Journal of Visualized Experiments

In Vivo Evaluation of Mucociliary Clearance in Mice

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61929R1
Full Title:	In Vivo Evaluation of Mucociliary Clearance in Mice
Corresponding Author:	Maliha Zahid, MD, Ph.D. University of Pittsburgh Pittsburgh, PA UNITED STATES
Corresponding Author's Institution:	University of Pittsburgh
Corresponding Author E-Mail:	maz7@pitt.edu
Order of Authors:	Kyle Feldman
	Maliha Zahid, MD, Ph.D.
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
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.	Pittsburgh, PA, USA
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TITLE:

In vivo Evaluation of Mucociliary Clearance in Mice

AUTHORS AND AFFILIATIONS:

Kyle S. Feldman[1], Maliha Zahid[1]

[1]Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Corresponding Author:

Maliha Zahid (maz7@pitt.edu)

Email Address of Co-Author:

Kyle S. Feldman (ksf23@pitt.edu)

KEYWORDS:

Mucociliary clearance, motile cilia, respiratory function, in vivo.

Summary:

In this publication, we describe protocols for assessing airway mucociliary clearance (MCC) in mice in vivo utilizing dual-modality radionuclide imaging. This protocol is designed for a single photon emission computed tomography (SPECT) and computed tomography (CT) acquisition protocol using mouse whole body (MWB) collimators in a dual SPECT/CT system.

Abstract:

Respiratory motile cilia, specialized organelles of the cell, line the apical surface of epithelial cells lining the respiratory tract. By beating in a metachronal, synchronal fashion, these multiple, motile, actin-based organelles generate a cephalad fluid flow clearing the respiratory tract of inhaled pollutants and pathogens. With increasing environmental pollution, novel viral pathogens and emerging multi-drug resistant bacteria, cilia generated mucociliary clearance (MCC) is essential for maintaining lung health. MCC is also depressed in multiple congenital disorders like primary ciliary dyskinesia, cystic fibrosis as well as acquired disorders like chronic obstructive pulmonary disease. All these disorders have established, in some case multiple, mouse models. In this publication, we detail a method using a small amount of radioactivity and dual-modality SPECT/CT imaging to accurately and reproducibly measure MCC in mice in vivo. The method allows for recovery of mice after imaging, making serial measurements possible, and testing potential therapeutics longitudinally over time. The data in wild-type mice demonstrates the reproducibility of the MCC measurement as long as adequate attention to detail is paid, and the protocol strictly adhered to.

Introduction:

Cilia are microtubule based cellular organelles conserved across evolutionary history from algae to humans. They emanate from cell surfaces and have a number of functions[1], ranging from recognition of local environmental sensory signals to motility, functions which can be traced back from humans to early unicellular eukaryotic organisms[2,3]. Cilia can be non-motile and single serving as a cell's specialized antenna to process environmental signals; or motile and multiple, beating in synchronized, metachronal waves to generate fluid flow, such as in the lining of the fallopian tubes and the upper and lower airways, except for the terminal bronchioles leading to the alveoli[1,2].

The extensive epithelial surface of the respiratory tract is exposed to a constant barrage of contamination in the form of a variety of potentially hazardous inhaled pollutants and pathogens, necessitating a defense. One key defense mechanism is the mucociliary apparatus of the tracheobronchial tree, where a continuous flow of secreted mucus is mechanically transported out of the airway by the beating of multiple motile cilia lining the apical surfaces of the tracheo-bronchial epithelial cells. These function to entrap inhaled contaminants, and through their continuous, synchronal beating, transport them cephalad[4,5].

Cilia have been demonstrated to have key roles such as in the development of left-right patterning in developing embryos, where motile cilia at the embryonic node break symmetry[6]. Mutations in cilia related genes have been linked to diseases such as congenital heart disease (CHD) due to the asymmetric structure of the heart[6]. Recent studies have reported a high incidence of ciliary dysfunction in the respiratory tracts of patients with CHD, as well as an increased prevalence of post-operative respiratory complications and chronic respiratory tract symptoms in the upper and lower airways[7-10]. Patients with CHD and ciliary dysfunction, with or without heterotaxy, have been demonstrated to have increased risk of respiratory complications and negative respiratory outcomes post-operatively[5,8,10]. Beyond their roles in signaling and development, the importance of airway cilia has been demonstrated by ciliopathies, of which a prime example is primary ciliary dyskinesia (PCD). PCD is a congenital disorder resulting from a number of mutations affecting the motile respiratory cilia, leading to recurrent lung infections, bronchiectasis, and potentially the need for lung transplantation[11]. Additionally, even though cilia are normal in cystic fibrosis (CF), most common congenital disorder in the Caucasian population, MCC is impaired due to thick, viscous mucus resulting from mutations in the CFTR gene[12]. There are multiple mouse models of PCD and CF, as well as an ever-increasing number of models of CHD. Ultimately cilia are versatile structures with many key roles, and a method to assess the function of motile respiratory cilia in vivo can be valuable for pre-clinical study, and assessing effects of mutations as well as drugs on mucociliary clearance (MCC)[13]. The method would also be valuable in assessing effects of novel drugs, gene therapy or interventions on MCC in these mouse models.

There are many different models that have been used to assess MCC. One notable method involves the use of methylene blue dye that has been instilled into the bronchus, with clearance measured by fiberoptic measurement of dye movement[14]. This method is limited by the ability

to observe the movement of the dye, which is more routine in humans than in pre-clinical mouse models. Another notable method is synchrotron phase-contrast X-ray imaging (PCXI), which can be used to track individual particles in an airway. This method is relatively new and not widely accessible[15]. There are numerous ex vivo methods of assessing the airway by excising a trachea for video-microscopy, however these models provide little utility in human patients[16]. High resolution techniques for cilia imaging such as optical coherence tomography are limited in the same way[17].

In this article, we present a reproducible method to measure MCC in vivo that has been used to measure lung clearances in myriad animal models, as well as study MCC in chronic obstructive pulmonary disease and assess the effects of immunosuppressive drugs[18,19]. This method tracks the clearance of the radiopharmaceutical ^{99m}technetium-sulfur colloid (^{99m}Tc-Sc), an insoluble particulate radiotracer, after instillation into the lungs. The radionuclide can then be tracked using single photon emission computed tomography (SPECT)[18,20]. We have further refined this technique for measuring MCC by using dual modality SPECT and computed tomography (CT) imaging with co-localization of radioisotope counts to the lungs and measuring the decrease in these counts over 6 hours. Dual-modality imaging, with co-registration of CT and SPECT images allows for accurate localization of radiation counts to our region of interest, the lungs. Although we describe in detail the method for MCC measurement in mice, the protocol can be adjusted to study MCC in rats. The collimators would need to be adjusted as well as radiation dose. In our view, mouse MCC scans are more technically challenging due to the small animal size, but more useful than rats due to the large number of established mouse models of a number of human disorders. Additionally, due to their lower cost and cost of maintenance in animal colonies, a larger sample size is more feasible in mice.

Protocol:

The University of Pittsburgh's Institutional Animal Care and Use Committee approved all animal protocols specified in this publication prior to undertaking any of these animal experiments.

NOTE: This protocol details how to perform in vivo mucociliary clearance studies utilizing radionuclide imaging with a dual-modality SPECT/CT scanner. The techniques demonstrated are running system calibrations, anesthetizing mice, tracheal intubation of mice, instilling the isotope into the lungs, dual-modality imaging, co-registration of these images, and analysis.

1. SPECT/CT system setup

1.1. Design an appropriate workflow and set up prior to running experiments using living animals.

1.1.1. Use a SPECT acquisition consisting of 60 projections with a step size of 6^{o} between projections with a 40 cm radius of rotation. The CT acquisition consists of 220 projections with a 1.6^{o} angle between projections.

1.2. Ensure that the system has the correct MWB collimators for mice and SPECT imaging in place. If the inappropriate collimators are installed, use the collimator wizard to install the correct ones.

1.3. Run the necessary system calibrations to prepare the system for use.

NOTE: The SPECT and CT components of the scanner need calibration. Calibrate the CT components using a source conditioning and a Dark/Light (D/L) calibration once a day, a Center Offset (COS) calibration every 2 weeks, and evaluate the x-ray hardware every month. The SPECT components need to be calibrated once a year.

1.3.1. To evaluate the x-ray hardware, check the **Evaluate X-ray hardware** box during CT calibrations (**Supplemental CT Calibration Menu**).

1.3.2. To perform source conditioning, check the **Perform source conditioning** box during CT calibrations (**Supplemental CT Calibration Menu**).

1.3.3. To perform a D/L calibration, check the **D/L** box next to the CT acquisition protocol used during experiments during CT calibrations. Uncheck all other protocols (**Supplemental CT Calibration Menu**).

1.3.4. To perform a COS calibration, replace the bed with the calibration ring tool, adjust the bed type settings to match in the motion control settings, and check the **COS** box next to the CT acquisition protocol used during experiments during CT calibrations. Uncheck all other protocols (**Supplemental CT Calibration Menu, Supplemental Calibration Ring**).

2. Mouse Intubation and Instillation

2.1. Weigh the mice to be scanned. If scanning multiple mice, take care to mark the mice for identification purposes using methods such as ear punching or marking of the tail.

2.2. Anesthetize a mouse using 1.5% isoflurane with a gas flow of 2 L/min O_2 in a gas chamber for ~5 minutes to produce anesthesia of sufficient depth, until breathing slows to ~55-65 breaths per minute [16] (**Figure 1A**).

2.3. Remove the mouse from the chamber and suspend by the front incisors on an intubation stand at a 45° incline. Equip the intubation stand with a nose cone to ensure the mouse is anesthetized during the intubation (**Figure 1B**).

2.4. Connect one end of a 50 µm fiber optic wire to a light source and thread a 20-gauge cannula over it using the wire to act as a guide (**Figure 1C**).

2.5. Open the mouth of the mouse and pull the tongue forward using blunt forceps. Illuminate the guide wire and use it to visualize the vocal cords (**Figure 1D**).

2.6. Pass the guide wire through the vocal cords so that the wire is just beyond the vocal cords and resting in the upper trachea. Slide the 1 inch cannula forward along the wire to intubate the mouse, passing the cannula deep enough so that the hub of it is against the animal's incisors (**Figure 1E**). Remove the wire leaving the cannula in place.

2.7. Test the intubation by briefly plugging the cannula with a finger and checking for changes in breathing. Halted breathing or strained breathing while plugging and accelerated breathing upon release are signs of proper tracheal intubation. If there is no change in respiratory patterns upon plugging the cannula, the latter is likely in the esophagus.

2.8. Prepare 0.2 mCi of ^{99m}technetium-sulfur colloid (^{99m}Tc-Sc) in a volume of 10 µL, and pipette into the cannula. Allow the mouse to spontaneously inhale it into the lungs over 1-2 min (**Figure 1F**). Remove the cannula before transferring the mouse to the pallet of the scanner.

NOTE: The radionuclide was prepared and filtered by Cardinal Health.

3. SPECT/CT Imaging

3.1. Transfer the mouse to a 25 mm pallet with a nose cone and secure with tape, taking care to not tape the chest and abdomen too tightly to avoid impairing respiration. Take care to remove any metal ear tags attached to the mouse.

3.2. Prepare a radioactive phantom consisting of 0.05 mCi in 200 µL and place this amount in a 0.2 mL PCR tube. Position the tube by taping to the pallet under the lower abdomen of the mouse, avoiding overlap with the lungs.

NOTE: The phantom is used for the purpose of co-registering CT and SPECT images, as well as a negative control for clearance.

3.3. Insert the mouse into the SPECT/CT system, select the imaging workflow, and run **Setup**.

3.4. Set up the positioning of the detectors on the mouse, and run the imaging workflow.

3.5. Prepare a cage for mice that have received radioactivity post-procedure, with unrestricted access to food and water, and clear labeling using a radiation safety sticker.

3.6. Upon completion of the workflow, remove the mouse from the imaging pallet, and allow it to recover in the prepared cage for a duration of 6 hours between scans (end of scan 1 to beginning of scan 2) with ad libitum access to food and water. 6 h was chosen as it corresponds to the time period in which linear clearance depending on cilia function is taking place with very little alveolar clearance.

3.7. After 6 hours, re-anaesthetize the mouse and scan, along with the phantom, using the same workflow to measure the amount of isotope cleared from the airways.

NOTE: It is critical to allow the mouse to recover as uninterrupted anesthesia with isoflurane for 6 hours will lead to a significant cilia-depressant effect, resulting in near zero mucociliary clearances.

4. Analysis

4.1. After imaging, perform post-processing to reconstruct complete 3D stack images.

4.1.1. Histogram the SPECT images using the factory standard settings for ^{99m}Tc, and then reconstruct using a MAP3D algorithm and point spread function (PSF) reconstruction.

NOTE: The reconstruction was performed using 8 iterations and 6 subsets. An effective reconstruction needs a ratio of subsets to projections at 1:10 or divide evenly into the number of projections, so 6 subsets were used due to the acquisition using 60 projections.

4.1.2. Reconstruct the CT images using the Feldkamp algorithm and a Shepp-Logan filter.

NOTE: The reconstruction was performed using 4 iterations.

4.2. Process the CT and SPECT images in FIJI ImageJ[21] using the reslice tool to generate coronal view images from the default axial images. Then perform a z-stack sum projection on the SPECT image to add the count data from each slice and generate a single image for ease of analysis.

4.3. Resize and co-register the CT and SPECT images using the phantom Eppendorf tube as a reference (**Figure 2A,B**). Track and use consistent resize measurements across all samples.

4.4. Binarize the CT image using auto thresholding, followed by inverting the stack, and performing a z-stack sum projection to generate an outline of the lungs for analysis (**Figure 2C**).

4.5. Rotate the CT and SPECT images and merge the image using the channel tools. Calculate MCC by drawing an ROI around the right lung and measuring (**Figure 2D**).

NOTE: This measurement will be of the total counts in the right lung for the 0 and 6 hour time points, with the 6 hour images corrected for radioactive decay using the formula: $N(t) = N_0 e^{-t}$. ^{99m}Tc-Sc has a decay constant of $3.21e^{-5}$ per second with a half-life of ~6 hours. These values can then be used to calculate a percent clearance.

NOTE: Right lung is chosen for ROI drawing and measuring counts as the mucociliary clearance will transport the radioisotope out of the lungs to the pharynx from where it will be swallowed and end up in the stomach. Quite frequently, counts can be seen in the stomach that may overlap with the left lung and hence produce erroneous counts. This confounding can be avoided by measuring counts in the right lung only.

Representative Results:

Using this protocol, we anesthetized mice in an isoflurane chamber (**Figure 1A**). After achieving an adequate level of anesthesia, mice were placed onto vertical supports (**Figure 1B**) and the vocal cords were visualized using an illuminated guide wire (**Figure 1C-1D**). The mice were intubated and instilled with 0.2 mCi ^{99m}Tc-Sc in volumes of 10 µL through a cannula and mice allowed to spontaneously inhale into the lungs (**Figure 1E-1F**). After image acquisition and processing, the CT and SPECT images were colocalized (**Figure 2A**) using the phantom tube as a landmark (**Figure 2B**). Masks of the lungs were generated from the CT image (**Figure 2C**) and used to draw ROIs around the right lung for analysis at 0 (**Figure 2D**) and 6 hours (**Figure 2E-2F**). To test reproducibility of the protocol, a total of 8 mice were scanned twice on different days with identical experimental conditions, with analysis using a paired t-test showing no significant difference between the repeat scans (p-value=0.9904) (**Figure 3A**). An additional 2 mice were scanned three times on different days with identical experimental conditions, with analysis using one-way ANOVA showing significant matching between the repeat scans (p-value of 0.0041) (**Figure 3B**). A total of 8 mice were scanned and two representative images were displayed (**Figure 4**).

Figure Legends:

Figure 1. Mouse intubation and isotope instillation. Images of the steps needed to intubate and instill isotope into the airway. **A**) The mouse is anesthetized in a chamber. **B**) The anesthetized mouse is placed onto a vertical support, suspended by the front incisors. **C**) An illuminated 0.5 mm fiber optic wire serving as a guide wire is prepared by running it through a 20 G cannula. **D**) The mouth of the mouse is opened using forceps and illuminated using the lighted guidewire to visualize the vocal cords. **E**) The cannula is pushed across the vocal cords and guidewire is

removed. **F**) Soluble isotope is instilled into the cannula using a pipette and the mouse allowed to spontaneously inhale the isotope into the lungs.

Figure 2. SPECT/CT images of an MCC scan. A) A SPECT image that has been co-localized with a CT image. **B**) A CT image with a visible phantom tube that was used for co-localization. **C**) A mask of the airway derived by binarizing the CT image and performing a z-stack sum projection. **D**) The CT mask co-localized with the SPECT image. An ROI for analysis has been drawn around the right lung. **E**) A mask of the airway at 6 hours. **F**) A CT and SPECT co-localized image of the airway at 6 hours with an ROI for analysis.

Figure 3. Clearance measurements of the same mice across multiple scans. A) Two individual repeat clearance were measured for 8 mice with no changes in experimental conditions. A paired t test showed that there was no significant difference between the repeat scans with a p-value of 0.9904. **B**) Three individual repeat clearances were measured for two mice with no changes in experimental conditions. A one-way ANOVA showed there was significant matching between the repeat scans with a p-value of 0.0041.

Figure 4. Co-localized SPECT/CT images of the 0 and 6 hour airway in 2 mice with ROIs drawn at 0 and 6 hours outlining the right lung.

Supplemental Figure 1. A video of the vocal chords illuminated by a fiber optic wire with the effect of breathing visualized.

Discussion:

The role of motile respiratory cilia in both disease and development continues to evolve and be better appreciated. Synchronous, metachronal beating of multiple motile cilia on the apical surface of cells lining the tracheobronchial tree generate cephalad flow producing mucociliary clearance or MCC. MCC is compromised in ciliopathies like PCD[22], acquired diseases like COPD[18], and its importance being recognized in CHDs, not traditionally considered to be ciliopathies. Recent data has shown respiratory ciliary dysfunction in both CHD with heterotaxy[23] and without heterotaxy[7]. Such motile cilia dysfunction was shown to translate into greater respiratory symptoms[9] as well as greater post-operative morbidity[8]. Most, f not all, of these diseases, have mouse models available and our protocol for measuring MCC in mice is a valuable tool that can be utilized to test potential therapeutics.

Animal models provide utility for understanding diseases and the development of therapies. In vivo animal imaging provides further utility with the ability to acquire multiple data points from the same animals, without the need to sacrifice the animals, allowing investigators to follow longitudinal course of disease as well as study duration of treatment effects. The mouse model of MCC has been developed over the course of decades by multiple investigators, initially being performed on beagle dogs using planar scintigraphy, a two dimensional nuclear imaging technique[24]. The technique was adapted for use in mice a decade later, followed by adaptation

to SPECT imaging a decade after that[25,26]. The development of this technique in mouse models was a major development in the relevance of this technique, due to the availability of multiple mouse models of human diseases like PCD in which ciliary function is significantly altered. MCC has been assessed in mouse models of lung denervation and immunosuppression, and has the potential to be used in conjunction with other models[19,26]. MCC measurement studies in human patients with airway diseases such as CF, asthma, PCD, and ciliopathies associated with CHD have been conducted, and have yielded results that the technique can aid both studies of lung physiology and therapeutic efficacy[13].

An important part of this protocol is setting up acquisitions with the correct imaging parameters to acquire accurate images for quantification. A number of factors are key when designing SPECT acquisition settings, including which collimators are used, the number of projections to acquire per revolution, and rotation step size. Collimator selection is a major factor in the sensitivity and resolution of the acquisition, and acquisition settings may need to be tailored to the collimator being used[27]. Alternately, when using bigger animals like rats, the collimators would need to be adjusted. Multiple pinhole collimators for example are more sensitive, but care should be taken when selecting a step size in order to avoid overlapping projections and causing undesired multiplexing, which can further increase sensitivity of the acquisition at the expense of some image ambiguity that can cause reconstruction artifacts[25]. Reconstruction setup is also key to generate quantifiable images. MAP3D is a commonly used iterative reconstruction algorithm, and PSF is a common reconstruction model. Both are reliable for reconstructing images, but care should be taken when setting the number of iterations and subsets. A higher number of iterations will increase the computational time required for the reconstruction, and increase the quality of the reconstruction with diminishing returns upon further increase.

In order to quantify images in ImageJ, the ideal measurement tool to use is RawIntDen, which outputs the sum value of pixels in a selection. When quantifying SPECT data across differently sized lung ROIs, the use of RawIntDen provides an absolute measure of counts and avoids adjusting the measurement to the area of the ROI, like the mean measurement would[21].

This technique has a number of associated sources of error that the investigator should be cognizant of when applying this technique. A notable confounder is the use of anesthetic agents. Isoflurane is a fast acting, inhaled anesthetic that the mice recover from rapidly after completion of an acquisition. However, care should be taken to provide the mice with ample time to recover in their cages, and not kept anesthetized any longer than necessary. In our personal experience (unpublished data) mice that were kept anesthetized continuously using inhaled isoflurane between the 0 and 6 hour time-point showed negligible clearance. Likewise, a controlled dose of anesthetic is also necessary to ensure rapid recovery. When securing the animal to the pallet for imaging, the phantom tube used for co-registration should be kept low on the stomach to avoid artifacts from overlapping with the lungs. Likewise, to ensure a quality CT image, take care to remove any metal tags from the mouse to avoid artifacts from x-ray scattering.

The current MCC protocol can be applied to myriad animal models. This technique has a negligible effect on the health of the animal scanned, is well tolerated by mice, and because of this it can be used with disease models without risking the health of already delicate mice. The strength of this methodology comes from it being an in vivo technique, which allows for the acquisition of consistent and repeatable measurements of airway function without the sacrifice of animals to excise tracheas for video-microscopy, that ex vivo models require[26]. The consistency of this technique in producing repeatable measurements across multiple scans of the same animals, allows for the same animal to be treated with different agents or potential therapeutics, and statistical comparisons made between the same animal to reduce biological variability inherent in any animal model, thereby reducing the sample size needed to show statistically significant differences.

The assessment of airway function using the MCC technique can be adjusted to a variety of animal models and applied to many different models of airway health, as well as testing new therapies. The airways of mouse models of PCD can be assessed using this technique, as well as models of COPD. Our method can also be utilized to study differential effects of various anesthetics on MCC that are in common clinical use. Finally, the effects of therapeutic agents on the airway can also be assessed using this model. As previously stated but bears repetition, as it is an in vivo measurement it allows for repeat MCC assessments over the course of a disease, as well as test benefits of therapeutic interventions over time. Additionally, mice are the most common laboratory animals used to mimic/study human diseases, with, in some cases, multiple transgenic mouse models of human disease available to choose from.

Acknowledgements:
M.Z. and K.S.F. and this work was supported by a grant awarded under the Pitt Innovation Challenge (PInCh), through the Clinical and Translational Science Institute at the University of Pittsburgh, and NHLBI R01 grant HL153407, awarded to M.Z.

Disclosures:
None related to this work.

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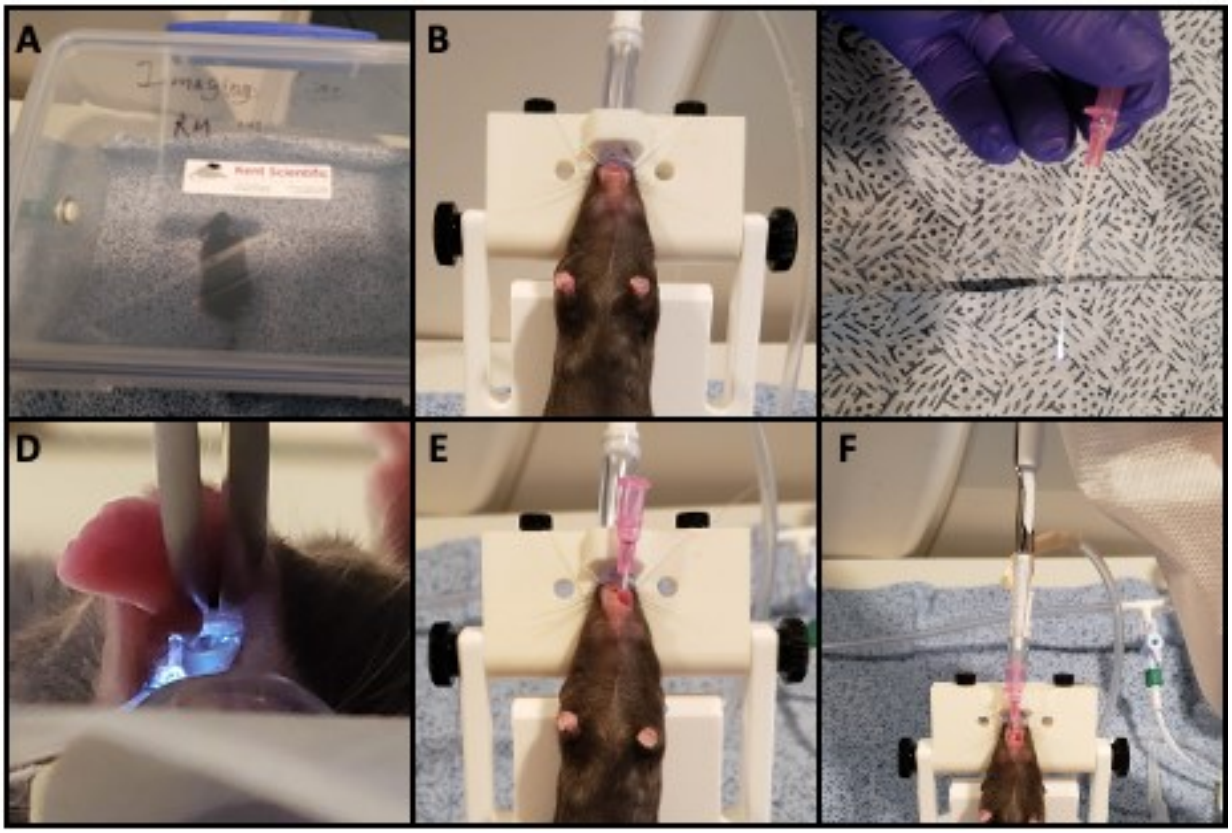
Figure 1.



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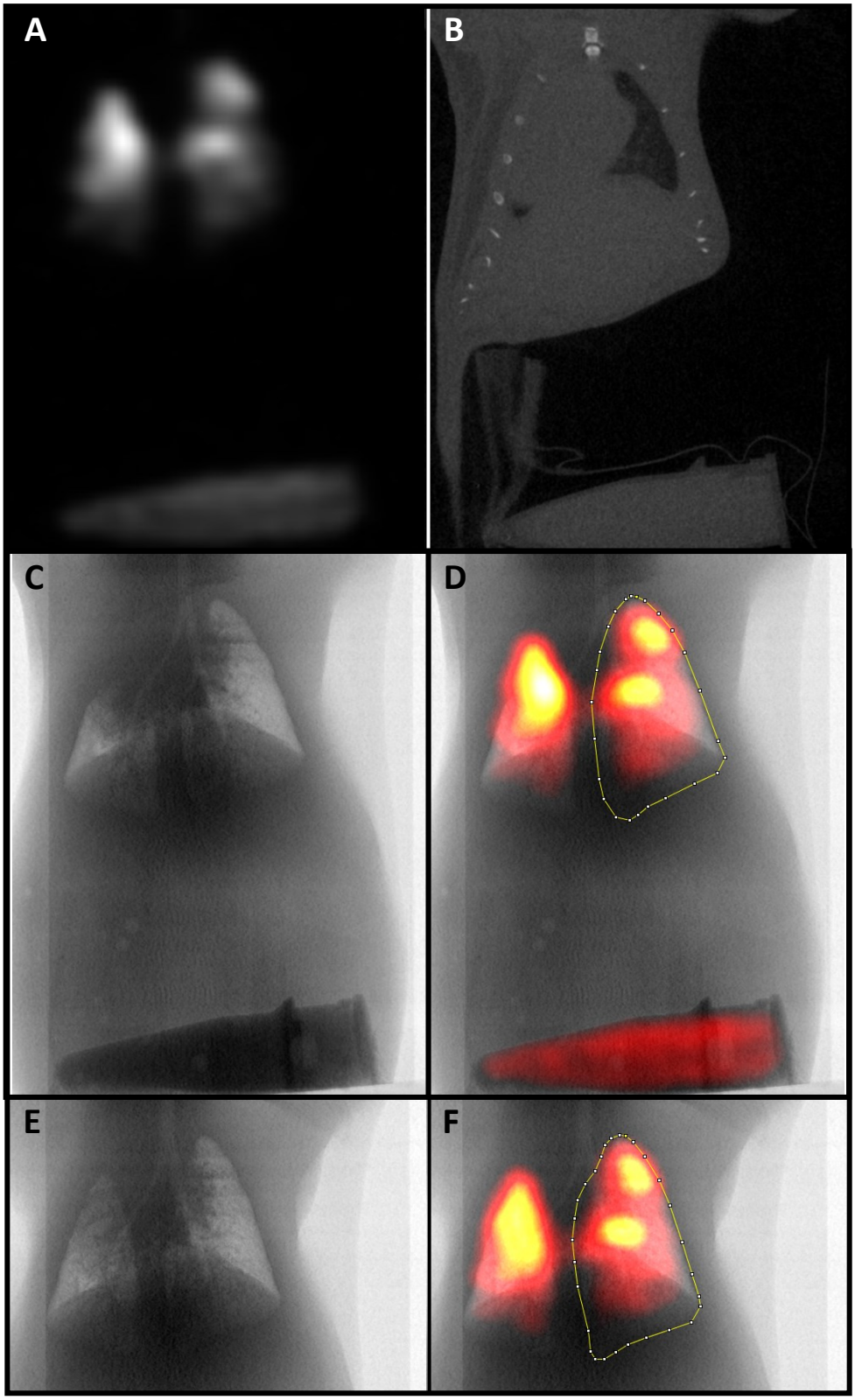

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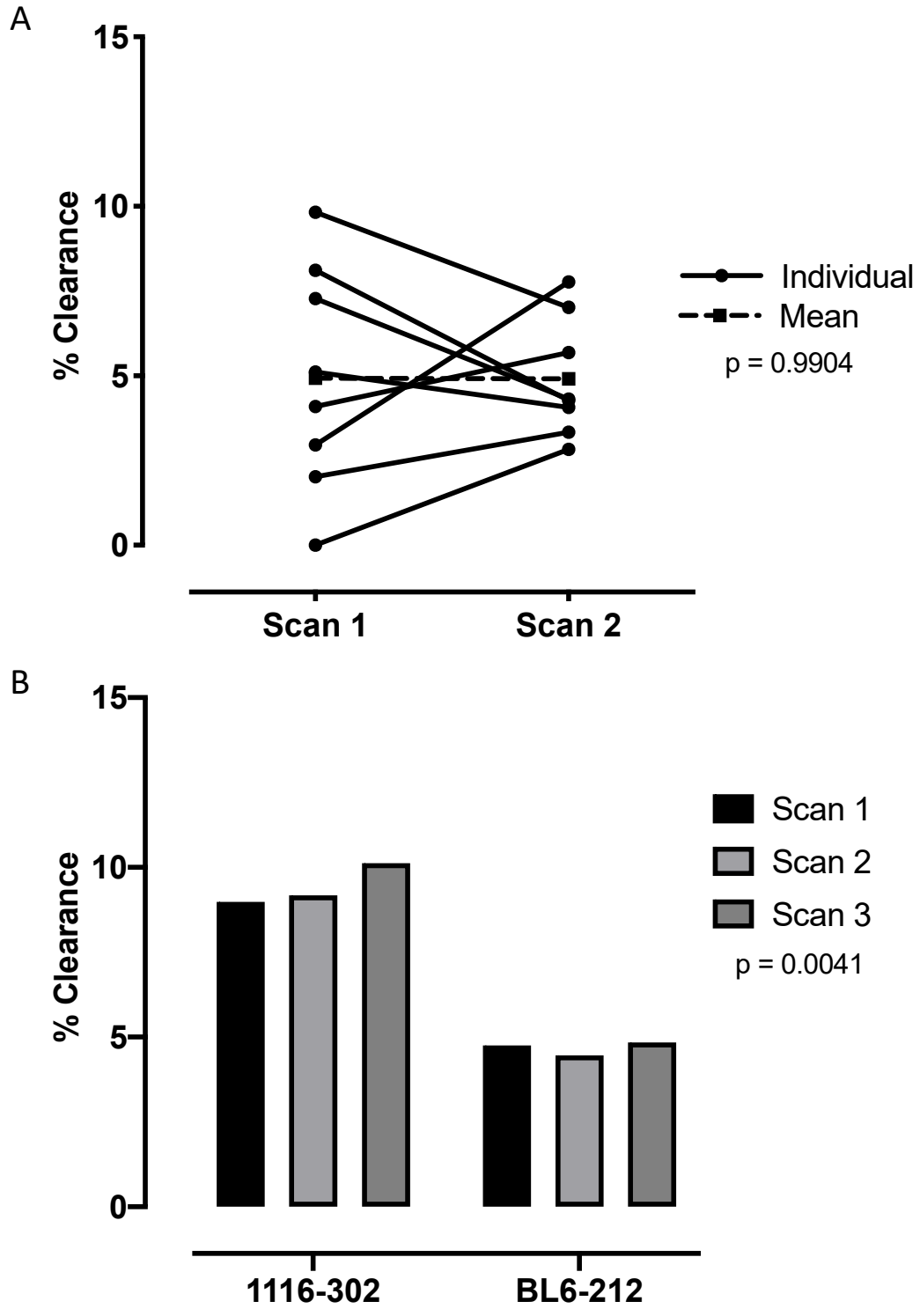
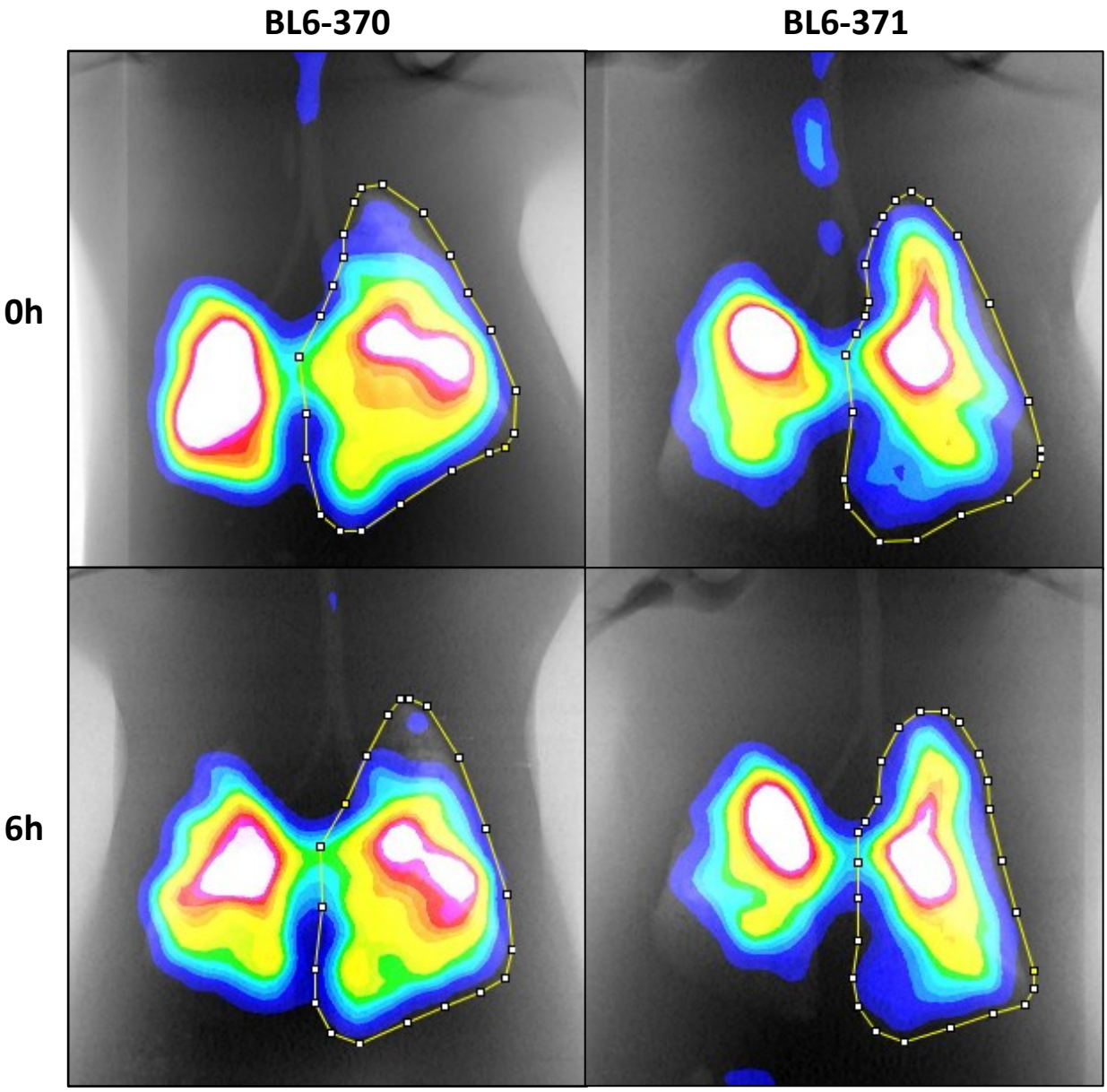
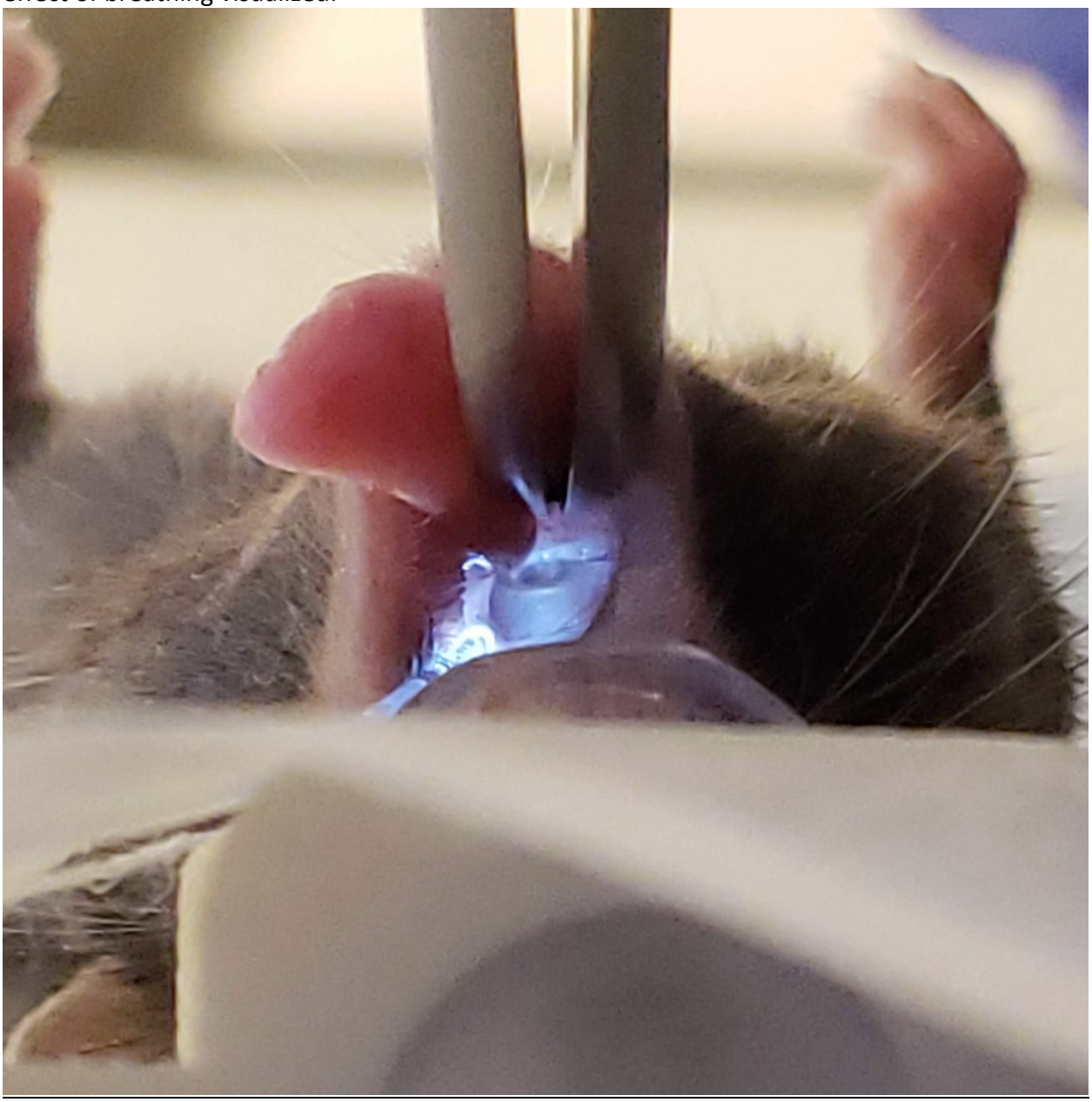


Figure 4.



Supplemental:

Supplemental Figure 1. A video of the vocal chords illuminated by a fiber optic wire with the effect of breathing visualized.



Name of Material/ Equipment	Company	Catalog Number
500 µm Unjacketed Fiber Optic Wire	Edmund Optics	02-532
99mTechnecium-Sulfur Colloid	Cardinal Health	
Anesthesia Vaporizer	Vetland Medical	A13480
Durmont #5 Forceps	Fine Science Tools	99150-20
FIJI ImageJ 2.0.0-rc-65/1.52p Software		
Introcan Safety Catheters 20G 1inch	Fisher Scientific	NC1534477
Isoflurane	Henry Schein	118-2097
Mouse Intubation Stand	Kent Scientific	ETI-MSE-01
Siemens Inveon dual-modality SPECT/CT	Siemens	
Single Channel Anesthesia Stand	Summit Anesthesia Solutions	22860

Comments/Description

Response to Reviewers' comments

We thank the Reviewers for their time and insightful comments. We have made extensive changes to the manuscript, as well as provided a point-by-point response to their comments below.

Reviewer #1:

Manuscript Summary:

This report by Feldman and Zahid describes an in vivo method for measuring mucociliary clearance in mice using a combination of SPECT and CT imaging. There is a need for better methods/standardization of measuring MCC in mouse models, as the authors point out, and a video demonstrating some of the technical aspects of these measurements would be a useful contribution to the field.

Major Concerns:

There are 2 major concerns with this report. First, the level of clearance in most of the mice is less than 5% of the radioactivity deposited. This is such a low level of clearance, that it is not clear the mice are actually clearing anything by MCC. From the images shown, almost all of the radioactivity is deposited deep in the lung (alveolar space), where mice have few ciliated cells. This is probably the result of using a fairly large volume of soln (10-25 ul) to deliver the radioisotope.

The clearance in mice of the same age, sex and strain varies by a substantial amount. In our studies, the clearances range from 5.2 to 7.2%. We have since the original writing of the manuscript, tried to keep volumes down to 10uL. We have made that change in the manuscript.

The second major issue is that there is no demonstration that the technique can actually be used to measure changes in MCC. Since even in control mice, only 5% of the radioactivity is cleared, and the range was ~0-10%, it is hard to see how this method would ever be useful to measure changes in MCC.

This is a very interesting point raised by this Reviewer. It is true that the clearance is not as high as one would like to see and there is a lot of variation from mouse to mouse. However, our protocol is extremely sensitive with little variation seen with repeat measurements in the same mouse. We have actually done studies of clearances in B6 mice at baseline and after ~90mins of deep anesthesia with various anesthetic combinations in clinical use. For an N=8 mice, we were able to show that Propofol and Fentanyl based anesthetic combinations do not decrease MCC significantly whereas Ketamine and Midazolam based anesthetics decrease MCC statistically significantly. That manuscript is being resubmitted to Anesthesiology.

Minor Concerns:

As a methods paper, there are too many places where specific details are left out. Exact volumes, lengths, etc. should be specified. For example, "0.2-0.5 mCi 99mTc-Sc in volumes of 10-25 µL" is not a very precise method. These numbers represent a 2.5 fold change in radioactivity and volume. This will likely have a significant effect on where the radioactivity is deposited and how it is cleared. (should this be adjusted, based on the weight of the animal?) Also, what is the length of the cannula? The guide wire? How long is the inhalation period? 5 minutes? 35 minutes? Should the cannula be removed before the mouse is placed on the pallet?

We have added the details and changes specific volumes/doses instead of ranges. The volume has been changed to 10uL with a dose of 0.2mCi of the radiation. The cannula is a standard 20G, 1 inch cannula. The guide wire is measure to go just beyond the tip of the cannula. The hub of the cannula should be against the incisors to avoid right bronchial intubation. The mouse is allowed to inhale it over 1-2mins. The cannula is removed before placing the mouse on the pallet. All of these details have been added to the protocol.

The paragraph "An important part of this protocol is setting up acquisitions with the correct imaging parameters to acquire accurate images for quantification…." Does not seem useful to someone who is not an imaging/radiation expert, and maybe is not helpful. It seems to be general in nature, and does not really give specific details about performing MCC measurements in mice.

We have changed that statement and emphasized use of the phantom tube to make sure correct registration of the CT and SPECT images are done post acquisition.

Figure 4. What is the scale of the color intensity? What is the outline, how was it drawn, and why is it not the same for the 0 and 6 hr time point?

The color intensity is controlled by the program and adjusted (without changing the counts) to optimize the SPECT image visually. For drawing the R01, the SPECT intensities are lowered significantly to be non-existent and ROIs drawn based on CT image of the right lung. The two time point ROIs are not exactly the same because the mouse is recovered between the two timepoints and allowed to move freely in its cage. Upon repositioning for the second image, the phantom is used again and a new ROI redrawn as the mouse will not be exactly in the same place it was the first time around.

4.5" MCC is then calculated by drawing an ROI around the right lung and measuring (Figure 2 D)." Measuring what? Calculated how? This sentence is not complete, or accurate.

Measuring the radiation counts collected from the ROI drawn over the right lung. We have completed the sentence and made it clearer.

Reviewer #2:

Manuscript Summary:

In general, I would comment that this manuscript is well written, and the instructions are clear. From the introduction it seems the novelty of this method is that it uses the CT in addition to the SPECT imaging, however I think a few sentences are needed to justify and make clear why the addition of the CT imaging is needed to improve this method.

Thank you for the suggestion. We have added the additional clarification to the introduction.

Minor Concerns:

Title:

* As there are different methods for measuring Mucociliary Clearance I think it would be useful to specify the use of the SPECT/CT imaging modality in the title of the methods.

We have changed the title to be more descriptive.

Introduction:

* There should be more justification as to why the CT imaging needs to be added to the SPECT imaging. What is the limitation of only using the SPECT imaging to measure MCC? This should be highlighted further to justify the addition of the CT imaging.

We have added additional justification to use of CT imaging in concert with SPECT images to the Introduction section.

Protocol:

* 1.1 - Does the term 'workflow' correspond to the experiment workflow (i.e. what the experiment protocol is and the actions of each participant), or the motion plan for the SPECT/CT device?

This refers to the acquisition workflow for the SPECT and CT. we have clarified that.

* 1.2 - The acronym MWB should be defined. It stands for mouse whole body.

We have defined it upon first usage.

* 1.3 - I'm not sure it's necessary to define all the calibration methods required for the CT and SPECT machines, particularly if they are routine calibrations that are not specific to this method.

As changes in calibration can create a world of difference in the results, we would like to leave them in for protocol completion. The familiar reader can skip, while the novice or a new user would be helped greatly by it.

* 2.7 - I understand that it is important that the cannula is positioned correctly, however ethically I am concerned that stopping the animal from breathing just to determine if the cannula is in the right position is unnecessary. An alternative could be to puff air through the cannula and observe any potential chest movement. Or if you have access to the raw projections from the CT device, it may be possible to use one projection (i.e an X-ray image) to confirm that the cannula is in the correct position.

The cannula is blocked for a mere second or two and changes in breathing observed. However, we have added other markers of assessment of placement, like the puff of air you suggested or seeing the bubble of liquid technetium move with every breath.

* 3.1 - Was the mouse/pallet aligned to the centre of rotation of the CT device? If so the method used to align the mouse and/or pallet should be explained.

Correct mouse positioning by looking at scout films will be demonstrated in the video. Center-offset calibration is performed for the system every 2 weeks and alluded to in the manuscript.

* 4.3 - Was the registration process conducted manually or automatically using a specific registration function on ImageJ? Also was the registration a rigid registration (rotation and translation only) or did the registration process involve deforming or warping the images?

The registration was a rigid registration using the phantom as guide. The registration process was conducted manually using a line coregistration tool for ImageJ. A rigid registration was used to rotate and resize the images using bicubic interpolation, with consistent settings used across all images.

* 4.5 If possible, an appropriate reference should be provided for the formula and/or decay constant used to calculate clearance.

The radiation decay formula is quite common and firmly established in Nuclear Physics. There are no recent, relevant references. There are multiple online calculators, with one included here. http://www.radprocalculator.com/Decay.aspx

Representative Results:

* Following on from my earlier comments, I think it would demonstrate the advantage of using the combination SPECT/CT method if results also generated results from a SPECT only scan. That is, attempt to calculate the MCC using only the SPECT images (without the ROI from the CT), and show how the SPECT/CT results are superior or how the SPECT only results highlight the limitation of the SPECT only setup.

Our protocol took almost 2 years to develop and finalize. We did do PLANAR imaging when we first started and quickly realized that though it has temporal accuracy, the spatial resolution, especially in a small animal, is lacking and therefore not useful. As this is a particularly challenging protocol that requires attention to detail, we did not want to detract the reader from the protocol by throwing in too much of techniques that did not work.

Discussion:

* The authors stated that "This technique has a negligible effect on the health of the animal scanned". Whilst this is true when one or two CT scans have been conducted on the animal, repeated CT scans of any animal over a short period of time will cause an accumulation of radiation dose. Whilst I imagine a number of CT scans would need to occur, this may require mentioning, particularly if longitudinal studies.

Thank you. That is indeed an important point. For repeated studies on the same mouse, we allow for at least 6 half-lives of Technetium sulfur colloid (T1/2 ~6hrs) to allow for complete radioactive decay of the isotope. We have clarified this in the manuscript.

Reviewer #3:

Overall comments

The manuscript describes a method of evaluating mucociliary clearance in mice, using a radiotracer deposited into the lung as a fluid bolus. The radiotracer method is relatively standard, having been described in the literature back at least as far as 2001 (10.1152/jappl.2001.90.3.1111). So I'm not really sure what new information is added here. Certainly the SPECT / CT equipment is newer than what other studies report, but the usefulness of this description over others is not clear. I understand that JoVE is a methods journal whose primary purpose is to demonstrate new (and challenging) techniques that cannot be adequately described in text. I'm not sure that this paper really fits into that category because the bulk of the parts to be filmed are related to setup of the imaging software and the use of FIJI to do the analysis. The animal work (anaesthesia / intubation) is a standard, well documented procedure. Many figures are dedicated to the intubation method, but this is already well covered by https://www.jove.com/t/60844/repeated-orotracheal-intubation-in-mice

I also have a few major concerns about the data and technique. The first is that all data presented is in normal animals. Is there any data to show that this technique can actually separate effects of disease? It's great that the measurements are repeatable (i.e. precise), but if they are not also accurate for discriminating different diseases then they are not very helpful. The second is how do you convert measurements into MCC rates that are provided by other direct assessment methods? The third is what's the effect of delivering Tc as a fluid, rather than inhalation as dry particulates.
The images provided were very low quality, making them difficult to assess. The provision of line numbers would also make review much easier. The grammar also needs to be improved throughout.

Thank you for your comments. Please see responses to Reviewers 1 and 2 above. We have indeed added/stressed the importance of dual-modality imaging as improving accuracy. Although we have not done MCC scans in mice harboring primary ciliary dyskinesia mutations, we have done extensive studies in mice after various anesthetic challenges and shown a differential effect of anesthetics on clearances (Manuscript under review). This method cannot be converted to other rates due to different time lengths over which clearances have been measured in the literature. There is literature showing that clearances are not linear throughout the measured period. We also have only used technetium sulfur colloid in fluid form as delivered by radiopharmacies for injecting into humans. We are unable to say how particulate matter would behave. As an aside, we did try using fluorescent beads inhalationally and use IVIS (in vivo imaging systems) to try and measure clearances but the signal was neither stron enough nor with enough spatial resolution to allow for these measurements. The grammatical mistakes have been rectified as well.
Specific comments
P3 para 1: Add nasal airways to this list, or alter tracheobroncheal tree to be upper and lower airways.

We have made the change.
P3: Probably also worth mentioning CF along with PCD.

We have added CF to the list of disorders.
P3: There should be a paragraph in here to describe the currently available methods of measuring MCC models, including dye transit, synchrotron PCXI of marker particles, CT of microdisks (in pigs), uOCT, and of course clearance of radiolabelled particles. The relationship of this work to articles such as these should be clarified: 10.1152/japplphysiol.00669.2009 and 10.1152/jappl.2001.90.3.1111

We have added the references and a paragraph to put out method in context of existing ones.
P5: Could these methods be applied to other animals such as rats?

We have mentioned rats along with the limitation of rat models.
P7: Any safety information to be aware of for Tc?

All radiation and radioisotopes need to be handled with appropriate precautions. All personnel need to be radiation safety training prior to handling isotopes. Beyond that, there is nothing specific aboiut Tc. We used clinical grade Tc in our MCC scans.
P7: Provide some information about why 6 hours is chosen. Can this time be varied? How critical is this time? What happens to the results if it is longer or shorter? Presumably measuring image acquisition times is also important for calculating clearance?

We have added the reasoning for the choice of 6 hrs. Please see reference Wanner, A., Salathé, M., O'Riordan, T.G. Mucociliary clearance in the airways. American journal of respiratory and critical care medicine. doi: 10.1164/ajrccm.154.6.8970383 (2012).

P8: Does this measurement automatically take into account the decay? I'm not clear how this relates to the note below. How do you convert the pixel intensities into a clearance measurement?

The measurement is corrected using the decay formula. We measure, correct for decay, and calculate the % change in pixel intensity.

P9: Were these all normal mice? What do the results look like in a disease model?

The methods paper outlines the technique in normal mice. We, and other investigators, have seen reduction in MCC in mouse smoke models of COPD. We have also studied a differential effect of anesthetics on MCC and that body of work is the subject of another manuscript in preparation.

P11 para 1: This is all a repeat of what's in the introduction. Remove or reduce.

We have reduced the paragraph.

P11 para 2: There is no one model of MCC. I assume you are talking about measurement methods here? In any case, this information should be in the introduction, not the discussion.

We have clarified this further.

P11: "MCC has been used to assess" should be "MCC has been assessed in"?

We have changed this line as per your suggestion.

P11: When you say "Studies in human patients", do you mean MCC assessments?

Yes, and we have clarified this.

P12 para 2: Is this normal mice? Some strains may have trouble with 25 ul of fluid instilled into the lung, depending on their pathology.

We have reduced volumes to 10uL.

P12: What are mouse models of anaesthesia? Do you mean that this method can be used to assess the effects of different anaesthetics on MCC?

Yes, and we have clarified this.

P12: Deflection? Do you mean scattering? Or beam hardening?

Beam scattering. We have changed the sentence.

P12: Any other weaknesses? Global measure, so can't tell regional effects? Also seems like you're saying you can't do left lung? What's the sensitivity of the method?

Yes, our MCC method is a global measure. We cannot assess regional differences in clearance. Left lung can be used but due to occasionally finding radioisotope in the stomach which would overlap with counts form the left lung, we prefer to measure only in the right lung to avoid confounding. Where no counts in the stomach are seen, especially on the 6hr images, clearances can reasonably be measured from either the right or left lung. We have no data to suggest that the two lungs differ in their clearances.

P18: What's the difference between A and B? Was it just that for 2 animals you did it 3 times rather than 2?

Figure 3A is data on 8 mice that were scanned twice. Figure 3B are two different mice that were scanned three times each a suitable time apart, to show that there is very little repeat measurement variability.
P18: In B what do these codes mean? Are these the same strain of mice? If so, why are there statistically significant differences between the two animals?

These are different strains of mice. Our point in showing the data was to show the consistency of the method on repeat measurements.