Compensation: Basic Principles

Bonus topic:
Daytime Stargazing

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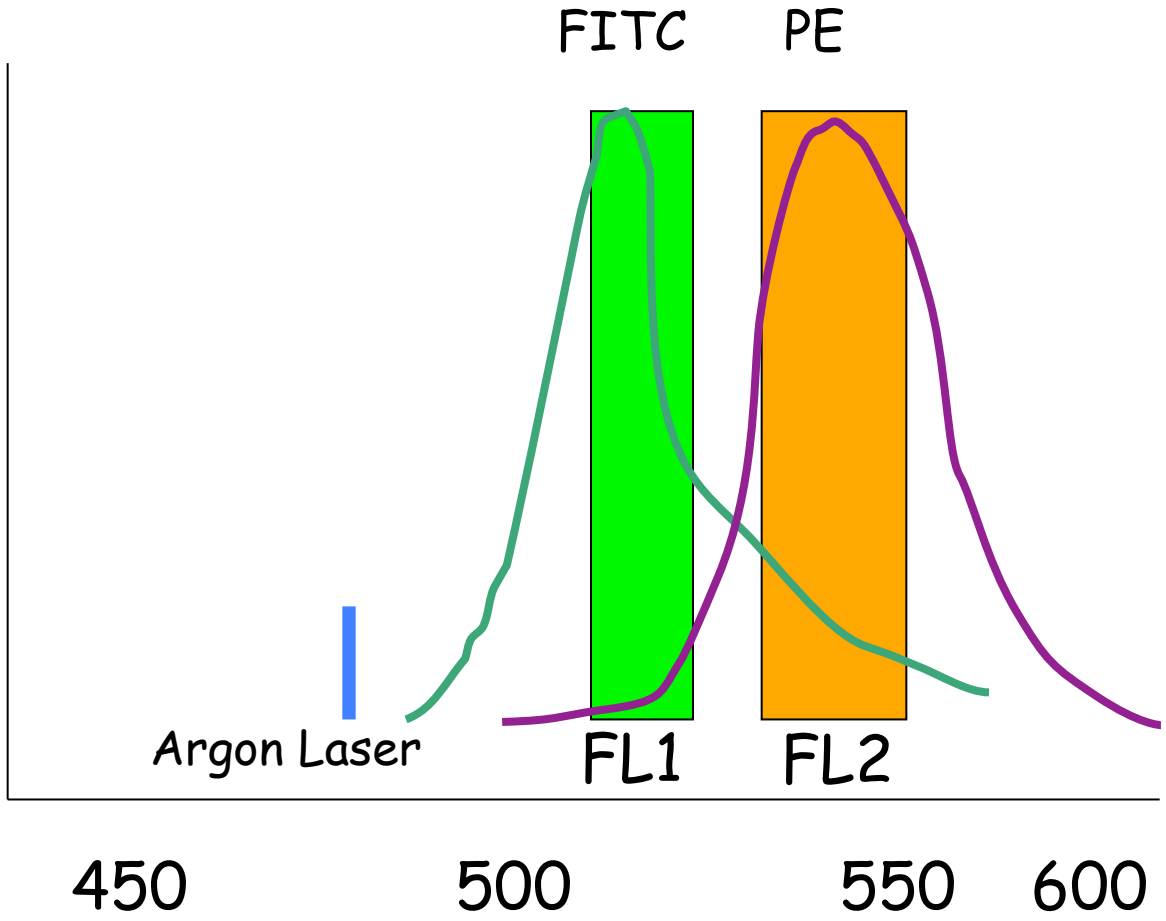
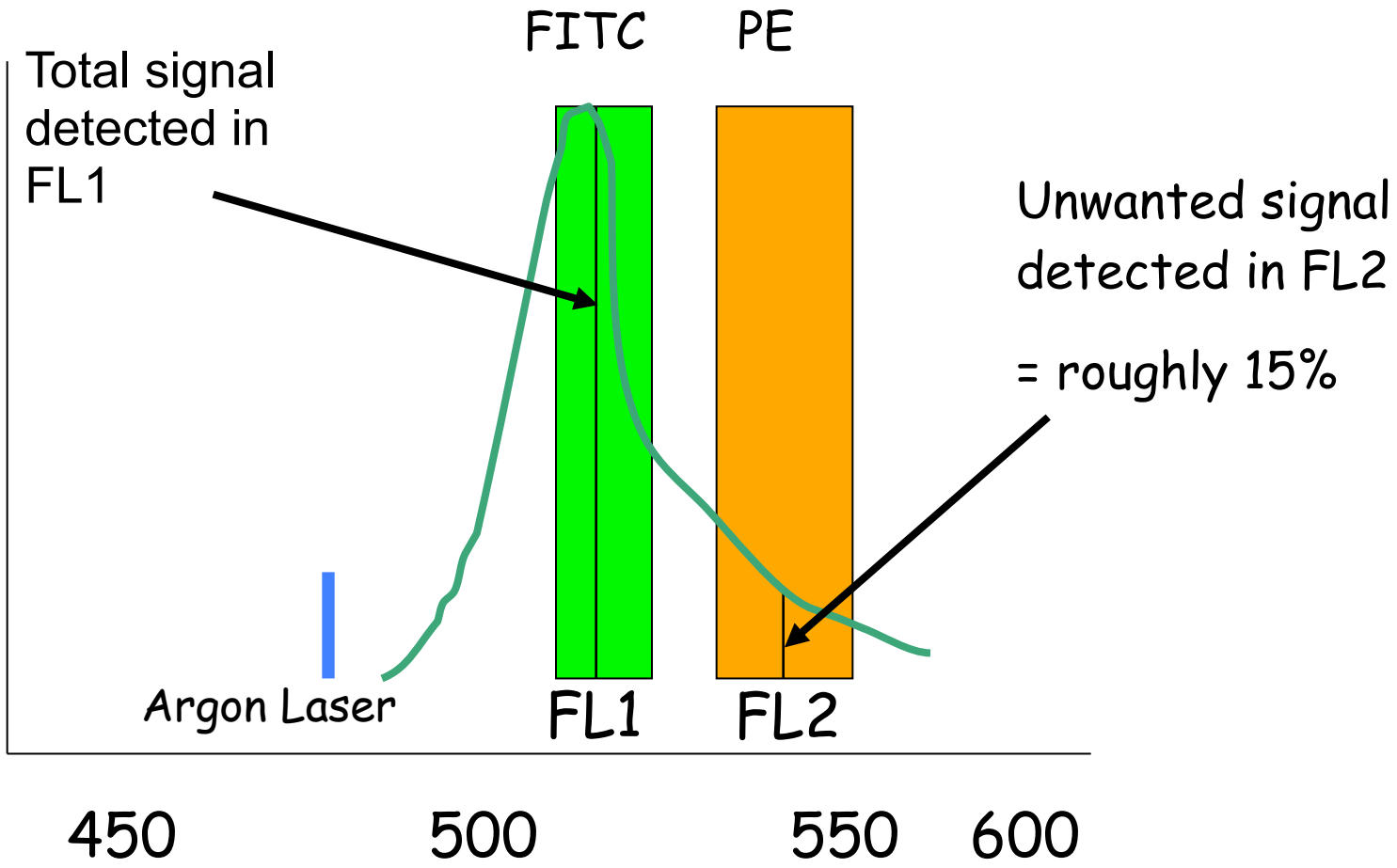
Spectral Spillover Necessitates Compensation



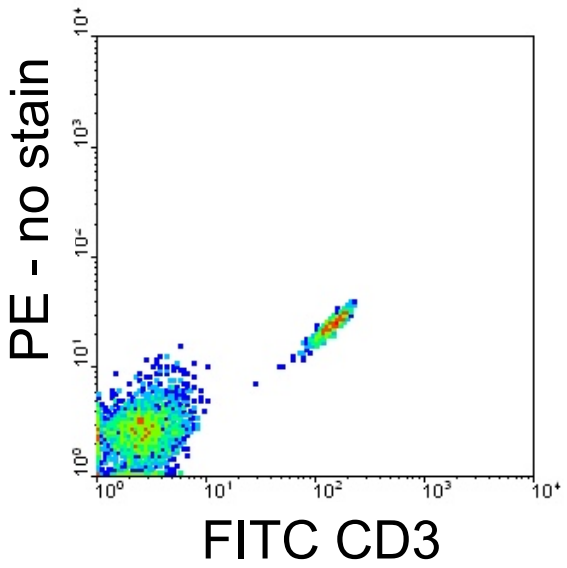
Spectral Spillover Necessitates Compensation



True PE = Total FL2 – 15% FL1

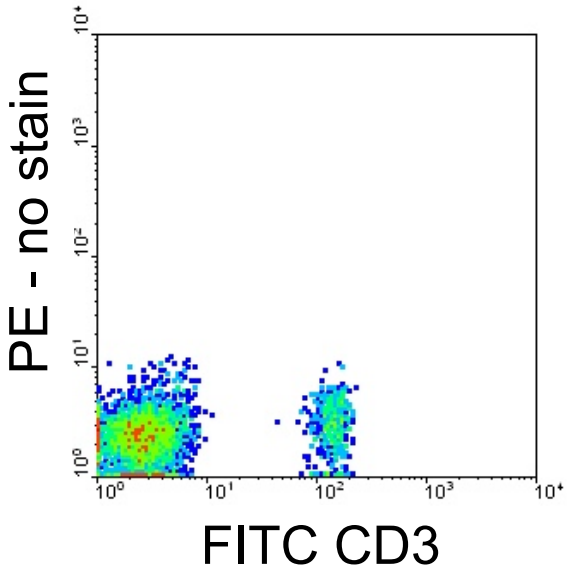
Compensation Converts From Measurement to Expression

Uncompensated



FL2-15%FL1

Compensated

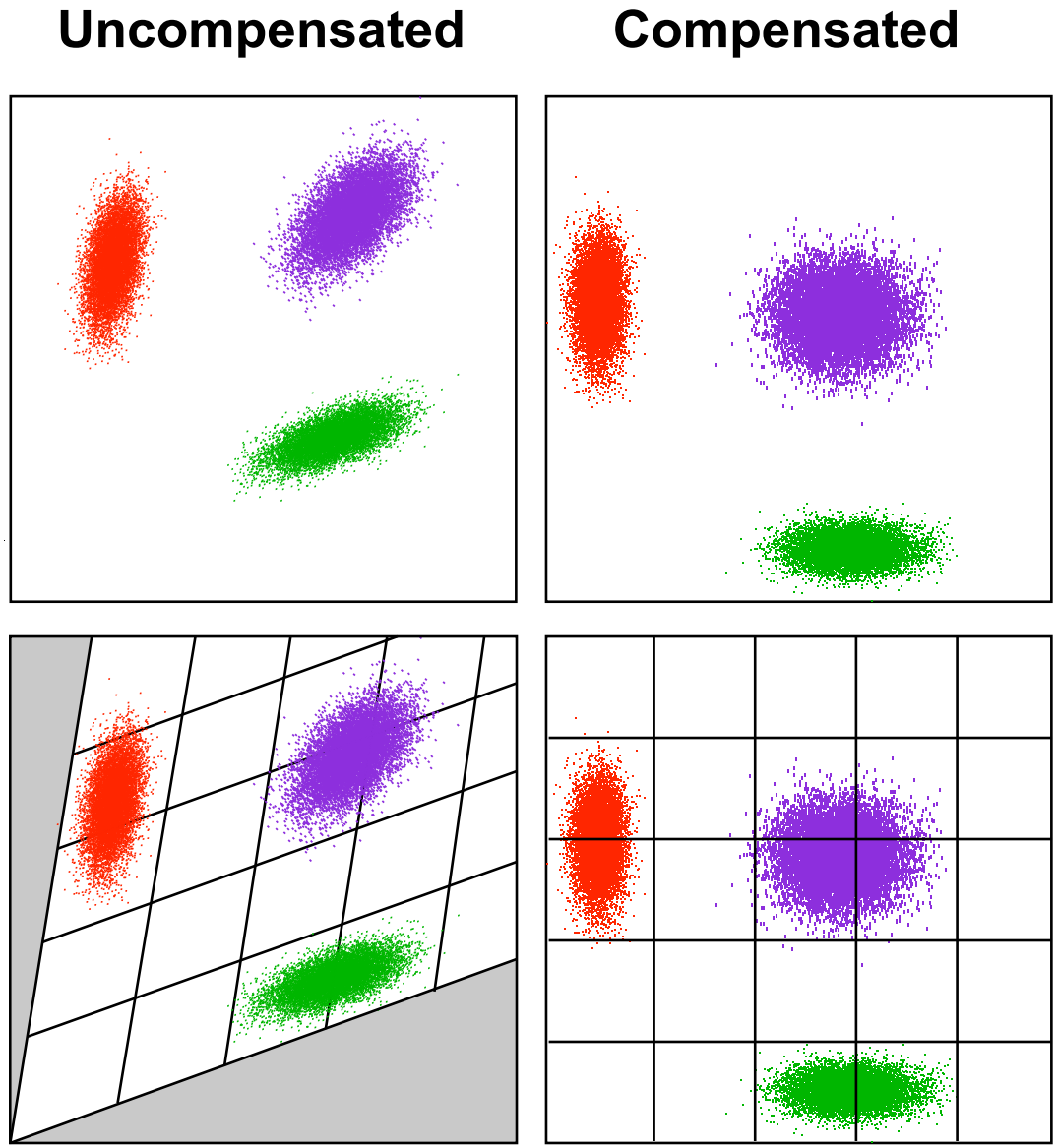


Measured Fluorescence

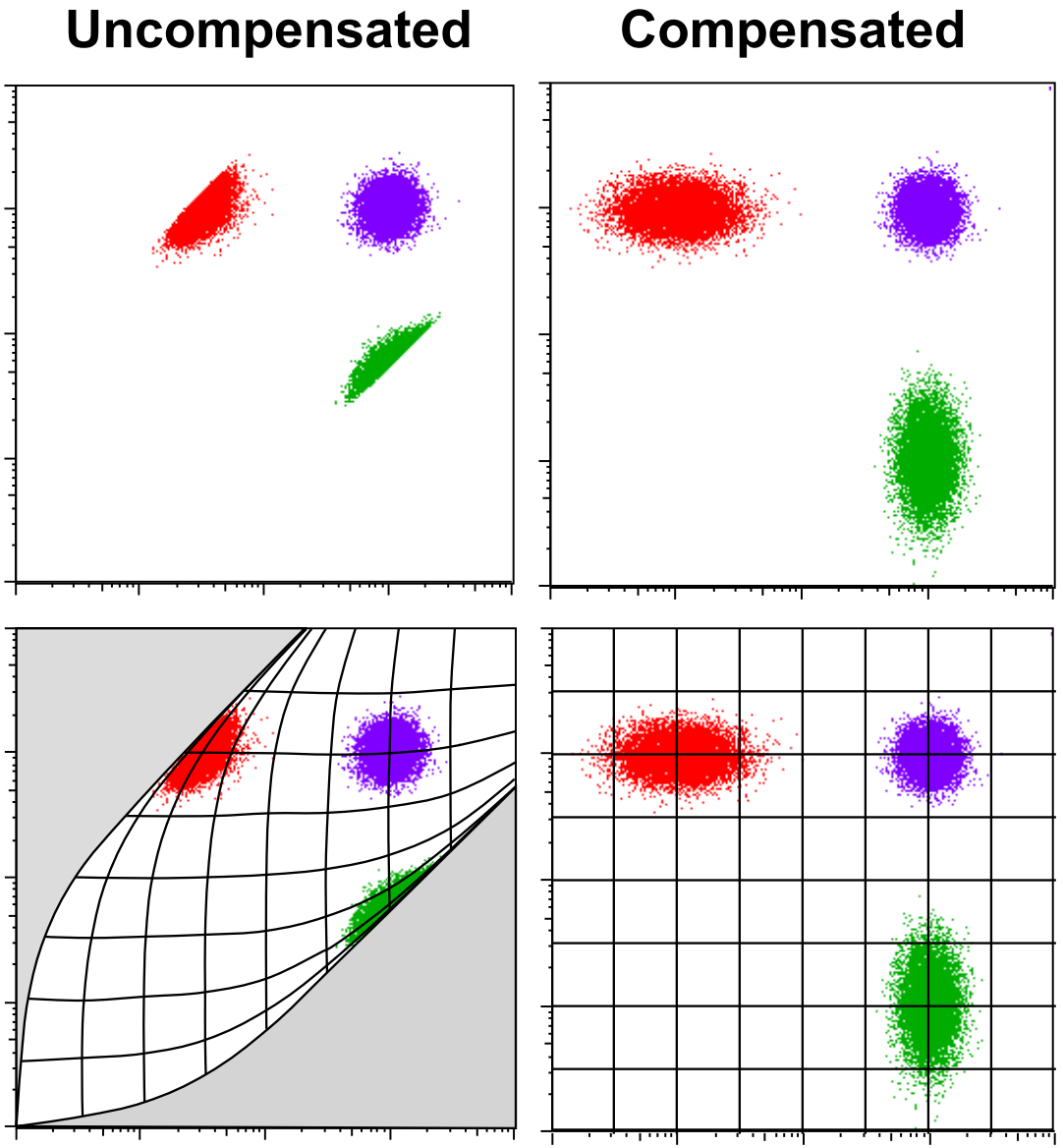
Calculated Expression

Compensation in Linear Domain

Lines indicate cells that express equal amounts of an antigen



Compensation in Log Domain



Lines indicate cells that express equal amounts of an antigen

Compensation is a Transformation

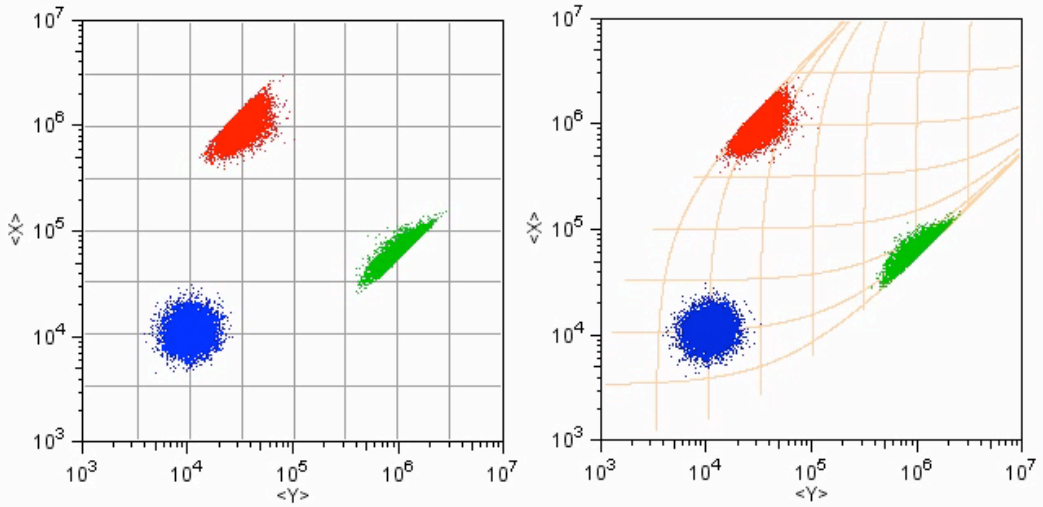
Bivariate Plots

Histograms of each measurement

Grid Lines

Equal Measurement

Equal Expression



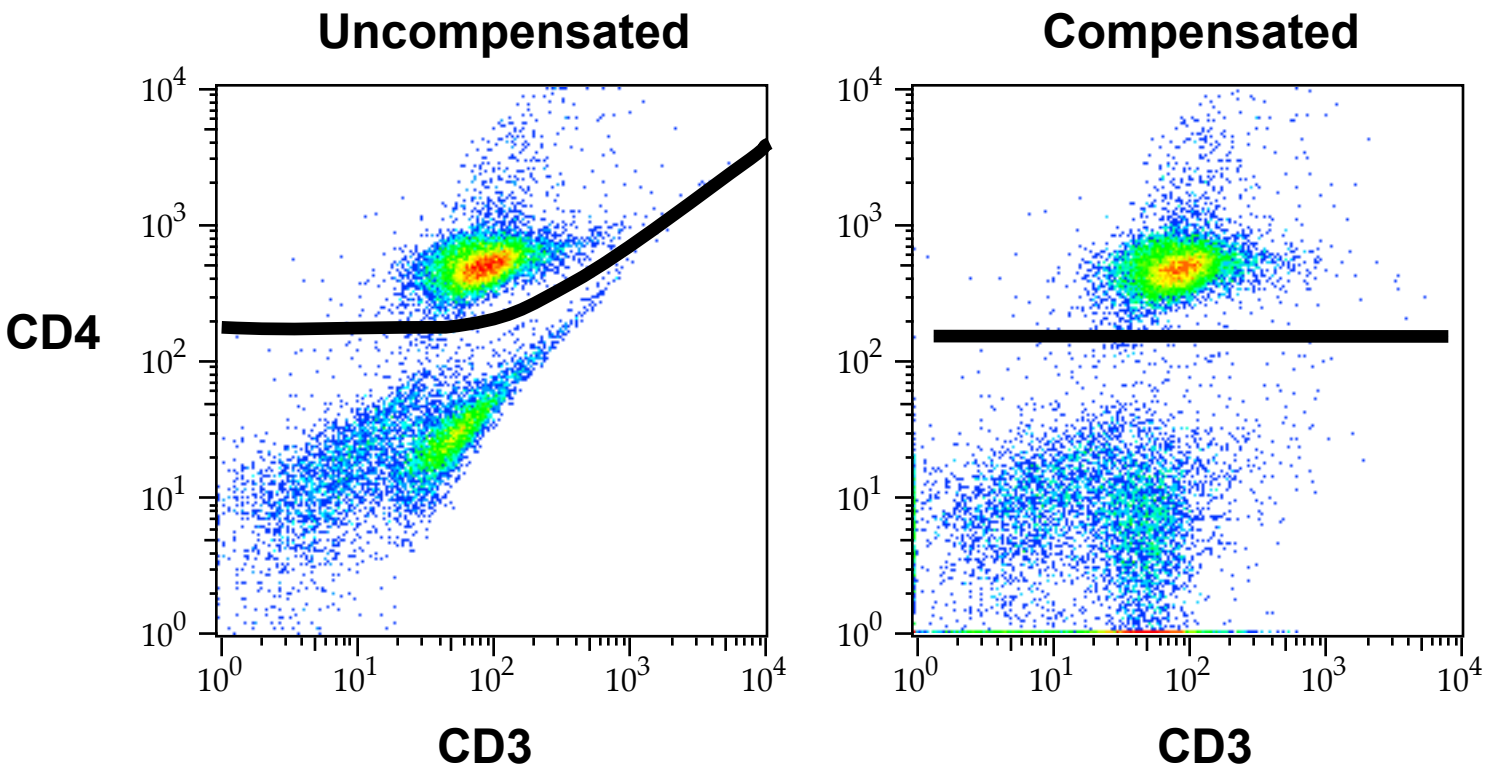
Compensation settings

X → Y: 0%

Y → X: 0%

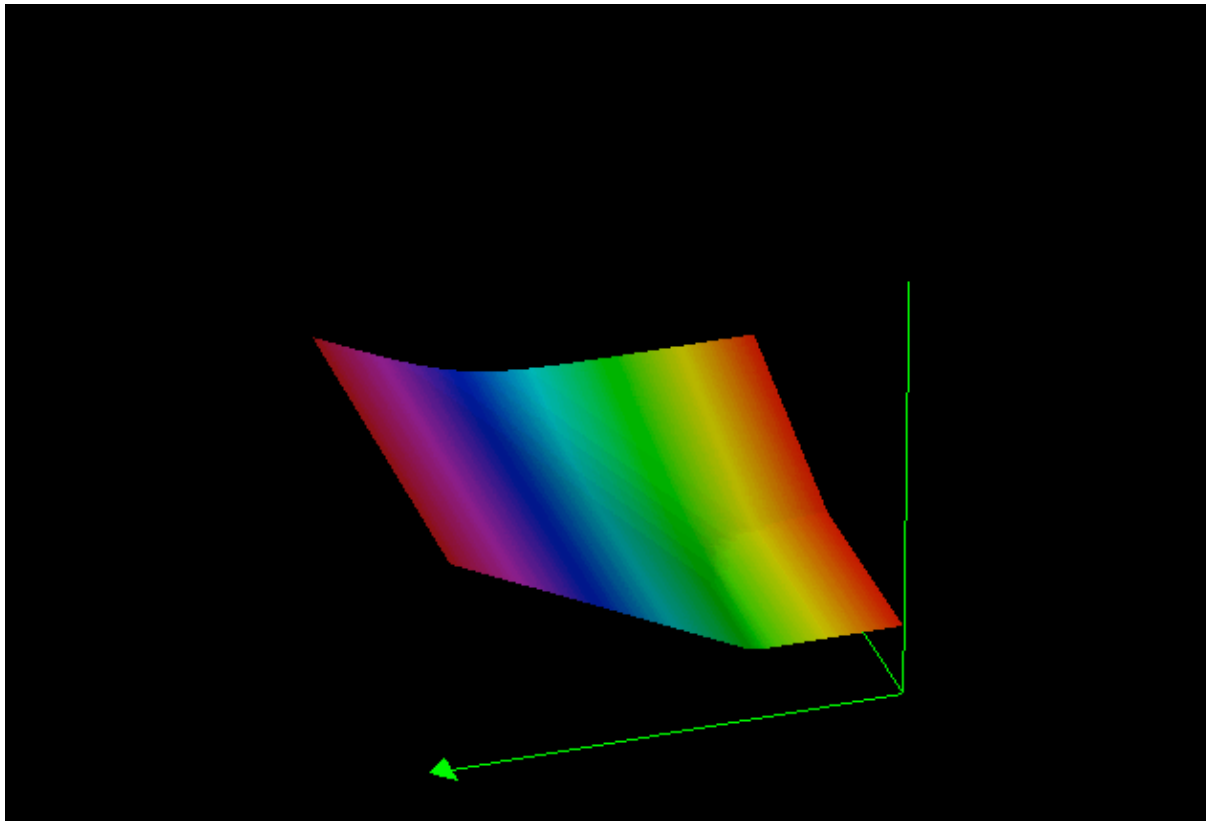
Compensation in 2 colors: Mostly aesthetic?

Accurate identification and enumeration of subsets is still possible in two color experiments (but not visual estimation of expression levels)



Compensation with 3 colors

Accurate separation of populations can require a curved, two-dimensional surface



This gets worse with more colors...

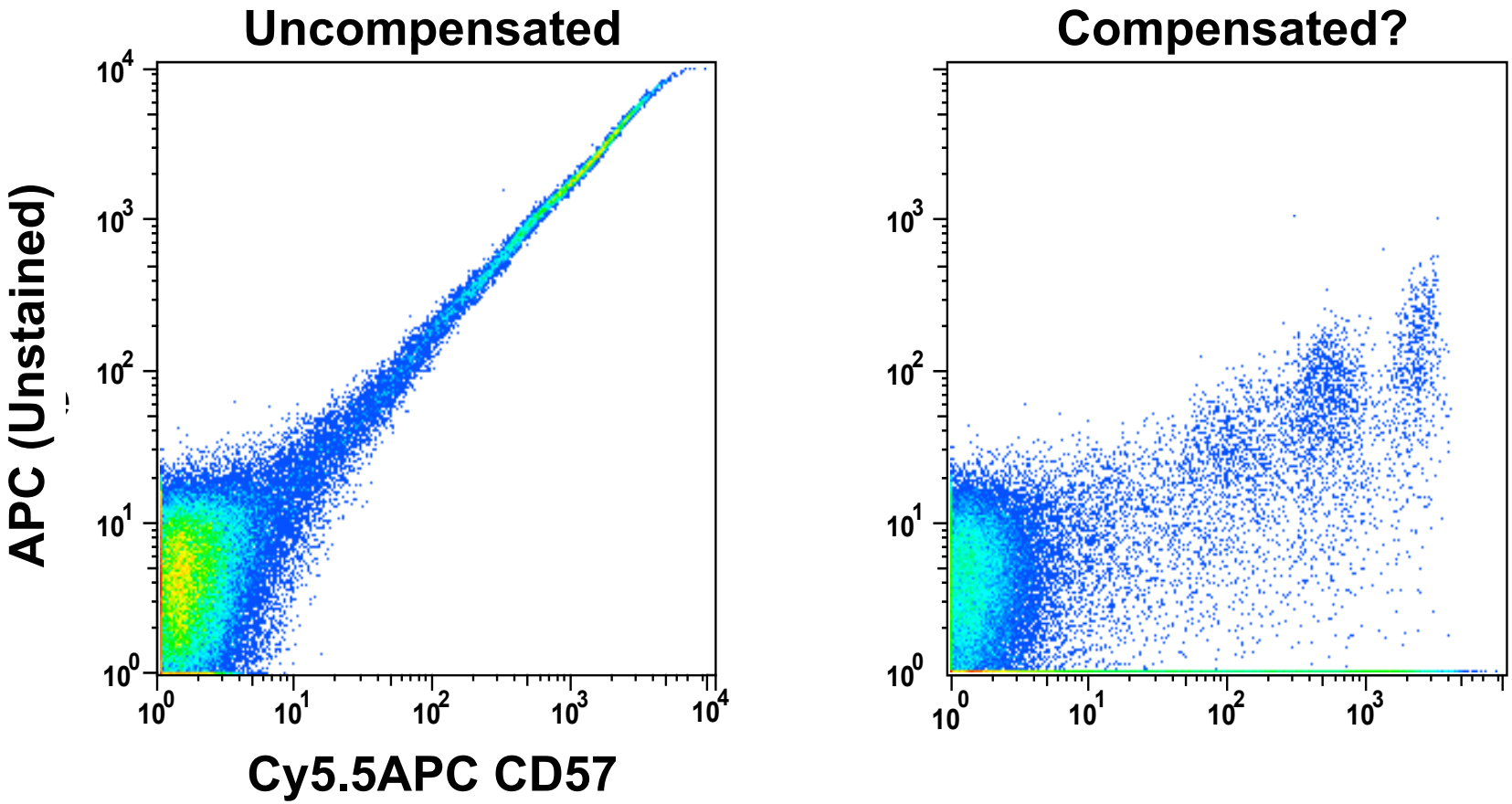
Compensation: Not just aesthetic

- 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)
- Separation of populations may require multi-dimensional surfaces.

Compensation for more colors: It's not just pretty pictures

- Spillover from unviewed measurement channel can alter event positions– without obvious visual evidence (no diagnostic diagonals!)
- Thus, gate positions may depend on unviewed measurement channels and be different for various tubes in a panel
- Gates could also be highly dependant on which subset is being analyzed!

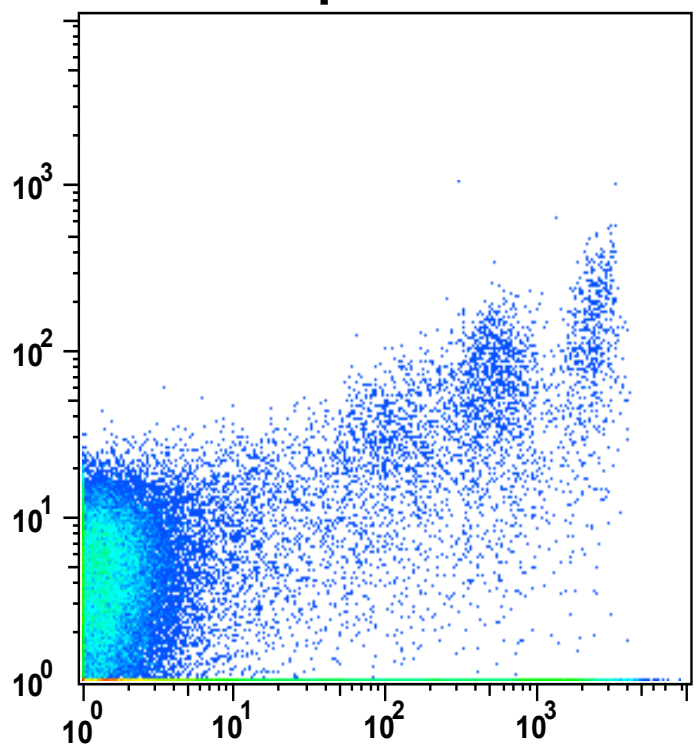
Compensation in the Polychromatic World



Compensation in the Polychromatic World

But… is it *correctly* compensated?

Compensated?



Compensation in the Polychromatic World

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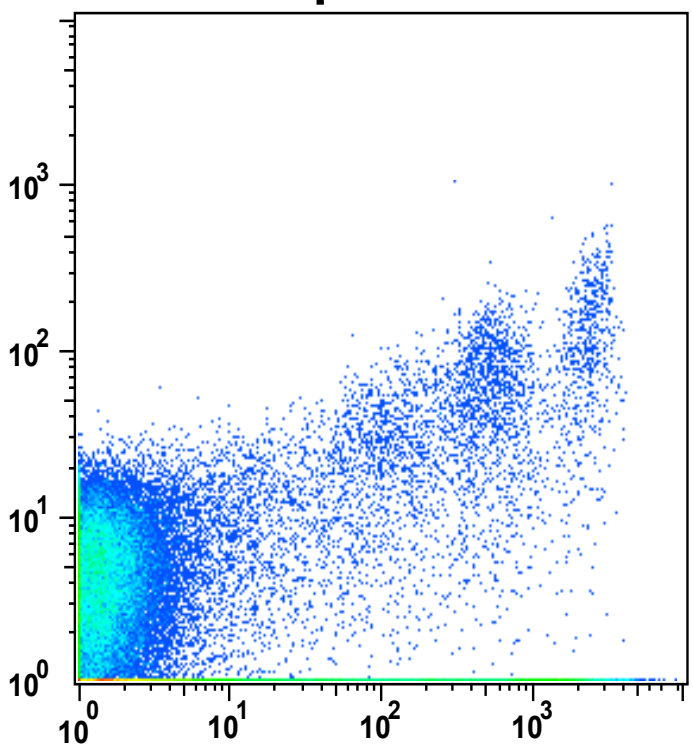

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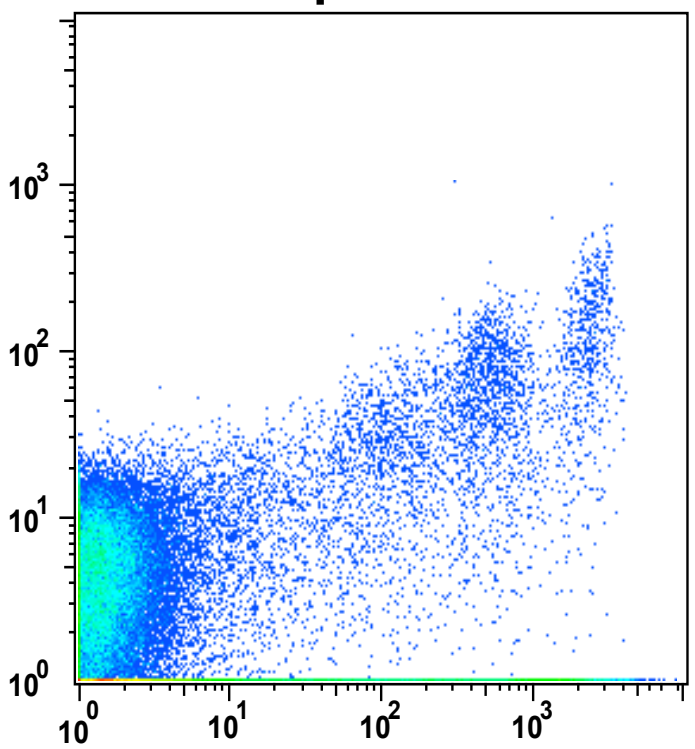

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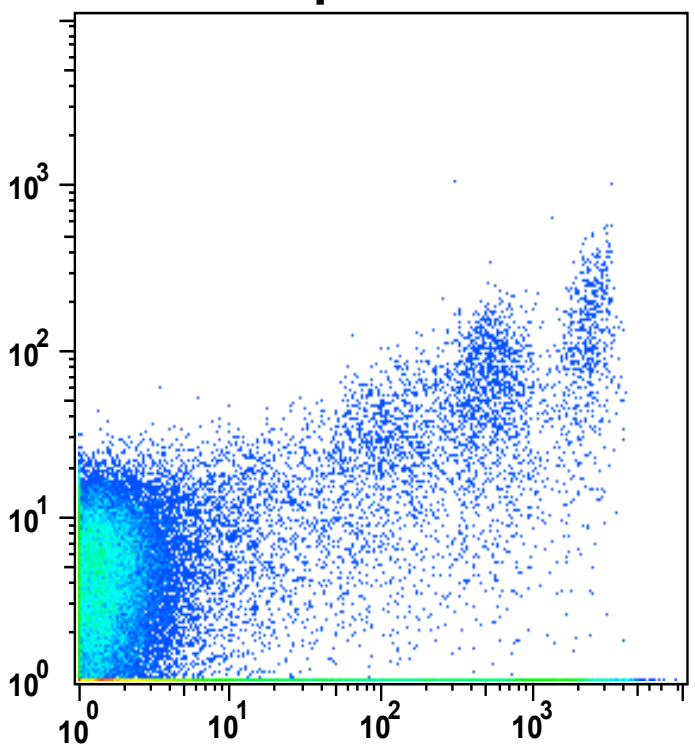

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~~Yes!~~

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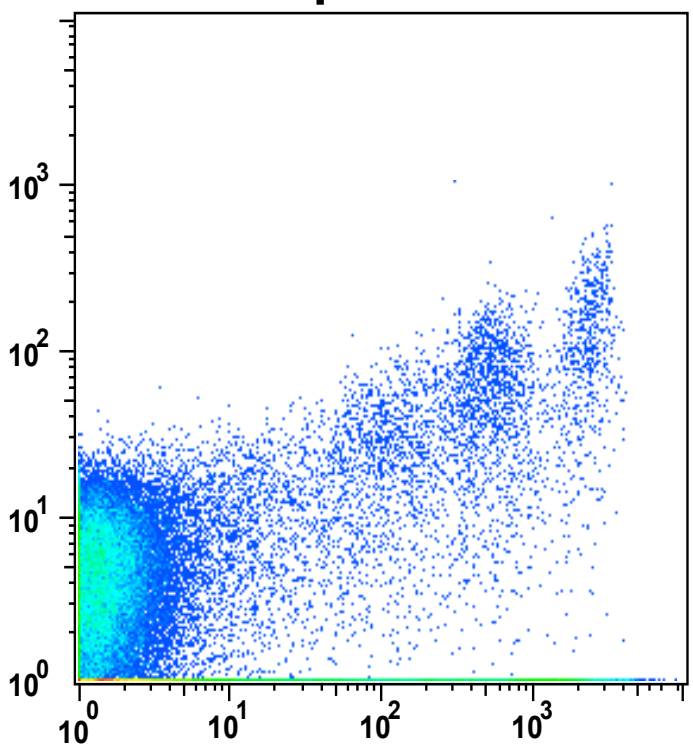


Compensation in the Polychromatic World

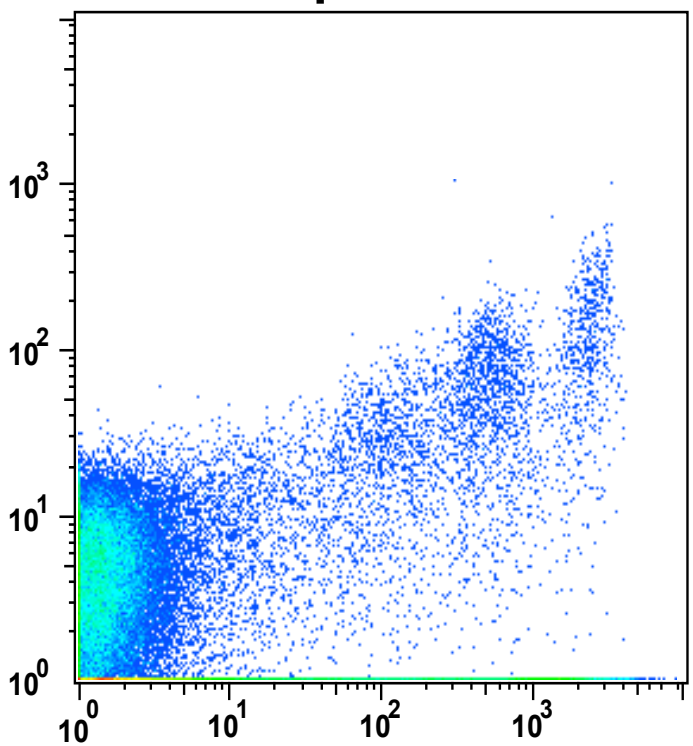
But… is it *correctly* compensated?

~~**No!**~~

~~**Yes!**~~

We can't tell just by looking!

Compensated?



What's Missing From This Picture?

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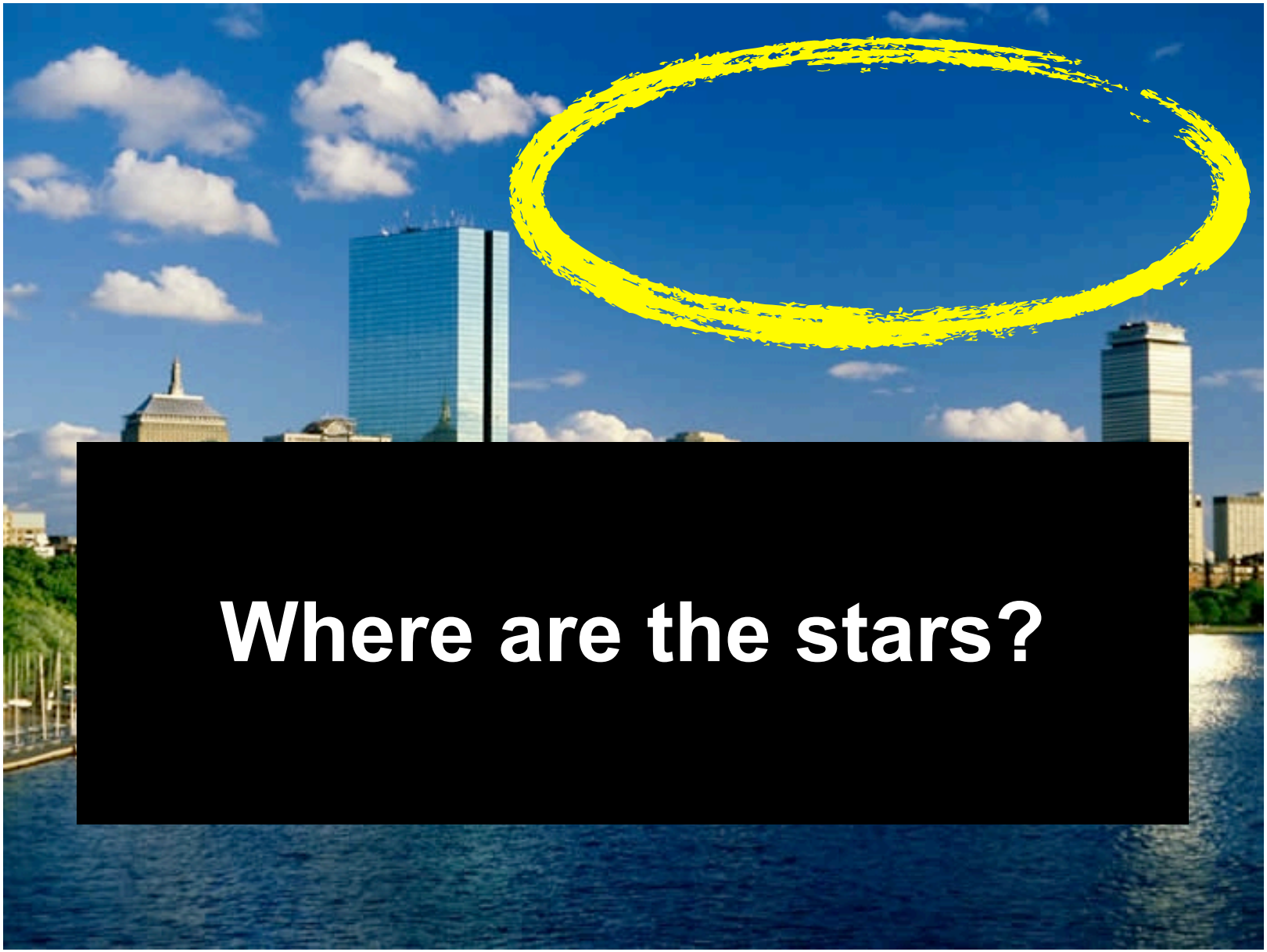

Why can't we see stars during the day?

On cloudless nights, we see lots of stars.
On cloudless days, we rarely see stars.

We call this the "Blue Sky Effect."

But….. Why?

Nowhere on the web is this commonly-asked question answered correctly.

The Blue Sky Effect

The stars are easily visible at night (well, in most places). When the sun rises… the sky becomes blue and the stars are no longer detectable.

The Blue Sky Effect

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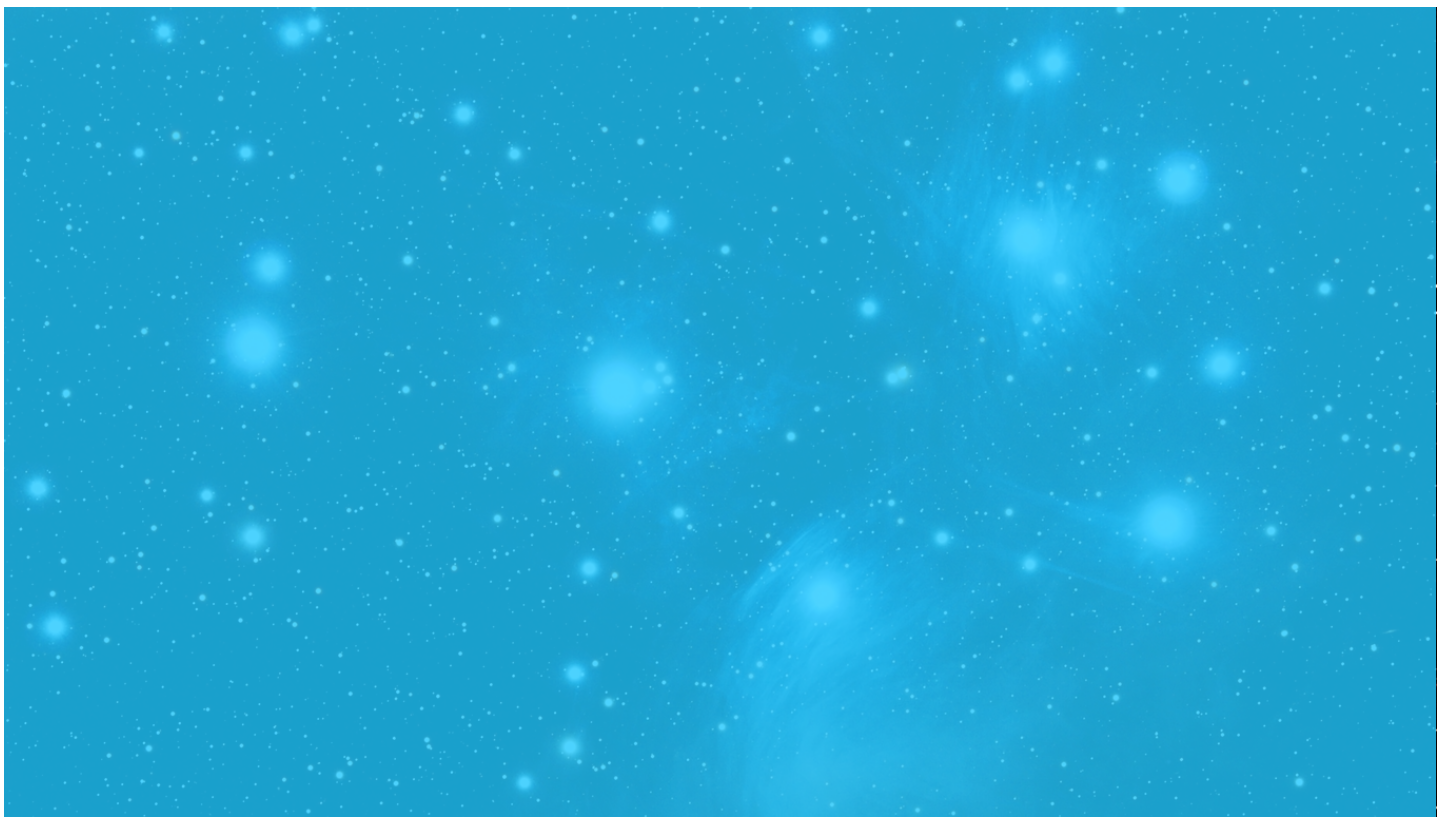
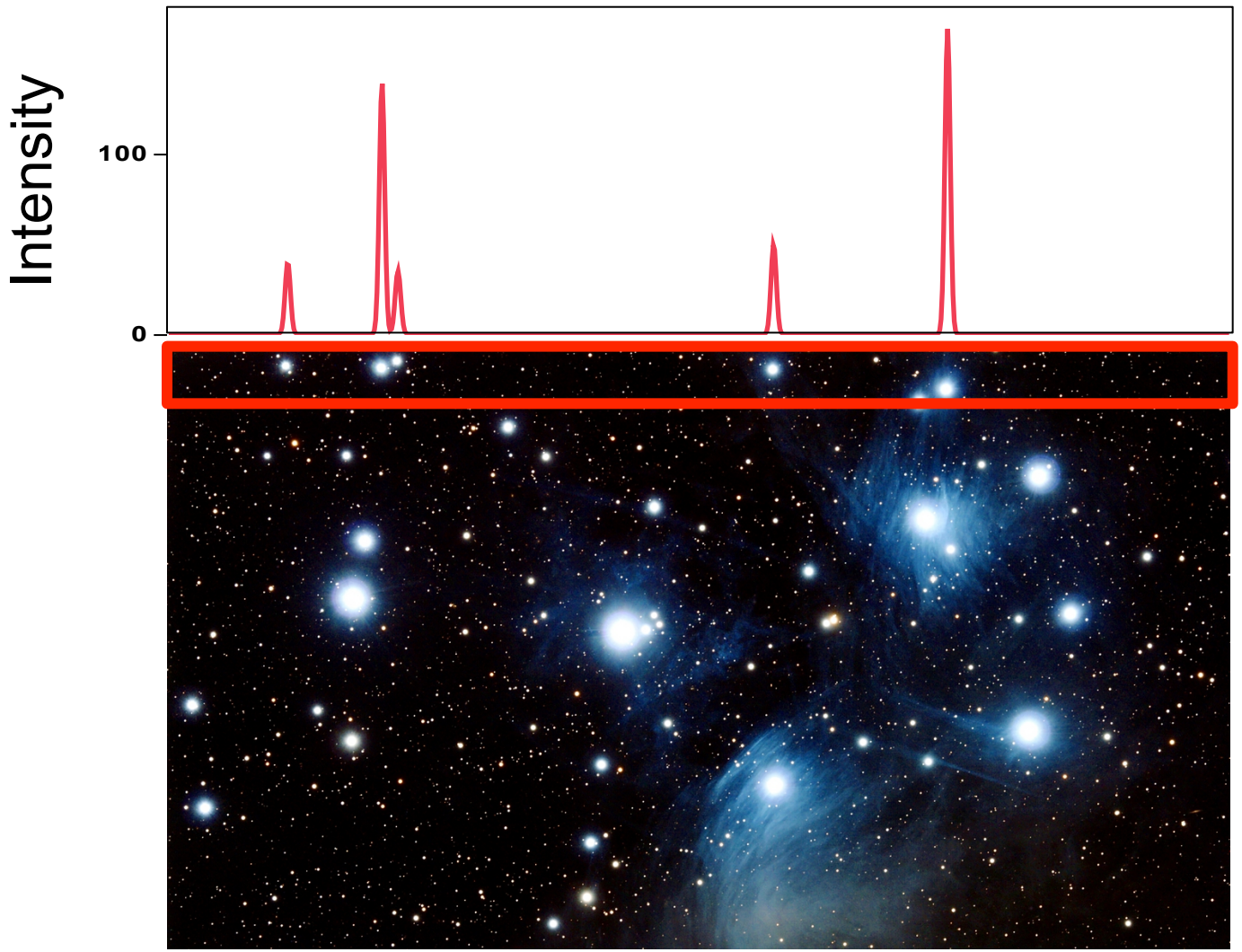
Why Doesn't it Look Like This?

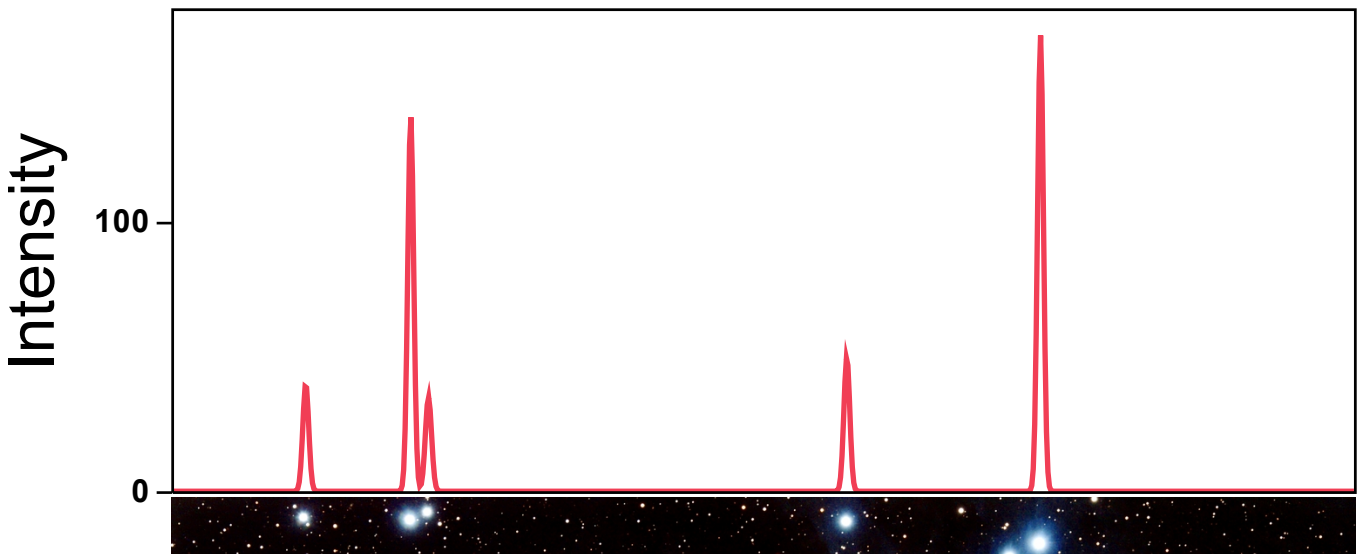
But light is additive. The white light from the stars should add to the blue scattered light from the sun… why don't we see white pinpricks against the blue background?



Signal Quantification

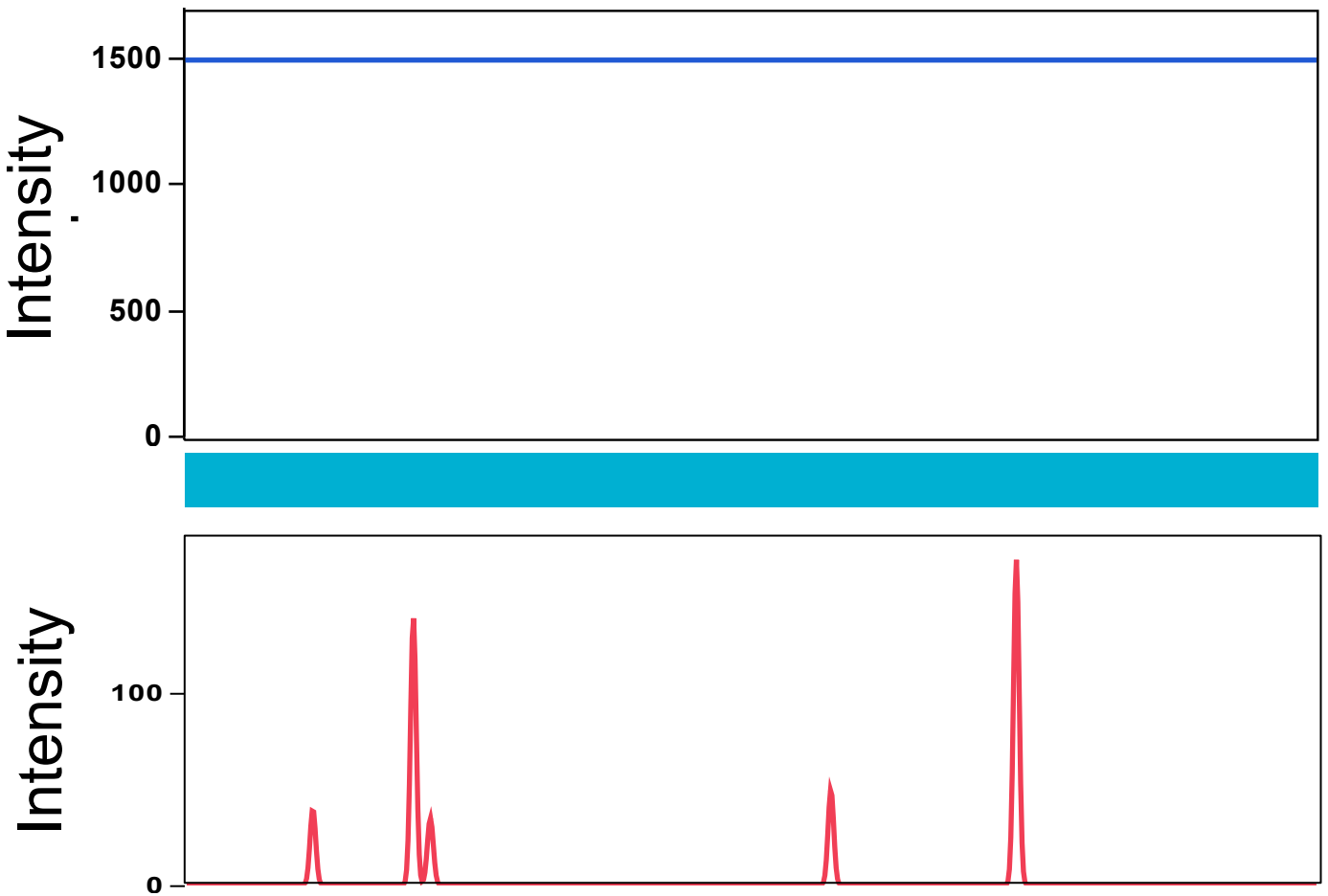


How Bright Are the Stars?

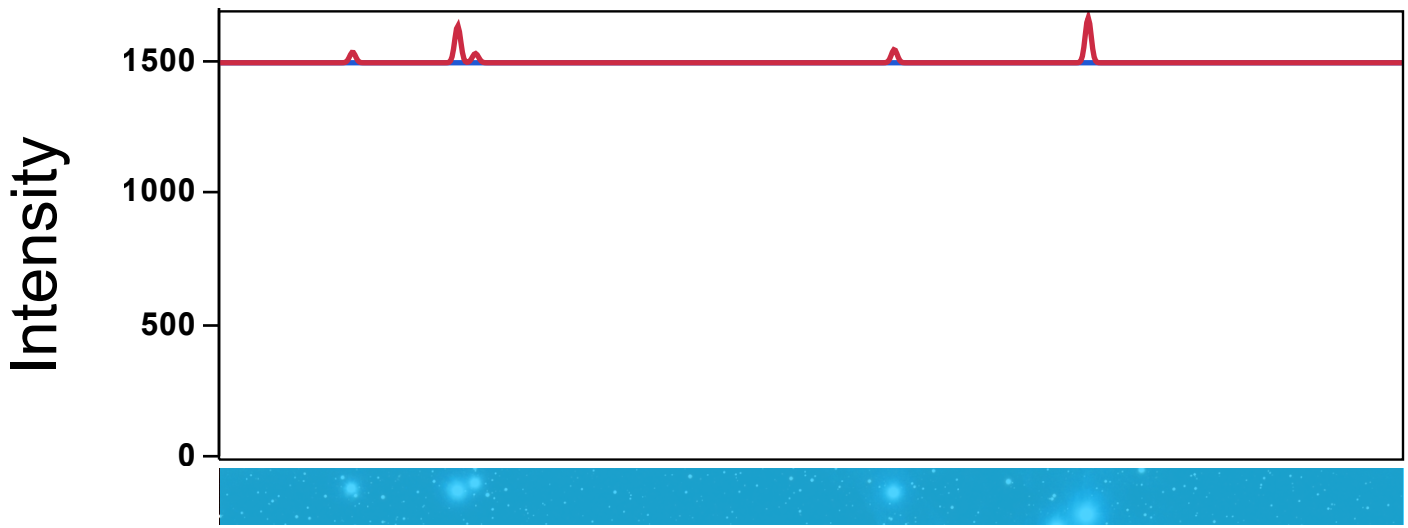


A star's intensity is the area under the histogram (i.e., counting the number of photons coming from the star).

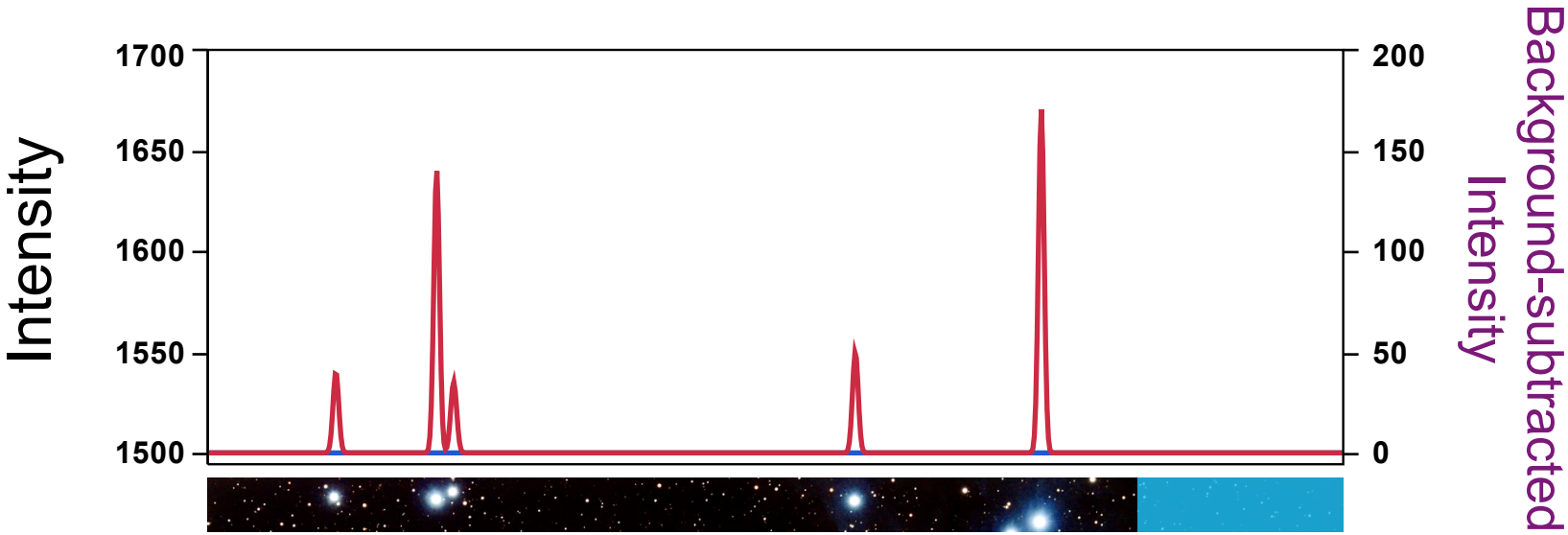
How Bright Is The Sky?



Light Signals Are Additive



Star Light Brightness Is Unchanged



We determine the intensity of a star by **subtracting the background**. Here, the sky (background) is 1500 units, so subtract 1500 units from the measurements.

A star's intensity is now the area under the histogram (# of photons only from the star)

But Measurements Are Not Perfect

Every measurement we make has an error associated with it.

The variance in a measurement is the distribution of values that you would get if you made the *same measurement* on exactly *the same* object time and again (and the object did not change over time).

Variances can arise from a variety of sources: e.g., inherent variability, measurement error. The final variance is the *sum* of all of these.

Measurement error can arise from many sources. When counting photons, for example, *the minimum error* is governed by Poisson statistics.

Measurement Errors

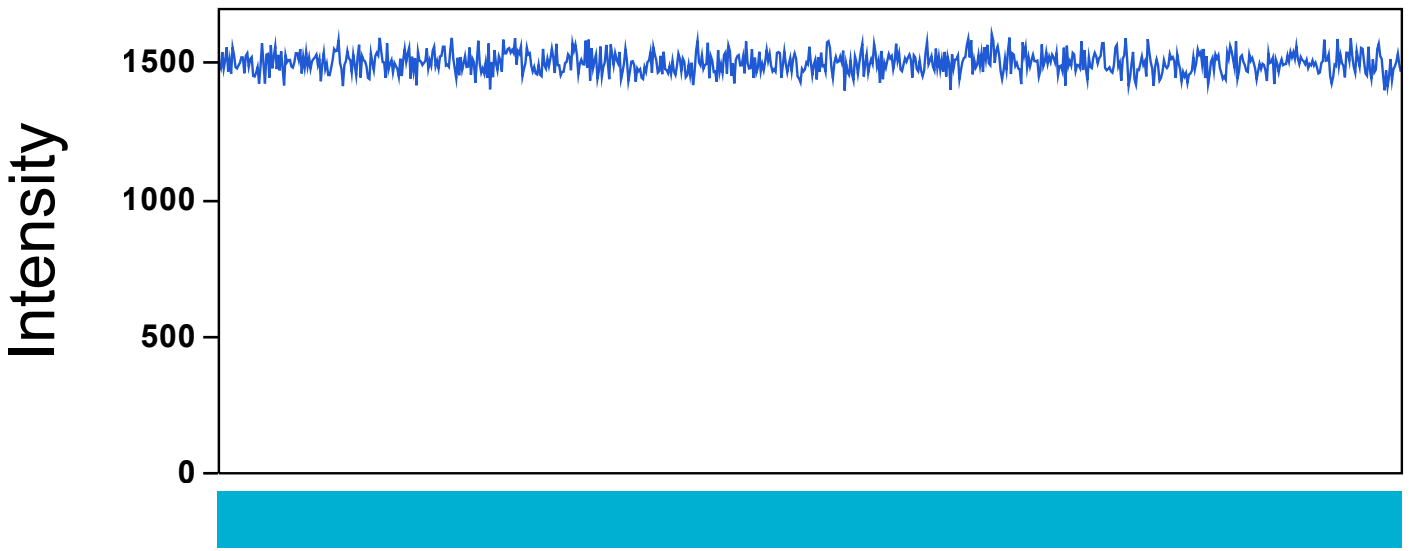
Light intensity measurements are, essentially, photon counting.

The *minimum* error will be $\pm\sqrt{n}$, where n = # of photons counted.

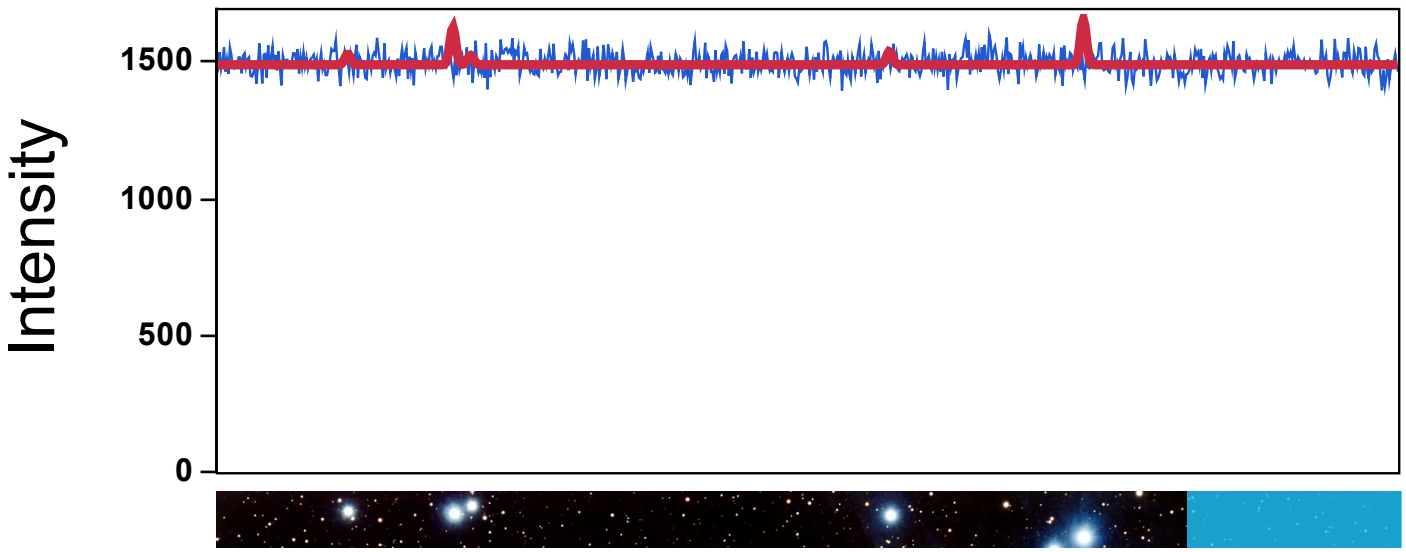
Number of counts	Error	Precision
100	±10	10%
10,000	±100	1%
1,000,000	±1,000	0.1%

As we increase the signal level, while the *precision* (CV) is getting better (smaller), the *absolute error* is increasing.

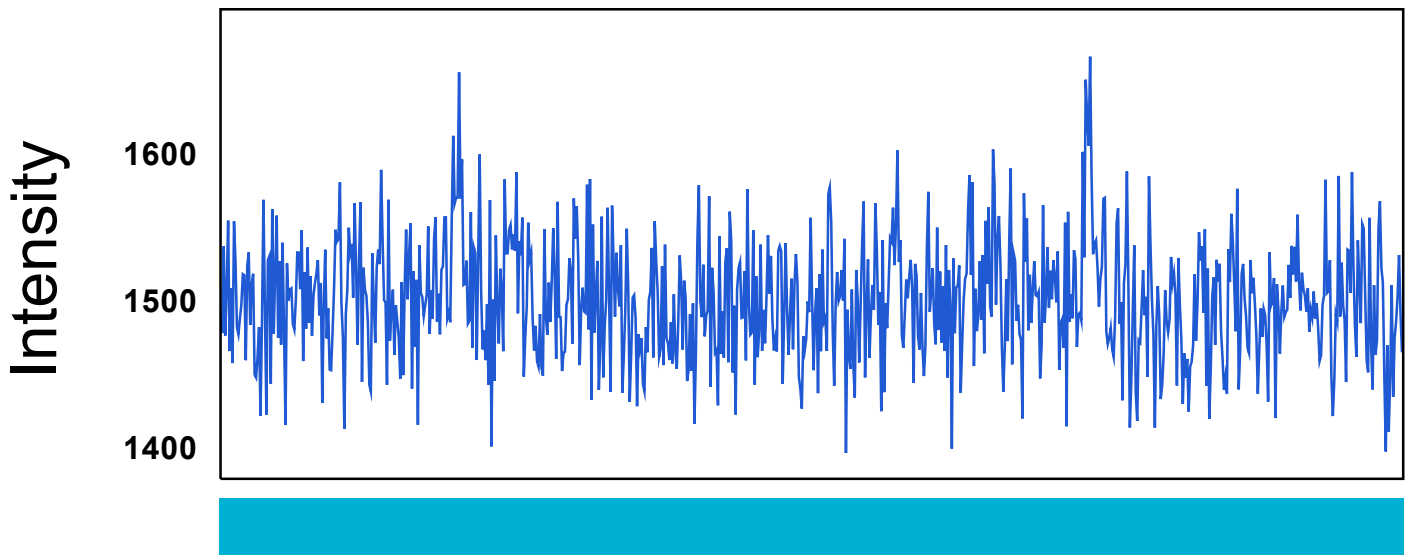
Measuring Sky Intensity



Star Light is Still Additive



Variation in Intensity



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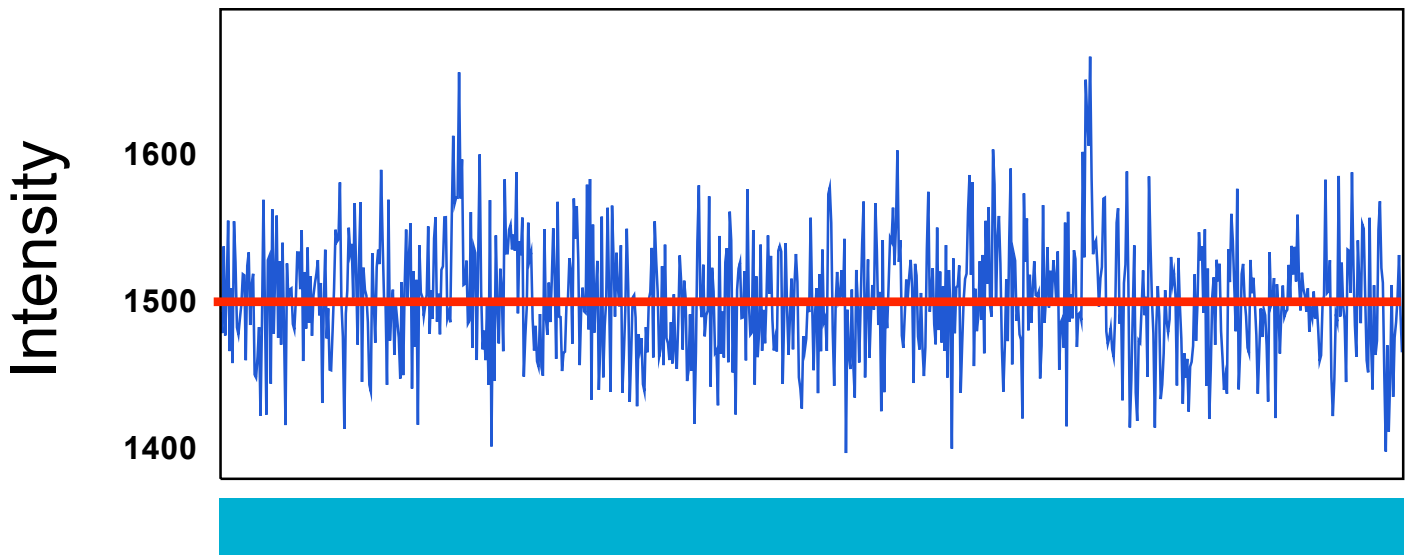
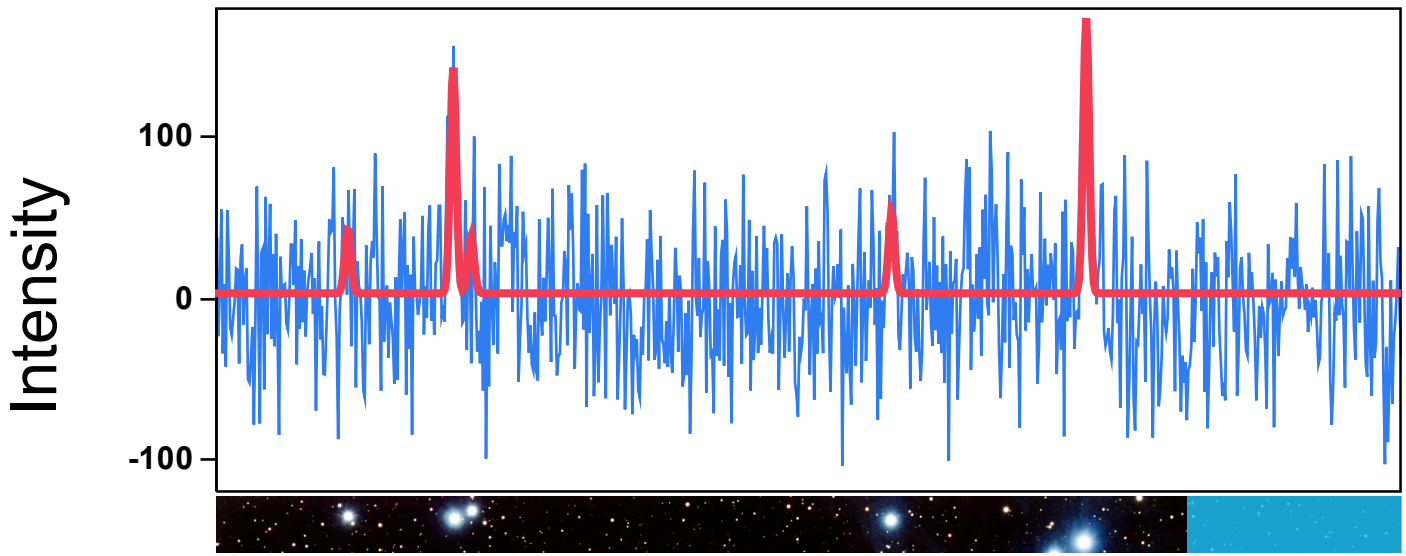
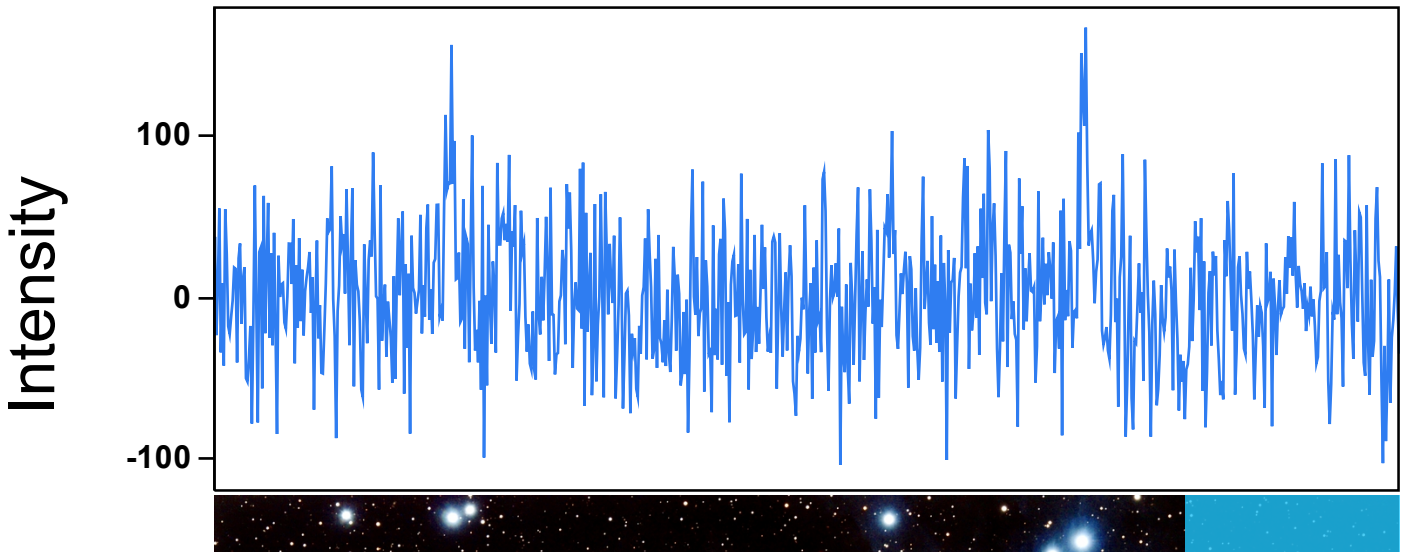


We estimate the intensity of a star by subtracting the background. Here, the sky (background) is, ***on average***, 1500 units, so subtract 1500 units from the measurements.

Estimating Star Light Intensity



Estimating Star Light Intensity

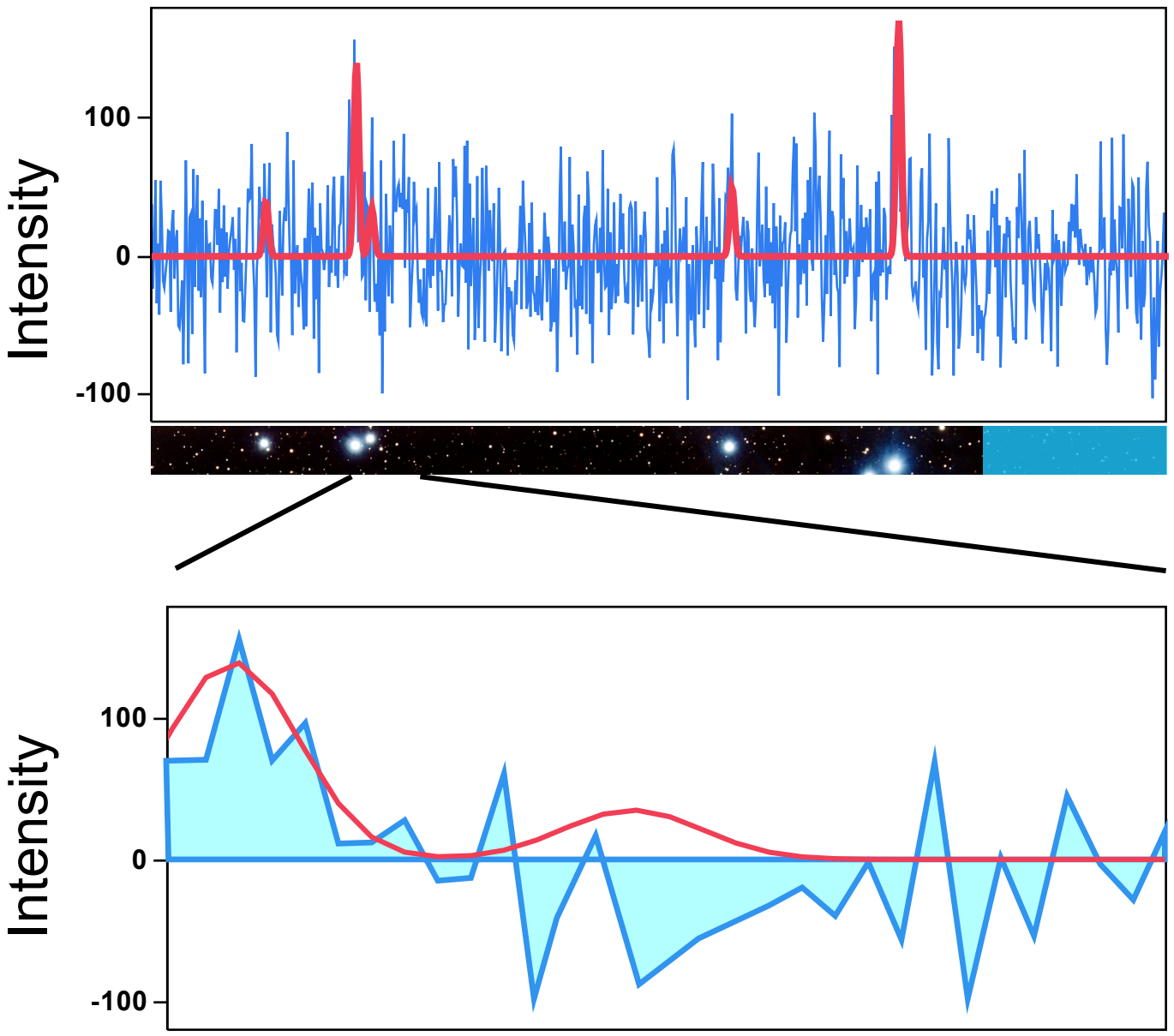


A star's intensity is, as before, the area under the histogram (# of photons only from the star)

Background Variance

Ideal

Measured



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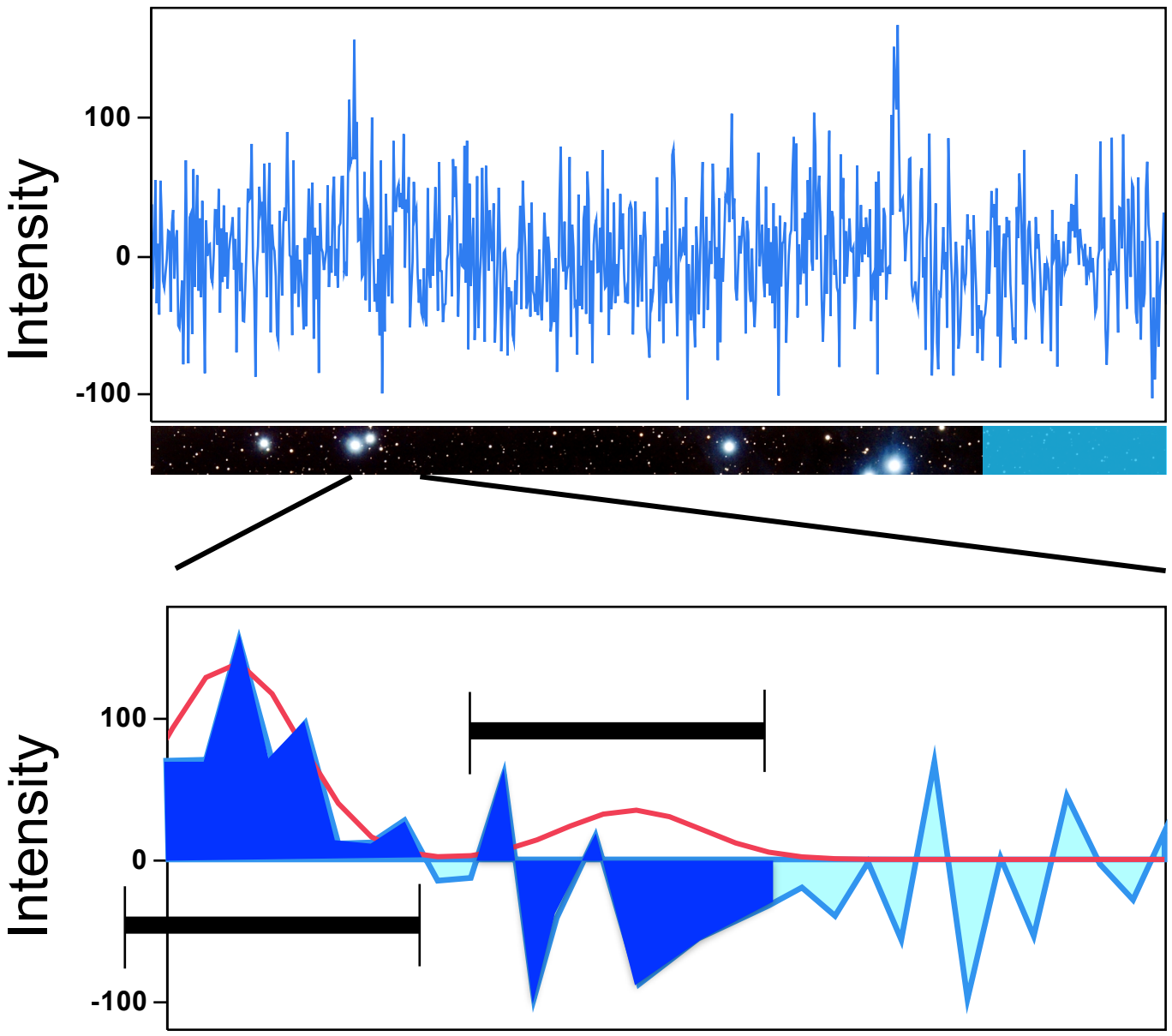
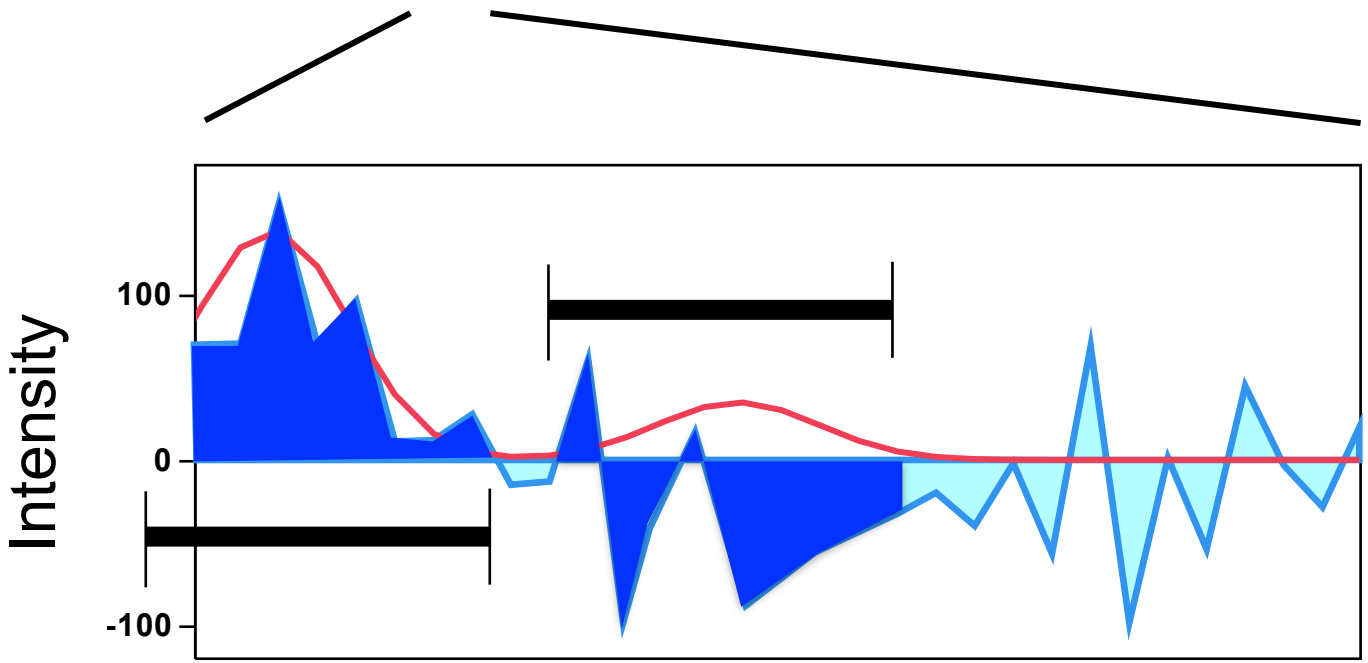


Background Variance

Is this “negative light intensity?”

No. It is a negative *estimated* light intensity! And arbitrarily setting it to zero would introduce a systematic bias.



Why Can't We See Stars During the Day?

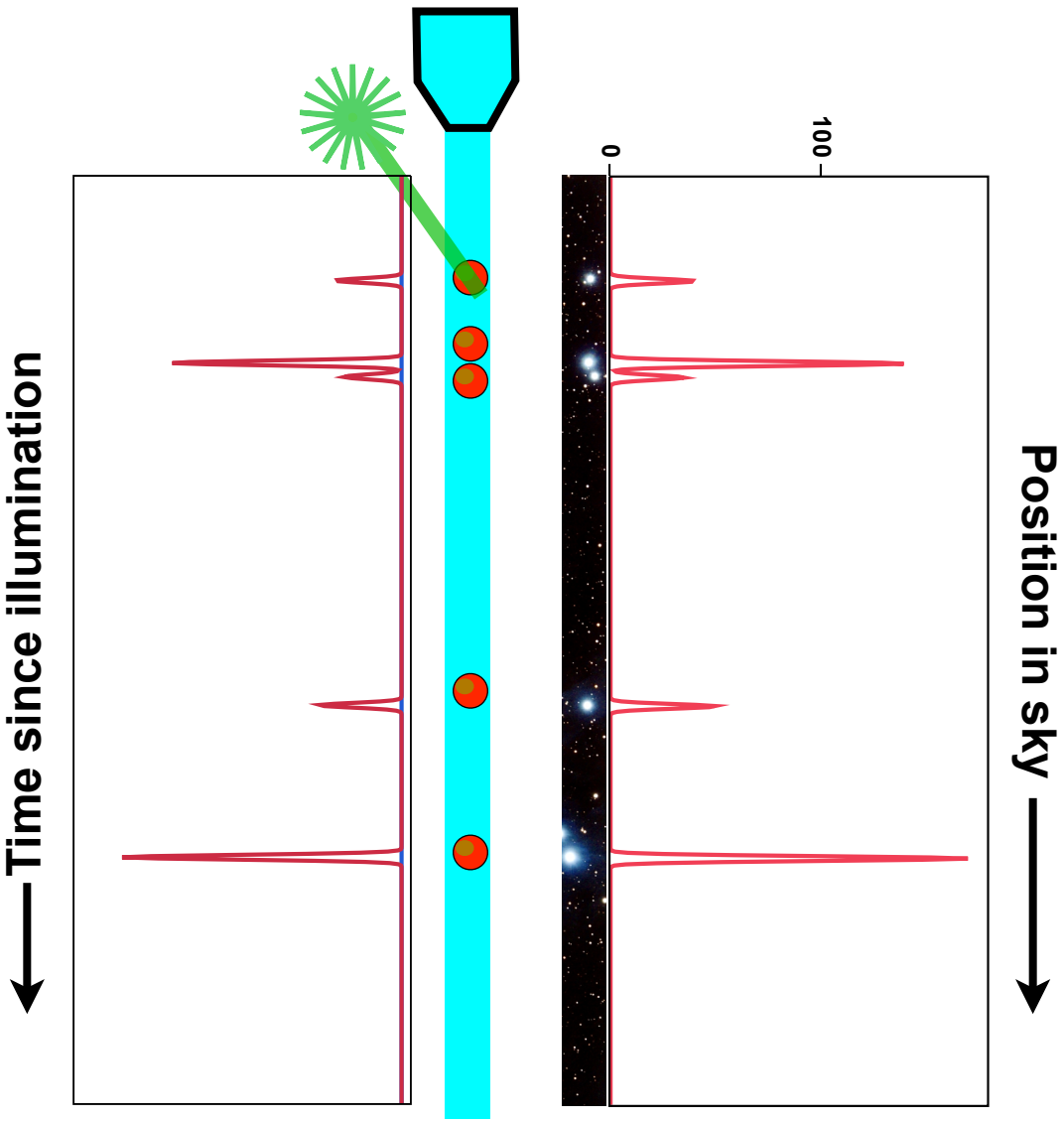
Because the intensity of the star light is less than the *variance* of the intensity of the day light.

Stars that are brighter than this variance can still be seen.

The error on estimating the intensity of the star light will be at least as great as the variance of the day light... and can sometimes be <0.

Is This Related to Compensation?

Completely.



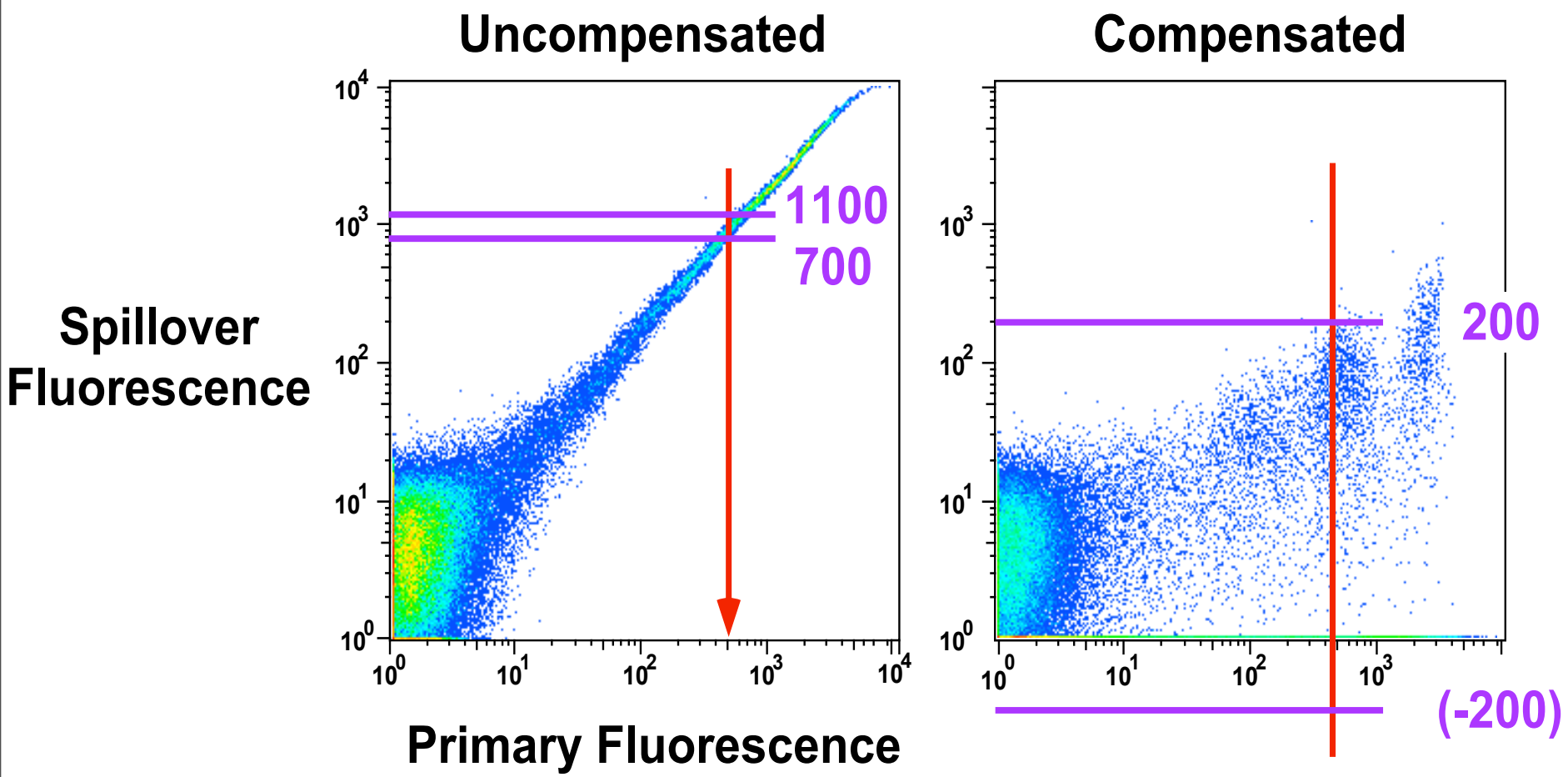
Is This Related to Compensation?

Completely.

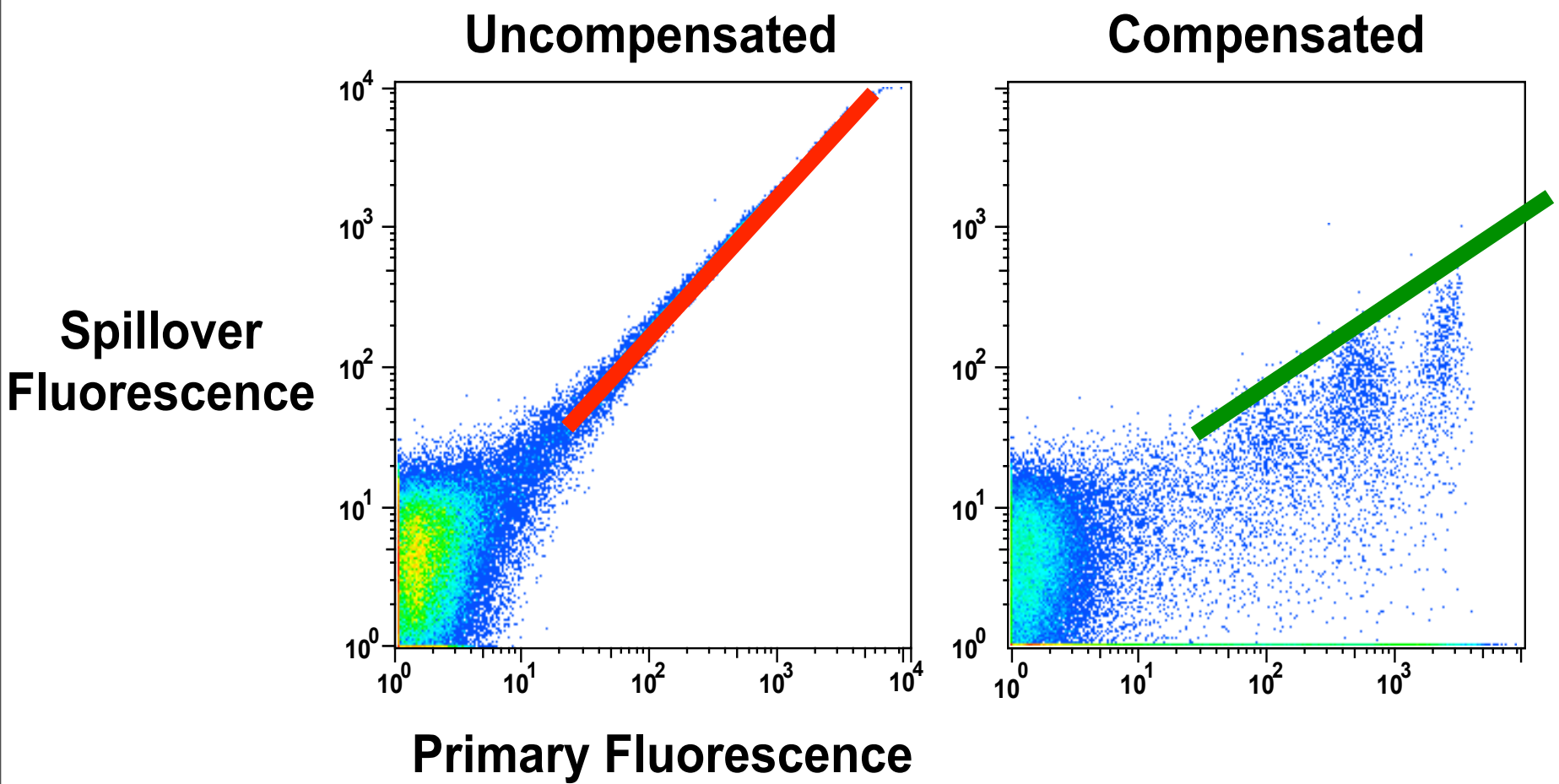
Compensation is the process by which we estimate a probe's contribution to a signal by subtracting estimated contributions from other sources.

All the same principles apply: errors propagate; you cannot distinguish dim objects against high variance; you can get negative estimated signal intensities.

Imperfect Measurement Leads to Apparent Spread in Compensation



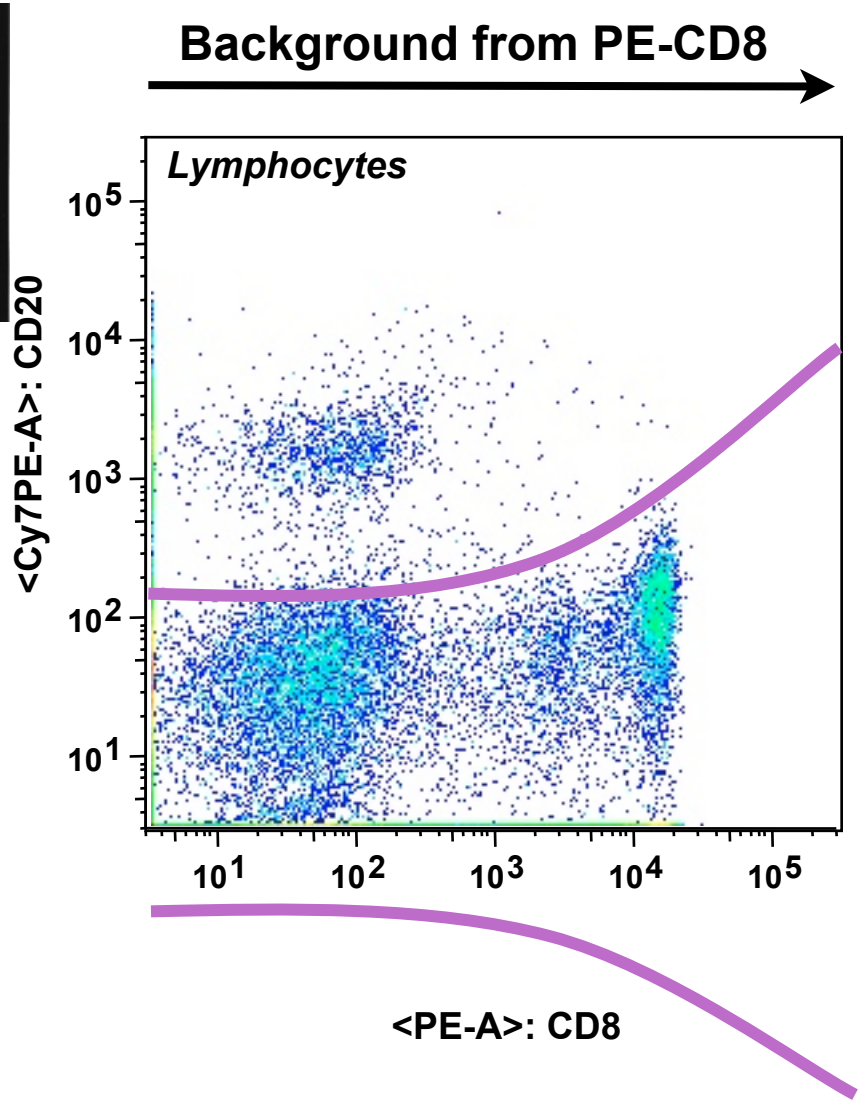
Imperfect Measurement Leads to Apparent Spread in Compensation



Spreading manifests with a 1:2 Log:Log slope: square root relationship!

Undercompensation manifests with a 1:1 Log:Log slope: linear relationship

The Actual Spread



Variance contributed from background

Compensation Does NOT Introduce nor Increase Error:

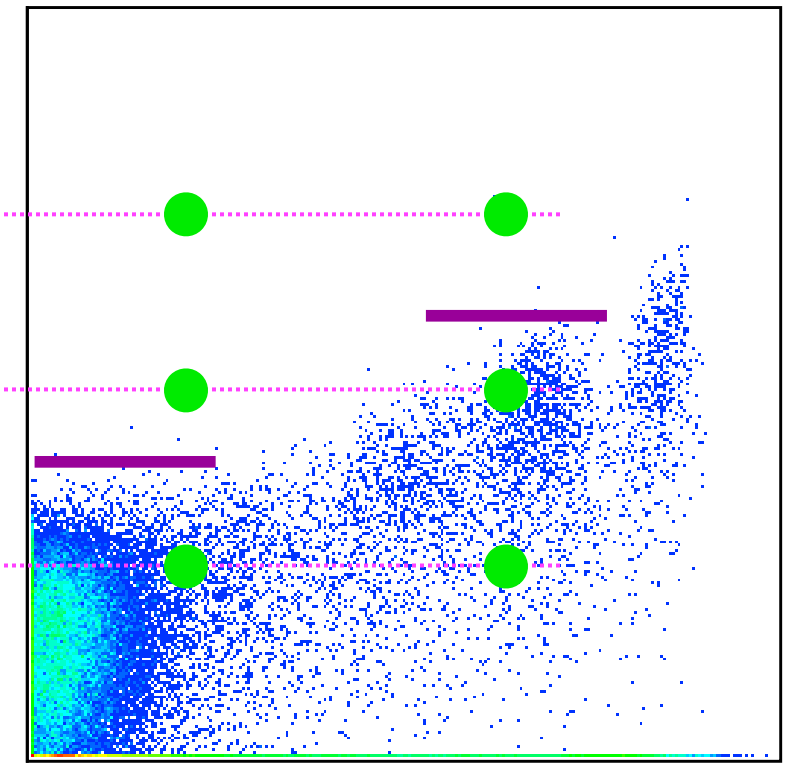
Compensation Only Reveals It!

- 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.
- Staining controls are necessary to define gate placement!

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!



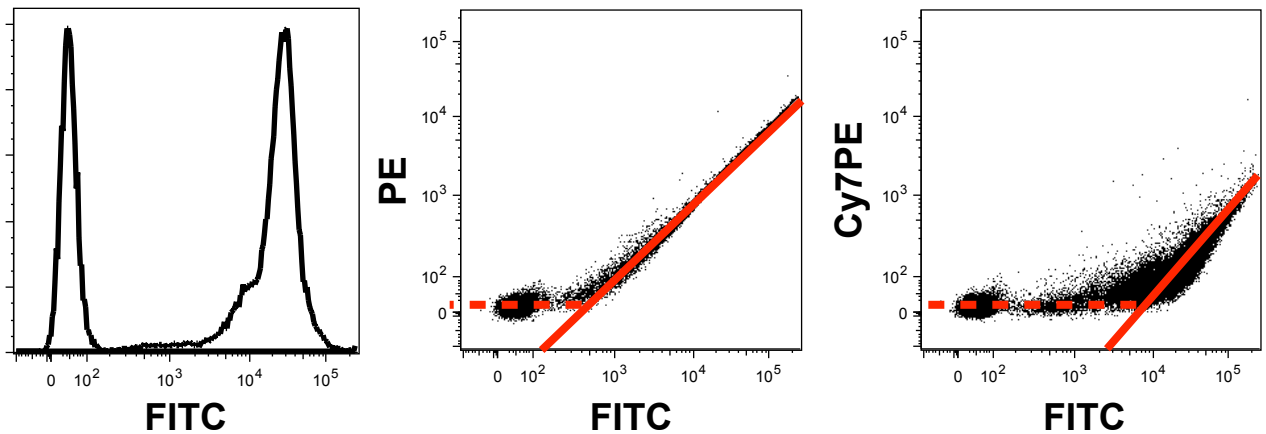
FMO Controls

- 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

Compensation Controls

The purpose of a compensation control is to allow software to estimate how much spillover is happening. This needs to be as precise as possible!

Precise and accurate compensation requires determining the slope as precisely as possible.



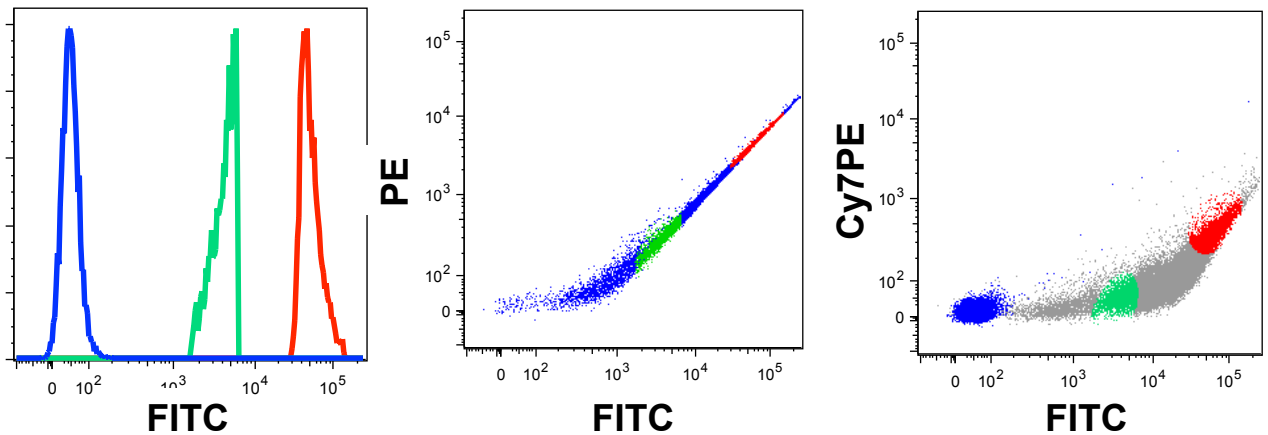
Compensation = 7.4% **= 0.56%**

Compensation Controls

The purpose of a compensation control is to allow software to estimate how much spillover is happening. This needs to be as precise as possible!

Precise and accurate compensation requires determining the slope as precisely as possible.

“Small absolute errors” can be “large relative errors” in compensation and dramatically impact data



Compensation = 0.56%

Compensation = 0.86%

Compensation Controls

There are only three rules to follow to ensure ***accurate*** compensation. All errors in compensation can be traced to a problem with one of these.

1. **The experimental samples and control samples must be matched.**
   - Identical fluorochrome
   - Identical instrument sensitivity (*not settings*)
2. **The background (autofluorescence) for any given control's positive and negative events must be identical.**
3. **Each control's positive must be at least as bright as any experimental sample.**

To ensure ***precise*** compensation, collect enough events to precisely estimate median fluorescences in *all* channels:

Beads: 5-10K *Positive* cells: 20-50K