Appendix C: Bronchial Biopsy Dissociation and Sorting Protocol

Bronchial Biopsy Dissociation and Sorting Protocol

MATERIALS

- DPBS (no calcium, no magnesium) (Gibco 14190-250 or Corning CellGro #21-031-CV)
- FBS (ThermoFisher 10438018)
- HEPES 1M (Gibco 15630-080)
- UltraPure™ 0.5M EDTA (Gibco 15575)
- RPMI- no phenol red (ThermoFisher 11835055)
- Complete RPMI - with 10% FBS (filtered).
- Falcon 70um cell strainers (Falcon 352350)
- Petridishes 60mm (any brand)
- 3-ml syringe plunger with flat back (or equivalent device)
- Liberase TL Research Grade (Roche), (Millipore Sigma 05401020001)– reconstitute 1 vial with 300 uL sterile H_2O. Per biopsy need 50 uL Liberase in 2 ml RPMI. Aliquot 50 uls and keep at -20C.
- 5mL sterile polystyrene round-bottom culture tubes with cap (VWR 60818-565).
- Filter bottles (0.2 um), sterile, any brand or style to fit the volume.
- Sort buffer and Wash buffer:

Sort Buffer (store at 4 °C)
PBS (Ca/Mg free)
5mM EDTA
25mM HEPES
1% FBS
0.2um filter sterilize

Wash Buffer (store at 4 °C)
PBS (Ca/Mg free)
5mM EDTA (5 ml/ 500 ml)
25mM HEPES (12.5 ml / 500 ml)
0.2um filter sterilize

Cell Staining

Note: the fluorochrome combination is dependent on your instrument configuration so these choices are just for guidance. Do not omit anti-CD235a.

- APC anti-human CD235a (Glycophorin A) antibody (BioLegend 306607) – Lot B242428. Titrated use: Dilute 1:100 to make working stock, from this use 5 ul per sample (<5 million total cells). If the sample appears bloody and a lot of RBCs are visible in the pellet, double or triple the amount.
- APC-Fire750 anti-human CD326 (EpCAM) (BioLegend 324234) – Lot B223438. Titrated use: Dilute to ¼ to make working stock, from this use 5 ul per sample (or use 1.25 ul directly).
- BV510 Anti-Human CD45 (BD Horizon 563204) – as titrated (lots vary).
- DAPI (Sigma Cat. #D9542) 0.5 ug/ml in ddH_2O.
- Filter cap FACS tubes (BD Falcon 352235).

Cell Sorting

- NP-40 collection solution for RNA plates:
  - 1% NP-40 SurfactAmps™ Detergent Solution (ThermoFisher 85124, 10% stock)
  - 5% RNaseOut™ Recombinant Ribonuclease Inhibitor (ThermoFisher 10777019, 5000U/vial, 40U/ul, 125ul/vial).
  - UltraPure™ DNase/RNase-free Distilled Water (ThermoFisher 10977023)
  - Mix: 60 ul NP-40 solution + 30 ul RNaseOut + 510 ul UPwater (600 ul total) to fill two 96-well plates using 2 ul/well. For each additional plate use 200 ul more (a trough needs ~200 ul dead volume with this solution, 150 ul is usually not enough).
- REPLI-g Single Cell Cryo-Protect Reagent for DNA plates (Qiagen 150370).
- Trizole (or Qiazole) or equivalent.
- MicroAmp® Optical 96-Well Reaction Plate with Barcode (ThermoFisher 43-067-37, originally Applied Biosystems).
- Microseal 'F' foil seals (Biorad MSF1001, rated to -70C).

Equipment

- Cell sorter (BD FACSAria II) with Diva 8.0 (capable of index sorting) – or equivalent
- Mini Plate Spinner (Fisher Scientific #14100143)
- Multichannel pipettor (p50 or smaller, accurate in range 2-10 ul).
- Dry ice

PROCEDURE

Sample Collection:

- Collect sample in 1-2 mL of RPMI in 1.5mL cryovial. * Post-collection, if waiting is required before sample prep (1-4 hours), keep sample at 4 °C. Samples can be stored overnight IF the cryovial is filled up to the top with complete medium (RPMI/10% FBS added to the existing medium) immediately upon receipt. This may not work for very small biopsies.

Sample Dissociation:

1. Remove most of the storage medium from the biopsy in the original cryovial (be careful not to remove pieces of biopsy) and transfer to a clear tube (i.e. 5ml polystyrene FACS tube) using a P1000. Inspect the solution and put any visible not-bloody clumps back into the original vial. If any clumps are mainly bloody, they can/should be added to this tube.
2. Transfer the solid biopsy pieces to another clear tube with 1 ml RPMI (no FBS). Use some of the RPMI to wash out the original cryovial to make sure there are no more fragments in the vial. Pipet up and down to wash the biopsy of loosely associated blood cells, but make sure that the biopsy does not get lodged in the pipet tip. Then transfer all medium but not the biopsy pieces to the other clear tube that contains the storage medium. This together is called the “Wash”.
3. Add 1 ml **Liberase** in RPMI to the tube with the biopsy pieces.
4. Using the same P1000, gently transfer the biopsy pieces in Liberase to a 15 ml tube labeled with the sample number and “BT” (for Biopsy Tissue).
5. Incubate in waterbath at 37C for 20 mins – swirl up every few minutes (original protocol states to use a shaking waterbath but this works too).
6. Meanwhile, spin the “Wash” tube (all spins 1500 rpm ~400 g x 5 min at RT).
7. Remove most of the sup from the “Wash” tube by aspiration, add 1 ml Liberase in RPMI, resuspend, and transfer to a second 15 ml tube labeled with the sample number and “W” (for Wash). Also place this tube in the waterbath for the remainder of the original 20 minutes (usually 10-15 minutes).
8. After incubation, add 10 ml Complete RPMI (with serum) to both tubes, mix gently by inversion.