Cytometry tutorial:
The impact of adjusting PMT voltages on spillover and compensation

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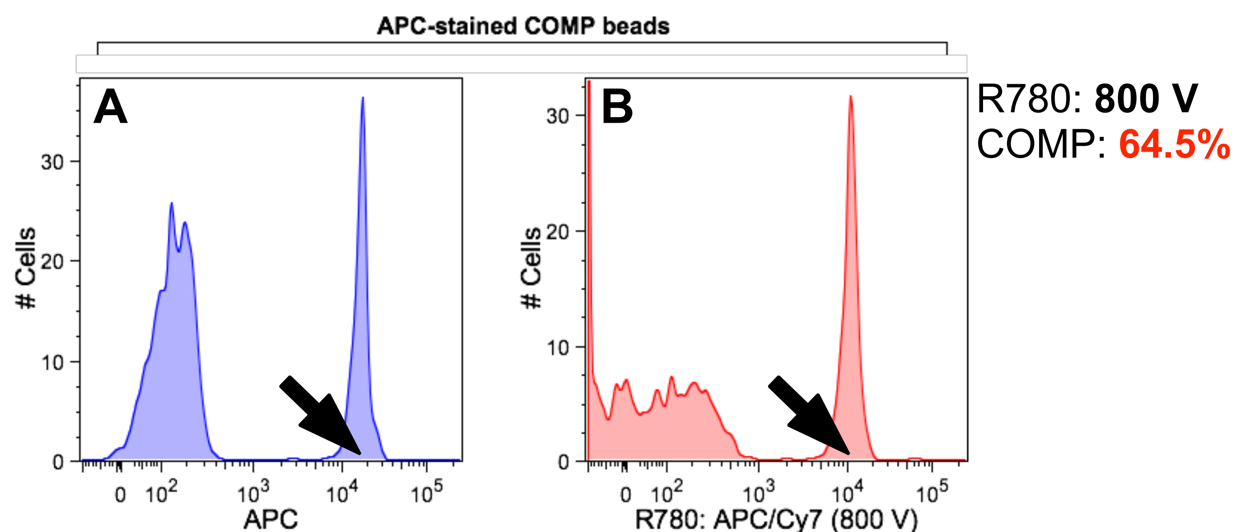
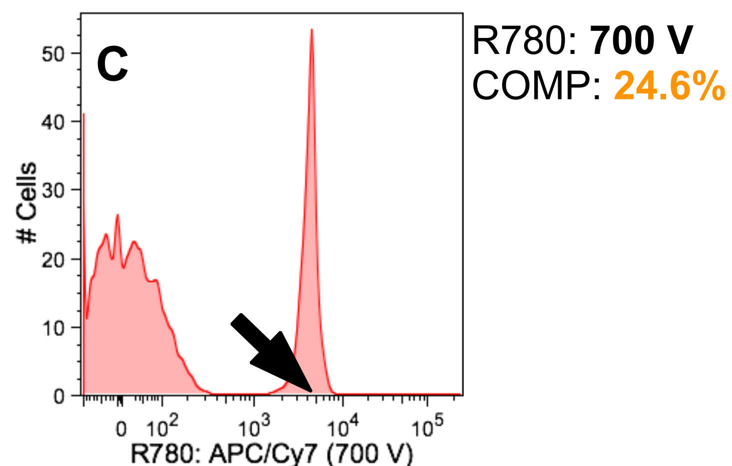
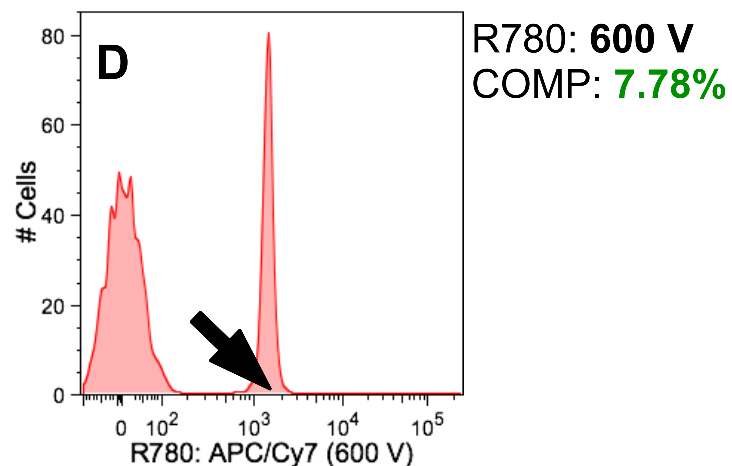
Voltage adjustment to reduce spillover?

Misconception: *"I need to adjust my voltage to lower the compensation required between two overlapping fluorophores."*







Case study

- My APC comp bead (A) spills into the APC/Cy7 detector (R780) (B)
- The compensation value is 64.5% (B)
- My supervisor thinks this is too high and wants me to reduce the compensation value by lowering the voltage of R780
- If I reduce the voltage from 800V to 700V or 600V I can reduce the compensation value
  - from 64.5% (high) (B)
  - to 24.6 (medium) (C)
  - or 7.78% (low) (D)

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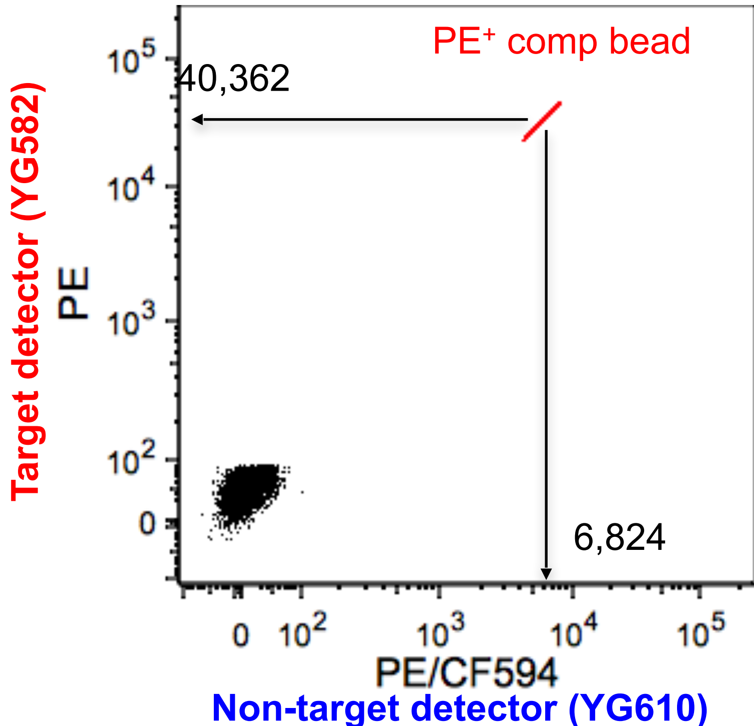
Refresher: what is compensation?

Compensation is “correcting” fluorescence spillover



Compensation value (extracting **PE** from the **PECF594 (YG610)** detector):
6,824 / 40,362 x 100

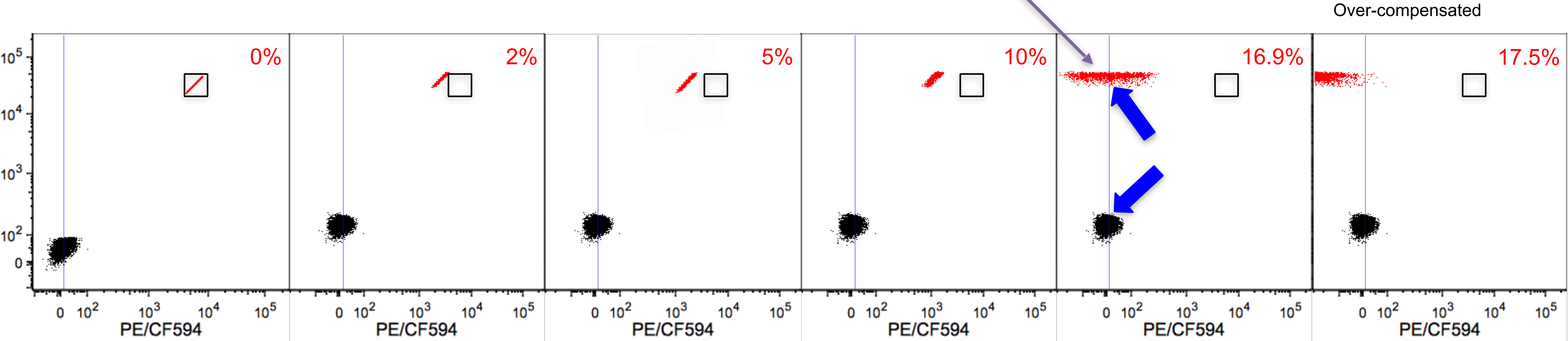
= 16.9%

This compensation value is dependent on voltages

Comp(YG610) = Measure(YG610) - %Measure(YG582)

Why is this spreading?

Correct compensation:
*Median Fluorescence Intensity (MFI) of **PE+** and **PE-** beads in the **PECF594 (YG610)** detector are equal*



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What is spreading error?

Spreading error is what causes problems for resolving true positive populations due to spreading of the negative population.

Diagrammatic example:

- ***PerCP/Cy5.5 (with marker "A")*** *spreads into the* ***BV711*** *and* ***BV650*** *detectors, leading to a loss of resolution in both.*
- ***Figure 1:*** ***BV711*** *is bright enough, and* ***marker "B"*** *is expressed highly enough that* ***A+B+ cells*** *can be resolved from* ***A+B- cells****.*
- ***Figure 2:*** *However,* ***BV650*** *is <u>not bright enough</u> to resolve* ***A+B+ cells*** *from* ***A+B- cells.***

Figure 1: resolvable

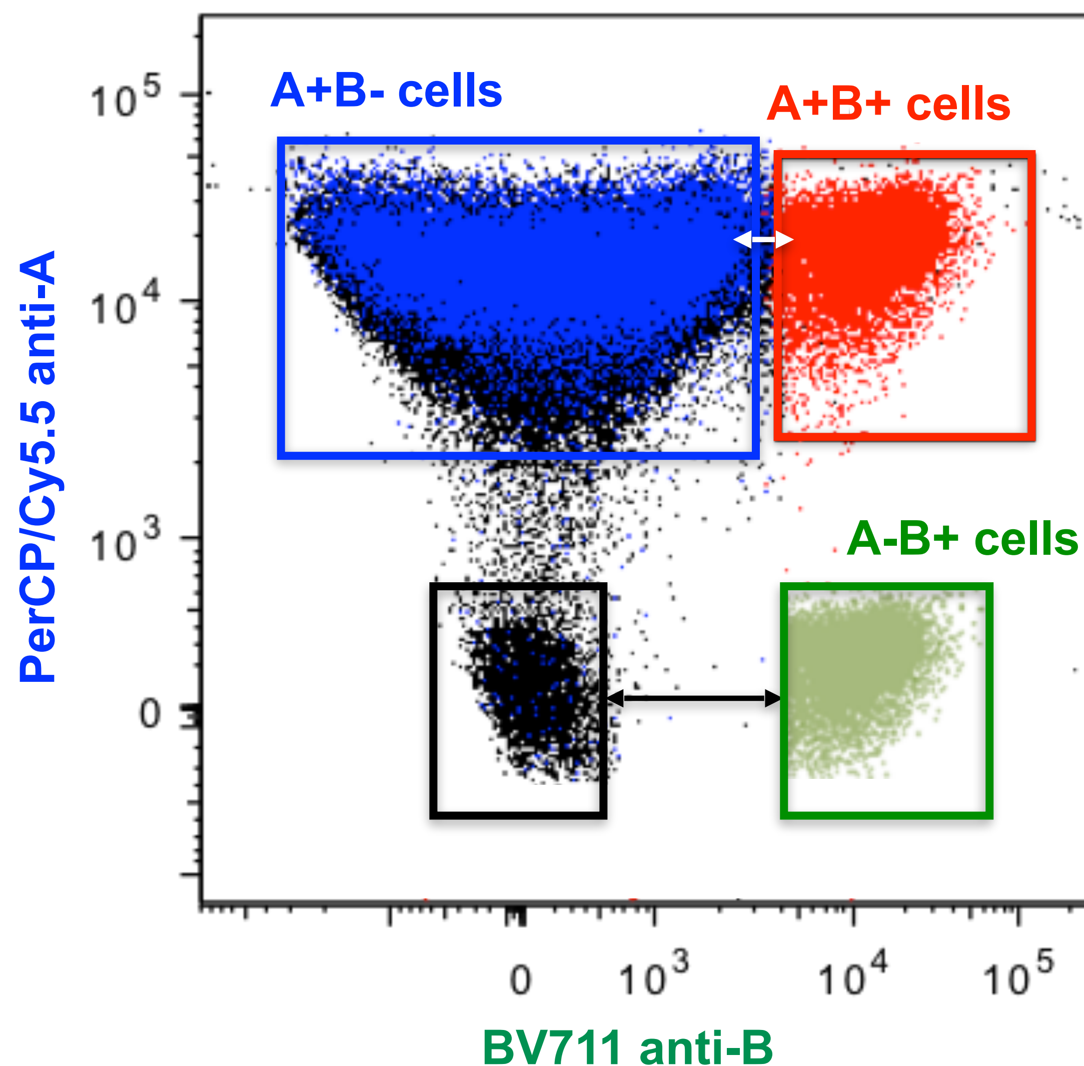
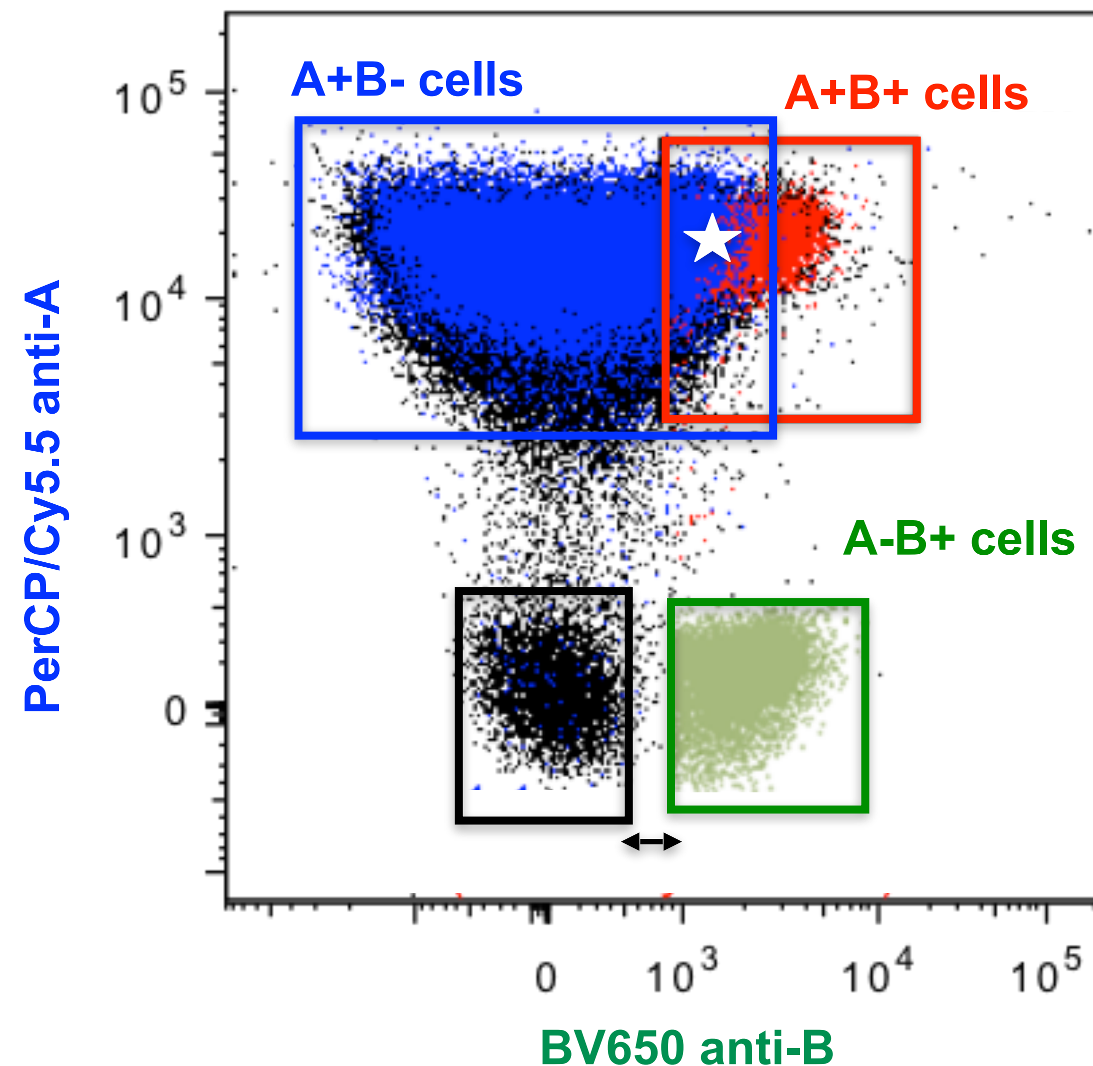


Figure 2: not resolvable



The process of correct compensation reveals (not creates) measurement error, known as spreading error (SE)

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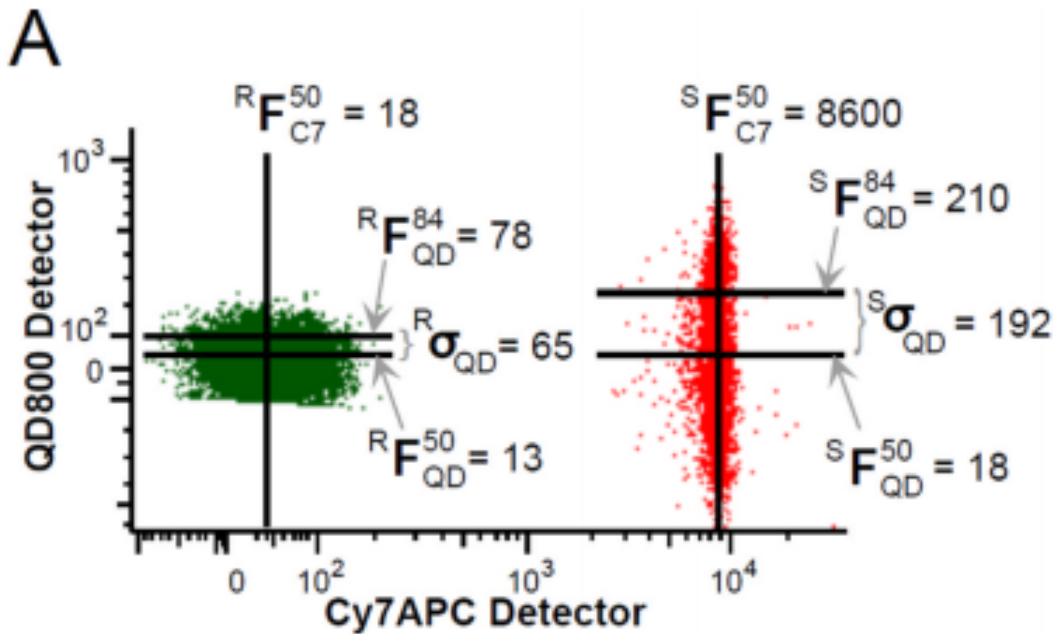
What is spreading error?

For more information on spreading error, see the following publication

Quantifying Spillover Spreading for Comparing Instrument Performance and Aiding in Multicolor Panel Design

Richard Nguyen[1], **Stephen Perfetto**[1], **Yolanda D. Mahnke**[2], **Pratip Chattopadhyay**[2], and **Mario Roederer**[1,2,*]
[1]Flow Cytometry Core, Vaccine Research Center, NIAID, NIH, Bethesda, MD
[2]ImmunoTechnology Section, Vaccine Research Center, NIAID, NIH, Bethesda, MD

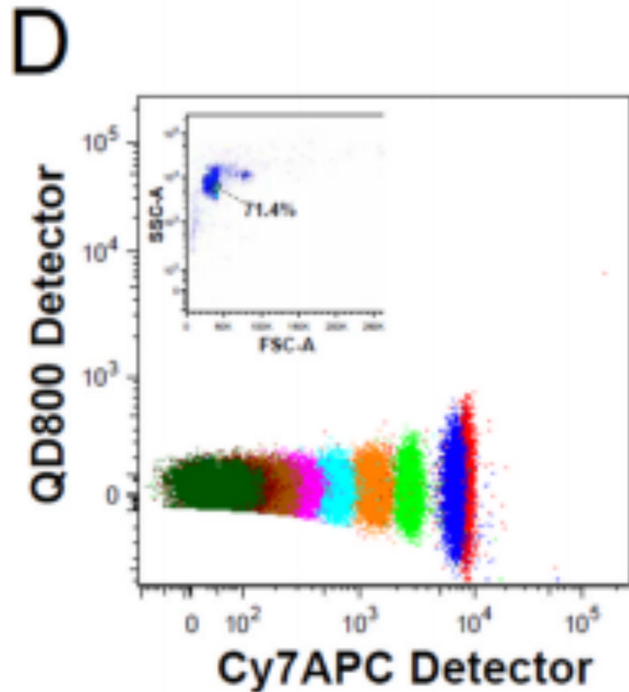


$$SS_C^P = \frac{\Delta\sigma_C}{\sqrt{\Delta F_P}}$$

$$\Delta\sigma_C = \sqrt{{}^S\sigma_C^{\,2} - {}^R\sigma_C^{\,2}}$$

$$\Delta F_P = {}^SF_P^{50} - {}^RF_P^{50}$$

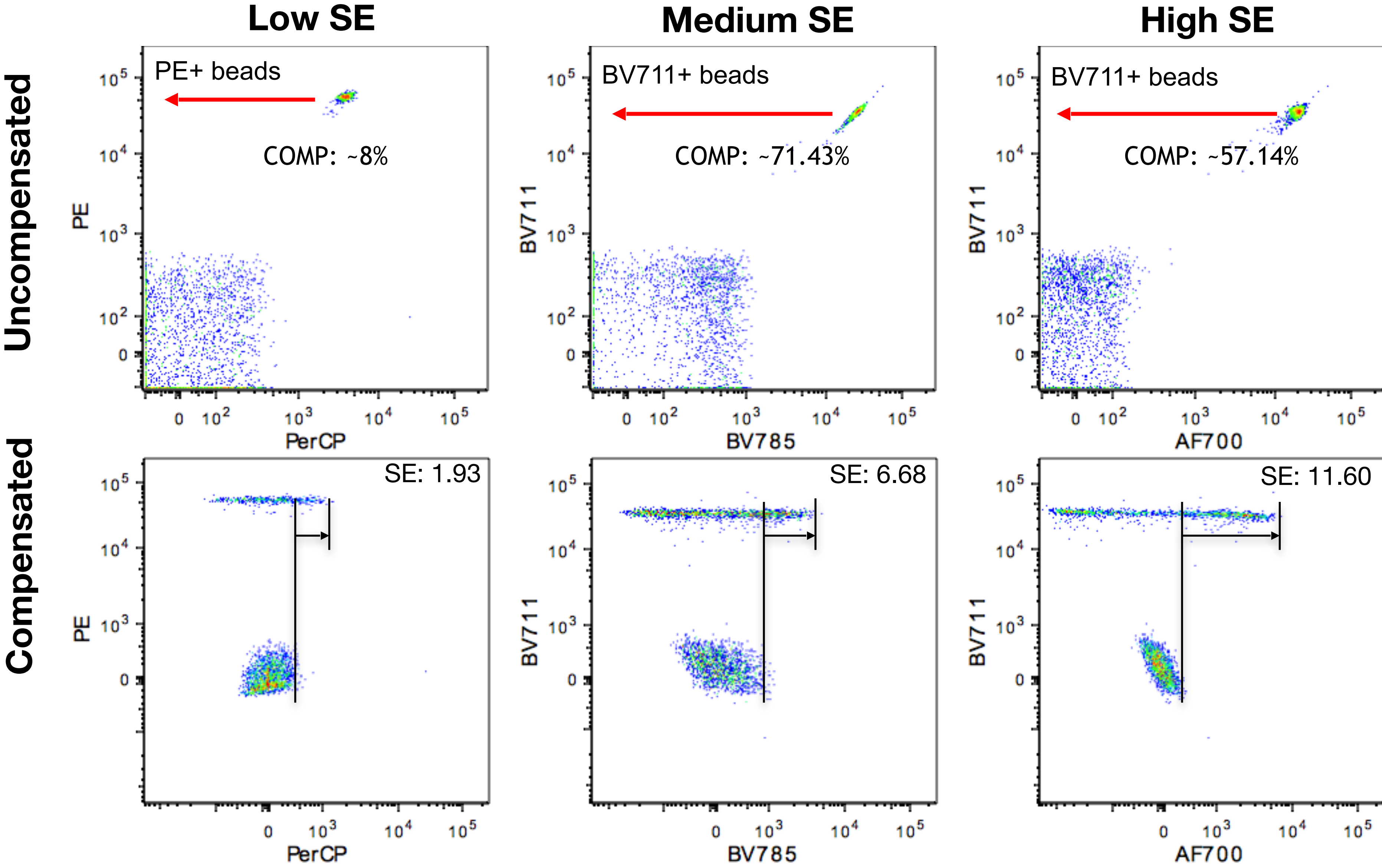
$${}^R\sigma_C = {}^RF_C^{84} - {}^RF_C^{50}$$



Reference: Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P., Roederer, M. 2013. Quantifying spillover spreading for comparing instrument performance and aiding in multicolor panel design. Cytometry A, 83(3): 306-315

What is spreading error?

The process of correct compensation reveals (not creates) measurement error, known as spreading error (SE)



Example:

- Higher compensation values *tend* to correlate with higher spreading error values (i.e. more spillover, more the measurement error will spread)
- However, higher compensation values cannot predict higher spreading error values

Example:

- BV711 into BV785 detector
  - Comp = **71.43**%
  - SE = **6.68**
- BV711 into AF700 detector
  - Comp = **57.14**%
  - SE = **11.60**

Despite the higher compensation value, the SE is lower

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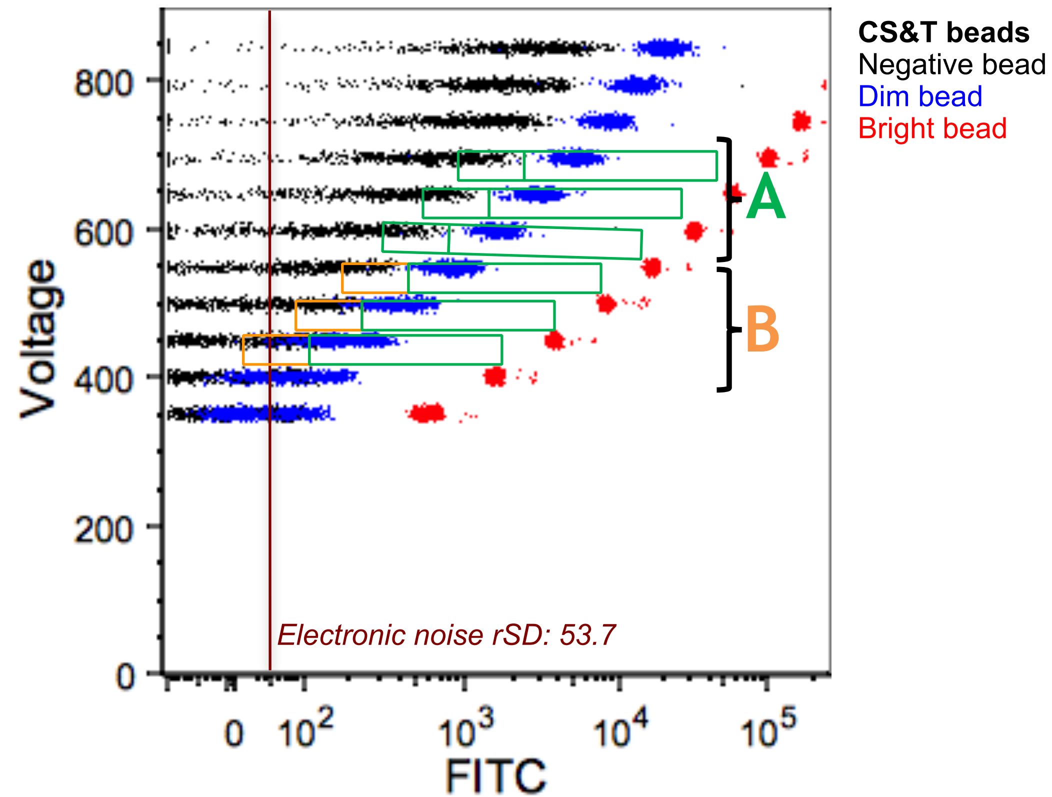
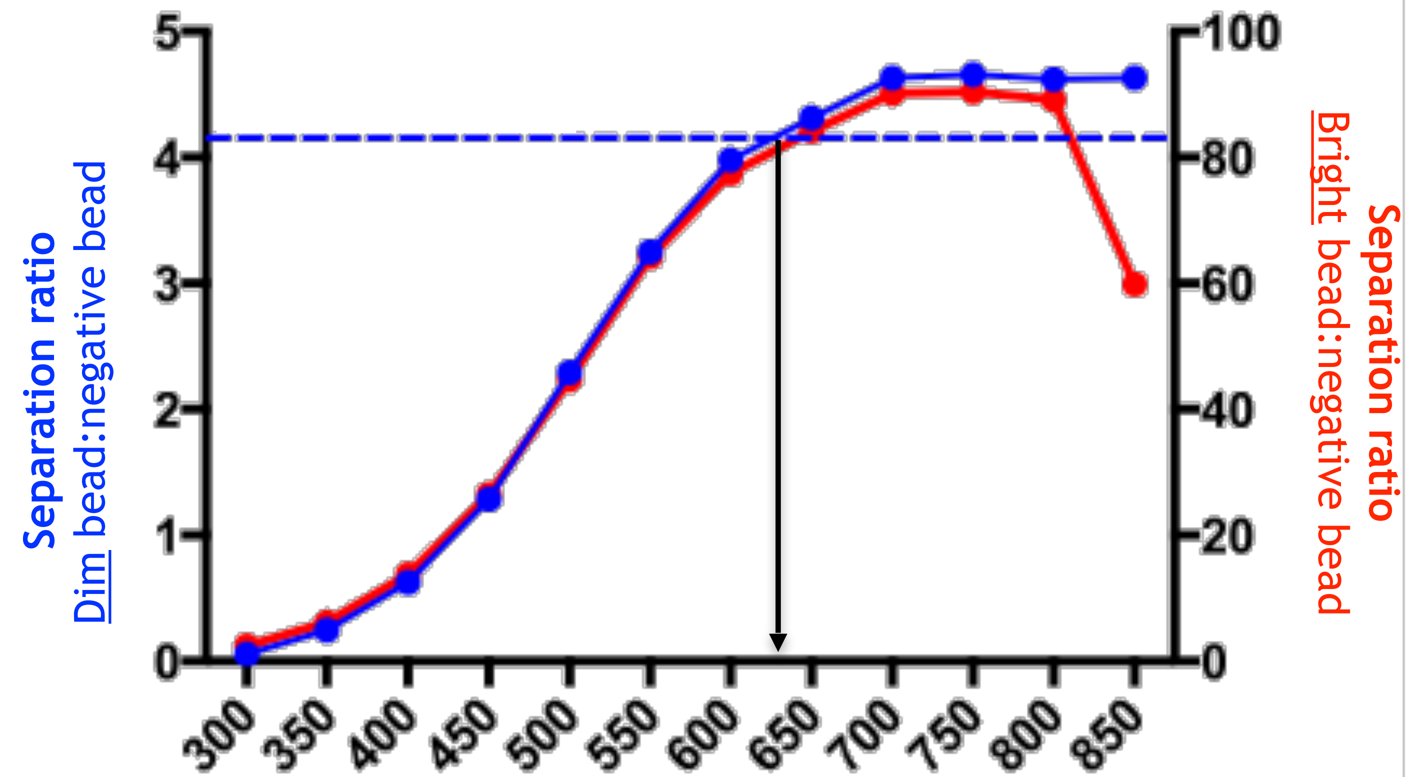
Detector voltages

What happens when we adjust the voltages for a given detector

CS&T beads (neg, dim, and bright) run at various voltages



FITC detector



- Each detector uses a photomultiplier (PMT) that amplifies any signal picked up in that detector. The voltage applied dictates the level of amplification
- At a specific voltage (~**600 volts for the FITC detector in this example**), fluorescence signal is
  - a) detectible above *background*, and
  - b) is *consistently* and *proportionately separated*
- Voltages **above** this value do not increase the separation of signal (**A**) (including dim-neg, dim-bright, bright-neg)
- Voltages **below** this value compromise the detection of low-end (dim) signal (**B**)
- This is true of **any signal** (target fluorophore or spillover fluorophore) appearing in the detector

'Voltage scan' approach to determine minimum voltage:

Perfetto, S. P., Ambrozak, D., Nguyen, R., Chattopadhyay, P. K., & Roederer, M. (2012). Quality assurance for polychromatic flow cytometry using a suite of calibration beads, 7(12), 2067–2079. doi:10.1038/nprot.2012.126i

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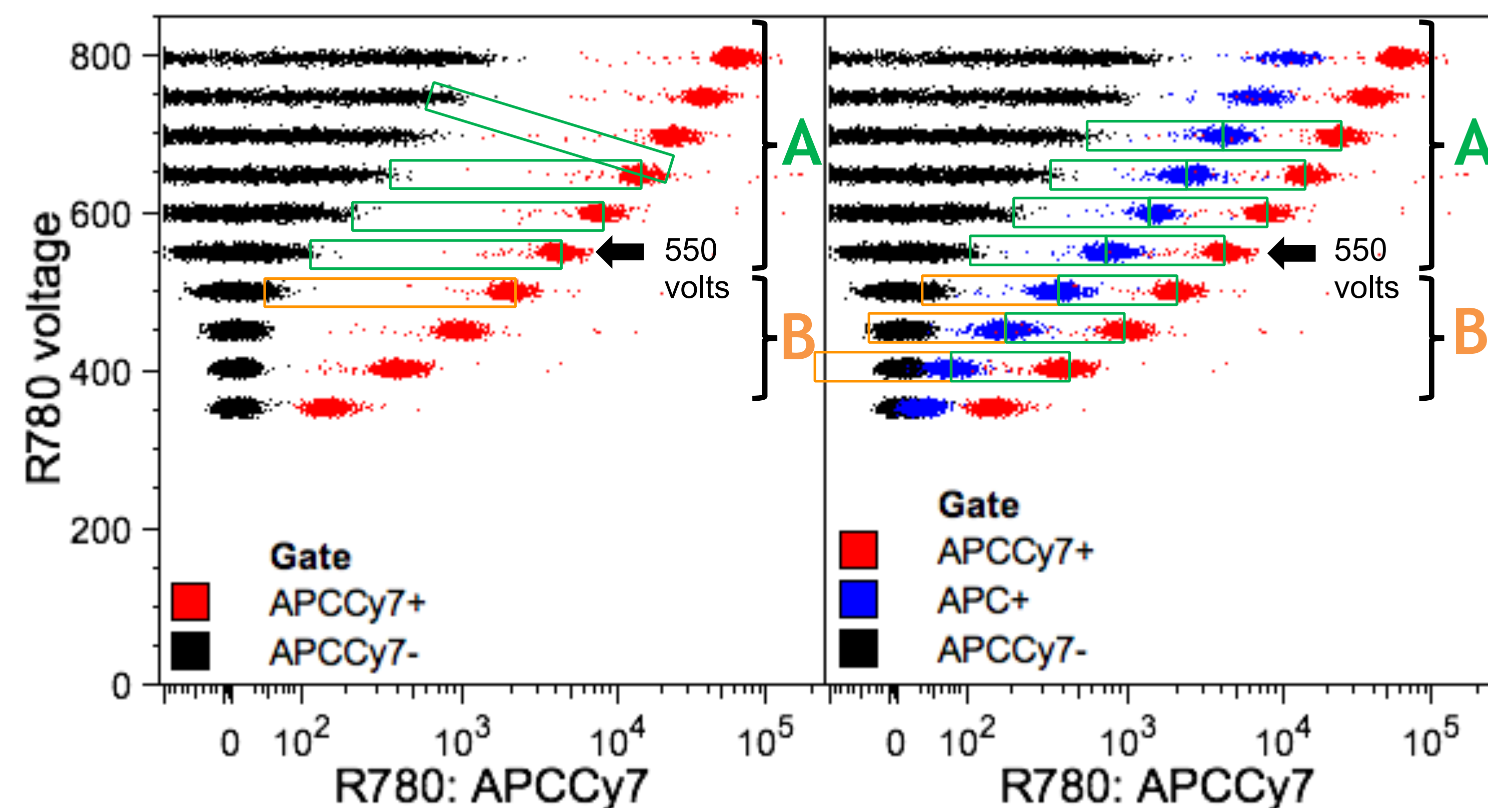
APC/Cy7 and APC in the R780 detector

Let's take a look at the performance of the APC/Cy7 detector (R780)



- COMP beads were recorded, where the R780 voltage was increased in 50V increments from 350 V up to 800 V
- At a specific voltage (**550 volts in this case**), fluorescence signal is
  - a) detectible above background, and
  - b) is consistently separated for any voltages above this
- This is clearly seen with the **APC/Cy7 comp bead** in the R780 detector
- However, this is also true of contaminating signals, such as the uncompensated **APC comp bead** in the R780 detector

- The minimum voltage required for optimal separation of **signal from noise** is between **500-550 volts**
- Voltages **above** the minimum for separation (550 volts) do not increase the separation of signal (**A**)
- Voltages **below** the minimum for separation (550 volts) compromise the detection of low-end (dim) signal (**B**)
- The separation of bright from dim signal occurs at lower voltages (~350 volts)
- This is true of **any signal** (target fluorophore or spillover fluorophore) appearing in the R780 detector

- In this case lowering the voltage below 500-550 volts would result in the **loss** of **dim APC/Cy7** signal

'Voltage scan' approach to determine minimum voltage:

Perfetto, S. P., Ambrozak, D., Nguyen, R., Chattopadhyay, P. K., & Roederer, M. (2012). Quality assurance for polychromatic flow cytometry using a suite of calibration beads, 7(12), 2067–2079. doi:10.1038/nprot.2012.126i

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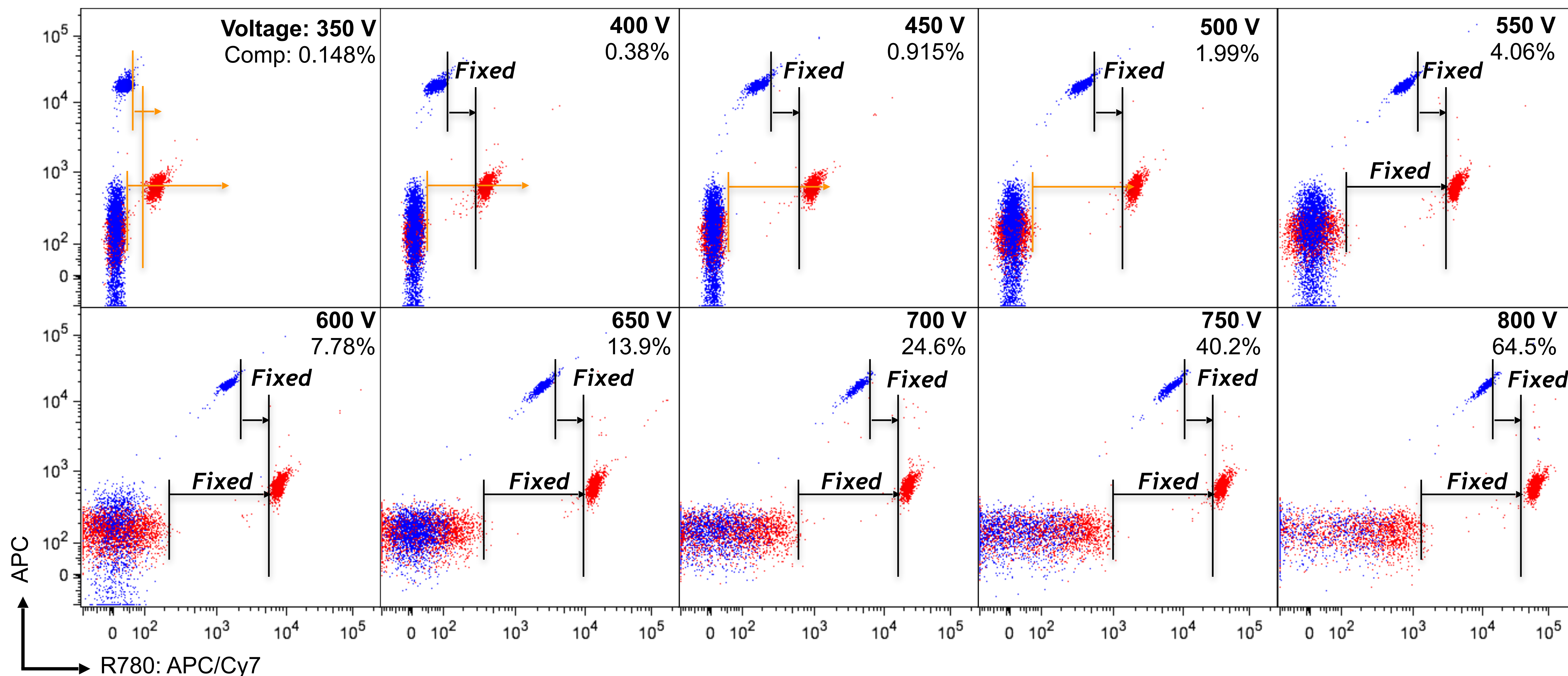
APC/Cy7 and APC in the R780 detector

*Looking at the **uncompensated** plots individually: above the 550 volt cut off the separation of the target fluorophore (APC/Cy7) from the spillover fluorophore (APC) is **fixed**, even though the compensation value increases. This is also true for the separation of APC/Cy7 from the background.*



Consistent with the principles of our previous slide

- The minimum voltage required for optimal separation of **signal from background** is between **500-550 volts** (APC/Cy7+ signal and APC+ signal above background)
- The separation of **bright from dim signal** occurs earlier (separation of APC+ from APC/Cy7+ signal is consistent above above 350V)

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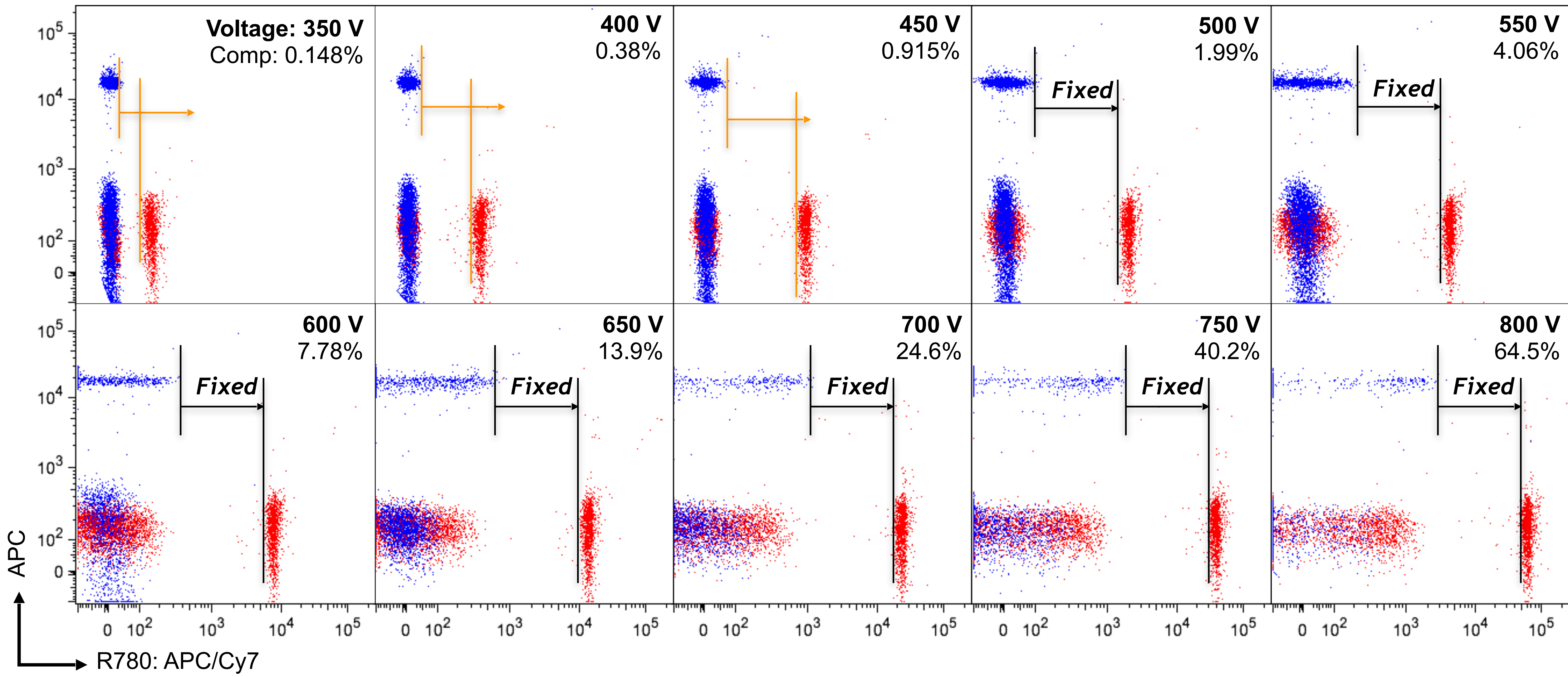
APC/Cy7 and APC in the R780 detector

*Looking at the **compensated** plots individually: above the 550 volt cut off the separation of the target fluorophore (APC/Cy7) from the spread of the compensated spillover fluorophore (APC) is **fixed**, even though the compensation value increases.*



- The minimum voltage required for optimal separation of **signal from background** is between **500-550 volts**
- The separation of compensated APC+ (now in the negative/low region scale of the R780 detector) from APC/Cy7+ signal is consistent above above 550 V

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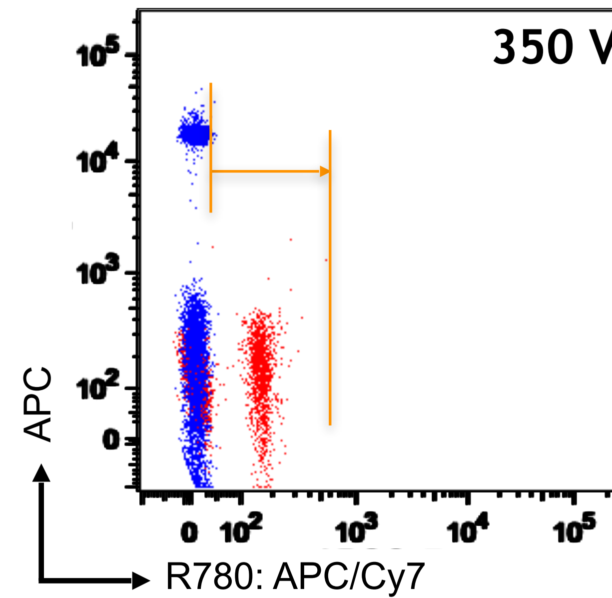
APC/Cy7 and APC in the R780 detector

Overlay of the compensated data: 350-800 volts in R780



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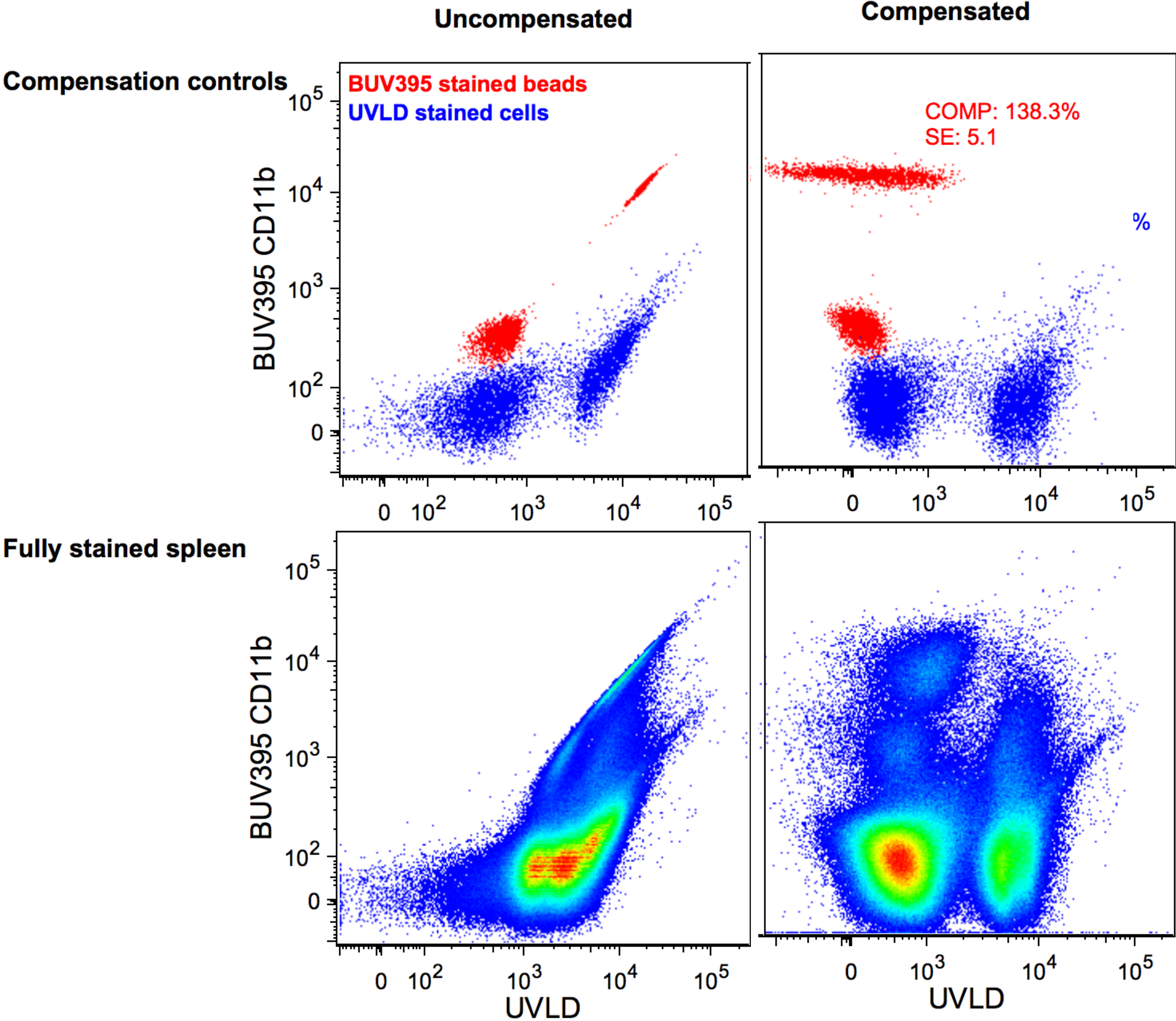
So what does compensation >100% look like?

Despite compensation values of ~140%, the spreading error, and resolution of target (UVLD) from non-target (BUV395) signal is reasonable.



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So how do I set my voltages?

On-the-fly method for setting voltages (not a full optimisation procedure)

- Start with the CS&T assigned voltages (this will set an approximate minimum for separation of dim signal)
- Run through your full set of compensation beads/cells — reduce voltages for any detector where the signal is off-scale/ in the non-linear range (typically reduce if the peak is above 10^5). Even if reducing the voltage compromises the resolution of dim signal, it is more important to have linear signals (signal on-scale), as it is absolutely required for correct compensation
- Run through the compensation controls again and check the spillover values. ***Voltages should not be modified to reduce these values, as this will not impact resolution***
- Check each of your sample types (especially samples from any stimulation or inflammatory situation) to ensure signals are all on scale/in the linear range
- Record compensation controls, apply compensation, and record samples

Full optimisation procedure: see the following resources

- Sydney Cytometry Facility optimisation and tracking (email thomas.ashhurst@sydney.edu.au or a.smith@centenary.org.au)
- BD Application settings using unstained cells (https://www.bdbiosciences.com/documents/BD_FACSDiva_Stndrd_App_Setup_TechBulletin.pdf)
- VRC bead suite optimisation method (http://www.nature.com/nprot/journal/v7/n12/abs/nprot.2012.126.html)

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Summary and application

Summary: changing voltages does not impact resolution

- Users are often concerned by **high compensation values**, especially values above 100%, and want to reduce the voltage of the detector receiving spillover to reduce the compensation value
- However, decreasing/increase voltages will only modify the **compensation value**, and will **not change** the impact of spillover/spreading on the resolution of positive signal

PMT voltages: properly optimised

- Each detector will have a minimum voltage where signal is consistently and proportionally detected above background
- Decreasing the voltage for a given PMT below this minimum, then resolution of dim populations will be lost.
- Increase the voltage for a given PMT above this gives no advantage to population resolution.
- Often the cause for abnormally high compensation values (values >100% in particular) are a result of improperly optimised PMT voltages, for example, such as unnecessarily high default voltages being set by the CS&T program in some detectors.
- Use a proper optimisation protocol (or at least an on-the-fly method) to optimise PMT voltages

What do I do if I have spillover/spreading error causing a loss of resolution in my data

- Firstly, optimise your instrument
- If spillover/spreading error is causing a loss of resolution in your data on an optimised system, the solution is to **modify** your panel, not to modify the voltages

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