Staining Protocol

\*On ice or cold plate\*

Extracellular Staining:

1. Pipette 1-5 x 106 cells of samples into wells of a V-bottom plate
2. Prepare master mixes for 1X more samples than necessary
   1. For Single Colors: 100 μL PBS-serum + 0.5 μL 24G2 blocking Ab per sample
   2. For Samples: 100 μL PBS-serum + 0.5 μL 24G2 + 0.2 μL each mAb (unless otherwise noted) per sample
3. Spin down V-bottom plate at 4 °C until the speed comes to 1650 RPM
4. Flick off supernatant and resuspend samples in 100 μL Single Color or Stain Set.
   1. For single color controls individually add 0.2 μL of each mAb in the stain set (unless otherwise noted)
5. Incubate cells for 20 minutes, on ice in the dark. If using a tetramer, stain for 15 minutes at RT in the dark and then 15 on ice in the dark.
6. Wash 2X in PBS-serum
7. If performing intracellular staining proceed to Step 8, otherwise resuspend cells in 200 μL PBS-serum and run samples.

Intracellular Staining:

1. Intranuclear staining (*e.g.* transcription factors): \*\*If samples contain fluorescent protein see step 14\*\* Prepare master mix of a 1X Fixation/Permeabilization solution using 4X Fixation/Permeabilization Concentrate and Fixation/Permeabilization Diluent.
   1. Use 100 μL of 1X + 0.5 μL 24G2 per sample to be fixed

Intracytoplasmic staining (*e.g.* cytokines): use 100 μL of Fixation and Permeabilization solution 1X + 0.5 μL 24G2 per sample to be fixed

1. Resuspend samples and incubate for 30 minutes, on ice in the dark
2. Prepare 1X Permeabilization Buffer using 10X Permeabilization Stock + ddH2O for all samples
   1. For Intracellular Stain Set: 50 μL of 1X Buffer + 0.5 μL 24G2 + 0.2 μL intracellular mAb (unless otherwise noted) per sample, including single color controls
   2. For samples not being stained, resuspend in 50 μL 1X Permeabilization Buffer + 0.5 μL 24G2
3. Wash 2X in PBS serum, then resuspend in intracellular stain set
4. Incubate cells for 20 minutes, on ice in the dark
5. Wash 2X in 1X Permeabilization Buffer and 1X in PBS. Resuspend samples in 200 μL PBS-serum and run samples

YFP preservation during intracellular staining:

1. Incubate samples with 100 ul of fresh 4% PFA for 15 min. Wash 2X.
   1. PFA is prepared using 10X PBS and water to make 1X PBS
2. Resuspend samples in 100 ul of Miltenyi Solution 2 (from Foxp3 kit) and incubate 40 minutes. Wash 2X.
3. Resuspend samples in 50 ul of 1% triton X100 (diluted in PBS) and incubate 15 minutes. Wash 2X
4. Stain samples with intracellular antibodies in PBS-serum for >30 min.