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Volume Determination with an Electron Microscope

A human blood cell was measured as 20 cubic micrometers.

Introduction

ELECTRON MICROSCOPES of various kinds have been used in the biological and other sciences for quite some time. Its use in Virology, e.g., is well established. During the past several decades the main objective of microscope research has been higher resolution. As a result, the presently available *transmission* electron microscopes are capable of resolving less than 0.5 nm. However, observed in thin specimens in terms of the gradually disappearing contrast. This gradual disappearance of contrast depends also on the angle of incidence the electron beam makes with the plane of the specimen. By knowing the thickness of the thin specimens (with, say, interference microscopy) and the angle of tilt between two photographs of the same specimen, along with the knowledge of the magnification, one can obtain some information on

ABSTRACT: *Using a scanning electron microscope to obtain photographs, the volume of a human blood cell was determined to be 20 cubic micrometers, and a platelet as 2 cubic micrometers. Because of the nature of the system, in order to provide three-dimensional stereoscopic views, two separate photographs had to be taken, the second one with the specimen tilted with respect to the first (10° in this instance). The blood cells were contoured at an interval of 0.2 micrometers with a Zeiss Stereotope. The scale of the photos was 5,000:1, and "field" control consisted of a spherical ball known to be 2 micrometers in diameter placed in the field of view.*

these instruments are very restricted in their use insofar as the information content is considered. To quote Hayes and Pease,[1] "The image of a high resolution Transmission Electron Microscope cannot contain three-dimensional spatial information; it cannot contain information directly relative to the chemical (as compared to the elemental) makeup of the specimen; it cannot contain information based on the interaction of highly specific molecular stains such as is available to the light microscope; it cannot contain information related to electrical properties of the specimen; and it cannot contain information obtained directly from living, physiologically intact specimens."

In these conventional electron microscopes the specimens are ultra-thin sections which render two-dimensional specimens and naturally lack specific information on the size and shape of the fibers or lamelles embedded in the sample. Occasionally, lines can be size and shape of the object, so indispensable for meaningful scientific studies.

Against all these, such information can be found in the images of a *scanning* electron microscope. However, this great information content is, unfortunately, accompanied by a loss of resolution, which is approximately 20 nm if operated in such multi-informational modes.

Very recently some researchers in the area of Medicine at the Ohio State University (Drs. Stanley Balcerzak and Nye Larrimer) indicated their interest in studying the volumes of human blood cells and platelets which constitute vital parts of the all important red liquid in the human body.

For the stereoscopic effect the specimen in question may be photographed from two directions. This gives two images that correspond to each other, and points of the object lying at different locations would show parallactic differences. This can be very easily
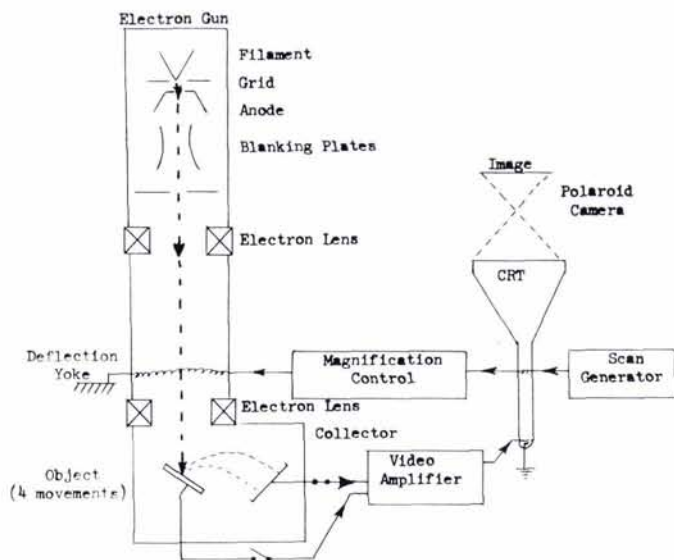
FIG. 1. Schematic diagram of the Scanning Electron Microscope.

accomplished by tilting the specimen between the first and the second photographs. Three-dimensional information can be obtained as long as there are parallaxes (both X and Y) to be seen and optically, or otherwise, be eliminated in the stereo-model. Such information, however, needs to be checked for scale and orientation for proper evaluation of the sample.

In view of the comparative simplicity and ease of operation, and eventual fewer complications, a scanning electron microscope (Model: Cambridge MK2) owned by Battelle Memorial Institute at Columbus, Ohio, was used in these studies.

IMPORTANT FEATURES OF THE SYSTEM

The Scanning Electron Microscope system is described schematically in Figure 1.

A fundamental advantage of the imaging system of a Scanning Electron Microscope is that it uses electron radiation for localization and visible light radiation for information transfer. The high resolving power is dictated by electron optical consideration and the rich information content dictated by the interaction of visible light in matter.

In this instrument electron radiation is used for the two synchronous scanning beams: (1) a point source of radiation sweeping over the specimen in a well defined pattern of lines with a well defined velocity; and (2) a corresponding second point source of radiation over a fluorescent screen. The radi-

ation used to transfer information is the visible light eventually recorded with a Polaroid Land Camera.

A one-to-one correspondence in time and space exists between the points on the specimen and points on the image. However, here this is achieved not by optical focusing, but by time sequencing. This means that the contract mechanism has a wider choice and it need not give rise to information that must be focused optically (e.g., scattering of electrons).

The magnification of the microscope is given by the ratio of the scan amplitude on the CRT face to that on the specimen. The polaroid camera registers the image in a scale 0.85 times that on the CRT screen. However, the total magnification is always referred to the image recorded finally by the camera.

The projection distance in this instrument is about 2 feet. However, this distance with respect to the small area of the specimen (object) gives the geometry of projection which at best can be described as parallel (orthogonal), and is distinctly different from central (perspective) projection so common in the world of photogrammetry. On the other hand, a three-dimensional object photographed in a parallel electron beam cannot be measured with reliability by means of divergent rays representing a central projection because this causes perspective errors which cannot be compensated easily. In this

respect, no conventional precision stereo-photogrammetric instrument can be used in studies with these photographs. This means either a purely computational technique has to be developed which, necessarily, becomes much too complicated to the eventual user (e.g., the biological and medical scientists), or a simple but accurate analog technique needs to be developed.

Considering all the pros and cons, the use of a so called third-order stereoplotter surprisingly offers possibilities unmatched by any other system known so far.

The magnetic lens in use at these microscopes tend to twist the electron beam which, with increasing distance from the central beam, becomes more and more curved (Figure 2). The stage plate containing the specimen (object) has four degrees of freedom, viz.,

1. Tilt, uniaxial, around an axis parallel to the Y-axis corresponding to ϕ tilt in conventional photogrammetry.
2. Rotation, around an axis parallel to the general direction of flow of the electron beam (similar to κ rotation in conventional photogrammetry).
3. X-translation in a direction at right angles to the two rotation axes.
4. Y-translation in a direction parallel to the tilt axis.

Resolution of the system plays a major role in the metric evaluation of the photographs. In the reported application, the microscope could not be intimately tested for resolution. It is, however, more than 10 nm. This may be the limiting factor for precision measurements. Ten nanometers with a magnification of approximately 5,000 times corresponds to 0.05 mm on the photograph. The measuring tool having a standard error of less than 0.05 mm at the photo should then be
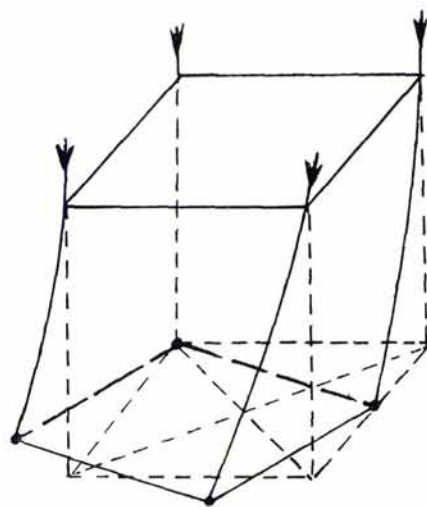

FIG. 2. Distortion of a cube by the twisting course of the beam.

considered as adequate. Higher magnification is considered to be of no help in this respect; it simply tends to obtain an unsharp image. Lower magnification would give sharp photographs but would demand precision measuring instrument and a stable photo base, etc. Therefore, considering all aspects, 5,000 times magnification and the use of Zeiss Stereotope was considered the optimum under the circumstances.

Provision of good and dependable control in the system to check the scale and deformation of the stereomodel imposed a difficult problem. This was partly solved in this instance with a latex particle placed in various locations of the field in several test samples. The latex particle is known to have a spherical
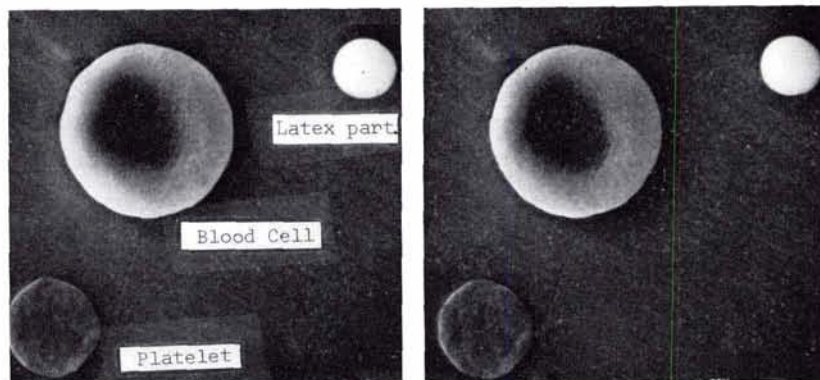

FIG. 3. Stereo pair from a scanning electron microscope. The scale is approximately 5,000:1. The left photo was untilted; the right one was tilted 10°.

Fig. 4. Contour map of the stereo pair of Figure 3 plotted with a Zeiss Stereotope (blood cell only). Compilation scale 10,000:1. The contour values are in micrometers.

shape with diameter between 1.8 μm and 2.2 μm. Therefore, assuming an exactly spherical shape of 2 μm diameter, not only the scale could be checked for accuracy and consistency, but also local deformations could be studied. Its shape being spherical, its dimensions along X, Y, and Z measured separately would provide control on affine scale deformation to some extent. (See Figure 3 for a sample Latex particle.)

A stereo pair of each sample was created by using tilts (0° and 10°) and not using the rotation at all. The tilt axis being away from the stage plate, tilting necessitated translations. By trial and error a stage was reached where only one translation (in X) was required to bring the sample (object) in the field of view after tilt was introduced for the second photo of each pair. This means that only two degrees of freedom, viz., tilt and X-translation were used finally. This provided comparatively simpler geometry and a complication-free system that can be easily handled with a third order stereoplotter. An example of such a stereo pair is in Figure 3.
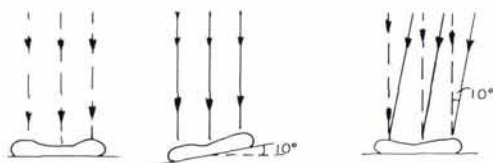
Contoured maps (Figure 4) can be plotted

Fig. 5. Simplified geometry of the system depicting the left photo, the right photo in the center, and the combined photos on the right.

TABLE 1. EXAMPLES OF VOLUME DETERMINATIONS
IN CUBIC MICROMETERS

		Vol. of Blood Cell	Vol. of Platelet
Method 1:	Sample I	—	—
	Sample II	24.5	—
Method 2:	Sample I	19.0	1.8
	Sample II	21.5	1.2
Method 3:	Sample I	20.4	2.3
	Sample II	24.2	1.3

with simple devices like a so-called third-order plotter. The geometry of the projection in creating the stereomodel is depicted in Figure 5.

DATA PROCESSING

The volume of the object can be computed from measurements in any one of the following basic methods:

1. First prepare a contoured map using a third-order plotter (Zeiss Stereotope was used in this study). Volume may be calculated from the expression

$$V = \Sigma a \cdot \delta H$$

where a is the area bounded by a contour and δH is the contour interval.
2. Divide the planimetry of the object into a number of equal sections (squares) and determine the height of each of these sections (i.e., consider the volume as the summation of innumerable columns). Then,

$$V = A \ \Sigma H$$

where A is the area of each square and H is the height of each section.
3. Divide and approximate the sample into simple mathematical cubical forms, determine the volume of each and find the total volume, e.g., the blood cell (see Figures 3 and 4) can be approximated into two parts, an irregular doughnut and a central cylinder, and the platelet can be approximated as a cylinder.

Some examples of such volume determinations using these methods are given in Table 1 where the volumes are in cubic micrometers. Disparity in the scale of the stereomodel was noticed as indicated in Table 2.

TABLE 2. SCALE DETERMINATIONS

	In Horizontal Direction	In Vertical Direction
Sample I	5752:1	5658:1
Sample II	5746:1	5205:1

The average scale factor is thus 5,590:1, which is discussed presently.

Conclusions and Comments

• The scale determination plays a very important role. In the present instance, although the microscope scale indicated an enlargement factor of $5,000\times$ based on the dimensions of the latex particle, we arrived at an average scale of 5590:1.

• For satisfactory stereo measurements, the parallactic angle of 10° as was used in these studies may not be the best. On the other hand, a greater tilt causes to delay in exposing the second picture with the corresponding risk of degenerating or deforming the sample. Thus an optimum angle of tilt has yet to be determined.

• It may be possible to determine the volumes by a purely computational approach where a program could be written to give the required volume directly from the observations taken with a comparator. But the development of such a program may be time-consuming and initially very expensive.

• The electron beams are considered to be projected parallel. Their distortions would tend to deform the image, resulting in a deformed stereo model.

• The resolution with the scanning electron microscopy is 10 nm or more. This could be easily the limiting factor for precision measurement.

• The parallactic angle is obtained from a very crude scale. With an analytical approach this will have to be known with precision, otherwise the mathematical model may be considered erroneous. By using a third-order plotter and by providing some control in the field, this problem was handled empirically with a simplified system.

• Lack of fiducial marks on the photographs would force the coordinates system for measurement (for a computational approach) to be established with respect to the photo corners, which are not always very distinctly sharp.

• The axis of rotation (κ-axis) of the sample cannot be located with precision without calibrating the system. This calibration would mean continued and time consuming tests. The same comment can be made of all the other three degrees of freedom and the system as a whole. Such studies are contemplated at this university. Such studies should involve checks for deformations in the stereo model and linear or non-linear scale errors in the system also.

• Sometimes the lack of surface texture made stereovision difficult. This problem may be overcome by tinting the samples by some means.

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