METHOD OF OBTAINING CLEAR IMAGES OF BIOLOGICAL OBJECTS IN THE SCANNING ELECTRON MICROSCOPE

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UDC 57.086.3

The quality of the image is affected not only by fixation of the material, but also by the conditions of its study in the scanning electron microscope. If the sprayed layer of carbon or heavy metal is thin there is the risk of charging the specimen during rapid scanning with the electron beam, and this interferes with the quality of the image. The study of biological objects is best carried out at two nominal values of the accelerating voltage (for example, 10 and 4 kV), for in this way errors during interpretation of the results can be avoided. Neither the thickness of the layer of carbon or heavy metal or different nominal values of the accelerating voltage can affect the reliability of the information obtained if in each concrete case the physical basis of image formation and the experimental conditions are allowed for in the analysis. Museum material fixed in ordinary formalin can be used for study in the scanning electron microscope.

KEY WORDS: scanning electron microscope; accelerating voltage.

To obtain a high-quality image of a biological object in the scanning electron microscope (SEM) a number of specific difficulties have to be overcome that are not met with by workers using the SEM in other fields of science and technology. To prevent charging, films of carbon or a heavy metal (gold, silver, palladium) are usually sprayed on its surface [2]. It is recommended that the thickness of the layer sprayed on the surface must not exceed 200-300 Å otherwise the film would distort the microrelief of the surface of the object. However, spraying a heavy metal on the surface of a biological object does not only prevent charging of its surface. Even assuming that the surface of the object is not charged, the probing beam, as it penetrates much deeper into the organic object than into the metals, causes distortion of the surface relief it is intended to investigate. Consequently, another reason making it essential to form a film of heavy metal on the surface of the object is the attempt to increase the resolving power of the instruments and to reduce distortion during the study of biological objects on account of an increase in the emission of secondary electrons from the surface.

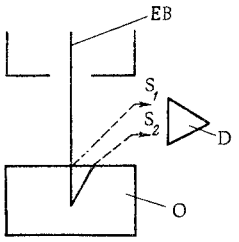

Fig. 1. Diagram showing emission of electrons from an object under the influence of a probing electron beam. S_1) Emission of secondary electrons induced by probing electron beam; S_2) emission of secondary electrons induced by electrons reflected from the depth of the object; EB) electron beam; D) detector, O) object.

The object of this communication is to discuss certain problems connected with distortion of the true microrelief of the surface during a change in the conditions of emission and, in particular, a change in the accelerating voltage and thickness of the film sprayed on the surface of the object, i.e., problems that have been encountered during the study of human and animal cells and tissues.

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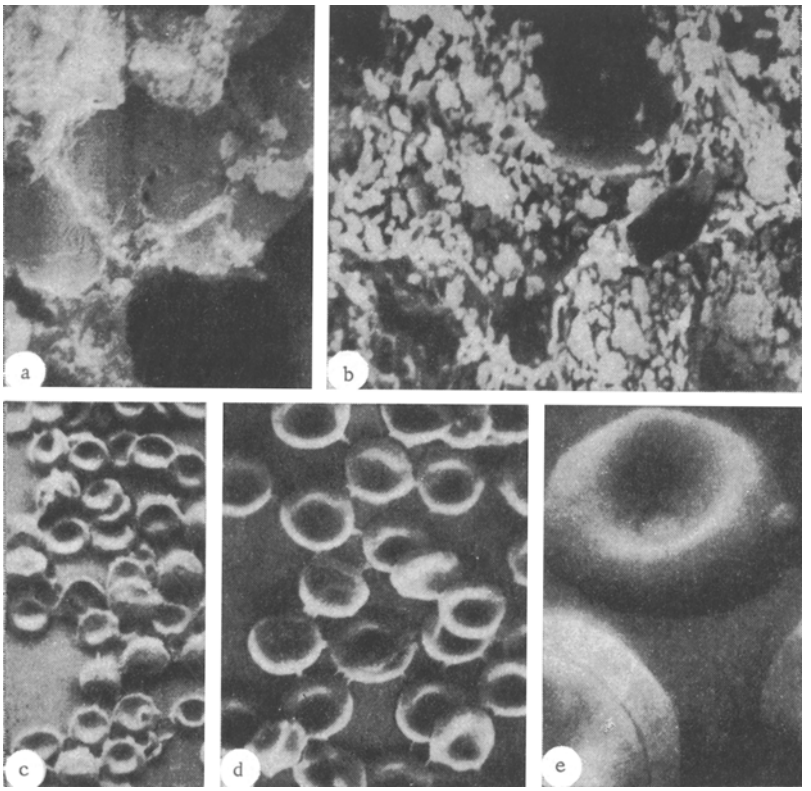

Fig. 2. Biological objects in the raster electron microscope: a) alveolus, REM, 600×. Accelerating voltage 4 kV; b) alveolus, SEM, 600×. Accelerating voltage 10 kV. Aluminum particles of conducting glue lying beneath the object examined; c) under an accelerating voltage of 4 kV the red cells appear flattened, SEM, 2000×; d) under an accelerating voltage of 20 kV the red cells appear biconcave, SEM, 3000×; e) red cells shaped like a pessary, SEM, 10,000×. Accelerating voltage 10 kV.

To examine the causes of distortion of the surface microrelief of biological objects in the SEM it must be recalled that other types of radiation arising when the probing beam acts on the objects will also affect the image quality. The intensity of the secondary radiation is a function of the atomic number of the element of the object and the energy of the interacting electron. Usually depths from which secondary electrons are emitted is less than 500 Å, in the case of reflected electrons a 1000 Å (1 μ), and for x rays 5000 Å (5 μ). The greater the energy of interaction between the electrons and the lower the atomic weight of the material, the deeper the electron will penetrate into the specimen.

Penetration of electrons into the depth of biological objects is considerable. For example, for an organic substance such as celluloid it is 6 μ. [1]. The penetration of electrons inside an object considerably reduces the degree of contrast of the image and the resolving power of the instruments, in which an image is formed in secondary electrons. A decrease in the resolving power of the instrument is an undesirable phenomenon, but one that does not lead to error in the interpretation of the image for the investigator, who simply does not find the individual details because of the lack of contrast of the electron micrographs. A more serious danger is a false interpretation of the image as obtained. The cause of the false information is the appearance of additional secondary radiation as a result of elastic-scattered (reflected) electrons from the depth of the object (Fig. 1).

The deeper the probing beam penetrates, the more electrons are reflected, and the more information is added on account of emission from the surface caused by the reflected electrons.

The results of a study of lung tissue using two nominal values of the accelerating voltage (4 and 10 kV) can serve as an illustration to these facts. Tissues section 10 μ thick were used. The thickness of the sprayed layer did not exceed 100-150 Å. An alveolus is shown in Fig. 2a under an accelerating voltage of 4 kV, and in Fig. 2b under an accelerating voltage of 10 kV. The considerable difference between the images

is explained by the fact that the subjacent structures, i.e., aluminum particles contained in the conducting glue used to fix the section to the metal grid, can be seen in Fig. 2b. Distortion of the surface of the biological object when studied in the SEM is difficult at present to illustrate, for biological objects are not exact likenesses of each other. However, as even the simple analysis of the image of a red blood cell in Fig. 2c, d, under different accelerating voltages, shows the apparent perforation through the red cell (Fig. 2d) is the result of distortion caused by the fact that the contrast of the image is due more to the emission from the grid than to the small depth of the depression of the bioconcave disc of the red cell.

The different shapes of the image of red cells studied under accelerating voltages of 4 and 10 kV is determined not only by differences in the signal, but also by the degree to which the subjacent structures are visualized. The distinctive shape of the red cell (Fig. 2e) could be the result of the arrangement of the hemoglobin inside it. Where there is no hemoglobin the membrane appears "translucent."

To avoid a misinterpretation of the secondary electron image obtained in the SEM, on the discovery of a new form or an interesting surface defect, it is extremely important to compare the results of the study under low and high accelerating voltages.

If the sprayed layer is too thin, this could also lead to a reduction in quality of the image. Poor quality of spraying is easily detected when objects are studied on a television screen (with rapid scanning). The appearance of luminous enlarged areas in the result of charging of the specimen, and if slow scanning is used, poor quality of spraying may be expressed as a loss of clarity of the image, or it may not be noted at all. The reason for the differences in this manifestation of poor quality spraying is that if the subjacent structures are charged and fast scanning is used, the charge is unable to "leak away," whereas at a low scanning speed the charge on the internal structures can lead only to worsening and instability of the image or to the appearance of artefacts.

An improvement of contrast can be obtained by observing the objects at an angle of 30-45°; this is particularly important when isolated cells are studied, for the three-dimensional quality of the image is improved.

Unsprayed objects can be studied under an accelerating voltage of 4 kV. Observations in this way does not detract from the quality of the image at magnifications of up to 700 times.

We found no significant difference in image quality when different materials were used for spraying (gold, silver, carbon), and this is extremely important because of the tremendous difference in cost of these noble metals and carbon. However, if only carbon is sprayed on, and no noble metal is sprayed over it, the contrast of the resulting image is slightly reduced and the risk of visualizing the internal structures of the cells and tissues arises. However, this does not affect the results obtained by the examination of biological objects, for in each concrete case the reasons for obtaining a particular image of the cells and tissues was analyzed from the standpoint of the physical basis of image formation and allowing for the experimental conditions. By analysis of this type it was also noticed that heart valves and blood vessels, if fixed in formalin and kept for a long time (1-2 years) were perfectly suitable for examination in the SEM within a range of magnifications of 100 to 3000 times.

Pathologists, histologists, and morphologists are well aware that museum material, fixed in ordinary formalin, can be used for study in the SEM after a long period of preservation.

Image quality is thus affected not only by fixation of the material but also by the conditions of its study in the SEM. With a thin layer of carbon of heavy metal sprayed on it there is the risk of charging the specimen during rapid scanning with the electron beam, and this spoils the quality of the image. Biological objects are best studied with two nominal values of the accelerating voltage (for example, 10 and 4 kV), in order to avoid mistakes when the results are interpreted.

LITERATURE CITED

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