Length Estimation from the Past to the Future: A Brief Review

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ABSTRACT

The quantification of linear biological objects is crucial in many biological studies. A number of different methods have been used in the past for the length and length density estimation of linear features. Due to the progression of science and technology, some of these methods have been replaced by computerised analysis methods. The objective of this article is to describe briefly the length estimation methods used in the past and to review them as a basis for future developments in this area.

Key Words: Length estimation, Cavalieri principle, vertical section, isotropic uniform sectioning, stereology

Introduction

Unbiasedness in any sort of quantitative analysis requires a high level of objectivity and methodological discipline. Stereology is the only complete methodological framework that meets this requirement in sectional morphometric studies. Stereological tools enable us to derive three-dimensional information from two-dimensional planar sections (Gundersen, 1986; Altunkaynak et al., 2012; Kaplan et al., 2012a, 2012b). The main application logic of stereology is relying on observable intersections between geometric probes and the object (or quantity) of interest. Stereology is the only way of obtaining unbiased quantitative results from misleading sectional profiles of complex structures, like we see in the biological material (Baddeley, 2001).

The term ‘unbiased’ refers to the measurements or estimations that approach the true value with increased number of repeated experiments. It generally indicates that the results obtained by means of a measurement method have no systematic deviation from the true value, which is generally hidden in most cases (Gundersen, 1986; Gundersen and Jensen, 1987). Utilisation of some special geometrical probes in stereology requires a careful design in order to obtain valid and unbiased results. Probe is a kind of geometric question that has to be asked on the structure of interest in order to get the desired quantity (Howard and Reed, 1998). For example, if we had a rectangle applied on a tissue section and we count cell profiles contained only in that rectangular area, our rectangle becomes a ‘planar (2-D) probe’ in terms of stereology. Stereological probes can be one, two or three-dimensional.

Probability of Probe-Object Intersection in Length Estimation

All parametric estimates in modern stereology are based on the probability of probe-object intersection. For length estimation, this probability of random intersections between a
probe and an object is illustrated by the needle problem of George Leclerc Buffon in 1777 (Figure 1). Buffon proposed that the randomly thrown needles will intersect with the lines on a surface with a predictable probability:

\[ P = \left( \frac{2}{\Omega} \right) \left( \frac{l}{d} \right) \]  
(Eq. 1)

\( p \): the probability

\( l \): the length of the needle (cm)

\( d \): the distance between the lines on the floor

\( 2/\Omega \): isotropic intersections between the lines on the floor and the needle

For the unbiased estimation of ‘l’, repeated tosses of the needle in the air will cause an observable number of intersections with the lines on the floor. The fraction of needle–line intersections (\( \Sigma l \)) of the total number of tosses (\( \Sigma N \)) gives the probability ratio (\( \Sigma l / \Sigma N \)) (Calhoun and Mouton, 2000).

While using 2D probes in length estimation, the probe hits the object in proportion to the height that is normal to the section plane. The chance of being hit by a test line is proportional to the height of the object with respect to the section plane (Figure 2). The border complexity of the object is also important (Figure 3). While a simple polygonal object profile on a section intersects with the test lines fewer times, the convoluted border generates several intersections in a similar situation (Howard and Reed, 1998). Therefore, it is clear that the number of the intersection of the linear feature per unit area of the probe is in some way related to the length of the feature packed into a unit volume of the reference space. With this knowledge, if we adjust Buffon’s principle to the length density of linear objects (as Smith and Guttman did in 1953) the formula will be:

\[ L_v = \frac{\text{Length of feature } Y \text{ in reference space}}{\text{volume of reference space}} \]  
(Eq. 2)

Where \( \Sigma Q \) is the number of random intersections and \( \Sigma A \) is the total area of sampling probe (Calhoun and Mouton, 2000). Nonetheless, we cannot ignore the orientation of the feature with respect to the probe. In Figure 4, a series of parallel rods are intersected with planes of differing orientation. We see that the number of intersections per unit area of the plane is related to the plane orientation. Sections should be generated isotropically, but this is not adequate to solely achieve an unbiased estimation, since we actually do not know the orientation distribution of the linear features. To solve this problem, the planar probes should also be isotropic (Howard and Reed, 1998).

In summary, in order to obtain an accurate estimate of \( L \), either the object or the probe orientation must be isotropic. However, it is not easy to achieve object isotropy because nearly all of the structures tend to be anisotropic with a few exceptions. Hence, scientists either randomise the orientation of the object or the probe to solve this problem. In this review, we will discuss the methods used in the object and probe orientation.
Figure 2. Schematic representation of the objects with different heights and their probability of being hit by a series of planar probes (Kaplan et al., 2012b).

Figure 3. Importance of feature convolution: While a simple plane intersects with a straight line only once, the same plane is intersected with a more convoluted feature several times (three times in this schematic example). Thus, the probability of intersection with a random plane is dependent on the three dimensional complexity of the feature of interest (modified from Howard and Reed, 1998).

Isotropic-Uniform Random Sections
Isotropic section is a plane that is perpendicular to an isotropic orientation. If this plane also has a uniform random position, it is known as isotropic uniform random plane. For isotropic structures like liver, isotropic uniform random (IUR) sections are obtained by cutting the block and randomising the orientation. However, if the structure is anisotropic, the orientator is needed (Matthfeld et al., 1990) to obtain isotropic samples. For smaller objects, the isector method is generally preferred (Nyengaard and Gundersen, 1992).

IUR Sectioning in Anisotropic Material: The Orientator and the Isector Method

As a first step, a uniform random slab of the object is cut and the vertical direction is defined for further reference. The slab is then placed in uniform random position and in arbitrary rotation on a uniformly divided Q clock. While placing the slab on the clock, the original vertical axis of the slab should be normal to the plane of the clock. Numbers between zero and nine is generated with a random number table and a number is randomly chosen. The slab must then be cut along this random direction as defined by the selected random number. One of the two pieces of the cut section is taken and is placed in uniform random position and in a face down position on the clock. After generating a random number between zero and nine, the final cut is generated along this random direction. This final cut face is an isotropic uniform random plane section through the structure (Matthfeld et al., 1990; Larsen et al., 1998).

The Isector Method
The object is placed on a spherical mould and the tissue is hardened within a paraffin block or plastic embedding media. The small spherical block is then rolled on a flat surface, such as a laboratory bench, to ensure that the random rotation and the block is re-embedded in a rectangular mould. After this step, the tissue block is sectioned in a systematic random manner. Now, these sections represent the IUR sections through the object (Nyengaard and Gundersen, 1992; Larsen et al., 1998).

Length Estimation Using the IUR Sections
After generating the IUR sections, straight line probes are superimposed on the projections of
sectional profiles. Moreover, the numbers of object-line intersections are counted. Meanwhile, the volume of the structure is estimated using the Cavalieri method. With these two results, \( L_v \) can be estimated with the formula given above (Eq. 2). After estimating the object total volume and \( L_w \) using the Cavalieri method, the total length of the object can be found by multiplying these two estimates. In 1997, Tang et al. (1997) estimated the total length and size of the myelin fibres in the white matter of the human brain using IUR sections. They estimated the volume of the cerebral white matter using Cavalieri’s principle and the fibre length density was obtained from IUR sections generated by the isector technique.

**Problems in IUR Sectioning**

The main problem in estimating length with the IUR sections is randomising the orientation of the linear object. Since the orientation (coronal, sagittal, and so on) has also been selected during the preparation of the IUR sections, we should not expect to have a truly isotropic section in such a preferred direction. Highly layered structures identified only by tissue landmarks, in particular, may not be identifiable in IUR sections. Thus, this method does not seem suitable for length estimation (Nyengaard and Gundersen, 1992).

**Vertical Sections**

As an alternative to IUR sections, Baddeley and co-workers introduced vertical uniform random (VUR) sections in 1986. In VUR sections, instead of randomising all axes, as performed with the IUR sections, one of the axes of the section is selected as the ‘vertical’ axis and the tissue is then rotated randomly around this particular axis to generate the VUR sections. Afterwards, the tissue is sectioned perpendicular to this vertical axis in a systematic and random manner. Choosing a specific and predefined axis enables the observer to recognise the interested landmarks in the tissue (Baddeley, et al., 1986; Howard et al., 1992).

In the length estimation procedures on VUR’s, a special type of line probe must be used. It is a sine-weighted curve called the cycloid. The main purpose of using a cycloid as a probe is to compensate the possible bias that might arise from the pre-selected vertical axis. The minor axis of cycloid must be orientated parallel to the vertical axis when placed on a VUR section. The length of a cycloid, \( d L (\theta) \) in a given infinitesimal orientation range, \( \theta \) to \( (\theta + d \theta) \) is directly proportional to the sine of the angle \( \theta \) between the vertical axis and the tangent to the line element \( d L (\theta) \). A cycloid that has its minor axis parallel to the vertical axis can be viewed as a series of connected line segments, such that the length of any one segment is proportional to the sine of its angle with the vertical axis (Figure 4). Moreover, the length of a cycloid is twice the length of its minor axis (Baddeley, et al., 1986).

The efficiency of the VUR sampling can be enhanced by appropriately choosing the vertical axis and by scanning the tissue with multiple virtual cycloids at each sampled x-y location (Baddeley et al., 1986; Gokhale, 1990).

**Length Estimation Using Vertical Sections and Cycloids**

To estimate length from vertical sections with cycloid probes, systematic random samples of the reference space at any convenient orientation should be prepared. After the reference volume is estimated, a suitable cycloid grid is applied to the sections with a random start at each x-y location to count intersections between the linear feature of interest and the cycloid lines. Finally, \( L_v \) is calculated from the above equation (Eq. 2). If the thick vertical section completely contains the entire linear feature, then its total length can be estimated directly without the section thickness information (Cruz-Orive and Howard, 1991).

Stocks et al., (1996) gave the example of the application of the Gökhaile method that was used to estimate the total length of the immunocytochemically stained epidermal nerve fibres in punch skin biopsies. In another
work, McMillan et al., (1994) used the Gökhale approach to estimate the capillaries in the cerebral cortex. Roberts et al., (1991) used the TVP method in magnetic resonance imaging (MRI) studies of the length of the arteries in the brain.

Problems in Vertical Sections
As already discussed, length estimation with both IUR and a combination of VUR sections with sine-weighted line probes require random rotation of the objects around at least one axis. Such rotations may cause the anatomical landmarks of the tissue to be lost. Moreover, they may not allow the non-rotated parts of the tissue to be observed. Hence, the recent approaches have demonstrated that instead of the linear feature, stereological probes can be rendered isotropic (virtual isotropic planes/spheres) (Nyengaard and Gundersen, 1992).

Virtual Planes
It may sometime be difficult to define the region of interest in the methods described previously unless the tissue is sectioned in a specific non-random orientation. Thus, instead of rotating the tissue, new methods prefer to use isotropic planes or spheres to ensure unbiased length estimation. In length estimation with virtual planes, a suitable stereological test system is superimposed on the live video images of the microscopic fields (Calhoun and Mouton, 2000). A simple device called the microcator reads the displacement of the microscope stage along the vertical (or ‘z’) axis and the computer receives this information as a measure of position through the section thickness. As the focal plane moves through the thickness of the physical section, a virtual plane is seen on the screen as a moving random line with respect to depth reading from the microscope. This line represents the intersection between the focal plane and the virtual plane (Calhoun and Mouton, 2000), (Figure 5).

This method is especially useful in strongly anisotropic but spatially homogeneous structures. In such tissues, it is more efficient to analyse different orientations within a few sampling box rather than analysing many sampling boxes, each with a new orientation. In some tissues, the reference space boundaries are difficult and in some cases, the physical cutting procedure can be very time-consuming (Calhoun and Mouton, 2000). The use of isotropic virtual planes, therefore, reduces the requirements for physical sampling. Sometimes, the linear structures are orientated close to a particular orientation (i.e. anisotropic). If this is the case, the previous estimators may exhibit a higher estimator variance. Hence, the use of isotropic virtual planes reduces the estimator variance by probing the specimen with arbitrarily determined systematic orientation in arbitrarily defined sampling boxes. Larsen et al., (1998) used such an approach in estimating the $L_V$ and $L$ of microvessels in the rat cerebellum.

2-D Disector
The number of transects of a linear structure can be estimated using 2D dissectors (Larsen et al., 1998), (Figure 6). These dissectors have two lines separated by a distance (h). The lowermost line is an exclusion line of the length ($l$) with a left-handed forbidden line. The uppermost line is also an exclusion line. The profiles intersecting the inclusion line are sampled.

Figure 5. Schematic view of an isotropic virtual plane application (redrawn from Calhoun and Mouton, 2000).

Possible Disadvantages of the Method
For length estimation, using the virtual isotropic planes, the linear feature of interest must be stained completely throughout thick, transparent sections. In addition, a high power oil immersion objective with a high numerical aperture should be used to obtain sufficiently thin focal planes. The necessity to use a set of
equipment that is capable of integrating correct information of z axis displacement with suitable software may be regarded as a disadvantage of the method. The difficulty of the decision of the counting rule makes the method less applicable as well (Larsen et al., 1998).

Figure 6. 2D disectors used to estimate the number of transects of a linear structure (redrawn from Larsen et al., 1998).

Spherical Probes
The isotropic surface of a sphere encompasses all possible orientations in 3-D space. Therefore, this property makes it an ideal probe to estimate the length parameter of anisotropic structures (Gökhaile, 1990). Spherical probes ensure isotropic probe-object intersection. A probe can be visualised as a series of concentric circles of increasing and decreasing diameter. In the z axis at each focal plane, the linear feature and the probe intersections are counted (Calhoun and Mouton, 2000). One important requirement is that the total surface area of the probe must be large enough with respect to the diameter of the linear feature (at least five times or more) (Calhoun and Mouton, 2000).

Virtual isotropic probes allow the investigator to select a convenient section orientation and to preserve the anatomy of the object. The other advantage of the method is that there are no artificial edges when using such a spherical probe. As such, the method does not need complex counting rules to avoid the ‘edge effect’, which is a result of the artificial edges generated by the cutting procedures. Hence, such probes are easier to apply on several cases. Likewise, the method does not require different random orientations from one sampling area to another (as in the case of virtual planes), which can cause problems in the recognition of the probe–object intersection (Calhoun and Mouton, 2000). Mouton et al., (1999) demonstrated a practical application of this method by estimating the mean total length of thin fibres, primarily axons, in a brain region that is important for cognitive information.

Confocal Microscopy and Object Rotation
Confocal microscopy adds enormously to the power of stereological approaches. It reduces the difficulties and labour involved in obtaining suitable images. Moreover, when it is used in conjunction with novel analysis software, it allows the convenient application of stereology to small samples, particularly in cases where it is essential to maintain a specific orientation for correct interpretation (Howell et al., 2004). Kubinova et al., (2001) suggested that virtual section planes could be constructed through the tissue in randomly chosen orientations by collecting optical three-dimensional reconstructions. They also suggested that one can count the intersection between the planes and the structure of interest. Sets of images at a consecutive z-axis allow the investigator to virtually manipulate the orientation of the images (Howell et al., 2004). Placing a linear probe with a random rotation on the image also provides the object probe intersection for length estimation. Thus, the problem of anisotropy can easily be solved by confocal microscopy.

Conclusion
It seems that the best method to estimate length density nowadays is to use the virtual spherical probe approach. Using a computerised stereology system, it is now easier to estimate several sophisticated geometrical parameters. Especially with the developments in confocal microscopy, stereological applications are now easier and more efficient than ever.

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