

Phenotyping of Live Human PBMC using CyTOF™ Mass Cytometry

Michael D. Leipold* and Holden T. Maecker

Human Immune Monitoring Center, Stanford University, Stanford, USA

*For correspondence: mleipold@stanford.edu

[Abstract] Single-cell analysis has become an method of importance in immunology. Fluorescence flow cytometry has been a major player. However, due to issues such as autofluorescence and emission spillover between different fluorophores, alternative techniques are being developed. In recent years, mass cytometry has emerged, wherein antibodies labeled with metal ions are detected by ICP-MS. In order for a cell to be seen, a metal in the mass window must be present; there is no analogous parameter to forward or side scatter. The current mass window selected is approximately AW 103-196, which includes the lanthanides used for most antibody labeling, as well as iridium and rhodium for DNA intercalators.

In this protocol, we use a cocktail of antibodies labeled with MAXPAR metal-chelating polymers to surface-stain live PBMC that have been previously cryopreserved. Many of these markers were taken from a standard fluorescence phenotyping panel (Maecker *et al.*, 2012). No intracellular antibodies are used. We use a CyTOF™ (Cytometry by Time-Of-Flight) mass cytometer to acquire the ICP-MS data. Subsequent analysis of the dual count signal data using FlowJo software allows for cell types to be analyzed based on the dual count signal in each mass channel. The percentage of each cell type is determined and reported as a percent of the parent cell type.

Materials and Reagents

1. Peripheral blood mononuclear cells (PBMC) (fresh, or frozen after Ficoll isolation procedure)
2. RPMI (HyClone, catalog number: SH30027.01)
3. FBS (heat-inactivated before use) (Atlanta Biologicals, catalog number: S11150)
4. Pen-Strep-Glutamine (100x) (HyClone, catalog number: SV30082.01)
5. Benzonase (25×10^5 U/ml) (Pierce Antibodies, catalog number: 88701)
6. MilliQ water [no contact with beakers or bottles washed with soap (due to barium content of most commercial soaps)]
7. PBS (10x stock) (Rockland, catalog number: MB-008)
8. Bovine serum albumin (BSA) (30% w/v in 0.85% NaCl) (Sigma-Aldrich, catalog number: A7284)
9. 0.5 M EDTA (pH 8.0) (Hoefer, catalog number: GR123-100)

10. Sodium azide (10% w/v solution) (Teknova, catalog number: S0209)
11. 2% para-formaldehyde (PFA) (16% w/v aqueous stock, methanol-free, diluted to 2% in 1x CyPBS) (Alfa Aesar, catalog number: 43368)
12. Maleimide-DOTA (Macrocyclics, catalog number: B-272)
13. 139-Lanthanum (chloride salt) (Sigma-Aldrich, catalog number: 203521)
14. 115-Indium (chloride salt) (Sigma-Aldrich, catalog number: 203440)
15. Phenotyping antibodies (filtered with 0.1 μm spin filters) (Millipore, catalog number: UFC30VV00)
16. Ir-intercalator stock solution from Fluidigm (catalog number: 201192B; Rh103-intercalator catalog number: 201103B can be used)
17. 10x saponin-based permeabilization buffer (eBiosciences, catalog number: 00-8333-56)
18. Complete RPMI (see Recipes)
19. CyPBS (see Recipes)
20. CyFACS buffer (see Recipes)
21. Live-dead stain (see Recipes)

Equipment

1. 37 °C water bath
2. 96-well plate: 300 μl well volume (250 μl washes) or 1.2 ml well volume (500 μl washes)
Note: If using a 300 μl plate, do an additional wash step (resuspension and centrifugation) at each point.
3. Biosafety cabinet
4. Centrifuge
5. Calibrated pipettes
6. ViCell (Beckman Coulter) or Hemocytometer cell counter
7. CyTOF™ version 1 mass cytometer (Fluidigm Sciences)

Software

1. FlowJo software
2. Microsoft Excel

Procedure

- A. Thaw PBMC

1. Warm Complete RPMI media to 37 °C in water bath. Each sample will require 20 ml of media with benzonase. Calculate the amount needed to thaw all samples, and prepare a separate aliquot of warm media with 1:10,000 benzonase (25 U/ml final concentration; benzonase removes reduces viscosity and background from free DNA from lysed cells). Thaw no more than 10 samples at a time.
2. Remove samples from liquid nitrogen and transport to lab on dry ice.
3. Place 10 ml of warmed benzonase media into a 15 ml tube, making a separate tube for each sample.
4. Thaw frozen vials in 37 °C water bath.
5. When cells are nearly completely thawed, carry to hood.
6. Add 1 ml of warm benzonase media from appropriately labeled centrifuge tube slowly to the cells, then transfer the cells to the centrifuge tube. Rinse vial with more media from centrifuge tube to retrieve all cells.
7. Continue with the rest of the samples as quickly as possible.
8. Centrifuge cells at (RCF = 483) for 10 min at room temperature.
9. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
10. Gently resuspend the pellet in 1 ml warmed benzonase media. Filter cells through a 70 micron cell strainer if needed (*i.e.* if you observe any clumps). Add 9 ml warmed benzonase media to the tube to make volume 10 ml altogether.
11. Centrifuge cells (RCF = 483) for 10 min at room temperature.
12. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
13. Resuspend cells in 1 ml CyFACS buffer.
14. Count cells with Vicell (or hemocytometer). To count, take 20 μ l cells and dilute with 480 μ l CyPBS in Vicell counting chamber. Load onto Vicell as PBMC with a 1:25 dilution factor.
15. Calculate the resuspension volume needed to obtain 1 million viable cells.

Note: It is typical to recover 4-8 x 10⁶ cells from a vial frozen at 10 x 10⁶ cells/vial.

B. Stain cells

Day one

1. Add 1 million viable cells from a donor into a well of the 96 well plate. Repeat for all samples. Add CyFACS buffer to approximately 600 μ l and centrifuge cells (RCF = 483) for 10 min at room temperature.
2. Flick or aspirate to remove supernatant, and repeat wash step and centrifugation with 500 μ l CyFACS.
3. Make cocktail in CyFACS buffer of metal-chelating polymer-labeled antibodies according to previously determined titration. Make sufficient volume for each sample to have 50 μ l of cocktail. Pipet into 0.1 μ m spin filter and centrifuge in a tabletop microcentrifuge (RCF =

- 14,000) for 10 min at room temperature.
4. Flick or aspirate to remove supernatant from second wash step B2. Add 50 μ l antibody cocktail to each sample. Pipet up and down to mix. Incubate on ice for 60 min.
 5. Add 500 μ l CyFACS buffer, then centrifuge cells (RCF = 483) for 10 min at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 μ l CyFACS, and centrifuge cells (RCF = 483) for 10 min at room temperature.
 6. Make 1:3,000 dilution in CyPBS of 5 mg/ml live-dead maleimide-DOTA stain. Add 100 μ l to each sample, pipetting to mix. Incubate on ice for 30 min.
 7. Add 500 μ l CyFACS buffer, then centrifuge cells (RCF = 483) for 10 min at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 μ l CyFACS, and centrifuge cells (RCF = 483) for 10 min at room temperature.
 8. Add 100 μ l of 2% PFA (in CyPBS); pipet to mix. Place at 4 °C overnight. PFA fixation is required due to permeabilization and MilliQ water wash osmotic stress in Day two.

Day two

9. Add 500 μ l CyFACS buffer, then centrifuge cells (RCF = 805) for 10 min at 4 °C. Flick or aspirate to remove supernatant, resuspend pellet in 500 μ l CyFACS, and centrifuge cells (RCF = 805) for 10 min at 4 °C.

Note: It is common to increase RCF after fixation, particularly during permeabilization steps.

Live cells in Day 1 cannot take the stress.

10. Make 1x saponin permeabilization buffer in CyPBS. Flick or aspirate to remove supernatant from step 9. Add 100 μ l of 1x saponin permeabilization buffer to each sample, pipet to mix. Incubate on ice for 45 min.
11. Add 500 μ l CyFACS buffer, then centrifuge cells (RCF = 805) for 10 min at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 μ l CyFACS, and centrifuge cells (RCF = 805) for 10 min at 4 °C.
12. Make 1:2,000 dilution in CyPBS of Ir-intercalator. Add 100 μ l of diluted Ir-intercalator solution to each sample, pipet to mix. Incubate at room temperature for 20 min.
13. Add 500 μ l CyFACS buffer, then centrifuge cells (RCF = 805) for 10 min at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 μ l CyFACS, and centrifuge cells (RCF = 805) for 10 min at 4 °C. Flick or aspirate to remove supernatant, and repeat at least twice with 500 μ l of MilliQ water. The MilliQ water washes are critical to remove buffer salts, which can cause the Current setting on the CyTOF to drift over the course of your experiment.
14. Acquire samples on the CyTOF, after standard instrument setup procedures. Collect at least one “push” of 500 μ l per sample.

C. Analyze data

1. Analyze data using FlowJo software using the standard CyTOF template. Adjust gates on a donor-specific basis, if necessary, to control for any differences in background or positive staining intensity. Export the statistics for each gated population to an Excel spreadsheet.

Recipes

1. Complete RPMI

Note: Combine and 0.2 μ m filter to sterilize before use.

RPMI 500 ml

50 ml heat-inactivated FBS

5 ml Pen-Strep-Glutamine 100x stock

2. CyPBS

Dilute 10x PBS to 1x with MilliQ water

3. CyFACS buffer

1x CyPBS with 0.1% BSA

2 mM EDTA and 0.05% Na azide

Made in MilliQ water

Note: Do not use FBS!

4. Live-dead stain

5 mg/ml maleimide-DOTA loaded with 139 -Lanthanum* or 115 -Indium*

*Natural-abundance metal chloride salt used; >95% specified isotope; trace-metal pure 99.99%

Dissolve solid DOTA-Maleimide at 41.5 mg/ml in L-buffer (Fluidigm) or MilliQ water acidified with nitric acid (100 μ 3% v/v nitric acid per 10 ml water) containing 25 mM Metal (III) salt. Incubate at room temperature for 15 min, then aliquot and freeze at -20 °C.

41.5 mg/ml is 50 mM for the DOTA Maleimide. The 2:1 molar ratio of DOTA: Metal is intended to reduce/eliminate unchelated metal in the barcoding reagent.

Note: Each new batch of metal-loaded maleimide-DOTA should be titered for best resolution of live vs dead cells, with minimal background. Therefore, final working concentration and final dilution may vary slightly from batch to batch.

Acknowledgments

We would like to acknowledge funding from NIH grants S10RR027582, 5U19AI057229, and 5U19AI090019. We would also like to thank Dr. Evan Newell for initial protocol development (Newell *et al.*, 2012).

References

1. Maecker, H. T., McCoy, J. P. and Nussenblatt, R. (2012). [Standardizing immunophenotyping for the Human Immunology Project](#). *Nat Rev Immunol* 12(3): 191-200.
2. Newell, E. W., Sigal, N., Bendall, S. C., Nolan, G. P. and Davis, M. M. (2012). [Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8⁺ T cell phenotypes](#). *Immunity* 36(1): 142-152.