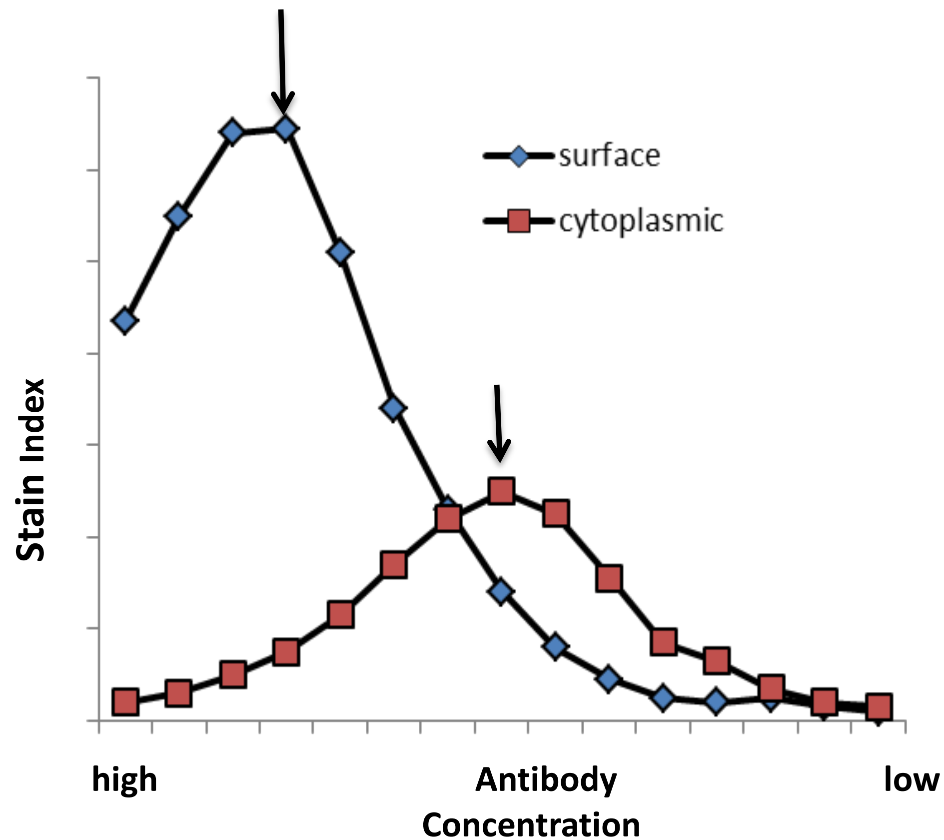
4. Calculate stain index for each dilution

Titration


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

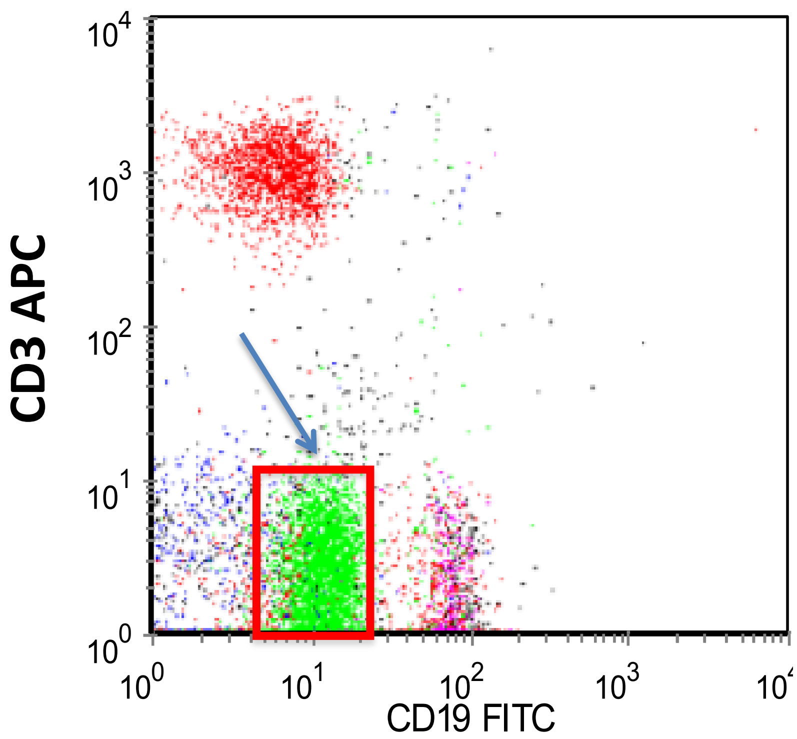
Titration CD3 surface vs CD3 cytoplasmic





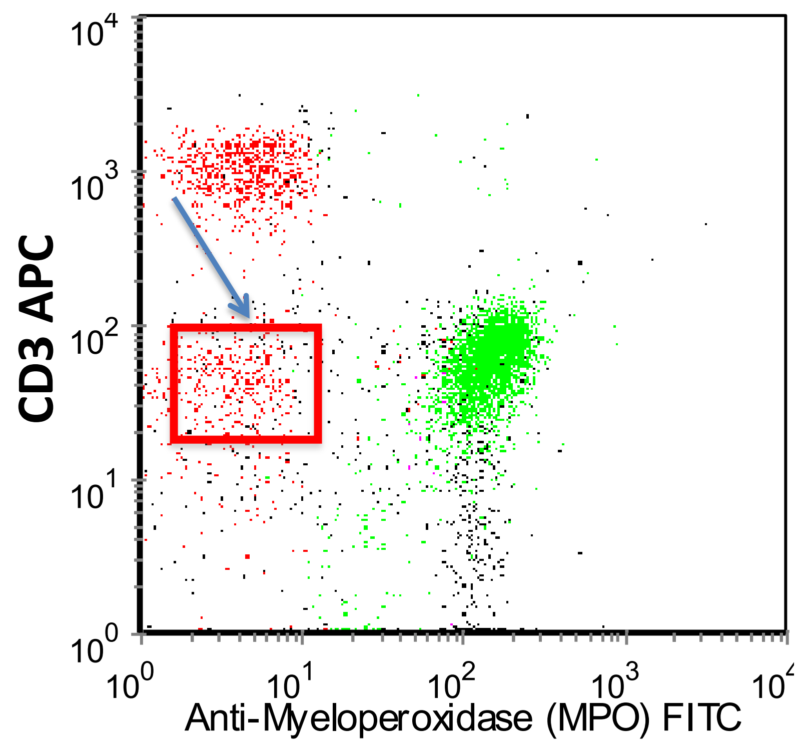
Increased Background in Fixed Cells

Surface CD3 APC (3ul): low background



Cytoplasmic CD3 APC (3ul): high background

due to sample processing

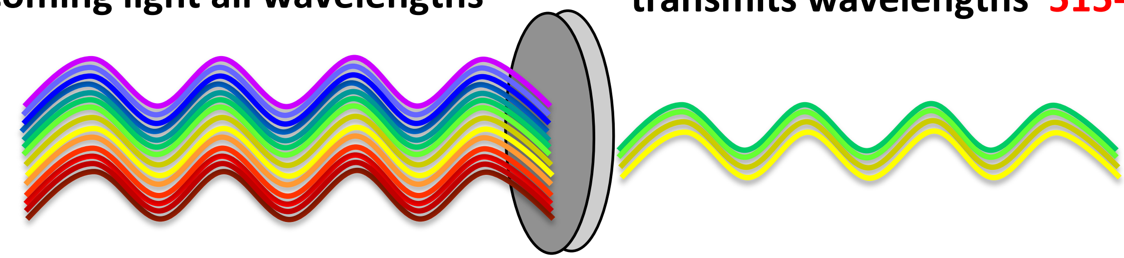


Spectral Overlap, Spillover and Compensation

Optical Filters: Band Pass

Band Pass Filter BP530/30

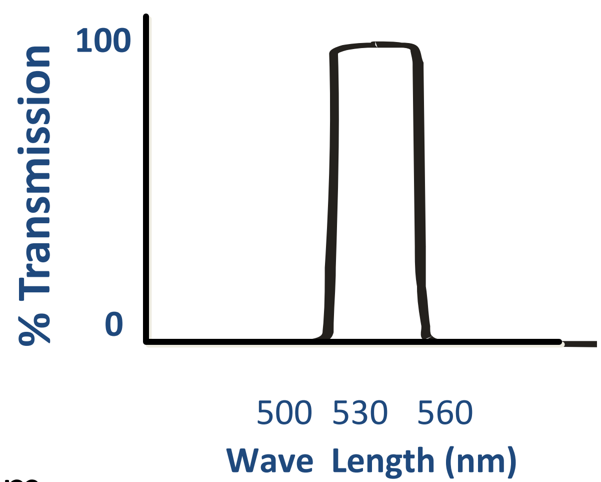
incoming light all wavelengths **transmits wavelengths** **515-545nm**



Bandpass → BP 530/30 nm

The first number refers to the center wavelength of the filter.

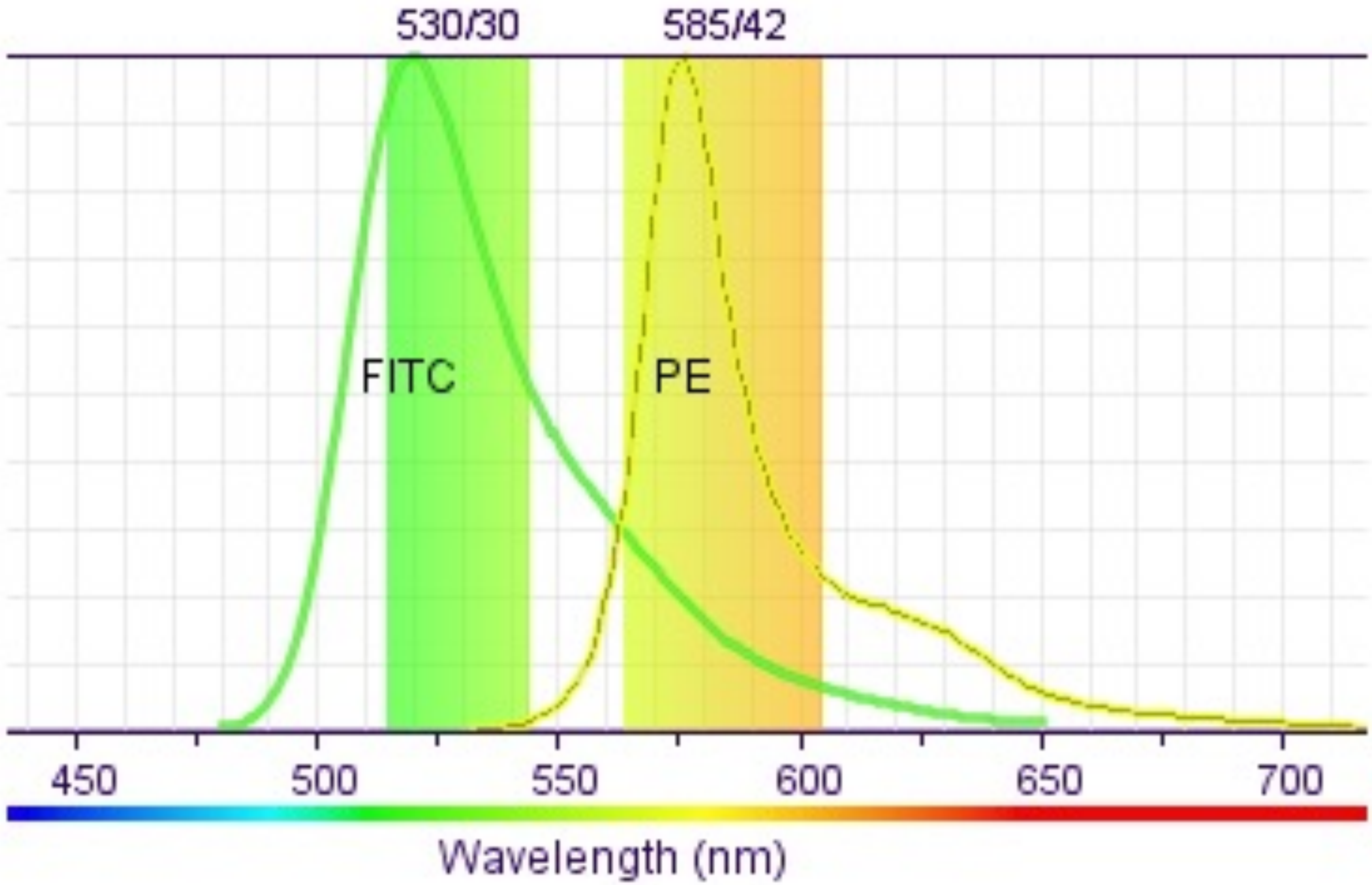
The second number refers to the size of the filter window.



This means it transmits 530+/-15 or 515-545 nm

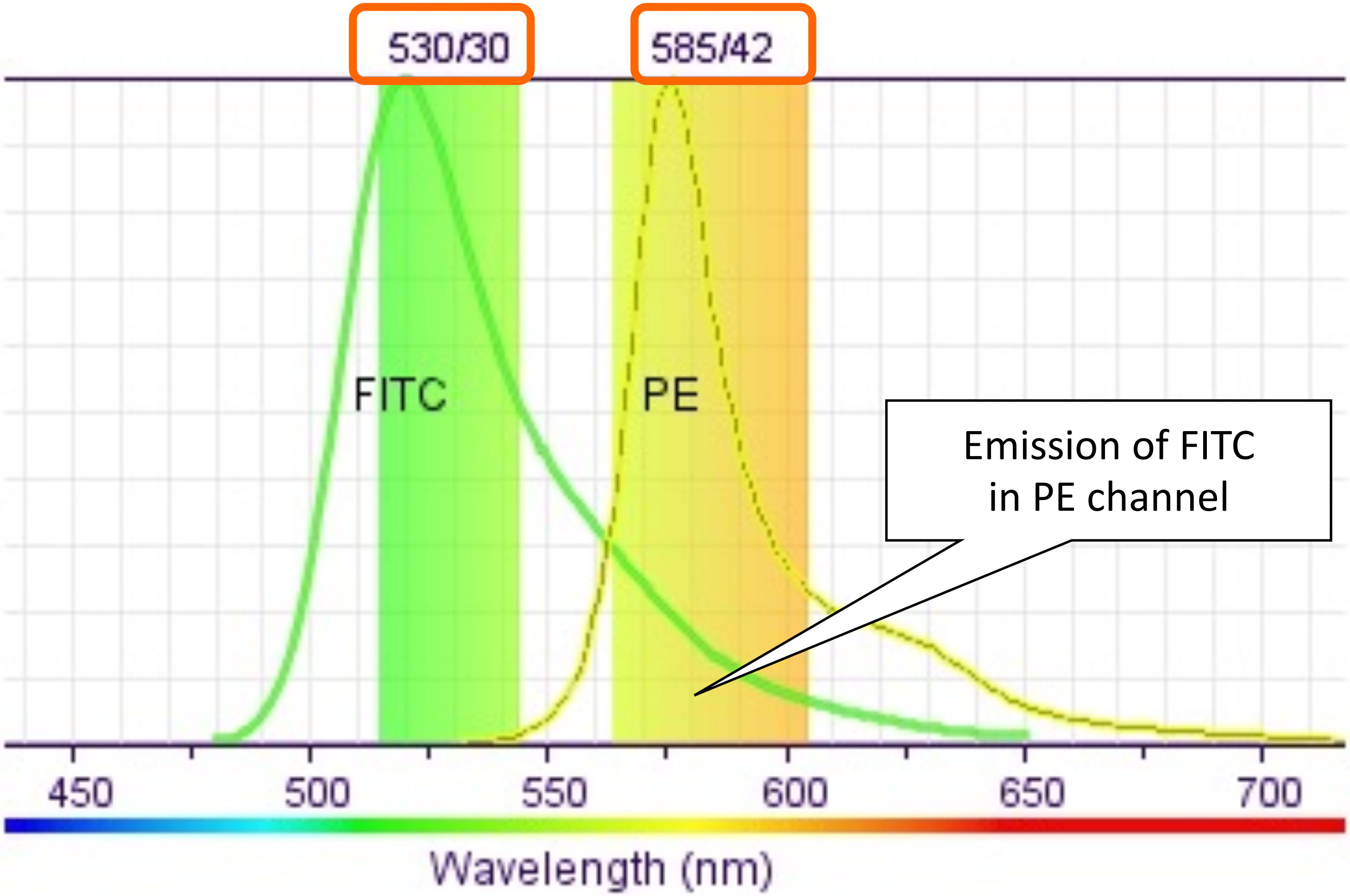
Fluorochrome Emission Range

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



Fluorescence Spillover

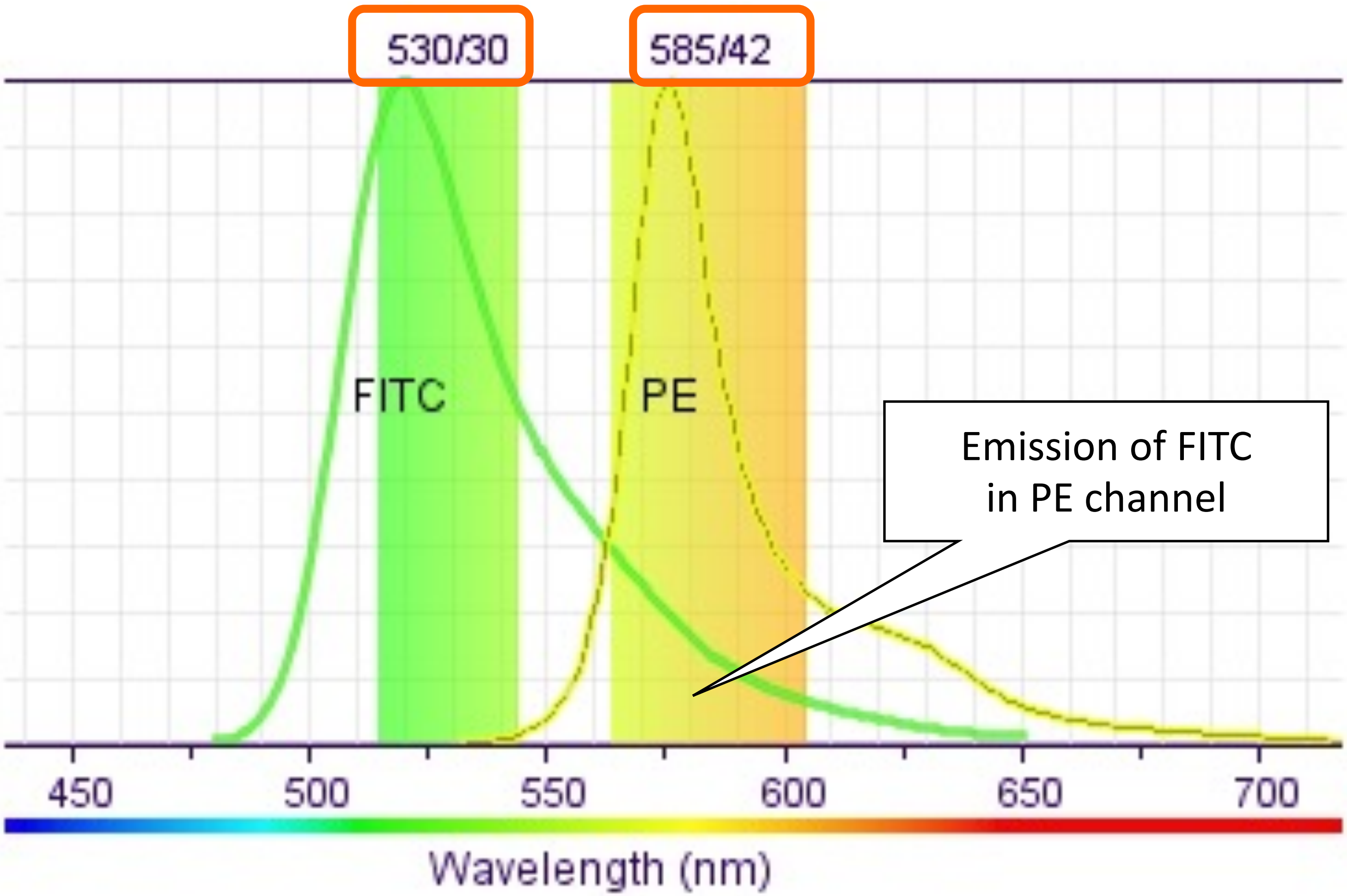
These are the optical filters in which each fluorochrome is detected: the longer wavelength emission from FITC is seen in the PE detector



Emission

Fluorescence Spillover

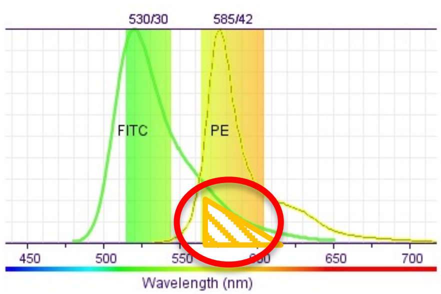
Thus part of the emission measured in the PE detector is due to FITC emission
But we need to measure each fluorochrome separately



Emission

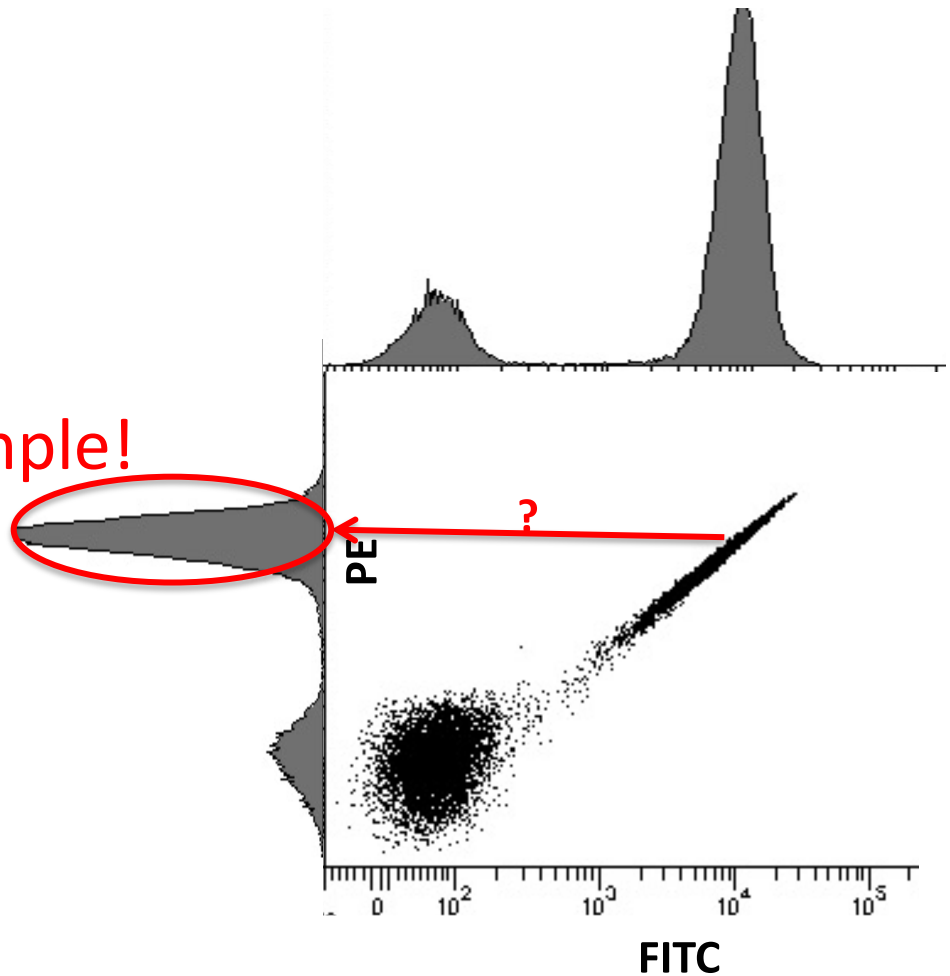
The Spillover Effect: FITC into PE

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?

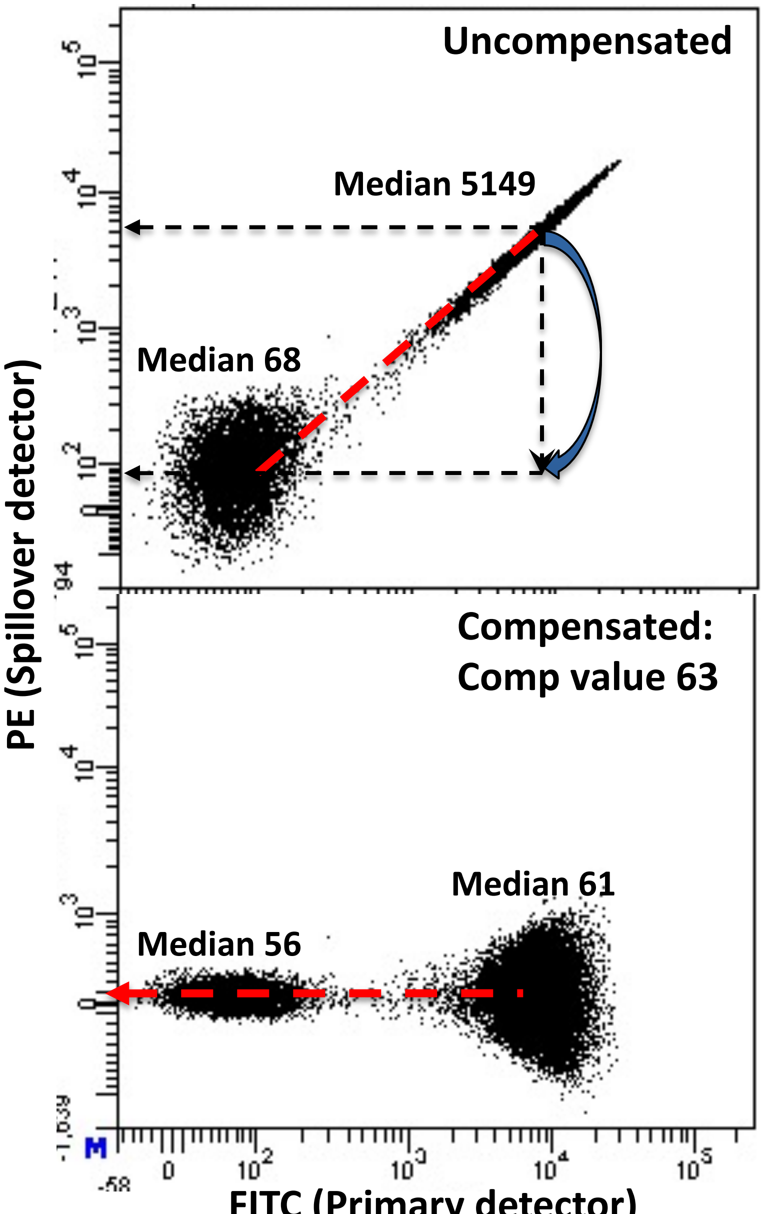
We compensate!

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

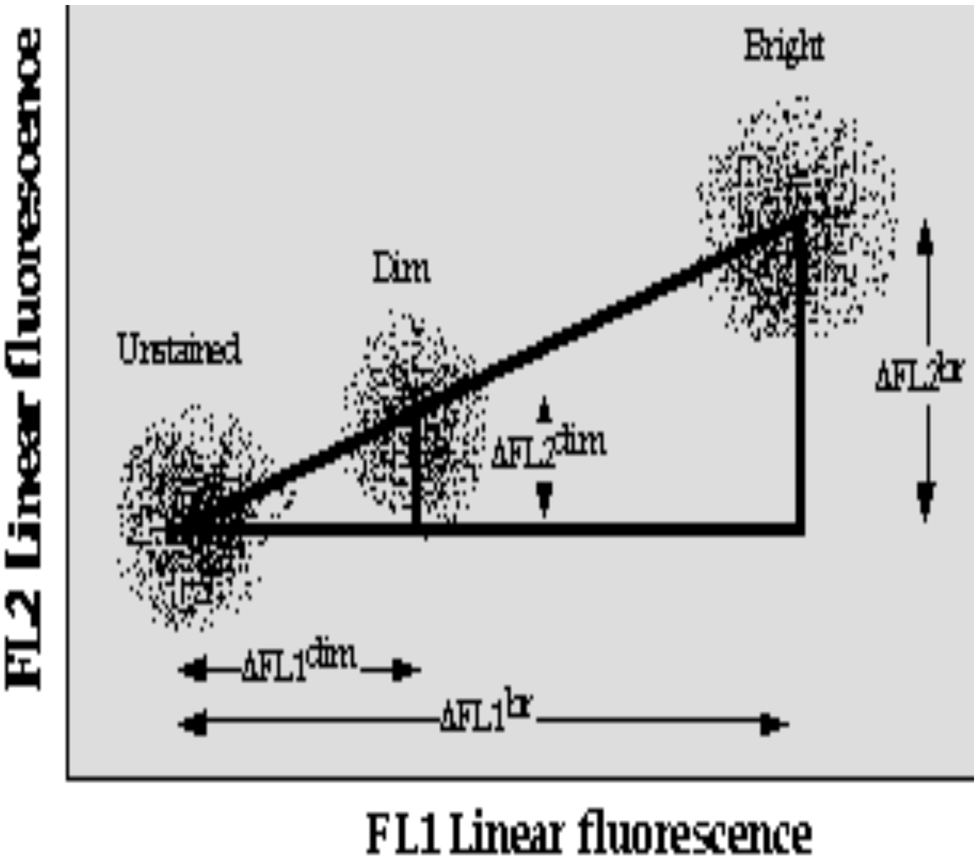
Spillover is corrected by Compensation



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

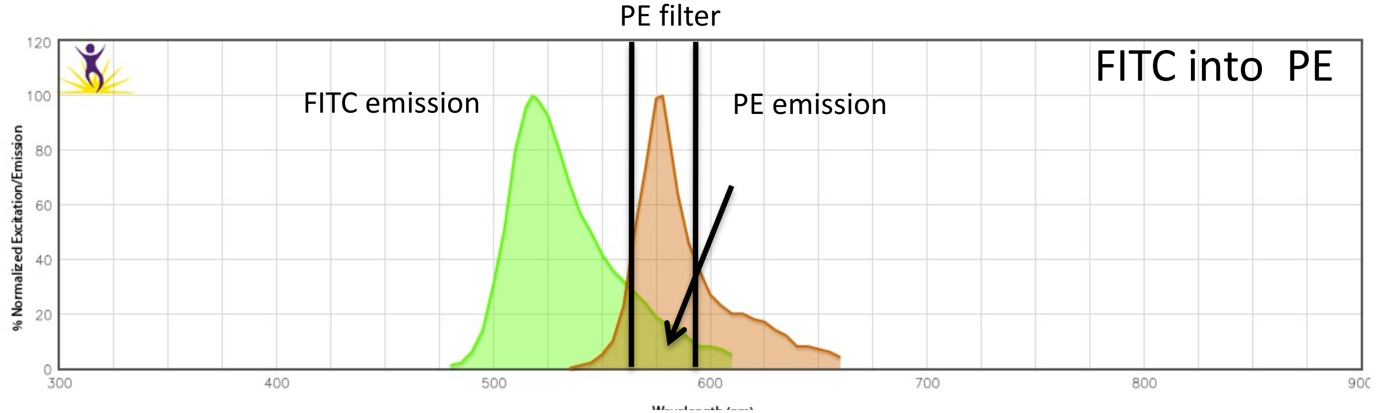
Compensation is not a simple percentage:

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

Three Sources of Spillover

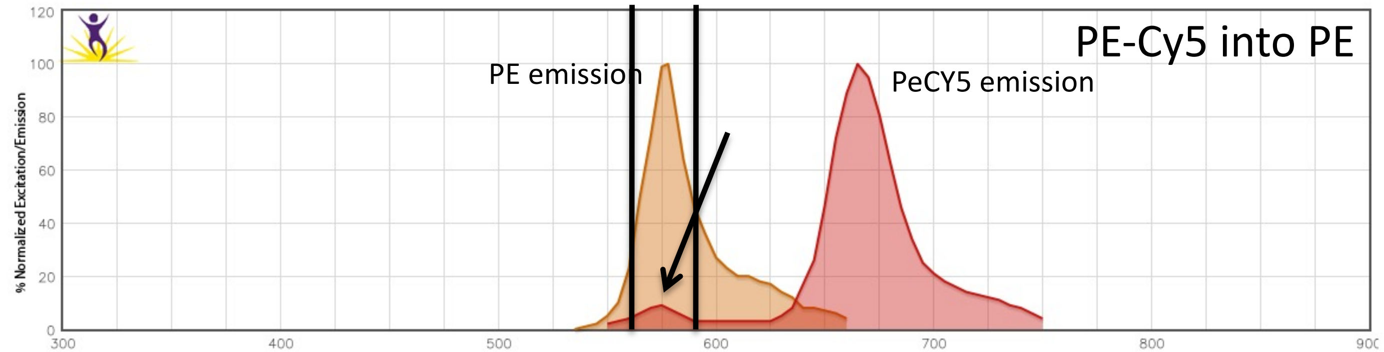
1

Adjacent spectra



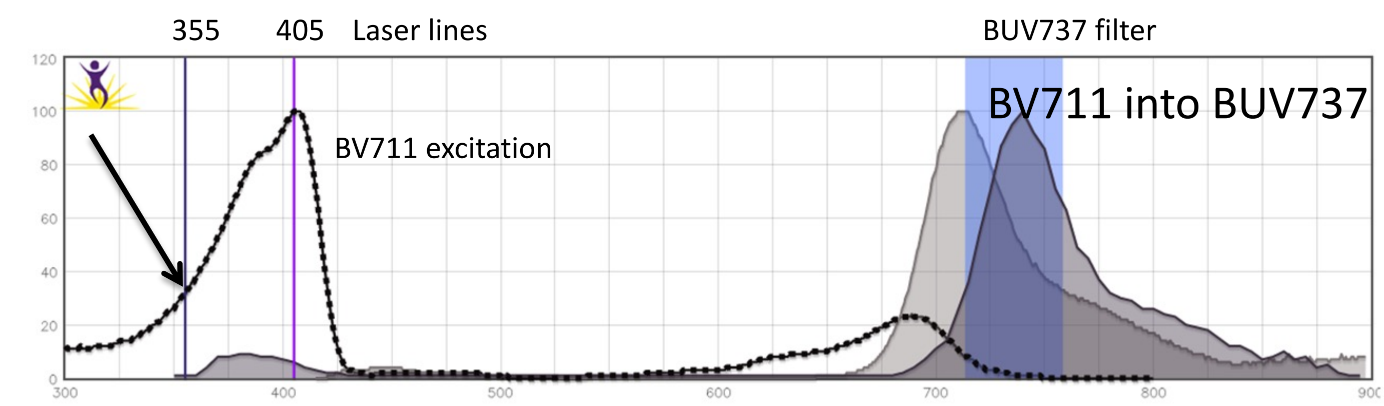
2

Tandems and their bases

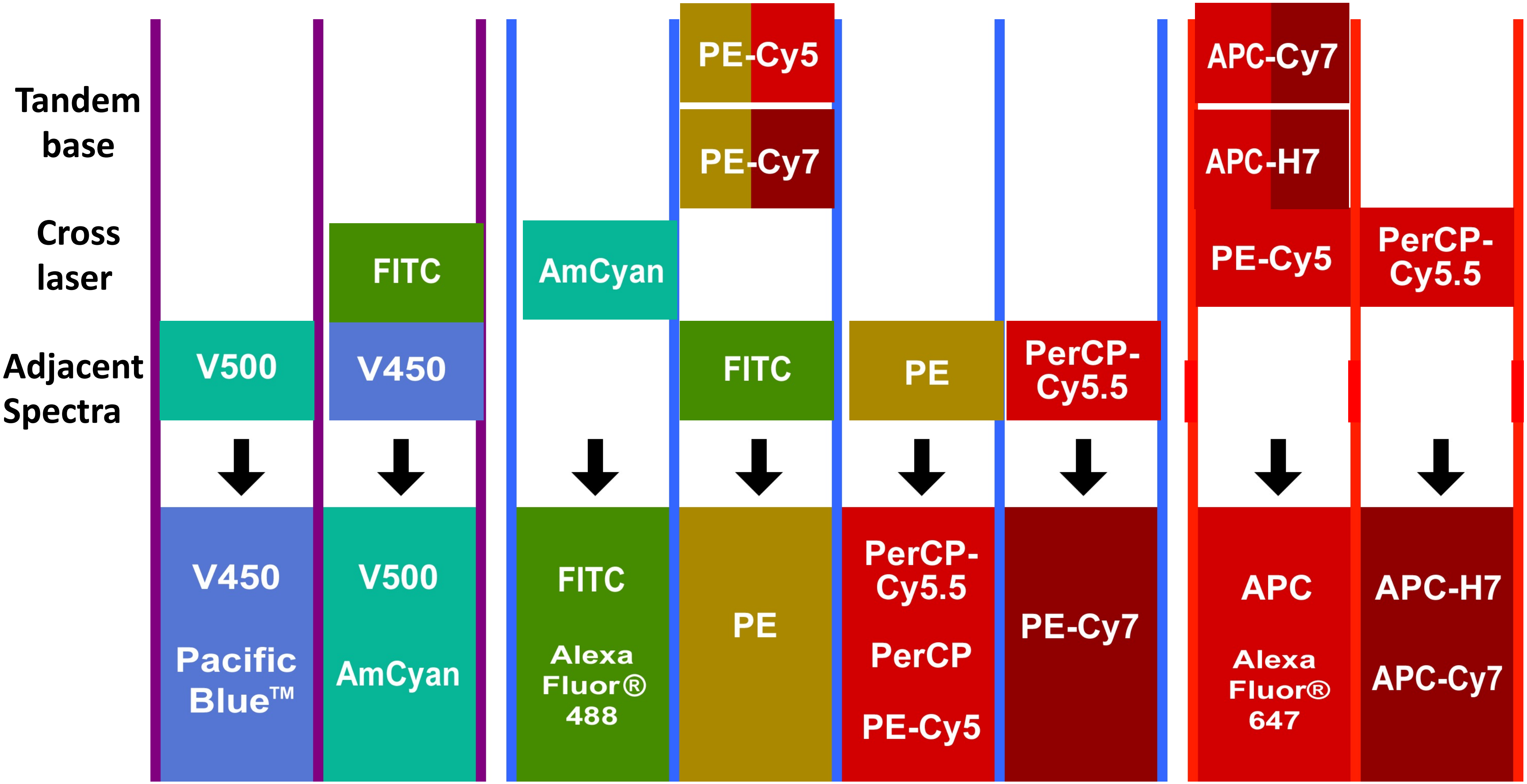


3

Cross laser excitation

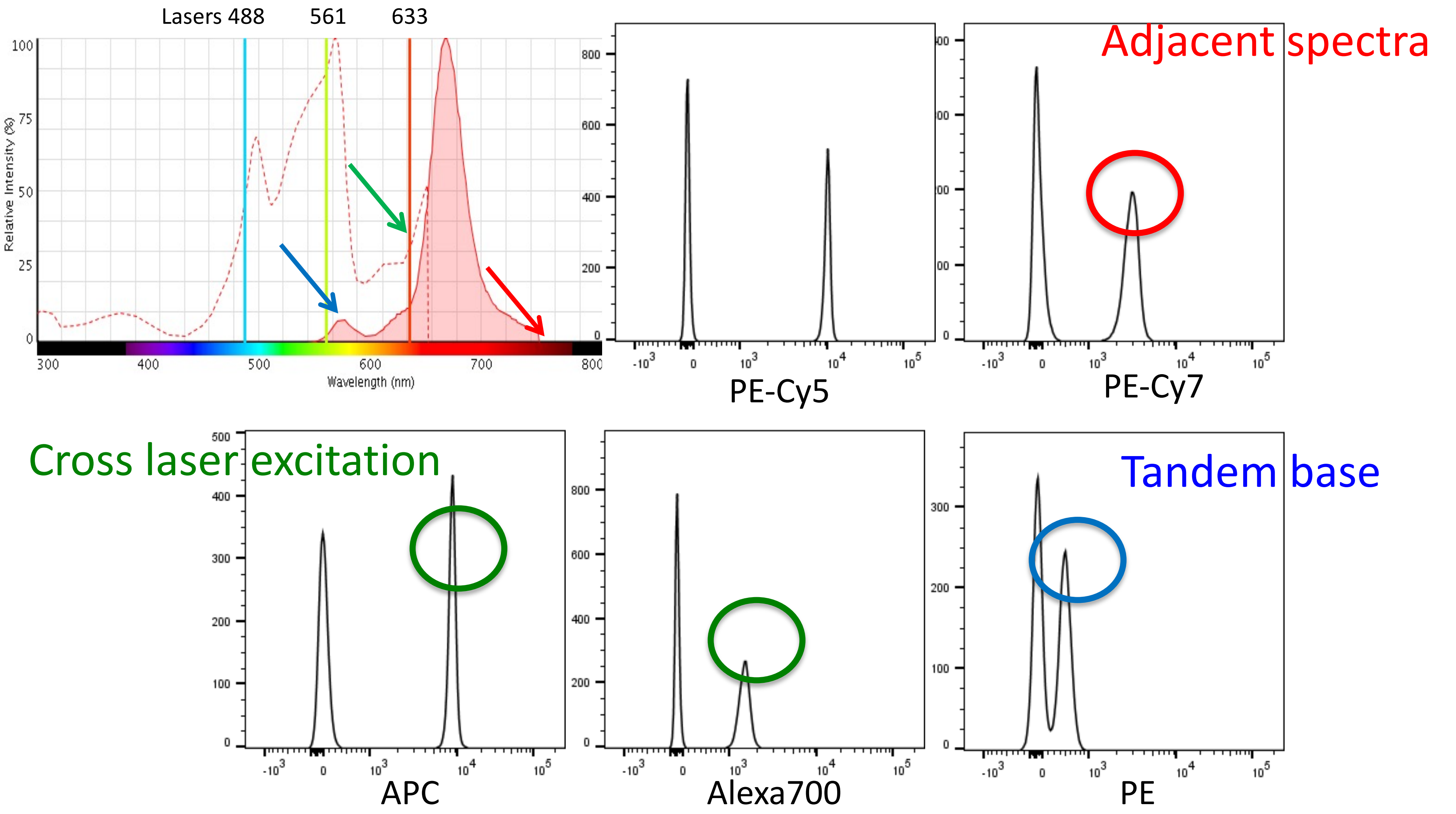


Who spills into what?



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



Summary

- Know your cytometer, know what lasers and filters you have available. Filters on many cytometers can be easily swapped out if needed

- Characterize your instrument. Determine the best instrument detector settings to maximise sensitivity. Set up a template for data acquisition with these settings.

- Titrate your antibodies! Also every time you get a new antibody lot!

- Choose your fluorochromes carefully for excitation, emission and brightness. More on that in panel design!

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

THANK YOU

धन्यवाद