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3-D Stereological Probes Using 'Optical Sections' in Scanning Light Microscopy

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The tandem scanning reflected light microscope has the property of being able to obtain information from 'inside' solid objects by taking a thin optical section at the focal plane of the objective lens. This plane can be focussed up and down through the specimen.

The microscope works in reflectance and fluorescence modes, providing high contrast dark field images. The microscope can be used on living tissue. Because of its 'serial section' capability the counting of number, collection of 3-D coordinate data and assessment of connectivity are far simpler than in conventional microscopy.

A thorough discussion of the possibilities in stereological research using TSRLM is be given.

INTRODUCTION

The past two years have seen a major shift from model-based biased estimators in stereology, dependant upon stringent geometrical assumptons for their validity, to design-based unbiased estimators which are free from such constraints.

It is now possible to obtain estimates of the number of arbitrarily-shaped particles in a volume from pairs of sections, a known distance apart, provided that the distance is not greater than the height of the smallest particle (Sterio: 1984). This has been called the disector. From single isotropically uniform random sections (IUR) unbiased estimates of mean particle volume-weighted volume may be made, using point-sampled intercept measurements (Gundersen and Jensen: 1985). On 'vertical' sections (i.e sections taken in a preferred orientation) it is now possible to obtain an estimate of the surface of an anisotropic structure (Baddeley et al., 1986). By combining the measurements available on single sections with those from the disector, unbiased estimates of mean particle surface become available in addition to the mean particle volume in the number-weighted distribution. For an extensive review of these methods see Gundersen (1986).

In parallel with these developments there has been a resurgence of interest in scanning light microscopy, which started with the 'flying spot' microscope (Young and

Roberts:1951). This was followed by reports of other developments and, in particular, the advent of the Tandem Scanning Reflected Light Microscope (TSRLM) was reported by Petran et al. (1968). The confocal scanning light has been described by Sheppard and Choudhury (1977) and Brackenhoff (1979). More recently the laser-scanning microscope has been introduced (Wilke: 1983). The theory and practice of scanning light microscopy has been thoroughly reviewed by Sheppard & Wilson (1981).

After the initial activity of the 1950s the attention of the scientific community was diverted by the rapid development of electron microscopy, which offered previously unimaginable resolving power. Progress in scanning light microscopy has, however, seen a resurgence and this has largely been stimulated by the realisation, among biologists, that the microscopes can be used to examine the internal structure and function of living organisms, i.e. for 'vital' microscopy.

The property of 'optical sectioning' possessed by these instruments makes them potentially very useful as tools for collecting 3-D stereological information. This is particularly true of TSRLM, which can be used to examine very large specimens (i.e. intact animals). Most other forms of scanning light microscopy impose a severe mass limitation on the size of the specimen (of the order of 5 grams), which is itself scanned mechanically. Furthermore it appears that this scanning motion of the specimen stage makes the prospect of simultaneous imaging of a cell while making intracellular recordings seem impracticable in anything other than TSRLM. The realisation of the potential of TSRLM in the biological field is largely due to the work of Boyde et al. (1983, 1985). The measurements reported in this paper were made on Professor Boyde's TSRLM at University College, London.

When a conventional light microscope is used for examining very thick sections, a blurred image usually results. The information from the focal plane of the objective lens is present but it is swamped by multiple reflections and refractions from above and below that plane. In scanning light microscopy information from above and below the focal plane of the objective lens may be 'removed', leaving a high contrast image from an 'optical section' about 0.5 microns in thickness. In the TSRLM this is achieved by the use of a mechanical scanning device, the Nipkow disc (1884). TSRLM works in the reflectance or fluorescence modes. The 'optical section'may be racked up and down through the specimen to produce, in effect, an infinite number of serial sections, without physically disturbing the tissue. For a lucid explanation of the physics see Petran et al. (1985).

TSRLM has already been used for counting osteocyte lacunae (Howard et al: 1985) in mineralised bone. This technique is applicable to particles of any arbitrary shape, provided that they can be imaged in reflectance or fluorescence. The technique will be discussed briefly below and some preliminary results of an analysis of 3-D coordinates of the centroids of osteocyte lacunae given.

ESTIMATION OF NUMBER.

A 3-D counting rule using TSRLM has been described by Howard et al. (1985). Although it had been mentioned in a theoretical context before (Gundersen:1981), as an extension of his unbiased 2-D counting rule (Gundersen: 1977),there appears to have been no practical use for such a rule prior to the arrival of TSRLM.

The rule is illustrated in Fig. 1. If an object intersects the 'brick' , but does not intersect any of the hatched surfaces, then it is counted. In this way no particle will be counted twice in a tesselation of such 'bricks'. Clearly if a particle is totally inside the brick, it will be counted.

A schematised implementation of the above rule, using a machine capable of optical sectioning, is illustrated in Fig. 2. A sampling universe containing particles of arbitrary shape is depicted. Within that universe a sampling volume is described by the passage of an unbiased counting frame (Gundersen, 1977) from plane A, through a distance h, to plane B. Any particle cutting plane A at any place (inside or outside the counting frame) will not be counted. As the frame moves, its fully drawn edges describe the 'forbidden surfaces'. These are shown in hatched relief, with the exception of the nearest face (to do so would have obscured the figure). Note that the 'forbidden surface' parallel to A extends, in this figure, to the left of the forbidden line in the plane of B. Note also that the 'forbidden surfaces' described by the forbidden line extend to infinity both into and out of the plane of the picture. In practice it is necessary to have a 'guard volume' at the sides of the sampling volume and below plane B, large enough to inspect the full extent of every particle intersecting the counting frame. In Fig. 2 only particles c and f qualify for inclusion. Furthermore, particle f would have been incorrectly counted as two particles if it were not inspected in the guard volume below B. Particles d and e would be erroneously included if a guard volume was not present. It is evident from this figure that the height of the brick need not be restricted to the height of the smallest particle.

For the physical details of how data were collected using TSRLM the reader is referred to to the paper of Howard et al. (1985). The numerical density of osteocyte lacunae in mineralised compact monkey skull bone was estimated in 4 animals. A coefficient of error of the estimate within animals of the order of 3% was obtained after 30 minutes for each specimen. Four animals were studied and the inter-animal variation was found to be 18%.

SPATIAL DISTRIBUTION

If features under investigation can be assumed to have a point location then there is a body of statistical methods available to investigate the nature of their distribution in space. These methods can lead to valuable insights about the nature of the process which has caused a particular spatial pattern to occur. The main application of spatial point pattern analysis hitherto has been to 2-D data sets. Much of the early work was done by Ripley (1977). An excellent treatise of the

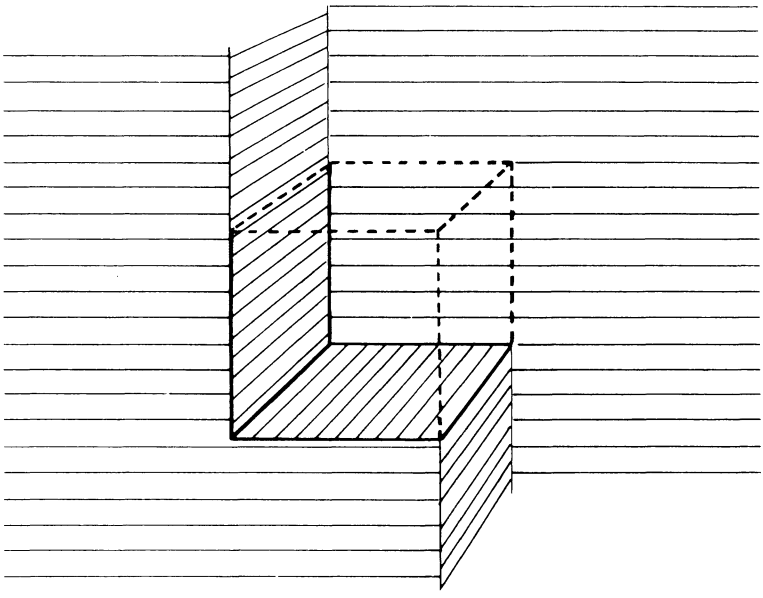

Fig 1. An unbiased 'brick'. See text.

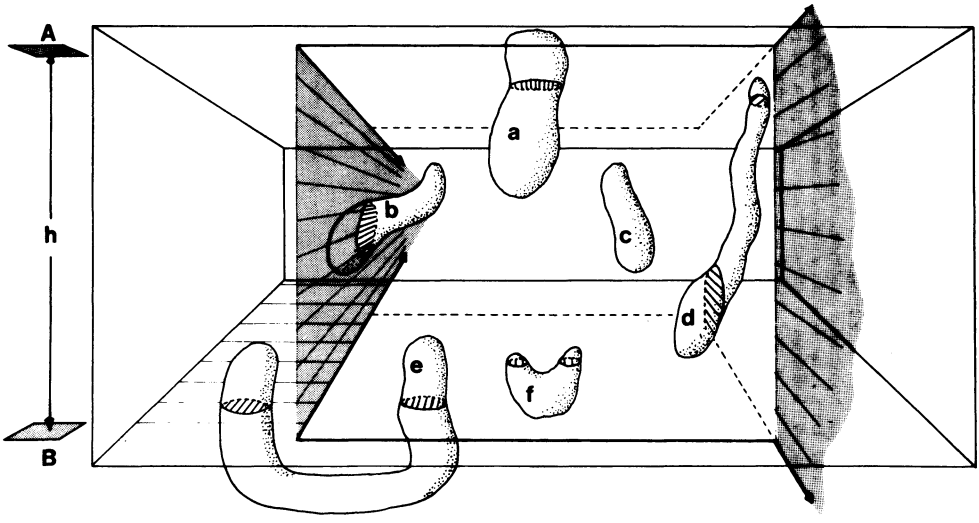

Fig. 2. Schematised counting rule on TSRLM. See text.

state of the art is contained in the book by Diggle (1983). The statistical theory has been extended to higher dimensions but there has, until recently, been a dearth of 3-D data to which to apply these methods.

A large collection of 3-D spatial point co-ordinates of osteocyte lacunae in compact monkey bone has been made using TSRLM (Baddeley et al.: 1986). A TV image of the osteocytes was overlayed with a graticule, graduated on the x and y axes. The x and y co-ordinates of the centroids of individual lacunae were read off the screen while the z co-ordinate was read from the fine focus mechanism of the TSRLM. For an example of the nature of the image from TSRLM the reader is referred to the article by Boyde (1985).

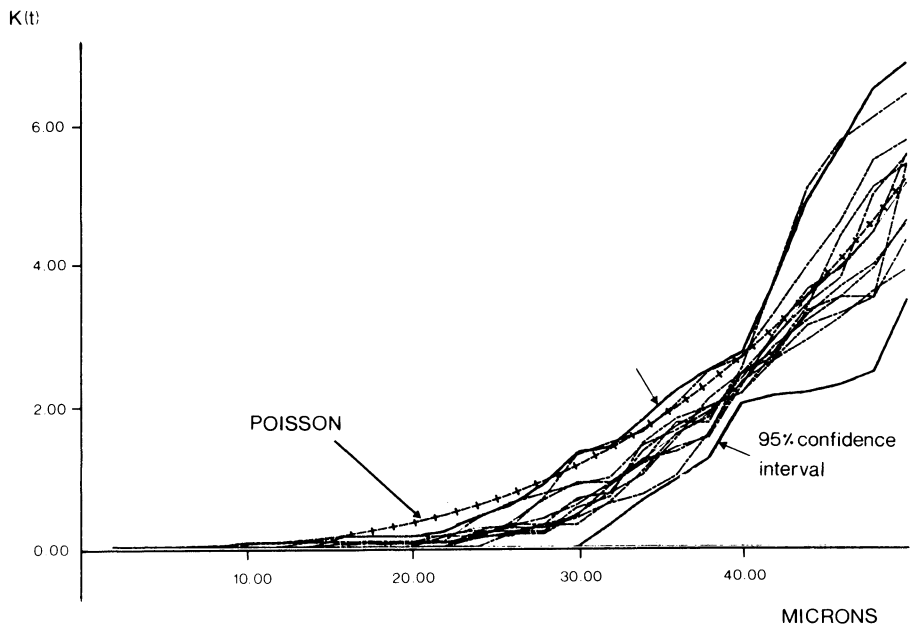

Fig. 3. See text.

Fig. 3 shows an example of the application of the K function to several sets of data collected from one animal. On the x axis the distance t from an event (in this case an osteocyte centroid) is indicated. The y axis shows the concentration parameter K(t),

which is the number of other events within a sphere of diameter t. A random point process is modelled by a Poisson field whose theoretical distribution is shown by the smooth curve. The 95% confidence envelope for the data, based on the t distribution, is shown in heavily drawn lines.

Note that the data departs below the Poisson distribution at 27 microns, indicating a hard-core packing with a 'repulsion' between osteocytes in 3-D. Had the data departed above the Poisson distribution then a spatial process which was more clustered than would be expected from a purely random process would be present. The computer software for this procedure has been developed by Baddeley et al. (1986).

With the 3-D spatial coordinates of features stored in computer memory it is a relatively easy step to produce 'stereo-pairs' of both the data and the model. These serve to illustrate qualitatively the objective statistical inferences described above. With TSRLM it will be a relatively straightforward procedure to obtain large 3-D data sets because the tedious task of correlating serial section data is removed.

OTHER 3-D STEREOLOGICAL QUANTITIES.

A preview of quantitative serial sectioning technique has been written by De Hoff (1983). In it he gives a list of those geometric properties that cannot be estimated in the plane, i.e that must be approached in 3-D. His list was:

1) Number of features (general)
2) Connectivity of features (general)
3) Size distributions (by volume, area, diameter)
4) Spatial distribution information (covariograms)
5) Real feature shape

We have seen that an estimate of the number of particles of arbitrary shape can be obtained from pairs of sections (Sterio:1984) and to that extent De Hoff's list is already out of date (the reader is urged to read that paper to discern how much progress has been made in stereology since 1983. Of the four properties that De Hoff defined at that time as needing geometrical assumptions only one now remains, the degree of anisotropy!). However the other 3-D properties are still firmly out of reach of measurements made solely on 2-D sections.

CONCLUSIONS

When compared with model-based estimators, where many hundreds of measurements are usually required to obtain a biased estimate of particle size or number, the new design-based methods are extremely efficient, requiring only tens of measurements to achieve robust unbiased estimates. This efficiency is increased again when these methods are combined with TSRLM, where tissue preparation time is minimal or non-existent. If the data of Howard et al. (1985) is examined, it is clear that too much work was performed on each animal and that a coefficient of variation of the estimate of 10% within animals would have been adequate when compared with a CV between animals of 18%. In practice that

means that a stable estimate of the number of osteocyte lacunae in that investigation could have been achieved by expending 10 minutes per animal!

When employing optical sectioning for particle counting, the distance between the planes (A and B in Fig. 2) can be varied when optimising the experiment. In the disector (Sterio: 1984) the distance between the planes should not exceed the height of the smallest particle in the direction of sectioning. That rule applies under ideal conditions but in practical circumstances the distance has to be rather less. This does not mean that the disector is inefficient (far from it!) but simply that the depth of the disector is dictated by the feature under scrutiny. No such restriction applies to TSRLM studies and the dimensions of the 'brick' can be optimally designed for each experiment in much the same way that a 2-D test probe is in classical stereology.

The disector principle can only be applied if it is possible to unambiguously identify the planar transects through an individual particle" (Cruz-Orive 1980). If there is any doubt about the provenance of profiles then it is necessary to perform serial sectioning without reconstruction (Cruz-Orive 1980) to establish that provenance. With TSRLM this presents no problems because features can be traced up and down and inspected in the guard volume at the sides of and below the 'brick'.

With respect to number estimation in TSRLM, the novel prospect exists that the same volume of tissue could be re-examined, non-destructively , on a number of occasions in a time sequence. Therefore it should be possible to study a developing cell population.

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3-4
Q: Is there no inclination of waving in the empirical lines you have shown? (I. Higuti)

A: The Poisson distribution is a smooth function. It can be reduced, in this case, to a straight line if $K(t) = 4/3\pi r^3$ is plotted. The data is subject to the normal effects of biological and sampling variance. We could not detect any regular waves from our examination of this data set.

Q: I have been struck by the performance of the microscope you have introduced. But I just wonder whether your microscope can be applied not only on such hard tissues as bone or tooth you have shown, but equally to soft tissues that are not stained or stained by usual methods. (T. Takahashi)

A: I must stress again that the hard tissue that has been shown is the work of Professor Alan Boyde of University College London, who has been largely responsible for the development of applications using TSRLM. The Microscpe was originally developed by Professor M. Petran for looking at unstained nerve cells in living tissues (J. Opt. Soc. Amer(1968)58, 661-664). This is to some extent possible but I see the real future of this machine as a fluorescence microscope. Alan Boyde has been responsible for realising this potential to date. I can envisage a neurophysiologist being able to perform intra-cellular electrode recordings while, by the introduction of a small amount of an intra-cellular fluorescent marker, being able to characterize the cell's morphology a form.

The short answer to your question is yes - soft tissues can be examined in TSRLM if the objects of interest reflects a fluoresce light.

Q: Please could you give the main references relating to the TSLM? (R. Miles)

A: Petran M., Hadravsky M., Egger M. D. and Galambos R. (1968) J. Opt. Soc. Amer. 58:661-664.

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Q: How much would a TSLM cost, and what would the waiting period be? (R. Miles)

A: The current price is about $30,000. I am not sure of the waiting period, but it is probably in the order of months. Those interested should contact:

SLUZBA VYZKUMA
Konevova 131
Prague 3,
CZECHOSLOVAKIA

Q: It's excellent to learn about an equipment (the tandem scanninglight microscope) for measuring the K-function, i.e.the reduced second moment function, for 3-D random homogeneous and isotropic point structures.
These are some questions:
1. What is the behavior of the measured K-function as t tends to zero?
2. Which statistical procedure (distribution) did you use for getting the confidence intervals?
3. How can you quantify the orientation of anisotropic point structures with the help of the equipment and the K-function or another quantity?
4. Is it possibleto determine the suitable K-function for fibrous structures by your method? (D. Koenig)

A:
1. The function tends again towards the Poisson. There are rare occurrences where these cells do appear close together. No biological system is completely uniform and we would not expect it to be so. However, there is little doubt that we are looking here at a hard core packing process.
2. t-distribution.
3. These cells are ellipsoidal (usually) and we have recorded their orientation in the X-Y axes, though not the Z. We have not yet attempted this analysis.
4. Certainly, in general, if the numerical density of points is replaced by the length density of fibres then the K-function could be applied. It would <u>not</u> relate to the <u>number</u> of fibres. I believe that Stoyan has performed some work on this problem. In particular, the 'fibres' seen on the micrographs are infact canaliculi in the bone. We have not made any measurements on them. Any serious study of these canaliculi would require serial reconstruction.