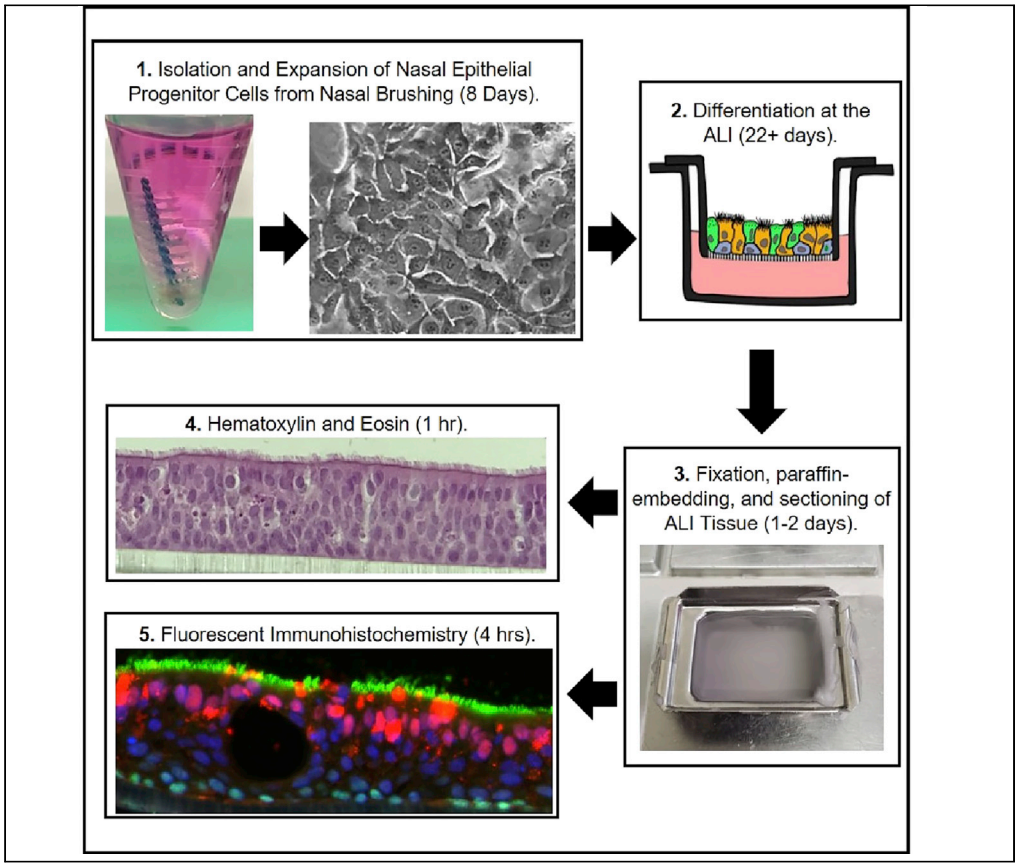
STAR Protocols

CellPress
OPEN ACCESS

Protocol

# Isolation, expansion, differentiation, and histological processing of human nasal epithelial cells




This protocol is intended as a guide for implementing or refining the usage of the air-liquid interface (ALI) model system to generate airway mucociliary tissue *in vitro*. We present a streamlined protocol for isolating the stem cells from inferior nasal turbinates of donors, allowing for a simple and low-cost supply of primary cells for research. We also provide our detailed protocols for ALI tissue processing and immunofluorescence to aid in the standardization of these techniques between research groups.

Vincent Manna, Salvatore Caradonna

MannaV6@Rowan.edu (V.M.)
Caradonn@Rowan.edu (S.C.)

## Highlights

Rapid isolation of human nasal epithelial cells via nasal brushing

Generation of airway mucociliary tissue *in vitro* using the air-liquid interface

Detailed description of formalin fixation and paraffin embedding procedures

Antigen retrieval and immunohistochemistry of *in vitro*-generated airway tissue

Manna & Caradonna, STAR Protocols *2*, 100782 December 17, 2021 
https://doi.org/10.1016/j.xpro.2021.100782

STAR Protocols

CellPress
OPEN ACCESS

Protocol

# Isolation, expansion, differentiation, and histological processing of human nasal epithelial cells

Vincent Manna[1,2,3,*] and Salvatore Caradonna[1,*]

[1]Department of Molecular Biology, Graduate School of Biomedical Sciences and School of Osteopathic Medicine, Rowan University, Stratford, NJ, 08084, USA

[2]Technicial contact

[3]Lead contact

*Correspondence: MannaV6@Rowan.edu (V.M.), Caradonn@Rowan.edu (S.C.)
https://doi.org/10.1016/j.xpro.2021.100782

## SUMMARY

**This protocol is intended as a guide for implementing or refining the usage of the air-liquid interface (ALI) model system to generate airway mucociliary tissue *in vitro*. We present a streamlined protocol for isolating the stem cells from inferior nasal turbinates of donors, allowing for a simple and low-cost supply of primary cells for research. We also provide our detailed protocols for ALI tissue processing and immunofluorescence to aid in the standardization of these techniques between research groups.**

**For complete details on the use and execution of this protocol, please refer to Hussain et al., (2014) Yang et al., (2016) Im et al., (2019).**

## BEFORE YOU BEGIN

Protocols for the isolation of human nasal epithelial cell (HNEC) specimens from human subjects require approval by the Institutional Review Board.

### Isolation and expansion of nasal epithelial cells from nasal turbinates

Timing: 20–30 min

1. Pre-warm a water bath or heating block to 37°C.
2. Prepare PneumaCult-Ex plus medium (STEMCELL Technologies inc, Vancouver, Canada) as per manufacturers protocol, supplement with Penicillin (100 I.U./mL), Streptomycin (100 μg/mL) and Amphotericin B (2.5 μg/mL) (Antibiotic Antimycotic Solution 100×, VWR, Radnor, PA).
3. Bring the Animal Component-Free (ACF) Cell Dissociation kit (STEMCELL) solutions to 22°C–25°C before usage.
4. Warm PneumaCult-Ex plus medium to 37°C before usage.

### Differentiation at the ALI

Timing: 20–30 min

5. Prepare complete PneumaCult-ALI differentiation media (STEMCELL) as per manufacturers protocol (ALI Basal Medium with ALI 10× Supplement and 100× ALI Maintenance Supplement). Supplement complete ALI medium with Penicillin (100 I.U./mL), Streptomycin (100 μg/mL), and Amphotericin B (2.5 μg/mL).
6. Media and PBS should be warmed to 37°C before usage.

### Fixation, paraffin-embedding and sectioning of ALI tissue

Timing: 20–30 min

7. Prepare molten paraffin at 55°C–60°C.
8. Prepare containers for xylene and graded alcohols (70%, 80%, 95%, 100%) for washes.
9. Pre-warm a water bath to 55°C–60°C for floating histological sections.

### Rehydration of ALI4 tissue

Timing: 10–15 min

10. Prepare slide containers with xylene and graded alcohols (70%, 80%, 95%, 100%) for washes.
11. Pre-warm heat block to 55°C–60°C.

### Antigen retrieval and fluorescent immunohistochemistry of ALI tissue sections

Timing: 20–30 min

12. Prepare blocking solution of 5% BSA in TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween-20), 100 μL/section.
13. Prepare primary antibody in 1% BSA in TBST (100 μL/section).
14. Prepare secondary antibody in 1% BSA in TBST (100 μL/section).
15. Prepare antigen retrieval buffer.
16. Prepare a humidification chamber for slides.
    a. Soak a sponge in water and place into an open container.
    b. Place container with sponge in a secondary container that can seal.
    c. The sealed secondary container will act as a humidification chamber, slides should be placed inside this container but not inside the sponge container.
17. Devise a way to block light from entering the humidification chamber, we recommend molding aluminum foil over the outside of the chamber.

### Hematoxylin and eosin staining of ALI tissue sections

Timing: 10–15 min

18. Prepare slide container with graded alcohols, hematoxylin and eosin.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Anti-rabbit IgG (H+L) F(ab')2 Fragment (Alexa Fluor 488 Conjugate) #4412, working dilution 1:1000 | Cell Signaling Technology | Product#4412S |
| P63 Polyclonal antibody, working dilution 1:100 | Proteintech | Cat#12143-1-AP |
| CoraLite488-conjugated acetylated tubulin(Lys40) monoclonal antibody, working dilution 1:3000 | Proteintech | Cat#CL488-66200 |
| Uteroglobin/CC10 Polyclonal antibody, working dilution 1:300 | Proteintech | Cat#10490-1-AP |
| MUC5AC Monoclonal Antibody (45M1), working dilution 1:400 | Invitrogen | Cat#MA5-12178 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| FoxJ1 Monoclonal Antibody (2A5), eBioscience, working dilution 1:100 | Invitrogen | Cat#14–9965-82 |
| Anti-mouse IgG (H+L) F(ab')2 Fragment (Alexa Fluor 594 Conjugate) #8890, working dilution 1:1000 | Cell Signaling Technology | Product#8890S |
| **Chemicals, peptides, and recombinant proteins** | | |
| Antibiotic Antimycotic Solution 100× | Corning | Ref#30-004-Cl |
| PneumaCult -Ex Plus Basal Medium | STEMCELL Technologies | Cat#05041 |
| PneumaCult -ALI Basal Medium | STEMCELL Technologies | Cat#05002 |
| DPBS/MODIFIED -Calcium -Magnesium | HyClone | Cat #SH30028.03 |
| 10% Neutral Buffered Formalin | VWR | Cat#10790-714 |
| Xylenes | VWR | Cat#MK866816 |
| Ethyl Alcohol 200 Proof | Pharmco | Cat#111000200 |
| Sodium citrate dihydrate | VWR | Cat#470302-528 |
| TWEEN 20 | Sigma Life Science | SKU P9416-100ML |
| EDTA Disodium Salt | Amresco | M101-1KG |
| Bovine Serum Albumin (BSA) | VWR | Cat#97061-420 |
| Tris Base, ULTROL Grade | EMD Millipore Corp | Cat#648311-1KG |
| ProLong Gold antifade reagent with DAPI | Invitrogen | Ref#36935 |
| Surgipath, SelecTech Hematoxylin 560 | Leica | Ref#3801570 |
| VWR Eosin | VWR | Cat#95057-848 |
| **Critical commercial assays** | | |
| Animal Component-Free Cell Dissociation Kit | STEMCELL Technologies | Cat#05426 |
| **Biological samples** | | |
| Brushing from human nasal turbinate (Our study utilized HNECs isolated from a male donor age 34) | n/a | n/a |
| **Other** | | |
| 5 mL MacroTube, Sterile | VWR | Cat#470225-020 |
| GUM Proxabrush – Moderate | Sunstar | UPC 670875250741 |
| 1000 µL Universal Pipet Tips | VWR | Cat#76322-522 |
| Tissue Culture Plate, 6-Well | Corning | Ref#353046 |
| Transwell Permeable Support 12 mm Insert, 12 Well Plate, 0.4 µm Polyester Membrane | Costar | Ref#3460 |
| Processing/Embedding Cassette w/Attached Lid | VWR | Cat#18000-130 |
| Histoplast PE Paraffin | Thermo Scientific | Cat#83-30 |
| Sterile Carbon Steel Surgical Blades, No 11 | Sklar Instruments | Manufacturer #06–3011 |
| Mega Base Molds | Sakura | Product Code 4166 |
| Cytoseal XYL | Thermo Scientific | Cat#8312-4 |
| MX35 Premier Microtome Blasé 34/80mm | Thermo Scientific | Cat#3051835 |
| SureBond Charged Microscope Slides | Avantik | SL6332 |
| Humidification Chamber (Made from plastic storage bins) | Sterilite | B01MR7L1VD |
| VWR Mini Block Heater | VWR | Cat#10153-318 |
| Revolve Fluorescent Microscope | Echo | Model: RVL-100-G |
| Tissue Floating Bath | Premiere | Model XH-1001 |
| Tissue-Tek Embedding Module | Sakura | Model: TEC 5 EM A-1 |
| Tissue-Tek Cryo Module | Sakura | Model: TEC 5 CM A-1 |
| Shandon Finesse 325 microtome | Thermo Scientific | Part No: A78100101 |
| IHC PAP (mini) pen | Enzo Life Sciences | ADI-950-232-0001 |
| Kimwipes | Kimberly-Clark Global Sales | Product Code 34120 |
| Xtreme Wear Nail Color, invisible | Sally Hansen | Item#4860-01 |
| Microscope Cover Glass, 22 × 22mm | VWR | Cat#16004-094 |

## MATERIALS AND EQUIPMENT

| Sodium Citrate Antigen Retrieval Buffer (store solution at 4°C for up to 1 month) | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Sodium Citrate | 10mM | 2.9g |
| Tween 20 | 0.05% | 0.5mL |
| $ddH_2O$ | n/a | Up to 1000mL |
| pH 6.0 | n/a | n/a |
| **Total** | **n/a** | **1000 mL** |

| TBST Buffer (store solution at 4°C for up to 1 month) | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris base | 50mM | 6.06g |
| NaCl | 150mM | 8.77g |
| Tween-20 | 0.1% | 1mL |
| $ddH_2O$ | n/a | Up to 1000mL |
| pH 7.6 | n/a | n/a |
| **Total** | **n/a** | **1000 mL** |

## STEP-BY-STEP METHOD DETAILS

### Isolation and expansion of human nasal epithelial cells (HNECs) from nasal turbinates

Timing: 8 days

In this step a donor will swab their own inferior nasal turbinate with an interdental brush and the tissue on the brush will be removed, digested with a protease solution and plated for incubation, this process will take around 30 min. The cells will then be expanded in culture over an 8-day period. Our protocol is a streamlined adaptation from previously established protocols (Hussain et al., 2014) (Yang et al., 2016).

1. Have donor blow nose to remove any excess mucous from the nasal cavity.
2. The donor should slowly insert the interdental brush into the nasal cavity, following the nostril to the inferior turbinate and gently spin the brush for 10 s.
3. Remove the brush from the handle using wire cutters and place the brush into a 5 mL Eppendorf tube containing 1 mL of ACF dissociation solution at 22°C–25°C (Figure 1).
4. Repeat steps 1–3, having the donor brush the opposite nostril, place the second brush into the same 5 mL Eppendorf tube containing the first brush.
5. Use a P1000 micropipette tip to remove the cellular material from the brush by trimming 2–3 mm from the pipette tip and inserting the cut wire end of the brush into the cut end of the pipette tip. Gently push the pipette down so the entire brush enters the pipette tip, you should see cellular material and mucous accumulate at the end of the pipette tip. Press and spin the tip against the side of the Eppendorf tube until the cellular material and mucous are suspended in ACF solution. Discard the pipette tip and brush (Figure 2).
6. Incubate the Eppendorf at 37°C for 15 min, gently flicking the tube halfway through and at the end of incubation.

⚠ CRITICAL: To avoid cell death do not vortex samples.

7. Add 1 mL of ACF inhibition solution, mix by gently inverting Eppendorf multiple times.

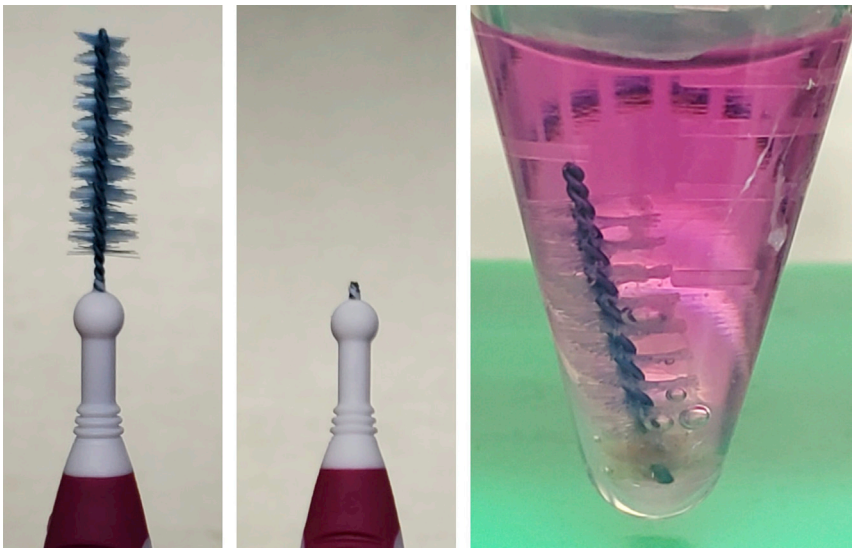


**Figure 1. Interdental brush before and after nasal brushing**
Left-to-right; Interdental brush intact, handle with brush removed, Eppendorf tube with brush submerged in ACF dissociation solution

8. Centrifuge at 22°C–25°C for 5 min at 200 × *g*.
9. Carefully remove and discard supernatant.
10. Using a P1000 pipette, resuspend pellet in 1 mL PneumaCult-Ex Plus media.

⚠ CRITICAL: Slowly pipette pellet to avoid cell death.

11. Plate cells into one well of a 6-well culture plate containing 1 mL pre-warmed complete Expansion Plus media for a total of 2 mL media.
12. Incubate at 37°C, 5% CO2. Colonies will be visible by day 2–4; if no colonies are visible by day 4 then it was likely an unsuccessful isolation (Figure 3).
13. Media should be refreshed when colonies are first observed (day 2–4), and again every two days.
14. On day 5–6 of culture the colonies must be dissociated and passaged for maximal expansion. To do so, remove media from wells and wash cells briefly with warmed PBS.
15. Apply 1 mL of ACF Dissociation Solution and incubate plates at 37°C for 6–8 min. Gently tap on plates to fully dissociate cells.
16. Apply 1 mL of ACF Inhibition solution to cells and gently swirl the plate to mix.
17. Transfer the cells to a 15 mL conical tube or 5 mL Eppendorf tube and centrifuge for 5 min at 200 × *g*.

⚠ CRITICAL: Be very gentle when transferring the cell solution and use a 1–2 mL serological pipette or slowly transfer with a P1000 micropipette.

18. Carefully remove and discard supernatant.
19. Resuspend pellet in 1 mL complete Expansion Plus media using a P1000 micropipette.

⚠ CRITICAL: Slowly pipette pellet to avoid cell death.

20. Transfer the cell solution to a T25 flask containing 2 mL of complete expansion-plus media for a total of 3 mL media. Incubate at 37°C, 5% CO2.
21. Cells should be transferred to transwell inserts after 8 days of culture to ensure maximal differentiation, collect cells by repeating steps 14–19.

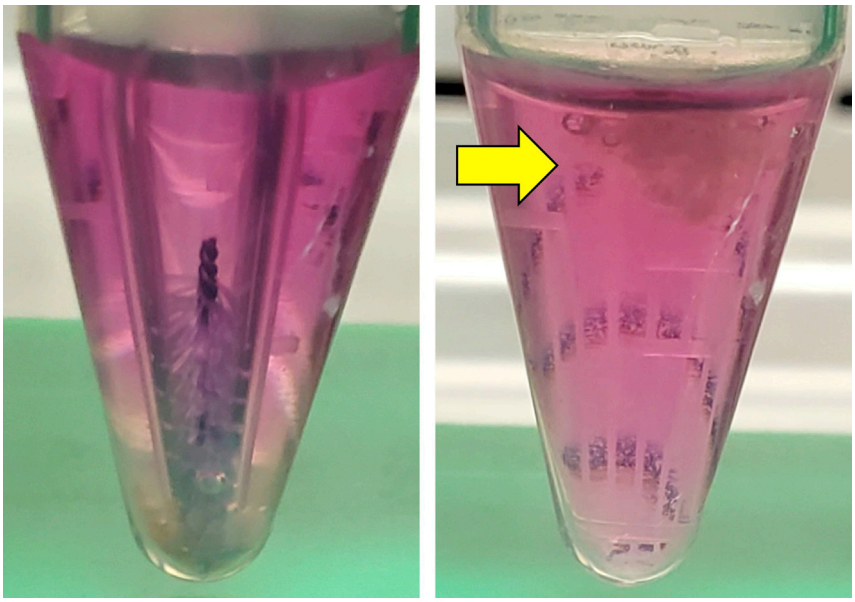


**Figure 2. Removal of tissue from interdental brush**

Left-to-right; Removal of tissue from brush using a 1000uL pipette tip; tissue and mucosubstance (yellow arrow) in ACF dissociation solution after removal from brush.

## Differentiation at the ALI

Timing: 22–30 days

In these steps the HNECs will be seeded onto 12 mm polyester transwell inserts and cultured to 100% confluence before initiating the ALI to trigger HNEC differentiation. After 20–25 days of ALI culture the HNECs will have differentiated and formed complete airway mucociliary tissue. Our protocol for ALI differentiation is adapted from published protocols which recommend coating transwell inserts with type I collagen (Lam et al., 2011) (You et al., 2002). We find that ALI cultures achieve robust differentiation without the addition of collagen coating and therefore have removed this step from our protocols.

22. Apply 1 mL of complete expansion plus media to each well containing a transwell insert.
23. Seed the apical chamber of each transwell insert with 2×10^5 HNECs in 0.5 mL of complete expansion plus media. Incubate cultures at 37°C, 5% CO2.
24. Confluent monolayers should be established within 24–48 h after seeding chambers. Visually confirm monolayer formation via phase contrast microscopy.
25. Initiate the ALI by removing the media in the apical and basal chambers and replenishing only the basal chamber with 1 mL of ALI media, incubate cultures at 37°C, 5% CO2.
26. Replenish the 1 mL of ALI media in basal chamber every 48 h. When media is being replaced also wash the apical chamber with 0.5 mL PBS to remove cell debris and prevent over-accumulation of muco-substances which negatively impact multiciliated cell differentiation.
27. Formation of mucociliary epithelium will be observable by day 20–25 and can be confirmed via phase contrast microscopy.

*Note:* ALI tissue can be maintained in culture for prolonged experiments (40+ days) by continuing to replenish ALI media and washing the apical surface with PBS every 48 hrs. Long-term ALI cultures are at an increased risk for contamination by bacterial and fungal

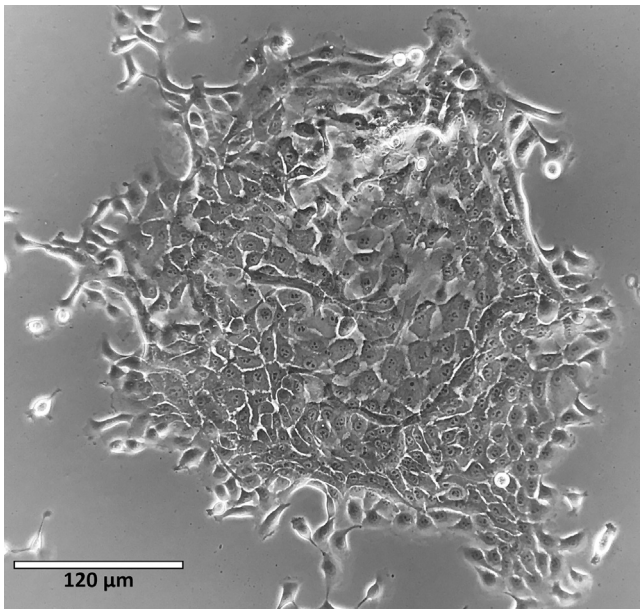



**Figure 3. Phase contrast image of HNEC colony formed after 3 days of culture**
Scale bar, 120 µm.

sources, therefore special consideration should be given to these types of experiments and sterile procedures utilized while handling cultures.

### Fixation, paraffin-embedding and sectioning of ALI tissue

Timing: 2 days

ALI tissue will be fixed in formalin, removed from the plastic transwell inserts, dehydrated via graded alcohol washes, and embedded into paraffin wax. Histological sections will then be obtained from the paraffin blocks for downstream analysis of ALI tissue.

28. Rinse apical and basal chambers with PBS.
29. Apply 1 mL of 10% buffered formalin to the basal chamber and 0.5 mL to the apical chamber.
30. Wrap plate in parafilm.
31. Place at 4°C for 12–16 h.
32. Rinse inserts with PBS.
33. Remove the polyester membrane from the transwell inserts using a #11 scalpel blade.

*Note:* Cut the membrane into 2 equal halves by inserting the scalpel blade from the underside of the membrane ~3–4mm deep and cutting a vertical line down the center of the insert. Next, using the transwell insert as a guide, cut in a circular motion to remove the two halves of the membrane (Figure 4).

*Note:* Use fine forceps to move ALI tissue by gripping the membrane at the circular edges as opposed to the center cut.

⚠ CRITICAL: Insert the scalpel blade from the underside of the membrane otherwise ALI tissue will be damaged during cutting. (Figure 5)

34. Place membrane into 70% ETOH and wash on slow rocker for 15 min at 22°C–25°C.

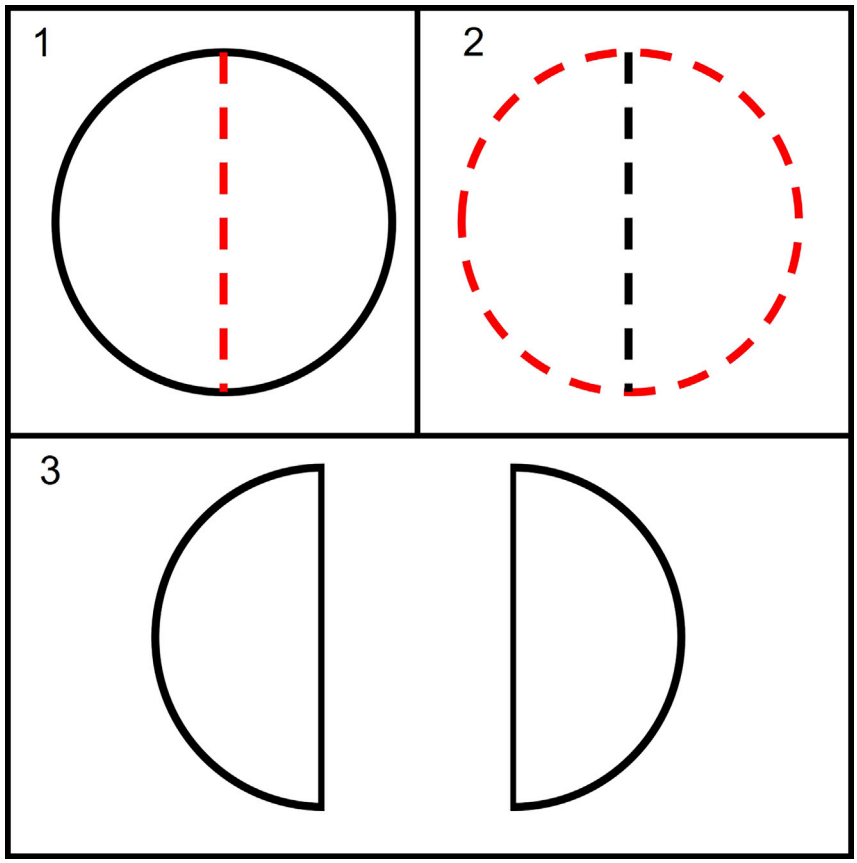



**Figure 4. Steps for removing the membrane from the transwell insert prior to embedding and sectioning**
1. Make a linear cut down the center of the insert (red line). 2. Cut in a circular motion using the plastic insert as a guide (red line). 3. Final product is membrane cut into two mirrored halves.

*Note:* Washes should be performed with volumes sufficient to completely submerge membranes.

35. Wash membrane in 80% ETOH on slow rocker for 15 min at 22°C–25°C.
36. Wash membrane in 95% ETOH on slow rocker for 15 min at 22°C–25°C.
37. Wash membrane in 100% ETOH on slow rocker for 15 min at 22°C–25°C.
38. Wash membrane in Xylene on slow rocker for 15 min at 22°C–25°C.
39. Place inserts into 55°C molten paraffin wax for 25 min.

⚠ CRITICAL: Prolonging the paraffin infiltration step beyond 30 min can result in irreversibly damaged ALI tissue.

*Note:* Polyester membranes will roll up on themselves when in molten paraffin. Therefore, they must be manually molded into flat shapes as they cool.

40. Using forceps, remove the ALI tissue from molten wax and gently press tissue in-between thumb and forefinger (wearing latex or nitrile gloves) to cool the wax/membrane into a flat shape to create a wax 'chip'.
41. Using forceps to grip the wax chip from the edge, briefly dip in and out of molten paraffin to add a layer of wax. Keep using thumb and forefinger to cool/maintain flat shape of chip after dipping into molten wax.

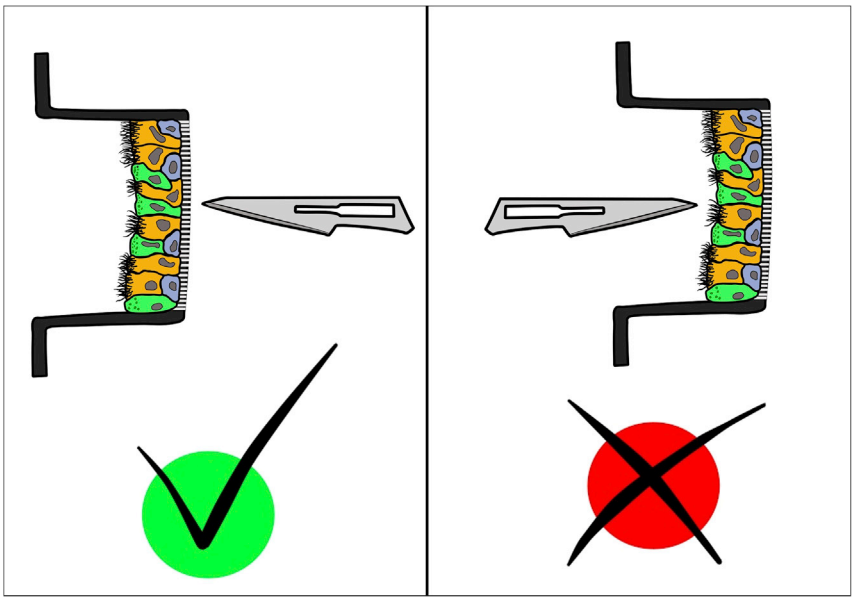


**Figure 5. The direction to insert scalpel blade into ALI tissue when cutting tissue in half**
Depiction of correct (Green circle) and incorrect (Red circle) ways to perform scalpel cuts on intact ALI tissue.

⚠ CRITICAL: Be sure to track which side of the wax chip is the flat edge of the polyester membrane half and which is the rounded edge (Figure 6).

42. Repeat step 41 at least 5 times to increase the thickness of the wax chips to between 2–4 mm.

⚠ CRITICAL: The membrane needs to be coated in a sufficient amount of wax to ensure that it remains flat when the wax chip is embedded into the mold.

43. Fill an embedding mold with molten wax and place mold onto cryo plate or ice pack.
44. When the molten wax in the mold has turned opaque and is partially cooled, insert the wax chip into the mold with the flat side of the membrane facing down.

⚠ CRITICAL: If the wax in the mold is too hot when the chip is inserted then it will re-melt the wax chip before the block solidifies, if this happens the membrane will begin to curl and will no longer maintain a flattened orientation.

⚠ CRITICAL: The orientation of the membrane when inserted into the mold should be so that it is perpendicular to the cutting blade, not parallel (Figure 7).

45. After inserting the wax chip into the mold, let the mold cool on a cryo plate or ice pack until solidified.

**Pause point:** Wax block can be removed from the mold and stored at room temperature until future sectioning.

46. Use microtome to collect 10 uM sections.

*Note:* Wax block should be at room temperature before sectioning to reduce cracking of wax during sectioning.

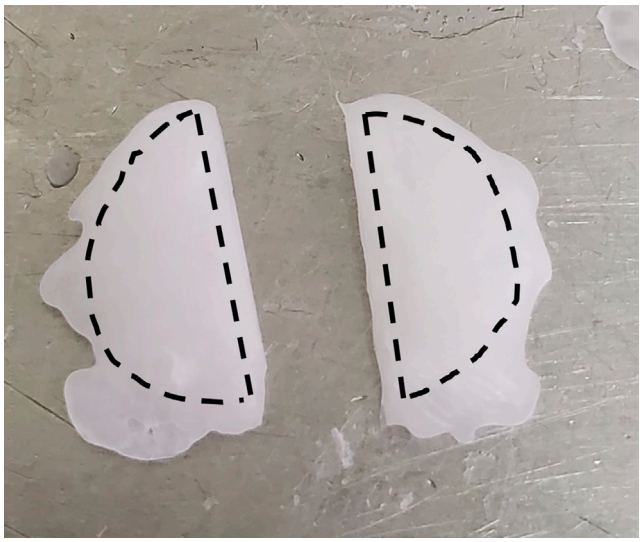


**Figure 6. ALI membrane halves embedded in paraffin wax**

Wax chips containing each half of an ALI membrane (dotted line), formed by briefly dipping the membrane in wax 4–6 times.

*Note:* Each cross section will likely curl up on itself as it is generated. Use a small paintbrush to prevent the section from curling.

47. Transfer each section to a 55°C water bath using fine forceps.
48. Pick up floating section using a charged slide.

⚠ CRITICAL: Paraffin sections must be mounted on charged slides otherwise tissue sections will fall off slides during the antigen retrieval process.

49. Dry at room temperature for a minimum of 12 h before staining.

**Pause point:** Dried sections can be stored at room temperature for future analysis.

## Rehydration of ALI tissue

**Timing: 30 min**

Sections of ALI tissue will be de-paraffinized with xylene and rehydrated via graded alcohol washes. After rehydration sections are ready for immunohistochemistry or special stains.

50. Place slides on a 55°C heat block for 10 min.
51. Submerge slide in xylene for 5 min at 22°C–25°C.
52. Submerge slide in a second container of Xylene for 5 min at 22°C–25°C.

*Note:* The two xylene washes are performed in separate containers.

53. Submerge slide in 100% ETOH for 5 min at 22°C–25°C.
54. Submerge slide in 95% ETOH for 5 min at 22°C–25°C.
55. Submerge slide in 80% ETOH for 5 min at 22°C–25°C.
56. Submerge slide in 70% ETOH for 5 min at 22°C–25°C.

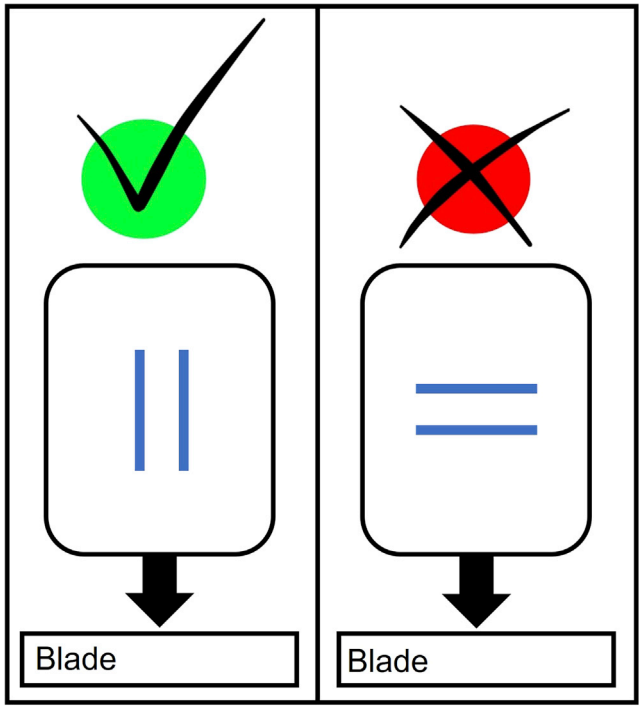



**Figure 7. The proper direction to embed ALI tissue in wax block**

Depiction of the correct (green circle) and incorrect (red circle) orientation of the membrane halves (blue lines) within the wax block regarding the cutting blade and direction of sectioning.

## Antigen retrieval and fluorescent immunohistochemistry of ALI tissue sections

Timing: ~4.5 h

Before immunohistochemistry, antigen retrieval is performed to unmask antigenic sites within the ALI tissue. Our protocol utilizes a commercial electronic pressure cooking to perform heat induced antigen retrieval. The slide will be submerged in a container of antigen retrieval buffer that sits within the pressure cooker chamber. The pressure cooker chamber will contain distilled water which will be boiled to produce the heat and pressure (Figure 8). After antigen retrieval the sections will be processed for fluorescent immunohistochemistry using protocols modified from standard formalin fixed paraffin embedded methods (Im et al., 2019). There are alternate antigen retrieval methods and buffers that we do not cover in this protocol (Shi et al., 2010). For example, a microwave may be used as an alternative method of heat induced antigen retrieval, although laboratories should perform optimization experiments with timing and power level parameters for adequate antigen retrieval. We find the advantages of using an electronic pressure cooker to be the consistency of results within large batches of slides in addition to the reproducibility between multiple pressure cookers in the laboratory.

57. Submerge slide in distilled $H_2O$ for 5 min at 22°C–25°C.
58. Place slide into a pressure-cooker safe container, fill container with enough antigen retrieval buffer to completely submerge slide.
59. Place antigen retrieval container containing the slide inside the pressure cooker chamber.
60. Fill pressure cooker with distilled water. Follow the pressure cookers' manual for the minimum volume of water required for safe operation.
61. Set pressure cooker to 15 min on High setting.


**Figure 8. Antigen retrieval setup using an electronic pressure cooker and pipette tip box. Photo of pressure cooker setup for antigen retrieval. Left: Outside of pressure cooker with lid removed. Right: Inside of pressure cooker is a secondary container (we use a micropipette tip box) containing the slide submerged in antigen retrieval buffer. The secondary container is placed on the steam rack to keep it stable during antigen retrieval. The inner chamber is filled with distilled H2O until it touches the bottom of the secondary container.**

⚠ CRITICAL: This antigen retrieval protocol is optimized for the 'Instant Pot DUO Mini' commercial pressure cooker, changing the pressure cooker used for this step may alter results.

62. Carefully vent pressure cooker and remove slide from antigen retrieval buffer.
63. Submerge slide in distilled $H_2O$ for 5 min at 22°C–25°C.
64. Submerge slide in TBST for 5 min at 22°C–25°C.
65. Carefully dry the slide using a Kimwipe, being sure not to touch the section of ALI tissue.
66. Use a hydrophobic pen to draw a barrier around the section of ALI tissue with 5 mm margins.
67. Pipette 50–100 μL of Blocking Solution directly onto the section of ALI tissue and incubate for 60 min at 22°C–25°C in a humidified chamber.

*Note:* Humidified chamber is to protect against evaporation of blocking/antibody solutions. We recommend creating a humidification chamber using a resealable plastic container and a wet sponge (Figure 9).

68. Remove blocking solution from sections by gently tapping the side of the slide on a paper towel and use a Kimwipe to remove any remaining solution, being sure not to touch ALI tissue.

⚠ CRITICAL: Do not use a vacuum manifold to suction solutions off sections, this can move and/or damage ALI tissue.

69. Dilute primary Ab to desired concentration in 1% BSA-TBST solution. Apply 50–100 μL of primary antibody solution directly onto the section of ALI tissue and incubate for 60 min at 22°C–25°C in a humidified chamber.
70. Remove primary antibody solution from sections by gently tapping the side of the slide on a paper towel and use a Kimwipe to remove any remaining solution.
71. Submerge slide in TBST for 5 min at 22°C–25°C.
72. Carefully dry the slide using a Kimwipe, being sure not to touch the section of ALI tissue.
73. Dilute fluorescent secondary antibodies to desired concentration in 1% BSA –TBST solution. Apply 50–100 μL of secondary antibody solution directly onto the section of ALI tissue and incubate for 60 min at 22°C–25°C in a darkened humidified chamber.

*Note:* We recommend molding aluminum foil over the humidified chamber to block light from reaching the samples, this protects against photobleaching of fluorophores.

**Figure 9. Humidification chamber used for blocking, primary antibody, and secondary antibody incubations**
Top: Humidification chamber with lid off, containing a secondary container filled with distilled water and a sponge completely soaked in distilled water, placed next to two slides. Bottom: Sealed humidification chamber.

74. Remove secondary antibody solution from sections by gently tapping the side of the slide on a paper towel and use a Kimwipe to remove any remaining solution.
75. Submerge slide in TBST for 5 min at 22°C–25°C.
76. Carefully dry the slide using a Kimwipe, being sure not to touch the section of ALI tissue.
77. Place one small drop of ProLong Gold antifade with DAPI directly onto the ALI tissue section.
78. Gently apply glass coverslip over the sample and let it settle naturally over the section of ALI tissue.

⚠ CRITICAL: Pressing down on the coverslip during this process can cause the ALI tissue section to move on the slide, possibly detaching from the membrane and/or folding on itself in a 'ribbon-like' fashion. If this happens it will become difficult to locate continuous sections of ALI tissue for imaging.

79. Seal the coverslip by applying clear nail polish around the edges.
80. Visualize with a fluorescent microscope, store slides away from light at 4°C.

### Hematoxylin and eosin staining of ALI tissue sections

Timing: ~20 min

Sections of rehydrated ALI tissue (Step 56) will be stained with hematoxylin and eosin to visualize tissue structure and gross cell morphology.

81. Submerge slide in hematoxylin for 1 min.
82. Gently rinse slide with tap-water for 5–7 s.

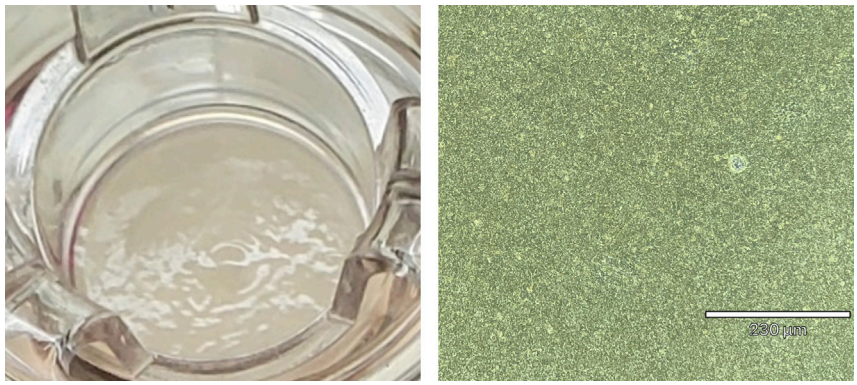



**Figure 10. Fully differentiated ALI tissue**

Left: Photo of day 30 ALI tissue in transwell insert displaying opaque color with a smooth and uniform surface. Right: Phase contrast image (10×) of the surface of day 30 ALI tissue demonstrating homogeneous appearance. Scale bar, 230 µm

⚠ CRITICAL: Do not use distilled water for step 82, this will affect hematoxylin color.

*Note:* The length of time for rinsing will determine the intensity of the hematoxylin stain, the slide can be briefly examined under a light microscope after the water rinse to determine if tissue is too dark and requires more rinsing. We recommend starting with 5 sec of water and examining slide to determine if further rinsing is necessary.

83. Submerge slide in 80% ETOH for 5 min at 22°C–25°C.
84. Submerge slide in 95% ETOH for 5 min at 22°C–25°C.
85. Submerge slide in 100% ETOH for 5 min at 22°C–25°C.
86. Submerge slide in eosin for 30 s.
87. Gently rinse slide with 95% ETOH.
88. Submerge slide in 95% ETOH for 30 s.
89. Submerge slide in 100% ETOH for 30 s.
90. Apply mounting media and cover slip.
91. Visualize tissue with a light microscope.

## EXPECTED OUTCOMES

The surface of well differentiated ALI tissue will be smooth and homogenous both to the visible eye and under a microscope (Figure 10). Histological cross sections should reveal uniform tissue structure and thickness, with a well ciliated apical surface (Figure 11). The apical cell population will contain multiciliated cells, secretory cells, and mucous producing cells, while the basal layer will mostly be composed of progenitor cells (Rock et al., 2010, Brooks and Wallingford, 2014) (Figure 11). Each millimeter of embedded ALI tissue may yield 75+ sections at 10 µM thickness (depending on the skill level of the microtome user), making it possible to harvest hundreds of histological sections from ALI tissue grown on a 12 mm transwell insert. Slides stained with fluorescent antibodies should be stored at 4°C away from light, the lifespan of the signal will vary from days to weeks depending on the antibody used. Optimal antigen retrieval times may vary once wax blocks are over 3 months old and often require more time for sufficient antigen retrieval.

## LIMITATIONS

Successful isolation of HNECs is dependent on the donor's ability to both locate the inferior nasal turbinate and brush it sufficiently. In our experience, HNEC donors who are successful in proper brushing of nasal turbinates are also successful on subsequent future isolation attempts.

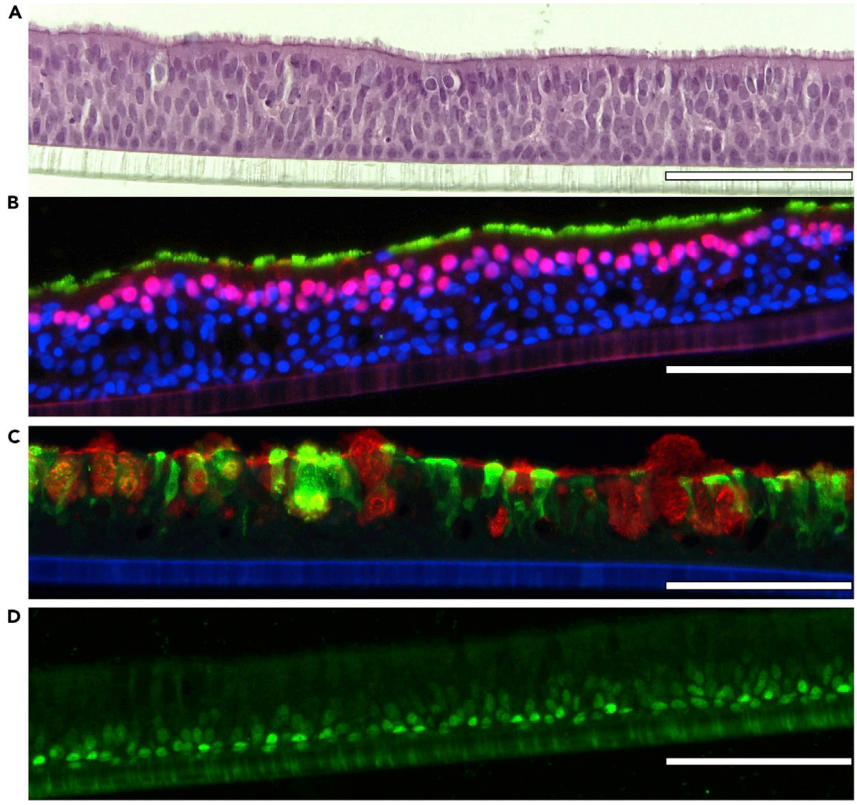



**Figure 11. Histological analysis of ALI tissue**

(A) Brightfield image of hematoxylin and eosin-stained day 30 ALI tissue displaying a well ciliated epithelial surface and uniform tissue structure.

(B) Fluorescent immunohistochemistry on ALI tissue differentiated for 20 days, multiciliated cells visualized using antibodies against the ciliary constituent acetylated tubulin (green, 1:1000 dilution) and the ciliated cell-specific transcription factor FoxJ1 (Red, 1:100 dilution). DAPI (Blue) was used as a nuclear counterstain.

(C) Fluorescent immunohistochemistry on day 20 ALI tissue, secretory cells were visualized with antibodies against the secretoglobin Scgb1A1 (Green, 1:300 dilution), mucous producing cells visualized with antibodies against the mucin MUC5AC (Red, 1:500 dilution).

(D) Fluorescent immunohistochemistry on day 30 ALI tissue, visualizing the basal progenitor cell population with antibodies against the transcription factor p63 (Green, 1:100 dilution). All secondary antibodies were used at 1:1000 dilution. Scale bars, 110 μm.

## TROUBLESHOOTING

### Problem 1

Cannot remove supernatant without disturbing cell pellet because cell pellet is stuck to strings of mucous within the supernatant (Step 9).

### Potential solution

Add 3 mL of Pneumacult-ex plus medium to bring the total volume to 5 mL, centrifuge for 10 min at 200 × *g* at 22°C–25°C. The increased volume and centrifugation time allows for greater separation between the buoyant mucous and dense cellular material. If this step is still insufficient to separate mucous and cellular material the sample can be transferred to a 15 mL conical tube and the volume can be raised to 10–15 mL using Pneumacult-ex plus media or PBS, centrifuge samples for 10 min at 200 × *g* at 22°C–25°C.

### Problem 2

Freshly isolated HNECs are forming spheroids instead of attaching to the culture plate (Step 12).

### Potential solution

Pellet the spheroids by centrifugation for 10 min at 200 × *g*. Resuspend the pellets in 1 mL ACF dissociation solution and incubate at 37°C for 10 min, add 1 mL ACF inhibition solution and pellet cells by centrifugation for 10 min at 200 × *g* at 22°C–25°C. Remove and discard supernatant, resuspend cell pellet in 2 mL fresh Pneumacult-ex plus medium. Incubate at 37°C, 5% CO2. Attached cells should be visible within 24 h.

### Problem 3

Media from the basal chamber leaked into the transwell insert 12–24 h after initiation of ALI (Step 25).

### Potential solution

Inspect the integrity of cell cultures under phase contrast microscope and look for open spaces in the cell monolayer. Remove the media from the transwell insert and return cultures to the incubator for 12–16 h. Open spaces in the monolayer should fill in and leaking should stop by day 3–4 ALI, if not then it is likely that cells will not differentiate well.

### Problem 4

ALI tissue is torn and/or twisted and disconnected from the membrane after sectioning (Step 46).

### Potential solution

ALI tissue can be damaged by mildly used microtome blades, switch to a fresh microtome blade.

This can be caused by residual paraffin from incomplete deparaffinization. Use fresh reagents for the rehydration of ALI tissue (steps 50–56).

### Problem 5

ALI sections detach from slides during antigen retrieval (Steps 58–62).

### Potential solution

This can be caused by residual paraffin from incomplete deparaffinization. Use fresh reagents for the rehydration of ALI tissue (steps 50–56).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vincent Manna (Mannav6@Rowan.edu).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate/analyze data sets/code.

## ACKNOWLEDGMENTS

We thank Dr. Susan Muller-Weeks (Rowan University) for comments and edits on the manuscript. We thank Dr. Renee Demarest (Rowan University) for edits on the manuscript and access to necessary histological equipment. We thank Katie Centritto for help with paraffin wax embedding procedures. This work was funded by the Rowan Graduate School of Biomedical Sciences and supported in part by a grant from the New Jersey Health Foundation.

## AUTHOR CONTRIBUTIONS

V.M. adapted, modified, and implemented the technique for isolating HNECs from nasal passages of human subjects. He also developed modified procedures for optimally growing and

differentiating these progenitor cells. V.M. developed all the techniques listed here for histochemical and immunochemical analysis of differentiated airway tissue. S.C. advised and assisted with the development of optimal protocols for growth and differentiation of airway-derived stem cell epithelia. Protocols for isolation of HNEC specimens from human subjects have been approved by our Institutional Review Board and all donors provided informed consent.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

Hussain, R., Hugosson, S., and Roomans, G.M. (2014). Isolation and culture of primary human nasal epithelial cells from anesthetized nasal epithelia. Acta Oto-Laryngol. *134*, 296–299.

Yang, D.P., Jim, S., Kim, C.H., Yoon, J.H., and Kim, H.J. (2016). alternative method for primary nasal epithelial cell culture using intranasal brushing and feasibility for the study of epithelial functions in allergic rhinitis. Allergy Asthma Immunol. Res. *8*, 69–78.

Lam, H.C., Choi, A.M.K., and Ryter, S.W. (2011). Isolation of mouse respiratory epithelial cells and exposure to experimental cigarette smoke at air liquid interface. JoVE *48*, 2513.

You, Y., Richer, E.J., Huang, T., and Brody, S.L. (2002). Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am. J. Physiol. Lung Cell. Mol. Physiol. *283*, L1315–L1321.

Im, K., Mareninov, S., Diaz, M.F.P., and Yong, W.H. (2019). An introduction to performing immunofluorescence staining. Methods Mol. Biol. *1897*, 299–311.

Shi, S.R., Shi, Y., and Taylor, C.R. (2010). Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J. Histochem. Cytochem. *59*, 13–32.

Rock, J.R., Randell, S.H., and Hogan, B.L.M. (2010). Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. Disease Models Mech. *3*, 545–556.

Brooks, E.R., and Wallingford, J.B. (2014). Multiciliated cells: a review. Curr. Biol. *24*, R973–R982.