Journal of Visualized Experiments

Collection, Expansion, and Differentiation of Primary Human Nasal Epithelial Cell Models for Quantification of Cilia Beat Frequency

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63090R2
Full Title:	Collection, Expansion, and Differentiation of Primary Human Nasal Epithelial Cell Models for Quantification of Cilia Beat Frequency
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Medicine
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Sydney, NSW, Australia
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TITLE:

Collection, Expansion, and Differentiation of Primary Human Nasal Epithelial Cell Models for Quantification of Cilia Beat Frequency

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SUMMARY:

This protocol describes nasal epithelial cell collection, expansion, and differentiation to organotypic airway epithelial cell models and quantification of cilia beat frequency via live-cell imaging and custom-built scripts.

ABSTRACT:

Measurements of cilia function (beat frequency, pattern) have been established as diagnostic tools for respiratory diseases such as primary ciliary dyskinesia. However, the wider application of these techniques is limited by the extreme susceptibility of ciliary function to changes in environmental factors e.g., temperature, humidity, and pH. In the airway of patients with Cystic Fibrosis (CF), mucus accumulation impedes cilia beating. Cilia function has been investigated in primary airway cell models as an indicator of CF Transmembrane conductance Regulator (CFTR) channel activity. However, considerable patient-to-patient variability in cilia beating frequency has been found in response to CFTR-modulating drugs, even for patients with the same *CFTR* mutations. Furthermore, the impact of dysfunctional

CFTR-regulated chloride secretion on ciliary function is poorly understood. There is currently no comprehensive protocol demonstrating sample preparation of *in vitro* airway models, image acquisition, and analysis of Cilia Beat Frequency (CBF). Standardized culture conditions and image acquisition performed in an environmentally controlled condition would enable consistent, reproducible quantification of CBF between individuals and in response to CFTR-modulating drugs. This protocol describes the quantification of CBF in three different airway epithelial cell model systems: 1) native epithelial sheets, 2) air-liquid interface models imaged on permeable support inserts, and 3) extracellular matrix-embedded three-dimensional organoids. The latter two replicate *in vivo* lung physiology, with beating cilia and production of mucus. The ciliary function is captured using a high-speed video camera in an environment-controlled chamber. Custom-built scripts are used for the analysis of CBF. Translation of CBF measurements to the clinic is envisioned to be an important clinical tool for predicting response to CFTR-modulating drugs on a per-patient basis.

INTRODUCTION:

Measurements of Cilia Beat Frequency (CBF) and pattern have been established as diagnostic tools for respiratory diseases such as Primary Ciliary Dyskinesia (PCD)[1]. In Cystic Fibrosis (CF), dysfunction of the CF Transmembrane conductance Regulator (CFTR) chloride channel causes dehydration of the airway surface liquid and impaired mucociliary clearance[2]. Ciliary function has been investigated *in vitro* in primary airway cell models as an indicator of CFTR channel activity[3]. However, considerable patient-to-patient variability exists in CBF in response to CFTR-modulating drugs, even for patients with the same *CFTR* mutations[3]. Furthermore, the impact of dysfunctional CFTR-regulated chloride secretion on ciliary function is poorly understood. There is currently no comprehensive protocol demonstrating sample preparation of *in vitro* airway models, image acquisition, and analysis of CBF.

Nasal epithelial sheets isolated from nasal mucosal brushings are directly used for measurements of ciliary function for PCD diagnosis[4]. Yet, while there is no control over the size or quality of the nasal epithelial sheets obtained, CBF varies depending on whether it is measured on single cells or cell sheets and on epithelial sheet ciliated edges that are disrupted or undisrupted[5]. As such, secondary dyskinesias caused by damage to cells during the collection of nasal mucosal brushings may influence CBF. Primary cell culture of nasal epithelial cells and their differentiation at Air-Liquid Interface (ALI) or in three-dimensional basement membrane matrix into ciliated airway epithelial organoids give rise to cilia that are free from secondary dyskinesias[4,6–8]. Airway epithelial cells differentiated at ALI (henceforth termed ALI models) have been deemed an important secondary diagnostic aid that replicate the ciliary beat patterns and frequency of *ex vivo* nasal mucosal brushings[6] and enable analysis of ciliary ultrastructure, beat pattern, and beat frequency while retaining patient-specific defects[9]. Yet, discrepancies exist in the methodologies used to create these pseudostratified, mucociliary differentiated cell models. Different culture expansion or differentiation protocols could induce distinct epithelial phenotypes (ciliated or secretory)[10] and result in significant differences in CBF[11]. CBF has been quantified in nasal epithelial brushings[4,6,12–16], airway epithelial organoids[14,17,18] and ALI models[3,4,6,13,19–21]. Yet, amongst these protocols, there are large variabilities, and often many parameters are not controlled for. For example, in some studies, CBF is imaged *in situ* while the cells of the ALI model remain on the permeable support insert[3,19–21], yet others scrape the cells from the permeable support insert and image them suspended in media[4,6,13].

Furthermore, the wider application of techniques that measure ciliary function is limited by the extreme susceptibility of ciliary function to changes in environmental factors. Environmental factors such as temperature[22], humidity[23,24], and pH[25,26] influence ciliary function and must be regulated to quantify CBF accurately. The various physiological parameters used across different laboratories and how they influence CBF has been reviewed previously[27].

Various imaging technologies and approaches to CBF measurements are reported in the literature. For PCD diagnostics, video microscopy is used to measure ciliary function[28,29]. Recently, a video analysis algorithm based on differential dynamic microscopy was used to quantify both CBF and cilia coordination in airway epithelial cell ALI models[3,30]. This method enables the characterization of ciliary beating in airway epithelial cells in a fast and fully automated manner, without the need to segment or select regions. Various methods for imaging and quantification of CBF may add to the differences reported in CBF in the literature (**Supplementary File 1**).

A protocol from culture to quantification to streamline existing methods, standardization of culture conditions, and image acquisition, performed in strict environmentally controlled conditions, would enable consistent, reproducible quantification of CBF within and between individuals.

This protocol provides a complete description of the collection of epithelial cells, expansion and differentiation culture conditions, and quantification of CBF in three different airway epithelial cell model systems of nasal origin: 1) native epithelial sheets, 2) ALI models imaged on permeable support inserts and 3) Extracellular Matrix (ECM)-embedded three-dimensional organoids (**Figure 1**). Nasal epithelial cells obtained from nasal inferior turbinate brushings are used as representatives of the airway epithelium since they are an effective surrogate for bronchial epithelial cells[31] while overcoming the invasive procedure associated with collecting bronchial brushings. The Conditional Reprogramming Cell (CRC) method is used to expand primary airway epithelial cells for the creation of ALI models and three-dimensional organoids. Conditional reprogramming of airway epithelial cells to a stem cell-like state is induced by co-culture with growth-arrested fibroblast feeder cell system and Rho-associated kinase (ROCK) inhibitor[32]. Importantly, the CRC method increases population doubling in airway epithelial cells while retaining their tissue-specific differentiation potential[33,34]. In all airway epithelial cell models, the ciliary function is captured in a temperature-controlled chamber using a high-speed video camera with standardized image acquisition settings. Custom-built scripts are employed for the quantification of CBF.

[Place **Figure 1** here]

PROTOCOL:

Study approval was received from the Sydney Children's Hospital Network Ethics Review Board (HREC/16/SCHN/120). Written consent was obtained from all participants (or participants' guardian) prior to the collection of biospecimens.

1. Preparations for establishing airway epithelial cell models

1.1. Prepare nasal cell collection media by combining 80% Dulbecco's Modified Eagle Medium and 20% Fetal Bovine Serum. Supplement with 1 µL/mL of Penicillin/Streptomycin. Store at 4 °C for up to 3 months.

1.2. Coat the flasks or permeable support inserts with Collagen solution on a per-need basis following steps 1.2.1–1.2.4. Do not store collagen-coated vessels long-term.

1.2.1. Make a 1:100 dilution of Type I Collagen solution (3 mg/mL stock) with phosphate-buffered saline (PBS) to an end concentration of 0.03 mg/mL. Mix well.

1.2.2. Coat the cell culture flasks (section 4) with 160 µL/cm^2 (i.e., 4 mL per T25 flask) and permeable support inserts (section 5) with 455 µL/cm^2 (i.e., 150 µL per 6.5 mm insert) of the prepared Collagen solution.

1.2.3. Incubate at 37 °C for 2–24 h.

1.2.4. Remove the Collagen solution by pipette or vacuum aspirator prior to seeding cells. Do not wash the vessel prior to seeding cells.

1.3. Prepare Conditional Reprogramming Cell (CRC) media by combining components[32] listed in **Table 1**. Filter sterilize using a bottle-top vacuum filter system. Store at 4 °C for up to 2 months.

1.4. On the day of use, add human epidermal growth factor, ROCK inhibitor and antibiotics as indicated in **Table 1**.

[Place **Table 1** here]

2. Collection of nasal inferior turbinate brushings

NOTE: This section of the protocol requires a collection tube (50 mL) with nasal cell collection media, cytology brushes, tissues, and appropriate Personal Protective Equipment. Avoid brushing during an upper respiratory tract infection. There is a small risk of bleeding, which is increased if inflammation is present. If the purpose of the brushing is to obtain airway epithelial sheets for *ex vivo* CBF measurements brushing should occur a minimum of 6 weeks post any upper respiratory infection; ideally, more than 10 weeks post infection[35].

2.1. Prepare the nasal cell collection media (section 1) and keep the tube on ice.

2.2. Describe the procedure to the participant as uncomfortable. Explain that a full sensation is felt in the nostril during the brushing, similar to jumping into the ocean/pool and water rushing into the nasal passage. Advise participants that the procedure will induce the production of tears as a reflex.

2.3. Assess which positioning is appropriate for the participant. Lay the participant in supine position if an examination couch is available since supine positioning prevents movement of the participant's head away from the brush during the procedure. Alternatively, seat the participant next to a wall, which they can press their head back against.

2.4. Inspect the nasal passage. Note septal deviation, polyps, and any other anatomical abnormalities that may affect the passage of the brush in the nasal passage and increase bleeding risk.

2.5. Clean the nose of excess mucus by asking participants to blow their nose into a tissue.

2.6. Ask the participant to breathe through their mouth. Take a cytology brush in the dominant hand. While resting the fifth digit on the participant's chin to anchor the hand, insert the cytology brush into the participant's nasal passage (**Figure 2)**. Insert the brush at ~45° to the participant's face to pass through the nasal meatus.

2.7. Pivot the brush upright so that it is perpendicular to the participant's face. Advance the brush gently but firmly against the lateral wall of the nose beneath the inferior turbinate until it is at the mid to posterior part of the inferior turbinate.

NOTE: Avoid over-insertion; if a sudden drop in resistance is felt, the nasal pharynx has been entered, and the brush should be retracted until resistance is again felt by the proceduralist.

2.8. Rotate the brush 360° up to three times. Remove the brush gently in reverse of the insertion maneuver, so cells are not dislodged from the brush.

2.9. Place the brush into the prepared collection tube with nasal cell collection media. Place the collection tube on ice.

2.10. Repeat the brushing in the second nostril if the participant is agreeable/a large number of cells are required (e.g., to initiate cell culture).

NOTE: The same nostril can be brushed again if there were no visible blood cells on the brush, noting, however, that the risk of bleeding is slightly increased with a second brushing in the same nostril.

[Place **Figure 2** here]

3. Preparation of airway epithelial sheets

NOTE: This section of the protocol requires collection tube (cytology brush(es) + 1 mL of nasal cell collection media) (section 2) and 96-well flat-bottomed plate. If collecting nasal turbinate brushings for the purpose of imaging airway epithelial sheets, only use 1 mL of antibiotic-free nasal cell collection media; otherwise, epithelial sheets will be too dispersed for imaging.

3.1. Gently swirl the collection tube containing the cytology brush(es) to dislodge the airway epithelial sheets from the brush(es).

3.2. Collect all media and cells with a P1000 pipette. Dispense 5–6 drops into a well of a 96-well flat-bottomed plate. Repeat for approximately seven wells.

3.3. Transfer plate to the microscope as per step 7.1.4 and follow the remainder of section 7 to image cilia beat frequency.

3.4. Image epithelial sheets (**Figure 1**) and not single unattached cells since it has been demonstrated that ciliary function differs between epithelial sheets and single unattached cells[5].

4. Airway epithelial cell expansion and maintenance

4.1. Airway epithelial conditional reprogramming cell expansion culture

NOTE: Collagen solution coated vessel (section 1), Irradiated mouse embryonic feeder cells (NIH-3T3), Conditional Reprogramming Cell (CRC) media (section 1), cytology brush(es) in nasal cell collection media (section 2).

4.1.1. Plate irradiated feeder cells into prepared Collagen solution coated culture vessel(s) at a seeding density of 8,000 cells/cm^2 at least 2 h and no more than 72 h prior to co-culture with airway epithelial cells (see[36] for feeder cell culture and irradiation).

4.1.2. Transfer the brushed cells in the collection tube (cytology brush(es) + nasal cell collection media) to the vortex on ice. On a low speed, vortex tube 10 s on, 10 s off (keep on ice in between) to dislodge cells from the brush(es). Vigorous vortexing may decrease cell viability. Inspect the brush(es) to check if the mucus is still adhered. If so, repeat the vortexing.

4.1.3. Transfer the tube(s) on ice back to the biosafety cabinet. Use a serological pipette to transfer the media from the collection tube to a new tube (Tube B), leaving behind the cytology brushes. Centrifuge Tube B at 300 × *g* for 7 min at 4 °C.

4.1.4. Remove Tube B from the centrifuge, discard the supernatant. If the mucus is visible, wash the pellet with another 5 mL of nasal cell collection media and centrifuge again.

4.1.5. Add 1 mL of CRC media to resuspend the cell pellet in Tube B. Using a 5 mL serological pipette, pass cells through a cell sieve placed on top of a 50 mL tube (Tube C) in a circular motion.

4.1.6. Repeat multiple times to form a single cell suspension. Collect the residual media from the bottom of the sieve and incorporate it with the media. Discard the cell sieve.

4.1.7. Using a 5 mL serological pipette, take 1 mL of media from Tube C and transfer it into a microcentrifuge tube.

4.1.8. Take 10 µL of this cell suspension and add it to the microcentrifuge tube pre-aliquoted with 10 µL trypan blue. Mix well and immediately use an automated cell counter to record cell count and viability.

4.1.9. Seed the airway epithelial cells into the T25 flask pre-seeded with irradiated feeder cells.

4.2. Airway epithelial cell maintenance and dissociation

NOTE: CRC media must be warmed to 37 °C by placing it in a temperature-controlled laboratory water bath or a bead bath device before it is added to the cells.

4.2.1. Check cells under the cell culture microscope (4× objective lens) regularly for attachment, contamination, morphology, and confluence.

4.2.2. Change CRC media every second day. When reprogrammed cells are observed (**Figure 1**) and there is no contamination present, reduce or withdraw antibiotics.

4.2.3. When cells reach 90% confluency, use a double trypsin method[32] to dissociate the cells and perform a cell count as described in step 4.1.8 (refer to **Supplementary File 2** for cell dissociation and freezing).

5. Seeding and differentiation of airway epithelial cells and maintenance of differentiated ALI models

5.1. Seeding airway epithelial cells to permeable support inserts

5.1.1. Transfer the Collagen solution coated permeable support inserts (section 1) from the CO_2 incubator to the biosafety cabinet. Aspirate the Collagen solution and discard. Add 750 µL expansion medium (antibiotic-free) to the basal compartment of the permeable support inserts.

5.1.2. Transfer the dissociated cells or thawed cells on ice to the biosafety cabinet. Add the volume of expansion medium needed to seed 200,000–250,000 cells in 150 µL to the apical compartment of each permeable support insert.

5.1.3. Being careful not to create bubbles; mix well to ensure the cells are homogenous and in suspension. Add 150 µL of the cell suspension to the apical side of each permeable support insert.

5.1.4. Resuspend the cells after seeding every three permeable support inserts to maintain a homogenous cell suspension.

5.1.5. Every second day until a confluent cell monolayer is formed (usually by Day 4 post seeding), discard the media and add fresh expansion medium warmed to room temperature (RT, 15–25 °C).

5.2. Differentiation of airway epithelial cells at the air-liquid interface

5.2.1. Warm ALI media (antibiotic-free) to RT (15–25 °C).

5.2.2. Remove the expansion medium and change to differentiation media (ALI) on both apical and basal compartments.

5.2.3. After 2 days of culture in submerged ALI media, aspirate and discard the media.

5.2.4. Add 750 µL of ALI media to the basal compartment only to create an air-liquid interface.

NOTE: If after 1 week of culture, the monolayer is not confluent and holes are still observed, cells may no longer have the capacity to expand into the void regions, consider discarding of the airway epithelial cells.

5.3. Maintenance of differentiated ALI model and mucus removal

5.3.1. Change the apical and basal media every second day until full differentiation (day 21–25 post air-liquid interface establishment).

5.3.2. Once per week, wash mucus from the apical side following steps 5.3.3–5.3.4.

5.3.3. Warm PBS to RT (15–25 °C).

5.3.4. Add 200 µL of PBS to the apical compartment. Incubate in the CO_2 incubator for 10 min. Use an aspiration device or pipette to remove the PBS.

6. Three-dimensional airway epithelial organoids

6.1. Preparations for airway epithelial organoid culture

6.1.1. Place 24-well plate(s) in a CO_2 incubator to warm to 37 °C overnight.

6.1.2. Thaw a 10 mL vial of ECM (**Table of Materials**) on ice as per the manufacturer's instructions. Prepare 500 µL aliquots (one-time use) to minimize the number of freeze-thaw cycles.

NOTE: Use ECM with protein concentration >10.5 mg/mL for the best culture outcomes is recommended. Lower concentration will accelerate the disintegration of the ECM dome and increase the occurrence of apical-facing-outwards organoids.

6.1.3. Use the Airway Organoid Kit (**Table of Materials**) to prepare Airway Organoid Seeding Media (AOSM) and Differentiation Media (AODM) as per the manufacturer's instructions.

6.1.4. Prepare airway organoid basal media as per **Table 2**.

[Place **Table 2** here]

6.1.5. Use the number of live airway epithelial cells dissociated in section 4.2 to calculate how many wells can be seeded at a seeding density of 10,000 cells (see **Table 3**).

6.1.6. Calculate the total volume of ECM and AOSM needed to create 1 x 50 µL of 90% ECM dome (45 µL of ECM and 5 µL of AOSM) per well.

NOTE: The recommended seeding density of 10,000 cells per well is for CRC-expanded nasal epithelial cells at passage 1. Later passage cells may require higher seeding density to achieve the formation of the same number of organoids.

[Place **Table 3** here]

6.2. Seeding airway epithelial cells in ECM domes

NOTE: Keep ECM on ice at all times and perform all steps involving ECM on ice, since ECM will start to solidify at temperatures >10°C.

6.2.1. Resuspend the airway epithelial cells dissociated in section 4.2 with the calculated volume of 90% ECM as per **Table 3**.

6.2.2. Holding the pipette at a 90° angle (vertical) as close to the bottom of the well as possible, dispense 50 µL (to the first stop to avoid creating bubbles) of the ECM cell suspension to the center of the well. Avoid touching the wall of the well.

6.2.3. Incubate plate at 37 °C for 20 min until the ECM solidifies. While the ECM is solidifying, warm AOSM to RT (15–25 °C) to prevent it from causing re-liquification and disintegration of the ECM dome upon addition.

6.2.4. Add 500 µL of warmed AOSM to each well by dispensing down the wall of the well. Do not pipette media directly onto the ECM dome.

6.2.5. Change media every 2 days for 4–7 days. To aspirate media, tilt the plate at a 45° angle and aspirate from the bottom edge of the well away from the ECM dome.

6.2.6. After 4–7 days, initiate organoid differentiation by adding 500 µL of AODM (15–25 °C) to each well and change media every 2 days for 7 days.

6.3. Replating airway epithelial organoids at Day 7 of differentiation

NOTE: Replating airway epithelial organoids is necessary because the edge of the ECM domes gradually disintegrate over the 2-week culture period. Airway epithelial organoids at the edge of the dome may be lost (dislodge into the media) or have apical-facing-outwards orientation when not fully embedded in ECM. The replating step also “cleans up” the ECM dome by removing cells/debris which does not successfully form organoids.

6.3.1. Aspirate the media from each well. Add 500 µL of cold airway organoid basal media (henceforth termed basal media) to each well.

6.3.2. Use the P1000 pipette since this pipette tip has the largest orifice and will reduce the likelihood of organoids bursting during pipetting. Adjust the pipette to 350 µL to avoid creating bubbles, then pipette up and down gently to disrupt the ECM dome in each well. Collect all ECM/basal media into a 15 mL centrifuge tube.

6.3.3. Rinse each well with 500 µL of cold basal media. Collect the basal media containing any remaining ECM and organoids into the same 15 mL centrifuge tube as above.

6.3.4. Centrifuge at 300 x *g* for 5 min at 4 °C. Of the three layers visible following centrifuging – (1) supernatant, (2) ECM containing cellular debris (fluffy) and (3) pellet containing organoids – discard the supernatant and ECM layer and preserve the organoid pellet.

6.3.5. Add 1 mL of cold basal media to the organoid pellet and pipette up and down gently to separate any remaining ECM. Add 6 mL of cold basal media to the tube and mix gently.

6.3.6. Centrifuge at 300 × *g* for 5 min at 4 °C. Discard the supernatant.

6.3.7. If excess ECM is still visible, repeat steps 6.3.5– 6.3.6 to perform another wash.

6.3.8. Resuspend the organoid pellet with an appropriate volume of 90% ECM (use AODM instead of AOSM) to plate ~30 organoids per 50 µL of the dome.

6.3.9. Check the density of organoids under the cell culture microscope (4x objective lens) after plating the first dome. If too dense, add an additional 90% ECM to achieve the desired density of ~30 organoids.

6.3.10. Follow steps 6.2.3– 6.2.4 to solidify ECM and feed cells every second day with 500 µL of warmed AODM to each well for another 14 days until they reach maturity (after 21 days of differentiation) with lumen formation surrounded by inward-facing pseudostratified epithelium containing basal cells, ciliated cells, and goblet cells.

NOTE: The airway epithelial organoids described here are terminally differentiated and cannot be passaged or cryopreserved.

7. Imaging cilia beat frequency

NOTE: This section of the protocol requires a live-cell imaging microscope with a heating and humidity environmental chamber, a fast frame rate (>100 Hz) scientific camera, a 20x long working distance objective, and imaging software (refer to **Table of Materials** for recommended equipment used in this protocol).

7.1. Microscope set up

7.1.1 Ensure the microscope heating system is turned on and equilibrated to 37 °C. Turn on the microscope. Adjust the gas to 5% CO_2 via the CO_2/air gas mixer.

7.1.2 Top up the humidity module bottle that the CO_2 passes through with purified water. Set the relative humidity to 85% via the stage top controller so that the water is heated and the cells are supplied with humidified air. Equilibrate the chamber for 30 min.

7.1.3 Place the microscope plate insert into the microscope holder.

7.1.4 Transfer the airway epithelial cell models from the incubator to the microscope on a heat block or thermal beads equilibrated to 37 °C to maintain the sample at a physiological temperature.

7.1.5 Place the culture plate containing the airway epithelial cell models into the microscope plate insert. Close the microscope environmental chamber.

7.1.6 Allow the sample to equilibrate in the pre-warmed 37 °C, 5% CO_2-filled microscope chamber for 30 min.

NOTE: A shorter equilibration time may be sufficient. This can be determined by performing an experiment to identify the time required for the stabilization of CBF (refer to **Figure 3**).

[Place **Figure 3** here]

7.1.7 During the equilibration period, at the computer, open the acquisition software. Select the 20x long working distance objective lens.

7.1.8 At the microscope eyepiece, focus on the cell model (~Z = 8000 µm).

7.1.9 Ensure that the microscope is set up for Kohler illumination so that transmission light source bulb filaments are not focused on the sample plane, avoiding artifacts in the imaging. For this follow steps 7.1.10–7.1.13

7.1.10 Completely close the field iris diaphragm above the condenser. Slowly open the field iris diaphragm and move the condenser up/down until an octagon shape appears.

7.1.11 If the field iris diaphragm is not aligned (i.e., the octagon is not in the center of the field of view (FOV)), align it to the center using Allen keys.

7.1.12 Once the field iris diaphragm is aligned, adjust the condenser focus to bring the octagon into sharp focus.

7.1.13 Open the field iris diaphragm until it can no longer be seen within the FOV.

7.1.14 Using the acquisition software, click on **L100** to switch the light path to the port where the camera is mounted. Click on the green play (**Run**) button to visualize the microscope FOV via the software. Check that the cilia are in focus and adjust if required.

7.1.15 Using the acquisition software, set up the microscope with the following settings: Filters: empty; Condenser: empty; Format: no binning; Exposure time: 0.003 s; Readout mode: rolling shutter; ROI: 512 × 512 pixels.

NOTE: Exposure time is based on the highest frequency that needs to be measured since 1/exposure time must be at least twice this frequency. E.g., if the maximum physiological range of cilia beating = 30 Hz, then 1/exposure time = 60, and exposure time must be ≤ 0.016 s. ROI depends on camera frame rate specifications. Select an ROI that captures frame rates >100 Hz.

7.2. Image acquisition

7.2.1 To acquire time-lapse images from the menu, click on **Acquire** and then click on **Fast Time Lapse**. In the pop-up window, select a save location and file name. Acquire 1000 frames.

7.2.2 Click on **Apply**. Click on the green play (**Run**) button to preview the cilia in the microscope FOV and adjust the Z focus if required. Click **Run Now** to capture the fast time-lapse.

7.2.3 Once the fast time-lapse has been captured, click on the green play (**Run**) button to visualize the microscope FOV. Using the microscope joystick, move along the X/Y axis to another FOV.

7.2.4 Adjust the Z focus to bring the cilia into focus. Click on **Run Now** to capture another fast time-lapse.

7.2.5 Repeat steps 7.2.3–7.2.4. For ALI models and airway organoids, image 6x FOV in each of 3x replicate samples. For airway epithelial sheets, image a minimum of 4x replicate images per participant.

8. Data analysis and quantification of CBF

8.1. Preparations for data analysis

NOTE: This section of the protocol requires custom analysis scripts (**Supplementary File 3**), raw image files (acquired in section 7.2), a computing software, and analysis software.

8.1.1. Install the computing software, preferably the latest version, on the analysis computer. Ensure that standard computing software toolboxes (elmat, ops, datafun, uitools, datatypes, iofun, iotools, audiovideo) and Image and Signal Processing toolboxes are installed.

8.1.2. Copy the custom analysis scripts 'BeatingCiliaBatchOMEfiles_JOVE.m' and 'LoadRawDataExportFilteredMovies_JOVE.m' and 'support scripts' folder to the local drive of the computer.

8.1.3. On the computing software, click on the **Home** tab. Then click on **Set Path** (**Figure 4A–B**).

8.1.4. In the pop-up window, click on **Add With Subfolder**s (**Figure 4C**). Under 'MATLAB search path', select the folders shown in **Figure 4D**, then click on **Save** and **Close** (**Figure 4E–F**).

8.1.5. Confirm that the analysis scripts are linked to the computing software by checking that they appear in the left-hand panel (**Figure 4G**).

8.1.6. Transfer the raw image files (open microscopy environment (OME) format) acquired in section 7.2 to the computer's local drive.

NOTE: Example raw image files may be accessed at: https://doi.org/10.6084/m9.figshare.16649878.v1.

[Place **Figure 4** here]

8.2. Quantification of CBF by peak detection of the spectrum of intensity of single pixels

8.2.1. Open the computing software. Click on the 'BeatingCiliaBatchOMEfiles_JOVE.m' analysis script file (**Figure 5A**).

8.2.2. Click on the **Editor** tab, and then click on the green play (**Run**) button to run the script (**Figure 5B–C**). In the prompt window, select the raw image files to be analyzed (**Figure 5D**).

8.2.3. Enter the exposure time from step 7.1.15 into the prompt window for acquisition time per frame, then click on **OK** (**Figure 5E**).

8.2.4. Wait ~15 min per file while the script computes and outputs the CBF in the 'AveSpectrum' file (**Supplementary File 4**), which is automatically saved to the same folder as the raw image files. Visualize the progress via the progress bar (**Figure 5F**).

[Place **Figure 5** here]

8.2.5. Run the 'GetFirstAmplitude.m' script on the folder that contains the 'AveSpectrum' files using the process in steps 8.2.1–8.2.2. Wait for the script to output the 'FirstAmplitudeStacked.xlsx' file, which contains the frequency that has the highest amplitude and is within the physiological range of airway epithelium cilia beating, ≥3 and <30 Hz.

8.2.6. Copy the frequency values from the 'FirstAmplitudeStacked.xlsx' file and plot using a scientific analysis software.

NOTE: An explanation of how the custom analysis script quantifies CBF is provided in **Supplementary File 5**. Example analyzed datasets may be accessed at: https://doi.org/10.6084/m9.figshare.16649815.

8.3. Exporting a video of cilia beating

8.3.1. Open the computing software. Click on the 'LoadRawDataExportFilteredMovies_JOVE.m' script file (**Figure 5A**) to load the script.

8.3.2. Click on the **Editor** tab, and then click on the green play (**Run**) button to run the script (**Figure 5C**). In the prompt window, select the raw image files to be exported to movie files (**Figure 5D**).

8.3.3. Input the settings detailed in **Table 4** into the 'Make Movie' pop-up window (**Figure 5G**).

8.3.4. Wait ~8 min per file while the script creates the movie files and outputs them to the location of the raw image files. Visualize the progress via the progress bar (**Figure 5H**).

[Place **Table 4** here]

REPRESENTATIVE RESULTS:

To demonstrate the efficiency of this protocol in quantifying CBF, the results of CBF measured in airway epithelial cell ALI models derived from three participants with CF and three healthy control participants are presented. On Day 14 of culture differentiation, beating cilia were present (**Figure 6**). From Day 14 to 21 of culture differentiation, a statistically significant ($P < 0.0345$) increase in CBF was observed within both cohorts. On Day 21 of culture differentiation, the mean CBF for healthy control participants (7.61 ± 0.11 Hz) was significantly higher than that of participants with CF (6.75 ± 0.17 Hz). To understand the extent to which mucus accumulation and removal impact CBF, CBF was imaged in the same cell models following the removal of mucus. In ALI models of both healthy individuals and those with CF, there was a statistically significant ($P < 0.0001$) increase in CBF when mucus was removed (**Figure 6**).

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of workflow. Following brushing the participants' nasal inferior turbinate, airway epithelial cells are utilized in one of two ways. Either airway epithelial sheets are isolated, and cilia beat frequency is imaged immediately, or airway epithelial cells are expanded via the conditional reprogramming cell method. CRC-expanded airway epithelial cells are differentiated to establish airway epithelial cells at an air-liquid interface or airway epithelial organoid cultures. Imaging of ciliary beat frequency is acquired using a live-cell imaging microscope with a heating and humidity environmental chamber and a fast frame rate (>100Hz) scientific camera. Data analysis is performed using custom-built scripts.

Figure 2: Collection of nasal epithelial cells. Illustration of the location of the cytology brush at the mid to posterior part of the inferior turbinate. This position is reached by inserting the brush through the nares, pivoting the brush to a 90° angle to the face and guiding the brush along the nasal passage below the inferior turbinate.

Figure 3: Stabilisation of ciliary beat frequency in live-cell imaging microscope. Dot plots of mean cilia beat frequency (CBF) in airway epithelial cells at the air-liquid interface (ALI models) following transfer into a live-cell imaging microscope with an environmental chamber. Chamber was equilibrated and maintained at 37 °C, 5% CO_2 and relative humidity of 85% for 30 min prior to opening the chamber door and placing the culture plate into the microscope plate insert. Cell models were imaged for 60 min at indicated intervals. ALI models were derived from two participants with CF. Six field of view (FOV) images were acquired per ALI model. Each dot (blue) represents the mean CBF in 12–36 FOV images. Data are represented as mean ± SEM, with mean connected by a dotted line. One-way analysis of variance (ANOVA) was used to determine statistical differences. **** $P < 0.0001$, ns: no significance.

Figure 4: Setting up computing software for data analysis. (**A**) Open the **Home** tab. (**B**) Select **Set Path**. (**C**) Select **Add with Subfolders**. (**D**) Select folders containing the analysis scripts. (**E**) Select **Save**. (**F**) Select **Close**. (**G**) The analysis scripts will appear in the left-hand panel.

Figure 5: Running analysis scripts using computing software. (**A**) Open the script for analysis of CBF ('BeatingCiliaBatchOMEfiles_JOVE.m') or creation of cilia beating movie ('LoadRawDataExportFilteredMovies_JOVE.m'). (**B**) Open the **Editor** tab. (**C**) Select the green play (**Run**) button to run the analysis script. (**D**) A prompt window will require the selection of files for analysis or movie creation. (**E**) While running the 'BeatingCiliaBatchOMEfiles_JOVE.m' script, a prompt will appear to manually input the acquisition time per frame (s) in case the file-reading script does not read the metadata properly. (**F**) Progress bar indicating cilia beat frequency being computed. (**G**) While running the 'LoadRawDataExportFilteredMovies_JOVE.m' script, a prompt will appear to manually input the type of movie to be outputted (mp4 or avi), the movie frame rate (fps), whether the immobile component is removed from the movie data ('y' or 'n'), the frame time (s), and the pixel size (microns) of the data exported into the movie. It is recommended to use 'y' for immobile filtering as it will remove mucus or any other obstructing immobile layers in the data. (**H**) Progress bar for indicating movie being exported.

Figure 6: Effect of mucus removal on cilia beat frequency. Dot plots of mean cilia beat frequency (CBF) in airway epithelial cells at the air-liquid interface (ALI models), acquired pre- and post-mucus washout on day 14 and 21 of culture differentiation. Mucus washout was performed by washing the apical cell surface with 200 µL of warmed PBS. ALI models were derived from healthy participants (n=3) and participants with CF (n=3). Data are represented as mean ± SEM. Each dot represents a single field of view image. Six field of view images were acquired in three replicate ALI models. Each participant is coded with a different color. One-way analysis of variance (ANOVA) was used to determine statistical differences. **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

Table 1: Components for 500 mL of conditional reprogramming cell media

Table 2: Components of airway organoid basal media

Table 3: Calculations for seeding airway epithelial cells in ECM domes

Table 4: Input settings for movie creation

Supplementary File 1: Summary of 18 publications showing the diversity of culture and live-cell imaging parameters used to quantify cilia beat frequency in organotypic models of the airway epithelium.

Supplementary File 2: Airway epithelial cell dissociation for differentiation or cryopreservation

Supplementary File 3: Custom analysis scripts

Supplementary File 4: Data files outputted by analysis script

Supplementary File 5: Description of the CBF analysis algorithm

DISCUSSION:

There are multiple factors that could obscure the quantification of CBF in nasal epithelial sheets. Epithelial sheets should be imaged within 3-9 hrs of sample collection since the ciliary function is most stable during this time[37]. Less red blood cells and debris are most optimal for imaging since these interfere with data acquisition. When selecting an ROI for imaging, it is important to select an epithelial sheet that edge has not been damaged or disrupted during the collection of the sample, and not a single unattached epithelial cell, since these variables have been demonstrated to influence CBF[5]. It is also necessary that the epithelial sheet is stationary because movements may obscure data acquisition. Epithelial sheets in media often orientate in different directions[27]. Since it has been previously demonstrated that variances in sample quality, such as disrupted epithelial edges, impact CBF[5], it is likely that the orientation of the epithelial sheet may also influence CBF. One limitation of this protocol is that the impact of the epithelial sheet orientation has not been assessed. This is anticipated to be an important area of study for further standardization.

Mature ALI models have a pseudostratified epithelium with the presence of goblet cells that produce mucus[34]. The abundance and viscosity of mucus impact ciliary function[2,38]. It was recently shown that removing accumulated mucus from nasal epithelial ALI cell models caused an increase in CBF[3]. A cyclical process was described, wherein the regeneration of the mucus over a 24-h period compromised CBF until the mucus was removed and CBF increased again. To demonstrate that the repeatability of CBF measurements is contingent on the regulation of the physiological environment of cilia, the impact of mucus removal on CBF was assessed. A statistically significant 3.5 Hz change in CBF was observed following mucus removal. In comparison to these results, a recent study testing cilia response to CFTR-modulating compounds[3] reported a change in CBF no greater than that caused by mucus removal in our model system. This emphasizes the importance of regulating environmental variables that influence CBF, especially if this model system is to be established as a platform for studying the patient-specific response to treatments in the future. As such, it is recommended to be consistent in either removing mucus or not removing it prior to imaging in order to control the influence of this environmental variable of quantifications of CBF.

Temperature is the dominant factor that causes fluctuations in CBF. Cilia have previously been shown to be sensitive to fluctuations in physiological temperature in mouse lung slices and nasal biopsies[39,40]. As such, it is critical to observe steps that minimize environmental temperature fluctuations when handling samples for image acquisition and ensure cells are stabilized in the 37 °C microscope chamber prior to imaging. When imaging cilia, the cilia should come into focus just above the cell monolayer. If cilia beating is not observable via light microscopy, removing accumulated mucus by washing with warmed PBS may increase cilia beating since it is known that mucus obstructs cilia beating[3]. Another suboptimal situation will be if there is a movement of mucus over the cells, as this will obstruct data acquisition. A solution would be to select an ROI without the visible movement of mucus. However, in the situation where this is unavoidable, removing the mucus by washing is recommended.

An important caveat to consider is selecting an appropriate camera and objective lens to fulfill the temporal and spatial Nyquist sampling. The long working distance lens employed in this study protocol allows a relatively large field of view to be captured. This enables CBF to be imaged in intact ALI cultures, with a spatial resolution of ~500 nm (NA0.45). As such, the ciliary bundle can be spatially resolved. Yet, a limitation of this protocol is that the whole ALI model cannot be imaged at a resolution amenable to analysis. As a result, an ROI must be selected for data acquisition. To limit the bias associated with selecting an ROI, it is recommended that six ROIs are selected from different zones within each permeable support insert. This is important as it has been previously demonstrated that cilia do not beat synchronously within a sample, and CBF varies between different edges and ROI[16,41], implying that different ROIs likely have different average CBF values. Moreover, it is essential to have access to fast-speed cameras with a frame rate of at least 100 Hz so that any temporal event happening at a rate of 50 Hz can be resolved by the Nyquist sampling criterion. A fast sCMOS camera with extremely low noise allowing single-molecule measurement is recommended. However, this protocol is not limited by the use of this type of camera, so long as the camera fulfills temporal sampling requirements and captures the pixels intensity fluctuations resulting from ciliary beating.

ACKNOWLEDGMENTS:

We thank the study participants and their families for their contributions. We appreciate the assistance from Sydney Children's Hospitals (SCH) Randwick respiratory department in the organization and collection of patient biospecimens – special thanks to Dr. John Widger, Dr. Yvonne Belessis, Leanne Plush, Amanda Thompson, and Rhonda Bell. We acknowledge the assistance of Iveta Slapetova and Renee Whan from the Katharina Gaus Light Microscopy Facility within the Mark Wainwright Analytical Centre at UNSW Sydney. This work is supported by National Health and Medical Research Council (NHMRC) Australia (GNT1188987), CF Foundation Australia, and Sydney Children's Hospital Foundation. The authors would like to acknowledge Luminesce Alliance - Innovation for Children's Health for its contribution and support. Luminesce Alliance - Innovation for Children's Health is a not-for-profit cooperative joint venture between the Sydney Children's Hospitals Network, the Children's Medical Research Institute, and the Children's Cancer Institute. It has been established with the support of the NSW Government to coordinate and integrate pediatric research. Luminesce Alliance is also affiliated with the University of Sydney and the University of New South Wales Sydney. KMA is supported by an Australian Government Research

Training Program Scholarship. LKF is supported by the Rotary Club of Sydney Cove/Sydney Children's Hospital Foundation and UNSW University postgraduate award scholarships.

DISCLOSURES:

The authors declare that they have nothing to disclose.

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Figure 1

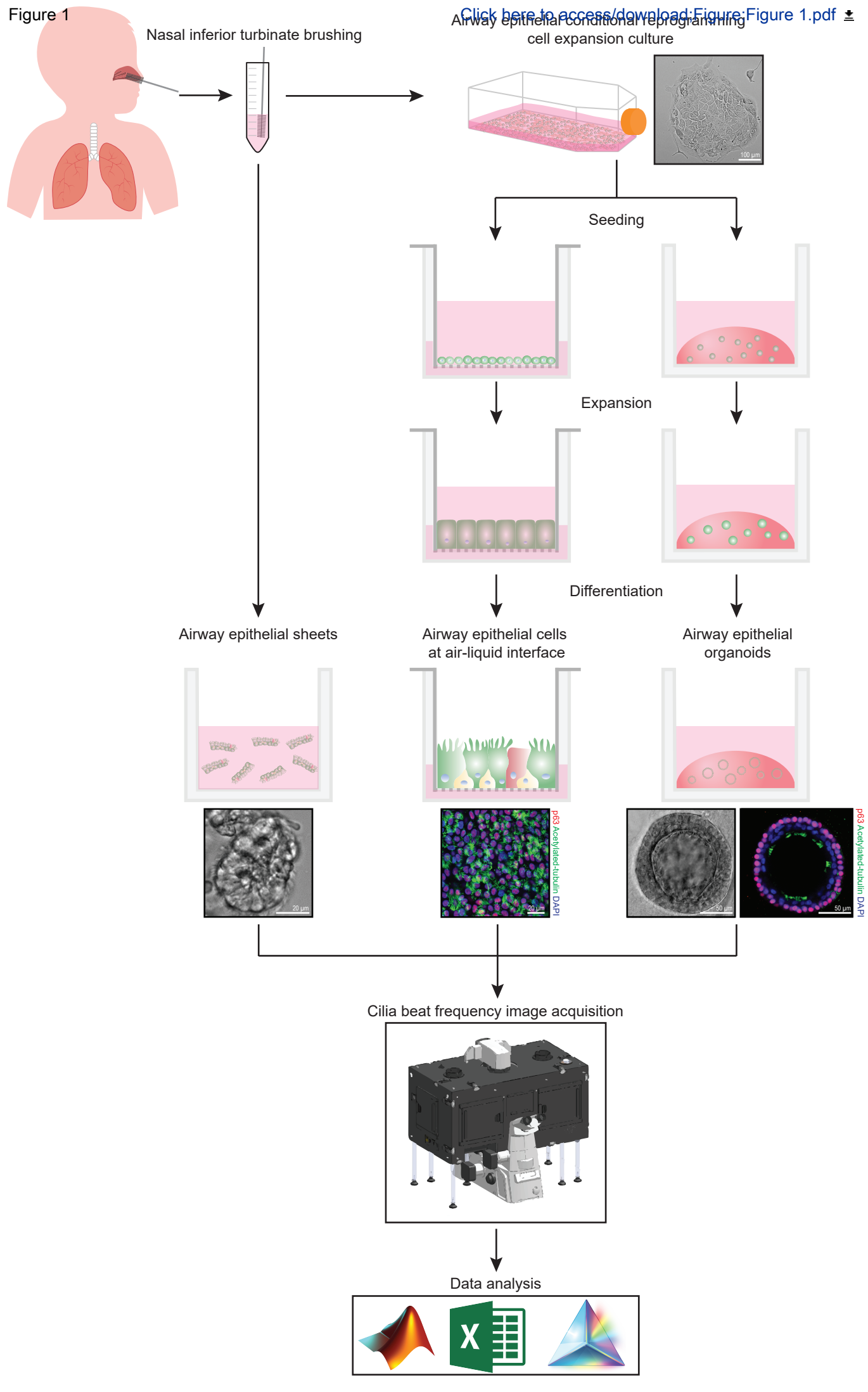

Figure 2

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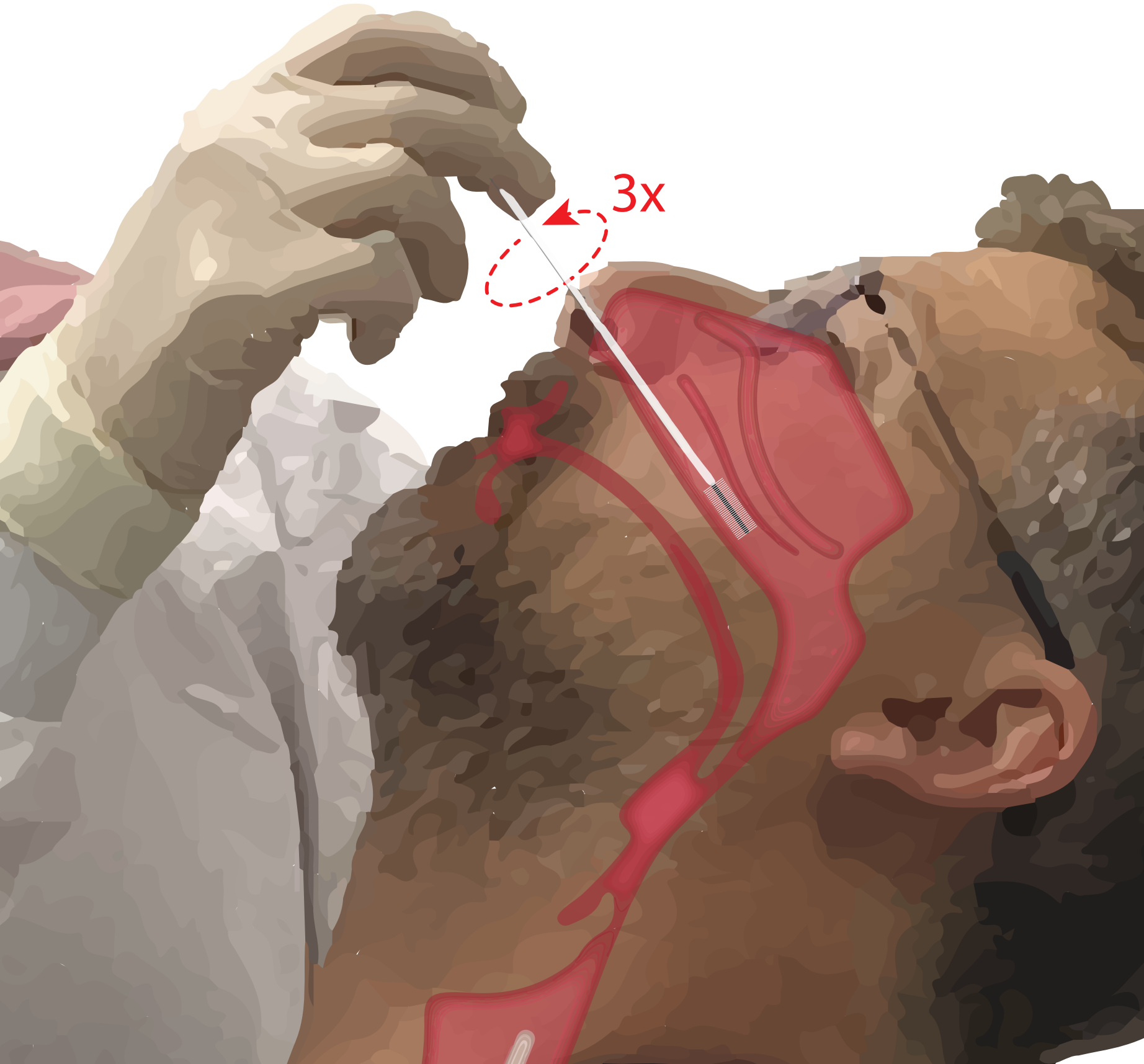

Figure 3

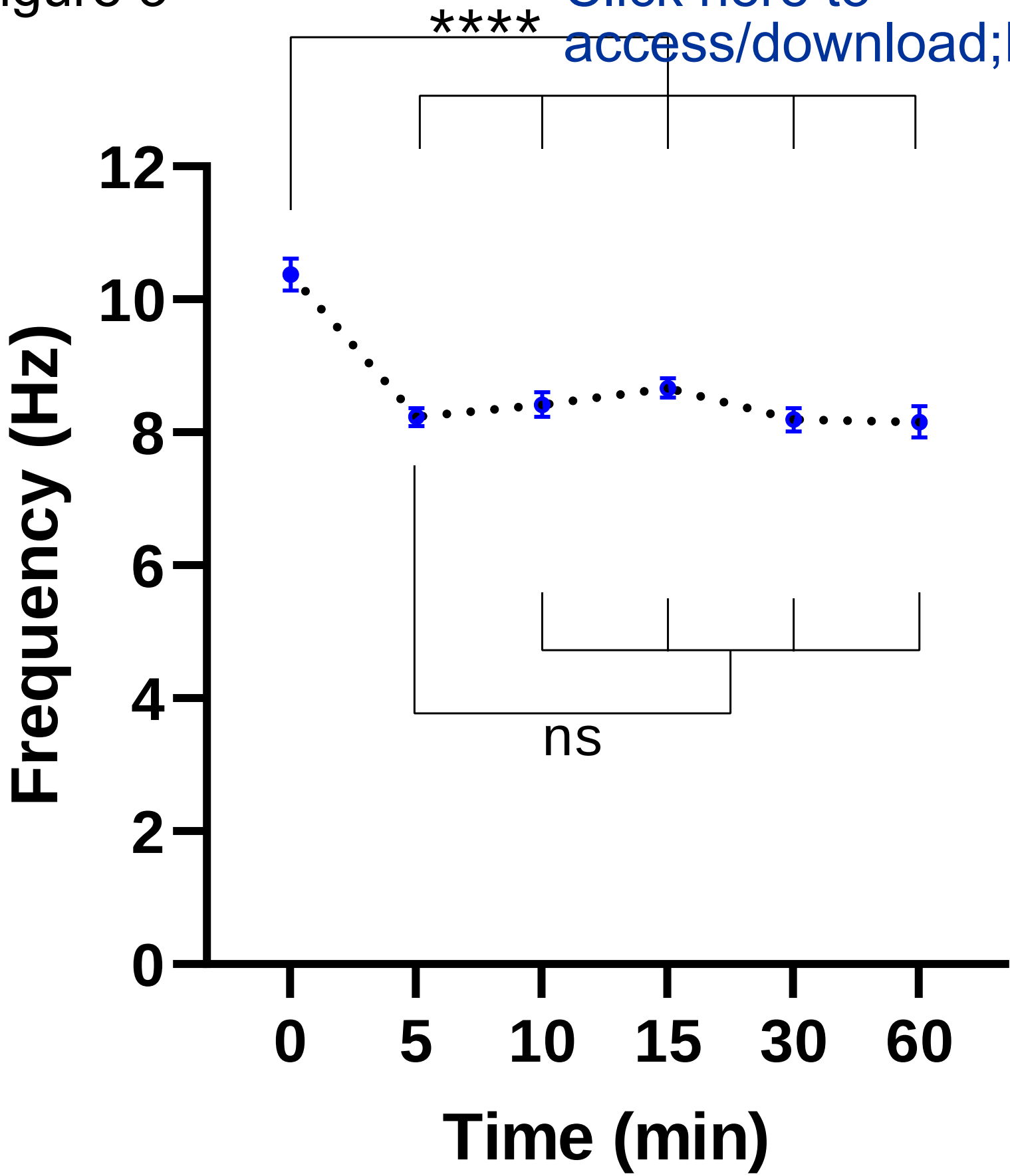

Figure 4

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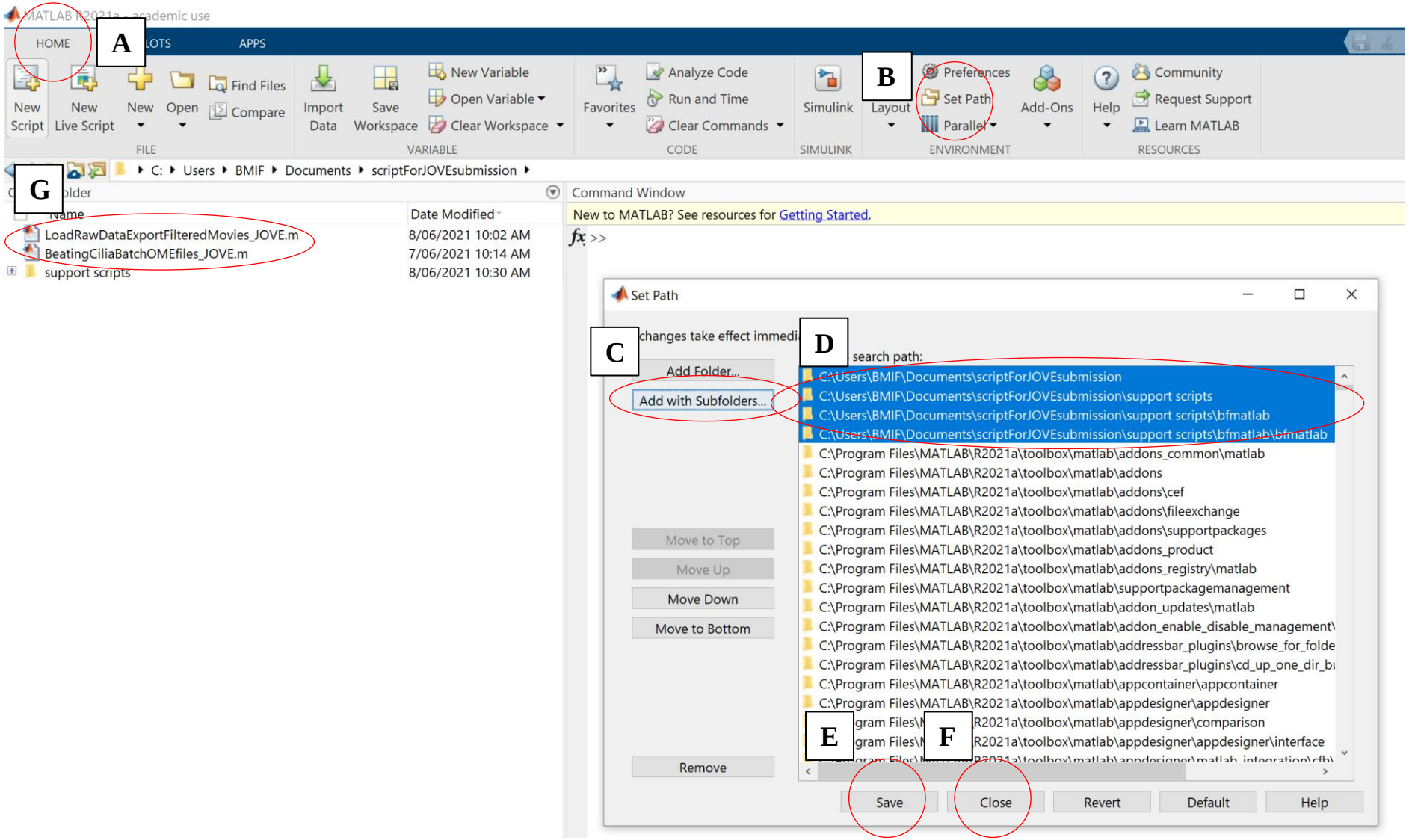

Figure 5

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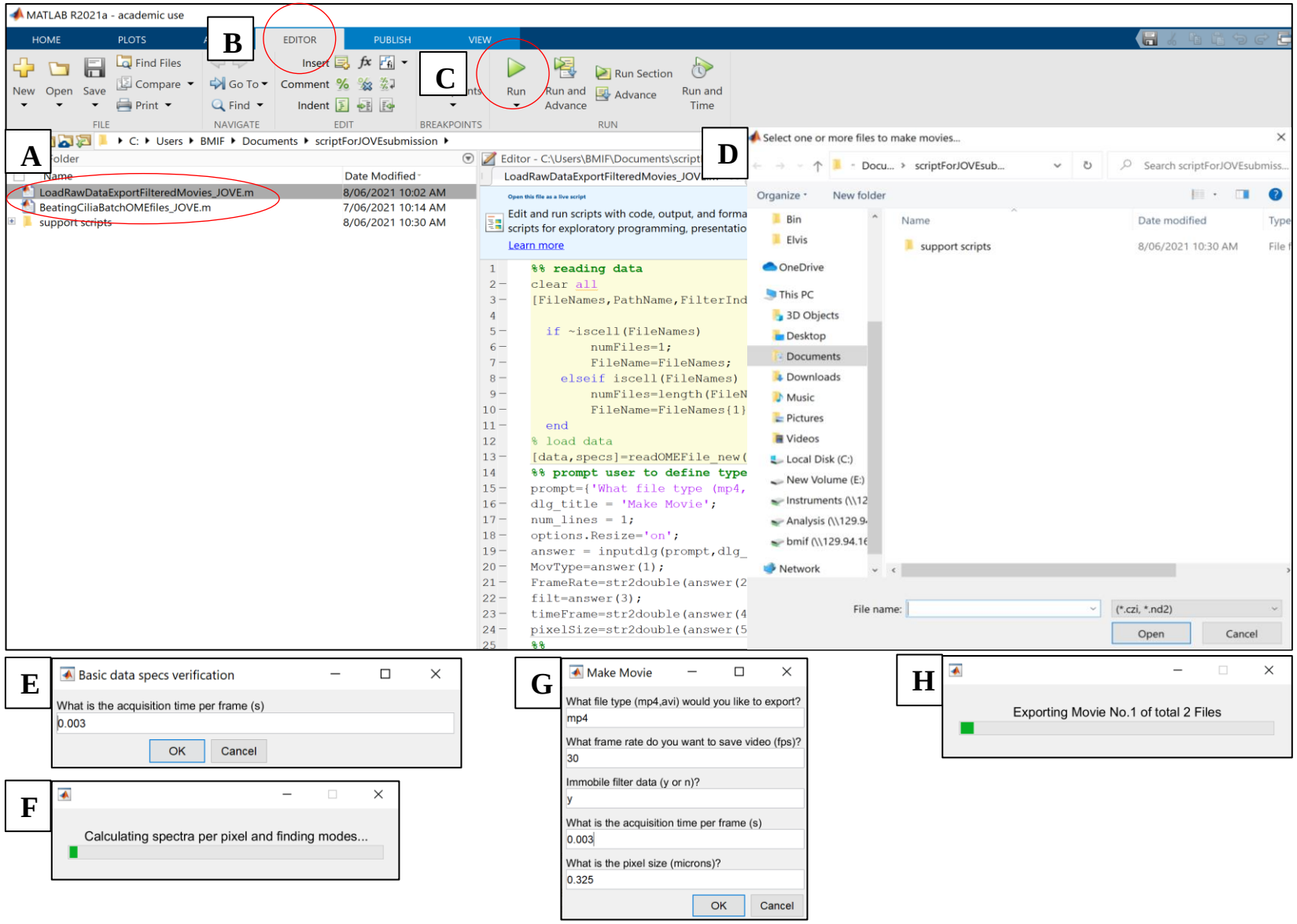

Figure 6

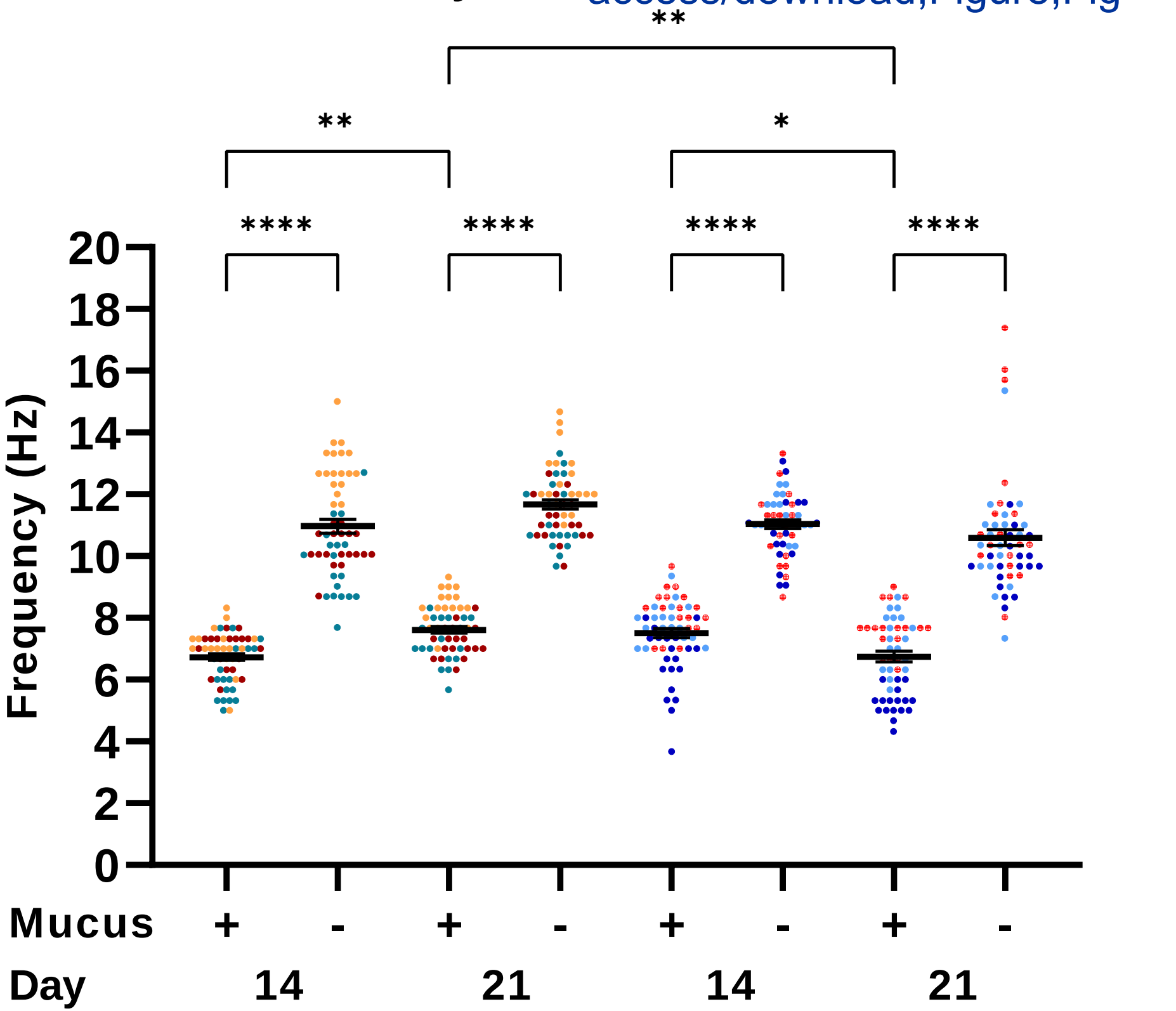

Table 1

Component	Volume
DMEM, high glucose	156.7 mL
DMEM/F-12, HEPES	313.3 mL
Hydrocortisone	55.6 µL
Insulin	1.25 mL
Cholera toxin	21 µL
Adenine	1.2 mL
HI-FBS	25 mL
Penicillin-Streptomycin	5 mL
Human epidermal growth factor	1 µL/mL
ROCK inhibitor	1 µL/mL
Fungizone	2 µl/ml
Tobramycin	2 µL/mL
Ceftazidime hydrate	4 µL/mL
Gentamicin solution	1 µL/mL

Component	Volume
Advanced DMEM/F-12	500 mL
HEPES	5 mL
Alanyl-glutamine	5 mL
Penicillin-Streptomycin	5 mL

Table 3

Number of wells	Number of cells	Number of domes	Vol of Matrigel ECM	Vol of AOSM
1	10,000 cells	1	45 µL x 1.1	5 µL x 1.1
2	20,000 cells	2	90 µL x 1.1	10 µL x 1.1
5	50,000 cells	5	225 µL x 1.1	25 µL x 1.1
……	……cells	……	……µL x 1.1	……µL x 1.1

Table 4 Click here to access/download;Table;Table 4-63090R2.xlsx

Movie inputs	Description
File type	Input the file type you would like to export (mp4 or avi).
Frame rate	Input the frame rate at which the movie should be exported. If you have ~1000 frames per time series acquired, it is recommended to set frame rate ~30 fps.
Immobile filtering	Options are 'y' or 'n'. Default is 'y', and the time filtering script removes, using Fourier space, any immobile components from movie data. Typically, any layers of cells under cilia or immobile mucus will contribute a zero-frequency offset component or time invariant component in the signal that can be filtered out.
Acquisition time per frame	The acquisition time per frame of acquired data. It is used to display a time stamp in the movie in seconds.
Pixel size	The pixel size in micrometres is used to display a scale bar in the movie in micrometres.

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Table of Materials

Table of Materials-63090R2.xlsx

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Thank you. This has been done.

2. Please ensure that abbreviations are defined at first usage.
This has been corrected.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Manuscript has been checked and this language removed.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Nikon Eclipse Ti2-E, Orca Flash 4.0, Hamamatsu, NIS Elements AR, Natick, MA, GraphPad Prism (San Diego, CA), Sigma-Aldrich CLS430829, Advanced BioMatrix 5015, Selleckchem S1049, McFarlane 33009, PureCol, STEMCELL Technologies 05040, CoolCell LX Cell Freezing Container (Sigma-Aldrich CLS432002), PneumaCult-Ex Plus Medium (STEMCELL Technologies 05040), etc.
All trademark symbols, commercial and company names removed and added to the Table of Materials.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
Numbering amended and bullet points removed.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Language changed so that all steps are given as actions and discussion removed from protocol.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
Addressed.

8. 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.
More detail has been added to protocol steps as described under comment 9 below.

9. Please add more details to your protocol steps.
Line 146: Please include the details of the acquisition software in the Table of Materials.
All software and equipment added to Table of Materials.

Line 150: Why the Kohler illumination was necessary? Please include a brief NOTE on that.
Line 162 has been amended:
"*1.1.9 Ensure that the microscope is set up for Kohler illumination so that transmission light source bulb filaments are not in focus at the sample plane, avoiding artefacts in the imaging."*

Line 376: How was the coating done?
This is explained in detail starting from line 293:
"*3.0.2 Coat flasks or permeable support inserts with Collagen solution, on a per need basis. Do not store collagen-coated vessels long-term. To do this:*

3.0.2.1 Make a 1:100 dilution of Type I Collagen solution (3 mg/ml stock) with phosphate buffered saline (PBS) to an end concentration of 0.03 mg/ml. Mix well.

3.0.2.2 Coat cell culture flasks (Section 6.0) with 160 µl/cm2 (i.e., 4 ml per T25 flask) and permeable support inserts (Section 7.0) with 455 µl/cm2 (i.e., 150 µl per 6.5mm insert) of the prepared Collagen solution.

3.0.2.3 Incubate at 37°C for 2-24 hrs.

3.0.2.4 Remove the Collagen solution by pipette or vacuum aspirator prior to seeding cells. Do not wash the vessel prior to seeding cells."

Please provide volume of all the solutions added: ROCK inhibitor, fungizone, tobramycin, etc.
Volumes are now indicated in Table 2.

Line 504: How were the cells counted?
Following amendment has been made on Line 417:
"*6.0.6 Using a 5 ml serological pipette, take the 1 ml of media from Tube C and transfer into a microcentrifuge tube. Take 10 µl of this cell suspension and add it the microcentrifuge tube pre-aliquoted with 10 µl trypan blue. Mix well and immediately* ***use an automated cell counter to record cell count and viability***."

The details for the automated cell counter that was used in this protocol is provided in the Table of Materials.

Line 564, 616: Please provide all microscope parameters and settings.
Cell culture microscopes do not have any input settings. The steps for operating are simply switching it on and focusing on the sample. Live-cell imaging microscopes require multiple settings which are provided in the protocol.

Following sentences have been added to indicate use 4x objective lens for cell culture microscopes.
Line 430:
"*6.1.1 Check cells under the cell culture microscope (****4× objective lens****) regularly for attachment, contamination, morphology, and confluence*."
Line 573:
"*8.2.9 Check the density of organoids under the cell culture microscope (****4× objective lens****) after plating the first dome. If too dense, add additional 90% ECM to achieve the desired density of ~30 organoids*."

Line 761: How was the aspiration done? A pipette was used?
Following changes made on line 305:
"*3.0.2.4 Remove the Collagen solution* ***by pipette or vacuum aspirator*** *prior to seeding cells. Do not wash the vessel prior to seeding cells*."

10. Please provide the outputs of the OME data files (Line 259-273) in a separate Supplementary document. Please remove them from the Protocol as there are no action steps involved.
Removed from protocol. Supplementary File 3 created with outputs of the OME data files.

11. Please mention warming temperature and method for various media like PBS, AODM, etc.
Following notes have been included:

- Line 427: "*NOTE: CRC media must be **warmed to 37°C by placing it in temperature-controlled laboratory water bath or a bead bath device** before it is added to the cells.*"
- Line 456: "*7.0.4 Every second day until a confluent cell monolayer is formed (usually by Day 4 post seeding), discard media and add fresh expansion medium **warmed to room temperature (15-25°C).***"
- Line 461: "*7.1.1 Warm ALI media (antibiotic-free) to room temperature (15-25°C).*"
- Line 528: "*8.1.3 Incubate plate at 37°C for 20 min until the ECM solidifies. While the ECM is solidifying, **warm AOSM to room temperature (15-25°C)** to prevent it causing re-liquification and disintegration of the ECM dome upon addition.*"

12. The protocol length is 10-page maximum (with proper formatting). Please revise the protocol to be up to 10 pages.
Manuscript has been thoroughly revised to meet 10-page limit.

13. Please include all the button clicks, command lines, etc. in the softwares and on the instruments. Please also ensure that the button clicks are bolded throughout.
All button clicks for software and instruments checked. Changed all commands to "*click '**X**'*" to ensure clarity. Bolded the name of buttons to be clicked.

For example, line 177 now reads:
*"1.1.10 Using the acquisition software, click **'L100'** to switch the light path to the port where the camera is mounted. Click the green **'play'** button to visualise the microscope FOV via the software. Check the cilia is in focus and adjust if required."*

14. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
3 pages of essential protocol (including headings and spacing) highlighted for video.

15. Figure 1: For the microscopy images, please include scale bars.
Scale bars added to microscopy images in Figure 1.

16. Please do not abbreviate journal name in references.
Reference list updated to include full journal names.

17. Please provide the details of all materals/reagents/equipments in the Table of materials. Please remove them from the Protocol section.
All materials/reagents/equipment removed from the Protocol section and added to the Table of Materials.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
"This protocol describes the quantification of CBF in three different airway epithelial cell model systems: 1) native epithelial sheets, 2) three-dimensional organoids and 3) air-liquid interface models. The latter two replicate in vivo lung physiology, with beating cilia and production of mucus."

Major Concerns:
The manuscript is not prepared appropriately. It appears to be an excerpt from a project thesis. It appears that the protocol is highly specialised to this group and would not produce a solution for standardisation of CBF measurements for all. It requires highly prescriptive work up and fails to acknowledge other well described and less involved methods. The authors propose that there are no other definitive

protocols to allow for cilia (CBF) analysis of culture airway epithelium, which is not accurate see manuscripts: e.g (spheroids) Jorissen 1989, Castillon 2002, Willems 2004, Marthin 2017, Aydin 2021; e.g. (ALIs) Hirst 2010, Hirst 2014, Rubbo 2019, Coles 2021, Dabrowski 2021. Therefore the protocol presented in not a definitive go to method and I am concerned that the results section is extremely light.

The manuscript has been prepared and revised as per the JoVE author guide.

The following sentences have seen added to the introduction (line 77) to acknowledge other methods as suggested by the reviewer:
Line 77: *"CBF has been quantified in nasal epithelial brushings*[4,6,12-16]*, airway epithelial organoids*[14,17,18] *and ALI models*[3,4,6,13,19-21]*. Yet, amongst these protocols there are large variabilities, and often many parameters are not controlled for. For example, in some studies CBF is imaged in situ while the cells of the ALI model remain on the permeable support insert*[3,19-21]*, yet others scrape the cells from the permeable support insert and image them suspended in media*[4,6,13]*."*

I am also concerned that the protocol includes use of aminoglycosides that are not appropriate for some studies looking at drug effects on cilia, particularly nonsense mutations and are not necessary for non-infected cultures. It appears to be more a specialised procotol for infection-prone CF samples.

While aminoglycosides are used during the expansion CRC reprogramming phase, the differentiation phase is antibiotic free. Following lines have been amended to clarify this further.

Line added (line 433) to note that antibiotics used for stem cell culture from participant are gradually dropped and omitted if not needed during the expansion CRC reprogramming phase:
"*6.1.2 Change CRC media every second day.* ***When reprogrammed cells are observed (Figure 1) and there is no contamination present, reduce or withdraw antibiotics***.

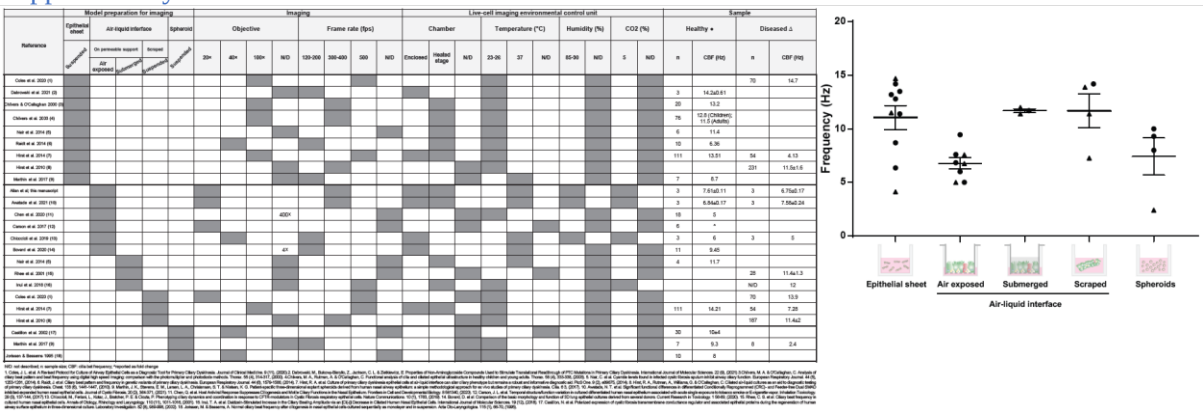
We have clarified that nasal collection media is antibiotic-free for the purpose of *ex vivo* CBF imaging.
Line 372 reads:
"NOTE: If collecting nasal turbinate brushings for the purpose of imaging airway epithelial sheets, only use 1 ml of ***antibiotic-free*** *nasal cell collection media, otherwise epithelial sheets will be too disperse for imaging."*

We have clarified that the 27 days of air-liquid interface culture is antibiotic-free.
Line 442:
"*7.0.1 Transfer the Collagen solution coated permeable support inserts (Section 3.0) from the CO2 incubator to the biosafety cabinet. Aspirate the Collagen solution and discard. Add 750 µl expansion medium (****antibiotic-free****) to the basal compartment of the permeable support inserts."*
Line 461:
*"7.1.1 Warm ALI media (****antibiotic-free****) to room temperature (15-25°C)."*

This protocol is arguing that normal CBF at 37oC is 7.6 Hz in healthy cells. With over a decade of post culture analysis in healthy and diseased airway cultures and with the publications described above 7.6 Hz is below normal range and therefore brings into question the heating method.

Source of tissue, type of culture, media, length of culture, and imaging technologies (presence of environmental chamber/controls) and quantification techniques is variable between different labs and could contribute to variability in CBF results. We have created Supplementary File 1 which brings together 18 publications (including those suggested above with CBF data). As evident CBF values ranges from 2.4 Hz to 14.7 Hz. CBF and seem primarily dependent on culture model and its preparation for imaging. The 7.61±0.11 Hz CBF reported by our protocol for ALI cells imaged while on the membrane is within the normal range of CBF reported in the literature with similar protocol (Awatade et al. 2021, Chen et al. 2020, Chioccioli et al. 2019, Bovard et al. 2020; ALI air exposed: 6.78±0.52 Hz).

Supplementary file 1:



Reviewer #2:

Manuscript Summary:

This manuscript details various acute and culture methods for airway epithelial cells to measure ciliary beat frequency. This is a very specialized technique and more discussion of how to perform such measurements in the literature is worthwhile. Overall, the topic of this manuscript is important. There is a lot of very useful information in the manuscript and I applaud the authors for compiling it.

Major Concerns:

The discussion of the culturing models, cell sampling, etc. are excellent. That's a worthwhile contribution to the literature in and of itself. I have no problems with those protocols. I think that's great.

Thank you for the positive and constructive review.

However, the discussion of CBF acquisition and what the custum scripts are doing is very limited and as a result this part of the protocol is somewhat limited in terms of usefulness.

Supplementary File 4 has been provided to explain how CBF is calculated via algorithm:

"*To quantify cilia beating frequency (CBF), image series were analysed using a custom-built script in MATLAB (MathWorks, Natick, MA) as previously described*[1].

BeatingCiliaBatchOMEfiles_JOVE.m algorithm uses Open Microscopy Environment (OME) package of scripts (bfmatlab) to load the microscopy files, in this case an .nd2 file. The user has choice to run a single or multiple (batch) processing. User is prompted to input a frame time in seconds and then algorithm proceeds to calculate CBF for each pixel in the field of view (FOV) as well as an average value per FOV.

One of the first and essential steps in the analysis is to use Fourier space filtering to remove the immobile (static) component from each pixel in the time series. This is usually seen as stationary elements of cell bodies and mucus that do not move during the 1000 frames of the acquisition. Next the algorithm proceeds to compute CBF pixel by pixel. At each pixel, a temporal spectrum is computed using Fast Fourier Transforms (FFT). Then peaks in the spectrum are detected using MATLAB function 'findpeaks' and setting the allowed peak prominence to 0.05 and peak signal-to-noise cut-off (see MATLAB 'findpeaks' for details) at 5, so that noisy peaks in spectrum are filtered out. For each pixel, the main 3-5 peaks with highest amplitudes are saved with their corresponding frequencies. From these 3-5 peaks, the highest amplitude peak within the physiological range of 3-30 Hz is assigned the CBF for this pixel. The average spectrum of all pixels in the FOV is computed."

There is no discussion of image acquisition rate/sampling frequency in terms of Nyquist sampling. This is critical to acquiring images that arcuately reflect changes in CBF.

Paragraph added to the discussion (line 705) to address image acquisition rate/sampling frequency in terms of Nyquist sampling and choice of camera:

"*An important caveat to consider is selecting an appropriate camera and objective lens to fulfill the temporal and spatial Nyquist sampling. The long working distance lens employed in this study protocol allows a relatively large field of view to be captured. This enables CBF to be imaged in intact ALI*

cultures, with the spatial resolution of ~500 nm (NA0.45). As such, the ciliary bundle can be spatially resolved."

A OrcaFlash4.0 via USB3 images at approximately 60 fps only if a small quadrant of the chip is used. A OrcaFlash 4.0 with integrated board images ~100 fps, which is considered the bare minimum standard for imaging CBF, but there is no discussion of choosing a camera for imaging or what is needed to make valid measurements. As the low light level sensitivity of an sCMOS camera is not really needed here, this could be replaced with a camera costing ~10% of the Hammamatsu provided it images fast enough.

Paragraph added to the discussion (line 715) which discusses choice of camera:

"*Moreover, it is essential to have access to fast speed cameras with frame rate of at least 100 Hz so that any temporal event happening at a rate of 50 Hz can be resolved by Nyquist sampling criterion. A fast sCMOS camera with extremely low noise allowing single molecule measurement is recommended. However, this protocol is not limited by use of this type of camera, so long as the camera fulfills temporal sampling requirements and captures the pixels intensity fluctuations resulting from ciliary beating.*"

There was a mistake in the camera description since we have many similar systems in our facility. We used Andor Zyla 4.2 sCMOS camera. This has been amended in the Table of Materials. Both OrcaFlash 4.0 and Zyla 4.2 sCMOS cameras have ~400 fps readout for the 512x512 field of view acquired in this protocol (https://andor.oxinst.com/downloads/uploads/Zyla_hardware_user_guide.pdf).

The steps of image acquisition are also presented as instructions with somewhat limited generalizability due to the lack of explanation of what their MatLab scripts are actually doing. I think more explanation of (1) imaging parameters for CBF and (2) more generalizable description of steps in analyzing the images to better explain what these software scripts are doing. It would not be good for others who read this paper to be using these scripts without a better concept of what or why they are doing what they are doing and thus generating garbage data because they are imaging too slow, analyzing incorrectly, etc. I think what the authors are doing is great, but I think they need better explanation to allow these procedures to translate to those who may have never done CBF analyses before. Otherwise, a simple plug-and-play description without some description of requirements and caveats may result in people using these scripts incorrectly. More basic information is required here for what and why the authors are doing what they are doing to educate the uninitiated who may read this.

Thank you for raising these concerns and for the suggestions made. We believe the supplement document provided to explain how CBF is calculated via algorithm addresses these concerns. Furthermore, we added a paragraph to the discussion (see above) which discusses both temporal Nyquist (frame rate) and spatial sampling (NA lens and lateral resolution) and how this relates to whether we can spatially see beating of separate cilia or bundles of cilia.

A detailed description of the steps for image analysis is given as per the editors request to "*Please include all the button clicks, command lines, etc. in the softwares and on the instruments.*"

Minor Concerns:

Example datasets should also not be presented on a personal Google Drive site but rather some more stable archived site like the journal site (e.g., supplementary material) or a site like FigShare that is designed to maintain this data in perpetuity. Linking to Google Drive is not ideal.

Thanks for this suggestion. Example datasets have been put on Figshare and link provided in the protocol at the beginning of section 2.0: "*NOTE: An example dataset has been provided with examples of the outputs that ought to be acquired by following this protocol. The example dataset and outputs may be accessed here:* ***https://figshare.com/account/home#/projects/123208.***"

Reviewer #3:

Manuscript Summary:

This protocol describes the excellent details of the methodology of the cilia beat frequency quantification, nasal epithelium isolation, and its culture in vitro. The protocol is well-outlined, -

described in detail, and concise. Except for the major concerns related to the title and the samplings, this reviewer is overall satisfactory. This reviewer also found it challenging to understand the specific terminologies, which the JoVE video production can resolve as a minor concern. It would be great to show the step-by-step videos for the cell cultures and cilia beating measurement.

Major Concerns:
1. Title should be changed because it describes the comprehensive protocol not only for cilia beating measurement but also the collection and culture of the nasal epithelial cells. Otherwise, it should be separately submitted.
Thank you for this suggestion. The title has been changed to be inclusive of the collection and culture of nasal epithelial cells:
"A protocol for collection, expansion and differentiation of primary human nasal epithelial cell models for quantification of ciliary beat frequency"

2. Line416~420: "Describe the procedure to participants as uncomfortable....."; Is there any way to describe those sentences to justify the harmful procedure for the patients? Ex. Under the informed consent of patients and IRB approval, we perform nasal epithelium harvests. Absolute discomfort as if stimulating the deep inside of the nose by seawater shall be felt, necessary for enough sampling from the middle to the posterior part of the inferior turbinate region.
Thank you. We considered your suggestion. However, the written style aligns with the Journal and editorial request to *"ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible"*

3. To understand the required positions described in the Line427~488, it is better to have the additional figure illustrating the anatomical position of the middle to the posterior part of the inferior turbinate region for the readers.
Figure added showing the anatomical position of the collection target, the middle to the posterior part of the inferior turbinate (**Figure 5**).

"Figure 5. Collection of nasal epithelial cells. *Illustration of the location of the cytology brush at the mid to posterior part of the inferior turbinate. This position is reached by inserting the brush through the nares, pivoting the brush to a 90° angle to the face and guiding the brush along the nasal passage below the inferior turbinate."*

4. To avoid any accidents by following this protocol in section 4.0, it would be great to suggest how to prevent the over-insertion of the nasal brushing, which may cause penetration or rupture of the nasal walls.
Note added line 350:
"*NOTE: Avoid over-insertion, if a sudden drop in resistance is felt the nasal pharynx has been entered and the brush should be retracted until resistance is again felt by the proceduralist."*

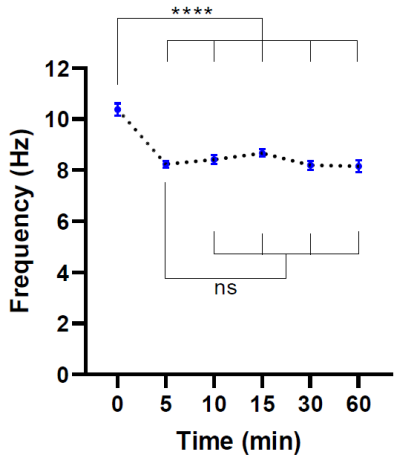
We believe Figure 5 will further aid the clinician to perform the nasal brushing using the correct technique.

Minor Concerns:
1. Line 177: The incubation time for 10min to stabilize the temperature for live-cell imaging generally seems too short, although it depends on the distance between microscopy and the cell culture incubators. Typically, live-cell imaging requires a minimum of 30min pre-incubation for stabilizing the temperature and other parameters such as humidity and CO2 gas concentrations. Since CBF will be significantly affected by those parameters, it would be great to have some annotations for this matter.
We agree with the reviewer that the standard stabilisation time for equilibrating the environmental chamber for live-cell imaging is typically 30 min.

In this protocol we have recommended pre-stabilisation of the environmental chamber for 30 min prior to placing the cells into the chamber. As such, reducing the time needed to equilibrate the sample once the cells are transferred into the microscope chamber (Section 1.1 microscope set up steps 1- 3). Furthermore, the culture plate is transferred on a heat block or beads equilibrated to 37°C to maintain the sample at a physiological temperature (step 1.1.4 – Line 143).

A note has been included to prompt the user to empirically determine the optimal stabilisation time, and representative data has been provided from an experiment which we have performed to determine how long it takes for our system to stabilise. This time was determined to be 5 min following transfer of the culture plate to the already equilibrated chamber (see **Figure 2** stabilisation data).

"*NOTE: A shorter equilibration time may be sufficient. This can be determined by performing an experiment to identify the time required for stabilisation of CBF (refer to **Figure 2**).*"



"Figure 2. Stabilisation of ciliary beat frequency in live-cell imaging microscope. *Dot plots of mean cilia beat frequency (CBF) in airway epithelial cells at air-liquid interface (ALI models), following transfer into a live-cell imaging microscope with an environmental chamber. Chamber was equilibrated and maintained at 37°C, 5% CO2 and relative humidity of 85% for 30 min prior to opening the chamber door and placing the culture plate into the microscope plate insert. Cell models were imaged for 60 min at indicated intervals. ALI models were derived from two participants with CF. Six field of view (FOV) images were acquired per ALI model. Each dot (blue) represents mean CBF in 12-36 FOV images. Data are represented as mean ± SEM, with mean connected by a dotted line. One-way analysis of variance (ANOVA) was used to determine statistical differences. ****P<0.0001, ns: no significance."*

2. Related to line 77: Any measurement for the humidity during the imaging in this protocol?
Yes, an OKOLAB incubator system was used in this protocol. It has been added to the Table of Materials. In this system, humidity is maintained by CO_2 passing through a bottle of purified water

heated to 37°C. The CO_2 is then dispensed into the microscope stage chamber. Relative humidity is measured via a humidity sensor module within the stage chamber. For this protocol, relative humidity was set to 85% and maintained at this level.

A step was added for humidity set up (line 137):
"1.1.2 Top up the humidity module bottle that the CO2 passes through with purified water. Set the relative humidity to 85% via the stage top controller so that the water is heated, and the cells are supplied humidified air. Equilibration of the chamber typically takes 30 min."

3. Line381: After the incubation, no need to wash out the collagen? How do you store the plastics after the coating?
Collagen solution is removed, but not washed. Storage of collagen-coated vessels is not recommended, rather coat on a per need basis.

Line 293: Section now reads:
"*3.0.2 Coat flasks or permeable support inserts with Collagen solution, on a per need basis. Do not store collagen-coated vessels long-term. To do this:*

3.0.2.1 Make a 1:100 dilution of Type I Collagen solution (3 mg/ml stock) with phosphate buffered saline (PBS) to an end concentration of 0.03 mg/ml. Mix well.

3.0.2.2 Coat cell culture flasks (Section 6.0) with 160 µl/cm2 (i.e., 4 ml per T25 flask) and permeable support inserts (Section 7.0) with 455 µl/cm2 (i.e., 150 µl per 6.5mm insert) of the prepared Collagen solution.

3.0.2.3 Incubate at 37°C for 2-24 hrs.

3.0.2.4 Remove the Collagen solution by pipette or vacuum aspirator prior to seeding cells. Do not wash the vessel prior to seeding cells."

CBF acquisition by the camera is described in detail but difficult to follow up because of the specific terminologies (such as MATLAB) lack of robustness that may influence the efficacy of reproducibility of the described protocol. A clear demonstration by video movie would resolve this issue.
Thank you for raising this concern. We have added Supplementary File 4 explaining how CBF is calculated via algorithm. We believe this will aid understanding of the user to give a better idea what is occurring, therefore improving ease of reproducibility. All specific terminologies (e.g. MATLAB) were removed as per editorial instruction. We agree with the reviewer that a clear demonstration by video will also help resolve this.

5. Where can the reader obtain the BeatingCiliaBathOMRfiles JOVE.m scripts? Is it common resources available or specifically designed programs in the lab? If not, is there any open resource available alternatively that can help to standardize the described protocol?
Custom analysis scripts are provided as Supplementary File 2 as instructed in the author guide.
This has been clarified on Line 209:
"*Materials:* ***Custom analysis scripts (Supplementary File 2)*** *...*".

The **bolded** words were added to line 217:
"Copy the **custom analysis scripts** 'BeatingCiliaBatchOMEfiles_JOVE.m' and 'LoadRawDataExportFilteredMovies_JOVE.m' and 'support scripts' folder to the local drive of the computer.

6. Regarding the nasal epithelium collection, the authors, should briefly state that all human specimens were collected after the IRB approval in section 4.0.
Line 121 includes detailed study approval information as per author guide:

"*Study approval was received from the Sydney Children's Hospital Network Ethics Review Board (HREC/16/SCHN/120). Written consent was obtained from all participants (or participants' guardian) prior to collection of biospecimens.*"

7. In section 8.2, Matrigel is used. Matrigel will come with different concentrations, depend on the lot#. If any, the authors should provide the required range of the Matrigel concentration.
Note on line 494 indicates the recommended Matrigel concentration:
Line 494 "*NOTE: Use ECM with protein concentration >10.5 mg/ml for the best culture outcomes is recommended. Lower concentration will accelerate the disintegration of the ECM dome and increase the occurrence of apical-facing-outwards organoids*."

Supplementary File 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary File 1.pdf

Reference	Model preparation for imaging					Imaging								Live-cell imaging environmental control unit										Sample			
	Epithelial sheet	Air-liquid interface			Spheroid	Objective				Frame rate (fps)				Chamber			Temperature (°C)			Humidity (%)		CO2 (%)		Healthy •		Diseased Δ	
	Suspended	On permeable support		Scraped	Suspended	20×	40×	100×	N/D	120-200	300-400	500	N/D	Enclosed	Heated stage	N/D	23-26	37	N/D	85-90	N/D	5	N/D	n	CBF (Hz)	n	CBF (Hz)
		Air exposed	Submerged	Suspended																							
Coles et al. 2020 **(1)**	■							■				■					■				■		■			70	14.7
Dabrowski et al. 2021 **(2)**	■								■	■				■			■				■		■	3	14.2±0.61		
Chilvers & O'Callaghan 2000 **(3)**	■							■			■				■		■				■		■	20	13.2		
Chilvers et al. 2003 **(4)**	■							■			■				■		■				■		■	76	12.8 (Children); 11.5 (Adults)		
Nair et al. 2014 **(5)**	■								■				■		■		■				■		■	6	11.4		
Raidt et al. 2014 **(6)**	■						■			■					■		■				■		■	10	6.36		
Hirst et al. 2014 **(7)**	■							■				■		■	■		■				■		■	111	13.51	54	4.13
Hirst et al. 2010 **(8)**	■								■		■					■	■				■		■			231	11.5±1.6
Marthin et al. 2017 **(9)**	■								■	■						■		■					■	7	8.7		
Allan et al; this manuscript		■				■					■			■	■					■		■		3	7.61±0.11	3	6.75±0.17
Awatade et al. 2021 **(10)**		■				■					■			■	■					■		■		3	6.84±0.17	3	7.58±0.24
Chen et al. 2020 **(11)**		■							400×		■		■			■	■				■		■	18	5		
Carson et al. 2017 **(12)**		■				■							■			■			■		■		■	6	^		
Chioccioli et al. 2019 **(13)**		■					■			■				■				■		■		■		3	6	3	5
Bovard et al. 2020 **(14)**		■							4×	■				■				■			■		■	11	9.45		
Nair et al. 2014 **(5)**			■						■				■		■			■			■		■	4	11.7		
Rhee et al. 2001 **(15)**			■						■				■			■			■		■		■			28	11.4±1.3
Inui et al. 2018 **(16)**			■						■			■			■		■				■	■				N/D	12
Coles et al. 2020 **(1)**				■		■						■				■	■				■		■			70	13.9
Hirst et al. 2014 **(7)**				■					■			■		■	■		■				■		■	111	14.21	54	7.28
Hirst et al. 2010 **(8)**				■					■		■					■	■				■		■			187	11.4±2
Castillon et al. 2002 **(17)**					■		■						■			■			■		■		■	30	10±4		
Marthin et al. 2017 **(9)**					■				■	■						■	■				■		■	7	9.3	8	2.4
Jorissen & Bessems 1995 **(18)**					■		■						■			■					■		■	10	8		

N/D: not described; n: sample size; CBF: cilia bet frequency; ^reported as fold change

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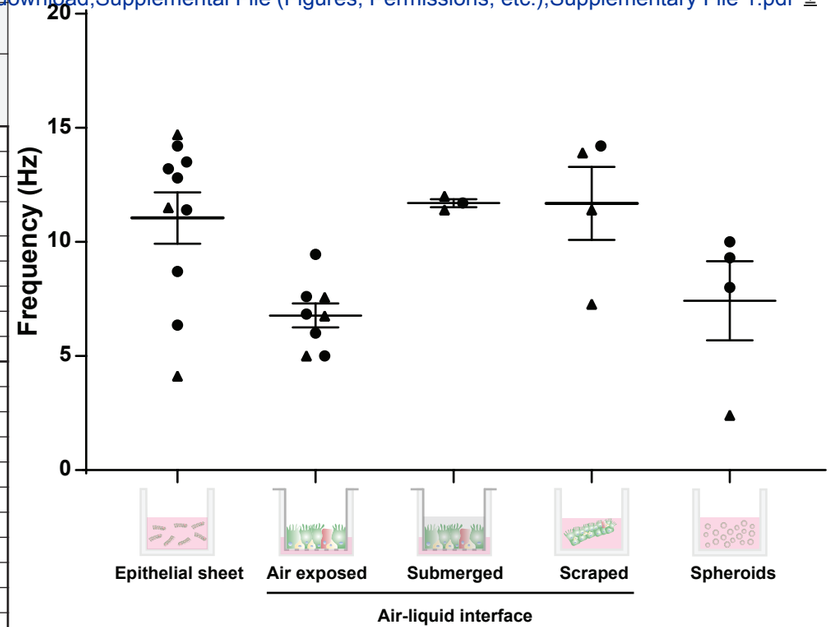

Supplementary File 1. Summary of 18 publications showing diversity of culture and live-cell imaging parameters used to quantify cilia beat frequency in organotypic models of the airway epithelium.

Supplementary File 2. Airway epithelial cell dissociation for differentiation or cryopreservation

Materials:

- Phosphate buffered saline (PBS)
- 1:100 trypsin-EDTA. Make 1:100 dilution of trypsin-EDTA (below) in PBS.
- Hanks' Balanced Salt Solution (HBSS)
- Trypsin-EDTA
- Trypsin Neutralising Solution (TNS)
- PneumaCult-Ex Plus Medium
- Countess Cell Counting Chamber Slides
- PneumaCult-Ex Plus Medium
- Cryogenic vials

Preparations

- Prepare PneumaCult-Ex Plus Medium according to manufacturer's instructions.

1. Remove the NIH-3T3/airway epithelial co-culture flask from the CO_2 incubator and transfer to the biosafety cabinet. Remove the media from the flask and discard.

2. Rinse the cells with **5 mL** of **warmed PBS**. Aspirate the PBS and discard.

3. Add **2 mL** of **warmed media 1:100 trypsin-EDTA**. Incubate at 37 °C for 1 min. When the NIH-3T3 feeder cells round up and begin to detach from the substrate, gently tap the cultures and remove all detached NIH-3T3 feeder cells by aspiration. The epithelial colonies should remain tightly adherent.

4. Rinse the epithelial cells with **2 mL** of **warmed HBSS** to remove any loose fibroblasts. Aspirate the HBSS and discard.

5. Perform cell dissociation by adding **2 mL** of **warmed trypsin-EDTA**. Incubate at 37 °C for 5 min.

6. After 5 min, under the microscope check what percentage of the cells have lifted.

    6.1 If a large proportion of cells are still attached, gently tap the side of the flask to help dislodge more of the cells. Check again under the microscope. If most cells are lifted, go to **step 13**.

    6.2 If the cells are still adherent, return to the CO_2 incubator for a further 2 min. Check under the microscope. Most of the cells should have now detached. Go to **step 7**.

NOTE: Do not incubate the cells with trypsin for more than 7 mins or the cell viability will be compromised.

7. To inactivate the trypsin-EDTA, add **2 mL** of **warmed TNS** to the cell suspension. Aspirate all cells and transfer to a 15 ml centrifuge tube.

8. Under the microscope check what percentage of cells remain adherent to the flask. If the cells are still adherent, add 1 mL of CRC media to the flask and gently scrape in one direction.

NOTE: This decreases viability, so always remove those that have detached prior to scraping and scrape cells in a new aliquot of CRC media.

9. Transfer the cell suspension to the 15 mL centrifuge tube from **step 7**.

10. Rinse the flask with 2 mL of CRC media. Pool the cells into the same 15 ml centrifuge tube.

11. Centrifuge at 300 × *g* for 7 mins at 4°C.

12. Once the centrifuge is complete, transfer the 15 mL centrifuge tube containing the cells on ice to the biosafety cabinet. Aspirate the supernatant and discard.

13. Resuspend the cell pellet with **1ml chilled Ex Plus media.** Take 10 µL of this cell suspension and add it to the microcentrifuge tube pre-aliquoted with 10 µl trypan blue. Mix well.

14. Add 10 µL of the cell/trypan blue suspension to one side of a Countess Cell Counting Chamber Slide. Use a Countess Automated Cell Counter to perform a cell count. Record the cell count and viability.

Airway epithelial cell cryopreservation

1. Make up freezing media containing 90% FBS, 10% DMSO and 1µL/mL ROCK inhibitor.

2. Resuspend the cell pellet with freezing media at 500,000 cells/mL.

3. Mix well. Aliquot 1 mL cell suspension per cryogenic vial.

NOTE: Once cells are added to DMSO, work quickly to transfer the cells into -80 °C storage because DMSO is toxic to cells.

4. Transfer the cryogenic vials in a CoolCell LX Cell Freezing Container. Balance the CoolCell with balance tubes (empty cryogenic vials) for even cooling of CoolCell. Immediately transfer the CoolCell containing the cells to -80 °C.

Supplementary File 4. Data files outputted by analysis script

Output files	Description
'Modes' excel file	Data is given per pixel. For each pixel, the signal in time is used to output all the modes and corresponding amplitudes from the 512 × 512 pixel raw image data file. In order to extract the dominant mode, use the 'AveSpectrum'. 'f1' is the frequency sheet of the most dominant mode, with highest amplitude in the spectrum. 'f2' is the frequency of the next highest mode in the spectrum. For each mode there will be 512 × 512 values (i.e., one value per pixel in the 512 × 512 FOV). Amplitudes of each mode are outputted in separate excel sheets and can be recognised in importance by their numbering.
'AveSpectrum' excel file	This excel averages all the modes in the 'Modes' excel to output the dominant modes. 'f1' is the frequency of the dominant peak of the average spectrum and is assigned as the CBF.
'AverageSpectrum' png file	A plot of the average spectrum obtained by averaging all 512 × 512 spectra obtained in the analysis of one FOV.
'workspace' MATLAB file	Contains all outputs from the MATLAB analysis so the user can revisit for further analysis (beyond this protocol) if desired.

Supplementary File 5. Description of the CBF analysis algorithm

To quantify cilia beating frequency (CBF), image series were analysed using a custom-built script in MATLAB (MathWorks, Natick, MA) as previously described[1].

BeatingCiliaBatchOMEfiles_JOVE.m algorithm uses Open Microscopy Environment (OME) package of scripts (bfmatlab) to load the microscopy files, in this case an .nd2 file. The user has choice to run a single or multiple (batch) processing. User is prompted to input a frame time in seconds and then algorithm proceeds to calculate CBF for each pixel in the field of view (FOV) as well as an average value per FOV.

One of the first and essential steps in the analysis is to use Fourier space filtering to remove the immobile (static) component from each pixel in the time series. This is usually seen as stationary elements of cell bodies and mucus that do not move during the 1000 frames of the acquisition. Next the algorithm proceeds to compute CBF pixel by pixel. At each pixel, a temporal spectrum is computed using Fast Fourier Transforms (FFT). Then peaks in the spectrum are detected using MATLAB function 'findpeaks' and setting the allowed peak prominence to 0.05 and peak signal-to-noise cut-off (see MATLAB 'findpeaks' for details) at 5, so that noisy peaks in spectrum are filtered out. For each pixel, the main 3-5 peaks with highest amplitudes are saved with their corresponding frequencies. From these 3-5 peaks, the highest amplitude peak within the physiological range of 3-30 Hz is assigned the CBF for this pixel. The average spectrum of all pixels in the FOV is computed.

Reference

1 Awatade, N. T. *et al.* Significant functional differences in differentiated Conditionally Reprogrammed (CRC)- and Feeder-free Dual SMAD inhibited-expanded human nasal epithelial cells. *Journal of Cystic Fibrosis.* **20** (2), 364-371, (2021).

Supplementary File 3

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Supplemental Coding Files

Supplementary File 3.zip