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Publishing flow cytometry data

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Alvarez DF, Helm K, DeGregori J, Roederer M, Majka S. Publishing flow cytometry data. *Am J Physiol Lung Cell Mol Physiol* 298: L127–L130, 2010. First published November 13, 2009; doi:10.1152/ajplung.00313.2009.—Cellular measurements by flow cytometric analysis constitute an important step toward understanding individual attributes within a population of cells. Assessing individual cells within a population by protein expression using fluorescently labeled antibodies and other fluorescent probes can identify cellular patterns. The technology for accurately identifying subtle changes in protein expression within a population of cells using a vast array of technology has resulted in controversy and questions regarding reproducibility, which can be explained at least in part by the absence of standard methods to facilitate comparison of flow cytometric data. The complexity of technological advancements and the need for improvements in biological resolution results in the generation of complex data that demands the use of minimum standards for their publication. Herein we present a summarized view for the inclusion of consistent flow cytometric experimental information as supplemental data. Four major points, experimental and sample information, data acquisition, analysis, and presentation are emphasized. Together, these guidelines will facilitate the review and publication of flow cytometry data that provide an accurate foundation for ongoing studies with this evolving technology.

flow cytometric analysis

BACKGROUND

The advent of cellular measurements by flow cytometric analysis constituted an important step toward understanding individual attributes within a population of cells. Initially developed in the 1960s, flow cytometry made automated separation of cells based on the unique recognition of cellular patterns within a population feasible (5). Using such a separation approach, cellular patterns can be identified by assessing, in individual cells within a population, protein expression using fluorescently labeled antibodies and other fluorescent probes (1, 4). In the early 1980s, an approach to characterize cells by analyzing the expression of more than one protein was possible by the simultaneous use of two different fluorophore-conjugated antibodies (3). The ability to analyze the expression of multiple proteins has since been markedly extended. Not only can an extensive number of surface marker analyses be performed on single cells, but descript intracellular functions can also be studied. Currently, up to 20 different parameters can be analyzed by using a combination of different fluorophores and scatter light measurements, an approach known as polychromatic flow analysis (2, 11).

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The technology for accurately identifying subtle changes in protein expression within a population of cells has not come without a price. Reproducibility of results following multiparameter analysis has been controversial and can be explained at least in part by the absence of standard methods to facilitate comparison of flow cytometric data. Lack of such standard methods has further led to conflicting data regarding cellular phenotypes that represent minor or rare fractions within populations. Examples of rare subpopulations include very small stem cells (VSEL), a population of pluripotent stem cells, and side population (SP) cells, a rare population of less than 1×10^{-2} cells of total bone marrow cells (12, 18, 21). Similarly, identification of endothelial progenitor cells from resident tissues or circulation also comprises a variable but small population. These require the integrated analysis of multiple parameters to define the small populations as well as a single cell (20). In the case of endothelial progenitor cells, initial findings of putative phenotypes based on the ability of cells to express certain markers have been reevaluated.

The complexity of technological advancements and the need for improvements in biological resolution results in the generation of complex data that demands the use of minimum standards for their publication. In the case of findings utilizing flow cytometric analysis, comprehensive guidelines to outline fundamental information to publish flow cytometry data have been generated (6, 7, 14, 16). Herein, we present a summarized view for the inclusion of consistent flow cytometric experimental information within the manuscript and the opportunity to further strengthen its interpretation with supplemental data. Four major points, experimental and sample information, data acquisition, analysis, and presentation are emphasized.

EXPERIMENTAL AND SAMPLE INFORMATION

A detailed description of the experimental design and preparation of cell suspension samples to be analyzed by flow cytometry is key to interpreting the first steps of data analysis. Such a description may include the number of independent experiments performed and the number of samples analyzed

Table 1. *Example of reagent list*

Instrument: Beckman Coulter Cyan ADP		
Antibody/Probe	Fluorochrome	Vendor/Cat. No./Clone
Anti-mouse CD45	APC	BD Pharmingen/#559864/30F11
Anti-mouse GR-1	PE	BD Pharmingen/#553128/RB6-8C5
Anti-mouse Thy1.2(CD90)	FITC	BD Pharmingen/#553003/53-2.1
Live-dead discriminator	Propidium iodide	Sigma/# P-4864

Table 2. *Example of flow cytometer setup*

Instrument: Beckman Coulter Cyan ADP				
Laser lines	488 nm			635 nm
Emission filters	530/40	575/25	613/20	665/20
Fluorochrome	FITC	PE	PI	APC

within each experiment (e.g., duplicates, triplicates, etc.). Details of how cell suspensions were prepared for analysis are necessary to judge what the corresponding appropriate controls may be for flow cytometry analyses, and to ensure a greater degree of reproducibility between laboratories. Such details should include specific proteases used in cell isolation, filtration approaches to ensure single cell suspensions, red blood cell lysis reagents, permeabilization reagents and procedures, as well as fixatives utilized. All fluorescent reagents used should be included in the Methods, including vendors, catalog numbers, and clone designations using a list or table (see example in Table 1).

DATA ACQUISITION

The parameters set during data acquisition using a flow cytometer are as important as sample preparation. Methodology should include a description of the flow cytometer instrument used including the manufacturer, model, and software. The laser lines and optical emission filters used for the corresponding fluorescent reagents should also be listed. Examples are given in Tables 1 and 2, which may be included in a Supplemental Methods section. As multiple fluorochromes are used, the need for distinguishing them and avoiding spectral overlap is increased (13). Therefore, there is a need for com-

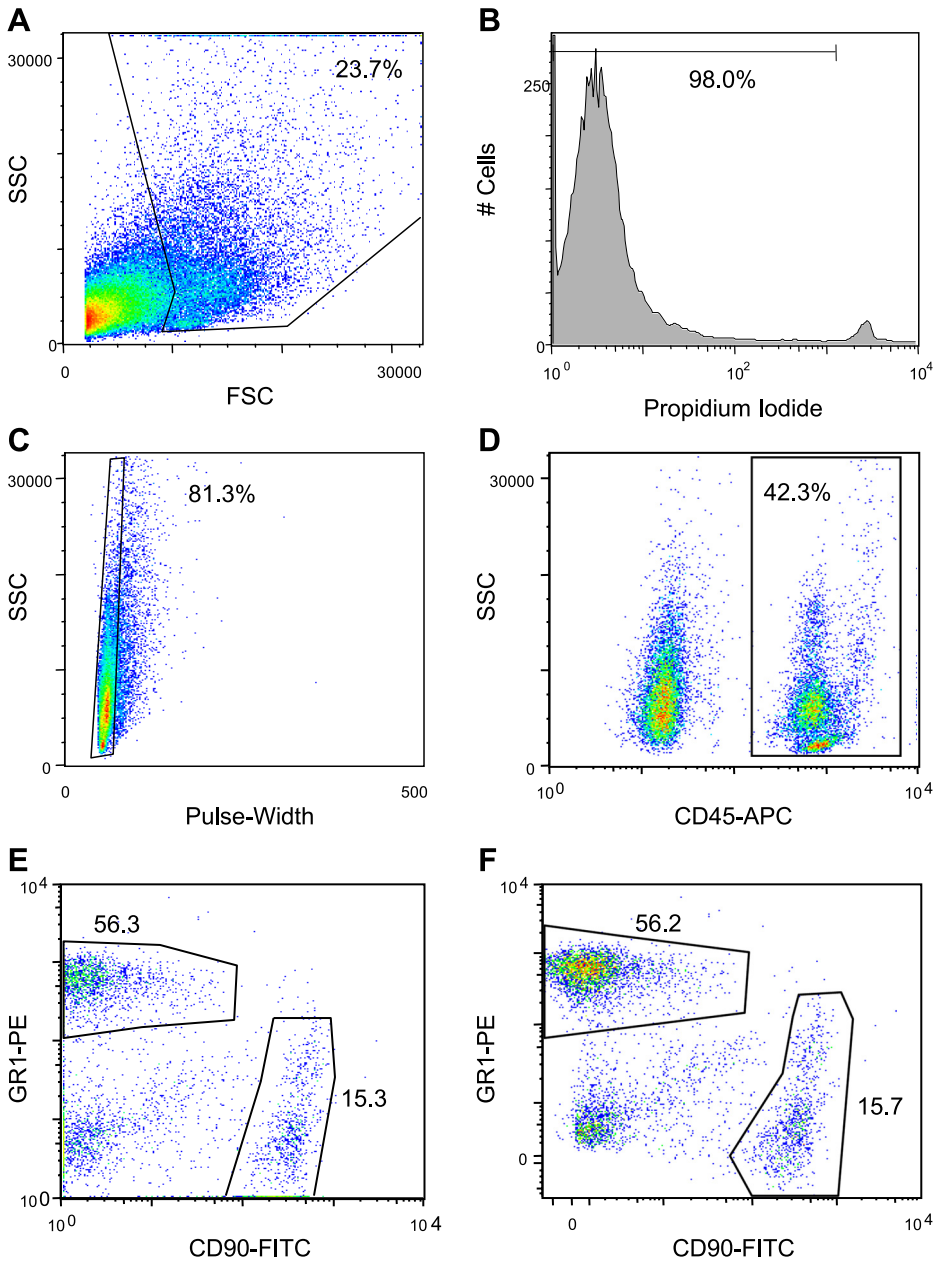

Fig. 1. Example analysis of mouse bronchoalveolar lavage (BAL) fluid. BAL fluid was collected from mice with acute lung injury by lavaging lungs with 300 μl of PBS. The cellular components were isolated by centrifugation, and red blood cells were lysed using an eBioscience red blood cell lysis buffer according to the manufacturer's instructions. Remaining cells were collected by centrifugation and incubated for 10 min on ice with antibodies using staining buffer (PBS + 2% FBS). The antibody combinations for this experiment were CD45-APC/GR-1-PE/CD90-FITC (Table 1). Compensation was performed using CD45-APC and GR-1-PE and CD90-FITC with antibody capture beads (Anti Rat k; BD Pharmingen, #552844). Propidium iodide was used as the live/dead discriminator. Acquisition and analysis was performed on a Beckman Coulter Cyan ADP using Summit Software 4.3. Analysis was performed using Flow Jo v8.5. The gating tree was set as follows. *A*: FSC/SSC (represents the distribution of cells in the light scatter based on size and intracellular composition, respectively) to *B*: live gate (PI negative, which represents the fraction of viable cells within the sample analyzed) to *C*: SSC/pulse width (excludes events that could represent more than 1 cell) to *D*: SSC/CD45 positive to *E*: GR-1-PE/CD90-FITC (identifies selective subsets) or *F*: biexponential GR-1-PE/CD90-FITC (which more accurately displays the selective subsets). A total of 7,535 cells were analyzed, and the result is presented in *E* and *F* (19); however, a SSC pulse-width gate was used to exclude potential doublets. Greater than 8% of events fell on the axis (*E*), so biexponential scaling was used (*F*).

pensation, a mathematical process that corrects for spillovers or spectral overlap when the emission of at least two fluorophores are examined on a single cell to ensure that the output of a channel represents only the fluorescence arising from the fluorophore the channel is designated to detect (8, 13, 15). Uncompensated or improperly compensated samples result in measurement artifacts and improper quantification of antigen density (13). When identifying and quantitating rare and dim event populations, proper compensation is important for optimal estimation of frequency. That being said, poor compensation can have a significant impact on the field of progenitor biology in which multiple fluorochromes are used to distinguish small populations of cells. Therefore, it is necessary to specify how multi-color compensation was performed by including the antibodies, cells, or beads used. Additionally, the number of events analyzed for each sample or a minimum number of events for a target population should be provided.

When considering compensation, it is also important to consider statistics to determine the collection of a large enough sample size to ensure a precision of measurement for labeled populations of interest, including rare event populations. The distribution of events collected and presented for analysis is described by Poisson statistics. The precision of the data is dependent on the sample size or number of cells counted per population of interest. The sample size must be increased to decrease the coefficient of variance to an acceptable level. Following the determination of appropriate sample sizes, fluorescence expression or intensity within subpopulations of cells in a sample may be determined.

DATA ANALYSIS AND PRESENTATION

Data analyses and presentation to represent the cell population of interest are as important as acquisition. The gating scheme used should be outlined and include all light scatter gates, live-dead gates, doublet gates, and fluorescence-detecting gates (Fig. 1). The method used to define gates (thresholds) should be stated (17). This might include unstained controls, biological controls (wild-type vs. GFP), isotype controls, fluorescence minus one controls, or the use of an internal negative population present within the sample. Information should be presented about approaches to compensation. Specify how multi-color compensation was performed including antibodies, cells, or beads used. If statistical analysis is presented, it needs to be assessed on the scale values exclusively and needs to determine whether it is the fluorescence intensity of the gated population or the proportion of cells within a specific gate that is examined. Report whether the statistical analysis represents a comparison among mean, median (e.g., in the case of fluorescence intensity measurements), or percentage (in the case of proportion of cell measurements) values. When multiple data sets are compared for fluorescence intensity, verify that the flow cytometer was calibrated to exclude the possibility of instrument-related fluorescence intensity changes over time. Report the software package utilized to perform the statistics and the number of replicate analysis (n).

While data presentation in a manuscript is selected to highlight the cell populations of interest and convey research findings in a simple and understandable fashion, tables and bar graphs do not provide the information necessary for an adequate interpretation of flow cytometry data. Flow cytometry data plots should be included in the body of the text or the Supplemental data section, and stylistic choice of color scheme should be left to the authors' discretion unless a comparison between two distinct populations is depicted within a single figure, in which case the use of color is necessary. The following are suggestions for formatting plots (9). Both axes of the plot should be labeled, and proper quantitation for linear or logarithmic scales should be displayed. It is useful for the reader if plots are labeled with the antibody and fluorochrome used rather than instrument-specific parameter descriptions. For example, CD45-FITC rather than FL1-height. Percentages should be listed in gates. These data may also be compiled (in addition to plots) into table format for ease of interpretation. Plots should avoid piling up events on the axis. Adjust scale if necessary or provide a different scale if appropriate [e.g., changing logarithmic to biexponential scale (10)]. The number of total events in a plot should be displayed or listed in the figure legend. One-, bi-, or multidimensional displays are acceptable. However, select a plot that better displays the point the author is trying to convey. For bivariate displays, use contours or density dot plots rather than single dot displays.

CONCLUSIONS

Recent advancements in flow cytometry allow multiparametric analysis for better identification and functional assessment of individual cells within a population. However, the malleability of the technique has resulted in a vast array of approaches in its use that leads to conflicting data reproducibility. A clear solution to the problem is the establishment of standard methods to facilitate data comparison. To achieve this goal, systematic flow cytometry data description is described. The application of fundamental guidelines described in this paper will most certainly result in the advancement of our field and the promotion of new strategies that enhance the quality of experimentation during planning stages.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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