Evaluation of Multiple Techniques for PMT Optimization

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ABSTRACT
To obtain high quality fluorescent flow cytometry data, a well-optimized instrument is required. The most common type of detector is the photomultiplier tube (PMT), which amplifies signal from emitted light photons by applying a voltage to the PMT. As the voltage is increased the fluorescent signal is increasingly separated from background, providing greater resolution of the positive signal. However, at a certain voltage, the increasing separation of fluorescent signal from background will plateau and the separation of fluorescent signal from background will remain constant. This is called the minimum voltage required (MVR); an ideal minimal voltage will amplify dim signal above background, but is not so high that the fluorescent signal exceeds the upper range of PMT linearity. In most cases, adjusting voltages above the minimum voltage required does not increase the separation, while voltages below this voltage compromise detection of dim fluorescent signal. A variety of methods have been proposed to optimize the PMT voltage (PMTv) with most using a technique called the voltage walk, or voltolysis. This study compares a variety of techniques and calculations, using different types of hard-dyed beads that are detected in all detectors but do not include aqueous fluorophores used typically for flow cytometry applications; antibody-capture beads labeled with fluorophores specific to a detector; cells labeled with fluorophores specific to a detector; evaluation of the Electronic Noise (EN) of the PMT, and methods combining these approaches.

GENERAL MATERIALS AND METHODS


Antibodies:

Other Reagents: Attune™ Focusing Fluid (Thermo Fisher Scientific P/N 4449791) Gibco™ PBS pH 7.4 (Thermo Fisher Scientific P/N 10010-023)

Instrumentation: Flow Cytometers (Thermo Fisher Scientific) each configuration has 14 fluorescent detectors:
-Blue/Red/yellow/violet configuration (P/N A243585)
-Blue/Red/yellow/violet 6 configuration (P/N A29004)

Sample Acquisition:
The samples were acquired on the Attune NxT Flow Cytometer (BRFY and BRVY configurations) at a flow rate of 200 μL/min using a FSC threshold. A gate was placed around the main bead or cell population, and a stop criteria of 10,000 gated events was used. Area, Height, and Width parameters were collected for all data points. Samples were recorded over a range of voltage settings, in 1 mV and from 50 to 650 mV, recorded at 50 mV increments for each detector.

Sample Preparation:
Hard-dyed beads were vortexed briefly before adding 2-3 drops to 1 mL Attune Focusing Fluid or PBS.
CYTO-TROL Control Cells were reconstituted according to kit instructions; 100 μL reconstituted CYTO-TROL Control Cells were diluted in 1 mL with PBS, or 100 μL reconstituted CYTO-TROL Control Cells were labeled with 5 μL, antibody conjugate, incubated for 15 minutes at room temperature protected from light and then resuspended in 0.5 mL PBS. Then one drop of negative beads (component B) was added. Separate samples of the labeled capture beads and the negative beads were also prepared.
UltraComp eBeads were briefly vortexed, 1 drop of UltraComp eBeads (containing both capture and negative beads together) was dispensed, 5 μL antibody conjugate was added and mixed well. Beads were incubated for 15 minutes at room temperature protected from light, then resuspended in 0.5 mL PBS.

RESULTS I

Calculation of Standard Deviation of the Electronic Noise (Sd0)

The data was exported to CSIV and analyzed in Microsoft Excel to determine the minimum voltage required as discussed in the subsequent sections specific to each method.

RESULTS II

Comparison of Staining Index (SI), Alternative Staining Index (Alt SI), and Votolysis (VI) using beads, cells, and combination of beads and cells with detector-specific fluorophores.

RESULTS III

Linearity of PMT Response

A variety of methods were evaluated to determine the Minimum Voltage Requirement on the Attune NxT Flow Cytometer. A summary of the results is displayed below for the B1 (FITC) detector:

SAMPLE
<table>
<thead>
<tr>
<th>%CV-TOTL</th>
<th>%CV-Dim</th>
<th>Sd0 D650</th>
<th>Sd0 D450</th>
<th>IL + VI</th>
<th>Alt SI</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYTO-TROL Lymphocytes (neg+pos)</td>
<td>350</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>AbC Beads (neg+pos)</td>
<td>350</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>UltraComp eBeads (neg+pos)</td>
<td>NA</td>
<td>410</td>
<td>445</td>
<td>445</td>
<td>445</td>
<td>445</td>
</tr>
<tr>
<td>CYTO-TROL (neg + AbC Bead)</td>
<td>350</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>CYTO-TROL (neg + Peak 3 2-pos)</td>
<td>350</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>CYTO-TROL (neg + Peak 3 1-pos)</td>
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<td>450</td>
<td>450</td>
<td>450</td>
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</tr>
<tr>
<td>Attune Performance Tracking beads</td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>

The Staining Index, Alternative Staining Index, and Voltolysis Index can all be equally used to determine the MVR on the Attune NxT Flow Cytometer. All of these methods yield approximately the same MVR (400-450 mV for BL1) regardless of the particles used for the assessment (similar results were obtained for each of the 14 fluorescence channels). The %CV infection method should not be used as this method underestimates the MVR. The area measurement must be used for the Sd0 methods. The particles used for the 1×Sd0 and 2×Sd0 methods were found to vary depending on the particle, making this method less universal.

The SL, Alt SI, and VI methods provide a working range of voltages where the signal is optimal and in some cases provide a clear upper limit in addition to the MVR. Robust statistics were compared to standard statistics (data not shown) for all methods and were found to be the preferred statistic in all cases. Further work is required to assess the resulting Spillover Spreading Matrix and the ability to resolve dim targets when using MVRs attained using these methods.

REFERENCES

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