Journal of Visualized Experiments

Intratracheal aerosolization of viral vectors to newborn pig airways

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60725R1
Full Title:	Intratracheal aerosolization of viral vectors to newborn pig airways
Section/Category:	JoVE Medicine
Keywords:	porcine, gene transfer, microsprayer, intubation, viral vector delivery, gene therapy
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Iowa City, IA, USA

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August 29, 2019

Dear Editors,

Enclosed is our manuscript reporting a method for achieving widespread viral vector distribution in a large animal model. Specifically, we describe a detailed method for aerosolizing viral vector delivery to the airways of newborn pigs. Efficient lung gene transfer could be beneficial for a broad range of applications, even beyond gene therapy. We believe these findings would be of significant interest to the readers of the *Journal of Visualized Experiments.*

Sincerely,

Patrick L Sinn, PhD
Associate Professor of Pediatrics
Associate Professor of Microbiology and Immunology
Director, University of Iowa Viral Vector Core

TITLE:

Intratracheal Aerosolization of Viral Vectors to Newborn Pig Airways

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KEYWORDS:

porcine, gene transfer, microsprayer, intubation, viral vector delivery, gene therapy

SUMMARY:

To maximize the potential benefits of pulmonary gene therapy, widespread and uniform topical delivery of a viral vector across the surface epithelium is an important goal. Here, we demonstrate an aerosolization technique using a microsprayer positioned intratracheally to deliver a viral vector to newborn pig airways.

ABSTRACT:

Gene therapy for airway diseases requires efficient delivery of nucleic acids to the intrapulmonary airways. In small animal models such as mice, gene delivery reagents are commonly delivered as a bolus dose. Routes of delivery may include either nasal sniffing or direct tracheal instillation. However, using a large animal model for preclinical applications is relevant for translation to human trials. Widespread and uniform distribution of transgene expression is critical for developing a successful lung gene therapy treatment. Aerosolizing viral vectors to the lungs of large animals, such as pigs or sheep, is a strategy to maximize gene transfer efficiency and results in greater airway distribution than a liquid bolus dose. Here we demonstrate a technique for direct aerosolization of a viral vector to the airways of newborn pigs. Briefly, a pig is anesthetized, intubated with an endotracheal tube, and a microsprayer is passed through the endotracheal tube. A syringe is used to push the vector through the microsprayer, resulting in a fine mist being released into the distal trachea. The microsprayer produces ~15–16 μm size particles that deposit across the proximal and distal regions of the lung. Using a microsprayer to deliver an adenoviral-based vector, we previously observed ~30–50% of surface epithelial cells transduced in both the large and small airways of newborn pigs.

INTRODUCTION:

Gene transfer to the lung holds great potential for treating many genetic diseases, such as

cystic fibrosis or alpha-1 antitrypsin deficiency. However, developing gene therapy approaches to successfully deliver genes of interest to the airways has been challenging. Animal models play a major role in driving innovation of viral vector design and delivery strategies to the intrapulmonary airways. Indeed, we and others have developed methods to overcome many gene delivery hurdles using large animal models. Many examples of delivery challenges have been previously reviewed[1–5]. Using pigs as a large animal model, we have refined a protocol to achieve widespread airway distribution following intratracheal aerosol delivery.

Here we demonstrate how to achieve efficient viral vector delivery to a pig lung through aerosolization. Conceptually, topical delivery of a vector encoding a therapeutic transgene to the lung is simple. However, in practice, achieving efficient delivery is a challenge. Important considerations include the viral vector, the appropriate vehicle for the vector, and the aerosolization method. In general, devices for generating airborne vectors can be categorized as follows: aerosolizing catheters, atomizers, and nebulizers. All devices convert liquids into particles small enough for respiration. Aerosolizing catheters convert liquids into particles at expulsion. For these studies, we use a syringe-mounted aerosolizing catheter named a microsprayer. We selected a microsprayer as our aerosolization device in part because of its ease of use and because of its ability to effectively aerosolize a viral vector in a particle size that can reach all areas of the lung. We quantified droplet geometric size by laser diffraction and obtained consistent measurements of 15–16 μm for each droplet. The microsprayer works by generating an aerosol at its tip that results from the force generated by depressing a syringe plunger. We validated this delivery method for both adenoviral (Ad)- and adeno-associated virus (AAV)-based viral vectors[6,7].

Alternatively, there are aerosolizing catheter devices that utilize pressurized delivery through compressed air. Particle sizes as small as 4–8 μm may be possible with pressurized delivery. Such a device was used to aerosolize helper-dependent adenovirus vectors to rabbit airways[8,9] and Sendai virus vectors to sheep[10]. Atomizers are a type of aerosolizing catheters that deliver large sized particles (~30–90 μm diameter). We have observed that this type of atomizer is effective for delivering multiple viral vectors, including lentiviral vectors, especially when formulated with a viscoelastic material such as methylcellulose[11]. Nebulizers first convert the liquid into a mist that is passively inhaled. Using this strategy, a plasmid-based vector was delivered to the airways of CF patients in a phase IIB gene therapy trial[12]. Nebulization requires a large volume of concentrated material and is therefore the least economical option for delivery of viral vectors.

Prior to developing this protocol, we tested multiple different delivery methods in newborn pigs. We evaluated localized delivery via a pediatric bronchoscope lined with either a PE20 catheter delivered as a bolus liquid dose, or through a drug infusion balloon[13]. Additionally, we tested an atomizer[14] and a pressurized aerosolizing catheter (unpublished). The pressurized aerosolizing catheter delivery was effective but required extra equipment and the pressurized delivery occasionally resulted in injury to pig tracheas. Based on ease of use and reproducibility, we now routinely opt for the syringe-mounted microsprayer for delivery of encapsidated viral vectors such as adenoviral and adeno-associated viral vectors. The atomizer gives the most

comparable lung expression to the microsprayer without needing to pass through an endotracheal tube. Although our focus has been on developing a delivery method for efficient lung gene transfer to correct cystic fibrosis, this method could be adapted for other applications. The aerosolization device and droplet size may play an important role in the efficiency and distribution of vector mediated transgene expression. Here, we focus on the procedure of intubation in newborn pigs and passing an aerosolizing catheter through an endotracheal (ET) tube to deliver vector.

PROTOCOL:

All animal experiments performed following this protocol must be approved by the respective Institutional Animal Care and Use Committee (IACUC). All procedures described here were approved by the University of Iowa IACUC.

1. Prepare the procedure space and vector delivery materials.

1.1. Place a heating pad covered by a disposable underpad to warm the procedure area.

1.2. Set up the pulse oximeter to measure the heart rate and peripheral capillary oxygen saturation (SpO2). Prepare a rectal thermometer by coating with a lubricating jelly.

1.3. Pass the microsprayer nozzle through a 2.0 mm inner diameter ET tube and mark the base of the microsprayer when the tip exits the ET tube by ~1 mm. This will serve as a guide for how far to insert the microsprayer into the ET tube once it is placed in the animal's airway.

NOTE: Not all ET tubes are the same length, so this step should be repeated for every new ET tube used in a procedure.

1.4. Remove the microsprayer from the ET tube and screw the microsprayer onto to a luer locking syringe loaded with 1–2 mL of viral vector. Set aside.

NOTE: A test spray through the microsprayer is recommended prior to setup. Prefilling the spray nozzle with viral vector is not necessary.

1.5. Insert a stylet into the ET tube to support intubation.

1.6. Pigs will be anesthetized using 2–4% isoflurane. Assemble an isoflurane vaporizer.

1.6.1. Connect an O_2 tank with a pressure regulator and flowmeter to the vaporizer. Connect the vaporizer through tubing to deliver the isoflurane through an anesthesia mask and an anesthesia gas filter canister to collect the waste anesthesia gas from the operating room environment.

1.7. Within the procedure area, arrange a laryngoscope with a 4 in elongated blade, the

microsprayer with the syringe containing the vector, and an ET tube lined with a stylet. Precoat the ET tube tip with lubricating jelly.

2. Sedate the pigs.

2.1. Use a pulse oximeter to measure the pig's oxygen saturation and the heart rate. Place a wraparound SpO_2 sensor around the pig's hind leg and ensure the readings register on the pulse oximeter. Record the pre-anesthetic reading.

NOTE: Animals must fast prior to sedation to prevent aspiration during delivery.

2.2. Turn on the O_2 tank (flowmeter set to 2 L/min) and the isoflurane vaporizer to begin the flow to the anesthesia mask.

2.3. Place the anesthesia mask over the pig's snout and hold the pig until sedated. This may take approximately 4–5 min but will vary depending on the age and weight of the animal. Begin by holding the pig during the initial stages of anesthesia. Once the pig is sedated, lay it on the prepared procedure space (i.e., the underpad over a heating pad). Confirm anesthesia by testing the pedal reflex.

NOTE: The animal should never be left unattended.

2.4. Record the rectal temperature and the respiratory rate.

2.5. Continue the procedural monitoring every 15 min throughout sedation (i.e., SpO_2, heart rate, temperature, and respiratory rate).

3. Intubate the sedated pigs with an endotracheal tube.

3.1. Confirm that the ET tube with the stylet has been lightly coated with lubricating jelly to facilitate intubation (step 1.6).

3.2. Remove the anesthesia mask from the pig and turn off the flow of isoflurane.

3.3. Lay the pig supine on the procedure space and visualize the larynx using a laryngoscope.

3.4. Pass a 2.0 mm ET tube through the vocal folds of the larynx and into the trachea (**Supplemental Movie 1**). If the pig is properly intubated, the SpO_2 levels will start to decline.

NOTE: The exact ET tube placement will vary depending on the size of the animal. Placement can vary from just beyond the larynx for larger animals (~3–4 kg) to near the carina for smaller animals (~0.8–1 kg). In small animals, there is risk of one side intubation and trimming 3–5 cm from the ET tube may be warranted.

3.5. Remove the stylet.

4. Aerosolize the viral vector using the microsprayer.

4.1. Pass the microsprayer connected to the viral vector-containing syringe through the ET tube until reaching the mark at the base of the microsprayer.

4.2. Spray the solution intratracheally by pressing the syringe plunger with a firm and consistent force to generate a mist. This will take approximately 3–4 s.

NOTE: The appropriate pressure should be practiced beforehand. Too little pressure will result in a stream instead of a spray.

NOTE: For our studies, we limited the volume delivered to ~1 mL/kg.

4.3. A post-spray "air chaser" of ~500 µL will help ensure complete delivery of the vector from the syringe and nozzle.

4.4. Gently remove the ET tube and microsprayer from the intratracheal intubation at the same time. Successful delivery will typically result in the sound of crackles when breathing.

NOTE: A typical procedure time from initiated anesthesia time to extubation is 10–15 min.

5. Monitor the pigs as they come out of sedation.

NOTE: Apnea is a common response to intubation in newborn pigs. Sporadic breathing may last 2–3 min. Gentle chest compressions can help facilitate normal breathing.

5.1. Monitor SpO_2 levels until they return to 95–100% then remove the SpO2 sensor from the pig's hind leg.

5.2. Continue with the postprocedural monitoring every 15 min until the pig is alert, sternal, and walking. Typically, pigs will recover within 15 min.

REPRESENTATIVE RESULTS:

We previously validated this technique for delivering gene transfer vectors to pig lungs and showed widespread and uniform airway distribution following delivery of an adenoviral vector expressing green fluorescent protein (GFP)[6]. To assess transduction, all six lung lobes were separated into two to four segments. From each segment, tissue was designated for DNA or mRNA isolation and transduction was quantified by real-time PCR to detect the GFP sequence. GFP-positive cells were counted and graphed as a function of the airway diameter. Remarkably, we achieved abundant gene transfer throughout large and small airways. We observed a variety of cell types transduced, including surface epithelium (ciliated, non-ciliated), basal cells, and submucosal gland cells[6]. Droplet particles can reach the most distal regions of the lung,

expressing a transgene in epithelial cells of both the cartilaginous and noncartilaginous airways.

There are multiple strategies to confirm successful intubation and vector delivery. When first learning this technique, it is helpful to practice on a euthanized pig. After intubation, the trachea can be visualized via dissection and successful intubation can be immediately confirmed. When appropriate in our studies, we employ computerized tomography (CT) to confirm placement of the ET tube in the trachea (**Figure 1**) and the distribution pattern of delivered material throughout the lung (**Figure 2**). As shown, pre- and post-delivery CT images (**Figures 2A** and **2B**, respectively) confirm material delivery to the pulmonary airways. "Cloudy" CT images result from the aerosolized liquid and indicate successful delivery. Contrast agents are not necessary. In past experiments, we used X-ray imaging to confirm airway catheter placement in rabbits[15]. X-rays are a less expensive and more accessible alternative to CT imaging.

To confirm successful viral vector-mediated gene transfer to the airways, we use viral vectors that express standard reporter genes such as GFP. In the example shown in **Figure 3**, an Ad vector expressing GFP (Ad-GFP) formulated with 0.1% lysophosphatidylcholine (LPC) was aerosolized to the airways of a newborn pig. LPC is a natural airway surfactant that will transiently disrupt tight junctions to allow Ad to access its basolateral receptor[16]. Five days later, we collected the lungs and used low power fluorescence microscopy to examine the fresh, unfixed tissue. We observed abundant GFP expression spread uniformly throughout the lung lobes (**Figure 3**). Low power epifluorescent and bright field views of the right middle lobe are shown (**Figure 3A**, **3B**). Such low power views mainly reveal alveolar expression. As we previously reported[5], this tissue should be fixed and sectioned so that high power microscopy can be used to confirm the number and abundance of transduced epithelial cells in the conducting airways.

In addition to low power images of whole tissue, which largely represent alveolar expression, gene expression in conducting airways can be confirmed by manual dissection. In this example from our Ad-GFP experiment, parenchymal tissue from transduced pig lungs was removed and airway branches were imaged using a fluorescent dissecting microscope (**Figure 4**). GFP expression indicates widespread distribution in the conducting airways. In this low power image, GFP expression appears fainter in the large (cartilaginous) airways because the thicker tissue masks the epithelial signal on the luminal surface.

FIGURES AND LEGENDS:

Figure 1: CT confirmation of the tracheal placement of the ET tube. While under isoflurane anesthesia, a newborn pig was intubated with an ET tube. The pig was subsequently imaged using a CT scanner. Proper placement of the ET tube (yellow arrow) was confirmed in the trachea (red arrow).

Figure 2: Example of widespread aerosol delivery confirmation. CT images of pig lungs were collected (**A**) pre- and (**B**) post-vector aerosolization. (**A**) Following intubation, but before vector

delivery, the lungs were imaged. (**B**) Ad-GFP formulated with 0.1% LPC was aerosolized through the microsprayer and the pig was imaged again. The resultant "cloudy" appearance is indicative of successful aerosol delivery.

Figure 3: Representative images from a pig transduced with Ad-GFP showing high levels of gene transfer. Ad-GFP formulated with 0.1% LPC was aerosolized intratracheally to newborn pigs. Five days later, the lungs were collected and GFP expression was visualized by fluorescence microscopy of fresh, unfixed tissue. Fluorescent (**A**) and bright field (**B**) images of the right middle lobe were captured using a fluorescent dissecting microscope. The white arrows indicate untransduced regions of the lobe.

Figure 4: Gene transfer in conducting airways. Five days following aerosol delivery of Ad-GFP, the lungs were collected. The parenchymal tissue was removed through manual dissection. As shown by the photo montage, GFP expression in the conducting airways was confirmed using a fluorescent dissecting microscope.

Supplemental Movie 1: Video of a bronchoscope passing through the vocal folds of the larynx and into the trachea. A 4-week-old pig was sedated using isoflurane and intubated using a bronchoscope. The bronchoscope was guided through the vocal folds of the larynx to demonstrate proper intubation. The tracheal rings visualized indicate that the trachea was properly intubated.

DISCUSSION:

Widespread airway distribution of a viral vector would help ensure the success of a gene therapy approach for treating pulmonary diseases. Here, we demonstrate an aerosolization technique that leads to whole lung expression of large and small pig airways. We describe the steps for sedating a pig, intubating with an ET tube, and aerosolizing a viral vector through the microsprayer aerosolization device. This technique is important as a preclinical approach to testing viral vector efficacy.

There are multiple critical steps involved in this procedure. First, the most critical step is ensuring the correct placement of the ET tube in the trachea and not the esophagus. The intubation technique can be improved with practice, beginning with intubating a euthanized pig and immediately checking for proper ET tube placement by dissecting a tracheal window. Multiple examples of verifying ET tube placement are described in the Representative Results. However, with practice, intubation will become reliable and routine. A CT scan can not only verify ET tube placement before vector delivery but pre-and post-delivery CT imaging is also a potential strategy to determine general deposition patterns. Second, the microsprayer tip should just slightly exit the ET tube. If the microsprayer tip does not extend past the end of the ET tube, the aerosolization will pool within the ET tube and exit as a bolus dose. If the microsprayer tip extends too far beyond the ET tube, there is a risk of the microsprayer tip puncturing the wall of the trachea and causing a pneumothorax. Using the described protocol in this manuscript, we have never observed a pneumothorax or serious complication. However,

if the animal displays an extended period (>20 min) of labored breathing or signs of distress, it should be euthanized for autopsy. Third, an appropriate plane of anesthesia will facilitate successful delivery. Newborn pigs typically recover quickly (<10 min) from isoflurane so the procedure should be performed with care for time management: If a pig is too lightly sedated, it may kick or clench its jaw. However, too much sedation will needlessly prolong the procedure and recovery time.

There are multiple options for vector delivery to the airways. This procedure may be adapted for different devices. When deciding which aerosolizing device to use for any application, it is helpful to know how each device works. In mice, a bolus dose results in high levels of expression in the lung[11,17]. However, to achieve these levels as a bolus dose in a large animal, a small region or specific lobe would need to be targeted. Such targeting was previously described in rats[18]. Delivery to a small region within the lung requires visual guidance by a bronchoscope. We validated successful gene delivery to 4-week-old pig airways using a bronchoscope lined with a PE20 catheter[13]. However, that delivery method is limited to a specific region of the lung, which may be useful for particular applications. An atomizer is a good choice for whole lung viral vector delivery[14]. The atomizer results in widespread delivery at a particle size of 30–90 µm and is effective at delivering lentiviral vectors formulated with viscoelastic gel[10,13]. The atomizer we used previously (see "Alternative Aerosolization Devices" in the **Table of Materials**) has a bulb-shaped tip that can be challenging to pass through the vocal folds of a newborn pig[14]. A pressurized aerosolizing catheter leads to high levels of gene transfer by a lentivirus in sheep lungs[10] and has been used to deliver liposomal formulations[19]. It is important to note that the microsprayer used in this manuscript and the pressurized aerosolizing catheter previously screened (see **Table of Materials**) are no longer available for purchase. Suitable substitutions will need to be identified.

This method requires skill in the intubation technique. Practice and hands-on training will facilitate success. Alternate methods such as nasal instillation or nebulization are less invasive, do not require full sedation, and are easier to learn. However, viral vectors delivered via nasal instillation may not reach the distal airways in large animal models, and nebulization requires a volume of viral vector that is often cost prohibitive. Using intratracheal aerosolization to newborn pig airways, we can rapidly and efficiently deliver vector directly to the large and small airways. From practical experience we know that 1 mL/kg of adenoviral vector (~10^{10} transducing units/kg) is sufficient to reach all regions from the trachea to the small bronchioles in newborn pigs.

ACKNOWLEDGMENTS:

We thank Christian Brommel for aiding with the airway dissection and Raul Villacreses Rada with the supplemental movie. We thank the University of Iowa Office of Animal Resources and the animal caretakers. We thank the Viral Vector Core for vector production. This work was supported by the National Institutes of Health [NIH P01 HL-51670, NIH P01 HL-091842, NIH R01 HL-133089, NIH R01 HL-105821], the Center for Gene Therapy of Cystic Fibrosis [NIH P30 DK-054759], and the Cystic Fibrosis Foundation [SINN19XX0] and [COONEY18F0].

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

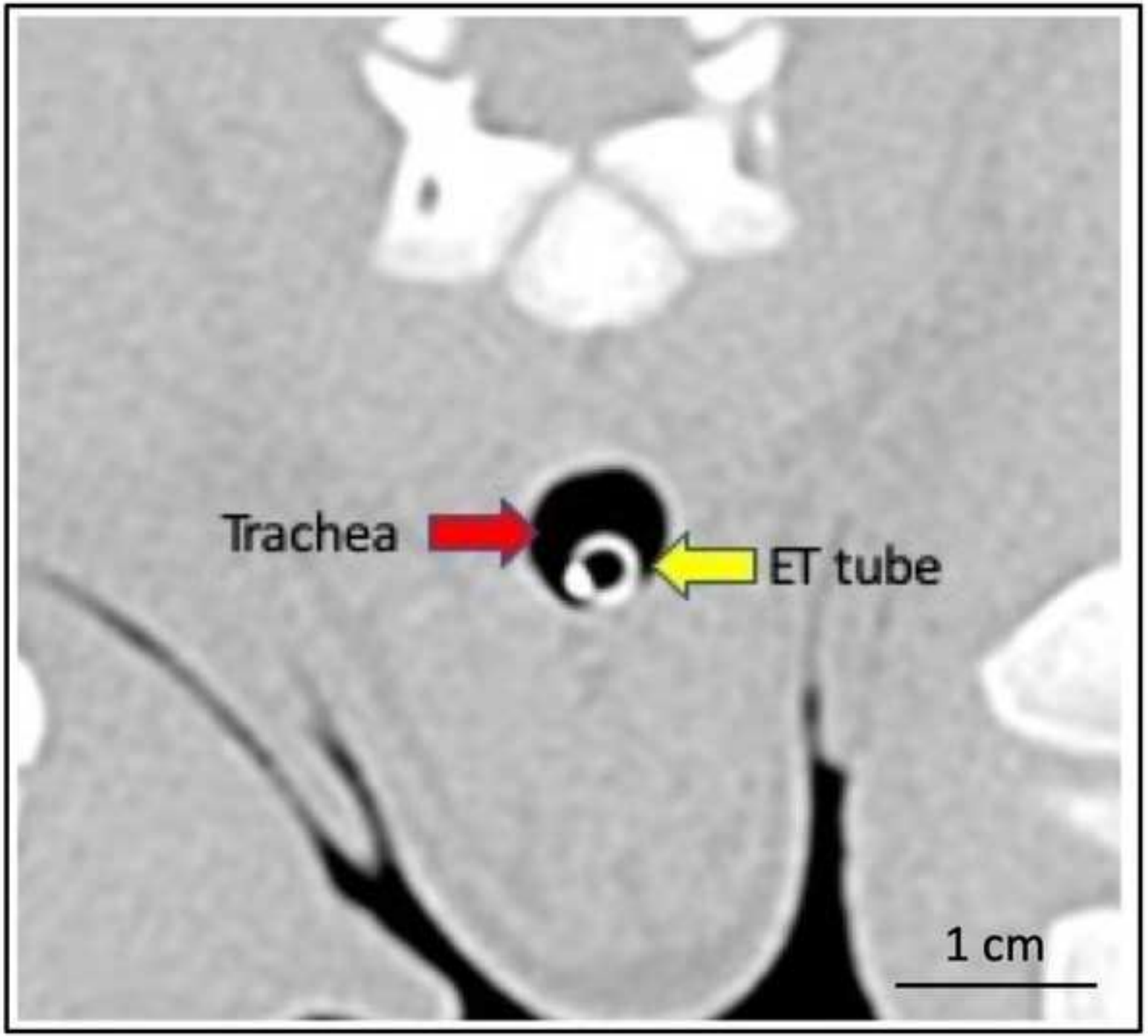

Figure 2

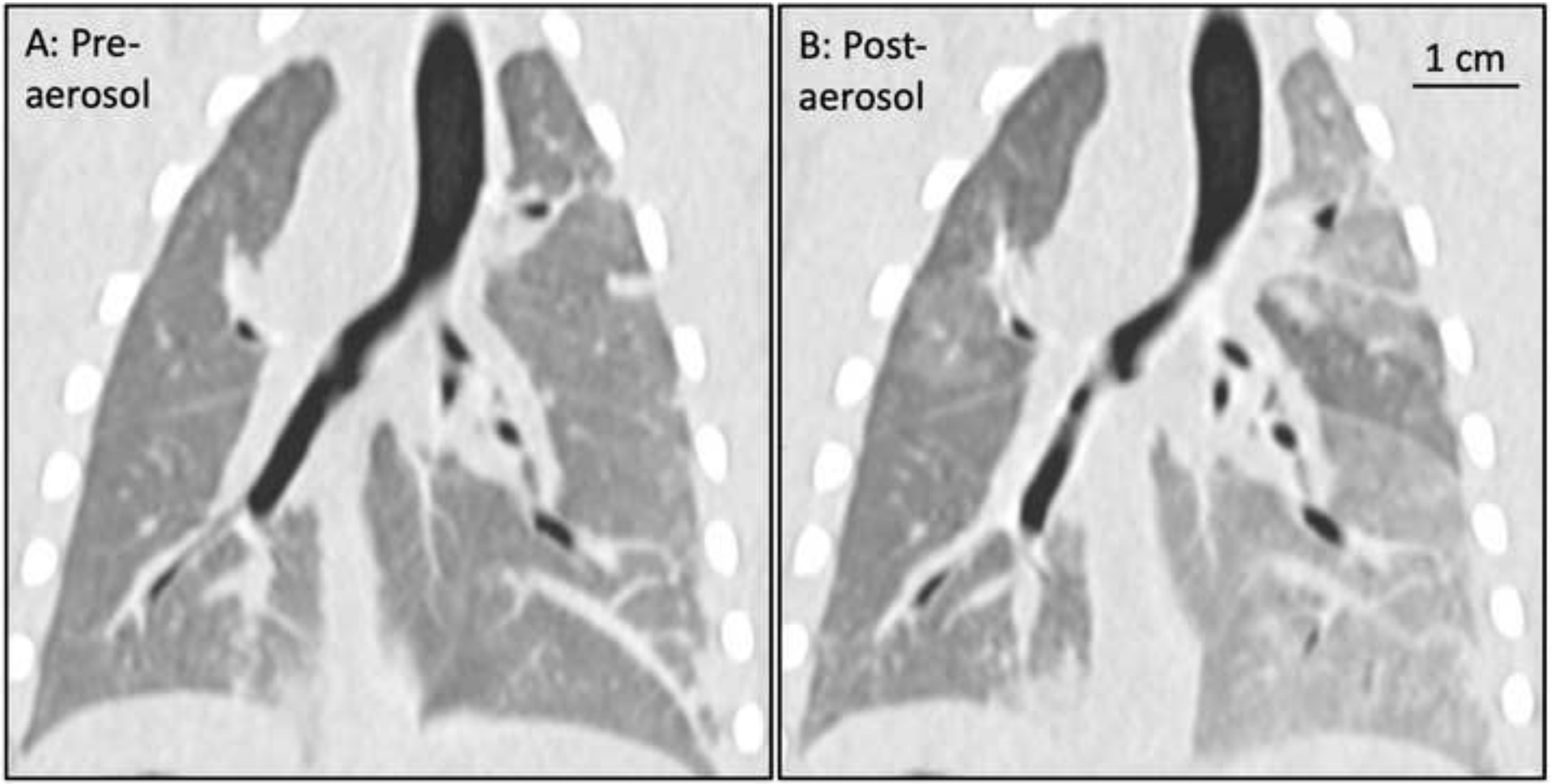

Figure 3

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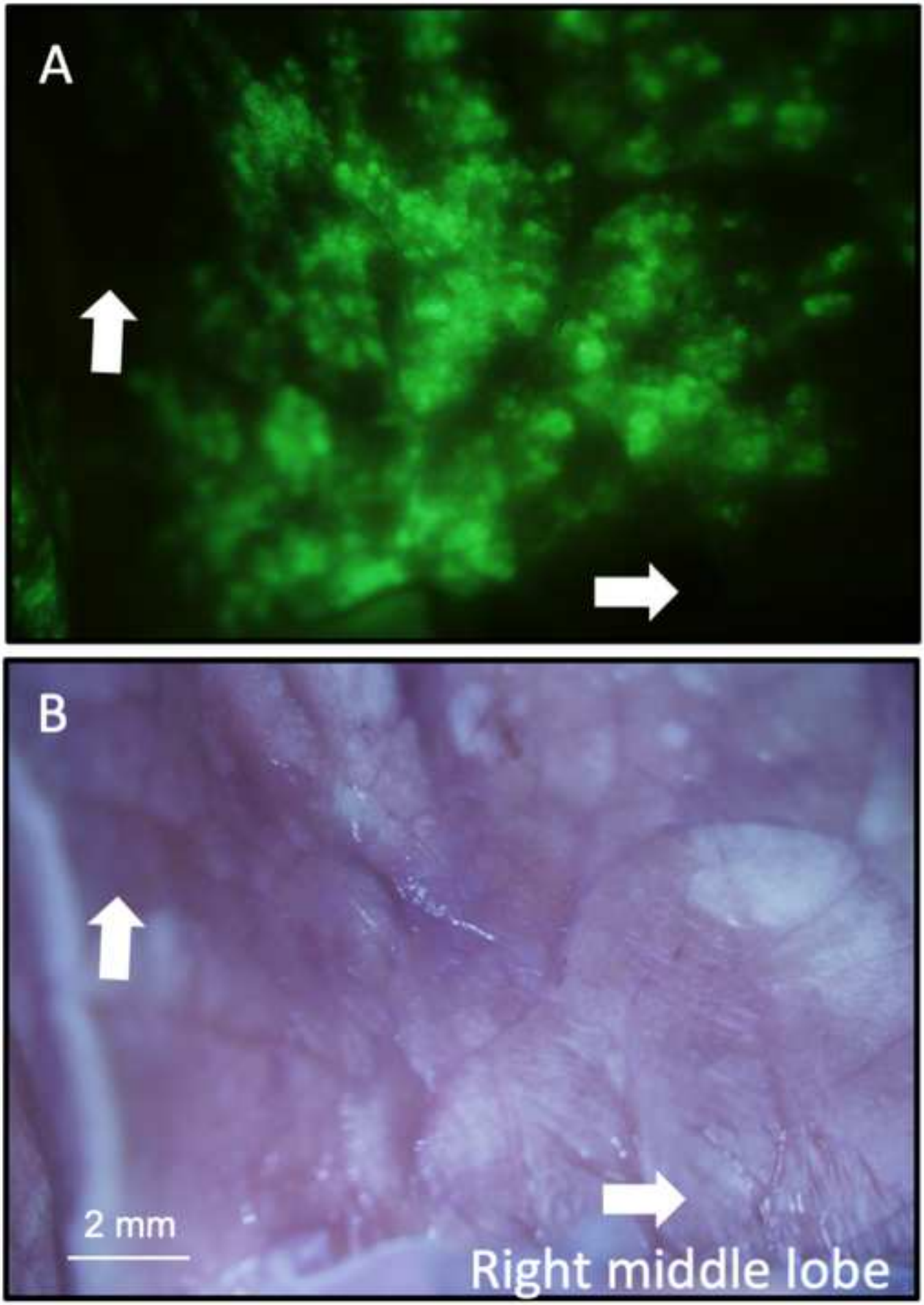

Figure 4

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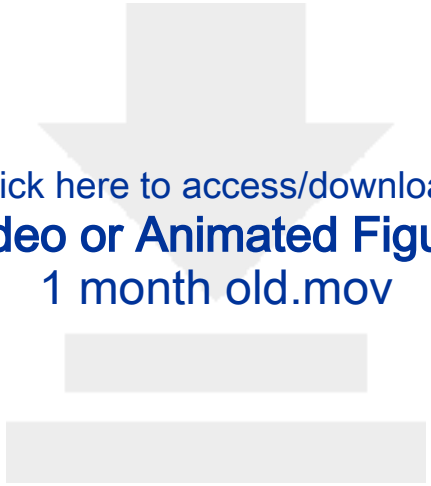
Supplemental Video



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Video or Animated Figure

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Atomizer	Teleflex	MAD700	MADgic Laryngo-Tracheal Mucosal Atomization
Compressed Oxygen (O2) gas	Praxair		Also need pressure regulator and flowmeter
Disposable underpad	General stores		
Endotracheal (ET) tube 2.0 mm I.D.	Teleflex Medical	5-10404	Hudson RCI; Sheridan Uncuffed
Fluorescent Dissecting Microscope	Leica	MDG41	
Heating pad	General stores		
Isoflurane	Pharmacy		
Isoflurane F/Air Filter Canister	Vetamac Anesthesia	SKU VAD020	
Isoflurane regulator (vaporizer)	Vetamac Anesthesia		
Laryngoscope traditional set	DarvallVet	#8070	4" blade used for delivery
Leur locking syringe (3 ml)	General stores		
Lubricating jelly	General stores		
Microsprayer	PennCentury		Aerosolizing catheter; No longer available
Pressurized aerosolizing catheter	Trudell Medical Corporation		Aeroprobe; No longer available
Pulse oximeter	Pacific Medical Supply	UQNE4600	
SpO2 sensor band	Hospital stores		
Stylet	Hospital stores		5 Fr (1.7 mm O.D.)
Thermometer (Digital)	General stores		
Veterinary anesthesia mask	Hospital stores		
Viral vectors	University of Iowa Viral Vector Core		Adenoviral vector; fee for service

Device

We thank the reviewers for their comments. We have created a line-by-line response and addressed each in turn.

Editorial Comments:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response 1: The manuscript has been proofread.

Comment 2: **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response 2: We have done our best to comply with this requirement.

Comment 3: **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please bear in mind that anesthesia steps will not be filmed.

Response 3: The appropriate protocol steps have been highlighted.

Comment 4: **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. (paragraph 2)

Response 4: We have done our best to comply with this requirement.

Comment 5: **Figures:** Add scale bars to fig 2, fig 3, fig 4

Response 5: Scale bars were added to figures 1- 4.

Comment 6: **References:**
1) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
2) Please spell out journal names.
3) Minimum of 10 references is required.

Response 6: We now have >10 references that are appropriately formatted.

Comment 7: **Commercial Language:**
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1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Response 7: Brand names were deleted throughout the manuscript. When appropriate, the brand names of devises used in this study are named in the Materials list.

Comment 8: **Table of Materials:**
Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as viral vector, microscope, etc.

Response 8: Essential supplies, reagents, and equipment are listed in the Table of Materials.

Comment 9: Please define all abbreviations at first use.

Response 9: Non-standard abbreviations are defined.

Comment 10: Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

Response 10: Standard abbreviations are used.

Comment 11: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response 11: All figures are original.

Reviewer Comments:

Reviewer #1:

Comment 12: Newborn pigs as a large animal model. In many experimental models, juvenile or adult pigs are used to test novel therapies. Have the authors tested airway gene transfer in different animal ages? Or are newborn animals used for showing better results?

Response 12: We have experience delivering viral vector to 4-week old pigs and observed high levels of gene transfer (1). As now briefly discussed, (lines 314-315), in larger animals, vector can be delivered through the working channel of a pediatric bronchoscope. For many of our studies, we focus on newborn pigs for economic and logistical reasons: less vector is required; 4-week old pigs are ~30 kg and rambunctious; and housing pigs (especially CF pigs) is expensive. We have not directly compared newborn to older animals in a controlled experiment; however, we suspect that similar vector/kg doses would result in similar levels of gene transfer. In this report, our goal is to show how viral vector can be aerosolized to the whole lung to a 1-2 kg animal without the use of a bronchoscope.

Comment 13: Type of viral vectors. While adenoviruses may achieve a greater but shorter gene expression time pattern, AAVs may be preferred due to a longer, sustained gene expression profile. This protocol shows GFP transduction suing adenoviruses, but a potential reader trying to apply this protocol may wonder if similar results are expected using AAVs, and which serotype. Do the authors have experience with AAV airway delivery to provide a representative example of successful gene transfer? Another issue is the timing for gene transfer quantification, that is related to the viral vector used. In this example, the authors analyze gene expression 5 days later, but longer latency may be expected for AAVs.

Response 13: This technique is applicable to AAV and was used in our recent manuscript (2). For pig airways, our serotype of choice is an evolved capsid termed H22 that was developed in the laboratory of Joe Zabner (3). We now include the following sentence (lines 65-66) to include AAV: "We validated this delivery method for both adenoviral (Ad) and adeno-associated viral (AAV) vectors." In our experience, 4 days

for Ad and 2 weeks for AAV is sufficient for gene transfer quantification in pigs. We feel that a comparative study of Ad and AAV is outside the scope of this report.

Comment 14: The authors show GFP expression in whole lung samples. Does expression occur only at epithelial cells, or may other cells (vascular) express GFP? Maybe a histology example will illustrate this point.

Response 14: We previously published the details of the cell types transduced following this method (4). Epithelial cell types include: ciliated and non-ciliated cells, basal cells, and submucosal glands. Endothelial cells were not noted as being positive. We now include the following sentence (lines 216-218) to clarify: "We observed a variety of cell types transduced, including surface epithelium (ciliated, non-ciliated), basal cells, and submucosal gland cells."

Comment 15: In Figure 2, what causes the CT scan "cloudy" images, is diluted contrast added to the viral vector solution?

Response 15: The "cloudy" images in the CT scan is simply the liquid aerosol that was deposited, no contrast has been added. The following statement had been added to line 227-229: ""Cloudy" CT images result from the aerosolized liquid and indicative a successful delivery. Contrast agents are not necessary."

Comment 16: What safety measures are taken into consideration in this protocol? It is expected that a small % of vectors contact may contaminate the endotracheal tube or may even flow back to the proximal end and get into the respirator.

Response 16: The endotracheal tubes are disposable, so we can use a new one for each animal to avoid contamination between animals. The anesthesia mask is removed prior to intubation. Removal of the anesthesia mask prior to vector delivery also prevents contamination between animals.

Comment 17: In the introduction, please briefly explain how previous strategies for gene therapy in target diseases have failed and how the new approach may overcome previous hurdles.

Response 17: The editors and journal guidelines have directed us keep the manuscript method focused. However, in the revised manuscript we added the following statement (line 48-49): "Many examples of delivery challenges have been previously reviewed."

Comment 18: The fact that some of the devices are no longer available will limit the replication of these experiments. Is any of the currently available options a good choice for prospective users?

Response 18: We and others are actively searching for alternatives sources of a similar devise. The MADgic atomizer produces a larger particle size but confers comparable results. We now include in lines 88-89: "However, without needing to pass through an

endotracheal tube, an atomizer gives the most comparable lung expression to the microsprayer."

Reviewer #2:
Comment 19: Line 31. "Is improved" does not describe what the improvement is compared to. In addition, not all vectors and all aerosolisations work well in lung. Claim should be modified appropriately.

Response 19: The statement was modified as follows (lines 30-32): "Aerosolizing viral vectors to the lungs of large animals, such as pigs or sheep, is a strategy to maximize gene transfer efficiency and results in greater airway distribution than a liquid bolus dose."

Comment 20: Line 39: Having noted % transduction in the Abstract from studies that are not part of this paper, and describing the technique for general purposes, there should be a clear note here that this paper has used and Adv vector and Penn Century device to demonstrate the method.

Response 20: Lines 37-38 now read: "Using a microsprayer to delivery an adenoviral-based vector,…"

Comment 21: Line 45: "..understand the limitations".. There are many successes from use of animal models, this should be added.

Response 21: The indicated statement was modified as follows: "Animal models play a major role in driving innovation of viral vector design and delivery strategies to the intrapulmonary airways." (Lines 45-47)

Comment 22: Line 97: ET tube manufacturer and type/model is not listed in the Materials / Equipment.

Response 22: Line 4 of the Materials list now reads:

Endotracheal (ET) tube 2.0 mm I.D.	Teleflex Medical	5-10404	Hudson RCI; Sheridan Uncuffed

Comment 23: Line 105: Comment could be made here about whether there is need for an "air chaser" to fully expel vector, and /or prefilling of the delivery device is also done.

Response 23: Line 121 now reads: "NOTE: Prefilling the spray nozzle with viral vector is not necessary."
The following note as added (line 190-191): "4.3. A post-spray "air chaser" of ~500 ml will help ensure complete delivery of vector from the syringe and nozzle."

Comment 24: Line 125: Note here what a setting of '2" means in liters per minute.

Response 24: Line 143 now reads “(flowmeter set to 2 L/min)”.

Comment 25: Line 132: The comment about supervision of the anaesthetised pig (although appreciated and important) is an ethical / care issue rather than a vector delivery procedural issue; consider if it is needed here.

Response 25: The statement “Never leave the pig unattended” is now included as a separate Note (line 151). We feel that this is an important detail to include.

Comment 26: Line 148: Important to note here how deeply into the trachea the ET tube is placed. Deeply placed (near the carina) would be expected to deliver more effectively to the deeper lung while if placed just through the chords the trachea and initial airways would presumably be better targeted. This issue could be a point for later discussion as well, especially if there is any evidence of different effects across the author's earlier studies.

Response 26: The following note was added to the protocol (line 170-172): NOTE: The exact ET tube placement will vary depending on the size of the animal. Placement can vary from just beyond the larynx for larger animals (~3-4 kg) to near the carina for smaller animals (~0.8-1 kg).

Comment 27: Line 159: "sufficient force" is somewhat vague. Does this mean that any force past "sufficient" produces the same aerosols, and transduction distribution / effects)? Was the sufficiency of the aerosols produced for the aerosol sizing the optimal / minimum / supra sufficiency?

Response 27: “Sufficient” was replaced with “firm and consistent,” and followed with the following note (Line 185-186): “NOTE: The appropriate pressure should be practiced beforehand. Too little pressure will result in a stream instead of a spray.”

Comment 28: Line 161/2: A typical procedure time, from pig being started on isolfuorane to extubation to recovery from anaesthesia would be useful.

Response 28: Line 196 now reads: “NOTE: A typical procedure time from initiated anesthesia time to extubation is 10-15 minutes.” Line 185 states “Typically, pigs will recover within 10 minutes.”

Comment 29: Line 177: CT is clearly helpful but may not be available to many groups. The ET tube placement is clear this way, and the change in contrast can show the success of delivery. Two lobes in Figure 2 (upper right, and between ribs 4 and 5 on the left side) do not appear to be aerosolised based on the contrast levels. Is this amount of differential / failed delivery typical? This could be commented on. Images like this could also be used to advantage - to justifiably remove them from later processing and assessment of lobe or whole lung analysis, or to use as comparator "almost-control" lobes?

Response 29: We agree that the hypothesis that the change in contrast pre- and post-CT imaging may correlate with gene transfer efficiency (e.g. as determined by GFP expression). However, we do not have the data to support or refute this hypothesis. The following statement was added to the Discussion (lines 297-298): "Pre-and post-delivery CT imaging is also a potential strategy to determine general deposition patterns." We would not advise using CT results to establish "almost-control" lobes.

Comment 30: Line 185: State reason for use of lysophosphatidylcholine (and reference)

Response 30: Lines 236-237 now reads: "LPC is a natural airway surfactant that will transiently disrupt tight junctions to allow Ad to access its basolateral receptor."

Comment 31: Line 188: Could be called "epi-fluorescent" to differentiate from the more common compound microscope fluorescence for GFP.

Response 31: The text was edited as suggested (line 240).

Comment 32: Line 231: I had not expected that there would be challenges telling if the ET placement was in the trachea or the oesophagus. Are the tracheal rings not visible past the chords through the ET tube when the ET tube is placed? Is it a common problem that the oesophagus is intubated by mistake? Another option that would avoid the need for CT for ET placement is to use a cheap Ebay-type smartphone inspection camera (e.g. https://www.ebay.com/itm/USB-Earpick-Visual-Otoscope-Camera-Ear-Inspection-Endoscope-Camera-Wax-Borescope/372631803139?hash=item56c2961503:g:u6gAAOSwDcJckWiU) to visualise the ET tube going into the chords from a close parallel position insertion into the mouth nearby. Could the method used to check rodent lung intubations (a puffer bulb of proper volume) be used in these newborn pigs?

Response 32: Certainly, tracheal rings can be observed by a bronchoscope, and presumably by the suggested smartphone camera, in older (2-3 week) pigs. However, in both cases, the diameter of the camera is greater than the 2.0 mm inner diameter of the ET tube necessary to intubate newborn pigs. A puffer bulb is an idea we have not pursued. Intubating newborn pigs will become reliable and routine with practice. However, if an investigator tries to self-teach intubation in newborn pigs, we suspect that the animal will be gavaged >90% of the time, even if the researcher has experience with intubating mice.

Comment 33: Line 237: This is related to the line 231 - cutting a window would work of course, but it becomes a surgical procedure for intubation which one would typically like to avoid.

Response 33: A tracheal window is only recommended as a practice method on a euthanized animal to visualize appropriate placement of the ET tube. (lines 292-294)

Comment 34: Line 248: Such lobe targeting has recently been reported in rats - McIntyre 2018 Human Gene Therapy, this could be cited.

Response 34: Lines 314-315 now read: “Such targeting was previously described in rats.”

Comment 35: Line 254: The viscoelastic gel method should be referenced.

Response 35: Viscoelastic gel method has been referenced as recommended (line 318).

Comment 36: Line 260: add "..reported on in this paper " after "microsprayer" to reinforce the device used in the method.

Response 36: This has been added as suggested (line 321).

Comment 37: Figure 3 and 4: These figures need some data or text discussions about auto-fluorescence, so that GFP images 3 and B and Figure 4 can be referenced to a control setting. GFP auto-fluorescence can be high in some tissues.

Comparing 3B and 3D, it appears there is no GFP present in the large airways there, which is the same in Fig 4 if the pale brown yellow is taken to be the background (?) auto-fluorescence. The different exposures in the montage in Figure 4 leave me not sure of what GFP positive parts are there. I interpret there are GFP highly-expressing regions (the ~10 short brighter-yellow lines) that elsewhere, but again I am not sure if the general smooth pale-green airways are GFP-transduced, or displaying background / auto-fluorescence.
This Figure needs a lot more on-image labelling and legend detail to be acceptable.

Response 37: Figures 2, 3, and 4 are all examples of successful viral vector delivery to the lung. The GFP expression shown in Figures 3 and 4 is quite remarkable and unmistakably different from auto-fluorescence. We now include white arrows in the figure to differentiate transduced and untransduced regions of the tissue. The left caudal lobe pictures were deleted from the revised manuscript to avoid redundancy. In regards to Figure 4, the following statement is now included (lines 250-252): “In this low power image, GFP expression appears fainter in the large (cartilaginous) airways because the thicker tissue masks the epithelial signal on the luminal surface.”

Reviewer #3:

Comment 38: While this protocol describes an important method for airway delivered gene therapy, the major limitation is that the device described is no more commercially available. This is of crucial importance, since the choice of the aerolization device plays an important role in the efficiency and distribution of the gene therapeutic target. The current evidence indicates that the size of the particles, which are generated through the aerolization device, plays a major role in the deposition pattern in the airways.

Larger droplets will tend to stay in the proximal airways, while smaller can reach the distal airways. Therefore, the change in the device used will affect the results obtained (efficiency, homogenicity, distribution). This, as well as the other factors affecting the efficiency of airway gene delivery should be discussed (e.g. particle size, hygroscopic properties of the vector etc).

Response 38: We discussed the fact that the Penn Century microsprayer is no longer commercially available with the editors prior to submitting this manuscript. We tried to focus this Methods paper on the procedure of intubation to newborn pigs and passing an aerosolizing devise through an ET tube to deliver vector. It is not intended to be a comparison of aerosolizing devises. In the revised manuscript we removed the use of brand names (as per Journal style). Furthermore, we added details concerning different aerosolizing devises and particle sized in the Introduction (paragraphs 2 and 3). We are certainly motivated to identify alternatives to the Penn Century microsprayer and a formal comparison could be the subject of a future publication.

The following statement was added to the Introduction (lines 91-94): "The aerosolization device and droplet size may play an important role in the efficiency and distribution of vector mediated transgene expression. Here, we focus on the procedure of intubation in newborn pigs and passing an aerosolizing catheter through an ET tube to deliver vector."

Comment 39: Another important factor which plays a role in the airway gene delivery in large animals is the ventilation pattern. The depth and rate of breathing affect the deposition pattern of the particles. If a ventilator is not used and the pig retains the own breathing pattern, it should be mentioned that the aerolization is given during inhalation.

Response 39: The respiration rate of a newborn pig under anesthesia is ~30-40 breaths/minute (1-2 breaths/2 seconds) and the delivery takes ~4 seconds. As such, some vector will be delivered during inspiration and expiration. In addition, as now mentioned (line 200-201): "Apnea is a common response to intubation in newborn pigs. Sporadic breathing may last 2-3 minutes. Gentle chest compressions can help facilitate normal breathing."

Comment 40: Introduction: Lines 61-69: the authors may consider including this paragraph to the representative results and not in the introduction.

Response 40: The paragraph was moved as suggested (lines 210-219).

Comment 41: Preparation:
Since a larynx mask is used, it is essential to mention that the animals have to fast overnight in order to prevent aspiration.

Response 41: Line 141 now reads: "NOTE: Animals must fast prior to sedation to prevent aspiration during delivery."

Comment 42: Intubation:
The way to assess the success of intubation in alive animals is of importance and should be mentioned. The visualization of the tube in CT is not the gold standard. The immediate test of successful intubation is the use of an ambu bag to ventilate, auscultate both lungs and confirm the movement of both the left and right thorax parts during inhalation. Also it is important to mention the one-side intubation as a potential complication. Besides the clinical signs, a simple x-ray would visualize the endotracheal tube. This method is much easier and cheaper than a CT scan.

Response 42: The following was added to the revised manuscript (line 229-231): "In past experiments, we used X-ray imaging to confirm airway catheter placement in rabbits. X-ray is a less expensive and more accessible alternative to CT imaging." (Lines 172-173) "In small animals, there is risk of one side intubation and trimming 3-5 cm from the ET tube may be warranted."

We have no experience using an Ambu bag as a confirmatory method but it will be a strategy to try in future studies.

Comment 43: Aerolization:
More details are needed. What is to the experience of the authors the best volume to be used for which animal weight? Too much fluid can cause respiratory insufficiency.

Response 43: Line 188 now reads: "NOTE: For our studies, we limited the volume delivered to ~1 ml/kg." We suspect that a greater volume is possible without causing respiratory insufficiency. We were typically limited by the practicality of vector production.

Comment 44: The sprayer should be tested in advance in order to find the appropriate pressure for pushing the syringe plunge to generate aerosol.

Response 44: Line 119 now reads: "NOTE: A test spray through the microsprayer is recommended prior to setup."

Comment 45: What are the potential side effects of the sprayer and how can they be managed. The experimenter should be prepared to deal with the complications of the procedure (resp. Insufficiency, tracheal bleeding etc).

Response 45: We have seen no side effects to the sprayer; however, apnea is a very common in newborn pigs in response to intubation. Interestingly, such a response is not seen in humans. The following information was added to the protocol (line 200-201): "5.1. Apnea is a common response to intubation in newborn pigs. Gentle chest compressions can help facilitate normal breathing." The apnea response is observed regardless if any material is delivered.

Comment 46: Representative Results:
What vector and what vehicle was used for these CT images? Adenovirus with LPC as

in Figure 3?

Response 46: The following information was added to Figure 2 figure legend (line 267): "Ad-GFP formulated with 0.1% LPC was aerosolized through the microsprayer and the pig was imaged again."

Comment 47: The method to assess the efficiency of the vector delivery should be described in more detail (How to section the lung, prepare for microscopy etc). Are the lungs perfused after euthanasia? Are they inflated before embedding?

Response 47: We added the following statement (line 238-239): "we collected the lungs and used low power fluorescence microscopy to examine the fresh, unfixed tissue." The method we used to fix and embed the tissue for high power microscopy is described in the cited manuscript (4).

Comment 48: Discussion:
Line 230-242: the critical steps should be analyzed in more detail:
1) successful ET placement should be clinically tested (s. previous comment)
2) Pneumothorax is a potential side effect. How do you diagnose? How do you treat? Bleeding is a much more common complication. How do you diagnose and treat (e.g. aspiration, cold saline?)
3) Anesthesia: please discuss the cough reflex associated with shallow anesthesia.

Response 48:
1) The following statement was added (lines 294-296): Multiple examples of verifying ET tube placement are described in the Representative Results; however, with practice, intubation will become reliable and routine.
2) Our only experience with pneumothorax was using the pressurized Aeroprobe in CF animals. The pigs were under propofol anesthesia. After several minutes, they struggled to breath and were euthanized. The pneumothorax was discovered after autopsy. This has never happened using a microsprayer. The following statement was added (lines 302-305): "Using the described protocol in this manuscript, we have never observed a pneumothorax or serious complication. However, if the animal displays an extended period (>20 min) of labored breathing or signs of distress, it should be euthanized for autopsy."
3) I've never experienced a cough reflex in newborn pigs. In my experience, a clenched jaw is the best indication that it needs more isoflurane.

Comment 49: The animals can cough and the virus will be distributed to the immediate environment. What are the precautions? Is there a filter in the ventilatory circuit for filtrating the virus in the exhalation part, in order to avoid cross contamination between the treated and untreated animals?

Response 49: I discussed this comment with colleagues. As a group, we have never observed a pig cough or sneeze as a result of intratracheal vector delivery. We have seen coughing and sneezing as a result of respiratory infections or syringe feeding, but

not vector delivery. In this protocol, the animals are not ventilated. The nose-cone delivering isofluorane is removed just prior to intubation. For Ad-based vectors, the delivery is performed in an ABSL-2 setting where personnel wear proper personal protective equipment (PPE) as a precaution. Shedding of viral-vector has never been demonstrated (although it is difficult to prove a negative). It is of our opinion that detectable cross contamination between treated and untreated animals as a result of co-housing is extremely unlikely.

Comment 50: minor spelling errors (e.g. it is a luer lock and not leur lock syringe)

Response 50: “leur” has been changed to “luer”. Accurate spelling was rechecked throughout.

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2. Cooney AL, Thornell IM, Singh BK, Shah VS, Stoltz DA, McCray PB, Jr., et al. Novel AAV-mediated gene delivery system corrects CFTR function in pigs. Am J Respir Cell Mol Biol. 2019.
3. Steines B, Dickey DD, Bergen J, Excoffon KJ, Weinstein JR, Li X, et al. CFTR gene transfer with AAV improves early cystic fibrosis pig phenotypes. JCI Insight. 2016;1(14):e88728.
4. Cooney AL, Singh BK, Loza LM, Thornell IM, Hippee CE, Powers LS, et al. Widespread airway distribution and short-term phenotypic correction of cystic fibrosis pigs following aerosol delivery of piggyBac/adenovirus. Nucleic Acids Res. 2018;46(18):9591-600.

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