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Numerical Density Estimation of Mitochondria in Thick Slice by Transmission Electron Microscopy

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A stereological formula is derived which allows estimating the numerical density (N_V) of random curved lines. This formula is obtained by the way that the numerical density (M_V) of terminal points and the length density (L_V) for a system of the lines are related to the numerical density (m_A) of terminal points and the length density (l_A) for the projection of a thick slice taken from this system onto a projection plane. Method using the formula is extended to include a system in which the particle shape may be modeled by curved cylinders each with two hemispherical caps and has been applied to estimate the numerical density of rat liver mitochondria in thick slice by 200kV electron microscopy. Then we can determine all the usual morphometrical and stereological parameters per mitochondrion.

INTRODUCTION

A stereological parameter, particle numerical density is frequently employed for the quantitative morphological evaluation of cells and subcellular structures. This stereological estimations — when measurements are made on random plane section — require information about shapes and size frequency distributions and assume that the biological components being quantitated can be likened to geometrically defined models(DeHoff: 1968, Weibel & Gomez: 1962). This restriction severely limits the utility of the methods when dealing with biological structure such as mitochondria. Inspection of the profiles of mitochondria suggests us their heterogeneity and non-convex features (Boulender: 1978).

As usual in transmission electron microscopy, an ultra thin section is a slice of finite thickness T whose entire content is projected onto the micrograph. The slice thickness is accounted for major sources of error to those applied in analysing biological components by stereological formulas for infinitely thin section (Weibel & Paumgarter: 1978). But two-dimensional projected image of a thick slice usually contains much information (Miyamoto: 1984), when some structures have adequate contrast to recognize the features which may be hidden by projection overlaps and reentrant folds (Cahn & Nutting: 1959).

In this paper, we describe a method which permits the

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numerical density (N_V) of random curved lines to be derived from measurements made on the projection of slice. It is applied here to an estimation of the numerical density of rat liver mitochondria in thick slices by electron microscopy.

THEORY

Before we undertake an analysis of mitochondria images, it might be well to examine theoretically the basic stereological relationships between the system of ideal objects and the projection of a slice taken from it.

Projection of Curved Lines in a Slice

Let us consider a system, consisted of straight, curved, continuous or broken lines, oriented and positioned randomly throughout a three-dimensional space. It is assumed that there is no loop and branch. We intercept the system of lines with a test slice of finite thickness T and project the entire content onto the projection plane (Fig.1). The projection of the spatial lines appear as lines in the plane, some of which are truncated by the upper and lower slice surfaces. The apparent terminal points may arise from intersection of lines with slice surfaces and from actual terminal points of lines inside the slice. The numerical density m_A' of terminal points which arise from upper and lower slice surfaces can be estimated as (Saltykov: 1958, Smith & Guttman: 1953)

$$m_A' = L_V, \tag{1}$$

where L_V is the length density of the lines. The numerical density m_A'' of projected terminal points which arise from actual

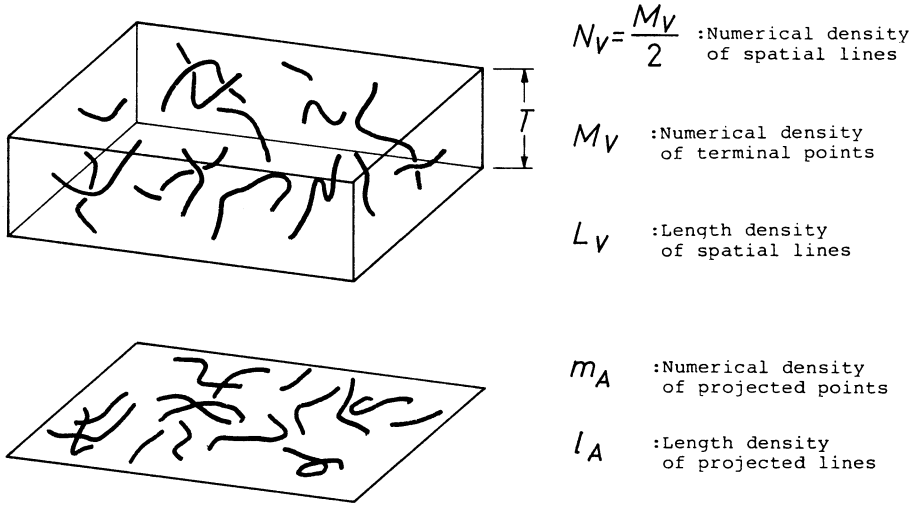

Fig. 1. Projection of curved lines in slice of thickness T onto a projection plane.

terminal points can be estimated as (Underwood: 1970)

$$m_A'' = TM_V, \tag{2}$$

where M_V is the numerical density of terminal points of spatial lines. Since the sources of the projected points cannot be distinguished and the probability of overlap is negligible for the projected lines, the apparent numerical density of terminal points in the projection plane is shown by a simple summation as

$$m_A = m_A' + m_A'' = L_V + TM_V. \tag{3}$$

The length density l_A of the projected lines is simply related to the length density L_V of the lines by (Underwood: 1970)

$$l_A = \frac{\pi TL_A}{4} . \tag{4}$$

Substituting Eq.(4) into Eq.(3) and using relationship $N_V = M_V/2$, we obtain

$$N_V = \frac{m_A}{2T} - \frac{2l_A}{\pi T^2} . \tag{5}$$

This simple formula shows that the numerical density of random curved lines in a slice of thickness T is related to the numerical density of terminal points and the length density for the projected lines. Since T cannot be obtained from the projection plane alone, an independent measurement by a particular method is required. It must be noted that the numerical density of curved lines in the slice can be obtained by the simple measurements and calculations without any information about curvature nor length of each line.

The Model of Mitochondria

To estimate the numerical density of mitochondria, we shall examine the model system, made of geometrically well-defined particles chosen in such a way that they simulate the mitochondria configurations and distributions in cells as faithfully as possible.

In many types of cells, mitochondria are so irregular in configuration that it becomes difficult or at times impossible to

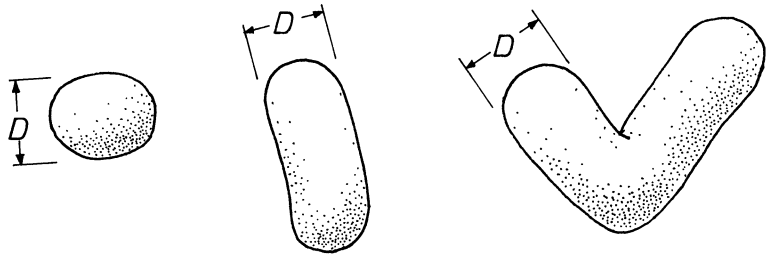

Fig. 2. Shape variations of mitochondria model.

define their size and shape adequately by mere inspection of profiles generated by random thin sectioning. In hepatocytes, for example, attempts have been made to estimate N_V by treating the average mitochondrion as a cylinder or an ellipsoid (Loud: 1968). However, microreconstruction of serial sections have suggested that mitochondria in these cells fall into two major shape classes, rod- and V-shapes (Berger: 1973).

It therefore will be adequate assumption that mitochondria have shape of a curved cylinder with two hemispherical caps as shown in Fig. 2. The model particle may have variable length and curvature, but it must have a constant diameter D along the major axis of the cylinder. Model systems are constructed by a large number of such particles scattered by a random process which allows all orientations and all positions, but without any overlap of particles. The particles are assumed to have medium contrast i.e., translucent in a transparent matrix.

Consider a center line along the major axis in each cylinder of the particles. When a slice of thickness T taken from the system of these particles is projected, these axial lines appear to us as the center lines inside the constant width D of particle image. The particle projection of width narrower than D, caused from grazing slices where only a shallow cap profile without the center line was included in the slice, allows us to avoid it from the measurements. The mathematical relationship between the axial lines in the particles and the center lines in the projected particle images is the same as that of the system of curved lines. If we can extract the center lines correctly from the mitochondria images, we may use Eq.(5) to estimate the numerical density of mitochondria.

In realistic cases in which overlap of particles with each other and reentrant fold of particle itself become appreciable, it is necessary to resolve individual particle images. If the slice thickness is less than that of the near particle dimension, however, the hidden parts of the particle image may be deduced readily by inspection of the amount of overlap and morphology of the outline of reentrant fold image.

ESTIMATION OF NUMERICAL DENSITY OF MITOCHONDRIA

To demonstrate the practical procedures of this method, we have analyzed rat liver mitochondria images and determined the actual thickness of the slice.

Material and Methods

Two adult wister rats were used. The animals were fed standard laboratory chow and water. They were anesthetized by ether and performed abdominal surgery. Syringe needle was inserted into hepato porta and 2.5% glutaldehyde in 0.1M phosphate buffer (pH 7.4) was perfused through the liver for 20 minutes according to the method described by Kanamura et al (1984). And then, blocks for electron microscopy were dissected from the prefixed liver and were rinsed in 0.1M phosphate buffer containing 0.05M sucrose at 4℃ for 1 hour. The blocks were post fixed in buffered 1% osmium tetroxide for 3 hours, dehydrated in graded ethanols and embedded in Epon 812 resin. At 70% ethanol dehydration, the blocks were stained with 2% uranyl acetate for 3 hours. A slice of set thickness 0.8μm was cut with a diamond knife on an LKB 4800 Ultorotome from the surface portion of each block and stained with lead citrate.

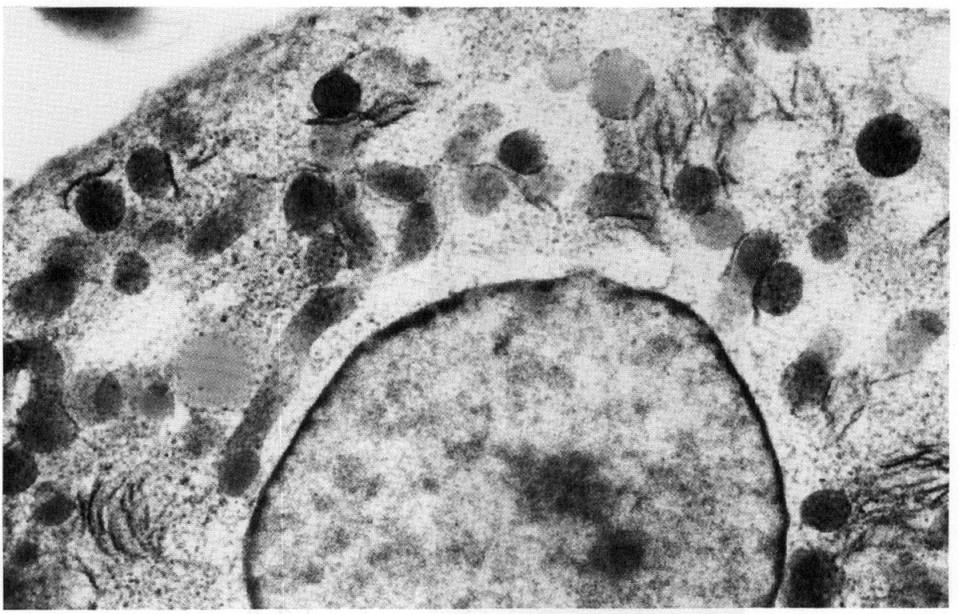

Fig. 3. Typical electron micrograph of thick slice (1.42μm) of rat liver. x7,200.

The specimen was examined with a JEM-200CX (JEOL, Japan) electron microscope at accelerating voltage 200kV. Electron micrographs at original magnification of 6,000 were obtained from the slice. Thus, 50 photographs enlarged to a final magnification of 10,200 which contained several mitochondria were prepared. We selected 10 photographs with mitochondria images of clearly defined contrast (Fig. 3). (The rest of the photographs contained some mitochondria images of unclear contrast.)

The outline of each mitochondrion image was traced with black ink. The average diameter of mitochondria was estimated from the width of mitochondria images which were not likely to have suffered grazing by slice surfaces. The way of drawing the center line was different for mitochondria images depending on its shape; so they were separated into three types: (a) no overlapping image, (b) overlapping image and (c) reentrant folding image. The examples of tracing the outline and drawing the center line for each type are shown in Fig.4. The number of terminal points was counted and the length of center lines was measured by a ruler, and then the numerical density m_A of terminal points and the length density l_A of center lines were obtained.

Thickness of the slice was measured by the method of the slice re-embedding. About ten slices selected randomly were re-embedded in Epon 812. Ultrathin sections at a set thickness of 0.07μm were obtained by slicing perpendicular sections to the surface of the re-embedded slice and they were stained twice with uranyl acetate and lead citrate. On electron micrographs, the thickness at 20 points from 4 sections were measured and the mean values were calculated. The data obtained for each photograph by using Eq.(5) were averaged and the standard error was calculated.

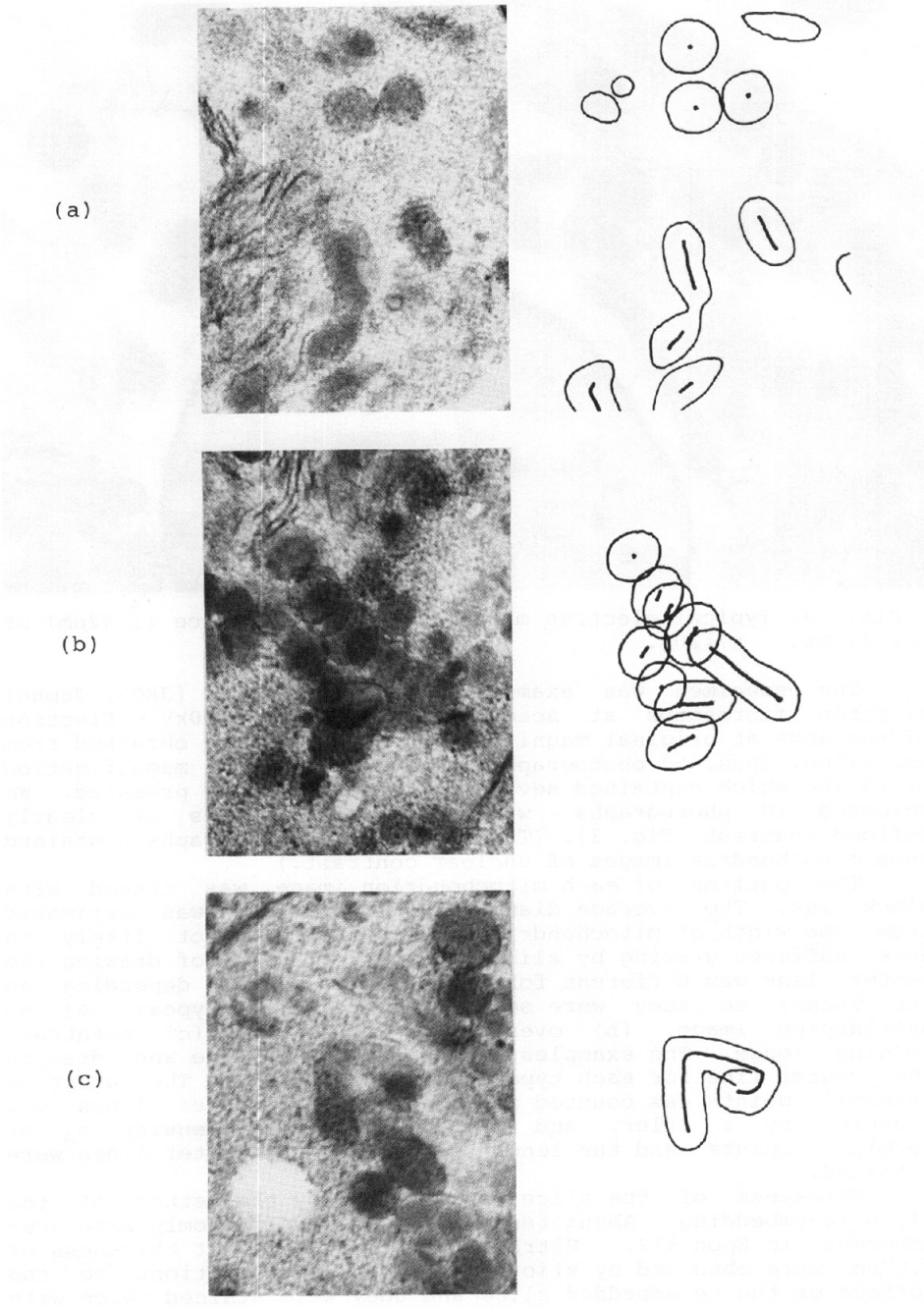

Fig. 4. Tracing the outlines and drawing the center lines onto the electron micrograph of mitochondria. (a) No overlapping image. (b) Overlapping image. (c) Reentrant folding image. x7,200.

Table 1. Estimation of numerical density of rat liver mitochondria. These data are mean values refered to the observation area $1.99 \times 10^3 \mu m^2$.

Numerical density of terminal points m_A (no./μm^2)	Length density of center lines l_A ($\mu m/\mu m^2$)	Slice thickness T (μm)	Numerical density of mitochondria N_V (no./μm^3)
0.499	0.106	1.420	0.142

Table 2. Stereological data characterizing mitochondria in rat liver.

Parameter	Expression	Stereological data mean ± SE
Average diameter (μm)	D	0.750 ± 0.157
Average length (μm)	$\frac{L_V^*}{N_V} + D$	1.419 ± 0.210
Average surface area (μm^2)	$\pi D \frac{L_V}{N_V} + D^2$	3.303 ± 0.695
Average volume (μm^3)	$\frac{\pi D^2 L_V}{4 N_V} + \frac{\pi}{6} D^3$	0.505 ± 0.120
Length per diameter		1.914 ± 0.495
Surface area per volume ($\mu m^2/\mu m^3$)		6.541 ± 2.076

* Calculated by the formula $L_V = 4 l_A / \pi T$ (Eq.(4) modified).

Results

The results of the measurement and the numerical density of mitochondria N_V estimated from Eq.(5) are provided in Table 1. The results represent overall means for the measurements and calculations on well contrasted electron micrographs. We have calculated the morphometric data characterizing average mitochondria size and shape. These are provided in Table 2. These values are calculated using the equations listed in the second column on this table.

DISCUSSION

Our formula is derived for the particles modeled by curved cylinder of any length and any curvature with two hemispherical caps. The method is very efficient and yeild reliable results by simple calculation, if the numerical density of particles is required first of all, without precise knowledge of distributions of the particles. In the estimates N_V of mitochondria, the magnitude of bias is governed partly by the shape approximation selected, being less for the present model than for the sphere or ellipsoid model. In application of a stereological method for biological tissues, the first question must be, whether the investigated components can be assumed as geometric objects. The accuracy of the results will be affected first of all by the

degree to which these assumptions are valid in the given case.

Recently, it is possible to observe cell organelles in thick slices, because high voltage electron microscopes have become popular in many laboratories. High voltage electron microscope gives us sharp images of thicker specimen, but reduces contrast that accompanies increasing accelerating voltages. It should be noted that the estimation procedures proposed are valid only if the measurements were done on well contrasted specimens where all objects in the slice are recognized on the projection plane. We are looking forward to a more reliable staining method that constantly provide good contrast to mitochondria throughout the depth of the slice. Theory and technique must go hand in hand to make a useful method.

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12-3
Q: Is there any way of getting the standard deviation or other measure of variation of the parameters associated with indivisual particles in a single sample? There are statistical ways to estimate this but can you make a direct estimation with a formula? (Y. Collan)

A: No, the standard errors were obtained indirectly and these were calculated from the values of the 10 electron micrographs by an ordinal statistical way. These values are just the parameters associated with each indivisual mitochondrion. Therefore, the mean value in Table 2 represents the mean of the mean mitochondia and the standard error is the measure of the variation of values obtained from these electron micrographs.

Q: How long does it take to make such an analysis and how many mitochondria would you have to examine? (V. Howard)

A: About one thousand terminal points were counted and about four hours were spent in total. Most of the time for the analysis was spent on tracing the outlines and drawing the center lines on the mitochondria images.