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Methodology article



# Performance of plate-based cytokine flow cytometry with automated data analysis

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## Abstract

**Background:** Cytokine flow cytometry (CFC) provides a multiparameter alternative to ELISPOT assays for rapid quantitation of antigen-specific T cells. To increase the throughput of CFC assays, we have optimized methods for stimulating, staining, and acquiring whole blood or PBMC samples in 96-well or 24-well plates.

**Results:** We have developed a protocol for whole blood stimulation and processing in deep-well 24- or 96-well plates, and fresh or cryopreserved peripheral blood mononuclear cell (PBMC) stimulation and processing in conventional 96-well round-bottom plates. Samples from both HIV-1-seronegative and HIV-1-seropositive donors were tested. We show that the percent response, staining intensity, and cell recovery are comparable to stimulation and processing in tubes using traditional methods. We also show the equivalence of automated gating templates to manual gating for CFC data analysis.

**Conclusion:** When combined with flow cytometry analysis using an automated plate loader and an automated analysis algorithm, these plate-based methods provide a higher throughput platform for CFC, as well as reducing operator-induced variability. These factors will be important for processing the numbers of samples required in large clinical trials, and for epitope mapping of patient responses.

## Background

Cytokine flow cytometry (CFC) assays use short-term *in vitro* stimulation and intracellular cytokine staining to quantitate potentially rare populations of antigen-specific T cells (see [1,2] for reviews). Because of the multi-parametric readout of flow cytometry, CFC is an attractive alternative to ELISPOT assays, allowing the dissection of responses by various phenotypes of T cells [3,4]. However, ELISPOT assays have traditionally been more amenable to high-throughput analysis due to their plate-based nature and the availability of automated instrumentation to analyze ELISPOT plates.

In order to provide similar advantages to the CFC platform, we optimized the ability to stimulate, process, and acquire CFC samples in 96-well or 24-well plates, using stimulation with the superantigen *Staphylococcal* enterotoxin B (SEB), as well as cytomegalovirus (CMV) or HIV antigens. The result was two protocols optimized for stimulation and processing of whole blood samples (in deep-well 24- or 96-well plates), and one protocol optimized for stimulation and processing of PBMC samples (in conventional round-bottom 96-well plates). These optimized protocols compared favorably with tube-based methods, and, when combined with automated acquisition and analysis software, resulted in a rapid and highly standardized assay with greatly reduced hands-on time.

## Results and Discussion

### *Comparison of CFC responses in tubes and plates*

Both whole blood and PBMC samples are frequently used for CFC assays. While conditions need to be optimized for these two sample types [5–8], comparable results can be obtained between whole blood and PBMC stimulations [9]. We therefore optimized protocols for each sample type. Processing of whole blood requires lysis of erythrocytes, so deep-well plates were employed to allow for addition of the required volume of lysis buffer. Whole blood samples from HIV-1-seropositive donors can potentially have lowered $CD4^+$ T cell counts, so 24-well deep-well plates were used. These can accommodate up to 1 ml of blood per sample, plus up to 9 ml of lysis buffer.

A representative comparison for each type of assay is shown in Figure 1. Whole blood (200 μl per sample) was stimulated in conical-bottom 96-well deep-well plates. PBMC ($10^6$ cells in 200 μl) were stimulated in conventional round-bottom 96-well plates. Finally, whole blood (1 ml per sample) from an HIV-1-seropositive donor was stimulated in round-bottom 24-well deep-well plates. Each sample was stimulated for 6 hours in the presence or absence of a CMV pp65 or HIV-1 Gag p55 peptide mixture. Replicate samples for each assay type were incubated in parallel in 15-ml conical polypropylene tubes, using a standard CFC protocol [5,6]. As seen in Figure 1, the plate and tube assays give qualitatively and quantitatively similar results.

In order to more accurately quantify the percentage of antigen-specific T cells identified by plate-based versus tube-based assays, a larger number of donors were tested by each method used in Figure 1. The results (Figure 2) show a close correlation between tube- and plate-based assays for the percentage of cytokine-producing cells. Strong correlations were seen for both SEB and CMV or HIV peptide stimulation, and in both CD4 and CD8-gated T cells. The slope of the linear regression lines approximated 1, indicating no particular bias toward higher results in either tubes or plates.

### *Comparison of staining intensity in tubes and plates*

Next, we compared the mean fluorescence intensity of cytokine staining in tube-based versus plate-based assays, using the same samples as above. As seen in Figure 3, the staining intensity for IFNγ compared favorably between tubes and plates, with plates yielding similar or even higher fluorescence intensities than tubes. This was true for both whole blood (deep-well) and PBMC (shallow-well) assays. The differences in the ratio of tube-to-plate staining intensity could be related to staining volume, which is a function of the residual volume after aspiration. This volume tends to be lowest in shallow-well 96-well plates (using our multichannel aspiration manifolds), and highest in 24-well plates.

### *Effect of overnight resting on cryopreserved PBMC*

For cryopreserved PBMC, we tested the effect of an incubation period after thawing but before antigen stimulation in 96-well plates. While we had previously reported that such a "rest" period did not significantly affect the result (percent of cytokine-positive cells) [10], we now show that it does affect the fluorescence intensity of cytokine-positive cells. As shown in Figure 4, we found that IFNγ staining intensity was significantly increased after an overnight incubation of thawed cells in medium, prior to stimulation with CMV pp65 peptide mix. This increased cytokine staining intensity correlated with an increase in functional avidity as measured by responses to lower concentrations of peptide antigen in titration experiments (data not shown).

### *Comparison of cell recovery in tubes and plates*

We also compared cell recoveries in tube- versus plate-based assays. Cell loss in CFC assays is likely to be associated with centrifugation and washing. By using a 12-channel vacuum manifold with custom-length prongs (see Methods), we were able to aspirate supernatants after centrifugation in a rapid and reproducible manner, without risk of aspirating the cell pellets. As such, cell recoveries for both PBMC and whole blood were similar in plates and tubes (Figure 5). For PBMC, recoveries in plates appeared to be slightly better than in tubes, perhaps because of the need to incubate PBMC tubes on a slant [7], and associated difficulties with recovering adherent cells from the side of the tube. Also, in the tube protocol, cells are transferred from a 15-ml stimulation tube to 5-ml staining tubes, which may account for some cell loss. While 5-ml polystyrene tubes can be used for stimulation, they result in reduced cell recovery and slightly reduced activation efficiency with protein antigens (our unpublished data). The plate protocol has the advantage that

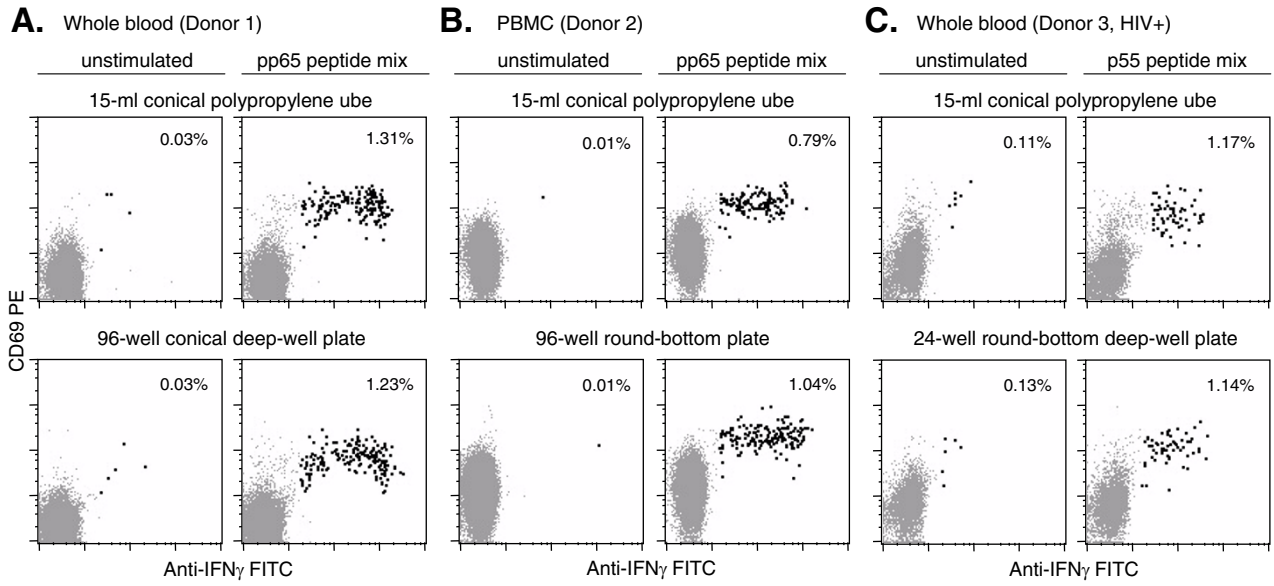


**Figure 1**
Representative examples of tube- and plate-based CFC results. (A) Whole blood from a CMV seropositive donor was stimulated (or not) with CMV pp65 peptide mix in 15 ml conical polypropylene tubes (top panels) or a deep-well 96-well polypropylene plate (bottom panels). (B) PBMC from another CMV seropositive donor were stimulated as above in 15 ml conical polypropylene tubes (top panels) or a 96-well round-bottom tissue culture plate (bottom panels). (C) Whole blood from an HIV-seropositve donor was stimulated (or not) with HIV p55 gag peptide mix in 15 ml conical polypropylene tubes (top panels) or a deep-well 24-well round-bottom polypropylene plate (bottom panels). Backgrounds and response to peptide mix were essentially equivalent in tubes and plates in each case. All data are gated on $CD3^+CD8^+$ cells.

cells are stimulated, processed, and acquired in a single plate.

### *Automated gating algorithm*

In addition to errors that may have been introduced by differential washing, another frequent source of variation in CFC assays is analysis. Relatively minor differences in gating can quantitatively affect the results [2], and the prospect of subjectivity in gating is a concern for many investigators. Analysis also tends to be time-consuming using most conventional software packages. However, using software that contains a cluster-finding algorithm, we were able to build templates for automated CFC gating that identify the required cell populations and report a percentage of CD69-positive, cytokine-positive cells. Samples could then be processed by these templates using batch analysis, and results automatically exported to a spreadsheet. By using the cluster-finding algorithm, much of the subjectivity in gating is avoided.

An example of such a template is shown in Figure 6A. When compared to expert manual gating, the automated template of Figure 6A yielded highly correlated results for a set of 25 whole blood samples stimulated with CMV pp65 peptide mix or SEB (Figure 6B).

The template of Figure 6A also yielded good correlation with manual gating for a set of 39 HIV-1-seropositive

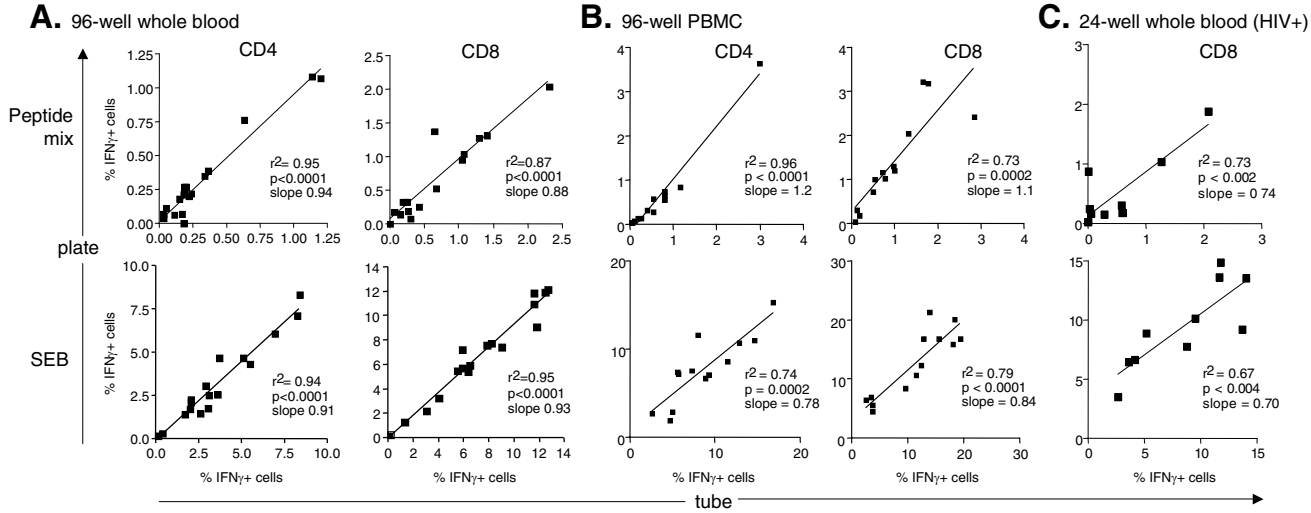


**Figure 2**
Correlation of tube- and plate-based CFC assays. Stimulation vessels and antigens were as in Figure 1. (A) Correlation of whole blood CMV and SEB responses in 17 CMV seropositive donors, done in tubes versus deep-well 96-well conical polypropylene plates. (B) Correlation of PBMC CMV and SEB responses in 13 CMV seropositive donors, done in tubes versus 96-well round-bottom tissue culture plates. (C) Correlation of whole blood HIV and SEB responses in 10 HIV seropositive donors, done in tubes versus deep-well 24-well round-bottom polypropylene plates.

whole blood samples stimulated with CMV pp65 peptide mix, HIV p55 peptide mix, or SEB (Figure 7A). However, it was noted that for some $CD4^+$ responses in the HIV-1-seropositive dataset, the automated template under-counted $IFN\gamma^+$ cells relative to manual gating, resulting in a slope of slightly less than 1 for the linear regression line (Figure 7A, CMV pp65 and SEB responses). In particular, samples with very few $CD4^+$ T cells tended to result in undercounting of $IFN\gamma^+$ cells by the automated template, due to suboptimal $CD4^+$ cluster identification. Such samples were better analyzed using a template that created a fixed region for $CD3^+CD4^+$ gating, tethered to the $CD3^+CD4^-$ population (Figure 7B). This fixed region was set to avoid the exclusion of $CD4^{dim}$ cells when the total number of $CD4^+$ cells was very low. When this revised template was applied to the determination of $CD4^+$ responses for the HIV-1-seropositive dataset, the resulting linear regressions yielded higher slopes, very close to 1 (Figure 7C). Thus, changes in the gating scheme employed by the automated template can improve the quality of data generated under certain circumstances. In particular, samples containing very few $CD4^+$ cells may be best handled by the modified template of Figure 7B.

Another alternative to the template of Figure 6A that was advantageous in certain circumstances involved changing the initial gating of lymphocytes. Datasets containing poorly defined lymphocyte clusters were best gated by

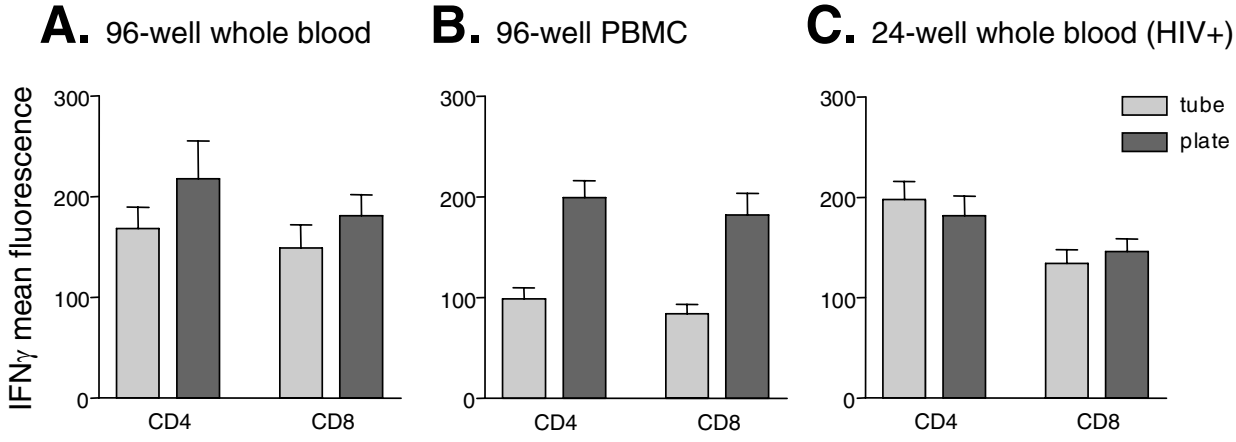


**Figure 3**
Mean fluorescence intensity of IFNγ staining using tube- or plate-based CFC. Data are taken from the samples of Figure 2. Error bars represent SEM.

using an initial automated region to identify $CD3^+$ lymphocytes in a CD3 versus side scatter plot, rather than using forward versus side scatter gating (data not shown).

The key features of any of these automated templates include: (a) the ability to track populations that "roam" as a result of donor, reagent, or instrument variations; (b) the ability to create a maximally sized region that will include activated T cells that have partially down-modulated the marker in question (CD3, CD4, and/or CD8); and (c) the ability to tether a manually drawn region to another population cluster to allow identification of potentially rare populations of cells (i.e., cytokine-positive cells).

### *Gains in assay throughput and standardization*

As a result of applying the above methodology, the throughput of CFC assays is increased in a manner proportional to the number of samples per experiment. For example, a complete plate of 96 samples can be processed with significantly less time than an equivalent number of tubes (saving perhaps 1–2 hours of pipetting time). Since acquisition on a plate loader can be unsupervised, this saves an additional 3–5 hours of technician time. Finally, batch analysis with an automated template might save at least one more hour of technician time as compared to manual analysis.

The other major benefit of the methodology described here is standardization. The more rapid sample handling associated with plate-based methods would be expected to reduce sample-to-sample differences in incubation

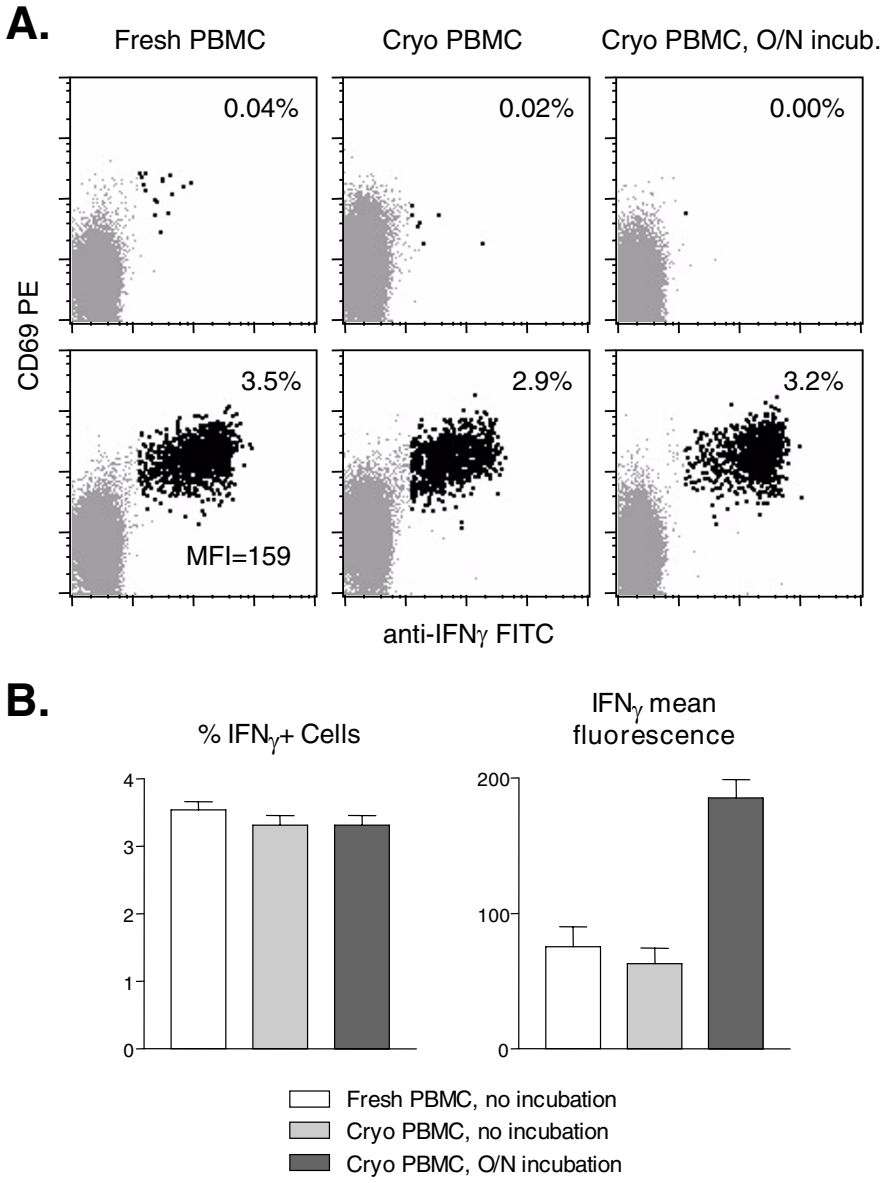


**Figure 4**
Effect of overnight "resting" on cryopreserved PBMC. (A) Representative comparison of fresh PBMC, cryopreserved PBMC, and cryopreserved PBMC incubated overnight prior to stimulation (all from the same $CMV^+$ donor). Unstimulated samples (top row) and CMV pp65 peptide mix-stimulated samples (bottom row) show similar results, but the IFNγ fluorescence intensity (MFI) is greatest in the cells rested overnight prior to stimulation. (B) Results of triplicate samples stimulated with CMV pp65 peptide mix and analyzed for percentage of $IFN\gamma^+$ cells or IFNγ mean fluorescence intensity. Error bars represent SEM. All data were gated on $CD3^+CD4^-$ cells. Similar results were obtained for $TNF\alpha^+$ cells (not shown). Results are representative of two similar experiments.

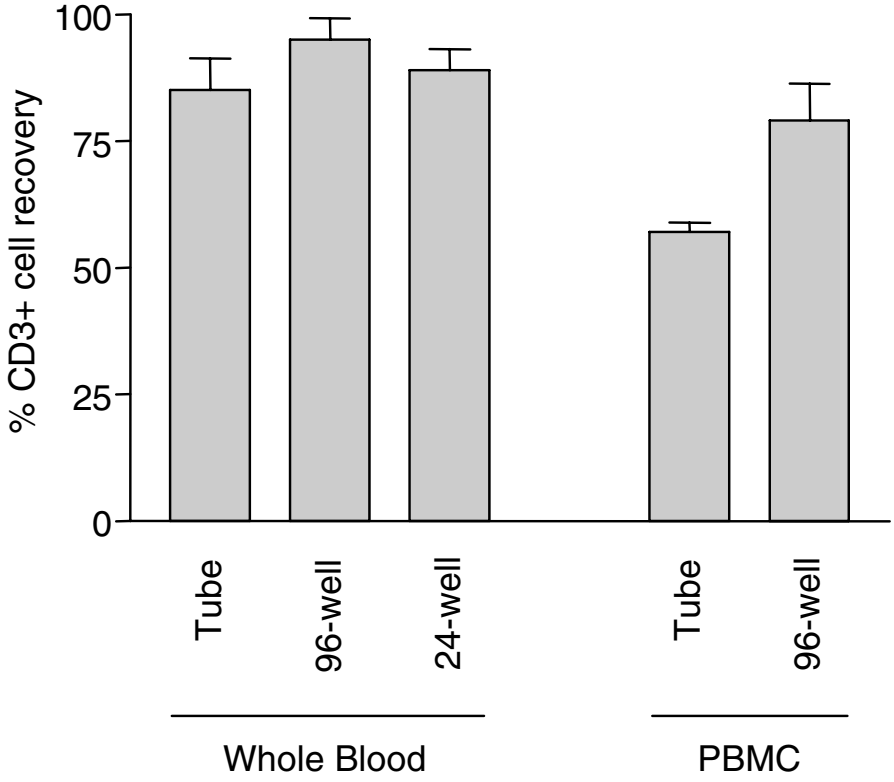


**Figure 5**
Cell recovery in tube- and plate-based CFC assays. Recovery was calculated by addition of a known number of beads to each CFC sample at the end of sample processing. Data are shown as a percentage of cells counted in fresh whole blood or PBMC from the same donor. Error bars represent SEM of 3 donors for each assay.

times for each processing step. Thus, variability in results associated with these time differences should also be reduced. Perhaps more significantly, automated analysis using a standard template should standardize differences in gating that occur between users. It is known that gating differences can make relatively large contributions to result variability [2]. In particular, the exclusion of cells with down-modulation of CD4 or CD8 staining due to activation can negatively impact the accurate reporting of cytokine-positive cells. The automated templates described above have been engineered to include these down-modulated cells in a reproducible fashion.

### *Comparison to ELISPOT assays*

By providing plate compatibility and a degree of automation to CFC assays, they become attractive relative to competing platforms such as ELISPOT. These two assays both measure cytokine production, but CFC can provide multiparameter information that clearly distinguishes T cell subsets such as CD4 and CD8 as well as a number of other phenotypic markers on the responding cells. While ELISPOT assays have been reported to have lower limits of detection than CFC assays [11], CFC generally reports higher numbers of positive cells [9,12–14]. Thus, plate-based CFC assays can provide the benefits of multiparameter information, with higher efficiency of detection and comparable throughput to ELISPOT assays.

## Conclusions

We conclude that CFC assays can be performed with increased throughput using plate-based methodology. By optimizing the type of plate used for either PBMC or

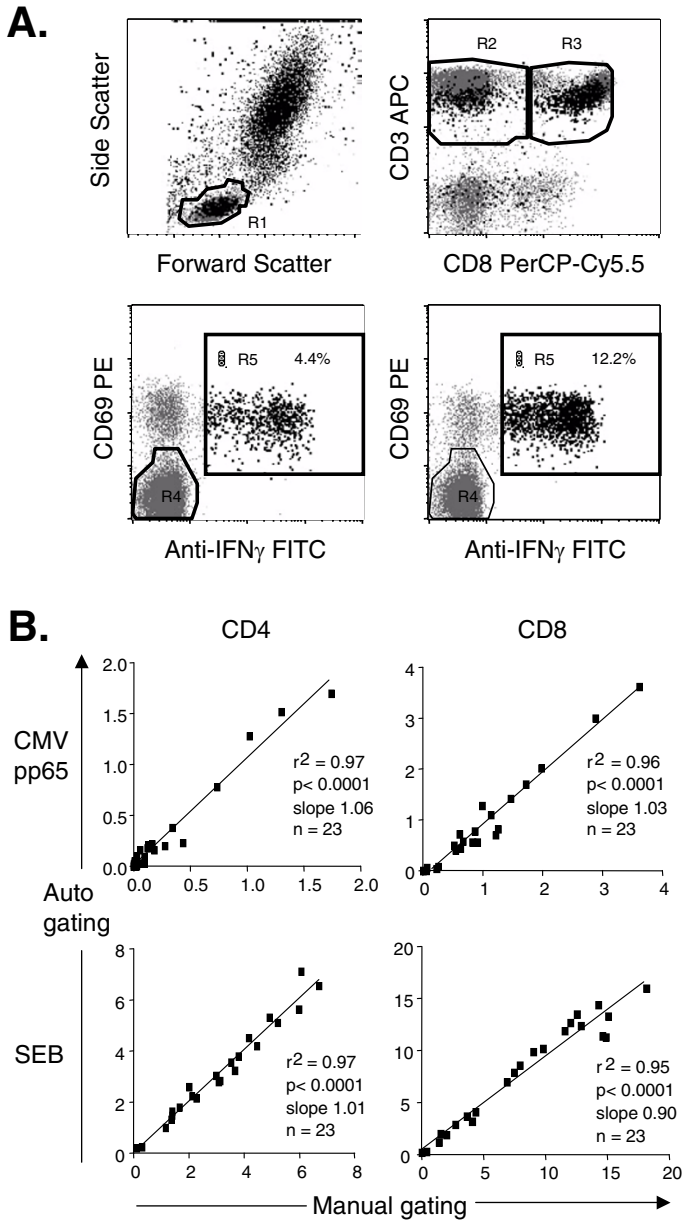


**Figure 6**
Automated gating of CFC samples. (A) Example of automated gating. A cluster-finding algorithm is employed to automatically identify small lymphocytes in forward versus side scatter (R1, top left panel; this region can also be drawn manually during sample acquisition). A similar automated region is calculated for $CD3^+CD8^-$ and $CD3^+CD8^+$ lymphocytes (R2 and R3, top right panel). R2 and R3 are set for maximal size to allow inclusion of activated cells that have down-modulated CD3 and CD8 (bold dots). The lower left plot, gated on regions R1 and R2 ($CD3^+CD8^-$ lymphocytes), uses the cluster-finding algorithm to identify the $CD69^-IFN\gamma^-$ population (R4). This population is tethered to a rectangular region that identifies the $CD69^+IFN\gamma^+$ cells (R5). The lower left plot is similar, but gated on R1 and R3 ($CD3^+CD8^+$ lymphocytes). The percentage of gated events in R5 is reported. (B) Correlation of the automated gating template to expert manual gating. Whole blood from 23 CMV seropositive donors was stimulated in deep-well 96-well plates, then analyzed manually or using the automated template in (A). Using a batch analysis protocol, many CFC samples can be analyzed and the resulting data downloaded to a spreadsheet in a rapid fashion.

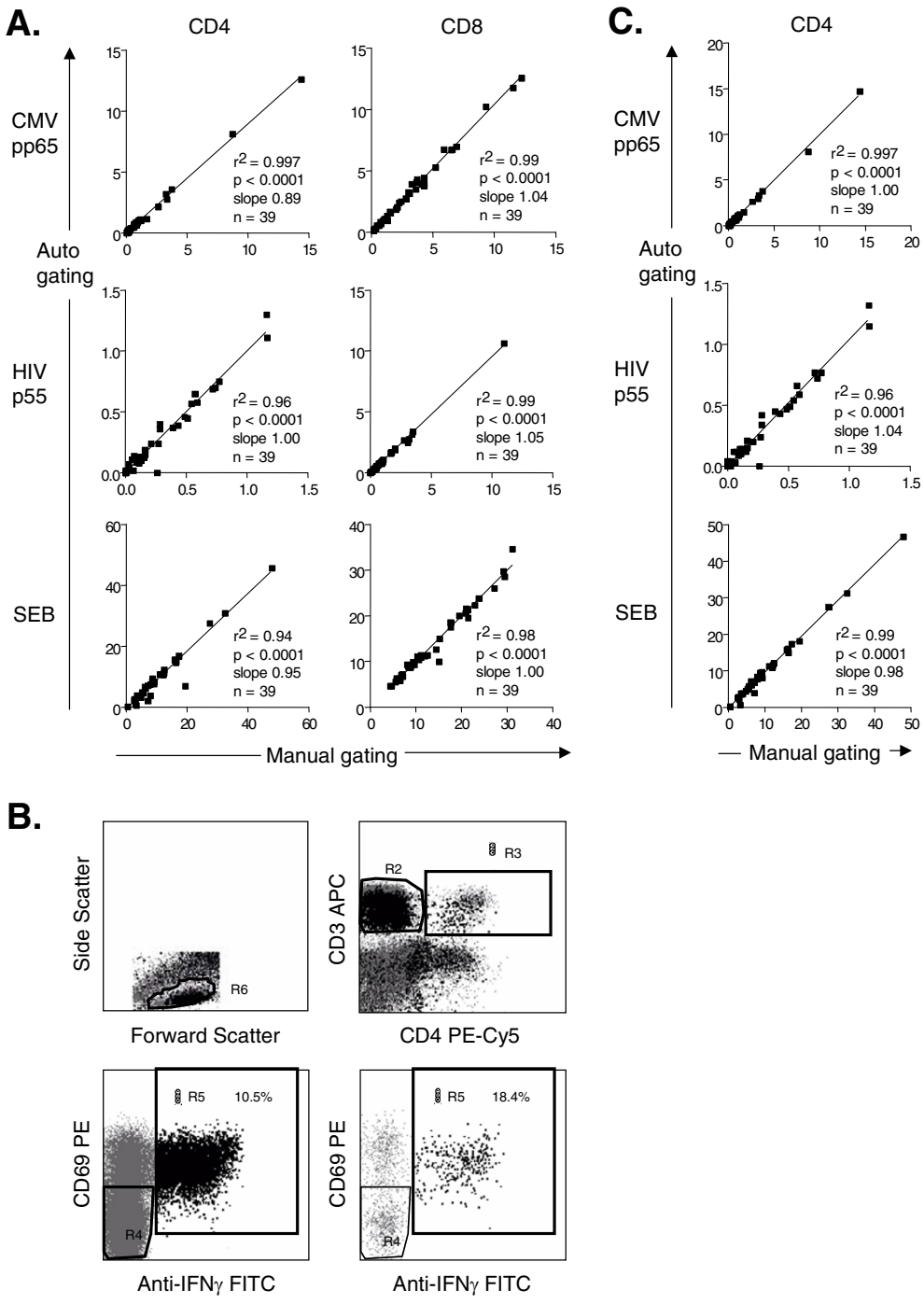


**Figure 7**
Automated gating of HIV-1-seropositive CFC samples. (A) Correlation of automated gating to expert manual gating. Whole blood from 39 HIV-1-seropositive donors was stimulated using the antigens shown, then analyzed manually or with the automated template of Figure 6A. (B) Revised template designed for samples with very low numbers of $CD4^+$ cells. A fixed region for $CD3^+CD4^+$ cells (R3) has been created and tethered to the automated region R2. (C) Improved performance of the revised template for calculating $CD4^+$ responses from the dataset of (A). Note that the slope of the linear regression line for pp65 peptide mix and SEB stimulation is now closer to 1.

whole blood, we are able to activate, process, and acquire samples in a single plate. This avoids labor and cell loss associated with sample transfers. We also show that data analysis can be streamlined using an automated template that uses cluster-finding algorithms. The data generated from such semi-automated plate-based assays are equivalent to data generated using tube-based methods and manual analysis. In addition, these plate-based methods and automated analysis templates are expected to increase standardization of CFC, such as would be required for its use in large clinical trials. Other applications include epitope mapping using a matrix of overlapping peptide pools [15], where high throughput and an array format are desirable features.

## Methods

For additional details of procedures and notes on critical parameters, please see reference [16].

### *Stimulation reagents*

SEB (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile PBS at 0.5 mg/ml and stored at 4°C. It was used at a final concentration of 1 μg/ml of blood or PBMC suspension. CMV pp65 and HIV p55 gag peptide mixes (15 amino acid residues in length, overlapping by 11 amino acid residues each) have been described previously [10]. They were stored in small aliquots at -80°C, and diluted in sterile PBS on the day of use to achieve a final concentration of approximately 2 μg/ml/peptide. In some experiments, costimulatory antibodies to CD28 and CD49d (FastImmune, BD Biosciences, San Jose, CA) were added at a final concentration of 1 μg/ml each [8]. Brefeldin A (FastImmune or Sigma) was stored in small aliquots at -20°C and diluted in sterile PBS on the day of use to achieve a final concentration of 10 μg/ml.

### *Plates and accessories*

Whole blood was activated in deep-well polypropylene plates, using 96-well plates (BD Discovery Labware, Bedford, MA; or E&K Scientific, Campbell, CA) for 200 μl samples, or 24-well plates (Qiagen, Hilden, Germany) for up to 1 ml samples. PBMC were activated in standard 96-well round-bottom polystyrene tissue culture plates (Falcon, BD Discovery Labware). 12-channel vacuum manifolds with 7 mm prongs (for standard plates) or 35 mm prongs (for deep-well plates) were purchased from V&P Scientific, San Diego, CA. Centrifuge holders compatible with deep-well plates were purchased from Sorvall Instruments, Newtown, CT.

### *Collection and cryopreservation of PBMC*

Informed consent (approved by either the UCSF or the BD Biosciences Institutional Review Board) was provided by all volunteer blood donors. For PBMC preparation, whole blood was collected in Cell Preparation Tubes (CPT™) containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ). PBMC were isolated following the manufacturer's instructions, and washed with RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotic/antimycotic solution (cRPMI-10, all components from Sigma). Fresh PBMC were resuspended in a volume of cRPMI-10 equivalent to the original blood volume. In some experiments, PBMC were resuspended for cryopreservation at $2 \times 10^7$ cells/ml in RPMI-1640+12.5% human serum albumin (Sigma). An equal volume of cold RPMI-1640 medium+10% human serum albumin+20% dimethyl sulfoxide (Sigma) was slowly added. Cells were transferred to freezing vials (1 ml/vial) and placed at -80°C in a freezing container (Mr. Frosty, Nunc, Naperville, IL). Cryopreserved cells were thawed briefly in a 37°C water bath, then 1 ml of warm (37°C) cRPMI-10 medium was added dropwise to the vial, and the cell suspension transferred to a 50 ml conical polypropylene tube (Falcon) containing 8 ml of warm cRPMI-10 medium. The cells were centrifuged for 7 minutes at 250 × G, then resuspended in cRPMI-10 at $5 \times 10^6$ cells/ml.

### *CFC of tube-based specimens*

For comparison to plate assays, heparinized whole blood or PBMC in cRPMI-10 were activated in 15 ml conical polypropylene tubes (Falcon) and processed in 12 × 75 mm round-bottom polystyrene tubes (Falcon). A standard method, previously described [5,6], was followed.

### *Cell activation in plates*

200 μl of heparinized whole blood or PBMC in cRPMI-10 medium were plated per well in 96-well plates; 0.5 ml or 1 ml of whole blood was plated per well in 24-well plates. Cryopreserved PBMC were then rested at 37°C overnight, while fresh PBMC or whole blood samples were activated immediately. Activation reagents as described above were prepared in a master mix to allow their combined addition to each well in a volume of 20 μl per 200 μl of cell suspension. The cells were incubated at 37°C for 6 hours. In some experiments, the plates were then held overnight at 18°C, using a programmable water bath.

### *Cell processing in plates*

20 μl of 20 mM EDTA were added per 200 μl of cell suspension, and incubated for 15 minutes at room temperature, followed by vigorous pipetting to dislodge adherent cells. For PBMC, the plates were then centrifuged at 250 × G for 5 minutes, and supernatants aspirated using a vacuum manifold (V&P Scientific, see above). Cells were resuspended in 100 μl/well of FACS Lysing Solution (BD Biosciences). For whole blood, 8–10 volumes of FACS Lysing Solution were added directly to the EDTA-treated blood. For both PBMC and whole blood, cells were incubated in FACS Lysing Solution for 10 minutes at room temperature, then centrifuged for 5 minutes at 500 × G.

Supernatants were aspirated using an appropriate-length vacuum manifold (described above). Cells were resuspended in FACS Permeabilizing Solution 2 (BD Biosciences) (200 μl for PBMC; 1 ml for deep-well 96-well plates; 2 ml for 24-well plates). After an additional 10 minute incubation at room temperature, sample wells of deep-well plates were filled with wash buffer (PBS+0.5% bovine serum albumin+0.1% $NaN_3$) and all plates were centrifuged for 5 minutes at 500 × g. Supernatants were aspirated as above, and the cells washed a second time. PBMC plates were subjected to a third wash, then all samples were resuspended in 20 μl of antibody cocktail containing anti-IFNγ FITC/CD69 PE/CD4 or CD8 PerCP-Cy5.5/CD3 APC (FastImmune, BD Biosciences). In experiments of Figure 7, CD4 PE-Cy5 (Beckman Coulter, Miami, FL) was used in place of CD4 PerCP-Cy5.5. Samples were incubated for 60 minutes at room temperature in the dark, then washed twice as above before resuspending in a final volume of 200 μl of 1% paraformaldehyde in PBS (250 μl for 24-well plates).

### *Flow cytometric analysis*

Samples were acquired within 24 h of staining using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences). Plate-based samples were acquired on the FACSCalibur using a Multiwell Autosampler and Multiwell Plate Manager software (BD Biosciences). In general, 40,000 $CD4^+$ or $CD8^+$ events were collected. For plate-based samples, sample volume was set to 200 μl and mixing volume to 100 μl in Multiwell Plate Manager. Acquisition criteria were set to 40,000 events of interest ($CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes), or 180 seconds. Manual analysis was done in CellQuest Pro or FloJo software (Tree Star, Inc., San Carlos, CA) by gating on both small lymphocytes and $CD3^+CD4^+$ (or $CD3^+CD8^+$) cells. A gated dot plot displaying CD69 versus IFNγ from an SEB-stimulated sample was then used to set a "response region" around double-positive cells. This response region was then applied to all samples to determine the percentage of cytokine-positive cells. Automated analysis was done as described in Figure 6A or 7B, using "Snap-to" gating and tethered region tools in CellQuest Pro 5.0.1.

### *Determination of cell recovery*

The absolute counts of $CD3^+$ T cells in whole blood and PBMC were determined using TruCOUNT Control beads and TriTest CD4 FITC/CD8 PE/CD3 PerCP antibody cocktail (BD Biosciences). For the samples representing 100% cell recovery, 50 μl of uncultured whole blood or PBMC were aliquoted by reverse pipetting into 12 × 75 mm polystyrene tubes containing 20 μl of TriTest CD4 FITC/CD8 PE/CD3 PerCP. Each cell sample was stained in triplicate. The samples were mixed well and incubated for 30 minutes at room temperature in the dark, and then lysed/fixed using 450 μl of FACS Lysing Solution. After a 15-minute incubation at room temperature, 50 μl of TruCOUNT "Low" or "Medium" beads were added by reverse pipetting. The samples were mixed well and acquired immediately using a FACSCalibur flow cytometer.

CFC samples were also stained in triplicate. EDTA-treated activated whole blood in deep-well plates and tubes (200 μl) or 24-well plates (1000 μl) and PBMC in shallow-well plates and tubes (200 μl) were lysed/fixed and permeabilized as described above. The samples were stained with 20 μl of TriTest CD4 FITC/CD8 PE/CD3 PerCP for 30 minutes at room temperature in the dark, washed twice and resuspended for acquisition in 200 μl of wash buffer. TruCOUNT "Low" or "Medium" beads were then added by reverse pipetting at 50 μl per sample. The samples were mixed well and acquired immediately.

Samples were acquired on a FACSCalibur flow cytometer using an FL3 (for CD3 PerCP) threshold. The $CD3^+$ lymphocytes were identified in FL3 vs SSC and region R1 was drawn to include all $CD3^+$ cells. The acquisition was carried out until 10,000 $CD3^+$ events were collected, however, all events passing the FL3 threshold were stored in order to include the beads, which were then identified and gated (R2) in FL1 vs FL2. The absolute numbers of $CD3^+$ lymphocytes were calculated as follows: (# of events in R1)/(# of events in R2) × (# of beads per test)/(test volume).

## Authors' contributions

MAS, HSD, PLO, RDL, and ES did the experiments. SAG designed the automated gating tools and edited the manuscript. BMB, JFD, and VCM supervised the work and edited the manuscript. HTM prepared figures and co-wrote the paper with MAS.

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