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Title: An Air-liquid Interface Bronchial Epithelial Model for Realistic, Repeated Inhalation Exposure to Airborne Particles for Toxicity Testing

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   No

   If **No**, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Will, model Willovert, serial number 200586

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps.

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

   If **Yes**, how far apart are the locations? N/A

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Eric Gremmer:** This protocol describes how to culture and expose human bronchial epithelial cells via the air. This is a more realistic model for studying exposure of the lung to inhaled particles.

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

1.2. **Eric Gremmer:** The lung cells prepared with this method resemble the real environment in the lung. The cells will produce mucus and form a tight barrier, remaining viable for several weeks.

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

OPTIONAL:

1.3. **Paul Fokkens:** This method can be used to study toxic effects of airborne particles. It can also be used to study infectious diseases of the airways and drugs taken by inhalation.

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

1.4. **Paul Fokkens:** When attempting this protocol for the first time, keep in mind that generating an aerosol is complicated and requires specialist knowledge. It also requires dedicated machinery for measurements.

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

Protocol

2. Seeding Calu-3 Cells onto Culture Inserts

2.1. Begin by diluting the cells in prewarmed cell culture medium, or CCM, to a concentration of 500,000 cells per milliliter for 6-well inserts and 250,000 cells per milliliter for 12-well inserts **[1]**.

2.1.1. WIDE: Establishing shot of talent diluting cells, with the CCM container in the shot.

2.2. Place a cell culture plate with inserts under aseptic conditions **[1]**, and fill the basolateral side with prewarmed CCM **[3-TXT]**. After mixing the cell suspension, pipette it on top of the membrane in the cell culture insert **[4-TXT]**. Cover the plate and incubate it at 37 degrees Celsius and 5% carbon dioxide **[5]**. *Videographer: This step is difficult and important!*

2.2.1. Talent placing a plate under aseptic conditions. NOTE: 2.2.1., 2.2.3. and 2.2.4. shot in one go, split as follows: 2.2.1. = 0.0-0.6, 2.2.3. = 0.20-1.12, 2.2.4. = 1.14-end of time))

~~2.2.2. Talent taking the insert out of the plate.~~

2.2.3. Talent filling the basolateral side of the plate with CCM. **TEXT: Use 2 mL for 6 well insert and 1 mL for 12 well insert**

2.2.4. Talent pipetting cell suspension on top of the insert membrane. **TEXT: 1 mL for 6 well insert and 500 µL for 12 wells inserts**

2.2.5. Talent placing the plate in the incubator and closing the door.

2.3. Culture the cells under submerged conditions for 7 days to allow them to reach confluency, changing the CCM every 2 to 3 days **[1]**. To measure the trans epithelial electrical resistance, use a charged Epithelial Voltohmmeter supplemented with a Chopstick Electrode Set **[2]**. Clean the electrodes with 70% ethanol **[3]**.

2.3.1. Talent taking the cells out of the incubator.

2.3.2. Voltohmeter with chopstick electrodes.

2.3.3. Talent cleaning the electrodes.

2.4. Place the longer electrode in the external culture media until it touches the bottom of the dish **[1]** and the shorter electrode in the media without touching the membrane **[2]**. *Videographer: This step is important!*

2.4.1. Talent positioning the longer electrode. NOTE: The electrodes are fixed together, so this is one step, 2.4.1. and 2.4.2. are one clip!

2.4.2. Talent positioning the shorter electrode.

2.5. Begin collecting measurements on the empty insert without cells. Wait until the measurement stabilizes **[1]** and write down the resistance in Ohms. This measurement is the resistance of the insert membrane without any cells, or blank resistance **[2]**.

2.5.1. Talent collecting measurement on the insert without cells. *Videographer: Obtain multiple usable takes because this shot will be reused in 3.5.3 and 4.6.1.*

2.5.2. Talent writing down the measurement.

2.6. Repeat the measurement for each insert and subtract the blank resistance to obtain the true resistance **[1]**. Remove the medium from the apical side of the inserts **[2]** and add prewarmed CCM to the basolateral side of the well **[3-TXT]**. The medium should touch the membrane from the bottom, but not leak onto the top of the insert **[4]**.

2.6.1. Talent collecting measurement on an insert with cells.

2.6.2. Talent removing medium from the apical side.

2.6.3. Talent adding medium under the insert. **TEXT: TEXT: 1.5 mL for 6 well insert and 1 mL for 12 wells inserts**

2.6.4. Plate with added CCM, with the medium at the appropriate level.

2.7. Culture the cells at the air-liquid interface in the incubator at 37 degrees Celsius and 5% carbon dioxide for 7 days, changing the basolateral medium every 2 to 3 days **[1]**.

2.7.1. Talent placing the plate with cells in the incubator and closing the door.

3. Preparing the Exposure Setup

3.1. Shortly before particle exposure, prepare a 1% nanoparticle suspension: Prewet the NP's with 96% ethanol **[1]**. Add pure water to a final concentration of 1% NP's **[2]**, put the vial in a beaker on ice **[3]**, sonicate the suspension for 16 minutes **[4]**, transfer the suspension to a bottle with a stirrer **[5]**, then connect the bottle to a peristaltic pump via a small tube and adjust the flow to 25 milliliters per hour **[6]**.

3.1.1. Added shot: Prewetting the NP's with 96% ethanol

3.1.2. Added shot: Add pure water to a final concentration of 1% NP's

3.1.3. Added shot: Putting the vial in a beaker on ice

3.1.4. Added shot: Sonicate the solution for 16 minutes

3.1.5. Talent transferring the suspension to a bottle with a stirrer.

3.1.6. Talent connecting the bottle to a pump and adjusting the flow.

3.2. One day before exposure, turn on the automated exposure station, or AES, and allow the cabinet to reach a temperature of 37 degrees Celsius **[1]**.

3.2.1. Talent turning on the AES and setting the temperature.

3.3. Two hours before the start of exposure, turn on the air flow and the humidity in the cabinet and set it to 85% humidity **[1]**. Then, turn on the heating of the exposure chambers in which the inserts with the cells will be placed **[2]**.

3.3.1. Talent turning on the air flow and humidity.

3.3.2. Talent turning on the heating of the exposure chambers.

3.4. Finally, turn on the quartz microbalance and set the initial value to 0 using the software **[1]**. Start logging the mass, which is measured every 10 seconds and expressed as nanograms per centimeter squared **[2]**.

3.4.1. Talent turning on the QCM, with a representative graph on the screen.

3.4.2. Talent logging the mass.

3.5. To prepare the cells for exposure, warm the CCM to 37 degrees Celsius in a water bath **[1]**. Wash cells once with CCM, check cell morphology **[2]**, and measure the trans epithelial electrical resistance of the cell model. The cells should form a tight monolayer without gaps **[3]**.

3.5.1. Talent placing medium in a water bath.

3.5.2. SCOPE: Cells with a tight monolayer.

3.5.3. *Use 2.5.1.*

3.6. Add HEPES buffered CCM without FCS to the basolateral side of fresh 6 or 12-well plates **[1-TXT]** and transfer the culture inserts with cells to the new plates **[2]**. If the transport of cell cultures to the AES takes more than 5 minutes, put the cells in a portable 37-degree Celsius incubator **[3]**.

3.6.1. Talent adding medium to a new plate. **TEXT: 1.5 mL for 6-well insert/1 mL for 12-well insert**

3.6.2. Talent transferring an insert to a new plate.

3.6.3. Talent putting the plate in the incubator for transport.

4. Handling the AES During an Exposure

4.1. At the AES, fill the exposure modules with HEPES-buffered CCM without FCS **[1]**. Use sterile tweezers to transfer the inserts with cells from the plates to the exposure modules, making sure that there are no air bubbles at the basolateral side of the cells **[2]**. *Videographer: This step is difficult and important!*

4.1.1. Talent filling the exposure modules with media.

4.1.2. Talent transferring the inserts to the exposure modules.

~~4.1.3. Talent removing CCM from the apical side.~~

4.2. If there are air bubbles at the basolateral side, gently turn the inserts until they are removed **[1]**. Keep the plates with CCM in an incubator for transfer after exposure **[2]**.

4.2.1. Talent turning inserts to remove bubbles.

4.2.2. Talent putting the plates back in the incubator.

4.3. Use the touchscreen display to choose exposure duration, air flow rate, and electrostatic deposition enhancement, and to check humidity and temperature **[1]**. Conduct a leak test before starting exposure, making sure that the leakage is less than 5 milliliters per minute **[2]**. If a leak is detected, check the tubing **[3]**. *Videographer: This step is difficult and important!*

4.3.1. Talent adjusting settings on the touchscreen display.

4.3.2. Talent starting a leak test.

4.3.3. Talent checking the tubing.

4.4. After the leak test, run the exposure **[1]**. When finished, open the door of the AES module, open the exposure modules **[2]**, transfer the cell culture inserts back to the cell culture plates **[3]**, and transfer the plates to the portable incubator **[4]**. *Videographer: This step is important!*

4.4.1. Talent starting exposure.

4.4.2. Talent opening the door of the module and opening the module.

4.4.3. Talent transferring the insert back to the plate.

4.4.4. Talent putting the plate in the portable incubator.

4.5. Collect the media from the modules and from the plates for later analysis **[1]**. Incubate the plates until the next exposure or until analysis **[3]**.

4.5.1. Talent collecting media from modules.

~~4.5.2. Talent transferring the inserts into fresh plates.~~

4.5.3. Talent putting the plate in an incubator and closing the door.

4.6. On the day after the final exposure, add CCM to the apical side and measure the trans epithelial electrical resistance **[1]**. Collect both the apical and the basolateral CCM separately for analysis of cytokines **[2]**. Then, remove all CCM and perform a cell viability assay by adding a proliferation reagent to the apical side **[3]**.

4.6.1. *Use 2.5.1.*

4.6.2. Talent collecting the CCM.

4.6.3. Talent adding a proliferation reagent to the apical side of the cells.

Results

5. Results: Cell Model Selection

5.1. Out of 4 different human bronchial epithelial cell lines, only the Calu-3 cells displayed all characteristics of a suitable cell model for this protocol **[1]**.

5.1.1. LAB MEDIA: Figure 3.

5.2. The cells formed a monolayer with tight junctions that remained stable over time **[1]**, whereas the other cell lines either did not form a barrier **[2]** or showed a decrease in barrier function when cultured at the air-liquid interface **[3]**.

5.2.1. LAB MEDIA: Figure 4. *Video Editor: Emphasize the Calu-3 data points and line.*

5.2.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the H292 and BEAS-2B data points and lines.*

5.2.3. LAB MEDIA: Figure 4. *Video Editor: Emphasize the 16HBE data points and line.*

5.3. Air-liquid culturing can cause stress to cell models **[1]**. Cell lines 16HBE, H292, and BEAS-2B *(pronounce 'bee-as-2-B')* all showed an increased LDH release when cultured at the air-liquid interface **[2]**, while Calu-3 cells showed only a slight LDH release **[3]**.

5.3.1. LAB MEDIA: Figure 5.

5.3.2. LAB MEDIA: Figure 5. *Video Editors: Emphasize the 16HBE, H292, and BEAS-2B bars.*

5.3.3. LAB MEDIA: Figure 5. *Video Editors: Emphasize the Calu-3 bars.*

5.4. To test how the Calu-3 model responds to substance exposure, lipopolysaccharide was administered via nebulization to the apical side of the model **[1]**. The WST-1 conversion **[2]**, LDH release **[3]**, and TNF-alpha release were quantified after exposure to the lipopolysaccharide **[4]**.

5.4.1. LAB MEDIA: Figure 6.

5.4.2. LAB MEDIA: Figure 6. *Video Editor, emphasize A.*

5.4.3. LAB MEDIA: Figure 6. *Video Editor, emphasize B.*

5.4.4. LAB MEDIA: Figure 6. *Video Editor, emphasize C.*

5.5. Finally, the Calu-3 monolayer was exposed to quartz silica, or DQ12, for 3 weeks **[1]**. The DQ12 induced no significant effects on trans epithelial electrical resistance **[2]**, cell viability **[3]**, and MCP1 release compared to the clean air controls **[4]**.

5.5.1. LAB MEDIA: Figure 7.

5.5.2. LAB MEDIA: Figure 7. *Video Editor, emphasize A.*

5.5.3. LAB MEDIA: Figure 7. *Video Editor, emphasize B.*

5.5.4. LAB MEDIA: Figure 7. *Video Editor, emphasize C.*

5.6. As more toxicity of DQ12 was expected, the reactivity of the particles was checked using an acellular assay **[1]**. The reactivity of the DQ12 batch was orders of magnitude lower than the carbon black positive control particles **[2]**, which might explain the absence of a toxicity response **[3]**.

5.6.1. LAB MEDIA: Figure 8.

5.6.2. LAB MEDIA: Figure 8. *Video Editor: Emphasize the DQ12 data points*.

5.6.3. LAB MEDIA: Figure 8.

Conclusion

6. Conclusion Interview Statements

6.1. **Eric Gremmer:** When performing this protocol, work systematically and orderly. No stress, no hurry. This holds especially when operating the automated exposure station.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.2, 4.1.3.*

6.2. **Paul Fokkens:** We have also developed a co-culture model where macrophages are added to the Calu-3 cells. This should better resemble the in vivo situation.

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