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Compensation: selected topics

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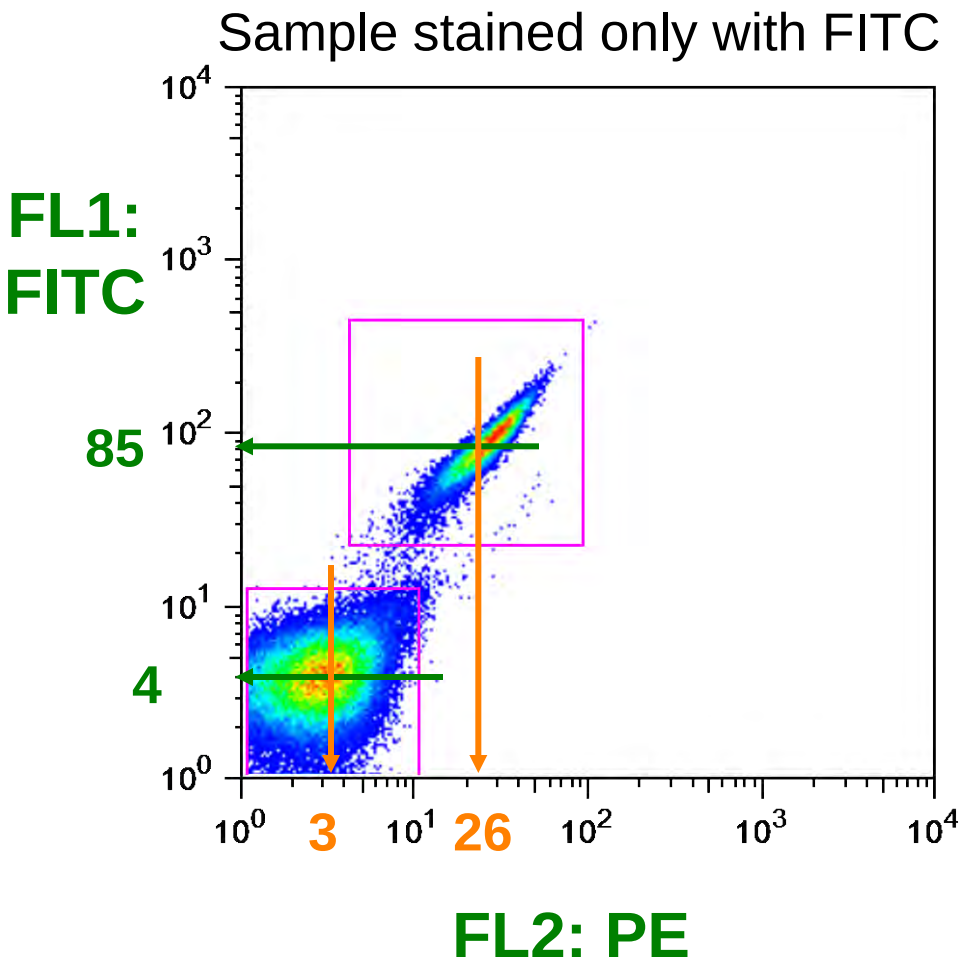
HIV VACCINE TRIALS NETWORK

Compensation

- Spectral overlap between dyes results in the detection of the fluorescence from one dye in one or more other detectors (primary vs. spillover fluorescence)
- The spillover fluorescence must be subtracted from the total fluorescence detected in the secondary detector
- This spillover fluorescence is proportional to the level of the primary fluorescence
- For each cell, a specified percentage of the primary fluorescence is subtracted from the total fluorescence detected in the secondary detector

FITC into PE



% spillover of FITC into PE=

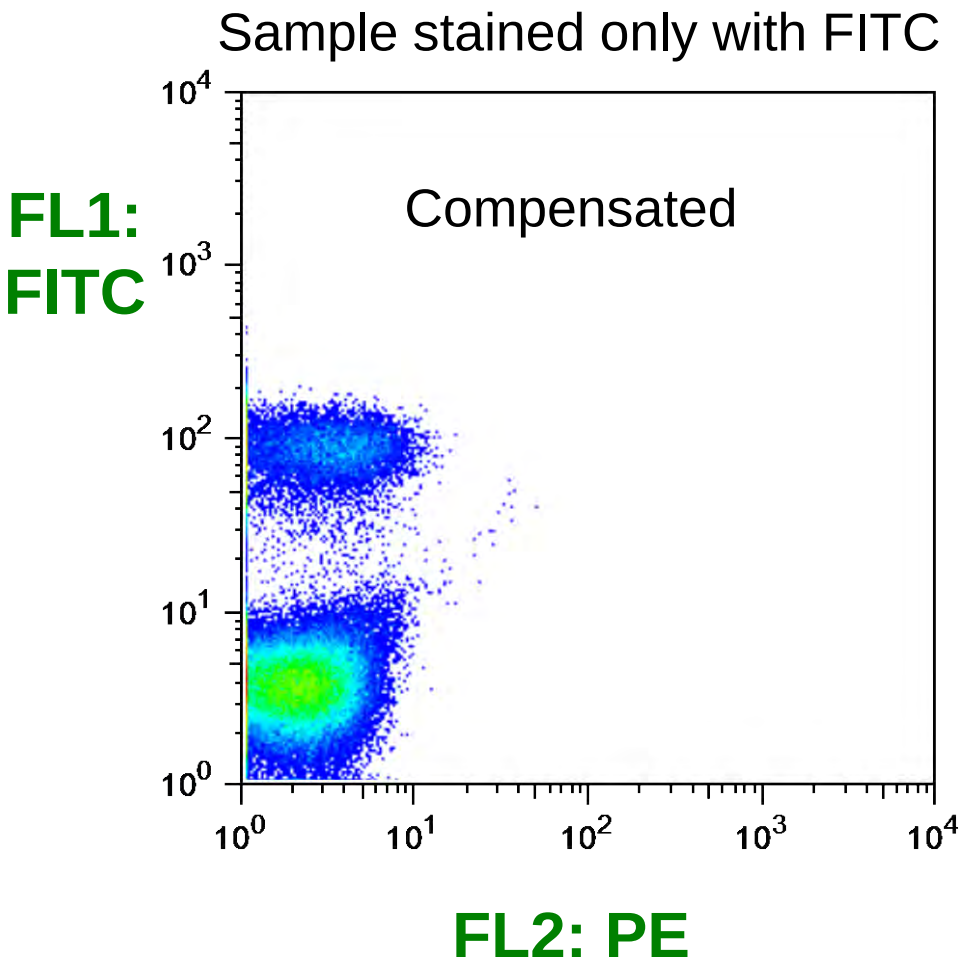
$$\frac{\text{Fluorescence}_{FL2}}{\text{Fluorescence}_{FL1}} \times 100$$

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

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

Data provided by J. Stucky, 041012

FITC into PE: Compensated



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Compensation….

- For digital data collection, compensation on-line and compensation post-collection are identical.
  - This may not be true for analog data
- One advantage of compensating at collection is that you can then view compensated data during collection.
- However, if multiple staining panels are used requiring different compensation requirements, then it can be confusing at collection to assign the different comp requirements for the different panels

A few practical considerations for setting compensation

1. Be sure PMT voltages are set properly before collecting compensation samples
   - Changing PMT voltages will change compensation requirements
2. Make singly-stained compensation controls
   - One control for each fluorochrome
   - Must be as bright or brighter than the reagents used on that color in the multicolor panels (always is safe to use same reagent for comp as for multicolor panel)
   - Can use comp “beads” (note: check species and isotype)
   - Dyes like PI, EMA, CFSE require special comp samples

Practical considerations - tandems

Different lots of tandem dyes have different compensation requirements:

- Prepare single-stained controls for each tandem lot
- If tandem lot is unknown, then make a separate comp sample for each tandem-Ab conjugate
- When creating the comp matrix after collection, match the single-stained controls with the appropriate multi-stain panels

Another note on tandems

- Spectral properties change over time due to exposure to light and to fixation reagents
- Minimize exposure to light during staining and store stained samples in the dark
- Minimize concentration of fixative in final resuspension (0.5 to 1% PFA)
- Consider using tandem “stabilization” buffer?
- Ensure that compensation controls and test samples are treated the same, e.g., all receive fixation/permeabilization

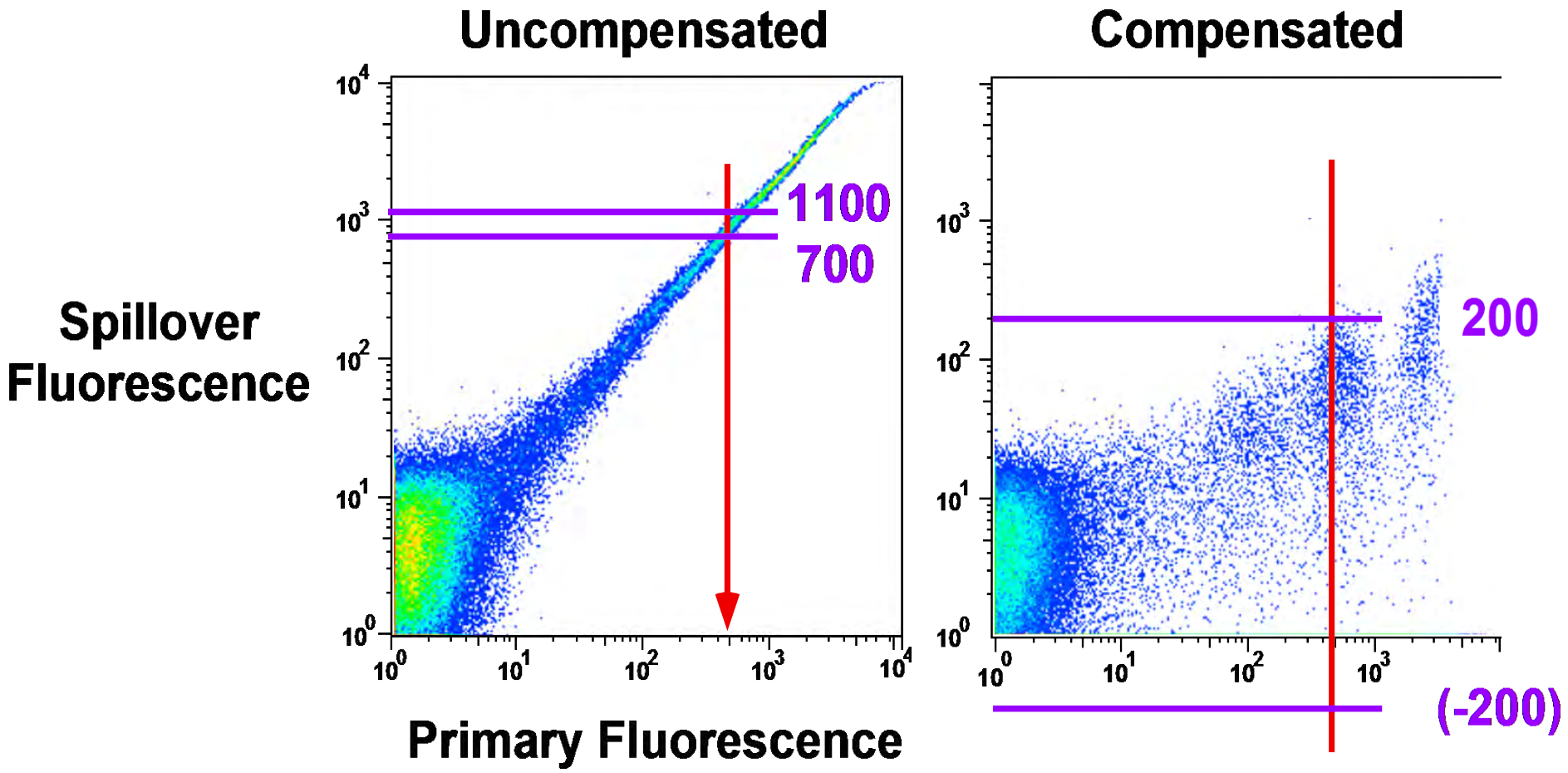
Advanced topics in Fluorescence Spillover Compensation

Mario Roederer, Cytometry 45:194-205 (2001)

Spectral Compensation for Flow Cytometry: Visualization Artifacts, Limitations, and Caveats

Nicole Baumgarth and Mario Roederer
Journal of Immunological Methods 243:77-97 (2000)
A practical approach to multicolor flow cytometry for immunophenotyping

Imperfect Measurement Leads to Apparent Spread in Compensation



Slide provided by M. Roederer, NIH

“Errors” in Compensation

- Properly compensated data may not appear rectilinear (“rectangular”), because of measurement errors.
- This effect on compensated data is unavoidable, and it cannot be “corrected”.
- It is important to distinguish between incorrect compensation and the effects of measurement errors.

Slide provided by M. Roederer, NIH

Compensation Does NOT Introduce or Increase Error:

Compensation Only Reveals It!

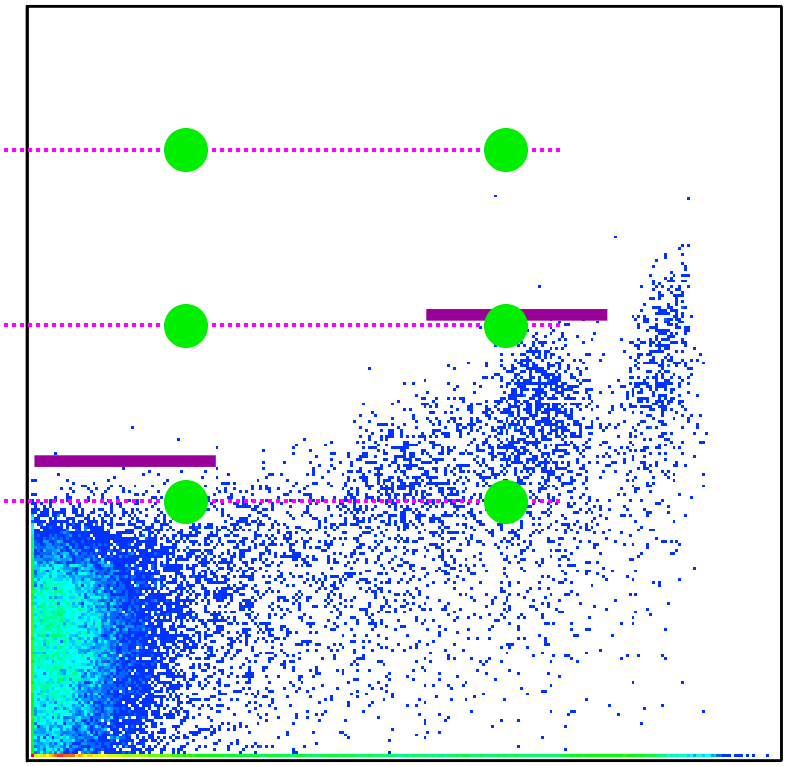
- The measurement error is already present. Compensation does not increase this error, it does not change it, it does not introduce any more error.
- Compensation simply makes the error more apparent by shifting it to the low end of the log-scale.

Slide provided by M. Roederer, NIH

Staining Controls

- Staining controls are necessary to identify cells which do or do not express a given antigen.

- The threshold for positivity may depend on the amount of fluorescence in other channels!



Slide provided by M. Roederer, NIH

Staining Controls

- Unstained cells or complete isotype control stains are *improper* controls for determining positive vs. negative expression in multi-color experiments.
- The best control is to stain cells with all reagents *except* the one of interest.

FMO Control

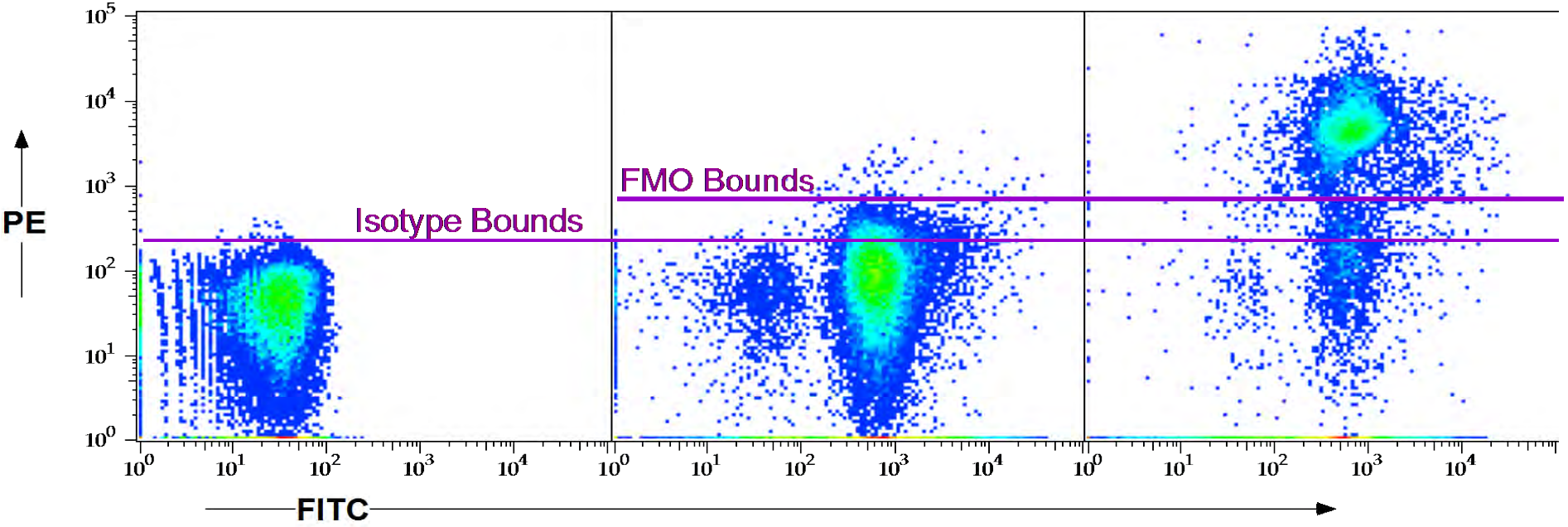
“Fluorescence Minus One”

Slide provided by M. Roederer, NIH

Identifying CD4 cells with 4 colors

PBMC were stained as shown in a 4-color experiment. Compensation was properly set for all spillovers

	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO



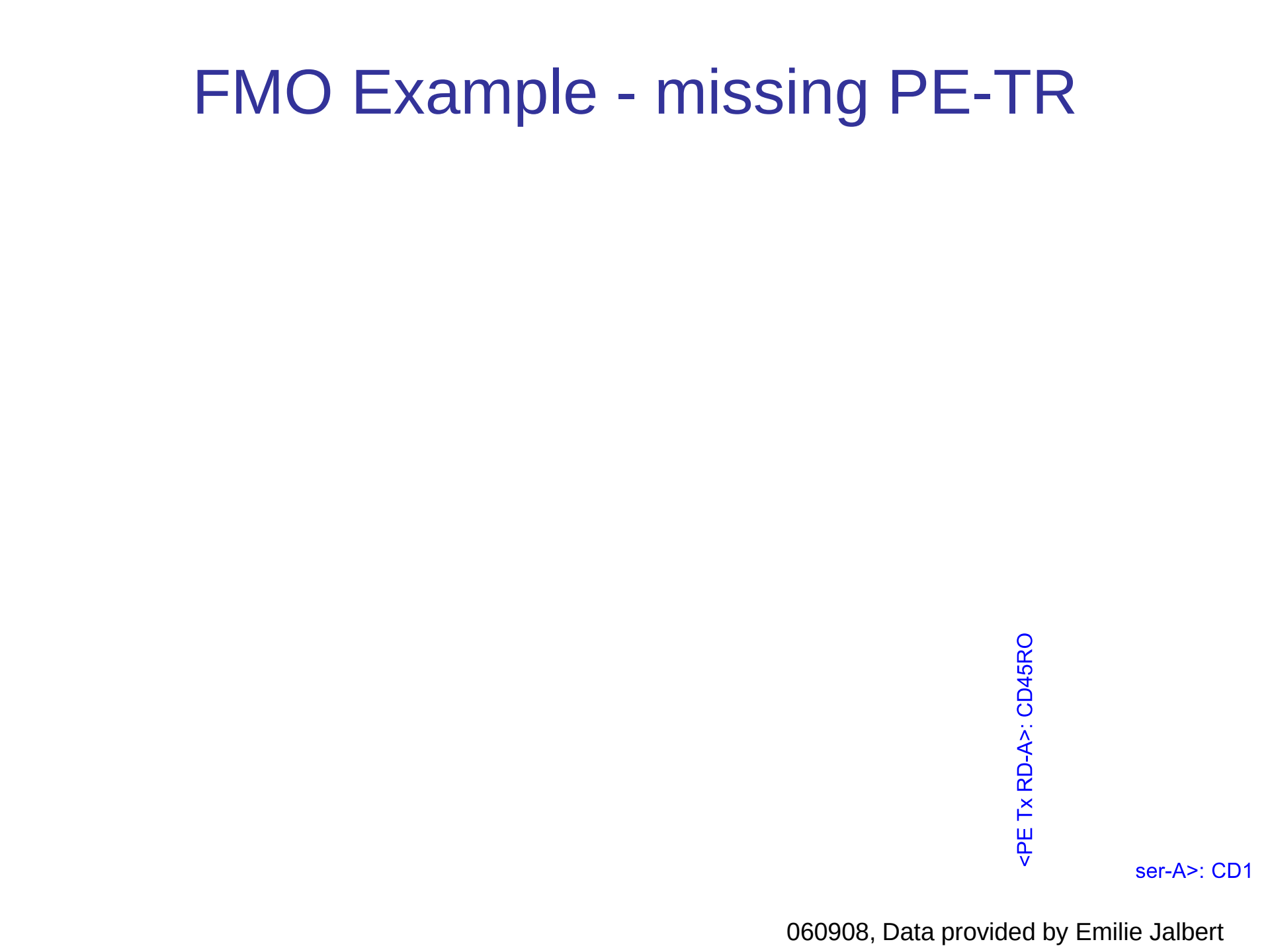
Slide provided by M. Roederer, NIH

FMO Controls

- FMO controls are a much better way to identify positive vs. negative cells

- FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

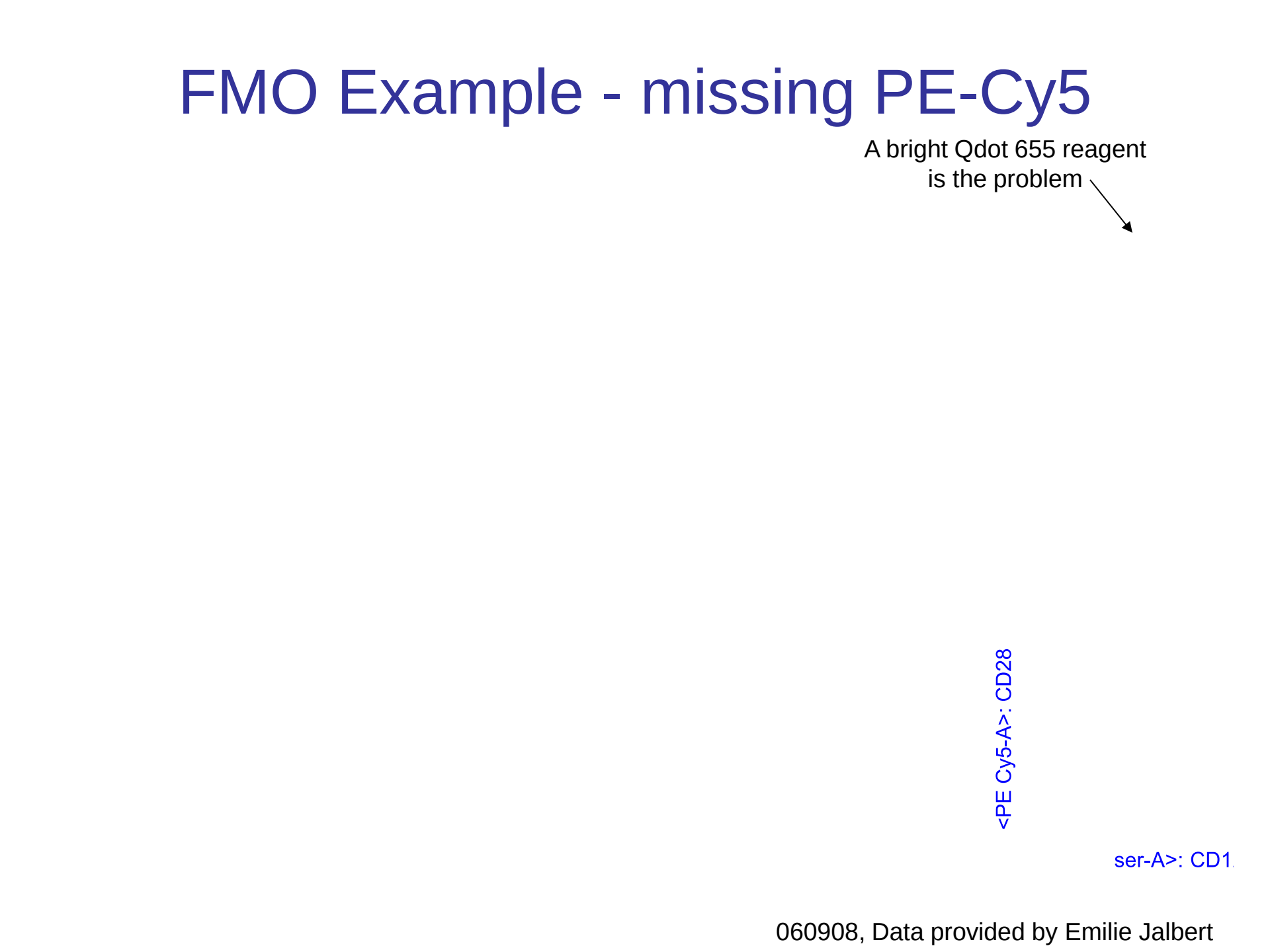
FMO Example - missing PE-TR



060908, Data provided by Emilie Jalbert

FMO Example - missing PE-Cy5

A bright Qdot 655 reagent
is the problem



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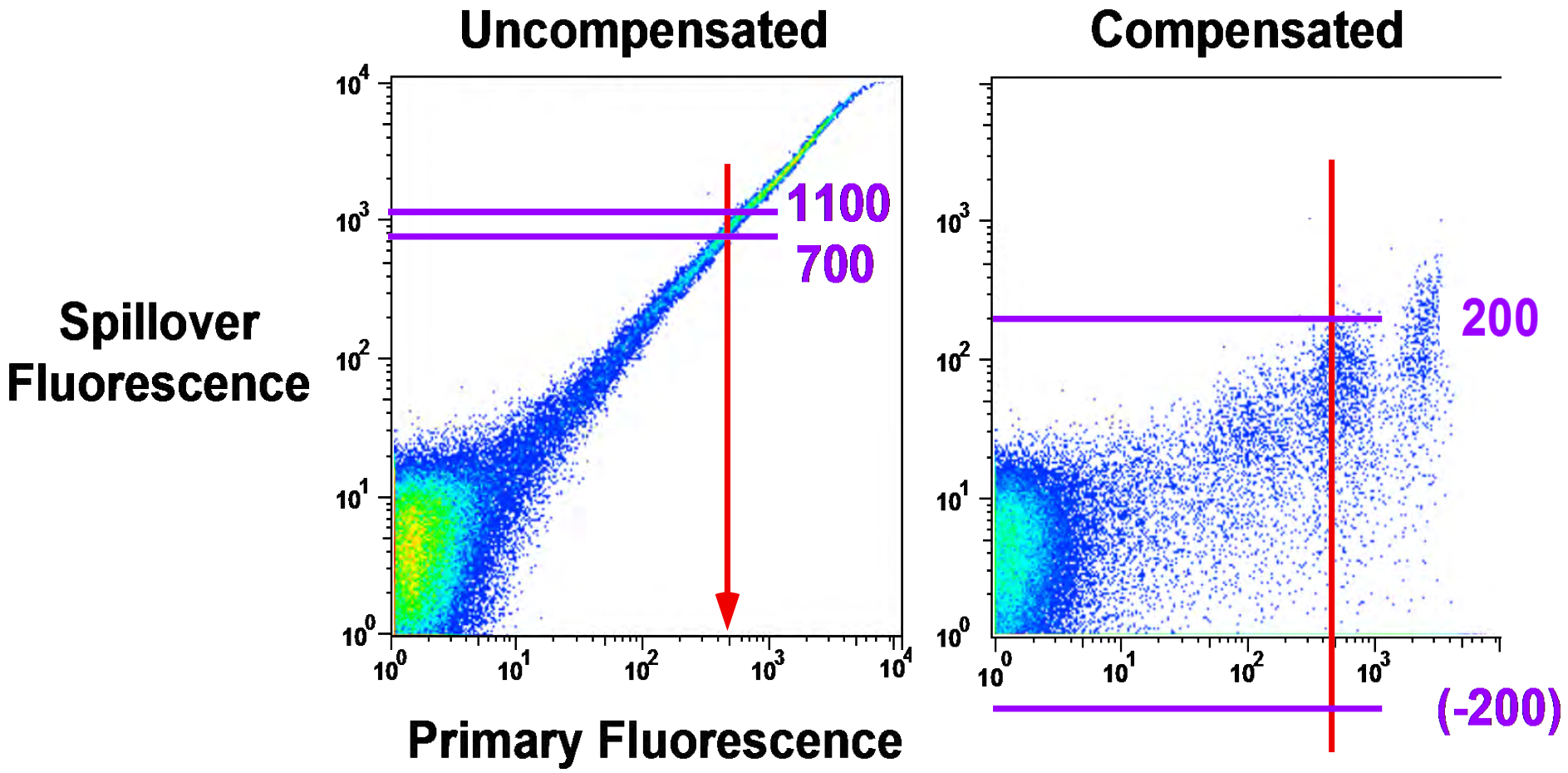
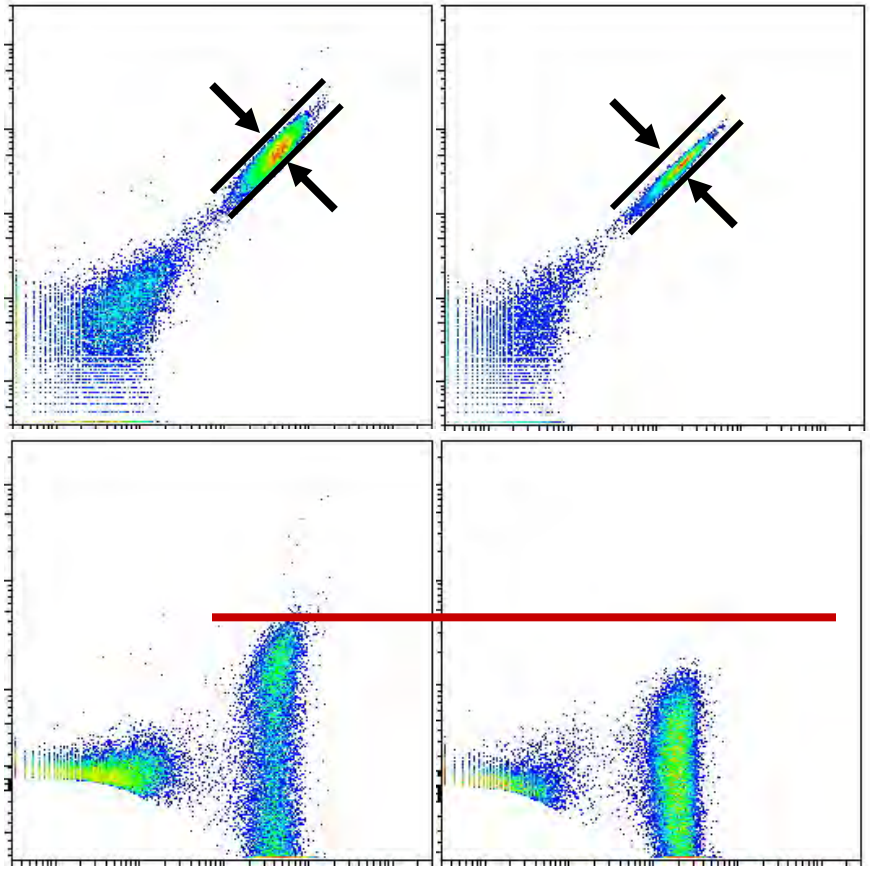
Imperfect Measurement Leads to Apparent Spread in Compensation



Slide provided by M. Roederer, NIH

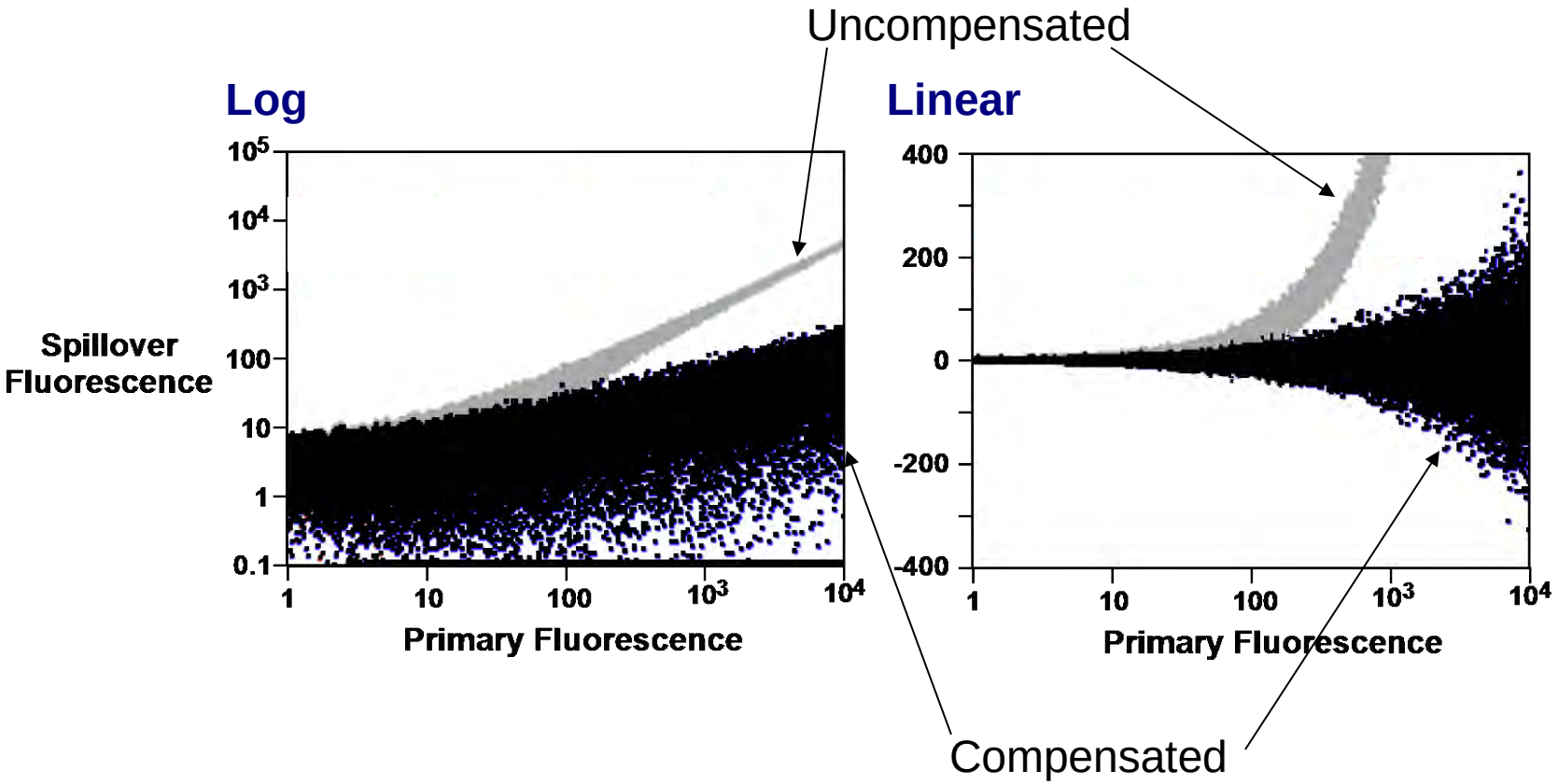
Good Instrument Alignment Is Critical!



While the amount of compensation did not differ, the measurement error (correlation) decreased leading to much better visualization of the population!

Slide provided by S. Perfetto

Log Transformation of Data Display Leads to Manual Overcompensation



Slide provided by M. Roederer, NIH

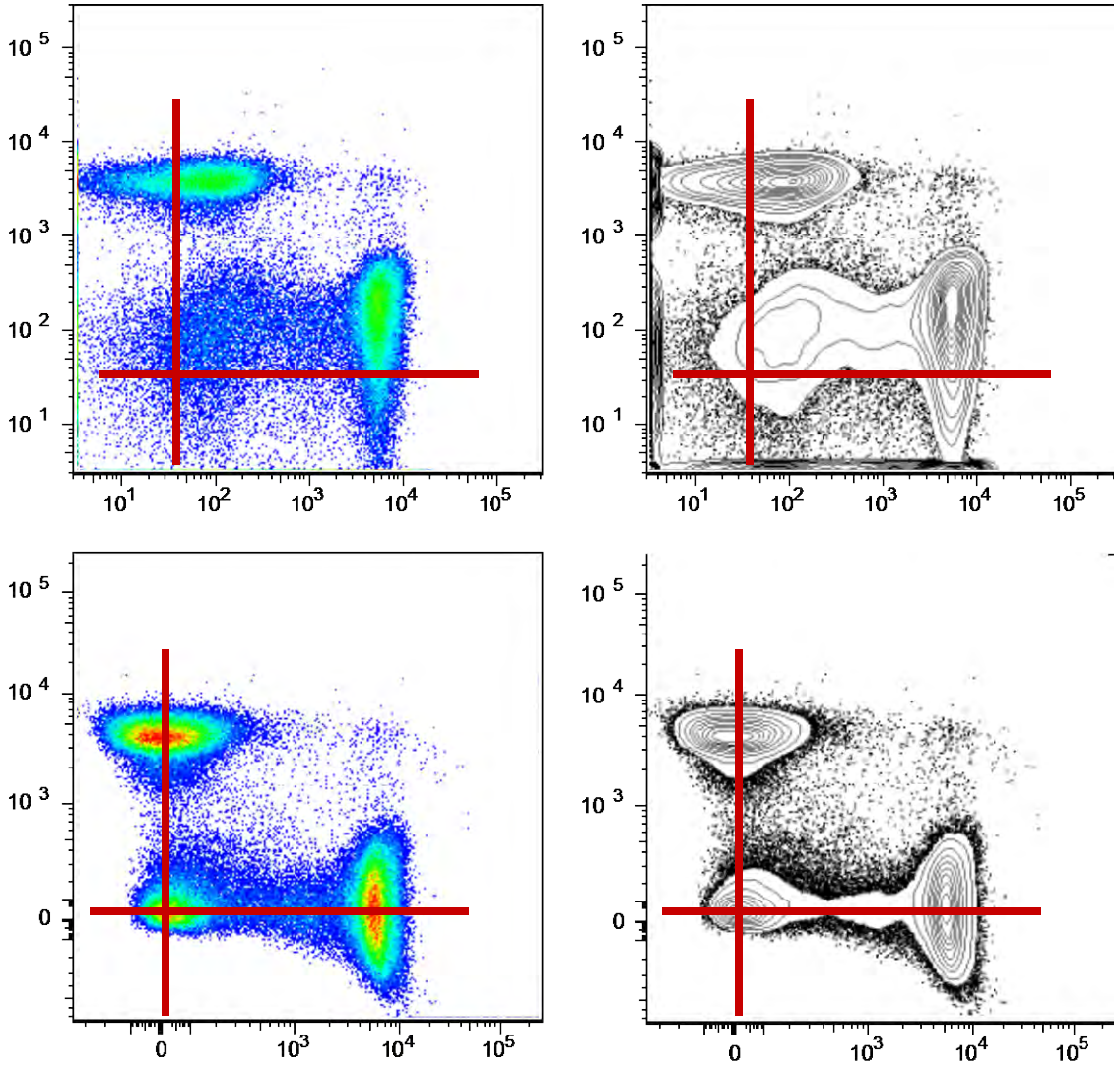
Bi-exponential or logicle transformation

- Transforms the log scale to display values below zero
- This allows better visualization of populations centered around zero
- This display feature is now available in most FACS analysis software

Transformation Confirms Compensation

Not Transformed

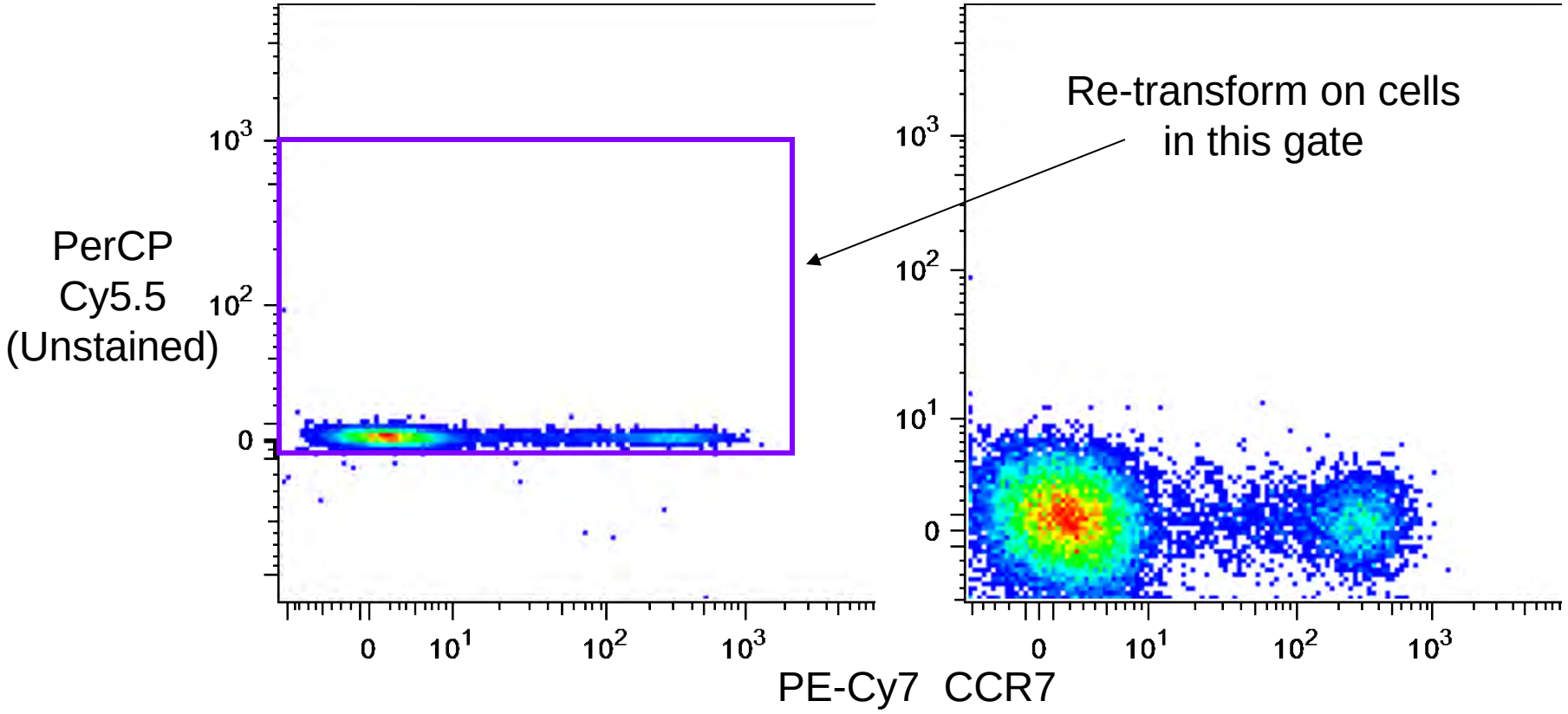
Transformed



Slide provided by M. Roederer, NIH

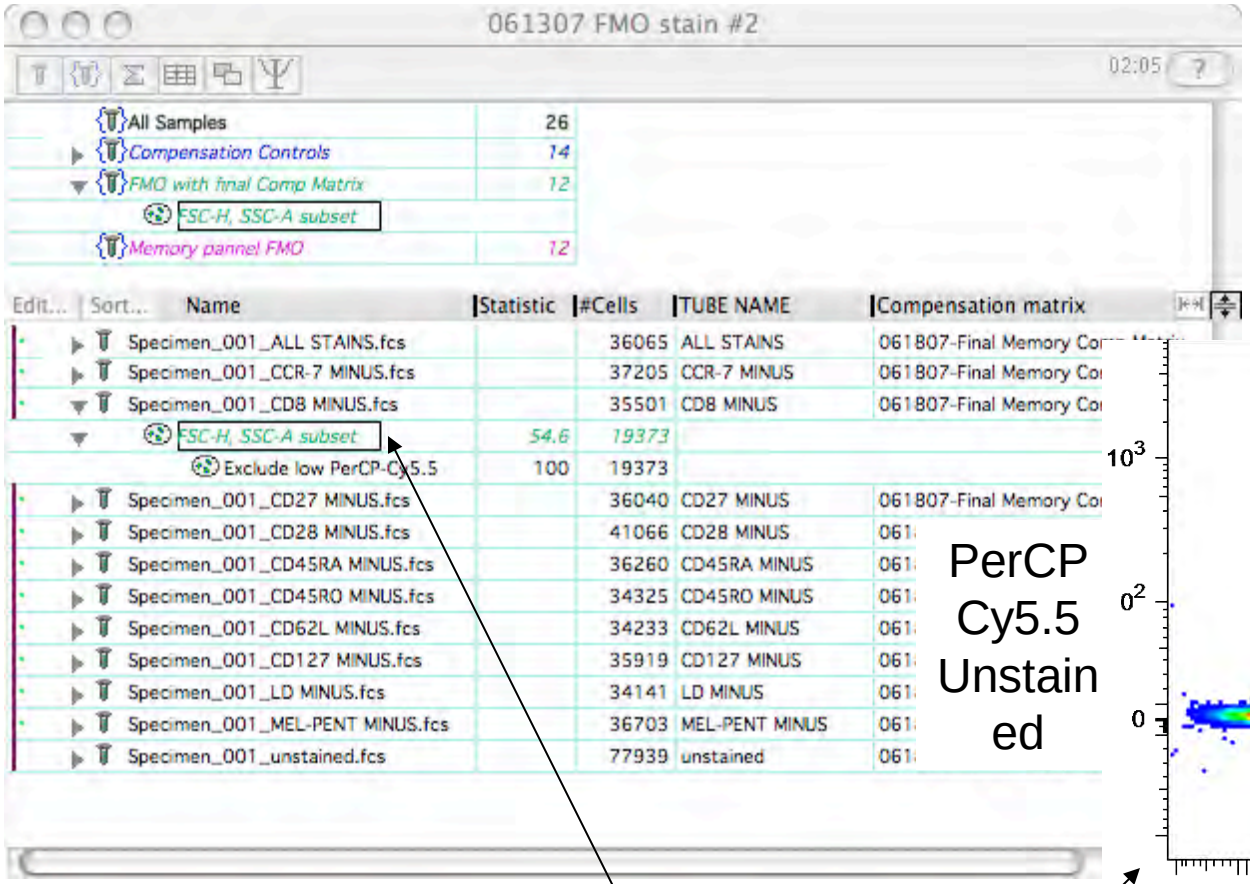
Re-Transformation

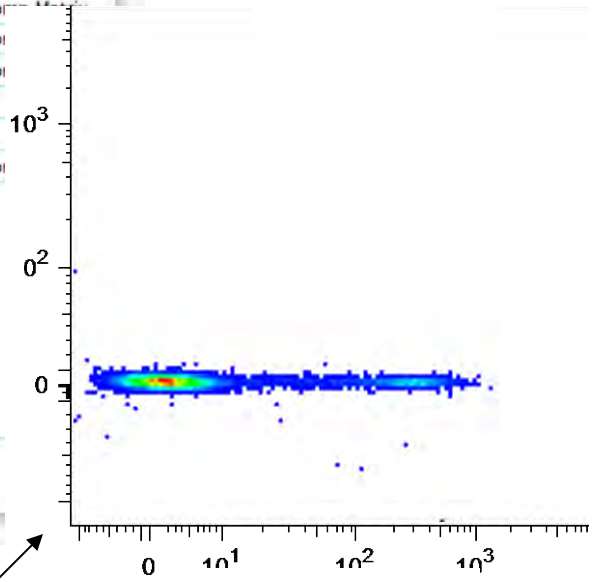
- Cells with large negative fluorescence values affect transformation
- Excluding these cells and re-transforming produces better results



Data provided by Jeff Pufnock, 061307, CD8 minus FMO

In FlowJo, choose the appropriate gate when defining transformation

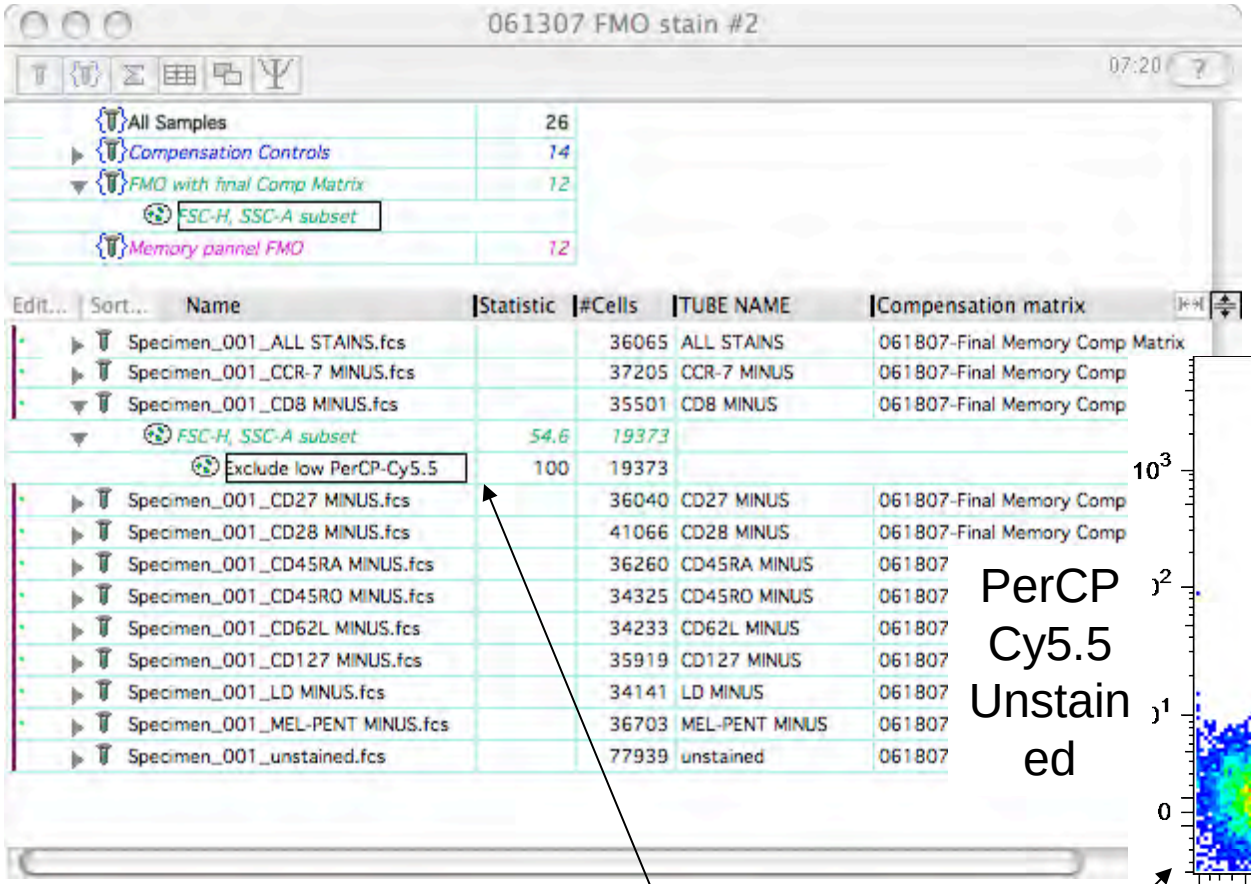




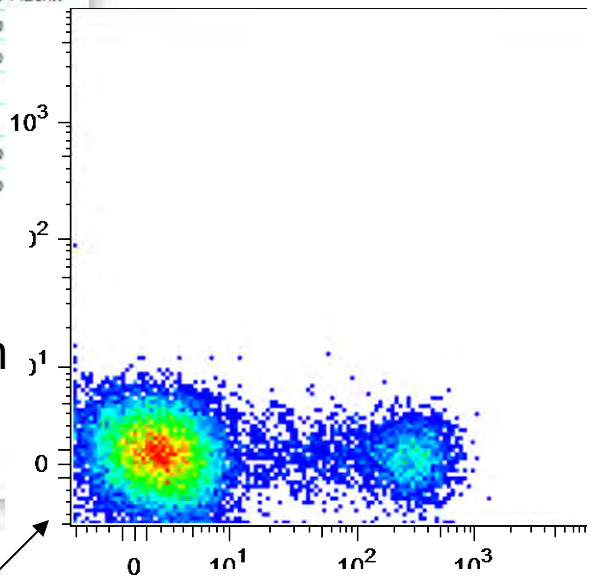
Transforming when this gate is selected produces this result

Data provided by Jeff Pufnock, 061307, CD8 minus FMO

Re-Transformation



PerCP Cy5.5 Unstained



PE-Cy7 CCR7

Transforming when this gate is selected produces this result

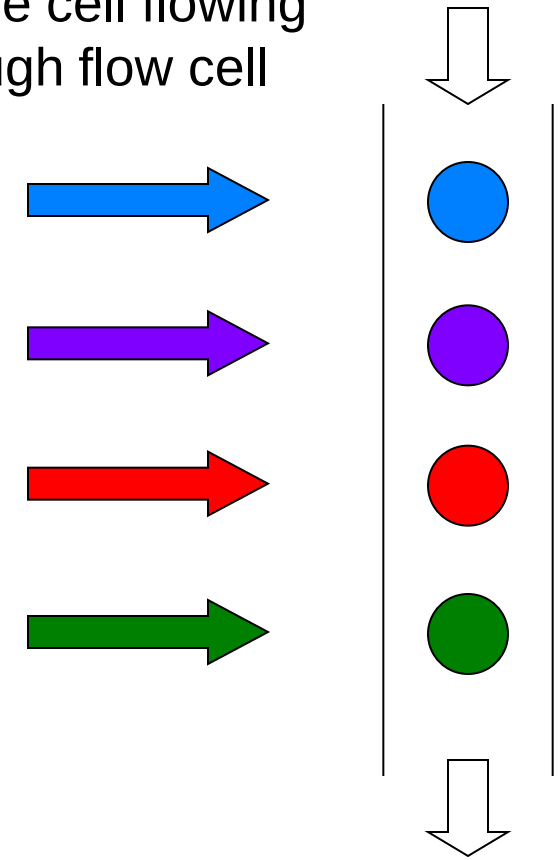
Data provided by Jeff Pufnock, 061307, CD8 minus FMO

Cross-Laser Excitation

- Cells are exposed to light from each laser at different times
- Electronic signals due to the excitation of dyes by each laser are only collected during the time that the cells are exposed to that laser
- If a dye is excited by only one laser, then even if the emission spectrum for this dye overlaps with another, the signals are distinguished in time
- This is not the case when a dye is excited by more than one laser

Laser Delay

A single cell flowing through flow cell



Signal collected at:

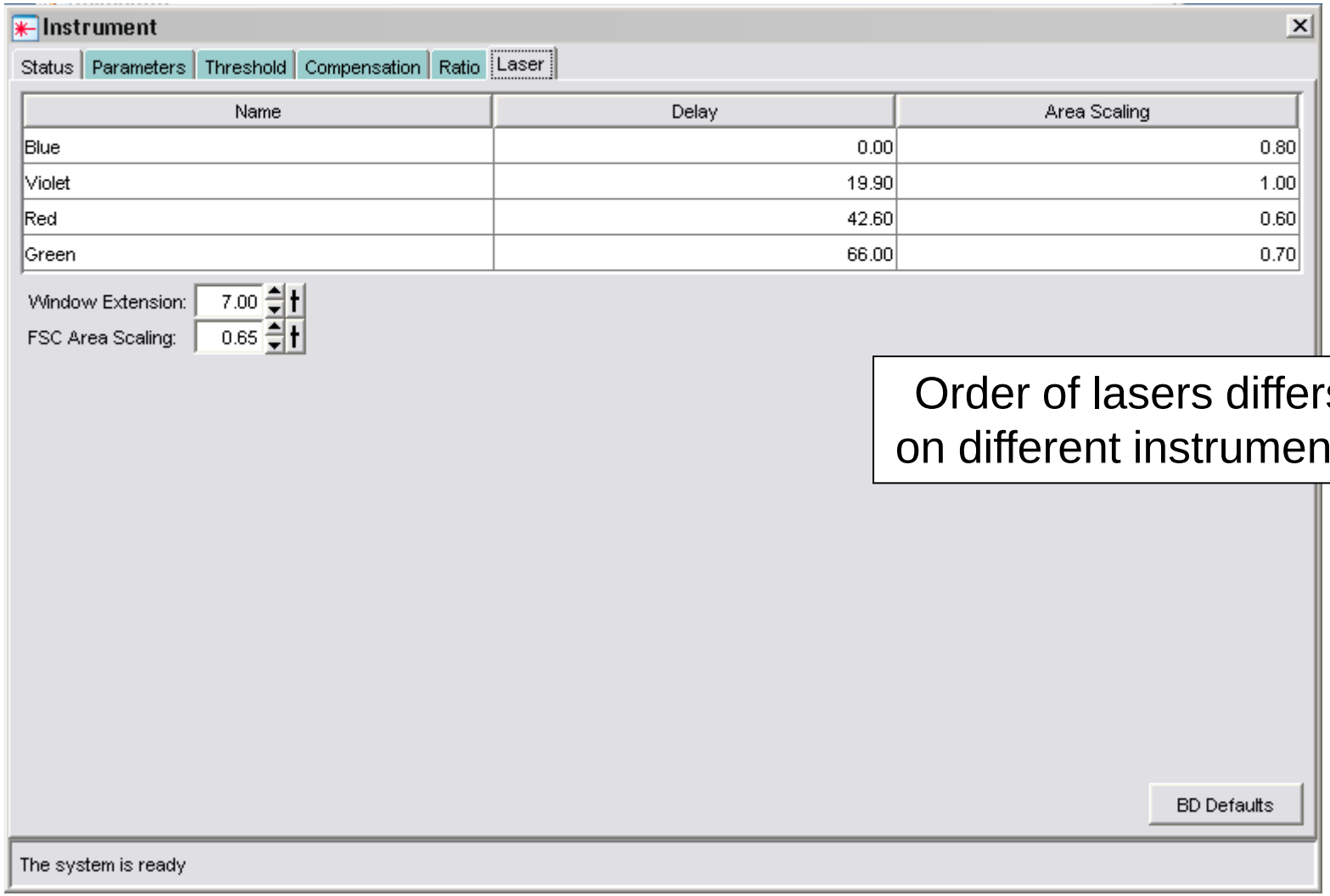
time T_{blue}

time T_{blue}+violet delay

time T_{blue}+red delay

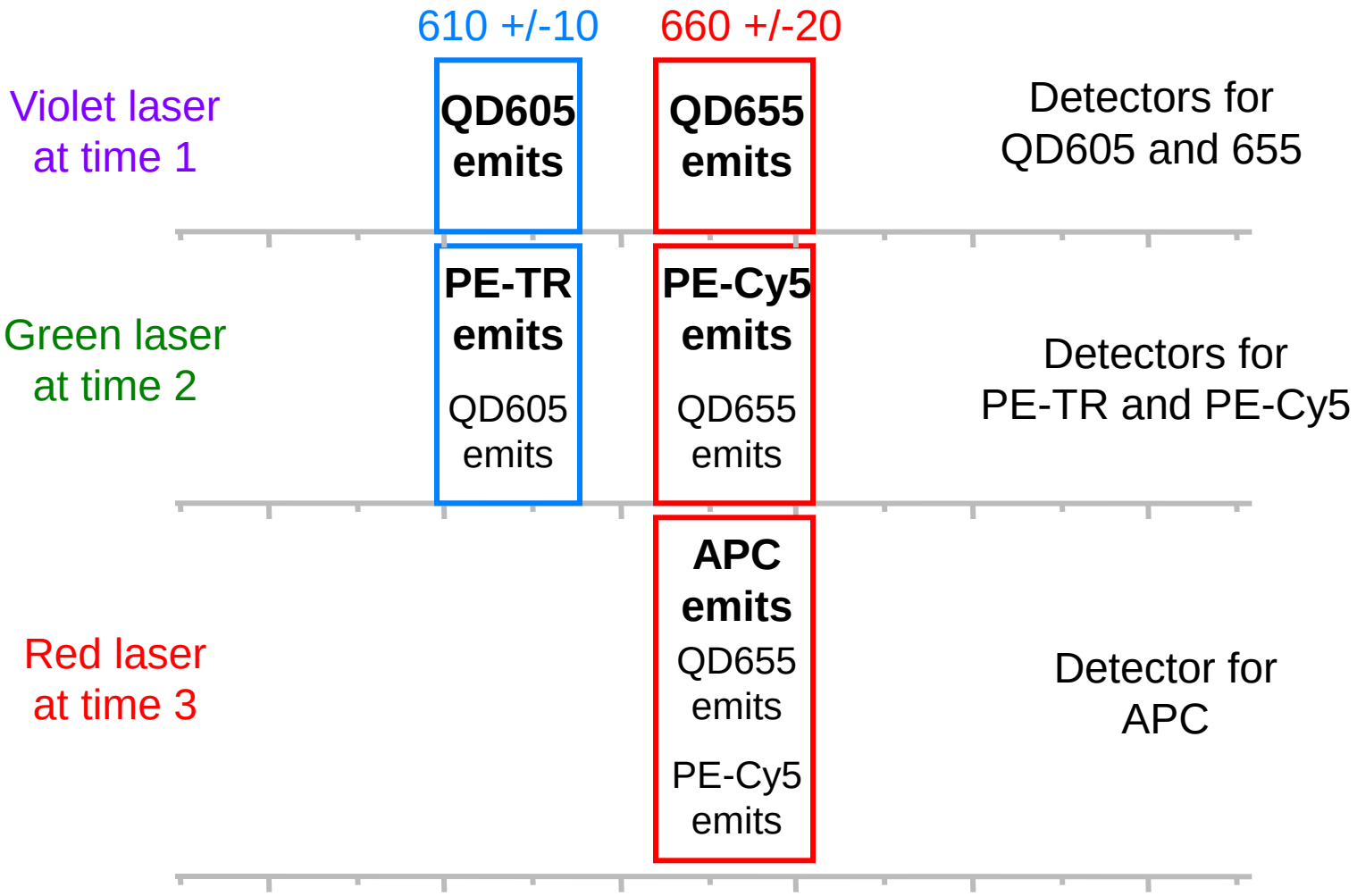
time T_{blue}+green delay

Laser Delay Settings



Order of lasers differs on different instruments

Cross-laser compensation



References

Multicolor flow, FMO, Compensation

- Baumgarth, N. and Roederer, M. (2000) A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods 243, 77-97.
- Roederer, M. (2001) Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry 45, 194-205.

Compensation errors: diagnosis and practical considerations (1)

- Consider an error in compensation:
  - Diagonal staining populations (except for two markers with correlated expression, e.g., IFN-γ vs. TNF-α)
  - Unexpected positive population (e.g., high frequency of cells expected at low frequency, CD25, CD69, IL-4….)
  - Overcompensated cells over the axis

Compensation errors: diagnosis and practical considerations (2)

- Steps to investigate potential compensation errors:
  - Apply custom transformation to visualize negative events and to assess medians
  - Visualize each parameter vs. all others to search for unobserved compensation issues (multigraph overlay, N by N plot)
  - Apply compensation to comp samples. Does compensation matrix need to be re-calculated or is the comp matrix not working for the test samples, but ok for the comp samples.
  - Is each comp sample bright enough, enough cells

Compensation errors: diagnosis and practical considerations (3)

- Steps to resolve compensation issues:
  - Import selected compensation controls from another experiment
  - Apply a higher gate to a compensation sample and remake matrix
  - It may be necessary to manually adjust compensation matrix

Quantum dots, Amine reactive viability dye

- Chattopadhyay, P.K., Price, D.A., Harper, T.F., Betts, M.R., Yu, J., Gostick, E., Perfetto, S.P., Goepfert, P., Koup, R.A., De Rosa, S.C., Bruchez, M.P. and Roederer, M. (2006) Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. Nat Med 12, 972-7.
- Perfetto, S.P., Chattopadhyay, P.K., Lamoreaux, L., Nguyen, R., Ambrozak, D., Koup, R.A. and Roederer, M. (2006) Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. J Immunol Methods 313, 199-208.

Transformation, data display

- Parks, D.R., Roederer, M. and Moore, W.A. (2006) A new "Logicle" display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. Cytometry A 69, 541-51.
- Herzenberg, L.A., Tung, J., Moore, W.A. and Parks, D.R. (2006) Interpreting flow cytometry data: a guide for the perplexed. Nat Immunol 7, 681-5.

Historical References: First FACS and First 2-color FACS publications

- Hulett, H.R., Bonner, W.A., Barrett, J. and Herzenberg, L.A. (1969) Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. Science 166, 747-9.
- Loken, M.R., Parks, D.R. and Herzenberg, L.A. (1977) Two-color immunofluorescence using a fluorescence-activated cell sorter. J Histochem Cytochem 25, 899-907.