MODERN
FLOW CYTOMETRY

Antibody Panels

Data Analysis

Controls

Cell Sorting

Statistics

EXCYTE
EXPERT CYTOMETRY

MODERN
FLOW CYTOMETRY

MODERN FLOW CYTOMETRY
CONTENTS

WHAT IS MODERN FLOW CYTOMETRY?

Flow cytometry is a tool in biotechnology that is used to evaluate physical and chemical characteristics of single cells at the whole cell level. The technology was introduced in the 1930s, but it wasn't until technological advancements in instrumentation that flow cytometry was able to expand its usefulness beyond basic cell calculating and sorting. Today, researchers can use the technology to perform complex experiments to work toward solving some of the world's greatest mysteries in health science. In addition to impacting how cells are analyzed, the commercial innovations of flow cytometry have also had an important impact on how researchers conduct experiments. This book provides instructions and guidelines for performing modern flow cytometry that should render experiments that are more successful.

In modern flow cytometry, the way you start an experiment remains the same. You must define the hypothesis or biological question that you want to answer. This initial step helps to confirm that flow cytometry is the correct technique for answering the question. One major determination that you need to make is whether the cells should be purified using sorting or if the experiment is simply for analytical purposes. In Chapter 1, "Controlling Your

Experiments", we consider the controls that can help ensure the experiment accurately tests the hypothesis.

Once you've established that flow cytometry is a suitable tool for your experiment, you next need to design your flow cytometry panel. An important part of this step is identifying the reagents that are needed for the experiment. In immunophenotyping experiments, this involves knowing which subpopulations need to be identified and determining the antibodies that are needed for the experiment. Not all experiments will involve complex phenotyping. For example, cells may be transformed to express green fluorescent protein (GFP) or other fluorescent proteins as a measure of the expression of some gene. Other experiments may involve measuring biological processes such as reactive oxygen, proliferation, or cell cycle. Regardless of your experiment parameters, understanding how to develop the flow cytometry panel is a very critical step. Chapter 2, "Creating the Right Panel", provides strategies for designing a panel that can save you time and ensure the experiment is efficient.

A final precursor to conducting an experiment using modern flow cytometry is understanding the output data and how it will be analyzed. This is accomplished by understanding the statistical analytical process that will be used to prove or disprove the hypothesis determined in the first step. Output data in flow cytometry is discussed in Chapter 3, "Flow Cytometry Statistical Analysis".

Once you understand your experiment, you are ready to conduct the experiment. This is the part of the process that varies significantly in modern cytometry in comparison to the processes of old. It involves knowing the protocols that will be used, setting up the instrument, and collecting the data. Core managers and their staff that run facilities have expertise in these areas and can offer assistance. In modern cytometry, cell sorting continues to be a central focus of conducting an experiment. In Chapter 4, "Cell Sorting", we explore how this process has advanced.

Next in the process is analyzing the data. This step consists of using the flow

cytometry software application of your choice to identify the populations that are relevant for the experiment. This should line up with the reagents you identified earlier in the process. Then, you extract the information necessary to test the hypothesis and answer the question you posed in the initial step. In Chapter 5, "Data Analysis", we discuss techniques for gathering data analysis for your flow cytometry experiments.

The final step in a flow cytometry experiment is to relax and enjoy. Flow cytometry is a powerful tool for answering biological processes at the whole cell level. A well-designed and executed experiment can provide a wealth of data, and being the first to unravel some secret about the biology of an organism is a very powerful feeling. Repeat the process as needed. Flow cytometry is constantly evolving and researchers must stay informed of the changes. Chapter 6, "Advanced Topics", provides an introduction to some of the topics you may want to consider to learn more about modern flow cytometry.

CONTROLLING A FLOW CYTOMETRY EXPERIMENT

Controls in a flow cytometry experiment serve to manage points in the process that could steer it from achieving accurate assessment of its hypothesis. You should apply these controls to each and every flow cytometry experiment. The following techniques can help you control your flow cytometry experiments and make sure they accurately test your hypotheses:

➤ **Autofluorescence Control**
➤ **Isotype Control**
➤ **FMO Control**
➤ **Cell Counting**
➤ **Compensation**

AUTOFLUORESCENCE CONTROL

Autofluorescence is the term used to describe the natural fluorescence that occurs in cells. The common compounds that give rise to this fluorescence signal include cyclic ring compounds such as NAD(P)H, Collagen, and Riboflavin. Aromatic amino acids such as tyrosine, tryptophan, and phenylalanine also

have a fluorescence characteristic. These compounds absorb in ultraviolet to Blue range (355-488 nm) and emit in the Blue to Green range (350-550 nm). The consequence of autofluorescence is the loss of signal resolution in these light ranges and a decrease in signal sensitivity.

Autofluorescence typically increases with cell size. Large cells have more autofluorescence than smaller ones because larger cells often contain more autofluorescent compounds. You can control this variability by aligning your instrument to compensate for excess excitation. This involves investigating the laser lines to determine if they are able to minimize autofluorescence.

ISOTYPE CONTROL

Isotype control refers to the genetic variation in the heavy and light chains that make up the whole antibody moiety. In mammals, there are nine possible heavy-chain isotypes and two light-chain isotypes. Every antibody will have a specific isotype, and you can find this information on the technical information specification sheet. For example, you might have an antibody with an isotype of IgG1, kappa. This indicates the heavy chain is a IgG1 isotype.

Isotypes can have different non-specific binding affinity to cells, which lead researchers to use isotype controls to identify what cells were positive or negative. Even though some researchers (and reviewers) continue to insist on using isotype controls to set positivity, they can only be used to identify potential blocking problems. Thus, isotype controls serve a purpose in early stage development of panels and in revealing blocking issues, but must never be used as the basis of determining positivity.

FMO CONTROL

Fluorescence Minus One (FMO) control is a type of control that you can use to properly interpret flow cytometry data. It is used to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel. An FMO control contains all the fluorochromes in a panel, except for the one that is being measured. For example, in a four-color panel there

would be four separate FMO controls, as shown in **Figure 1**.

Antigen	FITC	PE	Cy5-PE	APC
CD3 FMO	---	CD4	CD8	CD19
CD4 FMO	CD3	---	CD8	CD19
CD8 FMO	CD3	CD4	---	CD19
CD19 FMO	CD3	CD4	CD8	---

Figure 1: A 4-color panel with FMO Controls

The FMO control ensures that any spread of fluorochromes into the channel of interest is properly identified. The proper use of a single FMO control in a 3-color experiment is illustrated in Figure 2 below.
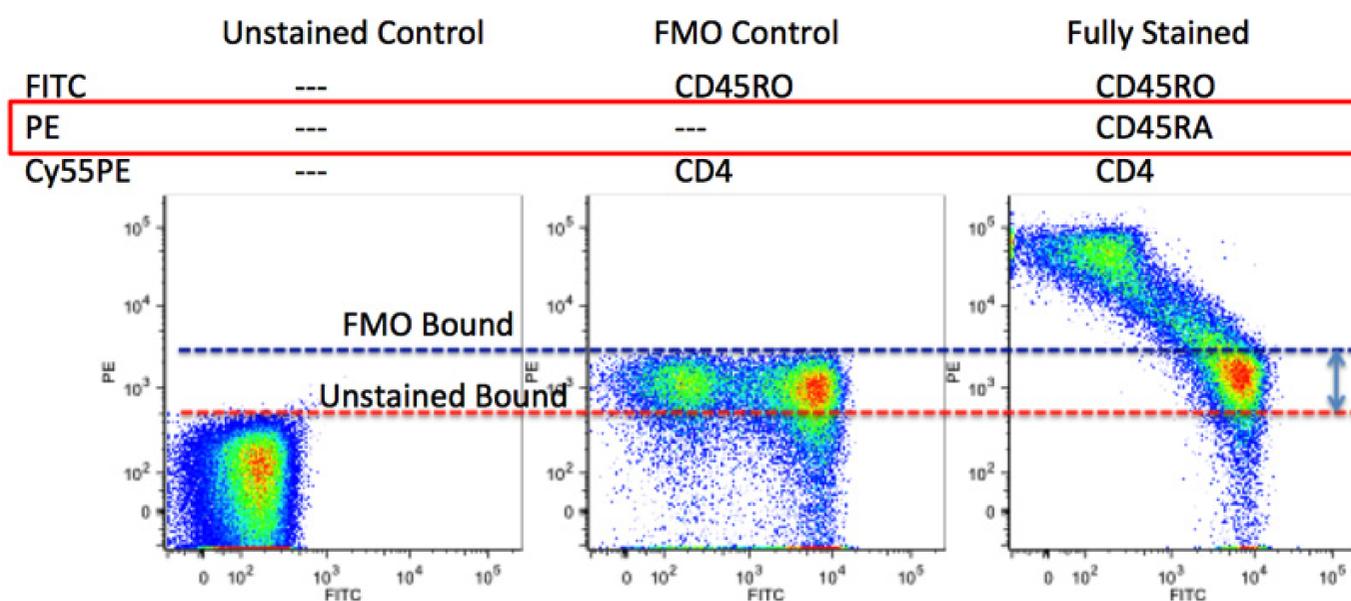
The image in Figure 2 shows the difference between the isotype bound and the FMO bound represents the spread of the data due to the fluorescence spread in the PE channel.

CELL COUNTING

The hemocytometer is considered the gold standard for cell counting. Invented by Louis Charles Malassez, this precision-etched microscope slide allows researchers to count cells under a microscope with high accuracy. This microscope is inexpensive in comparison to other methods, but that does not mean that it is the most efficient or fastest method.
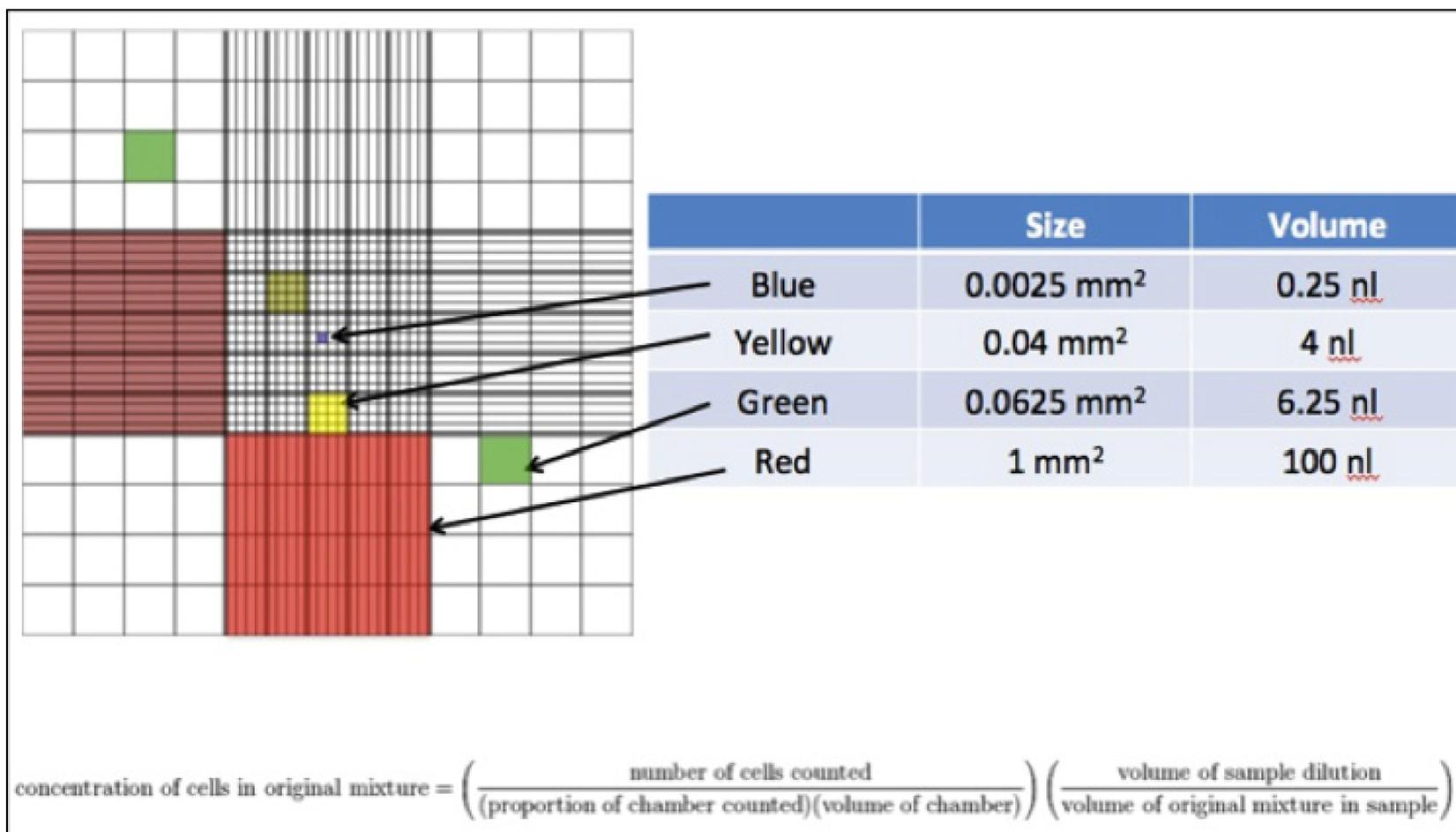
ExpertCytometry.com

	Size	Volume
Blue	0.0025 mm²	0.25 nl
Yellow	0.04 mm²	4 nl
Green	0.0625 mm²	6.25 nl
Red	1 mm²	100 nl

$$\text{concentration of cells in original mixture} = \left(\frac{\text{number of cells counted}}{(\text{proportion of chamber counted})(\text{volume of chamber})} \right) \left(\frac{\text{volume of sample dilution}}{\text{volume of original mixture in sample}} \right)$$

Figure 2: Hemocytometer Cell Counting

The key to using a hemocytometer is training. Since you will be visually inspecting the cells within a boundary, the rules of what cells to count and what to exclude on those boundaries becomes critical. If you are counting more than one sample, proper cleaning of the hemocytometer is a second critical step. Failure to completely remove the cleaning solution can cause cell lysis and lower than expected cell numbers.

The bias that an investigator brings to the hemocytometer and the slow speed for counting cells is the reason many users are moving toward automated counting methods. These can be divided into three major categories: image-based, impedance-based, and cytometry-based. Image-based methods such as the one implemented in the Cellometer, the T20, and the Countess use a system that takes a picture of a defined area using a proprietary slide and identifies the cells based on the relative size. These systems can also count "dead" cells using Trypan Blue, which is similar to what can be done with the hemocytometer. All of these systems are relatively fast at counting the cells and accurate within certain size ranges.
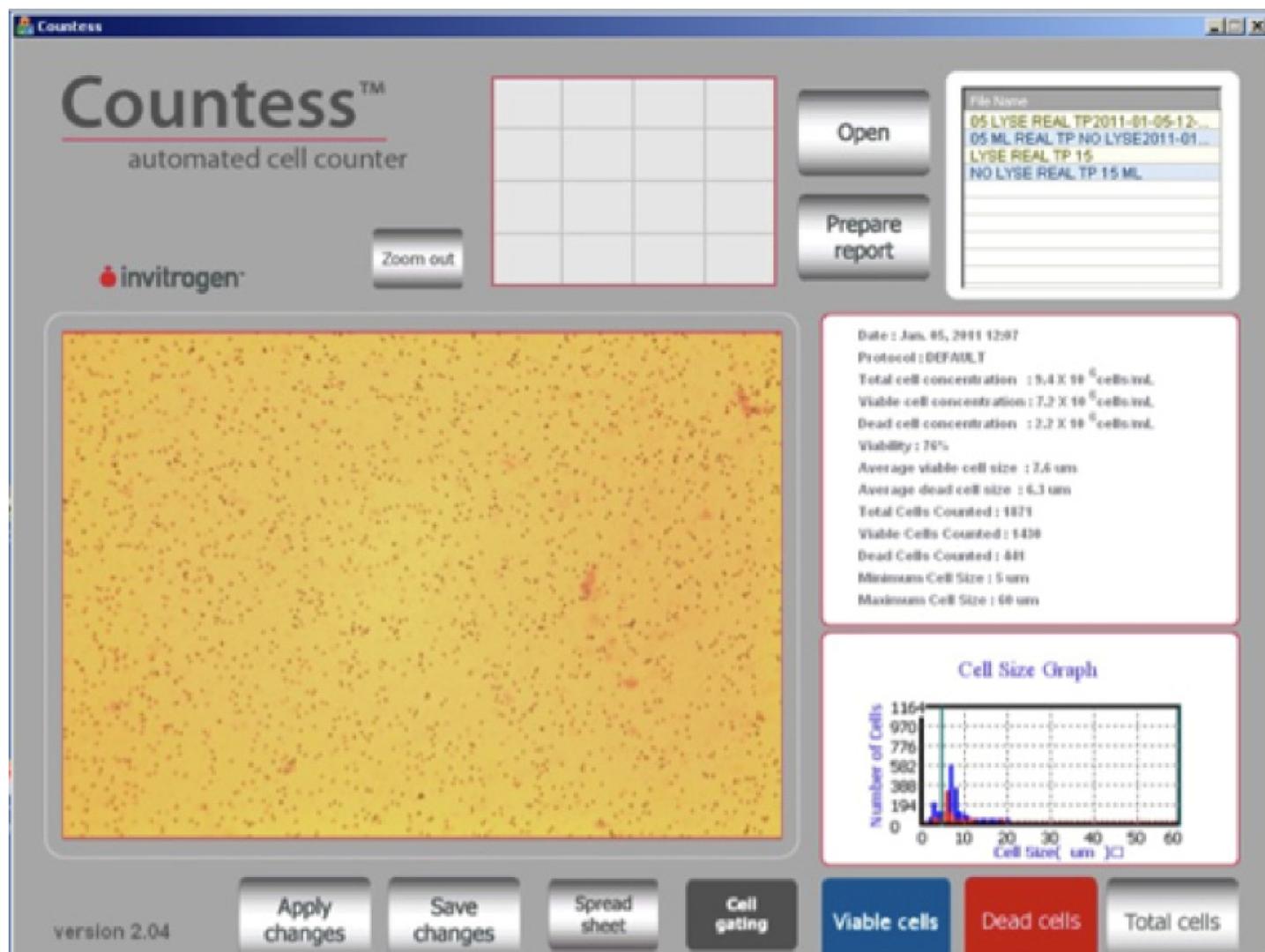
Figure 3: Countess Automated Cell Counter

Wallace Coulter discovered and patented the impedance principle for measuring cells in solution. This technique is still used in a clinical setting in cell counters today. It is also a very accurate way to measure the number and size of cells. Impedance measurements, as commercialized in the Coulter counter, the Scepter, and the Casy counter are also very accurate and require a diluted sample (especially the Scepter). Dead cells are measured based on their size (smaller than normal cells). The Scepter has the advantage of being a hand-held, pipet like device, making it amenable to rapid cell counting in a tissue hood.

The use of the flow cytometer as a cell counter requires a pump-driven system, which provides an accurate measurement of the volume of sample. It is a simple calculation to determine the concentration of the sample.

Instruments such as the Accuri and the Guava are excellent tools for counting cells. In addition, you can use a cell impermeant dye such as PI or 7AAD to measure the dead cells. This is more accurate than using Trypan Blue and visually inspecting the "blueness" of the cells.
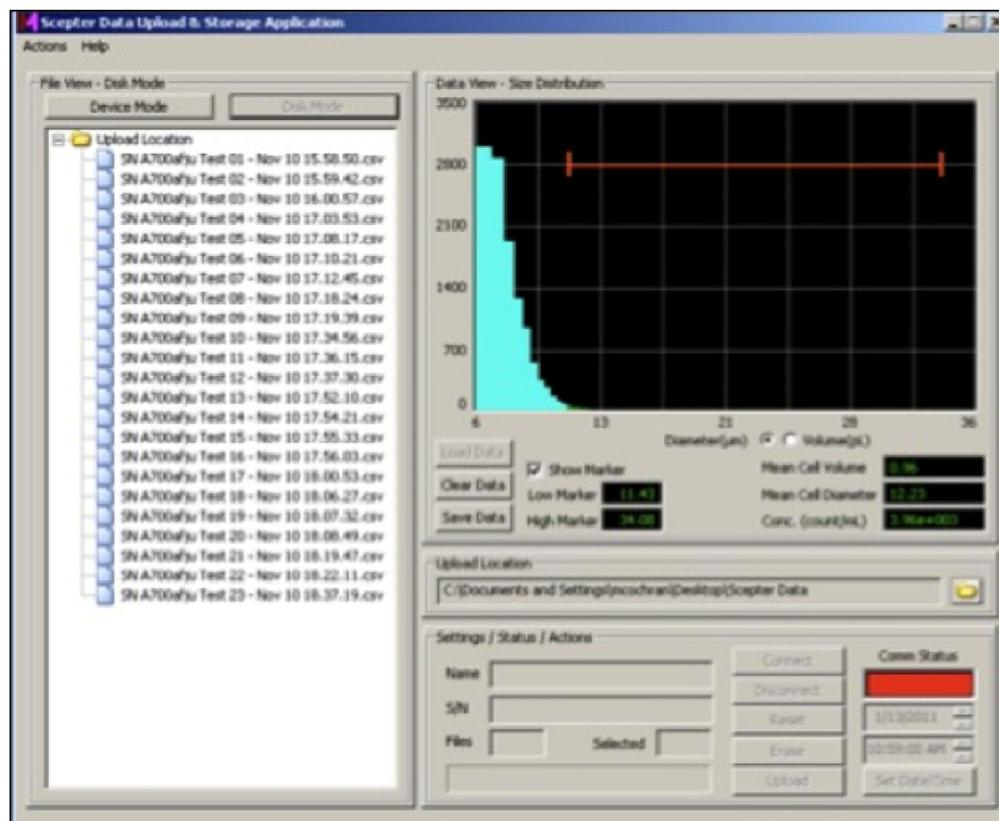
Figure 4: Scepter Counter

With a displacement or pressure system that is typically available in instruments, an extra step is required. Accurate volume measurement is not possible with this method of cell counting. To compensate, you must add a counting particle to the sample. A very accurate pipet is required to dispense the counting particle into the sample. Once the sample is run on the flow cytometer, you can then measure the number of counting particles and the ratio of collected particles to total particles to determine the original count in the sample. This method is suitable for high throughput applications that are typically integrated into the sample at the end, rather than a simple counting method at the beginning of an assay.
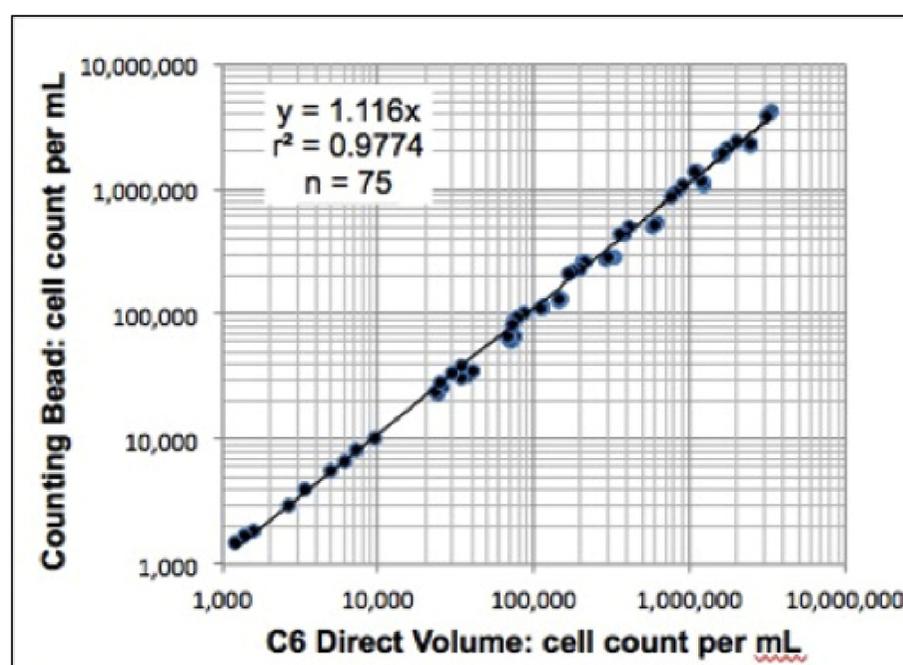

Figure 5: Example of Cell Concentrations Comparison.
Reprinted with permission from Accuri Cytometers (Accuri Cytometers, 2010).[1]

ExpertCytometry.com

The bottom line of cell counting is that the method you use is unimportant. You should, however, use a method that is consistent and reproducible.

COMPENSATION

One of the most important controls in proper flow cytometry is compensation. Compensation is the process used to correct spillover, the overlap of a fluorochrome into a second channel caused by the physics of fluorescence. Compensation is a mathematical value that makes sure that contributions from the fluorochromes that are not being displayed are not affecting the distribution of the data being displayed.

Manual compensation is the process of adjusting compensation based on the visual appearance of the data. Make sure you remove any manually compensated data from your lab notebook. Manual compensation results in overcompensated data, which yields incorrect conclusions. The best practice is to use automatic compensation algorithms that are available in current versions of flow cytometry software that are based, in part, on the work by C. Bruce Bagwell and Earl G. Adams that describes the mathematics behind multi-parameter compensation (Bagwell & Adams, 2006).[2]

Successful automatic compensation is based on the following three rules:

1. The controls must be at least as bright as the samples they will be applied to. Brighter is better, but not off scale.
2. Background fluorescence should be the same between the negative and positive population. Avoid using the universal negative for compensation.
3. The compensation color must be the same as the experimental color. For example, don't use Alexa488 to compensate for FITC.

If you follow these rules and the compensation doesn't appear accurate, resist the temptation to edit the compensation matrix to produce better data results. Instead, troubleshoot the problems and work to resolve them.

CREATING AN EFFECTIVE FLOW CYTOMETRY ANTIBODY PANEL

Designing a flow cytometry panel that is accurate and effective is similar to solving a puzzle. In the eyes of the flow cytometrist, the puzzle is trying to optimize the ability to make a sensitive measurement to answer the biological question the researcher has set out to answer. It is very common for the process to take a month or longer. This is mainly because panel design requires a delicate balance of biology and physics. Understanding the biology of the system and the physics of flow cytometry are critical to success. Despite antibody panel design and flow cytometry experimental design in general being a complicated process, there are ways you can simplify the process.

ANTIBODY RANKING

You should start the antibody panel design process by ranking your antibodies based on cellular expression level and importance in answering the biological hypothesis. For example, CD3 is a highly expressed antigen on T-cells and is important in making primary gating decisions, while CD86 is a dimly expressed (or emergent) marker on cells undergoing activation and may be critical to answer the biological hypothesis.

Knowing the approximate antigen density will help you rank them in proper order. You can use common categories such as high density, intermediate density, low density, an unknown density. For some common antigens, these values are known. Downloading an antigen density chart from the Internet can help.

FLUOROCHROME BRIGHTNESS

Fluorescence brightness can be measured and different fluorochromes compared to each other. Fluorescence brightness has two very important characteristics: laser power and detector efficiency. For each instrument, fluorescence brightness can be different, and should be part of the development process of any panel. New fluorochromes are shaking up many brightness charts. The addition of new brilliant violet dyes, for example, are spectacularly bright compared to traditional fluorochromes. Use a chart that ranks fluorochromes from brightest to most dim. Based on the brightness of fluorochromes and the expression density of the antigen on the cells, you want to pair highly expressed antigens such as CD3 with dimmer fluorochromes. You should then pair lower expression antigens with brighter fluorochromes.

INSTRUMENT CONFIGURATION

You must understand the configuration of the instrument that is being used in the experiment. This is a significant factor in designing an accurate and effective antibody panel. This means learning the instrument's excitation light sources and its sensitivity and quality control specifications. Instrument excitation light sources refers to the lasers that are available and whether they are co-linear or parallel. The laser sources and the pathways determine the fluorochrome choices that can be used. Especially with co-linear lasers, some fluorochrome choices may have to be eliminated.

Regarding the instrument's sensitivity and quality control specifications, pay particular attention to where the most sensitive measurements can be made on the system. Everything doesn't boil down to just fluorescence

intensity. Sometimes, it is better to use a less bright fluorochrome if the channel does not receive a lot of error. Figure 8 is an example Excel analysis of the spillover of fluorochromes into different detectors on a 4-laser instrument (405, 488, 532, 633). Adding the values across results in the error that that a given detector receives from the fluorochromes in the panel. Adding the values down the columns results in the amount of error a given fluorochrome contributes to the panel in question.


Figure 6: Example of Fluorochrome Spillover
(Courtesy of University of Rochester's Flow Cytometry Resource)

Using the above analysis (Nguyen, 2013)[3], you could make an informed determination of where the best fluorochrome choices are to make sensitive measurements.

This stage of panel design is where everything is pulled together. Start by paring the high antigen expression with dimmer fluorochromes. Those targets of low or unknown antigen expression should be paired with brighter fluorochromes. An additional consideration is to minimize the spread of error, especially in channels where sensitive measurements are made. Methods for determining the spread of error into different detectors has been published in several publications (Perfetto, Chattopadhyay, & Roederer, 2004).[4]

The data would look something like this:
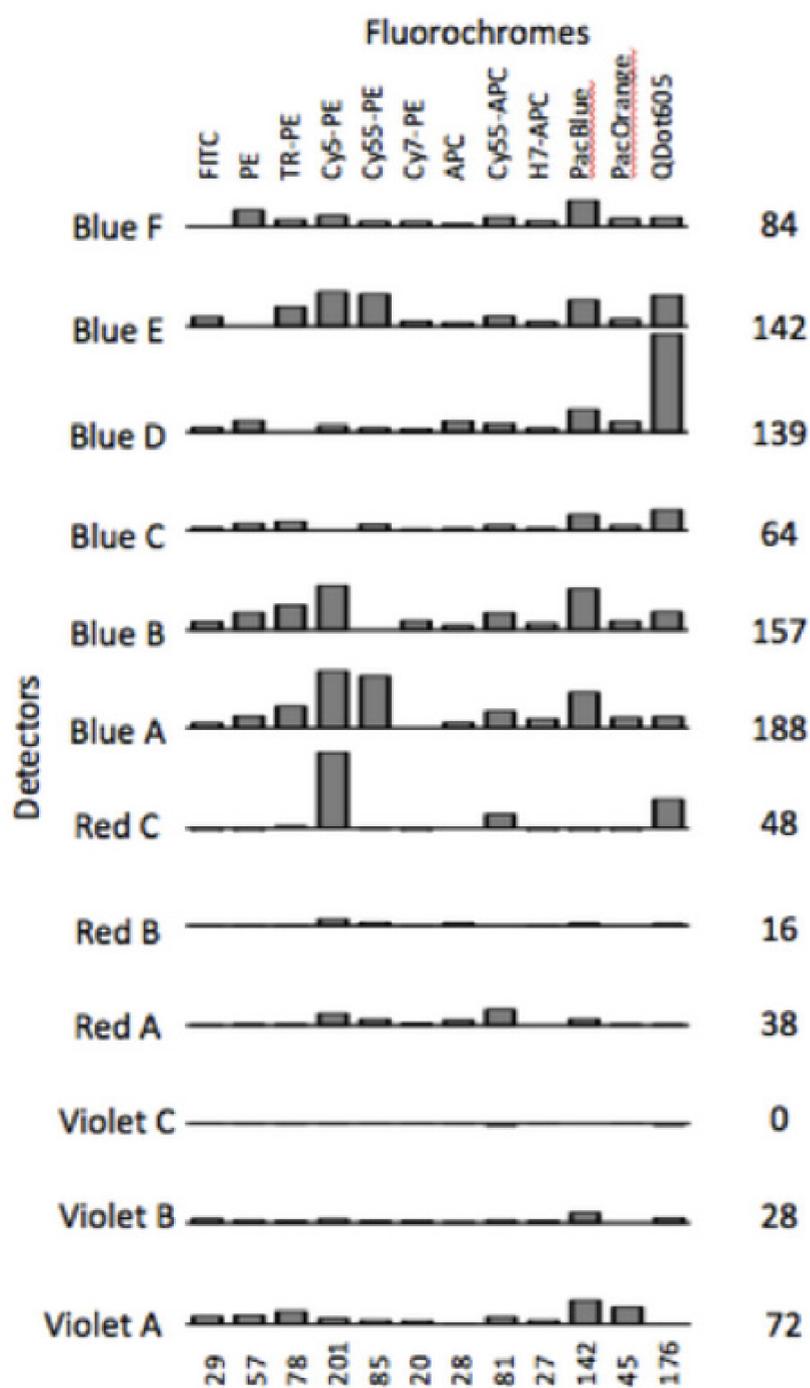
In Figure 9, the sum across the detectors reveals the amount of error that each detector receives. Summing down the fluorochromes reveals the amount of error that each fluorochrome contributes to the total panel. This type of chart can help identify where it is best to make the most sensitive measurements in the context of where the greatest spillover is, which reduces the sensitivity.

Figure 7: Example of Paring Antigen Expression with Fluorochromes (Courtesy of University of Rochester's Flow Cytometry Resource)

PANEL BUILDING SOFTWARE

As stated in the introduction to this book, advancements in technology have been a significant contributor to modern flow cytometry. Using a panel building program to design your antibody panel can save you valuable time. A critical step in the design process is finding all the antigen-fluorochrome pairs that are available. While it is possible to search through catalogues and use popular search engines such as Google, using one of the many software applications that are available can help you in the selection process. Three of the most popular applications are listed below (in alphabetical order):

> **Chromocyte** – a web-based system panel building system and a resource website (Home Page: chromocyte, n.d.).[5]
> **Fluorish** – a resource with a free downloadable panel building tool (Home Page: Fluorish, n.d.).[6]

➤ **FluoroFinder — a new resource with a web-based interface for panel building (Home Page: FluoroFinder, n.d.).**[7]

STAIN INDEX

Knowing how to optimize the reagents in your experiment is crucial. This is a multi-step process, first of which is titration of the antibodies. This process, discussed in more detail in the next section, ensures that that the optimal antibody concentration is used. If there are too much antibodies, sensitivity is reduced by increasing background (SI is decreased). Use too few antibodies and sensitivity is also reduced by decreasing the positive signal.
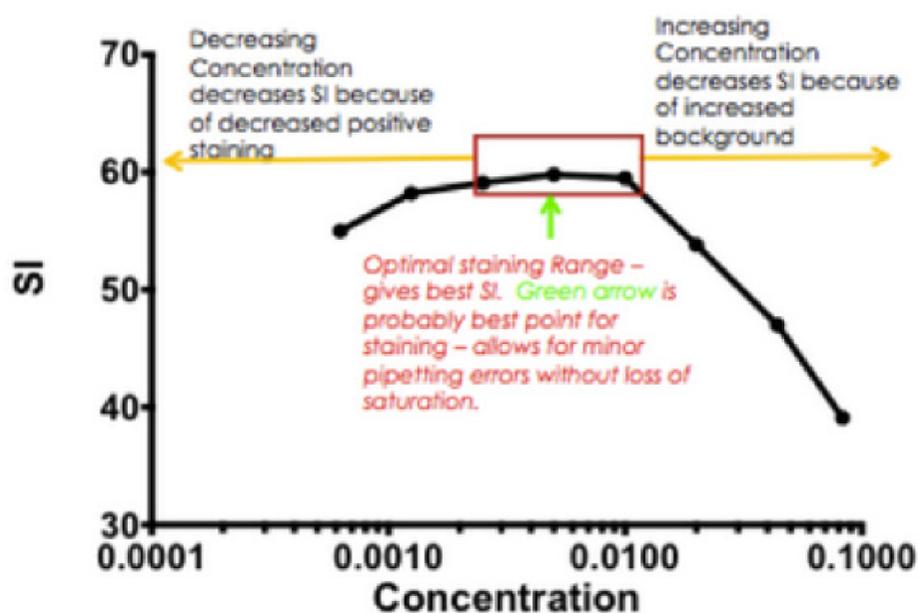
Figure 8: Results in Changes in Antibody Concentration

The second step is optimizing the voltage on the instrument for each fluorochrome. Staining the cells with optimal antibody concentration, then run a voltage series to determine if increasing the voltage will improve the Staining Index (SI).
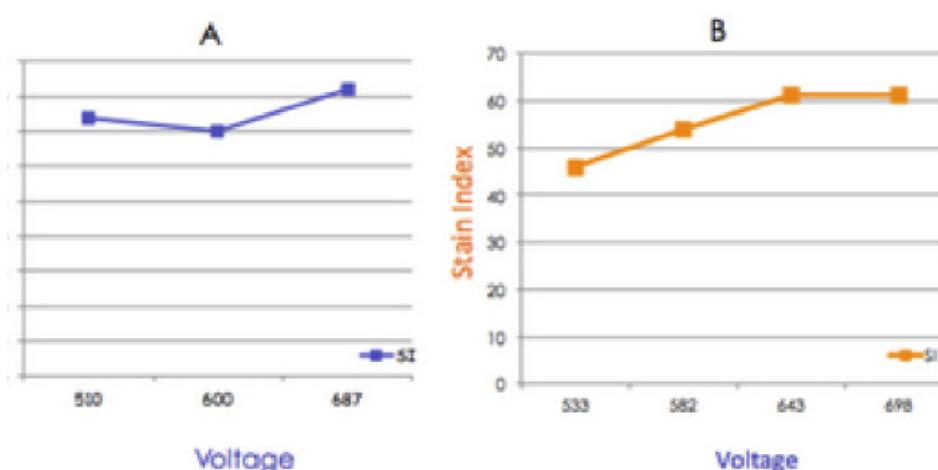
Figure 9: Changing Voltage to improve S/N

In the panel on the left, increasing the voltage doesn't change the staining index significantly. In the right panel, increasing the voltage shows an improvement to the staining index. Pairing highly expressed antigens (like CD3) with dimmer fluorochromes, and the antigens of interest with the brightest fluorochromes, is a key part of panel design with few tools to help.

With early generation instruments, pairing the antigens of interest with the brightest fluorochromes was relatively easy to determine since fluorochrome choice was limited. With the advent of instruments capable of measuring more than 4 fluorochromes, there is a need to characterize the relative brightness of different fluorochromes under actual experimental conditions, rather than as free fluors.

$$SI = \frac{\mu_{pos} - \mu_{neg}}{2\sigma_{neg}}$$

Figure 10: Formula for Stain Index

This equation, shown in Figure 12, compares the differences between the means of the positive and negative and corrects this value by dividing by two times the spread (as measured by the standard deviation) of the negative population (Bigos, 2007)[8] (Maecker H. T., 2004)[9]. This measurement, called the Staining Index (or SI), allows for the comparison of the relative brightness of fluorochromes. As shown in Figure 13, if different fluorochromes are attached to the same antigen, cells can be stained and the SI compared. Failing to consider SI values is a significant mistake that can negatively impact your flow cytometry experiments.

Reagent	Clone	Filter	Stain Index
PE	RPA-T4	585/40	356.3
Alexa 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1
PerCP-Cy5.5	Leu-3a	695/40	92.7
PE-Alexa 610	RPA-T4	610/20	80.4
Alexa 488	RPA-T4	530/30	75.4
FITC	RPA-T4	530/30	68.9
PerCP	Leu-3a	695/40	64.4
APC-Cy7	RPA-T4	7801/60	42.2
Alexa 700	RPA-T4	720/45	39.9
Pacific Blue	RPA-T4	440/40	22.5
AmCyan	RPA-T4	525/50	20.2

Slide Courtesy of Holden Maecker, Ph.D.

Figure 11: Stain Index Calculations

The above calculations reveal that PE > APC > FITC > PerCP, which provides the researcher with critical information necessary to design the polychromatic antibody panel (Maecker & Trotter, 2006)[10].

In early flow cytometry, there were some limitations to SI. The most notable limitation was the fact that the data was corrected by the standard deviation of the noise. With so many fluorochromes on digital instruments, at least part of the negative signal is background noise that can be attributed in part to the electronic noise in the flow cytometer. A modification of the SI was pulsed in 2009 (Telford, Hawley, Subach, Verkhusha, & Hawley, 2012)[11]. The equation for this correction is shown in Figure 14.

$$SI = \frac{Med_{pos} - Med_{neg}}{[(84\%_{neg} - Med_{neg})/0.995]}$$

Figure 12: Stain Index Correction Equation

In this equation, the difference between the medians of the positive and negative populations and divides that by the right hand of the negative distribution, as reflected by the 84% of the negative minus the median of the negative. This Separation Index (or SI) provides a similar metric to the Bigos/Maecker Staining Index as shown in Figure 4.

Fluorochrome	Separation Index	Staining Index
PE-TR	230.77	142.09
PE-CY5	216.26	133.00
PE	214.22	126.29
PE-CY7	181.33	110.75
FITC	180.52	104.28
PERCP CY5-5	177.82	107.25
PE-CY5-5	166.73	105.76
AF 488	158.38	94.26
APC	92.80	48.02
CY 5	61.04	32.02
AF 700	52.53	26.98

Figure 13: Stain Index Matrix

Whether you use staining or separation, SI is a critical parameter and the experiment is straightforward, and can be performed using any highly-expressed antigen that is available in many fluorochromes.

TITRATION

After you've added all the critical elements to the antibody panel, you should optimize it. Proper antibody titration is critical to ensure that the panel works as you expect. Titration is the process of identifying the best concentration to use an antibody for a given assay. While the vendor will provide a specific concentration to use, this may not be appropriate for your assay.

Performing titration is a simple process: fix cell concentration, time of incubation, volume of reaction, and temperature. Figure 16 illustrates how titration works. The graph displays an antibody from Leinco Technologies that was used to stain 1×106 cells for 20 minutes on ice. To identify the best concentration to use, the modified Staining Index was calculated (Telford, Hawley, Subach, Verkhusha, & Hawley, 2012)[11] and plotted against the concentration, as shown in Figure 16.
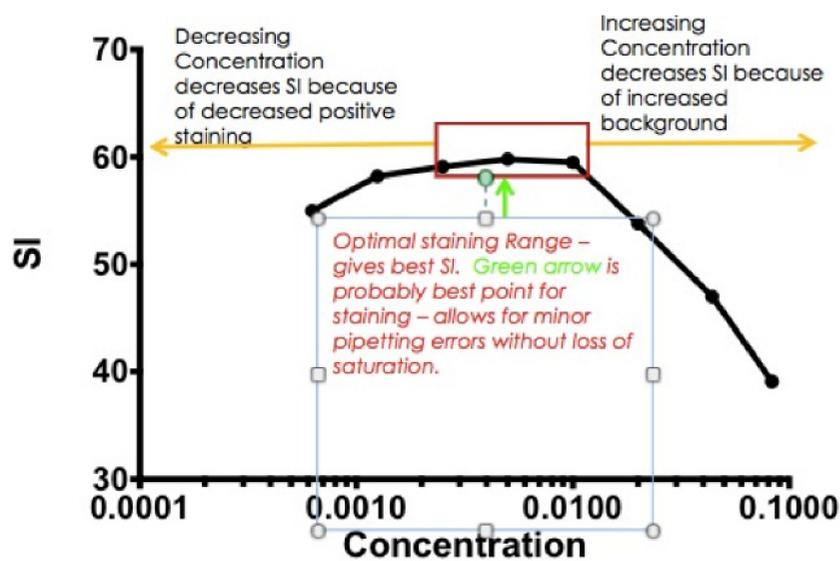
Figure 14: Optimal Staining Range

As is shown again in Figure 16, as concentration increases above 0.5 µg/ml, SI decreases. This is due in part to the increase in the background (non-specific staining). At concentration levels below 0.25 µg/ml, SI decreases because the antibody is no longer at a saturating concentration. Thus, the best concentration to use is between 0.25-0.5 µg/ml.

Titration helps save money and reagents, ensures the optimal concentration of reagent is being used, and avoids background due to high concentration of antibodies.

OMIPS

The elements of panel design mentioned above can simplify the experimentation process, but there is still some knowledge and understanding that is required. If you lack this background, an excellent resource for creating an antibody panel is an Optimized Multicolor Immunofluorescence Panel (OMIP). An OMIP is a peer-reviewed, optimized flow panel. The benefit of using an OMIP is that the work is already done for you. This includes the antigens, fluorochromes, and analysis template to use.

There are currently more than 23 OMIPs, all of which can save you the headache of building a panel from scratch. OMIPs are published in the journal Cytometry A, which reports the results of researchers who have developed multicolor panels (Roederer & Tarnok, OMIPs — Orchestrating multiplexity in polychromatic science, 2010)[14]. These results are the basis of developing and modifying fluorescent panels.

Of course, there are still an uncountable number of antibody panels for which no OMIP exists. If you're designing a new antibody panel, or are stuck after several attempts to optimize an existing panel, get expert help. Don't keep wasting time and resources on an incorrectly designed panel.

FLOW CYTOMETRY STATISTICAL ANALYSIS

Understanding statistics and flow cytometry statistical analysis is critical to understanding flow cytometry data. One of the powers of flow cytometry is the fact that we generate large amounts of data that are amenable to statistical analysis of our populations of interest. Using the standard set of statistical analysis tools allows for hypothesis testing and ultimately determining if there is statistical significance in the datasets.

FLOW CYTOMETRY STATISTICS

There are two basic classes of questions that are typically asked in flow cytometry. The first class relates to changes in the number or percent of a specific population on treatment or disease state. A hypothesis in this class might look like this:

CASE 1: IN PATIENTS SUFFERING FROM BOWDEN'S MALADY, TREATMENT WITH PESCALINE D CAUSES NO CHANGE IN THE PERCENTAGE OF CD86+ MEMORY T CELLS.

The second class of questions asked in flow cytometry relate to the changes

in expression of a given antigen upon treatment or disease state. A hypothesis in this class might be phrased similar to the following:

CASE 2: IN PATIENTS SUFFERING FROM BOWDEN'S MALADY, TREATMENT WITH PESCALINE D CAUSES NO CHANGE TO THE EXPRESSION INTERFERON GAMMA ON CD86+ MEMORY T CELLS.

Once you determine these questions, you are ready to perform an experiment with sufficient replicates (as determined by a power calculation), to extract the correct data for statistical analysis.

In Case 1, the data would be the percent of CD86+ memory T cells in patients with Bowden's Malady +/- treatment. This data would be compared using a T-test to determine significance. To perform the T-test, you would define the threshold (the a value), and calculate the P value.

> When $P < a$ – reject the null hypothesis and the difference is statistically significant.

> When $P > a$ – can't reject the null hypothesis, and the difference is not statistically significant.

In Case 2, the data that needs to be extracted is the central tendency of the expression of Interferon gamma on the CD86+ memory T cells. This is best represented as the Median Fluorescent Intensity (MFI). Additionally, the robust Standard Deviation (rSD) should be calculated, as it measures the spread of the data around the Median.

Before you move to hypothesis testing, it is often best to convert the data to a fold-over background, or resolution metric (R_D) value. This is especially important when you are performing multiple experiments. The R_D is better as it accounts for the spread of the data, not just the separation between experimental and control, as seen in Figure 17 below.

$$R_D = \frac{Median_{pos} - Median_{Ctl}}{rSD_{pos} + rSD_{Ctl}}$$

Once you calculate R_D, you can then move to hypothesis testing using a T-test against a hypothetical mean. In this case, the hypothetical mean would be *0*. Again, you need to define the threshold (the *a* value), and calculate the *P* value. The caveat for the T-Test is that the data follows a Gaussian distribution. If you do not have Gaussian distributed data, there are similar non-parametric tests that you can perform. They will result in a *P* value being reported and identification of statistical significance.

These basic pair-wise comparison tests allow you to determine statistical significance of two populations. If you have more than two populations or more complex questions, there are additional statistical tools that can be used, such as regression analysis and ANOVA analysis.

APPLYING THE RIGHT STATISTICAL TEST TO YOUR FLOW CYTOMETRY DATA

Flow cytometry data is numbers-rich. Data from experiments can be population measurements (percent of CD4+ cells, for example), or it can be expression level (median fluorescent expression of CD69 on activated T cells). Researchers are often content to show histograms to illustrate their point after a flow experiment. This approach misses the opportunity to take that content-rich data and extend the analysis into statistical analysis.

To properly perform statistical analysis, the first step is to understand the hypothesis. The hypothesis will guide the statistical analysis by identifying the correct test to be performed. There are several things that need to be considered when beginning the statistical analysis of the data. Statistical power answers the question of what is the probability of correctly rejecting the null hypothesis when the null hypothesis falls. There are three factors that influence the power of an experiment: the sample size, the spread of the data, and the number of replicates.

The power of the experiment is related to the ability of the experiment to avoid statistical errors. False positives (Type I errors) are when a true null hypothesis is incorrectly rejected. False negatives (Type II errors) are when the test fails to reject a false null hypothesis. In fact, the power of the experiment is defined as the b, which is equal to the True positive / (true positive + false negative).

The biological hypothesis and experimental design will determine the appropriate test for the data. The distribution of the data is also important to consider. How best to determine the correct test? This table can help you determine which test is most appropriate.

	H_0 is true	H_0 is false
Rejected H_0	Type 1 Error 'False Positive'	Correct outcome True Positive
Failed to reject H_0	Correct outcome True Negative	Type 2 Error 'False Negative'

Figure 18: Classes of Statistical Errors

The a value is the threshold that will be used to determine in the data is statistically significant or not. For historical reasons, this value is usually set at 0.05. This can be interpreted as the chance of finding significance where there is none; for example, the chance of committing a Type I error.

Once the a value is set, if the P-value is below that value, the data is statistically significant. The data is not more significant if the P-value is 0.01 and the threshold is 0.05 than if the P-value is 0.04. If there is an expectation, and a desire to decrease the Type I error, the threshold should be set to a more stringent level (0.01 or more).

In the case where the experimental design has Drug X, Drug Y, and the combination of Drug X and Y, to be compared to an untreated sample, what is the best test? Pairwise comparisons should not be performed in this case for the following reason. With the a set to 0.05, there is a 5% change of committing a Type one error. With each comparison, the change

of committing a Type I error increases, as shown Figure 18.

Number of pairwise comparisons	Changes of a Type I error
2	10%
3	15%
4	19%
5	23%

Figure 19: Pairwise Comparisons to Changes of Type 1 Errors

At the end of the day, statistical analysis of your flow cytometry data is a critical step for proving the validity of the hypothesis that was being tested. With careful and considered approach to performing the correct testing, the published data will stand up to the rigors of peer review and help lead to another discovery.

HOW TO PERFORM A T-TEST

With the ability to capture expression data at the single-cell level through many thousands of cells in a short time, flow cytometry generates rich data. The importance of those numbers and how to use them in hypothesis testing is critical to ensure the robustness of the analysis. After establishing the null hypothesis for the experiment, the type of statistical test, and the numbers necessary will become obvious. For example, if the null hypothesis states that the 'treatment of B cells with thiotimoline does not change the expression of CD221B in normal patients.

Based on this null hypothesis:

1. Assuming a Gaussian distribution, a T-test will be performed.
2. The median and robust Standard Deviation will need to be known for the untreated and treated samples.
3. Additionally, the threshold needs to be establish. In this experiment the threshold is set to 0.01.

The data is shown in Figure 19.

Control		Treated	
MFI	rSD	MFI	rSD
452	45	1254	119
521	52	1345	125
497	50	1059	100
388	28	888	89
601	58	1529	157
784	75	1789	184
488	50	1297	133
398	40	799	75
524	51	1404	139
544	52	1399	140

Figure 15: Threshold Example Data

Using a statistical tool such as Graphpad Prism, the T-test can be performed. Based on that result, the P value is less than 0.0001, which is below our threshold. Therefore the data is significant. There are some limitations with directly comparing the median fluorescent intensities, especially in the case of a multi-center study with different instrumentation and such. In that case, the conversion of the data to a resolution metric, such as the RD is recommended. In this case, a one-sample T-test can be performed on the data.

The R_D is defined as follows in Figure 20 below.

$$R_D = \frac{Median_{pos} - Median_{Ctl}}{rSD_{pos} + rSD_{Ctl}}$$

Figure 20

If the above data were to be converted, A would be defined as the treated sample and B as the control sample. That would result in the following data:

R_D
4.890243902
4.655367232
3.746666667
4.273504274
4.31627907
3.88030888
4.420765027
3.486956522
4.631578947

Figure 21: Another Threshold Example

A one-sample T-test allows the comparison of a single sample to be compared to a known population mean. In this case, the mean would be 0. This is because the assumption is that the treatment causes no effect, so MedianA-MedianB would be 0. When the T-test is run, the results show as expected that this data is statistically significant.
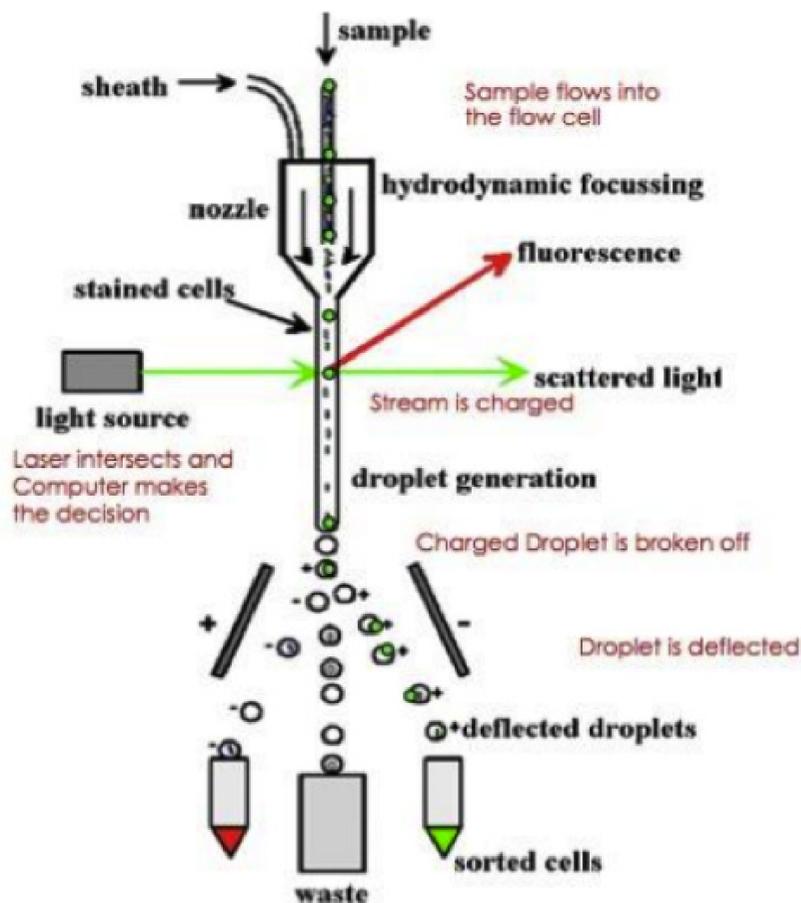
The advantage of the RD is that issues with the experimental system such as labeling and cell number and the instrument can be smoothed out, which makes comparisons easier to interpret and understand. This is a simple case of the power of statistics in flow cytometry.

CELL SORTING

Cell sorting remains the best tool to isolate and purify cellular populations that can be phenotypically defined. This is especially true for rare-event detection and purification. Successful rare event detection and purification requires some attention to ensure the best yield and purity.

Cell sorting is the process of isolating cells after identification of the cells using the principles of flow cytometry. The upstream components of the cell sorter are common to all flow cytometers. The difference is what is done with the cells after they have been interrogated and identified. The stream is vibrated to generate thousands of individual droplets (as many as 90,000 or more), a fraction of which contain a cell. Those droplets that contain a cell of interest can be electrically charged as they pass into an electric field, are deflected to the final receptacle, as shown in Figure 21 (Applied Cytometry, 2010)[12].

The two most common types of cell sorters are the Jet-in-Air and the Cuvette. The primary difference between the two is where the cells are interrogated. In the Jet-in-Air sorter, the cells are interrogated as the stream exits the nozzle. Cuvette-based systems interrogate the cells inside a cuvette before the

Figure 22: Cell Sorting Process

stream exits and droplets are formed. Regardless of the type of cell sorter you use, there are some steps you should take to ensure success.

A success measure you should take is to keep the core stream tight. The tighter the core stream (low differential pressure), the tighter the CV is on resulting data. That is, the easier it is to determine the best placement of gates for sorting. Tight core streams also reduce coincident events (two cells passing through the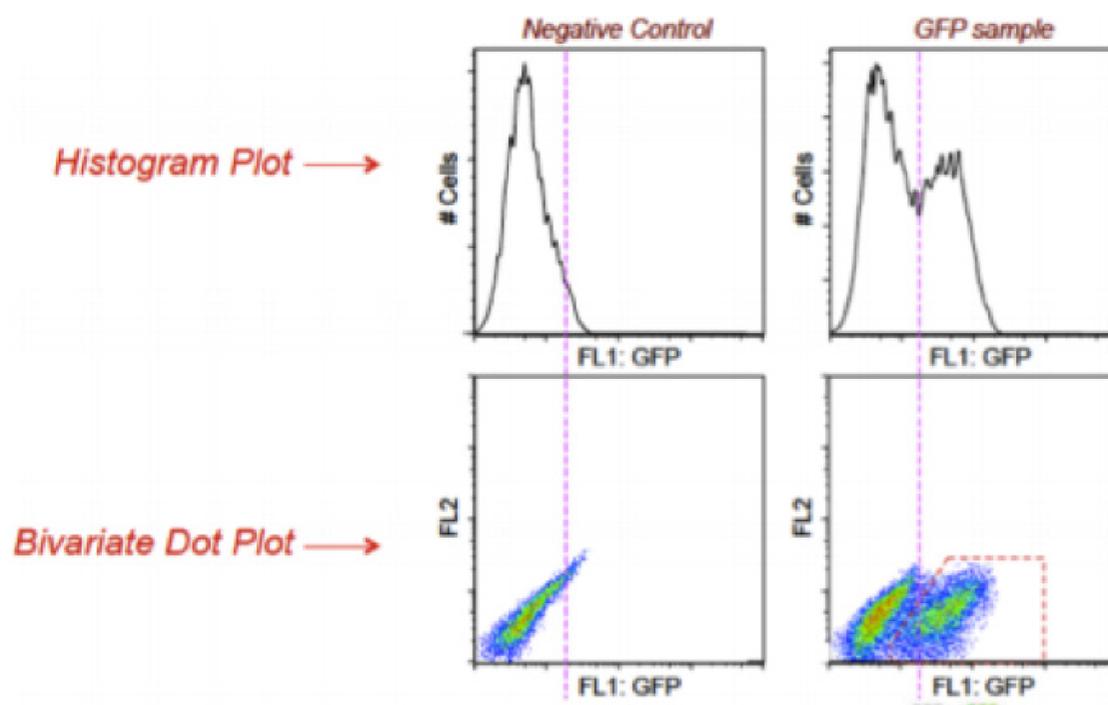 intercept at the same time). Since flow cytometry requires single cells for proper analysis, the more doublets, the fewer sorted cells.

You should also try to eliminate aborts. Aborts arise from a cell arriving at the laser intercept while the electronics are processing the previous pulse. This new event is lost (aborted). This results in loss of cells as well as decreased purity. Optimizing the window extension for the cell type can have a dramatic effect in improving the quality of the sort (and reducing the abort rate).

Avoiding artifacts is another method to increase success with cell sorting. You can accomplish this using viability dyes, avoiding aggregates, and gating for success. In reference to viability dyes, it cannot be stressed enough that a simple viability dye improves the quality of the sort for downstream applications by reducing the mis-sorted false-positive cells that are dead. In establishing a gating strategy, consider adding a pulse gate (height vs area, for example) to eliminate cells that are aggregated. Using the appropriate controls for gate placement and avoiding the use of the histogram. Histograms mask data and make it impossible to find rare events. Review the plots in Figure 22, courtesy of Jennifer Wilshire, for measuring GFP fluorescence.

Figure 23: Example Histogram and Dot Plots

Looking for the GFP population with a histogram is very difficult. Plotting a bivariate plot (against a PE signal, in this case) helps identify the cells that are truly GFP positive (bottom right). It also assists in demonstrating that the dim positive GFP cells can be separated from autofluorescence.

Finally, using a low threshold is recommended for cell sorting. When you set up a threshold, the cytometer is blinded to any signal that is below that threshold value. So while it is possible to eliminate debris and such from the data using a high threshold, if the cell sorter cannot see the debris, it will find its way into the sort tube and contaminate the cells for downstream applications.

IMPROVING CELL RECOVERY

Cell sorting can be a scary proposition. A precious sample is introduced into a machine that pressurizes the cells to 70 PSI, moves them past one or more lasers, and vibrates the stream at 90 kHz. This all happens before decelerating the cells to atmospheric pressure before they hit an aqueous surface. Many cells survive this journey. But some do not. A smart way to improve your cell recovery is to incubate your plastic tubes with a buffer solution containing protein. This will help reduce and possibly eliminate the charge on the plastic. Since the droplet containing the cell is charged, it can be attracted to the charge on the plastic. This results in the droplet hitting the side of the tube wall, and the cell dying as the small volume of liquid evaporates. To prevent

this, pre-coat the tube with protein buffer to neutralize the plastic charge. Even better, make sure that your tubes are not polystyrene.

The cells in your experiment will be traveling in a buffered saline. This is not very conducive for keeping cells alive for long periods of time. The good news is that you can improve cell recovery by ensuring that the catch buffer has some, but not too much, protein in it. Typically only 10-50% protein in the catch buffer is sufficient.

If you're sorting into media, make sure the media is buffered with HEPES. Buffers like RPMI are formulated to buffer in a CO_2 atmosphere (like the atmosphere found in your lab's incubator) and, don't buffer well in our normal atmosphere.

Making sure you maintain proper temperature for the cells is important for cell recovery. Smart scientists know how their cells respond to temperature differences. Some cells do not like to be kept cold and will die quickly if sorted into 4-degree Celsius buffer.

Sorting adherent cells adds a level of complexity to an experiment. The cells have to be disassociated to pass through the sorter, and this is often done with trypsin. The quickest and most common neutralization method is to add FBS to the cells. Be careful of this. While it neutralizes the trypsin effectively, it also adds back all the components that cells need to re-adhere to each other. Try soybean trypsin inhibitor instead.

Nothing is worse than a clogged nozzle when sorting. It adds time to the sort and reduces efficiency (and annoys the sort operator). Just before sorting, make sure to pass the cells through an appropriate sized filter to remove clumps and debris. The smartest scientists go as far as looking at their cells under a microscope to ensure that there are no clumps prior to sorting.

Make sure to include a viability dye in your staining panel. This will help eliminate dead cells. Using a viability dye is always a smart decision.

When trying to define a cell population, make sure you include both positive and negative markers in your antibody panel. The use of dump channels, negative markers and multiple positive markers will help ensure that the sorted cells are what they are supposed to be.

Know the cell count at the time the cells are going onto the sorter – NOT when you first began preparing them. Since an optimal sort speed is typically ¼ the droplet generation frequency, overconcentration will reduce purity at the backend. Bring some dilution buffer with you just in case the cells are too concentrated.

The higher the threshold, the easier it is to visualize the specific cell population. But this doesn't eliminate the fact that the debris and junk are still present within the cell population you're visualizing. It means that the cytometer is ignoring it. Here's the key: whatever the cytometer ignores will end up in the final sorted population. For example, someone may send a "pure" population of sorted mature B cells in for genomic sequencing only to learn that hemoglobin (from contaminating red blood cells) is the most abundantly expressed gene in their sample.

MEASURING CELL SORTING PERFORMANCE

One of the most important metrics to cell sorting is the sort purity. Purity is defined in Figure 23.

$$P = \frac{\# \textbf{\textit{target cells}} \textit{ in sorted tube}}{\# \textbf{\textit{total cells}} \textit{ in sorted tube}}$$

Figure 4: Purity Definition

Unfortunately, purity is not a good measure of instrument performance. Purity can be good when the instrument is performing well and not well. If the drop delay is not calculated correctly, the purity can be good, but the recovery will suffer. There have been a few publications and posters on this, but it has gone largely unnoticed that measuring purity is not the best way to measure sort performance or to calculate the drop delay, but it is the method that

most commercial sorters use to do this. How do we do a better job?

$$\text{Recovery:} \quad R = \frac{\text{\# target cells in \textbf{sorted tube}}}{\text{\# target cells sorted as \textbf{indicated by the instrument}}}$$

$$\text{Yield:} \quad Y = \frac{\text{\# target cells in \textbf{sorted tube}}}{\text{\# target cells in \textbf{original sample}}}$$

CALCULATING RECOVERY, YIELD, AND PURITY

If you want to calculate yield, there are a lot of counting methods such as cell counters and Neubauer chambers, but these are associated with a fairly high level of error. As long as you sort similar particles that show the same Poisson behavior when sorted, the following holds: at any given time, after a fraction α of the original sample is sorted, the absolute number of target particles *(t)* in this fraction $(\alpha.Ot)$ is distributed between the sorted tube *(St)* and the center stream catch *(Ct)* collected during the sort:

$$\alpha O_t = S_t + C_t$$

The same applies for non-target particles where:

$$\alpha O_{nt} = S_{nt} + C_{nt}$$

We can estimate the maximum recovery for a given set of sort conditions as follows:

$$Rmax = \frac{S_t}{\alpha O_t} = \frac{\dfrac{C_{nt}}{C_t} - \dfrac{O_{nt}}{O_t}}{\dfrac{C_{nt}}{C_t} - \dfrac{S_{nt}}{S_t}}$$

If sort purity approaches 100%, this simplifies to the following (Riddell, Gardner, Perez-Gonzalez, Lopes, & Martinez, 2015)[13]:

$$Rmax = 1 - \frac{O_{nt}}{O_t} \cdot \frac{C_t}{C_{nt}}$$

ADJUSTING DROP DELAY FOR PURITY VERSUS RECOVERY

You set up a sort with two different particle types, and then measure the proportions of each in the sort tube and the waste. The data is entered into the spreadsheet provided, and the maximum recovery can be predicted. By comparing the recovery and purity at the drop delay calculated by the instrument and sorting slightly above and below this value, you should see that the calculated drop delay is rarely the ideal for recovery. As shown in the graphs in Figure 24 for two different instruments, the calculated delay works very well for purity, but for maximal recovery, the delay should be shifted.
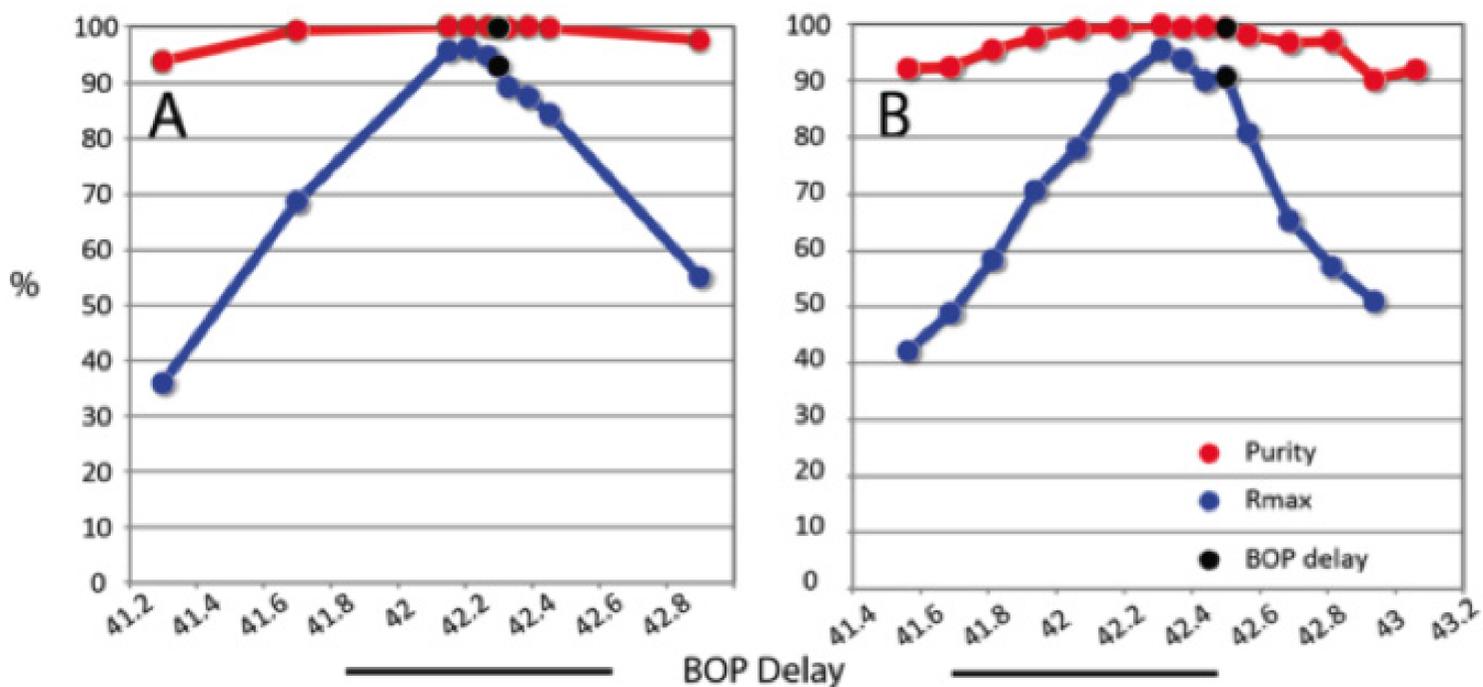
Figure 25: BOP Delay

In summary, recovery is much more sensitive to the correct calculation of the drop delay than the purity, and you should be sure you choose the correct metric for measuring sort performance.

FLOW CYTOMETRY DATA ANALYSIS

After completing the perfect staining and cytometry run, the hard work begins – data analysis. To properly identify the cells of interest, it is critical to pull together knowledge of the biology with the controls run in the experiment to properly place the regions of interest that will be dictate the final results. Gating is an all-or-nothing data reduction process. Cells inside the gate move to the next checkpoint, while cells outside the gate – even by a pixel, are excluded.

Before you begin, you must know the populations of interest. While it may sound funny, knowing the cells that are the target of the experiment is critical. How these cells are identified in the literature or past experience should guide the experiment. Check this first to make sure the proper stains are being used and that the proper controls are in place to analyze the data.

Size isn't everything. The reliance on forward and side scatter gates as a way to identify lymphocytes from other cells can be rife with peril. Blasting lymphocytes are larger than resting cells, and can be missed if there is a tight forward vs side scatter gate. It is best to use the scatter gate to remove the debris on the left size of the plot, as well as the small, pyknotic cells that

are often FSC small and SSC complex.

You should also check the stability of the run. Plot a time versus a scatter plot to see how even the flow was during the run. Using a plot like this will help eliminate artifacts caused by poor flow. Review the plot in Figure 25. The left plot shows good even flow while the right plot shows poor flow.
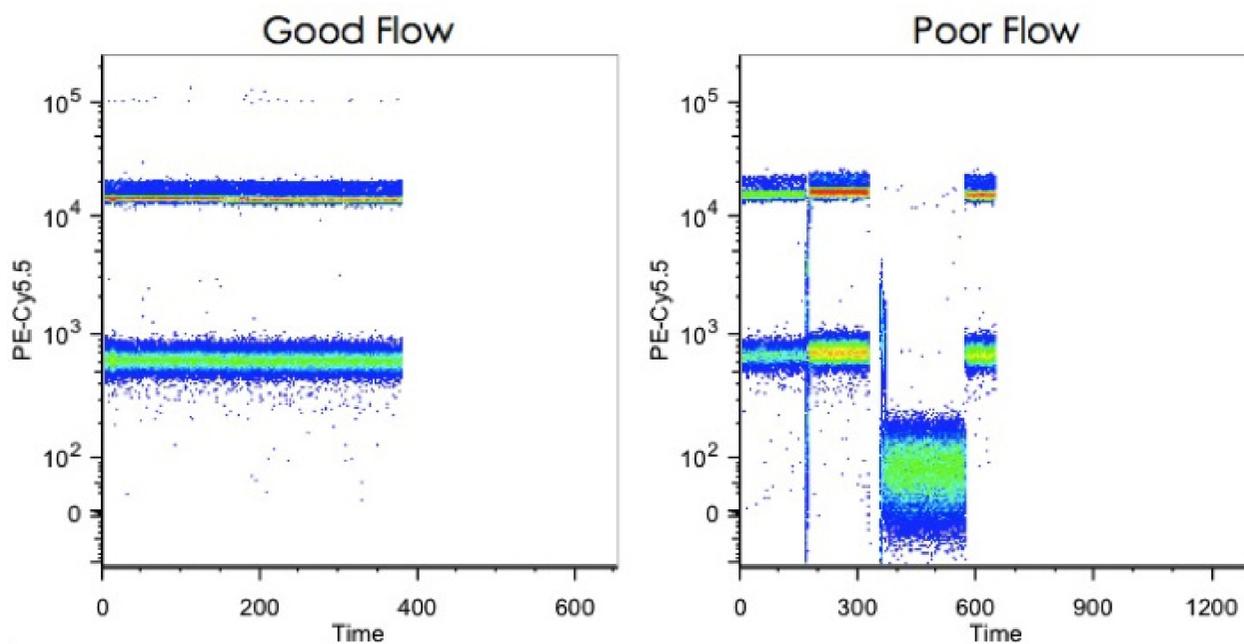

Figure 26: Good and Poor Flow Plots

As shown in Figure 26, cell clumps will take longer than single cells when they pass through the laser intercept. This affects the area of the signal. Using a pulse geometry gate such as FSC-H x FSC-A can easily eliminate doublets.
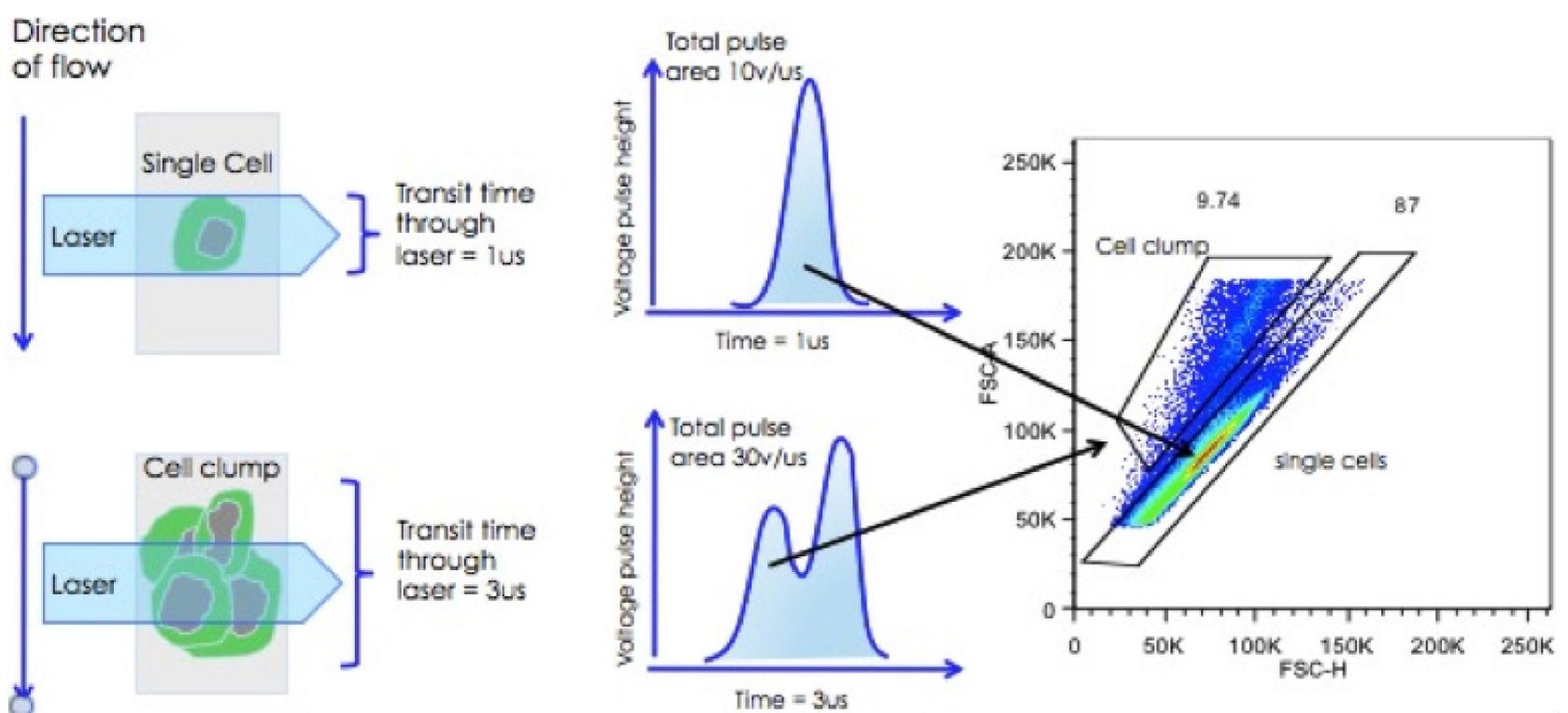
Figure 27: Cells through Laser Intercept

Let your controls be your guide. As explained in Chapter 1, the controls that are run in an experiment are critical for ensuring that the proper cells are identified. An FMO control, for example, is critical for identifying the proper placement of a gate in a polyclonal experiment. The spread of the data due to the fluorochromes in the panel cannot be corrected by using an isotype control, for example. As shown in Figure 27, the cells in the red circle represent cells that are in this spread region, and thus should be excluded. Without the FMO control, these cells would have been included in the analysis. Without the FMO control, these cells would have been included in the analysis.
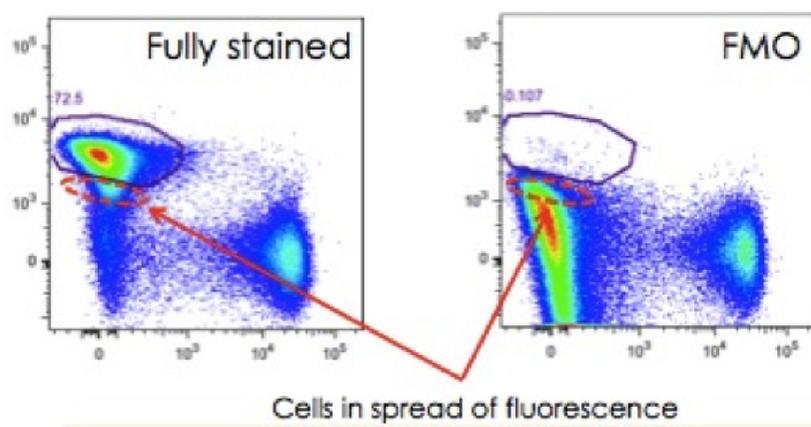
Figure 28: Cells Spread of Fluorescence

The back gating tool allows you to inspect the data to determine which cells will fall in the final population, assuming the gate of interest was not used in the gating scheme. In the third panel, especially, there a lot of cells that would be included in the final gate, assuming the gate was not used. Knowing that those cells are positive for a viability marker (and thus, should be excluded) helps confirm the placement of the gate.
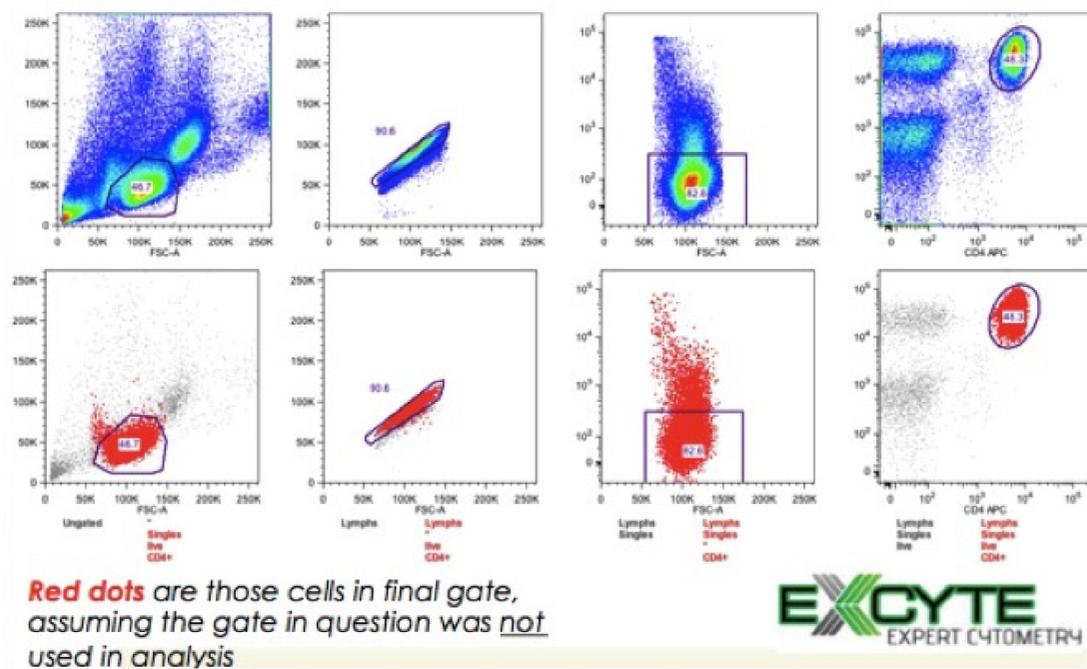

Figure 29: Final Gate Example

FLOWJO VERSION X TIPS

As we have discussed, software applications for flow cytometry are growing in numbers. While they offer a lot of value, reaching that value often takes time and practice. One such example is FlowJo, a software application that is designed specifically to analyze flow cytometry data. The FlowJo X tips provided in this section should help you publish your flow cytometry data.

If you have only a few events, select the "Use Large Dots" option.

When you only have 4-5 events in a population, it can often be difficult to see. If you turn off the high resolution, you can see the data better. Double-click a plot in the layout editor, and under the Specify tab, select the "Use Large Dots" checkbox, as shown in Figure 29.
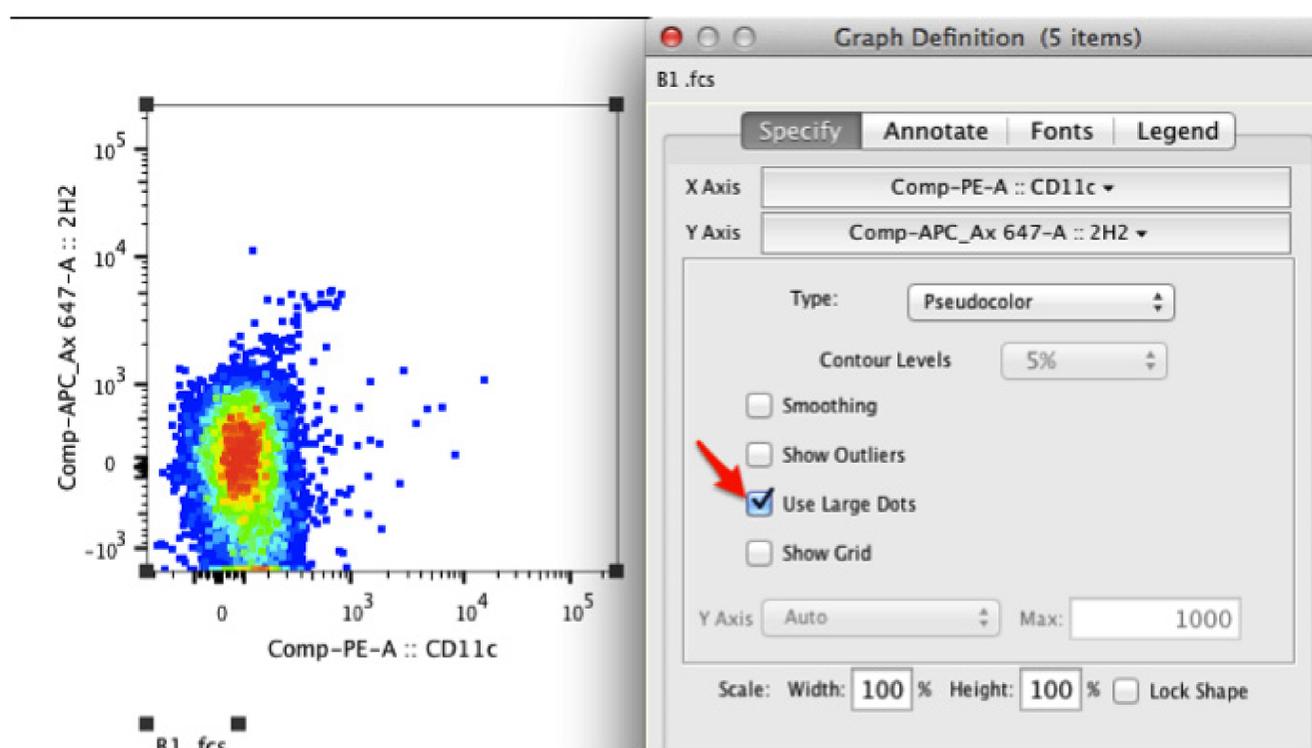

Figure 30: FlowJo X Graph Definition

Make sure your axes are labeled properly and cleanly with something that makes sense.

Many cytometers have a default axis label like FL1, or 525/50, as two examples. Some scientists doing flow are savvy enough to use the $PnS keywords to enter in their own axis labels during acquisition. However, if you don't want to or don't have time, you can edit the labels right in FlowJo. To do this, double-click a plot and select the Annotate tab to edit the text, and then select the Fonts tab to edit the font style. In the Annotate tab you can enter

the text you want for each label. In the Fonts tab, you can make the text bigger, bold, or even change the color.
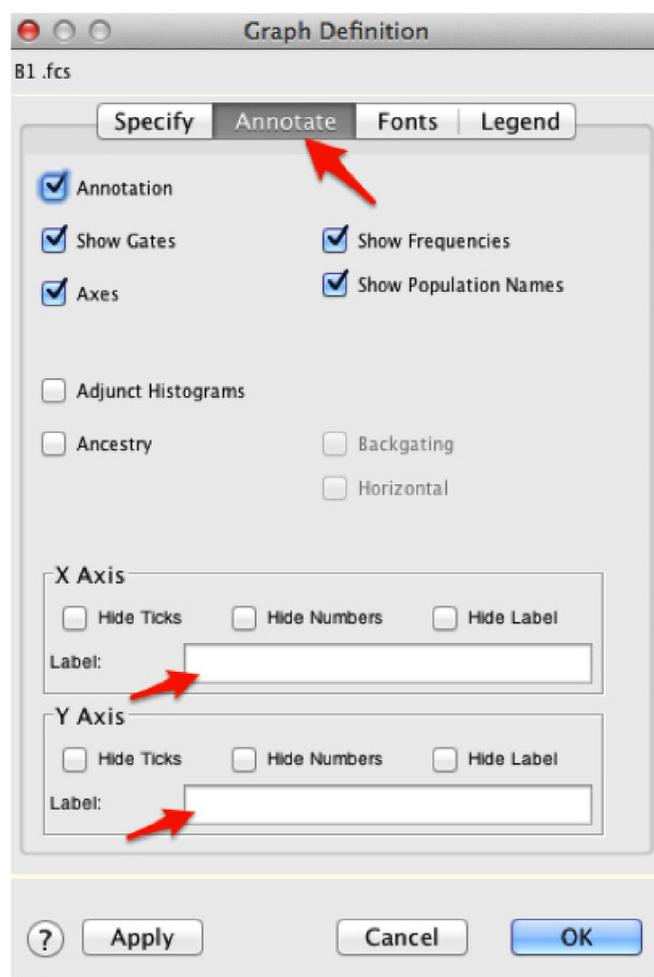
Figure 31: Labeling Axes in FlowJo X

Add a textbox to embed keywords and into a figure.

To add a textbox to your layout, right-click on it and select "Insert Keyword" from the list of options. You can also drag a statistic from the workspace into the layout editor. Embedding keywords and statistics right into the layout when generating your figures will ensure that your figure is annotated properly with useful information. You can then double-click the textbox and use the slider on the right to edit the text size and style. You can even create equations from statistics by selecting the statistics, right-clicking, and selecting "Equation."

Use the SVG format for exports.

Scalable Vector Graphics (SVG) are high-resolution, high-compression images that can be ungrouped in Adobe Illustrator, Photoshop, Intaglio, and Canvas. If you want to manipulate your graphics, you should use a program such as the ones I just named. These programs are designed for graphics manipulation and management. PowerPoint (PPT) is great for presenting, but not for graphic editing. If you must use PowerPoint for graphic editing, export your files as EMF. While the
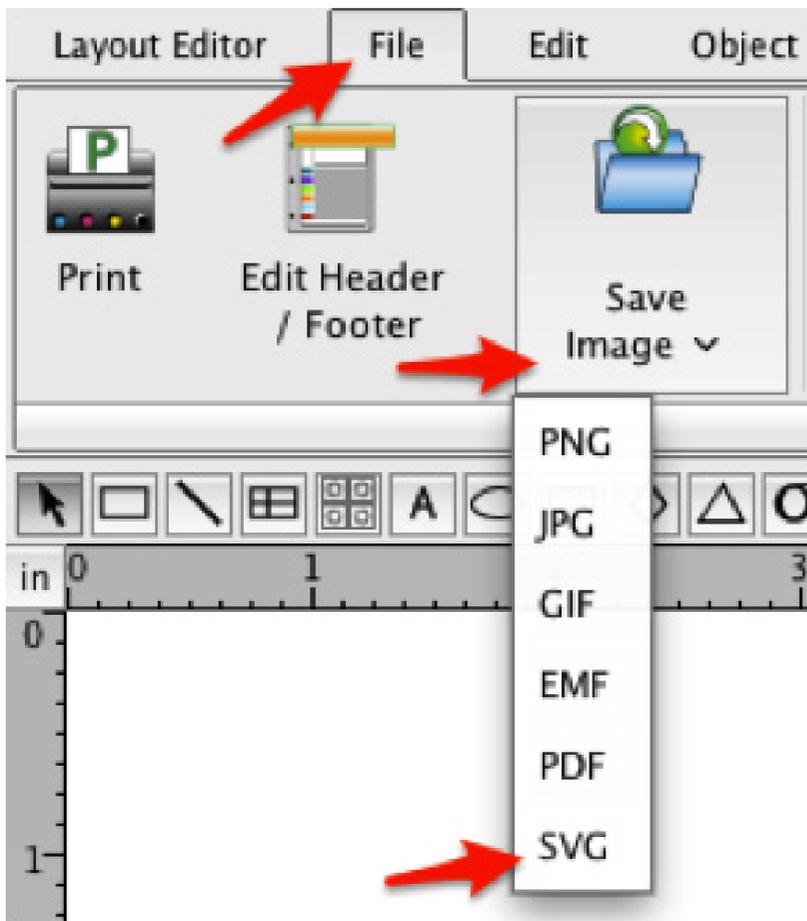
Figure 32: FlowJo X SVG Selection

quality is not as good as SVG, EMF will allow you to edit the file in PowerPoint.

PDF provides the best resolution out of FlowJo (300 dpi), but cannot be ungrouped. For comparison, all other formats are at 70 dpi. So, it may be best (or easiest) to try and do all of your graphic manipulations in FlowJo's layout editor and then save as a PDF. If FlowJo doesn't provide all the tools you need, use Illustrator or Canvas, but you'll have to export your files as an SVG to ungroup in those programs.

Change the width basis.

Use the T button in the graph window to adjust the width basis if you have any events on the axis, or if your negative population is dispersed. Increasing the width basis (making it more negative) will tighten the data up and give it a cleaner appearance. Be careful to not increase the width basis too much. You don't want to add a lot of white space below the data, as this is lost space.
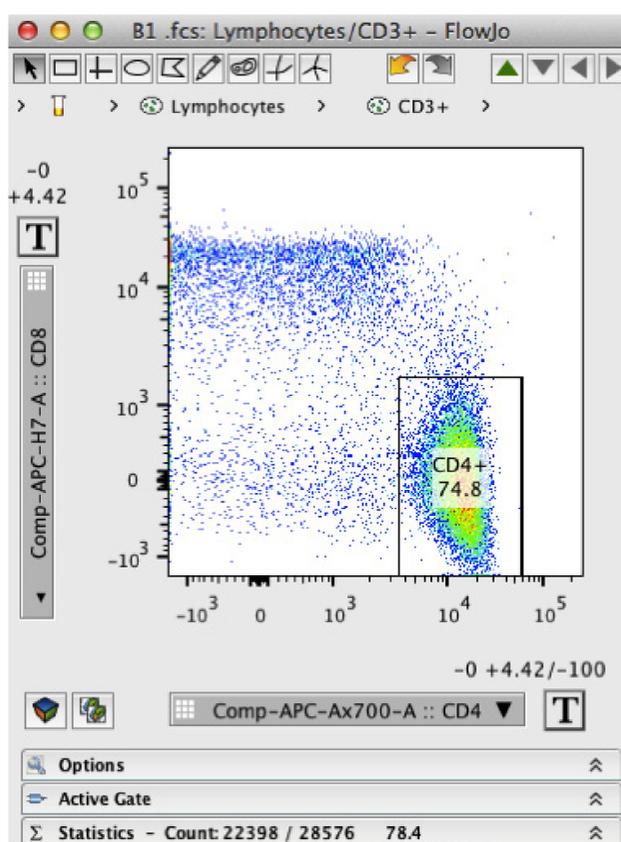

Figure 33: FlowJo Width Basis Setting

Offset your overlays.

Histogram overlays are a great way to show changes in expression profiles between samples. However, sometimes it is difficult to visualize differences using a histogram, especially when the plots are right on top of each other. Right-click on an overlay, scroll down to "Histograms," and select "Stagger Offset." Then, in the back-right corner you will be able to edit the perspective to make a "3D" view of the data.
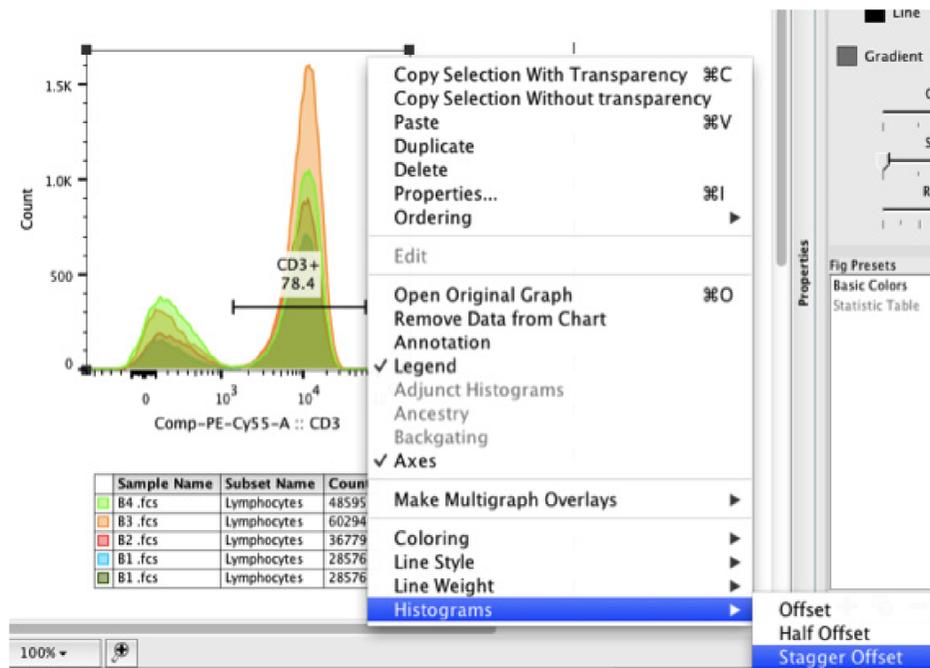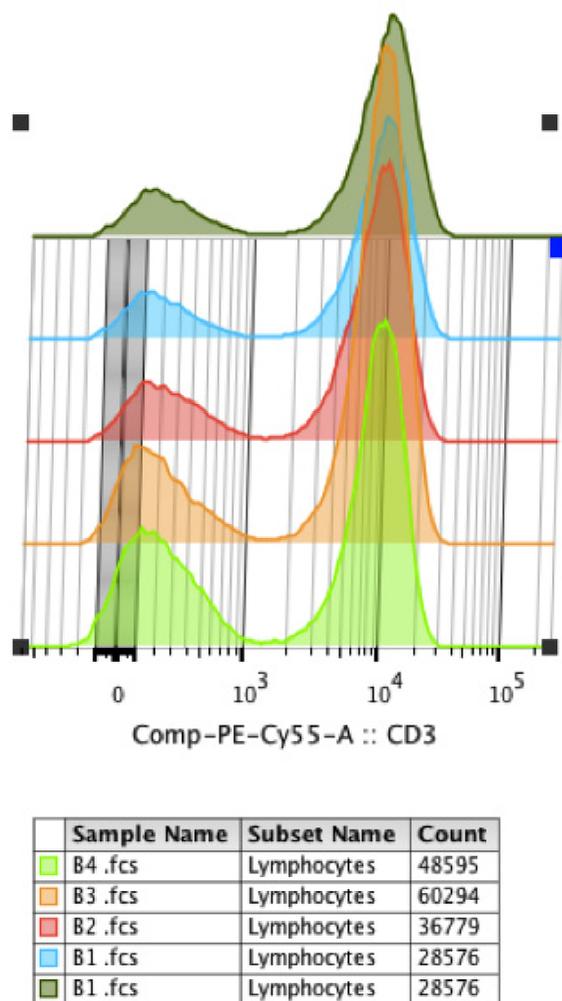

Figure 34: Stagger Offset Option

Figure 35: Sample Histogram with Overlays

Select "Guides" to align your plots.

There is a "Snap to Guides" option in the FlowJo version 10 layout that allows you to quickly align your plots. Of course, you can also use the align features. Go to the Edit tab in the layout editor to turn on the guides. The Arrange tab in the layout editor allows you to edit and align the tops, bottoms, and centers.
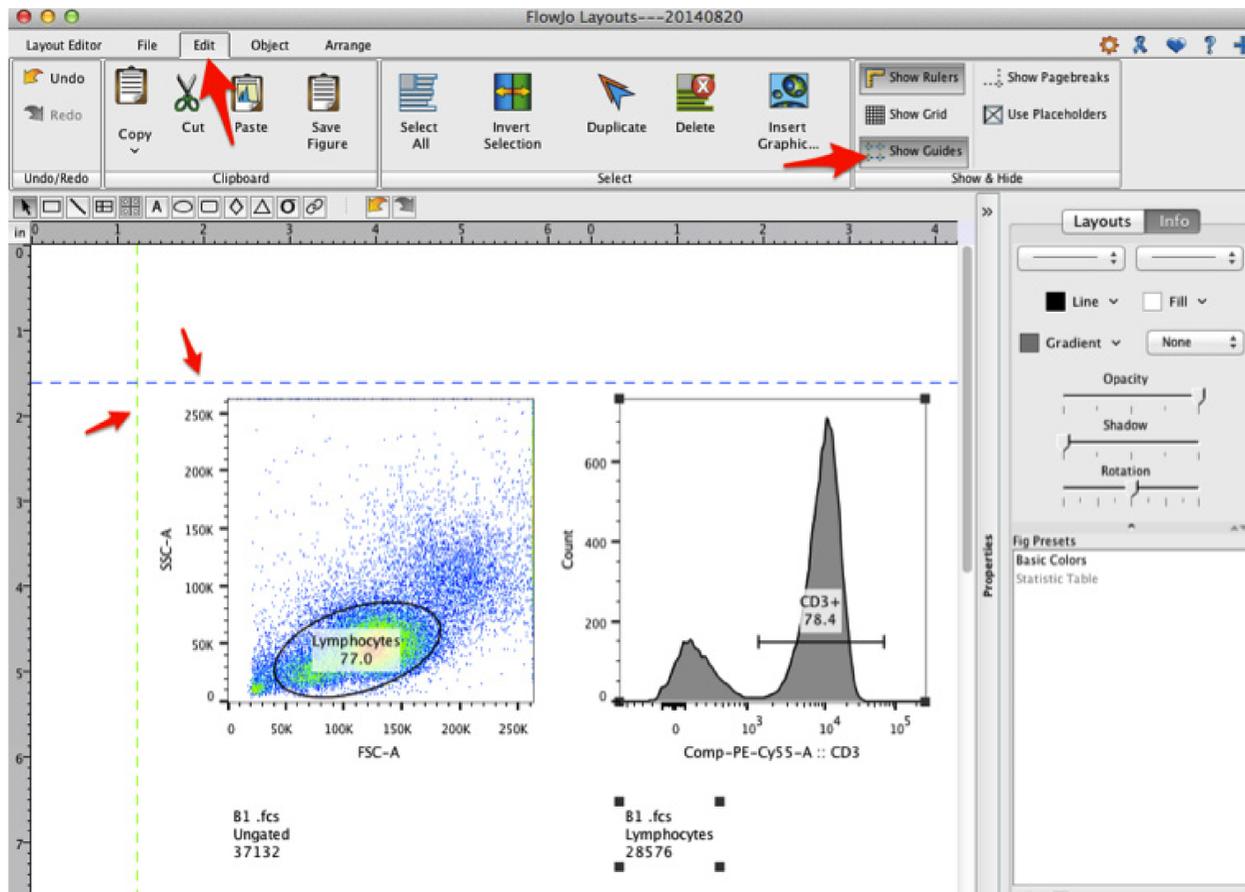

Figure 36: FlowJo X Layouts

Try the batch to "Web Animation" feature.

The Web Animation feature is especially useful when you are trying to visualize changes in the fluorescence profile over a time series or when titrating any treatment to your samples.
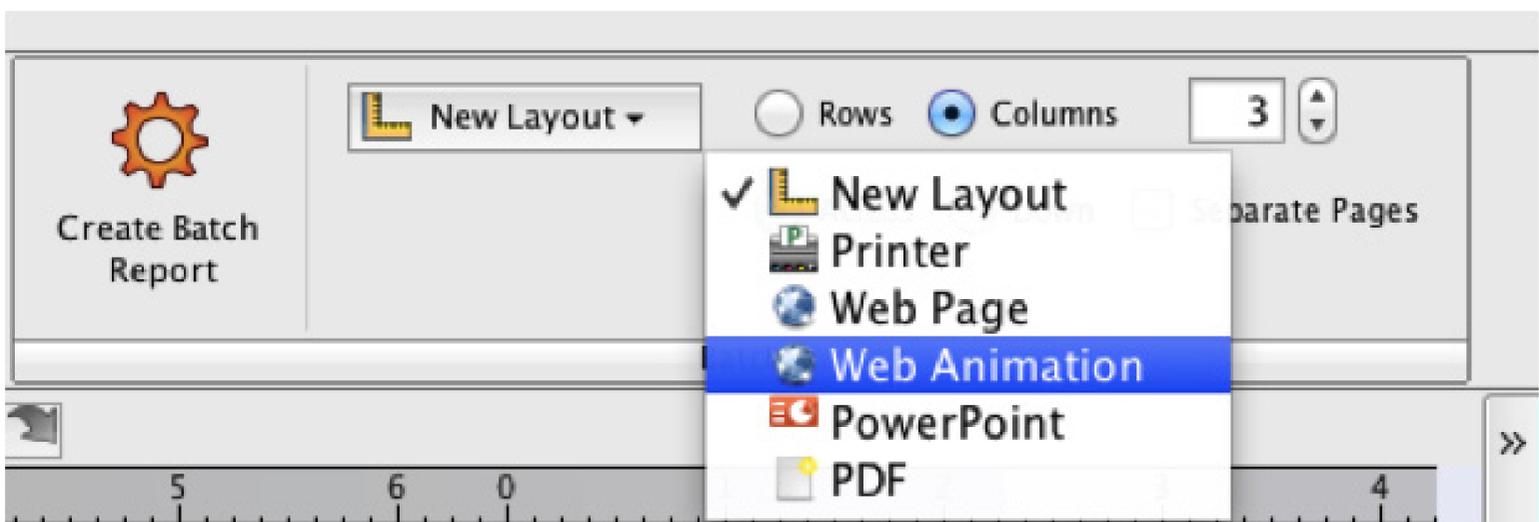

Figure 37: FlowJo X Web Animation Option

ExpertCytometry.com

Use the Ancestry and Backgating features to get your point across quickly.

To access the Ancestry or Backgating features, right-click on a plot and select Ancestry or Backgating from the menu. Ancestry shows the derivation or gating strategy used to isolate the population that is being viewed. Backgating can be used to indicate how each gate in your hierarchy contributed to the final population.
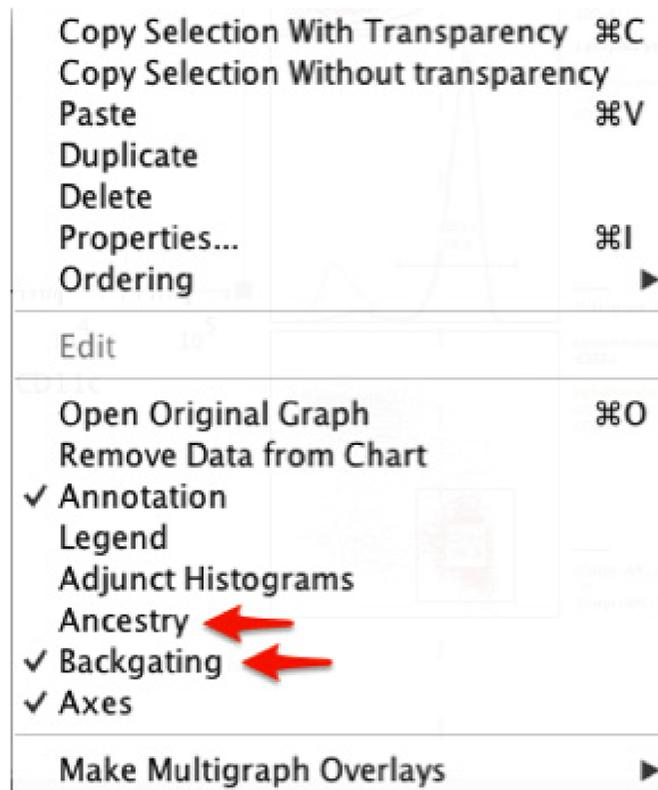

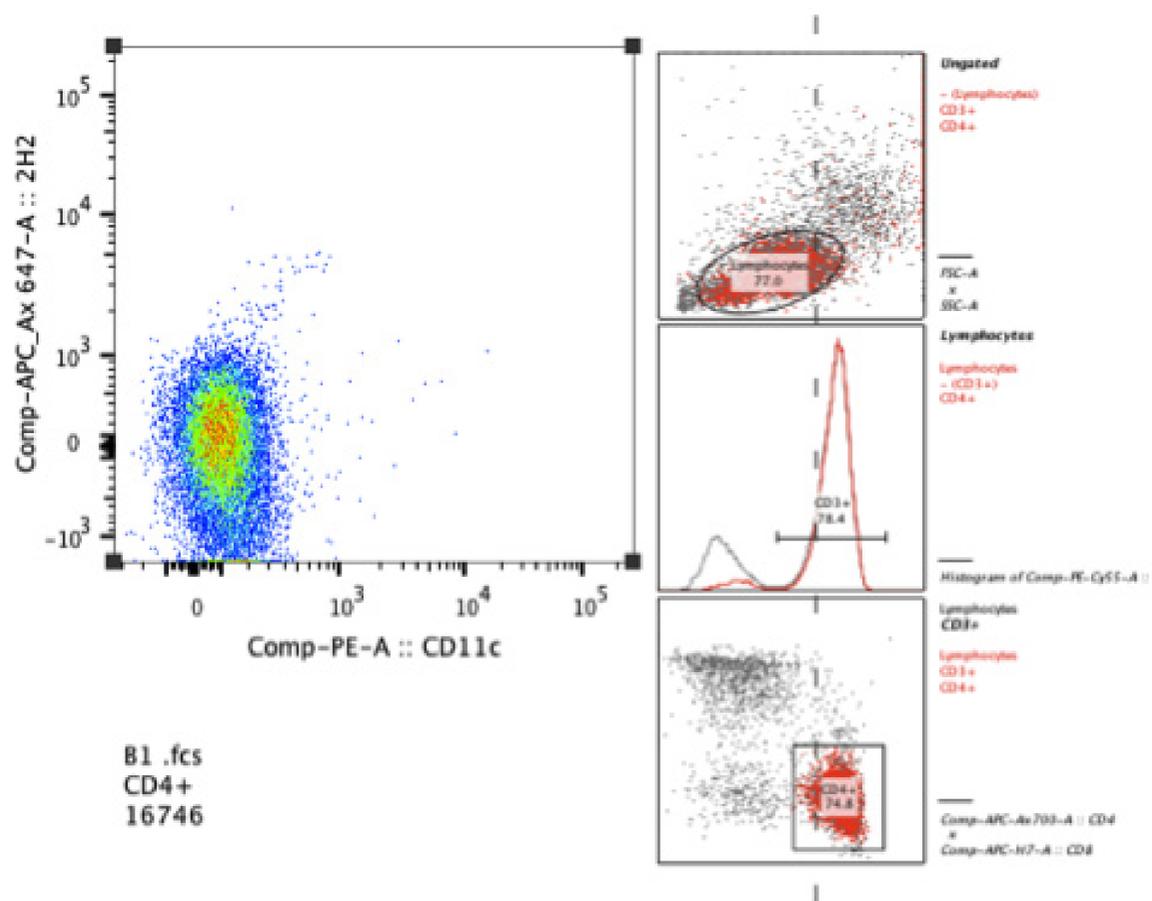**Figure 38:** FlowJo X Ancestry and Backgaiting Menu Options


Figure 39: FlowJo Backgaiting Example

Explore your data with multigraph overlays.

Overlaying some samples can reveal some fascinating information and key data trends and reviewers might want to see it. Maybe you want to show it for lab a meeting or a departmental seminar. There can be some interesting data in the multigraph view that you may miss otherwise. To make a multigraph overlay view in FlowJo X, right-click on a plot in the layout. Then, scroll down and select the "Make Multigraph Overlays" menu option as shown in Figure 39. You can choose to view all the histograms, all parameters by Y, or NxN. Explore these features and you'll quickly see their utility. Try making an overlay and then do a multigraph overlay view.
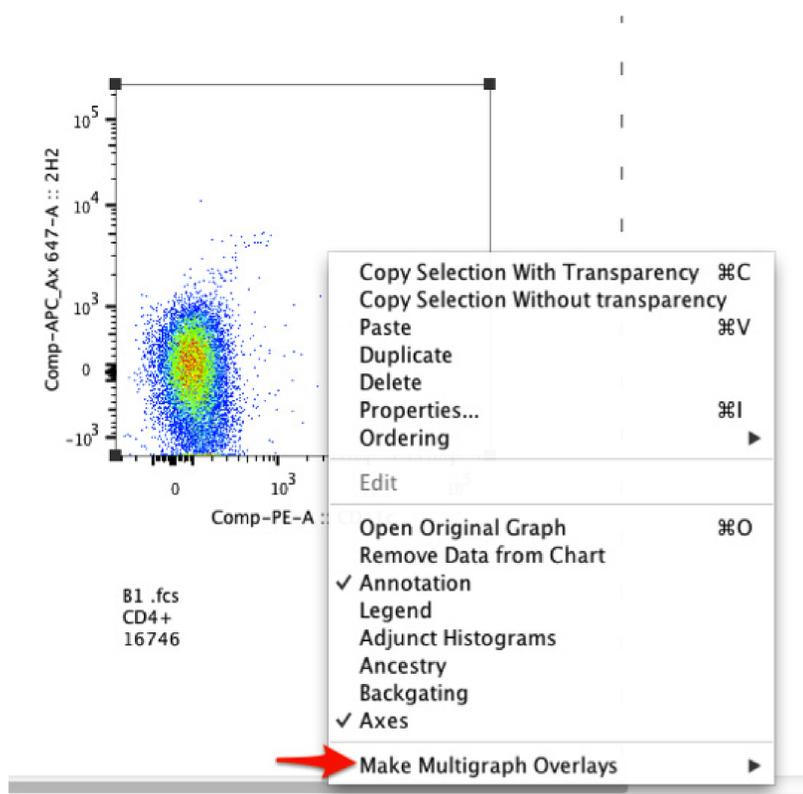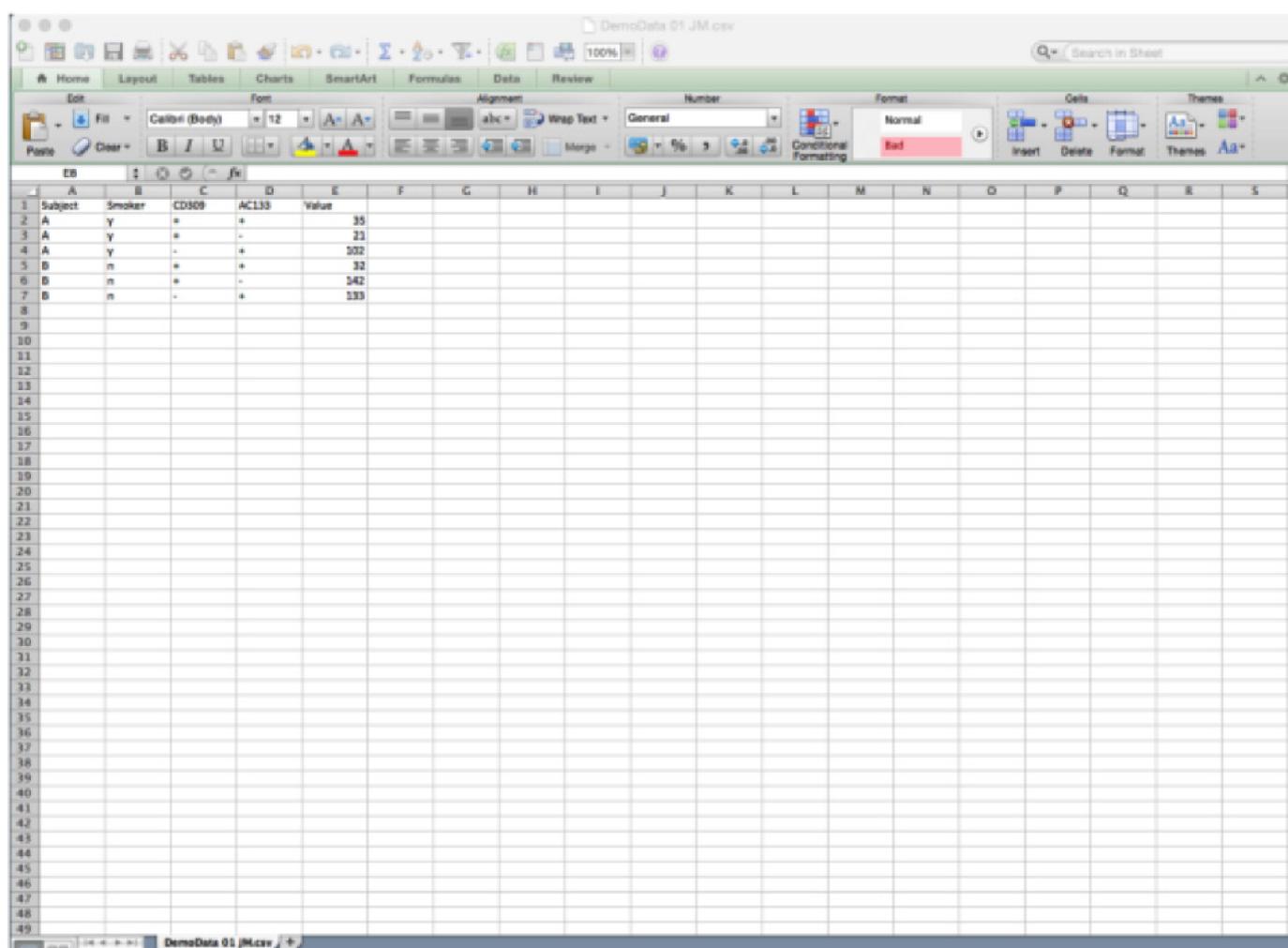

Figure 40: Make Multigraph Overlays
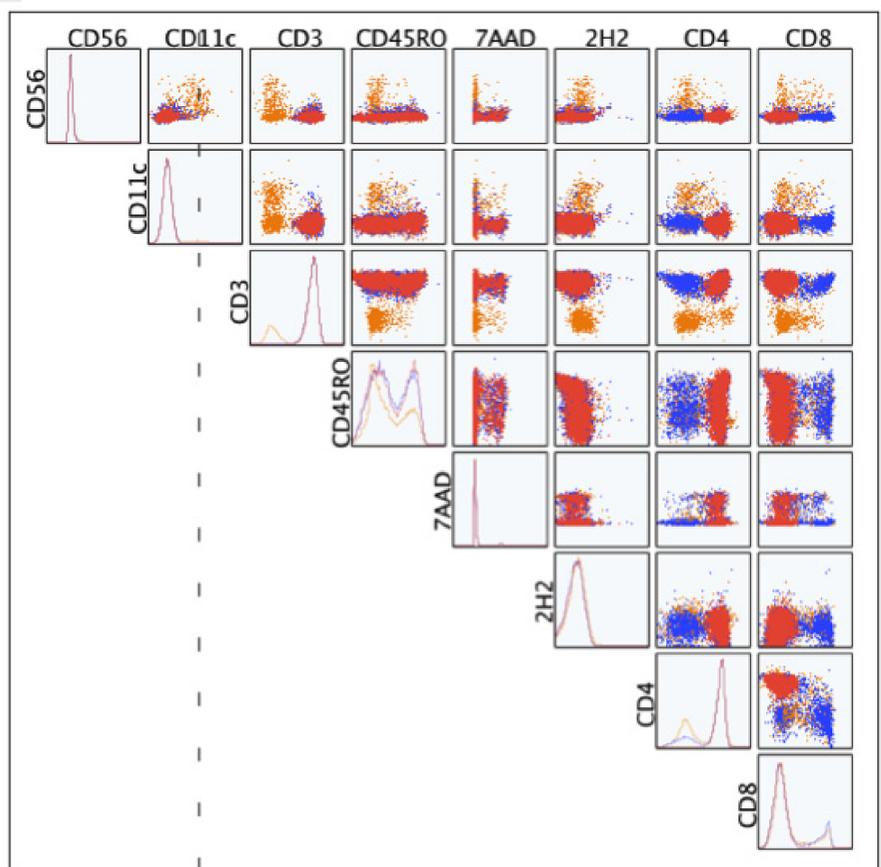
Figure 41: Sample Multigraph Overlay

PERFORMING A SPICE ANALYSIS WITH FLOWJO

Flow cytometry data analysis is becoming more complex. Gone is the rule of 2-3 color experiments. Even beginners are starting with more than five color assays, and the adoption of mass cytometry has the potential to increase our headaches even more. Current data analysis methods are good for single tubes or small cohort studies. What do you do when you have a large dataset with multiple sampling conditions and multiple outcome measurements?

With data complexity of this nature, you could export the numerical data to a third party analysis package, but even then the analysis could be difficult to perform. To overcome this limitation, and to allow for better discovery science, Mario Roederer and his colleagues have developed a solution. SPICE was developed to make sense of the increasingly complex datasets that modern flow cytometric methods can produce (Roederer, Nozzi, & Nason, 2011)[15].

SPICE is an acronym for Simplified Presentation of Incredibly Complex Evaluations, and it is designed to look at complex multidimensional datasets. Take, for example, research that is interested in CD34+ cell counts. At the individual level, that's easy and what standard data analysis packages can do.

Now take that same dataset and the comparison of a couple of conditions (smoker vs non-smoker), age and gender with over 100 different patients. This is where SPICE can assist your analysis without being tied to large spreadsheets and endless meeting with biostatisticians. With SPICE you can escape endless spreadsheets and their less-than-intuitive graphing and statistical interfaces and use a tool that is designed for flow cytometric data from the ground up.

If you are like 90% of researchers, you are likely using FlowJo for your starting data analysis. Luckily, you can pretty easily get data into a format that SPICE will like and let you get started with SPICE.
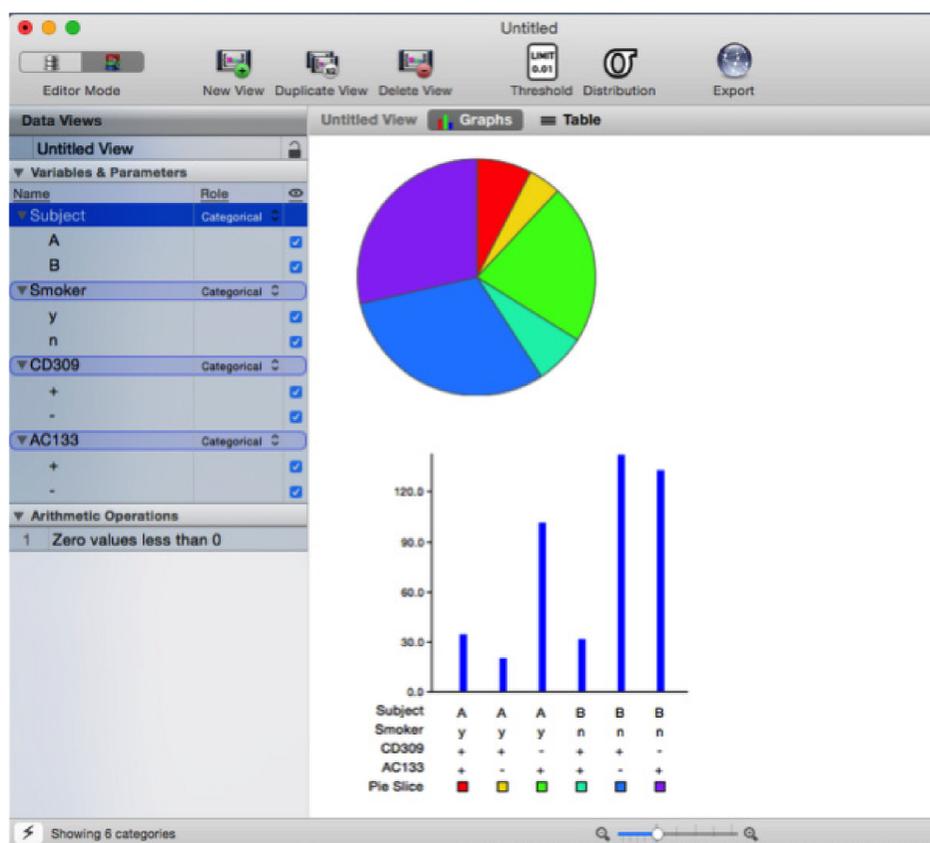
Figure 42: SPICE

Start by gating down to your base population of interest. If you are interested in percentage of CD34+ cells that also express AC133 and/or CD309, create gates for your CD34+ population and individual gates for the dependents. Don't worry about the negatives. Make sure you use Boolean gates to define all possible subsets. In this simple example you make two gates for CD309+ and AC133+. Boolean gating then creates 4 gates.

Next, create your table. Don't forget to include Keywords! For this example, I would add on a keyword about whether a sample came from a smoker or non-smoker, as well as an identifier.

Then, paste your tabular data into Excel, or any software that will allow you to save your data as a comma-separated value file (CSV). Be careful with formatting. SPICE is very particular about that. Read the help file for details on formatting data. Import the CSV file into SPICE.

Finally, use the SPICE commands on the left to show averages by patient, or overlay comparison of smokers versus non-smokers, or whatever question fits your work.

This is a very simple case. However, it is a good example of where to begin with SPICE, which is to start from data you already have with minimal manipulation.

TIPS FOR USING FACSDIVA SOFTWARE

BD Biosciences brand of digital flow cytometers, including the FACSCanto, the LSR-II, FACSAria and Fortessa, use a software acquisition program called FACSDiva. Diva, as it is called, is aptly named as it can be a difficult program to master. However, Diva has come a long way in the past 10 years and many improvements have been made to help end-users.
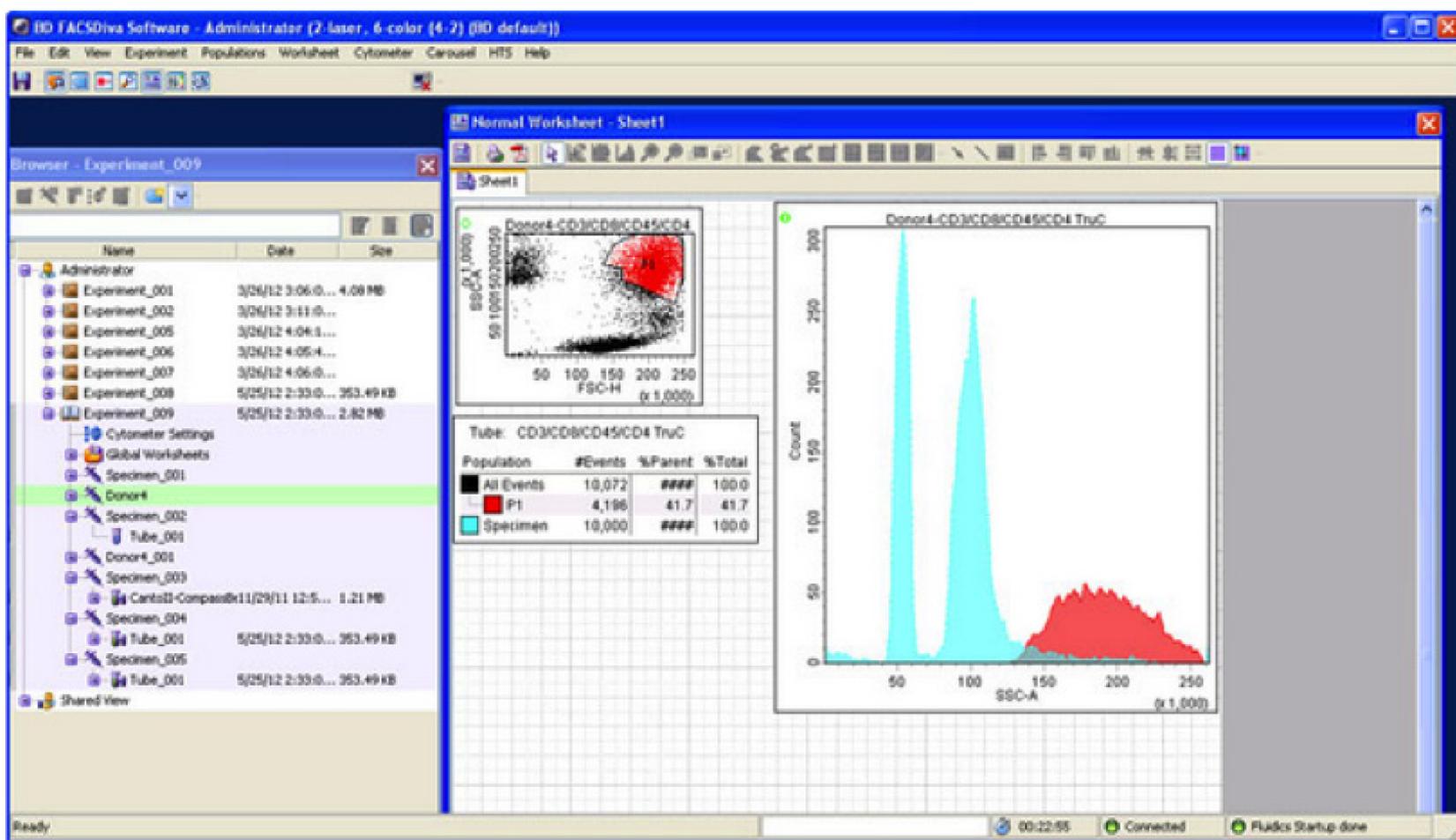

Figure 43: FACSDiva

Taking time to learn the changes to FACSDiva will improve the reproducibility of your data, the chances of your data getting published, and your overall experience on the cytometer. These changes will also save you time. The following are time-saving FACSDiva tips.

Compensation doesn't require the "universal negative."

It is important that you remember that the background of the positive and negatives should be matched. Using a universal negative will cause problems in compensation, especially if cells and beads are used to generate the compensation matrix. In Diva you can unselect the use a separate tube for negative control option when you're setting up compensation. This will allocate the placement of a P3 gate on the single-stained histograms. Diva will then use the P3 gate as the negative population when calculating compensation.

Make multiple matrices.

In the case where you are measuring several different antigens in the same channel, especially where the label is a tandem dye, it is critical to have a different compensation matrix for each tandem combination. In Diva, when you create a compensation matrix, you can add additional columns of the channel in question and label each one with the appropriate antigen name. Then, after collecting the controls and performing compensation, when you are finally collecting your samples, make sure each one is labeled with the same name. This will ensure that the proper compensation matrix is applied to each sample.

Collect more events.

When you calculate compensation, don't let the default of 5,000 events be your guide. When using compensation particles, 10,000-20,000 events is better. When using cells, 30,000-50,000 is better. A simple change in your DIVA layout can ensure that sufficient events are collected.

Create keywords.

Annotation of the data with keywords will help in finding data weeks and months after the experiments are run. Keywords are also useful when performing analysis in third party software for grouping and batch analysis. If you don't

know how to add keywords to your experiment, you are wasting valuable time that will affect your ability to reproduce and publish your data.

Keep the Diva database clean.

Diva is based in the Java programming language, and the database size can have a negative impact on the speed of the software. It's best to keep the data base seize well under 15 gigabytes. This means that you need to export your experiments on a regular basis. It also means that you need to back up your database before making major changes to your computer or the software program itself.

Limit the size of your data files.

With the ability to measure a million events in a few minutes, and with the occasional need to look at rare events, it is important to remember the size of file should be kept to a minimum. The best practice is to collect three or four 1-2 million event files versus, for example, one 10 million event files. Later, you can join the data (concatenated) in third party software platforms. However, during acquisition, don't let the file size get to big to keep processing times reasonable.

Read the Cytometer Setup & Tracking (CS&T) baseline report.

Diva provides the CS&T baseline report, which is full of useful information. The report includes suggestions for starting voltages, PMT dynamic ranges, and more. When you set up a new experiment, review these settings, especially the linear dynamic range of the PMTs. If your signals are outside of this range, then any compensation you apply to your cells will not be correct.

Use application specific settings.

When performing longitudinal studies, it's essential that you maintain the optimal voltage of the instrument. This will ensure that any differences discovered will be the result of the biology of your cells, not the settings of your instrument. Application specific settings should be finalized when the experiment is started. These settings are linked to the daily CS&T reports and, if the settings are correct, the software will make the appropriate adjustments in response to any instrument changes.

ADVANCED TOPICS

The information presented in this guide thus far is to prepare you to properly set up and perform effective and accurate experiments using flow cytometry. Once you understand that information, you are ready to extend your knowledge to include the advanced topics discussed in this chapter.

MEASURING AND REPORTING APOPTOSIS IN FLOW CYTOMETRY

Cell death is a fact of biological life. How, when, where, and most importantly, why cells die, can have huge biological consequences on the path an organism may take. Apoptosis, or programed cell death, can result in a selective advantage for an organism. Fingers, for example, are the result of apoptosis of cells during development.

Next to immunophenotyping, measuring apoptosis using flow cytometry is one of the most common assays. The reason may be stem from the many different ways that exist to measure the process, many of which can be easily performed in a high-throughput manner, or combined with other assays to determine if specific cellular subsets are sensitive to a given drug or treatment.

This subject was so critical to understanding cellular functions that the Nobel Prize committee recognized the study of apoptosis as seminal and in 2002. The prize in Medicine for that year was awarded to Sydney Brenner, Robert Horvitz and John Sulston for their work on the subject.

Annexin V is a calcium dependent protein that binds preferentially to phosphaditylserines. These phospholipids typically are inward facing, but are flipped to the extracellular side of the membrane as one of the early signals of apoptosis. Coupled with a membrane impermeant dye (like 7AAD or PI), early and late apoptosis can be characterized.

The following are tips for performing apoptosis assay using Annexin V and a membrane impermeant dye.

Make sure you use the correct buffer.

Annexin V is dependent on calcium for binding. When performing this assay, make sure to use a calcium containing buffer during the labeling process.

Annexin V binding is not stable.

Unlike antibody binding, the Annexin binding is not that stable. It also doesn't fix well. Thus, it is critical to read these samples shortly after labeling – typically between 1 to 3 hours from the point of labeling.

Pick the right assay.

Inducing apoptosis can be done with a host reagents. Make sure the reagent being used induces apoptosis and not necrosis. Sub-G1 fragmentation is an often misused method for detecting apoptosis. In this assay, low-fragment DNA is measured by standard DNA binding dyes, appearing to the left of the G1 peak on a DNA histogram. However, if the assay is not performed correctly, estimates will be off.

Watch your fixation.

Formaldehyde fixatives tend to cause the small DNA fragments to be retained, and thus underestimating the apoptosis.

Consider TUNEL.

When you use terminal deoxynucleotidyltransferase (TdT), the ends of DNA breaks are labeled with dUTP that can be later detected using an anti-BrdU antibody. In this case, you do need to use formaldehyde fixation. You also don't need to perform DNA denaturation, as the smaller fragments are readily accessible to the antibody. Loss of mitochondrial membrane potential is another early hallmark of early apoptosis. In cells undergoing apoptosis, the mitochondria will release cytochrome C and the apoptosis inducing factor – both of which are necessary of caspase activation (another critical step in apoptosis).

Choose your dye carefully.

Three common dyes Rhodamine 123, 3,3'-dihexyloxacarbocyanine iodide, and JC-1. R123 should be coupled with a dye like PI so that live (cells staining with R123), early apoptotic (cells lost the ability to accumulate R123), and late apoptotic (PI positive cells). JC-1, on the other hand goes from a green fluorescence to an orange fluorescence in cells undergoing apoptosis.

Don't over-interpret the data.

Some scientists have written biotechnology papers that suggest that the collapse of the mitochondria membrane potential may not be a critical step in apoptosis. The list of possible assays goes on. Detection of activated caspases, either directly with antibodies, or by activity, can be a very informative assay as well. In the end, each assay offers strengths and weaknesses. You need to understand what the output is and how the assay is to be combined with other readouts. Likewise, knowing a bit about the process of apoptosis in the cells of interest is critical.

PHOSPHO-FLOW CYTOMETRY

Researchers often enter a core wanting to look at the activation and downstream signaling events that occur in different immune cells. These events occur in response to signals such as cytokines, chemokines, various receptor ligands, and the engagement of the T cell or B cell receptors. The signaling events are also characterized by the initiation of several phosphorylation events. When this is the case, it is recommend that the

researchers set up a phospho-specific flow cytometry, or phospho-flow, experiment. These types of experiments measure the phosphorylation state of intracellular proteins at the single cell level.

Phospho-flow allows the simultaneous analysis of many phosphorylation events along with cell surface markers. These types of experiments enable the experimenter to resolve complex biochemical signaling networks in heterogeneous cell populations. Phospho-flow has been applied to numerous areas of biology, including antigenic stimulation and microbial challenge, high-throughput and high-content drug discovery, as well as the characterization of signaling pathways in both normal and disease-altered immune responses.

In phospho-flow, immune cells or other cell populations are stimulated with signaling receptor ligands or antagonists for a certain period of time. Following stimulation, the cells are fixed using paraformaldehyde-based buffers. The fixation process locks the cells in their induced states of phosphorylation so they can be permeabilized and stained with fluorescently-labeled antibodies against the phosphorylated proteins. The cells can also be stained with antibodies against cell surface markers and other proteins of interest at the same time. The final step is to analyze the stained populations with a flow cytometer or cell sorter.

When performing and optimizing a phosphor-flow experiment, there are several things to consider. The following are ways you can optimize your phospho-flow experiment.

Run all of your samples at the same time.

When analyzing phoso-flow data, there are two things to keep in mind. First, the fluorescence intensities of each population will serve as a measure of the magnitude of the protein target's phosphorylation levels. Second, the staining intensities will allow you to calculate the percentage of cells able to respond to a given signal. The problem is that you will often need to stimulate different cell populations on different days and at different times. This will create variability between staining intensities. The best way to limit this variability is to use a buffer that allows you to freeze all of your populations

before adding an antibody. Then, an hour or two before you're ready to run your samples on a flow cytometer, thaw all of your samples at once. Then, simply stain them and run them as one large batch.

Select the right permeabilization method.

Many protocols use 100% methanol for permeabilization following fixation. The advantage of this is that following methanol permeabilization, the cells can be stored for an extended period of time at -20°C to -80°C prior to staining. This means that you can stimulate different samples on different days and then run them altogether in one big batch to get more accurate results. All you have to do is keep freezing your cells after every stimulation.

Select the right target antigens.

Not all protein targets are created equal. Some antigens will not survive the permeabilization process, even if you're using methanol. The best way to determine which antigens you should use is to review the literature and see which proteins remain stable during permeabilization. You can also use online resources such as Fluorish and Cytobank to identify potential protein targets on your cell populations on interest.

Make sure you're targeting the right event.

To measure phosphorylation events uniquely, you have to use antibodies that are specific to the phosphorylated form of a protein. These antibodies are usually raised using short phosphorylated peptide immunogens that are coupled to carrier proteins. However, sometimes you'll want to use several antibodies against the same phosphorylated protein. The key is that each antibody will target a different phospho-residue within the protein. The advantage of this is that you can gain insight into which residues are important for particular signaling events.

Make sure you're ONLY targeting the right event.

Once you've ensured that your antibody is targeting the right phospho-residue, you'll want to confirm that your antibody is only targeting that residue. In other words, you'll want to confirm phosphor-specificity. There are several ways to confirm this. First, you can compare the staining

intensities of resting versus stimulated cell populations. Second, you can treat your samples with phosphatases prior to flow analysis. Third, you can compare phosphorylated peptides to non-phosphorylated peptides. Fourth and finally, you can compare phospho-protein levels to total protein content (Krutzik, Hale, & Nolan, 2005)[16].

AVOIDING PITFALLS OF FLOW CYTOMETRY PROLIFERATION EXPERIMENTS

You can measure cell proliferation in a number of ways. Figure 43 shows the tried and true method of counting cells. This straightforward assay can help determine if the cells are proliferating by comparing counts. Here, you can determine that the experimental treatment is increasing cell growth.
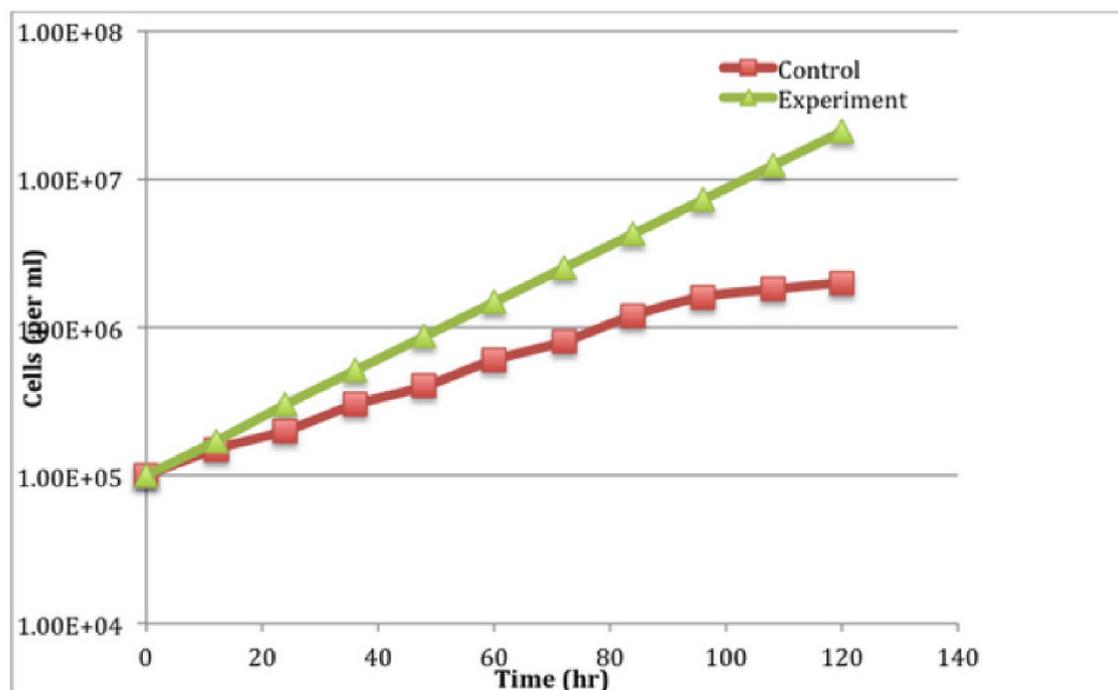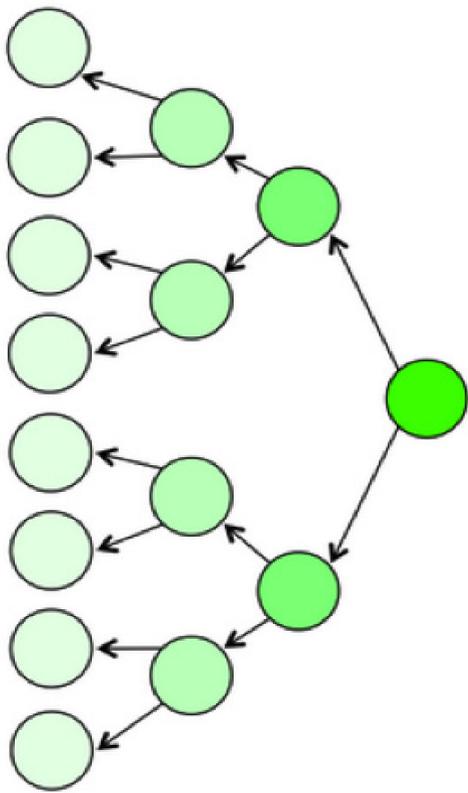

Figure 44: Cell Counting Cell Proliferation Example

A second method of measuring proliferation involves using a radioactive tracer such as 3H-Thymidine. In this assay, the amount of the isotope taken up by the cells correlates to the amount of DNA synthesis, and therefore growth. Of course this requires using radioactivity and all that entails.

A third method is using a something like yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (also known as MTT). Here the MTT is reduced by the mitochondrial enzyme succinate dehydrogenase. Cells are treated with an organic solvent and the purple formazan product of the MTT is measured with a spectrophotometer.

These three methods, unfortunately, only give results of bulk population growth. Many researchers are interested in knowing how different cell populations are proliferating based on different stimulation conditions. Some even want to isolate cells after a specific number of cell divisions. The solution to that assay is the dye-dilution method. This technique was first introduced in 1994 by Lyons and Parish and has been heavily cited. In this technique, cells are labeled with a dye (CFSE was the first used) that intercalates into the cells in such a way that as the cells divide, the dye is segregated roughly equally into the daughter cells. As the cell divides, the dye is diluted and by counting the peaks, (or modeling the pattern) the number of original dividing cells can be calculated.

To run a successful proliferation assay, there are mistakes that you must avoid. These are outlined below.

Figure 45: Proliferation Example

Not knowing the qualities of the dye you're using.

Dyes used for proliferation must be bright, readily taken up by the cells, and distributed equally between daughter cells. CFSE and its derivatives intercalate into the cell where they label intracellular proteins. One limitation of the CFSE dye is that there is a proliferation-independent loss of fluorescence in the first 24-36 hours. Thus interpretation on early proliferation has to take this into account. Alternatively, lipophilic dyes like PKH26, can be used. These dyes are membrane bound, and are a good alternative to the CFSE. However, they can be trickier to label cells as cell size and mixing can affect the labeling. Unlike CFSE, there is not a loss of signal after labeling, making these dyes better for short proliferation. If the cells are going to be fixed, it is important to avoid using organic solvents when using membrane dyes.

Forgetting to titrate your dye.

Too much of a cell tracking dye can negatively impact cell function and viability. Thus, titrating to the highest level that doesn't affect the cells is critical. Usually, this titration can be one at a fixed cell concentration –

thereby reducing the complexity of the testing.

Not using the proper controls.

Both positive and negative controls are critical, as well as the optimization of the instrument. Once the titrated concentration is optimized, performing a voltage optimization is critical. Using the unstimulated concentration, place the labeled cells at the highest decade.

Forgetting to account for viability or doublets.

As with every assay, identifying the live cells are important. Cells lose the proliferation dye as they undergo apoptosis, so it is important ensure that these cells are removed by viability. Likewise, removing doublets using pulse geometry is important to ensure that the true proliferation rates are measured.

Not collecting enough cells to accurately model the data.

Too few cells collected and the data models used for proliferation cannot accurately model the process. As with most flow experiments, more cells are better than less (Lyons & Parish, 1994)[17].
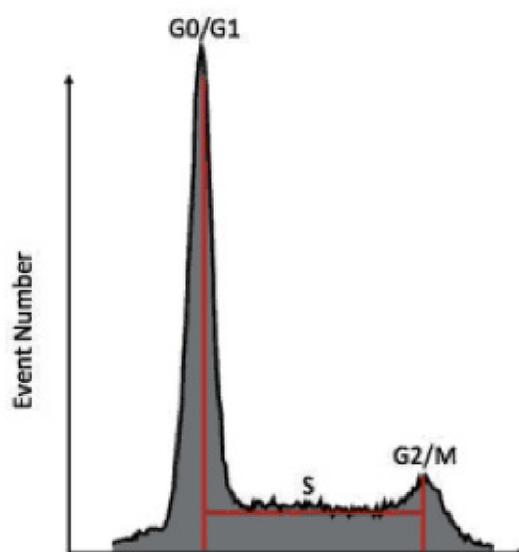
CELL CYCLE ANALYSIS

Cell cycle analysis by flow cytometry uses a DNA binding dye, such as propidium iodide (PI), 7- aminoactinomycin D (7-AAD) or 4', 6-diamidino-2phenylindole (DAPI), to determine the cell cycle state of a cell population. The Gap1 (G1) phase of an eukaryotic cell is defined as having 2C DNA. The synthesis (or S) phase is where the DNA is synthesized going from 2C->4C. Cells then spend some time in the Gape 2 (G2) phase before completing mitosis and the whole cycle starts over again. Since the cells in G0/G1 and G2/M have defined amounts of DNA, with the S phase having an increasing amount of DNA, an idealized single parameter cell cycle plot looks like the graph in Figure 45.
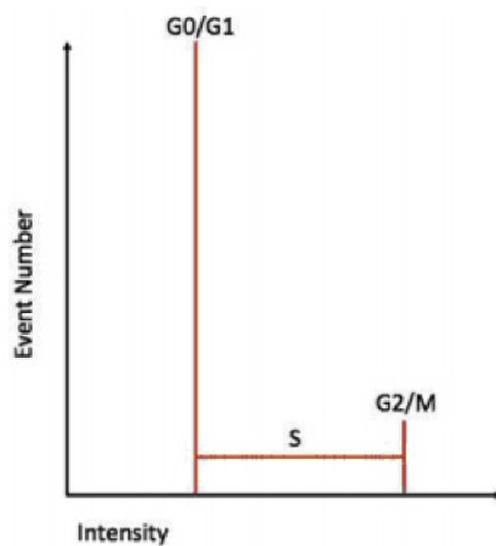
Figure 46: Single Parameter Cell

However, due to measurement error that is inherent in the flow cytometer, this diagram usually looks like the one shown in Figure 46.

Figure 47: Single Parameter Cell (2)

Cell cycle analysis is a powerful tool in the flow cytometrist toolkit. It can robustly determine the ploidy of a cell population that can be used to understand the aggressiveness of a cancer tumor, the ploidy of a plant population, or how a drug affects the growth of a cell.

CRITICAL STEPS IN DNA CELL CYCLE ANALYSIS

DNA cell cycle analysis is a very powerful technique in flow cytometry. It is deceptively easy, but there are several critical things to remember to ensure successful analysis. Collect enough events. Cell cycle analysis involves fitting the data using one of several mathematical models that describe the behavior of the data. These models make different assumptions about

the S phase as well as the G1 and G2/M phases. To have enough data, you should collect 100 events for each channel between the beginning of the G1 peak and the end of the G2/M peak. Thus, if the G1 peak starts at 40,000 and the G2/M phase ends at 110,000, the dataset should contain (110,000-40,000)*100 = 7,000,000 events. It is important to note, as has been shown by Bagwell reduction of the data to 256 channel resolution does not negatively impact the DNA histograms, and ensures there is sufficient numbers of cells in each channel[2]. Thus, in the above example the data reduction from 262,144 resolution to 256 resolution will reduce the number of events required to approximately 7,000.

Remember the stoichiometry.

The concentration of the DNA dye must be sufficient so that it binds in proportion to the amount of the DNA in the cell. Thus it is essential to have a good cell count to ensure the correct amount of DNA dye is added to the sample.

Fixation choice is also important.

Crosslinking agents like formaldehyde will lower the dye binding because they introduce chromatin crosslinking. Dehydrating fixatives like methanol and ethanol are better, but at high concentration can cause cell clumping. Dehydrating dyes can also negatively impact fluorescent dyes if the DNA is being stained in association with surface maker stains. Don't forget, a little detergent can help improve the access of the DNA dye. Also, don't forget the RNA. Some dyes (PI, for example) will bind to both DNA and RNA. If using PI, it is critical to add an RNAse to the staining buffer. Failure to do so will result in messy DNA histograms.

Watch the CV.

The CV of the G0/G1 peak is a measure of the quality of the DNA histogram. This can be affected by flow rate and laser alignment. The lower the CV the better, so it is critical to run DNA samples at low flow rates (narrow core streams) on a well aligned instrument.

Beware of the doublets.

Doublets can masquerade as cells in the G2/M phase. It is critical to have good single cell prep for DNA cell cycle analysis. Watch the fixation steps and remember to filter the sample before running on the cytometer. Make sure to collect the pulse geometry measurements (H, W and A) to ensure that doublet discrimination gating can be performed on the sample.

Control the cell cycle.

It is a good practice to include a DNA cell cycle control into all experiments. Doing this allows for better characterization of changes in DNA cell cycle over time, as well as comparisons between samples/machines/days, which improves repro-ducibility and confidence. The most common are chicken RBC and trout RBC.

Figure 48: Low and High Flow Rate Examples

In summary, cell cycle analysis is a powerful tool in the flow cytometrists toolbox, but there are many optimization steps necessary for this deceptively easy assay. Don't assume that you can add some PI to a sample and get a good DNA histogram. Choosing the best fixative for the

Figure 49: Fluorescence Intensity Example

assay, the right dye, and a well behaved instrument are all critical for successful DNA cell cycle analysis.

CYTOF

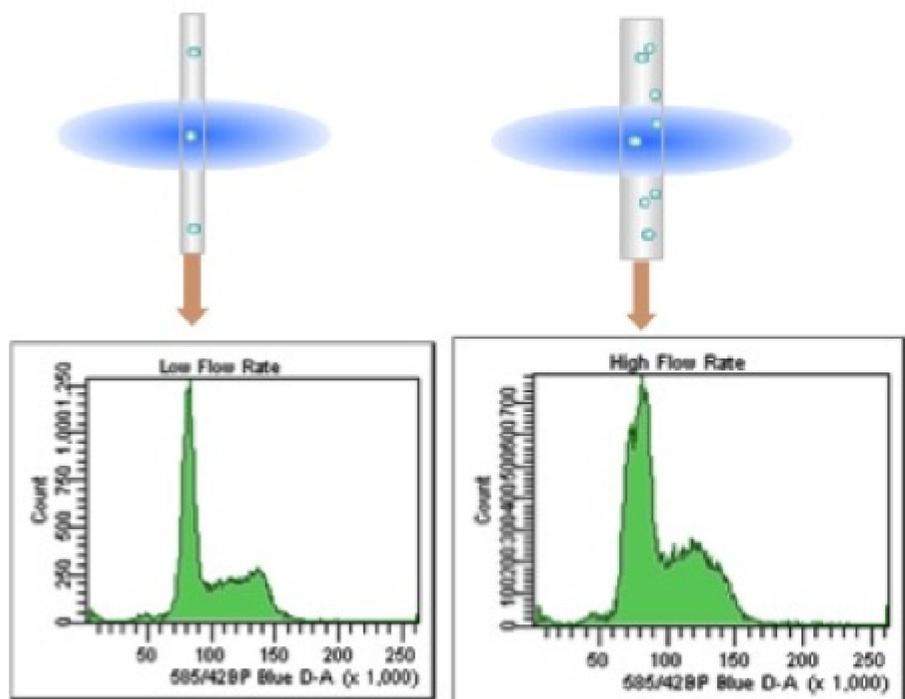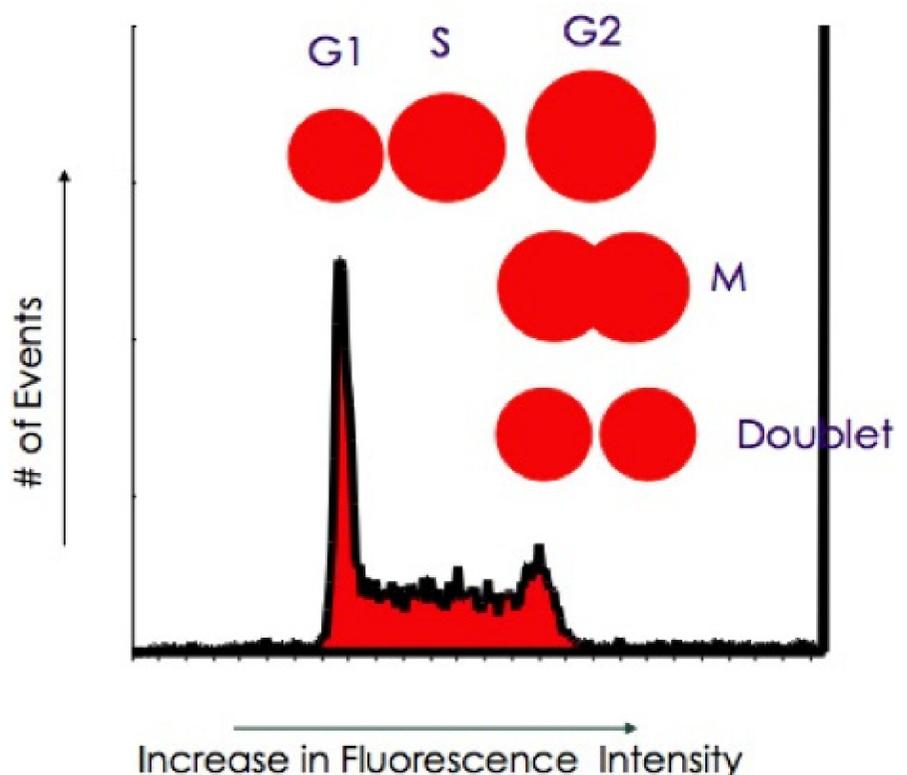Mass cytometry, commercialized by the company DVS Sciences, in the instrument called the CyTOF is a newly emerging technology in the field of flow cytometry. This technology replaces traditional fluorescent labeled antibodies with highly purified, stable isotopes with very well characterized mass values. This extends the power of flow cytometry from 14-18 fluorochromes to over 40+ simultaneous parameters.

The advantage of mass cytometer include the following:

- **Increased number of parameters measured**
- **No equivalent autofluorescence – cells do not contain stable lanthanide ions**
- **Minimal "compensation," mostly due to oxidation of some metals, which is predictable**

The following are a few limitations of the mass cytometer:

- **No equivalent forward or side scatter**
- **Slower acquisition rate (1,000 cells/second vs 10,000 cells/second on conventional flow cytometers)**
- **Cells are destroyed (vaporized by plasma)**
- **High content data needs new analysis techniques**

At the heart of the CyTOF is a very sensitive Time of Flight (TOF) mass spectrometer. This detection system can resolve 143Nd from 144Nd or 152Sm from 153Eu and so on. Cell preparation for CyTOF analysis is similar to traditional flow cytometry, except at the end the cells must be fixed and resuspended in very pure water. These cells are introduced into the CyTOF, where they are vaporized by an induced charge coupled plasma and ionized before being sent to the TOF. There the ion cloud is interrogated and, like in traditional fluorescent flow, the signal intensity detected is proportional to the amount of antibody on the surface of the cell.

CONCLUSION

Decades have passed since the start of modern flow cytometry. The numerous advancements that have taken place since this time have provided researches with a powerful tool for research experiments with hypotheses that are becoming more complex and important to substantiate. From a process standpoint, modern flow cytometry can easily be seen as a transition to a series of automated steps that carry fewer burdens of manual effort that allow researchers to focus on the experiment at hand — a benefit, of course. However, as exposed in this book, the offerings provided by the advancements in flow cytometry have not eliminated the significance of scrutiny, forethought, and review. These are valuable assets that researchers must continue to supply to flow cytometry throughout its continuous evolution.

[1] Accuri Cytometers. (2010). A Guide to Absolute Counting on the Accuri C6 Flow Cytometer. Accuri Cytometers.

[2] Bagwell, C. B., & Adams, E. G. (2006). Fluorescence Spectral Overlap Compensation for Any Number of Flow Cytometry Parameters. Annals of the New York Academy of Sciences, 167-184.

[3] Nguyen, R. P. (2013). Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P., & Roederer, M. Cytometry. Part A: The Journal of the International Society for Analytical Cytology, 306-315.

[4] Perfetto, S. P., Chattopadhyay, P. K., & Roederer, M. (2004). Seventeen-Colour Flow Cytometry: Unravelling the Immune System. Nature Reviews Immunology, 648-655.

[5] Home Page: chromocyte. (n.d.). Retrieved from chromocyte Web site: https://www.chromocyte.com/

[6] (n.d.). Retrieved from Home Page: Fluorish: https://www.fluorish.com

[7] Home Page: FluoroFinder. (n.d.). Retrieved from http://fluorofinder.com/

[8] Bigos, M. (2007). Separation Index: An Easy-to-Use Metric for Evaluation of Different Configurations on the Same Flow Cytometer. Current Protocols in Cytometr, 40:1.21:1.21.1–1.21.6.

[9] Maecker, H. T. (2006). Selecting Reagents for Multicolor Flow Cytometry. HotLines Platinum Edition, 27-30.

[10] Maecker, H., & Trotter, J. (2006). Selecting Reagents for Multicolor Flow Cytometry. HotLines Platinum Edition, pp. 27-30.

WORKS CITED

[11] Telford, W. G., Hawley, T., Subach, F., Verkhusha, V., & Hawley, R. G. (2012). Flow cytometry of fluorescent proteins. Elsevier, 318-330.

[12] Applied Cytometry. (2010). Resources: Flow Cytometry. Retrieved from Applied Cytometry: http://www.appliedcytometry.com/flow_cytometry.php

[13] Riddell, Andrew; Gardner, Rui; Perez-Gonzalez, Alexis; Lopes, Telma; Martinez, Lola. Rmax: A systematic approach to evaluate instrument sort performance using center stream catch. Flow Cytometry Methods and Apporaches, 64-73.

[14] Roederer, M., & Tarnok, A. (2010). OMIPs—Orchestrating multiplexity in polychromatic science. Cytometry, 811-812.

[15] Roederer, M., Nozzi, J. L., & Nason, M. C. (2011). SPICE: Exploration and analysis of post-cytometric complex multivariate dataset. Cytometry Part A, 167-174.

[16] Krutzik, P. O., Hale, M. B., & Nolan, G. P. (2005). Characterization of the Murine Immunological Signaling Network with Phosphospecific Flow Cytometry. Journal of Immunology, 175:2357-2365.

[17] Lyons, A. B., & Parish, C. R. (1994). Determination of lymphocyte division by flow cytometry. Journal of Immunological Methods, 171:131-137.

MODERN FLOW CYTOMETRY

INDEX