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# Stereoscopic Method of Depth Measurements under the Microscope

A stereoscopic method of depth measurements under the microscope has been developed. Different variants of this method have been studied, and the results obtained show that vertical measurements in microscopic specimens can be made with the accuracy equal to about 0.1 to 0.05 of focus depth of the microscope.

Depth measurements in microscopic specimens are usually performed by focusing the microscope on the top and bottom details, and reading the rotation of a previously calibrated fine-focusing screw. The sensitivity and accuracy of this procedure are limited to the visual depth of focus of the microscope. This is, of course, a serious shortcoming. Moreover, some inaccuracies in measurements arise from defects (nonlinearity, free movement) of the fine-focusing mechanism. In the case of high precision microscopes the fine-adjustment screw enables an experienced microscopist to measure the depth with accuracy $\pm 1$ μm or worse. This is unsatisfactory, since — as regards many microscopic works, and particularly in biology and medicine — it is necessary to determine linear parameters of specimen structures, the thickness of which is equal to, or exceeds slightly, the depth of focus of the microscope.

Much greater possibilities of depth measurements in microscopic specimens lie in stereoscopic microscopes. These possibilities are justified by the following formula derived from the point of view of first order geometrical o tics:

$$\delta_z = \frac{250\sigma}{\Gamma \tan\gamma} \text{ [mm]}, \tag{1}$$

where $\delta_z$ is the smallest difference in depth, which can be detected through a stereoscopic microscope by an experienced observer, $\Gamma$ is the total magnifying-power of the microscope, $\gamma$ is the angle of convergence, under which the left and right eyes of the observer view the object under examination, and $\sigma$ is the stereoscopic acuity of binocular vision. This latter is found to be 6″ to 60″, and its mean value equal to 30″ (= 0.000145 rd) is generally accepted.

For typical stereoscopic microscopes the angle of convergence $\gamma = 14°$, and the total magnifying power $\Gamma$ can be equal up to 200x. Then, a value of $\delta_z$ as small as 1 μm results from Eq. 1. Taking into consideration a mono-objective stereoscopic microscope, whose condenser aperture (or exit pupil of the objective) is divided into two halves, the angle of convergence $\gamma$ can be as great as 60° (for an immersion objective 100x/1.3), and the total magnification can be equal up to 2000x. Then, the minimum detectable difference in depth $\delta_z$ perceived with such a microscope is equal to about 0.02 μm.

The above mentioned theoretical data have been proved by using a conventional stereoscopic microscope (a PZO instrument, model MST 131) and special mono-objective phase contrast stereoscopic microscope developed by the author and described elsewhere [1]. This latter is manufactured by Polish Optical Works — PZO, and is commercially available as the biological Stereophase microscope, model MB30S. Both these instruments were adapted for depth measurements as shown in Fig. 1, where $E_1$ and $E_2$ denote left and right eyes of the observer; $O_1$ and $O_2$ are eyepieces of a binocular head; $P_1$ and $P_2$ are glass plates with stereometric marks $M_1$ and $M_2$, placed in the front focal plane of the eyepieces: $B$ is the specimen under examination, $B_1$ and $B_2$ refer to two details laying at different depths and $B'$ denotes the stereoscopic image of object $B$. (The objective and remaining elements of the microscope are omitted in this figure.)

If the microscope is properly adjusted for stereoscopic observation, then an observer with the normal eyes views three-dimensional image $B'$ of the object

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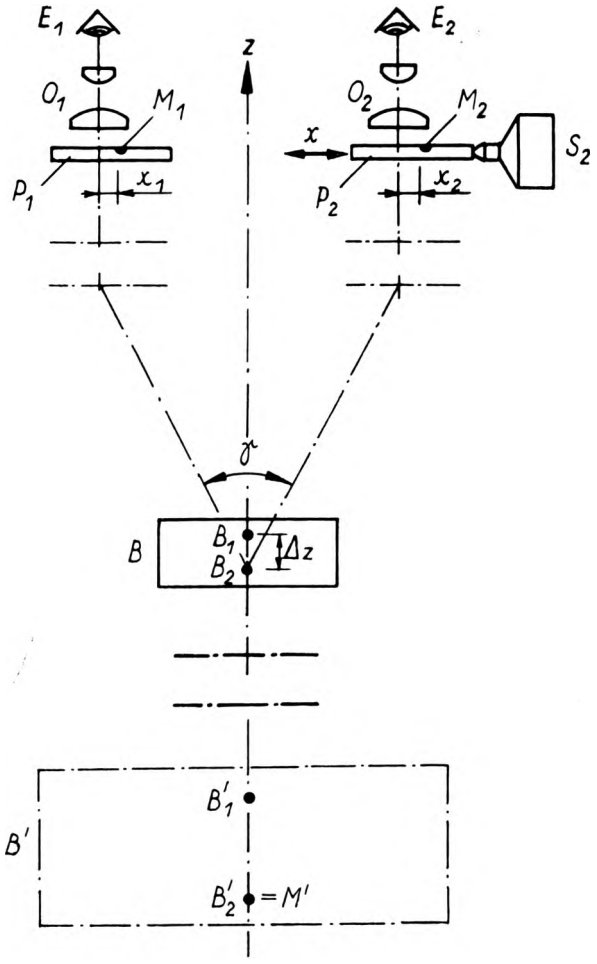


Fig. 1. Schematic diagram showing the principle of stereoscopic depth measurements under the microscope

$B$ together with images of the stereometric marks $M_1$ and $M_2$, and if the distance $x_2$ between $M_2$ and the optical axis of the eyepiece $O_2$ is the same (regarding both the absolute value and direction) as the distance $x_1$ between $M_1$ and the optical axis of the eyepiece $O_1$, then the observer perceives a single image $M'$ of the marks $M_1$ and $M_2$ instead of two ones. Only one image $M'$ is also received by the observer if $x_2$ is slightly greater or smaller than $x_1$. However, when the difference $\Delta x = x_2 - x_1$ is varied, the image $M'$ floats along the optical axis of the microscope objective ($z$-axis), and if one of the focal plates, e.g. $P_2$, is translated in the transverse direction $x$ by means of a micrometer screw $S_2$, then the image $M'$ can be arbitrarily plunged at different depths in the spatial image $B'$ of the object $B$ to be investigated. Thus, the image $M'$ can be matched without parallax with any specimen details $B_1$ and $B_2$, between which the difference $\Delta z$ in depth is to be determined.

Using some standards of depth (micrometric scales set obliquely to the objective axis, piles of glass plates, and others) it was experimentally proved that the location change $\Delta z$, related to the object space of the floating image $M'$ is, within the visual depth of the field of the microscope, a linear function of the transverse displacement $\Delta x$ of the stereometric mark, i.e.:

$$\Delta z = h \Delta x, \tag{2}$$

where $h$ is an instrumental constant. This constant expresses the vertical translation of the floating image $M'$, which corresponds to the unit transverse displacement of the stereometric mark.

When the total magnifying power $\Gamma$ of the microscope, and the angle of convergence $\gamma$ are changed, the value of the factor $h$ changes as well. This is illustrated in Fig. 2, which shows the dependence (determined experimentally) between $h$ and $\Gamma$ for a typical stereoscopic microscope with a magnification-changing system, having the angle of convergence $\gamma = 14°$, front objective magnification 1x, eyepiece magnification 12.5x and interpupillar distance 67 mm. The total magnifying power $\Gamma$ of this microscope was changed by means of its magnification-changing system comprising Galilean telescopes with magnification coefficients $W = 0.65$, 1.6, 2.6 and 3.86. The dependence between $h$ and $W$, or $\Gamma$, has therefore a hiperbolic form, i.e.:

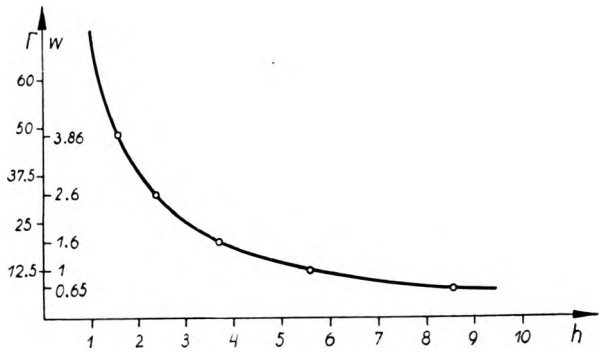


Fig. 2. Dependence between the factor $h$ and total magnifying power $\Gamma$ (or magnification coefficient $W$) for a typical stereoscopic microscope

$$h = \frac{h_0}{W}, \tag{3}$$

where $h_0$ is the value of $h$ for an initial total magnification $\Gamma_0$ arbitrarily fixed (e.g. $\Gamma_0 = 12.5$x).

A situation almost contrary to the previous one — illustrated in Fig. 3 — shows the dependence between the total magnification of microscope and a conventional parameter $k$, defined as the transverse displacement $\Delta x$ of the stereometric mark corresponding to the vertical translation of the floating image $M'$ by a constant or unit value $\Delta z$, related to the object space. In the case of Fig. 3, $\Delta z = 0.2419$ mm. As seen, the experimental points lie very well in a straight

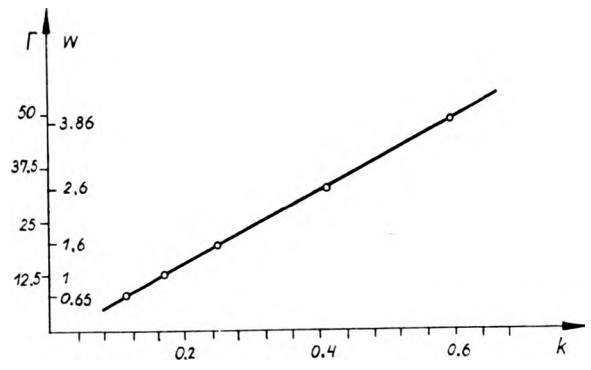


Fig. 3. Parameter $k$ plotted against the total magnifying power $\Gamma$ (or magnification coefficient $W$) of a typical stereoscopic microscope

line. Such a linear dependence results after all from formulae (2) and (3), which give the following relation:

$$\Delta x = \frac{\Delta z}{h_0} W = \frac{\Delta z}{h_0} \frac{\Gamma}{\Gamma_0}, \tag{4}$$

and suppose $\Delta z = \text{const}$ one has a linear dependence between $\Delta x$ and $\Gamma$ ($h_0 \cdot \Gamma_0$ is a constant).

The experimental confirmation of the facts expressed by formulae (2) to (4) is of great importance, since it supplies a simple way for an accurate calibrating of the micrometer screw $S_2$ (Fig. 1) connected with the stereometric plate $P_2$. To this effect it suffices to determine, as precisely as possible, the value $h_m$ of factor $h$, which corresponds to the maximally great total magnification $\Gamma_m$, and next to calculate all the others values of $h$ from the formula:

$$\frac{h_m}{h} = \frac{\Gamma}{\Gamma_m} = W, \tag{5}$$

where $\Gamma$ denotes the remaining total magnifications of the microscope.

The relations referred are approximately applicable to the mono-objective phase contrast microscope MB30S. This is confirmed by the graphs in Figs. 4 and 5, which show the variation of the factor $h$ and parameter $k$ with the total magnification $\Gamma$. In this case $k$ is, however, related to $\Delta z = 1$ μm, $\Gamma$ is varied by applying objectives of different magnifications $\Gamma_{ob}$, and the occular magnification $\Gamma_{ok}$ is invariable (= 18.75x). Asseen the graph 1 in Fig. 5 is not a straight line. This is caused by the fact that — as regards the microscope MB30S — the angle of convergence $\gamma$ changes together with changing of the objective magnification $\Gamma_{ob}$. Each phase contrast objective has a suitable annular condenser diaphragm of different diameters $D$. These diaphragms show the convergence angles $\gamma = 15°$, $22°$, $31°$ and $62°$ for the objectives of magnification $\Gamma_{ob} = 10x$, $20x$, $40x$ and $100x$, re-

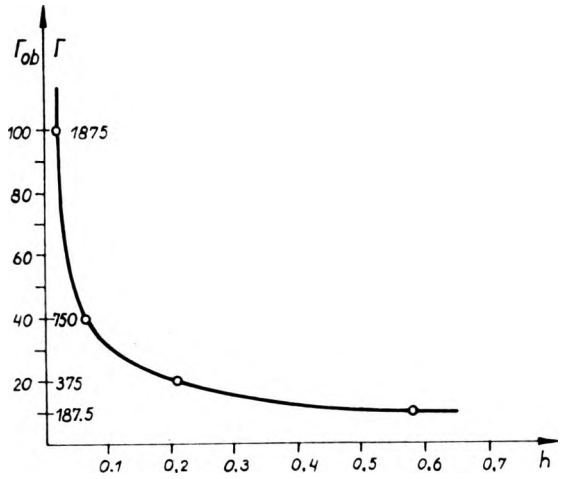


Fig. 4. Dependence between the factor $h$ and total magnifying power $\Gamma$ of the mono-objective phase contrast stereoscopic microscope MB30S ($\Gamma_{ob}$ — objective magnification)

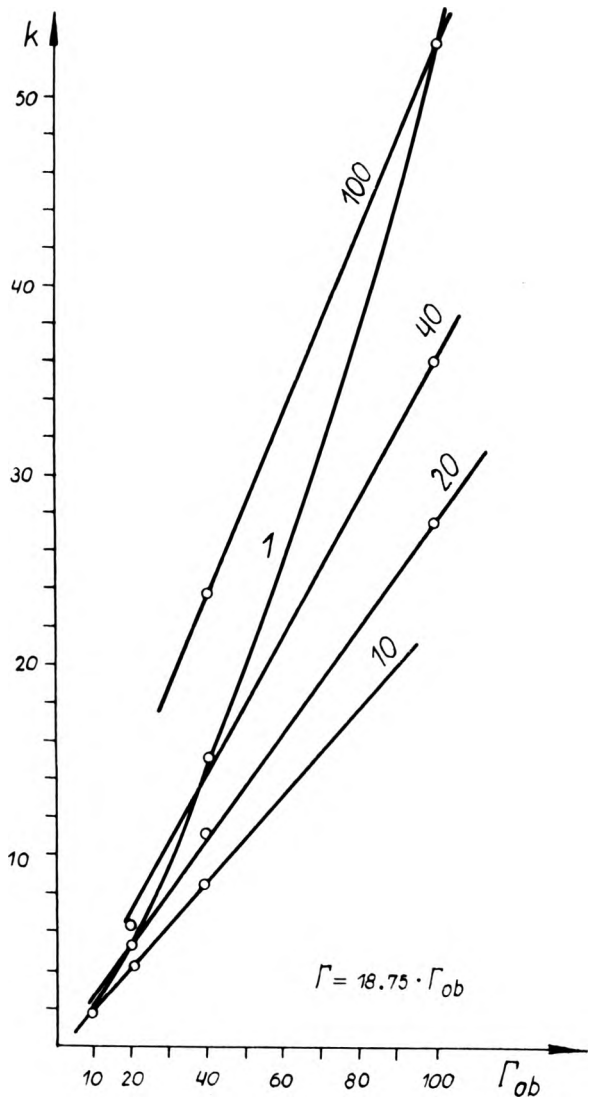


Fig. 5. Parameter $k$ plotted against the total magnification $\Gamma$ and condenser aperture of the mono-objective phase contrast stereoscopic microscope MB30S

spectively. If, however, this microscope is used for observation of amplitude objects, only one annular condenser diaphragm of suitable diameter can be applied with all the objectives. Then, the relation between $k$ and $\Gamma$ is linear, as shown in Fig. 5 by experimental graphs 10, 20, 40 and 100 (these numbers denote condenser diaphragms matched with phase contrast objectives of magnification 10x, 20x, 40x and 100x, respectively). Moreover, each objective can be used with different annular diaphragms giving various angles of convergence. Then, a variation of the parameter $k$ is observed. This variation has a linear form as shown in Fig. 6.

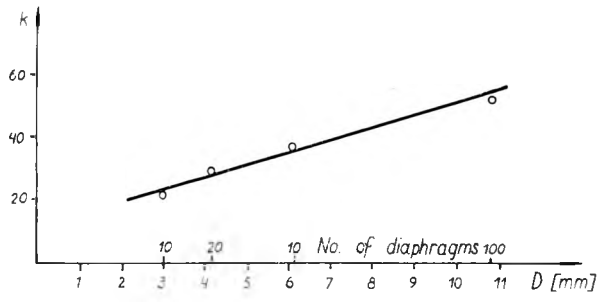


Fig. 6. Dependence between the parameter $k$ and diameter $D$ of annular condenser diaphragms of the mono-objective phase contrast stereoscopic microscope MB30S (objective 100x/1.3)

For the sake of brevity the above described method of the depth measurement will henceforth be termed "method I". It enables to measure differences in depth only within the visual depth of the field of the microscope. Thus, specimens thicker than the depth of the field should be measured by stages. Such a procedure is, of course, inconvenient and rather inaccurate. This inconvenience can be removed by applying another measuring method which will be termed "method II". It requires the setting of the floating image $M'$ of the stereometric marks (Fig. 1) at a fixed depth and next the matching of images $(B_1', B_2')$ of specimen details $(B_1, B_2)$ without parallax with $M'$ by using fine-focusing adjustment. The range of positions of the fine-focusing knob, for which the object details appeared at the same level as the floating image $M'$, expresses immediately the difference in depth $\Delta z$ being measured. As regards sensitivity and accuracy, method II is equivalent to method I when the factor $h = 1$, but it is less accurate if $h < 1$, and vice versa, it is more accurate if $h > 1$. Figure 2 shows that the factor $h$ is greater than 1 in a range of microscope magnifications up to about 75x. Such a range of magnification is typical for standard stereoscopic microscopes (without applying auxiliary objective lenses, which can increase the total magnification up to 150x or at most to about 200x). In the case of the mono-objective stereoscopic microscope MB30S the factor $h$ is, however, smaller than 1 (Fig. 4), the second method is, therefore, generally less advantageous than the first one. This disadvantage is caused by the fine-focusing screw, which does not permit to measure the axial displacements of the objective or microscope stage more precisely than 1 μm. Attempts to overcome this limitation have been made by introducing an optical-path changing device, which enables an experienced observer to focus fine object details with a standard deviation equal to about 0.01 μm. The device (experimental prototype) is shown, together with the microscope MB30S, in Fig. 7. Its basic elements are

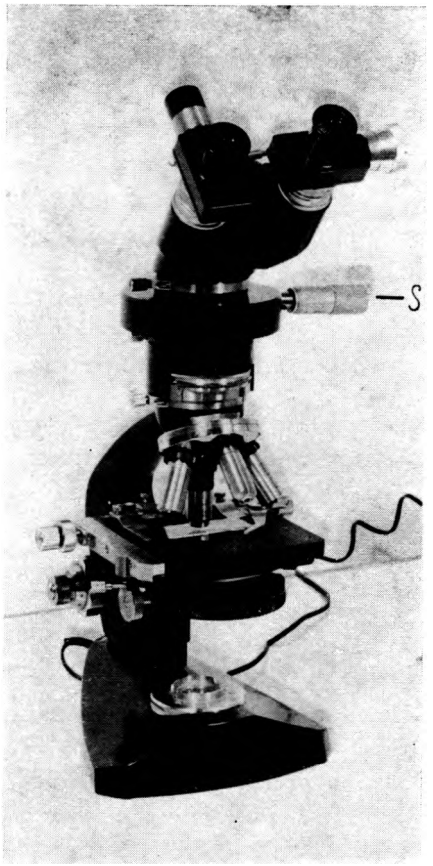


Fig. 7. Photograph of the mono-objective phase contrast stereoscopic microscope MB30S fitted with the microstereometric device described in the text

two glass wedges placed between the nosepiece and the binocular head. The wedges are set opposite each other, thus forming a plane parallel plate. One of these wedges is connected with a micrometer screw ($S$) and can be slid in the transverse direction to the objective axis, thus the optical path and image focusing are varied. In order to overcome a lateral floating of the object image, the other wedge is simultaneously shifted along the objective axis, and the space between both wedges remains constant. The lateral displace-

ment of the wedge by 0.01 mm defocuses the object image by about $\Delta z = 0.001$ $\mu$m. The total wedge displacement alters the optical path by about 10 mm. Such an optical path variation causes an undesired change of the total magnification $\Gamma$ of the microscope. This change is, however, small and can be completely neglected in measurements of little difference in depth.

In conclusion, the stereoscopic method of depth measurements presented here appears to have many advantages and can be useful for quantitative investigations of different microscopic speciments. In favourable conditions one can achieve an accuracy of measurement (mean quadratic error) equal to the value of $\delta z$ which results from Eq. (1) with $\sigma = 20''$ to $40''$. Such an accuracy corresponds to 0.02 – 0.04 $\mu$m, when the mono-objective phase contrast stereoscopic microscope MB30S with the maximum total magnification $\Gamma \approx$ 1800x (immersion objective 100x/1.3) is used.

## Reference

[1] PLUTA M., Microscope, **16**, 32 (1968).