A. SAMPLE PREPARATION

Prepare cells according to cell type.

Blood (Human, Mouse or Rat)

- For each 1 ml of blood, add 14 ml of room temperature **FCM Lysing solution** (sc-3621) to lyse the red blood cells. The cells will not lyse correctly if the solution is cold.
- Incubate for 5 minutes at room temperature on a rotator. Do not exceed 5 minutes, as the white blood cells will begin to lyse beyond 5 minutes.
- Centrifuge for 5 minutes at 1000 RPM for human blood, 2000 RPM for mouse or rat blood.
- Carefully aspirate supernatant, then resuspend pellet in approximately 50 ml cold 1X PBS. Take a small sample to perform a cell count.
- Centrifuge for 5 minutes at 1000 RPM for human blood, 2000 RPM for mouse or rat blood.
- Aspirate supernatant.

Mouse Spleen or Other Tissue

- Harvest organ or tissue and prepare single cell suspension.
- Pass cell suspension through a 70 µM cell strainer.
- Centrifuge for 5 minutes at 1000 RPM.
- Discard supernatant and add 5 ml of room temperature **FCM Lysing solution** (sc-3621).
- Incubate for 2–3 minutes at room temperature, allowing larger pieces to fall to the bottom of the tube.
- Carefully pipette the suspension out and deposit into a clean tube. Take a small sample to perform a cell count.
- Centrifuge for 5 minutes at 1000 RPM.
- Aspirate supernatant.

Suspension Cell Line

- Pipette off cells, rinsing plate to ensure maximum recovery. Take a small sample to perform a cell count.
- Centrifuge for 5 minutes at 1000 RPM.
- Aspirate supernatant.

Monolayer / Adherent Cell Line

- Vacuum off media. Rinse plate once with 1X PBS. Vacuum off PBS.
- Add approximately 5 ml of 0.2% EDTA (in PBS) to plate. Using a Trypsin/EDTA solution in the place of 0.2% EDTA may compromise any cell surface staining.
- Wait for cells to "round up." Placing the cells in an incubator may speed up this process. Check the plate(s) every 5 minutes.
- Add approximately 5 ml of media to neutralize EDTA.
- Pipette off cells, rinsing plate to ensure maximum recovery. Take a small sample to perform a cell count.
- Centrifuge for 5 minutes at 1000 RPM.
- Aspirate supernatant.

B. CELL STIMULATION

Stimulate cells as necessary.

C. STAIN PREPARATION

Fix cells or prepare live cells for staining.

NOTE: It may be desired to block Fc receptors for certain cell types including, but not limited to, mouse and rat blood, mouse spleen, mouse bone marrow, etc. A blocking reagent contains a high concentration of immunoglobulin that will bind to the Fc-receptors on cells. The blocking reagent should ideally be immunoglobulin from the species whose cells you are staining. Recommended blocking reagent: 1 µg normal immunoglobulins per 1 million cells.

Live Staining

- Once supernatant is aspirated from cell preparation, resuspend pellet in enough 1X PBS to have a final cell concentration of 10 million cells/ml.
- Block by incubating the cell suspension with blocking reagent for 10 minutes. Do not rinse. Proceed with staining.

Fixed and Permeabilized Cells for Intracellular Staining

- Once supernatant is aspirated from cell preparation, resuspend pellet in enough 1X PBS to have a final cell concentration of 10 million cells/ml.
- Block by incubating the cell suspension with blocking reagent for 10 minutes.
- Resuspend pellet in approximately 50 ml 1X PBS to wash away any excess blocking antibody.
- Centrifuge for 5 minutes at 1000 RPM.
- Once supernatant is aspirated from cell preparation, resuspend pellet in **FCM Fixation Buffer** (sc-3622). Use 1 ml per million cells.
- Incubate for 30 minutes at room temperature on a rotator.
- Centrifuge for 5 minutes at 1500–2000 RPM. Cells get more buoyant after fixation. If pellet is too small, spin again at a higher RPM, but do not exceed 3000 RPM.
- Pour off supernatant. Cells may be lost if aspirating from this point on, so always decant. Use a quick motion and don't allow the supernatant to wash back and forth over the cells.
- Resuspend pellet in approximately 50 ml 1X PBS to wash away any excess Fixation Buffer.
- Centrifuge for 5 minutes at 1500–2000 RPM.
- Decant supernatant. At this point, cells can be resuspended in a small amount of PBS and stored for up to 1 month at 4° C. To permeabilize at this time, proceed to next step.

NOTE: You should only proceed with permeabilization if you can stain immediately afterwards.

- If cells have been stored in PBS, centrifuge for 5 minutes at 1500–2000 RPM and decant supernatant.
- Break up cell pellet and dropwise add the same amount of COLD (stored at -20° C) **FCM Permeabilization Buffer** (sc-3623) at 1 ml per 1 million cells. Vortex while adding.
- Incubate for 5 minutes only at RT on a rotator.
- Immediately centrifuge for 5 minutes at 2000–2500 RPM. Cells are more buoyant after permeabilization and much care must be excercised to maintain volume of cells.

NOTE: Important: If a pellet is not recovered at this step, be sure to spin again and try to recover more cells.

- Decant supernatant and add approximately 50 ml 1X PBS to wash away any excess Permeabilization Buffer.
- Centrifuge for 5 minuntes at 2000–2500 RPM.
- Decant supernatant and resuspend pellet in enough **FCM Wash Buffer** (sc-3624), for a final cell concentration of 10 million cells/ml. In the staining steps, use FCM Wash Buffer in place of 1X PBS.

D. STAINING

Follow protocol for direct or indirect staining.

DIRECT STAINING

(With Fluorochrome-Conjugated Antibodies)

- Label tubes.
- Add 1 µg of fluorochrome-conjugated antibodies to tubes.
- Add 100 µl of the prepared cell suspension (equal to 1 million cells) to each tube.
- Vortex and incubate for 15–30 minutes in a covered ice bucket.To wash off excess antibody following staining, add 1.5–2 ml of 1X PBS to each tube.
- Centrifuge in tabletop microfuge for 5 minutes at 2000 RPM. This speed should be increased to 3000 or 4000 RPM for intracellular staining.
- Aspirate supernatant, being careful not to disturb pellet.
- Resuspend pellets in 500 µl of 1% paraformaldehyde. Tubes can be stored in the dark for 24 hours (maximum for intracellular staining) to 1 week (maximum for surface staining).

INDIRECT STAINING

(With Unconjugated Primary Antibodies and Fluorochrome-Conjugated Mouse IgG Binding Proteins or Secondary Antibodies)

- Label tubes.
- Add unconjugated primary antibodies to tubes. Use approximately 1 µg per tube.
- Add 100 µl of the prepared cell suspension (equal to 1 million cells) to each tube.
- Vortex and incubate for 15–30 min in a covered ice bucket.
- To wash off excess antibody following staining, add 1.5–2 ml of 1X PBS to each tube.
- Centrifuge in tabletop microfuge for 5 minutes at 2000 RPM (or 3000–4000 RPM for intracellular staining).
- Aspirate supernatant, being careful not to disturb pellet.
- Add 100 µl of 1X PBS to each tube. Add fluorochrome-conjugated Mouse IgG Binding Proteins or secondary antibodies to tubes. Use 0.5–1 µg of binding protein/antibody.
- Vortex and incubate for 15–30 minutes in a covered ice bucket.
- To wash off excess antibody following staining, add 1.5–2 ml of 1X PBS to each tube.
- Centrifuge in tabletop microfuge for 5 minutes at 2000 RPM (or 3000–4000 RPM for intracellular staining).
- Aspirate supernatant, being careful not to disturb pellet.
- Resuspend pellets in 500 µl of 1% paraformaldehyde. Tubes can be stored in the dark for 24 hours (maximum for intracellular staining) to 1 week (maximum for surface staining).

E. ACQUIRE

Acquire within 24 hours.