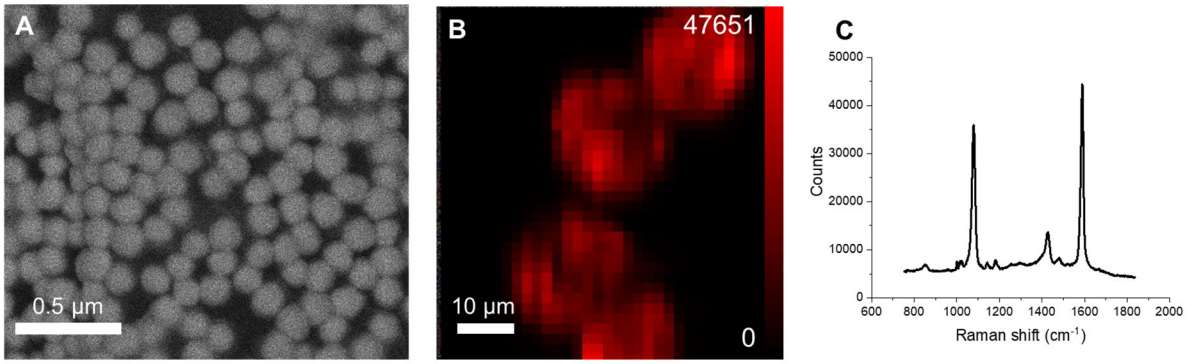

Fig. S1 A. SEM image of gold nanoparticle monolayer on SERS-MS shown in Figure 1. Both SEM images were collected with a Zeiss Crossbeam 550. **B.** Raman heat-map of SERS-MS in cell culture media collected on a Renishaw InVia with 785 nm laser, 0.5s exposure time, 0.165 mW laser power and a 60× water immersion objective. **C.** Unprocessed spectrum from SERS-MS in cell culture media. The spectrum was collected with a 785 nm laser, 0.165 mW laser power and 0.5s acquisition time using a 60× water immersion objective.

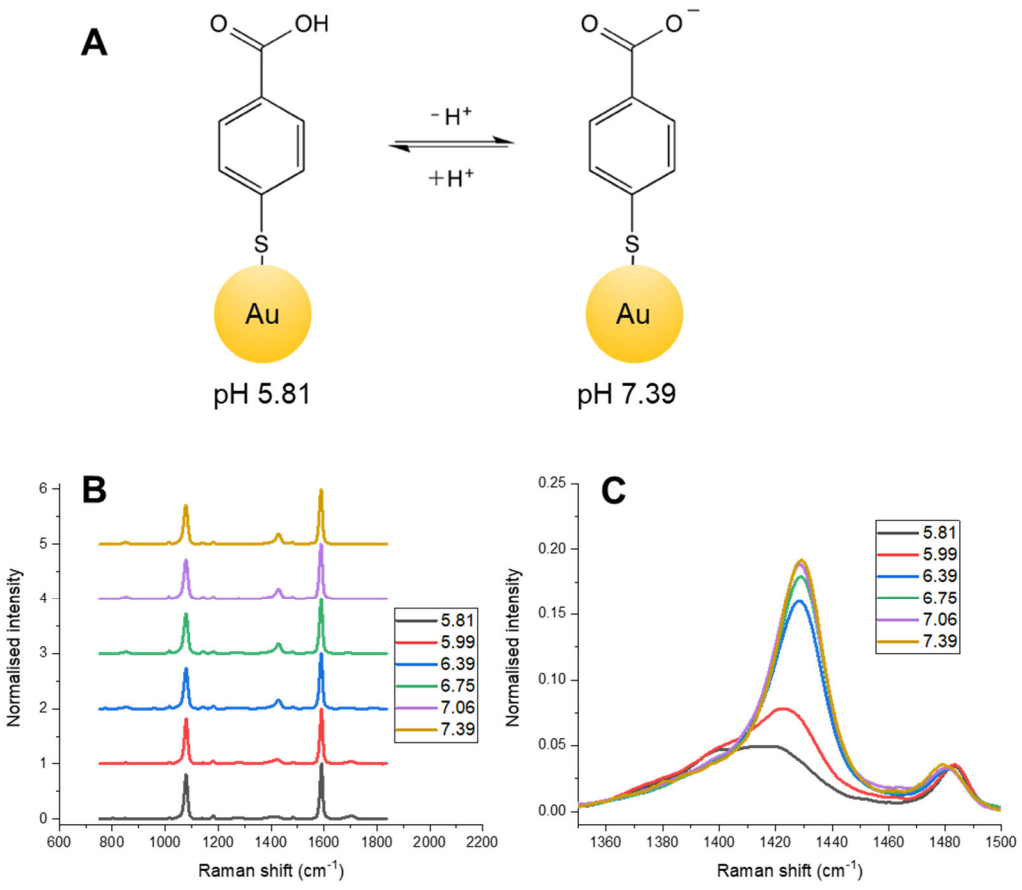

Fig. S2 A. The working principle of the MBA reporter molecule. When SERS-MS embedded in ECM are exposed to cell culture media at pH 5.81, the majority of MBA molecules at the gold surface are protonated and the intensity of the carboxylate peak (ν_s(COO)) at ~1430 cm^{-1} in the SERS spectrum is low relative the aromatic ring deformation peak (ν(ref)) at ~1590 cm^{-1}. At higher pH, more MBA carboxylic acid groups are deprotonated and the intensity of the ν_s(COO) peak relative to ν(ref) increases. **B.** The mean normalised spectrum of SERS-MS following 14 days embedded in cell-free ECM in pH adjusted cell culture media (10% FBS). Spectra were smoothed, baseline corrected and normalised as described in the spectral processing section. **C.** Expanded view of the ν_s(COO) peak of the spectra presented in panel A.

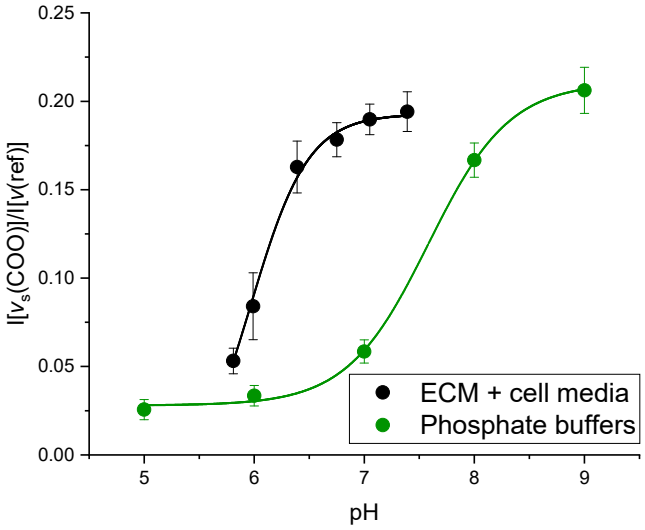

Fig. S3. Black data points indicate pH calibration curve of SERS-MS in cell-free ECM after 14 days of simulated cell culture conditions submerged in pH buffered cell culture media (10% FBS). **Green** data points indicate the pH calibration curve of SERS-MS suspended in phosphate buffer solutions. Each data point represents the mean intensity ratio (±SD) calculated across all SERS-MS spectra analysed. In 'ECM + cell media' each data point represents the mean of ≥37 SERS-MS spectra. In 'Phosphate buffers' each data point represents the mean of ≥13 SERS-MS spectra. In each case multiple particles in a single batch of SERS-MS were analysed.

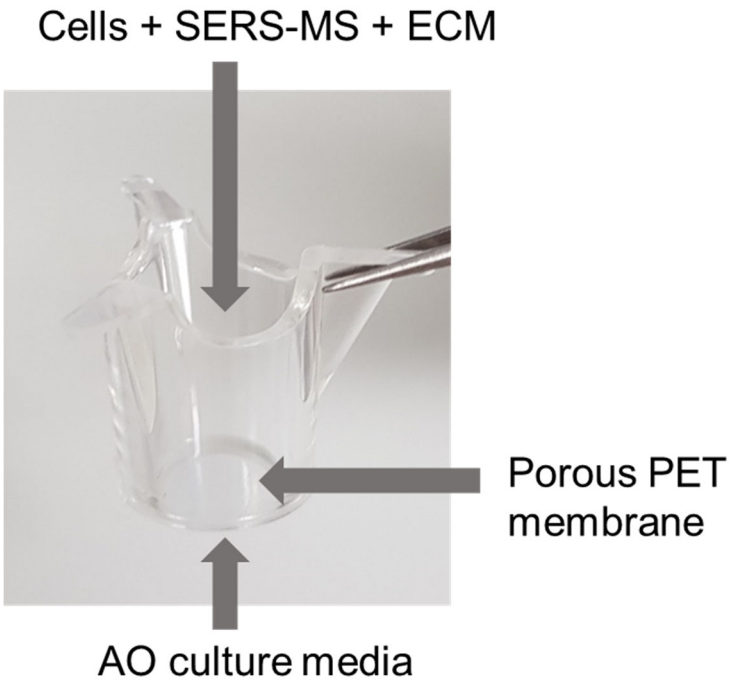

Fig. S4 Porous membrane tissue culture insert (Sarstedt, Inc. 83.3932.041). The liquefied ECM containing basal epithelial cells and SERS-MS were added to the upper compartment of the insert. The insert was then lowered into a reservoir of AO seeding media (0.5 ml).

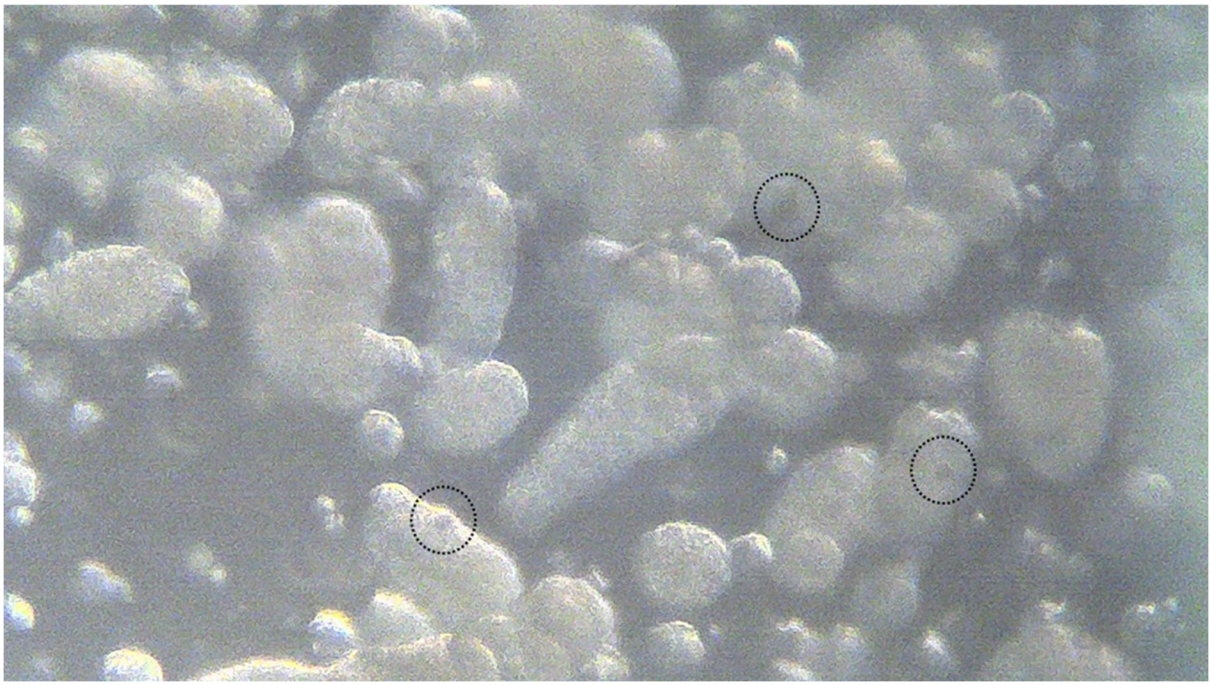

Fig. S5 Bright-field microscope image (4x magnification) of an AO culture after the complete cell culture period in the presence of SERS-MS. Selected SERS-MS are

circled for clarity. SERS-MS were identified by their size, circular shape and darker contrast against the surrounding cellular strucutres.

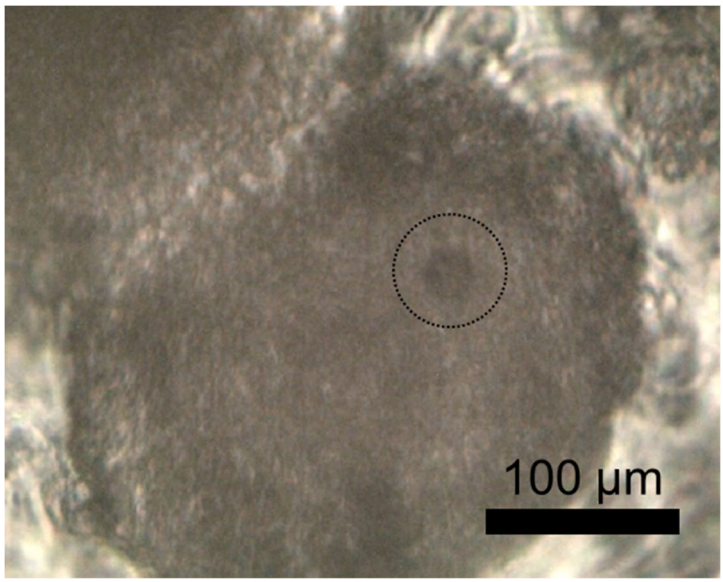

Fig. S6 Bright-field microscope image of SERS-MS inside an organoid. Image take with a 20× objective lens.

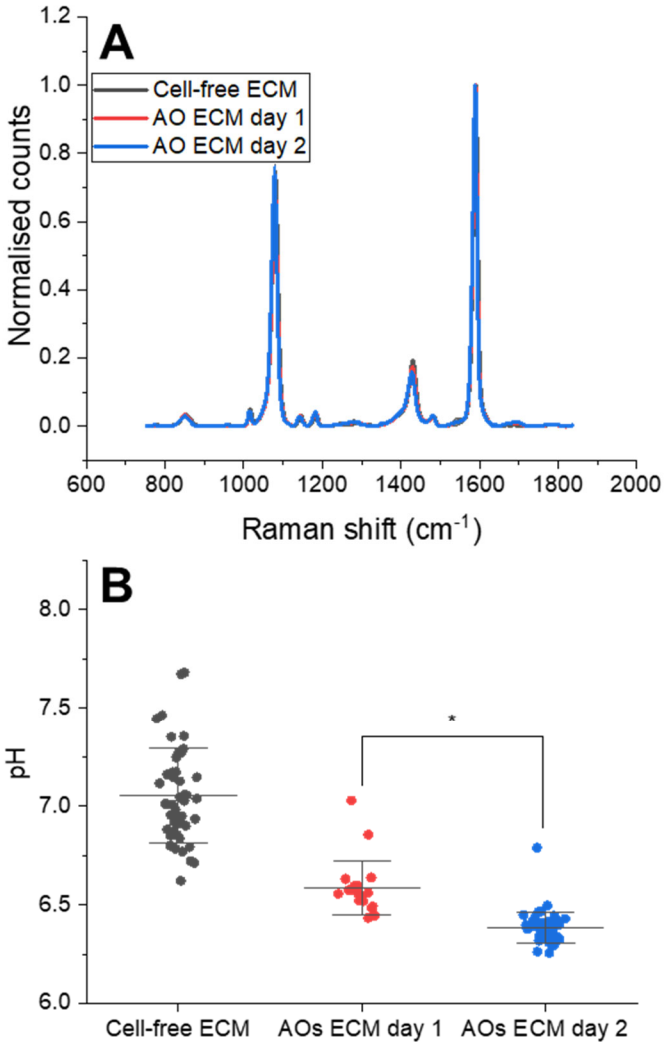

Fig. S7 A. Mean, smoothed, baseline corrected and normalised SERS-MS spectra from cell-free ECM, AO ECM day 1 and AO ECM day 2. 50 spectra from 10 SERS-MS were collected from a cell-free ECM sample, 20 spectra from 3 SERS-MS were collected from AO ECM day 1 and 50 spectra from 7 SERS-MS were collected from AO ECM day 2. Spectra were collected with a 785 nm laser, 0.033 mW laser power and 1s acquisition time and a 60× water immersion objective. **B.** pH values calculated from SERS-MS spectra collected from the cell-free ECM and two AO cultures 1 and 2 days after refreshing the basolateral cell culture media. The 'Cell-free ECM' data points represent 61% of the spectra collected from the sample, the remaining 39% of spectra were excluded because the peak ratio I[ν_s(COO)]/I[ν(ref)] > 0.19275 (variable B in Table S4) indicated the sensor was saturated. Hence, the pH of the 'Cell free ECM' is likely an underestimate. Each data point represents the pH calculated from a

single SERS-MS spectrum. Error bars are centred on the mean and extend to ± SD. A two-sample t-test was used to compare pH values collected from the ECM of AOs on day 1 and day 2 (* = p<0.05).

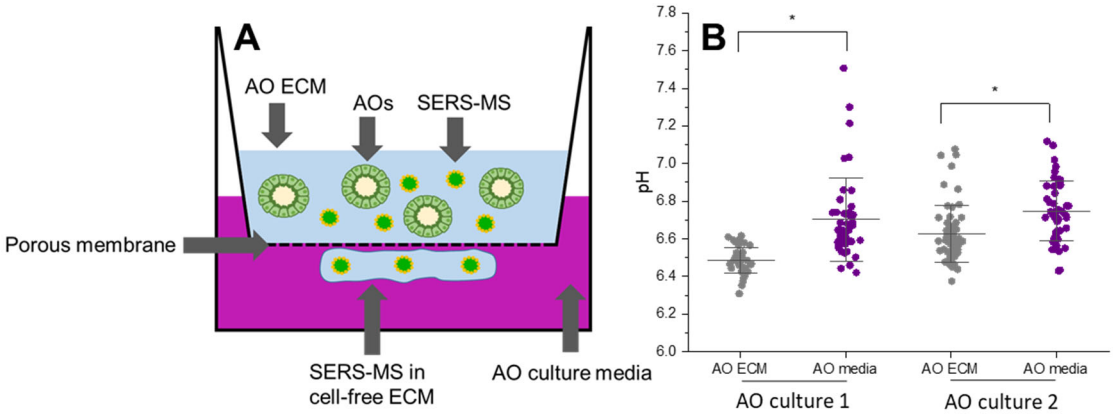

Fig. S8 A. Schematic of AO culture (not to scale). In the upper compartment of the tissue culture insert SERS-MS are shown only in the ECM next to AOs because only SERS-MS adjacent to AOs were selected for the pH measurments presented in panel B. The pH of the AO media reservoir below the insert was measured with SERS-MS encapsulated in cell-free ECM so that SERS pH measurements between the upper compartment and the media reservoir could be directly compared. The ECM is represented by a different colour to the AO culture media for clarity. **B.** pH measurements in the ECM exterior to AOs and in the AO culture media. Two AO cultures from the same donor were analysed the day after refreshing the AO media. Each data point represents a single SERS-MS spectrum. In AO culture 1, 42 spectra from 8 SERS-MS in the AO ECM, and 43 spectra from 6 SERS-MS in the AO media, were analysed. In AO culture 2, 58 spectra from 7 SERS-MS in the AO ECM, and 47 spectra from 9 SERS-MS in the AO media, were analysed. Within both cultures, the pH in the ECM was significantly lower than the pH in the basolateral culture media (* = $p < 0.05$ by two-sample t-test). Spectra were collected with a 785 nm laser, 0.033 mW and 1s acquisition time using a 60× water immersion objective.

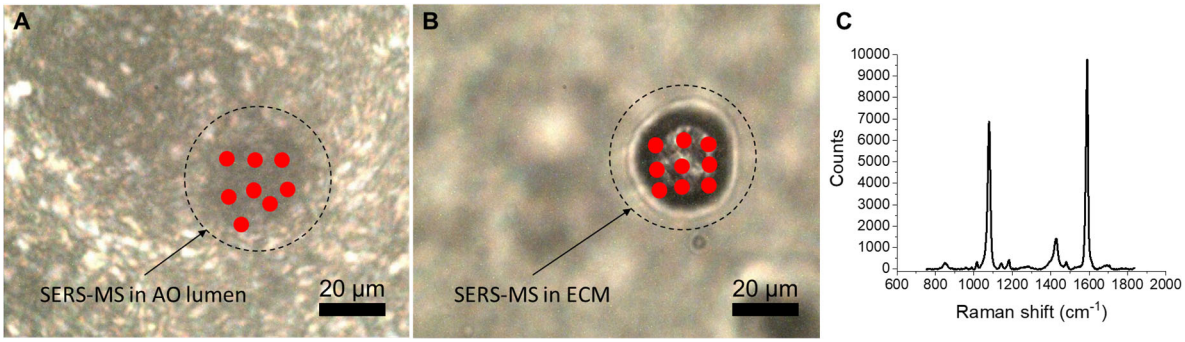

Fig. S9. A. Bright-field microscope image of SERS-MS determined to be inside the lumen of an AO. The SERS-MS outline is blurred by the light scattering of the AO 3D cellular structure. **B.** Bright-field microscope image of SERS-MS in the ECM surrounding AOs. Both images were taken using a 60× water immersion objective. The red circles illustrate how multiple spectra were collected on each SERS-MS. Between 5 and 9 spectra were collected per SERS-MS from non-overlapping locations on the SERS-MS. **C.** A SERS-MS spectrum collected from the lumen of AO in panel A shows the high quality spectrum obtainable from SERS-MS inside the AO lumen. The spectrum was smoothed and baselined as described in Section 1.2.

Table S1. AO seeding media formulation

Solution	**Supplier**	**Final volume or concentration in media**
Pneumacult Airway Organoid Basal Media	Stemcell	9 ml
Pneumacult Airway Organoid Seeding Supplement	Stemcell	1 ml
Penicillin / Streptomycin	Invitrogen	100 mg/ml
Primocin	Invitrogen	50 mg/ml

Table S2: AO differentiation media formulation

Solution	**Supplier**	**Final volume or concentration in media**
Pneumacult Airway Organoid Basal Media	Stemcell	36 ml
Pneumacult Airway Organoid Differentiation Supplement	Stemcell	4 ml
Penicillin / Streptomycin	Invitrogen	100 mg/ml
Primocin	Invitrogen	50 mg/ml
Hydrocortisone	Stemcell	480 ng/ml
Heparin 0.2%	Stemcell	80 µl

Table S3. Number SERS-MS (and number of individual spectra) analysed in the lumen and ECM of AO cultures in Fig. 5.

		SERS-MS	Spectra
AO culture 1	Lumen ECM	6 6	41 35
AO culture 2	Lumen ECM	3 9	19 45
AO culture 3	Lumen ECM	3 3	23 25

Experimental Methods

1.1 SERS-MS fabrication

1 mg of TentaGel® M NH_2 powder (Rapp Polymere GmbH, M30202, $2.4x10^8$ particles/g) was added to 3 ml of citrate-buffered 150 nm gold nanoparticle suspension (Sigma-Aldrich, 746649), the suspension sonicated for 10 minutes and left at 4 °C overnight before use. The nanoparticle-microparticle mixture was stored at 4 °C as prepared for up to 1 month before use. The day before seeding SERS-MS into AO cultures, the particles were pelleted via centrifugation and resuspended in a 100 µM solution of MBA (Sigma-Aldrich, 662534) of 1% ethanol (EtOH) (≥99.8%, Sigma-Aldrich) in deionised (DI) water. The MBA solution was prepared by first dissolving 1.54 mg of MBA in 1 ml of 100% EtOH and then adding 9 ml of DI water, 100 µl of this solution was then added to 900 µl of DI water. The SERS-MS were left in the functionalisation solution overnight at 4 °C. The following day the SERS-MS were pelleted and washed with 70% EtOH in water before two successive washes with sterile phosphate-buffered saline (PBS) and suspended at a final concentration of 2000 SERS-MS/µl in PBS. We confirmed our protocol functionalised the AuNPs surface with MBA by exposing colloidal 150 nm AuNPs to the same MBA functionalisation solution and measuring a redshift in the surface plasmon reasonance

(Fig.S10).

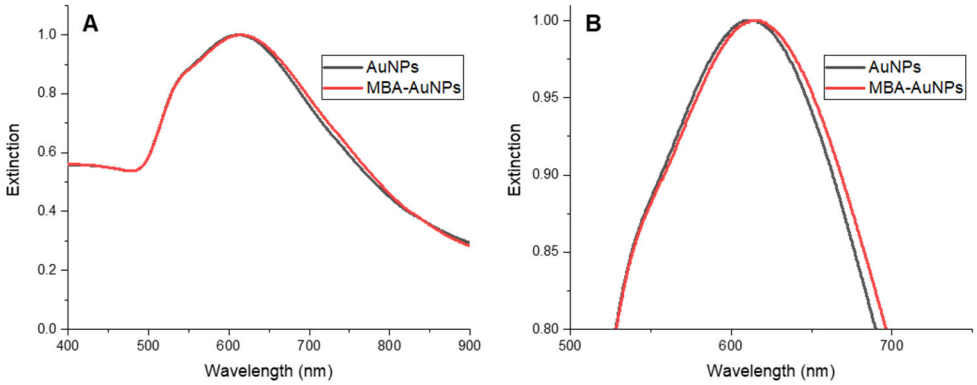

Fig. S10. A. The extinction spectrum of cirate-capped 150 nm AuNPs colloid (AuNPs) and MBA functionalised 150 nm AuNPs colloid (MBA-AuNPs). **B.** Expanded plot of panel A showing a red shift in the surface plasmon reasonance when AuNPs are functionalised with MBA. Spectra in both panels were normalised to 1 at their maximum intensity.

1.2 pH calibration of SERS-MS encapsulated in extracellular matrix

The extracellular matrix (ECM) used in this work was Cultrex® Reduced Growth Factor Basement Membrane Extract Type 2 (Trevigen Inc.). The ECM was thawed on ice, then SERS-MS were suspended in the liquefied ECM (77,000 SERS-MS/ml) and 50 µl of the SERS-MS in ECM suspension pipetted into the upper compartment of tissue culture (TC) inserts (Starstedt, Inc., 83.3932.041). The tissue culture inserts were then lowered into 0.5 ml of AO media in a 24-well plate. The samples were incubated for 1 week in AO seeding media (Table S1) and then for 1 week in AO differentiation media (Table S2) at 37ºC and 5% CO_2. For the calibration experiment, 200 µl of ice-chilled PBS was added to the apical compartment of the TC insert and the ECM was broken up with repeated aspirations. The solution of ECM and SERS-MS was transferred into a microcentrifuge tube and placed on ice for 1 hour. The solution was then centrifuged

at 4800G for 30 seconds and 200 µl of supernatant removed from the surface. The remaining liquified ECM (containing SERS-MS) was kept on ice until the calibration experiment.

Phosphate-buffered Leibovitz's L-15 medium (ThermoFisher, 11415064) containing 10% foetal bovine serum was used to calibrate SERS-MS encapsulated in ECM. Solutions of L-15 medium were adjusted to pH 5.81, 5.99, 6.39, 6.75, 7.09, 7.39 with KH_2PO_4 solution (0.1 M) and the pH measured with an Orion™ 9110DJWP double junction pH electrode. At each pH, 5 µl of the SERS-MS suspension in liquefied ECM was added to 200 µl of buffered L-15 media. The ECM immediately gelled in the room-temperature buffer and spectra were collected from SERS-MS within the areas of gelled ECM. SERS spectra were collected using a water immersion objective lens (Olympus, LUMPlanFL N 60×/1.00, working distance 2 mm) on a Renishaw InVia Raman microscope. At each pH, at least 40 spectra were collected across 7 individual SERS-MS encapsulated in the ECM. Spectra were collected with a 785 nm laser, 0.033 mW power and 1s acquisition time.

Spectra were smoothed and baseline corrected in WiRE 4.4 with 9-point Savitsky-Golay smoothing (polynomial order 3) and WiRE intelligent fitting baseline subtraction. In spectra used for analysis, all peaks were assigned to MBA vibrational modes and and the intensity of the ν(ref) peak at ~1590 cm^{-1} was greater than 1000 counts. The intensities of the ν_s(COO) peak at ~1400 cm^{-1} and ν(ref) at ~1590 cm^{-1} were extracted using MATLAB and the ratios for each spectrum calculated in Microsoft Excel. Origin 2021b was used to plot the calibration data and fit a Boltzmann curve (Equation 1). The values from the fit are presented in Table S4. The inverse of Equation 1 was used to calculate pH from SERS-MS spectra in all subsequent experiments.

$$Y = \frac{(A-B)}{1+e^{\frac{pH-c}{dx}}} + B$$ Equation 1

Table S4. Value of each variable in Equation 1 following fitting

Variable	*Value from fit*
A	-0.02287
B	0.19275
c	5.97042
dx	0.2609

1.3 Basal epithelial cell harvesting and expansion

Ethical approval was gained (North of Scotland Research Ethics Committee) and participants were recruited and consented. Cells were obtained via nasal brushing. The inferior nasal turbinate was located using an otoscope with a 9 mm specula attachment. A sterile cytology brush was passed through the operating channel to brush under direct vision. The brush was transferred into a 15ml Falcon tube containing 5ml of RMPI media (Gibco) with 1% Penicillin/Streptomycin, 10% FBS and 1% L glutamine.

The Falcon tubes were vortexed before removing the brush and centrifuging the media at 300G for 5 minutes at 20^{o}C. The supernatant was discarded, and the cell pellet resuspended in 10 ml Pneumacult Ex-Plus (StemCell) basal cell expansion media with supplements (Table S5). The suspension was transferred to a T75 tissue culture flask (Corning) which had been pre-coated with matrix proteins from 804G media. The Pneumacult Ex-Plus media was refreshed every 48-72 hours and cells passaged at 90% confluence using TrypLE Express (Gibco).

Table S5. Basal cell expansion media formulation, with supplier and concentration

Solution	Supplier	Final volume or concentration in media
Pneumacult™ Ex-Plus Media	Stemcell™	490 ml
Pneumacult Ex Plus 50x Supplement	Stemcell	1x
DMH-1	Tocris	200 nM
CHIR99021	Tocris	500 nM
A83-01	Tocris	500 nM
Y-27632	Abmole	5 mM
Amphoteracin B	Sigma	250 ng/ml
Gentamicin	Sigma	10 µg/ml
Penicillin / Streptomycin	Invitrogen	100 µg/ml / 100 mg/ml
Primocin	Invitrogen	50 mg/ml
Hydrocortisone	Stemcell	96 ng/ml

1.4 Airway organoid culture and SERS-MS addition

AOs were cultured in the upper compartment of porous tissue culture inserts (24-well plate, Starstedt Inc. 83.3932.041) (area 0.3 cm^2, pore size 0.4 µm and pore-density 2×10^6 pores/cm^2). For AO seeding in the presence of SERS-MS, basal epithelial cells (1.16×10^6 cells/ml) and SERS-MS (77,000 SERS-MS/ml) were suspended in ice-chilled ECM and 50 µl of this suspension added to the upper compartment of TC inserts. The inserts were then immersed in 0.5 ml of AO seeding media (Table S1)

and cultured for 7 days, during which time the AO seeding media was replaced every 24-72 hrs. On day 7, the AO seeding media was replaced with 500 µl AO differentiation media (Table S2) and samples cultured for a further 7-10 days, with the differentiation media replaced every 24-72 hrs.

1.5 Airway organoid staining and confocal fluorescence

AOs were fixed in 4% paraformaldehyde for 1 hour at 4 °C and then washed and stored in 70% EtOH in DI for 4 weeks. On the day of fluorescence imaging, the gel was removed from the TC insert by detaching the membrane and using a flexible plastic spatula to transfer it to a microcentrifuge tube containing 1 ml of PBS. Two drops of NucBlue™ (Live ReadyProbes™) were then added to the PBS and the solution incubated for 30 minutes at room temperature. The gel was then transferred to an imaging chamber fabricated from two layers of 0.2 mm acrylic (Fig. S11) and a droplet of Oil 10 S (VOLTALEF®) was placed on the surface of the gel before sealing the chamber with a glass coverslip and double-sided tape.

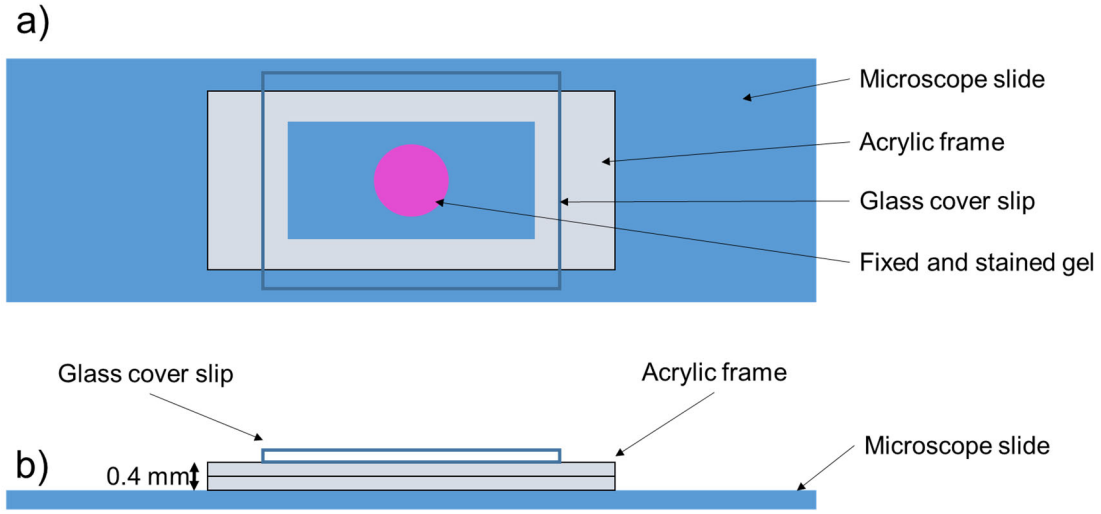

Fig. S11. a) vertical view of imaging chamber on a microscope slide. b) Side view of the imaging chamber.

Fluorescence z-stacks were collected on a Leica TCS SP8 confocal microscope using a 10× objective (Leica, HC PL APO 10x/0,40 CS2). NucBlue was imaged using a 405 nm diode laser with emission detection set to 408-495 nm. A 633 nm HeNe laser with emission detection set to 642-777 nm was used to image the SERS-MS. The z-step interval was 3.5 µm and the z-stack was constructed in ImageJ with the Bio Formats Plugin.[1, 2]

1.6 pH measurements in the extracellular matrix and the AO media

AOs were analysed 1-2 days after refreshing the cell culture media reservoir. For spectral acquisition on the Raman microscope, the TC insert was inverted and 100 µl of AO media (from the reservoir below the insert) was pipetted between a 60× water immersion objective and the TC insert (Fig. S12). The solution was allowed to equilibrate with the 5% CO_2 atmosphere in the imaging chamber for 5 minutes before spectra were collected from SERS-MS in the ECM adjacent to AOs. SERS-MS were selected at random in the cell-free ECM sample. Spectra were collected from SERS-MS adjacent to AOs in the ECM with a 785 nm laser, 0.033 mW and 1s acquisition time. To measure pH in the AO culture media, 5 µl of SERS-MS suspension in liquefied ECM (prepared as described in Section 1.2) was added to the droplet of media between the TC insert and the microscope objective lens. The SERS-MS encapsulated in ECM were allowed to settle on the porous membrane before spectra were collected.

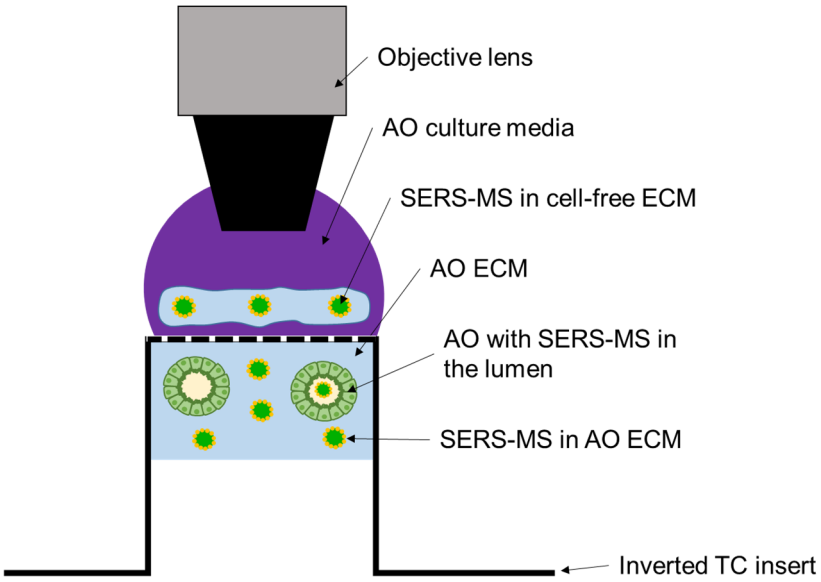

Fig. S12. Schematic of experimental set-up (not to scale). The inverted TC insert contained SERS-MS dispersed amongst AOs. A droplet of AO media was placed between the insert and the objective lens. SERS-MS in cell-free ECM were added to the droplet of AO media.

1.7 Airway organoid lumen pH measurements

The cell culture media in the AO cultures was refreshed the day before measuring lumen pH. For SERS acquisition, the TC insert was inverted and 100 µl of day-old AO media was pipetted between the 60× water immersion objective and the bottom of the TC insert. The culture was allowed to equilibrate with the 5% CO_2 atmosphere in the imaging chamber for 5 minutes before using the stage-top microcontroller and the bright field microscope to search for SERS-MS which were judged by eye to be inside the AO lumen. When a SERS-MS located in the lumen of an AO was found, multiple SERS spectra were collected from different points on the SERS-MS with a 785 nm laser at 0.033 mW power and 2s acquisition time or 0.165 mW power and 1s acquisition time. Spectra were then collected from the nearest SERS-MS in the ECM surrounding the AOs.

References

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2. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nature Methods*, 2012, **9**, 671-675.