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Evaluation of Voltration Approaches for Optimal Data Acquisition in Flow Cytometry

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Flow cytometry is a technology widely used to analyze biophysical and biochemical characteristics of cells and small particles quickly with high sensitivity at the single cell level. Due to its high sensitivity, broad dynamic range of quantification, robust reproducibility, and acquisition of multiplexed data at single cell level, flow cytometry has seen appreciable utilities in scientific research, biomedical industry and clinic. However, despite its increasing popularity, the quality of flow cytometry data can vary substantially, either between experiments, individuals, or laboratories. This prevalent problem can be attributed to multiple sources such as instrument standardization practices, reagent standardization, equipment configuration, user operation, quality of data acquisition, and data processing. Overcoming these challenges has been a major goal for the flow cytometry community, however not all sources of flow cytometric experimental error are equally obvious to even experienced users. In this thesis work, I systemically evaluated how the standardization method used for assessing instrument electronic noise and configuring operation voltages can contribute to observed experimental variances. Through side-by-side comparisons between these eight methods, I provide comprehensive operational suggestions to preserve both data quality and real-world practicality.

Dedication

This thesis is dedicated to Robert Abbott who always pushes me to reach for the stars.

Acknowledgments

I would like to express my gratitude to my family who has been there by my side every step of the way. I would also like to present my special thanks to my fiancé Robert Abbott who has pushed me and helped me every step of the way through the whole process. I would also like to thank Shoutian Zhu for his careful reading, feedback on this entire project and without who it was impossible to accomplish this work. I would also like to give a special thanks to my flow cytometry friends who convinced me to go back to school and have supported me through everything.

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Chapter I

Introduction

Flow cytometry is an experimental method to analyze the biophysical and biochemical properties of a cell in a liquid suspension. Cells are suspended as individual particles and passed through analyzing chambers one-by-one and analyzed for the parameters of interest. Photonic radiations, spanning the spectrum from ultraviolet to visible lights and infrared, are generally the readout signal of choice. Light signals could be from different sources and carry rich information. For example, a beam of light of a given wavelength can be absorbed, reflected and deflected when it encounters a particle, resulting in out coming light with altered strength, wavelength and path. These changes can carry information such as morphology and irregularity of the particles. Luminescence emitted from certain enzymatic reactions and fluorescence from naturally existing sources (e.g. fluorescent proteins) or fluorophore-labeled analytes are widely used to detect and as well as quantify the abundance of certain enzymes, proteins and other biological analytes of interests in flow cytometry studies. The availability of fluorophores of distinct excitation and emission wavelengths makes multiplexed analysis possible. In many cases the fluorophores are attached to monoclonal antibodies that can bind to a variety of different proteins on the cell surface or intracellularly. Components of the cells and/or particles can be fluorescently labelled and excited by lasers so emitted light can be collected at a multitude of wavelengths. These chemical and physical characteristics can

give information about the phenotype and/or function of the cell or particle. Today, up to twenty-six different fluorescence markers can be measured and analyzed simultaneously using modern flow cytometers. Besides commonly analyzed cells such as mammalian, plant, algae, yeast and bacterial cells, other particles including nuclei, chromosomes and manmade beads can also be analyzed using flow cytometry (Shapiro, 2005; Ormerod & Novo, 2008).

The History of Flow Cytometry

The original idea behind currently widely used commercial flow cytometers has been credited to Dr. Louis Kamentsky in the mid 1960s in Los Alamos National Laboratory (LANL). Dr. Kamentsky developed a system based on the microscopic spectrophotometer pattern of Moldavan, which was used in cervical cytology screening by monitoring how the cells absorbed UV light and scattered blue light. By 1967 Dr. Kamentsky and Myron Melamed expanded this idea by using a syringe-like system to separate cells in a fluidic flow according to the ratio between absorbed and scattered light signals. During this time Lenora and Leonard Hertzenberg at Stanford University were using the particle sorting idea developed at LANL to develop a fluorescence-based sorting system. Meanwhile, Wolfgang Dittrich and Wolfgang Gohde were the first to look at DNA histogram using ethidium bromide stained cells. At interim, Mack Fulwyler was using the idea of the inkjet Sweet to develop a charged stream deflection for particle sorting in the mid 1960s. Concurrently, LANL was working on a system combining laminar flow and laser-based light source (argon ion), which allowed for the measurement of multiple parameters including scattered light for morphology and irregularity, fluorescent intensity for markers of DNA and the volume of the collected

cells. By the early 1970s commercial cytometers were available on the market. The continuous development of flow cytometry technology has led to the production of equipment with various levels of capabilities from benchtop push-button analyzers to highly complex multi-stream high speed sorters, which allowed for a broad range of applications practiced daily in flow cytometry facilities all over the world (Diamond, 2012; Shapiro, 2005).

Flow Cytometry and its Utility

Thanks to its capacity of acquiring data at single cell level and richness in information (multiplexed analysis for many markers in each individual cell and the distribution of entire cell population), flow cytometry has become widely used in studying complex biological systems. For example, in immunology over thirty percent of most recent published studies include flow cytometry analyses (Ormerod & Novo, 2008). Besides biological research, flow cytometry also saw increased utility in clinics. For instance, chimeric antigen receptor T (CAR-T) cell-mediated immunotherapy is a promising cancer treatment that flow cytometry is applied at many stages of the therapeutic cell production. CAR-T cells are engineered T cells that can be programmed to kill certain cancer cells that express the antigen the T cells are designed to recognize. Flow cytometry is used to confirm that the T cells have been engineered correctly. Furthermore, clinicians can use flow cytometry to track the CAR-T cells in the patients' system and see how well the T cells are integrated and fighting the cancer cells, and evaluate the health of the white blood cells at the same time (Lim & June, 2017). However, despite the fact that the phenotype or function of a given cell population can be determined by flow cytometry, due to the need for the cells to be in suspension, the

information about tissue architecture, spatial distribution or cell-to-cell interactions is normally lost during the process of cell preparation (Shapiro, 2005; Ormerod & Novo, 2008). Thus, other technologies such as microscopic imaging are complementary to flow cytometry and also widely used in analyzing biological samples.

How a Flow Cytometer Works

A flow cytometer is an instrument analyzing various biophysical and biochemical properties of certain populations of cells in single cell liquid suspension. In most cases, the cells are fluorescently labeled, for example by expressing fluorescent proteins (e.g. green fluorescent protein, GFP) or binding to a fluorophore-conjugated antibody to help detect the cells of interest. To analyze these cells at single cell level, the flow cytometer contains three parts: the fluidics, the optics and the electronics. The fluidics use sheath fluid to deliver the cells to the laser intercept, which is called the interrogation point. The shape of the flow cell allows for hydrodynamic focusing that aligns the suspended cells along their axis. The optics focus excitation lasers onto the fluorescence-labeled cells and collect the emitted fluorescence lights, which are detected in photodetector. The photodetector converts the energy of photons into electric pulses, which are detected and processed by the electronics and analyzed by computer. A schematic of a flow cytometer is depicted in figure 1. Given all of these different parts and processes of a flow cytometer it is important that flow cytometry operators understand optics, fluid mechanics, biology, chemistry, biochemistry, electronics, and mathematical statistics. "This heterogeneity of technologies made flow cytometry a field where there were a million ways things could wrong and only one way they could go right." (Snow, 2003)

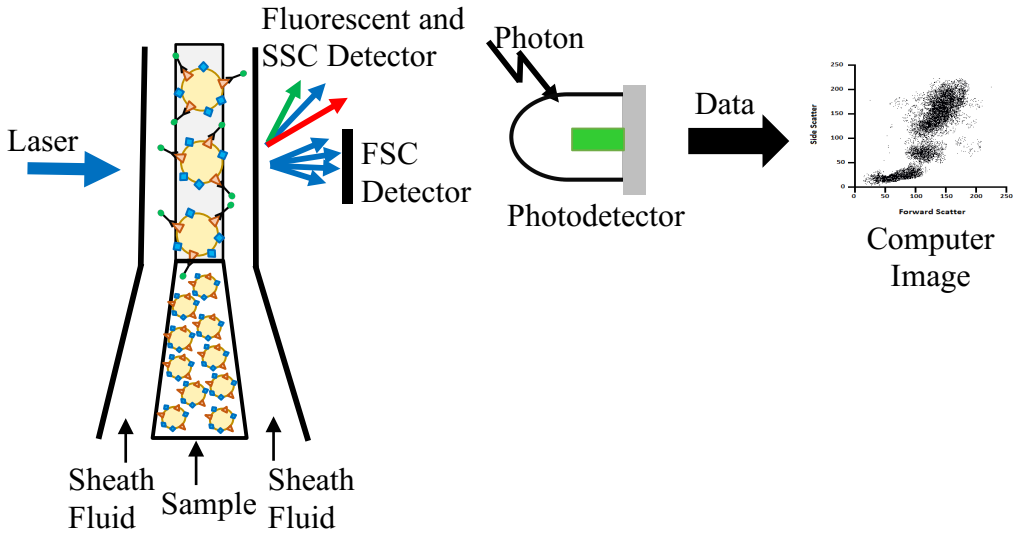


Figure 1. Flow cytometer schematic.

This figure depicts the basics of how a flow cytometer works.

The Fluidics of a Flow Cytometer

The power of flow cytometry to provide population scale analysis of cellular compartments relies on the fact that single cells, and not multiple cells, are being interrogated one at a time. The fluidic system of a flow cytometer enables this by focusing and moving the sample through the laser beam. The sample is initially injected into the center of a stream of sheath fluid. The sheath fluid creates a laminar flow which keeps the sample from mixing with the sheath fluid. The flow cytometer is under constant

pressure which allows the sheath fluid to continually move through the instrument, the pressure can be adjusted to allow the sample to move faster or slower. The sheath fluid moves the sample through the instrument into the flow cell where the shape of the flow cell and the fluid use hydrodynamic focusing to align the cells as they pass through the laser beams at the interrogation point. (Shapiro, 2005; Ormerod & Novo, 2008)

Flow Cytometry Optics

Once the cells move through the laser beams, scattered lights and emitted light from fluorochromes are collected. The light source is a laser or multiple lasers that pass through a focusing lens and then through the flow cell at the interrogation point. On the far side of the flow cell there is a bar that blocks the laser beam from entering the forward scatter detector which sits directly behind the blocking bar. The forward scatter detector detects light that is scattered at small angles in the forward direction. Forward scatter light is detected in a photodiode (PD). Scattered light signal shows the morphology and irregularity of the cells whereas, fluorescent signal shows what markers are present on or in the cell. A collecting lens is placed at a right angle to the laser beam. A series of dichroic mirrors then sort out light of different wavelengths. The light emitted from the fluorochromes is collected in photomultiplier tubes (PMTs). In front of the PMTs there are a group of longpass and bandpass filters to collect light with desired wavelength. The PMTs and PDs are known as the detector electronics that collect emitted photons and convert them into electronic signals and transcribed into the data seen on a computer-generated image (Shapiro, 2005; Snow, 2004; Steen, 1992; Perfetto, et al, 2014).

Flow Cytometry Electronics

The purpose of the electronics in a flow cytometer is principally to convert photons, detected by either fluorescence or scattered light, into an electronic measurement. This is done using two kinds of detectors as mentioned previously; PDs and PMTs. PMTs are particularly sensitive for detecting small changes in fluorescence, generally not visible by standard fluorescence microscopy.

These detectors electronics are so sensitive that even when no cells are passing through the lasers some light still gets through to the detectors producing what is called background current output, which must be taken in to account. The background current fluctuates at a certain baseline value above zero. Once a particle passes through the lasers it will cause a temporary influx in the current output, known as a current pulse, at each detector. Information about the cells is derived from these pulse signals. In immunofluorescence analyses, some positively labeled cells can produce several orders of magnitude brighter fluorescent signals than dim ones, which makes it impractical to plot all the data points on a linear scale. To this end, logarithmic amplifiers (also known as a log amp) are widely used in flow cytometers in the signal path that increase the dynamic range. A logarithmic amplifier creates an output signal that is directly proportional to the logarithm of the input signal. Most flow cytometers now exhibit a dynamic range spanning five to seven decades by taking advantage of the logarithmic amplifiers. Once the signal has passed through the log amp a voltage is applied and the signal processor uses the information from the log amp to create an analog pulse, which is then detected by the peak detectors, and transformed into a digital signal that is further processed by a computer. In most digital flow cytometers, an analog-to-digital converter

is used to digitize the signals (Shapiro, 2005; Snow, 2004; Zilmer, 1995). See figure 2 for a graphical repression of the electronics of a flow cytometer.

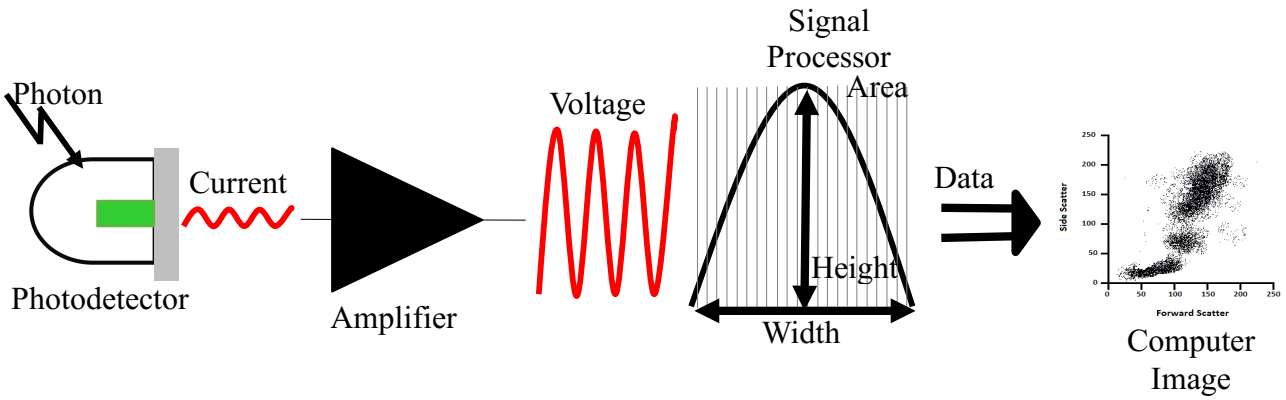


Figure 2. Flow cytometer electronics schematic.

This figure depicts details of the electronics of a flow cytometer.

Electronic Noise

Same as any other bioanalytical methodologies, the signal-to-noise ratio is a major indicator of assay performance of flow cytometry, and the optimization of which is a prerequisite for any successful experiment. In flow cytometry, one major source of noise is within the detector electronics where a large amount of electronic noise can be generated. The electronic noise may be generated in the photon to photoelectron conversion, current to voltage conversion, baseline restoration, amplification, DC

restoration and discrimination. The electrons flow through the solid amplifiers, which can heat up to cause molecules and atoms to vibrate thus creating noise. Noise can also come from Raman light scattering, laser light and fluorescence of the sheath fluid. Electronic noise can be seen in every channel on a flow cytometer and some channels have higher levels of noise than others, and does not always follow a consistent pattern. The effects of electronic noises are more obvious for low signals in a channel with high electronic noise or background. The uncertainty of the electronic noise affects the detection limit of weak signals (Shapiro, 2005; Snow, 2004; Steen, 1992; Perfetto, et al, 2014). Thus, it is important to measure the electronic noise in each channel in use, to make sure the voltage is properly adjusted to set the signal above the electronic noise so that weak signals can be effectively amplified.

Voltration

Voltration is the practice of adjusting the voltage or gain of the detector until an optimal signal above the noise is obtained. The voltration approaches include setting the negative control or population at the first decade or quartile (Maecker, & Trotter, 2006), using Cytometer Setup and Tracking (CS&T) values or Quality Control (QC), using 2.5 times the rSD_{EN} of unstained cells from the instrument QC (Meinelt, et al. 2012), using 8 peaks beads, optimizing the stain index based on compensation control beads, optimizing stain index based on single stained cells (Perfetto, et al, 2014; Chase et al, 1998;Volkman et al, 2016).

Each of these different techniques exhibits different advantages and limitations. Some were developed years ago accompanied the then state-of-the-art instruments. With the instruments advancing over time from analog to digital, and the dynamic range

expanded from four logs to upwards of seven logs, instrument set-up and calibration also need to evolve to keep up with the ever-evolving instrumentation. Unfortunately, it is still unclear which voltration technique is the most effective or which works the most consistently, and whether some of the older techniques still work on newer digital instruments.

Based on many conversations at the International Society for Advancement of Cytometry (ISAC) annual conference, cytometry mailing lists and cytometry blogs it seems clear that there is no standard practice consented among flow cytometer operators on how to set up the instruments. Configuration and calibration choices are generally made based on personal preference rather than on data. This may contribute to the inconsistent results between different groups and facilities that are sometimes observed. This study will focus on examining the electronic noise and comparing different methods of amplifying the signal above the electronic noise. The CS&T beads were used to determine instrument generated as well as independently calculated electronic noise values. Once the electronic noise was determined, eight voltration methods were evaluated to determine which methods are preferred for amplifying the fluorescence signal above the electronic noise. Each method of voltration will be tested using samples with bright, moderate and dim signals. Figure 3 depicts the experimental design of this study. Upon the completion of this study, a side-by-side comparison between all voltration methods will not only evaluate the performance of each individual method but also provide a reference to researchers in selecting the appropriate approaches for flow cytometry instrument set-up, and hopefully, reduce the data variation between different operators.

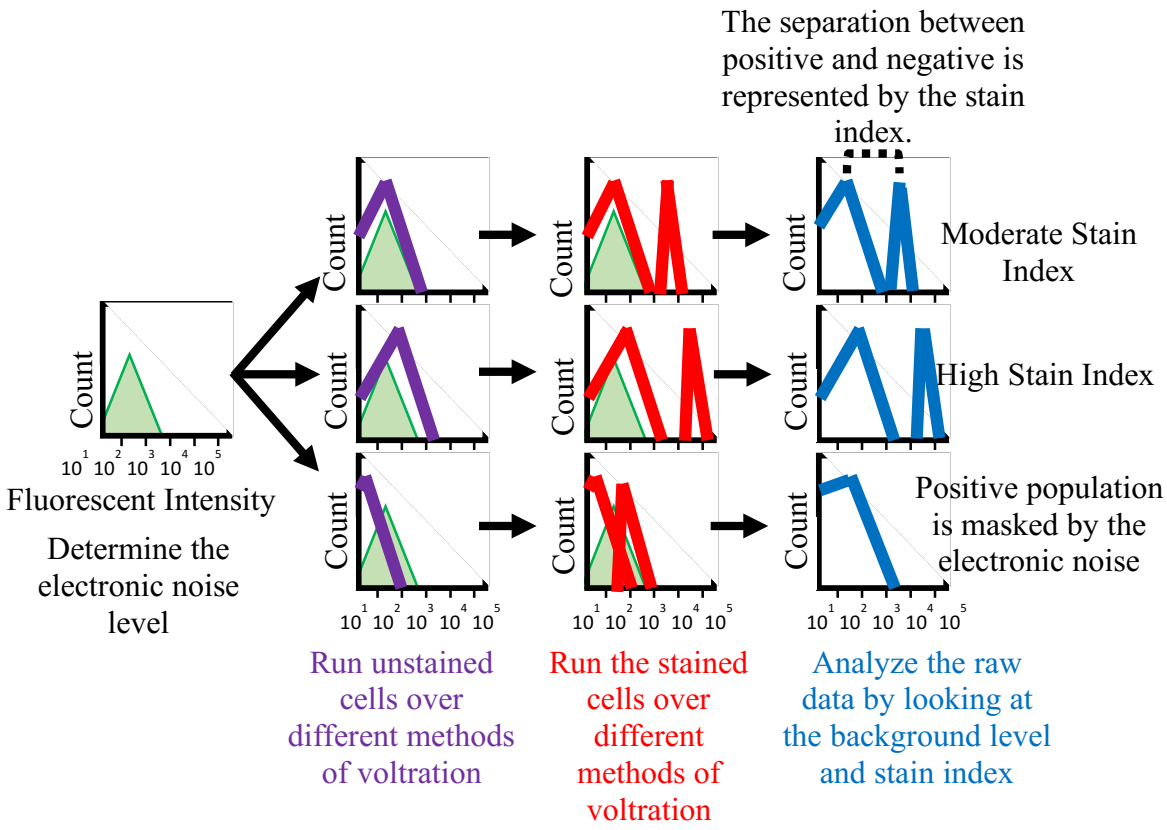


Figure 3. Schematic of experimental design.

This figure depicts the experimental design of this project. Initial the electronic noise level is determined. Then unstained cells are run over different methods of voltration. Stained cells are then run over the different methods of voltration. The raw data is analyzed by looking at the stain index and the background level to determine which method of voltration is the most effective.

Chapter II

Materials and Methods

The following section details the materials and techniques used throughout the study. Briefly, the electronic noise within the flow cytometers were calculated. Then different methods of voltration were performed on different markers. Finally, a preferred method was determined that is the most effective for multiple different markers.

Instrumentation

A Becton Dickinson (BD) LSRII (San Jose, CA) was used for this study, the lasers, filter sets and Cytometer Setup and Tracking (CS&T) generated voltages are listed in Table 1. This BD LSRII is maintained by the Stanford Shared FACS Facility.

Table 1. BD LSRII Configuration.

Laser	Channel Name	Filter Set	CS&T Generated Voltage	Channel Detector
488nm Blue	Alexa Fluor 488	505LP 525/50	347	Blue B
	PerCP-Cy5.5	685LP 710/50	611	Blue A
640nm Red	APC	670/30	552	Red C
	Alexa Fluor 700	690LP 730/45	425	Red B
	APC-Cy7	750LP 780/60	443	Red A
405nm Violet	Pacific Blue	450/50	392	Violet B
	BV510	505LP 525/50	415	Violet A
355nm Ultra Violet	DAPI	450/50	430	UV B
	Hoechst Red	505LP 525/50	674	UV A
561nm Yellow-Green	PE	585/15	475	YelGr E
	PE-CF594	600LP 610/20	609	YelGr D
	PE-Cy5	635LP 660/20	580	YelGr C
	PE-Cy5.5	685LP 710/50	565	YelGr B
	PE-Cy7	750LP 780/60	599	YelGr A

The configuration of the BD LSRII is listed in this table. This table includes the filters for each channel as well as the CS&T generated voltage and the detector.

Lymph node dissociation

Lymph nodes were excised and manually dissociated using 28-gauge insulin syringes and the blunt end of a 3ml syringe in FACS buffer (5% FCS in PBS). Preparations were filtered through a 70-micron mesh filter and lymphocytes were enumerated on a hemocytometer.

Lymphocyte staining

The cells were pelleted and resuspended in a 100μL of FACS buffer. All the samples were stained with the indicated antibodies at appropriate dilutions listed in Table 2. The cells were stained for twenty minutes at room temperature, then washed twice with FACS buffer and analyzed using the flow cytometer listed above. The APC-Fire750 antibodies were analyzed using the APC-Cy7 channel, while the BV510 antibody was analyzed using the BV510 channel.

Table 2. Antibody Information.

Antibodies	Clone	Dilution	Company
Anti-CD4 APC-Fire750	GK1.5	1:200	Biolegend, Inc.
Anti-CD3 APC-Fire750	17A2	1:200	Biolegend, Inc.
Anti-CD8a BV510	53-6.7	1:100	Biolegend, Inc.

The antibody along with the clone, diluation and company are listed in this table.

Brightness Index of Fluorochromes

This study used anti-CD3 APC-Fire750, anti-CD4 APC-Fire750 and anti-CD8a BV510 antibodies. The BV510 channel is known to show the autofluorescence of the cells. The BV510 channel on this specific instrument also has a high level of electronic noise. The other channel used in this study was the APC-Cy7 channel, which is known for having little to no autofluorescence. Due to the long wavelength of APC-Fire750 it

can be very sensitive to instrument changes and adjustments in PMT voltage (Maecker & Trotter, 2006). This channel also has a moderate to high level of the electronic noise. Besides knowing what the electronic noise is in this channel it is important to look at the brightness index of these colors. These two colors are considered moderate to dim, as indicated by the brightness index (scale 1-5 with 5 being the brightest) and BV510 is rated a three while APC-Fire750 is rated a two (Figure 4). Dimmer markers were used in this study with the thought of getting more information on the lower end of the scale, which is closer to the electronic noise levels.

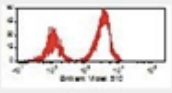
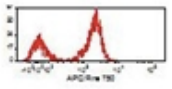
Fluorophore	Ex (nm) Max	Em (nm) Max	Filter Used	Brightness	Histogram
Brilliant Violet 510™	405	510	510/50	3	
APC/Fire™ 750	650	787	780/60	2	

Figure 4. Brightness index chart.

Figure from Biolegend, Inc. -- brightness index chart.

Beads Preparation

BD Cytometer Setup and Tracking beads were prepared by adding one drop of beads into 250μL of PBS. 8 Peak Rainbow beads (Spherotech, IL) were prepared by adding one drop of beads into 250μL of PBS. OneComp compensation beads (ThermoFisher, CA) were prepared by adding one drop of beads into 250μL of PBS and mixed with 1 μL of each antibody.

Electronic Noise

Method 1 – Instrument Generated Electronic Noise

The instrument generated values were determined using BD's CS&T software. CS&T beads were prepared and loaded into the system, the software adjusted the threshold and voltage while performing the calculations for the electronic noise.

Method 2 – Calculated Electronic Noise

Electronic noise of a flow cytometer is normally below the threshold so that most users do not realize that it exists. Threshold is a value that the signal must be above to be considered an event. By lowering the threshold, it will allow the electronic noise to be visualized and examined. In the current studies, the threshold was lowered to 200 on the Forward Scatter channel (FSC) to make the electronic noise visible.

Once, the threshold was lowered, negative beads were analyzed at various voltages. In these experiments, the negative beads were analyzed in each channel at voltages between 100 and 850. The data from the negative beads were collected and

analyzed using FlowJo software to calculate the robustCV (rCV) and median values of the beads at every voltage. Individual beads were gated using FSC and SSC, then statistics of the rCVs and median of fluorescence were calculated. The rCV^2 versus $1/median^2$ was then graphed as a scatter plot, and a linear trend line was fitted for the data points. The slope of the trend line represents the variance of the electronic noise. The square root of the slope was calculated to render the robust Standard Deviation of the Electronic noise (rSD_{EN}).

Voltration

Voltration is the process of adjusting voltages of a given fluorescence channel until the optimal separation of positive and negative populations is obtained. Eight different methods of voltration were applied and evaluated in the current study.

Method 1 – Negatives are Centered Within the 1st Decade

The voltage was adjusted based on the signal of the negative sample so that the median signal was centered within the 1st decade.

Method 2 – Negatives are Centered at 10^2

The voltage was adjusted based on the signal of negatively stained cells so that the median signal was centered at 10^2.

Method 3 – Using the Instrument Generated Voltages

The voltage was adjusted such that it matched the voltages defined by the CS&T daily performance check. The CS&T software generates the voltage by adjusting the

signal of the negative beads until the robust standard deviations (rSD) are 10 times the rSD_{EN}.

Method 4 – 2.5 times rSD_{EN} (Instrument Generated)

The rSD_{EN} generated by the instrument as described in method one of electronic noise determination was used to find the proper voltage. The voltage was adjusted based on the signal of the negative sample until the robust standard deviations (rSD) are 2.5 times the rSD_{EN}.

Method 5 – 2.5 times rSD_{EN} (Calculated)

The rSD_{EN} calculated in method two of electronic noise determination was used to find the proper voltage. The voltage was adjusted based on the signal of the negative sample until the medians are 2.5 times the rSD_{EN}.

Method 6 – Optimize with Stained Cells

Single stained cells were analyzed at multiple voltages. The stain indexes were calculated. The voltage was adjusted to that with the highest stain index.

Method 7 – Optimized with Stained Capture Beads

Single stained antibody capture beads were analyzed at multiple voltages. The stain indexes were calculated. The voltage was adjusted to that with the highest stain index.

Method 8 – Stain Index of Peak Two and Six Beads

Eight peak rainbow beads were analyzed at multiple voltages. The stain indexes using peak two and peak six signals were calculated. The voltage was adjusted to that with the highest stain index.

Chapter III

Results

Electronic noise was successfully analyzed and identified, showing that it is prominent in flow cytometers and should be taken into consideration. With electronic noise identified different voltration methods were preformed to determine which method gives the best signal to noise ratio over the background electronic noise. The data collected from these experiments are described in detail in this section.

Detection of Electronic Noise

Electronic noise of a flow cytometer is normally hidden below the threshold so that most users do not realize that it exists. To detect the electronic noise, the threshold needs to be lowered so that the noise is visible. To this end, the threshold was set at 200 in the current studies. Two methods were used to calculate the electronic noise for each channel tested.

Method 1 – Instrument Generated Electronic Noise

The CS&T beads (BD) contain three different types of beads in one vial: negative, non-fluorescent beads; beads with dim fluorescence; and beads with bright fluorescence. A range of voltages were scanned, and the signals from the negative bead population in each channel were used to determine the electronic noise. Upon completing the

calibration, the CS&T software generates a report with the rSD_{EN} value and a graph for each channel (an example graph is shown in Figure 5). The software uses the rCV and rSD to determine the optimal voltage for each channel and calculate the rSD_{EN}.

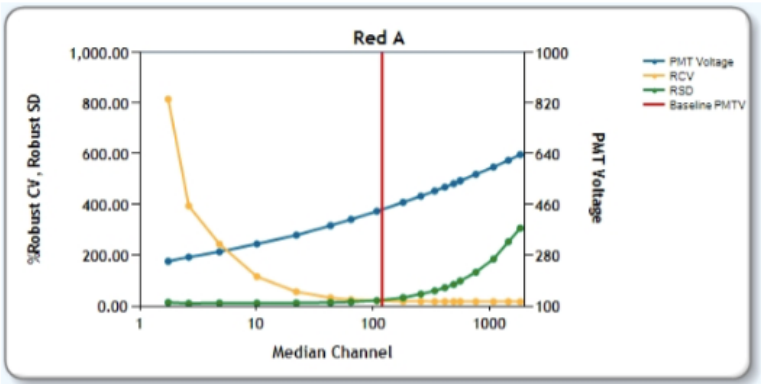


Figure 5. Electronic noise determination by CS&T software

This is an example of the graph produced by the CS&T software for each channel depicting the electronic noise as well as the CS&T generated voltage.

Method 2 – Calculate Electronic Noise Levels

Negative beads were analyzed at various voltages in all channels. The rCVs and medians were determined for each voltage using FlowJo software, a scatter plot of rCV^2 versus $1/median^2$ was graphed and a linear fitted trend line generated. Figure 6 shows a linear regression for a portion of the voltages. The linear portion of the line shows the

electronic noise effecting the rCVs and the medians, then at the point of inflection the voltage is high enough that the electronic noise is no longer effecting the rCV and median of the negative beads. The linear fit line shows the median at of the electronic noise. The square root of the slope of the fitted line is calculated as the median of the rSD_{EN}.

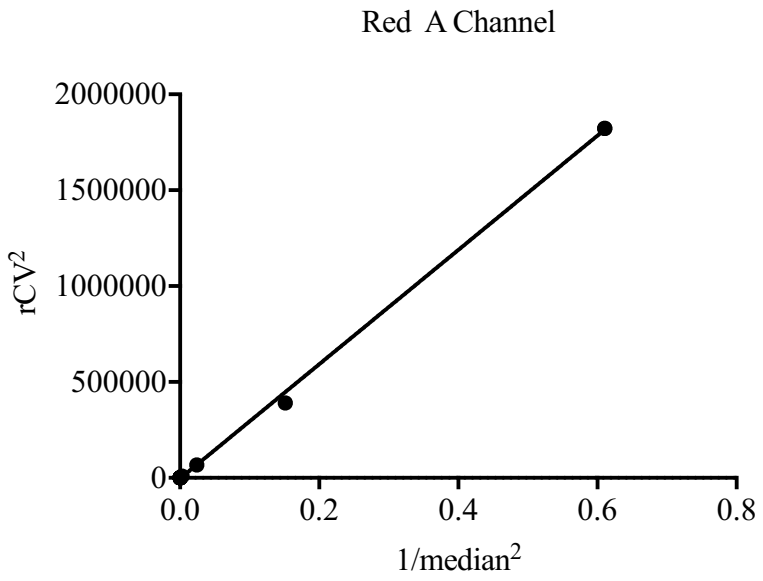


Figure 6. Calculated Electronic Noise

This shows the calculated electronic noise by looking at the best fit line of the rCV^2 verses $1/median^2$.

Using the abovementioned two different methods, the machine generated and calculated electronic noises were detected for all channels tested. Even though the

numbers generated by the two methods are different, the general trends correlate, as shown in Table 3. It is important to note which channels have the high levels of electronic noise. For example, the PE channel, exhibited the highest level of electronic noise in the current study. PE is one of the more favorable fluorochromes with good resolution in the low signal range that researchers, often use to detect low expressing markers. However, with a high level of electronic noise it may be hard to resolve low level signals. Thus, it may be advised to use other fluorochromes such as APC to detect analytes of low expression levels taking advantage of the significantly lower electronic noise in that channel.

Table 3. rSD_{EN}s for Every Channel

Channel	Calculated rSD_{EN}	Instrument generated-Electronic Noise rSD
Alexa Fluor 700	1405.3	13
APC	1541.5	14
APC-Cy7	1726.2	13
BV510	1973.3	14.1
DAPI	1024.6	9.4
Alexa Fluor 488	2080.0	17.8
Hoechst Red	1210.6	10.7
Pacific Blue	1532.3	14.1
PE	120437.2	19
PE-Cy5.5	3311.3	11.7
PE-Cy5	1411.9	12.6
PE-Cy7	1218.3	14.3
PE-CF594	1655.0	13.2
PerCP-Cy5.5	1535.2	15.2

The calculated rSD_{EN}s and the instrument generated rSD_{EN}s are listed here.

Voltration

Voltration is the process of finding the optimal voltage that provides the maximum separation between the positive and negative populations. This is generally performed using one of the eight methods discussed in the following sections. To systemically evaluate these methods, primary lymphocytes were labeled with following fluorophore-conjugated antibodies including anti-CD3 APC-Fire750, anti-CD4 APC-Fire750 and anti-CD8a BV510 CD3, CD4 and CD8 are proteins expressed on lymphocyte surfaces at different levels with CD3 at low levels (Ginaldi, L., 1996), CD8 at moderate levels (Takada, S., 1987) and CD4 at high levels (Davis, K., 1998). The stain intensity of a given marker is the result of multiple variables including brightness of the fluorophore, power of laser, affinity of antibody, number of surface molecules per cells, and number of fluorophores per antibody, just to name a few. The current study used each method of voltration to assess whether the three selected marker proteins can be properly detected in their corresponding channels. The staining index was calculated for each marker and used to evaluate the performance of each method.

Method 1 – Negatives Centered Within the 1st Decade

This method was a popular method in flow cytometry during the 1990s. This method was primarily used with analog instruments when the signal range only spanned four decades. Though this method is older, some people still use it even though they are no longer using analog instruments instead they are using digital instruments which can have up to seven decades signal span. Since, flow cytometry has evolved over the years that digital instruments become the norm this method has been gradually phased out of use. This method uses no calculations but relies on the visualization of the negative

population. Figure 7 (A&B) shows that negative sample is centered with the 1st decade for both APC-Fire750 and BV510 with voltages at 300 and 250 volts respectively. With the proper voltage determined based on the negatives, the three samples were analyzed. The CD4 and CD8 populations were visualized successfully but the CD3 population was undetectable. As seen in table 4, there is a small stain index for CD3 channel, even though the population is not visualized there still appears to be a small shift in the stain (Figure 7E). The CD4 stain exhibits a significantly higher stain index, which is expected since it is the brightest marker. The CD8 stain does not seem to be affected by this method. Thus, this method works for markers with mid to high brightness.

Table 4. Voltration Method 1

	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	300	18.7	16.49	Very low background with a low stain index
CD4 APC-Fire750	300	18.3	89.52	Very low background with a low stain index
CD8a BV510	250	16.8	68.57	Very low background with a high stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 1 are listed.

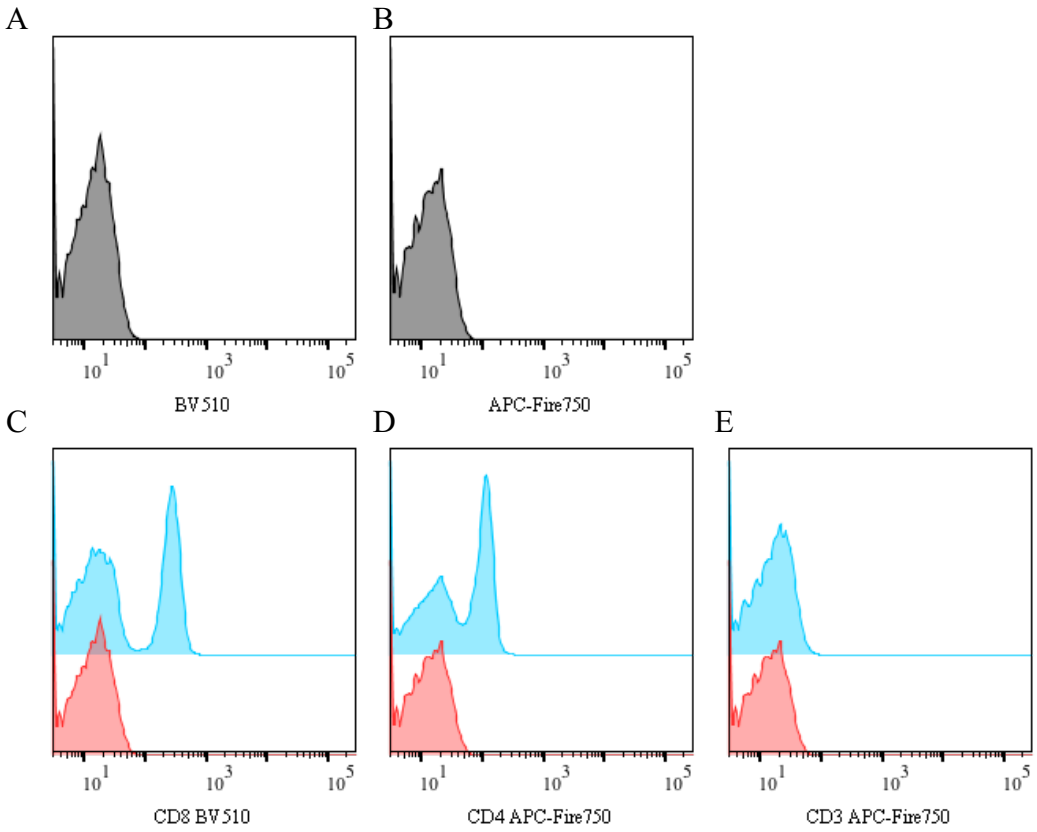


Figure 7. Voltration Method 1

A&B) Show the negative sample is centered at the 1st decade. C) In the red is the negative sample and the blue shows the CD8 BV510 sample. D) In the red is the negative sample and the blue shows the CD4 APC-Fire750 sample. E) In the red is the negative sample and the blue shows the CD3 APC-Fire750 sample.

Method 2 – Negatives are Centered at 10^2

Method two is a more commonly used technique where the negative is centered around 10^2. This method became popular when cytometers moved from an analog 4-log scale to a digital 5-log scale, like many of the cytometers in use today. The second method is very similar to the first method though it is more appropriate for digital instruments. The voltage is adjusted until the negative population of cells is centered around 10^2, with a voltage of 615 for APC-Fire750 and a voltage of 374 for BV510 (Table 5). As seen in Figure 8, proper separation and visualization of all the populations were achieved. Unlike method one, there is a clear CD3 positive population separated from the negatives. This method also allows for compelling stain indexes for all the markers (Table 5).

Table 5. Voltration Method 2

	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	615	132	38.32	High background with a high stain index
CD4 APC-Fire750	615	139	235.23	High background with a high stain index
CD8a BV510	374	38.7	69.37	High background with a high stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 2 are listed.

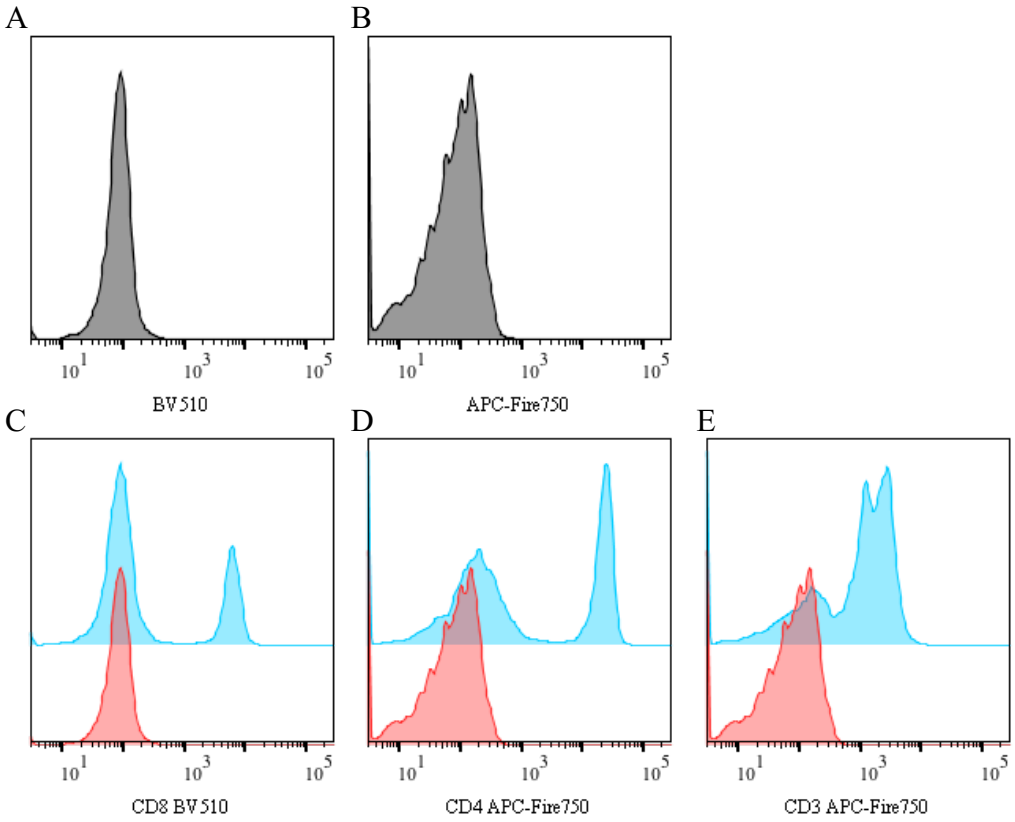


Figure 8. Voltration Method 2

A&B) Show the negative sample is centered at 10^2. C) In the red is the negative sample and the blue shows the CD8 BV510 sample. D) In the red is the negative sample and the blue shows the CD4 APC-Fire750 sample. E) In the red is the negative sample and the blue shows the CD3 APC-Fire750 sample.

Method 3 – Using Instrument Generated Voltages

Method three is the one of the most popular methods currently. This method uses the instrument generated voltages from the instrument standardized quality control (QC).

BD CS&T software is a quality control software that also generates standard voltages. These voltages are generated by adjusting the voltage of the negative beads such that it is ten times the rSD_{EN} generated by BD's software, taking into consideration the electronic noise of the system but using beads instead of cells to set the voltage. These voltages are evaluated every day so that if a laser starts to lose power it will increase the voltage in those channels to help compensate for the loss of laser power. This method allowed for clear separation of both CD4 and CD8a populations, however, CD3 population, although detectable, does not have a clear separated population when the data are visualized (Figure 9). This shows that ten time rSD_{EN} of beads does not allow for optimization of weakly stained cells (Table 6).

Table 6. Voltration Method 3

	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	438	32	15.80	Moderate background with a low stain index
CD4 APC-Fire750	438	23.2	175.16	Moderate background with a low stain index
CD8a BV510	414	75.8	67.21	Moderate background with a high stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 3 are listed

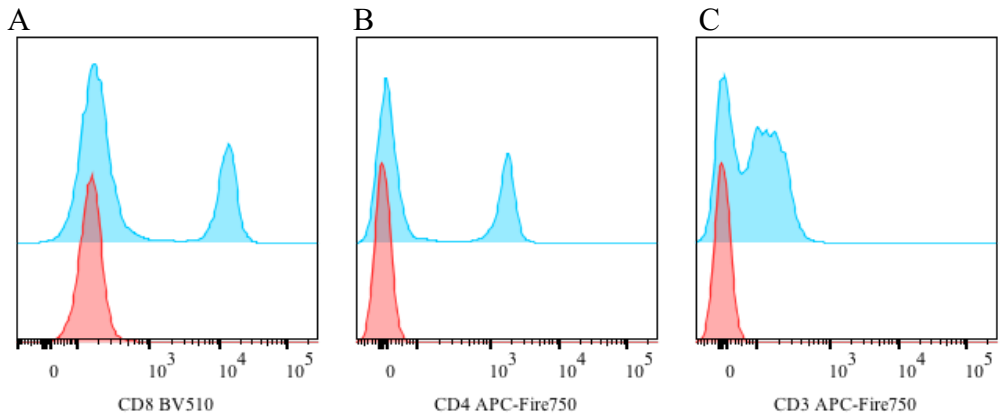


Figure 9. Voltration Method 3

A) In the red is the negative sample and the blue shows the CD8 BV510 sample. B) In the red is the negative sample and the blue shows the CD4 APC-Fire750 sample. C) In the red is the negative sample and the blue shows the CD3 APC-Fire750 sample.

Method 4 – 2.5 times rSD_{EN} (Instrument Generated)

Method four is considered the gold standard of voltration. However, this method is not the most commonly used since it is tedious to set up and evaluate. The rSDs of the negative sample needs to be evaluated while running and the voltage needs to be adjusted until the rSDs of the negatives are 2.5 times the instrument generated rSD_{EN}. Figure 10 (A, B &C) shows the results generated by the instrument, the separation in the CD4 and CD8a populations are very clear, while the CD3 population also shows two clearly separate populations (Table 7).

Table 7. Voltration Method 4

	rSD_{EN}	2.5* rSD_{EN}	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	13	32.5	514	42.4	29.60	Moderate background with a moderate stain index
CD4 APC-Fire750	13	32.5	514	44.7	211.34	Moderate background with a higher stain index
CD8a BV510	14.1	35.25	315	20	68.53	Moderate background with a high stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 4 are listed.

Method 5 – 2.5 times rSD_{EN} (Calculated)

Method five uses a less common way to determine the rSD_{EN} for voltration. The medians of the negative samples need to be evaluated while running and the voltage needs to be adjusted until the medians of the negatives are 2.5 times the calculated rSD_{EN}. Figure 10 (D, E &F) shows the CD4 and CD8 were so well separated that the positives were no longer on scale, with the positives off scale we cannot generate statistics such as percentage positive or median of the population. Meanwhile, the CD3 population was nicely separated with a high stain index (Table 8). With the calculated rSD_{EN} it is important to notice that the negative population has moved up in the axis creating high background, which explains why the CD4 and CD8 populations are off scale.

Table 8. Voltration Method 5

	rSD_{EN}	2.5* rSD_{EN}	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	1726.2	4315.5	1000	4981	34.49	High background but high stain index
CD4 APC-Fire750	1726.2	4315.5	1000	5391	77.81	High background but a low stain index
CD8a BV510	1973.3	4933.3	639	2097	50.74	High background but a lower stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 5 are listed.

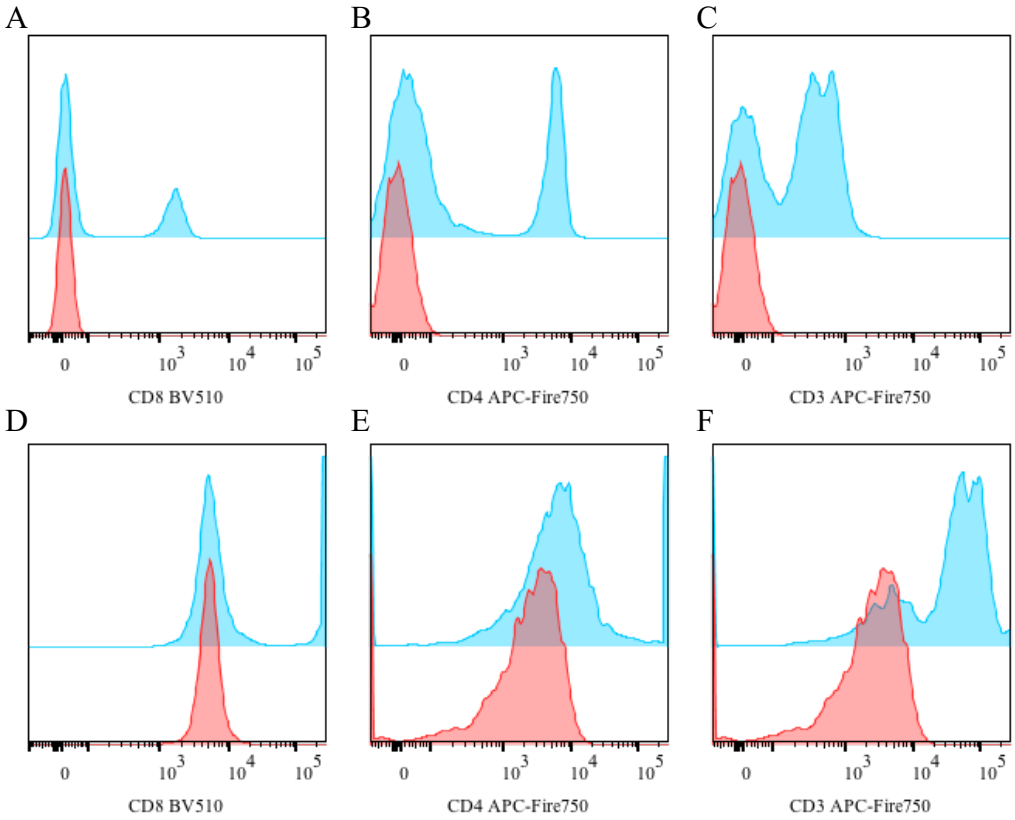


Figure 10. Voltration Method 4 and 5

A) Method 4 instrument generate rSD_{EN} times 2.5, in the red is the negative sample and the blue shows the CD8 BV510 sample. B) Method 4 instrument generate rSD_{EN} times 2.5, in the red is the negative sample and the blue shows the CD4 APC-Fire750 sample. C) Method 4 instrument generate rSD_{EN} times 2.5, in the red is the negative sample and the blue shows the CD3 APC-Fire750 sample. D) Method 5 calculated rSD_{EN} times 2.5, in the red is the negative sample and the blue shows the CD8 BV510 sample. E) Method 5 calculated rSD_{EN} times 2.5, in the red is the negative sample and the blue shows the CD4 APC-Fire750 sample. F) Method 5 calculated rSD_{EN} times 2.5, in the red is the negative sample and the blue shows the CD3 APC-Fire750 sample.

Method 6 – Optimize with Stained Cells

The sixth method uses stained cells in every channel to optimize the voltage based on the stain index on the CD4 cells. In the APC-Fire750 channel the CD4 single color control was run at multiple voltage points and the highest stain index was determined at a voltage of 525 (Table 10). With this information both the CD4 and CD3 stained samples were analyzed, the CD3 stained sample did have a nicely visualized separate population as well as a substantial stain index (Figure 12). The separation in the CD3 sample is clear without increasing the background, which is a benefit of looking at the stain indexes (Table 9). The BV510 channel was evaluated with the CD8a only control, and a high stain index and visualized separation was accomplished.

Table 9. Voltration Method 6

	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	525	54.8	36.14	Moderate background with a high stain index
CD4 APC-Fire750	525	55.5	218.34	Moderate background with a moderate stain index
CD8a BV510	375	44.4	69.44	Moderate background with a high stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 6 are listed.

Table 10. Stain Indexes of Stained Cells

APC-Fire750 CD4 Voltage	APC-Fire750 CD4 Negative Median	APC-Fire750 CD4 Positive Median	Stain Index= Positive Median/Negative Median
200	0.41	n/a	n/a
300	-0.83	107	-128.9
400	5.39	863	160.1
425	8.71	1330	152.7
450	11.6	2080	179.3
475	17.4	3056	175.6
500	23.2	4656	200.7
525	**30.4**	**6663**	**219.2**
550	43.7	9101	208.3
575	65.7	13530	205.9
600	86.9	17624	202.8
625	123	24614	200.1
700	273	58109	212.9
800	797	153518	192.6

Voltages, medians of the negative and positives populations are listed to calculate the stain index. The voltage with is the highest stain index determines what the optimal voltage is for Method 6.

Method 7 – Optimized with Stained Capture Beads

Method seven uses a technique similar to method six except that instead of using cells it uses antibody capture beads to determine the proper voltage. This method though just as prolonged is more elementary because there is no need to dissociate and stain the cells; antibody capture beads can be prepared in fifteen minutes. This method allowed for nice visualization and stain indexes for all channels and markers (Table 12). All the markers have clear separation and look similar to the results of method 6 (Figure 13). However, an obvious difference is that the background was about doubled in the APC-

Fire750 channel (Table 11). This could be potentially problematic with a more autofluorescent cell type.

Table 11. Voltration Method 7

	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	575	91.2	41.96	High background with a high stain index
CD4 APC-Fire750	575	96.7	235.85	High background with a high stain index
CD8a BV510	500	346	68.09	High background with a high stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 7 are listed.

Table 12. Stain Indexes of Compensation Beads.

APC-Fire750 CD4 Voltage	APC-Fire750 CD4 Negative Median	APC-Fire750 CD4 Positive Median	Stain Index= Positive Median/Negative Median
200	2.07	n/a	0.0
300	2.9	248	85.5
400	9.54	1837	192.6
425	17.4	2917	167.6
450	15.8	4613	292.0
475	39.1	7096	181.5
500	41.2	10502	254.9
525	83	15216	183.3
550	101	21660	214.5
575	**120**	**30950**	**257.9**
600	239	43403	181.6
625	252	57394	227.8
700	689	140401	203.8
800	1221	261124	213.9

Voltages, medians of the negative and positives populations are listed to calculate the stain index. The voltage with is the highest stain index determines what the optimal voltage is for Method 7.

Method 8 – Stain Index of Peak Two and Six Beads

The eighth method uses eight peak rainbow beads to determine the right voltage for each channel. This method does not require antibodies or compensation beads but a set of standard beads in most flow cytometry labs. This method calculates the stain index between the second and sixth peaks of the eight peak beads. This method worked moderately well for the CD4 and CD8a cells but did not work for the CD3 cells (Figure

11 & Table 13). Though there was a small population shifted away from the negative it was not as defined as other methods.

Table 13. Voltration Method 8

	Voltage	rSD of the Background	Stain Index	Conclusions
CD3 APC-Fire750	400	30.3	6.99	Low background with a low stain index
CD4 APC-Fire750	400	20.5	175.22	Low background with a moderate stain index
CD8a BV510	300	20	64.97	Low background with a moderate stain index

Voltages, rSDs, stain indexed and conclusions for voltration method 8 are listed.

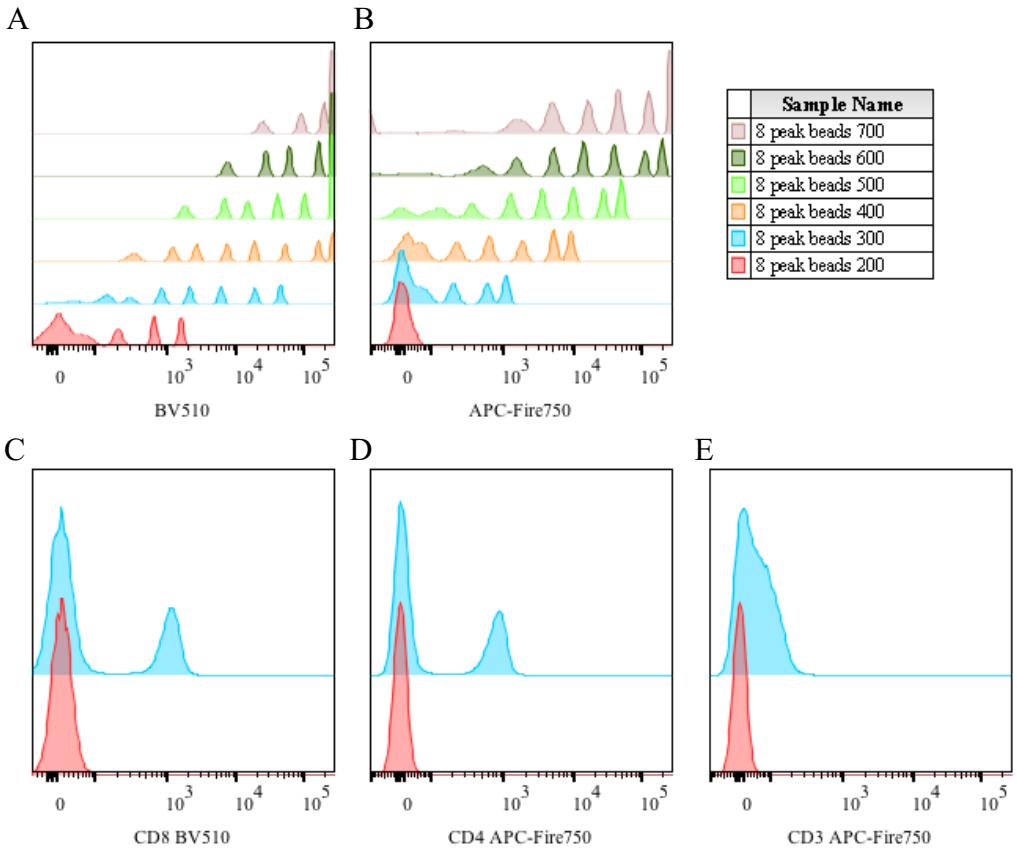


Figure 11. Voltration Method 8

A) BV510 single stained control over multiple voltage points to calculate the best stain index and voltage. B) APC-Fire750 single stained control over multiple voltage points to calculate the best stain index and voltage. C) In the red is the negative sample and the blue shows the CD8 BV510 sample, at the optimal voltage calculate from A. D) In the red is the negative sample and the blue shows the CD4 APC-Fire750 sample, at the optimal voltage calculate from B. E) In the red is the negative sample and the blue shows the CD3 APC-Fire750 sample, at the optimal voltage calculate from B.

All Methods Compared

By just looking at the stain indexes for each marker it is easy to pull out certain methods that work better than others (Table 15). For example, overall method two and seven seem to work the best for all channels. Method six also had considerable stain indexes. We also know from looking at the visualized data that method six actually worked better than method seven, due to method seven's increased background. We also know from looking at the stain indexes and the visualization that method one, three, five and eight did not work. While method five worked for CD3 it did not work for CD4 or CD8. The other three methods had the opposite problem, those methods did not work for CD3 but worked for CD4 and CD8. It is also interesting that BV510 CD8a seems to be less affected by these methods than the APC-Fire750 channel and markers. As expected though CD3 was the most affected by the different methods and was the hardest to detect and get a clear signal.

Chapter IV

Discussion

The aim of this study was to determine the most effective method to analyze electronic noise, and to establish the method of voltration that optimizes the signal above the noise. With flow cytometry becoming heavily utilized and an important tool in day to day research, it is imperative to ensure that scientists are aware of the limitations of the instruments. This study will help scientists to amplify their signal above the noise, while providing everyone with the tools needed to make sure they have quality data. While there is a variety of techniques to measure and assess noise limitations in current cytometers, there is a relative paucity of information of how to choose which techniques to apply.

Research has shown that there is some level of electronic noise in all flow cytometers, and different methods can be applied to amplify signals above the electronic noise (Shapiro, 2005; Snow, 2004; Steen, 1992; Perfetto, et al, 2014). The most basic way to set PMT voltages is to adjust the voltage such that the unstained sample falls within the first decade (or first quartile) of each fluorochrome being measured. This technique can be specifically troublesome when dealing with long wavelength emissions, such as APC-CY7, as demonstrated in this study. The issues associated with long wavelength channels are due to the little to no auto-fluorescence from the cells, and most signals from unstained cells will fall into the electronic noise range. When adjusting the

voltages in these channels it becomes rather subjective, which can jeopardize the optimal detection of the true signals (Maecker, & Trotter, 2006). However, these authors showed optimization and separation of positive cell populations from the negative cells using intracellular cytokine marker IL-2 by setting the voltage centered at 10^2.

A more convenient and consistent way to set the voltages of the PMTs with the intention of maximizing resolution sensitivity is by using the CS&T beads and software(BD). However, these voltages are set with beads rather than cells, this can be an issue since beads are perfectly spherical and their background auto-fluorescence is much lower than a cell leading to potentially lower voltages than what is optimal for cells (Nomura et al, 2008). By using the CS&T voltage, the author was able to clearly separate cytokine staining from the negative population, despite being detectable only at a low level. Another technique that uses information from the CS&T settings is to set the voltages at 2.5 times the rSD_{EN} (Meinelt et al, 2012) this technique was used in methods four and five of voltration. Meinelt's study showed that using 2.5 times the rSD_{EN} would ensure that the electronic noise will not interfere with the signal at the low end of the scale, and allow low level signals to be optimized. Voltages can also be optimized by using stain index or Q to B ratio. B is the background or noise of the system set by the negative population, while Q is the signal of the positive population. Both the stain index and Q to B ratio look at the signal to noise ratio. The voltage can be then adjusted so that the optimal ratio is achieved (Perfetto, et al, 2014; Chase et al, 1998). Using this method, Perfetto et al, were able to convincingly detect the expression of CCR7, a gene whose expression is known to be at low levels. Finally, 8 peaks beads have been used; this technique can give a read out of 8 peaks and obtain tighter CVs of Q and help determine

the optimal PMT voltage (Chase et al, 1998; Volkman et al, 2016). When instruments are not properly calibrated above the electronic noise, important information in the study could be missed due to the lack of sensitivity in the instrument (Chase & Hoffman, 1998). A body of literature suggests that there is a need for proper amplification of the signal above the electronic noise, and improper amplification can lead to false or no signal, thus negatively affecting the outcome of the experiments.

In my study, different methods of voltration drastically affected the detection of the signals and the separation between positive and negative cell populations. These different approaches of voltration made the difference between seeing no positive CD3 cells to having significant separation of CD3 positive and negative cells. Additionally, this study showed that if the negative population is set too low within the range of electronic noise (as method one of voltration did), the positive signal can become completely masked by the electronic noise of the system, and dimly stained cells will look negative. Even though the brighter markers were amplified above the background using method one, they did not have high stain indexes. I know from Maecker and Trotter's work in 2006 that fluorophores with the long wavelengths like APC-Cy7 (or APC-Fire750) would have even more issues amplifying above the noise as I saw in method one. Due to BV510 short wavelength it maintained a consistent stain index within a range of 20 points throughout each of the methods (Maecker, & Trotter, 2006).

As this study tested different methods of voltration, it is important to properly evaluate and compare different methods. I decided it was instrumental to look at the stain index, visualized data, and the rSD of the background to determine the method that effectively amplifies the signal over the noise. First, I applied two different methods to

investigate electronic noise, method one used CS&T software to generate rSD_{EN} (Meinelt, 2006); and method two calculated the rSD_{EN} using rCV and medians of negative beads or cells (Bushnell, 2017). The first method was less demanding, since the rSD_{EN} was determined using CS&T software and beads with default instrument settings (Meinelt, 2006), which was proven to be effective in the following voltration studies. Method two required certain levels of data analysis for rSD_{EN} calculation. When the rSD_{EN} determined by method two was used to calculate $2.5*rSD_{EN}$ in method five of voltration, the positive populations were off scale, which suggests that method two may over-estimate the electronic noise of the system in certain cases. Thus, method one of electronic noise determination appears to be more effective and user-friendly, and is widely used by most flow cytometry facilities. Table 14 shows a summarization of both methods.

Next, eight different voltration methods were evaluated by looking at the stain indexes of markers with different signal levels (e.g. CD3 with dim signal, CD8 with moderate signal and CD4 with strong signal, respectively), visualized data and the rSD of the background (Table 16). Some methods performed poorly by examining the stain index of the CD3 population, such as methods one, three and eight; some, including methods one, three, five and eight, performed poorly in analyzing stain index of the CD4 or CD8 populations. Methods two, four, six and seven all exhibited favorable stain indexes for different signal levels. However, the rSD of the background signal was high in method seven, in BV510 channel while the rSD of the background signal of method two was high for APC-Fire750 channel.

Method four took into consideration the electronic noise of the system, and the CD3 population was still readily detectable with two separate peaks, even though it did not produce the highest stain index for the CD3 population. Furthermore, this method did not increase the background so there was room available on the high end of the signal axis to accommodate markers stained brighter than CD4, which can occur with macrophage and granulocyte markers such as GR1, CD11b or MHCII. When comparing methods four and six I see that both produced similar results. Method four is more convenient because it does not use stained cells but beads and unstained cells for calibration. On the other hand, method six uses CD4 stained cells in every channel for calibration, which not only requires certain level of labor but maybe costly due to the antibodies used. Based on the performance, ease of operation and potential cost benefit, the author concluded that method four of voltration, as summarized in Table 16 should be the method of choice under most circumstances.

Table 14. Evaluation of Electronic Noise Methods

	Pros	Cons	Conclusions
Method 1– Instrument generate Electronic Noise Levels	Easy-no need to do anything No calculation necessary	No way to personally examine data	Worked well. Allows for quick analysis.
Method 2 – Calculate Electronic Noise Levels	Allows for personal examination	Time consuming Calculations can be tricking	Over-estimated the level of electronic noise.

Electronic noise methods compared with Pros, Cons and conclusions.

Appendix 1

Standard Operating Procedure for Analyzing Electronic Noise and Voltration

1. Preform preventative maintenance
2. If using a BD cytometer run a CS&T baseline. If using another cytometer run the QC software and determine the rSD_{EN}.
3. Run negative cells adjust all the voltages to match the rSDs to $2.5*rSD_{EN}$ in every channel.
4. Run rainbow beads as a standard for every channel.
5. Make a template either using gates for the standards or by matching medians.
6. Repeats steps three through 5 for as many different cell types as you have.

Appendix 2

Additional Figures

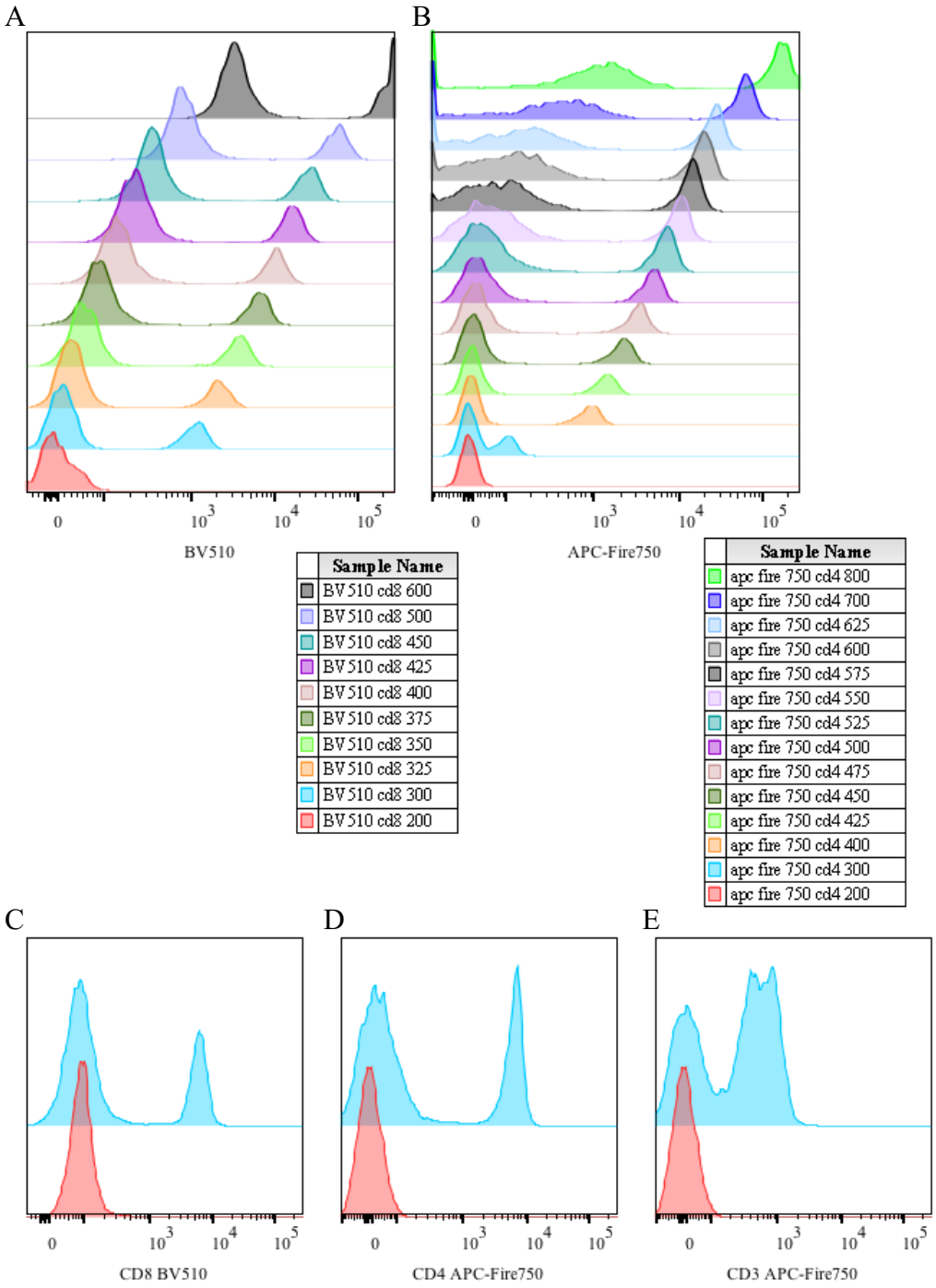


Figure 12. Voltration Method 6

A) BV510 single stained control over multiple voltage points to calculate the best stain index and voltage. B) APC-Fire750 single stained control over multiple voltage points to

calculate the best stain index and voltage. C) In the red is the negative sample and the blue shows the CD8 BV510 sample, at the optimal voltage calculate from A. D) In the red is the negative sample and the blue shows the CD4 APC-Fire750 sample, at the optimal voltage calculate from B. E) In the red is the negative sample and the blue shows the CD3 APC-Fire750 sample, at the optimal voltage calculate from B.

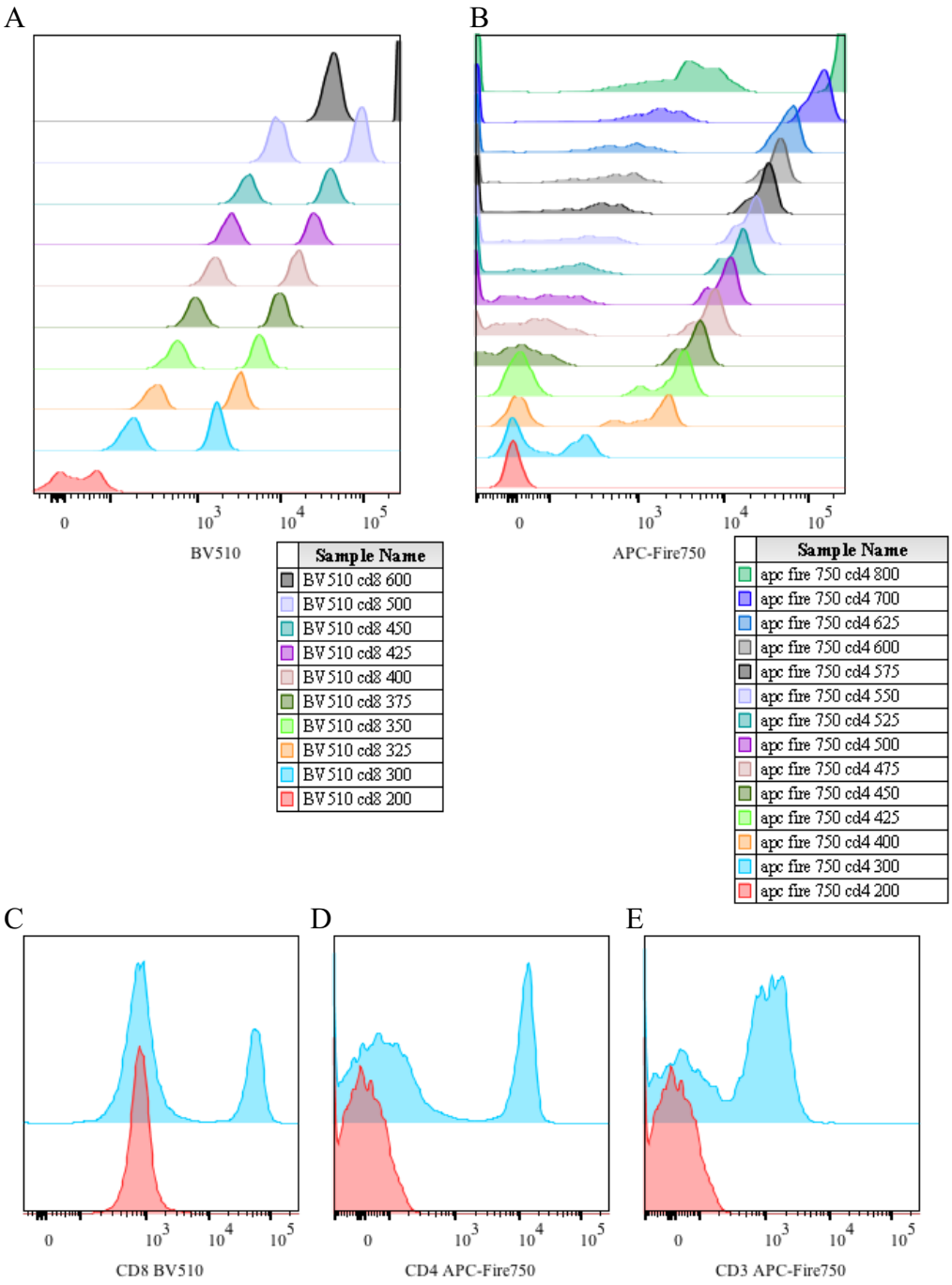


Figure 13. Voltration Method 7

A) BV510 single stained control over multiple voltage points to calculate the best stain index and voltage. B) APC-Fire750 single stained control over multiple voltage points to calculate the best stain index and voltage. C) In the red is the negative sample and the blue shows the CD8 BV510 sample, at the optimal voltage calculate from A. D) In the red is the negative sample and the blue shows the CD4 APC-Fire750 sample, at the optimal voltage calculate from B. E) In the red is the negative sample and the blue shows the CD3 APC-Fire750 sample, at the optimal voltage calculate from B.

Appendix 3

Additional Tables

Table 15. All Voltration Methods Compared

APC-Fire750 CD3				
	Stain Index	Voltage	rSD of the Background	Conclusions
Method 1	16.49	300	18.7	Very low background with a low stain index
Method 2	38.32	615	132	High background with a high stain index
Method 3	15.80	438	32	Moderate background with a low stain index
Method 4	29.60	514	42.4	Moderate background with a moderate stain index
Method 5	34.49	1000	4981	High background but high stain index
Method 6	36.14	525	54.8	Moderate background with a high stain index
Method 7	41.96	575	91.2	High background with a high stain index
Method 8	6.99	400	30.3	Low background with a low stain index
APC-Fire750 CD4				
	Stain Index	Voltage	rSD of the Background	Conclusions
Method 1	89.52	300	18.3	Very low background with a low stain index
Method 2	235.23	615	139	High background with a high stain index
Method 3	175.16	438	23.2	Moderate background with a low stain index
Method 4	211.34	514	44.7	Moderate background with a higher stain index
Method 5	77.81	1000	5391	High background but a low stain index

Method 6	218.34	525	55.5	Moderate background with a moderate stain index
Method 7	235.85	575	96.7	High background with a high stain index
Method 8	175.22	400	20.5	Low background with a moderate stain index
BV510 CD8a				
	Stain Index	Voltage	rSD of the Background	Conclusions
Method 1	68.57	250	16.8	Very low background with a high stain index
Method 2	69.37	374	38.7	High background with a high stain index
Method 3	67.21	414	75.8	Moderate background with a high stain index
Method 4	68.53	315	20	Moderate background with a high stain index
Method 5	50.74	639	2097	High background but a lower stain index
Method 6	69.44	375	44.4	Moderate background with a high stain index
Method 7	68.09	500	346	High background with a high stain index
Method 8	64.97	300	20	Low background with a moderate stain index

Stain Indexes, voltages, rSDs and conclusions for each method and antibody.

Table 16. Evaluation of Voltration Methods

	Pros	Cons	Conclusions
Method 1 – Negatives Centered within the 1st Decade	Easy	Used on analog instruments, not idle for newer digital instruments Does not take into account electronic noise	Produced low background in both channels. Produced low stain indexes for CD4 and CD8, and it was almost detectable for CD3. Not an optimal method.
Method 2 – Negatives are centered at 10^2	Easy-no calculations needed Fast	Does not take into account electronic noise	Produced high background in both channels. Produced high stain indexes. Not an optimal method but this method is effective.
Method 3 – Instrument Generated Voltages	Easy-no calculations needed Fast Takes into account electronic noise	Voltages may be too high for some more autofluorescent cell types May increase the background higher than needed	Produced moderate background in both channels. Produced low stain indexes for CD4 and CD3, while producing a high stain index for CD8. Not an optimal method.
Method 4 – 2.5*rSD_{EN} using instrument generated rSD_{EN}	Takes into account electronic noise	Time consuming	Produced moderate background in both channels. Produced high stain indexes for CD4 and CD8, while producing a moderate stain index for CD3. This is an optimal method.
Method 5 – 2.5*rSD_{EN} using calculated rSD_{EN}	Takes into account electronic noise	Time consuming Relies on the assumption that the initial calculation was correct	Produced high background in both channels. Produced a high stain index for CD3, while

			CD4 and CD8 were off scale. Not an optimal method.
Method 6 – Optimized with Stained Cells	Takes into account electronic noise	Time consuming Relies on the assumption that the initial calculation was correct	Produced moderate background in both channels. Produced high stain indexes for CD3 and CD8, while producing a moderate stain index for CD4. This is an optimal method.
Method 7 – Optimized with Capture Beads	Takes into account electronic noise	Time consuming Relies on the assumption that the initial calculation was correct	Produced high background in both channels. Produced a high stain index for CD3, CD4 and CD8. Not an optimal method.
Method 8 – Optimized with 8 Peak Beads	Takes into account electronic noise	Time consuming Relies on the assumption that the initial calculation was correct	Produced low background in both channels. Produced a moderate stain indexes for CD4 and CD8, while producing a low stain index for CD3. Not an optimal method.

Voltration methods compared with Pros, Cons and conclusions.

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