Published in final edited form as:

Clin Lab Med. 2007 September ; 27(3): 453–v.

Modern Flow Cytometry: A Practical Approach

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Abstract

In this presentation, we outline ways in which current users of Fluorescence Activated Cell Sorting (FACS) can get more from their FACS work without undue effort. FACS technology development, and the emergence of new software support for various aspects of this technology are now cooperating in this effort.

The demonstration that CD4 T cell counts can be used to monitor HIV disease progression opened the way to the first clinical FACS application; the demonstration that stem cells can be sorted and transferred to appropriately pre-treated recipients now opens the way to new and constructive FACS uses in the future.

We hope that our readers join us in helping to achieve the goal of seeing more and better FACS data in the future.

The use of Fluorescence Activated Cell Sorting (FACS) instruments and methods for clinical purposes dates almost to the time that this unique technology was first introduced (1,2). However, the widespread application of FACS in clinical research and practice really began with the development of monoclonal antibodies that recognized surface proteins or other markers that distinguished functional subsets of peripheral blood lymphocytes from one another. Once this was accomplished, the problem was not whether FACS would be used but how to produce the reagents and refine the technology so that clinically significant subsets could be identified, counted, sorted and even transferred to appropriate recipients. The demonstration that CD4 T cell counts can be used to monitor HIV disease progression opened the way to the first clinical FACS application (3,4); the demonstration that stem cells can be sorted and transferred to appropriately pre-treated recipients now opens the way to new and constructive FACS uses in the future (5). There are many types of FACS instruments made by many different manufacturers, just as there are a multitude of FACS reagents served by a host of different suppliers. The term “FACS”, which we introduced in our initial FACS papers (2, 6,7), was later trademarked by the company that translated our breadboard instrument into a commercially distributable one and is used commercially refer to the instruments and reagents produced by this company (now BDBiosciences, Inc., BDB.com). However, in common usage, “FACS” is widely understood to refer to flow cytometry instrumentation and technology, regardless of the source. We use it here in this sense.

For a technology that has been considered mature for some time, FACS has gone through an amazing growth spurt in the last few years. Substantial improvements have been made in the hardware and software available for data collection and analysis; the array of monoclonal and other reagents available for staining surface and intracellular markers continues to broaden;

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and, innovative tools have now been introduced to help with managing and using reagent inventories to plan FACS analyses, and with annotating and archiving FACS data to meet modern standards. This chapter provides a relatively practical overview of these changes, written both to help readers interpret contemporary literature and to decide when and where to incorporate the newer technology in their own work.

Overall, the newer technologies make it easier to distinguish lymphocyte and other subsets from one another and to more accurately characterize the frequencies and staining properties of the subsets. Thus, they make it easier to achieve the goals of FACS analyses. However, perhaps because the older technology is so difficult to learn and hard to work with, most FACS users who have achieved some level of competence with this older technology are very cautious about trying to enlarge the scope of their skills. "I have enough trouble managing to do just what I know how to do!", is the oft-heard comment.

This chapter is dedicated to these beleaguered users who, like it or not, will soon have to cope with the need for clinically relevant FACS assays that require measurements of intracellular levels of cytokines, phosphor-proteins and other functional markers in individual subsets of naïve and memory T cells in peripheral blood samples from patients and healthy individuals. Hopefully, the insights we present here will ease this transition and will help make current work easier as well.

Collecting FACS data

FACS instruments measure the amount of light emitted by fluorescent molecules associated with individual cells. Lasers are used to excite the fluorescent molecules, which are excited at one range of wavelengths and emit at a second range. Filters in front of each of a series of detectors restrict the light that reaches the detector to only a small range of wavelengths. Newer FACS instruments have up to 4 lasers and 18 or more detectors, commonly referred to as "channels"(8). The older FACS instruments may have only a single laser and only three fluorescence channels. In addition, most of the instruments have a pair of light scatter channels that provide a rough measure of cell size and granularity.

Most of the cell-associated fluorescence detected in a given channel is emitted by fluorochrome-coupled monoclonal antibodies or other fluorescent reagents used to reveal particular aspects of cells of interest. However, some of the fluorescent light comes from fluorescent molecules that are native to the cell and define its background fluorescence. Furthermore, some of the light comes from "spill-over" fluorescence emitted by a fluorescent reagent that is being measured in a different channel. This spill-over fluorescence can seriously compromise the intended measurements on a given channel. Fortunately, though, its contribution can be minimized by applying "fluorescence compensation" corrections based on data from singly-stained samples (or microspheres) that reveal the amounts of spill-over that occurs in each channel in the absence of other fluorochromes(9).

Fluorescence compensation

Most FACS instruments have fluorescence compensation hardware that can be set to correct for spill-over. This utility, which enables real-time visualization of subsets in the format that approximates (or is) the way that they are usually viewed, is crucial for setting gates for cell sorting. However, it has also been used for many years to generate FACS data sets to which compensation corrections are already applied, and it is still used by many laboratories today.

In early versions, these hardware compensation utilities provided the only way to obtain compensated FACS data. However, they have a major pitfall: errors or biases in the way the compensation settings are established during data collection cannot be corrected later, because

the compensated rather than the primary data is recorded in the data file. Further, it is not uncommon for such errors and biases to be introduced, since the methods for setting the online compensation correction commonly involve the arcane twiddling of knobs or other ways of “moving the data” until it falls in the “right” place on the screen. Therefore, collection of compensated FACS data has always posed a problem that, until recently, had to be “lived with”.

Happily, in a move that has substantially improved the quality of FACS data, modern FACS data analysis software has introduced easily accessible compensation utilities that simply make fluorescence compensation the first part of the analysis procedure with any primary FACS data set. Since primary data can be collected with any FACS instrument just by avoiding the compensation step, the new software opens the way to more accurate and reliable data processing. However, to facilitate this process while providing a real-time view of the data, modern FACS instruments have introduced capabilities for recording primary data simultaneously with the visualization of compensated data. Thus, regardless of which instrument is used for data collection, today’s FACS technology readily supports the collection of primary (rather than compensated) data and frees investigators to do better and more accurate analyses.

Logicle vs. Log visualization

Logarithmic scales have been used for years to visualize FACS data, both for data collection and data analysis. However, because logarithmic scales are asymptotic to zero, they cannot be used to correctly represent values for cells whose fluorescence values fall at or below zero. They are the result of background subtraction and fluorescence compensation. This has always caused a problem because negative values for FACS data points are real. Therefore, a display method that provides an accurate place for these points is essential for correctly viewing FACS data (Figure 1).

Logicle (bi-exponential) scales for visualizing FACS data were introduced to remedy this problem (10–12). The *logicle* scale approximates the typical logarithmic scale at the high end but transitions to a linear region around zero that is suitable for displaying data points that fall near or below zero. Thus it allows visualization of the zero and negative data points collected for uncompensated data with the newer FACS instruments. However, *Logicle* visualizations are also the correct way to display compensated data, regardless of the instrument used to collect it, because the subtraction of spill-over data that occurs during the compensation process results in values that fall at or around zero.

In fact, *Logicle* visualization provide a means for evaluating whether compensation has been correctly applied (10–12). Thus, when compensation is correct, cells that have not bound any of the fluorescent reagent detected in a given channel will distribute symmetrically around zero or around their autofluorescence level if that is above zero. Overcompensation centers the distribution of these cells below the autofluorescence level while undercompensation centers the distribution above zero.

Roadmap

In the sections that follow, we summarize procedures that we have established/adopted to make the collection and analysis of FACS data easier and more accurate. We then present examples of High-Dimensional (Hi-D) FACS analyses of human peripheral blood cells stained with reagent sets that reveal typical leukocyte and lymphocyte subsets of interest in the clinical world. Finally, we close with a brief discussion of new software support for experiment planning, data annotation and data archiving.

Staining peripheral blood cells: proper controls and more colors result in better subset resolution

Properly staining cells with fluorochrome-conjugated antibodies, fluorescent compounds and/or substrates is clearly the key to successful FACS analyses. Thus, we suggest:

1. Choose the appropriate reagent combination for your study

Even when they have access to FACS instruments that can collect data for 12 or more fluorescence colors, many investigators continue to do 2-or 3-color FACS analysis. Reasons vary, but this is often because the smaller number of colors seems simpler to manage and/or because the methods used are based on published stain combinations (which often were established years before). However, although the properties and frequencies of many currently targeted cell subsets can be *inferred* by combining data from several 2–3 color stains in this way, a single stain that combines more reagents, and hence more colors, can often be used to unambiguously identify subsets and increase the scope of the analysis. Basically, with the increased availability of monoclonal antibodies and fluorochromes and increased capabilities of today's FACS instruments, and with the software support now available for experiment planning and data analysis (see below), the deprivation imposed by the earlier FACS technology is unnecessary and, in some case, downright counterproductive.

For example, despite current practice in many laboratories, unambiguous identification of naïve T cells in human peripheral blood requires at least 6-color staining to distinguish CD4⁺ and CD8⁺ naïve T cell subsets from the various CD4⁺ and CD8⁺ T cell memory subsets(13). The commonly used 3-color method (a combination of antibodies to CD45RO and CD45RA plus either anti-CD4 or anti-CD8) is clearly inadequate to resolve these subsets (Figure 2). In fact, even with 6 colors, choices have to be made. For example, CD62L/CD45RA combination is better at resolving different memory populations than the CD11a/CD45RA and CD45RO/CD45RA combination (Figure 2). For these reasons, we usually use an 11-color T cell stain allows distinction and characterization of the properties (additional surface markers, internal staining) of the naïve and memory T cell subsets.

2. Add the correct compensation controls

Fluorescence compensation, which corrects for spectral overlap (spillover) of one fluorescence color into the channel in which another color is detected, is paramount to correct analysis of FACS data. The computations required to compensate the FACS data are readily done by several FACS data analysis packages. However, this can only be done if data is collected in the experiment for single-stain “compensation controls” for each reagent used in the experiment. That is, each reagent in each stain must be used separately to stain cells or antibody-capture beads that will report the amount of this reagent detected in each fluorescence channel. A negative, unstained control is also needed. Data collected for these samples are used to compute the “compensation matrix” or the instrument settings that will be applied to correct for fluorescence spillover. The Cytogenie experiment planning software (<http://www.ScienceXperts.com>) automatically specifies the correct compensation controls necessary for the experiment being planned (see below).

3. Include stains for Live/Dead discrimination

Dead cells non-specifically trap fluorochrome-conjugated antibodies. Therefore, it is imperative to include stains that enable elimination of dead cells during FACS analyses. In most standard staining protocols, propidium iodide (PI) is included for this purpose. However, because PI will stain any cell whose membrane is compromised, it cannot be used to stain cells that have been permeabilized to allow entry of stains that detect intracellular cytokines or other

proteins. Using PI with fixed cells can also cause problems, which is important since many of the clinical bio-safety protocols require human samples to be fixed prior to running on the FACS instruments. Thus, reagents other than PI are preferable for live/dead discrimination when fixation is necessary.

A series of “live/dead” discrimination kits have recently become available commercially. The fluorescent dyes supplied in these kits are added to the samples prior to fixation or permeabilization, to identify cells that are dead at this point in the staining procedure(14). These dyes stain both viable and dead cells. However, they stain dead cells much more brightly, making them easily distinguishable during analysis. We find that staining with Invitrogen Live/dead[®] kit (<http://www.Invitrogen.com>) is as good staining with PI for distinguishing live cells from dead cells in unfixed samples (Figure 3).

4. Include Fluorescence-minus-one (FMO) controls when needed

Defining the boundary between positive and negative cells has always been a challenge when dully-staining subsets need to be resolved. FMO controls reveal the maximum fluorescence expected for a given subset in a given channel when the reagent used in that channel is omitted from the stain set. Thus, these controls allow a simple decision as to where to place the upper boundary for non-staining cells in the channel (9,15).

The reasoning underlying the use of these controls is as follows: the compensated values displayed for the cell-associate fluorescence in a given channel include the intrinsic autofluorescence of the cell and the fluorescence due to binding of the reagent detected in that channel. The variation in these measurements, which tends to be most visible in cells with little or no associated fluorescence, is influenced by a variety of factors, including fluorescence compensation. Since the compensation corrections differ according the amounts of the various reagents present on cells in different subsets, it is important to independently determine the boundary between positive and negative cells for each subset. This is done by including FMO controls for all fluorescence channels in which this boundary will be at issue (see figure 2 and the section that follows).

For example, to determine the positive boundary for CD45RA expression in CD62L⁺CD4⁺ and CD62L⁻CD4⁺ populations, an FMO is included that has omits the fluorescence reagent that recognizes CD45RA (Figure 2b, bottom row). In this example, the associated fluorescence is determined in the FMO stain for these two populations and allows assignment of the positive/negative boundary for CD45RA expression. Note that the associated fluorescence seen in the CD45RA channel is different between the CD62L⁺ and CD62L⁻ populations.

5. Use automated protocol design tools if available

Engineers and architects routinely use Computer Aided Design (CAD) tools that provide the information and infrastructure necessary for the efficient planning of both simple and highly complex buildings. The CytoGenie[™] system that we mentioned above provides similar tools that “know about” fluorescence compensation, fluorochrome-coupled reagents, cell samples and other aspects of FACS technology and provide the information and infrastructure necessary for the efficient design of protocols for FACS experiments. The knowledge provided by these tools helps user to choose appropriate reagent combinations and to include appropriate controls. Basically, Cytogenie operates in the background. Without burdening users with unnecessary detail, it guides reagent and other choices necessary to create an experiment plan that is compatible with the intended FACS instrument and the locally available reagent inventory. Cytogenie also prompts for inclusion of relevant controls, axis labels, sample descriptions and other annotation information needed for data analysis, and maintains all of the information

internally so that it recycled and used in later experiments. CytoGenie Basic™, which has nearly all of these capabilities, is available free at <http://www.scienceXperts.com>.

Collect uncompensated data with a well-standardized instrument

1. Set the instrument up correctly

The hardware design for FACS instruments has progressed much in the last few years. With the incorporation of digital detection, linear data collection, and software-computed compensation matrix and overlay, the data quality collected in these digital instruments has increased dramatically over the data acquired using the analog FACS instruments. Importantly, the new digital instruments correctly record data points that fall at or below zero after the instrument background is subtracted.

Logicle visualization, when available on the instrument, is very useful for this purpose because it allows visualization of cells with “negative” fluorescence values. Analog instruments, in contrast, can only record data points as positive values. Thus, they typically record values that fall at or below zero as the lowest value on the logarithmic scale that is used by the instrument. This results in the “pile up” of the data points on the axes to an extent that cannot be readily estimated by inspection (see Figure 2). To avoid this pile-up without sacrificing too much dynamic range for the positive measurements, the instrument can be adjusted so that values for cells without any cell-associated fluorescence fall just above the axis, i.e. are mainly visible at the low end of the scale rather than piling up on the axis.

Prior to data collection, standard reference particles (e.g., Spherotec #197 fluorescent microspheres, <http://www.spherotech.com>) should be used to adjust the PMT so that the beads fall in approximately the same location for each color. Hint: adjusting (“standardizing”) the instrument to the established setting each time data is collected will help make the data from different experiments comparable.

2. Collect uncompensated data

FACS data should always be collected prior to application of the fluorescence compensation correction. If possible, the uncompensated data should be collected on an instrument that has a digital amplifier. However, uncompensated data should also be collected on instruments that only have a logarithmic amplifier. Hardware settings for fluorescence compensation are available on most instruments but, except for sorting, should not be used for data collection. Even then, uncompensated data should be collected for later analysis, and should be collected for the unsorted sample and for the sorted samples, once acquired. The digital compensation utility available on some instruments can be used to generate a compensation matrix and to visualize the compensated data during data collection. Uncompensated data should still be collected, since errors can only be corrected with this primary data. To save time, the matrix constructed during data collection can be recorded and transferred to some analysis programs, where it can be touched up if necessary and applied to the uncompensated data. We cannot emphasize more strongly that collecting compensated data can severely compromise data quality and hence should not be considered a viable option!

3. Safely store and archive the data once collected

If FACS data is worth collecting, it is most likely worth saving. In many situations, regulatory agencies demand that the data be available for a set number of years after it is collected. But even without this prod, most laboratories want to keep their FACS data accessible for at least several years so that it can be used for the usual scientific and legal purposes. Experience has shown, however, that preserving FACS data require more disk space and better disk and computer organization than is available in most laboratories. Therefore, most laboratories wind

up holding on to data until the person who can find it leaves or the disk it was preserved on “disappears”.

To prevent this data loss, and to facilitate locating data for analysis either immediately after an experiment is completed or years later, we built a data storage system at Stanford that records the data immediately after collection, emails an internet-accessible link to the data to the person who collected it, keeps the data on line for several years but writes it to DVD for archival storage almost immediately after collection, and at intervals sends each individual who collects data a CD with copies of all the data files the individual collected. A commercial version of this data storage system, ScienceDataStore™, can be found at <http://www.ScienceXperts.com>.

Use Logicle (bi-exponential) data displays to view compensated data

1. Compute the fluorescence compensation matrix and apply it to the data

Choose a data analysis program that has a compensation utility. Import the data into the program and use the utility provided to specify the data sets collected for the singly compensation controls that are to be used to compensate the experiment data for each stain set (staining combination). After the matrices are computed, apply each to the data for the appropriate samples. We use the Flowjo™ data analysis package (<http://www.Treestart.com>) for this purpose.

Note that data for compensation controls should always be collected together with the data for the samples. Except in dire circumstances (e.g., the dog ate the tubes containing the compensation controls), matrices from previous experiments should not be used, since compensation corrections are dependent on the calibration setting for each channel in the instrument. With the current instrumentation, these settings cannot be set and reset with the accuracy necessary to assure that fluorescence compensation will be accurate from one data collection session to another. In addition, changes in reagent fluorescence may occur even when the same reagent conjugation lot is used. Therefore, data accuracy demands that compensation controls be part of the each experiment for which data is collected.

2. Use Logicle displays to view the data

After fluorescence compensation correction has been applied, FACS data should be visualized on Logicle (bi-exponential) scales to obtain the clearest separation of subsets and the best view of subsets at the low end of the scale, i.e., with little or no cell associated fluorescence. FlowJo was the first FACS data analysis package to provide a commercial version of this utility, which we developed initially at Stanford (16,17). We use this software for all of our analyses. Like the logarithmic visualization methods that have been used for many years for flow cytometry data, *Logicle* visualization methods do not alter raw data in any way. They simply provide a method of visualizing data that enables display of data points that are obscured by logarithmic visualization methods and a more intuitive way for visualizing data in the region around zero. However, Logicle visualization offers an additional benefit: it provides a flexible scan that can be altered to enable the best visualization for a population of interest. This process, referred to as Logicle or bi-exponential “transformation”, is analogous to changing the scale on any graph to spread out the points of interest. However, in Logicle displays, it serves to increase the ability to resolve populations at the low end of the scale.

3. Use “transformation” to distribute the data as broadly as possible in the logicle display

Flowjo software provides a default setting for viewing FACS data in *Logicle* visualization. However, this default visualization setting is often sub-optimal as fluorescence values for some cells may fall below the default, forcing pile-ups at the low end of the scale during the initial

input into FlowJo that cannot be corrected except by re-importing the data. To overcome this problem, we routinely set the FlowJo “negative width” default for data import to -50 (and sometime find we have to reset it to -100). When these pileups occur even with these broad “width” settings, it usually means that the PMT was set too high during data collection. Once the data is imported, we then do an initial series of gatings to select non-clumped, live, size-gated lymphocytes, and to view this population in a window of its own (see Figure 2). This allows us to define a broad population that can be used to reset the Logicle visualization scales in a way that will put all cells in the population on scale in all channels. Resetting the scales in this way is referred to as “transformation” and can be repeated during the analysis whenever the scale becomes too compressed, i.e., there are very few points in the region below zero but the zero is position well above the graph origin. In these cases, the values surrounding zero are highly compressed and it is difficult to resolve populations that fall within that regions.

Figure 4 shows a data set before and after transformation. Note that the logicle transformation may increase or decrease length of the linear region that surround zero on the logicle scale. In Figure 4, it “stretches this region, and hence helps to resolve cells that were initially crowded into a much small part of the graph. Scale transformations such as these are more familiar in settings where a scale is changed from log to linear to separate points that would otherwise be crowded together. We developed the logicle scale to serve the same purpose in a way that is well suited to the types of data collected in flow cytometry.

4. Sequentially set gates to define subsets of interest

A combination of cell surface markers can be used to identify various human lymphocyte and leukocyte populations. The gating path (strategy) that is followed can make a great difference in the ease with which individual subsets can be teased out of the overall data set. We start by gating out dead cells and scatter gating to remove small debris and large clumps of cells. After this, we routinely try several strategies before deciding on one that is useful. Two of the strategies on which we have settled for routine work are shown in Figures 2 and 5.

Figure 2 shows an example of the 9-color stain combination and gating strategy that we use to characterize the properties of memory and naïve human peripheral blood T lymphocytes in the CD4 and CD8 T cells subsets(13). Six of the colors are routinely used to identify the subsets; the remaining colors are used for experiment purposes. Figure 5 shows the 10-color stain combination and gating strategy that we routinely use to identify human peripheral blood eosinophils, neutrophils, basophils, NK cells, monocytes, T cells, and B cells; 6 colors are used for subset discrimination(18). The reagents in the stain sets used for each of the staining combinations are presented in Table 1.

Note that, although quadrant gating is used quite commonly, this method most often forces the inclusion of unwanted cells in one or another of the gates. Further, it relies on the use of the upper boundary for “negative” cells in the lower left quadrant as a threshold with which to distinguish negative from positive cells at other locations. As we have indicated above, FMO controls are far more appropriate and rewarding for such purposes (see Figure 2B). In essence, unless the populations in all four quadrants are well separated, use of the quadrant gating method should be avoided.

Conclusion

Considering the amount of time, effort, money and patient sample material that goes into FACS studies every year, it is surprising (to us) that FACS studies have for so long relied on methodology developed in what might reasonably be termed “the dark ages of FACS”. In our discussion here, we have attempted to outline ways in which current FACS users can get more from their FACS work without undue effort. Fortunately, FACS technology development, and

the emergence of new software support for various aspects of this technology, are now cooperating in this effort. We look forward to seeing more and better FACS data in the future, and hope that our readers join us in helping to achieve this goal.

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Figure 1. *Logicle* (bi-exponential) visualizations provide more accurate data displays than logarithmic visualizations across the entire scale

In the figure, data are shown for live CD3⁺ lymphocytes from human peripheral blood. In the logicle display (left panel), CD4 single positive (CD4 SP) cells appear as a single subset centered near zero on the CD8 axis. In the logarithmic view (right panel), this population is artifactually split: the cells with the lowest fluorescence values in the CD8 channel pile-up on the CD8 axis, while the cells with statistically equivalent but somewhat higher values are separated into a population that appears to express low amounts of CD8. With more complex collections of subsets, the Logicle displays also provide better resolution for subsets at the low end of the scale.

Figure 2. Memory T cell subsets are best resolved by stains that include CD62L

Compensated data are shown for freshly isolated human peripheral blood mononuclear cells (Ficoll-isolated PBMC) stained with a 9-color reagent combination. **A)** Cells are initially gated to define live CD3⁺ T lymphocytes from dead and clumped cells. **B)** Three commonly used strategies for resolving naïve and memory subsets are shown. The CD62L and CD45RA combination resolves both the CD3⁺CD4⁺ and the CD3⁺CD8⁺ memory T cells into the M1, M2 and M3 subsets shown in the figure. These subsets are not resolvable by the CD45RA versus CD45RO staining method that is common used to distinguish memory T cells from naïve T cells. Mainly, the lie along the diagonal “swatch” that connects the two visible subsets. The fluorescence-minus-one (FMO) control stain (bottom row of the figure) identifies the boundary for CD45RA expression in the naïve and M3 CD4⁺ T cells. Note that this boundary cannot accurately be established by reference to the boundaries for CD45RA⁺ cells in the CD62L-CD45RA⁺ subset. This is a good illustration of the dangers of using quadrant-type gating.

Figure 3. Live/dead discrimination

Ficoll-isolated human PBMC normally do not contain very many dead cells. Therefore, to demonstrate how to gate these cells, we have added a small percentage on heat-killed cells to the Ficoll-isolated sample. Data for two common methods for discriminating live from dead cells are shown: the upper left panel shows cells stained with the Invitrogen Live/dead™ kit ([Http://Invitrogen.com](http://Invitrogen.com)) and the lower left panel shows cells stained with propidium iodide (PI). Both reagents are equivalent for distinguishing live from dead cells. However, the Invitrogen Live/dead kit is adaptable for analyzing human samples that require fixation, either immediately (for safety reasons) or prior to permeabilization for intracellular staining.

Figure 4. Transformation improves subset visualization in Logicle displays

We routinely do an initial *Logicle* transformation initially based on all live lymphocytes (upper panels) because our initial data input is set to include “cells” with large negative values. This initial transformation resets the *Logicle* scales in all data dimensions to eliminate regions where no data points exist. However, for individual subsets, the region below zero may be very sparsely populated even if data points exist in this region for other subset. Therefore, we sometimes do retransformations to obtain the best views for these subsets.

It is often useful to eliminate regions below zero where no data points exist because these “white-space” regions tend to compress the regions containing important fluorescence values. For example, in the upper panels in the figure, the region between -100 and 100 on the CD11a axis (shown in the figure as a shaded region) is compressed such that it is difficult to resolve subset details in this region. A better view of these data can be obtained by calling for a retransformation of the displayed data. This results in a new display in which the CD11a data for all cells in the subset being viewed are now appropriately distributed along the CD11a axis. Note the disappearance of “white space” and the expansion of the region between -100 and +100 fluorescence values on this axis in the retransformed data (bottom panels). In other analyses, retransformation often resolves subsets that have low cell-associated fluorescence from those with autofluorescence levels (not shown).

Importantly, retransformation based on the one subset (e.g., CD3⁺ CD4⁻ CD8⁺) may not be optimal for viewing a different subset (e.g., CD3⁺ CD4⁺ CD8⁻) cells, because the two subsets have bound different reagents and hence have different fluorescence compensation correction. Thus, additional retransformation may be required based specifically on individual subsets. Note that any transformation, including the original one, can be recovered by gating on the appropriate subset and calling for another retransformation.

Figure 5. Simultaneous analysis of granulocytes, monocytes and lymphocytes in human whole blood
Common procedures for the analysis of human blood include gradient density centrifugation-based or magnetic separation of subsets of interest. However, multiparameter flow cytometry enables examination of leukocytes subsets in whole blood, without any prior purification or manipulation. This figure illustrates stepwise gating, based on 6-color staining, to resolve neutrophils and other leukocyte populations in human peripheral blood. Other markers can be added to this 6-color combination to investigate functional properties (e.g., intracellular kinase activity or cytokine production) of cells in the identified subsets. The populations identified in this stain set include eosinophils (Eo), basophils (Bas), neutrophils (Neu), natural killer (NK), T (LT), and B (LB) cells.

Table 1**Stain combinations used for identifying leukocyte subsets in human blood**

The fluorescence detection channel denotes the fluorescence of the primary fluorochrome being detected. Reagents such as PerCPCy5.5 and Pacific Blue have overlapping emission spectra with PECy5.5 and Cascade Blue, respectively, and thus are detected in the same channels in place of the primary fluorochrome.

Fluorescence detection channel	Stain sets	
	Naïve and memory T	Leukocyte subsets
Fluorescein	CD45RO	CD63
PE	CD45RA	CD80
PE-TR		Live/Dead
PECy5	CD3	
PECy5.5	CD8-PerCPCy5.5	CD209
PECy7	CCR7	
Alexa594	CD62L	
APC	CD11a	CD294
APCCy5.5	CD28	
APCCy7	CD4	HLA-DR
Cascade Blue	Monochlorobimane	CD16-Pacific Blue
Quantum dot 605		CD66b