Chapter 21

Air–Liquid Interface Cell Exposures to Nanoparticle Aerosols

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Abstract

The field of nanomedicine is steadily growing and several nanomedicines are currently approved for clinical use with even more in the pipeline. Yet, while the use of nanotechnology to improve targeted drug delivery to the lungs has received some attention, the use of nanoparticles for inhalation drug delivery has not yet resulted in successful translation to market as compared to intravenous drug delivery. The reasons behind the lack of inhaled nanomedicines approved for clinical use or under preclinical development are unclear, but challenges related to safety are likely to contribute. Although inhalation toxicology studies often begin using animal models, there has been an increase in the development and use of in vitro air–liquid interface (ALI) exposure systems for toxicity testing of engineered nanoparticle aerosols, which will be useful for rapid testing of candidate substances and formulations. This chapter describes an ALI cell exposure assay for measuring toxicological effects, specifically cell viability and oxidative stress, resulting from exposure to aerosols containing nanoparticles.

Key words Air-interfaced culture, SPIONs, Iron oxide, Nanoparticles, Aerosol

1 Introduction

Traditionally, the toxicity of inhaled substances, including engineered nanoparticles, is determined with animal experiments, usually following guidelines proposed by the Organization for Economic Co-operation and Development (OECD). The testing strategies used in animals include subacute to chronic inhalation testing conducted over a month to several months, and acute inhalation testing that involves short-term, 4 h exposures. Acute inhalation testing following OECD test guidelines 403 and 436 starts with a maximum obtainable concentration of 5 mg/L for aerosols, which is a very unrealistic exposure scenario, and reveals toxicity data including gross response (behavioral changes and/or mortality), histopathology, and lethal concentration estimates (LC_{50}) [1, 2]. For nanoparticle aerosols, it is difficult to achieve

Sarah Hurst Petrosko and Emily S. Day (eds.), *Biomedical Nanotechnology: Methods and Protocols*, Methods in Molecular Biology, vol. 1570, DOI 10.1007/978-1-4939-6840-4_21,

such extreme concentrations, and several published studies do not report acute inhalation toxicity at maximum obtainable concentrations [3–6]. Other limitations include the fact that, when conducting these tests, animals could experience distress or pain, and little is revealed about the pharmacokinetics or mechanism(s) of action of the materials. In addition, it is generally recognized that, despite their necessity before human testing, responses measured in animal models may not accurately predict the responses in humans. Taken together, it is arguable that in vitro testing could be used to verify if maximum obtainable concentrations result in acute toxicity. Animal testing can then commence directly at realistic exposure concentrations to assess the more human relevant subacute (28 days) inhalation toxicity. In this chapter, we discuss recent progress in the field in using air–liquid interface (ALI) exposure systems to assess the in vitro toxicity of aerosolized nanoparticles, and we also provide step-by-step instructions to measure cell viability and oxidative stress following cellular exposure to aerosolized nanoparticles using an ALI exposure system.

Aerosols are tested in vitro using air–liquid interface (ALI) exposure, which involves directly introducing aerosols to cells cultured on permeable membrane supports. This allows for cells to receive nutrients from culture medium touching the basolateral side while exposing the apical side to air as shown in Fig. 1. The rationale behind moving toward ALI cell exposures is comparable to the rationale behind the increase in mouth/nose controlled breathing exposure versus intratracheal instillation in animal studies: particle deposition and distribution patterns differ greatly when delivered by a bolus suspension versus by aerosol. Although suspensions deliver a defined dose instantaneously, inhalation is a

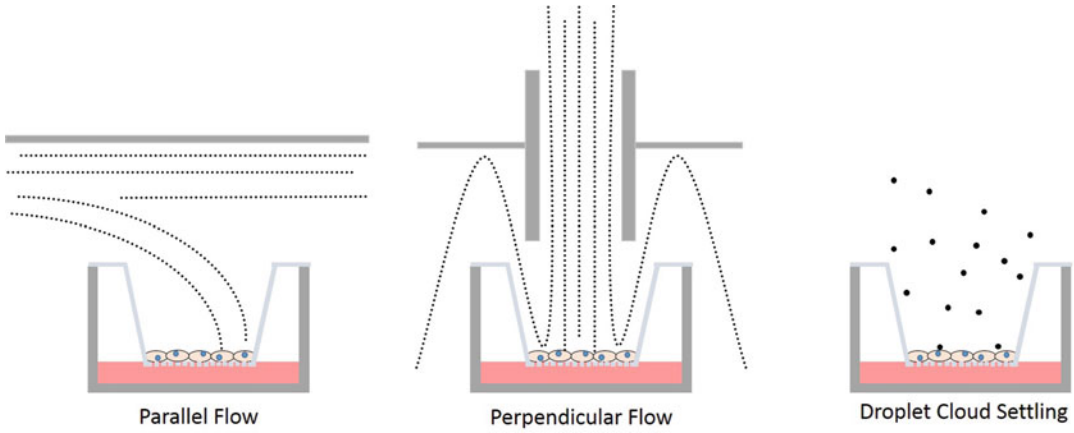

Fig. 1 Pictorial representation of the aerosol flow profiles of the MEC (*left*), Vitrocell or NACIVT (*middle*), and ALICE (*right*) exposure chambers with aerosol in *blue*. The MEC, NACIVT, and Vitrocell system work with aerosols of a wide size range, while the ALICE system relies on gravitational sedimentation, which requires aerosol droplets in the μm range

dynamic not static process, and the dose deposited in the lungs is related to the amount of air inhaled and the breathing rate. In addition, particle concentration, size distribution, and morphology are all influenced by nanoparticle agglomeration, which occurs differently in liquid and gas phases. An additional benefit of ALI models is that cells cultured at the ALI develop more differentiated cell monolayers that more closely resemble the in vivo lung epithelium when compared to cells cultured under media [7–9].

Several ALI exposure systems have been developed, which allow for nanoparticle exposure of cells cultured on transwell membranes. The essential components for ALI exposure systems are as follows: "(1) complex pulmonary cell systems, which can be cultivated for at least several hours at the ALI, (2) direct contact between the cultivated cells and the inhalable substances without interfering medium, (3) uniform exposure of the entire cell layer, (4) temperature and humidity conditioning of the air to maintain cell integrity (T ~ 37 °C; relative humidity >85%), and (5) precise control of the substance concentration and purity for accurate dosimetry" [10]. Equally important are the methods of test aerosol generation and characterization. For particles dispersed in liquid, nebulizers are used to generate aerosols and a variety of studies test nebulized nanoparticle suspensions with ALI cell exposure systems [11–13]. The three referenced systems rely on methods of diffusion and/or sedimentation as deposition mechanisms (Fig. 1). An important difference is the direction of aerosol flow toward the cells, which is either parallel, perpendicular, or without flow (droplet cloud sedimentation), and has implications on the deposition efficiency for different particle sizes and the stress to the cells from passing air. The systems also differ regarding the maximal number of transwell samples that can be exposed per experiment.

This chapter describes an air–liquid interface (ALI) cell exposure assay for nanoparticle aerosols using the multiculture exposure chamber (MEC) system [11]. We selected the MEC system for this protocol for its ease of use (well plates containing transwells can be placed directly in the chamber) and versatility (up to 144 transwells can be exposed at once); however, the methods could easily be adapted for other ALI exposure systems. First, we describe how to culture human lung cells at the air–liquid interface and perform in vitro exposure to nanoparticle aerosols using the MEC system. Then we explain how to analyze the toxicological effects of the nanoparticle aerosol using a reactive oxygen species (ROS) assay, which indicates oxidative stress due to redox imbalance, and a lactate dehydrogenase (LDH) release assay, which indicates cellular necrosis as LDH is released from cells upon loss of plasma membrane integrity. We describe these assays using ferumoxytol iron oxide nanoparticles as model nanoparticles, and we also provide optional methods to analyze iron content in cells following aerosol exposure. The reader could adapt these methods for use with other types of nanoparticles as desired.

2 Materials

2.1 Culture of Lung Cells at the Air–Liquid Interface

1. A549 human alveolar type II-like lung epithelial cell line (*see* **Note 1**).
2. 1× Hank's balanced salt solution (HBSS), pH 6.7–7.8.
3. 1× phosphate-buffered saline (PBS), pH 7.2.
4. Complete cell culture medium: 500 mL of Dulbecco's Modified Eagle Medium (DMEM) with Glutamax 5 g/L D-glucose. Supplemented with 50 mL of Fetal Bovine Serum (FBS) and 5 mL of Penicillin–Streptomycin (10,000 units/mL of penicillin and 10,000 μg/mL of streptomycin).
5. 1× 0.25% Trypsin–EDTA.
6. 6- or 24-well plates and polyethylene transwell inserts with 3 μm pore size (*see* **Note 2**).
7. Forceps.
8. Biological safety cabinet.
9. CO_2 incubator.
10. Light microscope.
11. Hemocytometer.
12. Micropipettes.
13. Pipetman.
14. Aspiration system.
15. Volt–Ohm meter with chopstick electrodes (*optional*).

2.2 In Vitro Aerosol Exposure

1. In vitro air–liquid interface (ALI) exposure system: This protocol describes the use of a laboratory developed multiculture exposure chamber (MEC) [11] (*see* **Note 3**).
2. Nanoparticle suspension in ultrapure water: This protocol describes the use of ferumoxytol (*see* **Note 4**).
3. Air supply (respirable air cylinder and/or technical house air).
4. 1-jet Collison nebulizer.
5. Conductive tubing (inner diameter 8 mm).
6. Digital thermal mass flow controller.
7. Thermal mass flow meter.
8. Aerosol particle counters: This protocol describes the use of the Scanning Mobility Particle Sizer (SMPS) (*see* **Note 5**).

2.3 Toxicity Assays

1. Microplate reader.
2. Flat-bottom 96-well plates.
3. LDH Assay Kit: This protocol describes the use of the CytoTox-ONE Homogenous Membrane Integrity Assay Kit.

Other commercial kits that are comparable include lactate, NAD^+, diaphorase, and resazurin as the components of the assay mixture.

4. DCFH-DA working solution: Dissolve 24.4 mg of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) in 50 mL methanol to make 1 mM DCFH-DA concentrated stock solution. This solution can be stored at −20 °C for up to 4 months. Dilute 1 mM DCFH-DA stock solution 100×s, or mix 0.1 mL of DCFH-DA stock solution with 9.9 mL HBSS for each 96-well plate, to make 10 μM DCFH-DA in HBSS.
5. Hydrogen peroxide (H_2O_2) stock solution: In a 50 mL volumetric flask, add 10 mL of Milli-Q water. Then add 0.5 mL H_2O_2 and fill up to the mark with Milli-Q water to achieve a final concentration of 0.1 M H_2O_2. This solution can be stored at 4 °C.
6. H_2O_2 working solution: Dilute 0.1 M H_2O_2 stock solution to 200 μM by adding 20 μL to a 10 mL volumetric flask and diluting with Milli-Q water.
7. Prussian blue iron stain kit (*optional*): This protocol describes the use of the Iron Stain Kit. Other commercial kits that are comparable include solutions of 4% w/v potassium ferrocyanide, 1.2 mM hydrochloric acid, and 1% w/v pararosaniline.
8. 50 mL conical tubes.

3 Methods

Safety note: This is a pressurized system with the potential to break and release hazardous materials. Have your safety and health staff verify the setup is safe for operation. All cell culture work should be conducted in a biological cabinet with standard personal protection equipment (lab coat, gloves, goggles). During testing and operation of the aerosol system, respiratory protection (N100 or FFP3 filter masks) is needed whenever accidental exposure cannot be excluded.

3.1 Culture of Lung Cells at the Air–Liquid Interface

Note: Cell cultures should be maintained in an incubator at 37 °C, 5% CO_2, and >80% relative humidity. The reader is assumed to be familiar with aseptic culture technique.

1. Harvest A549 cells cultured under liquid cover from culture flask. Remove medium and rinse cells with 3 mL PBS. Remove PBS and add 3 mL of trypsin. Incubate cells at 37 °C for 5–10 min. Examine flask using a light microscope to confirm cells have detached. Add 7 mL of complete medium to neutralize trypsin. Collect cells using a serological pipette with pipetman and transfer to a 15 mL conical tube.

2. Count cells using a hemocytometer to determine cell concentration. Remove 10 μL of cell suspension from the 15 mL conical tube using a micropipette and add to hemocytometer. Count cells in four 1 × 1 mm squares. Average and multiply by 10,000 to calculate number of cells per mL in harvested cell suspension. Repeat this step two additional times for higher statistical accuracy.
3. Prepare well plates with culture medium. For 6-well plates, add 1 mL to each well. For 24-well plates, add 0.3 mL to each well.
4. Seed transwell membrane inserts on apical side. For A549 cells at a seeding density of 1×10^5 cells/cm^2, add 3.5×10^4 cells to 24-well transwell or 5×10^5 cells to 6-well transwell.
5. Add complete medium to apical side of transwell to reach the recommended volume of 2 mL for 6-well plates and 0.5 mL for 24-well plates.
6. Culture cells under liquid cover for 7 days, replacing the medium every 1–2 days. (*Optional*: Monitor transepithelial electrical resistance (TEER) using a volt–ohm meter (*see* **Note 6**). Measure TEER of a cell-free transwell for the baseline.)
7. After 7 days, remove apical medium and culture cells for at least 1 day in ALI conditions, replacing basolateral medium every 1–2 days. Replace medium before exposing cells to aerosol. (*Optional*: Continue to monitor TEER in ALI conditions. Add 0.1 mL (6 well) or 0.02 mL (24 well) of prewarmed medium to apical side and incubate for 30 min before TEER measurement. Measuring TEER before conducting oxidative stress assay is not recommended (*see* **Note 7**).)

3.2 In Vitro Aerosol Exposure

1. Prepare nanoparticle suspensions at desired concentration in a minimum of 10 mL and a maximum of 200 mL ultrapure water.
2. Load nanoparticle suspension in nebulizer and ensure that nozzle tip is adequately submerged in suspension without blocking jet.
3. Connect nebulizer to aerosolization system. *See* Fig. 2 for an example system configuration. Ensure valve to SMPS is open and value to MEC is closed.
4. Load the exposure chamber with lung cell samples. Inside a biological safety cabinet, first disinfect MEC by wiping interior surfaces with 70% ethanol. After allowing ethanol to dry, line interior with aluminum foil to reduce electrostatic deposition of aerosol onto the MEC inner surface. Place the well plates containing the lung cells on transwells grown at the ALI for 7 days into the well plate holders with lids removed. Close MEC and remove from biological safety cabinet.

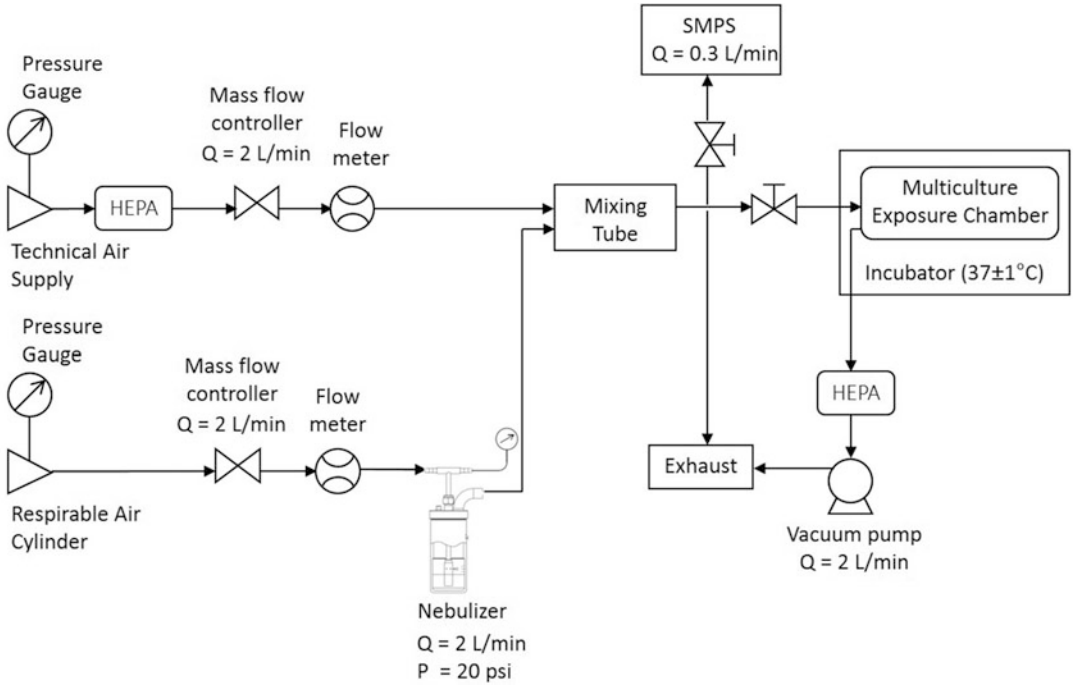

Fig. 2 Example aerosolization system configuration for in vitro air–liquid interface exposures

5. Place MEC inside incubator preheated to 37 °C. Connect all fittings and tubing. Ensure valve to the MEC inlet is opened.
6. Turn on the air supply, set the pressure using the gas regulator, and set the flow rate using the mass flow controller. The 1-jet Collison nebulizer should be supplied with air at a flow rate of 2 L/min and pressure of 20 psig (*see* **Note 8**). Turn on the vacuum pump to the exposure chamber to start the exposure. Nebulize the nanoparticle suspension for a 60 min period.
7. Physicochemical characterization of the generated aerosol should be conducted during the experiment. At a minimum, particle size should be measured using an aerosol particle counter, such as a scanning mobility particle sizer (*see* **Note 5**). For the physicochemical characterization of aerosolized ferumoxytol [14], particle size was determined using transmission electron microscopy, scanning mobility particle sizing, particle correlation spectroscopy, and nanoparticle tracking analysis. Metal analysis was conducted using atomic absorption spectroscopy and inductively coupled plasma optical emission spectroscopy.
8. After the 60 min exposure duration, turn off the vacuum pump to the exposure chamber and then shut the aerosol inlet valve to stop the exposure.

9. Disconnect the MEC from the aerosolization system setup. Remove the MEC from the incubator and transfer it to a biological safety cabinet. Open the MEC, immediately remove lung cell culture samples, and begin toxicity assays.

3.3 Toxicity Assays

This protocol describes two different toxicity assays. The first describes how to measure reactive oxygen species generation, which is indicative of oxidative stress, using DCFH-DA (*see* **Note 9**). The second describes how to measure lactate dehydrogenase (LDH) release from cells, which indicates disruption in cell membrane integrity and, indirectly, cell death.

3.3.1 Measuring Reactive Oxygen Species Generation Using DCFH-DA

1. Prepare new well plates containing 10 μM DCFH-DA working solution in each well. For 6-well plates, add 1 mL of DCFH-DA working solution per well. For 24-well plates, add 0.3 mL of DCFH-DA working solution per well.
2. Using sterile forceps, transfer transwells containing nanoparticle-exposed lung cells to the prepared well plates containing DCFH-DA dye. Save basolateral medium for the lactate dehydrogenase (LDH) release assay.
3. Cover the plate with aluminum foil to prevent photoactivation and incubate the cells with the DCFH-DA working solution at 37 °C in a humidified CO_2 incubator for 30 min.
4. Following incubation, load the well plate into the microplate reader and measure the fluorescence of DCF using excitation/emission wavelengths of 485/530 nm (*see* **Note 9**).
5. Report ROS production as the fluorescence intensity increase of treated cells relative to the baseline measurements, as shown in Fig. 3.

3.3.2 Measuring Lactate Dehydrogenase (LDH) Release

1. From the well plate containing the saved basolateral medium, transfer 100 μL of supernatant to a 96-well plate.
2. Equilibrate this plate to room temperature for approximately 20–30 min.
3. Add 100 μL of CytoTox-ONE Reagent to each well and mix or shake gently by hand for 30 s.
4. Incubate at room temperature for 10 min.
5. To each well, add 50 μL of stop solution (3% w/v sodium dodecyl sulfate) provided in the CytoTox ONE assay kit.
6. Shake the plate for 10 s and record the fluorescence within 1 h with an excitation wavelength between 560 nm and an emission wavelength between 590 nm (*see* **Note 10**).
7. Report the LDH release as a percentage based on the negative control after background correction (Fig. 4). This sets the

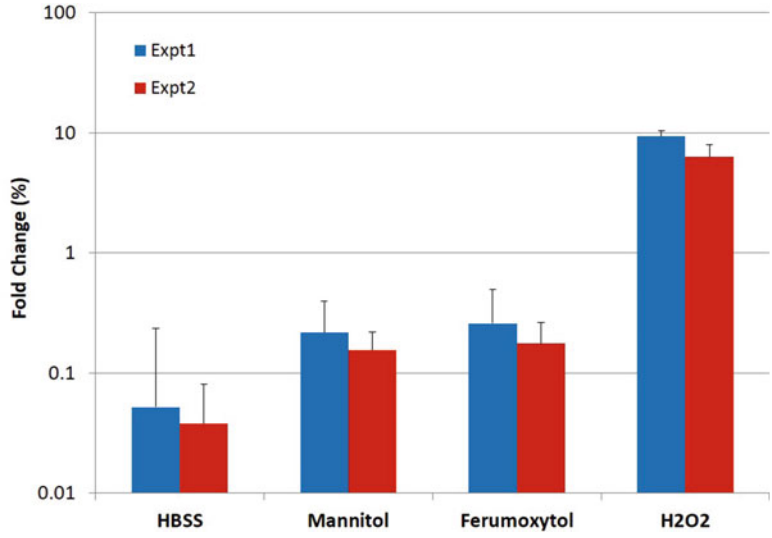

Fig. 3 Example result for DCFH-DA assay after 1 h aerosol exposure. Here, cells were analyzed after different aerosol exposure conditions, including HBSS (1×), mannitol (0.59 mg/mL), and ferumoxytol (0.4 mg Fe/mL). H_2O_2 (1 mM) was added to the cells to serve as a positive control and to ensure dye activation

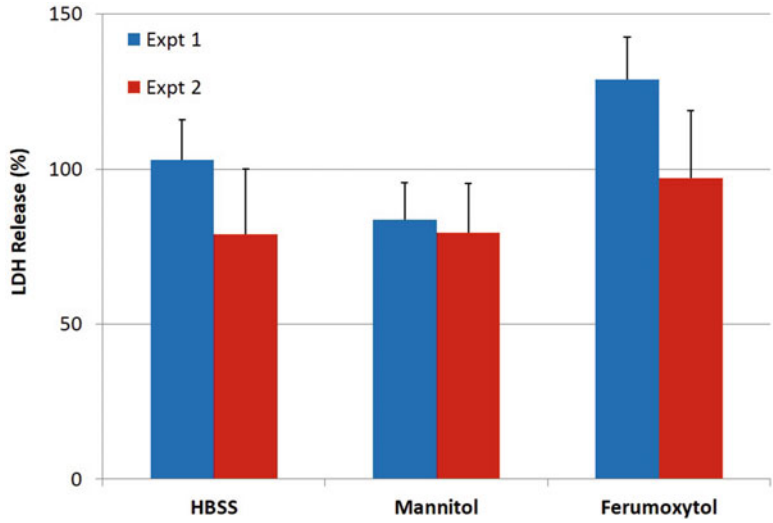

Fig. 4 Example result for LDH assay after 1 h aerosol exposure. Here, cells were analyzed after different aerosol exposure conditions, including HBSS (1×), mannitol (0.59 mg/mL), and ferumoxytol (0.4 mg Fe/mL)

untreated cells (negative control) at 100% LDH release, which is used as the reference for no leakage. Alternatively, cytotoxicity can be reported as a percentage based on the positive control (e.g., adding 1% Triton-X 100 solution to lyse cells

for maximum LDH release) after background correction (*see* **Note 11**). This sets the lysed cells (positive control) at 100% cytotoxicity, which is used as the reference for 100% cell death.

3.3.3 Iron Visualization (Optional)

This protocol can be used to visualize iron-containing nanoparticles, such as those in ferumoxytol, deposited on the cell monolayer after exposure.

1. After measuring ROS production (Subheading 3.3.1), transfer well plate to a chemical fume hood then add 100 μL of 4% formaldehyde to the top of the membrane to cover the cells. Fix cells for 15 min at room temperature.
2. Remove the transwell membranes from the inserts using a scalpel.
3. Dip the membranes in a freshly prepared 1:1 solution of 4% w/v potassium ferrocyanide and 1.2 mM hydrochloric acid and incubate for 10 min at room temperature. This stains the membranes for iron-containing compounds.
4. Rinse the membranes in deionized water and incubate in freshly prepared 2% v/v pararosaniline–water solution for 5 min at room temperature. This counterstains the membranes for polysaccharides.
5. Rinse the membranes in deionized water again.
6. Mount the membranes on coverglass and image with a bright-field microscope. A representative image showing the appearance of stained cells is shown in Fig. 5.

4 Notes

1. While this protocol describes the use of A549 lung epithelial cells, several types of lung cell monocultures and cocultures have been reported. Reported monocultures include established cell lines (Calu-3 human bronchial epithelial, BEAS-2B human bronchial epithelial, 16HBE14o- human bronchiolar epithelial-like, A549 human alveolar type II-like epithelial, LK004 human lung fibroblast) and primary lung cells (human bronchial epithelial—normal or diseased). Reported cocultures include laboratory developed [15–17] and commercially available (MucilAir, EpiAirway) tissues. Any of these model systems could be utilized with this protocol.
2. Transwell manufacturers provide excellent technical guidance on material and pore size selection for different applications. In general, in vitro lung models using transwells with pore sizes ranging from 0.4, 1, and 3 μm have been reported. Transwells

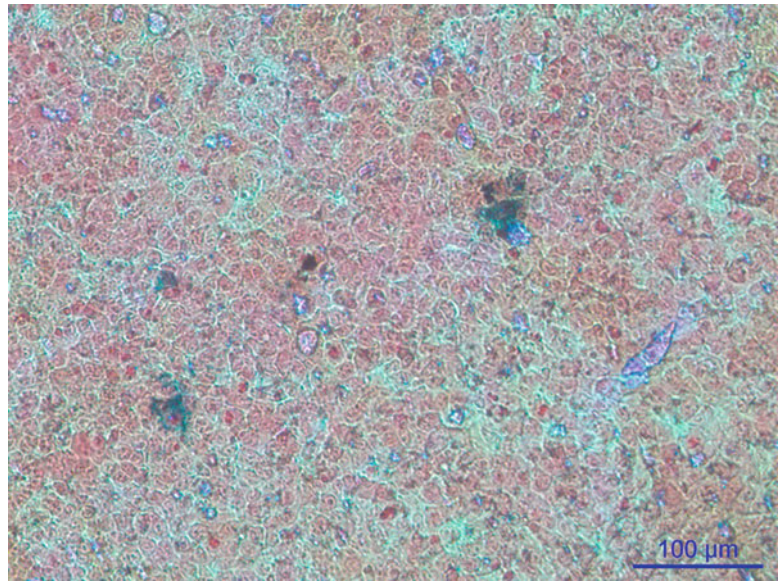

Fig. 5 Iron stained A549 cells exposed to ferumoxytol aerosol for 1 h. The *dark blue/black stain* indicates the presence of the iron oxide nanoparticles, while the pink counterstain reveals polysaccharides

with 0.4 µm pore size have been demonstrated to result in the least cell translocation and tracer dye leakage [16, 18].

3. Over 30 different ALI cell exposure systems have been developed. These include two commercially available systems (VitroCell, Cultex). These could be utilized instead of the MEC system described in this protocol.
4. While this protocol describes the use of ferumoxytol, it may be adapted for use with other types of nanoparticles that are of interest to the reader.
5. Nanoparticle aerosol concentrations are commonly measured using size mobility particle sizer/condensation particle counters (SMPS/CPC). Other online particle counters that can be used include fast mobility particle sizers (FMPS) and diffusion size classifiers (e.g., DiscMini).
6. TEER measurements indicate cell monolayer integrity and permeability. TEER values for A549 cells can be measured with values ranging between 20–60 $\Omega \cdot cm^2$ [19] and 140–180 $\Omega \cdot cm^2$ after 7 days of culture [17]. Note that TEER values for other cell lines (e.g., Calu-3) or primary lung cell models (e.g., MucilAir) that form functional tight junctions and a "tight" monolayer can be 3–10 times larger.
7. After cells are placed under ALI culture conditions, addition of liquid to the apical side can induce temporary oxidative stress.

As oxidative stress is an endpoint measured in this protocol, it is recommended to take TEER measurements after conducting the oxidative stress assay.

8. The 1-jet Collison nebulizer can be operated at pressures between 20 and 100 psig and flow rates of 2–7 L/min. According to the manufacturer, at 20 psig the droplets produced have a mass median diameter of 2 μm, and the liquid use rate is 1.5 mL/h.
9. DCFH-DA is a popular fluorescent probe for the detection of oxidative stress in cells. Since the probe can be prone to autooxidation, the use of carboxy-2′,7′-dichlorodihydrofluorescein-diacetate (carboxy-H_2DCF-DA), which is more stable, is also reported. Upon crossing the cell membrane, esterases hydrolyze DCFH-DA to DCFH, which remains trapped within cells. The oxidation of DCFH yields DCF, a fluorescent, fluorescein-based compound that can be measured using excitation/emission wavelengths of 485–495/520–530 nm.
10. The absorbance peak of resorufin is broad allowing the use of excitation/emission wavelengths in the ranges of 530–570/580–620 nm.
11. It is recommended to optimize the microplate reader settings to the positive control in order to ensure that the microplate reader's maximum readable value is not exceeded.

Acknowledgements

This work was supported by the Whitaker International Program through a postdoctoral grant to NAL, the Wagoner Foreign Study Scholarship through a summer research grant to NJL, the Institute for Work and Health in Lausanne, Switzerland, and Virginia Commonwealth University.

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