Intracellular cytokine optimization and standard operating procedure

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We describe here a method for optimizing the use of polychromatic flow cytometry (with up to 17 fluorochromes simultaneously) in surface and intracellular staining of human T lymphocytes. We will highlight and discuss how to procedurally optimize key steps in the experimental process before an intracellular cytokine staining assay protocol is finalized. These include but are not limited to the titration of monoclonal antibodies, use of a dead-cell discriminator and 'dump' channel, selection of a cytokine secretion inhibitor, selection of fixation and permeabilization reagents, and inclusion of compensation controls. Building on this basic protocol, we then establish a polychromatic assay designed to detect five separate functions of T lymphocytes (production of three cytokines and one chemokine, and degranulation) while simultaneously identifying multiple surface markers on the responding cells.

INTRODUCTION

Recent advances in multicolor flow cytometry, such as (i) the increase in the number of detection channels, (ii) the development of more user-friendly software for both instrumentation and analysis and (iii) the increase in the variety of available markers and fluorescent conjugates, enable users to expand the number of markers tested simultaneously. Because of these advances, more laboratories are using polychromatic flow cytometry to quantify a myriad of T-cell functions. However, with the addition of these new tools, the methods have become more complicated. Thus, there is a need for an algorithm or process by which protocols can be systematically and logically evaluated, so that they can benefit from these advances.

The principles discussed here are applicable to multiple-color assays ranging from 3 to 18 colors or more. When moving from a panel of three or four colors to a panel of six or more colors, it is advantageous to first test one or more four- to five-color panels to confirm that the fluorochrome-antibody combinations are compatible. Some fluorescent conjugates, for reasons that are not always understood, do not perform harmoniously with each other when grouped together. It is for this reason that small subsets of panels should be tested before attempting larger multicolor panels[1].

If it is appropriate to use a four-color panel, users may choose to combine makers on one fluorochrome to expand the number of functions evaluated. For example, in testing for antigen-specific responses in early HIV vaccine trials, the goal was to determine whether there was any cellular response at all. In this case, it was acceptable to use a four-color panel that included separate surface markers for CD3, CD4 and CD8 while placing the cytokine markers interferon (IFN)-γ and interleukin (IL)-2 on the same fluorescent dye. Again, all of the basic experimental principles of flow cytometry apply, although the application of additional parameters, such as the use of a 'dump' channel or viability dye, may not be possible when the user is restricted to four colors. Therefore, because of the limitations of four- or five-color assays, and because it is now known that vaccines can elicit broad immunological responses, we strongly recommend that experimenters progress toward a polychromatic evaluation of antigen-specific T-cell responses.

This protocol illustrates one approach to optimizing and performing an intracellular cytokine staining (ICS) assay using previously frozen peripheral blood mononuclear cells (PBMCs). Basic ICS procedures have been described previously in the literature, but our assay uniquely focuses on the detection of low-frequency cell populations, which may be responsible for protective immunity in vaccine trials[2–4]. As an example, a HIV-1 DNA vaccine candidate has been found to be safe and well-tolerated in healthy uninfected adults, as has a HIV-1 recombinant adenoviral vector vaccine[5,6]. Both vaccine candidates have also been proven to elicit CD4+ and CD8+ T-cell immune responses to HIV-1 envelope antigens from clades A, B and C. In addition, longitudinal analyses of cellular responses from the HIV-1 DNA vaccine trial show persistent immunogenicity throughout the first year. Notably, the patterns of response change over time, with the vaccine-induced CD4+ IFN-γ-producing cells decreasing over time and the CD4+ IL-2-producing cells remaining stable. IFN-γ- and IL-2-producing cells dominate the CD8+ response, with an increase in cells producing only IL-2 occurring at 12 weeks after vaccination. These CD4+ and CD8+ T-cell response patterns remain consistent until the 52-week time point, at which time the numbers of cells that produce cytokines other than IFN-γ and IL-2 increase substantially. These data reveal that the analysis of two functional markers alone is not enough to identify and understand the complexity of the T-cell immune response. Hence, polychromatic flow cytometry aids in the understanding of vaccine immunology and may be required to determine a correlate of protection.

Here we present a list of items that we addressed during optimization of the ICS protocol, which included (i) choice of which markers to use, (ii) choice of which fluorochromes to use with each marker, (iii) titration of antibodies to optimize signal-to-noise ratio and minimize background, (iv) inclusion of an overnight rest for the PBMCs, (v) testing of surface antibody markers with the fixation-permeabilization (fix-perm) reagents to ensure conjugate stability, (vi) addition of DNase to the thaw medium and the stimulation portion of the ICS procedure to prevent clumping of cells, and (vii) choice of cytokine secretion inhibitors. This discussion is followed by a step-by-step description of the specific

12-color biological ICS assay currently used in our laboratory, which is readily adaptable for use with smaller experimental panels. A discussion of additional parameters to be addressed during the analysis portion of the experiment is also included.

Marker choice

The choice of markers is determined mainly by the objective the user wishes to achieve. For example, at the Vaccine Research Center, our goal is to develop an effective AIDS vaccine[5,6]. To this end, our lab evaluates antigen-specific immune responses to vaccine candidates, and therefore it is critical for us to include functional markers. Because we do not yet know what the correlates of protection are and because previous studies have shown that vaccination in humans generates primary immune responses comprising complex cytokine profiles, we include three antibodies to the cytokine markers IFN-γ, IL-2 and tumor necrosis factor (TNF)-α and one antibody to a chemokine marker, MIP1β, in our panel to assess T-cell function[7].

Another functional marker that we include in our panel, anti-CD107a, assesses the potential of CD8^{+} T cells to mediate the killing of target cells. Surface expression of CD107a is found only in cells that have degranulated[8,9]. Upon stimulation of PBMCs, cytotoxic granules that contain CD107a and CD107b (LAMP-1 and LAMP-2) in their membranes migrate toward the cell membrane, whereupon the membranes fuse and the CD107a and CD107b are transiently exposed on the cell surface. At this point, CD107a and CD107b are available for binding to fluorescence-tagged antibodies before being reinternalized into the endosomal spaces. Previous experiments have shown that CD107a exposure on the cell surface is necessary but not sufficient for perforin-dependent killing by cytotoxic T lymphocytes, with sufficient amounts of stored perforin and granzymes in cytotoxic granules being the other prerequisite for immediate cytotoxicity[10].

Cell-surface markers were chosen for their ability to provide information about certain cell subsets. Discrimination of CD3-, CD4- and CD8-expressing populations is a prerequisite in any evaluation of T-lymphocyte responses. Staining with both antibodies to CD4 and antibodies to CD8 yields four populations: CD4^{+}CD8^{−}, CD4^{−}CD8^{+}, double positive and double negative. If only antibodies to either CD4 or CD8 are included, the double-positive cells will be included in the positive gate and the double-negative cells will be included in the negative gate, possibly contributing to erroneous results. Likewise, antibodies to CD3 must be included because natural killer cells may have low expression of CD8, making it impossible to precisely distinguish between CD8^{+} T cells and natural killer cells. The inclusion of natural killer cell antibodies does not necessarily solve this problem, as some T cells will stain with these antibodies[11].

Because scatter patterns alone are insufficient to define a gate for removal of small monocytes and B cells from the T-lymphocyte population, antibodies to CD14 and CD19 can be added to provide a 'dump' channel to eliminate nonspecific binding that might be attributed to these subsets. Similarly, a lymphocyte gate is not sufficient to fully eliminate dead cells, which may increase the background of the antigen-specific T-cell functional response. Our solution is to add a dead-cell discriminator, ViViD, to the surface-staining mix, which enables identification of dead cells[12]. We use conjugates of our 'dump' antibodies that fluoresce in the same channel as ViViD, allowing a single detector to be used to eliminate dead cells and unwanted subsets.

In addition to knowing a cell's functional profile, it may be important to determine the phenotypic profile of that response, such as central memory, effector memory or terminal effector. There are several options to consider, and more thorough discussion is found in other papers[1,13–16], but we chose to include CD45RO, CD27 and CD57 in our panel.

Selection of marker fluorochrome

The choice of fluorochromes is dependent in part on the choice of markers. Generally, bright fluorochromes are used with dim markers, meaning those markers that are present at low density or that identify rare events. Therefore, brightly emitting fluorochromes such as phycoerythrin (PE) and allophycocyanin (APC) are often reserved for cytokines. Fluorescein isothiocyanate (FITC), although somewhat dimmer, may also be chosen because it provides good separation of populations. Conversely, dimmer fluorochromes may be matched with markers that identify populations that can be easily separated, such as CD4^{+} and CD8^{+}.

The properties of certain fluorochromes, such as Cascade Blue, make them less than ideal for use with certain markers. Because Cascade Blue binds nonspecifically to dead cells, we use it with markers that define the 'dump' channel, such as CD14 and CD19, during the surface staining step (before cell permeabilization). It is also prudent when choosing staining panels to examine the total amount of spreading error, or light contamination, into possible detectors so that channels may be chosen in which less spreading error occurs, maximizing the detection sensitivity. A more detailed explanation may be found elsewhere[17].

Tandem dyes, which include the cyanine and Alexa conjugates of PE and APC, are an option to consider when expanding the immunophenotyping panel. However, tandem dyes tend to degrade more quickly over time, causing compensation issues when contaminating light from the APC portion of the molecule contributes error into the APC channel. It is also important to use the same lot of dye for the compensation controls as is used in the staining panel. Of course, this can be accomplished by using the same antibody conjugate for both the control and the experiment.

Quantum dots (QDots) are another choice when using instruments equipped with violet lasers. QDots are nanocrystals comprising a cadmium selenide core with an outer shell of zinc sulfite that is coated with polymers, which allow labeling with antibodies[18]. QDots emit light dependent upon the size and composition of the core: small QDots emit light at shorter wavelengths; large QDots emit light at longer wavelengths. This basic principle enables the user to measure multiple fluorescence wavelengths from one light source[19].

Antibody titration

Titration of all of the antibodies of interest must be done under the assay conditions to determine the optimal concentration. For example, antibodies used after permeabilization of cells (even to epitopes on the cell surface) must be titrated against permeabilized cells, because the fix-perm chemicals can sometimes alter the epitope and therefore the avidity of the antibody-antigen reaction. Antibody titrations determine the best concentration of antibody to use by examining both the separation of the positive and the negative populations and the position of the negative population

Figure 1 | Selection of optimal antibody volume. (**a**) Typical titration curve with the ratio of the median fluorescence intensity (MFI; positive MFI/negative MFI) graphed on the *y*-axis against the antibody concentration (µg ml^{-1}) on the *x*-axis to illustrate the proper selection of the optimum titration point. Note the rise of the curve from right to left owing to the increasing signal-to-background ratios, with a plateau at the volume of 7.5 µg ml^{-1}. This point is the position on the curve where the minimum amount of antibody volume gives the greatest signal-to-background ratio. (**b**) Comparison of dot plots with different concentrations of the same antibody reagent, IFN-γ–PE, represented in **a**. Each plot of antibody (*y*-axis) versus side scatter (SS; *x*-axis) is compared for positive fluorescence intensity, separation of positive and negative populations, and distance of the negative population from the *x*-axis. The maximum separation between positive and negative populations is seen at the concentration of 7.5 µg ml^{-1} with the negative population positioned just off the axis. Higher background would be observed if the antibody concentration were increased, whereas a decrease in positive fluorescence intensity would be seen if a lower antibody concentration were chosen. (**c**) In lieu of polychromatic panels, smaller four-color panels can be used to provide functional data. Example of a histogram in which the nonspecific staining (background) for all three cytokines conjugated to the same fluorochrome overlay as one peak. (**d**) Histogram in which one cytokine, IFN-γ, contributes significantly more to the total background.
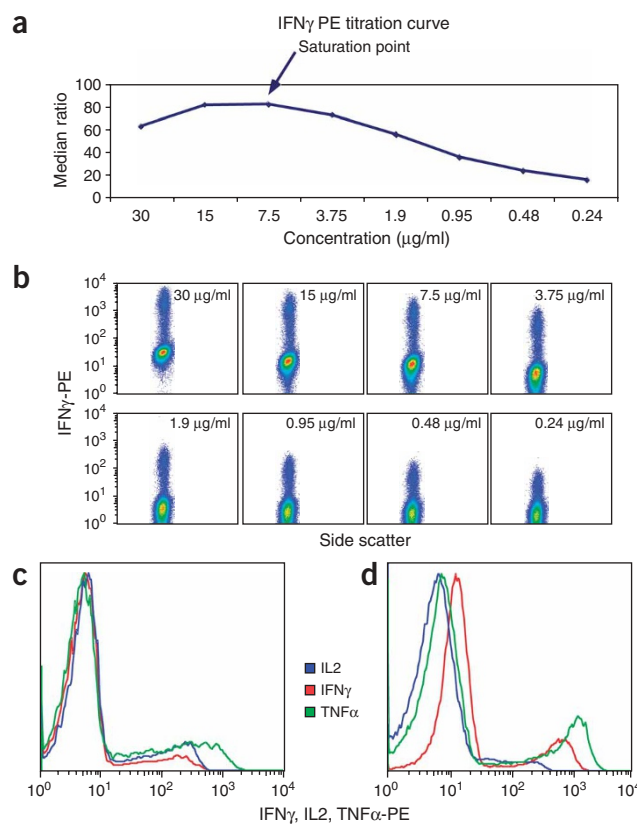
on the axis. The median intensities of the positive and negative populations are graphed as a ratio against the antibody concentration to determine the peak signal-to-background ratio (**Fig. 1a**). In addition, the antibody may be plotted against side scatter to view not only the degree of separation between positive and negative populations but also the position of the negative population against the axis, helping determine the concentration at which minimal background is seen (**Fig. 1b**). This is especially important when determining the optimal titration point of intracellular stains, because the best titer may not be the saturation titer if there is an increase in background at that point on the titration curve.

If it is necessary to incorporate in a panel multiple cytokine markers that are conjugated to one fluorochrome, because of inaccessibility to systems with multiple detectors, then one other histogram, in which the fluorescence pattern of one marker is overlaid onto the fluorescence pattern of a second or third marker, should be examined. These fluorescence patterns are from the optimal concentrations chosen for each cytokine marker as determined by titration experiments and, as such, the concentrations may differ from one another. **Figure 1c** and **d** provide examples of overlays. Note that **Figure 1c** depicts three different cytokines, IFN-γ, IL-2 and TNF-α, conjugated to the same fluorochrome, PE, and indicates that their individual optimal concentrations all yield approximately the same amount of nonspecific binding. However, **Figure 1d** illustrates another example in which the optimal titration concentration of IFN-γ contributes more nonspecific staining than the other two cytokines. Overlays allow the user to examine the position of the negative-staining populations (backgrounds) of all reagents in tandem, to ensure that the choice of optimal reagent volumes does not result in one cytokine contributing more significantly to the background than another. If necessary, the user may adjust the concentration of a cytokine marker to achieve this result.

When creating an antibody panel, titration of antibodies with several fluorochromes may help determine not only which concentration of antibody is optimal but also which antibody-fluorochrome conjugates are best for the assay. In fact, when

constructing a panel of six or more colors, it is advantageous to test several small panel combinations first to ensure compatibility of antibody conjugates.

PBMC 'rest' and the addition of DNase to the culture medium
The condition of the PBMCs after being thawed is extremely important to the accuracy of the assay. We have found that allowing the cells to 'rest' in culture overnight after thawing reduces the cytokine background and thereby increases the specificity of response. Also, any PBMCs that are apoptotic or necrotic partly as a result of the mechanical damage of the freeze-thaw process are destroyed overnight, removing their potential contribution to background.

Previous studies have shown that the addition of DNase to medium used to wash freshly thawed PBMCs prevents the cells from adhering to each other by dissolving the DNA that is released by dead and dying cells and causes 'stickiness'[20]. We have taken this one step further by adding culture medium with DNase not only when washing freshly thawed PBMCs but also during the stimulation portion of the ICS procedure. Once the PBMCs are thawed, as described in the procedure below, the cells are placed in prewarmed culture medium that contains DNase and are centrifuged. After being washed once more in culture medium alone (without DNase), the cells are placed in culture medium overnight and are held at 37 °C until the next morning, when they are washed in culture medium, counted once more and plated in medium containing DNase. Samples that clump immediately after being thawed result in reduced numbers of cells available for plating owing to the need for manual removal of any cell aggregates, and if clumping continues to occur throughout the stimulation, it may

also cause clogging of the flow cytometry aspiration device, resulting in loss of sample. The addition of DNase to the culture medium reduces sample loss due to cell adhesion. Side-by-side experiments with and without DNase in the culture medium have shown no difference in T-cell subset percentages or cytokine responses.

Choice of cytokine secretion inhibitors and fix-perm reagents

Brefeldin A (BFA) and monensin are the cytokine secretion inhibitors most commonly used in ICS procedures. If peptides are used for stimulation, either inhibitor may be added at the time of initial stimulation because processing of peptide through the endosome is not required. Several characteristics of each compound should be kept in mind when choosing an inhibitor. Monensin-treated cells are less viable when incubation periods are more than 18 h. CD8 downregulation occurs with both inhibitors but to a lesser extent when using BFA. BFA inhibits CD69 surface expression but not its intracellular expression, whereas monensin does not inhibit CD69 surface expression but results in less expression of CD69 intracellularly. Probably the most important characteristic of monensin, however, is that it does not sufficiently inhibit TNF-α secretion from the cell[21]. Therefore, BFA must be included in the mix when examining TNF-α production. However, because monensin is required to prevent acidification of lysosomes and endosomes when reinternalization of CD107a occurs during T-cell stimulation, if analysis of surface CD107a mobilization is warranted, monensin must also be included in the protocol. Both inhibitors can be used simultaneously without detriment to the assay.

Fix-perm reagents should be prepared, used and stored according to the manufacturer's instruction. If specific surface monoclonal conjugates have been used in the past and are selected for an intracellular cytokine assay, it is important to test them using the fix-perm reagents that will be used in the assay. This is an important consideration because the optimal titer of antibody for intracellular staining may be different from the titer used for surface staining.

Compensation

Fluorescence compensation is a necessary part of any multicolor experiment; there are no special requirements for ICS assays. Cells or antibody-capture beads may be used for compensation, but beads offer a distinct advantage in that they are convenient, provide a uniform binding capacity and will bind nearly all antibodies used in experiments (including antibodies to cytokine that may stain only a very small fraction of stimulated cells). For those antibodies not captured by the beads, standard cellular compensation controls must be used.

There are several critical issues to consider when performing proper compensation: (i) use of single-stained samples, including an unstained tube if using beads, (ii) use of compensation controls that are at least as bright as the stained sample, (iii) assurance that both the positive and negative stained populations are the same, i.e., both are lymphocytes or both are beads, (iv) use of the same photomultiplier tube (PMT) voltages for both the PBMC samples and the compensation samples and (v) performance of compensation each day of testing. There are several in-depth discussions of compensation in the literature that provide more information[22,23].

MATERIALS

REAGENTS
· PBMCs
· Culture medium with DNase (see REAGENT SETUP)
· Culture medium (without DNase)
· Peptide negative control and positive control mixes (see REAGENT SETUP)
· Wash buffer (see REAGENT SETUP)
· Surface staining mix (see REAGENT SETUP)
· Antibody to CD107a (clone H4A3; PharMingen, cat. no. 555798, or similar)
  ▲ CRITICAL This antibody is added to the cells at the beginning of the stimulation step. ▲ CRITICAL All antibodies must be titrated before use under the same experimental conditions to be used in future testing, as described in the Introduction.
· Cytofix-Cytoperm reagent kit (Becton Dickinson–PharMingen, cat. no. 554714), including Cytofix-Cytoperm reagent and Perm/Wash buffer (prepare buffer according to the manufacturer's instructions) ▲ CRITICAL The Perm/Wash buffer must be used after the Cytofix-Cytoperm reagent because it contains the saponin additive that maintains the permeabilized state. Do not use other wash buffers.
· 10% paraformaldehyde (PFA) solution (see REAGENT SETUP)
· Anti–mouse κ chain beads (Becton Dickinson, cat. no. 51-90-9001229) for compensation setup; use according to the manufacturer's instructions
· Violet viability dye (ViViD) amine-reactive dye kit for dead cell discrimination[12] (see REAGENT SETUP)
· Amine-modified microspheres for compensation of ViViD[12] (see REAGENT SETUP)

EQUIPMENT
· 96-well V-bottomed
· Multichannel pipette with reservoir

REAGENT SETUP
Amine-modified microspheres (amine beads) Dilute amine-modified microsphere stock (Bangs Laboratories, cat. no. PAO5N/5874) in wash buffer to a final concentration of 40×10^6 microspheres per ml.

BFA Dilute BFA (Sigma, cat. no. B7651) to a concentration of 10 mg ml⁻¹ in dimethyl sulfoxide (DMSO, ACS reagent grade; Fisher, cat. no. D128-500) and store in 250-μl aliquots at 4 °C. ❗ CAUTION BFA is an irritant, poisonous and highly flammable. Avoid contact with skin or eyes; use in areas of good ventilation. ▲ CRITICAL BFA poisons the Golgi apparatus, preventing secretion of cytokine. It is needed for adequate detection of TNF-α production. If TNF-α is not a cytokine of interest, monensin alone is sufficient[21].

Culture medium Prepare RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated FBS (Invitrogen), 100 U ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin and 2 mM L-glutamine (Gibco).

Culture medium with DNase Prepare a 1:100 solution of DNase working solution in culture medium.

DNase working solution Dilute DNase stock (Sigma, cat. no. DN-25) to a concentration of 2,000 μg ml⁻¹ with sterile PBS. Divide into aliquots in desired number of vials and store at 4 °C.

Intracellular staining mix Add appropriate amounts of intracellular antibodies, as predetermined by titration experiments, to 1× Perm/Wash buffer, ensuring that there is 100 μl of total volume per well. Spin at 15,700g for 3 min to remove aggregates. ▲ CRITICAL The spin step is critical for removing antibody aggregates that may interfere with staining. ❗ CAUTION Reagents are toxic. Avoid contact with eyes, skin and mucous membranes.

Monoclonal antibodies Obtain fluorescence-conjugated monoclonal antibodies from manufacturers. All antibodies, whether obtained commercially or conjugated in the laboratory, must be titrated to determine optimal conditions.

10% PFA solution Dilute PFA (Electron Microscopy Solutions, cat. no. 15712-S) to a concentration of 1% in PBS and store in a dark container at room temperature (18–28 °C).

Peptides Peptides should be 15 amino acids in length, overlapping by 11 amino acids, synthesized to more than 85% purity as confirmed by HPLC. Dilute to a concentration of 100 mg ml⁻¹ in DMSO or deionized H₂O.
▲ CRITICAL If DMSO is used as a diluent, prepare peptide stock so that

the final concentration of DMSO in each culture well does not exceed 1% (refs. 24,25). DMSO is toxic to the PBMCs at concentrations above 1%.

Peptide pools Pool individual peptides for each protein to a final concentration of 500 μg ml^{-1} of each peptide (5 μl of each peptide should be diluted to 1 ml in DMSO; thus, each mix can contain up to 200 individual peptides). Peptides are used at a final concentration of 2.5 μg ml^{-1}.

Peptide negative control and positive control mixes Prepare as indicated in **Table 1**. Volumes prepared are for eight samples, with additional volume allowing the user to use a reservoir and multichannel pipette for delivery into plate wells. Anti-CD28 and anti-CD49d (co-stimulatory antibodies) are both from Becton Dickinson, cat. nos. 340975 and 340976, respectively, each at a concentration of 1 μg ml^{-1}. Monensin (GolgiStop) is from Becton Dickinson, cat. no. 51-2092KZ (554724). The other components needed to prepare the medium are described elsewhere in this section. **! CAUTION** Monensin is a known toxin and is highly flammable. Avoid contact with skin, eyes and mucous membranes; keep away from sources of ignition. ▲ **CRITICAL** Monensin neutralizes the pH of the endosomal and lysosomal spaces into which the

CD107a is reinternalized after trafficking to the cell surface upon stimulation[9,26].

Staphylococcus enterotoxin B (SEB) Dilute SEB (Sigma, cat. no. S-4881) to a concentration of 200 μg ml^{-1} in deionized H$_2$O and store in 100-μl aliquots at –30 °C. **! CAUTION** SEB is toxic; avoid inhalation, ingestion or contact with skin, eyes or mucous membranes.

Surface-staining mix Add appropriate amounts of surface antibodies as predetermined by titration experiments to 1% PBS (Gibco, cat. no. 14190144), ensuring that there is 100 μl of total volume per well. Spin at 15,700g for 3 min to remove aggregates. ▲ **CRITICAL** The spin step is critical for removing antibody aggregates that may interfere with staining.

LIVE/DEAD Fixable Dead Cell Stain Kit (Lab name is ViViD) Place the DMSO provided with the ViViD kit (Molecular Probes, cat. no. L34955) in a 37 °C water bath until it is completely thawed. Add 12.5 μl to a vial of lyophilized dye (25 μg) provided and mix thoroughly. Store at –20 °C. ▲ **CRITICAL** Each lot of dye must be titrated before use.

Wash buffer Add FBS (Invitrogen) and sodium azide to final concentrations of 1% and 0.02% (vol/vol), respectively, in PBS (Gibco, cat. no. 14190144).

PROCEDURE
Preparation of cells

1| Determine the total number of PBMCs needed for the experiment, taking into account the number of stimulations to be performed, including a negative and positive control well for each sample. Select cell vials to be thawed accordingly.

2| Thaw cell sample vials in a 37 °C water bath until there is a pea-sized pellet of ice remaining.

3| Transfer the cell suspensions to a correspondingly labeled 15-ml conical tube containing prewarmed culture medium with DNase.

4| Centrifuge at 863g for 5 min at room temperature (18–28°C), decant supernatant and resuspend cells in culture medium.

5| Repeat centrifugation.

6| Add appropriate amount of medium without DNase and count cells to ensure an adequate number is available for the ICS experiment.

7| Place cells in labeled T-25 culture vials at density of 2×10^6 PBMCs per ml and culture overnight in an incubator at 37 °C and 5% CO$_2$.

8| Count cells the next morning to determine overnight recovery and viability.

9| Dilute cells to a density of 2×10^6 PBMCs per 100 μl in culture medium with DNase.

10| Add appropriate volume of anti-CD107a to the cells as predetermined by titration experiments.

Plate preparation and stimulation

11| Label the plate cover with sample and peptide identification.

12| Pipet 100 μl of cells into their appropriate wells.

13| Pipet 100 μl of peptide negative control and positive control mixes to the appropriate wells on the plate. (**Table 1** lists appropriate volumes for eight samples, with additional volume such that a reservoir and multichannel pipette may be used to deliver the medium.)

14| Mix cells and peptide negative control and positive control mixes with a multichannel pipette.

15| Incubate for 6 h in an incubator at 37 °C and 5% CO$_2$.
■ **PAUSE POINT** After incubation, plates containing cell samples may be stored in the refrigerator at 1–9 °C for up to 18 h.

Staining
16| After stimulation, remove plates from the incubator.

TABLE 1 | Preparation of peptide negative and positive control mixes.

Component	Amount (μl)
Peptide, DMSO or SEB	10
Anti-CD28	2
Anti-CD49d	2
BFA	2
Monensin	1.4
Culture medium with DNase	982.6
Total volume	1,000

17| Centrifuge the plate at 863*g* for 4 min at room temperature.
▲ **CRITICAL STEP** After each centrifugation step, check to ensure there is a visible pellet. If no pellet is seen, centrifuge the plate again. Once the Cytofix-Cytoperm reagent has been added, the pellet may look more diffuse but should still be visible.

18| Flick supernatant into biohazard waste container under a laminar flow hood and immediately and quickly blot plate on a paper towel.

19| Add 200 μl of wash buffer to each of the wells, mixing well.

20| Centrifuge the plate at 863*g* for 4 min at room temperature.

21| Flick supernatant into biohazard waste container under a laminar flow hood and immediately and quickly blot plate on a paper towel.

22| Add 100 μl of surface staining mix to each of the wells, mixing well.

23| Incubate for 20 min at room temperature in the dark.

24| Add 100 μl of wash buffer to each of the wells.

25| Centrifuge the plate at 863*g* for 4 min at room temperature.

26| Flick supernatant into biohazard waste container and immediately blot the plate on a paper towel.

27| Add 200 μl of wash buffer to the wells and repeat centrifugation.

28| Flick supernatant into biohazard waste container and immediately blot the plate on a paper towel.

29| Add 100 μl of Cytofix-Cytoperm reagent to each of the wells, mixing well.

30| Incubate for 20 min at room temperature in the dark.

31| Add 100 μl of Perm/Wash buffer to each of the wells.

32| Centrifuge plate at 863*g* for 4 min at room temperature.

33| Flick supernatant into biohazard waste container and immediately blot plate on a paper towel.

34| Add 200 μl of Perm/Wash buffer to the plate wells.

35| Centrifuge the plate at 863*g* for 4 min at room temperature.

36| Flick supernatant into biohazard waste container and immediately blot plate on a paper towel.

37| Add 100 μl of intracellular staining mix to the wells, mixing well.

38| Incubate for 20 min at room temperature in the dark.

39| Add 100 μl of Perm/Wash buffer to the plate wells.

40| Centrifuge plate at 863*g* for 4 min at room temperature.

41| Flick supernatant into a biohazardous waste container and immediately blot the plate on a paper towel.

42| Add 200 μl of Perm/Wash buffer.

43| Centrifuge plate at plate at 863*g* for 4 min at room temperature.

44| Flick supernatant into biohazard waste container and immediately blot plate on a paper towel.

45| Resuspend PBMCs in 250 μl of 1% paraformaldehyde. Hold plate(s) at 4 °C, protected from light with aluminum foil, until analysis.

46| Prepare compensation controls using single-stained compensation beads (such as anti–mouse κ-chain beads).
▲ **CRITICAL STEP** An unstained control must also be included when using compensation beads.

47| Process samples by flow cytometry within 48 h.

48| Analyze sample files using an analysis program such as FlowJo, Verity or BD DiVa software.

● TIMING
Preparation of cells (Steps 1–10): 1–2 h plus overnight incubation
Plate preparation and stimulation (Steps 11–15): 6–7 h
Staining (Steps 1–46): 2–3 h

? TROUBLESHOOTING
See **Table 2** for troubleshooting advice.

TABLE 2 | Troubleshooting table.

Problem	Possible reason	Solution
Clumping of samples in wells or clogging of instrument probe	Cell aggregation has occurred as a result of release of DNA from dead or dying PBMCs.	Add DNase to the thaw and stimulation media.
High backgrounds	A dead-cell discrimination dye was not used.	Incorporate dead-cell discriminator such as ViViD.
	Cells were not cultured overnight after thaw.	Try culturing (resting) PBMCs for a minimum of 6 h or overnight.
	Antibodies were not added at optimal concentrations.	Titrate antibodies (see INTRODUCTION) using the procedural assay conditions described here.
No or poor (dim) cytokine response with SEB control samples	BFA was not added at beginning of stimulation or was used at an incorrect concentration.	Add BFA at 0 h stimulation at a concentration of 10.0 μg ml^{-1}.
	SEB was not added, was outdated or was used at an incorrect concentration.	Add SEB that is not outdated and is at the correct concentration of 1.0 μg ml^{-1}.
	Antibodies were not added at optimal concentrations.	Titrate antibodies using the procedural assay conditions described here.
	Outdated fix-perm regent was used.	Ensure reagent is not outdated.
	A wash reagent other than Perm/Wash buffer was used.	Ensure use of correct wash buffer.
	An incorrect concentration of Perm/Wash buffer was used.	Ensure use of the correct concentration stated in manufacturer's instructions.
	Outdated Perm/Wash buffer was used.	Use within the 1-month expiration date stated in the manufacturer's package insert.
No CD107a signal in SEB well	Monensin was not added at the beginning of the stimulation or was added at the wrong concentration.	Ensure the addition of monensin at the time of stimulation and at a concentration of 0.7 μg ml^{-1}.
	BFA was added as a substitute.	Use of monensin is required for visualization of CD107a signal.
No cytokine response with known HIV-positive sample	Peptide was not added or incorrect peptide concentration was used.	Ensure the addition of peptide and titrating peptide.
	Co-stimulation reagents were not added or incorrect concentrations were added.	Ensure the addition of co-stimulatory reagents at 1 μg ml^{-1}.
Antibody responses in negative control well	Contamination with SEB occurred.	Repeat experiment, placing SEB wells on another plate if necessary.
Cell aggregates at time of analysis	Antibody mixes were not spun to remove aggregates.	Remove aggregates by spinning and aspirating the supernatant.
Inability to compensate tandem dyes	Different lots of tandem dye were used for compensation.	Use the same lot of tandem dye in the compensation tube as used in the antibody mix for samples.

ANTICIPATED RESULTS

Vaccination of humans has been shown to provide broad T-cell responses, and thus the assessment of multiple functional markers ensures maximum identification of responding T cells. Minimal background, which is crucial in discriminating dim and rare responses from negative responses, is ensured by the use of an overnight 'rest' for freshly thawed PBMCs and the use of optimal volumes of antibodies determined by titration experiments before the experimental setup. In addition to these measures, the use of the violet viability dye (ViViD) allows the identification and elimination of dead cells that nonspecifically stain with many antibodies. High backgrounds affect the results of the assay, making it less sensitive; therefore, the importance of adding a dead-cell discriminator cannot be overstated. **Figure 2** provides an example of a sample stained with and without the inclusion of ViViD. Because dead cells can nonspecifically bind reagents, they may artifactually increase a potential response. The elimination of dead cells can increase the specificity and sensitivity of the assay.

Although other dead-cell markers may be used, amine-reactive dyes have several advantages. First, there are three dyes to choose from—green, red or violet—all with different excitation and emission wavelengths, allowing the user flexibility when designing staining panels. Second, unlike ethidium monoazide (EMA) or propidium iodide (PI), which need a reliable dead-cell source for use in compensation panels, the amine-reactive dyes are incubated with beads precoated with active amine groups, which may be purchased from the manufacturer. Third, the use of fix-perm reagents precludes the use of PI in ICS assays because permeabilization

Figure 2 | Inclusion of ViViD excludes dead cells, decreasing background. After being subjected to the ICS procedure described in this paper, PBMCs were incubated with co-stimulatory molecules alone to provide a negative control. Under these conditions, any measurable functional responses would be considered background and would be subtracted from the 'true' antigen-specific responses. (**a**) Without the addition of ViViD to the stain, dead cells cannot be excluded and contribute nonspecifically to the CD107a response (5.35%). (**b**) Overlay of the positive CD107a population onto a dot plot of ViViD versus FS reveals that most of these cells are not viable. (**c,d**) Using a live cell gate (**c**), dead cells may be excluded, reducing the background to 0.061% (**d**).

of the cell membrane causes PI to leak out of the cells, causing a loss of fluorescence. Fourth, although this problem can be avoided when using EMA, the covalent binding of EMA to DNA requires exposure to UV light for a specified amount of time. Too little exposure results in little or no binding of EMA to DNA; too much exposure may result in a loss of fluorescence of other cell surface markers.

The inclusion of quality-control procedures is critical when performing any experiment to ensure accuracy and reproducibility of results. ICS experiments mandate the use of a negative and positive control well for each subject and time point. These controls help ensure no false-negative and false-positive results will be obtained. The negative control well (cells plus all reagents minus an antigen stimulus) guarantees the PBMCs are not responding to a nonspecific stimulus and also provides a 'background' control that is subtracted from all of the peptides and stimulants of choice. The positive control ensures that all other reagents are performing to standard and that a universal stimulus generates positive signals for all parameters of interest. Because SEB binds to T-cell receptors carrying certain Vβ chains found in all humans and induces broad T-cell functions, it is often used as the positive control stimulus.

In addition to the above controls, users may consider including a known positive sample for the peptides being tested. For example, for our human clinical trials, we include cells from a known HIV+ donor whose results have been statistically analyzed to produce an expected range of values. This donor sample is included with each day's run. If the results of this control sample fall within the expected range of values, this provides added assurance that the test sample results are accurate in response to the specific peptides added to each well. The known-donor sample also helps track any trends or shifts over time, alerting the user to any problems with reagents.

Polychromatic flow cytometry provides a powerful tool for identifying the phenotypes and functional responses of antigen-specific T cells. However, the user is then confronted with the question of how best to distinguish and identify these populations. An example of our gating strategy for 12-color flow cytometry, which may also be applied to panels using fewer colors, is shown in **Figure 3a,** and encompasses data from one of our vaccine subjects whose PBMCs were stimulated with peptides from a clade A HIV envelope. The top panel shows the progressive gating used to identify the CD8+ T-cell population. Once the CD8+ T cells were identified, the cytokines could be examined against one another. The lower panel shows IFN-γ, IL-2 and MIP1β plotted against TNF-α, with gates placed around each of those respective cytokines. TNF-α was then plotted and gated against CD107a. Finally, CD107a was plotted and gated against IFN-γ.
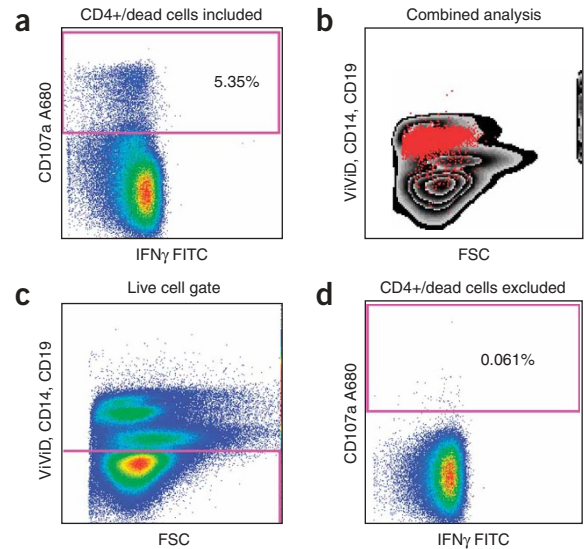
Figure 3 | Twelve-color gating strategy with representative Boolean gating in graphical format. (**a**) Gating scheme for identification of multifunctional CD8+ T-cell responses. Data shown are from a multiclade HIV-1 DNA vaccine subject whose 10-week-post-vaccination sample after recombinant adenoviral boost was stimulated with HIV-1 envelope B peptide. Top, the progressive gating strategy used to identify the CD8+ population. Bottom, results of the five functional responses of the CD8+ T cells. (**b**) Graph representation of the same volunteer's CD8+ T-cell multifunctional responses to HIV-1 envelope B peptide subsequent to Boolean gating. The five functions, CD107a, IFN-γ, IL-2, MIP1β and TNF-α, are listed along the x-axis with each of their respective 32 possible combinations. The blue bars represent the CD8+ T cell percent response to HIV-1 envelope B for each of the 32 possible combinations. The five horizontal bars of different colors below the x-axis depict the populations of five, four, three, two or one functional responses. Responses shown are background subtracted; i.e., the frequency of the cells in that particular category for cells that were cultured without stimulant is subtracted from the same frequency in the stimulated population. (**c**) Each pie chart represents the frequency of each of the 32 possible combinations of the 5 functional responses of the same vaccine volunteer to HIV-1 envelope peptides. The first three pie charts show the CD8+ T cell responses to the envelope A, B and C peptides and the second three show the CD4+ T-cell responses to the same envelope peptides, respectively. Note the similarities between the CD8+ T-cell responses to the different envelope antigens and between the CD4+ T-cell responses to the different antigens; this is indicative of the reproducibility of this kind of assessment even for low-frequency responses. The mean response of subjects to individual peptide pools may be calculated and charted in this way to study functional profiles between various vaccine protocols. (**d**) Responses are grouped by the number of functions and are color-coded to match the horizontal bars in **b**. The majority of responses comprise more than one function, and less than 25% include four or more functions. However, in all but one chart, 50% of the responses comprise at least three functions.
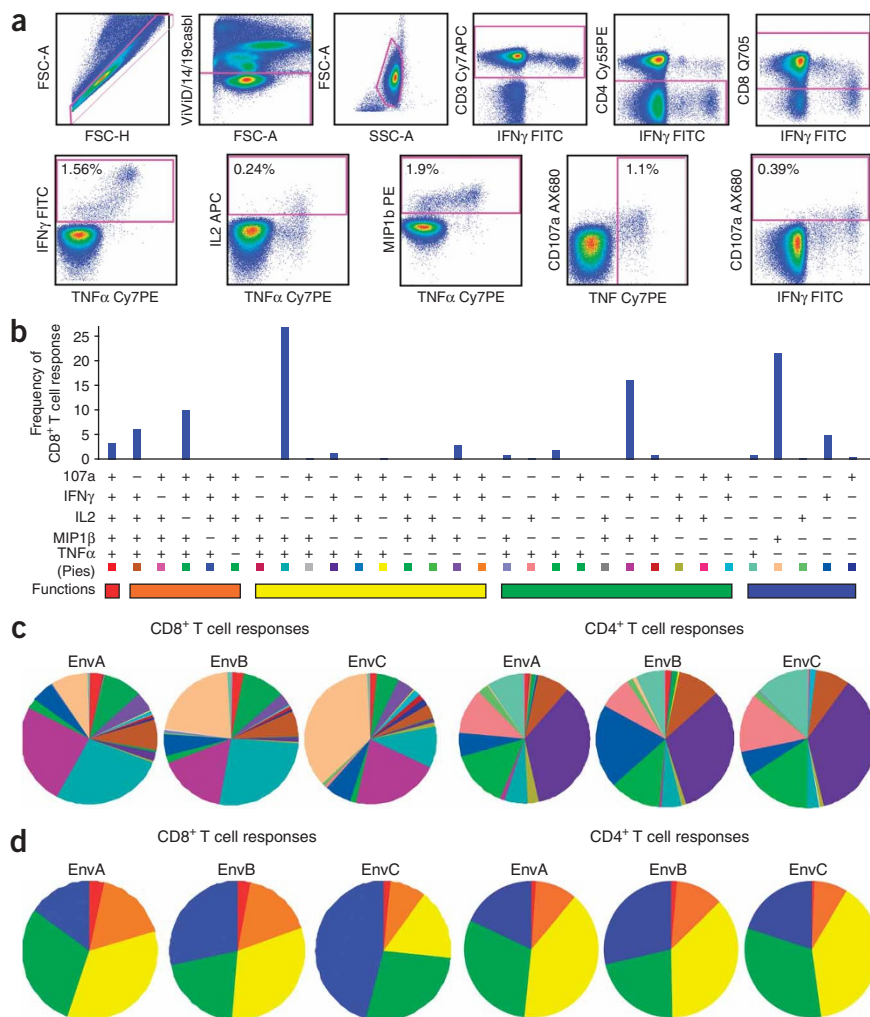
To measure the complexity of the antigen-specific T-cell response, an algorithm using Boolean gating was applied to the five measurements of functional data, revealing 32 possible profiles (**Fig. 3b**). The frequency of each of these profiles was then determined and plotted as a pie chart (**Fig. 3c**). Previous experiments examining antigen-specific responses between HIV progressors and nonprogressors have shown that nonprogressors maintain a more polyfunctional CD8+ response in the blood[27]. Hence, it is important to also group and color-coordinate the responses according to the number of functions seen (five functions versus four functions, etc.) to evaluate the polyfunctional profile (**Fig. 3d**). This is useful for comparison of polyfunctional responses between vaccine protocols and for comparison with vaccines such as smallpox that are known to provide immunity.

Analysis of multiple functional parameters measured on specific subsets is a complex undertaking. Others have described the use of Boolean gating strategies to aid in simplifying this process[27]. Furthermore, the use of new software analysis tools such as Simplified Presentation of Incredibly Complex Evaluations (SPICE, version 3.0, Mario Roederer, Vaccine Research Center (VRC), NIAID, NIH), organizes the data from the Boolean analysis into a graph format that enables the user to display the functional profiles in a variety of ways. This allows 'simplification' of data presentation in a manner that allows it to be interpreted more easily.

In conclusion, whether a basic 4-color panel or 12-color polychromatic multifunctional panel is chosen, critical steps need to be identified and addressed empirically before a standard operating procedure for ICS is established. It is worth the extra time and effort to ensure a reliable working protocol.

PROTOCOL

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