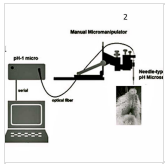

Version 2

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Extracellular and intracellular pH measurements in mice respiratory tract in vivo V.2

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1 Works for me Share dx.doi.org/10.17504/protocols.io.bu4knyuw

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ABSTRACT

In the last century, novel respiratory viruses with pandemic potential have emerged in human populations. Interference with HA acid stability of pandemic H1N1 significantly decreases pathogenicity in mice, ferrets, and swine. We propose that pH is the key determinant of viral fitness in the respiratory tract of mice. Fiber-optic pH sensing has been used *in vivo*in animal species such as dogs, mice, and swine and in humans. In vivo measurements of extracellular and intracellular pH of the respiratory tract after influenza virus infection and of baseline pH of the respiratory tract are not accurately studied. Here, we measured the pH in mice respiratory tract *in vivo* under anesthesia, using the fiber-optic phase detection system and by inserting the 140-µm diameter microsensor tip into the nasal cavity, soft palate, and trachea of mice. To the best of our knowledge, our study is the first to use pH sensing technology in the respiratory tract of infected and non-infected mice. In addition, individual cell properties differ significantly in size, protein levels, and expression. Therefore, we performed single intracellular pH analyses to better understand the cellular responses in different respiratory tissues and complex environments in response to IAVs, especially pandemic viruses. We report potentials in local extracellular pH in different parts of the mouse respiratory tract in correlation to the intracellular measurement of a single cell the local immune response.

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KEYWORDS

Respiratory tract pH, extracellular pH, intracellular pH, Influenza viruses, pandemic H1N1, HA acid stability, lysozyme, cystol, endosome, acidic, alkaline

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MATERIALS TEXT

A microfiber optic pH meter with needle-type housed pH Microsensor ((PreSens Precision Sensing GmbH, Regensburg, Germany)

PreSens Precision Sensing software GmbH, Regensburg, Germany

Computer

six colorless pH buffer solutions (pH 4, 5, 6, 7, 8, 9) prepared from isotonic solution containing different concentration of NaH_2PO_4(mmol), NaH_2PO_4 (mmol), NaCl (mmol)

Isoflurane

Acrylan

ABSL2+ facility for influenza viruses.

Six-week-old female DBA/2J mice

lamellar tissue culture hood.

sterile surgical instruments suitable for small-animal surgery.

70% ethanol

Sterile surgical scissors and scalpel.

Petri dishes

50 mL conical tube containing 30 mL PBS supplemented with 5% FBS and antibiotics, on ice.

100 mm Petri dish containing 10 mL buffer

sterile forceps and surgical scissors.

50 mL tube containing 10 mL 0.15% Pronase solution

Centrifuge

1 mL DNAse solution (100–200 µL/tissue)

Primaria Plates (Falcon)

37°C incubater an atmosphere of 95% air, 5% CO_2

40 µM strainer

cell counter

trypan blue vital staining

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pH-sensitive dyes of different wavelengths

Cytosolic pH, microtubule pH, cell membrane pH, and lysozyme pH were measured using pHrodo™ Red AM (Invitrogen, P35372), Paclitaxel, Oregon Green™ 488 (Invitrogen P22310), Oregon Green™ 488 1,2-dihexadecanoyl-sn-glycerol-3-phosphoethanolamine (Oregon Green™ 488 DHPE, O12650), and LysoSensor Blue, respectively.

Intracellular pH was quantified by the Intracellular pH Calibration Buffer Kit (Cat. No. P35379).

Live Cell Imaging Solution A14291DJ.

pHrodo™ Green and Red Amine-Reactive labels (P35369 and P36600)

EE1 antibody as a marker for early endosome (Abcam).

RAB7 as a marker for late endosome (Abcam).

glucose-regulated protein 94 (GRP94)

4',6-diamidino-2-phenylindole (DAPI)

flow cytometry.

Light scatter and DAPI

PowerLoad™ Concentrate (Fisher Scientific).

LCIS (Cat. no. A14291DJ)

BD FACSymphony A5 cytometer (BD Biosciences) with 355, 405, 445, 488, 561, and 640nm lasers and equipped with 30 fluorescence detectors was utilized for flow cytometric measurements throughout this study.

GraphPad software v9 (GraphPad, San Diego, CA).

1 Extracellular pH measurements in live mice

1.1 The fiber-optic microsensor, with a tip diameter of 140 µm and a tip length of 3 mm, was calibrated with six colorless pH buffer solutions (pH 4, 5, 6, 7, 8, and 9) according to the manufacturer instructions to generate a calibration curve.

1.2 Optimizing extracellular pH measurements in live mice: Ten healthy mice given different anesthetic compounds to select the best anesthetic that does not interfere with homeostatic pH. The pH was first

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measured in nostrils, and then the sensor was carefully introduced into the nasal cavity with precise localization to achieve a stable pH reading and prevent bleeding and/or irritation. We then measured the soft palate pH and the tracheal pH with a tracheal catheter.

1.3 We performed in-house hospital sterilization of the fiber-optic microsensor with low pH buffer (4) and Acrylan that did not considerably influence the calibration curves generated by the pH microsensor but adequately killed the viruses.

1.4 Procedures for monitoring in vivo extracellular pH in the respiratory tracts of IAV infected and uninfected mice: Female DBA2J mice (n = 25) aged 4 to 6 weeks and weighing 12–15 g were obtained from Jackson Laboratories and adapted to standard vivarium conditions, with temperatures of 21°C–24°C and relative humidity of 50%–65%. Mice were fed standard chow and drank only autoclaved tap water. We divided the mice into three groups of five and intranasally inoculated them with 30 µL of the acid-stable 2009 pandemic H1N1 virus (WT 750), acid-destabilized H1N1 virus (HA1-Y17H 750 or HA1-Y17H 375K), or PBS.

1.5 To monitor the pH of the respiratory tract at different time points post infection, five mice were randomly selected and anesthetized with isoflurane at 2, 5, 7, and 10 DPI and euthanized by cervical dislocation.

1.6 Salinity and temperature were measured with the pH microsensor.

1.7 Tissues were immediately collected into sterile microtubes for further analysis of the intracellular pH by flow cytometry.

1.8 Data were exported via the instrument software and evaluated with GraphPad Prism, v9.0. Statistical comparisons were made with two-way ANOVAs and appropriate posthoc analyses.

Preparation of pH dyes and calibration

2 Intracellular pH dyes:

2.1 pH-sensitive dyes of different wavelengths and the Intracellular pH Calibration Buffer Kit were used according to manufacturer's instructions.

2.2 Cytosolic pH, microtubule pH, cell membrane pH, and lysozyme pH were measured using pHrodo™ Red AM (Invitrogen, P35372), Paclitaxel, Oregon Green™ 488 (Invitrogen P22310), Oregon Green™ 488 1,2-dihexadecanoyl-sn-glycerol-3-phosphoethanolamine (Oregon Green™ 488 DHPE, O12650), and LysoSensor Blue, respectively.

2.3 Intracellular pH Calibration Buffer Kit P35379 Live Cell Imaging Solution A14291DJ was used to quantify intracellular pH.

2.4 pHrodo™ Green and Red Amine-Reactive labels (P35369 and P36600) were used to label specific antibodies for early endosome, late endosome, and secretory vesicles.

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3 Intracellu Intracellular.png lar pH measurements:

Single-cell preparations of live, primary mouse nasal, soft palate, and trachea epithelial cells:

3.1 Mice were euthanized by cervical dislocation after isoflurane exposure. In a lamellar tissue culture hood and using sterile surgical instruments suitable for small-animal surgery, mice were pinned down to the dissecting tray, sprayed with 70% ethanol, and the nasal part was cut with clean surgical scissors and scalpel. Using a suture loop around the front incisors, the head was immobilized, the soft palate was extracted, the skin around the tracheal area was removed, and dissection from its attachment of fat and salivary glands was performed.

3.2 Tissues were immediately placed in small Petri dishes containing collecting medium on ice. Each tissue was placed into a 50 mL conical tube containing 30 mL PBS supplemented with 5% FBS and antibiotics, on ice. Then, the tissue was transferred to a sterile 100 mm Petri dish containing 10 mL buffer to dissect connective tissues with sterile forceps and surgical scissors.

3.3 Tissues were washed twice and transferred to a 50 mL tube containing 10 mL 0.15% Pronase solution and incubated overnight at 4°C.

3.4 On the second day, tissues were removed from Pronase solution, washed twice, and centrifuged at 1400 rpm for 10 min at 4°C.

3.5 Pellets were gently resuspended in 1 mL DNAse solution (100–200 µL/tissue) and incubated for 5 min on ice and centrifuged at 1400 rpm (390 ×g) for 5 min at 4°C, and supernatant was discarded.

3.6 The cell pellet for each tissue was resuspended in PBS with FBS and plated on Primaria Plates (Falcon) and incubated at 37°C in an atmosphere of 95% air, 5% CO2 for 5 hr for a negative selection step for fibroblasts.

3.7 The cell suspension was then collected from plates and rinsed twice with 4 mL PBS containing 10% FBS.

3.8 The suspension was filtered through a 40 µM strainer, followed by cell counting using the trypan blue vital staining method and staining with pH dyes, and fluorescence activated cell sorting (FACS) staining and sorting.

Optimization of the intracellular pH measurement by flow cytometry

4 Approximately 2×106 cells isolated from each tissue were centrifuged at 550×g at 4°C for 8 min, followed by resuspension of cells in 1 ml of Sort Buffer and preparation of 107 cells/mL.

4.1 Three different staining of cells by using the chosen combination of antibodies, pH markers, and isotype controls were performed, and samples were incubated for at least 30 min at 4°C.

4.2 Cells were washed twice with cold PBS.

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4.3 To detect the gate for live cells for each tissue, common antibodies such as CD4, CD8, and 4',6-diamidino-2-phenylindole (DAPI) were used as markers for dead cells.

4.4 The FACS sorting strategy for isolating viable cells from each separate tissue was quantified by flow cytometry.

4.5 Light scatter and DAPI identified cell-sized events.

4.6 DAPI was not used in further tests due to its effect on cellular pH.

Staining

5 Intracellular pH staining and measurements:

5.1 For intracellular pH staining, ~200,000–500,000 cells were stained as per manufacturer's instructions. Briefly, after adding 10 µL of the pH dye to 100 µL of PowerLoad™ Concentrate (Fisher Scientific).

5.2 The dye solution was diluted with 10 mL of LCIS (Cat. no. A14291DJ) and incubated at 37°C for 30 min.

5.3 Cells were washed with LCIS if needed and analyzed by using A BD FACSymphony A5 cytometer (BD Biosciences) with 355, 405, 445, 488, 561, and 640nm lasers and equipped with 30 fluorescence detectors was utilized for flow cytometric measurements throughout this study.

5.4 Intracellular pH was quantified by the Intracellular pH Calibration Buffer Kit (Cat. No. P35379).

Optimization of the intracellular pH measurement by flow cytometry

6 Calibration and measurement of intracellular pH

6.1 The Intracellular pH Calibration Buffer Kit (Cat. no. P35379) was used to clamp the intracellular pH with extracellular buffer.

6.2 Four buffers are included in the kit, with pHs of 4.5, 5.5, 6.5, and 7.5. An average of three datapoints was plotted in the graph, and a linear trend line was fitted to obtain the pH standard curve error.

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6.3 The percentage of mean fluorescence intensity relative to three different pH buffers was used to create a linear regression to obtain the cellular pH.

6.4 A higher fluorescence intensity correlates with a lower pH value.

6.5 Statistical analysis. The pH values of each compartment were obtained by interpolation using the GraphPad software v9 (GraphPad, San Diego, CA). Measurements were repeated three times for each tissue and animal.

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