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U937 Phospho Flow Protocol

This experiment is an excellent way to familiarize yourself with the phospho flow protocol steps of stimulating, fixing, permeabilizing, and staining cells. The U937 cell line responds robustly and consistently and is recommended for your first experiment.

Materials

Cell line (U937 cells)
Stimuli (human IL-4 and human IFN-γ)
Phospho-specific antibodies (Ax647-conjugated anti-Stat1(pY701) and Ax488-conjugated anti-Stat6(pY641), Becton Dickinson)
Tissue culture media
Staining media (PBS + 0.5% BSA)
5-mL polystyrene FACS tubes (BD Falcon)
16% paraformaldehyde in water (PFA), EM grade (Electron Microscopy Sciences)
100% methanol, cooled to 4°C
Tabletop centrifuge with 5ml tube holder
Flow cytometer with 488 and 633 laser lines (e.g., Becton Dickinson FACSCalibur)

Methods

Stimulate

1. Grow U937 cells to 10^6 cells/ml. Add 1 ml to each of four FACS tubes.

   After adding the cells to FACS tubes, it is important to quickly add the cytokines to avoid cooling the cells far below 37 degrees.

2. Quickly add cytokines to stimulate cells as described in the following table:

Tube #	1	2	3	4
Stimulus	None	10ng IL-4	10ng IFN-γ	10ng IL-4 + 10ng IFN-γ

3. Vortex or pipette cells to mix, and then incubate for 15 min in a 37ºC, 5% CO2 incubator.

   15 minutes works well for cytokine stimulations. However, you should test different time points for your stimulus of interest.

Fix

4. Remove tubes from incubator and fix cells by adding 100 µL of fresh 16% PFA to each ml of media (for a final concentration of 1.5%); vortex to mix, and incubate for 10 min at room temperature. Centrifuge for 5 min at 375 x g, 4°C, and decant the supernatant.

Permeabilize

5. Resuspend the cells in the residual volume left after decanting by vortexing vigorously. Then add 1 mL of cold methanol (4ºC) to each tube, and vortex to mix.

   It is important to resuspend the cells prior to adding methanol to avoid clumping.

6. Incubate cells at 4ºC for 20 min.

   At this point, cells can be stored at -80 ºC for many months without significant loss of signal. Cells can be stored at -20 ºC overnight.

Intracellular staining

7. Wash the cells by adding 3 mL of staining media, pelleting (>375 x g, 5 min, 4°C), and decanting the supernatant. Repeat this wash.
8. Resuspend cells at 10^6 cells / 60 µL in staining media. Transfer 60 µL from each tube to a fresh tube.

   It is important to have the exact same volume in each tube so that the antibody concentration in the next step is consistent between samples.

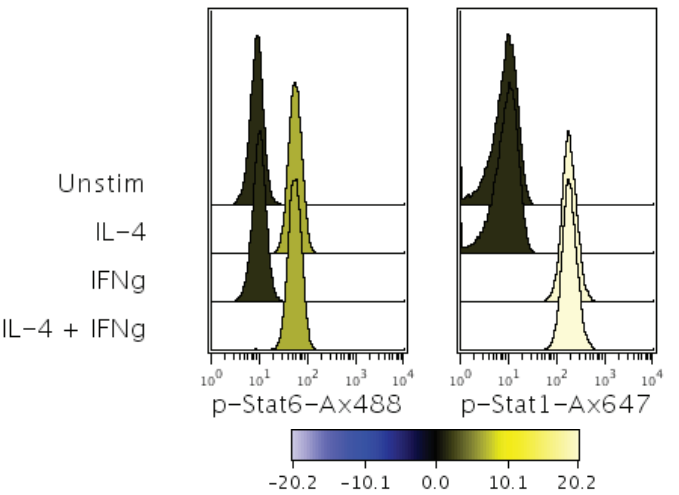
9. Add 20 µL of pStat1-Ax647 and 20 µL of pStat6-Ax488 antibodies and vortex to mix.

   Titrate phospho antibodies for optimal signal to noise ratios between unstimulated and stimulated samples.

10. Incubate cells at room temperature for 30 minutes to 1 hour in the dark. Wash the cells twice in staining media, as in step 7. Analyze by flow cytometry.

Expected Results

The expected results are shown in the Figure below. This figure was generated in Cytobank software, with histograms colored according to the fold change in phosphorylation relative to unstimulated.



Interact with sample data from this experiment at:
https://www.cytobank.org/cytobank/experiments/61

(If you are not yet registered to use Cytobank software, register and use it for free at www.Cytobank.org)

References

Schulz KR, Danna EA, Krutzik PO, Nolan GP. Single-cell phospho-protein analysis by flow cytometry. *Curr Protoc Immunol* (2007) Chapter 8: Unit 8.17.

Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry* (2003). 55(2):61-70.