Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm

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Mass-tag cell barcoding (MCB) labels individual cell samples with unique combinatorial barcodes, after which they are pooled for processing and measurement as a single multiplexed sample. The MCB method eliminates variability between samples in antibody staining and instrument sensitivity, reduces antibody consumption and shortens instrument measurement time. Here we present an optimized MCB protocol. The use of palladium-based labeling reagents expands the number of measurement channels available for mass cytometry and reduces interference with lanthanide-based antibody measurement. An error-detecting combinatorial barcoding scheme allows cell doublets to be identified and removed from the analysis. A debarcoding algorithm that is single cell–based rather than population-based improves the accuracy and efficiency of sample deconvolution. This debarcoding algorithm has been packaged into software that allows rapid and unbiased sample deconvolution. The MCB procedure takes 3–4 h, not including sample acquisition time of ~1 h per million cells.

INTRODUCTION

Barcode multiplexing

As a general approach, pooled sample analysis has been used to improve the efficiency and comparability of a diverse range of biological assays, including microsphere-based ELISA[1] and high-throughput DNA sequencing[2,3]. For these applications, assay-specific identifiers such as fluorochrome combinations or oligonucleotide sequences are used as barcodes to uniquely label each sample, and the barcoded samples are pooled together for processing and measurement. Multiplexing in this manner eliminates sample-to-sample assay variability, increases assay throughput and reduces reagent consumption. After pooled measurement, the uniquely identifiable barcodes are used to recover the individual samples for further analysis.

This multiplexing strategy was adapted to flow cytometry by use of the fluorescent cell barcoding (FCB) technique, which uses unique combinations of cell-reactive fluorophores to covalently label cell samples before pooled antibody staining and flow cytometry analysis[4]. Mass cytometry, a recently developed variation of flow cytometry, uses rare earth metal isotopes instead of fluorophores as detection reagents, allowing over 40 simultaneous antibody-based measurements at the single-cell level[5]. The principles of FCB were extended to mass cytometry by the MCB technique, which uses cell-reactive metal chelators to covalently label cell samples with combinatorial barcodes[6].

Advantages and disadvantages of MCB

Both FCB and MCB use a single antibody cocktail to stain all samples simultaneously within a single tube, ensuring that all samples are exposed to the same antibody concentration at the same cell density. This uniform antibody exposure removes tube-to-tube variability from the assay, and it is especially important when antibodies are used at nonsaturating concentrations, as is often the case with mass cytometry because antibody concentrations must be titrated low enough to prevent ion detector saturation.

Analysis of multiplexed samples offers additional benefits that are specific to mass cytometry. The ion detection sensitivity of a mass cytometer will drift during instrument use and vary after each maintenance, and although this effect can be mitigated by normalization using bead standards[7], measuring samples after pooling further reduces intersample variability. In addition, the sample introduction loop of a mass cytometer is a potential source of carryover between samples, but the possibility of sample cross-contamination is bypassed by MCB because the samples are individually labeled with a unique barcode. Further improvements to MCB described here include (i) palladium-based cell labeling reagents, (ii) a combinatorial doublet-filtering scheme and (iii) an improved barcode deconvolution algorithm implemented as a software application, all of which markedly improve the quality of mass cytometry data.

One drawback to the previously described MCB method is that paraformaldehyde (PFA) fixation and methanol permeabilization must be performed before the antibody staining step, which can adversely affect the quality of antibody staining for some epitopes. In our experience, ~50% of cell surface epitopes are adversely affected by methanol treatment, and <5% are adversely affected by PFA treatment. To address this problem, we have recently modified the MCB protocol to permit barcode staining before methanol permeabilization, allowing methanol-sensitive surface markers to be assessed in combination with MCB multiplexing[8].

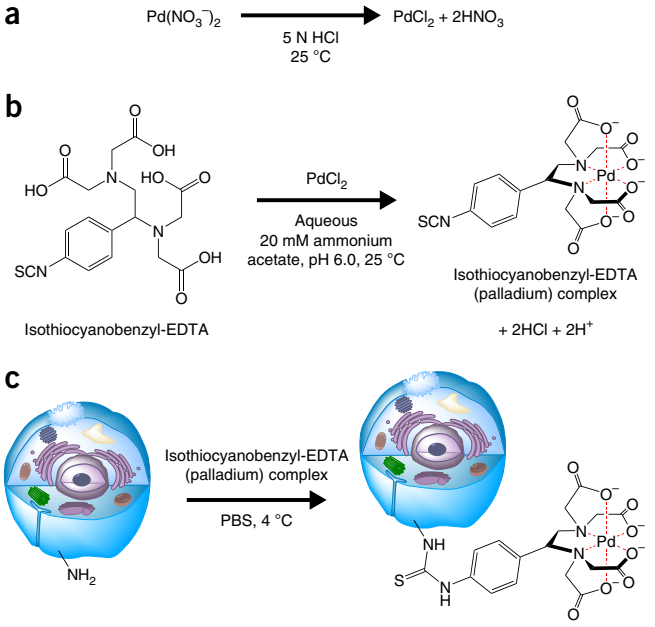


Figure 1 | Isothiocyanobenzyl-EDTA(palladium) MCB cell labeling reagent. (**a**) Palladium nitrate is converted to palladium chloride after dissolving in 5 N HCl. (**b**) Palladium chelation by isothiocyanobenzyl-EDTA. (**c**) Cell labeling by the isothiocyanobenzyl-EDTA(palladium) chelate.

This modified protocol relies on transient permeabilization with saponin, and it is included here as an alternate procedure. If PFA-sensitive epitopes must be used for an experiment, there are two options that still allow for MCB multiplexing. One option is to identify another antibody clone that recognizes a different, PFA-compatible epitope on the same marker of interest. A second option is to perform an initial stain with antibodies against all PFA-incompatible epitopes before PFA fixation, followed by the remaining MCB protocol as described here, therefore still gaining the advantages of sample multiplexing for all PFA-compatible antibodies.

Palladium-based MCB reagents

Lanthanide-based MCB reagents perform well as cell labeling reagents[6], but their utility for mass cytometry analysis is limited in two ways. First, lanthanides are used as antibody tags for mass cytometry, and therefore their use as MCB reagents reduces the number of antibody-based measurement parameters available. Second, MCB lanthanide reagents may interfere with other measurement channels owing to isotopic impurity of enriched lanthanide isotopes and owing to mass spectrometry effects. These effects include the '+1 effect,' which is due to the time-of-flight (TOF) mass trace distribution being skewed toward larger mass and overlapping with the next mass unit integration window, and the '+16 effect' of oxidation during ionization in the plasma ion source. These contaminating effects are typically minor, but they become more pronounced when a high-intensity MCB signal spills over into a low-intensity antibody signal.

In order to avoid both measurement channel cross talk and the reduction of antibody measurement channels, palladium was identified as a potential mass tag for MCB. As palladium is not compatible with the diethylene triamine pentaacetic acid (DTPA)-based polymer that is used for antibody labeling, it is not used for mass cytometry antibody measurements[9]. Palladium has six stable isotopes with masses of 102, 104, 105, 106, 108 and 110 amu, which are commercially available with enriched purities of 91%, 96%, 98%, 99%, 99% and 99%, respectively. These isotopes fall well below the 139–176 amu mass range of the lanthanides, and therefore their use in MCB reagents does not impinge upon lanthanide-based antibody detection. However, Pd110 does overlap with the Cd110 present in quantum-dot–conjugated antibodies, which are therefore not recommended for use in combination with the palladium-based MCB protocol. All six palladium isotopes were obtained in their 2+ charge state as nitrate salts, and dissolved in 5 N HCl (**Fig. 1a**). The addition of the bifunctional molecule isothiocyanobenzyl-EDTA yielded a palladium chelate (**Fig. 1b**), which may be subsequently used to covalently label cells (**Fig. 1c**).

For MCB multiplexing, a binary labeling strategy is used in which each sample is either positive or negative for each of the six palladium isotopes (**Fig. 2a**), and unique combinations of these positive and negative labels are used as sample-identifying barcodes. The isothiocyanobenzyl-EDTA(palladium) chelates label cells rapidly in PBS, reaching completion between 0.5 and 1 min at 4 °C (**Fig. 2b**). Care must be taken to wash excess FBS or BSA away from the samples before MCB labeling, because

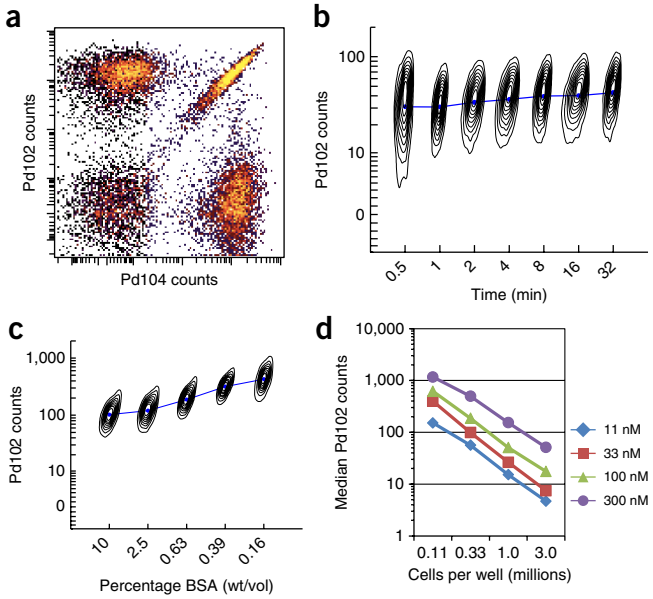


Figure 2 | MCB cell labeling by the isothiocyanobenzyl-EDTA(palladium) chelate. (**a**) Binary MCB labeling of PFA-fixed, methanol-permeabilized cells. (**b**) One million PFA-fixed, methanol-permeabilized cells were incubated with 100 nM isothiocyanobenzyl-EDTA(palladium) at 4 °C for the indicated times. Median counts are shown as connected blue circles, and they are overlaid on individual contour plots for each sample with Ir-intercalator along their hidden *x* axes. (**c**) One million PFA-fixed, methanol-permeabilized cells were mixed with the indicated concentrations of BSA before incubation with 300 nM isothiocyanobenzyl-EDTA(palladium) at 4 °C for 30 min. Median counts are shown as connected blue circles, and they are overlaid on individual contour plots for each sample with Ir-intercalator along their hidden *x* axes. (**d**) PFA-fixed, methanol-permeabilized cells were incubated at the indicated cell densities with the indicated isothiocyanobenzyl-EDTA(palladium) concentrations at 4 °C for 30 min.

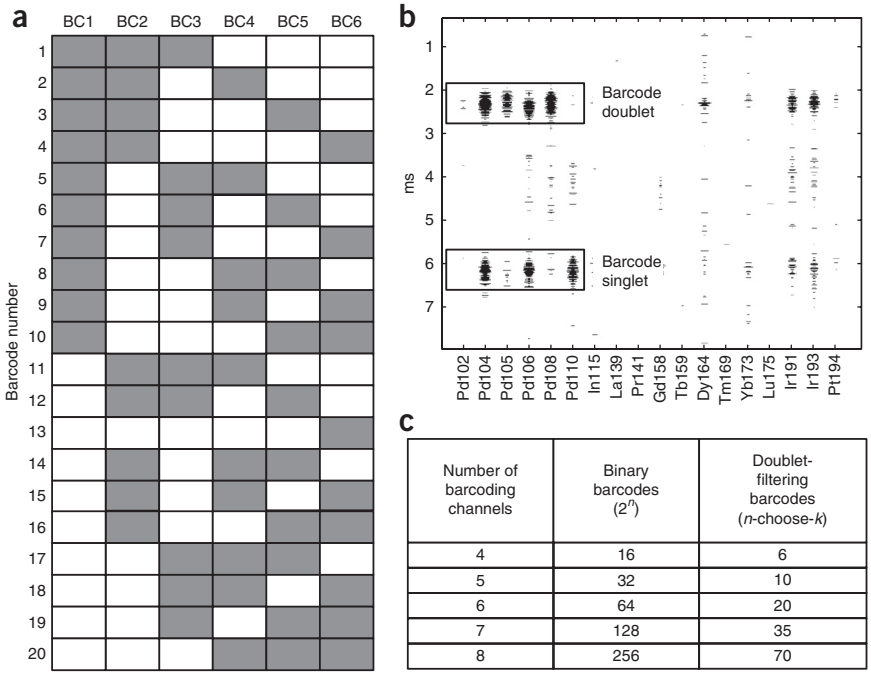


Figure 3 | Doublet-filtering MCB scheme. (**a**) The 6-choose-3 doublet-filtering barcode scheme. Each well is positive (gray) and negative (white) for exactly three out of the six MCB reagents. (**b**) Examples of a barcode singlet (three positive barcode channels) and a barcode doublet (>3 positive barcode channels) as seen in the time-of-flight spectra used to visualize cells while acquiring data at the instrument. (**c**) Maximum number of available barcodes as a function of the number of barcode channels for both doublet-filtering *n*-choose-*k* schemes and for the nonredundant 2^n binary scheme.

c

Number of barcoding channels	Binary barcodes (2^n)	Doublet-filtering barcodes (*n*-choose-*k*)
4	16	6
5	32	10
6	64	20
7	128	35
8	256	70

nucleophile-containing proteins will compete for isothiocyanate reactivity and decrease cell labeling intensity (**Fig. 2c**). In addition, care must be taken to quantify the number of cells in each sample to be labeled, because the isothiocyanobenzyl-EDTA(palladium) labeling reaction reaches completion rapidly in a stoichiometric manner, and therefore it is highly sensitive to differences in cell number (**Fig. 2d**). Although the EDTA-palladium dissociation rate is permissible for metal exchange between chelating groups on the timescale of days, in practice this effect and any resulting cross-contamination between labeled cell samples is observed to be negligible.

Doublet-filtering barcode scheme

In mass cytometry, physical or coincident doublets will result in the false interpretation of two or more cells as a single cell. Physical doublets occur when multiple cells are physically attached owing to some form of cell-cell interaction or owing to incomplete enzymatic separation of cells derived from tissue or adherent culture. Coincident doublets occur when physically separate cells pass through the instrument in too quick a succession to be identified as separate events by the mass cytometer's cell detection software. Cell doublets will confound any single-cell analysis, including algorithms such as SPADE[10], visNE[11] and Citrus[12], and they are especially problematic for the investigation of rare or uncharacterized cell types.

To improve doublet removal from mass cytometry data sets, a doublet-identifying barcode scheme was developed. Error-correcting codes such as Hamming codes have been used in barcode design for error detection and correction in multiplexed high-throughput sequencing[13]. This strategy relies on redundancy in the sample barcode: if one measurement is incorrect, the error is detected and it may even be corrected depending on the level of barcode redundancy. Here, an *n*-choose-*k* barcoding scheme was chosen as the minimally redundant code that allows doublet identification and removal while maximizing the number of unique combinatorial identifiers, where *n*-choose-*k* equals $n!/(k!(n-k)!)$.

With six palladium isotopes available for MCB labeling, a 6-choose-3 barcoding scheme was used, in which each of the 20 individual barcodes are positive for exactly 3 of the 6 possible palladium MCB

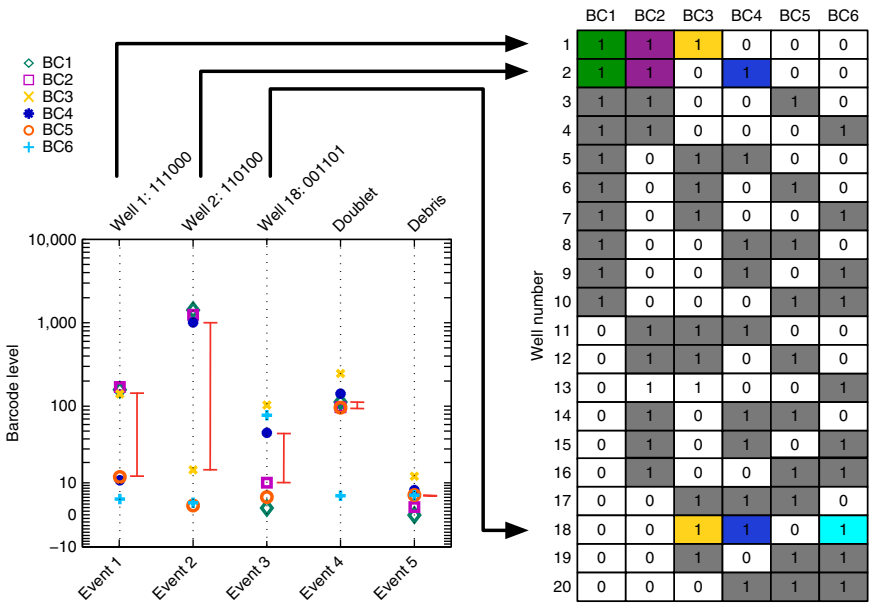


Figure 4 | Single-cell barcode deconvolution. Five events from a 6-choose-3 MCB-multiplexed FCS file are shown in single-cell format displayed on a vertical dashed line. Events 1–3 correspond to barcode singlets as indicated by the barcode key, Event 4 is a barcode doublet and Event 5 is classified as debris. The red line segments indicate 'barcode separation', assuming the 6-choose-3 scheme, which is always set as the distance between the third- and fourth-highest barcode intensities. Without this assumption, the last two events would have larger barcode separations but would still be discarded because their barcodes would not match any in the 20-sample scheme.

reagents (**Fig. 3a**). In this scheme, the combination of any two barcodes resulting from a cell doublet yields an 'illegal' barcode with at least four barcode channels that are positive—i.e., one that cannot belong to a single-cell event (**Fig. 3b**). This scheme cannot detect coincident doublets between two cells from the same sample, but every other combination can be detected and removed. By this reasoning, 95% of the coincident doublets can be removed when using the 6-palladium 20-sample scheme, because for any single cell within a coincident doublet the other cell within the doublet will have a different barcode 19 out of 20 times. However, in practice, the actual doublet removal rate may be <95% depending on the number of physical doublets present in the samples.

One tradeoff with this doublet-filtering scheme is a reduced number of cell samples that can be multiplexed by MCB. For every n metals used for barcoding, only n-choose-k combinations are available, which is maximized when $k = \text{floor}(n/2)$, rather than the 2^n that are available with a nonredundant binary scheme (**Fig. 3c**). With six palladium MCB reagents, the n-choose-k scheme results in 20 possible samples rather than 64. An appropriate barcode scheme must be chosen for each experiment on the basis of (i) the number of measurement channels available for barcoding, (ii) the desired number of samples to be multiplexed, and (iii) the importance of doublet removal for sample analysis.

Single-cell deconvolution algorithm

Traditionally, individual samples have been recovered from FCB and MCB data sets using Boolean combinations of manually drawn gates, but this method is not ideal because cell events that fall outside these gates must be discarded. This problem is exacerbated, and cell yield is made even lower, when there is

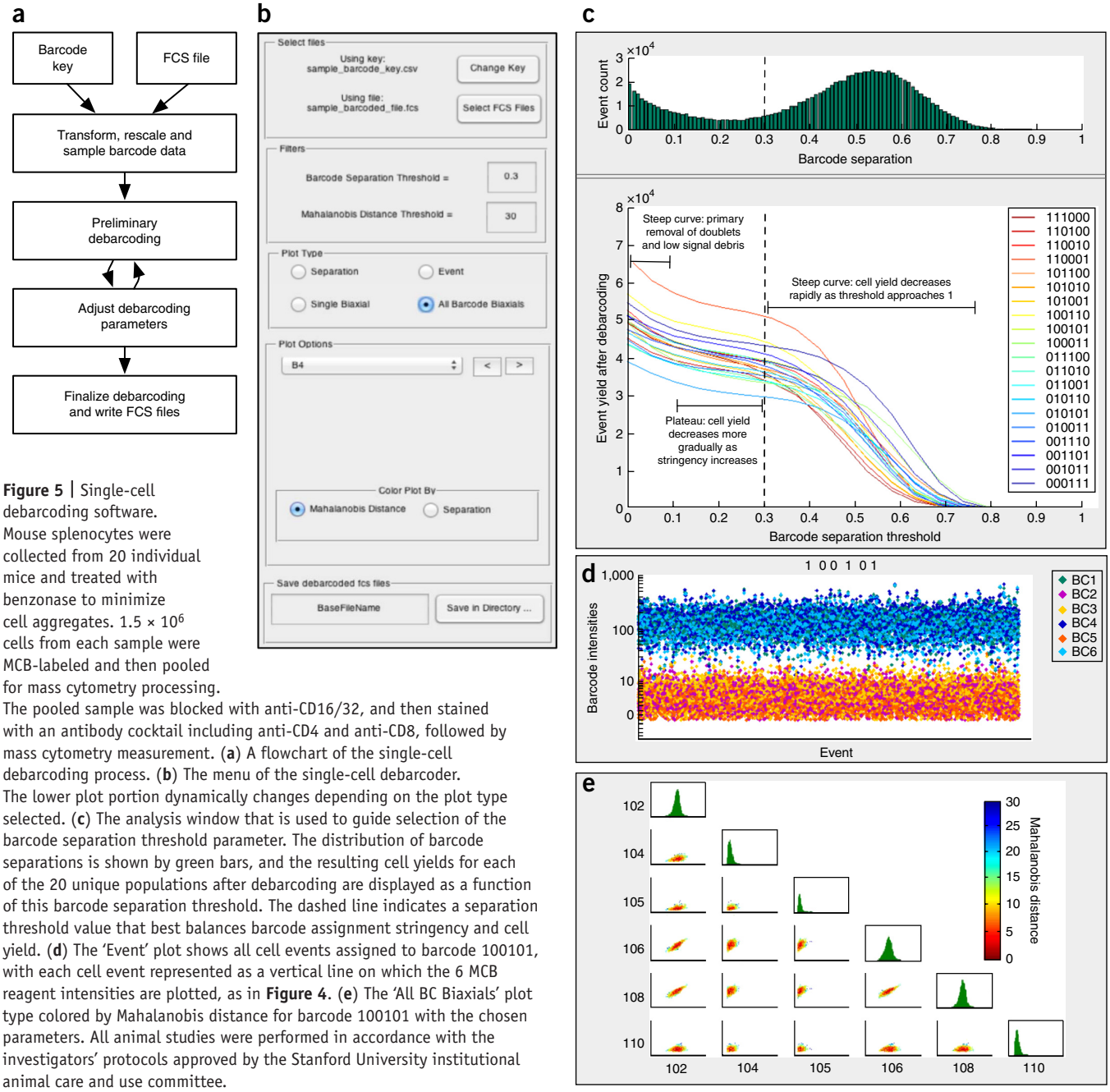


Figure 5 | Single-cell debarcoding software. Mouse splenocytes were collected from 20 individual mice and treated with benzonase to minimize cell aggregates. 1.5 × 10^6 cells from each sample were MCB-labeled and then pooled for mass cytometry processing. The pooled sample was blocked with anti-CD16/32, and then stained with an antibody cocktail including anti-CD4 and anti-CD8, followed by mass cytometry measurement. (**a**) A flowchart of the single-cell debarcoding process. (**b**) The menu of the single-cell debarcoder. The lower plot portion dynamically changes depending on the plot type selected. (**c**) The analysis window that is used to guide selection of the barcode separation threshold parameter. The distribution of barcode separations is shown by green bars, and the resulting cell yields for each of the 20 unique populations after debarcoding are displayed as a function of this barcode separation threshold. The dashed line indicates a separation threshold value that best balances barcode assignment stringency and cell yield. (**d**) The 'Event' plot shows all cell events assigned to barcode 100101, with each cell event represented as a vertical line on which the 6 MCB reagent intensities are plotted, as in **Figure 4**. (**e**) The 'All BC Biaxials' plot type colored by Mahalanobis distance for barcode 100101 with the chosen parameters. All animal studies were performed in accordance with the investigators' protocols approved by the Stanford University institutional animal care and use committee.

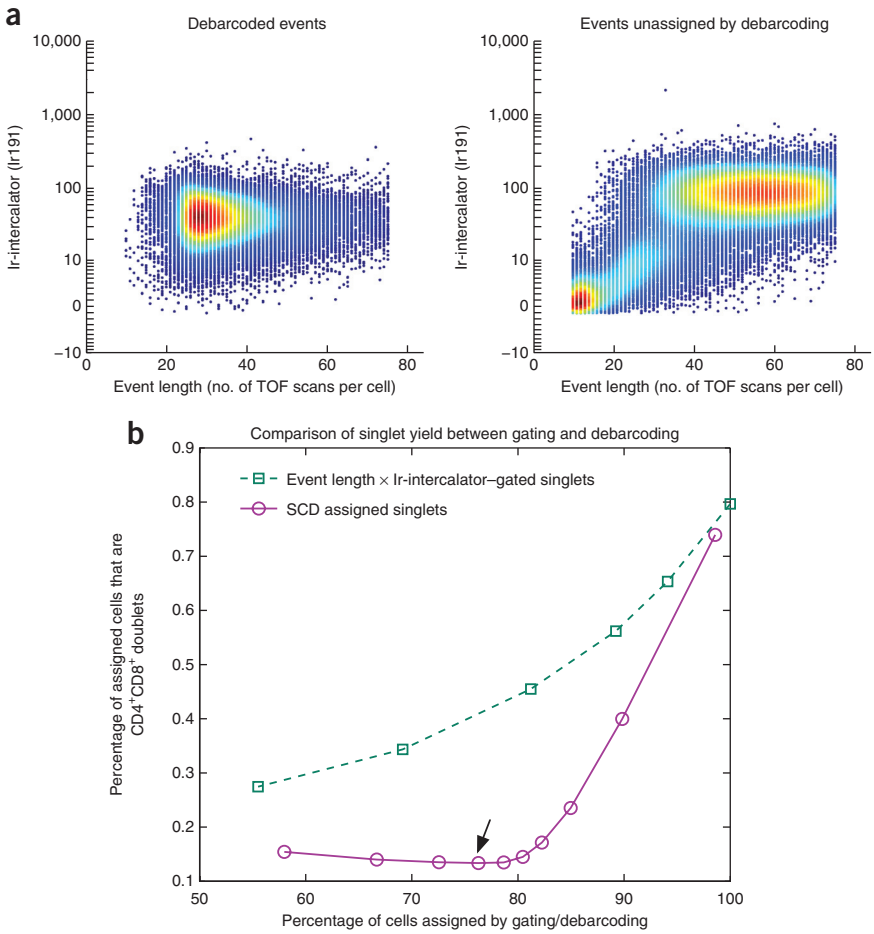


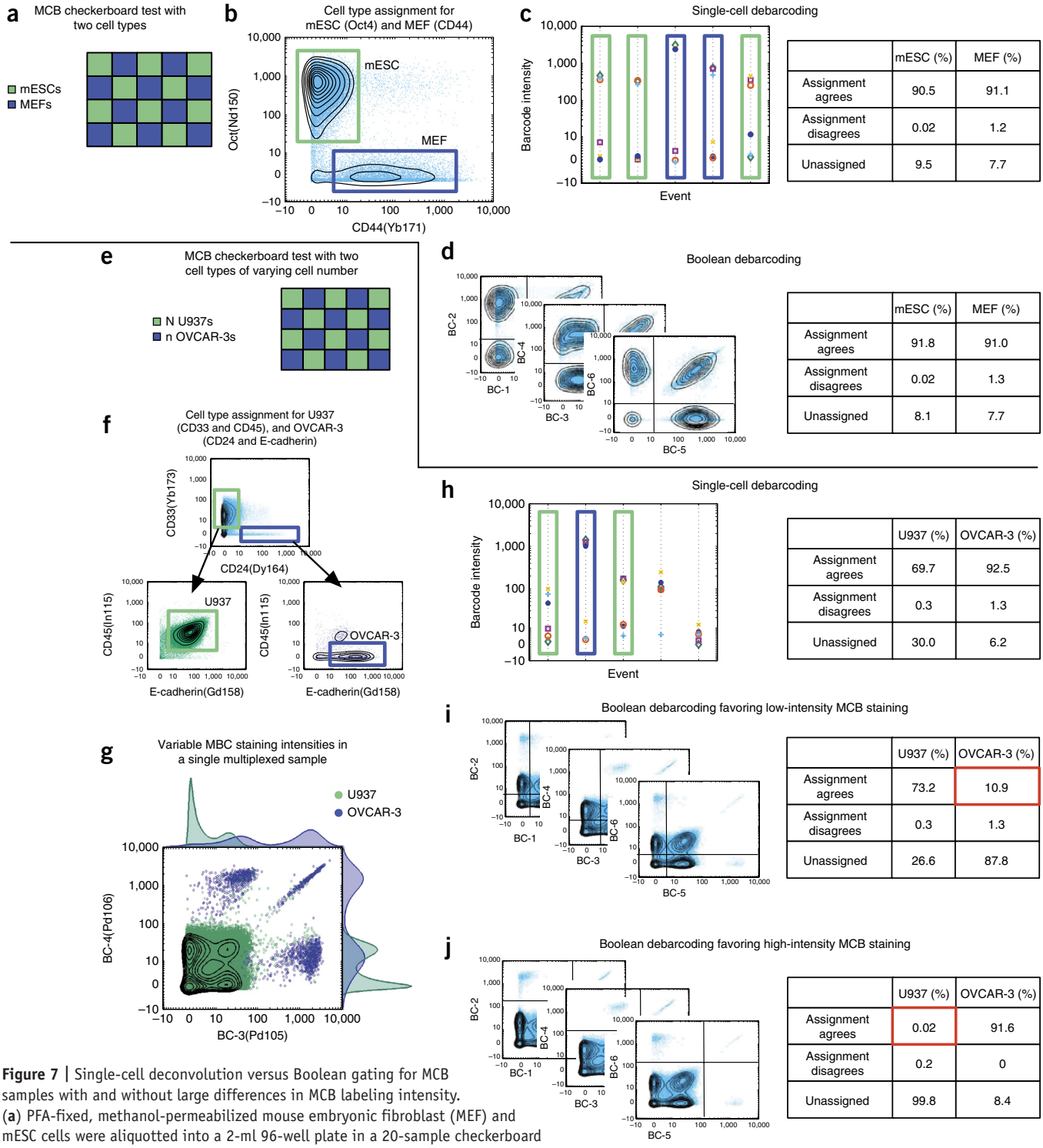
Figure 6 | Doublet removal with the 6-choose-3 MCB scheme and single-cell debarcoding. (**a**) Biaxial plot of event length × Ir-intercalator of events that were assigned a barcode, and of events that were left unassigned. (**b**) Percentage of cells assigned by gating (green squares) or debarcoding (purple circles) versus percentage of assigned cells that are $CD4^+CD8^+$ doublets. The different yields were acquired by variable event length × Ir-intercalator gates (green squares) or debarcoding threshold stringency (purple circles). The arrow indicates the debarcoding parameters used in **Figures 5** and **6a**.

variability in barcode staining intensity between pooled samples. This variability can be caused either by differences in cell number between samples without appropriate adjustment of MCB reagent (**Fig. 2d**) or by differences in cell size within samples (**Supplementary Fig. 1**), and this variability may lead to systematic depletion of certain cell types or samples from the data set during deconvolution. To address this problem, an alternative deconvolution algorithm was developed that treats each cell individually instead of using gates to demarcate populations of cells. This deconvolution strategy is termed single-cell debarcoding (SCD).

The SCD algorithm first rescales and then sorts the barcode intensities for each cell, in order to identify the largest barcode separation between adjacent barcode levels. This barcode separation is used as a boundary to define which barcode channels are 'positive' and 'negative' for each individual cell (**Fig. 4**). Cell events are assigned to a sample when they contain (i) a barcode separation that is larger than a threshold value and (ii) positive barcode channels corresponding to a combination used in the barcode scheme. Cell events remain unassigned if they contain (i) a barcode separation that is lower than the defined threshold, such as low signal debris, or (ii) positive barcode channels that do not correspond to a barcoded population, such as doublets. When an *n*-choose-*k* doublet-filtering barcode scheme is used, the SCD algorithm uses the *k* highest and *n*-*k* lowest barcode channels instead of the largest barcode separation to assign positive and negative barcode values, and the barcode separation threshold is applied to the difference between the *k*th and (*k*–1)th highest normalized barcode intensities. Once preliminary barcodes have been assigned, outliers are filtered out by applying a Mahalanobis distance threshold to each barcode population, which takes into account the covariance of the barcode populations. Finally, each barcode population is output to a corresponding Flow Cytometry Standard (FCS) file, and the cells that were discarded by the algorithm are output to a separate FCS file.

To facilitate deconvolution of barcoded data sets, the SCD algorithm was implemented as a standalone MATLAB application. By using the MATLAB Compiler Runtime (MCR), this application does not require a MATLAB installation or license. A flowchart describing the debarcoding workflow is shown in **Figure 5a**. The inputs are an FCS file that contains a barcoded data set and a spreadsheet in CSV format that defines the barcoding scheme, referred to as the 'Barcode Key'. After selection of the input FCS file and Barcode Key in the control panel of the GUI (**Fig. 5b**), a preliminary round of barcode assignment is performed for a range of barcode separation thresholds. A histogram of cells binned by barcode separation and a plot of the number of total events yielded for each barcoded sample as a function of the separation threshold are then displayed in the top right panel of the GUI (**Fig. 5c**). This view of yield versus separation threshold, as well as a single-cell view for each resulting barcode population (**Fig. 5d**) and biaxial scatter plots (**Fig. 5e**), aid the choice of deconvolution parameters that can favor either barcode stringency or cell yield.

In most cases of MCB debarcoding, there is no tradeoff necessary between stringency and yield, and high stringency settings may be used without substantially lowering the cell yield. In certain exceptional cases with large differences in cell number, cell size or the amount of debris between samples, the MCB staining may not be uniform and the researcher may then adjust the parameters according to his or her desire for barcode stringency versus cell yield. A valuable internal control for the MCB protocol that provides an estimation of the deconvolution error rate is to leave a single well empty—for example, only use 19 cell samples with the 20-sample 6-choose-3 MCB scheme. This allows for an estimation of incorrect sample assignment if any cells are assigned



	mESC (%)	MEF (%)
Assignment agrees	90.5	91.1
Assignment disagrees	0.02	1.2
Unassigned	9.5	7.7

	mESC (%)	MEF (%)
Assignment agrees	91.8	91.0
Assignment disagrees	0.02	1.3
Unassigned	8.1	7.7

	U937 (%)	OVCAR-3 (%)
Assignment agrees	69.7	92.5
Assignment disagrees	0.3	1.3
Unassigned	30.0	6.2

	U937 (%)	OVCAR-3 (%)
Assignment agrees	73.2	10.9
Assignment disagrees	0.3	1.3
Unassigned	26.6	87.8

	U937 (%)	OVCAR-3 (%)
Assignment agrees	0.02	91.6
Assignment disagrees	0.2	0
Unassigned	99.8	8.4

Figure 7 | Single-cell deconvolution versus Boolean gating for MCB samples with and without large differences in MCB labeling intensity. (**a**) PFA-fixed, methanol-permeabilized mouse embryonic fibroblast (MEF) and mESC cells were aliquotted into a 2-ml 96-well plate in a 20-sample checkerboard pattern at 0.2×10^6 and 0.5×10^6 cells per well, respectively. The cells were incubated with MCB reagents in a 6-choose-3 combinatorial scheme at 300 nM isothiocyanobenzyl-EDTA(palladium). (**b**) After MCB labeling and pooling of the checkerboard-arranged samples, the MEF-specific antibody against CD44 and the mESC-specific antibody against Oct4 were used to differentiate between the two cell types. (**c**,**d**) Single-cell debarcoding (**c**) and Boolean gate debarcoding (**d**) produce similar cell yields and accuracies. (**e**) PFA-fixed, methanol-permeabilized U937 and OVCAR-3 cells were aliquotted into a 2-ml 96-well plate in a 20-sample checkerboard pattern at 30,000 and 100,000 cells per well, respectively. A large percentage of the OVCAR-3 cells were lost during the PBS wash steps before MCB labeling, which resulted in unusually high MCB staining intensity for these samples. The cells were incubated with MCB reagents in a 6-choose-3 combinatorial scheme at 30 nM isothiocyanobenzyl-EDTA(palladium). (**f**) After MCB labeling and pooling of the checkerboard-arranged samples, U937-specific antibodies against CD33 and CD45 and OVCAR-3-specific antibodies against CD24 and E-cadherin were used to differentiate between the two cell types. (**g**) Gating based on CD33, CD45, CD24 and E-cadherin reveals the difference in MCB-labeling intensity between the U937 and OVCAR-3 cells. (**h**) Single-cell debarcoding successfully recovers both the U937 and OVCAR-3 populations. (**i**) Boolean gates bisecting the populations at a low MCB intensity primarily recover U937 cells. The low percentage of recovered OVCAR-3 cells is highlighted in red. (**j**) Boolean gates bisecting the populations at a high MCB intensity primarily recover OVCAR-3 cells. The low percentage of recovered U937 cells is highlighted in red.

to the empty well after sample deconvolution, and it is useful whether Boolean gating or the single-cell deconvolution algorithm is used.

Doublet identification and removal

Unlike fluorescence-based flow cytometry, mass cytometry does not have scatter measurements to identify cell doublets for removal. Previous attempts to eliminate cell doublets from mass cytometry data sets used gating to remove events that are high for both Ir-intercalator (iridium-intercalator) staining intensity and the number of TOF scans per cell (event length). This gating strategy is imperfect for doublet removal because the doublet and singlet populations substantially overlap on the Ir-intercalator × event-length scatter plot, as revealed by *n*-choose-*k* barcoding (**Fig. 6a** and **Supplementary Fig. 2**). In addition, this gating strategy may result in the systematic removal of specific cell types, because event length is dependent on the total amount of metal labeling each cell, which in turn is cell type– and staining panel–dependent. When the stringency of the Ir-intercalator × event length gate matches the stringency of the SCD separation threshold—i.e., the two methods produced the same cell yield—the SCD algorithm consistently filtered out more doublets than the 'singlet' gate (**Fig. 6b**). The 'singlet' gates used for this analysis are shown in **Supplementary Figure 3**; to achieve a similar range of yields, the SCD separation threshold was varied from 0 to 0.7, with the Mahalanobis distance threshold set to 30. In addition to its application for mass cytometry as presented here, this doublet-identifying barcode scheme may be applied to other single-cell analysis methods, and it may be especially useful when experimental analysis requires high-confidence discrimination between unique cells and removal of cell doublets.

Comparison of SCD with Boolean gating

Although Boolean gating has been used successfully for MCB deconvolution[6], it relies on distinct populations with consistent positive and negative MCB labeling intensities across all samples and cell types. If high-intensity, low-background staining is achieved consistently in all samples, the performance of Boolean gating and SCD is essentially the same (**Fig. 7a–d**). However, consistent MCB labeling can be difficult to achieve for several reasons, such as if cell number varies unexpectedly, if BSA washout is incomplete, or if one or more samples contain substantial amounts of debris. An important attribute of the SCD algorithm is that it does not depend on uniform MCB labeling across samples or even within samples, and it performs well on suboptimally MCB-multiplexed samples (**Fig. 7e–h**) in which deconvolution by Boolean gating fails (**Fig. 7i,j**). Because the SCD algorithm assigns samples by identifying the highest MCB measurements from each cell individually rather than on a population basis, it works for multiplexed samples with a continuous distribution of MCB labeling intensity, as well as for multiplexed samples with well-separated bimodal distributions.

It may not be possible to achieve equal levels of barcode staining across all wells when comparing multiple patient samples or different tissue types, yet it is precisely these situations in which the benefits of barcoding, in particular uniform antibody staining, will have the greatest benefit on the quality of the data. Performing barcode deconvolution with the single-cell, rather than population-based, method overcomes the challenges associated with variable barcode staining levels. In addition, in contrast to the variability of manually chosen gate boundaries, the results of the single-cell method are solely determined by the chosen distance parameters, and therefore the deconvolution is reproducible.

Experimental design

The main PROCEDURE describes how to prepare cell samples, label with MCB reagents and analyze the obtained results. **Box 1** describes how to prepare MCB labeling reagents. **Box 2** describes how to titrate MCB labeling reagents. **Box 3** describes how to make combinatorial plates. **Box 4** describes how to test the combinatorial plates. All the procedures in the boxes must be performed before performing an MCB labeling experiment, as they are all important components of the required setup (see also **Supplementary Figs. 4–6**).

Box 1 | Preparation of palladium MCB cell labeling reagents ● TIMING overnight

Reagents

Isotopically purified palladium nitrate. Pd102, Pd104, Pd105, Pd106, Pd108 and Pd110 nitrates are commercially available as a custom order from Trace Sciences International ▲ **CRITICAL** Palladium isotopes should be obtained with as high purity as possible, because debarcoding performance correlates positively with reagent purity, although in theory the MCB protocol would function reasonably well with only 60% pure reagents. The purities of the palladium reagents used to generate the example results presented were Pd102 >91%, Pd104 >96%, Pd105 >98%, Pd106 >99%, Pd108 >99% and Pd110 >99%. ! **CAUTION** Palladium nitrate is an oxidizer. Keep it away from heat and avoid contact with skin and eyes.

HCl (Fisher Scientific, cat. no. A466) ! **CAUTION** HCl is corrosive. Avoid contact with skin and eyes

Isothiocyanobenzyl-EDTA (Dojindo, cat. no. M030-10)

Eppendorf tubes, 1.5 ml (Fisher Scientific, cat. no. 14-222-155)

10× Pd102, Pd104, Pd105, Pd106, Pd108 and Pd110 solutions; see Reagent Setup in the main text

20 mM Ammonium acetate, pH 6.0; see Reagent Setup in the main text

Liquid nitrogen or dry ice

Needles, 18-gauge (BD, cat. no. 305195)

DMSO (Sigma-Aldrich, cat. no. D2650)

Ziploc quart-size freezer bags (VWR, cat. no. 82027-628)

Drierite desiccant (VWR, cat. no. 22891-050)

(continued)

Box 1 | (continued)

EQUIPMENT
Vortex mixer (GeneMate, cat. no. S-3200-1)
Tabletop microcentrifuge (5424, Eppendorf)
Lyophilizer (FreeZone 4.5, Labconco)

Reagent Setup
10× palladium solution
Dissolve isotopically pure palladium nitrate to 100 mM in 5 N HCl, giving a dark brown transparent solution. Store the solution indefinitely at room temperature.

Procedure
1. Remove isothiocyanobenzyl-EDTA powder from the –20 °C freezer and warm it to room temperature before opening to prevent condensation (water-sensitive).
2. For each palladium isotope MCB reagent to be prepared, tare a balance with a 1.5-ml polypropylene tube, dispense ~1.0 mg of isothiocyanobenzyl-EDTA and then weigh the tube to 0.1 mg accuracy to determine the precise amount of reagent dispensed. Use this mass in the next step.
3. Calculate the volume x of 10 mM palladium solution required to create a 2:1 isothiocyanobenzyl-EDTA:palladium molar ratio using the following formula, where a is the mass of isothiocyanobenzyl-EDTA in the tube from the previous step and 439.33 mg/mmol is the molecular weight of isothiocyanobenzyl-EDTA:

$$x\,\mu\text{l} = a\,\text{mg} \times \frac{1\ \text{mmol}}{439.33\ \text{mg}} \times \frac{1}{2} \times \frac{1\ \text{l}}{10\ \text{mmol}} \times \frac{1{,}000{,}000\ \mu\text{l}}{1\ \text{l}}$$

The isothiocyanobenzyl-EDTA chelator is used in excess to ensure complete palladium chelation and to remove all free palladium from the final reagent, because the free metal will react with the cells in a reversible manner that is inappropriate for cell barcoding.

4. Prepare the volume of 10 mM palladium solution calculated in the previous step by diluting 10× palladium solution at a 1:10 ratio in ammonium acetate buffer, and then adding this entire volume to the isothiocyanobenzyl-EDTA and vortexing to mix. Multiple rounds of vortexing and spinning in a tabletop microcentrifuge may be necessary to completely dissolve the isothiocyanobenzyl-EDTA solid, but it should dissolve completely within 15 s. Although the ammonium acetate is insufficient to buffer the HCl, we have observed no benefit from increasing (or decreasing) the ammonium acetate buffer concentration.
5. Snap-freeze the isothiocyanobenzyl-EDTA:palladium mixture in liquid nitrogen or in an ethanol-dry ice bath. Be sure to keep the tubes upright during freezing so that the frozen mixture will be at the bottom of the tube.
6. Prepare a vented cap for each tube by piercing the cap of an extra 1.5-ml tube with an 18-gauge or similar needle, and then by cutting off the cap. Warm the hinges of the frozen tubes with your fingers to prevent breaking, and then open the tubes and insert the vented caps, leaving the original caps attached. Work quickly to prevent thawing, and return the tubes to dry ice after capping to ensure that they are thoroughly frozen before lyophilization.
7. Lyophilize the mixture overnight to yield a brown/orange film.
8. Dissolve the lyophilized isothiocyanobenzyl-EDTA:palladium in DMSO to 10 mM, by using the same volume as calculated in **Box 1**, step 3. Note that this solution is 20 mM isothiocyanobenzyl-EDTA, with approximately half of the EDTA-chelating groups occupied by palladium.
9. Divide the 10 mM MCB reagent DMSO solutions into aliquots and store them at –80 °C with desiccant to protect from moisture. For example, place the aliquots in a 9 × 9 freezer box, and then place this box in a Ziploc bag containing desiccant. Remove as much air as possible from the Ziploc bag before sealing.
■ **PAUSE POINT** 10 mM Isothiocyanobenzyl-EDTA:palladium DMSO stocks may be stored up to 1 year at –80 °C with desiccant. Cell labeling activity may decrease with extended storage or with multiple freeze-thaw cycles.

Box 2 | Titration of palladium MCB reagents ● TIMING 4–6 h

Titrate each MCB reagent on bulk cell samples to identify the optimal concentration for cell labeling. Typically, the optimal concentration is between 50 nM and 1 μM for PFA-fixed/methanol-permeabilized cells, depending on the number of cells per labeling reaction, the amount of residual BSA remaining after PBS wash and the extent of isothiocyanate hydrolysis in the MCB reagent DMSO stock. PFA-fixed cells that have not been methanol-permeabilized typically require threefold more barcoding reagent for optimal staining.

Perform each measurement in triplicate to ensure accurate titration. For a five-point titration of six MCB reagents in triplicate, perform 90 cell labeling reactions. 96-well format eases pipetting and sample handling. Although this protocol is written for 0.5×10^6 cells per well here, MCB multiplexing can be performed on higher cell numbers, at least up to 5×10^6 cells per well. When varying the cell number, it is crucial to titrate the barcoding reagent concentration for the number of cells (and fixation method) that will be used in the final experiment (**Fig. 2d**).

(continued)

Box 2 | (continued)

Reagents

Bulk PFA-fixed or PFA-fixed/methanol-permeabilized cell sample; see **Box 1** for reagent preparation
PBS; see Reagent Setup in the main text
CSM; see Reagent Setup in the main text
Deep-well microplates, 96 well, polypropylene, 2.0-ml nonsterile (VWR, cat. no. 40002-012)
10× palladium solutions; see Reagent Setup in the main text
Intercalator solution; see Reagent Setup in the main text

Equipment

Tabletop 96-well format refrigerated centrifuge (Allegra X-22R, Beckman Coulter)
Aspirator, 96 well (VP Scientific, cat. no. VP 177A-1)
P20 and P1200 multichannel pipettes (Rainin, cat. nos. L8-20 and L8-1200)

Procedure

1. Prepare a sufficient number of bulk cells to perform the MCB reagent titration, as described in Steps 1–8 of the main PROCEDURE. Any cell type may be used for this bulk sample, but suspension cell lines such U937 are preferred for ease of passage and collection. Prepare either PFA-fixed or PFA-fixed/methanol-permeabilized bulk cells depending on the cells that will be used in the final experiment.
2. Bring PBS and two 2-ml 96-well plates to 4 °C on ice. If PFA-fixed (non-methanol-permeabilized) cells will be used, bring PBS-S to 4 °C on ice as well. For one of the 2-ml 96-well plates, add 1 ml of PBS or PBS-S to each well that will be used for titration, using the plate layout shown in **Supplementary Figure 4a**. These wells will be used to prepare the MCB reagent DMSO stock dilutions before adding to the cells.
3. Transfer bulk PFA-fixed or PFA-fixed/methanol-permeabilized cells from –80 °C into a single suitable container (15-ml or 50-ml conical tube) to wash before aliquotting, as described in Step 10 of the main PROCEDURE section. For a five-point titration of six MCB reagents in triplicate, use 4.5×10^7 cells. Note that these intensity versus concentration curves are specific for the number of cells per well (500,000 in this protocol), because MCB intensity varies with cell density, as described in **Figure 2d**. Adjust the number of bulk cells used in this MCB reagent titration to best match the expected cell densities of the final experimental samples.
4. Centrifuge the cells at 600*g* for 5 min at 4 °C, and then aspirate the supernatant.
5. Resuspend the cell pellet to a concentration of 0.5×10^6 cells per ml (1.0 ml per titration point) with CSM, and then add 1.0 ml per well into the empty, prechilled 96-well plate in the wells that will be used for the MCB labeling titration, using the plate layout shown in **Supplementary Figure 5a**.
6. Wash the cell-containing 96-well plate, as described in Step 12 of the main PROCEDURE.

▲ **CRITICAL STEP** Residual BSA competes with cells for MCB reagent. Failure to adequately wash away BSA results in decreased and inconsistent MCB labeling intensity.

7. Thaw 10 mM MCB DMSO stocks, and dilute each to 1.1 µM with fresh DMSO. Perform a 1:3 serial dilution on this 100× working solution in a 96-well PCR plate by mixing 10 µl with 20 µl of fresh DMSO at every step. Starting with a 1.1 µM 100× working solution yields final titration concentrations of 1,000, 333, 111, 37 and 12 nM. Perform the serial dilution in triplicate, by using the plate layout shown in **Supplementary Figure 5a**.
8. Pipette 10 µl of each 100× MCB working solution with a multichannel P20 pipette into the 4 °C PBS/PBS-S-containing 2-ml 96-well plate, mix by pipetting up and down five times with a multichannel P1000 pipette set to 900 µl and then transfer 900 µl to the 2-ml 96-well plate containing resuspended PBS/PBS-S-washed cells. Mix immediately by pipetting up and down five times to ensure uniform mixing, and incubate the mixture at 4 °C for 15 min.

▲ **CRITICAL STEP** The isothiocyanate group is unstable in PBS/PBS-S owing to hydrolysis, so work as quickly as possible to move MCB-containing PBS/PBS-S to the cell sample. Ensure that the PBS/PBS-S used for dilution is 4 °C instead of room temperature to further slow isothiocyanate hydrolysis.

9. Add 0.5 ml of CSM to each well of the MCB cell labeling reaction, and then centrifuge at 600*g* for 5 min at 4 °C and aspirate the supernatant.
10. Wash with CSM two additional times, and resuspend in 0.1 ml of CSM.
11. Pool the wells as shown in **Supplementary Figure 4b**, resulting in three tubes for each concentration point containing all MCB reagents.

▲ **CRITICAL STEP** In addition to reducing sample acquisition time, pooling each of the MCB reagent–labeled titration point samples into a single tube at this step allows for detection of any cell-to-cell transfer of the MCB label during the incubation in subsequent steps. Improperly prepared MCB reagent may exchange readily between cells, hindering its use as a barcoding reagent.

12. Centrifuge the cells for 5 min at 600*g* and 4 °C, and then aspirate the supernatant, leaving a residual volume of 100 µl.
13. Resuspend the cell pellet in residual volume and incubate it at room temperature for 60 min. This step simulates an antibody staining incubation and is necessary to test for cell-to-cell transfer of the MCB reagent.
14. Wash the cells once with CSM, and then incubate them for 15 min at room temperature with 1:10,000 Ir-intercalator in 1.6% PFA-containing PBS.

■ **PAUSE POINT** Intercalating cell samples may be stored for up to 1 month at 4 °C.

(continued)

Box 2 | (continued)

15. Wash the cells once with CSM and twice with water, and then analyze them on a mass cytometer. Run the samples in ascending order of MCB reagent concentration, and stop measuring the samples before the MCB signal reaches the mass cytometer detection limit, which is ~10,000 counts per cell for any measurement channel. The maximum MCB concentration sample measured will usually be 111 or 333 nM for the titration described in this procedure. The higher MCB cell labeling concentrations in the titration are included in case the MCB reagent stock has lower-than-expected cell labeling activity.

! CAUTION Measuring samples with MCB staining intensity above the mass cytometer's detection limit may substantially shorten the life span of the instrument's ion detector.

16. By using the measured median intensities for each MCB reagent, and by including only titration points that fall within the linear range of measurement (excluding points with a median intensity <10 or >5,000 counts per cell), derive a curve to determine the cell counts observed versus nM for each of the MCB reagents. This value will be used to determine the appropriate concentrations of each MCB reagent to use during preparation of the combinatorial MCB plates in **Box 3**.

Box 3 | Preparation of combinatorial MCB plates ● TIMING ~2 h

The combinatorial MCB plates are used as a 100× working solution, but they are initially prepared at 1,000× in order to conserve the –80 °C freezer space, and to allow for dilution to different concentrations for use with different numbers of cells or to compensate for lost activity during extended storage. If freezer space is not limiting and the number of cells to multiplex will not change substantially, then the combinatorial MCB plates may be initially prepared at 100× concentration for storage and direct use.

Reagents

10 mM MCB reagent DMSO stocks; see **Box 2** for reagent preparation
DMSO (Sigma-Aldrich, cat. no. D2650)
PCR plates, 96 well (Fisher Scientific, cat. no. 14-230-237)
Foil covers, 96 well (Fisher Scientific, cat. no. 14-222-342)
Dry ice
Ziploc quart-size freezer bags (VWR, cat. no. 82027-628)
Drierite desiccant (VWR, cat. no. 22891-050)

Equipment

P20 and P1200 multichannel pipettes (Rainin, cat. nos. L8-20 and L8-1200)
Aluminum 96-well block (GeneMate, cat. no. R-2027-S)

Procedure

1. Plan the MCB combinatorial scheme in advance using a spreadsheet program such as Microsoft Excel, and print out the layout to use as reference while pipetting each MCB reagent during **Box 3**, step 3. **Supplementary Figure 5** shows the six pipetting layouts required for a 6-isotope 20-sample combinatorial MCB plate, and the subsequent steps are written for this scheme.
2. Thaw the 10 mM MCB reagent DMSO stocks, and dilute each MCB reagent with fresh DMSO to produce 660 µl at 3,000× the concentration required for optimal cell labeling, as determined by reagent titration in **Box 2**. Optimal cell labeling intensity is ~500 counts per cell, although this number may vary from instrument to instrument depending on ion detector sensitivity. The optimal cell labeling intensity should be determined for each instrument as ~5-fold below the level at which the +1 TOF effect, isotopic impurity and event length elongation become problematic owing to high palladium signal intensity. If the 3,000× concentration is chosen to be 3 mM, then mix 198 µl of the 10 mM MCB stock with 462 µl of fresh DMSO.
3. Pipette an equal volume of each DMSO stock into the appropriate wells of a 96-well PCR plate following the printed key to create the 1,000× combinatorial MCB master mix. For the 6-metal 20-sample doublet-filtering scheme that contains exactly three MCB reagents per well, pipette 60 µl of each MCB 3,000× DMSO stock per well for a final volume of 180 µl in each well. We typically use the middle wells C4-F9 of the 96-well plate in a 5 × 4 grid so that the final working plates are better protected from moisture when sealed for long-term storage at –80 °C.

▲ CRITICAL STEP Visually inspect the PCR plate after all 3,000× DMSO stocks have been pipetted, and check that every well contains the same volume of DMSO solution. If a well was missed or pipetted twice by mistake, the volume will be noticeably different: either 120 or 240 µl instead of the desired 180 µl. Take extra care to ensure correct pipetting at this step, because any mistake will result in unusable 1,000× plates that must be discarded.

4. Inspect the 96-well PCR plate for bubbles present at the bottom of the wells. Remove any bubbles present with a P200 pipette, and then thoroughly mix each well of the 1,000× combinatorial MCB master mix by pipetting up and down 10–20 times with a P200 multichannel pipette set to 120 µl.
5. Replicate by aliquotting the 1,000× combinatorial MCB master mix plate into multiple 96-well PCR plates. For example, transfer 18 µl from each well to new plates using a multichannel P20 pipette or pipetting robot.
6. Seal each aliquotted PCR plate with foil adhesive, bring it to –80 °C with dry ice and store it in Ziploc bags with as much air removed as possible and with desiccant at –80 °C to protect from moisture.

▲ CRITICAL STEP Work as rapidly as possible to minimize DMSO absorbing water from the air, which can hydrolyze the MCB reagent isothiocyanate group.

■ PAUSE POINT 1,000× Combinatorial MCB plates may be stored for up to 1 year at –80 °C with desiccant. Cell labeling activity may decrease with extended storage or with multiple freeze-thaw cycles.

(continued)

Box 3 | (continued)

7. Thaw one 1,000× combinatorial MCB plate containing 18 µl of MCB reagent per well. An aluminum 96-well block may be used to increase the thaw speed.
8. Dilute each well at a 1:10 ratio by adding 162 µl of fresh DMSO to each well with a multichannel pipette or pipetting robot.
9. Mix each well of the 100× combinatorial MCB master mix by pipetting up and down 10–20 times with a P200 multichannel pipette set to 120 µl.
10. Transfer the 100× combinatorial MCB master mix plate into multiple 96-well PCR plates. For example, divide 12 µl from each well to new plates using a multichannel pipette or pipetting robot.
11. Seal each aliquotted PCR plate with foil adhesive, bring it to –80 °C with dry ice and store it in Ziploc bags with as much air removed as possible and with desiccant at –80 °C to protect from moisture.

▲ **CRITICAL STEP** Work as rapidly as possible to minimize DMSO absorbing water from the air, which can hydrolyze the MCB reagent isothiocyanate group.

■ **PAUSE POINT** 100× combinatorial MCB plates may be stored for up to 1 year at –80 °C with desiccant. Cell labeling activity may decrease with extended storage or with multiple freeze-thaw cycles.

Box 4 | MCB combinatorial plate validation ● TIMING ~2 h

Before using the MCB combinatorial plates for experimental samples, it is useful to first test them on bulk cells. The purpose of this test is twofold: (i) to confirm that the MCB cell labeling intensity from the 100× combinatorial plate is at an appropriate level, and (ii) to confirm that there are no errors in sample assignment owing to pipetting errors from **Box 3**, step 3. The fidelity of sample assignment is tested with multiple cell sample layout patterns such as checkerboard, striped and random arrangements to ensure that each of the 20 samples is correctly assigned. Although this protocol is written for 1×10^6 cells per well here, MCB multiplexing can be performed on higher cell numbers, at least up to 5×10^6 cells per well. When varying cell number, it is crucial to titrate the barcoding reagent concentration for the number of cells (and fixation method) that will be used in the final experiment (**Fig. 2d**).

Reagents

Bulk cells; see **Box 1** for reagent preparation
PBS; see Reagent Setup in the main text
CSM; see Reagent Setup in the main text
96-Well deep-well microplates polypropylene, 2.0 ml nonsterile (VWR, cat. no. 40002-012)
100× combinatorial MCB plates; see **Box 3** for reagent preparation
Intercalator solution; see Reagent Setup in the main text

Equipment

Tabletop 96-well format refrigerated centrifuge (Allegra X-22R, Beckman Coulter)
Aspirator, 96 well (VP Scientific, cat. no. VP 177A-1)
P20, P200 and P1200 multichannel pipettes (Rainin, cat. nos. L8-20, L8-200 and L8-1200)
Aluminum 96-well block (GeneMate, cat. no. R-2027-S)

Procedure

1. Prepare a sufficient number of cells, 2×10^7, for the procedure as written here. Any cell type may be used for this bulk sample, but suspension cell lines such as U937 are preferred for ease of passage and collection.
2. Add 1×10^6 cells per well into 20 wells of a 2-ml 96-well plate, corresponding to the 20 positions of the combinatorial MCB plate to be tested.
3. Label the 20 wells of bulk cells with the 100× combinatorial MCB plate to be tested, according to Steps 11–17 described in the main PROCEDURE, wash them two times with CSM and then resuspend the cells and fill the wells with CSM.
4. Arrange P200 pipette tips in empty pipette tip boxes into the eight patterns shown in **Supplementary Figure 6**.
5. By using a P200 multichannel pipette set to 150 µl, use the pipettes arranged in the previous step and pool MCB-labeled cells into eight groups corresponding to the eight patterns shown in **Supplementary Figure 6**. Use a 70-ml reservoir to pool each group before transferring to a 50-ml conical tube.
6. Centrifuge 50-ml conical tubes for 5 min at 600*g* and 4 °C, and then aspirate the supernatant.
7. Resuspend the eight pooled samples in CSM and transfer each sample to a labeled 4-ml FACS tube.
8. Centrifuge the 4-ml FACS tubes for 5 min at 600*g* and 4 °C, and then aspirate the supernatant.
9. Resuspend the cell pellet in residual volume and incubate it at room temperature for 60 min. This step simulates the antibody stain and is necessary to test for cell-to-cell transfer of the MCB reagent.

For each of the eight pooled samples, intercalate, measure using a mass cytometer and debarcode as described in Steps 20–28 of the main PROCEDURE. Confirm that only the expected MCB combinations are present in each pooled sample.

MATERIALS

REAGENTS

- Cells or tissue sample. We have successfully used this protocol for suspension and adherent cell lines; mouse tissues including blood, spleen, bone marrow, spinal cord and brain; and human tissues including blood, bone marrow, leukemia and ovarian tumor biopsies
  ! CAUTION All experiments should adhere to relevant institutional and governmental ethics guidelines and regulations. Informed consent should be obtained from donors of human blood or tissue. All of our animal studies were performed in accordance with the investigators' protocols approved by the Stanford University institutional animal care and use committee.
- Palladium MCB cell labeling reagents, prepared as described in **Box 1**
- Cell culture medium (cell type appropriate)
- Cell dissociation reagent (cell type appropriate, such as: PBS-EDTA/Versene (Life Technologies, cat. no. 15040-066), trypsin (Life Technologies, cat. no. 25200-056), TrypLE (Life Technologies, cat. no. 12605-010), Accutase (Innovative Cell Technologies, cat. no. AT 104) and collagenase (Life Technologies, cat. no. 17100-017) (only required for adherent cell types and solid tissue samples)
- Nylon mesh cell strainer, 40 μm (Corning, cat. no. 352340), 70 μm (Corning, cat. no. 352350) or 100 μm (Corning, cat. no. 352360) (only required for adherent cell types and solid tissue samples)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- Methanol (Fisher Scientific, cat. no. A412-4) **! CAUTION** Methanol is flammable. Keep it away from heat. Avoid contact with skin and eyes. Avoid inhalation.
- Sodium hydroxide (Sigma-Aldrich, cat. no. S-8045) **! CAUTION** Sodium hydroxide is corrosive. Avoid contact with skin and eyes.
- Sodium phosphate dibasic heptahydrate (Sigma-Aldrich, cat. no. S9390)
- Potassium phosphate monobasic anhydrous (Sigma-Aldrich, cat. no. P0662)
- Potassium chloride (Fisher Scientific, cat. no. P330)
- Sodium chloride (Fisher Scientific, cat. no. S271)
- Ammonium acetate (Sigma-Aldrich, cat. no. A7330)
- Saponin (Sigma-Aldrich, cat. no. S-7900)
- Tween-20 (Sigma-Aldrich, cat. no. P-9416)
- BSA fraction V (Sigma-Aldrich, cat. no. A-2153)
- Sodium azide (NaN_3; Sigma-Aldrich, cat. no. S-8032)
  ! CAUTION NaN_3 is highly toxic. Avoid contact with skin, eyes and clothing. It is fatal if swallowed.
- 16% Paraformaldehyde (PFA), electron microscopy (EM) grade (Electron Microscopy Sciences, cat. no. 15710) **! CAUTION** PFA is an irritant. Avoid contact with skin and eyes. Avoid inhalation.
- Cluster tubes, 1.2 ml polypropylene (Fisher Scientific, cat. nos. 07-200-319 and 07-200-317)
- Deep-well microplates, 96 well, polypropylene, 2.0 ml nonsterile (VWR, cat. no. 40002-012)
- Aluminum block, 96 well (BioExpress, cat. no. R-2027-S)
- Combinatorial MCB plate prepared as described in **Box 3** and validated as described in **Box 4**
- Hydrochloric acid (HCl; Fisher Scientific, cat. no. A466)

EQUIPMENT

- Multichannel pipettes, P20, P200 and P1200 (Rainin, cat. nos. L8-20, L8-200 and L8-1200)
- Vortex mixer (GeneMate, cat. no. S-3200-1)
- Tabletop refrigerated swinging-bucket centrifuge (Allegra 6R, Beckman Coulter)
- Hemocytometer (Hausser Scientific, cat. no. 3200)
- Tabletop microcentrifuge (5424, Eppendorf)
- Tabletop 96-well format refrigerated centrifuge (Allegra X-22R, Beckman Coulter)
- Aspirator, 96 well (VP Scientific, cat. no. VP 177A-1)
- Lyophilizer (FreeZone 4.5, Labconco)
- Mass cytometer (CyTOF, Fluidigm)
- Aluminum 96-well block (GeneMate, cat. no. R-2027-S)
- Debarcoding software, available at https://github.com/nolanlab/single-cell-debarcoder/releases/latest

REAGENT SETUP

HCl, 5 N Dilute concentrated HCl solution to 5 N in ddH_2O and store it indefinitely at room temperature (20–25 °C).

Sodium hydroxide, 5 N Dissolve sodium hydroxide to 5 N in ddH_2O and store it indefinitely at room temperature.

PBS, 10× Dissolve 320 g of sodium chloride (1.37 M), 8 g of potassium chloride (27 mM), 46 g of sodium phosphate dibasic heptahydrate (43 mM) and 8 g of potassium phosphate monobasic anhydrous (15 mM) in 3 liters of ddH_2O; adjust the pH to 7.4 with 5 N sodium hydroxide, and then bring the final volume to 4 liters and filter the solution using a 0.8-μm filter. Store it for up to 5 years at room temperature.

PBS Dilute 10× PBS at a 1:10 ratio in ddH_2O and store it for up to 5 years at room temperature.

PBS-S Dissolve solid saponin powder in PBS to 0.02% (wt/vol), and store it for up to 6 months at room temperature.

Fixation solution, 2× Dilute 16% (wt/vol) PFA at a 1:5 ratio (to 3.2%) into PBS and store it at 4 °C shielded from light for up to 2 weeks.

Cell staining medium Dissolve BSA to 0.5% (wt/vol) and NaN_3 to 0.02% (wt/vol) in PBS. Store it for up to 6 months at 4 °C.

Ammonium acetate buffer Dissolve ammonium acetate to 20 mM in ddH_2O, and then confirm that the solution is at pH 6.0. Store it for up to 1 year at 4 °C.

Intercalator solution Dilute 16% PFA at a 1:10 ratio (to 1.6%) into PBS, and then add Ir-intercalator diluted at a 1:10,000 ratio. Store the solution at 4 °C shielded from light for up to 2 weeks.

PROCEDURE

Cell sample preparation ● TIMING ~1 h

1| For each cell sample to be multiplexed, prepare a single-cell suspension concentrated at 1×10^8 cells per ml or less. Previously established protocols that are optimized for the cell type of interest should be followed, but here we provide generalized instructions. Follow Step 1A for suspension cell lines and blood samples, Step 1B for adherent cell lines or Step 1C for solid tissue samples.

(A) Suspension cell lines or blood samples

(i) Centrifuge the cell suspension at 300*g* for 5 min at room temperature.

(ii) Aspirate or decant the supernatant.

(iii) Resuspend the cell pellet in appropriate culture medium.

(B) Adherent cell lines

(i) Detach the cells from the culture plate by incubation with an appropriate dissociation reagent according to the cell line–optimized protocol.

(ii) Pipette the suspension up and down with a P1000 tip or a plastic transfer pipette to break up the cell clumps and to obtain a single-cell suspension.
(iii) Transfer 10 µl of the suspension to a microscope slide or to a hemocytometer to confirm single-cell suspension. Repeat the previous step until a single-cell suspension is obtained.
(iv) Filter the suspension with a nylon mesh (40, 70 or 100 µm, as appropriate) to remove any remaining clumps if necessary.

(C) Solid tissue samples

(i) Homogenize the tissue sample with tissue-optimized mechanichal or enzymatic treatment.
(ii) Transfer 10 µl to the microscope slide or to the hemocytometer to confirm single-cell suspension. Repeat the previous step until a single-cell suspension is obtained.
(iii) Filter the suspension with a nylon mesh (40, 70 or 100 µm as appropriate) to remove any remaining clumps if necessary.

2| Dilute all cell suspensions with culture medium to a uniform volume and transfer them to an appropriate container for PFA fixation. If all cell samples to be multiplexed are available simultaneously, then dilute each sample to 0.5 ml and transfer to a 2-ml 96-well plate to facilitate multichannel pipetting in subsequent steps. If samples are not available simultaneously, then transfer the cell suspensions to sealable individual containers such as 50-ml conical tubes, 15-ml conical tubes or 4-ml capped FACS tubes before PFA fixation, and scale all volumes appropriately.

3| Dilute 2× fixation solution twofold into the cell suspension for a final concentration of 1.6% PFA. Mix by pipetting up and down or by vortexing, and then incubate the mixture at room temperature for 10 min. For a 0.5-ml cell suspension in a 2-ml 96-well plate, add 0.5 ml of 2× fixation solution.

4| Slow the PFA fixation reactions by diluting twofold with ice-cold cell staining medium (CSM). This step can help equalize the sample fixation times by staggering CSM addition with the same timing as PFA addition. For a 1-ml PFA-fixation reaction in a 2-ml 96-well plate, add 1 ml of CSM.

5| Centrifuge PFA-fixed cells at 600*g* for 5 min at 4 °C.

6| Aspirate or decant the supernatant, and leave enough residual volume to resuspend cells into a single-cell suspension.

7| Vortex or pipette the suspension up and down to completely resuspend the cell pellet in the residual volume, and then bring the cell container to 4 °C on ice.

8| After PFA fixation, cells may either be permeabilized with methanol or frozen as is to allow saponin-mediated MCB staining[8]. If antibody staining will include any methanol-sensitive surface markers, cells should be frozen as is. If antibody staining will be only for intracellular markers and methanol-insensitive surface markers, cells should be methanol-permeabilized.

(A) Freeze PFA-fixed cells as is

(i) Wash the cells once with CSM.
(ii) Resuspend the cells in CSM containing 10% (vol/vol) DMSO.
(iii) Remove 10 µl of the suspension and pipette it onto a hemocytometer to determine the cell density.
▲ **CRITICAL STEP** Cell density must be determined for each sample because MCB labeling intensity is dependent on the cell number.
(iv) Freeze the cells at –80 °C.

(B) Methanol-permeabilize the cells

(i) Aspirate or decant the supernatant, and leave enough residual volume to resuspend the cells into a single-cell suspension.
(ii) Vortex or pipette the suspension up and down to completely resuspend the cell pellet in the residual volume, and then bring the cell container to 4 °C on ice.
(iii) Add 4 °C methanol to the resuspended cell pellet so that the final concentration of methanol is >80% (vol/vol).
▲ **CRITICAL STEP** If the cell pellet is not completely resuspended when methanol is added, cells will clump together and be lost. Adding methanol while vortexing the tube of cells works well, but take care to not let the methanol suspension overflow while vortexing.
(iv) Remove 20 µl of cell/methanol suspension and dilute it into 180 µl of CSM in a 1.1-ml cluster tube. Vortex the suspension to mix, and then pipette 10 µl onto a hemocytometer to determine the cell density. If the cell density is

too low to accurately determine, scale up the volumes of methanol and CSM, centrifuge for 5 min at 4 °C at 600*g*, aspirate the supernatant and resuspend the cells to an accurately determined smaller volume and transfer 10 µl to a hemocytometer to determine the cell density.

▲ **CRITICAL STEP** Cell density must be determined for each sample because MCB labeling intensity is dependent on the cell number.

■ **PAUSE POINT** Store PFA-fixed and PFA-fixed/methanol-permeabilized samples at –80 °C. PFA-fixed samples frozen in CSM/DMSO may be stored at –80 °C for 2 years or longer, with little to no epitope degradation. PFA-fixed/methanol-permeabilized samples may be stored at –80 °C for 12 months or longer with little to no epitope degradation. Epitope behavior during methanol storage is not uniform, but in general epitopes are stable in methanol at –80 °C (years), –20 °C (weeks), 4 °C (overnight) or at room temperature (minutes). Because cell samples in methanol are most stable at –80 °C, dry ice may be used for immediate storage of methanol-permeabilized cell samples before transfer to a –80 °C freezer.

MCB labeling and pooled mass cytometry analysis ● **TIMING ~3 h (plus ~1 h for every million cells being analyzed)**

9| Bring PBS and prelabeled cell containers to 4 °C on ice.

10| Transfer the cell samples from –80 °C storage to prelabeled cell containers. For large numbers of samples, it is efficient to use cluster tubes in a 96-well format or in a 2-ml 96-well plate. Subsequent steps of this procedure will be written for 20 samples in a 2-ml 96-well plate. Follow Step 10A for PFA-fixed cells, and follow Step 10B for PFA-fixed/methanol-permeabilized cells.

(A) PFA-fixed cells

(i) Thaw the samples on ice or in a cold water bath.

(ii) Vortex the cells to resuspend, and then pipette a volume of the cell suspension that contains 500,000 cells (counted in Step 8) into each well.

(B) PFA-fixed/methanol-permeabilized cells

(i) Store the samples on bench in dry ice.

(ii) Add 1.2 ml of CSM to each prelabeled tube per well.

▲ **CRITICAL STEP** CSM helps prevent cell loss in subsequent wash steps.

(iii) Vortex the cells to resuspend, and then pipette a volume of the cell suspension that contains 500,000 cells (counted in Step 8) into each well. Pipette the suspension up and down five times or vortex to mix. Methanol can cause the BSA in the CSM to precipitate. This typically occurs if methanol is >50% of the total solution. In addition, if the methanol and CSM are not completely mixed, then BSA will precipitate at the CSM/methanol interface. The BSA precipitate does not appear to have an adverse effect on cell labeling or antibody staining. It dissolves and washes away with subsequent washes.

▲ **CRITICAL STEP** Equalizing the cell number for each sample ensures equal MCB labeling intensity, which is important for optimal barcode deconvolution. If samples cannot be equalized for cell number because some have too few cells, then adjust the concentration of MCB combinatorial mix added to the wells with fewer cells in Step 15.

? TROUBLESHOOTING

11| Fill each well to the top with CSM, and then centrifuge the cells at 600*g* for 5 min at 4 °C and aspirate the supernatant.

12| Wash the cells and prepare for the MCB labeling reaction. Follow Step 12A for PFA-fixed cells, and follow Step 12B for PFA-fixed/methanol-permeabilized cells.

(A) PFA-fixed cells

(i) Wash the cells once with 4 °C PBS, centrifuging at 600*g* for 5 min at 4 °C.

(ii) Wash the cells once with 4 °C PBS-S (with 0.02% (wt/vol) saponin), centrifuging at 600*g* for 5 min at 4 °C, leaving a residual volume of ~100 µl.

(iii) Divide 1.0 ml of ice-cold PBS-S (with 0.02% (wt/vol) saponin) into 20 wells of the prechilled 2-ml 96-well plate corresponding to the wells of the MCB combinatorial plate.

(B) PFA-fixed/methanol-permeabilized cells

(i) Wash the cells twice with 4 °C PBS, centrifuging at 600*g* for 5 min at 4 °C, leaving a residual volume of ~100 µl.

(ii) Transfer 1.0 ml of ice-cold PBS into 20 wells of the prechilled 2-ml 96-well plate corresponding to the wells of the MCB combinatorial plate.

? TROUBLESHOOTING

13| Vortex or pipette the mixture to resuspend the cell pellet in residual volume and store it at 4 °C on ice.

14| Thaw a 100× combinatorial MCB plate (prepared with individual MCB reagents as described in **Box 1** and titrated as described in **Box 2**; combined as described in **Box 3**; and validated as described in **Box 4**). An aluminum 96-well block may be used to increase the thaw speed.

15| If any wells have an unequal number of cells, adjust the concentration of each well corresponding to cell samples that are not cell number–equalized with fresh DMSO.

16| Transfer 10 µl of reagent from each well of the 100× combinatorial MCB plate into the 4 °C PBS-S or PBS-containing wells of the 2-ml 96-well plate using a P20 multichannel pipette. Mix by pipetting up and down three times with a P1000 multichannel pipette set to 0.9 ml, and then transfer 0.9 ml of the ice-cold MCB mix to the resuspended cell samples. Mix the cells and MCB reagent immediately by pipetting up and down 5–10 times to ensure uniform mixing, and then incubate the mixture for 15 min at room temperature.
▲ **CRITICAL STEP** The isothiocyanate group hydrolyzes rapidly in PBS, so work as quickly as possible to move MCB-containing PBS to the cell sample. In addition, ensure that the PBS is at 4 °C when first mixed to slow isothiocyanate hydrolysis.
▲ **CRITICAL STEP** The volume of MCB reagent added to each well may be adjusted to accommodate changes in cell number. The MCB reagent volume may be approximately scaled 1:1 with cell number (**Fig. 2d**), although for best results a new titration should be performed with the same number of cells per well as in the final experiment.
? TROUBLESHOOTING

17| Fill each well to the top with CSM, and then centrifuge at 600*g* for 5 min at 4 °C and aspirate the supernatant.

18| Wash with CSM two additional times, and then pool the cell samples into a single tube for antibody staining. Depending on the sample numbers, residual volumes and desired wash volumes, it may be necessary to pool the samples in a 15- or 50-ml conical polypropylene tube before transferring them to a smaller-volume tube for antibody staining.
? TROUBLESHOOTING

19| Perform antibody staining on the MCB-multiplexed sample. Use the same antibody concentrations and incubation time as for nonbarcoded cell samples, but adjust the staining volume according to the pellet size and/or cell number as would normally be done for larger cell sample sizes to keep the cell density below any cell type–specific maximum threshold. Follow Step 19A for PFA-fixed cells, and Step 19B for PFA-fixed/methanol-permeabilized cells.

(A) PFA-fixed cells

(i) Perform surface marker antibody staining on the MCB-multiplexed sample.

(ii) If desired, permeabilize the MCB-multiplexed sample with methanol (Step 8B), wash the cells into CSM and perform intracellular antibody staining.

(B) PFA-fixed/methanol-permeabilized cells

(i) Perform antibody staining for intracellular markers and methanol-resistant surface markers on the MCB-multiplexed sample.
? TROUBLESHOOTING

20| After antibody staining, wash the cells twice with CSM, and then incubate them for 15 min at room temperature with intercalator solution.
▲ **CRITICAL STEP** The Ir-intercalator acts as a cell-identifying reagent during CyTOF analysis. The 1.6% PFA in the intercalator solution fixes the cell samples to prevent loss of metal and antibody when they are suspended in ddH_2O in the next step before mass cytometry analysis.
■ **PAUSE POINT** Intercalating cell samples may be stored for up to 1 month at 4 °C.

21| Wash cells once with CSM and twice with ddH_2O, and then analyze them on a mass cytometer, collecting all pooled samples as a single FCS file.

Sample deconvolution by single-cell debarcoder ● TIMING ~30 min

22| Modify the sample names in the barcoding key CSV template file with text-editing software or Microsoft Excel, and then save the modified version.

23| Open the Single Cell Debarcoder, and when prompted by the dialog box select the correct saved barcode key.

24| Click the 'Select FCS File' button in the 'Select files' panel and choose the FCS file containing the pooled samples. When the preliminary debarcoding is done, which may take several seconds depending on the size of the FCS file, a plot of the cell counts of the barcode populations will appear. To determine which barcode population corresponds to a particular line on the plot, click on that line and the label from the barcode key will appear.

25| Enter a 'separation threshold' in the input box in the 'Filters' panel. This is a number between 0 and 1 that defines the minimum distance after normalization between the 'positive' and 'negative' barcode channels that is required for a cell to be assigned to a barcode channel. The goal is to filter out uncertain barcode assignments but to still retain sufficiently large barcode populations; a suggested starting point is a value just below which the cell count of the barcode populations markedly decreases (**Fig. 5c**). A barcode well that has been left blank can provide guidance as to where to set the threshold, and it also provides an estimate of false assignment rate. In a well-stained sample, the false assignment rate calculated from cells assigned to wells that are left blank is typically <0.5%.

26| Evaluate the barcode separation threshold by browsing through the 'Event' plots of different barcode populations. It may be useful to use the 'zoom' and 'pan' tools. Adjust the threshold if necessary, settling on the smallest value for which all the populations consist of cells in which positive and negative barcodes are sufficiently separated.

27| Check the barcode deconvolution with biaxial plots of the barcode channels. To filter outliers from the populations, decrease the Mahalanobis threshold (**Fig. 5d**). This process of adjusting the separation threshold and the Mahalanobis threshold may be iterative until the appropriate balance between cell number and deconvolution confidence is reached. Examples of choosing the parameters can be found in the manual.

28| Create a separate FCS file for each barcode population by pressing the 'Save Debarcoded Files' button, which will give a prompt to select the folder in which to save the files. By default, the names of the wells as entered in the barcode key are appended to the original file name, but that may be adjusted before saving by editing the base file name next to the 'Save Debarcoded Files' button.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	Uneven MCB staining between multiplexed samples	Unequal cell numbers input into the MCB staining reaction	Recount the cell density for each cell sample and adjust volumes added for multiplexing to equalize the cell number
			If the cell number cannot be equalized because some samples have too few cells, then decrease the MCB reagent cocktail concentration for these samples with fresh DMSO. MCB staining intensity is approximately linear in relation to cell number, and thus for fivefold fewer cells dilute the MCB reagent cocktail fivefold with fresh DMSO
		Samples with large amounts of cellular debris will show reduced MCB labeling, because the debris behaves as additional cells and soaks up MCB reagent, reducing the amount available to label the cells of interest	If possible, re-prepare the sample and minimize debris generation with gentler mechanical trituration
			Debris may be removed from cell samples by density-gradient centrifugation
			Scale up the amount of MCB reagent added to high-debris samples, or reduce the cell number added for multiplexing

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	Lower-than-expected antibody staining intensity	Exceedingly high MCB staining intensity will reduce observed antibody staining intensity by ion-detector saturation/shutoff and by ion suppression	Adjust the cell number and MCB reagent volume so that MCB staining intensity is 200–5,000 counts per cell
12	Lower-than-expected MCB staining intensity	Quenching of MCB cell labeling by residual BSA	Increase the number and/or stringency of PBS washes before MCB cell labeling
	Cell loss during PBS wash steps	Small pellet size and PBS-only washing steps can lead to excessive cell loss	PBS in Step 12B(iv) may be supplemented with 0.1% (vol/vol) Tween-20 without adversely affecting MCB labeling. This should be tested on bulk samples to ensure that antibody staining is not adversely affected
16	Lower-than-expected MCB staining intensity	MCB reagent has been hydrolyzed by multiple freeze-thaws, desiccant failure or storage at elevated temperatures	Test additional 100× MCB combinatorial plates diluted from the same 1,000× stock plate on bulk cells. If these also look bad, dilute additional 1,000× MCB combinatorial plates prepared from the same batch and re-test with bulk cells. If 1,000× stock looks bad, test the individual MCB reagent stocks on bulk cells. Discard and re-prepare MCB combinatorial plates and reagents as necessary
18	Lower-than-expected cell yield (number of events collected)	Cell loss during sample handling and pooling, caused by loss of residual liquid and by cells sticking to the plastic of sample wells and pipette tips	During sample pooling, add an additional wash step for every well to collect residual cells
			Minimize the plastic surface area encountered by the cells by using a single pipette tip to transfer all samples, and by mixing cells by pipetting rather than by vortexing
19	Lower-than-expected antibody staining intensity	Increased cell number relative to unpooled samples resulting in high cell density	Increase the antibody staining volume so that the cell density is ≤ 50 million cells per ml

● TIMING

Steps 1–8, preparation of fixed, permeabilized cells: ~1 h
Steps 9–20, MCB labeling and antibody staining: ~3 h
Step 21, mass cytometry measurement: ~1 h for every million cells
Steps 22–28, sample deconvolution by single-cell debarcoder: ~30 min
Box 1, preparation of palladium MCB cell labeling reagents: overnight
Box 2, titration of palladium MCB reagents: 4–6 h
Box 3, preparation of 1,000× and 100× combinatorial MCB plates: ~2 h
Box 4, MCB combinatorial plate validation: ~2 h

ANTICIPATED RESULTS

The MCB protocol allows multiple samples to be pooled for antibody staining and mass cytometry analysis. The use of the six palladium MCB reagents and the doublet-filtering *n*-choose-*k* barcoding scheme allows 20-sample multiplexing. Although cell yield depends on several factors, including cell type, container type, starting cell number and procedures for liquid transfer and mixing, the typical yield for MCB multiplexing as described is ~50%; if the procedure is begun with 500,000 cells per well at Step 10 (1×10^7 total from 20 individual samples), then ~5×10^6 cells will remain in the pooled, antibody-stained sample at Step 21 for mass cytometry analysis. We observe cell loss to be especially pronounced when the initial cell number is low (**Fig. 7e–g**), and therefore we have found the MCB protocol to improve cell yield greatly for

low-abundance samples owing to the early sample pooling step, which results in a single high-abundance sample rather than multiple low-abundance samples.

After pooled sample processing and measurement on the mass cytometer, a single FCS file is produced for each multiplexed cell sample. The 20 individual samples are recovered from this pooled FCS file by the debarcoding software, which produces 20 FCS files plus an additional FCS file that contains the cell events that were unassigned by the deconvolution algorithm. The MCB protocol and software are flexible enough to allow for additional MCB reagents and barcode schemes. If a researcher desires to multiplex >20 cell samples, a nonredundant barcoding scheme may be used at the expense of doublet filtering, or with additional MCB reagents using lanthanide chelates[6] but at the expense of additional antibody measurement channels. The procedure described here is designed to enable a researcher to barcode and to antibody-stain four groups of 20 samples in 3–4 h. The attributes of MCB described in this report will enhance the quality of mass cytometry data.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS E.R.Z. developed the palladium MCB reagents and labeling protocol, implemented the doublet-filtering barcode scheme, developed the single-cell debarcoding algorithm, designed and performed the experiments, analyzed the data and wrote the manuscript; R.F. developed the single-cell debarcoding algorithm, designed and implemented the debarcoding software, designed the experiments, analyzed the data and wrote the manuscript; G.K.B. developed the palladium MCB reagents and labeling protocol, developed the saponin-mediated MCB labeling and wrote the manuscript; E.D.A. developed the doublet-filtering barcode scheme; S.K. assisted with developing barcode deconvolution methods; V.D.G. performed experiments; C.G.L. performed experiments; Z.B. performed experiments and wrote the manuscript; M.H.S. performed experiments; B.B. developed the palladium MCB reagents and labeling protocol; W.J.F. designed the experiments and wrote the manuscript; D.P. developed the doublet-filtering barcode scheme and wrote the manuscript; G.P.N. designed the experiments and wrote the manuscript.

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