Cryofixation of biological specimens – electron spectroscopic imaging of calcium and phosphorus of osteoblastic cell cultures and an elegant way to produce section ribbons with the help of a newly developed micromanipulator.

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Transmission electron microscopy has provided most of what is known about the ultrastructural organization of tissues, cells and organelles. Most samples were prepared according to the classical protocol of so called chemical fixation. During fixation with aldehydes, postfixation with heavy metals, dehydration in a solvent and subsequent embedding into a resin samples are preserved in such a way that they are easy processed for electron microscopic evaluation. However chemical fixation cannot preserve the native ultrastructural details in a satisfactory way. Quite some artefacts are introduced by this procedure (precipitations, loss of molecule, swelling, 1,2,3). Furthermore due to the recording of heavy metal staining patterns only an indirect visualization is possible. Subcellular structures of biological samples are preserved close to native state by cryofixation. Bulk specimens (thicker than some micrometre) are only well frozen or vitrified when high pressure freezing is applied. Subsequent cryosectioning and investigation in the cryo-electron microscope (CEMOVIS) leads to a structural preservation which is close to the native state of the sample (4).

A compromise is freezing followed by freeze-substitution. The samples show better structural preservation in comparison to samples chemically fixed (1,2,3). However freezing leading to vitrification is only possible in small samples: experience shows that sample thickness of a biological sample should not exceed a thickness of 200μm. Exceptions are based on the fact that some biological samples contain much more (or less) intrinsic cryoprotectants (eg solutes, sugars etc).

As a recent improvement in elemental analysis of temporally synchronized UMR106-01 osteoblastic cell cultures we discuss calcium and phosphorus distributions which were optimal only when cultures were processed using high pressure freezing (HPF), freeze-substitution and dry ultrathin sectioning with an oscillating knife (5). Three types of culture conditions were analyzed: mineralized, non-mineralized (limiting exogenous phosphate), and inhibited mineralization (treated with protease inhibitor AEBSF). UMR 106-01 cells were grown on fibronectin-coated sapphire discs and processed conventionally or for high pressure freezing (6; EMPact, Leica-microsystems, Vienna). HPF specimen holders containing sapphire discs with frozen cells were transferred to the freeze-substitution apparatus. Specimens were washed in anhydrous acetone and embedded in Epon Araldite resin. Dry 50 nm sections were produced with an oscillating diamond knife (Ultra sonic, Diatome, Nidau, Switzerland). With the help of electron spectroscopic imaging (ESI; Leo 912 with omega filter, Zeiss, Oberkochen, Germany) we could localize calcium and phosphorus in micrographs.

To facilitate the application of cryomethods the improvement and development of tools is necessary. Due to tremendous advances in crystallography and magnetic resonance imaging, almost any protein can now be modeled at atomic resolution. To fully understand the workings of biological “nanomachines” it is necessary to obtain images of intact macromolecular assemblies in situ. Cryofixation by high pressure freezing followed by cryosectioning circumvents many of the artefacts related to staining, dehydration and embedding. The approach immobilizes complex macromolecular assemblies in their native state in situ and the images in some cases reach such a good resolution that macromolecule structure can be fitted into them (7). Sophisticated instruments and software packages are needed, e.g. high voltage electron microscopes equipped with precise goniometers (for high resolution tomograms) that work at low temperature and digital cameras of high sensitivity and pixel number to get this structural information. However first of all good cryosections have to be produced. To get them is quite a challenge. Very steady hands are a prerequisite. To facilitate cryo-sectioning we developed a tool with two micromanipulators. One to guide the ribbon of cryo-sections produced during the sectioning process and the other one to put in place the grid for adsorbing the

ribbon with the help of an ionisation unit developed by Pierson et al. (8, Crion, Leica- microsystems, Vienna, Austria)

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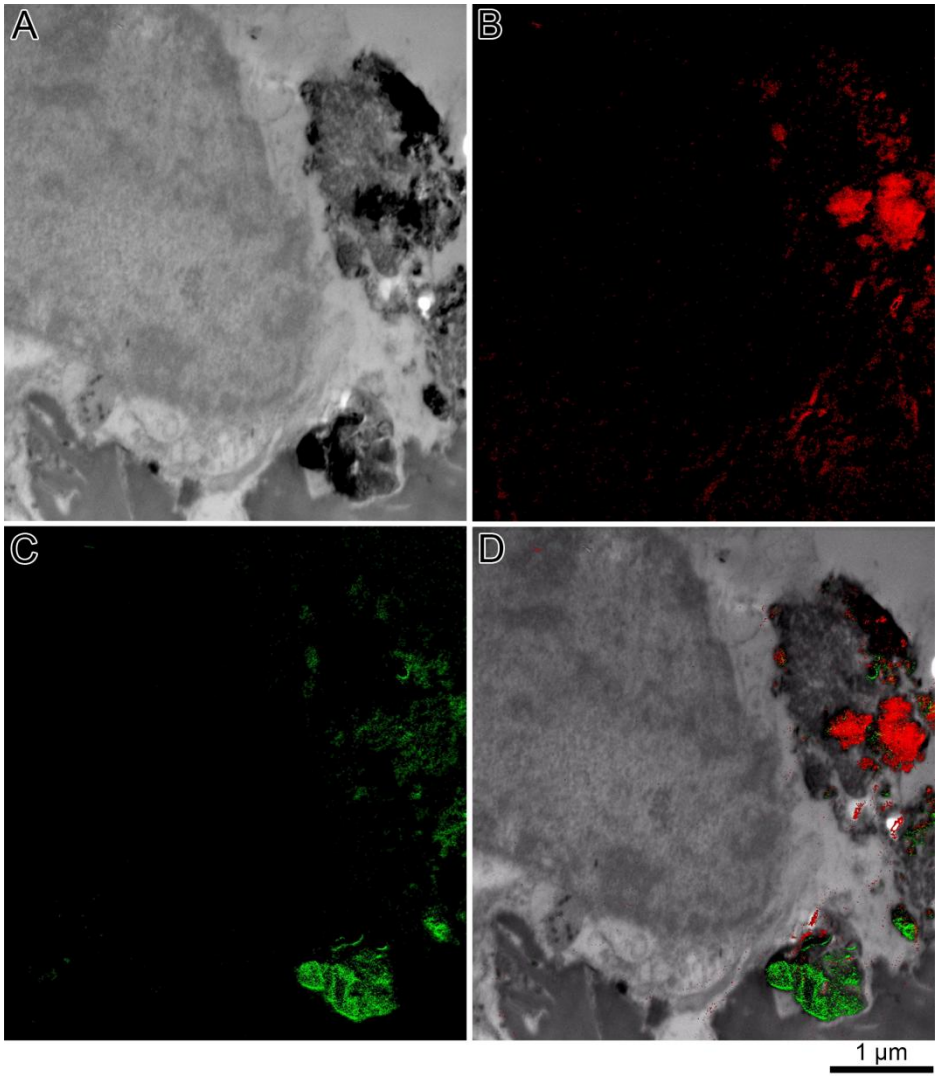

Figure 1. Part of a high pressure frozen osteoblastic cell is shown with portions of closely associated extracellular biomineralization foci containing separated calcium and phosphorus (explanation see above). The sample was freeze-substituted in acetone containing 2% of osmium tetroxide, embedded in Epon and dry ultrathin sectioned with an oscillating knife. In the electron spectroscopic imaging mode we recorded in Fig. 1A the zero loss image (nicely focused sample because no inelastically scattered electrons are present). Fib 1B shows the very same location recorded with inelastic electrons having suffered an energy loss of 346 eV corresponding to calcium (red) and in Fig. 1C inelastic electrons have lost 132 eV corresponding to phosphorus (green). Finally in Fig. 1D all three images are superimposed.