A. Morton / Nov 2010

CBDM Laboratory

BrdU incorporation and detection *in vivo* protocol 022410

Method

A. BrdU injections

1. Inject *n* mice with 1.6 mg BrdU in 200 µl i.p. (stock 20 mg/ml, 1:2.5 dilution = 8 mg/ml => 1.6 mg/200 µl).
2. 10-12 hrs later inject same mice with 1.2 mg BrdU in 200 µl i.p. (1:3.33 dilution of 20 mg/ml stock).

B. BrdU detection – Day 1

3. 24 hrs later sac and **perfuse (40 ml PBS/mouse)** mice **+ ctrl uninjected mouse** and harvest pancreata, PLN, CLN, thymi & spleen. **Make sure to pick pancreas clean of all LN.**

NB. Process all tissues in the same manner (cutting with scissors into 1 mm pieces and placing in digestion buffer etc).

4. Prepare fresh digestion buffer (collagenase IV @ 1 mg/ml + DNAseI @ 10 U/ml + ~1% BSA).
    - Weigh out 0.05g collagenase IV (Sigma # C5138) and dissolve in 50 ml DMEM.
    - Add 1 ml DNAseI (Sigma # DN25) from -20 frozen stock (1 mg/ml) to 50 ml collagenase.
    - Add ~1 ml 35% BSA (Sigma # A7409).
5. Prepare single cell suspensions by cutting up tissues using scissors (1 mm pieces of pancreas) and placing into freshly prepared digestion buffer as follows:
    - Pancreas – 25 ml in 50 ml tube
    - Thymus - 1 ml in 1.5 ml eppendorf tube
    - LN – 0.5 ml in 1.5 ml eppendorf tube
6. Digest for 20 min at 37^0C at 150 rpm in a shaking waterbath.
7. Filter digest (blue cell strainer (BD with 40 µm nylon) into fresh 50 ml tube. Undigested pieces, smash through blue cell strainer.
   Add DMEM up to 50 ml.
8. Centrifuge at 1400 rpm for 10 min.
9. During spin, process rest of samples and add 1 ml ACK lysis buffer to spleen samples for 2 min at RT.
10. Resuspend pancreas pellet in 2 ml DMEM and pass through Nitex into eppendorf tube.
11. Centrifuge all samples at 1400 rpm for 5 min.
12. Resuspend in appropriate Ab or control substance.
13. Incubate for 20 min on ice.
14. Add 1 ml DMEM/EDTA per tube and centrifuge at 1400 rpm for 5 min.

Proceed directly to step # 17 if no 2^0 Ab stain required.

15. Resuspend apt samples in 2^0 Ab if required and incubate on ice for 10 min.
16. Add 1 ml DMEM/EDTA per tube and centrifuge at 1400 rpm for 5 min.

Intracellular BrdU staining:

17. Resuspend cells in 100 µl eBioscience fix buffer (cat # 00-5123-43; diluted 1 part stock : 3 parts diluent in eBioscience dilution buffer (cat # 00-5223-56) for 30 min at room temp or on ice.
18. Wash cells in BD Perm/Wash buffer (cat # 51-2090KZ (554722); dilute stock 1:10 in dH_20 for use) & resuspend pellet in 300 µl 1X BD CytoFix/CytoPerm buffer (cat # 51-2091KZ (554723)).
19. Incubate **overnight** at 4^0C.

Day 2

20. Wash x 1 in 200 µl Perm/Wash buffer.
21. Resuspend in 100 µl **1% formaldehyde/0.5% Tween-20/PBS** for 10 min on ice**.**
22. Wash x 1 in 200 µl Perm/Wash buffer.
23. Re-fix in **BD Fix/Perm** buffer for 5 min.
24. Wash x 1 in 200 µl Perm/Wash buffer.
25. Resuspend in 100 µl of **BD DNAse** (cat # D4513) & incubate for 20 min (10 min for PLN) at 37^0C (*make up DNAse **immediately** prior to use. Each stock vial contains 300 µl of a 1 mg/ml soln of DNAse in DPBS. When staining 10 or more samples, thaw entire vial and add 700 µl DPBS (or PBS w/o Ca^{2+} or Mg^{2+}) to make working solution of 300 µg/ml. Unused **stock** DNAse can be refrozen **once**)*.
26. Wash x 1 in 200 µl Perm/Wash buffer.
27. Resuspend in 50 µl Perm/Wash buffer containing α-BrdU-APC mAb & incubated for 45 min at 4^0C.
28. Wash x 1 in 1 ml Perm/Wash buffer & resuspended in 300 µl DMEM/tube. *Alternatively, resuspend cells in 50:50 mix of DMEM/1% formalin/PBS if not running cells right away.*
29. Run samples on LSRII.