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A general method for bead-enhanced quantitation by flow cytometry

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Abstract

Flow cytometry provides accurate relative cellular quantitation (percent abundance) of cells from diverse samples, but technical limitations of most flow cytometers preclude accurate absolute quantitation. Several quantitation standards are now commercially available which, when added to samples, permit absolute quantitation of CD4⁺ T cells. However, these reagents are limited by their cost, technical complexity, requirement for additional software and/or limited applicability. Moreover, few studies have validated the use of such reagents in complex biological samples, especially for quantitation of non-T cells. Here we show that addition to samples of known quantities of polystyrene fluorescence standardization beads permits accurate quantitation of CD4⁺ T cells from complex cell samples. This procedure, here termed single bead-enhanced cytofluorimetry (SBEC), was equally capable of enumerating eosinophils as well as subcellular fragments of apoptotic cells, moieties with very different optical and fluorescent characteristics. Relative to other proprietary products, SBEC is simple, inexpensive and requires no special software, suggesting that the method is suitable for the routine quantitation of most cells and other particles by flow cytometry.

Keywords

Flow cytometry; Cell quantitation; T cell; Eosinophil; Apoptotic body

1. Introduction

Since its inception more than 30 years ago, flow cytometry has become an essential tool for investigators requiring high throughput analysis of single cells. Initial studies with early flow cytometer instruments focused on analysis of DNA for determination of neoplasia and cell-associated proteinases (Dolbeare and Smith, 1977; Gray et al., 1977; Jensen, 1977). However, the advent of monoclonal antibodies permitted the detection and rapid isolation of discrete lymphocyte subsets, propelling the technology firmly into the domain of immunology, where

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it remains one of the principal tools for the analysis of leukocytes (Hoffman et al., 1980). In addition to cell surface analysis, numerous cellular functions can now be probed with the flow cytometer and the technology is standard in the clinical diagnostic laboratory.

Although theoretically capable of providing absolute quantitation of cells, in practice the flow cytometer provides only relative quantitation (percent abundance) and not true counts. This is because typically only a fraction of the specimen (i.e., sample volume) is sampled by the forward scatter-activated data acquisition system. Moreover, the type of cell and its abundance likely affect the accuracy of “counts”, with further compromises at higher data acquisition speeds. Thus, because individual cytometer events only estimate the counts of cells based on light scatter, not volume, characteristics, absolute quantitation of cells using conventional flow cytometry may be unreliable unless the cytometer counts are adjusted relative to a standard of pre-determined concentration.

Relative cellular quantitation (percent abundance) is clearly adequate in many circumstances. However, such data can be misleading, especially when assessing the change in a population of cells over different experimental or clinical conditions. For example, the relative abundance of a specific subset of leukocyte in a complex biological sample may not change over different conditions, yet increase dramatically in absolute quantity. This occurs when compensatory changes in the abundance of other cells masks the change in relative abundance that would otherwise have occurred within the population of interest. It is also possible for the absolute quantity to actually change in opposition to relative abundance. Recently, we reported that particles from maternal plasma, which may be derived from apoptotic cells, stained positive for acridine orange (AO), a nucleic acid dye (Bischoff et al., 2004). We sorted the AO+ apoptotic bodies and PCR amplified male specific Y sequences, demonstrating that they contain male fetal DNA (Bischoff et al., 2004). For such analysis of subcellular apoptotic bodies, accurate quantitation is needed to determine the optimum amount of staining reagent required. Thus, these examples alone suggest that flow cytometers should routinely offer the option for absolute quantitation.

Recently, several proprietary technologies have emerged that permit absolute quantitation of CD4+ T cells using the flow cytometer (Table 1). Several products offer cellular quantitation based on the addition to samples of known quantities of dual fluorescent standardization beads (DBEC) (Barnett et al., 1996; Barnett et al., 1999). However, these products are limited by their cost and a reagent repertoire that permits analysis of only selected subsets of cells, especially T cells. Moreover, although the technology is relatively straightforward, additional computer software is suggested to facilitate the computations required (TruCOUNT, BD Biosciences), which further adds to the overall cost.

An alternative method for quantitation of cells is to use a flow cytometer pre-calibrated to express cytometer events as absolute cell counts (CD4 Easy Count and CyFlow SL; Partec, Inc.). Alternatively, the flow rate of any flow cytometer may be calibrated and the derived calibration factor used to convert cytometer events into absolute counts (Storie et al., 2003). The calibration procedure is more technically complex compared with the addition of fluorescent beads, at least in part due to the need to account for the usually different viscosities of sample and reference standard (Walker et al., 2006). Nonetheless, flow rate calibration offers the advantage that relatively expensive standardization beads need not be routinely added to samples. Flow rate calibration has become somewhat simplified by the use of stabilized biological standards with the same viscosity as samples, but the technology is best suited to clinical flow cytometers that analyze only a single sample of defined viscosity, usually blood (Walker et al., 2006).

For the typical research flow cytometer, issues such as cost and widely varying sample compositions conspire to limit the applicability of proprietary fluorescent counting beads and flow rate calibration. Moreover, the applicability of absolute cellular quantitation by flow cytometry to diverse cell and particle populations that are likely to be encountered in research settings has not been adequately assessed. For this study, we sought to develop an inexpensive, reliable method for absolute quantitation by flow cytometry applicable to samples with widely varying cellular and particulate compositions. The described method, which we term single bead-enhanced cytofluorometry (SBEC) utilizes inexpensive reagents that are readily available, requires no special software or calibration and can be applied to any flow cytometer.

2. Methods

2.1. Reagents

Fluorescent beads (Flow-Check™ Fluorospheres, 10 µm average diameter, Beckman Coulter, Miami Lakes, FL) were washed twice in double filtered (0.2 µm) phosphate buffered saline (PBS) by centrifugation and re-suspended in PBS at a concentration of 1650 beads/µl (as determined by a haemocytometer). Polypropylene tubes were used throughout all experiments to minimize adherence of counting beads and cells.

2.2. Cell culture and induction of cell death

A human trophoblastic cell line, JEG-3 (American Type Culture Collection, Rockville, MD), was cultured in MEM supplemented with 10% fetal calf serum, 20 U/ml penicillin/streptomycin, 5 mM L-glutamine, 2.5 mM sodium pyruvate, 0.025 mM HEPES, and 0.25 mM non-essential amino acids (Gibco, Carlsbad, CA). The cultures were maintained at 37 °C in an atmosphere of 5% CO₂. Cells were harvested, cultured at a concentration of 1.3×10⁵ cells/ml and treated with 50 µM rotenone, an hypoxia mimicking reagent (Sigma, St. Louis, MO) for 48 h.

2.3. Preparation of apoptotic bodies

Apoptotic bodies were collected by transferring supernatant from the adherent cells to 14×95 mm polyalomer centrifugation tubes (Beckman # 331374, Fullerton, CA, USA), followed by two consecutive ultracentrifugations at 28,200 rpm (100,000 ×g) and 40,800 rpm (200,000 ×g) for 1 h at 4 °C (SW 40 Ti swinging-bucket rotor and SW50.1 swinging-bucket rotor in a Beckman Coulter L8-M, Class H, ultracentrifuge) and apoptotic bodies were re-suspended in 3.5 ml PBS immediately prior to enumeration.

2.4. Flow cytometric analysis of JEG-3 cells

Cells were harvested using trypsin with 0.25% EDTA (Gibco, Carlsbad, CA), washed in 10% MEM and re-suspended in PBS at a concentration of 4×10⁶ cells per ml. To demonstrate differences in Forward Scatter (FS) between beads and cells, a standard concentration (as determined by hemocytometer) of 7 µm beads (FLOW-CHECK Fluorospheres, Beckman Coulter, Miami Lakes, FL) in 50 µl was added to 200 µl PBS plus 250 µl cells for a total volume of 500 µl. Ten thousand beads were acquired on an EPICS XL-MCL Flow Cytometer (Beckman Coulter, Miami Lakes, FL).

2.5. Mice

Female BALB/c mice were kept under specific pathogen-free conditions at the Baylor College of Medicine Transgenic Mouse Facility. All mice were used within 4–8 weeks of age according to Federal and Institutional guidelines.

2.6. CFSE labeling of CD4⁺ murine splenocytes

Mice were euthanized with pentobarbital followed by cervical dislocation. Spleens were removed and mechanically dispersed through 40 µm nylon cell strainers (BD Biosciences, USA) to obtain single-cell suspensions. CD4⁺ splenocytes were positively selected by immunomagnetic column cell sorting (MACS, CD4 MicroBeads, Miltenyi, USA) yielding >95% CD4⁺ T cells. These cells were then labeled with carboxyfluorescein-diacetate-succinimidyl-ester according to the manufacturers directions (CFSE, CellTrace, Molecular Probes, USA). CFSE-labeled CD4⁺ T cells were enumerated by a haemocytometer and used for subsequent experiments.

2.7. Murine airway inflammation

Allergic airway allergic inflammation was induced in mice as described (Kheradmand et al., 2002). Briefly, mice were anesthetized with isoflurane vapor (IsoFlo, Abbot-USA) and allowed to inhale 50 µL of a PBS suspension containing 5 µg of *Aspergillus oryzae* proteinase (Sigma) and 25 µg of ovalbumin (Sigma). Intranasal challenges were performed every 3 days for a total of 4 challenges. Mice were euthanized with pentobarbital followed by exsanguination 24 h after the last intranasal challenge. Single-cell suspensions were prepared from spleens and lungs and used in subsequent experiments.

2.8. Adoptive T cell transfer

10×10⁶ CD4⁺ CFSE labeled cells obtained from spleens of allergen-challenged mice were injected intraperitoneally into naïve wild type mice. Reconstituted mice were then challenged intranasally with allergen every 24 h for three consecutive days. Mice were euthanized 24 h after the third intranasal challenge, single-cell suspensions were prepared from lungs and CFSE labeled cells were enumerated by flow cytometry.

2.9. Collection and surface labeling of whole lung cells

Lungs were removed, dissected free of lymph node and thymic tissue and dispersed through 40 µm nylon filters to obtain single-cell suspensions. Red blood cells (RBC) were lysed with ACK lysis buffer and the remaining leukocytes were centrifuged once (1200 rpm× 5 min) and re-suspended in 0.5 ml of labeling buffer. 100 µl aliquots (1/5 volume) of these cells were used for cellular quantitation. Surface fluorescence staining was performed by adding the appropriate fluorochrome conjugated antibodies (CD3 PE, CD4 PE-Cy5; or MHC-II FITC, SiglecF PE; 1 µg/10⁶ cells, all from BD Biosciences, Palo Alto, CA) to iced cells followed by 20 min incubation. Cells were washed twice with labeling buffer and re-suspended in a volume of 500 µl.

2.10. Quantitation by haemocytometer/histology

The total number of whole lung leukocytes was determined by conventional haemocytometer. Approximately 10⁵ whole lung cells were placed on glass slides by a cytocentrifuge, fixed and stained with hematoxylin and eosin for visualization by light microscopy. Eosinophils were identified by characteristic morphology and staining characteristics and their percent abundance out of 500 total cells was determined.

2.11. Quantitation of cells and apoptotic bodies by flow cytometry

To determine the number of apoptotic bodies or cells, a standard concentration of 10 µm beads in 50 µl was added to either 450 µl PBS (control counts) or 350 µl PBS plus 100 µl apoptotic bodies or cells (Total Count) for a total volume of 500 µl (1/5 dilution). Five thousand gated beads were counted via fluorescent channel 4 (FL4) per 500 µl sample on an EPICS XL-MCL flow cytometer (Beckman Coulter, Miami Lakes, FL). To rule out intrinsic apoptotic body

contamination induced by the beads, a control bead count was determined prior to the apoptotic body plus beads count (total body count). The specific apoptotic body count in the 500 μ l sample was determined by subtracting the control bead count from the total body count as described below:

$$\begin{aligned} &\text{Specific apoptotic body count} \\ &= (\text{total body count}) - (\text{control bead count}) \end{aligned} \quad (1)$$

The cell or apoptotic body concentration in the 500 μ l sample was then determined as follows:

$$\begin{aligned} &\text{Cell/apoptotic body concentration} \\ &= [(\text{specific apoptotic body or cell count}) \\ &\quad \times (\text{bead concentration})] / (\text{bead count}) \end{aligned} \quad (2)$$

The total number of cells or apoptotic bodies from the original supernatant was determined by the following equation:

$$\begin{aligned} &\text{Total \#cells/Apoptotic bodies} \\ &= (1/5 \text{ Dilution factor}) \\ &\quad \times (\text{Cell or apoptotic body concentration}) \\ &\quad \times (\text{re - suspension volume}) \end{aligned} \quad (3)$$

2.12. Statistical analysis

Data are presented as means $\pm$ standard error of means (SEM) and are representative of two or three independent experiments that used at least four mice in each group. Statistical differences were considered significant if $P \leq 0.05$ using the Kruskal–Wallis test for multiple group comparisons.

3. Results

3.1. Physical and optical characteristics of fluorescent beads and labeled cells

Washed standardization beads were analyzed by flow cytometry to evaluate their size, purity and fluorescence. By forward and side light scatter characteristics, beads were highly uniform in size and easily distinguished from cells and microparticles in even the complex lung samples that include lymphocytes, granulocytes and macrophages (Fig. 1A, B). Peak bead fluorescence was very broad, ranging from 525 to 700 nm when excited at 488 nm, permitting their detection under a broad range of analytical conditions (Fig. 1C). CFSE labeled cells were readily detected at an emission of 517 nm when excited at 488 nm (Fig. 2).

3.2. Quantitation of CFSE+ T helper cells

To determine if CFSE labeled cells could be reliably quantitated by SBEC, we added known amounts of CFSE-labeled CD4+ T cells (>95%) to lung single-cell suspensions ($n=15$ duplicates). Despite somewhat variable forward and side scatter characteristics (Fig. 2A), the purified, labeled CD4+ T cells showed uniform fluorescence (Fig. 2B). After the addition of CFSE+ T cells to lung cells, the number of CFSE+ cells in each sample was calculated using Eqs. (2) and (3). The absolute and derived quantities of CFSE+ cells were then plotted to understand their relationship (Fig. 2C). By linear regression analysis, this comparison revealed

that over a broad linear range of cells, SBEC provided extremely accurate cellular quantitation ($r^2 = 0.96$, $P < 0.0001$).

SBEC was further evaluated in a more realistic experiment in which adoptively transferred CD4⁺ CFSE⁺ T cells were enumerated from mice that were intranasally challenged with either allergen or vehicle. Our prior studies and those of others have shown that allergen challenge in this experimental context results in recruitment to lung of far greater numbers of adoptively transferred cells compared to vehicle-challenged animals (Corry et al., 1998; Mathew et al., 2002). Despite three days of in vivo allergen challenge, the CFSE⁺ T cells were readily detected from whole lung homogenate cells (Fig. 3A, B). Data from nine CFSE-reconstituted mice are summarized in Table 2, showing that nearly eightfold more CD4⁺ CFSE⁺ T cells were recruited to the lung after allergen challenge compared to vehicle-challenged animals, a magnitude of difference that far exceeded that for percent abundance (1.54).

3.3. Characterization and quantitation of lung eosinophils

The preceding studies demonstrated that SBEC was suitable for enumerating relatively small and uniform CD4⁺ T cells from complex whole organ samples. These data indicated that any differences in physical and optical characteristics between lung CD4⁺ T cells and the beads had a negligible impact on this quantitative method. To assess the utility of SBEC for enumeration of a cell with markedly different physical and optical characteristics (Fig. 4A), we quantitated from allergen-challenged mouse lung the absolute number of eosinophils using SBEC and an independent method based on enumeration of total lung leukocytes by haemocytometer and determining the fraction of these cells that are eosinophils based on histologic staining.

Although a variety of cell surface markers may be used to identify mouse eosinophils (De Heer et al., 2004), we found that the combination of an anti-MHC class II antibody and SiglecF (Zhang et al., 2004) provided the best discrimination between eosinophils and other lung cells with similar forward and side light scatter characteristics such as neutrophils and especially macrophages. Based on typical staining characteristics of sorted cells from allergen-challenged mouse lung, MHCII[−]/SiglecF⁺ cells were >99% eosinophils, whereas MHCII⁺/SiglecF[−] cells were <5% eosinophils, the majority being macrophages (Fig. 4A, B).

Using the haemocytometer and SBEC methods, we quantitated MHCII[−]/SiglecF⁺ eosinophils from whole lungs of mice after each of 4 intranasal challenges with *A. oryzae* allergen. Regardless of the method used, the number of eosinophils detected increased with each subsequent challenge, ultimately encompassing a range of 10^4 to 6×10^6 eosinophils (Fig. 4C, Table 3). In comparing the two enumeration methods, there was no statistical difference between any of the simultaneously obtained counts ($P > 0.05$ for all points; Fig. 4C). At no point did the counts obtained at the same time differ by more than a factor of 2.6, with most counts differing by less than a factor of two (Table 3). These findings demonstrate that, across a wide dynamic range, SBEC is an accurate method for quantitating eosinophils from complex cell samples.

3.4. Quantitation of apoptotic bodies from a trophoblastic cell line

To enumerate apoptotic bodies generated in vitro from a trophoblastic cell line, we applied SBEC. Apoptotic bodies were collected from the supernatant of four million JEG-3 cells undergoing hypoxia-induced apoptosis for 48 h and counted using an appropriate concentration of beads (as determined by previous flow assays). Our data show a clear distinction between apoptotic bodies (67%), beads (22%) and cells (4%) as shown by light scatter and density plots (Fig. 5A, B, panels 1 and 2). Using Eqs. (1)–(3), we calculated that hypoxia-induced apoptosis produced 37.7×10^6 apoptotic bodies or 9 apoptotic bodies per cell as follows:

500 μ l sample total Apo-Body count = 66,444

500 μ l sample control bead count = 1067

500 μ l sample specific Apo-Body count
 $= (66,444) - (1067) = 65,377$ Apoptotic bodies

Apo-Body concentration of the 500 μ l sample was determined as follows: 50 μ l of beads were added (at a concentration of 1650 beads/ μ l) to 400 μ l or 450 μ l of PBS, with or without Apo-Bodies, respectively, for a total volume of 500 μ l and a final bead concentration of 165 beads/ μ l.

5000 beads were counted by the flow cytometer.

Apo – Body concentration (500 μ l sample)
 $= [(65,377 \text{ Apo-Bodies counted by cytometer})$
 $\times (165 \text{ beads}/\mu\text{l})] / (5000 \text{ beads})$
 $= 2157 \text{ Apo – Bodies}/\mu\text{l}$

The total number of apoptotic bodies in the supernatant (Eq. (3)):

Dilution factor = 5

Apo-Body concentration (500 μ l sample)
 $= 2157 \text{ Apoptotic bodies}/\mu\text{l}$

Re-suspension volume = 3500 μ l

Total # of apoptotic bodies in the supernatant
 $= (5) \times (2157 \text{ Apoptotic bodies}/\mu\text{l}) \times (3500 \mu\text{l})$
 $= 37,747,500$

Finally, to determine the number of apoptotic bodies produced per starting cell (Total # of bodies in the supernatant) / (Starting # of cells):

Total # of Bodies in the supernatant
 $= (5) \times (2157 \text{ Apoptotic bodies}/\mu\text{l}) \times (3500 \mu\text{l})$
 $= 37,747,500$

Starting # of cells = 4,000,000

Bodies produced per starting cell
 $= (37,747,500) / (4,000,000)$
 $= 9 \text{ Bodies/starting cell}$

4. Discussion

We have demonstrated a simple method for absolute quantitation of diverse cells and particles from complex samples using flow cytometry that requires the addition of only a single type of fluorescent bead. As expected based on studies using dual bead-based methods, CD4+ T cells were readily enumerated even from complex samples. However, eosinophils, which exhibit less uniformity in size and have very different optical properties, were enumerated with equivalent accuracy by SBEC across a broad range of relative abundance. Apoptotic bodies were also successfully counted, which are much smaller than cells and possess unique light scatter properties. Our findings thus broaden the applicability of flow cytometry-based cellular quantitation using added standards, but indicate that only a single fluorescent bead is required. Our method of SBEC provides additional advantages compared to other commercially available reagents and techniques.

A notable finding from this study is derived from the comparison between percent abundance and absolute quantity of CD4+ T cells from the same sample (Table 2). Although as expected T cell percent increased in lung samples containing inflammatory cells, the small magnitude of the change was not statistically different compared to the naïve lungs ($P>0.05$). In contrast, absolute quantitation of the same cells disclosed the full magnitude of the change—more than 7-fold, a highly significant difference ($P<0.01$). In some individual mice challenged under these experimental conditions, we observed that the percent abundance of CD4+ T cells actually decreased following allergen challenge, whereas absolute quantity invariably increased. The reason for the discrepancies when comparing these two methods of quantitation is that apparent increases in one cell population, i.e., Th2 cells, can be masked by much greater increases in other cell types, i.e., eosinophils, when assessing only relative abundance. In contrast, even small absolute increases in cell abundance are readily determined using methods such as BEC (single or dual bead) because the results do not depend on the abundance of other cell types. Because potentially confounding shifts in multiple cell populations often cannot be foreseen in complex samples, this example suggests that in many instances absolute quantitation should be preferred over relative quantitation.

In addition to CD4+ T cells, SBEC accurately quantified eosinophils, cells with distinct size and optical characteristics. SBEC also quantified apoptotic bodies, which differ further in terms of their physical properties from both T cells and eosinophils. However, an independent procedure was not available to establish the accuracy of SBEC for quantifying apoptotic bodies. Specifically, these subcellular structures are too small to be quantified by the haemocytometer and technical issues precluded calibrating our flow cytometer for volumetric enumeration. Nonetheless, because quantitation of apoptotic bodies is based on the same principles that provide accurate enumeration of T cells and eosinophils, it is reasonable to assume that the apoptotic body counts were equally accurate. Regardless of its reliability in this instance, it is noteworthy that SBEC was the *only* method available to us for quantifying apoptotic bodies. Thus, in distinction from other proprietary products and methods, we have shown that SBEC is suitable for quantitation not just of CD4+ T cells and closely related cells, but also of diverse cells and particles.

The described method of SBEC has other notable advantages. Because the flow cytometer provides extremely accurate data on relative abundance, the absolute quantity of cells from any sample can be derived knowing the total number of cells in the sample multiplied by the relative abundance. However, this relatively time consuming approach to cellular quantitation becomes less practical with increasing numbers of samples. The single step method described obviates the need for independent enumeration of total cells in cytometer samples, reducing the chances for technical error without sacrificing accuracy.

In addition, commercial standardization bead-based methods are relatively expensive, adding a substantial “tariff” to each sample analyzed (Table 1). These reagents include beads with two different sedimentation rates, which aids in correcting for varying bead/cell ratios during data acquisition. Although theoretically useful, correcting for differential sedimentation in this way adds substantially to the overall cost of the technique; furthermore, our studies indicate that such correction is not necessary as long as care is taken to vortex samples prior to data acquisition. Using SBEC, we estimate the minimum added cost to each sample analyzed to be \$0.12—a negligible increase in cost, especially if flow cytometer time must be purchased (Table 1).

Finally, a special software is not required to analyze samples. Calculation of cell quantities using Eqs. (1)–(3) is facilitated, especially when analyzing large numbers of samples, by using spreadsheet programs that accompany word processing software readily available in most laboratories.

Users of SBEC should be aware of the technical issues that may affect absolute cell counts. Measurements of adoptively transferred lung CD4⁺ T cells and lung eosinophils required the removal of RBC by lysis from whole lung homogenate cells. Multiple RBC lysis methods have been shown to reduce recovery of leukocytes from whole blood, suggesting that the results in Fig. 4 and Tables 2 and 3) may underestimate the total number of recovered cells (Greve et al., 2003; Greve et al., 2006). Although our studies did not involve whole blood and our RBC lysis protocol has been optimized to ensure minimal disruption of leukocytes, the possibility that SBEC might underestimate absolute cell counts due to an RBC lysis effect should be considered. In addition, high-speed centrifugation has the potential to disrupt apoptotic bodies and affect their enumeration. Our own studies suggest that a modest degree of apoptotic body disruption is to be expected, resulting in an overestimation of apoptotic body counts of 20–30% (A. Orozco, D. Lewis, data not shown).

In summary, absolute quantitation of diverse cells and particles from complex samples by flow cytometry is readily and inexpensively performed with the addition of a single reagent. Our findings are in agreement with others that indicate that in many instances, absolute quantitation of cells should replace relative abundance measurements that are currently the standard output of most flow cytometers (Barnett et al., 1999).

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Fig. 1. Identification of fluorescent polystyrene beads by flow cytometry. Beads are distinguished from lung cells in FS versus SS plots in density (A) and pseudo-color (B) plots. The number of beads analyzed is obtained from the corresponding bead histogram gate for fluorescence channel 3 (FL3; C).

Fig. 2.

CFSE labeling and quantitation of CD4⁺ T cells. CD4⁺ T cells were positively selected from splenocytes (A) and labeled with CFSE (B). CFSE-labeled T cells were then enumerated and added in increasing defined quantities to a mixed population of lung cells derived from allergen-challenged animals. Linear regression analysis was then performed to compare manual versus SBEC enumeration of CFSE⁺ CD4⁺ T cells (C). Numbers represent absolute number of CFSE⁺ cells per 1 ml volume of mixed lung cell suspension. $r^2 = 0.96$; $P < 0.0001$.

Fig. 3.

Identification of lung cell subpopulations. Ovalbumin-specific CFSE+ CD4+ T cells were transferred intraperitoneally to naïve syngeneic mice that received daily intranasal challenge with ovalbumin for three days. Lungs were removed and single cells were isolated. A. Forward versus side light scatter plot showing lymphocyte and granulocyte gates as well as the added fluorescent microbeads. B. Identification of CFSE+ CD4+ T cells from the lymphocyte gate (red square). C. Identification of SiglecF+MHCII- eosinophils from the granulocyte gate (red square). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Representative MHCII-SiglecF+ (A) and MHCII+SiglecF+ (B) cells isolated by flow cytometry. After fluorescent staining and appropriate gating, the indicated cell populations were isolated by FACS, counted and centrifuged onto glass slides for identification by histological staining. (C) Comparison of two methods for the enumeration of lung eosinophils. Wild type mice were challenged intranasally every three days with ovalbumin/*A. oryzae* allergen for a total of 4 challenges. 12–18 h following each allergen challenge, lungs were removed, single cells prepared and eosinophils quantitated using both haemacytometer and SBEC methods. Data are representative of two independent experiments.

Fig. 5.

Quantitation of apoptotic bodies using fluorescent beads. In this example, four million JEG-3 cells were treated with 50 μ M Rotenone. After 48 h, apoptotic bodies were collected and concentrated. A known quantity of fluorescent beads was added to samples which were then analyzed by flow cytometry. (A) and (B) were analyzed on different FS versus SSLOG settings to compare beads with both apoptotic bodies and JEG-3 cells. Apoptotic bodies (67%), which display the lowest Forward Scatter (FS) and variable Side Scatter (SS LOG), are clearly distinguished from both fluorescent beads (22%) and JEG-3 cells (4%), which display both high FS and SS as shown in light scatter and density plots (panels 1 and 2).

Table 1

Proprietary reagents for quantitation of cells by flow cytometry

Product Name	Cells enumerated	Minimum additional cost/sample ^a	Counting method	Comments
TruCOUNT tubes, BD Biosciences	CD4+ T cells	\$8.67	Dual fluorescent BEC	Additional software for computations available
Perfect Count; Caltag Counting Beads; others	CD4+/CD8+ T cells, CD34+ hematopoietic progenitor cells	≈\$1.65	Dual fluorescent BEC	
CD4 Easy Count	Human CD4+ T cells	\$1.75	Volumetric absolute counting of fluorescently labeled cells	Specially calibrated proprietary flow cytometer
Flow-Check™ Fluorospheres, Beckman Coulter	T cells, eosinophils, others ^b	\$0.12	Single fluorescent BEC	

^aManufacturers suggested retail price as of 5/2006.^bSee text; applicability to unique conditions should be verified using an independent quantitative method, where possible.

Table 2

Percent abundance versus absolute quantity of CFSE-labeled CD4+ T cells in lungs of naïve (Ag-) and allergen-challenged (Ag+) mice previously reconstituted with CFSE-labeled CD4+ T cells

	Ag+	Ag-	Fold difference
% CFSE+ CD4+ cells	0.20±0.045	0.13±0.089	1.54
No. of CFSE+ CD4+ cells/lung	7385±1327	944±700	7.8

Table 3
Summary of the two methods for quantitating eosinophils from lungs of allergen-challenged mice

No. of allergen challenges	Haemocytometer		SBEC		Fold difference, means
	Mean	SEM	Mean	SEM	
0	14,041	4024	27,388	6929	1.95
1	35,020	13,015	46,740	11,174	1.33
2	386,983	12,579	147,543	24,706	2.62
3	1,859,667	534,606	3,125,946	973,403	1.68
4	6,811,100	3,064,416	5,157,090	2,353,308	1.32

Representative of 3 independent experiments.