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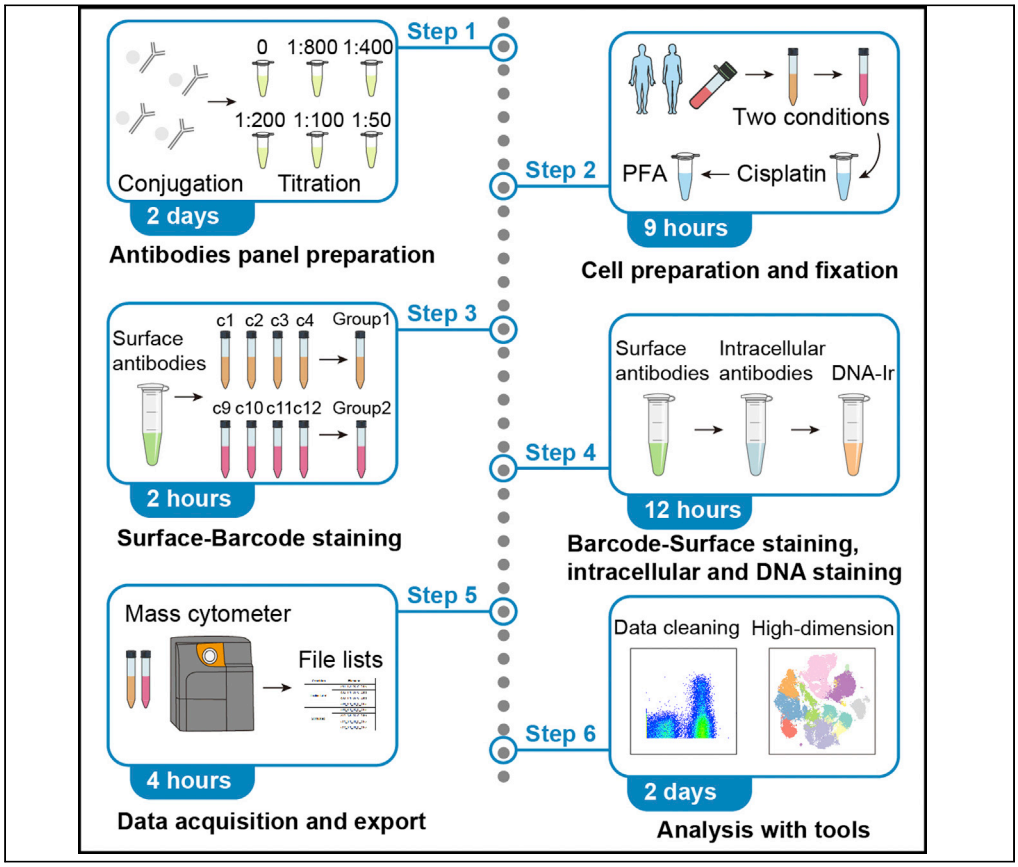
Functional analysis of human circulating immune cells based on high-dimensional mass cytometry



With the advantages of high resolution and high dimension, mass cytometry is implemented to analyze the blood complex immune system in clinical settings. However, long-term clinical sample collection may cause batch effects that mask true biological results. Here, we present a validated and streamlined mass cytometry workflow that features fixed staining for clinical use and optimized barcode staining patterns. The reagents and approaches used in this workflow can help reduce batch effects, thereby extending the application range and advantages of mass cytometry.

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Highlights

Detailed protocol for the detection of blood immune cells using mass cytometry

Suitable for clinical needs by cryopreserving samples after cell fixation

Optimizing the strategy of sample barcode staining for reducing intersample variation

Insights on high-dimensional analysis for mass cytometry data

Liu et al., STAR Protocols 3, 101310
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101310

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Functional analysis of human circulating immune cells based on high-dimensional mass cytometry

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https://doi.org/10.1016/j.xpro.2022.101310

SUMMARY

With the advantages of high resolution and high dimension, mass cytometry is implemented to analyze the blood complex immune system in clinical settings. However, long-term clinical sample collection may cause batch effects that mask true biological results. Here, we present a validated and streamlined mass cytometry workflow that features fixed staining for clinical use and optimized barcode staining patterns. The reagents and approaches used in this workflow can help reduce batch effects, thereby extending the application range and advantages of mass cytometry.

BEFORE YOU BEGIN

This protocol provides reagents and detailed approaches for testing the phenotypes and functions of human blood immune cells. In this protocol, 37 antibodies were used to recognize immune cells and detect secreted cytokines in the blood. Therefore, different combinations of antibody panels can be applied to other immune cell function analyses. The major improvement in this study lies in fixing samples before staining and optimizing the staining in the barcoding strategy.

Institutional permissions

Before performing this protocol, the study should be approved by the relevant institution and informed consent should be obtained from participants. The experiments using human samples in this protocol were approved by the Ethics Committee of Zhongshan Ophthalmic Center, and informed consent was obtained from all subjects.

Preparation of reagents

Timing: 30 min

Prepare the following reagents according to the number of samples and antibodies used in the experiments.

1. Fixation solution (2×) by adding 1-part phosphate buffer solution (PBS) into 4-parts of 4% paraformaldehyde to a final concentration of 3.2% and store at 4°C for up to 2 weeks.
2. Cisplatin stock solution (50 μM) by adding 1-part 5 mM cisplatin solution into 99-parts PBS, and store at −20°C for up to 6 months.
3. The Cell Staining Buffer (CSB) was directly purchased from Fluidigm (Cat#201068). The composition of this buffer is 0.02% BSA in PBS and store at 4°C for up to 2 weeks.
4. Antibody stabilization buffer containing 0.05% sodium azide, store at 4°C for up to 3 months.

Note: Due to the toxicity and low photostability of cisplatin, attention should be paid to protection from light and ventilation during storage and subsequent use. The preparation of some additional buffers please refer to materials and equipment section. Notably, the cell stimulation cocktails used in this protocol have a bias in lymphocyte activation, so appropriate cell stimulants can be selected for different experimental needs (see troubleshooting problem 1 for more details).

Conjugate antibodies to metal isotopes

Timing: 6 h

Although antibodies for mass cytometry can be purchased directly from commercial companies, researchers can design the antibody panel with more flexibility by customizing personalized antibody-metal combinations. MCP9 antibody labeling kits are suitable for labeling antibodies with cadmium (Cd), whereas X8 antibody labeling kits are suitable for labeling antibodies with lanthanide (Ln). Select the appropriate Maxpar labeling kit and protocol for use in the experiment. The X8 kit was used as an example.

5. Preload the polymer with metal.
   a. Thaw the polymer and Ln to room temperature (about 24°C).
   b. Add 95 μL L-Buffer to each X8 polymer tube to resuspend the polymer and mix well.
   c. Add 5 μL Ln metal solution (50 mM) to each X8 polymer tube and mix well.
   d. Incubate the mixture of polymer and metal at 37°C in a warm water bath for 40 min.
6. Wash and partially reduce the antibody.
   a. Add 100 μg stock antibody to each labeled 50 kDa filter. Adjust the volume in the filter to 400 μL with R-Buffer and centrifuge at 12,000 × *g* at 24°C for 10 min.
   b. Prepare a fresh 4 mM TCEP solution by diluting 8 μL of 0.5 M TCEP stock with 992 μL of R-Buffer.
   c. Add 100 μL TCEP solution (4 mM) to each antibody in the filter and mix well.
   d. Immediately incubate at 37°C in a warm water bath for 30 min.
7. Wash and purify the metal-conjugated polymer.
   a. Retrieve the metal-loaded polymer mixture from step 5 and transfer the mixture to a 3 kDa filter containing 200 μL L-Buffer.
   b. Centrifuge at 12,000 × *g* at 24°C for 25 min.
   c. Wash polymer with 400 μL C-Buffer, centrifuge at 12,000 × *g* at 24°C for 30 min, and then remove the centrifuged liquid.
8. Wash and purify the partially reduced antibody.
   a. After 30 min of antibody reduction, add 300 μL C-Buffer to each 50 kDa filter and mix well to carefully wash the antibody.
   b. Centrifuge at 12,000 × *g* at 24°C for 10 min and remove the centrifuged liquid.
   c. Repeat the wash once more with C-Buffer (2 washes in total).
9. Conjugate the partially reduced antibody with metal-loaded polymer.
   a. Retrieve the 3 kDa filter unit and 50 kDa filter.
   b. Resuspend the metal-loaded polymer in a 3 kDa filter in 60 μL of C-Buffer.
   c. Transfer the resuspended solution to the corresponding partially reduced antibody in the 50 kDa filter.
   d. Mix gently by pipetting and incubate at 37°C in a warm water bath for 90 min.
10. Wash the metal-conjugated antibody.
   a. Add 200 μL W-Buffer to each 50 kDa filter containing a metal-conjugated antibody and mix well to carefully wash the antibody.
   b. Centrifuge at 12,000 × *g* at 24°C for 10 min and remove the centrifuged liquid.
   c. Repeat the wash twice more with W-Buffer (3 washes in total).
11. Measure the protein level of antibody.
   a. Following 3 washes, the 50 kDa filter has approximate 20 μL of conjugate solution.

b. Add 80 μL W-Buffer to each 50 kDa filter and carefully rinse the walls of the filter to mix well.
c. Determine the protein level by measuring the absorbance at 280 nm using the NanoDrop spectrophotometer. The W-Buffer is set as blank.
d. Centrifuge at 12,000 × *g* at 24°C for 10 min and remove the centrifuged liquid.

12. Recover and store the metal-conjugated antibody.
    a. Prepare antibody stabilization buffer containing 0.05% sodium azide.
    b. Calculate and add the volume of antibody stabilization buffer required to obtain a final concentration of 0.5 mg/mL of antibody.
    c. Invert the 50 kDa filter over the new and clean collection tube, and centrifuge the assembly of the inverted filter and collection tube at 1,000 × *g* at 24°C for 2 min.
    d. Store the metal-conjugated antibody at 4°C until ready to titrate.

Note: MCP9 antibody labeling kits label antibodies by using Cd isotopes, while X8 antibody labeling kits label antibodies by using Ln isotopes. Because of the detection at a lower relative sensitivity, the Cd metal isotopes should be labeled with the antibody candidates featured by high expression and antibody sensitivity, such as CD3 and CD19. In contrast, several antibodies with low expression antigens and lower sensitivity, such as IL-17 and FOXP3, can be labeled by Ln using X8 antibody labeling kits. In addition, there are some matters needing attention in metal labeling; see limitations for more details.

Note: In this protocol, antibodies purchased from Fluidigm are directly labeled with metal, while antibodies purchased from other companies are manually labeled with metal by using the X8 or MCP9 antibody labeling kits.

⚠ CRITICAL: Polymer is an important factor influencing the quality of the antibody and metal conjugation, so it is necessary to confirm that the right polymer is applied to the labeled metal. Therefore, it isn't recommended to perform MCP9 and X8 labeling protocol at the same time. The differences in reagents and procedures between the kits are likely to lead to user errors or failure of the labeling reaction. There are some potential "noise" sources, such as oxidation products, metal impurities, and environmental contaminants, may influence the signals of specific metals, please refer to troubleshooting problem 2 for more details.

Titrate the conjugated antibody

Timing: 2 days

Determining the appropriate dilution factor of certain conjugated antibody is a key quality control step after labeling, which reflects the efficiency of labeling antibodies and the concentration of antibodies used in subsequent experiments.

13. Selection of cells.
    a. The sample is required to contain both positive and negative cells for this antibody (positive cells for signal, negative cells for background). Peripheral blood mononuclear cells (PBMCs) isolated from human blood samples were used.
    b. Count cells and record viability; determine the volume of PBS needed to resuspend the samples to 3 million cells/mL.
    c. Prepare 6 flow tubes and fill each tube with 1 mL PBS containing 3 million PBMCs.
    d. Centrifuge cells at 300 × *g* at 4°C for 5 min and remove supernatant.
    e. Gently vortex to resuspend cells in residual volume.
14. Viability stain and fix cells.
    a. Prepare 0.5 μM cisplatin working solution by adding 10 μL stock solution into 1 mL PBS.
    b. Add 1 mL of cisplatin working solution to each sample and incubate at 24°C for 2 min to label the dead cells.

c. Centrifuge cells at 300 × *g* at 4°C for 5 min, carefully remove the supernatant and then re-suspend cells in 1 mL of CSB.
d. Add 1 mL of 2× fixation solution to each tube and incubate at 24°C for 10 min.
e. Centrifuge cells at 800 × *g* at 4°C for 5 min, and carefully remove supernatant.
f. Repeat wash one more time with CSB (2 washes in total).
g. Gently vortex to resuspend cells in residual volume (50 μL).

15. Fc receptor (FcR) blocking.
    a. Add 5 μL of FcR blocking solution to each tube.
    b. Incubate the tubes at 24°C for 10 min.
16. Prepare serial dilutions of the antibody being titrated in microcentrifuge tubes. Prepare antibody serial dilutions in five microcentrifuge tubes at concentrations of 1:800, 1:400, 1:200, 1:100, and 1:50.
17. Add 50 μL of each antibody dilution into the corresponding flow tube containing PBMCs, and 50 μL of CSB directly to the last tube as a negative control. Stain according to the surface antibody staining protocol.
18. Data acquisition on Helios (WB Injector).
19. According to the results, select the dilution with low background and maximum separation between positive and negative populations (see the examples in Figure 1). The dilutions listed in Table 1 are used in the final experiments.

Note: When titration of an antibody results in an ideal dilution range, the minimum concentration should be selected to reduce dosage and background staining (see the examples in Figure 1).

⚠ CRITICAL: If the labeled antibody is an intracellular protein, stain with the appropriate protocol (see step-by-step method details for more details).

Pause point: The conjugated and titrated antibodies can be stored for up to 6 months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CCR4 (1:100)	BioLegend	Cat#359402
CCR7 (1:400)	BioLegend	Cat#353237
CXCR4 (1:200)	Fluidigm	Cat#3156029B
CD56 (1:400)	Fluidigm	Cat#3176008B
CD279 (1:100)	Fluidigm	Cat#3155009B
CD3 (1:200)	Fluidigm	Cat#3154003B
CD16 (1:400)	Fluidigm	Cat#3209002B
CD25 (1:200)	BD	Cat#555430
CD27 (1:400)	BD	Cat#555439
CD123 (1:200)	BD	Cat#555642
CD1C (1:200)	BioLegend	Cat#331502
CD57 (1:400)	BioLegend	Cat#359602
CD8 (1:400)	BioLegend	Cat#301002
CD4 (1:200)	BioLegend	Cat#300502
CD45RO (1:400)	BioLegend	Cat#304239
CD19 (1:200)	BioLegend	Cat#302202
HLA-DR (1:200)	BioLegend	Cat#307651
CCR2 (1:400)	BioLegend	Cat#357202
CCR6 (1:200)	BioLegend	Cat#353427

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD11C (1:200)	BioLegend	Cat#301602
CD69 (1:200)	BioLegend	Cat#310939
CX3CR1 (1:200)	BioLegend	Cat#341602
CXCR5 (1:200)	BioLegend	Cat#356902
CD14 (1:400)	BioLegend	Cat#301843
CD45RA (1:200)	BioLegend	Cat#304143
IFN-γ (1:200)	BioLegend	Cat#506521
IL-10 (1:200)	BioLegend	Cat#501423
IL-17A (1:50)	BioLegend	Cat#512331
IL-2 (1:100)	BioLegend	Cat#500339
IL-4 (1:100)	BioLegend	Cat#500829
IL-6 (1:100)	BioLegend	Cat#501115
TNF-α (1:200)	BioLegend	Cat#502941
FOXP3 (1:50)	BioLegend	Cat#320102
GM-CSF (1:100)	BioLegend	Cat#502315
IL-1β (1:200)	Novus	Cat#MAB201-100
IL-22 (1:100)	Novus	Cat#AF782
IL-23 (1:100)	Novus	Cat#MAB17161
Biological samples		
Human Blood	Healthy volunteers from Zhongshan Ophthalmic Center, 2 males and 2 females, average age 45 years	N/A
Chemicals, peptides, and recombinant proteins		
Cell-ID Cisplatin—5 mM, 100 μL	Fluidigm	Cat#201064
Cell-ID Intercalator-Ir—125 μM, 25 μL	Fluidigm	Cat#201192A
4% paraformaldehyde—500 mL	Beyotime	Cat#P0099
Human TruStain FcX™ (FcR Blocking Solution)	BioLegend	Cat#422301
Maxpar Cell Staining Buffer—500 mL	Fluidigm	Cat#201068
Maxpar Fix and Perm Buffer	Fluidigm	Cat#201067
Maxpar PBS—500 mL	Fluidigm	Cat#201058
Maxpar Fix I Buffer (5×)	Fluidigm	Cat#201065
Maxpar Barcode Perm Buffer (10×)	Fluidigm	Cat#201057
Maxpar Perm-S Buffer	Fluidigm	Cat#201066
Tuning Solution—250 mL	Fluidigm	Cat#201072
EQ™ Four Element Calibration Beads—100 mL	Fluidigm	Cat#201078
Maxpar Cell Acquisition Solution—200 mL	Fluidigm	Cat#201240
Antibody Stabilizer PBS (antibody stabilization buffer)	CANDOR Bioscience	Cat#131 050
RPMI-1640 Medium	Gibco	Cat#11875093
Fetal Bovine Serum (FBS)	Gibco	Cat#16140071
Sodium Pyruvate (100 mM)	Gibco	Cat#11360070
HEPES (1 M)	Gibco	Cat#15630080
Penicillin-streptomycin (100×, 10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin)	Gibco	Cat#15140122
Ficoll-Paque PLUS solution	Cytiva	Cat#17144003
Red Blood Cell Lysing Buffer	Sigma-Aldrich	Cat#R7757
Dimethylsulfoxide (DMSO)	MP Biomedicals	Cat#196055
Phorbol Myristate Acetate	Sigma-Aldrich	Cat#P8139
Ionomycin	Sigma-Aldrich	Cat#I3909
Brefeldin A	Sigma-Aldrich	Cat#B5936
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution, pH 7.0 (10 × 1 mL, 0.5 M)	MilliporeSigma	Cat#646547
HRP-Protector™ peroxidase stabilizer	Boca Scientific	Cat#222050
Critical commercial assays		
Maxpar MCP9 Antibody Labeling Kit- 111Cd	Fluidigm	Cat#201111A
Maxpar MCP9 Antibody Labeling Kit- 112Cd	Fluidigm	Cat#201112A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Maxpar MCP9 Antibody Labeling Kit- 113Cd	Fluidigm	Cat#201113A
Maxpar MCP9 Antibody Labeling Kit- 114Cd	Fluidigm	Cat#201114A
Maxpar MCP9 Antibody Labeling Kit- 116Cd	Fluidigm	Cat#201116A
Maxpar X8 Antibody Labeling Kit- 149Sm	Fluidigm	Cat#201149A
Maxpar X8 Antibody Labeling Kit- 167Er	Fluidigm	Cat#201167A
Maxpar X8 Antibody Labeling Kit- 150Nd	Fluidigm	Cat#201150A
Maxpar X8 Antibody Labeling Kit- 162Dy	Fluidigm	Cat#201162A
Maxpar X8 Antibody Labeling Kit- 143Nd	Fluidigm	Cat#201143A
Maxpar X8 Antibody Labeling Kit- 170Er	Fluidigm	Cat#201170A
Maxpar X8 Antibody Labeling Kit- 165Ho	Fluidigm	Cat#201165A
Maxpar X8 Antibody Labeling Kit- 161Dy	Fluidigm	Cat#201161A
Maxpar X8 Antibody Labeling Kit- 153Eu	Fluidigm	Cat#201153A
Maxpar X8 Antibody Labeling Kit- 141Pr	Fluidigm	Cat#201141A
Maxpar X8 Antibody Labeling Kit- 146Nd	Fluidigm	Cat#201146A
Maxpar X8 Antibody Labeling Kit- 144Nd	Fluidigm	Cat#201144A
Maxpar X8 Antibody Labeling Kit- 172Yb	Fluidigm	Cat#201172A
Maxpar X8 Antibody Labeling Kit- 171Yb	Fluidigm	Cat#201171A
Maxpar X8 Antibody Labeling Kit- 163Dy	Fluidigm	Cat#201163A
Maxpar X8 Antibody Labeling Kit- 168Er	Fluidigm	Cat#201168A
Maxpar X8 Antibody Labeling Kit- 166Er	Fluidigm	Cat#201166A
Maxpar X8 Antibody Labeling Kit- 169Tm	Fluidigm	Cat#201169A
Maxpar X8 Antibody Labeling Kit- 158Gd	Fluidigm	Cat#201158A
Maxpar X8 Antibody Labeling Kit- 142Nd	Fluidigm	Cat#201142A
Maxpar X8 Antibody Labeling Kit- 147Sm	Fluidigm	Cat#201147A
Maxpar X8 Antibody Labeling Kit- 175Lu	Fluidigm	Cat#201175A
Maxpar X8 Antibody Labeling Kit- 159Tb	Fluidigm	Cat#201159A
Maxpar X8 Antibody Labeling Kit- 174Yb	Fluidigm	Cat#201174A
Maxpar X8 Antibody Labeling Kit- 173Yb	Fluidigm	Cat#201173A
Maxpar X8 Antibody Labeling Kit- 164Dy	Fluidigm	Cat#201164A
Maxpar X8 Antibody Labeling Kit- 160Gd	Fluidigm	Cat#201160A
Cell-ID 20-Plex Palladium (Pd) Barcoding Kit	Fluidigm	Cat#201060
Software and algorithms		
CyTOF Software Version 6.5.358	Fluidigm	https://www.fluidigm.com/software
FlowJo	Becton, Dickinson & Company	https://www.flowjo.com/solutions/flowjo/downloads
Cytobank	Cytobank Inc.	https://www.cytobank.org/
FlowCore R package Version 2.0.1	N/A	http://www.bioconductor.org/packages/release/bioc/html/flowCore.html
CATALYST R package Version 1.12.2	N/A	http://www.bioconductor.org/packages/release/bioc/html/CATALYST.html
FlowSOM R package Version 1.20.0	N/A	http://www.bioconductor.org/packages/release/bioc/html/FlowSOM.html
Seurat R package Version 4.0.5	N/A	https://satijalab.org/seurat/
Other		
Corning® polypropylene round-bottom tubes, 5 mL capacity, 12 × 75 mm	Corning	Cat# 352063
Polypropylene round-bottom tubes with 35 µm cell-strainer cap, 5 mL capacity,12 × 75 mm	Corning	Cat# 352235
1 mL Norm-Ject® latex-free syringes and compatible 0.1 µm syringe filters	NORM-JECT	Cat# 53548-001
Amicon® Ultra-0.5 Centrifugal Filter Unit, 0.5 mL V-bottom	MilliporeSigma	Cat#UFC500308 (3 kDa)/Cat#UFC505008 (50 kDa)/Cat#UFC510008 (100 kDa)
Microcentrifuge Tubes, 0.2 mL	Eppendorf	Cat#0030124332
Microcentrifuge Tubes, 1.5 mL	Eppendorf	Cat#0030108442

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Microcentrifuge Tubes, 5 mL	Eppendorf	Cat#0030119401
Centrifuge Tubes, 15 mL	Jet	Cat#CFT011150
Centrifuge Tubes, 50 mL	Jet	Cat#CFT011500
6-well cell culture plate	Jet	Cat#TCP-011-006
BD Vacutainer® glass blood collection tubes with sodium heparin	BD Vacutainer	Cat#366480
Cryogenic Vials	Corning	Cat#430659
Pipettes (P10–P1000)	Eppendorf	N/A
Aerosol barrier (filter) tips	Eppendorf	N/A
NanoDrop One	Thermo Fisher Scientific	N/A
Two tabletop refrigerated centrifuges	Eppendorf	N/A
Mini-centrifuge compatible with 1.5 mL tubes	Eppendorf	N/A
Vortexer	Thermo Fisher Scientific	N/A
Forma CO2 incubator 3111	Thermo Fisher Scientific	N/A
Water bath capable of 37 ± 1.5°C	Thermo Fisher Scientific	N/A
Cellometer Auto 1000 Cell Counter	Nexcelom Biosciences	N/A
4°C and −80°C storage	Haier	N/A
Fluidigm Helios Mass Cytometer	Fluidigm	PN#400250
Helios WB Injector	Fluidigm	Cat#107950

MATERIALS AND EQUIPMENT

Human samples

Human blood samples come from volunteers that meet the following requirements: physical and psychological health and no clinically significant abnormalities in blood chemistry. Whole blood from the volunteers is collected in heparin anticoagulant tubes. PBMCs are isolated using a Ficoll-Hypaque solution according to standard density gradient centrifugation methods (Fuss et al., 2009). Cell viability is tested, and the cell viability of all used samples is required to be greater than 80%.

Note: If the cell viability of the sample is less than 80%, a low-speed centrifugation or Dead Cell Removal Kit (Cat#130-090-101, Miltenyi Biotec) can be used to remove partial dead cells and improve the viability. Several notes on improving cell viability please refer to trouble-shooting problem 3 for more details.

⚠ CRITICAL: This protocol includes experimental procedures that may cause harm to humans, such as cisplatin staining and cell fixation. We recommend that these operations be performed in fume hoods to reduce unnecessary injuries. In addition, blood residues after PBMCs separation should be properly disposed of in accordance with the management measures of the unit on discarded medical objects.

RPMI-1640 culture medium

Reagent	Final concentration	Amount (mL)
RPMI-1640	n/a	43.5
FBS	10%	5
Sodium Pyruvate (100 mM)	1 mM	0.5
HEPES (1 M)	10 mM	0.5
Penicillin-streptomycin (100×)	1×	0.5
Total	**n/a**	**50**

Stored at 4°C for up to 2 weeks.

Cell stimulation cocktails		
Reagent	Final concentration	Amount (mL)
RPMI-1640 culture medium	n/a	8.5
Phorbol myristate acetate (1 μg/mL)	50 ng/mL	0.5
Ionomycin (10 μg/mL)	500 ng/mL	0.5
Brefeldin A (20 μg/mL)	1 μg/mL	0.5
Total	n/a	10
Store at 4°C for up to 2 weeks.		

STEP-BY-STEP METHOD DETAILS

Prepare cells and viability stain

Timing: 8 h

1. PBMCs isolation and red blood cells (RBCs) lysis.
   a. Perform the PBMCs isolation from human blood samples by using Ficoll-Hypaque density gradient centrifugation methods.
   b. Add 1 mL RBCs lysing buffer to each sample and incubate at 24°C for 1 min.
   c. Add 10 mL culture medium to each sample to stop the lysis process.
   d. Centrifuge cells at 300 × *g* at 4°C for 5 min, carefully remove supernatant.
   e. Repeat the wash once more with culture medium (2 washes in total).
   f. Add 1 mL of culture medium to each tube to resuspend cells.
2. Count cells and determine the cell viability.
   a. Perform Trypan Blue staining by mixing 10 μL cell suspension with 10 μL trypan blue.
   b. Add the mixture into the counting slides and count using Cellometer Cell Counter.
3. Prepare cells for two different conditions.
   a. Prepare and add 3 million cells to the cell culture plate.
   b. Add the cell stimulation cocktails to each sample and incubate at 37°C in a 5% CO_2 environment for 5 h.
   c. Collect cells from culture plates and wash with PBS (name: stimulated group).
   d. Name the cells without cocktails treatment as the unstimulated group.
4. Viability stain.
   a. Count cells and determine the cell viability of each sample.
   b. Prepare 0.5 μM cisplatin working solution by adding 10 μL stock solution into 1 mL PBS.
   c. Add 1 mL of cisplatin working solution to each sample and incubate at 24°C for 2 min to distinguish between dead and living cells.
   d. Add 5 mL PBS to each sample and mix well to stop the staining reaction.
   e. Centrifuge cells at 300 × *g* at 4°C for 5 min, carefully remove supernatant.
   f. Add 1 mL of CSB to each tube to resuspend cells.

Note: The cell stimulation cocktails used in this step can influence the expression of several surface markers (please refer to troubleshooting problem 1 for more details).

Optional: Cell-ID cisplatin can be replaced by cell-ID intercalator-Rh (Cat#201103A).

Fixing cells

Timing: 30 min

Cell fixation is very important for cell preservation and subsequent surface and intracellular staining because of the long-time span of clinical sample collection. Before fixation, the CSB should be prepared at 24°C and ice-cold separately.

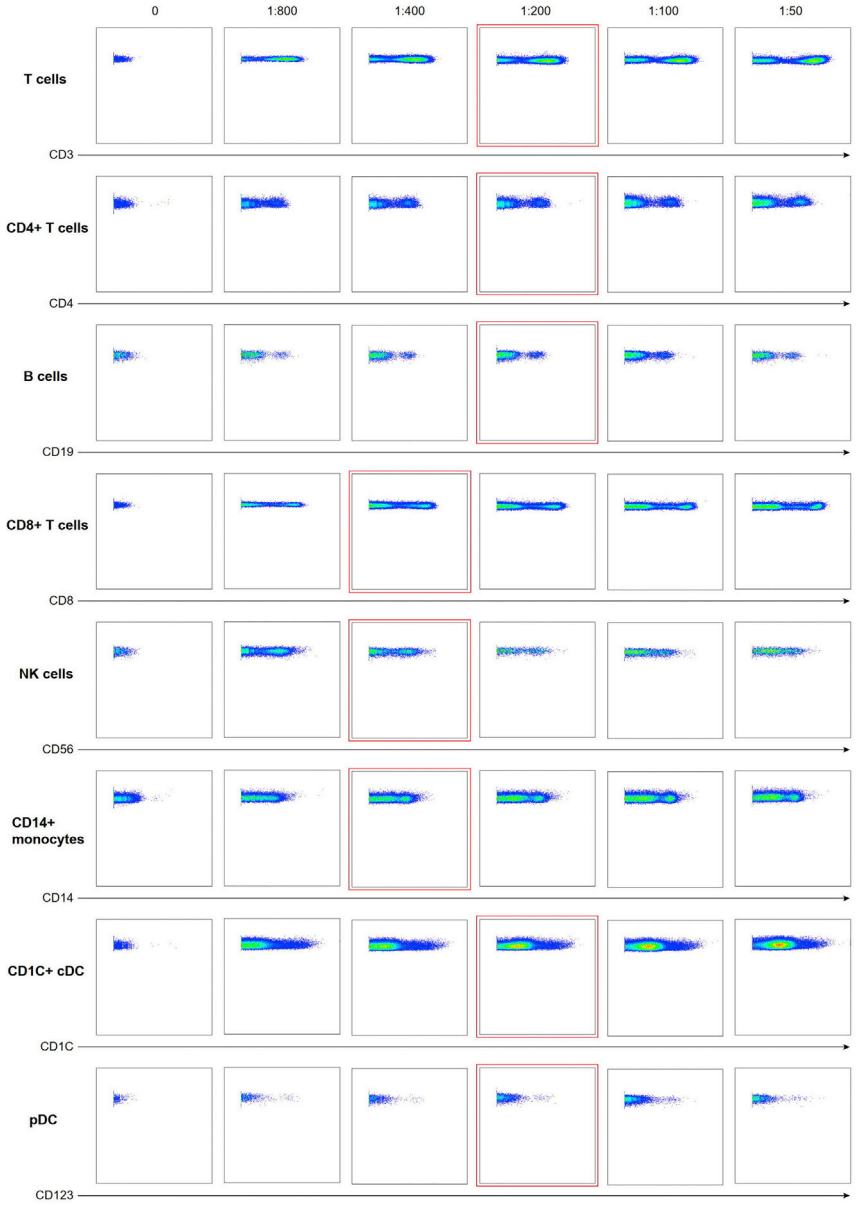

Figure 1. Titrate the surface antibody in the panel

Cells are stained with a mixture of surface antibodies with multiple concentration gradients, followed by data acquisition on Helios Mass Cytometer. Flowjo was used to perform the gating strategy and obtain viable and single-cell events for showing the negative and positive populations of certain markers (see expected outcomes and Figure 3 for more details). Note that the background staining of negative populations decreases with increasing dilution. Titration results show that the ideal dilution factors of antibodies are 1:200 or 1:400. For example, the optimal dilution factor of CD3 is 1:200, because the separation of negative and positive populations is not obvious when it is higher than 1:200, and the background staining of negative populations is stronger when it is lower than 1:200. For two dilutions with similar low background and clear staining separation, the higher dilution should be selected to reduce antibody dosage and background staining, like the dilution of 1:200 for CD4.

5. Add 1 mL of the 2× fixation solution to each tube and mix well. The final fixation working concentration should be 1.6% to reduce the damage of fixation to surface targets.
6. Incubate the tubes at 24°C for 10 min. Then, add 4 mL of ice-cold CSB to each sample to slow down the fixation reaction.

Table 1. Antibody panel for mass cytometry

Target	Clone	Label	Localization	Dilution
CCR4	L291H4	149Sm	Surface	1:100
CCR7	G043H7	167Er	Surface	1:400
CXCR4	12G5	156Gd	Surface	1:200
CD56	NCAM16.2	176Yb	Surface	1:400
CD279	EH12.2H7	155Gd	Surface	1:100
CD3	UCHT1	154Sm	Surface	1:200
CD16	3G8	209Bi	Surface	1:400
CD25	M-A251	150Nd	Surface	1:200
CD27	M-T271	162Dy	Surface	1:400
CD123	9F5	143Nd	Surface	1:200
CD1C	L161	170Er	Surface	1:200
CD57	HNK-1	114Cd	Surface	1:400
CD8	RPA-T8	111Cd	Surface	1:400
CD4	RPA-T4	116Cd	Surface	1:200
CD45RO	UCHL1	165Ho	Surface	1:400
CD19	HIB19	161Dy	Surface	1:200
HLA-DR	L243	112Cd	Surface	1:200
CCR2	K036C2	153Eu	Surface	1:400
CCR6	G034E3	141Pr	Surface	1:200
CD11C	3.9	146Nd	Surface	1:200
CD69	FN50	144Nd	Surface	1:200
CX3CR1	2A9-1	172Yb	Surface	1:200
CXCR5	J252D4	171Yb	Surface	1:200
CD14	M5E2	163Dy	Surface	1:400
CD45RA	HI100	113Cd	Surface	1:200
IFN-γ	B27	168Er	Cytokine	1:200
IL-10	JES3-9D7	166Er	Cytokine	1:200
IL-17A	BL168	169Tm	Cytokine	1:50
IL-2	MQ1-17H12	158Gd	Cytokine	1:100
IL-4	MP4-25D2	142Nd	Cytokine	1:100
IL-6	MQ2-13A5	147Sm	Cytokine	1:100
TNF-α	MAb11	175Lu	Cytokine	1:200
FOXP3	206D	159Tb	Transcription factor	1:50
GM-CSF	BVD2-21C11	174Yb	Cytokine	1:100
IL-1β	8516	173Yb	Cytokine	1:200
IL-22	Polyclonal	164Dy	Cytokine	1:100
IL-23	727753	160Gd	Cytokine	1:100

7. Centrifuge the cells at 800 × *g* at 4°C for 5 min. Carefully remove the supernatant.
8. Gently vortex to resuspend cells in residual volume.
9. Prepare the cryopreservation solution by adding 1-part DMSO into 9-parts CSB.
10. Add 1 mL of cryopreservation solution to resuspend cells and place them on ice.
11. Count cells of each sample.
12. Calculate the volume of the cryopreservation solution required to obtain a final density of 3.5 million/mL of cells.
13. Add the calculated volume of cryopreservation solution in the cells.
14. Add 1 mL cell suspension to the cryogenic vials for separate packing and store at −80°C.

Optional: The incubation duration can be adjusted by adjusting the time of adding ice-cold CSB.

Note: The greater cell recovery can result from increased centrifuge speed after cell fixation. The centrifugal speed of this protocol is 300 × *g* before fixation and 800 × *g* after fixation.

Pause point: The samples can be stored at −80°C for up to 3 months.

⚠ CRITICAL: It is necessary to thoroughly disrupt the pellet by vortexing before and after being incubated with the fixation solution.

Sample preparation and staining of cells with the surface antibody

Timing: 1 h

When sufficient clinical samples are collected, and antibodies and reagents are prepared, cell resuscitation and staining preparation can be performed.

15. Recover PBMCs from −80°C storage.
    a. Preheat the water bath to 37°C.
    b. Thaw cells of each sample from liquid nitrogen storage at 37°C in a warm water bath immediately.
    c. Transfer cells to a 15 mL centrifuge tube.
    d. Slowly add 10 mL CSB by drop to minimize damage from concentration changes.
    e. Centrifuge cells at 800 × *g* at 4°C for 5 min, and carefully remove the supernatant.
    f. Add 2 mL CSB to each tube to resuspend cells.
    g. Count cells and record viability.
    h. Centrifuge cells at 800 × *g* at 4°C for 5 min, and carefully remove the supernatant.
    i. Gently vortex to resuspend cells in residual volume (50 μL).
    j. Resuscitate four samples from the unstimulated group and four from the stimulated group for subsequent experiments.
16. Prepare the mixture of surface antibodies CCR4 and CCR7 according to the titration results.
17. Add CSB to the antibody mixture for a total volume of 50 μL.
18. Add 50 μL of the antibody mixture to each tube.
19. Gently pipette to mix well and incubate at 24°C for 15 min.
20. Gently vortex samples and incubate at 24°C for an additional 15 min.
21. Add 2 mL CSB to each tube to resuspend cells.
22. Centrifuge cells at 800 × *g* at 4°C for 5 min, carefully remove supernatant.
23. Repeat wash one more time with CSB (2 washes in total).
24. Gently vortex to resuspend cells in residual volume (50 μL).

Note: The resuscitation of the PBMCs is very important for subsequent experiments, so it is recommended that two experimenters prepare the cells together.

Barcoding samples with Cell-ID 20-plex palladium (Pd) kit

Timing: 1 h

The Cell-ID™ 20-Plex Pd Barcoding Kit can be performed with barcoding staining of 20 samples.

Each sample is tagged with a unique label so that they can be mixed into a multiplex group for subsequent staining and data acquisition. Because multiple samples are mixed, the kit reduces sample-specific staining and intersample manipulation errors (see Figure 2 for barcoding technical strategy). This protocol is adapted from the Cell-ID 20-Plex Pd Barcoding Kit User Guide, with the major changes listed below:

25. Fixing and Permeabilizing cells.
    a. Prepare the 1× Fix I Buffer by adding 1-part 5× Fix I Buffer into 4-parts PBS.
    b. Resuspend cells in 1 mL Fix I Buffer and incubate at 24°C for 10 min.

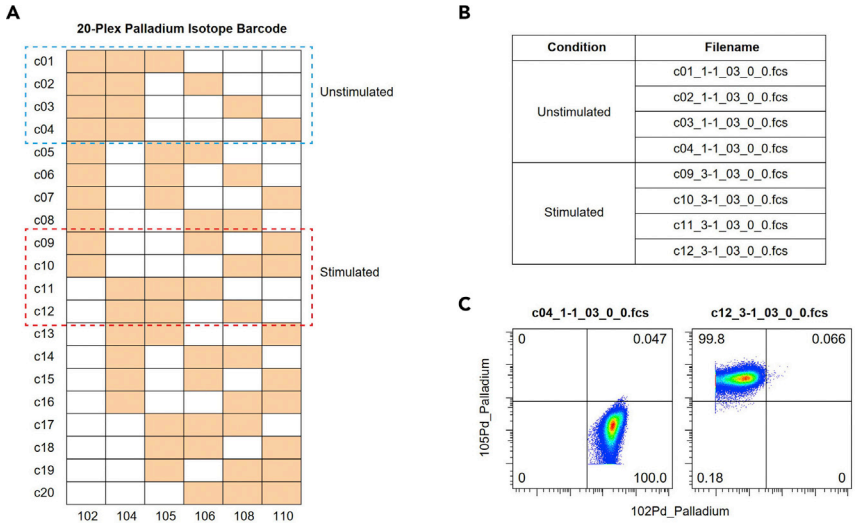

Figure 2. Barcoding technical strategy and results

(A–C) There are 20 Barcode Plex in the Cell-ID™ 20-Plex Pd Barcoding Kit, enabling unique barcoding of 20 samples. With a specific code number (c#), each plex is labeled with three specific palladium isotopes (A). The debarcoding process is performed in the Helios software based on the Pd-Plex combination and then exported file will be numbered with plex (B). For example, c04 is positive for 102Pd and negative for 105Pd, while c12 is the opposite (C).

c. Add 1 mL CSB to each tube to slow down the fixation reaction.
d. Centrifuge cells at 800 × *g* at 4°C for 10 min, and carefully remove the supernatant.
e. Prepare the 1× Barcode Perm Buffer by adding 1-part 10× Barcode Perm with 9-parts PBS.
f. Repeat the wash once more with Barcode Perm Buffer (2 washes in total).

26. Fully resuspend barcodes in 100 μL Barcode Perm Buffer.
27. Fully resuspend each sample to be barcoded in 800 μL Barcode Perm Buffer.
28. Transfer the barcodes to the corresponding samples, labeled with the respective barcoding group.
29. Mix the sample completely and incubate at 24°C for 30 min.
30. Centrifuge cells at 800 × *g* at 4°C for 5 min and carefully remove the supernatant.
31. Add 2 mL CSB to each tube to resuspend cells.
32. Centrifuge cells at 800 × *g* at 4°C for 5 min and remove supernatant.
33. Repeat wash one more time with CSB (2 washes in total).
34. Add 1 mL CSB to each tube to resuspend cells.
35. Count cells of each sample and calculate the volume of CSB containing 0.75 million cells.
36. Combine the four barcoded samples (0.75 million cells per sample) into one tube to obtain the barcoded group.
37. Centrifuge cells at 800 × *g* at 4°C for 5 min and remove supernatant.
38. Gently vortex to resuspend cells in residual volume (50 μL).

Note: Because barcoding with Cell-ID 20-Plex Pd can affect surface antibody staining, previous studies emphasize that this step must be performed after surface staining (Thrash et al., 2020). In this protocol, we improved the staining method of barcoding (see troubleshooting problem 4 for more details).

FcR-blocking cells and surface stain

Timing: 1 h

Following barcoding staining, samples can be mixed as a barcoded group. Then each barcoded group is performed with an FcR-blocking step and a surface-staining step for specific surface staining.

39. Add 5 μL of FcR Blocking Solution to each tube and mix well.
40. Incubate the tubes at 24°C for 10 min.
41. Prepare the mixture of surface antibodies according to the titration results.
42. Add CSB to the antibody mixture to a total volume of 50 μL.
43. Add 50 μL of the antibody mixture to each tube.
44. Gently pipette to mix well and incubate at 24°C for 15 min.
45. Gently vortex samples and incubate at 24°C for an additional 15 min.
46. Add 2 mL CSB to each tube.
47. Centrifuge cells at 800 × *g* at 4°C for 5 min and remove the supernatant.
48. Repeat wash one more time with CSB (2 washes in total).
49. Gently vortex to resuspend cells in residual volume.

Optional: The FcR blocking solution can be replaced by the reagent purchased from Miltenyi Biotec (Cat#130-059-901).

Intracellular stain

Timing: 1.5 h

50. Fixing and Permeabilizing cells.
    a. Prepare the 1× Fix I Buffer by adding 10 mL of 5× Fix I Buffer into 40 mL PBS.
    b. Resuspend cells in 1 mL Fix I Buffer, and incubate at 24°C for 20 min.
    c. Add 2 mL Perm-S Buffer to each tube.
    d. Centrifuge cells at 800 × *g* at 4°C for 5 min and remove the supernatant.
    e. Repeat the wash once more with Perm-S buffer for (2 washes in total).
    f. Gently vortex to resuspend cells in residual volume (50 μL).
51. Prepare the mixture of intracellular antibodies according to the titration results.
52. Add Perm-S buffer to the antibody mixture for a total volume of 50 μL.
53. Add 50 μL of the antibody mixture to each tube.
54. Gently vortex samples and incubate at 24°C for 30 min.
55. Add 2 mL CSB to each tube.
56. Centrifuge cells at 800 × *g* at 4°C for 5 min and remove the supernatant.
57. Repeat the wash once more with Perm-S buffer for a (2 washes in total).
58. Gently vortex to resuspend cells in residual volume (50 μL).

Note: When there are too many antibodies, the mixture of surface and intracellular antibodies must be performed by two people simultaneously to reduce the operating error. In certain cases, you can skip this step if you are not concerned with intracellular proteins.

Stain cells with Cell-ID Intercalator-Ir

Timing: 10 h

After cells are stained with surface antibodies and intracellular antibodies, cells also need to undergo nuclear staining to identify single cells and cell fragments.

59. Prepare 2 mL cell intercalation solution for each barcoded group by adding 2 μL Cell-ID Intercalator-Ir (125 μM) into 2 mL Fix and Perm Buffer to obtain a final concentration of 125 nM.
60. Add 1 mL cell intercalation solution to each tube and gently vortex.

61. Leave overnight (8–10 h) at 4°C.

Optional: If time is sufficient, the cells can be prepared for data acquisition after incubation in the cell intercalation solution at 24°C for 1 h.

Note: Frozen aliquots of Cell-ID Intercalator-Ir should be used only once and immediately after thawing.

Pause point: At the end of this major step, cells can be left in the cell intercalation solution at 4°C for up to 48 h.

Prepare cells for acquisition

Timing: 4 h (for 2 barcoded groups)

62. Rewarm the cells from the 4°C storage.
63. Centrifuge cells at 800 × *g* at 4°C for 5 min and remove the supernatant.
64. Add 2 mL of CSB in each tube to resuspend cells.
65. Centrifuge cells at 800 × *g* at 4°C for 5 min and carefully remove the supernatant.
66. Gently vortex to resuspend cells in residual volume.
67. Add 2 mL of cell acquisition solution (CAS) to each tube to resuspend and count cells.
68. Centrifuge cells at 800 × *g* at 4°C for 5 min and carefully remove the supernatant.
69. Repeat the wash once more with CAS (2 washes in total).
70. Calculate the volume of CAS required to obtain a final density of 1 million/mL of cells.
71. Prepare a sufficient volume of 0.1× EQ beads solution by adding 1-part beads to 9-parts of the CAS.
72. Add the calculated volume of 0.1× EQ beads solution to resuspend cells.
73. Filter cells into cell strainer cap tubes.
74. Acquire each sample in Helios™ (WB Injector) with an acquisition rate of 300–350 events/s.
75. After the acquisition, the files can be normalized, processed, and debarcoded using Helios software.
76. Eight Flow Cytometry Standard (FCS) files can be exported for subsequent analysis (see expected outcomes for more details).

Note: The start-up and tuning of the mass cytometer can be performed with sample washing at the same time. In addition, filtering cells with cell strainer cap tubes is necessary for the normal operation of the Helios™ Mass Cytometer.

⚠ CRITICAL: This protocol is specific for Helios™ mass cytometer with a WB injector and sample acquisition in CAS. It is known that the WB injector has the advantage of superior data quality compared to the High Throughput (HT) injector (Thrash et al., 2020).

EXPECTED OUTCOMES

After being obtained from a CyTOF system Helios with WB Injector, data was then processed with Helios software (version 6.5.358; Fluidigm), including deconvoluting barcoded samples and filtering cross-sample doublets. The Helios mass flow cytometer (Fluidigm) was quality controlled and tuned every time. The stimulated and unstimulated groups had 4 FCS files respectively, which were exported for subsequent analysis.

Cytometry software, like Flowjo, was used to perform the following gating strategy and cell analysis with the example of cells from donor 4. Gating cellular events with a dual-axis plot of Time versus Event Length to check the stability of cell acquisition in a cytometer. Then, the effects of EQ Beads and Gaussian calculations are removed. Finally, we eliminated the dead cells, and debris to identify

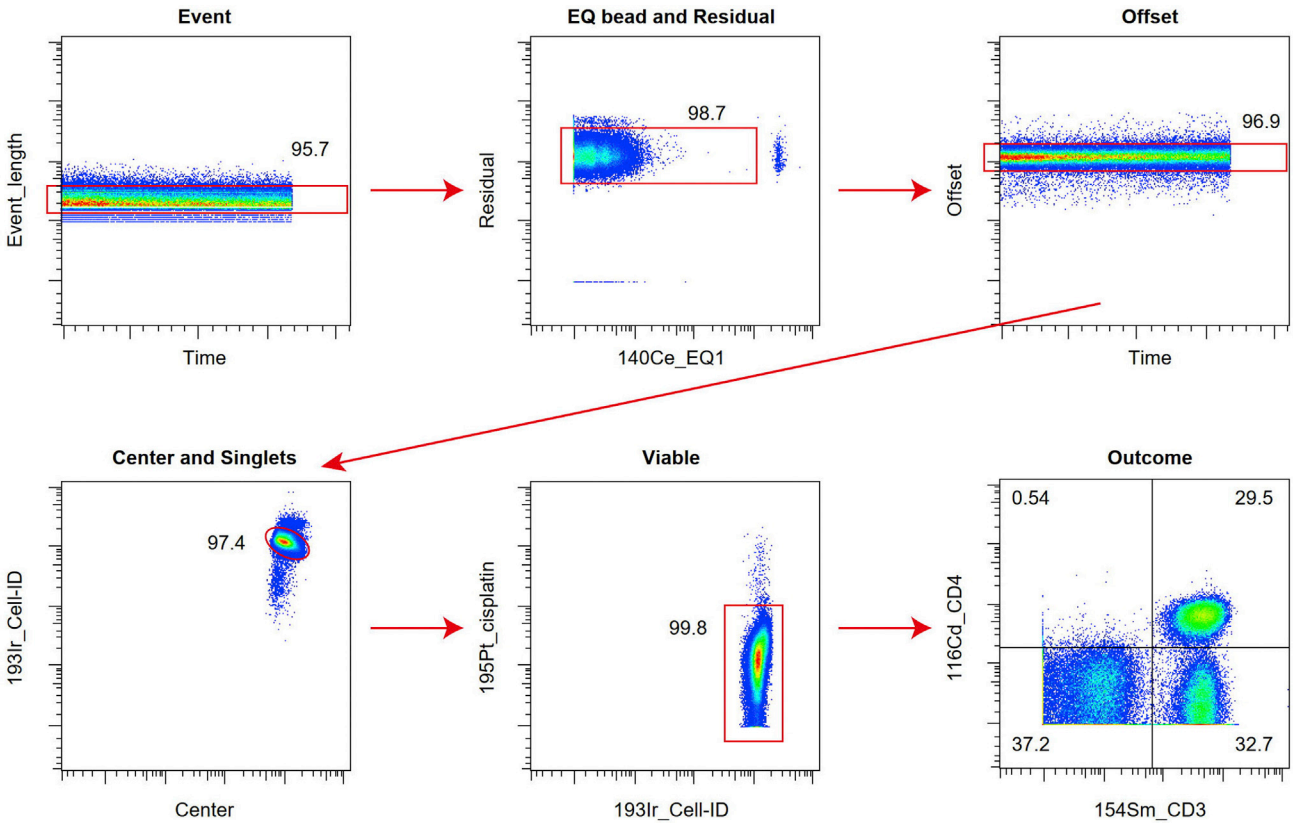

Figure 3. The gating strategy of data cleaning

As an example, the data in the #c04 FCS file is imported and performed data cleaning in Flowjo. The viable singlet cell events are obtained by removal of EQ Beads, Gaussian calculations, dead cells, and debris.

viable, singlet cell events based on event length and live-cell (195Pt) and DNA (191Ir and 193Ir) channels (see Figure 3 for more details). Finally, the single-cell events can be further manually gated to find the population frequencies of certain cell subsets. For example, the cells expressing IL-2, IFN-γ, GM-CSF, TNF-α, and IL-17A were increased after stimulation (see Figure 4).

In addition, the data cleaning can be performed on the Cytobank website for the subsequent analysis with high-dimensional algorithms as described in (Nowicka et al., 2017; Zheng et al., 2020; Liu et al., 2021). The FCS files were exported and processed by the FlowCore R package. For samples over 12,000 cells, we randomly selected 12,000 cells to ensure equal representation of the samples. The data were transformed and integrated using the CATALYST R package with the default parameters. All FlowSOM-based clustering was performed on the data to identify specific populations with default parameters (see Figures 5A–5B). In addition, we assessed the expression of multiple cytokines and receptors in different leukocyte lineages with the "FeaturePlot" function of Seurat R package, highlighting a complicated pattern of cytokine and receptor co-expression in different immune cell subsets (see Figure 5C).

Note: 19 of 37 antibodies were used to identify specific populations in high-dimensional analysis (See troubleshooting problem 5 for more details).

LIMITATIONS

This protocol has been optimized for human blood immune cells processed from peripheral blood using Ficoll density gradient centrifugation. By fixing samples before staining, and optimizing the staining mode of barcoding strategy, this protocol is only for this sample and immune types.

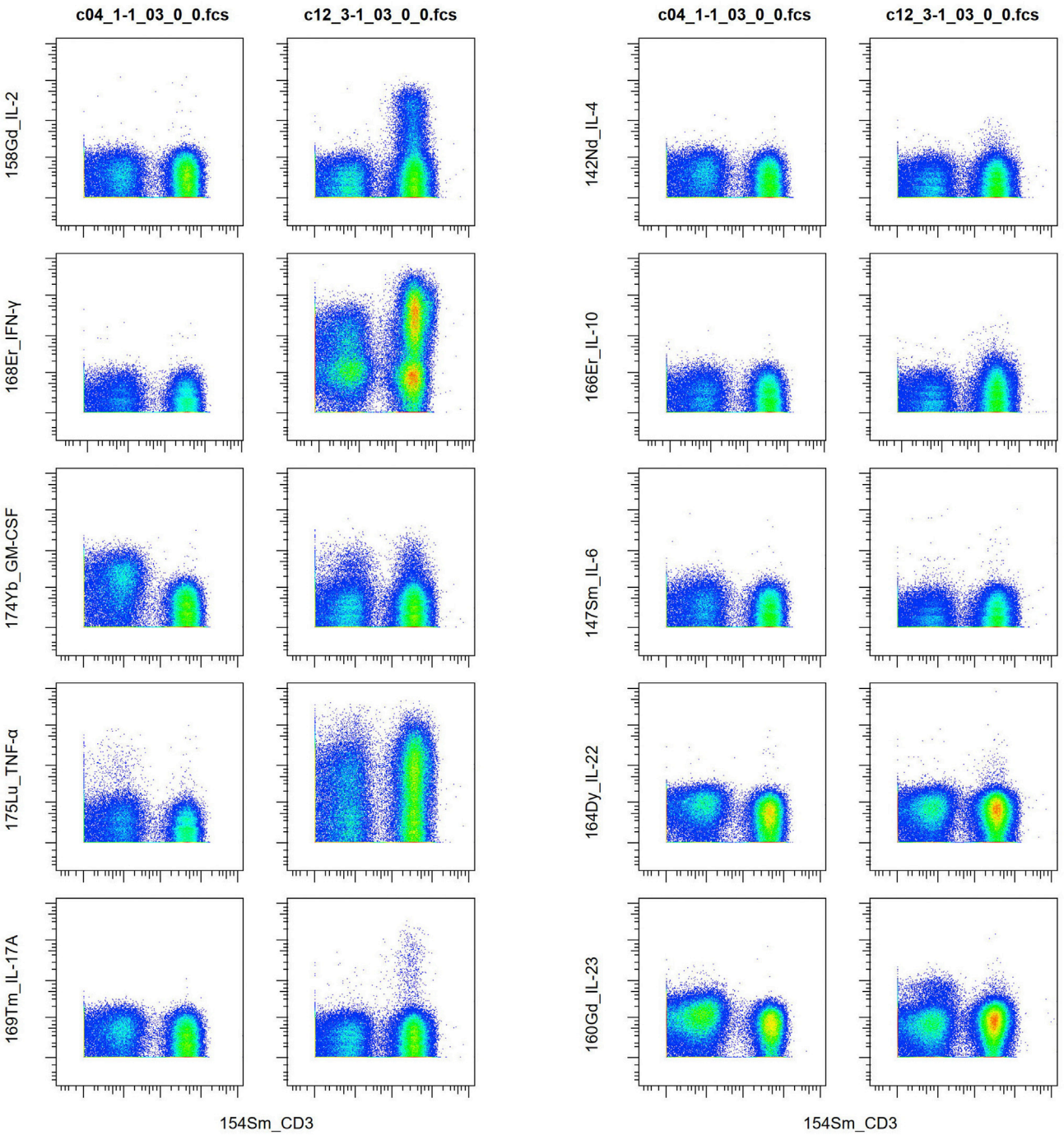

Figure 4. Measuring the expression of intracellular factors

The scatter plots show the expression of ten intracellular factors (besides IL-1β, see troubleshooting problem 1 and Figure 6 for more details) between unstimulated and stimulated groups.

Partial reduction of the antibody and loading of the polymer should be carried out at the same time. The incubation temperature and time of antibody reduction should be strictly controlled. Therefore, it is recommended that at least 2 experimentalists participate in this process, otherwise may lead to user error or program delays that may result in variable or poor results.

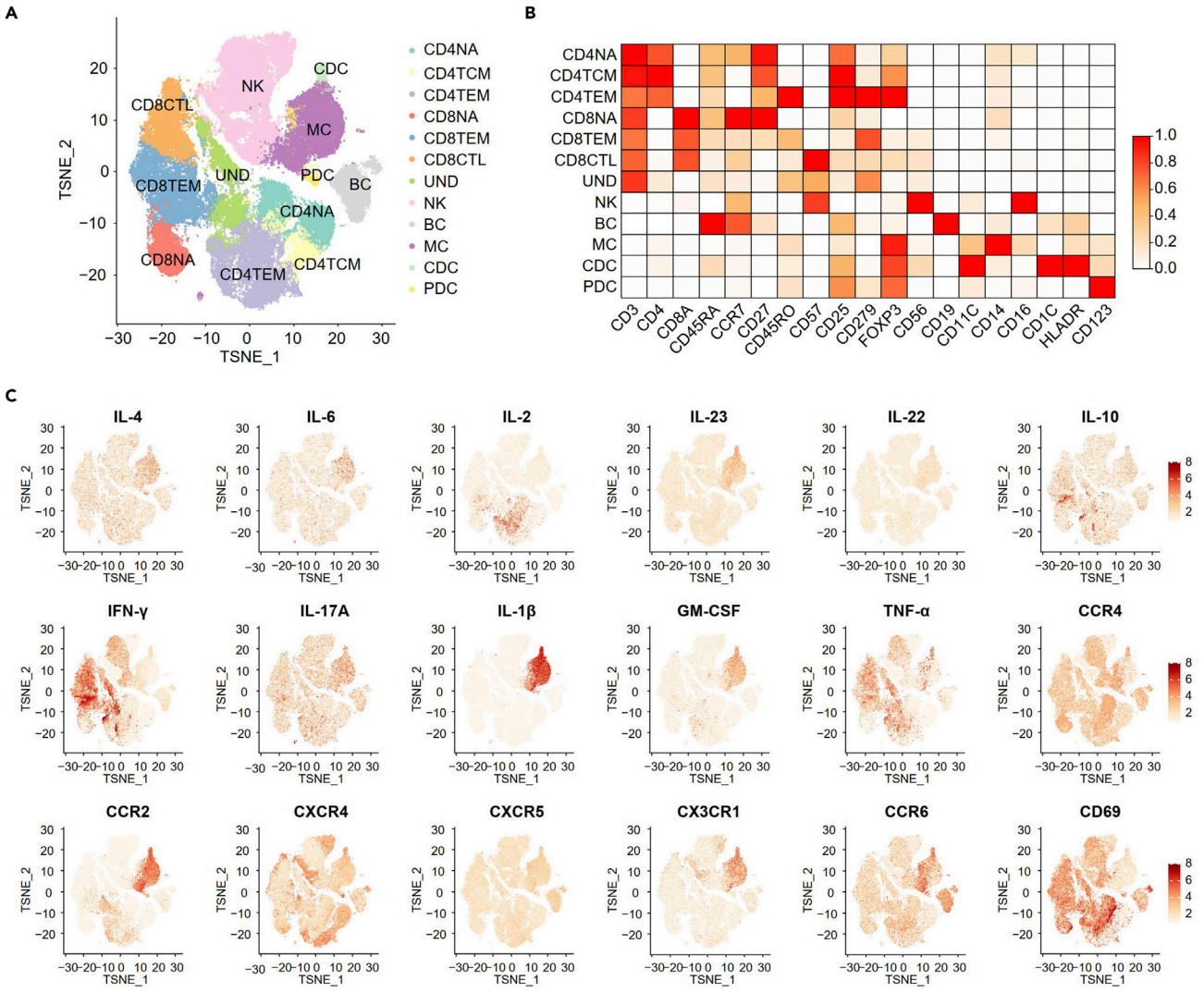

Figure 5. Profiling the blood immune cells using high-dimensional algorithms

(A–C) The clustering strategy of major immune cell populations identifying immune cell subtypes (A) based on the scaled expression heatmap of the discriminative gene for each cluster (B). The t-SNE map shows the co-expression of cytokines and receptors (C).

In addition to the metal-conjugated antibodies obtained from Fluidigm, we also conjugate antibodies to metal isotopes to custom conjugations. This makes researchers be more flexible in panel design by implementing personalized antibody-metal combinations. However, some metal ions can be affected by other ions or environmental pollution. Oxides from 111Cd staining may spill into the 127I (iodine) channel when using the Cell-ID 127 IdU labeling reagent. In addition, these two metals, I127 and Ba138, are susceptible to environmental pollution, which can change the signals of metals and affects the results.

TROUBLESHOOTING

Problem 1

The cell stimulation cocktails used in this study (see prepare cells and viability stain step) has side effects and influences certain immune cell populations.

Potential solution

In this study, we mainly focused on the functional analysis of human blood immune cytokines secreted by PMA and ionomycin stimulation. As a common method of stimulating cells, PMA

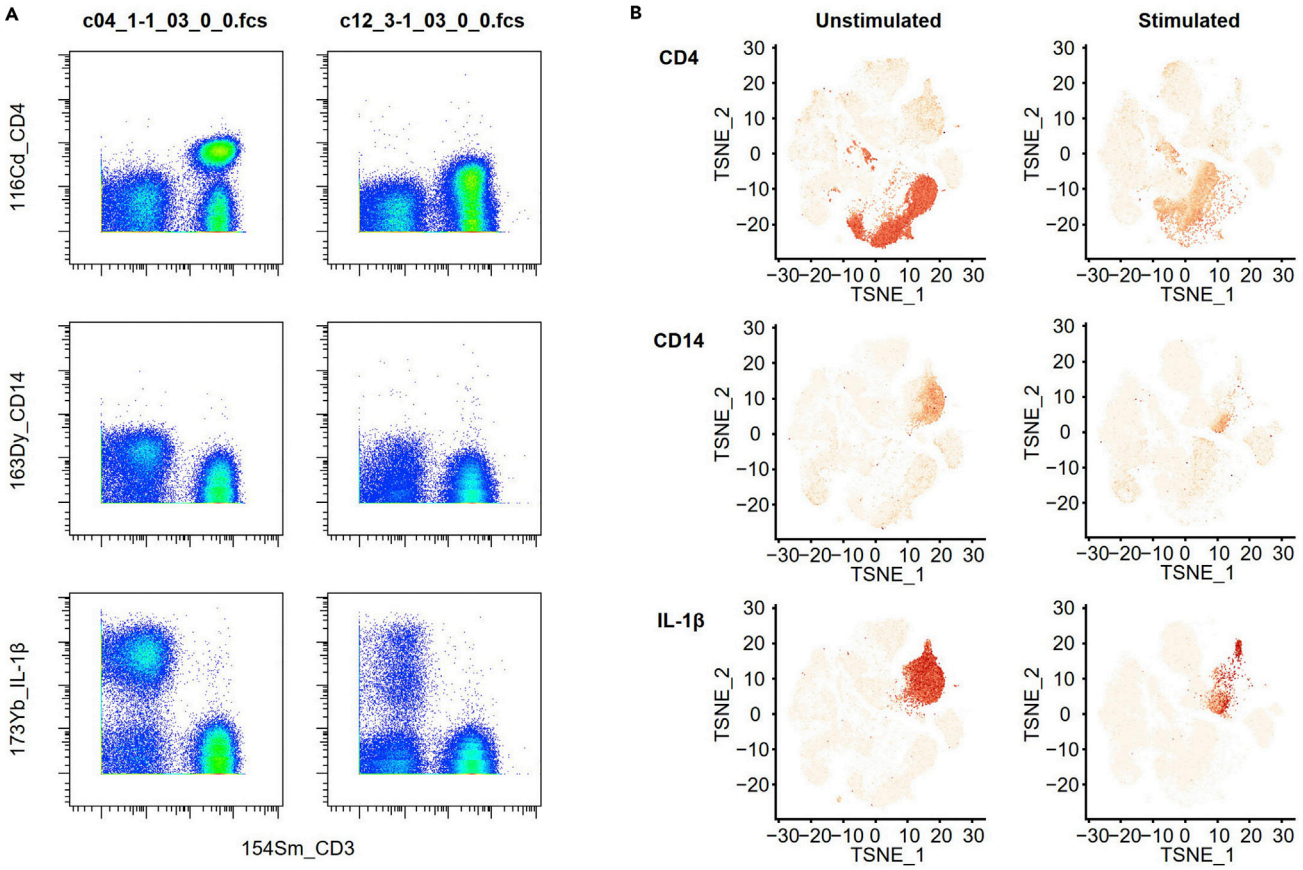

Figure 6. The stimulation condition affects several immune cell populations

(A and B) Certain markers are highlighted to represent the side effects of stimulation condition. The scatter plots (A) and t-SNE map (B) show the expression of CD4, CD14, IL-1β between unstimulated and stimulated groups.

stimulates the secretion of cytokines by activating the PKC pathway and ionomycin by activating the calcium ion pathway, both primarily stimulate lymphocyte activation. In addition, PMA stimulation of human cells has been reported to lead to endocytosis of CD4 molecule (Hsu et al., 2015), thereby affecting CD4 staining results. As shown in Figure 6, the stimulation induced changes in CD4 expression and reduced the proportion of CD14+ monocytes. In addition, stimulation with the two cell stimulation reagents decreased the expression of IL-1β (**A**). In the distribution of the t-SNE diagram, there is heterogeneity in the location of unstimulated and stimulated CD4+ TC and MC, which may be due to the difference in CD4, CD14, and IL-1β expression (**B**). Collectively, stimulation with PMA and ionomycin promotes lymphocyte activation and decreases monocyte activation.

Therefore, the stimulation conditions can be changed according to the purpose of the experiment: PMA can be selected when lymphocyte function is focused; LPS is recommended when cytokine secretion of monocyte is focused (Schnabel et al., 2021), because the latter can simulate the bacterial wall components of G-bacterium, promoting monocyte phagocytosis and antigen presentation. When studying the secretion function of CD4+ T cytokine, it can be considered to select CD4+ T cells by magnetic beads or flow separation, and then carry out the subsequent experimental steps of this method.

Problem 2

The performance of antibodies panel may be affected by several potential "noise" sources, such as oxidation products, metal impurities, and environmental contaminants.

Potential solution

When purchasing the pre-labeled antibodies or metal isotopes, please check their purity with commercial companies to reduce the confounding effects of metal impurities. Signal conflicts among metal isotope channels are mainly due to isotope promiscuity and oxides, such as the direct mass overlap of metal isotopes in the 20-Plex Pd Barcoding Kit, or the spillover of 111Cd oxides into the 127I channel. It is recommended to design the antibody panels using an interactive tool, "Panel Design Helper", which can identify irregularities or problems within the antibody panel. The tool can be obtained by consulting the technical staff of Fluidigm.

In order to make the results with high accuracy and sensitivity, it is recommended to establish an independent experimental environment for mass cytometry. Notably, iodine-containing alcohol and water cannot be used for cleaning of the operating bench. Before conducting any experiments, a test by obtaining unstained cells should be done to confirm that this environmental contaminant doesn't exist. Finally, it is recommended to use filter tips during pipetting steps to prevent cross-contamination between metal isotopes and reagents.

Problem 3

In this protocol, the required cell number for antibodies staining and data acquisition is 3 million. The cell viability of all used samples is required to be greater than 80% (The steps before fixing cells). However, cell loss and decreased viability may occur during the experiment.

Potential solution

Firstly, the clinical samples should be processed or placed at 4°C (within 12 h) as soon as possible after acquisition. Next, the cell resuscitation should be performed on ice with a slow drip of buffer to minimize damage from temperature and osmotic pressure changes. Thirdly, set centrifuge parameters in advance to ensure that the centrifuge step is performed at 4°C. Finally, resuspend cells with a wide-mouth pipette tip or directly vortex to reduce cell damage. If the cell viability is less than 80%, it is recommended to perform a low-speed centrifugation or use a Dead Cell Removal Kit (Cat#130-090-101, Miltenyi Biotec) to remove partial dead cells and improve the viability.

Problem 4

As used in this protocol, palladium mass-tag cell barcoding can mitigate the variable of staining samples separately (see Barcoding samples step). However, the intensity of staining for some surface markers may be reduced because it partially permeabilizes cells and can lead to staining issues for certain surface markers.

Potential solution

To effectively reduce the influence of barcoding on antibody staining, it is necessary to optimize the staining patterns of barcoding. We compared the intensity of surface antibodies before and after barcoding staining. As shown in Figure 7, we found that the vast majority of antibodies do very well in both conditions. On the other hand, we found that post-barcoding staining affected the results of two receptors CCR4 and CCR7. Therefore, in this experiment, the two antibodies were stained first, followed by barcoding, surface staining, and intracellular staining. Therefore, it is recommended to test all surface antibodies to better reduce sample error and optimize the experimental process based on ensuring accurate staining.

Problem 5

Some markers used for clustering can affect the t-SNE map distribution and subsequent bioinformatic analysis (see expected outcomes step).

Potential solution

In the high-dimensional analysis of data, the goal is usually to find real biological differences between groups but the real biological results are often confused by a variety of factors, including

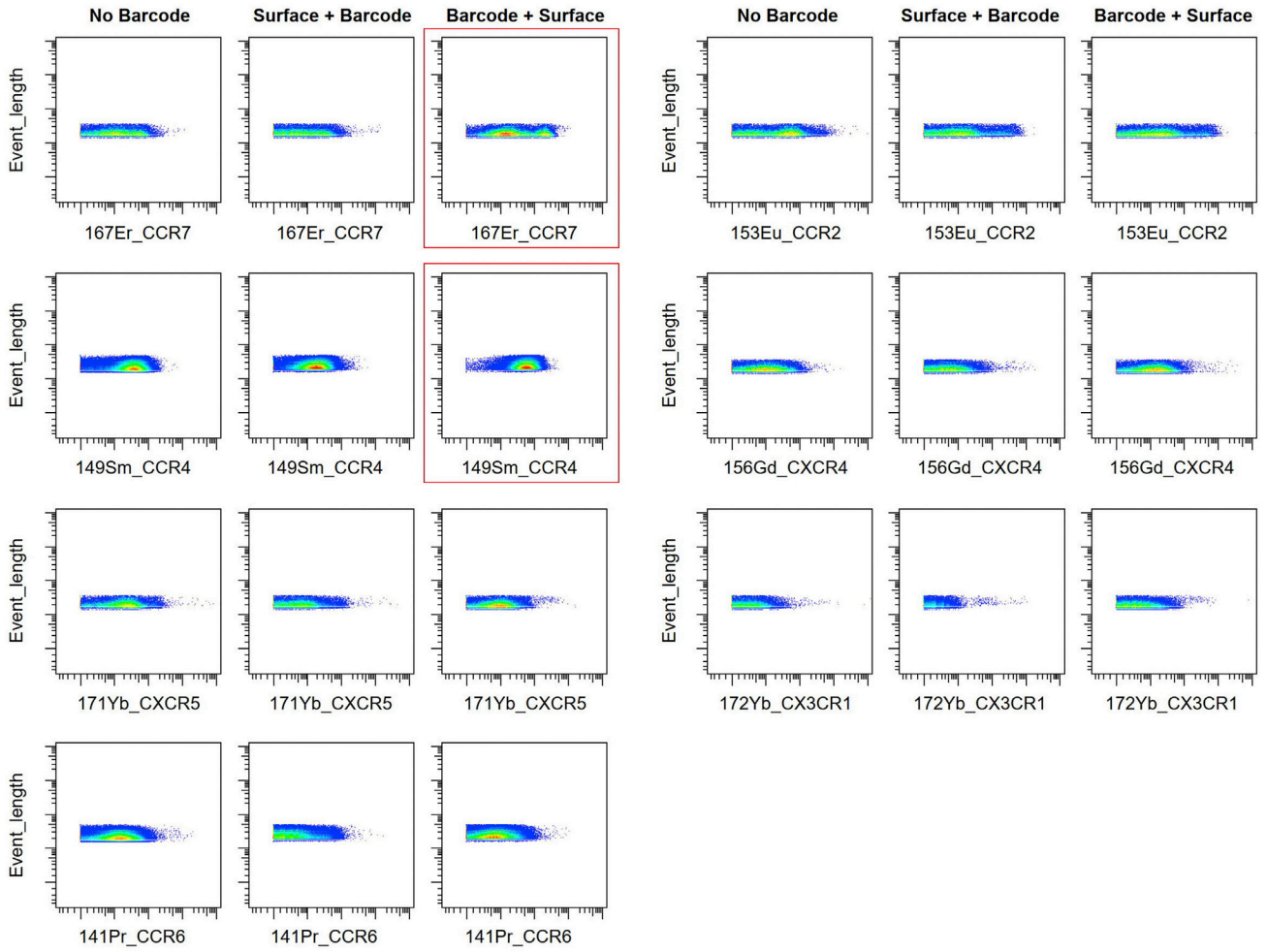

Figure 7. Optimize the staining mode of Barcoding

Cells are grouped by two staining modes, Surface-Barcode and Barcode-Surface staining. The scatter plots show the staining results of receptors in the panel.

stain process or different treatment. The use of barcoding can reduce sample-specific staining and data collection variation, but the batch effects induced by the treatment conditions affect the identification of cell subsets. In describing troubleshooting problem 1, we discussed the effects of cell stimulation cocktails on the phenotype and function of CD4+ T cells and monocytes. Therefore, we should select the suitable markers for clustering to reduce the batch effects. As shown in Figure 8, three panel markers are used for clustering and the results of intergroup fusion was compared. The commonalities between the markers of the three panels are necessary clustering markers, while the differences are CCR2 and CD69, which are mainly expressed in monocyte and CD4+ T cells, respectively. In the panel1, the data from the two groups don't combine well to influence the clustering and proportions of cell populations. The removal of CCR2 in panel2 resulted in a better fusion of monocytes between the two groups, but the regions of CD69 expression are only specific for the treatment group. This is because the cell stimulation cocktails promote lymphocyte activation and increase the expression of activation marker CD69. Thus, we discard CD69 in panel3 and performed subsequent bioinformatic analysis finally. Therefore, selecting the right markers used for clustering is a necessary way to diminish batch effects.

Marker	Panel1	Panel2	Panel3
CD3	√	√	√
CD4	√	√	√
CD8	√	√	√
CCR7	√	√	√
CD45RA	√	√	√
CD45RO	√	√	√
CD25	√	√	√
FOXP3	√	√	√
CD279	√	√	√
CD27	√	√	√
CD57	√	√	√
CD56	√	√	√
CD19	√	√	√
CD11C	√	√	√
CD14	√	√	√
CD16	√	√	√
HLA-DR	√	√	√
CD1C	√	√	√
CD123	√	√	√
CD69	√	√	
CCR2	√		

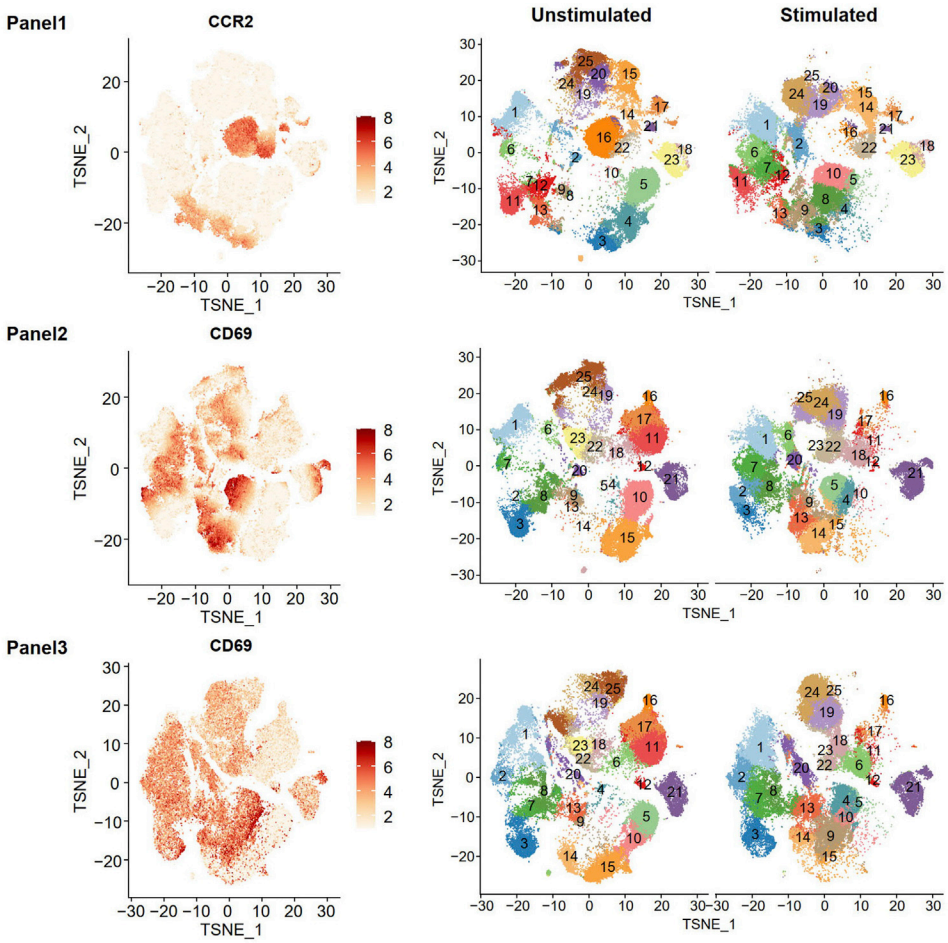

Figure 8. Select suitable markers used for clustering

The data is performed a bioinformatic analysis based on 3 different panels. CCR2 and CD69 are highlighted to show some markers that can affect the t-SNE map distribution and clustering.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wenru Su (suwr3@mail.sysu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data that support the findings of this study are available from the corresponding author upon request.

ACKNOWLEDGMENTS

This study was supported by the National Outstanding Youth Science Fund Project of China (no. 8212200477) and the National Key Research and Development Program of China (2017YFA0105804). We thank the study volunteers for their participation. We also thank the research group staff for their technical assistance and cooperation.

AUTHOR CONTRIBUTIONS

W.S. and Y.Z. designed and supervised the study. X.L. and J.L. took care of subjects, provided the clinical information, and performed the bioinformatic analyses. X.L. and H.W. performed the experiments. X.L., J.L., and H.W. wrote the paper. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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