Published in final edited form as:

Methods Mol Biol. 2014 ; 1121: 205–221. doi:10.1007/978-1-4614-9632-8_18.

Electroporation-mediated Delivery of Genes in Rodent Models of Lung Contusion

David Machado-Aranda, MD and Krishnan Raghavendran, MBBS

Division of Acute Care Surgery, Department of Surgery, University of Michigan, Ann Arbor, MI, 48109

Abstract

Several of the biological processes involved in the pathogenesis of acute lung injury and acute respiratory distress syndrome after lung contusion are regulated at a genetic and epigenetic level. Thus, strategies to manipulate gene expression in this context are highly desirable not only to elucidate the mechanisms involved but also to look for potential therapies. In the present chapter, we describe mouse and rat models of inducing blunt thoracic injury followed by electroporation-mediated gene delivery to the lung. Electroporation is a highly efficient and easily reproducible technique that allows circumvention of several of lung gene delivery challenges and safety issues present with other forms of lung gene therapy.

Keywords

Lung contusion; Acute lung injury; Acute respiratory distress syndrome; Electroporation

1 Introduction

Blunt chest trauma-induced lung contusion (LC) is a major public health problem, as it remains a primary cause of mortality following blunt chest trauma and blast explosions [1–3]. It constitutes an independent risk factor for the development of acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and ventilator-associated pneumonia. A significant gap still exists in our knowledge of the factors that are responsible for deterioration of 25–30 % of patients with LC into severe respiratory failure such as seen with ALI/ARDS. Despite several decades of intense research, most of the current clinical care still remains supportive, and no specific molecular targets for the treatment of LC exists or therapy has been developed [4–11].

Despite philosophical criticisms in the use of animal models of experimentation as surrogates of human diseases, we have developed an easily reproducible model of mouse and rat LC that recapitulates all the key pathological features that are observed during clinical practice [12–15]. These include the disruption of the alveolar epithelial and endothelial barrier with subsequent permeability injury, increased work of breathing, and

ventilation/perfusion mismatch, which causes severe hypoxemia. Simultaneously, a robust inflammatory response is also seen and is characterized by translocation and accumulation of neutrophils in the lung interstitium and alveolar spaces [16–18]. There is an increasing body of evidence that suggest that activation of neutrophils and the production of different inflammatory mediators will amplify and perpetuate the original insult and if left unchecked will lead to ALI/ARDS. Some of these events are determined by genetic and epigenetic regulation, and therefore, gene therapy could be an ideal modality of study and treatment [11, 12, 19–21] (Fig. 1).

Transfer of genes to the lung has considerable challenges as this tissue is designed to be naturally resistant to most forms of gene transduction. This property has been problematic for some methods of gene therapy. Additionally, inflammation and disease also impact gene transfer efficiency and side effects associated with certain forms of gene transduction, which could limit their use in LC and ALI/ARDS. Others and we have found that electroporation-mediated gene transfer is a highly efficient and reproducible method of gene delivery that could be used safely in the context of models of LC and lipopolysaccharide-induced lung injury [12, 22–24]. The following chapter describes the mouse and rat models of lung contusion and subsequent electroporation.

2 Materials

2.1 Plasmid DNA and DNA Delivery

1. Plasmid DNA is propagated in *Escherichia coli* and purified using Qiagen Gigaprep kits as described by the manufacturer.
2. These plasmids contain a pUC-19 backbone with CMV immediate-early promoter/enhancer (CMV_{iep}) driving expression of gene of interest. We have used the α_2 and β_1 subunits of the Na⁺/K⁺-ATPase as therapeutic genes in our models of LC.
3. An SV₄₀ DNA targeting sequence (DTS) is placed behind the open reading frame (ORF) to allow ubiquitous cell expression (*see Note 1*).
4. Plasmids are stored at 10 mM Tris–HCl and 1 mM EDTA pH 8.0 then diluted in phosphate-buffered saline (PBS) to their desired concentrations.
5. Doses between 50 and 200 µg for mice and 100 and 500 µg of plasmid DNA for rats are ideal, and concentrations below 1 mg/mL have adequate viscosity to allow excellent distribution in both lungs.
6. 1 mL non-Luer lock syringes containing DNA. Multiple doses can be administered with one filled syringe in mice, while a rat will use one complete syringe.
7. Delivery in mice requires different size needles (25 ga, 27 ga, 31 ga) for direct DNA injection into the trachea.

¹Modifications of the promoter sequences and DTS can produce important advantages in terms of duration of expression and cell specificity. As an example, long-term expression can be achieved by substituting the CMV_{iep} with the ubiquitin A promoter. Predominant alveolar type II (AT-II) cell expression can be achieved by exchanging the SV-40 sequence to small regions along the surfactant protein C (SPC) promoter.

8. Delivery in rats occurs via oral tracheal intubation using 1.25-inch 14–16-ga Angiocatheter™ (Becton Dickinson, Franklin Lakes, NJ).
9. Small-animal laryngoscope handle or equivalent (pediatric-neonatal) with Macintosh blades 0, 00, or 000 can be used for rat intubation.
10. Spring-Wire Guide™ guide wire (Arrow International Inc., Reading, PA) is used to facilitate intubation by catheter over wire technique.
11. A long (1.5–2-inch) 16-ga Angiocatheter™ (Becton Dickinson, Franklin Lakes, NJ) attached to mid-barrel cut 5 mL syringe with an incorporated Pasteur pipette bulb is used to aspirate and clear secretions from the oropharynx.
12. Telescopic, flexible fiber-optic light source connected to high-power lamp is essential for illuminating targets (neck in mice, oropharynx in rats).

2.2 Animals, Anesthesia, and Survival Surgery Materials

1. Mice of any gender, between 18 and 30 g, wild type as well as transgenic can be used.
2. Adult male rats between 250 and 300 g from different strains can be used (Long-Evans, Sprague–Dawley).
3. Appropriate anesthetic method should be used to ensure adequate depth of anesthesia during the creation of LC injury and post-injury analgesia should be provided after recovery.
4. For mice, we use intraperitoneal injection of ketamine/xylazine, which provides sufficient anesthesia length for both LC and post-contusion electroporation at the same setting (*see Note 2*).
5. For rats, we have successfully used inhaled agents such as halothane and isoflurane or intraperitoneal injection of pentobarbital.
6. Warming pads and warming lights are recommended for perioperative recovery.
7. High-flow oxygen should be available using chamber or nose cone delivery.
8. Minor surgical equipment (sharp Iris scissors, fine point angle tweezers, ringed needle holder).
9. 3-O Vicryl (Ethicon™) or any other absorbable suture for neck closure.
10. Over-the-counter Betadine solution for neck antisepsis.
11. Over-the-counter bacitracin ointment for neck wound dressing.

2.3 Contusion Devices

1. For mice, we have adopted the use of an Electrical Cortical Contusion Impactor® (Custom Design and Fabrication, Richmond, VA) of ample utilization and

²Inhaled and nose cone anesthesia using halothane and isoflurane can also be used if only LC is to be performed and electroporation to be done at a later date.

validation for neurotrauma studies [25–27] (Fig. 2). Different impactor heads can be exchanged to create a smaller or larger area of unilateral injury. Piston velocity is set at 5.8 m/s and adjusted to a depth of 10 mm. This allows a consistent lung contusion usually located in the right upper and middle lobes of the mouse lungs (see **Note 3** and Fig. 3). Energy released on impact is 1.682 J based on the equation

$$E = \frac{1}{2} mV^2.$$

2. For rats, a simple custom-made contusion apparatus is constructed and consists in a metal hollow tube with a sand-filled aluminum falling weight, impacting onto a precordial shield (Fig. 4).
3. The energy equivalent of 2.45 J by a hollow cylindrical weight dropped can be defined by the height and mass of impact onto a precordial shield that minimized associated cardiac trauma. The impact energy E (in Joules, J) of the falling weight was calculated from the equation:

$$E = mgh$$

In this equation, m is the mass of the aluminum weight (in kilograms), g is gravitational acceleration (9.8 m/s), and h is the height of weight above the Lexan[®] platform (in meters). Calculations assumed that all the potential energy of the weight was transferred to the animal, neglecting frictional dissipation. The heights for the hollow cylindrical weight above the chest were calculated to generate external chest impact energies of 2.2–2.45 J.

4. The path of the falling weight is directed by the cylindrical tube and Teflon guides on the shield. A key feature of the model is the precordial protective shield (Plexiglas), which is attached to the undersurface of the Lexan[®] platform and in direct contact with the chest. This shield protects the heart from contusion, directing the impact energy to the lateral aspects of the chest (Fig. 5).

2.4 Electroporator Generator, Electrodes, and Electroporation Settings

1. A BTX ECM 830 square wave pulse generator (Harvard Apparatus, Cambridge, MA) is used and set at 200 V/cm per impulse.
2. A total of eight square wave pulses of 5–10 ms of duration separated by 1 s are used during mouse electroporation.
3. Different electrodes have been used to vary the form and surface of the electrical field. Dean et al. [22] originally described the use of a 10-mm round Tweezertrode (Genetronics, San Diego, CA). Modifications of this protocol using larger electrodes as shown in Fig. 6 achieve better results.

³This position and target of impact is extremely critical as if it is too high it may cause traumatic transection of the axillary artery and if it is too low it may provoke hemoperitoneum with liver fracture. It is important to limit the number of animals to less than ten animals per setting as the device contains electromechanical parts that may heat up, decreasing the velocity of firing of the piston and varying the energy transmitted to the right chest and thus the degree of contusion.

4. *In mice*, soft transcutaneous electrical nerve stimulation (TENS) electrodes are cut to size and ensure larger contact surface. Hair clipping is unnecessary; water-based electroconductive gels (i.e., K-Y jelly—Johnson and Johnson, Cincinnati, OH) are used before application of electrodes to the chest will mat hairs down and ensure conductance (*see Note 4*). *In rats*, pediatric cutaneous pacemaker pads (Quik-Combo RTS; Medtronic Physio-Control Corporation, Redmond, WA) are cut to 3 × 4 cm squares and placed on either side of the chest under the forelimbs. Because their coat is coarser, small square areas should be clipped prior to electroporation.
5. A 250–300-g animal will have a chest cross section of approximately 3–4 cm.
6. Adjusting electrical field strength to 200 V/cm will automatically use the high-voltage (HV) capacitors and will only allow a time pulse between 10 and 600 microseconds (μs).
7. We have successfully used different pulse lengths (10, 50, 100, and 500 μs) (*see Note 5*).
8. A small amount of K-Y jelly (Johnson and Johnson, Cincinnati, OH) is placed on the skin, and the electrodes are held in place gently using surgical tape (*see Note 6*).

2.5 Measurements of Inflammatory Attributes

2.5.1 Pressure–Volume Curves

1. Different size blunt tip metallic cannulas 18–20 ga for mice and 14–16 ga for rats.
2. 3-O Silk sutures for fixation and tying cannula to the trachea.
3. Minor surgical set as described above.
4. A flexiVent™(SCIREQ®, Montreal, Canada) ventilator and data capture, with species-specific graphite piston modules and positive end-expiratory pressure (PEEP) water trap (*see Note 7*).
5. Tilt table animal platforms and soft tape restraints.

2.5.2 Mouse and Rat Bronchial Alveolar Lavage (BAL) and Cell Counts

1. Ice-cold phosphate-buffered saline.
2. Swinging bucket or tabletop centrifuge with refrigeration.
3. RBC hypotonic lysis buffer.

⁴It is important not to use alcohol-based conductive gels as these may pose a fire hazard and cause animal hypothermia with consequent injury and death.

⁵We have noticed that the longer the pulse length is, the more marked is the period of post-electroporation apnea. In essence, oxygen supplementation and ventilatory support after electroporation may be necessary to decrease the periprocedural mortality.

⁶However, operator may have to gently hold and push the electrodes in place as they do tend to slip, thus not allowing to create a parallel electrical field. As long as you are pushing the electrode from the exterior, you will not receive any electrical shock.

⁷The experimental algorithms should be written within the ventilator software and provided by the vendor. These most likely will follow previous published and validated protocols. Highly recommended is the in-service training provided by the selling company. It should include also on how to write controller software.

4. Trypan Blue vital dye.
5. 1.5–2-mL Eppendorf tubes and 15-mL conical tubes.
6. Countess automatic cell counter.
7. Cytospin II centrifuge, rotor, and slides.
8. Diff-Quik stain.
9. Bright-field microscope, with immersion lenses.

2.5.3 ELISA Determination of Albumin Concentration in BAL

1. Capture antibody is a polyclonal rabbit anti-mouse albumin (used both in mice and rat experiments).
2. Secondary antibody is a horseradish peroxidase-labeled goat anti-rabbit IgG.
3. Reactions are run in a polycarbonate 96-well microtiter plates.
4. ELISA Coating Buffer = 0.05 M carbonate–bicarbonate pH 9.6.
5. ELISA Wash Solution = 50 mM Tris–HCl, 0.14 M NaCl, 0.05 % Tween-20, pH 8.0.
6. ELISA Blocking Solution = 50 mM Tris–HCl, 0.14 M NaCl, 1 % bovine serum albumin, 0.05 % Tween-20, pH 8.0.
7. TMB ELISA peroxidase substrate.
8. ELISA Stop Solution, 0.18 M H₂SO₄.

2.5.4 Histopathology

1. 16-ga blunt needle tip.
2. Minor surgical procedure kit.
3. 3-O Silk suture ties.
4. 100 mm Petri dish.
5. Ice-cold phosphate-buffered saline.
6. 20-ga needle attached to 5-mL syringe.
7. Vertical stand with clamp holder sustaining a 20-mL syringe with a three-way stopcock.
8. 10 % Formalin, neutral buffered (*see Note 8*).
9. 0.14 M NaCl.

⁸An optional solution is 4 % paraformaldehyde (4 % PFA). Both fixatives are known to interact with lung tissue giving off auto-fluorescence, which may be problematic if immunohistochemistry experiments are to follow. Thus frozen sections may be a better alternative.

10. 1:1 admixture Tissue-Tek® Optimal Cutting Temperature (O.C.T.) and 30 % sucrose for frozen sections.

3 Methods

3.1 Mouse Unilateral Lung Contusion (LC)

1. Make sure several cages are available to separate injured from uninjured animals.
2. Anesthesia is administered; adequacy of depth is confirmed by toe pinch and corneal reflex methods.
3. Mice are positioned on a left lateral decubitus (left side down) on a movable platform. A plastic 50-mL conical tube can be cut, and limbs can be positioned and fixed with soft tape to ensure adequate positioning and exposed the left chest.
4. Target of impact is located along the posterior axillary line at 1.3 cm cephalad to the border of the rib cage, hair should be clipped, and targeted should be marked.
5. Subject is placed under the Electrical Cortical Contusion Impactor® (Custom Design and Fabrication, Richmond, VA).
6. Piston is manually lowered with a wheel lever to make contact with the right chest, and the set target depth and piston velocity are selected.
7. Press trigger button to arm piston and press again to fire piston against the chest.
8. Following contusion, mice are placed in prone position on a warming pad for recovery. If immediate electroporation is to follow, mice are placed in supine position on the tilt table to access the neck for gene delivery.

3.2 Bilateral Rat Lung Contusion

1. Animals are pre-oxygenated in an O₂ chamber for several minutes prior to inducing contusion.
2. Anesthesia is induced, confirming adequate depth.
3. The subject animal is placed in supine position with limbs fixed with soft tape underneath contusion apparatus.
4. The Lexan® shield is lowered and placed in contact with the chest, and weight is allowed to drop by gravity impacting the shield.
5. The rat is rapidly retrieved from the apparatus and placed in prone recovery position with frequent manual stimulation.

3.3 Mouse Electroporation

1. After adequate anesthesia and immediately following LC injury, mice are placed in supine position on an angled table with limbs and tail extension taped with soft tape. Maxilla is extended using silk suture across the hard palate creating gentle neck extension.
2. Skin along the neck is prepared with Betadine solution.

3. A 10-mm midline incision at skin level is performed in the cervical area followed by blunt dissection along the midline raphe separating salivary glands and strap muscles and exposing the tracheal rings.
4. Next, the angle of animal table is elevated almost to a vertical position. This maneuver helps with the delivery of the liquid bolus.
5. Using a small-gauge needle, DNA is delivered by steady injection, selecting a point of entry between the third and forth tracheal ring.
6. Bolus should be large enough that with its specific weight combined with the inspiratory effort will disperse it uniformly into the lungs and not be coughed. A 50 μ L bolus is ideal.
7. Several breaths are allowed during which the midline cervical incision can be closed with single separate absorbable suture.
8. A nose cone with high-flow oxygen can be used to improve the survival of the animals during electroporation.
9. Electrodes are applied under each forelimb, and generator is activated to deliver square wave impulses.
10. Animal is then retrieved from the surgical table and placed on a warming mat for full recovery.
11. Before their return to vivarium, antibiotic ointment is applied to the cervical incision.
12. If using disposable TENS electrodes, these can be reused up to 15 times during the experiment, after which some wear and tear can be noted, especially in the active electrode.
13. By convention, we apply same direction of current in all experiments (“red to right”).

3.4 Rat Electroporation

1. Right after contusion, rat is placed in supine position with neck extension on a tilting table that will be used initially in a complete horizontal position for intubation followed by 45° incline for DNA delivery.
2. Maxilla is loosely fixed by silk suture against the flat surface of the table, while all limbs and tail are secured gently with tape.
3. Using fiber-optic lighting illuminates the head and neck area.
4. With the small animal/pediatric handle laryngoscope and Macintosh neonatal blade, lift the mandible and tongue in an anterior fashion.
5. Using a small Q-tip or bulb syringe with Angiocath tip aspirates excess saliva that has accumulated in the posterior oropharynx.

6. The larynx and moving vocal cords can be visualized at this point. Light may be used to shine the anterior cervical region to increase the contrast of the glottis opening and surrounding soft tissue.
7. The soft end of the 0.025-inch Spring-Wire guide is advanced in-between vocal cords using the maxillary incisors to act as a fulcrum.
8. The animal may cough reflexively; however, if appropriately sedated, it should not encounter any respiratory distress.
9. The 16-ga Angiocatheter is advanced over the guide wire and slid very gently through the vocal cords.
10. Once the catheter is in place, the guide wire is removed, and the end of the tube is occluded to confirm placement.
11. Blocking the tube will provoke vigorous activation of inspiration effort. The release of the occlusion will lead to several large inspirations followed by regular respiratory efforts.
12. Allow the animal to recuperate for 1 min, time during which supplemental oxygen can be delivered via nose cone or whole animal chamber.
13. Tilt the table to a 45° angle to have gravity assist DNA distribution.
14. Place pre-cut soft electrodes underneath the forelimbs, making sure that they are parallel and will provide a transthoracic field.
15. Taking advantage of airway blocking reflex occludes the end of the catheter with a syringe pre-filled with a 100–500 µL bolus of DNA plasmid.
16. Inject while the animal is forcefully trying to inhale air.
17. After DNA is delivered, inject several times of 500 µL of air to clear the tube and allow return to regular respirations.
18. Activate the generator and deliver EP pulses.
19. Provide ventilation support if longer pulse lengths have been used and there is evidence of apnea. This can stop once evidence of spontaneous breathing has returned.
20. Allow animal to recover in a warm environment. We remove the airway catheter once the animal is fully recuperated and moving.

3.5 Pressure–Volume Curves

1. At specific time points, euthanized the mouse or rat with anesthetic overdose followed by transection of abdominal great vessels to ensure exsanguination and death.
2. During the surgical exposure of the trachea, be careful not to enter the chest cavity as this will create a pneumothorax and will change lung compliance, modifying drastically measured pressures and volumes.

3. Have the subject placed in supine position with only the maxilla fixed against the surgical table. Leave limbs and tail loose.
4. Expose the trachea using a midline cervical incision.
5. Insert a metal cannula in between the third and fourth tracheal ring with the tip ending right at the sternal notch or at the level of the collarbones. Secure cannula tightly with a 3-O silk suture around the trachea.
6. Connect the animal to the ventilator circuit provided by the corresponding animal module on the SCIREQ[®] flexiVent[™] and start preset ventilation.
7. For mice, we use the following postmortem ventilation settings: tidal volume (TV) of 10 mL/Kg, respiratory rate (RR) of 150 breaths per minute, and PEEP of 2 cm H₂O.
8. For rats, we use the following postmortem ventilation settings: tidal volume (TV) of 6 mL/Kg, respiratory rate (RR) of 60 breaths per minute, and PEEP of 2 cm H₂O.
9. Before data capture and execution of experimental algorithms, animal lung volumes are “normalized” once by performing a 6-s inspiratory hold of 0.6 mL for mice and 3 mL for rats at a pressure of 30 cm H₂O.
10. The SCIREQ[®] flexiVent[™] captures force oscillation measurements using a computer-controlled graphite piston which varies the circuit pressure and volume displacement. Proprietary software drives a fully automated pressure–volume respiratory maneuver to create pressure–volume curves (PV curves) and calculate the maximal vital capacity at 30 cm H₂O of inspiratory pressure (TLC-30) both measures of lung compliance.

3.6 Bronchial Alveolar Lavage Fluid and Cell Counts

1. *For mice*, after lung mechanics measurements, using the previously placed metallic cannula, inject 1 mL of ice-cold PBS using a 1-mL syringe. *For rat* BAL recollection, use a 5-mL instillation of PBS instead in suitable syringes and single conicals for each animal.
2. Without detaching the syringe, gently aspirate the fluid (bronchial alveolar lavage —BAL).
3. Depending on the severity of inflammation, it should come mildly pink in color with foaming coat.
4. Place BAL fluid into an appropriate Eppendorf tube on ice while all other animals are being harvested.
5. Centrifuge all samples at $1,500 \times g$ for 3 min at 4 °C.
6. A resulting cell pellet should form at the bottom of the tube. Naïve animals will have a very small pellet with almost no red blood cells, whereas acutely injured animals will have large hemorrhagic pellets that can be easily disturbed.

7. Try to separate by decantation or aspiration the supernatant and store at -80°C for further biochemical analysis.
8. Resuspend the cell pellet using 250 μL of PBS.
9. Dilute a 50 μL aliquot 1:1 into RBC hypotonic lysis buffer followed by vital staining using Trypan Blue at 1:10 dilution.
10. Cells and live/dead cells are counted manually using a hemocytometer or using a Countess automatic cell counter. However, cell counts may require serial dilutions from severely injured animals, as cells may be too many to count.
11. The rest of cells are spun $400 \times g$ at 4°C for 5 min onto Cytospin II slides.
12. Blots on slides are stained using commercially available modified Giemsa Diff-Quik stain (Dade Behring Inc., Newark, DE).
13. Morphology is determined, and differential formula count is performed at high-power fields (HPF) to assess for the number of macrophages and neutrophils present in BAL.

3.7 Determination of Albumin Concentration in BAL

1. Albumin concentrations serve as surrogates of permeability injury in models of lung contusion.
2. Albumin concentrations in BAL are best measured using enzyme-linked immunoabsorbent assay (ELISA) on 96-well plates prepared a day prior of the experiment precoated with 100 μL of polyclonal rabbit anti-mouse albumin antibody (this method serves for both mouse and rat albumin detection).
3. Incubate these plates overnight at 4°C with the capture antibody.
4. On the day of experiment, remove capture antibody and wash ($\times 5$) with ELISA Wash Buffer and air-dry at room temperature under a hood.
5. Apply 200 μL of ELISA Blocking Solution for 30 min at room temperature.
6. Aspirate blocking solution and dispense 100 μL per well of undiluted BAL samples, curve of standards, and controls. Perform by duplicate or triplicate.
7. Aspirate and wash each well ($\times 5$) times with ELISA Wash Buffer.
8. Dispense 100 μL of secondary HRP-labeled goat anti-rabbit IgG in each well and incubate at room temperature for 1 h.
9. Aspirate and wash each well ($\times 5$) times with ELISA Wash Buffer.
10. Dispense 100 μL of TMB ELISA peroxidase substrate and develop in dark room for 15–30 min at room temperature.
11. Stop peroxidase reaction by adding 100 μL ELISA Stop Solution without removing substrate that should be showing colorimetric change.

12. Known concentrations of mouse or rat albumin prepared from dry powder can be used as control and diluted in ELISA Blocking Solution.
13. At times, the severity of injury may require the use of dilutions so that measurements may stay within the curve of standards.

3.8 Cytokine and Chemokine Assays

1. Injury-related variations of distinct cytokines can be measured from samples of BAL supernatant fluid.
2. Cytokines can also be measured from lung tissue lysates and serum; however, cytokine values obtained from these tissues are highly inconsistent and not necessarily correlated with the degree of regional inflammation seen in the lung. These differences are most likely related to problems during tissue collection, handling, homogenization, extraction, and collection.
3. We have used commercially available ELISA cytokine kits both for mice and rats following manufacturers' instructions using undiluted BAL samples.

3.9 Histopathology

1. A separate group of treated mice are dedicated for histological analysis for each specific time point, as BAL collection and ventilation may alter histology in these lungs.
2. At time of harvest, euthanasia is achieved in subjects as previously described.
3. The trachea is exposed surgically and cannulated between the second and third tracheal ring with a 16-ga blunt needle tip secured snugly with 3-0 Silk ties.
4. The chest cavity is open by midline sternotomy. The diaphragms on each side are also cut open to expose the entire content of the chest.
5. The heart and lungs are removed en bloc by dividing the trachea proximally using the Iris scissors and dissecting en bloc the soft tissue off the posterior chest by directing the scissor tips towards the spine in a caudal direction.
6. Special attention is performed not to puncture the pulmonary tissue or injured the trachea.
7. Once separated from the rest of the body, the heart and lungs are placed in a Petri dish, and the right heart ventricle is identified (usually is a thin walled and darker, half is located at the tip of the heart).
8. Using a 20-gauge needle, the right ventricle is punctured, and 5 mL of ice-cold PBS is injected to flush the lung circulation free of blood. If performed optimally, a change in coloration should be seen (lungs turn from pink to white).
9. Attach the needle to the three-way stopcock located on the vertical hanging stand.
10. Allow 10 % formalin to flow into the lung by gravity (*see Note 9*).

11. Once the fluid starts to overflow from the needle hub, place a second tie underneath the cannula end, and the metallic cannula should be slid out and can be reused for the next animal after several washes with saline.
12. Drop tissue into the sufficient fixative (ten times the approximate volume of lung tissue ~10–15 mL) and store overnight at 4 °C prior to further processing and embedding.
13. Alternatively, tissues may be fixed using 4 % paraformaldehyde.
14. In case of frozen sections, use 1:1 admixture of O.C.T. embedding media with 30 % sucrose, prepared the night before, and store at 4 °C to allow bubbles to be removed from media. Solution is introduced in a similar fashion as above. Further cryoembedding is achieved by placing O.C.T.–sucrose-inflated lungs in O.C.T.-filled cryomold or alternatively 15/50 mL conicals. Perform solidification by placing molds sequentially in a –20 °C unit for 1 h followed by storage at –80 °C allowing bubbles to slowly rise to the surface and not be trapped within the media as this may cause problems during cryosectioning.

References

1. Kollmorgen DR, Murray KA, Sullivan JJ, Mone MC, Barton RG. Predictors of mortality in pulmonary contusion. *Am J Surg.* 1994; 168:659–663. [PubMed: 7978014]
2. Miller PR, Croce MA, Kilgo PD, Scott J, Fabian TC. Acute respiratory distress syndrome in blunt trauma: identification of independent risk factors. *Am Surg.* 2002; 68:845–850. [PubMed: 12412708]
3. Wu J, Sheng L, Ma Y, et al. The analysis of risk factors of impacting mortality rate in severe multiple trauma patients with posttraumatic acute respiratory distress syndrome. *Am J Emerg Med.* 2008; 26:419–424. [PubMed: 18410809]
4. ARDS_Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The acute respiratory distress syndrome network. *N Engl J Med.* 2000; 342:1301–1308. [PubMed: 10793162]
5. Network ARDS. Ketoconazole for early treatment of acute lung injury and acute respiratory distress syndrome: a randomized controlled trial. The ards network. *Jama.* 2000; 283:1995–2002. [PubMed: 10789668]
6. Blank R, Napolitano LM. Epidemiology of ards and ali. *Crit Care Clin.* 2011; 27:439–458. [PubMed: 21742210]
7. Bone RC, Fisher CJ Jr, Clemmer TP, Slotman GJ, Metz CA. Early methylprednisolone treatment for septic syndrome and the adult respiratory distress syndrome. *Chest.* 1987; 92:1032–1036. [PubMed: 3315478]
8. Brower RG, Lanken PN, MacIntyre N, et al. Higher versus lower positive endexpiratory pressures in patients with the acute respiratory distress syndrome. *N Engl J Med.* 2004; 351:327–336. [PubMed: 15269312]
9. Gallagher DC, Parikh SM, Balonov K, et al. Circulating angiopoietin 2 correlates with mortality in a surgical population with acute lung injury/adult respiratory distress syndrome. *Shock.* 2008; 29:656–661. [PubMed: 18091573]
10. Matthay MA, Brower RG, Carson S, et al. Randomized, placebo-controlled clinical trial of an aerosolized beta-agonist for treatment of acute lung injury. *Am J Respir Crit Care Med.* 2011; 184:561–568. [PubMed: 21562125]

⁹Injecting by hand may cause over or under distension of the lung, thus obtaining inconsistent results.

11. Matthay MA, Zimmerman GA, Esmon C, et al. Future research directions in acute lung injury: summary of a national heart, lung, and blood institute working group. *Am J Respir Crit Care Med*. 2003; 167:1027–1035. [PubMed: 12663342]
12. Machado-Aranda DA, Suresh MV, Yu B, Raghavendran K. Electroporation-mediated in vivo gene delivery of the na⁺/k⁺-atpase pump reduced lung injury in a mouse model of lung contusion. *J Trauma Acute Care Surg*. 2012; 72:32–39. [PubMed: 22310113]
13. Raghavendran K, Davidson BA, Helinski JD, et al. A rat model for isolated bilateral lung contusion from blunt chest trauma. *Anesth Analg*. 2005; 101:1482–1489. [PubMed: 16244015]
14. Raghavendran KDB, Helinski JD, Marschke CM, Woytash JA, Notter RH, Knight PR. A new rat model for isolated bilateral lung contusion-reversal of hypoxia by 24 hours. *Crit Care Med*. Poster presentation at Society for Critical Care Medicine. 2004
15. Suresh MV, Yu B, Machado-Aranda D, et al. Role of macrophage chemoattractant protein 1 in acute inflammation following lung contusion. *Am. J. Respir. Cell Mol. Biol*. 2012
16. Hoth JJ, Hudson WP, Brownlee NA, et al. Toll-like receptor 2 participates in the response to lung injury in a murine model of pulmonary contusion. *Shock*. 2007; 28:447–452. [PubMed: 17558351]
17. Hoth JJ, Stitzel JD, Gayzik FS, et al. The pathogenesis of pulmonary contusion: an open chest model in the rat. *J Trauma*. 2006; 61:32–44. discussion 44–35. [PubMed: 16832247]
18. Hoth JJ, Wells JD, Hiltbold EM, McCall CE, Yoza BK. Mechanism of neutrophil recruitment to the lung after pulmonary contusion. *Shock*. 2011; 15:15.
19. Bauer HC, Traweger A, Zweimueller-Mayer J, et al. New aspects of the molecular constituents of tissue barriers. *J Neural Transm*. 2011; 118:7–21. [PubMed: 20865434]
20. Lin X, Dean DA. Gene therapy for ali/ards. *Crit Care Clin*. 2011; 27:705–718. [PubMed: 21742224]
21. Howrylak JA, Dolinay T, Lucht L, et al. Discovery of the gene signature for acute lung injury in patients with sepsis. *Physiol Genomics*. 2009; 37:133–139. [PubMed: 19174476]
22. Dean DA, Machado-Aranda D, Blair-Parks K, Yeldandi AV, Young JL. Electroporation as a method for high-level nonviral gene transfer to the lung. *Gene Ther*. 2003; 10:1608–1615. [PubMed: 12907953]
23. Machado-Aranda D, Adir Y, Young JL, et al. Gene transfer of the na⁺, k⁺-atpase beta1 subunit using electroporation increases lung liquid clearance. *Am J Respir Crit Care Med*. 2005; 171:204–211. [PubMed: 15516538]
24. Mutlu GM, Machado-Aranda D, Norton JE, et al. Electroporation-mediated gene transfer of the na⁺, k⁺-atpase rescues endotoxin-induced lung injury. *Am J Respir Crit Care Med*. 2007; 176:582–590. [PubMed: 17556717]
25. Gatson JW, Liu MM, Abdelfattah K, et al. Resveratrol decreases inflammation in the brain of mice with mild traumatic brain injury. *J Trauma Acute Care Surg*. 2013; 74:470–475. [PubMed: 23354240]
26. Mouzon B, Chaytow H, Crynen G, et al. Repetitive mild traumatic brain injury in a mouse model produces learning and memory deficits accompanied by histological changes. *J Neurotrauma*. 2012; 29:2761–2773. [PubMed: 22900595]
27. Sangiorgi S, De Benedictis A, Protasoni M, et al. Early-stage microvascular alterations of a new model of controlled cortical traumatic brain injury: 3d morphological analysis using scanning electron microscopy and corrosion casting. *J Neurosurg*. 2013; 118:763–774. [PubMed: 23350772]

Fig. 1.
Biological attributes involved in lung contusion injury and possible targets of study and therapy using genetic manipulation techniques

Fig. 2.
Position of mouse, target of impact. Outline shows border of rib cage

Fig. 3.
Measuring target depth of electrical cortical contusion impactor

Fig. 4.
Rat lung contusion apparatus

Fig. 5.
Detail of Lexan[®] shield and Teflon[®] guides with impact surface

Fig. 6.
Electrodes for mouse electroporation