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Original Article

A review of state-of-the-art stereology for better quantitative 3D morphology in cardiac research

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Abstract

The aim of stereological methods in biomedical research is to obtain quantitative information about three-dimensional (3D) features of tissues, cells, or organelles from two-dimensional physical or optical sections. With immunogold labeling, stereology can even be used for the quantitative analysis of the distribution of molecules within tissues and cells. Nowadays, a large number of design-based stereological methods offer an efficient quantitative approach to intriguing questions in cardiac research, such as "Is there a significant loss of cardiomyocytes during progression from ventricular hypertrophy to heart failure?" or "Does a specific treatment reduce the degree of fibrosis in the heart?" Nevertheless, the use of stereological methods in cardiac research is rare. The present review article demonstrates how some of the potential pitfalls in quantitative microscopy may be avoided. To this end, we outline the concepts of design-based stereology and illustrate their practical applications to a wide range of biological questions in cardiac research. We hope that the present article will stimulate researchers in cardiac research to incorporate design-based stereology into their study designs, thus promoting an unbiased quantitative 3D microscopy. © 2010 Elsevier Inc. All rights reserved.

Keywords: Stereology; Myocardium; Quantitative microscopy; Number; Surface; Length; Volume; Immunogold labeling; Electron microscopy

1. Current status

Qualitative histological and ultrastructural images often accompany cardiac research articles focused on physiological, biochemical, or molecular biological techniques. While the latter aspects are usually dealt with in great detail, the microscopic images are often merely illustrative. A lack of awareness of the pitfalls related to microscopy, as well as prejudices about the scientific value of microscopic observations [1], tends to limit the use of more advanced and quantitative microscopic methods. Even worse, the ineffectual application of quantitative microscopic methods may introduce unpredictable errors leading to erroneous biological interpretations and conclusions. Table 1 provides a number of typical sources of bias in quantitative microscopy.

Ideally, quantitative microscopic methods provide hard, biologically useful information (e.g., about the total number, length, surface, or volume of structures within a well-defined reference or containing space), without making assumptions about the size, shape, orientation, or distribution of structural features. A design-based study, coupled with appropriate quantitative tools, can provide unbiased or minimally biased data. Furthermore, the methods should be simple and provide a precise estimate of the real value at low cost; that is, they should be efficient. Finally, the results should allow testing of a relevant scientific hypothesis by appropriate statistical methods. Design-based stereology [2] provides a portfolio of methods that fulfill these criteria and is, therefore, the gold standard in quantitative microscopy.

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Table 1
Typical sources of bias in quantitative morphology

Typical sources of bias in quantitative morphology	Recommended methods
Assumptions about shape, size, or distribution of structures (e.g., "mitochondria are evenly distributed within cardiomyocytes")	Avoid any assumptions
Anisotropy (e.g., "cross-sectioned papillary muscle was used for estimation of sarcolemmal surface area")	Apply the isector or orientator to generate IUR samples Randomize orientation
Tissue shrinkage (e.g., "myocardial tissue shrinkage was assumed to be equal in young and old mice")	Choose an appropriate embedding medium, measure tissue shrinkage, use the fractionator for number estimation
Reference trap (e.g., "the volume fraction of connective tissue was higher and this indicates that the volume of connective tissue was higher")	Use the Cavalieri principle or liquid displacement to estimate total volume ("never ever not measure the reference space") and then calculate total volumes, surface areas, length or number
Choice of samples, sections, test fields (e.g., "this area serves well for analysis" or "representative fields were selected")	Apply systematic uniform random sampling or the fractionator principle Give every part of the reference volume an equal chance of being included in the analysis
Overprojection	Make sections as thin as possible. As a rule of thumb, make section thickness <1/10 the size of structure
Volume-weighted mean volume ("e.g., total volume was divided by volume-weighted mean volume to obtain the number of...")	The volume-weighted mean volume includes information about size and size variability Estimate both number- and volume-weighted volume

Although quantitative morphology is a traditional and theoretically well-founded approach in cardiac research [3–7], the development of new stereological techniques has not yet replaced the use of other morphometric methods carrying potential sources of bias. Despite the fact that, in other areas of research (e.g., pulmonary research [8,9], kidney research [10], placental research [11], and neuroscience [12]), these new methods are already routinely applied or have been incorporated into journal policies [13,14], the use of modern stereology in cardiac research is still rare. This may be explained by the fact that several unbiased methods, for example, number estimation of cardiomyocytes [15] or quantification of immunogold labeling [16,17], have only been available for a few years. In addition, the use of stereological methods requires certain knowledge about sampling theory that is often thought of as being sophisticated. The latter, we believe, is at least in part caused by the fact that there is no comprehensive article that relates the new stereological tools to their practical applicability in cardiac research. Therefore, the present article aims to provide a practical guide for the estimation of volume, surface, length, and number of cells and organelles as well as the intracellular distribution of antigens labeled with immunogold particles.

2. Problems in quantitative microscopy and their solutions

2.1. Sample size reduction and sampling strategies

One of the major problems in microscopy is size reduction, which occurs as a consequence of taking small pieces of a large organ. However, the necessity to pick a

Fig. 1. Illustration of stereological work flow. A rabbit heart fixed as a whole (A) is used to measure the length of capillaries, the luminal surface area of the capillary endothelium, and the volume of cardiomyocyte mitochondria in the left ventricle. The left ventricle including the interventricular septum is embedded in agar and placed in a tissue slicer (B). Starting at a random position outside the ventricle, the tissue is totally cut into slabs of defined thickness, in this case, 3 mm (C). The nine slabs are placed with the same side up (D) and a random number is chosen between 1 and 2, which determines which slabs are used for light or electron microscopy. Here, the chosen number is 1. In a systematic uniform random fashion, the slabs sampled for light microscopy are 1, 3, 5, 7, and 9. Before further processing for microscopy, a point grid (area per point, 14.90 mm^2) is placed onto the samples and points hitting the cut surface of each slab are counted and summed, in this case, 95 (E). According to the Cavalieri principle, the volume of the left ventricle is calculated to be V(lv):=95×3 mm×14.90 mm^2=4246.5 mm^3. After randomization of the orientation (see Fig. 3) and processing for microscopy, test fields are obtained by systematic uniform random sampling. An unbiased counting frame (with an area of 25,920 μm^2) is projected on light micrographs, and capillary profiles within the counting frame are counted, in this case, 38 (F). From the number of profiles, the area of the counting frame, and the volume of the left ventricle, the length of capillaries in the left ventricle is estimated as L(cap, lv):= 2×(38/25,920 μm^2)×4246.5×10^9 μm^3=12.46 km. A line grid projected onto the test fields is used for counting intersections of the lines with the luminal surface of capillary endothelium, in this case, 20 (G). From the number of intersections, the total length of the test line (the length of one test line times the number of lines hitting the reference space; here, 21), and the volume of the left ventricle, the luminal surface area of the capillary endothelium is calculated as S(cap, lv):= 2×(20/622.22μm)×4246.5×10^9 μm^3=0.27 m^2. For estimation of the volume of cardiomyocyte mitochondria, both light and electron microscopy are needed. The two points at the end of each test line in Panel G are regarded as points and the number of points hitting cardiomyocytes are counted, in this case, 29. A point grid is projected onto randomly sampled electron micrographs and points hitting cardiomyocyte mitochondria are counted, in this case, 5. From the points hitting cardiomyocytes, mitochondria, and the reference volume (in Panel G, cardiac tissue; in Panel H, cardiomyocyte) as well as the volume of the left ventricle, the volume of mitochondria in the left ventricle is calculated as V(mi, lv):=29/42×5/12×4246.5 mm^3=1221.71 mm^3.

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