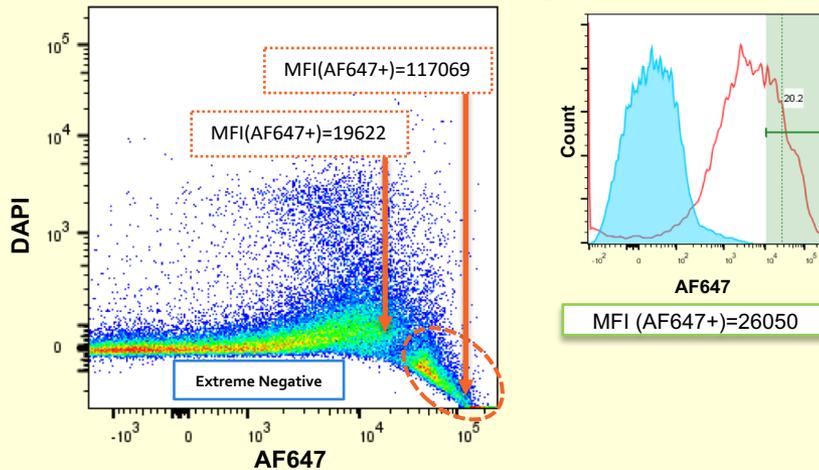


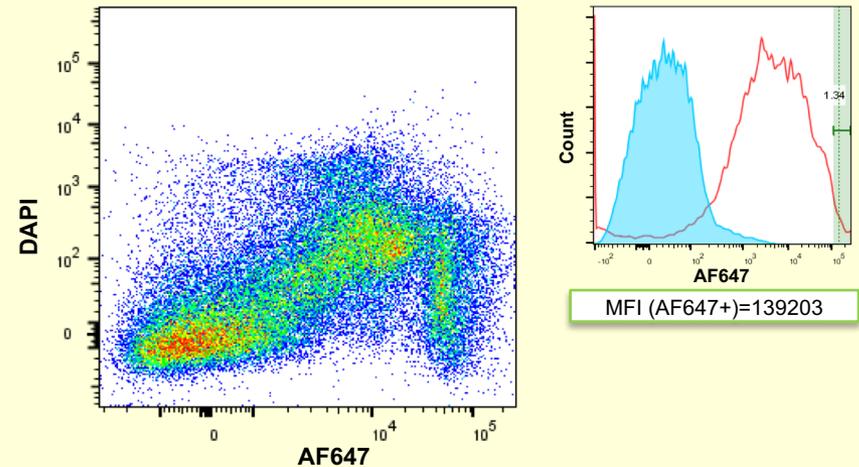
The use of the appropriate single stain controls is critical to successfully correct fluorescence spillover. **Gating** the positive and negative populations **correctly** to calculate unmixing or compensation **is crucial**. The single color controls need to have a **Median** Fluorescence Intensity (MFI) of equal intensity or higher than experimental sample. *Including a wider range of fluorescence in the positive gate will lower the overall MFI(+) value and it can lead to overcompensation or underestimation in unmixing.*

Extreme Negative Identified



- An extreme negative population is seen below the autofluorescence of the unstained population of DAPI as a result of overcompensation.
- All rules of compensation controls were thought to be obeyed.
- AF647 compensation control histogram on the right shows the **gate** for the **positive** population used to calculate compensation is including everything above autofluorescence of the **unstained**, giving an AF647 MFI value of **26050**.

Root Cause Found



- Adjusting the AF647+ **gate** to only include the brightest portion of population, the MFI of the positive is now **139203**.
- Once compensation was recalculated using this gating, the overcompensation is no longer seen, resulting in improved population representation.

Flow cytometry data must be evaluated to guarantee it shows the expected results. When extreme negatives are seen in flow cytometry data, troubleshooting needs to be done in order to determine the root cause so that it may be corrected. Here, it was found that the single color positive gate used to calculate compensation was including a wide range of fluorescence, resulting in a lower AF647 MFI and subsequent overcompensation.

By adjusting the positive gate of the same single color control to include the brightest AF647 population, the MFI of the positive population used to correct for spillover is now as bright or brighter than the experimental sample, allowing for a more accurate compensation matrix and improvement of data.