Original article

Application of stereological methods in Health sciences

Batigália, F.[1]*, Boer, NP.[2], Boer, ALR.[3] and Marcatto, G.[3]

[1]Head of Discipline of Clinical Anatomy, São José do Rio Preto Medical School – FAMERP, CEP 15090-000, São José do Rio Preto, SP, Brazil
[2]Head of the Department of Anatomy, Fernandópolis Educational Foundation – FEF, CEP 15600-000, Fernandópolis, SP, Brazil
[3]Medical Student, Camilo Castelo Branco University – UNICASTELO, CEP 15600-000, Fernandópolis, SP, Brazil
*E-mail: batigalia@netsite.com.br

Abstract

Stereology is defined as a Discipline consisting of a set of statistical-geometric procedures in order to obtain quantitative information of three-dimensional structures from their two-dimensional images. It becomes a methodological system for the determination of three-dimensional structures by means of mathematical interpretation of histological sections, which uses Mathematics, Geometry, Probabilistic Statistics, and above all, common sense as its main tools. This paper focuses on explaining stereological principles, techniques, grids, sample size and programs. Stereology is a quantitative and comparative method that uses planes, lines and points for the estimation of three-dimensional parameters of morphological structures from two-dimensional parameters, using isotropy and randomness as prerequisites. Stereology seems to be an applicable tecnique in Health Sciences to mathematically demonstrate the relationship among the components density of a three-dimensional structure.

Keywords: stereology, sample, program, Health sciences.

1 Introduction

In the past, a technical persistent problem in Histopathology would lie in the difficulty in obtaining quantitative data on the spatial distribution of morphological tissue components and their changes during growth, functional activity and disease (CHALKLEY, 1943). The French geologist Delesse mathematically demonstrated in 1847 that the relationship between the area occupied by a structure in the study area and another two-dimensional structure that it contains is equal to the volume density of the structure under study, thus, it empirically demonstrates the possibility of using areas to estimate volumes (MANDARIM-DE-LACERDA, 1995).

Stereology is defined as a Discipline consisting of a set of statistical-geometric procedures in order to obtain quantitative information of three-dimensional structures from their two-dimensional images (ELIAS, 1971). Thus, Stereology performs interpretations of flat images (represented by sections or projections) from precise criteria of geometric probability. Stereology is also a branch of Applied Mathematics (ELIAS, PAULY and BURNS, 1978) that provides comparative three-dimensional quantitative parameters of morphological structures through two-dimensional counts (TOMKEIEFF, 1945). In Histology, Stereology becomes a methodological system for the determination of three-dimensional structures by means of mathematical interpretation of histological sections (ELIAS, PAULY and BURNS, 1978), which uses Mathematics, Geometry, Probabilistic Statistics, and above all, common sense (TOMKEIEFF, 1945) as its main tools.

2 Stereological technique foundations

The objects of sterelogical research at histological level are the tissues, which are consisted of compact solid structures, configuring a set of three-dimensional bodies composed of a volume, surface and some geometric properties. The concept of density is fundamental in Stereology due to the nature of the work material: cuts or sections. A cut results from the intersection of a plane with a structure and its components, and the two-dimensional image of the structure cut or its components is called a profile. Stereology seeks to determine the relations of a profile with the dimensions of their respective three-dimensional structure, and its ultimate goal is to mathematically demonstrate the relationship between the density of the component profiles to the profile of the continent structure, and the density of the components in three-dimensional structure (MANDARIM-DE-LACERDA, 1995).

Stereology depends on the transference of the contours of the microscopic image of a tissue section component to the printed paper, by means of a light camera or a photograph (CHALKLEY, 1943). Three-dimensional reconstruction is also a part of the resources of Stereology, and it may be solid or graphic, and assisted or not by the computer (TOMKEIEFF, 1945). In the stereological method, an organ is called isotropic when it has the same structural feature in all directions, therefore, isotropy is a synonym of homogeneity (MANDARIM-DE-LACERDA, 1995). When evaluating many stereological parameters, there is a need for isotropic random sections as a prerequisite (TOMKEIEFF, 1945), expressing a condition of homogeneity in histological sections.

Two basic assumptions of Stereology must be obeyed in any relevant scientific research: Cavalieri and Delesse principles (MANDARIM-DE-LACERDA, 1995). The first principle concludes that there is a need to know all the histological sections of an organ and, counting such sections in a systematic interval way is necessary, depending on the microscopic magnification, the geometrical arrangement of the considered tissue and the thickness of the sections. In the second principle, there is a similarity between partial and absolute parameters of organs or tissues preferentially isotropic universe in which the sampling universe has been selected correctly (TOMKEIEFF, 1945).

3 Stereological grid

Once measuring means to compare as precisely as possible the investigated object with another one (used as a unit), such comparison seems to be governed by strict rules in Stereology. A random stereological standard is a well-defined test system, in which the confrontation (also randomly) of the stereological standards with the researched object becomes possible to estimate the dimensions of the investigated object. In a stereological study, three basic types of patterns can be used: planes, lines and points (MANDARIM-DE-LACERDA, 1995).

The planes are imaginatively considered on the tissue when it is sectioned in a paraffin block with a sharp scalpel. The section surface comes to be regarded, then, as a test plan in a stereological grid, as it travels through the various solid body tissues. The section on solid body tissues begin to be represented as specific areas, the cut surface as lines and through these points are characterized as the Stereological Principle of Dimensional Reduction (ELIAS, 1971).

A three-dimensional object can thus be represented by a line segment that crosses a certain tissue, with the two-dimensional surfaces marked by the intersection points of this line with the cutting surface, setting up a test line system. Thus, by placing a test line and points system of a stereological grid on a certain tissue, a two-dimensional image can be analyzed as a three-dimensional structure. Thus, a test plan of a stereological grid allows us to estimate lengths of lines in space, whereas the surface can be estimated by planes or lines, and volumes by planes, lines, or points (MANDARIM-DE-LACERDA, 1995).

A test system is a set of lines (straight or curved) and points to be superimposed on the morphological image for the stereological counting. The test systems can either be constructed with a regular arrangement or not (in other words, it is random) (ELIAS and HYDE, 1983). They can be printed on transparencies placed on photomicrographs or on screen video monitors, and can also be adjusted within the eyepiece of a microscope for stereological counting (ELIAS and HYDE, 1983; TOMKEIEFF, 1945). The cuts to be drawn or photographed must have the same thickness, and the drawings or photographs to be quantified must be made on a single cut. The standard reference system should also be randomly placed on the drawing or photograph (ELIAS and HYDE, 1983).

Every test system can be obtained by starting from the considered plane, which can be generated by cutting through a tissue block. The histological section, therefore, represents the ideal basic sample for the application of stereological studies. There are several ways to superimpose a test system in a sample cut. In the light microscope, structures with defined areas, lines and points can be adjusted to the focal plane of the eye1 and drawn on a screen where the histological section is projected, or even a device of this plane (DUNNILL and WHITEHEAD, 1972). The results obtained by stereological methods are estimates of the parameters investigated and the quality of this estimate should be tested by available statistical techniques (HALLY, 1964).

4 Randomness and sample size

The most important phases of the stereological research consist in determining the appropriate sample and the right tissue to be sectioned and studied (TOMKEIEFF, 1945). The stereological determinations, based on geometric-statistical principles, are derived from statistical profiles of a section that coincide with the standard system (or reference) chosen. The material samples should be carefully determined at random, from the choice of subjects and tissue blocks to the obtention and analysis of drawings or photomicrographs, as well as being representative of the entire material to be studied. It is always necessary to define sampling criteria for the collection of material for the sections to be drawn or photographed and the regions to be quantified. Among many possible criteria for adopting the sampling at random (taking the risk of studying cuts only of a single area of the material), systematic random sampling (dividing the organ to be studied in fragments uniformly distributed, establishing a analysis criterion so that all regions have the same probability to be analyzed), and stratified random sampling (dividing the organ into fragments, being randomly considered, in each fragment, many areas) (AHERNE, 1967). From the three types mentioned above, the systematic random sampling seems to be the most effective one, followed by the stratified sampling, which is, however, a more practical method, and, therefore, more frequently used in daily practice (WEIBEL, 1969).

The minimum sample size for a stereological study must have 5 individuals, because in Stereology there is always interest in evaluating the increase or decrease of a certain parameter, which fits a binominal statistical distribution with a probability of p = 0.5, that such an event will occur (CRUZ-ORIVE and WEIBEL, 1990; McGILL JUNIOR, McMAHAN, ZIESKE et al. 2000). Thus, if 5 individuals are analyzed, the probability that a certain parameter will increase in all 5 individuals will be $\rho = (0,5)^5$ or $\rho = 0,03$, which is considered statistically significant. The calculation of sample size can be obtained from the following formula (HALLY, 1964; MANDARIM-DE-LACERDA, 1995):

$$RSE = \sqrt{\frac{1-V_v}{n}}$$

where SRE is the relative standard error, Vv is the volume density of the structure and *n* is the number of points that should be counted.

5 Stereological program for compilation of data and statistical analysis

The dataset derived from the analysis of several drawings or photographs (images) related to a tissue block should be introduced in stereological formulas to calculate the desired parameters. The average values for each parameter in several blocks of each considered organism is calculated. The values calculated for each individual are then statistically evaluated, obtaining the mean, standard deviation and standard error of the mean or the mean of the confidence interval, and the results could be presented in tables or graphs (MANDARIM-DE-LACERDA, 1995). Currently, the computerized statistical programs easily allow the researcher to establish the sampling type distribution. Moreover, these programs calculate the measures of central tendency (arithmetic and geometric means) and measures of variability (variance, standard deviation, standard error of the mean and coefficient of variation). Even with the use of computer programs for stereological studies, we must respect the following methodological assumptions: observe Cavalieri and Delesse principles, obtain isotropic and random sections and establish the number of points that should be counted and the correct sample size (TOMKEIEFF, 1945).

In histological studies, the lack of statistical significance is probably a result of a small number of examined cases and, conducting a pilot study may be necessary to plan the sample size (SIMON, 1999). However, even samples of patients selected in non-addictive ways may not be representative of events in a larger population of such patients, due to the effects of random variation in its components. Individual studies have a higher risk of reporting a false-positive result when many analyses of subgroups are performed, and have a higher risk of false-negative result when it lacks statistical power, usually for including an insufficient number of patients or because the outcomes are unusual (FLETCHER, FLETCHER and WAGNER, 1996).

6 Discussion

In specialized literature, there is some confusion about the nomenclature of quantitative studies. Morphometrics is the direct measurement of structures with scales of metric system, and it should be reserved for microscopic studies. However, in cases of macroscopic studies, the term Biometrics is sometimes used instead of Morphometry by French anatomists (TOMKEIEFF, 1945), and also the terms Morphometry and Stereometry are often used as synonyms. In fact, Morphometry is the measurement of structures, while Stereometry refers to Solid Geometry, or the part of mathematics that calculates volumes and surface areas of defined solid structures, such as cubes, spheres and cylinders (ELIAS, PAULY and BURNS, 1978). Direct measurements on structures sections – first adopted by Morphometry – seem to be ineffective and sometimes generate unreliable results. On the other hand, the stereological methods allow the obtention of useful information from simple counting operations. Such techniques were used for many years in Mineralogy and Geology, and have been introduced into Biology in the past decades. With the development of reliable quantitative techniques in Physiology, stereological methods have become increasingly important, and a number of useful techniques have been developed (SALA, MATHEUS and VALERI, 1980). Stereology is a quantitative method much more powerful than the Morphometrics one (CRUZ-ORIVE and WEIBEL, 1990; TOMKEIEFF, 1945).

The importance of the quantitative results is to allow an objective diagnosis, and the data acquisition in most studies in Stereology is directly obtained from images, often in light microscopy or transmission electron microscopy. As a result, the quantitative composition of the organs, tissues and cells may be known. The stereological method can be applied in the organ analysis as a whole or its parts. No assertion is intended to be made about the number of cells or other components in Stereology. Two or more volume fractions or volume fractions in relation to a total volume were compared in this method. This proposal seems to be preferable, since it has physiological, chemical and physical significance to the component studied by relating it to their proportional volume in a certain organ, avoiding only considering the number of compartments in which such volume can be divided (although such information can also be obtained, if necessary) (TOMKEIEFF, 1945).

However, we must highlight that in every experiment in which a random sampling method is used, extreme care must be taken for the design of the study to provide an adequate sample (CHALKLEY, 1943). In fact, all measurements of the stereological method are relative measures, in other words, are the ratio between at least two joint measurements: a reference to the components of a structure, and the other one relating to the structure (MANDARIM-DE-LACERDA, 1995). Thus, Stereology does not intended to ensure the area or volume of tissue components measured, but the ratio of volumes or areas, and presents a high degree of accuracy when applied to the analysis of histological preparations fixed and stained (CHALKLEY, 1943).

When planning a stereological study and processing biological material, it is necessary to bear in mind that the quantitative parameters obtained from the processed material will only be significant if they are representative of vital conditions. The preservation should also be suitable for all tissue components, since "well-preserved" areas cannot be chosen (MANDARIM-DE-LACERDA, 1995). Therefore, an important step to ensure the data integrity of a scientific work is to have control over the randomness process. The manual stereological methods currently used to estimate relative volumes (determination of areas by cutting and weighing drawings, linear integration and point counting) provide equally similar and reliable results. However, when comparing the total time required to make the determinations by different manual methods, the point counting technique seems to be simpler and and faster (YUGOSHI, IYOMASA, MARTINS et al., 1996). The point counting method seems to be as fast, economical and safe as the linear integration method. Moreover, the point counting method (with their various subdivisions) should be preferred for general application, since it provides the study with larger samples (HENNING, 1958).

Clinical Science reaches its peak when the measurements are quantitative, because the information allows better numerical confirmation, more precision in communication among clinicians and between clinicians and patients, as well as allowing an estimation error (FLETCHER, FLETCHER and WAGNER, 1996). Stereology determines quantitative information and is particularly useful in analyzing the normal and abnormal tissue growth normal, such as in tumors or neovascularization. Stereology is also a fundamental tool in studies of comparisons of normal or pathological anatomical and functional states, resulting in proportions of cell number and size. Stereology has provided great technological and scientific development, with current use in various fields of knowledge such as Histology, Petrography, Metallography and Ceramics. A consistent increase in publications has been noted over the past decade involving Stereology in the most prestigious scientific medical and biological journals, as in studies with light microscopy as with transmission electron microscopy (TOMKEIEFF, 1945).

7 Conclusion

Stereology is a quantitative and comparative method that uses planes, lines and points for the estimation of three-dimensional parameters of morphological structures from two-dimensional parameters, using isotropy and randomness as prerequisites. Stereology seems to be an applicable tecnique in Health Sciences to mathematically demonstrate the relationship among the components density of a three-dimensional structure.

References

AHERNE, W. Methods of counting discrete tissue components in microscopical sections. *Journal of the Royal Microscopical Society*, 1967, vol. 87, n. 1, p. 493-508. PMid:5582822. http://dx.doi.org/10.1111/j.1365-2818.1967.tb04529.x

CHALKLEY, HW. Method for the quantitative morphologic analysis of tissues. *Journal of the National Cancer Institute*, 1943, vol. 4, n. 1, p. 47-53.

CRUZ-ORIVE, LM. and WEIBEL, ER. Recent stereological methods for cell biology: a brief survey. *American Journal of Physiology*, 1990, vol. 258, n. 1, p. 148-56.

DUNNILL, MS. and WHITEHEAD, R. A method for the quantitation of small intestinal biopsy specimens. *Journal of Clinical Pathology*, 1972, vol. 25, n. 1, p. 243-6. PMid:5018715 PMCid:477271.

ELIAS, H. Three-dimensional structure identified from single sections. *Science*, 1971, vol. 174, n. 1, p. 993-1000. PMid:4941041. http://dx.doi.org/10.1126/science.174.4013.993

ELIAS, H., PAULY, JE. and BURNS, ER. *Histology and human microanatomy*. 4. ed. Padova: Piccin Medical Books, 1978. p. 195-8, 533-79.

ELIAS, H. and HYDE, D. *A Guide to practical stereology*. New York: Karger, 1983. p. 305.

FLETCHER, RH., FLETCHER, SW. and WAGNER, EH. *Epidemiologia clínica*: Elementos essenciais. 3. ed. Porto Alegre: Artmed, 1996. p. 13-6, p. 238-63.

HALLY, AD. A counting method for measuring the volumes of tissue components in microscopical sections. *Quarterly Journal of Microscopical Science*, 1964, vol. 105, n. 1, p. 503-17.

HENNING, A. Kritische betrachtungen zur volumen und oberflächenmessung in der mikroskopie. *Zeiss-Werkschr*, 1958, vol. 30, n. 1, p. 78-86.

MANDARIM-DE-LACERDA, CA. *Métodos quantitativos em morfologia*. Rio de Janeiro: EdUERJ, 1995. p. 71-109.

McGILL JUNIOR, HC., McMAHAN, A., ZIESKE, AW., TRACY, RE., MALCOM, GT., HERDERICK, EE and STRONG, JP. Association of coronary heart disease risk factors with microscopic qualities of coronary atherosclerosis in youth. *Circulation*, 2000, vol. 102, n. 1, p. 374-9. PMid:10908207.

SALA, MA., MATHEUS, M. and VALERI, V. Estimation of the average diameter of human placental villi by a stereologic method. *Microscopica Acta*, 1980, vol. 82, n. 4, p. 339-42. PMid:7382841.

SIMON, R. The role of statisticians in intervention trials. *Statistical Methods in Medical Research*, 1999, vol. 8, p. 281-6. PMid:10730334. http://dx.doi.org/10.1191/096228099673618608

TOMKEIEFF, SI. Linear intercepts, areas and volumes. *Nature*, 1945, vol. 155, p. 24. http://dx.doi.org/10.1038/155024a0

WEIBEL, ER. Stereological principles for morphometry in electron microscopic cytology. *International Review of Cytology*, 1969, vol. 26, n. 1, p. 235. PMid:4899604.

YUGOSHI, LI., IYOMASA, MM., MARTINS, MP., LOPES, RA. and SALA, MA. Estudo comparativo de métodos estereológicos para estimação de volumes relativos. *Revista Regional de Ciências*, 1996, vol. 6, n. 1, p. 9-12.

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