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## Generation of Human Nasal Epithelial Cell Spheroids for Individualized Cystic Fibrosis Transmembrane Conductance Regulator Study --Manuscript Draft--

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To the Editors of the Journal of Visualized Experiments:

We are excited to submit our manuscript "Generation of Human Nasal Epithelial Cell Spheroids for Individualized CFTR Study" for your review. All co-authors have seen and approved this submission. In this paper, we describe our novel method to form a non-invasively acquired human nasal epithelial cell-based three-dimensional model of CFTR function.

The introduction of modulator therapies to directly improve CFTR function marks an exciting new method of treatment in CF. These drugs are prescribed in a genotype-directed way that, while personalized, is imperfect. Represented genotypes only include those mutations that occur in a high enough frequency to facilitate large, randomized clinical trials, excluding many individuals. Additionally, as more modulators enter the market, the ability to individually choose the ideal therapy will become increasingly complex. These difficulties can be circumvented by individualized preclinical model systems, using patient-derived samples to quantify drug response. Individualized human cell-derived models of CFTR function are not novel; human bronchial cells and intestinal cells have both been used extensively for such models. Both remain invasive to acquire, however. As such, we generated the nasal cell-based model described within this manuscript. This three-dimensional "spheroid" model reliably quantifies CFTR function through a swelling-based assay.

This work represents is a significant step towards a non-invasive, personalized model of CFTR function to aid in clinical selection of modulator therapies on the individual level. We believe that this line of research will directly impact patient care in CF, and hope that sharing our method will continue to advance this work towards the clinic.

We appreciate your consideration, and look forward to hearing your comments. The authors have no conflicts of interest to declare; this work was funded in part by grants from the CF Foundation as indicated in the manuscript.

Sincerely,



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**TITLE:**

Generation of Human Nasal Epithelial Cell Spheroids for Individualized Cystic Fibrosis Transmembrane Conductance Regulator Study

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**KEYWORDS:**

Cystic Fibrosis, CFTR, Precision Medicine, Organoid, Spheroid, Nasal Cell, Cell Culture

**SUMMARY:**

Here we describe a method to generate three-dimensional spheroid cultures of human nasal epithelial cells. Spheroids are then stimulated to drive Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-dependent ion and fluid secretion, quantifying the change in the spheroid luminal size as a proxy for CFTR function.

**ABSTRACT:**

While the introduction of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) modulator drugs has revolutionized care in Cystic Fibrosis (CF), the genotype-directed therapy model currently in use has several limitations. First, rare or understudied mutation groups are excluded from definitive clinical trials. Moreover, as additional modulator drugs enter the market, it will become difficult to optimize the modulator choices for an individual subject. Both of these issues are addressed with the use of patient-derived, individualized preclinical model systems of CFTR function and modulation. Human nasal epithelial cells (HNEs) are an easily accessible source of respiratory tissue for such a model. Herein, we describe the generation of a three-dimensional spheroid model of CFTR function and modulation using primary HNEs. HNEs are isolated from subjects in a minimally invasive fashion, expanded in conditional

reprogramming conditions, and seeded into the spheroid culture. Within 2 weeks of seeding, spheroid cultures generate HNE spheroids that can be stimulated with 3',5'-cyclic adenosine monophosphate (cAMP)-generating agonists to activate CFTR function. Spheroid swelling is then quantified as a proxy of CFTR activity. HNE spheroids capitalize on the minimally invasive, yet respiratory origin of nasal cells to generate an accessible, personalized model relevant to an epithelium reflecting disease morbidity and mortality. Compared to the air-liquid interface HNE cultures, spheroids are relatively quick to mature, which reduces the overall contamination rate. In its current form, the model is limited by low throughput, though this is offset by the relative ease of tissue acquisition. HNE spheroids can be used to reliably quantify and characterize CFTR activity at the individual level. An ongoing study to tie this quantification to *in vivo* drug response will determine if HNE spheroids are a true preclinical predictor of patient response to CFTR modulation.

## INTRODUCTION:

Cystic Fibrosis (CF) is a fatal, autosomal recessive disease affecting over 70,000 people worldwide<sup>1</sup>. This life-shortening genetic disease is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator protein (CFTR)<sup>2</sup>. CFTR is a member of the adenosine triphosphate-binding cassette family, and functions as an ion channel allowing movement of chloride and bicarbonate across the apical membranes of multiple polarized epithelia including the gastrointestinal tract, sweat gland, and respiratory tree, among others<sup>3,4</sup>. As such, dysfunctional CFTR leads to multisystem epithelial dysfunction, with most mortality stemming from the respiratory disease<sup>1</sup>. In the CF lung, loss of CFTR-driven airway surface liquid (ASL) regulation and mucus release leads to thickened mucus, airway obstruction, chronic infection, and progressive airway remodeling and loss of lung function<sup>1,5</sup>.

Despite the identification of CFTR dysfunction as the cause of disease, therapies in CF traditionally focused on mitigation of symptoms (*e.g.*, pancreatic enzyme replacement therapy, airway clearance therapies)<sup>1</sup>. This approach was recently revolutionized by the advent of novel therapeutics, termed "CFTR modulators," that directly target dysfunctional CFTR. This approach has shifted the clinical landscape from symptom management to disease-modifying care but carries several limitations<sup>6-10</sup>. Modulator activity is specific to the protein defect accompanying each CFTR mutation and limited to select genetic populations<sup>11</sup>. This limitation is driven by the heterogeneous nature of protein defects, but also by the impracticality of clinical trials in rare mutation groups. In addition, among subjects with well-studied genotypes (*e.g.*, F508del/F508del CFTR, the most common CFTR mutation), there is wide variability in disease burden and modulator response<sup>6-9,11</sup>.

To overcome both of these issues, investigators have proposed the use of personalized models for preclinical testing<sup>12</sup>. This concept utilizes patient-specific tissue to generate an individualized *ex vivo* model system for compound testing, predicting *in vivo* subject response to therapies in a personalized fashion. Once validated, such a model could be used by clinicians to drive precision therapy regardless of the patient's underlying CFTR genotype.

Human bronchial epithelial (HBE) cells obtained from explant tissue at the time of lung transplant

established the possibility of such a model for CF<sup>13,14</sup>. HBEs grown at an air-liquid interface (ALI) allow for functional CFTR quantification directly through electrophysiologic testing or indirectly through measures of ASL homeostasis<sup>13,15</sup>. This model has been critical to understanding CFTR biology and was a key driver in the development of CFTR modulators<sup>16</sup>. Unfortunately, HBE models are not tenable as a personalized model due to the invasive nature of acquisition (lung transplant or bronchial brushing) and lack of samples for those with rare mutations or mild disease. Conversely, intestinal tissue, obtained from rectal or duodenal biopsy specimens, can be used for intestinal current measurement (ICM) or a swelling-based organoid assay to study individualized CFTR function<sup>17-19</sup>. Organoid assays, in particular, are very sensitive models of CFTR activity<sup>20-22</sup>. Both models are limited by the tissue source (intestinal tissue, while most disease pathology is respiratory) and the semi-invasive method of acquisition. Alternatively, several investigators have studied human nasal epithelial (HNE) cells to model CFTR restoration<sup>23-25</sup>. HNEs can be safely harvested by brush or curettage in subjects of any age and, when cultured in ALI, recapitulate many characteristics of HBEs<sup>25-28</sup>. HNE monolayer cultures have traditionally been limited by squamous transformation and a long time to maturity<sup>29</sup>. Moreover, reported short-circuit current measurements in HNEs are smaller than those in HBEs, suggesting a smaller window to detect therapeutic efficacy<sup>25</sup>.

Given the need for a personalized, non-invasive, respiratory tissue culture model of CFTR function, we sought to merge the sensitivity of a swelling-based, organoid assay with the non-invasive and respiratory nature of HNEs. Here, we describe our method of generating 3-dimensional “spheroid” cultures of HNEs for individualized CFTR study in a swelling-based assay<sup>30</sup>. HNE spheroids polarize reliably with the epithelial apex towards the sphere center, or lumen. This model recapitulates numerous characteristics of a lower respiratory epithelium and matures more quickly than ALI cultures. As a functional assay, HNE spheroids reliably quantify a range of CFTR function, as well as modulation in well-studied mutation groups (*e.g.*, F508del CFTR). This swelling-based assay capitalizes on the ion/salt transport properties of CFTR, indirectly measuring fluid influx into the spheroid as water follows apical salt efflux. In this fashion, stimulated spheroids with fully functional CFTR swell robustly, while those with dysfunctional CFTR swell less or shrink. This is quantified through image analysis of pre- and 1-hour post-stimulation spheroids, measuring luminal area and determining the percent change. This measure can then be compared across experimental groups to screen for drug bioactivity in a patient-specific fashion.

## **PROTOCOL:**

HNE samples were procured from subjects recruited through the Cincinnati Children’s Hospital Medical Center CF Research Center. All methods described here have been approved by the Institutional Review Board (IRB) of Cincinnati Children’s Hospital Medical Center. Written consent was obtained from all subjects prior to testing.

### **1. Prepare Expansion Media and Antibiotic Media**

1.1. Gather media components listed in **Table 1**. Thaw frozen materials at room temperature and make necessary stock solutions as indicated in **Table 1** under “Expansion Media”.

NOTE: Use care when handling cholera toxin, which is toxic if ingested, and is a skin, eye, and respiratory irritant. Make and combine all solutions and media under clean conditions in the biosafety cabinet.

1.2. Using a 50-mL serologic pipette, remove and discard 50 mL of base medium (Dulbecco Modified Eagle Medium/F12) from one container to compensate for the addition of other materials. Pour all base media by hand into an autoclaved 1 L glass bottle.

1.3. Using a 50-mL serologic pipette or 1-mL pipette as appropriate, transfer all remaining components from the “Expansion Media” section of **Table 1** into the autoclaved glass bottle containing the media. Gently swirl the bottle by hand to mix.

1.4. Filter media into a fresh, sterile 1 L, 0.22 µm polyethersulfone (PES) filter flask using the manufacturer’s instructions and annotate with “Expansion Media” and date.

1.5. Prepare Antibiotic Media. Make necessary antibiotic stock solutions as indicated in **Table 1** under “Antibiotic Media.”

1.5.1. Using a 50-mL serological pipette, transfer 150 mL of Expansion Media to a fresh 250-mL autoclaved glass bottle.

1.5.2. Using a 1-mL pipette, add all components from the “Antibiotic Media” section of **Table 1** into the autoclaved glass bottle containing the media. Swirl by hand to mix until the media is uniform in appearance.

1.5.3. Filter media into a fresh, sterile 250 mL, 0.22 µm PES filter flask using the manufacturer’s instructions and annotate with “Antibiotic Media” and date.

1.6. Store media at 4 °C for up to one month, discarding if cloudy, suggesting contamination.

## **2. Prepare Differentiation Media**

2.1. Gather media components listed in **Table 2**. Thaw frozen materials at room temperature and make necessary stock solutions as indicated in **Table 2**.

NOTE: Use caution when handling retinoic acid, which is a reproductive toxin, can be toxic if swallowed, and causes skin irritation. Make, aliquot, and combine all solutions and media under clean conditions in the biosafety cabinet.

2.2. Remove and discard 52 mL of the base medium from one container to compensate for the addition of other materials. Pour all base media into an autoclaved 1 L glass bottle.

2.3. Using a 25-mL serological pipette or 1-mL pipette as appropriate, transfer all remaining

components from **Table 2** to the autoclaved glass bottle containing the media and swirl gently by hand to mix.

2.4. Filter media into a fresh, sterile 1 L, 0.22  $\mu$ m polyethersulfone (PES) filter flask using the manufacturer's instructions and annotate with "Differentiation Media" and date.

2.5. Store media at 4 °C for up to one month, discarding if cloudy, suggesting contamination.

### **3. Coat Culture Dishes and Plate Feeder Fibroblasts**

NOTE: Perform all steps under clean conditions in the biosafety cabinet.

3.1. Mix 0.5 mL of collagen stock and 37.5 mL of sterile water in a 50-mL conical tube.

3.2. Pipette 4-5 mL of collagen solution into each clean P100 culture dish, ensuring the entire surface is covered.

3.3. Cover dishes with lids and incubate overnight at 37 °C and 5% CO<sub>2</sub>.

3.4. In the biosafety cabinet, remove all dish lids. Swirl solution to cover dish, then aspirate.

3.5. Sterilize by exposure to ultraviolet (UV) light for 1 h. Place lids back on the dishes, stack and cover dishes with aluminum foil.

3.6. Store coated dishes at 4 °C for 3-6 months, re-sterilizing under UV light 15-30 min before cell culture use.

3.7. 24 h before obtaining a HNE sample, sterilize a pre-coated dish and label as Mouse Embryonic Fibroblast (MEF) and the date.

3.8. Add the Expansion Media to cover the plate (approximately 5 mL) with a serologic pipette.

3.9. Thaw a vial of 2 x 10<sup>6</sup> irradiated MEFs in a 37 °C waterbath. As soon as the MEFs are thawed, add half of the vial (approximately 10<sup>6</sup> cells) to the dish with a 1-mL pipette, swirling by hand to disperse.

3.10. Incubate at 37 °C and 5% CO<sub>2</sub> overnight in preparation for HNE culture.

NOTE: If necessary, steps 3.7–3.10 can be performed as late as 60 min before plating cells, though overnight is ideal.

### **4. Obtain and Process HNE Sample**

4.1. Ensure IRB-approved consent/assent is obtained for all subjects.

221  
222 4.2. Have patient blow nose to clear loose mucus.  
223

224 4.3. Visualize inferior turbinate using a rhinoscope. Ensure no lesions or polyps are present that  
225 may preclude sample collection.  
226

227 4.4. Collect nasal cells from the first nostril by curettage or brushing.  
228

229 4.4.1. Curettage: Bend curette at its midpoint to create a slight,  $\sim 135^\circ$  angle with the apex away  
230 from the curette cup. Insert the curette into the nostril and advance the curette cup under the  
231 inferior turbinate. Gently press the curette cup against the inferior turbinate and scrape the  
232 turbinate by pulling the curette forward, repeating 3-4 times total.  
233

234 4.4.2. Brush: Pass the cytology brush below the inferior turbinate, then rotate and move the  
235 brush in-and-out slightly 4-5 times, without exiting the nostril.  
236

237 4.5. Place curette/brush in a 15-mL conical filled with Antibiotic Media. Repeat steps 4.3–4.4 for  
238 the other nostril, placing the curette/brush into the same conical tube.  
239

240 4.6. Store conical on ice until ready for processing. Ensure that the processing occurs within 4 h  
241 of sample acquisition but can occur as late as 24 h after acquisition if necessary.  
242

## 243 **5. Process and Expand HNE Cells in Dishes** 244

245 NOTE: Perform all steps under clean conditions in the biosafety cabinet.  
246

247 5.1. Using a 1-mL pipette and media from the sample conical, wash all cells off of cures /  
248 cytology brushes into the same conical tube. Once clean, discard cures / brushes.  
249

250 5.2. Centrifuge conical at  $360 \times g$  and  $4^\circ\text{C}$  for 5 min.  
251

252 5.3. Aspirate supernatant, taking care not to disturb the cell pellet.  
253

254 5.4. Re-suspend the cell pellet in 3 mL of cell detachment solution, pipetting with a 1-mL tip to  
255 homogenize the mixture.  
256

257 5.5. Centrifuge conical at  $360 \times g$  and  $4^\circ\text{C}$  for 5 min.  
258

259 5.6. Repeat steps 5.3–5.5 once.  
260

261 5.7. Aspirate remaining supernatant, taking care not to disturb the cell pellet.  
262

263 5.8. Re-suspend the pellet in 1 mL of Antibiotic Media and count cells with a hemocytometer.  
264



5.9. Using a 1-mL pipette, plate cells dropwise onto the coated, fibroblast-seeded dish prepared in step 3.10. Add Antibiotic Media to fully cover the dish and disperse the cells across the surface. Label dish with contents and date and incubate at 37 °C and 5% CO<sub>2</sub>.

5.10. After 48 h, ensure that the cells are attached to the culture dish. Aspirate the media and replace with enough fresh Antibiotic Media to cover the plate.

5.11. Change media 3x weekly, aspirating old media with a vacuum line and Pasteur pipette, and replacing with enough fresh media to cover the plate. After 5 days on Antibiotic Media, change the media to Expansion Media, continuing to replace three times weekly.

NOTE: Contamination risk is high in the first week of culture. Keep fresh samples in a separate incubator to minimize the risk of cross-contamination.

5.12. Check the cell growth several times weekly. Once cells are approximately 70-80% confluent, proceed to passage cells.

## **6. Passage HNE Cells**

6.1. Prepare 0.1% Trypsin solution in ethylenediaminetetraacetic acid (EDTA).

NOTE: Use caution handling trypsin, which is a skin, respiratory, and eye irritant.

6.1.1. Weigh 15 mg of trypsin from the porcine pancreas in a conical tube.

6.1.2. Weigh 56 mg of EDTA in a separate conical tube.

6.1.3. In the biosafety cabinet, transfer trypsin and EDTA into an autoclaved 250-mL glass bottle. Use a small aliquot of sterile phosphate-buffered saline (PBS) to rinse the trypsin/EDTA tubes to ensure complete transfer.

6.1.4. Add sterile PBS to bring the solution total to 150 mL. Close lid tightly.

6.1.5. Incubate trypsin solution in a 37 °C waterbath until fully dissolved. Check every 15 min and remove promptly once dissolved.

NOTE: Do not leave unchecked for prolonged periods of time (*e.g.*, overnight), as trypsin in solution will denature if heated for too long.

6.1.6. Clean bottle with 2-3 sprays of 70% ethanol and return to the biosafety cabinet.

6.1.7. Filter 0.1% Trypsin-EDTA solution into a fresh, sterile 250 mL, 0.22 µm PES filter flask using the manufacturer's instructions and annotate with contents and date.

6.1.8. Store trypsin-EDTA solution at 4 °C for up to one month, discarding if cloudy.

6.2. Bring Differentiation Media, 0.1% Trypsin-EDTA solution, and sterile PBS to room temperature over 30 min. Clean with 70% ethanol and transfer to a clean biosafety cabinet.

6.3. In the biosafety cabinet, use a vacuum line and Pasteur pipette to aspirate and discard media from tissue culture dish. Wash the dish by adding, swirling, and aspirating 5 mL of PBS using a clean serological pipette.

6.4. Using a 5-mL serological pipette, add 5 mL of 0.1% Trypsin-EDTA solution to the tissue culture dish and return to the incubator at 37 °C and 5% CO<sub>2</sub> for 5 min.

6.5. Visualize cells on an inverted microscope at 4-10X. Check that the cells detach from the dish, become round, and float. Gently tap the side of the culture dish to dislodge cells, and/or re-incubate for an additional 5 min until most cells are detached.

NOTE: Take caution to quickly remove cells once detached from the culture dish; prolonged exposure to 0.1% Trypsin-EDTA will impair cell viability.

6.6. Using a 10-mL serological pipette, add 5 mL of Expansion Media to the dish and collect all liquid contents (10 mL total) into a labeled, sterile 50-mL conical tube.

6.7. If cells remain adherent to the culture dish, repeat steps 6.4 through 6.6 up to two times, collecting contents in the same conical tube.

6.7.1. If cells remain adherent to the culture dish after three rounds of Trypsin solution exposure, use a sterile cell scraper gently remove the remainder. After scraping the dish, wash the scraper and dish with 5 mL of Expansion Media and collect in the same labeled conical tube.

NOTE: If passaging to spheroids, modify this procedure to perform a differential trypsinization. After adding trypsin solution (step 6.4.), check the dish frequently until fibroblasts have detached (typically 1-2 min), leaving only polygonal epithelial cells attached to the dish. Collect and set aside this supernatant, which can be passaged forward for expansion. Repeat steps 6.4–6.6 using the same trypsin solution, and collect only the remaining epithelial cells for spheroid culture.

6.8. Centrifuge conical at 360 x g for 5 min and 4 °C. Aspirate media from conical.

6.9. Resuspend cell pellet in 1 mL of Expansion Media and count cells using a hemacytometer.

6.10. Once quantified, cells can be divided if necessary and either passaged forward onto a new culture dish (including preparation of fibroblast co-culture, step 3, and plating cells for expansion, steps 5.9–5.12) or cultured as spheroids (step 7).

NOTE: If passaging to a new culture dish for expansion, a seeding density of 0.5-1 × 10<sup>6</sup> cells in a

P100 dish is recommended.

## 7. Seeding Cells for HNE Spheroid Cultures

NOTE: Carry out all procedures in this step, other than centrifugation, in a clean biosafety cabinet.

7.1. Thaw the basement membrane matrix on ice per manufacturer's instructions (100  $\mu$ L per spheroid well).

NOTE: Consider making sterile 500  $\mu$ L aliquots of the basement membrane matrix on receipt; this amount will seed a single 4-well plate.

7.2. Separate an appropriate number of differentially trypsinized, passaged cells (from step 6.9) for a final concentration of 500,000 cells per mL of the matrix. For a 4-well plate, use 200,000 cells. Collect in a 1.5 mL tube.

7.3. Centrifuge cell and media mixture at 360 x g for 5 min at 4 °C. Completely, but carefully aspirate and discard media using a 1-mL pipette, taking care not to disturb the cell pellet.

7.4. Using a 1-mL pipette tip, add 100  $\mu$ L per planned well of the basement membrane matrix to the 1.5 mL tube containing the cells. Keep the tube on ice.

7.5. Pipette up and down to carefully and thoroughly resuspend cells in the basement membrane matrix. Move quickly to avoid solidification of the matrix. Avoid completely emptying the pipette tip to ensure air bubbles are not introduced into the matrix/cell mix.

7.6. Seed 100  $\mu$ L matrix aliquots into each well of a 4-well IVF culture plate.

7.6.1. Using a clean pair of scissors, trim off the distal 3-4 mm of a 200  $\mu$ L pipette tip.

7.6.2. Using the trimmed tip, carefully pipette 100  $\mu$ L of the cell/matrix mixture into the center of each well. Pipette slowly, with the goal of making a single matrix "drop" in the center of each well. The drop should remain spherical, and should not touch the sides of the well. When moving the plate, do so carefully as to avoid disturbing these drops.

7.6.3. Incubate the basement membrane matrix drops at 37 °C and 5% CO<sub>2</sub> for 30 min, until solid.

7.6.4. Carefully pipette 500  $\mu$ L of Differentiation Media into each well, taking care not to disturb the matrix drop. Ensure that the media just cover the matrix drop and add more if necessary.

## 8. Differentiate and Mature HNE Spheroids

8.1. Using a 1-mL pipette tip, gently aspirate all media from well; avoid aspirating the matrix, which will destroy spheroids in that portion of the matrix, though any spheroids left behind may

397 remain viable.

398  
399 8.2. Using a fresh 1-mL pipette tip, gently add 500  $\mu$ L of differentiation media to the well around  
400 the matrix. Do not touch the matrix with the pipette and avoid disturbing the matrix drop.

401  
402 8.3. Return spheroids to the incubator at 37 °C and 5% CO<sub>2</sub> for ongoing growth. Repeat steps 8.1  
403 through 8.3 three times weekly.

404  
405 8.4. Evaluate spheroid morphology daily under an inverted microscope at 20x. Spheroids should  
406 form within 3-5 days of plating, and reach maturity within approximately 10 days.

## 407 408 9. Pretreat, Stimulate, and Image HNE Spheroids for Analysis

409  
410 9.1. Pretreat spheroids as desired prior to imaging as previously described<sup>30</sup>.

411  
412 NOTE: For VX809 pretreatment, add 3  $\mu$ M of VX809 to the media for 48-72 h prior to imaging.  
413 Mix 1  $\mu$ L of 10 mM VX809 stock (see **Materials Table**) in 3.3 mL of media for this concentration.

414  
415 9.2. Change each well of spheroids to 1 mL of fresh Differentiation Media, including pretreatment  
416 (steps 8.1–8.3) before imaging spheroids.

417  
418 9.3. Prepare fresh stimulation drugs solutions (Forskolin, 3-isobutyl-1-methylxanthine [IBMX],  
419 VX770, and CFTR Inhibitor 172 [Inh172]) from stocks (see **Materials Table**). For forskolin / IBMX,  
420 add 1  $\mu$ L of each stock to 98  $\mu$ L of sterile water in a single labeled 1.5 mL tube. For VX770 and  
421 Inh172, add 1  $\mu$ L of each stock to 99  $\mu$ L of sterile water in separate labeled 1.5 mL tubes. Make a  
422 total volume allowing for 50  $\mu$ L of solution for each well tested. Prepare solutions under sterile  
423 conditions in the biosafety cabinet.

424  
425 9.4. Transfer spheroid plate to an incubated chamber set to 37 °C and 5% CO<sub>2</sub>, mounted on an  
426 inverted microscope equipped with electronic image capture software and a mechanical stage  
427 for XY adjustment. Turn on the microscope and camera, as well as the imaging computer.

428  
429 9.5. Capture baseline images of spheroids in one well.

430  
431 9.5.1. Open the image capture software (Slidebook 5.5 for directions below). Once initialized,  
432 open a new file by clicking "**File**", then "**New**". Name the file accordingly and click "**Save**".

433  
434 9.5.2. Carefully remove the culture plate lid and center one matrix drop over the objective.

435  
436 9.5.3. Starting at the top of the matrix drop, move in a grid to find spheroids at 20x magnification  
437 with the microscope eyepiece. Focus up and down to capture the sphere at its widest point.  
438 Annotate the location of each spheroid on a map of the matrix drop to re-identify after imaging.

439  
440 9.5.4. In the software, click "**Image-Capture**". For Exposure, select "**Manual**" and enter 50 ms.

Ensure other settings are appropriate (Bin Factor: 1x1, W: 512, H: 512, X and Y Offset: 0). Enter an appropriate name for each image in the “**Name**” box (e.g., Spheroid 1 Baseline). Click “**Start**” to view a live image, and “**Save**” to capture a baseline image.

9.5.5. Repeat steps 9.5.1–9.5.4 until goal number is reached, or entire matrix drop is imaged.

NOTE: Group differences can be detected with as few as 3-5 spheroids per condition, however, it has become our practice to image 10 or more spheroids per condition to increase power. Variability of the assay is demonstrated in **Figure 2** of the “Representative Results” section, with samples of 8-10 spheroids per subject as an example.

9.6. Stimulate spheroids.

9.6.1. Using a 200  $\mu$ L pipette, add 50  $\mu$ L of the appropriate stimulation drug into the media within the well, taking care not to disturb the matrix.

NOTE: After this 10-fold dilution (50  $\mu$ L into 500  $\mu$ L of media), the final drug concentration will be: forskolin 10  $\mu$ M, IBMX 100  $\mu$ M, VX770 1  $\mu$ M, Inh172 10  $\mu$ M.

9.6.2. For cAMP only, add only forskolin / IBMX.

9.6.3. For cAMP + VX770, add forskolin / IBMX first, then VX770 after 2 min.

9.6.4. For inhibited conditions, add Inh172 first, then forskolin / IBMX after 2 min.

9.7. Immediately after stimulation, monitor spheroid swelling by timelapse imaging for 1 h. In the software, click “**Image-Capture**”. Click “**Timelapse**”, and set the interval to 30 s, with a duration of 60 min. Enter an appropriate name and click “**Save**” to start timelapse.

NOTE: Standard timelapse is to capture images of a single spheroid every 30 s to ensure a high-quality video. Alternatively, perform lower-magnification capture of the entire well (e.g., 4x), and fewer images captured if desired. If an automated stage is used, perform timelapse of all spheroids using predefined coordinates; otherwise, use timelapse of a subset of spheroids to provide a qualitative review of swelling and to ensure no systematic issues arise during imaging (e.g., matrix detachment from the well).

9.8. Immediately upon completion of the 1 h timelapse image, capture post-stimulation images of all spheroids (per step 9.5) using the map created in step 9.5.3.

9.8.1. Refer to pre-stimulation images to ensure the same plane of focus is used for follow-up images (the focal quality of surrounding cells, spheroids, or debris greatly facilitate this process).

9.8.2. Save files with a paired annotation from step 9.5.4 (e.g., Spheroid 1 Post-Stimulation).

NOTE: Complete this step immediately after the timelapse, as spheroids may continue to swell.

9.9. Replace media in the imaged well with fresh Differentiation Media.

9.10. Repeat steps 9.5 through 9.9 for each well / condition, adjusting stimulation drugs as indicated for experimental conditions.

9.11. Once all imaging is completed, return spheroids to the incubator at 37 °C and 5% CO<sub>2</sub>.

NOTE: Depending on the sterility of the incubated microscope chamber, post-stimulation contamination of spheroids can be quite common. Either keep imaged spheroids in a separate incubator, to minimize cross-contamination risk, or discard spheroids immediately after imaging.

## 10. Analyze HNE Spheroid Images

10.1. Export all captured images to a format compatible with image analysis software. In the software, click **“View”**, hover over **“Export”**, and select **“Default Views of All Images as TIFF...”**. Save to the hard drive or a flash drive for analysis.

NOTE: A commercial analysis software (**Table of Materials**) and .tiff files perform well and are described here, though analysis can also be performed using other platforms. When naming files, staff analyzing images should be blinded to the experimental conditions, but aware of which images are paired (pre- and post-stimulation) to ensure equivalent analysis.

10.2. Using analysis software, delineate the luminal area of each spheroid image and export into a data analysis software.

10.2.1. Open analysis software. Click **“File”**, then **“Open”** and navigate to the file folder containing spheroid images. Select all images for analysis and click **“Open”**.

10.2.2. Click **“Measure”** and select **“Region Measurements”**. Within the Region Measurements dialog box, select the droptab **“Configuration”** and ensure **“Image Name”** and **“Area”** boxes are checked.

10.2.3. Click **“Log”** and select **“Open Data Log”**. Click **“OK”** on the Data Log dialogue box and name file accordingly (e.g., Condition X, Date). This will transfer all measurements to a spreadsheet.

10.2.4. Select **“Trace Region”** tool button and carefully trace the lumen of the spheroid in the first image for analysis. In the Region Measurement dialogue box, click **“Log Data”** to log the measurement into Excel.

10.2.5. Repeat steps 10.2.2–10.2.4 until all spheroids are analyzed.

10.3. Calculate the percent change ( $100 * [\text{Post-stim} - \text{Baseline}] \div \text{Baseline}$ ) in the luminal area from baseline for each individual spheroid image pair and compile data for analysis.

#### REPRESENTATIVE RESULTS:

HNEs should attach to the culture dish and form small islands of cells within 72 h of seeding; examples of good and poor island formation at one week are shown in **Figure 1A** and **1B**, respectively. These islands should expand to cover the dish over the course of 15-30 days. Small or suboptimal samples may take longer, and often will not yield useful spheroids. Contamination with infectious agents is evidenced by deep yellow/cloudy media, failure of the cells to attach to the culture dish, and/or direct visualization of fungi/bacteria. Any contaminated cultures should be immediately discarded to avoid cross-contamination.

Within the first 3-4 days of culture in the basement membrane matrix, small cystic structures should begin to form in the matrix. These will mature over approximately 10 days into intact spheroids demonstrating a thin wall and a luminal surface. If plated at the described density, successful cultures will contain 50-100 spheroids per matrix drop. The lumens may be relatively clear (**Figure 1C**) or filled with cellular debris and mucus (**Figure 1D**); the former is more common in spheroids with wild-type CFTR (wtCFTR), and the latter in CF spheroids. Masking to delineate the luminal area of the spheroids in **Figure 1C** and **1D** is demonstrated in **Figure 1E** and **1F**, respectively. Examples of poorly formed/unsuccessful spheroid cultures are provided in **Figure 1G** and **1H**.

Representative functional data for wild type and F508del CFTR homozygous HNE spheroids is shown in **Figure 2A** and **2B**, respectively; this data is representative of >10 unique HNE samples in wild type and F508del CFTR homozygous subjects<sup>30</sup>. In short, spheroids with functional CFTR swell, while those with dysfunctional CFTR swell significantly less, or may shrink. Specifically, spheroids from subjects with wtCFTR should swell over an hour when stimulated, and should swell less or shrink if stimulated in the presence of the CFTR inhibitor Inh172. Conversely, spheroids from a subject homozygous for F508del CFTR should either shrink or swell very slightly, with increased swelling (or less shrinking) when pharmacologically corrected with the CFTR modulators VX809 and VX770. Previous analyses of spheroid reliability both within and between subjects of the same genotype demonstrate functional segregation of CFTR genotypes and modest variability in repeated measures<sup>30</sup>.

#### FIGURE AND TABLE LEGENDS:

**Table 1: Components of Expansion and Antibiotic Media.**

**Table 2: Components of Differentiation Medium.**

**Figure 1: HNE Expansion and Structural Characteristics of HNE Spheroids.** Brightfield images of HNE expansion cultures taken seven days after plating demonstrate successful (white arrow, panel **A**) and unsuccessful (panel **B**) HNE colony formation on a feeder fibroblast background. Successful wtCFTR and F508del homozygous spheroids are shown in panels **C** and **D**, respectively. Masking to delineate the luminal area of spheroids from C/D is provided in panels **E** and **F**,

respectively. Unsuccessful spheroid cultures are characterized by small, disorganized cellular debris (panel **G**) and/or disorganized clumps of cells (panel **H**). Scale bar: 100  $\mu$ m.

**Figure 2: Functional Characteristics of HNE Spheroids.** Representative functional responses of wtCFTR spheroids from a single donor, when stimulated with forskolin/IBMX with/without the presence of the CFTR inhibitor Inh172 are shown in panel **A**. Each point represents the response in a single spheroid. Representative functional responses of F508del homozygous spheroids from a single donor, when stimulated with forskolin/IBMX with/without the presence of VX809 and VX770 are shown in panel **B**. Error bars: SEM. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## DISCUSSION:

This protocol describes the generation of patient-derived nasal cell spheroid cultures able to produce an individualized, specific model of CFTR function. There are several key steps in the process that should be closely attended to avoid difficulty. First is a good sample acquisition from the patient's nose. A good sample should have >50,000 cells, limited mucus/debris, and be ready for processing within 4 h (though success is also easily achievable with overnight shipping on ice). Practice acquiring samples with curette or brush by study staff is necessary, which is facilitated by focusing early sample acquisition into the hands of one or two providers to maximize their comfort. Second, good clean/sterile technique in all tissue culture steps is essential. The primary mode of failure, in our experience, for all HNE cultures is bacterial or fungal contamination, which can complicate both the primary sample and other growing cultures in the incubator. This risk only increases with cultures of subjects with chronic infection (*e.g.*, CF), therefore success hinges largely on the ability to keep cultures clean and separate. Our practice is to keep one incubator separate only for infection-prone cultures within the first 5-7 days, protecting older, successful cultures from inadvertent contamination. Third, it is important to closely watch cells during the expansion phase and avoid overconfluence. If the cells are allowed to reach full confluence on the plate, there is a risk that the culture will either become senescent or cells will begin to die and detach. Finally, when plating cells as spheroids, it is important to thoroughly disperse the cells through the matrix and to plate an evenly distributed sample. Either over- or under-population of the matrix drops will lead to culture failure, and large clumps of cells will not produce successful spheroids.

While implementing this protocol, investigators may encounter a few common problems. Contamination, as mentioned above, is best avoided by procuring a good initial sample without mucus and clean culture techniques. If cultures are successful through expansion, but no differentiated spheres are formed, several issues may have occurred. If the matrix appears mostly empty, it is most likely that the cells were seeded at too low of a density; increase the seeding density of subsequent attempts by approximately 20%. Conversely, if the matrix appears to be "dirty" with copious cell debris, it is likely that the cells were seeded at too high of a density, and subsequent attempts should use a seeding density reduced by approximately 20%. A common complication of post-seeding feeding, and maintenance is the detachment of the matrix from the culture vessel. This is caused by overly aggressive media exchange and can be avoided by cautiously and manually changing media with a 1-mL pipette, not wall suction. If encountered, spheres can still be stimulated and imaged, though this may be difficult if the matrix "floats" in



the well during imaging, necessitating cautious mapping of spheroids within the well.

Investigators may pursue several modifications to the protocol, depending on the needs of their lab. For higher-resolution imaging of spheroids, we have previously substituted the 4-well plates with optical glass options, including 35-mm glass-bottom dishes or chamber slides. This allows for high-resolution, live imaging of spheroids, but may reduce throughput. Alternatively, to increase throughput, smaller aliquots of cells in the matrix can be seeded into other vessels (*e.g.*, 24-well culture plates). In our experience, spheroids grow best with the matrix in a “droplet” form as opposed to forming a sheet on the bottom of the well; as such, we have had the best success with droplets of at least 25  $\mu$ L. Investigators may wish to alter the density of matrix by diluting with media. This reduces the total amount of matrix necessary, improving the cost of the assay. This may also, however, alter the spheroid composition. In our previous experience, lower concentrations of the basement membrane matrix lead to altered spheroid structure, with either partial or complete formation of spheroids in a “cell-apex-out” morphology. As such, spheroids generated through any protocol alterations in matrix concentration should be cautiously evaluated for morphology before functional testing is attempted. Finally, different stimulating or inhibitory drugs, or different concentrations of these drugs, could be employed. Forskolin, IBMX, and Inh172 were chosen for our studies at these concentrations based on previous experience in ALI cultures<sup>31</sup>. Using other drugs (*e.g.*, isoproterenol for stimulation, GlyH101 for inhibition) may better apply to an investigator’s study. Similarly, using different concentrations of forskolin, IBMX, or Inh172 may alter the dynamic range of the assay, however, we have not systematically tested these options.

Given the number of existing *ex vivo* patient-derived CFTR assays, the described model is notable for several key reasons. First, it capitalizes on nasal cells as an easily obtained source of respiratory tissue. HNE procurement can be performed safely with minimal training in almost any setting (clinic, OR, research visit) in all age groups<sup>25</sup>. This facilitates robust sample procurement, and repeat sampling if necessary due to growth difficulty or contamination. Compared to intestinal organoids, HNE spheroids are less well characterized and appear to have a smaller dynamic range in the present form, however, use of respiratory instead of GI tissue may be a key benefit, as several CF disease drivers (*e.g.* mucociliary clearance, epithelial sodium channel expression) are not equivalent in the gut. As opposed to planar, ALI cultures of HNE cells, spheroids are grown faster and may be more representative of *in vivo* conditions, measuring a physiologic process (fluid homeostasis) that may be more relevant than electrophysiology alone<sup>32</sup>. By improving the time from sample procurement to testing, contamination potential is reduced, therefore increasing the likelihood of culture success. Finally, the 3-dimensional nature of the model may be amenable to the novel and/or complementary lines of research in airway development or morphology that are not feasible in ALI cultures (*e.g.*, luminal mucus tracking, rapid studies of differentiation, *etc.*).

There are several key limitations to HNE spheroids as an assay of CFTR function. First, this assay remains relatively low-throughput. Using the current methods, image acquisition takes over an hour for each culture condition, and analysis takes an additional 20-30 min per condition. This is compounded by the degree of overlap between certain conditions (such as demonstrated in

**Figure 2A**), which necessitates a larger number of measurements (this overlap in itself may also represent a limitation of the sensitivity of this assay). As such, a single experiment with 4-6 conditions requires almost two days for completion. Throughput can be improved by employing methods of automated image capture and analysis; such adaptations are currently in progress. Secondly, this model requires a large amount of matrix, which can become expensive. As mentioned above, dilution of the matrix may help overcome this barrier, but must be taken with caution, as alterations in the growth matrix will likely result in alterations in spheroid morphology. Third, this model relies on spheroid measurements in an XY plane only, and disregards swelling in the Z plane, which may introduce bias. While previous reproducibility analyses have been reassuring, this shortcoming could be overcome by use of automated imaging with Z-plane scanning to calculate volume<sup>30</sup>. Finally, and most importantly, extensive work to tie spheroid responses to the individual subject's *in vivo* drug response is yet to be completed. In absence of this correlation, the predictive value of the model – while promising – is unclear.

Generation and analysis of HNE spheroids allow for *ex vivo* analysis of individual CFTR activity and modulation, which may ultimately be useful as a pre-clinical model of drug response. Moreover, given the extensive variability in CF disease severity, such an individualized model can provide insights into a subject's unique airway microenvironment. In this way, such a model may be useful for studies of broader CFTR biology and aid in understanding disease heterogeneity. Future use of this model, as well as other similar models of individualized CFTR function, holds promise to better understand CFTR biology in the lab, and to drive personalized and precision medicine in the clinic.

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#### DISCLOSURES:

The authors have nothing to disclose.

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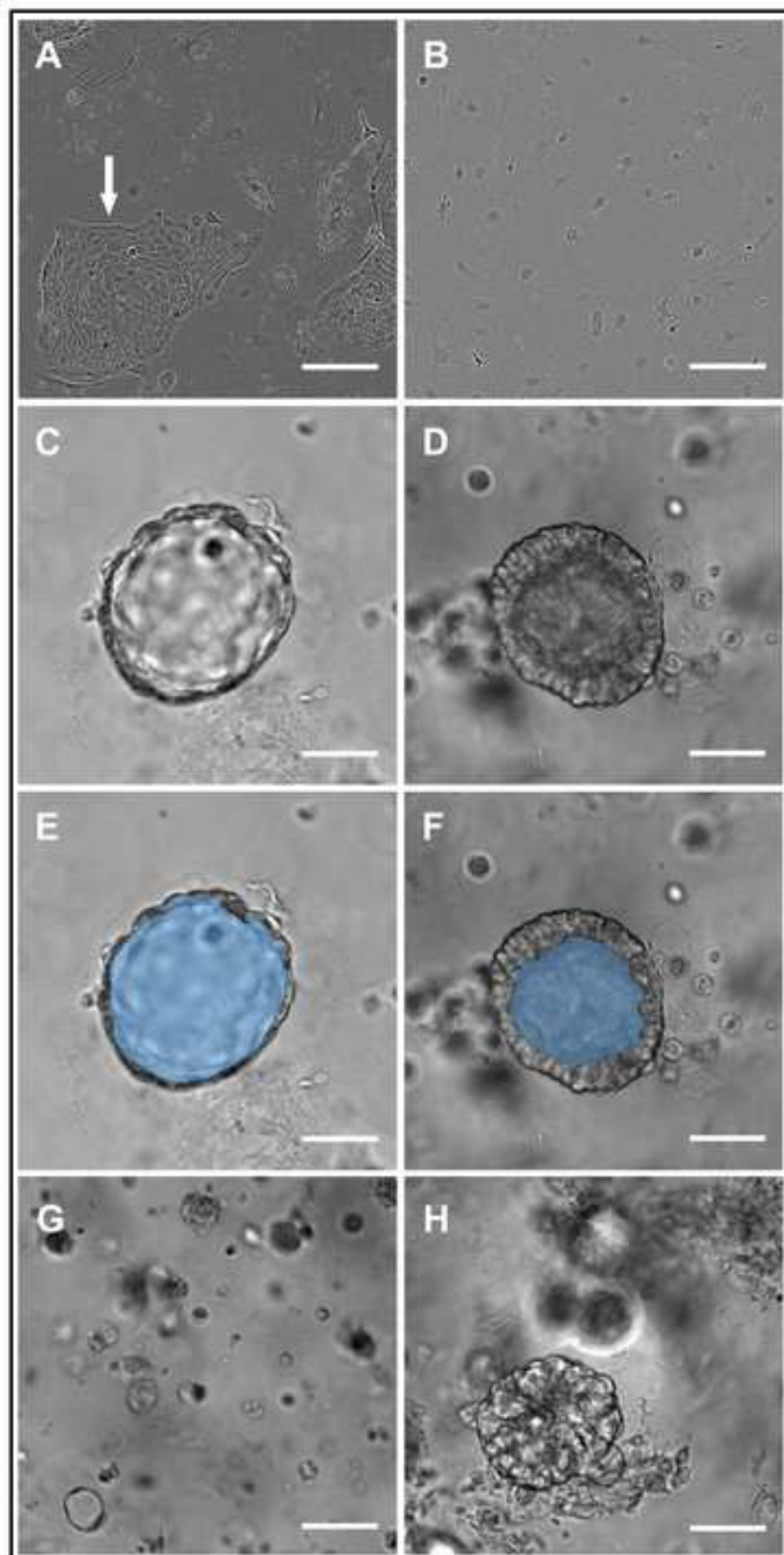
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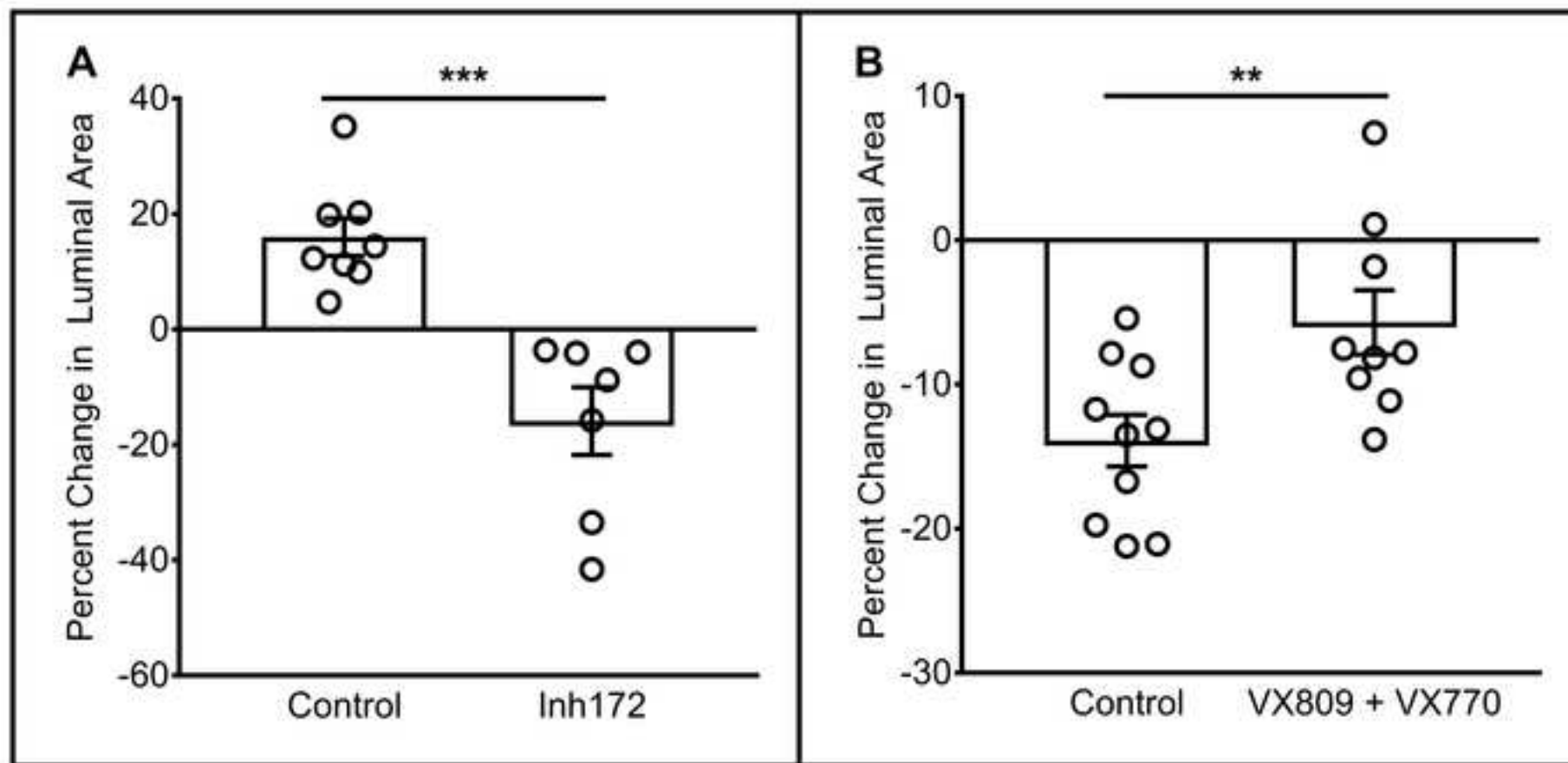
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Media Component	Stock Solution	Amount	Storage
<b>Expansion Media</b>			
DMEM / F-12 Nutrient Mixture "Base Media"	Use as is	2x 500 mL containers	Store at 4 °C up to manufacturer expiration date
Fetal Bovine Serum	Use as is	50 mL	Store at -20 °C up to manufacturer expiration date
Cholera Toxin	10 mg in 1 mL of sterile water	1 µL	Store stock at -20 °C up to six months
Epidermal Growth Factor	500 µg in 1 mL of sterile water	20 µL	Store stock at -20 °C up to six months
Hydrocortisone	0.4 mg in 400 µL of sterile water	Entire 400 µL aliquot	Make fresh with each batch. Store powder at room temperature up to manufacturer expiration date
Adenine	24 mg in 1 mL of sterile water	Entire 1 mL aliquot	Make fresh with each batch. Store powder at room temperature up to manufacturer expiration date
Y-27632	3.2 mg in 1 mL of sterile water	Entire 1 mL aliquot	Make fresh with each batch. Store powder at -20 °C up to manufacturer expiration date
<b>Antibiotic Media</b>			
Amphotericin B	Use as is	1.2 mL	Store at 4 °C up to manufacturer expiration date
Ceftazidime	15 mg in 1 mL of sterile water	Entire 1 mL aliquot	Make fresh with each batch. Store powder at -20 °C up to manufacturer expiration date
Tobramycin	15 mg in 1 mL of sterile water	Entire 1 mL aliquot	Make fresh with each batch. Store powder at -20 °C up to manufacturer expiration date
Vancomycin	15 mg in 1 mL of sterile water	Entire 1 mL aliquot	Make fresh with each batch. Store powder at -20 °C up to manufacturer expiration date

Media Component	Stock Solution	Amount	Storage
DMEM / F-12 Nutrient Mixture "Base Media"	Use as is	2x 500 mL containers	Store at 4 °C up to manufacturer expiration date
Ultrosor-G	20 mL of sterile water in a single, 20 mL bottle of lyophilized Ultrosor-G	Entire 20 mL aliquot	Make fresh with each batch. Store powder at 4 °C up to manufacturer expiration date
Fetal Clone II	Use as is	20 mL	Store at -20 °C up to manufacturer expiration date
Pen Strep	Use as is	10 mL	Store at -20 °C up to manufacturer expiration date
Bovine Brain Extract	Use as is	2.48 mL	Store at -20 °C up to manufacturer expiration date
Transferrin	Use as is	250 µL	Store at -20 °C up to manufacturer expiration date
Insulin	Use as is	250 µL	Store at -20 °C up to manufacturer expiration date
Ethanolamine	Use as is	15 µL	Store at room temperature up to manufacturer expiration date
Epinephrine	2.75 mg in 1 mL of sterile water	Entire 1 mL aliquot	Make fresh with each batch. Store powder at 4 °C up to manufacturer expiration date
Triiodothyronine	8.4 mg in 50 µL of DMSO	Entire 50 µL aliquot	Make fresh with each batch. Store powder at -20 °C up to manufacturer expiration date
Hydrocortisone	7.24 mg in 1 mL of ethanol	1 µL	Store stock at -20 °C up to six months
Phosphorylethanolamine	35.25 mg in 1 mL of sterile water	1 µL	Store stock at -20 °C up to six months
Retinoic Acid	3 mg in 1 mL of DMSO	1 µL	Store stock at -20 °C up to six months



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL Eppendorf Tube	USA Scientific	4036-3204	
150 mL Filter Flask	Midsci	TP99150	To filter Media
15 mL Conical Tube	Midsci	TP91015	
1 L Filter Flask	Midsci	TP99950	To filter Media
35 mm Glass-Bottom Dish	MatTek Corporation	P35G-0-20-C	Optional
3-Isobutyl-1-Methylxanthine (IBMX)	Fisher Scientific	AC228420010	Prepare a 100 mM stock solution of 22.0 mg in 1 mL of DMSO
50 mL Conical Tube	Midsci	TP91050	
Accutase	Innovative Cell Technologies, Inc.	AT-104	Cell detachment solution
Adenine	Sigma-Aldrich	A2786-25G	See Table 1
Amphotericin B	Sigma-Aldrich	A9528-100MG	See Table 1
Bovine Brain Extract (9mg/mL)	Lonza	CC-4098	See Table 2
Ceftazidime hydrate	Sigma-Aldrich	C3809-1G	See Table 1
Cell Scrapers 20 cm	Midsci	TP99010	
CFTR Inh172	Tocris Bioscience	3430	Prepare a 10 mM stock solution of 4.0 mg in 1 mL of DMSO
Cholera Toxin B (From Vibrio cholerae)	Sigma-Aldrich	C8052-.5MG	See Table 1
CYB-1	Medical Packaging Corporation	CYB-1	Cytology brush
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D5879-500ML	
Dulbecco's Modified Eagle Media (DMEM)/F12 Hepes	Life Technologies	11330-057	Base Medium; See Tables 1 and 2
Epidermal Growth Factor (Recombinant Human Protein, Animal-Origin Free)	Thermo Fisher Scientific	PHG6045	See Table 1
Epinephrine	Sigma-Aldrich	E4250-1G	See Table 2
Ethanol	Fisher Scientific	2701	
Ethanolamine	Sigma-Aldrich	E0135-500ML	16.6 mM solution; See Table 2
Ethylenediaminetetraacetic Acid (EDTA)	TCI America	E0084	
Fetal Bovine Serum (high performance FBS)	Invitrogen	10082147	See Table 1
Forskolin	Sigma-Aldrich	F6886-50	Prepare a 10 mM stock solution of 4.1 mg in 1 mL of DMSO

Growth Factor-Reduced Matrigel	Corning, Inc.	356231	Corning Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free, LDEV-Free, 10 mL.
Hemocytometer	Hausser Scientific	1483	
Human Collagen Solution, Type I (VitroCol; 3 mg/mL)	Advanced BioMatrix	5007-A	Collagen solution
HyClone (aka FetalClone II)	GE Healthcare	SH30066.03HI	See Table 2
Hydrocortisone	StemCell Technologies	07904	See Tables 1 and 2
Insulin, human recombinant, zinc solution	Life Technologies	12585014	4 mg/mL solution; see Table 2
IVF 4-Well Dish, Non-treated	NUNC (via Fisher Scientific)	12566350	4-well plate for spheroids; similar well size to a 24-well plate
MEF-CF1-IRR	Globalstem	GSC-6001G	Irradiated murine embryonic fibroblasts
Metamorph 7.7	Molecular Devices		Analysis Software; <a href="https://www.moleculardevices.com/systems/metamorph-research-imaging/metamorph-microscopy-automation-and-image-analysis-software">https://www.moleculardevices.com/systems/metamorph-research-imaging/metamorph-microscopy-automation-and-image-analysis-software</a> for a quote
Olympus IX51 Inverted Microscope	Olympus Corporation	Discontinued	Imaging Microscope. Replacment: Olympus IX53, <a href="https://www.olympus-lifescience.com/pt/microscopes/inverted/ix53/">https://www.olympus-lifescience.com/pt/microscopes/inverted/ix53/</a> for a quote
Pen Strep	Life Technologies	15140122	See Table 2
Phosphorylethanolamine	Sigma-Aldrich	P0503-5G	See Table 2
Retinoic Acid	Sigma-Aldrich	R2625-50MG	See Table 2
Rhinoprobe	Arlington Scientific, Inc.	96-0905	Nasal curette
Slidebook 5.5	3i, Intelligent Imaging Innovations	Discontinued	Imaging Software. Replacement: Slidebook 6, <a href="https://www.intelligent-imaging.com/slidebook">https://www.intelligent-imaging.com/slidebook</a> for a quote
Sterile Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific	20012050	
Sterile Water	Sigma-Aldrich	W3500-6X500ML	

Tissue Culture Dish 100	Techno Plastic Products	93100	Tissue culture dish for expansion
Tobramycin	Sigma-Aldrich	T4014-100MG	See Table 1
Transferrin (Human Transferrin 0.5 mL)	Lonza	CC-4205	See Table 2
Triiodothyronine	Sigma-Aldrich	T6397-1G	3,3',5-Triiodo-L-thyronine sodium salt [T3]; See Table 2
Trypsin from Porcine Pancreas	Sigma-Aldrich	T4799-10G	
Ultroser-G	Crescent Chemical (via Fisher Scientific)	NC0393024	20 mL lypophilized powder; See Table 2
Vancomycin hydrochloride from <i>Streptomyces orientalis</i>	Sigma-Aldrich	V2002-5G	See Table 1
VX770	Selleck Chemicals	S1144	Prepare a 1 mM stock solution of 0.4 mg in 1 mL of DMSO
VX809	Selleck Chemicals	S1565	Purchase or prepare a 10 mM stock solution of 4.5 mg in 1 mL of DMSO
Y-27632 Dihydrochloride ROCK inhibitor	Enzo LifeSciences	ALX-270-333-M025	See Table 1





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Generation of Human Nasal Epithelial Cell Spheroids for Individualized

Author(s):

John Brewington, Erin Filbrandt, FJ Shalaby, Jessica Mancini, Alicia Costanzo, Lauren Strecker, J.P. Clancy CTR Study

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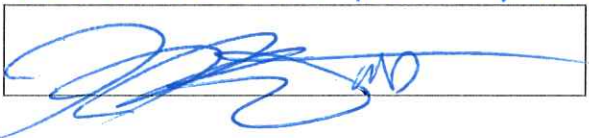
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### CORRESPONDING AUTHOR:

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Institution: Cincinnati Children's Hospital Medical Center  
Article Title: Generation of Human Nasal Epithelial Cell Spheroids for Individualized CFTR Study  
Signature:  Date: 10.20.2017

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To the Editors of the Journal of Video Experimentation:

We appreciate your ongoing consideration of our manuscript entitled “Generation of Human Nasal Epithelial Cell Spheroids for Individualized Cystic Fibrosis Transmembrane Conductance Regulator Study,” ID# 57492\_R1. Both reviewer and editorial comments have aided us in generating what we believe is a stronger manuscript that will be of key interest to numerous investigators in the Cystic Fibrosis research community and beyond. Please see our responses to the editorial comments below, and the attached, revised manuscript.

All responses to editorial comments are highlighted in **red text**, and changes in the manuscript have been tracked for review.

### **Editorial comments:**

1. The manuscript has been modified. Please read it carefully and revise if necessary. Enclosed please find the top copy. Please do not change the current format/font.

**We have read and reviewed the manuscript carefully, and approve of the current format and content.**

2. Please avoid using any abbreviation in the title.

**We have changed the abbreviation “CFTR” to its full name, “Cystic Fibrosis Transmembrane conductance Regulator.”**

3. Please define all abbreviations before use.

**We apologize for the oversight; after review of the manuscript and according modifications, we have defined all abbreviations before the first use.**

4. 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 and Reagents. For example: VitroCol, Matrigel, etc.

**We have removed/minimized all commercial language from the manuscript as requested, and appropriately referenced these items in the table of materials (see item #5 below, as well).**

5. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "matrigel" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

**We have removed the word “matrigel” from the manuscript as requested, leaving only the initial mention of the cell matrix for clarity, and apologize for the oversight in the initial revision.**



6. 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.

We have reviewed and revised key portions of the protocol, and believe the question of “how” is appropriately answered.

7. Protocol: 3.1: Please avoid using any commercial language, please use a generic term instead.

We have removed the commercial term as requested.

8. Protocol: 4.1: Please clearly describe the actions or refer to appropriate references.

In the manuscript, item 4.1 is to ensure IRB-approved consent is acquired for all subjects; while we are willing to expand upon this process, we have instead assumed that the editors were referring to step 4.4.1, nasal curettage. As such, we have expanded this step accordingly. Please note; if this assumption is incorrect, we will gladly expand upon step 4.1.

9. Protocol: 8.3: What are the conditions of the incubator?

We apologize for the oversight, and have added the requested information: 37°C and 5% CO<sub>2</sub>.

10. Currently, about 3 pages are highlighted. Please highlight 2.75 pages or less 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.

In an effort to ensure we stay within this limit and maximize the utility of the video, while ensuring all necessary steps are covered (item 11, below), we have removed the spheroid imaging steps (step 9) and improved the detail on cell passaging, spheroid generation, and spheroid maintenance/maturation. This reduces the total highlighted portion to just over 2 pages, and we believe will provide robust detail on the most critical portion of the protocol, while only removing a segment that will likely be carried out differently at different institutions, dependent upon their imaging capabilities. We appreciate the opportunity to improve on this section, and apologize for the excessive amount in the previous version.

11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Please see the response to item 10, above.

12. Please avoid sectioning the Representative Results.

We apologize for this; sectioning was performed in response to a reviewer request. We are glad to remove sectioning from the Representative Results text.

13. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

These symbols have been removed as requested.