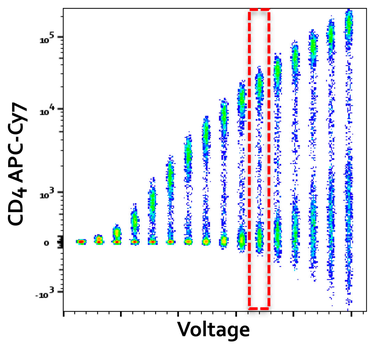
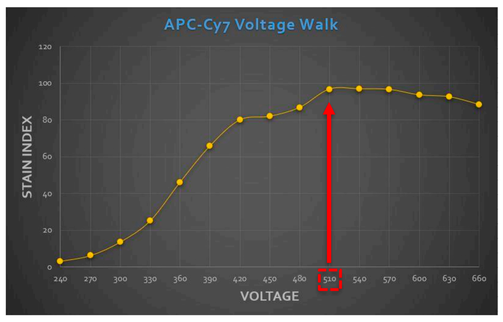
Flow Cytometry Platform CB UTechS

Flow Cytometry Technology

Optimizing Voltages & Gains

Setting PMT voltages or APD gains appropriately is a critical step for a successful flow cytometry experiment. If voltages/gains are set too low, it can result in loss of resolution of populations, especially for populations that have a very low level of expression. Alternatively, if set too high, populations may be off scale resulting in inaccurate statistics. Carrying out a **voltage/gain walk up** is one method to determine the **minimum PMT voltage or APD gain** that will provide **the best resolution** of populations in your experiment.





Lymphocytes were stained with CD4 APC-Cy7 and ran on a BD Fortessa. FSC and SSC settings remained constant while APC-Cy7 detector was increased in increments of 30V until signal was off scale. Stain Index (SI) was calculated to determine optimal voltage.

At a voltage of 510V, maximum SI was seen. Increasing the voltage does not benefit the data, but reduction of voltage can result in loss of population resolution.

Helpful Hints

- Optimized settings are instrument specific. Cross-instrument standardization will need to be done in order to have comparable settings across multiple instruments.
- Alternative methods to determine optimal starting voltages/gains are available. i.e. "Peak 2 method"
- Voltage walks help ensure that the autofluorescence is above electronic noise for your experimental samples. When working with cell types with differing autofluorescence, experiment specific voltage walks should be done.
- *Suggested Reading:* Mair, F. and Tyznik, A. 2019 doi: 10.1007/978-1-4939-9650-6_1

Stain Index*

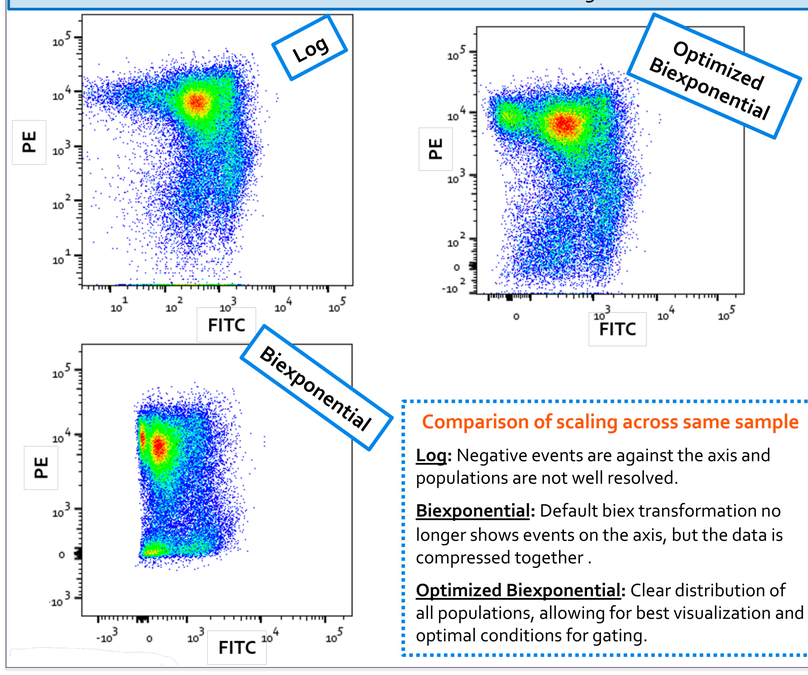
$$\frac{MFI_{pos} - MFI_{neg}}{2 * rSD_{neg}}$$

*Calculated using the Stain Index Plugin in FlowJo v.10.7.2

When setting up your flow cytometry experiment, consideration should be taken at the instrument to optimizing the acquisition settings. **Through a voltage walk up and data analysis, the voltage or gain at which the optimal separation of populations occurs can be determined.** Settings will differ across experiments when working with cells that have different levels of autofluorescence, requiring a separate optimization.

Scaling Matters

In flow cytometry, the most common data scaling methods are **linear** and **logarithmic.** When presenting data where there is a wide range of fluorescent signal, data is typically displayed in log so all events can be visualized. Often, when analyzing data in log scale, there can be a large portion of events stacked against the axis at zero. Through **biexponential log transformation,** we can visualize the distribution of these negative events around zero in a better way.



Comparison of scaling across same sample

Log: Negative events are against the axis and populations are not well resolved.

Biexponential: Default biex transformation no longer shows events on the axis, but the data is compressed together .

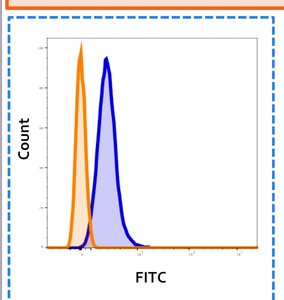
Optimized Biexponential: Clear distribution of all populations, allowing for best visualization and optimal conditions for gating.

Helpful Hints

- Scaling transformation does not alter the data, it simply changes the way the data is visualized.
- Distribution of events around or below zero can be a result of baseline restoration or compensation.
- Biexponential scaling needs to be optimized for better visualization.
- Incorrect scaling of data can hide poorly compensated or unmixed data.
- Scaling adjustments made in acquisition software do not carry over into analysis software.
- Other methods of log transformation can include Hyperlog and ArcSinh.

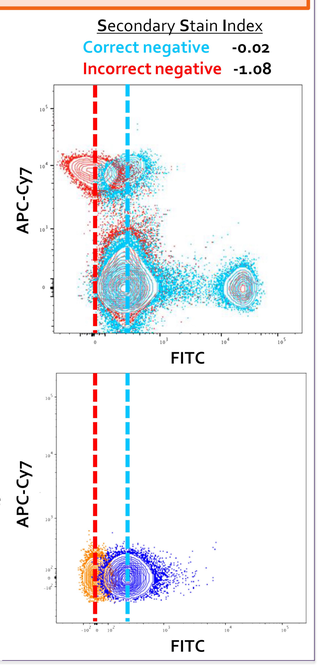
Autofluorescence and Compensation

When carrying out **compensation** or **unmixing** in multicolor Flow Cytometry experiments, the use of the appropriate controls is critical. Both beads and cells are often used in these corrections for fluorescence spillover, often times in combination. It is necessary in these cases to have the appropriate **autofluorescence** referenced for each single color for acquisition and analysis softwares to accurately correct for spillover.



Overlay of **unstained cells** and **unstained beads** show a clear difference in autofluoresence.

Example to the right depicts an experiment where FITC single color was beads and the APC-Cy7 single color was cells. Compensation calculated with an incorrect **universal negative** of unstained cells for all controls is in red. In blue, the correct **sample specific autofluorescence** was used for each single color. **SSI** was used to determine success of compensation results for experimental sample.



Helpful Hints

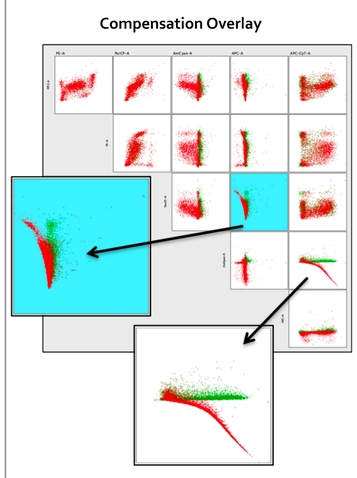
- Fixation/permeabilization can have an impact on autofluorescence. Control cells and beads should be treated the same as your experimental samples to account for this. (*Exception*: Brilliant or Super Bright staining buffers should not be used on compensation beads.)
- Different cells can have different autofluorescence signatures. The same considerations should be taken when using multiple cell types as single color controls.

Important Note

The calculation itself was correct in both cases. As seen here, the calculation correctly matched the spillover channel MFI to the negative control that it was referenced to.

« Recycling » Compensation

One of the most critical components to a successful flow cytometry experiment is the use of the **appropriate controls**. Single color controls allow for us to compensate or unmix data in order to correct for **spectral overlap**, enabling us to confidently identify our populations of interest. These controls should be freshly made and run at each experiment to account for any changes in the instrument or reagents*. **Reusing old compensation matrices from old experiments will lead to incorrect data**.



Compensation Overlay

- Overlay of **Recycled** and **Same Day** compensation show clear discrepancies. If not corrected, MFI values, population percentages and data interpretation would be incorrect.

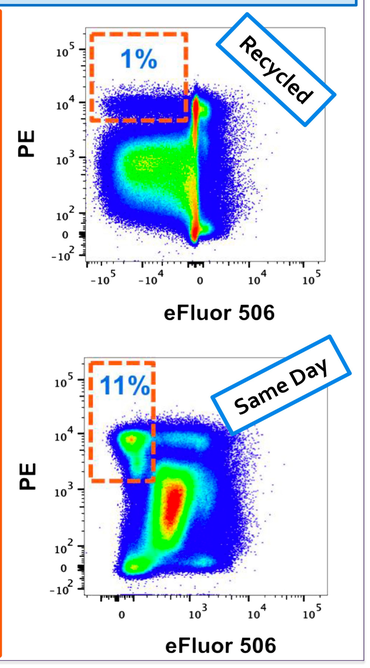
Recycled Compensation

- Extreme negatives seen. This is typically indicative of overcompensation.
- Poor resolution of populations.
- Gated population shows 1%.

Same Day Compensation

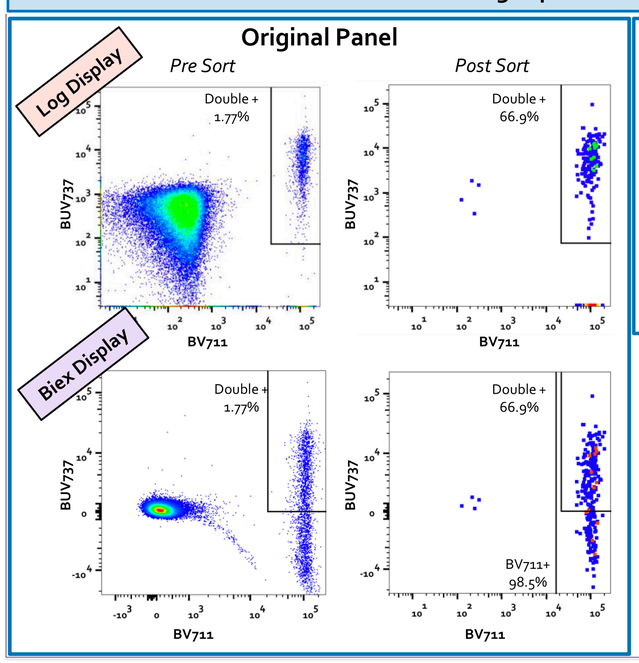
- No extreme negatives are noted.
- Resolution of populations shows significant improvement.
- Gated population now shows 11%.

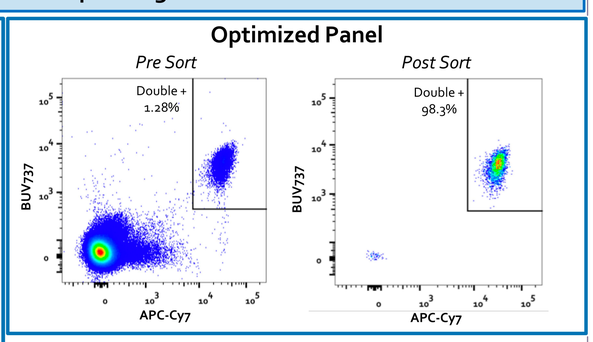
**Instrument drift*:* laser drift or replacement, flow cell degradation, changes in optical filters, etc.
**Reagent drift*:* Tandem dye degradation, experimental signal intensity change (i.e. expression level increases), etc.



Impacts of Spread

When designing and executing multiparameter flow cytometry experiments, instrument **configuration** and **panel design** both play a very important role. Just because a cytometer can detect fluorochromes does not mean that it will work with your experiment. Care must be taken when designing your panel to **avoid** fluorochrome combinations that introduce **high spread** with **coexpressing markers**.





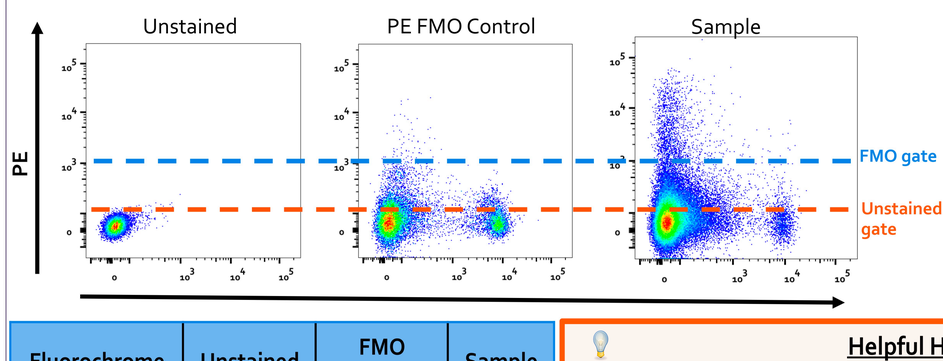
Experiment Comparison

Original: Double positive population of BV711 and BUV737 needed to be isolated for sorting. A gate was set on what was believed to be this population. Post sort purity revealed **purity of 66.9%**. During troubleshooting, samples were visualized in biexponential display and it was identified that there was **high spread** of BV711 into BUV737, resulting in no clear BUV737+ population. Sorter proved to be operational as purity of 98.5% was observed when gating on BV711+ population.

Optimized: Panel was adjusted with to minimize impacts of spread. New combination of BUV737 and APC-Cy7 was used. Double positive population resolved well and post sort check showed **purity of 98.3%**

FMO Controls

In multicolor Flow Cytometry experiments, spillover spreading is seen across detectors. This spread is fluorochrome dependedent and can impact resolution, which cannot be improved by changing the voltages or gains. In multicolor panels where spreading is seen, **F**luorescence **M**inus **O**ne (FMO) controls become essential to set appropriate gates, particularly when the expression is dim or a smear



All illustrated to the left, establishing the cut-off between positive and negative populations using the unstained control is not as accurate as using the FMO.

The FMO control allows us to account for the additive effect of spreading from multiple fluorochromes into the detector of interest, resulting in a more precise gating of positivity.

Fluorochrome	Unstained	FMO Control	Sample
APC	-	✔	✔
APC-Cy7	-	✔	✔
FITC	-	✔	✔
PE	-	-	✔
PE-Cy7	-	✔	✔
PerCP-Cy5.5	-	✔	✔

Helpful Hints

- FMO controls are the **same cells** as in the experiment **stained with all the fluorochromes in the panel except one**.
- Beads cannot be used for FMO controls.
- FMO controls **do not assess non-specific binding** of antibodies Antibody titration is still necessary.
- The use of isotype controls to define positivity is not correct. FMOs and other appropriate controls are recommended.
- Spreading will differ depending on fluorochrome combinations. Panels should be designed accordingly to reduce spread.

Reference:

Flow Post-its Memorial Sloan Kettering Cancer Center

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