General overnight staining protocol

Protocol date: 13 February 2020

Written by: Oliver Burton

Reagents required:

FACS buffer: 1 liter 1x PBS, 25ml FCS, 2ml 5% sodium azide (toxic!). Store at 4C.

Tissue Harvest buffer: 1 liter 1x PBS, 25ml FCS, 4ml 0.5M EDTA. Store at 4C.

1x eBioscience Foxp3 Fix/Perm buffer: prepare as needed just prior to use by mixing 1 part fix concentrate plus 3 parts diluent.

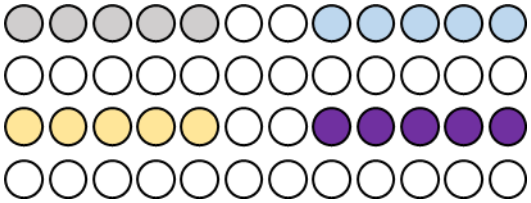
1x eBioscience permeabilization buffer: 5ml 10x buffer concentrate, 45ml ddH_2O. Store at 4C.

2.4G2 hybridoma supernatant. Store at 4C.

Fixable viability dye eFluor780 (aliquots at -80). Thaw just before use.

Stage 1: Surface staining

1. Prepare cell suspensions, lyse red blood cells if present. In order to retain CD62L, we recommend using the EDTA-containing Tissue Harvest buffer.
2. Count cells on Countess, adjust cell concentration to 2x10^7/ml in FACS buffer.
3. Plate cells in a V-bottom (conical) 96-well plate. You will need one well per sample. Plate the samples in order to minimize cross-contamination between sample types. For example, in the following plate, four types of samples are separated by empty wells.



4. Add 100ul 2.4G2 Fc block to the plate and transfer to the fridge while you prepare the staining mix. Incubate for at least half an hour for best results.
5. Collect plate from the fridge, transfer to a refrigerated centrifuge, and spin for 5min at 4C at 400g.
6. If you have administered a biotinylated anti-CD45 to your samples, first stain with Streptavidin-Qdot545 and viability-eFluor780 in 100ul FACS buffer (no 2.4G2).
7. Incubate 15min at RT in the dark (drawer).
8. Using a multi-channel pipette, add 150ul cold FACS buffer.
9. Centrifuge.
10. Flick out the supernatant into the sink.
11. Add 100ul surface stain to all wells.
12. Gently pipette up and down once using a multi-channel pipette. Do not cross-contaminate wells or introduce bubbles. Check that the cell pellets have been fully resuspended.

13. Incubate 60min at 4C in the dark (fridge).
14. Using a multi-channel pipette, add 150ul cold FACS buffer.
15. Centrifuge.
16. Flick out the supernatant into the sink.
17. Using a multi-channel pipette, add 200ul cold FACS buffer.
18. Gently pipette up and down once using a multi-channel pipette. Do not cross-contaminate wells or introduce bubbles.
19. Centrifuge.
20. Flick out the supernatant.

***Note**: In this protocol, I recommend using lots of tips to resuspend the samples at each wash step. The point of this is to break up the cell pellet so that you get effective washing and even staining. However, if you position the multi-channel directly aligned with the wells, then the ejected volume usually disrupts the pellet thoroughly without requiring the tips to contact the cells. In this case, you can reuse the tips, so you only need a couple boxes rather than 20-30. This takes practice. Done incorrectly, the cells can jump out of the wells, or you will have uneven staining. If you do this, you must examine the plate (hold it up to the light) to check for any visible clumps, and those clumps have to be dispersed.*

Stage 2: Fixation

1. Using a multi-channel pipette, add 200ul eBioscience Foxp3 fix/perm buffer.
2. Gently pipette up and down once using a multi-channel pipette. Do not cross-contaminate wells or introduce bubbles. Check that the cell pellets have been fully resuspended.
3. Incubate 30min at 4C in the dark with a lid.
4. Centrifuge.
5. Flick out the supernatant.

Stage 3: Intracellular staining

1. Using a multi-channel pipette, add 200ul cold 1x eBioscience permeabilization buffer.
2. Gently pipette up and down once using a multi-channel pipette. Do not cross-contaminate wells or introduce bubbles.
3. Centrifuge.
4. Flick out the supernatant.
5. Repeat steps 1-4.
6. Prepare intracellular staining cocktail. Dilute the antibodies in 1x eBioscience permeabilization buffer with 20% 2.4G2 supernatant added. You will need 100ul stain per well, but prepare a 20% excess.
7. Mix wells up and down gently with the multi-channel pipette.
8. Cover the plate with the lid, and place in the fridge for overnight incubation.
9. The next morning, add 120ul cold 1x eBioscience permeabilization buffer.
10. Gently pipette up and down once using a multi-channel pipette. Do not cross-contaminate wells or introduce bubbles.
11. Centrifuge.
12. Flick out the supernatant.
13. Repeat steps 10-13 using 200ul cold 1x eBioscience permeabilization buffer.
14. Add 200ul cold FACS buffer to all wells. Gently pipette up and down once using a multi-channel pipette. Do not cross-contaminate wells or introduce bubbles.
15. Centrifuge.

16. Flick out the supernatant.
17. Resuspend in 200ul FACS buffer. For experiments involving tissue preps, add 10ul CountBright beads (vortex before using) to the FACS buffer prior to adding to your cells.
18. Filter through Nytex immediately prior to acquiring or while acquiring the previous sample.

Overnight surface staining protocol

Protocol date: 31 January 2020

Written by: Oliver Burton

Prepare single cell suspension.

Count cells. Plate 2x10^6 or appropriate number in V96-well plate.

Block 30min with 100ul 2.4G2.

Wash with 130ul FACS buffer.

Stain 20min at RT with e780 at 1:4000 (and streptavidin-Qdot545 at 1:1000) in FACS buffer (no 2.4G2).

Wash with 130ul FACS buffer.

Stain overnight at 4C in 100ul antibody cocktail prepared in at least 20% 2.4G2.

For best results, stain in DMEM with 5-10% FCS.

Wash with 130ul FACS buffer.

Wash with 200ul FACS buffer.

Resuspend in 200ul FACS buffer containing 10ul counting beads (if desired).

Filter before acquiring.