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Ice Contamination Issues in the Visualization of the Ultrastructure of the Nuclear Envelope by Freeze-Fracture Technique

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The freeze-fracture technique and processing of the metal replicas of fractured structures is an excellent option for visualization of details in the nuclear membrane and for study of the ultrastructure of nuclear pores. In the replica the number of nuclear pores per unit area and the distribution of them can be evaluated, as well as the number of membrane proteins and their size [1], [2].

The process of replica preparation includes: freezing the biological sample, fracturing the sample under low temperature and in a high vacuum, shadowing fractured structures with metal (platinum) and stabilization of the metal by carbon deposit, and after melting the sample the residue of the biological material is removed by chemical agents. The replicas were fixed on cupper grids and observed in a transmission electron microscope. In these measurements replicas of HL-60 cells (human leukemic cells) were used. A holder with the samples was kept at the temperature -100°C in the vacuum chamber (Freeze Etching System BAF060, BAL-TEC). Time of etching: the ice contaminated sample was not etched, the sample without ice contamination was etched 15 min.

The most critical part of a sample processing is freezing. Biological material in a culture medium or in a buffer can be frozen in cryogen but in most cases this leads to ice crystal production. The solid ice crystals occur in the exoplasmic face of the inner or outer nuclear membrane, figure 1A. This means that the ice contamination originates from the perinuclear space. Removal of ice crystals from a membrane could be processed by etching (ice sublimation). The ice is removed not only from the membrane of the organelles but also from the cell cytoplasm and the extracellular space. This leads to the exposure of a large surface of organelles and it can cause complications with replica production.

Another option is to use cryoprotectives. The usual cryoprotectant used in the freeze-fracture technique is glycerol. Glycerol replaces a certain portion of water in the sample and prevents the formation of ice crystals. In addition, in the case of contamination by frost due to the transfer of the sample from a cryogen into a high vacuum chamber of Freeze Etching System, the ice crystals can be removed by sublimation from the sample surface without losing the sample material or exposing a large surface of organelles, figure 1B. The reason is that the cryoprotectant does not sublimate as fast as water.

In order to evaluate pore distribution or size of pores it is necessary to detect individual pores in the membrane. In the recorded micrograph, the large and solid crystals can appear so close to a pore that it could be difficult to distinguish them from the edges of the pore or position in the membrane, figure 1A.

Thus, the evaluation of protein distribution in the membrane and the exact number of them can be problematic. The number of proteins per unit area can be variable but there are some limits. With ice contamination the mean number of particles rapidly increases. The number of particles in the membrane contaminated by ice crystals is 490 ± 40 per 1 μm^2, and in the membrane without ice crystals 242 ± 24 per 1 μm^2. These results indicate that due to the contamination of the sample by ice crystals the number of particles in the membrane doubled.

In figure 2 the histogram shows the size of particles in the exoplasmic face of the nuclear membrane. The mean size of particles in the contaminated sample is 12 ± 8 nm and in the membrane without ice contamination is 9 ± 5 nm. The number of particles with size from 15 nm to 30 nm is higher in the contaminated membrane and there are some particles with size above 35 nm. In the ice-free membrane the largest particle has size of 32 nm.

It can be concluded that the ice contamination influences the results of the evaluation. Ice crystals can be mistaken for membrane structures and complexes of proteins. On one hand using cryoprotectants and long etching can prevent mistakes in evaluation on the other hand their application may not be consistent with the aim of the experiment.

References:
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[3] The research was supported by MEYS CR (LO1212) and its infrastructure by MEYS CR and EC (CZ.1.05/2.1.00/01.0017).

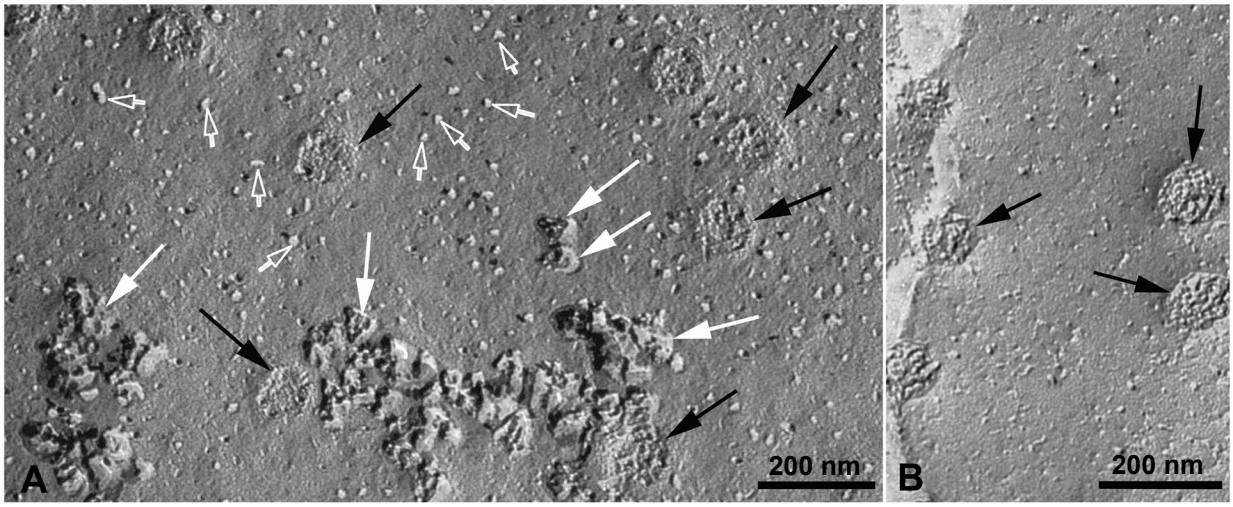

Figure 1. Exoplasmic face of a nuclear membrane: A) the ice contaminated membrane, B) the ice-free membrane. Black arrows show nuclear pores, white arrows show solid ice crystals in the membrane and small white unfilled arrows show particles in the membrane which could be small ice crystals.

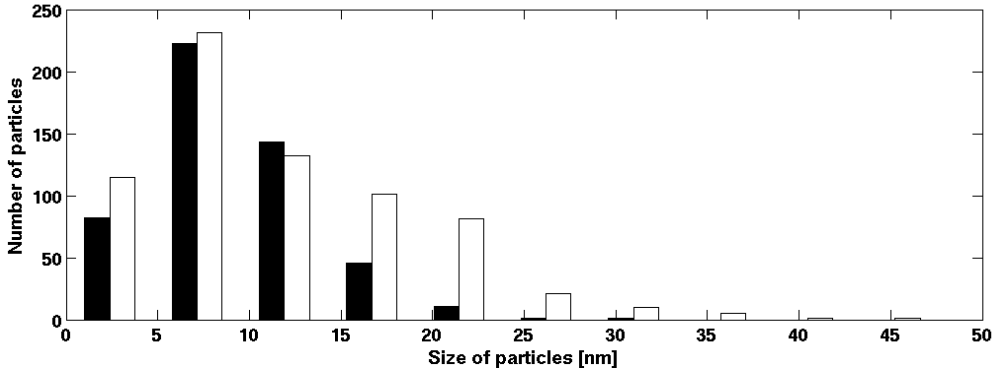

Figure 2. Histogram shows size of particles in the ice contaminated membrane (white boxes) and in membrane without ice contamination (black boxes). This shows that larger particles exist in the contaminated sample in contrast to the contamination-free sample.