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Transient Partial Permeabilization with Saponin Enables Cellular Barcoding Prior to Surface Marker Staining

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Abstract

Fluorescent cellular barcoding and mass-tag cellular barcoding are cytometric methods that enable high sample throughput, minimize inter-sample variation, and reduce reagent consumption. Previously employed barcoding protocols require that barcoding be performed after surface marker staining, complicating combining the technique with measurement of alcohol-sensitive surface epitopes. This report describes a method of barcoding fixed cells after a transient partial permeabilization with 0.02% saponin that results in efficient and consistent barcode staining with fluorescent or mass-tagged reagents while preserving surface marker staining. This approach simplifies barcoding protocols and allows direct comparison of surface marker staining of multiple samples without concern for variations in the antibody cocktail volume, antigen-antibody ratio, or machine sensitivity. Using this protocol, cellular barcoding can be used to reliably detect subtle differences in surface marker expression. © 2014 International Society for Advancement of Cytometry

Key terms

fluorescent cellular barcoding; mass-tag cellular barcoding; mass cytometry; permeabilization; saponin

Fluorescent cellular barcoding (FCB) and mass-tag cellular barcoding (MCB) methods are used to covalently stain cell populations with either fluorescent or mass tags to allow several samples to be combined into a single tube for simultaneous antibody staining and cytometric analysis (1,2). These methods enable high throughput analysis of multiple samples while minimizing inter-sample variability due to procedural limitations (sample processing and instrument variation), and reduce reagent consumption. FCB and MCB have been used in large-scale screening experiments for both drug discovery and immunology research (1,3–6).

Methods for FCB and MCB have been developed for dye or mass-tag labeling after cell permeabilization with alcohol. However, alcohol permeabilization can disrupt surface marker epitopes, necessitating that the surface staining be performed prior to barcoding or that surface marker assessment be limited to those epitopes that are not disrupted by alcohol fixation (7). This study describes a method of barcoding paraformaldehyde-fixed cells that employs transient partial cell permeabilization using a low concentration of saponin. This transient partial permeabilization allows for barcode labeling and accurate surface marker staining without significant interference due to staining of intracellular pools of surface epitopes (that might lead to incorrect immunophenotypic profiles). The method simplifies the process of barcoding samples when surface marker characterization is required and allows for extremely accurate comparison of surface marker expression levels among samples within a given experiment.

Materials and Methods

Antibodies

Antibodies, manufacturers, and concentrations used for labeling cells are listed in Supporting Information Table S1. For mass cytometry studies, primary antibody transition metal-conjugates were either purchased or conjugated in-house using 100-μg antibody lots combined with the MaxPAR antibody conjugation kit (DVS Sciences, Toronto, Canada) according to the manufacturer's recommended protocol. Following conjugation, antibodies were diluted to 100X working concentration in Candor PBS Antibody Stabilization solution (Candor Bioscience GmbH, Wangen, Germany) and stored at 4°C.

Cell Culture

U-937 cells and Jurkat cells (ATCC, Manassas, VA) were cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin. All cell culture was performed at 37°C in a humidified cell culture incubator at 5% CO_2. Cultured cells were fixed by addition of paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) to a final concentration of 1.5% and incubated for 10 min at room temperature. Cisplatin viability staining was performed as previously described (8).

Human Samples

Fresh bone marrow aspirates were collected into heparinized tubes and immediately fixed using a fixation/stabilization buffer (SmartTube, Palo Alto, CA), according to the manufacturer's instructions for 10 min at room temperature and then frozen at −80°C for up to 12 months prior to analysis by mass cytometry. Human peripheral blood samples were collected into heparinized tubes, fixed using the SmartTube system according to manufacturer's instructions, and stored at −80°C for 3–6 months prior to analysis. For both sample types, cells were thawed just prior to analysis in a 4°C water bath, and red cells were lysed using a hypotonic lysis buffer (SmartTube) following the manufacturer's recommended protocol. Cells were then washed twice in cell staining medium (CSM; 1XPBS with 0.5% bovine serum albumin and 0.02% sodium azide) at room temperature. All human samples were collected in accordance with a human research protocol approved by the Stanford University Institutional Review Board.

Barcoding

Barcoding was performed on approximately 1 million fixed cells placed into racked, 1.1-mL microtubes (BioExpress, Kaysville, UT) using a multichannel pipette and a multichannel aspirator. Fixed cells were washed once in CSM and then washed once in PBS, followed by a second wash in PBS plus 0.02% saponin (Sigma-Aldrich, St. Louis, MO), or PBS plus the indicated saponin concentration for titration experiments (all pre-barcoding saponin washes were performed at 4°C). Each wash step (resuspension of cells, centrifugation of cells for 5 min at 600*g*, and aspiration of supernatant) was completed in ∼10 min. After these washes, cells were resuspended in a residual volume of ∼60 μL PBS plus 0.02% saponin (or the indicated saponin concentration) prior to the application of the barcoding dye and maintained at 4°C. A 100X DMSO stock of the fluorescent or mass tag barcoding reagent was then rapidly (<20 s) diluted into 1 mL ice-cold PBS plus 0.02% saponin (or the indicated saponin concentration) and then quickly (<20 s) applied to the resuspended cell pellets. Cells were incubated for 15 min (at room temperature) to allow covalent reaction of the barcode dyes with the cells. After barcoding, cells were washed twice with CSM and then combined in a single tube or into separate tubes for the saponin titration experiments. After completion of barcoding, cells were not re-exposed to saponin in subsequent manipulations or antibody staining steps (once the barcoding reagent is added to the cells, the subsequent washes are not temperature sensitive and were performed at room temperature).

For fluorescent barcoding, stock solutions of the NHS/succinimidyl ester formulations of DyLight 800 (Thermo Scientific, Rockford, IL) and Pacific orange (Life Technologies, Grand Island, NY) were prepared from dried aliquots as described (2,9). Dye stock solutions were then made at 100X concentration in DMSO and used at final concentrations of 0.5 μg/mL DyLight800 and of 0, 0.1, 0.5, or 2 μg/mL Pacific orange. The fluorescent barcoding dye was incubated for 15 min on ice. For mass cytometry experiments, mass-tagged barcoding reagents were created as described (1), except that isotopically purified palladium nitrate (Trace Sciences International, Richmond Hill, Ontario, Canada) was used as the chelated metal and isothiocyanobenzyl-EDTA (Dojindo Molecular Technologies, Rockville, MD) was used as the chelator. The mass tag barcoding reagent was diluted into 100X concentrated stock in a 96-well PCR plate and frozen at −80°C for up to 12 months and thawed immediately prior to use. Mass tag barcoding was performed at a final metal concentration of 300 nM. The mass tag reagent was incubated with the cells for 15 min at room temperature. Mass tag barcoding was performed using a pattern of three of the six stable Pd isotopes (102, 104, 105, 106, 108, 110) for each sample; staining was equivalent for all Pd isotopes. This barcoding protocol allows removal of doublet events and is fully described in Zunder et al. (submitted).

Antibody Staining

Cells were incubated with surface marker antibodies in 100 μL CSM for 50 min with continuous mixing. Cells were then washed twice with CSM, pelleted by centrifugation, and resuspended with vortexing in ice-cold methanol. After a 15-min incubation at −20°C, cells were washed twice with CSM prior to incubation with antibodies against intracellular signaling proteins for 50 min at room temperature (with continuous mixing) as previously described (7,10). For detergent treatment of Jurkat cells, Tween 20 was added to a final concentration of 0.2% during CD3 antibody staining (these cells were not permeabilized with methanol).

Fluorescent Flow Cytometry

Fluorescence cytometry analysis was performed on a BD LSRII cytometer (BD Biosciences, San Jose, CA) equipped with 405, 488, and 633 nm lasers. Cells were washed with CSM and then blocked with mouse immunoglobulin (10 μg/mL) for 10 min. Antibody staining was then performed in 100 μL CSM for 50 min at room temperature. Compensation was performed using protein A/G compensation bead standards for each fluorochrome. A compensation matrix was made using FlowJo (v8.8.6). Single cells were gated based on FSC-A versus SSC-A and FSC-A versus FSC-W. Rainbow calibration particles (RCP-30-5; Spherotech, Lake Forest, IL) were used to QC the LSRII cytometer before each experiment.

Mass Cytometry

Mass cytometry staining and measurement was performed as previously described (11). Briefly, after completion of antibody staining cells were washed twice with CSM and then incubated for 20 min in PBS with a 1:5,000 dilution of iridium intercalator pentamethylcyclopentadienyl-Ir(III)-dipyridophenazine (DVS Sciences, Toronto, Canada) and 1.5% paraformaldehyde (to fix antibodies to cellular antigens). Excess intercalator was then removed with one CSM wash and two washes in distilled, deionized water. Cells were then resuspended in distilled, deionized water at approximately 1 million cells per mL and mixed with mass standard beads at concentration yielding approximately two beads events per second (DVS Sciences). Cell events were acquired on the CyTOF^{TM} mass cytometer (DVS Sciences) at an event rate of 100–300 events per second with instrument calibrated dual count detection (12,13). Noise reduction was used and cell extraction parameters were as follows: cell length 10–90, lower convolution threshold 200. The cell subtraction value was set to -100 (thereby adding 100 counts to the signal of each measured mass channel). After acquisition, the effect of the cell subtraction setting was negated by subtracting a value of 100 from every channel of each FCS file using the flowCore package for R (10). The above manipulations were performed to better estimate the effect of background subtraction and experimental noise for cells with low signal by allowing negative values to be displayed (11). After data acquisition, the mass bead signal was used to correct short-term signal fluctuation during the course of each experiment (14). Bead events were then removed from the final data files.

Data Analysis

All mass cytometry data are displayed with an arcsinh transformation and a scale argument of five (except for linear scales used for Ir intercalator and cell length parameters). All fluorescent cytometry data is displayed with an arcsinh transformation and a scale argument of 150. To perform analysis on this dataset, mass cytometry data was first singlet gated in Cytobank using a cell length by DNA (Ir intercalator) gate (Supporting Information Fig. S1). Immunophenotypic assignments were based on previous studies from our laboratory (11,15) and others (16). ViSNE analysis was performed using the CYT software tool as described previously (17), data files were down-sampled to 10,000 events each and all surface markers (Supporting Information Table S1) were used for the analysis.

Results

Low Concentrations of Saponin Allow Partial Permeabilization and Barcoding of Fixed Cells

Methods for cellular barcoding by fluorescence and mass cytometry have been previously described. These methodologies required methanol permeabilization prior to barcoding to obtain consistent barcode signals (7). As a result, alcohol-sensitive surface marker epitopes can either not be assayed or must be antibody-stained prior to cell barcoding, thereby adding additional complexity to the experiment and reducing certain benefits of barcoding. To address this, labeling of PFA-fixed U-937 cells was performed using traditional FCB or MCB reagents prior to cell permeabilization. Without permeabilization, cell barcoding using either fluorescent or mass tag reagents occurred with low efficiency and resulted in highly variable staining (Fig. 1).

We reasoned that the barcode staining could be improved by partially permeabilizing the cell membrane to allow the small molecule barcoding reagents (but not antibodies) to enter the cell and react with intracellular proteins. To implement this concept, cells were washed with PBS alone or with PBS plus increasing concentrations of saponin prior to and during incubation with fluorescent or mass-tag barcoding reagents. As shown in Figure 1, concentrations of saponin greater than or equal to 0.01% (the approximate critical micelle concentration (18)) applied during a single wash step ($\sim$10 min) and the barcode staining incubation (15 min) resulted in efficient cellular staining with either the fluorescent or mass tag barcoding reagents. On the basis of this titration, 0.02% saponin was chosen as the concentration for further experiments (this increase was made to ensure that the assay would not be sensitive to small changes in saponin concentration). The 0.02% saponin concentration is one-tenth that typically used in protocols where saponin is used for intracellular antibody staining. Transient treatment with this low concentration of saponin lead to a 3 to 4-fold increase in median barcode staining intensity and an approximately 3-fold reduction in the coefficient of variation (CV) of barcode staining by both MCB and FCB (Fig. 1). The improvement in the resolution of the barcoded populations was even more pronounced when two or more barcoding reagents were employed (Supporting Information Fig. S2). The high CV of the barcode signal in untreated cells was primarily due to a fraction of cells that were stained by the barcoding reagent in the absence of permeabilization. Although dead and dying cells did consistently demonstrate higher barcode staining (if saponin permeabilization was not performed), the majority of brightly labeled cells were not dead or dying as indicated by viability staining and staining for cleaved caspase-3 and cleaved poly-ADP-ribose polymerase (PARP; Supporting Information Fig. S3).

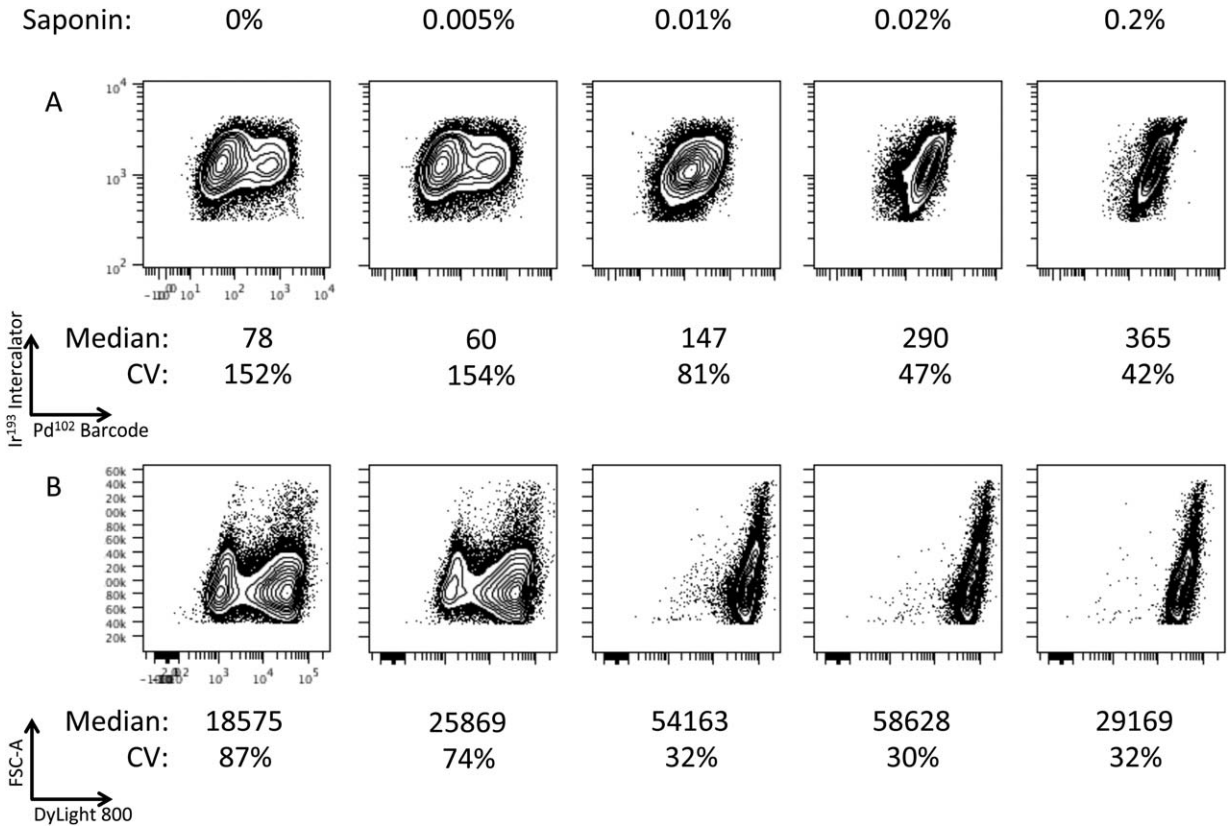

Figure 1. Transient exposure to low concentrations of saponin allows consistent fluorescent or mass-tag cellular barcoding. U-937 cells were fixed and then barcoded in the presence of the indicated concentration of saponin. Barcoding was performed with either (**A**) isotopically purified Pd isotopes or (**B**) a combination of DyLight 800 and Pacific orange.

Partial Permeabilization with Saponin Does Not Enable Significant Intracellular Antigen Staining and Yields Equivalent Surface Marker Staining Compared to Untreated Cells

As many cell types contain intracellular pools of surface marker proteins (19), it was important to confirm that transient partial permeabilization with 0.02% saponin did not result in increased antibody staining of intracellular proteins. U-937 cells were fixed and barcoded without saponin exposure or after transient permeabilization with a range of saponin concentrations. These cells were then stained for the intracellular phosphorylated epitope of Rb (S807/811; pRb) either before or after methanol permeabilization. As shown in Figure 2A, the level of staining of pRb in cells treated with 0.02% saponin was not significantly different from that of untreated cells (median mass cytometry counts of 6.6 vs. 10, respectively). Increasing the saponin concentration to 0.2% increased the median staining of pRb to 29.0 prior to methanol permeabilization. However, this small increase was far below the staining intensity achieved after methanol permeabilization (median mass cytometry counts of 1,145). In the mass cytometry experiment, multiple additional markers were also tested simultaneously. CD33, CD45, CD99, H3K9ac, pATM (S1981), and Ki-67 demonstrated similar median staining intensities with and without 0.02% saponin partial permeabilization (Supporting Information Fig. S4). The same experiment was performed using fluorescently barcoded U-937 cells and an Alexa Fluor 647-labeled antibody against pRb and yielded similar results (Fig. 2B). In addition, Jurkat cells were tested to confirm that cytoplasmic (intracellular) CD3 would not be stained as a result of partial permeabilization with 0.02% saponin. Jurkat cells have previously been shown to have significant pools of cytoplasmic CD3 antigen (in addition to surface CD3 antigen), and the presence of cytoplasmic CD3 is an important diagnostic criterion for certain T cells malignancies. As shown in Supporting Information Figure S5, neither saponin treatment nor barcoding increased staining of CD3 as compared to detergent treatment, which has previously been shown to allow simultaneous staining of both surface and cytoplasmic CD3 staining in Jurkat cells (20).

Unexpectedly, a small fraction (<2%) of cells stained intracellularly with several antibodies (particularly pRb, H3K9ac, and Ki-67) in the absence of methanol permeabilization or saponin treatment (with minimal effect on the median staining intensity of the population; Supporting Information Fig. S4). These cells were not apoptotic or necrotic at the time of fixation (as evidenced by staining for cisplatin (8), cleaved PARP, and cleaved caspase3), but did stain more intensely with the Pd barcode (Supporting Information Fig. S6). Thus,

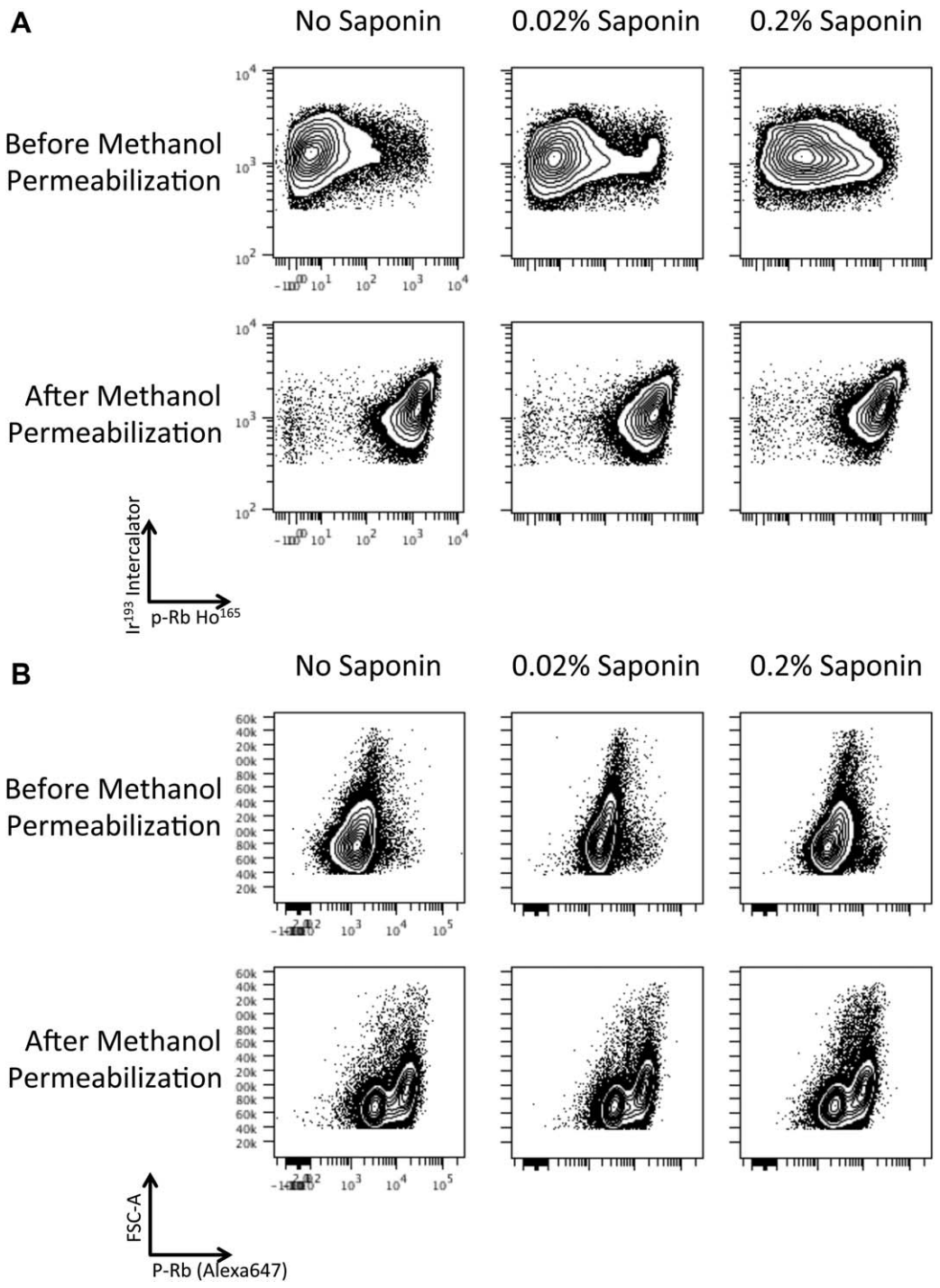

Figure 2. Transient partial permeabilization with saponin does not result in significant intracellular antibody staining with fluorescent or mass-tagged reagents. U-937 cells were fixed and treated with indicated saponin concentration and barcoded (representative plots from one of three experiments are shown). After barcoding and washing with cell staining medium, cells were stained with anti-pRb (S807/811) either before or after alcohol permeabilization with 100% methanol. (**A**) Barcoding with isotopically purified Pd isotopes, followed by staining with Ho165 conjugated anti-pRb (S807/811). (**B**) Barcoding with DyLight 800 and Pacific orange, followed by staining with Alexa 647 conjugated anti-pRb (S807/811).

this variability may be the result of membrane disruption during the washes performed after cell fixation. This staining variability was not observed for any of the intracellular markers following methanol permeabilization.

To confirm that partial permeabilization and barcoding did not affect surface immunophenotypic analysis, a freshly fixed and cryopreserved healthy human bone marrow sample was split into three aliquots each of which was subjected to

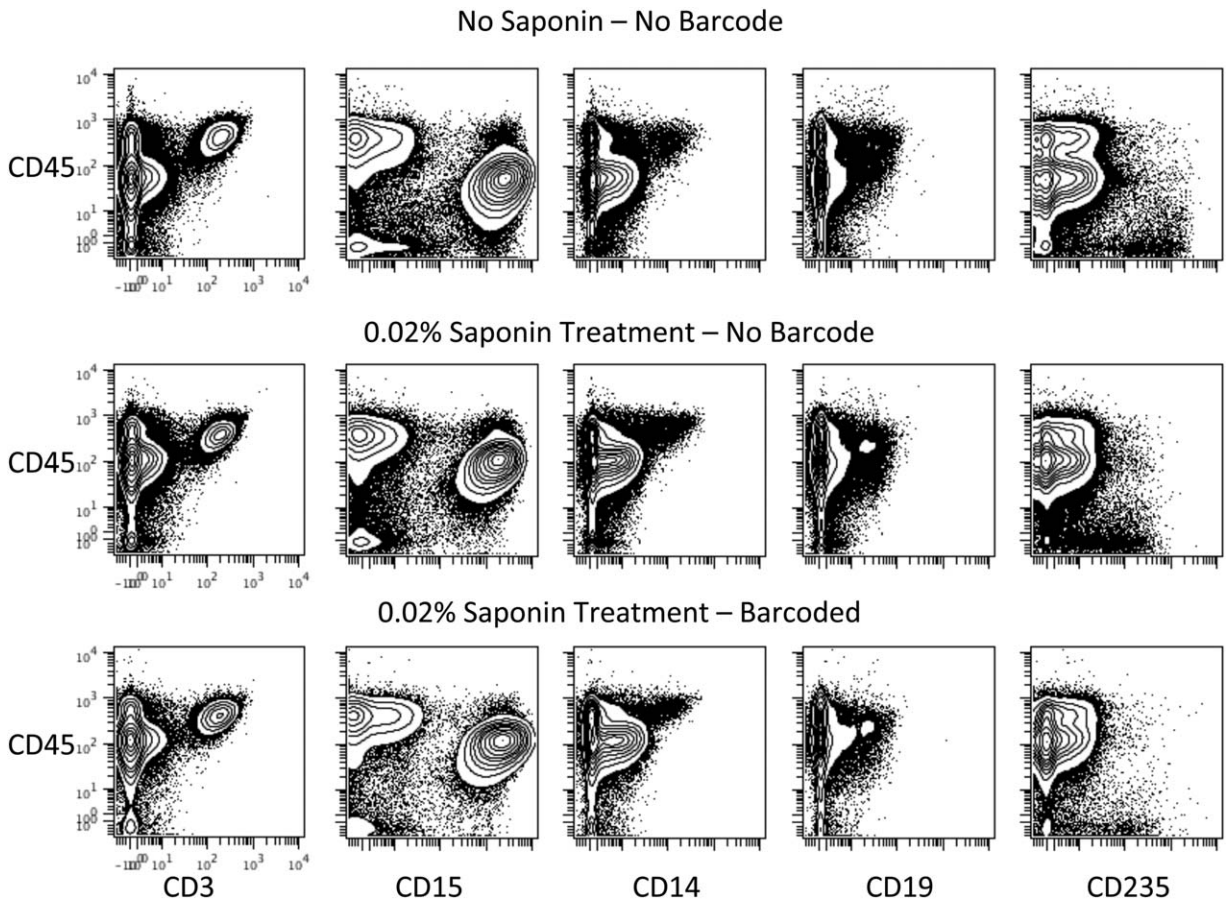

Figure 3. Surface staining of human bone marrow is equivalent with or without partial permeabilization and mass-tag barcoding. A single aliquot of freshly fixed and frozen human bone marrow was split into three tubes and then stained with a panel of 27 surface markers after (**A**) no treatment, (**B**) washing in 0.02% saponin, or (**C**) Pd isotope barcoding in 0.02% saponin.

three conditions: (i) PBS washes, (ii) partial permeabilization with 0.02% saponin without barcoding, and (iii) partial permeabilization with 0.02% saponin and Pd isotope barcoding. Each aliquot was then stained with the same 27-antibody panel. As shown in Figure 3 and Supporting Information Figure S7, all surface marker stains gave similar patterns under all three conditions with the exception of CD235, for which a decrease in the number of CD235 positive events was observed after saponin treatment, compared with the untreated sample. This decrease was most likely due to an increase in the lysis of residual red cells and red cell precursors in the presence of saponin (19). For some markers (CD45, CD44, CD71, CD16, and CD11b), small increases or decreases in their absolute staining intensity could be observed, but these did not significantly alter the staining pattern, frequency of cells expressing these markers, or the gating. To validate these findings further across the three conditions, viSNE plots of individual cells were generated. As shown in Supporting Information Figure S8, the viSNE plots for each marker were nearly identical across the different conditions and confirmed that no large changes in marker expression had occurred in rare cell populations (17).

To confirm that cell frequency within each gated population was not altered, each major cell lineage was gated from each sample under each of the three conditions (after gating out red cells). As shown in Table 1, cell frequencies were similar regardless of whether or not cells were partially permeabilized by saponin or partially permeabilized and then barcoded. However, there was a slight decrease in the frequency of mature granulocytes after barcoding (but not after saponin treatment without barcoding). This decrease was only found after barcode deconvolution and appeared to be due to the removal of granulocyte doublet events, which were somewhat more common than doublets of cells from other cell lineages (data not shown). The frequencies of each of the gated populations were very highly correlated between the three treatments. The population frequencies in the 0.02% saponin treated cells compared to the untreated cells revealed a (Pearson's *r*) correlation of 0.9995. The population frequencies in the 0.02% saponin-treated and barcoded cells compared to the untreated cells revealed a (Pearson's *r*) correlation of 0.9918.

Cell Surface Marker Staining After Barcoding Enables Detection of Subtle Immunophenotypic Differences Across Samples

To demonstrate the utility of this approach, peripheral blood leukocytes from four healthy donors were partially

Table 1. Frequency of gated immunophenotypic populations from normal human bone marrow with and without 0.02% saponin treatment and/or Pd isotope barcoding

GATE	NO SAPONIN	0.02% SAPONIN – NO BARCODE	0.02% SAPONIN – Pd BARCODE
T cells	14.5%	14.8%	16.6%
-CD8+ T cells	5.0%	5.0%	5.7%
B cells	7.7%	8.4%	8.1%
NK cells	4.2%	4.2%	4.9%
Mature monocytes	2.7%	3.5%	3.2%
Mature granulocytes	39.2%	40.3%	34.6%
Red cells	3.1%	2.7%	1.7%

permeabilized with 0.02% saponin and barcoded with a unique combination of three Pd isotopes. After barcoding, the donor samples were combined into a single tube, stained with a panel of 21 antibodies against surface markers, and analyzed by mass cytometry. Analysis of the mature monocyte population for the markers CD33, CD14, and HLA-DR revealed significant differences among donors as shown in Figure 4. Despite uniform sample collection and fixation conditions and simultaneous staining and data acquisition, median expression levels of CD33 varied by more than 10-fold between donor no. 116 and donor no. 103, and similar variation was observed in HLA-DR expression with median expression levels differing by more than 3-fold across donors (Fig. 4). There were also significant variations in CD11b (maximum median staining difference of 1.8 fold) but no significant differences in median cell length or median expression of CD45, CD4, or CD45RA (maximum fold differences 1.68, 1.35, 1.43, respectively; data not shown).

To confirm that these variations in expression were not due to technical issues, a second experiment was performed with four healthy human bone marrow samples. Each sample was aliquoted into 20–80 parts. Aliquots of each were analyzed on 2 consecutive days once a week for 3–4 weeks (donor no. 6 was analyzed eight times, while the others were each analyzed six times). As shown in Supporting Information Figure S9, analysis of the mature monocyte population demonstrated that this methodology has a high degree of technical precision. The coefficient of variation for the positive markers (CD33, CD11b, CD45, HLA-DR, CD38, and CD14) averaged 13.5% across the replicate samples. This small variation was largely the result of inter-day variation in staining intensity, as the relative expression levels of the samples compared to one another was highly consistent on any given day (Supporting Information Fig. S9). As with the first experiment, similar differences were observed across donors for CD33, HLA-DR and CD14 staining. For CD33, donor no. 6 displayed a nearly two-fold increase in average median CD33 staining compared to the other three samples (52 vs. 26, 28, and 30 counts; $P = 0.00067$ for each). For HLA-DR, highly significant differences were observed between each of the samples (all $P < 0.003$) with donor no. 4 having the highest average median expression at 120 counts and donor no. 5 having the lowest at 49 counts. Differences were also noted in median CD14 expression level. The high precision of this methodology, allowed the detection of statistically significant ($P < 0.05$)

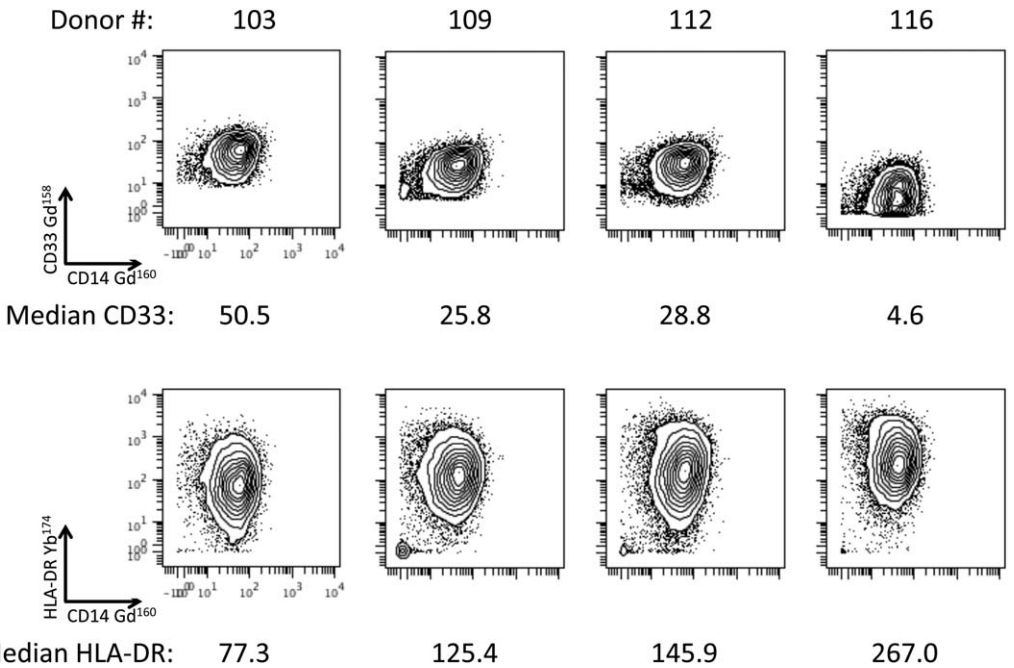

Figure 4. Mass-tag cellular barcoding prior to surface staining allows for characterization of subtle differences in monocyte surface marker expression between donors. Peripheral blood samples from four donors were barcoded using Pd isotopes in 0.02% saponin and then stained with a panel of 20 surface markers. Staining for the indicated markers is shown for the gated monocyte populations from each patient.

differences in average median CD14 expression as small as 23% between the monocytes of donor no. 5 and donor no. 6 (70 vs. 86 counts) and 26% between donor nos. 3 and 5 (88 vs. 70 counts; Supporting Information Fig. S9D). As in the peripheral blood experiment, CD45 expression was comparable between samples, confirming that the observed differences were not simply due to differences in cell size or sample quality. Thus, barcoding prior to surface epitope staining enabled the direct comparison and quantitation of both large and subtle differences in surface marker expression across samples at a level of detail not previously possible for samples stained and analyzed individually.

Discussion

The inclusion of barcoding in the experimental workflow of flow cytometry experiments offers significant advantages that enhance the quality of the data. These include a reduction in inter-sample variation, reduction in reagent consumption, as well as increased sample throughput. However, previously used protocols perform barcode staining only after alcohol permeabilization (1–6). We demonstrate here that transient partial permeabilization cellular barcoding (TPPCB) enables barcoding prior to alcohol permeabilization. Critically, this approach does not disrupt surface marker staining and does not result in staining of intracellular epitopes.

The optimal conditions determined in this study are a transient treatment of fixed cells with one wash of PBS plus 0.02% saponin, followed by barcode staining in a solution of PBS plus 0.02% saponin. After this barcoding step, multiple cell samples are combined into a single tube for surface antibody staining, permeabilization with methanol, and intracellular antibody staining. This methodology resulted in efficient cellular barcoding with either fluorescent or mass tag reagents with a low degree of staining variability (Fig. 1). By contrast, barcoding of fixed cells without partial saponin permeabilization resulted in a low level of barcode staining and a high degree of variability due to a small subset of cells that become brightly labeled (Fig. 1). This brightly stained population does not appear to be solely composed of dead or dying cells and may be the result of post-fixation membrane disruption (Supporting Information Fig. S3). Additionally, this population of cells (barcoded prior to saponin permeabilization) also demonstrates staining of intracellular antigens prior to permeabilization of any kind (Fig. 2, Supporting Information Figs. S4D, S4E, S4F, and S6A). Taken together the data suggest that these cells likely experienced some type of membrane disruption after fixation but prior to antibody staining. This protocol is focused on performing a transient saponin permeabilization in order to minimize any possible disruption of surface epitope staining. For protocols in which both surface and intracellular staining are performed in the presence of saponin, however, we have successfully maintained saponin in the cell staining media and wash buffers after barcoding and during the staining step with acceptable results.

Notably the TPPCB protocol described in this report minimized, to a considerable degree, variability in barcode dye staining (due to cell death or other membrane disruption), and treated cells did not exhibit significant differences in surface marker staining prior to alcohol permeabilization (Supporting Information Figs. S4A–S4C) or significant variation in intracellular staining performed after alcohol permeabilization (Supporting Information Figs. S4D–S4F). The TPPCB method described does not result in significant staining of intracellular antigens (Fig. 2, Supporting Information Figs. S4 and S5) and staining across a broad panel of markers was equivalent to cells that were not saponin treated or barcoded (Fig. 3, Supporting Information Figs. S7 and S8). Taken as a whole, these data demonstrate that TPPCB is superior to barcoding of untreated fixed cells and enables barcoding prior to alcohol treatment.

It is important to note that this protocol is designed for use with paraformaldehyde-fixed cells. The focus on fixed cells was intentional as the time required for the barcoding procedure ~1 h is significant relative to the biologic processes being studied in our typical experiments. In addition, as the procedure is best utilized when staining multiple samples (10–20) simultaneously, the time required to collect these samples and prepare them for the start of the experiment could also be significant. More importantly, the biological effects of exposing live cells to functionalized fluorescent barcoding dyes, or the heavy metals and functionalized chelators needed for mass-tag barcoding would be difficult to experimentally control. In addition, live cells could internalize or modify the barcoding reagent and this effect could potentially be cell type specific. For these reasons, this protocol has been developed for cells that have been biologically "frozen" by fixation, thereby avoiding these concerns at the expense of some decrease in the staining quality of certain surface markers (21).

The ability to detect aberrant expression of surface markers or activation markers is essential in clinical flow cytometry and in pre-clinical studies analyzing immune system function (22–25). The TPPCB protocol described in this report allows simultaneous staining and analysis of surface marker expression by fluorescence or mass cytometry and thereby enables direct comparison of the surface marker expression of multiple samples in a single experiment. By performing the barcoding step prior to staining for markers of interest, non-biologic differences in surface marker staining between samples are eliminated and the subsequent alcohol fixation and intracellular staining can also performed for all samples simultaneously in the same tube, eliminating any inter-sample variation at these steps as well.

To demonstrate the utility of this approach, we compared the expression of CD33 and HLA-DR in the mature monocytes of four different human donors (Fig. 4). Since each sample was fixed in a tightly controlled and consistent manner and then simultaneously stained and analyzed by mass cytometry, the differences in marker expression between them could be confidently attributed to expression differences in the samples (without concern for variation in staining cocktail volume, antigen-antibody ratio, or machine sensitivity). By directly comparing samples in a single experiment, both large and subtle differences in median marker expression could be

reliably quantitated. Additionally, cell frequencies within gated populations can be measured using the exact same gates for each sample (G.F., B.G., and G.K.B. unpublished observations), reducing the subjectivity of such comparisons. As a result of these attributes, this protocol results in highly reproducible antibody staining (Supporting Information Fig. S9) and small differences in expression levels can be detected with confidence. This TPPCB approach could thus allow for the detection of much smaller aberrant surface marker expression differences than is currently possible by traditional flow cytometry techniques.

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