Spectral Overflow Compensation in Flow Cytometry

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EMBL - Monterotondo

What is Compensation?

What is Compensation?

Simply, it corrects for spillover/overflow of one fluorochrome's emission into the detector/channel meant for another

Host	Rat
Isotype	IgG2b, kappa
Reactivity	Human, Mouse
Conjugate	PE
Laser	Blue Laser, Green Laser, Yellow-Green Laser
Emit	575 nm
Excite	488 - 561 nm
Reported Applications	Flow Cytometric Analysis

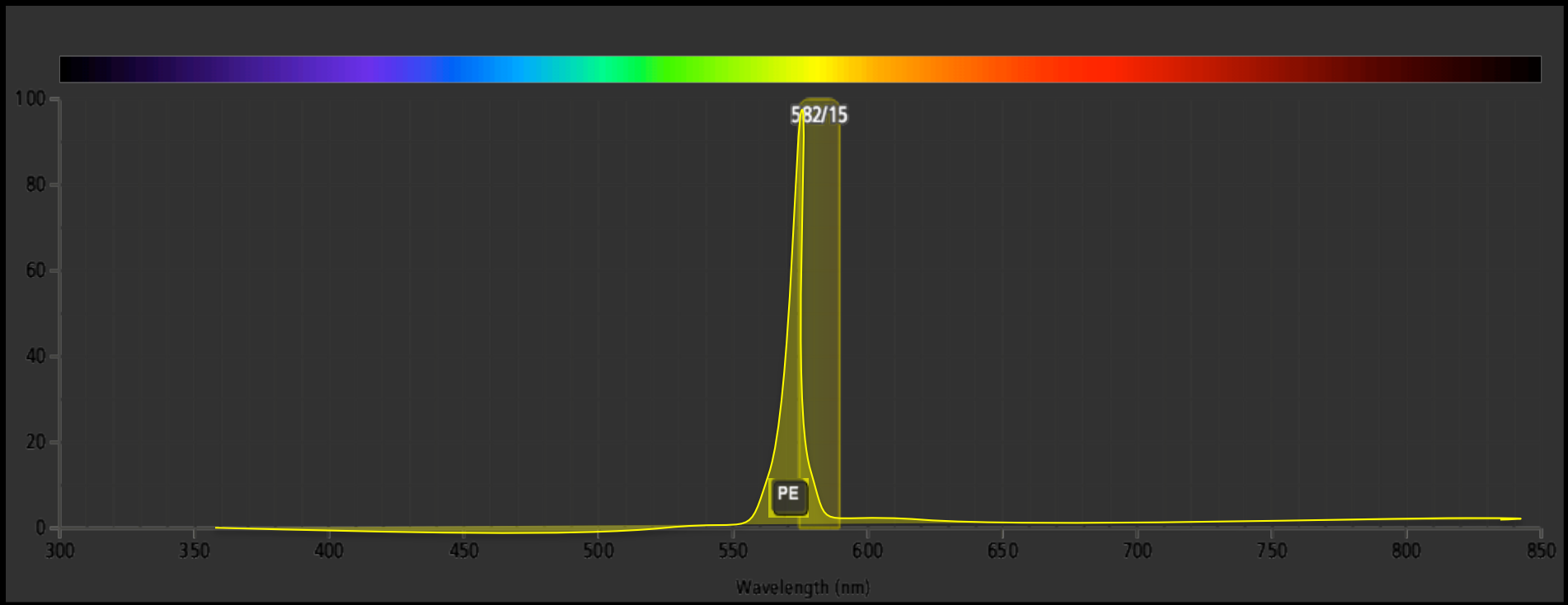
Host	Rat
Isotype	IgG1, kappa
Reactivity	Mouse
Conjugate	APC
Laser	Red Laser
Emit	660 nm
Excite	633 - 647 nm
Reported Applications	Flow Cytometric Analysis

Specifications

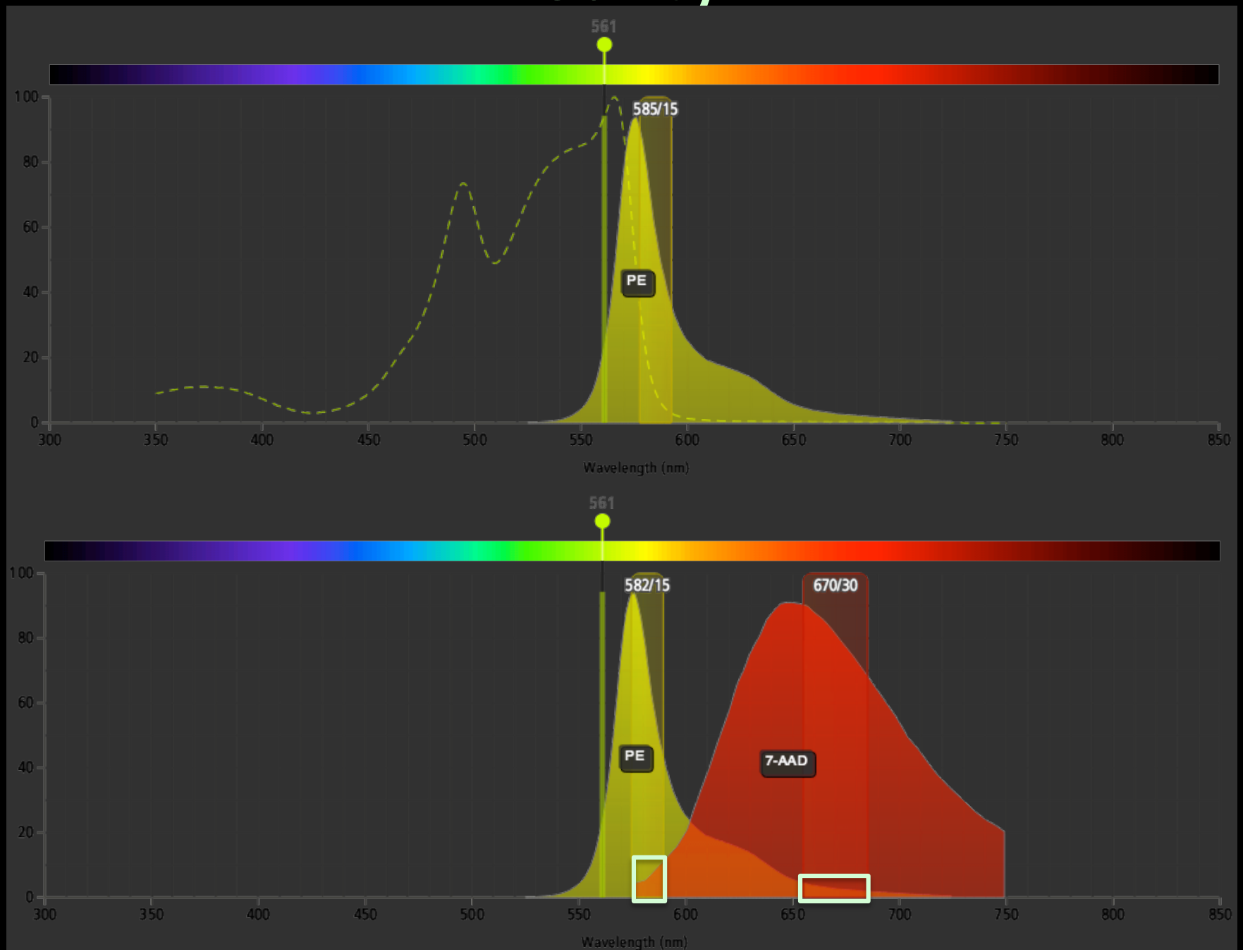
For Use With (Equipment):	Fluorescence Microscope, Flow Cytometer
Cell Permeability:	Cell-Impermeant
Sub-Cellular Localization:	Nucleus, Nucleic Acids
Form:	Solid
Solubility:	DMSO (Dimethylsulfoxide)
Label or Dye:	7-AAD
Product Size:	1 mg
Detection Method:	Fluorescent
Flow Cytometer Laser Lines:	488
Excitation/Emission (nm):	546/647
Shipping Condition:	Room Temperature

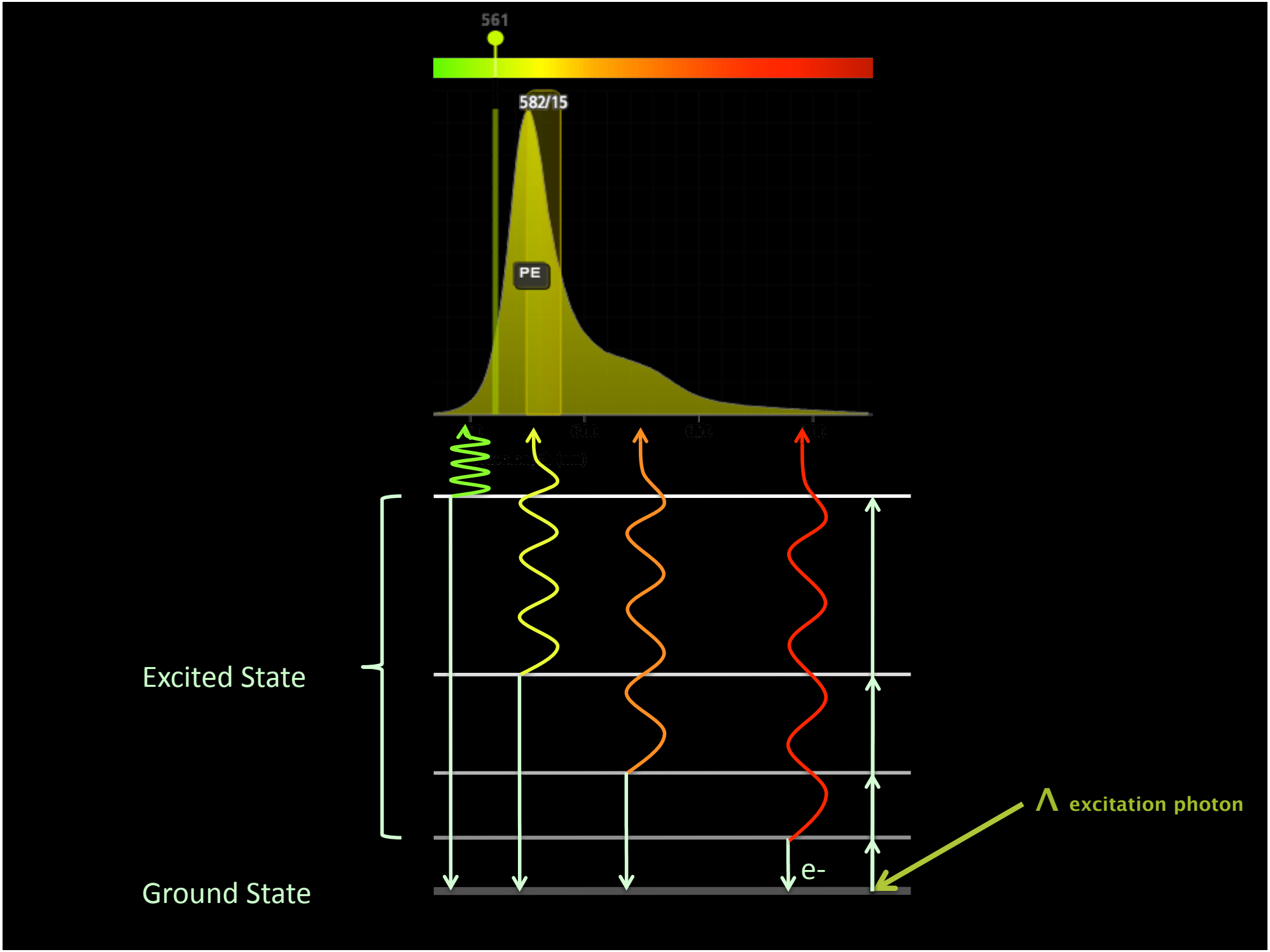
Expectation?

Host	Rat
Isotype	IgG2b, kappa
Reactivity	Human, Mouse
Conjugate	PE
Laser	Blue Laser, Green Laser, Yellow-Green Laser
Emit	575 nm
Excite	488 - 561 nm
Reported Applications	Flow Cytometric Analysis



Reality


561
585/15
PE
100
80
60
40
20
0
300
350
400
450
500
550
600
650
700
750
800
850
Wavelength (nm)
561
582/15
670/30
PE
7-AAD
100
80
60
40
20
0
300
350
400
450
500
550
600
650
700
750
800
850
Wavelength (nm)

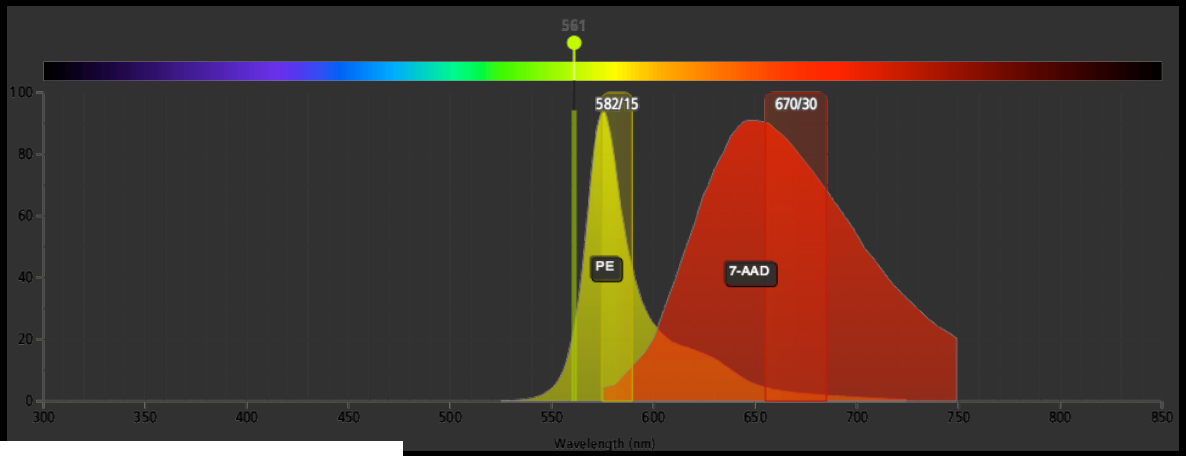

561
582/15
PE
Excited State
Ground State
e-
λ excitation photon

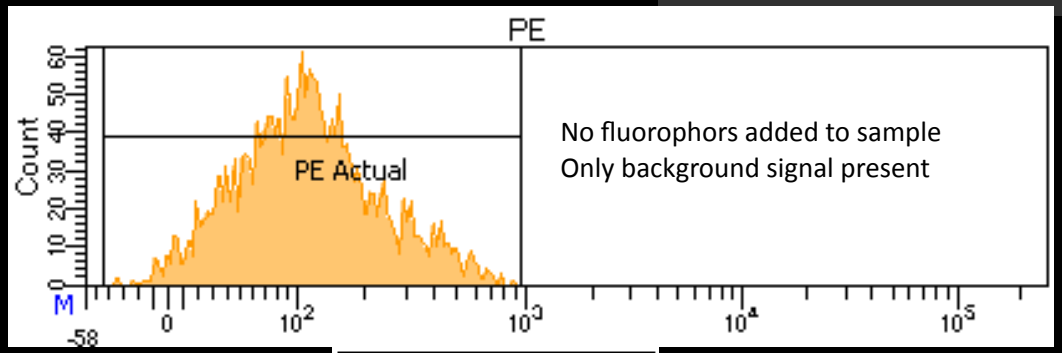
Auto and Manual Compensation

$CH1_A = CH1_O - (CH2_O)\ (\%_S 2 \rightarrow 1)$

$PE_A = PE_O - (7AAD_O)\ (\%_S)$

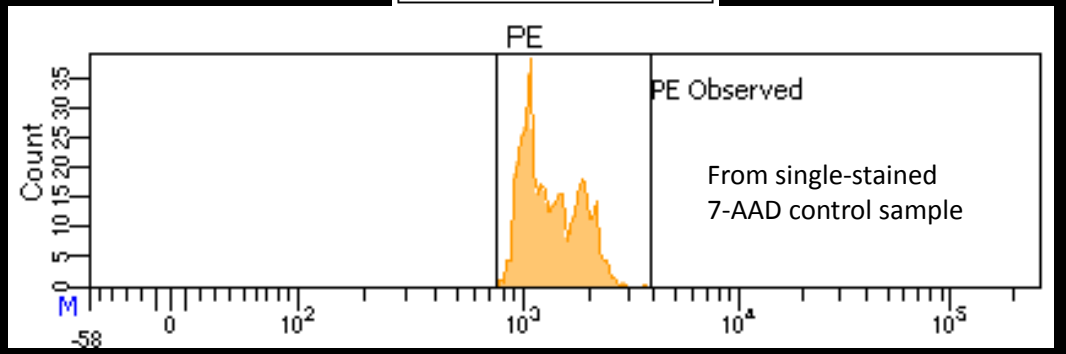
$\%_S = -\ (PE_A - PE_O) / 7AAD_O$



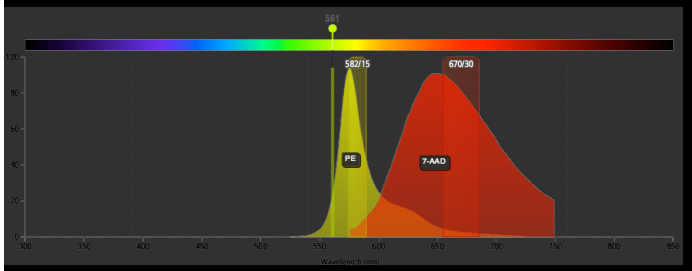


Population	PE-A Mean
PE Actual	139

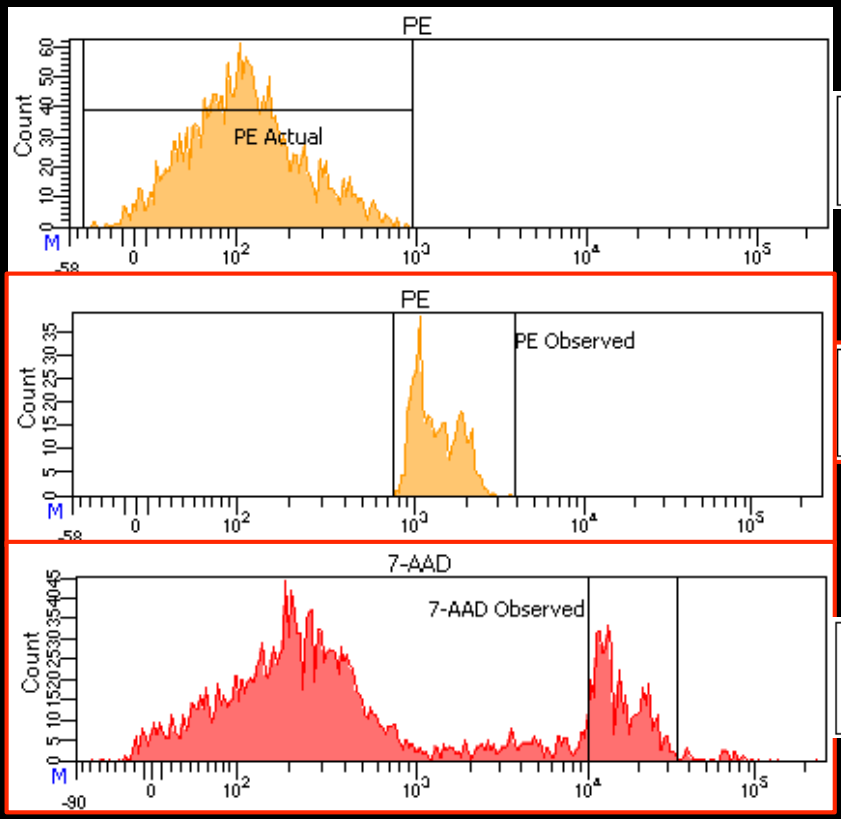
Population	PE-A Mean
PE Observed	1,337



Manual Compensation



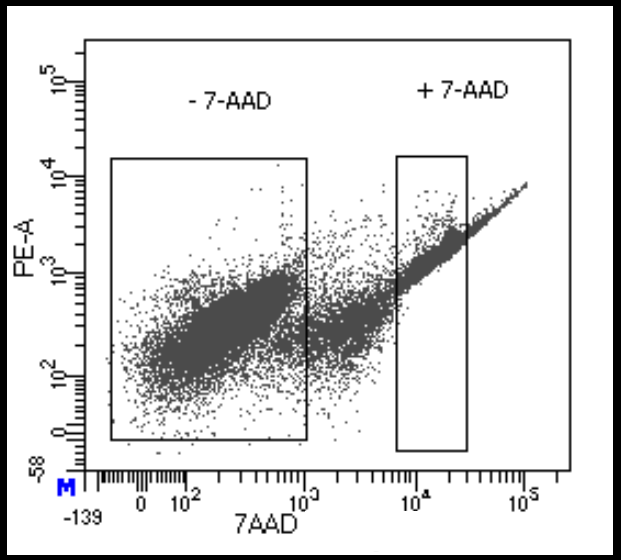
$$PE_A = PE_O - (7AAD_O)\ (\%_S)$$

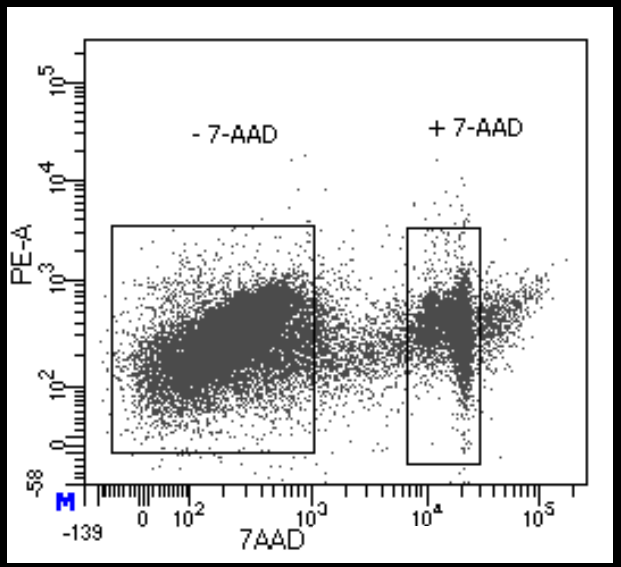


Population	PE-A Mean
PE Actual	139

Population	PE-A Mean
PE Observed	1,337

Population	7AAD ... Mean
7-AAD Observed	15,785

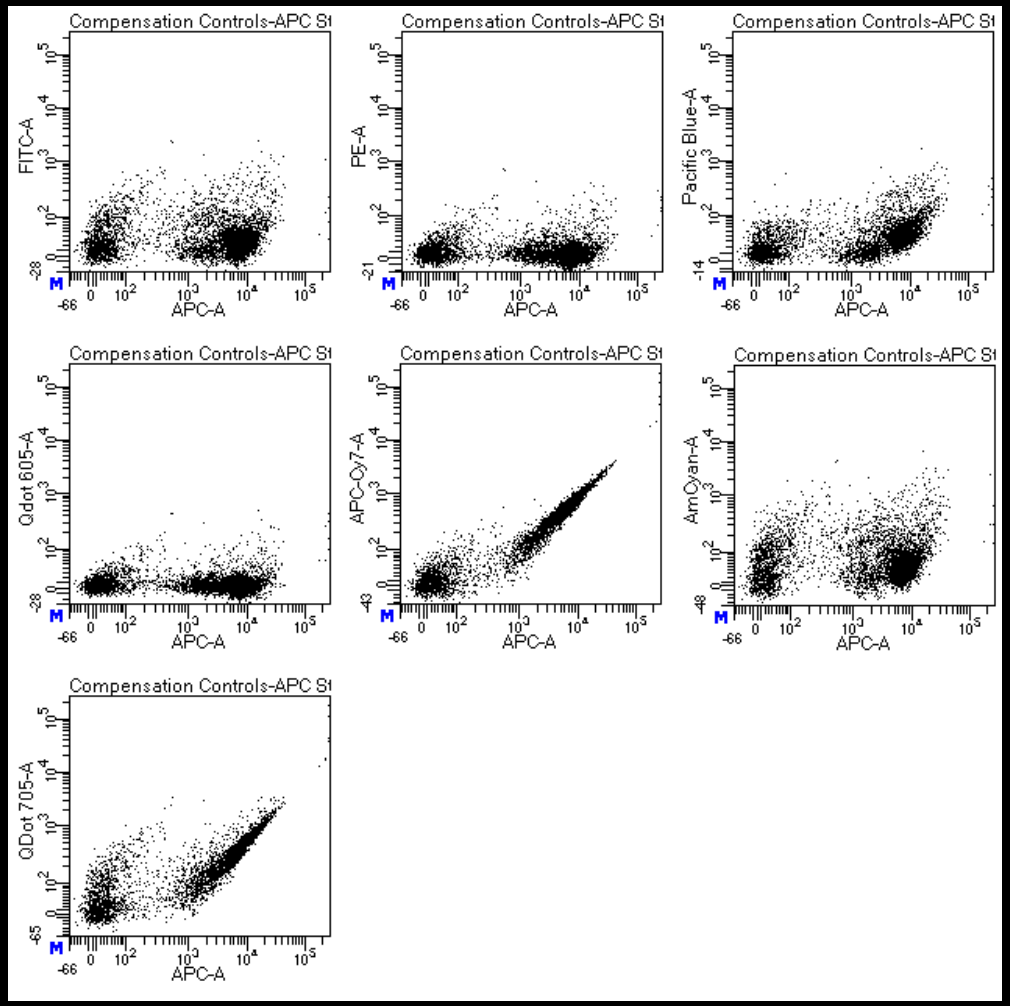




Population	PE-A Mean
- 7-AAD	459
+ 7-AAD	1,657

Population	PE-A Mean
- 7-AAD	393
+ 7-AAD	397

Manual Compensation for APC on 8 Color Panel



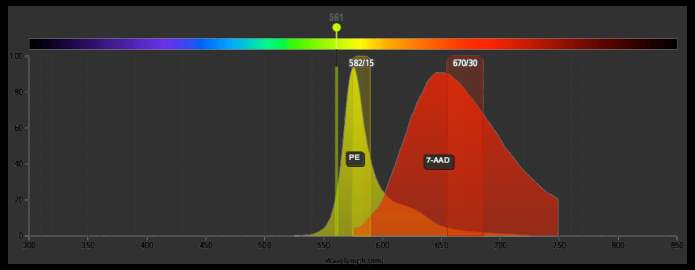
An experiment with 8 colors will require 56 instances of manual compensation

Auto Comp Values

Fluorochrome	- % Fluorochrome	Spectral Overlap
FITC	APC-Cy7	0.00
PE	APC-Cy7	0.00
Pacific Blue	APC-Cy7	0.28
Qdot 605	APC-Cy7	0.00
AmCyan	APC-Cy7	0.19
QDot 705	APC-Cy7	0.84
APC	APC-Cy7	6.55
APC-Cy7	FITC	0.08
PE	FITC	0.00
Pacific Blue	FITC	0.34
Qdot 605	FITC	0.08
AmCyan	FITC	6.85
QDot 705	FITC	0.08
APC	FITC	0.00
APC-Cy7	PE	0.00
FITC	PE	1.54
Pacific Blue	PE	0.50
Qdot 605	PE	1.27
AmCyan	PE	1.12
QDot 705	PE	1.62
APC	PE	0.49
APC-Cy7	Pacific Blue	0.07
FITC	Pacific Blue	0.63
PE	Pacific Blue	0.00
Qdot 605	Pacific Blue	2.18
AmCyan	Pacific Blue	343.61
QDot 705	Pacific Blue	2.90
APC	Pacific Blue	0.14

Show All	FITC_Alexa 4...	PE-A	PE-Cy55-A	PE-Cy7-A	CD3 CD14 Li...	APC_Ax 647-A	APC-Ax700-A
FITC_Alexa 488-A		16.64	0.96	0.15	0.00	0.02	0.00
PE-A	0.74		11.69	2.35	0.01	0.07	0.06
PE-Cy55-A	0.03	0.03		37.43	0.00	4.90	76.53
PE-Cy7-A	0.08	0.85	0.29		0.00	8.61	10.91
CD3 CD14 Live_Dead-A	0.54	0.18	0.00	0.00		0.00	0.00
APC_Ax 647-A	0.01	0.00	0.27	0.06	0.00		105.35
APC-Ax700-A	0.02	0.01	0.29	0.24	0.00	0.26	
APC-H7-A	0.08	0.00	0.01	1.71	0.04	5.15	17.93

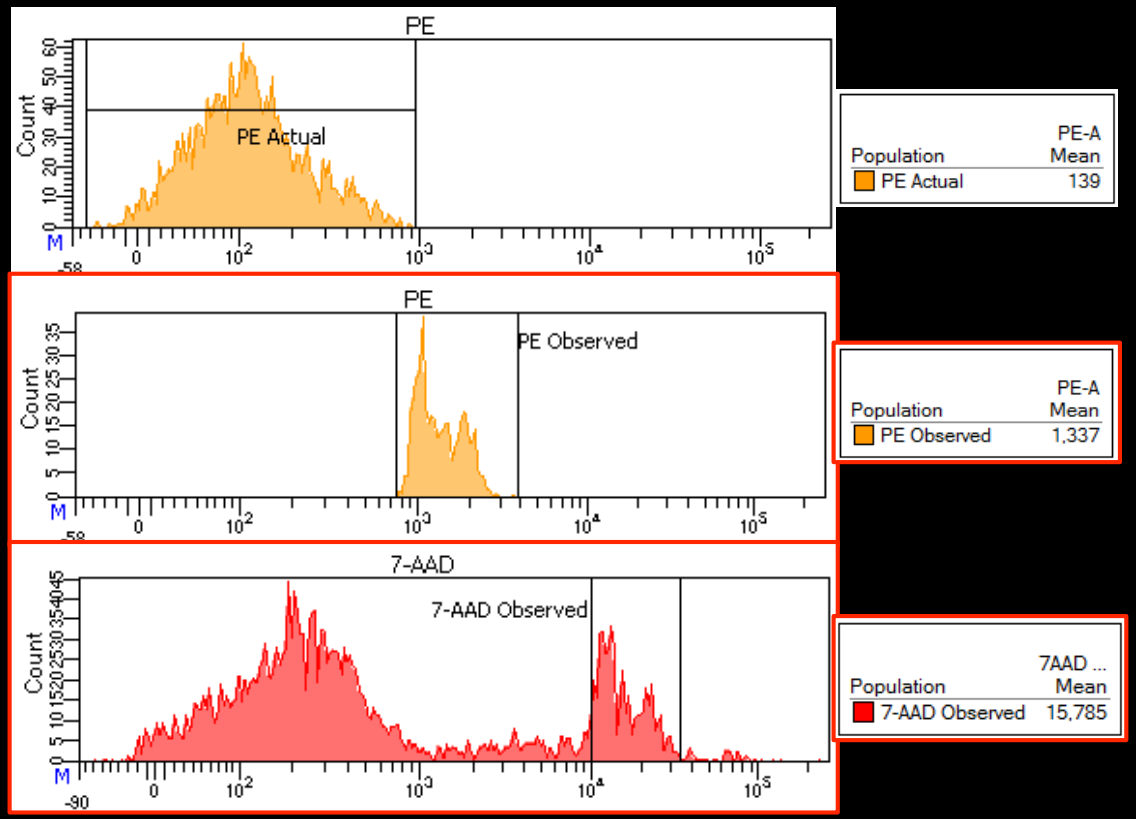
http://docs.flowjo.com/vx/experiment-based-platforms/plat-comp-overview/plat-comp-matrixwindow/

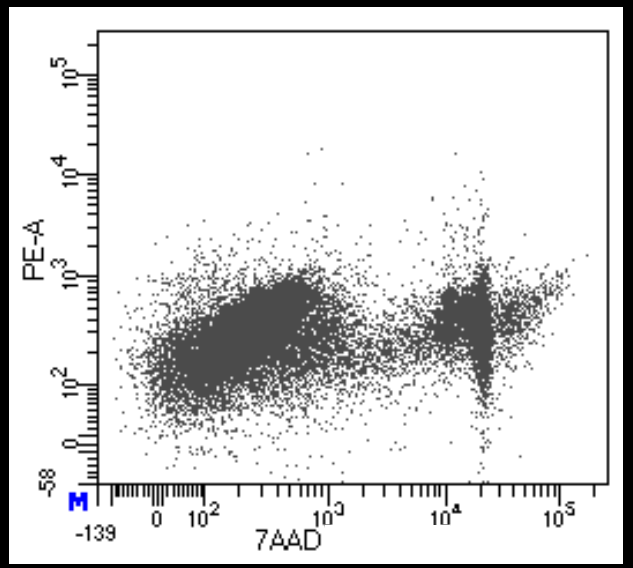


$PE_A = PE_O - (7AAD_O)\ (\%_S)$

$\%_S = -\ (PE_A - PE_O) / 7AAD_O$

Sample w/only 7-AAD Membrane Impermiable DNA Dye

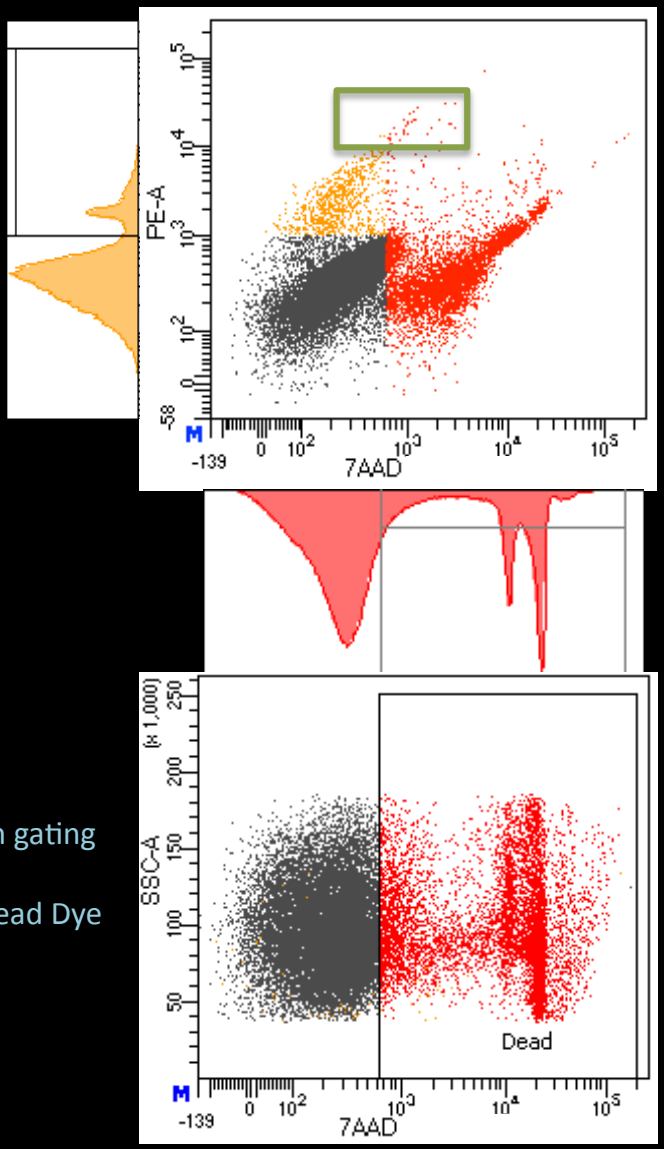




Spillover of 7-AAD into PE channel

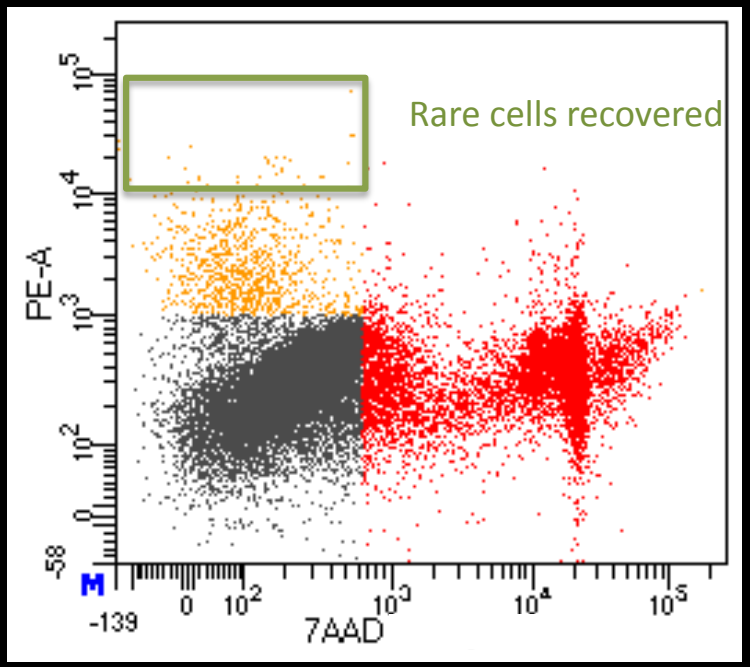
$\%_S = -\ (139 - 1337) / 15785$

$= 7.59\%$



Rare cells lost w/out compensation applied

Common gating strategy
•SSC v Dead Dye

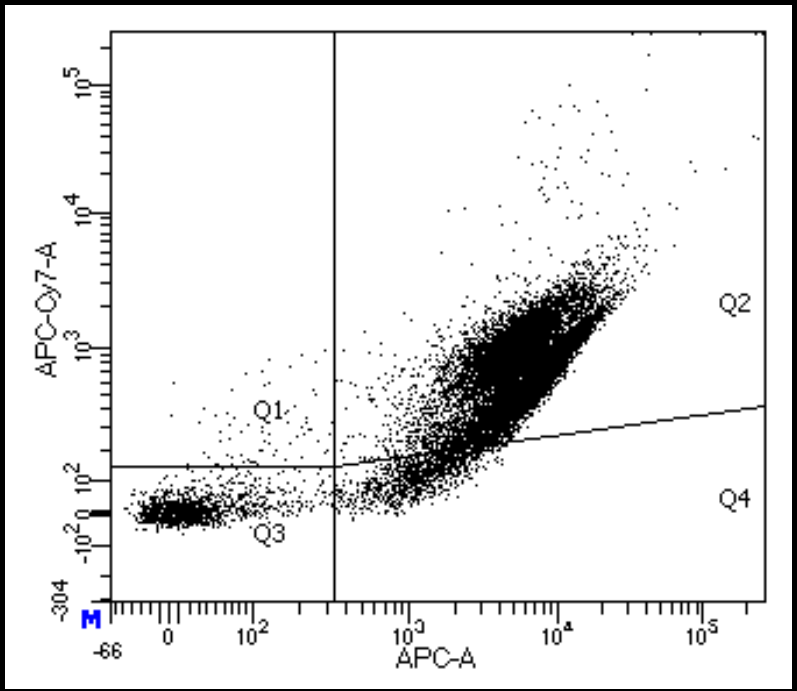


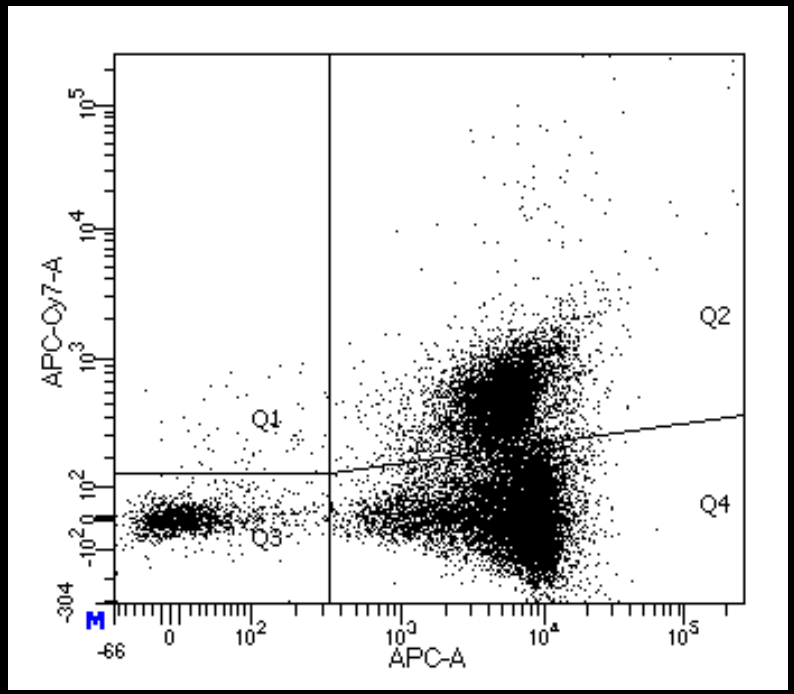
Fully Compensated

Reasons to Perform Compensation

•Multicolor (> 3 color panels) flow experiments – too may parameters to track to guarantee accurate analysis and/or cell sorting – or simply too time consuming

•Low surface antigen density





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- Low surface antigen density

- Reporting MFI (mean/median fluorescence intensity)

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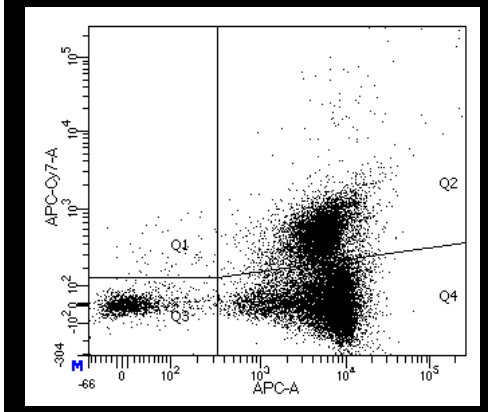
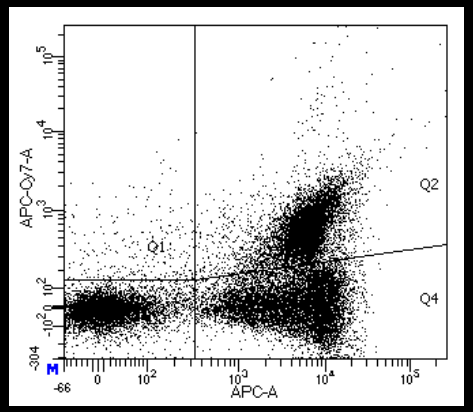
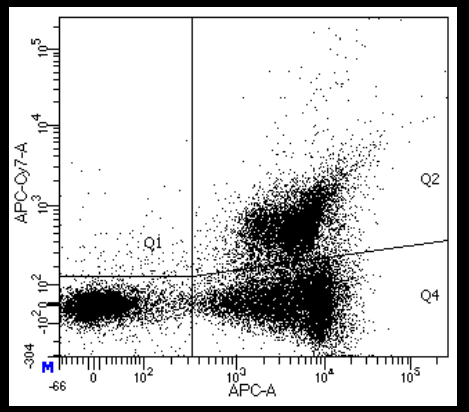
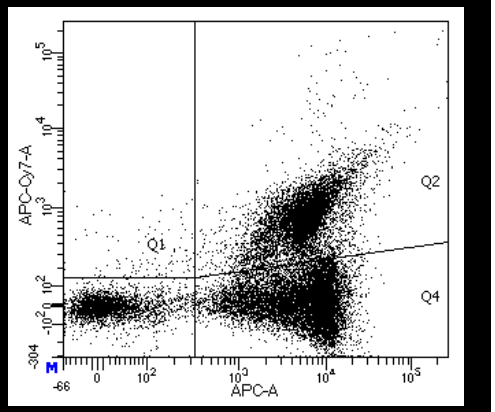
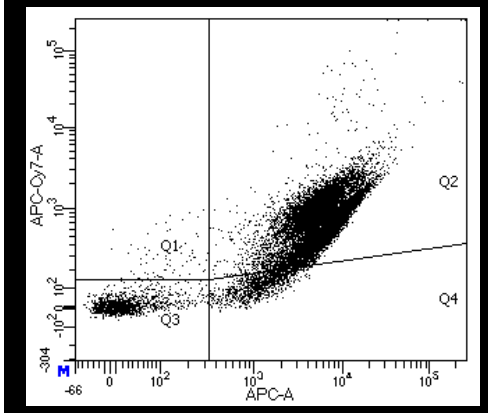
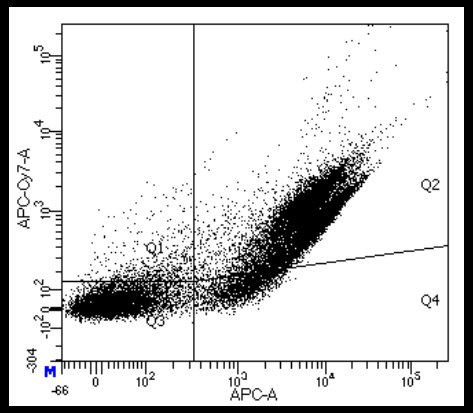


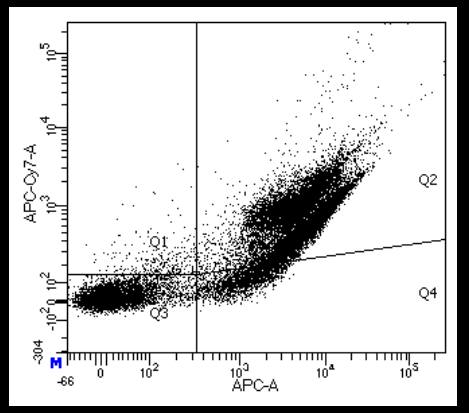


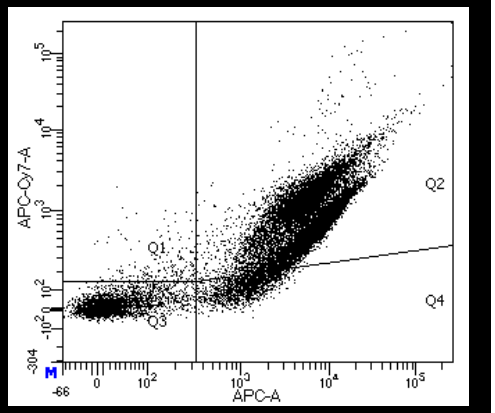












	No Tx MFI		Tx 1 MFI	% Change	Tx 2 MFI	% Change	Tx3 MFI	% Change
Compensated	533	Compensated	615	15.4%	616	15.6%	782	46.7%
Uncompensated	802	Uncompensated	978	21.9%	858	7.0%	944	17.7%

Reasons to Perform Compensation

•Multicolor (> 3 color panels) flow experiments – too may parameters to track to guarantee accurate analysis and/or cell sorting – or simply too time consuming

•Low surface antigen density

•Reporting MFI (mean/median fluorescence intensity)

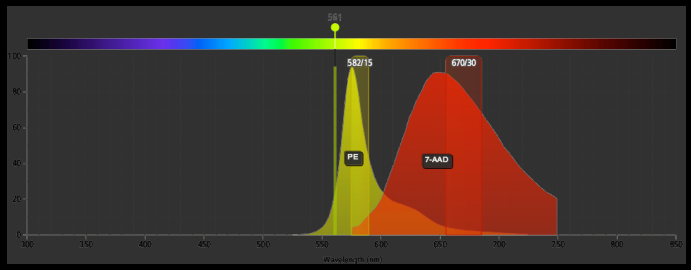
*Aesthetically pleasing figures for publication or supplemental data that will not make other flow cytometrists question your experimental methods

3 Rules of Compensation

1. Single-color controls must be as bright or brighter than samples that compensation will be applied to
2. Single color compensation controls must use the same fluorochromes as are used in the experiment
3. Each individual control must have the same background/autofluorescence

$$CH1_A = CH1_O - (CH2_O)\,(\%_S 2 \rightarrow 1) - (CH3_O)\,(\%_S 3 \rightarrow 1) \ldots - (CHn_O)\,(\%_S n \rightarrow 1)$$

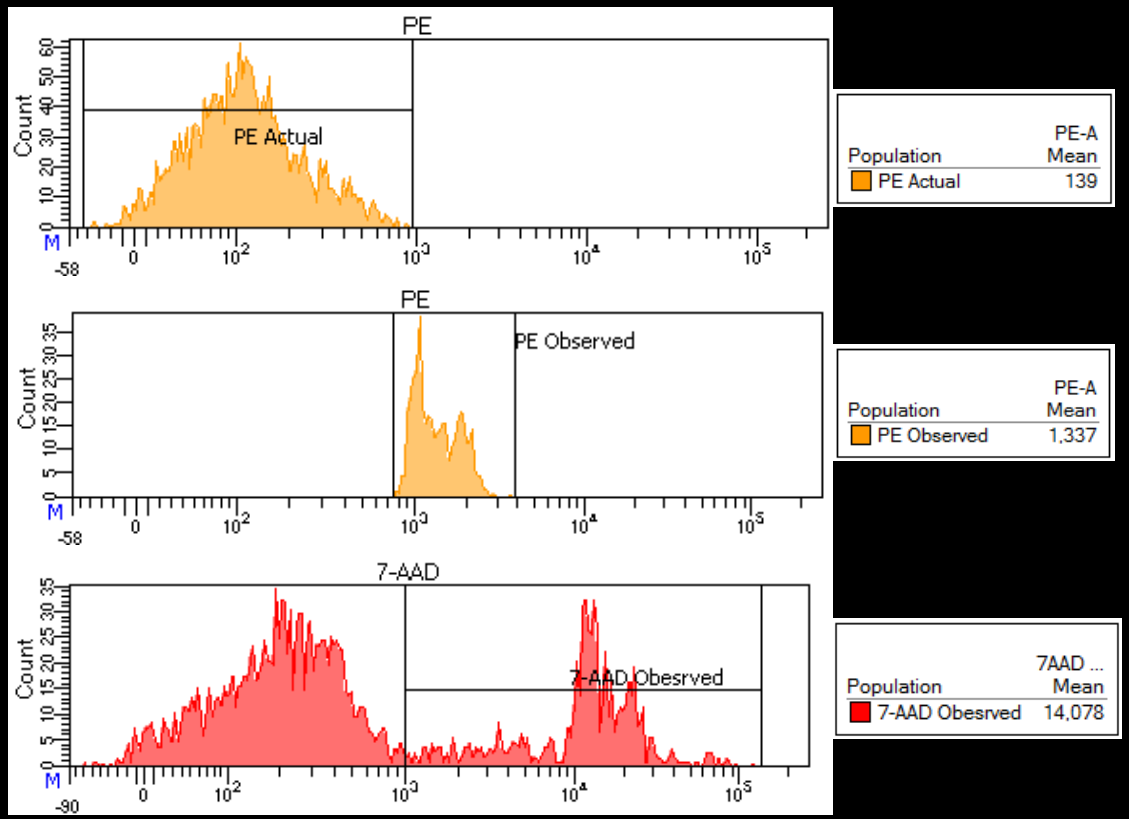
1. Single-color controls must be as bright or brighter than samples that compensation will be applied to

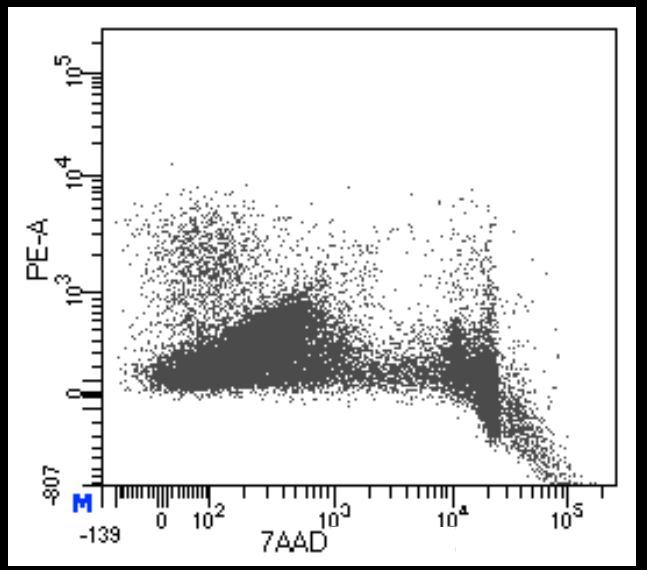


$PE_A = PE_O - (7AAD_O)\ (\%_S)$

$\%_S = -\ (PE_A - PE_O) / 7AAD_O$

Sample w/only 7-AAD Membrane Impermiable DNA Dye



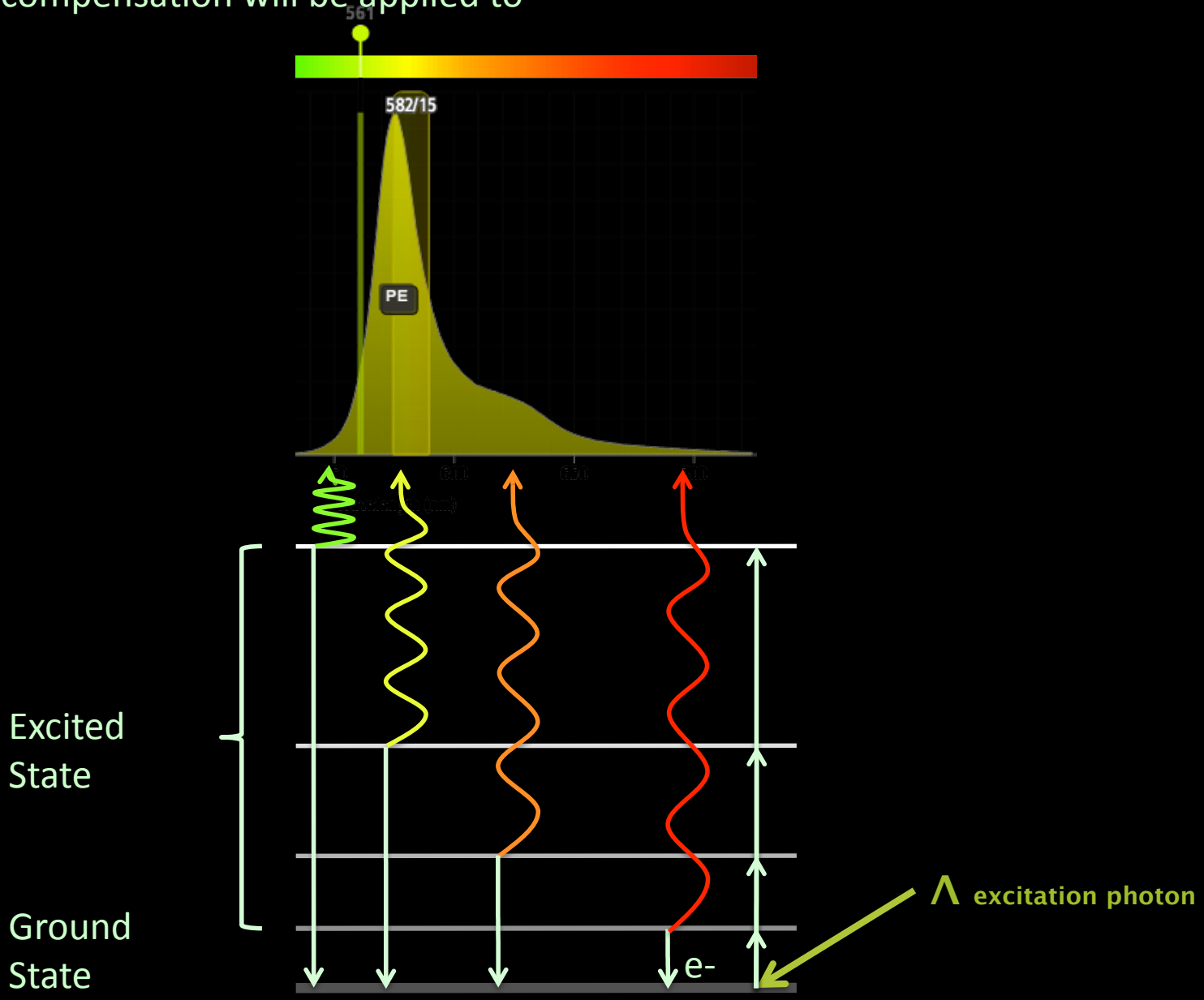


Spillover of 7-AAD into PE channel

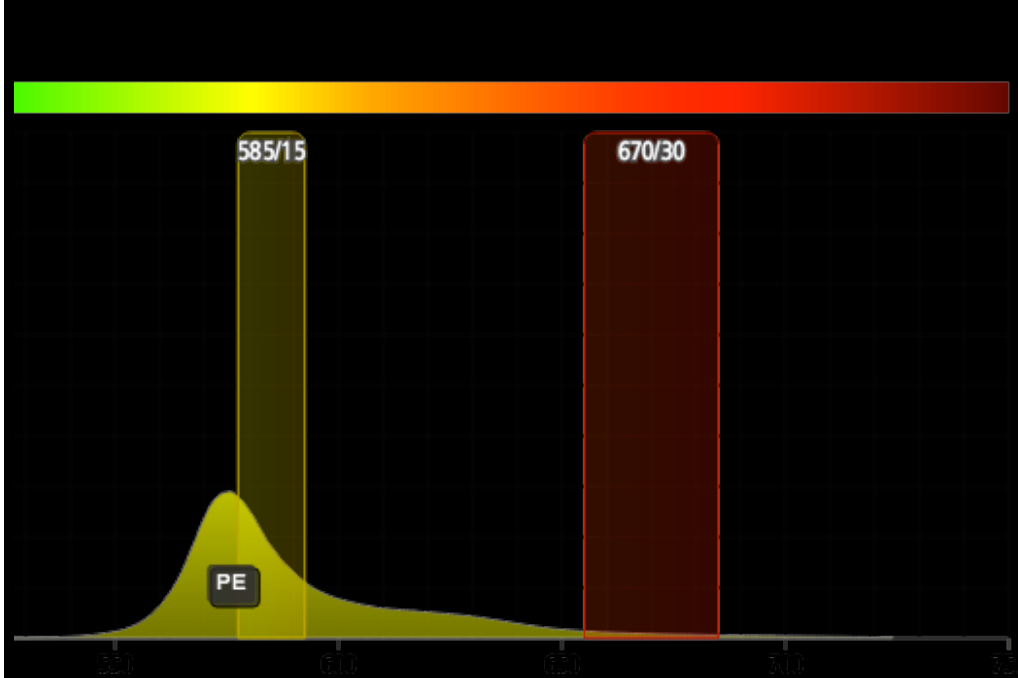
% = 7.59% w/specific gate

= 8.51% w/broad gate

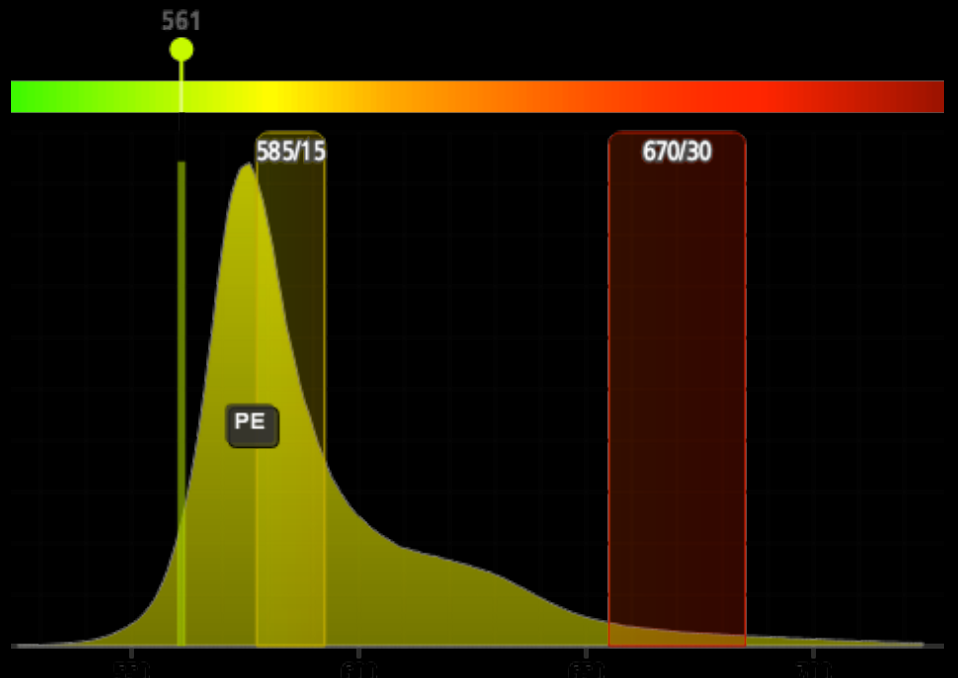
1. Single-color controls must be as bright or brighter than samples that compensation will be applied to



1. Single-color controls must be as bright or brighter than samples that compensation will be applied to
   - Fluorophore emission probability will not change



Dim Compensation Control

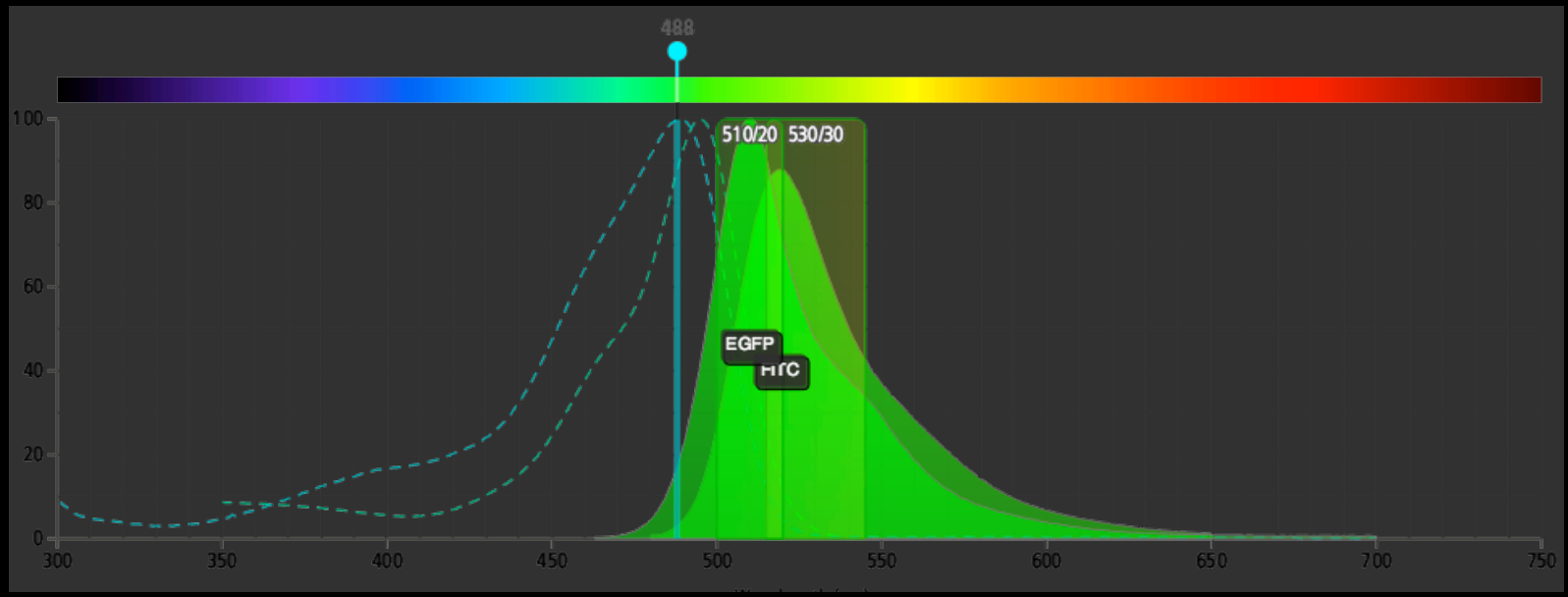


Bright Sample

1. IBS

2. Single color compensation controls must use the same fluorochromes as are used in the experiment

$CH1_A = CH1_O - (CH2_O)\ (\%_S 2 \rightarrow 1) - (CH3_O)\ (\%_S 3 \rightarrow 1) \ldots - (CHn_O)\ (\%_S n \rightarrow 1)$

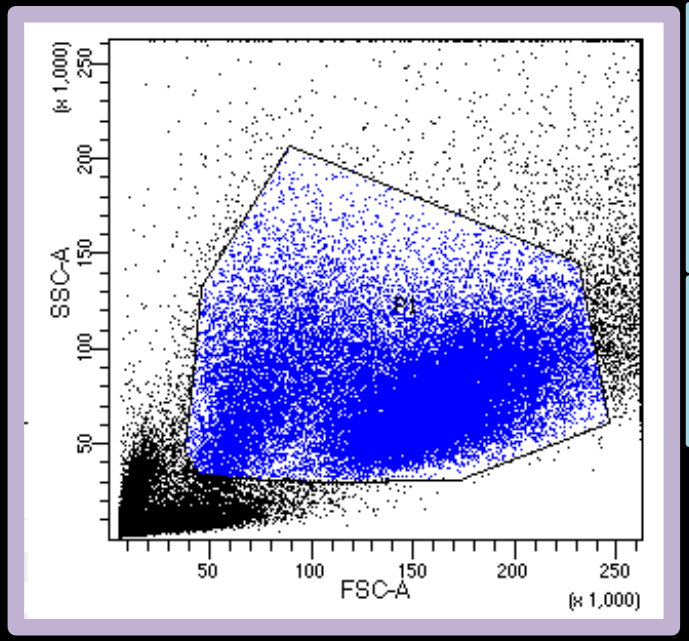


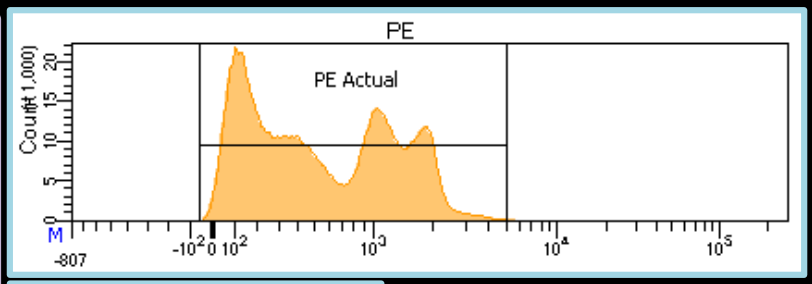
Note:
Compensation controls and samples using tandem fluorophores must use the same lot ID

2. Single

3. Each individual control must have the same background/autofluorescence

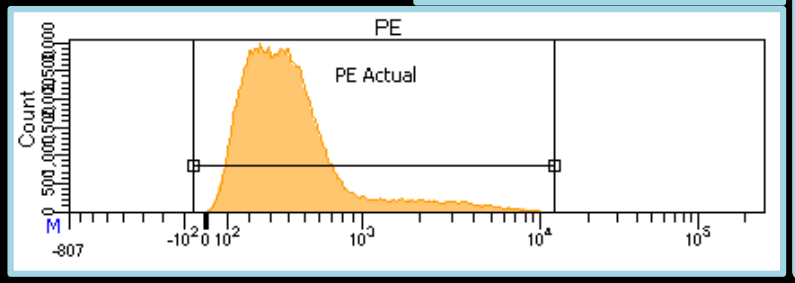
$CH1_A = CH1_O - (CH2_O)\ (\%_S 2 \rightarrow 1)$

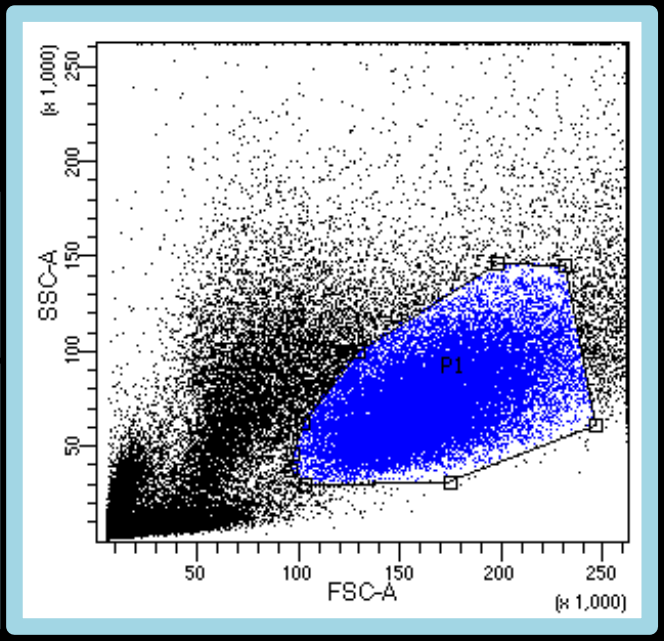




Population	PE-A Mean
☒ PE Actual	716

Population	PE-A Mean
☒ PE Actual	626





Avoid Many Problems and Simply Use Compensation Beads

Beads and the 3 Rules

1. Single-color controls must be as bright or brighter than samples that compensation will be applied to
   - Tend to be very bright and stain well with antibody
     - On rare occasion they will be more dim but bright beads are sold and a mix of cells for some controls and beads for other controls can be used
2. Single color compensation controls must use the same fluorochromes as are used in the experiment
   - Use same antibody on sample and control beads
     - *Note: not all species available
3. Each individual control must have the same background/autofluorescence
   - Compensation bead kits always contain unstained beads or can be purchased separately
   - Some cell types have very high autofluorescence and so it will not be possible to keep beads and sample on scale with matching detector voltage gain settings

$$CH1_A = CH1_O - (CH2_O)\,(\%_S 2 \rightarrow 1) - (CH3_O)\,(\%_S 3 \rightarrow 1) \ldots - (CHn_O)\,(\%_S n \rightarrow 1)$$

Final Note:
NEVER change laser voltage or detector gain/voltage after setting compensation controls