

Cytometry tutorial:

The impact of adjusting PMT voltages on spillover and compensation



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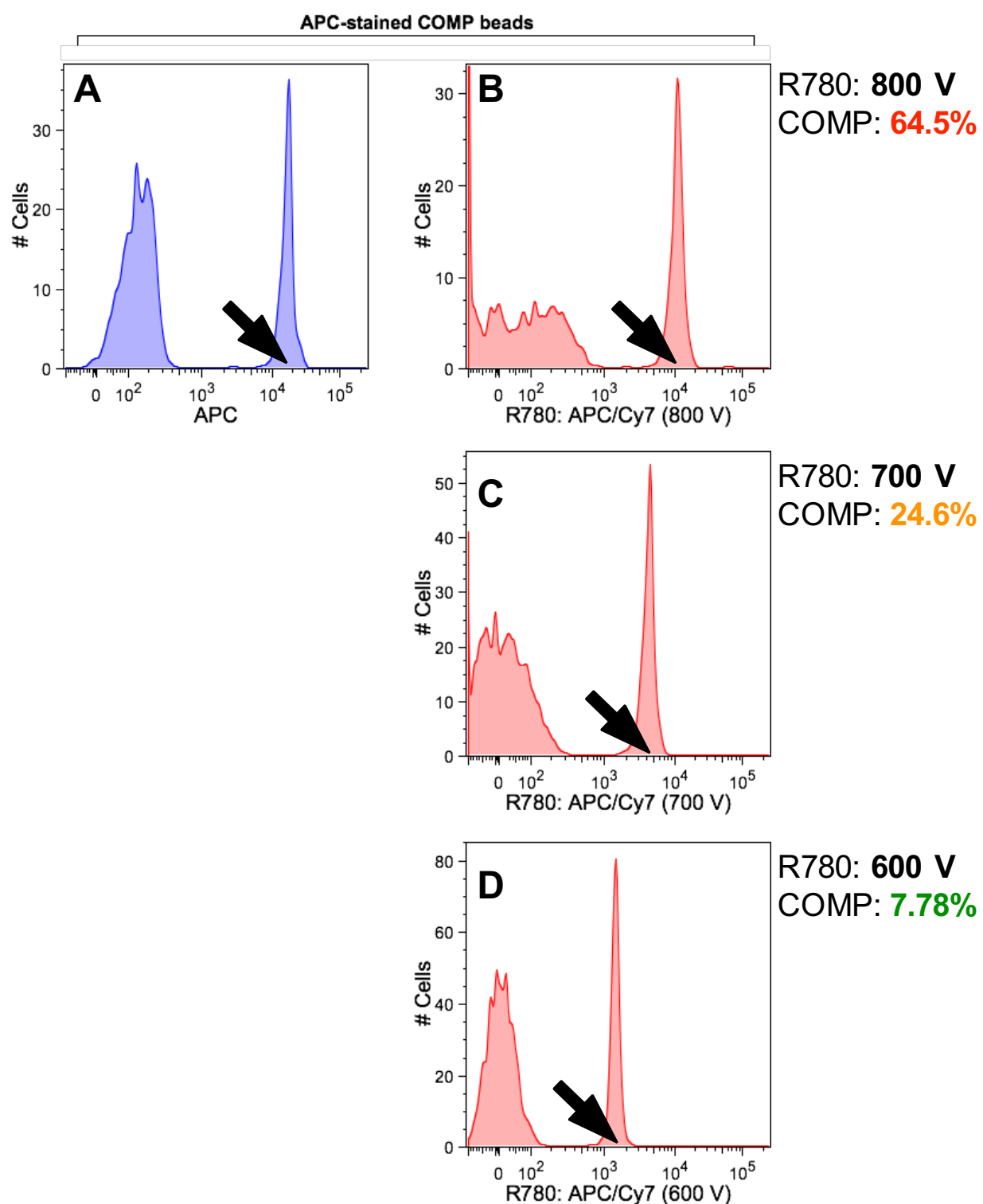
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Voltage adjustment to reduce spillover?

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

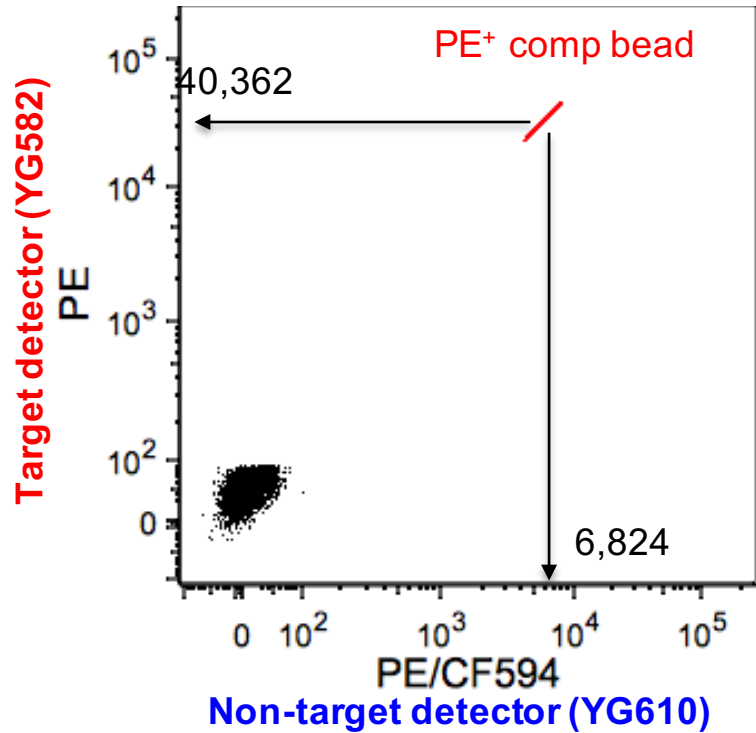


Case study

- 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)

Refresher: what is compensation?

Compensation is “correcting” fluorescence spillover



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

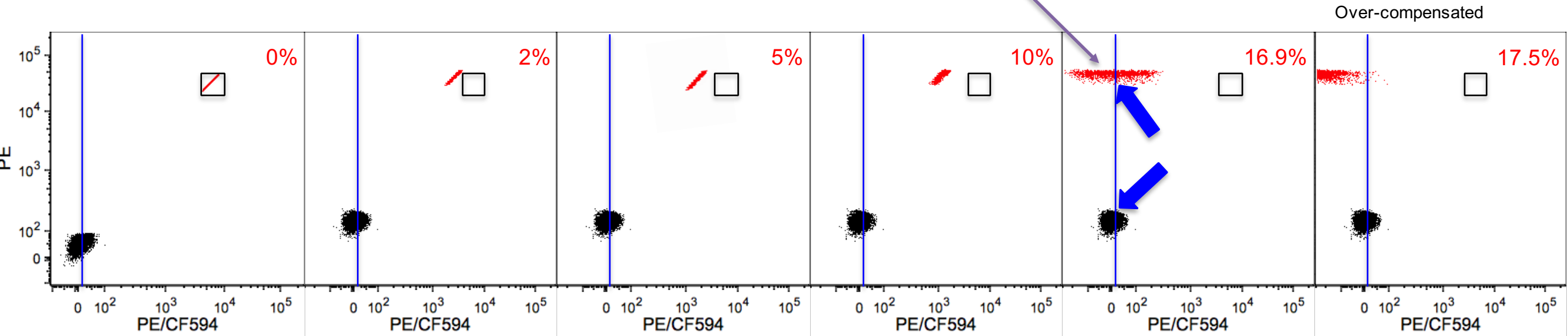
= 16.9%

This compensation value is dependent on voltages

$$\text{Comp}(\text{YG610}) = \text{Measure}(\text{YG610}) - \% \text{Measure}(\text{YG582})$$

Why is this spreading?

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



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 not bright enough to resolve A+B+ cells from A+B- cells.*

Figure 1: resolvable

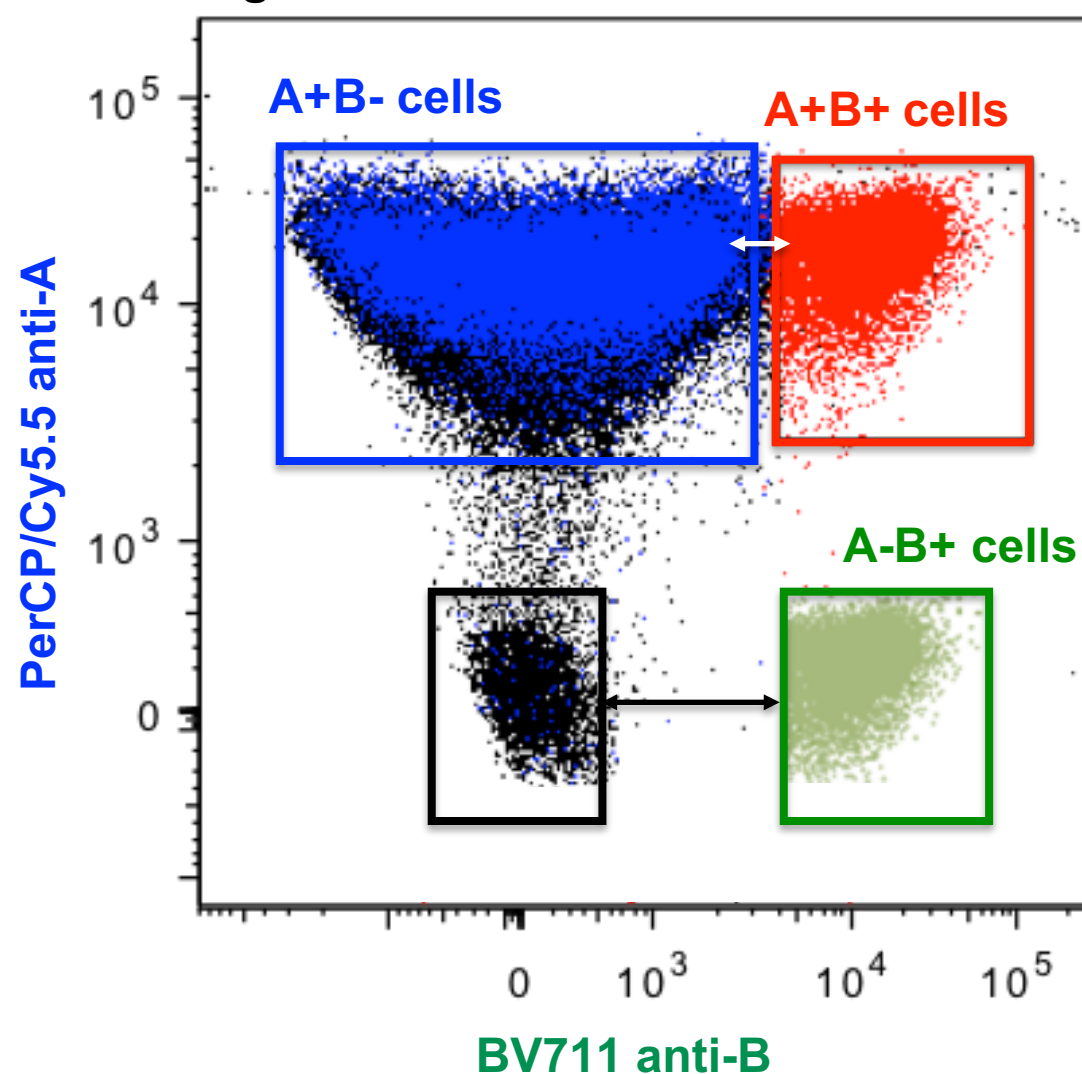
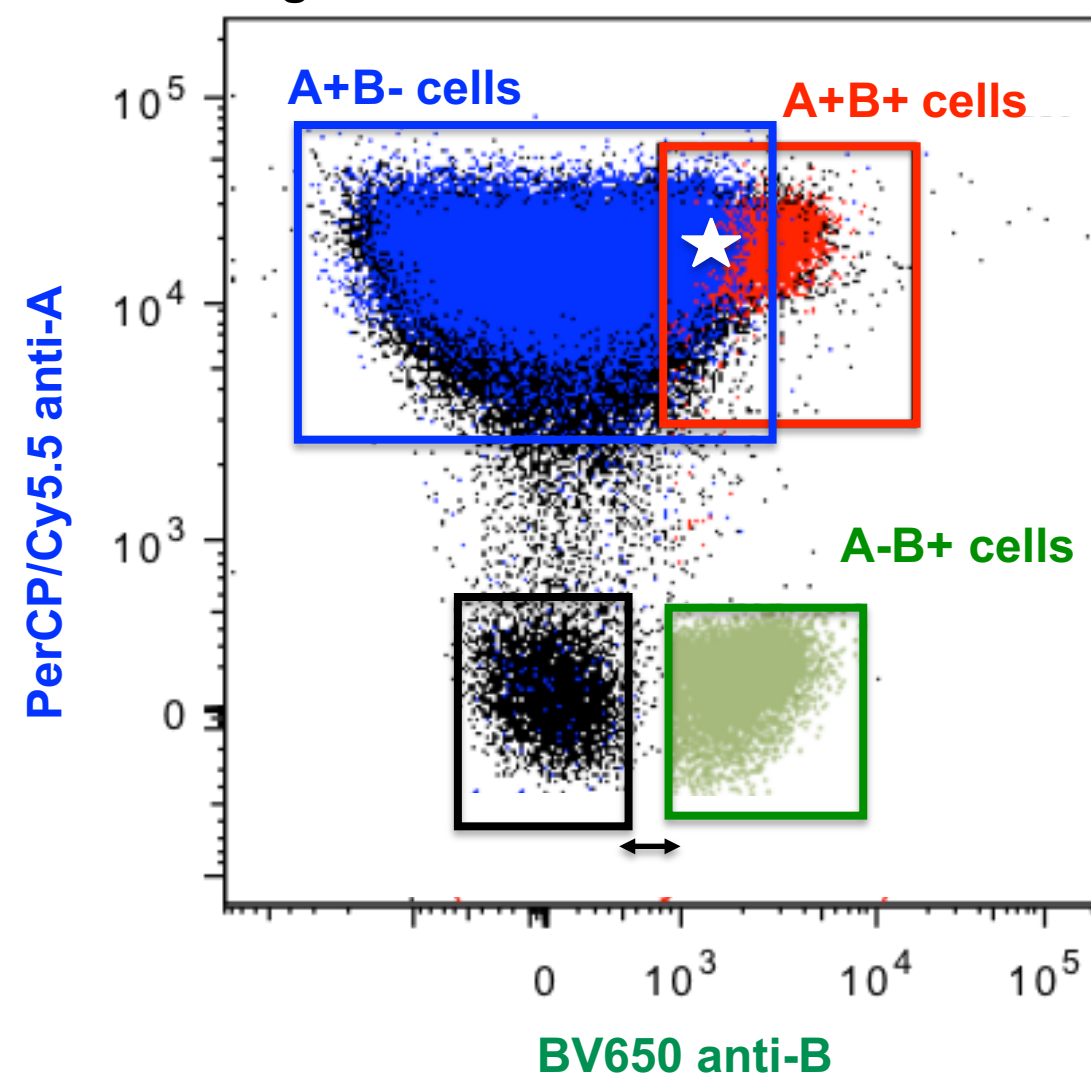


Figure 2: not resolvable



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

What is spreading error?

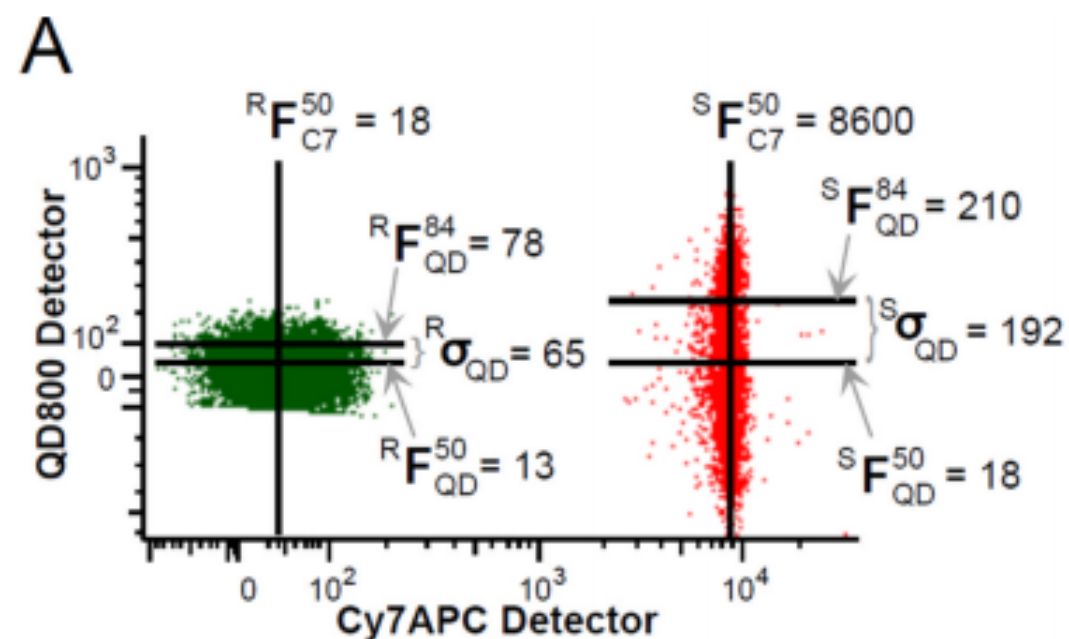
For more information on spreading error, see the following publication

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

Richard Nguyen¹, Stephen Perfetto¹, Yolanda D. Mahnke², Pratip Chattopadhyay², and Mario Roederer^{1,2,*}

¹Flow Cytometry Core, Vaccine Research Center, NIAID, NIH, Bethesda, MD

²ImmunoTechnology Section, Vaccine Research Center, NIAID, NIH, Bethesda, MD

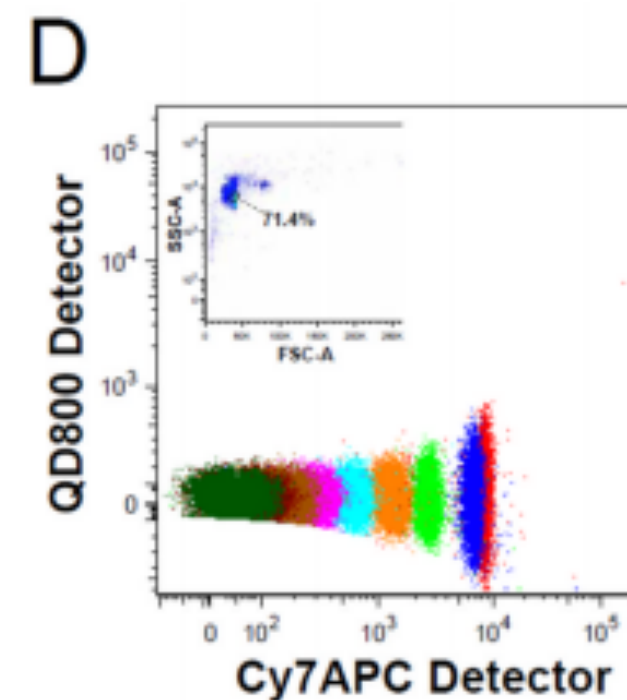


$$SS_C^P = \frac{\Delta \sigma_C}{\sqrt{\Delta F_P}}$$

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

$$\Delta F_P = S F_P^{50} - R F_P^{50}$$

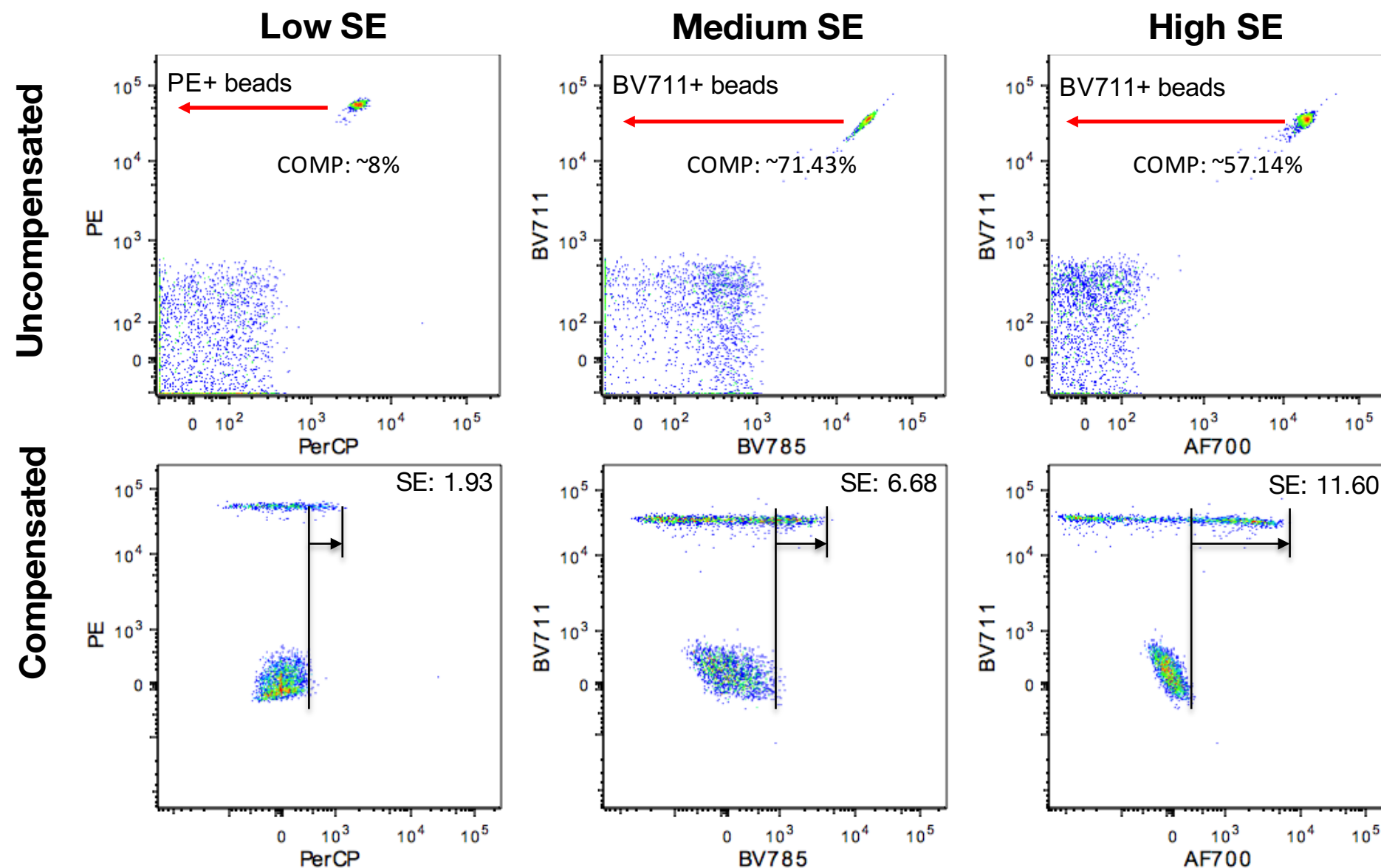
$$R \sigma_C = R F_C^{84} - R F_C^{50}$$



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:

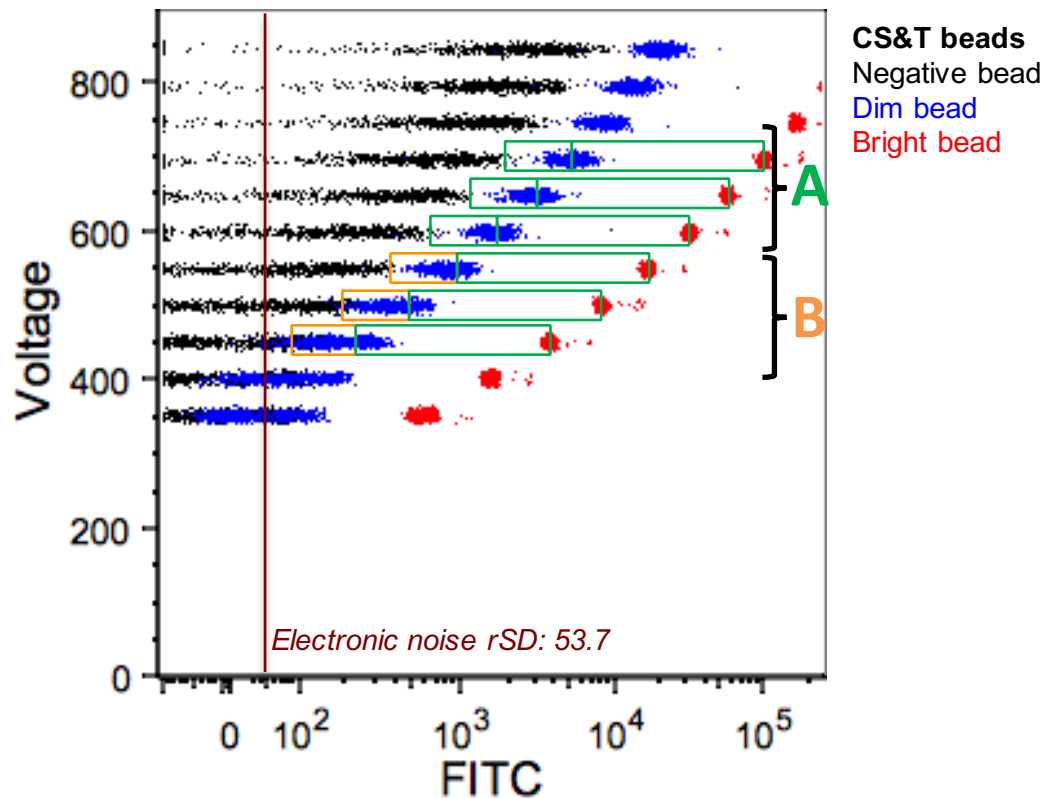
- BV711 into BV785 detector
 - Comp = **71.43%**
 - SE = **6.68**
- BV711 into AF700 detector
 - Comp = **57.14%**
 - SE = **11.60**

Despite the higher compensation value, the SE is lower

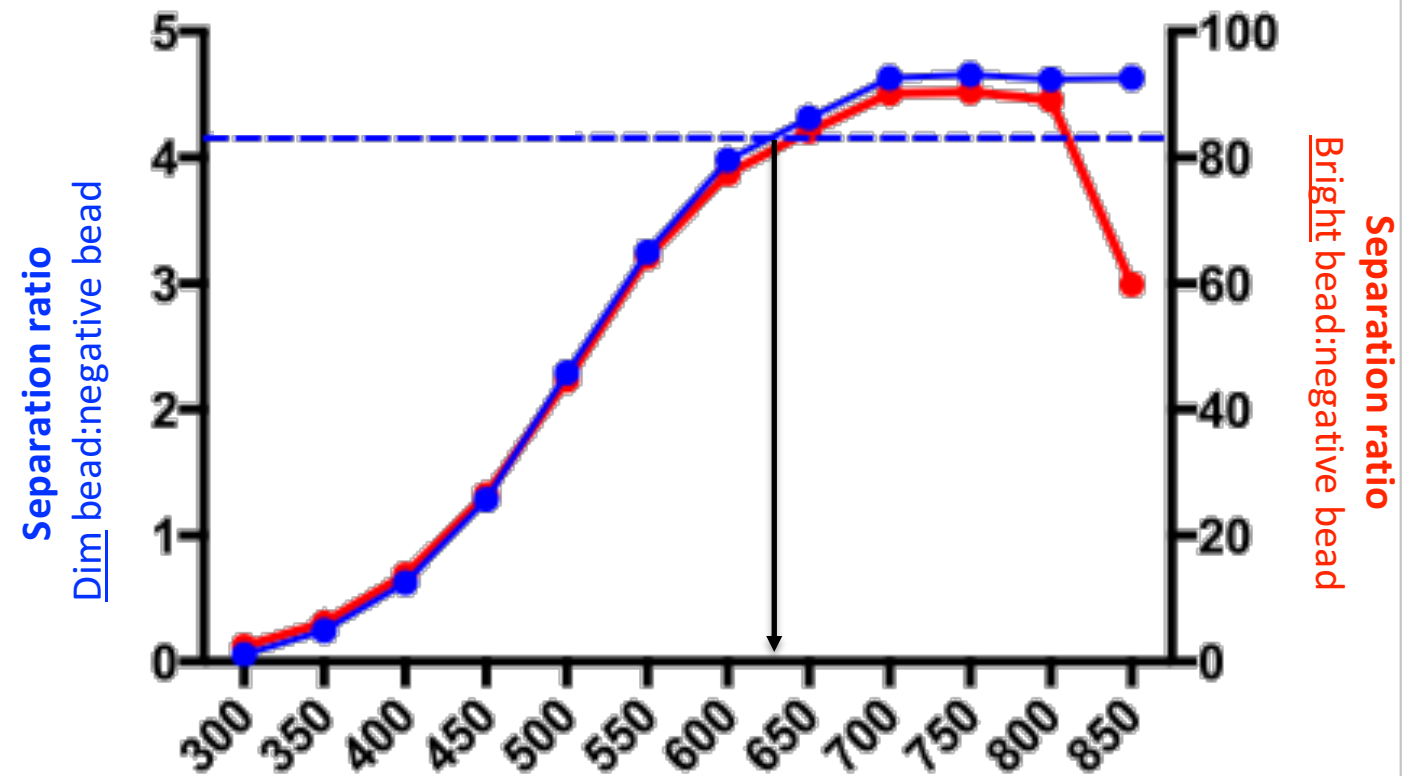
Detector voltages

What happens when we adjust the voltages for a given detector

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



FITC detector



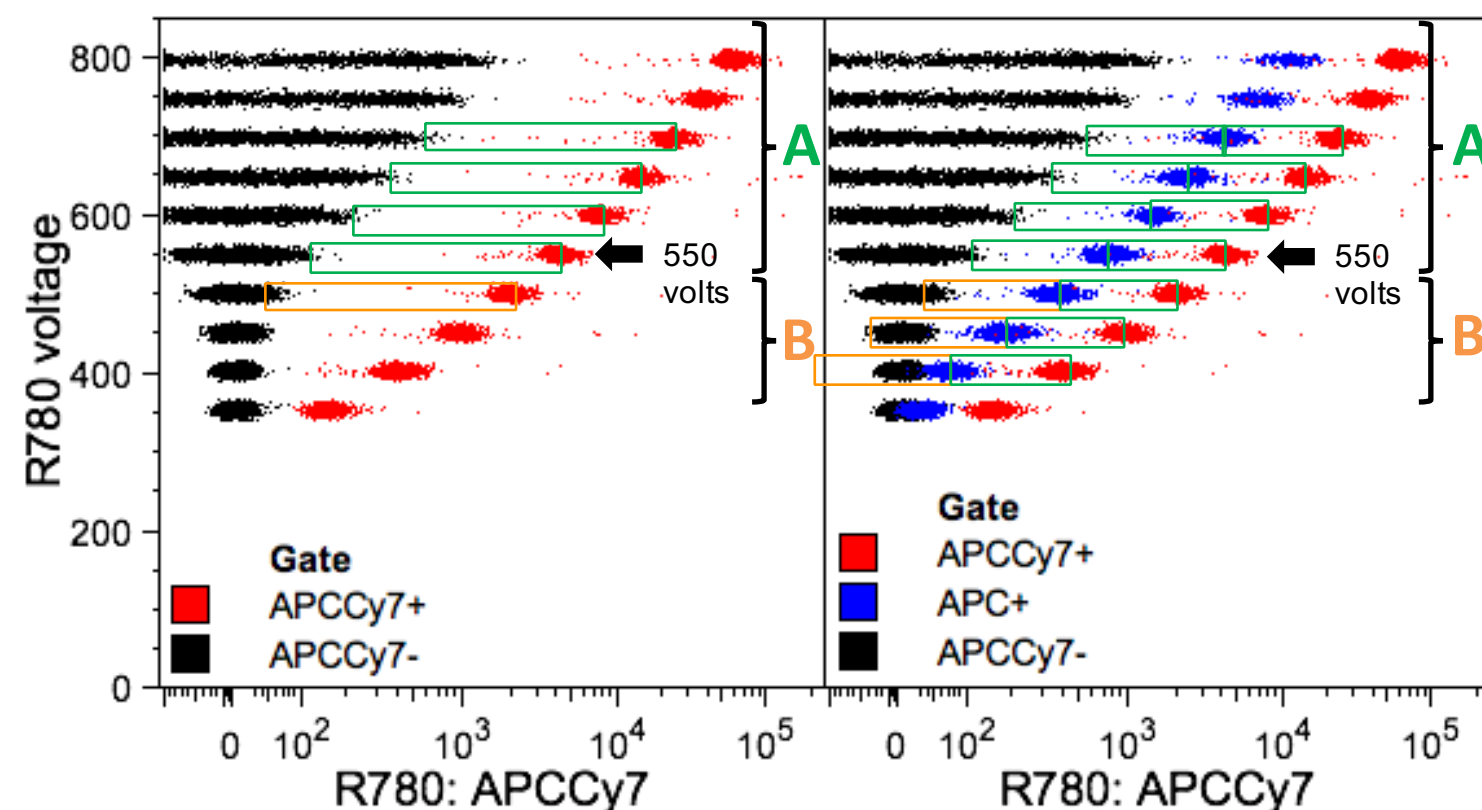
- Each detector uses a photomultiplier (PMT) that amplifies any signal picked up in that detector. The voltage applied dictates the level of amplification
- At a specific voltage (~600 volts for the FITC detector in this example), fluorescence signal is
 - a) detectable above *background*, and
 - b) 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

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) detectable 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

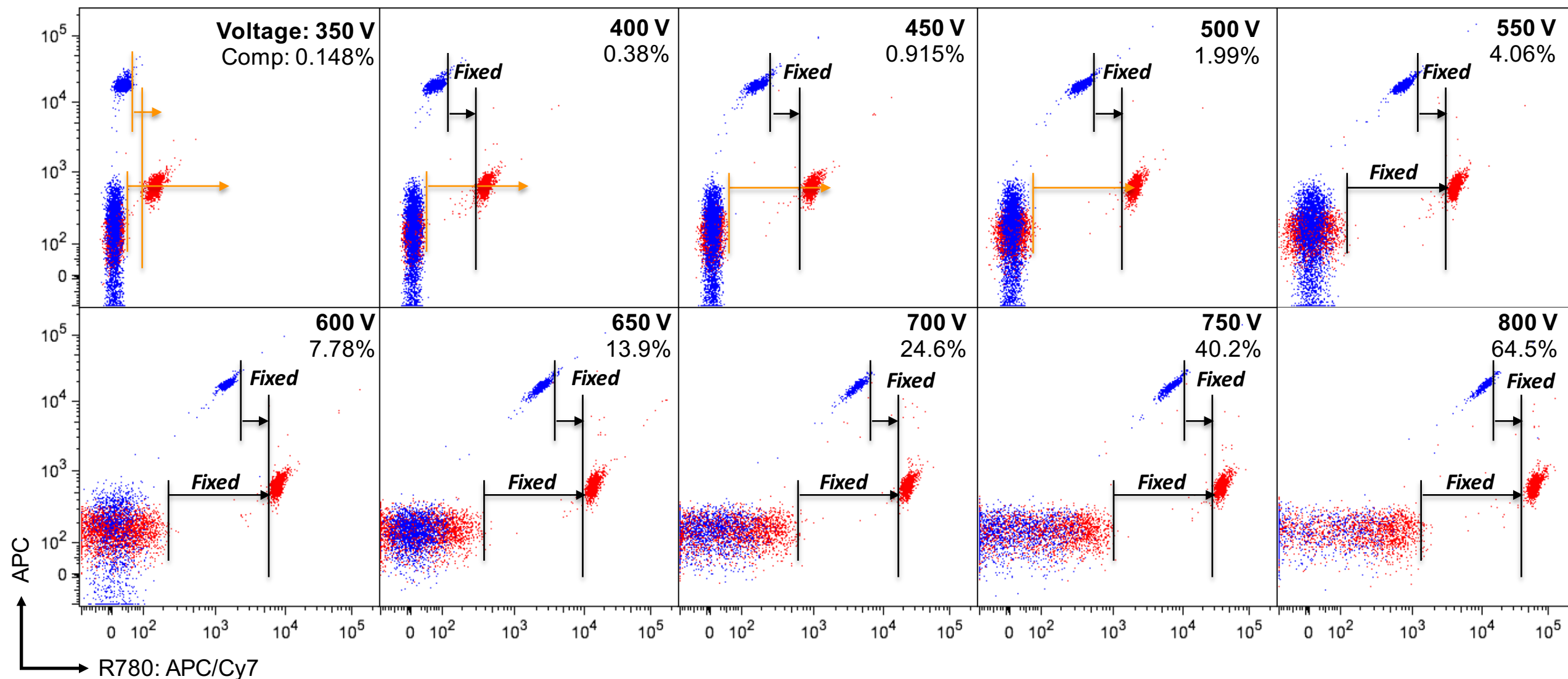
- The minimum voltage required for optimal separation of **signal from noise** is between **500-550 volts**
- Voltages **above** the minimum for separation (550 volts) do not increase the separation of signal (A)
- Voltages **below** the minimum for separation (550 volts) compromise the detection of 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

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.

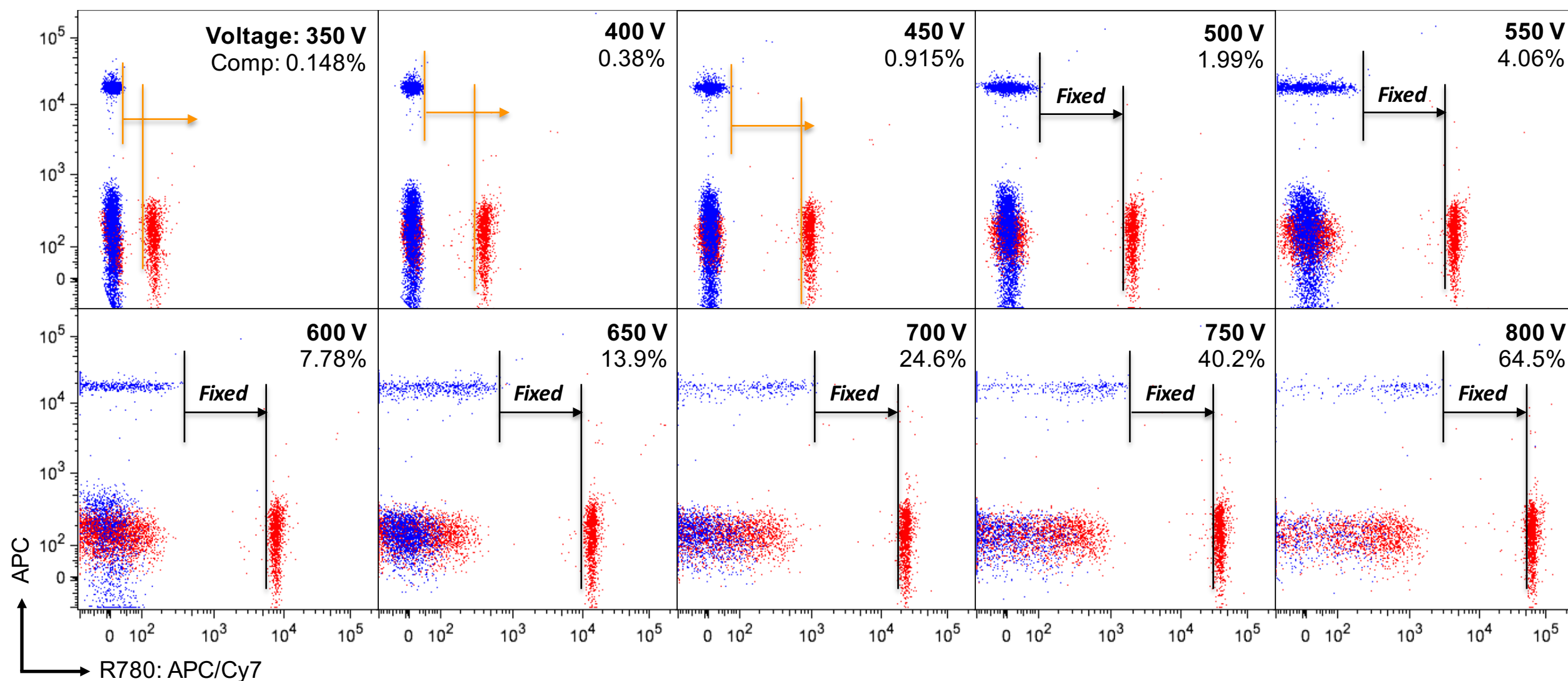


Consistent with the principles of our previous slide

- 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 350V)

APC/Cy7 and APC in the R780 detector

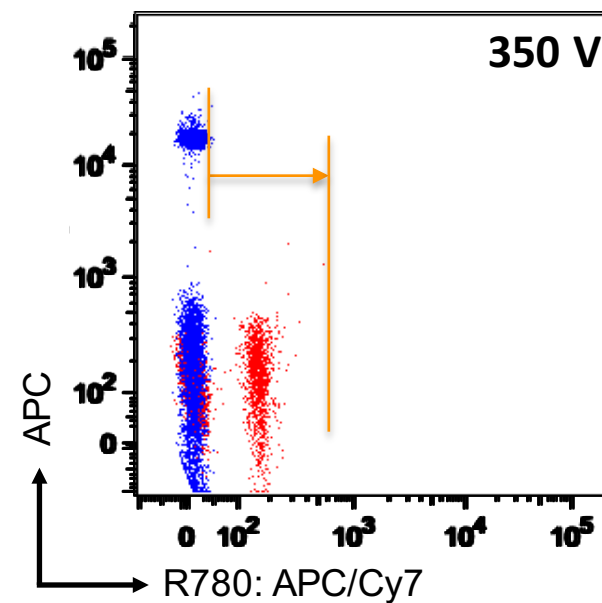
Looking at the **compensated** plots individually: above the 550 volt cut off the separation of the target fluorophore (APC/Cy7) from the spread of the compensated spillover fluorophore (APC) is **fixed**, even though the compensation value increases.



- 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 550 V

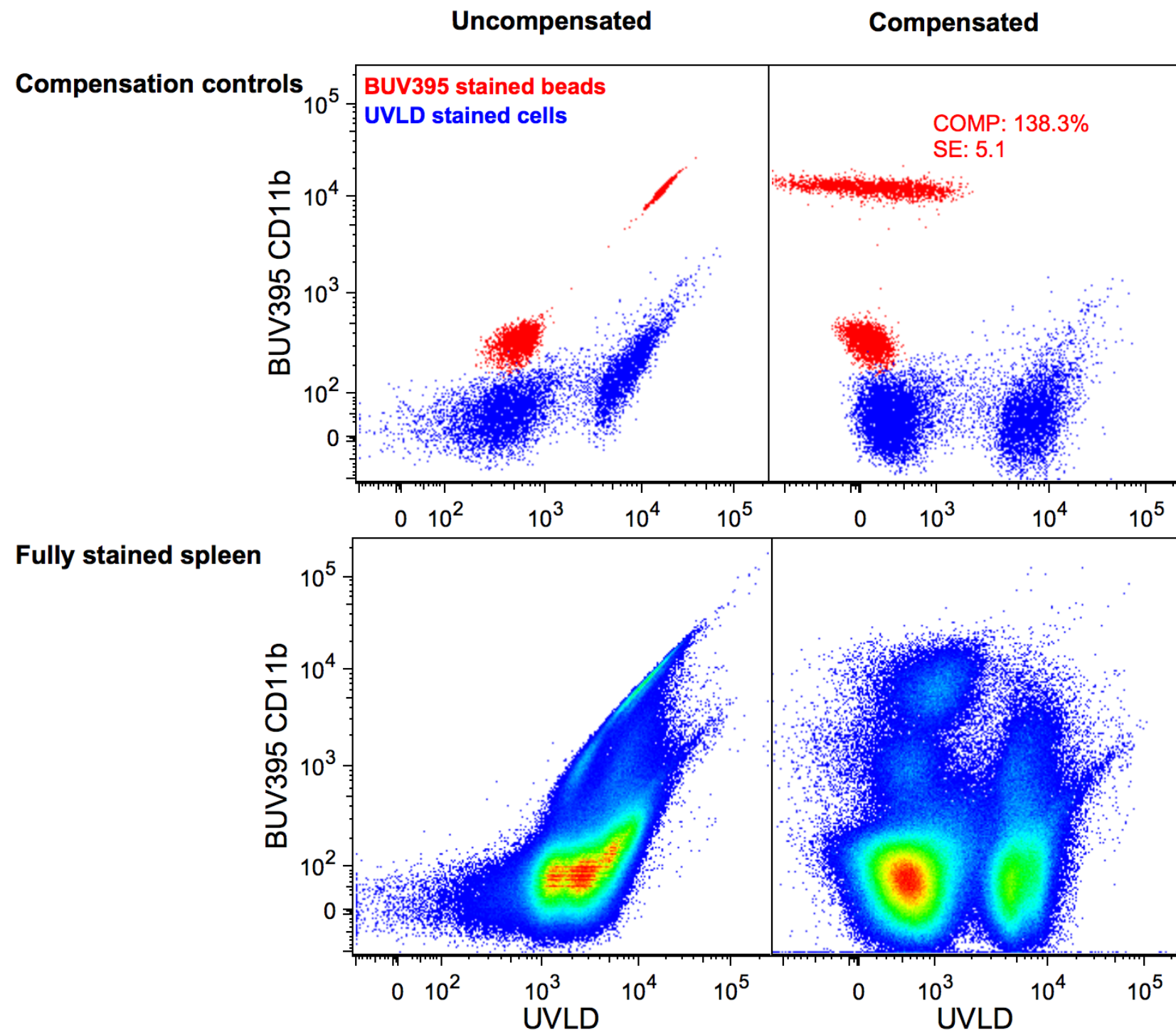
APC/Cy7 and APC in the R780 detector

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



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.



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_Stdndr_App_Setup_TechBulletin.pdf)
- VRC bead suite optimisation method (<http://www.nature.com/nprot/journal/v7/n12/abs/nprot.2012.126.html>)

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