

Phenotyping of Human PBMC using CyTOF™ Mass Cytometry

1. Principle

In this protocol, we use a cocktail of antibodies labeled with MAXPAR metal-chelating polymers to surface-stain PBMC that have been previously cryopreserved. No intracellular antibodies are used. We use a CyTOF™ mass cytometer to acquire the ICP-MS data. The current mass window selected is approximately AW 103-203, which includes the lanthanides used for most antibody labeling, as well as iridium and rhodium for DNA intercalators. 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. 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.

2. Materials and Equipment

- 2.1. PBMC, fresh or thawed frozen
- 2.2. Complete RPMI (RPMI with 10% FBS, P/S, glutamine)
- 2.3. Benzonase (25×10^5 U/ml)
- 2.4. 2% Para-formaldehyde (PFA), made in 1x CyPBS
- 2.5. CyPBS (1x PBS without heavy metal contaminants, such as 10X PBS from Rockland; made in MilliQ water)
- 2.6. CyFACS buffer (1x CyPBS with 0.1% BSA, 2 mM EDTA and 0.05% Na Azide; made in MilliQ water). Do NOT use FBS!!
- 2.7. MilliQ water: no contact with beakers or bottles washed with soap (due to barium content of most commercial soaps).
- 2.8. Phenotyping antibodies, filtered with 0.1 um spin filters
- 2.9. Live-dead stain: 5 mg/mL maleimide-DOTA (eg, Macrocylics B-272) loaded with 139 -Lanthanum* or 115 -Indium* (*: natural-abundance metal chloride salt used; >95% specified isotope; trace-metal pure 99.99%)
- 2.10. Ir-intercalator stock solution from DVS (Rh103-intercalator can be used).

- 2.11. Saponin-based permeabilization buffer: 10x (eg, eBiosciences ca# 00-8333-56)
- 2.12. 37°C water bath
- 2.13. 96-well plate; 300 uL well volume (250 uL washes) or 1.2 mL well volume (500 uL washes). If using a 300 uL plate, do an additional wash step (resuspension and centrifugation) at each point.
- 2.14. Biosafety cabinet
- 2.15. Centrifuge
- 2.16. Calibrated pipettes
- 2.17. ViCell or Hemocytometer cell counter

3. Procedure

Thaw PBMC

- 3.1. Warm 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 1:10000 benzonase (25 U/mL). Thaw no more than 10 samples at a time.
- 3.2. Remove samples from liquid nitrogen and transport to lab on dry ice.
- 3.3. Place 10 mL of warmed benzonase media into a 15 mL tube, making a separate tube for each sample.
- 3.4. Thaw frozen vials in 37°C water bath.
- 3.5. When cells are nearly completely thawed, carry to hood.
- 3.6. Add 1ml 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.
- 3.7. Continue with the rest of the samples as quickly as possible.
- 3.8. Centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature.
- 3.9. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
- 3.10. 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 10ml altogether.

- 3.11. Centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature.
- 3.12. Remove supernatant from the cells and resuspend the pellet by tapping the tube.
- 3.13. Resuspend cells in 1 mL CyFACS buffer.
- 3.14. Count cells with Vicell (or hemocytometer). To count, take 20 uL cells and dilute with 480 uL CyPBS in Vicell counting chamber. Load onto vicell as PBMC with a 1:25 dilution factor.
- 3.15. Calculate the resuspension volume needed to obtain 1 million viable cells. Note-it is typical to recover $4-8 * 10^6$ cells from a vial frozen at $10 * 10^6$ cells/vial.

Stain cells

Day One

- 3.16 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 uL and centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature.
- 3.17 Flick or aspirate to remove supernatant, and repeat wash step and centrifugation with 500 uL CyFACS.
- 3.18 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 uL of cocktail. Pipet into 0.1 um spin filter and centrifuge in a tabletop microcentrifuge (RCF=14,000) for 10 minutes at room temperature.
- 3.19 Flick or aspirate to remove supernatant from second wash step 3.17. Add 50 uL antibody cocktail to each sample. Pipet up and down to mix. Incubate on ice for 60 minutes.
- 3.20 Add 500 uL CyFACS buffer, then centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 uL CyFACS, and centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature.
- 3.21 Make 1:3000 dilution in CyPBS of 5 mg/mL live-dead maleimide-

DOTA stain. Add 100 uL to each sample, pipetting to mix.
Incubate on ice for 30 minutes.

- 3.22 Add 500 uL CyFACS buffer, then centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 uL CyFACS, and centrifuge cells at 1550 rpm (RCF=483) for 10 minutes at room temperature.
- 3.23 Add 100 uL of 2% PFA (in CyPBS); pipet to mix. Place at 4°C overnight.

Day Two

- 3.24 Add 500 uL CyFACS buffer, then centrifuge cells at 2000 rpm (RCF=805) for 10 minutes at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 uL CyFACS, and centrifuge cells at 2000 rpm (RCF=805) for 10 minutes at 4°C.
- 3.25 Make 1x saponin permeabilization buffer in CyPBS. Flick or aspirate to remove supernatant from step 3.24. Add 100 uL of 1x saponin permeabilization buffer to each sample, pipet to mix.
Incubate on ice for 45 minutes.
- 3.26 Add 500 uL CyFACS buffer, then centrifuge cells at 2000 rpm (RCF=805) for 10 minutes at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 uL CyFACS, and centrifuge cells at 2000 rpm (RCF=805) for 10 minutes at 4°C.
- 3.27 Make 1:2000 dilution in CyPBS of Ir-intercalator. Add 100 uL of diluted Ir-intercalator solution to each sample, pipet to mix.
Incubate at room temperature for 20 minutes.
- 3.28 Add 500 uL CyFACS buffer, then centrifuge cells at 2000 rpm (RCF=805) for 10 minutes at room temperature. Flick or aspirate to remove supernatant, resuspend pellet in 500 uL CyFACS, and centrifuge cells at 2000 rpm (RCF=805) for 10 minutes at 4°C.
Flick or aspirate to remove supernatant, and repeat at least twice with 500 uL 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.
- 3.29 Acquire samples on the CyTOF, after standard instrument setup procedures. Collect at least one “push” of 300 uL per sample.

Analyze Data

- 3.30 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.