Ex vivo stimulation and IFN-gamma staining

Cell culture:

1. Isolate lymphocytes from spleen and lymph nodes by mashing the organs through a 100µm cell strainer, and performing the red blood cell lysis.
2. Count your cells and plate 1 x 10^6 cells per well in a 48 well plate in 500µL culture medium. Culture medium is RPMI 1640 (10%FCS, 50µg/ml Pen/Strep, 1x Minimum essential amino acids (Gibco), 1x Sodium Pyruvate (Gibco), 0,05mM β-mercaptoethanol).
3. The medium should contain PMA 10ng/ml (Sigma Aldrich) and Ionomycin 1µM (Sigma Aldrich) for cell activation.
4. Incubate for 4 hours in a 37ºC incubator in 5% CO_2 and 99% humidity. At the 2h time point add 10µg/ml Brefeldin A (Sigma Aldrich).

Notes: You can scale how many cells depending on your needs. Don't forget to include a non-stimulated negative control.

Always spin the cells in 300g for 5mins.

FACS staining

1. Harvest the cells and move them in mini FACS staining tubes, then fill in with Sterile PBS untill the volume of 1ml.
2. Spin at 1500rpm, 4ºC, 5mins and re-suspend in 100µL L/D blue "UVB" (Life technologies). Remember to make a Live-Dead sample for compensation. Also work on ice at all times. (L/D blue is diluted 1:1000 in PBS, to make new stock dilute in 50µL DMSO).
3. Incubate in the fridge for 30mins.
4. Add 500µL ice cold PBS in each tube and spin at 1500rpm, 4ºC, 5mins.
5. Discard supernatant and re-suspend in 100µL extracellular antibody mix 1:1000. (Don't forget the single stains).
6. Incubate for 15mins at 4ºC in the dark.
7. Add 900µL of ice cold PBS in each tube and repeat the wash as in step 4.
8. Discard supernatant and re-suspend in 250µl of Fixation-Permeabilization solution (Miltenyi biotec) per tube. (From the Miltenyi FoxP3 staining buffer set)
9. Incubate in the fridge for 30mins.
10. Wash 1 time with 500µL of the 1x permeabilization buffer (10x diluted in MQ water) (Spin as in step 4.)
11. Stain with intracellular antibody: 100µl per tube IFN-gamma PE (ex: XMG1.2 eBioscience) antibody 1:400 in Perm buffer. Incubation 30min in the fridge.
12. Wash with 500µl of 1x permeabilization buffer by spinning as in step 4.
13. Re-suspend in 200µL of 1x permeabilization buffer. It is stable for at least three days in the fridge.