Stereological assessment of mouse lung parenchyma via nondestructive, multiscale micro-CT imaging validated by light microscopic histology

Dragoș M. Vasilescu,1,2 Christine Klinge,3 Lars Knudsen,3 Leilei Yin,5 Ge Wang,6 Ewald R. Weibel,4 Matthias Ochs,3 and Eric A. Hoffman1

1Department of Radiology, University of Iowa, Iowa City, Iowa; 2Department of Diagnostic Radiology, Philipps University, Marburg, Germany; 3Institute of Functional and Applied Anatomy, Hannover Medical School and Member of the German Center for Lung Research (DZL), Hannover, Germany; 4Institute of Anatomy, University of Bern, Bern, Switzerland; 5Beckman Institute, University of Illinois, Urbana, Illinois; 6Biomedical Engineering & Sciences, Virginia Tech University, Blacksburg, Virginia

Submitted 16 July 2012; accepted in final form 19 December 2012

Vasilescu DM, Klinge C, Knudsen L, Yin L, Wang G, Weibel ER, Ochs M, Hoffman EA. Stereological assessment of mouse lung parenchyma via nondestructive, multiscale micro-CT imaging validated by light microscopic histology. J Appl Physiol 114: 716–724, 2013. First published December 20, 2012; doi:10.1152/japplphysiol.00855.2012.—Quantitative assessment of the lung microstructure using standard stereological methods such as volume fractions of tissue, alveolar surface area, or number of alveoli, are essential for understanding the state of normal and diseased lung. These measures are traditionally obtained from histological sections of the lung tissue, a process that ultimately destroys the three-dimensional (3-D) anatomy of the tissue. In comparison, a novel X-ray-based imaging method that allows nondestructive sectioning and imaging of fixed lungs at multiple resolutions can overcome this limitation. Scanning of the whole lung at high resolution and subsequent regional sampling at ultrahigh resolution without physically dissecting the organ allows the application of design-based stereology for assessment of the whole lung structure. Here we validate multiple stereological estimates performed on micro-computed tomography (μCT) images by comparing them with those obtained via conventional histology on the same mouse lungs. We explore and discuss the potentials and limitations of the two approaches. Histological examination offers higher resolution and the qualitative differentiation of tissues by staining, but ultimately loses 3-D tissue relationships, whereas μCT allows for the integration of morphometric data with the spatial complexity of lung structure. However, μCT has limited resolution satisfactory for the stereological estimates presented in this study but not for differentiation of tissues. We conclude that introducing stereological methods in μCT studies adds value by providing quantitative information on internal structures while not curtailing more complex approaches to the study of lung architecture in the context of physiological or pathological studies.

mouse; fixed lung; high-resolution imaging; lung morphometry; quantitative CT

Micro X-ray computed tomography (μCT) offers the possibility of studying lung microstructure on the basis of the inherent contrast offered by the radiodensity differences of tissue structures to air. Using μCT, we have previously demonstrated considerable differences in the airway anatomy in multiple mouse models (36). A newly developed μCT scanner now allows scanning of biological samples at 1–2 μm ultrahigh resolution (HRES), which approaches the resolution of medium-power light microscopy (22, 23, 39). This technology therefore offers the ability to analyze the morphometry of parenchymal fine structures without the need for physical sectioning, thus maintaining an intact, three-dimensional (3-D) structure. Due to long exposure times, scanning living organisms at HRES is difficult. However, fixed organs can be readily studied. In previous studies, Vasilescu et al. (38, 39) demonstrated the possibility of imaging the internal structure of fixed whole mouse lungs down to the septal walls without the need for dissection or microtomy. Others have shown the ability to scan fixed lung samples with μCT, which allowed the measurement of 3-D structures down to the alveolar level (18, 32, 41). However, quantification of such microstructures in the context of the entire lung remains a challenge because the sample size is limited by the field of view (FOV), which is determined by the desired resolution.

These problems can be overcome by applying principles and methods of design-based stereology to μCT data sets because they allow accurate estimates of 3-D parameters of fine structure by measurements obtained of sections at different levels of magnification following rigorous sampling rules (2, 5, 13, 24, 29, 45). These methods have been extensively used for the quantitative assessment of lung structure by light and electron microscopy in conjunction with studies on physiological and pathological conditions of the lung (6, 14, 16, 17, 29, 46, 48).

In the present study we explore the application of design-based stereological methods to the analysis of the fine structure and architecture of mouse lung parenchyma by μCT. We followed the guidelines for the quantitative assessment of lung structures on the basis of the principles of design-based stereology (13) and applied them to data sets obtained by nondestructive μCT imaging of the internal parenchymal structure of fixed mouse lungs (39). We tested the validity of the estimates obtained by μCT by comparing them with those obtained via light microscopy on histological sections from the same mouse lungs. We show that stereological methods can be used successfully in CT-based studies to obtain accurate data on lung fine structure such as alveolar surface and number, and discuss the advantages and limitations of μCT compared with histology. Whereas histology has the advantages of higher resolution and the potential to differentiate tissue structures, nondestructive CT imaging offers the advantage of using sections for the measurement of internal structures while maintaining the 3-D data set intact, thus allowing the integration of morphometric data with studies of the spatial complexity of lung structure, such as by segmenting entire acini in context with their vascular supply (38).
**METHODS**

*Animal handling and lung preparation.* Four young (12 wk) C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME) with an average body wt of 27.25 g (SD 1.26) were used in this study. Animals were housed in an animal facility at the University of Iowa and provided food and water ad libitum. The described protocols were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Mice were weighed and anesthetized using a mixture of 87.5 mg/kg of ketamine and 12.5 mg/kg of xylazine, then tracheotomized and paralyzed (0.1 ml/kg of pancuronium) in preparation for mechanical ventilation. Mice used for this study were part of a larger study seeking to establish a normative, strain-specific mouse lung atlas. All mouse lungs were scanned in vivo (MicroCAT II, Siemens Preclinical Solutions, Knoxville, TN) at multiple fixed-inflation pressures utilizing two in house–built ventilator systems that automatically trigger the scanner while using our previously described (26, 39) intermittent iso-pressure breath-hold approach. Following in vivo scanning, all lungs were preserved in situ at a fixed inflation pressure of 20 cmH2O using vascular perfusion via the inferior vena cava at a hydrostatic pressure of 30 cmH2O. This was accomplished utilizing a newly developed and validated (39) fixation method suited for multiresolution μCT imaging. Lungs were excised and dried after perfusion to enable X-ray-based scanning.

*Imaging protocols.* Imaging of internal structures such as acini, alveoli, or the septal walls require a much higher resolution than the MicroCAT II scanner used in this study for in vivo imaging can provide. The thickness of septal walls is reported to be ~4 μm (12). To resolve such structures an image composed of isotropic voxels of 1–2 μm is required. In collaboration with L.Y. (Beckman Institute for Advanced Science and Technology, University of Illinois, Urbana, IL) and G.W. (SAM-CT, Virginia Tech, Blacksburg, VA), nondestructive, multiresolution imaging of the fixed lungs were performed on a μCT scanner capable of providing resolutions from 0.7 to 50 μm (MicroXCT-400, xradia, Pleasanton, CA).

The MicroXCT-400 scanner is equipped with a Hamamatsu W-anode microfocus tube emitting 20–90 KeV energy-adjustable X-rays. Because of the relatively “soft” X-rays at low energy levels, this system is suitable for imaging biological samples and, in the case of the lung, which has inherent contrast from air-tissue interfaces, yields excellent resolution down to the septal walls. The detector in this system is an Andor 4M-pixel cooled charge-coupled device (CCD) camera. There is one macro lens and four microscopic objectives installed in front of the CCD camera to give continuously adjustable magnification (objectives of 0.5×, 4×, 10×, 20×, and 40×) and isotropic voxel dimensions (therefore variable resolution). The sample stage of the scanner is capable of three-axial translation and rotation for acquiring multigauge projections. The precise sample stage and sophisticated control software allow any coordinate within the travel ranges of the sample stages to be assigned as the center of a scan. The combination of off-center-scan capability and adjustable imaging magnification allows one to first image the whole lung, then zoom in to particular regions of interest for higher-resolution scans. By optimizing the scanning configuration, high-quality data with reasonable scan time were obtained.

The MicroXCT-400 scanner requires a customized setup protocol to gather systematic, uniform random sample scans within the intact lung. The protocol is designed in two stages. The lungs were mounted apex down on a sample holder, held by the tracheal catheter as shown in Fig. 1A. A syringe needle inserted in the catheter and trimmed to the same length provided extra support. Because of the metal construction, the needle needed to remain outside the FOV to avoid reconstruction artifacts. Every lung was first scanned with a large field of view (LFOV) at a low magnification followed by a series of HRES scans at specific locations as determined by the sampling strategy (see below). The LFOV scans were performed at the highest magnification possible that allowed the whole lung to fit in the FOV (12 μm/voxel). At this setting, transitional bronchioles were visible and allowed the user to differentiate between airways, blood vessels, or alveolar space. With this information it was possible to determine different locations used for the further HRES scanning. The parameters used for this protocol were as follows: objective 0.495×, 40 kV, 8 watts, an exposure time of 2 sec/frame, binning of 1, source-to-object distance of 50 mm, detector-to-object distance of 65 mm, and 1,648 projections over 216 degrees. Due to the stability of the scanner components and long scanning times, reference images were acquired every 421 projections throughout the scan. The reference images were used for misalignment corrections such as source drift or stage drift.

The following parameters were determined to work best at all HRES locations: objective 10×, 40 kV, 8 watts, exposure time of 8 sec/frame, binning of 2, source-to-object distance of 55–62 mm, detector-to-object distance of 17–19 mm, and 1,227 projections over 184 degrees. The resulting voxel sizes for these scans were 2 ± 0.02 μm, depending on the slight source-to-object and detector-to-object variation. For centering on peripheral locations, the detector could not be placed too close because the sample would otherwise hit the

---

*Fig. 1.* Scanning cascade for stereological assessment. A: photograph showing a fixed lung, mounted on a sample holder, used for multiresolution scanning. B: sagittal view of LFOV scan (12 μm/voxel). Lines indicate the divisions of the scan into four slabs from which one random slice was chosen. C: randomly selected transaxial slice from LFOV scan with applied sampling grid. Red circle indicates location of randomly sampled coordinates. D: HRES scan (2 μm/voxel) clearly showing septal walls, airways, and blood vessels.

*J Appl Physiol* • doi:10.1152/japplphysiol.00855.2012 • www.jappl.org
objectives. Such an increase in distance resulted in a small variation in voxel size. The reconstructed datasets (LFOV and HRES) resulted in 1,024 slices, 16-bit gray-scale images each with a dimension of 1,024 by 1,024. Figure 1 shows an example for the scanning cascade. Figure 1A shows a mounted fixed lung on the scanning holder. The imaging system offers the ability to scan in a batch mode. A list of predefined coordinates and parameters created in a so-called recipe file is required at the beginning of a batch scan. The recipe is followed by the control software for each set of coordinates until the last volume scan is acquired. The parameters for each individual scan were set independently.

**Stereological sampling.** Stereology is a set of well-founded mathematical methods that allows the quantitative assessment of 3-D structures from measurements, generally counting events on test systems, performed on planar sections of the object of interest (4, 9, 10, 13, 20, 25). Micro-CT is an imaging modality that generates serial 2-D sections of the object that is scanned. Therefore it was possible to combine stereology and µCT imaging to assess the morphometry of the mouse lungs. The ability of the MicroXCT–400 scanner to scan the same sample at multiple magnifications offers the ability to implement the sampling cascade design described by Ochs (29). This is a major advantage over the traditional microscopy-based approach because embedding and cutting induced artifacts could be avoided. Another advantage of volumetric imaging is that it allows changing the orthogonal plane in virtually any orientation, whereas in microscopy, slicing is dependent on the initial orientation of the sample bloc.

Stereological sampling can be implemented as a cascade-like approach that enables the collection of information at multiple levels of magnification. Starting with a low-resolution scan (LFOV) that covers the whole lung, it is possible to estimate the whole lung volume and parenchymal fraction within the lung. For estimating parenchymal volume fraction, four slices from the LFOV scan are systematically sampled (Fig. 1). To avoid mostly empty scans at the next level of magnification, a few slices are excluded from the next sampling step at the top and the bottom of the lung. The remaining stack of slices is divided into four equal slabs. A random slice is selected within the first slab (top of the lung); three additional slices spaced at one slab-height apart are selected. These four slices (indicated as yellow lines in Fig. 1B) are used to estimate the parenchymal volume fraction by point counting on a grid that overlaps the entire slice area.

On each selected slice of the LFOV scan, one coordinate for an HRES scan had to be determined. The stereology sampling design implemented for our study was originally described by Cruz-Orive and Weibel (5). We designed a graphical user interface (GUI) implemented in Matlab (a commercial software package from The MathWorks, Natick, MA). The GUI provided a way for systematic, uniform random sampling of locations within the whole lung scan. To ensure that all areas within the lung could be sampled, a grid with an area corresponding to the FOV of an HRES scan (grid area = 3.7 mm²) was randomly oriented and displayed on top of each of the selected LFOV slices. One of the intersection points of the whole grid located on parenchyma was randomly selected, indicating the coordinates of the HRES scan. As a general rule for borderline cases, the lower right corner of the selected point had to be clearly located on parenchyma. The imaging cascade is depicted in Fig. 1, B–D.

The HRES scans cover a volume of 8 mm³ (2 × 2 × 2 mm) represented by 1,024 transaxial slices with an isotropic voxel size of 2 μm. This is a large enough FOV with a high enough resolution to allow estimation of parenchymal properties such as alveolar space, alveolar duct space, septal tissue, surface area density, mean linear intercept of air space, average thickness of the septum, and alveolar number using the disector principle (9, 35).

An advantage of 3-D imaging includes the ability to reslice the data sets at nearly infinite orientations. Previously, multiple methods (19, 27, 28) have been developed to avoid orientation bias, should the structure of interest require such orientation considerations.

**Estimating whole lung volume as reference parameter.** This study is concerned with determining volume fractions and numbers related to the whole lung. Therefore it is essential to determine the total lung volume that is used as the reference volume for the estimates. A semiautomatic image segmentation procedure [performed using a freely available image processing tool named FIJI (available from www.fiji.sc) utilizing the segmentation editor] provided a mask of the lung and was used to calculate the total lung volume as the product of number of voxels of the segmentation and the voxel size. The heart and upper airways that were not surrounded by lung tissue were excluded from the segmentation. This approach is comparable to the method used traditionally for the histological preparation, which is based on fluid displacement (34).

**Stereological counting principles.** The MicroXCT–400 scanner allowed imaging of the alveolar septum and small blood vessel in the parenchyma. Due to both spatial and contrast resolution limits, it was not possible to visualize structures within the septal walls. Thus only the measurements presented in Table 1 were performed. These parameters are classified as first-order properties.

All image processing and analyses including generating the various grids needed for counting were performed utilizing the FIJI image processing tool. A plug-in available in FIJI as part of the analysis tools named “Grid” provided a means to overlay a line or point grid on any open image. Two settings were required: the type of grid and the area the sampling cascade design described by Ochs (29). This is a major advantage over the traditional microscopy-based approach because embedding and cutting induced artifacts could be avoided. Another advantage of volumetric imaging is that it allows changing the orthogonal plane in virtually any orientation, whereas in microscopy, slicing is dependent on the initial orientation of the sample bloc.

On each selected slice of the LFOV scan, one coordinate for an HRES scan had to be determined. The stereology sampling design implemented for our study was originally described by Cruz-Orive and Weibel (5). We designed a graphical user interface (GUI) implemented in Matlab (a commercial software package from The MathWorks, Natick, MA). The GUI provided a way for systematic, uniform random sampling of locations within the whole lung scan. To ensure that all areas within the lung could be sampled, a grid with an area corresponding to the FOV of an HRES scan (grid area = 3.7 mm²) was randomly oriented and displayed on top of each of the selected LFOV slices. One of the intersection points of the whole grid located on parenchyma was randomly selected, indicating the coordinates of the HRES scan. As a general rule for borderline cases, the lower right corner of the selected point had to be clearly located on parenchyma. The imaging cascade is depicted in Fig. 1, B–D.

The HRES scans cover a volume of 8 mm³ (2 × 2 × 2 mm) represented by 1,024 transaxial slices with an isotropic voxel size of 2 μm. This is a large enough FOV with a high enough resolution to allow estimation of parenchymal properties such as alveolar space, alveolar duct space, septal tissue, surface area density, mean linear intercept of air space, average thickness of the septum, and alveolar number using the disector principle (9, 35).

An advantage of 3-D imaging includes the ability to reslice the data sets at nearly infinite orientations. Previously, multiple methods (19, 27, 28) have been developed to avoid orientation bias, should the structure of interest require such orientation considerations.

**Estimating whole lung volume as reference parameter.** This study is concerned with determining volume fractions and numbers related to the whole lung. Therefore it is essential to determine the total lung volume that is used as the reference volume for the estimates. A semiautomatic image segmentation procedure [performed using a freely available image processing tool named FIJI (available from www.fiji.sc) utilizing the segmentation editor] provided a mask of the lung and was used to calculate the total lung volume as the product of total number of voxels of the segmentation and the voxel size. The heart and upper airways that were not surrounded by lung tissue were excluded from the segmentation. This approach is comparable to the method used traditionally for the histological preparation, which is based on fluid displacement (34).

**Stereological counting principles.** The MicroXCT–400 scanner allowed imaging of the alveolar septum and small blood vessel in the parenchyma. Due to both spatial and contrast resolution limits, it was not possible to visualize structures within the septal walls. Thus only the measurements presented in Table 1 were performed. These parameters are classified as first-order properties.

All image processing and analyses including generating the various grids needed for counting were performed utilizing the FIJI image processing tool. A plug-in available in FIJI as part of the analysis tools named “Grid” provided a means to overlay a line or point grid on any open image. Two settings were required: the type of grid and the area of the individual squares of the grid. The user could choose to center the grid or to place it randomly on the image. A grid could also be applied to a stack of images and remained constant for all images in

<table>
<thead>
<tr>
<th>Table 1. Measurements performed on HRES images</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbreviation</strong></td>
</tr>
<tr>
<td>V(lung)</td>
</tr>
<tr>
<td>Vv(par,lung)</td>
</tr>
<tr>
<td>Vv(duct,par)</td>
</tr>
<tr>
<td>Vv(alv,par)</td>
</tr>
<tr>
<td>Vv(alvsep,par)</td>
</tr>
<tr>
<td>S(alv,par)</td>
</tr>
<tr>
<td>S(alv,par)</td>
</tr>
<tr>
<td>tf(alvsep)</td>
</tr>
<tr>
<td>Nv(alv,par)</td>
</tr>
<tr>
<td>N(alv,lung)</td>
</tr>
<tr>
<td>Nv(alv,par)</td>
</tr>
<tr>
<td>N(alv,lung)</td>
</tr>
<tr>
<td>Nv(alv,par)</td>
</tr>
<tr>
<td>P(cp), points on coarse parenchyma; Pv, total test points on parenchyma.</td>
</tr>
</tbody>
</table>
The basis of stereological assessment required the counting of well-defined events on 2-D sections. With increasing levels of magnification, more details could be observed and therefore counted. We started at the LFOV level when determining the parenchymal and nonparenchymal volume fractions applying a point grid that covered the entire section area. The total number of points falling on lung structures \([P_{sl}]\) were the reference point set. Separately counting points falling on parenchyma \([P_{p}]\) from the ones on nonparenchyma \([P_{np}]\) allowed the computation of parenchymal \([V_{p}]\) and nonparenchymal volume fractions \([V_{np}]\) at the lower magnification.

The volume fractions of the different parenchymal components were estimated by point-counting following the cascade sampling design (29, 47). The MicroXCT-400 scanner provided multiple levels of magnification, though only two levels were needed for the measurements presented here. Table 1 reflects the measurements performed on the HRES images. Figure 2 shows an example of the counting performed on the HRES images. From the 1,024 slices within the image stack of an HRES scan, the center slice (512) was used for counting at this level. The center slice represents the randomly chosen location in the LFOV scan. At this level of the stereological cascade a grid \((0.04 \text{mm}^2/\text{grid area})\) was randomly placed on the HRES slice. The intersection points falling on alveolar space (points surrounded by septal walls), alveolar ducts (air space surrounded by multiple alveoli), and points falling on septum were counted separately (see color coding in Fig. 2). Points falling on structures such as vessel or bronchial walls, within vessels or bronchioles, and outside the circular field of view were excluded from the counting (red points in Fig. 2).

Alveolar surface area density \([S_{v}(alv)]\), was estimated by counting intersections \([I_{alv}]\) with a set of test lines. To this end, line segments were systematically selected on the same grid that was used for point-counting. A random number between 1 and 4 was chosen to determine the first line segment used on the grid. Every fourth segment was then systematically selected by following a few sampling rules, such as the left end point had to be located on parenchyma. Based on the grid size, a line segment was 200 \(\mu\text{m}\) long and generally had two end points. Intersection points of the line segment with the surface of the septum were counted. This allowed calculation of the total surface area \([S_{alv,lung}]\) by multiplying \(S_{v}(alv)\) with total lung volume, and mean septal wall thickness \([f(alvsep)]\) as \(2 \times \) the ratio of septal volume density to surface density. The volume density of alveoli and ducts taken together and the total surface area of the alveolar epithelium per lung were used to calculate the mean linear intercept length of distal airspaces as \(L_{m} = 4[V_{v}(asp)]/S_{v}(alv)\) (3, 16).

The last level of the stereological cascade was used to estimate the alveolar density \([N_{alv,lung}]\), yielding the total number of alveoli in the lung \([N_{alv,lung}]\) by the method of Euler number estimation (14, 15, 30, 31, 35). For this, the disector principle was applied and counting was performed on HRES slice pairs with a distance (disector height) of 6 \(\mu\text{m}\) between them. On each slice pair, a grid \((0.04 \text{mm}^2/\text{grid area})\) was placed randomly (the grid had the same location on both slices of a slice pair). Grid frames were selected systematically to perform the counting at this level. The bottom right corner of a frame had to be located on parenchyma to be used for counting. Every fourth frame was selected for the counting. Figure 3 provides an example of this step. The boundaries of each frame that was used have two sides to indicate counting events that must be excluded if they intersect those lines (red lines, also known as the forbidden line, Ref. 29) and two sides that may be intersected by a counting event (green lines). An alveolus on 2-D sections is defined by the septal walls with only one opening formed by fiber tracts in the free edges (47). While visualizing consecutive sections of the septal walls, these walls appear to open and close between slices. A counting event is defined by the separation or unification of septal walls that can be observed switching from one slice of a slice pair to the other one. Such an event is also called a closing or opening of bridges (14, 31). To avoid misinterpretations of openings or closings in the \(\mu\text{CT}\) images, one can use the larger image stack and verify the observation while scrolling through the stack; this is equivalent to using the optical disector in light microscopy (9). The counting of bridges is a direct measure of the alveolar density and has been previously implemented.

![Fig. 2. Second level of the stereological assessment allows assessment of parenchymal components. Left: an example for point counting on parenchyma that differentiates between alveoli, alveolar duct, and tissue but excludes blood vessels and bronchioles. Right: an example for line intersection-counting that helps to determine the septal properties such as surface area, surface density, and average tissue thickness. Green lines are the selected segments of the whole grid. The first segment is determined randomly. On the basis of this starting selection, every fifth segment is used following a meandering shape. Yellow dots indicate the segment end points used. Segment size is 200 \(\mu\text{m}\) with two end points. If the line ends outside the FOV, then only half a segment (100 \(\mu\text{m}\)) with only one node is used for counting.](http://japphysci.org/)
by Vasilescu et al. for counting alveoli in individual acini (38). To achieve a statistically stable estimate of 200 counting events at this level of the cascade we used 8 slice pairs per lung within the 4 HRES scans. From each HRES scan, two evenly spaced slice pairs were used for counting. The first slice pair was located in the first quarter of the HRES stack, and the second slice pair was located in the last quarter.

Alveolar density multiplied with lung volume and parenchymal volume fraction yields an estimate of the number of alveoli within the whole lung. Dividing alveolar volume fraction in parenchyma by the number of alveoli per unit volume of parenchyma yields an estimate of the mean volume of an alveolus.

Validation by light microscopic assessment. To validate the \( \mu \)CT-based measurements, the same lungs were processed for microtome sectioning to be stereologically assessed with traditional light microscopy. For this, the lungs were embedded and sectioned following previously described sampling methods utilized for lung morphometry (13, 31). The lung volumes were estimated by water displacement (34). The same measurements as performed on the \( \mu \)CT data sets were made at comparable magnifications (5× for LFOV and 20× for HRES estimates) using the same counting principles by utilizing newCAST, a specialized system consisting of hardware and software (new CAST, Visiopharm, Hørsholm, Denmark).

A simple paired \( t \)-test was performed to evaluate the statistical significance (\( P < 0.05 \)) between the results achieved using the two imaging modalities, \( \mu \)CT and microscopy.

RESULTS

The stereological results are summarized in Table 2, which contains measurements obtained both from \( \mu \)CT images and from light microscopy; Fig. 4 presents a pairwise comparison of the estimates obtained for each lung. The statistical significance of the average results gathered with the two imaging modalities was determined using a simple paired \( t \)-test. The total lung volume was significantly lower when measured by...
means of fluid displacement. A significantly higher surface area density ($P < 0.05$) was determined in the lungs assessed via microscopy. No statistical difference was observed for any of the other parameters. The parenchymal ($V_{V_{\text{par, lung}}}$) and nonparenchymal ($V_{V_{\text{nonpar, lung}}}$) volume fractions were estimated using the LFOV (lower resolution) and these were similar when comparing the $\mu$CT and light microscopy derived measures. The HRES images were used to determine volume fractions of alveolar space ($V_{V_{\text{alv,par}}}$), ducts ($V_{V_{\text{duct,par}}}$), and tissue ($V_{V_{\text{tissue,par}}}$) using point counting as shown in Fig. 2. The volume fractions of the parenchymal subcomponents were also similar using either imaging modality.

The alveolar surface area density ($S_{V_{\text{alv}}}$), estimated by line intersection counts (Fig. 2, right), was 14% larger in the light microscopic estimate compared with $\mu$CT; this difference is observed in all lungs (Fig. 4). The surface area of the whole lung was estimated at 270.02 cm$^2$ (SD 38.03) and 268.89 cm$^2$ (SD 56.36) via $\mu$CT and light microscopy, respectively, showing no significant difference. The septal wall thickness shows slightly different values between the two modalities with a higher value based on the $\mu$CT images (12.9 $\mu$m; SD 3.63) than light microscopy (9.07 $\mu$m; SD 0.37), although this was not significant. The mean linear intercept showed no statistical difference between the two image modalities.

Using the disector principle, the alveolar density ($N_{V_{\text{alv}}}$) was estimated at $2.234.24 \text{ mm}^{-3}$ (SD 329.86) utilizing $\mu$CT imaging and $2.034.82 \text{ mm}^{-3}$ (SD 360.10) via microscopy, thus showing no significant difference ($P = 0.24$). Based on $\mu$CT imaging, the total number of alveoli ($N_{alv}$) was estimated at $1.65 \cdot 10^6$ (SD 0.40), whereas microscopy estimated a total of $1.27 \cdot 10^6$ (SD 0.15) alveoli in the whole lung with no significant difference between the values ($P = 0.07$). On the basis of these estimations the mean volume of an alveolus ($\bar{V}_{N_{\text{alv}}}$) was computed at $0.25 \cdot 10^{-6}$ $\mu$m$^3$ (SD 0.05) and at $0.22 \cdot 10^{-6}$ $\mu$m$^3$ (SD 0.04) using microscopy.

**DISCUSSION**

Stereology allows accurate quantification of lung structure based on measurements of sections. It is a tool with a long tradition in lung structure assessment and is used for a variety of morphometric measurements performed microscopically on histological preparations (4, 13, 20, 42, 43). Because it is also based on a sectioning process, multisresolution $\mu$CT imaging of perfusion-fixed lungs immediately allows for the application of stereological methods for quantitatively assessing lung structure by sampling the $\mu$CT images with geometric probes. Compared with histology, $\mu$CT has the advantage of being based on nondestructive sectioning, thus keeping the entire 3-D data set intact for more advanced analysis of lung architecture as in a recently published study showing differences in acinar structures between two different age groups of C57Bl/6J mice (38). The aim of this study was to examine the potential of using stereological methods to add the value of accurate quantitative assessment of internal lung structures, validating the $\mu$CT estimates against histological assessment of the same lungs using the same methods. This study followed closely the imaging and morphometry cascade presented by Ochs (29) and...
observed the standards issued by the American Thoracic Society/European Respiratory Society (13).

The generally good correlation between the estimates obtained utilizing two independent imaging modalities demonstrates the ability to perform stereology in the same manner on μCT images as it is possible to do traditionally via light microscopy. Our data also correlate with previously presented data on the same strain of mice as, for example, the estimated number of alveoli (1.22·10⁶; SD ~0.2) by Fehrenbach et al. in mice at 20 wk of age is within the range of our measurements (7). Voswinckel et al. (40) also reported stereological estimates on lungs of C57Bl/6 mice at a similar age. Their measurements of lung volumes (V = 0.72 ml) and parenchymal volume fractions [Vv(par,lung) = 0.86] are comparable to ours. However, there was a significant difference in alveolar surface area between the two studies [S(alv,lung) = 410 cm²; SD 13 by Voswinckel vs. 270 cm²; SD 38 in our study]. This can be explained by the difference in lung fixation: our lungs were fixed by vascular perfusion in a state of controlled air-filling that leads to a reduction of free alveolar surface due to the effect of surface tension (1) compared with instillation fixation as used by Fehrenbach and Voswinckel (7, 40) where fluid filling unfolds the alveolar surface completely. Our measurements of mean linear intercepts are higher (Lm = 82.45 μm) than previously reported by others (Lm = 38.9 μm, Ref. 16; Lm=54, Ref. 40); this is also explained by the effect of vascular perfusion fixation that results in smaller SV(alv) and larger volumes of alveolar ducts (1). These comparisons showing overlap in only a few parameters further underline the importance of standardized preparation methods for stereological investigations (13).

One limitation of the current study becomes evident when we compare the image resolution of the two imaging modalities. Figure 5 presents a comparison of the image resolution provided by the μCT scanner matching the magnification used with a microscope. Figure 5A is acquired with the same 10-fold optical magnification as the microscope. Zooming into the μCT images, after image acquisition, the resolution decreases and the structures become more pixilated and blurred. The slice thickness of the μCT scanner of 2 μm was not much greater than that of the histological sections of 1.5 μm. Furthermore, μCT images contain noise and sometimes other reconstruction artifacts. The image quality of the μCT scanner provided good definition of the structures of interest, down to the alveolar walls, yielding structural details suitable for performance of the desired measures. However, the histological preparation has the advantage of tissue discrimination provided by staining with different colors and higher resolution (different objectives), which enables visualization of the capillaries within the septa.

The difference in resolution between the imaging modalities led to a small difference in the surface area density, which is dependent on the ability to count intersection points of the septal surface outline with the overlaid line grid. As described by Weibel (44), the resolution of the images on which the line intercept counting is performed has a direct impact on the

Fig. 5. A–C: a zoomed-in series of an image acquired by μCT (2 μm/voxel). While magnifying the digital image, the limitations of the μCT at the selected scanning parameters become visible (optical magnification of 10×). D–F: the same area from the lung prepared for histology (embedded in JB4 and stained with toluidine blue) at three different magnifications (10×, 20×, and 40×) allowing the viewer to clearly distinguish between the finer structures at the higher magnifications.
counting results. More details are visible with a higher magnification, and this allows a more precise estimation of the surface area density. The sections visualized with a light microscope have a higher contrast, and more details of the septa are visible because they were images at a magnification of 20×, yielding a theoretical resolution of 1.1 μm. The reconstructed slices from projections acquired with a μCT scanner suffer from the partial volume effect caused by averaged information within one slice resulting in a voxel size of 2 μm. This suggests that resolution, defined as the smallest distance between two particles that can be seen, is inferior by at least a factor of 4 using μCT compared with light microscopy at similar magnification. Therefore, the septa can appear thicker in the μCT images than in the sections under a microscope. The effect of resolution on stereological estimates has been evaluated in detail by others. Paumgartner et al. (33) studied the membrane system of liver cells and showed the effect that resolution has on the stereological estimators of surface and volume interpreting the effect in terms of the theory of fractals. With respect to the lung, the effect of microscopic resolution on estimates of the alveolar surface area was discussed by Gehr et al. (8) when they found that the alveolar surface was very much larger when measured by electron microscopy compared with light microscopy (42).

A significant problem of the μCT technology compared with histology is the lack of differentiation between tissue types. This could have affected the counting of bridges in the disector for estimating the total number of alveoli by the appearance of alveolar entrance rings. In microscopy, these are unambiguously identifiable by the strong connective tissue fibers that constitute the fiber ring and can be stained differentially so that they are easy to recognize. This limited differentiation of tissues might have been the reason why the number of alveoli estimated via μCT was higher, because some edges in the alveolar structure that were not real entrance rings could have been counted as such.

On the basis of experience gathered in the course of developing this new imaging approach and implementing traditional assessment methodologies for μCT images, we propose multiple steps that must be taken into account when designing this type of study. The main message is that a multistage approach must be taken to assess the fine structure of alveolar architecture by the methods of stereology (5, 29). In the last step, various test grids can be implemented to efficiently and accurately assess the parameters that characterize these structures. It is beyond this report to develop detailed protocols to be followed, particularly as concerns the most efficient application of suitable stereological test systems. This has occurred from a general perspective in the recently published standards for the quantitative assessment of lung structure (13). Since the initiation of this study, a new tool for this type of approach has become available that can potentially facilitate this work: the STEPanizer (37), an on-line program for the stereological assessment of histological micrographs, whose applicability to μCT will have to be tested.

Novel methods for utilizing μCT coupled with stereology and unbiased sampling principles can provide a means by which lung morphometry can be assessed while maintaining 3-D lung anatomy and therefore spatial relationships intact. For larger lungs such as human lungs, a novel fixation and sampling approach such as recently published by McDonough et al. (21) could be included in our presented assessment cascade. In their study, McDonough et al. demonstrated that their fixation method is suitable for μCT imaging, allowing a characterization of the level of small-airway obstruction in chronic obstructive pulmonary disease. Our imaging and assessment methodology could provide an extension of McDonough’s study by combining their fixation and sampling method with our multisresolution imaging and assessment techniques. The assessment cascade of multisresolution, nondestructive μCT imaging and stereology demonstrated in this study ultimately opens new avenues for characterizing the architecture of the lung down to the alveolar septa.

Conclusions. When finally assessing the relative merits of the traditional histologic and the novel μCT approaches to lung morphometry, we must note that optical microscopy offers two clear advantages: 1) the resolution at which measurements can occur is much higher because it can be extended to electron microscopy, which achieves nanometer resolutions; and 2) the use of stains and molecular markers allows for the differentiation of structures by qualitative criteria. The disadvantage of this approach is, however, besides some usual artifacts of this technique, that the unavoidable physical sectioning process destroys the third dimension, rendering the analysis of spatial contexts or continuity very difficult if not impossible. Here lies the great advantage of CT, which is based on virtual sectioning: the resolution achievable today approaches that of medium-power light microscopy, satisfactory for many morphometric problems, but it maintains the 3-D data set intact, thus allowing the integration of detailed and accurate morphometric data obtained by stereological methods with studies of the spatial complexity of lung structure, such as by segmenting entire acini in context with their vascular supply (38). To introduce stereological methods in μCT studies adds value by providing quantitative information on internal structures while not curtailing more complex approaches to the study of lung architecture in the context of physiologic or pathological studies.

ACKNOWLEDGMENTS

The authors thank Kriti Sen Sharma from the Virginia Tech imaging laboratories for his assistance with the imaging equipment. We also acknowledge the help of Abhilash Kizhakke Puliakote from the University of Iowa for performing some of the stereological counting and data acquisition. We acknowledge Dr. Tillie L. Hackett for proofreading the manuscript.

GRANTS

This project was funded in part by National Institutes of Health Grants R01- HL-080285 and 1-S10-RR019242-01, and by National Science Foundation/Major Research Instrumentation Grant 0923297 awarded for “MRI: Development of the Next-generation Nano-CT system for ROI-focused Scanning and Exact Interior Reconstruction.”

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.M.V., L.K., L.Y., E.R.W., M.O., and E.A.H. conceived and designed the research; D.M.V., C.K., L.Y., and G.W. performed the experiments; D.M.V. and C.K. analyzed the data; D.M.V. and L.K. interpreted the results of experiments; D.M.V. prepared the figures; D.M.V. drafted the manuscript; D.M.V., L.K., G.W., E.R.W., M.O., and E.A.H. edited and revised the manuscript; D.M.V., C.K., L.K., E.R.W., M.O., and E.A.H. approved the final version of the manuscript.
REFERENCES


