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Human CD4$^+$ Lymphocytes for Antigen Quantification: Characterization Using Conventional Flow Cytometry and Mass Cytometry

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• Abstract

To transform the linear fluorescence intensity scale obtained with fluorescent microspheres to an antibody bound per cell (ABC) scale, a biological cell reference material is needed. Optimally, this material should have a reproducible and tight ABC value for the expression of a known clinical reference biomarker. In this study, we characterized commercially available cryopreserved peripheral blood mononuclear cells (PBMCs) and two lyophilized PBMC preparations, Cyto-Trol and PBMC–National Institute for Biological Standard and Control (NIBSC) relative to freshly prepared PBMC and whole blood samples. It was found that the ABC values for CD4 expression on cryopreserved PBMC were consistent with those of freshly obtained PBMC and whole blood samples. By comparison, the ABC value for CD4 expression on Cyto-Trol is lower and the value on PBMC–NIBSC is much lower than those of freshly prepared cell samples using both conventional flow cytometry and CyTOF^{TM} mass cytometry. By performing simultaneous surface and intracellular staining measurements on these two cell samples, we found that both cell membranes are mostly intact. Moreover, CD4$^+$ cell diameters from both lyophilized cell preparations are smaller than those of PBMC and whole blood. This could result in steric interference in antibody binding to the lyophilized cells. Further investigation of the fixation effect on the detected CD4 expression suggests that the very low ABC value obtained for CD4$^+$ cells from lyophilized PBMC–NIBSC is largely due to paraformaldehyde fixation; this significantly decreases available antibody binding sites. This study provides confirmation that the results obtained from the newly developed mass cytometry are directly comparable to the results from conventional flow cytometry when both methods are standardized using the same ABC approach.

• Key terms

quantitative multiparameter flow cytometry; CyTOF^{TM} mass cytometry; antibody bound per cell; cryopreserved peripheral blood mononuclear cells; whole blood; lyophilized peripheral blood mononuclear cell; Cyto-Trol; CD4 expression; cell membrane intactness; cell diameter; fixation effect

Introduction

Numerous methods for quantitative fluorescence calibration (QFC) have been developed to quantify receptor expression on various human cell populations because of the importance of the quantitation in diagnostic flow cytometry assays (1–3). However, the results from the use of these different QFC methods vary considerably in the literature (4–6). Though the ultimate goal of quantification is to measure the number of antigens or ligand binding sites on a cell, this task is performed practically by measuring the number of antibodies bound per cell (ABC). Out of the five QFC methods described earlier (7), the use of QuantiBrite PE calibration beads combined with unimolar anti-CD4 PE conjugates for quantitation of surface antigen expressions may appear more straightforward and less prone to binding related lim-

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itations, issues such as the lot to lot quality of unimolar PE antibody conjugates (8), and the availability of unimolar conjugates for surface markers other than CD4, CD20, CD38, and HLA-DR are not yet resolved. The calibration scheme that is used only deals with the quantitation associated with the PE fluorescence channel (FL2).

Alternatively, the use of CD4 on normal human T lymphocytes as a biological calibrator makes the quantitative immunophenotyping approach feasible (5,9). This biological calibrator with a known number of antibody binding sites for CD4 receptors (7,10–12) can be used to translate the linear fluorescence intensity scale obtained with fluorescent microspheres to an ABC scale (13). With this approach, it is highly desirable that a single clone of the antibody amenable to labeling with different types of fluorophores associated with various fluorescence channels is used for the scale conversion. Assuming that different antibodies against different antigens have the same average fluorescence per antibody, a direct measure of ABC can be obtained. A basic factor to consider is whether the effective number of fluorophores per antibody (effective F/P) is the same for the calibration antibody (CD4 antibody) and test antibodies. The ideal situation would be to use antibody conjugates that consist of only one fluorophore coupled to the antibody at a location that did not interfere with the ability of the antibody to bind to the antigen. It is also equally important that both the calibration antibody and any test antibodies should be purified to exclude unconjugated antibodies.

A novel technology of single cell analysis, mass cytometry, was included in this study. Mass cytometry is a real-time quantitative analytical technique whereby cells or particles are individually introduced into an Inductively Coupled Plasma Mass Spectrometer (ICP-MS), and each resultant ion cloud produced by a single cell is analyzed multiple times by time-of-flight mass spectrometry. The technology is premised on the use of elements, or stable isotopes, covalently attached to antibodies, as labels instead of fluorophores. The CyTOF™ instrument (DVS Sciences) has the ability to perform multiparameter assays of high order (up to 100) in single cells without the need for mathematical correction of signal overlap, and with large dynamic range both for a given target biomarker and between different biomarkers. It offers the specificity, dynamic range and quantitative capability of mass spectrometry in a format that is familiar to flow cytometry practitioners. Data is collected as dual counts, the combination of digital counting and analogue modes of ion detection, and is converted into the FCS 3.0 format which can be processed by any flow cytometry software (14–16). ABC values for cell samples stained with CD4 antibodies conjugated to tags with neodymium isotope 142 (142Nd) were obtained and compared with ABC values from conventional flow cytometry.

Potential cell reference materials include both cryopreserved and lyophilized peripheral blood mononuclear cells (PBMCs). The cryopreserved PBMCs can be stored at −80°C for long time periods, that is, a few years. They are widely available and can be produced commercially or in laboratories. A proper thawing protocol should be followed to ensure cell viability. Lyophilized PBMCs are stored at 0–4°C and are stable for at least half a year. In this study using both flow and mass cytometry, we characterized a commercially available cryopreserved PBMC and two lyophilized PBMC preparations, one commercially available and another produced at the National Institute for Biological Standard and Control (NIBSC, Hertfordshire, UK). These cells were compared to freshly prepared PBMC and whole blood samples. The characterization parameters include CD4 expression level by means of ABC value, $CD4^+$ cell size and morphology as well as the membrane intactness of the lyophilized cell preparations. Additionally, we investigated the fixation effect on the measured CD4 expression.

Materials and Methods

Reagents

Heparinized normal donor (ND) samples were obtained from NIH Department of Transfusion Medicine for the experiments carried out at NIST and FDA. Heparinized ND samples for the investigation performed at University of Toronto were from the University Hospital. Both sample sources are approved by the respective institutional review boards. Monoclonal antibodies, unimolar CD4-PE conjugate [clone Leu-3a (SK3), Catalog Number: 340586], purified CD4 (clone Leu-3a, Catalog Number: 346320), CD4-FITC (clone Leu-3a, Catalog Number: 340133), CD45-PerCP, CD3-APC, and QuantiBrite PE Quantitation kits were purchased from BD Biosciences (San Jose, CA)[1]. Purified CD3 (clone HIT3a, Catalog Number: 300302), CD4 (clone Leu-3a, Catalog Number: 344602), CD15 (clone W3D6, Catalog Number: 323002), and CD45 (clone HI30, Catalog Number: 304002) were obtained from BioLegend (San Diego, CA). Cyto-Trol Control Cells were obtained from Beckman Coulter (Fullerton, CA). Lyoph-

[1]Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

ilized PMBC produced at NIBSC, UK (ID 5022) were obtained and named as PBMC–NIBSC. Cryopreserved PBMC (Catalog Number: CTL-UP1) and antiaggregate wash supplement 20× (Catalog Number: CTL-AA-001) were purchased from Cellular Technology (Shaker Heights, OH). Rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14C10) monoclonal antibody labeled with Alexa Fluor 647 and rabbit (DA1E) monoclonal antibody IgG XP isotype control labeled with Alexa Fluor 647 were obtained from Cell Signaling Technology, (Danvers, MA). Additionally, Ultra Rainbow Calibration Microspheres that contain blank and five different fluorescence intensity population microspheres (Catalog Number: URCP-38-2K) were purchased from Spherotech (Lake Forest, IL).

Sample Preparations and Staining

The procedure for whole blood staining was described previously (7,8). Briefly, whole blood was washed twice with PBS and stained with a cocktail of the following three antibodies: unimolar CD4-PE conjugate, CD45-PerCP, and CD3-APC for 30 min at room temperature (RT). The cell suspensions were subsequently lysed with ammonium chloride (ACK Lysing Solution, Biowittaker). After washing twice with PBS, the obtained leukocytes were resuspended in 0.5 mL of PBS either with 1% fixative [Paraformaldehyde (PFA), Electron Microscopy Sciences, Hatfield, PA] serving as fixed whole blood samples or without the fixative serving as live whole blood samples.

PBMCs were obtained from whole blood (30 mL) using the Ficoll−Hypaque density gradient separation procedure [lymphocyte separation medium (LSM) from MP Biomedicals, Solon, OH] (17). A small amount of remaining red cells were removed by lysing with ammonium chloride. PBMCs were counted and then stained with the antibody cocktail for 30 min at RT. After washing twice with PBS, the stained cells were resuspended in 0.5 mL of PBS either with or without 1% fixative serving as fixed PBMC or live PBMC, respectively.

For cryopreserved PBMCs, a thawing protocol provided by Cellular Technology was closely followed using a thaw solution made of 1 mL of antiaggregate wash supplement and 19 mL of RPMI-1640 from Invitrogen (Carlsbad, CA). After the thawing procedure, PBMCs in the warm thaw solution were counted with trypan blue. The thawed PBMCs were centrifuged at 330 g for 10 min and the supernatant was discarded. PMBCs were washed once with PBS and resuspended in PBS followed by staining with the antibody cocktail in the same fashion as freshly prepared PMBCs described above to obtain both live cryopreserved PBMC and fixed cryopreserved PBMC.

For labeling lyophilized Cyto-Trol and PBMC–NIBSC with the antibody cocktail, a titration curve of the unimolar CD4-PE conjugates was first generated to ensure cell staining under saturation conditions. As a result, a 30 μL aliquot of unimolar CD4-PE conjugate was used for staining 100 μL of either reconstituted Cyto-Trol (an original volume of 1 mL of cells plus reconstitution buffer provided) or PBMC–NIBSC reconstituted in 1 mL of sterile distilled water at RT for 30 min. After washing with PBS, the stained cells were resuspended in 0.5 mL of PBS without fixative.

Intracellular Staining

The protocol for simultaneous surface and intracellular staining of whole blood is described as follows (18): Aliquot 100 μL of fresh whole blood to a 5 mL test tube followed by adding 65 μL of 10% formaldehyde. Vortex the sample and keep at RT for 15 min. Add 1 mL of Triton X in PBS to make up 0.1% Triton final concentration. After vortexing the mixture and keeping it at RT for 30 min, add 1 mL of PBS with 0.5% BSA as the incubation buffer. Pellet cells by centrifugation and aspirate supernatant. Resuspend cells in 1 mL cold 50% methanol in PBS stored at −20°C and incubate on ice for 20 min. Add 1 mL incubation buffer to the tube and centrifuge for 10 min. Repeat wash with 1 mL incubation buffer twice and pellet cells by centrifugation. Add 50 μL of incubation buffer to the tube followed by adding 20 μL CD4-FITC and 2 μL rabbit anti-human GAPDH monoclonal antibody labeled with Alexa Fluor 647 (100 μg/mL) to the tube. Incubate for 1 h at RT. Add 2 mL incubation buffer and pellet cells followed by resuspending cells in 0.5 mL PBS. Fresh whole blood lysed with ammonium chloride without fixation and permeabilization was stained simultaneously with both CD4-FITC and GAPDH-Alexa Fluor 647 conjugate serving as a negative control. Cyto-Trol cells and lyophilized PBMC–NIBSC were also stained directly with both CD4-FITC and GAPDH-Alexa Fluor 647 conjugate without prior treatment of fixation and permeabilization. To assess nonspecific antibody binding to GAPDH protein, rabbit (DA1E) Mab IgG XP isotype control labeled with Alexa Fluor 647 (2 μL, 100 μg/mL) was used instead of the rabbit anti-human GAPDH antibody in the staining procedure described above.

Preparation for Determining Fixation Effect

To evaluate the effect of fixation on the detected CD4 expression by flow cytometry, thawed cryopreserved PBMCs (1×10^6 cells) were first fixed with different amounts of PFA in PBS (0, 0.5, 1, and 4%) for different time periods, that is, 20 min, 2 h and overnight, followed by washing twice with PBS. Cells were pelleted by centrifugation and the supernatant was removed. Cells were resuspended in 100 μL of PBS with 0.5% BSA and stained with the labeled antibodies as described earlier. After washing with PBS, the stained cells were resuspended in 0.5 mL of PBS without fixative.

Data Acquisition and Analysis by Flow Cytotmery

The flow cytometric measurements were performed using both FACSCanto II and FACSAria II equipped with Diva software for data acquisition and analysis (BD Biosciences, San Jose, CA). For the measurements, lymphocyte populations were gated by using two-dimensional (2D) CD45 and side scatter plots. Back gating with CD3 versus CD4 was used to ensure the inclusion of the total T lymphocyte population. The geometric mean channel numbers obtained from fluorescence histograms were used for the determination of the equivalent reference fluorophore fluorescence values or number of PE molecules bound per cell. In the case of unimolar CD4-PE conjugates, an ABC value is calculated as a ratio of the number of PE molecules bound per cell and the number of PE mole-

Table 1. ABC values obtained for different cell samples by quantitative flow cytometry measurements using QuantiBrite PE beads and unimolar CD4-PE conjugates, and CyTOF™ mass cytometry using CD4 (SK3 clone) labeled with Nd142. Measurement coefficient of variation (CV) and the number of samples (*N*) are also indicated.

CELL SAMPLE	QUANTITATIVE FLOW CYTOMETRY (×1000)	CYTOF™ (×1000)[a]
Live whole blood	49 (CV = 4.0%, *N* = 18)	
Fixed whole blood	45 (CV = 5.5%, *N* = 10)	45 (CV = 11%, *N* = 5)
Live PBMC	51 (CV = 11%, *N* = 7)	
Fixed PBMC	43 (CV = 5.2%, *N* = 10)	Not done
Live cryopreserved PBMC	47 (CV = 6.9%, *N* = 3)	
Fixed cryopreserved PBMC	42 (CV = 3.0%, *N* = 3)	46 (CV = 12%, *N* = 5)
Lyophilized PBMC–NIBSC	21 (CV = 4.7%, *N* = 7)	14 (CV = 12%, *N* = 4)
Cyto-Trol	41 (CV = 3.1%, *N* = 9)	38 (CV = 4.9%, *N* = 5)

[a] Samples for CyTOF measurements are post-fixed with 1.6% formaldehyde after staining. This is necessary for the introduction of the DNA-binding Ir-intercalator that is membrane impermeable in live cells. The Iridium signal is used to identify the nucleated single cell event for mass cytometry.

cules per antibody which is one. The fluorescence properties of the PE molecule in CD4-PE conjugates were measured to be the same within the measurement uncertainties as the properties of cells stained with antibody through direct fluorimeter comparison of cells and CD4-PE solution (12).

Sample Preparations and Staining for Mass Cytometry

Cell samples for mass cytometric analyses were prepared according to published procedures (14,15). In brief, a 100 μL aliquot of whole blood washed twice with PBS was stained for 30 min at RT with metal-labeled antibodies prepared using the MAXPAR reagent from DVS Sciences (ON, Canada). Cell suspensions were subsequently lysed with ammonium chloride lysing solution (Stem Cell Technologies, 15 min at 4°C). After washing twice with PBS, the obtained leukocytes were resuspended in 0.5 mL 1.6% formaldehyde.

Cryopreserved PBMCs were thawed in RPMI-1640 containing 10% fetal bovine serum and counted with trypan blue. The thawed mononuclear cells were then stained with metal-labeled monoclonal antibodies for 30 min at RT. After washing twice with PBS, the stained cells were resuspended in 0.5 mL 1.6% formaldehyde fixative. Lyophilized cells were reconstituted in the same manner as described for flow cytometry. After staining, the lyophilized cells were washed once with PBS and resuspended in 0.5 mL 1.6% formaldehyde fixative. All samples described were stained with a mixture of metal-tagged antibodies (14,15): CD3(Sm152), CD45(Tb159), CD15(Er170), CD8(Nd146), and different amounts of CD4(Nd142) to perform titration from 300 ng/mL to 20 μg/mL. The various antibodies were labeled with different metals in house using the MAXPAR reagent kits. Cells were then washed twice with PBS/5% (w/w) BSA, fixed in 1.6% formaldehyde, and were counterstained with Iridium (Ir)-intercalator (19) that binds to nucleic acids and is used for cell event recognition during data acquisition.

Data Acquisition and Analysis by Mass Cytotmery

Data acquisition was performed using a CyTOF™ mass cytometer (DVS Sciences) (14,15). For the measurements, a primary gating on $CD45^+$ population on a 2D plot of CD15 versus CD45 was performed and then a sub-gating was carried out on $CD4^+$ population on a 2D plot of CD3 versus CD4 to assure the inclusion of the total T lymphocyte population. The acquired data was saved in FCS3.0 format and analyzed using FlowJo software (Tree Star, Ashland, OR). The mean values of each element were obtained within each gate and were used to plot titration curves (data not shown). The average number of metal atoms per cell was quantified for saturating concentrations. Quantification is dependent of the mean analog intensity multiplied by the intensity-to-count conversion factor, which is related to the detector-data acquisition system response and divided by the transmission coefficient for a given lanthanide ion (14). The transmission coefficient is defined as the number of ions that reach the detector per number of ions injected into the plasma, and is determined prior to experiment by tuning the instrument with a standard solution of pure elements. Thus, each element in the antibody mix is assigned a transmission coefficient which is used to calculate the number of metal atoms per cell. Specifically, in this series of experiments the transmission coefficient for Nd142 was 2.48E-05. Antibody concentration was determined by absorbance at 280 nm with a NanoDrop 1000 UV/VIS Spectrometer (Nanodrop, Wilmington, DE) using an average extinction coefficient for a human IgG of 1.3 OD_{280nm}/mg of protein (20). Additionally, an aliquot of antibody solution was diluted 1/100,000 in 2% HCl and analyzed by conventional ICP-MS (ELAN 9000, Perkin Elmer/PE/Sciex), from which concentration of lanthanide atoms per mL was determined. Dividing the lanthanide concentration by the antibody concentration yielded an average of 110 ^{142}Nd atoms per molecule of CD4 antibody. Table 1 includes the average number of CD4-Nd142 ABC at saturation for the gated population of $CD3^+$ lymphocytes obtained for different samples by mass cytometry.

Microscopic Imaging and Size Measurement of $CD4^+$ cells

Microscope slides (Superfrost, VWR Scientific, Radnor, PA) were cleaned by soaking in 1 N HCl for approximately 1 h, repeatedly rinsed with water and ethanol and then dried with a laboratory wipe. A number 1.5 glass coverslip was used

for all samples. Samples were diluted with 10 μL PBS and were allowed to adhere to the glass slides and the coverslip was then gently applied. For samples that were embedded in agarose, 10 μL of molten SeaPlaque Agarose (1% wt/vol in PBS solution at 37°C) was placed on a slide (37°C on a slide warmer) and then mixed with 10 μL of the cell sample (diluted in PBS) by pipetting up and down a few times. A coverslip was applied and the slide was held at 4°C for 20 min to solidify. Some of the samples were suspended in a buoyant density media to reduce settling of the cells to the slide surface. Cells (2 μL in PBS) were mixed with 8 μL LSM (density 1.077 to 1.080 at 20°C) and placed on a slide and a cover slip applied. The density media is based on the principle that human lymphocytes will not sediment through it (21).

Cells were visualized through an Olympus BX50 microscope (Center Valley, PA) equipped with phase-contrast illumination and objectives (40× dry and 100× oil immersion). Cells were checked for fluorescence staining using epi-illumination and an FITC filter cube. Images were captured with a SPOT RT monochrome camera (Diagnostic Instruments, Sterling Heights, MI) and saved as 12-bit TIF files using the manufacture's software (version 4.0). Image characterization was done using Image Pro software (version 4.5 or 6.2, Media Cybernetics, Bethesda, MD). The pixel size was calibrated using a stage micrometer (1 mm in 100 divisions, #KR0851, Klarmann Rulings, Manchester, NH) using the same setup for cell imaging.

Results

ABC Values Obtained for CD4$^+$ Cells in Different Cell Preparations

The anti-human CD4 antibody clone, Leu 3a (also named as SK3), was used to quantify the number of antibodies bound per CD4$^+$ cell for various sample preparations using both conventional quantitative flow cytometry and CyTOF^{TM} mass cytometry. Quantitative flow cytometry measurements were performed by implementing the QuantiBrite PE bead calibration scheme with the use of unimolar CD4-PE conjugates. Through accurate measurements of the number of metal ions per CD4$^+$ cell labeled with the same CD4 clone, and measurements of the number of metal ions per isotope-labeled CD4, the numbers of CD4 bound on the various cell preparations were determined by CyTOF^{TM} mass cytometry. Figure 1 shows the similar gating strategies for the two quantification methods. For flow cytometry, the primary gate was set on lymphocyte populations in the CD45-PerCP versus side scatter dot plot (top left). Moreover, gating with CD4-PE versus CD3-APC (top right) was used to ensure the inclusion of the total T lymphocyte population. The geometric mean channel value for CD4 was obtained from its histogram. For the mass cytometry measurements, the primary gate was set on CD45$^+$ cell populations shown in the CD45(Tb159)D versus CD15(Er170)D contour plot (bottom left). A second gate was established for quantification of Nd142 metal ions shown in the CD4(Nd142)D versus CD3(Sm152)D contour plot (bottom right). The ABC values for CD4 antibody binding resulting from the two methods are given in Table 1 for different sample preparations. As shown, the two quantification methods give fairly consistent ABC values for the four different cell samples measured: fixed whole blood, fixed cryopreserved PBMC, lyophilized PBMC–NIBSC and Cyto-Trol.

The ABC values obtained for freshly prepared whole blood (49K), PBMC (51K) and cryopreserved PBMC (47K) are in fair agreement with the consensus value published for freshly prepared PBMC, ~48K (7,10–12). It is well-known that lower ABC values are usually observed for cell samples fixed post-staining relative to fresh unfixed cell samples using quantitative flow cytometry as shown in Table 1 (7). The low CD4 values on Cyto-Trol control cells and lower CD4 values on lyophilized PBMC–NIBSC are reflected from the ABC values determined using both quantification methods. We hypothesize that the long lyophilization processes may damage the cell membrane and cause a significant decrease in cell size. These defects could ultimately affect antibody binding and therefore the measured CD4 expression.

Measurement of Membrane Integrity Using Simultaneous Surface and Intracellular Staining of Cell Samples

GAPDH is an abundant enzyme of ~37 kDa that catalyzes the sixth step of glycolysis in the cytosol and translocates to the nucleus when it is activated in T-lymphocytes (22). Hence, GAPDH was chosen along with the CD4 surface receptor for probing if CD4$^+$ cell membrane was compromised. Staining cell samples with rabbit (DA1E) Mab IgG XP isotype control labeled with Alexa Fluor 647 was used to evaluate nonspecific staining of GAPDH protein. The bivariate histograms of simultaneous surface and intracellular staining of different cell samples are shown in Figure 2. The CD4$^+$GAPDH$^+$ and CD4$^+$Isotype control are displayed in dot and contour plots, respectively. The results of the positive control (top left), fixed, and permeabilized ND whole blood and the negative control (top right), fresh whole blood without fixation and permeabilization demonstrate that staining protocol implemented was successful.

The simultaneous surface and intracellular staining experiment was performed using lyophilized Cyto-Trol control cells and PBMC–NIBSC without any fixation and permeabilization treatments. The CD4$^+$GAPDH$^+$ of Cyto-Trol show little difference relative to CD4$^+$ isotype (bottom left) control suggesting that the cell membrane is not compromised. By comparison, the CD4$^+$GAPDH$^+$ of PBMC-NIBSC exhibits slightly higher signal than the CD4$^+$ isotype control (bottom right). However, the GAPDH signal of PBMC–NIBSC is much lower than that of the positive ND control (note the log scale). This result indicates there is no significant damage of the cell membrane on PBMC–NIBSC cells.

Cell Size and Image Measurements

Phase contrast microscopy was used to characterize the size of CD4$^+$ cells that were fixed and then sorted using a FACSAria II flow cytometer sorter. The CD4$^+$ cells from newly thawed cryopreserved PBMC and fresh whole blood show av-

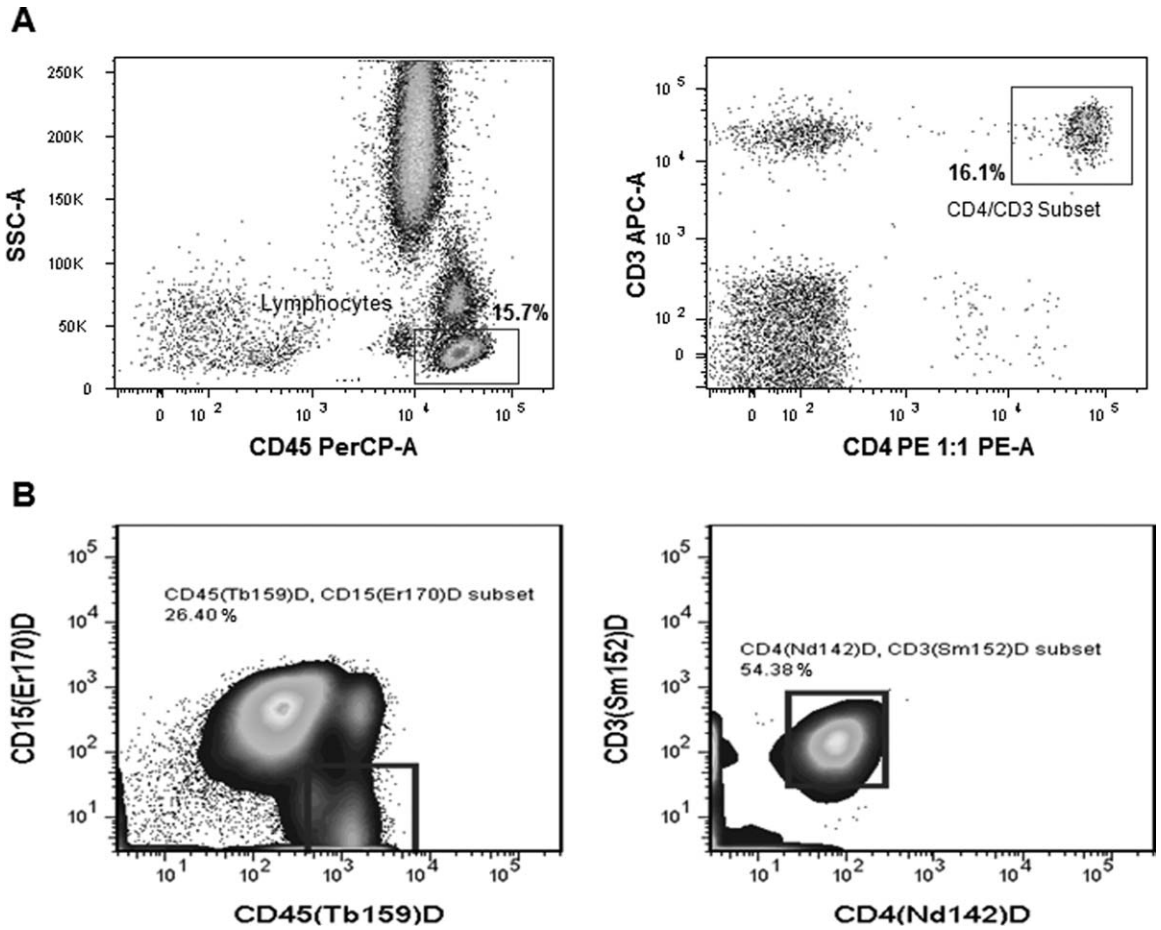

Figure 1. Gating strategies illustrated for the two CD4 quantification methods. For quantitative flow cytometry, the primary gate was set on lymphocyte populations in the CD45-PerCP versus side scatter dot plot (top left). Further gating with CD4-PE versus CD3-APC (top right) was used to ensure the inclusion of the total T lymphocyte population. For CyTOF^{TM} mass cytometry, the primary gate was set on CD45$^+$ cell populations in the CD45(Tb159)D versus CD15(Er170)D contour plot (bottom left). A sub-gating was then carried out for the quantification of Nd142 metal ions in the CD4(Nd142)D versus CD3(Sm152)D contour plot (bottom right).

erage diameters of 7.7 ± 0.8 μm and 8.1 ± 0.5 μm, respectively, when adhered to glass slides (Fig 3a). These cells suspended in PBS were placed on clean glass slides; cell size measurements were performed over a period of one to 2 h and no cell size increase was observed. When CD4$^+$ cells obtained from lyophilized Cyto-Trol and PBMC–NIBSC in PBS were placed on glass slides and allowed to adhere to the surface, a significant increase in cell diameter was observed over the same measurement time period. To overcome the apparent increase in cell diameter, we performed the cell size measurements using two different media, agarose and LSM. When the cells were suspended in these media, no increase in diameter with time was observed. The CD4$^+$ cells from newly thawed cryopreserved PBMCs display an average diameter of 7.5 ± 0.4 μm in LSM (Fig. 3b) consistent with that obtained in PBS (Fig. 3a) and the reported diameter in literature (23), assuring no medium effect present. Cells suspended in either medium did not undergo the flattening with time on the glass surface. Moreover, size measurements carried out in agarose and LSM are in excellent agreement for both Cyto-Trol and PBMC–NIBSC. For instance, the average diameters of CD4$^+$ cells measured in LSM are essentially the same, 6.3 ± 0.4 μm for Cyto-Trol and 6.5 ± 0.4 μm for PBMC–NIBSC, respectively (Fig. 3b).

Phase contrast CD4$^+$ cell images are shown in Figure 3 along with the average cell diameters. The phase contrast images of Cyto-Trol appear different from those of PBMC, whole blood and PBMC–NIBSC. The appearances of the images are likely due to changes in light refractive indexes of Cyto-Trol and PBMC–NIBSC as a result of the processing and lyophilization steps during cell preparations.

Effect of Fixation on Detected CD4 Expression

Prior to a three-day cycle lyophilization process to produce lyophilized PBMC–NIBSC, freshly obtained PBMCs were resuspended in a stabilization buffer containing a low level of PFA (0.5–1%) and stored at 4°C overnight. To mimic the treatment of PBMC–NIBSC cells, we designed an experiment using thawed cryopreserved PBMC to evaluate the fixation effect on the detected CD4 level. The PBMCs were first fixed

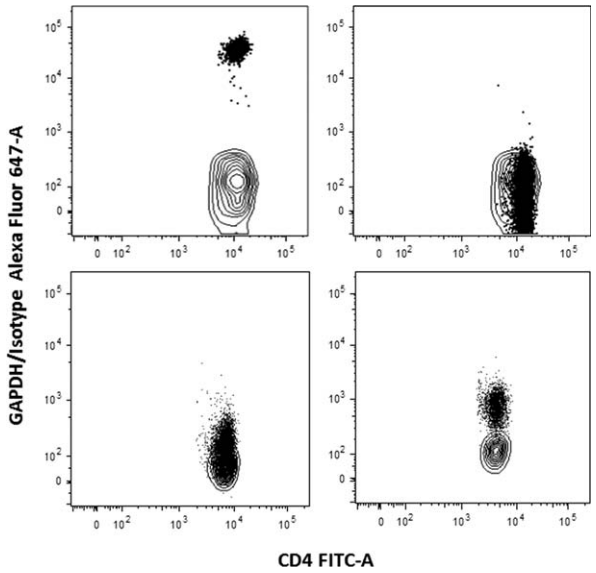

Figure 2. Bivariate histograms of the $CD4^+GAPDH^+$ population (dot) and $CD4^+$Isotype control (contour) from simultaneous surface and intracellular staining of different cell samples: fixed and permeabilized ND whole blood serving as the positive control (top left); fresh whole blood without fixation and permeabilization serving as the negative control (top right); Cyto-Trol control cells (bottom left); PBMC—NIBSC (bottom right). Reconstituted Cyto-Trol and PBMC-NIBSC were directly stained with labeled antibodies without fixation and permeabilization treatment.

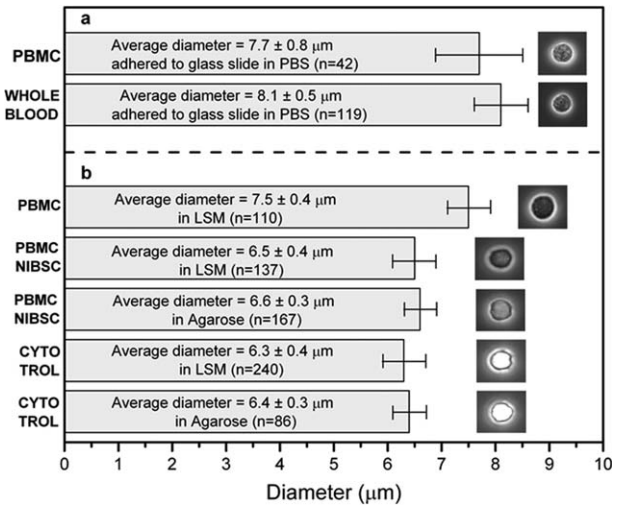

Figure 3. The average diameter and phase contrast image of the stained and then sorted $CD4^+$ cells from different cell samples characterized by phase contrast microscopy: (**a**) stained, fixed and then sorted $CD4^+$ cells from whole blood and cryopreserved PBMC, suspended in PBS were placed on clean glass slides; (**b**) size and image measurements were performed on $CD4^+$ cells obtained from cryopreserved PBMC, lyophilized PBMC—NIBSC and Cyto-Trol in two different media, agarose and LSM to overcome the cell enlargement issue. The "*n*" value refers to the number of cells examined for determining the average diameter.

with different amounts of PFA (0.5–4%), washed and then stained with the CD4-FITC. Figure 4 shows the results of the experiment displaying the detected CD4 expression levels in terms of ERF FITC. With increasing amounts of PFA, CD4 levels decrease monotonically relative to the no fixative control. For all levels of PFA, 0.5, 1, and 4%, a 2 h fixation time appears to be sufficient to fix the cells given that the detected CD4 levels are more or less consistent between 2 h and overnight fixation times. In fact, the CD4 levels decrease by 20–40% in the presence of 0.5–1% PFA with the 2 h fixation time. The similar effect of PFA on biomarker expressions has been reported on platelets and leukocytes (24,25).

Discussion

In this study, a single anti-human CD4 antibody clone, Leu 3a was used to quantify the number of antibodies bound per $CD4^+$ cell for various sample preparations using two different measurement methodologies: quantitative flow cytometry and CyTOF™ mass cytometry. The quantification scheme using QuantiBrite PE calibration beads and unimolar CD4-PE conjugates is fairly straightforward. Using the unimolar conjugates of PE to CD4 antibody (effective $F/P = 1$), ABC values of the $CD4^+$ cells stained with the unimolar CD4-PE conjugates can be determined directly according to the calibration curves generated with QuantiBrite PE beads. The ABC values obtained for freshly prepared whole blood, PBMC and cryopreserved PBMC are consistent with the consensus value of ~48K published for freshly prepared PBMC (7,12). Mass cytometry, conversely, uses isotope-tagged CD4 antibodies to quantify CD4 levels on helper T cells. Each CD4 antibody used in this study is tagged with ~110 Nd142 (14,26). The ABC value is calculated as a ratio of the number of isotopes per $CD4^+$ cell and the number of isotopes per labeled CD4 antibody. These two quantification methods give fairly consistent ABC values for the four different cell samples using a consist-

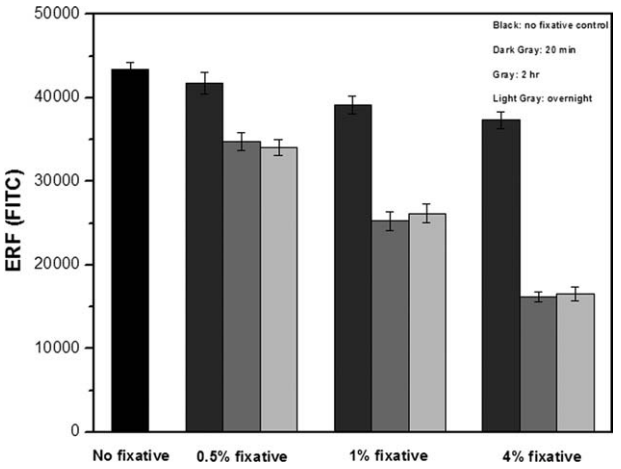

Figure 4. Fixation effect on the detected CD4 expression measured in terms of ERF FITC. Cryopreserved PBMCs were first fixed by different amounts of PFA in PBS (0, 0.5, 1, and 4%) for different time periods, 20 min, 2 h and overnight, followed by washing twice with PBS and then staining with CD4-FITC. The geometric mean channel numbers obtained from FITC histograms were converted to ERF FITC values based on the calibration curves generated by using ultra rainbow calibration microspheres (13).

ent staining protocol: fixed whole blood, fixed cryopreserved PBMC, lyophilized PBMC–NIBSC, and Cyto-Trol (Table 1). The lowest ABC values of CD4 were obtained for Cyto-Trol and PBMC–NIBSC cells.

We performed simultaneous surface and intracellular staining experiments on Cyto-Trol control cells and PBMC–NIBSC to evaluate the degree to which the cell membrane is compromised as measured by permeability of a large antibody. It is possible that long lyophilization processes could damage the cell membrane, that is, partial fragmentation of the cell membrane, and hence, reduce the actual number of CD4 receptors that can be detected through antibody binding. Figure 2 shows that the positive control, fixed and permeabilized ND whole blood, and negative control, fresh whole blood without fixation and permeabilization, work well as expected. When the same staining procedure was applied to the reconstituted Cyto-Trol and PBMC–NIBSC without any fixation and permeabilization treatments, the bivariate histograms show their profiles largely resemble that of the negative ND control. Though the GAPDH signal from PBMC-NIBSC is somewhat higher than the signal from the negative control, this signal is still lower by an order of magnitude than that from the positive ND control. These results imply no significant degree of disruption of the cell membranes of Cyto-Trol and PBMC-NIBSC samples. Hence, partial fragmentation of the cell membrane causing the reduction of the number of CD4 receptors is not likely the reason for the lower ABC values observed for the two lyophilized cell preparations.

We further hypothesize that long lyophilization processes could reduce cell size noticeably and create spatial hindrance for affinity binding of the CD4 antibody. The cell diameters measured by phase contrast microscopy are comparable for Cyto-Trol and PMBC–NIBSC as shown in Figure 3b. The average diameters of $CD4^+$ cells measured in LSM are 6.3 ± 0.4 μm for Cyto-Trol and 6.5 ± 0.4 μm for PBMC–NIBSC. The $CD4^+$ cell diameters of these two lyophilized cell preparations are smaller than that of thawed cryopreserved PBMC in LSM (7.5 ± 0.4 μm), suggesting the effect of cell size reduction due mostly to lyophilization processes. The diameter shrinkage of the lyophilized cells from 7.5 μm (cryopreserved PBMC) to 6.5 μm causes a decrease in surface area by 25% with respect to cryopreserved PBMC, assuming the cells are spherical and using the surface formula of (pi) $\times$ diameter^2 for a sphere. Because of the shrinkage, the surface of lyophilized cells is expected to become less smooth. This could hinder CD4 antibody binding even though lyophilized cells may still possibly retain the original number of CD4 receptors. This cell size reduction could account for the lower ABC values for CD4 antibody binding on Cyto-Trol relative to live blood lymphocytes (41 vs. 49 K).

An alternative explanation for the low ABC values of CD4 on lyophilized cells may be linked to the denaturation stresses of the lyophilization process even in the presence of stabilizers. Freeze-drying is known to destabilize and denature proteins to various degrees (27). This effect may alter the paratope of some CD4 antigens to the extent that the CD4 antibody fails to bind to it altogether or the affinity is greatly reduced.

Though no descriptive literature was found on the production of Cyto-Trol, a patent issued to Beckman Coulter (28) described a process that freshly obtained PBMCs were suspended in a stabilization buffer containing trehalose sugar without PFA prior to a 15 h cycle lyophilization process. If Cyto-Trol cells are prepared using this process, the lack of fixation may explain the higher level of CD4 antibody binding than that of PBMC–NIBSC (Table 1) and closer resemblance of the negative ND control profile shown in Figure 2.

It is apparent that the cell size reduction by lyophilization process is not the sole source for the low ABC values obtained for PBMC–NIBSC. For the production of lyophilized PBMC–NIBSC cells, freshly obtained PBMCs were resuspended in a stabilization buffer containing a low level of PFA (0.5–1%) and stored at 4°C overnight prior to a 3-day cycle lyophilization process. To mimic the cell treatment, an experiment was designed to evaluate the fixation effect on detected CD4 level using thawed cryopreserved PBMC. The PBMCs were first fixed with different amount of PFA, washed and then stained with anti-CD4 FITC. As shown in Figure 4, CD4 level decreases with increasing amounts of PFA when compared to the no fixative control. With 1% PFA treatments for 2 h or overnight, CD4 antibody binding decreased by 40%, likely due to cross-linking of protein induced by PFA preventing antibody binding. Our measurement results of the $CD4^+$ cell sizes and fixation effect suggest that the very low ABC values obtained for lyophilized PBMC–NIBSC are primarily due to the PFA fixation effect in addition to the cell size reduction caused by the lyophilization process.

Conclusion

In this study, we characterized a commercially available cryopreserved PBMC and two lyophilized human PBMC preparations, Cyto-Trol and PBMC–NIBSC, and compared them to freshly prepared PBMC and whole blood samples. It was found that the ABC values for CD4 expression on cryopreserved PBMC were consistent with those of freshly obtained PBMC and whole blood samples. By comparison, the ABC values on Cyto-Trol is lower and values on PBMC–NIBSC is much lower than those freshly prepared cell samples. Both quantitative flow cytometry and CyTOF^{TM} mass cytometry provided reasonably consistent antibody binding results for these two lyophilized cell preparations. By performing simultaneous surface and intracellular staining experiments on these two cell samples, we determined that both cell membranes are mostly intact. Moreover, $CD4^+$ cell diameters from both lyophilized cell preparations are smaller than those of thawed PBMC and whole blood. This observed cell size reduction could account for the 16% lower ABC values for CD4 antibody binding on Cyto-Trol (41 vs. 49 K). Further investigation of the fixation effect on the detected CD4 expression suggests that the low ABC values obtained for lyophilized PBMC–NIBSC are mostly due to the PFA fixation process. PFA fixation reduces significantly the available antibody binding sites. Additionally, the low ABC values could be due in part to the cell size reduction caused by the lyophilization process.

To serve as a biological calibrator for the transformation of a linear fluorescence intensity scale obtained with fluorescent microspheres to an ABC scale, a candidate cell reference material has to have a reproducible and tight ABC value for a known clinical reference biomarker expression, for example, CD4 in this study. Preferentially, chemical and physical properties of this cell reference material should resemble those of clinical blood samples. Moreover, the reference material should show a long-term stability under widely used and common storage conditions. From the present study of the three candidate reference cell preparations, cryopreserved PBMC and Cyto-Trol appear to fulfill reasonably these requirements though it would be of interest to investigate other cell preparations, such as stabilized blood (UK NEQAS). In addition, we are planning to study additional surface markers with high and low expression levels relative to CD4 for more accurate transformation of the linear fluorescence intensity scale to the more meaningful ABC scale.

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