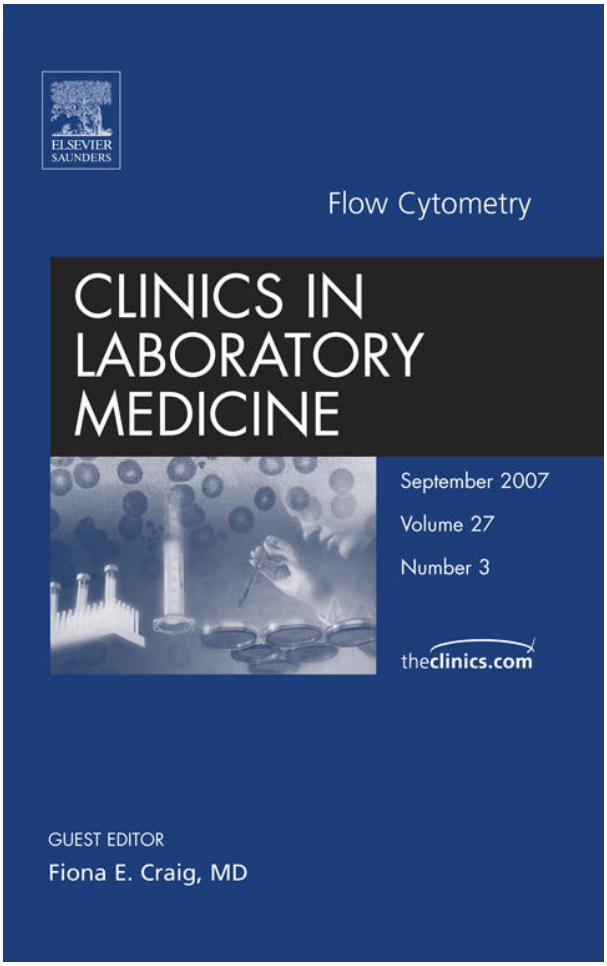

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Modern Flow Cytometry: A Practical Approach

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The use of fluorescence-activated cell sorting (FACS) instruments and methods for clinical purposes dates almost to the time that this unique technology was introduced [1,2]. The widespread application of FACS in clinical research and practice really began, however, 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 or not 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 pretreated recipients now opens the way to new and constructive FACS uses in the future [5].

There are many types of FACS instruments made by different manufacturers, just as there are a multitude of FACS reagents served by a host of suppliers. The term, "FACS," which the authors introduced in their initial FACS articles [2,6,7], later was trademarked by the company that translated the breadboard instrument into a commercially distributable one and is used commercially to refer to the instruments and reagents produced by this company (now BD Biosciences) [8]. In common usage, however, FACS widely is understood to refer to flow cytometry instrumentation

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and technology, regardless of the source. It is used in this article in this sense.

For a technology that for some time has been considered mature, FACS has gone through an amazing growth spurt in the past 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, and innovative tools have been introduced to help manage and use reagent inventories to plan FACS analyses and annotate and archive FACS data to meet modern standards. This issue provides a practical overview of these changes, written 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 characterize the frequencies and staining properties of the subsets more accurately. Thus, they make it easier to achieve the goals of FACS analyses. 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 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 an oft-heard comment.

This article is dedicated to these beleaguered users who, like it or not, soon will have to cope with the need for clinically relevant FACS assays that require measurements of intracellular levels of cytokines, phosphoproteins, 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 presented here will ease this transition and help make current work easier.

Collecting fluorescence-activated cell sorting 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 four lasers and 18 or more detectors, commonly referred to as channels [9]. Older FACS instruments may have only a single laser and three fluorescence channels. In addition, most of the instruments have a pair of light scatter channels that provide an approximate 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. Some of the fluorescent light comes from fluorescent molecules, however, that are

native to the cell and define its background fluorescence. Furthermore, some of the light comes from spillover fluorescence emitted by a fluorescent reagent that is being measured in a different channel. This spillover fluorescence seriously can compromise the intended measurements on a given channel. Fortunately, however, its contribution can be minimized by applying fluorescence compensation corrections based on data from singly-stained samples (or microspheres) that reveal the amounts of spillover that occurs in each channel in the absence of other fluorochromes [10].

Fluorescence compensation

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

In early versions, these hardware compensation utilities provided the only way to obtain compensated FACS data. They have a major pitfall, however: 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 are recorded in the data file. Further, it is not uncommon for such errors and biases to be introduced, because the methods for setting the online compensation correction commonly involve the arcane twiddling of knobs or other ways of "moving the data" until they fall in the "right" place on the screen. Therefore, collection of compensated FACS data has posed a problem that, until recently, had to be "lived with."

Happily, in a move that has improved the quality of FACS data substantially, 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. Because 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. 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 versus logarithmic visualization

Logarithmic scales have been used for years to visualize FACS data for data collection and data analysis. Because logarithmic scales are asymptotic

to zero, however, 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 always has 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 viewing FACS data correctly (Fig. 1).

Logicle (biexponential) scales for visualizing FACS data were introduced to remedy this problem [11–13]. 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. Logicle visualizations, however, also are the correct way to display compensated data, regardless of the instrument used to collect it, because the subtraction of spillover data that occurs during the compensation process results in values that fall at or around zero.

Logicle visualization provides a means for evaluating whether or not compensation has been applied correctly [12–14]. When compensation is correct, cells that have not bound any of the fluorescent reagent detected in a given channel distribute symmetrically around zero or around their

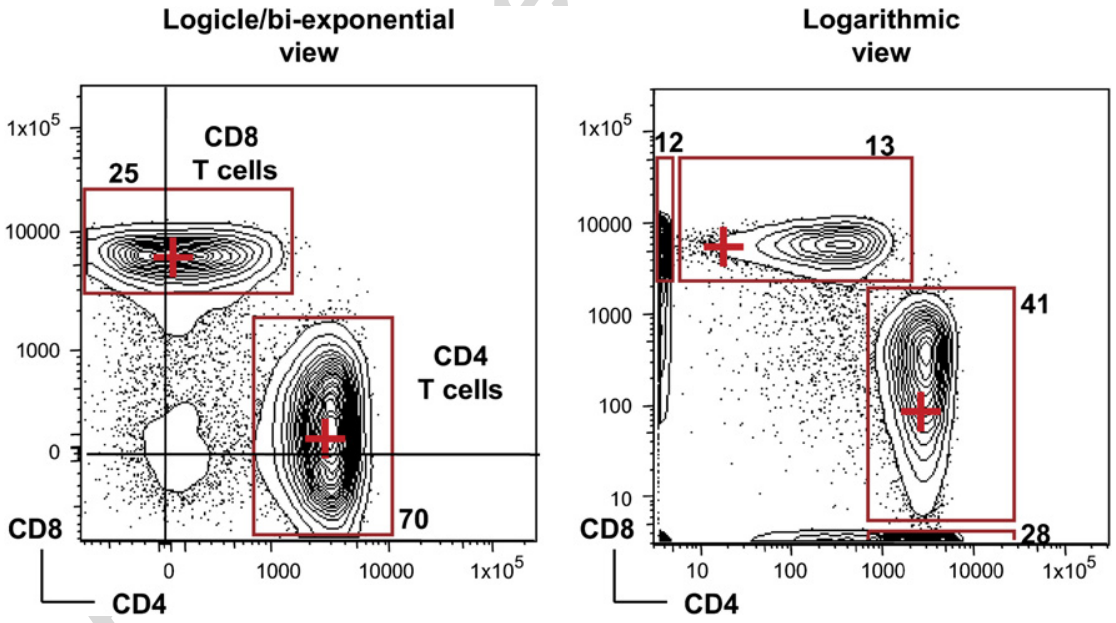

Fig. 1. Logicle (biexponential) visualizations provide more accurate data displays than logarithmic visualizations across the entire scale. Data are shown for live $CD3^+$ lymphocytes from human peripheral blood. In the Logicle display (*left panel*), CD4 single positive (CD4 SP) cells appear as a single subset centered near zero on the CD8 axis. In the logarithmic view (*right panel*), this population is split artifactually: the cells with the lowest fluorescence values in the CD8 channel pile up on the CD8 axis, whereas the cells with statistically equivalent but somewhat higher values are separated into a population that seems 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.

autofluorescence level if that is above zero. Overcompensation centers the distribution of these cells below the autofluorescence level, whereas undercompensation centers the distribution above zero.

Roadmap

The sections that follow summarize procedures that the authors have established or adopted to make the collection and analysis of FACS data easier and more accurate. Examples are presented of high-dimensional 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, in closing is 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, or substrates clearly is the key to successful FACS analyses. Following are suggestions.

Choose the appropriate reagent combination for a study

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

For example, despite current practice in many laboratories, unambiguous identification of naïve T cells in human peripheral blood requires at least six-color staining to distinguish $CD4^+$ and $CD8^+$ naïve T-cell subsets from the various $CD4^+$ and $CD8^+$ T-cell memory subsets [15]. The commonly used three-color method (a combination of antibodies to CD45RO and CD45RA plus anti-CD4 or anti-CD8) clearly is inadequate to resolve these subsets (Fig. 2). Even with six colors, choices have to be made. For example,

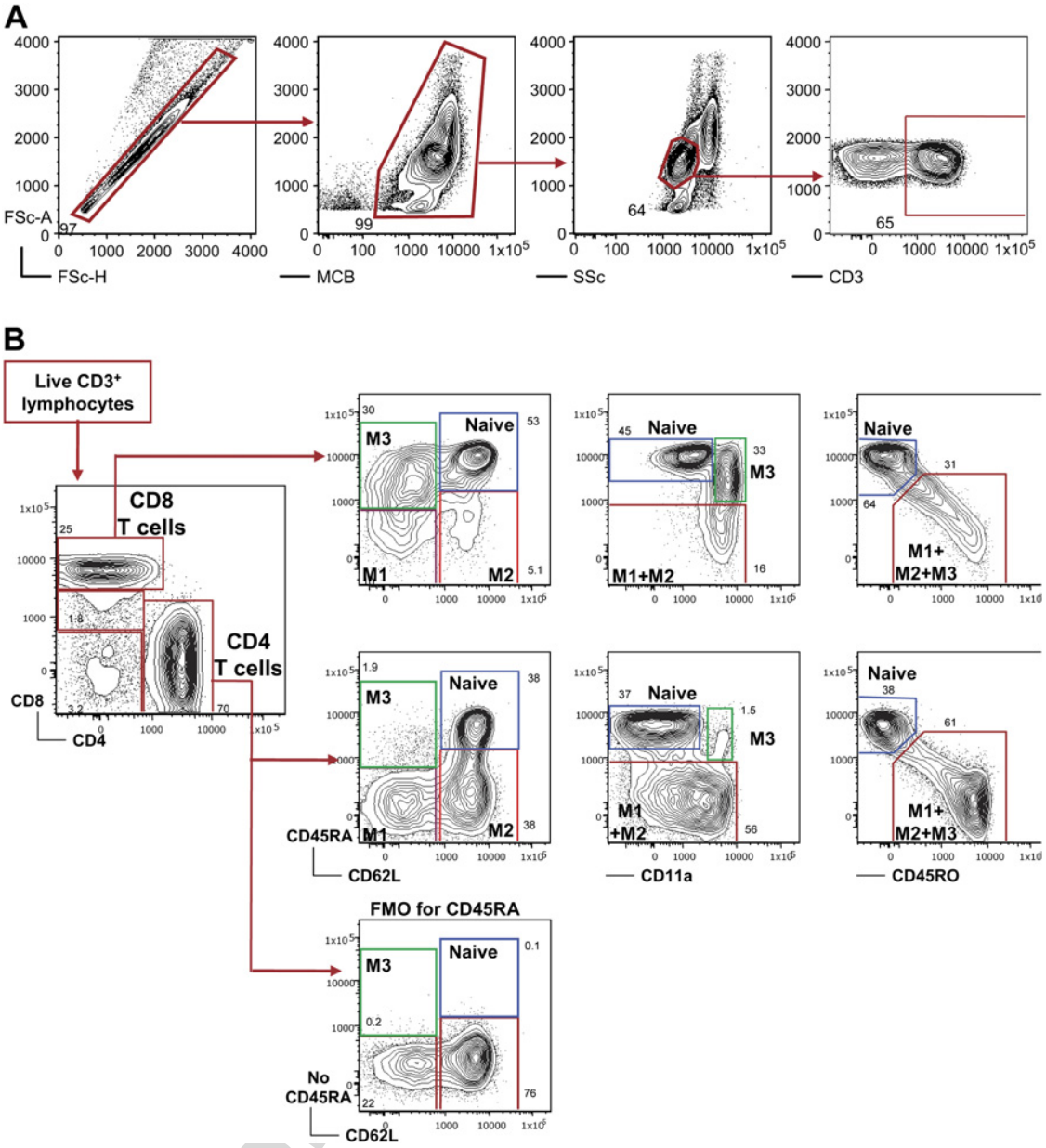

Fig. 2. Memory T-cell subsets are resolved best by stains that include CD62L. Compensated data are shown for freshly isolated human peripheral blood mononuclear cells (PBMC) (Ficoll-isolated PBMC) stained with a ten-color reagent combination. (*A*) Cells initially are gated to define live $CD3^+$ T lymphocytes from dead and clumped cells. (*B*) Three strategies used commonly for resolving naïve and memory subsets are shown. The CD62L and CD45RA combination resolves the $CD3^+CD4$ and the $CD3^+CD8$ memory T cells into the M1, M2, and M3 subsets shown. These subsets are not resolvable by the CD45RA versus CD45RO staining method that is used commonly to distinguish memory T cells from naïve T cells. Mainly, these memory subsets (M1, M2, and M3) lie along the diagonal "swatch" that connects the two visible subsets. The FMO control stain (*bottom row*) identifies the boundary for CD45RA expression in the naïve and M3 $CD4^+$ T cells. Note that this boundary cannot be established accurately by reference to the boundaries for $CD45RA^-$ cells in the $CD62L^-CD45RA^-$ subset. This is a good illustration of the dangers of using quandrant-type gating.

the CD62L/CD45RA combination is better at resolving different memory populations than the CD11a/CD45RA and CD45RO/CD45RA combination (see Fig. 2). For these reasons, the authors usually use an 11-color T-cell stain that allows distinction and characterization of the properties (additional surface markers and internal staining) of the naïve and memory T-cell subsets.

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 done readily by several FACS data analysis packages. This can be done, however, only if data are 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 report the amount of this reagent detected in each fluorescence channel. A negative, unstained control also is needed. Data collected for these samples are used to compute the ''compensation matrix'' or the instrument settings that are applied to correct for fluorescence spillover. The CytoGenie experiment planning software (www.scienceXperts.com) [16] automatically specifies the correct compensation controls necessary for the experiment being planned (discussed later).

Include stains for live/dead discrimination

Dead cells trap fluorochrome-conjugated antibodies nonspecifically. 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. Because PI stains any cell whose membrane is compromised, however, 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 also can cause problems, which is important because many of the clinical biosafety protocols require human samples to be fixed before 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 recently has become available commercially. The fluorescent dyes supplied in these kits are added to the samples before fixation or permeabilization to identify cells that are dead at this point in the staining procedure [17]. These dyes stain viable and dead cells. They stain dead cells more brightly, however, making them easily distinguishable during analysis. The authors find that staining with the Invitrogen live/dead kit (www.invitrogen.com) [18] is as good as staining with PI for distinguishing live cells from dead cells in unfixed samples (Fig. 3).

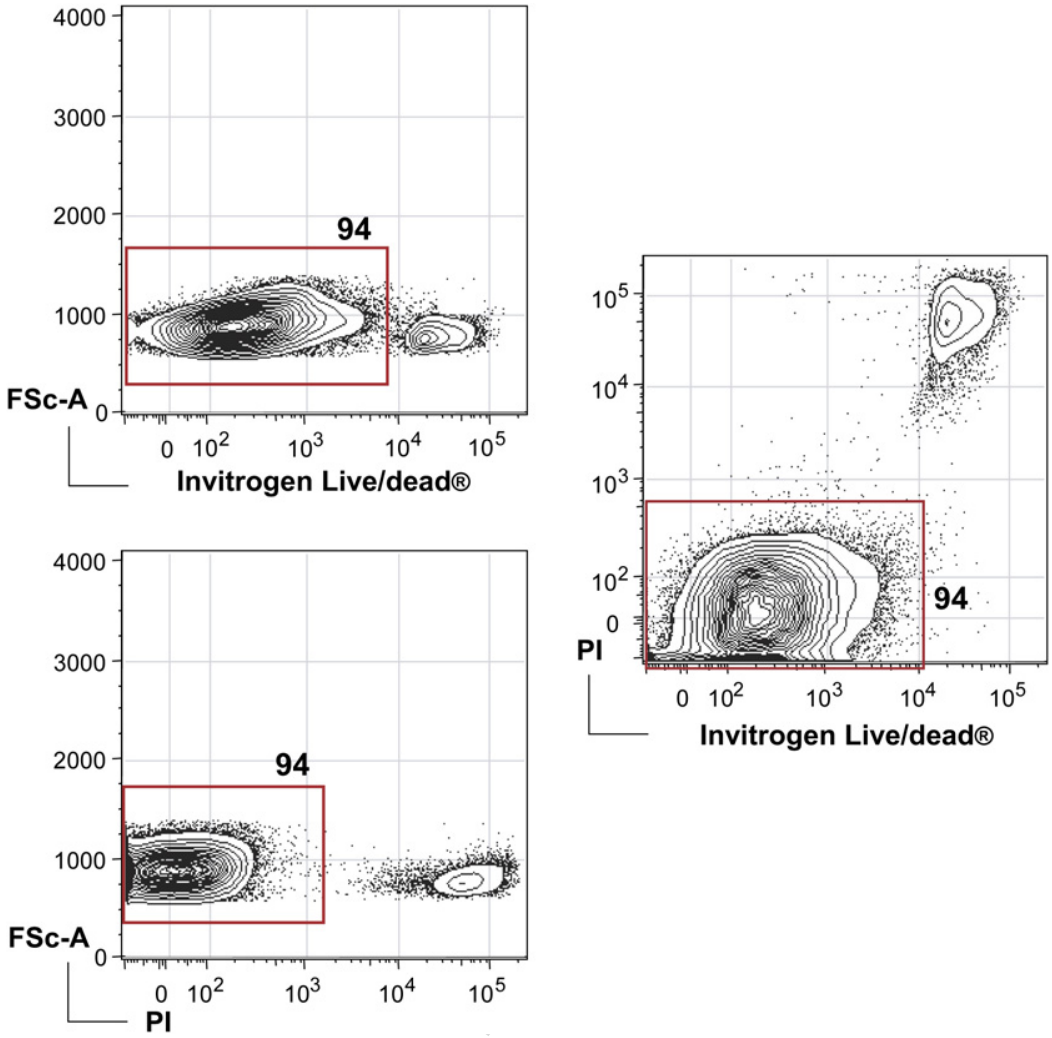

Fig. 3. Live/dead discrimination. Ficoll-isolated human PBMC normally do not contain many dead cells. Therefore, to demonstrate how to gate these cells, a small percentage of heat-killed cells to the Ficoll-isolated sample was added. Data for two common methods for discriminating live from dead cells are shown: the upper left panel shows cells stained with the Invitrogen live/dead kit (www.invitrogen.com.) and the lower left panel shows cells stained with PI. Both reagents are equivalent for distinguishing live from dead cells. The Invitrogen live/dead kit, however, is adaptable for analyzing human samples that require fixation, either immediately (for safety reasons) or before permeabilization for intracellular staining.

Include fluorescence-minus-one controls when needed

Defining the boundary between positive and negative cells always has been a challenge when dully staining subsets need to be resolved. Fluorescence-minus-one (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 nonstaining cells in a channel [10,19].

The reasoning underlying the use of these controls is as follows: the compensated values displayed for the cell-associated fluorescence in a given channel include the intrinsic autofluorescence of the cell and the fluorescence

as a result of 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. Because 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 is at issue (see Fig. 2 and the following discussion).

For example, to determine the positive boundary for CD45RA expression in $CD62L^{+}CD4^{+}$ and $CD62L^{-}CD4^{+}$ populations, an FMO is included that omits the fluorescence reagent that recognizes CD45RA (see Fig. 2B). 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. The associated fluorescence seen in the CD45RA channel is different in the $CD62L^{+}$ and $CD62L^{-}$ populations.

Use automated protocol design tools if available

Engineers and architects routinely use computer-aided design tools that provide the information and infrastructure necessary for the efficient planning of simple and highly complex buildings. The CytoGenie system (discussed previously) 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 users choose appropriate reagent combinations and 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 can be recycled and used in later experiments. CytoGenie Basic, which has nearly all of these capabilities, is available free [16].

Collecting uncompensated data with a well-standardized instrument

Set the instrument up correctly

The hardware design for FACS instruments has progressed much in the past 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

compared with the data acquired using the analog FACS instruments. 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 an instrument, is useful for this purpose because it allows visualization of cells with "negative" fluorescence values. Analog instruments, in contrast, can record data points only 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 estimated readily by inspection (see Fig. 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 (ie, are visible mainly at the low end of the scale rather than piling up on the axis).

Before data collection, standard reference particles (eg, Spherotec #197 fluorescent microspheres) [20] should be used to adjust the photomultiplier tube (PMT) voltage settings so that the beads fall in approximately the same location predetermined for each color. Adjusting (standardizing) the instrument to the established setting each time data are collected helps make the data from different experiments comparable.

Collect uncompensated data

FACS data always should be collected before application of the fluorescence compensation correction. If possible, the uncompensated data should be collected on an instrument that has a digital amplifier. Uncompensated data, however, also should be collected on instruments that have only 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 still should be collected, because errors can be corrected only 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. It cannot be emphasized more strongly that collecting compensated data can compromise data quality severely and, hence, should not be considered a viable option!

Safely store and archive the data once collected

If FACS data are worth collecting, most likely they are worth saving. In many situations, regulatory agencies demand that the data be available for

a set number of years after collected. But even without this prod, most laboratories want to keep their FACS data accessible for at least several years so they can be used for the usual scientific and legal purposes. Experience has shown, however, that preserving FACS data requires 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 the data leaves or the disk they were preserved on "disappears."

To prevent this data loss and to facilitate locating data for analysis immediately after an experiment is completed or years later, the authors built a data storage system at Stanford Shared FACS Facility that records the data immediately after collection, e-mails an Internet-accessible link to the data to the person who collected them, keeps the data on line for several years but writes them 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 www.sciencexperts.com [16].

Using Logicle (biexponential) data displays to view compensated data

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. The authors use the FlowJo data analysis package for this purpose [21].

Data for compensation controls always should be collected together with the data for the samples. Except in dire circumstances (eg, the dog ate the tubes containing the compensation controls), matrices from previous experiments should not be used, because 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 is 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.

Use Logicle displays to view the data

After fluorescence compensation correction is applied, FACS data should be visualized on Logicle (biexponential) scales to obtain the clearest

separation of subsets and the best view of subsets at the low end of the scale (ie, with little or no cell-associated fluorescence). FlowJo was the first FACS data analysis package to provide a commercial version of this utility, which the authors developed initially at Stanford [22,23] and use for all analyses.

Like the logarithmic visualization methods that have been used for many years for flow cytometry data [13,14] , 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. Logicle visualization, however, 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 biexponential transformation, is analogous to changing the scale on any graph to spread out the points of interest. In Logicle displays, however, it serves to increase the ability to resolve populations at the low end of the scale.

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. This default visualization setting often is suboptimal, however, 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 reimporting the data. To overcome this problem, the authors routinely set the FlowJo negative-width default for data import to −50 (and sometime find it has to be reset to −100). When these pile-ups occur, even with these broad width settings, it usually means that the PMT was set too high during data collection.

Once the data are imported, the authors then do an initial series of gatings to select nonclumped, live, size-gated lymphocytes and to view this population in a window of its own (see Fig. 2). This allows defining a broad population that can be used to reset the Logicle visualization scales in a way that puts 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 (ie, there are few points in the region below zero but the zero is positioned 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 region.

Fig. 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 Fig. 4, it ''stretches'' this region and, hence, helps to resolve cells that initially were crowded into a much small part of the graph. Scale transformations, such as these, are more

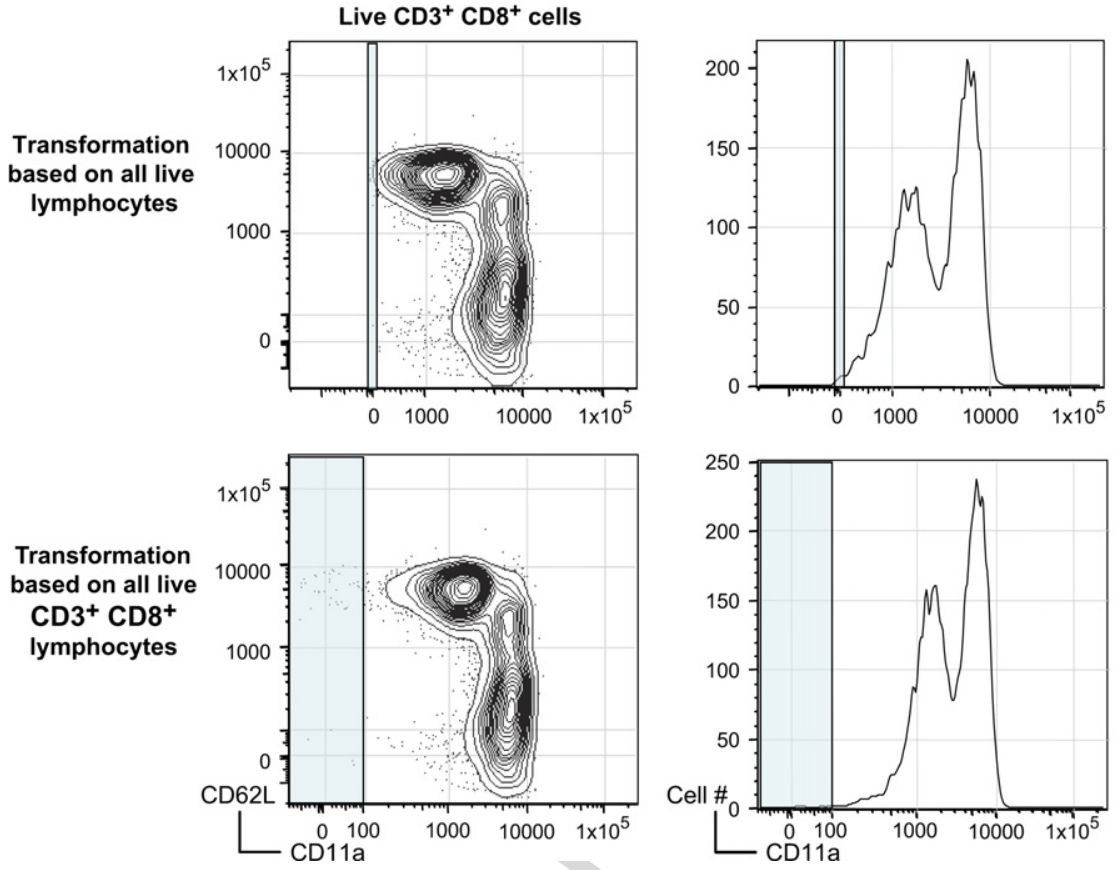

Fig. 4. Transformation improves subset visualization in Logicle displays. The authors routinely do a initial Logicle transformation based initially on all live lymphocytes (*upper panels*) because the initial data input is set to include "cells" with large negative values. This initial transformation resets the Logicle scales in all data dimensions to eliminate regions where no data points exist. For individual subsets, however, the region well below zero may be sparsely populated even if data points exist in this region for other subset. Therefore, the authors sometimes do retransformations to obtain the best views for these subsets that have very few events in the negative region. It often is 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 (*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 now are distributed appropriately 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). Retransformation based on the one subset (eg, $CD3^+$ $CD4^-CD8^+$) may not be optimal for viewing a different subset (eg, $CD3^+$ $CD4^+CD8^-$) 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. Any transformation, including the original one, can be recovered by gating on the appropriate subset a calling for another retransformation.

familiar in settings where a scale is changed from logarithmic to linear to separate points that otherwise would be crowded together. The authors 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.

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. The authors start by gating out dead cells and scatter gating to remove small debris and large clumps of cells. After this, they routinely try several strategies before deciding on one that is useful. Two of the strategies settled on for routine work are shown in Figs. 2 and 5.

Fig. 2 shows an example of the nine-color stain combination and gating strategy that the authors use to characterize the properties of memory and naïve human peripheral blood T lymphocytes in the CD4 and CD8 T-cell subsets [15]. Six of the colors are used routinely to identify the subsets; the remaining colors are used for experiment purposes. Fig. 5 shows the 10-color stain combination and gating strategy used routinely to identify human peripheral blood eosinophils, neutrophils, basophils, natural killer cells, monocytes, T cells, and B cells; six colors are used for subset discrimination [24]. The reagents in the stain sets used for each of the staining combinations are presented in Table 1.

Although quadrant gating is used commonly, this method most often forces the inclusion of unwanted cells in one or another of the gates.

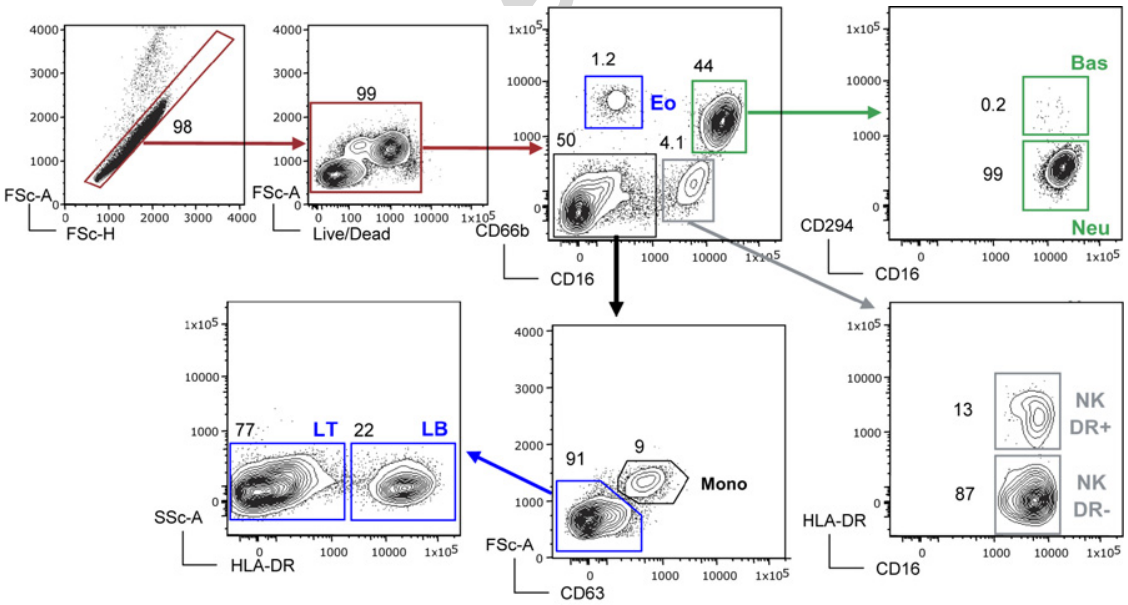

Fig. 5. Simultaneous analysis of granulocytes, monocytes, and lymphocytes in human whole blood. Common procedures for the analysis of human blood include gradient density centrifugation based or magnetic separation of subsets of interest. Multiparameter flow cytometry, however, enables examination of leukocytes subsets in whole blood, without any prior purification or manipulation. This figure illustrates stepwise gating, based on six-color staining, to resolve neutrophils and other leukocyte populations in human peripheral blood. Other markers can be added to this six-color combination to investigate functional properties (eg, 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

	Stain sets	
Fluorescence detection channel	Naïve and memory T cells	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

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.

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 discussed previously, FMO controls are more appropriate and rewarding for such purposes (see Fig. 2B). In essence, unless the populations in all four quadrants are well separated, use of the quadrant gating method should be avoided.

Summary

Considering the amount of time, effort, money, and patient sample material that goes into FACS studies every year, it is surprising that FACS studies for so long have relied on methodology developed in what might reasonably be termed, "the dark ages of FACS." This discussion has 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 now are 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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