Scanning Electron Microscopy

Volume 4
Number 1 *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*

Article 12

1985

# Preparation of Cryosections for Biological Microanalysis

Karl Zierold
*Max-Planck-Institut für Systemphysiologie*

Science of Biological Specimen Preparation (pp. 119-127)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507, U.S.A.

# PREPARATION OF CRYOSECTIONS FOR BIOLOGICAL MICROANALYSIS

Karl Zierold

Max-Planck-Institut für Systemphysiologie
Rheinlanddamm 201, 4600 Dortmund 1, FRG
Phone no.: 0231-1206455

## Abstract

The element distribution in biological cells and tissues can be revealed by electron probe microanalysis from ultrathin cryosections. In particular, the distribution of physiologically important and often mobile elements such as Na, Mg, P, S, Cl, K, and Ca can be studied in cryosections on an ultrastructural level. The cryopreparation technique required for this purpose consists of 1. cryofixation, 2. cryosectioning, 3. cryotransfer including freeze-drying and carbon coating if necessary, 4. energy dispersive X-ray microanalysis in a cold stage equipped scanning transmission electron microscope. The lateral analytical resolution of this method is less than 50 nm in freeze-dried ultrathin (about 100 nm thick) cryosections. The detection limit is about 12 mMol/kg dry weight for elements with an atomic number higher than 12. For sodium this value is about 48, for magnesium about 36 mMol/kg dry weight. Good cryofixation without or at least with very small ice crystals with a diameter of 50 nm or less is found to be necessary not only for recognition of ultrastructural details but also for reliable evaluation of X-ray spectra. Carbon coating of frozen-hydrated sections reduces the mass loss observed in uncoated frozen specimens.

KEY WORDS: Biological microanalysis, cryopreparation, cryofixation, cryosection, cryotransfer, cryomicroscopy, cryoultramicrotomy, frozen-hydrated specimen, freeze-drying, ion distribution.

## Introduction

The preparation of ultrathin cryosections from biological cells and tissues makes it possible to study the distribution of elements such as Na, Mg, P, S, Cl, K, and Ca in a defined physiological state on an ultrastructural level. Various experiences concerning X-ray microanalysis of cryosections are described in the literature, e.g., by Appleton (1974), Barnard (1982), Hagler and Buja (1984), Hall and Gupta (1983), Moreton et al. (1974), Roomans et al. (1982), Saubermann et al. (1981a and b), Sevéus (1980), Somlyo et al. (1977), Somlyo and Shuman (1982), Wendt-Gallitelli and Wolburg (1984), Zierold (1982). This paper describes the author's approach to prepare cryosections for energy dispersive X-ray microanalysis in a scanning transmission electron microscope (STEM). The preparation procedure is shown schematically in Fig. 1: It consists of cryofixation, cryosectioning, cryotransfer including freeze-drying or carbon coating, microscopy and energy dispersive X-ray microanalysis in a cold stage equipped STEM. In the following these preparation steps are described and discussed with respect to the related problems and perspectives.

The results reported here have been obtained with the help of a Reichert FC4 cryoultramicrotome and a Siemens ST 100 F scanning transmission electron microscope, equipped with a Link Systems X-ray analyser.

## Cryofixation

Cryofixation refers to quick freezing of biological cells and tissues in a way that preserves the ultrastructure and the distribution of substances as closely as possible to the living cell. The main problem in cryofixation is ice crystal growth that during freezing may damage the ultrastructural organization and cause displacement of diffusible elements and molecules. Therefore, freezing has to be performed with high velocity, preferably higher than 10,000 K/s, in order to keep the water in the uncrystallized vitrified state or to obtain ice crystals smaller than ultrastructural details (Moor, 1964; Dubochet et al., 1982). Due to the low thermal conductivity of water (2-4 W/m·K) this can be achieved only for thin specimens such as single cells or thin tissue layers. At present, plunging of the sample

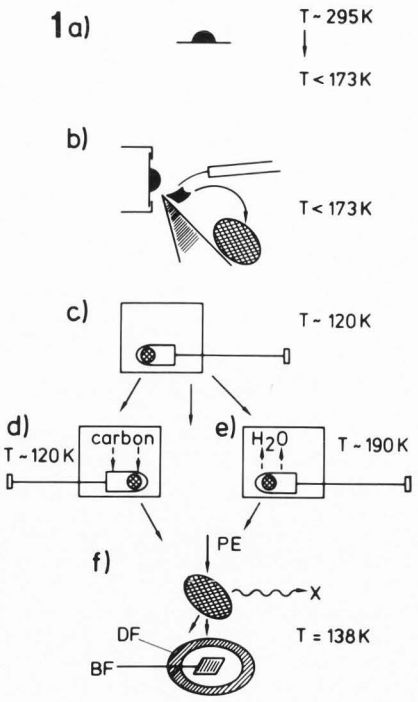



Fig. 1: Scheme of the preparation of cryosections: a) cryofixation, b) cryosectioning, c) cryotransfer, d) carbon coating, e) freeze-drying, f) STEM and X-ray microanalysis, BF = brightfield detector, DF = darkfield detector, PE = primary electrons, X = X-rays.

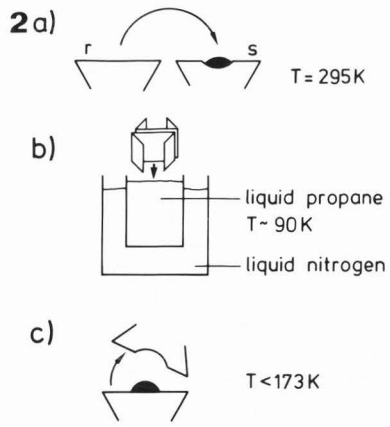
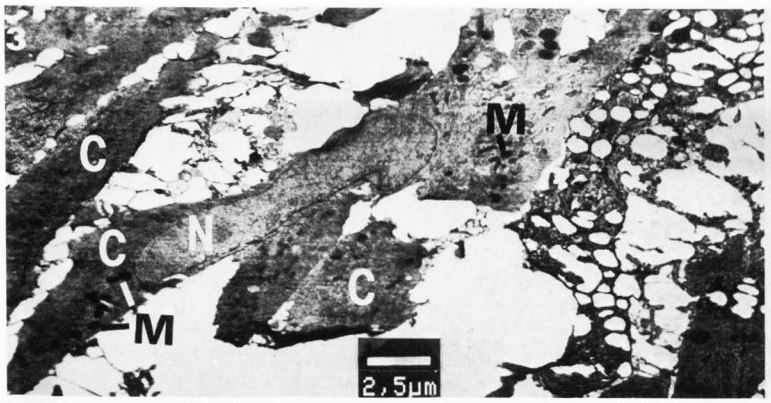


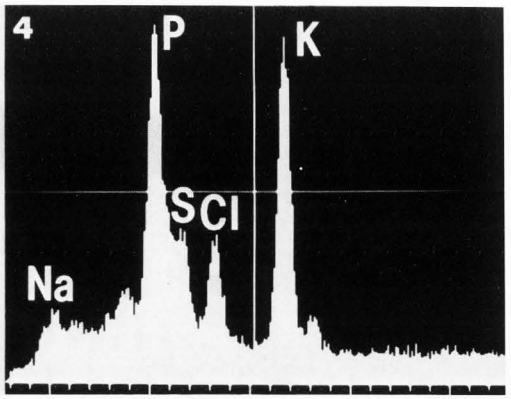

Fig. 2: Preparation of droplets for cryosectioning:
a) The droplet is placed into the cavity of a copper sheet with a smooth surface (s). A similar copper sheet with a plane rough surface (r) is laid on the droplet bearing sheet.
b) This device is plunged into liquid propane.
c) The copper sheets are detached from each other. The frozen droplet mostly remains on the copper sheet with the rough surface, appropriate for cryosectioning.

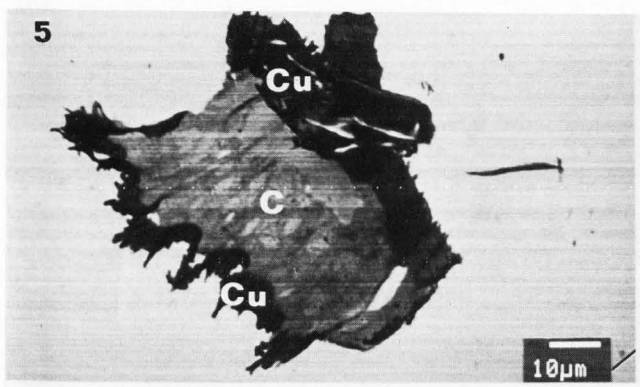
into liquid propane cooled by liquid nitrogen, propane jet fixation, or cold metal mirror fixation are the most established freezing methods. For a review of freezing techniques the reader is referred to Plattner and Bachmann (1982). According to present knowledge no particular freezing technique can be recommended which yields optimal results for all kinds of specimens. Each specimen requires a special approach depending on specimen type, geometry, and physiological boundary conditions. Droplets of cell suspensions or standard solutions as used for microanalytical calibration can for example be frozen by means of a copper sheet assembly, as demonstrated in Fig. 2. By this technique droplets suitable for further cryosectioning can be obtained without the risk of water evaporation during the time before freezing. All the techniques mentioned above provide cryofixation of the ultrastructure with acceptable ice crystal damage up to a specimen depth of about 30 µm. Significantly larger preservation areas can be achieved only by the high pressure freezing method (Müller and Moor, 1984), but this technique has, to our knowledge, not been applied to microanalysis.

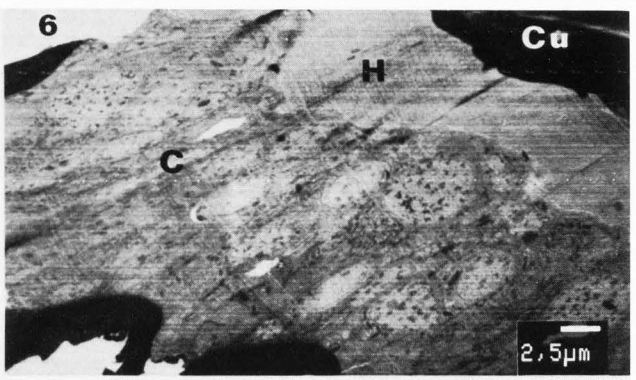
The limitation of cryofixation with respect to sample thickness makes tissue culture cells an ideal model for cryopreparation (James-Kracke et al., 1980; Zierold et al., 1984). Fig. 3 shows an ultrathin section of skeletal muscle cells which had been grown in vitro on a gold Balzers freeze-etch specimen support and frozen by the propane jet technique. The corresponding intracellular X-ray spectrum is given by Fig. 4. Other examples of good cryopreparation of tissue specimens are, for example, single fiber preparations of muscle (Hagler and Buja, 1984; Somlyo et al., 1977; Wendt-Gallitelli and Wolburg, 1984). The approximately 80 µm thick antennae of the silkmoth bombyx mori, plunged into liquid propane cooled by liquid nitrogen, also can be prepared successfully for cryosectioning. Figs. 5 - 8 illustrate the good ultrastructural preservation of the tissue. Even the rough endoplasmic reticulum can be easily recognized (Fig. 8). However, these are exceptional tissue specimens. The appropriate preparation of complex tissues in defined physiological states is one challenge in the future application of cryopreparation techniques.

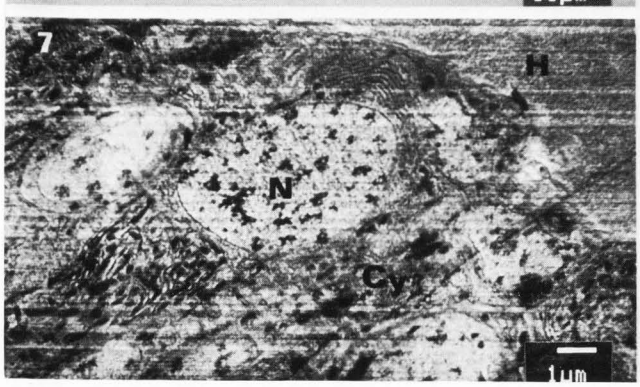
Good cryofixation is not only a prerequisite to preserve ultrastructural integrity, it is also necessary for reliable evaluation of X-ray spectra (Zierold, 1984a and b). As shown in Table 1 dry weight concentrations of potassium and phosphorus as measured in freeze-dried cryosections of droplets consisting of a mixture of dextran and $KH_2PO_4$-solution depend on the ice crystal size. The data indicate that the expected concentration of the elements is found only, if the ice crystal size is about 50 nm in diameter or less. This effect might be caused by problems in appropriate background correction for specimens with varying thickness. Although not yet completely understood it has to be taken into account in quantitative evaluation.



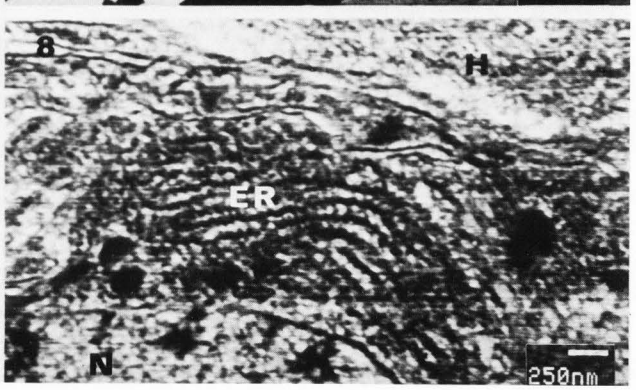




Fig. 3: Freeze-dried cryosection of skeletal muscle cells in vitro. C = cytoplasm, M = mitochondria, N = nucleus.

Fig. 4: Typical X-ray spectrum from the cytoplasm of a cell shown in Fig. 1.

Figs. 5-8: Freeze-dried cryosection of a silkmoth antenna in increasing magnification C = cells, Cu = cuticula, Cy = cytoplasm, ER = endoplasmic reticulum, H = hemolymph space, N = nucleus.

## Cryosectioning

Cryosectioning requires appropriate specimens: In particular, cryofixed specimens have to be mounted on cryoultramicrotome holders in such

Table 1: X-ray microanalysis of freeze-dried cryosections of droplets consisting of 20% dextran and 80% $KH_2PO_4$ in a concentration of 200 mMol/l, respectively 800 mMol/kg dry weight. The data are evaluated from X-ray spectra by means of the Link Quantem FLS program including background correction.

| mMol/kg dry weight ± standard error | ice crystal size 50 nm | ice crystal size 1/µm |
|---|---|---|
| phosphorus | 800 ± 49 | 357 ± 123 |
| potassium | 800 ± 120 | 343 ± 136 |

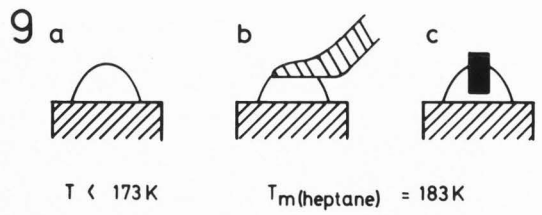



Fig. 9: Cryogluing of a frozen specimen by melting heptane: The heptane droplet is frozen on the specimen holder in the cryoultramicrotome chamber (a). The droplet is molten by a warm forceps (b). The specimen is placed in the molten heptane and then glued by the freezing heptane (c).

a way that they can be sectioned with a sufficiently small cutting plane of less than about 200 µm in any desired orientation. Thin frozen specimens can be glued on a specimen holder of the cryoultramicrotome with melting heptane (Steinbrecht and Zierold, 1984; e.g., thin antennae of silkmoth, Figs. 5 - 8) or copper sheets bearing frozen droplets can be glued by this method on holders for cryosectioning without raising the temperature of the specimen above 193 K, the recrystallization temperature of intracellular water. The cryogluing is illustrated by Fig. 9.

Cryosections are cut by means of a dry glass knife in the cold nitrogen gas atmosphere of the cryoultramicrotome chamber (Sevéus, 1980; Sitte, 1982). The cutting temperature is kept below 173 K, the cutting speed ranges preferably between 15 and 20 mm/s, the thickness of sections is between 70 and 100 nm. Transparent sections showing interference colours are obtained from well frozen specimens with small ice crystals. From specimens with large ice crystals, e.g., larger than 300 nm, brittle chips rather than clear sections are obtained. The same holds for specimens with large water-rich vacuoles such as plant tissues. The sections are transferred to Pioloform®-coated and carbon-evaporated 50 mesh grids by means of an eye lash (Christensen, 1971). A second grid coated in the same way is placed on top of the grid bearing the sections. Both grids are compressed together by means of a cold polished metal rod. Then they are detached, and the grids with adhering sections are mounted on electron microscope holders suitable for cryotransfer. The handling of cryosections is often complicated by static electricity, which can be reduced by using commercially available discharge devices (Gupta et al., 1977).

Cryosections show to a varying extent compression lines perpendicular to the cutting direction (Barnard, 1982; Chang et al., 1983; McDowall et al., 1983; Frederik et al., 1984a; Zierold, 1984c). Chatter lines with a period larger than one µm are caused by microtome vibration and can be avoided by lowering the cutting speed to 30 mm/s or less. The compression lines with a frequency of 3-6 lines/µm are always present in sections of well frozen specimens (Figs. 10 and 11). Even if not visible individually, the compression by cutting increases the section thickness by 20-40%. Only sections with ice crystals larger than 200 nm show no compression lines (Fig. 12), but these sections, of course, are not suitable for studying the ultrastructure and for microanalysis (see Table 1).

## Cryotransfer

Obviously, electron microscopy of frozen-hydrated specimens requires cryotransfer. This can be performed by means of a transfer chamber filled with nitrogen gas and equipped with a cold contact to the grid holder. Various cryotransfer systems and cold stages for electron microscopes have been described in the literature (e.g. Heide and Grund, 1974; Hutchinson et al., 1978; Talmon et al., 1979; Ross et al., 1981; Heide, 1982; Hax and Lichtenegger, 1982; Zierold et al., 1981). During transfer, the specimen temperature is kept below 130 K, in order to prevent sublimation of ice. The sections can be freeze-dried during transfer either by removing the cold contact from the grid holder or by counterheating the specimen holder in the transfer chamber.

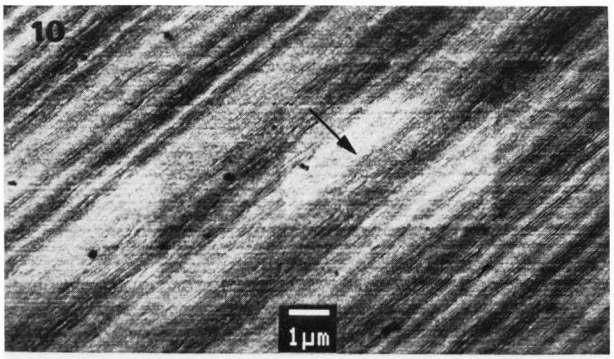
Cryotransfer turns out to be advantageous also for electron microscopy and microanalysis of freeze-dried sections. Freeze-dried sections stored at laboratory atmosphere are very hygroscopic. As a consequence of the contact with the humidity of the air the ultrastructure in these sections appears damaged (Figs. 13 and 14). Therefore, freeze-dried cryosections should be kept in a closed system without any contact with wet air, until they are studied in the electron microscope (Zierold, 1984b).

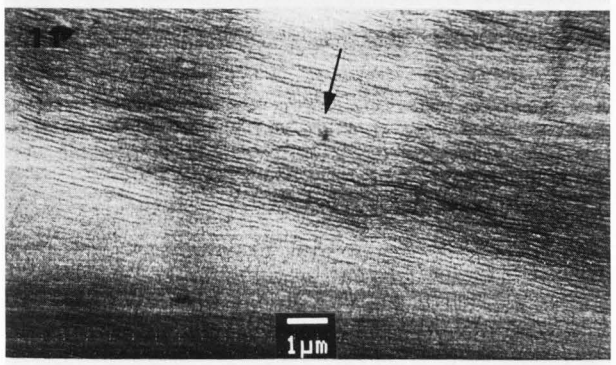
As indicated in Fig. 1, also carbon coating can be included in the cryotransfer system. A carbon layer on the frozen-hydrated section reduces mass loss caused by electron irradiation as discussed below (Zierold, 1984c).

## Cryomicroscopy and Microanalysis

Frozen-hydrated and freeze-dried sections are studied in a scanning transmission electron microscope equipped with a cold stage at a temperature of 138 K (Zierold, 1982 and 1983). Frozen-hydrated sections show a very low contrast in brightfield mode and a slightly increased contrast in darkfield mode. After superficial sublimation of ice cellular structures such as cell membranes can be recognized in frozen-hydrated sections in darkfield mode (Fig. 15). The low contrast is caused by the very similar electron scattering cross section of ice and organic material (Carlemalm and Kellenberger, 1982; Eusemann et al., 1982). Perhaps, the missing contrast in frozen-hydrated sections can be enhanced by Z-contrast imaging (Carlemalm and Kellenberger, 1982) or by electron spectroscopic imaging (Ottensmeyer and Andrew, 1980; Ottensmeyer, 1984; Colliex et al., 1984). In particular, electron energy loss spectroscopy and electron spectroscopic imaging require very thin cryosections of thicknesses less than 50 nm, which at present are very difficult to achieve.

Additionally, ultrathin frozen-hydrated sections are very sensitive to radiation damage resulting in mass loss during electron irradiation (Fig. 15). According to Heide (1984), at 140 K



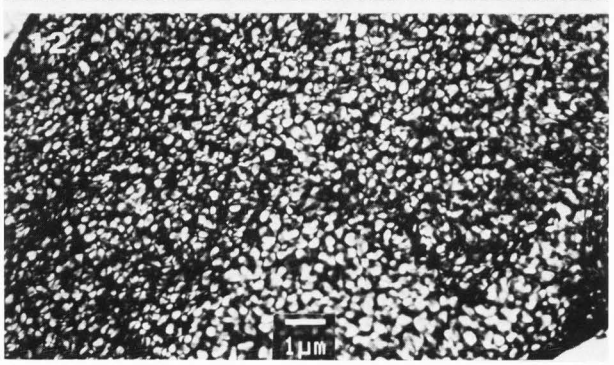
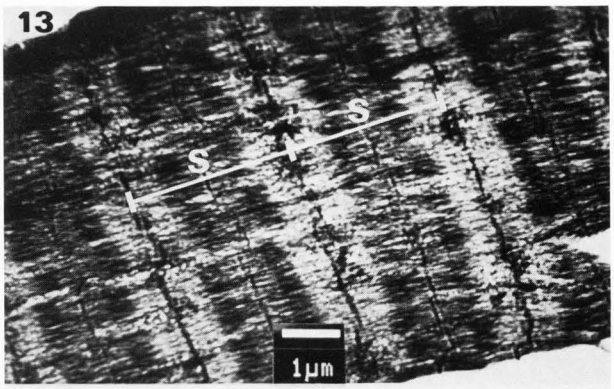




Fig. 10: Frozen-hydrated cryosection from a droplet containing 20% glycerol gelatin and 80% water cut at 50 mm/s. Bright areas are caused by radiation damage. Compression lines with a period of approximately 0.2 µm are superimposed by vibration and chatter lines with a period of 1 µm and about 3 µm. Arrow indicates cutting direction.

Fig. 11: Frozen-hydrated cryosection cut at 10 mm/s. Compression lines as in Fig. 10 are found without vibrations. Arrow indicates cutting direction.

Fig. 12: Freeze-dried cryosection with large ice crystals of about 300 nm in size showing no distortion lines.

this mass loss is 2 µg/As; respectively 1 water molecule is lost by irradiation of 94 electrons. Lowering the temperature to 9-90 K may decrease the mass loss to 0.8 µg/As, but this moderate



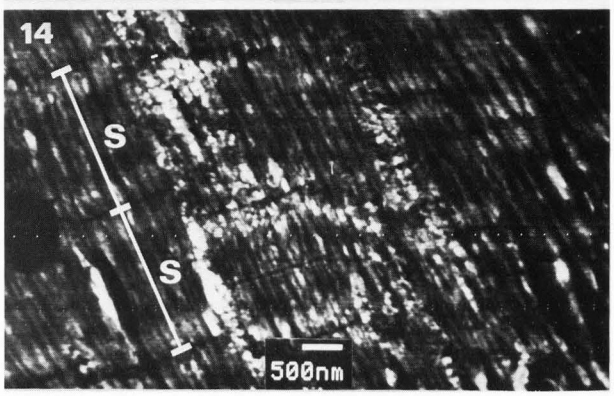




Fig. 13: Freeze-dried cryosection of frog skeletal muscle, cryotransferred into the STEM without contact with the air, S = sarcomeres.

Fig. 14: Freeze-dried cryosection of frog skeletal muscle, stored over night at the laboratory atmosphere, S = sarcomeres.

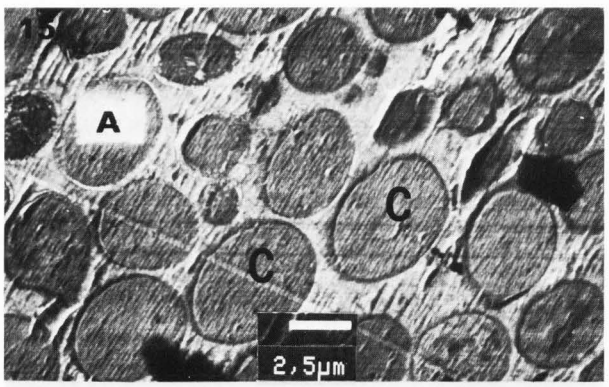
Table 2: Etch depth as expected in a frozen-hydrated section of ice irradiated by 100 keV electrons, based on the mass loss of 2 µg/As.

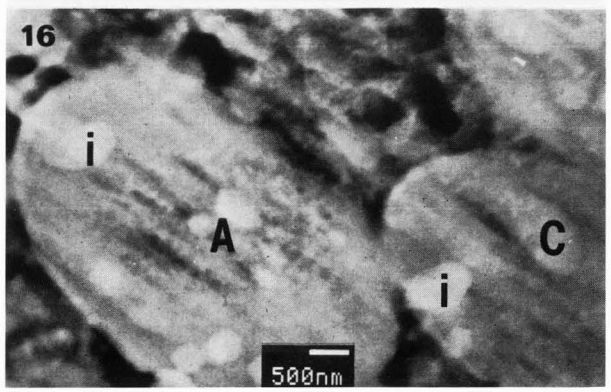
| Scanned area(/µm²) | electron current(nA) | time (s) | exposure (e/nm²) | etch depth (nm) |
|---|---|---|---|---|
| 10 | 1 | 10 | $6{,}25.10^3$ | 2,16 |
| 1 | 1 | 10 | $6{,}25.10^4$ | 21,6 |
| 1 | 1 | 100 | $6{,}25.10^5$ | 216 |
| 1 | 10 | 100 | $6{,}25.10^6$ | 2160 |

gain in cryopreservation is not sufficient for microanalytical purposes requiring high electron exposure. This is illustrated by the data given in Table 2, based on a mass loss of 2 µg/As. The upper two rows in this table rather hold for imaging conditions, whereas the lower two rows reflect parameters as used in X-ray microanalysis. According to Talmon (1984), this mass loss is even increased in the presence of organic material. Therefore, electron probe microanalysis of frozen-hydrated material is mainly done on bulk specimens

Table 3: Comparison of frozen-hydrated and freeze-dried ultrathin (100 nm thick) cryosections with respect to X-ray microanalysis. The detection limits given in the table are approximately 3 times higher for magnesium and 4 times higher for sodium.

| | frozen-hydrated | freeze-dried |
|---|---|---|
| ultrastructure | difficult to identify | visible/good, depends on ice crystal size |
| preparation artefacts | compression lines, ice crystal damage depending on cryofixation | + shrinkage |
| radiation damage | mass loss, can be reduced by carbon coating | negligible |
| lateral analytical resolution | $\leq$ 50 nm | $\leq$ 50 nm |
| relative detection limit for Z $>$ 12 | 10 mMol/liter cell water | 12 mMol/kg dry weight |
| absolute detection limit for Z $>$12 | 1500 atoms in 100x50x50 $nm^3$ | 500 atoms in 100x50x50 $nm^3$ |
| ion distribution | preserved close to the native state | mostly preserved, redistribution possible |




or thicker sections (Gupta and Hall, 1981; Saubermann et al., 1981a). Perhaps, coating the frozen-hydrated sections with carbon before analysis will improve the situation, because the mass loss caused by electron irradiation of ice is a surface phenomenon (Hren, 1979; Dubochet et al., 1982; Heide, 1984). As illustrated in Fig. 16 a 20 nm thick evaporation layer of carbon on frozen-hydrated sections may reduce the mass loss remarkably, depending on the thickness of the carbon layer and the contact of the section with the carbon coated support film (Zierold, 1984c). However, the exact relationship between the thickness of a superficial carbon layer and the preservation efficiency against mass loss remains to be clarified.

Fig. 15: Frozen-hydrated section of a suspension of yeast cells, superficially freeze-dried. C = cell, A = area scanned by 200,000 e/$nm^2$ resulting in complete mass loss.

Fig. 16: Frozen-hydrated section of yeast cells, coated by a 20 nm thick layer of carbon before transfer into the STEM, imaged in darkfield. C = cell, i = ice crystal, A = area scanned by 200,000 e/$nm^2$. Mass loss is much lower than in the example shown in Fig. 15.

Freeze-dried cryosections can be studied with much higher contrast and without mass loss. However, incompletely dried sections with superficial water layers on organic material also lose mass. The contrast in freeze-dried sections is caused mainly by differences in electron scattering between remaining organic material and vacuum. Thus, sections with visible ice crystal damage exhibit higher contrast than sections without recognizable freezing damage. Because of the good contrast in STEM and the stability with respect to radiation damage the freeze-dried cryosection turns out to be a versatile object for X-ray microanalysis of cryosections. During ice sublimation, diffusible substances presumably move not further than one ice crystal diameter to the next solid structure. Greater redistribution may occur in water-rich compartments such as vacuoles or vessels.

Cryosections undergo unidirectional shrinkage by freeze-drying of about 10-20% (Zierold, 1984c). According to Frederik et al. (1984b) this shrinkage can be prevented if sections are merely partly dehydrated and if "post-drying" is avoided. X-ray spectra can be obtained from any desired area with a lateral analytical resolution of about 40 nm, as found by contamination spots produced by electron irradiation of sections at room temperature. In principle, freeze-dried sections can be studied in the warm state. However, all diffusion processes increase with temperature. Therefore, the specimen temperature should be kept as low as possible from the cryofixation up to the analysis of the section in the electron microscope in order to avoid redistribution of solutes. Additionally, the cold stage and the even colder anticontamination trap near the specimen allow electron microscopy and microanalysis without any observable contamination.

The evaluation of X-ray spectra is done by comparison with freeze-dried sections from droplets of standard solutions mixed with organic material such as albumin, glycerol gelatin, or dextran (Hagler et al., 1983; Hall and Gupta, 1982; Rick et al., 1982; Roomans and Sevéus 1977; Saubermann et al., 1981b). Dextran standards are found to be very suitable, because dextran does not contribute characteristic peaks in X-ray microanalysis and solutions containing up to 50% dry weight can be prepared. Glycerol gelatin contains small amounts of sodium and sulfur, and albumin contains sulfur which may disturb the X-ray spectrum. The detection limit found is 12 mMol/kg dry weight for all elements with an atomic number higher than 12; for sodium and magnesium the detection limit is 48 and 36 mMol/kg dry weight, respectively. In a homogeneous section the X-ray spectra are independent of the size of the scanned area. This means that in a 50 nm x 50 nm area of a 100 nm thick section less than 2,000 characteristic atoms can be detected. In order to obtain the physiologically important wet weight concentrations of the elements, the local mass or the local water content has to be determined. This can be done for example by the method of Zeitler and Bahr (1962). Thus, electrolyte concentrations in terms of mMol/liter water should be available.

## Conclusion

The main features of frozen-hydrated and freeze-dried cryosections with respect to X-ray microanalysis are compiled in Table 3. Although freeze-dried sections are sufficient for investigations of many biological problems, the risk of ion distribution caused by freeze-drying leads to continuous efforts in improving electron probe microanalysis of frozen-hydrated states.

## Acknowledgement

I want to thank Miss S. Dongard for her very careful technical assistance. Some illustrations shown result from collaboration with colleagues: I thank Dr. F. Pietruschka for tissue culture cells, Dr. L. Edelmann for skeletal muscle cryosections and Prof. Dr. R.A. Steinbrecht for preparing silk moth antennae for cryosectioning.

## References

Appleton TC. (1974). A cryostat approach to ultrathin 'dry' frozen sections for electron microscopy: a morphological and X-ray analytical study. J. Microsc. 100, 49-74.

Barnard T. (1982). Thin frozen-dried cryosections and biological X-ray microanalyis. J. Microsc. 126, 317-332.

Carlemalm E, Kellenberger E. (1982). The reproducible observation of unstained embedded cellular material in thin sections: visualization of an integral membrane protein by a new mode of imaging for STEM. The EMBO Journal 1, 63-67.

Chang J-J, McDowall AW, Lepault J, Freeman R, Walter CA, Dubochet J. (1983). Freezing, sectioning and observation artefacts of frozen hydrated sections for electron microscopy. J. Microsc. 132, 109-123.

Christensen AK. (1971). Frozen thin sections of fresh tissue for electron microscopy, with a description of pancreas and liver. J. Cell Biol. 51, 772-804.

Colliex C, Jeanguillaume C, Maury C. (1984). Unconventional modes for STEM imaging of biological structures. J. Ultrastruct. Res. 88, 177-206.

Dubochet J, Lepault J, Freeman R, Berriman JA, Homo JC. (1982). Electron microscopy of frozen water and aqueous solutions. J. Microsc. 128, 219-237.

Eusemann R, Rose H, Dubochet J. (1982). Electron scattering in ice and organic materials. J. Microsc. 128, 239-249.

Frederik PM, Busing WM, Persson A. (1984a). Surface defects on thin cryosections. Scanning Electron Microsc. 1984; I: 433-443.

Frederik PM, Busing WM, Hax WMA. (1984b). Observations on frozen-hydrated and drying thin cryosections, in: Electron Microsc. 1984, Vol. 2, A. Csanády, P. Röhlich, D. Szabó (eds.), The programme committee of the 8th European Congress on Electron Microscopy, Budapest, 1411-1412.

Gupta BL, Hall TA, Moreton RB. (1977). Electron probe X-ray microanalysis, in: Transport of Ions and Water in Animals, BL. Gupta, RB. Moreton, JL. Oschmann, BJ. Wall (eds.), Academic Press, New York, London, San Francisco, 83-143.

Gupta BL, Hall TA. (1981). The X-ray microanalysis of frozen-hydrated sections in scanning electron microscopy: an evaluation. Tissue Cell 13(4), 623-643.

Hagler HK, Lopez LE, Flores JS, Lundswick RJ, Buja LM. (1983). Standards for quantitative energy dispersive X-ray microanalysis of biological cryosections: Validation and application to studies of myocardium. J. Microsc. 131, 221-234.

Hagler HK, Buja LM. (1984). New techniques for the preparation of thin freeze-dried cryosections for X-ray microanalysis, in: Science of Biological Specimen Preparation for Microscopy and Microanalysis, JP. Revel, T. Barnard, GH. Haggis (eds.), SEM Inc. AMF O'Hare (Chicago), IL 60666-0507, USA, 161-166.

Hall TA, Gupta BL. (1982). Quantification for the X-ray microanalysis of cryosections. J. Microsc. 126, 333-345.

Hall TA, Gupta BL. (1983). The localization and assay of chemical elements by microprobe methods. Quart. Rev. Biophys. 16, 297-339.

Hax WMA, Lichtenegger S. (1982). Transfer, observation, and analysis of frozen-hydrated specimens. J. Microsc. 126, 275-284.

Heide HG. (1982). Design and operation of cold stages. Ultramicroscopy 10, 125-154.

Heide HG. (1984). Observations on ice layers. Ultramicroscopy 14, 271-278.

Heide HG, Grund S. (1974). Eine Tiefkühlkette zum Überführen von wasserhaltigen biologischen Objekten ins Elektronenmikroskop. J. Ultrastruct. Res. 48, 259-268.

Hren JJ. (1979). Barriers to AEM: Contamination and etching, in: Introduction to Analytical Electron Microscopy, JJ. Hren, JI. Goldstein, DC. Joy (eds.), Plenum Press, New York and London, 481-505.

Hutchinson TE, Johnson DE, Mackenzie AP. (1978). Instrumentation for direct observation of frozen hydrated specimens in the electron microscope. Ultramicroscopy 3, 315-324.

James-Kracke MR, Sloane BF, Shuman H, Karp R, Somlyo AP. (1980). Electron probe analysis of cultured vascular smooth muscle. J. Cell Physiol. 103, 313-323.

McDowall AW, Chang J-J, Freeman R, Lepault J, Walter CA, Dubochet J. (1983). Electron microscopy of frozen hydrated sections of vitreous ice and vitrified biological samples. J. Microsc. 131, 1-9.

Moor H. (1964). Die Gefrier-Fixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z. Zellforsch. 62, 546-580.

Moreton RB, Echlin P, Gupta BL, Hall TA, Weis-Fogh T. (1974). Preparation of frozen hydrated tissue sections for X-ray microanalysis in the scanning electron microscope. Nature 247, 113-115.

Müller M, Moor H. (1984). Cryofixation of thick specimens by high pressure freezing, in: The Science of Biological Specimen Preparation for Microscopy and Microanalysis, JP. Revel, T. Barnard, GH. Haggis (eds.), SEM Inc, AMF O'Hare (Chicago), IL 60666-0507, USA, 131-138.

Ottensmeyer FP. (1984). Electron spectroscopic imaging: Parallel energy filtering and microanalysis in the fixed beam electron microscope. J. Ultrastruct. Res. 88, 121-134.

Ottensmeyer FP, Andrew JW. (1980). High resolution microanalysis of biological specimens by electron energy loss spectroscopy and by electron spectroscopic imaging. J. Ultrastruct. Res. 72, 336-348.

Plattner H, Bachmann L. (1982). Cryofixation: A tool in biological ultrastructural research. Int. Rev. Cytol. 79, 237-304.

Rick R, Dörge A, Thurau K. (1982). Quantitative analysis of electrolytes in frozen dried sections. J. Microsc. 125, 239-247.

Roomans GM, Sevéus LA. (1977). Preparation of thin cryosectioned standards for quantitative microprobe analysis. J. Submicr. Cytol. 9, 31-35.

Roomans GM, Wei X, Sevéus L. (1982). Cryoultramicrotomy as a preparative method for X-ray microanalysis in pathology. Ultrastruct. Pathol. 3, 65-84.

Ross A, Sumner AT, Ross AR. (1981). Preparation and assessment of frozen-hydrated sections of mammalian tissue for electron microscopy and X-ray microprobe analysis. J. Microsc. 121, 261-272.

Saubermann AJ, Echlin P, Peters PD, Beeuwkes R III. (1981a). Application of scanning electron microscopy to X-ray analysis of frozen-hydrated sections: I. Specimen handling techniques. J. Cell Biol. 88, 257-267.

Saubermann AJ, Beeuwkes R III, Peters PD. (1981b). Application of scanning electron microscopy to X-ray analysis of frozen-hydrated sections. II. Analysis of standard solutions and artificial electrolyte gradients. J. Cell Biol. 88, 268-273.

Sevéus L. (1980). Cryoultramicrotomy as a preparation method for X-ray microanalysis. Scanning Electron Microsc. 1980; IV: 161-170 and 26.

Sitte H. (1982). Instrumentation for cryosectioning, in: Electron Microscopy 1982, Vol 1, 10th International Congress on Electron Microscopy, the Organizing Committee (eds.), Deutsche Gesellschaft f. Elektronenmikroskopie, Frankfurt/Main, 9-18.

Somlyo AV, Shuman H, Somlyo AP. (1977). Elemental distribution in striated muscle and the effects of hypertonicity. J. Cell Biol. 74, 828-857.

Somlyo AP, Shuman H. (1982). Electron probe and electron energy loss analysis in biology. Ultramicroscopy 8, 219-234.

Steinbrecht RA, Zierold K. (1984). A cryoembedding method for cutting ultrathin cryosections from small frozen specimens. J. Microsc. 136, 69-75.

Talmon Y. (1984). Radiation damage to organic inclusions in ice. Ultramicroscopy 14, 305-316.

Talmon Y, Davis HT, Scriven LE, Thomas EL. (1979). Cold stage microscopy system for fast-frozen liquids. Rev. Sci. Instrum. 50, 698-704.

Wendt-Gallitelli MF, Wolburg H. (1984). Rapid freezing, cryosectioning, and X-ray microanalysis on cardiac muscle preparations in defined functional states. J. Electr. Microsc. Techn. 1, 151-174.

Zeitler E, Bahr GF. (1962). A photometric procedure for weight determination of submicroscopic particles quantitative electron microscopy. J. Appl. Physics 33(3), 847-853.

Zierold K, König R, Olech KH, Schäfer D, Lübbers DW, Müller KH, Winter H. (1981). A cooling chain for studies of cryofixed biological specimens by scanning transmission electron microscopy and X-ray microanalysis. Ultramicroscopy 6, 181-186.

Zierold K. (1982). Preparation of biological cryosections for analytical electron microscopy. Ultramicroscopy 10, 45-53.

Zierold K. (1983). X-ray microanalysis of frozen-hydrated specimens. Scanning Electron Microsc. 1983, II: 809-826.

Zierold K. (1984a). Quantitative X-ray microanalysis of biological cryosections depends on ice crystal damage. J. de Physique C2, 447-450.

Zierold K. (1984b). Experiences in X-ray microanalysis of freeze-dried cryosections. Scanning Electron Mircoscopy 1984; IV: 1867-1874.

Zierold K. (1984c). The morphology of ultrathin cryosections. Ultramicroscopy 14, 201-210.

Zierold K, Schäfer D, Pietruschka F. (1984). The element distribution in ultrathin cryosections of cultivated fibroblast cells. Histochem. 80, 333-337.