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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 photo-multiplier 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 voltration. 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 specific fluorophores used for typical experimentation; 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

Cells and Beads:
Attune™ Performance Tracking Beads, Thermo Fisher Scientific P/N 4449754
Ultra Rainbow Calibration Particles Kit, Spherotech P/N URCP-38-2K
Rainbow Calibration Particles - Peak 5, Spherotech P/N RCP-30-5A-5
Rainbow Calibration Particles - Peak 2, Spherotech P/N RCP-30-5A-2
Rainbow Calibration Particles - 8 Peaks, Spherotech P/N RCP-30-5A
ABC™ Total Antibody Compensation Bead Kit, Thermo Fisher Scientific A10513
UltraComp eBeads™ Compensation Beads, Thermo Fisher Scientific 01-2222-42
CYTO-TROL™ Control Cells, Beckman Coulter P/N 6604248

Antibodies:
CD4 (clone SK3) Super Bright 436, Thermo Fisher Scientific P/N 62-0047-41
CD4 (clone RPAT4) eFluor 506, Thermo Fisher Scientific P/N 69-0049-41
CD4 (clone SK3) Super Bright 600. Thermo Fisher Scientific P/N 63-0047-41
CD4 (clone SK3) Super Bright 645, Thermo Fisher Scientific P/N 64-0047-41
CD4 (clone SK3) Super Bright 702, Thermo Fisher Scientific P/N 67-0049-41
CD3 (clone UCHT1) Super Bright 780, Thermo Fisher Scientific P/N 780038-41
CD4 (clone SK3) FITC, Thermo Fisher Scientific P/N 11-0047-41
CD4 (clone RPAT4) PerCP-Cyanine5.5, Thermo Fisher Scientific P/N 45004941
CD4 (clone SK3) PE, Thermo Fisher Scientific P/N 12-0047-41
CD4 (clone S3.5) PE-Texas Red, Thermo Fisher Scientific P/N MHCD0417
CD4 (clone SK3) PE-Cyanine5.5, Thermo Fisher Scientific P/N 35-0047-41
CD4 (clone SK3) PE-Cyanine7, Thermo Fisher Scientific P/N 25-0047-41
CD4 (clone SK3) APC, Thermo Fisher Scientific P/N 17-0047-41
CD4 (clone RPAT4) Alexa Fluor™ 700, Thermo Fisher Scientific P/N 560049-41
CD4 (clone SK3) APC-eFluor 780, Thermo Fisher Scientific P/N 47-0047-41

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

Instrumentation
Attune™ NxT Flow Cytometers (Thermo Fisher Scientific)-each configuration has 14 fluorescent detectors:
- •Blue/red/yellow/violet configuration (P/N A24858)
- •Blue/red/yellow violet 6 configuration (P/N A29004)

Sample Acquisition
The samples were acquired on the Attune NxT Flow Cytometer (BRVY and BRV6Y 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, at 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 to 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 diluted with PBS to 1 mL.
- AbC Total Compensation Beads were briefly vortexed; 1 drop of capture beads (component A) and 5 µL antibody conjugate were mixed, 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.

GENERAL MATERIALS AND METHODS

Data Analysis
Data analysis was conducted using the Attune NxT software (v2.6). Gates were placed on populations of interest in detector specific histograms gated on the main population for both Area and Height measurements, and the following statistics for each gate were collected: Mean, Median, Standard Deviation (SD), robust Standard Deviation (rSD), Coefficient of Variation (CV), robust Coefficient of Variation (rCV).

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

RESULTS I

Calculation of Standard Deviation of the Electronic Noise (SD_{EN})

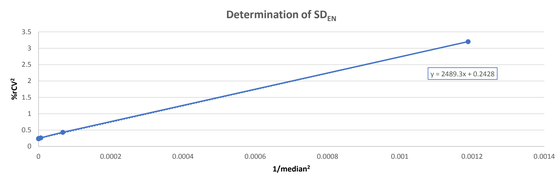


Figure 1. A dim/negative particle or cell was acquired over a voltage range and the median and rCV for the beads or cells were measured at each voltage. The slope of the line generated by plotting rCV^2 versus $1/median^2$ is the variance of the electronic noise. Standard deviation is the square root of the variance. On the Attune NxT instrument the SD_{EN} can be approximated by blocking the light at any detector and measuring the rSD of the signal in that detector (data not shown). In this example, the SD_{EN} was calculated to be approximately 50.

Determination of MVR based on SD_{EN} Using Unlabeled Cells, Unlabeled Compensation Beads, and Hard-Dyed Beads

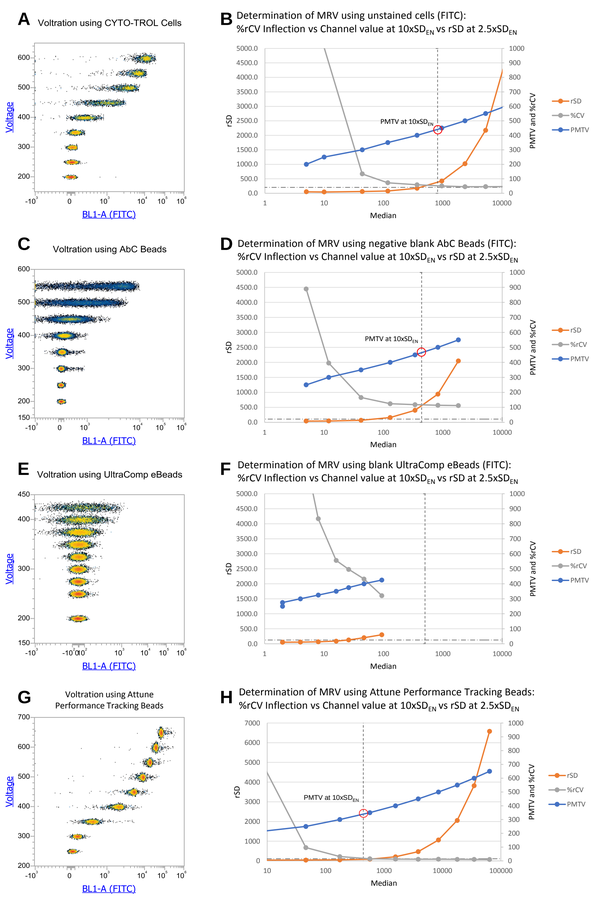
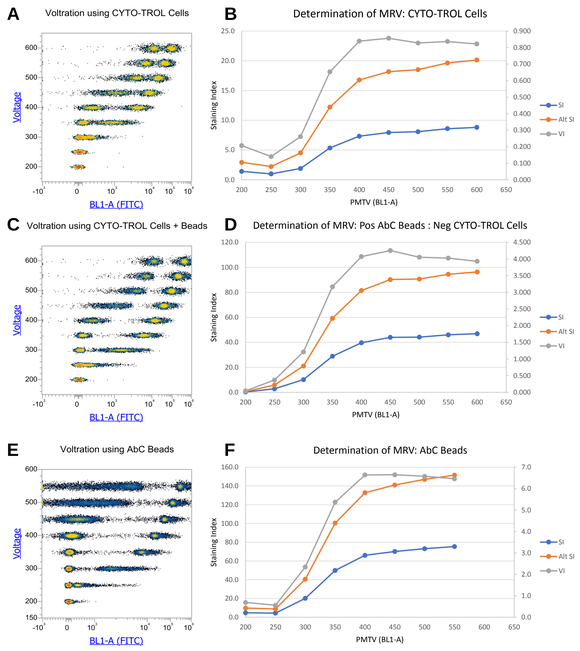


Figure 2. Samples were acquired at multiple voltages and data files were concatenated (**A,C,E,G**). The minimum voltage required (MVR) was assessed by plotting rSD, %rCV, and PMTV versus the median fluorescence signal for unlabeled CYTO-TROL cells **(A,B),** the negative AbC Total Compensation Beads (**C,D**), unlabeled UltraComp eBeads (**E,F**), and the dim bead in the Attune Performance Tracking beads (**G,H**). The minimum required voltage was determined using three methods: the rCV inflection point method, the voltage required to attain an MFI equal to $10xSD_{EN}$, and the voltage needed to set a population's rSD to be at least $2.5xSD_{EN}$. Unlabeled CYTO-TROL cells (**A**,**B**) and the negative AbC Total Compensation Beads (**C,D**) have similar results where the MRV was found to be 350 mV based on the %rCV inflection method, around 400 for the $2.5xSD_{EN}$, and around 450 mV for the $10xSD_{EN}$ method. The SD_{EN} methods cannot be used with the negative UltraComp eBeads (**E,F**) as the fluorescence of the bead could not be resolved from the SD_{EN}. The dim bead in the Attune Performance Tracking Bead (**G,H**) underestimates the MVR based on the $10xSD_{EN}$ and $2.5xSD_{EN}$ (350 mV) and %rCV inflection point method (300 mV).

RESULTS II

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



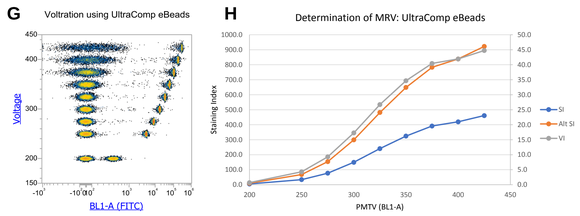


Figure 3. Samples were acquired at multiple voltages and data files were concatenated and plotted together (**A,C,E,G**). The minimum voltage required (MVR) was assessed by calculating the staining index, alternative staining index, and voltration index and plotting the results versus the median fluorescence signal for stained CYTO-TROL cells **(A,B),** stained AbC Total Compensation Beads with negative CYTO-TROL cells (**C,D**), stained AbC Total Compensation Beads with the negative AbC bead (**E,F**), stained UltraComp eBeads (**G,H**).

The staining index (SI) calculation: $\frac{Median\ Positive - Median\ Negative}{2xSD\ Negative}$

The alternative staining index (Alt SI) calculation : $\frac{Median\ Positive}{SD\ Negative}$

The "voltration index" (VI) calculation: $\frac{Alt\ SI}{\sqrt{Voltage}}$

The MVR determined for the CYTO-TROL cells (**A,B**) and AbC Total Compensation Beads with CYTO-TROL cells **(C,D)** were similar where the MVR was found to be around 450 mVs. The MVR for the AbC Total Compensation Beads (**E,F**) was found to be around 400 mVs. The MVR for the UltraComp eBeads (**G,H**) was not achieved within the acquired voltage range (200-425 mVs). All three methods (SI, Alt SI, VI) gave equivalent results but the voltration index gave a more definitive maximum in most cases.

Comparison of SI, Alt SI, and VI using Hard-Dyed beads

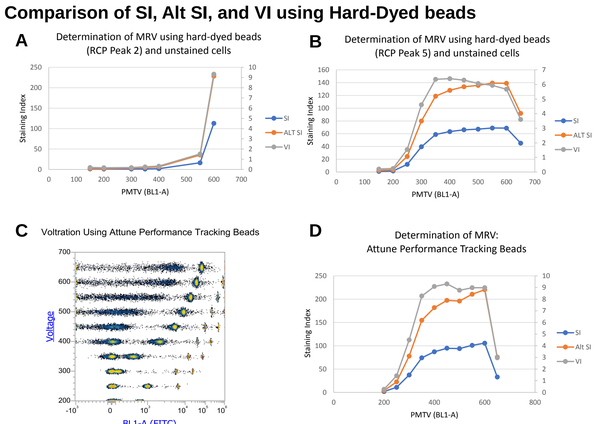


Figure 4. The minimum voltage required (MVR) was determined by the SI, Alt SI, and VI methods using Peak 2 (RCPs) combined with unlabeled CYTO-TROL cells (**A**), Peak 5 (RCPs) combined with unlabeled CYTO-TROL cells (**D**), and the mid and dim beads in the Attune Performance Tracking Beads (**C,D**). The MVR for the unstained CYTO-TROL cells with Peak 2 RCPs (**A**) was not identified within the acquired voltage range (150-550). The MVR for the unlabeled CYTO-TROL cells with Peak 5 RCPs and the Attune Performance Tracking Beads (comparing the mid with dim) was found to be 350 mV, and 450 mV, respectively.

RESULTS III

Linearity of PMT Response

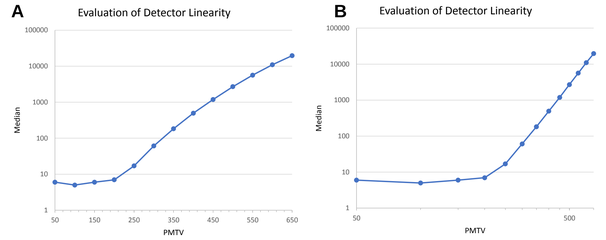


Figure 5. Median versus PMTV was plotted to determine the linearity of the detector response. The detector (BL1) is linear at voltages above 200. It is important that voltages are set within the linear range of the detectors. The relationship of signal to PTMV voltage is plotted as log versus linear (**A**) and log versus log (**B**).

CONCLUSIONS

A variety of methods were evaluated to determine the Minimum Voltage Required on the Attune NxT Flow Cytometer. A summary of the results is displayed below for the BL1 (FITC) detector:

Sample	%rCV	$10xSD_{EN}$	$2.5xSD_{EN}$	SI	Alt SI	VI
CYTO-TROL Lymphocytes (neg + pos)	350	450	425	425	450	450
AbC Total Comp Beads (neg + pos)	375	450	400	400	400	400
UltraComp eBeads (neg + pos)	NA	NA	400	>425	>425	>425
CYTO-TROL (neg) + AbC Bead (pos)	350	450	400	450	450	450
CYTO-TROL (neg) + Peak 2 (pos)	350	450	400	NA	NA	NA
CYTO-TROL (neg) + Peak 5 (pos)	350	450	400	350	350	350
Attune Performance Tracking Beads	300	350	350	450	450	450

The Staining Index, Alternative Staining Index, and Voltration Index can all be easily 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 %rCV inflection method should not be used as this method underestimates the MVR. The area measurement must be used for the SD_{EN} methods. The particles used for the $10xSD_{EN}$ and $2.5xSD_{EN}$ methods were found to vary depending on the particle, making this method less universal.

The SI, 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.

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