HIGHLIGHTED TOPIC | Physiological Imaging of the Lung

How much is there really? Why stereology is essential in lung morphometry

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Weibel ER, Hsia CC, Ochs M. How much is there really? Why stereology is essential in lung morphometry. J Appl Physiol 102: 459–467, 2007. First published September 14, 2006; doi:10.1152/japplphysiol.00808.2006.—Quantitative data on lung structure are essential to set up structure-function models for assessing the functional performance of the lung or to make statistically valid comparisons in experimental morphology, physiology, or pathology. The methods of choice for microscopy-based lung morphometry are those of stereology, the science of quantitative characterization of irregular three-dimensional objects on the basis of measurements made on two-dimensional sections. From a practical perspective, stereology is an assumption-free set of methods of unbiased sampling with geometric probes, based on a solid mathematical foundation. Here, we discuss the pitfalls of lung morphometry and present solutions, from specimen preparation to the sampling scheme in multiple stages, for obtaining unbiased estimates of morphometric parameters such as volumes, surfaces, lengths, and numbers. This is demonstrated on various examples. Stereological methods are accurate, efficient, simple, and transparent; the precision of the estimates depends on the size and distribution of the sample. For obtaining quantitative data on lung structure at all microscopic levels, state-of-the-art stereology is the gold standard.

WHY MEASURE LUNG STRUCTURE?

The first justification for lung morphometry derives from the need of structural information in setting up models that allow one to assess the importance of structural design for functional performance, such as 1) Do we really need an alveolar surface nearly the size of a tennis court to ensure adequate O₂ uptake in strenuous exercise? or 2) How is gas exchange affected by the arrangement of alveoli along the acinar airways? Such structure-function models can be exploited in attempts to understand the importance of variations in structural design among different species in comparative physiology. However, its greatest potential is in experimental morphology and physiology, including transgenic animals—without quantitative structural information, the analysis of gene functions at a systemic level remains incomplete—or in pathology, where the effect of structural changes, such as a loss of surface or a thickening of the tissue, leads to significant disturbances of functional performance, a good case being pulmonary emphysema and its consequences for gas exchange.

PITFALLS OF LUNG MORPHOMETRY

The structures of interest for such studies are very small and hence must be subjected to measurement under the microscope. Furthermore, in contrast to all solid organs, 85–90% of the volume of an inflated lung is air and only half of the remaining 10–15% is tissue. This introduces four problems that may become pitfalls of lung morphometry if not properly controlled.

Thin Sections

To look into the lung, the organ must be sectioned, resulting in a thin plane sample section, equivalent of a CT plane. Although the structures are three-dimensional (3-D) entities, their image on thin sections is two-dimensional (2-D). Measurements done simply on such sections therefore bear little meaning with respect to the real 3-D structure: if an alveolar profile is small, this does not mean that the sectioned alveolus is small; it could well mean that the section cut a large alveolus near its periphery. The tools to overcome this problem, i.e., to permit the derivation of real 3-D data from measurements done on 2-D sections, are provided by stereology, as outlined below. There is no real alternative. Measuring sections with sophisticated automatic image analysis systems cannot eliminate the bias introduced by the sectioning process, unless the programs are specifically designed for stereology.

Small Sample

The sections constitute a tiny, in fact, an infinitesimal sample of a large organ. If morphometric data are to be used in conjunction with functional information, both physiology and morphometry must be related to the same reference system. If physiological information relates to the whole organism, then morphometric information must be obtained for the whole lung as the entrance organ to the respiratory system. This means that
total lung volume is the starting point for sampling of tissue for microscopic analysis, but it is also the endpoint for obtaining functionally relevant data: we cannot be content with knowing only the alveolar surface density (surface per unit volume); we need to know the total alveolar surface as a determinant of diffusing capacity. Indeed, alveolar surface density in itself is not meaningful as it also depends on the degree of lung inflation at the time of fixation.

As a general strategic principle in morphometry, the reference space for the quantitative information sought must be clearly defined, and the sampling strategy must be designed to fit the requirements.

**Specimen Preparation**

Another potential bias relates to the preparation procedures, such as inadequate control of inflation during fixation, shrinkage due to tissue processing, or compression of the sections. These can alter tissue dimensions and therefore need to be controlled carefully. For example, if the tissue is not sufficiently hardened by the fixation procedure, it may contract when the lung is sectioned, thereby reducing the reference space. Adequate results are obtained by fixing the lungs with buffered glutaraldehyde by airway instillation under controlled pressure and maintaining the specimen in the inflated state for at least 24 h, preferably longer. This results in well-stabilized lung tissue that will not collapse in further processing, whereas the popular fixation with formaldehyde is inadequate in that respect (46, 53). Good results are also obtained by vascular perfusion fixation under controlled conditions (1, 7, 9, 46). It is advisable to measure lung volume after release of the instillation pressure, preferably by the Cavalieri method (31, 53). In this method the lung is serially sectioned at a known interval; the area of each slice is estimated by point counting and summed to yield a total volume estimate that most closely respects (46, 53). Good results are also obtained by vascular perfusion fixation under controlled conditions (1, 7, 9, 46). It is advisable to measure lung volume after release of the instillation pressure, preferably by the Cavalieri method (31, 53). In this method the lung is serially sectioned at a known interval; the area of each slice is estimated by point counting and summed to yield a total volume estimate that most closely represents the state of the tissue immediately before sampling and embedding, an essential condition for accuracy of the findings. The subsequent preparation steps, embedding, and sectioning must be well controlled, standardized, and monitored for shrinkage effects (42, p. 40–50). Embedding in paraffin usually leads to considerable shrinkage, which can be avoided by embedding in glycol-methacrylate or epoxy resins. If such artifacts are considered a serious problem, shrinkage factors must be estimated, which allows the derivation of correction factors to convert measurements obtained on sections to data pertaining to the lung in a physiological state (cf. 42, p. 45). Clearly, the control of such preparation bias is a precondition for the study of lung morphometry.

**Model Assumptions**

Another source of potential bias is introduced by relying on model assumptions about the structure, such as its shape (“this cell type is spherical”), orientation (“this surface is randomly oriented”), or spatial distribution (“this cell type is homogeneously distributed”). Such assumption-based or model-based methods often lead to wrong data and conclusions.

**OVERCOMING THE PITFALLS BY USING STEREOREOLOGICAL METHODS**

Stereology is the science of quantitative characterization of the physical properties of irregular 3-D objects based on measurements made on 2-D sections. Stereological methods do not require any assumption on the shape, size, orientation, or spatial distribution of the objects under study but rather utilize geometric probes to sample the structure and derive 3-D measurements in a framework of strictly unbiased sampling, thus relating estimates of structural parameters to the entire organ.

**Stereology as a Principle of Unbiased Sampling with Geometric Probes**

Let us begin with an example, the estimation of alveolar surface area. If we were to fictionally stick a thin needle of known length, $L$, randomly into the lung, it would cross the alveolar surface $I$ times. It is intuitively plausible that there will be more such intersections the more surface is packed into the unit lung volume; hence the ratio $I/L$ is proportional to the surface density $S/V$. In practice, the “needle” is a test line or a set of test lines of total length $L$ placed on a microscopic picture of sufficient resolution obtained on a random thin section. The proportionality factor linking $I/L$ and $S/V$ is 2 and is derived theoretically on the condition that the intersections between the test lines and the alveolar surface are random with respect to orientation and location within the lung. It does not mean that the alveolar surface must be “random”—it is of course not random—rather the sampling process must be “isotropic uniform random” (IUR). The “isotropic” part of this condition is ensured by allowing the tissue blocks from which the sections are cut to be embedded in any direction; to achieve this in a rigorous manner, one can use procedures such as the “orientator” (25) or “isector” (28), but with respect to the alveolar surface this is usually not necessary. The situation is different when attempting to measure the surface of bronchioles or blood vessels that are anisotropic; in this case one could make “vertical sections” for sampling and use a cycloid test line system for counting intersections (2).

The “uniform” part of IUR deserves special comment as it requires sampling from the entire lung, considering the possibility of uneven regional distribution of structures. The sampling strategy begins with the estimation of lung volume and obtaining tissue samples from all parts of the lung. We recommend using a stratified sampling strategy combined with a Cavalieri procedure (6, 13, 27); each stratum of the fixed lung is cut into slices of equal thickness using a random start (Fig. 1A); the area of the slices is measured, e.g., by point counting (Fig. 1B); tissue blocks are selected from these slices by systematic sampling with a random start, ensuring that all regions (e.g., lobes) are sampled in proportion to their volume (Fig. 1B). Sections are cut from these blocks (for light and electron microscopy) allowing for unbiased orientation, a single section is picked and a number of microscopic fields are chosen again by systematic sampling, and these fields are overlaid with a suitable test system for analysis (Fig. 1, C–F).

An important consideration is that the lung’s internal structures cover a wide range of magnitudes. An inflated human lung has a volume of several liters and a diameter that is on the order of 20 cm, whereas an acinus measures 5 mm, an alveolus 0.250 mm, an alveolar cell 5 μm, and the thickness of the air-blood barrier 0.5 μm. To do justice to all levels of magnitude, a cascade sampling strategy should be used as demonstrated in Fig. 1 and explained in its legend.
Stereology as a Set of Practical Methods

While many of the unbiased sampling conditions mentioned are common to all approaches to estimate quantitative properties, the use of geometric probes to measure 3-D structures introduces specific requirements. As we are not making any model assumptions about the nature of the structure, we must design a measuring strategy where the samples and the probes are randomized (design-based sampling; 3). This is particularly true at the last stage where probes are applied to sections or micrographs. It is here that stereological methods in the narrow sense come into play.

Classical stereology: the basic tools. The basic problem is to accurately quantify 3-D structures from measurements on sections. We want to know, for instance, the volume $V_{\text{alveolar septal tissue}}$ or of capillary blood, the surface area $S_{\text{alveolar epithelium}}$, the length $L_{\text{capillaries or elastic fibers}}$, or the number $N_{\text{alveoli}}$. The sectioning process reduces the image to a 2-D sample, i.e., one dimension is lost: volumes (3-D) appear as areas (2-D), surfaces (2-D) as boundaries [1-dimensional (1-D)], lengths (1-D) (of tubes or filaments) as transects or points [zero-dimensional (0-D)], and these can be measured; in contrast, the 0-D parameter “number” is not represented and therefore cannot be estimated on...
Table 1. Relationship between structural quantities and stereological methods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Appearance in 2-D</th>
<th>Probe</th>
<th>Event</th>
<th>Measurement</th>
<th>Density Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume V</td>
<td>Area A</td>
<td>Point P_{T}</td>
<td>Point “hits” volume</td>
<td>Point count P(x)</td>
<td>V_{(x)} = P(x)P_{T}</td>
</tr>
<tr>
<td>Surface area S</td>
<td>Boundary B</td>
<td>Line L_{T}</td>
<td>Line “hits” surface</td>
<td>Intersection count I(x)</td>
<td>S_{(x)} = 2I(x)L_{T}</td>
</tr>
<tr>
<td>Length L</td>
<td>Point Q</td>
<td>Plane A_{T}</td>
<td>Plane “hits” line</td>
<td>Transect count Q(x)</td>
<td>L_{(x)} = 2Q(x)A_{T}</td>
</tr>
<tr>
<td>Number N</td>
<td></td>
<td>Disector (volume) A_{T} \cdot t</td>
<td>Disector volume “hits” particle top</td>
<td>Top count Q’(x)</td>
<td>N_{(x)} = Q’(x)A_{T} \cdot t</td>
</tr>
</tbody>
</table>

Overview of basic structural quantities that can be estimated, their appearance in single thin histological sections, the appropriate geometric probes to measure them, the events generated by the interaction of the probe with the structure, the counts (measurements) that result, and the formulas permitting the calculation of densities in the reference volume (see text).

single sections. A further restriction is that we see only a small field of the section: the sample. All measurements must therefore be related to the containing space that we see on the section field; accordingly, we cannot estimate volumes or surface areas per se but rather volume densities V_{V} (volume per unit containing volume) or surface densities S_{V} (surface area per unit containing volume), etc.

This apparent limitation is, however, an advantage because it provides easy and straightforward ways to get this information by using simple probes: points, lines, and areas (44, 45, 47) (see Table 1 and Fig. 2). For the estimation of each of these parameters, there is one simple and highly efficient geometric probe that allows the measurement to be reduced to simple counting. By randomly placing a set of test points P_{T} on the section, one can directly estimate the volume composition of the structure by counting the number of points falling on each component structure (e.g., alveolus, capillary, tissue). As we have seen, a set of test lines L_{T} allows the estimation of surface areas. The length of linear features, such as connective tissue fibers, is estimated by counting the number of times they transect the section area A_{T}. Figure 1, D–F, and Fig. 2 show suitable test grids for this kind of counting. Stereological test systems are preferentially designed as a set of test points and test lines within a test area (ALP-sector) in such a way that the number of points, the length of the lines, and the area of the test frame are linked in a simple fashion; such test systems are called coherent (44) and facilitate the counting process.

In all this, there is a basic rule: the dimension of the structural parameter plus the dimension of the probe has to equal 3 (Table 1). This is why an unbiased estimation of particle number requires 3-D probes, i.e., test volumes, which can be generated by the so-called disector (37). A disector consists of two sections from the same tissue block a known distance t apart (hence the name “di-sector”); marking a test area frame A_{T} on each thus creates a test volume. Either two discrete, usually adjacent, sections (physical disector) or one

Fig. 2. Structural parameters and their stereological representation (see also Table 1). A structure (left) of total reference volume V(R) containing particles of volume V(x) and surface S(x), as well as threadlike features of length L(y), is randomly sectioned. On this isotropic uniform random section (right), the profiles of x are characterized by their area A(x) and boundary B(x), the feature y appears as a number of small transects Q(y), while the reference space is represented by the section area A(R). Applying a coherent stereological test grid (ALP-sector) with test points P_{T} = 16, test lines L_{T} = P_{T} \cdot 2d, and test area A_{T} = P_{T} \cdot d^{2} to the section allows one to assess volume, surface, and length densities per unit volume from point hits P(x) (marked by squares), intersection counts I(x) (arrowheads), and transect counts Q(y) (short arrows), whereby the reference area is estimated by the number of test points included in the section profile P(R), i.e., excluding the points falling outside (marked by triangle). In this example P(R) = 15; the actual test area is A(R) = P(R) \cdot d^{2}, and the length of test line included in the sample is L(R) = P(R) \cdot 2d. Using a second parallel section a distance t apart and the counting frame with area A(R) (disector), the numerical density of particles per unit volume can be assessed from counting particle tops Q’(x) in the disector volume A(R) \cdot t.
thick section where one can focus through in the z-direction (optical dissector) can be used. The counting event is the appearance of a particle within the dissector, i.e., particle tops are counted, and this leads to an estimate of number density.

Recent extensions of stereological methods. The introduction of the dissector method in 1984 significantly increased the flexibility of stereology (37). A new set of methods evolved for the quantitative characterization of particle number and size without need for model assumptions (for review, see 3, 5, 14, 15, 17, 26, 49). New methods include tools to account for anisotropy of structures when estimating their surface area or length (2, 25, 28); fractionator sampling, which makes number estimation independent of changes in tissue dimensions (12, 16); a whole family of mean particle size estimators (local stereological methods where particle size is estimated directly without having to measure the volume of the particle-containing compartment, e.g., nucleator, rotator, and point-sampled intercepts); estimators of barrier thickness distribution and their means; and second-order stereology for the characterization of spatial relationships (22, 35). Many of these new methods have been successfully applied to the lung (for review, see 33).

Using stereological methods. In summary, the measurements obtained by classical stereological methods are densities, or quantities per unit volume, and these can or must be converted to absolute/total values by multiplying them by the volume of the reference space. For this, it is all-important to design a sampling scheme that allows counts obtained on micrographs to be related back to the whole organ or the organism (Fig. 1) (6, 49): lung volume is the starting point for sampling and the endpoint for data analysis.

The power of stereological methods is based on the fact that they are both accurate and efficient. Sampling is accurate (unbiased) when each part of the organ is sampled with equal probability. Sampling is efficient when a sufficient precision is achieved within a reasonable amount of time and cost. While accuracy is independent from sample size, precision can be tuned by modulating the sampling effort.

To improve precision, i.e., to decrease the coefficient of error within one individual, one has to put more effort into the higher levels of the sampling cascade, e.g., by sampling more tissue blocks per individual, while reducing the effort at the lower levels of the sampling cascade, e.g., by decreasing the number of test points per field. This approach, known as Weibel’s principle “do more less well” (10), also improves efficiency. As the imprecision of the measurements, e.g., point or intersection counting, is usually inconsequential compared with the variability among tissue blocks, an exceedingly high precision offered by automatic or semiautomatic image analyzers is superfluous and inefficient (Fig. 3) (24).

As a rule of thumb, 100–200 well-distributed counting events (e.g., test points, intersections, dissector counts, or local measurements) per individual are sufficient to reach a precision for any estimator that ensures that the total observed variation within a group is dominated by the biological variation between individuals (the signal) and not by the variation due to stereological sampling (the noise) (13, 24). Figure 2 reveals that a square grid generates many more intersections than point hits, which is inefficient when surfaces and volumes must be estimated. To improve efficiency, one can use diluted test systems with fewer test lines, such as that shown in Fig. 1E on an electron micrograph of an alveolar septum. By using digital cameras and computer-assisted stereology systems, it is possible to fully analyze one lung in no more than one working day at the light microscopic level. At the level of electron microscopy, the requirements are similar; the effort to produce sections and micrographs in general surpasses the effort for counting.

PRACTICAL APPLICATIONS OF STEREOLOGY

Below we describe common problems in study design, measurement, or data interpretation encountered when assessing the role of lung structure in gas exchange, or the consequences of its loss, using emphysisma and postpneumonectomy compensatory lung growth as examples. In addition, we provide information on how to estimate the number of alveoli directly and how to characterize lung cells and surfactant by stereology.

![Graph showing cumulative volume density estimation by point counting with grids with different numbers of points](http://jap.physiology.org.org/)
Pulmonary Diffusing Capacity Estimated by Lung Morphometry

Pulmonary diffusing capacity is determined by the volume of alveolar capillary blood, \( V(c) \), the air-blood contact area that is measured by the alveolar and capillary surfaces, \( S(a) \) and \( S(c) \), respectively, and the harmonic mean thickness of the diffusion barrier between air and erythrocytes (43, 48). All these parameters except for the harmonic mean barrier thickness are estimated by classical stereological methods: the capillary volume density is estimated by point counting and the surface densities by intersection counting. The harmonic mean barrier thickness is estimated from the reciprocal lengths of random test lines that fall across the air-tissue interface to capillary erythrocytes (48). These measurements must be done on electron micrographs using a suitable test system of lines and points with the volume of lung parenchyma as reference space (Fig. 1E). To calculate the total surface areas and the total capillary volume, the volume density of parenchyma in lung volume is estimated at lower magnifications, and the lung volume measured before sampling, preferably by the Cavalieri method (Fig. 1, A and B). As an example, total alveolar surface area (in \( \text{cm}^2 \)) is the product of alveolar surface density in parenchyma (in \( \text{cm}^{-1} \)) times volume density of parenchyma in lung times lung volume (in \( \text{cm}^3 \)) (Fig. 1). This cascade approach has been used for assessing the morphometric determinants of diffusing capacity and their importance in maintaining adequate \( \text{O}_2 \) flux through the entire pathway for oxygen from rest to exercise (50, 51) in the human lung (8), as well as in comparative and experimental studies.

Emphysema

Emphysema is more than airspace enlargement. The 2004 American Thoracic Society-European Respiratory Society Standards for the Diagnosis and Management of Patients with chronic obstructive pulmonary disease (COPD) (http://www-test.thoracic.org/copd/pdf/copddoc.pdf) defines emphysema as “the presence of permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls and without obvious fibrosis.” This definition makes it clear that “airspace enlargement” is not synonymous with “emphysema”; the loss of functional surface is indeed more important. Airspace enlargement also occurs in the absence of tissue destruction, for example in developmental failure of alveolarization, or in normal ageing (4, 39). Patchy parenchymal fibrosis could also cause the appearance of heterogeneous airspace enlargement by imposing traction on airspace walls of adjacent uninvolved units. In addition, primary and secondary alveolar hypoplasia, seen in various gene knockout models and clinically in the Swyer-James syndrome, causes the appearance of enlarged airspace without actual destruction of their walls. Therefore, the diagnosis of emphysema implies the knowledge or assumption of normal alveolar development and histology before the onset of the disease or intervention under investigation.

Mean linear intercept does not measure airspace size. One hallmark of hypoplasia or destruction of airspace walls is a low surface-to-volume (\( S/V \)) ratio, which correlates with physiological abnormalities in elastic recoil and gas exchange (40, 52). The \( S/V \) ratio can be directly estimated by intersection and point counting using a coherent stereological test grid (Fig. 1D). A popular unbiased estimate of the \( S/V \) ratio is obtained from the mean linear intercept \( L_m \) (42). A test line is randomly overlaid on an image and the chord lengths or linear intercepts, \( L_I \), between intersections of the line with two adjacent airspace walls are measured with a ruler; the average \( L_I \) yields mean chord or intercept length \( L_m \), which is inversely related to \( S/V \) ratio. The apparent simplicity of this method underlies its widespread use, but its meaning is difficult to work out. In principle, \( L_m \) measures the “mean free path” within peripheral airways, alveoli and ducts taken together. One common error is to equate \( L_m \) with airspace size without knowledge of shape. For illustration, a sphere and an ellipsoid with the same volume differ by their surface area, which is larger in the ellipsoid; measuring linear intercepts from their random cross sections will yield a lower \( L_m \) for the ellipsoid, reflecting only a higher \( S/V \) ratio but not a difference in volume. However, the robustness of \( L_m \) estimates depends critically on the steps before measuring \( L_I \), i.e., standardization of inflation pressure, precise measurement of the reference lung volume; unbiased and sufficient sampling of tissue blocks, histological sections, and microscopic fields; as well as an accurate interpretation of the results. Alternative methods are available for assessing either the volume fraction of air in the distal lung region by simple point counting, as well as for assessing \( S/V \) by counting point hits and intersections with test lines. In fact, \( L_m \) is much more difficult to measure and fraught with danger of bias if the air space size is very variable. This method should be abandoned. Even the rapid and precise measurement of \( L_m \) with automatic image analyzers can be misleading: accuracy is more important than precision.

Postpneumonectomy Compensatory Alveolar Growth

Special considerations in sampling. Pneumonectomy is an example where the reference space, lung volume, differs grossly between the experimental and control groups (18, 19). Following surgical resection of one lung, the negative intrathoracic pressure causes the remaining lung to expand to twice its normal volume at a given transpulmonary pressure; redirection of blood flow causes a similar doubling of capillary blood volume in the remaining lung at a given cardiac output. For comparison with the corresponding control lung or lobe, proportionally more tissue blocks should be sampled and analyzed from the postpneumonectomy lung or lobe to ensure a constant sampling rate per unit of lung volume across different experimental groups. Owing to the asymmetric anatomy of mediastinal structures and ligaments and the elevated hemidiaphragm, the distribution of mechanical strain after pneumonectomy is not uniform throughout the remaining lung. For example, following left pneumonectomy in the dog, volume of the remaining right upper and cardiac (or accessory) lobes doubles but volume of the right middle and lower lobes does not increase (34). Disparity in regional strain may cause disparate regional structural response, evident even in rodent lungs (36) and further accentuated in large animals; these observations emphasize the need for a stratified sampling scheme that covers all regions of the remaining lung to prevent bias (Fig. 1A). This consideration is not unique to pneumonectomy but applies to other lung disease, injury, or intervention that result in differential regional effects.
Adequate resolution of alveolar tissue and blood compartments. Compensatory alveolar growth refers to the generation of new gas exchange tissue in response to an insult, leading to increased tissue volume and surface area and ultimately mitigating the loss of function caused by the primary insult. On the other hand, passive capillary distension per se, while contributing to gas exchange improvement by increasing capillary blood volume, is not evidence of active alveolar tissue growth. A magnification of approximately $\times 1,000$ is required to reliably distinguish septal tissue from capillary blood for quantification. At a lower magnification an increase in septal volume of the remaining lung due to capillary distension would be indistinguishable from that due to growth of new tissue; similarly, an increase in alveolar surface area caused by the unfolding of existing septa would be indistinguishable from that caused by the generation of new surfaces.

Alveolar-capillary surface area and the harmonic mean tissue-plasma barrier thickness are key structural determinants of the membrane resistance to oxygen diffusion. The alveolar blood-gas barrier is unique in its surface complexity, with irregular folds and projections that are lined with surfactant and blood-gas barrier is unique in its surface complexity, with membrane resistance to oxygen diffusion. The alveolar tissue-plasma barrier thickness are key structural determinants of that caused by the generation of new surfaces. Unfolding of existing septa would be indistinguishable from that due to growth of new tissue; similarly, an increase in alveolar surface area caused by the unfolding of existing septa would be indistinguishable from that caused by the generation of new surfaces.

Fig. 4. Comparison of pulmonary diffusing capacity for carbon monoxide (DLco) estimates obtained by a physiological (rebreathing) method at heavy exercise and by a morphometric method in dogs that had undergone left or right pneumonectomy (LPNX and RPNX, respectively) as puppies or adults and in their respective controls (Sham) in different experiments (18, 19, 38).

formed of the strong fiber tracts in the free edges of the alveolar septa. These entrance rings constitute the virtual wall of alveolar ducts and thus form a 2-D network in 3-D space. One can make use of this unique feature by assessing, as a surrogate for alveolar number, the number of “holes” in this network, which is directly related to the so-called Euler number, the topological expression of the number of connections in a network. Using a disector, one counts any new bridge (a connection of the free edges of alveolar walls) that appears, which signifies that there is an alveolar opening (21, 31). For the human lung, this new method yielded 480 million alveoli (31). This method has been used in several species, including gene-manipulated mice (23, 32); it is direct, unbiased, and independent of the shape, size, or orientation of alveoli. This opens new possibilities: if the number-weighted mean alveolar size (estimated by dividing total alveolar volume by alveolar number) and the volume-weighted mean alveolar size (estimated by point-sampled intercepts) are known, information about alveolar size heterogeneity can be assessed, since the volume-weighted mean volume equals the number-weighted mean volume amplified by the relative variance (for review, see 33).

Lung Cells and Surfactant

Many lung diseases are associated with hyperplasia and/or hypertrophy of certain cell types. Thus the number and size of a given cell type are highly important information. The total volume of any type of lung cell can be estimated via point counting in a cascade sampling design (Fig. 1). Cell number per lung can directly be estimated using dissectors (Table 1). Since the disector is not only a tool for counting of particles but also for sampling them with equal probability, direct estimation of mean cell size (and size distribution) is possible using local stereological methods, e.g., the nucleator. Alternatively, mean cell size can be estimated indirectly by dividing cell volume density by cell number density (Table 1). When the number- and volume-weighted mean cell size are known, information about size heterogeneity becomes also available, which is valuable for assessing the degree of cell differentiation (11).
Of particular importance is the assessment of the surfactant-associated compartments in the type II cells. Intracellular surfactant is stored in characteristic organelles termed lamellar bodies. The total volume of lamellar bodies, which can be used as a morphological measure of intracellular surfactant content, can be estimated by point counting in a cascade sampling design (Fig. 1F). If further information about the way type II cells handle a certain amount of surfactant is of interest, the number of lamellar bodies can be estimated using the disector at the electron microscope level. Their mean size is then estimated by dividing total volume by number (see 33).

Intra-alveolar surfactant consists of different subtypes that represent different stages, and thus different activity, within surfactant metabolism. The estimation of volumes of active (corresponding to lamellar body-like forms and tubular myelin) and inactive (corresponding to unilamellar vesicles) surfactant forms by point counting allows one to assess the functional status of intra-alveolar surfactant while preserving its microorganization and localization in the lung (29, 30).

**TAKE-HOME MESSAGES**

Methods for microscopy-based quantification of lung structure must be accurate and efficient, simple and transparent, and allowing as precise an estimate as the conditions permit and the question requires. To achieve accurate results, unbiased sampling is unavoidable at all levels, from tissue sampling to application of geometric probes on the sections. Stereological sampling is necessary at all levels, from tissue sampling to the measurement of arbitrary particles observed on random sections. J Microsc 138: 127–142, 1985.


27. **Michel RP, Cruz-Orive LM.** Sampling and unbiased stereology: or “Do more less well.” J Microsc 143: 3–45, 1986.


