| Flow Cytometry Facility | # Intracellular staining of transcription factors |
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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 $1 \times 10^6$ 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)
- Foxp3/Transcription Factor Staining Buffer Set (Thermofisher cat. no. 00-5523)
- FACS Buffer (1X PBS with 2% FBS and 0.02% $NaN_3$)

## Buffers and solution preparation

- Prepare fresh Foxp3 ***Fixation/Permeabilization working solution*** by mixing 1 part of Foxp3 Fixation/Permeabilization Concentrate with 3 parts of Foxp3 Fixation/Permeabilization Diluent. 100 $\mu$L or 500 $\mu$L of the working solution is required for each sample, if staining in 96-well plates or in 5 mL FACS tubes, respectively.
- Prepare a ***1X working solution of Permeabilization Buffer*** by mixing 1 part of 10X Permeabilization Buffer with 9 parts of distilled water. 100 $\mu$L or 500 $\mu$L of the working solution is required for each sample, if staining in 96-well plates or in 5 mL FACS tubes, respectively.

## Procedures

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 $\mu$L 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 $10^6$ cells/mL.

4. Transfer 100 $\mu$L cell suspension (<2 X $10^6$ cells) per well of a round-bottomed 96-well plate or 500 $\mu$L cell suspension (>2 X $10^6$ cells) to 5 mL FACS tubes.

5. 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.

6. 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.

7. Add 100 $\mu$L FACS Buffer per well (for plates) or 500 $\mu$L FACS Buffer to tubes.

8. 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.

9. Vortex the pellets and add 100 $\mu$L per well (for plates) or 500 $\mu$L to tubes of Foxp3 Fixation/Permeabilization working solution and homogenize by pipetting or by gently vortexing.

10. Incubate for 30-60 minutes at 2-8°C protected from light. Note: mouse samples can be incubated for up to 18 hours at 2-8°C in the dark.

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.

12. Vortex the pellets and add 100 $\mu$L 1X Permeabilization Buffer per well (for plates) or 500 $\mu$L Permeabilization Buffer to tubes.

13. 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 $\mu$L Fc Block (diluted in 1X Permeabilization Buffer) per well (for plates) or 80 $\mu$L to tubes.

15. Incubate for 15 minutes at 4ºC.

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

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

18. Add 100 $\mu$L 1X Permeabilization Buffer per well (for plates) or 500 $\mu$L 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 $\mu$L FACS Buffer (minimum volume recommended: 200 $\mu$L).

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