**Lederer Lab Standard intracellular CyTOF staining with barcoding**

*For washes, we recommend not flicking out supernatant.*

*Carefully pipette off or aspirate the supernatant after every centrifugation.*

**Prepare cells and Viability**

1. After sample preparation/thawing, filter each ~10mL sample through 70um smart strainer (Miltenyi, Cat# 130-110-916). Centrifuge samples according to sample preparation protocol (e.g. 100 x g/10min if post-thaw)
2. In general, try to start with 1-2 x 106 cells in 200μL.
   1. Samples are diluted to 5x106 cells/mL in Thaw media, i.e. 200μL = 1x106 cells
3. Pipette 200μL cells into 96 well polypropylene plates (Corning, Cat# 3365) or 1.5 mL Eppendorf tubes and spin 750 x g/3min
4. Prepare Rhodium viability reagent (103Rh) and 0.4% Paraformaldehyde (PFA), both found aliquoted in -80°C freezer
   1. 103Rh: 1:1000 dilution in culture media (C5) (Stock 2000μM, Working 2μM, 1μL in 1mL C5).
   2. Thaw 16% PFA stock and make up 0.4% stock PFA from the 16% PFA stock performing a 1:40 dilution of the 16% in CyTOF PBS (100μL into 3900μL CyTOF PBS)
5. Retrieve samples from centrifuge and remove supernatants from each sample with multi-channel pipette.
   1. Total volume in each well = 200μL, set multi-channel pipette to 190μL
   2. This step is repeated after every centrifugation step, with all subsequent steps leaving behind ~40uL of residual volume
6. Add 100μL of 2μM 103Rh solution with multichannel pipette to wells. Mix each sample well and incubate 5min at room temperature (RT).
7. Add 100μL of the 0.4% PFA directly to each sample for a final concentration of 0.2% PFA for 5min RT, mix cells and PFA well. Spin at 750 x g/3min
   1. If cells are not pelleting and/or sticking to sides of wells, transfer each row to a new row of wells, by transferring in 2 installments of 100ul of CSB, Spin at 750 x g/3min

**Apply surface staining antibodies**

1. Add 20μL Fc-Block (human or mouse) reagent per well (1:100 in CSB); incubate for 10min RT
2. Add 20μL of Metal- coupled surface Ab cocktail to each well (1:100 dilution of each surface Ab in CSB). Incubate for 30min RT
3. Wash with 150μL CSB, spin with 750 x g/3min

**Fix/Perm**

1. Add 100μL of culture medium to each sample. Mix cells and culture medium well.
2. Make up 0.8% stock PFA from 16% PFA aliquots and add 100μL to the 100μL of culture medium in each sample for a final concentration of 0.4% PFA for 10min RT, mix cells and PFA well; Spin 750 x g/3min
   1. ***Can stop here and store at 4°C overnight in 150μL of CSB***
   2. If cells are not pelleting and/or sticking to sides of wells, transfer each row to a new row of wells, by transferring in 2 installments of 100ul of CSB, Spin at 750 x g/3min
3. Prepare 1X eBioscience Fixation/Permeabilization buffer by diluting 4X eBioscience Fixation/Permeabilization concentrate 1:4 in eBioscience Fixation/Permeabilization diluent (e.g. 600μL of 4X concentrate into 1800μL of diluent)
4. Permeabilize the cells with 100μL of eBioscience Fixation/Permeabilization buffer and incubate for 30min at RT, spin 750 x g/3min

**Barcoding (final BcR concentration is highly dependent on cell type and cell number)**

1. Wash 2x with 150μL CyTOF PBS, spin 750 x g/3min
2. Barcode Reagent (BcR) preparation:
   1. Add 100μL of CyTOF PBS to corresponding wells in separate polypropylene Barcoding transfer plate
   2. Place PBS reservoir and Barcoding transfer plate on ice
   3. During spin, begin thawing 10X BcR at RT
3. After final CyTOF PBS wash and supernatant aspiration, Place resuspended cells on ice
4. Add 10μL of 10X BcR to the 100μL of CyTOF PBS in transfer plate (mix once with multi-channel pipette)
   1. Be sure to work quickly so BcR does not hydrolyze in CyTOF PBS
5. Use a multichannel pipette preset to 90μl to mix 4 times and transfer BcR + CyTOF PBS to corresponding cell samples
6. Mix Cells and CyTOF PBS/BcR suspension 7 times
7. Once all samples have been resuspended with BcR, incubate at RT for 15min
8. Wash 1x with 100μL of CSB to dilute excess BCR and spin with 750 x g/3min
9. Wash 3x with 150μL of CSB to dilute excess BCR and spin with 750 x g/3min
10. After final wash, combine and filter barcoded samples in a 5mL polypropylene snap cap tube (Sarstedt, Cat# 55.526.006) fitted with 40um Filter cap (BD Falcon #352235) at 150μl per sample, spin with 750 x g/3min
11. Prepare 4% PFA by diluting 16% PFA stock 1:4 in CyTOF PBS (e.g. add 500μL of 16% PFA to 1500μL CyTOF PBS)
12. Add 1mL of 4% PFA for 10min RT, mix sample well
13. Spin out PFA and store cells in 1mL of CSB at 4°C overnight
    1. ***Proceed directly to Intercalation in step 28 if not storing overnight***

**Intercalation and cell counts**

1. Spin out CSB and add 1mL of Intercalator solution (see below)
   1. MaxPar Intercalator-Ir 500uM stock solution is pre-diluted 1:4 in CyTOF PBS (i.e. 20uL of 500uM Ir 191/3 in 60uL of CyTOF PBS) and stored at 4C for the duration of the experiment. This 1:4 solution is then diluted 1:4,000 at the time of labeling (i.e. 1uL of 1:4 Ir 191/3 into 4,000uL of CyTOF PBS)
2. Incubate 20min RT, spin 750 x g/3min
3. Wash with 1mL CSB, spin 750 x g/3min
4. Wash 2x with 1mL Cell Acquisition Solution (CAS); spin 750 x g/3min
5. Resuspend cells in 1mL CAS containing 1:10 dilution of EQ beads (for normalization)
6. Count cells to get a final cell concentration of 0.75 x 106 cells/1 mL
7. Transfer cell suspension to a fresh 5 mL polypropylene tube through a 40um filter cap, 2 separate times
   1. Polypropylene tube’s (Sarstedt, Cat# 55.526.006) cap is saved for later, 40um filter cap (BD Falcon #352235) is used to filter cell suspension in fresh 5mL polypropylene tube 2 separate times, and then original polypropylene tube cap is replaced for safe transport of sample to CyTOF acquisition core
8. Add additional EQ Bead CAS to get final concentration of 0.75 x 106 cells/1 mL
9. Run CyTOF