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Dear Dr Cao,

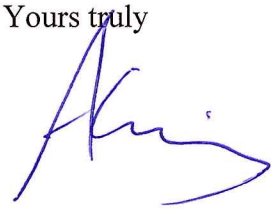
RE: Submission of revised manuscript **JoVE60565_R2**.

Thank you very much for your decision letter of September 26, 2019, and helpful suggestions for modification of our manuscript.

We have introduced the suggested corrections as indicated in the manuscript file. In addition, we have modified the Materials file according to your suggestion.

We hope that you find our revised manuscript suitable for publication in JoVE. Thank you.

Yours truly



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TITLE:

Detection of Lung Tumor Progression in Mice by Ultrasound Imaging

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KEYWORDS:

lung cancer, LSL-KRAS G12D mouse model, ultrasound imaging, 2D volume quantification, tumor formation, B-mode

SUMMARY:

This protocol describes the steps taken to induce KRAS lung tumors in mice as well as the quantification of formed tumors by ultrasound imaging. Small tumors are visualized in early timepoints as B-lines. At later timepoints, relative tumor volume measurements are achieved by the measurement tool in the ultrasound software.

ABSTRACT:

With ~1.6 million victims per year, lung cancer contributes tremendously to the worldwide burden of cancer. Lung cancer is partly driven by genetic alterations in oncogenes such as the KRAS oncogene, which constitutes ~25% of lung cancer cases. The difficulty in therapeutically targeting KRAS-driven lung cancer partly stems from having poor models that can mimic the progression of the disease in the lab. We describe a method that permits the relative quantification of primary KRAS lung tumors in a Cre-inducible LSL-KRAS G12D mouse model via ultrasound imaging. This method relies on brightness (B)-mode acquisition of the lung parenchyma. Tumors that are initially formed in this model are visualized as B-lines and can be

quantified by counting the number of B-lines present in the acquired images. These would represent the relative tumor number formed on the surface of the mouse lung. As the formed tumors develop with time, they are perceived as deep clefts within the lung parenchyma. Since the circumference of the formed tumor is well-defined, calculating the relative tumor volume is achieved by measuring the length and width of the tumor and applying them in the formula used for tumor caliper measurements. Ultrasound imaging is a non-invasive, fast and user-friendly technique that is often used for tumor quantifications in mice. Although artifacts may appear when obtaining ultrasound images, it has been shown that this imaging technique is more advantageous for tumor quantifications in mice compared to other imaging techniques such as computed tomography (CT) imaging and bioluminescence imaging (BLI). Researchers can investigate novel therapeutic targets using this technique by comparing lung tumor initiation and progression between different groups of mice.

INTRODUCTION:

As the leading cause of cancer-related deaths worldwide, lung cancer remains refractory to treatments, mainly due to lack of relevant pre-clinical models that can recapitulate the disease in the lab[1]. Around 25% of lung cancer cases are due to mutations in the KRAS oncogene[2]. KRAS-driven lung cancer is often associated with poor prognosis and low response to therapy, highlighting the importance of further studies in this disease[2].

We optimized a method that allows the relative evaluation of lung tumor growth in real time in KRAS lung cancer-induced immune-competent mice. We use Lox-Stop-Lox KRAS G12D (LSL-KRAS G12D) mice in which the KRAS G12D oncogene can be expressed by Cre lentiviral vectors[3,4]. These vectors are driven by carbonic anhydrase 2, allowing the viral infection to take place specifically in alveolar epithelial cells[5]. In addition, to accelerate the initiation and progression of lung tumors, the lentiviral construct also expresses P53 shRNA from an U6/H1 promoter (the lentiviral construct herein will be referred to as Ca2Cre-shp53)[6]. The biological relevance of this method lies in the natural course of lung tumor development in mice as opposed to xenografts of non-orthotopic tumors in mice. An obstacle using the orthotopic method is monitoring lung tumor growth without sacrificing the mouse. To overcome this limitation, we optimized ultrasound imaging to permit the analysis of lung tumor progression in two-dimensional (2D) mode in this mouse model. Initiating tumors at 7 weeks post-infection are reflected as B-lines in ultrasound images, which can be counted, but will not reflect the exact number of tumors present on the lung. B-lines are characterized by laser-like vertical white lines arising from the pleural line in the lung parenchyma[7,8]. Large tumors can be visualized after 18 weeks of infection. The relative volume of these tumors is quantified by 2D measurements done on ultrasound.

This method is optimal for researchers investigating the effect of pharmacological drugs on lung tumor growth in the LSL-KRAS G12D mouse model. In addition, lung tumor progression can be compared between mice with different genetic lineages, to examine the importance of the presence or absence of certain genes/proteins on the development of lung tumor volume.

PROTOCOL:

Animal studies were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of McGill University and procedures were approved by the Animal Welfare Committee of McGill University (animal use protocol # 2009-5754).

1. Generation of CA2Cre-shp53 lentiviral titre

NOTE: The following protocol is the same as that described in Xia et al.[6], with minor modifications.

1.1. Preparation of lentivirus (for 15 x 10 cm dishes)

1.1.1. On day 1, plate healthy HEK293T cells (7.5 x 10^6 cells per 10 cm dish) with 10 mL of Dulbecco's modified Eagle medium) (DMEM), 10% fetal bovine serum (FBS), and 1% pen/strep. Culture in a 37 °C, 5% CO_2 incubator.

1.1.2. Prepare the mix for calcium-phosphate transfection (mixture for 15 plates). Prepare tube A containing 225 µg (15 µg/plate) of the lentiviral vector (Ca2Cre-shp53), 75 µg (5 µg/plate) of PsPAX2 plasmid (containing HIV-1 gag and HIV-1 pol genes), 75 µg (5 µg/plate) of pMD2.G plasmid (containing VSV-G gene) for packaging, a final concentration of 0.15 M of $CaCl_2$ and fill the tube with distilled H_2O up to 3.75 mL. Prepare tube B containing 3.75 mL of 2x HEPES-buffered saline (HBS; 50 mM HEPES, pH 7.05, 280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, and 12 mM D-dextrose).

1.1.3. Vortex tube A and add it dropwise to tube B under continuous vortexing.

NOTE: The total volume will be 7.5 mL.

1.1.4. Incubate at room temperature in the dark for 20−30 min.

1.1.5. Approximately 9 h after plating the cells, add 500 µL of the transfection mixture dropwise into the cell medium (7.5 mL/15 dishes: 0.5 mL per dish).

1.1.6. Swirl gently each dish to mix, and incubate all dishes at 37 °C, 5% CO_2.

1.1.7. On day 2, 12−18 h after transfection, replace the media with 10 mL of antibiotic-free reduced serum media (**Table of Materials**) per 10 cm dish. Place dishes back in the 37 °C, 5% CO_2 incubator.

1.1.8. On day 3, collect the media containing lentiviruses expressing Ca2Cre-shp53 and filter through 0.45 µm filters. Replenish the media with fresh antibiotic-free reduced serum media.

NOTE: The collected media can be kept at 4 °C for no more than 3 days.

1.1.9. On day 4, collect, for a second time, the media containing the lentivirus and filter through a 0.45 µm filter. Keep at 4 °C (for no more than 3 days).

1.1.10. Combine the virus supernatants from steps 1.1.8 and 1.1.9. Concentrate them through centrifugal filter units (**Table of Materials**) by centrifuging at 1,372 x *g* for 30 min at 4 °C. Repeat the process until all the collected media has passed through the columns.

NOTE: After each centrifugation, 100−200 µL of concentrated filtrate can be harvested.

1.1.11. Collect and mix the concentrated filtrates in a 15 mL ice-cold tube. Mix the concentrated lentiviruses well and then aliquot (e.g., 100 µL/ tube). Store at -80 °C.

1.2. Lentiviral titration

NOTE: Immortalized mouse embryonic fibroblasts (MEFs) expressing a loxP-flanked allele of green fluorescent protein (GFP) are used in this protocol for the quantification of viral titer. However, any cell line with a loxP-GFP allele should be suitable for this step.

1.2.1. Culture cells expressing a loxP-flanked allele of GFP with DMEM, 10% FBS and 1% pen/strep at 37 °C, 5% CO_2.

1.2.2. Plate 2 x 10^5 cells in two 50 mm wells of a 6-well plate.

NOTE: The cells of one well will be used for lentiviral infection while that of the other will be used as a negative control.

1.2.3. The next day, replenish the cells with 2 mL of DMEM, 10% FBS, and 1% pen/strep 2 h before lentiviral infection.

1.2.4. Add 20 µL of the CA2Cre-shp53 lentivirus (the volume may vary when needed) in the lentiviral infection well.

1.2.5. After 3 days in culture, determine the frequency of the Cre-induced GFP-positive cells by flow cytometry, as shown in **Figure 1**. Wash cells with phosphate-buffered saline (PBS), detach by trypsinization and collect cells by centrifugation at 112 x *g*.

1.2.6. Wash the cells twice with PBS. Finally, suspend the cells in 100 µL of PBS. Determine the percentage of GFP positive cells by a cell analyzer (**Table of Materials**).

1.2.7. Calculate the lentivirus infectious/functional titer using the following formula:

$$\text{Infectious/functional titer [TU/mL]} = \frac{\mathrm{F} \times \mathrm{Cn}}{\mathrm{V}} \times \mathrm{DF}$$

where F is the frequency of GFP-positive cells and calculated by subtracting the frequency of GFP -positive cells after infection (Fi) from the frequency of GFP-positive cells (background) prior to infection (Fc) as shown in **Figure 1** (F = Fi-Fc), Cn is the number of plated cells (2 x 10^5), V is the volume of the inoculum (mL), and DF is the virus dilution factor.

NOTE: The infectious/functional concentration = ~2 x 10^6 TU/mL.

2. Intratracheal intubation of lentiviruses in LSL-KRAS^{G12D} mice

NOTE: The method of intratracheal intubation was used as described in the published protocol by Vandivort et al.[9]. In this protocol, mice LSL-KRAS^{G12D} mice in C57BL/6 background are used at age 6−8 weeks. A home-made working procedure board is used as described in Vandivort et al.[9]. The board is positioned in front of the experimenter in a convenient workspace (approximately 1 m^2).

2.1. Prepare a spirometer by removing the plunger of a 1 mL syringe and loading 60 µL of PBS into the syringe.

2.2. Attach a 22 G catheter tip into the syringe and set aside.

2.3. Anaesthetize the mice by intraperitoneal injection of a 1 µL/gram of mouse body weight of etamine (50 mg/mL)/xylazine (5 mg/mL)/acepromazine (1 mg/mL) cocktail. Ensure proper sedation of the mouse by a low respiratory rate (1 breath every 2 s).

2.4. Aspirate 20 µL of CA2Cre-shp53 lentivirus into a pipettor and set aside.

2.5. Position the sedated mouse on the working procedure board by hooking its upper incisors into the thread of the board.

NOTE: The mouse's dorsum should be flat against the platform.

2.6. Tape the caudal portion of the thoracic cavity to the platform to ensure alignment of the mouse during the procedure.

2.7. Adjust a gooseneck illuminator between 80−100% intensity and place the light 1−2 cm from the skin surface.

2.8. From behind the platform, draw the tongue out of the oral cavity of the mouse using sterile forceps.

2.9. While securing the tongue, insert a depressor into the oral cavity of the mouse then release the tongue.

2.10. Position the gooseneck on the main stem bronchi to illuminate trachea.

NOTE: The trachea may be visible through the action of respiration, causing fluctuation of the light.

2.11. When the trachea is clearly viewed, insert the spirometer prepared (syringe with PBS and catheter) into the tracheal path.

2.12. Remove the depressor and observe the rise and fall of PBS in the syringe with each breath.

NOTE: This is an indicator that the catheter is properly positioned into the trachea.

2.13. Remove the syringe containing PBS while maintaining the catheter inside the trachea as the previous position.

2.14. Deposit the 20 µL CA2Cre-shp53 lentivirus into the center of the catheter.

2.15. While keeping the catheter in place, inject 300 µL of air into the catheter using an empty syringe to ensure proper distribution of lentivirus in the lungs.

2.16. Keep the catheter in place and reinsert the spirometer into the catheter.

NOTE: The rise and fall of the PBS bubble will ensure that the procedure is successful.

2.17. Remove the catheter and tape. Place the animal in a warm and dry place until it is revived.

3. Ultrasound imaging of lung tumors in mice

NOTE: Ultrasound imaging was performed after 7 and 18 weeks of lentiviral intubation using the system listed in **Table of Materials**; however, any model can be used for the analysis.

3.1. One day prior to imaging, remove fur from the chest area of the intubated mouse.

NOTE: Mouse should be sedated during this step by placing them in an induction chamber of 3% isoflurane and 2.5% O_2.

3.2. On the day of imaging, set up the workspace as shown in **Figure 2**. Turn on the heating pump for ultrasound gel and the temperature monitor.

3.3. Set up a warm 33 °C incubator to place the mice in post-imaging.

3.4. Place the three-dimensional (3D) motor (**Figure 2F**) on the integrated rail system.

3.5. Make sure that the 3D motor and the transducer mounting system are securely in place.

3.6. Connect a preferred transducer (frequency: 40 MHz; **Figure 2E** and **Table of Materials**) for tumor measurements perpendicular to the 3D motor.

3.7. Start a new study on the ultrasound software.

3.7.1. Select **Study Browser**, then select **New** at the bottom of the screen.

3.7.2. Select **New Study**, a new window will appear that enables the addition of a **Study Name** in addition to further information about the study, i.e., date of study, name of researcher, etc.

3.7.3. Fill in the information in **Series Name**, i.e., Animal ID, Strain, Weight, Date of Birth, etc.

3.7.4. Select **Done**, the program will change for B-mode.

3.8. Put a heating lamp in a convenient position above the animal platform.

3.9. Place the mouse in the induction chamber (3.5% isoflurane).

NOTE: Proper anesthetization is confirmed by unconsciousness of the mouse, and slower respiratory rate of around 1 breath per 2 seconds.

3.10. When the mouse is sedated, change the connection of the anesthetic machine to be directed towards the animal platform, reduce isoflurane to 2.5%.

3.11. Place the mouse on the animal platform in decubitus ventral, with its oral cavity directed towards the anesthetics tube.

3.12. Apply lubricant on the eyes of the mouse.

3.13. Place the mouse in decubitus dorsal and tape its hands and feet firmly onto the animal platform.

3.14. Apply a small layer of ultrasound gel on the mouse chest.

3.15. Lower the acquisition probe using the height-control knob to touch the surface of the mouse chest. Position the probe such that the heart of the mouse is approximately centered.

3.16. Use the micro-knobs to acquire images of the whole chest, from both extremities, in the transverse orientation ideally gathering 500 frames per mouse (number of frames may vary depending on personal choice).

3.17. Once imaging is done, remove the gel from the chest of the mouse and place the mouse in the warm incubator.

4. 2D analysis of ultrasound images

4.1. After opening acquired frames on the ultrasound software, scan the frames for tumors.

4.2. For small initiating tumors, count the number of B-lines periodically every 10 frames for the full length of the 500 frames acquired.

Therefore, B-lines are counted in a total of 50 images, each image is separated by 10 frames. B-lines are characterized by longitudinal white straight lines fully traversing the screen.

4.3. For 2D measurements of large tumors, select the linear tool and measure the width and length of the tumor present.

4.4. To calculate the volume of the tumors, use the following formula:

$$\text{Volume } [mm^3] = \frac{L\,[mm] \times W^2[mm^2]}{2}$$

where L and W are the length and width of the tumor, respectively.

REPRESENTATIVE RESULTS:

After obtaining a lentiviral infectious titer of ~2 x 10^6 TU/mL (**Figure 1**), the Ca2Cre-shp53 lentivirus was intratracheally injected when LSL-KRAS G12D mice reached an appropriate age (6–8 weeks)[9]. Ultrasound imaging was performed after 7 weeks of infection upon initiation of tumors (**Figure 3B**). Imaging was done at 7 weeks in order to include the various types of precursor lesions that occur in the LSL-$KRAS^{G12D}$ mouse model, ranging from hyperplasia to adenoma[3] as shown in **Figure 4A** after 8 weeks of infection. Imaging earlier than 6 weeks will not ensure the inclusion of adenoma-forming tumors in the analysis[3]. These precursor lesions are visualized as B-lines in ultrasound and can be counted by eye since they can be identified as narrow beams of white light vertically traversing the screen and are the result of a strong reflection of ultrasound wave (**Figure 3B**)[10]. B-lines represent small tumors that are on the surface of the lungs; tumors being initiated in deeper structures of the lung such as close to the trachea will not be reflected as B-lines. The number of tumors being initiated on the pleural surface can be quantified by counting the B-lines, since they are too small for volume measurements. We acquired 500 frames spanning the full lung area in 5 mice. B-lines were counted every 10 frames; thus, they were counted in a total of 50 images per mouse that span the full 500 frames acquired. The scatter plot in **Figure 3D** shows the sum of B-lines counted in every 10 images (100 frames) for 5 mice. The number of B-lines per mouse is then summed up to include B-lines present in the full lung area. The mean quantity of B-lines representing tumors in the five mice was 141.6 ± 41.52 (**Figure 3E**). **Figure 3A** shows an ultrasound image acquired of a non-infected mouse lung, for comparison.

According to Jackson et al.[3], 16 weeks post-intubation includes adenoma as well as adenocarcinoma, which are tumor types of interest in KRAS lung cancer. To ensure inclusion of these types of tumors when analyzing volume measurements via ultrasound in our model, we imaged the lungs of mice at 18 weeks post-infection. In order to quantify the volume of the large tumors via ultrasound, we used 2D analysis on the ultrasound software. Large tumors appear as deep clefts interrupting the pleural surface as shown in the image of **Figure 3C**. When a tumor is spotted, we measure the length (L) and width (W) of the tumor, using the measurement tool of the ultrasound software. Once obtained, L and W are applied in the conventional formula used for calculating tumor volume (protocol step 4.4). We analyzed the tumor volume of 10 mice, as shown in **Figure 3F**, where the number of tumors formed in each mouse ranged from 5 to 26 tumors. The volumes of all tumors formed per mouse are then summed up to represent the tumor volume per mouse. For this analysis, we assume that the tumors formed in the lungs of mice are epileptic in shape, thus the width of the tumor is considered as perpendicular to the imaging plane. Relative volume quantification can be done when linear size of tumors reaches 0.3 mm (either length or width). After analyzing the lung parenchyma of 10 mice, the average tumor volume obtained was 31.8 ± 6.61 mm^3 (**Figure 3G**). Hematoxylin and Eosin (H&E) staining analysis on lung sections confirmed the formation of large tumors at 20 weeks post-infection (**Figure 4B**) and confirmed formation of adenoma and adenocarcinoma. It should be noted that as tumor growth progresses, different tumors might merge together to form large ones, as shown in **Figure 4B**. Although the ultrasound software permits 3D analysis of tumors, this type of analysis proved difficult in our model, mainly due to the high dynamic movement of the primary tumors formed that is consistent with lung sliding (movement of the pleural line with respiration) as well as cardiac beats[11]. We reasoned that 2D analysis would provide consistency in relative tumor volume measurements, especially when comparing lung tumor progression between different groups of mice.

FIGURE LEGENDS:

Figure 1: Flow cytometry analysis of MEFs with a GFP LoxP-allele. Representative flow cytometry images showing frequency of GFP-positive MEFs that are non-infected (Fc) (**A**) and infected (Fi) with the Ca2Cre-shp53 lentivirus (**B**).

Figure 2: Workspace of integrated rail system of ultrasound imaging. (**A**) X-axis micro-knob. (**B**) Y-axis micro-knob. (**C**) Animal platform. (**D**) Height control knob of scan head-probe. (**E**) Transducer. (**F**) 3D motor. (**G**) Heating lamp. (**H**) Anesthetics tube.

Figure 3: Representative ultrasound images of mouse lungs before and after lung tumor induction. (**A**) Image of a non-infected mouse lung section. Red arrows point to ribs (R) and the pleural surface (PS) of the lung. (**B**) Image of a mouse lung 7 weeks after tumor induction by intratracheal intubation of lentivirus. Arrows indicate B-lines reflecting small tumors on the lung surface. (**C**) Image of a mouse lung section 18 weeks after tumor induction. Blue lines indicate measurements of the length (L: mm) and width (W: mm) of the tumor shown. (**D**) Scatter plot showing B-line quantification in 10 images spanning 500 frames (each image is separated by 10

frames). (**E**) Quantification of relative tumor number in lungs at 7 weeks post-induction. (**F**) Scatter plot showing the relative volume of the different tumors formed in the lungs of 10 mice at 18 weeks post-infection. (**G**) Quantification of lung tumor volume in mice (n = 10).

Figure 4: Representative H&E staining of LSL-KRAS G12D lung sections. (**A**) Lung section at 8 weeks post-infection at 500 µm magnification. Arrows point at precursor lesions formed at this timepoint, which are reflected as B-lines in ultrasound. Black arrows point at magnified lesions at 60 µm. (**B**) Lung section at 20 weeks post-infection at 2 mm magnification. Arrowheads of the same color show different tumors that have merged together. Arrows show magnified tumors at 60 µm.

DISCUSSION:

We demonstrate a method that can assess lung tumor growth in the Cre-inducible LSL-KRAS G12D mouse model by ultrasound. This method can be used for evaluating the effect of pharmacological inhibitors on lung tumor growth. It can also be used to compare lung tumor growth between mice of different genetic backgrounds. Using this technique does not require specialized computational skills, however, it is important to be systematic in the number of frames used for analysis to allow for proper comparison if the method is used for comparing different groups of mice.

The intratracheal intubation of ~2 x 10^6 TU/mL Ca2Cre-shp53 in LSL-KRAS G12D mice led to the formation of precursor lesions after 7 weeks of infection. These appear as longitudinal white lines on sonographic images and are called B-lines (**Figure 3B**)[12]. The average number of B-lines per mouse was 141.6 ± 41.52 (**Figure 3E**). We chose to visualize initiating tumors at 7 weeks post-infection since this timepoint would include various types of precursor lesions; atypical adenomatous hyperplasia, epithelial hyperplasia and adenoma[3]. The formation of precursor lesions on the lung was verified by H&E staining after 8 weeks of lentiviral infection (**Figure 4A**). After 18 weeks of viral intubation, the tumors are large enough to allow 2D volume measurements in the ultrasound software (**Figure 3C**). Analysis of ultrasound images was done at the 18-week timepoint in order to include the quantification of well-developed adenoma and adenocarcinoma[3]. The formation of these types of tumors was confirmed at 20 weeks post-infection by H&E staining analysis (**Figure 4B**). 3D volume measurements are also possible by the ultrasound software but require high level of expertise. The titre described in this protocol is optimal for the formation of lung tumors that can be properly visualized and quantified in 2D.

Other imaging techniques can also be used to monitor lung tumor growth in mice, such as CT imaging and BLI[13]. However, CT imaging requires delivering a radiation dose to the tumor, which might affect tumor growth upon repeated assays[13]. Moreover, it requires the use of contrast agents for accurate tumor measurements[13]. BLI is an important imaging technique that is often used for quantifying tumor proliferation; however, its main limitation is its dependence on metabolic activities and the presence of ATP and O_2[13]. The advantage of ultrasound imaging lies in it being a non-invasive, non-irradiating, speedy and inexpensive technique for acquiring images of the lung parenchyma[13]. Ultrasound has been previously used to monitor growth of orthotopic human tumors xenografted in lung and pancreas of mice[13,14]. Raes et al. have been successful in

longitudinally monitoring the growth of a single human lung tumor implanted near the posterior diaphragmic surface in mice via ultrasound[13]. The difference in the LSL-KRAS G12D mouse model is having spontaneous development of lung tumors that can be monitored from initiation to progression and this development can be compared between mice groups. Also, there are numerous tumors being developed on the lung of the LSL-KRAS G12D mouse model, that eventually grow, and might even merge together to form large tumors (**Figure 4B**). This urged the need to use a method that can monitor lung tumor initiation and progression in this mouse model. We optimized the 2D brightness (B) mode analysis to allow quantitative assessment of lung tumors developed in this mouse model.

A possible limitation with ultrasound imaging is the presence of false positives[15]. These are seen when a highly reflective surface is present (diaphragm or liver) that interrupts the trajectory of the beam and might create a virtual object mimicking a true object[15]. Such images could be false tumors but can be bypassed upon proper examination; a large tumor is usually dynamic whereas a virtual object would be static.

Ultrasound imaging for the LSL-KRAS mouse model is ideal due to the non-invasive nature of this technique. The method of analysis used in our lab to monitor lung tumor progression in the LSL-KRAS mouse model is fast and user-friendly.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1.PNG

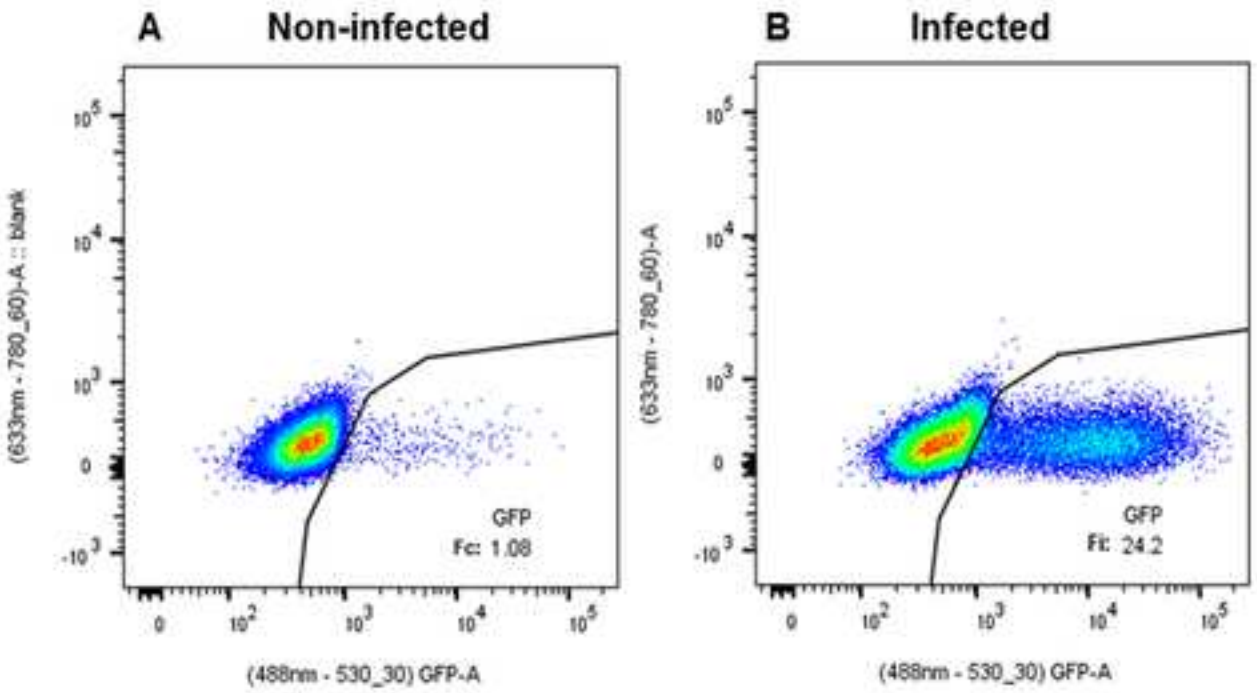


Figure 2

Click here to access/download;Figure;Figure 2.PNG

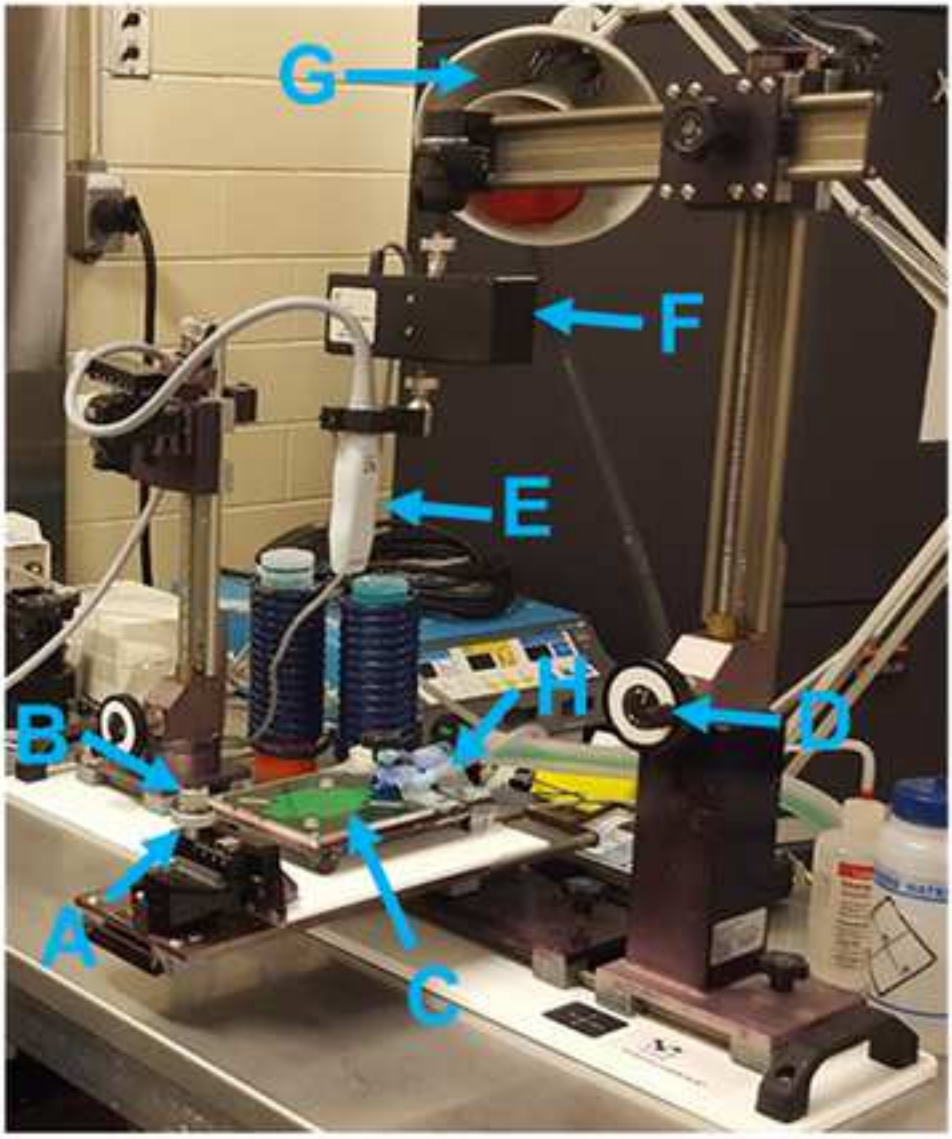


Figure 3

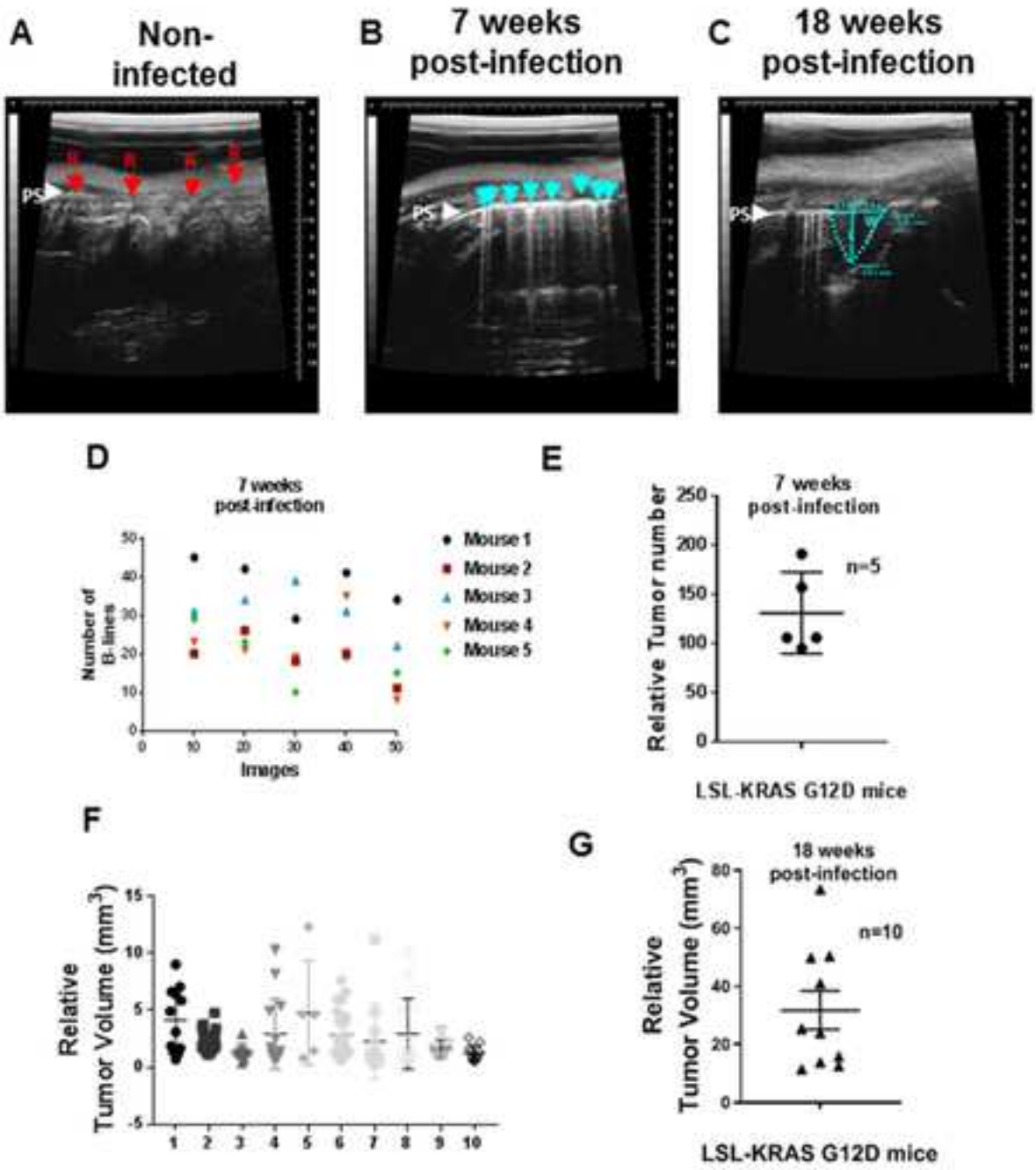
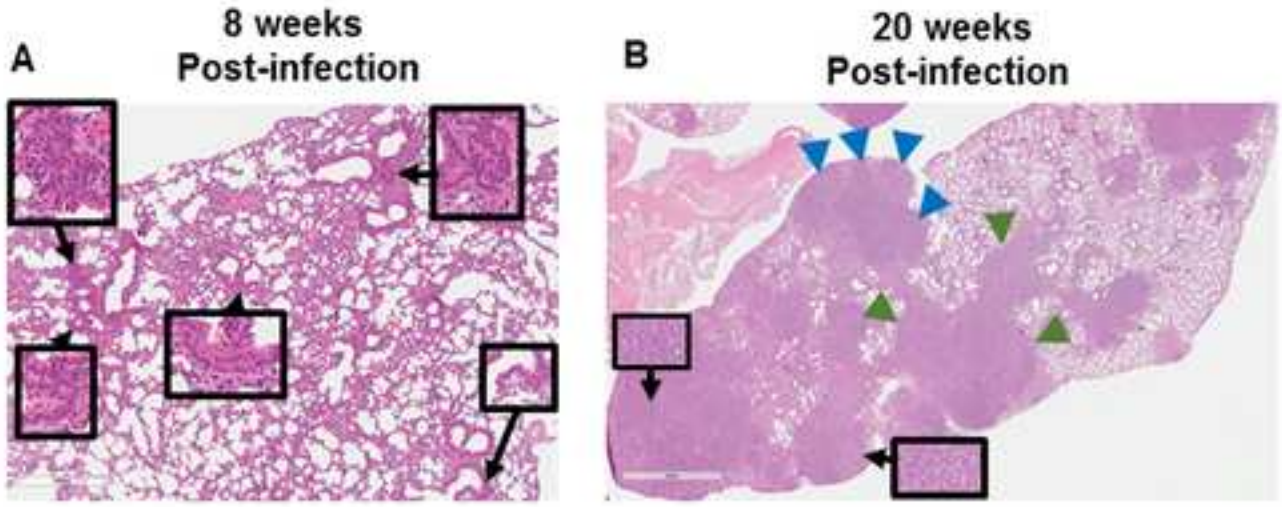


Figure 4



Name of Material/Equipment	Company	Catalog Number
0.45 µm Acrodisc Syringe Filters	Pall Corporation	PN 4614
100-mm Cell Cultre Plate	CELLSTAR	664 160
6-well Cell Culture Plate	CELLSTAR	657 160
Amicon Ultra - 15 Centrifugal Filter Units	Merck Millipore Ltd.	UFC910024
BD LSR-Fortessa	BD Biosciences	649225B 3024
CA2Cre-shp53 lentiviral vector		
DMEM	Multicell	319-005-CL
FBS	Multicell	80450
LSL-KRAS^{G12D} mouse	JAX Mice	8179
MX550S; Centre Transmit: 40 MHz	FUJIFILM VisualSonics	51070
OptiMEM	gibco	11058-021
Pen/strep	Multicell	450-201-EL
pMD2.G	Addgene	12259
PsPAX2	Addgene	12260
VEVO-3100	FUJIFILM VisualSonics	51072-50

Comments/Description

From Dr. I Verma Laboratory

Editorial comments:

1. Short Abstract: Please shorten it to no more than 50 words.
CORRECTED

2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.
CORRECTED

3. Please define acronyms/abbreviations upon first use in the main text.
CORRECTED

4. Please list all centrifugation speeds in terms of centrifugal g-force instead of rpm: 100 x g.
CORRECTED

5. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.
CORRECTED

6. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
CORRECTED

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: OptiMEM, Millipore, VEVO, etc.
CORRECTED

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.1.2.1.: Culture cells under what conditions? Please specify1.2.3: What media? Please specify. 1.2.5: Please describe how flow cytometry is done.
CORRECTED

9. Section 2: Please describe in imperative tense how intratracheal intubation is actually done.
CORRECTED

10. 3.1: Please specify the age, gender and strain of mice used here.
CORRECTED

11. 3.9: Please mention how proper anesthetization is confirmed.
CORRECTED

12. Please describe how lentivirus was intratracheally injected in the protocol.
CORRECTED

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
CORRECTED

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
CORRECTED

15. References: Please do not abbreviate journal titles; use full journal name.
CORRECTED
16. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.
CORRECTED

17. Table of Materials: Please remove any ™/®/© symbols. Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
CORRECTED

REVIEWERS'COMMENTS:

REVIEWER #1:
The manuscript by Ghaddar et al describes the method for the use of ultrasound to monitor and quantify the volume of lung tumors that spontaneously arise in the LSL-Kras lung cancer animal model. Ultrasound imaging is a technique that provides fast, easy, and inexpensive monitoring of lung cancer development, however, the manuscript does not clearly describe how accurately the ultrasound imaging can monitor cancer progression as compared with other techniques.When suggesting a technique or analysis that is better than the other known techniques in terms of speed, easiness, and cost to use, it is based on the assumption that the results obtained with the suggested technique are comparable to those using the pre-established techniques. To this end, comparing the number of tumors found by ultrasound with those obtained by other methods such as histological analysis is strongly recommended.

REPLY: Our protocol does not allow the exact quantification of the number of tumors present in the lungs of the mice, rather, it gives a relative tumor number based on B-line counting in 50 images (each image separated by 10 frames) of acquired Ultrasound film. The main purpose is to provide a method that can compare spontaneous development of lung tumors between different groups of mice, i.e.: Control compared to treatment group, wild-type versus genetically modified, etc. Therefore, the relative tumor number or the relative volume of tumors obtained by our ultrasound technique cannot be compared with other techniques since the quantification will vary greatly. Histological analyses have been incorporated in Fig. 4 to confirm validity of Ultrasound imaging. H&E staining of mouse lungs at 8 weeks post-infection shows various precursor lesions being formed, these are reflected by B-lines in ultrasound. H&E staining at 20 weeks post-infection shows mouse lungs with large tumors, mostly adenocarcinomas. These are reflected by clefts seen in ultrasound.

Histology shows the degree of damage, extension, etc, which cannot be directly told by ultrasound.
REPLY: Histology requires sacrificing the mice for analysis, whereas in ultrasound imaging, the relative tumor number and volume can be compared between mice groups without having to sacrifice mice. If the study requires comparing degree of damage and extension, histology should be done. But, ultrasound can suffice for comparison of tumor number and tumor volume quantifications in lungs between different groups of mice.

Also, after longer periods, if tumors merge, the 3D structure can vary immensely and a simple 2D measurement does not seem to reflect real volume. The ultrasound needs to be compared with other technique(s).

REPLY: 2D measurements of tumor volume in this protocol do not reflect real volume of tumors present on lungs, they are rather relative measurements, based on a scale on the ultrasound program, where the scale differs from actual measurements. We are mainly introducing a method that can compare tumor volumes formed in the LSL KRAS mouse model in different groups of mice.

The more comprehensive study comparing among different imaging techniques including ultrasound, micro CT, and photoacoustic imaging to analyze the presence/development of tumors was published by Raes et al in PLoS One, 2016 as cited in this manuscript as reference 10. In this published study, they used a mouse model of orthotopic transplantation of tumor cells. I understand that a manuscript in JoVE does not have to be novel, but it at least has to show and validate the technique that the authors present is valid and reliable for at least a particular condition to analyze.

REPLY: H&E staining of lung tumor sections have been incorporated in figure 4 to ensure validity of ultrasound imaging in our model.

From this point, this manuscript needs to emphasize the differences from the previously published paper that include the following 2 points; 1) it uses genetically modified mouse model where tumors spontaneously develop, suggesting that the way tumor develops is different from the one published, which can be well monitored by ultrasound technique that

the authors present (for details see below), and 2) how to use ultrasound technique to monitor tumors needs to be more precisely described, perhaps with showing the instrument. Protocol described is rather confusing and needs to be rewritten in a clearer way in more details, targeting people who have never seen or used the instrument (this is one of the objectives that the JoVE has; to help newcomers to carry out successful experiments without much problem).

REPLY: Comparison between the LSL-KRAS G12D model and the orthotopic model described in Raes et al. have been included in lines 438-442. An image of the ultrasound workplace has been included in figure 1.

Important features of tumor analysis in the present case are tumor numbers, tumor merging, ability to attach to the tissue, tumor volume, etc. In order to better understand and show the technique that works well for them, it would be good to increase the number of analysis performed instead of just 7 and 18 weeks (for instance, 1 ultrasound analysis every 1 or 2 weeks). More frequent analysis and possibly comparison with the histological results, may tell how development of tumor occurs, and if the proposed technique can clearly differentiate different tumor development phases such as small tumor appearance vs. their merge. This comparison would be particularly important for future uses and comparisons amongst different treatments in order to analyzed initial, middle and final aspects of these tumor features.As mentioned, it is not clear why only 7 and 18 weeks were used. Is there any previous data showing that these time points are crucial for tumor development such as many small tumors appear at 7 weeks, which merge and become large tumors at 18 weeks?

REPLY: A study by Jackson et al. (Genes Dev. 2001 Dec 15;15(24):3243-8) monitored histological changes in lung tumors of the LSL-KRAS G12D mouse model at 2, 6, 12 and 16 weeks post-infection. Tumors at 2 weeks post-infection were formed of 2 types of early lesions: atypical adenomatous hyperplasia (AAH) and epithelial hyperplasia (EH) whereas at 6 weeks more diverse types of early lesions were formed, ranging from AAH lesions to small adenomas. To us, imaging at 7 weeks was logical in order to include in our tumor number count the various types of precursor lesions formed in the lungs at earlier time-points of infection. In Jackson et al.'s study, 12 weeks post-infection included larger adenomas, whereas 16 weeks post-infection also included adenocarcinomas, which are types of tumors we wanted to include in our volume quantifications. We reasoned that 18 weeks post-infection will include large adenomas and adenocarcinomas, therefore including the various types of tumors formed in this model in volume measurements.

And why tumor numbers are not shown at 18 weeks?

REPLY: As shown in the histological images of figure 4, tumors merge at later timepoints of infection, and those are reflected as only one large tumor in ultrasound, which is quantified for volume measurements. Therefore, counting the tumors would be mis-represented, where one tumor at 18 weeks can account for two or three different merged tumors. This is why we fixed the quantification for relative tumor number at 7 weeks and relative tumor volume at 18 weeks. A

scatter-plot of lung tumor volume measurements has been included in figure 3F, where the number of tumors analyzed ranged from 5 to 26 tumors per mouse.

Further, how long the animals survive after intratracheal intubation? If they live longer than 18 weeks, it would be interesting to show and compare the lungs analyzed with ultrasound and other techniques at much later time point.

REPLY: According to our histological analyses, some lungs had an almost full-lung cancer at 20 weeks (Figure 4B), meaning the cancer might have begun to be saturated between different groups. We reasoned that 18 weeks is optimal for comparing tumor volume between different groups of mice, since more than that maybe the cancer will reach full-capacity and will be saturated, leaving no room for variation or comparison between one mouse and another or one group to another.

The authors showed that B-lines are present only on the pleural surface. How about deeper structures? Close to the trachea, for example and blood vessels? Do they have tumors too? Does the ultrasound can reach these deeper structures? For one analyzing tumor/metastasis development, or even using a different tumor induction technique (tail vein cancer cell injections for example), it is of extreme importance since cancer cells can arrive through blood.

REPLY: B-lines reflect only tumors present on the surface of the lungs. However, when tumors are large enough, they can be visualized in Ultrasound imaging even in deeper structures. The text has been modified to clarify this in lines 343-344.

When and where a false positive is more likely to occur? Which kind of body structure? What can be done to avoid this?

REPLY: A false positive occurs near the diaphragm region. It appears as an epileptic shape and might resemble a tumor to the inexperienced eye. False positives cannot be avoided but can be recognized as static objects, therefore, the researcher should overrule such objects as being tumors.

Minor comments. Please briefly describe the intratracheal intubation even though it is referred.
REPLY: We have done so.

Figure 2: Please use different symbols for different structures. In the current figure, arrows are used for all of them. Please, distinguish, for example, arrow for structures, arrow heads for b-lines, or use different colors. For Figure 2C, a dashed line can be used to indicate the limits of tumor (circle it with dashed line). The structures need to be clearly shown with no margin of doubt. How the B-lines are counted? By pixels? It should be described in the main text.
REPLY: Figure 3 showing ultrasound images has been modified. B-line counting is now described further in the text in lines 346-350.

Lines 192 to 194: Did the authors count B-lines in 50 frames of each animal, is that correct? This is not clear. If this is correct, please state that the total number of frames analyzed was

50.This means that each frame has a number. Maybe a graph can be presented as a scatter plot showing the value of each frame in different color for different animals…?

REPLY: Analysis of B-lines is now further described in lines 346-350. Since a scatter plot having 50 images will be too busy, we summed up the B-lines visualized every 10 images in the scatter plot.

Line 201 (Fig. 2E): Same as above is applicable here. Fig. 2E shows the volume of tumor at 18 weeks, but it does not say how many tumors were used for analysis per animal. Need to describe how many tumors an animal has at each time of analysis and compare with previous validated methods. If all the tumors were analyzed, maybe a scatter plot graph as described above can be provided with different volumes.

REPLY: A scatter plot has been included in figure 3F. The analyzed tumors range from 5 to 26 (lines 366-367).

REVIEWER #2:
Manuscript Summary: This is a very clear explanation of a quite simple and very potentially useful non-invasive method. Major Concerns: None Minor Concerns: None

REVIEWER #3:

The visualisation of B-lines is possible when the tumor reaches the lung surface, this should be acknowledged in the manuscript. This may imply that tumours developed deeper into the lung will not be identified by the presented method.

REPLY: The text has been edited to include this point (lines 343-344). It is true that B-lines reflect initiating tumors present only on the surface of the lung. Larger tumors at later time-points can be visualized in deeper structure of the lungs.

Why is a 40 MHz probe the preferred transducer for tumor measurements. Please provide an explanation or a reference.

REPLY: 40 MHz probe is optimal for Ultrasound imaging of mice. The high frequency has a lower focal point (few mm) and is perfect for imaging the small body of the mouse, as opposed to human bodies, where 7-10 MHz probe has a better penetration for deeper tissues (cm) and is sufficient for ultrasound.

The volume estimation techniques assumes a specific geometry of the tumor, since the measured Width is assumed to hold also in the direction perpendicular to the imaging plane. This assumption and the consequent error should be acknowledged in the manuscript

REPLY: We do assume that the tumor follows an epileptic shape, which is why the Width is considered to be perpendicular to the imaging plane. This has been updated in the text in lines 364-365.

What is the minimum tumor size measurable? At which size is there the transition between simply spotting the presence of the tumor by B-lines and the possibility to perform the measurement?

REPLY: Measurements for volume are considered when linear measurements are 0.3 mm. This has been updated in the text. (lines 365-366)

The following papers should be included in the reference list as they contain the latest developments in terms of the understanding of B-line artifacts and in terms of dedicated lung ultrasound imaging L. Demi, et al , "Determination of a potential quantitative measure of the state of the lung using lung ultrasound spectroscopy", Scientific Reports, Vol 7, 12746, 2017. K. Mohanty et al, "Characterization of the Lung Parenchyma Using Ultrasound Multiple Scattering", Ultrasound in Medicine and Biology, Vol 43, pp. 993-1003, 2017. G. Soldati, et al, "On the Physical Basis of Pulmonary Sonographic Interstitial Syndrome", Journal of Ultrasound in Medicine, Vol 35, pp. 2075-2086, 2016.

REPLY: The references are now included.