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An accurate and rapid single step protocol for enumeration of cytokine positive T lymphocytes

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ABSTRACT

Accurate determination of cellular subsets that secrete particular cytokine(s) is a significant parameter for functional characterization of an immunological response. The present study was conducted to develop a method for simultaneous measurement of intracellular cytokine positive CD4 and CD8 positive T lymphocytes in a single tube, with a no-wash protocol. We report here the development of a simplified, rapid procedure for precise enumeration of cytokine positive T lymphocytes using BD Trucount tubes. This single step protocol for accurate enumeration of cytokine positive T lymphocytes, will allow for better characterization of immune cell phenotype and function.

1. Introduction

Inflammatory cytokines are pivotal immune mediators for several cellular processes such as activation, proliferation, acute phase and first line pyrogenic defence responses.[1,2] Due to their pleiotropic functions, intracellular cytokines are crucial indicators of immune function and competence. One of the limitations in enumeration of cytokine positive cells is the small numbers of these cells, which makes flow cytometric quantification of cytokine positive cells quite challenging.[3,4] Using freeze-dried peripheral blood mononuclear cells (PBMC) reference materials, we report here the proof-of-principle for a rapid and accurate enumeration of cytokine positive T lymphocytes, by a flow cytometry-based method.

2. Materials and methods

A freeze-dried preparation[5] of unstimulated (NIBSC code: SS570) and phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulated PBMC, NIBSC code:15/272), obtained from healthy donors was used as the cellular reference material for the evaluation. Cell stimulation was carried out for 6 h in the presence of Brefeldin A. Cells were harvested, washed and following overnight fixation in 20% vol/vol Transfix (Cytomark, U.K.), pooled in a (2x) PBS lyophilization buffer; and freeze-dried in type-1 glass, 3 ml ampoules using a 6 m^2 Serail CS-100 freeze dryer (Le Coudray St Germer, France), set to a 4 day cycle. Each ampoule contained approximately 0.5 g of cell suspension in buffer. The sample weight prior to lyophilization had a mean of 0.515 g with a coefficient of variation (CV) of 0.53%. After lyophilization, the ampoules were flame-sealed and the lyophilized cakes had a robust appearance with a mean dry weight of 0.0143 g (CV of 1.10%.), a mean residual moisture content of 0.46% w/w and a mean oxygen headspace of 0.26% as determined on 12 test ampoules. This material was designated as a positive reference material, 15/272. A negative cellular reference material, SS570, prepared from unstimulated PBMCs, was freeze dried similarly in a VirTis Advantage pilot dryer (provided by Biopharma Process Systems, Winchester, UK). The sample weight prior to lyophilization had a mean of 0.508 g (CV of 0.15%). Following lyophilization, the ampoules were flame-sealed and the lyophilized cakes had a robust appearance with a mean dry weight of 0.0143 g (CV of 2.7%.), mean residual moisture content of 0.47% w/w and a mean oxygen headspace of 0.37% based on evaluation of 3 ampoules.

While intracellular cytokine staining (ICS) can be used for simultaneous measurement of multiple cytokines at a single cell level, the present evaluation was performed using IFN-γ as a prototype cytokine. The study was carried out independently at three different sites utilizing a standardized protocol that included a common antibody panel and cell

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staining protocol. Sample acquisition was performed using respective in-house laboratory protocols for instrument setup at each of the sites. Performance of FACS machines was carefully monitored by regular maintenance and tracked by CS&T beads. Post-acquisition analysis was carried out at each site using FlowJo software. The number of independent ampoules analyzed are indicated for each figure. Cell percentage and count determinations results from all sites were compiled at NIBSC and statistical analysis was performed using Minitab18 software. A fixed effect model with laboratories and methods as factors, using log10 transformed data and Tukey's test with 95% confidence interval to counteract multiple comparisons, was employed to determine statistical significance.

3. Results and discussion

In order to determine whether staining in Trucount tubes has an impact on proportions of IFN-γ^+, $CD4^+$ and $CD8^+$ T-cells, we performed parallel staining of positive, 15/272 (Fig. 1 G, H) and negative, SS570 (Fig. 1 I, J) reference material in FACS tubes versus BD Trucount tubes with or without a permeabilization step. The staining antibody panel consisted of anti-CD3 perCPcy5.5, anti-CD4 FITC, anti-CD8 APC and anti-IFN-γ PE. Cells were stained for surface markers, followed by cytokine staining in the presence or absence of permeabilization buffer. Representative gating strategy involved gating $CD4^+$ and $CD8^+$ T cells on a lymphocyte gate, as depicted in Fig. 1A–F. Samples were acquired and absolute cell counts were determined following sample dilution with PBS. The dilution eliminates the necessity for further wash steps, that could lead to cell loss and inaccuracies in cell count determination. Antibody specificity was confirmed by simultaneous staining experiments performed with PE-conjugated mouse IgG1 isotype control antibody (Fig. 1H, J). Results obtained from evaluation and analysis of a total of at least 2 individual biological replicates across 3 sites were compiled for statistical analysis. Each symbol represents results obtained from evaluation at a particular site. No significant difference was observed in the total percentage of $CD4^+$ T cells, $CD8^+$ T cells and IFN-γ positive $CD4^+$ and $CD8^+$ T cells when the staining procedure was performed either in FACS tubes or BD Trucount tube (Fig. 1G–J). Staining with isotype control established specificity of the staining protocol (Fig. 1H, J). These results indicate that BD Trucount tubes can be used for simplified, reliable enumeration of cytokine positive cells in cellular samples. Interestingly while cell scatter profiles and total cell percentages remain comparable upon permeabilization, cytokine signals were detectable in both $CD4^+$ and $CD8^+$ T cells under unpermeabilized conditions (1G). This intriguing observation could be attributable to effects of cell processing methodology. A similar effect of freeze-drying, causing membrane permeabilization of cellular reference material has been reported earlier by Wang et al.[6] In addition, an increased membrane permeability has been observed in Transfix-treated leukocyte subsets.[7] We consider that a combinatorial effect of fixation followed by freeze drying could explain the observed effect on membrane permeability in the reference materials used for the evaluation. Further, detailed ultrastructural analysis by transmission or scanning electron microscopy, will help gain insight into the features of membrane integrity in these cells.

We further observed that permeabilization in Trucount tubes had no impact on either the proportions of cytokine positive cells or bead counts and therefore the procedure can be utilized as a simple method for determination of absolute cell counts (Fig. 1). Since acquisition is performed following dilution of the sample in Trucount tubes, the requirement of washing steps that may lead to sample loss is avoided, greatly strengthening the precision of absolute cell number determinations.

This methodology was next used for determination of absolute cell counts of cytokine-positive cells in the positive and negative cellular reference material,15/272 and SS570 respectively. Optimal detection of cytokines is normally achieved by using a combination of paraformaldehyde fixation and subsequent detergent-based permeabilization of cell membranes with agents such as saponin following staining with cell surface markers for immunophenotyping as described above. In order to simplify the procedure further, we performed a comparative staining analysis using the conventional 2- step method (surface staining followed by permeabilization and cytokine staining) versus a 1-step method which involved the simultaneous addition of antibodies for surface markers and IFN-γ in permeabilization buffer. A 1-step approach would not only reduce the time for staining, but also significantly simplify the method. Results obtained from evaluation and analysis of 8 individual biological replicates across 3 sites for 15/272 are represented as individual plots (Fig. 2A–C), and a compilation is depicted in Fig. 2D. Total lymphocyte, $CD4^+$ and $CD8^+$ T cell counts were comparable for both positive, 15/272 and negative, SS570 reference materials ($p \geq 0.8$). Similarly, cytokine positive $CD4^+$ and $CD8^+$ T cells were comparable for the 1-step and 2-step methods for both the positive ($p \geq 1.0$) (Fig. 2A–D), and negative cellular reference material ($p \geq 0.9$), (Fig. 2E).

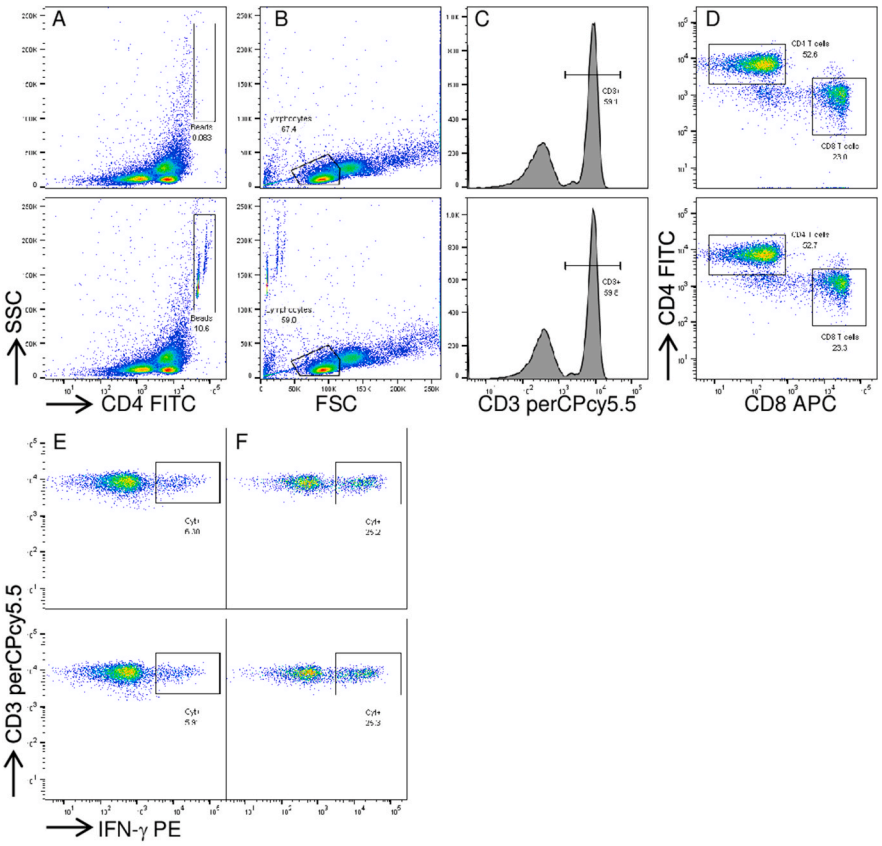
We conclude that precise flow cytometric enumeration of cytokine positive T cells in cellular samples can be performed reliably as a single step procedure in BD Trucount tubes. The cellular positive and negative reference materials included in the evaluation have been recently developed at NIBSC to provide a means for harmonization of enumeration of various cellular subsets in pre-clinical and clinical human samples. While the current study performed on freeze dried reference materials show intracellular cytokine signal in the absence of permeabilization step, it is anticipated that freshly cultured and fixed cells would require permeabilization to detect intracellular cytokine. However, our results confirm that the permeabilization step does not adversely impact surface marker staining and cell count determinations. Based on the results, we propose that inclusion of negative cellular reference materials in addition to isotype control in flow cytometric staining procedures would provide a robust determination of the negative baseline for staining methods. Though the current evaluation was based on IFN-γ as a readout for cytokine staining, the method has the potential to be applied for simultaneous measurement of multiple cytokines in clinical samples. We conclude that the method could be used for parallel analysis of several phenotypic and immunological parameters for a detailed functional characterization of T cell subsets, for potential use in clinical application as a comprehensive immune cell function test.

Credit author statement

Deepa Rajagopal: Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Linhua Tian:** Conceptualization; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Shiqiu Xiong:** Conceptualization; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Lili Wang:** Conceptualization; Formal analysis; Investigation; Methodology; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Jonathan Campbell:** Conceptualization; Formal analysis; Investigation; Methodology; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Luisa Saraiva:** Conceptualization; Investigation; Methodology; Resources; Validation; Visualization; Writing - review & editing. **Sandrine Vessillier:** Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence



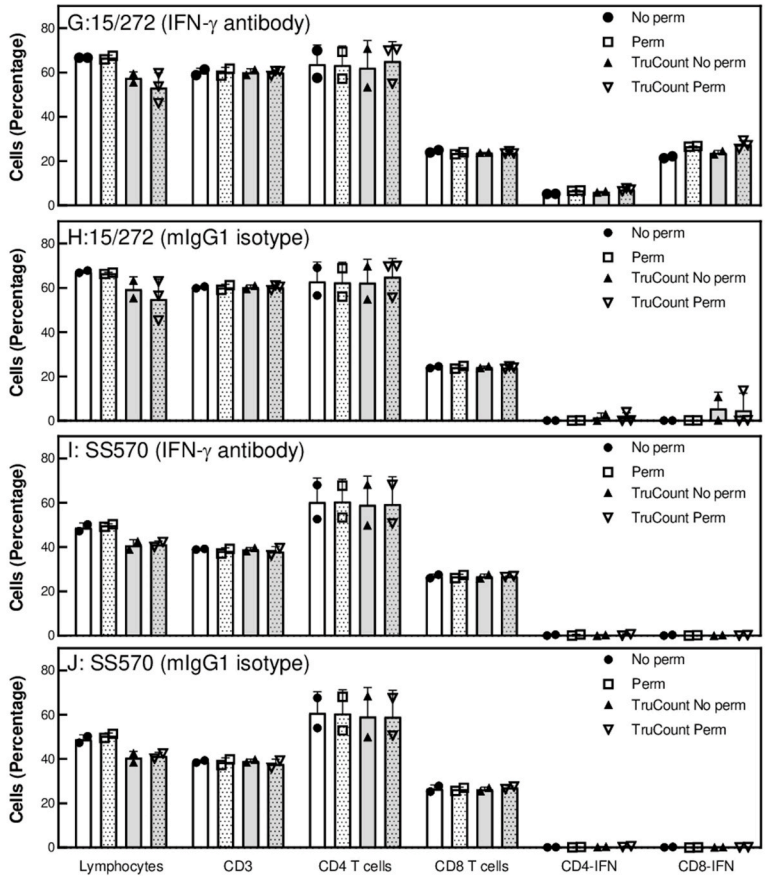


Fig. 1. Percentage of IFN-γ positive CD4$^+$ and CD8$^+$ T cells in FACS tube versus BD Trucount tubes. Cellular reference materials SS570 (unstimulated PBMC: 1I, 1J) and 15/272 (PMA/ionomycin stimulated PBMC: 1A- 1H) were stained with surface markers for CD3, CD4 and CD8 and then either permeabilized (1A- 1F and 1G- 1J: dotted bars and hollow symbols) or stained without permeabilization with anti-IFN-γ PE (1G, 1I: white and grey bars, filled symbols) in either polystyrene FACS tubes (1A- 1F: upper panel and 1G- 1J: white bars) or BD Trucount tubes (1A- 1F: lower panel and 1G- 1J: grey bars). Samples were diluted with PBS prior to acquisition. Cells were gated based on forward scatter versus FITC channel or forward scatter versus side scatter to determine bead (1A) or lymphocyte population (1B), respectively. CD4$^+$ (1D, 1E) and CD8$^+$ T cells (1D, 1F) were then gated as subsets of CD3$^+$ T cells (1C). IFN-γ positive gate (1E, 1F) was set based on staining with PE-mouse IgG1 isotype control.

1A-1F: Representative FACS plots and gating strategy for analysis of 15/272 reference material stained in FACS tube (upper panel) or BD Trucount tubes (lower panel) following cell permeabilization.

1G- 1H: Compiled cell percentages obtained from analysis of 15/272 (1G, 1H) and SS570 (1I, 1J) following staining with PE-IFN-γ antibody (1G, 1I) or PE-mouse IgG1 isotype control (1H, J). In all plots the dotted bars and hollow symbols indicate permeabilized samples. Data obtained from evaluation at each individual site are depicted as hollow symbols (permeabilized samples) or filled symbols (samples without permeabilization). Each symbol represents data from a particular site and error bars represent SD of values calculated from compilation of sample analysis for at least 2 biological replicates, between sites. Statistical analysis was performed on the compiled data, using Minitab18 software as described above and difference of the means between staining performed in FACS tubes versus staining performed in BD Trucount tubes was found to be statistically insignificant.

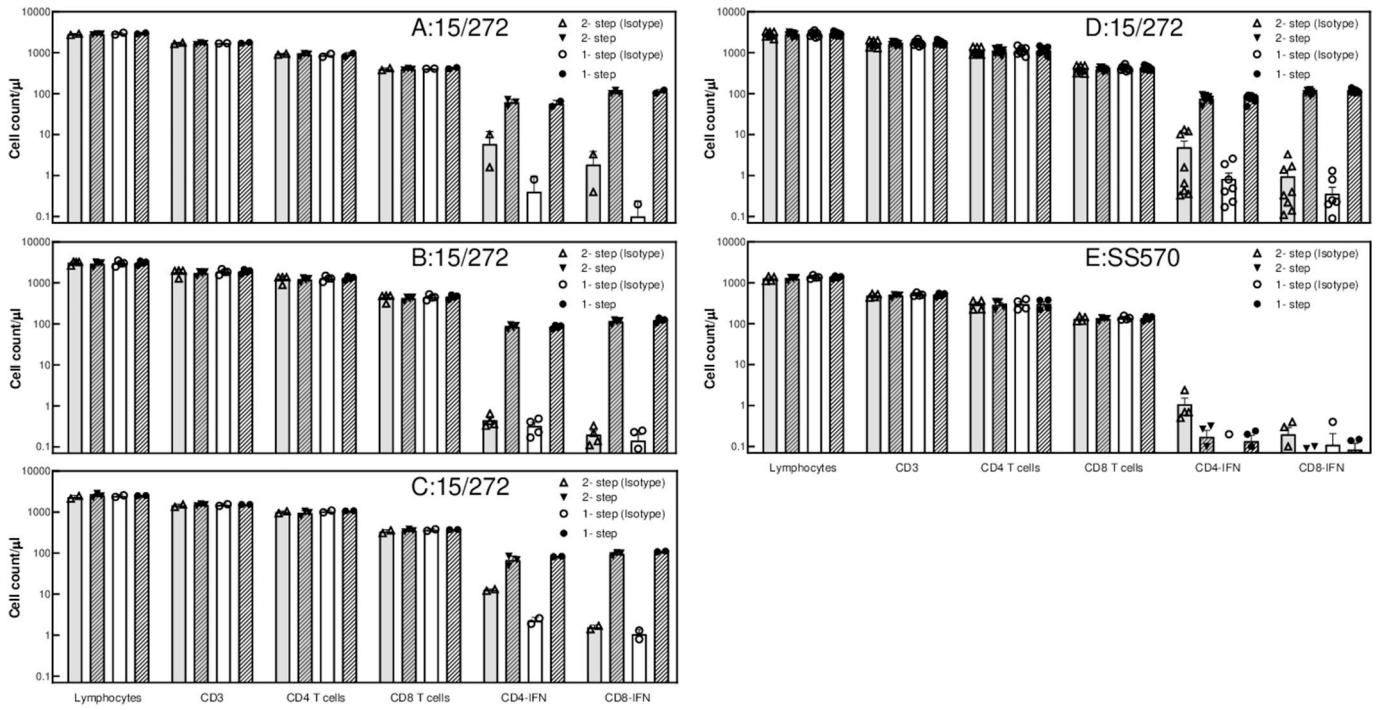


Fig. 2. Enumeration of IFN-γ positive CD4$^+$ and CD8$^+$ T cells in BD Trucount tubes using a 1-step or 2-step method. Cellular reference materials 15/272 (PMA/ionomycin stimulated PBMC: 2A-D) and SS570 (unstimulated PBMC: 2E) and were stained using either a 1-step (white bars) or 2-step method (grey bars). Samples were stained as described in the text. Absolute cell counts were determined for samples stained with IFN-γ antibody (striped bars and filled symbols) or isotype controls (plain bars and hollow symbols) following permeabilization as a 1-step or 2-step protocol. Fig. 2A–C represent data obtained from staining at each individual site and each symbol represents an individual evaluation. Compiled results for values obtained from all the sites for 15/272 (2D) and SS570 (2E) are depicted. Error bars represent SEM of values obtained from sample analysis of a total of at least 8 individual biological replicates across 3 sites for 15/272 and 4 individual biological replicates across 2 sites for SS570. Statistical analysis was performed using Minitab18 software as described above and difference of the means between staining performed by 1-step versus the 2-step method for 15/272 reference material stained with IFN-γ antibody was found to be statistically insignificant.

the work reported in this paper.

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