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A CD45-Based Barcoding Approach to Multiplex Mass-Cytometry (CyTOF)

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● **Abstract**
CyTOF enables the study of the immune system with a complexity, depth, and multidimensionality never achieved before. However, the full potential of using CyTOF can be limited by scarce cell samples. Barcoding strategies developed based on direct labeling of cells using maleimido-monoamide-DOTA (m-DOTA) provide a very useful tool. However, using m-DOTA has some inherent problems, mainly associated with signal intensity. This may be a source of uncertainty when samples are multiplexed. As an alternative or complementary approach to m-DOTA, conjugating an antibody, specific for a membrane protein present on most immune cells, with different isotopes could address the issues of stability and signal intensity needed for effective barcoding. We chose for this purpose CD45, and designed experiments to address different types of cultures and the ability to detect extra- and intra-cellular targets. We show here that our approach provides an useful alternative to m-DOTA in terms of sensitivity, specificity, flexibility, and user-friendliness. Our manuscript provides details to effectively barcode immune cells, overcoming limitations in current technology and enabling the use of CyTOF with scarce samples (for instance precious clinical samples).   © 2015 The Authors. Published by Wiley Periodicals, Inc.

● **Key terms**
CD45; multiplexing; CyTOF; barcoding

HIGH throughput, multiplexed approaches are increasingly employed to dissect and unravel the complex multidimensionality of the immune system. Among these tools, cytometry by time of flight mass spectrometry (CyTOF) has largely expanded the capability for deep interrogation of the immune system, previously eminently relying on fluorophores. The number of isotopes that are detectable and amenable to CyTOF is currently over 34 (1), far more than the number of fluorophores that the current state-of-the-art fluorescence flow cytometry can discern. Furthermore, up to 100 isotopes (2) are potentially available for the detection of phenotypic markers. This approach has enabled the study of the immune system with a complexity, depth, and multidimensionality never achieved before.

However, there are limitations that prevent the utilization of CyTOF to its full potential, particularly in the context of samples with scarce cells available. Indeed, so far CyTOF has a cell transmission rate of 30% (1), due to the mechanical limitation of the device itself. Therefore, a large amount of cells is required per batch run to achieve meaningful results, which can be a severe constraint when dealing with limited clinical samples. Also CyTOF can only process about 500 cells per second, which is 0.02% compared with flow cytometry, resulting in variation of signal intensity over the time, which can increase the bias when large data is analysed (1). Finally, experiments carried out by CyTOF can be costly.

Barcoding has been employed on other technological platforms (3,4) for the simultaneous analysis of multiple inputs or samples (multiplexing) to maximize information throughput. Its application is of great potential in CyTOF as it allows samples to be processed in a single tube. Cells treated differently (for instance different

stimulations) can be combined into a single sample to generate meaningful data (5). This approach results in a significant decrease in the number of cells required per batch run. Consistency of staining as well as reduced bias during CyTOF runs are also improved as each sample is processed in the same tube at the same time. Finally, there is significant cost saving through the reduction of reagent usage (6).

To date, barcoding for CyTOF has employed maleimido-monoamide-DOTA (m-DOTA; (7)). This has provided a very useful tool to address some of the issues listed above. However, the use of m-DOTA has its inherent problems. Signal intensity generated by m-DOTA for each isotope may be low, thus compromising the differentiation of each individual cell population. As preparation of m-DOTA is done with a lyophiliser, it is hard to standardize the final concentration of the substance. In addition, signal intensity is significantly reduced after several freeze-thaw cycles. This increases uncertainty when samples are multiplexed.

As an alternative or complementary approach to m-DOTA, we reasoned that a ubiquitous cell membrane protein can be employed as a barcoding target. The target's surface expression has to be stable under various experimental conditions with reasonable expression levels for effective barcoding. Since CD45 fulfils the above criteria, it was chosen as a model antigen for this approach (8).

Another hurdle in this work flow is represented by the fact that traditionally debarcoding of mass cytometry data is performed using interactive software such as FlowJo (Tree Star, Ashland, OR), which is relatively simple in application. However, it fails to address a number of key issues. First, it requires manually defined cutoffs for each step, which results in operator bias. In addition, it is time consuming if multiple batch experiments need to be analyzed. Finally, FlowJo is a commercial software and requires a paid license. We have developed and made available an open-source auto debarcoding function in R language that addresses these issues.

MATERIALS AND METHODS

Antibody Labeling

Purified antibodies (lacking carrier proteins) were purchased from BD Sciences (Franklin Lakes, NJ), Biolegend (San Diego, CA), eBioscience (San Diego, CA), Abcam (Cambridge, UK), Invitrogen (Carlsbad, CA), or R&D systems (Minneapolis, MN), 100 μg of each antibody was labeled with heavy metal-loaded maleimide-coupled DN3 MAXPAR chelating polymers (DVS Science, Sunnyvale, CA) using the recommended labeling protocol from DVS Science.

Cell Stimulation

All studies were approved by the SingHealth Centralised Institutional Review Board (Singapore). Venous blood was obtained from one healthy human adult (27 years old). Peripheral blood mononucleated cell (PBMC) was isolated and stored at −80°C. Frozen human PBMCs were thawed, resuspended in complete medium (RPMI-1640 containing 10% FBS, 10 mM HEPES). For stimulation, freshly prepared 150 ng/ml of Phorbol 12-Myristate 13-Acetate (PMA; Cat No:

P8139; Sigma-Aldrich, St. Louis, MO) and 100 ng/ml of ionomycin (Cat No: I0634; Sigma-Aldrich, St. Louis, MO) were added and incubated at 37°C for 6 h. Freshly prepared 1× Monensin (Cat No: 420701; Biolegend, CA) and Brefeldin A (Cat No: 4506–51; eBioscience, CA) solution was added for the last 4 h of incubation.

Barcoding and Staining

CD45 antibodies (Cat No: 304002; Clone HI30; Biolegend, CA) were labeled with Tb159, Ho165, Tm169, and Lu175 (DVS Science, CA) as described; 5 ng of barcoding antibodies were added to the desired wells containing 1–3 million cells and incubated for 20 min. Following a wash with staining buffer (2% BSA in PBS), all barcoded samples were pooled into a single FACS tube (BD Sciences, NJ). This was followed by the cell staining procedure, which employs the protocols provided by DVS Science, including cisplatin (Cat No: 201064; DVS Science, CA) for live/dead cell staining, surface biomarker cocktail staining, intracellular marker staining and DNA intercalator-Ir (Cat No: 201192; DVS Science, CA) staining.

m-DOTA (Cat No: B-272-100; Macrocyclics, Dallas, TX) based barcode was prepared following the protocol described by Zivanovic et al. (9). In summary, m-DOTA barcodes were prepared at 2:1 molar ratio of m-DOTA/metal in 20 mM acetate buffer, pH 5.5, lyophilized and resuspended in DMSO as a 10 mM stock. For barcoding, a final concentration of 250 nM to 1 μM was adopted based on the titration results (Fig. S2). After cisplatin staining, cells were fixed and permeabilized before the barcode reagents were added. The cells were transferred into one single tube, followed by full panel antibodies cocktail staining and DNA intercalator staining.

Data Acquisition and Analysis

After staining and washing with distilled water, cells were diluted to a final concentration of 0.5 million cells/ml. This was achieved by adding a diluted four element beads solution and then loaded into a 96-well plate for autosampler application. CyTOF setting was based on the manufacturer's default. Briefly, cell length was set to a range from 10 to 150 and dual counting in "Instrument" mode was used. Washing steps were skipped between wells in the same sample. "fcs" files generated from the same mixture were concatenated and normalized by the pre-installed software provided by DVS Science.

FlowJo vX10.0 (CyTOF settings) was used for data analysis. Singlets were gated out based on a plot of DNA intercalator Ir193 vs. cell length. Subsequently cells negative to cisplatin were gated as live cells. After which each specific barcoded population was gated against DNA. Boolean gating was performed to obtain subgroup separation.

Auto Debarcoding R Function for Mass Cytometry Data

All the cutoffs in each step were defined in an automated fashion to eliminate operator bias (Fig. S1). The detailed procedure is described as below (using 4 bar-coding channels as an example).

Single cells were gated by identifying major population in event length and DNA. Major population was defined as two
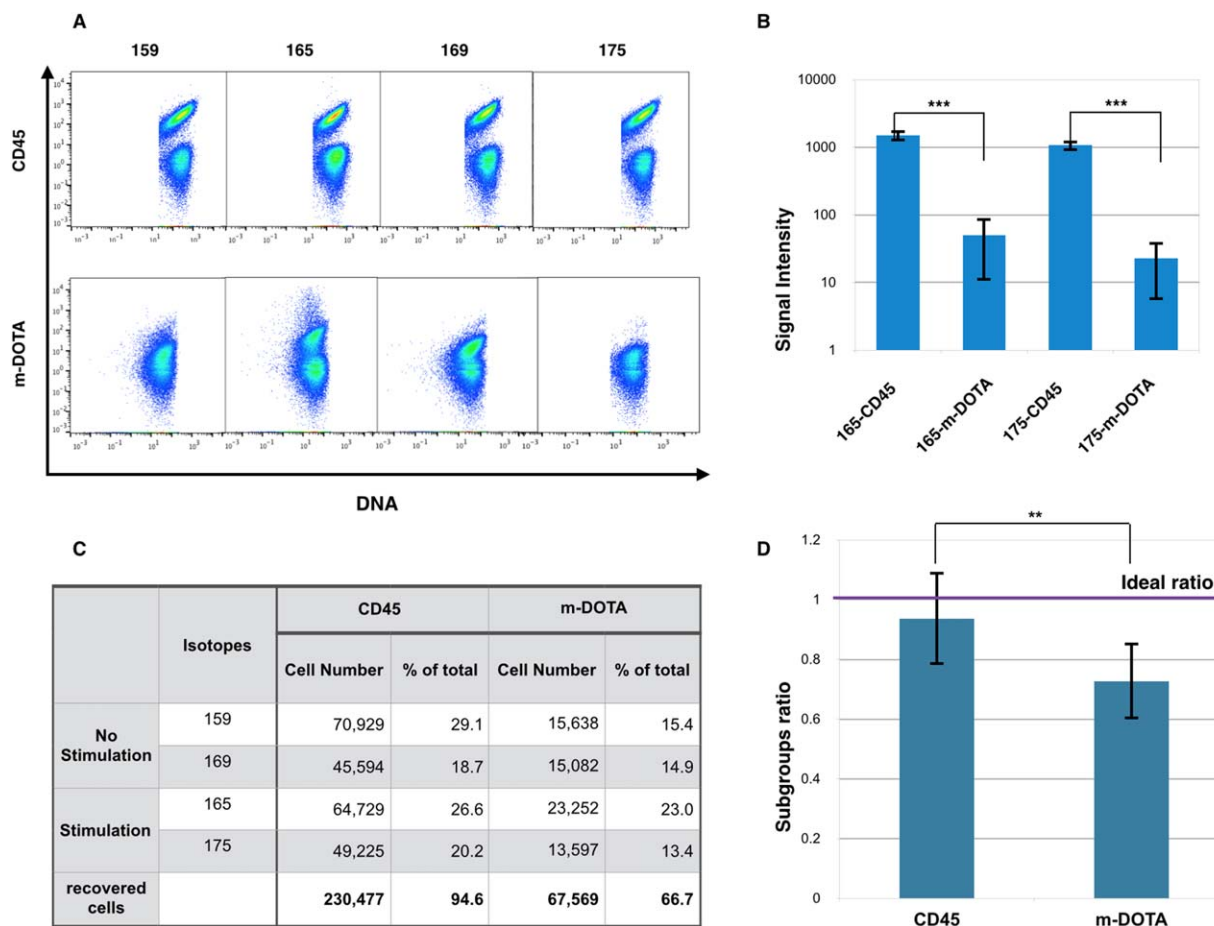
Figure 1. Ability of CD45 versus m-DOTA barcoding in subgroups differentiation in different channels. **A**: After singlets, live cells gating, the population positive to each isotopes. **B**: Cell number and population ratio of each subgroup in two barcoding methods. Data generated by Boolean gating in FlowJo. **C**: Log value of average signal intensity (five independent experiments) comparison between CD45 and m-DOTA barcodes in Ho165 and Lu175. CD45 intensity is 30× higher ($P = 0.00021$ in Ho165, $P = 0.00014$ in Lu175). The error bar (one standard deviation) in CD45 is consistently lower than mDOTA. **D**: Comparison of ratio of actual subgroup distribution versus ideal distribution between CD45 and mDOTA ($P = 0.0022$). Subgroup ratio is a function of actual subgroup proportion generated by Boolean gating divided by ideal proportion, that is, if n represents the number of equal population in an experiment, ideal proportion is $1/n$.

valley cutoff around highest peak in density plot. See details in Figure 3.

Live cells gated by identifying dead/live cutoff. The cutoff is defined as the first valley cutoff higher than highest peak.

Identify separate cutoffs in CD45A, CD45B, CD45C, and CD45D. The cutoff is defined as the lowest valley cutoff between two highest peaks. See details in Figure 3.

Gating all 16 samples (all combinations using CD45A-D) using DNA versus CD45A-D plot by all identified cutoffs.

The debarcoding R function is available on request.

Results and Discussion

CD45 Is Efficacious in Distinguishing Different Populations

The ability to resolve different cell populations was compared between the two barcoding methods. We found that, compared to m-DOTA, CD45 barcoding provided a highly distinct signal for each cell population resulting in a very clear differentiation (Fig. 1A). The average signal

intensity of two isotopes (Ho165 and Lu175) of five independent experiments using CD45 barcoding was at least 30 times higher than that of m-DOTA barcodes ($P < 0.001$, T test; Fig. 1B).

CD45 Barcoding Is Highly Efficient

The starting numbers of cells employed in each barcoding protocols were the same. However, we were able to demonstrate that CD45 barcoding successfully labeled 340% more cells (230,477 vs. 67569; Fig. 1C) than the same cells barcoded with m-DOTA. This is likely due to the difference in staining steps, as we are able to process the cells at an earlier time point, thus reducing cell loss during washing steps. This capability of preserving cell numbers is crucial in any CyTOF experiment that has to work with scarce clinical samples.

CD45 Barcoding Allows for the High Resolution Capability of Each Subpopulation

CD45 barcoding can effectively distinguish each subpopulation, achieving up to a ratio of 0.935 vs. the ideal
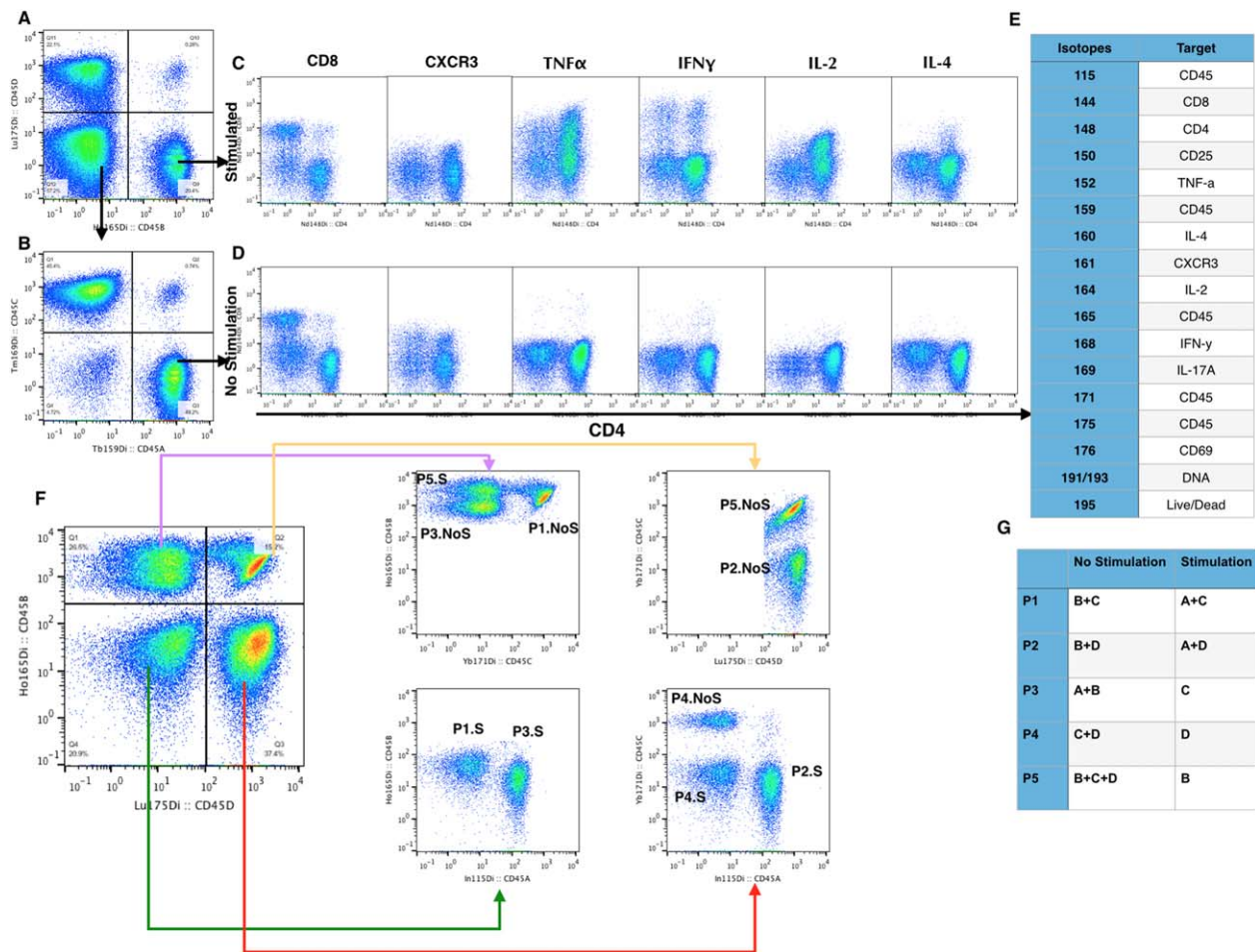
Figure 2. Efficacy in differentiating of subgroups. **A**: Three distinct PBMC populations were gated by two out of four CD45 barcodes. **B**: The population negative to two CD45 barcodes in (A) was further gated by another two CD45 barcodes. **C, D**: CD8, CXCR3, TNFα, IFNγ, IL-2, and IL-4 were gated from nonstimulated and stimulated population, respectively. **E**: Panel used for CD45 barcodes and targets labeling. **F**: Resolution of ten different cell populations employing four barcodes. **G**: Multiplexing strategy using four CD45 barcodes.

distribution of 1.0 (Fig. 1D). Comparatively, m-DOTA barcodes has a ratio of 0.72 ($P = 0.002$, T test). This difference may also be a consequence of a much lower background for CD45 barcoding. CD45 barcoding was performed under stimulated and unstimulated conditions with different surface cell markers as well as intracellular markers. We were able to obtain consistent results for intra and extra cellular markers, in both stimulated and unstimulated conditions (Figs. 2A–2D). Multiplexing samples by CD45 barcoding is a prepermeabilization method, allowing the cells to be multiplexed at the beginning of the whole staining procedure. Thus, surface markers expression will not affected by permeabilization (Figs. 2C and 2D).

CD45 Barcoding Works Well Under Multiplex Conditions

We subsequently employed the use of four CD45 isotope barcodes to differentiate ten individual cell populations and managed to debarcode them successfully (Fig. 2F and Table S1). The same high degree of resolution was maintained even with the expansion of use of CD45 barcoding under multiplexing conditions. CD45 barcoding performs well under

multiplex conditions, with the potential to barcode up to 2^N (N = Number of barcodes) samples (7).

The versatility of CD45 barcoding allows the researcher to employ this method to barcode a large variety of samples with ease. CD45 barcoding is suitable in most channels, providing a greater degree of freedom for panel arrangement. Stability of conjugated CD45 eliminates the need for tedious titration, which is usually required for m-DOTA barcoding.

Importantly, the high resolution and efficiency in cell labeling allows CD45 barcoding to be extended for use in multiple scenarios where samples may be scarce, that is, multiple patient samples, time points, or different conditions.

It might be argued that the use of the same CD45 clone conjugated to different metals in multiplexed barcoding is likely to result in an overall lower signal per channel as the different metal bound antibodies will be competing for the same epitope on the receptor. However, as demonstrated, the signal differentiation is still very clear despite employing multiplex barcoding strategy. This is likely due to the large amount of CD45 receptors available for binding, thus mitigating the reduction in signal strength when multiple barcodes are used.
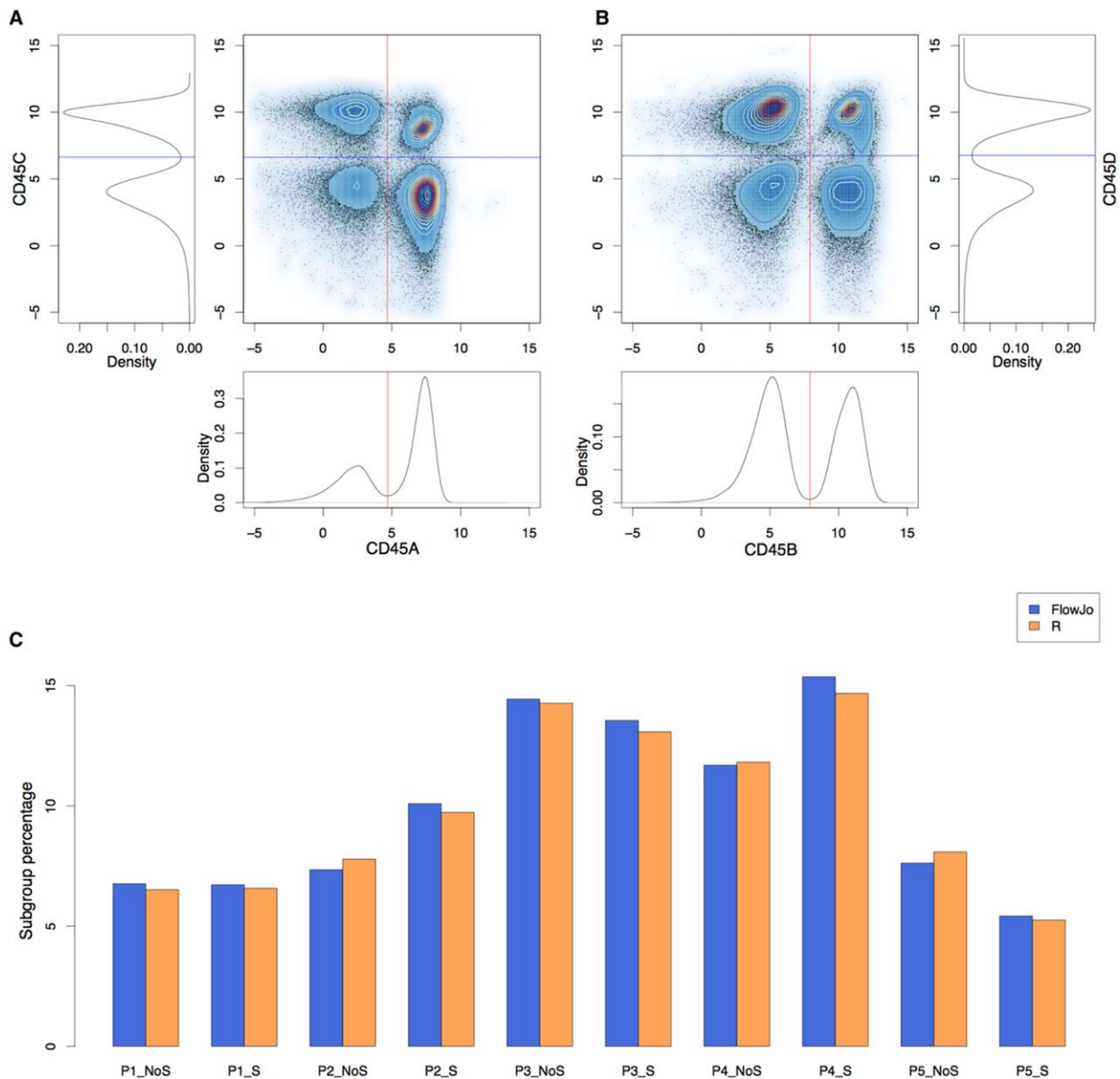
Figure 3. Diagram illustrates the use of automated gating in R. **A, B**: Scatter plots of CD45A versus CD45C and CD45B versus CD45D. The red and blue lines are auto-generated gates based on R. Each individual population has been gated out effectively by R auto generated gates. The density plots demonstrate how the auto gates are generated in R. The valley between the two peaks is detected and used as the reference cutoff for gating. **C**: Bar chart compares subgroup percentages generated by FlowJo Boonlean gating to R auto debarcoding for five subjects with stimulation (S) and without stimulation (NoS). R debarcoding is robust and can generate similar results as FlowJo.

Comparison of CD45 Barcoding Versus Other Barcoding Strategies

m-DOTA barcoding requires the presence of free thiol groups on cell for binding, which can pose an issue in mass cytometry experiments as this binding to free thiol groups might also inhibit binding of other antibodies. In addition most free thiol groups are found intracellularly, thus it becomes necessary to carry out permeabilization on cells before barcoding. This reduces the reagents saved as surface staining still has to be carried out before barcoding.

A recently developed technique of using different polymers (i.e., m-DOTA, m-EDTA, and m-DTPA) conjugated to palladium has been used for barcoding. By using nonlanthanide metals, it preserves the full spectrum of channels for CyTOF interrogation. In addition, it can be used in non PBMCs, such as lung and liver cell types. However, one major issue with palladium barcoding is the preferential binding of barcodes to dead cells (10). In addition, as it works on the same principle as lanthanide m-DOTA barcoding, it contends with the same issues as described earlier.

Automatic Debarcoding Function in R Language

Employing auto debarcoding in R language allowed us to resolve each individual target population well (Fig. 3).

The data obtained was comparable to that obtained via use of manual Boolean gating in FlowJo. The use of auto debarcoding in R language demonstrates an objective, repeatable gating strategy that can be rapidly completed and which can easily handle multiple batches of experiments. By having automatically generated cut-off gates, it eliminates operator bias. For this to work well, it requires a barcoding methodology that can provide a clear distinction between each subgroup population, which was successfully accomplished by the use of CD45 as a target antigen for barcoding. The debarcoding R function is fully available for use.

CONCLUSION

We would like to propose CD45 barcoding as a technically simple and easily reproducible method to achieve high sensitivity of differentiation in a multiplex setting, particularly when sample is scarce, including translational research settings with precious human samples.

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