ACCURATE MEASUREMENT OF THE THICKNESS OF ULTRATHIN SECTIONS BY INTERFERENCE MICROSCOPY

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In quantitative electron microscopy, a knowledge of the thickness (θ) of the sections is often required. For example, when objects smaller than θ (e.g. ribosomes) are counted in sections, θ must be known in order that the observed numbers may be related to the unit volume of the section. Even when comparatively large structures are submitted to morphometric analysis, the section thickness cannot be disregarded if the size of the object is of the order of a few times θ (5).

Several techniques have been used to measure section thickness, but they suffer important limitations, which can be of three kinds: (*a*) Their accuracy is not satisfactory, at least for θ ranging from 30 to 60 nm. In published interferometric methods, the accuracy is not better than 10% (2, 7). (*b*) Thickness measurement and electron microscopic examination cannot be performed on the same section, mounted on the usual microscopy grids (6). (*c*) The method is restricted to special cases, for example when certain folds are present in the section (4) or when standard objects are embedded together with the material (3). The interferometric method proposed here obviates the limitations (*b*) and (*c*) and its accuracy is better than 1 nm.

We used a Zeiss Standard GFL POL microscope, equipped with a Jamin-Lebedeff

interference system, to which a photomultiplier was adapted (Fig. 1, *a–e*). In this interference microscope, a beam of plane-polarized light (*a*) is split by means of a birefringent plate. The two beams emerging from the beam splitter are plane-polarized with their vibration plane (*b*) at a right angle to one another and at 45° to the vibration plane of the polarizer. The beams pass through a $\lambda/2$ plate, which rotates by 90° their vibration plane (*c*). One of the beams (the measuring beam, *MB*) passes through the specimen, whereas the other (the reference beam, *RB*) is propagated in the reference medium. The two beams are recombined by another birefringent plate (*d*) and made to interfere in the vibration plane of the analyzer (*e*), perpendicular to that of the polarizer. The interferometric system can be adjusted so that, in the absence of object, and with monochromatic light of $\lambda = 546$ nm, there is no optical path difference (Γ) between the two components of the recombined wave. Since these components are in phase, the resultant wave is plane-polarized and is extinguished by the analyzer.

When the measuring beam passes through an object of refractive index n (different from that of the reference medium, n_0), its optical path changes. A phase difference is generated and the recombined wave is now elliptically polarized. As a result, a component of the wave is transmitted by the analyzer and the object is visualized against a dark background.

Ultrathin sections give rise to small optical path differences, which are suitably measured by means of a Brace-Köhler compensator designed for Γ up to $\lambda_{546}/10$. The point of compensation is reached when, after rotation of the compensator by an angle β, the light intensity of the object image is minimal. (At the same time, the background becomes brighter). Γ is derived from β according to the formula: $\Gamma_{nm} = 54.6 \sin 2\beta$. The thickness of the object is then calculated as: $\theta = \Gamma/(n - n_0)$. If the reference medium is air, $n_0 = 1$.

In practice, the grids supporting the sections were placed on a perforated perspex plate and they were examined with the 40 × condensor-objective assembly. By means of the rotating stage of the microscope, they were oriented so that the reference field for the area under observation was free from grid bars and from any other object (Fig. 2).

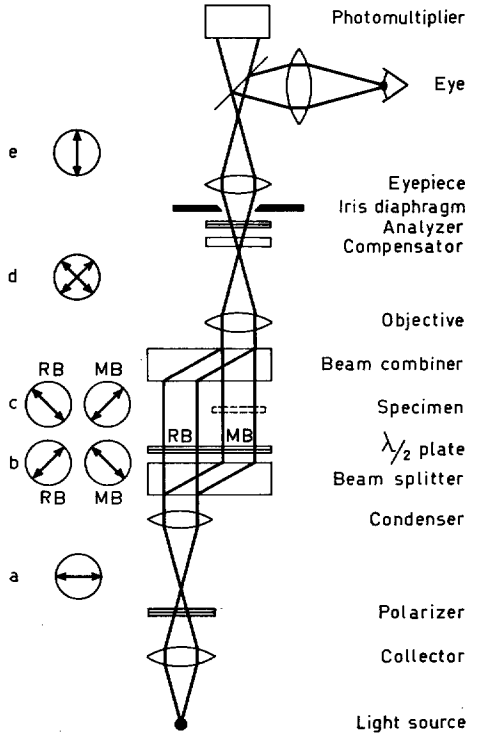

FIGURE 1 Path of the light rays in the Zeiss interference microscope equipped with a photomultiplier. The plane of vibration of the light wave at various levels is represented on the left side (see text for explanation).

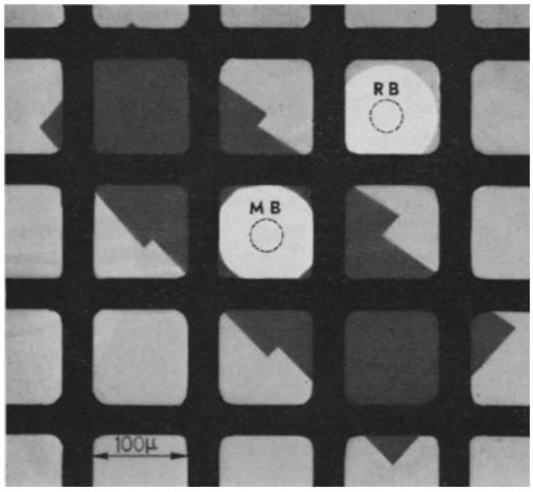

FIGURE 2 200-mesh grid supporting three sections, observed with the 40 × condensor-objective assembly. The measuring beam (*MB*) passes through a section, whereas the reference beam (*RB*) is propagated through an empty square. After maximum closing of the iris-diaphragm, the area "seen" by the photomultiplier is that delimited by the dotted circle (700 μm^2).

This requirement precludes the use of some types of grids, since the center-to-center distance between the reference beam and the measuring beam is fixed to 175 μm for the 40 $\times$ condensor-objective assembly.

In order to perform correctly the adjustment of the interferometric system in the absence of object ($\Gamma = 0$) and the compensation in the presence of an object, the maximum extinction of the light in the field of view must be accurately determined. Accordingly, the microscope was fitted with a photomultiplier, which was placed where a camera would ordinarily be located. An iris-diaphragm (Fig. 1) situated in the image plane was closed to block out stray light surrounding the area tested (e.g., diffraction fringes at the edges of the grid bars), and the transmitted light intensity was monitored by the photomultiplier.

The reproducibility of the method was checked by repeated measurements of Epon sections, prepared as described by Baudhuin et al. (1), except that Epon was supplied by another manufacturer (Ladd Research Industries, Inc., Burlington, Vt.). For each section tested, β was determined five times and the corresponding thicknesses were calculated by taking n as equal to 1.518. (This value was obtained as follows: small scraps of polymerized resin were immersed in kerosene-chloronaphthalene mixtures of increasing n, calibrated with an Abbé refractometer, until equality of the refractive indexes was observed, as indicated by disappearance of the Becke's fringes). The means and standard deviations are listed in Table I. The error of individual measurements rarely exceeds 1 nm. This accuracy is attributable to the great sensitivity of the photomultiplier used for monitoring light transmission. With such a system, β is measured with an accuracy of 0.25°, whereas the accuracy is not better than 1° when the minimum of light intensity is judged by eye.

TABLE I

Reproducibility of the Thickness Measurements

Thickness (mean of five determinations*)	Standard deviation
nm	*nm*
40.6	0.38
46.4	0.28
49.3	0.66
63.6	0.62
82.3	0.65

* Before each determination, the object was removed from the field and the zero of the interferometric system was checked.

The described method seems to be the most accurate way of measuring the thickness of individual sections before electron microscopic examination.

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REFERENCES

1. BAUDHUIN, P., P. EVRARD, and J. BERTHET. 1967. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J. Cell Biol.* **32**:181.
2. COSSLETT, A. 1960. Some applications of the ultraviolet and interference microscopes in electron microscopy. *J. Roy. Microsc. Soc.* **79**:263.
3. SILVERMAN, L., B. SCHREINER, and D. GLICK. 1969. Measurement of thickness within sections by quantitative electron microscopy. *J. Cell Biol.* **40**:768.
4. SMALL, J. V. 1968. Measurement of section thickness. Abstracts Fourth European Regional Conference on Electron Microscopy, Rome. 609.
5. WEIBEL, E. R. 1969. Stereologic principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* **26**:235.
6. WILLIAMS, M. A., and G. A. MEEK. 1966. Studies on thickness variation in ultrathin sections for electron microscopy. *J. Roy. Microsc. Soc.* **85**:337.
7. ZELANDER, T., and R. EKHOLM. 1960. Determination of the thickness of electron microscopy sections. *J. Ultrastruct. Res.* **4**:413.