A review of recent developments and applications of morphometry/stereology in lung research

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Mühlfeld C, Jan, Hegermann, Wrede C, Ochs M. A review of recent developments and applications of morphometry/stereology in lung research. Am J Physiol Lung Cell Mol Physiol 309: L526–L536, 2015. First published July 17, 2015; doi:10.1152/ajplung.00047.2015.—Design-based stereology is the gold standard of quantitative morphology in lung research. Approximately 50 yr after the beginning of Weibel’s research on the structure-function relationships of the lung (160, 162), the American Thoracic Society and the European Respiratory Society published a joint research policy statement on the use of stereology for the quantitative assessment of lung structure (51), thereby providing a coherent framework for quantitative morphology in lung research. Still, the implementation of this theoretical framework into a practical research question remained challenging. This led us to provide a more practically and problem-oriented approach to lung stereology recently by addressing the pathology of various lung diseases and by making practical recommendations of useful stereological parameters for these diseases (101, 111).

The current review article intends to provide an update on lung stereology over the last 2 yr, first by analyzing the use of stereological methods in the two basic lung research journals with the highest impact factor in recent years. Here, we discuss what stereological methods are currently most widely used and where there is still space for further improving the quality of quantitative morphology in lung research. In the second part of this article, we address recent methodological developments and advances, and we highlight new biological observations made by the use of stereology.

Analysis of the Current Use of Stereology in Lung Research

Selection of journals and articles. Among the journals dedicated to pulmonary research, we chose the American Journal of Physiology Lung Cellular and Molecular Physiology and the American Journal of Respiratory Cell and Molecular Biology for a literature review. The two journals were selected because they have the highest impact factor of those journals publishing basic lung science and because their impact factor has proven to be in the same range over the last years (87). Another important reason is the fact that both journals or their corresponding societies have made substantial efforts to increase the quality of morphometry in lung research by publishing research guidelines (51) and review articles (10, 101, 111) as well as editorials (47, 89, 162) dedicated to this topic. To identify target articles, all articles (except reviews) published in the print issues in 2013 and 2014 were searched for the terms “morphometry/morphometric” and “stereology/stereological.” The selection of articles was then closely looked at to analyze which species, diseases, and lung compartments were investigated by morphometry and which morphometric methods were implemented (Tables 1–4). The aim of this analysis was to gain a general overview on the current use of morphometric methods in lung research. Thus it may not be complete (e.g., articles making use of morphometry but do not contain the search terms morphometry or stereology) and in some cases it may be subjective (e.g., when the term morphometry was only used for microscopic scoring the article was excluded). However, we feel that this survey provides a realistic picture of the current use of lung morphometry. In total 99 articles out of 715 were included with 56 of 384 in the Am J Respir Cell Mol Biol (1, 3, 8, 15–17, 19, 21, 23, 26, 27, 31–35, 37, 38, 40, 45, 46, 53, 57, 58, 60, 61, 63, 64, 69–71, 74, 75, 78, 79, 81, 85, 98, 99, 108, 114, 117, 118, 122, 128, 129, 131, 134, 135, 139, 141, 142, 144, 146, 150, 159), and 43 of 331 in the Am J Physiol Lung Cell Mol Physiol (4–7, 11, 14, 20, 25, 36, 48, 49, 54, 59, 73, 76, 77, 80, 83, 84, 86, 93–95, 97, 106, 116, 119–121, 125, 127, 130, 133, 136, 137, 140, 143, 155–158, 163, 165). Thus a...
fraction of 10–15% of articles on the lung make use of morphometric/stereological methods, which underlines the impact of structural quantification in lung research.

Analysis of the articles. Overall, the articles could be grouped into one of three categories: 1) method development or improvement; 2) stereology/morphometry contributes a major part to the conclusion of the article; and 3) stereology/morphometry is used as an additional source of information. Not surprisingly, more than 75% of the articles deal with rodent models whereas other species including humans and nonhuman primates make only a minor proportion (Table 1). Besides the naturally limited availability of human samples due to ethical reasons, their use for morphometric studies is difficult as the biopsy specimens will always deliver relative values, thus limiting the conclusions that can be drawn from the data. However, the quantitative analysis of these samples is still encouraged because animal experiment-based hypotheses can be evaluated with respect to their transferability to humans. In such cases, a suitable internal reference parameter needs to be defined to which the parameters of interest are related, e.g., the surface area of epithelial basal lamina in airway biopsies (126, 164).

The investigated pathology is relatively homogeneously distributed over the main lung diseases (lung injury/inflammation, chronic obstructive pulmonary disease/emphysema, pulmonary hypertension, and pathological development/bronchopulmonary dysplasia). For several of these diseases, a set of well-suited stereological parameters has been available for a long time, such as acute lung injury (103, 112) and emphysema (113). In other cases, the application of stereology is just emerging such as fibrosis (9, 82) or bronchopulmonary dysplasia (83). In pulmonary hypertension, various morphometric methods have been used since many years which relate to the thickness of the vessel media (%medial thickness) and the amount of non-, partially, or fully muscularized vessels. The latter is usually based on the counting of vessel profiles related to a certain area and has to be treated with caution for reasons of relativity and the representation of structures in two dimension (2D; see below). It will be of great interest to see the first study applying design-based stereological methods to pulmonary hypertension and comparing these methods with the currently used techniques (148). Of note, 6–7% of the articles deal with method development/improvement, in particular with the use of morphometry from nondestructive imaging (Table 2).

More than 50% of the articles use morphometry to investigate the alveolar region whereas airways and vasculature share the remaining 50% with a few articles on other compartments (e.g., innervation, subcellular composition, etc.; Table 3). As the pioneering work on lung stereology mainly addressed the alveolar region, this distribution may still have traditional reasons. Another reason may be related to the anisotropy of nonalveolar airways, blood vessels, and innervation and the problems associated with this. A simple solution to this is to make the whole sample isotropic by applying suitable methods for randomization of the orientation (88, 107).

When looking at the implemented methods, it becomes apparent that the majority of articles used nonstereological morphometric methods whereas the classical point and intersection counting for volume and surface area estimation (161) and the disector for number estimation (138) were much less frequently used. The most frequently used method was the mean linear intercept/chord (MLI) length (Table 4). The group of other morphometric methods included the counting of profiles per area (e.g., cells/field of view or vessels per area), 2D image analysis-based planimetry, automated or manual segmentation, direct measurements of distances from 2D images or projections, semiquantitative scoring systems, model-based calculations, medial wall thickness, and radial alveolar counts

Table 1. Species investigated by morphometry

<table>
<thead>
<tr>
<th>Species</th>
<th>AJP</th>
<th>AJRCMB</th>
<th>Sum</th>
<th>Relative Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>26</td>
<td>43</td>
<td>69</td>
<td>57.5</td>
</tr>
<tr>
<td>Rat</td>
<td>13</td>
<td>10</td>
<td>23</td>
<td>19.2</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>Hound</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Sheep</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>Human or nonhuman primate</td>
<td>5</td>
<td>6</td>
<td>11</td>
<td>9.2</td>
</tr>
<tr>
<td>Horse</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
<td>1</td>
<td>9</td>
<td>7.5</td>
</tr>
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</table>

Table 2. Biomedical topics addressed by morphometry

<table>
<thead>
<tr>
<th>Subject</th>
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<th>AJRCMB</th>
<th>Sum</th>
<th>Relative Fraction, %</th>
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</thead>
<tbody>
<tr>
<td>Normal lung development</td>
<td>8</td>
<td>4</td>
<td>12</td>
<td>12.1</td>
</tr>
<tr>
<td>Pathological development, bronchopulmonary dysplasia</td>
<td>9</td>
<td>11</td>
<td>20</td>
<td>20.2</td>
</tr>
<tr>
<td>Injury, inflammation</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>15.2</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>9</td>
<td>6</td>
<td>15</td>
<td>15.2</td>
</tr>
<tr>
<td>COPD, emphysema</td>
<td>4</td>
<td>14</td>
<td>18</td>
<td>18.2</td>
</tr>
<tr>
<td>Asthma, airway hyperreactivity</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>9.1</td>
</tr>
<tr>
<td>Compensatory lung growth, postpneumonectomy</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Method development</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>3.0</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>6.1</td>
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</table>

Table 3. Lung compartments addressed by morphometry

<table>
<thead>
<tr>
<th>Lung Compartment</th>
<th>AJP</th>
<th>AJRCMB</th>
<th>Sum</th>
<th>Relative Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airways</td>
<td>9</td>
<td>11</td>
<td>20</td>
<td>16.3</td>
</tr>
<tr>
<td>Vasculature</td>
<td>17</td>
<td>11</td>
<td>28</td>
<td>22.8</td>
</tr>
<tr>
<td>Alveoli</td>
<td>27</td>
<td>41</td>
<td>68</td>
<td>55.3</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Table 4. Contribution of morphometric methods

<table>
<thead>
<tr>
<th>Method</th>
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<th>AJRCMB</th>
<th>Sum</th>
<th>Relative Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Point counting (volume)</td>
<td>12</td>
<td>7</td>
<td>19</td>
<td>12.7</td>
</tr>
<tr>
<td>Intersection counting (surface area)</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>Disector counting (number estimation)</td>
<td>6</td>
<td>1</td>
<td>7</td>
<td>4.7</td>
</tr>
<tr>
<td>MLI/MCL</td>
<td>13</td>
<td>34</td>
<td>47</td>
<td>31.3</td>
</tr>
<tr>
<td>Other</td>
<td>32</td>
<td>40</td>
<td>72</td>
<td>48.0</td>
</tr>
</tbody>
</table>

MLI/MCL, mean linear intercept/chord.
Table 5. List of minimum methodological information

<table>
<thead>
<tr>
<th>Methodological Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects (total, per group)</td>
</tr>
<tr>
<td>Technique of lung fixation</td>
</tr>
<tr>
<td>Technique of reference volume estimation</td>
</tr>
<tr>
<td>Sampling of tissue (and down to the field of view)</td>
</tr>
<tr>
<td>Number of samples investigated (blocks, sections, fields of view)</td>
</tr>
<tr>
<td>Method of randomization for location and spatial orientation</td>
</tr>
<tr>
<td>Tissue processing including embedding medium</td>
</tr>
<tr>
<td>Physical and optical sections (thickness, staining, etc.)</td>
</tr>
<tr>
<td>Test systems used for counting</td>
</tr>
<tr>
<td>Number of counting events per subject</td>
</tr>
<tr>
<td>Data reporting and statistical methods</td>
</tr>
</tbody>
</table>

(RAC). In general, morphometric methods were used to investigate one of the following structural events: air space enlargement (MLI), loss of alveoli/alveolar surface (surface area estimation, RAC), increase/decrease of (muscularized) vessels (profile counting), wall thickness (direct measurement, arithmetic and harmonic mean barrier thickness, and calculation from perimeters), and increase/decrease of cells (profile counting and disector).

These data demonstrate that, although there is an apparent need for morphometric methods in lung research, their application can and should be improved. In the following, we address several issues that are of particular importance.

Problems associated with the current use of morphometric/stereological methods. Lack of methodological information. It has become a habit that detailed methodological information is missing, which often makes it impossible to understand how the data were exactly obtained. Partly, this is caused by journals seeking to reduce the number of printed pages and moving detailed method information to an online data supplement. Although in principle the online data files have no strict size limit, this policy assigns a minor value to this type of information, which certainly changes the way scientists report how data were gathered. Another reason is that researchers may not always be aware of which information is necessary to assess the quality of the reported data. Therefore, Table 5 shows a list of minimum information that should be reported for morphometric/stereological methods to provide scientific transparency.

Use of ratios. A frequent problem remains the reporting of relative values (volume densities, surface area densities, or number densities) in spite of absolute data. When these data are treated like total values and when it is ignored that changes may result both from changes in the enumerator and the denominator, false conclusions may easily occur because of unawareness of the “reference trap” (12, 56). It is therefore of utmost importance to provide total data or to relate the data to an appropriate reference structure.

MLI as the sole parameter to quantify air space enlargement and emphysema. It has been repeatedly stated that emphysema is characterized by air space enlargement and destruction of alveolar septa (28, 101, 110). Thus the sole description of air space enlargement is not in line with the definition of emphysema. In principle, the MLI may be used as an indirect measure of air space enlargement as it measures a mean diffusion distance “from wall to wall” in acinar air spaces but should at least be combined with a measure of total alveolar surface area or total alveolar number. However, one has to be aware that the MLI strongly depends on the inflation status and the elastic properties of the lung and is therefore prone to pitfalls (68, 100). If the lungs of two experimental groups are fixed by instillation at the same hydrostatic pressure, for example, a decrease in the elastic recoil within one group will automatically result in a stronger inflation and therefore greater MLI values in that group. The number of alveoli and the gas-exchange surface area may nevertheless be the same in both groups.

Use of nonstereological methods when unbiased methods are available. There is still a large number of articles using morphometric methods that are not unbiased. As mentioned before, many authors use the counts of cellular (or vessel) profiles related to a certain area as a measure for the number of the cells within the organ. This approach carries two potential sources of bias: the first one is that a ratio is reported (see above). The second one is that due to the loss of one dimension in the cutting process the number of structures in three dimensions (3D) is not represented within a single 2D microscopic section. It is often argued that this criticism is equally valid for different groups that are compared; however, an easy example shows that this may lead to false conclusions. As the probability of a cell to be sectioned and therefore of its profile to be contained in the section is related to its height perpendicular to the section plane, larger sized cells have a higher chance of being cut and counted in a single 2D section. If an experimental intervention leads to hypo- or hypertrophy of a certain cell type, this will therefore necessarily affect the number of cell profiles per area although the total number of cells in the reference volume may be the same. By the same token, RAC are often used as a measure of the number of alveoli or the alveolarization. In this method, the number of alveolar profiles traversed by a perpendicular line “placed from the junction of conducting and respiratory epithelia to the nearest connective tissue septum or pleura” (18). If the number of alveoli related to one acinus remains the same but the number of acini in the lung is different between two experimental groups, the RAC will not be able to detect this difference. The only accurate and efficient way for gaining information on the number of objects in 3D is to count them in 3D, which in microscopy is achieved by the disector method (138). The disector has been established in lung research both for particles (such as cells) and for more complex structures (such as alveoli) (55, 109, 113). Another example is the direct measurement of distances (e.g., wall thickness) on 2D sections although the orthogonal intercepts method has been shown to be superior (30). In general, it should be mentioned that for most morphometric questions an unbiased stereological method is available and should be used instead of assumption-based methods or relative values which are prone to bias or misinterpretations.

Taken together, this analysis shows that morphometric methods have a clear justification in lung research but also that there is still space for improving the use of morphometry/stereology in practice. In some areas of research, plenty of morphometric approaches are available, and other fields may benefit from new methodological developments or from the consistent implementation of already existing approaches.
Digital quantification of airway innervation. Quantitative studies on the innervation of airways are rare, partly because the nerve fibers are rare, difficult to visualize, and even more tedious to quantify. Although unbiased stereological methods are available (104) and have been applied to airways (39), additional morphometric approaches are desirable. Two remarkable papers by Scott et al. (134, 135) have provided new ways of quantifying nerve fibres in the respiratory tract. Although the light microscopic approach does not differentiate between nerve fibres containing one or more axons, it provides comprehensive information on length, diameter, branching points, and the number of ganglia. In the first paper of this group (134), airways were prepared by microdissection and computer-based morphometry was used to quantify structures. In the second paper (135), the authors applied tissue clearing to whole mouse lungs and thus enabled the visualization of airway innervation in 3D and correlating with, for instance, the airway generation.

Tissue deformation. One of the frequently ignored problems in stereology is the tissue deformation that occurs during processing of the samples (22). The problem can shortly be illustrated by the following example: a lung is fixed by tracheal instillation of the fixative and its volume is determined by Archimedes’ principle (132). Then, the lung is sampled according to one of the accepted sampling regimes, say systematic uniform random sampling (43, 90), and stereological parameters are estimated at the microscopic level subsequently. All stereological estimates are related to the volume measured before tissue processing. However, between lung volume estimation and the final microscopic analysis, the samples are subjected to deformation by cutting and to various chemicals, which may result in swelling or shrinkage of the samples. This tissue deformation is difficult to control, but it may be of great importance when experimental groups with a different degree of tissue deformation are compared using stereological parameters. In a recent study, Schneider and Ochs (133) compared the shrinkage of mouse lung samples subjected to various embedding protocols and media. Their data show that both the preembedding protocol and the embedding medium influence the degree of tissue shrinkage. While paraffin embedding according to standard protocols led to global tissue volume shrinkage of ~50%, embedding in glycol methacrylate also caused a shrinkage of ~40% if the protocol did not contain postfixation with osmium tetroxide and uranyl acetate. However, when the lung samples were embedded in glycol methacrylate including these postfixation steps, hardly any shrinkage occurred. When paraffin embedding cannot be avoided (e.g., when certain immunohistochemical stains need to be performed), several solutions are possible, such as the use of the (smooth) fractionator for number estimation (41, 42) or the integration of a correction factor (22, 105). In the latter case it should be kept in mind that it is only possible to estimate global shrinkage. Differential shrinkage, e.g., different degrees of shrinkage between cells and extracellular matrix, cannot be measured or controlled.

Nondestructive lung imaging. With the advance of imaging techniques, a new focus of quantitative morphology in lung research has been the use of 3D data sets from nondestructive lung imaging modalities instead of the traditional microscopy-based quantification. Here, a whole fixed lung (or parts of it) is scanned ex vivo, thus leading to 3D image stacks without physically sectioning it (65, 66, 91, 92). The major advantage of this approach is that stereological data can be obtained within the 3D environment, thus facilitating a correlation between quantitative data and 3D geometry. In addition, the image stacks in z direction provide virtual disectors, thereby significantly facilitating the estimation of particle (e.g., alveolar) numbers. Using micro-computed tomography (CT), Vasilescu et al. (153) visualized whole mouse lungs and applied design-based stereology to estimate various parameters to characterize the gas-exchange region of the lung (e.g., number and mean volume of alveoli, surface area and thickness of alveolar septa). Importantly, the authors compared their data to stereological data obtained from the same lungs by light microscopy. While most parameters were nicely correlated, the higher surface area in the light microscopic data illustrates the limitation of the micro-CT approach: the current resolution limit at 1–2 μm. A different approach based on high-resolution X-ray tomography was chosen by Haberthür et al. (44). These authors performed a semiautomatic method to extract information on isolated acini from their tomographic data sets and performed design-based stereology afterwards. Thus they were able to provide data on the volume, surface area, and number of alveoli per acinus. An interesting aspect of this technique is that it allows relating the stereological data to the acinus as a reference structure which may be of particular usefulness for studies where only a part of the lung can be investigated. Again using micro-CT, Kumar et al. (72) performed registration-driven in vivo scanning to obtain whole lung information under varying conditions. Focusing on the acini and the alveoli, the authors used morphometry to estimate volume and surface area of alveoli under different expansion pressures. This method allows to relate lung mechanics to geometrical features of the alveoli/acini.

Biological Observations Obtained by Stereological Methods

Alveolarization. Three papers on the postnatal development and aging of the rat, the nonhuman primate, and the human lung will be addressed (48, 49, 147). All of the three papers used state-of-the-art stereology and present a portfolio of detailed methods to assess lung alveolarization or age-associated loss of alveoli. Tschanz et al. (147) investigated the alveolarization process in the rat lung through a prolonged postnatal period and showed that a biphasic pattern of alveolar development exists with the major part of alveoli developing between days 4 and 21 of rat lung development and a second phase of “continued alveolarization” between 21 and 60 days of postnatal life. Herring et al. (49) provided the first investigation of postnatal human alveolarization using design-based stereology. Their data clearly show that the major process of alveolarization takes place exponentially through the first 2 yr of postnatal life but continues at a much slower rate during adolescence. The loss of alveoli during aging was studied by the same group in the rhesus macaque and differentiated between male and female monkeys (48). Their data demonstrate a more pronounced loss of alveoli in the female monkeys compared with the males. This interesting finding warrants further research into the mechanisms involved in the stronger aging-associated loss of alveoli in females.
**Compensatory lung growth.** The occurrence of compensatory lung growth (CLG) after pneumonectomy has been known for many years and can be induced in various species (29, 50, 154). Ravikumar et al. (123, 124) addressed various interesting mechanistic questions of CLG by assessing the structures of the remaining lung tissue by design-based stereology. In the first paper (123), the authors investigated the effects of two mechanical stimuli on the extent of CLG, namely the increased perfusion of the remaining lung and the lateral expansion due to mediastinal shift. To decipher between the influences of these stimuli they replaced the right lung by an inflatable prosthesis, which was either kept inflated until the experiments or was acutely deflated 4 mo after pneumonectomy. The data on volume expansion by high resolution CT and on various alveolar components by stereology enabled the authors to attribute certain portions of CLG to either the increased lung perfusion or the lateral expansion. The second paper (124) analyzed the degree of CLG after different amounts of lung resection (42, 58, and 65–70%) to see if a stimulus-response relationship exists. The stereological data showed that CLG peaks with 58% and is less extensive after 70% lung resection, which the authors hypothesized to be related to mechanical stress that threatens the integrity of alveolar septa. Both papers contain an exemplary and detailed methods section that may help other researchers when planning own stereological studies on the amount (volume, surface area) and composition of alveolar septa (volumes of various septal compartments).

**Bronchopulmonary dysplasia.** As demonstrated in Table 2, bronchopulmonary dysplasia (BPD) is a frequent topic among the articles using morphometry. However, this complex pathology does not have a long tradition of state-of-the-art stereology, which explains why most authors resorted to less suitable and less reliable morphometric methods. In that respect, it is worth mentioning the article by Madurga et al. (83). Here, the authors used a mouse model of BPD induced by hyperoxia to investigate the effect of systemic hydrogen sulfide administration on alveolarization. Various design-based stereological parameters were applied to quantify characteristics of the alveolar region of the lungs (number, surface area, volume, septal thickness, and MLI, among others). The methods are described clearly and in detail and are therefore easy to replicate by others. This paper solves the problem of the not yet established methods for assessing parameters in BPD and sets a new standard in this field of research.

**Pulmonary fibrosis.** Very recently, two studies applied design-based stereology to rodent models of diffuse parenchymal disease and human lungs with idiopathic pulmonary fibrosis (9, 82). In one of the articles, the authors correlated pulmonary function testing with the structural alterations in the rat bleomycin-model of pulmonary fibrosis over a sequence of 14 days after injury. Using various stereological techniques, the authors investigated the number and surface area of open alveoli as well as the preservation of intracellular and intra-alveolar surfactant, among others. The data provide convincing evidence that at the early stages after the injury alveolar derecruitment is associated with a loss of surfactant function but that the collapsed alveoli can still be recruited by positive end-expiratory pressure. At later stages, collapse induration (defined as the irreversible collapse of alveoli) occurs and is characterized by hyperplastic alveolar epithelium covering the collapsed alveoli, thus limiting the fraction of alveoli that can be recruited by ventilation strategies (82). In the second paper, the authors used the mouse amiodarone model of pulmonary fibrosis to analyze the correlation between changes of the intracellular surfactant pool and the degree of fibrotic remodeling. The investigation shows that amiodarone leads to progressive

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**Fig. 1.** Illustration of a focused ion beam scanning electron microscope (FIB-SEM) data set. A: illustration of the total volume contained in the data set. B: nucleus (green) and lamellar bodies (red) were manually or automatically segmented, respectively, and used to visualize the 3-dimensional characteristics of these organelles.
changes of alveolar epithelial type II cells during the first 2 wk after the injury, which are characterized by cellular hypertrophy and increased content of larger sized lamellar bodies. These alterations were closely correlated with the increased collagen content in the septal walls (9). Both studies also compared their findings with lung tissue from patients with idiopathic pulmonary fibrosis and demonstrated that the pathology was similar in human lung tissue.

Cell types. Finally, an example of design-based stereology applied to a single cell type comes from our own laboratory (143). Lipid body-containing interstitial cells or lipofibroblasts have come into focus of lung research in recent years because they have been placed into connection with various important physiological functions of the lung, such as surfactant synthesis (145) and lung development (149). Using a comparative approach (14 species), Tahedl et al. (143) investigated the volume of lipid bodies in interstitial cells of the alveolar septa by stereology and showed that the main species possessing lipofibroblasts were found to be rodents whereas the presence of this cell type, when defined by the presence of lipid bodies, in the other investigated species is arguable. These findings were discussed in an excellent and well-balanced editorial focus putting the stereological observations into a broader perspective (2). From a methodological point of view, this article may serve as a source of information when stereology is performed from the light microscopic down to the subcellular level.

Outlook: Volume Electron Microscopy

Finally, we would like to draw attention to a new electron microscopic technique that is of particular interest for lung research and may facilitate the integration of stereology of subcellular structures as it overcomes the problems of the small section thickness in traditional transmission electron microscopy. This relatively new approach is an integral part of “volume electron microscopy” and is already frequently used in neurosciences (13, 52, 115). The technique is based on the alternating scanning of the face of a tissue block and the removal of a few nanometers from the surface of the tissue block. This is realized by the integration of an ultramicrotome (SBF-SEM) or a focused ion beam source (FIB-SEM) into a more or less conventional scanning electron microscope (67, 96). The subsequently (automatically) scanned block face images form a z stack that contains the ultrastructural information of the whole scanned tissue block. Both approaches, microtome or focused ion beam, of generating new block faces are complimentary since they differ in the sample volume that can be scanned and the z-resolution that can be achieved. Besides containing high-resolution 3D information (Fig. 1) these image stacks provide ideal material for estimating the number of intracellular particles (e.g., lamellar bodies, autophagosomes, or nanoparticles) using the disector technique (Fig. 2), which previously relied on manual serial sectioning or electron tomography (24, 62, 102, 151, 152).

Closing Remarks

The present review was meant to provide an analysis of the current use of morphometry and stereology in lung research. It shows that despite the generally accepted need for morphometry, the methodological approaches that are used in practice can be improved. While there is a wealth of information available on the alveolar region of the lungs, further work is needed to establish quantitative structural characteristics of airways and vasculature. In particular, further correlative and mechanistic studies are needed addressing the functional impairment and the underlying structural pathology of the lung in diffuse parenchymal diseases and pulmonary hypertension. Methods to characterize airways and vasculature in an unbiased fashion are available, in principle, but development of additional parameters and evaluation of their analytical power still needs to be done. Major technical advances were made in the combination of nondestructive lung imaging and design-based stereology as well as computer-based morphometry. Volume electron microscopy may facilitate stereology both at tissue and single cell level in the near future. Several articles have used stereology as the major source of information in their studies and have provided interesting results in various fields of lung research. The methods sections in these articles are recommended to those who wish to apply stereology in their own future studies.

![Fig. 2. Disector pair taken from a z-stack generated by FIB-SEM. After each scan (i.e., each image) a slice of ~10 nm tissue was removed with a focused ion beam. Here, A is the 9th section of the z-stack and B is the 19th section; thus 100 nm are between the surface of A and B. According to the disector principle, particles are counted if they are present in one section and absent in the other section. Here, lamellar bodies are counted if they are completely within the counting frame (black arrows) or touch the dashed inclusion line. If they are partly within the counting frame but touch the full exclusion line (white arrows), they are not counted.](http://ajplung.physiology.org/)

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

AUTHOR CONTRIBUTIONS

Author contributions: C.M. and M.O. conception and design of research; C.M., J.H., and C.W. analyzed data; C.M., J.H., C.W., and M.O. interpreted results of experiments; C.M. and M.O. drafted manuscript; C.M. and M.O. edited and revised manuscript; C.M., J.H., C.W., and M.O. approved final version of manuscript; J.H. and C.W. performed experiments; J.H. and C.W. prepared figures.

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RECENT DEVELOPMENTS IN LUNG STEREOLOGY

Review


