Optimized murine lung preparation for detailed structural evaluation via micro-computed tomography

Dragoș M. Vasilescu,1,2 Lars Knudsen,3 Matthias Ochs,3 Ewald R. Weibel,4 and Eric A. Hoffman1

1Department of Radiology, University of Iowa, Iowa City, Iowa; 2Department of Diagnostic Radiology, Philipps University, Marburg; 3Institute of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany; and 4Department of Anatomy, Bern University, Bern, Switzerland

Submitted 2 May 2011; accepted in final form 3 August 2011

Vasilescu DM, Knudsen L, Ochs M, Weibel ER, Hoffman EA. Optimized murine lung preparation for detailed structural evaluation via micro-computed tomography. J Appl Physiol 112: 159–166, 2012. First published August 4, 2011; doi:10.1152/japplphysiol.00550.2011.—Utilizing micro-X-ray CT (μCT) imaging, we sought to generate an atlas of in vivo and intact/ex vivo lungs from normal murine strains. In vivo imaging allows visualization of parenchymal density and small airways (15–28 μm). Ex vivo imaging of the intact lung via μCT allows for improved understanding of the three-dimensional lung architecture at the alveolar level with voxel dimensions of 1–2 μm. μCT requires that air spaces remain air-filled to detect alveolar architecture while in vivo structural geometry of the lungs is maintained. To achieve these requirements, a fixation and imaging methodology that permits nondestructive whole lung ex vivo μCT imaging has been implemented and tested. After in vivo imaging, lungs from supine anesthetized C57Bl/6 mice, at 15, 20, and 25 cmH2O airway pressure, were fixed in situ via vascular perfusion using a two-stage flushing system while held at 20 cmH2O airway pressure. Extracted fixed lungs were air-dried. Whole lung volume was acquired at 1, 7, 21, and >70 days after the lungs were dried and served as validation for fixation stability. No significant shrinkage was observed: +8.95% change from in vivo to fixed lung (P = 0.12), −1.47% change from day 1 to day 7 (P = 0.07), −2.51% change from day 1 to day 21 (P = 0.05), and −4.90% change from day 1 to day 70 and thereafter (P = 0.04). μCT evaluation showed well-fixed alveoli and capillary beds correlating with histological analysis. A fixation and imaging method has been established for μCT imaging of the murine lung that allows for ex vivo morphometric analysis, representative of the in vivo lung.

perfusion-based lung fixation; high-resolution imaging; lung morphology; quantitative computed tomography

MURINE MODELS HAVE BEEN WIDELY used to investigate lung anatomy, physiology, and pathological processes and are of great interest because of the ability, through genetic manipulation, to create models of lung diseases, including asthma (16), emphysema (5, 9), and lung cancer (6, 8). These models are traditionally evaluated through postmortem, histological evaluation of a small number of thin sections. To minimize bias, sampling and morphometric methods have been developed and were recently reviewed (14). Significant limitations or disadvantages of the histological approach lay in the fact that, once sampled, the lung cannot be resampled to alternate orientation, sampling may induce distortions, as well as contextual relationships between extracted portions of the lung, and the remaining lung is lost, as is the relationship of the evaluated portion of excised lung and the in vivo lung anatomy. By imaging the in vivo and ex vivo anatomy in full three dimensions (3D), one can be mapped to the other. Micro X-ray CT (μCT) imaging (13, 24, 26) has begun to provide solutions to some of these limitations. The lung can be imaged in vivo with resolutions on the order of 50 μm (isotropic voxel dimensions on the order of 25 μm). Because the lung is imaged volumetrically, it is possible to assess 3D anatomy, and sections can be evaluated at any obliquity. Intermittent breath-hold techniques can be used in conjunction with imaging, such that details of in vivo lung anatomy are detectable down to small bronchioles (7, 20, 31). Methods are available to nondestructively interrogate the interior of the ex vivo fixed lung by μCT at an isotropic voxel dimension of 1–2 μm and 3D fields of view of 2 mm. The potential of such high resolutions using μCT has been shown by others (18, 19). To take advantage of this new imaging technology, we have been motivated to revisit the need to develop a method of lung fixation, such that μCT can be exploited to better understand the morphology of the lung in health and disease. Here, we describe a newly developed method that aims to preserve the murine lung structures in the most natural and physiological state for accurate radiological and histological analysis while, at the same time, leaving air spaces filled with air, such that contrast differences between components are adequate for X-ray imaging.

Various methods of lung fixation have been developed to produce anatomic specimens for microscopic evaluation (2, 4, 12, 14, 23, 33). Methods can be categorized as 1) instillation-based and 2) perfusion-based. Instillation-based methods of fixation consist of instillation of a fixation solution via the trachea into the lungs. With this approach, the lungs are inflated, either in situ or excised, to the level of pressure of the fixative. Renne et al. (25) discussed the optimal method for formalin-based fixation of lungs and concluded that instillation fixation is the most efficient way to fix the lungs for histological investigations. Blumler et al. (4) published a lung fixation method for MRI studies based on formalin instillation and, to avoid collapse, dried the lungs while maintaining inflation pressure. Although formalin fixation is the most popular method in histology and is relatively inexpensive, it has significant disadvantages, such as severe shrinkage, which is difficult to correct (17). Studies employing instillation fixation at lower pressures (28) result in higher tissue density with smaller and potentially distorted alveoli (30), as well as smaller airways, which are potentially not visible in imaging obtained using whole lung μCT.

In contrast, perfusion-based fixation preserves the alveolar septal walls in a physiological way (2). As summarized recently (14), the method has several advantages. State-of-the-art...
stereological assessment of the lung is discussed, along with the advantages and disadvantages of lung fixation methods. None of the methods satisfied all desired criteria for an ideal stereological assessment. Gil et al. (10) discussed the alveolar volume-surface area relation in air- and saline-filled lungs and showed that, because of nonexistent surface tension in liquid-filled lungs, septal walls are not fixed in a natural and smooth way.

Recently, a new cryogenic-based method (13) for lung fixation was presented as an alternative to fixation of large whole lungs, including those from humans. The method is relatively complex and labor-intensive but has produced better results. Others used cryosampling for histology (29, 32, 34), and such methods have been recently recommended (14) for specific histological assessments. Such cryogenic-based methods are not suitable for imaging of whole lungs with long scanning times and a requirement that the air spaces remain filled with air. Other than the cryogenic-based fixation method, none of the methods provide a suitable way to preserve a whole lung for extended periods of time for study via \( \mu \)CT at multiple resolutions.

To achieve our study goals of identifying and validating a fixation method for the nondestructive ex vivo assessment of the murine lung via \( \mu \)CT, we have set the following criteria. 1) The preparation should be suitable for multiresolution \( \mu \)CT as well as conventional histological sectioning. 2) The lungs should be fixed at a controlled inflation state. 3) In the final state for imaging, the air spaces must be air filled. 4) Anatomic geometry should remain close to the in vivo state from central airways and vasculature to alveoli and capillary beds. 5) The volume and geometry of the air-filled fixed lung should remain stable over \( \geq 1-2 \) mo to allow time for evaluation. 6) The lung and tissue architecture must be reasonably well and homogeneously preserved in all parts of the lung to allow unbiased analysis.

Nondestructive imaging modalities allow us to resolve septal walls within the murine lung at 2 \( \mu \)m/voxel. The shapes of alveolar walls are detectable at this level of magnification; therefore, we need a fixation method that fixes the septal walls closest to the in vivo state. To accomplish all these goals, a perfusion-based fixation method has been developed, with the notion that perfusion fixation is a requirement to study the alveolar septal walls and make accurate conclusions about the gas exchange regions of the lung. Fulfillment of these requirements will allow us to use design-based stereological techniques, in combination with multiresolution \( \mu \)CT imaging, without the need to section the fixed lungs, to provide insights into the morphometry of the lung.

We have validated our fixation method by first scanning the mice in vivo and then fixing the lungs and scanning the fixed lungs again after they are dried. The volume was computed on the basis of image segmentation of the \( \mu \)CT images of the whole lung. The ex vivo volumes are reported in relation to the in vivo scans, with airway distending pressures applied similarly in vivo and ex vivo. The present gold standard for evaluating fixed tissue samples is histology. This required embedding the samples and cutting them into thin slices that could be viewed via different microscopy techniques. The samples were inspected using light microscopy for possible damage or artifacts induced by the fixation method. We demonstrate that our fixation method not only provides an in vivo-like lung structure preservation but that radiological and microscopy-based assessments are possible.

**MATERIALS AND METHODS**

The flowchart in Fig. 1 is an overview of the methods and validation utilized in the study. After the animal was prepared, in vivo \( \mu \)CT scans were carried out at multiple static intermittent airway pressures (Paw) (20); then the lungs were perfusion-fixed in situ. After the lungs were extracted and dried, we scanned the preparations over time to determine volume changes from the desired fixation pressure. Histology was performed after all needed scans were acquired to validate the fixation quality.

**Animals and Preparation**

Six 12-wk-old male mice of a commonly used inbred strain (C57Bl/6J, Jackson Laboratories) and six 91-wk-old male mice of the same strain were used for the study. The weight of the young and the old mice at the time of the study was 26.0 g (SD 1.3) and 26.1 g (SD 2.0), respectively. Animals were housed in an animal facility at the University of Iowa, with food and water provided ad libitum. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Iowa.

The animals were weighed and then anesthetized using a mixture of ketamine and xylazine (87.5 and 12.5 mg/kg ip, respectively; Ratiopharm). A tracheostomy was performed, and a 20-gauge catheter was placed and tied, so that it could not slide out. Each mouse was paralyzed with an injection of pancuronium (0.1 ml/kg ip) and placed on mechanical ventilation (Scireq Flexivent, ON, Canada). The ventilator system also allowed for the performance of pulmonary function tests. We acquired compliance, resistance, and elastance measurements, which allowed us to determine noninvasively the state of the lungs. Subsequent to the ventilator used for pulmonary function tests, a second ventilator (model SAR 830/A, CWE, Ardmore, PA, modified to enable respiratory gating) was utilized during imaging to permit intermittent isopressure imaging, as previously discussed (20).

An ECG monitoring system (Biovet, M2MMImaging, Newark, NJ) was used to track the heart rate and signal characteristics while the animal was in the scanner. For this purpose, three leads were attached to the paws of the mouse (front paws and left hindpaw).

**In Vivo \( \mu \)CT Imaging Protocol**

**\( \mu \)CT scanners.** In vivo imaging was performed using a MicroCAT II \( \mu \)CT scanner (Siemens Pre-Clinical). Ex vivo fixed lung scanning

---

Fig. 1. Workflow chart. PFTs, pulmonary function tests.
was performed on the same scanner, as well as on a high-resolution μCT scanner (MicroXCT 400, XRadia, Concord, CA), located at the Beckman Institute for Technology and Science (Urbana, IL), for biological samples.

The MicroCAT II scanner allows in vivo and ex vivo scanning of small animals and samples. Our system uses a DALSA camera, which incorporates a charge-coupled device (CCD) detector of 3,072 × 2,480 pixels and a KeVex X-ray source (40–120 Vp) with variable focal spot size (8–20 m).

For the in vivo scanning protocol, the following parameters were used: 60 kV, 500 μA, 600-ms exposure time, detector binning of 2,720 projections over 270°, and 100 dark and 100 light images prior to each scan for calibration purposes. Using a 10-μm tungsten wire phantom at these parameters, we determined a system resolution of 50 μm at 10% modulation transfer function with a voxel size of 28 μm.

Each mouse was scanned at three lung volumes: 15, 20, and 25 cmH2O Paw. To ensure a constant inflation during image acquisition, an intermittent isopressure breath-hold sequence was implemented using LabView to control the CWE ventilator, similar to the method employed by Namati et al. (20). The scanner was under computer control by way of the LabView-based control software and was paused during breathing. Just prior to imaging, the mouse was given an intermittent isopressure breath-hold sequence was implemented using LabView to control the CWE ventilator, similar to the method employed by Namati et al. (20). The scanner was under computer control by way of the LabView-based control software and was paused during breathing. Just prior to imaging, the mouse was given two deep inspirations to 30 cmH2O Paw. During the second expiration phase, the lungs are held at the desired fixed Paw while several angles of view were collected by the μCT scanner. The scanner was paused, and the cycle was repeated until a complete set of images was gathered for CT image reconstruction. The mice were ventilated with a mixture of O2 and 1–2% isoflurane to ensure a constant sedation level.

In Vivo Fixed-Lung Imaging Protocol

The fixed excised lungs were scanned on the MicroCAT II scanner using the following parameters: 50 kV, 200 μA, 2,500-ms exposure time, detector binning of 2,720 projections over 270°, and 100 dark and 100 light images prior to each scan for calibration purposes. The lungs were placed inside a plastic tube suspended by the tracheal tube in a position similar to that used during the in vivo scan.

MicroXCT-400: specifications. The high-resolution X-ray CT scanner is an Xradia MicroXCT 400 (20–90 keV). Five microscopic objectives installed in front of the CCD camera allow continuous adjustable magnification and resolution (pixel size). The combination of off-center scan capability and adjustable magnification of the camera allowed us to image the whole lung and then zoom in on particular spots of interest for higher-resolution scans.

Ex vivo fixed-lung high-resolution imaging protocol. A large field-of-view scan with the MicroXCT 400 scanner was performed using the following parameters: 40 kV, 200 μA, 2,000-ms exposure time, detector binning of 1, and a step size of 0.125°/projection over 180° using a ×0.5 lens. The data sets were reconstructed with a voxel size of 12–17 μm. Each scan took ~4.5 h.

For the high-resolution scanning, we set the scanner to the following parameters: 40 kV, 200 μA, 8- to 10-s exposure time (depending on the size of the lung), detector binning of 2, and a step size of 0.125°/projection over 184° using a ×10 lens. The data sets were reconstructed with a voxel size of 2 μm with an approximate resolution of 2.5 μm. Each high-resolution scan took ~4 h.

Perfusion Fixation Process

The general arrangement of the apparatus used for the perfusion fixation process is depicted in Fig. 2. Two containers, 60-ml syringes, were connected to a distributor, which led the fixative via a tube to a bubble trap. The distributor helps switch between the two solutions. The first container was used for the preflush solution and the second for the fixation solution. A bubble trap is essential, since the smallest bubbles could block small vessels or capillaries, which could lead to only a partial perfusion with the fixative. From the bubble trap, another tube leads to the perfusion cannula.

After an in vivo scan, the mouse was removed from the scanner, placed on an absorbing mat, and fixed to the mat with tape on all four paws to prevent involuntary movement. Ventilation and anesthesia were maintained. The abdomen was opened, and the inferior vena cava was freed of the intestines and connective tissue as much as possible. A 22-gauge catheter was placed and ligated to the vein. A few drops of blood were aspirated through the cannula, which was then flushed and filled with the preflush solution to prevent clotting and air bubble formation prior to connection of the flushing system. Adja-

Fig. 2. Fixation setup scheme. Two-stage perfusion system was connected via a bubble trap and through a catheter to the inferior vena cava (IVC). Manometer was connected to the tracheal tube to allow alveolar recruitment and setting of precise inflation pressure of lungs.
Throughout this procedure, the mouse was maintained alive/anesthetized.

Before the preflush was started, an outflow route for the perfusion solutions was established via the abdominal aorta or one of its branches near the liver.

The perfusion method is based on two steps: a preflush and a fixation flush. The perfusion pressure was initially set to 30 cmH2O. It is important to maintain the perfusion pressure at a constant level of 30 cmH2O to ensure flow through the inflated lungs, which received a constant Paw of 20 cmH2O once fixative perfusion was established.

Preflush. Our preflush solution consisted of 94.5% isotonic Ringer (with 5% dextran), 5% procaine (10%), and 0.5% heparin. The initial flush with this solution helped prevent blood clots due to blood coagulation or blood that was fixed during the fixation. Furthermore, vasodilation by procaine ensures a homogeneous perfusion of the entire lung.

Fixative. The second solution is responsible for the fixation of the tissue and consisted of 25% polyethylene glycol 400, 10% ethyl alcohol (95%), 10% formaldehyde (37%), and 55% double-distilled water. This fixation solution was originally proposed by Heitzman (12) in the early 1980s as an instillation solution that provided lung fixation with a radio density similar to in vivo imaging. Because it preserves radiographic contrast of tissue, we used this fixation solution as a perfusate.

Specifications of the fixation process. The preflush took on average ~12–15 min to run through and wash out all the blood. After an initial 40 ml of preflush, the ventilator was stopped. To achieve a standardized volume history, we performed three consecutive deep inspirations (from 10 to 30 cmH2O). A manometer was connected to the tracheal catheter. A 25-ml syringe was connected in-line with the manometer to control the breaths. During the deflation of the third deep inspiration, the pressure was maintained at 20 cmH2O, and the lungs were kept inflated at this constant pressure throughout the remaining fixation process. The preflush was continued until >40 ml of the solution ran through the lung and the fluid leaving the transected artery was completely clear. At this point, we switched to the fixation solution.

Approximately 50 ml of fixative were let through the vasculature. This took ~25 min. It was slower than the preflush, because all the tissue along the perfusion path became stiff. After the perfusion was finished, the tubing was removed from the inferior vena cava catheter.

Lung extraction procedure. Removal of the lungs from the chest required extra care to avoid injury to the pleura. A lung free of injuries is important for the subsequent drying process. It is necessary to separate the lungs from connective tissue that cannot be pulled off; i.e., the connective tissue must be cut using very small surgical scissors (we used microsurgery tools available from various vendors). The lungs and heart were removed en bloc. The first part of the extraction consisted of detachating the lungs from the ribs. During separation of the diaphragm from the lung base, the areas entering and exiting vessels and the esophagus require extra attention. The last extraction step consisted of separating the lungs from the spine, where it is connected over the entire length of the lung. At this point, Paw was released to avoid tears of the trachea due to the rigid catheter and tubing. Since the lungs were completely fixed at this time, they did not change their shape. In only a few cases, we observed a small deflation of the lungs, which indicated a partial fixation. These lungs were excluded from the study.

To achieve the necessary contrast between tissue and airway paths using X-ray imaging, it is necessary to ensure that the airways are air-filled. We achieved this by drying the lungs. To avoid shrinkage, we reconnected the lungs to the same constant pressure of 20 cmH2O and placed them in an oven, where the temperature was kept constant at 28–30°C for 3 days. The pressure ensures that, during the drying process, possible shrinkage forces are compensated. The constant temperature helps remove moisture from the oven to accelerate the drying process and compensates for room temperature fluctuations caused by air conditioners. After the drying process, the lungs were removed from the oven, photographed, and scanned using the MicroCAT II scanner in a position similar to that used during the in vivo scan (supine).

Analysis tools. For quantification of our fixation method, we used whole lung and partial lung volume comparisons, as well as microscopic analysis of tissue samples. The lung volumes were computed from 3D segments of the images acquired with the μCT scanner. Microscopic analysis was performed by L. Knudsen and M. Ochs. Image segmentation. In vivo lung segmentation was performed using an automatic 3D segmentation algorithm (15). The resulting segments were visually inspected for accuracy and corrected if needed. An example for such segmentation is depicted as a 3D surface rendering in Fig. 3. Lobe segments were obtained by manual lobe separation using Pulmonary Workstation (PW2, Vida Diagnostics, Coralville, IA).

Ex vivo segmentation was accomplished using a manual tracing-based approach. An initial threshold-based segmentation including the heart, which was part of the extracted fixed block, was obtained using Fiji, an ImageJ-based software package. The heart and blood vessels and connective tissue outside the lung were deleted from the initial segmentation using the in-house-developed image analysis tool PASS (Pulmonary Analysis Software Suite) (11).

To evaluate possible time-related shrinkage of the fixed lungs, we rescanned the fixed lungs 7, 21, and >70 days after the drying process was finished. The final time point was determined by the availability of the off-site MicroXCT 400 scanner and ranged from 70 to 120 days. Image processing for those scans was performed as described above.

Image analysis. The total lung volume was computed as the sum of voxels contained by the lung segmentation, including the upper airways, multiplied by the voxel size of a scan. For the volume computations and visual inspections, we used PASS.

For localized information within a lung, we separated the lung segments into the individual lobes. For the lobar separation, we used PW2. The lobe volumes were determined in the same way as the total lung volume.

Tissue preparation and embedding for microscopic evaluation. The fixed and dried lungs were stored in 0.15 M HEPES buffer for 72 h. Then the total lung volume was determined using a fluid displacement method (27), and a systematic uniform randomization was performed to give every part of the lung the same chance to be sampled for microscopic analysis. Three to four slabs of the lung were obtained and processed for light microscopy. Tissue slabs were osmicated, immersed in half-saturated aqueous uranyl acetate, dehydrated in an acetone solution with rising concentration (e.g., 70%, 90%, and 100%), and embedded in glycol methacrylate according to the manufacturer’s instructions (Technovit 8100, Heraeus Kulzer, Wehrheim, Germany). The plastic-embedded tissue was cut into 1.5-μm-thick sections and stained with toluidine blue.

RESULTS

Lung compliance measurements prior to in vivo scanning were 0.043 ml/cmH2O (SD 0.005) and 0.062 ml/cmH2O (SD 0.007) for young and old mice, respectively. The lung resistance was determined at 1.303 cmH2O·s·ml⁻¹ (SD 0.577) and 0.837 cmH2O·s·ml⁻¹ (SD 0.103) for young and old mice, respectively. A significant difference in compliance (P = 0.002) between young and old mice was calculated using a two-sample t-test with equal variance for a two-tailed distribution. Using a two-sample t-test with an unequal variance and a two-tailed distribution, we observed no significant difference in lung resistance (P = 0.204) between the two age groups.

Visual inspection of all the fixed lungs analyzed for this study shows only small changes over a long period of time.

J Appl Physiol • doi:10.1152/japplphysiol.00550.2011 • www.jap.org
Overall shrinkage occurred slowly, ~1–2%/mo, and was therefore difficult to observe. Figure 3 shows the time-dependent changes. In vivo lung volumes were directly compared with volumes of the fixed lungs acquired at multiple time points. In addition to the μCT-based images, photographs of the fixed lungs were obtained at the same time points. No obvious time-related changes in structure or color were observed (Fig. 3).

The lungs were inspected further at multiple magnification levels. Figure 4 shows the imaging cascade of in vivo imaging from low to high resolution of the structural integrity of the parenchyma. Coarse internal structures visible at the lower resolutions were visualized in detail at higher magnification using the nondestructive interior tomography technique. Since no cutting- or embedding-related deformations were introduced by the sampling, a correlation between in vivo and high-resolution ex vivo imaging could be established. The high-resolution images show well-preserved parenchymal structures with no obvious distortions at the level of the septal walls.

Accumulation of interstitial fluid in the septal walls or collapse of alveoli or single ducts (atelectasis) occurred in only two specimens and appeared in the μCT images as inhomogeneous high densities scattered throughout the parenchyma. In addition, noticeable shrinkage and deformation were observed after the drying process. These two lungs were excluded from the studies for evaluation of the stability of a successful fixation.

---

**Fig. 3.** Images of 1 lung over a period of ~100 days. From top to bottom: in vivo and ex vivo coronal section of whole lung scans, 3-dimensional rendering of lobe segmentation, and photos of fixed and dried lung before each ex vivo scan. Calculated whole lung volumes reflect stability of fixation (SD of manual segmentations = 7 mm³). Lobe names were adopted from the official statement of the World Association of Veterinary Anatomists (1).

<table>
<thead>
<tr>
<th>Total lung volume</th>
<th>In-vivo at 20cmH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.862mL</td>
<td>Day 1</td>
</tr>
<tr>
<td>0.872mL</td>
<td>Day 7</td>
</tr>
<tr>
<td>0.852mL</td>
<td>Day 21</td>
</tr>
<tr>
<td>0.863mL</td>
<td>Day 98</td>
</tr>
</tbody>
</table>

---

**Fig. 4.** Imaging series of in vivo (left), ex vivo (middle), and high-resolution (right) images. In vivo imaging can be correlated to findings in images of fixed lungs visually and by means of registration. Homogeneous fixation of lung tissue can be observed in randomly selected high-resolution micro-CT imaging.
In vivo, whole lung volume at 20 cmH2O Paw was determined volumes that were consistently smaller in young than old mice. We plotted both time series together in Fig. 6. We observed lung age groups. To facilitate comparison of the two age groups, we influence on the fixation process, we split the data into the two lung volume on each time point was compared with the corresponding fixed volume at the same pressure after drying was completed (day 1). We did not have precise control over the exact time of the postfixation. The last scans were performed off-site; therefore, however, combined lung volume of all lungs shows significant shrinkage only after 70 days (P = 0.07), there is no statistically significant difference between in vivo and fixed ex vivo lung volume in the old mice. The fixed lungs in both age groups show minor shrinkage over time. Using image-based whole lung segmentation, we determined the lung volumes at 7, 21, and 70–100 days postfixation. The last scans were performed off-site; therefore, we did not have precise control over the exact time of the scans. Comparing the lung volumes in the two age groups with the volume on day 1 postfixation, we found no significant shrinkage in either age group after any time point. However, the small number of animals precluded statistical analysis for the young mice (n = 4). For statistical analysis, we compared the combined fixed lung volumes in all mice combined. On the basis of a simple paired t-test on the 10 lungs, we determined a significant average shrinkage only after 70 days (P = 0.04), leading us to believe that the best correlations between measurements on the fixed lungs and in vivo imaging can be achieved within the first 4 wk of fixation (we do not have detailed measures between 4 wk and 70 days).

**DISCUSSION**

**Observations**

The lung fixation method is intended to allow accurate representation of mouse lung fine structure down to the level of acini, with air spaces remaining air-filled to provide the needed contrast for μCT imaging. This requires fixation of the lung in situ in an air-filled state and under controlled inflation conditions. Challenges related to the delicacy of the organ and its physical size.

Image-based volumetric measures obtained from the preserved lungs matched corresponding measurements from the in vivo lungs, and visual assessment showed close preservation of structural relationships. Additionally, ultra-high-resolution μCT images allow for correlation of fine structure (acinar-level anatomy) to the whole lung fixed anatomy, which in turn can be correlated with the in vivo images. This offers a great advan-
Because of the lack of fluid, the X-ray attenuation is decreased entirely eliminated from the vasculature. During the drying volume and under physiological conditions (3, 10). in lungs fixed by vascular perfusion at relatively high inflation capillaries in the planar parts of the septum, as to be expected in lungs fixed by vascular perfusion at relatively high inflation and under physiological conditions (3, 10).

As shown by the histological assessment, the blood cells are entirely eliminated from the vasculature. During the drying process, the fixative in most of the larger vessels is cleared. Because of the lack of fluid, the X-ray attenuation is decreased by ~200 Hounsfield units. This does not impose difficulties for image analysis, since vessels can be easily tracked and recognized in volumetric data sets on the basis of localization, size, and shape.

Evaluation of the Preparation Methods

Comparison of lung volumes over time shows the stability of the fixed lungs. Comparison of the lung volume of the fixed lungs with the desired in vivo volume showed that the initial small overinflation worked to our advantage after the lungs stabilized in shape and volume. Noticeable shrinkage effects occurred only after a few months. We recommend acquiring any volume-sensitive data within the first 2 mo after fixation. Shrinkage was noticeable within the first 2wk after drying, however, if a lung was not fixed well because of improper perfusion. No shrinkage was noted during the drying process if the visceral pleura and upper airways were intact after removal from the thorax. Very small and peripheral injuries of the pleura did not cause shrinkage. Only in cases of large cuts into the pleura or the upper airways did we notice distortions of the lungs during and after drying. These are caused mainly by contraction of the elastic fibers in the regions in which inflation could not be maintained during drying.

For fixation, we adhered to the well-established Heitzman’s formula but applied it by perfusion, rather than by airway instillation. The solution contains 10% alcohol. Alcohol may contribute to reducing the elastic recoil due to elastic fibers (23) but cannot eliminate it completely at this concentration. So it is possible that this did mitigate tissue shrinkage to some extent by facilitating drying; elastic fibers lose their elasticity when dry, as shown by Oldmixon et al. (23). The embedding for the histological assessment was accomplished using glycol methacrylate (22), which causes minimal shrinkage of the tissue (see page 3 of the online supplement of Ref. 22 for further information).

To further evaluate the effects of shrinkage, we subjected a set of lungs that were not air-dried to our fixation process. On the basis of the histological sections, we did not observe differences in the histological sections of dried lungs. However, it was not possible to scan those lungs with the μCT scanner, since no contrast between tissue and air spaces could be seen because of some liquid accumulation at the alveolar level.

Glutaraldehyde is the ideal fixative for perfusion fixation of the lung for electron microscopy, but not necessarily for all other purposes (14). Initial attempts to modify the formulation designed by Heitzman by replacing the formaldehyde with glutaraldehyde did not succeed. The lungs were well preserved in situ. However, the extraction was problematic, because the visceral pleura had become tightly adherent to the thoracic wall as a result of penetration of the fixative into the parietal pleural tissue. For this reason, an injury-free lung extraction was nearly impossible. In addition, in the few cases of successful extraction of lungs fixed with the modified solution including glutaraldehyde, up to 50% shrinkage occurred during and after drying within a short period of time. Therefore, the formaldehyde-based Heitzman fixative was retained as the only option that worked for μCT. Samples resulted in tissue preservation that was acceptable for light microscopy but inadequate for transmission electron microscopy.

For accurate assessment of lung structure using ex vivo fixed lungs, the fixation method must maintain the air spaces air-filled and the lung volume must remain similar to that used for the in vivo lung imaging (20). This is the focus of our method. Drying is indispensable for X-ray-based imaging. Therefore, our combination of the Heitzman fixative and the perfusion technique allowed preservation of the lungs at the desired state, correlating well with the in vivo inflation and shape.

Formaldehyde-based fixation as used here does not adequately preserve cell structure, but this was not among our goals with this fixation method. Current μCT scanners are not capable of imaging and resolving cellular structure. Histology so far has been used for comparison and validation of μCT-based morphometry of the lung, and our goal has been to use μCT to follow acinar- and alveolar-level lung structure.

Summary

We have developed a new method for in situ, perfusion-based lung fixation that permits an in vivo-like preservation of the murine lung that can be imaged by X-ray-based imaging systems and can also be used for histological assessment following embedding and sectioning.

Our perfusion fixation method for murine lung allows a homogeneous and stable preservation of the entire lung in any desired degree of air inflation. The apparatus and procedures are described in detail. Results show very good correlation between in vivo and ex vivo volumes. The fixed lungs represent very closely the in vivo state and allow morphometric assessment at the microscopic level within the intact lung. X-ray-based CT imaging at multiple resolutions is possible. The histological quality of the specimens fixed using our procedure is very good.

The unique capability of the MicroXCT 400 scanner to resolve the alveolar structures within the intact fixed lung permits random sampling within the lung without loss of the spatial information. A stable whole lung fixation in combination with this imaging technique will allow us to assess the lung parenchyma in new ways. We plan to perform stereology-based morphometry of the whole lung, as well as acinar analysis of the intact lungs, and to compare them with corre-
lated morphometry on histological sections. Such structural analysis using conventional microscope methodologies requires destructive preparation of the tissues for obtaining thin sections, but it affords higher resolution of fine structure. By maintaining lung structure, we provide a tool for linking lung structure from the central airways to the peripheral alveoli. Such linkage will allow for the differential assessment of central vs. peripheral pathological processes, the use of computational fluid dynamics to better understand the role of structure in influencing susceptibility to inhaled pollutants, and better investigation of heterogeneous disease processes.

ACKNOWLEDGMENTS
We thank Beat Haenni and Anh Tu (Bern University) for expert assistance in histological specimen preparation, as well as Christine Klinge, who recently joined the team of Matthias Ochs at the Hannover Medical School for her doctoral thesis. We especially thank Leilei Yin (Beckman Institute, Urbana/Champaign, IL), where sample high-resolution images were taken for testing µCT compliance of the fixation method. We also thank Abiblih Kizhakke Puliyakote and Tim Eggleston (Department of Radiology, University of Iowa) for help with experimental setup and data analysis and Randall W. Grout for advice on the statistical analysis.

GRANTS
This work was funded in part by National Institutes of Health Grants R01-HL-080285 and S10 RR-019242-01.

DISCLOSURES
E. A. Hoffman is founder and Shareholder of Vida Diagnostics, a company commercializing lung imaging analysis software developed at the University of Iowa.

REFERENCES