Quantitative microscopy of the lung: a problem-based approach. Part 2: stereological parameters and study designs in various diseases of the respiratory tract

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Mühlfeld C, Ochs M. Quantitative microscopy of the lung: a problem-based approach. Part 2: stereological parameters and study designs in various diseases of the respiratory tract. Am J Physiol Lung Cell Mol Physiol 305: L205–L221, 2013. First published May 24, 2013; doi:10.1152/ajplung.00427.2012.—Design-based stereology provides efficient methods to obtain valuable quantitative information of the respiratory tract in various diseases. However, the choice of the most relevant parameters in a specific disease setting has to be deduced from the present pathobiological knowledge. Often it is difficult to express the pathological alterations by interpretable parameters in terms of volume, surface area, length, or number. In the second part of this companion review article, we analyze the present pathophysiological knowledge about acute lung injury, diffuse parenchymal lung diseases, emphysema, pulmonary hypertension, and asthma to come up with recommendations for the disease-specific application of stereological principles for obtaining relevant parameters. Worked examples with illustrative images are used to demonstrate the workflow, estimation procedure, and calculation and to facilitate the practical performance of equivalent analyses.

design-based stereology; acute lung injury; diffuse parenchymal lung disease; emphysema; pulmonary hypertension; asthma

EXPERIMENTAL STUDIES addressing the pathobiology or the possible therapeutic intervention in the setting of a specific disease of the respiratory tract rely on accurate (unbiased) methods that allow evaluating a particular hypothesis by appropriate statistical methods. Ideally, these methods are simple to apply and deliver data at reasonable cost, i.e., they are efficient. As outlined in the first part of this review (73), design-based stereology offers a comprehensive set of methods that are regarded as the gold standard for quantitative morphology of the respiratory tract (37). In the second part of this review, we propose sets of useful parameters for various respiratory diseases to accurately analyze the alterations quantitatively. Worked examples with illustrative images are provided to facilitate the practical performance of equivalent analyses. The choice of diseases [acute lung injury (ALI), fibrosis, emphysema, pulmonary hypertension (PH), and asthma] was based on the consideration that they include most of the pathological lesions observed in pulmonary diseases so that investigators of other pathologies may gather information from related pathological entities; e.g., investigators of chronic obstructive pulmonary disease will be able to benefit from the information provided in the sections of emphysema and asthma. As such, we hope that this review facilitates and furthers the use of stereological methods in most areas of pulmonary research.

ALI

Pathobiology of ALI. ALI is characterized by a typical and time-dependent pattern of pathological events evoked by various types of alveolar injury (44, 60). According to the sequence of pathological lesions, this diffuse alveolar damage (DAD) is divided into 1) the exudative phase with a duration of ~1 wk, and subsequently 2) the fibroproliferative phase lasting about 2 wk (1, 4, 103). The histopathological characteristics of the exudative phase are interstitial and intra-alveolar edema, intra-alveolar hemorrhage, neutrophil accumulation, and hyaline membranes. In the transmission electron microscope, capillary endothelial cells and alveolar epithelial type I (AE1) cells show swelling and necrosis, the intra-alveolar surfactant is shifted to morphological forms corresponding to inactive surfactant (74), and the intracellular surfactant pool is decreased (16, 52). As the chronic lesions of the fibroproliferative phase are morphologically distinct from that of the exudative phase and finally result in a fibrotic remodeling, they will be explicitly dealt with below. Taken together, investigators of the exudative phase of ALI will most likely be interested in the quantification of intra-alveolar and interstitial edema, damage of the air-blood barrier (ABB), and changes in intra-alveolar and intracellular surfactant.
**Quantitative parameters of ALI.** Previous work has demonstrated that the volume of edema fluid estimated by stereology correlates well with functional data such as blood/perfusate oxygenation and that intra-alveolar edema becomes functionally relevant when the volume fraction of edema in the lung exceeds 3% (20, 23). In addition, the quantitative microscopic analysis allows the assessment of edema volumes in different compartments, intra-alveolar, septal, and peribronchovascular (64), and is therefore superior to the global parameter wet-to-dry ratio (20). The damage of the ABB (consisting of AE1 cells, capillary endothelium, and interstitium in between) can be evaluated by estimating the surface area fraction of normal, swollen, and fragmented epithelium/endothelium or by estimating the arithmetic mean barrier thickness of the ABB and its constituents (23, 65, 66). Interestingly, there is no clear regional correlation between the occurrence of a strong damage of the ABB and the formation of edema (23). The changes of the intra-alveolar surfactant can be described by the volume of the different intra-alveolar subtypes that can be distinguished based on their ultrastructure (tubular myelin, lamellar-body-like, unilamellar vesicle, multilamellar vesicle); the intracellular surfactant can be characterized by the volume, size, and number of the lamellar bodies in AE2 cells (52, 67). It has been shown that the volume fraction of inactivated intra-alveolar surfactant correlates inversely with the perfusate oxygenation and that the volume of tubular myelin is related to the functional preservation of the lung in an extracorporeal ischemia-reperfusion rat lung model (19, 67, 75). Furthermore, the inactivation of the intra-alveolar surfactant does not seem to be a consequence of intra-alveolar edema but rather the cause of edema formation (74). In line with Ochs (72), Table 1 provides an overview of recommended stereological parameters to estimate these changes. Depending on the underlying hypothesis or question, we recommend a hierarchical procedure starting with evaluation of edema in the various compartments by light microscopy (LM), followed by estimation of the damage of the cellular components of the blood-air barrier/alveolar septa by transmission electron microscopy (EM). The EM analysis of the surfactant system will most likely be limited to special scientific questions.

**Example.** In the following worked example (Table 2, Figs. 1 and 2), the sequence of stereological estimations in the setting of ALI is taken from an extracorporeal ischemia/reperfusion model of the rat lung (66). At the end of the experiments, the left lung was fixed by vascular perfusion (a prerequisite for analysis of the intra-alveolar compartment, including intra-alveolar edema and surfactant). The volume was measured by Archimedes’ principle (87), i.e., the lung is completely sub-

**Table 1. Recommended stereological parameters in the exudative phase of ALI**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Primary Parameter (recommended notation)</th>
<th>Total Value (recommended notation)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-alveolar edema</td>
<td>Volume density of intra-alveolar edema per parenchyma ( (V_{(ed/par)}(V)) )</td>
<td>Volume of intra-alveolar edema in the lung ( (V_{(ed/lung)}) )</td>
<td>Point counting, LM</td>
</tr>
<tr>
<td>Septal edema</td>
<td>Volume density of alveolar septa per parenchyma ( (V_{(sept/par)}(V)) )</td>
<td>Volume of alveolar septa in the lung ( (V_{(sept/lung)}) )</td>
<td>Point counting, LM</td>
</tr>
<tr>
<td>Peribronchovascular edema</td>
<td>Volume density of the peribronchovascular compartment per nonparenchyma ( (V_{(pbv/nonpar)}(V)) )</td>
<td>Volume of the peribronchovascular compartment in the lung ( (V_{(pbv/lung)}) )</td>
<td>Point counting, LM</td>
</tr>
<tr>
<td>Thickness of air-blood barrier</td>
<td>N/A</td>
<td>Arithmetic mean thickness of epithelial, interstitial and endothelial</td>
<td>Point and intersection counting, EM</td>
</tr>
<tr>
<td>Damage of air-blood barrier</td>
<td>Surface density of normal, swollen, or fragmented parts of the air-blood barrier in relation to its total surface area ( (S_{(normal/ABB)}, S_{(swollen/ABB)}, S_{(fragmented/ABB)}) )</td>
<td>Total volume of intra-alveolar surfactant compartments in the lung ( (V_{(alvsurf/lung)}, V_{(tm/lung)}, V_{(mlv/lung)}, V_{(ulv/lung)}) )</td>
<td>Intersection counting, EM</td>
</tr>
<tr>
<td>Intra-alveolar surfactant subgroups</td>
<td>Volume density of intra-alveolar surfactant, lamellar-body-like structures, tubular myelin, unilamellar vesicles, unilamellar vesicles per parenchyma, or alveolar surfactant, respectively ( (V_{(alvsurf/par)}, V_{(tm/algsurf)}, V_{(mlv/algsurf)}, V_{(ulv/algsurf)}) )</td>
<td>Volume of lamellar bodies per AE2 cell ( (V_{(lb/ae2)}(V)) ) in the lung ( (V_{(lb/lung)}) )</td>
<td>Point counting, EM</td>
</tr>
<tr>
<td>AE2 cells and intracellular surfactant (basic)</td>
<td>Numerical density of AE2 cells per parenchyma ( (N_{(ae2/par)}(N)) )</td>
<td>Number of AE2 cells in the lung ( (N_{(ae2/lung)}) )</td>
<td>Disector, LM</td>
</tr>
<tr>
<td></td>
<td>Volume of individual AE2 cells sampled by disector ( (N_{(ae2)}(V)) )</td>
<td>Number-weighted mean volume of AE2 cells per lung ( (V_{(lb/ae2)}(V)) ) in the lung ( (V_{(lb/lung)}) )</td>
<td>Disector, rotator, LM</td>
</tr>
<tr>
<td>AE2 cells and intracellular surfactant (extended)</td>
<td>Volume density of lamellar bodies per AE2 cell ( (V_{(lb/ae2)}(V)) ) or per lung ( (V_{(lb/lung)}) )</td>
<td>Volume of lamellar bodies per AE2 cell ( (V_{(lb/ae2)}(V)) ) or in the lung ( (V_{(lb/lung)}) )</td>
<td>Point counting, EM</td>
</tr>
<tr>
<td></td>
<td>Numerical density of lamellar bodies per AE2 cell ( (N_{(lb/ae2)}(N)) )</td>
<td>Number of lamellae per AE2 cell ( (N_{(lb/ae2)}(N)) ) or lung ( (N_{(lb/lung)}(N)) )</td>
<td>Disector, EM</td>
</tr>
<tr>
<td></td>
<td>Volume density divided by numerical density of lamellar bodies ( (V_{(lb)}(V)) )</td>
<td>Number-weighted mean volume of lamellar bodies ( (V_{(lb)}(V)) )</td>
<td>Point counting, EM</td>
</tr>
<tr>
<td></td>
<td>Intercept length of lamellar bodies sampled by points ( (l_{i}) )</td>
<td>Volume-weighted mean volume of lamellar bodies ( (V_{(lb)}(V)) )</td>
<td>Point sampled intercepts, EM</td>
</tr>
</tbody>
</table>

ALI, acute lung injury; LM, light microscopy; EM, electron microscopy.
Table 2. Worked example for stereological parameters in the exudative phase of ALI illustrated with an extracorporeal rat ischemia/reperfusion injury model

<table>
<thead>
<tr>
<th>Structure</th>
<th>Counts and Test System Information for Injured Lung (control)</th>
<th>Calculation/Result for Injured Lung (control)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung volume (V(lung))</td>
<td></td>
<td>7950 mm³ (4540 mm³)</td>
<td>Reference volume Data are needed for further calculation</td>
</tr>
<tr>
<td>Volume of parenchyma and nonparenchyma (V_v(par/lung), V_v(nonpar/lung))</td>
<td>( \Sigma P(par) = 894 ) (936)</td>
<td>V_v(par/lung) = ( \Sigma P(par)/\Sigma P(lung) ) = 0.9058 (0.8595)</td>
<td></td>
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<tr>
<td></td>
<td>( \Sigma P(nonpar) = 93 ) (153)</td>
<td>V_v(nonpar/lung) = ( \Sigma P(nonpar)/\Sigma P(lung) ) = 0.0942 (0.1405)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(lung) = 894 + 93 = 987 ) (1089)</td>
<td>V_v(par/lung) * V_v(lung) = 7201 mm³ (3902 mm³) V_v(nonpar/lung) * V_v(lung) = 749 mm³ (638 mm³)</td>
<td></td>
</tr>
<tr>
<td>Intra-alveolar and septal edema ( (V_v(ed/par), V_v(sept/par), V_v(sept/par)) )</td>
<td>( \Sigma P(ed) = 77 ) (0)</td>
<td>V_v(ed/par) = ( \Sigma P(ed)/\Sigma P(par) ) = 0.0631 (0.00)</td>
<td>Intra-alveolar edema is present in injured but not control lung; the higher total volume of septal tissue in the injured lung may be due to injury or different lung size</td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(sept) = 141 ) (123)</td>
<td>V_v(sept/par) = ( \Sigma P(sept)/\Sigma P(par) ) = 0.1155 (0.1054)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(par) = 1221 ) (1167)</td>
<td>V_v(ed/lung) = ( \Sigma P(par)/\Sigma P(par) ) * V_v(par/lung) * V_v(lung) = 454 mm³ (0.00 mm³) V_v(sept/par) * V_v(par/lung) = 832 mm³ (411 mm³)</td>
<td></td>
</tr>
<tr>
<td>Peribronchovascular edema ( (V_v(pbv/nonpar), V_v(pbv/lung)) )</td>
<td>( \Sigma P(pbv) = 141 ) (152)</td>
<td>V_v(pbv/nonpar) = ( \Sigma P(pbv)/\Sigma P(par) ) = 0.4448 (0.4967)</td>
<td>The peribronchovascular space was not different between control and injured lung</td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(nonpar) = 317 ) (306)</td>
<td>V_v(pbv/lung) = V_v(pbv/nonpar) * V_v(nonpar/lung) * V_v(lung) = 333 mm³ (317 mm³)</td>
<td></td>
</tr>
<tr>
<td>Thickness of the air-blood barrier ( (without\ partitioning\ of\ its\ components) )</td>
<td>( \Sigma P(ABB) = 330 ) (412) ( \Sigma I(ABB) = 62 ) (74)</td>
<td>( \tau(ABB) = (I_v/2<em>P)/(2</em>I) ) = 2.66 µm (2.78 µm)</td>
<td>The thickness of the air-blood barrier was not different between control and injured lung</td>
</tr>
<tr>
<td>Damage of air-blood barrier ( (S_s(normal/ABB), S_s(swollen/ABB), S_s(fragmented/ABB)) )</td>
<td>( \Sigma I(normal) = 207 ) (793)</td>
<td>S_s(normal/ABB) = ( \Sigma I(normal)/\Sigma I(ABB) ) = 0.1568 (0.7759)</td>
<td>Injured lung contains a higher fraction of swollen and fragmented parts of blood-air barrier surface area</td>
</tr>
<tr>
<td></td>
<td>( \Sigma I(swollen) = 894 ) (229)</td>
<td>S_s(swollen/ABB) = ( \Sigma I(swollen)/\Sigma I(ABB) ) = 0.6773 (0.2241)</td>
<td></td>
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<tr>
<td></td>
<td>( \Sigma I(fragmented) = 219 ) (0)</td>
<td>S_s(fragmented/ABB) = ( \Sigma I(fragmented)/\Sigma I(ABB) ) = 0.1659 (0.000)</td>
<td></td>
</tr>
<tr>
<td>Intra-alveolar surfactant subgroups ( (V_v(alvsurf/par), V_v(bl/lalvsurf), V_v(tm/alvsurf), V_v(mlv/alvsurf), V_v(alvsurf/lung), V_v(bl/lalvsurf), V_v(tm/lung), V_v(mlv/lung), V_v(ulv/lung)) )</td>
<td>( \Sigma P(bl) = 13 ) (21)</td>
<td>V_v(alvsurf/par) = ( \Sigma P(alvsurf)/\Sigma P(par) ) = 0.0004 (0.0025)</td>
<td>Inactive surfactant forms (unilamellar vesicles) are increased and active forms (lamellar-body like, tubular myelin) are decreased in injured lung compared with control lung</td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(tm) = 15 ) (113)</td>
<td>V_v(blalvsurf) = ( \Sigma P(bl)/2*P(alvsurf) ) = 0.0243 (0.0440)</td>
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<tr>
<td></td>
<td>( \Sigma P(mlv) = 144 ) (214)</td>
<td>and accordingly for ( V_v(tm/alvsurf) = 0.0280 (0.2369), V_v(mlv/alvsurf) = 0.2692 (0.4486), V_v(ulv/alvsurf) = 0.6785 (0.2704)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(ulv) = 363 ) (129)</td>
<td>V_v(alvsurf/lung) = V_v(alvsurf/par) * V_v(par/lung) * V_v(lung) = 2.880 mm³ (9.756 mm³)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(alvsurf) = 535 ) (477)</td>
<td>V_v(bl/lung) = V_v(bl/lalvsurf) * V_v(alvsurf/par) * V_v(par/lung) * V_v(lung) = 0.080 mm³ (2.311 mm³), V_v(mlv/lung) = 0.7754 mm³ (4.377 mm³), V_v(ulv/lung) = 1.954 mm³ (2.638 mm³)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \Sigma P(par) = 1337500 ) (190800)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued
The volume fraction of lamellar bodies within AE2 cells as well as the volume fractions of the different intra-alveolar surfactant subtypes are estimated by point counting.

As illustrated by Table 2, the general estimation procedure and calculation of these parameters is rather simple; however, a few practical issues may be mentioned here. The data in Table 2 frequently relate a compartment with a small volume fraction to a comparably large reference volume. Thus, to achieve a sufficient degree of precision, a total of 100–200 points (or counting events in general) per compartment and animal should be counted from five to six sections. In the case of parenchyma/nonparenchyma, this means that, for 100 points hitting nonparenchyma, about 900 points have to be counted on parenchyma. This workload can be reduced by using an integral point grid with points of different order; here, a grid with 24 normal (fine lattice) and four encircled points (coarse lattice) was used. It is highly recommended to design the test systems according to the biological needs because this greatly increases the efficiency. Another issue that needs to be mentioned here is that surface area estimations generally rely on the use of isotropic uniform random sections. In case of the pulmonary parenchyma, it seems acceptable to assume tissue isotropy, so we abstained from randomizing the orientation of the samples.

**Diffuse Parenchymal Lung Diseases/Pulmonary Fibrosis**

Pathobiology of diffuse parenchymal lung diseases/pulmonary fibrosis. Diffuse parenchymal lung diseases (DPLDs) are a highly heterogeneous group of diseases that lead to fibrosis of the alveolar interstitium, thereby decreasing lung compliance and gas exchange. Among the large group of underlying diseases, three basic etiologies are accepted (96): 1) exogenous lung exposure by inhalation, radiation, or drugs; 2) part of systemic disease; and 3) idiopathic. Among the idiopathic DPLDs, the term idiopathic pulmonary fibrosis (IPF) nowadays should be exclusively used for a “specific form of fibrosing interstitial pneumonia referred to as usual interstitial pneumonia” (26), as this disease differs from other forms of interstitial pneumonias in histopathology, prognosis, and re-

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**Table 2.—Continued**

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>AE2 cells and intracellular surfactant (basic)</td>
<td>n(dis) = 436 (795)</td>
<td>Nv(AE2/par) = 2.75*10^-7 * Nv(AE2) * h(dis)</td>
<td>The size and lamellar body content of the AE2 cells were similar in both lungs, the number of AE2 cells and the total volume of lamellar bodies was higher in the injured lung, probably due to an increased overall lung volume.</td>
</tr>
<tr>
<td>(Nv(AE2/par), Sv(AE2), Vv(lb/AE2), Vv(lb/lung), Nv(AE2,lung), V(lb,AE2), V(lb,lung))</td>
<td>ΣQ’(AE2) = 79 (118)</td>
<td>Vv(lb/AE2) = 0.0994 (0.1123) * V(lb,AE2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>h(dis) = 3 μm, at(CF) = 1098.8 μm²</td>
<td>Vv(lb/AE2) = 18.14 μm³ (19.87 μm³) * Sv(AE2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΣV(AE2) = 14416.71 μm³ (20874.23 μm³)</td>
<td>Vv(lb/AE2) = 18.14 μm³ (19.87 μm³) * Sv(AE2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΣP(lb) = 124 (134)</td>
<td>Vv(lb,AE2) = 18.14 μm³ (19.87 μm³) * Sv(AE2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ΣP(AE2) = 1247 (1193)</td>
<td>Vv(lb,AE2) = 18.14 μm³ (19.87 μm³) * Sv(AE2)</td>
<td></td>
</tr>
</tbody>
</table>

ΣP, sum of points hitting a structure of interest; ΣI, sum of intersections of test lines with a structure of interest; l; length of individual test line segment; n(dis), number of dissectors used for counting; h(dis), disector height; ΣV(AE2), sum of AE2 cell volumes estimated by rotator. For further details refer to Table 1.

merged in a beaker glass filled with water, placed on a balance, and the weight change of the beaker glass with and without the lung is divided by the density of water to provide the volume of the lung. Note that the lung must not lie on the bottom, touch the walls of the glass or float at the top of the water. Devices that fulfill these criteria can easily be made from inexpensive materials (22, 69). A detailed discussion of the advantages and disadvantages of this method is provided by Hsia et al. (37). The lung is then submerged in 4–5% fluid agar. When the agar has hardened, the lung is cut from apex to base into slabs of approximately equal thickness using a tissue slicer. The resulting slices are placed into a row with the same side up, which means that either the first or the last slab has a natural surface. At this point, as an alternative to Archimedes’ principle, the lung volume can also be estimated using the Cavalieri estimator (115). The integration of the Cavalieri estimator into the sampling process can either be performed by simultaneous point counting or by taking a digital photo of the slabs and later estimation. In general, the Cavalieri estimator is considered to possess various advantages over Archimedes’ principle (37). The slices are further assigned to LM or EM by systematic uniform random sampling. After embedding in suitable embedding media [in this case, glycol methacrylate (GMA) for LM and epoxy resin (EP) for EM], sections are generated from the tissue blocks, namely 1-μm-thick sections from the GMA-embedded samples, disector pairs (e.g., the first and the fourth of a consecutive row of 1-μm semithin sections, thus resulting in a disector height of 3 μm), and ultrathin sections from the EP samples. At a low magnification (e.g., ×5–10), the volume density of parenchyma and nonparenchyma is determined using point counting. For estimation of intra-alveolar, septal, and peribronchovascular edema, point counting is performed at a higher magnification, e.g., ×20–40. Using the disector sections from the EP blocks, the numerical density of AE2 cells is determined using oil immersion at ×63–100. In the same working step, the number-weighted mean volume of AE2 cells can be determined by the rotator or the nuclearator (28, 108). At the EM level (~×20,000 for all measurements), the arithmetic mean thickness and the degree of damage of the ABB is determined by counting of intersections of test lines with the ABB. The volume fraction of lamellar bodies within AE2 cells as well as the volume fractions of the different intra-alveolar surfactant subtypes are estimated by point counting.

As illustrated by Table 2, the general estimation procedure and calculation of these parameters is rather simple; however, a few practical issues may be mentioned here. The data in Table 2 frequently relate a compartment with a small volume fraction to a comparably large reference volume. Thus, to achieve a sufficient degree of precision, a total of 100–200 points (or counting events in general) per compartment and animal should be counted from five to six sections. In the case of parenchyma/nonparenchyma, this means that, for 100 points hitting nonparenchyma, about 900 points have to be counted on parenchyma. This workload can be reduced by using an integral point grid with points of different order; here, a grid with 24 normal (fine lattice) and four encircled points (coarse lattice) was used. It is highly recommended to design the test systems according to the biological needs because this greatly increases the efficiency. Another issue that needs to be mentioned here is that surface area estimations generally rely on the use of isotropic uniform random sections. In case of the pulmonary parenchyma, it seems acceptable to assume tissue isotropy, so we abstained from randomizing the orientation of the samples.
response to treatment. Pathological hallmarks of this disease include first the adjacent occurrence of peripheral fibrotic zones with scarring and honeycombing next to relatively unaffected areas and second the presence of fibroblastic foci and collagen deposition. As these lesions represent different stages of lung remodeling, the spatial and temporal heterogeneity is a diagnostic criterion (46). Other forms of DPLDs feature a more pronounced inflammatory state and a more uniform distribution of pathological alterations. Among the pathophysiological hypotheses of IPF, the old paradigm of chronic inflammation has been replaced by the conception that repeated stimuli cause sequential lung injury and that defective wound healing causes chronic injury (26). Recent work has emphasized a key role of AE2 cell apoptosis in the pathogenesis of IPF (31, 53, 94), thus paving the way for mesenchymal cells either by epithelial-mesenchymal transition (48), loss of control over this cell compartment (114), or by attracting circulating fibrocytes (79). Given the role of AE2 cells in surfactant metabolism, it is not surprising that surfactant changes are also part of IPF and experimental pulmonary fibrosis (30, 35). The presently available mouse models of IPF only represent parts of the pathological features of human IPF (62) although new developments may come closer than previous ones (13, 90). The single-dose bleomycin model, for example, rather represents the fibroproliferative phase of ALI (see above). During this phase, AE2 cells proliferate but contain abnormally large and shaped lamellar bodies (84), and alveolar septa are thickened by the proliferation of fibroblasts and myofibroblasts which, however, also migrate into the alveolar lumen. The fibroblasts start to reorganize the exudate and the alveolar septum with increasing

![Fig. 1. Demonstration of quantitative assessment of light microscopic parameters in acute lung injury. A: estimation of parenchymal (P) and nonparenchymal (NP) volume using a coarse lattice (encircled points) for the reference volume and a fine lattice (all points) for nonparenchyma. The points of the coarse lattice are enhanced by a 3/4 circular arch. The exact test point is the intersection of the line edges in the open (upper right) quadrant of the circle. This counting rule avoids uncertainties due to the thickness of the test lines. 3 out of 24 points of the fine lattice are hitting nonparenchymal structures; all points of the coarse lattice are hitting lung tissue/reference space. Hence, the volume density of nonparenchyma is 3/24 = 0.125 and of parenchyma 1–0.125 = 0.875, respectively. Note that the number of encircled points hitting the reference space has to be multiplied by 6 to match coarse and fine lattice. B: estimation of intra-alveolar (E) and septal (S) edema and alveolar (A) luminal volume related to parenchyma. Points hitting nonparenchyma are not counted (NC). C: estimation of peribronchovascular space (PBV) and bronchovascular lumen (L) volume related to nonparenchyma. Points hitting parenchyma are not counted (NC). D: estimation of the number-weighted mean volume of AE2 cells. An AE2 cell sampled by the disector (see E and F) is subjected to a volume estimation using the rotator. Using computer-assisted tools, the center of the cell (nucleolus) is marked as well as several intersections of computer-generated lines with the cell border. E and F: estimation of AE2 cell number using the disector. 2 semithin sections (of known thickness), a known distance apart, are used as a physical disector. An unbiased counting frame with known area is used for counting. If a certain cellular feature, here the nucleolus (arrows), is present in one of the 2 counting frames but not in the other, it is counted. Note that the feature of interest must not touch the solid exclusion line or its extensions but may touch the dashed inclusion line. Here, both AE2 cells fulfill the criteria of a counting event. D–F have the same magnification.](http://ajplung.physiology.org/doi/10.1152/ajplung.00427.2012)
amounts of extracellular matrix (ECM). Collapsed alveoli thus become sealed with epithelial cells directly appositioned to each other, so-called collapse induration (9, 10, 45, 102). Within the collapsed alveolar walls, abundant basal lamina material without endothelial or epithelial cell contact is present. Finally, the lung can be completely remodeled with only limited numbers of cells contained between collagen tissue.

Quantitative parameters of DPLDs/pulmonary fibrosis. Given the diversity of DPLDs and the resulting pulmonary fibrosis, in line with Knudsen and Ochs (50), we propose a number of basic stereological parameters as useful determinants (Table 3). First of all, the volume of nonfunctional parenchyma (collapsed or already remodeled) can be estimated and related to the volume of ventilated parenchyma. Quantification of the luminal volume of blood vessels within the collapsed areas may provide information on the shunt volume. Within the ventilated parenchyma, the thickness and surface area of the septa should provide a first impression of the degree of fibrosis and the functional impairment. Using EM, the volume of various septal compartments (collagen, ECM, fibroblasts, etc.) can be estimated. Within the collapsed areas, the surface area of the basal lamina without cell contact may provide an estimate of the perished gas exchange area. The number of various cell types (AE2 cells, fibroblasts, inflammatory cells), the volume of AE2 cells, and their subcellular compartments give important pathophysiological information. Finally, additional parameters depending on the disease or disease model being used may be useful, such as the volume of honeycomb zones or the number of fibroblastic foci, as the latter are correlated with the prognosis and survival in IPF (49) (Table 3).

Example. A rat lung treated with bleomycin to induce pulmonary fibrosis was investigated using stereology (Table 4). After lung volume measurement by Archimedes’ principle and systematic uniform random sampling, lung samples were embedded in GMA, and thin sections (1.5 µm) were cut for light microscopy. First, the volume fraction of functional and nonfunctional (collapsed) parts of the air-blood barrier to the total surface area of the air-blood barrier. Intersections of the line segments with the epithelial surface (arrows) are counted and assigned to 1 of the 3 categories. At high magnification, it may not always be easy to distinguish between capillary (cap) and alveolar (alv) lumen, particularly if red blood cells (B) or edema fluid (C) have entered the alveolar lumen. D and E: estimation of subtypes of intra-alveolar surfactant. Points hitting lamellar-body-like structures (lbl), tubular myelin (tm), unilamellar vesicles (ulv), or multilamellar vesicles (mlv) are counted and related to the surfactant as reference volume. F: estimation of lamellar body volume. Points hitting lamellar bodies (lb) and points hitting other compartments of AE2 cells (AE2) are counted and used to calculate the volume fraction of lb in relation to the AE2 cell. Here, the volume fraction is 2/10 = 20%. In combination with number and cell volume estimation (see Fig. 1), the total lamellar body volume can be calculated per cell and per lung (see Table 2). All panels were taken at the same magnification.

Fig. 2. Demonstration of quantitative assessment of electron microscopic parameters in acute lung injury. A–C: estimation of the contribution of normal (A), swollen (B), and fragmented (C) parts of the air-blood barrier to the total surface area of the air-blood barrier. Intersections of the line segments with the epithelial surface (arrows) are counted and assigned to 1 of the 3 categories. At high magnification, it may not always be easy to distinguish between capillary (cap) and alveolar (alv) lumen, particularly if red blood cells (B) or edema fluid (C) have entered the alveolar lumen. D and E: estimation of subtypes of intra-alveolar surfactant. Points hitting lamellar-body-like structures (lbl), tubular myelin (tm), unilamellar vesicles (ulv), or multilamellar vesicles (mlv) are counted and related to the surfactant as reference volume. F: estimation of lamellar body volume. Points hitting lamellar bodies (lb) and points hitting other compartments of AE2 cells (AE2) are counted and used to calculate the volume fraction of lb in relation to the AE2 cell. Here, the volume fraction is 2/10 = 20%. In combination with number and cell volume estimation (see Fig. 1), the total lamellar body volume can be calculated per cell and per lung (see Table 2). All panels were taken at the same magnification.

Review

L210 STEREOLOGICAL PARAMETERS IN VARIOUS LUNG DISEASES

Emphysema

Pathobiology and current use of morphometry in emphysema. As defined by the American Thoracic Society, pulmonary D

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B

C

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emphysema is "the abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls" (2), with cigarette smoking being the most important pathogenetic factor. Although this clearly states that two pathological lesions (airspace enlargement and destruction of alveolar septa) define this disease, it has become a common "scientific" practice to address only airspace enlargement in experimental studies of emphysema (21). The reason for this is that mean linear intercept and mean linear chord length are stereological parameters that are particularly easy and quick to determine. Unfortunately, both parameters can be influenced by various factors (e.g., inflation pressure during fixation, tissue shrinkage) that will not be recognized if they are not extremely closely controlled (51, 110). In other words, the most widely used morphometric parameter to assess pulmonary emphysema quantitatively can be easily misinter-

Table 3. Recommended stereological parameters in DPLD

<table>
<thead>
<tr>
<th>Structure</th>
<th>Primary Parameter (recommended notation)</th>
<th>Total Value (recommended notation)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collapsed alveoli</td>
<td>Volume density of functional and nonfunctional parenchyma per lung (V_funct-par/lung), V_funct-par/lung, V_nonfunct-par/lung</td>
<td>Volume of functional and nonfunctional parenchyma in the lung (V_funct-par/lung), V_nonfunct-par/lung</td>
<td>Point counting, LM</td>
</tr>
<tr>
<td>Functional gas-exchange area</td>
<td>Surface density of functional alveolar septa per parenchyma (S_sept/funct-par))</td>
<td>Surface of functional alveolar septa in the lung (S_funct-lung))</td>
<td>Intersection counting, LM</td>
</tr>
<tr>
<td>Septal thickening</td>
<td>N/A</td>
<td>Arithmetic mean thickness of alveolar septa (T_sept))</td>
<td>Point and intersection counting, LM</td>
</tr>
<tr>
<td>AE2 cell hyperplasia</td>
<td>Numerical density of AE2 cells per parenchyma (N(AE2/par))</td>
<td>Number of AE2 cells in the lung (N(AE2, lung))</td>
<td>Disector, LM</td>
</tr>
<tr>
<td>Fibrosis (fibroblasts/myofibroblasts)</td>
<td>Numerical density of fibroblasts/myofibroblasts in the lung (N(fibro,lung))</td>
<td>Number of fibroblasts/myofibroblasts in the lung (N(fibro,lung))</td>
<td>Disector, rotator, LM</td>
</tr>
<tr>
<td>Fibrosis (fibroblast foci)</td>
<td>Numerical density of fibroblast foci per parenchyma (N(fibrofoci/par), V(fibrofoci/par))</td>
<td>Number of fibroblast foci in the lung (N(fibrofoci,lung))</td>
<td>Disector, LM, Point counting, LM</td>
</tr>
<tr>
<td>Fibrosis (alveolar septa)</td>
<td>Volume density of collagen, ECM, and fibroblasts related to alveolar septa (V(collagen,sept), V(ECM,sept), V(fibro,sept))</td>
<td>Volume of alveolar septal collagen, ECM, and fibroblasts in the lung (V(collagen,lung), V(ECM,lung), V(fibro,lung))</td>
<td>Point counting, EM</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Numerical density of apoptotic cells per parenchyma (N(apopt,par))</td>
<td>Number of apoptotic cells in the lung (N(apopt,lung))</td>
<td>Disector, LM, Immunostaining</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Numerical density of inflammatory cells per parenchyma (N(inflam,par))</td>
<td>Number of inflammatory cells in the lung (N(inflam,lung))</td>
<td>Disector, LM, Immunostaining</td>
</tr>
</tbody>
</table>

DPLD, diffuse parenchymal lung diseases; ECM, extracellular matrix.

Table 4. Worked example for some stereological parameters in DPLD illustrated with a rat model of bleomycin-induced pulmonary fibrosis

<table>
<thead>
<tr>
<th>Structure</th>
<th>Counts and Test System Information for Fibrotic Lung (control lung)</th>
<th>Calculation/Result for Fibrotic Lung (control lung)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung volume (V(lung))</td>
<td></td>
<td></td>
<td>Reference volume</td>
</tr>
<tr>
<td>Volume of nonparenchyma, functional and nonfunctional parenchyma (V_nonpar/lung), V_funct-par/lung, V_nonfunct-par/lung</td>
<td>( \Sigma P(nonpar) = 147 ) (124)</td>
<td>( V_{nonpar}(lung) = \Sigma P(nonpar)/\Sigma P(lung) = 0.1238 ) (0.089) and accordingly ( V_{funct-par}(lung) = 0.7355 ) (0.911) and ( V_{nonfunct-par}(lung) = 0.1407 ) (0.0)</td>
<td>Nonfunctional parenchyma is increased in fibrotic compared with control lung</td>
</tr>
<tr>
<td>Volume of alveolar septa (S(sept))</td>
<td>( \Sigma I(sept) = 239 ) (435)</td>
<td>( S_{sept}(function) = 2*\Sigma I(sept)/\Sigma P(par)) (415.7 cm(^{-1}))</td>
<td>Surface area of functional alveolar septa is decreased in fibrotic lung</td>
</tr>
<tr>
<td>Thickness of alveolar septa (T(sept))</td>
<td>( \Sigma P(sept) = 197 ) (201)</td>
<td>( V_{sept}(function) = \Sigma P(sept)/\Sigma P(par) = 0.2998 ) (0.1957)</td>
<td>Thickness of alveolar septa is increased in fibrotic lung</td>
</tr>
</tbody>
</table>

\( \Sigma P \), sum of points hitting a structure of interest; \( \Sigma I \), sum of intersections of test lines with a structure of interest; \( l(p) \), length of test line per line point. For further details refer to Table 3.
interpreted, and we recommend not to use it as a single parameter for this purpose. Alternative methods have been suggested (78) and discussed by leading experts in the field (109). One of the main problems identified in this discussion was that 2D images are used to extract information on the 3D size of the airspaces without considering the higher probability of large structures to be represented in a 2D section than smaller structures. In addition, the loss of elastic recoil as a pathogenic factor in the development of airspace enlargement adds further challenge to the estimation of airspace size. At the same inflation pressure during instillation fixation, the airspaces of a lung with reduced elastic recoil will naturally be more expanded than those of a control lung, which should also result in a thinning of the stretched alveolar septa. Changes in the elastic properties of the lung may therefore result in different airspace sizes of fixed tissue independent of the presence of emphysema. Due to these restrictions in the interpretation of data on airspace size, we strongly recommend that quantitative studies on pulmonary emphysema include both information on airspace enlargement and destruction of alveolar walls.

**Quantitative parameters of emphysema.** The destruction of alveolar walls may be assessed by a set of basic parameters such as total volume and surface area of alveolar septa (55, 106, 111) and should be accompanied by information on the septal thickness, which may directly be derived from volume and surface area (89), as the mean thickness is directly related to the volume-to-surface ratio. Additionally, a disector-based approach allows the unbiased estimation of the number of alveoli based on the connectivity of the alveolar elastic entrance rings (38, 76). As recently demonstrated, technical innovations have opened the possibility to estimate the number of alveoli on image stacks obtained by microcomputed tomography (107). More laborious stereological methods like the selector (12) have also been used to assess alveolar number (59). Remarkably, not all of the parameters are necessarily altered to the same extent as observed by Massaro and Massaro (59), where the number of alveoli was decreased, whereas the surface area was not. Other studies have shown both decreased alveolar surface area and alveolar number in elastase or genetic models of pulmonary emphysema (43, 77, 116). Also, studies on smoking-induced emphysema are available that have detected a loss of alveolar surface area not (11, 58). As such, a combination of the above mentioned parameters appears to be the safest approach to decipher destruction of alveolar walls (21).

Two parameters of airway enlargement can easily be obtained if the total volume of the alveoli is determined in addition. Dividing the total volume of alveoli by their total number provides an unbiased estimate of the number-weighted mean volume of alveoli. The number-weighted mean volume does not, however, contain information on the size heterogeneity, which is definitely present in emphysema. Therefore, this parameter should be combined with the volume-weighted mean volume of alveoli, and the size variation can be extracted because the relationship between number- and volume-weighted volume is $\bar{V}_N(alv) = \bar{V}_V(alv) \times (1 + CV_v^2(v))$, where $CV(v)$ is the coefficient of variation of the volume. Still, it should be kept in mind that any information on airway enlargement is prone to the confounding influence of the degree of inflation (Table 5).

**Example.** A genetic model of emphysema-like pathology is used (Table 6, Fig. 3), namely the lung of a surfactant protein D-deficient mouse. After instillation fixation, lung volume estimation and systematic uniform random sampling for light microscopic analysis were performed the same way as described in the ALI example. Again, at a low magnification ($\times 5$), the volume of parenchyma and nonparenchyma was estimated to use the parenchymal volume as the reference volume for the subsequent estimations. Estimations of the volume and surface area of alveolar septa were performed at higher magnification ($\times 20$) and used to calculate the arithmetic mean thickness of alveolar septa. Volume and number of alveoli were estimated using point counting and the disector (first and fourth section of a consecutive row of sections of 1-μm thickness), respectively, and further used to calculate the number-weighted mean volume of alveoli. In addition, the point-sampled intercepts method was used to estimate the volume-weighted mean volume of alveoli.

**PH**

**Pathobiology of PH.** PH is a disorder with elevated mean pulmonary artery pressure (>25 mmHg) and consecutive pressure-induced right ventricular hypertrophy and failure (112). According to the Dana point classification, five major different types of PH can be distinguished (101), which have different pathophysiology and pathology, namely pulmonary arterial hypertension (PAH), PH associated with left heart disease, with interstitial/hypoxic lung disease or emphysema, PH due to

### Table 5. Recommended stereological parameters in emphysema

<table>
<thead>
<tr>
<th>Structure</th>
<th>Primary Parameter (recommended notation)</th>
<th>Total Value (recommended notation)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Destruction of alveolar walls (basic)</td>
<td>Volume and surface density of alveolar septa per parenchyma ($V_s(sept/par)$, $S_v(sept/par)$)</td>
<td>Volume and surface area of alveolar septa in the lung ($V(sept,lung)$, $S(sept,lung)$)</td>
<td>Point and intersection counting, LM</td>
</tr>
<tr>
<td>Destruction of alveolar walls (extended)</td>
<td>Numerical density of alveoli per parenchyma ($N_v(alv/par)$)</td>
<td>Arithmetic mean thickness of alveolar septa ($t(sept)$) derived from volume and surface area</td>
<td>Disector, Euler number estimation, LM</td>
</tr>
<tr>
<td>Alveolar enlargement (number-weighted)</td>
<td>Volume and numerical density of alveoli per parenchyma ($V_n(alv/par)$, $N_n(alv/par)$)</td>
<td>Number of alveoli in the lung ($N(alv,lung)$)</td>
<td>Point counting, disector, Euler number estimation, LM</td>
</tr>
<tr>
<td>Airway enlargement (volume-weighted) and heterogeneity</td>
<td>Intercept length of alveoli sampled by points ($l$)</td>
<td>Volume, number and number-weighted mean volume of alveoli in the lung ($V(alv,lung)$, $N(alv,lung)$, $t_0(alv)$)</td>
<td>Point sampled intercept method</td>
</tr>
</tbody>
</table>
### Table 6. Worked example for stereological parameters in emphysema illustrated with SP-D knockout mouse lung

<table>
<thead>
<tr>
<th>Structure</th>
<th>Counts and Test System Information for SP-D (/−−−− Lung (control lung))</th>
<th>Calculation/Result for SP-D (−−−−−− Lung (control lung))</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung volume (V(lung))</td>
<td>—</td>
<td>( V_{\text{v(par/lung)}} = \Sigma P_{\text{par}} = 988 (1122) )</td>
<td>( V_{\text{v(par/lung)}} = \Sigma P_{\text{par}}/\Sigma P_{\text{lung}} = 0.12 (0.084) )</td>
</tr>
<tr>
<td>Volume of nonparenchyma and parenchyma</td>
<td>( \Sigma P_{\text{par}} = 988 (1122) )</td>
<td>( V_{\text{v(par/lung)}} = \Sigma P_{\text{par}}/\Sigma P_{\text{lung}} = 0.12 (0.084) )</td>
<td>Nonparenchyma is increased and parenchyma is decreased in SP-D knockout lung.</td>
</tr>
<tr>
<td>Volume of alveoli ((V_{\text{alv}}))</td>
<td>( \Sigma P_{\text{par}} = 135 (103) )</td>
<td>( V_{\text{v(alv)}} = 780 \text{ mm}^3 (790 \text{ mm}^3) )</td>
<td>Volume and surface area of alveolar septa are decreased in SP-D knockout lung but the mean thickness of the septa is similar.</td>
</tr>
<tr>
<td>Volume, surface area and arithmetic mean thickness of alveolar septa ((V_{\text{alv}}, S_{\text{alv}}, A_{\text{alv}}))</td>
<td>( \Sigma P_{\text{par}} = 988 (1122) )</td>
<td>( V_{\text{v(alv)}} = 780 \text{ mm}^3 (790 \text{ mm}^3) )</td>
<td>Volume and surface area of alveolar septa are decreased in SP-D knockout lung but the mean thickness of the septa is similar.</td>
</tr>
<tr>
<td>Number of alveoli ((N_{\text{alv}}))</td>
<td>( \Sigma I = 2 (0) )</td>
<td>Euler number: ( \Delta X = \Sigma I - \Sigma B = -139 (-165) )</td>
<td>The number of alveoli is smaller in SP-D knockout than in control lung.</td>
</tr>
<tr>
<td>Volume-weighted mean volume of alveoli ((V_{\text{v(alv)}}))</td>
<td>( \Sigma P_{\text{par}} = 356 (257) )</td>
<td>( V_{\text{v(alv)}} = 0.824 (0.673) \text{ mm}^3 )</td>
<td>Total volume of alveoli is higher in SP-D knockout than in control lung.</td>
</tr>
<tr>
<td>Number-weighted mean volume of alveoli ((V_{\text{n(alv)}}))</td>
<td>—</td>
<td>( V_{\text{v(alv)}} = 0.824 (0.673) \text{ mm}^3 )</td>
<td>Total volume of alveoli is higher in SP-D knockout than in control lung.</td>
</tr>
</tbody>
</table>

\( \Sigma P \), sum of points hitting a structure of interest; \( \Sigma I \), sum of intersections of test lines with a structure of interest; \( \Sigma P_{\text{l}} \), sum of points of a line grid; \( l(p_l) \), length of test line per point of line grid; \( \Sigma B \), sum of bridges (islands) counted with the disector; \( a(p) \), area per point of counting frame; \( a(CF) \), area of counting frame; \( h(\text{dis}) \), disector height; \( \Sigma l^3(\text{alv}) \), sum of intercept lengths of alveoli raised to the power of 3. For further details refer to Table 5.

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chronic thromboembolism, and PH without a clear underlying cause or a multifactorial pathogenesis (112). Because of the diversity of the etiology, the pathology of the different forms of PH presents with a spectrum of lesions (plexiform, obliterator, concentric) more or less specific for the type of disease (104, 105). Animal models of the various forms of PH are mostly available but have to clarify which form they are thought to represent (25, 86, 97). For example, both the chronic hypoxia model as well as the monocrotaline model present with medial thickening but absent endothelial alterations, which are typical for the human phenotype (97). New therapeutic approaches to PH require morphometric tools for assessing the vascular remodeling quantitatively to allow proper statistical testing and correlation with functional parameters (95). The importance of gathering quantitative data is emphasized by theoretical considerations that at least 80% of the vascular luminal area has to be compromised in PH (104). In PAH, the focus is on the distal pulmonary arteries, which show medial/smooth muscle cell (SMC) hypertrophy with fibrotic and/or proliferative alterations of the intimal layer. Also, the adventitial layer is thickened and shows signs of inflammation (88). Although PH associated with high left heart filling pressures also affects the distal pulmonary arteries, the focus is more on the consequences of left heart failure here (extravasation of fluid and red blood cells, enlargement of veins, capillaries, and lymph vessels). In DPLDs or chronic hypoxia, rarefaction of arteries occurs, but distal arterial changes may be similar to the ones observed in PAH. In addition, increased amounts of pulmonary capillaries have been reported in animal models (36), but the matter of vascular gain/rarefaction is still controversially debated (32). Also, in chronic thromboembolism, similar changes as with PAH are present in distal arteries, but the occurrence of
thrombi and remodeling of larger pulmonary arteries is in the focus.

Quantitative parameters of PH. Here, we focus on the alterations of the distal pulmonary arteries and capillaries. To break down the pathological changes to quantities, which may be of relevance in PH studies, we can distinguish between a general change in the amount of the vascular compartment and changes of the vascular wall composition. We recommend to start with the changes in the overall content of the vessels to obtain an overview of the degree of the PH before resorting to the more time-consuming analysis of the wall composition. Suitable parameters to quantify the amount of capillaries include the volume and surface area, whereas length is not a suitable parameter, as shown in a recent study (68). Other

Table 7. Recommended stereological parameters in pulmonary hypertension

<table>
<thead>
<tr>
<th>Structure</th>
<th>Primary Parameter (recommended notation)</th>
<th>Total Value (recommended notation)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of alveolar capillaries</td>
<td>Volume and surface density of alveolar capillaries per parenchyma (V_V(cap/par), S_S(cap/par))</td>
<td>Volume and surface area of alveolar capillaries in the lung (V(cap,lung), S(cap,lung))</td>
<td>Point and intersection counting, LM (or EM for surface area)</td>
</tr>
<tr>
<td>Amount of precapillary arteries</td>
<td>Volume and length density of precapillary arteries per lung (V_V(cap/par), L_L(cap/par))</td>
<td>Volume and length of precapillary arteries in the lung (V(cap,lung), L(cap,lung))</td>
<td>Point and profile counting, LM, immunohistochemistry</td>
</tr>
<tr>
<td>Vascular wall remodeling (composition)</td>
<td>Volume densities of wall layers or components, e.g., of intimal layer per unit artery wall volume (V_V(intima/art))</td>
<td>Volume of wall layers of components, e.g., of intimal layer in the lung (V(intima,lung))</td>
<td>Point counting, LM or EM depending on the structures of interest</td>
</tr>
<tr>
<td>Vascular wall remodeling (cell types)</td>
<td>Numerical densities of specific cell types, e.g., of smooth muscle cells per unit artery wall volume (N_S(SMC/art))</td>
<td>Number of specific cell types, e.g., of smooth muscle cells in the lung (N(SMC,lung))</td>
<td>Disector, LM, immunohistochemistry</td>
</tr>
</tbody>
</table>

Note that the parameters may also be referred to as an internal reference structure, such as the basement membrane of the endothelium. SMC, smooth muscle cell.

Fig. 3. Demonstration of quantitative assessment of parameters in emphysema. A: combined estimation of alveolar luminal (A), ductal airspace (D), and septal (S) volume as well as alveolar septal surface area. The distinction between alveolar and ductal airspace is important when the mean alveolar volume is calculated from the volume and number of alveoli. For the latter, the number of intersections of the line segments with alveolar septa (asterisks) is counted and related to the parenchyma as the reference volume. B: point-sampled intercepts method. A point grid with 4 points (dashed line) is used to sample alveoli. Every time a point hits an alveolar space, the distance between the alveolar septa is measured (red line). Here, for each point, a random angle was chosen using a random number table. In the case of isotropic uniform random samples, this is not necessary. Measurements can be performed from “wall to wall”, thus comprising alveoli and alveolar ducts (volume-weighted mean volume of acinar airways). Alternatively, they can be restricted to alveoli proper by virtually closing them at their openings with a straight line (green line) (volume-weighted mean volume of alveoli). C and D: estimation of the Euler number. The connectivity of alveolar opening rings is estimated in an unbiased counting frame. If an open alveolus is present in one section but closed in the other section, the counting event is a bridge (arrow). Islands are extremely rare events with alveoli being present in one section but not in the other. All panels are taken at the same magnification.
Table 8. Worked example for stereological parameters in pulmonary hypertension illustrated with rat lung treated with vascular endothelial growth factor receptor 2 inhibitor and chronic hypoxia

<table>
<thead>
<tr>
<th>Structure</th>
<th>Counts and Test System Information for Hypertensive Lung (control lung)</th>
<th>Calculation/Result for Hypertensive Lung (control lung)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung volume (V(lung))</td>
<td>$\Sigma P$ (par) = 498 (458)</td>
<td>$V_V$ (nonpar/lung) = $\Sigma P$(nonpar) $\Sigma P$(lung) = 0.166 (0.073) and accordingly $V_V$(par/lung) = 0.834 (0.927)</td>
<td>Reference volume</td>
</tr>
<tr>
<td>Volume of nonparenchyma and parenchyma (V_m(lung), V_V(par/lung), V_V(par/lung))</td>
<td>$\Sigma P$(lung) = 597 (494)</td>
<td>$V_V$(nonpar/lung) = $V_V$(par/lung) * V(lung) = 820 mm$^3$ (525 mm$^3$) and accordingly $V_V$(par/lung) = 4119 mm$^3$ (6665 mm$^3$)</td>
<td>Nonparenchyma is increased and parenchyma is decreased in hypertensive lung</td>
</tr>
<tr>
<td>Volume of arterial wall layers (lumen, intima, media, adventitia) and surface area of endothelial basement membrane (V_V(lumen/nonpar), V_V(intima/nonpar), V_V(media/nonpar), V_V(advent/lung), V_V(lumen, lung), V_V(intima, lung), V_V(media, lung), V_V(advent, lung), S(BM, lung))</td>
<td>$\Sigma P$(intima) = 35 (22)</td>
<td>$V_V$(lumen/nonpar) = $\Sigma P$(lumen)/$\Sigma P$(lung) = 0.0413 (0.0974) and accordingly $V_V$(intima/nonpar) = 0.0106 (0.0252), $V_V$(media/nonpar) = 0.0379 (0.0458) and $V_V$(advent/lung) = 0.0419 (0.0447)</td>
<td>Hypertensive lungs have increased media and adventitia volumes and a decreased lumen volume. This can only be seen from the total values as the volume densities per nonparenchyma are similar (adventitia) or may even show changes in the opposite direction (media; an example of the reference trap).</td>
</tr>
<tr>
<td>Volume of arterial wall layers (intima, media, adventitia) in relation to surface area of endothelial basement membrane (V_V(intima/lung), V_V(media/lung), V_V(advent/lung), S(BM, lung))</td>
<td>$\Sigma P$(media) = 125 (40)</td>
<td>$S_B$(BM/nonpar) = 2*$\Sigma I$(BM)/$\Sigma P$(lung)*l(pL) = 0.003399 mm$^{-1}$ (0.01059 mm$^{-1}$)</td>
<td>Hypertensive lungs have increased media and adventitia volumes compared with control lung.</td>
</tr>
<tr>
<td>$\Sigma P$(advent) = 138 (39)</td>
<td>$S_B$(BM/lung) = $S_B$(BM/nonpar) * $V_V$(par/lung) * V(lung) = 2786 mm$^2$ (5558 mm$^2$)</td>
<td>Hypertensive lungs have increased media and adventitia volumes compared with control lung.</td>
<td></td>
</tr>
<tr>
<td>$\Sigma I$(BM) = 109 (90)</td>
<td>l(pL) = 19.47 mm</td>
<td>Hypertensive lungs have increased media and adventitia volumes compared with control lung.</td>
<td></td>
</tr>
<tr>
<td>Volume of arterial wall layers (intima, media, adventitia) in relation to surface area of endothelial basement membrane (V_V(intima/lung), V_V(media/lung), V_V(advent/lung), S(BM, lung))</td>
<td>$V_V$(intima, lung)/S(BM, lung) = 3.12 mm$^3$ (2.38 mm$^3$)</td>
<td>Hypertensive lungs have increased media and adventitia volumes compared with control lung.</td>
<td></td>
</tr>
<tr>
<td>Length of arteries (L_V(art/nonpar), L_V(art,lung))</td>
<td>$\Sigma Q$ (art) = 78 (50)</td>
<td>L_V(art/nonpar) = (2*$\Sigma Q$(art))*l(pL) = 80.60 mm$^{-2}$ (195.60 mm$^{-2}$)</td>
<td>Both length density and total length of arteries are decreased in hypertensive compared with control lung.</td>
</tr>
<tr>
<td>$\Sigma P$(nonpar) = 212 (56)</td>
<td>L_V(art,lung) = L_V(art/nonpar) * $V_V$(par/lung) * V(lung) = 66.41 mm (102.87 mm)</td>
<td>Hypertensive lungs have increased media and adventitia volumes compared with control lung.</td>
<td></td>
</tr>
<tr>
<td>$a(p) = a(CF)/4 = 9129.5$</td>
<td>V(lumen, lung)/L_V(art,lung) = 510 mm$^2$ (497 mm$^2$)</td>
<td>Mean cross-sectional area of arterial lumen</td>
<td>The mean cross-sectional area of an artery profile was not different between hypertensive and control lung.</td>
</tr>
</tbody>
</table>

BM, basement membrane; $\Sigma P$, sum of points hitting a structure of interest; $\Sigma I$, sum of intersections of test lines with a structure of interest; l(pL), length of test line per point of line grid; $\Sigma Q$ (art), sum of profiles of arteries; a(p), area per point of counting frame; a(CF), area of counting frame. For further details refer to Table 7.

vascular compartments (e.g., precapillary arteries) do not share the characteristics of the alveolar capillary network, which prohibit the estimation of length so that length adds to volume and surface area estimations here. In many articles, the term “number of arteries” is used for counts of arterial profiles referred to the area used for counting, but this is rather proportional to their length than their number and should therefore be named length density accordingly if and only if the sections are isotropic. In general, for highly anisotropic structures, such as blood vessels, the use of isotropic sections is indispensable when surface area or length estimations are planned. Obviously, the length density should also be related to the reference volume to make sure no erroneous interpretation due to the use of a ratio is made (reference trap). It is indeed possible to estimate the number of vessel loops in a vascular network using the Euler Poincaré characteristic (63, 65, 99), but this has not been applied to the pulmonary vasculature so far. A classical feature of PH is the muscularization of previously only partially muscularized or nonmuscular arteries (80). Again, this is most usually reported as the “number of fully,
partially or non-muscularized” arteries, a denomination that should be avoided and replaced by reporting the length density and total length of these arteries. One frequently used parameter to characterize the arterial wall thickening is percentage of medial thickness, which relates external and internal diameter (83, 95). Thus it may be altered by changes in wall thickness as well as luminal diameter. As an alternative approach, we recommend relating the volume of the wall or its components to the surface area of the endothelial basal lamina. When immunohistochemical methods and/or EM are applied, information about the mechanisms of vascular wall remodeling can be obtained, e.g., by estimating the volume of the different wall layers (intima, media, and adventitia) or components (endothelium, SMCs, ECM, etc.) or the number and mean volume of a specific type of cells (e.g., SMC number and mean volume to distinguish between hyperplasia and hypertrophy) (Table 7).

Example. In this example (Table 8, Fig. 4), we use rat lungs treated by a vascular endothelial growth factor receptor 2 antagonist (SU5416) under chronic hypoxia (100). Controls received diluent under normoxic conditions. Samples were kindly provided by Rubin Tuder, Denver. The left lungs were fixed and cut. The volume was estimated using the Cavalieri estimator, and the samples were randomized for orientation using the isector (70) and embedded in paraffin. Sections (5 μm thick) were cut and stained with hematoxylin-eosin. At low magnification (×10), the volume of parenchyma and non-parenchyma was estimated. At a higher magnification (×40), the volume fraction of arterial lumen and wall layers (intima, media, and adventitia), as well as the endothelial basement membrane, were determined. In addition, at ×40, the length of arteries was estimated by counting the number of profiles per test area. As an estimator of luminal size, the mean cross-sectional area of the lumen was calculated from the volume of arterial lumen and the total length of the arteries.

Airway Hyperreactivity/Asthma

Pathobiology of airway hyperreactivity and asthma. In humans, asthmatic airway remodeling is a highly complex process with remarkable qualitative and quantitative changes in the composition of the bronchial/bronchiolar wall (34). The complexity of the disease and the bronchial wall (40), the remarkable differences between species, and the challenge of properly phenotyping animal models of asthma (6, 39) make the use of quantitative data particularly useful in this situation. An additional challenge in characterizing the pathological changes in asthma is induced by the site-specific changes, which may vary within one airway generation, between different airway generations, and between animals (3, 39).

The remodeling of the airways in asthma has several hallmark characteristics, which can well be assessed quantitatively, and the use of stereology has been explicitly recommended by Jeffery et al. (41). A prominent feature is the so-called reticular basement membrane (RBM) thickening, a phenomenon characterized by subepithelial fibrosis and replacement of the loose connective tissue by a dense connective tissue network of different composition (17, 85). It is still a matter of debate whether this feature correlates with the severity of the disease and functional parameters (7); however, it is not specific for asthma and occurs also in chronic obstructive pulmonary disease (57). Another important characteristic is the increase in airway SMC/myofibroblasts of the proximal airway wall, which is thought to be a major contributor to the functional consequences of airway hyperresponsiveness (61). The inflammatory pathogenic component is reflected by the increased occurrence of immune cells, such as eosinophils, neutrophils, and mast cells in the airway wall (8, 93). In the bronchial epithelium, increased amounts of goblet cells lead to hypersecretion, and damaged and fragile epithelial cells are thought to occur because of loosened adherens junction contacts between ciliated and basal cells (54, 91, 92). Vascular remodeling with increased number and size of blood vessels resulting from vascular endothelial growth factor signaling contributes to airway wall thickness, inflammation, and edema (14, 56).

Quantitative parameters of airway hyperreactivity/asthma.

As such, the most straightforward stereological approach to characterize asthma pathology includes the estimation of cell numbers (SMC/myofibroblasts, epithelial cells, subepithelial fibroblasts, and inflammatory cells), the volume and/or the mean thickness of the subepithelial layer and its components, the diameter of the airway lumen, and the volume of blood vessels surrounding the airways (Table 9). The hierarchical order of the recommended parameters will most likely start with the estimation of RBM thickening and the composition of the airway walls in terms of volume, followed by number estimation of inflammatory cells and SMC. In case of the RBM thickening, a comparison between two usually employed methods (85, 98) and a stereological approach has documented the superiority of stereological methods for this purpose with better reproducibility (24). The stereological approach is based on the orthogonal intercepts method (42) and requires the use of isotropic uniform random sections, which can be generated easily using the isector method (70). According to the concept of the epithelial-mesenchymal trophic unit, each airway generation of the tracheobronchial tree may differ from the ones before or after, both in terms of histological and functional reaction to hypersensitizing stimuli (18, 33). Therefore, in contrast to more diffuse diseases or pathologies affecting the gas-exchange region of the lung, the reference space containing the structures of interest (or to which the stereological parameters are related) needs to be defined within certain limits, e.g., the trachea or a specific airway generation. In
Table 9. Recommended stereological parameters in asthma/airway hyperreactivity

<table>
<thead>
<tr>
<th>Structure</th>
<th>Primary Parameter (recommended notation)</th>
<th>Total Value (recommended notation)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subepithelial fibrosis/reticular basement membrane (RBM) thickening</td>
<td>Volume density of epithelium, RBM, subepithelial connective tissue, cartilage, SMC, adventitial layer per unit airway volume ( V_{V(str/airway)} )</td>
<td>Volume of airway epithelium, RBM, subepithelial connective tissue, cartilage, SMC, adventitial layer in the lung ( V(V_{str/lung}) )</td>
<td>Point counting, LM</td>
</tr>
<tr>
<td></td>
<td>Measured thickness of RBM ( d(RBM) )</td>
<td>Arithmetic or harmonic mean thickness of RBM ( \bar{t}_{harm}(RBM) )</td>
<td>Orthogonal intercepts method, LM</td>
</tr>
<tr>
<td>Increased airway smooth muscle mass</td>
<td>Volume density of airway SMC per unit airway volume ( V_{V(SMC/airway)} )</td>
<td>Volume of airway SMC in the lung ( V(SMC,lung) )</td>
<td>Point counting, LM</td>
</tr>
<tr>
<td></td>
<td>Numerical density of SMC per unit airway volume ( N_{V(SMC/airway)} )</td>
<td>Number of airway SMC in the lung ( N(SMC,lung) )</td>
<td>Disector/fractionator, LM</td>
</tr>
<tr>
<td>Inflammatory infiltration</td>
<td>Volume density divided by numerical density of SMC ( \bar{V}(SMC) )</td>
<td>Number-weighted mean volume of SMC ( \bar{V}(SMC) )</td>
<td>Point counting, disector, LM</td>
</tr>
<tr>
<td></td>
<td>Numerical density of specific cell type per unit airway volume ( N_{V(inflam/airway)} )</td>
<td>Number of specific cell type in the lung ( N(inflam/lung) )</td>
<td>Disector/fractionator, LM, immunohistochemistry</td>
</tr>
<tr>
<td>Epithelial changes</td>
<td>Volume and numerical density of epithelial cells per unit airway volume ( V_{V(epi/airway)}, N_{V(epi/airway)} )</td>
<td>Volume and number of airway epithelial cells in the lung ( V(ves,lung), N(ves,lung) )</td>
<td>Disector/fractionator, LM, possibly immunohistochemistry</td>
</tr>
<tr>
<td>Peribronchial/-bronchiolar vascular remodeling</td>
<td>Volume and length density of peribronchial/-bronchiolar blood vessels per unit airway volume ( V_{V(ves/airway)}, L_{V(ves/airway)} )</td>
<td>Volume and length of peribronchial/-bronchiolar blood vessels in the lung ( V(ves,lung), L(ves,lung) )</td>
<td>Point counting, LM</td>
</tr>
</tbody>
</table>

Note that the parameters may also be referred to as an internal reference structure, such as the basement membrane of the epithelium. RBM, reticular basement membrane.

In the worked example (Table 10, Fig. 5), we use a single mouse trachea to illustrate the use of the fractionator for estimating the number of SMC and the volume of various wall compartments. A mouse trachea was cut into a number of pieces (here: 12), of which three were systematically randomly selected. Thus the block sampling fraction was 1/4. The selected pieces were embedded in EP with known orientation to allow a good observer orientation on the sections. As these pieces were only used for number and volume estimation, there

Table 10. Worked example for some stereological parameters in asthma/airway hyperreactivity using the mouse trachea as a reference

<table>
<thead>
<tr>
<th>Structure</th>
<th>Counts and Test System Information for Trachea</th>
<th>Calculation/Result for Trachea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of epithelium, RBM, subepithelial connective tissue, cartilage, smooth muscle cells, adventitial layer ( V_{V(epi/trachea)}, V_{V(subepi/trachea)}, V_{V(cart/trachea)}, V_{V(SMC/trachea)}, V_{V(adven/trachea)}, V_{V(epi/trachea)}, V_{V(subepi/trachea)}, V_{V(cart/trachea)}, V_{V(SMC/trachea)}, V_{V(adven/trachea)} )</td>
<td>( \Sigma P(epi) = 126 ) ( \Sigma P(subepi) = 72 ) ( \Sigma P(cart) = 186 ) ( \Sigma P(SMC) = 72 ) ( \Sigma P(adven) = 132 ) ( \Sigma P(trachea) = 588 ) ( a(p) = 17.943 \mu^2 ) ( d(sec) = 1 \mu ) ( bsf = 1/4 ) ( Q \times (SMC) = 381 ) ( bsf = 1/4 ) ( ssf = 100 ) ( asf = 1/2 )</td>
<td>( V_{V(epi/trachea)} = \Sigma P(epi) / \Sigma P(trachea) = 21.43 % ) and accordingly ( V_{V(subepi/trachea)} = 12.24 % ) ( V_{V(cart/trachea)} = 31.63 % ) ( V_{V(SMC/trachea)} = 12.24 % ) ( V_{V(adven/trachea)} = 22.45 % ) ( V_{V(epi/trachea)} = \Sigma P(epi) * a(p) * d(sec) * bsf / ssf / asf = 1.356 ) mm(^3) and accordingly ( V_{V(subepi/trachea)} = 0.7751 ) mm(^3) ( V_{V(cart/trachea)} = 2.002 ) mm(^3) ( V_{V(SMC/trachea)} = 0.7751 ) mm(^3) ( V_{V(adven/trachea)} = 1.421 ) mm(^3)</td>
</tr>
</tbody>
</table>

\( \Sigma P \), sum of points hitting a structure of interest; \( a(p) \), area per point; \( d(sec) \), section thickness; \( bsf \), block sampling fraction; \( ssf \), section sampling fraction; \( Q \times (SMC) \), sum of smooth muscle cells counted with the disector; \( asf \), area sampling fraction. For further details refer to Table 9.
was no need to randomize the orientation. EP was chosen as embedding medium because it allows the precise cutting of 1-μm-thick sections and therefore a good structural evaluation at the LM level. If the aim is to quantify cell numbers, which can only be identified by immunostaining, paraffin embedding is also fine with the fractionator, as the results are not influenced by the shrinkage that occurs with paraffin embedding (15). After embedding, the three tissue blocks were exhaustively sectioned into 1-μm-thick sections, and physical dissector pairs with a dissector height of 3 μm were mounted on glass slides. For example, the first and the fourth, the second and the fifth, etc., section could be used. The dissector pairs should be taken systematically randomly from the tissue blocks. Starting with a random number between 1 and k, every kth section is used as a start of a new dissector pair, e.g., k = 100, random number 43, sections 43, 143, 243, 343, etc. are used. For the volume estimation, either one of the dissector sections is used, or additional sections are gathered. Here, the section sampling fraction for the volume estimations was 1/150 and for the number estimation 1/100, so the sampling fractions were now 1/4 * 1/150 = 1/600 for volume and 1/4 * 1/100 = 1/400 for number. The sections were then stained with toluidine blue and analyzed with a light microscope and a stereology system. For volume estimation, a point grid with a known area per point [a(p)] was projected onto the randomly sampled fields of view, and the number of points hitting one of the following compartments was counted: epithelium, subepithelial layer, cartilage, smooth muscle, and adventitial tissue. The total number of points for each compartment was multiplied by the section thickness (1 μm) and the area per point. As this volume was estimated within a 1/600 fraction of the volume of the trachea, the multiplication with 600 yields an estimate of the total tracheal compartment volume. This procedure can be regarded as a combination of Cavalieri estimator and fractionator. For the estimation of the number of SMC, an unbiased counting frame was projected onto the fields of view with an area sampling fraction of 50%. SMC were counted if their nucleus was present in the sampling section but not in the look-up section. For reasons of efficiency, the sampling and the look-up section were then used vice versa, which means that each dissector was used twice, hence the area sampling fraction had to be multiplied by 2. The total sampling fraction is then 1/400 * 1/2 * 2 = 1/400. The total number of SMC in the whole trachea is therefore calculated by multiplying the number of counting events by 400.

**Summary**

We have tried to define, critically evaluate, and illustrate quantitative morphological parameters for a number of important pulmonary diseases. These parameters are useful estimators that, depending on the underlying pathology, may also be applied to other lung diseases. In general, a reduction to only one or two parameters is not sufficient for a comprehensive characterization of the structural aspects of a complex disease. Instead, coherent sets of relevant parameters are necessary, which can usually be estimated simultaneously following one multipurpose sampling design. Experimental lung research critically depends on the ability to quantitate the degree of structural alterations in disease and their prevention or reversal by prophylactic or therapeutic interventions. The silver bullet to achieve this is long known: design-based stereology.

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