Advanced Topics in Compensation & Panel Design

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January 30, 2020

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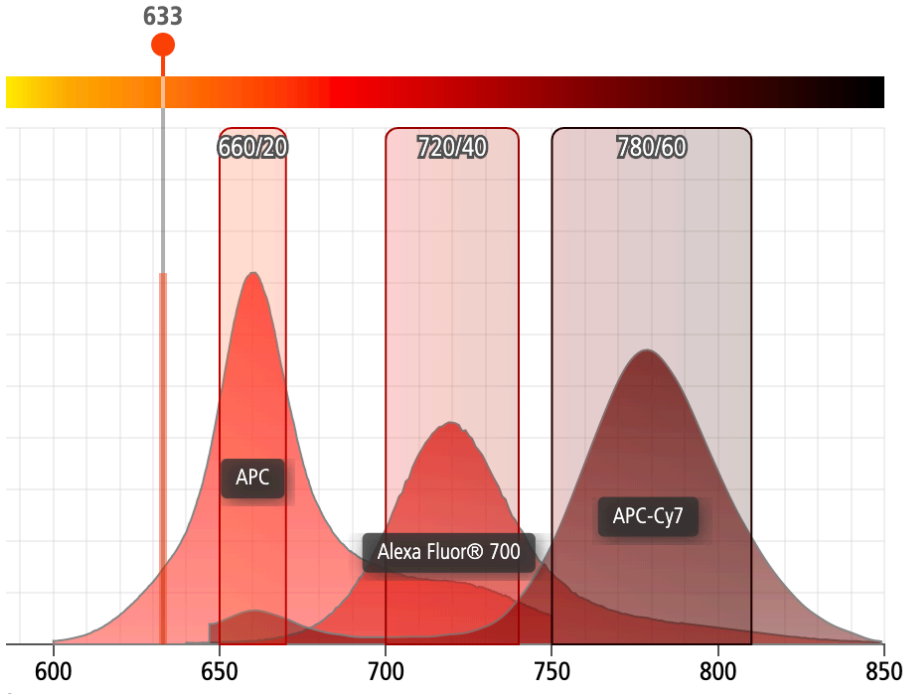
OVERVIEW

- Compensation
- Spillover/spreading error
- Panel Design

Compensation – Why is it Necessary?

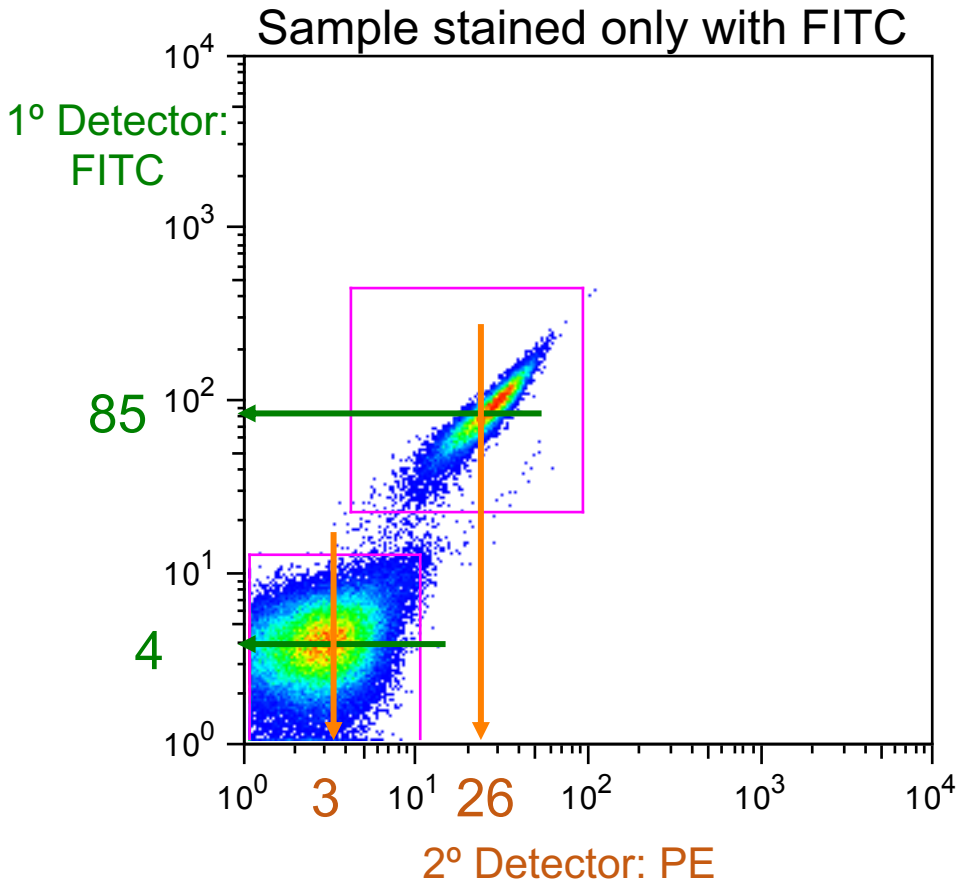
- Light is not discrete
  - Spectral overlap between dyes results in the detection of the primary fluorochrome in one or more secondary detectors
  - Spillover fluorescence must be subtracted from the total fluorescence detected in the secondary detector(s)



Spillover characteristics of dyes excited by the red laser

Compensation – Uncompensated Data

- Spillover fluorescence is proportional to the level of the primary fluorescence
- The percent spillover of the primary fluorescence is subtracted from the total fluorescence in the secondary detector, <u>on a per cell basis</u>



% spillover of FITC into PE=

$$\frac{\text{Fluorescence}_{FL2}}{\text{Fluorescence}_{FL1}} \times 100$$

$$\frac{MFI_{FL2}(pos) - MFI_{FL2}(neg)}{MFI_{FL1}(pos) - MFI_{FL1}(neg)} \times 100$$

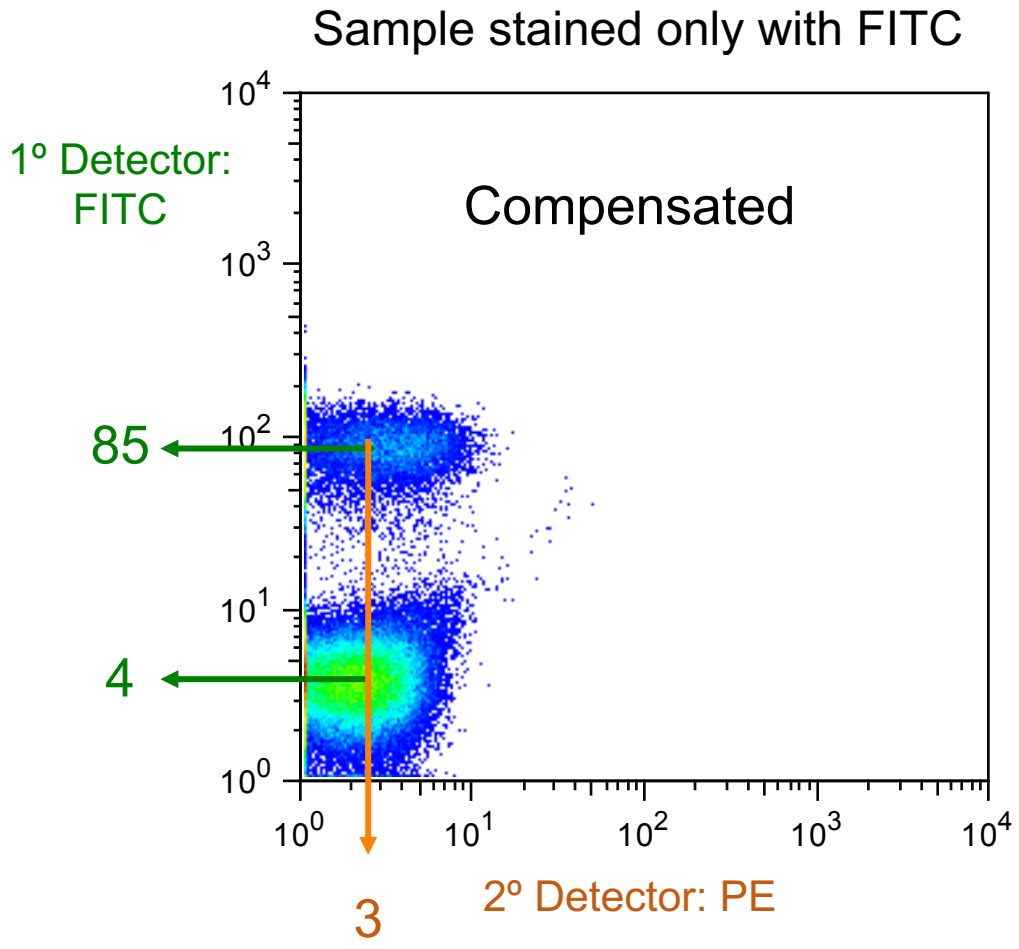
$$\frac{26 - 3}{85 - 4} \times 100 = 28\%$$

*we are only considering the signal of the FITC fluorochrome

Data provided by J. Stucky, 041012

COMPENSATION – COMPENSATED DATA

- 28% of the total signal measured in the secondary (PE) detector is removed
- After compensation, the median fluorescence signal of the primary antibody as seen in the secondary detector is the same for both the negative and positive populations
- Repeat for every detector
- Repeat for every single stained antibody



Data provided by J. Stucky, 041012

Compensation – example of a 17 colour compensation matrix

Detector

Fluorochrome

	B515-A	B710-A	G575-A	G610-A	G780-A	R660-A	R710-A	R780-A	V450-A	V510-A	V570-A	V610-A	V655-A	V710-A	V780-A	U395-A	U730-A
B515-A		0.7944	0.7875	0.2066	0	0	0	0	0	6.014	2.67	0.9578	0.1865	0.05617	0	0	0
B710-A	-0.2832		0	-0.07375	60.36	52.87	85.76	26.43	0	0	-0.385	-0.3417	9.501	93.98	26.16	0	30.22
G575-A	0.1528	3.821		36.61	1.501	0	0	0	0.05965	0.215	19.3	9.509	2.184	1.003	0.1002	0.06125	0.3351
G610-A	-0.1434	12.9	16.1		6.653	0.6604	0.2865	0	-0.3857	-0.5716	1.9	17.58	6.52	4.078	0.6893	-0.08108	1.402
G780-A	0.1134	0.5811	1.442	0.5426		0	0.5166	4.278	0	0.1177	0.1467	0	0	0	7.621	0	0.7713
R660-A	0.4102	0.35	0.1561	0.2527	2.526		48.95	12.46	0.3762	1.95	0.5256	0	8.681	3.646	0.5578	0.4889	3.455
R710-A	0.1444	1.404	0.2299	0.1871	3.635	2.79		22.98	0.2996	0.7349	0.5548	0.5021	0.325	7.304	3.364	0.1122	5.657
R780-A	0	0	0	0	16.22	1.656	6.718		0	0.07643	0	0.08833	0.1529	0.1376	15.01	0	1.058
V450-A	0	0	0	0	0	0	0	0		35.9	6.594	2.012	0.3288	0.1027	0	0	0
V510-A	0.4265	0.1497	0.3632	0.3411	0.1531	0.241	0.2389	0.08493	7.772		61.35	27.3	5.614	2.313	0.4725	0	0.6766
V570-A	0.5978	0.9308	33.57	19.12	1.369	0.4577	0.3353	0.1449	11.79	2.97		67.03	20.12	10.81	2.194	0.361	1.786
V610-A	-0.7727	0.6499	3.975	23.89	2.782	0.4005	0.1677	0	4.046	0	3.719		42.01	23.03	5.687	-0.1008	6.145
V655-A	-0.06703	0.439	0.1453	1.153	1.046	30.23	16.65	3.808	6.005	1.181	0.3542	11.03		56.3	10.25	0	11.31
V710-A	0.8312	5.733	0.1382	0.07761	1.796	3.192	38.65	12.7	7.025	1.491	0.7546	1.216	2.311		48.69	0	41.09
V780-A	0	0	0	0	4.591	0	0.7995	5.395	3.927	0.749	0.1963	0.1698	0.1273	0.8749		0	4.389
U395-A	0.06651	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1034		0.07176
U730-A	0	5.159	0	0	1.335	0.312	29.83	12.27	0	0	0	0	0	2.066	2.965	3.504	

COMPENSATION – PRACTICAL CONSIDERATIONS(1)

- PMT voltages must be set properly before acquiring compensation samples and remain unchanged
  - ➢ Changing PMT voltages will change compensation requirements

- Make single stained compensation controls – one for each fluorochrome in the panel
  - ➢ Each sample must be stained with ONLY ONE antibody
  - ➢ Control must be **as bright or brighter** than the experimental sample
  - ➢ Ideally use the same reagent as used in the staining panel
    - Utilize compensation beads (check species reactivity and isotype)
  - ➢ Stain comp control with CD4 in the same fluorochrome

COMPENSATION – PRACTICAL CONSIDERATIONS(2)

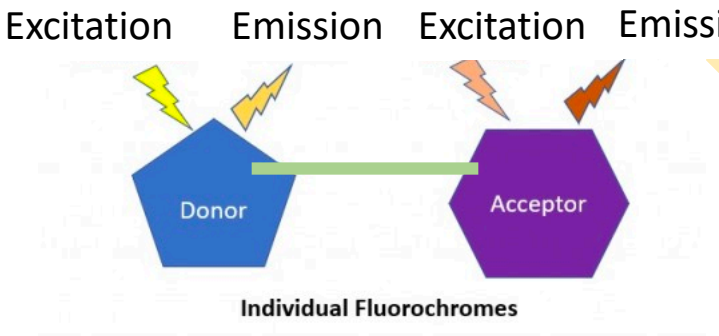
- Treat the compensation controls **exactly** like the experimental sample
- Positive and negative populations within a compensation control must be **of the same kind** (i.e. have the same autofluorescence)
  - Do not use a positive bead and a negative cell in combination

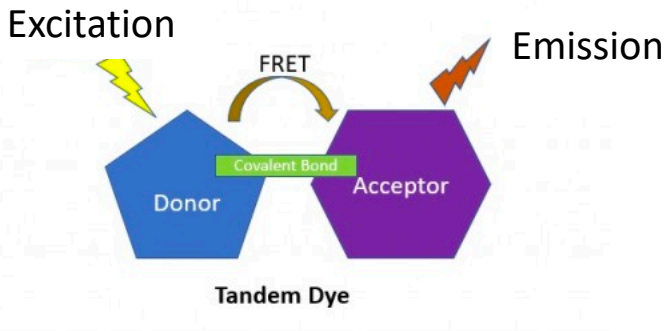
COMPENSATION – TYPES OF SINGLE STAINED CONTROLS

- Antibody capture beads
  - Test ahead for intensity/binding – isotype and species reactivity
  - Titrate reagent on beads if too bright
  - Be sure that the negative beads are the same as the positive beads
  - Not all fluorophores accurately compensate on beads (see TDS from manufacturer)
- Single stained cells
  - Give the most accurate compensation matrix (especially for larger panels)
  - Some markers may not stain “normal” cells – require stimulation (activation markers, cytokines)
  - Positive and negative cells must have the same autofluorescence
    - Proper negative for CD14 expressed on monocytes is the scatter gated monocytes on an unstained sample or the single stained control spiked with unstained cells prior to acquisition

COMPENSATION – TANDEM DYES

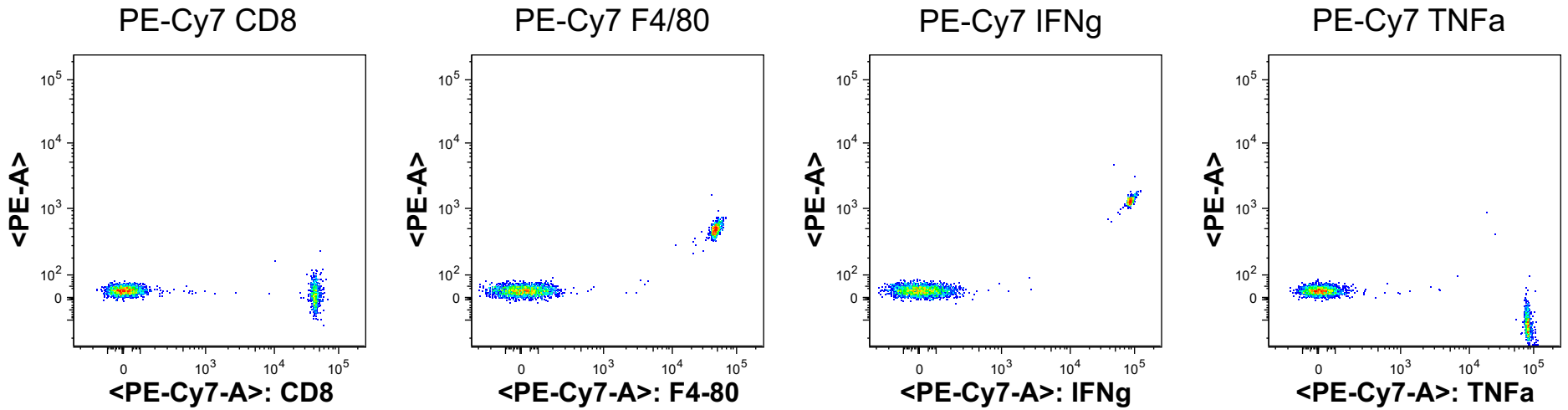
- A tandem dye consists of a donor and acceptor fluorochrome that are covalently bonded
- Donor molecule transfers excitation energy to the acceptor molecule via FRET (fluorescence resonance energy transfer)
- Acceptor molecule gives off light
- Usually the first molecule excited by a laser line serves as the “base” for the remainder of the fluorophores excited by the same laser
  - i.e.: Green laser fluorophores: PE, PE-CF594, PE-Cy5, PE-Cy5.5, PE-Cy7





COMPENSATION – TANDEM DYES

- Tandem dyes will differ:
  - In their spillover characteristic between different antibody conjugates
  - **Lot to lot** for the same antibody conjugate
  - Between manufacturers
- Be aware that many BV (brilliant violet) and BUV (brilliant UV) dyes are also tandems!



All are compensated with the single stained control for CD8-PE-Cy7

Images courtesy of Florian Mair

COMPENSATION – ADDITIONAL NOTES ON FLUOROCHROMES

- Spectral properties change over time due to exposure to light and fixation reagents
  - Tandem dyes are susceptible to degradation over time
- Minimize exposure to light during staining and store stained samples in the dark
- Minimize concentration of fixative in the final resuspension volume (0.5 to 1% PFA) or wash out and resuspend in wash buffer for longer term storage

Compensation – log scale versus linear scale

- Fluorescence is usually displayed on a log scale
- Log display may skew perception of the data and lead to manual overcompensation
  - Events at zero are squished against the axis (log scale does not go below zero)

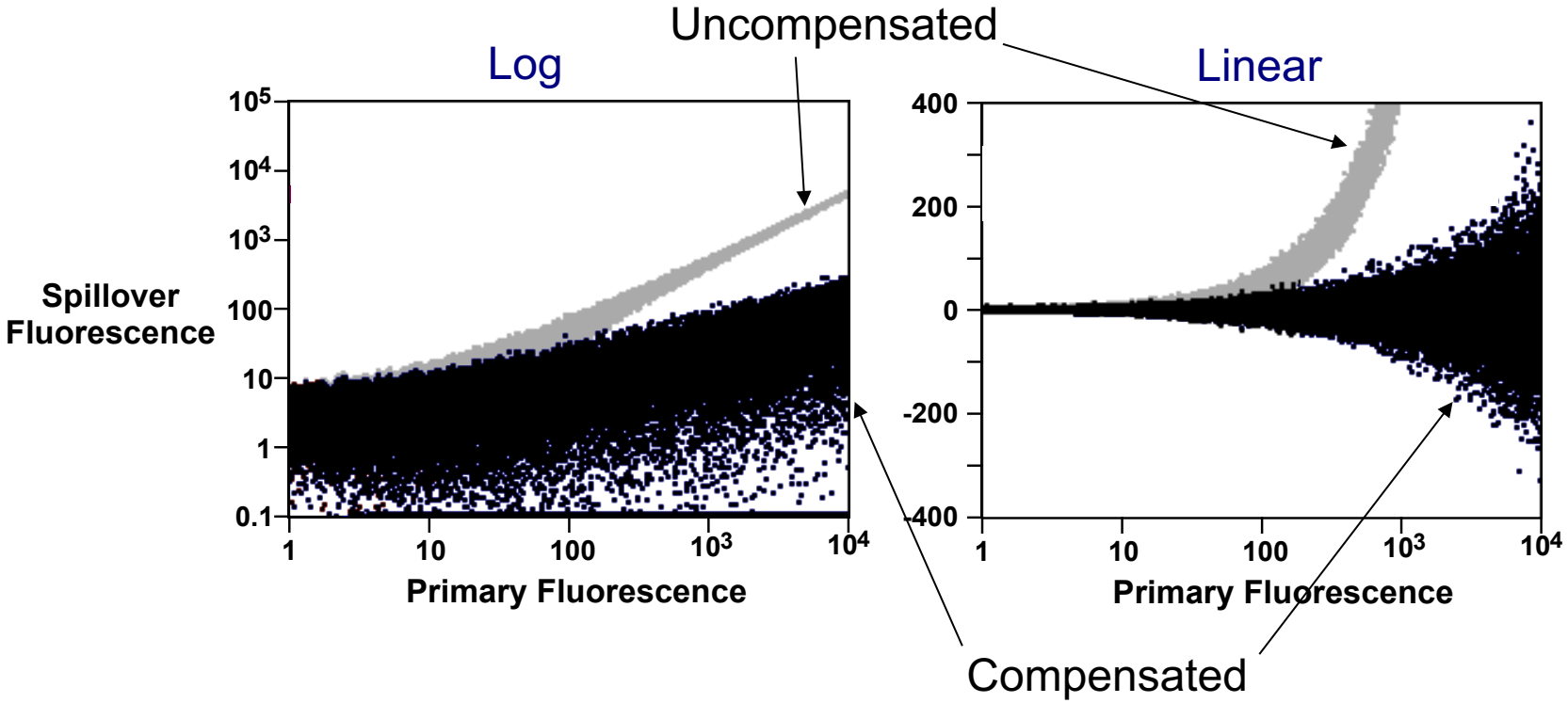


Image courtesy of M. Roederer, NIH

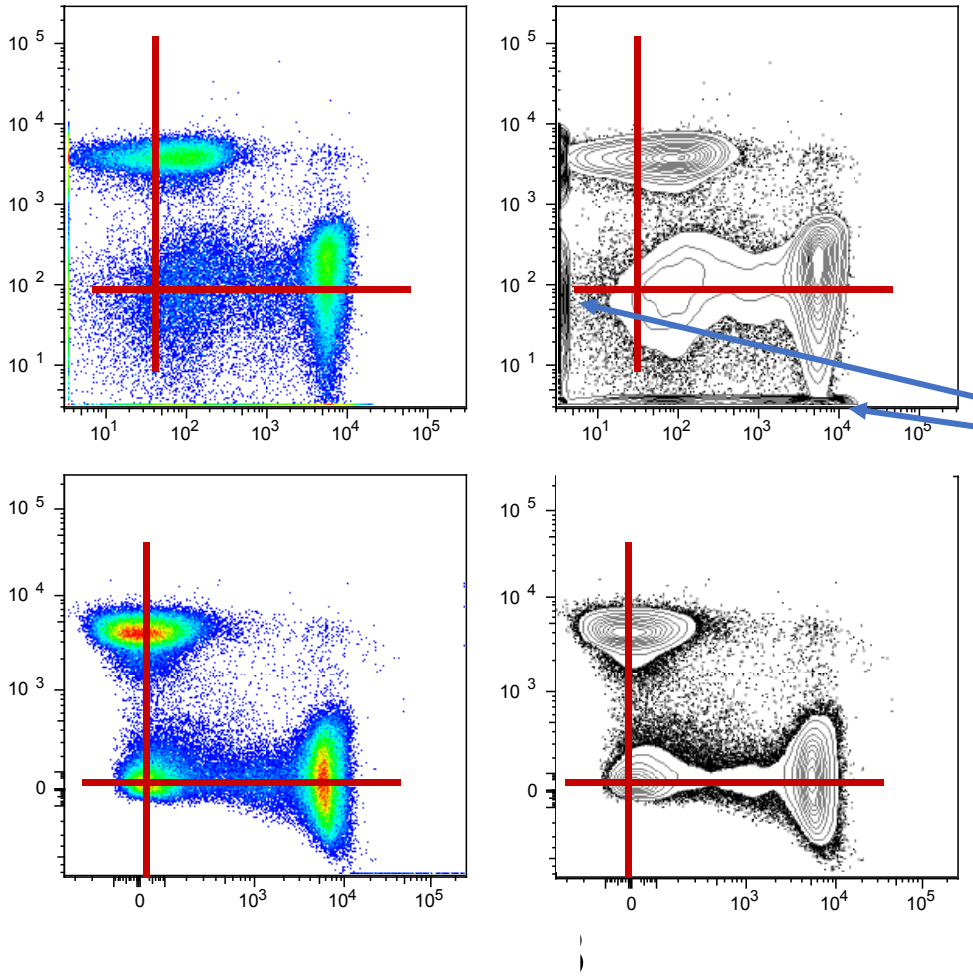
Bi-exponential or logicle transformation

- Transforms the log scale to display values below zero
- Allows for better visualization of populations centered around zero
- Feature is available in most FACS analysis software
- **Always analyze properly transformed data!**

TRANSFORMATION CONFIRMS CORRECT COMPENSATION

Not Transformed

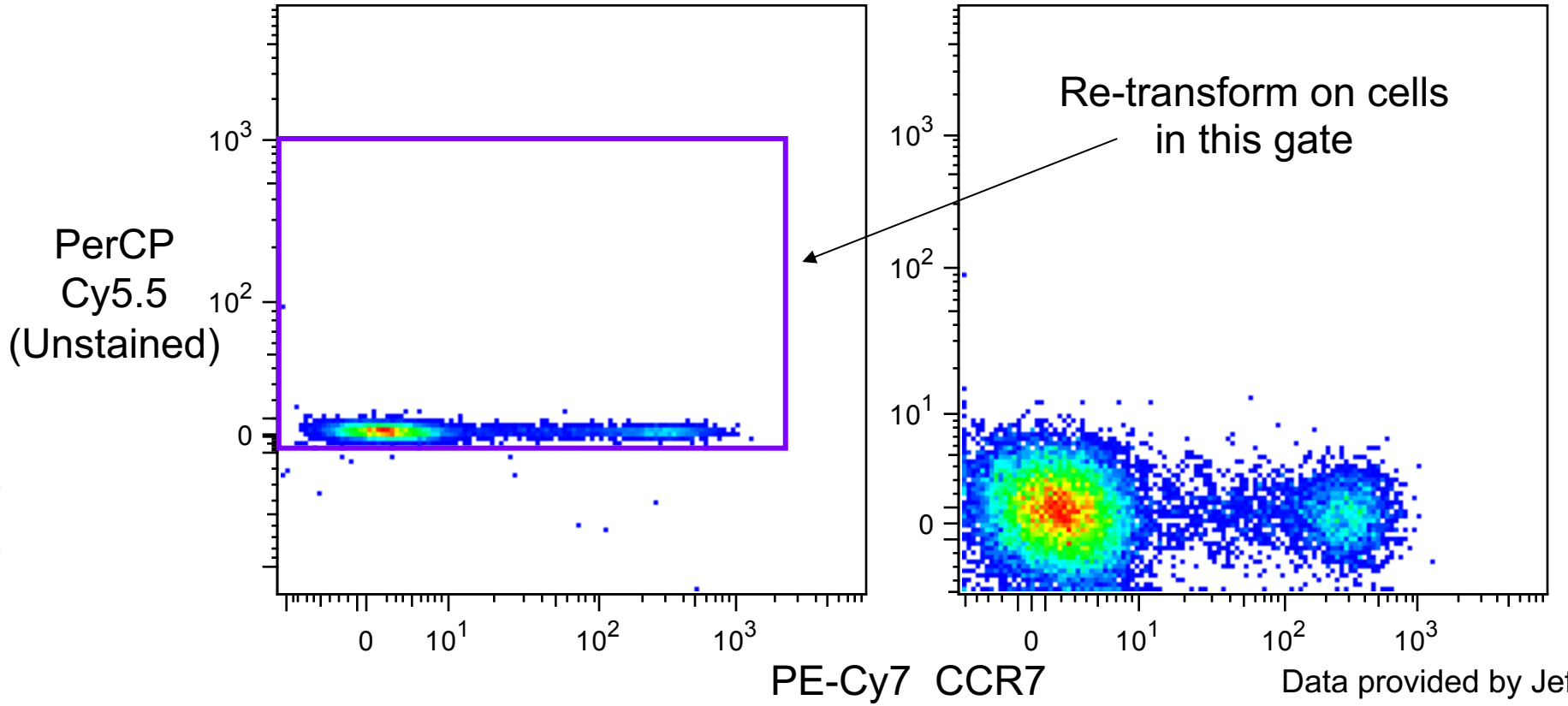
Transformed



Slide courtesy of M. Roederer, NIH

RE-TRANSFORMATION

- Cells with large negative fluorescence values affect transformation
- Excluding these cells by drawing a temporary gate and retransforming produces better results



Data provided by Jeff Pufnock, 061307, CD8 FMO

RE-TRANSFORMATION – INCORRECT GATE CHOSEN IN FLOWJO

061307 FMO stain #2

02:05

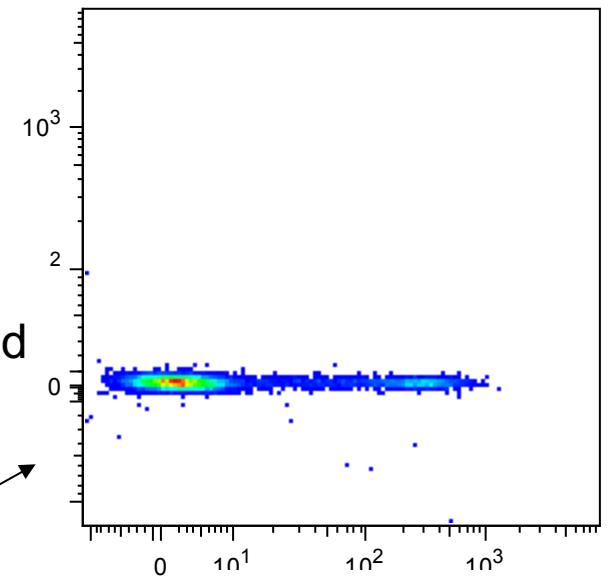
All Samples	26
Compensation Controls	14
FMO with final Comp Matrix	12
FSC-H, SSC-A subset	
Memory pannel FMO	12

Edit... Sort...

Name	Statistic	#Cells	TUBE NAME	Compensation matrix
Specimen_001_ALL STAINS.fcs		36065	ALL STAINS	061807-Final Memory Comp Matrix
Specimen_001_CCR-7 MINUS.fcs		37205	CCR-7 MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD8 MINUS.fcs		35501	CD8 MINUS	061807-Final Memory Comp Matrix
FSC-H, SSC-A subset	54.6	19373		
Exclude low PerCP-Cy5.5	100	19373		
Specimen_001_CD27 MINUS.fcs		36040	CD27 MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD28 MINUS.fcs		41066	CD28 MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD45RA MINUS.fcs		36260	CD45RA MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD45RO MINUS.fcs		34325	CD45RO MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD62L MINUS.fcs		34233	CD62L MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD127 MINUS.fcs		35919	CD127 MINUS	061807-Final Memory Comp Matrix
Specimen_001_LD MINUS.fcs		34141	LD MINUS	061807-Final Memory Comp Matrix
Specimen_001_MEL-PENT MINUS.fcs		36703	MEL-PENT MINUS	061807-Final Memory Comp Matrix
Specimen_001_unstained.fcs		77939	unstained	061807-Final Memory Comp Matrix

Transforming when this gate is selected produces this result

PerCP Cy5.5 Unstained



PE-Cy7 CCR7

Data provided by Jeff Pufnock, 061307, CD8 minus FMO

RE-TRANSFORMATION – CORRECT GATE CHOSEN IN FLOWJO

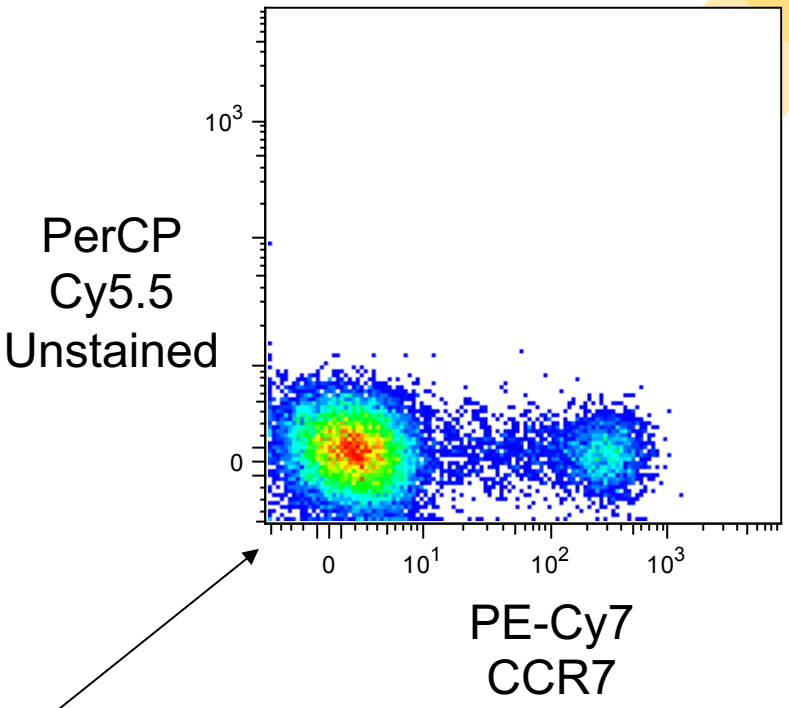
061307 FMO stain #2

07:20

All Samples	26				
Compensation Controls	14				
FMO with final Comp Matrix	12				
FSC-H, SSC-A subset					
Memory pannel FMO	12				

Edit... Sort...

Name	Statistic	#Cells	TUBE NAME	Compensation matrix
Specimen_001_ALL STAINS.fcs		36065	ALL STAINS	061807-Final Memory Comp Matrix
Specimen_001_CCR-7 MINUS.fcs		37205	CCR-7 MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD8 MINUS.fcs		35501	CD8 MINUS	061807-Final Memory Comp Matrix
FSC-H, SSC-A subset	54.6	19373		
Exclude low PerCP-Cy5.5	100	19373		
Specimen_001_CD27 MINUS.fcs		36040	CD27 MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD28 MINUS.fcs		41066	CD28 MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD45RA MINUS.fcs		36260	CD45RA MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD45RO MINUS.fcs		34325	CD45RO MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD62L MINUS.fcs		34233	CD62L MINUS	061807-Final Memory Comp Matrix
Specimen_001_CD127 MINUS.fcs		35919	CD127 MINUS	061807-Final Memory Comp Matrix
Specimen_001_LD MINUS.fcs		34141	LD MINUS	061807-Final Memory Comp Matrix
Specimen_001_MEL-PENT MINUS.fcs		36703	MEL-PENT MINUS	061807-Final Memory Comp Matrix
Specimen_001_unstained.fcs		77939	unstained	061807-Final Memory Comp Matrix



Transforming when this gate is selected produces this result

Data provided by Jeff Pufnock, 061307, CD8 minus FMO

COMPENSATION ERRORS - DIAGNOSIS

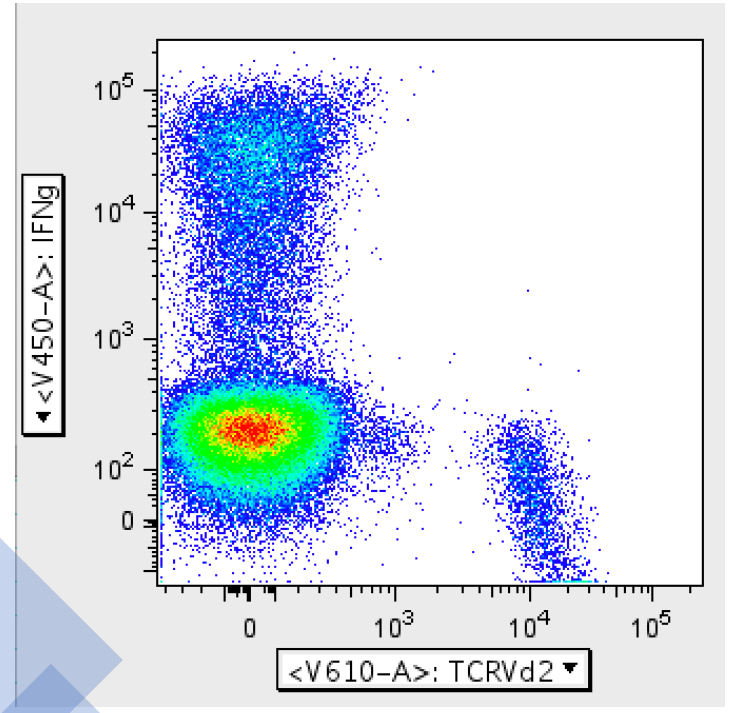
- Consider an error in compensation in the following situations
  - Diagonal staining populations (except for two markers with correlated expression, e.g. IFNg vs TNFa
  - Unexpected positive populations (e.g. high frequency of cells expected at low frequencies, CD25, IL-4…)
  - Cells leaning over the axis

Compensation errors – Investigative options

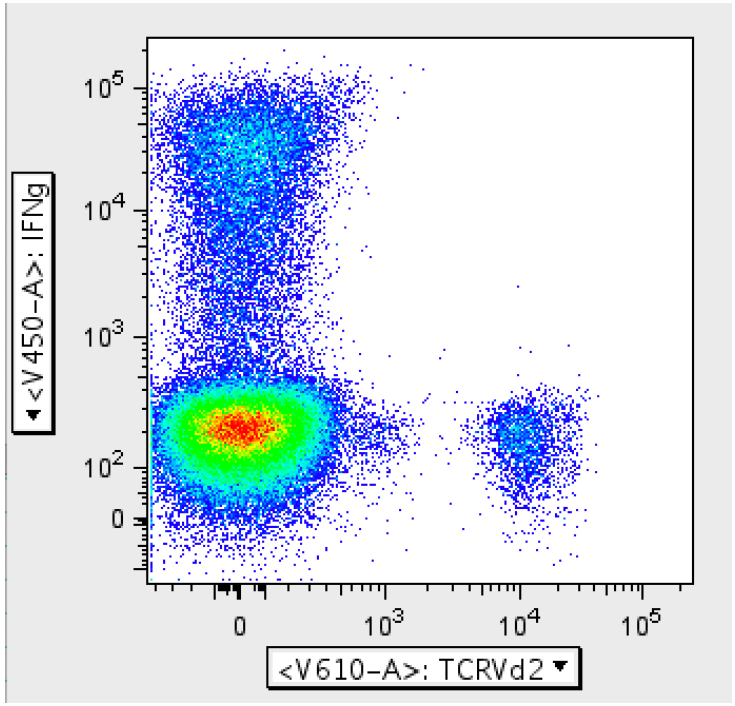
- Steps to investigate potential compensation errors:
  - Apply custom transformation to visualize negative events and to assess medians
  - Visualize each parameter versus all others to search for unobserved compensation issues (multigraph overlay, N by N plot)
  - Apply compensation to the compensation samples
    - Does the compensation matrix need to be re-calculated or is the comp matrix not working for the test samples but okay for the comp samples
  - Ensure that the compensation sample is bright enough and/or that the gate is placed high enough
    - Calculation is based on the median fluorescence in the positive gated population
    - Higher gates useful for markers with continuous distribution
  - Ensure that there are enough events in the positive population
  - Use a compensation control(s) from another experiment and remake the matrix

EXAMPLE PLOTS OF OVER, UNDER, AND CORRECTLY COMPENSATED DATA

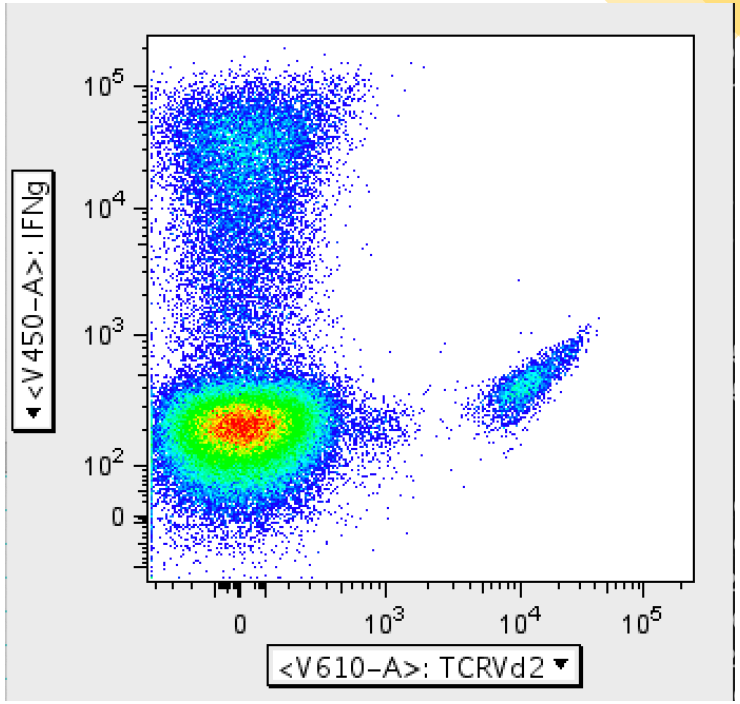
Primary fluorochrome on x-axis, secondary detector on the y-axis



Over Compensated
V450 MFI on V610+ cells is <u>less than</u> V450 MFI on V610- cells



Correctly Compensated
V450 MFIs of V610+/- cells are idential



Under Compensated
V450 MFI on V610+ cells is <u>greater than</u> V450 MFI on V610- cells

MANUAL ADJUSTMENT OF THE COMPENSATION MATRIX

- Only to be done if the reason for the matrix failure cannot be identified

AND

- It is certain that there is an error in compensation
  - ➢ Do not overcompensate to attempt to correct for spreading error!
- Each row shows the percent signal of a fluorochrome subtracted from each detector, listed in columns
  - ➢ Overcompensated – decrease number
  - ➢ Undercompensated – increase number

Detector

Fluorochrome	B515-A	B710-A	G575-A	G610-A	G780-A	R660-A	R710-A
B515-A		0.7944	0.7875	0.2066	0	0	0
B710-A	-0.2832		0	-0.07375	60.36	52.87	85.76
G575-A	0.1528	3.821		36.61	1.501	0	0
G610-A	-0.1434	12.9	16.1		6.653	0.6604	0.2865
G780-A	0.1134	0.5811	1.442	0.5426		0	0.5166
R660-A	0.4102	0.35	0.1561	0.2527	2.526		48.95
R710-A	0.1444	1.404	0.2299	0.1871	3.635	2.79	
R780-A	0	0	0	0	16.22	1.656	6.718
V450-A	0	0	0	0	0	0	0
V510-A	0.4265	0.1497	0.3632	0.3411	0.1531	0.241	0.2389
V570-A	0.5978	0.9308	33.57	19.12	1.369	0.4577	0.3353
V610-A	-0.7727	0.6499	3.975	23.89	2.782	0.4005	0.1677
V655-A	-0.06703	0.439	0.1453	1.153	1.046	30.23	16.65
V710-A	0.8312	5.733	0.1382	0.07761	1.796	3.192	38.65
V780-A	0	0	0	0	4.591	0	0.7995
U395-A	0.06651	0	0	0	0	0	0
U730-A	0	5.159	0	0	1.335	0.312	29.83

A NOTE ABOUT COMPENSATION PERCENTAGES

- Actual percentages required to compensate for the spillover are arbitrary
- Compensation percentages depend on the PMT voltage settings in the primary and secondary detectors
- Compensation values over 100% are not necessarily wrong!
  - A compensation value over 100% indicates a “brighter” signal in the secondary rather than primary detector
  - Voltages can be adjusted to avoid this
  - BUT
  - It is always better to set each detector to its optimal voltage even if it results in a compensation value of over 100%

Compensation does NOT introduce or increase error…

Compensation only reveals it!!!

Additional Resources

Basic Multicolor Flow Cytometry

UNIT 5.4

Zofia Maciorowski,[1] Pratip K. Chattopadhyay,[2] and Paresh Jain[3]

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[2]Laura and Isaac Perlmutter Cancer Center, NYU-Langone Medical Center, New York, New York
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Andrea Cossarizza et al. DOI: 10.1002/eji.201646632 Eur. J. Immunol. 2017. 47: 1584–1797

European Journal of Immunology HIGHLIGHTS

Guidelines for the use of flow cytometry and cell sorting in immunological studies

In Review

- Compensation
  - What is compensation and why it is necessary
  - Compensation controls
  - Transformation of data to confirm compensation
  - Diagnosis of compensation errors and how to fix them
- Spillover/spreading