Compensation:

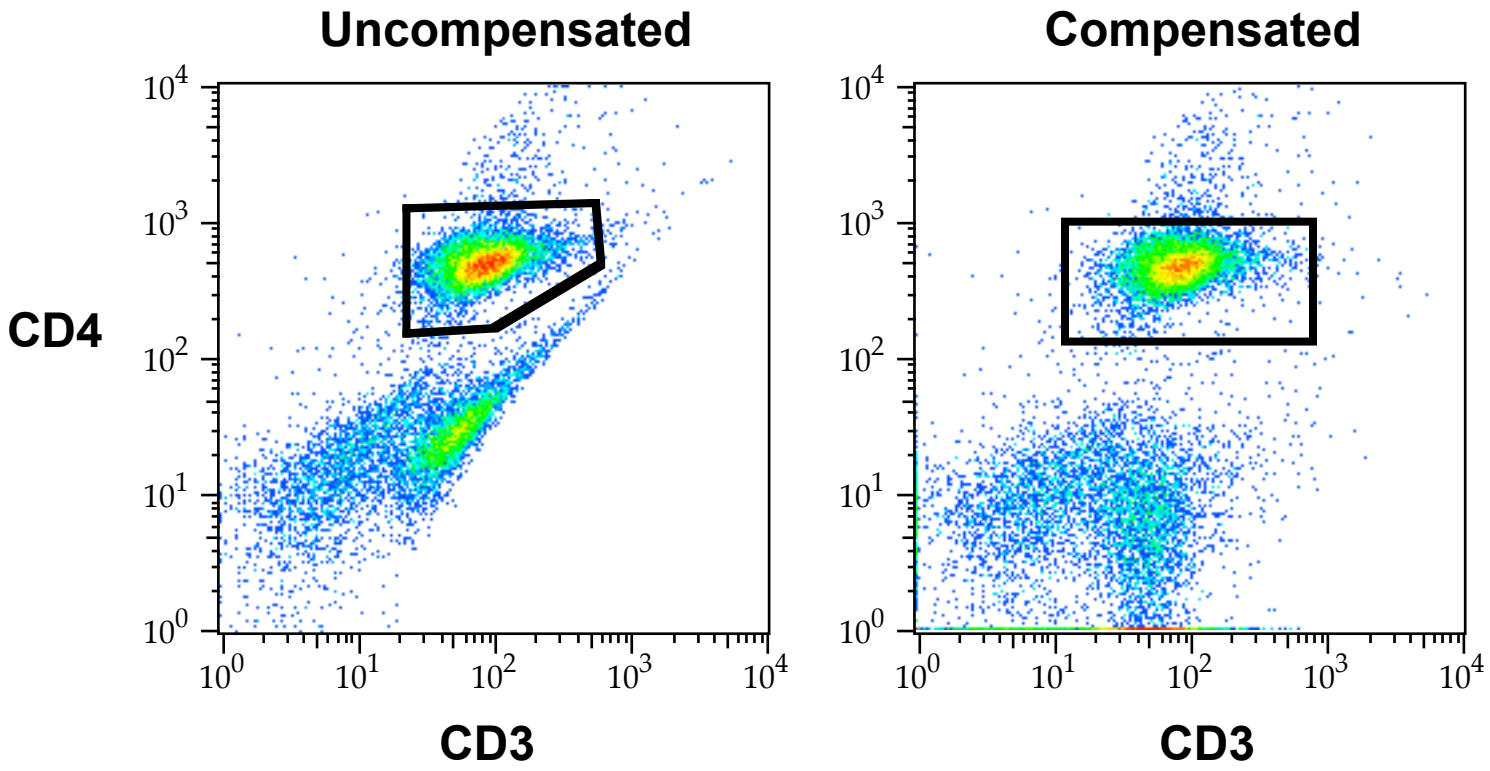
It’s not just for pretty pictures

Fluorescence Spillover Compensation

- **Simple in concept…**
  - Correct spillover into different parameters
- **Straightforward in execution…**
  - Proper settings based on controls
- **…But a lifetime to understand?**
  - Profound impact on visualization
  - Nonintuitive aspects
  - Subtle interactions that can be hard to diagnose

Compensation in 2 colors: Mostly aesthetic

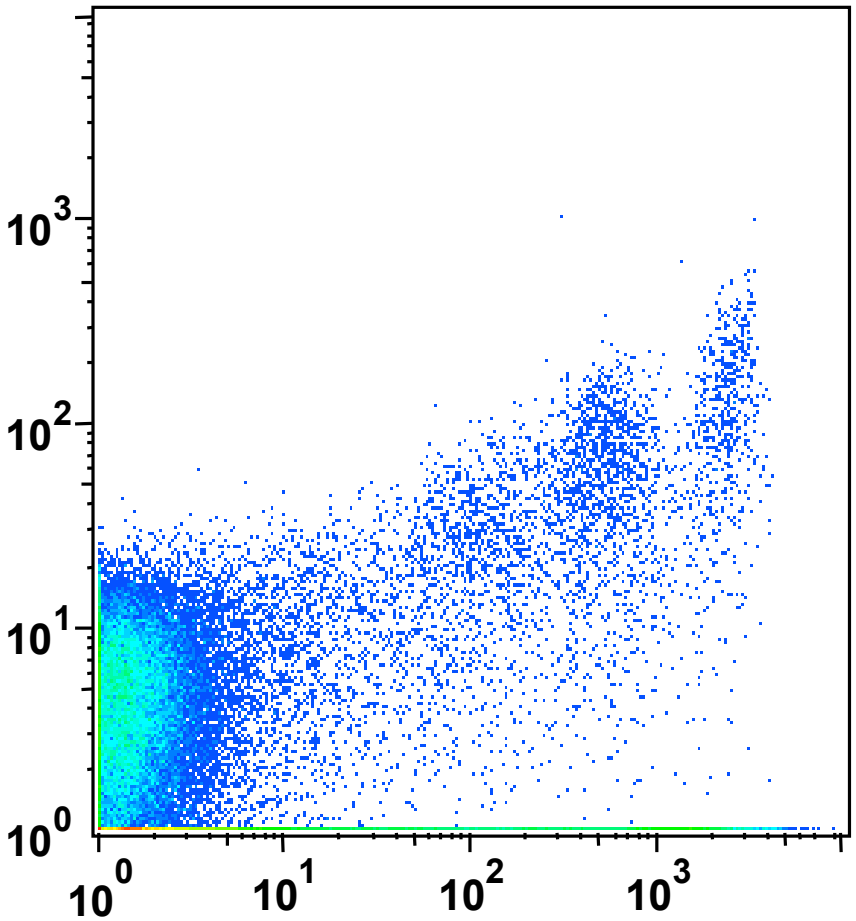
Accurate identification and enumeration of subsets is still easy in two color experiments



Compensation: Mostly aesthetic

- Accurate discrimination of subsets is possible with uncompensated data
- However, this is true only when the expression of all antigens is uniform on each subset (e.g., CD45 / CD3 / CD4 / CD8)
- Otherwise, it may not be possible to gate on subsets (with current tools)

Is this data properly compensated?

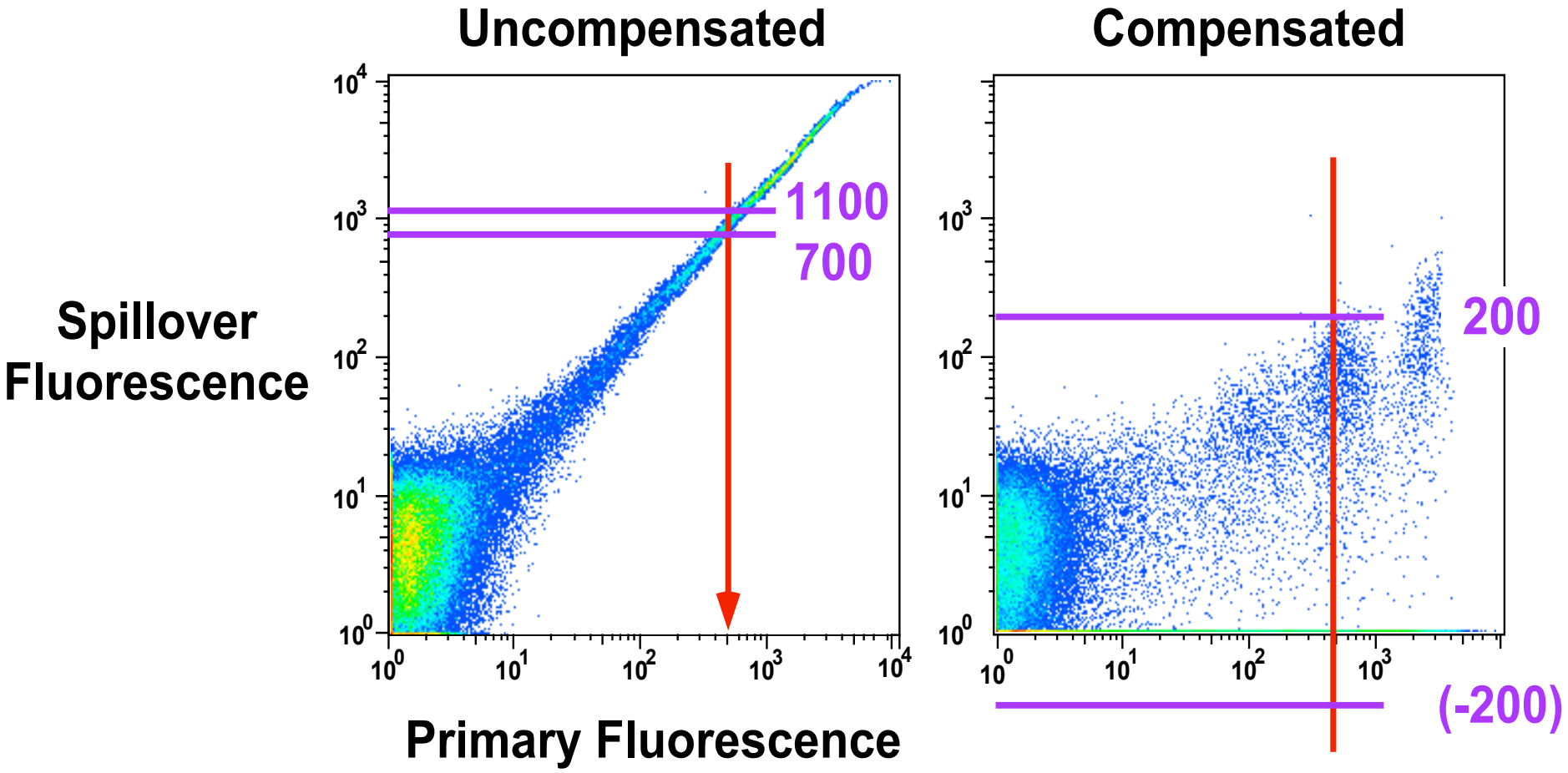


~~No~~

~~Yes~~

Can't Tell

Imperfect Measurement Leads to Apparent Spread in Compensation



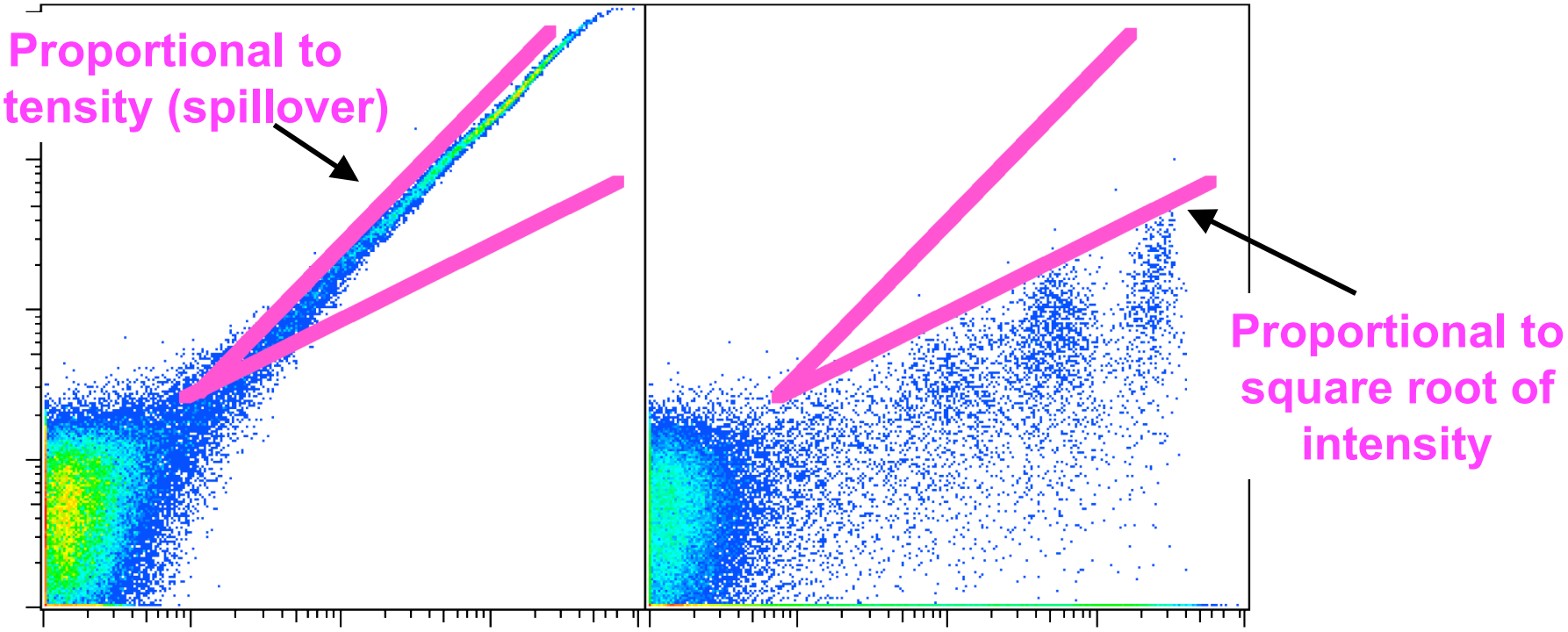
Why is there a 400-unit spread? Photon counting statistics.

Spread of Compensated Data

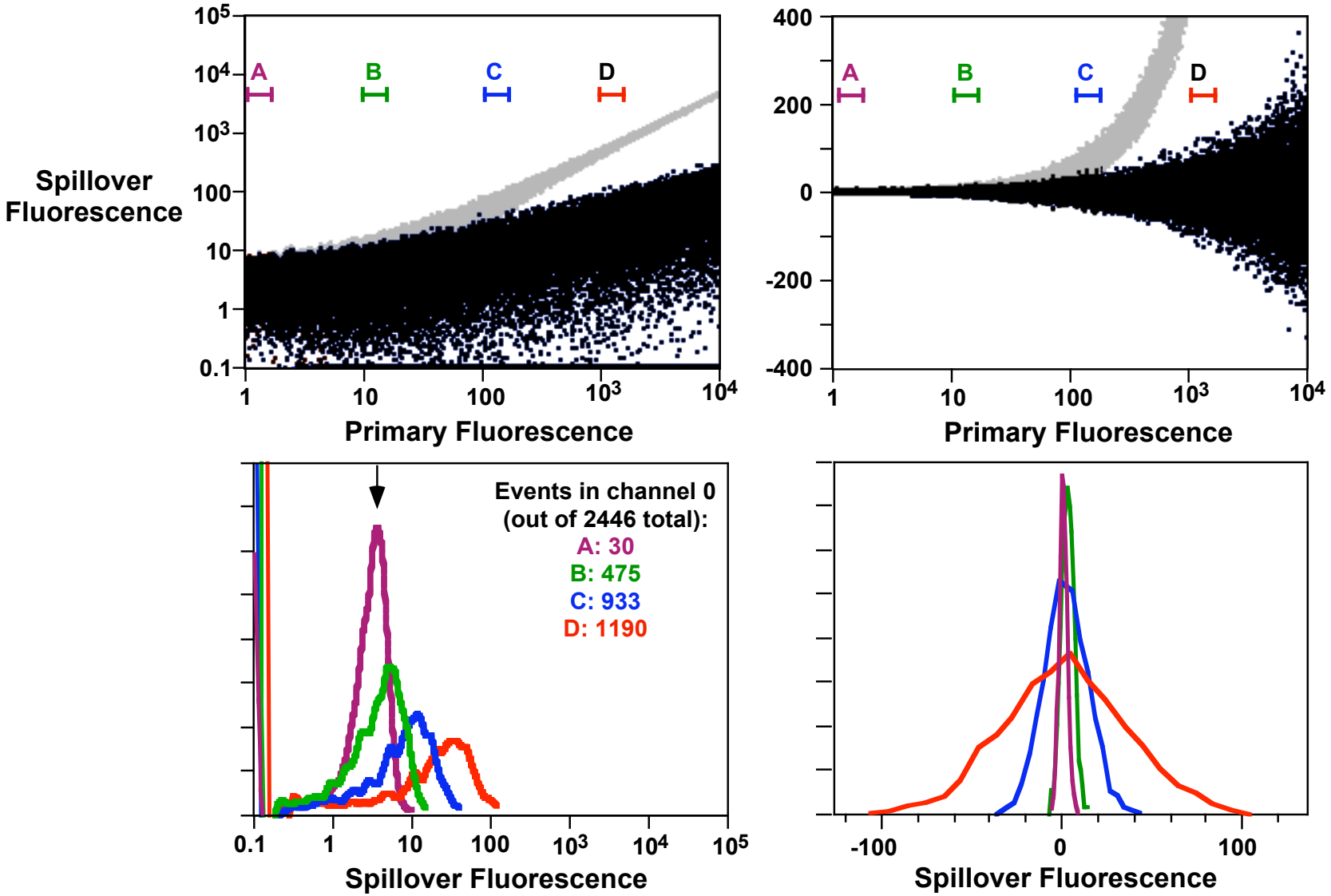
- **Properly compensated data may not appear rectilinear (“rectangular”), because of measurement errors.**
- **This effect on compensated data is unavoidable, and it cannot be “corrected”.**
- **It is important to distinguish between incorrect compensation and the effects of measurement errors.**

How can we identify undercompensated data?

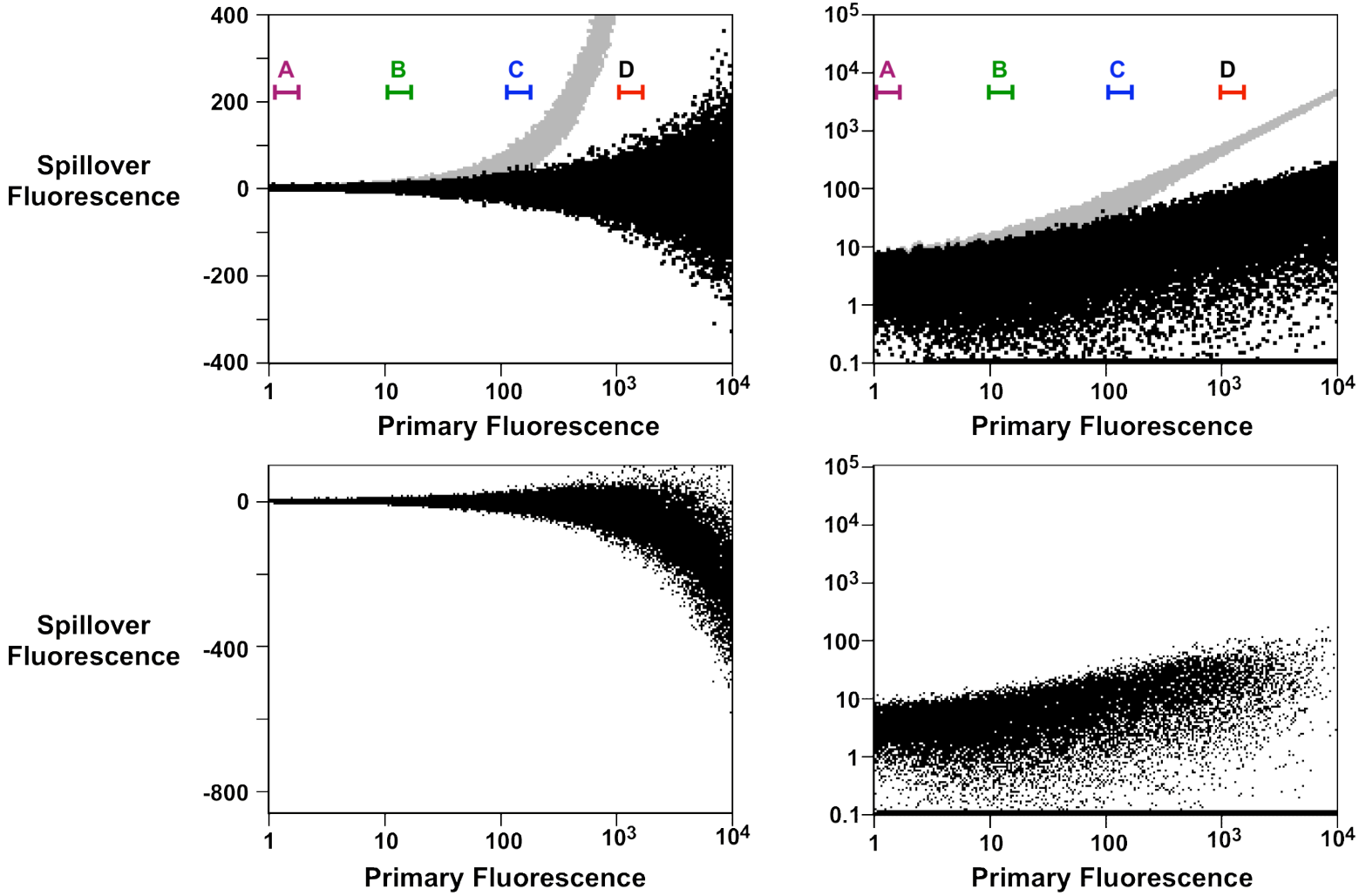
Diagonals in the data indicate poorly compensated data: but **ONLY AT 45˚**! Other slopes indicate nonlinear correlations that have nothing to do with compensation. Visual estimation is very difficult.



Log Transformation of Data Display Leads to Manual Overcompensation



Overcompensation Cannot Correct Error-induced Spread



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.

Controls

Staining controls fall into three categories:

- **Instrument setup and validation (compensation, brightness)**
- **Staining/gating controls (Viability, FMO)**
- **Biological**

Compensation Controls

Single-stained samples…must be at least as bright as the reagent you are using in the experiment!

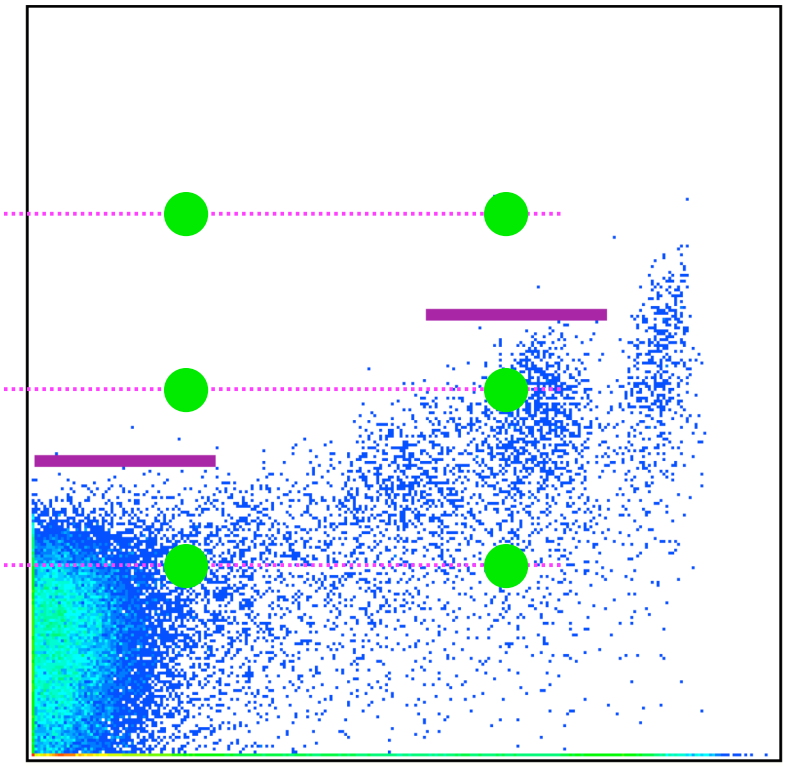
Can use *any* “carrier”, as long as the positive & negative populations have the same fluorescence when unstained:

Cells (mix stained & unstained)
Subpopulations (CD8 within total T)
Beads (antibody-capture)

One compensation for every color… and one for each unique lot of a tandem (Cy5PE, Cy7PE, Cy7APC, TRPE)

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!



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.

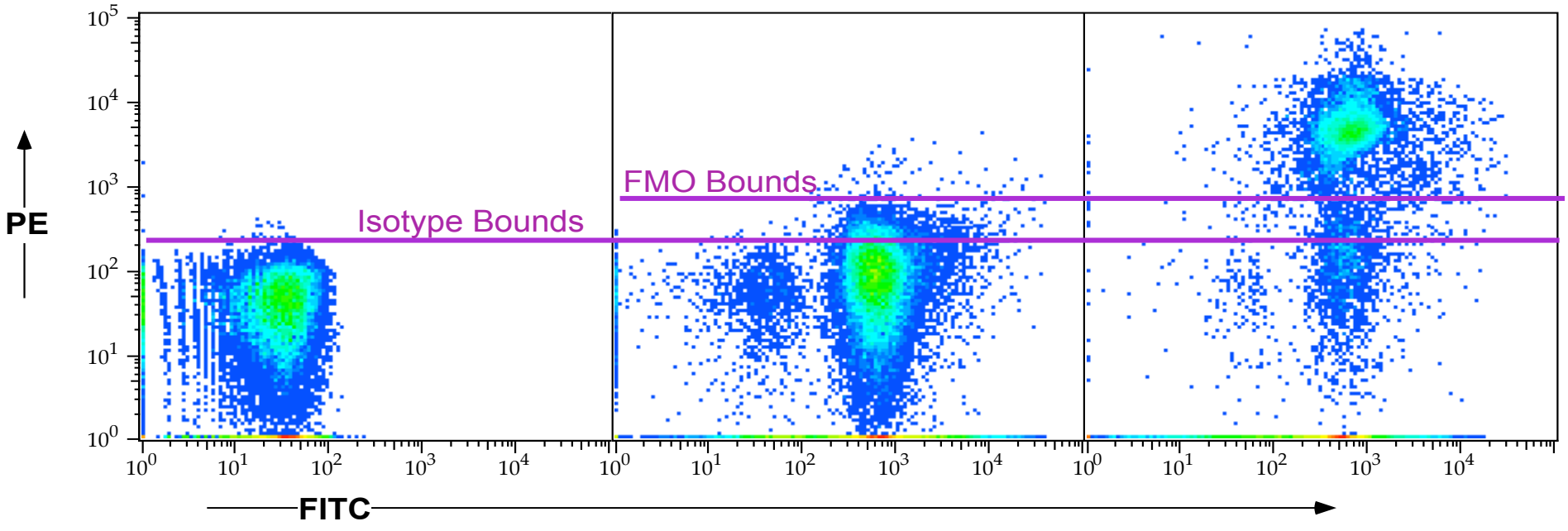
FMO Control

"Fluorescence Minus One"

Identifying CD4 cells with 4 colors

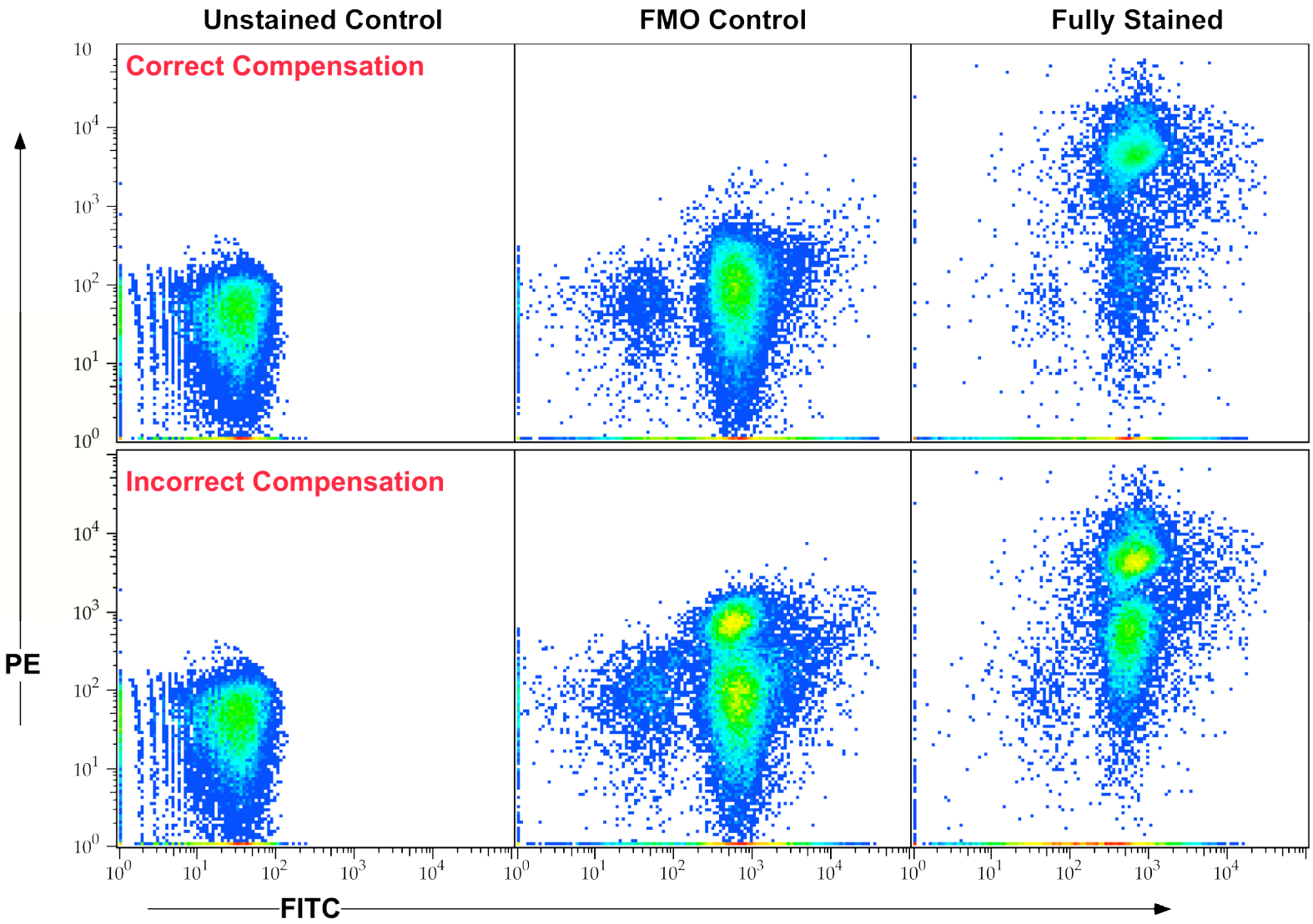
PBMC were stained as shown in a 4-color experiment. Compensation was properly set for all spillovers

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



Complex Interactions in Compensation

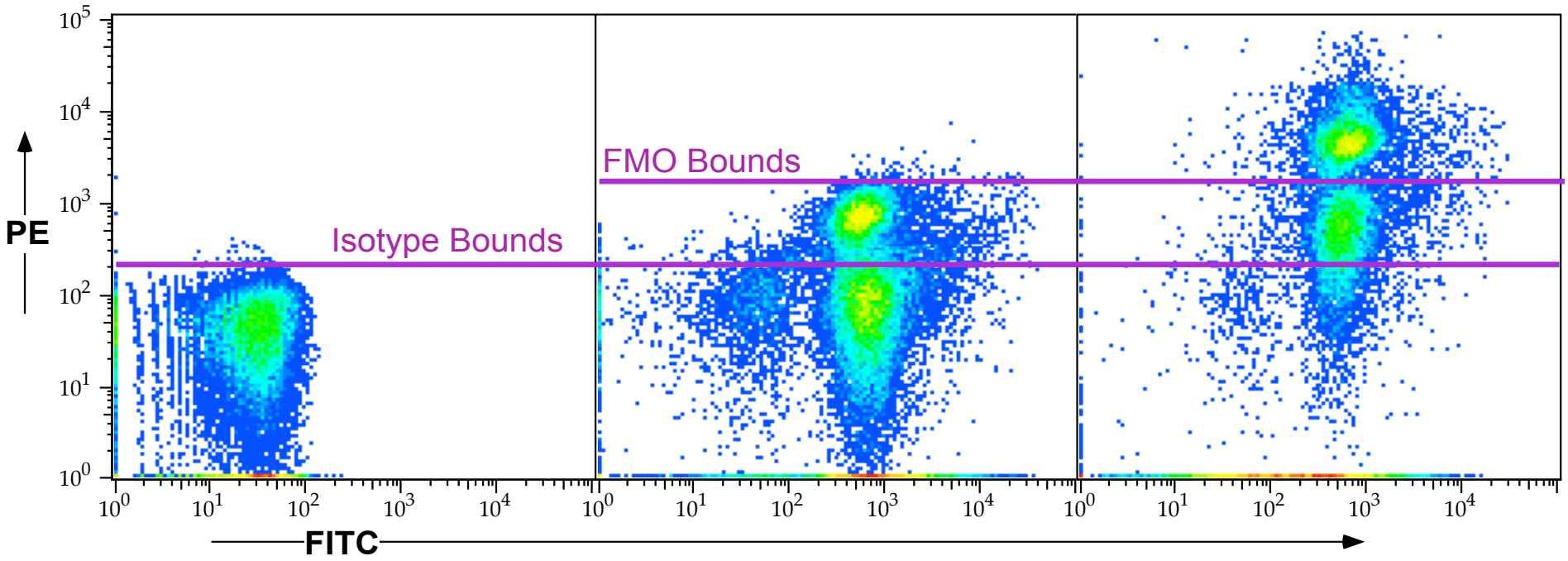
The same data is shown with correct or wrong Cy5PE->Cy7PE comp setting. Note that neither of these channels is shown here!



FMO controls aid even when compensation is improper

Incorrect Cy5PE into Cy7PE compensation

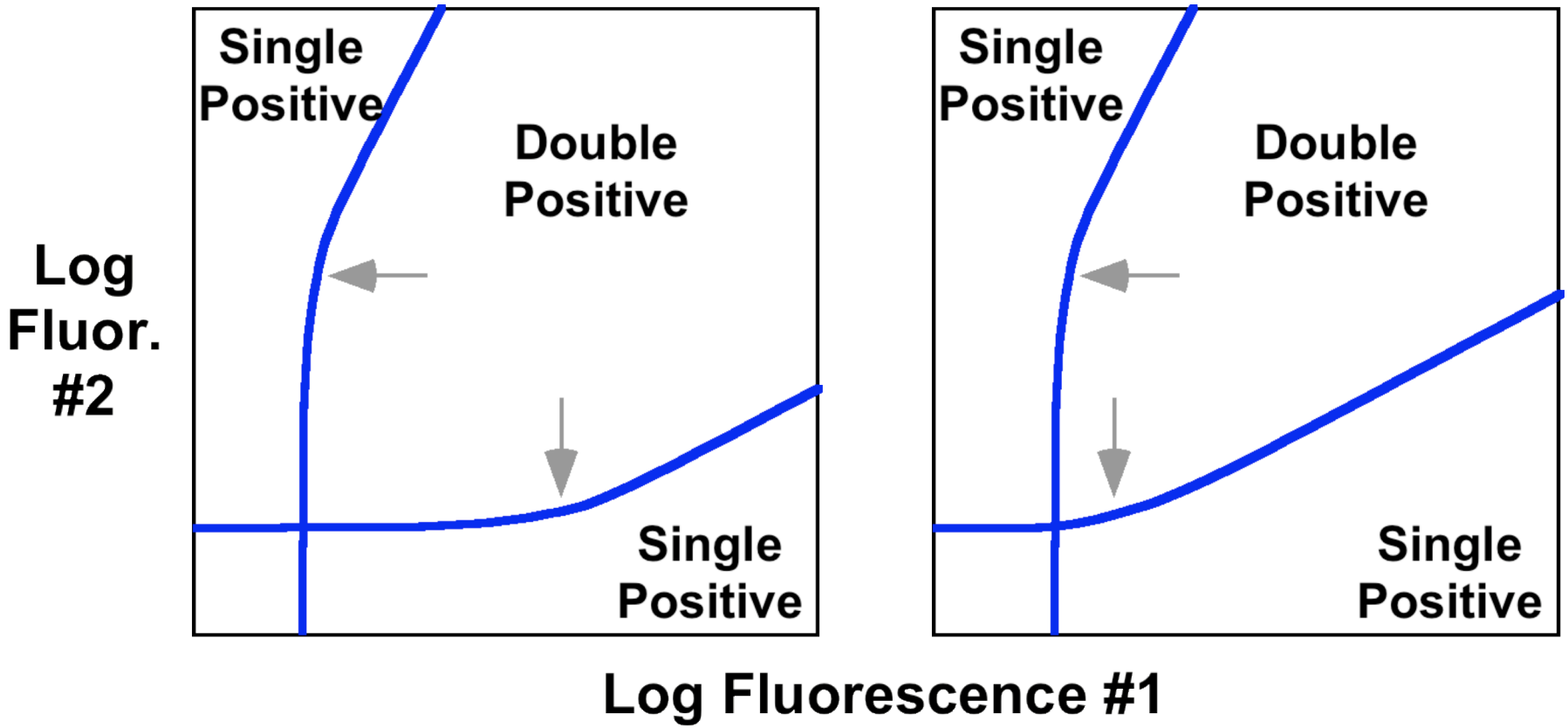
	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO



FMO Controls

- FMO controls are a much better way to identify positive vs. negative cells
- FMO controls can also help identify problems in compensation that are not immediately visible
- FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

Quad-Gates of the ~~Future~~ *Present*

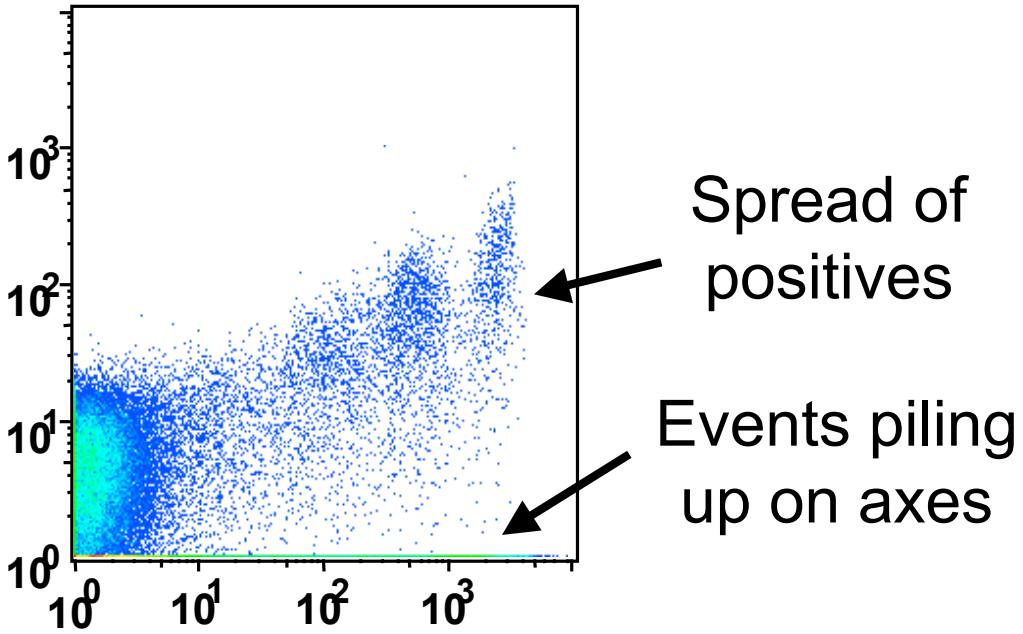


Compensation & Data Visualization

These “new” distributions are much more frequently seen nowadays, with the use of red dyes (Cy7PE, Cy7APC) and with more precise instruments.

Some users have questioned the correctness of these distributions, leading some manufacturers to try to provide “corrections”.

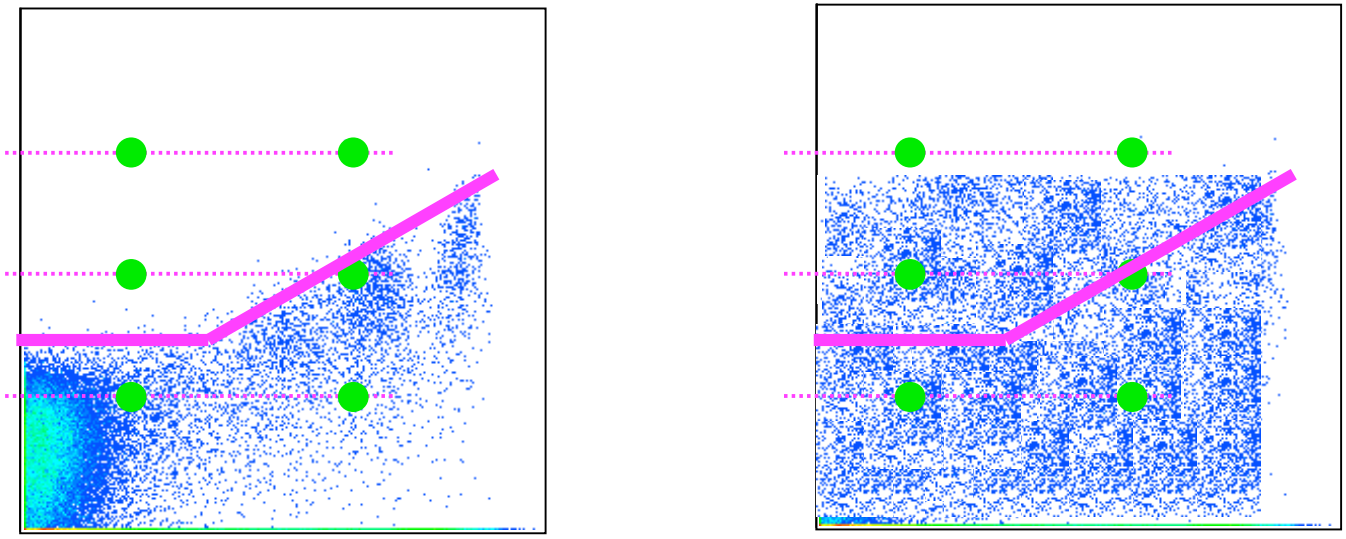
However, this cannot be “corrected”–what is needed is education!



Compensation Offset???

- At least two FACS data analysis software programs offer a “feature” to turn on a “compensation offset”–to try to make data look more like what we have expected.
- The term “Compensation Offset” simply means that ***random noise*** is added to the data to hide the “spread” in compensated data.
- This “feature” ***reduces*** sensitivity!

Compensation Offset???



- Artificially increase error does make distributions rectangular again--but at a significant expense!
  - Low level antigen expression will be lost
  - In >4 color experiments, problems multiply!
- **Turn OFF this “feature” in your software**

Is There A Solution?

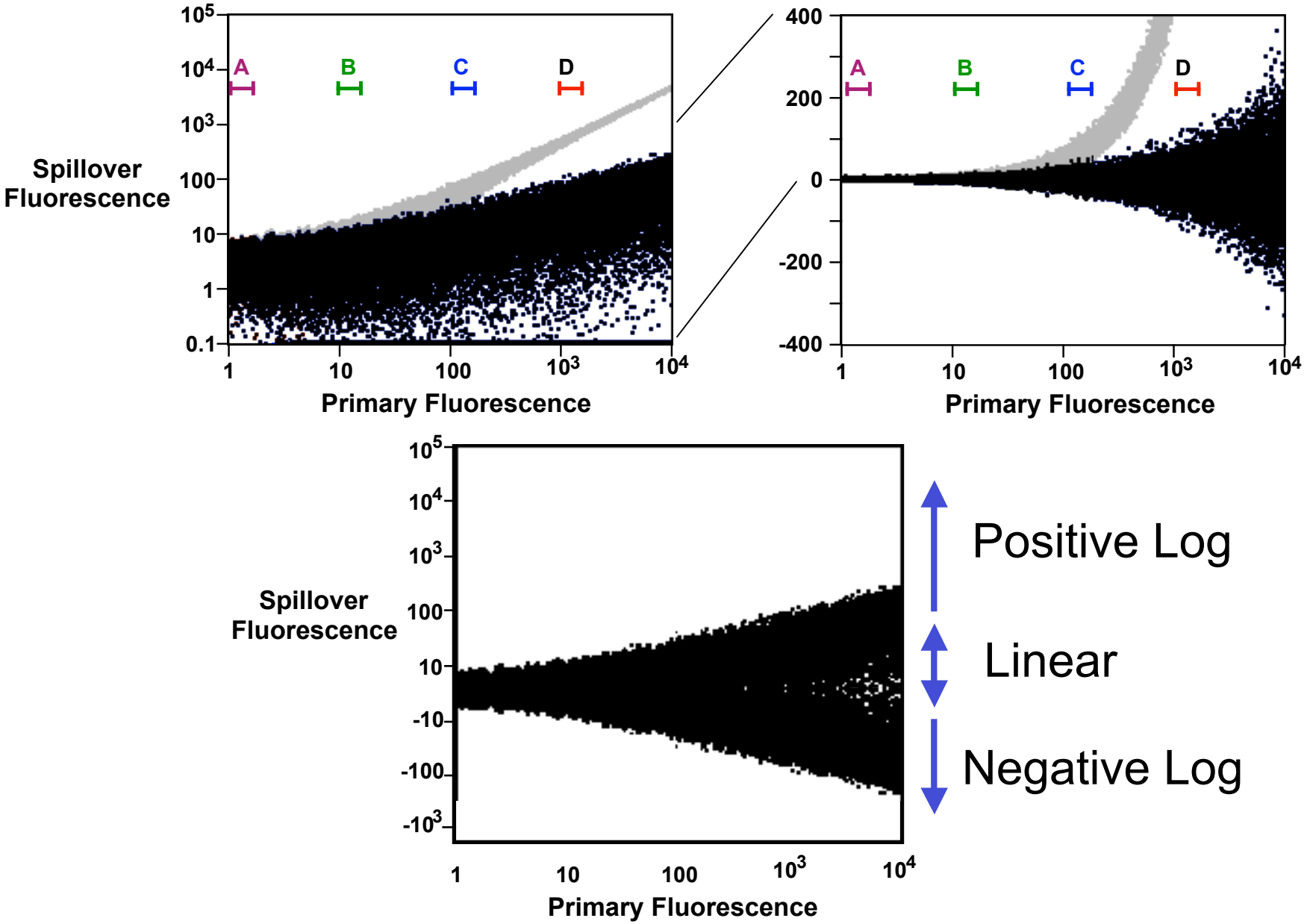
- The spread in compensated data is unavoidable (basic physics)
- Can we visualize data so that the distributions are more intuitive?
- Nearly all immunophenotyping data is shown on a logarithmic scale… why?
  - Dynamic range of expression (4 logs)
  - Often, distributions are in fact log-normal

Alternatives to a Log Scale

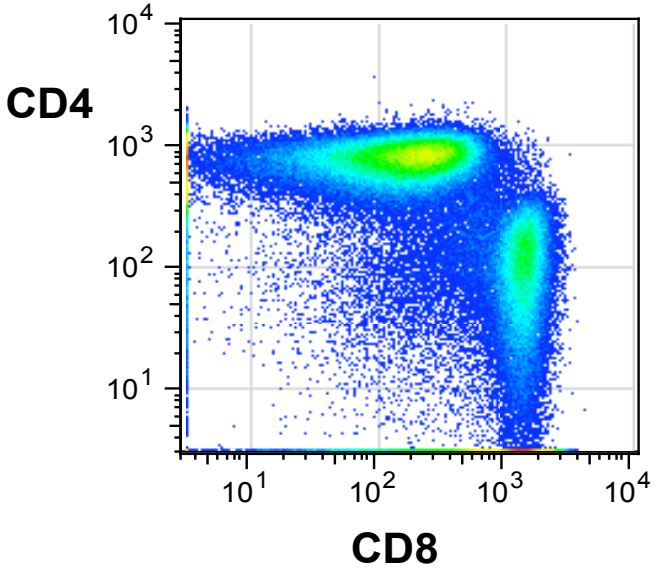
- Compensation reveals a linear-domain spreading in the distribution.
- This is most obvious at the low end of fluorescence, because the measurement error is small compared to bright cells.
- Can we re-scale the low end of the fluorescence scale to effect a different compression in this domain?
- What about **negative values**?
  - *Remember, this is just a fluorescence from which we subtract an estimated value with measurement error*

“Bi-exponential” Scaling

Wayne Moore
Dave Parks



“Bi-exponential” Transformation Makes Compensated Data More Intuitive

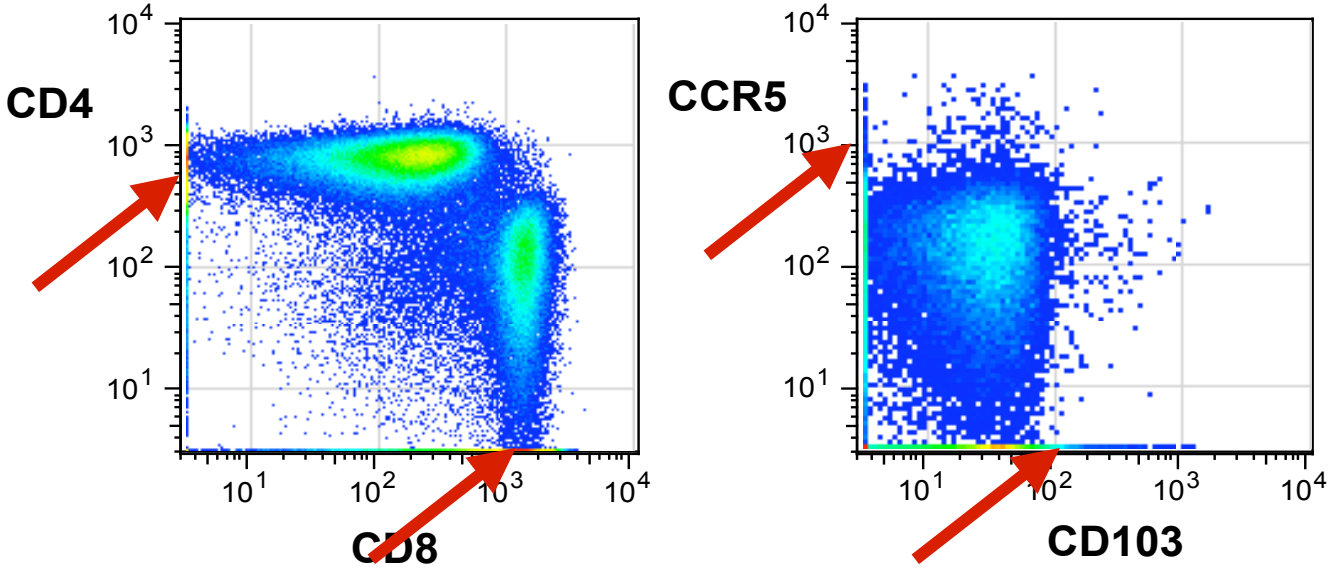


Gated for CD3+ Lymphocytes;

Stained for CD3, CD4, CD8, CCR5, CD103, and 7 other reagents

Question: What is the expression of CCR5 and CD103 on CD4 Lymphocytes?

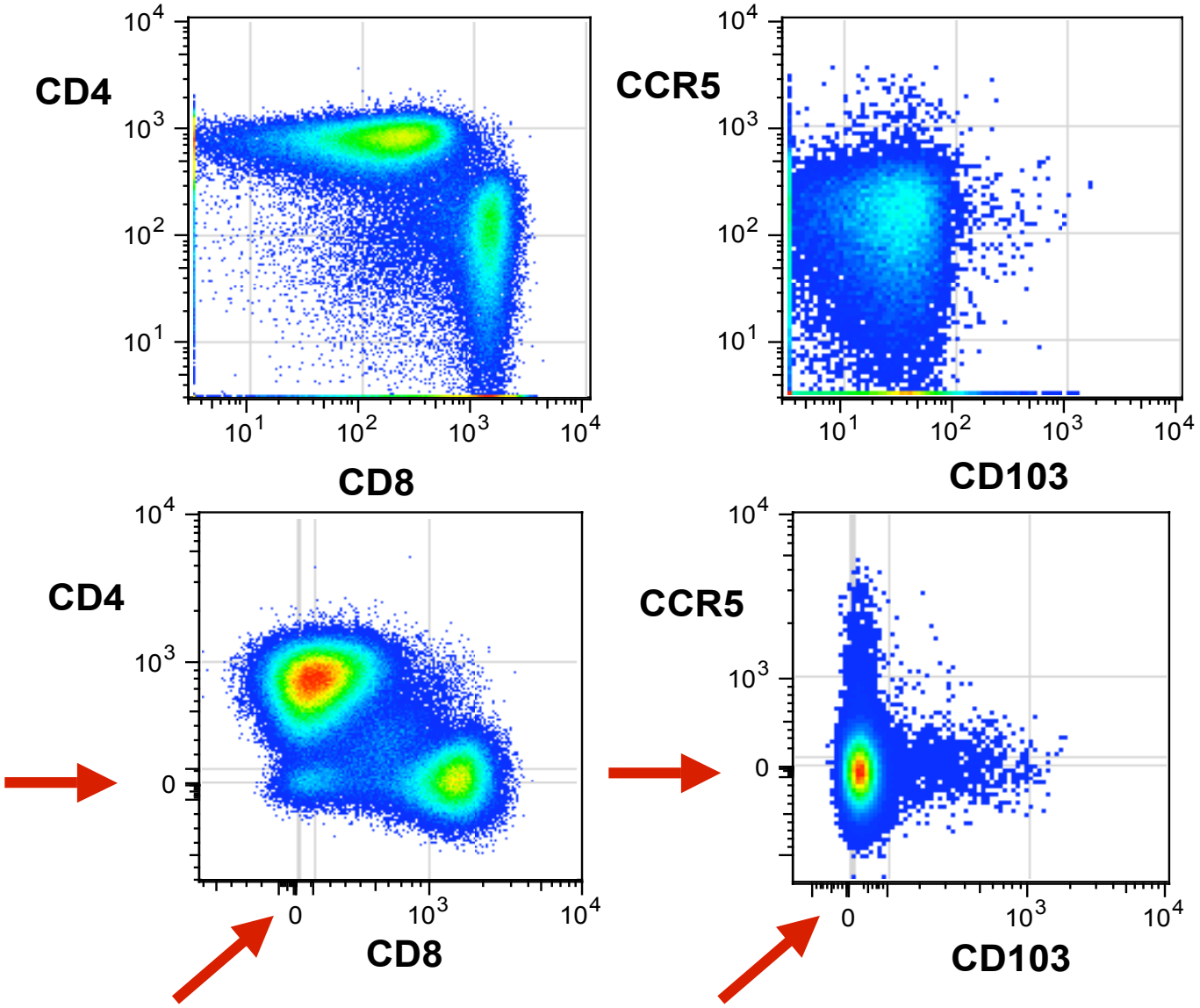
“Bi-exponential” Transformation Makes Compensated Data More Intuitive



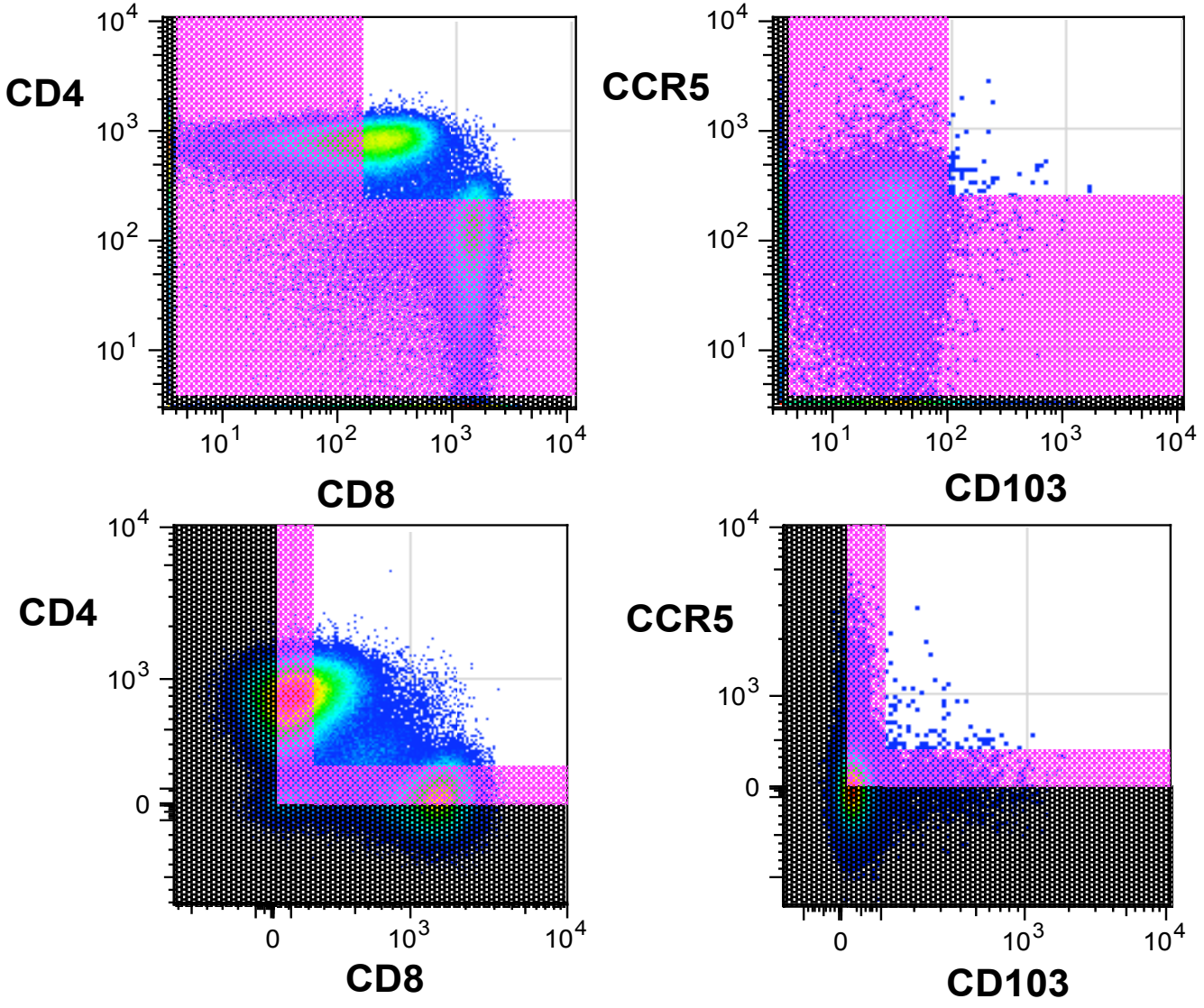
How many events are on the axis?

LOTS!

“Bi-exponential” Transformation Makes Compensated Data More Intuitive



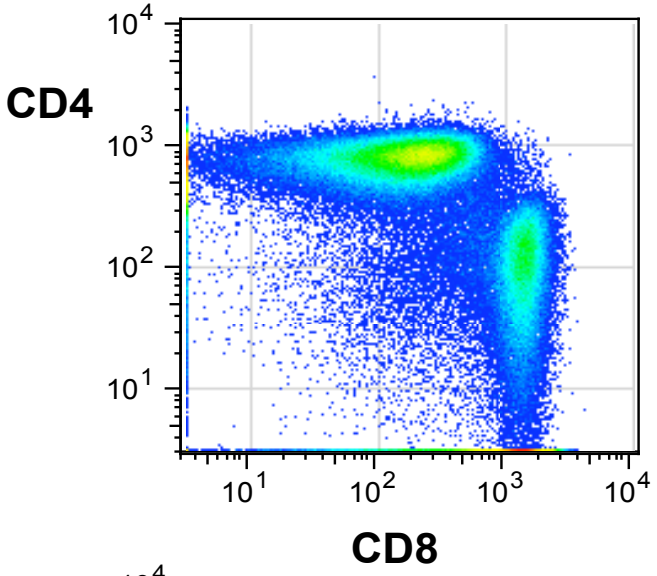
“Bi-exponential” Transformation Makes Compensated Data More Intuitive

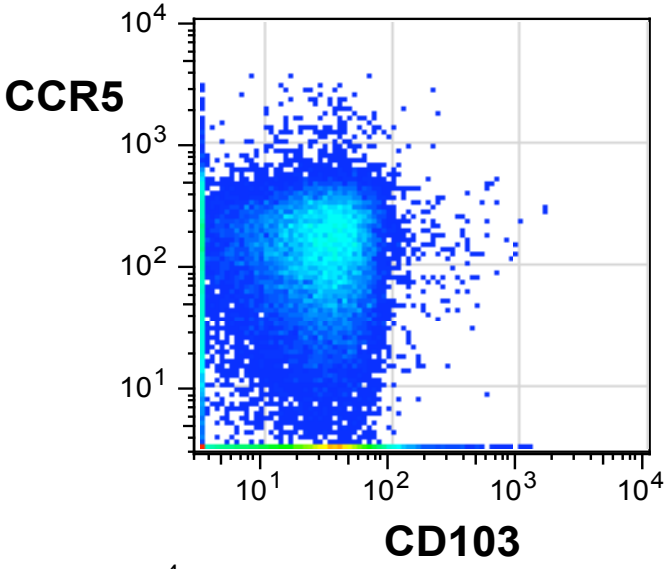


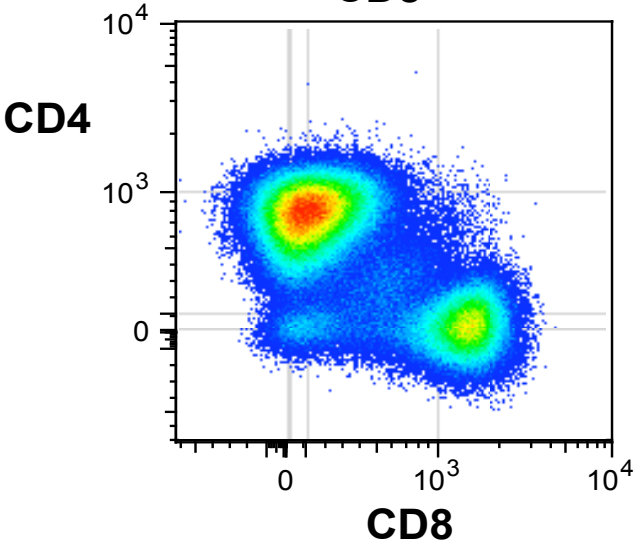
Negative Log Scale: Events with measured fluorescence < 0

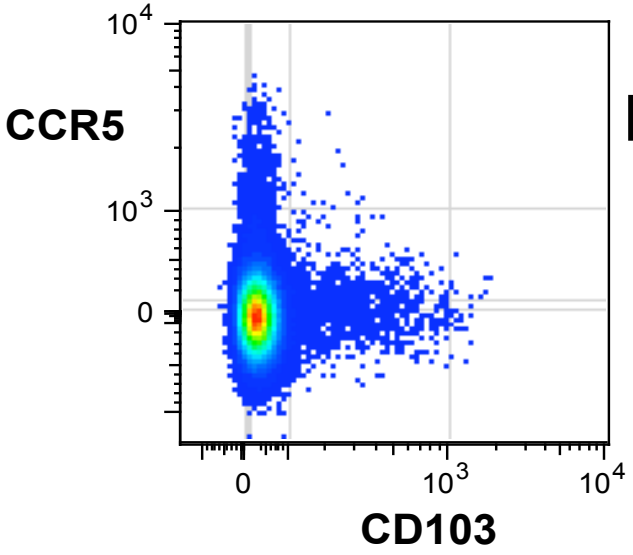
Linear Scale: Compression of the amount of visual space devoted to this range

“Bi-exponential” Transformation Makes Compensated Data More Intuitive









Result:

No events hidden on the axes

Populations are visually identifiable

“Bi-exponential” Transformation Makes Compensated Data More Intuitive

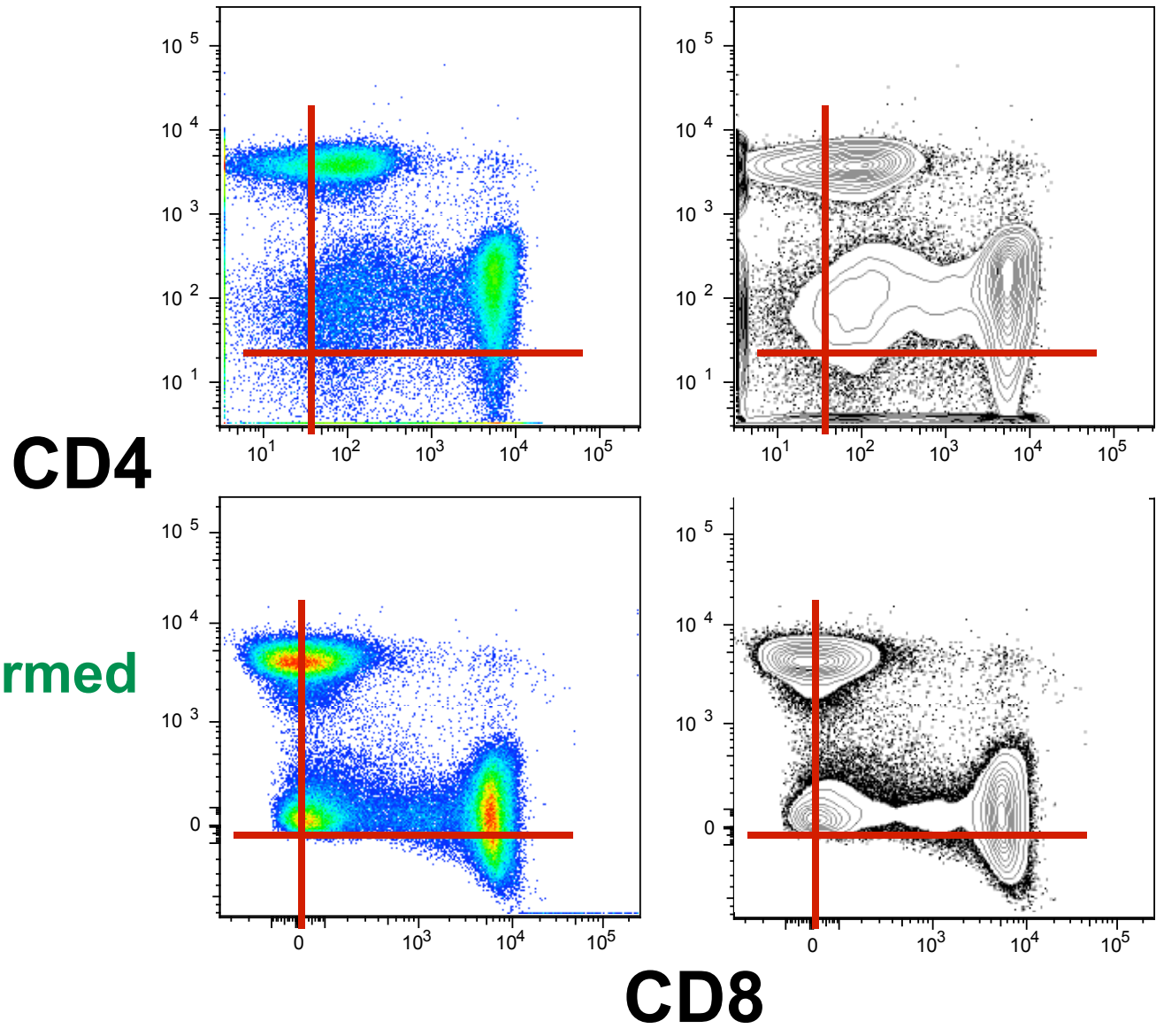
Only changes the visualization of data

- **Does not affect gating or statistics**
- **Cannot change the overlap (or lack thereof) of two populations.**

Supports the basic goal of graphing data: showing it in an intuitive, aesthetic manner

Note: the transformation is complex: it is different for each measurement channel and compensation matrix, and depends on the autofluorescence distribution. ***However, these parameters can be automatically selected by the software.***

Transformation Confirms Compensation

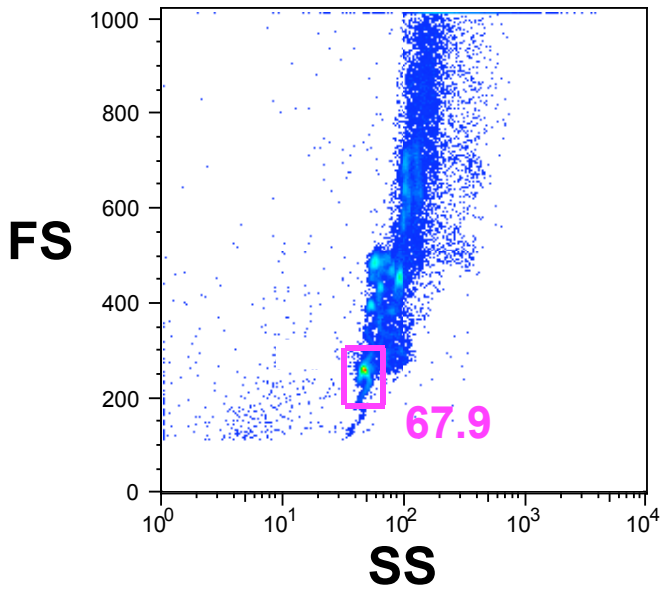


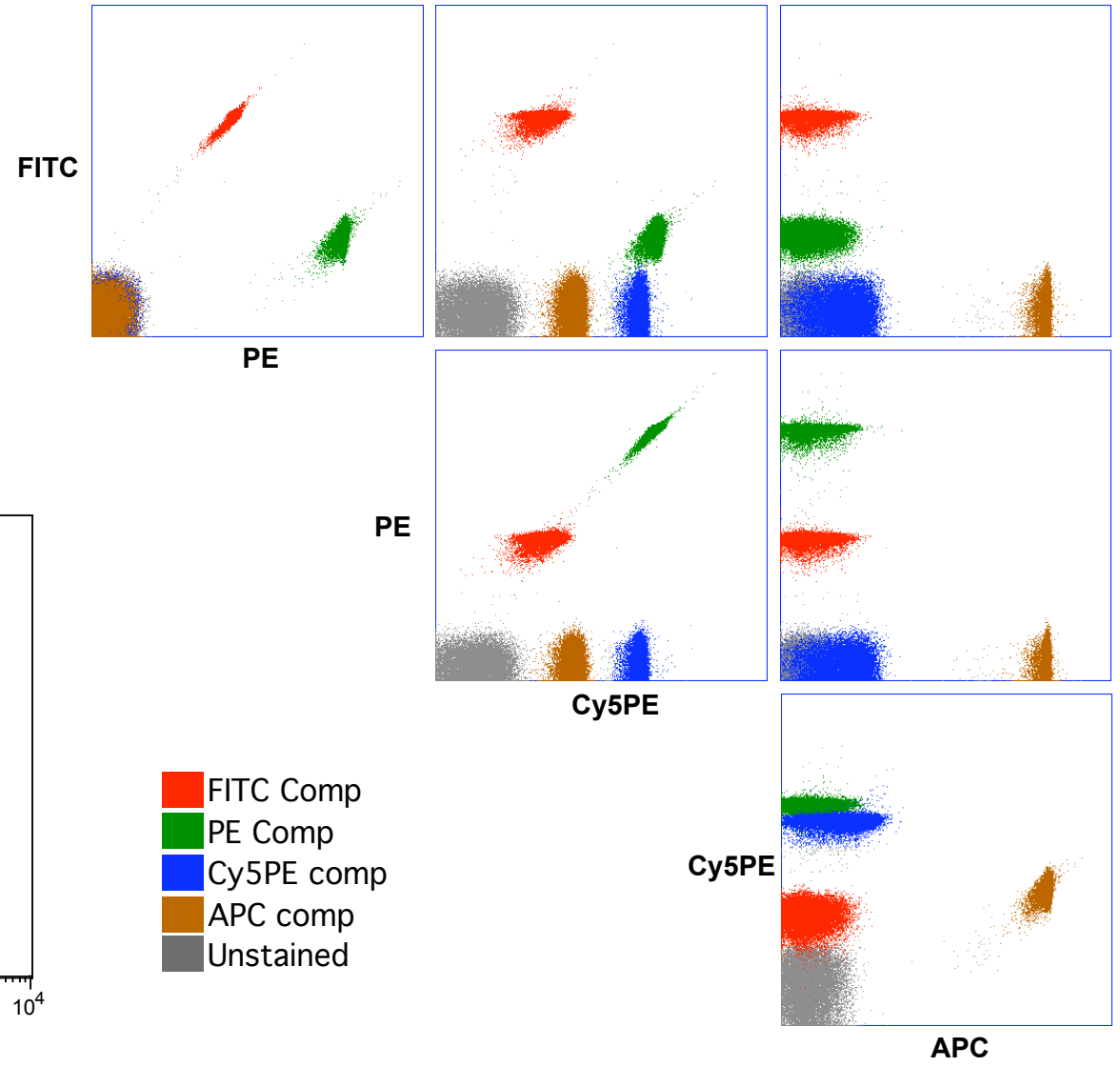
Using Beads to Compensate

- Antibody-capture beads
- Use reagent in use
- Lots positive
- Small CV, bright
- Sonicate
- Some reagents won’t work (IgL, non mouse, too dim, EMA/PI)--mix with regular comps

Using Beads to Compensate

Gate on "Singlets"; then gate on single-stained beads.

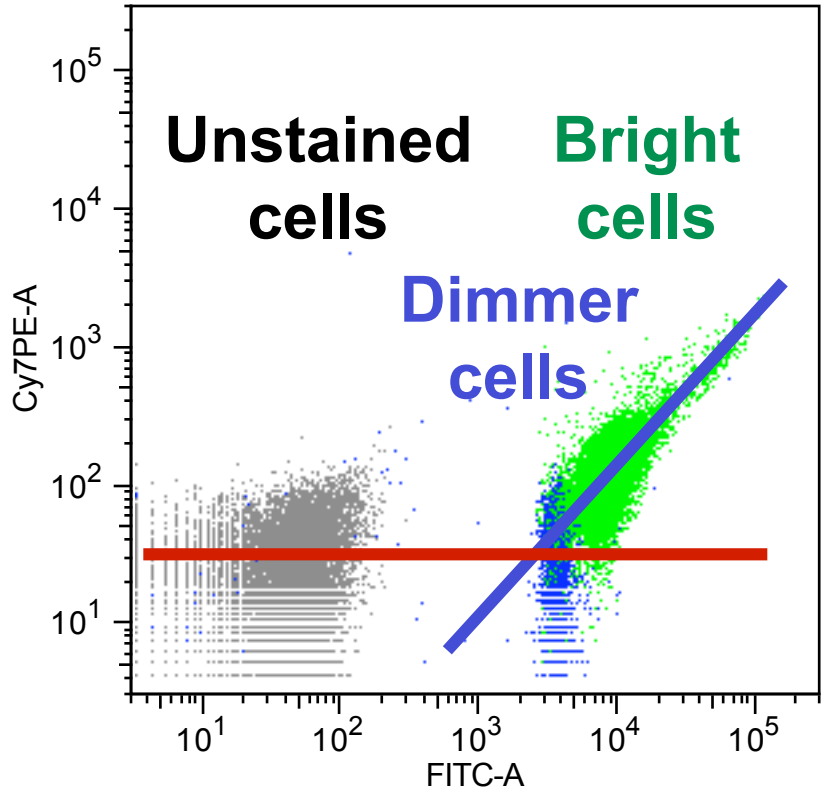




Why Bright Comp Controls?

Estimating a low spillover fluorescence accurately is impossible (autofluorescence).

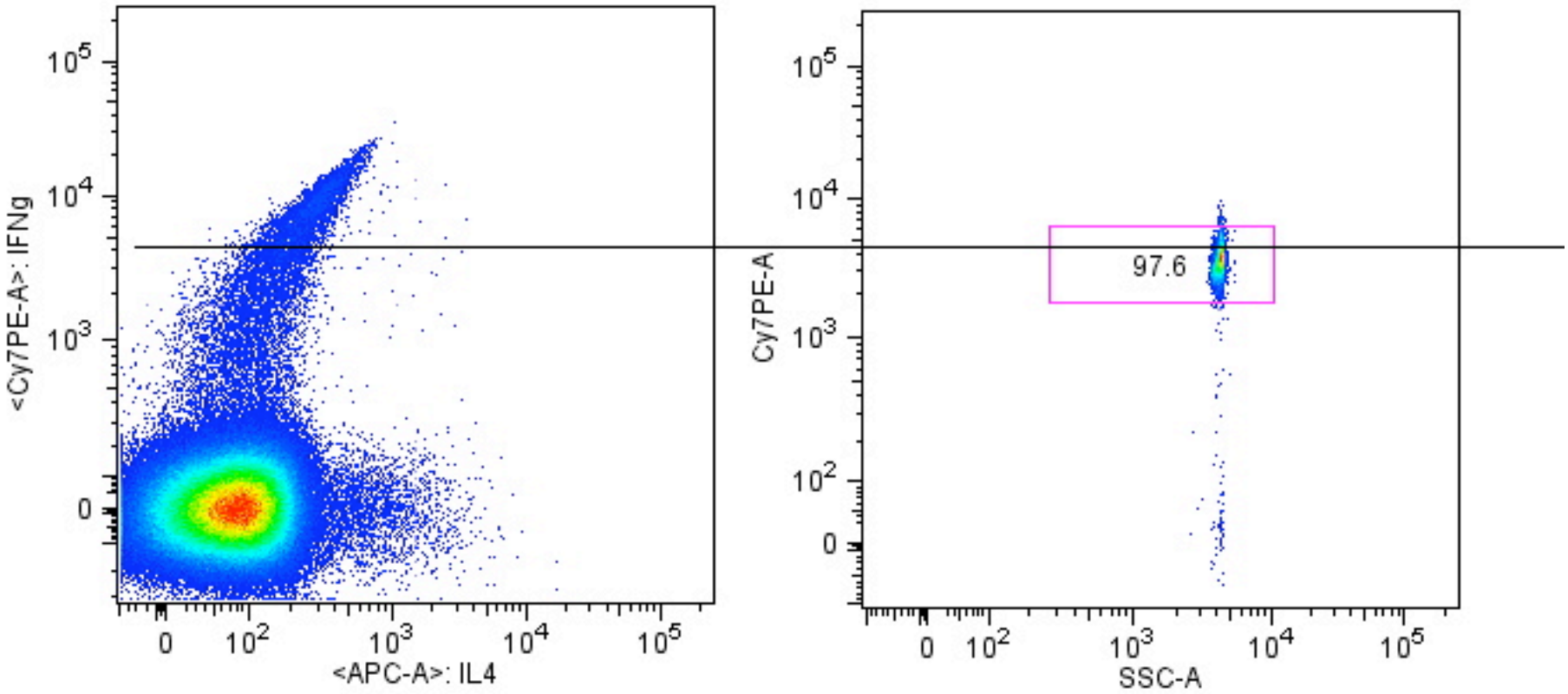
Therefore, compensation is generally only valid for samples that are duller than the compensation control.



FITC spillover into Cy7PE (1%)

Autofluorescence

Insufficiently-Bright Comp Control Is …. Bad!

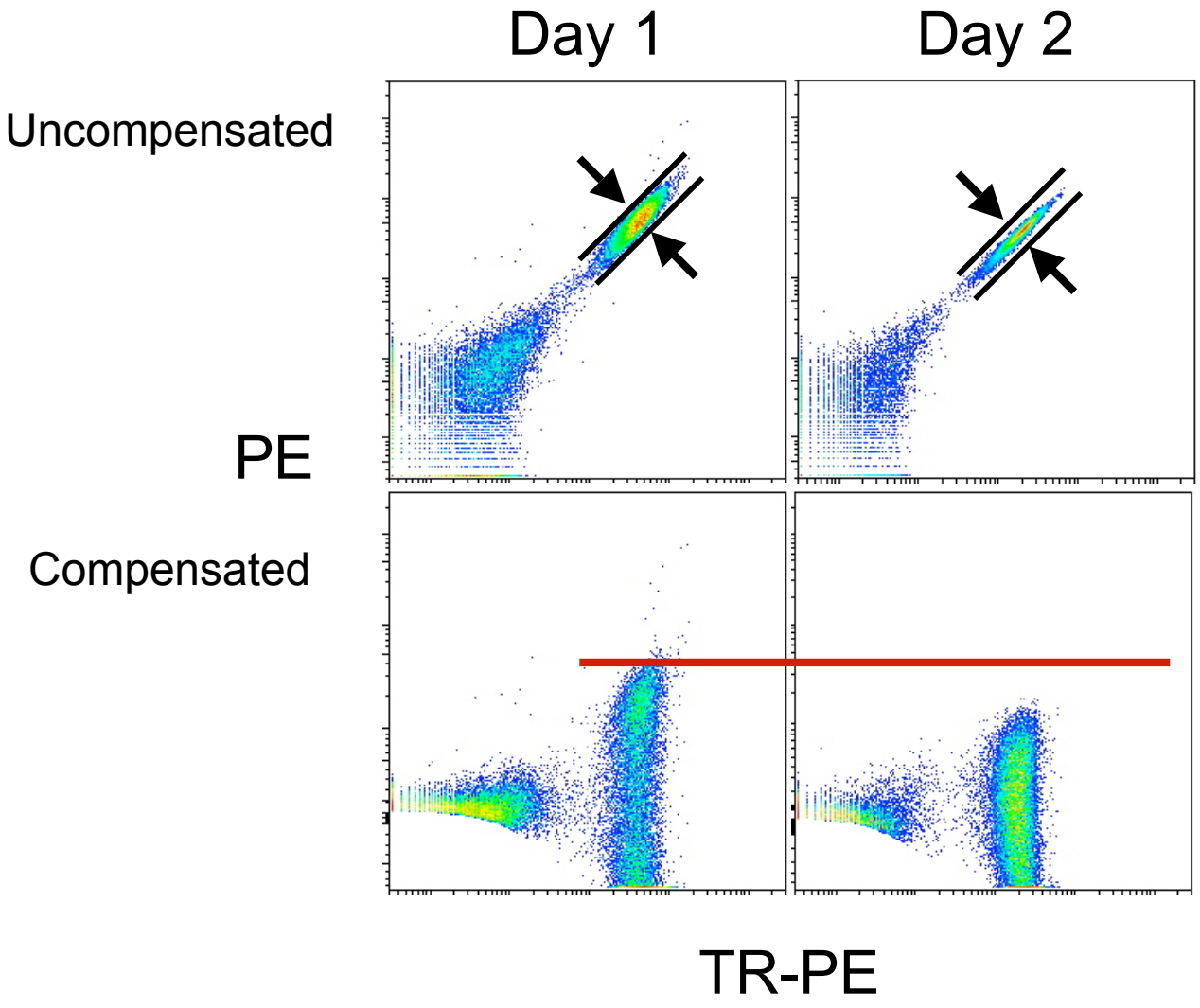


Note that either under- *or* over-compensation can result from using comp controls that are too dim!

Compensation of Multicolor FACS Data

- It is impossible to set proper compensation using visual guides (dot plots, histograms).
  - **Use statistics (medians of gated cells)**
  - **Use automated compensation tools**
- Antibody-capture beads are an excellent way to set compensation!
- Compensation controls must be matched to your experiment and at least as bright as any of your reagents.

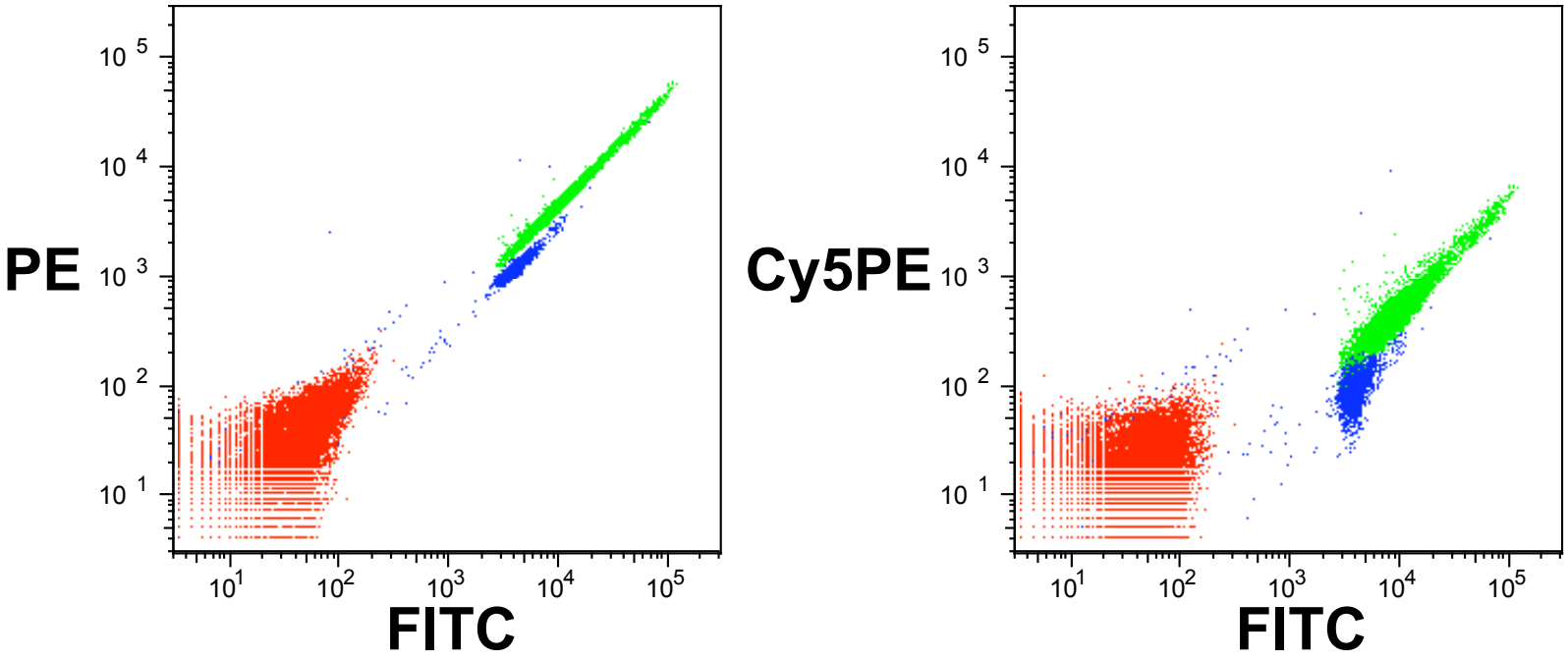
Good Instrument Alignment Is Critical!



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

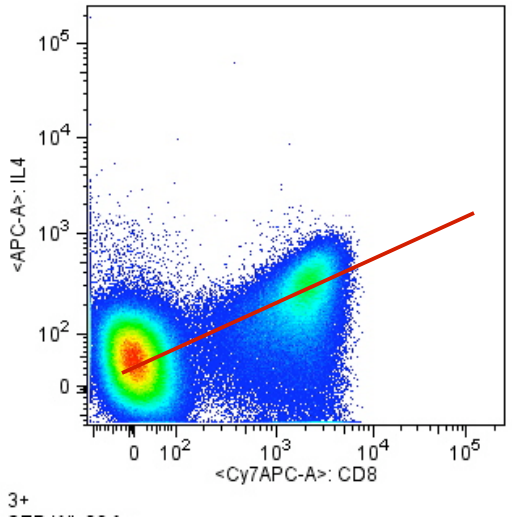
CFSE: A Special Case

Cells stained with CFSE, CD8, or unstained were collected uncompensated.

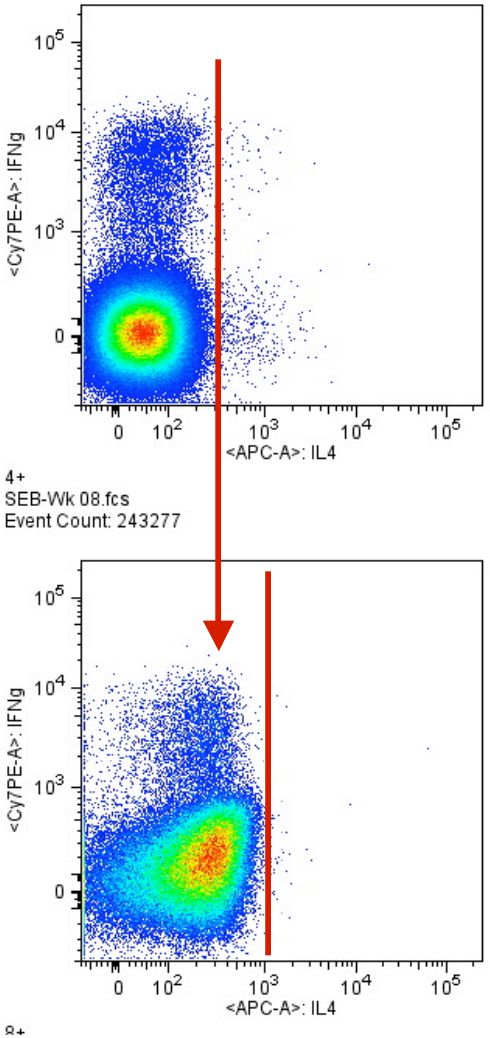


CFSE has a slightly redder spectrum than FITC… must use CFSE as a comp control!

Fix/Perm Changes Cy7APC Compensation Requirement



The longer Cy7APC is in fixative, the more it "falls apart", leading to more APC compensation

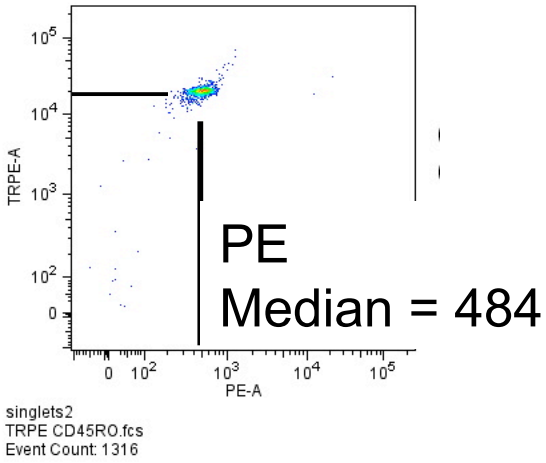


Note that this exacerbates the higher "IL4+" gate required for CD8 cells.

The undercompensation would not have been detected except by looking at the APC vs. Cy7APC graphic…

Different lots of tandems can require different compensation!

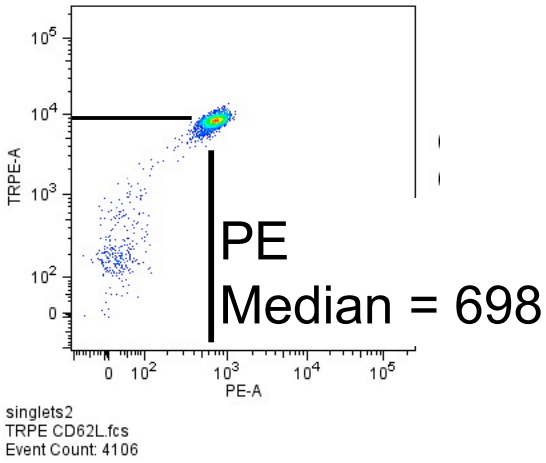
TR-PE reagent 1
Median = 21,100



Compensation Required
(ΔPE / ΔTRPE)

2.3%

TR-PE reagent 2
Median = 8,720



8.0%

Compensating with the wrong TRPE

Wrong TR-PE comp control

Right TR-PE comp control