Basic Guideline for Staining Cells:

- Pre-staining: Prepare antibody cocktail mix by adding the volumes of each antibody/staining buffer according to the amounts determined by antibody titration. Keep at 4°C protected from light until ready to use.
  Note: Make any FMO antibody mixes required by adding all antibodies except the one the tube is an FMO for.

1. Add 4 mL of DPBS (NO protein in buffer) to each sample.
2. Centrifuge cells at 350 RCF at room temperature for 6 minutes.
3. Carefully remove the supernatant and resuspend cells in appropriate mixture of Live/Dead stain based on manufacturer's instructions.
4. Incubate cells in Live/Dead per manufacturer's instructions.
5. To each aliquot, add 4 mL of room temperature FACS buffer (2% BSA in DPBS). (Or follow manufacturer's instructions if different)
6. Centrifuge cells at 350 RCF at 4°C for 6 minutes.
7. Carefully remove the supernatant from each and resuspend cells in 5 µL of cold (4°C) Fc block plus 50 µL cold staining buffer. Incubate cold for 5 minutes protected from light.
8. Add appropriate volume of 4°C antibody cocktail mix or FMO cocktail mix.
9. Incubate at 4°C for 30 minutes, protected from light.
10. Add 2 mL cold FACS buffer and centrifuge at 350 RCF at 4°C for 5 minutes.
11. Carefully remove supernatant and resuspend cells in 2 mL cold FACS buffer, and centrifuge as in previous step.
12. Remove supernatant and resuspend cells in 200 µL of cold fixation buffer.
13. Incubate at 4°C for 30 minutes, protected from light. (Follow fix buffer manufacture instructions if different)
14. Add 2 mL cold FACS buffer and centrifuge at 350 RCF at 4°C for 5 minutes.
15. Remove supernatant and resuspend cells in 2 mL cold FACS buffer, and centrifuge as in previous step.
16. Remove supernatant and resuspend cells in 200 µL of cold FACS buffer. Store at 4°C protected from light until ready to collect data.

Guide for Compensation Controls:

1. Label tubes and add cells or beads so that you have one tube for each color you stained cells plus a tube for an unstained control. (ie if you have a 9 color panel you should have 10 tubes. **If every tube has both stained and unstained cells/beads you can skip the unstained control**)
2. Make unstained control - leave 1 tube of beads/cells unstained with any antibody or fluorescent marker.
3. Add antibody to appropriate beads/cells based on amounts determined to give a clear positive population with antibody staining of equal/slightly higher staining than actual sample.
4. Incubate for 30 minutes, protected from light at 4°C (Double check your live/dead – manufacturer instructions may require a different temperature).

5. Then add 1 mL of FACS buffer and centrifuge for 5 min at 800 RCF for beads (or for cells- 350 RCF) at 4°C.
6. Carefully remove supernatant and resuspend in 1 mL cold FACS buffer.
7. Centrifuge again for 5 min at 800 RCF for beads (or for cells- 350 RCF) at 4°C.
8. Remove supernatant and resuspend all compensation controls in 200 µL of cold fixation buffer.
9. Incubate at 4°C for 30 minutes, protected from light.
10. Centrifuge for 5 min at 800 RCF for beads (or for cells- 350 RCF) at 4°C.
11. Remove supernatant and resuspend beads/cells in 1 mL cold FACS buffer, and centrifuge again as in previous step.
12. Remove supernatant and resuspend beads/cells in 150 µL of cold FACS buffer. Store at 4°C protected from light until ready to collect data.