Preparation of primary lung cancer cells

Reagent:

Dispase/Collagenase IV solution:

Dissolve 1gram of Dispase II in 100ml HBSS. Dissolve 0.2 gram of Collagenase IV to 10ml PBS. Dilute even further with the culture medium to a final concentration of 1U/ml. Sterilize through a 0.22um filter membrane. Aliquot to 1ml tubes, store at -20C.

10x Dispase/Collagenase is in Neil's box

Deoxyribonuclease I solution

Get aliquot from Nico. 0.5mg/ml DNase, 10mg/ml BSA in Hams F12

Labeling buffer (same as Separation buffer)

PBS supplemented with 2mM EDTA and 0.5% biotin-free BSA. Keep buffer cold. Remove any air bubbles before use.

Red Blood Lysis Buffer

Ammonium chloride lyse (10X concentration), NH4Cl (ammonium chloride) 8.02gm, NaHCO3 (sodium bicarbonate) 0.84gm, EDTA (disodium) 0.37gm

QS to 100ml with Millipore water. Store at 4C for six months.

Working solution: Dilute 10ml 10X concentrate with 90 ml Millipore water. Refrigerate until use.

DMEM Culture medium

DMEM + 10% FBS + PS

Preparation of the Mouse Lung

1. Sacrifice the mouse by CO_2 asphyxiation. **Note**: Do not perform cervical dislocation as this will injure the trachea so that following steps of the protocol, such as installation of the lung with liquid, cannot be performed successfully. Ensure loss of nociceptive reflexes.
2. Spray the mouse with ethanol and make a long cut along the ventral midline of the body. Pull the ventral fur and skin and subsequently also carefully cut and remove the peritoneum.

3. Exsanguinate the mouse by cutting the left and right jugular vein (*Vena jugularis*) as well as the renal artery (*Arteria renalis*). Remove flowing blood with tissue. Only remove the surface blood, don't perfuse the animal.
4. Carefully puncture and then remove the diaphragm to expose the heart and lung by cutting away the ribs. Take special care not to injure the lung.
5. Cut and remove the salivary glands to expose the trachea. Also carefully cut the muscle surrounding the trachea.
6.

Enzymatic Digestion of the Lung tumor tissue

7. Cut out the whole lung, wash with PBS twice on ice.
8. Isolate individual tumors under the stereoscope, trim away the surrounding normal tissue. Put each tumors into 15ml Falcon tubes with 2ml PBS on ice. Number the tumors based on their size (large -> small: T1, T2…). Check metastases on the dorsal side of the lung, isolate mets before starting cutting out the primary tumors.
9. Cut the lung tissue into small pieces on ice using scissors, add 200ul 10x Dispase/Collagenase in 15ml Falcon tubes, incubate for 60 min at 37C on TC shaker. Check until the edge of the tissue piece start to become fuzzy.
10. Add 2ml of PBS + 1 mM EDTA to stop digestion. (*EDTA stops the protease reaction as it is a collagenase inhibitor*)
11. Use the syringe with 19G needle to completely homogenize the tissue.
12. Filter the cell suspension through nylon meshes with 40µm pores. Take care to rinse each mesh as well as the tubes and dishes thoroughly with DMEM to minimize loss of cells and maximize yield. Renew the filter in case of clogging.
13. Depending on the volume, transfer the filtrate to one or more 50 ml tubes and centrifuge for 15 min at 250 x g at 4 °C. Remove the supernatant.
14. Supernatant is discarded. Pellet containing cells is incubated for 10 min at RT with complete media plus DNaseI (final concentration 10 µg/ml).
    Note: Often as a result of cell damage, DNA leaks into the dissociation medium increasing viscosity

and causing handling problems. Purified DNase is included in cell isolation procedures to digest the nucleic acids without damaging the intact cells.

15. Samples are centrifuged at 300 *x g* for 5 min at at 4 °C.
16. Resuspend the cell pellet in 2 ml 2xRBC Lysis buffer (room temperature) for 5 minutes and quickly terminate lysis by addition of 13 ml of DMEM medium. Take 100ul for cell counting to estimate the cell number. Transfer the solution to a 15 ml tube and centrifuge for 12 min at 250 x g and 4 °C.

Isolate Tumor associated fibroblast

17. Media is removed and cells are resuspended in complete media and seeded in a dish (2ml medium/each well, 6-wellplate). Place samples in the incubator.
18. After 30 min, media in all samples is replaced with fresh complete media and place back in the incubator.

    Note: After 30 min, most of the fibroblasts would have adhered to the dish, whilst other cell types will remain in suspension. Using this tip will help you enrich for the fibroblastic population.
19. After 2-3 days, isolated cells can be enriched for the fibroblastic population using the enhanced adherence to plastic of fibroblasts.

    - Media is removed.
    - Cells are washed with PBS once.
    - Cells are covered with Trypsin/EDTA and placed in the incubator for 10 min.
    - Cells are resuspended in complete media, seeded into a new dish and placed back in the incubator for 30 min.
    - Media is replaced with fresh new complete media.

Select EpCAM+ epithelial tumor cells by Flow Sorting

Contact Hector hectorno@berkeley.edu in advance to schedule sorting time. He has the parameter I used for sorting.

1. Collect the supernatant from 17 above which contains the tumor cell, determine the cell number
2. Centrifuge the supernatant at 300 *x g* for 5 min at at 4 °C. Aspirate supernatant completely.

3. Resuspend up to 10^6 nucleated cells per 45 µL of buffer.
4. Add 5 µL of the EpCAM-PE antibody (CD326 (EpCAM)-PE, mouse, clone: caa7-9G8, Miltenyi)
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2−8°C).
   Note: Higher temperatures and/or longer incubation times may lead to nonspecific cell labeling. Working on ice requires increased incubation times.
6. Wash cells by adding 1−2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in 1ml buffer for analysis by flow cytometry or fluorescence microscopy. Pass the cells through 5ml tube with cell-strainer cap to get rid of cell clumps (Falcon, 352235). Transfer the single cell suspension to 5ml PP tube for FACS (Falcon, 352063), keep the sample on ice covered with foil.
8. For RNA sample collection, sort EpCAM+ cells directly into commercial Trizol.

Selection of EpCAM+ Epithelial tumor cells using MACS beads

1. Collect the supernatant from 17 above which contains the tumor cell, determine the cell number
2. Centrifuge the supernatant at 300 x g for 5 min at at 4 °C. Aspirate supernatant completely.
3. Make labeling solution by mixing 10ul anti-EpCAM-biotin (CD326 (EpCAM)Biotin, 130-102-033, Miltenyi.) and 100 ul labeling buffer. Cell number can range from 10^5 to 10^8 cells/test. Keep a small cell aliquot for FACS
4. Resuspend cell pellet with the antibody solution and keep on ice for 10 minute.
5. Wash cells to remove unbound primary antibody by adding 2ml of labeling buffer per 10^7 cells and centrifuge at 300 x g, 4C for 10 minutes. Aspirate supernatant completely.
6. Repeat washing step once.
7. Resuspend cell pellet in 90ul labeling buffer per 10^7 cells. Add 10ul of Streptavidin microbeads per 10^7 total cells.
8. Mix well and refrigerate for 15 minutes.
9. Wash cells by adding 1-2 ml of labeling buffer per 10^7 cells and centrifuge at 300xg for 10 minutes, aspirate supernatant completely.

10. Resuspend up to 10^8 cells in 500ul separation buffer.
11. Place MS column in the magnetic field of a MACS Separator. Prepare the column by rinsing with 500ul separation buffer.
12. Apply cell suspension onto the column.
13. Collect the unlabeled cells that pass through and wash the column with 3x500ul separation buffer. Only add new buffer when the column reservoir is empty.
14. Remove column from the separator and place it on a collection tube.
15. Pipette 1ml of separation buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column and collect the cells in 1.5 ml Eppendorf tube. The cells can be cultured directly in DMEM medium.
16. If the cells will be used for RNA-seq, spin down the cells 2000 RPM 5min 4C, aspirate the supernatant and resuspend the cell pellet in 1ml commercial Trizol.