The Optical Measurement of Depth

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Summary

The formula for vertical measurement by means of the fine adjustment of the microscope,

$$n_2/n_1 \times \text{measured depth} = \text{real depth},$$

is true, where n_2 is the refractive index of the *object* and n_1 is that of the immersion medium of the *objective lens*. The refractive index of the mountant is unimportant.

Several additional complications of this method of measurement suggest that it should not be used unless it cannot be avoided, or rough figures are required quickly.

Introduction

THE measurement of depth in microscopical preparations has many applications, the most recent being the determination of refractive index, by means of the interference microscope, of cytoplasm and cell inclusions (for instance, Ross, 1954). This instrument permits measurement of the retardation of the light waves, and from this may be calculated the refractive index, provided that the thickness is known.

Optical depth measurements are taken by focusing successively the upper and lower surfaces of the object, and reading off the travel of the tube of the microscope by means of the calibration on the fine adjustment knob. Brattgard (1953) treats in detail of the accuracy with which these readings may be made.

The present paper is concerned with difficulties in calculating the actual depth from the readings so obtained, by consideration of the optical path.

Correction of Depth Measurements

The accepted formula for obtaining depth is to multiply the distance through which the tube of the microscope must be moved to focus the upper and lower surfaces of the object, by the refractive index of the medium. It can be seen from the diagram that

$$\frac{\text{actual depth}}{\text{apparent depth}} = \frac{AC}{AB} = \frac{\tan \theta_2}{\tan \theta_1}$$

where AC is the real depth, and AB is the apparent depth, since $\angle ACD = \theta_1$ and $\angle ABD = \theta_2$. For small angles, $BA = BD$ and $CA = CD$, so that

$$\frac{\tan \theta_2}{\tan \theta_1} = \frac{\sin \theta_2}{\sin \theta_1}, \text{ and}$$

$$\frac{\text{actual depth}}{\text{apparent depth}} = \text{refractive index of the medium below the coverslip.}$$

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This is the usual formula found in elementary physics textbooks for the apparent depth of water seen with the naked eye, i.e. with small angles. The application of this formula to microscopy is seen in the paper by Addey (1922),

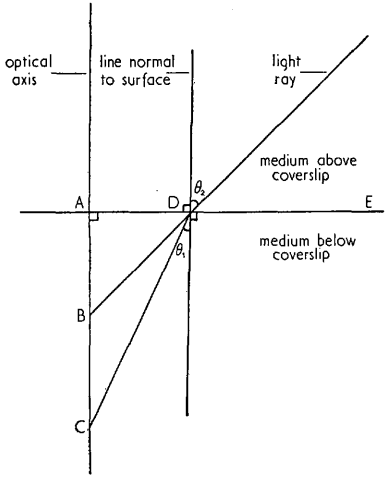

FIG. 1. Diagram showing the relationship between the apparent depth *AB* and the actual depth *AC*. For the sake of simplicity the upper side of the object is supposed to touch the coverslip. The coverslip is represented by a line *AE* because a flat plate at right angles to the optical axis does not affect the calculations.

in which appears the rather remarkable statement that 'If we consider a fairly narrow cone of rays, *such as we should be concerned with in microscopy*,

$$\frac{\tan \theta_1}{\tan \theta_2} = \frac{\sin \theta_1}{\sin \theta_2}$$

because the angles are small.' (The italics are mine.) θ_1 and θ_2 are again as in the figure, which is taken from Addey, and the formula is inverted because Addey approached the problem differently.

At first, then, it appears that we should use a correction, based not on the sines of the angles, as the refractive index correction is, but on their tangents.

LENS CORRECTIONS

Calculation shows that the correction will not be the same figure for all angles, but will increase from the refractive index figure at the paraxial rays, as shown in table 1, where the refractive index of the medium below the coverslip is taken as 1·52.

This is of course equivalent to spherical aberration of the objective lens, due to the medium in which the object is mounted.

Now, lens corrections for spherical aberration take the form of approximating the marginal rays to the paraxial focus, so that the paraxial rays alone need to be considered; and for these, which are of small angle, the refractive index correction applies. It is evident, therefore, that the right correction is used, but for the wrong reason.

TABLE I

θ_2	θ_1	*Correction factor:* $\frac{\tan \theta_2}{\tan \theta_1}$
5°	3° 17′	1·524
10°	6° 34′	1·531
20°	13° 0′	1·577
30°	19° 12′	1·659
40°	25° 2′	1·797
50°	30° 16′	2·042
60°	34° 44′	2·499
70°	38° 12′	3·491
80°	40° 23′	6·688

Refractive Indices

When applying the correction, the refractive index in question is that of the substance through which the rays travel a different distance on altering the focus; that is, the reading should be multiplied by the refractive index of the *object*, not of the mounting medium. For biological tissues in balsam, the difference is roughly 0·1%, and since Brattgard gives the accuracy of measurement as $\pm 0{\cdot}1\ \mu$, this would be unimportant except for objects of about 100 μ or more in thickness.

For cells in saline, however, the difference is roughly 1·0% (see Ross's figure of 1·3535 for the cytoplasm of the spermatid of *Locusta*). The difference is therefore greater than the error of the readings for objects of about 10 μ and upwards.

It is known that this correction should not be applied for homogeneous immersion systems, or alternatively the more general formula may be applied, by multiplying by n_2/n_1, where n_2 is the refractive index of the object, and n_1 is the refractive index, not of the mountant (for example, Canada balsam), but of the immersion medium (lens immersion oil, water, or air). This is not quite clear in Brattgard's paper.

It is important to realize that the movement of the tube of the microscope in focusing the top and bottom of the object is equal to the thickness of the object only if the refractive index of the lens immersion medium is identical with that of the object.

Spherical Aberration

Another source of error arises from the fact that the spherical correction is calculated for a particular thickness of intervening substance, i.e. coverglass and medium, and vertical measurements therefore necessarily involve using

the lens at depths for which it is wrongly corrected. The tube length can be adjusted to give spherical correction for only one of the two foci. If the tube length be adjusted for each in turn, then an additional correction will be required, involving the tube lengths and the focal length of the objective lens.

Corrections are calculated to bring the marginal rays to the paraxial focus, with which the position of the best focus does not quite coincide. When the microscope is racked up and down, the best focus moves in relation to the paraxial focus, in a way that depends on the characteristics of the particular objective lens in use. The error, however, is likely to be small for small vertical travel, only assuming measureable proportions in the approximate region of 100 μ.

Discussion

A dilemma therefore arises, because to measure the thickness it is necessary to know the refractive index, and to calculate the refractive index from the wave retardations obtained by the interference microscope, it is necessary to know the thickness.

Therefore it is suggested that whenever possible, it is preferable to make horizontal measurements, either of the same object if it is spherical or cylindrical, or of the similar objects lying in a different orientation—for instance on a differently cut section.

In this paper the object has been regarded for simplicity as a plane, parallel-sided layer. Refraction at the surfaces of curved objects complicates measurement still more, and adds weight to the above suggestions, because it is obvious that all rays leaving the bottom of a spherical or horizontal cylindrical object must pass through it if they are to reach the objective. The rays will therefore be refracted at the upper surface of the object, and in the latter case will be refracted differently in the two different axes.

In the special case of the measurement of an object which approximates to a vertical line, for example a flagellum, n_2 should be taken as the refractive index of the *mountant*, i.e. saline or Canada balsam, since rays from the bottom of it will not pass through the object itself.

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