Protocol: Intracellular Staining (post-Activation)

Steps:

1. Detaching adherent cells:
   a. Add EDTA at a final concentration of 2 mM (see stock preparation table), incubate 15 m at room temperature. Centrifuge at 250 x G for 5 m, aspirate supernatant.

2. Viable cell staining (optional):
   a. Resuspend in 100 μL of 2.5 μg/mL amine-reactive dye in PBS (or concentration as titered for dye lot). Incubate 20 m at room temperature, then add 100 μL of wash buffer and wash as in step 1.

3. Surface antibody staining (for markers sensitive to fixation and permeabilization):
   a. PBMC: Resuspend in 50-100 μL of wash buffer containing optimal titers of all surface antibody stains. Incubate 30-60 m at room temperature, then add wash buffer up to at least 200 μL and wash as in step 1.
   b. Whole blood: Treat as above, but instead of wash buffer, directly add BD FACS Lysing Solution.

4. Fixation and permeabilization:
   a. For IL-10 and TGFβ: use BD Cytofix/Cytoperm kit according to manufacturer's instructions.
   b. For most other markers: use either BD FACS Lysing Solution+FACS Permeabilizing Solution 2, or BD Cytofix/Cytoperm, according to manufacturer's instructions.

5. Intracellular antibody staining:
   a. After permeabilization and thorough washing, resuspend in 50-100 μL of wash buffer containing optimal titers of all intracellular antibody stains. Incubate 30-60 m at room temperature, then add additional wash buffer up to at least 200 μL and centrifuge at 500 x G for 5 m. Repeat wash step a second time for optimal staining, particularly when using plates.

6. Final fixation (optional):
   a. For assays with Cy7 tandems, but NOT AmCyan: Resuspend in at least 200 μL of BD Stabilizing Fixative and store at 4C in the dark. Acquire within 24 h if possible.
   b. For other assays: Resuspend in at least 200 μL of 0.5-2% paraformaldehyde in PBS and store at 4C in the dark. Acquire within 24 h if possible.

Notes:

1. Use a fixed-length vacuum manifold to reproducibly aspirate supernatant from plates.

2. CD3, CD4, and CD8 can be used as intracellular markers with most fluorochromes. Most other antibodies to cell-surface determinants should be used prior to fixation and permeabilization.

3. Viability dye staining can also be done together with surface Ab staining, but the required concentration will be higher, and staining may not be as clean.

4. Samples can be frozen at -80C in BD FACS Lysing Solution, then batched for later processing. Whole blood should be washed once to remove red cell debris before freezing.

5. After fixation, cells become more bouyant, and need to be centrifuged at higher G force (500 x G).

6. Be sure to wash thoroughly (2-3 times when using plates) between fixation/permeabilization and addition of intracellular Abs, to reduce background.

7. Fixation of stained samples can degrade staining of some sensitive markers. Resuspending in wash buffer is preferable, even if cells will be stored overnight prior to acquisition (keep dark at 4C).

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Stock Solution Recommendations:

Solution	**Stock Concentration**	**Intermediate Dilution**	**Final Concentration**
EDTA	20 mM in PBS	None	2 mM (1:10)
Wash Buffer	0.5% BSA+0.1% NaN_3 in PBS	None	Use neat
Final fixative	0.5% paraformaldehyde in PBS	None	Use neat