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Stereology, morphometry, and mapping: the whole is greater than the sum of its parts

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Abstract

The latest developments in computer-based stereology build upon the similarities of classical stereology and computer microscopy to provide refined and effective spatial analyses that also permit mapping of anatomical regions. Classical stereology and computer microscopy have developed along independent pathways as methodologies to provide a quantitative understanding of the structure of the brain. They approach brain morphology and brain morphometry from different points of view. On one hand, stereology has concentrated upon the unbiased numerical estimation of parameters, such as length, area, volume, and population size that characterize entire regions of the brain, e.g. hippocampus, as well as individual elements within them, e.g. cell volume. On the other hand, computer microscopy has concentrated upon providing accurate three-dimensional maps of the morphology of entire regions of the brain as well as of individual elements within them, e.g. neuronal dendrite and axon systems. The differences in point of view are not so extensive as to keep the two methodologies separate. They share, after all, a similar manner of controlling microscope data input and analyzing the images the microscope provides. The incorporation of data archiving permits easier access to previous studies, as well as the sharing of stereological findings and their related maps throughout the scientific community. Some of the stereological systems now integrate spatial mapping with stereological analyses to provide more comprehensive methods to analyze brain tissue.

Keywords: Brain mapping; Computer microscopy; Unbiased stereology; Neurostereology

1. Introduction

The state-of-the-art in contemporary computer based stereology is a fusion of the application of classical stereology (Weibel, 1979) and morphological mapping as performed by computer microscopy (Glaser and Van der Loos, 1965). The fusion of stereological methods with techniques developed for computer assisted morphological mapping has resulted in the implementation of efficient methods that now make it practical to make unbiased estimates on a wide variety of biological material. A second benefit of this fusion is the integration of stereological estimates within an anatomically meaningful framework. In addition, recent developments in contemporary stereology include the emergence of new stereological techniques and analyses that have become possible only with the introduction of computers and imaging instrumentation.

Due to differences in their methodological approaches to the analysis of 3D structures, it has taken more than two decades for this fusion of stereology and computer microscopy to occur. It is interesting to review the historic differences between these two approaches. Stereology's fundamental development has been the unbiased estimation of geometric properties, such as length, area, volume, and population size. Stereological methods have recently become more widespread because there has been serious concern for the validity of measurements arrived at by largely ad hoc methodologies. Various investigators could arrive at startlingly different estimates of these parameters, even when they were outcomes of very similar experimental situations. Which estimate (of population size, for example) was credible, or were any of them? To answer this question, the advancement of stereological theory was principally devoted to developing unbiased statistical techniques that were practical to apply to many types of tissue and that could yield estimates of how precise the resulting estimates were.

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Computer microscopy, on the other hand, has had an entirely different goal — to map the morphology of individual structures accurately in three dimensions without regard to statistical issues of parameter estimation. The stimulus for this was the seminal work of Sholl (1956) who postulated that knowledge of the morphology of neurons and their connectivity was fundamental to the understanding of brain organization. Neurons that could be stained in their entirety (usually by a Golgi related method) were considered individually, so that no statistical concepts needed to be involved except, perhaps, the general idea of selecting specific neurons for study in an unbiased manner. An illustration of the differences of these two points of view is illuminating. Ask a stereologist about the properties of the cerebral cortex and the response is likely to be numerical summaries of the total number of neurons, total dendritic length, total number of spines, etc. Ask a neuroanatomist who employs computer microscopy the same question and the answer is likely to be sketches of pyramidal, stellate, and multipolar cells showing the shapes of their somata; the general shape and orientation of their dendrites and axons, and in what layers they are found.

It has taken a number of years for these two points of view to merge, for stereologists to appreciate that statistical estimates unassociated with morphological insights are inadequate, and for the morphologist to recognize that accurate 3D depictions of subjectively selected specimens tend by themselves to be anecdotal and fall short of providing statistically verifiable morphometric information. The result is the development of a powerful analytical system concept that combines 3D mapping and morphometry with stereology; so that the researcher can first make detailed reconstructions of regions in a selected population of subjects, and then perform a variety of stereological analyses on selected regions in this population.

2. Stereology in brief

The motivating principle of stereology since the 1970s has been the concept of design-based testing. Design-based stereology, as contrasted with the older model-based stereology, relies on systematic sampling procedures that are independent of the properties of the tissue. It discards the early testing principles that were based upon models of the tissue structure. The term 'assumption-free' has sometimes been used as a synonym for design-based to denote stereology's independence of assumptions on tissue morphology, e.g. spherical cell shape. It imposes almost total control over the user, removing subjective choices and options.

An underlying principle of design-based stereology is the systematic random sampling of the specimen. Systematic random sampling assures that a set of sampled measurements will be acquired efficiently and without bias. Computers are well suited to perform systematic random sampling by automating procedures for selecting random origins for coordinate systems and for locating sites where sampling is to be performed. A researcher selects a particular stereological probe depending on what the structures he wishes to quantify are and how he wishes to quantify them. The parameters for using the probe are dictated by the estimated precision he is interested in achieving. A stereological probe is a geometrical entity, e.g. an array of points, lines, cycloids, etc. with a set of counting rules that describe what actions can occur with the interaction, e.g. intersections, of the geometrical entities with the structure(s) of interest (Howard and Reed, 1998). The only decisions the user needs to make are those such as whether the structure is in or out of focus, in or outside a particular boundary, or whether the probe and the structure satisfy defined rules for an intersection. These procedural constraints are imposed so that statistical sampling procedures are properly adhered to. Different modifications of protocol can occur according to the particular probe.

A major topic of interest in stereology has been the establishment of criteria for how much sampling needs to be done to achieve a specific variability in the estimate of a quantity. It is important to select a probe and its associated sampling protocol that will yield enough data to achieve the desired precision of the estimate, while making the work efficient, i.e. by making the fewest measurements needed to achieve this precision. In some cases, e.g. volume estimation, the same data that leads to the estimate of the quantity can also be used to estimate the variability of the quantity estimate. Once the protocol has been fixed, the user is constrained to adhere to it lest the results of the analysis be compromised. These issues have been well discussed in the stereological literature (Gundersen et al., 1988), and the properties of a wide variety of probes and their protocols have been established for dealing with many types of morphology. There is no need to go into detail here.

The result of performing a stereological probe is a number or a set of numbers that represent estimates of the quantity being sampled. Unfortunately, the estimates are not linked in any explicit way to the images of the tissue studied. Consequently, it is difficult to easily replicate the results of a study. Another shortcoming is that estimates of separate tissue regions are considered independently, so there is no convenient way to associate results from one tissue region to those of another that may be of interest. This makes conventional stereological studies difficult to integrate into a broader program of tissue investigation.

3. Computer microscopy in brief

Computer microscopy was developed to quantify the 3D morphology of structures seen in light microscopy, ranging in size from individual cells to larger anatomical entities, e.g. cerebral cortex, and its laminae. The researcher uses a complex set of criteria, which may or may not involve statistical concepts, to select the parts of the tissue that are to be analyzed. The goal is to obtain accurate representations of the anatomical structures. The specific instances of morphology are of interest, not their abstract statistical properties. To achieve this goal, computer microscopy gives almost total control of spatial data acquisition to the user. Early in the evolution of computer microscopy (Glaser and Van der Loos, 1965; Wann et al., 1973; Lindsay, 1977), it was appreciated that the user's powers of observation were the foundation of morphometric analysis; and that automated image analysis was not likely to replace human intelligence for many years in this regard. This is still true for the vast spectrum of neuroanatomical material. As a result, computer microscopy concentrated on providing the user with interactive hardware and software to minimize tasks that interfered with the optimal use of the microscope. In particular, interactive systems that employed computer controlled stages were developed to give the user complete control of microscope stage position either by single translations of the stage or by programmed sequences of movement. This was coupled with computer-graphic overlays of the microscope's image, video images of the tissue sections, and data file structures that facilitated the acquisition, analysis, and storage of morphometric data. From the stored data, 3D reconstruction of morphology could then be performed.

Computer microscopy's aim is accurate 3D mapping and quantification of biological tissue whether it be of individual cells, groups of cells, or entire histological regions. We use the term mapping rather than imaging because the spatial properties are of importance, not pictorial images with color or gray levels that are of no direct importance unless they serve to delineate boundaries and other morphometric features of the tissue. In computer microscopy, the user selects those features of the tissue that are to be studied and then uses a mouse to make an accurately traced reconstruction of the tissue section, or sections if serial section analysis is involved. The reconstruction is in the form of linked 3D boundary contours and/or sequences of isolated points. The contours and points are coded for identification purposes. This can be whatever the user selects, from an individual neuron with its dendrites and axons, to collections of neurons, to anatomical nuclei, to entire brains. The spatial properties of the sample can be analyzed by a variety of techniques (Glaser et al., 1979; Glaser and McMullen, 1984), though these analysis techniques are specialized and may pertain only to the specific type of sample studied. Line lengths and closed areas are measured by tracing and standard computer numerical integration techniques. Populations are exhaustively counted. These are non-stereological measurements and are subject to the shortcomings that stereologists have described. Computer microscopy is not per se a statistically oriented methodology and was not intended to perform sampling operations. Computer microscopy's mapping capability complements stereology's basic shortcomings and provides it with a far broader set of capabilities. The following sections introduce them. Note that the purpose of this paper is to describe how stereological methods are implemented in computer-based systems and not to explain their theoretical basis. Readers interested in acquiring the latter type of information are encouraged to consult such sources as Howard and Reed (1998) and the representative references provided.

4. Advanced computer-based stereology

The integration of classical stereology and computer microscopy has resulted in a flexible system that permits the combination of rigorous stereological measurements with elective user-controlled morphometric maps and studies. Thus, a tissue specimen can be utilized first for anatomical mapping and then as a sample in a strict stereological study. The result is that the mapping serves as a reference framework for the stereology (Fig. 1). The framework allows all aspects of the study, such as the placement of systematic random probes, to be recorded for possible further analysis extensions. In this way, the user can combine particular samples with general sampling results.

4.1. Map-integrated stereology

The title of this section is indicative of a new direction in stereology that emphasizes the importance of morphological information contained in 3D maps. The inability to specifically characterize distinguishing morphological features of a specimen has been one of classical stereology's most serious shortcomings. Now, with the recognition that morphology can reveal more information about a specimen than is contained in simple magnitude measurements of morphometric parameters, mapping is recognized as increasing the value of these simple statistical estimates. Furthermore, mapping provides the framework for the combination of stereological and anatomical data and improves the ability to evaluate the outcomes of separate studies and obtain a better understanding of how variations in estimates can arise.

To provide for the compatibility of stereology with mapping, what is needed is the development of probes and sampling schemes that can be jointly used for estimation and for mapping.

4.2. Point counting versus tracing

Point counting is used generally in stereology, e.g. in area and volume estimation, where counts are made of randomly overlaid lattice points that fall within a closed boundary area. The area and volume estimates are unbiased (Gundersen and Jensen, 1987) but the points' coordinates are not recorded and so no spatial information is preserved, only point count accumulations. For shapes commonly encountered in biology, only tens of points need be counted to attain an estimate with a precision of several percent (Gundersen and Jensen, 1987; Cruz-Orive, 1993; Roberts et al., 1994). This task does not require much time. The primary source of error in this technique is decision errors about whether a point is inside or outside an area's boundary. Earlier, Gundersen et al. (1981) indicated that tracing boundaries around areas was both expensive and time consuming. However, the subsequent development of low cost mouse and data tablet technology has eliminated the cost aspect of this claim. Furthermore, with the advent of new software it is now fast and easy to do tracings of video images displayed on computer.

Though there is no comprehensive study on the errors and biases associated with tracing, the report of Mercer et al. (1990) indicates that with a modest investment of time tracing can yield relative errors of less than 0.5% in area (and volume) estimation. Consequently, in planning a stereological study the user should consider whether the benefits of boundary tracing are worth the extra time investment. The benefits are that it can yield higher precision in area and volume measurements and, perhaps more important, it permits area and volume mapping, which preserves the shapes of structures for subsequent analysis. We point out that the additional time required is usually insignificant in comparison with the large expenditures of time already required to prepare tissue specimens for stereological testing.

4.3. Mapping without overlap

In stereology, the estimation of a region's area and volume is usually considered in a form of isolation — the user's interest is in a particular region to the exclu-

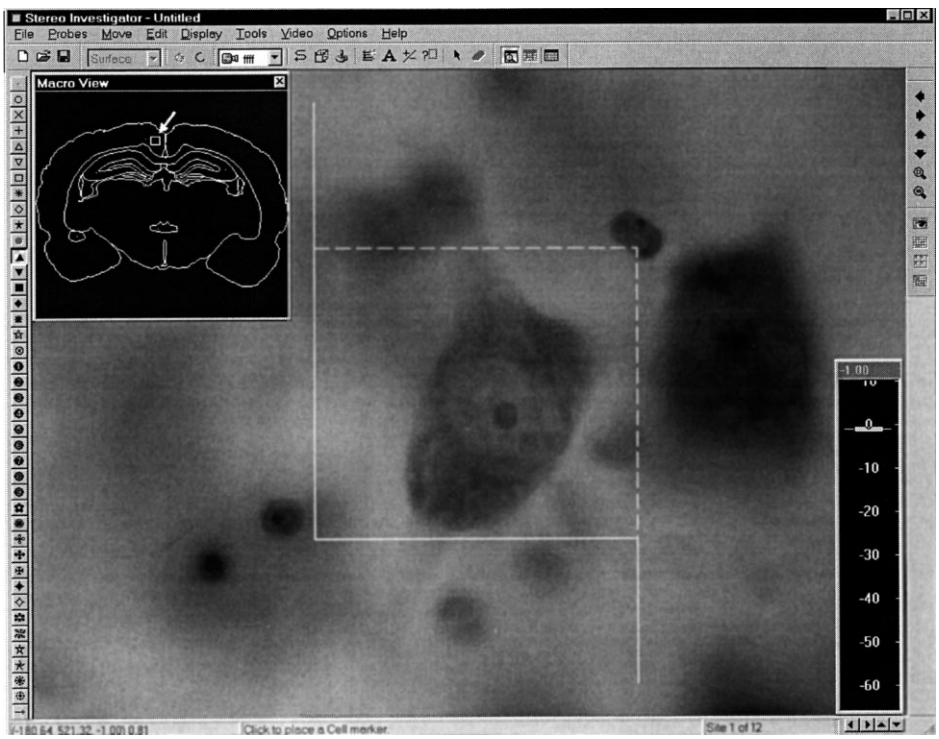

Fig. 1. A high magnification microscope image is shown overlaid with the computer display generated by the software. The software's control functions are located along the edges of the microscope image. A counting frame overlays the image as it would be seen while performing the optical fractionator protocol to estimate population size. At the upper left of the display is a map of the entire section with boundary lines outlining various histological regions. The outline of the area that is in view is indicated there by a rectangle near the dorsal midline. At the lower right is a meter indicating the focal position of the microscope relative to the top of the section. This and the two subsequent figures are from Stereo Investigator™ displays.

sion of all other regions that may border it. There are many situations, however, where the spatial relationships within a group of neighboring regions are of importance. In such situations, it is useful to be able to map such a group of regions jointly and to estimate areas, volumes, and perhaps population sizes as well. On the one hand, the usual lattice point counting method is likely to be unsatisfactory for such a task because, for one thing, lattice sizes that are designed to produce a desired estimation precision for one region may not do so when applied to another region of different size. On the other hand, conventional boundary tracing is done without regard to the boundaries of bordering regions. This means that boundaries traced for one region may overlap those for other regions, or there may be lacunae between the separate regions. This long-standing shortcoming of boundary tracing compromises the accuracy of the maps. It also reduces the accuracy of the stereological estimates that are associated with these maps. However, new 'zero-overlap tracing' algorithms have been developed that guarantee that the boundaries of bordering regions always abut one another and that the individual boundaries are separable so that regions can still be analyzed in isolation.

5. An overview of advanced stereology systems

We now discuss in more detail the general features of computer-based stereology systems that are currently commercially available. They integrate a three axis motor driven stage with a computer in order to acquire data from 3D structures. By and large, they implement the standard stereological probes and estimation procedures for length, area, volume and population size. Some also incorporate a variety of newly implemented analytical tools that can only be performed with the use of a computer, as well as, enhanced versions of earlier protocols that did not specifically require computer assistance. These systems make obsolete the simple translations and rotations of manual stereology and the use of plastic transparent overlays on photomicrographs. It should be pointed out that the features in the various stereological systems vary, sometimes substantially, from one to another, as to which specific stereological probes are performed and how they are implemented. The commercially available stereology systems include: CAST Grid, Olympus Denmark; Digital Stereology, Kinetic Imaging, Ltd; Stereologer, SPA, Inc.; Stereology Toolkit, R & M Biometrics, Inc. However, only MicroBrightField, Inc.'s Stereo Investigator© system, offers all the advanced hardware and software features discussed in this paper.

5.1. Microscope stage control

Tissue specimens are viewed through a light microscope equipped with a three axis computer-controlled motorized stage. The motorized stage is an important component of a stereology system. It is used to map objects that are larger than a single field-of-view, to rapidly access specific locations throughout the entire tissue specimen regardless of optical magnification, and to perform systematic random sampling of the specimen. The stage is positioned by stepper motors having a resolution as small as 0.1 μm. Backlash error can be reduced to a fraction of a micrometer by software correction and position feedback transducers. Scanning speed can be as high as tens of mm/s thus permitting rapid access to all parts of large sections. Stage motion is commanded by either direct user activation or by program control, e.g. scanning, which is established by the user to suit a particular form of data input. We note, however, that though the precision of a stage (step size) in the horizontal (XY) plane is usually in accord with the manufacturer's specification, position repeatability of the stage should be verified by the user for the particular stage in use. Test slides with calibration grids are available for this purpose. The microscope's focus mechanism (Z-axis) can also be equipped with a motor. Typically, this motor is externally attached onto the fine focus knob of the microscope, though now some microscope manufacturers offer microscopes with internal focus motors that can be computer controlled. The measurement accuracy of the microscope's Z-axis is particularly important when performing 3D probes. To achieve the required accuracy for these probes it is often necessary to equip the microscope with a Z-axis position encoder (sometimes referred to as a microcator). This encoder is used to measure the actual focal position of the microscope stage. It is used in conjunction with a focus motor to obtain closed-loop Z-axis operation. If a focus motor were to be used without a focus encoder, the Z-axis measurements would be dependent on the repeatability and accuracy of the microscope's focus mechanism. This is an important consideration since most microscope manufacturers have not designed focus mechanisms to make repeatable measurements. The importance of the Z-axis accuracy can be shown in the following example: if a user specified an optical disector 15 μm in depth to make population size estimates, a depth inaccuracy of 1.5 μm could result in an error of 10% for the total population estimate.

5.2. Data input

When performing stereological analyses all data input is under the direct control of the user. While the major biological use of stereology has been in light

microscopy, its applications now extend to confocal microscopy (Peterson, 1999), electron microscopy, computed tomography (CT) (Pakkenberg et al., 1989), and magnetic resonance imaging (MRI) (Roberts et al., 1993). In keeping with this expanded range of application, some commercial stereology systems provide image file readers that are capable of accepting 3D confocal and MRI image sets, as well as the file formats generated by a variety of electron microscopes and flatbed scanners. Once these image files are read, they can be analyzed by the system's analysis procedures. New types of stereological analyses are constantly being incorporated into the software as applications for them are found. For brevity, we restrict this discussion to classical microscopic applications.

The tissue specimen is viewed on a computer monitor either via a video camera or directly through the eyepieces of the microscope. Direct viewing provides the highest degree of optical resolution and color accuracy, even compared with the high-resolution video cameras that are now available. When using the direct viewing method, computer graphics that provide user control of data input can be overlaid directly on the optical image. This is accomplished with a high intensity, high-resolution miniature display (Glaser et al., 1983). The only commercially available display of this type is the *Lucivid*™ of MicroBrightField. However, many users find it more ergonomic to view a video image on a computer monitor. Also, under the low light conditions that are associated with fluorescence or other advanced microscopy techniques video cameras may be preferable. Typically, most 3D stereology, i.e. the optical fractionator (West et al., 1991), is performed using analog CCD video cameras rather than digital cameras. The reason for this is the probe's counting rules require the identification of the location where an object comes into focus. This requires real-time observation, which is obtained with live, 30 frames per s (fps), analog NTSC or PAL video cameras. While digital video cameras can provide higher resolution images, with frame rates that run from 10 to 0.1 fps, they are typically much slower in image acquisition than analog cameras. In order to work with digital cameras, it is often necessary to include an extra step in the analysis process, an image collection step. This step involves the acquisition of a Z-series of digital images and the storage of the images in the computer's memory. The software can then display the stored Z-series images in real-time to the user for rapid analysis. When using any of these visualization methods, the user views the tissue specimen with the probe's geometric overlay superimposed upon it. With some systems the overlay provides the user with the visual feedback necessary to control the data acquisition. The user then applies the counting rules of the probe by clicking on the sites of interaction of the specimen with the probe's overlay. After the execution of a probe, the results may be presented together with the superimposed maps. Further options are available to the user to edit and otherwise modify data acquisition.

5.3. Data storage organization

Certain stereological systems permit saving all of the acquired data in an object oriented file that contains the 3D locations of all points entered into the file and their relationship to one another, e.g. as a contour or a set of points of a particular type. Other systems save only the raw counts obtained, e.g. the number of cells counted. Several advantages are found in a system that saves all of the acquired data. Using this storage technique permits maps to be easily generated in any desired orientation, stereological computations performed, and other forms of analysis and reconstructions carried out. It follows that large databases associated with specimen populations can be preserved and analyzed by ancillary methodologies according to the user's needs.

6. Stereological procedures

With these basic system features described, we now present the particular stereological procedures as a user would employ them to design and carry out a stereological analysis using an advanced computer-based stereology system.

6.1. Procedural step 1 — selection of the probe

6.1.1. Library of probes

A comprehensive library of probes is required to estimate the variety of parameters associated with different specimen morphologies. In addition, the implementation of these probes must be adaptable and flexible to meet the wide variety of conditions that occur when quantifying neuroanatomical specimens. For instance, it is often desirable to have exclusion regions within larger regions. It is also desirable to implement several probes simultaneously. Among the extensive set of probes that have been developed for arriving at unbiased parameter estimates are the optical fractionator, the physical disector, the Cavalieri probe, combined slope intercept, cycloids, nucleator, and rotator. The more commonly used ones are described here.

6.1.1.1. Population size and density. Probably the most popular probe for estimation of population size is the optical fractionator (West et al., 1991). To varying degrees, the current commercial systems assist the user throughout all the steps of this procedure, including determination of the correct sizing and spacing of the optical disectors to meet preliminary estimations of the

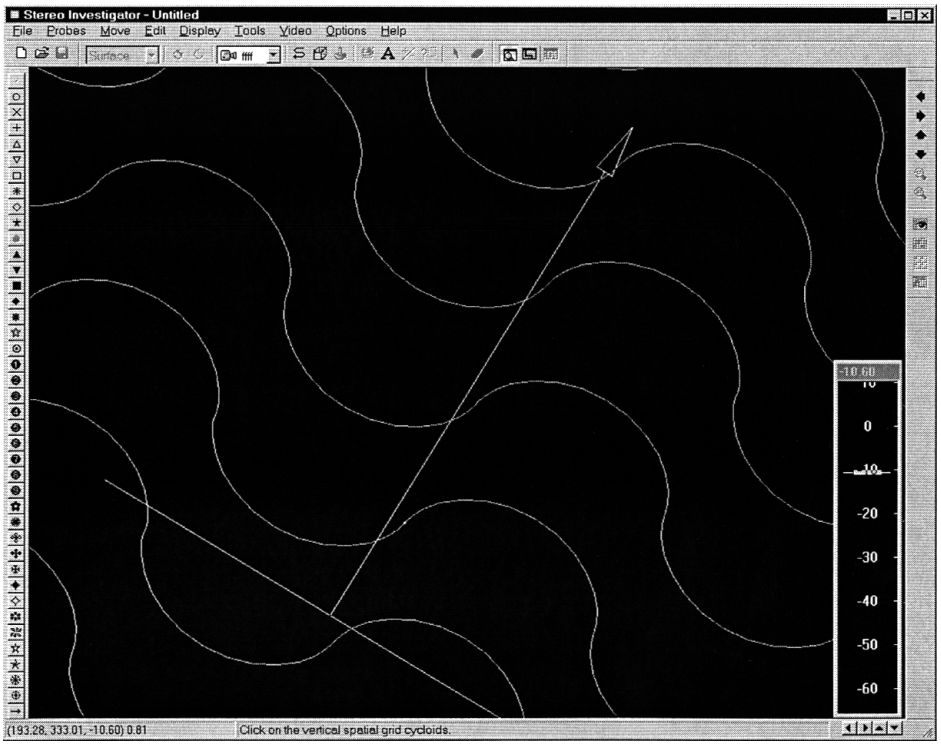

Fig. 2. A cycloidal probe (the vertical spatial grid) is shown as it would be displayed when overlaid on a tissue section (not shown). The vertical axis is oriented in a direction that is selected by the user. The meter at the lower right indicates the depth value of the microscope.

number of disectors that need to be examined to achieve a desired coefficient of error (CE). Advanced systems automatically move from one sampling site to another, selecting only the sampling sites that lie within the area of interest. Graphical depth indicators allow the user to clearly determine if an object lies within each optical disector. Some systems display a Z-axis depth indicator that is visible while the optical fractionator is in use (Fig. 1). The depth indicator is calibrated in μm s and corrected for the index of refraction of the objective lens in use. As a result, the user has more confidence in the reliability of the disector data. Once the probe has been completed, the system automatically calculates the estimated population size using the appropriate estimation formulae. It is important also to provide estimates of the CE in order to allow researchers to choose the one most appropriate for their work, whether it be that of Gundersen et al. (1999), Schmitz (1998), Schmitz and Hof (2000), or Glaser and Wilson (1998).

Another method used for the estimation of population size is the physical disector (Sterio, 1984). This method has not been widely used in light microscopy because it is cumbersome, originally implemented by a pair of microscopes viewing adjacent sections. Several systems use new computer based implementations that eliminate the need for the second microscope. The technique is performed by displaying a properly rotated video image of the first (reference) section with the live image of the second (lookup) section. The simplification of the procedure for the physical disector produced by the elimination of the second microscope has awakened renewed interest in this probe. We believe that its use will now become more widespread.

6.1.1.2. Length. Length estimation can be performed with several types of probes. The choice of probe is dictated by the specimen and the sectioning method used. Contour tracing can also yield accurate length measurements (Mercer et al., 1990). To obtain accurate length estimations well-established stereological techniques use vertical uniform random (VUR) sections and isotropic uniform random (IUR) sections. Both methods are implemented in some current software systems. The preparation of VUR and IUR sections is well described in Howard and Reed (1998). The VUR-based method is implemented by the generation of cycloidal overlays whose orientation is determined by the vertical axis of the section. In earlier software implementations that use cycloids to estimate length in VUR sections it is necessary to align the vertical axis with an edge of the image. This can be time consuming and labor intensive. Recent software refinements allow the user to graphically specify the vertical axis (Fig. 2). This speeds up and simplifies analysis of vertical sections.

An example of a recent stereological probe that can be implemented by only using computer-based techniques, is the method of global spatial sampling with

isotropic virtual planes (Larsen et al., 1998; Calhoun and Mouton, 2000). This addresses a key need of neuroscientists — the estimation of length in preferentially sectioned material. A shortcoming of the IUR or VUR requirement was that this type of sectioning was often impractical when analyzing specimens that required a form of preferential sectioning, i.e. coronal or sagittal, to identify the anatomical region containing the objects of interest. When using isotropic virtual planes, the computer generates a set of isotropic random planes and projects them on to the specimen. The lines formed by the intersection of these planes with the focal plane of the microscope are calculated. The user then uses a set of counting rules and identifies intersection of these calculated lines with the objects in the specimen (Fig. 3).

6.1.1.3. Area. The area of a region in a single section is estimated by using the traditional lattice point probe (Gundersen and Jensen, 1987) or by the area enclosed by a contour traced around the region. Additionally, area fractions can be calculated when integrating traditional point counting with a fractionator.

6.1.1.4. Surface area and surface density. As with length, a number of the well-established techniques for estimating these quantities are based upon the use of vertical uniform random (VUR) sections and isotropic uniform random (IUR) sections. Before the advent of computer-based stereological systems some probes were so difficult to perform manually as to be impractical, like the surfactor; or impossible to perform without the use of a computer based system, i.e. the isotropic fakir method (Kubínová and Janáček, 1998). This is no longer the case. Although the fakir method has not yet come into wide usage, we believe its availability will enhance stereological studies in general.

6.1.1.5. Volume. The Cavalieri method, based on serial section analysis, remains the method of choice. Section areas are estimated by the earlier discussed point grid overlays, or the included areas in boundary contour tracings. Regardless of the probe employed, the volume estimate is obtained from the section area estimates and the spacing of sections. The CE of the volume estimate is also estimated at the same time. When contour tracing is used to estimate a section's area, the maps of the section are saved for later 3D reconstruction of the volume's shape.

6.1.2. Making preliminary population estimates

The determination of which members of a population to sample is beyond the scope of stereology software; but the determination of how many members to sample is not because it requires making a preliminary estimate of inter-subject and across-subject variability, and how the estimates will combine to yield a desired overall estimated variability for the test. The procedure for making a preliminary estimate within a subject is similar to that of an estimate itself, although shortcuts can be taken, e.g. using one section instead of a set of sections, in keeping with the approximate nature of the

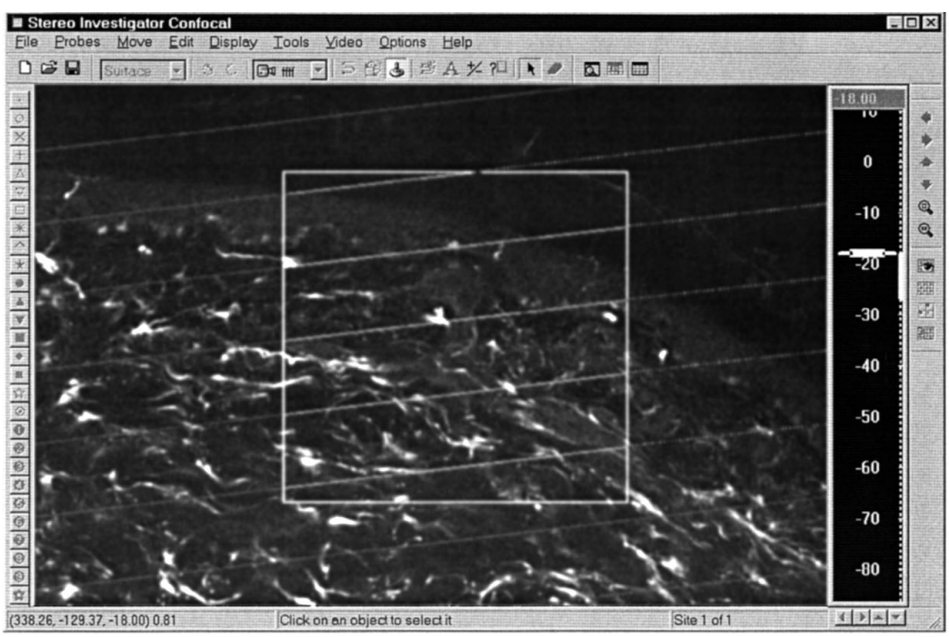

Fig. 3. This confocal image stack is analyzed using the isotropic virtual planes probe to estimate the total length of epidermal nerve fibers obtained by skin biopsy. This probe, which can be used to analyze length in arbitrarily oriented sections, is an example of a technique that could not be implemented without the use of a computer-based stereology system. The confocal tissue image is used with permission of William R. Kennedy, University of Minnesota.

estimate. The procedure for a rigorous estimate made from an individual subject follows in step 2.

6.2. Procedural step 2 — specifying the sampling protocol

The governing principle throughout stereology is that sampling be performed in a statistically appropriate way: random or systematic. To satisfy these requirements, the user must work with one of the basic types of section, random, isotropic uniform random (IUR), vertical uniform random (VUR), or arbitrary. The user must choose at an early stage of the study which type of sectioning to use based on the tissue's properties and the parameter to be estimated. This decision is beyond the scope of stereological software. However, since the user usually knows beforehand the parameter to be estimated, the choice of sectioning will be governed by that knowledge. For example, if the user were interested in estimating surface area or density, his choice would likely be to prepare VUR sections. Only after the sections have been prepared does data acquisition begin and stereological software come into play.

6.2.1. The fractionator

The fractionator principle is a basic component of population size and density estimation. It is a sampling procedure that systematically partitions the tissue that is contained in a single section or in a uniformly spaced series of sections into equal sized volumes (disectors). In each of the sections the disectors are spaced in rows and columns at constant distances from one another. The fractionator exists in two versions, the optical and the physical. In the optical version, each section of tissue is scanned in depth, i.e. from top to bottom, identifying those elements which fit the rules for inclusion within the optical disector and therefore, to be counted. In the physical version, each disector is examined in section pairs for the presence of elements that satisfy the inclusion rules. The factors that determine how to establish disector arrays that will provide a population estimate of a desired precision are: the size of a disector, the vertical and horizontal spacing between disectors, and the random placement of the disector arrays over the region of interest. Some software systems perform these tasks automatically once the user makes a preliminary estimate of population density. Instructions on how to obtain the preliminary estimate are also provided so that the entire population estimation procedure becomes primarily a task of identifying the population elements that are included within a disector. This is a matter of using one's observational skills, skills that automatic image analysis systems still do not possess.

6.3. Procedural step 3 — estimating the precision of an estimate

While the sine qua non of a stereological estimate is its freedom from bias, minimum variability in the estimate is the second most important requirement. The general properties of statistical probes have made the prediction of variability a difficult task to deal with. That is, the statistical properties cannot be explicitly described. Because of this, the technique most resorted to in describing an estimate's variability is that of the coefficient of error or CE (Gundersen et al., 1999; Cruz-Orive, 1999). This approach describes variability in terms of the predicted variance of the estimate and its magnitude with respect to the estimate itself. A proper understanding of CE is essential for planning the size of an experimental animal population and the amount of data to be acquired from each animal. The concept unfortunately has proven somewhat difficult for the typical, non-statistically oriented users of stereology to deal with. Therefore, it is appropriate to seek other more commonly used methods of analysis to describe the variability of a stereological estimate; in particular, the more commonly used confidence limits. How much more useful and revealing it would be to state that the population size of a region is between 95 and 105, with a confidence limit of 5%, rather than that the population size is 100 with a CE of 5%. It is too early to know whether this approach will prove generally practical for stereological probes.

6.3.1. Predicting the precision of a volume estimate

Most of the stereological parameters of interest are obtained with probes that extract information from systematically selected sections. The data obtained from these sections can be considered independent. Straightforward averaging techniques are used to arrive at estimated values.

Volume estimation of an organ or other large structure that is contained in a sequence of sections is one example where the estimation procedure relies on measurements that are correlated across sections. The Cavalieri method described earlier is the favored procedure for estimating volume. Volume estimation can be distinguished from other stereological estimators because it permits the user to estimate the precision of a volume estimate from just a single systematic sample from a subject. Since only a single systematic sample is required, the procedure is applicable to MRI studies of individual patients. This is because it systematically estimates the cross sectional areas of the volume as seen in a set of systematic samples of serial sections through the region of interest. The method (Gundersen and Jensen, 1987; Roberts et al., 1994) for predicting the precision of the volume estimate has been referred to earlier in this paper and has been implemented in some

stereological software. Note that the Gundersen and Jensen equations assume the cross section areas are measured without error while the Roberts et al. equations, use estimated areas that are obtained by lattice point counting. Since the precision of a volume estimate is a prediction, its estimate is itself a statistically varying quantity whose distribution is known only approximately. Some preliminary unpublished studies we have made indicate that if a predicted CE is of the order of 0.1, the CE of the prediction will have a CE of about 0.1. Thus if an estimate's predicted CE is 0.1, the true CE of the estimate is likely to be between 0.09 and 0.11. The controlling factors on precision are the number of sections used and the precision of the cross sectional area estimates of the organ in each of the sections. The implementation assists the user in choosing these factors to arrive at the desired predicted precision. This information is useful in the early stages of planning an experiment.

6.3.2. Estimating the precision of ratio estimates

Ratio estimates are employed in a variety of stereological estimates: cell population density expressed as cells per volume, surface area per reference volume, etc. These ratio estimates are obtained from an estimate of the numerator and an estimate of the denominator. It is important to note, however, that ratio estimates are to be used with caution when dealing with populations (West, 1999). The statistics of ratio estimators are known and are built into the protocols in order to yield the estimate and its estimated precision. State-of-the-art stereology systems use the appropriate equations for calculating the estimates and their precision within each estimation protocol. The equations show explicitly how the various factors contribute to the result.

6.3.3. Estimating the precision of a population size estimate

The estimation of population size requires systematic sampling when the population of interest is so large that it cannot be counted by counting every population member. The accepted procedure of systematic sampling involves: first, systematically sampling a set of serial sections, and then sampling within sections by using some form of the fractionator. The time required to arrive at a population estimate depends upon both the way the population is distributed through the region of interest and the size of the sample taken. It is now clear that, in the case of fractionator estimates, the sample size is more critical in controlling the precision of the estimate than is the population's spatial distribution throughout a sequence of sections (Glaser and Wilson, 1998; Schmitz, 1998; Schmitz and Hof, 2000). There is a simple relationship between sample size and the predicted precision of the population size estimate. The calculation for this estimate and the earlier population size precision estimators of Gundersen and Jensen (1987), Gundersen et al. (1999) and Cruz-Orive (1999), are provided in some of the software systems. Users can easily compare the estimates.

6.3.4. Estimating the precision of group studies

By a group study, we mean a stereological study involving a group of animals or subjects. In such a study the results are pooled to yield an overall estimate of a stereological parameter and an estimate of the precision of this estimate. A large number of studies have discussed the relationship between the precision of an estimate made within a single subject and the precision of an estimate made from a group of subjects. The studies emphasize that inherent biological variability across subjects limits the precision that can be expected from a group study regardless of the precision obtained from a single subject. Some software contain equations that indicate the relationship between individual and group precision and show how to plan stereological studies so as to not attempt to achieve useless within-animal precision. The convenient access to these equations provides another tool to help the user achieve maximal benefit from a stereological analysis.

6.4. Second generation stereology

In its current state stereology provides us with a variety of tools for obtaining unbiased estimates of average or total length, area, volume and population size. It can be referred to, therefore, as mean value stereology or as first order stereology (Howard and Reed, 1998). However, biological structures have far more morphological complexity than can be described by these first order properties. It is now time for stereology to go beyond its first order limitations and deal with estimating parameters of more complex spatial attributes. This will open a more advanced form of stereology. Stereologists refer to it as second order stereology, which seems to imply that the new stereology will be much like first order stereology except in incorporating the estimation of spatial gradients of length, area, volume, etc. We favor the term second-generation stereology because we believe that the new stereology will depart from classical stereology in major ways, including the nature of its probes and its sampling techniques. One particular example of structural complexity that has been resistant to analysis with first order stereology is the neuron. The stereology of its length, surface area, and volume only partially reveal what there is to be known about neuron morphology. For example, Glaser et al. (1979) and Glaser and McMullen (1984) provide non-stereological analyses of the orientation of dendrite systems. As for current stereology, it tells us nothing about the regional variability of cell population density or of the orientation

of neuronal dendrites. Having said this and acknowledging the role that stereology can play in dealing with such analyses, it seems clear that a great deal of work is needed in developing stereological probes and statistical sampling concepts to address these issues. Similar considerations are encountered when dealing with cell populations when more than population size is of interest. Such properties as the spatial gradients of cell density and nearest neighbor distances require methodological advances beyond the conceptual models of current stereology. Some examples of steps in this direction can be found in the work of Duyckaerts et al. (1994) and Duyckaerts and Godefroy (2000). Similar statements can be made with respect to estimating organ shape in general. However, this is only the simplest aspect of what is needed in achieving a better understanding of the spatial properties of tissue. It seems inevitable, however, that the new stereology with its emphasis on quantifying spatial structure will need to employ and extend the mapping techniques that are currently available.

7. Summary

With the advent of advanced computer based systems, stereology has now attained maturity as a practical laboratory methodology. Its range of application extends from light microscopy to other forms of imaging that provide data on the 3D properties of biological tissue. While stereology can still be practiced in its traditional form with simple microscopes and a minimum of computer assistance, its greatest advantages are obtained when it is integrated into computer based microscopy systems that expedite data collection, storage, and analysis. Further benefits are now available when stereology is coupled with the morphometry of individual structures and the 3D mapping of assembled structures.

One of stereology's shortcomings at present is that its estimation techniques are limited to acquiring simple numeric data concerning averages of, volume, area, length, and population size. Its range of analysis needs to be expanded to deal more intimately with image content and how the structures revealed in these images are distributed in space. This new phase of stereology, second order or second generation, will greatly extend our understanding of biological structure and its variability, whether the stereology be applied to individual structures or to assemblies of them.

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