Review

Standards for Quantitative Assessment of Lung Structure: The Dawn of Stereopneumology

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- biopsy
- anatomy
- histology
- microscopy
- imaging

SUMMARY. The lungs are complex 3D structuresstudied in the clinic and the laboratory using histologic or imaging sections. Although such 2D analyses of lung structure are considered "gold standards", the information conveyed is often insufficient and does not represent the whole organ. Stereology, the mathematical approach to the analysis of 3D structures via 2D sampling and morphometry, the practical application of stereology, provide solutions to this problem, but had until recently not been systematically adoptedin pneumology. In an effort of minimizing the above-mentioned methodological problems and of standardizing the quantitative assessment of lung structure, the American Thoracic Society and European Respiratory Society formed a task force, which recently published its findings. The task force aimed at comprehensively reviewing current stereologic methods for lung morphometry, formulating practical guidelines for using unbiased methods for basic and translational research of lung structure, and examining the extensions of stereologic methods on non-invasive imaging of lung architecture. In the statement of the task force are included useful directives with important application in the laboratory and the clinic, the most pertinent of which are discussed in the present mini-review. *Pneumon 2010, 23(2):147-152.*

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INTRODUCTION

The problem

The lungs are complex 3D structurescomposed of various cell types, extracellular matrix, air, and blood. Compared with other organs (e.g. kidney), the complexity of lung spatial distribution is further aggravated by the relative movement of lung components during respiration and by the heterogeneity and anisotropythat characterizes lung parenchyma, which contains solid and fluid mater as well as various cell types in proportions that vary between different lung regions (e.g. central versus distal lung regions). Understanding

normal lung function, embryologic development and post-natal growth of the lungs, mechanisms and effects of respiratory diseases, as well as the impact of various therapeutic interventions prerequisites the acquisition of data on 3D lung structure. This information is currently derived from 2D histologic or imaging lung sections and is extrapolated to assumptions on global lung structure and function. Although structural (histologic or imaging) lung analyses are considered the "gold standards" for assessment of disease severity or the impact of therapeutic or experimental interventions, the information obtained from 2D sampling of the moving 3D structure called lung is often incomplete and may lead to false conclusions.

Stereology

Solutions to these problems are provided by stereology, a systematic mathematical approach to the analysis of 3D structures via 2D sampling. Although the method was first applied to the lungs 40 years ago[1-3] and has developed a coherent and complete set of analytical tools[4], no methodologic standards had been adopted until recently, as has occurred in the cases of the kidney and the brain. In an effort of minimizing the aforementioned methodological problems and of standardizing the quantitative assessment of lung structure, the American Thoracic Society and European Respiratory Society formed a task force, which recently published its findings in an Official Joint Research Policy Statement[5]. The task force aimed at comprehensively reviewing current stereological methods for lung morphometry, articulating practical guidelines for using unbiased methods of basic and translational research of lung structure, and examining the extensions of stereologic methods on non-invasive imaging of lung architecture. In the findings of the task force are included useful directives with important application in the laboratory and the clinic, the most pertinent of which are discussed in this mini-review. Pulmonary, radiology, and pathology specialists clinically or research-wise primed to further dwell on the subject are referred to the above-referenced and other more detailed documents[4-14].

REVIEW

Stereologic principles

Asmentioned above, stereology refers to the mathematical methods employed for the determination of the physical properties of irregular 3D structures using physical or optical 2D sections. The term morphometry refers to measuring form and mainly consists of the practical applications of stereology. The parameters of a 3D structure like the lungs may feature three (volume or size), two (surface area), one (length or thickness), or zero (number) dimensions and are estimated by*sampling*and*estimation*, the two main steps of stereology. *Sampling*should be completely random and unbiased, and its results should be reproducible by different sampling procedures. Stere ologic*estimation*often relies on simple measurement of the number of interactions between structures of interest and simple geometric probes and is governed by the following principles: a) The probe used determines the estimatedparameterso that the sum of the dimensions of the parameter and the probe equals three (Figure 1 and

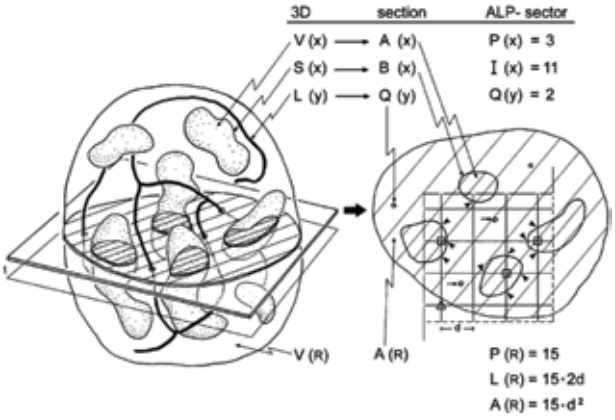

FIGURE 1. Structural parameters and their stereological representation. A structure (left) of total reference volume V(R) containing particles of volume V(x) and surface S(x) as well as thread-like features of length L(y) is randomly sectioned. On an isotropic uniform random (IUR) section (right) the 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= PT · 2d, and test area A_T= 15 · $d^2$to the section allows 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), that is, excluding the points falling outside (marked by triangle). In this example P(R) = 15; the actual test area is A(R) = P(R) · d^2, and the length of test line included in the sample is L(R) = P(R) · 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) · t. Reproduced with permission from reference (5).

Table 1). b) Stereologic measurements consist of densities or ratios and should refer to a reference volume (e.g. lung or alveolar volume) in order to be meaningful. c) Particle counting (zero dimensions) cannot be performed on 2D sections and requires the use of paired sections, physical (histologic) or optical, separated by a known distance (disector), creating a 3D study volume that equals the product of the section surface area times the distance between the two sections.

Processing of lung tissue

Preparation of lung tissue for stereologic morphometry aims at optimal preservation of lung volume at a given phase of the respiratory cycle, of lung parenchymal architecture, of pulmonary biological fluids (e.g. blood, epithelial lining fluid), as well as the molecular identity of lung cells for subsequent labeling of proteins (immunohistochemistry) and/or nucleic acids (in situhybridization). No method achieves all above goals in order to be considered the "gold standard", but some goals are better achieved by certain methods (Table 2). A preferred method that allows for optimal preservation of alveolar structures and blood, but not of epithelial lining fluid, *airway instillation*, is best performed via transtracheal rapid infusionof 2,5% glutaraldehyde (osmotic pressure 350 Osm; pH 7,4) under 20-25 cmH_2Opressurefor 24 hours. *Vascular perfusion*achieves preservation of alveolar and capillary structures, as well as of epithelial lining fluid, but not of blood, and is preferentially performed by infusion into the pulmonary artery of a combination of 2,5% glutaraldehyde– 3% dextran (osmotic pressure510 Osm; pH 7,4), sequentially followed by 1% osmium tetroxide,0,5% uranium acetate, and 70-100% ethanol. Finally, as an optimal method allowing for both stereologic morphometry and immunohistochemistry is recommended the infusion of 4% formaldehyde with 0,1% glutaraldehyde in 0,2 M HEPESvia the trachea or pulmonary artery followed by freeze substitution using 0,5% uranium acetatein methanol.

Sampling

For the examined samples of lung tissue to be representative of the whole organ, all lung segments should have the same probability of being sampled and examined. This is best achieved by randomization of the sampling procedure according to, among others, the methods of systematic, stratified or isotropic, uniform random sampling and vertical sections. For small rodent

TABLE 1. Basic parameters for lung morphometry and the stereological methods to estimate them. Reproduced with permission from reference (5). D = dimensions.

Parameter (Dimension)	Example	Method	Test System (Dimension)
Volume (3D)	Lung parenchyma Alveolar septal tissue	Point counting (Figure 6a)	Test points (0D)
Surface area (2D)	Alveolar epithelium Capillary endothelium	Intersection counting (Figure 6b)	Test lines (1D)
Length (1D)	Fibers	Transect counting (Figure 1)	Test planes (2D)
Particle number (0D)	Alveoli Type II cells	Top counting (Figure 7)	Disector (3D)
Mean particle size (3D)	Alveoli Type II cells	Derived from volume and number or Local stereology (nucleator etc.)	Test points (0D) and disector (3D) Test lines (1D)
Mean linear intercept (chord) (1D)	Airspace size mean free distance	Chord measurement (Figure 8) or Derived from volume and surface area	Test lines (1D) Test points (0D) and the lines (1D)
Barrier thickness (1D)	Alveolar septum Blood-air barrier	Derived from volume and surface area (arithmetic mean barrier thickness) or Intercept length measurement (harmonic mean barrier thickness)	Test points (0D) and lines (1D) Test lines (1D)

TABLE 2. Comparative qualification of results of different methods of lung fixation for morphometry. Reproduced with permission from reference (5). GA = glutaraldehyde, OsO4 = osmium tetroxide, UrAc = uranium acetate, FA = formaldehyde, PFA = paraformaldehyde, EA = ethyl alcohol.

	Airway Instillation		Vascular Perfusion	Rapid Freezing
GOAL to preserve	**2.5% GA → OsO4, UrAc**	**FA, PFA**	**GA / OsO4 / UrAc / EA**	**Freeze substitution**
Lung volume	++	-	+++	-
Internal architecture	++	-	+++	-
Parenchyma	++	-	+++	-
Airways and vessels	++	-	+++	-
Tissue fine structure	+++	+	++	-
Capillary blood	+++	+	-	-
Cell structure	+++	-	++	-
Surface lining and edema	-	-	+++	+
Cells: molecular identity	-	+	-	+
LM	+	+	+	+
TEM	+	-	+	-
SEM	+	-	+	-
LSM	-	+	-	+

lungs, 100-200 measurements on 50 fields of view on 10 tissue samples from each lung pair usually suffice.

Reference parameters

Morphometric methods usually yield results expressed in units of density or ratios (percentages), which are meaningful only when they refer to a reference volume, e.g. lung or alveolar volume. For determination of lung volume, the physical immersion method and the histologic or imaging Cavalieri method are available. According to the immersion method, the lungs are submerged in normal saline and the displaced fluid volume is determined, whereas according to Cavalierithe lungs are sliced into parallel sections of equal thicknesst and total lung volume V is estimated as the productof slice thickness tmultiplied by the sum of the surface area A of each section ($V = t \cdot \Sigma A$). the former method overestimates lung volume by 10-15% but is technically easier and more suitable for small lungs (e.g. rodent lungs).Reference sub-volumes that are included in total lung volumeare stereologically estimated at the microscopic level, as detailed below (Figure 2).

Lung parenchyma morphometry

Serial examination of the lungs is recommended at gradually increasing magnification, from the macroscopic to the hypermicroscopic level, in a cascade procedure. For estimation of volumes (e.g. tumor, interstitial tissue, or vascular volume) point counting is employed, for surface estimation (e.g. alveolar surface) counting of the length of standard linesencompassed in the structure of interest is used, whereas for the estimation of the particlenumber (e.g. tumor or alveolar number) the dissector method is employed. Based on these determinations, mean particle size (e.g. of tumors or alveoli) can be estimated as the ratio of total particle volume and particle number, as well as complex measures such as alveolar-capillary barrier thickness and pulmonary diffusing capacity. Using similar methods, analysis of hypermicroscopic, airway, vascular, and other structures is possible.

Biopsies

Stereologic analysis of open, transbronchial, and transthoracic lung biopsies is hampered by several problems and should be undertaken using additional caution. Biopsies are not obtained at random sites, biopsy tissue is often crushed, fixation technique is often suboptimal, and lung volume can be only determined by imaging.

Quantitative structural analysis andin vivo imaging

Stereology can be combined with contemporary imaging techniques such as computed tomography. Imaging should be complemented and validated by

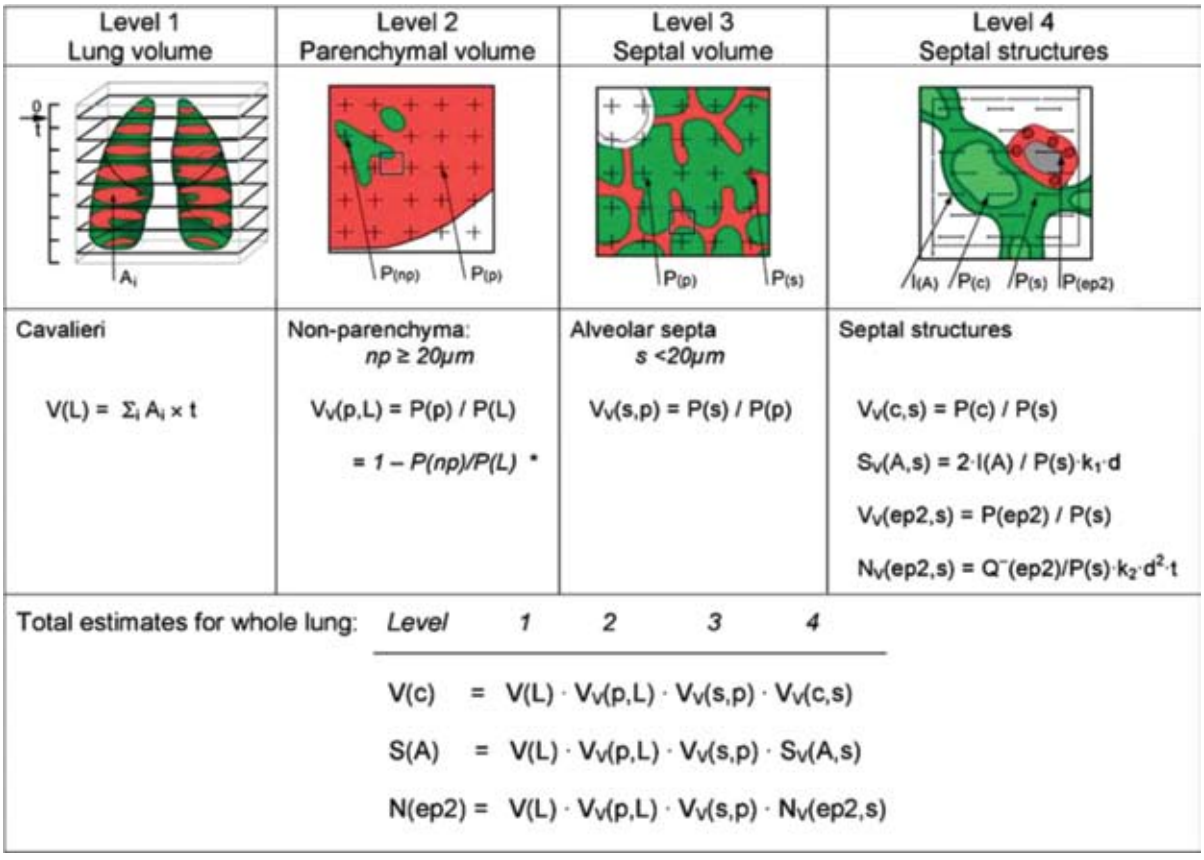

FIGURE 2. Estimating morphometricparameters of lung parenchymausing multistagestratified sampling at fourlevels of increasing magnification.The parameter estimatedat one level becomes the referenceparameter at the nexthigher level. This approachallows calculation of total estimatespertaining to the wholelung and permits efficientsampling. Level 1 is Cavalierisampling, allowing estimationof lung volume. Level 2 andlevel 3 sections are overlaidwith a simple point grid toestimate volume fractions,whereas at level 4 an electronmicrograph is overlaid witha multipurpose test systemcomprising a set of test linesegments within an unbiased-counting frame (SECTION 2). *Because nonparenchyma occupiesa small fraction of thelung, it may be more efficientto estimate VV(np). Reproduced with permission from reference (5).

microscopic stereometry. In addition, more dense lung components (e.g. interalveolar septa, tumors) are possibly over-projected and over-estimated by imaging.

CONCLUSIONS

Stereology is characterized by precision, objectivity, and efficiency, and provides a set of tools for sampling andmeasuring irregular structure. These tools are flexible in thatthey can be applied to a variety of imaging approaches. Forthese reasons, this approach has become the gold standard inquantitative structural analysis of different organs, including thelung.The recent establishment of standards for lung stereology by the American Thoracic Society and the European Respiratory Society is anticipated to significantly contribute to the more efficient and precise analysis of lung structure in the clinic and the laboratory and to further developments in the researchinto respiratory diseases.

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Conflict of interest: The author does not report and perceived or real conflict of interest with the article matter.