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Multiparameter Flow Cytometry Monitoring of T Cell Responses

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Abstract

HIV vaccine research increasingly uses polychromatic flow cytometry as a tool to monitor T cell responses. The use of this technology allows for the analysis of highly defined subsets of cells with unique phenotypes and functions. Ultimately, such studies may identify surrogate markers of protection from disease progression. However, this powerful technology comes with a number of technical hurdles, and there is a need to standardize the assays and protocols used in clinical trial monitoring. Here an optimized protocol, with variations for specific circumstances, is presented. This protocol covers the analysis of multiple cytokines, cell surface markers, and other functional markers such as perforin, CD107, and CD154. While the protocol can be adapted to various numbers of fluorescence parameters, optimized panels of 8–10 colors are presented.

Keywords

Antigen-specific; intracellular staining; multicolor; polychromatic; fixation; permeabilization; AIDS vaccine research

1. Introduction

HIV vaccine researchers have been among the first to adopt polychromatic flow cytometry (more than four colors) as a tool to dissect T cell responses to HIV infection and to HIV vaccines. There is little doubt that a successful HIV vaccine will need to induce a strong cellular immune response, as well as neutralizing antibodies (1). However, the nature of a protective cellular immune response to HIV is only beginning to be elucidated, largely by studies in the SIV model (2–8), and by examination of HIV+ long-term nonprogressors (9 – 11). The ability of polychromatic flow cytometry to interrogate multiple subsets of immune cells for their functional capacities makes it a powerful tool for discovering potential surrogates of protection. In fact, it could be argued that this technology provides the most in-depth view currently possible into the workings of the human immune system at a cellular level.

What are the potential surrogates of T cell-based protection from HIV progression? Obviously, no definitive answers are yet available, as the only information comes from animal models or correlative studies in humans. However, current evidence suggests that HIV+ nonprogressors maintain proliferative capacity of their HIV-specific CD4+ and CD8+ T cells (9, 10), at least in part by maintenance of the ability to produce IL-2 (10–12). In fact,

recent studies using polychromatic flow cytometry suggest that a greater proportion of HIV-specific CD8+ T cells in nonprogressors are “multifunctional” (11), being able to produce several cytokines (e.g., IFNγ, TNFα, IL-2, and MIP-1β) and to degranulate (as evidenced by cell-surface CD107 expression).

Phenotypic studies in SIV models suggest that protected animals maintain T cells of a “central memory” phenotype (CD28+, CD95 +) in contrast to nonprotected animals (7, 8). HIV+ progressors are known to show altered differentiation of HIV-specific T cells, as seen by staining for markers such as CCR7, CD62L, CD27, CD28, CD45RA, and CD127 (13–19). Very recent work indicates that upregulation of a death receptor, PD-1, on HIV-specific T cells may lead to dysfunction of those cells and consequent disease progression (20, 21). And extensive literature has correlated an elevated expression of activation markers such as CD38 and HLA-DR on CD8+ T cells with poor prognosis [reviewed in (22)].

Some researchers may believe that functions (e.g., cytokines and degranulation capacity) are more important than phenotypes (e.g., memory/effector markers) in categorizing antigen-specific T cells. But the emergence of markers such as PD-1 suggests that cell-surface proteins may hold important prognostic value as well. Thus, researchers find themselves in the position of wanting to monitor an ever-growing number of phenotypic and functional markers; and polychromatic flow cytometry is the best available tool to meet that goal.

1.1. Instrumentation Considerations

Polychromatic flow cytometry has been made practical only recently by the availability of commercial instruments with digital signal processing and detectors for up to 18 colors. Digital processing is important in that it allows for more precise calculation of optical spillover between detectors, and thus more precise compensation than was possible with analog systems (23,24). Equally important, software routines are now available that will automate the compensation process when presented with a set of single-color fluorescent controls. This makes it possible to collect data in eight or more colors with nearly the same ease as traditional two to four color experiments, despite an exponentially more complex spillover matrix.

Much of the instrumentation for polychromatic flow cytometry is customized, which means that each user’s system can have different lasers and optical filters. This in turn can alter the efficiency of detection of particular fluorochromes, such that the same antibody panel on one instrument will not yield identical results on another instrument. Some degree of standardization, or at least awareness of these variables, is beginning to occur, with the emergence of groups sharing their experiences (see for example http://maeckerlab.typepad.com). A minimum level of instrument standardization will need to be defined in order to achieve comparable results with polychromatic antibody panels on different instruments. Such standardization is a prerequisite to doing multicenter trials in which polychromatic flow cytometry will be performed at more than one site; and it is certainly a prerequisite to comparing results from different vaccine trials.

Even with two identical cytometers, setup of the instrument is still a variable that can vastly alter results. There has been recent progress in automated software routines using

standardized particles to create optimal instrument setups (e.g., CST, BD Biosciences). For users not equipped with such automated setup paradigms, manual procedures need to be defined that optimize photomultiplier tube (PMT) voltage gains (25), as well as optimize instrument performance in general (26). Finally, monitoring of certain cytometer parameters over time is an important quality control tool to ensure consistent data and to anticipate and/or identify potential problems that might require service.

1.2. Reagent Considerations

The mere possession of an instrument capable of detecting, for example, 12 fluorescence parameters, does not guarantee success in 12-color flow cytometry. Choice of fluorochromes, antibody specificities, and the combination of these into an optimized reagent panel, are important considerations. In many cases, increasing a panel by one additional fluorochrome detracts so severely from the resolution sensitivity in other detectors, that it is not warranted. This occurs because of fluorescence spillover, the fact that each fluorochrome contributes some signal to neighboring detectors as well as to its primary detector. Because the optical spectrum is limited, the addition of new fluorescent reagents becomes more and more difficult without creating severe spillover problems, as the number of fluorochromes in an experiment increases. This is clearly a case where more is not always better.

Selection of fluorochromes and antibody conjugates based on brightness and minimal spillover has been recently reviewed (24). In general, one should start by selecting a set of fluorochromes that offer the greatest brightness, within the constraints of the user's instrument, while minimizing spectral overlaps between detectors. Suggestions of fluorochrome sets to use for common instrument configurations are given in Table 25.1.

Next, one should assign antibody specificities to particular fluorochromes by matching the dimmest specificities with the brightest fluorochromes. Further adjustments should then be made to minimize potential spillover issues. For example, two fluorochromes with significant spectral overlap might be used to identify non-overlapping cell populations, thereby negating their spillover. Conversely, one should avoid compromising a reagent for which high sensitivity is required by having a reagent in a neighboring detector that brightly stains the same cell population. For example, use of CD8 APC-Cy7 with anti-IL-2 APC is a potential problem, if IL-2 is to be detected on CD8+ cells. In this case, not only does APC-Cy7 cause spillover into the APC detector, but the tandem dye can also degrade, resulting in false positive signals in APC (24). Among tandem dyes, APC-Cy7, followed by PE-Cy7, are most sensitive to such degradation, which is catalyzed by light, increased temperature, and exposure to fixative (24). Finally, all of the above considerations need to be tempered by what antibody conjugates are commercially available or can be made by the investigator.

Once an antibody panel is selected, titration of certain reagents is often required to achieve an optimal signal:noise ratio. While many reagents are sold pre-titered, this does not always mean that the specified titer is optimal in a given application. Since polychromatic experiments already compromise sensitivity due to spillover between detectors, the need for optimal signal:noise is critical. It should also be noted that the optimal titer for cell-surface staining with an antibody is often higher than that for intracellular staining (after fixation

and permeabilization). For many antibody specificities, resolution is compromised so drastically by fixation and permeabilization that these antibodies need to be used prior to application of a fixative.

Given the many considerations in optimizing a reagent panel for polychromatic flow cytometry, it makes sense to take advantage, wherever possible, of panels already validated by others. Table 25.2 shows some staining panels successfully used in the author's laboratory. These can be taken as a starting point, given that small variations are still likely to be successful, as opposed to starting from "scratch" in the design of a new panel.

The basics of intracellular cytokine staining have been reviewed elsewhere (27, 28), and tips for optimizing protocols have also been recently published (29). The main focus of the protocol presented here is to show how intracellular cytokine staining can be adapted to a polychromatic format, which may include readout not only of phenotypic markers, but also of functional markers such as CD107 or CD154, in addition to cytokines. Variables in the stimulation and processing steps that apply to these markers and others are summarized in Table 25.3.

2. Materials

2.1. Reagents

1. Freshly isolated or cryopreserved PBMC, isolated by Ficoll gradient centrifugation or via Cell Preparation Tubes (CPT; BD Vacutainer, Franklin Lakes, NJ) or equivalent.
2. RPMI-1640 medium with 20 m*M* HEPES, 10% fetal bovine serum, and antibiotic/antimycotic solution (cRPMI-10, components from Sigma Chemical Co., St. Louis, MO).
3. Stimulation antigens, e.g., peptide mixes [*see* **ref**. (30)], or SEB as a positive control. Optional: Preconfigured plates containing lyophilized stimulation reagents and secretion inhibitor(s) can be purchased [BD Lyoplate, BD Biosciences, San Jose, CA (31)].
4. Recommended: costimulatory antibodies to CD28 and CD49d, 0.1 mg/mL each in sterile PBS (FastImmune, BD Biosciences).
5. Brefeldin A, 5 mg/mL in DMSO (Fast Immune, BD Biosciences); or brefeldin A +monensin, 2.5 mg/mL each in 50% DMSO+50% methanol. For the latter stock, combine brefeldin A and monensin (Golgistop, BD Biosciences) 1:1. Aliquot and store both stocks at −20°C.
6. EDTA, 20 m*M* in PBS (pH 7.4) (FastImmune, BD Biosciences)
7. Cell fixation reagent, e.g., BD FACS Lysing Solution (BD Biosciences) or equivalent.
8. Cell permeabilizing reagent, e.g., BD FACS Permeabilizing Solution 2 (BD Biosciences) or equivalent.

9. Fluorescent-labeled antibodies (*see for example* Table 25.2). Optional: Preconfigured plates containing lyophilized antibody cocktails [BD Lyoplate, BD Biosciences (31)].
10. Wash buffer: 0.5% bovine serum albumin +0. 1% NaN_3 in PBS.
11. Recommended: BD CompBeads [anti-mouse Ig κ, anti-rat Ig κ, or anti-rat/hamster Ig κ (BD Biosciences)], for creating single-color compensation controls.
12. Optional: To reduce biohazard potential, or if samples will be stored > 24 h prior to acquisition: 1% paraformaldehyde in PBS [dilute 10% paraformaldehyde (EM Science, Gibbstown, NJ) 1:10 in PBS]; or BD Stabilizing Fixative (BD Biosciences).

2.2. Equipment

1. 96-well conical bottom polypropylene plates with lids [e.g., BD Falcon (Bedford, MA) or equivalent] (*see* Note 1).
2. 12-channel aspiration manifold with 7-mm prongs (V&P Scientific, San Diego, CA).
3. Plate holders for table-top centrifuge [e.g., Sorvall Instruments (Newtown, CT)].
4. Polychromatic flow cytometer with digital signal processing, e.g., BD LSR II (BD Biosciences) or Dako Cyan ADP (Dako Corporation, Fort Collins, CO).
5. Optional: 96-well plate loader for flow cytometer.

3. Methods

3.1. Sample Collection

1. For fresh PBMC (*see* Note 2): Resuspend at 5×10^6 to 1×10^7 viable lymphocytes/mL in warm (37 °C) cRPMI-10 (*see* Note 3).
2. For cryopreserved PBMC (*see* Note 4): Thaw briefly in a 37 °C water bath, then slowly dilute up to 10 mL with warm (37 °C) cRPMI-10 and centrifuge for approximately 7 min at 250 × *g*. Resuspend in a small volume of warm cRPMI-10,

[1]Plates vs. tubes: Cells can also be stimulated in 15 mL conical polypropylene tubes, with staining in 12 × 75 mm polystyrene tubes (BD Falcon). However, plates are preferred for ease of handling multiple samples, and results for human PBMC are equivalent to those in tubes (32).

[2]Fresh PBMC: If PBMC are not to be cryopreserved, they should ideally be prepared on the day of blood draw, then either stimulated the same day, or rested at 37 °C in cRPMI-10 overnight and stimulated the following day. Overnight resting at 37 °C increases the staining intensity of cytokines, but the effect is more pronounced with cryopreserved samples. Overnight shipping of whole blood or PBMC at ambient temperatures can cause a variable decrease in cell function and should be avoided if possible, though shipping PBMC is preferable to shipping whole blood.

[3]Higher cell concentrations (1×10^7/mL, 2×10^6/well) should be used when possible, especially when response levels are low and/or there are many cell subsets to enumerate.

[4]Cryopreserved PBMC: If cells cannot be stimulated within 24 h of blood draw, they should be cryopreserved by a validated protocol (33). Upon thawing, recoveries of > 60% and viabilities of > 80% should be obtained to minimize loss of functional responses. The method of thawing is equally as important as that of cryopreservation (33). Thawed cells should be rested in cRPMI-10 for 6–18 h at 37 °C to maximize cytokine staining intensity (32). Some cell loss may occur during this period, so plating a slight excess of cells is desirable. Alternately, the cells can be rested in bulk (e.g., in a slanted 15 mL conical polypropylene tube), then recounted and resuspended at the desired concentration after resting.

perform a viable cell count, and dilute to a final concentration of 5×10^6 to 1×10^7 viable lymphocytes/mL (*see* Note 3).

3. Add 200 μL of cell suspension per well to a 96-well plate (*see* **Section 2.2.1** for appropriate plates). For cryopreserved PBMC, incubate at 37 °C for 6–18 h prior to stimulation (*see* Note 4).

3.2. Cell Activation

1. For assays not involving CD107 or CD154: Thaw an aliquot of 5 mg/mL brefeldin A stock (*see* Note 5). Dilute 1:10 in sterile PBS to make a 50× working stock.
2. For assays measuring CD107 and/or CD154: Thaw an aliquot of 2.5 mg/mL brefeldin A+2.5 mg/mL monensin stock (*see* Note 5). Dilute 1:10 in sterile PBS to make a 50× working stock.
3. For assays using preconfigured lyophilized stimulation reagents in plates: Add 200 μL of cell suspension directly to the appropriate wells, let sit for a few minutes, then pipet up and down thoroughly to mix. Skip to **step 3.2.7**.
4. Resuspend peptides or peptide mixes in DMSO at a concentration of 500 μg/mL/peptide or greater (*see* Note 6). Store resuspended peptides in aliquots at −80 °C. Dilute peptide stocks in sterile PBS, if necessary, to achieve a 50× working stock that is between 50 and 100 μg/mL/peptide (when diluted 1:50, this will yield a final concentration of 1–2 μg/mL/peptide).
5. Prepare a 50× SEB stock of 50 μg/mL in sterile PBS. Store this stock at 4 °C (aliquoting is not necessary).
6. For each stimulation condition, prepare a “master mix” of the 50× working stocks and costimulatory antibodies as follows:

   4μL/well peptides, SEB (positive control), or PBS (negative control).

   4μL/well brefeldin A or brefeldin A+monensin.

   4μL/well CD28 + CD49d Ab stock (*see* Note 7).
7. Pipet 12 μL of the appropriate master mix into each well containing cells. Mix by gently pipetting.

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[5]Brefeldin A vs. monensin: Secretion of most cytokines of interest (IFNγ, IL-2, etc.) is best inhibited by brefeldin A at 10 μg/mL cells. However, CD107 and CD154 are transiently expressed on the cell surface. Therefore, staining Abs to CD107 and/or CD154 are added to the stimulation culture to bind the antigen(s) as soon as they are expressed. Monensin increases the intensity of staining under these conditions by preventing the acidification and degradation of lysosomal vesicles that contain the recycled CD107 and CD154. Thus, for combined cytokine and CD107 or CD154 detection, 5μg/mL each of brefeldin A and monensin is recommended.

[6]Peptide mixes: Peptide mixes can be prepared and lyophilized as premixed pools of up to several hundred peptides (30). These can then be resuspended in DMSO at high concentration per peptide, avoiding DMSO toxicity. The total concentration of DMSO in the assay should be kept at < 0. 5%.

[7]Costimulatory antibodies: Antibodies to CD28 and CD49d can increase the cytokine response to protein antigens, peptides, and SEB by amplifying the signal for low-affinity T cells (34). In occasional donors, they increase cytokine production in the absence of antigen (TNFα is usually most affected).

8. For assays involving CD107 and/or CD154, also add the recommended titer of the antibody conjugate(s) to each well. Minimize exposure to light, particularly for tandem dye conjugates (*see* Note 8).

9. Incubate covered plate for 6–12 h at 37 °C (*see* Notes 9 and [10]).

3.3. Sample Processing

1. To halt activation and detach adherent cells, add 20 μL per well of 20 mM EDTA in PBS and mix by pipetting.

2. Incubate 15 min at room temperature, then mix again by vigorous pipetting to fully resuspend adhered cells.

3. Centrifuge plate at 250 × *g* for 5 min. Aspirate supernatant with 7 mm vacuum manifold (*see* Note 11).

4. For assays using amine-reactive dye for staining non-viable cells: Resuspend the amine dye at optimum concentration in PBS (usually around 2. 5 μg/mL, but this should be determined for individual lots of dye). Resuspend each well with 100 μL of this solution, incubate 20 min at room temperature, then add 100 μL wash buffer, and wash as in **step 3.3.3** above.

    a. For assays using liquid reagents and cell-surface markers other than CD3, CD4, and CD8: Resuspend each well in 100 μL wash buffer and add optimal titers of all Abs to cell-surface markers (*see* Note 12), incubate 30–60 min at room temperature, then add 100 μL wash buffer, and wash as in **step 3.3.3** above.

    b. For assays using preconfigured lyophilized staining reagents and cell-surface staining Abs: Resuspend the appropriate wells of the surface Ab plate with 50 μL of wash buffer. Let sit for a few minutes, then pipet up and down thoroughly to mix. Transfer the solution to appropriate wells of the cell plate, incubate 30–60 min at room temperature in the dark, then add 100 μL wash buffer, and wash as in **step 3.3.3** above.

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[8]Adding staining Abs during stimulation: As described in Note 5, staining Abs to CD107 and CD154 are best added during stimulation, to capture the transiently expressed antigen. Fluorochrome conjugated Abs are sensitive to light exposure, so they should be handled in low light and, once added, the samples should be incubated in the dark. Certain tandem dyes such as APC-Cy7 and PE-Cy7 are particularly sensitive to light and temperature (24) and are not optimal choices for use in stimulation cultures.

[9]Stimulation time: A minimum of 5–6 h allows adequate detection of most proinflammatory cytokines like IFNγ, TNFα, and IL-2 (35). Increasing the time of incubation (in the presence of brefeldin A) increases cytokine staining intensity, but is not recommended for CD107 or CD154. For whole proteins requiring intracellular processing, a preincubation of 2 h prior to adding brefeldin A and/or monensin is recommended (35). CD8 responses to whole protein antigens can sometimes be detected, and are increased with longer incubation in antigen alone, but not in all donors (36).

[10]Automating incubation times: A programmable heat block, incubator, or water bath can be used to time activation, cooling the samples to 4–18 °C at the end of a specified period at 37°C, and holding them for later processing.

[11]A fixed-length vacuum manifold helps achieve consistent washing without undue cell loss in microtiter plates. Because of the small wash volume, a sufficient number of washes and efficient removal of supernatant are essential.

[12]CD3, CD4, and CD8 can be stained either before or after fixation and permeabilization. Down-modulation of these antigens occurs to a variable degree depending upon the stimulus. Cells that have down-modulated these antigens can be better detected by intracellular staining (postfixation and permeabilization) (30), although the overall staining intensity is usually decreased. Most other cell-surface antigens are optimally stained before fixation.

5. Resuspend cell pellets with 100 μL of 1× BD FACS Lysing Solution per well. Incubate at room temperature for 10 min (*see* Notes 13 and [14]).

6. Add 100 μL wash buffer to each well, then centrifuge plate at 500 × *g* for 5 min (*see* Note 15). Aspirate supernatant with 7 mm vacuum manifold.

7. Resuspend cell pellets with 200 μL of 1× BD FACS Permeabilizing Solution 2 per well. Incubate at room temperature for 10 min (*see* Note 14).

8. Centrifuge plate at 500 × *g* for 5 min (*see* Note 15). Aspirate supernatant with 7 mm vacuum manifold.

9. Add 200 μL wash buffer to each well, and wash as in **step 3.3.8** above.

10. Again add 200 μL wash buffer to each well, and wash as in **step 3.3.8** above.

11. a. For assays using liquid reagents: Resuspend pellet in 100 μL wash buffer and add optimal titers of all Abs to intracellular markers. Incubate in the dark at room temperature for 60 min, mixing by pipetting or gentle agitation every 15–20 min.

    b. For assays using preconfigured lyophilized intracellular staining reagents: Resuspend the appropriate wells of the intracellular Ab plate with 50 μL of wash buffer. Let sit for a few minutes, then pipet up and down thoroughly to mix. Transfer the solution to the appropriate wells of the cell plate, and incubate at room temperature in the dark for 60 min, mixing by pipetting or gentle agitation every 15–20 min.

12. Add 200 μL of wash buffer to each well, and wash as described in Sample Processing step 8 above.

13. Again add 200 μL wash buffer to each well, and wash as described in Sample Processing step 8 above.

14. Resuspend pellets with 150 μL wash buffer. Store at 4 °C in the dark until ready for data acquisition, which should be performed within 24 h. Optional: resuspend pellets with 150 μL of 1% paraformaldehyde in PBS or BD Stabilizing Fixative (*see* Note 16).

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[13]Freezing of activated samples: Samples can be frozen at −80 °C directly in FACS Lysing Solution (35,37). This allows for samples to be sent to another laboratory for processing, or for longitudinal samples to be accumulated for batch processing. Lysed whole blood should be washed once prior to freezing.

[14]Fixation and permeabilization steps: Solutions for these steps should be stored and used at 22–25 °C. FACS Lysing Solution simultaneously lyses erythrocytes and fixes leukocytes. While erythrocyte lysis is not required for PBMC samples, fixation is still helpful to prevent cell loss prior to permeabilization.

[15]Centrifugation speed: All centrifugation post-fixation should be done at higher *g* force (500 × *g*) due to increased cell buoyancy.

[16]Use of paraformaldehyde is only helpful when samples are stored for more than 24 h prior to acquisition, or to ensure neutralization of potentially biohazardous samples. In addition to subtle effects on cell scatter and fluorescence, storage in paraformaldehyde can cause degradation of tandem dyes such as APC-Cy7 and PE-Cy7. An alternative fixative is available that protects these tandems from degradation (BD Stabilizing Fixative, BD Biosciences), but it is not compatible with AmCyan staining.

3.4. Data Acquisition and Analysis

1. First determine optimal PMT settings for the instrument and reagent panel in question. If automated software for this purpose is not available, follow guidelines as described in reference (24). In brief:
   a. Establish minimum baseline PMT settings for the instrument by acquiring a set of dim particles at various voltages, and choosing the lowest voltage for each PMT for which the CV of these particles is minimized.
   b. Run a sample stained with the full reagent cocktail in question, and adjust baseline PMT voltages as needed so that events are mostly above zero but do not register in the highest fluorescence channel.
2. Create a set of compensation controls consisting of single-stained cells or beads (*see* Note 17). Acquire these controls and use the software's automated algorithm to calculate compensation (*see* Note 18).
3. Create a template for acquisition that displays the relevant parameters in the test samples in the form of dot plots. This template need not be the same as that used for analysis, i.e., it does not need to specify all gates or regions of interest. In fact, a simplified acquisition template will allow faster processing of data. However, the template should show any gates used to define the saved population of cells or the stopping criteria (e.g., CD3+ cells).
4. Set an appropriate threshold, usually on FSC, to eliminate small debris, and set the stopping and storage criteria. It is usually safest to store all events (rather than a gated subset) to allow exploration of all data on analysis. However, sometimes a threshold or gate on CD3+ cells may be employed in order to reduce file sizes (*see* Note 19). When using a plate loader, be sure to set a stopping criterion based on time, so that samples with insufficient cells will not run dry.
5. Acquire data from a fully stained sample to verify that the settings chosen are appropriate. If any changes to PMT voltages are made, be sure to re-run compensation samples and recalculate compensation based on the new voltages.
6. Record data from samples.
7. Analyze data using the acquisition software or compatible third-party software. Be sure to define all regions of interest and report the desired statistics on these (*see*

[17]Compensation controls: Where possible, anti-immunoglobulin coated capture beads (BD Biosciences) are preferred as compensation controls, because they provide a bright and homogeneous population of events stained with the antibody conjugate of interest. Ideally, the same lot of antibody should be used for compensation as is used in the experiment. In practice, however, this is only important for certain tandem conjugates, such as APC-Cy7 and PE-Cy7. The compensation controls should ideally be treated identically to the experimental samples in terms of fixation, etc., although this too is only important for the above tandem dyes.
[18]When to apply compensation: While compensation can be calculated and changed at any time by software packages such as FloJo (TreeStar, Ashland, OR) or FACSDiva (BD Biosciences), it is helpful to perform compensation before sample acquisition, so that any setup problems can be more readily detected.
[19]Number of events to collect: Because multiparameter ICS assays tend to divide responding populations of cells into ever-smaller subsets, it is important to process and collect enough cells per sample to allow statistically significant differences between samples to be detected. The number of events required will depend upon the anticipated levels of responses and background, as well as the number of subsets of responding cells being identified. Statistical tools for sample size calculation can be found at http://maeckerlab.typepad.com.

Note 20 and Fig. 25.1). Where possible, use a batch analysis function to analyze all samples from a given experiment or study and export the statistical data to a spreadsheet (*see* Note 21).

8. For large studies, it is helpful to create a database to accept the statistical output files from batch analysis. This database can then be queried to create data tables from subsets of the data, allowing rapid graphing, statistical analysis, background subtraction, conversion to absolute counts, etc.

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[20]Gating of down-modulated cells: Be sure that gates set on CD3, CD4, and CD8 parameters include dim-positive cells, since down-modulation of these markers occurs with activation. When using dynamic gating (*see* Note 21), set the region size to the maximum value possible without causing inclusion of neighboring populations. Some donors have a significant population of CD4 + CD8dim T cells. This population contains a disproportionate number of cells specific for chronic antigens such as CMV and HIV, and should be included in the CD4+ T cell gate to avoid under-reporting of responses.

[21]Batch analysis: Dynamic gating tools such "Snap-To" gates in FACS Diva (BD Biosciences) can be used to accommodate staining differences between samples for populations such as CD3+, CD4+, and CD8+ cells (*see* Fig. 25.1). This in turn allows use of a single analysis template and batch analysis across multiple samples in an experiment or study. However, dynamic gates are not always useful for rare populations, and their specifications (size and movement) need to be adjusted for the data set being analyzed. Batch analysis and dynamic gating thus do not replace the need for visual inspection of all data.

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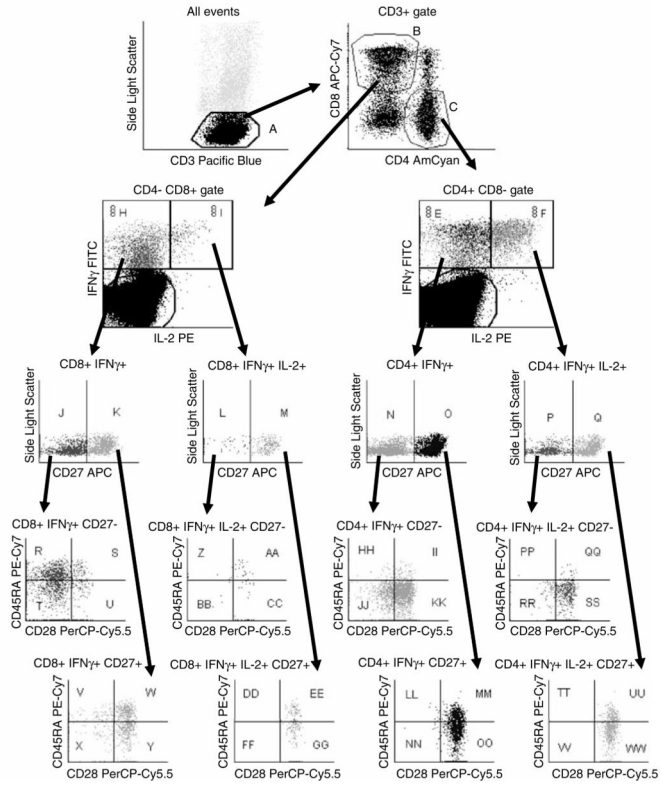

Fig. 25.1.

Gating scheme for eight-color ICS study [reproduced from reference (19)]. An acquisition threshold was used to exclude CD3-negative cells. Dynamic gates were used for defining CD3+, CD4+, and CD8+ lymphocytes, tethered regions were used to define the cytokine-positive cells, and conventional (static) regions or quadrants were used to define all other (rare) subsets. Note that all possible combinatorial subsets of CD27, CD28, and CD45RA were reported for each subset of cytokine-positive cells, resulting in a set of 32 populations whose frequencies describe a response “fingerprint” for that sample.

Table 25.1

Suggested fluorochrome configurations

Laser	6-color	8-color	10-color	12-color
488 nm	FITC	FITC	FITC	FITC
	PE	PE	PE	PE
488 nm or 532 nm			PE-Texas Red, PE-Alexa 594 or PE-Alexa 610	PE-Texas Red, PE-Alexa 594 or PE-Alexa 610
	PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5
	PE-Cy7	PE-Cy7	PE-Cy7	PE-Cy7
	APC	APC	APC	APC
633 nm			Alexa 680 or Alexa 700	Alexa 680 or Alexa 700
	APC-Cy7	APC-Cy7	APC-Cy7	APC-Cy7
		Pacific Blue	Pacific Blue	Pacific Blue
405 nm		AmCyan	AmCyan	AmCyan
				Qdot 655
				Qdot 705

Table 25.2

Some suggested multicolor antibody panels

8-color[1]	**10-color**[2]	**10-color**[3]
Anti-IFNγ FITC	CD27 FITC	Anti-IFN FITC
Anti-IL-2 PE	CD154 PE	Anti-IL-2 PE
	CD107 PE-Alexa 610	Anti-TNFα PE-Alexa 610
CD28 PerCP-Cy5.5	CD4 PerCP-Cy5.5	CD28 PerCP-Cy5.5
CD45RA PE-Cy7	Anti-IFNγ PE-Cy7	CD45RA PE-Cy7
CD27 APC	Anti-IL-2 APC	CD27 APC
	Anti-TNFα Alexa 700	CD3 Alexa 700
CD8 APC-Cy7	CD8 APC-Cy7	CD8 APC-Cy7
CD3 Pacific Blue	CD3 Pacific Blue	CD4 Pacific Blue
CD4 AmCyan	CD14 AmCyan	CD14 AmCyan

[1] Used in ref. (19).

[2] Used for International Flow Cytometry School (IFCS) 2006, Florence, Italy. Note that readout of IL-2 on CD8+ cells could be compromised by the use of CD8 APC-Cy7 with anti-IL-2 APC (*see* **Section 1.2**).

[3] Used for Multicolor ICS Users Group standardization studies (see also http://maeckerlab.typepad.com for additional panel suggestions and details).

Table 25.3

Procedural variables for different functional markers

Variable	Covered by this protocol IL-2, IL-4, IL-5, IL-10[2], IL-13, IFNγ, MIP-1β, TNFα	CD107, CD154	Not covered[1] TGFβ
Stimulation conditions	6–12 h	5–6 h in the presence of staining antibodies for these markers	16–24 h in serum-free medium[3]
Secretion inhibitor	brefeldin A	monensin[4]	monensin
Fixation/permeabilization system	FACS Lysing Solution, FACS Permeabilizing Solution 2[5]	FACS Lysing Solution, FACS Permeabilizing Solution 2[5]	Cytofix, Cytoperm[6]

[1] This marker would be difficult to combine with those in the first two columns, mainly due to the longer optimal stimulation time.

[2] We have performed IL-10 staining with some positive results under the listed conditions, but have not attempted to optimize conditions for IL-10 detection.

[3] Serum-free medium (e.g., AIM V, Invitrogen, Grand Island, NY) produces much stronger TGFβ responses, presumably because serum contains free TGFβ that blocks staining for this marker.

[4] CD107 and CD154 are taken up by endocytic vescicles and degraded. Monensin blocks this degradation by preventing acid-ification of these vescicles. When doing combined assays with cytokines, a monensin+brefeldin A combination is recommended (see Note 5).

[5] BD Biosciences, San Jose, CA. Note that the Cytofix/Cytoperm system (BD Biosciences, San Diego, CA) is also used successfully for these markers by many investigators.

[6] BD Biosciences, San Diego, CA.