Basic Application of Stereology in Histology and Medical Sciences

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The science of stereology is newfound, extremely active and rapidly developing. Stereology has a special place in three-dimensional studies of quantitative biology and histology. Thus, studies that have used proper stereological techniques, have particular value. This scientific method relies on applied mathematics and statistics with the help of a set of rules, which enable various parameters such as volumes, dimensions, components and the number of components to be estimated. Although measuring parameters in histology such as length, weight and counting parts seems easy, when these parameters are evaluated at the microscopic level, practice approaches are not simple and time efficient. For determining these parameters under microscopic conditions, direct measurement or use of routine techniques are not an option and microscopic techniques are required.

Stereology provides knowledge about shapes, images and stereograms, and has pragmatic methods to recognize images and enables calculation of volumes and volume ratio, the area of samples, the number of particles per unit volume, particle size, unit volume and etc. Thus, this technique is very important to obtain reliable quantitative data for various researches in the field of histology.

Stereology is generally three-dimensional measurements of microscopic structures. In histological methods we encounter two-dimensional sections or microscopic images. The science of stereology is used to obtain quantitative information based on the observation and analysis of two-dimensional tissue sections or microscopic images in three-dimensional space (1). In fact stereology relies on the knowledge of mathematics and statistical analysis, to obtain extract three-dimensional data from two-dimensional images. Numerous stereological methods have been developed and published, each being specific for different histological tasks. Methods defined in this technique are essential for proper understanding of the structure of tissue, cells, cellular organelles, and other particles in the body of living organisms. On the other hand, understanding the above content is extremely important for studies of histology, embryology and pathology. Today, the science of stereology is the best option for obtaining three-dimensional information from two-dimensional images. It is based on geometric probability and integral geometry of the test feature (2).

Stereology is derived from a Greek term and means the study of objects in 3D. Analysis of 3D goes back to ancient Egypt and was developed with the introduction of Euclidean geometry (3, 4).

Bonaventura Cavalieri (1598-1647), an Italian mathematician and astronomer, was a precursor of infinitesimal calculus. In 1635, during the height of the Italian Renaissance, he provided the basis for volume estimation of biological objects from their areas on tissue sections. Cavalieri in his book, Geometria Indivisibilibus Continuum: Nova Quadam Ratione Promota (1635) used what is now known as Cavalieri's principle and discussed and strengthened his principle of indivisibles. He stated that "the probe can be used for imaging of a line, lines can be an image of a region, and regions can image size. In this regard a line is made of inDnite number of points, a plane is made of inDnite number of lines, and a solid is made of inDnite number of planes, each indivisible being capable of generating a continuum of a next higher dimension by continuous motion"(5).

At that time there was another problem when calculating the length of objects. George Leclerc Buffon in 1777 explained this subject by introducing the 'Needle Problem'. He explained how to calculate the surface area and length of biological objects in an unbiased and accurate manner (2, 4).
In fact, stereology was found by a French geologist named August Delesse in 1847 AD. In his article it was stated that, the characteristics of mineral components in two-dimensional cross sections are directly proportional to its three-dimensional volume. Subsequently, it was demonstrated that, using Delesse principle, the volume fraction of a test component could be estimated by placing a network or point grid over the sample section and by counting the points lying over the test component. Similarly, we can measure the surface density of a test component from the number of intersection points composed of membrane traces of the test body with a test line system placed on the sample sections.

Historical researches continued, when finally Professor Hans Elias introduced stereology in 1961 as a scientific discipline. In the 1960s, for the first time, biologists could see biological objects with high resolution and much clearer because of the new powerful microscopes (high-resolution light microscope and corrected electron-microscope) and histological procedures (like immuno-based methods and immunocytochemistry) for visualization of specific structures. With these facilities and mathematical-based methods, biologists focused on obtaining authentic 3-D information from 2-D figures of biological objects. In the first decade of stereology (1961-1971), the ‘Needle Problem’ and Cavalieri and Delesse Principles were commonly used by biologists. Since biological structures did not fit classical models (Euclidean formulas based on classical shapes such as spheres), from 1971 to 1981, biologists used stochastic geometry and probability theory and unbiased sampling strategies for accurate quantification of these structures. With this strategy it could be possible to quantify number, length, surface area and volume without further information about the size, shape, or orientation of the objects. By the 1980s, the main question was how to make reliable counts (3, 4).

One of the most important tips for accurate counting of particles in certain structures of the body is to have knowledge of the principles that have substantial importance in these counts. In other words, the counts and the numbers obtained from the research must be trustworthy in order to produce reliable scientific and practical use (2).

Given that arbitrary-shaped 3-D objects do not have the same chance of being sampled by a 2-D sampling probe, thus the number of profiles per unit area in 2-D does not equal the number of objects per unit volume in 3-D. This matter was first introduced as the Corpuscle Problem (Wick sell, 1925). Since 1925, a large number of correction methods have been proposed. Formulas based on these correction methods can cause bias (systematic error) in the results. One of the important features of modern stereology (unbiased stereology) is its ability to partially solve this problem (3, 4).

Before 1984, methods used to estimate the size and number of particles were dependent on assumptions made by that study. Because of these assumptions, the traditional methods of measurement were bias. These methods are called model-based methods. New methods are without assumptions about the subject of the study, and in addition to better efficiency, they are unbiased. These approaches are called design-based methods. The most important issues during estimating the total number are bias and inefficiency. When a method has bias, correct estimation is not possible. In this case, even if extensive work is done for the study the results are unimportant, because the performed estimation has been diverted from the actual amount and this deviation cannot be corrected with further work (8). After introduction of the Dissector Principle in 1984, biologists were able to count objects with an unbiased method, thus there was no need for correction factors and assumptions; when using dissector and unbiased counting rules (Gunderson, 1977), biologist could estimate the number per unit volume of tissue while double counting (bias) was avoided. Also to remove the effects of tissue shrinkage during counting, the fractionator method could be used. Other techniques such as the nucleiator and rotator are used for unbiased estimation of object sizes (3, 4). One of the most important benefits of modern stereology in histology is reduced workload with proper and reliable sampling, so that the information obtained from the study can be generalized for the whole structure (1). Since biological structures are three-dimensional, measurements in two-dimensional histological slides are biased (7, 10). Thus, our challenge is to extract the unbiased three-dimensional information from two-dimensional slides. This is what stereology enables us to do in histology.

Due to the variety of unbiased stereological methods, planning of the entire stereological workflow is the most important prerequisite for obtaining unbiased quantitative data from microscopic images. The key to a successful stereological study is the deliberation made during the planning phase; on the other hand all steps of the quantification workflow are interdependent as each step is built on the previous one. By considering unbiased tissue sampling and unbiased probing of microscopic images, a biologist will succeed and have accurate and precise estimation of all stereological parameters.

Today, computerized stereology systems have increased the speed of data collection and the stereology software has allowed for analysis of tissue at any orientation. The advent of this system leads to an accurate assessment of stereological parameters with minimal human intervention that allows testing of biological hypotheses (11-15).

Researchers in the field of biology have introduced unbiased stereology as the best practical method for quantitative histology. Stereology is used in determining the area and volume of biological structures, length of fibers or the number of cells. Stereology has an important role in authenticating and denying experimental hypothesis in a biological research. With this technique, we can answer the following questions: How much a tissue has been affected by a factor? Has the volume of a tissue changed? Has the length of blood vessels changed? Is the number of cells in a tissue from test subjects different compared to controls?

Nowadays, in medical sciences, stereology assists in diagnosis and treatment through the study of tissues, cells, organelles and spatial location of cells during the disease and treatment by modern microscopic calculations. Also it contributes to the development of science in the field of histology during health and disease through the study of volume, surface, length and number of the biological objects.

Non-invasiveness, speed, simplicity and lack of bias in stereology makes this method widely used in the medical field. Stereology is extensively applied in cell biology and medical image analysis. In the recent years, many researchers have used stereology for quantitative analysis, for example, in the fields of cancer cells, tumor grade and prognosis of patients. This method is also extremely valuable in oncology, radiology, neurology, dermatology, and etc. Tumor characteristics including tumor size, tumor cell density, three-dimensional structure of cancer cells and nuclei can be investigated with stereology (9, 16). Quantitative radiology calculations can be performed using the principles of Stereology and radiology images. Proper sampling, image segmentation, image registration, data base exploration, 3D reconstruction, non-invasiveness, finite resolution and image artifacts of medical images must be considered for reliable quantitative analysis. Considering to these principles we can obtain a deeper understanding of the structure and function of the human body and a more objective diagnosis of disease and assessment of its response to treatment. These goals are possible with stereological methods (17, 18). For example the structure of the skin has been studied extensively using stereological techniques. Stereology has made it possible to quantify structures in each layer of the epidermis, while volume fractions and surface densities of intracellular organelles and their variation throughout the layers of the epidermis have been established (19). Quantitative morphology of the central nervous system has undergone major developments. Several approaches known as design-based stereological methods have become available and have been applied for neuro-morphological studies. Stereology can analyze numbers of neurons and glial cells within brain regions, lengths of brain regions, lengths of vessels and nerve fibers within brain regions and so on (20). In many studies, effects of different drugs on volumetric parameters of brain regions such as cerebellum were investigated using stereological methods. Also modern stereological techniques suggest approaches for quantifying neuropathological changes associated with neuropsychiatry, aging and degenerative diseases like Alzheimer and schizophrenia (22-24).

Stereology is also used in the study of infectious and physiological diseases. For example diabetes and chronic periodontitis are major public health problems. Scholars are looking for treatments to control blood glucose and cure diabetes. Some studies investigated the protective effect of compounds like sodium tungstate, *Eucalyptus globulus* and *Tamarindus indica* against streptozotocin (STZ)-induced beta-cell damage by means of stereological methods. These studies by stereological estimation of volume density and total volume of islets and beta cells, volume weighted mean islets volume, mass of beta cells, islets, and pancreas and total number of islets suggested that these compounds partially restore pancreatic beta cells and repair STZ-induced damage (25-27). In another group of studies stereological methods were used as a complementary method in diagnosis of diabetes and oral complications. On the other hand, in studies done on periodontitis, stereological indices of dental pulp (28) and the level of tissue destruction and periodontal disease progression (29) had been investigated. Stereological parameters such as volume fractions, volume per unit volume of the gingival components had been analyzed using Cavalier's point counting method. Results showed that periodontal disease affects stereological parameters of pulp.
Stereological methods can be used by understanding what is being measured. We must be proficient during the entire process however there is no need to extract methods from basic geometry. Often, the results of the measurements are used to compare one structure with another and in some cases data are needed to assess the relationship between geometric properties of a structure and its functions and characteristics. Stereological fundamental tools are used in biological research, materials science, etc. There are differences in sampling techniques and cutting of materials and how to describe the results, however, the basic procedures are the same. Design-based stereology can provide valuable information about histological, physiological, functional and biochemical properties of organs. It has statistical background and can produce accurate information. In addition, it allows researchers to achieve appropriate precision in their studies. With detailed planning we can overcome the current pitfalls of stereological evaluation. To achieve successful planning, we need to know how many samples are needed, and definition of parameters is essential to handle the desired hypothesis. Moreover, the method of further tissue processing is critical for a successful study. Stereology is not difficult but requires an appropriate design for sampling, data collection and statistical analysis.

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Learning of histology and histopathology by undergraduate students in Medicine and Science at UNSW was limited by the technical and practical barriers imposed by conventional microscopy such as difficulty with mastering optimum focus and illumination, students being isolated at their own microscope limiting collaboration with peers and demonstrators, problems with loss or breakage of glass slides, differential staining and tissue section variability, many.

4. Counterstaining is the application of a second stain to the section to stain additional components of the tissue. Usually the second stain produces a complementary colour reaction to that of the first: e.g. Eosin after Haematoxylin.