Collecting cells

## Adherent cells

1. Detach cells with 1mL cell stripper (15 min at RT or **5 min at 37C**). If cells not detaching, scrape well with pipette tip or cell scraper.
2. Collect in 1.5 mL tubes, spin down, and wash with 1 mL RPMI. Proceed to step 3.

## Suspended cells (for tissue-derived cells, skip to step 3)

1. Transfer suspended cells to 15mL falcon tube, wash well with 1mL appropriate media.
2. Spin down cells, resuspend in 1mL RPMI. Proceed to step 3.
3. Resuspend cells in 100 ul blocking buffer
   1. Mouse: 2.4G2 antibody 1:500 in FACS buffer
   2. Human: Human FC block 1:500 in FACS buffer
4. Transfer cells to (pre-labelled) wells of 96-well round bottom plate
5. Proceed to cell labeling

# Labeling for flow cytometry

## Extracellular staining

1. Spin down cells
2. Dilute conjugated antibody to 1:200 unless otherwise specified. Add extracellular antibody (with viability dye 1:1000) solution (50 ul per sample)
3. Use beads for compensation controls: 1 drop of beads, stained in the same manner as the sample
4. Incubate on ice for 30 min, in the dark
5. Wash 3x with FACS buffer

## Fixation (if doing FACS next day)

1. Resuspend cells in 100uL 4% formaldehyde. Fix for 15 min at 4oC in the dark
2. Wash cells 2x with FACS buffer, resuspend in FACS buffer and store overnight at 4oC

## Intracellular staining (panel-dependent)

1. Add 100ul/sample Fix/Perm buffer
2. Fix cells at 4C for 40-60 minutes (in the dark).
3. Meanwhile, prepare intracellular antibody staining mix in Perm wash.
4. Wash samples with 150ul Perm Wash 2x
5. Dilute conjugated antibody to 1:200 in perm wash unless otherwise specified. Add intracellular antibody solution (50 ul per sample) to samples

Add perm wash to compensation controls

1. Incubate for 30 min at 4C, in the dark
2. OPTIONAL: During labeling, prepare Countbright absolute counting beads:
   1. Vortex the bottle for 30 seconds
   2. Calculate the amount of beads that you will need (I use 20000 beads per sample) and dilute in FACS buffer to have 100ul per sample.
   3. Add beads (100ul) to flow cytometry cluster tubes
3. Wash samples with 150ul perm wash 3 times

## Proceeding to FACS

1. Resuspend the cells in 100ul FACS buffer or medium and transfer to cluster cytometry tubes (which should have 100ul of beads)
2. Proceed to analysis, or analyze the following day.
3. During analysis, make sure you acquire at least 500k events for the TIL samples. Untreated controls may need many more events acquired, since they don’t have many lymphocytes.

# Reagents

\*\*\*Prepare Fix/Permeabilization buffer from FoxP3 staining buffer set (1 part fixation: 3 part Permeabilization diluent).

\*\*\*Prepare Permeabilization Wash (dilute concentrate 1:10 in H2O)

1. RPMI-10 +abx
2. RPMI-0 (serum-free RPMI)
3. FACS buffer: PBS+ .5% FCS + 1mM EDTA

For labeling

FoxP3 staining kit

24G.2

All antibodies

Blocking buffer:

FACS buffer with 1:500 2.4G2 antibody

96 well round-bottom plates.

All spins at 1500RPM/4oC/2 min unless otherwise indicated

\*\*spin 96-well plates 2 min at 2000RPM and 4oC