Journal of Visualized Experiments

Culture and Imaging of Human Nasal Epithelial Organoids

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63064R2
Full Title:	Culture and Imaging of Human Nasal Epithelial Organoids
Corresponding Author:	Jennifer Guimbellot UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	jguimbellot@uabmc.edu
Order of Authors:	Zhongyu Liu
	Justin Anderson
	Jennifer Natt
	Jennifer Guimbellot
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Birmingham, Alabama, United States
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please confirm that you have read and agree to the terms and conditions of the video release that applies below:	I agree to the Video Release
Please provide any comments to the journal here.	

TITLE:

Culture and Imaging of Human Nasal Epithelial Organoids

AUTHORS AND AFFILIATIONS:

Zhongyu Liu[1,2], Justin D. Anderson[1,2], Jennifer Natt[1,2], Jennifer S. Guimbellot[1,2]*

[1]Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham (UAB), Birmingham, AL, USA

[2]Department of Pediatrics, Division of Pulmonary and Sleep Medicine, UAB, Birmingham, AL, USA

Email addresses of the authors:

Zhongyu Liu (zhongyu@uab.edu)
Justin D. Anderson (jda0027@uab.edu)
Jennifer Natt (jenjen95@uab.edu)
Jennifer S. Guimbellot (jguimbellot@uabmc.edu)

*Email address of the corresponding author:

Jennifer S. Guimbellot (jguimbellot@uabmc.edu)

SUMMARY:

A detailed protocol is presented here to describe an *in vitro* organoid model from human nasal epithelial cells. The protocol has options for measurements requiring standard laboratory equipment, with additional possibilities for specialized equipment and software.

ABSTRACT:

Individualized therapy for cystic fibrosis (CF) patients can be achieved with an *in vitro* disease model to understand baseline Cystic Fibrosis Transmembrane conductance Regulator (CFTR) activity and restoration from small molecule compounds. Our group recently focused on establishing a well-differentiated organoid model directly derived from primary human nasal epithelial cells (HNE). Histology of sectioned organoids, whole-mount immunofluorescent staining, and imaging (using confocal microscopy, immunofluorescent microscopy, and bright field) are essential to characterize organoids and confirm epithelial differentiation in preparation for functional assays. Furthermore, HNE organoids produce lumens of varying sizes that correlate with CFTR activity, distinguishing between CF and non-CF organoids. In this manuscript, the methodology for culturing HNE organoids are described in detail, focusing on the assessment of differentiation using the imaging modalities, including the measurement of baseline lumen area (a method of CFTR activity measurement in organoids that any laboratory with a microscope can employ) as well as the developed automated approach to a functional assay (which requires more specialized equipment).

INTRODUCTION:

Introduction to the technique

Ex vivo culture-based assays are an increasingly utilized tool for precision medicine and the study

of disease pathophysiology. Primary human nasal epithelial (HNE) cell culture has been used in numerous studies of cystic fibrosis[1–13], an autosomal recessive disease that affects epithelial cell function in multiple organs. HNE culture provides a renewable source of airway epithelia that may be obtained prospectively and recapitulates electrophysiological and biochemical qualities to test Cystic Fibrosis Transmembrane conductance Regulator (CFTR) activity. HNE cells can be sampled with minimal side effects[14], similar to common viral respiratory swabs. Research work describing a model for cystic fibrosis study derived from HNE brush biopsies has been recently published[11,13]. While similar to other models using primary HNE[2,3] and intestinal tissue[15–19], detailed characterization of the differentiation and imaging of this model are described here for use in CF research and for aiding in the studies of other airways diseases[13]. The organoid model is not unlimited like immortalized cell lines but can be expanded by conditional reprogramming (using irradiated and inactivated feeder fibroblasts and Rho-kinase inhibitors) to a more stem cell-like state[20–23]. The processing of HNE brush biopsies using this method yields large numbers of epithelial cells for use in multiple applications at higher throughput while still retaining the ability to differentiate fully. While this protocol was developed using feeder cells, other methodologies may be used by investigators wishing to avoid feeder cell technology[14,24].

Importance of the technique to pulmonary biology

A significant study has been devoted to understanding how the absence of regular, functioning CFTR in the cell membrane of epithelial cells results in dysfunction in the lungs, pancreas, liver, intestine, or other tissues. Dysfunctional epithelial ion transport, particularly that of chloride and bicarbonate, results in a decreased volume of the epithelial lining fluids and changes in mucous secretions, leading to mucous stasis and obstruction. In other airway diseases, such as primary ciliary dyskinesia, altered ciliary motion impairs mucociliary clearance and leads to mucous stasis and obstruction[25]. Therefore, the current HNE organoid model has been developed for various applications, depending on the investigator's experimental design and resources. This includes live-cell imaging using live-cell stains; fixation and sectioning to characterize the morphology; immunofluorescence staining with antibodies and whole-mount confocal imaging to avoid disrupting intraluminal structures; and bright-field imaging and micro-optical coherence tomography for quantitative measurements of ciliary beat frequency and mucociliary transport[13]. To facilitate expansion to other investigators, commercially available reagents and supplies were used for culturing. A functional assay was developed that used common microscope techniques and more specialized equipment. Overall, while the present model was designed to assess CFTR activity at baseline or in response to therapeutics, the techniques described in this protocol can be applied to other diseases involving epithelial cell function, especially epithelial cell fluid transport.

Comparison to other methodologies

Recently the utility of this organoid model was developed by correlating *in vitro* CFTR modulator responses of patients' organoids with their clinical response[11]. Notably, it is also demonstrated that the present model paralleled short-circuit current responses, the current gold standard for assessing CFTR function, in the same patients. Short-circuit current differs from the swelling assay because the former measures CFTR function *via* ion transport[26]. In contrast, this assay measures a more downstream effect with fluid transport, providing additional information about the

overall function of CFTR[27–32]. Short-circuit current measurements have continued to be a common and reliable method for determining CFTR chloride channel activity[1,33]. These electrophysiological assays require specialized, expensive equipment, require many times more cells for each experimental replicate than the organoid assay, cannot be easily automated, and are not amenable to scaling up for higher throughput applications. Another organoid model derived from intestinal epithelia has additional advantages[15–18], such as more excellent replicative capability, but is neither derived from an airway tissue nor is universally available. HNE brushings are obtained with inexpensive cytology brushes without the need for sedation and at minimal risk. Getting the brushing does not require a clinician and can be performed by trained research coordinators and other research staff[14]. The HNE organoid model can be cultured by any laboratory with primary cell culture capabilities, and some of the applications can be performed with standard microscopy techniques. Altogether, these advantages provide additional access to technology for assessing airway epithelial function that might otherwise be unavailable to some laboratories. Furthermore, HNE organoids can be utilized to study other disease states that affect the airway, such as primary ciliary dyskinesia[25] or viral infection, which intestinal organoids cannot.

PROTOCOL:

HNE samples were collected at the Children's of Alabama hospital. All procedures and methods described here have been approved by the IRB University of Alabama at Birmingham (UAB IRB #151030001). To facilitate the expansion and improve the function of human nasal epithelial cells (HNEs), the present culturing methods are adapted from the well-known air-liquid interface (ALI) culture method[28,34]. HNEs were initially collected by brush biopsy as previously described[12,14], with the only difference being the use of a cytology brush. All sample processing steps and cell culture were performed in the biosafety cabinet.

1. Cell culture and expansion of nasal epithelial cells

1.1. After brushing, store the nasal biopsy sample in 8 mL of RPMI media in a 15 mL conical tube on ice and transfer it to the lab within 4 h (not more than 24 h).

1.2. Dissociate the nasal brush biopsy into 8 mL of RPMI media in a 15 mL conical tube by passing the cytology brush through a 1 mL large bore pipette tip (cut off the tip) several times until the brush is clear of tissue.

1.3. Centrifuge the cells at 500 x *g* for 5 min at 4 °C, remove the supernatant, and then re-suspend the cell pellet in 3 mL of cell detachment solution (see **Table of Materials**) and incubate at room temperature for 16 min to digest.

1.4. Use 5 mL of expansion media (**Table 1**) to wash the cells twice. Then, add cells to a T75 flask pre-seeded with irradiated and inactivated 3T3 fibroblasts[22] (50%–60% confluence) to co-culture the cells and expand in the expansion media for 7–14 days (see **Figure 1** for the appropriate colony). Before use, test the irradiated 3T3 fibroblasts to ensure they cannot proliferate, which will negatively influence the epithelial cell expansion.

NOTE: Discard the cells if the nasal epithelial cell confluence does not reach 70% within 14 days.

1.5. For the cells derived from patients with CF, introduce four antibiotics (100 µg/mL of tobramycin, 2.5 µg/mL of amphotericin B, 100 µg/mL of ceftazidime, 100 µg/mL of vancomycin, see **Table of Materials**) into the expansion media for disinfection of the cells for the first 3 days of culture, and then replace the media with antibiotic-free expansion media changing the media every 2 days.

NOTE: This limited use of antibiotics is intended to reduce culture loss due to bacterial colonization while encouraging proliferation after the additional antibiotics are removed. These antibiotics can be tailored to the patient's specific microbiology results, with sensitivities, if needed.

1.6 Harvest HNEs from co-culture using the double trypsinization method[22] once the cells reach approximately 80% confluence. This method ensures that the irradiated and inactivated 3T3 fibroblasts are removed from the flask, and they do not contaminate subsequent organoid seeding.

1.6.1. Wash the cells with 1x DPBS, and then add 1.5 mL of 0.05% trypsin-EDTA into the T75 flask for 4 min at 37 °C to remove the irradiated and inactivated 3T3 fibroblast from culture.

1.6.2. Rinse the T75 flask with 1x DPBS twice to thoroughly wash away any remaining 3T3 fibroblasts; add 1.5 mL of 0.05% trypsin-EDTA into the T75 flask for 10 min at 37 °C to detach HNEs.

1.6.3. Neutralize the trypsin with soybean trypsin inhibitor (see **Table of Materials**) at a 1:1 ratio. Centrifuge the cells at 500 x *g* for 5 min, and then remove the supernatant. After washing the cells with 5 mL of expansion media once, the cells are ready for seeding to grow organoids.

NOTE: HNEs with a passage number of three are recommended to be used for further experiments.

2. Growth and differentiation of organoids in slides and culture inserts

2.1. Thaw the organoid culture extracellular matrix (ECM) overnight at 4 °C. Cool pipette tips to 4 °C and 15-well angiogenesis slides (see **Table of Materials**) overnight at 4 °C.

NOTE: ECM should be put at 4 °C the night before the cell harvesting needs to be done.

2.2. Coat the slides with 5 µL of cold 100% ECM on ice (pipette 5 µL of cold 100% ECM with a cold pipette tip into each well of the 15-well slide), and then place them into a cell culture incubator at 37 °C for at least 30 min for consolidation.

2.3. Count the HNEs harvested from co-culture using a hemocytometer and dilute the cells to 500 cells/µL in total number with 20% ECM diluted by differentiation media (**Table 2**) on ice. Then, seed 5 µL of the cold cell/ECM mixture into each well of the slides coated with ECM.

2.4. Immediately transfer the slides into a culture incubator at 37 °C for 1 h to consolidate the cell/ECM mixture.

2.5. Feed the cells in each well of the 15-well angiogenesis slides with 50 µL of differentiation media. Change the media every other day until the organoids are ready for further experiments.

NOTE: Organoids can usually be visualized after 1–2 days. There are 20–90 organoids commonly formed in each well of the slides. The organoids can typically survive for 40–60 days in ECM with feeding every other day when kept in a humidified incubator at 37 °C.

2.6. Culture the organoids in the culture inserts (see **Table of Materials**) for greater quantity for specific applications (such as sectioning for histology or immunofluorescence) as per the steps mentioned below.

2.6.1. To grow organoids in the culture inserts, prepare the organoid culture ECM, pipette tips, and inserts as mentioned in steps 2.1–2.2. Coat the inserts with 100 µL of cold 100% ECM.

2.6.2. Seed 60 µL of cell/ECM mixture (500 cells/µL with 20% ECM in differentiation media) on top of the ECM coating in the insert.

NOTE: All the other steps for making organoids in the inserts are the same as those conducted in the slides, steps 2.4–2.5.

2.6.3. Add 600 µL of the differentiation media into the bottom part of the insert. Change the media every alternate day until the organoids are used for experiments, typically 2–3 weeks.

3. Preparation and isolation of organoids for whole-mount immunofluorescence

3.1. Isolation and fixation of organoids

3.1.1. Pre-treat 8-well glass-bottom chamber slides with cell adhesive (see **Table of Materials**) for 30 min following Reference[35]. After discarding the solution, air-dry the wells for 30 min.

3.1.2. To harvest the organoids, remove the media from the top of the ECM, and then add 50 µL of cold 1x PBS into each well of the 15-well slides on ice (1:1 with ECM volume).

3.1.3. Pipette up and down 3–5 times using 200 µL of the large-bore pipette tip, and then dispense the solution onto the center of a well of the 8-well chamber slides.

3.1.4. Immediately remove excess liquid from the wells by a fine-tip pipette. Then, place the

chamber slide into a 37 °C incubator for 40 min to enhance the organoid adhering to the glass bottom.

3.1.5. After gently washing with 1x PBS twice, fix the organoids with 300 µL of 4% paraformaldehyde in each well for 30 min at room temperature (RT).

3.1.6. Wash twice with 1x PBS and store the organoids in 1x PBS at 4 °C for immunostaining for up to 2 weeks.

3.2. Immunofluorescence staining

3.2.1. To reduce auto-fluorescence, add 250 µL of 50 mM NH_4Cl in 1x PBS into each well of the slides at RT for 30 min while gently shaking on a shaker at 20 rpm.

3.2.2. After washing with 1x PBS twice, permeabilize the cells with 0.1% Triton X-100 for 30 min at RT while gently shaking at 20 rpm.

3.2.3. After washing with 1x PBS twice, add 300 µL of blocking solution, including 5% BSA and 0.1% Triton X-100 in 1x PBS, into each well for 1 h at RT.

3.2.4. Following washing, add primary antibody into the appropriate wells followed by secondary antibodies (see **Table of Materials**). Prepare primary and secondary antibody solutions in 2% BSA and 0.3% Triton X-100 in 1x PBS. Incubate all the primary antibodies at 4 °C for 2 days and all the secondary antibodies at 4 °C for 1 day.

NOTE: The final concentrations are dependent on the protein desired for the experiment. Please check the stock concentration on the manufacturer's datasheet. Then, calculate the final concentrations based on the dilutions used in the **Table of Materials**.

3.2.5. After incubation, wash the wells thoroughly with 1x PBS and add DAPI in 2% BSA and 0.3% Triton X-100 into each well for nuclear staining.

3.2.6. Image the organoids using a confocal laser scanning microscope, with a 20–60x oil immersion objective (see **Table of Materials**). Use Z-stack mode to set the upper and lower bounds of the image and use the recommended optimal Z-step size determined by the confocal software.

NOTE: The following four confocal laser excitation wavelengths were used: 408.7 nm, 489.1 nm, 561.7 nm, and 637.9 nm.

4. Preparation and isolation of organoids for histological sectioning

4.1. To harvest the organoids for histological studies, remove the media from the culture and add 50 µL of cold 1x PBS into each well of the slides on ice.

4.2. Pipette up and down three to five times using a 200 µL large-bore pipette tip, combine all the solutions from the 15-well slide or culture inserts into a 15 mL conical tube on ice. Adjust the total solution volume in the tube to 10 mL by adding additional cold 1x PBS.

4.3. Centrifuge the tube at 4 °C, 300 x *g* for 5 min; aspirate out the supernatant and add 60 µL of warm histogel (see **Table of Materials**) to mix with the organoid pellet using a 200 µL of the large-bore pipette tip.

4.4. Immediately transfer the suspension into a histology mold. After the consolidation of the histogel at room temperature, put the mold block into 4% paraformaldehyde for fixation overnight at 4 °C.

4.5. After embedding in paraffin, cut the histogel block into 5 µm cross-sections (for example, with a microtome), fix the sections onto glass slides, and stain using hematoxylin and eosin (H&E) or immunofluorescence-labeled antibodies. Take images using a bright-field microscope or inverted epi-fluorescence microscope (see **Table of Materials**).

5. Imaging of live organoids

NOTE: The following steps are carried out using an automated imaging system (see **Table of Materials)**. Different imaging systems need to adapt these steps following their specific manufacturer's instructions. Regardless of the equipment utilized, imaging live organoids require a temperature-controlled and humidified environmental chamber with an accompanied CO_2 gas controller.

5.1. To monitor the differentiation of organoids before performing a functional swelling assay[36], capture the whole slide images manually with any bright-field microscope or with an automated imaging system, as detailed below.

5.1.1. Turn on the power to the automated imaging system and the CO_2/O_2 gas controller and allow the system to complete the automated calibration (~30 min).

5.1.2. After completion, set the imaging system temperature to 37 °C; add 15 mL of sterile water to the humidification reservoir, open the CO_2 valves, close the lids, and let the imaging system pre-incubate for a minimum of 30 min before imaging.

5.1.3. Open the automated imaging software to set up a protocol for imaging and choose the location to save the raw experimental data.

NOTE: An example protocol file (**Supplementary File 1**), specific to the imaging system, has been provided as a template for the automated imaging of organoids to monitor organoid differentiation.

5.1.4. Once the environmental settings are met, immediately transfer up to two 15-well slides with lids into the environmental chamber in the automated image system's slide holder insert (see **Table of Materials**).

5.1.5. Select the wells to be imaged and start imaging to complete imaging of the desired wells.

NOTE: The basic recommended settings are: 4x and 10x air objective, bright-field channel, 2 x 2 montage for 4x objective to cover the whole well area, 4 x 4 montage for a 10x objective. Z-stack settings: three to six Z-stack slices, Z-step size = 50–100 µm, one to two slices below autofocus point, and three to five slices above.

5.2. To image live organoids for use in a forskolin-induced swelling (FIS) assay, use a microscope with an automated stage equipped with an environmentally controlled imaging chamber that allows temperature and CO_2 control.

5.2.1. Begin with steps 5.1.1–5.1.4, substitute the protocol file in step 5.1.3 with the one provided in the example protocol file (**Supplementary File 2**) containing settings specific for performing a FIS assay.

5.2.2. Before each experiment, adjust the exposure settings by evaluating at least three wells for each slide (far left, middle, and far right). Apply the X and Y coordinate offsets to ensure that the objective will be in the center of the well for all the wells.

NOTE: The basic recommended settings are: 4x air objective, Channel 1 = Bright Field, Channel 2 = DAPI, 2 x 2 montage (four image tiles). Z-stack settings: three to four Z-stack slices, Z-step size = 50–100 µm, one slice below autofocus point and two to three slices above. Image acquisition time: 8 h with 20 min intervals (Total reads = 25; Read 1 is T = 0).

5.2.3. Once all the settings are appropriately selected, choose the wells to image for both slides, or select all, and begin the run as per the equipment's instructions.

5.2.4. After running, save the experiment, close the imaging software, and shut down the imaging system[37].

6. Baseline lumen measurements

NOTE: This is done using manual imaging analysis software (see **Table of Materials**). A similar methodology can be followed using an open-source software[38] or any software that can measure the area of a region on an image.

6.1. In the software, open the **Automated Measurement Panel** by right-clicking the bottom of the screen, select **Measurement,** and then **Automated Measurement Results**. The area of each region of interest (ROI) measured will appear there.

6.2. Open the organoid image in the software and select 5-10 organoids with visible lumens.

NOTE: Lumens will be a circular area in the middle of the organoid that is visibly different in color than the rest of the organoid (**Figure 2A**).

6.3. Using the polygon ROI measurement feature, hold right click on the image to open the menu and select **Polygonal ROI** to outline the full organoid to obtain the organoid's total surface area (TSA). Then using the same feature, outline the lumen area (LA) (**Figure 2B**).

6.4. Repeat for the remaining organoids in the well and all the wells in the assay.

6.5. Export the data to excel. Divide the LA by the TSA and average all the organoids from the sample to get the Baseline Lumen Ratio (BLR)[11,13].

NOTE: Typically, ~87% of non-CF organoids will have a BLR over 0.6, and 97% will be over 0.5, while only 14% of CF organoids will have a BLR over 0.6, and 31% will be over 0.5.

7. Pre-treatment and automated imaging of HNE organoids

NOTE: All pre-treatment steps are carried out in a clean biosafety cabinet. Pre-setup the automated imaging system and the software for recording the assay before step 7.1. The incubation with DAPI is optional but is recommended as a fail-safe if the quality of bright field images is compromised. The DAPI channel (377 nm) can be analyzed instead.

7.1. Pre-incubate the organoids in a well of 15-well slides with 50 µL of differentiation media containing DAPI with or without 100 µM of CFTRinh-172 (see **Table of Materials**) in a 37 °C incubator for 1 h. While the organoids incubate, perform step 5.2.1 using a swelling assay custom protocol following **Supplementary File 2.**

7.2. Remove the pre-incubation medium using a glass Pasteur pipette with aspiration. Add 10 µM of forskolin and 100 µM of IBMX (stimulation cocktails) (see **Table of Materials**) for a total volume of 50 µL differentiation media into each well.

NOTE: Ensure no bubbles are introduced into the wells. Bubbles on the images will affect the automated analysis.

7.3. Without delay, begin the FIS imaging protocol following step 5.2.2 to 5.2.4. Acquire images every 20 min in each well, with a total runtime of 8 h.

8. Automated analysis of forskolin-induced swelling assay on HNE organoids

8.1. Open the automated imaging analysis software, find the experiment previously saved from step 7.3, and bring up the images of the experiment.

8.2. Choose the vessel window to be evaluated and select the option containing the processed images that have been stitched and z-projected. This step should provide a picture of the entire well with all organoids within the imaging frame for masking assessment and measurements.

8.3. Choose the well image to assess. Select **Analyze**. Repeat this process for other images to ensure appropriate masking for all images included in the automated measurements. Save the setting parameters.

8.4. After completing the analyzing settings, apply the changes. The software will change the measurements based on the settings.

NOTE: After the initial image pre-processing is complete, quality control (QC) measures should be performed to ensure consistent masking. These include manually reviewing all wells to ensure organoids are within the imaging frame, bubbles or debris are not inappropriately masked, and checking the masking in bright field and DAPI channels.

8.5. Export the data for the summary analysis (including graphing and statistical analysis).

REPRESENTATIVE RESULTS:

HNEs expansion is essential for a thriving organoid culture. HNEs from a successful sample collection should expand to over 70% confluence around 10 days. An example of successful and unsuccessful samples is shown in **Figure 1A** and **Figure 1B**, respectively. The cells must be discarded if they cannot reach 70% confluence by 14 days after co-culture with irradiated 3T3 cells. Any contaminated cells are to be immediately discarded if unable to rescue with additional antimicrobial agents quickly.

The growth of the organoids was compared in 15-well slides and culture inserts. The culture inserts are thicker and further from the objective than the optically optimized slides, impacting the image and resolution. Despite this, no significant difference in the morphology was observed in these two culture methods, as shown in **Figure 2**. Morphological differences can be seen between non-CF and CF organoids, as shown in **Figure 3A**. Non-CF organoids tend to have a larger lumen containing more fluid within it. In contrast, CF organoids usually have a smaller lumen with less fluid and sometimes are filled with mucus and debris. Lumen size was measured manually (**Figure 3B**), and the baseline lumen ratio was calculated and shown in **Figure 3C**. Cross-sectioned organoids were characterized using H&E and immunofluorescence staining. The representative images are shown in **Figure 4A,B**. Airway epithelial markers such as cilia, mucus, and tight junction are demonstrated in organoids by whole-mount immunofluorescent staining shown in **Figure 5A–D**. Depending on the application, sectioned or whole-mount immunofluorescence can be employed. The whole-mount method maintains the three-dimensional nature of the organoid, keeping the organoid's interior intact, as shown in the previously published work[13].

CFTR function was assessed by forskolin-induced swelling (FIS) assay using an automated imaging system. Only 15-well slides are used for functional assays due to the better image resolution. A representative forskolin dose-response experiment of non-CF volunteers (n = 5 subjects) is shown

in **Figure 6A** to illustrate the rationale of the optimized imaging time and analysis. Data comparing non-CF and CF organoid responses are detailed in previous publications[11,13]. A dose-response shows the incremental change in CFTR activity to demonstrate the best approach to measurements. Assay duration of 1 h and 8 h were evaluated (**Figure 6B,C**) as well as analysis using average fractional change (AFC) versus area under the curve (AUC) is seen in **Figures 6C,D**. Based on our previous experience, swelling for most subjects and conditions plateau after 8 h, and in some cases, results in bursting of the organoids over that time. Therefore, assays were limited to 8 h only. At this extended assay length, swelling becomes non-linear. The use of the AUC also considers both the changes in size and the rate of change. Therefore, the AUC over 8 h was used for all the FIS assays in the final methodology.

FIGURE AND TABLE LEGENDS:

Figure 1: Bright-field images of HNEs in co-culture. HNEs expand in expansion media with irradiated and inactivated 3T3 fibroblasts for 10 days. An inverted bright-field microscope is used for imaging the cells. (**A**) HNEs grow well in a large cluster (black arrow). In contrast, in (**B**), the HNEs grow poorly in two small clusters (black arrows) surrounding irradiated 3T3 cells. Scale bar = 50 µm.

Figure 2: HNE organoid formation in a 15-well slide and culture insert. Bright-field images of organoids were captured using an inverted bright-field microscope over 21 days. Organoids in the 15-well slide (**A**) have more precise and sharper images than those in the culture insert (**B**). No morphological difference was observed between the organoids cultured in the slide and insert.

Figure 3: Organoid lumen size (panel A) and lumen measurements (Panel B and C). (**A**) Non-CF organoids typically have a larger lumen and more fluid than CForganoids. (**B**) A method to manually measure the total surface area (TSA) indicated by the red outline and lumen area (LA) indicated by the green outline in a single organoid. (**C**) An example for using the total surface area and the lumen area to calculate the baseline Lumen Ratio (LA: TSA) in organoids from a non-CF vs. a CF subject. Error bars represent standard deviation.

Figure 4: Cross-section of the organoids embedded in paraffin. (**A**) An example of H&E staining in organoids from a non-CF and F508del/F508del subject. (**B**) Immunofluorescent staining of cilia in an organoid. Green is the cilia (white arrow) stained with acetylated-tubulin and FITC labeled secondary antibody, and blue is the nuclei labeled with DAPI.

Figure 5: Confocal images of whole-mount immunofluorescence in organoids. (**A**,**C**) Maximum projection images of the two representative organoids. (**B,D**) Three-dimensional reconstruction images of (**A**) and (**C**), respectively. An 8-well glass-bottom slide was fitted on the platform of a confocal microscope, and the 40x lens was used to create the photomicrographs. Imaging analysis software was applied for imaging and reconstruction of the images. White arrows indicate mucus (in **B**) and cilia (in **C**) within the lumen of the organoids.

Figure 6: Rationale for the swelling assay length and analysis methods. Forskolin (FSK)-induced swelling (FIS) assay to test CFTR function on the primary nasal epithelial cells. Different dose of forskolin indicated in the figures was administrated into 21-day old organoids in the differentiation media; organoid swelling was immediately recorded with the automated imager for 8 h. After 8 h, swelling is shown in (**A**) (n = 5, non-CF subjects) using average fractional change (AFC). FSK dose-response is compared with AFC at 1 h (**B**) vs. at 8 h (**C**), which suggests that the 8 h assay can produce a more significant swelling difference among different FSK doses than those at 1 h. AFC (**C**) vs. the area under the curve, AUC (**D**) at 8 h are compared, indicating AUC can reflect a minor swelling difference than AFC. The X-axis in panels (**B–D**) represents the different treatment conditions corresponding with the symbols in the figure legend. All error bars in the figures indicate standard deviation.

Table 1: All the components for making the expansion media. The detailed information about reagent stock concentration, stock storage, amount of stock for making a 500 mL media, and final concentration have been described.

Table 2: All the components for making differentiation media. The detailed information about reagent stock concentration, stock storage, amount of stock for making a 500 mL media, and final concentration have been described.

Supplementary File 1: An example protocol file specific to the imaging system is provided as a template for the automated imaging of organoids to monitor organoid differentiation.

Supplementary File 2: An example protocol file containing settings specific for performing an FIS assay.

DISCUSSION:

This manuscript provides detailed methodologies for comprehensive live and fixed imaging of the airway epithelial organoids derived from HNE brush biopsy. It describes functional assays that can determine CFTR activity in an individual. HNEs provide a minimally invasive, primary tissue for a variety of applications. The expansion techniques offered here can be used for modeling airways disease, including organoids. Organoids can be used for precision therapeutic approaches and to monitor the stability of gene or mRNA-based therapies over time, for precision trial design, and to aid in resolving inconclusive diagnoses[39]. The current research is on CF, but these models have applications for other diseases affecting epithelial function.

The initial expansion of HNE after biopsy is essential. It has been observed that cytology brushes yield larger initial cell numbers and better results than other biopsy tools[14]. From previous experiences, we have concluded that combining brushings from both nares in a single sample and processing that sample within 4 h yields the best results. Other investigators have used more extended time frames from biopsy to processing with success[3]. The appropriate initial collection of biopsies is vital to subsequent expansion and seeding as organoids. High-quality irradiated and inactivated fibroblasts for co-culture are required, which are grown and treated in-house in our laboratory but may also be purchased commercially. Investigators are advised that not all 3T3

fibroblasts are on the same line and should be validated before use.

For specimens not expected to have pathogenic bacterial or fungal culture, antibiotic treatment is limited to standard penicillin and streptomycin treatment for the experiments described in this manuscript. For those known to have chronic colonization of the upper airway, an antibiotic cocktail described above is utilized for only 3 days because antibiotics seem to slow epithelial expansion and yield poorer results in the experiments described here. Three days were selected to balance contamination risk with providing a similar expansion rate for both CF and non-CF specimens. For individuals with unusual pathogens, tailored antimicrobial treatment can rescue contaminated cultures if recognized early or initiated *a priori*. For initial biopsies that are unusually slow to grow, results will typically be poor for organoid studies. Investigators need to monitor both growth rate and morphology daily. The in-house culture media and reagents used are most helpful for functional swelling assays, but other commercially available media may benefit other applications[12,25,40,41] depending on the experimental design. The type of ECM used can lead to different morphology and different results, and reproducible results are critical. All reagents used in this protocol are routinely tested before use for experiments. Despite this experience, some cultures will fail to expand or generate organoids for inexplicable reasons. Investigators are encouraged to consider these factors as they optimize this protocol for their applications.

A specific type of 15-well slide is used in this protocol optimized for optical imaging, utilizing minimal volumes and maximizing replicates while reducing the costs. These slides have a lower and upper chamber fixed on a polymer coverslip that minimizes menisci (which would otherwise impair imaging) and also media replacement without risk of dislodging the matrix and destroying the organoid cultures. These slides make bright-field imaging and live stain confocal microscopy straightforward, with initial seeding, growth, and imaging in the same dish. Organoids will be lost during the collection, fixation, and staining process, so meticulous care must be taken during each step, and sufficient starting numbers must be obtained to ensure success. Growing organoids in culture inserts can help as these techniques are developed.

Imaging techniques that utilize common laboratory microscopes are included. However, the automated functional assays use an imaging system that is complex and requires a well-trained user. This protocol has been developed for users with a basic level of experience using this microscope and its software. It is recommended to first train the Individuals on the primary use of the instrument and the software by representatives of the manufacturer; this same practice is followed in our lab. A minimum of 4 weeks of training was needed to use this microscope effectively for experiments.

As described above, this methodology has some limitations. Expertise in biopsy collection, quick processing time, and facility with feeder fibroblasts are needed to successfully expand and culture HNE organoids. This method was developed using specific reagents and equipment that may not be universally available. The methodology has proved useful for research in cystic fibrosis[3,11,13] and may not be as applicable to other disease processes[25]. However, other methods and equipment may be used to develop similar strategies.

ACKNOWLEDGMENTS:

We gratefully acknowledge the contributions of all the participants who donated HNE brush biopsies to develop this protocol. We thank Latona Kersh and Children's Research Unit staff for coordinating study volunteer recruitment and sample collections. We thank Lily Deng, Johnathan Bailey, and Stephen Mackay, former trainees in our laboratory, for technical assistance. We thank Zhong Liu and Rui Zhao for their technical help. Steven M. Rowe, Director of the CF Research Center at UAB, provides leadership and resources, without which this work would not be possible. We also would like to thank Sarah Guadiana at Biotek for assistance with instrument training, Robert Grabski for confocal microscopy assistance at the UAB High-Resolution Imaging Facility, and Dezhi Wang for histological assistance at the UAB Histology Core. This work was supported by the National Institutes of Health (NIH.) Grant K23HL143167 (to JSG), Cystic Fibrosis Foundation (CFF) Grant GUIMBE18A0-Q (to JSG), the Gregory Fleming James Cystic Fibrosis Center [NIH Grants R35HL135816 and DK072482 and the CFF University of Alabama at Birmingham (UAB) Research and Development Program (Rowe19RO)], and the UAB Center for Clinical and Translational Science (NIH Grant UL1TR001417).

DISCLOSURES:

JSG is listed as an inventor on a patent application 20170242033 from the University of North Carolina that describes a similar model. When licensed technology from UNC produces royalties, the inventors receive a share of the revenue. Otherwise, the authors declare no conflicts of interest. The funders had no role in the study's design, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

REFERENCES:

1. Brewington, J. J. et al. Brushed nasal epithelial cells are a surrogate for bronchial epithelial CFTR studies. *JCI Insight*. **3** (13) (2018).
2. Brewington, J. J. et al. Generation of human nasal epithelial cell spheroids for individualized cystic fibrosis transmembrane conductance regulator study. *Journal of Visualized Experiments: JoVE*. **134**, e57492 (2018).
3. Brewington, J. J. et al. Detection of CFTR function and modulation in primary human nasal cell spheroids. *Journal of Cystic Fibrosis*. **17** (1), 26–33 (2017).
4. Bridges, M. A., Walker, D. C., Davidson, A. G. Cystic fibrosis and control nasal epithelial cells harvested by a brushing procedure. *In Vitro Cellular & Developmental Biology*. **27A** (9), 684–686 (1991).
5. Bridges, M. A., Walker, D. C., Harris, R. A., Wilson, B. R., Davidson, A. G. Cultured human nasal epithelial multicellular spheroids: polar cyst-like model tissues. *Biochemistry and Cell Biology*. **69** (2-3), 102–108 (1991).
6. Collie, G., Buchwald, M., Harper, P., Riordan, J. R. Culture of sweat gland epithelial cells from normal individuals and patients with cystic fibrosis. *In Vitro Cellular & Developmental Biology*. **21** (10), 597–602 (1985).
7. Conger, B. T. et al. Comparison of cystic fibrosis transmembrane conductance regulator (CFTR) and ciliary beat frequency activation by the CFTR Modulators Genistein, VRT-532, and

UCCF-152 in primary sinonasal epithelial cultures. *JAMA Otolaryngology–Head & Neck Surgery*. **139** (8), 822–827 (2013).
8. de Courcey, F. et al. Development of primary human nasal epithelial cell cultures for the study of cystic fibrosis pathophysiology. *American Journal of Physiology-Cell Physiology*. **303** (11), C1173–1179 (2012).
9. Gruenert, D. C., Basbaum, C. B., Widdicombe, J. H. Long-term culture of normal and cystic fibrosis epithelial cells grown under serum-free conditions. *In Vitro Cellular & Developmental Biology*. **26** (4), 411–418 (1990).
10. Mosler, K. et al. Feasibility of nasal epithelial brushing for the study of airway epithelial functions in CF infants. *Journal of Cystic Fibrosis*. **7** (1), 44–53 (2008).
11. Anderson, J. D., Liu, Z., Odom, L. V., Kersh, L., Guimbellot, J. S. CFTR function and clinical response to modulators parallel nasal epithelial organoid swelling. *The American Journal of Physiology - Lung Cellular and Molecular Physiology*. **321** (1), L119–L129 (2021).
12. Guimbellot, J. S. et al. Nasospheroids permit measurements of CFTR-dependent fluid transport. *JCI Insight*. **2** (22) (2017).
13. Liu, Z. et al. Human nasal epithelial organoids for therapeutic development in cystic fibrosis. *Genes (Basel)*. **11** (6) (2020).
14. Muller, L., Brighton, L. E., Carson, J. L., Fischer, W. A., 2nd, Jaspers, I. Culturing of human nasal epithelial cells at the air liquid interface. *Journal of Visualized Experiments: JoVE* (2013).
15. Dekkers, J. F., van der Ent, C. K., Beekman, J. M. Novel opportunities for CFTR-targeting drug development using organoids. *Rare Diseases*. **1**, e27112 (2013).
16. Dekkers, J. F. et al. A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nature Medicine*. **19** (7), 939–945 (2013).
17. Okiyoneda, T. et al. Mechanism-based corrector combination restores DeltaF508-CFTR folding and function. *Nature Chemical Biology*. **9** (7), 444–454 (2013).
18. Schwank, G. et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*. **13** (6), 653–658 (2013).
19. Geurts, M. H. et al. CRISPR-based adenine editors correct nonsense mutations in a cystic fibrosis organoid biobank. *Cell Stem Cell*. **26** (4), 503–510 e507 (2020).
20. Bove, P. F. et al. Breaking the in vitro alveolar type II cell proliferation barrier while retaining ion transport properties. *American Journal of Respiratory Cell and Molecular Biology*. **50** (4), 767–776 (2014).
21. Chapman, S., Liu, X., Meyers, C., Schlegel, R., McBride, A. A. Human keratinocytes are efficiently immortalized by a Rho kinase inhibitor. *Journal of Clinical Investigation*. **120** (7), 2619–2626 (2010).
22. Liu, X. et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *The American Journal of Pathology*. **180** (2), 599–607 (2012).
23. Palechor-Ceron, N. et al. Radiation induces diffusible feeder cell factor(s) that cooperate with ROCK inhibitor to conditionally reprogram and immortalize epithelial cells. *The American Journal of Pathology*. **183** (6), 1862–1870 (2013).
24. Scudieri, P. et al. Ionocytes and CFTR chloride channel expression in normal and cystic fibrosis nasal and bronchial epithelial cells. *Cells*. **9** (9) (2020).

25 Marthin, J. K., Stevens, E. M., Larsen, L. A., Christensen, S. T., Nielsen, K. G. Patient-specific three-dimensional explant spheroids derived from human nasal airway epithelium: a simple methodological approach for ex vivo studies of primary ciliary dyskinesia. *Cilia*. **6**, 3 (2017).
26. Blouquit, S. et al. Ion and fluid transport properties of small airways in cystic fibrosis. *American Journal of Respiratory Cell and Molecular Biology*. **174** (3), 299–305 (2006).
27. Birket, S. E. et al. Combination therapy with cystic fibrosis transmembrane conductance regulator modulators augment the airway functional microanatomy. *The American Journal of Physiology - Lung Cellular and Molecular Physiology*. **310** (10), L928–939 (2016).
28. Birket, S. E. et al. A functional anatomic defect of the cystic fibrosis airway. *American Journal of Respiratory and Critical Care Medicine*. **190** (4), 421–432 (2014).
29. Chu, K. K. et al. Particle-tracking microrheology using micro-optical coherence tomography. *Biophysical Journal*. **111** (5), 1053–1063 (2016).
30. Chu, K. K. et al. In vivo imaging of airway cilia and mucus clearance with micro-optical coherence tomography. *Biomedical Optics Express*. **7** (7), 2494–2505 (2016).
31. Liu, L. et al. Method for quantitative study of airway functional microanatomy using micro-optical coherence tomography. *PLoS One*. **8** (1), e54473 (2013).
32. Tuggle, K. L. et al. Characterization of defects in ion transport and tissue development in cystic fibrosis transmembrane conductance regulator (CFTR)-knockout rats. *PLoS One*. **9** (3), e91253 (2014).
33. McCravy, M. S. et al. Personalised medicine for non-classic cystic fibrosis resulting from rare CFTR mutations. *European Respiratory Journal*. **56** (1), 2000062 (2020).
34. Mutyam, V. et al. Therapeutic benefit observed with the CFTR potentiator, ivacaftor, in a CF patient homozygous for the W1282X CFTR nonsense mutation. *Journal of Cystic Fibrosis*. **16** (1), 24–29 (2017).
35. Inc., C. *CORNING CELL-TAK CELL AND TISSUE ADHESIVE*. Discovery Labware, Inc, Bedford, MA (2013).
36. Anderson, J. D., Liu, Z., Odom, L. V., Kersh, L., Guimbellot, J. S. CFTR function and clinical response to modulators parallel nasal epithelial organoid swelling. *The American Journal of Physiology - Lung Cellular and Molecular Physiolo*gy. **321** (1), L119–L129 (2021).
37. *Lionheart FX Live Cell Imager Operator's Manual*. Biotek Instruments, Incorporated (2016).
38. Schneider, C. A., Rasband, W. S., Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. **9** (7), 671–675 (2012).
39. Simmonds, N. J. Is it cystic fibrosis? The challenges of diagnosing cystic fibrosis. *Paediatric Respiratory Reviews*. **31**, 6–8 (2019).
40. McGarry, M. E. et al. In vivo and in vitro ivacaftor response in cystic fibrosis patients with residual CFTR function: N-of-1 studies. *Pediatric Pulmonology*. **52** (4), 472–479 (2017).
41. Garratt, L. W. et al. Determinants of culture success in an airway epithelium sampling program of young children with cystic fibrosis. *Experimental Lung Research*. **40** (9), 447–459 (2014).

Figure 1

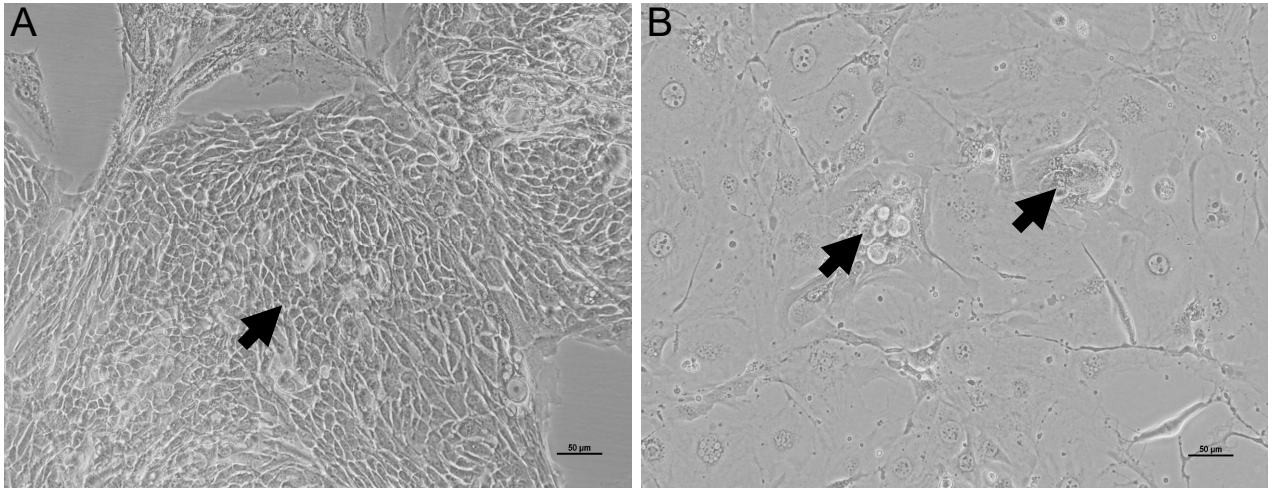


Figure 2

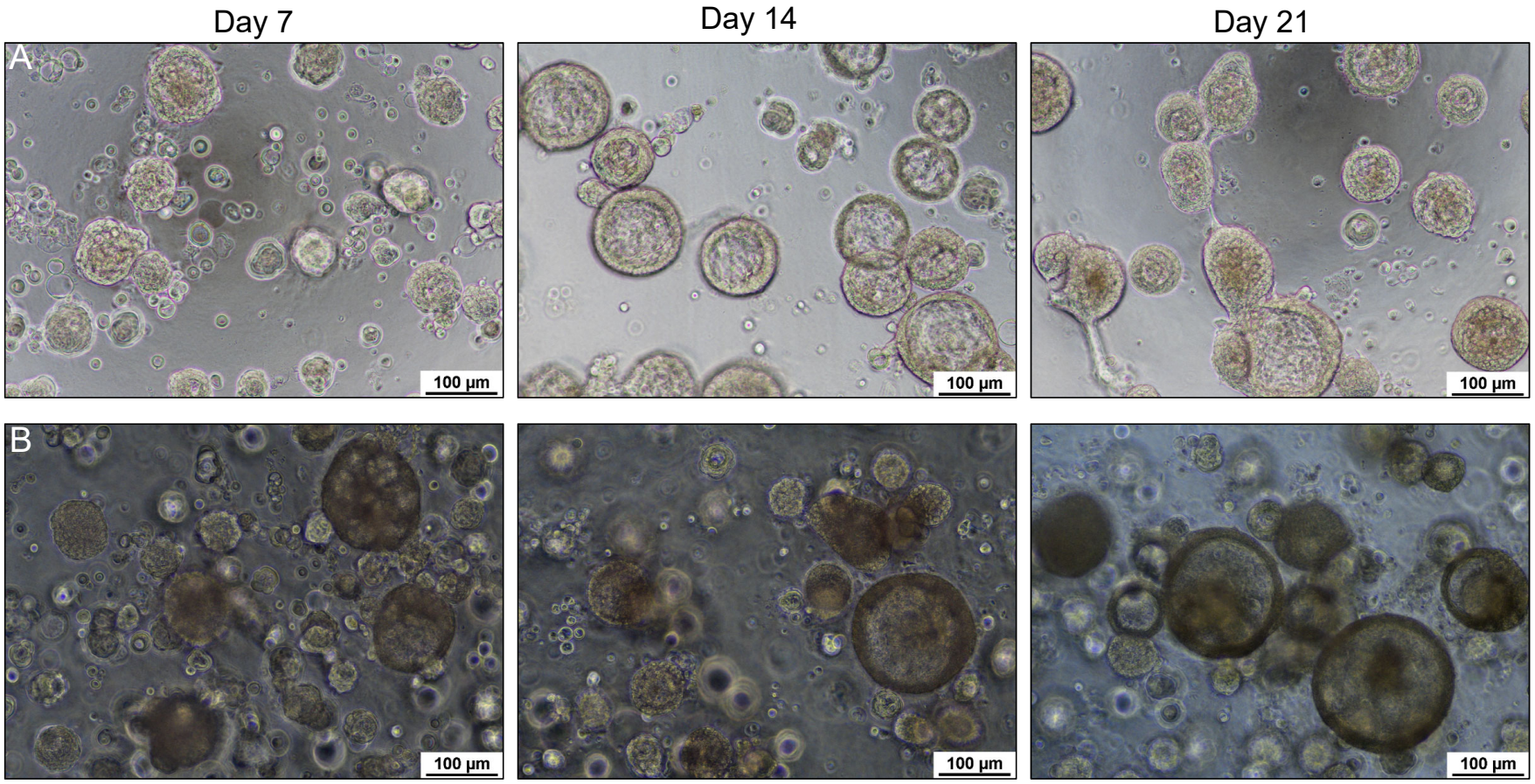


Figure 3

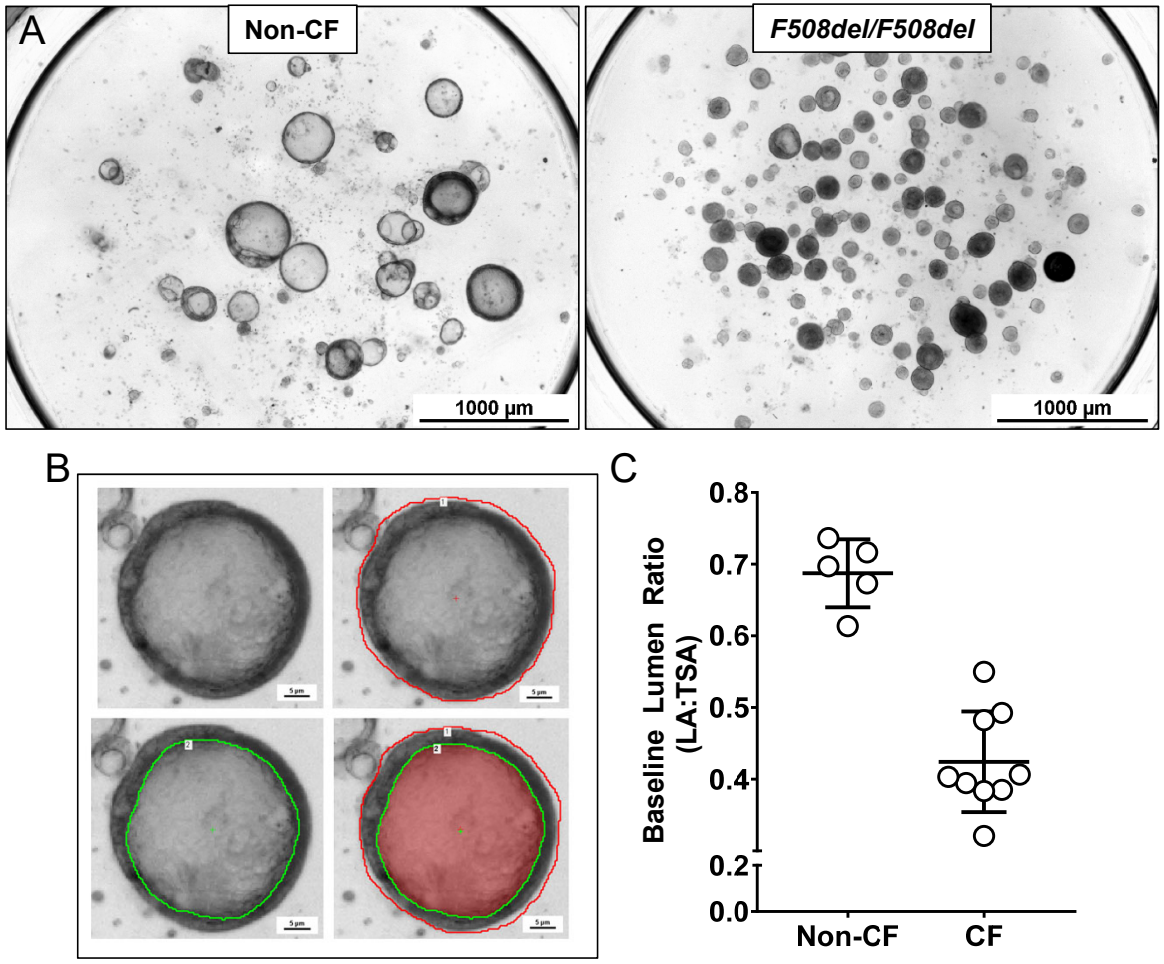


Figure 4

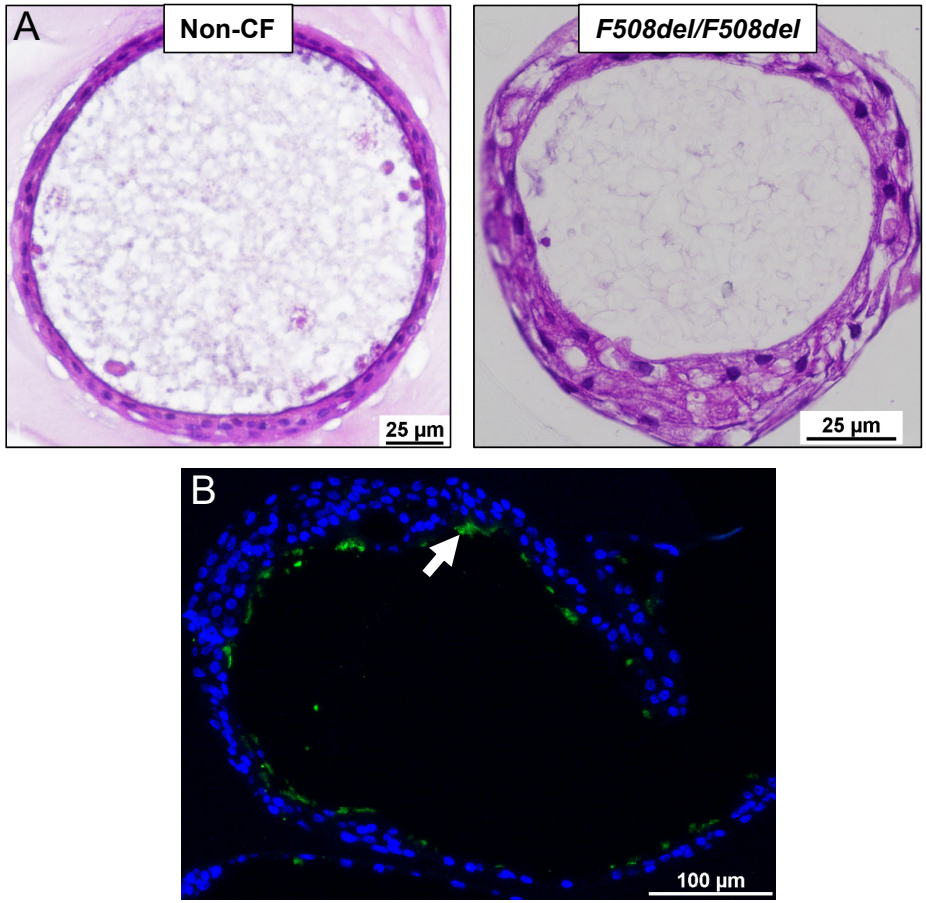


Figure 5

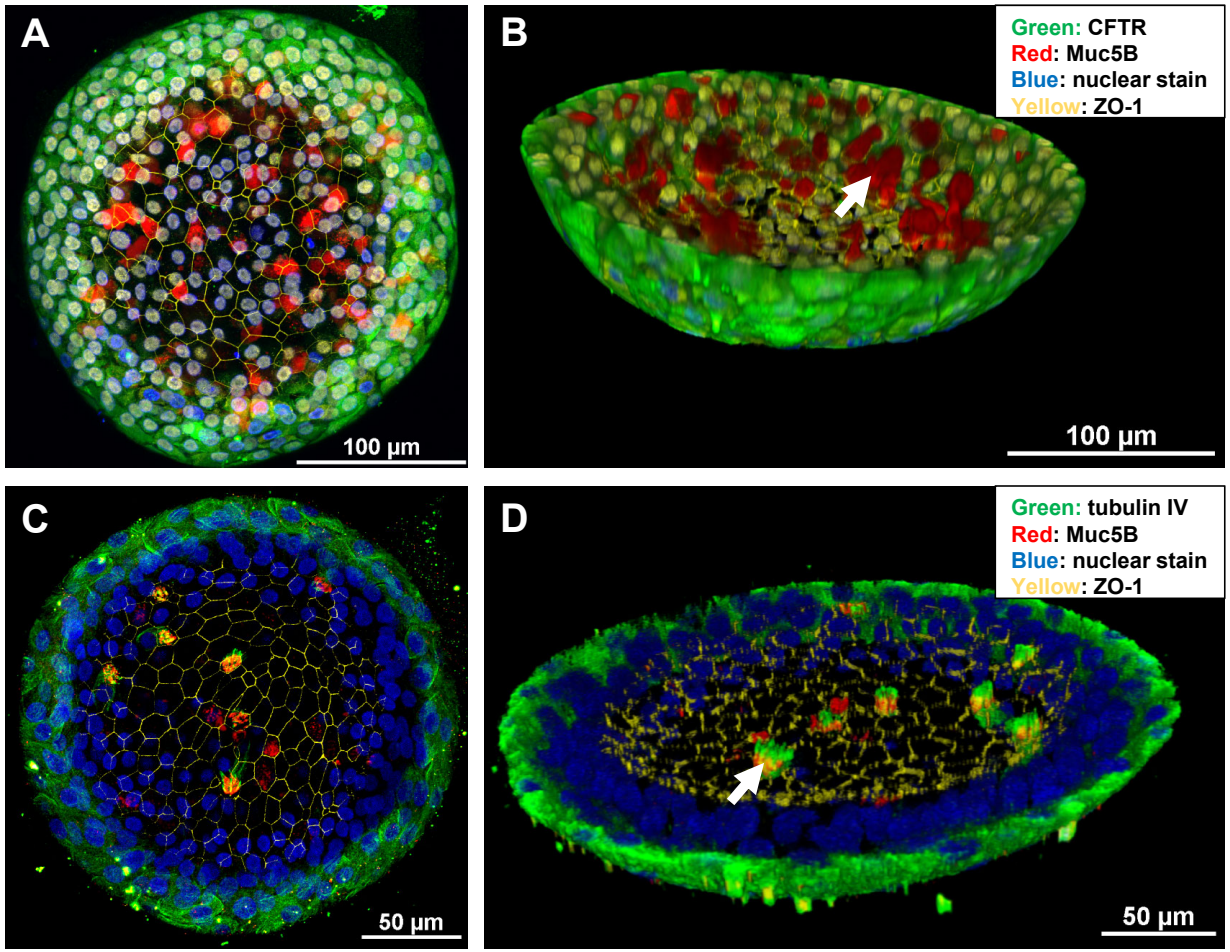


Figure 6

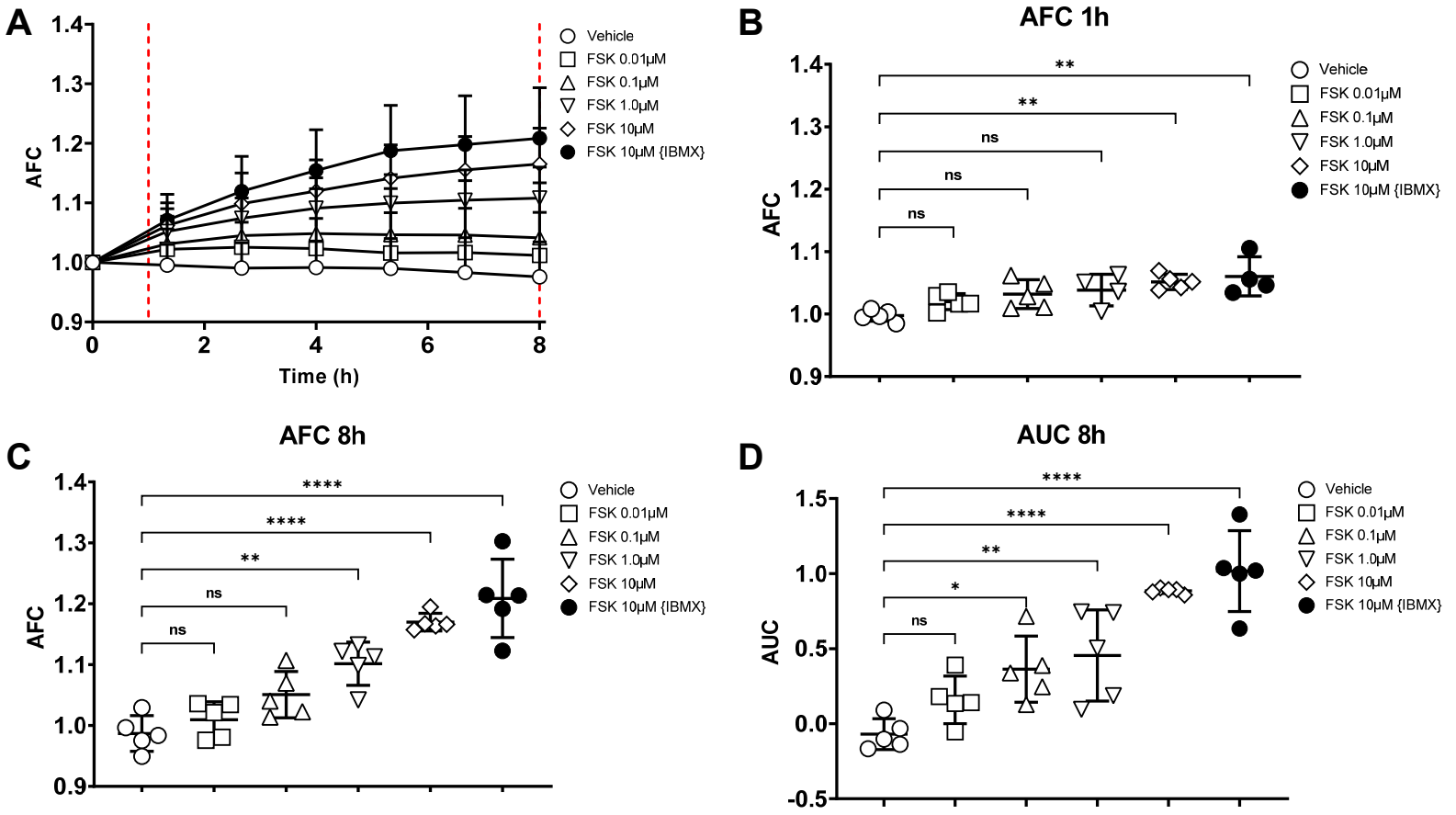


Table 1

Media Component	Stock Concentration	Stock Storage
Expansion Media		
DMEM	Use as is	Store at 4 °C
F12 Nutrient mix	Use as is	Store at 4 °C
Fetal Bovine Serum	Use as is	Store at -20 °C
Penicillin/Streptomycin	Use as is	Store at -20 °C
Cholera Toxin	1 mg/mL in sterile water	Store at 4 °C
Epidermal Growth Factor (EGF)	Powder, use as is	Store at -20 °C
Hydrocortisone (HC)	0.5 mg/mL in 100% ethanol	Store at -20 °C
Insulin	9.5-11.5 mg/L, use as is	Store at 4 °C
Adenine	Powder, use as is	Store at 4 °C, add powder to 500 mL media
Y-27632	10 mM, use as is	Make 50 uL of aliquots for 50 mL media, store at -20 °C
Antibiotic Media		
Ceftazidime	50 mg/mL in sterile water	Store at -20 °C
Tobramycin	50 mg/mL in sterile water	Store at -20 °C
Vancomycin	50 mg/mL in sterile water	Store at -20 °C
Amphotericin B	250 µg/mL in sterile water	Store at -20 °C

Amount of Stock for 500mL Media	Final Concentration

374 mL	
125 mL	
50 mL	10%
5.5 mL	1%
4.3 µL	8.6 ng/mL
Mix 1mL of 0.5 mg/mL HC with 19 mL of DMEM containing 2.5 µg EGF. Make 500 µL	0.125 ng/mL
	25 ng/mL
250 µl	5 µg/mL
12 mg	24 µg/mL
1:1000 dilution; Add 50uL aliquot just before using media.	10 µM

1:500 dilution	100 µg/mL
1:500 dilution	100 µg/mL
1:500 dilution	100 µg/mL
1:100 dilution	2.5 µg/mL

Table 2

Media Component	Stock Concentration	Stock Storage
DMEM/F-12 (1:1)	Use as is	Store at 4 °C
Ultroser-G	20 mL of sterile water in a bottle of lyophilized Ultroser-G	Store at -20 °C
Fetal Clone II	Use as is	Store at -20 °C
Bovine Brain Extract	Use as is	Store at -20 °C
Insulin	Use as is	Store at 4 °C
Hydrocortisone	10 mM in 100% ethanol	Store at -20 °C
Triiodothyronine	10 mM in DMSO	Store at -20 °C
Transferrin	30 mg/mL in sterile water	Store at -20 °C
Ethanolamine	Use as is	Store at room temperature
Epinephrine	50 mg/mL in 0.5 M HCl	Store at -20 °C
O -Phosphorylethanolamine	100 mM in sterile water	Store at -20 °C
Retinoic Acid	10 mM in DMSO	Store at -20 °C

Amount of Stock for 500 mL Media	Final Concentration
500 mL	
10 mL of stock solution	2%
10 mL of stock solution	2%
1.25 mL	0.25%
125 µL	2.5 µg/mL
1 µL	20 nM
25 µL	500 nM
42 µL	2.5 µg/mL
7.8 µL	250 nM
2.5 µL	0.25 µg/mL
1.25 µL	250 nM
0.5 µL	10 nM

UAB MEDICINE
PEDIATRICS

Children's of Alabama

September 23, 2021

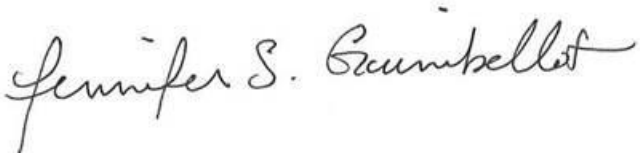
Nilanjana Das Saha, Ph.D.
Review Editor
JoVE

Dear Dr. Saha,

Thank you for the prompt and favorable review of our original research manuscript we have re-titled, "Culture and Imaging of Human Nasal Epithelial Organoids" for consideration for publication in the Journal of Visual Experimentation.

Your editorial team's constructive comments have significantly improved the manuscript. All of the critiques for the Reviewers are addressed in a point-by-point response that addresses all of the concerns raised in peer review. We appreciate your consideration of this revision.

Sincerely,



Jennifer S. Guimbellot, MD, PhD
Assistant Professor
Division of Pulmonary and Sleep Medicine
Department of Pediatrics
Associate Scientist
Gregory Fleming James Cystic Fibrosis Research Center
University of Alabama at Birmingham

Point-by-point response to Reviewers

Editorial Review Comments:

****Straightforward editorial critiques have all been addressed for items 1, 4, 5, 6, 8, 13, 14, and 16. Please note that we changed citation style to that on the journal website: https://www.jove.com/files/JoVE.ens.***

2. Please make the title concise and do not make it as a statement.
We have changed the title to "Culture and Imaging of Human Nasal Epithelial Organoids."

3. Do you need to film the sample processing steps, i.e., collecting HNEs by brush biopsy technique (line 111-113)? In that case, we need patients/volunteers for filming the Protocol by JoVE videographers in a single day. Please confirm. If patients/volunteers are not available for filming, please remove the highlighting from this step.
The brushing technique has been previously describe in two JOVE articles with accompanying video. We do not think that filming this part of the protocol will provide additional information, but we have ensured that these articles are cited in the appropriate location. Furthermore, with the COVID-19 pandemic, nasal brushing has become a high infection risk procedure given its potential for creating aerosols. It is not necessary to film this because of these reasons.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
The requested information has been added to each Step of the protocol and the protocol reviewed to ensure that everything necessary for replicating the protocol is provided.

9. Do you want to film the steps which involve the extraction of HNEs? If not, then please remove the highlighting from lines 109-114.
The specific steps for isolating the HNEs from the brush need to be filmed starting with line 119. The actual brushing does not need to be filmed.

10. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and is in line with the Title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
11. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action written in the imperative tense.
Protocol sections for the video have been highlighted.

12. Please include a paragraph on the limitations of the method in the Discussion section.

Several limitations are included in the Discussion already, including the necessity to develop expertise in the biopsy collection, the need for feeder fibroblasts, risk of contamination, and necessity to validate and test all reagents prior to use. We have summarized these limitations in a concluding paragraph.

15. Please include a title and a description of each table (similar to the Figure legends) in the Figure and Table legends section. Please remove the company name and catalog number from the tables. This should be provided in the table of materials.

Material and equipment table. All materials and equipment used in the experiments have been listed in the table. There are 4 sections in the table: 1) material/equipment 2) expansion media 3) differentiation media 4) primary and secondary antibodies. The name of material, vendor, and catalog number have been provided here.

Table 1. All the components for making expansion media have been summarized in the table 1. The detailed information about reagent stock concentration, stock storage, amount of stock for making 500mL media, and final concentration have been described here.

Table 2. All the components for making differentiation media have been summarized in the table 2. The detailed information about reagent stock concentration, stock storage, amount of stock for making 500mL media, and final concentration have been described here.

We have added the title and description to each table and removed the company name and catalog number from the tables to the table of materials according to editor's suggestions.

Reviewer #1 :

1) Lines 96-102: The authors mention other organoid models, such as intestinal organoids, as comparative methods to their HNE organoids. The rational behind the advantages of HNE compared to, in the present case, intestinal organoids is not entirely clear. We suggest the authors to motivate their specific advantages in a more compelling fashion.

The advantages of HNE compared to intestinal organoids are detailed in our prior publications which are cited, but we do include a detailed explanation in this section of the text. The main advantages of the nasal organoid over the intestinal organoid assay is that the intestinal organoid assay is not universally available, and in the United States where sedation for rectal biopsy is common in pediatrics, can be difficult to obtain (and get IRB approval for). The nasal brushing requires no sedation, and requires little specialized equipment other than a cytology brush. This allows nasal brushing to be performed by more laboratories who may not have the ability to conduct intestinal organoid culture. These advantages are already detailed in lines 96-102, which allows the HNE organoid model to be performed in any laboratory that cannot readily access intestinal tissues, thus expanding organoid technologies to additional investigators. Furthermore, airway organoids may be used for other disease states that primarily affect the airway, including primary ciliary dyskinesia and even respiratory viral infection (very common currently during the pandemic). We have added an additional statement to this effect at the end of the relevant paragraph "Furthermore, HNE organoids can be utilized to study other disease states that affect the airway, such as primary ciliary dyskinesia (1) or viral infection, which intestinal organoids cannot."

2) Lines 142-143: The removal of the feeder cells is poorly described. These feeder cells if left over in the HNE cells solution can have great influence on the organoid culture in BME.

We have revised the text to clarify in all steps that the feeder 3T3 cells are irradiated and inactivated. They are tested prior to use in our laboratory to ensure they cannot proliferate. The double trypsinization method is detailed and cited, and has been used with success by many laboratories. In our experience irradiated inactivated feeder 3T3s are easily removed from culture; occasionally some airway epithelial cells are lost in order to ensure that all fibroblasts are removed. In our experience, we have never seen any evidence that fibroblasts are

proliferating once seeding the airway epithelia into organoid culture. We have added to step 1.4 “Prior to use, test the 3T3 fibroblasts to ensure they cannot proliferate, which will negatively influence epithelial cell expansion.” and to step 1.6 “This method ensures that the irradiated and inactivated 3T3 fibroblasts are removed from the flask and do not contaminate subsequent organoid seeding.”

Lines 263-330, section 5, lines 332-361, section 6, lines 387-419, section 8: The authors are describing a set of methods to set up an automated imaging routine. We suggest to revise the entire three sections as the methods are linked to specific and proprietary softwares and is, thus, not broadly applicable to the audience. We suggest to rewrite the sections in a broader view, adding the key elements found on any brightfield/fluorescence microscope that need to be prepare/set up in order to perform the three methods successfully with any system.
We have revised this entire section to be more general, as best as possible to assist readers to apply similar methodology to their own equipment capable of automated imaging.

Lines 501-502: The time from biopsy to processing for achieving the best yield of HNE organoid generation is extremely short and thus could be a key challenge for a broader audience, i.e. proximity to biopsy site, etc…
We have concerns about loss of viability of cells and contamination of the sample when the sample is not processed within four hours. We did note that there was a typographical error (two hours instead of four), which we have corrected. We have not tested longer timeframes. This is an anecdotal observation and preference. We have also included this limitation in a new paragraph at the end of the document since we agree that this may be limiting to investigators. We have also added a statement that other investigators using a similar protocol report that samples can be stored on ice up to 24 hours and included that reference in paragraph 2 of the discussion.

Lines 503-506: The use of feeder cells is a clear disadvantage of the technique and can introduce very high variability over time. We suggest the authors to comment on the possibility to avoid the use of feeder cells.
We have addressed this critique in two places. In the paragraph “Introduction to the technique” we added a sentence at the end “While this protocol was developed with the use of feeder cells, other methodologies exist that may be used by investigators wishing to avoid feeder cell technology.(1-3)” In addition to revision of the statement in the Discussion approximately “The culture media and reagents we use…other commercially available media *and methods* may be useful for other applications…” we also added additional references to other methodologies that do not rely on the use of feeder cells.

Minor Concerns:
Line 124: The digestion step is poorly described , digestion solutions and manipulations can have a significant impact on the resulting organoids quality.
To address this critique, we will include a video segment on the dissociation step. The digestion step, in section 1.3, the “cell detachment solution” is a brand that (due to journal requirements) cannot be specifically stated in the text but is detailed in the Table of Materials (including brand and catalog number) and is an alternative to traditional trypsin. This step is prior to expansion of epithelial cells in the flask, and would not be expected to affect organoid quality which is many steps and several days later.

Line 157: The authors should specify what they mean by: "organoid culture artificial extracellular matrix"
We have revised this term to “extracellular matrix” or “ECM” throughout the text, as the manufacturer describes the commercial agent. Matrigel, by Corning, is an extracellular matrix based hydrogel, which was also

added to the text. This is detailed in the Table of Materials including brand and catalog number, as required by the journal.

Reviewer #2:
1) The Authors show a CFTR swelling assay for healthy individuals. As is described in sachs et al: https://www.embopress.org/doi/full/10.15252/embj.2018100300 and Geurts et al: https://www-sciencedirect-com.proxy.library.uu.nl/science/article/pii/S1934590920300199 airway organoids derived from people with Cystic Fibrosis, without a functional CFTR channel, retain quite a strong forskolin induced swelling response. Authors should also include data of a swelling response experiment using organoids from people with CF to show the need for this protocol.

The JOVE editors requested representative results for each of the major steps to demonstrate the methodology with a focus on the development and characterization of the model. The cystic fibrosis response is detailed with sufficient supporting data in our publications that are cited in the text. We have added a statement to the text in the final paragraph of Representative Results (line 456-457) "Data comparing non-CF and CF organoid response are detailed in prior publications" including citations for the two manuscripts where this data is shown.

The referenced materials from Sachs *et al* details a different tissue type from bronchoalveolar resections or lavage of lower airways, which most likely explains some of the difference in response, particularly those that may include alveolar material (which our model does not). As shown in our prior studies, we also see some swelling in CF organoids, but the degree of swelling is much less than non-CF. We were unable to determine which manuscript of Geurts *et al* was referenced, as the link is specific to the university.

2) In general the protocol has 8 parts, of which the first 4 can be performed without any additional specialist, other than cell culture, equipment. Thus for people that do not have these specific imaging tools available only these first 4 parts are of direct interest. Addition of swelling on organoids derived from individuals with CFTR and perhaps a drug screen using orkambi/tricafta would be a great addition to this protocol.

We agree that certain section may not be accessible to all investigators. Section 6 requires only a bright field microscope, with which most laboratories capable of cell culture are equipped, but that readers may wish to have an option for scaling up these experiments. We have revised section 5, 7, and 8 as requested by Reviewer 1 to provide more general detail that can be applied to other kinds of specialized equipment. We have added additional references to detail assays of CF organoids including a modulator screen in the final paragraph of the Representative Results section: "Data comparing non-CF and CF organoid response are detailed in prior publications. (11, 13)"

3) I would recommend to focus more on the easy of replication of the first 4 parts to make it easy for readers to understand in a first read. One example can be seen in line 124: "cell detachment solution". I would recommend to specify these things more clearly in text to make sure readers do not have to consult your equipment table as much.
We agree that inclusion of the specific reagents would be helpful; unfortunately the journal cannot include brand names in the text and so readers must consult the Table of Materials. This particular reagent is Accutase, which calls this reagent "cell detachment solution". We have gone through the manuscript in detail and provided additional information to help with this as much as possible.

4) The protocol now starts with a description of how to put nasal brush samples into culture.

However, no description is given about the nasal brushing itself. The manuscript would benefit from a short step-by-step protocol of how the brush biopsy is performed, including needed equipment.

The nasal brushing is well detailed already in other manuscripts including a video of the basics of the procedure in a prior JOVE article by other investigators as detailed in the response above. We have cited the relevant manuscripts and added to the sentence " HNEs were initially collected by brush biopsy as previously described, with the only difference being the use of a cytology brush". The manufacturer and catalog number of this brush added to the Table of Materials.

Minor Concerns:

5) The authors state that brush biopsies have to be transported to the lab within 4 hours of the procedure. Can they provide any data that supports this or is this anecdotal?

We have concerns about loss of viability of cells and contamination of the sample when the sample is not processed within four hours. We have not tested longer timeframes. This is an anecdotal observation and preference. We have also included this limitation in a new paragraph at the end of the document since we agree that this may be limiting to investigators. We have also added a statement that other investigators using a similar protocol report that samples can be stored on ice up to 24 hours and included that reference in paragraph 2 of the discussion.

6) In step 1 of the protocol "cell culture and expansion of nasal epithelial cells" there is a note regarding confluence. Could the authors provide brightfield images of samples that you would and would not use for further organoid establishing?

We have added a new figure (Figure 1) to show confluence that we would accept, as long as the cells reached that stage within the specified length of time. Longer times may yield the appropriate confluence but in our experience, cells that are slow to proliferate do not form good organoids.

7) Is there a specific reason for not using standard cell culture plates for the 3D propagation of HNEs?:

The rationale for the plates used (for swelling assays and most imaging) is detailed in the fourth paragraph of the discussion. Standard cell culture plates come in many varieties. Most are made of plastic that is not optimized for optical imaging, or are thick and thus place organoids away from the objective. They may also be large and use a lot of ECM, which is very expensive. Other investigators have used such plates for droplet technique organoids. This technique is useful but can create variation in the number and size of droplets, a meniscus that is not ideal for optical imaging (as the menisci create artifact in the image), and organoids can form in different planes, making imaging very challenging in general and impossible for automated approaches. Furthermore, the lower and upper chamber allow the use of small amounts of media, minimizing costs. These slides also come as 96 well plates for scaling up.

Reviewer #3:

Line 51: Source of [airway] epithelia; Line 60: using [inactivated] feeder; Line 116. 1.1 - Indicate at what temperature samples are kept during this step; Line 123: Indicate % of confluence of pre-seeded irradiated fibroblasts; Line 165: Indicate if 500 cell/ul is 500 live cells or total.

Each of these critiques have been addressed as requested.

Line 361 and reflected in discussion: Indicate if organoids with multilumen form in your experience and if so how they are measured

Rarely we do see these. For the automated imaging, multilumen organoids may be included as the total surface area of change is the outcome measurement. For BLR measurements, multilumen organoids are irregular and are not easily measured objectively, so when these are encountered for BLR measurements they are excluded.

line 369: Why is DAPI added to media in this step?
DAPI provides a quality control step. Most of the time, the bright field images are sufficient to ensure appropriate masking of organoids in the automated imaging. If a bright field image cannot allow us to confirm that the organoids are properly masked by the automated software, DAPI is used to confirm. The following lines have been added to the text, to clarify the purpose of DAPI.

Lines 409-410: “The incubation with DAPI is optional but is recommended as a fail-safe in case quality of bright field images is compromised in which case DAPI channel can be analyzed instead.”

Supplementary File 1

Click here to access/download

Supplemental Coding Files

Supplemental_File_1.prt

Supplementary File 2

Click here to access/download

Supplemental Coding Files

Supplemental_File_2.prt