Compensation

Controls

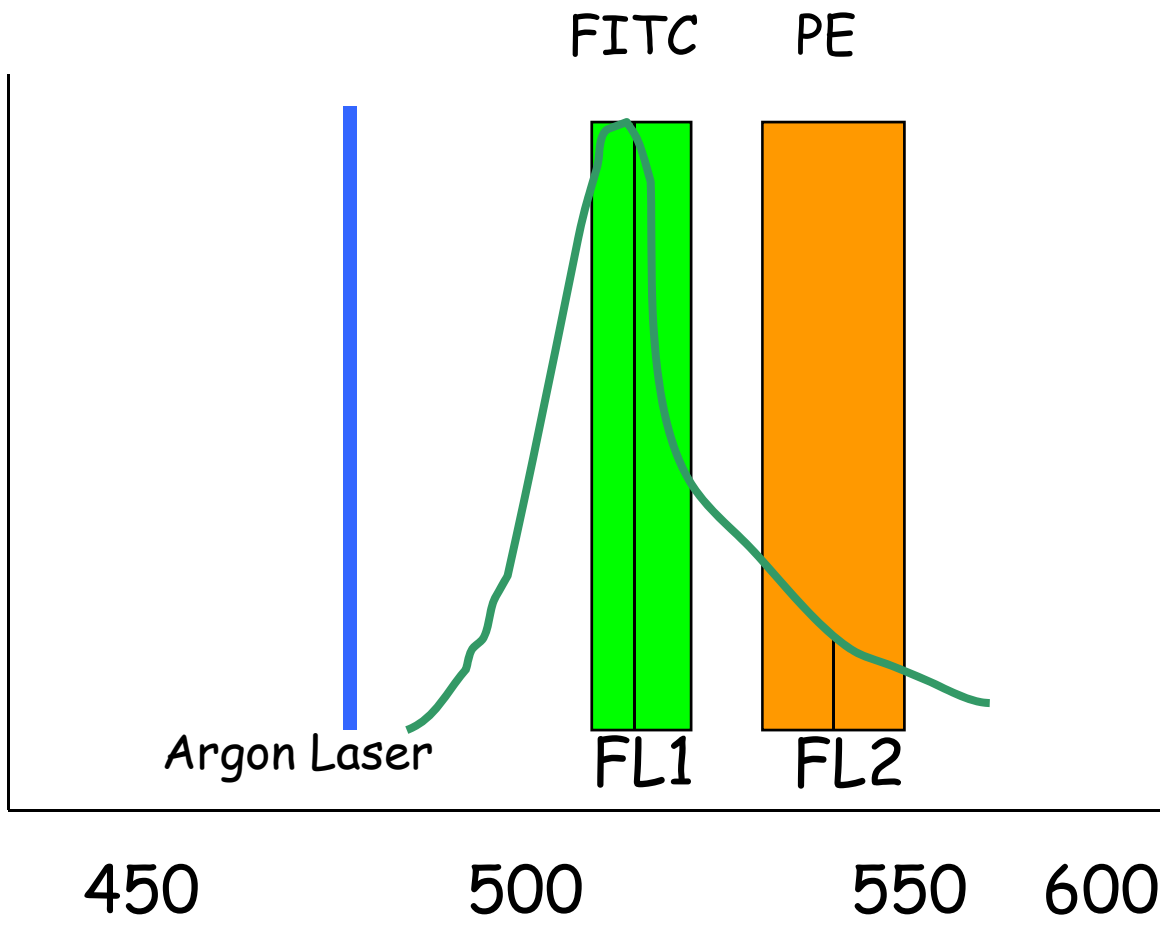
Data Visualization

Panel Development

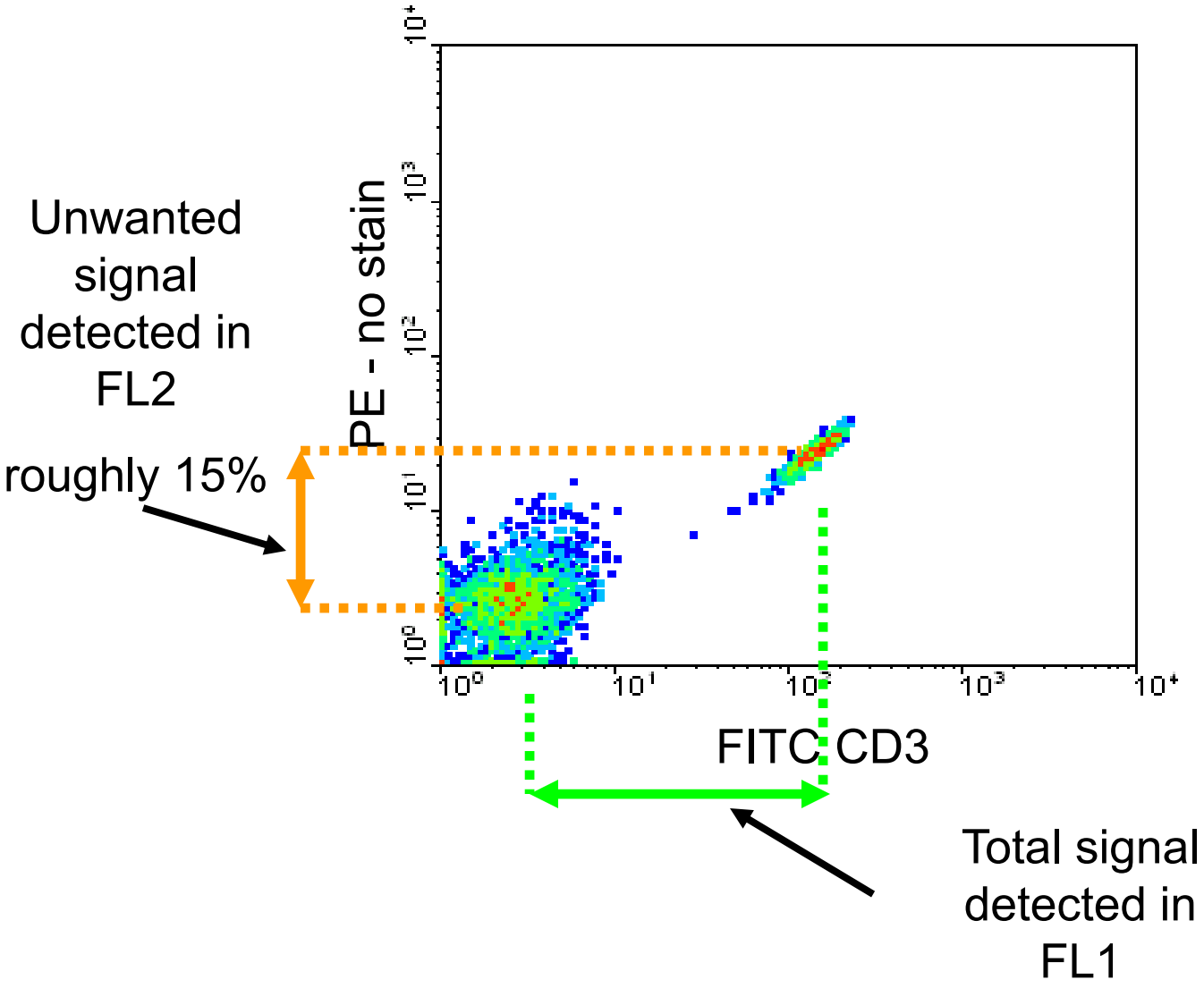
Gating

Quantum Dots

FITC Single Stain Control

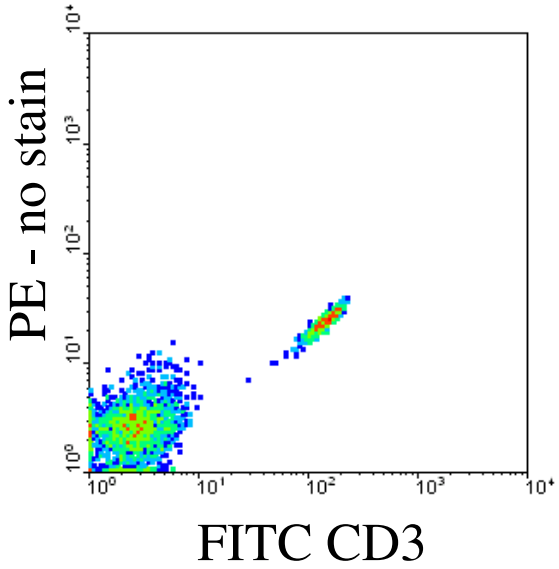


FITC Compensation Control



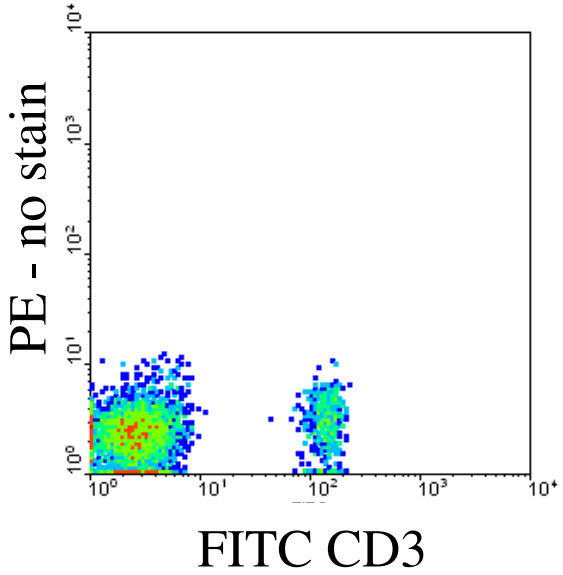
FITC Compensation Control

Uncompensated



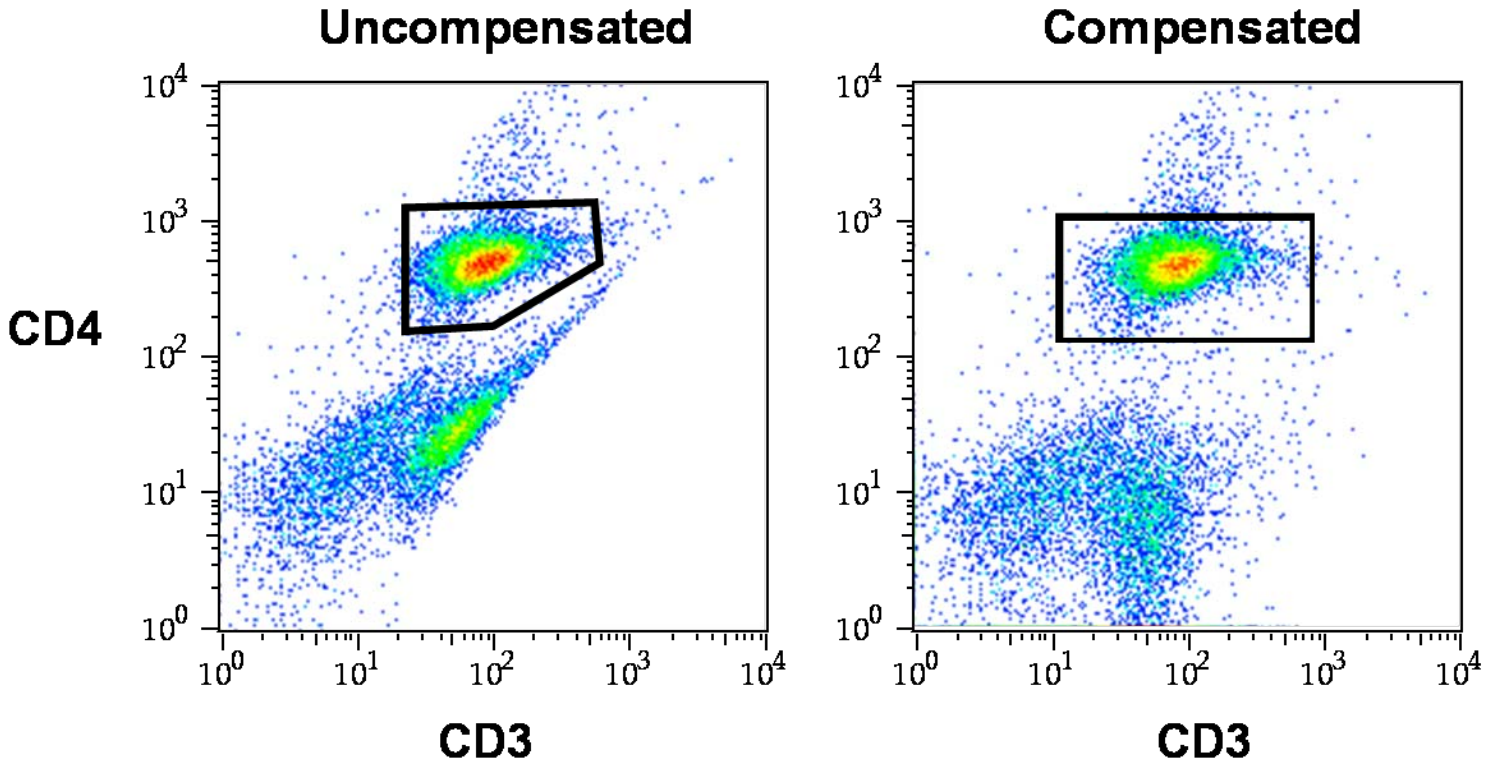
FL2-15%FL1

Compensated



Compensation in 2 colors: Mostly aesthetic

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



Compensation: Mostly aesthetic

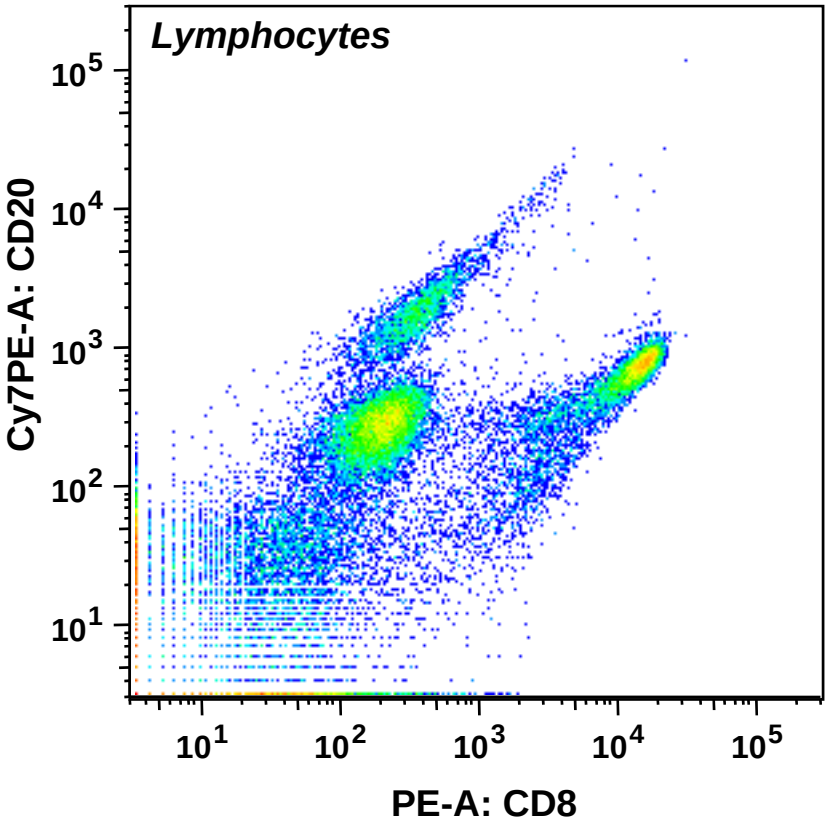
- **Accurate discrimination of subsets is possible with uncompensated data**
- **However, this is true only when the expression of all antigens is uniform on each subset (e.g., CD45 / CD3 / CD4 / CD8)**
- **Otherwise, it may not be possible to gate on subsets (with current tools)**

Spreading due to Measurement Error

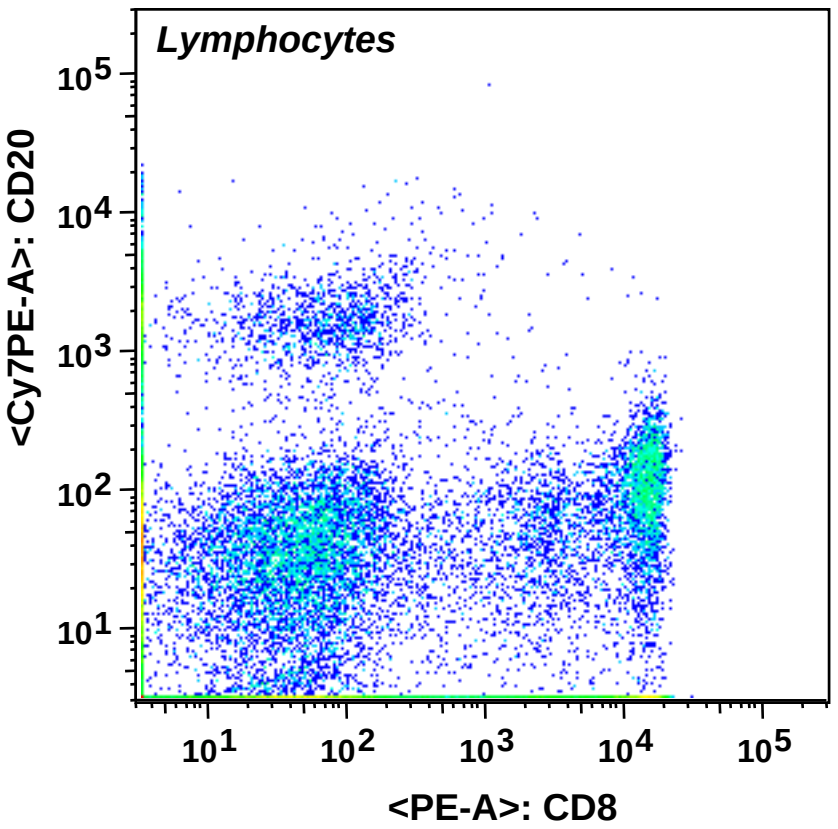
Why do these populations look funny?

Multicolor Compensation

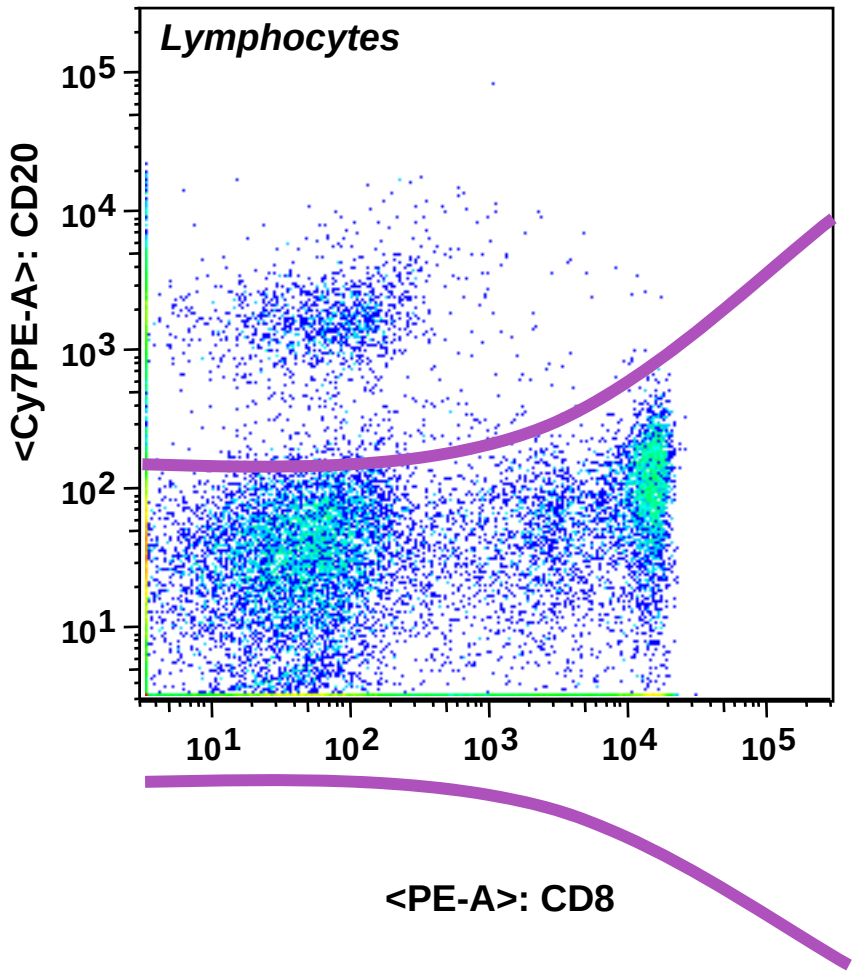
Uncompensated



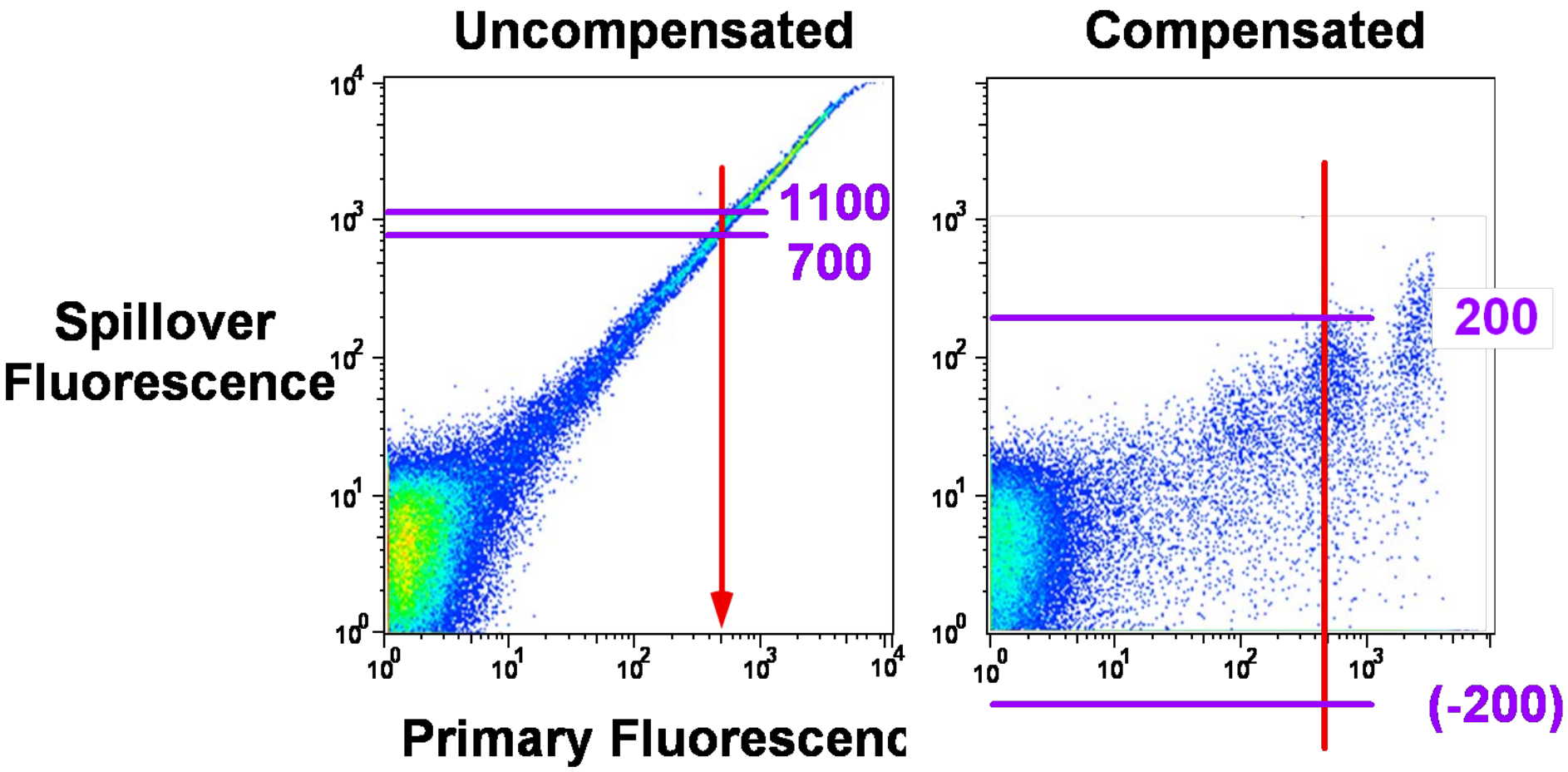
Compensated



The Actual Spread

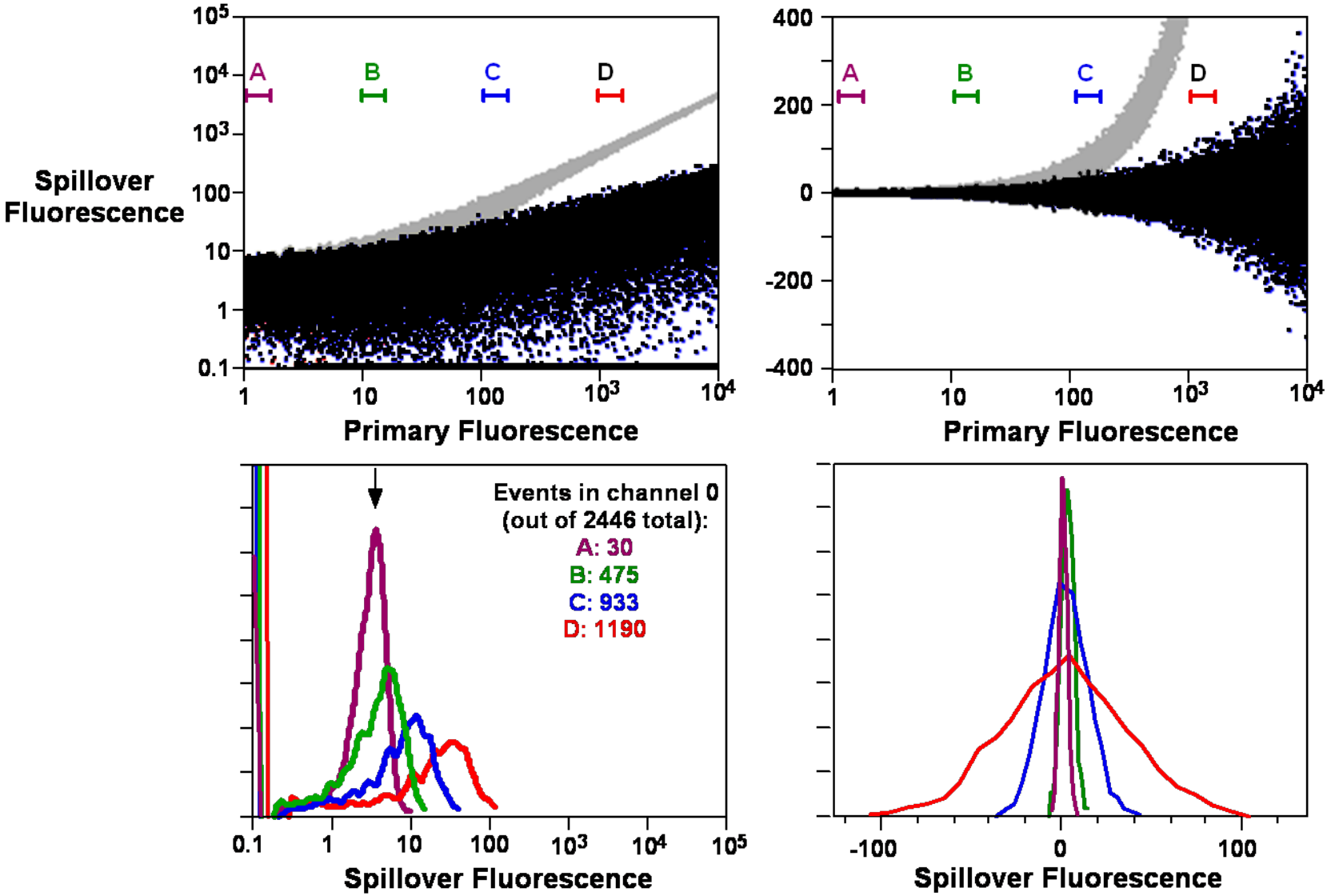


Imperfect Measurement Leads to Apparent Spread in Compensation



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

Log Transformation of Data Display Leads to Manual Overcompensation



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.

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.**

Compensation

Controls

Data Visualization

Panel Development

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Quantum Dots

Controls

Staining controls fall into three categories:

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

Instrument Setup Controls

Typically, fluorescent beads… with a range of fluorescences from “negative” to very bright.

Use these to validate:

- Laser stability & focusing
- Filter performance
- PMT sensitivity (voltage)
- Fluidics performance
- Daily variability

Consider setting target fluorescences for alignment: this allows for greatest consistency in *analysis* (gating) between experiments.

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)

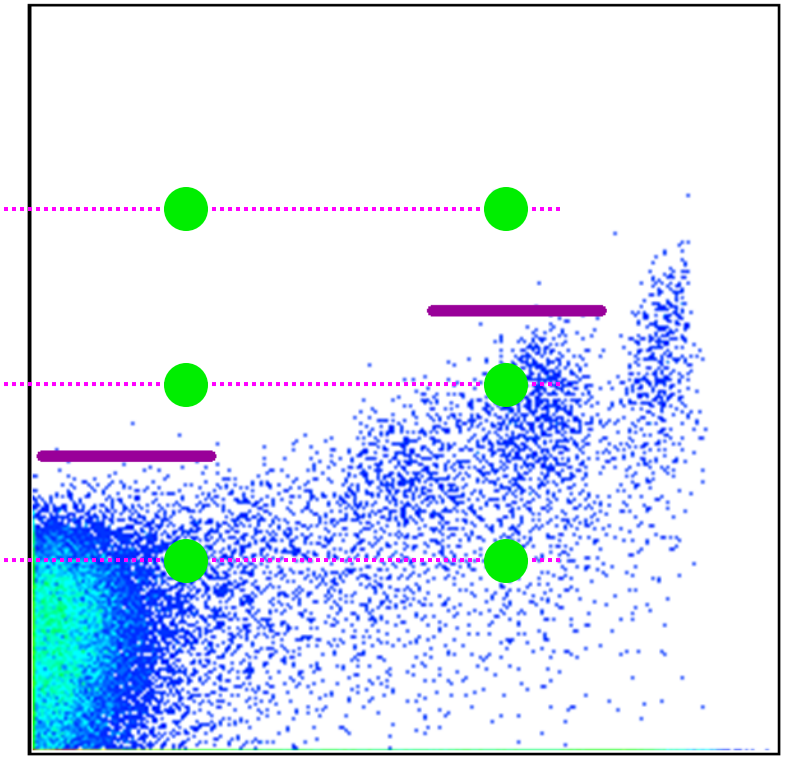
Using Beads to Compensate

- 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

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.

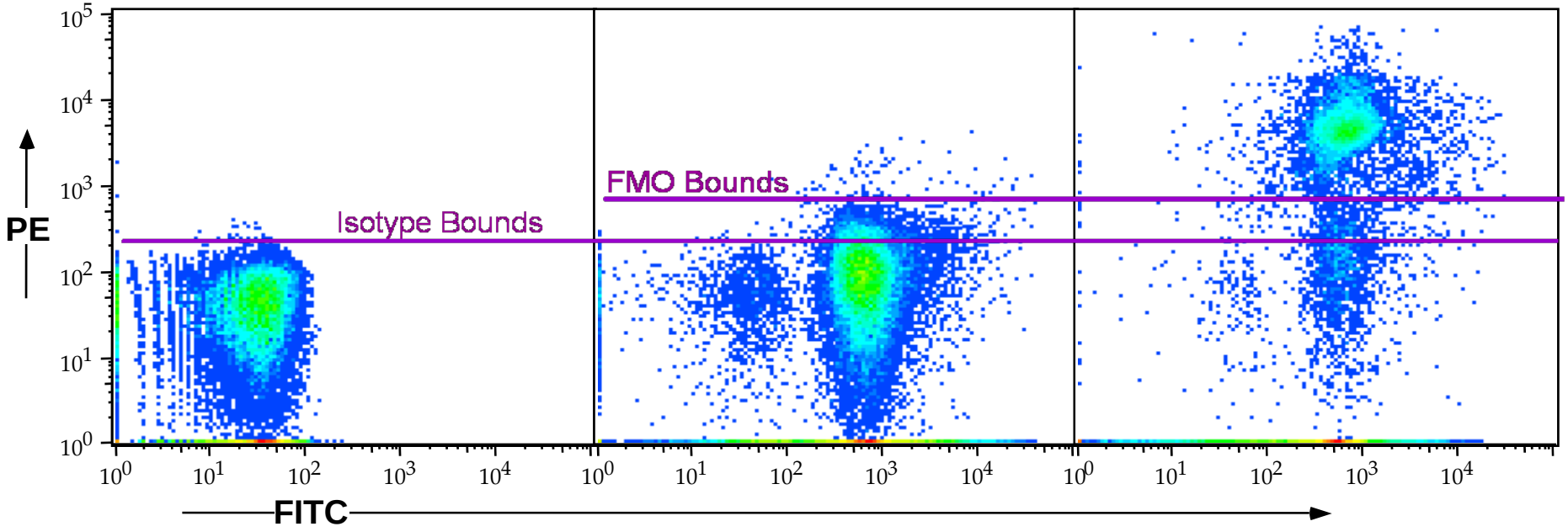
FMO Control

"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



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

3 Color Experimental Setup

Example staining setup of a 3 color experiment:

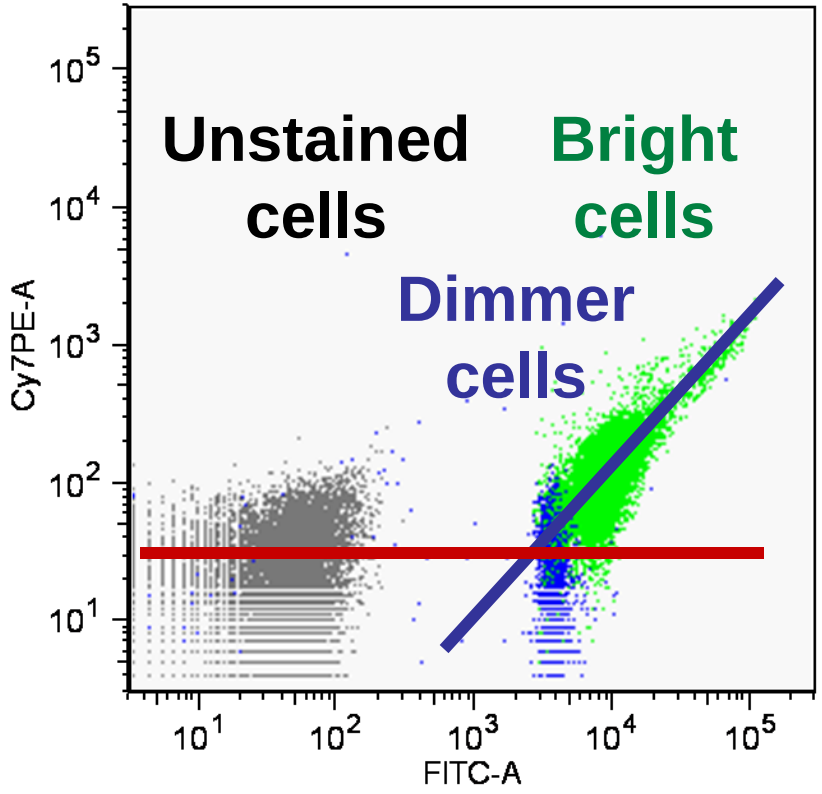
Tube #	Description	FL1	FL2	FL3
1	*Unstained Sample*	-	-	-
2	*Experimental Sample*	CD3 FITC	CD4 PE	CD8 Cy5PE
3	*Compensation Controls* (Single stains – one for each fluorochrome used in the experiment)	CD3 FITC	-	-
4		-	CD4 PE	-
5		-	-	CD8 Cy5PE
6	*Gating Controls* (FMO – leave out one fluorochrome at a time)			
7		CD3 FITC	-	CD8 Cy5PE
8		CD3 FITC	CD4 PE	-
9	*Experimental Controls* (fully stain healthy or untreated samples to compare to experimental sample)	CD3 FITC	CD4 PE	CD8 Cy5PE
10		CD3 FITC	CD4 PE	CD8 Cy5PE
11		CD3 FITC	CD4 PE	CD8 Cy5PE

* no stain added or add isotype matched control stain.

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.



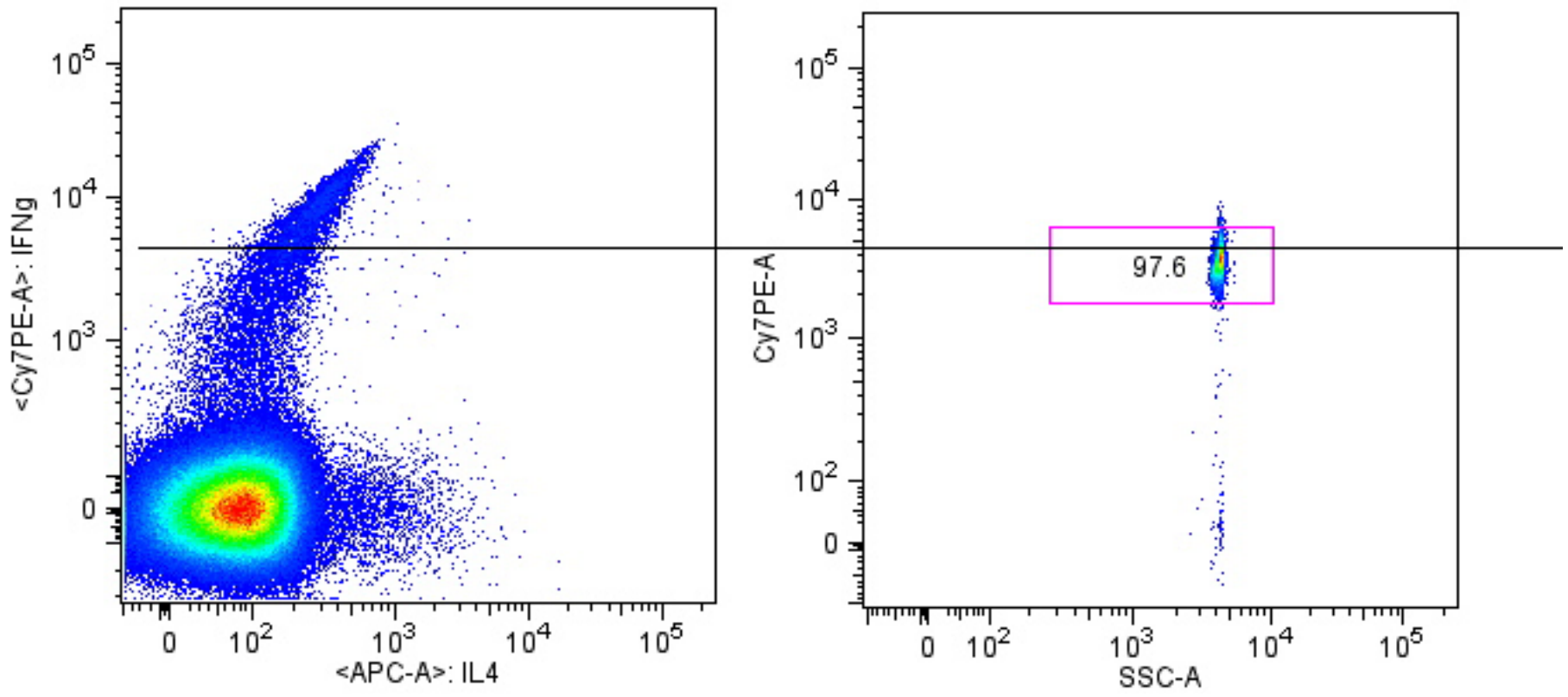
FITC spillover into Cy7PE (1%)

Autofluorescence

Some Examples of Problems

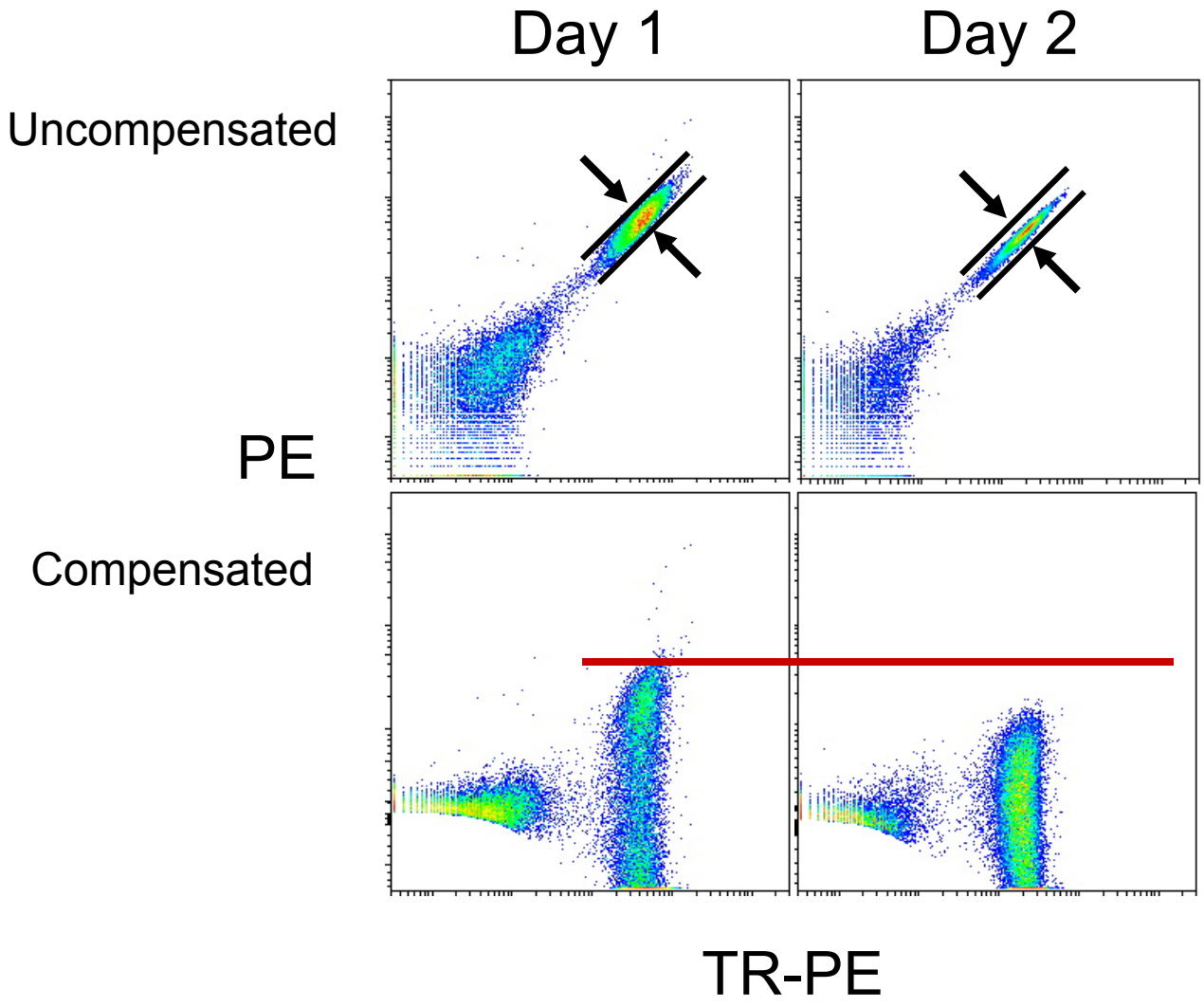
- The following four examples illustrate some types of problems that can be occur related to compensation.
- In each case, compensation itself is not the problem: there is an underlying reagent, instrumentation, or analysis problem.
- However, the manifestation of this problem is an apparent incorrect compensation!

Insufficiently-Bright Comp Control Is …. Bad!



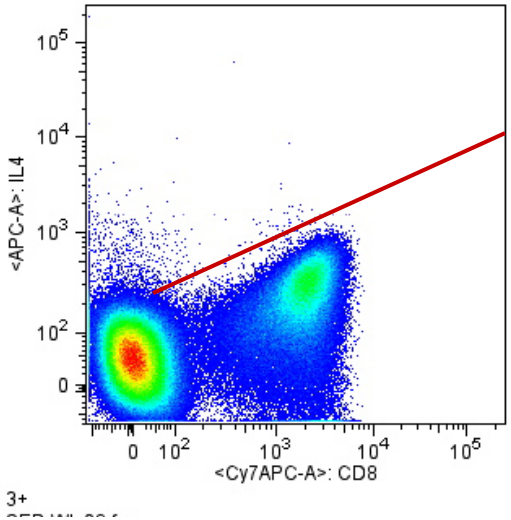
Note that either under- *or* over-compensation can result from using comp controls that are too dim!

Good Instrument Alignment Is Critical!

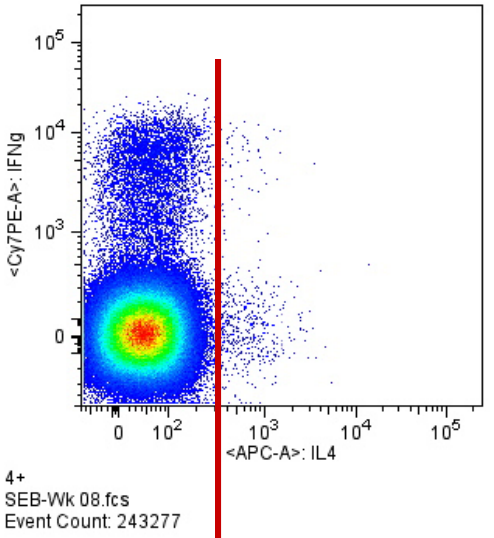


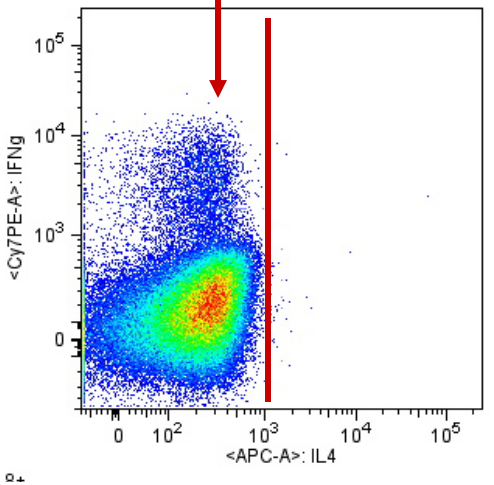
While the amount of compensation did not differ, the measurement error (correlation) decreased leading to much better visualization of the population!

Fix/Perm Changes Cy7APC Compensation Requirement



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



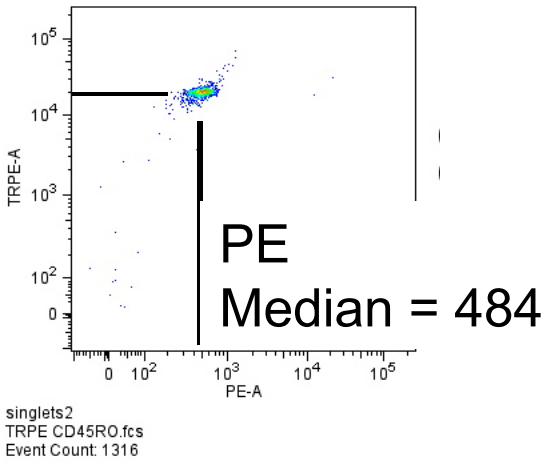


Note that this exacerbates the higher "IL4+" gate required for CD8 cells.

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

Different lots of tandems can require different compensation!

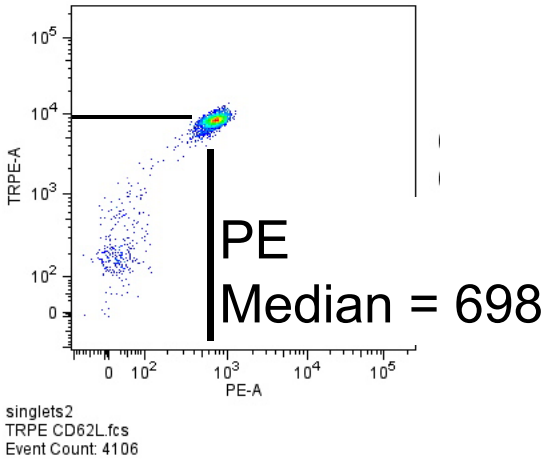
TR-PE reagent 1
Median = 21,100



Compensation Required
(ΔPE / ΔTRPE)

2.3%

TR-PE reagent 2
Median = 8,720

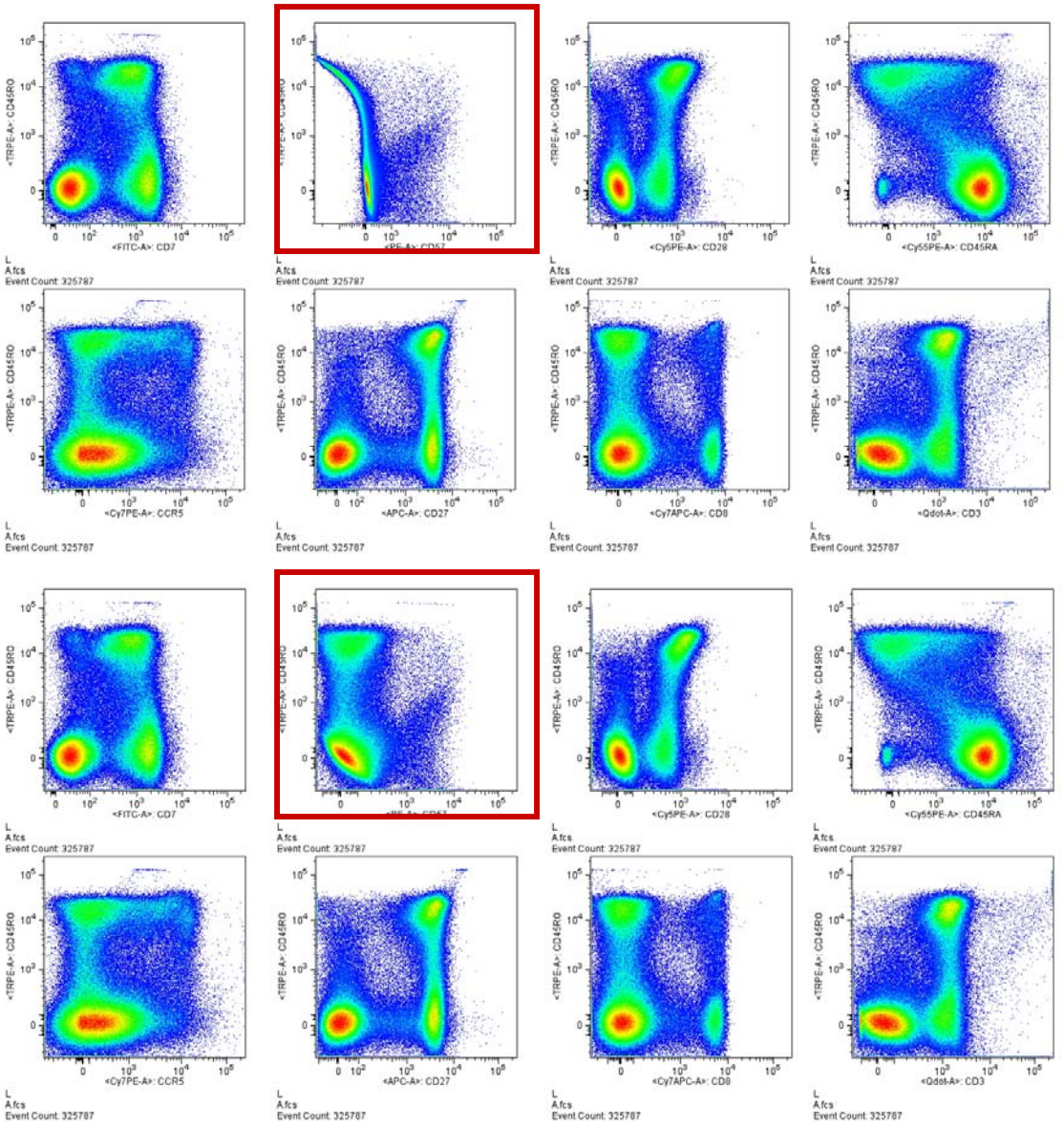


8.0%

Compensating with the wrong TRPE

Wrong TR-PE comp control

Right TR-PE comp control



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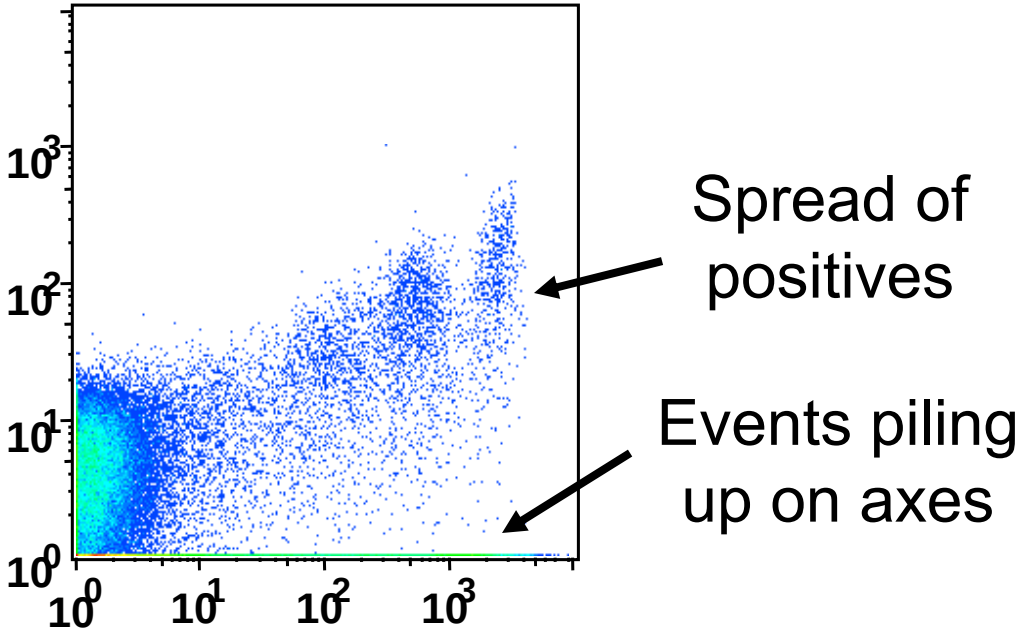
Compensation & Data Visualization

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!



Is There A Solution?

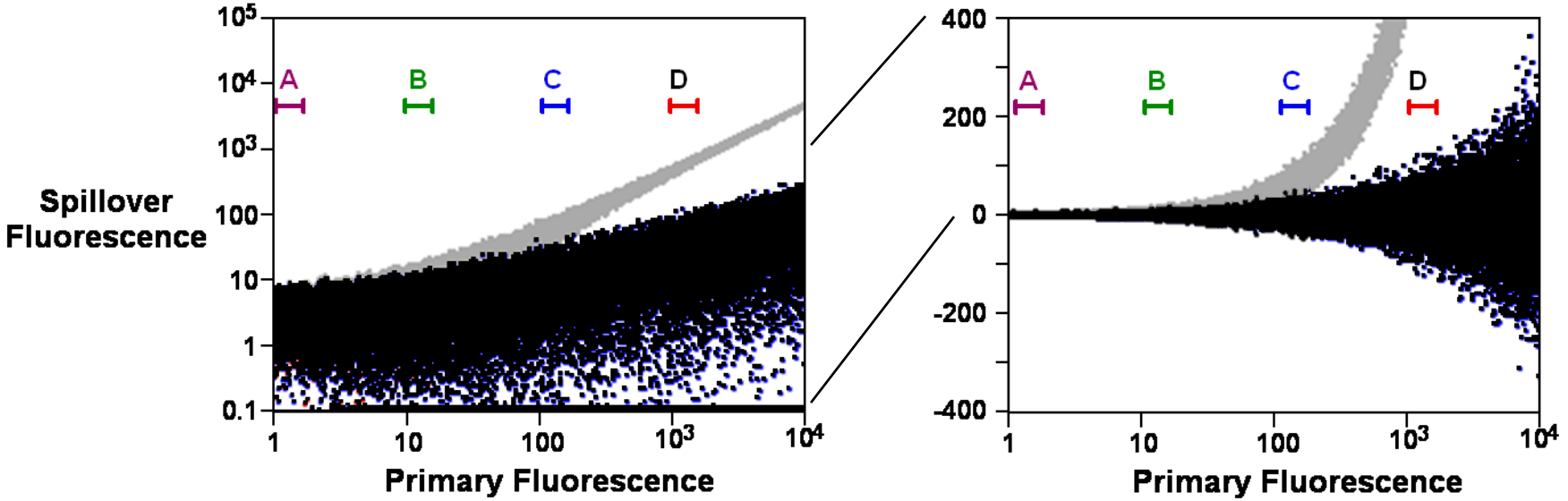
- The spread in compensated data is unavoidable (basic physics)
- Can we visualize data so that the distributions are more intuitive?
- Nearly all immunophenotyping data is shown on a logarithmic scale… why?
  - Dynamic range of expression (4 logs)
  - Often, distributions are in fact log-normal

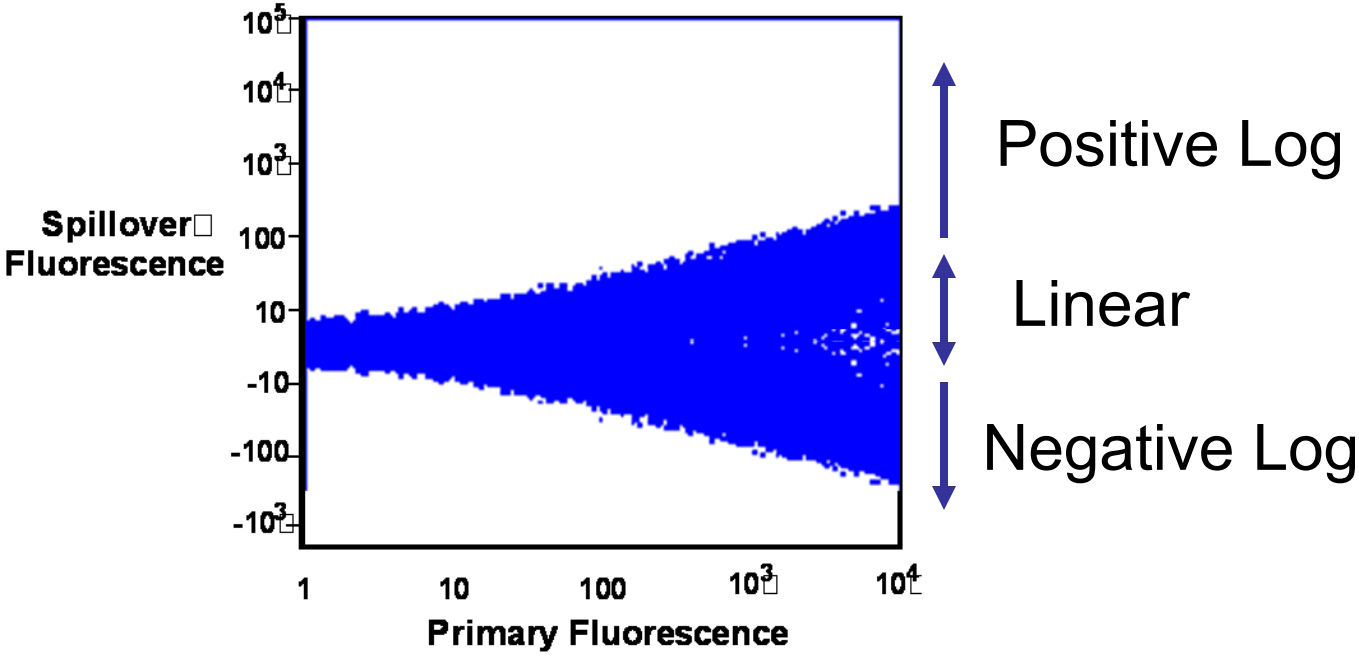
Alternatives to a Log Scale

- 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*

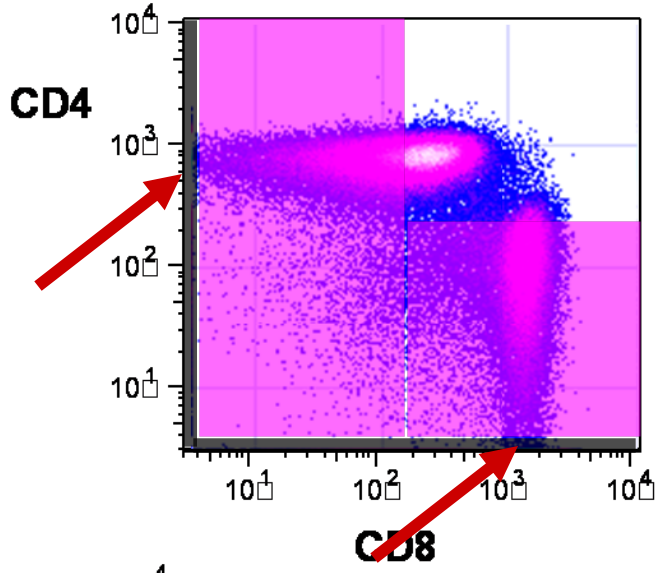
“Bi-exponential” Scaling

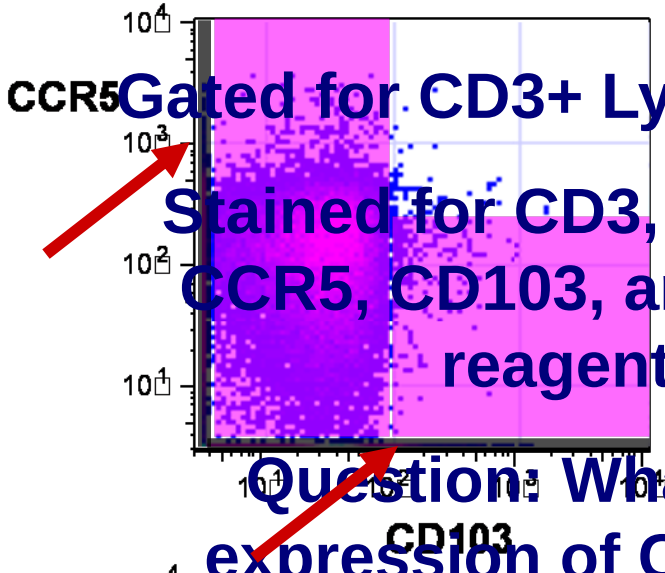
Wayne Moore
Dave Parks





“Bi-exponential” Transformation Makes Compensated Data More Intuitive

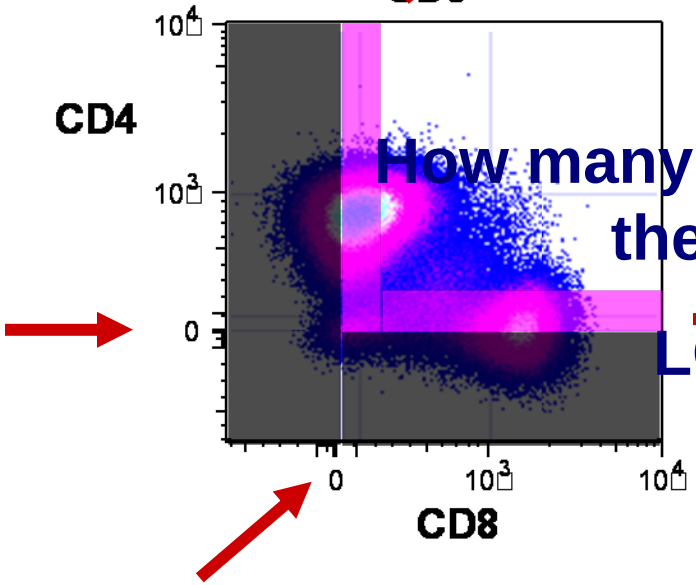




Gated for CD3+ Lymphocytes; Stained for CD3, CD4, CD8, CCR5, CD103, and 7 other reagents

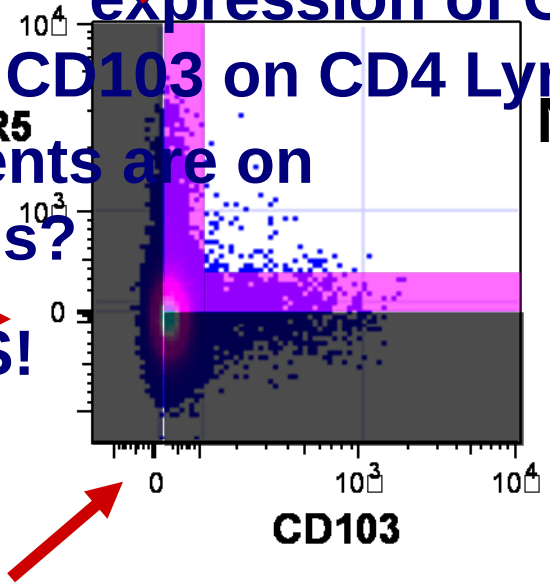
Question: What is the expression of CCR5 and CD103 on CD4 Lymphocytes?

Result:



How many events are on the axis?

LOTS!



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

No events hidden

Transformed Distributions

Populations are visually identifiable

“Bi-exponential” Transformation Makes Compensated Data More Intuitive

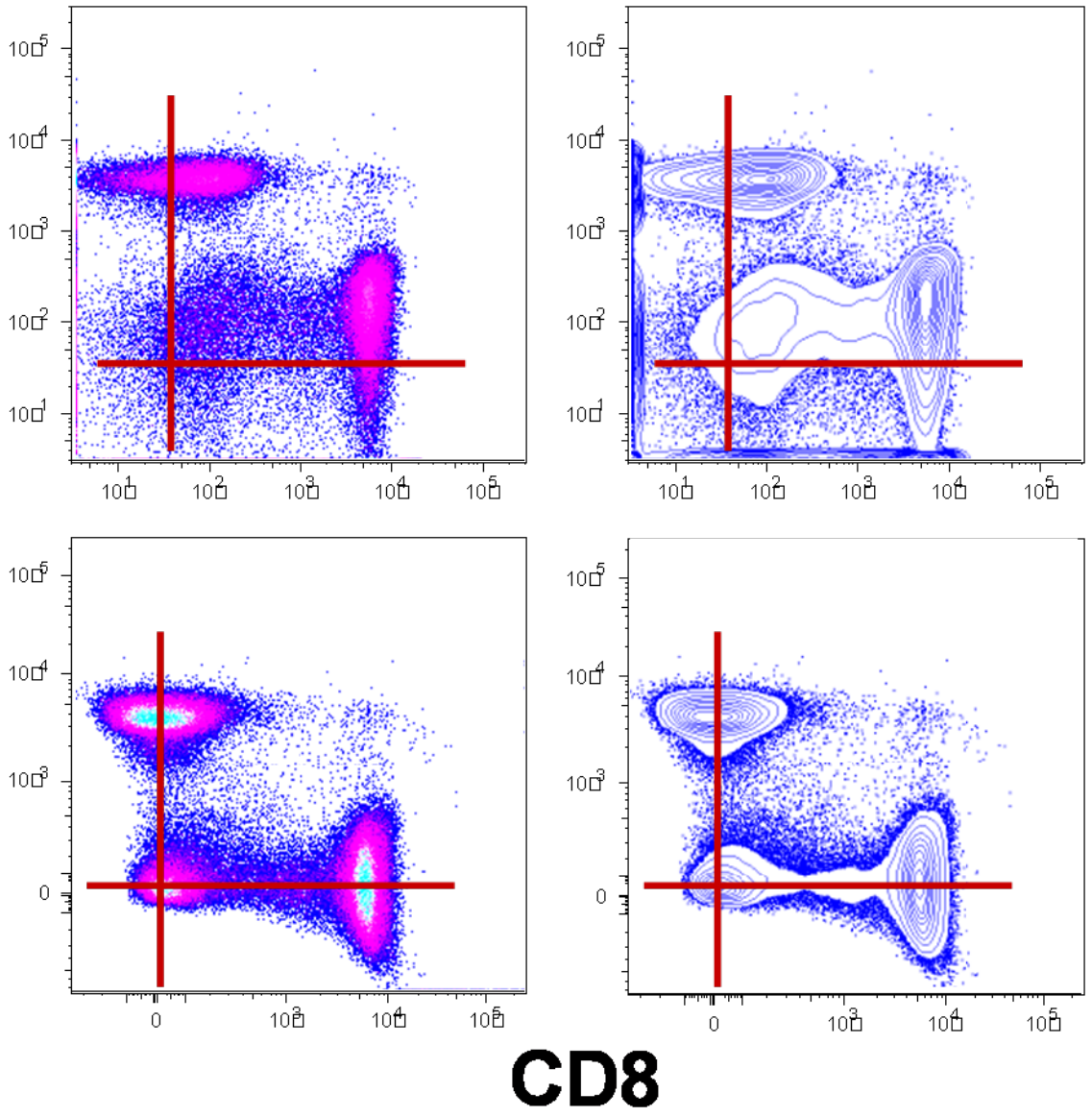
Only changes the visualization of data

- **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



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Designing a Multicolor Panel

Considerations:

1. **What do you want to identify?**
    - Minimum set of necessary markers
    - Multiple panels vs. single panel
2. **What do you want to exclude?**
    - Dump channel
    - Negative markers
3. **What additional markers might you use?**
    - Rank: Is it useful, or is it luxury?

How Many Markers to Use?

It is always tempting (and in fact desirable) to use as many markers as possible.

However, this must be balanced against the overriding tenet of multicolor flow cytometry

The more colors you use, the more problems you will have

Problems include:

- Loss of sensitivity (from spectral crossover)
- Unwanted FRET
- Reagent interactions

How Many Markers to Use?

Divide your potential reagents into three groups:

(1) Absolutely necessary

(2) Important

(3) Luxury

Always consider splitting panels if the information content not overlapping (for example, if you are separately interrogating B cells and T cells).

You will optimize in same order as your list, being careful to validate each step against the previous.

Advantage of More-Than-Minimal Markers

Two extremes of gating strategy:

“Liberal” - gates are drawn to include much larger areas than visually appear to belong to a subset.

- Greatest sensitivity
- Greatest chance of contamination

“Conservative” - gates are drawn to be very “tight” around the visually-defined populations

- Greatest purity of subset
- Lowest sensitivity

Note that multiple rounds of “Liberal” gating (based on multiple parameters) often results in excellent purity.

Advantage of More-Than-Minimal Markers

When designing your panels, try to include reagent combinations that will allow you a combination of positive and negative expression gates for every subset of interest.

Note that there is almost never a downside to including additional markers that are negative gates--the lack of this fluorescence signal on your cells of interest cannot alter the sensitivity of your measurements.

“Dump” channels and viability channels are virtually always a good thing!

Selection of Marker/Color Combinations (1)

All colors are not created equal.

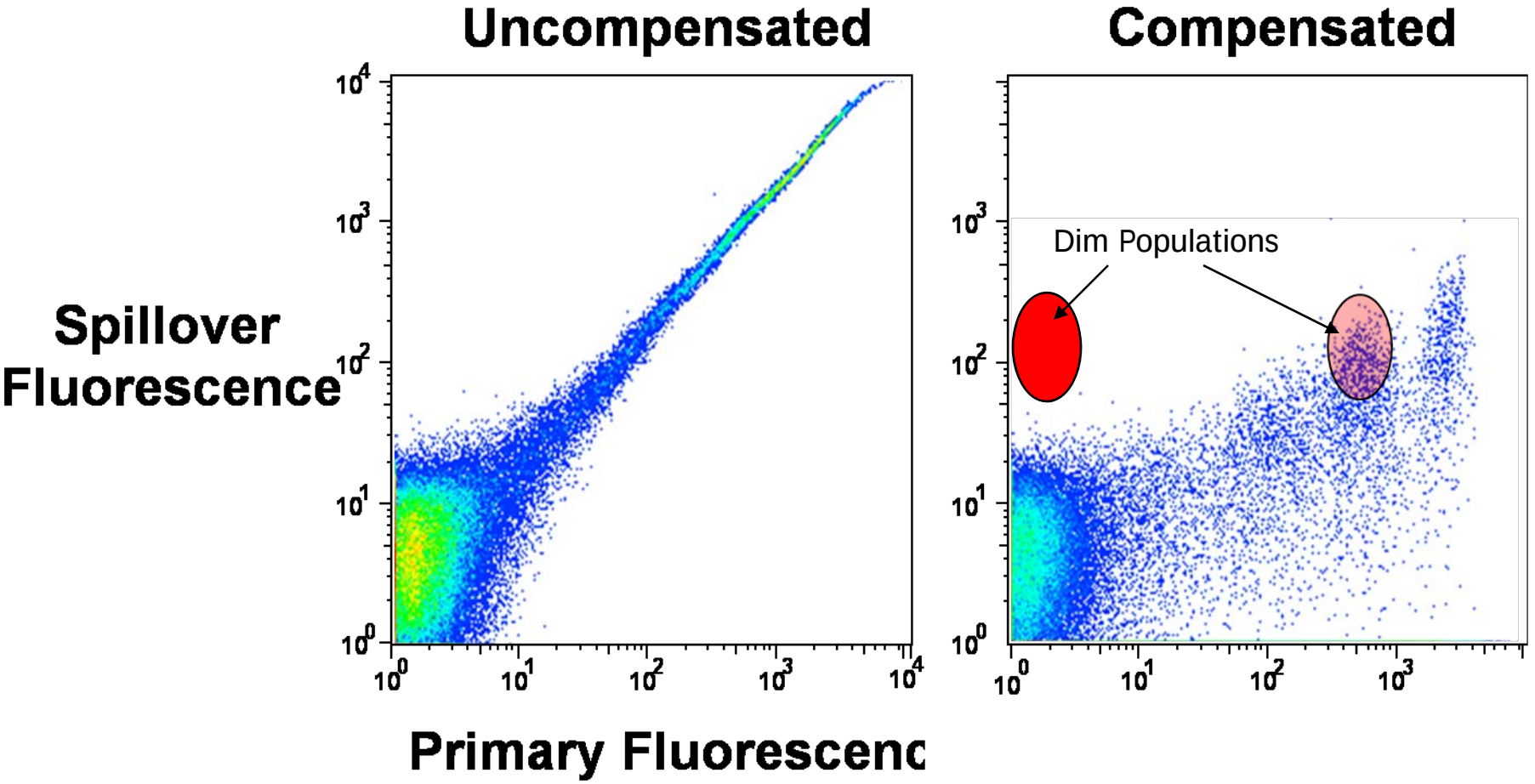
The same monoclonal antibody conjugated to FITC, PE, Cy5PE, APC, Cy7APC can show apparently different distributions on singly-stained cells.

Two facets contribute to this:

Reagent brightness: Compared to autofluroescence, dimly stained cells may resolve with some colors but not others (combination of brightness, AF, sensitivity)

Absolute signal: PE yields many more photons per antibody-conjugate than Cy7PE, hence the *width (CV)* of distributions is narrower, providing better separation even for brightly-stained cells.

Panel Development: Effect of Spreading Error



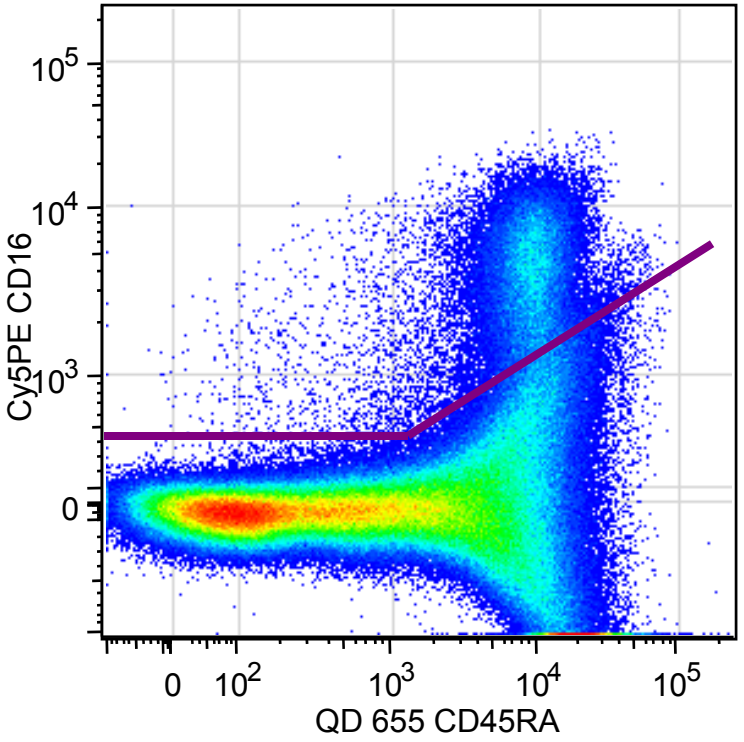
This spreading error makes it difficult to detect dimly-staining populations.

Selection of Marker/Color Combinations (2)

All colors are not created equal.

The same monoclonal antibody conjugated to FITC, PE, Cy5PE, APC, Cy7APC can show apparently different distributions on multiply-stained cells.

This is due to spectral-spillover, and the propagation of the error in those measurements.



Selection of Marker/Color Combinations (2)

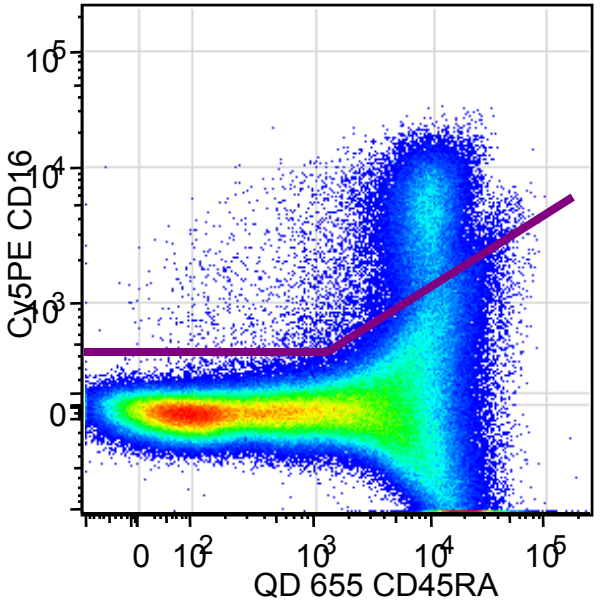
Prediction of the spillover effect is very difficult. You need to know three different aspects:

(1) The brightness of the *other* reagents in your panel

(2) The spillover of these reagents into your channel

(3) The absolute brightness of every measurement

The amount of spread in your measurement channel is equal to the sum of all other reagents' brightnesses multiplied by their spillover coefficient and by the inverse square root of the absolute brightness….



Selection of Marker/Color Combinations

Given the difficulty in predicting how color selection for each reagent will perform in the final panel, it is necessary to perform panel optimization *empirically* and *iteratively*.

The iterative process should be performed *step-wise*: begin with a subset of the the reagents in the panel, and then add the other reagents one or two at a time.

At each step, validate the combination to make sure the performance is what you expect.

Fortunately, this process is not pure guess-work…

Selection of Marker/Color Combinations

We divide reagents into three categories:

"Primary" Well-characterized, identify broad subsets of cells, expression is usually on/off.

e.g., CD3, CD4, CD8, CD14, CD19, CD20
Typically used as "parent" gates in analysis

"Secondary" Well-characterized, bright expression patterns

e.g., CD27, CD28, CD45RA/RO, γIFN, perforin
Expression levels can be a continuum

"Tertiary" Low-expression levels or uncharacterized

e.g., CD25, CCRs, "X"

Selection of Marker/Color Combinations

"Primary" Well-characterized, identify broad subsets of cells, expression is usually on/off.

e.g., CD3, CD4, CD8, CD14, CD19, CD20
Typically used as "parent" gates in analysis

These reagents are usually assigned to "dimmer" colors and colors that exhibit the greatest spillover problems

e.g., Cy5.5PE, Cy7PE, Cy7APC, AmCyan

Selection of Marker/Color Combinations

"Secondary" Well-characterized, bright expression patterns

e.g., CD27, CD28, CD45RA/RO, γIFN, perforin
Expression levels can be a continuum

These are usually assigned to the next tier of colors, those that perform well with little spillover problems

e.g., FITC, TRPE, Cy5PE/PerCP, Alexa 405, Alexa 690

Selection of Marker/Color Combinations

"Tertiary" Low-expression levels or uncharacterized

e.g., CD25, CCRs, "X"

These require the absolutely brightest colors, with the least spillover problems possible

e.g. PE, APC, QD655

Reagent Inventory

In order to test multiple combinations and iteratively improve your panels, you will need to have multiple colors of each conjugate available!

This is expensive. (Hopefully, the reagent manufacturers will help).

Our approach is to have as many combinations of Primary reagents as possible, less for Secondary, and only one or a few for Tertiary.

Example Optimization

In this example, we wished to evaluate the expression of CXCR3 and CCR4 on naïve ($CD62L^{+}CD45RA^{+}CD45RO^{-}$) CD4 T cells.

- What fraction of naïve T cells express these molecules?
- If possible: are those cells “truly” naïve ($CD28^{+}CD11a^{dim}CD27^{+}$)?

Requirements:

CD4, CD3 = Primary reagents

CD45RO/RA, CD62L = Secondary (need excellent separation)

CXCR3, CCR4 = Tertiary reagents

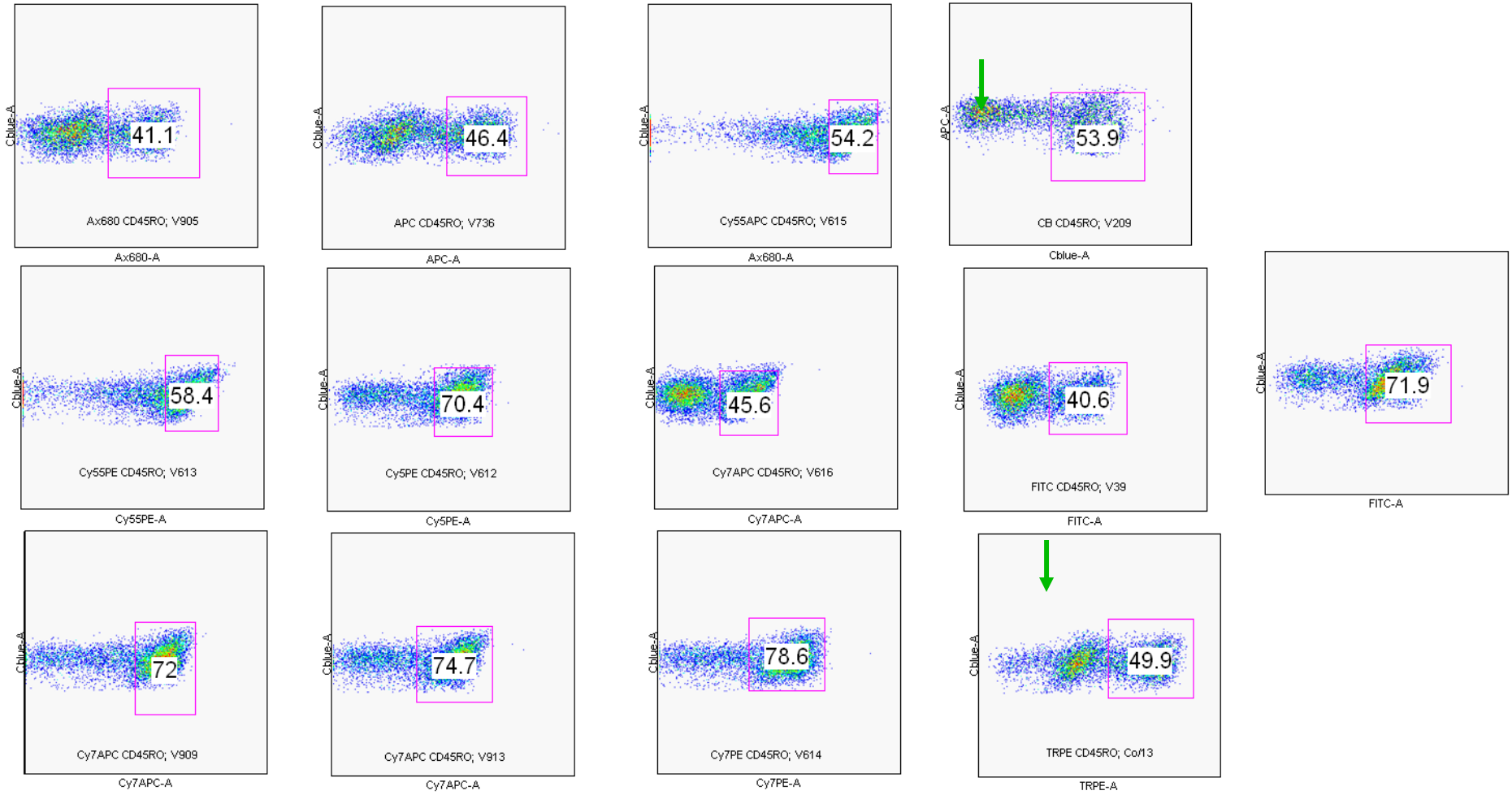
CD27, CD11a, CD28 = Luxury reagents

General Approach

1. Test all conjugates of Secondary reagents to determine how good they are.
2. Choose 3-4 best conjugates, and construct panels with Primary reagents “slotted” in.
3. Evaluate expression patterns to ensure appropriate identification of naïve/memory subsets.
4. Evaluate potential sensitivity of FITC and PE channels (where CXCR3 and CCR4 will be used).

CD45RO Example Stains

For panel



Design of panels

Since optimal sensitivity was desired, I tried to minimize reagents that would have spillover-spreading into FITC and PE.

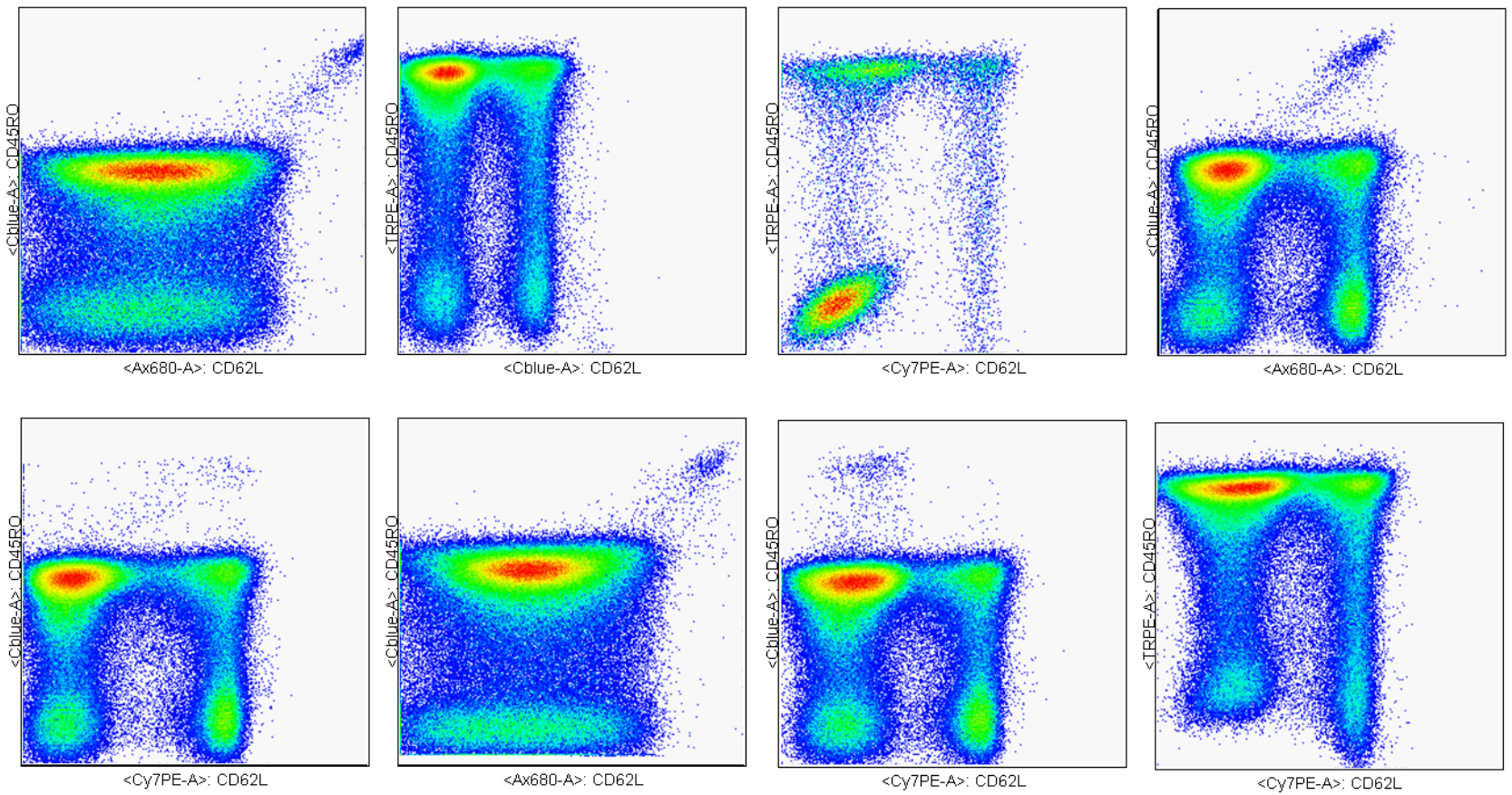
Optimal separation of CD62L and CD45Rx was required.

Other memory markers were less important: therefore, some panels were designed to test minimal requirements, and others were part of the “wish list”.

First set of panels

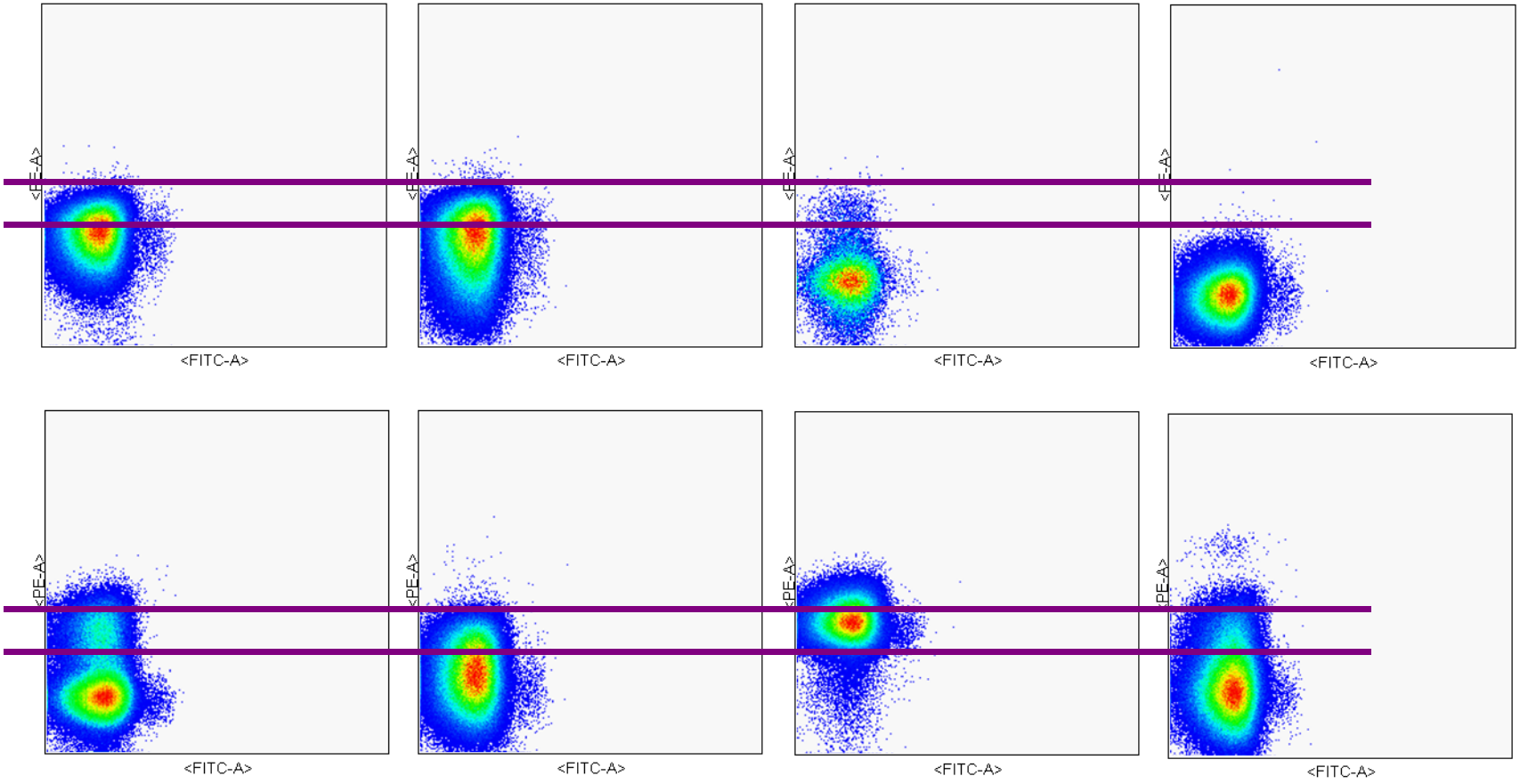
	TRPE	Cy5PE	Cy55PE	Cy7PE	APC	Cy55APC Ax680	Cy7APC	CB	QD655
1			CD45RA	CD4	CD27	CD62L	CD11a	CD45RO	“CD3”
2	CD45RO			CD4	CD27	CD45RA	CD11a	CD62L	“CD3”
3	CD45RO		CD45RA	CD62L		CD27	CD4	CD11a	“CD3”
4			CD45RA			CD62L	CD4	CD45RO	“CD3”
5				CD62L	CD4	CD45RA		CD45RO	“CD3”
6		CD45RA		CD11a	CD27	CD62L	CD4	CD45RO	“CD3”
7	CD4	CD45RA		CD62L	CD27	CD28	CD11a	CD45RO	“CD3”
8	CD45RO	CD3		CD62L	CD28	CD11a	CD4	CD27	CD45RA

Panel Evaluation: CD45RO vs. CD62L



Cy55APC CD62L: Too much smearing in some panels. CD45RO: Looks good in all panels

Sensitivity for FITC, PE



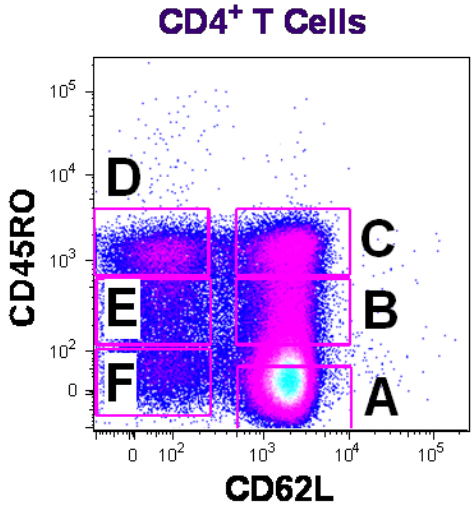
Final Panels

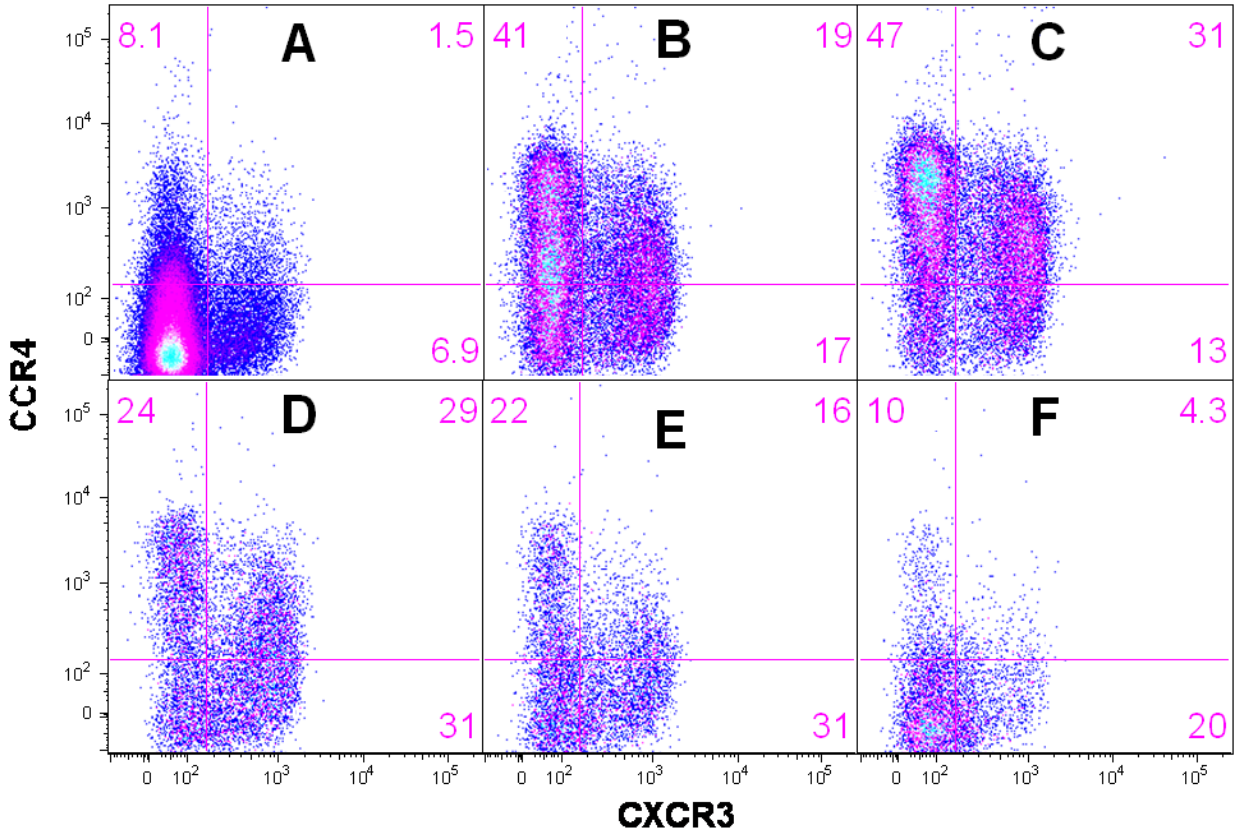
Based on the evaluation of the first sets of panels, certain combinations were eliminated. The good aspects of other combinations were combined and fine-tuned.

	TRPE	Cy5PE	Cy55PE	Cy7PE	APC	Cy55APC Ax680	Cy7APC	CB	QD655
1						CD62L	CD4	CD45RO	CD45RA
2	CD45RO	CD3		CD62L	CD28	CD11a	CD4	CD27	CD45RA
3	CD45RO				CD27	CD4	CD11a	CD62L	CD45RA

Note: CD3 was dropped from 1 & 3 as CD4 staining was deemed good enough to identify CD4 T cells.
Panel 2 will validate this assertion!
Panels 2 & 3 add more memory markers to verify the final phenotype of the chemokine-expressing cells.

Result





Final panel worked very well--in fact, identified expression of CCR4 not previously seen on FACSCalibur!

Panel Optimization

Is a long, complicated, iterative process.

Plan to spend 5 experiments minimum.

(1): Survey range of reagents

(2): Construct 8-12 possible multicolor combinations

(3): Rank each combination, deriving rules about reagents and combinations. Construct 4-6 derivative combinations

(4): Repeat step 3, winnowing down the combinations.

Record the process as you go along!

Compensation
Controls
Data Visualization
Panel Development
Gating
Quantum Dots

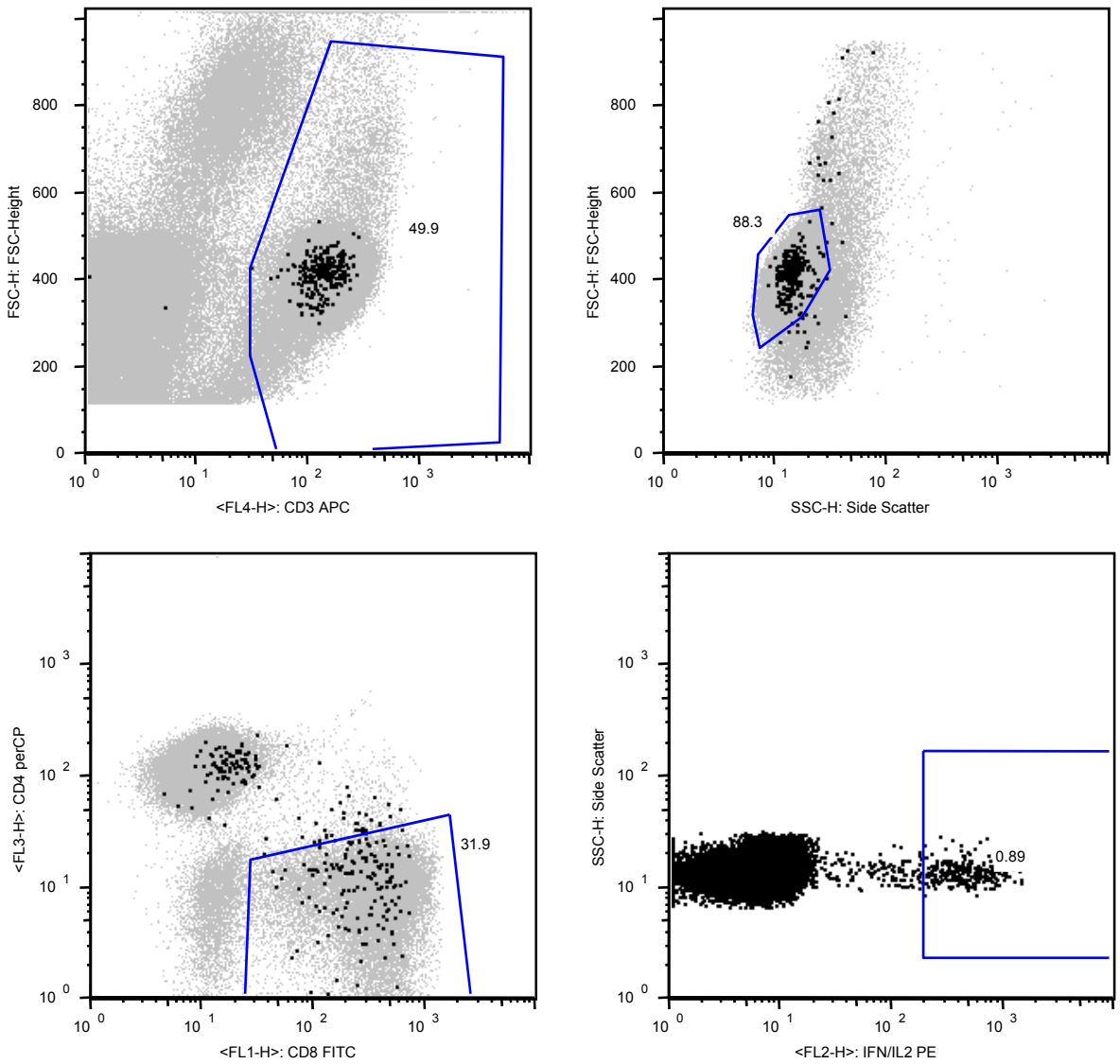
Know where your cells are!

Remember… often we make decisions about where cells are based on way more cells than we're analyzing!

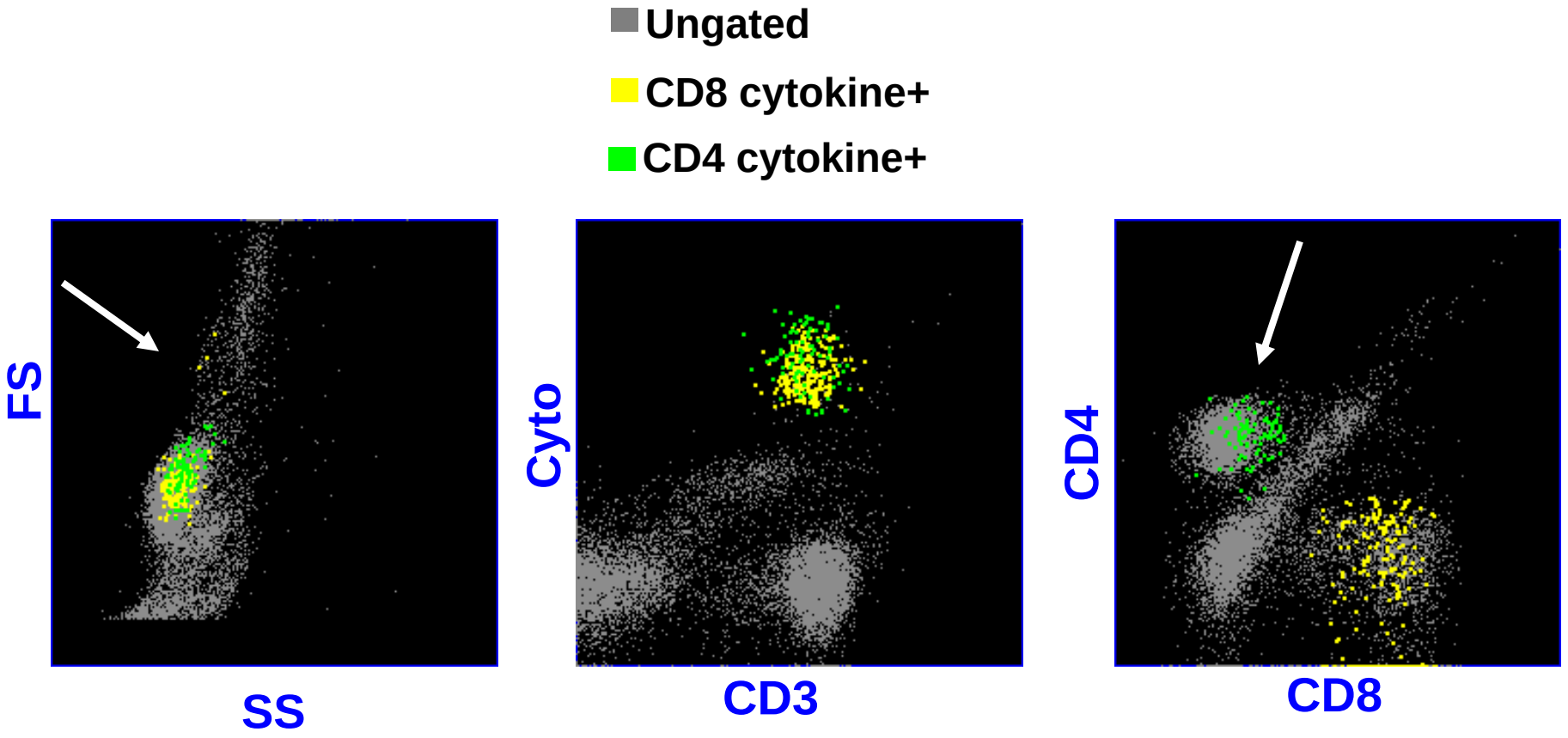
Do not assume that the distribution of your rare population (e.g., antigen-specific cells) in "parent" gates is the same as for the bulk population of cells.

Using "back-gating" approaches to verify that the cells of interest have been fully identified.

Know where your cells are!



Know where your cells are!



Backgating reveals some cytokine+ cells are high in FS (outside “classical” lymphocyte gate), and tend to be higher in CD8 on CD4+ T cells

Gating Considerations

Two extremes of gating strategy:

“Liberal” - gates are drawn to include much larger areas than visually appear to belong to a subset.

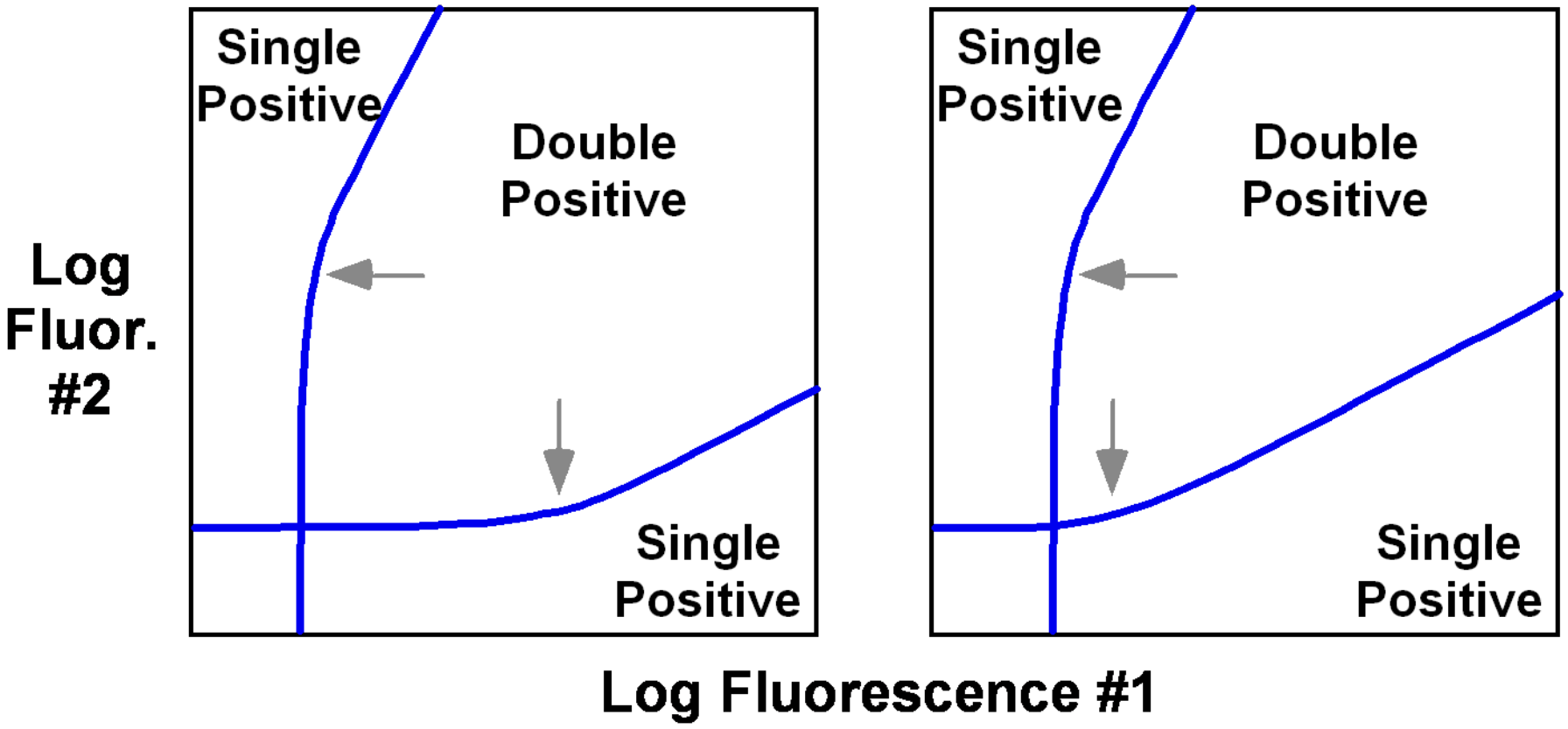
- Greatest sensitivity
- Greatest chance of contamination

“Conservative” - gates are drawn to be very “tight” around the visually-defined populations

- Greatest purity of subset
- Lowest sensitivity

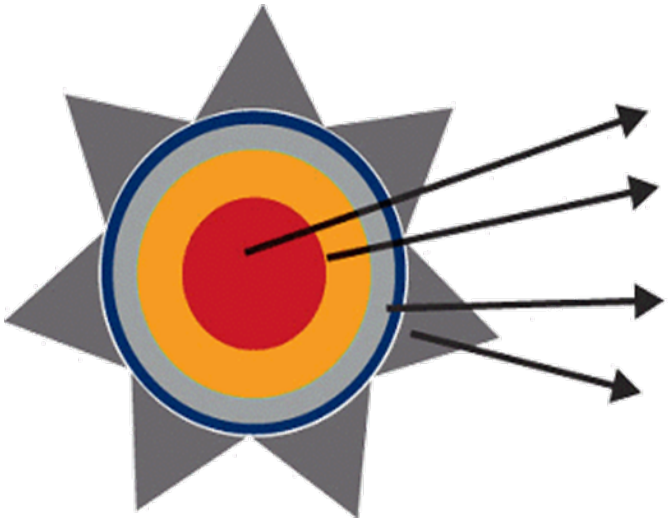
Note that multiple rounds of “Liberal” gating (based on multiple parameters) often results in excellent purity.

Quad-Gates May Need to be Curly



Compensation
Controls
Data Visualization
Panel Development
Gating
Quantum Dots

What are Quantum Dots?



Core Nanocrystal - Core synthesis using Cd(II) v. Cd(0) precursors.

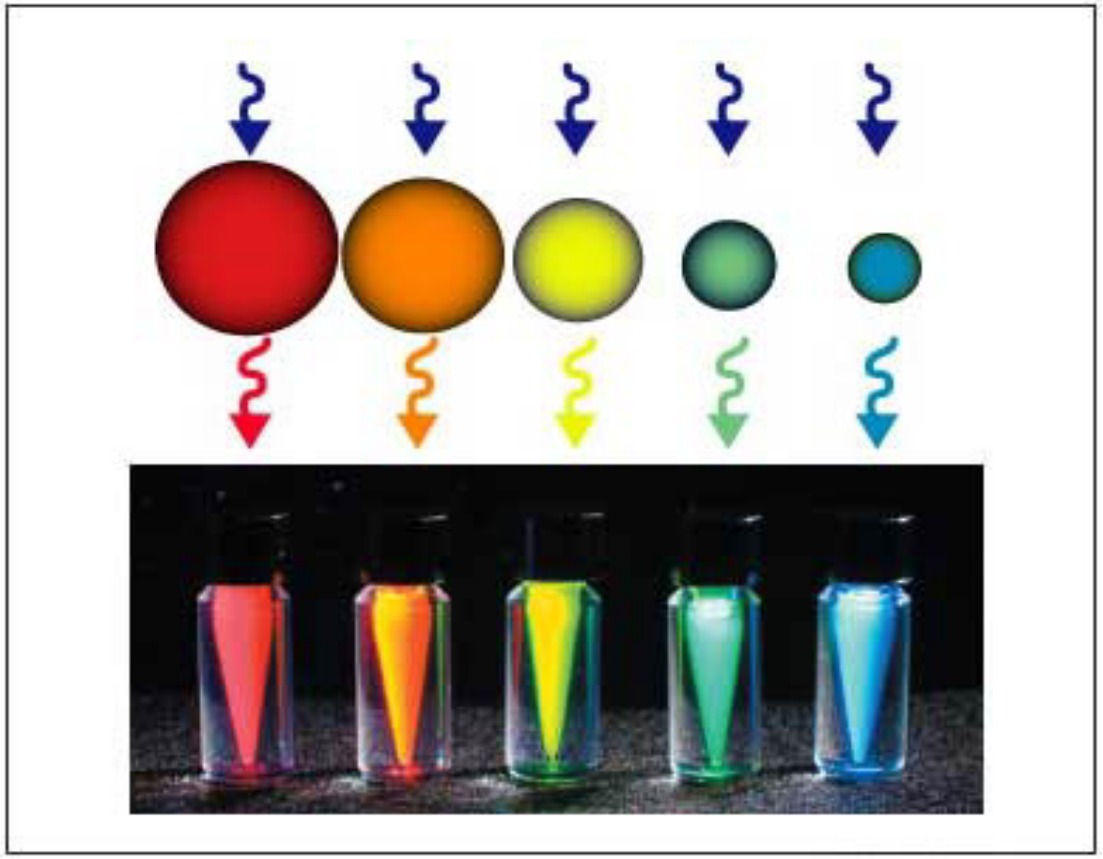
Inorganic Shell - improved stability.

Organic coating - New polymer for water solubility and conjugation. Available functional groups for conjugation.

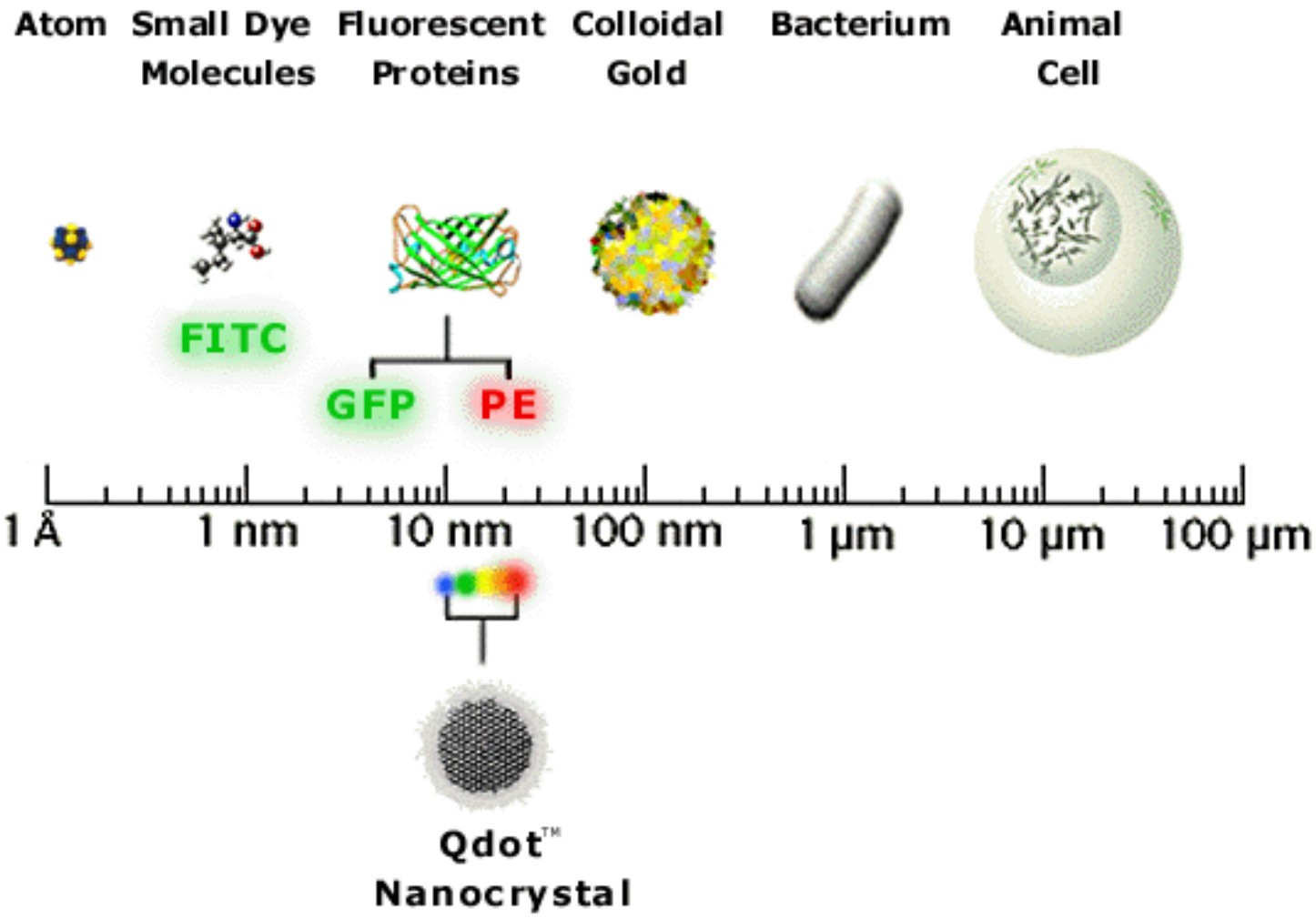
The core nanocrystal has fluorescent properties useful for flow cytometric and imaging applications

Quantum Dot Emissions

Different core sizes produce different emission spectra:



Quantum Dots for Immunofluorescence



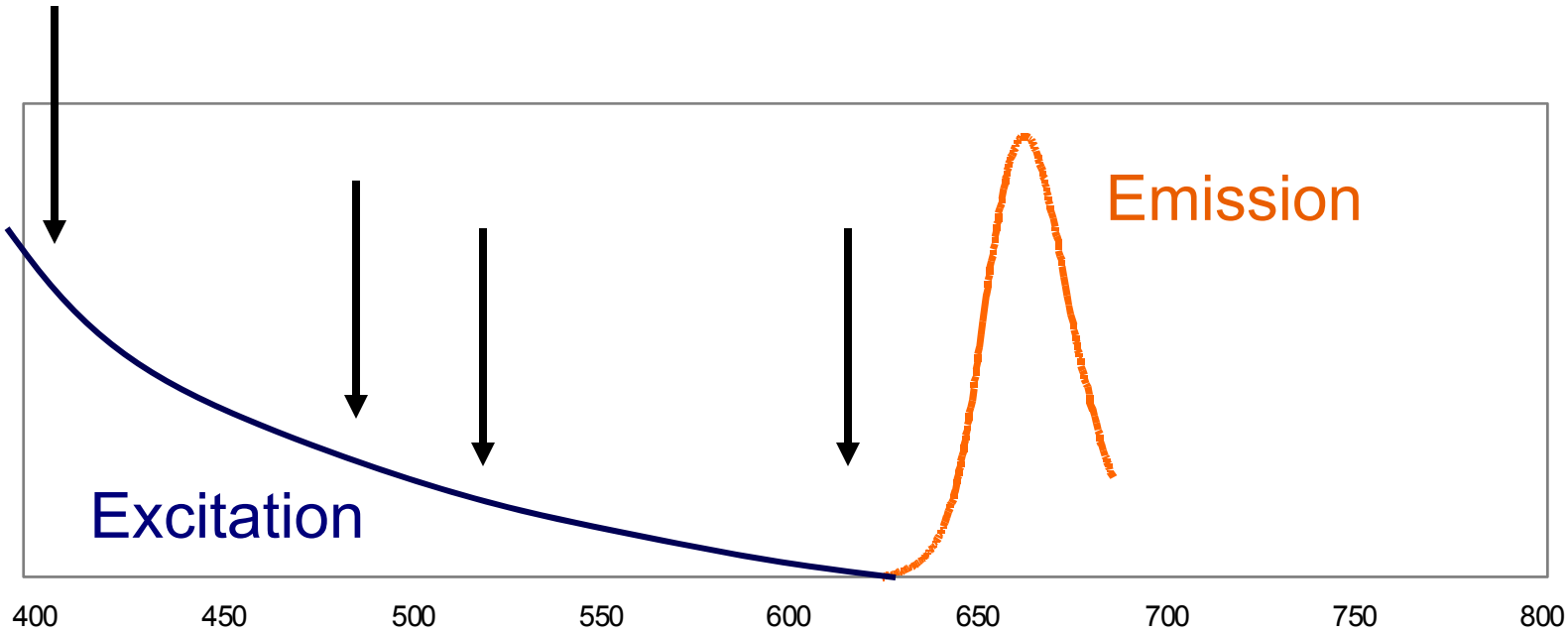
Quantum Dots for Immunofluorescence

The cores that emit red are substantially larger than the cores that emit blue.

However, after coating the core with the shell and the polymer, the size difference between the different dots is minimized.

The visible-spectrum quantum dots are approximately the same physical size the phycobiliproteins. Hence, conjugates of quantum dots are expected to have similar biophysical properties.

Quantum Dot Emissions



The further from the emission peak, the higher the absorbance.

Emission spectrum is independent of excitation

Quantum Dot Spectra

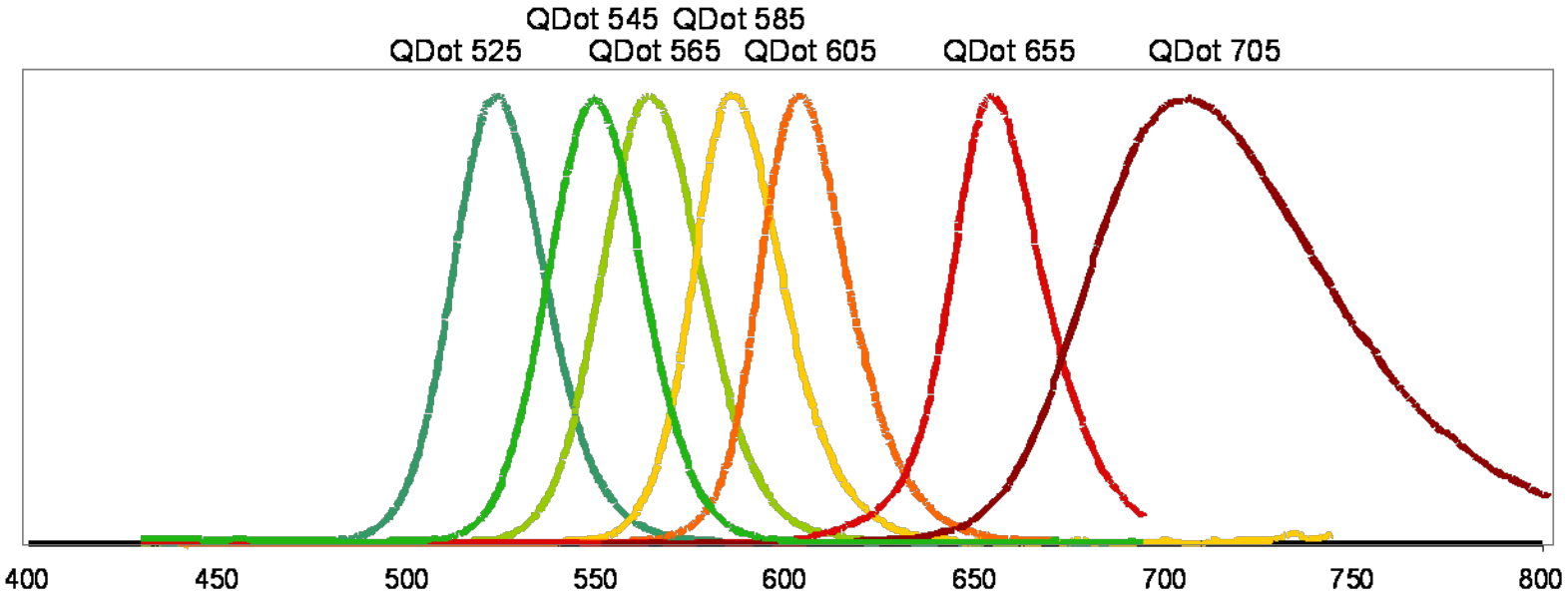
The further from the emission peak, the higher the absorbance. Therefore:

The brightest signal will be obtained by using the shortest wavelength excitation possible (e.g., 355 > 405 > 488)

The dots will be excited by all (shorter wavelength) lasers on the system. Therefore, the QD655 will be excited by the 633 laser (and look like APC), by the 488 (like Cy5PE/PerCP), and by the 405 (and look like nothing else).

However, the APC-like emission is very low… so low compensation is predicted; Cy5PE should be fairly high.

Quantum Dot Emissions

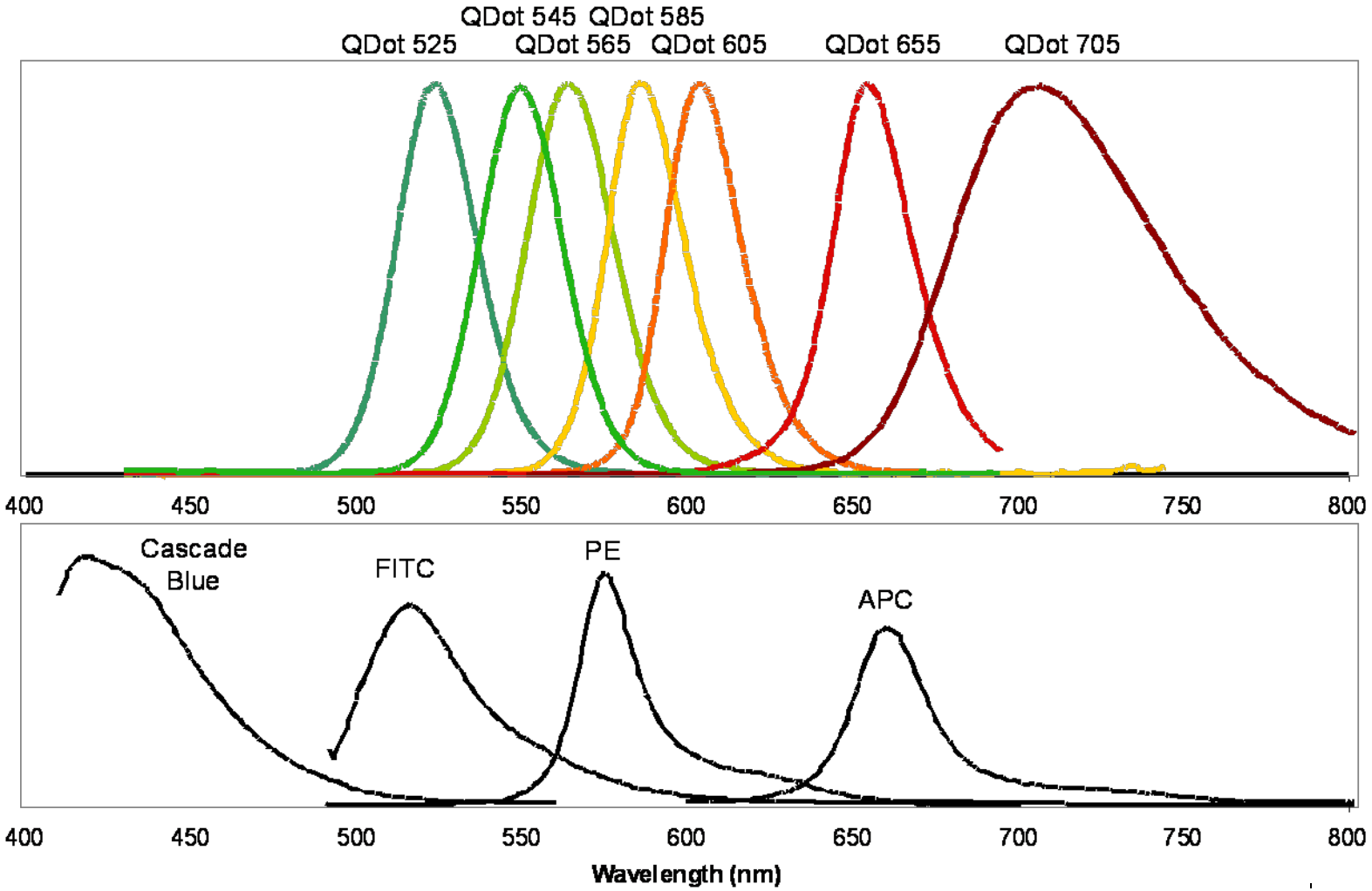


Quantum dot emission spectra are *fairly* narrow. Theoretical minimum width is ~30 nm (FWHM); significant narrowing of most emissions is unlikely.

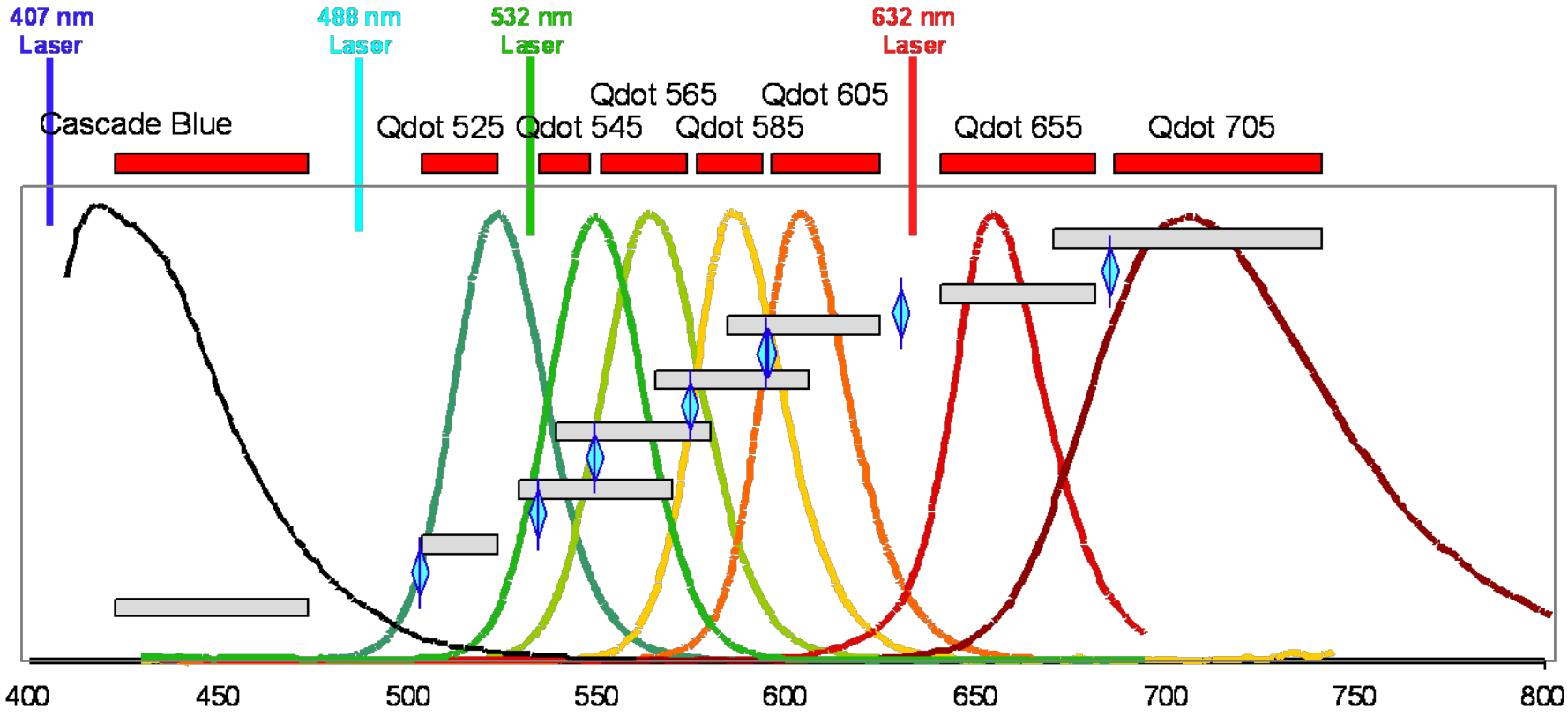
Quantum dots have no red emission trail

Adjacent dots have much overlap; but alternate dots do not

Quantum Dot Emissions

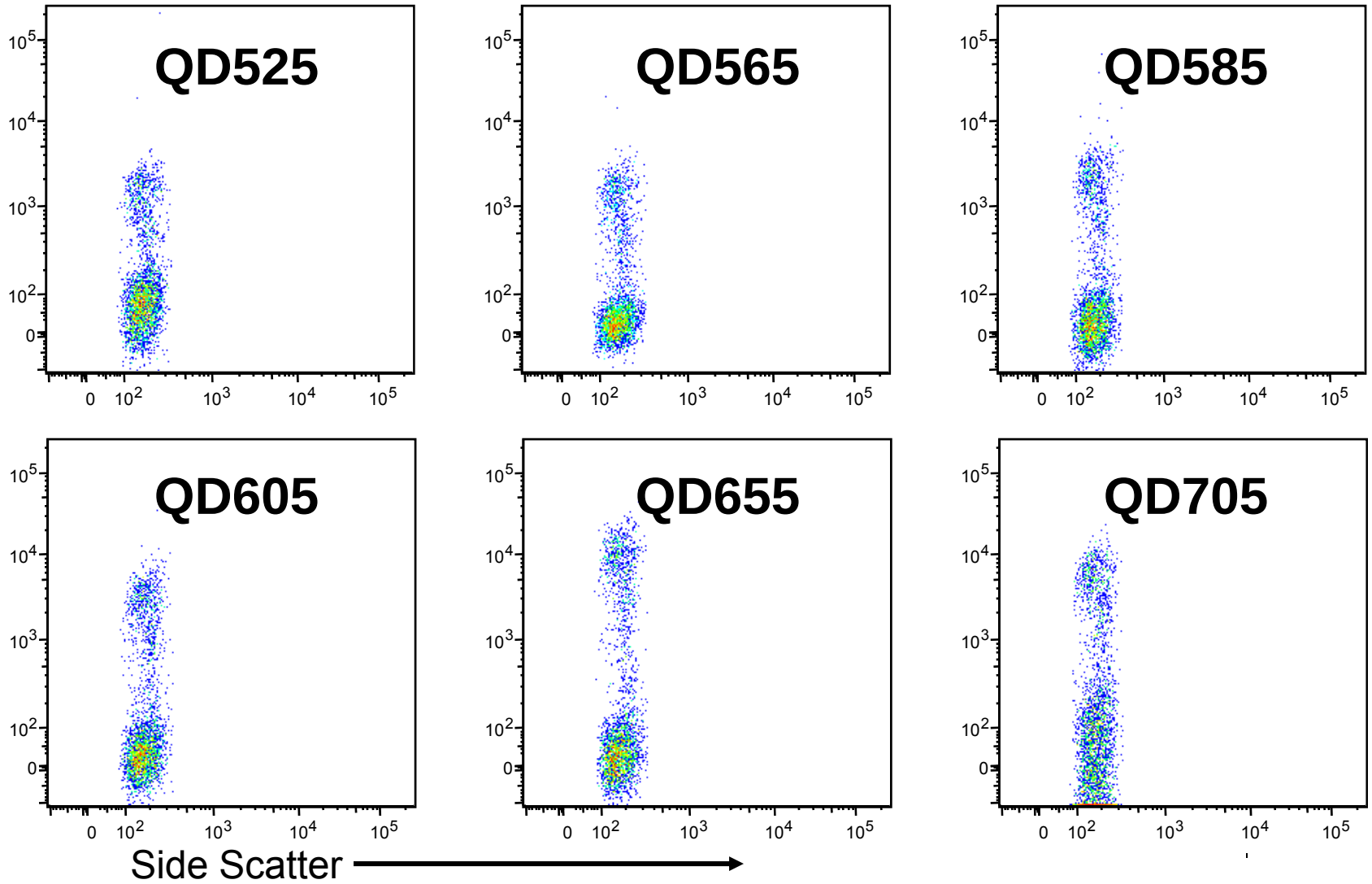


Detecting Quantum Dots: LSR II



Use wide bandpass filters; most of the “work” is done by the dichroics. Optimization remains to be done…

Quantum Dot Conjugation to CD8



Quantum Dots

Quantum dots are NOT a panacea

Quantum dots are comparable to existing fluors in terms of brightness (and even compensation)

There are still issues of stability, resistance to perm/fix reagents, and aggregation that crop up

They DO provide additional tools and solutions for multicolor flow cytometry