Journal of Microscopy, Vol. 202, Pt 3, June 2001, pp. 468–472.
Received 24 January 2001; accepted 9 March 2001

SHORT COMMUNICATION

Cylindrical diameters method for calibrating section thickness in serial electron microscopy

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Key words. Alignment, mitochondria, serial sections, stereology, three-dimensional reconstruction, ultrathin sections.

Summary

An estimate of section thickness is required for measuring structures in serial section microscopy. Mean section thickness is estimated reliably by averaging the ratios of the diameters of cylindrical objects, such as mitochondria, to the number of sections they span. This cylindrical diameters method improves the accuracy of section thickness as inferred from the colour of sections floating in water. The cylindrical diameters method gives the same answer as that obtained by the minimal folds method. It is preferable because it can be done in a series that has no folds that can distort and obscure the objects that are being measured.

Introduction

The availability of computer-based tools for reconstructing serially sectioned objects and the development of unbiased stereological techniques requiring three-dimensional probes (Fiala & Harris, 2001) has led to more widespread use of serial electron microscopy (EM). A critical parameter for these methods is section thickness.

Actual section thickness differs greatly from the nominal value indicated by ultramicrotome settings. Section thickness can be better judged by the interference colour of sections floating on water (Peachey, 1958). However, variability in the conditions of observation, and in the thickness of individual sections, can reduce confidence in this value as well. The minimal folds method reliably measures section thickness as judged by interferometry (Small, 1968; De Groot, 1988). However, it has the disadvantage of requiring the presence of folds in sections. For purposes of accurate analysis of ultrastructure, one needs series without the distortions introduced by folds. Hence, this method is not suitable for determining section thickness in the same images that one plans to analyse.

Here we demonstrate the accuracy and reliability of a practical method for estimating mean section thickness in a series based on cylindrical objects, such as mitochondria (Harris & Stevens, 1988). The estimate is shown to be equal in accuracy to more complicated or destructive methods of checking section thickness (De Groot, 1988), and is repeatable across independent observers.

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Materials and methods

A series was prepared from a hippocampal slice rapidly fixed with microwave processing in mixed aldehydes (2% paraformaldehyde, 6% glutaraldehyde) and processed for serial electron microscopy using standard procedures (Kirov *et al.*, 1999). The tissue was manually trimmed to a region containing only area CA1, processed in potassium ferrocyanide-reduced osmium, osmium and aqueous uranyl acetate, and dehydrated in a sequence of increasing acetone concentrations using microwave processing (Feinberg *et al.*, 2001). Infiltration began with equal amounts of acetone and a mixture of 1 : 1 Epon : Spurr's resins for 15 min in a microwave oven. This was followed by two more changes in 100% resin mixture, each microwave processed for 15 min. Two days in a 60 °C oven produced the final resin polymerization. Some of the comparative measurements were made on additional series prepared from blocks of perfusion-fixed hippocampus that were infiltrated with Epon only (Kirov *et al.*, 1999).

The cured block was trimmed to a trapezoidal region in the middle of stratum radiatum of area CA1, approximately 40 μm high by 70 μm wide, using a Diatome Trim Tool (Electron Microscopy Sciences, Fort Washington, PA). Serial

sections were obtained on a Reichert Ultracut S ultramicrotome (Leica Inc., Allendale, NJ) at grey to silver colouring (nominally 50 nm). Ribbons of 17–27 serial sections were mounted on Pioloform-coated Synaptek slot grids (Ted Pella Inc., Redding, CA) and stained with saturated ethanolic uranyl acetate and Reynold's lead citrate, for 5 min each.

Grids were loaded into grid cassettes (Advanced Microscopy Techniques, Danvers, MA) and stored in numbered gelatin capsules (Ernest Fullam Inc., Latham, NY). A grid cassette was mounted in a rotating holder to obtain consistent orientation of sections on the grid, and across adjacent grids during photography on the JEOL 1200EX electron microscope (JEOL, Peabody, MA). The series was photographed at 10 000× magnification. A diffraction grating replica grid (0.463 μm per square, Ernest Fullam Inc.) was photographed at the same magnification as the series for calibration.

The calibration grid and serial section negatives were scanned at 1000 dpi using a SprintScan45 film scanner (Polaroid, Cambridge, MA). Digitized images were aligned using sEM Align software (http://synapses.bu.edu/), and the final magnification expressed in pixels per μm was determined from the calibration grid (Fiala & Harris, 2001). Measurements were made on the computer using IGL Trace software (http://synapses.bu.edu/). Computer-assisted alignment of sections is convenient but not required, as the same methods were applied successfully to undigitized series of photographic prints.

Minimal folds method

One commonly used method for estimating section thickness is measuring the width of minimal folds in the sections (Small, 1968). The thinnest fold occurs when a section adheres to itself in a low ridge (Fig. 1). Section thickness is half the measured width of this ridge.

Cylindrical diameters method

A cylinder sectioned longitudinally has its diameter appear in the series in two directions (Fig. 2). In the section passing through the middle of the cylinder, the diameter can be measured in the plane of the section. The diameter also extends perpendicular to the plane of sectioning, causing the cylinder to appear across several sections. By counting the number of sections in which the cylinder appears, section thickness can be estimated as the ratio of the diameter to the number of sections. In the case of biological ultrastructure, cylindrical objects such as mitochondria are often sectioned longitudinally in a typical series. By measuring the diameters (d_i) for N mitochondria distributed evenly throughout the series, and counting the number of sections (s_i) each mitochondrion spans, multiple independent estimates of section thickness are obtained:

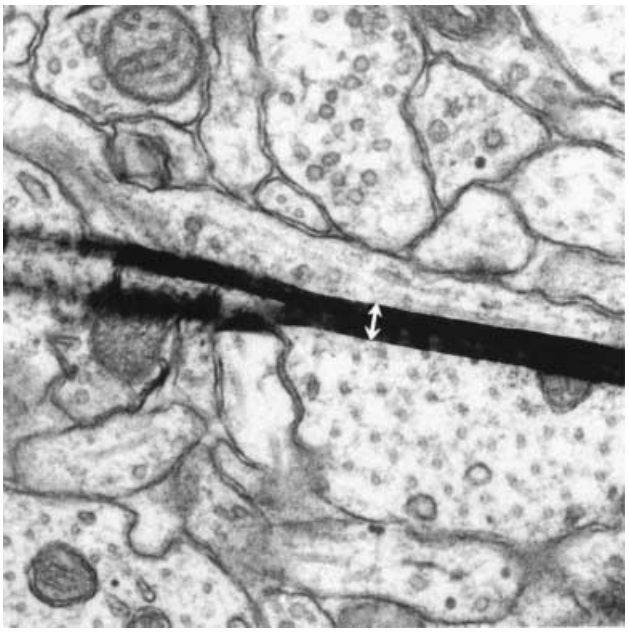

Fig. 1. Minimal fold on an ultrathin section. The fold width is twice the section thickness where indicated (arrows).

$$t_i = \frac{d_i}{s_i}, \qquad for \quad i = 1..N$$

The mean section thickness ($\bar{t}$) for the series is thus:

$$\bar{t} = \frac{1}{N} \sum \frac{d_i}{s_i}$$

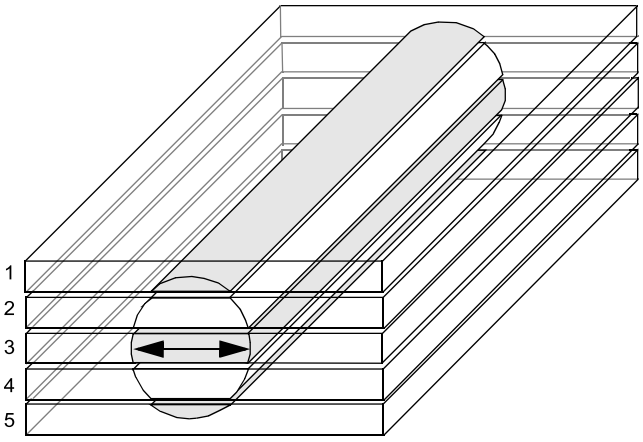

Fig. 2. A cylinder sectioned longitudinally by serial sections. The cylinder appears in five sections. The diameter can be measured directly in section 3 (arrows).

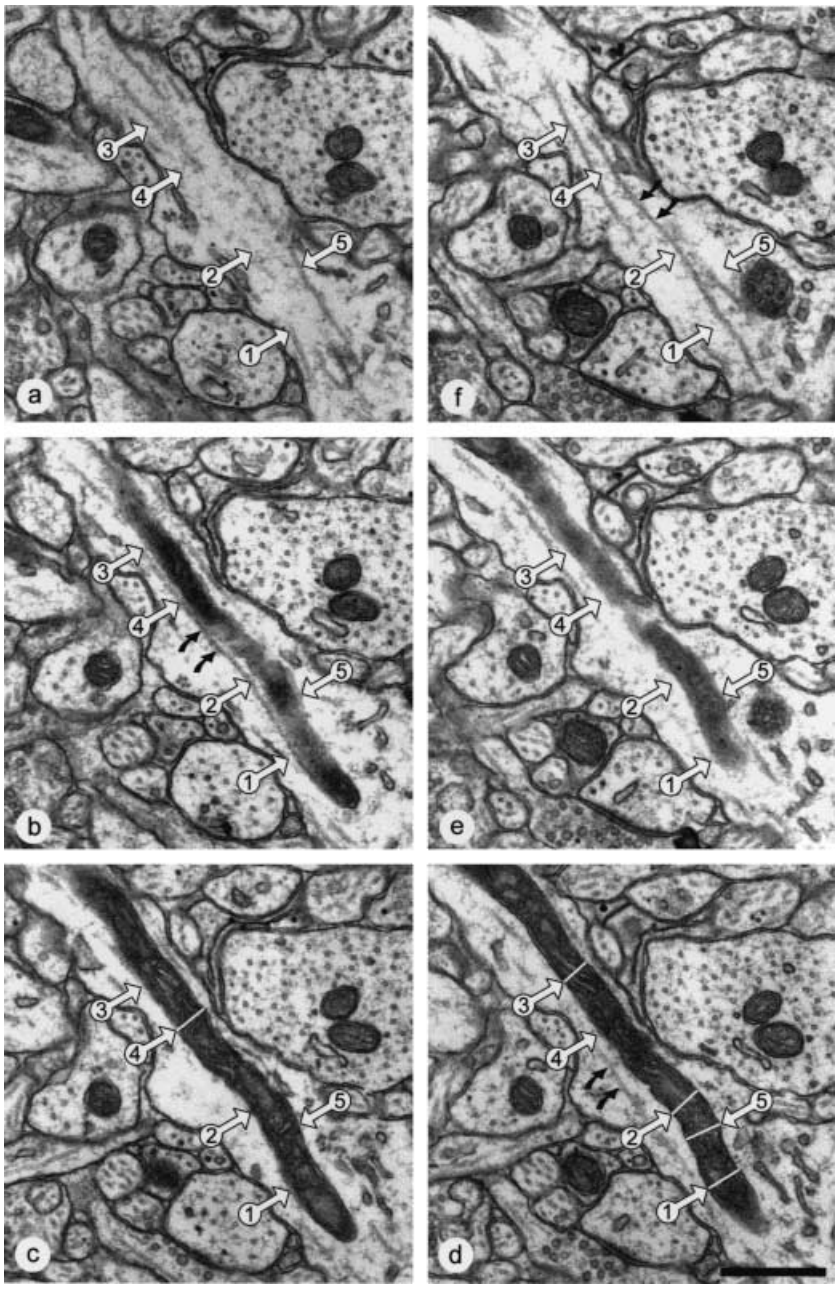

Fig. 3. A set of six serial sections through a longitudinally sectioned mitochondrion: (a) is the first section, and (f) is the last section in the series, proceeding counterclockwise. This mitochondrion is recognized as longitudinally sectioned by the presence of long microtubules beside it (black arrows). The numbered arrows depict locations of five different measurements. The diameters were measured by the thin white lines on section (c) for measurement 4, and on section (d) for the other measurements. The results of these measurements are summarized in Table 1, yielding a mean section thickness of 50 nm. Scale bar in (d) is 0.5 μm.

Procedurally, begin by making sure that the mitochondrion is longitudinally sectioned. The mitochondrion should disappear all at once from the sections (Figs 3(a) and (f)), usually with a long grey shadow in the final sections (Figs 3(b) and (e)). If the mitochondrion instead gradually disappears along its length, it is obliquely sectioned and should not be used. Because the long axes of mitochondria are typically orientated parallel to microtubules, longitudinally sectioned microtubules nearby are another indicator of proper orientation. In many cases, mitochondria may be only partly parallel to the plane of sectioning. In these cases, just the longitudinal portion is used.

Pick a location along the length of the mitochondrion where the measurement of diameter will be made. Then locate the section on which the diameter is widest. Typically, the membranes are also most distinct on this section. Measure the diameter perpendicular to the long axis of the mitochondrion at the chosen location on this section. As an example, consider location 1 on the mitochondrion in Fig. 3. It is widest on the section of Fig. 3(d), where all of the mitochondrial membranes are distinct. The diameter for location 1 measures 168 nm.

Determine how many sections the mitochondrion traverses at the point where the diameter was measured.

The first and last sections through the outer membrane of a mitochondrion are scored as 1, 0.75, 0.5 or 0.25 section, depending on the image. If cristae are visible at the measured location on an edging section, it is given a full score of 1. A dark grey shadow receives a score of 0.75, a medium grey shadow receives a score of 0.5 and a faint grey shadow receives a score of 0.25 section. These edge scores are then added to the number of intervening sections to get the full section count. For the example of the measurement at location 1, the scoring is 0.5 on Fig. 3(b) and 0.5 on Fig. 3(e). With two intervening sections (Figs 3(c) and (d)), the section count is 3.0. The section thickness for the measurement at location 1 is thus $168/3 = 56$ nm. Several other possible measurements through different parts of the mitochondrion are illustrated in Fig. 3 and Table 1.

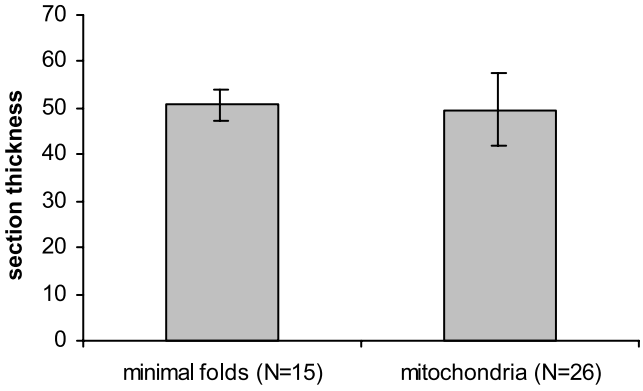

Fig. 4. Mean section thickness estimated from minimal folds measurements are not significantly different from those obtained by the cylindrical diameters measurements obtained from longitudinally sectioned mitochondria in the same set of serial EM sections. Error bars depict standard deviations.

Results

The series prepared from the hippocampal slice had many folds and hence was not suitable for serial EM reconstruction. However, it was useful for comparing estimates of section thickness obtained by the minimal folds and cylindrical diameters methods in the same set of serial EM sections. Mean section thickness was determined by the cylindrical diameters method using 26 mitochondria distributed throughout the series. One of the mitochondria used is shown in Fig. 3. This method produced an estimated section thickness of 50 nm, which was within 2.5% of the minimal folds estimate (Fig. 4).

Estimates obtained using the cylindrical diameters method are repeatable by independent observers. In Kirov *et al.* (1999), the method of measuring mitochondrial diameters was applied by making hand measurements using calipers on photographic prints. We repeated the section thickness estimation for three of the same series used in Kirov *et al.* (1999) with independent sets of mitochondrial measurements on scanned, aligned and digitally calibrated series using the computer. These new measurements were made by investigators other than those making the original measurements. The section thickness estimates agreed to within 2.5% for all three series (Fig. 5).

Discussion

The cylindrical diameters method obtains the same estimate of section thickness as the method of minimal folds, which agrees with interferometric measurements (De Groot, 1988). However, it has the advantage of being applicable to series without undesirable folds. This method is relatively quick on digitized series, and can be easily performed using freely available software for serial EM.

The method can be applied to any spherical or cylindrical serially sectioned objects of sufficiently uniform diameter. Rod-shaped mitochondria, abundant in most eukaryotic cells, are a particularly attractive candidate for ultrastructural work. In our laboratory, the method applied to longitudinally sectioned axons and dendrites has produced comparable section thickness estimates to that obtained using mitochondria. We anticipate that the method could

Table 1. Cylindrical diameter measurements made from Fig. 3.

Measurement point	First section with mitochondrion	Score on first section	Last section with mitochondrion	Score on last section	Number of intervening sections	Section count	Section of diameter measurement	Diameter (nm)	Section thickness (nm)
1	b	0.5	e	0.5	2	3	d	168.1	56.0
2	b	0.5	e	0.75	2	3.25	d	163.8	50.4
3	b	0.75	e	0.5	2	3.25	d	143.8	44.3
4	a	0.25	e	0.25	3	3.5	c	174.8	49.9
5	b	0.75	e	0.75	2	3.5	d	171.2	48.9

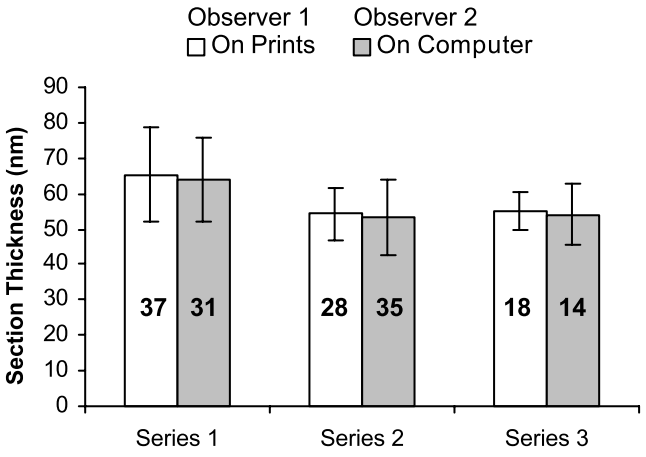

Fig. 5. Cylindrical diameters measurements of mitochondria made by hand on serial EM prints as compared to independent measurements made on digitized and computer-aligned series from the same micrographs. The similarity of these measurements demonstrates both the reliability of the cylindrical diameters measurement between observers, and between hand measurements on prints and computer-assisted alignment and measurement. Error bars depict standard deviations. The number of mitochondrial measurements is superimposed on each bar.

receive wider application in serial-section microscopy at all levels of resolution. All that is required is regularly shaped objects visible in at least three sections.

A number of factors may contribute to the variance in section thickness measurements obtained using the cylindrical diameters method. These include errors in placing the diameter measurement, accuracy of counting edging sections, and deviations in object shape from that of a perfect cylinder. Although less precise than a minimal folds measurement, an individual cylindrical diameters measurement is unbiased. With sufficient numbers of measurements an accurate estimate of section thickness is obtained. One must also consider that there are real variations in section thickness from section to section, and sometimes even within a section. The best mean estimate of section thickness for a series is obtained with multiple measurements evenly spaced throughout the series, which is more easily achieved using the cylindrical diameters approach.

We normally use 25–35 mitochondria to estimate section thickness in a series of 100 sections. Mitochondria in stratum radiatum typically span three to five sections when section thickness is approximately 50 nm. In this way, every section is sampled by at least two mitochondria. It is also important to choose cylindrical objects that span a few sections so that the edges do not dominate the counts. Hence, in series with greater than 60 nm section thickness, we recommend including some objects larger in diameter than mitochondria, such as longitudinally sectioned axons or dendrites. In our experience, 40–50 nm sections produce images that are easier to analyse than thicker sections, especially when counting small objects such as synaptic vesicles.

In conclusion, the cylindrical diameters method provides a reliable estimate of section thickness that can be used to determine volumetric dimensions of objects that span serial sections.

Acknowledgements

Many thanks to Marcia Feinberg for preparation of the series with folds. Thanks also to Sergei Kirov and James Cooney for making some of the mitochondrial measurements. This work was supported by Human Brain Project grant MH/DA 57351 funded jointly by NIMH and NIDA, and NIH grants NS21184 and NS33574.

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