Fluorescence Compensation and Panel Design

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Overview

- What is compensation?

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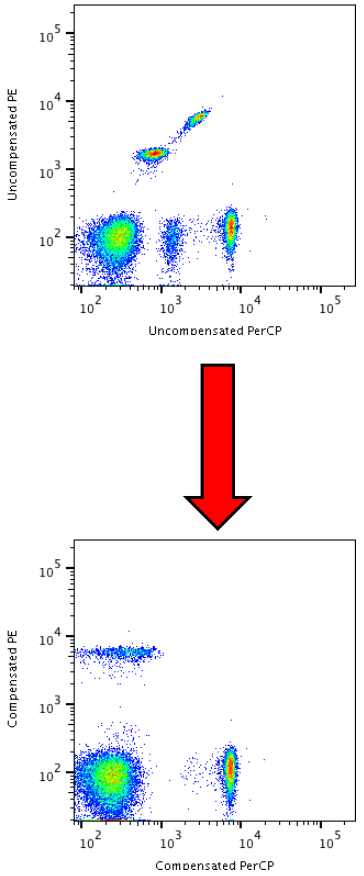
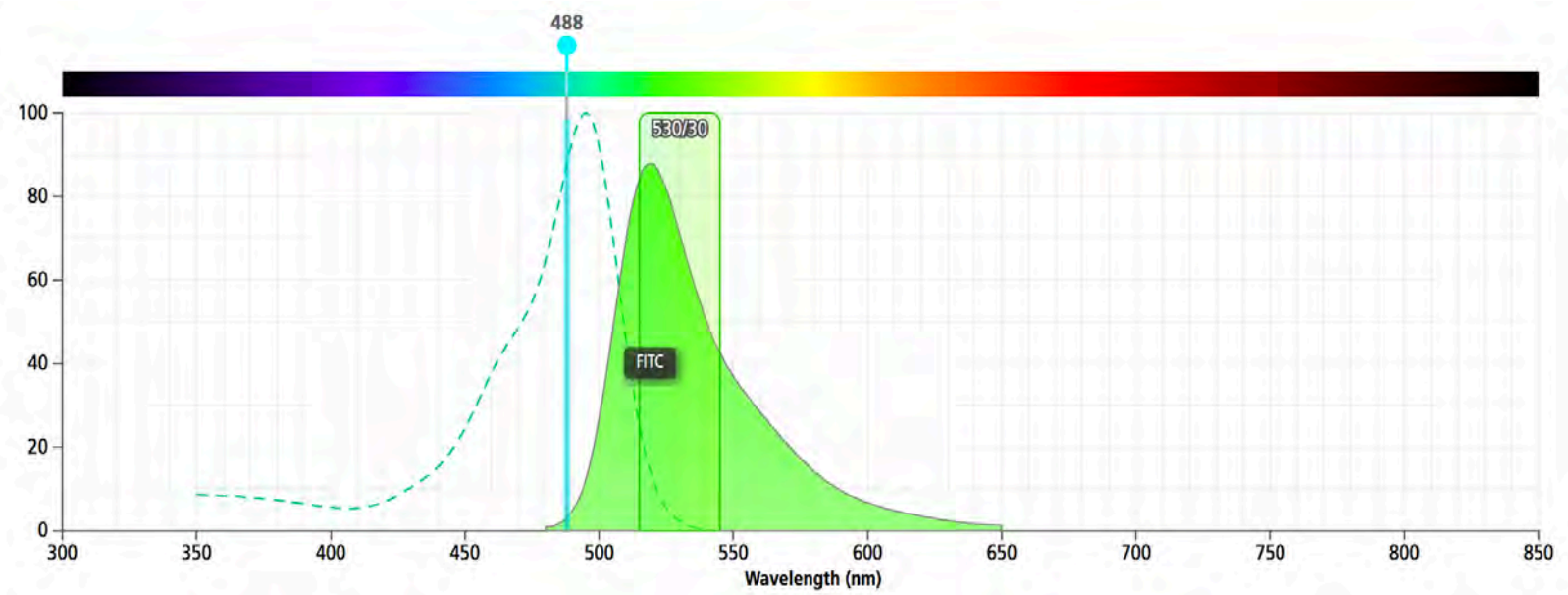
Overview

- What is compensation?
- Why do we need it?
- How is it accomplished?
  - Theory
  - Rules
  - Practice
- A brief overview of panel design
  - Fluorochromes
  - Cytometer
  - Antigens



Fluorescence: FITC

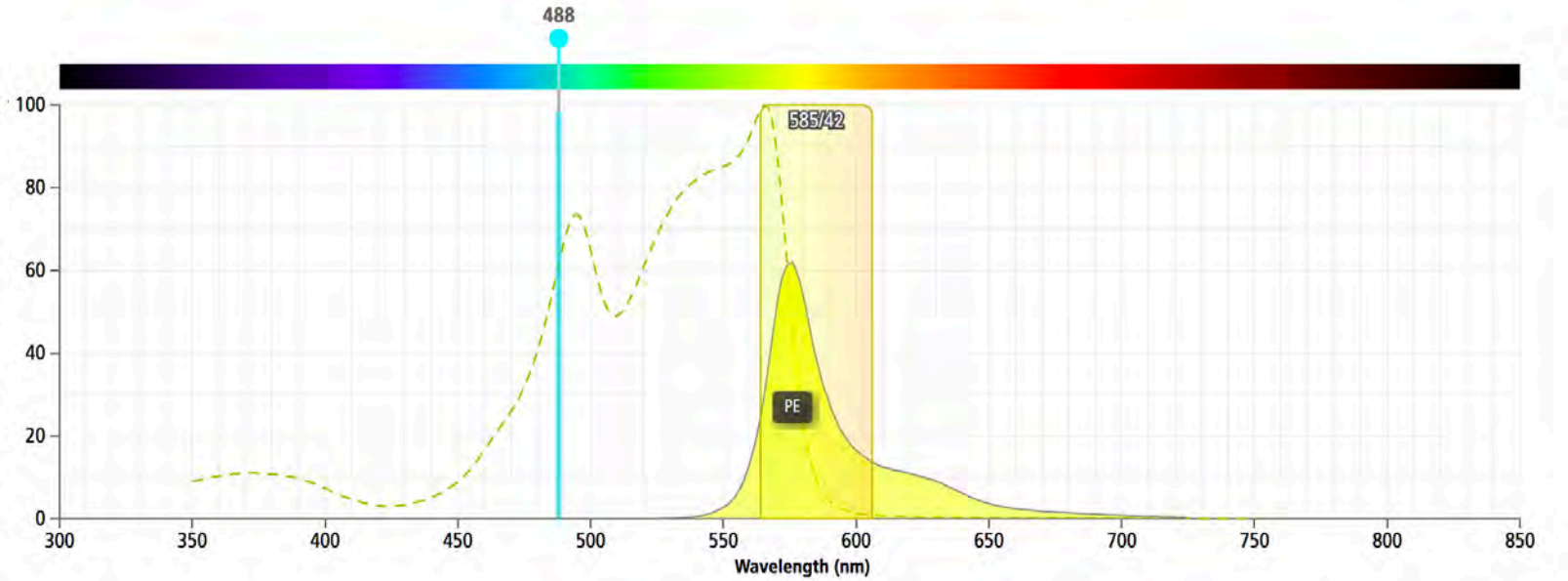
But remember this?



Emission is a SPECTRUM. We choose to measure a part of it.

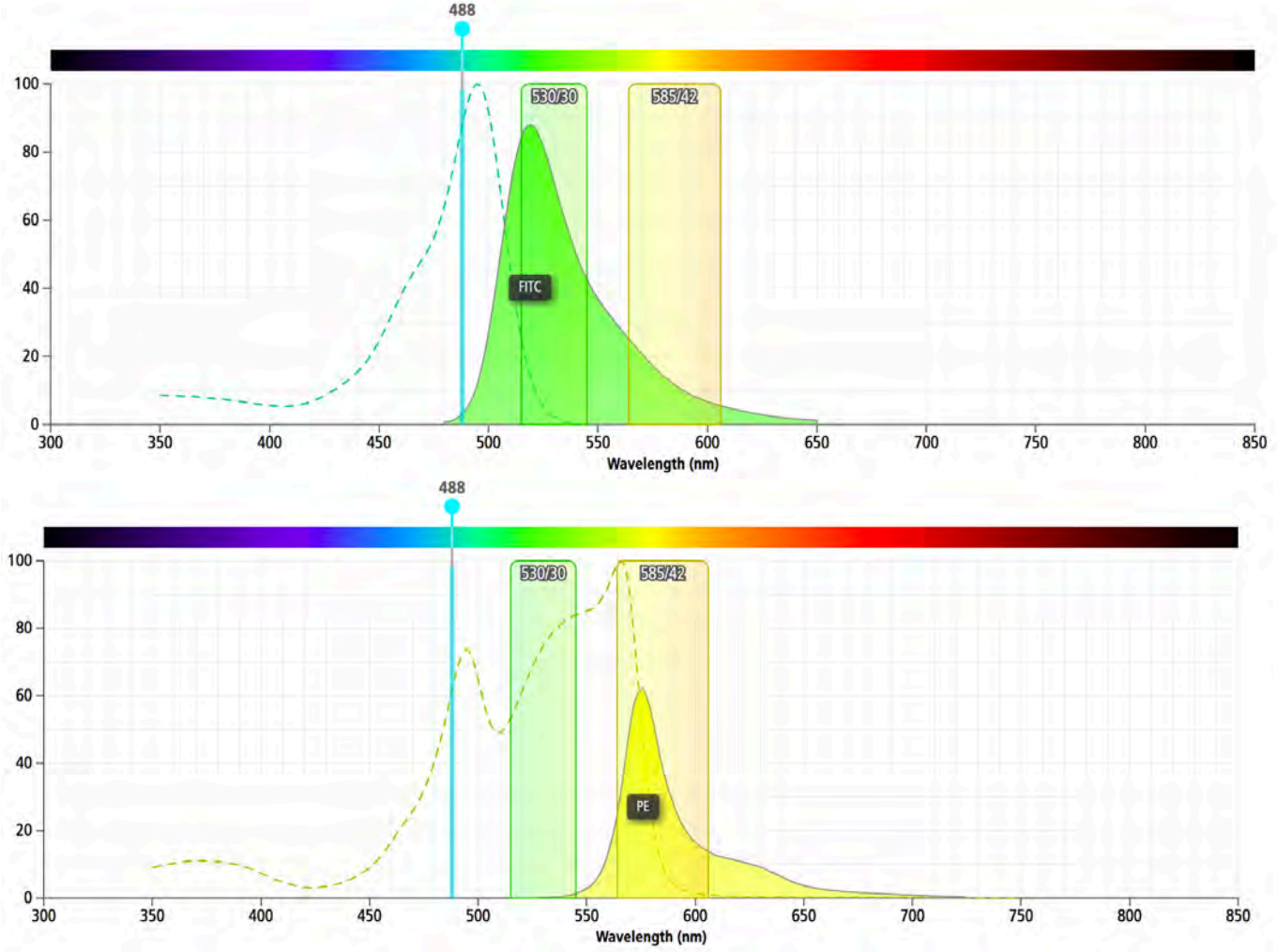
Fluorescence: PE

But remember this?

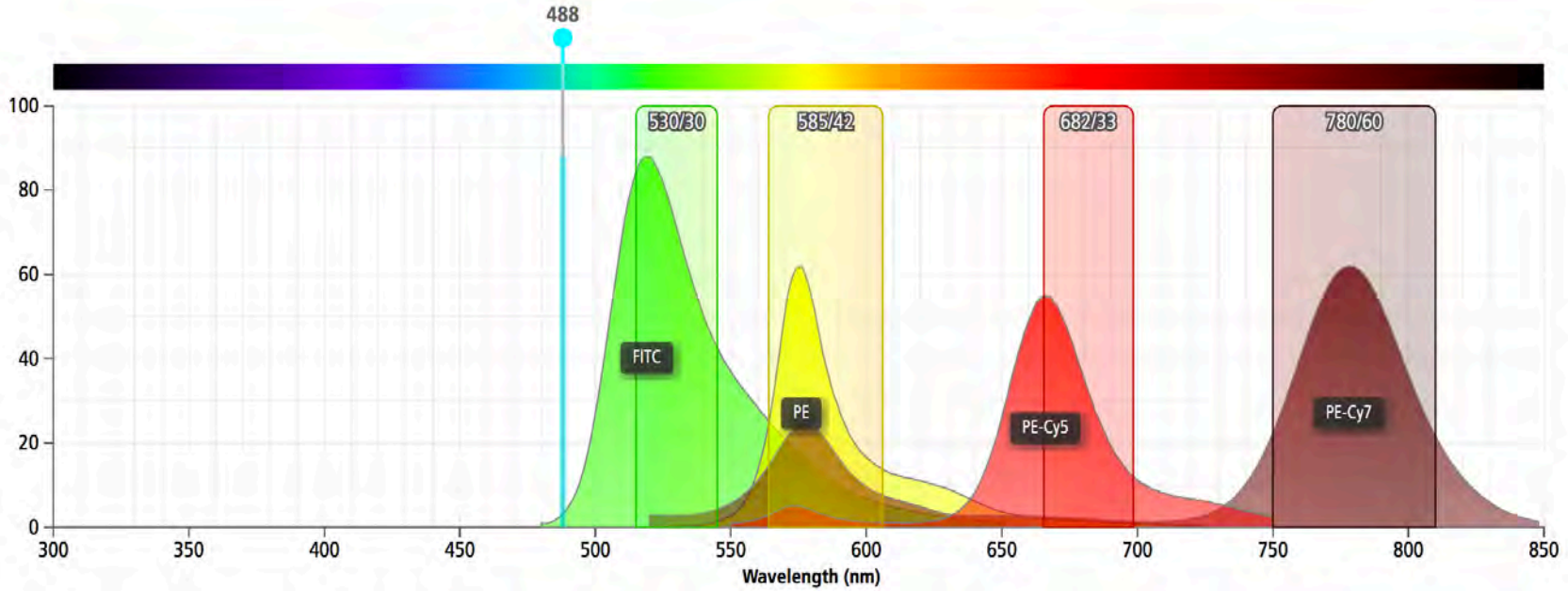


Emission is a SPECTRUM. We choose to measure a part of it.

Fluorescence: FITC and PE

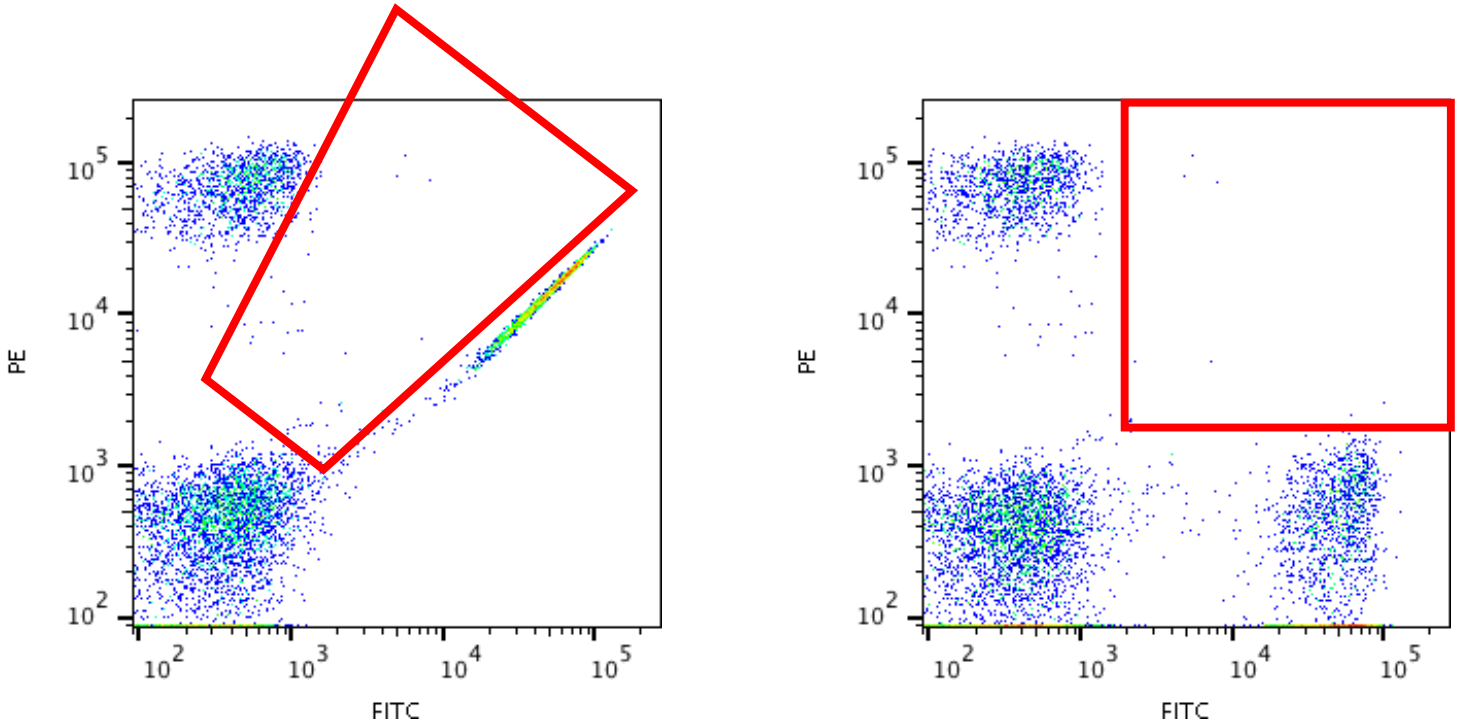


Fluorescence: Overlap



Each detection channel receives photons from the specific fluorochrome but also autofluorescence, and unwanted photons from other fluorochromes.

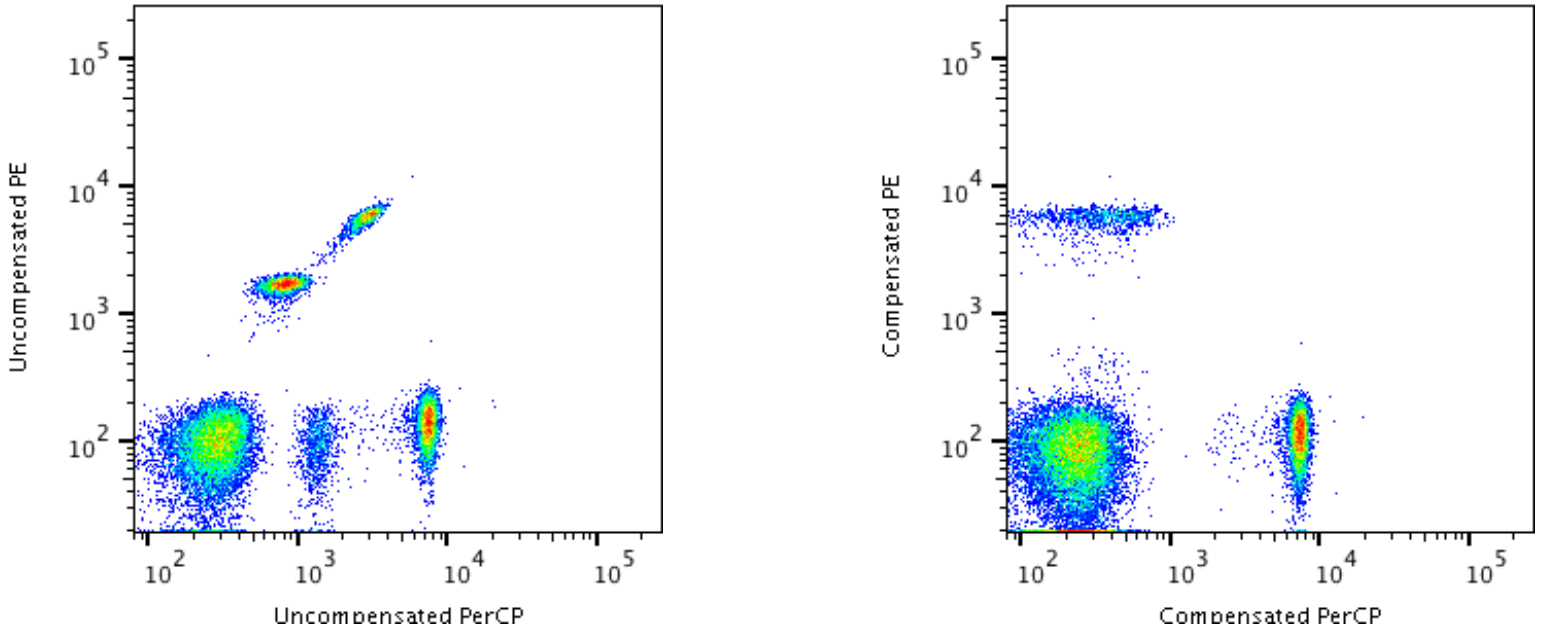
Uncompensated v compensated



Negative, FITC and PE only

Uncompensated v compensated

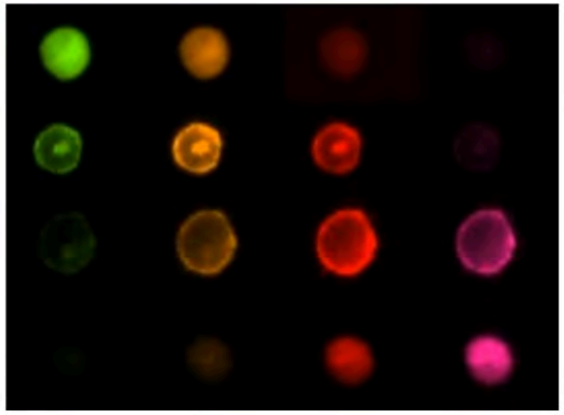
However, to determine what is truly positive and what is negative, compensation is required.

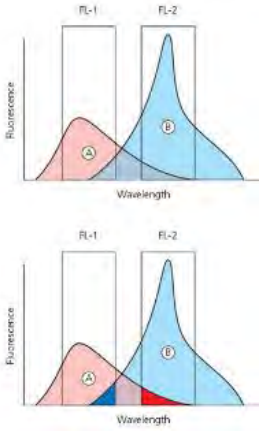


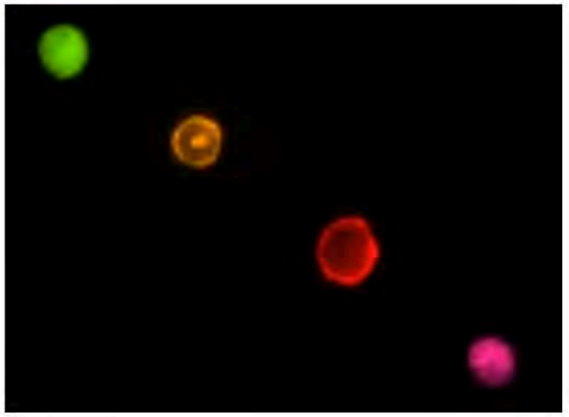
Negative, FITC, PE, PerCP and APC

All fluorochromes overlap with all others

- Conceptually a very simple procedure
- Look at a control sample stained with a single fluorochrome
- See where that flourochromes photons are seen and account for them = COMPENSATION!

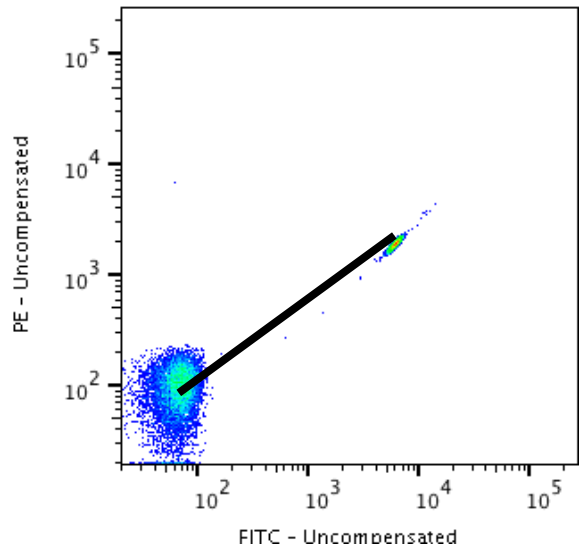


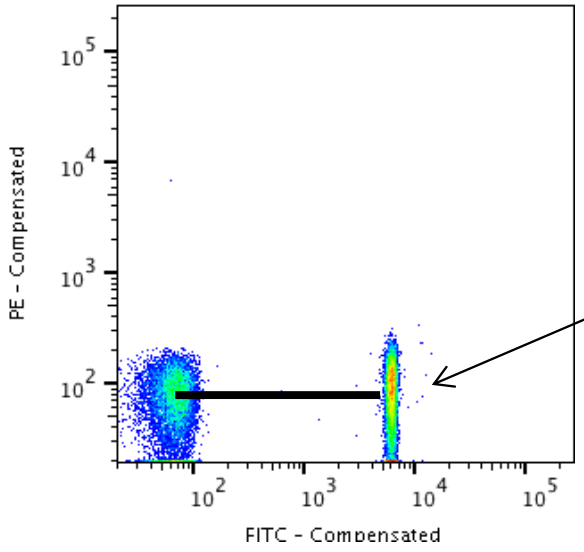




How do we compensate?

How do we know when compensation is correct?
What should be the final outcome?
The negatives and positives in the FITC axis should have the same intensity in the PE (or spillover) channel



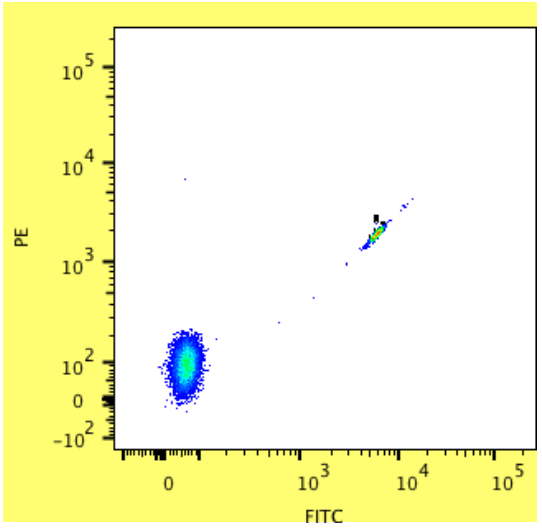


Medians of populations

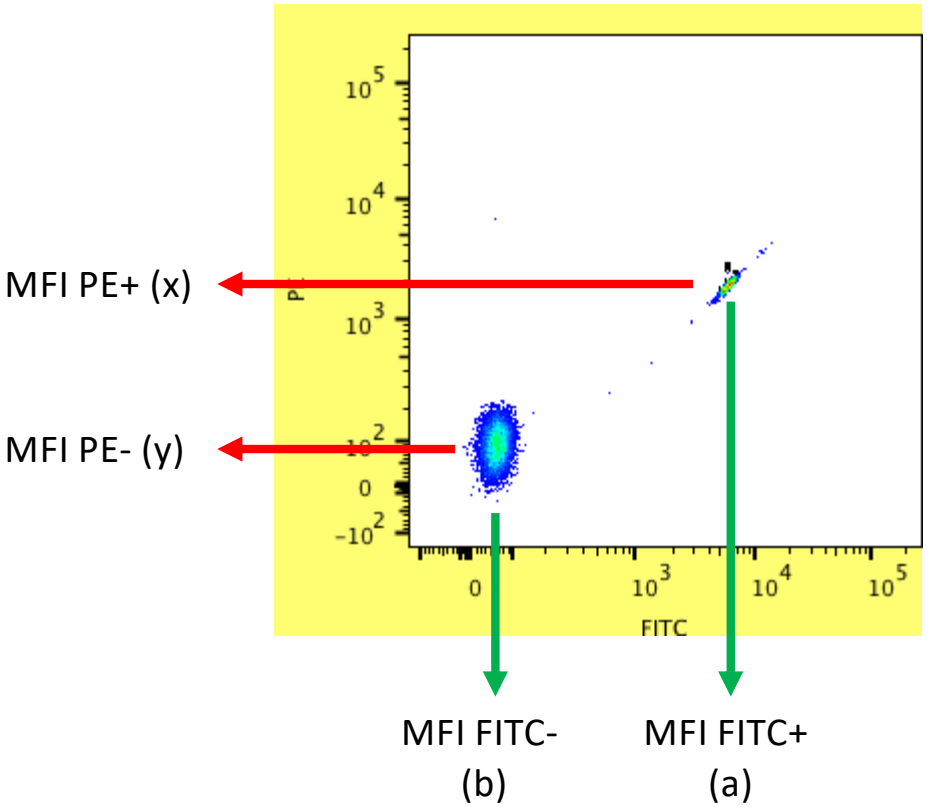
How do we compensate?

Compensation is expressed as a percentage.

We work out the ratio of staining in the spillover channel related to the true signal. Once we have that % (x), for every PE signal we reduce it by x% of the FITC signal.



How do we compensate?

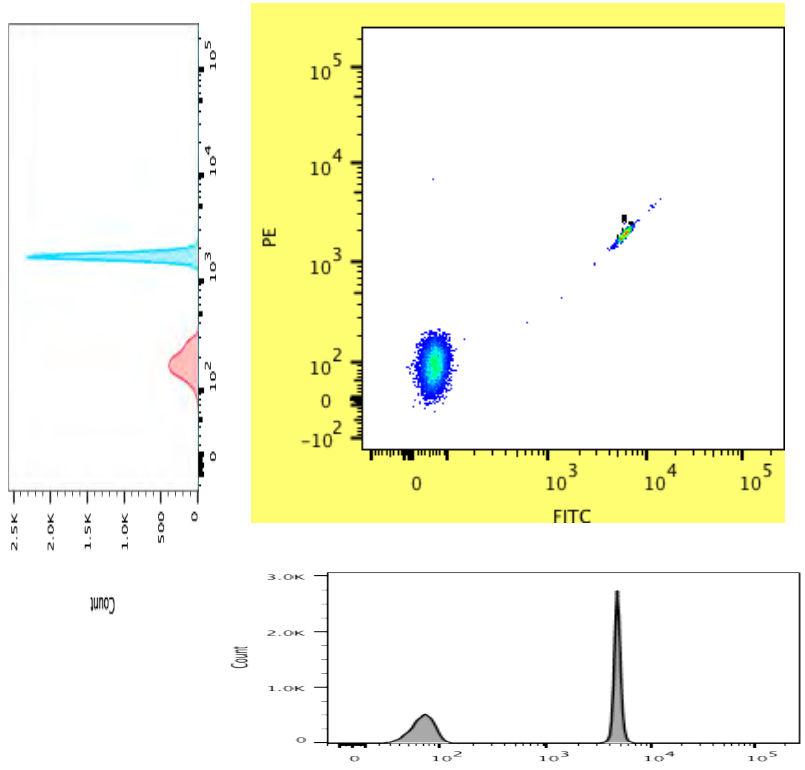


Compensation

$$\frac{x-y}{a-b} \times 100 = \%$$

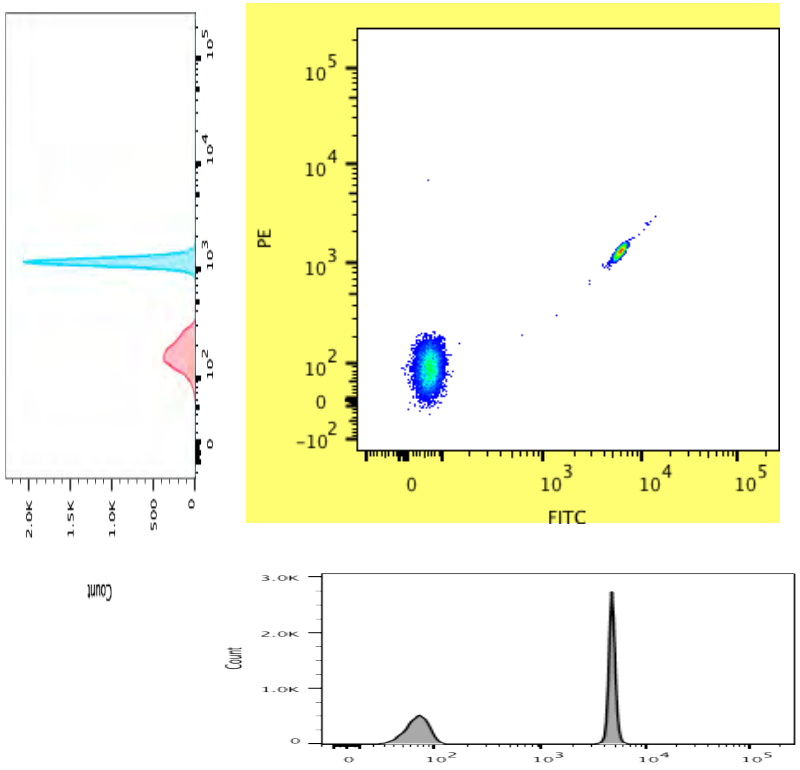
How do we compensate?

No compensation



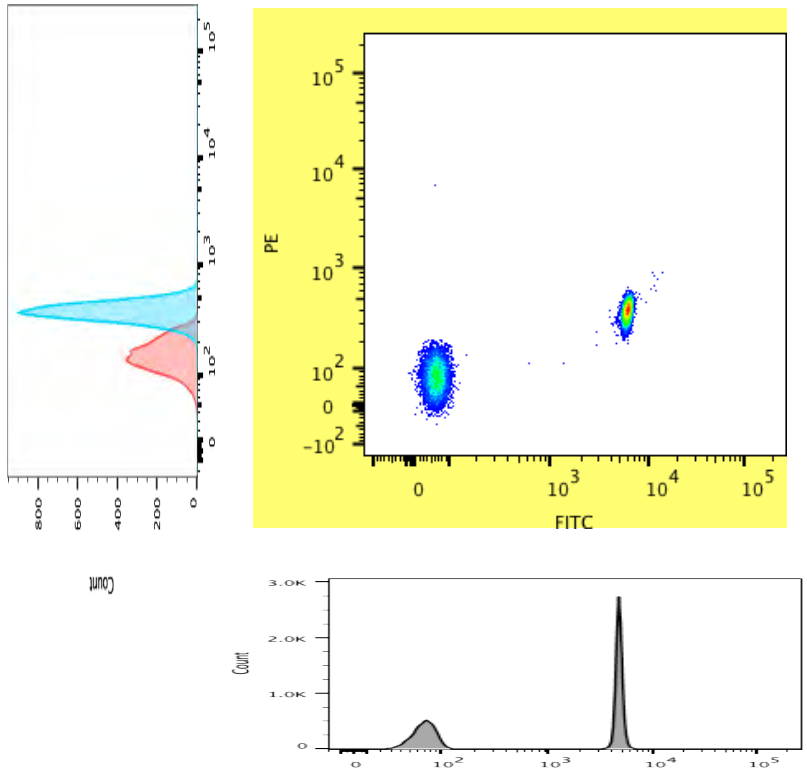
How do we compensate?

10% compensation



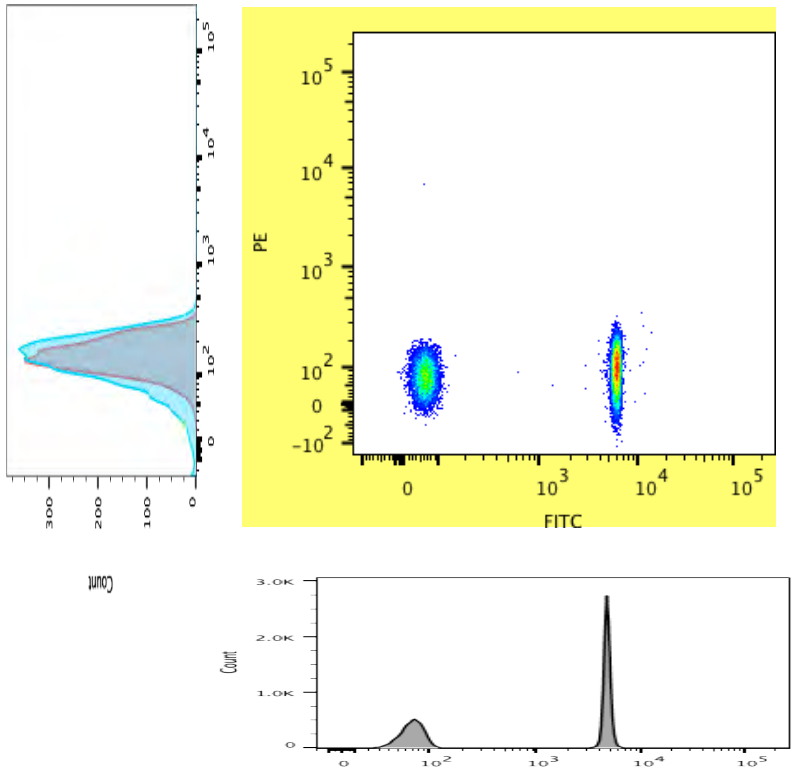
How do we compensate?

20% compensation



How do we compensate?

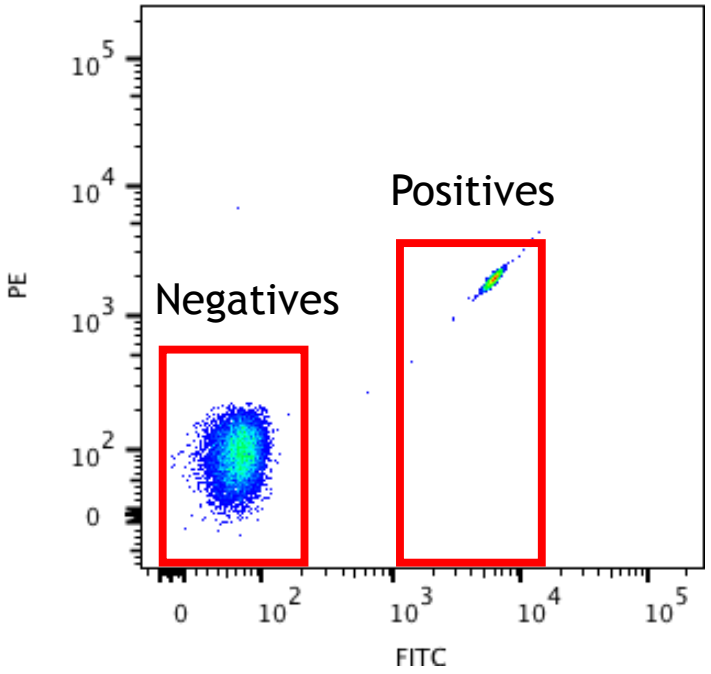
30% compensation



Fluorescence Compensation

How do we set the correct level of compensation?

By aligning the middle of the positive and negative populations in the spillover channel



[M] Edit Save Matrix SSM

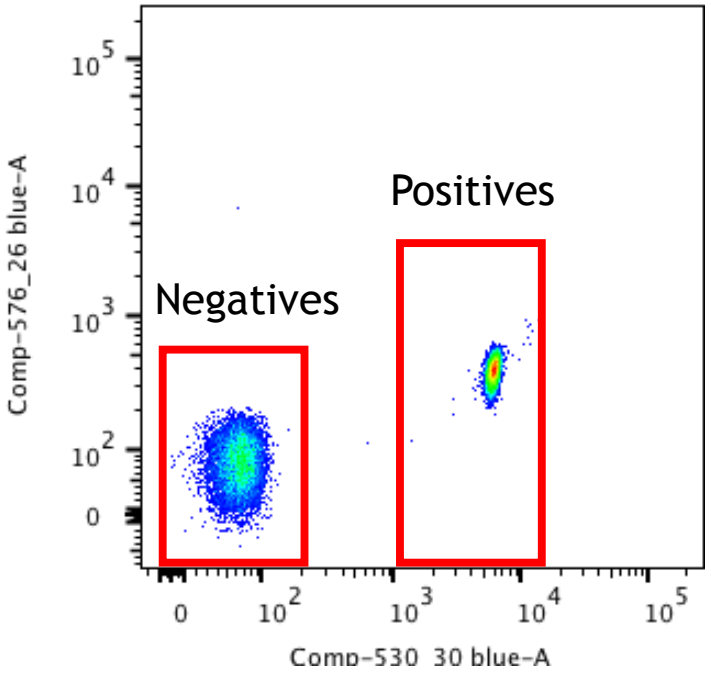
Show All	530_30 blue-A	576_26 blue-A	695_40 blue-A	670_14 red-A
✓ 530_30 blue-A	100	0	0	0
✓ 576_26 blue-A	0	100	0	0
✓ 695_40 blue-A	0	0	100	0
✓ 670_14 red-A	0	0	0	100

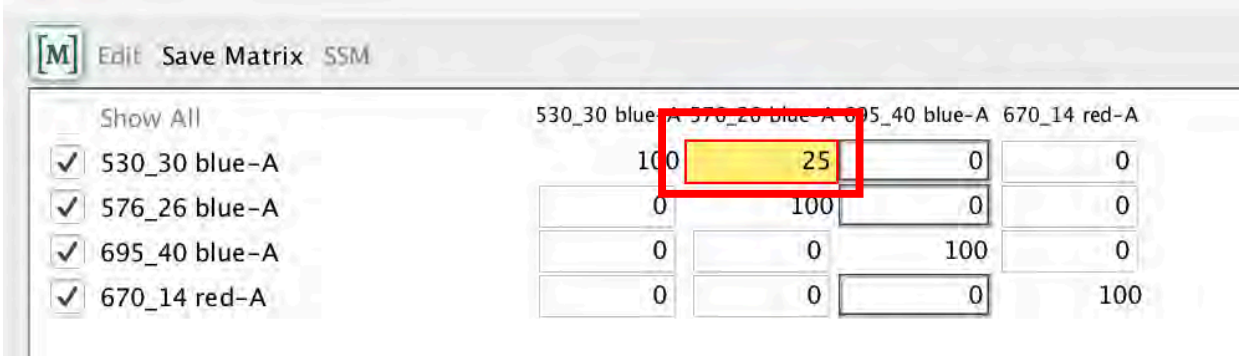
Name	Statistic
Specimen_001_FITC_002.fcs	
Beads	93.650
Negative	42.074
Σ Median : Comp-576_26 blue-A	93.793
Positive	57.885
Σ Median : Comp-576_26 blue-A	1877.8

Fluorescence Compensation

How do we set the correct level of compensation?

By aligning the middle of the positive and negative populations in the spillover channel



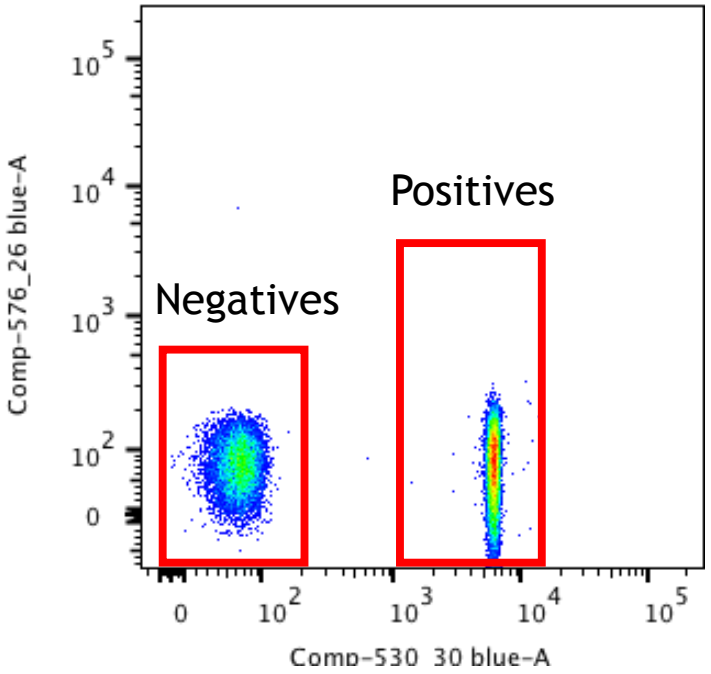


Name	Statistic
Specimen_001_FITC_002.fcs	
Beads	93.650
Negative	42.074
∑ Median : Comp-576_26 blue-A	77.508
Positive	57.885
∑ Median : Comp-576_26 blue-A	391.68

Fluorescence Compensation

How do we set the correct level of compensation?

By aligning the middle of the positive and negative populations in the spillover channel



Edit Save Matrix SSM

Show All

	530_30 blue-A	576_26 blue-A	695_40 blue-A	670_14 red-A
✓ 530_30 blue-A	100	30.4	0	0
✓ 576_26 blue-A	0	100	0	0
✓ 695_40 blue-A	0	0	100	0
✓ 670_14 red-A	0	0	0	100

Name	Statistic
Specimen_001_FITC_002.fcs	
Beads	93.650
Negative	42.074
∑ Median : Comp-576_26 blue-A	74.004
Positive	57.885
∑ Median : Comp-576_26 blue-A	72.392

When do we compensate?

Compensation can be performed LIVE or OFFLINE

Compensation can be MANUAL or AUTOMATIC

Fluorescence Compensation

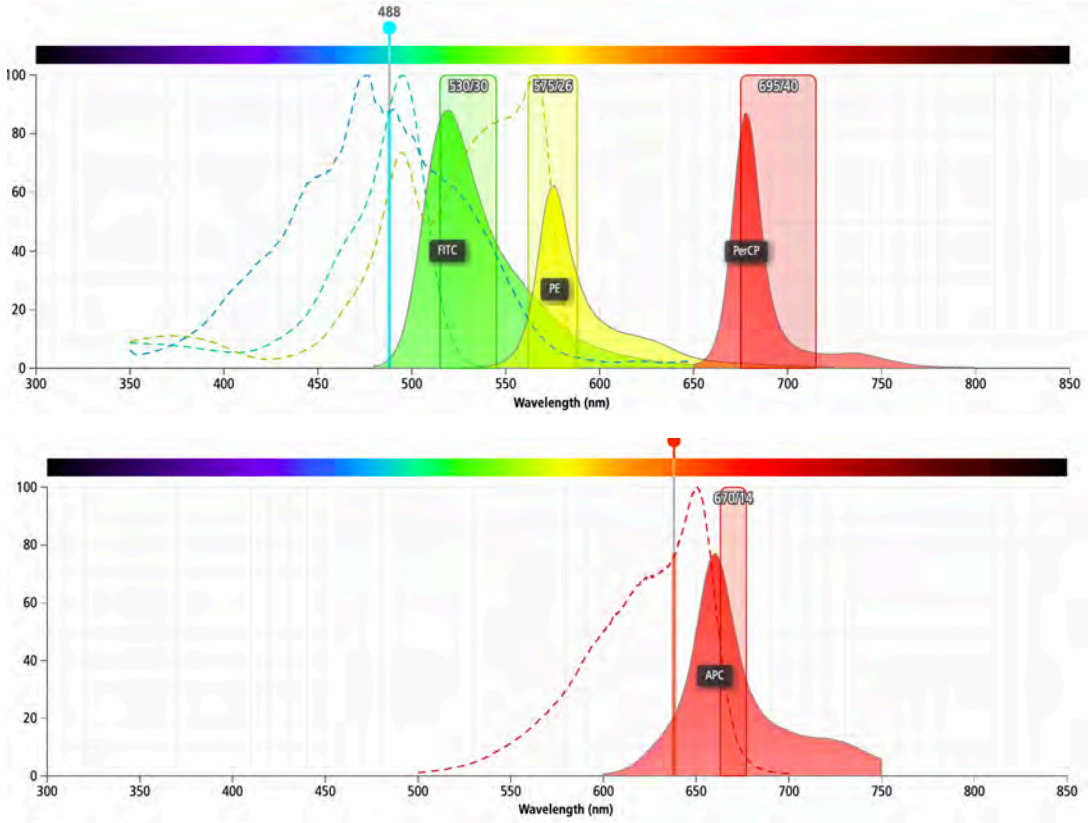
Let’s see how this is done live!

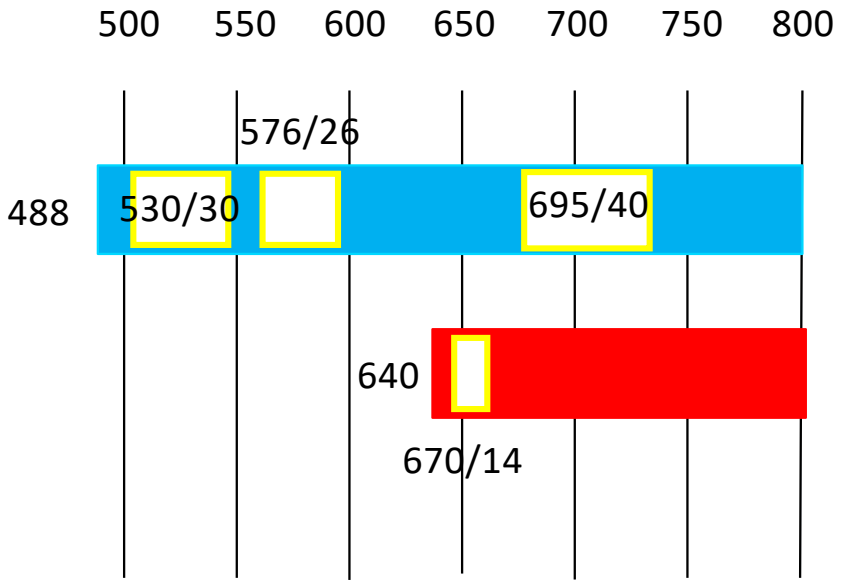
Will be using FlowJo v10.7.1 on Mac OS

Beads: FITC, PE, PerCP, APC

Cytometer: BD LSR Fortessa

Fluorescence Compensation

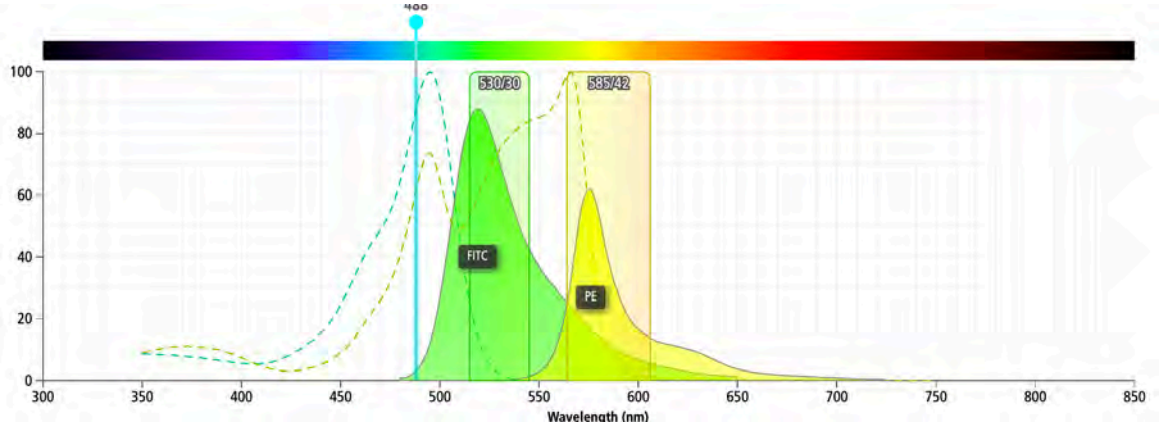




Fluorescence Compensation

We compensate the spillover

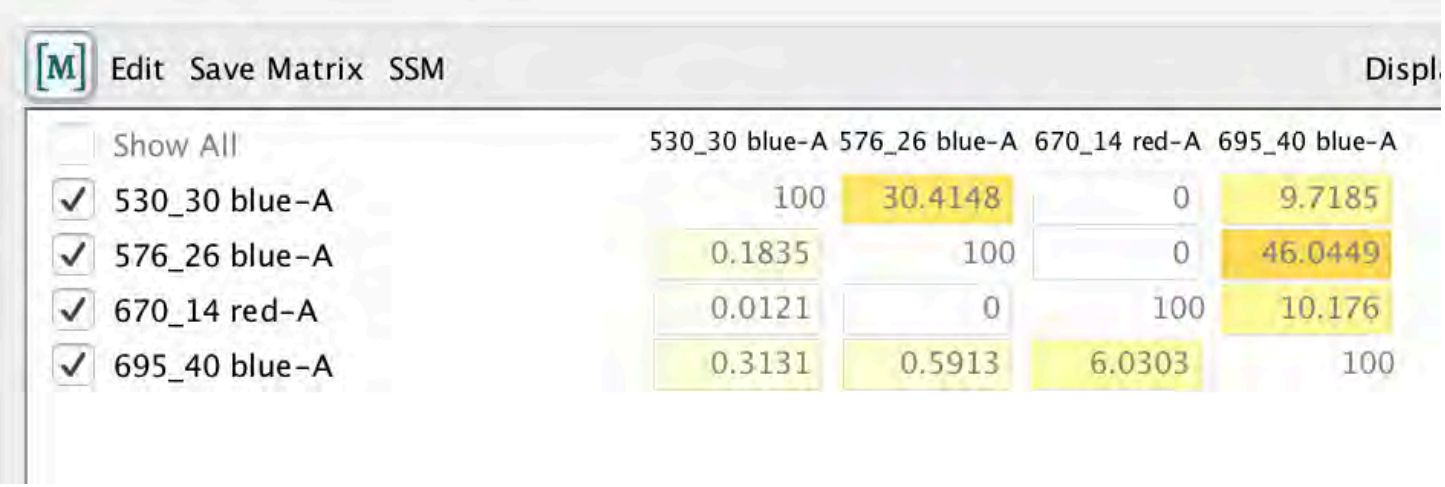
- Do NOT change the voltage of any channel after setting compensation
- If we do, we need to re-calculate the compensation value
- Be prepared to take some time to do this properly!



Fluorescence Compensation

You can perform compensation ‘live’ or off-line.

I recommend using automatic compensation



	530_30 blue-A	576_26 blue-A	670_14 red-A	695_40 blue-A
530_30 blue-A	100	30.4148	0	9.7185
576_26 blue-A	0.1835	100	0	46.0449
670_14 red-A	0.0121	0	100	10.176
695_40 blue-A	0.3131	0.5913	6.0303	100

Can I trust it? Yes, if you follow the rules…..

Fluorescence Compensation

What do I need to bring to the cytometer to successfully set compensation?

Single stained control (to adjust overlap) – ideally with Positive and negative populations

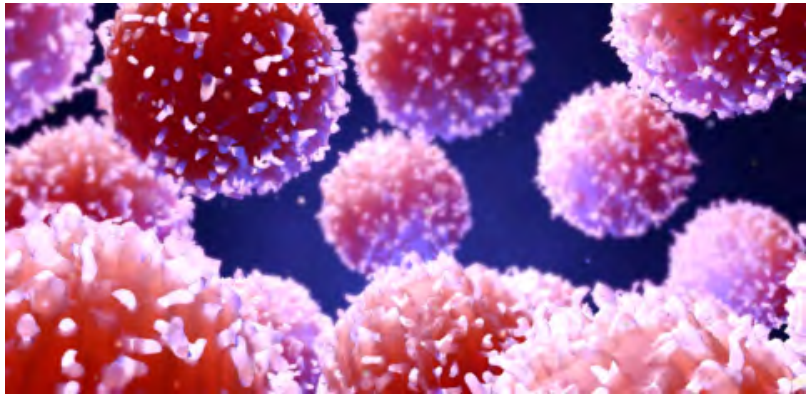
Unstained sample (to set autofluorescence) or act as a Universal Negative

Fluorescence Compensation

There are three ‘rules’ of compensation.

We are always thinking about fluorescence and fluorochromes but we need to attach them to something.

This could be CELLS or BEADS

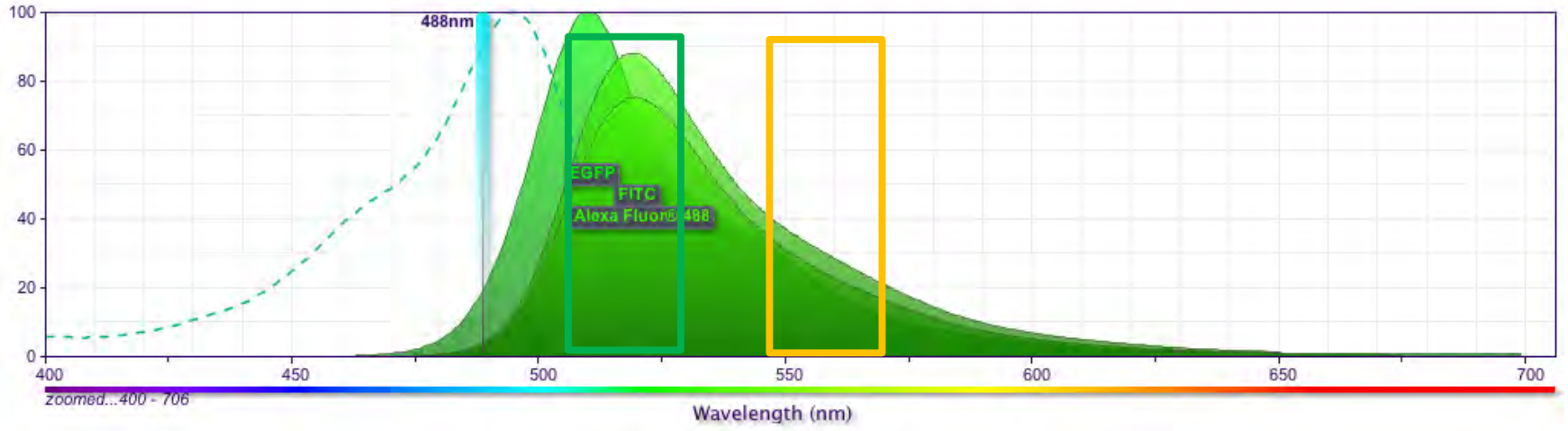


Fluorescence Compensation: Rule 1

Compensation must be performed using the **same fluorochrome** that is in the sample and controls must be **treated in the same way**.

Fluorescence Compensation: Rule 1

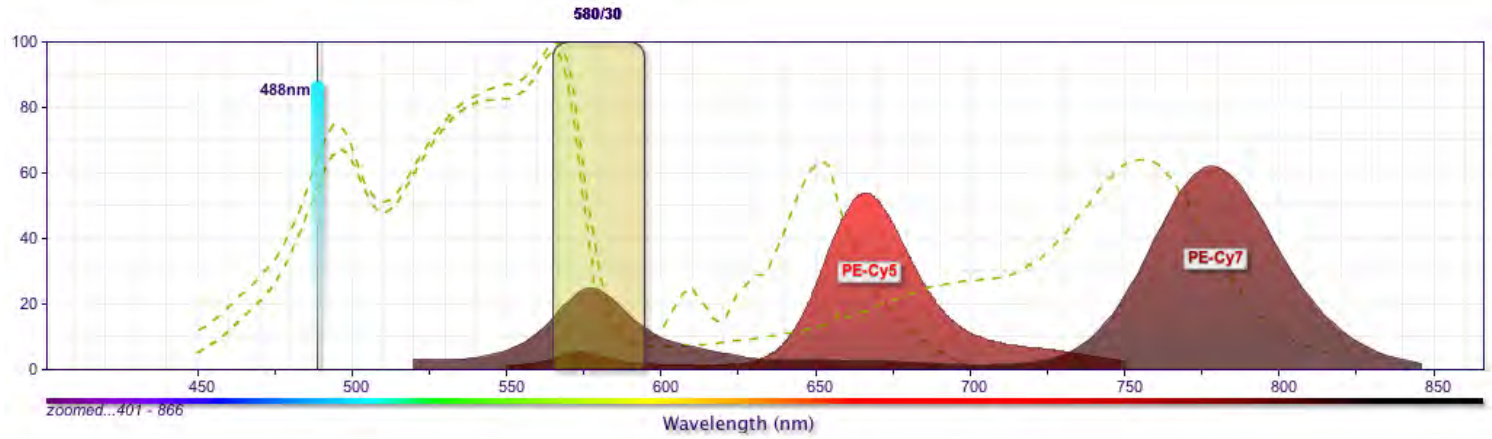
Why? FITC, AF488 and GFP are not the same fluorochrome. Their emission spectra are different, so they will have different amounts of spillover into other channels.



Fluorescence Compensation: Rule 1

Beware tandem dyes.

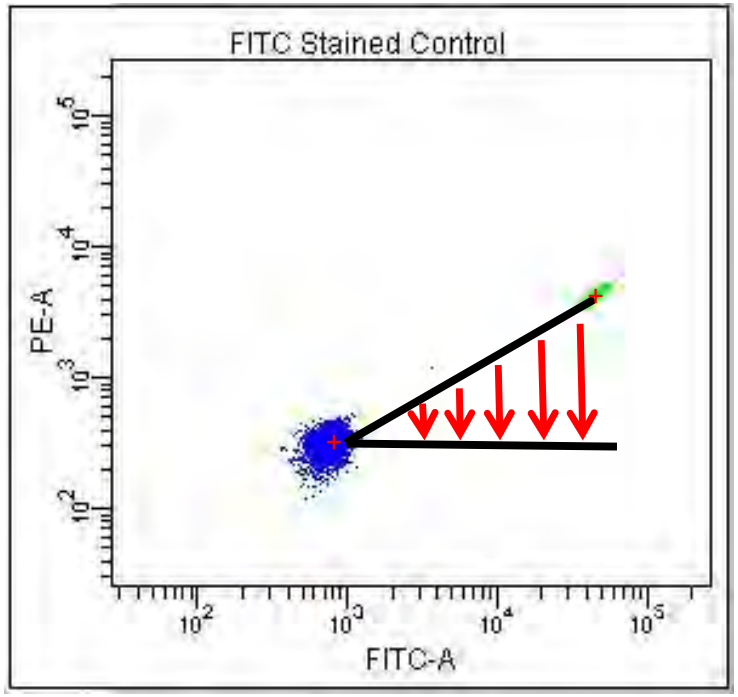
There is batch and manufacturer variation.



Use the exact antibody used in the experiment.

Fluorescence Compensation: Rule 2

The positive cells should be **at least as bright** as anything in your samples and **sufficient events** should be collected.



Fluorescence Compensation: Rule 2

Why? We are determining the slope of the line between the positive and negative cells, so the further apart they are the easier this is.

But, can be problematic if the positives are:

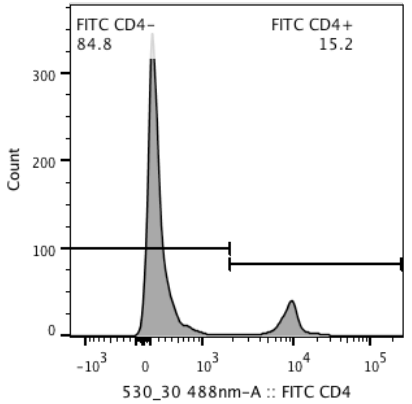
- Low in expression
- Low in number

We can use beads or cells

Compensation Controls - Cells

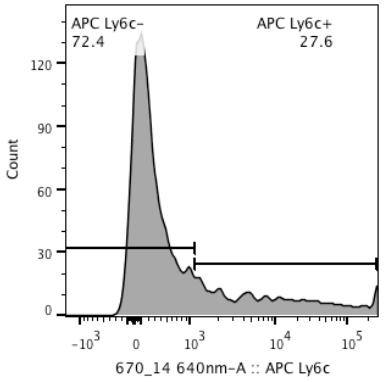
Advantages

Autofluorescence of background and sample match
No extra reagents needed
Actual target is measured



Disadvantages

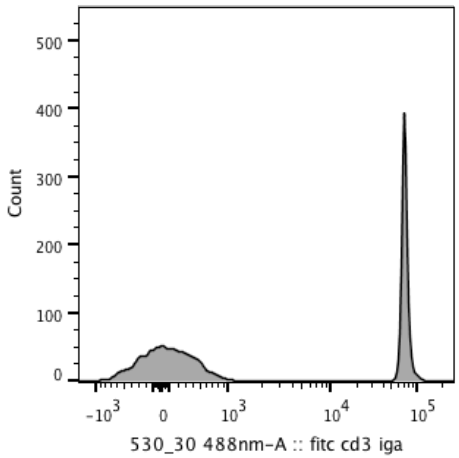
CVs may be broad
Peaks may not be easily distinguished
Not ideal for rare events
Uses precious sample



Compensation Controls - Beads

Advantages

Cells not wasted on controls
Easy to use
Capture maximum antibody
Low CV for peak



Disadvantages

Isotype specific
Not all species supported
Not useful for FPs/probes

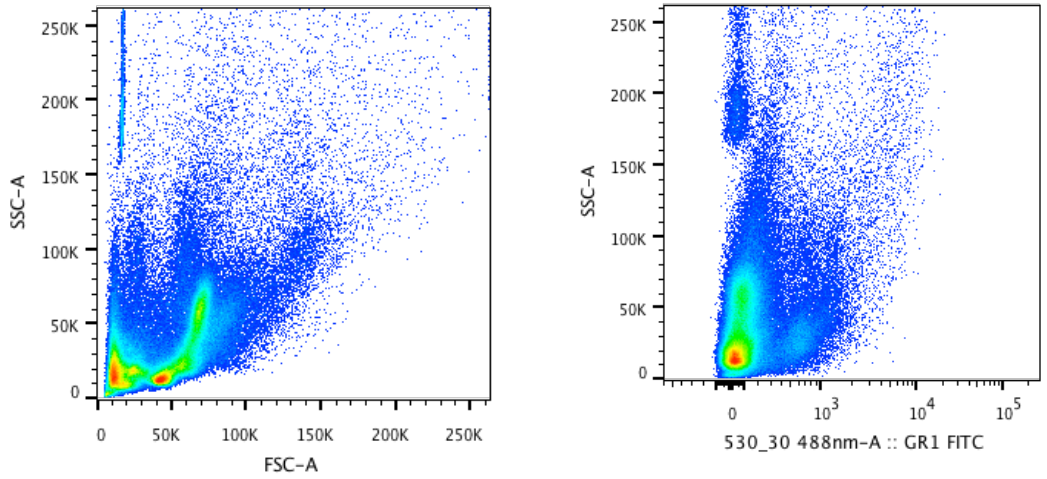
Fluorescence Compensation: Rule 3

The positive cells/beads must have the **same autofluorescence** when unstained as the negative cells/beads.

Fluorescence Compensation: Rule 3

Why? Because we want to ensure that we correct for the right level of fluorescence.

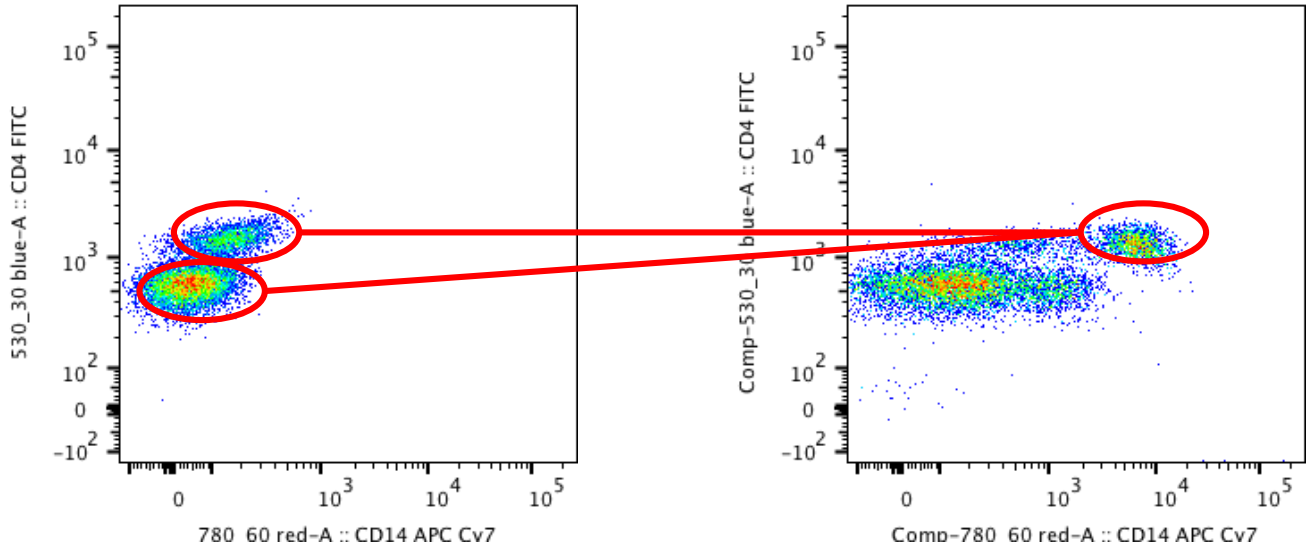
Monocytes do not have the same level of autofluorescence as lymphocytes.



Unstained Bone Marrow (with enumeration beads) showing different autofluorescence in FITC channel

Fluorescence Compensation: Rule 3

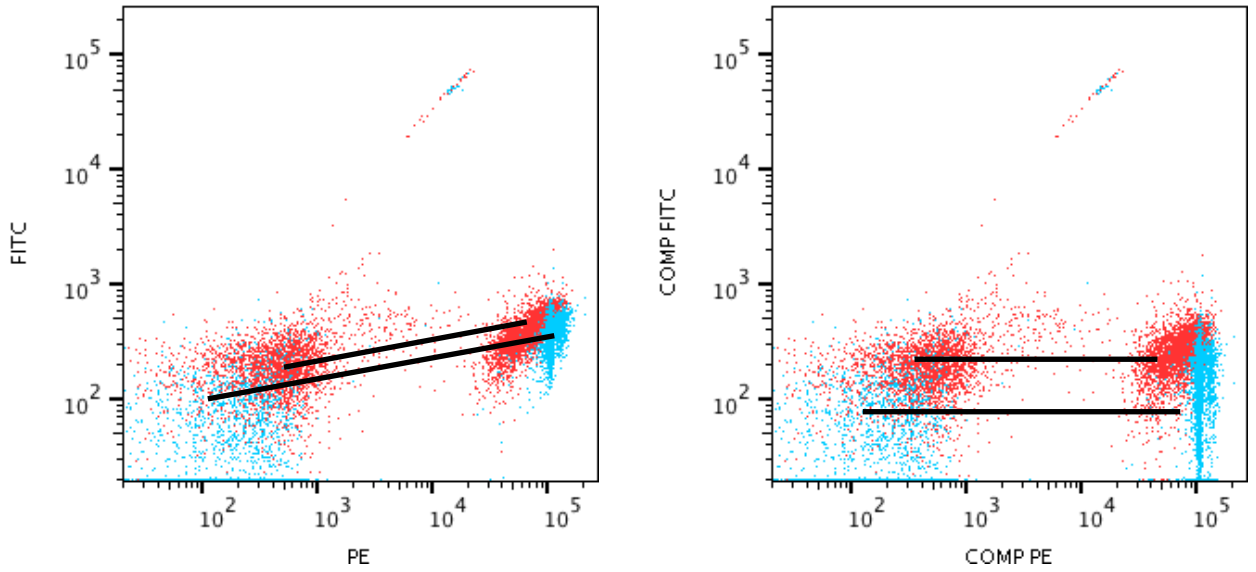
Here we use a monocyte marker, so we need to compensate to the autofluorescence of the monocytes.



At the same voltage, the compensation would also be correct for a lymphocyte marker.

Fluorescence Compensation: Rule 3

As long as voltages remain the same, we can use either cells or beads



Fluorescence Compensation

Run compensation controls every time. Why?

- Instrument changes over time
- Cellular autofluorescence may change
- A new tandem batch may be introduced

Use fresh samples every time

Panel Design rules

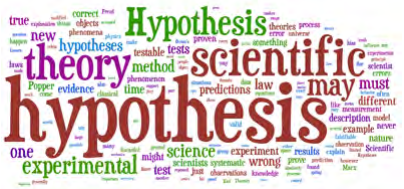
Instruments → Lasers and filters

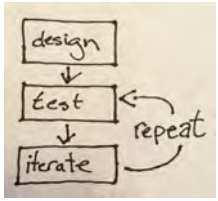
Fluorochromes → Brightness

Understanding your antigens → Density and expression pattern

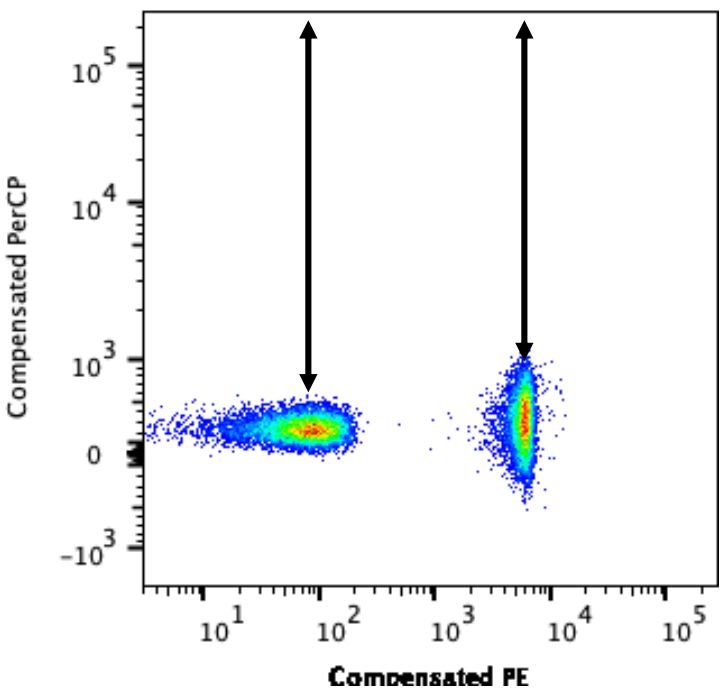
Maximising resolution → Signal to noise ratio

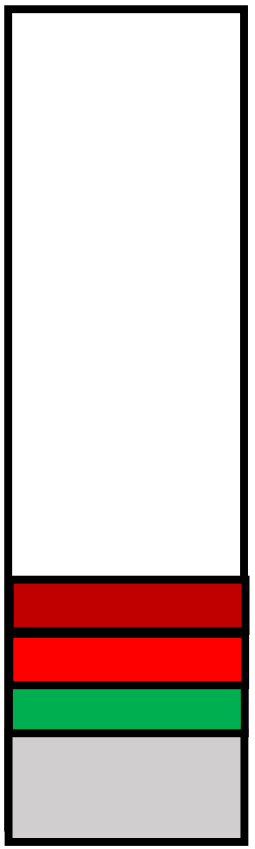
Biology → What is your question?
Where is your target?





Maximising resolution – why is it important?





Good news – compensation accounts for spillover

Bad news – spillover affects resolution

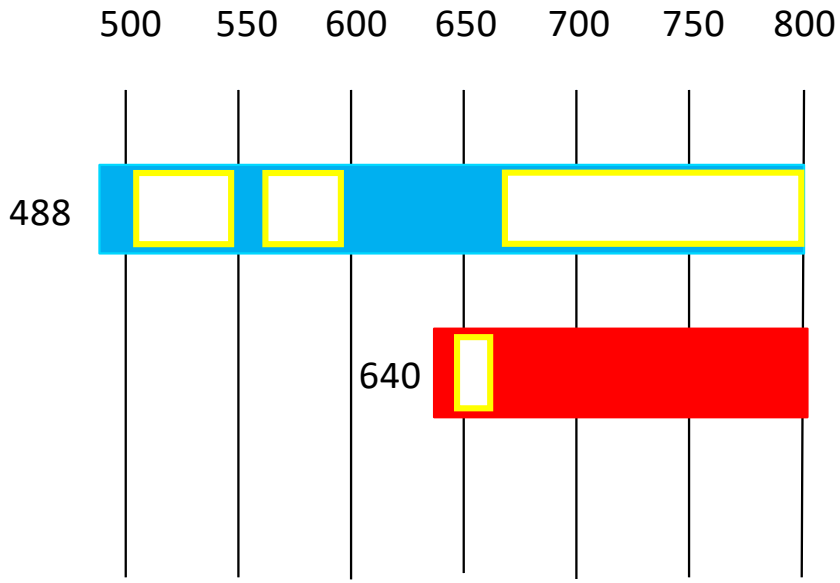
Panel Design rules

Spread fluorochromes within laser

Spread fluorochromes across lasers

Weak antigens on bright fluorochromes

Avoid channels with large spillover

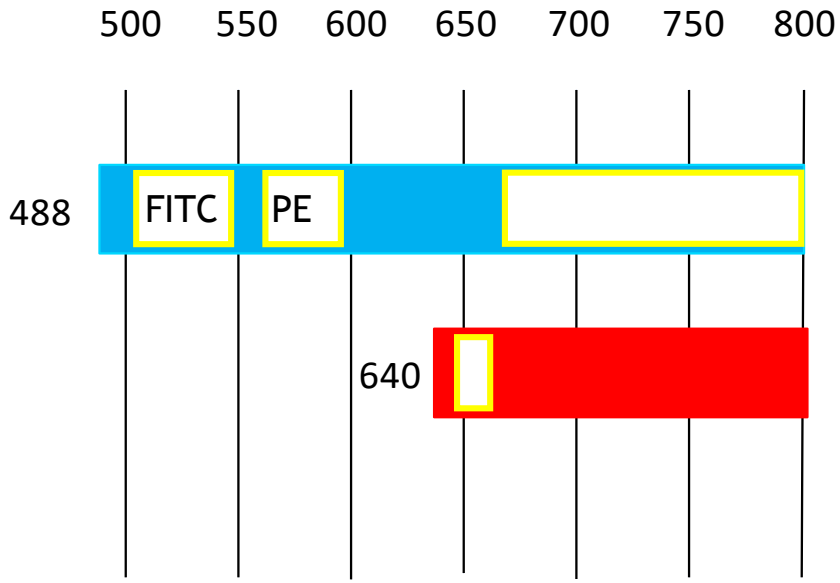


Sources of spillover

Same laser adjacent channels

Tandem base fluorescence

Cross-laser excitation



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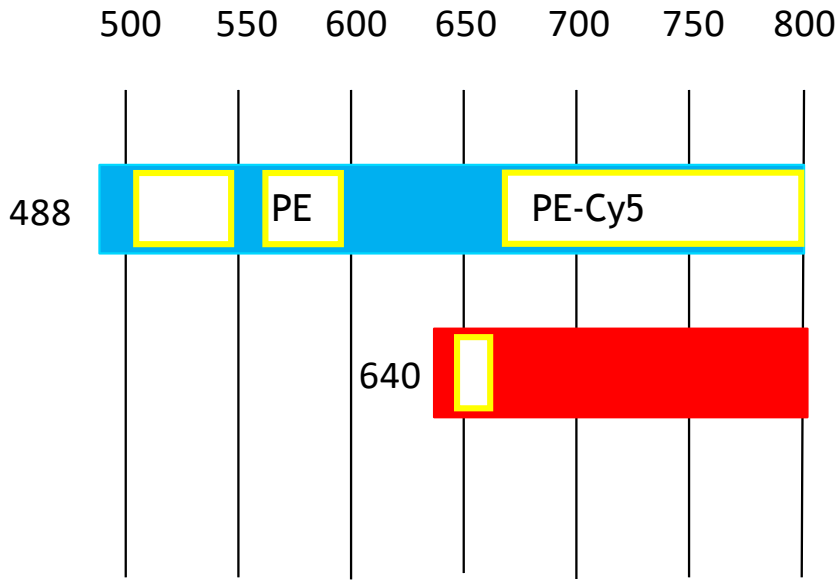

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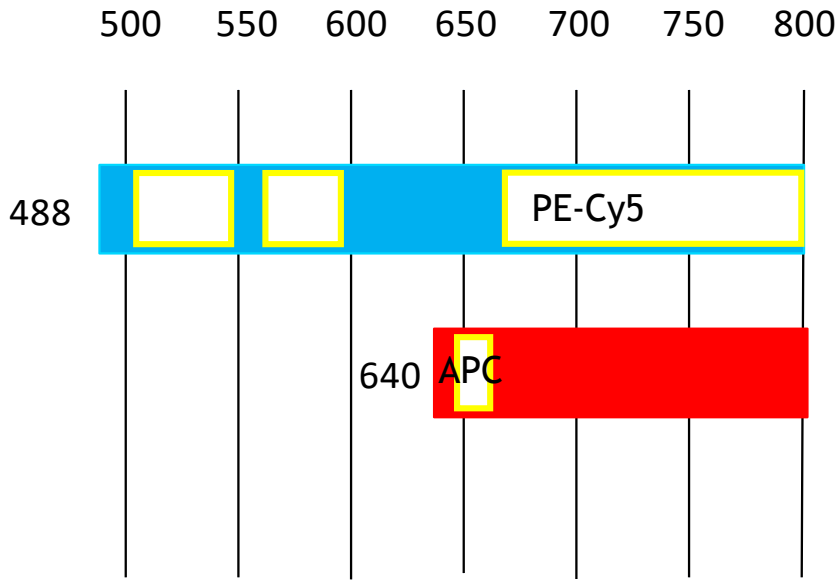
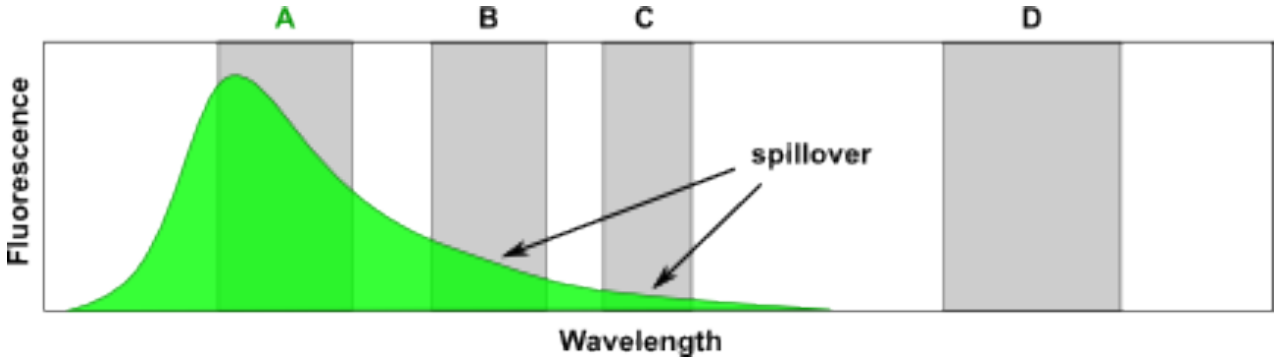


Summary

Compensation is inherent in multicolour experiments (flow or microscopy)

It is a logical process – follow the rules and it will work!

Spillover will impact on population resolution and experimental design



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https://www.youtube.com/playlist?list=PLpAyGqPO0aHrmHgwSV4oiU0M9G_j4wCXs