SCANNING OPTICAL AND ELECTRON MICROSCOPES
WITH COMPUTER IMAGE ACQUISITION*

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A b s t r a c t. A Tandem Scanning Reflected Light Microscope (TSRLM) with computer image analyser and with a structure quantimeter (hardware, software and rotary microtome) are presented and compared to other microscopic methods with respect to new possibilities in visualisation of biological material. Scanning optical microscopes are the most recent constructions in optical microscopy. They offer the rejection of out-of-focus noise and higher contrast than the conventional imaging. The only allowed to reach a detector is the light emitted from the objective focal plane. This cuts off any out-of-focus image blurring. A short history of confocal microscopy from Marvin Minsky to Tandem Scanning Reflected Light Microscope (TSRLM) and Confocal Laser Scanning Microscope (CLSM) has been presented. The use of scanning optical and electron microscope method for the investigation of biological materials is estimated and compared.

K e y w o r d s: confocal microscopy, scanning microscopes, computer image analysis

INTRODUCTION

An optical microscope appeared as a tool for the observation of live organism structures impossible to see with a naked eye. However, both the construction of the microscope and the structure of most of organisms caused that it was an imperfect tool. On one hand an optical microscope has resolution that cannot be exceeded so we are not capable to notice objects smaller than half the wavelength of the light illuminating a preparation, and on the other, biological structures are often transparent for visible light and therefore invisible in a microscope.

The latter problem has been managed with by working out drying procedures, which however, reduced the resolution capability and in majority required the work with a dead material being substantially changed. Reaching the limit of capabilities of the time combined with the appearance of an electron microscope caused that an optical microscope slowly became an auxiliary instrument. Only the appearance of a confocal microscope gave the beginning of a Renaissance in optical microscopy.

The principle of operation of a confocal microscope has been presented in a patent bureau by Marvin Minsky in 1957, but the first, fully satisfactory construction appeared in the market about thirty years later.

CONFOCAL MICROSCOPY

The construction of Minsky's microscope is very simple [5,6,10] (Fig. 1). Traditional condensing lens has been replaced with an optical system identical with an object glass system. A diaphragm with a very small pinhole has been placed between the light source and a condensing lens, at the optical axis of the instrument.
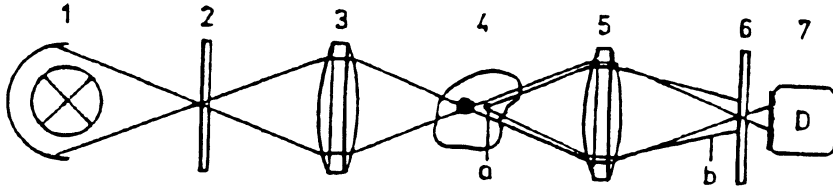
Fig. 1. Diagram of the simplest confocal microscope. 1 - light source; 2 - diaphragm producing an illumination spot; 3 - condenser; 4 - object; 5 - object glass system; 6 - diaphragm; 7 - detector; a - point out-of-focus, b - light stopped by diaphragm.

A diminished image of this diaphragm has been projected onto a preparation through the condensing lens. The range of view of the object glass system has been limited by a second, twin diaphragm placed symmetrically to the first one and separating the object glass system from a photodetector. Thus, both diaphragms and the point on the object plane being observed have been located in the foci of two optical systems, and, at the same time, the focus located in the field of observation has been common to both optical glass system and condenser. The diaphragms had common foci, i.e., they have been confocal, with the plane of observation and this is the origin of the name of the entire optical system. As a result of such construction, an image of one point from one plane has been project onto the detector; the smaller the light of the diaphragms, the more precise the projection. The images of the points located out of the focus level were not points in the diaphragm plane but were of a surface nature. Therefore, most of the light creating them has been cut off and has not reached the photodetector. Now, when an object was moved and subsequent states of the detector registered, it has been possible to reproduce, point by point, the image of one layer of a preparation, separated from others, with the clarity of image that had not been achieved before.

The construction of Minsky's microscope had been faced with two problems. The first was that it was necessary to put the optical elements together very precisely so that the condenser and object glass system were confocal; the problem was substantially growing with an attempt to use a changing enlargement, which had been connected with changing both condenser and object glass system at the same time. The second prob-

lem was how to move the preparation. The precision of stage movement had to be comparable with the resolution capability of a microscope and at the same time it had to have a big movement range and practical, i.e., not very low, movement speed.

The first of the problems has been solved quite quickly: they resigned of the light passing through the preparation for the benefit of the construction of a microscope with epiilluminescence. In this illumination system, the object glass system acts as a condenser at the same time, which indicates the object glass system to be a natural construction basis for a confocal microscope. However, this decision had limited the application of confocal microscopy to the methods, which take advantage of epiilumination, i.e., first of all to epifluorescence and reflected light microscopy. Resignation of the penetrating light in confocal microscopy had not been as painful as it had seemed at first. The definition obtained in a conventional microscope equipped with Nomarski's DIC contrast is comparable with that obtained in a confocal microscope [6]. If we combine CLSM with Nomarski's contrast in one instrument, which can be done [3], then we should satisfy any requirement.

The second problem appeared to be much more difficult. A satisfactory system of stage movement has not been constructed till present although much improvement is observed. In 1967 the first attempts at dealing with the problem concerning the movement of the object by inducing the movement of a light spot project onto it were undertaken. This experiment has been performed by Mojmir Petran and Milan Hadravsky from Prague, who has constructed a confocal tandem microscope [9, 10]. This basis of this microscope is Nipkov's

disc, named so by the name of its inventor, Paul Nipkov, a German student, who constructed it in 1934 as an instrument for telegraphic image transmission. The movement of the disc by one cycle causes that a number of pinholes mapping subsequent lines of the image being processed pass by the detector. If one set of pinholes is there on the disc, then the cycle is equal to one turn of the disc. If there are more sets of pinholes, then many cycles are contained in one turn. An even number of pinhole sets is contained on a disc used for tandem confocal microscope. Half of them is used as diaphragms for the illuminating beam and the remaining ones - for the light coming from a preparation. At appropriately high disc r.p.m. an image characteristic for a confocal microscope is obtained. It can be observed directly, with no need to use a detector and further processing of an electronic signal. It allows to carry out observations in a real time. Recently, Gordon Kino [9] from Stanford University has modified this construction so that one set of pinholes, through which light passes in both directions, is enough. The basic problems, which arise while using Nipkov's disc in practice are: necessity to make the disc itself very precisely and to place it very precisely in the light beam combined with high r.p.m. speed. Such a disc has about 200 000 precisely located pinholes and is rotating at about 2 000 r.p.m.

The Tandem Scanning Reflected Light Microscope (Fig. 2) is unique in the sense that it allows one to see objects in real time directly, i.e., without destructive sample preparations (e.g., living cells and their components in animals and plants, or the shape of non-planer surfaces) with enhanced contrast and with unmatched resolution to depth, depending on the transparency of the object. It gives a new visualization of objects and a possibility to follow motions in structures in real time, which cannot be obtained by any other microscope. If we look at a living tissue, or a non-polished surface or three-dimensional structure with a reflected light microscope, we cannot see much more than a general pink blur. The reason for this is that the light is scattered within the entire volume of the illuminated specimen. The image we want to see is usually very
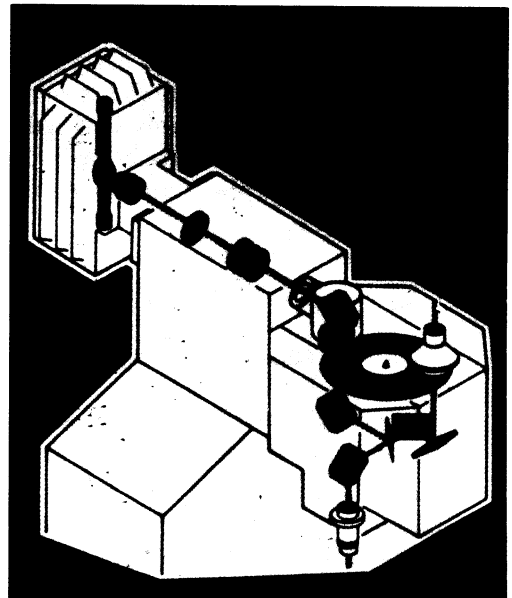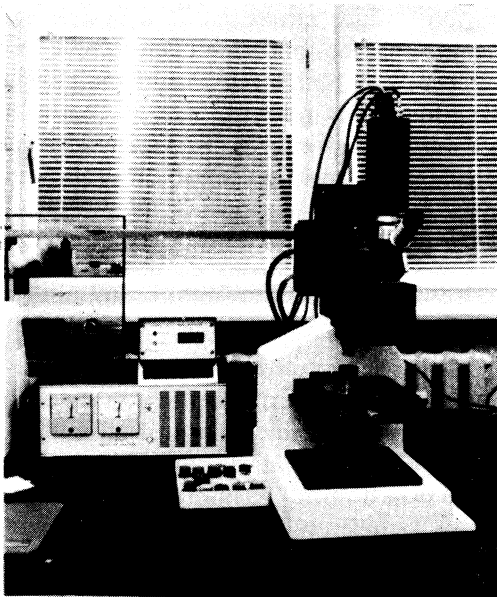

Fig. 2. Tandem scanning reflected light microscope (TSRLM).

weak and it does not show much contrast. Countless reflections from out-of-focus layers and from optical surfaces of the microscope modulate the image brightness more than a weak reflection from the thin layer, which ought to be in the focus, so that the resulting contrast is very low. The contrast can be increased by narrowing the illuminated field but, in this case, the details cannot be seen in the context. The field observed will be surrounded by a halo. The images observed by conventional optical microscopes are improved with the best dark ground illumination, which can approach the ideal situation of self-illuminous object. A better situation is to use the fluorescence principle. In neither case, however, we can observe the image of a thin layer, nor we can penetrate a significant distance into a semi-infinite specimen which is our main purpose. Coming back to the idea of limiting the illuminated field we can put together a composition of small fields which would be a time consuming, non-practical and the halos of surrounding fields would interfere. The halos can be eliminated by making sure that the light seen by the eye or another detector comes from the most strongly illuminated layer the objective is focused upon, i.e., if we have a point light source and a point detector - a confocal microscope like TSRLM. Our microscope is the fastes confocal microscope, i.e., it needs 20 ms only to capture the image in the computer analysing system, which makes it possible to observe the motions in real time.

The microscope was design to overcome the following general problems:
- to have an enhanced contrast;
- to observe the microscopic image of a thin layer in a non-destructed object without any preparations (e.g., living tissue or non-planar, non-polished surfaces) with enhanced;
- to capture the image in real time for the investigation of motion and computer analysis using CRT video-system;
- to penetrate a significant distance into a semi-infinite specimen with the resolution to depth dependent on the transparency of the object.

The object plane is focused-on illuminated in several very fine light spots by a light beam travelling over the specimen. This is the first or the illumination scanning. The light reflected from this object plane goes to the image plane where it hits a moving diaphragm with openings, which allow the light coming from the illuminated 'points' of the object plane focused - on only to go through. Almost all the light dispersed in the deeper or shallower parts of the object, or reflected on the microscope lenses intercepted by the opaque portions of diaphragm. This is the second, or image scanning. The third scanning is performed in the computer image analysis system.

COMPUTER IMAGE ANALYSER

The image from microscope undergoes acquisition and analysis. Structure analyser is a complete image processing computer system for image analysis in laboratory, industrial or hospital environments, where images are an ideal way of conveying or storing informations. It is the open system, which makes rebuilding and changing of configuration or destination possible. This is 'user's friendly program', which means that it is flexible for changes and does not require any special experience with computers. Many possibilities allow to use this system in scientific research or industrial applications. This system makes it possible to complete image analysis from image acquisition to graphical representation of results. Provides a versatile tools for particle size analysis allowing the user to choose the functions and features necessary to suit his particular needs (Fig. 3).

This includes powerful routines for preparation, separating and extracting objects and their parameters to count interesting quantities. Flexibility of system enables work in wide range materials from a micro- to macro-scale. Program feature include: calibration, shading corrections, grey and pseudo-colour image processing, wide range objects measurement, result output options, storage of work results, the system enables particle size, shape and statistical analysis [1].
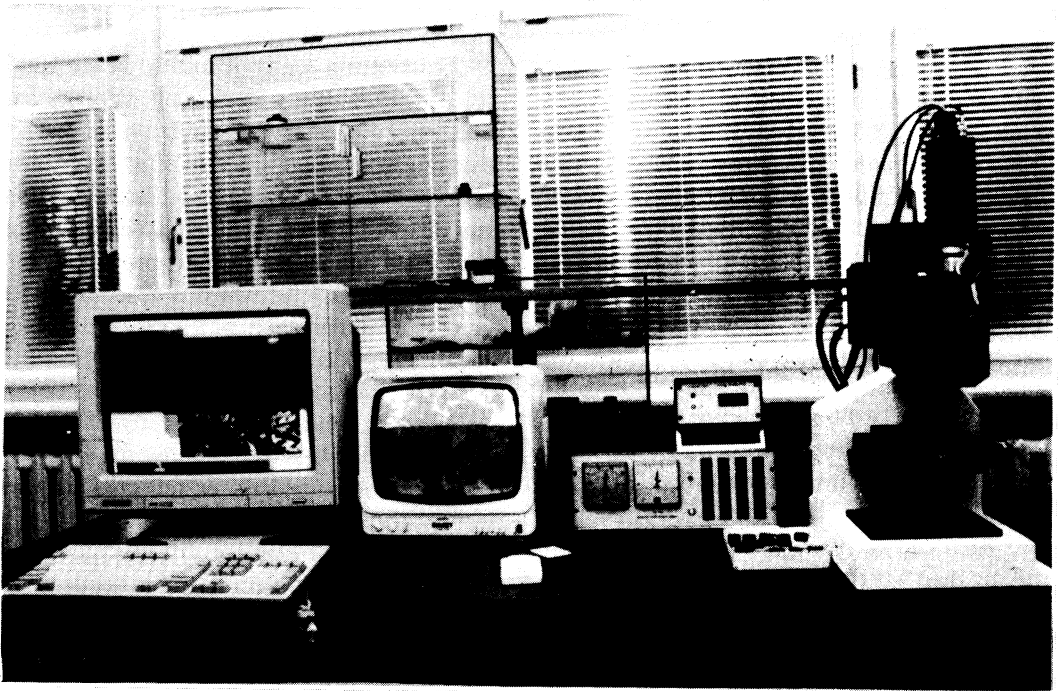
Fig. 3. Scanning optical microscope and computer image acquisition.

ROTARY MICROTOME

Rotary Microtome is a device with a rotating blade for smoothing and slicing to make precise slices of live, soft tissues (Fig. 4). It is specially useful for cutting samples for the Scanning Reflected Light Microscopes and Electrophysiology. This device can make slices of most of the living tissues except for bones and teeth. Furthermore, it works well on fixed tissues, plant tissues, other fibrous tissues and even on paper

Fig. 4. Rotary microtome.

and silicone. It is equipped with a special chamber where the tissue can be fed before observation, e.g., using fine bubbles of O_2 or CO_2.

Tissue slices of quickly dissected soft tissues such as the brain, can be obtained easily and efficiently. After fixing the tissue on the agar stage, the system provides slices of material.

SCANNING ELECTRON MICROSCOPY

The scanning electron microscopy (SEM) makes it possible to observe the external structure of the objects being investigated, whereas transmission electron microscopy (TEM) enables us to observe the inner structure of a preparation [2,4].

The invention and development of these technics has been caused by the limitations of resolution, magnification and depth of field in case of optical microscopes, which result from the light diffraction and interference effects on the objects smaller than half the wavelength of the incident light.

The discovery of dual nature of elementary particles by Louis Victor de Broglie in

1924 resulted in the idea than an electron beam can be used to 'illuminate' an object being observed.

The length of an electromagnetic wave, corresponding to a beam of electrons accelerated with 100 kV is $1 \cdot 10^{-11}$ m , i.e., theoretically the resolution capability yielded by such microscope is $5 \cdot 10^{-12}$ m. Unfortunately, due to the imperfections of electromagnetic lenses, which are similar to those of optical lenses, i.e., spherical and chromatic aberrations, and astigmatism, the best transmission electron microscopes manufactured at present have resolution capability of $1.2 \cdot 10^{-10}$ m and magnification of 800 000 times (Philips, Jeol, Opton). Scanning electron microscopes have the resolution of ca. $6 \cdot 10^{-9}$ m and magnification of 200 000 times.

As it can bee seen in Fig. 5 electron beam falling onto a preparation can:

- reflect from the preparation,
- knock secondary electrons out of the preparation,
- cause the emergence of a characteristic X-ray radiation,
- cause the luminescence of a preparation in the visible range of the spectrum,
- go through a preparation.

All these effects but the last one are used in scanning electron microscopy, while the last of them, i.e., analysis of electron going through a sample constitutes the foundation for transmission electron microscopy.

The electron source is located in the upper part of the column - a glowing cathode, and underneath - an anode plate with a hole. The accelerating voltage of 300 V to 40 kV is applied between cathode and anode. Accelerated electrons, due to inertion, go through the hole in the anode plate into the interaction zone of a (magnetic) condenser lens, which is responsible for appropriate 'illumination' of a preparation.

Then, the electron beam runs into the interaction zone of another magnetic lens - an objective (-F) lens, whose task is to focus this beam on a preparation in such a way that the optimum conditions for secondary, or reflected primary electrons detection are obtained.

An important element differentiating the design of scanning electron microscope from transmission electron microscope is the presence of two scanning magnetic lenses, which are to brush the surface of a preparation with a focused electron beam according to the work conditions selected by the operator. Both reflected and secondary electron detectors are located close to the C-F lens, over a preparation. The detectors are multiplier phototubes, which, together with power discriminators, selectively choose and analyze point by point reflected or secondary electrons.
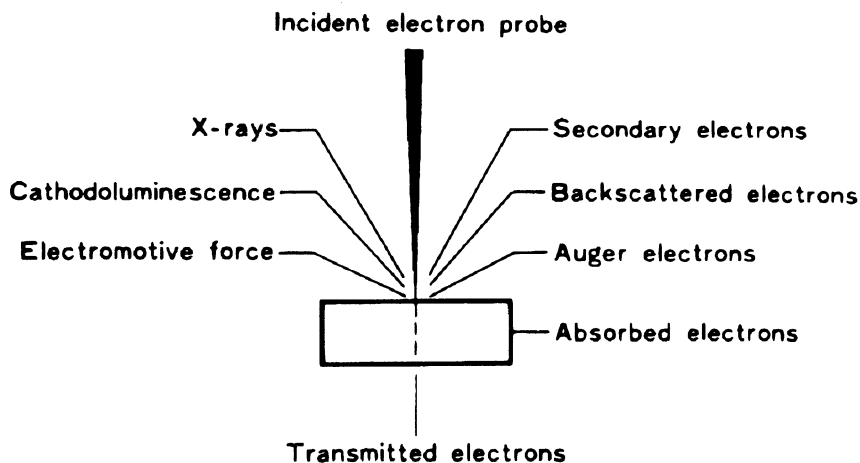

Fig. 5. Interaction of an electron beam with a preparation.

THE IMAGE IN REFLECTED ELECTRONS

Although reflected and secondary electrons present the topography of the surface, as it can be seen in Fig. 6 the image obtained with secondary electron detection is more varied and legible as compared with the image obtained with reflected electrons [7,8].

We can imagine this in a diagram form looking at Fig. 6, where in case of secondary electrons we obtain a shadeless illumination of a preparation; in case of reflected electrons we obtain a shaded image of a preparation.

The image of the object being observed can be seen on a TV monitor screen. Photographic documentation is obtained by taking pictures of a special high-resolution screen. As the image is obtained by electronic processing of detector signals, contrast and brightness of the image is adjustable.

One should remember that the entire process of beam focusing and its detection must take place in perfect vacuum, so that the electrons not collide with the particles of air gases and do not change their track. This fact enforces specific requirements concerning the preparation of biological material sample for observation in a scanning electron microscope.

Like in routine histological investigations, a preparation must be fixed in a buffer solution of glutar aldehyde. A subsequent stage is to dehydrate the sample in alcohol or acetone series. It appears, however, that the most dramatic moment in preparation is to replace the liquid phase of the solvent in a cell with a gas phase.

Figures 7 and 8 present the same structure. Figure 7 presents the material from which the solvent vapourized in a natural manner, while Fig. 8 presents the very same structure dried in the so-called 'critical point'. Such drying consists in the replacement of a liquid solvent with a liquid-solid-gas CO_2 or with freon 13 in critical conditions. Such conditions correspond to the phase balance of a solid, liquid and gas states. For CO_2, the critical temperature is 31 °C, and critical pressure 7.5 MPa. Contrary to the expectations that such high pressure may cause the deformation of the structure, as we can see in Fig. 5 the structure is maintained properly.

The small size of this apparatus draws attention but it is not quite normal considering small capacity of a drying chamber. The drying process itself is time-consuming. It consists in introducing the sample to be dried into a drying chamber, air-tight closing of the chamber, slow pumping of CO_2 into the drying chamber, and adjusting the temperature and pressure to the critical conditions. Then, after flushing with
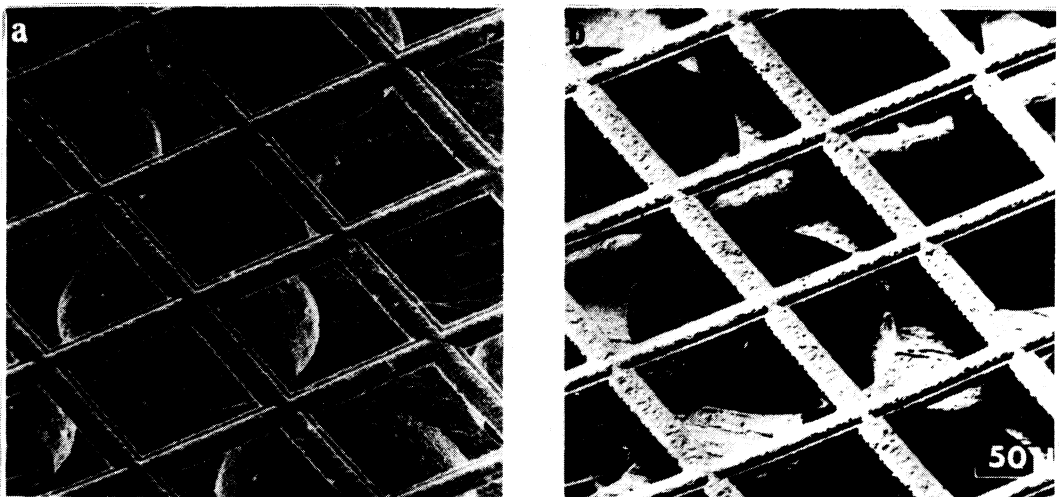

Fig. 6. Difference in illumination effect between the secondary electron image (left) and backscattered electron image (right).

Fig. 7. Image of a tissue after natural drying.

Fig. 8. Image of a tissue after critical point drying.

subsequent portions of CO_2, which remove the solvent from the preparation, the pressure is being decreased very slowly, which, depending on a preparation may take even a few hours.

From the preparation dried in this way, gases are being removed in the microscope chamber only. However, in order to be able to observe a biological object, which as a rule is a bad electricity conductor, such preparation should, after drying, be coated with a thin film of a good electricity conductor - usually gold. Usually, this process is being conducted in the so-called sputtering depositors.

It is a special type of depositors specially appropriated for scanning electron microscopy. Perhaps, you could observe the coating process in the incadescent depositors. The coating of a surface with a required metal takes place in effect of vaporizing metallic atoms, with which a given surface is to be coated in a perfect vacuum. However, the atoms of such metal, like a light beam, travelling along straight lines, shade the surface and do not coat it with a uniform film, whereas in sputtering depositors the preparation most often is placed at the bottom of the chamber. Over the preparation, removed by some 15 cm, there is a gold plate. Air is being pumped out of this chamber, and it is replaced with a neutral gas - most often argon. Voltage of ca. 1.5 kV is applied between the gold plate and the base on which the preparation is placed.

The gas in the chamber undergoes partial ionization and the positive ions Ar accelerated in an electric field strike the gold plate knocking gold atoms out of it, and those atoms fall down due to the earthpull. Due to Brown's motion of argon atoms, the gold atoms colliding with other atoms do not travel along straight lines and therefore even the hollows of the preparation are being coated with a uniform film.

On the other hand, the scanning electron microscope made by a Czech company Tesla named BS 340 has a guaranteed resolution of 7 nm and magnification of 10 to 200 000 times. This microscope is possessed by Institute of Agrophysics, Polish Academy of Sciences in Lublin.

Additionally, it is equipped with a magnification marker, possibility of preparation description, division of an image into two parts, one of which is an enlarged, selected part of the image of the second one, in the following proportions: 1:2, 1:5 or 1:10. The preparation is being moved using a special joystick or mechanically. At this disposal, the operator also has a microcomputer storing 100 of programmed positions of a preparation.

Subsequent picture (Fig. 6) presents a perfect anti-bacteria filter, i.e., the shell of a bird egg. Another example is a amazing structure of human heel bone - Fig. 7. Equally well the examples from the world of plants are being photographed.

TRANSMISSION ELECTRON MICROSCOPY

The incident electron beam passing through a preparation and detection of these electrons constitutes the image in the transmission electron microscopy. The structure of the beam in the transmission electron microscopy is presented in Fig. 5.

Like in the previous type of a microscope, the source of electrons is made by a glowing cathode and underneath an anode plate is located. Accelerating voltage of 40 to 200 kV is applied between cathode and anode. Under the anode plate there are magnetic condenser lenses. Then, the beam goes through a preparation and comes into the zone of interaction of a magnetic objective lens, which creates the first magnified image of the object being observed. Below the intermediate and projective lenses are located. The final effect is made by the image observed on a fluorescent screen. The resolution capability of transmission electron microscopes is much higher and reach ca. $1.4.^{-10}$ m with the magnification of up to 800 000 times.

In contrast to a scanning electron microscope, the thickness of the preparation plays an important role with regard to the quality of the image. Therefore, the technique of preparation for this type of microscopy is different. As the inner structure of a cell is being observed, the thickness of the preparation should not exceed 60 nm. In order to comply with this requirement, the preparation after previous fixing and contrasting in osmium oxide (VIII) OsO_2 must be hardened in special resins and only the sample prepared in this way can be sliced into microscopic preparations of a required thickness using an ultramicrotome. Using this apparatus one can get the slices with the thickness of ca. 0.5 nm. So thin the slices can be obtained cutting the preparations fixed in resin using self-prepared glass cutters or company-made diamond cutters.

Similarly to a scanning electron microscope, the entire beam focusing process is carried out in perfect vacuum. However, this vacuum does not have any impact on the preparation placed in the column as it is hardened much earlier in the preparation process. Often a questions is asked if it is possible to obtain a colour image in electron microscopes. The very principle of electron microscopy, and what follows the maximum reduction of chromatic aberration, the image is obtained in varied shades of grey only, depending on the number of electrons falling on too the screen. However, the shades of grey can electronically by converted into specific colours.

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