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Review Article

Considerations for the Control of Background Fluorescence in Clinical Flow Cytometry

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Accurate measurement of antigen-positive cells by flow cytometry can be hampered by background fluorescence of antigen-negative cells and other particles (e.g., debris). This article focuses on three major causes of background (autofluorescence, spectral overlap, and undesirable antibody binding) by reviewing individual aspects of flow cytometric measurements that contribute to these causes. The appropriate use of controls facilitates a thorough understanding of these contributing factors as well as the development of robust cell labeling protocols intended for routine flow cytometric analysis. We present a set of recommendations that enables the user to develop an optimized cell labeling protocol that minimizes background and maximizes the ability to reliably distinguish between a positive and a negative population of cells. These recommendations are also intended to augment existing guidelines designed to aid in the formulation of a consensus regarding the utility of flow cytometry for the analysis of clinical samples. © 2009 Clinical Cytometry Society

Key terms: cell labeling; antibody; flow cytometry; controls; recommendations

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A persisting concern in flow cytometric analysis is the ability to reliably distinguish between antigen-positive and antigen-negative populations of cells, and to accurately measure the population of positive cells (1). Although flow cytometry only permits measurements above a defined threshold of signal intensity and may therefore not be able to measure cells that are truly negative for a particular antigen, we will apply the term "antigen-negative," or simply "negative cells" to cells that express antigen levels below the detection limits of a flow cytometer (see Table 1 for definitions of terms). Parameters like the resolution index (2,3) and the staining index (4) are useful tools to describe quantitatively the degree of separation between two populations. Both tools require the ability to determine median and standard deviation (SD) of fluorescence intensities of cell populations (see formulas in Table 1). When the degree of separation between "positive" and "negative" is only minimal, statistical tests are available to assess the likelihood of one population being significantly different from another (5). However, when populations overlap considerably and neither the median nor the SD can be determined, the resolution or staining index cannot be calculated.

A prerequisite for exploiting these tools is proper use of control samples to confirm that the test sample was prepared correctly and, if necessary, to establish the level of background. Ignoring instrument-derived background signal (generally called noise), the cause of background fluorescence can be categorized into three groups: (I) autofluorescence, (II) spectral overlap, and (III) undesirable antibody binding. The level of background in each of these groups depends on, and may be

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Table 1
Table of Terms

Term	Description
Antigen	A substance that induces the production of antibodies.
Autofluorescence	Self-fluorescence; inherent fluorescence of an object.
Background	Extraneous signals that can be confused with the phenomenon to be measured.
Bleed-through (emission)	Spillover; part of an emission spectrum that overlaps with the peak intensity of another spectrum.
Crossreactivity	Binding to an identical or similar epitope than that the antibody was generated against and present on different antigens (Ref. 23).
Epitope	Portion of the antigen that an antibody is generated against (Ref. 23).
Event	(Light-derived) electronic signal processed by a flow cytometer and added to the dataset (list-mode file).
FMO control	Fluorescence-minus-one; a control that includes all antibodies involved in the experiment, except one (Ref. 20).
Internal negative control	Population of cells that does not express the antigen of interest in a sample that also contains a population of cells that does.
Isoclonic control	Mixture of a fluorochrome-conjugated and excess of an identical, but unconjugated antibody.
Isotype control	Antibody of the same class (isotype) of immunoglobulin as the specific antibody, but generated against an antigen that is not present on or in the cells under study.
Negative cells	Cells that express antigen levels at or below the detection limits of the measurement technology.
Nonspecific antibody binding	Binding of an antibody to something that it was not generated against.
Relative fluorescence intensity	Amount of fluorescence relative to the instrument settings of the flow cytometer.
Resolution index	Degree of separation between a positive and negative population, described as follows: $(X_{pos} - X_{bckgrnd})/\sqrt{(SD_{pos}^2 + SD_{bckgrnd}^2)}$ (Refs. 1, 2).
Specific antibody binding	Binding of an antibody to the epitope it was generated against.
Spectral compensation	Mathematical method to correct for bleed-through emission.
Spectral overlap	Spillover between two (or more) fluorescence emission spectra.
Spreading error	Error due to imprecise measurements and spectral compensation.
Staining index	Describes how much a positive population is separated from the negative population, as follows: $[(X_{pos} - X_{bckgrnd})/2] \times SD_{bckgrnd}$ (Ref. 4).
Undesirable antibody binding	User-defined term, but in general: Any type of bond between an antibody and a cell that obscures correct interpretation of the data.

minimized by, several components of the measurement (e.g., antigen of interest, choice of antibody, choice of fluorochrome, cell labeling protocol, and optical configuration of the flow cytometer) as described later. With a thorough understanding of how each component may contribute to the background level, sets of control samples can be designed to reliably tell truly positive from negative and other detected signals (i.e., events) in a flow cytometric data set. This review discusses the causes of background fluorescence and the methods used to control for this background. We provide recommendations for the use of controls to identify background fluorescence in clinical flow cytometry such as unstained cells, cells that do not express the antigen of interest in an antibody-labeled sample, as well as fluorescence-(or full)-minus-one (FMO), isotype and isoclonic controls. In addition to previously published guidelines (6–9), this document is intended to contribute to formulation of consensus regarding the utility of flow cytometry for analysis of clinical samples.

BACKGROUND CAUSED BY AUTOFLUORESCENCE

In addition to the fluorescence emission of the antibody-bound fluorochrome, excitation sources in flow cytometers (specifically 488 nm) also excite other naturally occurring cellular components (e.g., NADPH and flavins) (10) resulting in levels of autofluorescence that may obscure detection of target-specific antibodies. Since it has been shown that the use of a 532 nm laser is less problematic in this regard (11), it may be worth considering the excitation source prior to purchasing a new cytometer or upgrading an old one.

The level of autofluorescence is also influenced by the cells' biological and physiological conditions and is also cell type specific (10). Because of their high content of granule-associated flavoproteins, autofluorescence of granulocytes is generally higher than that of lymphocytes (12). As cells age, or are subject to other manipulations (e.g., tissue culture, thermocycling, and in-situ hybridization) autofluorescence of these cells usually increases although this may be reduced by the addition of dithiothreitol (DTT) (13), trypan blue (14), crystal violet (15), or η-octyl-β-D-galactopyranoside (16). In addition, several tools have been developed to correct for autofluorescence in flow cytometric data to increase sensitivity and thus the ability to detect weakly positive cell populations. Steinkamp and Stewart described a method utilizing electronic circuitry that subtracts autofluorescence-derived signals from the total signal measured by means of dual, specific and nonspecific, laser excitation (17). Alberti et al. developed an alternative method requiring only single laser excitation (18). Since neither of these inventions can be applied to a wide spectrum of applications, cell-by-cell autofluorescence correction as described by Roederer and Murphy (19)

appears to be a more accessible alternative (see recommendations in this article). Similar to spectral compensation, this method subtracts the signal from a detector with no spillover (but dedicated to measure autofluorescence) from the detector that measures the emission from a specific fluorochrome, by means of software.

BACKGROUND CAUSED BY SPECTRAL OVERLAP

The spectral ranges of most fluorochrome emissions are so large that the emission of a particular dye is generally measured by many detectors rather than by the one solely designated to measure the emission peak of that dye. This overlap of emission spectra in the various detection regions (also called "bleed-through" or "spillover") contributes to background fluorescence as well and can be corrected for by utilizing spectral compensation. When applied appropriately, the emission bleed-through intensity measured by a particular detector is mathematically reduced to the same median intensity as the negative population measured by that detector. However, if the SD of the "bleed-through population" is high, a so called "spreading error" is added to the negative population in the compensated dataset, resulting in decreased sensitivity (20). This phenomenon becomes significant when more than four colors are used.

Clearly, spectral overlap can be minimized at the outset by choosing combinations of fluorochromes that have little to no overlap with each other (21) or by choosing multiple fluorochrome-specific, independent excitation sources. For example, spectral overlap between FITC and PE-Cy7 using one excitation source (488 nm) is significantly higher compared with that between FITC and APC-Cy7 when excited by two excitation sources (488 and 633 nm). At the risk of reducing sensitivity, spectral overlap can also be minimized by choosing optical filters with narrower bandpass characteristics (22).

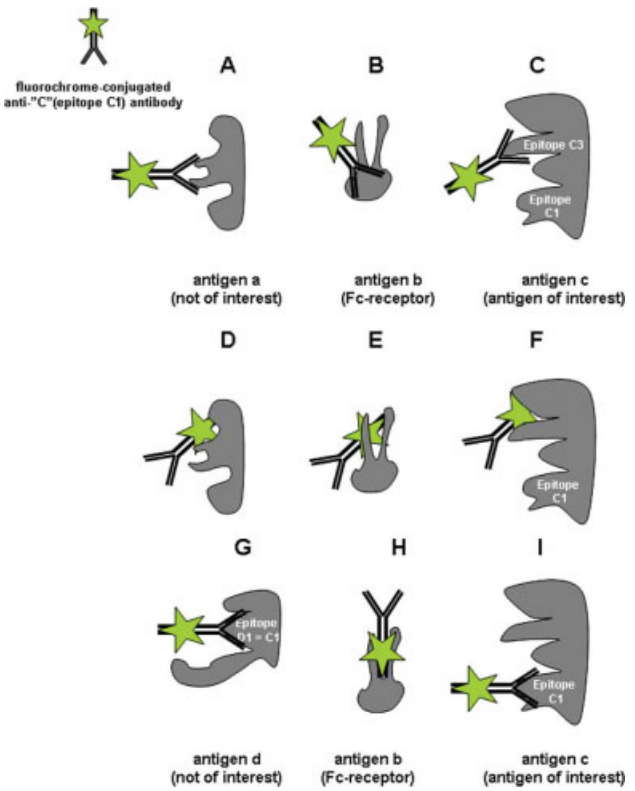

FIG. 1. Schematic representation of modes of antibody binding. (**A**–**F**) Nonspecific antibody binding: (A) to an antigen that is not of interest in the study; (B) to an Fc-receptor, but not as a receptor-ligand interaction; (C) to the antigen of interest, but not to the epitope to which the antibody was generated against; (D–F) through its conjugated fluorochrome to an antigen that is not of interest, Fc-receptor or the antigen of interest, respectively. (**G**–**I**) Specific antibody binding: (G) to an epitope that is shared by a different antigen from what it was generated against; (H) to an Fc-receptor as part of a receptor-ligand interaction; (I) to the antigen and epitope of interest, the antibody was generated against. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BACKGROUND CAUSED BY UNDESIRABLE ANTIBODY BINDING

The term undesirable antibody binding describes a user- or application-defined phenomenon (Table 1). Although it is generally applied to nonspecific antibody binding, specific binding can sometimes be undesirable as well. The term nonspecific antibody binding means binding of an antibody to a different epitope than the one it was generated against. It includes several ways of antibody binding as schematically represented in Figure 1 (Panels A–F). For example, an antibody that has been generated against epitope c1 of antigen c can nonspecifically bind to a similar epitope present on antigen a (Panel A). Thus, it is plausible that nonspecific binding in fact occurs at the antigen of interest, yet to a different epitope from the one it was generated against (Panel C). Specific binding to the epitope of an antigen of interest occurs through the antibody binding and/or the $F(ab)_2$ domains of the variable regions of an antibody molecule (Panel I) (23). Various studies have indicated an epitope can be shared by different antigens (24–26). Depending on the investigator's interest, this phenomenon (sometimes referred to as cross-reactivity) (23) may result in specific antibody binding that is undesirable (Panel G).

The Fc-regions of many antibodies also interact with Fc-receptors of various specificities (CD16, CD32, CD64, CD89) functioning as ligand for the activation of the cells upon which they are expressed (Panel H). Although classified as receptors, Fc-receptors can also be considered as antigens (with epitopes that antibodies can be generated against). Fc-receptors are expressed on a variety of unfixed, live cells, including neutrophils, monocytes, macrophages, B-cells, natural killer cells, and some T-cell subsets (27). Fc-mediated binding to cells that do not express the antigen of interest is in this context considered undesirable antibody binding. The level of this type of undesirable antibody binding can be reduced by blocking Fc-receptors with anti-Fc-receptor antibodies (28), Fab or $F(ab)_2$ fragments thereof, or with pooled immunoglobulins, or even with whole serum.

In addition to undesirable specific binding to Fc-receptors, undesirable nonspecific antibody binding can occur

as well (Panel B). Such binding is of much lower affinity than the specific antigen-antibody binding and can be controlled with blocking reagents like nonimmune serum. However, nonspecific binding is usually best eliminated by optimizing antibody concentration and antibody amount with titration assays (29,30).

Undesirable binding may occur through the fluorochrome that is conjugated to the antibody (Panels D–F), an issue most commonly observed in the context of energy-coupled or tandem dyes (31,32). Fluorochrome conjugation can change the overall charge of the antibody. For example, FITC-conjugated anti-CD34 monoclonal antibodies are more negatively charged than their PE-conjugated counterparts. Consequently, some CD34 epitopes are poorly detected, if at all, by FITC-conjugated anti-CD34 antibodies (reviewed in Ref. 33).

The choice of conjugated form can have other undesirable effects. For example, the PE conjugated form of the IgG1 anti-glycophorin A antibody KC16 (clone 11E4B-7-6) induces large-scale aggregation of red blood cells which highly express this mucin-like molecule. In contrast, the more negatively charged FITC conjugates of the same monoclonal antibody induce much less aggregation of red blood cells (34).

Finally, for intracellular antibody labeling protocols, the fixation/permeabilization procedure used may result in the intracellular entrapment of antibodies. The choice of conjugated form is also important in this context. For example, a high number of FITC molecules per immunoglobulin (i.e., a high *F/P* ratio) results in a highly charged antibody that binds nonspecifically to cytoplasmic elements and is extremely difficult to remove by washing.

From the earlier discussion, it is obvious that the degree of undesirable antibody (conjugate) binding depends not only on the antibody's specificity per se, but on a wide variety of other interactions, as well as the physical and biological conditions that the target cell populations encountered during the assay. Knowledge of the molecular nature of the antigen and/or epitope under investigation when selecting specific monoclonal antibodies and specific conjugates for use can therefore be advantageous.

Typically a set of specific control samples (isotype, isoclonic, internal negative, and FMO) is measured and analyzed to determine which category observed background is caused by. Levels of background may be assessed by comparing fluorescence intensities between unstained cells and that of these specific control samples.

ISOTYPE CONTROLS

Isotype controls are antibodies of the same class (isotype) of immunoglobulin as the specific antibody, but are either myeloma-derived antibodies of unknown specificity, or are raised against an antigen (e.g., T2-mycotoxin or dinitrophenyl) that is presumed not to be present on or in the cells under study (35). The ideal isotype control should "match" the specific antibody not only in heavy chain (IgA, IgG, IgD, IgE, or IgM), subclass and light chain (kappa, lambda) class but also in fluorochrome type and number of fluorochrome molecules per immunoglobulin (*F/P* ratio). It should also have been derived by the same manufacturing process as the specific conjugate under investigation (36) and be presented in the same formulation (buffer, concentration, preservative, etc.). Such a "perfectly matched" isotype control represents one way to determine whether there is undesirable antibody binding through Fc-receptors and/or fluorochrome binding (37). However, whether the more commonly used, but less rigorously selected, isotype controls are sufficient to determine nonspecific binding has been under debate for over a decade (38,39). Since the V_H and V_L regions that determine the antibody binding sites of the isotype control are different from those of the specific antibody in the experimental sample, one can argue that the two antibodies may also show different levels of nonspecific binding through this part of the protein. Indeed, it has been noted that an isotype and fluorochrome-matched control showed more cells nonspecifically stained than specifically stained by the corresponding CD34 conjugate used (38,40). In addition, similar characteristics of nonspecific binding can be expected through the C_H and C_L regions of an isotype control and a specific antibody. Isotype controls may have utility in specific settings such as postcultured cells where there may be a generalized increase in cellular "stickiness"; consequent to such unavoidable manipulations (reviewed in Ref. 37), isotype controls should be restricted to qualitative assays. Differences in antibodies present the impractical requirement of including a matched (concentration, *F/P* ratio, etc.) isotype control for each antibody used in a multicolor assay (41). We do not recommend isotype controls for quantitative (cell counting) assays (see recommendations later).

ISOCLONIC CONTROLS

An isoclonic control consists of a mixture of fluorochrome-conjugated antibody and an excess amount of the same, unlabeled antibody (41). An example of such a control (42) was incorporated into Coulter-Immunotech's Stemkit™ (43). An isoclonic control is specifically designed to determine undesirable antibody binding through fluorochrome-mediated binding. Since all specific and nonspecific binding of the fluorochrome-conjugated antibody is blocked by the large excess of unconjugated antibody, any increase in fluorescence over unstained control cells cannot be due to Fc-receptor-mediated binding or any other nonspecific antibody binding. Instead, it can only be due to fluorochrome-induced binding. However, to the best of our knowledge, it has never been reported that this control resulted in significant numbers of "positive" events (8,9,38,43). We do not therefore, recommend the use of isoclonic controls quantitatively (i.e., subtract the isoclonic control-positive events from the sample-positive events that is under study) but instead solely as an

indicator for whether antibody binding through fluorochrome binding in the sample of interest occurs. If so, alternative fluorochrome-conjugates should be tested (see recommendations later).

INTERNAL NEGATIVE CONTROLS

An internal negative control is a population of cells that does not express the antigen of interest and thus remains unlabeled in an antibody-labeled cell suspension that also contains a population of cells that does express this structure. The advantage of an internal negative control is that it is a population of cells that has been exposed to identical conditions (including exposure to the antibody directed to the antigen of interest) as the cell population under study. In an ideal world, the fluorescence intensity of the internal negative control should be the same as that determined by autofluorescence only (i.e., unstained cells). In practice, however, this is not always the case because of nonspecific binding of excess antibody molecules. The expected fluorescence intensity can be determined (and minimized) by proper titration techniques when the assay is being developed and/or optimized. Such procedures are of fundamental importance to good assay design (see recommendations later). Figure 2 shows that the level of nonspecific antibody binding is determined by the total absolute amount of antibody rather than the antibody concentration (also described in Ref. 29). Panel A illustrates that the fluorescence intensity of the antigen-negative cell population rapidly increases as the assay volume is kept the same in a typical antibody titration assay where both antibody concentration and the absolute amount increase. Using a constant absolute amount of antibody but changing the assay volume to test different antibody concentrations (Panel B), mostly avoids the increase in background. This indicates that antibody concentration and antibody amount need to be considered separately. Both nonspecific binding by excess antibody molecules and autofluorescence may be cell type-specific (10). Furthermore, the large array of variables combined in a sample preparation procedure may also increase or decrease background fluorescence of specific cell types (41). Thus background fluorescence is only properly assessed if the negative population of a particular cell type is compared with the positive population within that same cell type (Fig. 3). Unfortunately, not every application contains a positive as well as a negative subpopulation of the same cell type (37). Specifically, in the analysis of malignant cells where the specific and/or nonspecific antibody binding characteristics may be different from normal cells, a matching negative subpopulation is not present. The introduction of an additional marker that allows one to exclude either the malignant or normal cells by means of Boolean gating (see recommendations in this article) can improve the analysis. Comparing the intensity of the cell type-specific, internal negative control to the unstained control of the same cell type allows for an estimation of the level of nonspecific antibody binding (see recommendations later).

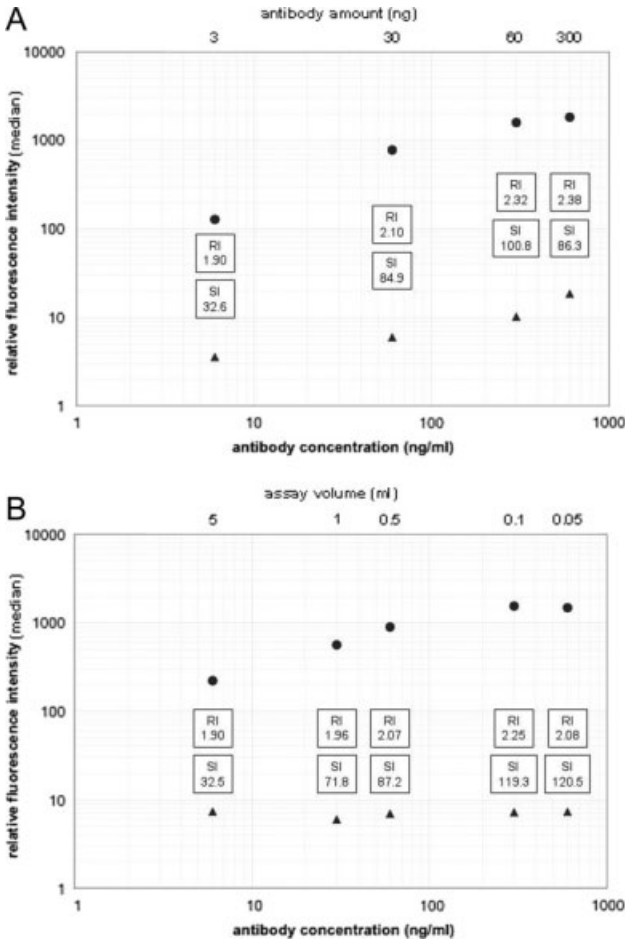

FIG. 2. Antibody titration assays to establish high separation between positive and negative cell populations while maintaining a low level of background. (**A**) Conventional antibody (PE-conjugated mouse anti-human CD3) titration assay in a fixed (0.5 ml) assay volume to determine optimum antibody concentration, using 10^6 peripheral blood mononucleated cells. Resolution index (RI) = $(X_{pos} - X_{bckgrnd})/\sqrt{(SD_{pos}^2 + SD_{bckgrnd}^2)}$; staining index (SI) = $X_{pos} - X_{bckgrnd}/2 \times SD_{bckgrnd}$; SD = $(CV \times X)/100$, where X_{pos} and SD_{pos} are, respectively, mean intensity and standard deviation of the positive population, and $X_{bckgrnd}$ and $SD_{bckgrnd}$ are the mean intensity and standard deviation of the negative population. In this assay, the fluorescence intensity of the negative population (background) increases with increasing antibody concentration due to an increase in total amount of antibody. (**B**) Antibody (PE-conjugated mouse anti-human CD3) titration assay using a fixed (30 ng) amount of antibody and variable amounts of assay volume to determine optimum antibody concentration, using 10^6 peripheral blood mononucleated cells. RI, resolution index; SI, staining index. These data show that when the total amount of antibody is fixed, the background remains low as the antibody concentration increases.

FMO CONTROLS

FMO controls are samples labeled with all antibodies of the multicolor test sample except one (44,45). As such, a five-color test sample requires five different FMO control samples. They can be considered the counterpart of singly stained positive controls in multicolor experiments and include an internal negative control.

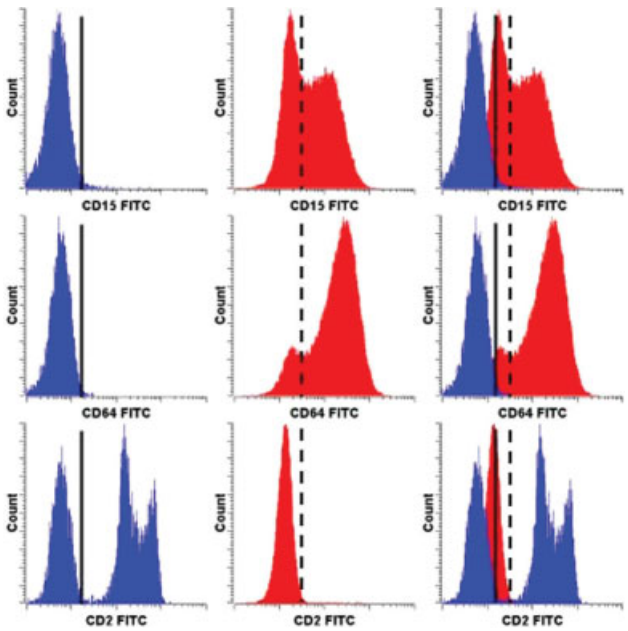

FIG. 3. Human mononuclear peripheral blood cells labeled with FITC-conjugated anti-CD15, CD64, or CD2 to illustrate that the level of background in internal negative controls is cell type-specific. Lymphocytes (black, solid line) show significant less background fluorescence than blast cells (gray, dotted line). Note that conditions for sample preparation and labeling are of key importance when specific information is to be obtained from immunofluorescence assays. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

However, aside from undesirable antibody binding due to nonspecific binding, the intensity of the negative population in FMO controls is also determined by compensation (or lack thereof) for spectral overlap. Background as a result of compensation for spectral overlap is proportional to the SD and mean fluorescence intensity of a positive population and the relative amount of bleed-through into the detector in which the fluorochrome of interest is measured (44). As a result, the coefficient of variance (CV) of a negative population may appear larger in compensated data than in uncompensated data. Since FMO controls are labeled with all fluorochromes involved except one, they show (unlike the singly positive controls) the same apparent increase in CV of the negative population as the experimental sample. Furthermore, FMO controls help to determine positivity and set regions in samples that contain multilabeled subpopulations. As illustrated in the example of Figure 4, the CD25-positive subpopulation of CD4-expressing cells in the experimental sample (blue, d events in the circled population) can accurately be identified by comparing it to the FMO control sample that is labeled with PE-conjugated anti-CD127 and PECy7-anti-CD4 but not labeled with PECy5-conjugated anti-CD25 (green, c). Since background levels in FMO controls are determined by undesirable antibody binding as well as spectral overlap, they cannot be used to specifically expose any type of undesirable binding.

RECOMMENDATIONS

Aside from applying suitable instrument QA/QC procedures (46–48), the following steps can be taken to increase the ability to distinguish positive and negative populations of cells. The recommendations described later are particularly important for antigens that are expressed at low levels and in cell labeling procedures that easily result in overlapping antigen-positive and antigen-negative cell populations. Although it may not always be feasible in an established clinical laboratory to act on the entire set of recommendations, each individual recommendation provides valuable facts that improve the utility of flow cytometry for clinical samples. These recommendations do, therefore, apply to a wide variety of circumstances. These range from setting up a clinical laboratory in which an entire process needs to be developed, to well-established clinical laboratories in which a cell labeling protocol requires an update.

Avoid or Correct for High Autofluorescence

The degree of autofluorescence (generally most prominent in mature myeloid-series cells, post cultured primary cells, and cell lines) is determined by measuring an unstained, but fully processed, cell sample on the flow cytometer using the same settings used during measurements of the experimental samples. The relative amount of autofluorescence in each detector can be compared with reference intensities of nonbiological particles (i.e., beads) measured with the same instrument settings. However, if evaluated on a stable flow cytometer with sufficient dynamic range to allow preset, fixed detector voltages, the relative amount of autofluorescence can be measured directly, without the need for reference particles. At the onset of protocol

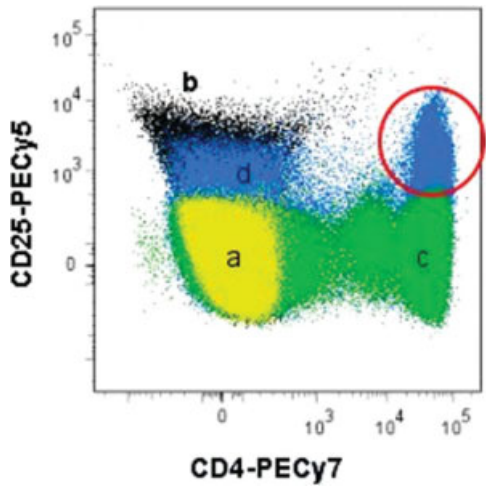

FIG. 4. Overlay bivariate histograms displaying compensated data from unstained control (yellow, a), single positive (CD25-PECy5) control (black, b), FMO (no CD25-PECy5) control (green, c), and three-color (CD4-PECy7, CD25-PECy5, and CD127-PE) sample (blue, d) of human mononuclear peripheral blood cells. The circle indicates the CD25 positive fraction of CD4 expressing cells, illustrating how an FMO control rather than a single positive control allows for immediate identification of positive cells on subpopulations of cells.

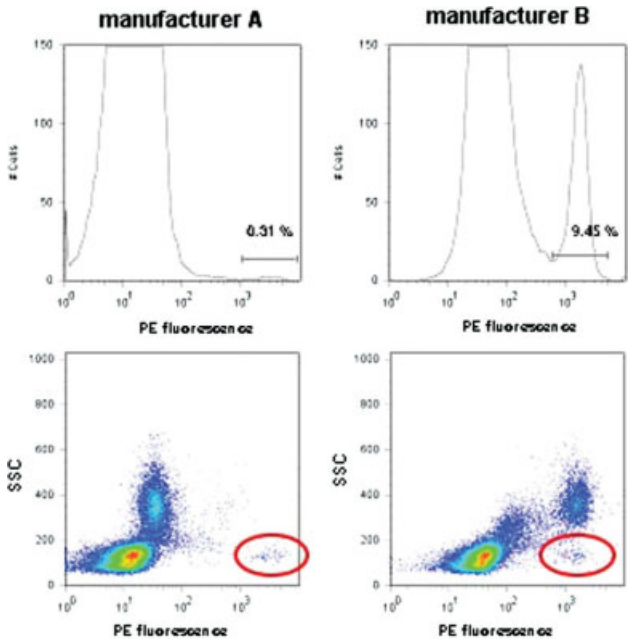

FIG. 5. PE-conjugated mouse IgG1 anti-human CD34, clone 581 produced by different manufacturers can show different binding characteristics. Top panel: univariate histograms of PE-fluorescence. Labeling a sample of human peripheral blood mononucleated cells with antibody from manufacturer B results in a more than 30-fold larger positive population than when labeled with antibody from manufacturer A. the lower panel shows through bivariate histograms of side light scatter versus PE-fluorescence that the antibody from manufacturer B displays a high degree of undesirable binding. The cell population of interest is circled. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

development, it might be possible to consider avoiding excitation by a 488 nm laser line to minimize autofluorescence (most 488 nm excitable fluorochromes can be excited by a 532 nm laser line as well). Alternatively, autofluorescence can be corrected for by means of the same procedure as routinely applied in spectral compensation by regarding it as an additional fluorochrome measured in an assigned detector (19,49).

Establish Antibody Specificity

It is important to first ensure the specificity of the antibody intended to identify the protein of interest (50). Although commercially available antibodies for clinical applications are expected to have undergone extensive QA/QC programs to meet the manufacturer's strict release criteria, antibodies with poor performance characteristics do sometimes enter the market (Fig. 5) (reviewed in Ref. 51). It has always been the user's responsibility to verify the manufacturer's expected antibody binding characteristics (even for FDA/CE approved kits where comparison with previous lots is required) and thus all, rather than only one, of the aforementioned controls may need to be incorporated in the development of a cell labeling protocol to reliably call a cell population truly positive (52). Equally important in establishing a reliable antibody labeling protocol is verification by other technologies. Since flow cytometers measure fluorescence as total intensity of a cell, information about the signal's (i.e., antibody) distribution on the cell is lost. Fluorescence microscopy can be used to examine the distribution of the fluorescence signal on, or in a cell and often assists in distinguishing background from truly positive cells. In some cases, Western blot can be used to obtain confidence about the antibody's specificity (53).

Minimize Background Through Antibody Concentration and Amount

After antibody specificity has been established, a proper titration assay should be applied to determine the antibody concentration resulting in the highest signal of the positive population and the lowest signal of the negative population (29). When the positive and negative population can be easily distinguished, the separation between the two can be expressed quantitatively by means of the resolution or staining index. These data can be used as a reference in future antibody titration assays and in monitoring other lot numbers of the same antibody-conjugate. In addition, maintaining small volumes and high-cell concentrations during cell labeling procedures avoids high background. Here, the use of an unstained cell sample acts as a good reference to establish the degree of nontargeted antibody binding (background) observed in the internal negative control population of the antibody-labeled cell sample.

Understand and Minimize Undesirable Antibody Binding

Most samples sent to the clinical flow laboratory for routine assays (lymphocyte subset analysis, CD34 enumeration, and leukemia/lymphoma testing) contain sufficient negative and positive cell populations to visually estimate the degree of undesirable antibody binding by comparing the intensity of the internal negative population to that of the unstained control. If the background is unexpectedly high, an isotype control may provide some insight regarding its cause. The fluorescence intensity of the "perfectly matched" isotype control should be identical to that of the internal negative control. If the isotype control signal is different from that of the internal negative control, the isotype antibody is either at a different concentration than that of the target-specific antibody, has nonspecific binding characteristics that are different from those of the target-specific antibody, has a different *F/P* ratio, or is not an "irrelevant" antibody. The potentially different nonspecific binding characteristics of an isotype antibody, the possible pitfalls in the interpretation of the data (41) and the notion that it is virtually impossible to manufacture the perfectly matched isotype antibody, have lead to the reconsideration of earlier recommendations (54,55). Over the last decade or so, it has become more widely accepted that isotype controls are of little value in distinguishing positive from negative (6,30,41,44,52,56) and should therefore not be used to set positive gating regions for test antibodies. Isotype controls are not recommended for

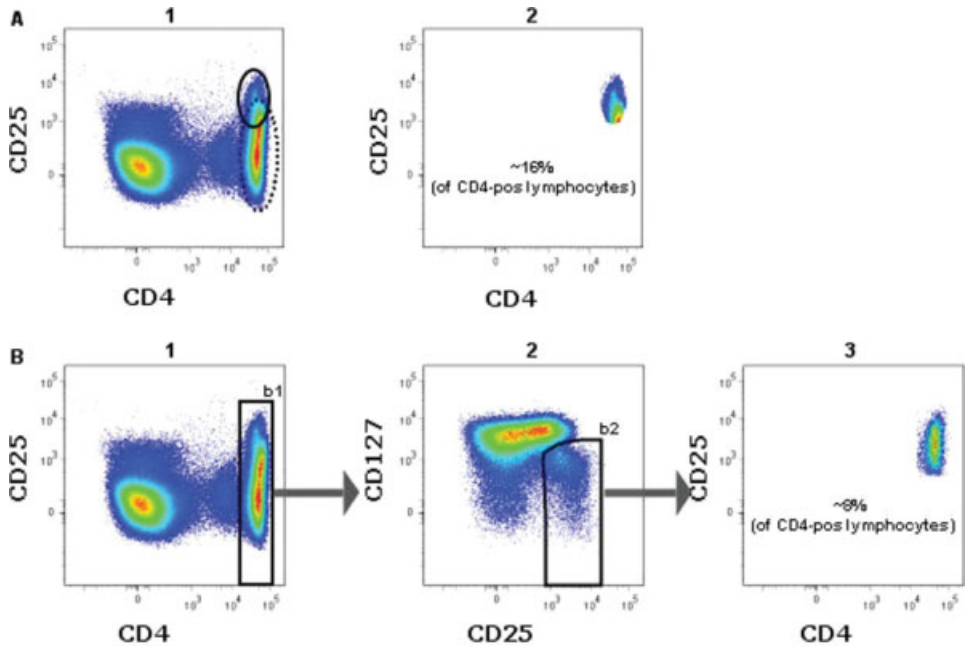

FIG. 6. Human mononuclear peripheral blood cells labeled to identify regulatory T cells by cell surface markers. Regulatory T cells reside within the CD25-bright subpopulation of CD4-expressing lymphocytes only (solid region), comprising only 5–10% of CD4+ T cells (58). (**A**) Using a two-color flow cytometric assay, identification of the CD25-bright subpopulation of CD4-expressing lymphocytes may result in much higher percentages than expected due to overlap with CD25-mid CD4-expressing cells (dotted region). (**B**) By including an additional marker (CD127) and Boolean gating (region b1 selected events are displayed in histogram B2, regions b1 + b2 selected events are displayed in histogram B3), a significant proportion of the overlapping CD25-mid expressing cell population can be excluded. As a result, it becomes easier to distinguish the entire population of CD25-bright CD4 expressing lymphocytes.

use in quantitative (cell counting) assays to determine nonspecific binding (7–9,38,43,57). Similarly, isoclonic controls should not be used in quantitative assays because they block both specific and nonspecific binding due to the potential presence of targets that fluorochrome-conjugated and unconjugated antibodies may share. Some cell labeling protocols benefit from a step that involves blocking agents to reduce Fc-receptor and nonspecific binding of antibodies and thus background.

Minimize the Need for Spectral Compensation

The wide variety of fluorochrome-conjugated antibodies available today makes it easier for the user to select combinations with minimum spectral overlap to avoid a decrease in sensitivity during analysis of multicolor cell samples. In addition, it is worth spending time on optimizing combinations of fluorochrome-conjugated antibodies such that the low-antigen-density targets are labeled with antibodies conjugated to bright fluorochromes and targets expressed in abundance are labeled with "less bright" conjugates (44,56). Multiple-laser instruments offer the advantage of minimizing spectral overlap by choosing less fluorochrome-conjugated antibodies per laser. Singly labeled controls (or antibody-binding particles) should be used to determine the degree of spillover fluorescence into other detectors to apply spectral compensation. FMO controls are appropriate to set regions in multicolor samples, whereas both singly labeled and FMO controls can be used to assess the level of background due to spectral overlap.

Identify the Population of Interest Through Sequential Boolean Gating

When dealing with weakly positive cell populations, the aforementioned steps may not result in the ability to distinguish positive from negative cells. Given that isoclonic and isotype controls are not recommended for quantitative use, but only as QA/QC tools in cell labeling protocols, these controls cannot be used to accurately delineate weakly positive cell populations from negative ones. One strategy to accurately distinguish overlapping cell populations (i.e., when neither resolution index nor staining index can be determined) is to seek an additional, specific parameter that allows the cell population of interest to be "pulled" out of the overlapping population through sequential Boolean gating strategies. An additional benefit of this approach (increasing the number of parameters used) is that the statistical likelihood of an undesirable cell meeting the same criteria as the cell of interest is significantly diminished. The ISHAGE protocol for CD34+ cell enumeration (8,43) is an example of such an approach in a clinical flow assay. In the example shown in Panel A in Figure 6, the CD4-expressing $CD25^{bright}$ subpopulation significantly overlaps with the $CD25^{mid}$ subpopulation which may result in overestimating the percentage of cells of interest. Setting a more specific region to exclude most of the $CD25^{mid}$

population will also exclude many $CD25^{bright}$ cells. As shown in Panel B, using an additional marker (CD127) allows one to "pull out" and separate the entire $CD25^{bright}$ population of CD4+ T cells (regulatory T cells) resulting in data that represent expected values better (58). When there is no such parameter available, a choice has to be made between the identification of all cells of interest (enriched, impure population) or to focus on cells of interest (small, pure population). However, such a choice will always come at the expense of reduced assay accuracy.

The ability to reliably distinguish between positive and negative populations of cells is an essential aspect of clinical flow cytometry. To improve and maintain consistency in the interpretation of flow cytometric data, it is important to develop a cell labeling protocol that maximizes this ability. A thorough understanding of the role and appropriate use of control samples is a prerequisite for optimizing cell labeling protocols. Furthermore, appropriate use of control samples, reliable cell labeling protocols and the resulting accurate measurement of positive cells, facilitate formulation of a consensus regarding the utility of flow cytometry for the analysis of clinical samples.

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LITERATURE CITED

1. Hassett J, Parker J. Laboratory practices in reporting flow cytometry phenotyping results for leukemia/lymphoma specimens: Results of a survey. Cytometry 1995;22:264–281.
2. Brando B, Sommaruga E. Nationwide quality control trial on lymphocyte immunophenotyping and flow cytometer performance in Italy. Cytometry 1993;14:294–306.
3. Martini ED, Hautcourt JL, Brando B, Lawry J, O'Connor JE, Sansonetty F, editors. First European Quality Control of Cellular Phenotyping by Flow Cytometry. Paris: Frison-Roche; 1990.
4. Maecker TH, Frey T, Nomura LE, Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. Cytometry Part A 2004;62A: 169–173.
5. Young IT. Proof without prejudice: Use of the Kolmogorov-Smirnov test for the analysis of histograms from flow systems and other sources. J Histochem Cytochem 1977;25:935–941.
6. Stelzer GT, Marti G, Hurley A, McCoy P, Lovett EJ, Schwartz A. U.S.-Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: Standardization and validation of laboratory procedures. Cytometry 1997;30: 214–230.
7. Borowitz MJ, Bray R, Gascoyne R, Melnick S, Parker JW, Picker L, Stetler-Stevenson M. U.S.-Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: Data analysis and interpretation. Cytometry 1997;30: 236–244.
8. Sutherland DR, Keeney M, Gratama JW. Enumeration of CD34+ hematopoietic stem and progenitor cells. In: Robinson JR, Darzynkiewicz Z, Dean PN, Rabinovitch PS, Stewart CS, Tanke HJ, Wheeless LL, editors. Current Protocols in Cytometry. New York: Wiley; 2003. Unit 6.4, pp 1–23.
9. Gratama JW, Kraan J, Keeney M, Mandy F, Sutherland DR, Wood BL. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry: Approved Guideline, 2nd ed (H42-A2, Vol. 27, No. 16). Wayne, PA: Clinical and Laboratory Standards Institute; 2007.
10. Monici M. Cell and tissue autofluorescence research and diagnostic applications. Biotechnol Annu Rev 2005;11:227–256.
11. Perfetto SP, Roederer M. Increased immunfluorescence sensitivity using 532 nm laser excitation. Cytometry Part A 2007;71A:73–79.
12. Mayeno AN, Hamann KJ, Gleich J. Granule-associated flavin adenine dinucleotide (FAD) is responsible for eosinophil autofluorescence. J Leukoc Biol 1992;51:172–175.
13. Hulspas R, Bauman JGJ. The use of fluorescent in situ hybridization for the analysis of nuclear architecture by confocal microscopy. Cell Biol Int Rep 1992;16:739–747.
14. Mosiman VL, Patterson BK, Canterero L, Goolsby CL. Reducing autofluorescence in flow cytometry: An in situ method. Cytometry 1997;30:151–156.
15. Hodge SJ, Hodge GL, Holmes M, Reynolds PN. Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease. Cytometry Part B 2004;61B:27–34.
16. Hed J, Hallden G, Johansson SG, Larsson P. The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. J Immunol Methods 1987;101:119–125.
17. Steinkamp JA, Stewart CS. Dual-laser, differential fluorescence correction method for reducing cellular background autofluorescence. Cytometry 1986;7:566–574.
18. Alberti S, Parks DR, Herzenberg LA. A single laser method for subtraction of cell autofluorescence in flow cytometry. Cytometry 1987; 8:114–119.
19. Roederer M, Murphy RF. Cell-by-cell autofluorescence correction for low signal-to-noise systems: Application to epidermal growth factor endocytosis by 3T3 fibroblasts. Cytometry 1986;7:558–565.
20. Roederer M. Spectral compensation for flow cytometry: Visualization, artifacts, limitations, and caveats. Cytometry 2001;45:194–205.
21. McLaughlin BE, Baumgarth N, Bigos M, Roederer M, De Rosa SC, Altman JD, Nixon DF, Ottinger J, Oxford C, Evans TG, Asmuth DM. Nine-color flow cytometry for accurate measurement of T cell subsets and cytokine responses. Part I: Panel design by an empiric approach. Cytometry Part A 2008;73A:400–410.
22. Baumgarth N, Bigos M. Optimization of emission optics. In: Darzynkiewicz Z, Roederer M, Tanke HJ, editors. Methods in Cell Biology, Vol. 75: Cytometry: New Developments, 4th ed. San Diego, CA: Academic Press; 2004. pp 3–22.
23. Decker JM. Introduction to immunology. In: Decker JM, editor. The 11th Hour Series, 3rd ed. Wiley-Blackwell; 2000. p 6.
24. Gebhardt BM. Evidence for cross-reactivity between herpesvirus and the acetylcholone receptor. J Neuroimmunol 2000;105:145–153.
25. Oliveira MF, Bijovsky AT, Carvalho TU, de Souza W, Alves MJ, Colli W. A monoclonal antibody to *Trypanosoma cruzi* trpomastigotes recognizes a myosin tail epitope. Parasitol Res 2001;87:1043–1049.
26. Kim MJ, Kim MK, Kang JS. An epitope shared by cellular cytokeratin and *Orientia tsutsugamushi*. Microb Pathog 2006;41:125–132.
27. Capel PJ, van de Winkel JG, van den Herik-Oudijk IE, Verbeek JS. Heterogeneity of human IgG Fc receptors. Immunomethods 1994; 4:25–34.
28. Gratama JW, Van der Linden R, Van der Holt B, Bolthuis RLH, Van de Winkel JGJ. Analysis of factors contributing to the formation of monoclonal cell aggregates ('escapees') in flow cytometric immunophenotyping. Cytometry 1997;29:250–260.
29. Srivastava P, Sladek TL, Goodman MN, Jacobberger JW. Streptavidin-based quantitative staining of intracellular antigens for flow cytometric analysis. Cytometry 1992;13:711–721.
30. Kantor AB, Roederer M. FACS analysis of leukocytes. In: Herzenberg LA, Weir DM, Herzenberg, LA, Blackwell C, editors. Handbook of Experimental Immunology, 5th ed. Cambridge, MA: Blackwell Scientific; 1996. Chapter 49.
31. Van Vugt MJ, van den Herik-Oudijk IE, van de Winkel JGJ. Binding of PE-Cy5 conjugates to the human high-affinity receptor for IgG (CD64). Blood 1996;88:2358–2361.
32. Roederer M, Kantor AB, Parks DR, Herzenberg LA. Cy7PE and Cy7APC: Bright new probes for immunofluorescence. Cytometry 1996;24:191–197.
33. Lanza F, Healy L, Sutherland DR. Structural and functional features of the CD34 antigen: An update. J Biol Regul Homeost Agents 2001;15:1–13.
34. Sutherland DR, Kuek N, Anderson T, Acton E, Barth D, Keeney M. Use of FLAER-based white blood cell assay in the primary screening of PNH clones. Am J Clin Pathol (in press).
35. Stewart C, Stewart S. Cell preparation for the identification of leukocytes. In: Darzynkiewicz Z, Robinson J, Crissman H, editors. Methods in Cell Biology. New York: Academic Press; 1994. pp 39–60.
36. Lowdell MW. Data analysis in flow cytometry. In: McCarthy DA, Macey MG, editors. Cytometric Analysis of Cell Phenotype and Function. Cambridge, UK: Cambridge Press; 2001. pp 89–99.

37. O'Gorman MRG, McNally A, Anderson D, Myones B. A rapid whole blood lysis technique for the diagnosis of moderate or severe leukocyte adhesion deficiency (LAD). Ann NY Acad Sci 1993;667:427–430.
38. Keeney M, Gratama JW, Chin-Yee IH, Sutherland DR. Isotype controls in the analysis of lymphocytes and CD34+ stem and progenitor cells by flow cytometry—Time to let go! Cytometry 1998; 34:280–283.
39. O'Gorman MRG, Thomas J. Isotype controls—Time to let go? Cytometry 1999;38:78–80.
40. Donnenberg AD, Donnenberg V. Understanding clinical flow cytometry. In: O'Gorman MRG, Donnenberg AD, editors. Handbook of Human Immunology, 2nd ed. Boca Raton, FL: CRC Press; 2008. pp 181–220.
41. Langweiler M, Givan AL. Flow cytometry and quality control: An uneasy alliance. In: Stewart C, Nicholson J, editors. Immunophenotyping. New York: Wiley-Liss; 2000. pp 23–49.
42. Kreissig C, Kirsch A, Serke S. Characterization and measurement of CD34-expressing hematopoietic cells. J Hematother 1994;3:263–289.
43. Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE Guidelines. Cytometry (Commun Clin Cytometry) 1998;34:61–70.
44. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods 2000; 243:77–97.
45. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: Unraveling the immune system. Nature Rev 2004;4:648–655.
46. Agrawal YP, Mahlamäki EK, Penttilä IM. Use of quality control standards in clinical flow cytometry. Ann Med 1991;23:127–133.
47. Owens MA, Vall HG, Hurley AA, Wormsley SB. Validation and quality control of immunophenotyping in clinical flow cytometry. J Immunol Methods 2000;243:33–55.
48. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay P, Roederer M. Quality assurance for polychromatic flow cytometry. Nat Protoc 2006;1:1522–1530.
49. Sebestyén Z, Nagy P, Horváth G, Vámosi G, Debets R, Gratama JW, Alexander DR, Szöllősi J. Long wavelength fluorophores and cell-by-cell correction for autofluorescence significantly improves the accuracy of flow cytometric energy transfer measurements on a dual-laser benchtop flow cytometer. Cytometry 2002;48:124–135.
50. Mason D, André P, Bensussan A, Buckley C, Civin C, Clark E, de Haas M, Goyert S, Hadam M, Hart D, Hořejši V, Jones Y, Meuer S, Morrissey JA, Schwartz-Albiez R, Shaw S, Simmons D, Turni L, Uguccioni M, van der Schoot E, Vivier E, Zola H, editors. Leucocyte Typing VII. New York: Oxford University; 2002.
51. Couchman JR. Commercial antibodies: The good, bad and really ugly. J Histochem Cytochem 2009;57:7–8.
52. Schultz C, Rott C, Temming P, von Puttkammer J, Bucsky P. Influence of specimen age and use of different negative controls in determination of intracytoplasmic levels of cytokines after whole-blood culture assay. Clin Diagn Lab Immunol 2002;9:295–298.
53. Saper CB. A guide to the perplexed on the specificity of antibodies. J Histochem Cytochem 2009;57:1–5.
54. Landay AL, Muirhead KA. Procedural guidelines for performing immunophenotyping by flow cytometry. Clin Immunol Immunopathol 1989;52:48–60.
55. McCoy JP, Carey JL, Krause JR. Quality control in flow cytometry for diagnostic pathology. I. Cell surface phenotyping and general laboratory procedures. Am J Clin Pathol 1990;93:S27–S37.
56. Mahnke YD, Roederer M. Optimizing a multicolor immunophenotyping assay. Clin Lab Med 2007;27:469–485.
57. Gratama JW, Sutherland DR, Keeney M, Papa S. Flow cytometric enumeration and immunophenotyping of hematopoietic stem and progenitor cells. J Biol Regul Homeost Agents 2001;15:14–22.
58. Le NT, Chao N. Regulating regulatory T cells. Bone Marrow Transplant 2007;39:1–9.