Intracellular staining of cytokines

This protocol is suitable to label murine cells isolated from the blood or from tissues, such as lymph nodes, spleen, liver or lungs, after tissue dissociation and eventual digestion. Please ensure to follow a protocol with optimized procedures for the dissociation of each tissue. Ensure all antibodies had been previously titrated and the optimal dilution of each one to stain 1 x 10^6 cells is known.

**Reagents & Materials**

- Trypan blue
- 5-mL round bottom FACS tubes or 96-well U-bottom plate
- [Optional] Fixable Viability Dye
- Fc Block (unlabeled CD16/32, clone 2.4G2)
- Fluorescently labeled antibodies (each one at its optimal dilution)
- eBioscience™ Intracellular Fixation & Permeabilization Buffer Set (ThermoFisher cat. no. 88-8824) containing:
  - IC Fixation Buffer (cat. 00-8222)
  - Permeabilization Buffer (10X) (cat. 00-8333)
- FACS Buffer (1X PBS with 2% FBS and 0.02% NaN₃)

*For cell stimulation:*

In sterility, prepare the following PMA/ionomycin + Brefeldin A & Monesin solution in complete RMPI or IMDM (for IL-17, it is better to use IMDM):

- Phorbol 12-myristate 13-acetate (PMA, stored at -80°C) at a final concentration of 50 ng/mL.
- Ionomycin (stored at -80°C) at a final concentration of 500 ng/mL.
- Brefeldin A (stored at -20°C) at a final concentration of 10 µg/mL.
- Monensin (BD GolgiStop™ stored at 4°C): 1.5 µL/mL

**Buffers and solution preparation**

- Prepare a 1X working solution of Permeabilization Buffer by mixing 1 part 10X concentrate with 9 parts distilled water. Each sample will require 100 µL or 500 µL of the working solution if staining in 96-well plates or in 5 mL FACS tubes, respectively.
Procedures

A. Cell stimulation

1. Count the cells in suspension using an Neubauer hemocytometer.
   1.1. When having several millions of cells, it is more accurate to have cells suspended in at least 5 mL.
   1.2. Take 20-50 µL of cell suspension and make a 1:1 dilution in Trypan Blue to assess only the viable cells.

2. Sediment the cells by centrifugation at 1400 rpm for 5 min and discard the supernatant by decantation.

3. Vortex the pellets and resuspend cells in FACS Buffer at 20 X 10⁶ cells/mL.

4. Transfer 5 X 10⁶ cells from each sample to a sterile tube (or 2 X 10⁶ to a well from a 96-well plate), pellet the cells by centrifugation and resuspend the cells in 500 µL (or 200 µL in case of a plate) stimulation medium, containing PMA/ionomycin + brefeldin A & monensin.

5. Incubate cells for 2 to 5 hours at 37ºC, 5% CO₂ (typically, innate lymphocytes require less time of stimulation, and T cells require longer stimulation periods).

B. Cell staining

1. Sediment the cells by centrifugation at 1400 rpm for 5 minutes (for plates, a quick spin up to 2500 rpm is sufficient) and discard the supernatant by decantation.

2. Vortex the pellets and add 100 µL FACS Buffer per well (for plates) or 500 µL FACS Buffer to tubes.

3. Sediment the cells by centrifugation at 1400 rpm for 5 minutes (for plates, a quick spin up to 2500 rpm is sufficient) and discard the supernatant by decantation.

4. Perform the cell surface staining. Refer to the “Antibody staining of surface antigens” protocol for details. It is highly recommendable to stain cells with a Fixable Viability Dye during this step.

5. Add 100 µL FACS Buffer per well (for plates) or 500 µL FACS Buffer to tubes.

6. Sediment the cells by centrifugation at 1400 rpm for 5 minutes (for plates, a quick spin up to 2500 rpm is sufficient) and discard the supernatant by decantation.

7. Vortex the pellets and fix the cells by adding 100 µL per well (for plates) or 200 µL to tubes of IC Fixation Buffer and homogenize by pipetting or by gently vortexing.

8. Incubate for 20-60 minutes at room temperature protected from light.

9. Sediment the cells by centrifugation at 1400 rpm for 5 minutes (for plates, a quick spin up to 2500 rpm is sufficient) and discard the supernatant by decantation.

10. Vortex the pellets and add 100 µL 1X Permeabilization Buffer per well (for plates) or 500 µL Permeabilization Buffer to tubes.

11. Sediment the cells by centrifugation at 1400 rpm for 5 minutes (for plates, a quick spin up to 2500 rpm is sufficient) and discard the supernatant by decantation.
14. Vortex the pellets and add 20 µL Fc Block (diluted in 1X Permeabilization Buffer) per well (for plates) or 80 µL to tubes.

15. Incubate for 15 minutes at 4°C.

16. Without washing, add 5 µL intracellular antibody mix (diluted in 1X Permeabilization Buffer at 1:20) per well (for plates) or 20 µL FACS Buffer to tubes.

17. Incubate for 30 minutes at room temperature, protected from light.

18. Add 100 µL 1X Permeabilization Buffer per well (for plates) or 500 µL 1X Permeabilization Buffer to tubes.

19. Sediment the cells by centrifugation at 1400 rpm for 5 minutes (for plates, a quick spin up to 2500 rpm is sufficient) and discard the supernatant by decantation.

20. Vortex the pellets and repeat step 19.

21. Vortex the pellets and resuspend in ~300-500 µL FACS Buffer (minimum volume recommended: 200 µL).

22. Filter cells before for flow cytometry analysis using cell-strainers or tubes with filter-caps to remove any clumps that may be present.