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Assessment of Ozone-Induced Lung Injury in Mice

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Abstract

Ozone is a major pollutant in the air we breathe, and elevated levels lead to significant morbidity and mortality. As the climate warms, levels of ozone are predicted to increase. Accordingly, studies to assess the mechanisms of ozone-induced lung diseases are paramount. This chapter describes mouse models of ozone exposure and methods for assessing the effects of ozone in the lungs. These include bronchoalveolar lavage, necropsy, and measurement of lung function. Lavage allows for assessment of cell infiltration, cytokine production, tissue damage and capillary leakage in the airspaces. Necropsy provides tissue for gene expression, histology, and protein assessment in the whole lung. Lung physiology is used to assess airway hyperresponsiveness, tissue and total lung resistance, compliance, and elastance.

Keywords

Ozone; Bronchoalveolar lavage; flexiVent; Lung function measurements; Inflammation

1 Introduction

Many infectious and environmental exposures induce lung injury, and many methods have been developed to assess exposure-induced lung injury in mice. In this chapter, we describe two such methods: (1) collection of bronchoalveolar lavage fluid, which can be used to assess inflammatory cell infiltration and inflammatory cytokine production, and (2) airway physiology measurements using the flexiVent apparatus. To demonstrate the utility of these assays, we also describe methods to expose mice to a key environmental toxin, ozone.

1.1 Ozone as an Important Environmental Toxin

Since the Clean Air Act of 1970, ambient ozone (O₃) has been identified as a major pollutant. Lung injury by ozone leads to airway inflammation and airway hyperresponsiveness (AHR). As a result, ozone is an important trigger of lung disease exacerbations. Well-conducted epidemiology studies suggest that in the United States, ozone leads annually to 800 deaths, 4500 hospitalizations, 900,000 school absences, and over 1 million restricted activity days at a cost of \$5 billion [1–5]. Each 10-ppb increase in 1-h daily maximum ozone levels is associated with a mortality increase in heart-lung disease patients by 0.39–0.87% [2, 4, 6, 7]. In children, 27,000 hospital admissions and 19,000 emergency room visits for asthma were attributed to ozone in 2005 [8]. The US Environmental Protection Agency (EPA) states that due to climate change, ozone levels and effects will increase substantially [9]. It is thus imperative to understand the mechanism of ozone-induced lung disease.

Mouse models of ozone-induced lung inflammation have aided us substantially in understanding the pathobiology of lung after ozone exposure. In the sections below, we describe the methods for ozone exposure; invasive measurement of airway physiology parameters in intubated, sedated, and paralyzed mice; bronchoalveolar lavage; and necropsy.

1.2 Bronchoalveolar Lavage

Bronchoalveolar lavage is widely used to study cellular and humoral components of the epithelial lining fluid in both humans and animals. Advantages of this procedure include the ability to reliably assess indices of inflammation and epithelial damage after lung injury, ease of performance, and high reproducibility. The main disadvantage is that the procedure is terminal in small rodents. Additionally, it should be noted that changes in lavage fluid do not directly reflect interstitial cellularity or cytokine expression. Lavage techniques vary considerably, and there is a paucity of studies comparing different techniques. Our unpublished observations, as well as personal communications by other investigators, suggest that euthanasia agents and lavage methodology subtly influence results. This may be important, particularly in low-grade injury models such as ozone-induced injury, described below. While performing lavage, it is crucial to avoid over- and underinflation of the lung, as both will lead to unreliable samples. Underinflation of the lungs during lavage leads to nonrepresentative sampling of the airspace. Conversely, overinflation of the lungs can lead to injury, artificial increases in cytokine levels, and emphysema-like changes in histology [10]. We believe these risks are best avoided by allowing for passive inflation of the lungs at a standard pressure equivalent to 20 cm H₂O pressure. In the interest of best utilization of tissue for studies, we provide a protocol for lavage, with subsequent snap freezing in of the right lung liquid N₂ for genomic or proteomic analyses, and paraformaldehyde fixation of the left lung for histologic assessment.

1.3 Airway Physiology Measurements

Measurement of airway physiology parameters is crucial for the assessing how environmental insults affect lung function. Several methods have been described [11], ranging from whole-body plethysmography in unrestrained animals to invasive measurement of airway pressures in anesthetized, paralyzed, and intubated animals. There are advantages and disadvantages to all methods, and the ideal approach depends on the purpose of the measurement. We have found the invasive measurement of airway pressures to be a reasonably reproducible and reliable method of obtaining relevant information on airways obstruction, airways hyperresponsiveness (AHR), and lung tissue compliance. This chapter does not intend to endorse a specific product. However, at present all airway physiology assessment methods are specific to available apparatuses. In the following we describe the use of a widely accepted and used product, fl(A. How. fl(AHR), measures respiratory mechanics by using volume and pressure measurements to assess lung function. The equipment has transducers on both the inspiratory and expiratory lines that record data as the ventilator cylinder delivers measured volume or pressure perturbations to the animal's lungs. In brief, the anesthetized animal is attached to the machine by a cannula inserted in the trachea. The animal is anesthetized, paralyzed, and ventilated, and when stable breathing is attained, the lungs are assessed using the chosen pressure or volume perturbations. Prior to a

perturbation, an airway constrictive agent (like methacholine) can be delivered through a nebulizer attached to the inspiratory lines, in order to evaluate airway responsiveness.

2 Materials

2.1 Ozone Exposure

1. Cork with hole.
2. Erlenmeyer flask, vented.
3. Humidity/temperature meter and probe.
4. 55-L Hinners-style exposure chamber [12].
5. Luer connectors.
6. Manometer.
7. Picopure distilled water.
8. Oxygen bottle (100%) with regulator and connector.
9. Stainless steel cage to house mice during exposure, custom-made.
10. Tape.
11. PVC Tubing (3/8 in. and 1/4 in.).
12. Teledyne 400A ozone analyzer (Teledyne API, San Diego, CA).
13. Ozone generator, Yanco model (Matheson Gas, Montgomeryville, PA).
14. Flow meter.

2.2 Bronchoalveolar Lavage

1. PVC tubing (1.27 mm).
2. 70% Ethyl alcohol.
3. Butterfly needles (any gauge).
4. Needles (21, 23 gauge).
5. Tuberculin syringe (if blood sampling is desirable).
6. Forceps (3" sharp-tip; 3" serrated).
7. Scissors (large, dissecting).
8. Hemostat.
9. 10 mL syringe.
10. Three-way stopcock.
11. Braided silk suture material.
12. Liquid N₂ with appropriate container.

13. Ice.
14. 4% paraformaldehyde.
15. Phosphate-buffered saline (PBS).

2.3 Airway Physiology Measurements: Equipment

1. Computer and monitor (minimum requirements: 2.8 GHz quad core, 8 GB RAM, 1 TB hard drive, Windows 7).
2. flexiVent Legacy System (Scireq respiratory equipment, Legacy System no longer available from Scireq, but newer systems are) including flexiVent base unit, flexiVent EC controller unit, flexiVent XC accessories controller, flexiVent module M1, Scireq FV-AN-A1 aerosol base, PEEP trap, or flexiVent FX with Module 1 or 2.
3. Scireq Aeroneb plug-in.
4. Scireq Aeroneb plug-in adapter cord.
5. Aeroneb pro aerosol cup.
6. PVC tubing (3/8 in.).
7. Luer connector kit (Harvard apparatus).
8. FlexiWare 7.6 software.
9. Manometer (Scireq).
10. Heating pad (42 °C).

2.4 Airway Physiology Measurements: Other Materials

1. Ethanol, 70%.
2. Methacholine (acetyl B-methacholine chloride).
3. Pancuronium bromide.
4. Saline, 0.9%.
5. Urethane.
6. Cannulae 18–20 ga.
7. Compressed air.
8. Forceps (3" sharp-tip; 3" serrated).
9. Kim wipes.
10. P200 pipette with tips.
11. Scissors (spring and dissecting).
12. Suture thread 4.0.
13. Syringes 1 cc with 26 gauge needles.

2.5 Airway Physiology Measurements: Stock Solutions

1. Anesthesia: Urethane (125 mg/mL stock solution)—add 12.5 g urethane into 100 mL ddH₂O. Store protected from light at room temperature.
2. Paralytic: Pancuronium bromide (8 mg/mL stock solution)—add 50 mg pancuronium bromide to 6.25 mL 0.9% saline and store protected from light at −4 °C. Working solution is 0.08 mg/mL, a 1:100 dilution of the stock solution.
3. Methacholine (100 mg/mL stock solution, prepared fresh each time): add 1 g of methacholine to 10 mL 0.9% saline. Working solutions are 100, 50, 25, and 12.5 mg/mL. Dose range can be changed to suit experimental setup.

3 Methods

3.1 Ozone Exposure

Ozone is a highly reactive gas, and exposure can be hazardous. Thus, it is important to avoid exposure of the operator. This is best achieved by placing the setup in a ventilated fume hood. Proper and undisturbed circulation of air in the exposure chamber is crucial in order to generate a uniform distribution of ozone throughout the chamber. We recommend placing the ozone monitoring tube in different positions within the chamber to ensure that ozone concentrations throughout the chamber are uniform.

1. Set up the ozone generator inside a fume hood using PVC tubing and Luer connectors as per Fig. 1.
2. Turn on the ozone generator and oxygen flow, which will direct 100% oxygen gas through an ultraviolet (UV) light ozone generator upstream from the exposure chamber.
3. Turn on air supply, and run it through a water source to maintain the relative humidity in the chamber (*see Note 1*).
4. Ensure the chamber is “pre-charged” to 2 ppm ozone.
5. Place mice individually in stainless steel cages and place cages in chamber (*see Note 2*).
6. Record the ozone reading, and adjust the air and ozone flow until the detector reads 2 ppm ozone (*see Note 3*).
7. Continue exposure for desired length of time (for AHR models: 3 h).

3.2 Bronchoalveolar Lavage

1. Euthanize mouse. This is best achieved pharmacologically through intraperitoneal pentobarbital injection (*see Note 4*).
2. Soak fur with 70% ethanol to ensure that it is matted and does not interfere with necropsy.

3. Perform a cross-shaped incision in the abdominal area, from the suprapubic area to the subxiphoid area, with cross snips in both directions in the mid-abdomen area. Gently push bowel loops aside and identify inferior vena cava (IVC).
4. If blood collection is desired, this can be now performed using a tuberculin (1 mL) syringe and 23-gauge needle (*see* Note 5). If no blood collection is desired, snip the IVC to decompress the vasculature.
5. Use scissors to thoroughly sever the diaphragm, aorta, and esophagus. The lungs are now collapsed, thus allowing safer handling of the thorax.
6. Use scissors to perform median sternotomy from the xiphoid process through the tip of the sternum. Carefully expand section through the skin of the neck until the mandible is reached.
7. Open the two exposed thorax flaps, and cut through the ribs on either side of the sternum, approximately midway between sternum and anterior axillary line to expose the mediastinum (heart, vessels, lungs).
8. Bluntly dissect the salivary glands and musculature anterior to the trachea using forceps.
9. Thread forceps under the trachea, bluntly expand the area under the trachea, and sever the esophagus using dissecting scissors (*see* Note 6). Visualize trachea from thyroid cartilage to carina.
10. Thread a suture (approx. 3" long) under the trachea.
11. Make small incision between two tracheal rings in the upper third of the exposed tracheal segment (*see* Note 7).
12. Cut PVC tube (approx. 1" long) to create a bevel at approximately a 45° angle to the axis of the tube. Connect this to butterfly tubing, from which you have previously removed the needle. Connect butterfly/PVC set-up to a plunger-free 10 mL syringe, which is secured vertically on a stand (Fig. 2). Flush tubing. A three-way stopcock ensures flow control (*see* Note 8).
13. Insert PVC tube bevel down into the trachea, such that the tip of the tube gently lifts the tracheal tissue and enables easy advancement into the trachea (*see* Note 9). Tie in place with a simple knot.
14. Measure the height of the PBS and paraformaldehyde fluid columns. These should be 20 cm above the trachea. Adjust if necessary.
15. Move stopcock to the "open" position to start lavaging the lungs with PBS. All lobes should inflate (*see* Note 10).
16. When the lungs have stopped inflating, disconnect butterfly tubing from 10 mL syringe/stopcock setup, and flip tubing around so that it can drain into your sampling tube. When done, pinch tubing to prevent air from entering, and reconnect to syringe/stopcock. Repeat lavage process as needed (*see* Note 11).

17. BAL sample can be used to measure: (1) cell counts using a hemacytometer, cytokines by ELISA, and tissue damage/leakage via total protein content.
18. Flush the pulmonary vasculature to remove the blood. Grasp right ventricle with serrated forceps, and gently provide traction. Insert a 21-gauge needle into the right ventricle (bevel pointing downward) (*see* Note 12). Flush with approximately 3–6 mL saline. The lungs should turn from pink-red to tan-white.
19. Grasp right lung lobes with forceps, and use hemostat to pinch the lung off at the hilum (*see* Note 13).
20. With dissecting scissors, cut right lung lobes close to the hemostat, place in cryo-resistant tubes and flash-freeze in liquid nitrogen. The flash-frozen tissue can be used to analyze gene expression, protein expression, and extracellular matrix composition.
21. Remove saline-containing butterfly tubing from PVC, and attach paraformaldehyde-containing tubing.
22. Open stopcock and allow the left lung to passively expand.
23. When the lung is fully expanded, stop flow at stopcock, and remove PVC tubing from the trachea while holding the suture threads under tension (*see* Note 14).
24. If lung histology is to be performed, sever the trachea above the suture, lift the trachea by the suture thread, and carefully dissect the left lung and attached heart from the posterior mediastinum. Place into sample container with 4% paraformaldehyde (*see* Chapter 20 for lung histologic methods).

3.3 Airway Physiology Measurement

3.3.1 Study Definition and Planning

1. Scireq provides detailed manuals and technical notes about the flexiVent on their website; these manuals can be accessed by requesting an account on their website. Read both the flexi-Vent manual and the flexiWare manual. Scireq also has a Jove Video for instruction on using the FX flexiVent [13].
2. Scireq provides premade scripts for different pulmonary assessments. These can be altered, or new scripts can be written in the Script Editor program.
3. flexiVent monitors four main types of computer-controlled pressure or volume waveforms that are described in-depth in the flexiVent manual. In brief, the primary perturbations are TLC, Snapshot, Prime waves (Quick Prime), and pressure- volume (PV) loops. TLC is total lung capacity. Snapshot is a volume-based perturbation that assesses total lung resistance and compliance. Quick Prime pressure perturbation allows for differentiation of tissue and airway contributions to lung resistance and compliance. Pressure-volume loops are perturbations that assess total lung function. The standard perturbations are listed in Table 1.

4. For airway hyperresponsiveness monitoring, Quick Prime and Snapshot with TLC should be used between methacholine doses to fully inflate the lungs.

3.3.2 Experimental Methods

1. Ensure apparatus is fully assembled (*see* Note 15).
2. For legacy model: fill PEEP trap with deionized water. Set interior tube 2–3 cm below water.
3. Weigh the experimental animal.
4. Follow program prompts for calibration.
5. Anesthetize experimental animal by injecting 1–2 g/kg urethane intraperitoneally.
6. Use a toe pinch to ensure the mouse is fully anesthetized, and secure it to the heating pad using lab tape.
7. Clean and wet fur of throat area with 70% EtOH.
8. With serrated forceps, lift the skin and make a vertical cut no lower than the clavicles up to larynx, exposing salivary glands.
9. To visualize the muscles around the trachea, bluntly separate salivary glands.
10. Using spring scissors and forceps, lift the muscle, and part it to expose the trachea (*see* Note 16).
11. Using sharp forceps, slide their tip around and under the trachea between the esophagus. Spread forceps, grasp a 2–4 in. piece of suture, and pull it under the trachea.
12. Make a horizontal incision between the second and third tracheal rings below the larynx, and insert the cannula, which is tied in place with suture thread (*see* Note 17).
13. Attach mouse to the flexiVent “Y” tubing via the cannula (*see* Note 18).
14. Turn on default ventilation.
15. Ensure that the mouse is at the same height and in a straight line from the “Y” tubing to the mouse’s lungs to ensure good ventilation and no blockage of cannula or trachea. (Fig. 3).
16. Run a single TLC (deep inhale).
17. Inject paralytic, 0.08 mg/mL pancuronium bromide intraperitoneally. This will stop the mouse from breathing on its own; breathing will now be controlled by the ventilator.
18. Observe the trace of P_{chl} until the trace is a smooth cyclic trace, thus assuring that the paralytic has taken effect (Fig. 4).

19. Activate desired script, and follow Software prompts for methacholine nebulization and measurements.
20. After the final dose, change the fluid in the nebulizer to PBS, and activate the nebulizer two times to clean the high-dose solution out of the tubing.
21. Stop the ventilation by pushing the stop ventilation button on the Task bar.
22. Remove the nebulizer from the stand and disconnect the Y tubing from the flexiVent.
23. Blow out all liquid in it with compressed air, dry, and reattach everything.
24. Assign next animal to the active site, and follow Software prompts to enter information about next experimental animal.
25. Clean out the cannula by blowing compressed air through it.
26. Reattach cannula to “Y” tubing, and repeat tube calibration.
27. Repeat process until all animals have been assayed.

3.3.3 Data Analysis

1. Review data by choosing “Review and Reporting” from welcome screen. Select an experiment to review. Data can be directly analyzed in this program; however, to enter numbers into other charting software or graphing programs, you will need to export the data, which puts it in excel format. To export data, click “Data” and then “export” from the toolbar. Export “Parameters” (the raw data) and “Dose Response” (a pre-calculated maximum dose response). Every time you export data, the software will use the same file names. To avoid problems, rename the files with the experimental identifier as soon as they are exported.
2. Determine if you want to analyze data as a specific peak point, average of three highest points or a fixed point after methacholine dose.
3. Data can also be normalized as % baseline average for each parameter if desired.
4. Data quality can be assessed by examining the coefficient of determination (COD) for each parameter read. The COD indicates how well the data fit the model. Parameter reads with COD closer to 1.00 are considered better quality.

4 Notes

1. Chamber air should be exchanged at the rate of 20 changes/h, with ~50% relative humidity and a temperature of 20–25 °C.
2. This will cause a temporary drop in ozone concentrations in the chamber. Start timing the exposure when the levels have stabilized back at 2 ppm ozone.
3. Ozone level should stabilize with a fluctuation of ± 0.05 ppm.
4. We find that pentobarbital causes much less vascular congestion and alveolar hemorrhage than euthanasia via CO₂ or cervical dislocation and is less likely to

alter results due to the presence of the blood and exudative immune cells in the lung parenchyma.

5. Larger-bore needles may lead to IVC perforation; smaller-bore needles may lead to hemolysis. We prefer IVC sampling to cardiac sampling, if possible, as this approach does not include puncturing the heart tissue and activating tissue factor.
6. The natural aperture of the forceps keeps the trachea under mild tension and allows for more precise handling.
7. Scissors can be used for this activity, but we prefer using the tip of a 19 gauge needle, which acts as a scalpel and prevents severing the trachea.
8. Placing the stopcock lever at 45° to the outlets ensures that there is no flow and no air enters the tubing.
9. Do not advance beyond the carina, as this is likely to sever the airway.
10. If a lung lobe is not inflating, try repositioning the PVC tube (usually pulling back will resolve this issue). Note that PBS may interfere with some assays such as flow sorting of cells. Some investigators prefer to use other solutions such as Hanks' Balanced Salt Solution or add 0.5% EDTA or 2% BSA or FBS to their PBS for this reason.
11. Usually three repetitions ensure a return of approximately 2–2.5 mL (for a 20–25 g mouse). If a more concentrated lavage fluid is desired, instead of draining to collection tube after the first pass, use tuberculin syringe to repeatedly draw and slowly re-instill fluid.
12. Flushing ensures that there are no artifacts from pooled blood elements in the pulmonary vasculature.
13. Some operators tie off the lung at the hilum to ensure there is no leakage of fixative, but this is not absolutely necessary. In our experience, the hemostat crushes the tissue such that the hilum remains collapsed and does not leak for inflation pressures of 20 cm H₂O pressure.
14. Thus, when PVC tube is removed, the suture knot ties the trachea off.
15. There are certain software-dependent steps required for data entry and storage. These are likely to change with Software upgrades and thus are not described here.
16. Do not cut too far down around the edges of the muscle around trachea because there is risk of cutting the carotid artery.
17. Ensure the cannula is placed so it only reaches the last tracheal ring.
18. Cannulae for flexiVent are available in several sizes ranging from 18 to 22 gauge. They also come ridged or smooth. We recommend obtaining endotracheal tube, 1.0 × 20 mm from Harvard apparatus since it has ridges for the suture to lock the cannula in place and reference lines to ensure proper placement.

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References

1. Dockery DW, Pope CA 3rd, Xu X, Spengler JD, Ware JH, Fay ME, Ferris BG Jr, Speizer FE (1993) An association between air pollution and mortality in six U.S. cities. *N Engl J Med* 329(24):1753–1759. 10.1056/NEJM199312093292401 [PubMed: 8179653]
2. Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F (2004) Ozone and short-term mortality in 95 US urban communities, 1987–2000. *JAMA* 292(19):2372–2378. 10.1001/jama.292.19.2372 [PubMed: 15547165]
3. Gryparis A, Forsberg B, Katsouyanni K, Analitis A, Touloumi G, Schwartz J, Samoli E, Medina S, Anderson HR, Niciu EM, Wichmann HE, Kriz B, Kosnik M, Skorkovsky J, Vonk JM, Dortbudak Z (2004) Acute effects of ozone on mortality from the “air pollution and health: a European approach” project. *Am J Respir Crit Care Med* 170(10):1080–1087. 10.1164/rccm.200403-333OC [PubMed: 15282198]
4. Katsouyanni K, Zmirou D, Spix C, Sunyer J, Schouten JP, Ponka A, Anderson HR, Le Moullec Y, Wojtyniak B, Vigotti MA et al. (1995) Short-term effects of air pollution on health: a European approach using epidemiological time-series data. The APHEA project: background, objectives, design. *Eur Respir J* 8(6):1030–1038 [PubMed: 7589368]
5. Hubbell BJ, Hallberg A, McCubbin DR, Post E (2005) Health-related benefits of attaining the 8-hr ozone standard. *Environ Health Perspect* 113(1):73–82 [PubMed: 15626651]
6. Ito K, De Leon SF, Lippmann M (2005) Associations between ozone and daily mortality: analysis and meta-analysis. *Epidemiology* 16(4):446–457 [PubMed: 15951662]
7. Levy JI, Chemerynski SM, Sarnat JA (2005) Ozone exposure and mortality: an empiric bayes metaregression analysis. *Epidemiology* 16(4):458–468 [PubMed: 15951663]
8. Fann N, Lamson AD, Anenberg SC, Wesson K, Risley D, Hubbell BJ (2012) Estimating the national public health burden associated with exposure to ambient PM_{2.5} and ozone. *Risk Anal* 32(1):81–95. 10.1111/j.1539-6924.2011.01630.x [PubMed: 21627672]
9. Grambsch A, Hemming BL, CP. Weaver Gilliland A, Grano D, Hunt S, Johnson T, Loughlin D, Winner D (2009) Assessment of the Impacts of Global Change on Regional US Air Quality: a synthesis of climate change impacts on ground-level ozone. www.epa.gov. 2016
10. Cilley RE, Wang JY, Coran AG (1993) Lung injury produced by moderate lung overinflation in rats. *J Pediatr Surg* 28(3):488–493. discussion 494–485 [PubMed: 8468667]
11. Hoymann HG (2007) Invasive and noninvasive lung function measurements in rodents. *J Pharmacol Toxicol Methods* 55(1):16–26. 10.1016/j.vascn.2006.04.006 [PubMed: 16793289]
12. Hinnert RG, Burkart JK, Punte CL (1968) Animal inhalation exposure chambers. *Arch Environ Health* 16(2):194–206 [PubMed: 4171547]
13. McGovern TK, Robichaud A, Fereydoonzad L, Schuessler TF, Martin JG (2013) Evaluation of respiratory system mechanics in mice using the forced oscillation technique. <https://www.jove.com/video/50172/evaluation-respiratory-system-mechanics-mice-using-forced-oscillation>. Accessed 24 July 2017

Fig. 1.

Ozone exposure setup. 100% oxygen is conducted through PVC tubing into the ozone generator, which contains a UV bulb. Ozone is then routed through PVC tubing into the exposure chamber in a mixed stream of air that has been humidified. Humidification can be simply achieved by bubbling the air (not the ozone!) through purified water. Humidification to a reading of approximately 50–60% should be targeted. The ozone level is sampled in the chamber by the ozone detector, which has its exhaust port attached to a carbon filter and vented to the room

Fig. 2. Mouse lung lavage setup. 10 mL syringes are held upright and connected to the cannula via a three-way stopcock (shown in locked position)

Fig. 3.
Mouse attached to the ventilator. The trachea is cannulated and in proper alignment with
“Y” tubing

Fig. 4.

Real-time data trace in flexiWare software. **(a)** The Pchl trace shows pressure fluctuations due to the incompletely paralyzed mouse breathing against the ventilator (arrows). **(b)** Pchl trace of a fully anesthetized and paralyzed mouse. Note the smooth, straight pressure curve rise

Table 1

Standard perturbations on flexiVent

Name	Purpose	Description
TLC	Open airspaces Standardize volume	Deep inflation of lungs to a pressure of 30 cm H ₂ O followed by a breath hold of a few seconds
Snapshot	Resistance and compliance data	Single frequency, sinusoidal forced oscillation waveform matched to respiratory rate
Quick Prime	Measure input impedance Distinguish between airways and tissues	Broadband (multifrequency) forced oscillation waveforms for fixed duration
PV loop	Assess nonlinearities in PV loops Measure quasi-static compliance	Slow stepwise or continuous inflation to total lung compliance (TLC) and deflation back to functional residual capacity (FRC), controlling either volume or pressure