THE USE OF ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY (Aquasem) IN COLOPROCTOLOGY

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Summary: Aquasem is the environmental scanning electron microscope (ESEM) - a device which permits the direct observation, manipulation and analysis of many materials in their natural and unaltered state. The microscope operates under low vacuum and fresh or unfixed biological specimens are maintained wet in a separate chamber filled with water vapour. The aim of this study was to examine the mucosa of small and large bowel and anal canal.

Different sample preparation methods were used in 34 speciemen and the results were compared with the use of both conventional scanning electron microscopy (SEM) and ESEM. In the future, we are to examine the tumor surfaces of colon, rectum and anus.

Key words: environmental scanning electron microscopy, coloproctology, mucosa

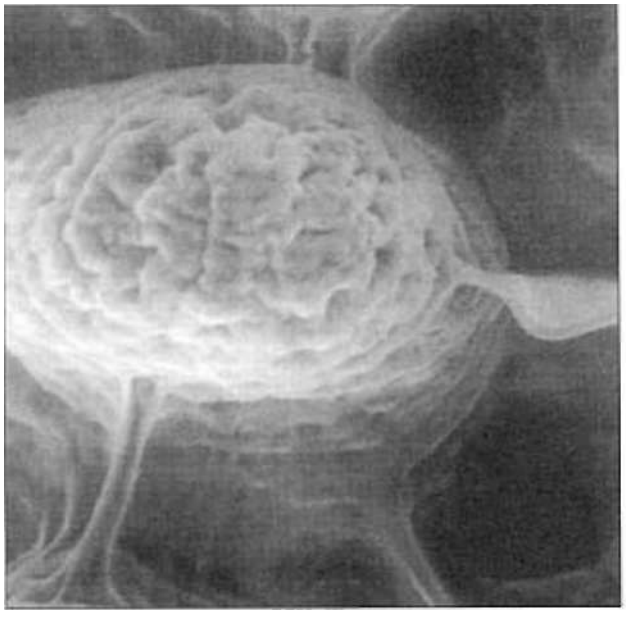


Fig.1
Ileal mucosa (x 520)

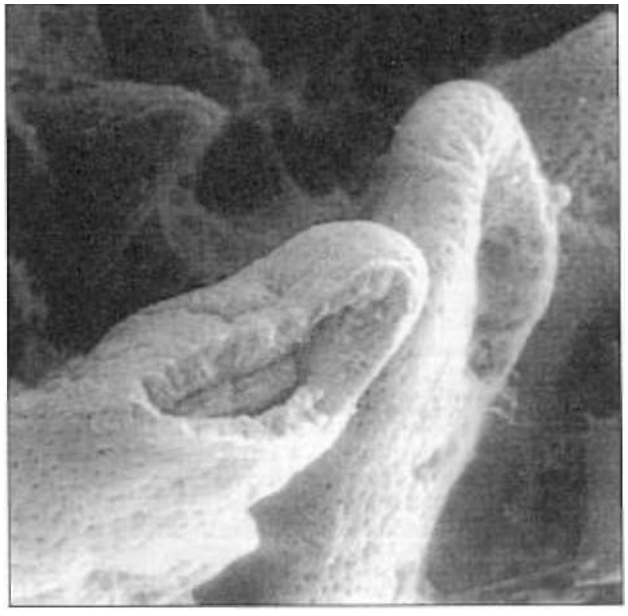


Fig. 2
Recta mucosa (x 370)

INTRODUCTION

In 1997 it was 100 years since the British physicist Thomson discovered one of the fundamental matterparticles - the electron. This discovery stimulated research activities in this field so much that, in a relatively short period, both electron mass and charge were determined and, using the laws of mechanics and optics, its movement was described. The finding that an electron beam of a defined energy behaved similarly to a light beam became the foundation for the construction of electron optical devices. In 1931 Ruska and Knoll put together the first transmission electron microscope and in 1939 its manufacture commenced. In 1938 Von Ardenne published his discovery of the scanning principle and when, in 1942, Zworikin used a scintillation counter for detection of secondary electrons, the first scanning electron microscope (SEM) in the world appeared *(Mráz and Polónyi,* 1988).

Initially, it was believed that, because of extreme conditions to which specimens are subjected, electron microscopy (EM) would not be a technique suitable for processing biological materials. In thel940s, preparation methods to separate specimens with high water content from the destructive effects of vacuum were still being sought. These trials, however, were not successful.

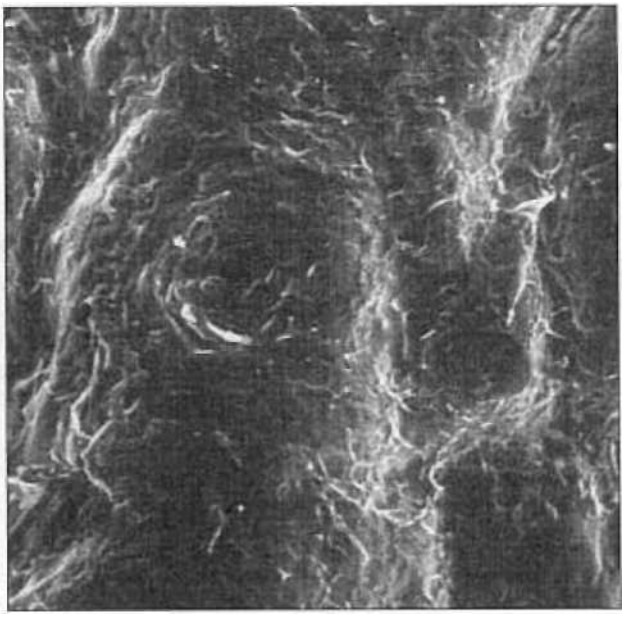
The development of S E M made remarkable progress with the advent of television. The S E M resolution power improved and the contruction of suitable detectors permitted the use of the great amount of information which comes from the interaction between electron beams and the specimen.

In spite of the fact that methods for preparation of biological specimens, including soft tissues and individual cells, have shown great advancement, their demands in terms of costs and time revived the idea of constructing a device which would allow the viewing of specimens in their natural state, i.e., with their water content *(Danilatos,* 1981).

The first attempts at this, conducted in the 1970s, were influenced by efforts to wrap the specimen in a thin film transparent to primary electron beams. This approach made it possible to produce electron micrographs of biological specimens at atmospheric pres-

Fig. 3
Meckel divericulum (x 300)

Fig.4
Unfixed wet specimen anal mucosa (x 370)



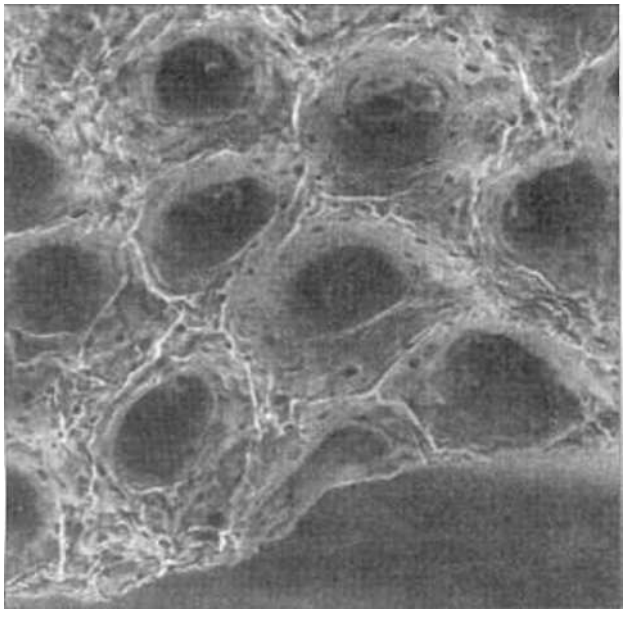


Fig. 5
Unfixed sample immediately after being placed in the microscope rectal mucosa (x 260)

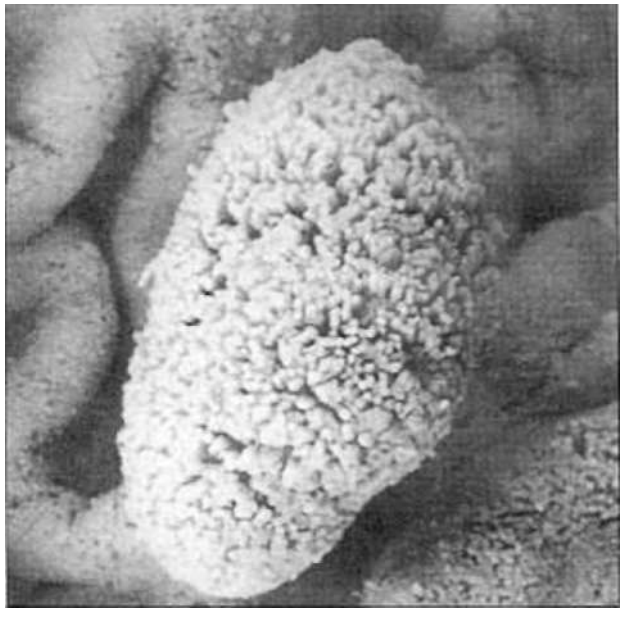


Fig. 6
PEG fixation rectal polyp (x 420)

sure *(Danilatos,* 1980). Useful results, however, were obtained only within the scanning mode, i.e., using transmission electrons *(Dupouy* et al., 1962; *Lane,* 1970).

Another approach was the construction of a specimen holder which permitted continuous diffusion of water vapour into the vacuum and production of a higher pressure zone around the specimen which is thus protected against deformation. Further improvement in this principle resulted in the construction of two-chamber vacuum systems. The high vacuum electron optics column is separated from the specimen chamber in which vacuum is regulated by a diaphragm with a minute aperture. These SEMs, termed High Pressure or, more often, Low Vacuum Scanning Electron Microscopes (LVSEM), do not facilitate direct insertion of specimens but have the advantage of allowing observation of non-conducting materials without metal shadowing *(Danilatos and Robinson,* 1979; *Danilatos,* 1991; *Shah and Durkin,* 1992).

The most accurate method for direct viewing of wet specimens has so far been achieved by separating the high vacuum chamber from the regulated vacuum chamber with the use of a differential pumping system. This principle has been developed, both theoretically and in practice, by the Australian scientists Robinson and Danilatos in the 1980s *(reference).* They introduced the designation Aenvironmentald' to emphasise that the viewed specimen is situated in a surrounding which is close to its natural environment. At the beginning of commercial use, some manufacturers tried to construct adapters to conventional electron microscopes. The first producer of environmental scanning electron microscopes (ESEM) was the PHILIPS Company which had bought the patent coverage from the American ELECTROSCAN Co. and, in 1995, introduced the SEN XL 30 ESEM on the market.

In the same year the TESCAN Company in Brno presented an environmental scanning electron microscope under the name AQUASEM. This was the result of joint research and development activities carried out by a team of workers from the Institute of Scientific Instruments of the Academy of Sciences of the Czech Republic and from the Department of Electrotechnology, Faculty of Electrical Engineering and Computer Science, Technical University in Brno, supervised by Professor Autrata *(Autrata* et al., 1997). Their ESEM was based on a TESLA BS 434-PERLA electron microscope and some their own patents. The manufacturer of these microscopes became PRECIOSA CRYTUR, Ltd., Turnov.

This paper reports on our initial experiences with the AQUEASEM.

MATERIALS AND METHODS

Environmental scanning electron microscopy facilitates the observation of specimen surfaces without any special treatment, thus allowing time consuming processes of fixation, dehydration, drying and metal shadowing to be eliminated.

In this study, biopsy samples of human digestive tract tissue were used.These were collected, together with tissues for histological examination, from patients being operated on at the Department of Surgery, Masaryk Memorial Cancer Institute, Brno. The samples were fixed with 5% formaldehyde to preserve them during the period between surgery and observation.

The other samples included in this investigation were selected organs from insects, worms and mice.

After the relevant processing, the samples were viewed

1. Tesla BS 300 scanning electron microscope (after fixation, drying] and metal shadowing)
2. AQUASEM scanning electron microscope
   a) unfixed and wet
   b) fixed and wet
   c) fixed, dried and without metal shadowing
   d) fixed, dried and metal shadowed
   e) fixed twice and wet
   f) fixed twice, dried and without metal shadowing
   g) immersed in polyethylene glycol (PEG)

RESULTS AND DISCUSSION

In August 1997, an AQUASEM scanning electron microscope, which is a property of the Masaryk Memorial Cancer Institute, Brno, was installed in the electron microscope laboratory of the Department of Histology and Embryology, Faculty of Medicine, Masaryk University in Brno.

This computer-controlled ESEM facilitates the viewing of the surfaces of conductive, non-conductive and wet specimens, recording and saving enlarged images in the standard format onto computer recording media and further computer processing of the image.

This ESEM operates on the basis of a three-step vacuum system in which the differential pumping chamber is controlled by ahigh-speed rotary air pump and, together with a regulatory valve, maintains the appropriate gas pressure in the specimen chamber. The maximum pressure level set by the manufacturer is 1500 Pa. The differential chamber is separated by a diaphragm from the electronic optics and by a special crystal (YAG) from the specimen chamber.

The Y A G single crystal has been developed by the Institute of Scientific Instruments in Brno and has a mutiple function as an aperture diaphragm for the differential chamber, a scintillating detector and a gaseous secondary electron detector *(Autrata et al,* 1997a). The latteris function is to detect the gas (water vapour) ionisation in the vicinity of the specimen, which results in a significant amplification of the number of electrons detected and also suppression of positive charge accumulation on the specimen surface.

The A Q U A S E M operates with a directly-heated tungsten cathode at an accelerating voltage of 10 to 20 kV. In our observations involving soft, wet tissues, the resolution claimed by the manufacturer to be 10 nm was actually lower, due to the penetration of high energy primary electron beams deeper under the tissue surface. This outcome can partly be avoided by treating the material to be viewed with a fixation agent containing heavy metals *(Procházka and Ilkovics,* 1998; *Horký and Skřička,* 1998).

The computer accessories include a software programme (WinTip) for image analysis; some of its applications can be seen in Fig. 2. Using the high quality printer, an image comparable with a conventional electron micrograph is immediately available.

Our experience showed that the pressure of 700Pa used at room temperature was not sufficient to maintain the saturated vapour environment and this resulted in considerable deformation of soft tissue surfaces during specimen observation. Some of these conditions have also been mentioned by *Robinson* (1978) and *Gilpin* (1997). It is expected that this disadvantage will be solved by installing a water vapour developer and the Peltier cooling stage, thus allowing the stage temperature to be regulated. A reduction in temperature and vapour access should improve environmental conditions for viewing soft, wet tissues.

In order to verify that the results of specimen imaging with the use of the A Q U A S E M were fully comparable with those obtained with the BS 300 R E M , different wet specimens were observed. Wet solid materials, such as bone or gallstones, presented no problem when viewed in the A Q U A S E M . The preparation procedure could be reduced to a mere rinsing of the surface, followed by fitting the specimen into the holder. Neither deformation nor charging of the surfaces occurred. Similarly, specimens which included chitin or keratin as their major component, did not require any special preparation. It appears that the majority of data so far published in the E S E M field deal with solid samples *(Uwins et al,* 1993; *Gilbert and Doberty,* 1993; *Kaufman et al,* 1992; *Kodaka et al,* 1991; *Neubauer and Jenning,* 1996).

Tissues with a greater content of firm connective tissue and a semi-fine surface epithelium were also easy to view. They are subject to deformation only after a prolonged exposure to vacuum *(Horký and Skřička,* 1998).

Some problems appeared when samples of wet soft tissue were observed. After the specimen was placed in the vacuum, rapid evaporation of water occurred in spite of the fact that, in the specimen chamber, the pressure was maintained above 600 Pa. At the beginning when the surface relief was still covered by water, the ionisation detector did not mediate any image. After the surface water had evaporated, the relief became apparent but quickly became deformed, particularly if fine surface structures were present (Figs 1,2,3,4). The period between the evaporation of surface water and the subsequent deformation of surface structures lasted only a few minutes. Therefore, only a very short time was left for selecting a site to be viewed, adjusting the selected section at the desired magnification and final focusing. If tissue surface deformation occurs during exposure , the photograph will be out of focus *(Fujimaki et al,* 1990). Moreover, the situation is complicated by the use of a small viewing field at a large initial magnification (x300). It has been shown that, in such specimens, the magnification cannot exceed x 500 *(Procházka and Ilkovics,* 1998). In our observations, the disappearance of fine surface structures resulted in the fact that, at higher magnifications, the image no longer corresponded to the real state. (Fig. 5)

An attempt was made to delay surface deformation by subjecting the specimen to double fixation. While a single, though prolonged, fixation did not produce results different from those obtained in untreated tissues, the double fixation brought some improvement. In addition to an increase in contrast, the surfaces showed better resistance to deformation. This allowed the viewing of wet soft tissues routinely at a magnification of x 1000, and occasionally at x3000, with good resolution *(Procházka and Ilkovics,* 1998; *Horký and Skřička,* 1998). Further approaches involved the immersion of specimens in different media. P E G proved to be the best agent because it blends with both ethyl alcohol and water. After immersion it was possible to remove excess P E G from the tissue surface. When this procedure was combined with the double fixation, it was possible to achieve a good contrast with the differentiation of details at a high magnification and a reduced surface deformation. (Fig. 6)

We are planning to use the A Q U A S E M for specifying the categories of biological materials suitable to be viewed in their natural state as well as for targeted investigation of surfaces in various implants. The parameters are improved after installation of the Peltier cooling stage.

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