**Lederer Lab one step 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**

1. Prepare cell stimulation buffer
	1. 1:1000 dilution of 103Rh in culture media with RPMI (Fisher, Cat#11875-085) **without** added FBS, added cell stimulant at required concentration
2. Stimulate whole blood (minimum of 200uL) or prepared cells (try to use 1-2 x 106 cells) according to experimental timing
	1. Standard protocol is 200uL of whole blood into 70uL 4x cell stimulation buffer
3. Dilute out the stimulant with Smart Tube proteomic stabilizer (Fisher, PROT-1, stored RT) at a 1:1.4 dilution (e.g. 380uL of proteomic stabilizer into 270uL of cells+stim) and incubate for 10 minutes at RT
	1. **After incubation cells can be transferred directly to -80°C, or proceeded directly to step 3**

**Cell thaw/lyse**

1. If cells stored at -80, thaw at room temperature, but do not allow to sit thawed for long periods of time
2. Add diluted Thaw/Lyse Buffer (Fisher, stored RT at 1000x) at a concentration of 1:4 (e.g. 1.2mL of diluted blood and 3.6mL diluted Thaw/Lyse Buffer) and lyse for 10 minutes RT
3. After incubation spin cells at 600 x g/5min, and discard supernatant
4. Resuspend in 10mL of Thaw/Lyse for 500uL of blood (if working with 1mL of blood to start use 20mL of Thaw/Lyse) and incubate for 10 minutes RT
5. After incubation spin cells at 600 x g/5min, and discard supernatant
6. Resuspend in 2mL of C5 and spin cells at 100 x g/10min, and discard supernatant
7. Transfer remaining liquid/cells to 96 well plate
8. Spin at 750 x g/3min
9. 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)
10. Permeabilize the cells with 100μL of eBioscience Fixation/Permeabilization buffer and incubate for 30min at RT
	1. During incubation, make up 1X eBioscience Permeabilization wash buffer (make at least 10mL of 1X buffer, need more for >20 sample experiment) from the 10X solution by diluting in Ultra-Pure/MilliQ water (e.g. 1mL of 10X eBioscience Permeabilization buffer diluted into 9mL of Ultra-Pure/MilliQ water)
11. Do not aspirate wells- Add 100μL of 1X eBioscience Permeabilization wash buffer directly to the cells and the F/P, spin 750 x g/3min

**Barcoding**

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 2x with 150μL of CSB to dilute excess BCR and spin with 750 x g/3min

**Heparin Blocking and Intracellular Stain**

1. Make 1X (100 Units/mL) heparin solution, dilute the 10X (1000 Units/mL) solution 1:10 in CyTOF PBS
2. Add 150μL 1X heparin solution to each well and incubate at RT for 15min
3. After 15min incubation, combine barcoded samples in a 5mL polypropylene snap cap tube (Fisher, #14-959-10AA) at 150μl per sample, spin with 750 x g/3min
4. To resuspended cells (leave 50-100μl residual volume), add 20μL of Fc-Block (human or mouse) reagent per sample (1:100 in Perm Buffer); incubate for 10min RT (e.g. for 10 samples add 200μL of Fc-Block to pooled sample)
5. Add 20μL antibody (1:100 in 1X eBioscience Permeabilization wash buffer) per sample (e.g. for 10 samples add 200μL of antibody to pooled sample), incubate for 60min at RT
6. Wash with 1mL eBioscience perm buffer, spin 750 x g/3min
7. Wash with 1mL CSB, spin 750 x g/3min
8. Prepare 4% PFA by diluting 16% PFA stock 1:4 in CyTOF PBS (e.g. add 500μL of 16% PFA to 1500μL CyTOF PBS)
9. Add 1mL of 4% PFA for 10min RT, mix sample well
10. Spin out PFA and store cells in 1mL of CSB at 4°C overnight
	1. ***Proceed directly to Intercalation in step 33 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 1:4000 in CyTOF PBS (e.g. 1μL of Ir191/3 stock to 4000μL 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
	1. Polypropylene tube’s (Fisher, #14-959-10AA) cap is saved for later, 40um filter cap (BD Falcon #352235) is used to filter cell suspension in fresh 5mL polypropylene tube, 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