**Begins after single cell suspension has been obtained-by digestion (enzymatic or mechanical)**

1. Add MACs Buffer to filtered (40-100μM filters depending on cell size) single cell suspension of cells (5-10mL) and centrifuge at 350xg for 5 minutes, discard supernatant.
   **For ACK Lysis Step-Used in Tissues with High RBC content**
2. Add 5mLs cold ACK lysis buffer, vortex lightly and centrifuge as above, discard supernatant.
3. Add MACs Buffer (5-10mLs) to wash out any remaining ACK Buffer, and centrifuge as above, discard supernatant.
   **Begin here if post-Ficoll-Paque isolation or Tissues with low RBC contamination.**
4. Re-suspend cells in MACs buffer (1-5mLs depending on density of pellet). Count viable cells with trypan blue exclusion or using a cell counter. (*If using counting beads-a rough estimate is needed here and the fraction of the total volume is what needs to be recorded to extrapolate the count back from the flow cytometer-if not using counting beads-this count is important for cell numbers, otherwise the only data you will be able to obtain is percentages. With cells from liquid components like peritoneal lavage or blood, it is important to denote the original amount of blood received, as it will be used to normalize the counts) (*May need to re-filter if notice increased death after this step or clumps)
5. Take recommended amount of cells in 200μL (max) and move to U-bottom plate (remember to make plate map) or move needed volume into flow tubes for staining.
   **For staining with fixable viability dye**
6. Wash cells with PBS, spin down, and discard supernatant. (*If using plates-one strong flick to remove the volume-never two. Then wipe plate with a kimwipe to remove any spray.)
7. Make a master mix (MM) of your viability dye in PBS. (*1/2 to 1/4 the recommended volume ends up staining efficiently.)

   # of samples +2 X amount of stain per sample = amount of stain in MM

   # of samples +2 X 50μL PBS= amount of PBS in MM
8. Add 50μL/tube of viability MM. (Re-suspend cells-Pipette up and down for plates, vortex lightly for tubes) Put tubes or plate in dark at room temperature for 10mins.
   **For Flow Cytometry Staining-begin with Fc Blocking step**
9. Add MACS buffer (1mL to tubes-100μL to wells). Centrifuge and decant.
10. Move tubes to ice bucket.
11. Add MM for Fc block. For mouse samples use 2.4G2 (in house isolations 1μL/sample). For human samples use Human TruStain (Biolegend) (5μL/sample). (Human TruStain is directed against CD16/32, but is compatible with clone 3G8 anti- human CD16 staining for flow.) After adding pipette up and down for plates or vortex lightly for tubes.

    # of samples +2 X amount of Fc block per sample = amount of block in MM

    # of samples +2 X 50μL MACs= amount of MACs in MM

    What to add per sample = 50μL MACs + amount of Fc block per sample
12. Allow a 5 minute incubation for maximum Fc blockage.
13. Add MM stain cocktail (200μL/sample). If using more than one BV or BUV stain, don't forget to add Brilliant Violet Staining Buffer to your cocktail. Pipette up and down for plates, vortex lightly for tubes. Incubate 15-20 minutes in the dark on ice. (BV stain

buffer protocol recommends 50µL/sample-10-15µL/sample is sufficient to provide the protection from these dyes sticking to each other)
(Also antibody recommendations by the manufacturer should be followed unless titrations have been performed. In the Martin laboratory ½ µL stain / 1 million cells for the majority of anti-mouse antibodies is sufficient for staining.)

# of samples +2 X amount of BV Stain Buffer/sample = amount of buffer in MM
# of samples +2 X amount of stain/sample (stain1)= amount of stain 1 in MM
# of samples +2 X amount of stain/sample (stain2)= amount of stain 2 in MM
# of samples +2 X amount of stain/sample (stain3)= amount of stain 3 in MM
# of samples +2 X amount of stain/sample (stain4)= amount of stain 4 in MM
Done for each stain…
MACs buffer/sample= (200µL total stain volume/sample-= - {Total amount of stain/sample (stain 1+stain 2+ stain 3+ stain 4…etc) + amount of BV stain buffer/sample})
# of samples +2 X MACs buffer/sample= amount of MACs buffer in MM

14. Wash cells with MACs buffer (1mL for tubes, 50µL for plates). Centrifuges and discard supernatant. Repeat this, so the cells are washed two times. (Second wash in plates is 200µL)
15. If a secondary antibody is needed, repeat steps 13 and 14.
    **For Fixation of cells that will not be immediately run (If doing intracellular staining will be followed with perm steps follow manufacturer's protocol. For nuclear or transcription factor staining use fixation buffer from True Nuclear Fix/Perm kit for this step)**
16. Re-suspend cells in Fixation buffer (300µL for tubes, 100µL for plates). Pipette up and down or vortex. Incubate for 15mins at room temperature.
17. Wash cells with PBS (1mL for tubes, 100µL for plates). Centrifuge, discard supernatant.
18. Re-suspend cells in PBS (Cyto-last buffer is highly recommended, if storage is going to be longer than 3 days). (300µL for tubes, 100µL for plates) **Can stop here before proceeding and leave in the fridge, covered with a plate sealer or capped tubes protected from light.**
    **For Preparation for running cells and adding Counting beads (optional)**
19. *To transfer from the u-bottom plate into tiny FACs tubes. (If using counting beads-the volume precision is important here). Spin plate, discard supernatant. Re-suspend cells by pipetting up and down in 200µL PBS and transfer exactly 200µL into 96-well holder filled with tiny FACs tubes.
20. * Add Counting Beads at this point. Vortex bottle of beads for 15 seconds. Make sure to record the lot and concentration of beads-as is varies per bottle. Add 100µL beads MM to each tube.

    # of samples +2 X 20µL beads/sample= amount of beads in MM
    # of samples +2 X 80µL PBS/sample= amount of PBS in MM
    100µL beads MM/tube
21. Run samples on Flow cytometer.

## Addendum

**MACs buffer recipe**
PBS pH 7.2
2mM EDTA
0.5% BSA

**Products**

Precision Count Beads Biolegend Catalog Number 424902 $220.00
Brilliant Violet Stain Buffer BD Catalog Number 566385 (1000 Tests) $612.85
Fixation Buffer Biolegend Catalog Number 420801 $50.00
Cyto-last Buffer Biolegend Catalog Number 422501 $65.00
Intracellular Staining Perm Wash (10x) Biolegend Catalog Number 421002 $80.00
True Nuclear Transcription Factor Buffer Set Biolegend Catalog Number 424401 $140.00
Human TruStain FcX Biolegend Catalog Number 422302 $195.00
ACK lysis buffer Quality Biological (VWR) 500mL Catalog number 10128-802 $49.64

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