OBSERVATION OF BIOLOGICAL SAMPLES WITH ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY

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ABSTRACT

This work deals with the study of wet biological samples using environmental scanning electron microscope. Unfortunately, high vacuum conditions in conventional scanning electron microscope disallow study ~~of~~ nature state of low conductive wet biological samples. Samples have to be fixed, dehydrated and coated before they can be observed. This treatments cause the undesirable biochemical and morphological changes of the sample. The influence of elimination treatments of the natural, unaffected sample surface is shown in this work.

1. INTRODUCTION

Scanning electron microscopy (SEM) is a very important diagnostic method which allows the study of morphological structure. With a few notable exceptions, most biological samples that are suitable for study in the SEM are poor conductors and are composed of beam sensitive light elements containing variable amounts of water. Biological samples are very sensitive to damage conditions in the microscope and produce weak signal. Therefore samples have to be treated before they can be observed. Biological sample preparation consists of following steps:

- obtaining and cleaning the sample
- fixation
- dehydration and drying
- coating

1.1. FIXATION

Typically, the first step is the chemical fixation of the sample to prevent autolysis and provide stabilization of the sample state closest to their natural one. This commonly used procedures are based on precipitation, denaturation and cross-linking, which immobilize the highly mobile solubilized state of cytoplasm, cells and tissues. The used solution contains fixative (formaldehyde or glutaraldehyde) and buffer (sodium cacodylate) for ensuring optimal conditions like osmolarity and pH. The pH of the fixative should be as close as possible to the natural environment of the cell and tissue fluids [1]. Osmolarity

affects changes in cells and is regulated by the concentration of buffer. Every kind of sample requires a different type of the fixative solution, its composition, temperature and time activation. The fixation solution has to be chosen to ensure the fidelity of the structure details without artifacts.

1.2. DEHYDRATION AND DRYING

Dehydration is used for removing water from samples. The water in the sample is replaced with an organic solvent, generally used in form of an ascending series of ethanol or acetone in aqueous solution up to an absolute dehydrating agent.

After complete replacement of the water, dehydrating agents has to be rid from the sample. Air draying is usually unavailable for biological sample. There is the force of surface tension which severely distorts the structural integrity of soft samples in the liquid/air interface. The critical point drying method is the most frequent technique used for sample drying. Critical point drying is based on the principle that there is the critical point on the isothermal for many liquids at which the density of the liquid and gaseous phases is identical. At this critical temperature-pressure point there is an equal exchange of molecules between the gas and liquid phases and surface tension is zero [1]. The liquid carbon dioxide is used to its optimal parameters like critical temperature (304 K), pressure (7,39 MPa) and miscibility with 100% ethanol and acetone. Then the samples totally immersed in one of these chemicals after dehydration can be transferred directly into liquid carbon dioxide. Although critical point drying eliminates deleterious effect of surface tension, this method can also cause artifacts.

1.3. COATING

Dried biological specimens are thermally and electrically non-conductive. These properties complicate SEM observation. The surface is charged during the imaging process. The accumulated charge causes image deformation and image definition loosing. The way how to overcome charging problems is to deposit a very thin (nm) conductive material layer on the sample surface. The function of this thin layer is elimination of electric field by conducting the charge to ground and increasing the SE and BSE signal from the sample. Materials which can be used for sample coating are platinum, gold or palladium/platinum alloy. There are two methods used for applying a thin coating layer. The first procedure is surface coating by vacuum evaporation which is based on condensation heated metal atoms on sample's surface. Another way to apply a thin layer of metal is sputtering. In this method the plasma is established between the substrate and the target and transposes the sputtered off target atoms to the surface of the substrate and there is made a thin layer of using metal [3]. Both described methods can cause problems such as thermal damage and surface contamination, which have to be minimized.

1.4. ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY

Procedures of treatments mentioned above are time consuming and precipitate formation cannot be prevented. Environmental scanning electron microscopy (ESEM) is an adaptation of the SEM which eliminates the need for many sample´s preparation treatments described above. The samples are observed in an environment of high pressure (1 – 3000 Pa) of gas or water vapour. These conditions avoid drying and gas ionization in the sample chamber removes the charge from uncoated surface. It is allowed by using a series of pressure-limiting apertures and differential pumping chamber. For biological

examination high relative humidity in specimen chamber is important throughout the observation period. This is important for dehydration and sample shrinkage avoiding. Desirable conditions in the specimen chamber can be obtained by adjusting chamber pressure or temperature of the cooling stage (Fig. 1). Ideal environment for the observation of the biological samples was determined on 4°- 6°C, 693 – 786 Pa and humidity of 85 % [2].

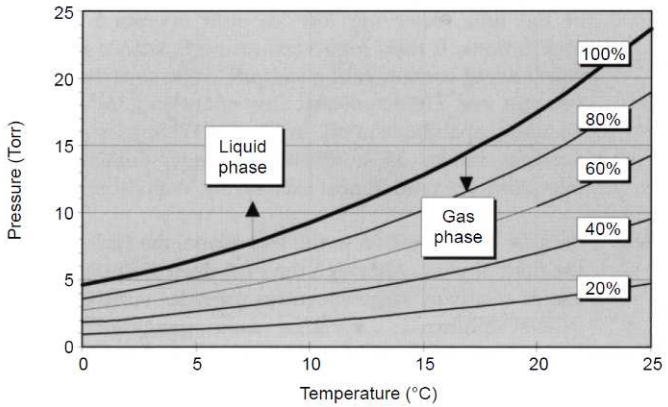
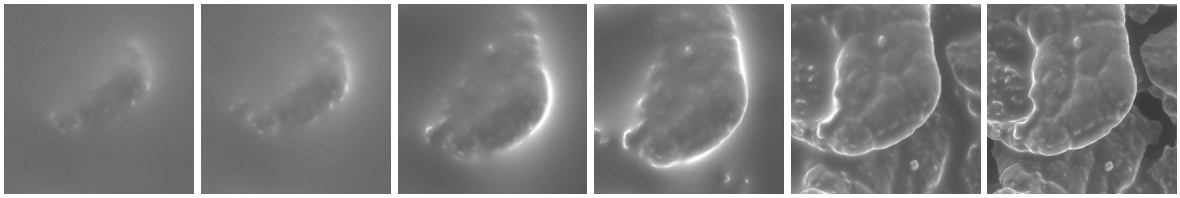


Figure 1: Relative humidity in specimen chamber. [2]

The small intestine of laboratory mice was used as the biological samples. The small intestine is the largest part of the digestive tract and its surface is covered with villi and microvilli that sensitively respond to dehydration. This work deals with study of the fixed and unfixed intestinal villi in high pressure conditions of environmental scanning electron microscope.

2. MATERIALS AND METHOD

Preparation and fixation was realized in cooperation with the Department of Histology and Embryology, Faculty of Medicine of Masaryk University, Brno. The part of samples was fixed in a 6% glutaraldehyde solution in a 0.1 M cacodylate buffer immediately after being obtained. Observations were made with experimental environmental SEM AQUASEM - II by Ionization and YAG - BSE detectors. The water vapour flow in the specimen chamber and the temperature of the sample was regulated to a relative humidity from 100 to 70 %, which ensured fully hydrated natural surface of intestine villi. Samples were placed on a Peltier cooled specimen holder to a drop of water. Consequently the water was slowly evaporated from the sample, see Fig. 2a – 2f.



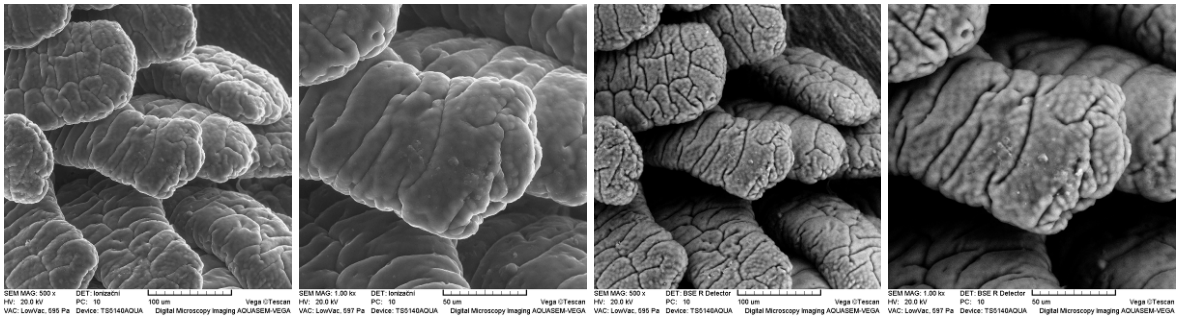
a) b) c) d) e) f)

Figure 2 a) – f): Controlled dehydration of intestine villi observed in experimental environmental SEM AQUASM II. The samples were observed in pressure of 900 Pa (Fig 2a) to 750 Pa (Fig. 2e and 2f), temperature on Peltier stage 3 °C, beam accelerating voltage 20 kV, and working distance 2,5 mm. Horizontal field of view 250 µm.

3. RESULTS AND DISCUSSION

3.1. FIXED INTESTINAL VILLI

Beginning of the observation process is illustrated in Figure 2a - 2f, where we can see fully hydrated surfaces of the intestinal villi. Figures 3a and 3b show slightly dehydrated sample surface recorded with ionisation detector of secondary electrons.



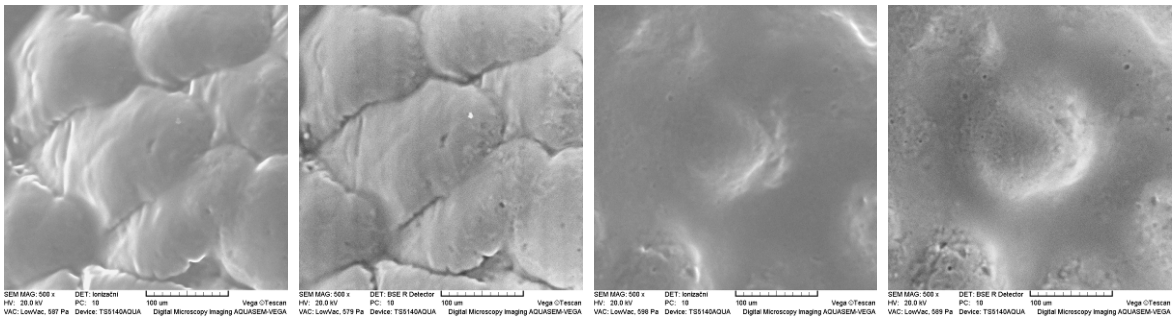
a) b) c) d)

Figure 3 : a), b) Fixed intestinal villi observed with ionization detector.
c), d) Fixed intestinal villi observed with YAG – BSE detector.
The sample was observed at a pressure of 600 Pa,
temperature on Peltier stage 3 °C and relative humidity about 80%

The fixation stopped secretion of mucus, intestinal villi are projected over the surface and the microstructure is well recognizable. Unfortunately, details of the microvilli on the apical surface of the enterocytes cannot be observed because the surface is covered with remains of mucus which is produced by goblet cells. This condition causes decrease of detection efficiency of ionization detector, because the passage of low-energy secondary electrons is prevented by a thin layer of water and mucus on the surface of the sample. On the other hand, enterocytes can be seen in figure 3c, d recorded with YAG - BSE detector.

3.2. UNFIXED INTESTINAL VILLI

The aim of the secondary experiment was observation of surface of small intestine in their native state without fixation (Fig 4 a – 4d).



a) b) c) d)

Figure 4 : a), b) Unfixed intestinal villi observed with ionization and BSE-YAG detector
c), d) Unfixed but damaged intestinal villi observed with ionization and BSE-YAG detector. The sample was observed at a pressure of 600 Pa, temperature on Peltier stage 3 °C, relative humidity about 80%.

To prevent autolysis, the samples had to be observed immediately after being obtained. The surface of sample is covered with hardly removable layer of mucous that is also formed during the examination in the microscope. Individual villi are recognizable, nevertheless even very short time of observation leads to irreversible degradation of the sample (Fig. 4 c,d). Also in this case, BSE-YAG detector allows to visualize the topography of the sample, but due to thick mucus on the surface, this feature is very limited (Obr. 4 b,d).

4. CONCLUSIONS

This study of fixed and unfixed intestinal mucosa demonstrates the impact of treatments on observed biological samples. It illustrates necessity of fixation in studying soft tissues like small intestine, which continuously renews its absorptive cells, even during the observation process. If fixation is omitted, mucus secretion continues and prevents any observation. Short-term chemical fixation allows using environmental scanning electron microscope and samples can be examined in fully hydrated state, but with fewer details in microstructure. Microstructures, e.g. microvilli visibility requires using treatments, especially dehydration and drying, which are suitable for conventional scanning electron microscopy. This work points out to the need of suitable methodology for the biological samples preparation before using environmental scanning electron microscope.

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