Three-dimensional imaging and morphometric analysis of alveolar tissue from microfocal X-ray-computed tomography

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Three-dimensional imaging and morphometric analysis of alveolar tissue from microfocal X-ray-computed tomography. Am J Physiol Lung Cell Mol Physiol 291: L535–L545, 2006. First published May 5, 2006; doi:10.1152/ajplung.00088.2005.—We evaluated microfocal X-ray-computed tomography (micro-CT) as a method to visualize lung architecture and to obtain morphometric data. Inflated porcine lungs were fixed by formaldehyde ventilation. Tissue samples (8-mm diameter, 10-mm height) were stained with osmium tetroxide, and 400 projection images (1,024 × 1,024 pixel) were obtained. Continuous isometric micro-CT scans (voxel size 9 μm) were acquired to reconstruct two- and three-dimensional images. Tissue samples were sectioned (8-μm thickness) for histological analysis. Alveolar surface density and mean linear intercept were assessed by stereology-based morphometry in micro-CT scans and corresponding histological sections. Furthermore, stereology-based morphometry was compared with morphometric semi-automated micro-CT analysis within the same micro-CT scan. Agreement of methods was assessed by regression and Bland-Altman analysis. Comparing histology with micro-CT, alveolar surface densities (35.4 ± 2.4 vs. 33.4 ± 1.9/mm, P < 0.05) showed a correlation (r = 0.72; P = 0.018) with an agreement of 2 ± 1.6/mm; the mean linear intercept (135.7 ± 4.5 vs. 135.8 ± 15 μm) correlated well (r = 0.97; P < 0.0001) with an agreement of −0.1 ± 3.4 μm. Semi-automated micro-CT analysis resulted in smaller alveolar surface densities (33.4 ± 1.9 vs. 30.5 ± 1/mm; P < 0.01) with a correlation (r = 0.70; P = 0.023) and agreement of 2.9 ± 1.4/mm. Non-destructive micro-CT scanning offers the advantage to visualize the spatial tissue architecture of small lung samples two and three dimensionally.

MATERIALS AND METHODS

Isolation of Porcine Lungs

Lungs were harvested from five healthy hybrid pigs (6 mo of age; weight 120 kg) at a local slaughterhouse. No animals were killed for the particular purpose of this study. Only left lungs with intact visceral pleurae were taken for investigation. Lungs were isolated by intersection of the left main bronchus close to the carina. The left main bronchus was subsequently intubated with a cuffed tube and inflated with a breathing bag.

Lung Fixation

Lungs were connected to a tube of a modified Engstrom-type respirator. After recruitment with a peak airway pressure of 40 cmH2O for 40 s, lungs were ventilated in a volume-controlled, pressure-limited mode with a positive end-expiratory pressure of 5 cmH2O. The peak pressure was limited to 35 cmH2O. Tidal volume was adjusted to reach full lung inflation, respiration rate was set at 10 breaths/min.

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12/min, and the inspiratory-to-expiratory ratio was 1:2. Fixation occurred by ventilation of the lung with formaldehyde vapor (27). The fixation process lasted 6 h. It resulted in an air-filled lung preparation with inflated alveoli (Fig. 1).

**Determination of Lung Volume and Weight**

To prove the reproducibility of the preparation technique, lung volume and weight were determined before preparation and after fixation.

Lung volume was measured by water displacement (33). Prior preparation volumes were taken in full inflation (near total lung capacity). To avoid deformation of the emerged lung (leading to incorrect values), the intubated main stem bronchus was clamped.

Weight was assessed by use of a common pair of scales.

**Assembling of Tissue Specimens**

Lungs were cut into parallel slices of 10-mm thickness. Tissue specimens were taken from the second slice counting from the costal surface of the lung. With the use of a sharp mechanical stamp, cylindrical samples (diameter 8 mm, height 10 mm) were gained from two subpleural locations.

**Staining of Tissue Specimens**

To enhance contrast for micro-CT scanning, tissue samples were immersed in 1% osmium tetroxide solution (1, 4) in tubes. Tubes were rotated gently for 3 h. Afterward, samples were washed threefold for 30 min with purified water to displace excess osmium tetroxide solution in the alveolar air space.

**Drying of Stained Tissue Specimens**

To remove remaining fluid inside the alveolar air space, the samples were subjected to a critical point drying procedure (15). The samples were incubated threefold with 10 ml of 2,2-dimethoxypropane for 30 min (10).

In the critical point dryer (Critical Point Dryer; Balzers, Witten, Germany), the dimethoxypropane was completely replaced by liquid carbon dioxide. Finally, the chamber was heated to 40°C with a pressure of 90,000 kPa. The critical point of carbon dioxide is reached at a temperature of 31°C and a pressure of 73,800 kPa when carbon dioxide changes abruptly from the liquid to the gaseous phase.

**Determination of Diameter and Weight of Tissue Specimen**

To test the effect of the preparation technique on the spatial dimensions, the diameter and weight of the tissue specimen were determined before and after osmium staining-critical point drying.

**Micro-CT Scanning of Tissue Specimens**

Micro-CT scanning of the lung samples was performed on a SkyScan-1072 system (Aartselaar, Belgium). The scanner is equipped with a tungsten microfocus X-ray tube (80 kV type L6731-01 Hamamatsu, Toyooka-village, Japan). It has a beam angle of 39°, reaching a focal spot size of 8 μm. The X-ray detector is a 1,024 × 1,024 12-bit digital charge-coupled device (CCD) camera with a fiber-optic 3:7:1 coupled to a P43 powder scintillator (Gd0) with 3-μm grains. The opacity of each voxel is represented by a 16-bit gray-scale value. Each 16-bit planar image consisted of a 1,024 × 1,024 array of pixels ranging from 0 to 255 (minimum to maximum) X-ray intensity scale.

Scanning was carried out with a tube voltage of 60 kV and a tube current of 100 μA. After performing a scout view, 400 projections of the specimen were scanned over a total rotation of 180° (rotation step was 0.45°). One projection image required 0.3 min, resulting in a total acquisition time of 2 h/sample. Out of the 400 projection images, ~900 continuous axial scans with a matrix of 1,024 × 1,024 were calculated with a modified Feldkamp reconstruction modus (12). According to the cone-beam geometry of the X-ray, the magnification as well as the pixel size were determined by the distance of the object to the X-ray source. The sample diameter of 8 mm permitted a magnification of 27.5, resulting in cubic (isotropic) voxels with a size of 9 μm.

**Preparation of Histological Sections For Light Microscopy**

After micro-CT scanning, the tissue specimens were embedded in paraffin. From this paraffin block, serial sectioning was performed (135 sections/sample with a thickness of 8 μm). Digital pictures (10-fold lens magnification) were obtained from all intact histological sections using a universal light microscope (Axioplan 2; Zeiss, Jena, Germany) attached to a CCD camera (DXC-3000P, Sony).

**Comparison Between Sections From Micro-CT and Histology**

The histological slices were matched with the corresponding micro-CT scans for comparison. Slice-to-scan registration of the different imaging modalities was performed with the custom Analyze Software version 4 (Biomedical Imaging Resource; Mayo Foundation, Rochester, MN).

Despite the most possible accuracy, complete agreement between corresponding images could not be accomplished within micrometers due to angular mismatching in the specimen orientation. Histological sections did not correspond exactly to the transversal micro-CT slices. Thus matching layers were extracted on the basis of anatomical structures (e.g., greater bronchiol, interlobular septae) with a threedimensional tool providing the facility to generate arbitrary orientated slices of the entire volume (Fig. 2, A and B).

**Three-Dimensional Postprocessing of Two-Dimensional Micro-CT Scans**

Three-dimensional postprocessing of the images was performed with the custom ANALYZE software (Mayo Foundation). Two-dimensional, eight-bit bitmap files were transformed into three-dimensional volumes and visualized by volume rendering (Fig. 3, A and B).

**Segmentation of Bronchiolar Tree and Pulmonary Acinus**

The three-dimensional structure of the conductive airway tree was reconstructed with a seeding technique, considering equal Hounsfield units and connectivity. The seed was placed in a bronchus, and automated segmentation was carried out under visual control of the growing seed. Thus segmentation was performed in a process that was based on an automated algorithm in addition to examiner supervision and guidance. Following the bronchiolar tree, the seeding technique was performed again starting at the terminal bronchus. The airways
were followed down to the alveolar walls (Fig. 4, A and B). Visualization of the segmented air space unit in a surface three-dimensional rendering is demonstrated in Fig. 4, C and D. The software employed in this step was Volume Tool of the Advantage Workstation 4.1 (General Electric Medical Systems Europe, Buc, France).

**Morphometric Data Determined By Semiautomated Micro-CT Analysis**

We chose alveolar surface density and the mean linear intercept (an estimate for the alveolar diameter) as morphometric parameters for comparison with stereology-based morphometry.

For semiautomated micro-CT analysis, a cubic volume of interest (side length 702 µm including 78 continuous scans; volume of interest = 0.346 mm³) had to be defined within the reconstructed micro-CT images of the samples that just covered alveoli (large bronchi and vessels were excluded by visual control; see Fig. 6A). The gray-scale images were transformed into binary images using a threshold plug-in according to the Canny method (8) (see Fig. 6, C and D). Morphometric analysis was performed with the custom ANALYZE software. The alveolar (tissue) volume and the alveolar surface area can be assessed directly from micro-CT scans; the alveolar surface density can be calculated from these morphometric parameters.

\[
\text{alveolar surface density} = \frac{\text{alveolar surface area}}{\text{volume of interest}} = \frac{\text{alveolar surface area}}{0.346 \text{ mm}^3} \quad (1)
\]

The mean linear intercept was calculated by the general equation

\[
\text{mean linear intercept} = k \times \frac{\text{alveolar air space volume}}{\text{alveolar surface area}} \quad (2)
\]

where \( k \) is a factor with the value 6 (see APPENDIX) and alveolar air space volume is the difference between the volume of interest and alveolar tissue volume.

**Histological Data Determined By Stereology-Based Morphometry**

For histomorphometry, the digital images acquired during the histological examination were processed by an IBAS 2 image proces-
sor (Kontron, Germany). A similar region of interest as previously defined in the micro-CT measurement was analyzed.

**Alveolar Surface Density**

According to a fundamental equation of stereology originally obtained by Saltykov (31), the surface density (surface area divided by tissue volume) can be estimated by

\[
\text{alveolar surface density} = 2 \times \frac{\text{number of intercepts}}{3.5 \times \pi \times \text{mm}^2}
\]

The number of intercepts was evaluated in 20 grids of Merz per sample (2 grids/section × 10 sections).

**Mean Linear Intercept**

An overlay image providing a measuring grid according to Merz (24) was used to define intercepts with the alveolar borders (Fig. 5). These intercepts were used to define the starting point for a two-point measurement of the intercept length. In total, 125 intercept lengths per sample were assessed (5 lengths/set × 5 sets/section × 5 sections). Only completely preserved closed alveolar structures were considered.
Stereology-Based Morphometric Data From Micro-CT

For micro-CT morphometry, the same grid of Merz was overlayed on the micro-CT scans.

Alveolar Surface Density

The surface density was calculated according to Eq. 3. The number of intercepts was evaluated in 20 grids of Merz per sample (2 grids/scan × 10 scans; Fig. 6B).

Mean Linear Intercept

The grid of Merz was used for a two-point measurement of the intercept length. In total, 125 lengths per sample were assessed by the two-point measurement described above (Fig. 6B).

Statistics

Data were tested by analysis of variance. Correlation was tested by bivariate regression analysis. \( P < 0.05 \) was considered as significant.

The agreement between alveolar surface density and the mean linear intercept obtained by semiautomated micro-CT analysis and stereology-based morphometry within the same micro-CT scans was assessed according to Bland and Altman (6). In addition, the agreement of the stereology-based morphometric data assessed in micro-CT scans and in histological slices was tested.

RESULTS

Volumes and Weights of Whole Lungs Before and After Preparation

The mean volume of inflated lungs decreased only by 3% during formaldehyde vapor ventilation. Mean lung volumes did not differ significantly before and after lung fixation (2,107 ± 309 ml vs. 2,047 ± 296 ml). In contrast, the mean lung weight decreased by 70% during fixation (364 ± 27 g vs. 109 ± 9 g; \( P < 0.05 \)).

Dimensions of Tissue Specimen Before and After Staining and Drying

Further preparation of the cylindrical lung tissue specimens by osmium tetroxide staining and critical point drying resulted in a sample shrinkage of 10.4% regarding the sample diameter and further weight loss of 37.1% in average (Table 1).

Comparison of Micro-CT Scans and Histological Sections

Micro-CT scanning resulted in an isotropic voxel size of 9 \( \mu \)m with a resolution of 18 \( \mu \)m. Realistic two- and three-dimensional images of the sample could be reconstructed.

The comparison of micro-CT scans to corresponding histological sections of the same sample showed a high agreement.

Fig. 6. For morphometric micro-CT analysis, a region of interest (ROI) was defined that covered only alveoli (square in A). B: ROI (side length 700 \( \mu \)m) of the gray-scale value image with the grid of Merz superimposed. Arrows indicate the linear intercepts. C: red line shows the border of the threshold-based segmentation of tissue and air. D: binary image. With threshold-based segmentation, some of the tissue structures are not included (B and D).
by visual control (Fig. 2). However, exact matching is not always possible on a microscopic level.

Basic Descriptive Statistic of Histological and Micro-Tomographic Parameters

Semiautomated micro-CT analysis. Air space volume and alveolar surface (primary morphometric parameters) assessed by micro-CT analysis are shown in Table 2. Column three in Table 2 is used to calculate the mean linear intercept (APPENDIX).

Comparison of Stereology-Based Results Between Micro-CT and Histology

To evaluate the accuracy of micro-CT, stereology-based morphometric analysis and by stereology-based measurement on micro-CT scans as well as on histological slices.

Alveolar surface density. Stereology-based measurements of the alveolar surface density (Table 3) differed significantly between histological and micro-CT images (35.47 ± 2.41/mm vs. 33.47 ± 1.91/mm). The regression analysis (Fig. 7A) showed a correlation of r = 0.72 (P = 0.018); the agreement was 2 ± 1.6/mm in the Bland-Altman plot (Fig. 7B).

Mean linear intercept. In contrast to alveolar surface density, stereology-based measurements of the mean linear intercept (Table 4) did not differ between histological and micro-CT images (135.7 ± 14.5 μm vs. 135.8 ± 15 μm). The correlation of mean linear intercepts was r = 0.97 (P < 0.0001), with an agreement of −0.1 ± 3.4 μm (Fig. 8, A and B).

Comparison Between Semiautomated and Stereology-Based Results Within the Same Micro-CT Image

To evaluate the accuracy of semiautomated micro-CT morphometric analysis, stereology-based measurements were compared with semiautomated micro-CT analysis within the same micro-CT scan.

Alveolar surface density. The alveolar surface density (Table 3) differed significantly between stereology-based measurement and semiautomated analysis within the same micro-CT volume of interest (33.47 ± 1.91/mm vs. 30.49 ± 0.95/mm). The regression analysis (Fig. 7C) showed a correlation of r = 0.7 (P = 0.023); the agreement was 2.9 ± 1.4/mm in the Bland-Altman plot (Fig. 7D).

Mean linear intercept. The mean linear intercept (Table 4), calculated by Eq. 2, did not differ significantly from mean linear intercept assessed by stereology-based morphometry (134.4 ± 13.06 μm vs. 135.8 ± 15 μm). The regression analysis showed a correlation of r = 0.97 (P < 0.0001); the agreement was 1.4 ± 3.3 μm (Fig. 8, C and D).

DISCUSSION

The classic approach to image alveolar tissue is based on histological serial sectioning. A major limitation of histology is the destructive nature of preparation. Even adjacent slices often

### Table 1. Diameter and weights of cylindrical tissue samples before and after osmium tetroxide staining and critical point drying

<table>
<thead>
<tr>
<th>No. of Specimen</th>
<th>Before Staining and Drying</th>
<th>After Staining and Drying</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diameter, mm</td>
<td>Weight, g</td>
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<tr>
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<td>8.0</td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
<td>8.3</td>
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<tr>
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</tr>
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<td>5</td>
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<td>0.038</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
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<td>0.034</td>
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<tr>
<td>8</td>
<td>8.2</td>
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</tr>
<tr>
<td>9</td>
<td>8.2</td>
<td>0.037</td>
</tr>
<tr>
<td>10</td>
<td>8.1</td>
<td>0.038</td>
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<tr>
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</tr>
<tr>
<td>SD</td>
<td>0.08</td>
<td>0.003</td>
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</table>

Diameter and weight of specimens were compared before and after osmium tetroxide staining and critical point drying. The difference between the diameter (and the weight) of the sample before and after staining and drying was significant (P < 0.05).

### Table 2. Primary morphometric parameters assessed by micro-CT analysis

<table>
<thead>
<tr>
<th>No. of Specimen</th>
<th>Air Space Volume</th>
<th>Alveolar Surface</th>
<th>Volume/Surface</th>
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<tbody>
<tr>
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<td>10.446</td>
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</tr>
<tr>
<td>2</td>
<td>0.246</td>
<td>10.254</td>
<td>0.0240</td>
</tr>
<tr>
<td>3</td>
<td>0.229</td>
<td>10.426</td>
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<tr>
<td>4</td>
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<td>10.686</td>
<td>0.0217</td>
</tr>
<tr>
<td>5</td>
<td>0.190</td>
<td>10.201</td>
<td>0.0186</td>
</tr>
<tr>
<td>6</td>
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<td>0.0249</td>
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<td>7</td>
<td>0.293</td>
<td>11.217</td>
<td>0.0261</td>
</tr>
<tr>
<td>8</td>
<td>0.236</td>
<td>10.713</td>
<td>0.0220</td>
</tr>
<tr>
<td>9</td>
<td>0.215</td>
<td>10.766</td>
<td>0.0200</td>
</tr>
<tr>
<td>10</td>
<td>0.226</td>
<td>10.680</td>
<td>0.0212</td>
</tr>
<tr>
<td>Mean</td>
<td>0.236</td>
<td>10.551</td>
<td>0.0224</td>
</tr>
<tr>
<td>SD</td>
<td>0.026</td>
<td>0.329</td>
<td>0.0023</td>
</tr>
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</table>

The mean air space volume was 0.236 ± 0.026 mm³ corresponding to 68% of the total volume (0.346 mm³). The mean alveolar surface area was 10.551 ± 0.329 mm². Volume/surface is used to calculate the alveolar diameter (see APPENDIX).

### Table 3. Alveolar surface density assessed by micro-CT morphometric analysis and by stereology-based measurement on micro-CT scans as well as on histological slices

<table>
<thead>
<tr>
<th>No. of Specimen</th>
<th>Histology (Merz grid)</th>
<th>Micro-CT (Merz grid)</th>
<th>Micro-CT</th>
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<td>33.47</td>
<td>30.49</td>
</tr>
<tr>
<td>SD</td>
<td>2.41</td>
<td>1.91</td>
<td>0.95</td>
</tr>
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</table>

The results for the alveolar surface density, measured by stereology-based morphometry (Merz grid) and semiautomated micro-CT analysis (micro-CT), are shown. Means differed significantly between methods (P < 0.05).
do not match exactly due to tissue deformation. In contrast, micro-CT offers the possibility to image tissue non-destructively by creating a continuous data set of the investigated object (13). However, alveolar architecture, in contrast to cancellous bone (20, 30), is rather difficult to study with micro-CT because of the low spatial stability and the inadequate intrinsic contrast of native lung tissue.

To solve this problem, preparation techniques must be applied that avoid alveolar collapse and furthermore enhance radiodensity. Various methods of preparing inflated lungs have been described; some employ vascular perfusion with fixatives (2), others employ rapid freezing followed by permeation with fixatives (23) or formaldehyde inflation (27, 32, 40). In our study, formaldehyde ventilation resulted in stable air-filled lungs with a mean volume of 2 l for the left lung. In adult pigs, total lung capacity was reported to be 4.3 l in average (35). However, there is no question that formaldehyde fixation alters lung tissue, despite constant total lung volumes in our study. Proteins (e.g., collagen, elastin) are denaturated and the tissue is dehydrated. As a consequence, lung tissue loses elasticity, and an average volume loss of up to 20% was described for formaldehyde fixation (22). Differences between native and

Table 4. Mean linear intercept assessed by micro-CT morphometric analysis and by stereology-based measurement on micro-CT scans as well as on histological slices

<table>
<thead>
<tr>
<th>No. of Specimen</th>
<th>Histology (Merz grid)</th>
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<td>SD</td>
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The results for the mean linear intercept, an estimate of alveolar diameter, measured by stereology-based morphometry (Merz grid) and semiautomated micro-CT analysis (micro-CT) are shown. There was no significant difference between means.
fixed lung volumes must be considered for morphometric analysis (41). In addition, lung preparations should be timely analyzed to avoid further changes by shrinkage over time (5). Another important issue is the constancy of alveolar volumes during preparation. Whether alveolar volumes behave comparable to total lung volume during fixation cannot be answered with certainty. Most investigators believe that alveoli unfold in the tidal breathing range and only become stretched at high volume (3). However, others believe that the alveolar volume has a rather persistent dimension. Using in vivo microscopy, Carney et al. (9) showed that the increase in lung volume from 20 to 80% of total lung capacity was based mainly on recruitment of previously deflated alveoli and was not accompanied by a significant change in the individual alveolar volume.

The main problem regarding micro-CT analysis of pulmonary microstructure is the inadequate intrinsic radiodensity of native lung tissue that prevents imaging with high resolution. Whereas very small samples of native tissue (~1 mm³) may be imaged, larger samples of unstained preparations do not have sufficient contrast between the thin alveolar walls and the air space for satisfying data acquisition. Initial attempts were made using silver nitrate as dye followed by gentle centrifugation to remove the staining solution from the air space (36). This procedure did not succeed in a homogeneous staining of the tissue. The centrifugation resulted in deformation of the alveolar architecture that interfered with morphometric analysis. Therefore, we used osmium tetroxide as dye and fixative (4). Osmium tetroxide binds covalently to unsaturated fatty acids of the cell membrane. This results in further tissue stabilization. Due to the high atomic number of 76, it offers excellent radiodensity. The most critical step in our preparation was to eliminate excessive osmium tetroxide solution from the air spaces. The best results were achieved using a critical point drying procedure. With this technique, effects of surface tension are avoided, and the entire fluid is eliminated without tissue deformation. However, some extent of shrinkage does occur (22). A total volume loss of about 10–14% in relation to the native lungs must therefore be taken into consideration. To calculate native lung parameters (e.g., mean linear intercept), a correction factor can be calculated from the relation of sample diameters before and after critical point drying (8.17 / 7.32 = 1.11).

Fig. 8. Mean linear intercept: regression analysis and Bland-Altman plots for the comparison between methods are shown. A and B: comparison of stereology-based measurements between histological and micro-CT images. C and D: comparison between stereology-based measurements and semiautomated micro-CT analysis within the same micro-CT scan.
We achieved two- and three-dimensional micro-CT images of alveolar tissue with a voxel size of 9 μm and resolution of 18 μm. Corresponding micro-CT scans and histological images showed an excellent matching of structures by visual control (Fig. 2, A and B). Perfect congruence on a microscopic level, however, is not feasible because of angular distortions between methods. In addition, it must be addressed that classification of tissues (e.g., cell types), which can be easily done by histology, is impossible with micro-CT.

One of the goals of this study was to establish micro-CT as a method for morphometric investigation of alveolar tissue. Stereology-based histological analysis of perpendicular slices can be considered as the gold standard for morphometric measurements (7, 38). To evaluate the accuracy of micro-CT imaging (transforming the different X-ray attenuations of the tissue into gray-value images), alveolar surface density measured by a modified stereology-based method within corresponding histological sections and micro-CT images was compared. Micro-CT resulted in consistently lower values with good correlation, however. This may be explained by the limited resolution of micro-CT analysis. Because alveolar septae are only 5–10 μm thick, which lies in the range of one voxel (9 μm), very thin walls are not sufficiently represented in micro-CT images.

In addition, we evaluated the accuracy of a semiautomated micro-CT analysis to obtain morphometric data. Alveolar surface density determined by a modified stereology-based method and semiautomated micro-CT analysis was compared within the same micro-CT scan. Semiautomated micro-CT analysis resulted in reasonable data, but the values were lower compared with the stereology-based measurements in micro-CT scans and histology, respectively. The critical step in quantitative assessment of semiautomated micro-CT analysis is to create a binary model of the tissue by using a threshold procedure. Although the tissue/air contrast of our preparation is very high (10:1), care must be taken that thick walls are not overrepresented while thin walls are lost using a global threshold procedure (Fig. 6, C and D). Feldkamp et al. (13) and Kuhn et al. (20) addressed this issue by pointing out that X-rays within heterogeneous material are inconsistently attenuated, not only between different specimens but also within the same sample. We set the threshold level by comparing the binary images with the histological slices. However, if the threshold level chosen is too high, alveolar surface density will be too large, whereas mean linear intercept will be too small, and vice versa. We believe that the smaller values for the alveolar surface density are due to loss of thin walls during transformation into binary images. To date, the resolution of histology is not achieved with micro-CT. Although the gray-scale value scans look very similar compared with the histological slices, the binary images do not. With a voxel size of 9 μm, a realistic binary tissue model is not feasible. In spite of the difference in attenuation between alveolar tissue and air, the thickness of alveolar walls is not sufficient enough to create a realistic binary tissue model. To some extent, this can be explained by the partial volume effect of CT imaging. Because the voxel size has a finite dimension, an error occurs if more attenuations than one (e.g., air and tissue) are present within a voxel. The most significant limitation of the threshold segmentation is to assume that each voxel contains one type of tissue. This segmentation is incompatible with volume averaging as voxels representing different types of X-ray attenuation cannot be classified correctly. The effect of volume averaging is greatest at tissue-surface interfaces. This geometric reality makes the accurate assessment of surfaces by means of threshold segmentation difficult. The effect of volume averaging can be reduced if the voxel size is decreased. The use of synchrotron radiation sources permits a high spatial resolution down to the micrometer level (26). With these advanced techniques, morphometric analysis may be enhanced.

In contrast to alveolar surface density, agreement for mean linear intercept as an estimate for the alveolar diameter was much better. This finding can be explained by the fact that the diameter is not as sensitive for limitations in resolution. Based on histomorphometric investigation, Lum and Mitzner (21) determined an alveolar diameter of 133 μm in pig. Their data are consistent with our results. Even semiautomated micro-CT analysis, which uses primary data as volume and surface for the calculation of alveolar diameter, resulted in reasonable data. However, a proportionality factor of 6 had to be incorporated into the formula (see APPENDIX).

It should be noted that the morphometric micro-CT analysis is not a fully automated procedure. Micro-CT morphometry still depends on human judgment, since the program does not distinguish between anatomical structures. The inclusion of bronchi or vessels in the volume of interest will result in errors in alveolar morphometric data.

The advantage of micro-CT is that relying on the isotropic data set imaging of three-dimensional structures like bronchioli and acini can be easily performed. The traditional anatomical concept holds that the acinus begins at the last airway without alveoli (the transitional bronchiole) (16). All airways proximal to the transitional bronchiole are purely of conducting nature. Segmentation of the acinus can be done by tracking down a terminal bronchiole to the alveolar air space. The segmentation process started in placing a seed in a section of a bronchus. Under visual control of the growing seed, a model of the conductive airway was constructed. As shown in Fig. 4A, there was a clear transition in the wall thickness of the conductive airways and the alveolated airways. Thus defining a transitional bronchiole (the last airway without alveoli) as the starting point for the segmentation of the acinus was reliable. In a second segmentation, the airways were followed down distally to the alveolar walls, resulting in the visualization of the acinus (Fig. 4, C and D). To validate the correct calculation, the borders of the segmentation (Fig. 4, A and B) were checked in the two-dimensional scans by the investigator. Therefore, the segmentation still depends on human judgment. To optimize the segmentation, further work needs to be done to improve the resolution of the micro-CT scanning process.

In conclusion, we showed that semiautomated micro-CT analysis is feasible in fixed and radiological-enhanced lung tissue samples. However, the accuracy of morphometric measurements is restricted to the applied voxel size that determines resolution. Because thickness of alveolar septae is only 5–10 μm, a voxel size <3 μm is required for precise tissue quantification.

In contrast to histology, the isotropic continuous data set allows the reconstruction of three-dimensional images and may be used for segmentation of the pulmonary acinus. As the preparation technique can be applied in human lungs, there may be an interesting field for morphometric investigations of human pulmonary pathologies (e.g., fibrosis, emphysema, or pneumoconiosis) performed on postmortem lungs.
APPENDIX

Grid of Merz: Length of Test Line

The grid of Merz is composed of ten lines. Each line is composed of 10 semicircles (corresponding 5 circles) with the diameter (d). The d of one circle is the tenth part of the side length (700 μm) of the square (d = 70 μm). The circumference of a circle is π × 70 μm. The total length of the test lines:

Total length of test lines = 10_lines × 5 Circles(line)
× (π × 70 μm)/length(circle) = 3500 × π μm = 3.5 × π mm (A1)

Alveolar Diameter

Under the assumption that alveoli are ideal spheres, d of a single alveolus can be calculated when the air space volume and the surface of the alveoli are known.

\[ V_{sphere} = \frac{\pi \times d^3}{6} \]  \hspace{1cm} (A2)

\[ A_{sphere} = \pi \times d^2 \] \hspace{1cm} (A3)

The d can be calculated by:

\[ d = k \times \left( \frac{V}{A} \right) \] \hspace{1cm} (A4)

\[ k = \left( \frac{d \times A_{sphere}}{V_{sphere}} \right) \] \hspace{1cm} (A5)

By inserting Eqs. A2 and A3 into Eq. A4:

\[ k = 6 \times \left( \frac{(d \times \pi \times d^2)/(\pi \times d^3)}{6} \right) \] \hspace{1cm} (A6)

In this case the proportionality factor (k) gives the number 6.

Under the assumption that the alveoli in our volume of interest can be considered as a group of spheres with different diameters, the situation is more complex. k depends not only on the distribution of the spheres but also on the variation of individual diameters.

Homogeneous Distribution of Spheres: Example 1: Different Diameters

In the case of five small spheres (d = 1 mm) and five large spheres (D = 2 mm), the mean diameter (d_{mean} = 1.5 mm) can be calculated by Eq. A4.

The air space volume (V) is the sum of the volume of the small spheres \[ V_d = \frac{5 \times (\pi \times d^3)}{6} \] and the volume of the five large spheres \[ V_D = \frac{5 \times (\pi \times D^3)}{6} \]:

\[ V = \frac{5 \times (\pi \times d^3)}{6} + \frac{5 \times (\pi \times D^3)}{6} \] \hspace{1cm} (A7)

\[ V = \frac{5}{6} \times \pi \times (d^3 + D^3) \] \hspace{1cm} (A8)

The surface area is the sum of the area of the five small spheres \( A_d = 5 \times \pi \times d^2 \) and the area of the five large spheres \( A_D = 5 \times \pi \times D^2 \):

\[ A = 5 \times \pi \times d^2 + 5 \times \pi \times D^2 \] \hspace{1cm} (A9)

\[ A = 5 \times \pi \times (d^2 + D^2) \] \hspace{1cm} (A10)

By inserting Eqs. A8 and A10 into Eq. A4:

\[ d_{mean} = k \times \left( \frac{(d^3 + D^3)/(d^2 + D^2)}{6} \right) \]

1.5 = k \times \left( \frac{(d^3 + D^3)/(d^2 + D^2)}{6} \right) \hspace{1cm} (A11)

1.5 = k \times \left( \frac{(1 + 8)/(1 + 4)}{6} \right)

1.5 = k \times 9/30

k = 5

In this example, k is 5.

Example 2: Similar Diameters

In the case of five small spheres (d = 1 mm) and five large spheres (D = 1.1 mm), the mean diameter \( d_{mean} = 1.05 \) mm can be calculated by Eq. A4.

By inserting Eqs. A8 and A10 into Eq. A4:

\[ d_{mean} = k \times [(V_d + V_D)/(A_d + A_D)] \]

1.05 = k \times \left( \frac{(d^3 + D^3)/(d^2 + D^2)}{6} \right) \hspace{1cm} (A12)

1.05 = k \times \left( \frac{(1 + 1.331)/(1 + 1.21)}{6} \right)

k = 5.97

In this example, k is 5.97.

Heterogeneous Distribution of Spheres: Example 3: Different Diameters

In the case of one small sphere (d = 1 mm) and nine large spheres \( D = 2 \) mm, the mean diameter \( d_{mean} = 1.9 \) mm can be calculated by Eq. A4.

Analog to Eq. A7 the sum of the volumes is:

\[ V = \frac{1 \times (\pi \times d^3)}{6} + \frac{9 \times (\pi \times D^3)}{6} \] \hspace{1cm} (A13)

Analog to Eq. A9 the sum of the areas is:

\[ A = \frac{1 \times (\pi \times d^2)}{6} + \frac{9 \times (\pi \times D^2)}{6} \] \hspace{1cm} (A14)

By inserting Eqs. A13 and A14 into Eq. A4:

\[ d_{mean} = k \times [(V_d + V_D)/(A_d + A_D)] \]

1.9 = k \times \left( \frac{(d^3 + 9 \times D^3)/(d^2 + 9 \times D^2)}{6} \right) \hspace{1cm} (A15)

1.9 = k \times \left( \frac{(1 + 9 \times 8)/(1 + 9 \times 4)}{6} \right)

k = 5.78

In this example, k is 5.78.

If the variation of the d is small (e.g., <10%, k reaches 6 regardless of the distribution of the spheres.

ACKNOWLEDGMENTS

We thank Gerd Magdowski for expert technical advice in osmium tetroxide staining and critical point drying of the tissue samples. We are grateful to Gunhild Martels for micro-CT scanning and Tamara Papadakis for histological preparation.

REFERENCES


