

## **FACS cell prep for lungs—improved for isolation of AT2 cells and BASCs**

Carla Kim, Children's Hospital Boston

Updated 5/21/07

*Notes—this protocol may limit the number of Clara or other bronchiolar cells you get due to the agarose part; use of LMP agarose exposes the alveolar cells to the collagenase/dispase, improving AT2 cell yield; collagenase/dispase may cleave certain surface antigens, thereby limiting detection*

BEFORE BEGINNING SURGERY, you will need:

- Avertin (1X stock in 4°C under bench)
- Forceps, surgical scissors, pins, dissecting platform (Styrofoam lid wrapped in foil, covered with a paper towel)
- Cold PBS
- 10, 3, and 1 mL syringes
- 20G needles
- 10% Formalin (RT)
- Dispase (aliquots in -20°C; **\*\*THAW IN 50°C WB, remove immediately to ice once thawed\*\***)
- 1% LMP Agarose (RT, **\*\*THAW IN 50°C WB, leave in bath until ready to use**)
- Collagenase/dispase (-20°C below bench)
- DNase (-20°C below bench; 1% solution; check unit activity of each lot number)
- PF10 (make 50 mL by adding 5 mL FBS (4°C below bench) to 45 mL PBS)
- 100 and 40  $\mu$ m filters (above bench)
- Bucket with ice
- 50 mL conical tubes

- 1) Anesthetize mouse with avertin, test with forceps (toe pinch) to make sure under enough
- 2) Spray down mouse with 70% ethanol
- 3) Quickly cut into ribcage, perfuse 10 mL of Hank's balanced salt solution or PBS (ice cold) through right ventricle until lungs cleared of blood (cut a slit in left ventricle to allow blood to leave)
  - Use a butterfly needle and 10 mL syringe
- 4) Cut out heart to euthanize mouse

**\*\*If saving tissue for histology, dissect out about 1/3 of the left lobe (below the primary bronchus), place into a dish, inject ~200  $\mu$ L formalin directly into the tissue, and place into 50 mL tube with formalin\*\***

- 5) Expose trachea, place forceps under trachea to keep exposed
- 6) Inject dispase (BD, undiluted liquid, aliquots in -20°C) into trachea (go between the cartilage rings) just until the lungs inflate—you should be able to do this with less than 2 mL

- 7) Follow with tracheal injection of 0.5-1 mL of 1% LMP agarose (made in H<sub>2</sub>O)
  - Use a 20G needle to make sure the agarose goes through nicely; use a *different needle for each mouse*
- 8) Place a few pieces of ice on lungs, leave everything there for a few minutes as you prep needles, etc. for the next mouse
- 9) Dissect out the lungs by gently tugging on the trachea while snipping away the connective tissue; leave lungs intact and place in Petri dish on ice with PBS
  - Until ready to process/digest, keep all lungs on ice or proceed to step 9
- 10) Trim other tissues off lung, dissect off each lobe, and mince lung tissue into small pieces inside of a 50 mL conical tube. For each lung sample, add 6 mL PBS.
  - You may leave lung tissue on ice while dissecting other mice and before proceeding to digestion; if you do this, add 1 mL PBS
- 11) Add 120  $\mu$ L collagenase/dispase (from Roche, 100 mg/mL in H<sub>2</sub>O stock stored in -20°C below bench; powder in cold room) to PBS; rotate 45 min @ 37°C; final collagenase/dispase concentration is ~2 mg/mL
- 12) Place digested tissue on ice. Add 15  $\mu$ L DNase per 6 mL of digest solution (from 1% stock stored in -20°C, which is 10 mg/mL); final DNase concentration is ~0.025 mg/mL
- 13) Invert several times to mix. Leave on ice to cool for no more than 5 minutes.
- 14) Into a 50 mL tube, filter digested tissue through a 100  $\mu$ m filter, then a 40  $\mu$ m filter
- 15) Spin 6 min @ 800 rpm in Beckman centrifuge
- 16) Discard supernatant; resuspend pellet in 2 mL PF10 (10% FBS in PBS) and count/stain cells OR proceed to RBC lysis protocol if you need to eliminate RBCs

### Red blood cell lysis

- 1) Resuspend cell pellet from above in 1 mL of lysing solution (0.15 M NH<sub>4</sub>Cl, 10mM KHCO<sub>3</sub>, 0.1 mM EDTA, in 1L distilled H<sub>2</sub>O; filtered with 0.45  $\mu$ m filter and stored at RT)
- 2) Lyse 90 sec at RT
- 3) Add 6 mL of DMEM without serum (refrigerator below bench); mix
- 4) Add 0.5 mL FBS slowly to bottom of tube by inserting pipet tip all the way through the resuspended cell solution
- 5) Centrifuge undisturbed layers for 6 min @ 800 rpm

6) Aspirate the supernatant; resuspend in PF10 for counting or staining

### Surface staining

- 1) Stain 1 million cells in 100  $\mu$ L PF10 (~500  $\mu$ L to 1000  $\mu$ L per mouse) for 15 minutes with appropriate amount of antibody on ice; touch spin in microcentrifuge for ~10 sec, aspirate supernatant, wash with ~3x volume of stain solution PF10, touch spin, aspirate sup, resuspend in 300  $\mu$ L PF10 for FACS
  - Repeat for secondary antibody if necessary

\*Use single-stained controls for every antibody you use and a negative control (no staining) for every mouse/genotype/situation for which you will sort cells. This is for compensation. Cells for these should come from a control sample; dilute the original sample such that after 100  $\mu$ L aliquots are placed into each control tube, 500  $\mu$ L of the sample remain for staining.

- Stain with Sca-1 (usually FITC conjugated), CD45 Biotin (note it will depend on the strain of mouse which CD45 to use; for 129 and B6 strains, use CD45.2), Pecam Biotin, SA-PE (secondary), then 7AAD (see protocol below) to exclude dead cells
- Ab dilutions:
  - 1° Sca-1-FITC  $\rightarrow$  1:100
  - 1° CD31 (Pecam)-Biotin  $\rightarrow$  1:100
  - 1° CD45-Biotin  $\rightarrow$  1:100
  - 2° SA-PE  $\rightarrow$  1:200 (i.e. 2.5  $\mu$ L in 500  $\mu$ L)
  - may be better to use SA-APC instead of PE for better compensation (PE and FITC are more difficult to compensate than APC and FITC)

### 7AAD staining

- 1) Resuspend the cells in appropriate amount of PF10. Add 7AAD so that it is 1:300 dilution.

(7AAD comes as a powder from Molecular Probes in Oregon, A-1310, resuspend in 500  $\mu$ L DMSO (this makes a 2 mg/mL stock), store in freezer and thaw before each use)

- 2) Incubate cells on ice for 15 minutes
- 3) Touch spin, wash as before
- 4) Resuspend cells in PF10 for sorting
- 5) Filter the cell/PF10 suspension through the blue (40 $\mu$ ) filters, either directly into FACS tubes or into 50mL conical and then transfer to appropriate tube for FACS. (depends on the density of your cells)(be sure to use the #2063 tubes for Moflos, doesn't matter for Aria). This is very important so that you won't clog the FACS machine!

\*if you are just looking for GFP+ cells, you don't have to do any staining—just count and put approximately 1 million cells per 300 uL PI solution or PF10 solution for FACS

\*keep cells on ice as much as possible; bring tubes with media/solution you want to collect sorted cells into to the FACS facility

### Cell Population Isolation

For **BASCs** (Bronchio-alveolar stem cells), sort the Sca-1+ CD45- Pecam- population; this population also contains ciliated cells

For **clonal assays** (use of single cells), use Sca-1+ CD45- Pecam- and CD34+ cells (just add CD34 staining into the mix above)—this will enrich for your ability to isolate single cells capable of giving rise to colonies.

For **AT2 cells**, sort the Sca-1- CD45- Pecam- population that has high autofluorescence in FITC channel (in your negative control, you should be able to see 2 populations falling out—highly autofluorescent (still live) and low autofluorescent

### Sorting cells

For cultures, you can sort directly onto a plate or into a tube and then plate by hand

For sorting into tubes, prepare collection tubes ahead of time:

3% BSA in PBS, made fresh or filtered, fill up the collection tube and let stand at least one hour (or overnight in 4°C). Aspirate this solution, add about 200 uL of media into which to sort the cells.

\*\*\*It seems very important to reduce the pressure setting on the FACS machine for BASCs to grow. We have been using a Moflo sorter, 30Psi with success. Other machines/pressure settings have not yet been verified. When you make the FACS appointment, you must request the pressure setting to be lower, they cannot just change it at your appointment.

### Cytospin

Sorted or cultured cells can be stained after cytopspin at 600 rpm 3 min, fixation and permeabilization with CytoFix/CytoPerm (Pharmingen) for 20 min at 4°C (if on matrigel, fix at RT). Followed by washing in PBS/0.2% Tx-100, 3 x 5 min.; blocking and primary antibody. (\*\*See immunofluorescence protocol)

### Epithelial cultures

Cells can be plated in DME+/HEPES/10% FBS/Pen-Strep/L-glutamine on 96-well plates coated with 100  $\mu$ L Matrigel (for differentiation)(Becton Dickinson) or irradiated DR4 MEFs (for maintenance of undifferentiated state, colony formation assays, etc.). For Matrigel, best to plate at least 10,000 cells per 96-well; for feeders, plate 1000 cells per 96-well unless for limiting dilution. Coat the 96-well with 100  $\mu$ L Matrigel that has been thawed on ice, place plate in incubator for 20 minutes (don't do more or less time, important for the Matrigel to solidify correctly), then pipette the cell mixture on top of the Matrigel.

Cells do not do well on larger well formats, perhaps unless same cell density is maintained.

---

*Technique was based on:*

Bortnick, A.E., Favari, E., Tao, J.Q., Francone, O.L., Reilly, M., Zhang, Y., Rothblat, G.H., and Bates, S.R. (2003). Identification and characterization of rodent ABCA1 in isolated type II pneumocytes. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 285, L869–L878. *Epithelial Cultures*

Kim, C.F., Jackson, E.L., Woolfenden, A.E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R.T., and Jacks, T. (2005). Identification of Bronchioalveolar Stem Cells in Normal Lung and Lung Cancer. *Cell* 121, 823-835.