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High Throughput pSTAT Signaling Profiling by Fluorescent Cell Barcoding and Computational Analysis

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Abstract

Fluorescent cell barcoding (FCB) is a multiplexing technique for high-throughput flow cytometry (FCM). Although powerful in minimizing staining variability, it remains a subjective FCM technique because of inter-operator variability and differences in data analysis. FCB was implemented by combining two-dye barcoding (DyLight 350 plus Pacific Orange) with five-color surface marker antibody and intracellular staining for phosphoprotein signaling analysis. We proposed a robust method to measure intra- and inter-assay variability of FCB in T/B cells and monocytes by combining range and ratio of variability to standard statistical analyses. Data analysis was carried out by conventional and semi-automated workflows and built with R software. Results obtained from both analyses were compared to assess feasibility and reproducibility of FCB data analysis by machine-learning methods. Our results showed efficient FCB using DyLight 350 and Pacific Orange at concentrations of 0, 15 or 30, and 250 µg/mL, and a high reproducibility of FCB in combination with surface marker and intracellular antibodies. Inter-

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operator variability was minimized by adding an internal control bridged across matrices used as rejection criterion if significant differences were present between runs. Computational workflows showed comparable results to conventional gating strategies. FCB can be used to study phosphoprotein signaling in T/B cells and monocytes with high reproducibility across operators, and the addition of bridge internal controls can further minimize inter-operator variability. This FCB protocol, which has high throughput analysis and low intra- and inter-assay variability, can be a powerful tool for clinical trial studies. Moreover, FCB data can be reliably analyzed using computational software.

Keywords

fluorescent cell barcoding; variability; phosphoproteins; computational analysis; phenotyping

1. Introduction

Fluorescent cell barcoding (FCB), a high-throughput flow cytometry (FCM) technique for multiplexed assays, was first developed for single-cell phospho-specific FCM (phosphoFlow) [1–6], and subsequently optimized for detection of intracellular cytokines [7–8] and immunophenotyping [1–10]. FCB minimizes staining variability and decreases both antibody consumption and required sample volumes [1,11]. The advantage in performing large-scale studies by the FCB technique is to generate more reliable FCM data by minimizing technical variability, as all samples and controls are processed and acquired collectively [1]. For these reasons, FCB is particularly applicable to translational studies, and new barcoding methods are being developed using amine-reactive or fixable viability dyes [9–10,12].

The analysis of cell-to-cell variation in gene and protein expression obtained from high content single cell techniques, such as mass cytometry and FCB, is challenging in terms of time, reliability, and reproducibility [13–15]. Manual analysis is subjective and difficult to reproduce due to inter-operator variability, subjectivity in choosing the sequence of marker combinations to explore, and the shape and boundaries of gates [16–17]. By contrast computational FCM techniques are more objective, allowing users to reproducibly identify cell populations and measure protein expression levels [18]. Several software tools have been developed for computational data preprocessing, transformation, analysis, and visualization of FCM data including standalone programs or platforms such as Cytobank and packages for Matlab and RStudio [19–27].

Standardization of protocols and antibody panels in FCM for phenotyping is important for more reliable, comparable, and reproducible data, and, especially critical for samples collected during clinical trials [28–31]. The use of FCB minimizes inter-assay variations, as all samples are acquired together, but a rigorous standardization and optimization of the protocol is still required before implementation of the technique both in clinical and basic research settings [1–10]. However, analysis of FCB data is usually conducted by conventional manual gating strategies using available commercial software products, which

are designed to analyze individual samples by a gating sequence limited to one or two parameters at a time.

Here, we provide a method to assess intra- and inter-assay variability of FCB for immunophenotyping and phosphoFlow analysis, and for quantification and minimization of inter-operator variability. For FCB data analysis, we also compare percent of positive cells or median fluorescence intensity values obtained by conventional manual gating strategies to those calculated with machine learning methods in order to further minimize operator- and software-dependent variations, as well as significantly decrease analysis time.

2. Materials and methods

2.1 Reagents

The following FCB dyes were used: DyLight 350 NHS ester and Pacific Orange NHS ester (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies used for surface staining were: mouse anti-human CD3-PerCP-Cy5.5 (clone SK7), mouse anti-human CD4-PE-Cy7 (clone SK3), mouse anti-human CD8-FITC (clone RPA-T8), and mouse anti-human CD20-APC-H7 (clone H1) (BD Biosciences, San Jose, CA, USA); and CD14-PE (clone M5E2) from BioLegend (San Diego, CA). Antibodies used for phosphoproteins were: pSTAT1(pY701)-Alexa Fluor 647 (clone 4a), pSTAT3(pY705)-Alexa Fluor 647 (clone 4/P-STAT3), and pSTAT5(pY694)-Alexa Fluor 647 (clone 47/Stat5 pY694) (BD Biosciences). Phosflow Lyse/Fix Buffer 5X, Phosflow Perm Buffer III, and Phosflow Barcoding Wash Buffer 4X buffers (BD Biosciences) were prepared and used according to manufacturer's instructions. Phosflow Perm Buffer II (BD Biosciences) was diluted 1:1 with cold PBS and kept on ice before use. For PBMC stimulation, the following cytokines were used: recombinant human IL-10 (PeproTech, Rocky Hill, NJ); human Interferon- α (Cell Signaling Technology, Boston, MA); recombinant human IL-2 (Hoffmann-La Roche Inc, Nutley, NJ). The CTL Anti-Aggregate (CTL) wash buffer was from Cellular Technology Limited (Shaker Heights, OH).

2.2 PBMC isolation

Heparinized fresh whole blood was obtained from healthy donors of the NIH Clinical Center Department of Transfusion Medicine (n= 32) after informed consent was obtained in accordance with the Declaration of Helsinki [32] (NIH, Bethesda, MD, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (GE Healthcare, Chicago, IL) with Leucosep centrifuge tubes (VWR, Radnor, PA), according to manufacturer's instructions. Cells were frozen in medium containing 90% FCS and 10% dymethyl-sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and stored in liquid nitrogen until use.

2.3 pSTAT stimulation

PBMCs were stimulated with cytokines for phosphorylated Signal Transducer and Activator of Transcription (pSTAT) activity [33–35]. Cryopreserved human PBMCs were thawed in a water bath at 37°C, washed twice with 10 mL of CTL buffer, according to manufacturer's instructions, and counted with AO/PI (acridine orange/propidium iodide) staining solution.

Viability and number of PBMCs were determined using a cellometer (Nexcelom Bioscience, Lawrence, MA). A total of 1.5×10^6 PBMCs were seeded at 15×10^6 /mL in a deep 96 well round-bottom plate. Cells were stimulated with human Interferon- α (Cell Signaling Technology) at 200 ng/mL for pSTAT1 stimulation, recombinant human IL-2 (Hoffmann-La Roche Inc) at 200 U/mL for pSTAT5, or recombinant IL-10 (PeproTech) 200 ng/mL for pSTAT3 and incubated for 20 min at 37°C. Untreated PBMCs were processed following the same protocol and used as negative control.

2.4 FCB and phosphoflow staining

Each dye was dissolved in DMSO at a final concentration of 500 μ g/mL and stored at -80°C . Using the stock solution, FCB dyes were diluted with DMSO to have the following concentrations: 0, 30, and 250 μ g/mL for DyLight 350 and 0, 15, and 250 μ g/mL for Pacific Orange. A final volume of 40 μ L/well was used for barcoding by combining 30 μ L of sample and 5 μ L of each dye or DMSO in order to have the final concentrations of 0, 6.25 (for DyLight 350) or 3.125 (for Pacific Orange), and 31.25 μ g/mL. At the end of stimulation, untreated and treated PBMCs were fixed with 550 μ L of 1X Lyse/Fix Buffer for 10 min at 37°C, centrifuged at 500g for 5 min, and then permeabilized with 600 μ L of Phosflow Perm Buffer III on ice for 30 min. Subsequently, cells were washed twice with 600 μ L of Phosflow Perm Buffer II (1:1 with PBS), resuspended in 35 μ L of cold Phosflow Perm Buffer II and 30 μ L were added to appropriate wells (1.29×10^6 cells/ 30 μ L/well) with various concentrations of FCB dyes previously prepared in a U-bottom 96 well plate. After incubation on ice for 20 min in the dark, samples within each matrix were combined and washed twice with BD Phosflow Barcoding Wash Buffer (3 mL) by centrifugation at 500g for 5 min, followed by resuspension with BD Phosflow Barcoding Wash Buffer (50 μ L) for antibody staining. After antibody titration (Supplementary Figure 1), cells were stained with 20 μ L of CD14-PE (20 μ g/mL), 40 μ L of CD8-FITC (1.25 μ g/mL), 40 μ L of CD3-PerCP-Cy5.5 (0.6 μ g/mL), 40 μ L of CD20-APC-H7 (2.4 μ g/mL), 10 μ L of CD4-PE-Cy7 (0.3 μ g/mL), and 40 μ L of appropriate pSTAT-Alexa Fluor 647 (0.3 μ g/mL for pSTAT1 and pSTAT3; 0.6 μ g/mL for pSTAT5). Cells were incubated for 1 hr at room temperature in the dark with gentle shaking, washed with BD Phosflow Barcoding Wash Buffer (3 mL), and resuspended in 300 μ L of the same buffer for acquisition. As non-barcoded controls, 1.5×10^6 cells were directly stained without fixation, permeabilization and barcoding. For each treated and untreated donor, 1.5×10^6 fixed and permeabilized cells were stained without barcoding. After staining, all samples were washed and acquired using BD Phosflow Barcoding Wash Buffer.

2.5 Data acquisition and analysis

LSR Fortessa cytometer (BD Biosciences) equipped with 6 lasers (ultraviolet, 355 nm; violet, 405 nm; blue, 488 nm; green, 552 nm; orange, 592 nm; and red laser, 628 nm) and BD FACSDiva software (v.8.0.1, BD Biosciences) were used for sample acquisition. Compensation was calculated using bead standards for each fluorochrome (anti-Mouse Ig, κ /Negative Control Compensation Particles Set, BD Biosciences) and barcoded cells with the highest concentration of each dye. An unstained sample was used as negative control for setting PMT voltages, and all samples were run using the same PMT voltages. A minimum of 300,000 lymphocytes were recorded. FlowJo software (v.10.0.7b, Treestar, Ashland, OR,

USA) was employed for post-acquisition compensation and conventional flow cytometric analysis. Compensation matrix was exported from FlowJo as a CSV file (Supporting Information S1). FCM data are shown using biexponential transformation.

2.6 Conventional gating strategy

After post-acquisition compensation using FlowJo, lymphocytes and monocytes were identified using linear parameters (FSC-A vs SSC-A) and double cells were excluded (FSC-A vs FSC-H) (Supplementary Figure 1). Single cells were deconvoluted by plotting the FCB dye channels (Pacific Orange vs DyLight 350), identifying nine populations. On each barcoded lymphocyte sample, CD3 and CD20 expression were first investigated, and CD4 and CD8 expression was further studied on CD3⁺ cells. CD14⁺ cells were identified (CD14 vs FSC-A) on barcoded monocyte populations. pSTAT1, pSTAT3, or pSTAT5 expression was calculated on CD3⁺, CD4⁺, CD8⁺, CD20⁺, and CD14⁺ cells as median fluorescence intensity (MFI) values.

2.7 FCS file datasets and R packages

Computational workflows were built using datasets available on FlowRepository (FR-FCM-ZZZ2). The dataset included six FCS files from the current study: three unstimulated and three stimulated samples for each pSTAT. For computational analysis, RStudio (v. 1.1.456) and R software (v.3.5.1) were employed (RStudio, Inc., Boston, MA, USA) (28) and BiocInstaller (v. 1.32.1, Bioconductor) was used to update Bioconductor, CRAN, and GitHub packages (28–29). The following packages were used: flowType (v.2.20.0), ggcyto (v.1.10.0), ggplot2 (v.3.1.0), flowWorkspace (v.3.30.1), flowViz (v.1.46.0), flowClust (v. 3.20.0), and flowCore (v.1.48.0). R codes were deposited in GitHub (https://github.com/ValeGiu076/FCB_computational_analysis.git).

2.8 Computational gating strategy

R packages were loaded into R session and FCS files read using the *read.FCS* function from the flowCore package. We began by removing debris using rectangular gates (*rectangleGate* function in flowCore) and *subset* function, and then cell clusters were identified using t-mixture modeling (*flowclust* function in flowClust R package; Supplementary Figure 2). Lymphocyte and monocyte clusters were identified based on SSC.A values, since monocytes are bigger and more complex compared to lymphocytes. In details, clusters were first split in two flowFrame objects, ordered by SSC.A values and then identified as monocyte cluster if SSC.A values were higher than those present in the other cluster. After biexponential transformation, unsupervised deconvolution was performed using FCB dye channels (Pacific.Orange.A and BUV395.A) and set the number of clusters (*K*) at 9, as implemented in the *flowclust* function of flowClust package. Barcoded populations were split in separate flowFrame objects and included in a list. On each barcoded lymphocyte population, unsupervised clustering was carried out to identify CD3⁺ and CD20⁺ cells using *flowclust* function setting the fluorescence channel parameters (APC.H7.A and PerCP.Cy5.5.A) and the number of expected clusters (*K*=3) for modeling. The three clusters were then ordered by APC.H7.A and PerCP.Cy5.5.A values, and the population with the highest maximum value of PerCP-Cy5.5.A was assigned as CD3⁺ cells, and CD4⁺ and CD8⁺ cells were further gated using the same strategy. While, the cluster with the highest value of APC.H7.A was assigned

as CD20⁺ cells. Similarly, after deconvoluting monocytes, we identified CD14⁺ cells using linear parameters and CD14 expression by defining rectangular gates at the 1st quartile of the PE.A parameter.

2.9 Statistical analysis

Data were analyzed using Prism (v.8.0.1; GraphPad software, Inc., La Jolla, CA). Fluorescence values were reported as median fluorescence intensity (MFI), CV (CV = SD/mean of population), and MFI fold change. For each antibody tested, percentage of positive cells and MFI values were recorded for every barcoded population and matched non-barcoded controls. Variability between barcoded samples and matched controls, and sample-to-sample variability were defined and reported as previously described [9]. Percentages of positive cells or MFIs were correlated between barcoded and matched non-barcoded samples, or between conventional and computational methods by Pearson test and linear regression. Position variability was assessed using 1-way ANOVA with multiple comparisons corrected for FDR (False Discovery Rate). For inter-assay and inter-operator variability assessment, 1-way ANOVA and Pearson analysis were employed. A *P-value* < 0.05 was considered statistically significant.

3. Results

3.1 FCB efficiency

In this study, FCB was optimized using Pacific Orange and DyLight 350 dyes at concentrations of 0, 15 or 30, and 250 µg/mL to barcode nine human PBMC samples, and FCB efficiency was assessed by MFI fold increase calculation [9]. Lymphocytes were identified based on linear parameters (FSC-A vs SSC-A) and double cells were excluded (FSC-A vs FSC-H) (Supplementary Figure 3A). On single cells, FCB dyes were visualized separately using normalized cell number count histograms, and three peaks were shown according to FCB dye concentrations (Supplementary Figure 3B). For each peak of 12 separate matrices, MFIs and CVs were calculated in FlowJo software. Values were used to assess MFI fold increase as follows: $MFI\ fold\ increase = [MFI_{peak_2} - CV_{peak_2}] / [MFI_{peak_1} + CV_{peak_1}]$ [9]. MFI fold increase ranged from 4.1 to 11.3 achieving a complete peak separation without any overlap. CVs were higher in 0 µg/mL barcoded populations (21% and 58% for Pacific Orange and DyLight 350, respectively) compared to 15 or 30 and 250 µg/mL barcoded samples (15±1%) (Supplementary Figure 3C).

3.2 Surface marker intra-assay variability

After optimization of dye concentrations, intra-assay variability was assessed for combination staining with FCB dyes and surface and intracellular markers. Lymphocytes and monocytes were characterized using surface markers (CD3, CD4, CD8, CD20, and CD14), and phosphorylation levels of STAT1, STAT3, and STAT5 were measured in each T/B cell and monocyte population. One donor was used for barcoding all nine populations in a 3×3 matrix (p1 to p9; Figure 1A and Supplementary Figure 4) for each pSTAT (total of six matrixes: three untreated and three stimulated for each pSTAT). This experiment was repeated with three healthy donors. For each condition, a fresh sample without fixation,

permeabilization and barcoding was stained only with surface marker antibodies and used as a control.

In order to exclude that methanol permeabilization could affect reliability and reproducibility of FCB data, we first compared percentage of positive barcoded samples to matched non-barcoded controls by Pearson correlation. For each donor, the average percentage of positive cells for all stimulated or unstimulated barcoded samples was compared to matched non-barcoded stimulated or unstimulated controls. The two staining methods were highly correlated ($r = 0.993$; $P < 0.0001$) (Figure 1B), confirming that methanol did not affect the staining process by increasing non-specific binding.

Range and ratio of positive stained cells were calculated to determine variations within a matrix compared to the arithmetic mean of barcoded samples or to non-barcoded controls, respectively. The range of variability was calculated as average of percent of positive cells of all barcoded samples $\pm 2SD$. Average of percent of positive cells and SD were calculated for each position in a matrix (p1 to p9) among all matrices with unstimulated (total of nine values for each parameter) or stimulated samples. The ratio of variability was defined as the ratio between the average of barcoded samples in every position in a matrix and the average of non-barcoded samples (Figure 1A). Ratios within 0.80 and 1.20 were acceptable because a coefficient of variation $<20\%$ is related to physiological and technical variations [36–40]. A minor non-statistically significant variability was described for CD3⁺, CD8⁺ and CD20⁺ populations, and no ratios were <0.80 or >1.20 . No intra-assay variability was documented for CD4⁺ and CD14⁺ cells (Figure 1C and Supplementary Figure 5). We also compared values for each position to those in position 1 (p1, barcoded only with DMSO) to determine if variability in percent of positive cells was due to specific dye concentration. No variations were described (all $P > 0.1$).

We further assessed the effects of FCB dyes on fluorescence intensity and intra-assay variability of fluorochromes used. MFIs from barcoded samples were compared to those from matched non-barcoded samples in order to confirm that FCB did not significantly affect fluorescence intensity of the fluorochromes used for staining. As expected, the data from the two techniques were highly correlated ($r = 0.864$; $P < 0.0001$) (Figure 2B). Subsequently, ranges and ratios of variability were calculated for MFIs of PE-, PE-Cy7-, FITC-, APC-H7-, and PerCP-Cy5.5-conjugated antibodies as described above, and populations barcoded with 0 $\mu\text{g/mL}$ of dyes were used as controls (Figure 2A and Supplementary Figure 6). No MFIs were outside the ranges (Figure 2C). However, when ratios were defined, a statistically significant variability was described for FITC- and PE-conjugated antibodies. Position variability was also assessed by 1-way ANOVA corrected for multiple comparisons, and significant variations were confirmed in some positions for MFIs of FITC- and PE-conjugated antibodies. However, our FCB protocol produced a reproducible combination staining with Pacific Orange plus DyLight 350 with surface markers for T/B cell and monocyte identification.

3.3 pSTAT intra-assay variability

We assessed intra-assay variability of pSTAT staining in combination with FCB and surface marker antibodies. Samples were treated with appropriate stimuli as described above, and

MFIs of pSTAT1, pSTAT3, and pSTAT5 in CD4⁺, CD8⁺, CD20⁺, and CD14⁺ cells were obtained from barcoded populations of each stimulated and matched unstimulated sample (Supplementary Figure 7). Fold change (FC) was calculated as MFI of stimulated sample in pi / MFI of unstimulated sample in pi (matrix position, p1 to p9). Then, average and SD of all FC values in CD4⁺, CD8⁺, CD20⁺, and CD14⁺ cells were used to calculate the range of variability (average FC + 2SD). The ratio of variability was also defined as average FC in pi / average FC (Supplementary Figure 8). For pSTAT1, no intra-assay variability was described for CD4⁺ and CD14⁺ cells (Figure 3); while, minor variability was documented for CD8⁺ and CD20⁺ cells. For pSTAT5, there was no intra-assay variability for CD4⁺, CD8⁺, and CD14⁺ cells; a minor variability was documented for CD20⁺ populations. For pSTAT3, some variability was described for CD4⁺, CD8⁺, CD20⁺ and CD14⁺ cells. These variations were caused by an increased variability of MFI values in unstimulated samples (Supplementary Figure 6). Despite these variations in MFIs in specific populations, our FCB protocol remained highly reproducible for pSTAT signaling analysis in T/B cells and monocytes.

3.4 Inter-assay and inter-operator variability

After establishing minimal intra-assay variations of our FCB protocol, we investigated FCB reproducibility across different operators and days. PBMCs from eight different healthy donors (D1 to D8 in the same position across all matrixes; Figure 4A) were employed for FCB of a nine-sample matrix, and samples were appropriately stimulated for pSTAT1, pSTAT3, and pSTAT5. In position 9, one healthy donor used to measure intra-assay variability was placed in all matrices and considered an internal bridge control (Figure 4A). In addition, each of three operators performed FCB of one pSTAT by doing appropriate stimulation, fixation, permeabilization, FCB, and staining independently. This experiment was repeated four times on four separate days with a total of 32 healthy donors used. The same bridge control was used across the four days to assess inter-day variability.

The percentages of positive cells were first obtained from individual internal controls and compared by unpaired t-tests across operators. No significant differences were observed (Figure 4B). Similarly, percent of positive cells obtained from the other 32 donors were compared across operators by 1-way ANOVA, and no significant variations were described (Figure 4C). Pearson correlation analysis was performed between each stimulated and unstimulated sample, and high correlations were identified (Figure 4C–E and Supplementary Figure 9). FC and Log₁₀FC of pSTAT1, pSTAT3, and pSTAT5 for all samples were calculated using MFIs of appropriate stimulated and unstimulated samples. For each population, average and SD of Log₁₀FC were calculated and used to build heatmaps (Figure 4F). Total variability for pSTATs was 2.5%. Using an internal control across matrices, we were able to exclude technical variations between operators and, therefore, considered this variability as biological and not technical. In addition, stability and reproducibility of FCB was assessed by Pearson correlation of pSTAT values at different timepoints or assays in CD4⁺, CD8⁺, CD14⁺ or CD20⁺ cell populations (Supplementary Figure 10). Overall, the FCB protocol showed minimal variation across different operators and multiple assays, allowing greater sample collection and minimizing data acquisition variability for large data studies.

3.5 Intra-assay variability by computational analysis

To address the demand for rapid large-scale profiling, intra-assay variability was also assessed using semi-automated workflows. Results were compared to those obtained by conventional manual gating strategies in order to assess the feasibility of computational workflows for FCB data analysis. The percentages of positive cells determined by our computational analysis were highly correlated to those obtained from matched samples analyzed by conventional manual gating ($r = 0.968$; $P < 0.0001$; Figure 5A). The percentage of positive cells from computational gating was next compared to those from manual gating by Wilcoxon matched-pairs signed rank test and unpaired t-test (Figure 5B). No significant variations were observed for CD3⁺, CD4⁺, and CD8⁺ T cells; while, a significant difference was described for CD20⁺ and CD14⁺ cells. Intra-assay variability was assessed as explained above by first defining ranges and ratios of variability, and by using matched non-barcoded samples as controls (Figure 5C and Supplementary Figure 11). Since no statistically significant variabilities were detected, semi-automated workflows could be used for FCB data analysis with similar results of conventional analysis. The comparability of the computational gating to manual methods demonstrate the possibility of rapid and reproducible FCB data analysis by machine learning workflows. Therefore, our FCB protocol showed low intra- and inter-staining variability within an assay and across operators, and high reproducibility by computational data analysis which reduced workload and increased efficiency.

4. Discussion

FCB is a multiplexed high-throughput flow cytometry technique which has been used for phosphoFlow analysis [1–6], detection of intracellular cytokines [7], and immunophenotyping of human PBMCs and mesenchymal stem cells [9–10]. In recent years, mass cytometry has gained popularity for phosphoprotein profiling, and has been frequently used for studies aimed at quantifying relative changes. However, CyTOF's slow acquisition speed has created the need to control for time tracking and normalization compared to standard FCM rates. In addition, the costs associated with mass cytometry, including the costs of reagents, which are often not standardized if metal conjugation is done in-house, needs to be taken into account. Our efforts focused on precision, accuracy, and reproducibility of multiplexing of pSTAT profiling because we believe that the ability to combine standard FCM with multiplexed methods and cost efficiency makes FCB a feasible alternative. Therefore, we proposed and validated the use of a bridge control across FCB assays and showed a robust method to assess intra- and inter-operator variability of our FCB technique. Finally, we built a simple computational method to analyze FCB data in a faster and operator-independent way, which was also used to assess and verify the variability.

In previous work, we have optimized FCB for nine-sample barcoding using two sets of concentrations of three FCB dye combinations (0, 13 and 250 $\mu\text{g/mL}$ or 1.56, 50 and 500 $\mu\text{g/mL}$) which allowed a good deconvolution with MFI fold increase >2 [9]. In this study, we implemented FCB for phosphoprotein analysis using DyLight 350 and Pacific Orange for barcoding nine samples. We also developed a standardized method for a robust assessment of intra- and inter-assay and inter-operator variability that could allow FCB to be applied to

samples collected during clinical trials. Our FCB was optimized using Pacific Orange and DyLight 350 for surface and intracellular staining of human PBMCs. Dye concentrations were slightly changed to previous work (0, 15 or 30, and 250 µg/mL) in order to have MFI fold increase >3 (range, 4.1–11.3) for a complete and sharp separation of both lymphocytes and monocytes. As a result, FCB with DyLight 350 and Pacific Orange at 0, 15 or 30, and 250 µg/mL achieved complete deconvolution of both lymphocyte and monocyte populations.

FCM is a powerful tool for rapid phenotyping in clinical research; however, subjectivity and variability of FCM are well-known pitfalls, and many international organizations are working to improve comparability and reliability of FCM data across laboratories [16–17, 28–31]. Considering the need to standardize FCM techniques and minimize staining variability, here we provide a robust method for assessment of intra- and inter-assay variability of a nine-sample FCB using DyLight 350 and Pacific Orange co-stained with surface (CD3, CD4, CD8, CD20, and CD14) and intracellular (pSTAT1, pSTAT3, or pSTAT5) markers. We also assess different permeabilization methods which could allow sufficient permeabilization for pSTAT staining. Since antibody cocktails were added after permeabilization and barcoding, our method minimizes non-specific binding of surface marker antibodies to intracellular reservoirs. Our results show a high correlation between percent positive cells obtained from barcoded samples and those from matched non-barcoded samples stained only with surface marker antibodies used as controls. These results confirmed that FCB did not affect the staining process and different concentrations of methanol for permeabilization did not increase non-specific binding of intracellular reservoirs of surface markers [37]. For intra-assay variability assessment, one donor was barcoded for all nine populations in a matrix in order to exclude any variation in the percent of positive cells or MFIs due to biological differences between samples and not due to technical variations [13]. For each parameter, a range of variability was calculated, and values were considered as acceptable if within this range. Values obtained from barcoded samples were compared to those from matched non-barcoded specimens by calculating the ratio of variability which we utilized to directly compare to matched controls allowing only 20% of biological variation [36–40]. The use of two simple ranges for detecting outliers combined with standard statistical analysis could systematically and reliably identify intra- and inter-assay variability of FCB. Indeed, the employment of a single mathematical method could not find out variabilities within positions in a matrix or between barcoded and not-barcoded samples. In our case, some minor non-statistically significant variabilities were documented for CD3⁺, CD8⁺, and CD20⁺ cells for unstimulated specimens; no intra-assay variability was documented for MFIs of any fluorochrome studied. Significant variabilities in MFIs of FITC- and PE-conjugated antibodies were documented in positions 3 and 6, and also in position 9 for CD14-PE antibody. As CD14-PE was used to identify monocytes, variations in MFIs may be caused by an increased ability of this population to bind larger amount of FCB dyes and/or because of a higher cytoplasmic complexity [6,11]. These variations were described using other antibody clones and fluorochromes in both fresh and frozen PBMCs (e.g. BV650-conjugated CD14; data not shown). Due to this dye concentration-related variability of some antibodies, we suggest avoiding quantitative analysis of MFIs of antibodies that bind monocyte markers. However, we did not observe simultaneous variations in both range and ratio of variability for other antibodies. Intra-assay

variability was also assessed for pSTATs. We documented low variability for pSTAT1 and pSTAT5; while, pSTAT3 was the most variable mainly caused by an increased variability of MFIs of unstimulated samples, especially in CD20⁺ populations. Nonetheless, our data suggest that the current FCB protocol for phosphoFlow analysis in T/B cell subpopulations and monocytes showed high robustness, reproducibility and reliability.

During post-acquisition analysis, accurate identification of target cells and gate boundaries are the major cause of inter-operator and inter-laboratory variability of FCM data analysis [16–17]. In order to measure and minimize inter-assay and inter-operator variability, nine-sample FCB for phosphoFlow analysis was performed by three operators independently from sample stimulation to antibody staining. Moreover, one of the healthy donors used for intra-assay variability assessment was employed as an internal “bridge” control and added to every matrix in position 9. The use of “bridge samples” for data normalization has already been proposed by several authors and for different assays [38]. Here, we employed bridge samples for the determination of accuracy, reproducibility, and reliability of FCB data from different operators for the development of protocols for clinical tests. The presence of significant variations in percent of positive cells or MFIs in the bridge control was considered as a rejection criterion for a particular run and the experiment was repeated. However, the robustness of our protocol was confirmed by the absence of significant differences between percent of positive cells or FC of pSTATs across operators for both internal controls and barcoded samples in all experiments.

The large datasets generated by FCB studies makes conventional FCM analysis challenging and poorly reproducible, mainly because of inter-operator variability [10, 24–25]. Computational FCM is becoming increasingly utilized, given that machine learning analysis is more reproducible and efficient due to simultaneous exploration of more than two parameters [24]. In this study, we investigated the applicability of a semi-automated machine learning workflow for analysis of combination staining with FCB dyes and surface and intracellular antibodies. We provided a simple R code for semi-automated FCB data analysis using available packages, such as flowCore and flowClust, and without post-acquisition compensation, which is a source of inter-assay variability [16]. The only guided step was the identification of lymphocyte and monocyte clusters which was followed by unsupervised analysis for deconvolution and identification of CD3, CD4, CD8, and CD20 cells by flowClust. For CD14⁺ monocytes, we employed the *rectangleGate* function from flowCore package, and we set the 1st quartile of chosen parameters (e.g. CD14-PE) as non-arbitrary threshold for cluster identification. Using this approach, data obtained from semi-automated workflows and conventional manual gating strategy were highly comparable. Some variations were observed for CD20⁺ cells possibly because of the very small SD, and for CD14⁺ monocytes because the threshold for gating was higher in the computational analysis compared to manual gating. Intra-assay variability of the data obtained from computational workflows was assessed, and minor variability was described in position 9 for CD3⁺ and CD8⁺ populations that could likely be adjusted by applying a post-acquisition compensation. However, this approach could be further implemented in order to develop a dedicated fully automated R package for FCB data analysis with minimal variations, such as by building an algorithm for auto-compensation of single barcoded populations for autofluorescence adjustment that could likely decrease intra-assay variability.

5. Conclusions

Analysis of single-cell high-throughput data is applied in many fields, including FCM. Similarly, standardization of pre- and post-analytical procedures and minimization of intra- and inter-assay variability are required in order to have reproducible and reliable data. FCB is a multiplexed high-throughput FCM technique which allow large-scale studies with minimal intra- and inter-assay variability and may also reduce inter-operator variability by including a bridge sample across matrices used as a rejection criterion for a particular run. Our results demonstrated that FCB is a powerful tool especially for samples obtained during clinical trials, translational research and longitudinal studies. In addition, computational workflows may improve FCB data analysis by reducing time of analysis and inter-operator variability for gating boundaries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Surface marker intra-assay variability assessment for percentage of positive cells. One donor was employed for barcoding all nine populations in each of six matrixes (three with unstimulated samples and three stimulated for pSTAT1, pSTAT3, or pSTAT5). (**A**) Mean of percent of positive cells and SD were calculated for each position in a matrix (pi ; p1 to p9) among all matrixes with unstimulated samples (total of nine values for each parameter; N) or stimulated samples. The range of variability was calculated as average of % of positive cells of all barcoded samples $\pm 2SD$. The ratio of variability was defined as the ratio between the average of barcoded samples in every position in a matrix and the average of not-barcoded samples; values were considered as acceptable if between 0.8 and 1.2. (**B**) Percent of positive barcoded cells were compared to those from matched not-barcoded (w/o FCB) samples by Pearson analysis. (**C**) Ranges and ratios of variability were calculated for each population, and values outside the ranges ($< -2SD$ or $> +2SD$; or < 0.8 or > 1.2) were highlighted in black.

Figure 2. Surface marker intra-assay variability assessment for median fluorescence intensity (MFI) values.

One donor was employed for barcoding all nine populations in each of six matrixes (three with unstimulated samples and three stimulated for pSTAT1, pSTAT3, or pSTAT5). (A) Mean of MFIs and SD were calculated for each position in a matrix (pi ; p1 to p9) among all matrixes with unstimulated samples (total of nine values for each parameter; N) or stimulated samples. The range of variability was calculated as average of MFIs of all barcoded samples $\pm$ 2SD. The ratio of variability was defined as the ratio between the average of barcoded samples in every position in a matrix and the average of barcoded samples in $p1$ (only DMSO). (B) MFIs for each fluorochrome were compared to those obtained from matched not-barcoded (w/o FCB) samples by Pearson analysis. (C) Ranges and ratios of variability were calculated for each population, and values outside the ranges ($< -2SD$ or $> +2SD$; or < 0.8 or > 1.2) were highlighted in black.

Figure 3. pSTAT intra-assay variability assessment.

MFIs of pSTAT1, pSTAT3, and pSTAT5 in CD4⁺, CD8⁺, CD20⁺, and CD14⁺ cells were obtained from barcoded populations of each stimulated and matched unstimulated sample and fold change (FC) calculated. Mean and SD of all FC values in each population were calculated in order to identify the range of variability: Average FC $\pm$ 2SD. The ratio of variability was also defined as Average FC in *pi* (from p1 to p9) / Average of all FC. Values outside of these ranges were highlighted in black.

Figure 4. Inter-operator variability assessment.

(A) Each of three operators performed independently stimulation, fixation, permeabilization, FCB and staining for one pSTAT. Eight healthy donors (D1 to D8) were used for FCB in combination with surface marker and intracellular antibodies. An internal control sample (Int Ctrl) was added in position 9 of all matrixes and used as bridge sample to assess inter-assay and inter-operator variability. (B) Percent of positive cells from internal controls were compared between operators by 1-way ANOVA. Data are shown as mean±SD. (C) Percent of positive cells were also obtained from stimulated and unstimulated samples of each donor and compared by 1-way ANOVA in order to exclude variations between operators (Op1 to Op3). Pearson correlation analysis was carried out between percent of positive cells from stimulated samples (Stim) and those from unstimulated specimens (Unstim) in order to investigate inter-assay variability. A representative example of 1-way ANOVA (D) and Pearson analysis (E) for CD4⁺ cells is shown. (F) Fold change (FC) of each pSTAT in CD4⁺, CD8⁺, CD20⁺ or CD14⁺ cells were calculated for all 32 donors, and ranges of variability calculated as described in Figure 3. Values outside these ranges were highlighted in black.

Figure 5. FCB computational data analysis.

(A) Using semi-automated workflows, percent of positive cells were obtained and compared to those from matched barcoded samples analyzed by conventional manual gating strategy. (B) Percent of positive cells obtained using computational analysis (R) were also compared to those calculated by manual gating using FlowJo software (FJ) by unpaired t-test. (C) Intra-assay variability was assessed as described in Figure 1 by defining range and ratio of variability for each population and by using as controls matched not-barcoded samples. Values outside these ranges were highlighted in black.