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The 21st Century Anatomist and Histopathologist: Moving from "Smaller, Focal, Widespread, Bigger, Larger and Greater" to Numbers

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ABSTRACT

Traditionally, histologists and histopathologists have relied heavily on verbal and qualitative descriptions of tissue structure. Histological sections are used to define the normal appearances of cells, tissues and organs. These slides, therefore, also define pathological appearances which may be a consequence of a disease affecting an organ. The traditional approach to morphological analyses was mainly descriptive and therefore largely influenced by the researcher or observer and insensitive to minute changes. In certain pathological cases, quantitative analysis may be required to detect subtle morphological changes, such as small changes in cell number. Relatively new design-based methods provide the tools for obtaining accurate, precise quantitative structural data from tissue sections. These tools are sensitive enough to detect small changes by combining statistical sampling principles with geometric analysis of the tissue microstructure. The estimates yielded by these methods are statistically valid, truly three-dimensional, and referential of the entire organ. Though, the new unbiased methods (including computer software) have been in use for some years, these tools and their uses are still poorly understood by many researchers. These new tools are commonly referred to as unbiased stereological methods. Stereology being the quantitative study of three-dimensional structures from their two-dimensional images or profiles is ideally suited to the quantitative analysis of tissues and organs.A lot of quantitative estimations encountered in biological science(especially by histologists and histopathologists) often involve estimation of cell population and volume. This paper traces a little of the past of stereology, gives an overview of unbiased stereology and discusses some of the stereological approaches for obtaining unbiased estimation of cell population and volume in biological tissues in very simple and easy-to-apply formats by researchers (based on personal experience acquired in Nigeria teaching Stereology at the postgraduate level for over ten years at the College of Medicine of the University of Lagos and expertise acquired from training at the Morphometry and Stereology Laboratory, Charles R. Drew University of Medicine and Science, Los Angeles, California, USA).When used correctly, these tools offer a statistically relevant and practical approach to achieve number and volume estimation without bias from cell size, shape, or orientation.

Keywords: Stereology, Morphometry, Cell number, Volume estimation, 2-D, 3-D.

INTRODUCTION

Traditionally, histologists and histopathologists have relied on verbal descriptions of tissue structure. The traditional approach to morphological analyses was mainly descriptive. These previous approaches have seldom favoured a mathematical methodology. A major consequence therefore is that a correlation between structural and functional data has, in the past, been difficult. While functional studies have tended to produce quantitative data, structural studies (especially anatomy and pathological anatomy) have tended to be qualitative.

Conventional methods usually involve assumptions about the size, shape and distribution of cells in the organ or any other structure. Such assumptions are seldom true, and may lead to systematic deviations that cannot be corrected.

Morphological changes were largely reported qualitatively, and these are only fairly accurate. Histological and pathological descriptions used terms like "large", "small", "few", "many" "absent", "present" "focal", "widespread" or subjective judgments like '+ / ++ / +++'. These terms were helpful (especially, in the initial stages of many scientific studies) and in deed still useful as they are sometimes sufficient to describe the basic features of a section. However, they are not good enough to; for example, statistically test for significant changes of appearance, which may result from a disease, experimental treatments or other modulations. To do so, anatomists, pathologists and other biologists need to attach numbers to the more or less subjective terms used in the descriptions because answering more penetrating questions commonly requires quantitative data. A

correlation between structural and functional data is extremely difficult with these descriptive terms. These traditional approaches to counting, such as counting cell profiles, are biased. In addition, calculating relative density in sample regions may not reflect a change in cell number or be representative of the whole tissue, as a lot of investigators report, believing that errors cancel out, which very often are actually accentuated.

Moreover, the human visual system is highly optimized for pattern recognition[1], *i.e.*, morphological changes forming discrete patterns (*e.g.*, an inflammatory infiltrate or tissue necroses) are easily recognized. However, the human visual system is not optimized for spatial or density information, i.e., differences between objects in densities or sizes are difficult to detect, particularly if the objects cannot be viewed close together. Subjective assessment of primarily quantitative morphological changes is often poorly reproducible[2].

There is therefore a need to search for more objective methods of histological and histopathological descriptions. Stereology (a set of methodological tools-mathematical, statistical and geometric- for obtaining information about three-dimensional [3-D] objects from two-dimensional [2-D] profiles) is useful not only because it allows the study of the structure of entire cells and tissues based on tissue sections or photomicrographs of sections, but also because it allows the study of these structures quantitatively and objectively.

Though the importance of stereology to the anatomists and pathologists is enormous, yet very few of these professionals are using good stereology. Subjective commentary still pervades a lot of studies and diagnostic reports. These subjective assessments of primarily quantitative morphological changes are often poorly reproducible and inaccurate. There is therefore a need to embrace the more objective methods of histological and pathological descriptions. The aim of this paper is to highlight the shortcomings of subjective assessment of structures, the dangers of assumption-based stereology, emphasize the importance of good stereology as well as describe some simple and practical unbiased stereological methods for estimating number and volume of structures (number and volume estimations being the two parameters most frequently assessed) in biological science based on existing concepts.

Objectivity, Reproducibility and Sensitivity

Qualitative histopathology has limited sensitivity to detect changes in cell number. Depending on the tissue, the magnitude of the change in cell number must probably reach 25 to 40% before it can be appreciated by the pathologist[3]. For example, in a study by de Groot *et al.*[4], a 33% reduction in total hippocampal neuron number was not detected even with side-by-side comparison of photomicrographs. If subtle alterations in cell number are to be appreciated and reported, sensitive quantitative methods are required.

Objectivity refers to judgment, inferences, outcomes and conclusions based on observable phenomena and uninfluenced by personal emotions, individual perceptions, preconceptions or personal prejudices. Reproducibility, a major principle of scientific enquiry, is the ability of an entire experiment or study to be replicated, either by the researcher or by someone else working independently. If research is to be a process of discovering facts and creating knowledge through scientific methods by establishing evidences and theories, and reaching new conclusions, then studies must be both objective and reproducible.

To achieve objectivity and reproducibility in histological and pathological studies and reports, there is a need to attach numbers to the more or less subjective terms used in the descriptions - and almost all terms which could be called subjective can be associated with numbers. The use of quantitative techniques imposes a greater degree of objectivity and reproducibility in the assessment of morphological features. Detection of morphological changes that escape subjective judgment is possible by quantitation, and early stage lesions detected with quantitative morphological methods can provide new and frequently essential insights in the pathogenesis of disease processes[2,5,6].Stereological methods offer practical and scientifically valid approaches forobtaining accurate and precise quantitative estimates of subtle structural changes in tissues from histological sections[3]. These stereological methods are accurate (unbiased) and precise (reproducible) for counting cell numbers and estimating volume of structures.

Some common Misconceptions on Sections, Slides and Stereology

Some erroneously believe that human body is uniform and homogenous. Though this belief is very popular, it is a misconception. In reality the opposite is the case. Organs are not uniform in shape and consistency, and the concentration of cells changes throughout the body. In order to give a true representation of an organ's cellular distribution, appropriate stereological techniques must be deployed and used to garner3-Dinformation from 2-Dsections.

A 3-D structure looks completely different in a 2-D section: volume is seen as area, length is seen as dots and surfaces are seen as boundaries. Direct interpretation from a 2-D structure from the human body is most likely to be misleading.

Some scientists and researchers think that stereology is too time-consuming and expensive. This is not correct. Stereology is cost-effective and can be time-saving.

Some assume that an advanced mathematical

background is required for scientists to use stereological tools. This is far from the truth. No advance knowledge of mathematics is required to understand stereology.

There is also a misconception that the use of stereology always requires computerized hardware and software systems. Stereology can be done without these systems.

Sectioning and Dimensional Reduction

It is not usually possible to put geometrical probes into 3-D objects in a way that they can be observed. In the biological sciences and in the materials sciences many objects must be cut, in order to make them accessible to microscopic study of their internal structure. Sectioning, however, reduces solid bodies to flat, 2-D images. One slide or section is just a single, extremely small, 2-D sample of a tissue. Care and caution must be entertained when constructing a mental image of the 3-D structure of a tissue based on 2-D images. A tissue sliced in different ways can present two entirely different 2-D views.

All parts contained within a solid body are reduced in sections to formations one dimension less than their own. The results of this is that sections through solids appear as areas, sections through surfaces (interfaces or membranes)as lines, sections through lines (edges between three cells, or fibres) as points or dots. To state it concisely: a section through an n-dimensional object is, in general, an (n - 1)-dimensional figure. Conversely, an n-dimensional figure in a section results, in general, from cutting an (n + 1)-dimensional object. This statement is calledin stereology the principle of dimensional reduction[7].

Classical and Natural Objects

All objects can be broadly divided into one of two groups: natural or man-made. Both natural and man-made objects exist in three dimensions. Shape and form define objects in space. Shapes are 2-D (possessing height and width), and are usually defined by lines, unlike forms that exist in three dimensions (having height, width and depth). Almost all geometric forms used for constructing man-made objects belong to Euclidean geometry; they are comprised of lines, planes, rectangular volumes, arcs, cylinders, spheres, *etc*. Man-made objects are characterized by classical geometric shapes. Naturally formed objects show relatively high variability in morphological features within the same individual and among individuals in the same population[8].

An important breakthrough occurred in the 1970s when mathematicians joined the International Society of Stereology (ISS) to apply their unique expertise and perspective to problems in the field. These mathematicians (alsoknown as theoretical stereologists), recognized the shortcomings in the traditional approaches toquantitative biology based on modeling biological structures as classical shapes (spheres, cubes, pyramids, rectangles, straight lines, *etc*.), for the purpose of applying Euclidean geometry formulae, *e.g.*, area = πr^2.These formulae, they argued, only apply to objects that fit the classical models, which biological objects do not. They also rejected so-called 'correction factors' intended to force biological objects into Euclidean models based on false and non-verifiable assumptions. Instead, they proposed that stochastic geometry and probability theory provided the correct foundation for quantification of arbitrary, non-classically shaped biological objects[9].

Stochastic geometry is the study of random spatial patterns. At the heart of the subject lies the study of random point patterns. The term stochastic is used to describe random processes, objects, or variables. Even in the most extreme example of individuals following the same model, underlying stochastic processes lead to biological variability.

A stochastic process or event is governed by probabilistic laws. Such a process is one whose state is non-deterministic (*i.e.*, random) so that the subsequent state of the process is determined probabilistically. As a result of stochastic processes, variability is expected in the morphological parameters of individual organisms. Due to this expected variability, as a rule, classical geometry does not apply to biological objects[8].

Common Errors in the use of Classical Geometry in Biological Science

It is not proper to use the formula for the area of a circle (Figure 1) to calculate the cross-sectional area of any tubule in the body of any organism. There is no naturally-occurring tubule or cell or cell organelle that has a cross-sectional profile with this classical shape.

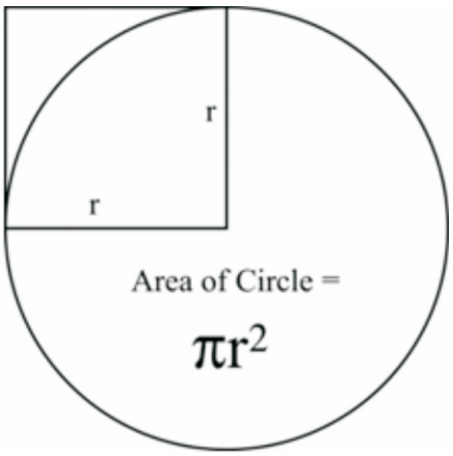

Figure 1: A circle and the formula for its area.

It is erroneous to estimate the surface area of a cell or the volume of an organ or gland by using the formulae for the area and volume of a sphere, respectively (Figure 2), simply because such structure appears spherical in histological slide. Certainly, there is no human structure or any other naturally-occurred object that fits into this classical shape.

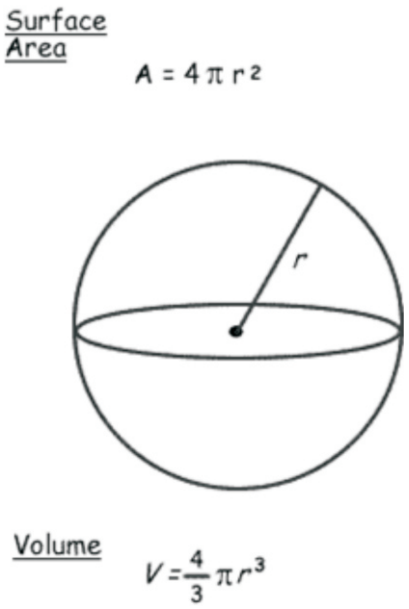

Figure 2: A sphere and the formulae for its surface area and volume.

It is a fundamental error to use the formulae (Figure 3) for the surface area and volume of a cylinder in the estimation of surface area and volume of any structure in the human body. There is no tubule or cell in any organism that fits into this classical geometry.

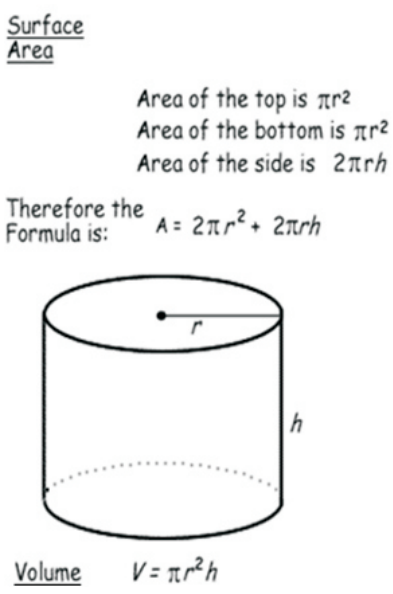

Figure 3: A cylinder and the formulae for its surface area and volume.

If the formulae (Figure 4) for surface area and volume of a cuboid are used to determine the surface area or volume of any naturally-occurring object, stereological bias would be introduced into the estimates. This is because these formulae attempt to force naturally-occurring objects to fit into the Euclidean geometry.

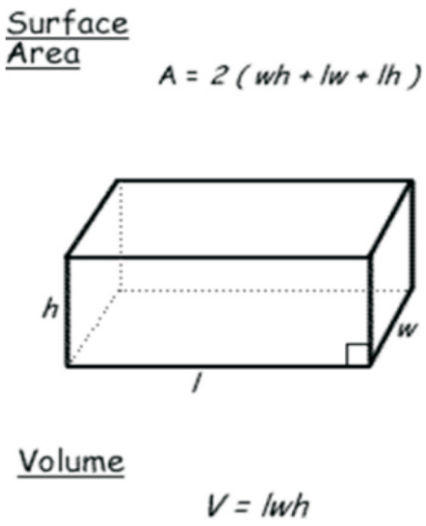

Figure 4: A rectangular cuboid and the formulae for its surface area and volume.

It will equally be erroneous to use the formulae (Figure 5) for the surface area or volume of a pyramid to estimate the surface area or volume of any structure in the human or animal body. To do this is to introduce bias as no naturally-occurring object fits into this classical shape.

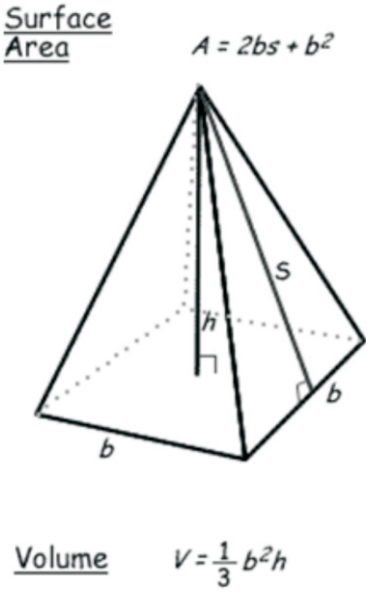

Figure 5: A pyramid and the formulae for its surface area and volume.

Meaning of Stereology

Several definitions will be given in this work for clarity, emphasis and to show the diversity in the use of stereology. Stereology is:

A set of methodological tools -mathematical, statistical and geometric- for obtaining information about 3-D objects from 2-D profiles.

The study of estimating geometrical quantities, the analysis of tissue in 3-D, and the spatial interpretation of

sections.
A set of techniques for extracting 3-D information from 2-D images or sections.

Extrapolation from 2-D to 3-D space, or 3-D interpretation of 2-D images, by methods of geometrical probability.

A set of geometrically and statistically founded methods designed to extract quantitative information about a 3-D structure, based on measurements on 2-D-cut sections. Simply put, it is the extraction or interpretation of 3-D data from 2-D data.

A technique that enables acquisition of data on number, volume, length and surface area of identifiable objects in a 3-D structure by sampling in 2-dimensions.

A body of mathematical methods relating 3-D parameters to 2-D measurements obtained on sections of the structure.
The science of the geometrical relationships between a structure that exists in 3-dimensions and the images of that structure that are fundamentally 2-D.
A set of techniques that combine mathematical statistics with 2-D observations obtained from sections through various objects, to provide quantitative information about the 3-D characteristics of these objects.

Stereometry, Histometry, Image Analysis, Morphometry and Histomorphometry
Stereometry refers to the science of determining the dimensions and estimation of volumes of solid objects, while histometry refers to measurement of tissue and tissue components (including cell counting) or measurement of the microscopic images of tissues.
In image analysis, a software program extracts (semi- or fully automatically) meaningful information from digital images. Image analysis deals with pixels (along a line or within an outlined area) that are converted into real units (*e.g.*,μm) after system calibration. It is essentially a 2-D procedure[10].

A frequent misconception is that stereology and morphometry are the same thing.Morphometry is the measurement of morphological structures. Morphometry is the quantitative description of a structure, *i.e.*, the use of quantitative data in the description of structural features. Thus, morphometry always means a quantitative, but not necessarily a 3-D analysis. Stereological analyses, on the other hand, are always 3-D analyses. Morphometry has a wider meaning than histometry and stereology in the context of biology as it encompasses measurement of size of cells, organisms, nuclei, subcellular components, area and volume fractions of tissue components. Also included in morphometry is a large range of measurements such as numbers, length, surface area, volume, angles and curvature. There are also measurements of shape ratios such as how round or box-like an object is. Distributions and textures can also be measured. Morphometry is mainly 2-D, in which either histological, confocal or electron microscopy images are analyzed.

Histomorphometry is broadly defined as the measurement of the shape or form of a tissue. Histomorphometry refers to the quantitative study (measurement and characterization) of the microscopic organization and structure of a tissue using a computer, manual or automated digital image analysis of images formed by a microscope. It typically involves measurements and comparisons of selected geometric areas, perimeters, length, angle of orientation, form factors, centre of gravity, coordinates, as well as image enhancement. Histomorphometry is not necessarily a 3-D analysis.

The International Society for Stereology (ISS)
There was a meeting of diverse researchers from fields of biology, geology, engineering and materials sciences held in 1961 at a resort on the Feldberg Mountain in the Black Forest of Germany (11th and *12th of May*, *1961*). This meeting was coordinated by a biologist Prof. Hans Elias. The aim of this meeting was to benefit scientists in several disciplines who had one thing in common: struggling with the quantitative analysis of 3-D images based on their appearance on 2-D sections. At this meeting, Prof. Elias suggested "stereology" as a useful term to describe their discussions[9].

Shortly after the first stereology meeting at the Feldberg Mountain, Prof. Elias sent an announcement on the proceedings to the journal Science. Soon thereafter, he received a large response from researchers in academia, government agencies, industries and other institutions around the world. They contacted Prof. Elias for information about the next stereology meeting. The suspicion of Prof. Elias had been right: Scientists across a broad spectrum of disciplines needed new approaches for the analyses of 3-D objects based on their appearance on 2-D sections. The following year, in 1962, the International Society for Stereology (ISS) was established with the 1st Congress of the International Society for Stereology in Vienna, Austria. At this congress, Prof. Hans Elias was elected the founding president. The Society was incorporated in 1963, and included members from the fields of mathematics, statistics, biology and materials science. The official journals of the Society are the Journal of Microscopy and Image Analysis and Stereology (formerly ActaStereologica).

Disciplines Employing Stereology
Stereology is an interdisciplinary field that is largely concerned with the 3-D interpretation of planar sections of materials or tissues. Some of the fields in which stereology is often used include: Anatomy, Neurobiology, Cell Biology, Pathology, Medicine, Marine Sciences, Ecology, Forestry, Metallurgy, Engineering, Geology, Petrology and Botany.

The Central Concepts of Modern Stereology

The following are the core concepts of modern stereology (modified from Mouton)[9]:

1. Modern stereology was developed by biologists, materials scientists, mathematicians and statisticians in the early 1960s.
2. Stereology estimates number, volume, surface area, length and their variability.
3. Stereology is based on stochastic geometry and probability theory.
4. Advanced mathematical background is not required for users.
5. Stereology is applicable to all biological structures, regardless of size, shape or orientation.
6. Stereology is appropriate for defined reference space, rather than arbitrary 'regions of interest'.
7. Values of parameters are inferred from a sample; this is statistical estimation.
8. Stereology uses highly efficient systematic-random sampling.
9. In stereology, objects of interest must be unambiguously defined and identified.
10. Stereology is unbiased for absolute parameters, not ratios, *e.g.*, density.
11. Tissue processing requirements for stereology are different from older methods.
12. Stereology avoids tissue-processing artifacts, *i.e.*, tissue shrinkage or expansion, lost caps, *etc.*
13. Accuracy (unbiasedness) is assured as modern stereology avoids models and assumptions.
14. Stereology does not use inappropriate correction formulae.
15. Sampling is optimized for maximum efficiency.
16. Efficient sampling is based on true biological variability.
17. Stereology does not require computerized hardware-software systems.
18. Appropriately applied computerized stereology systems are efficient.
19. In stereology, statistical power is cumulative for multiple studies on same populations.
20. Stereology is preferred by journal editors and grant reviewers since early 1990s.Stereological studies are increasingly more frequent in literature, particularly in the fields of morphological sciences, development, evolution, pathology and neurosciences.
21. Stereology offers great potential for dissemination of results.

Parameters and Probes

Stereological parameter is a geometrical structural characteristic. In stereology the index to be measured is called a parameter. For examples, the number of Leydig cells in testicular interstitium, the volume of adrenal cortex, the number of neurons in the brain and number of glomeruli in the kidney would be parameters. To measure a parameter (such as the number of Leydig cells), images or sections of the testis are needed. A stereological probe is then used to make the measurements on the images or sections. The probe is a set of points, lines, planes, or other geometrical shape used to quantify stereological parameters. In stereology, a probe is often a grid of points or a grid of lines that is placed over the images or sections. Probes were developed to avoid assumption- and model-based sources of bias when estimating stereological parameters of objects in 3-D.

The number of grid points that "hit" the object of interest is counted. Geometric probes, as they are sometimes referred to, intersect objects in a tissue with a probability that is directly related to the magnitude of parameter of interest. The number of counted points is then used in the appropriate stereology equation to estimate the value of the parameter. The equation or formula is sometimes called the stereological estimator, *e.g.*, Volume:= (distance between images) x (area of 1 grid point) x (Σ grid points count). This is an estimator of volume. The symbol ":=" means "estimate" instead of "equal". Due to the fact that we are sampling, we can only estimate the "truth" we cannot know the "truth". The stereological estimator is therefore, the mathematical equation which with the correct stereological design will provide an unbiased result. So the estimator is the "machine" which requires special sampling modalities and then provides the correct answer with the equation.

Stereological probes can be zero, one, two or 3-D. As a general rule, to be theoretically unbiased (*i.e.*, to get correct information about actual, 3-D structure), number of dimensions in parameter of interest and probe must sum to 3 (or at least 3) (Table 1). For example, to estimate total number (a 0-D parameter), a 3-D probe like dissector is used; for total volume (a 3-D parameter), a point grid (0-D probe) can be employed.

Table 1: Dimensions of Parameters and Probes.

Parameter	Probe
Number (0-D)	Volume/Disector (3 -D)
Volume (3 -D)	Points (0-D)
Length (1-D)	Area (2-D)
Area (2-D)	Lines (1-D)

Importance of Stereology in Histology and Histopathology

Histology occupies a central position in both anatomy and pathology; quantitation is especially valuable in studying structural and functional changes produced by physiological and disease states. Stereology allows the study of the structure of the entire cells and tissues based on thin sections or photomicrographs of sections. Stereology enables the study of these structures quantitatively and objectively. Stereology utilizes systematic, uniform, random sampling to provide unbiased, quantitative data on a number of areas, including the volume, area, numbers, length and size of an organ.

Stereology provides the cell biologist, histologist and the pathologist with powerful and robust tools for describing biological structure in quantitative terms at various levels of organization from the organelle to the organ.Minimal morphological changes can be detected by appropriate stereological tools. Though, visual analysis of microscopic images of cells and tissues has long been in practice for achieving diagnosis of pathological lesions, the pattern recognition may vary from person to person in subjective analysis. However, many definable quantitative parameters like diameter of cells and nuclei, numbers, width, surface area or length of tissue components, may be well analyzed through stereology.

Finally, organelles, cells and tissues, develop as 3-D objects. Due to the fact that these structures mature as 3-D objects, function as 3-D objects and react to toxicants, infectious agents and treatment as 3-D objects, they are best studied in 3-D.

Cell counts have been used to validate animal models of human neurological disorders[11,12]. In the field of neuroprotection, cell counts have been used to demonstrate efficacy of treatments[13], that impact the basis for starting or stopping human clinical trials. One neurological disease where cell counts play a significant role is in the development of models and treatments of Parkinson's disease.
Two-dimensional methods make assumptions about the organ, tissue, or structure of interest and generate data based on surrogates of 3-D tissue structure (*e.g.*, cell profile counts as a surrogate of cell number) and do not report quantitative data referent to the whole organ. Pathologists have historically relied on 2-D cell profile counts to assess effects on cell number; however this is a surrogate, assumption-based measurement, it is insensitive to subtle changes[3].

Cytoarchitectural changes in the testis induced by quinine administration in rabbits have been investigated by Osinubi *et al.*(2005)[14], using stereological techniques. An assessment of the effects of long-term administration of quinine on the morphology of rat testis has been stereologically conducted by Osinubi *et al*. (2005)[15], while a stereological approach was also used by Osinubi *et al*. (2005)[16] in studying the attenuation of quinine-induced testicular toxicity by ascorbic acid in rats.

Number Estimation

Dangers in Counting cells from Profiles

The huge disparities in the figures of many of the estimates done in the past with older methods of morphometry is largely partly due to the making of inferences from 2-D profiles, and examples of these abound in the literature. These examples illustrate the potential magnitude of the systematic error associated with 2-D methods. In a rodent model of testicularatrophy, Mendis-Handagama and Ewing (1990)[17] found 2-D and3-D estimates of Leydig cell number varied by 100%. The probability of particles being cut by single sections depends on several factors, including section thickness, section angle, particle size and particle shape. These are important considerations because a 2-D set of cell profiles does not provide a generally valid indication of the number of 3-D cells which, on sectioning, gave rise to them. Thus large cells (*e.g.*, megakaryocytes, osteoclasts) have more chance of being cut than small cells (*e.g.*, lymphocytes, plasmacytes). Similarly, mononucleate cells generate sectional images which comprise more cell profiles than nuclear profiles because cells are bigger than their nuclei.

Another good example is that a 2-D section through a 3-D kidney results in an irreversible loss of qualitative information and a reversible quantitative change of information[18]. A section through a single podocyte, which results in many unconnected profiles of the podocyte, results in loss of qualitative information (that all podocyte profiles represent just one podocyte). The apparent thickness of the glomerular basement membrane in two dimensions may be larger than the true thickness in three dimensions.

Dissimilar 3-D structures canproduce similar 2-D profiles, and similar 3-D structures canproduce dissimilar 2-D profiles. In addition, the position andorientation of the sectioning plane will influence size, shape,and frequency of these 2-D profiles.
Noteworthy of mentioning is the huge differences seen in the number of neurons and glial cellsin the human brain in many texts. For examples, estimates of neurons in the human brain have been reported various values, though many authors quote about 100 billion[19,20]. Glial cells have been reported to be 10 times more abundant in the mammalian brain than the neurons[21], while others have reported an equal proportion[22,23].

The Corpuscle Problem

Before the field of stereology could gain greater acceptance by the wider research community, stereologists would have to resolve one of the oldest, well-known, and most perplexing problems: How to make reliable counts of 3-D objects from appearance on 2-D tissue sections?[8]. The work of S.D. Wicksell in the early 20thcentury[24] demonstrated the Corpuscle Problem –that the number of profiles per unit area in 2-D observed on histological sections does not equal the number of objects per unit volume in 3-D; *i.e.*, $N_A \neq N_V$.

Causes of the Corpuscle Problem

The Corpuscle Problem arises from the fact that not all arbitrary-shaped 3-D objects have the same probability of being sampled by a 2-D sampling probe (knife blade). Larger objects, objects with more complex shapes, and objects with their long axis perpendicular to the plane of sectioning have a higher probability of being sampled (hit) by the knife blade and are therefore overcounted as shown in figure 6.

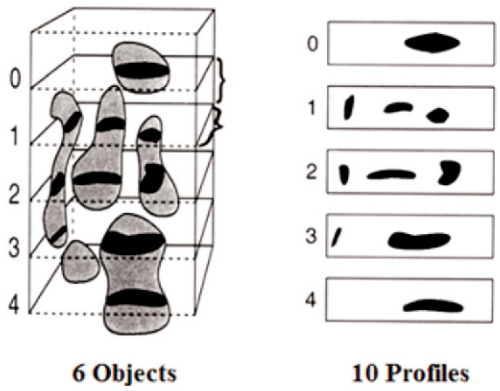

Figure 6: An illustration of how overprojection can result when counts are made directly on 2-D profiles of 3-D objects. (Source: http://www.disector.com)

Wicksell Problem in Summary
The probability of particles being hit by a section depends on:
Particle size
Section thickness
Particle shape
Particle orientation (sectioning angle)
Particle distribution

Attempts at Solving the Wicksell Problem
There have been many attempts to solve the Wicksell Problem, and these include the following:
Report number of profiles per area
Assume specific particle shape (model-based)
Assume all particles have same size and shape
Of course, none of these solved the problem!

The Correction Factors
A close examination of classical geometry reveals a number of attractive formulae that, if they could be applied to biological objects, would provide highly efficient but assumption- and model–based approaches for estimation of biological parameters of tissue sections. Since the work of Wicksell, many workers have proposed a variety of correction factors in an effort to "fit" biological objects into classical Euclidean formulae. This approach using correction formulae requires assumptions and models that are rarely, if ever, true for biological objects. These formulae simply add further systematic error (bias) to the results.

The Wicksell's Model
By modeling cells as spheres and ellipses, Wicksell proposed an early model- and assumption-based correction factor in an attempt to overcome the corpuscle problem.
$N_V = N_A/D$
N_V = number of objects per unit volume (μm^{-3}).
N_A = number of objects per unit area (μm^{-2}).
D = mean diameter of the object (μm).

The Abercrombie Formula
The Abercrombie formula(1946)[25]attempts to convert number of counted object segments to an estimate of the true number of objects in a sample of the sectioned volume.
It attempts to correct for over counting by adjusting the count by a factor based on the section thickness and the average height of the particles being counted.
Expressed mathematically, $N_V = N_A[T/(T+2r)]$ where, N_V = actual object number per unit volume; N_A = number of object profiles counted in area A; T = thickness of section; $2r$ = diameter of structure.
This is a biased formula and should be avoided.

The Floderus Equation
The work of Floderus (1944)[26] and Abercrombie (1946)[25] stemmed independently from Agduhr's correction formula (1941)[27]. Using a geometrical argument, Floderus arrived at a correction formula which, can be written

$N_V = N_A[T/(T+D-2k)]$
N_V = actual object per unit volume.
N_A = number of object profiles counted in area A.
T = thickness of section.
D = diameter of structure.
2k = smallest detectable part of the structure.

Challenges of Correction Factors
The equations are, on their own correct. However, the models that are needed to make the assumptions true do not occur in biological objects.
Very often, instead of correction factors rectifying the errors, the latter are accentuated.

The Basic Principles of the Disector
The year 1984 was a watershed in stereology as it witnessed the introduction of highly efficient and unbiased design-based methods for counting the number of arbitrary objects in 3-D space.
The disector principle is the first theoretically unbiased method used to estimate total number of objects per unit volume.The dissector principle is a technique that allows for unbiased estimation of numerical density (the number of particles per unit volume of reference space.) independent of particle size and shape.

The term disector comes from the composition of the terms *di* for two and *section*. The method developed by D. C. Sterio[28](pseudonym of a well-known stereologist) in 1984,uses two planes, the disector pair and two conventions introduced by Professor Hans JuergenGundersen in the late 1970s: an unbiased counting frame and unbiased counting rules.

Particles are counted with the unbiased 3-D counting rule using pairs of parallel planes separated by a known distance. The only requirement about particle shape is that it must be possible to identify all particle profiles on sections which belong to the same parent particle.

A unique feature of the object of interest is selected for the purpose of counting. For example, the nucleolus, the nucleus, or cell top can be used as the unique counting feature. Essentially what is done is to determine whether or not a unique point on each object, the "top", or the "trailing edge", lies within the volume sampled by the disector.

The Physical Disector

The physical disector consists of two thin sections separated by a known distance, typically consecutive sections for cell counts. The distance between the tops of consecutive physical sections is termed the disector height. The area of the section planes multiplied by the disector height constitutes a volume of tissue that fulfills the requirement for a 3-D probe. Particles are selected in the reference (sampling/counting) section and compared with profiles in the lookup section. If a particle appears in the reference section but not the look-up section, then the particle is counted. This rule ensures that cells are counted only once and avoids overrepresentation of larger cells.

The counting frame of known area with attendant counting rules ensures only cells that "belong" to the sampled field are counted and thus avoids overcounting. The physical disector is used to estimate number of objects in the reference volume on adjacent thin sections. Each disector pair is randomly located within the specimen. The rule is to count only those particles which appear in an unbiased counting frame on one plane (the reference plane) but not on its partner (the look-up plane).

The two planes must be close enough so that it is possible to infer what lies between the two planes. The optimal height of the disector is generally targeted at 1/4 to 1/3 of the object height or diameter. This is to ensure that objects showing on two consecutive planes are appropriately linked and so avoid profiles from same object being counted twice. It also eliminates the possibility that small objects do not escape both the reference and look-up planes, thereby ensuring thatsuch objects are at least profiled by one of the two consecutive planes.

Typically, the counting process is repeated by moving the unbiased counting frame to between 100 and 200 systematic-random locations on 8 to 10 systematic-random sections through the entire reference space. Sum of the objects counted divided by total volume of the dissector probes provides a sample estimate of total number of objects counted in known volume of reference space.

The disector alone yields numerical density (N_V) rather than number (N) itself. In consequence, estimates are sensitive to preparation artefacts such as fixation distortion (shrinkage or swelling).

The Optical Disector

The use of optical sectioning in conventional transmission light microscopy for counting has become known as the optical disector technique[29]. The optical disector consists of a stack of optical sections created by moving a focal plane through a known distance within a thick section[3]. The distance between the stack of focal planes, is termed the disector height. The optical disector is a stereological probe for counting objects in a thick tissue section. This is an extension to the basic disector method, which is applied to a thick section using a series, or stack, of disectors. Rather than using pairs of physical sections (the basic Disector method), optical sectioning is used by creating focal planes with a thin depth-of-field through the section. The focal plane (or optical section) can be moved a known distance through the thickness of the section, producing in effect, a continuous series of superimposed sections within which counting could be carried out with disector counting rules. Counting frame rules are applied when the particle first comes into focus. In practice this consists of counting the number of new objects that come into focus (or alternatively, disappear) as one focuses through a known volume of the tissue. The optical disector also uses a microcator, a device on the microscope for accurate measurement of stage movement in the z-axis.

The optical disector has one very important advantage over the physical disector: it eliminates the difficult and time consuming task of identifying corresponding parts of two physical sections (*i.e.*, determining whether particular object can be seen on one section and not the other). In optical disectors, the sections are always optimally positioned for comparisons compared with physical disectors[30]. However, the physical disectors are advantageous in that the use of thin sections mitigates several concerns encountered with thick sections for optical disectors including "lost caps," z-axis deformation and adequate stain penetration[3].

Guard Volume

A guard volume is a tissue space above and below the 3-D disector probe in which no counting is done. In this region, counting is avoided because as the leading edge of the knife pushes through relatively soft tissue, objects in the path of the knife can be cut, torn from the tissue, or pushed out of the way either above or below the sectioning plane.

A guard volume is implemented by defining the middle of the tissue section. A value of 20-40% is suggested, which should be preserved from damage by the knife. Only the cells that are present in that middle 20-40% are counted. In practice, this means identifying the optical sections within the confocal image stacks that are the top and bottom of the tissue section, and measuring only from the remaining middle 20-40% optical slices. Mouton (2002)[8], on the other hand, has suggested an optimal guard volume of one-fifth to one-fourth of the expected diameter of the object being counted for each

of the guard volumes above and below the disector height, for example, 4-5 μm above and below the disector height for a population of cells averaging 20 μm in diameter.

In essence, embedding the disector within the central portion of a relatively thick section's depth, with guard zones above and below the disector in which no quantification is performed, ensures that estimates of cell number are not biased by cells lost from the surface during the cutting and tissue-processing steps, a phenomenon known as "lost caps"[31]. Thus, the truly quantitative data in are in the central portion of the section depth[32].

The Fractionator

The disector principle solved the problem of how to obtain unbiased estimates of cell number in either thick sections orpairs of consecutive thin sections. In using the disector principle, total cell number is estimated in a two-step process by first estimating average numerical density (N_V) and then multiplying by the organ volume.

Although a robust approach, the dissector method can be cumbersome because it requires measurement of the total organ volume (reference volume) with careful monitoring of any changes in tissue volume or section deformation that occur asa consequence of histological procedures. Changes in the tissue volume that occur during histological processing can be quite extensive and may significantly affect density estimates[3].

Fortunately, a direct estimator, the fractionator, was introduced that obviates issues of tissue shrinkage and section deformation, and the need to estimate total organ or referencevolume[33]. In addition, if total particle number is the only result that is required then the fractionator technique offers a direct and robust method to estimate total number. This method is unaffected by shrinkage and the magnification does need to be known[29].The fractionator was first described in the context of quantitative microscopy by Gundersen (1986)[33]and later refined by West *et al*. (1991)[30]. The sampling principle underlying the fractionator is, however, a very widespread approach that is adopted in a number of scientific disciplines.

The basic principle of the fractionator is that a known fraction of an organ is sampled in one or more sampling steps and in the final sample, the total number of cellsis determined using either physical or optical disectors known respectively as the physical or optical fractionator. The total number of cells in the organ is then estimated by multiplying the number of cells counted in the final sample, by the inverse of the sampling fraction for each sampling step. If a known fraction (f_1) for instance, ½ of the whole organ like the pancreas is taken in a systematic random fashion, this fraction is then embedded into blocks and from the blocks exhaustive serial histological sections taken. The second step is to obtain a second level of a known fraction (f_2), of all the sections for instance, one-thousandth, (1/1000). The final step is to count all particles of interest say, the islets of Langerhans in that fraction of sections. A true estimate of the total number of islets, $N_{(total)}$ in the original organ would be

$$N_{total} = \sum Q \times \frac{1}{f_1} \times \frac{1}{f_2}$$

The only condition that must be met is that the fraction sampled should be explicitly known and that it should have been obtained with uniform random probability, that is, every part of the object had the same chance of being included in the sample before sampling began. The number of levels of fractionation can be extended especially for larger organs.

Disector/Fractionator

In the fractionator/disector method, objects of interests are identified by location[34]. In the fractionator portion of the method, a known fraction of sections through an object is typically selected to count particles within the object[33], while the disector aspect of the method provides a mechanism to count the number of 3D particles in 3-D space using 2-D images[28]. To eliminate sampling bias a random number table is used to choose the first image[35].In the disector/fractionator method, the exact distance between the sample and look-up sections is not required. However, the distance between the sample and look-up sections must be less than the height of the particles being counted. In addition, the estimate of the reference volume is not required. Required are a known fraction of sections (sample sections) and a second section for each sample section (look-up section). The profiles from particles that hit the sample section but not the look-up section (Q-) are counted. Number of objects or cells = (1/F) x ΣQ-, where F is the sampling fraction and Q-, is the number of profiles seen in the sample image but not in the look-up image.

Volume Estimation

Volume is a major stereological parameter of interest in a wide variety of biological and biomedical studies. Volumetric differences are used in research on development and aging, pathological conditions and experimental manipulations[8]. Volume differences caused by cancer cells and other abnormal growths can be estimated using stereology.

Regional volume estimation could yield very important information on the functional and structural units of vital organs of the body. Alveolar number, for example, is closely related to total lung volume, with larger lungs having considerably more alveoli. Volume estimations are also important in studies of comparative anatomy and the relation between structure and function. Volumes and functions of organs such as testes, ovaries and pancreas of various animals can be estimated and compared. In biological applications, researchers are frequently faced with reference volumes that appear as

reference areas on 2-D tissue sections. Classical geometry provides numerous estimators for volume, but these models are generally not appropriate for biological objects.

Estimating Volume of Objects From Weight and Specific Gravity

One way of avoiding assumptions about shape of an object in estimating its volume is to use its weight (W) and specific gravity (SG).If an organ is of constant and known density (specific gravity) then weighing gives a direct method of estimating volume. However, biological tissues often have varying densities, hence this method cannot be applied with any degree of certainty.

Archimedean Principle of Fluid Displacement

Another method of avoiding assumption bias about shape in estimating volume is by water immersion. This relies upon the Archimedean principle of fluid displacement which states that an object displaces its own volume when immersed in water. This method has been used to measure volumes of large organs.

As accurate and unbiased as this method is however, one problem with water immersion volumetry is that if the object has internal cavities that are open to the exterior (such as lungs heart and liver), when the organ is immersed, the cavities fill with water, leading to an underestimate of volume. Secondly this method is inappropriate for very small glands like the pituitary or rat adrenal gland.

Measurement of Volume of Arbitrarily Shape Objects: Cavalieri Principle

The Cavalieri method is a stereological probe for quantifying regional volumes. To estimate volume, the Cavalieri method of segmentation is combined with point-counting. The Cavalieri method involves systematic random sampling through the region of interest. In summary, an exhaustive series of parallel sections are cut through the object at a fixed distance. This section gives rise to a series of thick slices or slabs. Then on the sections that are randomly and systematically chosen, grids with points (associated with known area) are randomly "thrown" over. Each point has an area associated with it. If the points being laid out in a grid has a side length of 'x' then the area associated with each point is x*x, as shown in Figure 7.

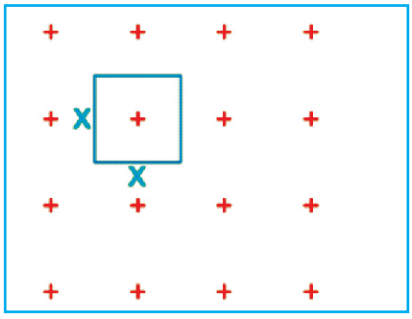

Figure 7: A diagram showing the illustration of points ("+") on a grid with associated dimension "x."

Since each point actually "represents" an area, it means that the area occupied by an object in the section can be estimated by placing a grid of points on the section and counting the points which fall onto the object. In addition, the area estimate for a given section is the number of points falling on the section times their associated area. This is in accordance to the Cavalieri principle, that the total volume of a reference space, $V_{(ref)}$ is proportional to the sum of the reference areas on the cut surfaces of systematic-random sections through the reference space. The formula for estimating volume in this method is the product of the distance between the slices and the sum of the areas on the cut surfaces of each slice as shown in Figure 8, where Volume of the organ = t x ΣA (t is interval between the slices and ΣA is sum of the area of slices A_{1-4}).

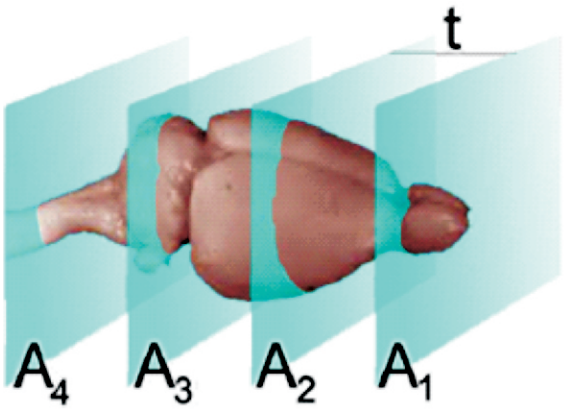

Figure 8: A Diagram Showing the Exhaustive Sectioning of Brain at A1 to A4 and at Intervals T. (Source: MBF Bioscience)

In practice, the Cavalieri approach requires an initial random cut through the reference space of interest, with subsequent cuts at consistent intervals, that is, systematic-uniform-random sampling.This approach allows volume to be estimated from as few as 6 sample slices of an object provided the sectioning and sample selection through the whole object is systematic-random, that is, all sections through the reference space have an equal probability of being sampled.

Estimation of Volume by Volume Fraction

Estimation of volume fraction using cut surfaces was first proposed by a French mining engineer, Delesse in 1847[36]. He noted that on polished sections the area of a phase of interest per unit area of the section was an excellent predictor of the volume of that phase per unit volume of a crushed rock. Delesse Principle is an unbiased method for estimating volume fraction from sections. Delesse's method was modified by fellow geologists who showed that for a randomly positioned test point grid, the number of points hitting the phase of interest, divided by the number of points hitting the whole section, gave an unbiased estimate of volume fraction. In summary, the Delesse Principle is that the fractional area of a component on a section is directly proportional to the fractional volume of that component in the reference space. This principle has been used

substantially in kidney research, for example in the estimation of the ratio of volume of glomerular capillary to glomerular volume. Unlike the Archimedes and Calvalieri estimators, both of which generate absolute values (total volume), the Delesse method estimates a ratio, volume fraction. The Delesse method assumes that changes in the area and volume of object of interest are in direct proportion to changes in the area and volume of the reference space.

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