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Title: Measurement of Nitric Oxide Production in Mouse Tracheal Epithelial Cell Cultures Differentiated at the Air Liquid Interface

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Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No, all the shots labelled as SCREEN were captured by the videographer, as the authors couldn't provide them.**
3. **Filming location:** Will the filming need to take place in multiple locations? **No**
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 15

Number of Shots: 62 (25 SC)

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Videographer's Note: Did each interview question at least twice.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Muddassar Iqbal:** Our research investigates how nitric oxide signaling regulates airway cilia function and contributes to respiratory disease.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Muddassar Iqbal:** Existing methods are often semi-quantitative, whereas our protocol enables sensitive nitric oxide detection in physiologically relevant airway cultures.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Muddassar Iqbal:** This method allows a reliable quantitative assessment of NO in apically secreted mucus from ALI-differentiated airway epithelium.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Muddassar Iqbal:** Background nitrite contamination is common in biological samples; therefore, we recommend including appropriate blank controls when performing this protocol.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Muddassar Iqbal:** Accurate in vitro measurement of NO enables mechanistic studies and therapeutic evaluation for motile cilia disorders.

- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Maryland

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Videographer's Note: A couple shots were tilted and panned to follow the action as talent needed to move things around the room. In these cases, an additional closeup of that step was done and labeled as the same shot number Close Up or CU on the slate.

2. Sample Collection from ALI Cultures for Nitric Oxide Quantification

Demonstrator: Muddassar Iqbal

2.1. To begin, remove the Transwell plates containing experimental groups [1] of differentiating airway epithelial cells at air-liquid interface, or ALI (A-L-I) for nitric oxide, or NO (N-O) analysis from the incubator [2]. Collect 750 microliters of basal media from each of the triplicate wells and pool them into a single tube [3]. Likewise, collect 750 microliters of blank control medium into a separate tube [4]. Store the labeled basal media and blank samples at minus 20 degrees Celsius until NO quantification using the nitric oxide analyzer, or NOA (N-O-A) [5].

2.1.1. WIDE: Talent removing the Transwell plates with cells from the incubator.

***Videographer:** Please obtain CU shot as well for this shot.*

2.1.2. TEXT ON A PLAIN BACKGROUND

Experimental Groups for NO Analysis:

- Untreated Control
- 250 μ M NO donor (DETA/NO)- treated
- 1 mM NOS (Nitric Oxide synthase) inhibitor (L-NAME)- treated
- Blank Control (Media without cells)

2.1.3. Talent collecting 750 μ L basal media from the transwell insert into a tube.

2.1.4. Talent adding 750 μ L of blank media into a single tube.

2.1.5. Talent placing the labelled blank and sample tubes into a -20 degrees Celsius freezer.

2.2. Next, to determine the NO in apical mucus of the epithelium, add 50 microliters of prewarmed 1X phosphate-buffered saline, or PBS (P-B-S) to the cells in each insert [1]. Gently rock the plate to evenly spread PBS over the monolayer [2]. Incubate the cells at 37 degrees Celsius for 30 minutes [3]. Collect the PBS from the apical side of three inserts per experimental condition into a single tube to obtain approximately 150

microliters of sample [4]. Store the PBS-mucus sample and a PBS blank solution at minus 20 degrees Celsius [5]. **Note:** The narration and numbers have been updated to reflect the added shot.

2.2.1. Talent adding prewarmed PBS onto the cells in each insert.

Added shot: Gently rock the plate to evenly spread the PBS over the monolayer.

2.2.2. Talent placing the plate with inserts in the incubator.

2.2.3. Talent collecting the PBS containing mucus from the apical side of the inserts into a single tube.

2.2.4. Talent placing the labelled apical wash sample tube and PBS blank tube into a -20 °C freezer.

2.3. To obtain the NO measurement from the cell lysate sample, wash the apical and basal sides of the cells in three inserts twice with 500 microliters of prewarmed PBS [1]. Add a total volume of 500 microliters of Accutase solution to the apical side of the inserts [2] and incubate until the cells completely detach from the membranes [3].

2.3.1. Talent washing the apical and basal sides of the inserts with phosphate-buffered saline.

2.3.2. Talent adding Accutase solution onto the apical side of the inserts.

2.3.3. Talent placing the inserts in the incubator until cell detachment.

2.4. Add 1.5 milliliters of serum-free RPMI-1640 (*R-P-M-I-sixteen forty*) media to the detached cells [1]. Pool the detached cells from the three inserts into a single tube [2]. Spin the cells at 4000 *g* for 5 minutes at 4 degrees Celsius [3]. Resuspend the cell pellet in 50 microliters of lysis solution containing RIPA (*REE-pah*) buffer and Halt 1X (*Halt-one-X*) protease inhibitor cocktail [4]. Incubate the lysate on ice for 45 minutes [5].

2.4.1. Talent adding serum-free RPMI-1640 media to the detached cells.

2.4.2. Talent pooling detached cells from three inserts into one tube.

2.4.3. Talent placing the tube into a centrifuge.

2.4.4. Talent resuspending the cell pellet in lysis solution.

2.4.5. Talent placing the lysate tube on ice.

2.5. Spin the lysate at 14,800 *g* for 20 minutes at 4 degrees Celsius [1]. After collecting the supernatant, use 3 microliters of lysate to determine the protein concentration using the bicinchoninic acid assay [2]. Dilute the remaining lysate at a one-to-one ratio in RIPA buffer to obtain 100 microliters of cell lysate sample [3]. Store the lysates at minus 80 degrees Celsius and the blank RIPA buffer at minus 20 degrees Celsius [4].

2.5.1. Talent placing the lysate tube into a centrifuge. *Videographer: Please capture the digital meter on the centrifuge showing the centrifuge speed and time*

- 2.5.2. Representative shot of talent analyzing the protein concentrations.
- 2.5.3. Talent diluting the lysate solution with RIPA buffer.
- 2.5.4. Talent placing the lysates and RIPA buffer into designated freezers.

3. Nitric Oxide Measurement by Nitric Oxide Analyzer

Videographer: Please record all shots labeled "SCREEN", as they cannot be provided by the authors.

- 3.1. Prepare and store triiodide reagent in a chemical fume hood [1] for use within two weeks of preparation [2]. To generate a standard curve, prepare a 1 millimolar stock solution of sodium nitrite in nitrite-free water [3]. Store at minus 20 degrees Celsius until use [4].

- 3.1.1. WIDE: Talent preparing the triiodide reagent inside a chemical fume hood while wearing personal protective equipment.

- 3.1.2. TEXT ON A PLAIN BACKGROUND

Triiodide Reagent Composition

Potassium Iodide	400 mg
Iodine	250 mg
Glacial Acetic Acid	28 mL
Ultrapure water	8 mL

- 3.1.3. Talent preparing the sodium nitrite stock solution in a tube.

- 3.1.4. Talent placing the sodium nitrite stock solution into a minus 20 degrees Celsius freezer.

- 3.2. Prepare fresh working standards of sodium nitrite stock in PBS immediately before injections [1-TXT]. Turn on the nitrogen carrier gas and initiate gas flow through the system [2]. Assemble the glass purge vessel and secure it with a septum and screw cap to ensure an airtight seal [3]. Add 4 milliliters of triiodide reagent with 20 microliters of diluted anti-foam solution into the vessel [4].

- 3.2.1. Talent preparing working standards in labeled tubes. **TXT: Concentration range: 0.125 μ M to 4.0 μ M**

- 3.2.2. Talent turning on the nitrogen gas supply connected to the system.

- 3.2.3. Talent securing the purge vessel with a septum and screw cap.

- 3.2.4. Talent adding triiodide reagent and anti-foam solution into the glass purge vessel.

- 3.3. Pour 10 milliliters of sodium hydroxide into the trap chamber [1]. On the NOA display screen [2], set the instrument to **MR2:50.00** (*M-R-Two-Fifty-point-oh-oh*) and select **Fast** for the filter setting [3]. Adjust the nitrogen gas pressure until the reactor pressure stabilizes between 27 and 29 pounds per square inch [4]. Open the **eDAQ** (*ee-dack*) **chart** software on the computer connected to the NOA [5].
- 3.3.1. Talent pouring sodium hydroxide solution into the trap.
- 3.3.2. SCREEN: Show the NOA display screen and select **Measurement** followed by **Select Gas and Range**. **Note:** At the time of filming, the prompt "Measurement does not appear on screen, so the shot is slightly modified."
- 3.3.3. SCREEN: Show the operator setting **MR2:50.00** and selecting **Fast** on the NOA screen.
- 3.3.4. Talent adjusting the nitrogen gas pressure regulator while monitoring the pressure reading. *Videographer: Please capture the pressure reading on nitrogen gas pressure guage*
- 3.3.5. SCREEN: Show the eDAQ chart software opening on the connected computer.
- 3.4. Set the acquisition range to 10 per second and the voltage to 10 volts in the software [1]. Navigate to **Setup > Channel Settings** and select **Channel 1** [2]. Set the display scale to **2:1** (*two-to-one*) on the NOA software [3]. Start the data acquisition on the NOA instrument and in the software [4]. Monitor the baseline until it stabilizes [5].
- 3.4.1. SCREEN: Show the acquisition settings panel with acquisition range and voltage values being entered.
- 3.4.2. SCREEN: Show navigation to **Setup > Channel Settings** and selection of **Channel 1**.
- 3.4.3. SCREEN: Show the display scale being changed to 2:1.
- 3.4.4. SCREEN: Show the operator starting data acquisition in the software and on the instrument.
- 3.4.5. SCREEN: Show the baseline signal stabilizing on the graph display.
- 3.5. Use a Hamilton syringe to inject 10 microliters of PBS through the septum into the purge vessel to confirm baseline stability [1]. Record the chemiluminescent signal for each injection [2]. Allow the signal trace to return fully to baseline before the next injection [3]. Inject 10 microliters of the 0.125 micromolar nitrite standard and subsequent standards in duplicates into the purge vessel [4].
- 3.5.1. Talent injecting PBS into the purge vessel using a Hamilton syringe.
- 3.5.2. SCREEN: Show the chemiluminescent signal trace being recorded in the software.
- 3.5.3. SCREEN: Show the signal trace returning to baseline on the graph.

- 3.5.4. Talent injecting the nitrite standard into the purge vessel.
- 3.6. Annotate each injection in the **Comment** tab with the corresponding sample information [1]. Between injections, wipe the syringe with a lint-free tissue [2] and rinse it 3 to 4 times with sterile nitrite-free water to remove excess triiodide [3]. Allow the signal to return to baseline between injections for 20 to 30 seconds [4].
- 3.6.1. SCREEN: Show the operator entering sample information into the **Comment** tab.
- 3.6.2. Talent wiping the syringe with a lint-free tissue.
- 3.6.3. Talent rinsing the syringe multiple times with sterile nitrite-free water.
- 3.6.4. SCREEN: Show the signal trace stabilizing at baseline between injections.
- 3.7. Open the Flow Analysis window [1]. Review peak height and time values for each injection in the analysis table [2]. Highlight each peak and adjust the selection to calculate the area under the curve [3]. Toggle the display from **S** to **C** to enable calibration mode [4]. Enter the known nitrite amounts for each standard in the **Amount** column [5].
- 3.7.1. SCREEN: Show the Flow Analysis window opening in the software.
- 3.7.2. SCREEN: Show the analysis table displaying peak height and time values.
- 3.7.3. SCREEN: Show individual peaks being highlighted for area under the curve calculation.
- 3.7.4. SCREEN: Show the display being toggled from **S** to **C**.
- 3.7.5. SCREEN: Show the operator entering nitrite standard values into the **Amount** column. **Videographer's Note: 3.8.1 (screen recording) was added to the 3.7 step and wasn't on the slate.**
- 3.8. Click the **Calibration curve** button to generate the standard curve and verify that the R-squared value is greater than or equal to 0.9 [1]. Next, thaw the biological samples collected from ALI cultures on ice [2]. Inject 50 microliters of each sample into the purge vessel [3] and record the chemiluminescence signal for each sample in duplicates as demonstrated with standard solutions previously [4].
- 3.8.1. SCREEN: Show the operator clicking the **Calibration curve** button and displaying the generated standard curve with $R^2 > 0.9$.
- 3.8.2. Talent thawing biological samples on ice.
- 3.8.3. Talent injecting blank cell-free medium into the purge vessel.
- 3.8.4. SCREEN: Show the chemiluminescence signal trace being recorded for sample injections.
- 3.9. Allow the chemiluminescent trace to return to baseline and stabilize between injections for 20 to 30 seconds [1]. At the end of sample injections, open the **Flow Analysis** window [2]. Highlight each sample peak to calculate the area under the curve [3].

- 3.9.1. SCREEN: Show the chemiluminescent trace returning to baseline between injections.
- 3.9.2. SCREEN: Show the Flow Analysis window opening after sample analysis.
- 3.9.3. SCREEN: Show sample peaks being highlighted for area under the curve calculation.
- 3.10. Determine nitrite concentrations for each sample using the calibration curve generated from the standard solutions [1]. Export the data, including peak height, area under the curve, and mean area under the curve from the **Flow Analysis** function in the eDAQ software to an Excel file [2]. Review the exported file showing the height versus time of the signal traces for the NOA experiment [3].
 - 3.10.1. SCREEN: Show calculated nitrite concentrations displayed using the calibration curve.
 - 3.10.2. SCREEN: Show the operator exporting data from the **Flow Analysis** function to an Excel file.
 - 3.10.3. SCREEN: Show the exported Excel file containing height versus time signal trace data.

Videographer: Please record all shots labeled "SCREEN", as they cannot be provided by the authors.

Results

4. Results

- 4.1. Triiodide-based chemiluminescent assay for the detection of nitrite-nitric oxide showed the differential levels of baseline NO (*N-O*) in the basal media [1], lysate [2] and apical wash samples obtained from ALI (*A-L-I*) differentiated airway epithelia [3].
 - 4.1.1. LAB MEDIA: Figure 3A. *Video Editor: In the table, please emphasize the value "0.208345" in the "Untreated" row and "Total Nitrite" column.*
 - 4.1.2. LAB MEDIA: Figure 3B. *Video Editor: In the table, please emphasize the value "0.069233" in the "Untreated" row and "Total Nitrite" column.*
 - 4.1.3. LAB MEDIA: Figure 3C. *Video Editor: In the table, please emphasize the value "0.221424" in the "Untreated" row and "Total Nitrite" column.*
- 4.2. In the basal medium, the amount of nitrite to nitric oxide is non-significantly altered upon NOS (*N-O-S*)-inhibitor, L-NAME (*L-name*) treatment compared to untreated controls [1]. In contrast, the NO (*N-O*)-donor, or DETA/NO (*D-E-T-A-N-O*) positive control treatment, shows an expected increase in NO release over the 24-hour incubation period [2].
 - 4.2.1. LAB MEDIA: Figure 3A. *Video Editor: Please show the bar graph on the extreme right side of the figure and emphasize the heights of orange and blue bars sequentially.*
 - 4.2.2. LAB MEDIA: Figure 3A. *Video Editor: Please show the bar graph on the extreme right side of the figure and emphasize the height of pink bar with labels corresponding to orange bar.*
- 4.3. In the cell lysate samples, nitrite-nitric oxide levels were similar across untreated cells, the L-NAME-treated cells, and the DETA/NO-treated cells [1].
 - 4.3.1. LAB MEDIA: Figure 3B. *Video Editor: In the bar graph on the extreme right side of the figure, please emphasize the heights of orange bar, followed by blue bar and then the purple bar.*
- 4.4. A significant decrease in nitrite-nitric oxide levels was observed in the apical washes from L-NAME -treated cells as compared to the untreated cells [1]. Additionally, DETA/NO treatment resulted in more than 6.5-fold increase in detected nitrite-nitric oxide [2].
 - 4.4.1. LAB MEDIA: Figure 3C. *Video Editor: In the bar graph on the extreme right side of the figure, please emphasize the heights of orange bar and blue bars simultaneously.*
 - 4.4.2. LAB MEDIA: Figure 3C. *Video Editor: In the bar graph on the extreme right side of the figure, please emphasize the height of purple bar relative to the orange bar.*

