Chapter 10

Well-Differentiated Primary Mammalian Airway Epithelial Cell Cultures

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Abstract

Well-differentiated primary airway epithelial cell (AEC) cultures have been widely used for the characterization of several human respiratory viruses including coronaviruses. In recent years, there has been an increase in interest toward animal AEC cultures and their application to characterize veterinary viruses with zoonotic potential, as well as studying host–pathogen interactions in animal reservoir host species. In this chapter, we provide a revised and improved protocol for the isolation and establishment of well-differentiated AEC cultures from diverse mammalian species and the use of the cultures for the characterization of veterinary coronavirus. We also describe immunohistochemistry protocols with validated antibodies for the visualization and identification of viral cell tropism in well-differentiated AEC cultures from human, swine, bovine, and feline origin.

Key words Veterinary coronavirus, Animal airway epithelial cell cultures, Cell tropism, Virus characterization

1 Introduction

Coronaviruses are important respiratory pathogens that can cause a large variety of diseases in mammalian species including humans, bovine, porcine, feline, and nonmammalians such as avian species. In humans, coronavirus infections are mainly associated with the common cold, however more recently also with several outbreaks of life-threatening respiratory infections, as illustrated by Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) cases [1, 2]. In addition to humans, coronaviruses are also notable for causing respiratory illnesses in animals, as in the case of porcine respiratory coronavirus (PRCoV) [3, 4], bovine respiratory coronavirus (BCoV) [5, 6], and feline coronavirus (FCoV) [7]. From a genetic perspective, many human and animal coronaviruses share a high degree of similarity; for instance, BCoV and human coronavirus OC43 (HCoV-OC43) share a nucleotide identity of 96%
Moreover, it is evident that some of the emerging human coronaviruses originate from animal coronaviruses that crossed the species barrier [9, 10]. This explains the increasing interest in veterinary coronavirus infections and efforts to study the pathogenesis of coronavirus in the authentic animal hosts.

One of the most commonly used in vitro platforms to study coronavirus infections is well-differentiated airway epithelial cell (AEC) cultures grown at an air–liquid interface. These organotypic cell cultures recapitulate many aspects of the respiratory tract, including airway cell heterogeneity, mucus production, ciliary beating activity, and the dynamics of the innate immune system toward pathogens. In the veterinary research field, there is an increasing demand for the use of more physiologically relevant systems in the study of veterinary pathogens. Hence, the availability of animal AEC cultures for the study of animal respiratory pathogens, especially coronaviruses, is highly desirable.

Previously our group has described a protocol to establish human AEC cultures and its use for human coronavirus replication studies [11]. In this chapter, we revise and adapt the previous version for the establishment of AEC cultures from various animal species. We outline optimized protocols on how to generate AEC cultures from bovine, porcine, and feline species, which have been successfully cultivated and are in use for veterinary coronavirus studies in our lab (see Figs. 1 and 2). We highlight that these methods are versatile and can be easily adapted for other animal species. In addition, we describe an updated immunofluorescence protocol with validated antibodies to visualize virus in the animal AEC cultures and to identify coronavirus tropisms. Other virus detection methods that are useful for virus detection such as quantitative reverse transcriptase (RT-qPCR) and plaque assays are not discussed in this chapter as they have been previously described [11]. Hopefully, these techniques will provide a more realistic model for coronavirus pathogenesis and host–virus interaction studies in the veterinary field and also contribute to reducing animal use (3Rs principle) in the veterinary virology field.

2 Materials

2.1 AEC Cultures

1. Whole tracheobronchial tissue resections for the isolation of primary tracheobronchial epithelial cells from mammals can be obtained postmortem, whereas for human origin, this can also be done from partial tracheobronchial tissue resections or bronchoscopy samples. It is mandatory that the procurement of the tissue material is in accordance with local ethical guidelines.
2. Isolating/transfer medium: minimum essential medium (MEM), 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

3. Sterile phosphate-buffered saline (PBS).

4. 10× Digestion solution: 1% (w/v) protease from *Streptomyces griseous* type XIV, 0.01% deoxyribonuclease I from bovine pancreas in isolating/transfer medium.

5. Bronchial epithelial growth medium (BEGM): LHC basal medium, supplemented with the required additives (*see* Tables 1 and 2).

6. Air–liquid interface (ALI) medium: LHC basal medium and Dulbecco’s modified MEM (DMEM) mixed in a 1:1 ratio, supplemented with the required additives (*see* Tables 1, 2, and 3).

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**Fig. 1** Schematic representation of the establishment of mammalian AEC cultures. Chronological representation of the different procedural steps for the establishment of AEC cultures from mammalian tracheal/bronchial tissue material that can be used for the characterization of respiratory coronaviruses. The experimental analysis shows a three-dimensional projection of Z-stacks from human AEC infected with HCoV-229E expressing GFP (green) for 48 h at 33 °C, and the visualization of nuclei (blue), tight junctions (yellow), and cilia (red).
7. 0.4 μm Pore polyester membrane inserts (diameter 6.5 mm) and 24-well cluster plates.

8. 3 mg/ml Collagen type I and III solutions from human fibroblasts.

9. 0.5 mg/ml Collagen type IV from human placenta. Reconstitute 10 mg in 5 ml filter-sterilized water with 0.25% acetic acid. Dissolve for a few hours at 37 °C, occasionally swirling. Once dissolved, add sterile water containing 0.25% acetic acid to the total volume of 20 ml, mix gently by pipetting. Filter-sterilize the solution through a 0.22 μm polyethersulfone (PES) filter, and store at −20 °C in aliquots of 800 μl per microfuge tube. The stock solution is stable for at least 1 year at −20 °C.

10. TEER solution: dissolve 4.5 g NaCl, 91.89 mg CaCl₂, and 1.194 g of HEPES in 400 ml of distilled water and stir for 10 min at room temperature. Add water up to 500 ml and sterilize the solution via autoclaving, 20 min at 121 °C.

11. Dissection sets: forceps, tweezers, scissors (see Note 9).

12. Cover sponge/drape.

13. Rocking platform/tube roller or magnetic stirrer.
Table 1
Common stock additives for BEGM and ALI medium

| Component                          | Stock concentration | Comment                                      |
|-----------------------------------|---------------------|----------------------------------------------|
| Bovine serum albumin (BSA)        | 300×, 150 mg/ml     | See Note 1                                   |
| Bovine pituitary extract (BPE)    | 1000×, -14 mg/ml    |                                              |
| Insulin                           | 2000×, 10 mg/ml     | Store at 4 °C                                |
| Transferrin (TF)                  | 1000×, 10 mg/ml     | Dissolve in PBS                              |
| Triiodothyronine (T3)             | 1000×, 0.067 mg/ml  | Dissolve in 1 mM sodium chloride (NaOH)      |
| Epinephrine (EP)                  | 1000×, 0.6 mg/ml    | Dissolve in 10 mM hydrochloric acid (HCl)    |
| Phosphorylethanolamine (PE)       | 1000×, 70 mg/ml     | Dissolve in PBS                              |
| Ethanolamine (EA)                 | 1000×, 30 μl/ml     | Dissolve in PBS                              |
| Stock 11 (S11)                    | 1000×, 0.863 mg/ml  | See Note 2                                   |
| Stock 4 (S4)                      | 1000×               | See Note 3                                   |
| Trace elements (TR)               | 1000×               | See Note 4                                   |
| Penicillin/streptomycin (P/S)     | 100× 10,000 U/ml penicillin and 10.000 μg/ml of streptomycin | Store at 4 °C |

All additives should be aliquoted and stored at −20 °C unless stated otherwise

Table 2
Stock additives for BEGM and ALI medium added differently (see Note 5)

| Component                          | Stock concentration | BEGM | ALI | Comment                                      |
|-----------------------------------|---------------------|------|-----|----------------------------------------------|
| Epidermal growth factor (EGF)     | 1000×–50,000×, 25 μg/ml | 25 ng/ml | Vary | Dissolve in 0.25 mM HCl                      |
| Hydrocortisone (HC)               | 720×–1000×, 0.48 μg/ml | 0.48 μg/ml | Vary | Dissolve in PBS                              |
| Retinoic acid (RA)                | 720× to 1000×, 5 × 10⁻⁵ M | 50 nM | Vary | See Note 6                                  |
| Y-27632                           | 2500×, 20 mM        | Yes  | No  |                                              |
| Isoprenaline                      | 333,000×, 100 mM    | Yes  | No  | Dissolve in sterile water                    |
| A83-01                            | 10,000×, 10 mM      | Yes  | No  |                                              |
| Primocin™                         | 500×, 50 mg/ml      | Yes  | No  | See Note 7                                  |
| DAPT                              | 1000×, 5 mM         | No   | Vary|                                              |

All additives should be aliquoted and stored at −20 °C unless stated otherwise
Table 3
Optimized EGF, RA, HC, and DAPT concentration in the ALI medium for differentiation of AEC cultures from several species (see Note 8)

| Components | Stock concentration | Human | Bovine | Porcine | Feline |
|------------|---------------------|-------|--------|---------|--------|
| EGF        | 25 µg/ml            | 0.5 ng/ml | 25 ng/ml | 25 ng/ml | 25 ng/ml |
| RA         | 5 × 10⁻⁵ M          | 50 nM | 50 nM | 70 nM | 50 nM |
| HC         | 0.48 mg/ml          | 0.48 µg/ml | 0.48 µg/ml | 0.072 µg/ml | 0.072 µg/ml |
| DAPT       | 5 mM                | No    | 2.5 µM | No      | No     |

2.2 Propagation of Coronaviruses from Mammalian Origin
1. Apical wash solution: Hank’s Balanced Salt Solution (HBSS), without calcium and magnesium.
2. Virus transport medium (VTM): MEM, 25 mM HEPES-buffered, 0.5% gelatin (see Note 10), 100 U/ml penicillin, and 100 µg/ml streptomycin.
3. Aerosol barrier pipette tips and 1.5 ml locking lid microfuge tubes.

2.3 Immuno-fluorescence Analysis
1. Fixation solution: 4% formalin solution, neutral buffered.
2. Confocal staining buffer (CB): 50 mM ammonium chloride (NH₄Cl), 0.1% saponin, and 2% IgG and protease-free BSA dissolved in 500 ml of PBS (pH 7.4). Filter-sterilize (0.2 µm PES filter) solution, prepare aliquots of 40 ml, and store at −20 °C (see Note 11).
3. Primary antibodies: see Table 4.
4. Secondary antibodies: see Table 5.
5. Fluorescent DNA dyes: 4′,6-diamidino-2-phenylindole (DAPI) 1 mg/ml (1000×).
6. Wash solution: PBS, pH 7.4, without calcium and magnesium.
7. Scalpel (No.10).
8. Rat-tooth forceps.
9. Aluminum foil or nontransparent plastic container.
10. Microscope slides and coverslips.
11. Hard setting mounting medium.
12. Gyro rocker.
### Table 4
List of example primary antibodies

| Antibody                  | Target                  | Dilution | Host    | Clone     | Supplier        | Species reactivity |
|---------------------------|-------------------------|----------|---------|-----------|-----------------|-------------------|
| β-Tubulin IV              | Cilia                   | 1:100    | Rabbit  | EPR16775  | Abcam           | H, B, P, F        |
| β-Tubulin                 | Cilia                   | 1:100    | Rabbit  | Clone 9F3 | Cell signaling  | H, B, P, F        |
| ZO-1                      | Tight junctions         | 1:100    | Mouse   | Clone 1A12| Thermofisher    | H, B, P, F        |
| Cytokeratin-5             | Basal cells             | 1:100    | Rabbit  | EP1601Y   | Abcam           | H, P              |
| Uteroglobin               | Club cells              | 1:200    | Rabbit  |           | Abcam           | H, P              |
| Muc-5 AC                  | Goblet cells            | 1:200    | Rabbit  | EPR16904  | Abcam           | H, P              |
| Vimentin                  | Non-epithelial cells    | 1:200    | Mouse   | RV202     | Abcam           | H                 |
| dsRNA                     | dsRNA                   | 1:500–1000 | Mouse   |           | Scicons         |                   |
| CD13                      | CD13/APN                | 1:200    | Sheep   |           | RnDsystems      | H                 |
| CD26                      | CD26/DPP4               | 1:100    | Goat    |           | RnDsystems      | H                 |
| CD26                      | CD26/DPP4               | 1:100    | Mouse   | OTI11D7   | Origene         | H, B              |
| ACE2                      | ACE2                    | 1:200    | Rabbit  |           | Abcam           | H                 |
| Intravenous immunoglobulins (IVIG) | Viral proteins | 1:1000 | | | | |

*H* human, *B* bovine, *P* porcine, *F* feline

Antibodies listed used successfully in our laboratory. Alternative antibodies and suppliers may be used after validation.

### Table 5
List of example secondary antibodies

| Antibody  | Conjugate     | Dilution | Host | Supplier               |
|-----------|---------------|----------|------|------------------------|
| Anti-mouse| Alexa Fluor® 488 | 1:400    | Donkey | Jackson ImmunoResearch  |
| Anti-goat | Alexa Fluor® 647 | 1:400    | Donkey | Jackson ImmunoResearch  |
| Anti-rabbit| Alexa Fluor® 594 | 1:400    | Donkey | Jackson ImmunoResearch  |
| Anti-human| Cy3           | 1:400    | Donkey | Jackson ImmunoResearch  |

Antibodies listed are used successfully in our laboratory. Alternative antibodies and suppliers may be used after validation.
3 Methods

Unless stated otherwise, carry out all the procedures in a biosafety cabinet according to local biosafety regulations.

3.1 AEC Cultures

3.1.1 Collagen Coating of Cell Culture Flasks for Cell Expansion

Cell culture flasks are coated with a mixture of type I and III collagen from fibroblasts. This is necessary to promote attachment and proliferation of primary AECs.

1. Use filter-sterilized water (0.22 μm PES membrane) to prepare a 1:75 dilution of collagen type I and III stock.
2. Use 6 ml per 75 cm², distribute the collagen solution by gently shaking the flask to make sure the entire surface is covered.
3. Incubate for 2 h at 37 °C or overnight at room temperature.
4. Aspirate remaining liquid and wash twice with 10 ml of PBS to remove traces of acetic acid.
5. Culture flasks can be directly used. Optional: store coated flasks at 4 °C for a maximum of 6–8 weeks.

3.1.2 Collagen Type IV Coating of Inserts

The inserts need to be coated with collagen type IV, which will support the development and long-term maintenance of differentiated AEC cultures.

1. Prepare 1:10 collagen solution by mixing 7.2 ml of filter-sterilized water with 800 μl of collagen type IV solution stock.
2. Apply 50 μl of diluted collagen type IV per insert. After completing one plate, make sure that the entire surface of each well is covered.
3. Incubate the plates for 2 h at 37 °C.
4. To remove traces of acetic acid, wash inserts twice with 200 μl of PBS.
5. After these steps, coated inserts can be used directly. Optional: store at 4 °C for a maximum of 6–8 weeks.

3.1.3 Isolation of Primary Tracheal and/or Bronchial Cells

Isolation of primary epithelial cells from tracheobronchial tissue resections from animals can be done in the same manner as for primary epithelial cells of human origin.

1. Transport of the tracheal/bronchial tissue resections should be done in isolating/transport medium. The tissue should be kept on ice to prevent degradation and to minimize the bacterial growth (see Note 12).
2. Isolation can be done in a flow cabinet or workbench in the Biosafety Level 2 (BSL-2) lab. Prepare a sterile working environment cleaned with 70% ethanol. Subsequently, cover the surface with a large sterile cover drape/sponge. For large animal tissues, first cut the large tissue into 5–10 cm segments.
3. Transfer the tracheal/bronchial tissue to a large petri dish and remove all excess of connective tissue, fat, and lymph nodes.

4. Wash the cleaned tissue three times with PBS.

5. Before the digestion step, take a sample of the trachea for histological analysis. Cut a 0.5 cm segment of the trachea tissue and fix the segment with 40 ml of fixation solution overnight. Wash the fixed tissue three times in PBS. For long-term storage, this sample can be stored in PBS at 4 °C.

6. Fill the desired number of sterile containers (50 ml tube for small trachea tissue or 1 L wide neck glass bottle with screw cap for large trachea tissue) with isolating/transfer medium up to 60% of its capacity (30 ml or 600 ml). Transfer as many tissue segments as possible into a single container, until the volume reaches 36 ml or 720 ml, respectively. Then add 10% (v/v) of 10 x digestion solution to each tube/bottle, to a final volume of 40 ml or 800 ml, respectively.

7. Place 50 ml tubes on a rocking platform/tube roller at 4 °C and incubate for 48 h (see Note 13). For the trachea tissue of large animals, place the 1 L bottles on magnetic stirrers with a low speed in a 4 °C cold room.

8. Place the 50 ml tube/1 L bottle containing the digested tissue on ice and add heat-inactivated FBS to each tube to a final concentration of 10% (v/v), to neutralize the protease activity. Invert tubes/bottle three times.

9. Pour solution along with the tissue into a large petri dish. Cut open the trachea cartilage side and gently scrape off the epithelium from the collagen–cartilage surface, using a scalpel in a parallel angle to the surface. Pool solutions containing dissociated cells into a 50-ml conical tube and wash the petri dish once with PBS to collect remaining cells.

10. Centrifuge for 5 min at 500 g. Wash cells once with HBSS and resuspend cells in warm BEGM to a concentration of, approximately, 5 x 10^6 cells/ml.

11. If not expanded directly, the cells can be cryopreserved for long-term storage.

3.1.4 Maintenance of AEC on Plastics for Expansion

1. To generate passage 1 and 2 cell lineages for subsequent subculture on porous supports, primary cells can be plated in BEGM on collagen-coated flasks at a density of 1–3 x 10^6 cells per 75 cm^2.

2. Change the growth media 24 h after seeding to remove red blood cells and any unattached epithelial cells. First, remove the old medium and wash the cell monolayer twice with 12 ml of HBSS. Lastly, add 15 ml of pre-warmed BEGM to the monolayer.
3. Incubate cultures at 37 °C, humidified with 5% CO₂.
4. BEGM growth media should be changed every 2–3 days until the cells reach 80–90% confluence.

3.1.5 Subcultivation of Primary Epithelial Cells on Porous Inserts for Differentiation

To generate differentiated AEC culture, the expanded AECs can be seeded on collagen type IV-coated porous supports once the cells in the collagen-coated flask reach 80–90% confluence.

1. Collect medium and wash the cell monolayer twice with 12 ml of HBSS.
2. Dissociate the cells with 5 ml of trypsin–EDTA solution per T75 cell culture flask and incubate the cells for 5–10 min at 37 °C, in a humidified 5% CO₂ incubator (see Note 14).
3. Pellet cells in previous collected medium by centrifugation for 5 min at 500 × g.
4. Remove supernatant and wash cells once with 25 ml of HBSS.
5. Pellet cells by centrifugation for 5 min at 500 × g.
6. Remove supernatant and resuspend the cell pellet in 6 ml of BEGM medium.
7. To generate differentiated AEC cultures, seed cells at a density of 3.3–8.25 × 10⁵ cells per cm², which is equivalent to 1.0–1.5 × 10⁵ cells in 200 μl per 6.5 mm insert. Count cells with trypan blue method and dilute the cells accordingly.
8. Fill the basolateral compartment of the culture plates with 500 μl of BEGM medium and transfer 200 μl of the diluted cell suspension to the upper chamber of the collagen-coated inserts. The cells are now in liquid–liquid interface.
9. Incubate cultures at 37 °C, in a humidified 5% CO₂ incubator.
10. The next day, change the media. Changing the media should be done in the following order: remove the medium in the apical compartment to remove any unattached cells. Wash the apical surface with 200 μl HBSS. Next, remove the old medium from the basolateral side. Apply 500 μl of pre-warmed BEGM medium to the basolateral side. Lastly, apply 200 μl of pre-warmed BEGM medium on the apical side.
11. Change the BEGM medium every 2–3 days until the cells reach confluence on the inserts (see Note 15).
12. Monitor the resistance of the cells using a Volt/Ohm meter for tissue culture. To measure the resistance, add 200 μl of TEER solution to the apical sides. Once the resistance reaches 500 Ω cm², replace BEGM with ALI medium.
13. Different animal species require a different proportion of several components necessary for differentiation. Adjust the concentration of several additives in the ALI medium as listed in
Table 3. Keep the cells at liquid–liquid interface in ALI medium for at least 2 days.

14. To establish air–liquid interface, aspirate the apical side medium, and wash the surface twice with HBSS. Remove the basolateral medium and add 500 μl of pre-warmed ALI (see Note 16).

15. To prevent acidification, the basolateral medium should be changed every 2–3 days.

16. The mucus, although protecting the cells, can accumulate over time and then can affect the cells negatively. Therefore, the apical surface should only be washed with HBSS every 7–14 days to maintain the homeostatic balance of the mucus and cells in the ALI. Differentiation of the animal AEC cultures can be assessed by the cilia appearance, mucus production, and the stability of the measured TEER resistance. After 3–4 weeks, depending on the donor, the well-differentiated animal AEC cultures are ready to be used for further experiments.

3.2 Virus Propagation

1. Wash the apical surface of the culture twice with 200 μl of HBSS solution prior to inoculation with the veterinary coronavirus specimen to remove excess mucus.

2. Dilute the clinical material or virus supernatant in HBSS and inoculate 100 μl dropwise to the apical surface and incubate for 2 h at either 33 °C or 37 °C (see Note 17), in a humidified 5% CO₂ incubator. Optional: centrifuge inoculum solution for 4 min at 1500 × g at room temperature to remove cell debris prior to inoculation.

3. Collect the inoculum, transfer it to a container and store at −80 °C for later analysis. Optional: transfer the collected inoculum into an equal volume of VTM.

4. Wash the apical surface three times with 100 μl HBSS. Incubate the infected cultures for the desired amount of time at the appropriate temperature in a humidified 5% CO₂ incubator, e.g., 48 h at 33 °C.

5. Apply 100 μl of HBSS dropwise to the apical surface 10 min prior to the desired collection time and incubate in the humidified 5% CO₂ incubator. After the 10 min incubation, collect the apical wash containing progeny virus and transfer it to a container. Store at −80 °C for later analysis. Optional: transfer the collected progeny virus into an equal volume of VTM.

6. Wash the apical surface once with 200 μl of HBSS solution.

7. To quantify the cellular viral RNA yield, apply a total of 350 μl of lysis buffer to the cells, divided over two steps, and incubate for 10 min at room temperature. Collect the cell lysate and store it at −80 °C if not analyzed directly.
3.3 Immuno-fluorescence Analysis

All incubation steps are performed at room temperature on a gyro rocker (20–30 rpm), unless stated otherwise.

1. After the apical wash containing progeny virus has been collected, the apical surface needs to be washed twice with 200 μl of HBSS (see Subheading 3.2, step 6), prior to cell fixation with formalin solution for later immunofluorescence analysis.

2. Apply 200 μl of 4% formalin solution to the apical compartment and 500 μl to the basolateral. Incubate for 15–30 min.

3. Remove the formalin solution and wash both compartments three times with equal volumes of PBS. This material can also be used for histology analysis together with the previously fixed ex vivo tissue (see Note 18).

4. Transfer the fixed AEC cultures to a new 24-well cluster plate.

5. Discard washing solution and apply 200 μl and 500 μl of confocal buffer (CB) solution to apical and basolateral compartments, respectively.

6. Incubate fixed cultures for 30–60 min to block non-specific binding of antibodies (see Note 19).

7. Remove the CB solution from the apical and basolateral compartments.

8. From this stage, one should only apply CB solution to the apical compartment.

9. Wash the apical surface once with 200 μl of CB solution for 5 min.

10. Apply primary antibodies (see Table 4) diluted in 50 μl CB solution dropwise to the apical surface and incubate for 120 min.

11. Wash the apical surface three times with 100 μl of CB solution for 5 min.

12. Apply the appropriately diluted conjugated secondary antibodies (see Table 5) in 50 μl CB solution dropwise to the apical surface and incubate for 60 min. From this step, cover the plates with aluminum foil to prevent bleaching of the fluorophores (see Note 20).

13. Wash the apical surface twice with 100 μl of CB solution for 5 min.

14. Incubate cells with nucleic acid counter stain solution (DAPI) diluted in 50 μl of CB solution for 5 min.

15. Wash the apical surface once with 100 μl of CB solution for 5 min.

16. Lastly, wash the apical surface twice with 100 μl of PBS for 5 min to remove residual saponin and restore cell membrane integrity.
17. Before removing the washing solution, apply mounting medium on a glass slide (use 1–2 drops). Remove any air bubbles.

18. Excise the membrane from the plastic holder and carefully place the basolateral side of the membrane on top of the mounting medium, without generating air bubbles.

19. Then slowly add one drop of mounting medium on top of each membrane.

20. Slowly place the coverslip, in a tilted fashion, on top of the membrane without generating air bubbles.

21. Allow the mounting medium to cure overnight at RT, after which the slide can be analyzed. At all times protect the slides from direct sunlight exposure, and keep at 4 °C for long-term storage.

4 Notes

1. Dissolve 5 g of BSA, globulin-free, powder in 20 ml PBS in a 50 ml tube (do not vortex). Place the tube on a shaker/roller bank for 2–4 h (max 24 h) at 4 °C, until BSA is completely dissolved. Add the volume up to 34 ml, mix gently by inverting the tube three times. Filter-sterilize the solution through a 0.22 μm PES filter, and store at −20 °C in aliquots of 3.5 ml in 15 ml tubes. Invert the tube three times before usage.

2. Dissolve 43.2 mg zinc sulfate in 50 ml water. Filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 1100 μl per microfuge tube.

3. Dissolve 42 mg ferrous sulfate heptahydrate, 12.2 g magnesium chloride hexahydrate, and 1.62 g calcium chloride dihydrate in 80 ml water, add 0.5 ml concentrated HCl, and fill up to 100 ml. Filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 1100 μl per microfuge tube.

4. Prepare seven separate 25 ml stock solutions (see Table 6) in water. Filter-sterilize (0.22 μm) each component after preparation. Afterwards, transfer from each separate component an aliquot of 50 μl in 49.6 ml filter-sterilized water (0.22 μm) and add a volume of 50 μl concentrated HCl solution. Mix the solution well through gentle vortexing, filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 1100 μl per microfuge tube.

5. Y-27632 and A83-01 are small molecules that act as a Rho-kinase and TGF-β pathway inhibitors, respectively. These molecules have been shown to enhance the proliferation of primary cells [12]. Dissolve 10 mg Y-27632 in 1.56 ml filter-
sterilized water and store at −20 °C in aliquots of 250 μl per microfuge tube. To make the A83-01 stock, dissolve 10 mg A83-01 in 2.37 ml DMSO. Store the stock at −20 °C in aliquots of 100 μl per microfuge tube. DAPT is a Notch pathway inhibitor molecule that has been shown to promote ciliary differentiation of epithelial cells [13]. DAPT stock should also be dissolved in DMSO. Add 2.31 ml DMSO to 5 mg DAPT. Store the DAPT stock at −20 °C in aliquots of 100 μl in dark microfuge tubes.

6. We recommend using EC23, a stable retinoic acid receptor ligand, to replace the commonly used all-trans retinoic acid in the BEGM and ALI medium. Prepare the stock solution by dissolving 25 mg EC23 in 2.5 ml dimethyl sulfoxide (DMSO). Store this stock at −20 °C in aliquots of 100 μl in 1.5 ml tubes. To prepare the 1000× stock, dilute 100 μl of EC23 stock in 60 ml of PBS containing BSA 7.5 mg/ml. The 1000× stock solution should be stored at −20 °C in aliquots of 1 ml per microfuge tube without the need to protect from light.

7. Additional antibiotics are important for the first 5 days after plating to minimize microbial contamination. Thereafter, the media should only be supplemented with P/S as prolonged use of the antibiotic combination can interfere with primary cell differentiation.

8. The concentration of the additives in the ALI medium has been optimized to generate animal AEC cultures with 40–80% of ciliated cells after 4 weeks of differentiation. To define the optimum conditions for other animal species, test several dilutions of EGF, HC, RA, and DAPT in the ALI medium.

9. Isolation of primary tracheal/bronchial cells from large animal such as bovine requires a larger size of dissection kit set.
10. Prepare a 10× stock solution of 5% gelatin by dissolving 5 g gelatin in 100 ml of distilled water. Autoclave the solution for 30 min at 121 °C. Cool down the 10× solution to RT before mixing with other VTM components.

11. Alternatively, autologous commercially available serum can be used although not recommended, due to the presence of potential virus-directed IgGs.

12. One can acquire tracheal/bronchial tissues from other geographical locations. For shipment, the tissue should be kept in isolating/transport medium in a sealed container and packed in an insulated box with cold packs.

13. For the trachea tissue of small animals (e.g., cats), protease/DNAse treatment can be reduced to 24 h.

14. Cells might take longer to dissociate from the bottom of the flask due to the collagen coating. If the cells are not dissociated after 5–10 min, additional rounds of 1 min incubations can be performed until all cells have detached.

15. The seeded primary cells in BEGM should reach confluence on the inserts within 1 week. If this takes longer, the success rate of establishing well-differentiated AEC cultures declines exponentially.

16. If the day after no leakage of medium to the apical side is observed, the cells can be maintained at air–liquid interface. Otherwise, put the cells back to liquid–liquid interface for another day. If at some point the cell layers form holes or a relatively large scratch is accidentally introduced during washing, the cells can be put back at liquid–liquid interface.

17. The temperatures of 33 °C and 37 °C are the incubation conditions of the upper and lower respiratory tract of humans, respectively. However, the incubation temperature can be adapted as some animals have a higher body and respiratory tract temperature.

18. For morphological comparison, vertical sections of both fixed animal ex vivo tissue and AEC culture from the same animal can be stained with hematoxylin and eosin using standards histological techniques. Sectioning 10–20 μm thickness of fixed AEC inserts is recommended to prevent the detachment of the insert membrane from the cells.

19. Formalin-fixed AEC cultures can be kept for 1–3 months at 4 °C if the CB is filter-sterilized (0.2 μm), and all the procedures are performed under sterile conditions. After storage, it is preferential to acclimatize the fixed cultures for 15 min to room temperature on a gyro rocker (20–30 rpm) prior to continuation of the staining protocol.
20. Alternatively, already conjugated primary antibodies for immunofluorescence staining are commercially available. In this case, **steps 12 and 13** can be omitted from the immunofluorescence protocol.

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