**Protocol CyTOF003.18: Mass Cytometry (CyTOF) Analysis – General Protocol**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Panel** | **Antigen** | **Atomic Symbol** | **Atomic Mass** | **Stock Conc. (mg/mL)** | **Final Conc. (μg/mL)** | **Vol. per Rxn. (μL)** | **Vol. for ½ BC plate (μL)** | **Vol. for BC plate (μL)** |
| **Surface (Pre-MeOH)** | CD235ab | In | 113 | 0.2 | 1 | 0.5 | 1.4 | 2.8 |
| CD61 | In | 113 | 0.2 | 0.5 | 0.25 | 0.7 | 1.4 |
| CD45 | In | 115 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| CD66 | La | 139 | 0.2 | 1 | 0.5 | 1.4 | 2.8 |
| CD7 | Pr | 141 | 0.2 | 4 | 2 | 5.5 | 11 |
| CD19 | Nd | 142 | 0.2 | 8 | 4 | 11 | 22 |
| CD11b | Nd | 144 | 0.2 | 10 | 5 | 13.8 | 27.5 |
| CD4 | Nd | 145 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| CD3 | Er | 170 | 0.2 | 1 | 0.5 | 1.4 | 2.8 |
| CD14 | Yb | 175 | 0.2 | 4 | 2 | 5.5 | 11 |
|  | **TOTAL** | | | | | | **46.3** | **92.3** |
| **CSM** | | | | | | **105** | **210.2** |
| **Master Mix** | | | | | | **151.3** | **302.5** |
| **Intracellular (Post-MeOH)** | pCREB | Sm | 149 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| pSTAT5 | Nd | 150 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| pp38 | Eu | 151 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| pSTAT1 | Eu | 153 | 0.2 | 1 | 0.5 | 1.4 | 2.8 |
| pSTAT3 | Sm | 154 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| pS6 | Gd | 155 | 0.2 | 4 | 2 | 5.5 | 11 |
| pMAPKAPK2 | Tb | 159 | 0.2 | 2 | 1 | 2.8 | 5.5 |
| IκB | Dy | 164 | 0.2 | 8 | 4 | 11 | 22 |
| pNFκB | Er | 166 | 0.2 | 1 | 0.5 | 1.4 | 2.8 |
| pERK1/2 | Er | 167 | 0.2 | 1 | 0.5 | 1.4 | 2.8 |
|  | **TOTAL** | | | | | | **34.7** | **68.9** |
| **CSM** | | | | | | **130.3** | **261.1** |
| **Master Mix** | | | | | | **165** | **330** |

**Panel Design**

The antibody panel to be used for any given experiment will depend on several factors, including preferences in identification of phenotypic and functional markers of interest, commercial availability of antibodies targeting markers of interest, and experimental question(s) being asked. Above is an example panel with a “backbone” phenotypic and functional readout. In addition, great care should be exercised in designing a CyTOF panel so that certain important considerations are taken into account:

1. **Spill-over.** Unlike traditional flow cytometry where fluorophores are covalently attached to antibodies, mass cytometry experiences far less “spectral overlap” since the peaks resulting from isotopes with discrete masses are far narrower in a mass spectrometry readout. However, a small amount of overlap (≤3%) does still occur, so caution should be exercised when deciding which channel should be used for each antibody. Specifically, +1 spillover is of the most concern, and an antibody with strong binding affinity that recognizes a target that is highly expressed should not be placed in a -1 position relative to an antibody with weak binding affinity and/or that recognizes a rare target, as the +1 spill-over from the highly expressed target may generate a false positive reading in the adjacent channel. Furthermore, metal oxides can cause a +16 effect, so the same caution should be exercised for highly expressed targets with respect to rare targets in the channel corresponding to an additional 16 amu.
2. **Brightness of Channels.** Certain channels are “brighter” than others, meaning the amplification of signal from certain channels is greater than with other channels. In general, channels in the center of the detectable range are the brightest, while channels at the periphery are the dimmest. It is therefore advisable to place antibodies that have weak binding affinity and/or are detecting rarer species in the center channels (150-176), while placing antibodies with strong binding affinity and/or detecting ubiquitously expressed species toward the periphery (89, 113, 115, 139-149, 194-196, 198, 209).
3. **Tandem Detection.** Because a mass cytometry readout is limited to the number of available channels within the detectable range, it is recommended to take advantage of the ability to “double-up” on certain channels where possible. For instance, if certain cell populations will be “gated out” (excluded) during analysis, it can be advantageous to place multiple markers for excluded populations on the same channel in order to free up other channels for markers of interest. A prime example is placing antibodies targeting both erythrocytes (CD235ab) and platelets (CD61) on 113In, so that both can be gated out using the 113 channel (see example panel above).
4. **Surface vs Intracellular.** Special care should be exercised when deciding in which staining step each antibody should be used. Antibodies targeting proteins expressed on the cell surface should be used in the “Pre-MeOH” surface stain, while antibodies targeting proteins expressed within the cytoplasm or nuclear envelope should be used in the “Post-MeOH” intracellular stain. On occasion, a surface marker may work better when stained with the intracellular cadre, since methanol may disrupt binding of certain antibodies that were used during the surface stain procedure. These instances will need to be determined on a case-by-case basis.
5. **Selecting Channels.** There are 46 channels that generally work well for mass cytometry and for which metal isotopes are commercially available. Anything beyond these channels should be tested empirically. Note that 120Sn, 127I, and 138Ba are common contaminants in municipal water supplies and buffers used for CyTOF, and may cause background signal in the corresponding channels. Furthermore, these species cannot be reliably eliminated with standard water purification techniques, including Milli-Q ultra-purification. Therefore, these channels should be avoided. The end user should focus on employing the following channels:
   * 89 (very dim)
   * 113 (dim)
   * 115 (dim)
   * 139-149 (weak-moderate)
   * 150-176 (strong)
   * 194-196, 198 (moderate)
   * 209 (very dim)
6. **In-house Conjugation.** Pre-conjugated antibodies that have been validated in flow cytometry are commercially available from Standard BioTools (SBT – formerly Fluidigm, South San Francisco, CA), but only certain combinations are produced, limiting the range of panel design capabilities when relying solely on SBT antibodies. These commercial preparations are also very expensive, and large projects requiring substantial volumes of antibody can become quite costly. In-house conjugation is therefore a powerful tool for broadening the scope of panel design and reducing costs. Labeling kits which include purified metal isotopes, metal chelating polymer, and appropriate buffers are commercially available from SBT. Furthermore, many companies (e.g. Biolegend, Cell Signaling Technology, BD, Thermo Fisher, etc.) offer a wide range of purified, unlabeled antibodies that can be used for in-house conjugations, many of which have been validated in flow cytometry. The end user therefore has the ability to choose which metal isotope will be conjugated to each antibody, allowing virtually endless freedom in panel design. Of course, this necessitates validation of each antibody that is conjugated in-house. For preparation of conjugated CyTOF antibodies, see Protocol CyTOF004.1 or CyTOF004.2.

**Preliminary Considerations**

General Note 1:The following protocol is intended for barcoding and staining cells isolated from whole blood samples prepared using Smart Tube Inc. (Las Vegas, NV) proteomic stabilizer (PROT1), but can also be used for PBMC preps. The protocol is optimized for processing cells isolated from 0.5mL of whole blood (~2x106 cells) per sample, up to 20 samples. Because the typical sample preparation is 1mL whole blood + stabilizer (2.4mL total) separated into 2 cryovials, only one cryovial per sample should be used (this provides the added benefit of retaining half of each sample in case problems arise during staining/analysis of the first half). If the intention is to process a different number of total cells, barcoding reagent and antibody concentrations should be scaled accordingly.

General Note 2: The following protocol is optimized for a total pooled cell population of ~2x107 cells. Because each mL of blood typically yields 4x106 cells, the total number of cells obtained from 20 samples (each isolated from 0.5mL of whole blood) will normally be in the vicinity of 4x107. It is therefore advisable to count the aggregate (pooled) cell population after barcoding, set aside 2x107 cells for staining, and store the remaining cells in CSM at -80°C. The stored cells will be stable for up to 1 year for subsequent staining and analysis, and will be pre-barcoded.

General Note 3:Older (>4 years) antibody stocks may have formed Ig aggregates over time. These aggregates precipitate from solution and can lead to “streaking” during analysis on the CyTOF if they are included in the staining master mix. These aggregates can be “spun down” from the stock tubes or filtered out of staining master mixes prior to staining. Regardless, caution should be exercised when using older stocks, as Ig aggregate formation and precipitation result in a decrease in dissolved antibody, necessitating an increase in the volume of the stock needed to achieve the desired final concentration. Disposal of old stocks or filtration and re-analysis of their protein concentrations is highly recommended.

**Procedure**

Necessary reagents:

1. CyTOF PBS:
   * Filter 1X PBS through a 0.2μm bottle-top filter and store at 4°C.
2. Cell Staining Media (CSM):
   * Add 10g Bovine Serum Albumin (BSA) and 0.4g Sodium Azide (NaN3) to 2L of 1X PBS (final concentrations: 0.5% BSA, 0.02% NaN3). Mix thoroughly and filter into four 500mL pre-labeled glass bottles using a 0.2µm bottle-top filter. Store at 4°C.
3. 2% Saponin/PBS:
   * Dissolve 800mg saponin in 40mL CyTOF PBS (see above) and mix thoroughly but gently to avoid generation of too many bubbles. Allow solution to sit at room temperature for at least 10 minutes. Aliquot at 1.05mL per tube into 1.5mL microcentrifuge tubes and store at 4°C.

Barcode plate layout:

The 1X barcode plate is a 96-well PCR plate in which 20 wells in the center of the plate are occupied, each containing 12µL of a unique combination of 3 Pd isotopes dissolved in DMSO. Because there are 6 available Pd isotopes (102, 104, 105, 106, 108, 110), a 6-choose-3 pattern results in 20 possible combinations according to the formula below:

The combinations are arranged in a pattern of 4 rows, with 5 wells per row:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A |  |  |  |  |  |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C |  |  |  | 102 104 105 | 102 104 106 | 102 104 108 | 102 104 110 | 102 105 106 |  |  |  |  |
| D |  |  |  | 102 105 108 | 102 105 110 | 102 106 108 | 102 106 110 | 102 108 110 |  |  |  |  |
| E |  |  |  | 104 105 106 | 104 105 108 | 104 105 110 | 104 106 108 | 104 106 110 |  |  |  |  |
| F |  |  |  | 104 108 110 | 105 106 108 | 105 106 110 | 105 108 110 | 106 108 110 |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

For de-barcoding after data acquisition, the barcodes are numbered 1-20, starting at well C4, proceeding across the first row, then to D4, etc:

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A |  |  |  |  |  |  |  |  |  |  |  |  |
| B |  |  |  |  |  |  |  |  |  |  |  |  |
| C |  |  |  | 1 | 2 | 3 | 4 | 5 |  |  |  |  |
| D |  |  |  | 6 | 7 | 8 | 9 | 10 |  |  |  |  |
| E |  |  |  | 11 | 12 | 13 | 14 | 15 |  |  |  |  |
| F |  |  |  | 16 | 17 | 18 | 19 | 20 |  |  |  |  |
| G |  |  |  |  |  |  |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

For preparation of barcode plates, see Protocols CyTOF006.1 and CyTOF006.2.

**Day 1**

Before Starting:

1. Thaw cryotubes containing lysed samples (see protocol CyTOF002.2). It is highly recommended to order sample tubes according to their planned arrangement in the deep-well block and to record the arrangement for future reference.
2. For each set of 20 samples, prepare 50mL of RT CyTOF PBS. Add 50mL CyTOF PBS (4°C fridge) to a 50mL conical tube and leave on lab bench to equilibrate to room temperature (RT).

Approximate Time: 5 hours

Barcoding of Cells (20 samples):

1. After sample thaw is complete, transfer samples to a deep-well block, arranging them in a 5x4 pattern according to how they are to be barcoded (see above barcode plate layout).

**Note:** There are 4 available quadrants that can each accommodate 20 samples in a deep-well block. Deep-well blocks can therefore be re-used until all 4 quadrants have been utilized.

1. Label deep-well block “Cells”.
2. Centrifuge deep-well block at 600xg for 5 minutes at 4°C. During spin, take 1X barcode plate out of -80°C freezer and place on benchtop to thaw.
3. Aspirate supernatant using 96-well multichannel aspirator and vortex deep-well block to loosen cell pellets.
4. Wash cells with 1mL PBS.
5. Centrifuge deep-well block at 600xg for 5 minutes at 4°C. During spin, add 500μL 2% saponin/PBS (see above) to 50mL RT CyTOF PBS (see above) to create 0.02% saponin/PBS. Using a reservoir and a multi-channel pipettor, dispense 1mL 0.02% saponin/PBS per well into the corresponding 20 wells of a separate deep-well block, and label deep-well block “Saponin”.

**Note:** if 2 sets (“plates”) of samples are being stained, the wells in the “Saponin” deep-well block can be “doubled up” since the barcode playout will be the same for both sets. Simply dispense 2mL 0.02% saponin/PBS per well.

1. Aspirate supernatant and vortex deep-well block to loosen cell pellet.
2. Wash cells with 1mL 0.02% saponin/PBS (see Step 7).
3. Centrifuge at 600xg for 5 minutes at 4°C.
4. Aspirate supernatant and vortex deep-well block to loosen cell pellet.
5. Using a multichannel pipettor, and working row-by-row (or column-by-column):
   1. Add 6μL of barcode reagent to corresponding wells in the “Saponin” deep well block. Mix thoroughly with a multichannel pipettor set to 980µL.

**Note:** for 2 plates of samples, dispense 12μL of barcode reagent in 2mL.

* 1. Transfer 980μL saponin/PBS/barcode solution from the “Saponin” deep-well block into the appropriate wells of the “Cells” deep-well block. Mix carefully 5X by pipetting up and down. Return to step 9a for next row (or column) and repeat until all 20 wells are processed.

**Note:** for 2 plates of samples, make sure to change tips between each plate.

1. Incubate cells with barcode reagent at RT for 15 minutes.
2. During incubation, prepare surface antibody master mix (see above table):
   1. Mix all antibodies listed under “Surface (Pre-MeOH)” in a 1.5mL microcentrifuge tube, and add the appropriate volume of CSM.
   2. **For older antibody stocks:** Prepare a centrifugal filter unit (Millipore Durapore 0.1µm PVDF) by adding 150µL CSM to the filter, and spin at 5,000xg for 2 min to prime the filter.

**Note:** centrifugal filter units can only accommodate 500µL, so use another filter unit if master mix volume exceeds 500µL

* 1. Remove CSM from the bottom of the capture tube by pipetting.
  2. Add antibody master mix to the filter, and repeat centrifugation.
  3. Store filtered antibody cocktail on ice until ready to use.

1. In a microcentrifuge tube, combine 5μL Fc block and 145μL CSM (10μL Fc block + 290μL CSM for 2 plates).
2. After 15 minute incubation is complete, spin down cells (600xg for 5 min at 4°C) and aspirate supernatant.
3. Wash and spin 2X with 1mL CSM per sample.
4. Re-suspend in residual volume by vortexing.
5. Pool all 20 samples from each plate into one FACS tube.
6. To “rescue” residual any cells, add 100μL CSM to each well of the deep-well block, vortex briefly, and add to corresponding FACS tube.

Surface Staining of Cells (Pre-MeOH):

1. Add sufficient CSM to FACS tube for ~3mL total volume.
2. Pellet cells (600xg for 5 min at 4°C), aspirate supernatant to ~50μL residual volume, and vortex briefly to dissociate pellet.
3. Re-suspend cell pellet in 1mL CSM and count pooled cells.

**Note:** if pooled cell number is ≤ 2x107, skip steps 5 and 6 below and proceed with staining the entire sample.

1. Pellet cells, aspirate supernatant to ~50μL residual volume, and vortex briefly to dissociate pellet.
2. Measure residual volume and add the appropriate volume of CSM to achieve 4x105 cells/μL.
3. Transfer excess cell suspension to a cryovial, leaving 50μL (2x107 cells) for staining. Store cryovial at -80°C.
4. Add 150μL diluted Fc block to each set of pooled cells and incubate with vigorous shaking (600rpm) at RT for 10 minutes.
5. Add 300μL Surface Antibody Master Mix (see above table) to each set of pooled cells and pipette up and down to mix.
6. Incubate with vigorous shaking (600rpm) at RT for 30 minutes.
7. Wash 2X with CSM, and aspirate as much supernatant as possible without disturbing cell pellet.

Permeabilization and Intracellular Staining of Cells (Post-MeOH):

1. Vortex cells to dissociate pellet.
2. Add 700μL 4°C MeOH to each set of pooled cells, and pipette up and down gently 3X to mix.
3. Incubate for 10 min at 4°C.
4. During incubation, prepare intracellular antibody master mix (see above table):
   1. Mix all antibodies listed under “Intracellular (Post-MeOH)” in a 1.5mL microcentrifuge tube, and add the appropriate volume of CSM.
   2. **For older antibody stocks:** Prepare a centrifugal filter unit (Millipore Durapore 0.1µm PVDF) by adding 150µL CSM to the filter, and spin at 5,000xg for 2 min to prime the filter.

**Note:** centrifugal filter units can only accommodate 500µL, so use another filter unit if master mix volume exceeds 500µL.

* 1. Remove CSM from the bottom of the capture tube by pipetting.
  2. Add antibody master mix to the filter, and repeat centrifugation.
  3. Store filtered antibody cocktail on ice until ready to use.

1. Add 3mL PBS to each tube to wash.
2. Pellet cells, aspirate supernatant, and vortex briefly to dissociate pellet.
3. Repeat steps 5 and 6.
4. Add 3mL CSM to each tube to wash.

**Note:** addition of CSM after methanol treatment can cause some cells to adhere to the sides of the polystyrene FACS tube. This is more likely to happen with smaller pooled cell masses. Check for patches of adherent cells after adding CSM and if any patches are detected, dislodge them by scraping with a pipette tip and frequently aspirating/dispensing.

1. Pellet cells, aspirate supernatant to ~50μL residual volume, and vortex briefly to dissociate pellet.
2. Add 150μL CSM to each set of pooled cells and pipette up and down to mix.
3. Add 300μL Intracellular Antibody Master Mix (see above table) to each set of pooled cells and pipette up and down to mix.
4. Incubate with vigorous shaking (600rpm) at RT for 30 minutes. During incubation, prepare intercalator (see below).
5. Wash 2X with CSM, and aspirate as much supernatant as possible without disturbing cell pellet.

Intercalator Staining:

1. Prepare intercalator:
   1. Dispense 2mL CyTOF PBS into a FACS tube.
   2. Dispense 1µL 250mM Ir intercalator stock into 500µL microcentrifuge tube.
   3. Transfer 9µL CyTOF PBS from FACS tube to microcentrifuge tube containing intercalator stock and mix thoroughly (1:10 dilution).
   4. Add 200µL 16% PFA (filtered) to CyTOF PBS in FACS tube.

**Note:** Filtered 16% PFA is stable for 2 weeks. A new batch should be filtered if current batch is older than 2 weeks.

* 1. Add 4µL 1:10 intercalator dilution to FACS tube and vortex to mix.

1. Vortex FACS tubes containing cells briefly to dissociate pellets.
2. Add 1mL intercalator to each FACS tube containing cells.
3. Incubate cells with intercalator overnight at 4°C.

**Note:** If samples are to be analyzed immediately after staining, cells can be incubated with intercalator for 20 min at RT.

**Day 2**

Approximate Time: 3 hours per plate of barcoded samples

Preparing Samples for CyTOF:

1. Prepare Normalization Beads (20μL stock per mL Milli-Q ddH2O) in a 15mL conical tube.

**Note:** Depending on the size of the cell pellet, the volume of Normalization Bead solution can be scaled accordingly. In general, an event rate of 800-1,000 events/sec is preferred. For a pooled cell population starting with 2x107 cells, a reasonable expectation is to recover up to 50% (1x107 cells) during analysis. Therefore, an event rate of 1,000 events/sec resulting in 1x107 events would suggest a cell concentration of 2,000 cells/µL (2x106 cells/mL) at a flow rate of 30µL/min, meaning for a pooled set of 2x107 cells, 5mL of Normalization Bead suspension should suffice.

1. Wash cells in FACS tube (see above) **2X** with 3mL Milli-Q ddH2O.
2. Aspirate supernatant.
3. Re-suspend cells in 900µL Normalization Bead solution, and strain cells through cell strainer cap into a new FACS tube.
4. Replace tip, aspirate strained cells, and dispense into 15mL conical tube containing 1X Normalization Bead solution.

Running samples on CyTOF – see protocols CyTOF005.1 and CyTOF005.2.