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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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SUMMARY:

Described is the cell culture and exposure method of an in vitro bronchial model for realistic, repeated inhalation exposure to particles for toxicity testing.

ABSTRACT:

For toxicity testing of airborne particles, air-liquid interface (ALI) exposure systems have been developed for in vitro tests in order to mimic realistic exposure conditions. This puts specific demands on the cell culture models. Many cell types are negatively affected by exposure to air (e.g., drying out) and only remain viable for a few days. This limits the exposure conditions that can be used in these models: usually relatively high concentrations are applied as a cloud (i.e., droplets containing particles, which settle down rapidly) within a short period of time. Such experimental conditions do not reflect realistic long-term exposure to low concentrations of particles. To overcome these limitations the use of a human bronchial epithelial cell line, Calu-3 was investigated. These cells can be cultured at ALI conditions for several weeks while retaining a healthy morphology and a stable monolayer with tight junctions. In addition, this bronchial

model is suitable for testing the effects of repeated exposures to low, realistic concentrations of airborne particles using an ALI exposure system. This system uses a continuous airflow in contrast to other ALI exposure systems that use a single nebulization producing a cloud. Therefore, the continuous flow system is suitable for repeated and prolonged exposure to airborne particles while continuously monitoring the particle characteristics, exposure concentration, and delivered dose. Taken together, this bronchial model, in combination with the continuous flow exposure system, is able to mimic realistic, repeated inhalation exposure conditions that can be used for toxicity testing.

INTRODUCTION:

The lungs are vulnerable to inhalation exposure to airborne particles. To assess the potential toxicity of airborne particles, progress has been made to develop air-liquid interface (ALI) exposure systems[1-5]. ALI exposure systems allow more relevant and realistic exposure models compared to traditional submerged exposure via culture medium that alters the characteristics and kinetics of the particles[6]. The ALI exposure systems place specific demands on the cell culture models, as the models lack culture medium and thus nutrients at the apical side. Many cell models are negatively affected by being cultured and exposed at the air (e.g., drying out) and only remain viable for a few days. This limits the exposure conditions that can be used in these models: usually relatively high concentrations are applied within a short period of time as a cloud (i.e., droplets containing particles, which settle down rapidly). Such experimental conditions do not reflect realistic long-term exposure to low concentrations of particles; thus, the relevance of the results can be questioned. To overcome these limitations, the culture and exposure protocol for a bronchial model consisting of the human bronchial epithelial cell line Calu-3[7] was optimized.

Most in vitro lung models used for ALI exposure contain other cell lines such as A549, BEAS-2B, and 16HBE14o- (16HBE) or primary cells as a basis[8]. These cell lines have the disadvantage that they remain viable for only a few days when cultured at the ALI. In addition, some of these cell lines overgrow when cultured for a period longer than 5 days. Finally, A549 cells miss functional tight junctions and can therefore not form a tight barrier that is needed to mimic the lungs[9,10]. Primary epithelial cells might be a good option for ALI exposure as they can be cultured at the ALI for weeks. However, primary cells differ from batch to batch, are more difficult to maintain, and are more expensive compared to cell lines, which makes them less suitable for toxicity testing and screening. When comparing different human bronchial epithelial cell lines (16HBE, Calu-3, H292, and BEAS-2B), only the Calu-3 cells fulfill all criteria needed for realistic, repeated ALI exposure: they remain viable for weeks while cultured at the ALI, provide a high barrier integrity, do not overgrow, and are easy to culture and maintain. Calu-3 cells originate from an adenocarcinoma and are able to produce mucus[11,12]. There are inconsistencies as to whether the cells can develop cilia[11,13]. Calu-3 cells are also a suitable model to study respiratory syncytial virus (RSV) infections that infect ciliated airway epithelial cells[14].

Besides the cell model, an automated exposure system (AES) is used for the air-liquid exposure to aerosols[15,16]. The AES has the advantage that it uses a continuous airflow to expose the cell model to aerosols. This is in contrast to other air-liquid exposure systems that usually use relatively high concentrations within a short period of time as a cloud (i.e., droplets containing

particles that settle down rapidly)[17-19]. These cloud systems do not reflect realistic long-term exposure to low concentrations of particles. By applying a continuous airflow using the AES, the cell model can be exposed to a low concentration of particles over a longer time period, reflecting realistic exposure conditions. Another advantage over cloud systems is that the AES has the option to connect particle characterization instruments, allowing measurement of particle size, number concentration, and mass over time. A limitation of the AES is that it uses relatively high airflows between 10 mL/min and 100 mL/min.

PROTOCOLS:

1. Preparing cell culture medium (CCM)

1.1. Prepare a bottle of 500 mL of minimum essential medium (MEM) supplemented with glutamine.

1.2. Add 5 mL of penicillin-streptomycin (i.e., 100 U/mL penicillin and 100 µg/mL streptomycin).

1.3. Add 5 mL of non-essential amino acids (NEAA) solution.

1.4. Add 10 mL of amphotericin B (optional).

1.5. Add 50 mL of FBS (heat inactivated, please follow the ATCC protocol for heat inactivation; (https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx, page 19)

2. Subculturing of Calu-3 cells

NOTE: Calu-3 cells are cultured in T75 or T175 cell culture flasks at 37 °C and 5% CO_2. Cells are passaged at 60–80% confluency every 7 days with CCM renewal every 2–3 days. CCM is poured off and fresh CCM (T25 = 5 mL, T75 = 15 mL, and T175 = 25 mL) is pipetted into the flask and the flask is placed back into the incubator. Cells should be passaged at least 2x after thawing, before using in experiments, or before freezing, and they should be passaged no more than 25x in total.

2.1. Confirm if flask is 60–80% confluent by checking under a light microscope.

2.2. Pour off the CCM from the flask.

2.3. Wash the cells 2x with 5 mL of 1x Hanks' Balanced Salt Solution (HBSS) without calcium and without magnesium. Discard the HBSS after each wash. HBSS removes serum, which inhibits trypsin.

2.4. Add 3 mL of trypsin-EDTA for a T75 (4 mL for a T175) and place the flask back into the incubator at 37 °C and 5% CO_2 for 10–15 min. Check after 10 min, ensuring the cells have become detached from the flask surface. In case the cells are grown to >80% confluency, they will not

detach using trypsin 0.05% and trypsin 0.25% could be used.

2.5. Add 6 mL (i.e., double the trypsin-EDTA volume originally added) of CCM to the flask and gently rock the flasks to ensure proper mixing. This is to ensure the trypsin has been neutralized by the FBS in the CCM and its activity on the cells halted. If trypsin is allowed to remain in contact with the cells for too long they will not reattach when put into a new cell culture flask.

2.6. Pour the complete contents of the flask into a 50 mL centrifuge tube.

2.7. Centrifuge the cells for 5 min at 130 x *g*, ensuring that the centrifuge is correctly balanced.

2.8. Return the vial containing the cells back to aseptic conditions and remove the supernatant gently, without disturbing the pellet. The supernatant can be poured off and the remainder pipetted off, ensuring the pellet is not disturbed.

2.9. Resuspend the cell pellet in 1 mL of CCM by pipetting up and down until all cells are suspended (i.e., no pellet or cell agglomerates are observed). Additional CCM can be added to dilute the cell suspension.

2.10. Count the cells, both dead and alive, in 1 mL of CCM using a hemocytometer. If needed for proper counting, dilute the cells in 3 or 4 mL.

2.11. Suspend the cells into the CCM volume required and add the cell suspension into each flask. To achieve about 80% confluency in a week, seed 2 x 10^6 cells in a T75 or 6 x 10^6 cells in a T175.

2.12. Gently rock the flask and then place it back into the incubator at 37 °C and 5% CO_2.

2.13. Replace with fresh CCM every 2–3 days and subculture when the cells reach 60–80% confluency.

3. Seeding Calu-3 cells onto culture inserts

NOTE: Inserts are available with different pore sizes. Small pore sizes (e.g., 0.4 µm) have the advantage that the cells grow more easily and can achieve a good barrier already after 5 days culturing under submerged conditions, as measured by Trans Epithelial Electrical Resistance (TEER). However, when interested in particle translocation, these pores are too small and will trap the particles. Therefore, larger pore sizes (e.g., 3 µm) are usually chosen to test particles. When using a larger pore size, the cells need longer time periods (e.g., 7–10 days culturing under submerged conditions) to achieve a good TEER.

3.1. Prepare cell suspension with known concentration following steps 2.1–2.10.

3.2. Dilute cells to a concentration of 5 x 10^5 cells/mL in prewarmed CCM for 6 well inserts or 2.5 x 10^5 cells/mL for 12 well inserts.

3.3. Take a cell culture plate with inserts and place under aseptic conditions.

3.4. Fill the basolateral side with 2 mL of prewarmed CCM for 6 well inserts, or 1 mL for 12 well inserts. While adding the culture medium, take the insert out using tweezers.

3.5. Carefully mix the cell suspension by pipetting up and down. Pipette 1.0 mL for 6 well inserts and 500 µL for 12 wells inserts (equivalent to 100,000 cells/cm^2) on the top of the membrane in the cell culture insert.

3.6. Cover the cell culture plate and incubate at 37 °C and 5% CO_2.

3.7. Change the CCM every 2–3 days.

3.8. Let the cells become confluent for 7 days under submerged conditions before continuing to culture at the ALI.

3.9. Measure TEER.

3.9.1. Take an Epithelial Voltohmmeter supplemented with Chopstick Electrode Set and charge the battery system overnight.

3.9.2. Disconnect the Voltohmmeter from the charger and connect the chopstick electrode.

3.9.3. Clean the electrode with 70% ethanol.

3.9.4. Place the electrode in the CCM by putting the longer electrode in the external culture media until it touches the bottom of the dish and putting the shorter electrode in the media without touching the membrane.

3.9.5. Start with an empty insert without cells. Wait until the measurement stabilizes and write down the resistance in Ohms. This measurement is the resistance of the insert membrane without any cells (i.e., blank resistance).

3.9.6. Repeat the measurement for each insert and subtract the blank resistance to obtain the true resistance.

3.9.7. For data analysis, multiply the resistance values by the surface area of the insert into Ω x cm^2. For a 6 well insert, the surface area is 4.67 cm^2. Thus, if a resistance of 600 Ohm is measured and the background is 120 Ohm, the resistance is 480 Ohm, which is then multiplied by the surface area of 4.67 cm^2 for a total of 2,241.6 Ohm x cm^2. The TEER should be >1,000 Ω x cm^2 to continue.

3.10. Remove the CCM from the apical side of the inserts.

3.11. Add 1.5 mL of prewarmed CCM for 6 well and 1 mL for 12 well inserts to the basolateral side of the well (i.e., under the cell culture insert). The CCM should touch the membrane from the bottom, but not leak onto the top of the insert.

3.12. At this point cells are apically exposed to air, which is referred to as culturing at the ALI.

3.13. Culture cells at the ALI in the incubator at 37 °C and 5% CO_2 for 7 days prior to exposure.

3.14. Change the basolateral CCM every 2–3 days. The cells can be used at the ALI for 6 weeks.

4. Preparing the exposure setup

NOTE: Sections 5–7 describe preparations for particle exposure using an automated exposure station (AES, see **Table of Materials**). The setup for particle nebulization and characterization is also compatible with other ALI exposure systems from other manufacturers. As an example, the exposure to particles is described below. Such systems can also be used for other exposures, such as sensitizers, cigarette smoke, and diesel exhaust. **Figure 1** shows the AES and an exposure module. **Figure 2** shows a schematic representation of the exposure setup including all other instruments.

4.1. Before starting an exposure using the AES, connect the system to several instruments to measure aerosol characteristics; these are measured in a side stream just before the aerosols enter the cabinet.

NOTE: A flow splitter is used to connect the side stream to the exposure characterization equipment. Generally, the following equipment is used: scanning mobility particle sizer (SMPS), optical particle sizer (OPS), condensation particle counter (CPC), tapered element oscillating microbalance (TEOM). The SMPS and OPS measurements are performed 1x per hour and use the same tubing from the flow splitter. The CPC and TEOM perform continuous measuring and data from both are logged on a Squirrel model 2020 data logger. In addition, gravimetric mass concentration is determined using a microbalance in controlled relative humidity (40–70%) and temperature (21–23 °C) conditions. Teflon filters are weighed before and after each exposure to confirm the exposure concentration. To capture the exhaust, a HEPA filter is used. The setup including engineered nanomaterial (ENM) suspensions and nebulizer are all placed in a flow cabinet to prevent any exposure to people. The AES can be used for testing many different types of ENMs, including metals and metal oxides.

4.2. Prepare a nanomaterial suspension shortly before exposure. Usually a 1% suspension is prepared as a stock solution. For example, suspend 100 mg of nanomaterial in 10 mL of pure water.

NOTE: For DQ12 exposure, 300 mg is used to achieve about 2 µg/cm^2. This amount can be used in a single exposure or divided over repeated exposures (e.g., 300 mg is suspended in 30 mL for

a single exposure or 21.5 mg is suspended in 2.15 mL freshly every day for 3 weeks of repeated exposure). The suspension is freshly prepared on each exposure day. The particle suspension is sonicated for 16 min using probe sonication. The volume of the 1% stock solution is adjusted to a total volume of 100 mL by adding pure water.

4.3. Put the ENM suspension in a small bottle with a cap and a magnetic stirrer to prevent settling of the particles. Connect the bottle to a peristaltic pump via a small tube and adjust the flow to 25 mL/h.

4.4. Connect the peristaltic pump to a spray nozzle and adjust the settings to allow continuous aerosolization.

4.5. The spray nozzle is mounted to a 60 cm long aluminium tube (mixing chamber, diameter 15 cm, heated to 60 °C). The setup is connected to the AES via a 1.5 meter long copper tube (diameter 15 cm). On top of the AES an impactor removes all aerosols larger than 2.5 µm.

4.6. Connect the spray nozzle to 3 bar compressed air through two mass flow controllers (MFC) to allow nebulization of suspensions. One flow of 14 L/min is used for the spray nozzle, the other MFC for mixing of the air in the tube.

4.7. The day before the start of the exposure, turn on the AES to allow the cabinet to reach a temperature of 37 °C.

4.8. Turn on the air flow and the humidity in the cabinet 2 h before start of the exposure, to reach 85% humidity. Turn on the heating of the exposure chambers in which the inserts with the cells will be placed.

4.9. Turn on the quartz microbalance (QCM) and set the initial value at 0 using the software. Start logging. Every 10 s the mass is measured and expressed as ng/cm^2.

4.10. Warm the cell culture media to 37 °C in a water bath (~20–30 min).

5. Preparing Calu-3 cells for exposure

NOTE: For a typical ALI exposure using the AES, 15–20 inserts with a confluent cell layer are needed. These consist of 3 clean air controls, 3 incubator controls that will be handled similarly to the other inserts without exposure in the AES, 6–8 inserts for aerosol exposure (depending on the use of 0, 1, or 2 microbalances), 1–3 inserts for control measurements (such as maximum LDH release), and 3 spare inserts in case the TEER of some of the inserts is not sufficient. The cells should have a TEER of >1,000 Ω x cm^2 to continue.

5.1. On the first day of exposure, wash cells 1x with CCM, check cell morphology, and measure the TEER of the cell model using a Voltohmmeter. The cells should form a tight monolayer without gaps.

5.2. Put 1.5 mL/1 mL of HEPES buffered CCM without FCS to the basolateral side of 6 well/12 well plates and transfer the culture inserts with cells to the new plates.

NOTE: During exposure, no CO_2 is present in the AES. Therefore, HEPES buffered culture medium (25 mM HEPES) is used during transport and exposure. This medium is used for both the exposed cells in the AES as well as the incubator control cells.

5.3. In case the time to transport the cell cultures to the AES is more than 5 min, put the cells in a portable incubator of 37 °C during transport.

6. Handling the AES during an exposure

6.1. At the AES, fill the exposure modules with HEPES-buffered CCM without fetal calf serum (FCS). The amount of CCM depends on the unit used, and the type of cell culture insert. Keep in mind that to keep cells at the ALI, the medium should reach the bottom of the membrane, but should not leak on top of the membrane. When using 6 well inserts, add 6 mL of HEPES-buffered CCM to the exposure modules.

6.2. Transfer the inserts with cells from the plates to the exposure modules using sterile tweezers. Check that there are no air bubbles at the basolateral side of the cells and remove any CCM on the apical side of the inserts. In case there are some air bubbles at the basolateral side, gently turn the inserts using the sterile tweezers until they are removed. Keep the plates containing CCM in an incubator for transfer after an exposure.

6.3. Use the touchscreen display to choose exposure duration, air flow rate, and electrostatic deposition enhancement. The display can also be used to check humidity and temperature. Usually, an exposure duration of 4 h is chosen, with a flow rate of 50 mL/min on the inserts at 37 °C and 85% humidity.

NOTE: The modules in the first level (**Figure 1**) are used for clean air exposure; inserts in this level are used as clean air exposure controls. The other modules in the second and third level can be used for aerosol exposure, including two modules for quartz crystal microbalances (QCM) to measure deposition online.

6.4. The leak test should be conducted before starting exposure. The leakage needs to be less than 5 mL/min. Follow the instructions on the AES display. When the leak test has finished, the exposure can be started. In case of a leak, the tubing should be checked.

6.5. At the end of exposure, open the door of an AES module, open the exposure modules, place the cell culture inserts back to the cell culture plates, and transfer the plates to the portable incubator.

6.6. Collect the media from the modules (i.e., exposed samples) and from the plates (i.e.,

incubator controls) for later analysis, such as lactate dehydrogenase (LDH) measurement.

6.7. Back at the cell culture lab, transfer the cell culture inserts to plates filled with fresh standard CCM. Put the cell culture plates in the incubator until the next exposure or until analysis.

6.8. After the final exposure day, put the inserts in the incubator until the next day.

6.9. At the day after the final exposure, add CCM to the apical side to measure TEER using a Voltohmmeter. Collect both the apical and the basolateral CCM separately for analysis of cytokines.

6.10. Remove all CCM and perform a cell viability assay by adding, for example, a proliferation reagent to the apical side.

7. Cleaning the AES

7.1. Fill the exposure modules with water, wait for 1 min, and remove the water. Next, fill the modules with 70% ethanol, leave it for 10 min, and remove the ethanol. Clean the exposure trumpets also with 70% ethanol.

7.2. Stop the 85% humidity control, but leave the temperature of the cabinet at 37 °C for the next experiment.

REPRESENTATIVE RESULTS:

This article provides a method for culturing and exposing human bronchial epithelial cells at the ALI that mimics realistic, repeated inhalation exposure conditions that can be used for toxicity testing. Characteristics of both the cell model and of the exposure system are essential for achieving a realistic inhalation exposure model that can be used for repeated exposures. Results on these characteristics are shown below.

Cell model requirements and selection

When selecting a suitable cell model, the following characteristics must be taken into account:

1. The cell model should be able to form a confluent monolayer with functioning tight junctions to mimic the lung barrier.

2. The cell model should show optimal performance when exposed repeatedly to conditioned (temperature and humidity) air.

3. The cell model should respond to an exposure.

This study started with four different human bronchial epithelial cell lines: 16HBE, Calu-3, H292, and BEAS-2B. These are all widely used for toxicity testing of nanomaterials and chemicals. Of the four cell lines, only the Calu-3 cells fulfilled all the above requirements. The cells formed a

monolayer with tight junctions (**Figure 3**) that remained a stable barrier over time as measured by TEER, whereas the other cell lines either did not form a barrier or showed a drop in barrier function when cultured at the ALI (**Figure 4**). In addition, H292 and BEAS-2B tended to overgrow into multiple cell layers when cultured for a longer time period. Traditional submerged cell culturing and ALI culturing differed greatly, because at the ALI nutrients were only available from the basolateral side and the cells were exposed to dry conditions at the apical side. These conditions can cause stress to the cell models, which could be observed by measuring the cell viability over time. Cell lines 16HBE, H292, and BEAS-2B all showed an increased LDH release when cultured at the ALI, while Calu-3 cells showed only a slight LDH release (**Figure 5**).

Next, the response of the Calu-3 model to substances was tested. As a positive control substance, LPS was administered via nebulization to the apical side of the model. The deposited dose was 0.25 μg/cm^2. The Calu-3 cells showed a reaction to lipopolysaccharide (LPS) by an increase in LDH release and in tumor necrosis factor alpha (TNF-α) release (**Figure 6**).

Finally, the Calu-3 monolayer was exposed to quartz silica (DQ12) nanomaterials (IOM, Edinburgh). Crystalline silica can induce silicosis and may also cause lung tumors. Therefore, the International Agency for Research on Cancer (IARC) has classified crystalline silica as a Group I human carcinogen[20]. The mechanism of action of crystalline silica is thought to be via the induction of persistent inflammation caused by its reactive surface[21-23]. Several in vivo studies in both rats and mice report the induction of inflammation and histopathology changes, including tumors and fibrosis, after inhalation exposure to crystalline silica[24-29]. These effects are all observed after repeated exposure and/or long-term follow-up. The Calu-3 model was used to investigate whether the observations from the in vivo studies could be mimicked using an in vitro model that could be exposed repeatedly at the ALI.

Calu-3 cells were exposed for 3 consecutive weeks, 5 days per week, 4 h per day to DQ12. The deposited dose was measured using a QCM. The average deposited dose was 120 ng/cm^2 per day, with a cumulative dose of 1.6 μg/cm^2, similar to the doses inducing an effect in vivo. Other particle characteristics are shown in **Table 1**. After 3 weeks of exposure, DQ12 induced no significant effects in TEER, cell viability, and monocyte chemoattractant protein 1 (MCP-1) release, compared to the clean air controls (**Figure 7**). As more toxicity of DQ12 was expected based on the in vivo data, the reactivity of the particles was checked using an acellular assay according to the protocol optimized within the EU-project GRACIOUS (deliverable 5.3). The reactivity of the DQ12 batch was lower than expected (**Figure 8**), orders of magnitudes lower compared to the positive control particles carbon black (CB). This lack of reactivity might explain the absence of a toxicity response in the Calu-3 model.

FIGURE AND TABLE LEGENDS:

Figure 1: The automated exposure station (AES). The left figure shows the outside of the cabinet with the touch panel. The AES has three levels with exposure modules: the top level for clean air exposures, and the middle and bottom level for aerosol exposures. The right figure shows the exposure module in which inserts with cells are placed.

Figure 2: Schematic representation of the exposure setup. From left to right: 1) the ENM suspension connected to the spray nozzle via a peristaltic pump; 2) Using compressed air the spray nozzle nebulizes the ENM suspension and via a mixing chamber the aerosols are led to the AES; 3) Just before entering the AES, aerosol characterization instruments are connected: SMPS, OPS, CPC, and TEOM.

Figure 3: Representative image of Calu-3 cells after culturing at the air-liquid interface (ALI) for 10 days. Fluorescence microscopy image of Calu-3 cells after culturing at the ALI for 10 days. Tight junction protein ZO-1 is stained in green, nuclei of the cells are stained in blue.

Figure 4: Transepithelial electrical resistance (TEER) of four different cell lines during a culture period of 21 days. TEER values of 16HBE, Calu-3, H292, and BEAS-2B when cultured for 21 days: first 7 days submerged, followed by 14 days at the ALI. TEER values were corrected for the background resistance of the insert and multiplied by the surface area of the insert. The symbols and error bars represent the average value and standard deviation of six inserts.

Figure 5: LDH release of four different cell lines during a culture period of 21 days. LDH release of 16HBE, Calu-3, H292, and BEAS-2B when cultured for 21 days: 7 days submerged, followed by 14 days at the ALI. LDH values shown are relative to the maximum LDH release per cell type. The symbols and error bars represent the average value and standard deviation of five inserts.

Figure 6: Cellular effects in Calu-3 cells exposed to LPS. Calu-3 cells were exposed via cloud nebulization to 0.25 μg/cm^2 LPS. (**A**) The WST-1 conversion. (**B**) LDH release. (**C**) TNF-α release after LPS exposure. The symbols and error bars represent average values and standard deviations of three inserts.

Figure 7: Cellular effects in Calu-3 cells exposed to DQ12 nanomaterials. Calu-3 cells were exposed for 3 weeks (4 h per day, 5 days per week) to DQ12 nanomaterials, about 120 ng/cm^2 per day, cumulative dose of 1.6 μg/cm^2. (**A**) TEER values. (**B**) LDH release. (**C**) MCP-1 release after DQ12 exposure. All symbols and error bars represent average values and standard deviations of three inserts for the controls and six inserts for the DQ12 exposure.

Figure 8: Acellular reactivity of DQ12. DQ12 was incubated with a 2',7'-Dichlorofluorescein Diacetate (DCFH-DA) probe to detects its surface reactivity. As a positive control, carbon black (CB) particles were included. Compared to CB, DQ12 has very low surface reactivity.

Table 1: DQ12 exposure characteristics. Values are shown as average with standard deviation in brackets.

DISCUSSION:

This paper describes a method for culturing human bronchial epithelial cells under ALI and exposing this bronchial model to aerosols or gases. The advantage of using Calu-3 cells is that they form tight junctions, remain a monolayer, are able to withstand the air flow, and can be cultured for weeks at the ALI, unlike many other cell types (e.g., 16HBE, H292, and BEAS-2B).

Using the VITROCELL® automated exposure station (AES) has the advantage that the cells can be exposed under realistic and relevant conditions as low concentrations can be applied using a continuous airflow.

Continued flow systems, such as the AES, have advantages compared to using cloud systems[3,32], which use a single nebulization of a suspension. The continuous flow is more realistic and many variables like flow rate, humidity, and temperature are controlled. In addition, deposition can be enhanced using an electrical field. Finally, aerosol characteristics like size, number concentration, and mass are monitored online. A disadvantage is that continued flow systems are more complex compared to cloud systems. Therefore, it is important to run preparatory experiments that focus on the particle characteristics of the aerosol and the delivered dose on the insert. The initial starting concentration of the particles and the AES settings can then be adjusted to achieve the desired dose on the cells[33]. Depending on the type of particles being tested, the aerosol generation method can differ. The use of electrostatic deposition depends on the particle type and works best for metallic particles. For particles with a positive surface charge a negative electrostatic field should be applied and vice versa.

Selection of exposure concentrations can be difficult for any air-liquid exposure experiment. For the DQ12 exposures, the aim was to achieve a total cumulative dose of 1 $\mu g/cm^2$ after 3 weeks exposure, 5 days per week, 4 h per day. This dose is similar to doses that induced an effect in vivo[21,25,27,32,33]. When performing the exposures, there was some variation between different exposure days. Although the actual deposited dose of 1.6 $\mu g/cm^2$ is higher than the 1 $\mu g/cm^2$ that was aimed for, the dose might have been too low to observe effects in the Calu-3 model. Only minor differences in TEER, viability, and cytokine response were observed between the clean air exposure and the DQ12 exposure, and these differences were not statistically significant. An explanation for the observation that DQ12 exposure for 3 weeks did not induce significant effects in the Calu-3 cells is that macrophages were lacking from the Calu-3 model. Possibly, after DQ12 uptake macrophages produce proinflammatory cytokines that may affect Calu-3 cells. Another explanation is that the DQ12 batch that was used for the experiments was not as reactive as expected. When using LPS as a positive control substance, Calu-3 does show a response, as measured by an increase in LDH release and an increase in TNF-α release. This indicates that the model can detect toxicity.

The Calu-3 cells model has many advantages, as discussed in the results section. Moreover, when cultured for a longer time at the ALI, the Calu-3 cells can grow cilia/cilia-like structures[13] and produce mucus[11-13]. Despite these advantages, the model has limitations with respect to its physiological relevance. The Calu-3 cell lines originates from an adenocarcinoma, whereas 16HBE and BEAS-2B originate from healthy tissue. Unfortunately, the latter two are not suitable for repeated ALI exposure as they do not remain a stable monolayer over time. Another limitation of the Calu-3 model is that it only represents a single cell type. In the human lung, multiple cell types that interact and respond differently to exposure are present. Inhaled particles will deposit in different regions of the lungs depending on their aerodynamic size. This is where the particles contact the epithelial cell barrier, as mimicked by the Calu-3 model. In the human lung, alveolar macrophages are attracted to the particles, engulf them, and clear them from the lungs.

Macrophages also play an essential role in the inflammation response to particle exposure. Therefore, efforts are being made to extend the Calu-3 model by adding primary macrophages to mimic the lung barrier more closely. The disadvantage of the macrophages is that they remain viable only for about 7 days when cultured on top of Calu-3 cells at the ALI. Therefore, macrophages should be readded weekly to transform the current Calu-3 model into a coculture model. The optimization of the coculture protocol is currently ongoing.

Given the above, the Calu-3 bronchial model is a suitable model for repeated exposure to aerosols of partly soluble substances such as chemicals from cigarette smoke and LPS. These soluble substances induce significant increases in cytokine responses in the Calu-3 cells. For testing insoluble particles such as diesel exhaust and DQ12, a coculture model is needed, because the macrophages play a crucial role in the induction of effects by particle exposure.

For the exposures described, insert membranes with 3.0 µm pores were used. The main reason for choosing this type of insert is that it is possible to test the translocation of nanomaterials. When using smaller 0.4 µm pore size, particle agglomerates will not be able to cross the insert membrane. The disadvantage of using a large pore size is that the cells need a longer time to grow confluent and that it is more difficult to visualize the morphology of the cells using light microscopy. To check that the cells do form a confluent monolayer, the TEER should be >1,000 Ω x cm^2 before starting an exposure.

Taken together, the Calu-3 bronchial model presented here is suitable to use for repeated exposure to aerosols, at least up to 3 weeks. The model can withstand being cultured and exposed via a continuous airflow and is capable to detect toxicity to the bronchial epithelium.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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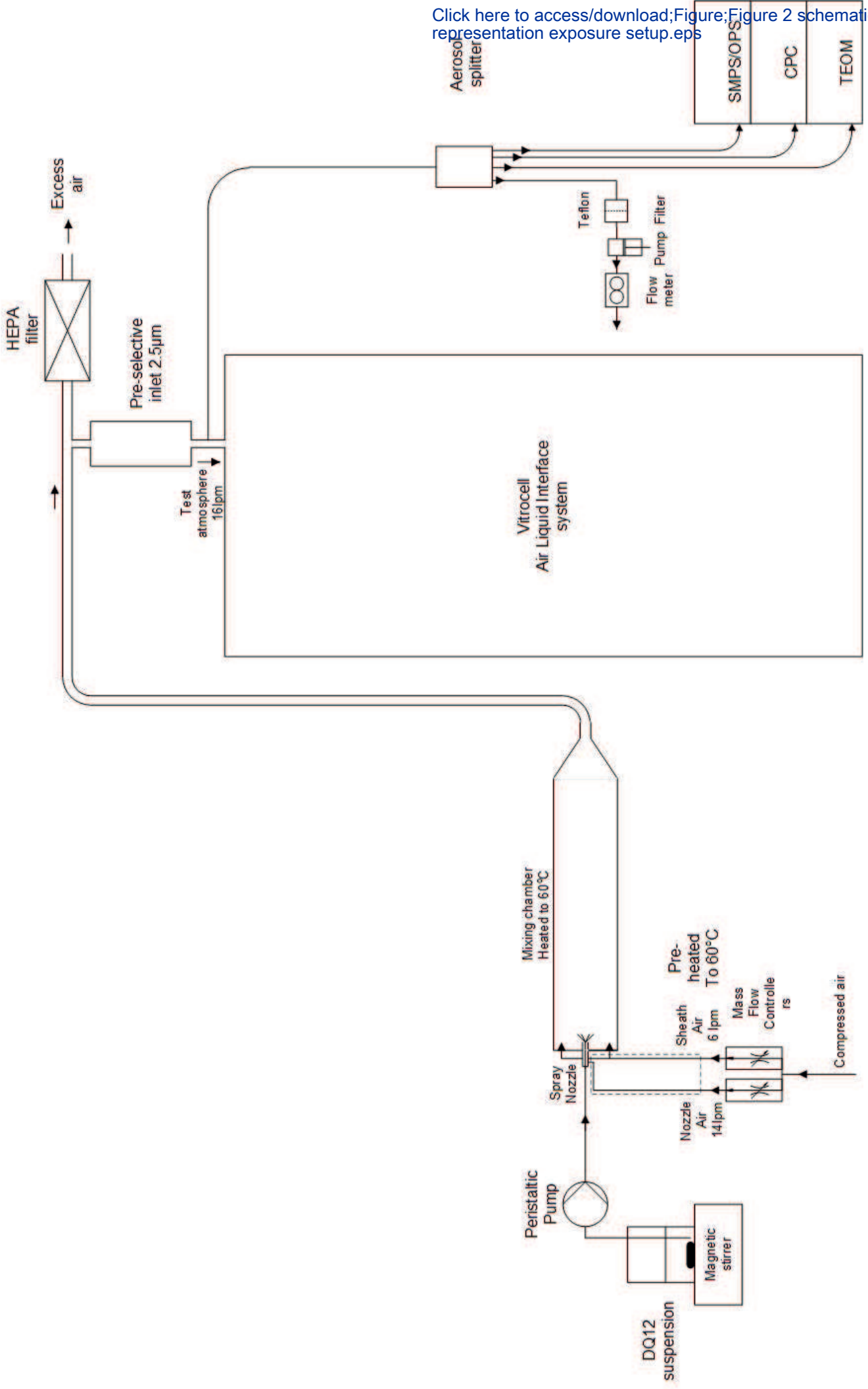
Figure

Click here to access/download;Figure;Figure 1 AES.eps

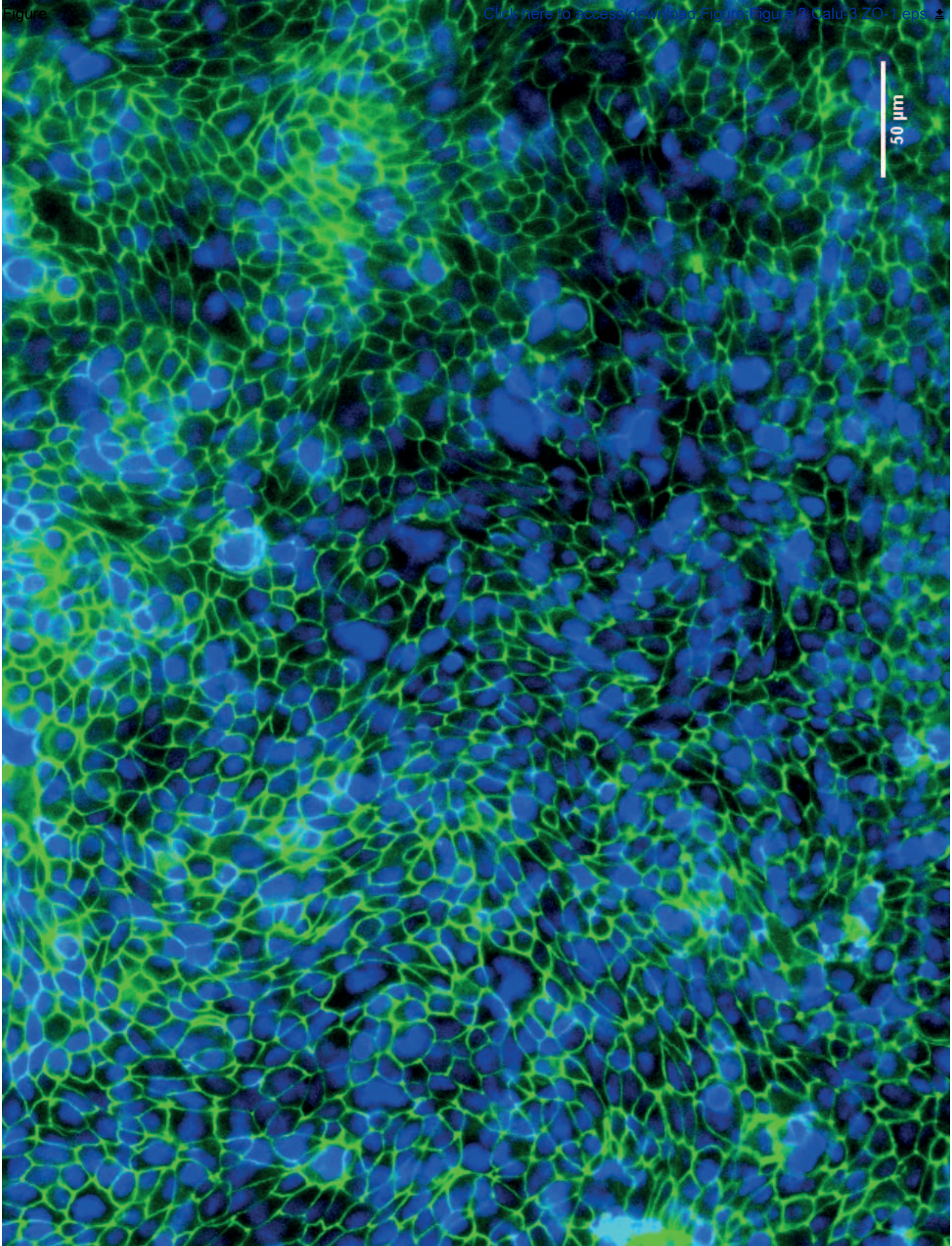
Figure

Click here to access/download;Figure;Figure 2 schematic representation exposure setup.eps



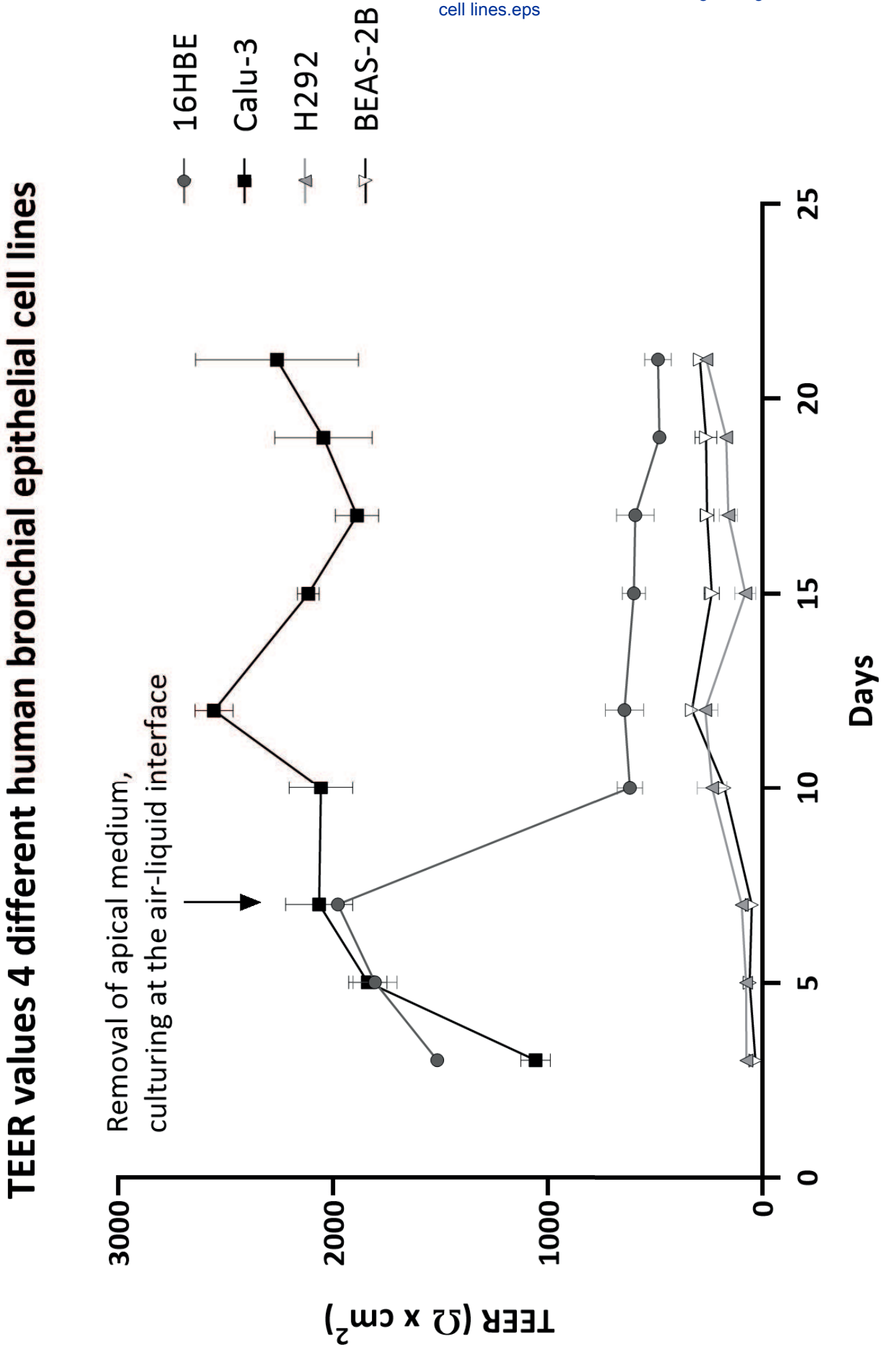
Figure

Click here to access/download;Figure;Figure 2 Calu-3 ZO-1.eps



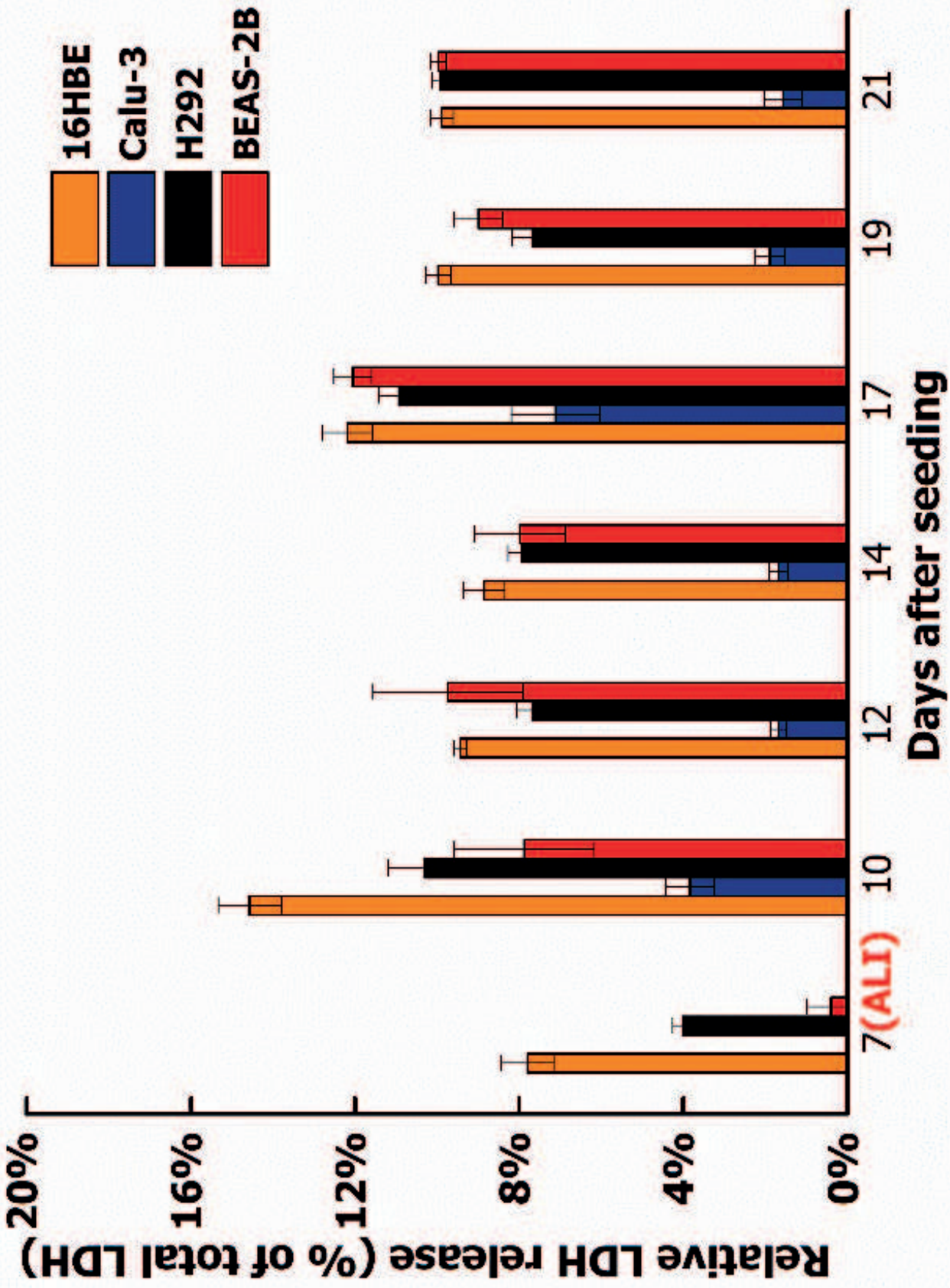
Figure

Click here to access/download;Figure;Figure 4 TEER values 4 cell lines.eps



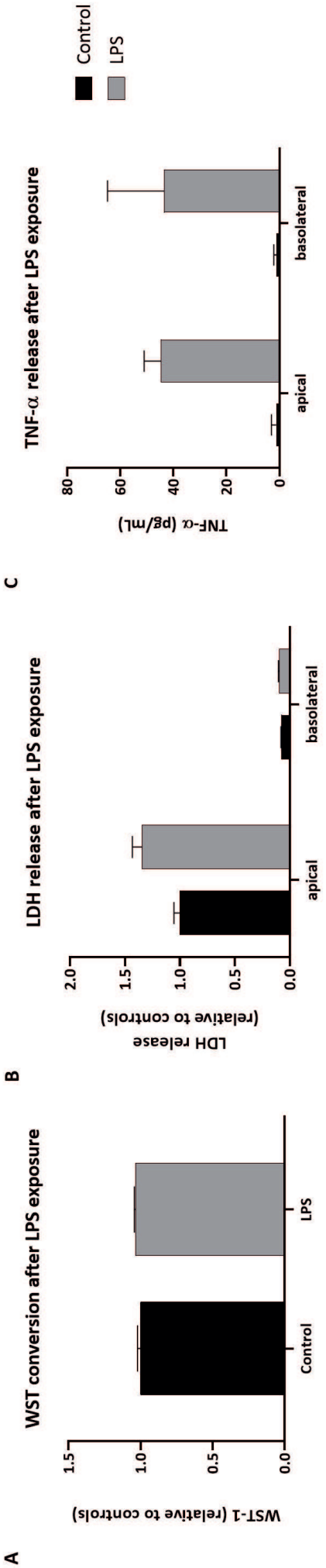
Figure

Click here to access/download;Figure;Figure 5 LDH release 4 cell lines.eps



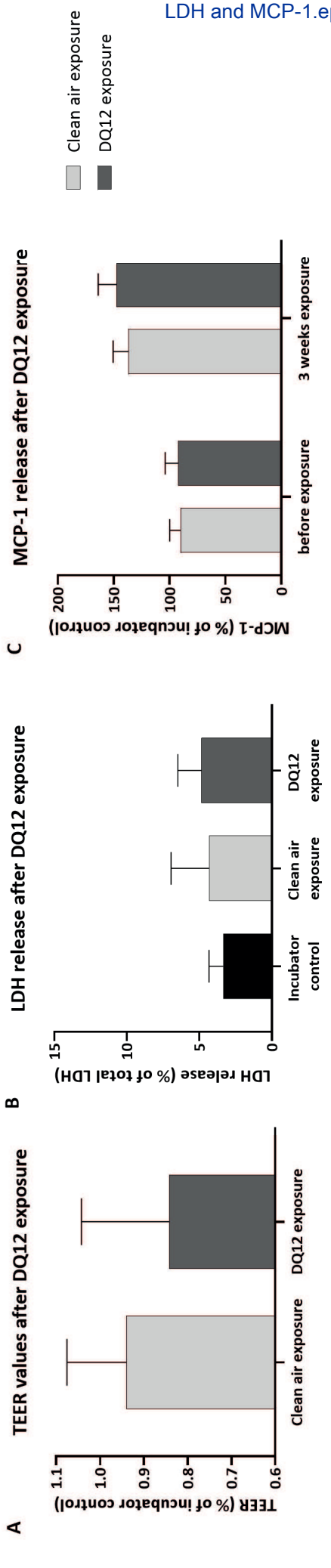
Figure

Click here to access/download;Figure;Figure 6 LPS exposure.eps



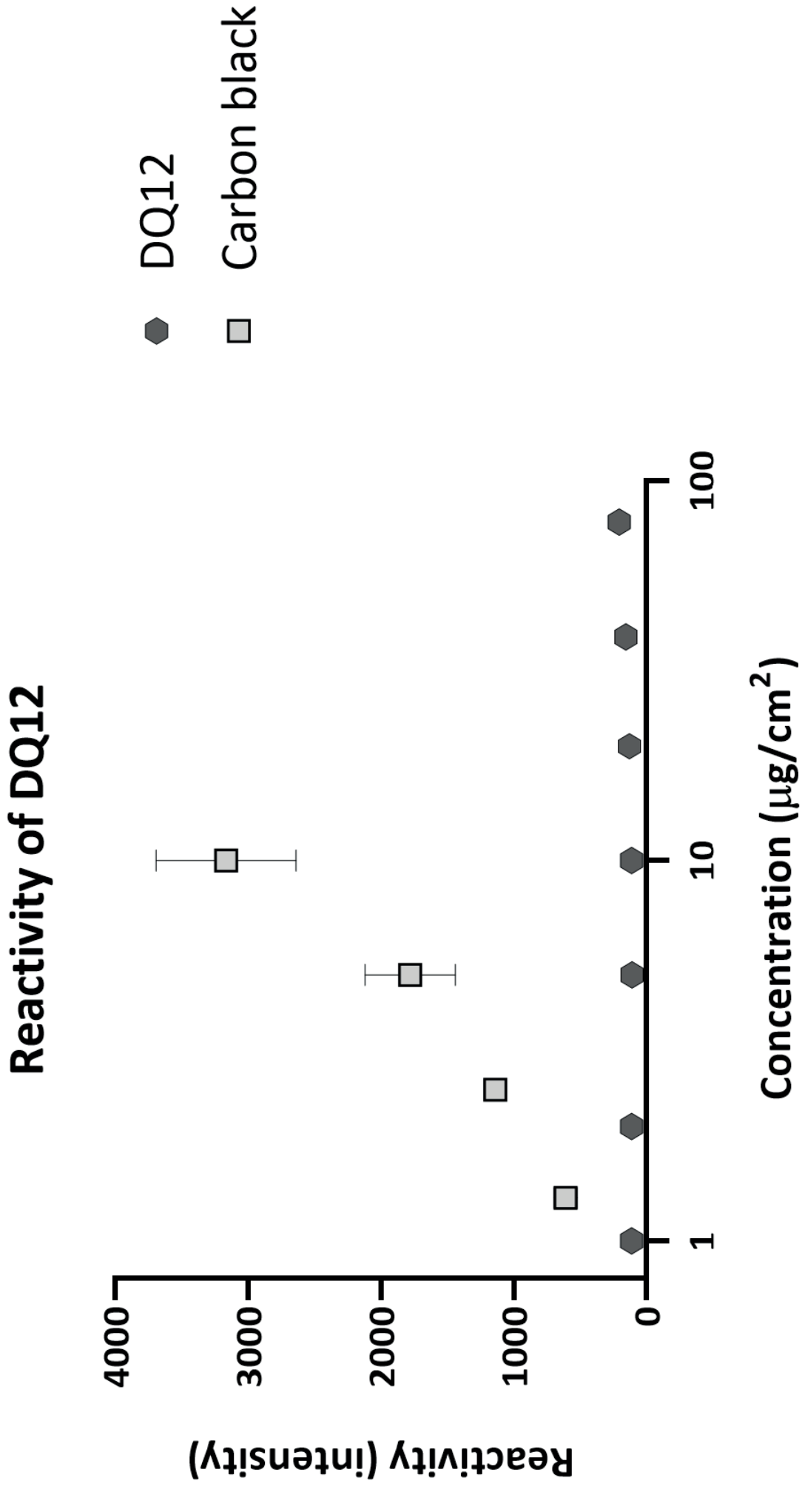
Figure

Click here to access/download;Figure;Figure 7 DQ12 TEER, LDH and MCP-1.eps



Figure

Click here to access/download;Figure;Figure 8 Reactivity.eps



Table

Particle mass ($\mu g/m^3$)	Particle number ($\#/cm^3$)	Mobility particle size (nm)	Geometric standard deviation
2969 (418)	83983 (10215)	66.6	2.5

Optical particle size (µm)
1.1 (1.3)

Name of Material/ Equipment

0.01 M NaOH
0.1M (x10) PBS
2',7'-dichlorodihydrofluoresin diacetate ($DCFH_2$-DA)
3-Morpholinosydnonimine hydrochloride (SIN-1 hydrochloride)
Amphotericin B
Automated exposure station
Cell proliferation reagent WST-1
Centrifuge
CPC
Cytotoxicity detection kit LDH
DQ12
ELISA Ready-SET-Go
EVOM2
FBS
Flow splitter
Fluorescein diacetate (F-DA)
HBSS
Light microscope
Mass flow controllers
Methanol (analytical grade)
Microbalance
Minimum essential medium (MEM) + GlutaMAX
NEAA
OPS
Pen/Strep
Phenol red free MEM
Pure water
SMPS
Spray nozzle
Teflon filters
TEOM
Tissue culture flask
Transwell inserts
Trypsin-EDTA
Tryptan Blue

Company

Sigma Aldrich
Gibco
Sigma Aldrich
Abcam
Thermo Fisher Scientific Inc.
Vitrocell
Roche
Eppendorf
TSI inc., St Paul MN, USA
Roche
IOM
Fischer Scientific
World Precision Instruments Inc., FL, USA
Greiner bio-one
TSI inc., St Paul MN, USA
Sigma Aldrich
Thermo Fisher Scientific Inc.
Olympus
MFC, Bronkhorst, the Netherlands
Sigma Aldrich
Sartorius, Goettingen, Germany
Thermo Fisher Scientific Inc.
Thermo Fisher Scientific Inc.
TSI inc., St Paul MN, USA
Thermo Fisher Scientific Inc.
Gibco
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758093
model 3708
F7378
14175
CKX41
34860
ME-5
41090
11140
3339
15140
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3460, 3462
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Comments/Description

nanomaterials

Dear Dr. Steindel,

Thank you for sending the comments of the reviewers. We would like to thank all reviewers for their time to critically evaluate our manuscript. We appreciate all comments and think they help to improve the quality of the manuscript. Please find below the response to each of the comments.

Best regards,
Hedwig Braakhuis

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have checked the manuscript thoroughly.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
We have checked the JoVE format.
3. Please include email addresses for all authors in the manuscript itself. done
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript (and figures) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Glutamax, EVOM2™, Sartorius, Teflon, MilliQ Commercial language has been removed from the manuscript.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Ok, we highlighted specific parts of the protocol
2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Table:

1. lease remove the embedded table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Ok

References:

1. Please do not abbreviate journal titles. Ok

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used,

especially those mentioned in the Protocol. Ok, we made a few additions to the table of materials.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript provides several protocols including culturing of Calu-3 cells and ALI exposures to airborne particles. Protocols miss some important information, and should be written more carefully.

Major Concerns:

Manuscript lacks highlighted text for future video recording. The discussion mentions 'preparatory experiments that focus on particle characteristics' -

I propose to demostrate it in the video. As the protocol should be general for this method, more materials should be presented. We believe that demonstrating the particle preparation is a good suggestion, we included this in the highlighted text for video recording. In addition, we understand the comments of the reviewer on preparatory experiments and including more materials. We had quite some discussion on this internally, because the particle generation is the most variable part of the protocol. Depending on the research question, the particle generation will change. Also, depending on the particles used, the protocol will change. In other words, for each experiment, a specific deposited dose on the cells is aimed for to test a hypothesis. To achieve the desired deposited dose, one needs to test different starting concentrations of the particle. Based on these preparatory experiments, the optimal particle concentration that will be nebulized can be chosen. For particle generation, there are several options: spark generation, nebulization, vapor aerosolization etc. Depending on the particle characteristics and the research question a specific generation method will be chosen. We believe that particle nebulization is used most often and that is why we included this in the paper. DQ12 is used as an example, as this particle is inducing fibrosis and tumors *in vivo*.

Protocol for culturing Calu-3 cells does not bring any hard or surprising steps compare to usual cell subcultivation, and therefore it does not bring any extra value to a paper. We agree the Calu-3 culturing is straight forward, but is essential to the preparation of the model.

Several times mentioned 'cells cultured at the air' - this is misleading information, as cells are cultured at the ALI. Ok, we changed this.

More references should be included in Introduction, specially ones referring to high concentrations applied with cloud, reference no. 8 is not the proper one for this statement. Ok, we included additional references.

Within the protocol, it is mentioned several times, that 'cells need longer period to achieve a good TEER', however it is not specified how much longer is accepted. We included more details.

Figures 1 and 2 should be merge Figure 1 shows some of the details of the AES, including an exposure module, while Figure 2 shows a schematic representation of the exposure setup. We

believe these are different and should be separate.

ENM abbreviation is not explained ENM: engineered nanomaterial, we included this in the manuscript.

Protocol for ALI exposures is insufficient and unclear, provide more details - why do you weight teflon filters, how does the QCM work, preparing particle suspension for single exposure and for 3 weeks of exposures - confusing, for 3 weeks of exposure, is it everyday a new suspension? Particle suspension is not sonicated prior use? How do you 'adjust setting'? Points 4.3 - 4.7 - when do you perform this, if 4.8 is day in advance? Does QCM need to equilibrate prior further use? What is QCM outcome? We provided more clarifications in the text.

5.1. how do you measure TEER at ALI? By adding culture media to the apical side of the insert.
6.3. how do you separate layers? The layers we refer to here are the levels in the AES. At the top level, the inserts are exposed to clean air. At the middle and bottom level, the inserts are exposed to aerosols. We changed the word layer to level for clarity.
7.2 how do you close the humidity? We mean here that 85% humidity does not have to be maintained overnight. We changed this to stop humidity control.

Results: the representative results do not correspond with the main focus of manuscript, it rather discuss choice of optimal cell line. What will be novelty of reference 9? We believe it is important to justify the choice for the Calu-3 cell line as a robust cell line that can be cultured at the air-liquid interface for weeks. The reference of He et al. will show more detailed results on the different cell lines.

Carbon black particles are not discuss in results, but are shown in Figure 8. CB are used as a positive control in the reactivity assay. We included this also in the results section.

Monolayer formation of Calu-3 is mentioned several times, but it is not shown. Protocol mentions culturing of Calu-3 cells for up to 6 weeks, why 10 days time point is presented at Figure 3? The Calu-3 cells remain a stable cell layer for weeks. We did not stain the tight junctions at all time points, but included the time points used mainly in our experiments up to 3 weeks at ALI condition. The picture of 10 days ALI culturing is a representative image of all these pictures.

Discussion: not statistically significant result - what statistical test was used? We used an unpaired t-test

Minor Concerns:

Why is Mr. Frosty mentioned in the list of equipments?We use Mr. Frosty to freeze cells. As this is not part of the manuscript, we removed this.
LDH, antibodies, etc. are not mentioned in material list, or in the text. We added the assays to the material list and more explanation to the protocol. Step 6.7 – 6.10.
Are the airflow values coorect? Can you include the reference? Yes, we checked the airflows thoroughly. These will be explained in more detail in the manuscript of He et al., which is in preparation. The AES is capable of applying airflows between 10 and 100 mL/min. We observed no effect of the airflow of 50 mL/min on the cells while having a optimal deposition rate.
HBSS abbreviation is not explained We included this.

Step 3.9.3. - electrode doesn't need any time to equilibrate after ethanol sterilization? No, it does not affect the electrode.
3.9.5. Is TEER value measured one time per insert?, blank resistance should be explained better Yes, TEER is measured once per insert. We included more details on the blank.
3.9.7. can you include an example of calculation of the TEER value? Yes
3.9.8. What should be done if TEER is < 1000? In case TEER is below 1000 Ohmxcm2, this means the barrier integrity is not ok and the monolayer is not intact. It could be that the cells are damaged during handling. In case this happens, a spare insert that has sufficient TEER should be taken to start experiments.
3.10. - wrong numbering, pour off CCM from where? CCM was added to the apical side of the inserts to be able to measure TEER. All steps at 3.9 refer to TEER measurements. Step 3.10 continues by removing the apical medium to allow culturing the cells at ALI.
3.15. 6 weeks + 7 days, or 6 weeks including 7 days for differentiation? 6 weeks from the moment the cells are cultured at the ALI.
5.2 where are cells floating at ALI? The cells transport a little bit of medium to the apical side. In general, there are no floating cells at ALI.
Please, explain abbreviations in Table 1 caption again Ok
Why is there a difficulty to visualize cell morphology using light microscopy, when using bigger pore sizes? I think it is because the reflection of the light is different.

Reviewer #2:

Manuscript Summary:
The authors describe a bronchial model in combination with a continuous flow exposure system for particle toxicity testing under repeated conditions.

Major Concerns:
There is no real effect of the DQ12 exposure on the cell model tested here. It would be better if the exposure resulted in significant effects. Yes, we agree on this. We were hoping to see a clear effect of the DQ12.

Minor Concerns:

Line 300 - We started with 4 different human bronchial epithelial cell lines: 16HBE, Calu-3, H292 and BEAS-2B. These are all widely used for toxicity testing of (nano)materials and chemicals. Of the 4 cell lines, only the Calu-3 cells fulfill all the above requirements.
It would be better to include in the introduction that actually 4 cell lines were also tested. We included this in the introduction.

Line 399 - In addition, deposition can be enhanced using an electrical field.
Line 405 - Depending on the type of particles that are being tested, the aerosol generation method can differ.
The use of electrical field needs to be elaborated (e.g., whether certain particles should not be applied using electrical field. We included some more information: The use of electrostatic deposition depends on the particle type and works best for metallic particles. For particles with a positive surface charge a negative electrostatic field should be applied and vice versa.

Line 415 - An explanation for the observation that DQ12 exposure for 3 weeks did not induce significant effects in the Calu-3 cells is that macrophages are lacking from the Calu-3 model.
It would be better to elaborate the type of situations that would be suitable for one to use Calu-3 model. Such information would be useful for readers to decide whether Calu-3 model would be fit for their studies. We agree with the reviewer and included the following: We believe the Calu-3 bronchial model is a suitable model for repeated exposure to aerosols of (partly) soluble substances such as chemicals from cigarette smoke and LPS. These soluble substances induce significant increases in cytokine responses in the Calu-3 cells. For testing non-soluble particles such as diesel exhaust and DQ12, a co-culture model is needed as the macrophages play a crucial role in the induction of effects by particle exposures.

Reviewer #3:

Manuscript Summary:

The authors selected the human bronchial epithelial cell line Calu-3 out of three other cell lines (16HBE, H292 and BEAS-2B) for culturing and exposure to test aerosols at the air-liquid interface. The selection criterion was the transepithelial electrical resistance (TEER), which was stable with the Calu-3 over three weeks but dropped down with the other cell lines. This property is necessary for a repeated continuous aerosol exposure at the ALI which can be compared to in vivo inhalation experiments.

In the protocol the culturing of the Calu-3 cells is described in detail. Furthermore, the VITROCELL automated exposure station and its preparation and handling for the exposure are described in detail.

For testing the response of the Calu-3 cells the authors used LPS and DQ12 quartz particles as positive controls and administrated these substances via nebulization. LPS induced as expected TNF-alpha release. Exposure to DQ12 over 3 weeks at a final cumulative dose of 1.6 µg/cm² was expected to induce an effect as it did in vivo. However, the effects were not significant.

In summary, the Calu-3 model is suitable for a long-term repeated ALI exposure in the VITROCELL automated exposure station and therefore suitable for comparison of in vitro and in vivo exposure to aerosols.

Major Concerns:
Page 10, Table 1: DQ12 quartz may contain a small nanoparticle fraction, but the main fraction is not in the nanometer range. How could it be that the particles in the aerosol are so small? On top of the AES, we used an impactor that removed all aerosols larger than 2.5 µm. In addition, the size distribution data is based on SMPS measurements. The SMPS we use, has the limitation that it can measure aerosol sizes up to 1 µm. So the particles in between 300 nm and 2.5 µm are measured using an OPS. According to the OPS data, the aerosols were about 1 µm on average. However, the variation was quite large with a range between 0.3 and 3 µm. We included this in the table with the DQ12 exposure characteristics.

Minor Concerns:

Page 3, line 72 - Please also cite the scientific publication where the development of the VITROCELL automated exposure station and its application has been first described and readers can retrieve relevant information (Mülhopt et al. Toxicity testing of combustion aerosols at the air-liquid interface with a self-contained and easy-to-use exposure system. J Aerosol Sci 96, 38-55, 2016). Of course we will include this reference. Thank you! Reference to only a commercial website used for advertisement of the system is probably not sufficient as a scientific reference. Also, much of the information which is described in the protocols of this submitted manuscript is already provided in Mülhopt et al. although in a more condensed version.
Page 7, line 241: please indicate which cell culture medium was used for the ALI exposure. Was it the same as for culturing the cells plus additional HEPES? Did you add FBS? It is the same culture medium as the cells are cultured in, with addition of HEPES and without FCS. You could also use standard RPMI culture medium with additional HEPES.

Page 9, line 327-335: it would be helpful to give a reference of an animal inhalation experiment using quartz particles at similar dose and appropriate endpoints. We included addition references.

Page 10, line 383: Please explain the abbreviations in the table (GMD, GSD). We adjusted the headings of the table.

Page 10, line 383: Please explain the abbreviations: MCP-1, ENM, HEPES, LPS, DCF-DA. Ok

Reviewer #4:

Manuscript Summary:

Clear and concise manuscript that is well written and informative.

Line:
50- need to mention this model is more physiologically relevant than a submerged model and some cells can produce surfactant like substances and therefore don't dry out. Ok
60 - 16HEB14o- This is incorrect in the manuscript (also 300, 309, 362, 355, 392) We corrected this.
63 - reference for this? Yes, we included 2 references
106 - pour off to where from the flask
162 - repeat how many times? Until each insert has been measured
153 - you haven't mentioned here that there needs to be media on the apical side of the insert to do these measurements The cells are still under submerged condition here.
203 - is this section sterile? Setting up the AES is not sterile. The modules in the cabinet and the trumpets are sterile. The measurements of aerosol characteristics are not in a sterile environment.
210 - what about metal ENM, or those with metal impurities Also metal oxide nanomaterials can be used for exposures. We included this in the manuscript.
253 - what about 12 wells? Either 6-wells or 12-wells inserts can be used.
275 - fresh CCM Ok
388 – bronchial Ok, thank you

Reviewer #5:

In this paper, the authors seek to demonstrate that the Calu-3 cell line is a good candidate for

repeated exposure (up to 6 weeks) to airborne particles at the air-liquid interface (ALI).

The system used to expose cells is the automated exposure system (AES) from Vitrocell, equipped with exposure modules for 6 or 12-wells inserts.

This system allows a dynamic exposure of cells at the ALI, with a continuous aerosol generation, obtained here by nebulization of a NP suspension and characterized by a set of equipment (SMPS/OPS, CPC, TEOM, microscopy…).

INTRODUCTION:
The context in which experiments are performed is an exhaustive and very clear summary of what is done currently in the field with the advantages and drawbacks of each technique (submerged versus ALI, static-i.e. Cloud versus dynamic) and of most used cellular models.

PROTOCOLS:
Protocols to achieve repeated inhalation exposure of Calu-3 at the ALI are well-described with adequate level of details for implementation.
Somme minor questions, comments however…
Line 107: wash the cells with 5 mL of HBSS. Usually, buffer is used without divalent ions (Ca and Mg) to facilitate cell detachment. Please specify if this is important. Yes, we use HBSS without calcium and without magnesium.
Line 109: usually trypsin 0.25 (instead of 0.05) is used for Calu-3. Maybe it is less important if they are trypsinized at 60-80% confluency. Please add clarification We have no problems using trypsin 0.05 for cell detachment. Indeed, in case the cells are grown to full confluency, they will not detach anymore using the 0.05 trypsin. We added the following: In case the cells are grown >80% confluency, they will not detach using trypsin 0.05% and trypsin 0.25% could be used.
Line 140: (…) the steps in 1.4. Please check for "1.4" Thank you, we adjusted this to 2.1 – 2.10
Line 154: Chopsticks are OK but consider also the use of an Endhom that should give more reproducible results. Yes, we recently purchased a cup holder for more stable TEER measurements.
Line 173: What is the rationale for leaving cells at the ALI for 7 days before exposure? Some studies let the cells at the ALI for 24h only before they get exposed. In our lab, we found that the cells need 7 days at ALI to adjust to the conditions, e.g. they grow an even more stable barrier and start to produce mucus. If you culture the cells longer at ALI, they even can grow cilia-like structures.
Line 238: if you measure TEER just before the first exposure, then you "break" the ALI conditions and remove mucus deposited on cells as you have to put medium at the apical side of the insert. Can you comment this point? We had some discussion on this point internally. We would like to know the TEER before exposure to be able to compare this to the TEER after exposure. In addition, the TEER is for us a quality check that the cell layer is ok. To not break the ALI conditions daily, we decided to only measure TEER prior to exposure and after the final exposure.
Line 262: Just a remark: The flow-rate of 50 mL/min is ten-fold the flow rate in Vitrocell modules used alone (i.e. without the AES). Seems quite high… Not sure that all kind of cells are able to stay as long as 4 hours in these conditions. Seems OK for Calu-3 so also OK for me You are very right. We had some debate on this and have been building several ALI systems to be able to use more realistic airflows. The AES we use here has the limitation that airflows between 10 mL/min and 100 mL/min can be used and not as low as 5 mL/min, which would be more realistic.

REPRESENTATIVE RESULTS
Line 328: It should be interesting to indicate the deposition rate also and if you used the electrostatic deposition enhancement. We did not use the electrostatic deposition enhancement in these experiments. The deposition efficiency is very low. When using a starting amount of 300 mg DQ12, we achieved a deposited dose of 1.6 µg/cm2. This is a total of 45 µg that is deposited out of 300 mg

input.
Line 333-335: Even if this is not the main issue, it should be useful to describe briefly the protocol for measuring particle reactivity with the DCF-DA probe. We included a reference to the protocol.

The low reactivity of DQ12 is a known phenomenon, probably since DQ12 have been collected a long time ago (surface passivation). It would have been interesting to test more reactive particles. Yes, we agree.

DISCUSSION
This section is very clear and well-written. This is a very good idea to consider adding macrophages on top of Calu-3 cells as they are one of the first cells in contact with inhaled particles and for their role in pro-inflammatory responses.
Line 431: instead of primary macrophages, why not considering the use of differentiated THP-1 cells (that should be easier to get than human primary macrophages)? We have tried both in our lab. The THP-1 cells have the disadvantage that there are different clones available between labs. In addition, their cytokine response is different from primary cells and for some cytokines the response is too low to measure in our setup. When using primary cells, we found significant induction of cytokines and optimizations in protocols gives us the opportunity to use frozen primary monocytes as a source, making the use of the primary cells relatively easy.

Reviewer #6:

Manuscript Summary:
This is an attempt to describe an ALI-model for realistic, repeated inhalation exposure to airborne particles for toxicity testing.

Major Concerns:

The authors have chosen Calu-3 cells and motivate this by the development of the highest TEER value as a marker for tight junctions. The presented cut-off TEER values are extremly high (<1000) but even lower TEER values are ok and often used in literature. In literature much lower TEER values have been described in different airway wall models, so the choice of this high cut-off value need to be discussed. They excluded models with more normal epithelial cells (BEAS-2B and 16HBE) due to lower TEER values. They also exclude A549, which are adenocarcinomic human alveolar basal epithelial cells, and constitute a cell line. So they are not reflecting normal bronchial epithelial cells at all and this need to be clarified better. However, the presented TEER value of 16HBE is still ok and might be a better option since these cells most likely represent a normal human mucosa to a higher extent than an adenocarcinoma cell line like CALU-3. Also, although clearly stated the poor size they have chosen to use (3µm) influence the TEER value, but they used this for optimal testing of particle translocation. However 0.4µm is often a better option with regard to tight junction development and differentiation of different cell types present in the bronchial epithelial mucosa (see below). How often does the smaller poor size influence particle translocation when working with nanoparticles although agglomeration is occurring. These are all very valid comments. As our aim was to develop a model that can be used for repeated ALI exposure, we focused on a model with a stable TEER when cultured at the ALI. As Calu-3 cells achieve a high TEER, we choose the cut-off of 1000 Ohmxcm2. For other cells, other cut-offs can be chosen and justified. The reviewer is right that at the smaller pore size of 0.4 µm the tight junctions develop better and a high TEER is easier achieved. Therefore, only for particle translocation studies we recommend the larger pore size. For other experiments, we recommend the smaller pore size. It is known that the smaller pore size does

affect the translocation rate.[1]

The cell´s characteristics is not described at all, since it is a adenocarcinoma cell line you can question how well it represent normal human bronchial mucosa. Are ciliated cells, mucus producing cells and basal cells present? This can be demonstrated by expression data or immmunohistochemical and confocal microscopy pictures after staining with ß-tubulin and MUC5AC for instance. Do the cells form psudostratified layer? How are these epithelial characteristics changed with increased passages? All cell lines have limitations. Indeed, the Calu-3 cell line originates from adenocarcinoma whereas the 16HBE and BEAS-2B originate from healthy tissue. The disadvantage of the latter two is that they seem not suitable for (repeated) ALI exposure.
When cultured for a longer period at the ALI, the Calu-3 cells can grow cilia/cilia-like structures[2] and produce mucus[2-4]. The occurrence of cilia appears inconsistent. They are also used as an infection models for RSV infection studies.[5] RSV infection primarily occurs in ciliated airways epithelial cells. We included more information in the manuscript.

Minor Concerns:
No information regarding which passage number that are optimal, they only mention that lower passage than 25. No comments if the cells start to change after so many passages is given. The optimal passage number is between passage 3 and 25. Between these passage numbers the cells show a very stable phenotype and stable response to an exposure.

1 Cartwright, L. *et al.* In vitro placental model optimization for nanoparticle transport studies. *Int J Nanomedicine.* **7** 497-510, (2012).
2 Grainger, C. I., Greenwell, L. L., Lockley, D. J., Martin, G. P. & Forbes, B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res.* **23** (7), 1482-1490, (2006).
3 Papazian, D., Wurtzen, P. A. & Hansen, S. W. Polarized Airway Epithelial Models for Immunological Co-Culture Studies. *Int Arch Allergy Immunol.* **170** (1), 1-21, (2016).
4 Jeong, M. H. *et al.* In vitro model for predicting acute inhalation toxicity by using a Calu-3 epithelium cytotoxicity assay. *J Pharmacol Toxicol Methods.* **98** 106576, (2019).
5 Harcourt, J. L., Caidi, H., Anderson, L. J. & Haynes, L. M. Evaluation of the Calu-3 cell line as a model of in vitro respiratory syncytial virus infection. *J Virol Methods.* **174** (1-2), 144-149, (2011).