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Expanding Mass Cytometry Discoveries and Applications with Cadmium-Labeled Antibodies

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Introduction

Mass cytometry, powered by CyTOF® technology, leads the field of high-parameter cytometric analysis with 135 independent channels (75–209 Da) available for signal detection. With the recent addition of seven cadmium (Cd) isotope tags (106, 110, 111, 112, 113, 114, 116 Da), there are now 56 commercially available tags for use in panel building and cell identification (Figure 1). We have previously established how Cd-labeled antibodies can be successfully integrated into existing high-parameter panels with ease[1]. Further, we have validated that Cd-labeled antibodies are compatible with existing Maxpar® cell staining protocols and products[2].

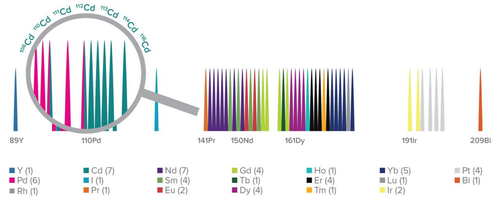


Figure 1. The advent of seven cadmium (Cd) isotopes has enabled mass cytometry to expand the multiparametric characterization of single cells to 56 parameters. This enables in-depth cellular analysis by cytometry and allows for enhanced panel expansion and greater design flexibility.

A particularly important integration and application of Cd-labeled antibodies into previously defined panels is seen with cell barcoding (BC). Cell barcoding enables sample multiplexing for single-cell analysis in mass cytometry (Figure 2), leading to improved data consistency and workflow efficiencies[3]. The Cell-ID™ 20-Plex Pd Barcoding Kit (Cat. No. 201060) utilizes six stable palladium (Pd) isotopes for barcoding up to 20 samples in a 6-choose-3 format. Importantly, a cell fixation and permeabilization step is required for this barcoding approach, and therefore barcoding after staining surface markers is recommended to preserve fixation- and/or permeabilization-sensitive marker expression (Figure 2B).

Live-cell barcoding is an attractive solution that allows for barcoding prior to marker staining while still maintaining optimal detection of these fixative-sensitive epitopes. Utilizing the Cd isotypes in a 7-choose-3 format would allow for barcoding up to 35 samples without interfering with the lanthanide mass-tag range (139–176 Da) for panel design. In this work, we have demonstrated that Cd-labeled antibodies may be used to support live-cell barcoding of human peripheral blood mononuclear cells (PBMC) with antibodies targeting the pan-leukocyte protein CD45.

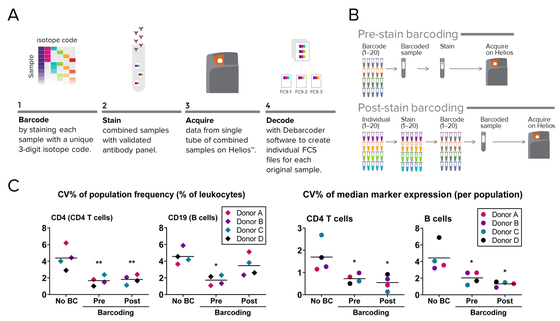


Figure 2. The benefits of cell barcoding. Cell barcoding leverages the high-parameter capabilities of mass cytometry by enabling sample multiplexing for single-cell analysis. **A)** Standard workflow for cell barcoding. **B)** Examples of 20-plex barcoding methods, illustrating the efficiency of pre-stain barcoding. **C)** Example of the impact of barcoding on staining and acquisition variability[3]. Population frequencies and median surface marker expression for four PBMC donors are shown. Each point represents the CV% of five technical replicates per donor, and the horizontal line represents the grand mean of CV per protocol. No BC = no barcoding protocol; Pre = pre-stain barcoding protocol; Post = post-stain barcoding protocol. While both pre- and post-stain barcoding methods enhance the efficiency of antibody staining and sample acquisition, the pre-stain barcoding protocol conserves reagent use and yields greater staining and acquisition consistency.

Materials and Methods

Sample Preparation, Staining and Analysis

- All seven cadmium isotopes were conjugated to purified anti-CD45 antibodies using the Maxpar MCP9 Antibody Labeling Kit (Figure 3A).
- Live-cell barcoding using cadmium-labeled anti-CD45 (Cd-CD45) antibodies was tested on frozen human PBMC from healthy donors (ePBMC® from Cellular Technology Limited).
- Thawed PBMC was resuspended in complete Gibco™ Advanced RPMI-1640 media (Thermo Fischer Scientific™) and stained with Cell-ID Intercalator 103Rh (Fluidigm) for live-dead cell discrimination.
- Prior to aliquoting 2 x 10^6 cells into 35 individual sample tubes, PBMC was first stained with pan-cell markers as internal controls: 170Er-beta-2-microglobulin (B2M) or 209Bi-CD47 for odd and even numbered samples, respectively.
- Cd-CD45 antibody barcoding mixtures were created according to the barcode key below and added to the corresponding sample tubes (Figure 3).
- After barcode staining, all 35 samples were combined into 1 tube and stained with the following counterstains according to the Maxpar Cell Surface Staining with Fresh Fix Protocol (PN 400276): 162Dy-CD66b, 154Sm-CD3, 142Nd-CD19.
- Samples were acquired on a Helios™ instrument running CyTOF Software v7.0.5189.
- The normalized FCS file was debarcoded using the open source software package Premessa (github.com/ParkerICI/premessa)[4].
- Debarcoded FCS files were analyzed using Cytobank (Cytobank.org).
- For additional information, contact your local Fluidigm field application specialist.

A

Fluidigm Cat. No.	Antibody (clone)	Isotope
3106001B	CD45 (HI30)	106Cd
3110001B	CD45 (HI30)	110Cd
3111001B	CD45 (HI30)	111Cd
3112001B	CD45 (HI30)	112Cd
3113001B	CD45 (HI30)	113Cd
3114001B	CD45 (HI30)	114Cd
3116001B	CD45 (HI30)	116Cd

Fluidigm Cat. No.	Antibody (clone)	Isotope
3170016B	B2-microglobulin (2M2)	170Er
3209004B	CD47 (CC2C6)	209Bi
3154003B	CD3 (UCHT1)	154Sm
3142001B	CD19 (HIB19)	142Nd
3162023B	CD66b (80H3)	162Dy

B

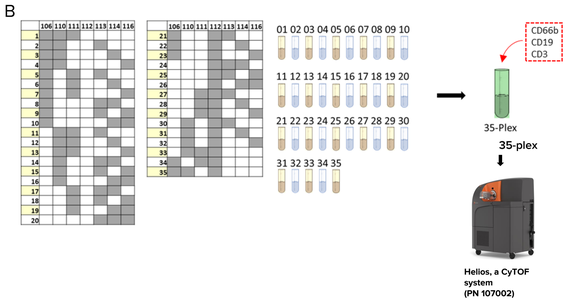


Figure 3. Experimental design for 35-plex live-cell barcoding using Cd-CD45 antibodies. A) List of antibodies that were used in this live-cell barcoding experiment. **B)** Barcode key selected for each sample. Gray boxes indicate the Cd-CD45 antibodies used for staining each sample. Prior to aliquoting into individual sample tubes, PBMC was stained with pan-cell markers, either 170Er-B2M (yellow shaded tubes) or 209Bi-CD47 (blue shaded tubes) as internal staining controls. After barcode staining, all 35 samples were combined into a single tube (green shaded tube) and stained with the following metal-tagged antibodies: 162Dy-CD66b, 154Sm-CD3, 142Nd-CD19.

[1] Fluidigm Canada Inc., Markham, Ontario, Canada

Results

Debarcoding the 35-Plex FCS File

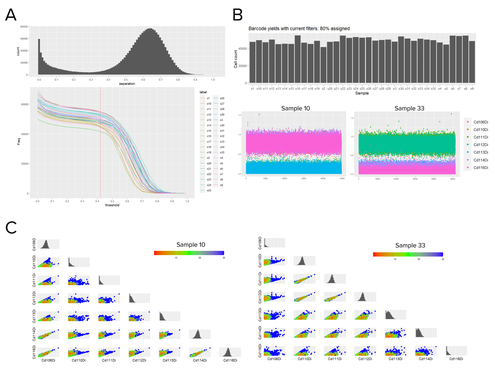
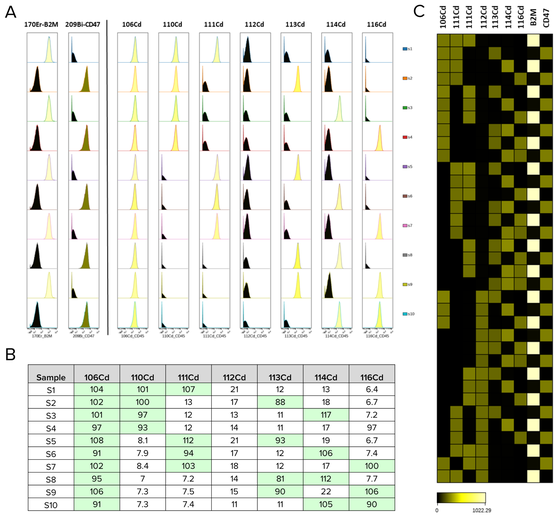


Figure 4. Premessa debarcoder results. A) We observed efficient separation between the positive and negative barcode channels as depicted in the histogram on the separation plot. We selected a minimum barcode separation of 0.42 and the maximum Mahalanobis distance (MD) was left as the default value of 30. **B)** These debarcoder settings resulted in an estimated barcode yield of 80%. Efficient visualization of the positive and negative barcode channel intensities for each event in the scatterplot was seen. Samples 10 and 33 are shown as representative samples. **C)** Finally, the expected staining patterns on biaxial plots were observed for each sample. Biaxial plot color scheme was set to heat map of MD values. Samples 10 and 33 are shown as representative samples.

Debarcoded Sample Analysis



B

Sample	106Cd	110Cd	111Cd	112Cd	113Cd	114Cd	116Cd
S1	104	101	107	21	12	13	6.4
S2	102	100	13	17	88	18	6.7
S3	101	97	12	13	11	117	7.2
S4	97	93	12	14	11	17	97
S5	108	8.1	112	21	93	19	6.7
S6	91	7.9	94	17	12	106	7.4
S7	102	8.4	103	18	12	17	100
S8	95	7	7.2	14	81	112	7.7
S9	106	7.3	7.5	15	90	22	106
S10	91	7.3	7.4	11	11	105	90

Figure 5. Debarcoded sample analysis. A) Histograms showing median signal intensity from all seven Cd-CD45, 170Er-B2M and 209Bi-CD47 antibodies on viable CD66b- PBMC (Samples 1–10 shown as representative samples). All debarcoded samples showed positive signal for the expected Cd-CD45 channels and had minimal spillover in the negative cadmium channels. Furthermore, 170Er-B2M and 209Bi-CD47 internal staining controls were observed as expected. **B)** Signal intensity analysis across the seven cadmium channels (Samples 1–10 shown as representative samples). Green shading indicates raw values from the 5th percentile for the Cd-CD45 markers used in the barcoded sample. No shading indicates raw values from the 95th percentile of unused Cd channels. A clear separation between the positive and negative values was observed and no overlap was seen for any given sample, indicating that the amount of spillover detected did not negatively impact the outcome of Cd-CD45 debarcoding. **C)** Heat map of all 35 debarcoded samples showing median signal intensities from all seven Cd-CD45, 170Er-B2M and 209Bi-CD47 antibodies on viable CD66b- PBMC. We observed the expected signal from Cd-CD45 compared to the barcode key in Figure 3 as well as the checkerboard signal for B2M and CD47 expression on odd and even samples that were used as internal staining controls.

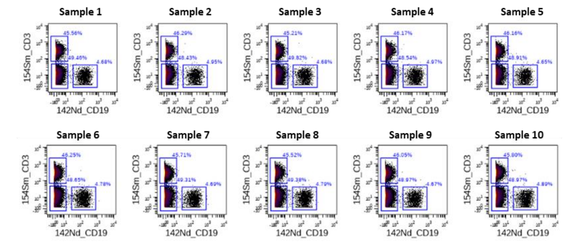


Figure 6. Functional population gating from debarcoded samples. CD3 vs. CD19 biaxial plots of viable CD66b- PBMC. Gates show percent populations of T cells (CD3+CD19-), B cells (CD3-CD19+) and non-T/B (CD3-CD19-) cell populations. We observed negligible differences in functional population gating for CD3+CD19- T cells and CD3-CD19+ B cells across all debarcoded samples, indicating that the 35 samples were effectively live-cell barcoded using Cd-CD45 antibodies. (Samples 1–10 shown as representative samples).

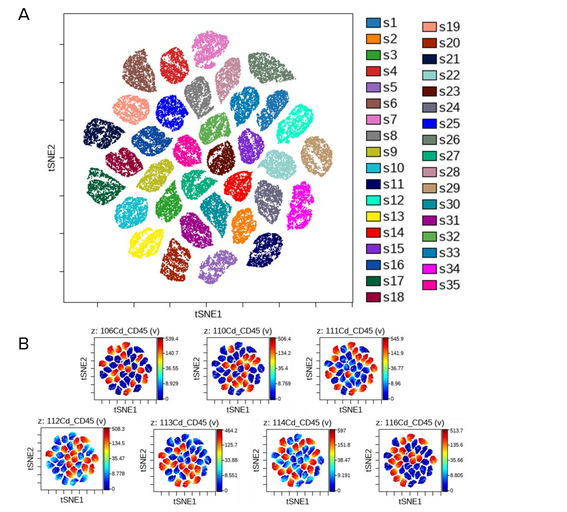


Figure 7. Visualization of debarcoded samples via high-dimensional viSNE analysis. As a means of visualizing all 35 debarcoded samples on a single plot, we used the viSNE dimensionality reduction tool. 100,000 events from the viable CD66b– PBMC population were proportionately sampled from all debarcoded files as well as the mixed, unbarcoded FCS file. All seven Cd-CD45 channels were used for the viSNE analysis. **A)** Color scheme showing overlay of all 35 debarcoded samples. We observed a compelling clustering of the debarcoded samples into 35 unique islands across the viSNE map. **B)** Color scheme showing signal intensity heat map of all seven Cd-CD45 channels of the mixed-unbarcoded sample. Note the expected Cd-CD45 signal is expressed in each particular island. For example, Sample 13 in lower-left corner of the viSNE map is positive for 110Cd, 111Cd and 116Cd.

Conclusions

- The advent of seven cadmium (Cd) isotopes has enabled mass cytometry to expand the multiparametric characterization of single cells to 56 parameters.
- The possibilities afforded by Cd-labeled antibodies can be seen in their ability to expand existing panels and support new applications, such as live-cell barcoding described in this work. Notably, this allows for pre-stain barcoding for fixation-sensitive targets.
- PBMC samples barcoded with all permutations of 7-choose-3 Cd-CD45 antibodies (35-plex) can be successfully mixed together and debarcoded using available open source software such as Premessa.
- Combined barcoded samples can be effectively stained in a single tube with equal functional gating outcomes across all debarcoded samples.
- Overall, we have demonstrated that using Cd-CD45 antibodies is an appealing choice for live-cell barcoding applications on PBMC and provides an alternative workflow to Pd barcoding for multiplexing mass cytometry samples.

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References

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2. Technical Note: Compatibility of Cadmium-Labeled Antibodies with Existing Protocols (FLDM-00086)
3. Application Note: The Benefits of Palladium Barcoding on Data Quality and Workflow (FLDM-00012)
4. Premessa Debarcoder method based on Zunder, E.R. et al. "Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm." *Nature Protocols* 10 (2015): 316–33