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Quantitative Monitoring of Murine Lung Tumors by Magnetic Resonance Imaging

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Abstract

Primary lung cancer remains the leading cause of cancer-related death in the western world and the lung is a common site for recurrence of extra-thoracic malignancies. Small-animal (rodent) models of cancer can play a very valuable role in the development of improved therapeutic strategies. However, detection of murine pulmonary tumors and their subsequent response to therapy, *in situ*, is challenging. We have recently described magnetic resonance imaging (MRI) as a reliable, reproducible, and non-destructive modality for the detection and serial monitoring of pulmonary tumors. Combining respiratory-gated data acquisition methods with manual and automated segmentation algorithms described by our laboratory, pulmonary tumor burden can be quantitatively measured in approximately one hour (data acquisition plus analysis) per mouse. Quantitative, analytic methods are described for measuring tumor burden in both primary (discrete tumors) and metastatic (diffuse tumors) disease. Thus, small-animal MRI represents a novel and unique research tool for preclinical investigation of therapeutic strategies for treatment of pulmonary malignancies and may be valuable in evaluating new compounds targeting lung cancer *in vivo*.

Keywords

Magnetic Resonance Imaging (MRI); Lung; Tumor; Mouse; Respiratory Gating; Segmentation

INTRODUCTION

Primary lung cancer remains a major cause of death in the Western world and the lungs are the most common site for systemic metastases¹⁻³. Murine models play a critical role in advancing our understanding of malignancies and testing therapeutic options prior to initiation of clinical trials. For example, recent clinical success in the blockade of cytotoxic

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AUTHOR CONTRIBUTION STATEMENT

ASK, VKT, and JRG designed the imaging experiments, performed the data analysis, and wrote the manuscript; JAE collected all the MR images; AEG and DK helped with experimental design; AN helped with data analysis; VVA provided technical support; MY and HGV provided lung-tumor-bearing animals and were instrumental in initial efforts to use MRI for monitoring primary murine lung tumors.

T lymphocyte antigen-4 for treatment of malignant melanoma^{4,5} can be linked directly to related preclinical small-animal studies demonstrating success of such therapy⁶. Most of these preclinical experiments, however, rely on subcutaneous injection of tumor cell lines, and response to therapy is evaluated by the direct measurement of tumor diameter^{6,7}. While this approach may be valid for malignancies of the skin or soft tissue, it does not translate directly to the physiology of tumors that develop and grow in the lung due to significant differences in immunosurveillance between the lung and subcutaneous sites^{8,9}.

Traditional models for the study of orthotopic murine lung cancer (i.e., tumors occurring within the lung) have relied on either the administration of carcinogen¹⁰ or intratracheal (i.t.) or intravenous (i.v.) injection of lung cancer cell lines, which result in intra-pulmonary growth of tumor^{11,12}. The recent development of genetically engineered mouse models of lung cancer (e.g., activation of oncogenic K-ras, loss of function of p53) has significantly furthered the range of experimental tools available for modeling lung cancer and offers a reproducible and reliable system for studying biologic and therapeutic strategies for treating lung cancer¹³⁻²⁰.

Many investigators have relied on destructive modalities, such as animal sacrifice and analysis of tumor burden by counting tumor nodules on lung blocks or histologic slides^{14,21}, for monitoring intra-cavitary malignancies, such as lung cancer, and their response to therapy. The use of non-destructive imaging modalities, such as computerized tomography scanning (CT)^{11,20}, bioluminescent imaging of tumor cells expressing a marker gene such as luciferase²², or metabolic imaging such as positron emission tomography scanning (PET) has also been reported²³(Table 1).

Computerized Tomography

Computerized tomography (CT) is the imaging modality of choice for screening, diagnosis, and monitoring of lung cancer in humans due to its high resolution, rapidity of data acquisition, and ability to detect sub-centimeter nodules^{24,25}. Based on this success in humans, multiple groups have adapted micro-CT to murine models of lung cancer and have utilized this technology for both detection and serial monitoring of disease^{11,26}. However, micro-CT can deliver radiation in doses ranging from 0.22 to 0.76 Gy per scan^{20,27}. While such radiation doses are several log-fold lower than therapeutic doses typically used to treat lung cancer^{28,29}, the possibility that radiation delivered by micro-CT can affect tumor growth, especially with serial imaging, must be considered. In addition the cumulative effects of serial CT scans on the immune system need to be taken into account when designing long-term drug studies. Doses of whole body irradiation as low as 2.7 to 4.2 Gy (5 to 6 micro-CT scans) can result in peripheral lymphopenia, leading to homeostatic proliferation of the remaining native T lymphocytes as they expand to repopulate the immune system³⁰⁻³². Since T-cell homeostatic proliferation can lead to a tumor-immune response³³, even low doses of radiation may confound therapeutic studies. While both PET scanning and bioluminescent imaging can provide information regarding tumor burden, either by uptake of a radioactive tracer (PET) or detection of tumors engineered to express luciferase (bioluminescence), the anatomic resolution of both modalities is low (Table 1)^{23,34} (e.g., typical spatial resolution of small-animal PET is 1 – 2 mm³⁵). Despite these limitations all three of these imaging modalities have played an important role in preclinical small-animal studies of tumor therapy^{20,23,26}. However, an imaging modality with high anatomic resolution that avoids ionizing radiation, and requires neither introduction of foreign genes into the tumor nor extraneous manipulation of a tumor cell line, may be advantageous for small-animal studies of lung cancer.

Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) is a powerful imaging tool for characterizing animal systems and animal models of disease. *In vivo* MRI is a non-invasive and non-destructive technique that enables a wide variety of longitudinal (time-course) studies that are not possible with other destructive analytical methods. The vast majority of clinical and pre-clinical MRI studies involve the detection of signal from hydrogen nuclei in water. The observed signal depends upon both the amount of water present in the sample and on the relaxation properties of that water. In magnetic resonance, spins are often characterized by two characteristic relaxation times: longitudinal (T1) relaxation, describing the return to equilibrium of the magnetization following RF excitation and transverse (T2) relaxation, describing the loss of signal following RF excitation. Further details about MRI, including T1 and T2 relaxation, can be found in a variety of standard nuclear magnetic resonance (NMR) and MRI textbooks³⁶⁻³⁸. For the practical use of MRI (and the purposes of this article), it is sufficient to know that the water molecules in different tissues and organs often have different relaxation times, as do healthy and pathologic tissues. These differences in either T1 or T2 (or both) can be exploited to provide the contrast necessary to generate high-resolution MR images.

MRI signal intensity, which depends on the density of water in soft tissue, has been well developed for the study of primary brain³⁹, prostate⁴⁰, and liver tumors⁴¹, as well as for evaluation of malignancies in many other organs and tissues. Lungs, however, present unique challenges for MRI, which required the development of new and innovative methods⁴²⁻⁴⁴. Among the unique challenges to MRI of lung are: (i) low tissue density and low water content within the lung which severely limits signal-to-noise; (ii) variations in magnetic susceptibility associated with the many air-tissue interfaces of the alveoli and bronchioles which result in short T2* and T2 relaxation time constants, thus further contributing to low signal-to-noise (T2 characterizes the decay of signal due to magnetic susceptibility in the absence of static magnetic-field inhomogeneity; T2* characterizes the loss of signal due to both magnetic susceptibility and static-field inhomogeneity³⁶; and (iii) respiratory and cardiac motions which lead to significant image blurring in the absence of motion-synchronized data acquisition. Nonetheless, the opportunities for characterization of lung tumors and pathology by MRI are significant.

Here we present a protocol for quantifying pulmonary tumor burden, of both primary lung cancer and metastatic disease to the lung, using small-animal MRI. These protocols have been used by our group in multiple settings for identification and serial examination of both individual, sub-millimeter lesions (minimum detection limit is ~0.5 mm diameter) as well as extensive tumor metastases, in which tumor tissue replaces a large portion of healthy lung parenchyma in one or both lungs^{42,43,45-48}. We have further defined and validated a nearly fully automated algorithm for segmenting mouse-lung MR images that allows for rapid estimation of metastatic lung-tumor burden⁴⁹. The lung is a particularly favorable case for the development of automated methods to estimate tumor burden, since tumors appear as bright features against a dark image background for healthy lung. The semi-automated method described herein could likely be extended to other organ systems (e.g., liver, brain), though this would require the (non-trivial) development of appropriate shape and signal models for healthy organ tissue and tumor. In total, the protocols in this manuscript will prove to be invaluable for the characterization of murine experimental models of lung cancer, advancing the development of new treatments for pulmonary malignancies.

Comment regarding the Equipment—The equipment described in the Material section represents our laboratory's preference. However, all equipment can be modified according to an investigator's individual needs and preferences. Photos of selected large equipment

(Supplemental Figure 1) and small-animal equipment (Supplemental Figure 2) required for the acquisition of MR images are supplied as supplementary figures.

Manual Segmentation/Volume Calculation of Primary (Discrete) Lung Tumors

Primary lung cancer in mice leads to the presence of one or more well-defined, discrete tumors within the lungs. Typically, primary lung tumors ranging in size from 0.5 to 4.0 mm in diameter. These tumors can be identified as bright spots against the dark, background image of healthy lung tissue. In these mice, lung-tumor volumes for each tumor can be calculated by drawing regions of interest (ROIs) around each of the observed nodules, calculating the cross-sectional areas within an imaging slice, multiplying by the slice thickness, and adding together volumes from contiguous slices corresponding to the same common tumor. The total tumor burden for a given mouse is then simply the sum of the volume of each individual tumor within that animal.

Manual Segmentation/Volume Calculation of Metastatic (Diffuse) Lung Tumors

The techniques described above are appropriate for quantitatively measuring tumor volumes in mice with primary tumors, in which multiple well-defined, discrete tumors are observed. However, for metastatic disease, in which tumors are diffuse and not amenable to being circumscribed by individual regions of interest, an alternate method for assessing total tumor burden is needed. We have recently demonstrated that for mice with diffuse tumor, *average* MR lung-image intensity provides an accurate measure of total lung-tumor burden⁴⁹. (Figure 1 is a plot demonstrating the correlation between *average* MR lung-image intensity and tumor burden, as derived from measured lung weight.) To account for small experiment-to-experiment differences in absolute image intensity due to variations in RF coil tuning or animal positioning, the *average* MR lung-image intensity is *normalized* to that of liver within each animal. In considering these measurements, it is useful to distinguish and discuss separately three different cases: I) Assess, qualitatively, lung-tumor burden in the same mouse at two or more time points (e.g., monitor tumor progression or therapeutic response); II) Measure *relative* lung-tumor burden across a series of mice; III) Measure *absolute* lung-tumor burden across a series of mice, as illustrated in Figure 2.

Automated Segmentation/Volume Calculation of Metastatic (Diffuse) Lung Tumors

In calculating average mouse-lung image intensity, the labor-intensive, time-consuming step is segmentation of the lungs. The throughput and efficiency of the analysis procedure would be greatly aided by the ability to accurately and automatically segment the lungs throughout the mouse. Our recent publication demonstrating the utility of average lung image intensity for quantitatively measuring lung tumor also validated a new algorithm developed for segmenting lung⁴⁹. This new algorithm was implemented using code written and developed within Matlab and has been used extensively by our laboratory to decrease both the time and effort necessary to quantitatively monitor metastatic pulmonary tumor burden in the mouse. The procedures for installing this Matlab code and for automated lung segmentation and tumor-mass calculation using this code are described below. The necessary Matlab *.m files are included as Supplementary Material to this article.

MATERIALS

Reagents

- Male C57BL6 mice, 6-8 weeks of age (stock # 000664), for establishment of lung metastases by intravenous (i.v.) tumor injection and A/J male mice, 6-8 weeks of age (stock #000646), which are susceptible to urethane-induced lung cancer, both from Jackson Labs (Bar Harbor, MA).

- B16 malignant melanoma cell line (Catalog #CRL-6475, American Type Culture Collection, Manassas, VA).
- Tumor growth media, consisting of RPMI1640 (Catalog #R0883), 10% fetal calf serum (Catalog #F2442), and Penicillin-Streptomycin solution (Catalog #P7539), all from Sigma Chemical Co. (St. Louis, MO).
- Trypsin-EDTA solution for liberation of adherent cells grown in culture (Catalog #T3924), Sigma Chemical Co.
- Urethane, for induction of primary mouse lung cancer (Catalog #U2500), Sigma Chemical Co., dissolved in sterile saline at a final concentration of 100 mg/cc, and injected intraperitoneally (i.p.) for a total dose of 1mg/g body weight, as previously described¹⁰.
- Multi-Hance (Gd-BOPTA MR contrast agent; Bracco Imaging; San Donato Milanese, Italy).

Equipment

MRI Acquisition Equipment

- 40-cm clear-bore 4.7 T Oxford Instruments magnet (Oxford, UK)(Figure S1A)
- Varian/Agilent MRI scanner (Agilent Technology, Santa Clara, CA), equipped as described in Experimental Setup.
- Haskris Model WW2 Water Chiller (Haskris Company; Elk Grove Village, IL)
- Morris RF Tuning Unit – Model # 505NV+ RF Sweeper: 1.6 to 505MHz (Morris Instruments, Inc., Ottawa, Ontario, Canada)(Supplementary Figure 2C)
- Tektronix Model TDS 1002, two-channel digital storage oscilloscope (Tektronix; Beaverton, OR) (Supplementary Figure 2B)
- RG-223 coaxial cable (Amphenol; Wallingford, CT)
- Animal tray (Custom design/construction) (Supplementary Figure 1D)
- Nose cone (Custom design/construction)

Anesthesia

- Drager (Model #19.1) vaporizer (DRE Veterinary Supply; Louisville, KY) (Figure S2B)
- Isoflurane (Isothesia™; Butler Animal Health Supply; Dublin, OH 43017)
- 1-qt. Tupperware container (Purchased at Target)

Miscellaneous Supplies

- 5 lb test fishing line (Purchased at Target)
- Milwaukee Heavy Duty, 3-Stage Heat Gun (Milwaukee Electric Tool Corporation, Brookfield, WI) for warming air
- Plastic tubing (Poolstyle 1.5-inch Deluxe Vacuum Hose) for delivering warm air to the magnet bore
- 3-position heating pad (Purchased at Walgreens)
- Surgical pads

- Masking tape, ¼” and ½” (Purchased at Office Depot)
- Tygon tubing, ½” (Saint-Gobain North America Performance Plastics; Valley Forge, PA)

MRI Analysis Hardware and Software

- Dell PC (Model #390) + LCD monitor
- ImageJ software (Java-based, public-domain software developed at the NIH; available at <http://rsbweb.nih.gov/ij/>)
- Matlab (Mathworks; Natick, MA). Code was developed and calculations performed using Matlab version 7.6.0, R2008a, with the Image Processing Toolbox. The programs have also been successfully tested in earlier versions of Matlab [7.0.4 (R2007a) and 7.5.0 (R2007b)], and will likely run in many other versions of the program.

REAGENT SETUP

Induction of Primary Tumors—The total time for carcinogen preparation and injection is about 2 hours per group of 10 mice. Tumors become visible by MRI and gross inspection 4-5 months after carcinogen administration.

1. Dissolve the urethane* in sterile saline to a final concentration of 100 mg/ml. (While urethane represents our laboratory's choice for induction of murine lung cancer, the use of other carcinogens, such as benzo[*a*]pyrene, has also been described ⁴⁵.)
2. Inject mice intraperitoneally, at a dose of 1 mg/kg. Using a 100 mg/ml solution, this corresponds to 10 µl per g weight (i.e., a 25 g mouse is injected with 250 µl of the 100 mg/ml urethane solution).

Intravenous Injection of Metastatic Tumors—(The total time for tumor cell-line injection - 1 hour). In our experience, a single cell-suspension injection, consisting of 2.5×10^5 B16 murine melanoma tumor cells, will yield tumors visible by MRI and visual inspection by 2 weeks post-injection, with requisite animal sacrifice ~3 weeks after injection. Tumor cell lines such as B16 melanoma demonstrate optimal *in vivo* growth when injected during the logarithmic phase of growth (at approximately 50% confluence, *in vitro*).

1. Lift off B16 melanoma from tissue-culture flask when it reaches 50% confluence, by incubating with 1× trypsin-EDTA solution at 37°C for 5 minutes.
2. Neutralize the trypsin-EDTA with fetal calf serum at a 1:1 ratio and wash the cells to remove all traces of trypsin-EDTA.
3. Re-suspend tumor cells in sterile saline at a concentration of 1×10^6 cells/ml and filter through a 40 µm cell strainer to obtain a single-cell suspension.
4. Inject 2.5×10^5 cells (250 µl) intravenously to establish uniform pulmonary metastases.

EQUIPMENT SETUP

- Varian/Agilent MRI scanner (Agilent Technology, Santa Clara, CA).
 - DirectDrive™ MRI electronics console (Figures S1B,C)
 - Magnex/Agilent 20 cm ID self-shielded, high-performance gradient coils (Figure S1D)

■ Max gradient strength: 28 G/cm

■ Rise time: 650 μ s

○ High-power RF amplifiers, installed within the DirectDrive™ MRI electronics console (Figure S1C)

■ 2 × American Microwave Technologies (Herley Industries, Anaheim, CA), Model M3200

○ International Electric Company, OY (Helsinki, Finland) Model GPA-200-350 gradient amplifiers (Figure S1E)

○ Dell (Round Rock, TX) Linux PC, Model #390 + LCD monitor

○ 2.5-cm “birdcage-style” RF coil and shield (Stark Contrast, Erlanger, Germany) (Figure S2A) – Note: a wide variety of different RF coils can be used for the acquisition of MRI data. Because of the need to image the full lungs of the mouse, a volume coil is preferred to a surface coil for this application. As a general rule, the smallest coil into which the mouse can be inserted and that covers the full chest cavity of the mouse should be selected.

- Respiratory-monitoring unit (Supplementary Figure 2B)

○ Designed/constructed – Washington University; Details in Garbow, et. al.⁵⁰

○ Alternatively, can purchase and use a commercial animal monitoring unit that includes respiratory monitoring, such as SA Instruments Small Animal Monitoring and Gating System, Model #1025 (SA Instruments, Inc.; Stony Brook, NY).

○ Requirement is that monitoring unit generate a 0/+5V transistor-transistor logic (TTL) signal that can be used to trigger data acquisition on the MR console.

PROCEDURE

MR Data Acquisition (Timing: 45 minutes per animal)

Anesthetize the mouse.

1. To anesthetize the mouse, place it into a Tupperware container attached to the vaporizer and induce anesthesia with isoflurane, 2-3 % v/v in O₂.
2. Route the output of the isoflurane vaporizer through the home-built respiratory monitoring/gating unit (Figure S2B). Attach the TTL output of the gating unit to the External Input BNC connector on the back of the Varian DirectDrive™ console.
3. While maintaining anesthesia with isoflurane/O₂ (1 – 1.5% v/v; O₂ flow of 50 – 100 cc/min), secure the mouse to the nose cone with a strand of fishing line looped around its upper front teeth. Gently pull the fishing line to draw the animal's snout into the cone, loop the line around the tubing used to deliver the anesthesia (to keep it from slipping), and secure it with masking tape (Figure 3A).

? Troubleshooting

Position the mouse.

- 4 Deliver contrast by injecting a 500 μ L bolus of MultiHance (Gd-based MR contrast agent) intraperitoneally using a 1 ml syringe with a 29 G needle.
- 5 Position the mouse within the RF coil.
- 6 Stretch the mouse's hind legs and tape them to its tail using 1/2-inch masking tape.
- 7 Position the mouse prone within the RF coil. (The details of securing the mouse are highly coil dependent, but the animal should be placed so that its chest cavity is at the center of the coil, as demonstrated in Figure 3B).
- 8 Position the RF coil assembly.
- 9 Insert the RF coil into the RF shield (Figure 3C)
- 10 Place the full assembly into the animal tray (Figure 3D).
- 11 Position the animal tray in the magnet, being sure to place the RF coil assembly at the center of the magnet field/gradients (Figure 3E).
- 12 Check the tuning of the RF coil, using the Morris tuning unit, observing reflected power at the hydrogen resonance frequency. Adjust coil tuning as necessary (the details of tuning are highly coil dependent, so no simple set of universal instructions can be provided).

Calibrate and plan mouse-lung images.

- 13 Maintain mouse body temperature at $37 \pm 1^\circ\text{C}$ throughout the imaging experiments by circulating warm air from a heat gun through the bore of the magnet.
- 14 Collect scout images and calibrate the RF coil.
- 15 Collect a gradient-echo scout scan (single slice, sagittal view along midline of animal), repetition time (TR) = 50 ms, echo time (TE) = 5 ms, field of view (FOV) = $3 \times 3 \text{ cm}^2$, slice thickness (THK) = 1 mm, data matrix = 128×128 ; to verify the proper positioning of the animal within the magnet. Slide the animal tray in or out of the magnet, as necessary, so that the mouse's chest cavity is positioned in the center of the sagittal image.

? Troubleshooting

- 16 Use the sagittal image to plan a second scout scan – Collect this scout scan (single slice, transaxial view through the center of the chest cavity) with the same experimental parameters used to collect the sagittal image (Step 15). Rotate the animal tray, as necessary, so that the mouse's spine is at the top of transaxial image.

? Troubleshooting

- 17 Using a one-pulse spectroscopy pulse sequence, shim the magnetic field manually or with an automated shim procedure to improve its homogeneity. At 4.7 T, we typically observe a proton linewidth (full-width at half maximum) for water *in vivo* of 100 Hz or less following shimming.
- 18 Perform a pulse calibration using a one-pulse spectroscopy pulse sequence -- e.g., at fixed transmitter power, measure the intensity of the water signal as a function of pulse length. Enter the appropriate values into the pulse calibration table.

Collect high-resolution, respiratory-gated spin echo images of mouse lung.

- 19 Modify the spin-echo pulse sequence, as necessary, to permit respiratory monitoring/gating with a variable number of image slices collected per respiratory period (**BOX 1**).
- 20 Plan a series of coronal slices to cover the lungs, typically 21 – 25 0.5 mm-thick slices; transfer the plan to a custom-written respiratory-gated spin-echo pulse sequence.
- 21 Shuffle the slice order to ensure that data from adjacent slices are not collected during the same breath (**BOX 1**).
- 22 Collect data with TR of ~ 3 sec (the exact value will be established by the breathing rate), TE = 30 ms, FOV = $25 \times 25 \text{ mm}^2$, THK = 0.5 mm, data matrix = 128×128 , scanner gain (GAIN) adjusted for proper data collection (e.g., on our scanner, GAIN = 48).
- 23 Gather respiratory-gated spin-echo MRI data, with images collected during post-expiratory periods.
- 24 Collect 4 single-scan data sets. Examine the images, and average them together, excluding scans that show obvious blurring due to motion.

? Troubleshooting

Manual Segmentation/Volume Calculation of Primary (Discrete) Lung Tumors

Manually Segment Primary Lung Tumors (Timing: 15 minutes per animal). Draw regions of interest around all discrete tumors within the lung.

- 25 Open ImageJ software.
- 26 We have demonstrated previously that coronal MR images provide useful visualization of lung tumors and facilitate measurement of tumor volumes^{42,43}. The quantitative correlation of MRI-derived tumor volumes with those measured by histology is shown in Figure 3 of Garbow, et. al.⁴² Using ImageJ, load a stack of contiguous, coronal-orientation MR images that span the entire mouse.
- 27 Using ImageJ, create a montage of these images on the screen: Image → Stacks → Make Montage. Accept the default values within the Make Montage dialogue box.
- 28 Set the distance scale to properly convert pixels on the screen into measurable distances (steps 28 – 32). Under the Analyze menu, select “Set Scale”.
- 29 In the Set Scale dialogue box, enter the number of pixels in the acquisition matrix into the field labeled “Distance in pixels”. (As described in Step 22, our default setting is 128.)
- 30 In the Set Scale dialogue box, enter the length dimension from the field of view (FOV) used in acquiring the images into the field labeled “Known distance”. (As described in Step 22, our default setting is 25 mm.)
- 31 In the Set Scale dialogue box, enter “mm” into the field labeled “Unit of length”.
- 32 Click OK or hit <Enter>. ImageJ will capture the information needed to convert square pixels, defined in the ROIs to be traced, into a cross-sectional area, measured in units of mm^2 .

- 33 Open the ROI manager: Analyze→Tools→ROI Manager
 Move the ROI Manger dialogue box to a convenient location on the screen that does not overlap the montage of images. In order to illustrate the method of defining ROIs, Figure 4 shows four out of the 20-24 images used to create a typical montage. A full montage, containing 24 coronal sections, is shown later in Figure 5.
- 34 Create ROIs in the images that define a tumor of interest (steps 34 – 37). Select the polygon selection drawing tool (third icon from the left) on the ImageJ menu (Figure 4A).
- 35 Outline the ROI through a series of left mouse clicks (each left mouse click specifies a vertex), until the tumor is completely outlined. Close the ROI by double-clicking at any point (ImageJ will automatically connect the “double-clicked” vertex to the point of origin for the ROI) or by left clicking a second time on the point-of-origin of the ROI. Figure 4B shows an ROI drawn around one tumor in a single image slice.
- 36 Add the ROI to the ROI Manager by clicking “Add” within the ROI Manager dialogue box (Figure 4C). This action will create an entry/label within the ROI manager corresponding to the outlined segment of the image.
- 37 Proceed to the next (contiguous) image containing the same tumor of interest and repeat steps 25 - 27 above (Figure 4D). Continue until ROIs defining the complete tumor have been drawn on a series of sequential images. Each ROI will have its own unique identification in the ROI manager.
- 38 Edit ROIs -- Individual ROIs can be easily edited at any time within ImageJ (steps 38 – 41). In the ROI Manager dialogue box, click on the ROI to be modified.
- 39 To reposition the entire ROI, place the cursor inside the ROI and drag it to a new position.
- 40 To reposition an individual vertex of the ROI, place the cursor on top of a vertex and drag it to a new position.
- 41 Save the edited ROI by clicking the Update button in the ROI Manager dialogue box.
- 42 **Save individual ROIs (steps 42 – 25).** Click on the ROI to be saved in the ROI Manager dialogue box.
- 43 Click on More→ Save.
- 44 Navigate to the desired Folder, enter a name for the ROI under “File name:” with file extension .roi, and click “Save”.
- 45 The ROI will be saved with the file extension .roi.
- 46 **Save a set of ROIs (steps 46 – 48).** Ensure that **no** individual ROIs are selected in the ROI Manager dialogue box.
- 47 Click on More → Save.
- 48 Navigate to the desired Folder, enter a name for the set of ROIs under “File name:” with file extension .zip, and click “Save”.

Estimate tumor volume from the ROIs drawn in 34 – 37.

- 49 In the ROI Manager dialogue box, left mouse click the first ROI from the selected tumor, then CTRL-left click all other ROIs associated with this same tumor, so that they are all highlighted in the ROI Manager (Figure 4E).
- 50 In the ROI Manager dialogue box, click “Measure”. A Results box will appear listed the area of each individual ROIs under the column labeled “Area”. The units will be specified by “Set Scale” (Steps 28 – 32) (Figure 4F).
- 51 Sum the areas of all of the ROIs associated with a given tumor. To properly account for partial-volume effects (Box #2), divide the areas of the first and last ROI by 2 before summing.
- 52 Calculate the tumor volume by multiplying the summed area of all of the ROI's by the slice thickness used in acquiring the images. As described above in Step 22, our preferred slice thickness is 0.5 mm. The resulting value will be the MRI-derived volume of a single tumor, in units of mm³.
- 53 Repeat Steps 33 – 37 and 49 – 52 for each individual tumor in the animal, in order to estimate its volume.

Manual Segmentation/Volume Calculation of Metastatic (Diffuse) Lung Tumors

- 54 **Manually Segment Metastatic Tumors** (Timing: 30 minutes per animal). Tumor burden can be assessed at three different levels of detail and quantitation, as described below: (A) Assess, qualitatively, lung-tumor burden in the same mouse at two or more time points (e.g., monitor tumor progression or therapeutic response); (B) Measure relative lung-tumor burden across a series of mice; or (C - D) Measure absolute lung-tumor burden across a series of mice.

Qualitative assessment of the lung-tumor burden in the same mouse at two or more time points. This application takes advantage of the previously discussed observation that in MR images of lung, healthy parenchyma appears dark, while tumor tissue is bright. Thus, as described below, progressive increase in tumor burden leads to an increase in average lung-image intensity, compared to (normalized to) that of liver within the same mouse (Steps 54.A.v and 54.A.vi below). Similarly, decrease (or stability) in average, normalized lung intensity indicates regression (or stability) of pulmonary tumor burden. At each time point, perform the steps described in Option A.

The measurement of *relative* lung-tumor burden across a series of mice requires that imaging data first be collected for a series of healthy (non-tumor-bearing), age-matched control mice (n=5). For each control mouse, perform the steps described in Option B. This procedure is limited to studying mice that are age- and genotype-matched to the cohort of control mice used to establish baseline image intensity for healthy lung. If one wishes to characterize tumors in a different species of mouse, lung images of an appropriate set of healthy control mice must be first collected and analyzed.

To measure *absolute* lung-tumor burden across a series of mice you first need to collect imaging data for both for a series of healthy (non-tumor-bearing), age-matched control mice (n=5) and a training series of tumor-bearing mice (n=10). Except as noted, the steps in Option C should be applied to **each** (control and tumor-bearing) mouse. To use this information to determine lung-tumor burden in the test series of mice, perform the steps in Option D.

A. Qualitative assessment of the lung-tumor burden in the same mouse at two or more time points (e.g., monitor tumor progression or therapeutic response)

- i. Collect respiratory-gated, spin-echo images of the lung (coronal orientation), as detailed in Steps 19 - 24. Be certain that all experimental parameters, including scanner gain, are the same at each time point.
- ii. Using ImageJ, load a stack of contiguous, MR images (coronal-orientation) that span the entire mouse and create a montage of these images, as detailed in Step 27 (Figure 5A).
- iii. Open the ImageJ ROI Manager as described in Step 33.
- iv. Set the distance scale to properly convert pixels on the screen into measurable distances, as described in Steps 28 - 32.
- v. Compute the average, normalized lung-image intensity as described in **Box 3**.
- vi. Tumor burden at two or more time points can be assessed by comparing values of average, normalized lung-image intensity – An increased value indicates increased tumor burden; a decreased value indicates decreased tumor.

B. Measurement of relative lung-tumor burden across a series of mice.

- i. Collect respiratory-gated, spin-echo images of the lung (coronal orientation), as detailed in Steps 19 - 24. Be certain that all experimental parameters, including scanner gain, are the same at each time point.
- ii. As detailed above in **Box 3**, for each image slice containing observable lung, manually segment the lungs (left and right) and save the resulting ROI. In drawing lung ROIs, be careful to avoid image slices containing the heart, and to exclude both the thorax and the spine from the resulting ROIs.
- iii. Compute the average, normalized (based on liver) image intensity for healthy lung, as detailed in **Box 3**.
- iv. Compute the average, normalized image intensity for healthy lung across all animals by averaging together (simple, arithmetic average) the values computed in Step 54.B.iii.
- v. Having established the average, normalized image intensity for healthy lung, average, normalized lung image intensity for each of the tumor-bearing mice is computed as described in Step 54.A.
- vi. For each tumor-bearing mouse, subtract the average, normalized image intensity for healthy lung, computed from the cohort of control animals, from its average, normalized image intensity. The resulting value provides a measure of *relative* tumor burden within the mouse. Values of *relative* tumor burden can be compared between animals and across time points of a longitudinal, time-course study.

C. Measurement of absolute lung-tumor burden across a series of mice.

- i. Collect respiratory-gated, spin-echo images of the lung (coronal orientation), as detailed in Steps 19 - 24. Be certain that all experimental parameters, including scanner gain, are the same at each time point.
- ii. Euthanize the mouse immediately following the imaging experiments
- iii. Remove and weigh the lungs.
- iv. For the entire cohort of **control** mice, compute the average lung weight (simple, arithmetic average).

- v. For each **tumor-bearing** mouse, subtract the average weight for healthy lung, computed from the cohort of control animals (Step 54.C.iv) from its measured lung weight, to obtain a **corrected** tumor weight.
- vi. Compute the average, normalized lung-image intensity, as described in **Box 3**.
- vii. For the cohort of **control** mice, compute the average, normalized image intensity for healthy lung by averaging together (simple, arithmetic average) the values computed in Step 54.C.vi.
- viii. For each **tumor-bearing** mouse, compute the absolute tumor burden, as described in **Box 4**.

D. Determination of absolute tumor burden in a test tumor-bearing mouse:

- i. Collect respiratory-gated, spin-echo images of the lung (coronal orientation), as detailed in Steps 19 - 24. Be certain that all experimental parameters, including scanner gain, are the same at each time point.
- ii. Calculate the average, normalized lung-image intensity, as described in Box 3.
- iii. Calculate the total lung volume, as described above in Step 54.C.viii.
- iv. Subtract the average, normalized lung intensity calculated for the cohort of **control** (non-tumor-bearing) mice [Step 54.C.vii] to yield a **corrected** average, normalized lung intensity.
- v. Multiply the **corrected** average, normalized lung intensity (Step 54.D.iv) and the total lung volume (Step 54.D.iii) to generate the total MRI-measured tumor burden.
- vi. Use the calibration curve generated in Step 54.C.viii, to convert this MRI-measured value into **absolute** tumor burden (mg).
- vii. As noted above for the measurement of *relative* lung-tumor burden across a series of mice, this procedure is limited to studying mice that are age- and genotype-matched to the cohort of control mice and the training set of tumor-bearing mice used to establish the calibration curve of Step 54.C.viii. If one wishes to characterize tumors in a different species of mouse, lung images of an appropriate cohort of healthy control mice and a training series of tumor-bearing mice must be first collected and analyzed⁴⁹.

Automated Segmentation/Volume Calculation of Metastatic (Diffuse) Lung Tumors
(Timing: 10 minutes per animal).

- 55 **Install MATLAB *.m files.** Download all of the MATLAB *.m files from Supplementary Materials and save them into a single new folder named lung_segment within the Matlab *toolbox* folder: Program Files→Matlab→R2008a → toolbox → lung_segment
- 56 **Process the MR images in MATLAB.** Supplementary Video #2 shows the steps in the automated segmentation/volume calculation, as described below. Open MATLAB (version 7.6.0 R2008a) and select as your current directory the folder into which the attached MATLAB *.m files were saved (Program Files/Matlab/R2008a/toolbox/lung_segment).
- 57 Run the file *Lung_Segmentation_Main.m*, which is contained in the supplied package of Matlab *.m files. An outline of how the program performs lung segmentation, together with information on how to run the *Lung_Segmentation_Main.m* file is provided in **Box 5**.

ANTICIPATED RESULTS

On the basis of our experience with these procedures over the last 4 years, we anticipate a learning curve of 24 animals (data acquisition) and 5 – 10 animals (image analysis) for any individual with a basic-science background. These procedures are routinely executed in our laboratory by a veterinary technologist with basic animal handling skills, but no previous training or background in MRI data acquisition or image analysis prior to working in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BOX 1 – Modifying the spin-echo pulse sequence and choosing experimental parameters for respiratory-gated mouse-lung imaging

- i. The spin-echo pulse sequence used to collect mouse-lung MR images must be modified for collection of respiratory-gated data. In general, most pulse sequences (including all of those supplied with the Varian DirectDrive™ console) contain a flag for an external input signal that can be used to trigger data acquisition. In the unlikely event that this flag is not present, it should be added at the beginning of the pulse sequence.
- ii. Nonetheless, even pulse sequences that contain a flag for triggering acquisition must be modified, as detailed below. Standard spin-echo pulse sequences divide the time period defined by the repetition time (TR) into N equal parts, where N is the number of slices of image data to be collected, and acquire data on an individual slice every (TR/N) seconds. However, this is not appropriate for respiratory-gated data, in which one would like to collect data from **several** imaging slices during each post-expiratory (PE) period, but gather **no** data during the time between PE periods.
- iii. To accomplish this, it is necessary to break up the loop in the pulse sequence used to collect data across slices into a pair of loops. The inner loop controls the number of slices (a grouping) to be collected during one PE period. (In our pulse sequences, we define the number of slices in a grouping by the variable *eprg*. The outer (existent) loop then controls the number of such groupings that are required to cover all of the slices. This scheme places modest limitations on the number of slices that must be collected (e.g., the total number of slices must be evenly divisible by *eprg*).
- iv. Let us consider a specific example: Assume that the mouse's respiratory rate is 2 s^{-1} , a normal rate for a warm animal under proper anesthesia. This respiratory rate produces a PE period of 250 – 300 ms per breath. With a typical echo time (TE) of 30 ms and allowing several ms for data collection, the time required to acquire data for one image slice is approximately 50 ms. Thus, one can collect a maximum of perhaps 5 slices of data (*eprg* = 5) per PE period (250 ms/50 ms = 5). Clearly, data for a smaller number of slices can also be acquired per PE period.
- v. Within this requirement, the values for total number of slices (*ns*) and *eprg* must be selected so that *ns* is an integer-multiple of *eprg*. Thus, if one needs to collect ~25 slices of data to cover the entire lungs, acceptable combinations of *ns* and *eprg* include: *ns* = 25, *eprg* = 5; *ns* = 24; *eprg* = 4; *ns* = 28; *eprg* = 4. While not seriously constraining, the user must be aware of this limitation when designing respiratory-gated experiments and choosing specific pulse-sequence parameters.
- vi. Finally, the order in which data for the slices is collected must be “shuffled” to ensure that images from adjacent slices are not acquired during the same PE period. (The frequency-selective pulses used to select individual slices are not ideal and there can be some “bleeding” of the RF excitation into adjacent slices. Since data from slices within a grouping are collected within the short time of a single PE period, this can lead to saturation and subsequent diminution of signal.) This shuffling is the equivalent of “interleaving” slices, a standard practice when acquiring non-respiratory-gated, spin-echo data. A simple macro can be written to accomplish the necessary shuffling of slices.

BOX 2 – Partial volume averaging

Each slice within an image represents the two-dimensional projection of all features within that anatomical slice onto the imaging plane. Observed imaging features may be completely or partially contained within that slice – from the image itself, there is no way of knowing. Partial volume averaging results when an imaging feature is only *partially* within the MR imaging slice. For an imaging slice at the edge of a tumor, there is no way to know, *a priori*, whether the tumor is fully or partially (and to what extent) contained within the slice. In calculating tumor volumes, we make the assumption that the tumor is “half in” and “half out” of the imaging slices at the edge of the tumor. Thus, for “edge” slices, we divide the ROI-derived cross-sectional area, as described above, by 2 prior to summing the areas across all slices.

BOX 3 – Computing average, normalized lung-image intensity

- i. For each image slice containing observable lung, manually segment the lungs (left and right) and save the resulting ROI for each lung in each image, as described in Steps 34 – 37 and Supplementary Movie 1. At the completion of this step each individual lung in each image will be its own ROI (Figure 5B shows segmentation of the left lung; Figure 5C demonstrates segmentation of the right lung.) In drawing lung ROIs, be careful to avoid image slices containing the heart (which, if visible, is highly blurred due to motion), and to exclude both the thorax and the spine from the resulting ROIs. (We typically omit two or three slices per lung image that include the heart.) Supplementary Video #1 demonstrates the manual segmentation of mouse lungs using ImageJ.
- ii. After segmenting all of the slices containing visible lung and saving the corresponding ROIs, click “Measure”. The ROI area, in units of mm², and the mean intensity for the ROI in each image slice will be displayed in the pop-up table.
- iii. Save the “Measure” table, if desired, by selecting “Save As” from the “File” menu.
- iv. Determine the average lung-image intensity across all of the selected images by computing the product (ROI area × mean intensity) for each slice, summing these values, and dividing by the total of the ROI areas summed over all the slices.
- v. Compute the average liver-image intensity and normalize the lung-image intensity based upon liver:
 - Select an image slice with clearly visible liver.
 - Draw an ROI around a large, homogeneous section of liver, as described in Step 54.A.v above, being careful to avoid obvious regions of either hyper- or hypo-intensity arising from pockets of lipid or vasculature, respectively.
 - Click “Measure” in the ROI Manager to calculate the average image intensity within the liver ROI.

Divide the average lung-image intensity, calculated in Step 54.A.viii, by the liver-image intensity to determine an **average, normalized** lung-image intensity.

BOX 4 – Computing absolute lung-tumor burden

- i. Compute the volume of the segmented lungs by summing the lung ROI areas for every image slice and multiplying by the slice thickness.
- ii. Subtract the average, normalized image intensity for healthy lung, computed from the cohort of control animals (Step 54.C.vii), from its average image intensity, to obtain a **corrected** average, normalized image intensity.
- iii. Multiply its **corrected** average, normalized image intensity by its measured lung volume to generate the total MRI-measured tumor burden.
- iv. Plot its total MRI-measured tumor burden vs. its **corrected** lung weight, as measured in Step 54.C.v.
- v. Fit a (linear, least-squares) straight line through all of the points plotted in D that passes through the origin (0,0). The resulting plot serves as a calibration curve that can be used to convert MRI lung-image intensity into **absolute** tumor burden. An example of such a calibration curve is shown in Figure 1.

BOX 5 Outline of algorithm for automated image segmentation

A. Description of algorithm.

- i. As described above, the automatic lung-segmentation process accepts as manual user input: i) the first and last (ventral-most and dorsal-most) image slices to segment, ii) a manually outlined single, initial slice of lung; iii) the upper and lower center points of the segmented lungs, and iv) a manually outlined area of liver in a single slice.
- ii. A parametric model of the lung shape is determined from the initial image and the manual segmentation of this initial slice. A locally-optimal set of parameter values is calculated using the Nelder-Mead simplex method.
- iii. The optimized parameter values for the initial slice are used as initial values for determining the locally-optimal parameter values for the two adjacent slices.
- iv. The parameter values for the adjacent slices are likewise propagated dorsally and ventrally until all lung tissue is segmented.
- v. The average lung-image intensity is calculated from all of the segmented slices and is then scaled by the average liver intensity, calculated from the manually-drawn liver ROI.

A more detailed discussion of the algorithm, and the objective function used for the optimization steps, can be found in our recently published paper ⁴⁹.

B. How to run the Lung_Segmentation_Main.m program.

Refer to Supplementary Movie 2.

- i. The program can be run with no inputs or arguments, by typing the following command into the MATLAB Command Window:


```
[Avg_intensity,Lung_Volume]=Lung_Segmentation_Main;
```
- ii. Execution of this command will cause the program to prompt the user to enter all of the necessary pieces of information:
 - the directory containing the MR lung images, in *.fdf format, which are to be segmented (Figure 6A);
 - a flag indicating whether the images must be rotated 90° prior to processing (necessary if the original images are oriented horizontally, rather than vertically) (Figure 6B);
 - the slice number of the ventral-most slice with sizable lung area (Figure 6C);
 - the slice number of the dorsal-most slice with sizable areas of both left and right lung (Figure 6C).
- iii. To aid in the input of these values, a single lung slice from the specified folder of MR images is displayed when the user is prompted for the rotation flag, and a montage of all of the lung images is displayed when the user is prompted for the slice-number inputs. In the displayed montage, images are tiled from ventral to dorsal, from left to right and top to bottom, beginning in the upper-left corner of the montage, and ending in the bottom-right corner.
- iv. Alternatively, the necessary information can be directly input as arguments in the MATLAB Command Window command line by typing:

[Avg_intensity,Lung_Volume]=Lung_Segmentation_Main(directory,rotate,ventral,dorsal),

where *directory* is the path of the directory containing the lung images (entered as a string, i.e., within single quotation marks), *rotate* has the value of 1 or 0, depending upon whether rotation of the image is required (1) or not required (0), *ventral* is the slice number of the ventral-most slice with sizable lung area, and *dorsal* is the slice number of the dorsal-most slice with sizable areas of both left and right lung.

- v. Details about the command options can be found by typing the command help Lung_Segmentation_Main
- vi. Following the program prompt, outline the exterior boundary of the lungs in the displayed image slice (Figure 6D).

The left and right lungs should **not** be separated, but rather should be outlined within one continuous boundary.

The lung is encircled with a region of interest (ROI) by left-clicking on a series of vertices that create an outline of the desired polygon.

The polygon can be closed by re-clicking the starting vertex, or by double-clicking when selecting the final vertex.

The ROI can be repositioned if necessary. To reposition the entire ROI, left-click inside the ROI and drag it to a new position. To reposition an individual vertex, left-click the vertex and drag it to a new position.

The polygon is finalized by double-clicking within the closed shape outlining the lungs.

Detailed instructions on this interface are available in the MATLAB help file for the *roipoly.m* function.

- vii. Following the program prompt, click on the upper and lower center points of the displayed, segmented lungs (Figures 6E, F). These points should both lie along the median line of the animal, which may be distinguishable due to small amounts of spine visible in the segmented slice. The upper center point should be the point nearest the top of the image that lies along the median line within the segmented area. Similarly, the lower center point should be the corresponding point closest to the bottom of the image.
- viii. Following the program prompt, outline a region of liver below the lungs in the displayed image slice (Figure 6G). The procedure for outlining liver is the same as that described for lung in vi. Double-clicking within the liver ROI initiates the automated lung segmentation routine.
- ix. The program displays a “Progress Bar” that allows the user to follow the status of the segmentation. The execution time for a 15-slice segmentation (128×128 voxel images) on an Intel Core 2 Quad, 2.4 GHz Dell computer, is approximately 3 minutes. An outline of how the program performs this segmentation is provided above.
- x. The program displays as output, within a Matlab Figure, a montage of lung images with automatic segmentation boundaries overlaid in red (Figure 6H). This montage can be saved from within the figure, if desired, in a variety of different formats, using the Matlab “Save As” command.

- xi.** The program also outputs (in the Matlab Workspace window) values of the liver-corrected lung intensity (variable name = *Avg_intensity*), averaged across all of the segmented slices, and the total segmented lung volume in voxels (variable name = *Lung_Volume*) (Figure 6I). This volume can be converted to physical units (e.g., mm³) by multiplying the voxel volume by the voxel size (as described in Step 22, our default setting is ~0.019 mm³).
- xii. Note:** In computing lung-tumor burden, manual (Step 54.A) and automatic (Steps 55 - 57) lung segmentations should not be mixed. All control, training, and test-animal data sets must be segmented automatically, since the average lung-image intensities based upon automatic and manual segmentation will not be the same (automatic segmentation does not exclude slices containing heart).

Figure 1. Anesthesia and RF coil/animal tray setup for MR acquisition: (A) Anesthetized mouse placed inside the nose cone and then inside the RF coil (B); (C) RF coil assembly, including an anesthetized mouse, placed inside the RF shield; (D) Entire coil assembly, placed within the animal tray; (E) Animal tray placed inside the magnet.

Figure 2.

Manual segmentation of discrete tumors: (A) Screen shot of a montage containing four segments and the ROI manager. In our experience, this organization of the screen is optimal for segmentation of discrete lung tumors. (B) As described in the text, after selecting the polygon tool, a discrete lung tumor is encircled through a series of left mouse clicks. (C) Clicking the “Add” button in the ROI manager creates an entry within the ROI manager uniquely associated with the encircled ROI (e.g., 0085-0135). (D) A new ROI is created by outlining the tumor on a contiguous image slice and is added to the ROI manager by pressing the “Add” button. (E) After all the ROIs have been added to the ROI Manager, selecting the “Measure” button produces the Results screen shown in (F). Data in Results include the area of the ROI, the mean intensity of the image within the ROI, as well as Minimum (Min) and Maximum (Max) intensity values. As described in the text, these values are subsequently used to estimate the tumor burden.

A) Progression of disease in the same mouse

B) Relative lung tumor burden in different mice

B) Absolute tumor burden compared to non-tumor bearing control

Figure 3.

Graphic, illustrating three different approaches to measuring diffuse tumor burden, as described in Module #3: (A) Monitor progression of disease (as illustrated here) or response to therapy in individual mice *via* serial imaging; (B) Compare relative lung-tumor burden between mice – Shown here are animals with heavy (left) and light (right) tumor burden; (C) Measure absolute tumor burden (in units of mg), by comparing average, normalized lung-image intensity to that of a cohort of non-tumor-bearing control mice. This measurement requires the development of a calibration curve, as detailed in Tidwell, et. al.⁴⁹

Polygon tool
(step 6A of module #2)

Figure 4.

Manual segmentation of diffuse metastatic tumors: (A) The montage is assembled and displayed as described in Figure 2 for segmentation of individual discrete tumors. (B) The ROI manager is opened and the left and right lungs in each image are outlined as a single ROI. (C) The lung ROIs are sequentially added to the ROI Manager, as in Figure 2.

Figure 5. Sample calibration curve for estimation of absolute tumor burden from a test MR image. The calibration curve was generated based on lung weights and automatic lung segmentations of four control mice and ten tumor-bearing mice, following the instructions provided in Step 54.C.

Figure 6.

Automated segmentation of diffuse metastatic tumors: (A) After opening Matlab and running the file named *Lung_Segmentation_Main.m*, the directory containing the files to be segmented is input. (B) If necessary, images oriented horizontally are rotated 90° to a vertical orientation by entering “1”, otherwise enter “0”. (C) The numbers of the image slices containing the most ventral and dorsal portion of the lung are entered into the program. This step is necessary, since not all images will contain lung tissue and some will contain images of spine or anterior chest wall (e.g., slices #1-4, 23 and 24, as illustrated). (D) To initiate automatic segmentation of all the displayed lung fields, the exterior boundary of the lungs in one representative slice is outlined. (E) Following the automated program prompt, the upper (E) and lower (F) center points of the displayed segmented lungs are identified. (G) After outlining the boundary of the lungs and identifying the upper and lower points of the lung, the user is prompted to outline a part of the liver. (H) The lungs are

segmented by the program and displayed as a montage plot. (I) Values of the liver-corrected, average lung intensity (*Avg_intensity*) and total segmented lung volume (*Lung_Volume*) are output to the Matlab Workspace window.

Table 1Comparison of Modalities for Non-Invasive *In vivo* Imaging of Pulmonary Tumors

Modality	Strengths	Weaknesses	References
MRI	Subject not exposed to ionizing radiation Excellent endogenous image contrast between tumor and healthy lung tissue High spatial resolution, with ability to quantify changes in spatial and temporal tissue morphology Visualization of vasculature using dynamic contrast enhanced imaging	Requires respiratory gating Requires access to a small-animal MRI scanner or purpose-built small-animal RF coil for clinical scanner Not high throughput (~40 minutes per subject)	23,42,43,45,46,48,51,52
Micro-CT	High spatial resolution No need for respiratory gating with free-breathing technology, but preferred for high resolution High throughput (~10 minutes per subject) Excellent endogenous image contrast between tumor and healthy lung tissue	Subject exposed to ionizing radiation Requires access to a small-animal CT scanner Visualization of vasculature requires a large dose of contrast agent	23,27,48,53
Micro-PET	Metabolic evaluation of primary tumor and systemic metastasis High signal detection sensitivity Quantitative measurement of uptake kinetics	Subject exposed to ionizing radiation Low spatial resolution, lack of anatomic information Requires synthesis and administration of radiolabeled tracers Requires access to a small-animal PET scanner	23,48,54,55
Optical	Subject not exposed to ionizing radiation Relatively inexpensive, compact benchtop or portable instrumentation High-throughput determination of tumor burden using bioluminescence imaging (BLI) Image specific metabolic pathways and protein expression with fluorescence imaging (FI) and fluorescent molecular probes High signal detection sensitivity of multiple fluorescent reporters within the same animal	Low spatial resolution at depths > 500 μm , lack of anatomic information BLI requires expression of biomarker (luciferase) by tumor cells FI requires expression of gene-encoded fluorescent protein or administration of tumor-selective fluorescence-tagged reporter probes to visualize metabolic pathway or enzyme activity	14,23,46,56-60

Table 2

Troubleshooting

Steps	Problem	Reason	Solution(s)
1-3	Uneven respiration	Over-sedation with anesthetic at induction	Turn off isoflurane; wait for respiration to recover; restore isoflurane to 1%, monitor respiration Decrease isoflurane in steps of 0.1%
19-24	Rapid respiration rate	Light anesthesia Overheated animal	Check isoflurane level in vaporizer and fill, if necessary Check O ₂ tank, replace if empty Check O ₂ flow rate and adjust as necessary Increase isoflurane in steps of 0.25% Adjust temperature/flow of heat gun
19-24	Depressed respiration rate	Cold animal Excessive oxygen flow/back pressure	Adjust temperature/flow of heat gun Reduce oxygen flow/pressure
19-24	Apparent loss of respiration	Animal separated from nose cone	Remove animal tray from magnet; re-attach nose cone
15	Lungs not in image FOV	Improper positioning of animal	Check position of coil within animal tray and tray within magnet
16	Mouse spine not vertical in image	Improper positioning of animal Animal awake and moving in RF coil	Rotate Animal tray to properly align the spine Remove animal tray from magnet; reposition animal; increase isoflurane
Box 4, B.i	MATLAB error message "Undefined function or variable 'Lung_Segmentation_Main'"	*.m files not installed in the proper directory	Verify that the provided files are installed into the directory specified in Step 12.A.
Box 4, B.x	Poor quality automatic segmentations	Improper manual initialization Improper positioning of animal Improper selection of imaging parameters	Check manual inputs, including the starting and ending slice numbers, the manual lung segmentation, and the selection of the center points, to verify that they comply with the instructions provided in Steps 13.B.vi-vii. Check that the mouse's lungs are roughly symmetric in the dorsal-most images. This corresponds to the mouse's spine being positioned at the top of the transaxial image. Check that the images are sharp and low in noise, with good contrast between healthy lung tissue and tumor. Reimage if necessary.
54.C.viii Box 4, B.xii	Poor correlation of calibration curve	Inconsistent imaging parameters Improper selection of liver regions for intensity normalization	Check that imaging parameters were identical for all control and tumor-bearing animals. Check that the liver region selected contains a large portion of the liver, contains only liver, and, for fully manual segmentation (Step 11), is relatively homogeneous.