

## **Supplemental Material to**

The mucin bundles responsible for airway cleaning are retained in cystic fibrosis and by cholinergic stimulation

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## **Material and Methods**

### **Video microscopy.**

The distal part of neonatal WT and CF pig trachea together with the most proximal part of the primary bronchi were mounted in a Petri dish coated with Sylgard. The tissue was covered in oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) Krebs-glucose buffer as described previously {Ermund, 2017 260 /id}. Tissue was heated to 37°C and stained with 0.4 mM Alcian blue 8GX pH 7.4, red (580/605) fluorescent beads (FluoroSpheres™ Carboxylate-modified microspheres, 0.04 μm, ThermoFisher Scientific, Waltham, MA) or fluorescein labeled *Lotus tetragonolobus* lectin (Vector laboratories, Burlingame, CA). Buffer without bicarbonate was prepared by omitting NaHCO<sub>3</sub>, adjusting the osmolarity with NaCl and adding 10 mM HEPES. Tissue was monitored through a stereo microscope (Nikon, Tokyo, Japan) and white light (Photonics, Pittsfield, MA) or a CoolLED pE-300ultra light source (CoolLED, Andover, UK). Time-lapse recordings were acquired using a 5.0-megapixel color CCD camera (DS-Fi2 or DS-Fi3, Nikon, Tokyo, Japan) or a monochrome cooled-CCD camera (DS-Qi1, Nikon, Tokyo, Japan) and NIS elements software (Nikon, Tokyo, Japan). *Pseudomonas aeruginosa* bacteria were

stained by 6-(Tetramethylrhodamine-5-(and-6)-Carboxamido) Hexanoic Acid, Succinimidyl Ester (TAMRA; ThermoFisher Scientific, Waltham, MA) Bundle transport velocity, Alcian blue bundle thickness and bead-strand thickness were calculated using NIS elements. To calculate transport velocity, the mean of the five fastest-moving points in each time-lapse was taken on moving bundles/strands.

### **Lectin staining of live tissue for confocal microscopy.**

WT and CF pig airway tissue was dissected into 1 cm pieces and opened to expose the luminal surface. Tissue was glued to a Petri dish using Vetbond tissue adhesive (3M, Sollentuna, Sweden) and a mixture of fluorescein labeled *Lotus tetragonolobus* Lectin (LTL), rhodamine labeled *Ulex europaeus* Agglutinin I (UEA1) or FluoroSpheres™ Carboxylate-modified microspheres, 0.04 µm, red fluorescent (580/605) beads (ThermoFisher Scientific, Waltham, MA) were mixed in Krebs-glucose buffer and added to the tissue. After approximately 30 min of incubation at ambient temperature, lectins were removed and fresh Krebs-glucose buffer was added. Tissue was imaged with a Plan-Apochromat ×20/1.0DIC water immersion objective and an upright LSM 700 Axio Examiner 2.1 confocal imaging system (Carl Zeiss, Oberkochen, Germany). Measurements of bundle volume, distance from the epithelium and number of attachment points were performed using the Imaris software (Bitplane, Zurich, Switzerland).

### **Immunofluorescence staining of fixed sections.**

Pig airway tissue was fixed in 4% formalin, embedded in paraffin and cut in 4 µm thick sections, which were dewaxed using Xylene substitute (Sigma, St. Louis, MO) and hydrated in decreasing concentrations of ethanol. Antigen retrieval was performed by microwave heating in 0.01 M citric buffer pH 6. Sections were blocked for 60 min with 3% donkey serum

in Tris-buffered saline (TBS) and permeabilized with 0.1% Triton X-100. Stainings were performed sequentially. Primary antibodies, mouse monoclonal MUC5AC clone 45M1 (Sigma Aldrich, St. Louis, MO) and MUC5B, kind gift from M. Kesimer (University of South Carolina, Chapel Hill, SC) in blocking solution were incubated over night at 4°C. For MUC5B, donkey anti-rabbit Alexa 488 and for MUC5AC donkey anti-mouse Alexa 647 (Thermo Fisher Scientific, Waltham, MA) secondary antibodies were incubated in blocking solution for two hours at room temperature in the dark. Nuclei were counterstained with Hoechst 34580 (Thermo Fisher Scientific, Waltham, MA). Slides were mounted with Prolong gold mounting medium (Thermo Fisher Scientific, Waltham, MA). Images were acquired with an upright LSM 700 Axio Examiner 2.1 confocal imaging system (Carl Zeiss, Oberkochen, Germany).

### **Electron microscopy.**

WT and CF pig airway tissue was fixed in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M, sodium cacodylate buffer, pH 7.2) for 24 h at 4°C followed by preparation for scanning electron microscopy (SEM). Postfixation was performed in 1% OsO<sub>4</sub> at 4°C three times with an intervening 1% thiocarbohydrazide step. The samples were dehydrated with increasing concentrations of ethanol followed by hexamethyldisilazane that was allowed to evaporate, samples were mounted on aluminum pins with carbon tabs and finally sputter-coated with palladium before examination at 3 kV in a field emission scanning electron microscope (Zeiss DSM 982 Gemini, Carl Zeiss, Oberkochen, Germany).

## Movies:

**Movie S1:** Alcian blue stained bundles on WT piglet trachea.

**Movie S2:** Beads gathering on WT piglet trachea.

**Movie S3:** Alcian blue staining in CF piglet trachea.

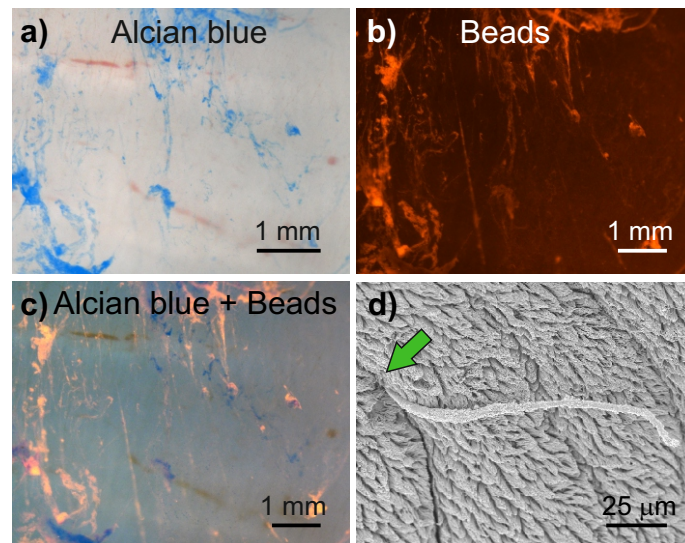
**Movie S4:** Beads on CF piglet trachea.

**Movie S5:** Alcian blue and beads on CF piglet trachea.

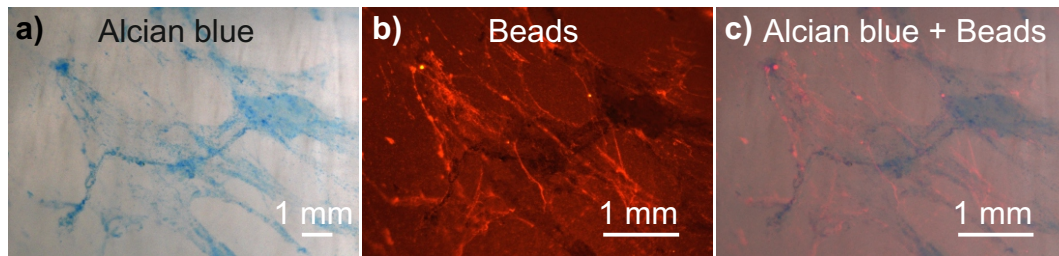
**Movie S6:** Stained *Pseudomonas aeruginosa* bacteria (red) added to WT piglet trachea with LTL-stained bundles (green).

**Movie S7:** Stained *Pseudomonas aeruginosa* bacteria (red) added to CF piglet trachea with LTL-stained bundles (green).

All movies collected at 1 frame per second and rendered at 16x original speed.



**FIGURE S1** Submucosal glands secrete mucus bundles separate from bead-gathering strands in WT piglet trachea. a) WT piglet tracheobronchial preparation stained with Alcian blue. b) WT trachea with fluorescent beads. c) Merge of a and b. d) Bundle exiting the submucosal gland (green arrow) observed with scanning electron microscopy (SEM) in WT piglet trachea. Scale bars in a, b and c 1 mm, in d 25  $\mu\text{m}$ .



**FIGURE S2** Submucosal glands secrete mucus bundles separate from bead-gathering strands in CF piglet trachea. a) CF piglet tracheobronchial preparation stained with Alcian blue. b) CF trachea with fluorescent beads. c) Merge of a and b. Scale bars in a, b and c 1 mm.