Optimize detectors for high-quality flow cytometry data

Evaluation of different techniques for PMT optimization using the Attune NxT Flow Cytometer.

In a flow cytometry experiment, high-quality data can only be obtained when the instrument and its individual components are optimized. The detector is one of the critical components of a flow cytometer. A detector captures the photons that are emitted by the excited fluorophores and scattered laser light and converts them into photocurrent, which is then passed to the electronics system.

The most commonly used detectors for flow cytometry are the photomultiplier tubes (PMT) that reside in each channel of the instrument (Figure 1). In addition to converting the photons to photocurrent, the PMT amplifies the signal, a process that requires the application of a steady-state voltage to the detector. PMT sensitivity is controlled by the material used to construct it and the wavelength of light entering it, as well as the amount of voltage applied to it. Therefore, optimization of that applied voltage (or gain) is required for each PMT detector in order to obtain the best-quality data in each channel of the flow cytometer.

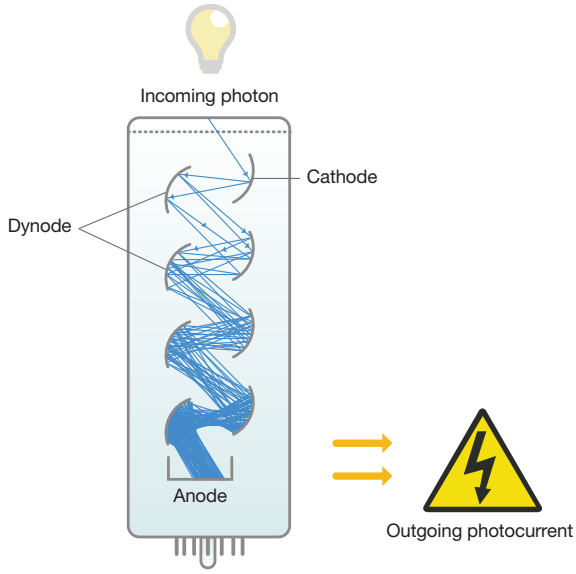

Figure 1. Schematic of a photomultiplier tube detector for a flow cytometer. An electron is emitted when a photon enters the photomultiplier tube (PMT) at the cathode. It then travels through the PMT, being amplified at the dynodes (electrodes) throughout and ending up at the anode, which is a collecting electrode.

Optimizing the voltage

As the voltage applied to the PMT is increased, the fluorescent signal is increasingly separated from background noise, providing greater resolution of the positive signal. At a certain voltage, however, the increasing separation of fluorescent signal from background will plateau, and the separation of fluorescent signal from background will remain constant. This voltage level is called the minimum voltage requirement (MVR); an ideal MVR setting will amplify dim signals above background but will not be so high that the fluorescent signals exceed the upper range of PMT detection linearity. Additionally, the MVR should allow the fluorescence signal of unstained and brightly stained cells or beads to be seen in plots on the same numeric scale. If the voltage is set too far above the MVR, the dim signal will still be separated from background but the positive signal may go off scale. Conversely, setting the voltage too far below the MVR will most likely result in compromised detection of dim signals.

Flow Cytometry Basics Module—Molecular Probes School of Fluorescence

What are the main components of a flow cytometer? How does a flow cytometer focus the sample stream and interrogate individual cells? How is the photocurrent from the detector digitized and processed? Find answers to these questions and more in the Invitrogen™ Molecular Probes™ School of Fluorescence—Flow Cytometry Basics module, available online. The Flow Cytometry Basics module is designed to help you understand the fundamentals of a flow cytometry experiment. This online content covers the three main components of a flow cytometer—fluidics, optics, and electronics—and was written by the bench scientists who created the *Molecular Probes Handbook*. Learn more at **thermofisher.com/mpsf-flow**.

Voltration

A common method for setting the MVR is the "Peak 2" method [1]. With this method, dimly fluorescent beads are run using a series of different voltage settings (also called voltration or voltage walk), and the spread of the signal (or the coefficient of variation, CV) is plotted against the voltage series. An example of a voltration experiment is shown in Figure 2. While this method does result in setting the MVR to get clear resolution of the dim fluorescent signal from the background noise of the detector, it does not address the need to ensure that the brighter fluorescent signals do not exceed the upper limit of the PMT detection range. This drawback has led to the development of a variety of methods in which unstained and brightly stained cells or beads are both used to determine the MVR [2-4].

Comparison of methods to determine MVR

Recently our scientists presented a study in which they compared different sample types as well as various calculated parameters to determine the MVR on the Invitrogen™ Attune™ NxT Flow Cytometer and any significant differences between the methods [5]. The samples tested included: different types of internally dyed microspheres (hard-dyed beads) that are detected in all detectors but do not include fluorophores used for typical experimentation; antibody-capture beads labeled on the bead surface with fluorescent antibody conjugates that are detected in a specific detector; lymphocytes that were either unstained or stained with fluorescent antibody conjugates; and a combination of the different samples.

The parameters derived from the data included calculation of the standard deviation of the electronic noise (SDEN), as well as of the staining index (SI), alternative staining index (Alt SI), and voltration index (VI) (Figure 3). In this article we will focus on a subset of these data, mainly the comparison of SI, Alt SI, and VI using antibody-capture beads (Invitrogen™ AbC Total Antibody Compensation Beads), lymphocytes (unstained and stained Beckman Coulter CYTO-TROL™ Control Cells), and a combination of the two (Table 1). You can view the scientific poster of the entire study at thermofisher.com/pmtposterbp78. →

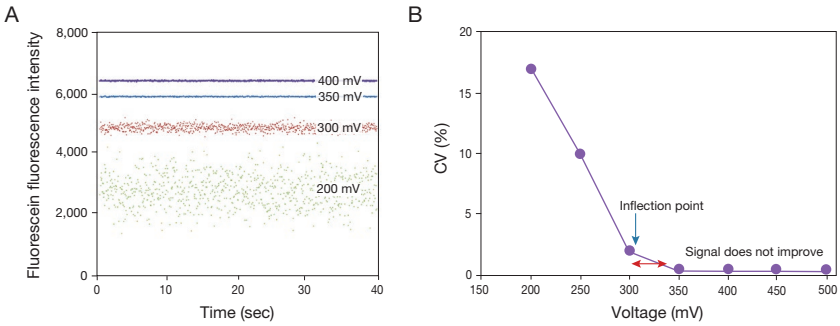

Figure 2. Principle of voltration using the Peak 2 method. Fluorescein-labeled beads were run at increasing PMT voltage settings. **(A)** The fluorescence events in the green channel were detected at different PMT voltages (indicated in the plot for each data set) and plotted against time. **(B)** The coefficient of variation (CV) for each data set in **A** was plotted against the PMT voltage setting. The point on the curve where the CV begins to level out is the inflection point, which marks where there is decreasing variation of the data at higher voltages. The red arrow indicates the optimal PMT voltage range for this fluorescence channel.

$$\text{Staining index (SI):} \quad \frac{\textit{(Median positive – Median negative)}}{\textit{(2 x SD negative)}}$$

$$\text{Alternative staining index (Alt SI):} \quad \frac{\textit{Median positive}}{\textit{SD negative}}$$

$$\text{Voltration index (VI):} \quad \frac{\textit{Alt SI}}{\sqrt{\textit{Voltage}}}$$

Figure 3. Equations used for MVR calculations. Three different equations were used to determine MVR. Median positive and median negative refer to the median fluorescence signal from the stained and unstained, respectively, beads or cells. SD negative is the standard deviation of the fluorescence measurements from the unstained beads or cells.

Table 1. Minimum voltage requirement (MVR) for the BL1 channel, determined with different parameters and different sample types.

Sample composition	Parameter used to calculate MVR for BL1*		
	Staining index	Alternative staining index	Voltration index
AbC Total Antibody Compensation Beads (unstained and stained beads)	400 mV	400 mV	400 mV
CYTO-TROL lymphocytes (unstained and stained cells)	425 mV	450 mV	450 mV
AbC Total Antibody Compensation Beads (stained) and CYTO-TROL lymphocytes (unstained)	450 mV	450 mV	450 mV

*The MVR determinations for the BL1 detector (fluorescein channel) using fluorescein-stained cells and beads are shown. The MVR for each detector must be determined separately.

The resulting MVR values

The results of the study are shown in Figure 4, and the MVR obtained for the BL1 detector (the fluorescein channel) using each method on three different sample types is summarized in Table 1. On the Attune NxT Flow Cytometer, the MVR for the AbC Total Antibody Compensation Beads alone (Figure 4A and 4B) using SI, Alt SI, or VI values was found to be ~400 mV. The MVR for the CYTO-TROL lymphocytes alone (Figure 4C and 4D) and for labeled AbC Total Antibody Compensation Beads with unstained CYTO-TROL cells (Figure 4E and 4F) was determined to be ~450 mV. All three methods (SI, Alt SI, VI) provide a working range of voltages that can be used to achieve high-quality flow cytometry data.

MVR methods and more

Instrument settings play a critical role when designing a flow cytometry experiment and must be refined for each application depending on the target specificity and abundance, specific antibodies, cell function probes, and fluorophores in the study. Using MVR settings that have been optimized for each PMT detector in each flow cytometer channel helps to minimize day-to-day variation in instrument performance and ensure that the resolution sensitivity for each parameter will be consistent from experiment to experiment. For a more complete description of all MVR methods evaluated, visit thermofisher.com/pmtposterbp78. ■

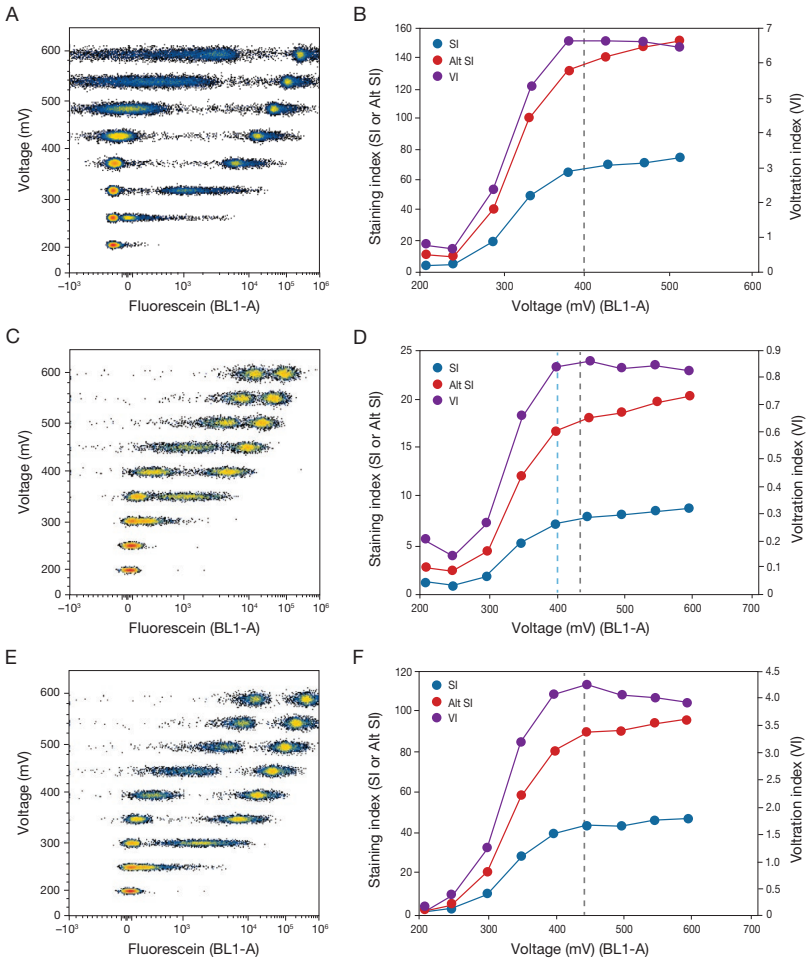

Figure 4. Comparison of different methods for MVR determination. The data for each sample type were acquired on the Invitrogen™ Attune™ NxT Flow Cytometer (BRVY and BRV6Y configurations) at a flow rate of 200 µL/min using a FSC threshold. A gate was placed around the main cell population, and a stop criterion 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. Data files were concatenated and plotted in the same graph **(A, C, E)**. The SI, Alt SI, and VI values were then calculated for each sample type and were plotted as a function of PMT voltage setting **(B, D, F)**. **(A, B)** Data were collected using Invitrogen™ AbC Total Antibody Compensation Beads labeled with a fluorescent antibody conjugate, and the unstained negative AbC beads. The black dashed line in **B** indicates the MVR for all three methods of calculation. **(C, D)** Data were collected using the autofluorescent signal of unlabeled CYTO-TROL™ lymphocyte cells (Beckman Coulter) and cells labeled with a fluorescent antibody conjugate. The blue dashed line in **D** represents the MVR for the SI calculations, and the black dashed line represents the MVR for both the Alt SI and the VI. **(E, F)** Data were collected using a combination of labeled AbC Total Antibody Compensation Beads and unlabeled CYTO-TROL lymphocytes. The black dashed line in **F** represents the MVR for all three methods of calculation (SI, Alt SI, and VI).

References

1. Maecker HT, Trotter J (2006) *Cytometry A* 69:1037–1042.
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3. Maciorowski Z, Chattopadhyay PK, Jain P (2017) *Curr Protoc Immunol* 117:5.4.1–5.4.38.
4. Perfetto SP, Ambrozak D, Nguyen R et al. (2012) *Nat Protoc* 7:2067–2079.
5. Shallice M, Diz R, Oxford C et al. (2018) *J Immunol* 200 (1 Supplement):120.37.

Product	Quantity	Cat. No.
Attune™ NxT Flow Cytometer, blue/red/violet6/yellow (BRV6Y configuration)	1 each	A29004
Attune™ NxT Flow Cytometer, blue/red/violet/yellow (BRVY configuration)	1 each	A24858
AbC™ Total Antibody Compensation Bead Kit	25 tests 100 tests	A10513 A10497