Advanced Instrumentation and Methodology Related to Cryoultramicrotomy: A Review

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ADVANCED INSTRUMENTATION AND METHODOLOGY RELATED TO
CRYOULTRAMICROTOMY: A REVIEW*

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Abstract

This review is concerned with the considerable progress in the field of cryo-ultramicrotomy (cryofixation, cryosectioning, investigation and analysis of cryosections) during recent years. This progress includes both more efficient instrumentation and methodology. The article is mainly directed to the investigation and analysis of frozen-hydrated sections in the low dose cryo-transmission electron microscopy (TEM) and cryo-energy filtered TEM (EFTEM). A general survey is followed by an evaluation of the different relevant procedures. Both cryo-ultramicrotomy for macromolecular cytochemistry (Tokuyasu technique) and cryo-ultramicrotomy for element analysis are only shortly mentioned without discussion of the chemical and analytical approach. Because of lack of first hand experience, cryo-sectioning for X-ray microanalysis in the frozen-hydrated state according to Hall and Gupta is not included into this review. The methods and instruments required for ultrathin sectioning at low temperatures are described and discussed in detail. This concerns the preceding cryofixation, the cryosectioning itself with special emphasis to the required stability and precision of the cryo-ultramicrotome, the characteristics of the knives, the charging phenomena due to sectioning and the subsequent TEM investigation including EFTEM with electron spectroscopic imaging (ESI) and the available accessories for digital low dose registration of signals.

Key Words: Cryo-ultramicrotomy, cryo-immobilisation, high-pressure freezing, ambient-pressure freezing, vitrification, cryoprotection, cryostabilisation, cryotransfer, low dose imaging, energy filtering (EFTEM), electron spectroscopic imaging (ESI), beam damage, radiolysis, hybrid methods.

*The paper is dedicated with some delay to my brother Prof. Peter Sitte on the occasion of his 65th birthday (December 8, 1994) with very best wishes and the highest fraternal respect.

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"Den Vorsatz, etwas zu verbessern muß mancher Forscher sehr verwässern. Die so erzeugte Wasserkraft treibt dann die Arbeit fabelhaft" [frei nach Eugen Roth, 1977 (translation page 460)]

Introduction

In most cases the real possibilities of new methods or instruments are considerably overestimated, a lot of difficulties and limitations overlooked or hidden. At the end it often turns out, that the power of the innovation is below the expectations and that the hurdles to be taken increase with each step forward. In the early fifties the "ultrathin serial sections in the thickness range of 100 Å" (see Porter and Blum, 1953; Sitte, 1955; Sjöstrand, 1953, 1954) turned out often to be "beefsteaks" ten times thicker, as clearly shown by Peachey (1958) or Bachmann and P. Sitte (1958, 1960). And even those "beefsteaks" were not collected
### Table 1. Important improvements 1985 - 1995 influencing cryo-ultramicrotomy

<table>
<thead>
<tr>
<th>New Method or Technology</th>
<th>Main Improvements</th>
<th>Literature</th>
<th>See this Review</th>
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<tr>
<td><strong>High-Pressure Freezing</strong> (Synchronisation of pressurisation and cooling by alcohol volume at ambient temperature, 1-hexadecane as intermediate between sandwich container and object, variable thickness 0.1 to 0.6 mm of specimen container, dialysis tubes for cell suspensions)</td>
<td>Yield of well frozen specimens often near 100%, minimum delay between pressurisation and freezing (≤ 13 msec for layers of 0.1 mm) → minimised pressure artefacts, prevention of blowing out of suspensions by use of dialysis tubes</td>
<td>Müller and Moor, 1984, Studer et al., 1989, Michel et al., 1991, Hohenberg et al., 1994. See also catalogues of Balzers (HPM010) and Leica (EM-HPF)</td>
<td>Sections &quot;Freezing and the Frozen State of Water&quot; and &quot;High Pressure Freezing&quot;, Fig. 12, Table 5</td>
</tr>
<tr>
<td><strong>Cryo-Ultramicrotomes</strong> (through-the-wall specimen arm, shell-mounted cryochamber, continuous refilling of LN₂)</td>
<td>Mechanical stability at least identical to standard ultramicrotomes for ambient temperature work, better reproducible section thicknesses (thinner sections) regularly available → minimising of crevasses</td>
<td>Catalogues of RMC (Cryochamber RMC 21) and of Leica-Reichert (Ultracut-S/PCS and Ultracut-UCT/ FCS)</td>
<td>Sections &quot;Cryo-Ultramicrotomes&quot; and &quot;Ultrathin Sectioning and Handling..&quot;, Figs. 13e,f, 14b, 15</td>
</tr>
<tr>
<td><strong>Adjustable Ioniser Spike inside the GN₂ of the cryochamber</strong></td>
<td>Minimising of charging phenomena (cryosections versus knife material), perfect diamond trimming and diamond knife sectioning of sugar protected material according to Tokuyasu, easier section transfer</td>
<td>Michel et al., 1992. See also catalogue of Diatome (Static Line II)</td>
<td>Sections &quot;Cryo-Ultramicrotomes&quot; and &quot;Ultrathin Sectioning and Handling..&quot;, Fig. 17</td>
</tr>
<tr>
<td><strong>(a) Low Angle Cryo-Diamond Knives and (b) Diamond Trimming Tools, in combination with an adjustable ioniser</strong></td>
<td>(a) Reduced section compression and formation of &quot;crevasses&quot;, smoother sections, lower section thicknesses available. - (b) Better geometry of sectioning surface by diamond trimming improves all results</td>
<td>Michel et al., 1992. See also catalogues of Diatome</td>
<td>Sections &quot;Cryo-Ultramicrotomes&quot; and &quot;Ultrathin Sectioning and Handling..&quot;, Figs. 17 - 21</td>
</tr>
<tr>
<td><strong>High-Performance Cryotransfer-Systems (cold stages)</strong> for low temperature TEM work down to -180°C coupled with efficient decontamination systems (cold traps) for TEM</td>
<td>Minimising of frost deposition during and after cryotransfer, good temperature stability → minimum drift (&lt; 0.1 nm/sec) → optimum resolution (≤ 0.34 nm)</td>
<td>Catalogues of Gatan (Model 626-DH) and of Oxford (CT 3500 and Anticontamination system)</td>
<td>Section &quot;Ultra-thin Sectioning and Handling ..&quot;, Fig. 22</td>
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<tr>
<td><strong>Freeze-Drying for EDX and EELS</strong></td>
<td>Freeze-drying considerably simplified and improved by cryosorption freezedryers</td>
<td>Sitte et al., 1994. See also catalogue Leica (EM-CFD)</td>
<td>Sections &quot;.. Electron Analysis&quot; and Discussion, Fig. 25</td>
</tr>
<tr>
<td><strong>Slow Scan CCD Cameras</strong> for digital on-line low dose recording of TEM images or diffraction patterns or autotuning/autofocus of TEM</td>
<td>≥ 10:1 reduction of e⁻ dose (beam damage), linear ratio between e⁻ dose and signal intensity, wide dynamic range, automatic TEM operation (focus, tomography, lens calibration, stigmator control etc.)</td>
<td>De Ruijter, 1995, Dierksen et al., 1993, Koster and De Ruijter, 1992, Krivanek and Mooney, 1993, Tietz, 1992. See also the catalogues of Gatan and LEO (Zeiss)</td>
<td>Section &quot;.. Frozen-Hydrated Ultrathin Sections&quot;, Fig. 27, Table 6</td>
</tr>
<tr>
<td><strong>Electron Stimulable Phosphorus Image Plates</strong> for digital off-line low dose recording of TEM images or diffraction patterns</td>
<td>≥ 10:1 reduction of e⁻ doses (beam damage), linear ratio between e⁻ dose and signal intensity, wide dynamic range, extremely large detector surface identical to photographic plates/films, compatible with all TEM models</td>
<td>Ayato et al., 1990, Burmester, 1992, Mori et al., 1988, 1990, Oikawa, 1990, Shindo et al., 1990, 1991. See also catalogue Fuji FDL-5000 system</td>
<td>Section &quot;.. Frozen-Hydrated Ultrathin Sections&quot;, Figs. 28 and 29, Table 6</td>
</tr>
</tbody>
</table>
Cryoultramicrotomy

| Electron Energy Filters (EFTEM) for Electron Spectroscopic Imaging (ESI) in "in column" or "post column" design | Minimising of underfocus < 0.1 μm needed in conventional TEM to obtain sufficient contrast by "zero loss imaging" (elimination of inelastically scattered el\(^{-}\)) → better contrast and resolution of frozen-hydrated specimens with high contents of elements with low atomic numbers | Bauer, 1988, Krivanek et al., 1995, Benner et al., 1994, Bihr et al., 1991, Schröder, 1992, Schröder et al., 1990. See also catalogues of LEO (Zeiss) and Gatan | Section ".. Frozen-Hydrated Ultrathin Sections", Fig. 30 |

as routinely as often claimed at that time. Pictures presented as results of daily work were often identified as extraordinary singles, selected from thousands of electron micrographs. Neither cryostats nor cryo-chambers for sectioning (see Appleton, 1974; Bernhard, 1965; Bernhard and Nancy, 1964; Bernhard and Leduc, 1967; Bernhard and Viron, 1971; Christensen, 1969, 1971; Dollhopf and Sitte, 1969; Leduc et al., 1967) reached the claimed minimal temperatures without severe drawbacks and cryowork was much more complicated than announced (see e.g. Echlin, 1992, pp. 138-140; Robards and Sleytr, 1985, p. 504; Sitte, 1982; Tokuyasu, 1980, p. 383; Zierold, 1987, p. 145). The 100:1 profit in "cryostabilisation" by extremely low temperatures with the LHe-cryostat-lens at 4.2 K (Dietrich et al., 1980; Knapek and Dubochet, 1980) in comparison to work at ambient temperature was shrinking to a small x 3 to x 10 stabilising factor for the observed organic crystals exposed to an electron beam at low temperature (Chiu et al., "International Experimental Study Group", 1986) already obtainable with a liquid nitrogen (LN\(_{2}\)) cold stage. But, very important for scientific progress in our field - and probably in most similar new areas of high-tech research: nobody with rational concepts would have continued to invest and waste precious time and a lot of money after a clear cut statement from the leading pioneers, that there exist tremendous difficulties to obtain the desired results. Nobody would have made further effort to run cryo-apparatuses which neither reached the wanted temperatures at a reasonable time nor worked correctly. And nobody would have been interested in cryo-scopes, which only cause big problems without really preserving precious samples from beam damage. Nevertheless: we should not worry about all the mistakes and misinterpretations made (and published) in scientific enthusiasm. They were mostly at the end coupled with remarkable real progress. It should for example not hamper, that high pressure freezers vitrify only 100 μm thick samples (instead of calculated 600 μm, see Moor, 1987; Sartori et al., 1993; Studer et al., 1995) if it becomes evident at the same time, that ambient pressure cryofixation also does not reach its goal (see Sitte et al., 1987a). High pressure remains still more than 10 times better. This makes the point. Therefore: progress is evident, and in cryo-ultramicrotomy and all directly associated fields of freezing and low dose electron microscopy just the recent years brought a lot of really important improvements as shown in Table 1. Of course, there exist a couple of excellent reviews covering this field (see e.g. Echlin, 1992; Dubochet et al., 1987, 1988; Menco, 1986; Morgan, 1995; Reid and Beesley, 1991; Robards and Sleytr, 1985; Roos and Morgan, 1990; Zierold, 1987). But they now have in some respects more "historical" character and do not cover the present state of the art. On the other hand, also the current literature is not always based on the best methods and instruments available today. Additionally a lot of comparative work is needed to evaluate, what is really possible with different specimen categories. The entire sense of this review is therefore, to close these gaps and to encourage interested colleagues to start again and to gain from those fascinating advances.

To avoid misunderstandings, it is important to differentiate precisely between the various fields of applications, in which cryo-ultramicrotomy nowadays is used. Besides the broad application in materials sciences (not considered in this article) the main application is immuno cytochemistry (or more general: macromolecular cytochemistry). As shown in Table 2 this specific application follows a completely different protocol in comparison to all other areas of application. Chemical prefixation with aldehydes, necessary for these freeze-thawing procedures, does not affect most of the biomacromolecules in respect of their antigenicity. Since even a weak aldehyde fixation opens the cell membranes, efficient intra- and intercellular cryoprotection is possible, which simplifies freezing and sectioning tremendously. It was the incredible merit of Tokuyasu (1973) to introduce sucrose as cryoprotectant, which makes bulk tissue samples so well sectionable at low temperature, that his protocol pushed cryo-ultramicrotomy into the field of well reproducible routine work not essentially different from standard ultramicrotomy of resin blocks (for further detail see e.g. Griffiths, 1993; Griffiths et al., 1983, 1984; Tokuyasu, 1986; Sitte et al., 1988). Nevertheless the Tokuyasu technique also took and
Table 2. Comparison between the pathways and protocols for cryo-ultramicrotomy (CUM), if macro-molecular histochemistry (e.g., Tokuyasu method), EDX or EELS for element analysis on ultrathin cryosections after freeze drying or direct imaging of frozen-hydrated ultrathin cryosections in the cryo-TEM is the goal. Note the considerable difference between macromolecular histochemistry (Tokuyasu technique), element analysis and frozen hydrated work.

<table>
<thead>
<tr>
<th>Step</th>
<th>Macro-molecular Histochemistry</th>
<th>Element Analysis on Ultrathin Sections</th>
<th>Frozen-Hydrated Investigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical prefixation</td>
<td>Low molarity aldehyde mixture</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cryoprotection</td>
<td>2.3 M sugar (only in exceptional cases lower molarity)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cryo-ultramicrotomy (CUM)</td>
<td>CUM at temperatures around -100°C with cryo-diamond knives and ioniser (easy sectioning after 2.3 M sugar protection even of surfaces around 0.5 x 0.5 mm²)</td>
<td>CUM at temperatures ≤ -160°C with cryo-diamond knives and ioniser (very difficult sectioning of the extremely brittle material even in surface areas below 0.1 x 0.1 mm²)</td>
<td>CUM at temperature of ≤ -160°C with cryo-diamond knives and ioniser (very difficult sectioning of the extremely brittle material even in surface areas below 0.1 x 0.1 mm²)</td>
</tr>
<tr>
<td>Follow-up procedures</td>
<td>Picking-up with droplets of concentrated sugar solution within wire loop → thawing → labelling → coating (e.g., methyl cellulose) → drying → TEM at +20°C</td>
<td>Dry transfer to grid → freeze drying ≥ 12 h at low temperature (start -80°C) → EDX or EELS in TEM/STEM at +20°C or low temperature</td>
<td>Dry transfer to grid → cryo-transfer ≤ -150°C to TEM → low dose imaging with SS-CCD or image plate in EFTEM (“zero loss mode”) at temperature ≤ -150°C</td>
</tr>
</tbody>
</table>

Takes advantage of the progress in cryosectioning, specifically of the new cryodiamond knives together with the discharging ionisers and of course of the improved cryochambers themselves (see separate Section "Cryo-Ultramicrotomy According to Tokuyasu" and Table 2).

The demands for the investigation or diffraction analysis of frozen-hydrated specimens in the cryo-transmission electron microscopy (TEM) and/or for energy-dispersive X-ray analysis (EDX) or electron energy loss spectroscopy (EELS) of ultrathin sections differ considerably from the needs of Tokuyasu work for immuno cytochemistry (see again Table 2). Chemically prefixed and sugar protected (2.3 M sucrose) specimens do not need a rapid cryofixation: LN₂ does the job mostly sufficiently and sometimes even better than ethane, since vitrification is easy obtainable and clefts or ruptures in the samples are not provoked by high cooling rates. Also sectioning of the "sugar embedded" material even in rather old cryo-ultramicrotomes poses no severe problems, since in the range around -100°C those specimens have a nice consistency: sections with a width up to 0.5 mm (in some cases up to 1.0 mm) are easily available. The collection of sections from the dry knife with a droplet of 2.3 M sugar solution (also an invention of Tokuyasu) is as simple and elegant as spreading and deposition of the sections on grids or cover slips.

The technical and methodological progress of the recent years (see Table 1) eliminated some of the severe draw-backs of the cryo-ultramicrotomy and investigation of fresh frozen samples both for element analysis and studies in the frozen-hydrated state by TEM/scanning transmission electron microscopy (STEM)/EDX/EELS. This progress started with the development of a better and far more efficient methodology of high pressure freezing (see e.g. M. Müller and Moor, 1984; Moor, 1987; Studer et al., 1989, 1995; Michel et al., 1991), which has advanced to a routine method at least for all cell suspensions (Hohenberg et al., 1994), some stable animal tissues (e.g., cartilage) and a lot of plant material not accessible for ambient pressure freezing due to their high water content. Now in those fields high pressure freezing allows to vitrify rather large samples. This is of great importance since clear evidence was presented recently, that microcrystalline freezing generates artefacts (McDowall et al., 1984; Studer et al., 1995), although the small crystals are not resolvable in the electron micrograph and do not influence the cryo-
sectioning process at the same extent as larger crystals of hexagonal ice (I_h). Besides the high tech pressure freezing also the simpler ambient pressure freezing methods should not be overlooked: they are now further developed, better understood and open new possibilities for subsequent cryosectioning. Dramatic improvements resulted in the design and operation of cryochambers since 1990: both the new "through-the-wall" operation of the specimen arm of the ultramicrotome, the "shell-mounted cryochamber" and the nowadays available systems for electronically controlled "continuous LN2-refilling" of the latest generation of cryochambers (Leica FC S and FC R, partially RMC CR 21) (see list of suppliers) reach or surpass the stability and precision of standard ultramicrotomes for ambient temperature work. Everybody struggling with a former system knows exactly, that especially stability and precision are of the greatest practical importance, since all cryosectioning artefacts are increasing with increasing section thicknesses. Irregular sectioning, that means thicker sections now and then, destroys all hopes and benefits of a stable system. The maximum precision (exclusion of outside thermal influences, further improved advance and drive systems) is therefore really needed in this field. The application of an adjustable ioniser and the use of the new cryodiamond knives for dry trimming and sectioning work act in the same direction (Michel et al., 1992): they indeed make cryosectioning of fresh frozen specimens more reproducible and efficient. Additional advantages will arise for sure from cryodiamond knives with facet angles below 35°: section compression will be lowered once more noticeably, as Jésior (1986, 1989) has demonstrated already for ambient temperature sectioning. Since frozen sections do not allow any spreading operation by heat or organic solvent vapours, this is a very important measure in order to reduce this disturbing artefact.

Finally, any progress to lower the needed electron dose for documentation of biostructures in the frozen-hydrated state or to minimise beam damage by other means, should be carefully realised. This concerns both screening and selection of useful areas in the frozen-hydrated sections, focusing and digital registration of all signals excited by the electron beam. Since the expected considerable stabilisation of sensitive biological ultrastructures by low temperature (The

Figure 1. Equilibrium phase diagrams for freezing of "pure" water (a) and of the eutectic binary system "water/glycerol" (b). As explained in this section ("Freezing and the Frozen State of Water..."), freezing processes are essentially kinetic events: the diagrams allow therefore no conclusion about the periods of time needed to reach the state of equilibrium. (a) Dependence of melting temperature T_m, temperature T_h of "homogenous nucleation" (broken line) and transition from one of the polymorphic modifications I ↔ II ↔ III into one another, on pressure. Ice I is a synonym for hexagonal ice I_h. Ice II and ice III are like ice IV to IX high pressure modifications (IV to IX stable at pressures above 3 kbar and not considered in this review, see e.g., Eisenberg and Kauzman, 1969). Cubic ice (I_c) is mostly generated by warming up amorphous (vitreous) ice (I_v) above the devitrification temperature T_d. Since both I_c and I_v are unstable modifications, they are not included in this equilibrium phase diagram. The diagram shows clearly, that T_m has a minimum slightly below -20°C at a pressure of 2.1 kbar. T_h for homogenous nucleation shows also the minimum at the same pressure. (b) Dependence of freezing point T_m in the binary system "glycerol/water" on the relative amount of glycerol/water: maximum depression occurs in the equilibrium at 67 % glycerol, where according to the diagram below -40°C (EP = eutectic point) a mixture of ice and solid glycerol ("eutectic mixture") should crystallise.
Figure 2. Typical bare thermocouple record from plunging into liquid ethane. The ethane was kept at -160°C. The motion record was realised with a photo diode on a black and white sequence moved and recorded together with the plunger. The plunge velocity was 2 m/sec. The cooling rate between 0 and 100°C was calculated according to the definition (approximately 9300°C/sec). The plunging motion in the cryogen was limited to 8 cm (modified Fig. 7 of Ryan, 1992, published with permission). Further explanation in the section on "Freezing and the Frozen State of Water..."

The term "cryostabilisation" used by Dubochet et al. (1981) seems to me much better than the previously used term "cryoprotection", which provokes some confusion with cryoprotection by sucrose or glycerol) was not reproducible for the whole range of structures and procedures (Chiu et al., 1986), the new possibilities of digital accumulation of signals at minimum electron doses are a precious tool for frozen-hydrated work. This low dose registration is now possible both with slow-scan charge-coupled-devices (SS-CCD-systems, see e.g., De Ruijter, 1995) and with image plates (see e.g., Burmester, 1992; Mori et al., 1988, 1990; Oikawa et al., 1990) already used in radiology and now just for the first time introduced and made commercially available for TEM-work by Fuji (FDL-5000 system). Both different systems besides their higher sensitivity have the big advantage of a precise linearity of signal intensity to the electron dose enabling an accurate quantification of the recorded signals. Finally "Energy Spectroscopic Imaging" (ESI) with energy filtering energy filtering TEMs (EFTEMs, see e.g., Bauer, 1988; Henkelmann and Ottensmeyer, 1974; Krivanek et al., 1995; Ottensmeyer and Andrew, 1980; Schröder, 1992; Schröder et al., 1990) opens a possibility to reduce underfocus, to improve signal-to-noise ratio and contrast of fresh-frozen material considerably. Since all above mentioned recent progress contributes to frozen-hydrated TEM work in a highly welcome manner, the different new approaches will be described and discussed in detail in the following sections. As far as necessary and useful, earlier (already forgotten) progress will also be mentioned and integrated. Since some instruments will not be well known, a short list of suppliers is added for interested colleagues.

<table>
<thead>
<tr>
<th>Cryogen</th>
<th>Tm</th>
<th>Tb</th>
<th>(Tb-Tm)</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethane</td>
<td>-171</td>
<td>-89</td>
<td>82</td>
<td>1.3</td>
</tr>
<tr>
<td>Propane</td>
<td>-190</td>
<td>-42</td>
<td>148</td>
<td>1.0</td>
</tr>
<tr>
<td>Freon 13</td>
<td>-185</td>
<td>-81</td>
<td>104</td>
<td>0.8</td>
</tr>
<tr>
<td>Freon 22</td>
<td>-155</td>
<td>-41</td>
<td>114</td>
<td>0.7</td>
</tr>
<tr>
<td>Freon 12</td>
<td>-152</td>
<td>-30</td>
<td>122</td>
<td>0.5</td>
</tr>
<tr>
<td>Isopentane</td>
<td>-160</td>
<td>+28</td>
<td>188</td>
<td>0.5</td>
</tr>
<tr>
<td>LN2 cooled to Tm</td>
<td>210</td>
<td>-196</td>
<td>14</td>
<td>0.2</td>
</tr>
<tr>
<td>Boiling LN2</td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Hyperbaric</td>
<td></td>
<td></td>
<td></td>
<td>&gt;1.3</td>
</tr>
<tr>
<td>LN2 (pressurised) at about 2 kbar</td>
<td></td>
<td></td>
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</table>
Freezing and the Frozen State of Water - Cryoprotection

It is indeed very encouraging for a biologist, to read the statement of two well known specialists in the field of the physicochemistry of water in an excellent review article (see Bachmann and Mayer, 1987, p. 4), that "water has some unusual properties which have made it one of the most investigated liquids whose structure is still not fully understood". But this comment should not be an excuse and some basics have to be understood or at least noticed and accepted before starting a cryopreparation.

As long as we work at "normal" ambient pressure of 1 bar, we know that water freezes (or melts) at 0°C (T_0). Unfortunately things are not as simple as expected, since freezing is a typical "kinetic" event. If we lower the temperature rapidly enough, e.g., with a cooling rate of several 100,000°C per second, cooling...
is more rapid than freezing. We will therefore probably observe liquid "supercooled" water with a temperature below 0°C, e.g. -10°C, which does not seem to be in agreement with the diagram in Fig. 1a (for physicochemical details see e.g., Bachmann and Mayer (1987), Eisenberg and Kauzmann (1969) or Franks (1972-1982). Under such conditions finally freezing mostly starts from an impurity (if "pure" H₂O is our liquid) which acts as "nucleus" or "crystallisation center". Since those nuclei are "foreign" particles in water, one speaks of a "heterogeneous nucleation". Freezing in this case is a very speedy process and leads to the well known normal hexagonal ice (Iₕ) - the only ice which we observe in our terrestrial environment: the ice in our deep freeze, the beautiful snow crystals, the ice of the increasingly melting glaciers and pole regions - all consisting of Iₕ. Provided that no "foreign", that means heterogeneous nucleus is present in our water, we reach by further cooling at ambient pressure a temperature Tₕ near -40°C, were the society of water molecules itself serves for nucleation: in this case one speaks of "homogeneous" or "spontaneous nucleation". Freezing now starts everywhere in the whole liquid phase and huge amounts of small ice crystals are formed.

Things change again if pressure is applied: according to the phase diagram in Fig. 1a both melting point Tₘ and the temperature Tₕ, where homogenous nucleation starts during cooling, decrease with increasing pressure. The cross-hatched area in the diagram represents the region within which supercooled water can exist. Also Tₕ is a limit, which follows kinetic laws: during an extremely rapid cooling the start of spontaneous crystallisation becomes delayed. But this is not the whole story: like all other liquids also water gets increasingly viscous with decreasing temperature. Like for very viscous sugar solutions crystallisation processes in other viscous liquids are very slow. For example honey tends to crystallise but often needs several years for this event due to its high viscosity. Also pure water at -100°C has already a remarkable viscosity and finally crystallisation becomes impossible if the temperature decreases below about -135°C. We have an extremely viscous glass-like fluid. In other words: we have vitrified our water to "amorphous" ice Iᵥ without a crystalline structure. This vitreous ice Iᵥ is stable over a long time at these temperatures. It de-vitrifies and changes into microcrystalline cubic ice Iₕ, if we heat it above this "devitrification temperature Tₖ" around -135°C (for physicochemical details see Bachmann and Mayer, 1987). If we now take a look at the phase diagram in Fig. 1a, the basics of pressure freezing should be evident and understandable: at the pressure of about 2,000 bar Tₘ is reduced to -20°C, Tₕ below -90°C. Homogenous nucleation is shifted into the range of more viscous water, since viscosity is strongly dependent on temperature and not changing with pressurisation. To produce Iᵥ at 2,000 bar we need therefore much lower cooling rates in comparison to ambient pressure.

Instead of applying pressure similar effects with respect to reduction (depression) of Tₘ and Tₕ result from suited additives (antifreezes or cryoprotectants) as visible in diagram Fig. 1b. If we add for example 50 % glycerol to pure water, freezing at ambient pressure starts at a lower temperature. The 50/50-mixture starts freezing below -20°C. Since Iᵥ is formed, concentration of glycerol is increasing and the system follows the Tₘ-curve to the "eutectic point EP" at the eutectic temperature near -50°C and a glycerol concentration of 67%. A further decrease of temperature should result in complete crystallisation of the residual liquid if we have the patience to wait for it. The kinetic character of this process is even more expressed than during rapid cooling of untreated fresh samples and in reality those "cryoprotected systems" react extremely slowly: reality is far away from all diagrams. Probably supercooled liquids result rather than the announced eutectic crystallisation, consisting theoretically of Iᵥ and frozen glycerol according to Fig. 1b.

The first successful cryoprotection and vitrification in biological cryopreparation used the mentioned cryoprotection by glycerol according to Fig. 1b (see Polge et al., 1949; Moor, 1964). Cell suspensions and tissues were frozen for subsequent freeze fracture followed by freeze etching and freeze replication, which offered for the first time an alternative to chemical fixation, dehydration and resin embedding by an exclusively physical process (see e.g., Benedetti and Favard, 1973; Moor and Mühlethaler, 1963; Steere, 1957, 1973). Glycerol made it possible to vitrify those specimens even by quite simple freezing procedures. For the first time disturbing ice segregation, that means separation of the cytoplasmic phase with its high water content between 50 and sometimes near 100 % firstly into pure Iᵥ and finally into an eutectic mixture could be suppressed. In the meantime a lot of different cryoprotectants for cells and tissues were checked (e.g., DMSO: dimethylsulfoxide, PVP: polyvinlypyrrolidone, sucrose; for detailed information see Meryman, 1971 or Skaer, 1982). The initially used glycerol made the frozen samples at lower sectioning temperatures around or below -100°C extremely brittle. It was therefore nicely suited for freeze fracturing but terrible for cryo-ultramicrotomy at low temperatures. Contrary to glycerol the already mentioned sugar protection according to Tokuyasu (1973) is
probably not suited for freeze fracture, but excellent for sectioning around -100°C. The drawback of this kind of intracellular cryoprotection is, that cell membranes (and of course also other cellular structures) have to be chemically damaged and by this treatment (chemical prefixation with aldehyde) opened. After this pre-treatment e.g., by aldehyde in low concentrations, which does not affect severely the protein conformation, sugar or glycerol molecules can freely enter through the leaky membranes and a complete protection is possible. Of course such a treatment changes all equilibria of living cells totally: ions and water are moving rapidly and practical nothing is preserved in the original state as far as smaller molecules and ions are concerned. Biomacromolecules (e.g., proteins, nucleic acids, macromolecular carbohydrates) retain most of their antigenicity and that is exactly the value of this pre-treatment, because cell destruction and changes in the conformation e.g., of proteins resulting from the much more aggressive dehydration by organic solvents for resin embedding together with OsO₄-stabilisation of membranes are avoided.

Often cryoprotection of the extracellular (extraplasmatic) compartments or fluids without preceding chemical fixation is advantageous, since mostly severe artefacts by segregation of Ih start around the cells. In such cases extraplasmatic protection by non poisoning agents (e.g., sugar in the suspension medium, see Moor and Mühlethaler, 1963; Dubochet et al., 1983; McDowall et al., 1984, and discussion in the next section) improves the results often considerably, even if the cryoprotectant does not penetrate or enters only slowly or in small amounts into the cytoplasmic phases.

We have to ask: what do we really gain by application of high pressure or physicochemical cryoprotection by glycerol or sucrose in freezing procedures? There is strong evidence, that water is by several cations of high pressure or physicochemical cryoprotection by glycerol or sucrose in freezing procedures. Such cooling rates are obtainable, if specimen thickness does not exceed 0.15 mm. If we cryoprotect our sample according to Tokuyasu (1973) with 2.3 M sucrose, even 1,000°C/sec vitrifies most structures and excludes Ih segregation despite the poor cooling by LN₂.

Rapid heat extraction from biological specimens ("cryofixation") results from direct contact between the specimen surface and a "cryogen". As already mentioned, both liquid and solid state cryogens are suited for this procedure. The most simple cryofixation is achieved by immersing the sample into a liquid cryogen: this procedure is called "immersion cryofixation" or "plunge freezing". It was observed a long-time ago (Hoerr, 1936, see also e.g., Pearse, 1961), that boiling liquids (e.g., LN₂ at its boiling temperature T_b, ~196°C) are not well suited. These "primary cryogens", which maintain their low temperature by continuous boiling (evaporation heat), develop immediately a gas layer (bubble) around the immersed warm specimen ("Leidenfrost phenomenon"). Since gas layers (e.g., gaseous nitrogen, GN₂) are very efficient thermal insulators, such layers prevent the desired rapid cool-
ing. Suited cryogens are therefore only "secondary cryogens", that means liquids with boiling temperatures $T_b$ around or slightly below ambient temperature and a very low melting / freezing temperature $T_m$, which results in a very big difference between their boiling- and freezing temperatures ($T_b-T_m$). Table 3 shows the data of some liquid cryogens proven for rapid freezing in comparison. To evaluate correctly the "cooling efficiencies, CE" it was necessary to cool small probes (microthermocouples) acting instead of specimens in the different cryogens under standardised conditions (see e.g., Costello and Corless, 1978; Ryan, 1991, 1992; Ryan et al., 1987; Schwabe and Terracio, 1980). By the same reason the "cooling rate" (cooling speed) was defined as the average speed measured during cooling such a probe from 0°C to -100°C (see Fig. 2). It is evident, that those "cooling rates" had practically nothing to do with the real cooling profiles within very small biological specimens. But they enabled elegantly to study the possibilities and limitations of different liquids. By similar measurements the importance of the different cooling parameters like the speed and the mount of the specimen or the influence of cold gas layers could be checked (see e.g., Ryan, 1992; Ryan and Purse, 1984, 1985). It became clear by these experiments that ethane is the best suited liquid cryogen of all hitherto studied liquids. Its cooling efficiency CE is 1.3 times higher than CE of liquid propane under identical experimental conditions (see Table 3). CE itself is influenced by a confusing variety of physical parameters like e.g. specific heat, heat conductivity, heat transfer on the phase border between specimen and cryogen, and viscosity (for details see Bald, 1984, 1985). But all this is only of theoretical importance. The message for the application remains simply: ethane is not only the best, but for subsequent cryo-ultramicrotomy of vitrified specimens the only suited cryogen with respect to its fairly high vapour pressure at those low temperatures. LN2 is only suited and then excellent, if a high pressure (e.g., 2 kbar) excludes the Leidenfrost phenomenon. Propane is a low cost and quite good coolant for daily work with hybrid technologies (freeze substitution and freeze drying) as far as the safety rules are strictly regarded (please read carefully the comments on safety e.g., of Sitte et al., 1987b or Robards and Sleytr, 1985, pp. 507-512). For subsequent cryo-ultramicrotomy at real low temperatures below -150°C propane is not applicable without special measures, since it does not evaporate sufficiently fast due to its low vapour pressure at those temperatures. It sticks therefore on all surfaces of an immersed specimen and disturbs sectioning by this liquid surface coat. Hyperbaric LN$_2$ should be an excellent cryogen also outside a high pressure freezer (Bald, 1984; Bald and Robards, 1978), but the results in practical use did not come up to the expectations.

Contrary to solid state cooling ("metal mirror" or "impact freezing") liquid cryogens extract heat in two spatial directions from a thin foil (e.g. "bare grid") according to Fig. 3a, see Adrian et al., 1984; Dubochet et al., 1982a, 1987; Lepault et al., 1983a; or "double jet" M. Müller et al., 1980; Th. Müller et al., 1989; see also Taylor and Glaeser, 1974, 1976) and in all spatial directions from a small droplet (e.g., "spray freezing" according to Bachmann and Schmitt, 1971; see also Bachmann and Schmitt-Fumian, 1973; Plattner et al., 1973) as shown in Fig. 3c. Under such favorable conditions not only is heterogeneous nucleation minimised by minimum specimen (mostly suspension-) volumes, but also maximum cooling rates are achieved by multidirectional heat extraction. It is a severe disadvantage of most biological cryopreparations, that bulk specimens (tissue samples with diameters > 0.5 mm) have no profit from these favourable conditions: here extraction of heat in the border layers of some micrometers in depth during the deciding microseconds after the start of the freezing process is strictly unidirectional. This precondition is also given for the freezing in contact with precooled solid state surfaces. According to Fig. 3c multidirectional heat extraction from small droplets with diameters <50 µm creates cooling profiles (see lower part of the diagram), where the maximum cooling rates result in the center of the sphere. This behaviour is not only visible by the state of preservation of the ultrastructure but can also be demonstrated by ice crystal diameters and by calculations (see e.g., Van Venrooij et al., 1975; Ryan et al., 1990). Similar profiles are estimated for double jet freezing, where heat is extracted only in two directions (Fig. 3b) : also in this case the diameter / thickness of the sheet is a deciding parameter. Only very thin layers (thicknesses _ 100 µm) show a useful improvement of cryopreservation in the center region. All specimens considerably thicker e.g., with diameters in the order <0.5 mm have no noticeable profit from this phenomenon, because freezing of the thin border layers is already finished before heat extraction through the opposite surface region is able to contribute to the cooling rate. Both immersion of such a bulk specimen into a liquid cryogen and surface contact with a cold solid state surface (Fig. 3d) result therefore at their beginning in a unidirectional heat extraction through the already frozen and thermally insulating border layers of the specimen. It is understandable that under these preconditions only very small surface layers (e.g., up to 5 µm) can be vitrified if an unprotected specimen is frozen under ambient
pressure. Only high pressure or sufficient intra- and intercellular protection (e.g., glycerol or sucrose) enable improvement in the situation.

Ambient Pressure Freezing

It has been shown several times, that also under ambient pressure well frozen specimens for subsequent cryosectioning are obtainable (see e.g., Plattner and Knoll, 1983; Sitte et al., 1987a). As far as liquid cryogens are used, this is at least valid for cell suspensions, which often can be cryoprotected partially, that means around the cells, by sugar or glycerol containing suspension media. As shown already by Dubochet and co-workers (Dubochet et al., 1983; McDowall et al., 1984; see also Moor, 1964; Moor and Mühlethaler, 1963), such extracellular protection does not affect different cells and is helpful for vitrification. Obviously this is true for some bacteria, yeast cells and a couple of other eucyte suspensions without the detrimental pre-treatment by chemical fixatives like aldehydes. The main advantage of this protection of the extracellular liquid is the more homogeneous freezing over larger areas, which results for sure in a much better overall sectioning consistency in comparison to completely unprotected samples.

The simplest cryofixation for subsequent cryoultramicrotomy results from immersion into propane or ethane, which is often done in home made laboratory set-ups (for details see e.g., Ryan, 1991, 1992; Sitte, 1979, 1984; Sitte et al., 1985, 1987a). Since these cryogens freeze above -196°C, direct cooling with LN₂ (Tc about -196°C) for longer periods of time is not possible. Either one needs thermostatic exchange between the warm center of the specimen and a couple of other eucyte suspensions without the detrimental pre-treatment by chemical fixatives like aldehydes. The main advantage of this protection of the extracellular liquid is the more homogeneous freezing over larger areas, which results for sure in a much better overall sectioning consistency in comparison to completely unprotected samples.

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Within studies of immersion technology several times cooling columns with heights exceeding 20 cm were used (see e.g., Ryan, 1991, 1992). In normal laboratory practice such columns do not offer advantages: mostly a height of 8 to 10 cm is absolutely sufficient if small specimens and light weight carriers are used as described above. Also the velocity of the specimen (entry speed and speed through the ethane) is not that important as one may conclude from some measurements or pictures (e.g., Fig. 5). Of course an extremely "forced convection" at the specimen surface increases the initial cooling rate, as Handley et al. (1981) clearly demonstrated. But if cryogen columns with a height of 10 cm or less are used for practical reasons and specimens with a diameter > 0.5 mm are frozen, such higher entry speeds do not offer a real advantage (Robards and Crosby, 1983): after standing still the specimen is not completely frozen and heat exchange between the warm center of the specimen

tainer as recommended by Elder et al. (1982) just before immersion (see again Fig. 4a). Finally it is very important, to freeze the specimen already on a carrier, which has sufficient mechanical stability together with a minimum heat capacity and fits into the chuck of the cryo-ultramicrotome used. Standard carriers ("pins", Fig. 4b) are not suited due to their relatively large heat capacity (see Ryan and Purse, 1984, or Ryan, 1992). A good compromise is given by the cone shaped hollow carrier developed for the FC-systems of Reichert-Leica (Fig. 4c). The wall thickness of this carrier is only 0.2 mm, the heat capacity according to the weight of 59 mg very small. Nevertheless the stability of the tube/cone design is absolutely sufficient for cryosectioning of very hard frozen specimens. Such carriers can be used for the application of microdroplets of suspensions or even for the mounting of small bulk samples with diameters less than 0.5 mm. By grinding with silica abrasive paper (finest grain size, control under the stereo microscope, see Fig. 4c) a small platform is produced for deposition of the microdroplet or the small specimen. Viscous cell suspensions or sticky tissue samples can be easily applied to such platforms on the tip of the cone under the stereomicroscope. Low viscosity cell suspensions in water or samples with smooth or rigid surfaces mostly do not adhere sufficiently to the ground platform. In such cases a coating of the rough aluminum surface with a viscous gelatine suspension is advantageous. As far as cell suspensions in water are concerned besides good adhesion an additional advantage of gelatine coating results, if a nearly dry gelatine coat is used: the gelatine in this case takes up liquid by swelling and enriches the cell concentration in the remaining surface layer without osmotic effects.
Figure 4. Essentials for immersion ("plunge") cryofixation and cryotransfer of plunge frozen specimens to the cryoultramicrotome. See Tables 3 and 4. Further explanations in the sections on "Ambient Pressure Freezing" and "Ultrathin Sectioning ...". (a) Plunging device (similar to Reichert KF 80 or CPC, see Sitte et al., 1987a, Fig. 1b, p. 90): a thermally insulating container IN is divided into two compartments by a cylindrical sleeve SL. The aluminum container AC inside SL is thermostatically heated by the cartridge HC. In AC a tube TU is inserted for the secondary cryogen CR (e.g., ethane), which is introduced through the capillary tube CT (connection TC to the container with pressurised propane or ethane). During liquefaction of the cryogen CR the level of LN$_2$ is lifted above h1 of SL, so that direct cooling of AC by LN$_2$ results. After liquefaction of a sufficient amount of CR (TU nearly completely filled) CT/TC are removed, LN$_2$-level again lowered below h1 and temperature of CR (AC/TU) set thermostatically slightly above its freezing temperature Tm (see Table 3). Immediately before immersion tube TU is lifted from h2 to h3, so that the specimen SP does not have to pass through cold GN$_2$. If larger bulk specimens with dimensions $\leq 0.5$ mm have to be frozen, a convection of the cryogen after stand still at the end of the movement is recommendable. Such a convection is realised either by a magnetic stirrer ST driven by a magnetic dipole MD connected with motor MO, or more efficient with a small light weight propeller PR on the injector rod IR. IR starts speedy revolution after the superficially frozen specimen reaches its final lowest position in tube TU. (b) Standard carrier SC ("pin") of a cryo-ultramicrotome normally made of aluminium: this carrier is only suited for sugar protected specimens according to Tokuyasu (see Fig. 16) and for mounting a part of the slam frozen tissue slice (see Fig. 23), but not suited for plunge freezing of unprotected fresh samples. (c) Hollow cone carrier HC offered by Leica-Reichert for FC-systems FC4/FCS/FCR: the wall thickness of approx. 0.2 mm guarantees a very low thermal capacity and conductivity. A groove GR enables handling with the forceps inside the plunging device and cryochamber. The pointed tip PT of the cone CO is carefully flattened with fine grain abrasive paper as shown in the encircled inset below. If non viscous liquids (e.g., suspensions in H$_2$O without protein content like blood plasm) have to be frozen, the cone must be coated with a "sticky layer" (e.g. gelatine GE) and only a very thin suspension layer has to be applied. Otherwise the suspension SU positioned on the ground platform PF is splashed away during speedy entry into CR. If larger bulk specimens with diameters exceeding 0.5 mm are to be frozen by immersion, a larger part of the cone has to be removed (see the right hand schematic diagram). In this case the hollow carrier HC' has to be filled with a low weight thermal insulator (e.g., polyurethane foam FO) to avoid entry of water into the cavity which would increase heat capacity. (d/e) Cryotransfer of HC is either realised in a small transfer container TR below LN$_2$, or just "dry" within GN$_2$ in precooled TR sealed with a precooled cover CV.
Table 4. Details influencing the quality of immersion ("plunge") cryofixation

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| **Cryogen**              | **Ethane** for all cryosectioning below devitrification temperature $T_d \sim -135^\circ C$ and if real vitrification is needed (analytical grade ethane is expensive, handling more complicated because of high pressure)  
**Propane** is not recommendable for low temperature work $< -135^\circ C$ with respect to residual surface films on the specimens. Approx. 25% lower cooling rates in comparison to ethane. There exists the risk of explosions due to incorrect handling (but less expensive and easier to handle)  
**Primary cryogens like LN2** are not suited under ambient pressure due to the "Leidenfrost phenomenon" (the same counts for partially frozen LN2 with respect to the small temperature difference between freezing and boiling temperature)  | See Table 3 and Sections 2 and 3 (this review), Bald, 1994, Ryan, 1992, Sitte et al., 1987a |
| **Cryogen temperature**  | Liquid cryogens are normally used at a temperature near the melting point $T_m$.  
Subcooled liquid cryogens below the melting temperature $T_m$ are more efficient, but mostly not recommendable (not stable: tendency to solidify)  | See Figs. 4 and 5 (this review) and Handley et al., 1981, Robards and Crosby, 1983, Robards and Sleytr, 1985, Ryan, 1992, Sitte et al., 1987a |
| **Cryogen container**    | Usually a **thermostatically heated container** of aluminium (wall thickness $\geq 5$ mm) with a height of $8$ to $10$ cm and a diameter of approximately $2$ cm guarantees reproducible temperatures without disturbing gradients. A height $>10$ cm does not pay. Stirring of the cryogen is not needed for small specimens (diameters $\leq 0.5$ mm) on low weight carriers  | See Figs. 4c and 5a (this review). See also Handley et al., 1981, Ryan and Purse, 1984, 1985, Ryan, 1992, Sitte et al., 1987a |
| **Specimen carrier**     | **Hollow cone carrier** or **4 µm-titanium foil sandwiches** deliver good results. Standard carriers ("pins" according to Fig. 4b) are not suited for unprotected highly hydrated biological specimens due to their high heat capacity (low cooling rates)  | See Figs. 4c and 5a (this review). See also Handley et al., 1981, Robards and Crosby, 1983, Robards and Sleytr, 1985, Ryan, 1991 and 1992 |
| **Immersion speed**      | For most specimens a **speed between 1.0 and 1.5 m/sec** is sufficient. Higher speeds up to $10$ m/sec offer only an advantage, if an aerodynamically shaped and very pointed edge is formed and if the sample is protected by a fairly rigid metal foil (e.g., $4$ µm-titanium) against the shock connected with the high-speed entry into the cryogen. Microdroplets on hollow cones according to Fig. 4c are mostly blown away at entry speeds approaching $10$ m/sec. Similarly sandwiches between planchettes according to Fig. 3b are not stable enough to withstand this impact. For sandwiches double jet freezing is a helpful alternative in most cases  | See Figs. 4c and 5a (this review). See also Handley et al., 1981, Robards and Crosby, 1983, Robards and Sleytr, 1985, Ryan, 1991 and 1992 |

and the surrounding areas results. Usually such phenomena cannot be avoided completely by a magnetic stirrer, since the relatively slow convection of the liquid cryogen is not comparable with the "forced convection" occurring by an entry speed of e.g., $10$ m/sec as used by Handley and co-workers. Only in such exceptional cases remarkable differences can be observed between slow and rapid movements of the sample (see legend of Fig. 5). Summarising all discussed facts one can conclude, that immersion cryofixation of fresh specimens for subsequent cryosectioning should be made under the experimental conditions listed in Table 4 in order to get optimal results.

The elegant spray-freezing method according to Bachmann and Schmitt-Fumian (1973, see also Fig. 3c) is a special modification of plunge freezing of
Figure 5. Under certain preconditions the results of immersion ("plunge") cryofixation depend on the speed of the specimen. See Handley et al. (1981), Sitte et al. (1987a). Further explanations in the section on "Ambient Pressure Freezing". Electron micrographs (b) and (c) reproduced with permission. (a) Specimen set-up of Handley et al. (1981): a blood sample BS is inserted between a V-shaped 4 µm thick titanium foil TF, mounted in a rigid low weight holder HO. By a spring loaded device HO/TF/BS is moved with adjustable speed into a secondary cryogen CR (Handley et al. used subcooled Freon 22 at -164°C). (b) Results obtainable with normal immersion speed of 1.25 m/sec. Arrow CP and dashed line show the position of the contact plane between CR and TF. Note the rapidly increasing ice segregation by insufficient cooling rate clearly visible in the red blood cells RB. Frozen suspension BS freeze substituted in OsO$_4$ acetone according to Van Harreveld and Crowell (1964) and embedded in epoxide. Ultrathin sections stained with uranyl acetate. (c) Cooling rate considerably enhanced by extremely high entry speed (10 m/sec) due to "forced convection". Note the absence of visible segregation artefacts in a border layer of approx. 20 µm. The conditions and results correspond closely to double jet cryofixation of sandwich specimens. It makes no sense to shoot larger bulk specimens with such a speed into a liquid cryogen of a height h$_2$ ≤ 10 cm (see Fig. 4a), since without special measures after stand-still rediffusion of heat causes secondary phenomena and changes the well frozen layer.

Figure 6. (on facing page) Slam freezing followed by freeze substitution in OsO$_4$/acetone and epoxide embedding ("hybrid method") according to Van Harreveld and Crowell (1964) is a very fast procedure, which delivers results already over night. This hybrid method is very well suited for morphology and morphometry on resin blocks sectioned at ambient temperature on a standard ultramicrotome. It is often not suited for histochemistry since OsO$_4$ tends to damage a lot of antigens. One of the criteria for a good freezing quality (extremely fast cooling rate) is the dense appearance and the sharp delineation of heterochromatin (arrows) in the nuclei of slam frozen samples. In both cases (a) and (b) the nuclei are located in a distance of approx. 7 µm from the border CP of the samples (contact area between sample and metal mirror plane). Both samples are frozen on a simple LN2 cooled copper mirror without vacuum protection. Sections are stained as usual with uranyl acetate and lead citrate. Further explanation in the sections on "Freezing and the Frozen State of Water", "Ambient Pressure Freezing" and Discussion. See also description and discussion in Sitte et al. (1987a). (a) Frog sartorius muscle frozen on the inverted slammer according to Edelmann (1989b). Specimen and electron micrograph courtesy of L. Edelmann. (b) Neutrophil granulocyte frozen on Reichert MM80-system according to Fig. 7. Specimen and electron micrograph courtesy of E. Morgenstern.
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Figure 7 (on facing page). Air damped slamming device with an LN₂ cooled metal mirror similar to the Leica-Reichert MM80. See also Figs. 3d, 7d, 8 and 9. Further explanations in the sections on "Freezing and the Frozen State of Water", and "Ambient Pressure Freezing". (a) Schematic diagram of the whole apparatus: the thermally insulated cryochamber CC within the base is partially filled with LN₂, which cools directly the aluminium cylinder AC with the metal block MB (mirror-like polished surface MM) to a temperature of approx. -190°C. After pushing the release button RB (arrow) the injector rod IR moves downward. The movement is accelerated by an adjustable helical spring HS to values between 1.5 and 2.5 m/sec at the impact of the specimen (e.g., tissue slice TS) on the mirror plane MM. Bouncing is suppressed by minimum inertia of the hollow cylinder HC sliding in IR and by "air bag" AB between IR and HC (see "b" and Fig. 8). (b) Position of HC just before first contact between tissue slice TS and MM: since IR due to inertia continues the downward movement, air inside AB becomes increasingly compressed and starts to escape through the holes HO (arrows). The specimen carrier consists of a stainless steel plate SS with a foam support FS for the specimen (tissue slice TS). The extremely thin separation foil SF of polypropylene (the thinner the better) between FS and TS facilitates the separation of the specimen from the foam support after freezing. The loaded specimen carrier SS/FS/SF/TS is simply attached to the magnetic plate MP on the bottom of the injector IR. (c) View from top on the mirror plane MM of MB after removal of the frozen tissue TS: the borderline of TS is clearly visible by a grainy frost layer GF formed by the water vapour escaping from the side walls of the warm specimen immediately before freezing. Similar frost GF² is deposited, if a bubble of GN₂ was trapped between the specimen TS and MM. (d) The corresponding complementary contact plane to (c) after turn of the specimen TS by 180° around horizontal axis BB shows a grainy surface GS on the area formerly covered by the GN₂ bubble. The remaining surface MS is a mirror-like shiny replication of MM. (e) Specimen TS broken along the line BB in schematic drawing (d) in side view shows bright reflections BR of hexagonal ice crystals and the dark amorphous border layer AB on the well frozen mirror replication surface, if intense dark field illumination by fibre optics and sufficient magnification of the stereomicroscope are used. The dark border layer AB of the amorphously frozen (vitrified) contact area with MM disappears completely in the area GS formerly covered by the GN₂ bubble. (f) Compression phenomena according to Fig. 9 can be reduced by a carefully adjusted spacer ring SR around the specimen SP placed on propylene separation foil SF. The specimen SP or a suspension is either mounted inside SR or positioned on an intermediate layer IL (e.g., liver homogenate, blood plasma, soft gelatine or β-gel according to Hanyu et al., 1992). Suspensions can be directly filled into SR instead of SP/IL. The optimum height h and consistency of SR and the best intermedium IL have to be found empirically by trial and error for each specimen. Often thin (e.g., h ≤ 0.5 mm) plastic foils with punched holes (diameter approximately 5 mm) are the best solution for slamming of suspensions (Warning: it is not easy to establish the best conditions, but this is the only way to obtain success reproducibly!).

H. Sitte

Microdroplets. To the best of my knowledge this method has not yet been used for subsequent cryosectioning: spray-freezing is therefore not included in this discussion, but probably remains an alternative worthy of consideration.

Immersion cryofixation is characterised by a travelling specimen which enters into a resting cryogen. In jet freezing (sometimes also called "spray-freezing", see e.g., Bald, 1985) the conditions are just the inverse: the specimen rests in a firm position and a liquid cryogen is moved. Using one or two jet nozzles and an extrusion of the cryogen by a rather high pressure much higher relative speeds "cryogen vs. specimen" are possible, than with immersion systems. This thermodynamic advantage is coupled with the drawback that the specimens have to be protected from the mechanical impact of the cryogen jet by protection foils. Of course these foils enhance the heat capacity and diminish the cooling rates, if they are not extremely thin (Handley et al., 1981; Ding et al., 1992). Jet freezing was used since 1978 mainly for freeze fracturing (M. Müller et al., 1980; Plattner and Knoll, 1983) and sometimes in studies using hybrid methods like freeze substitution or freeze drying. According to my own knowledge it has never been used for subsequent cryo-ultramicotomy. But it may be useful in future, since the recent generation of double jet systems (I refer exclusively to the Balzers JFD 030 - Jet-Freezer according to the principle of M. Müller et al., 1980) work really reproducibly and reach evidently extremely high cooling rates (see Th. Müller et al., 1989). Such double jet systems take advantage of the bi-directional heat extraction according to Fig. 3b. The precondition for this bi-directional heat extraction seems to be an exact synchronisation of both jet streams on a really thin specimen, which was according to obtainable second-hand information mostly not guaranteed by earlier or other double jet systems. According to Ding et al. (1992) the JFD 030 system "vitrified" plant material nicely within a thickness of
60 µm, if two titanium sandwich foils of minimum thickness (4 µm) in analogy to Handley et al. (1981) are used. Since Ding et al. used only freeze substitution, no evidence of real vitrification to I, could be shown, but in agreement with other reports and results one can expect a proper freezing. Although the gluing of the frozen sandwich onto a specimen carrier for cryo-ultramicrotomy (see the section on "cryo-ultramicrotomy") may be tricky it seems to be a promising alternative to immersion or impact cryofixation if one enters into the development of a suitable follow-up technology. This follow-up procedure for cryosectioning must include at least the elimination of propane traces (e.g., by liquid ethane) and a tight mounting of the thin frozen lamella (thickness approximately 60 µm) on a stable carrier similar to the gluing procedure for impact frozen layers on standard pins (see Fig. 23).

Probably the most powerful but also certainly the most tricky method for rapid freezing is the impact cryofixation, usually denominated as "slamming" or "metal mirror cryofixation". In this case heat extraction results from the contact between the specimen and a highly polished mirror-like solid state surface. This cooling method was already tried by Altmann in the last century without considerable success. It was applied by Eränkö 1954 for histochemistry and finally introduced by Van Harreveld and Crowell (1964) into electron microscopy. Most electron micrographs of slam frozen specimens were obtained with hybrid techniques (freeze substitution, freeze drying, resin embedding at ambient or low temperature, see e.g., Armbruster et al. (1984), Carlemalm et al. (1982, 1986), Edelmann (1989a,b, 1991a,b, 1994a,b), Hobot et al. (1985), Hummel and Müller (1986), Menco (1986), Sitte et al. (1988, 1994), Steinbrecht and Müller (1987), but also successful application for subsequent cryo-ultramicrotomy was demonstrated in a couple of papers (e.g., Edelmann, 1992; McDowall et al., 1983). The results are often fascinating (see e.g., Heuser et al., 1979; Sitte et al., 1987a, p. 96; Van Harreveld and Crowell, 1964), the drawbacks and difficulties mostly not known because they are not described: frustration of beginners reaches therefore a
rather high level. Besides the power and the specific advantages therefore mainly the diversity of drawbacks of this elegant, absolutely recommendable and necessary method will be mentioned. Additionally some current misunderstandings concerning slam freezing will be explained and corrected.

Not to discourage all prospective applicants I want firstly to mention the striking advantages and the necessary instrumentation. Mirror freezing is really the only method which is suited for larger specimens. This concerns both the surface area for rapid freezing and the total volume of the sample. Areas around 1 cm² can be frozen without severe problems, e.g., tissue slices in thicknesses between 0.1 and 1.0 mm (the thinner the better) sectioned either with a slicer (e.g., a modified Stadie-Riggs slicer, see Sitte et al., 1987a, p. 86, Fig. 4d) or with a vibratome (Bold, 1995). But also larger pieces of tissue e.g., a segment of a kidney or liver with a volume of 1 ml or even more can be frozen on the metal mirror. The reason, why this is possible contrary to all other rapid freezing methods is simple: the heat conductivity of the just frozen border layer of the specimen is extremely low in comparison to the excellent heat conductivity of the mirror material (usually copper). Since the heat capacity of the copper block in comparison to the sample is extremely high, the temperature of the border region of the copper mirror remains practically unchanged during the whole freezing procedure, that means around -190°C for an LN₂-cooled copper block. This is completely different from immersion cryofixation, jet and high pressure freezing, which all need very small specimen dimensions around 0.1 mm at least in one dimension. For some studies larger areas or volumes are of paramount importance: this concerns all cases, where with respect to changes of the object extremely speedy preparation is needed (e.g., kidney cortex, central nervous tissue) or where dissection of a sensitive specimen is not possible without severe mechanical damage (e.g., many soft tissues, which disintegrate if dissected into small pieces).

Similar to measurements during plunging, correct measurements of cooling rates at the border between specimen and mirror plane during slam freezing are not possible. But measurements in some distance from the border (Escaig, 1982, 1984; Escaig et al., 1977) and results tell us, that slam freezing enables extremely high cooling rates - probably the highest obtainable. By immersion cryofixation in our laboratory (jet freezing was not available and is not considered) we could never obtain the same beautiful preservation of chromatin and other sensitive structures (see Sitte et al., 1987a) as with slam freezing. These pictures (see Fig. 6) result obviously from extremely high cooling rates and could be obtained on LN₂-cooled copper mirrors with simple apparatuses (see Sitte et al., 1987a; Edelmann, 1989b). Fig. 7a shows the principle of one of the used instruments, which is basically identical with the Reichert KF 80/MM 80 (new version CPC/MM 80). The copper block in those instruments is directly cooled by LN₂. The specimen is mounted onto an air damped low weight injector (Fig. 7b), which excludes or at least diminishes bouncing (see Boyne, 1979; Phillips and Boyne, 1984). Following release of the injector the specimen approaches finally with a speed between 1.5 and 2.5 m/sec (adjustable) against the mirror plane. A simple return stop element prevents a reverse upward motion of the injector after the first contact between specimen and mirror. Finally: the pressure of the injector system with the specimen against the mirror surface is increased automatically after the first contact and superficial freezing with a delay of approximately 5 msec. The border layer is therefore already frozen before this pressure is developed. This measure prevents distortion (bending) of thicker specimens by one-sided cooling, which can result in a partial separation of the specimen surface from the mirror plane and a subsequent recrystallisation of those areas.

In practical use, some specific peculiarities have to be regarded. For example: a gas bubble (GN₂) can be trapped between the mirror and the specimen surface. This concerns especially larger areas exceeding 50 mm². Areas of such gas bubbles are easily recognised by frost deposition on the mirror surface and a grainy appearance on the corresponding complementary surface of the frozen sample as shown in Fig. 7c and d. After complete freezing (finished after 15 sec or less for tissue slices in thicknesses < 1 mm) the frozen specimen is separated from the mirror and from its support and turned upside down. Both complementary areas on the specimen and on the mirror plane are inspected carefully with the stereomicroscope using a bright cold illumination. The former contact area on the mirror surface is surrounded by a grainy frost layer resulting from water evaporation from the side walls of the warm specimen to the cold mirror immediately after the first contact between specimen and mirror. If gas was trapped by the specimen surface, those areas show the same grainy frost, too. The well frozen specimen surfaces represent a perfect mirror-like reflecting replica of the highly polished mirror surface. Only non-contacting areas due to gas inclusions have a grainy appearance which is completely different from the shiny replication and cannot be overlooked. Such areas have to be discarded since they are badly frozen and always show severe segregation artefacts. Another control of the quality of
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Figure 8. "Bouncing", i.e., repelling of a soft specimen SP from the rigid metal mirror surface MM is a common problem of slamming devices for impact cryofixation of biological samples. It can be recorded according to Boyne (1979). Further explanation in the section on "Ambient Pressure Freezing". (a) Set-up consisting of a voltage source VO connected by an extremely thin flexible wire WI to the surface of the specimen SP (see Fig. 7) and with the metal block MB via resistances R1 and R2. Recording of resistance or voltage with oscilloscope OS. (b) The oscilloscope OS registers a first contact C1 between wire WI and metal mirror MM at t1, followed by a separation due to bouncing (repelling) till C2 at t2 and so on ... C3 ➔ C4 ➔ • • • Cn. Between the very short periods of contact there are periods of separation in the msec-range. It is understandable, that under such preconditions optimum freezing is not possible. It is therefore worth checking any slamming device with the specific set-up of specimen and specimen support and to change all influencing parameters (e.g., speed of application, return stop, specimen support, spacer arrangement, specimen geometry) empirically by trial and error up to the moment that bouncing is reproducibly suppressed. This procedure "contact - interruption - contact - interruption..." (Fig. 8) is repeated several times in the msec-range. Finally the specimen remains in a continuous contact with the mirror plane. Since freezing of the border layer occurs in fractions of a msec this bouncing phenomenon may provoke several "freezing - thawing - freezing..." or "freezing - recrystallisation" procedures. Structural preservation by such a bouncing slammer is bad - and many systems bounce against all precautions and expectations. It is therefore worth checking each system, that bouncing is properly suppressed before use according to Fig. 8 and to modify the parameters (speed of application, specimen support, specimen geometry, return stop, spacer elements etc.) before the start of experimental work. This check has to be repeated if one of the important parameters is changed (e.g., freezing of another object with different consistency or geometry). According to my own experience and advice from literature and colleagues, pneumatic systems (see e.g., Boyne, 1979; Sitte et al., 1987a; Trachtenberg, 1993) have the best damping conditions and are to some extent bounce-proof.

The most severe drawbacks of slam freezing are bouncing and specimen compression. Both depend among other influences on the system used and the mounting of the specimen on the injection device. Boyne (1979) first observed and analysed the absolutely unexpected bouncing phenomenon with a simple set-up. He found, that the specimen after the first contact with the mirror is usually repelled, that means, that the contact between object and mirror plane is again interrupted. This procedure "contact - interruption - contact - interruption..." (Fig. 8) is repeated several times in the msec-range. Finally the specimen remains in a continuous contact with the mirror plane. Since freezing of the border layer occurs in fractions of a msec this bouncing phenomenon may provoke several "freezing - thawing - freezing..." or "freezing - recrystallisation" procedures. Structural preservation by such a bouncing slammer is bad - and many systems bounce against all precautions and expectations. It is therefore worth checking each system, that bouncing is properly suppressed before use according to Fig. 8 and to modify the parameters (speed of application, specimen support, specimen geometry, return stop, spacer elements etc.) before the start of experimental work. This check has to be repeated if one of the important parameters is changed (e.g., freezing of another object with different consistency or geometry). According to my own experience and advice from literature and colleagues, pneumatic systems (see e.g., Boyne, 1979; Sitte et al., 1987a; Trachtenberg, 1993) have the best damping conditions and are to some extent bounce-proof.

In contrast to the completely unexpected bouncing phenomenon, everybody was aware that considerable compression or distortion artefacts will occur, if a soft specimen hits the rigid mirror plane. If the specimen
Figure 9. Compression (schematical drawings "a" and "b") and ruptures (electron micrograph "c") by slam freezing. Further explanations in the section on "Ambient Pressure Freezing". (a) Against former expectations according to Bold (1995) the most severe compression artefacts occur just in the well frozen border layer of the specimen SP, that means in the immediate vicinity of the contact plane between SP and metal mirror surface MM on the metal block MB. The deformation of originally spherical inclusions SI change these elements to rotational ellipsoids SI' of identical volume. The extent of compression depends on size and rigidity of the structures: smaller structures like mitochondria or tiny vesicles show less deformation than larger inclusions. Structures with some kind of a skeleton like nuclei or small cells are more resistant against compression than free membrane systems without stabilising skeletons. Deeper layers show less compression than the border layer near the contact plane between specimen SP and the metal mirror MM. (b) According to Bold (1995) different structures within the same area (e.g., an outer envelope OE including small vesicles SV, original size before slamming in the upper half of the diagram) are compressed in different extents to OE' and SV' after slamming (lower half of diagram). They behave like a collection of rigid golf balls in a thin walled balloon. For example glomerula of the kidney cortex are deformed according to this schema. (c) Ruptures of the plasmalemma of single cells in suspension, which look like explosions in the direction of the pressure wave are not necessarily an artefact generated by the impact of the suspension on the metal mirror as argued formerly (see Sitte et al., 1987b; DA = Damage of plasmalemma, EP = exit of cell plasma, RC = red blood cell). A recent study of Frei (1992) gave some support that such "eruptions" may be a result of the freezing process itself, which runs also in the direction of the arrow. The electron micrograph shows a slam frozen human red blood cell in human blood after freeze substitution in OsO₄-acetone according to Van Harreveld and Crowell (1964), followed by epoxide embedding. Ultrathin section stained with uranyl acetate and lead citrate. Electron micrograph courtesy of E. Morgenstern.

The situation is somewhat confusing, since different structures are deformed to a different extent (see Fig. 9b). Generally smaller structures in the \( \mu \text{m-range} \) (e.g., granules, vesicles, mitochondria, ER-profiles, protocytes) or very rigid structures with a skeleton (e.g., nuclei, even some small cells) show less compression than larger structures. For example, the mainly spherical glomeruli in the kidney cortex are much more compressed than the smaller structures within or around those glomeruli. The situation corresponds to a collection of small rigid golf balls suspended in a thin walled balloon: if forces are applied, only the balloon is deformed - the small balls do not change their shape considerably. But the spatial relationships of the smaller spheres are completely altered. This should always be considered if mirror frozen...
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specimens have to be investigated.

Finally, sometimes after slam freezing in cell suspensions defects on the outer cell membrane (plasmalemma) are observed, where cytoplasm exits (Fig. 9c). These openings or "eruptions" are always located on the cell surface opposite to the contact area between suspension and metal mirror. It was therefore argued, that this phenomenon is a result of the shock wave generated by the impact of the suspension onto the rigid metal mirror surface (see Sitte et al., 1987a, Fig. 7 on p. 102). In the meantime this phenomenon was reinvestigated by Frei (1992). There are strong indications, that those ruptures are created by the freezing process itself, which has obviously the same direction as the pressure wave created by the impact shock. Further studies of this phenomenon including other freezing methods (e.g., sandwich freezing by jet or high speed immersion according to Handley et al., 1982) are needed, before definite statements concerning the generation of these artificial ruptures are possible.

At least the discussed phenomena of bouncing and compression are inherent to slamming. Concerning bouncing there is only a gradual difference between different systems, e.g., reduced bouncing tendency due to pneumatic damping or the application of β-gels according to Hanyu et al. (1992). The most important influence on compression phenomena is possible by a careful individual adaptation of spacer elements and support arrangements for each specimen: it seems that different specimens behave completely differently. It is also difficult to answer the question, which commercially available mirror freezer is the best or most convenient for a specific purpose. Probably the best will be to check different systems with one's own specimens of interest. In contradiction to several claims and calculations, our own practical experience and published pictures tell us that results obtainable with LHe cooled mirrors are not substantially better than results with LN₂ cooled mirrors [see in this respect the papers of Bald (1983, 1985), Escaig (1982, 1984), and Wendt-Gallitelli et al. (1980)]. Recently Hanyu et al. (1992) reported about an improvement by a 2.45 GHz microwave irradiation of the sample starting 50 msec before the first contact between specimen and mirror plane. They believe, that this irradiation before and during the freezing procedure may crack H₂O-pentamers responsible for homogeneous nucleation. Also this report does not allow a correct comparison and a definite conclusion on the benefits of that somewhat complicated set-up. Up to now all the results presented with LHe, vacuum and microwave technology does not differ significantly from our own results obtained with the above mentioned simple LN₂ systems according to Sitte et al. (1987a), or Edelmann (1989b), working without LHe, without vacuum and without microwave irradiation. But there may be some potential to improve the situation for the already mentioned surface layer of 5 to 10 μm, which can be important, if true ambient pressure vitrification is wanted with respect to cryo-ultramicrotomy of unpretreated samples frozen with an extremely high cooling rate above 10⁶°C/sec.

Concerning the other extreme: simplification of the slamming procedure by do-it-yourself instruments with a home made copper block according to Dempsey and Bullivant (1976) looks extremely convincing but is extremely frustrating in comparison with a commercial slammer. According to our own experience only in exceptional cases we could obtain useful results in this way, probably because a continuous sufficient contact between the mirror block and the specimen holder, operated with the right and left hand of the same
person, was not possible (see Sitte et al., 1987a, Fig. 4a, p. 96).

High Pressure Freezing

The basics of this method have already been discussed (see last part of the section "Freezing and the Frozen State...."). High pressure freezing was introduced by Moor and Riehle already in 1968 [see also Hunziker et al. (1984), Kaeser et al. (1989), Moor (1971, 1973, 1987), Moor and Hocchli (1970), M. Müller and Moor (1984), Riehle (1968), Studer et al. (1989)]. After long years of development and experiments the first commercial high pressure freezer of Balzers became available around 1985. In the meantime two slightly different high pressure systems are on the market [see M. Müller and Moor (1984), Sartori et al. (1993), and Studer et al. (1989, 1995)] and a lot of experience has been accumulated. Since 1985 this principle was successfully used to vitrify biological samples for subsequent cryo-ultramicrotomy and frozen-hydrated investigation in the TEM [see e.g., Hohenberg et al. (1994), Michel et al. (1991, 1992), Studer et al. (1995)] as well as preparations with hybrid methods (see e.g., Allenspach (1993), Hippe et al. (1989), Hippe-Sanwald (1993), Hohenberg et al. (1994), Hunzicker et al. (1994), Studer et al. (1989)]. In many respects high pressure freezing opened up a new era in frozen-hydrated work since for the first time homogeneously vitrified samples for cryosectioning were available. Within a short time it turned out, that comparatively large flat and astonishing perfect cryosections were much easier obtainable with such pressure frozen specimens (see Figs. 10 and 11).

The high pressure system is not as complicated as mostly expected (for physical and technical details see e.g., Moor (1987), and M. Müller and Moor (1984). The hydraulic generation of the pressure of 2 kbar by pistons represents standard pressure technology. The most difficult problem was correct synchronisation of the pressurisation and the cooling procedure, since it became evident that such a high pressure produces within short times artefacts in living systems [see e.g., Ding et al. (1992), Moor and Hoehli (1970), Moor et al. (1980), Studer et al. (1995)]. Firstly the pressure of approximately 2 kbar has to be developed by the hydraulic system to ensure the full reduction of $T_a$ and $T_b$ (see Fig. 1a) for subsequent freezing. Immediately after the pressurisation the rapid cooling by LN$_2$-double jet must start. Heat extraction needs some time - more time in the center of the specimen than in a border region, even if the LN$_2$-double jet starts immediately after complete pressurisation. A

**Figure 10 (on facing page).** Martin Michel obtained the first beautiful flat cryosections free of artefacts from high pressure frozen discs (diameter 2 mm) of Golden Delicious apple leaves. Sometimes good cryosections were obtained with H2O-coated balanced broken glass knives (Michel et al., 1991), but better reproducible cryosections were achieved with diamond knives (Michel et al., 1992) using an ioniser. The electron micrographs (a) and (b) of the amorphous frozen-hydrated cryosections show representative areas of such cells: VA = vacuoles filled with a network of thick strands are existent besides other vacuoles VA' without this internal structures. CH = chloroplasts with the typical compound membranes of the thylacoids TH, NU = nucleus, MI = mitochondrium, ER = endoplasmic reticulum. The nuclear envelope NE shows the well known pore complexes (arrows). The cell walls CW are well sectioned. All sections show a severe compression up to 50 % perpendicular to the direction of sectioning (arrows in circles). The most astonishing fact is the nice contrast of the biomembranes, ribosomes and other components without any heavy metal staining. Sectioning with Reichert Ultracut/FC-system with automatic mode: cutting speed 0.4 mm/sec, maximum return speed, minimum sized cutting window and cycle length, advance setting 80 nm, chamber kept at the lowest possible temperature near -170°C. Electron micrographs: Zeiss EM 902 EFTEM with ESI, cold stage temperature approx. -170°C; Bar: 1 µm. Preparation and electron micrographs by Martin Michel, Electron Microscopy I, ETH Zürich. Reproduction with permission. Further explanation in the sections on "High-Pressure Freezing", Cryo-Ultramicrotomes", "Ultrathin Sectioning ..." and "... Frozen Hydrated Ultrathin Sections". See also Figs. 11, 12, 17, 24, and 25.

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**Figure 12c** shows the sandwich elements for high proper synchronisation of pressurisation and cooling is therefore of great importance. This synchronisation is achieved in an elegant and very simple way by a certain amount of alcohol introduced into the tubing, which later on guides the LN$_2$ to both jet nozzles (see Fig. 12). If pressurisation starts, for 15 msec 2 ml of ethanol or isopropyl alcohol at ambient temperature is shot against the specimen container during the time that pressure is built up. With the delay of these 15 msec after the alcohol LN$_2$ enters through the nozzles into the pressure chamber and hits the specimen container from both sides. As mentioned before, LN$_2$ under this pressure does not boil and is therefore an excellent cryogen with a high specific heat and an extremely low viscosity (see Table 3).
Figure 11. Frozen-hydrated cryosection of amorphously frozen bovine articular cartilage after high pressure freezing. Preparation and electron micrograph similar to Fig. 10. The diffraction pattern (insert) of this area shows the characteristic diffuse fringes of vitreous ice IV. Chondrocyte CC with profiles of the granular endoplasmic reticulum gER (arrow) and a vacuole VA in the cytoplasmic matrix. Adjacent to the plasmalemma PL (arrow) of the chondrocyte CC the extracellular matrix EM with its typical ultrastructure only visible after perfect freezing to I. Sectioning direction: arrow in circle. Bar: 1 µm. Section preparation and electron micrograph by Martin Michel, M.E. Müller Institut für Biomechanik, Universität Bern, Switzerland. Reproduction with permission. Further explanation in the sections on "High-Pressure Freezing", "Cryo-Ultramicrotomes", "Ultrathin Sectioning ..." and "... Frozen Hydrated Ultrathin Sections". See also Figs. 10, 12, 17, 24 and 25, and Studer et al. (1989, 1995).

Pressure freezing in cross section diagrams. To guarantee an efficient heat extraction from the specimen to the outer wall, the space between those elements according to Studer et al. (1989) is completely filled with an inert medium of low viscosity (1-hexadecane). The same counts for cavities filled with gas inside the specimens [e.g., plant leaves; see Michel et al. (1991)], since air bubbles, due to bad heat conductivity and compression during pressurisation, reduce the yield of useful preparations considerably. With respect to a perfect freezing of normal biological specimens, the thickness of the sample should not exceed 0.1 mm. Only specimens with a good internal cryoprotection or an unusual low water content can be frozen in thicknesses up to 0.6 mm. The high pressure systems therefore allow an adjustment for thicknesses between 0.1 and 0.6 mm in steps of 0.1 mm by combination of two different sandwich elements. The circular discs of
Figure 12. Simplified schematic diagrams of the basic design and function of the Balzers and Leica high pressure systems for cryofixation, which are basically very similar. See Moor (1987); M. Müller and Moor (1984); Studer et al. (1989) Further explanations in the sections on "Freezing and the Frozen State of Water..." and "High Pressure Freezing". See also Figs 10 and 11, and Table 5. (a/b/c) Design of the high pressure part for freezing (front view "a" sectioned in plane S2, top view "b", sectioned in plane S1, specimen holders "c"); the specimen carrier SC holds the sandwich elements SE1/SE2. SC is inserted into the pressure chamber PC and held by the transverse bolt BO. The LN$_2$ tubes NT/NT' above valve VA1 are filled with alcohol AL from container CO through valve VA2. In this condition the system is ready for a freezing cycle, which can be started by push button operation and runs then completely automatically in the following sequence: LN$_2$ is introduced with a pressure $> 2.1$ kbar through pressure tube PT and valve VA1 (valve VA2 closes automatically) the alcohol AL in NT/NT' first hits the sandwich elements SE1/SE2 (initial period of approx. 40 msec) LN$_2$ enters through the nozzles NO/NO' the specimen is cooled to $-100^\circ$C within 13 msec, if the preselected thickness of the specimen in the sandwich chamber SE1/SE2 is $t = 0.1$ mm (see "c" and Table 5) the excess alcohol AL and nitrogen (GN$_2$) escape through the narrow apertures AP/AP' of the pressure chamber PC (see "b"). The whole procedure is finished within a period $<0.5$ sec. The system allows approximately 40 freezing cycles per hour. Each cycle needs $\leq 0.2$ liter LN$_2$. The thickness t of the specimen in the sandwich chamber (see "c") can be adjusted by combination of the different sandwich elements SE1 and SE2 between $t = 0.1$ mm and $t = 0.6$ mm in increments of 0.1 mm. Complete vitrification of unprotected highly hydrated specimens SP is only reproducibly possible with $t = 0.1$ mm (see Table 5). (d) Synchronisation of pressurisation and cooling: due to the small amount of alcohol AL at ambient temperature in the nitrogen tubes NT/NT' pressurisation from 0 to 2 kbar started at t1 is achieved without cooling. Cooling starts at t2 when subsequent LN2 hits the sandwich SE1/SE2. At t3 temperature of $-100^\circ$C is reached.
Table 5. Cooling rates in the centre of samples during high pressure freezing depend on the thickness $t$ of the specimen in the sandwich container according to Fig. 12c. The same counts for the time needed for complete freezing of the sample which may be responsible for pressure artefacts. Values according to Studer et al. (1995) and leaflet Leica EM HPF (6/94). See also Fig. 12.

<table>
<thead>
<tr>
<th>Total Thickness of the Sample +)</th>
<th>Cooling Rate in the Centre of the Sample</th>
<th>Time Needed for Complete Freezing of the Sample to -100°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µm</td>
<td>10,000 - 20,000 °C/sec</td>
<td>13 msec</td>
</tr>
<tr>
<td>200 µm</td>
<td>4,000 - 6,000 °C/sec</td>
<td>50 msec</td>
</tr>
<tr>
<td>600 µm</td>
<td>400 - 600 °C/sec</td>
<td>450 msec</td>
</tr>
</tbody>
</table>

+) Including intermedium (e.g., 1-hexadecane). Value corresponds to thickness "$t$" of the specimen in the sandwich chamber SE1/SE2 according to Fig. 12c.

With the overall thickness (specimen and intermedium, e.g., 1-hexadecane) of 0.1 mm the cooling rate in the center of the sandwich chamber remains between 10,000 and 20,000°C/sec. If the overall thickness is enhanced to 0.2 mm, this value drops already down to 4,000 to 6,000 °C/sec. With the formerly used value of 0.6 mm the rate in the center region is only around 400 to 600 °C/sec. Table 5 shows these values in comparison with the periods needed for complete freezing of samples in different thicknesses. True vitrification of normal biological samples is only possible with cooling rates between 10,000 and 20,000 °C/sec even under the optimum pressure of 2.1 kbar (Studer et al., 1995). This has to be considered especially if hybrid follow-up procedures are used, which do not allow checking of the frozen-hydrated state by electron diffraction patterns.

**Cryo-Ultramicrotomes**

Probably Fernández-Morán in 1951 was the first who tried ultrathin sectioning at low temperatures for the study of cell structures and element analysis in the electron microscope with some success [see also e.g., Robards and Sleytr (1985), Roomans et al. (1982), Roomans and Shelburne (1983), Sitte (1982), Sitte and Neumann (1983), Wendt-Gallitelli and Wolburg (1984), Zierold (1982, 1987)].

In the mid-sixties Wilhelm Bernhard used the cryostat technology of Linderström-Lang and Mogensen (1938) [cited by Pearse (1961) for studies on macromolecular cytochemistry with the TEM]: he simply installed a Porter-Blum ultramicrotome in a deep freeze box and sectioned cryoprotected material at temperatures around -40°C with remarkable results (Bernhard and Nancy, 1964; Bernhard, 1965, Bernhard and Leduc, 1967; Bernhard and Viron, 1971). On his recommendation LKB together with the group around Appleton at Cambridge UK developed a quite perfect cryostat system for cryosectioning at temperatures down to -80°C (Appleton, 1974). In the late sixties Dollhopf together with Hodson and Marshall in London (UK) and completely independently Christensen together with Blum in the USA introduced small cryochambers with a volume of approximately 1 liter [see Fig. 13 and Dollhopf (1968), Dollhopf and Sitte (1969), Christensen (1969, 1971), Blum (1970), Sitte (1982)]. Shortly afterwards LKB introduced a similar system ["LKB CryoKit", Persson (1972)] and for the first time a set of special "cryotools" for a more convenient and efficient handling of dry cut frozen sections for element analysis [Sevêu, 1977; Barnard and Sevêu, 1977]. The new cryochambers surrounded only the area of knife and specimen. They made it possible to...
Figure 13. Physical basics and different designs of cryochambers for cryo-ultramicrotomes. Further explanations in the section on "Cryo-Ultrotomes". See also Figs. 14, 15 and 17. (a) Simple cryochamber CC with a thermally insulating wall, partially filled with LN₂. The boiling LN₂ delivers continuously GN₂, which fills the chamber CC completely and flows away over the wall (arrows). Since cold GN₂ is considerably denser than room atmosphere, air and GN₂ do not mix, but form a very stable border layer BL similar to a phase border liquid/gas ("GN₂ lake"). Temperature sensors show, that within a few millimetres of height the temperature changes from $T_a < -100°C$ to $T_b > +10°C$. This physical behaviour is essential for cryo-ultramicrotomy in small chambers: since the temperature inside CC is very low and stable, room atmosphere cannot enter into CC and frost formation is completely prohibited similar to an open deep freeze in a supermarket. (b) Situation changes completely, if a hole HO is drilled in the wall CC' of the cryochamber: the dense LN₂ flows away through HO and the border layer between GN₂ and room atmosphere changes to a lower level BL'. The former cold wall of CC (see "a") now shows disturbing frost formation FF. The problem in the design of cryochambers is therefore to inhibit the outflow of cold GN₂ through the chamber wall and to introduce the specimen arm of the ultramicrotome in a suited manner. (c) Dollhopf found the first suited solution: the specimen arm SA with the specimen SP was introduced through the chamber wall CW. The hole HO was sealed by an extremely thin flexible foil TF mounted on the rear wall of the chamber and on the specimen arm SA (CW mounted on knife support KS, knife KN mounted on the bottom of CW). (d) Christensen and Blum found an alternative with the bridge BR. (e) H. Hagler used a "labyrinth sealing" by some thin resin foils of Mylar MY, sliding in slits of the chamber wall CW (RMC-21 cryochamber). Through the labyrinth only small amounts of GN₂ escape from the chamber, which do not affect the correct function. (f) Leica FCS-cryochamber for Ultracut-S/R/UCT: R. Lihl developed a contact free labyrinth sealing by complementary cylinder surfaces CP/CP' mounted on SA and CW. The cylinder radius corresponds exactly with the radius around the bearing of the ultramicrotome. Without doubt this solution is both foolproof and simple and guarantees functioning of the cryo-ultramicrotome without any risk of disturbing frictions.

use in this smaller volume lower temperatures down to -150°C by cooling with cold GN₂ or even LN₂. The main advantage of those chambers besides the lower temperatures urgently wanted for element analyses was the better function of all moving parts of the ultramicrotome (motor drive, bearings, mechanical advance). But the draw-backs of those first generation chambers were very frustrating and practically nothing worked as expected. Besides cryostat systems and first generation cryochambers in the same decade some extremely simple freezing heads as accessories for standard ultramicrotomes (e.g., Crudgington, 1966) were described. They were made and used exclusively for sectioning of rubberlike elastic or tough resins in material sciences and never worked successfully in biology.
Figure 14. The mounting of the cryochamber decides the stability of a cryo-ultramicrotome: Most cryochambers are developed as an accessory for already existing ultramicrotomes and therefore mounted simply on the knife support KS (a). This makes the cryochamber sensitive against unavoidable manipulation forces. The only help in this case according to a recommendation of R. Ornberg (personal communication) is a stable metal profile MP mounted on the table top TT and covered by a 3 mm styrofoam sheet SF for handrest. Correct mounting is realised in the purpose designed Leica-Reichert cryochambers FCS/FCR on the ultramicrotomes Ultracut-S/R/UCT (b), where the aluminium cast AC' of the FC-system is mounted on the "shell" SH' of the ultramicrotome. Only the knife carrier KC' is directly connected to the "iron core" IC'. This design allows to use the handrest HR on the chamber AC' without annoying distortion of the system. Further explanations in Section 5. (a) FC-chamber on former Reichert-Ultracut models 1980/90 (front view cross section, roughly schematic): the base of the ultramicrotome rests on the damping plate DP and shows a "shell construction" according to Kenzian et al. (1975), consisting of an insulating shell SH, a flexible intermediate layer of rubber foam RF and an extremely rigid "iron core" IC. All precision elements influencing the regularity of sectioning (knife support KS, advance and bearing system) are mounted on IC. The thermostatically heated (heating cartridges HC) aluminium cast AC of the FC4-cryochamber was mounted on the considerably fortified knife support KS by a flexible rubber layer RL similar to RF. Only the knife carrier KC was mounted directly to the knife support. LN\textsubscript{2} was stored in two tanks (interconnected twin tanks) right and left-hand of the open top sectioning chamber with KC. (b) Improved chamber mount of FCS/FCR on Ultracut-S/UCT (models 1990 ff): AC' is mounted exclusively on shell SH'. Only the knife carrier KC' is directly connected with knife support KS'. A labyrinth between AC' and cover plate CP on KC' guarantees free x/y-translation of KC' with KS'. LN\textsubscript{2} is only stored in one tank left-hand. Right-hand handrest HR as mentioned above.

The first real break-through in cryo-ultramicrotomy was achieved with a system, where a purpose-designed ultramicrotome was developed together with a new type of a cryochamber (Sitte et al., 1980; Sitte, 1982, 1984; Sitte and Neumann, 1983). This system (see Fig. 14a) worked with an open top sectioning chamber and direct LN\textsubscript{2} cooling. It reached a minimum temperature near -180°C and section preparation was simplified considerably even at lower temperatures. Nevertheless not all instrumental difficulties were properly eliminated. Like all former cryochambers also the heavy-weight FC4-chamber was mounted on the knife support, which was extremely rigid in comparison to former knife support constructions. But it turned out during practical work that this support was nevertheless sensitive in the nanometer-range. Also the specimen was mounted to a somewhat sensitive "bridge" according to the design of Christensen and Blum [see Fig. 13d and Christensen (1969, 1971), and Blum (1970)]. The automatic and electronically controlled refilling of LN\textsubscript{2} from a 35 l
Figure 15. Electronically controlled continuous refilling of microlitre-amounts of LN$_2$ allows to maintain a height "h" of the LN$_2$-level in the LN$_2$ tank of a cryochamber with a precision better than ± 0.5 mm. This is important for absolutely regular cryosectioning of fresh frozen (unprotected) specimens for frozen-hydrated investigations in the cryo-TEM/STEM. Two different systems (a) and (b) are available for Leica-Reichert cryochambers FCS and FCR. Further explanations in the sections on cryo-ultramicrotomes and cryosectioning and in Figure 14b. (a) Refilling by membrane pump and two valves: a rubber membrane ME is bent by a lever LE linked to the eccentric disc on the driving wheel of the motor MO. This membrane pump causes an oscillating up- and down-movement of the LN$_2$-column in the tube TU connected with a valve box VB containing two spheres VA1 and VA2. A downward motion of LN$_2$ in TU closes VA2 and opens VA1. Opposite an upward motion of LN$_2$ in TU opens VA2 and closes VA1 (insert). The described automatic operation of both elements VA1 and VA2 results in a stepwise upward motion of LN$_2$ in the refilling tube RT inside the dewar vessel DE and finally in a transfer of LN$_2$ into the tank of the cryochamber. The LN$_2$-flux is precisely controlled by the electronic system EL connected with motor MO and filling sensor FS: if FS is wetted by LN$_2$, the motor drive is slowed down. If the LN$_2$ level drops below FS, the speed of the motor drive increases with increasing distance between LN$_2$ level and FS (CW = chamber wall thermostatically heated by cartridge HC, FC = sectioning chamber with open top). (b) Refilling by slight pressurisation of the 4 liter Dewar can DC according to K. Neumann, H. Hässig and A. Kunz: DC is closed by the stopper ST (O-ring sealing OR), which contains an aperture AP adjusted for the escape of the small amount of GN$_2$, which evaporates from the LN$_2$ inside DC by static evaporation ("defined leakage"). Without heating of the pressurisation cartridge PC therefore no refilling of LN$_2$ results. If the LN$_2$ level in the cryochamber drops down below the sensor FS, the electronic control EL powers PC and additional GN$_2$ is evaporated. The pressure inside DC increases and LN$_2$ is transferred from DC to the cryochamber. The precision of LN$_2$ refilling operation is identical with system (a). Disadvantages of this simple system are the needed precooling of DC to reach a steady state for static evaporation before closing ST and the limited volume of DC (4 liter), but compare comments in the section on cryo-ultramicrotomes.
Dewar vessel into the twin tanks of the cryochamber was not continuous: refilling was started by a sensor, when the twin tanks of the chamber were nearly empty and interrupted as soon as a similar thermal sensitive diode sensor was wetted with LN₂. The consumption of LN₂ at higher temperatures above -100°C was increasing to 7 liter LN₂/h by counter heating, since this system was originally designed exclusively for extremely low temperatures and later on adapted for temperatures between -120 and -80°C needed for Tokuyasu work with sugar protected specimens. All of LN₂ when the twin tanks of the chamber were nearly empty was not continuous: refilling was started by a sensor, Dewar vessel into the twin tanks of the cryochamber.

Another important detail concerns the "shell-mounting" of the cryochamber. Since all former cryochambers were accessories for already existing standard ultramicrotomes for ambient temperature sectioning, the cryochambers were mounted simply instead of the standard knife holder assembly on the knife support. For the FC4-chamber in 1980 (see Fig. 14a) the knife support was considerably fortified by roller guides and a dovetail mounting. Additionally the outer wall of the chamber was mechanically separated from the knife stage by an elastic intermediate rubber layer similar to the "shell construction" of the ultramicrotome (Kenzian et al., 1975). Both measures reduced the sensitivity of the cryochamber against manipulation forces remarkably, but the chamber was still sensitive against touching and was for example not suited as handrest during the picking up of the sections. After this experience, it was understood that a complete separation of the outer chamber wall from the sensitive knife holder system was needed. A first successful attempt in this direction was made by LKB with the new model LKB Cryo-Nova: the molded poly-urethane cryochamber with incorporated cell-u-foam thermo-insulation was mounted directly on the heavy weight steelbase of the Ultrotome Nova. The cryoknifeholder of the Cryo-Nova system was independently mounted. The new design reduced sensitivity considerably and allowed to use the outer chamber as handrest for cryosection preparation. Within the same development of LKB the "cryotools" were further improved and a possibility for a direct cryotransfer of loaded grids from the cryochamber to the cryo-TEM included. A real "shell-mounting" of a cryochamber with a strict complete mechanical separation of the cryochamber from the steel-core of an ultramicrotome was finally realised with the system Ultracut-S/FCS as shown in Fig. 14b. Within the new design the thermostatically heated aluminum wall of the FCS-cryochamber is now rigidly mounted to the resin shell of the Ultracut-S or Ultracut-UCT. Only the cryo-knifeholder inside the chamber is mounted to the knife support of the ultramicrotome by a metal-metal connection without intermediate plastic parts. The thermal insulation between ambient temperature and cryo elements is realised both at the object holder and at
the knife holder system by intermediate tubes of stainless steel, which are a good compromise between heat resistance and mechanical stability. The backlight illumination works identically to the ambient temperature version through the tube of the knife holder. Besides the approach of the diamond knife against the mirror frozen and therefore perfectly reflecting specimen surface the special advantage of this backlight system for the cryework is the simple identification of vitrified areas compared to segregation artefacts with hexagonal ice (see Fig. 16c). In comparison with the preceding FC4-chamber the FCS-chamber contains only one LN₂-tank instead of the FC4-twintank system. This results in a considerable reduction of LN₂ consumption, which remains considerably below 2 liter LN₂/h for temperatures down to -170°C. Instead of the right-hand tank there is now a handrest, which remarkably simplifies all preparation procedures as well as the mounting of the cryochamber on the ultramicrotome (see Fig. 14b).

A further improvement of cryo-ultramicrotomes relates to the refilling of the chamber tank with LN₂, which was formerly discontinuous as mentioned above. In the meantime two perfectly working systems for continuous LN₂ refilling are known. The more sophisticated FCS-refilling system (Fig. 15a) uses a motor driven membrane pump and two simple valves consisting of stainless steel balls with corresponding circular openings. If the eccentric piece on the driving wheel of the motor is rotating, the LN₂-column in the connective tube is stepwise moving upward and LN₂ is transferred from the Dewar vessel into the tank of the cryochamber. The motor is electronically controlled by a level sensor in the cryogen tank of the chamber. If the LN₂-level in the chamber drops down below the sensor, the motor drive is activated and LN₂ is transferred from the Dewar vessel into the tank of the cryochamber. Minimal pressures are sufficient for this transfer. The system is foolproof, since the head of the refilling system with the pressurisation device (heating cartridge) is not tightly mounted in the neck of the Dewar can: if any failure (e.g., aperture closed by contamination) a slightly higher pressure is developed, the head is lifted up and excess GN₂ released. This system works with the same precision as the motor driven unit according to Fig. 15a and delivers precisely µl-amounts of LN₂ to the chamber tank. It works absolutely silently and will have probably an unlimited life span. There are only two disadvantages: Precooling and limited LN₂-volume. Precooling of the 4 liter Dewar is needed, because the evaporation rate of the still warm and just filled Dewar lies considerably above the "static evaporation" of the cold container. It is a simple measure, first to fill the can with LN₂, then to mount the cryochamber and finally to close the can and to connect the LN₂ supply tube between refilling device and chamber. The second disadvantage (4 liter volume) turned out to be an advantage in reality, since it is in comparison to a big Dewar vessel so easy to exchange and to refill such a light can. Since the new cryochambers consume only small amounts of LN₂, the volume of 4 liter enables work for at least 3 h and that is sufficient for most cases. If not: refilling is easy. The continuous LN₂-refilling is an advantage mainly for cryosectioning at extreme conditions, that means if absolutely regular cryosections of minimum thicknesses are needed for frozen-hydrated investigation, where crevasses are the big problem. For Tokuyasu work I cannot believe, that continuous refilling makes a big difference in comparison to discontinuous refilling: only the start of refilling with the former FC4/FC4D/FC4E-systems introduces a slight irregularity, that means a loss of 1 to 4 cryosections within a ribbon. But: better is better and we are happy that we reached the goal of continuous LN₂ refilling.

Cryosectioning According to Tokuyasu

The Tokuyasu method (Tokuyasu, 1973, 1978, 1980, 1986; Tokuyasu and Singer, 1976) is well introduced and some excellent reviews report on the technical and methodological details [see e.g., Griffiths et al. (1983, 1984), Griffiths (1993), Leunissen and Verkleij (1989)]. Cryosectioning of biological material protected with 2.3 M sucrose according to Tokuyasu

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protected materials change immediately, if the sugar content is reduced noticeably below 2.3 M, which is recommended for some special purposes [see e.g., Tokuyasu (1973, 1980), or Tokuyasu and Singer (1976)]. The changes concern both sectioning consistency and ice segregation artefacts. Mostly in such preparations a more rapid freezing by propane or ethane is needed. Generally one has to check carefully by backlight illumination (see Fig. 16d), if a "clear" vitrification is obtained, since also for protected specimens proper freezing is a precondition for convenient sectioning and reproducible results.

Usually freezing is done after presectioning the chemically fixed and sugar protected sample to a pyramid with a razor blade (Fig. 16a) and mounting of these pyramids on a standard carrier ("pin", Fig. 16b) of the cryo-ultramicrotome used. Such a carrier has a weight of approximately 0.1 g. Such a "heavy weight" carrier is not suited for freezing of non pre-treated fresh material, but sufficient for a specimen with a good cryoprotection. Of course the heat extracted from the pin causes a profile of cooling with considerably smaller cooling rates inside: sometimes as a result of insufficient sucrose impregnation or lower sucrose concentration there is a contact region between specimen and pin, which looks "milky" and contains hexagonal ice. In such cases only the border regions directly exposed to the coolant (LN$_2$ or propane or ethane) are absolutely clear and properly vitrified (Fig. 16c): these well frozen (vitrified) regions are easy to identify by backlight illumination (Fig. 16d) as mentioned above. And only these regions of the specimen block should be sectioned and studied. If LN$_2$ cooling is not sufficient and the whole specimen looks milky, freezing in a secondary cryogen like propane or ethane (see Table 3) is necessary. As already mentioned above, slower freezing at lower cooling rates, that means dipping into LN$_2$ according to Fig. 16c in an advantage, if fully (2.3 M) sucrose protected specimens have to be frozen: faster cooling in a "secondary cryogen" like propane or ethane provokes often tension (stress) cracks. The resulting clefts inside the sample may affect the cryosectioning procedure considerably.

Formerly it was the general opinion, that diamond knives are not suited for dry sectioning of protected material at low temperatures. It was also claimed that glass knives have to be broken very slowly over several seconds and that both balanced break and tungsten coating are striking advantages (e.g., Griffiths et al., 1983, 1984; Roberts, 1975). We compared several times tungsten coated, slowly broken or balanced broken glass knives (Leica balanced break Knife-maker) with normal glass knives broken in the usual

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**Figure 16.** Compared to the mounting of most fresh frozen (unprotected) samples, mounting of fully (2.3 M sucrose) protected specimens according to Tokuyasu is simple. Compare the section on "Cryosectioning according to Tokuyasu" and cited reviews of Tokuyasu and Griffiths. (a) The protected sample (broken line SA) is presectioned with razor blade RB before mounting and freezing to a suited pyramid SP below a stereomicroscope. (b) The pyramid SP is positioned on the standard carrier SC ("pin") in holder HO. (c) Freezing in LN$_2$ (styrofoam container CO) is probably an advantage, since faster freezing in propane or ethane seems to provoke tension cracks and clefts inside the sample SP, which may impede regular sectioning. Usually fully protected samples are vitrified to an absolutely clear and proper matrix CT by LN$_2$. Only in cases of insufficient sugar protection (e.g., period of impregnation too short) or at sugar concentrations considerably below 2 M a milky area MA indicates lower cooling rates in centre regions at the border between carrier SC and the bottom of the pyramid SP. (d) Milky areas MA are easy to identify under the darkfield ("backlight") illumination BL in the cryochamber: the carrier SC is investigated with the stereomicroscope of the cryo-ultramicrotome in horizontal position after mounting in the specimen holder SH on the specimen arm (SA, see Fig. 13). Only the clear and transparent part CT of the sample should be sectioned, since the milky area MA contains severe hexagonal segregation artefacts.

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(1973) as mentioned already in the "Introduction" (see Table 2) differs in most respects from cryosectioning of fresh frozen material described in the following sections of this review. This results mainly from "sugar embedding": the high amount of sugar reduces the amount of free water to an extent, that segregation of hexagonal ice is not possible even at the low cooling rates obtainable with immersion or dipping the samples into LN$_2$ (Fig. 16c). On the other hand, sugar changes the consistency of the sample considerably from a brittle to a somewhat jelly-like state. This enables sectioning of rather large areas with a width of 0.5 to 1.0 mm in most cases. The characteristics of
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Figure 17. Cryosectioning with low angle cryodiamond knife, diamond trimming tool and an adjustable ioniser (e.g. Diatome static line II) simplifies the work both with protected and with fresh frozen samples considerably. Further comments in the sections on cryosectioning and in the Discussion, in Table 2 and the paper of Michel et al. (1992). (a/b) Professional set up in an FCS/FCR-cryochamber: near the specimen SP in holder SH the knife holder KH is visible. Usually in one of the two positions a triangular low angle diamond knife DK (see "d"), in the other a cryodiamond trimming tool TT (see "e") is mounted. The ioniser spike IS is positioned approx. 30 mm behind the knife edge (held by the cryomanipulator system CM). (c) Static positive and negative electricity is generated, if two non conductive materials MA and MB (e.g., frozen water and diamond) slide in a good contact, which always creates mechanical friction. This corresponds to the well known production of static electricity by a rotating disc sliding over electrodes of non conductive material. Since opposite charges attract each other, electrostatic forces develop. Such forces must have annoying results, if one piece is very fragile like an ultrathin cryosection. Concerning the generation of static electricity, the couple "diamond/ice" seems to be much more efficient than the couple "glass/ice". (d) Triangular Diatome cryodiamond knife for "dry sectioning" with edge DE. The metal holder MH has the geometric shape of a triangular glass knife and fits therefore in all common knife holders. (e) Diatome diamond trimming tool TT with diamond edges TE1/TE2/TE3: the base BA corresponds to a triangular glass knife and fits in all knife holders like (d). The trimming tool TT has two oblique side edges T1/T2 for facing the pyramid and one normal edge T3 for presectioning the later cutting surface. (f) Diatome cryodiamond knife for "wet sectioning": the metal holder forms a section collection boat CB and is practical exclusively used in material sciences.

way with a well adjusted old LKB Knife Maker 7801A in our laboratory. In repeated experiments we could not find a reproducible difference between coated and uncoated, slowly and normally broken, or balanced and non-balanced broken glass knives: all delivered useful sections of the same quality as long as breaking was done with a well adjusted knife maker and care was taken not to damage the edge by improper handling. There was also no difference between freshly broken knives and edges stored up to several weeks in the laboratory, if care was taken, that the knife edges were not contaminated by dust or coating layers. But there was an evident difference between glass and diamond knives: sectioning with diamond knives in former times without special measures was frustrating because the sections seemed to stick to the dry diamond surface and it was practically impossible, to obtain good ribbons with the diamond knife. Especially at higher temperatures above -120°C and with fully protected samples after 2.3 M sucrose impregnation -
This phenomenon on diamond surfaces was evident: glass knives under these conditions made the job much better. That was a severe disadvantage, since the profit of the diamond edges in standard ultramicrotomy at ambient temperature was obvious, where only students and beginners in workshops are forced to use glass knives and practically all experienced users already switched over to the better, absolutely reproducible, and actually less expensive (if the costs for glass strips, knife maker, troughs and, not to forget, all wasted time of scientists and technicians are taken into consideration) diamond knives.

In the meantime the situation has changed completely and diamond edges are now usable with the same advantages as in standard ultramicrotomy for cryosectioning and trimming at low temperatures. Investigations of Helmut Gnägi (Diatome AG, Biel, Switzerland) around 1990 lead to the clear conclusion, that those detrimental phenomena result from charging phenomena of the specimen, the sections and the diamond surfaces, which can be eliminated by discharging ionisers. If such an ioniser is properly installed and used inside the chamber (see Fig. 17a and b), the diamond knife is clearly superior to the best glass knives. Discharging by antistatic pistols or ionisers was formerly recommended both for standard ultramicrotomy at ambient temperature (Nicholson, 1978; see also Mattheij and Dignum, 1975) and for cryowork with fresh frozen specimens (Sitte, 1982; Sitte and Neumann, 1983). But nobody had the idea, that sticking of the sections on the facet of a diamond knife could be caused by electric charges. It is the merit of Helmut Gnägi that this question is now clearly answered. Indeed: in retrospective this is easily understandable, since friction between two different nonconductive materials mostly provokes splitting of electrical charges (see Fig. 17c and Michel et al., 1992). After this charging, attraction of those materials results from the electrical field, which develops between positive and negative loads of the corresponding non conductive materials. The proper solution for discharging is an ioniser with a spike within the GN₂-atmosphere of the cryochamber (Fig. 17a and b) connected to a transformer with an adjustable voltage in the range between 1 and 10 kV. This AC-spike produces negatively and positively charged nitrogen particles which are able to discharge both the sections and the knife surface. The spike of the ioniser has to be mounted inside the GN₂ outside mounting in the room atmosphere (e.g., above the chamber) leads to a considerable turbulence at the border between ambient air and cold GN₂ at the open top of the chamber. Additionally such outside mounting results in severe frosting of both knife and specimen. Care has to be taken not to approach too close to temperature sensors (e.g., Pt 100 sensors for object, knife or GN₂ temperature) or other electric elements of the cryochamber, since such ioniser spikes at higher voltages can easily damage IC elements of the electrical control unit. As far as protected material is sectioned, the ioniser spike is positioned as shown in Fig. 17b. The improvement is striking and easy to demonstrate by switching on and
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**Figure 19.** A high performance stereomicroscope SM and a bright cold fibre illumination are essential for cryosectioning of fresh-frozen biological samples, since surfaces below 0.1 x 0.1 mm² have to be trimmed and cut. According to our own experience a flexible fibre system FF between the illumination box IL with the light source (IL mounted separated from ultramicrotome table, e.g., on the wall) and an intermediate piece IP mounted on a high performance stereomicroscope SM (for systems Ultracut-S/UCT the compatible Leica-Wild stereomicroscope M31 with wide field eyepieces 16 x /14B and 40 x /0.8 and mount WS and adapter lens 1.0 x /F = 89) or on the carrier of the stereomicroscope is the best solution. IP divides the light in two goose necks GN, which are bent right- and left-hand around the body of SM and remain always properly adjusted with respect to the working distance d and optical axis OO of SM. The advantage of the double system GN/FF is, that after turning away the optic holder OC around axis AA (e.g., for exchange of specimen or knife) the illumination with respect to SM is again correctly centred, after SM is switched once more into working position. Additional remarks in the section on "Ultrathin Sectioning .." and in the Discussion.

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off the ioniser during sectioning. With the ioniser long straight ribbons of incredible quality are obtainable from sugar protected samples with the dry diamond edge (I have never seen before such results !) and there is no question at all about the best suited methodology. In summary: the actual state-of-the-art is without doubt the sectioning with a triangular cryo diamond knife (best facet angle 35°) and an adjustable ioniser. One should forget all discussions about slow breaking, balanced breaking or coating of glass knives and change over to these considerably easier and better tools. Of course, glass knives remain the proper edges for all students, beginners and workshops for financial reasons (without tungsten coating and without all sophisticated breaking methods, but produced with a well adjusted knife maker and carefully checked).

**Figure 20.** "Crevasses" (cold fractures) in cryosections of fresh-frozen material at different magnifications according to Frederik et al., 1982 (reproduction with permission). The cryosections were freeze-dried, subsequently osmicated, reembedded in epoxide, cross sectioned and stained. It is clearly visible, that the crevasses form sharp edge like profiles. At higher magnification they look often like freeze fracture profiles FF of split membranes. See also Fig. 25b and further comments and discussion in the section on "Ultrathin Sectioning .." and in the Discussion.

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All follow-up procedures remain unchanged as described within the already initially cited review articles.

**Ultrathin Sectioning and Handling of Fresh Frozen Samples**

As already stated in the "Introduction" and the preceding section "Cryosectioning According to Tokuyasu", there is a considerable difference between sectioning of samples impregnated in 2.3 M sugar solutions after a chemical prefixation by aldehyde and specimens frozen without chemical pre-treatment for element analysis or fresh frozen investigation or diffraction analysis in the TEM/STEM (see Table 2). If specimens have to be sectioned, which are protected by some reasons with a lower amount of sugar (< 2.3 M, e.g., 1 M, see Tokuyasu, 1973) or with another cryoprotectant (e.g., according to Bernhard, 1965, see also Bernhard and Leduc, 1967), the methodology has to be changed and may have some similarities with fresh frozen sectioning described in this section. There may be also slight differences between the needs for EDX or EELS of freeze dried ultrathin sections and direct observation of frozen-hydrated ultrathin sections in TEM/STEM with respect to the solid state of water (I, see section "Freezing and the Frozen State of Water ...."). Nevertheless these pathways are now treated together, since it seems to be important to exercise also all freezing and cryosectioning for subsequent EDX or EELS at the best obtainable level of methodology in order not to run in the already mentioned pitfalls of misinterpretations. Finally it has to be mentioned, that ultrathin sectioning of fresh frozen material as described in this section and cutting of semithin sections for frozen-hydrated EDX in the STEM according to the methods of Hall and Gupta (see Gupta and Hall, 1981) are quite different: as mentioned already, I do not want to include this interesting but very special methodology needed for EDX of extracellular compartments not containing proteins (e.g., urinary space) into this review, since we have no first hand experience in our laboratory with the methods of Hall and Gupta. I refer therefore to the previously cited literature.

Already freezing as the first step of cryo-preparation deserves full attention, since proper vitrification is needed to exclude possible artefacts connected with any crystallisation process starting from a liquid phase. At the same time vitrification is the best precondition for an optimal sectioning consistency of frozen specimens. But remember: proper freezing makes no sense without a stable mounting of the frozen specimen to the carrier of the cryo-ultramicrotome, which will be described first. Sectioning also makes no sense without a diamond trimming tool and a low angle cryo diamond knife for sectioning together with an adjustable ioniser. Finally the mounting of the ultrathin sections on the grid and both the cryotransfer of the grids to the scope for direct studies in the frozen-hydrated state or to the freeze dryer for subsequent EDX are important steps which decide on success or failure of the whole effort. These steps are therefore also included here.

Proper vitrification at its best is achieved by high pressure freezing of sufficiently thin specimens. This means according to facts discussed in the previous section "High Pressure Freezing" mostly a thickness below 0.2 mm (see Table 5). In several cases vitrification is also possible by immersion or impact freezing (see preceding sections of this review). As far as immersion in ethane is used and the sample (cell suspensions or tiny bulks with diameters < 0.5 mm) is mounted on a gelatine coated hollow cone carrier (see Fig. 4c) no further mounting operation is needed. After immersion cryofixation the carrier has to be transferred to the precooled cryochamber (required minimum temperature around -170° C) in LN₂, following Fig. 4d. If this transfer has to bridge only a short distance in room atmosphere between the cryofixation unit and the cryo-ultramicrotome in the same laboratory a transfer in a LN₂-precooled but dry metal container closed with a similarly precooled metal cover is sufficient, because during this dry operation the container is warmed up only to a negligible extent while GN₂ from inside escapes by expansion (Fig. 4e): there is no risk. Care should be taken, that the cone shaped carrier has the correct geometry and that heat capacity near the top is really small. After inserting of the carrier, trimming with the Diatome diamond trimming tool is easily carried out at minimum temperature with the motor drive and automatic feed according to Fig. 18. It makes no sense to speed up trimming by manual operation of the ultramicrotome with respect to the risk of loosing the sample from the carrier. With the motor driven cryo-ultramicrotome (feed: 0.2 to 0.5 µm, cutting speed approximately 0.5 mm/sec) trimming is also finished elegantly within a few minutes. It is mostly restricted in such cases to the upper and lower edges with-out presectioning the sectioning plane. Normally both edges are set in a distance of 0.1 mm or less. Splinters and section fragments on the trimming knife or the specimen are easily removed according to Herbert Hagler by splashing LN₂ from a small cotton ball mounted to a cocktail stick or in a forceps: the rapid evaporation of GN₂ normally takes off all fragments and splinters. An ioniser is also helpful during trimming, since fragments and splinters
Cryoultramicrotomy do not stick to the charged surfaces. Trimming is very difficult without a good stereo microscope and a bright cold light illumination. As far as a Reichert Ultracut-S or UCT is used, the compatible Wild stereo microscope M3C with wide field eyepieces 16x/14B and 40x/OB is recommended instead of the standard optics. The mount WS for this stereo microscope is available for the optic carrier of these Ultracuts. Of course the 40x eyepieces together with the lens l.0x/F = 89 mm for the correct working distance on the Ultracut produce a magnification, which cannot increase the resolution compared to eyepieces with smaller magnification. But experience tells, that this high magnification is nevertheless an advantage for practical work in combination with a bright illumination. The brightness of the backlight illumination of the Ultracut-S/UCT together with an FCS cryochamber is absolutely sufficient for this high magnification. But the standard incident illumination (fluorescent tubes) of the Ultracuts has to be improved by a more powerful fibre optic illumination. In our laboratory we use the Schott cold light source KL 1500 with two flexible goose necks bent around the stereo microscope according to Fig. 20. For other ultramicrotomes a similar set-up is recommended. It makes no sense to enter into fresh frozen sectioning without this optimised optical equipment. It should be mentioned in this context, that the simpler cryosectioning system Ultracut R/FRC was developed mainly for Tokuyasu preparations (immuno-cytochemistry on sugar protected material), but is not suited for work below -120°C with fresh frozen samples directed to element analysis or frozen-hydrated investigations in the cryo-TEM.

Ultrathin sectioning is generally carried out for frozen-hydrated investigation in the TEM at the obtainable minimum temperature of the cryochamber. This temperature should be -165°C or below. If the temperature does not reach this value, check the temperature of the outside walls of the cryochamber and of the defroster at the specimen arm: the built-in heaters are meant to prevent condensation or frost. Therefore the temperature of the chamber walls and of the specimen arm defroster should be around room temperature, but not noticeable higher. When frozen suspension droplets according to Fig. 18 have to be sectioned, cutting is started at the original surface of the frozen droplet and continued up to the moment, that the first small ribbon is obtained. Proper setting of the cutting window and use of an adjustable ioniser (see preceding section and Fig. 17a and b) are preconditions for good sections. The cutting speed usually is set around 0.5 mm/sec, the advance as small as possible dependent on the specimen (e.g., approx. 50 nm) with respect to the formation of "crevasses" (see Fig. 20 and 25b), which are much more obviouls in thicker sections. The cycle length (time between two consecutive sections) is not that important with a new FCS cryochamber than with former systems (e.g., FC4/FC4D/FC4E). The formerly often recommended speedy manual operation to shorten the cutting cycle is a disadvantage with respect to the regularity of the section thicknesses and the adjustment of the needed ioniser, since high spike voltages introduce risks with respect to the electronic system (see preceding section "Cryo-Ultramicrotomes"). For cutting a clean triangular low angle cryo diamond knife (facet angle 35° or less, the smaller the better with respect to crevasses and irreversible compression) with minimum clearance angle is recommended. The transfer of the sections to the grids [according to McDowall et al. (1983), a carbon coated, according to Michel et al. (1991) an uncoated 600-mesh copper grid, e.g. G 600 TT, SPI, West Chester, PA, USA] is carried out using a precooled eyelash. The grids are either mounted in an adjustable holder (Fig. 21a), available as an accessory for the FCS cryochamber or positioned on a polished metal plate according to R.L. Omberg (personal communication), mounted on the knife holder of the cryoultramicrotome according to Fig. 21b. The Omberg-device makes it very easy to have a couple of grids available and to shift single grids for loading with cryosections immediately behind the cutting edge of the diamond knife. This Omberg-device is not offered commercially. But it may be possible to manufacture such a plate in one's own workshop and to adapt it carefully to the height of the knife used for cryosectioning. The device enables immediate first flattening and pressing of the cryosections with a precooled section flattening tool. Since a good thermal contact between the cryosections and the grid with respect to heat conduction under the electron beam is of greatest importance, efficient pressing of the sections onto the grid is an indispensable step even after a first pressing on the plate according to Fig. 21b. The best solution up to now remains the section press introduced by Dubochet and McDowall (see Fig. 21c), which is used in different versions by different groups and is available as accessory to the FCS-cryochamber. Pressing is carried out inside the chamber and the cold press may be used for a first cryotransfer operation to a loading station of a commercial cryotransfer system. An interesting home-made system was described by Tvedt et al. (1984) (see also Sitte, 1982, Fig. 2, p. 15). This system was especially designed for pressing and transfer of cryosections for subsequent freeze drying and EDX.

Cryo transfer units are offered for TEMs with side entry both by Gatan and Oxford Instruments. The
Gatan Model 626/626-DH (see Fig. 22a) is an improved version of the Philips transfer unit according to Hax and Lichtenegger (1982) with a protection shield ("shutter"), that can be moved from outside after the frost layer built up during the transfer operation through the humid room atmosphere has been evaporated in the vacuum of the scope. This evaporation process can be easily read on the vacuum indicator of the TEM. The new Oxford system Cryotrans CT 3500 according to R. Henderson uses an additional protection by inert GN₂ to avoid any frost formation during the transfer operation (see Fig. 22b). Both seem to work reproducibly according to second hand information. In our own laboratory we use successfully a Zeiss cryotransfer chamber for the top entry system of our Zeiss EM 902 filter lens scope (see Sitte, 1984), which was the only transfer system compatible to the former FC4/FC4D/FC4E-cryochambers. For the new FCS-cryochamber Ludwig Edelmann has designed an accessory (see Fig. 22c), which enables direct cryotransfer from the cryochamber to the EM 902: it consists of a polyurethane-foam profile mounted on the top of the FCS-chamber and is equipped with an entry port and holder for the Zeiss transfer chamber. Without doubt such a direct loading in the cryochamber of the ultramicrotome represents the simplest, most efficient and cheapest solution for this kind of cryo-work. But there is another very simple solution for side entry systems, which was used already in 1980 by Dubochet and co-workers [see McDowall et al. (1983) and Fig. 22d]. They transferred the sections with the old Philips cryo specimen holder PW 6591/100 for their Philips EM 400 without any modification, that means without protection shield (shutter) and workstation for loading. The grid with the sections was inserted under LN₂ into a small insulating container. Then the loaded cold stage with the grid below LN₂ was brought as close as possible to the opening of the side entry port on the column of the scope. Afterwards the rod of the cold stage was quickly moved through the ambient air and inserted. It is astonishing, that most of such simple transfer operations were successful: both the amorphous frozen state of the specimen is maintained and the contamination of the cryosections is within the usual limits - compared with much more sophisticated transfer operations and instruments. This fact is easily explained: since the rod of the cold stage with the grids is covered by a coat of LN₂ and has a temperature of -196°C, some seconds are necessary for evaporation of the LN₂ coat. Within these seconds ambient air cannot approach to the rod with the grids and evaporation of LN₂ acts as a heat sink providing a stable temperature even of the extremely thin grids with the cryosections. I agree with Jacques Dubochet that the most simple operation and set-up often is the best. Of course vitrified specimens have to be kept through the whole chain of cryopreparation according to the first idea of Heide and Grund (1974) always at a value below the devitrification temperature of ice, that means < -135°C (see section "Freezing and the Frozen State"). To be on the "safe side" temperature indication should never show values above -145°C in order to prevent devitrification to cubic ice. This precondition can be easily fulfilled with the cryochambers and the transfer systems mentioned above.

There remains only the question if the mechanical energy during the process of cryosectioning is transformed into heat to an extent, that may cause a "through-the-section-melting" of the knife edge as argued by Thornburg and Mengers (1957). Up to now a lot of serious evidence was collected against this hypothesis [see e.g., Frederik (1982), Frederik and Busing (1981, 1982), Hodson and Marshall (1972), Karp et al. (1982)]. The most striking evidence against a melting process during cryosectioning under the conditions given above is the preservation of the amorphous-frozen state of vitrified specimens and vitrified water, shown by electron diffraction of the sections in the TEM [see McDowall et al. (1983), Michel et al. (1991, 1992)] and the formation of cold fracture profiles (crevasses) during that sectioning procedure (see Fig. 20). In comparison to freeze cleavage before freeze etching, where the whole splitting energy is freed instantaneously (Sleytr and Robards, 1977), cryosectioning is a very slow and smooth continuous process, where heat is generated and spread at rather small rates. Therefore it seems evident, that it is really possible with the available methodology and instrumentation to produce proper cryosections in thicknesses between 50 and 100 nm from vitrified biological samples, which show correctly cellular structures embedded in amorphous frozen water in the TEM/STEM.

The whole preparation procedure changes somewhat, if slam or high pressure frozen specimens have to be sectioned. Slam frozen material has the advantage, that the surface of the well frozen border layer of the specimen is a replica of the highly polished mirror plane. The most important step is the mounting of the mirror-frozen specimen on the carrier of the cryo-ultramicrotome. If tissue slices of thicknesses of 0.5 mm or less are used (Sitte et al., 1987a, 1988), this mounting operation according to Fig. 23 is quite simple. Since the areas of such tissue slices mostly correspond to the cross section diameter of the sliced organ (e.g. a rat kidney with a diameter of approximately 1 cm), the frozen slice has to be divid-
Figure 21. Manipulation of cryosections of fresh-frozen material in the cryochamber: the dry transfer of the sections SE from the edge DE of the diamond knife DK to the grid GR is mostly done with a cold eyelash-probe EP and considerably simplified by a properly adjusted ioniser (see Fig. 17a and b). The grids are either mounted on a holder GH (a) or positioned on a polished metal plate (b). All schematic drawings in side view (upper half) and top view (lower half). Further explanations in the section on “Ultra-thin Sectioning ...”. (a) Grid holder GH is available as cryotool for FC-systems of Leica-Reichert: it takes up to 5 grids and is mounted on the bolt BO of the cryo-manipulator CM. By rotation around BO and x/y/z-translation of CM it is simply possible to adjust each grid GR exactly just below the edge DE of the knife DK or below the sections SE for the section transfer with EP. (b) Omberg mounted a metal plate PL on the knife holder KH in the cryochamber: the highly polished surface PS of PL is adjusted approx. 0.5 to 1.0 mm below the edge of DK. It is easy to move a grid on the polished plate against the edge just below the ribbon of sections SE. This simplifies the transfer considerably. Afterwards provisional affixing with precooled stamp ST is easy. The set-up is not commercially available but highly recommendable, if manufacturing in ones own workshop is possible. (c) Up to the moment the section press according to Dubochet and McDowall is the best tool to obtain the needed thermal contact between cryosections and gridbars or cryosections and support film. In the commercially available Reichert-version the section press consists of a base BA, a hardened and polished steel ring RI, a glass plate GP and the screw SC. If SC is loosened, the glass plate is lifted up by spring SP and the grids GR can be inserted with a forceps FO (open corner OC). The grids are moved below glass plate GP by turning ring RI and pressed by tightening the screw SC between base BA and glass plate GP. If needed, the whole set up can be used after closing for a short cryotransfer into a loading station of a transfer unit.

ed into smaller pieces after the control described in the former section "Ambient Pressure Freezing" (see also Fig. 7). The piece used for cryosectioning should not exceed the diameter of the object table of a standard pin. It is positioned with the well frozen side downwards on the LN2 cooled mirror of the slam freezer (Fig. 23a and b). The pin with a droplet of cryoglue is mounted on a plunge injector device and guided vertically downwards to the frozen specimen. After contact the glue hardens due to heat extraction by the mirror block. Since this heat is extracted through the frozen specimen, one may believe that at least recrystallisation could occur due to this heat transfer. But this is certainly not possible at least in the well frozen border layer, since that layer remains cooled by the mirror. The situation is identical to the application of the warm specimen to the mirror plane: also during slam freezing the whole heat of the specimen definitely is extracted through this well frozen border layer without detrimental effects. But "seeing is believing" : it is easy to check the well frozen layer again by dark field inspection (Fig. 7e and 16d). The cryoglue used in our lab for this mounting is simply soft soap. But there are a lot of very useful cryoglues described in the literature, which may serve as "more scientific" alternatives [see e.g., Bachmann and Schmitt-Fumian (1973), Karp et al. (1982), Michel et al. (1991), Steinbrecht and Zierold (1984)]. The special advantage of the described mounting operation is the exact perpendicular orientation of the mirror-like replicated surface of the slice with respect to the longitudinal axis of the pin (see again Fig. 23). A further advantage is the easy adjustment of the trimming diamond or the diamond
edge against the mirror-like surface which corresponds exactly the adjustment of a knife against a presectioned resin block with the aid of the backlight illumination reflection during standard ultramicrotomy at ambient temperature (see e.g., Sitte and Neumann, 1983, Fig. 31 and 32, pp. 64 ff and Fig. 81, p. 132). Finally it is certainly nice never to be confronted with the compression artefacts according to Fig. 9a and b, which are connected which each slam freezing: of course they still exist, but are not visible, since the sectioning plane always shows circles if formerly spherical structures are sectioned. But one should remember, that the diameters of such circles or spheres do not show the original size, since all x/y-dimensions are enlarged by the compression artefact due to slamming.

Trimming in principle is similar to the "drop-let trimming" described above (see Fig. 18). Since the slam frozen specimen has an absolutely plane surface, trimming is essentially a "mesa trimming". The trimming operation is made once more with a Diatome diamond trimming knife (see Fig. 23c) together with an ioniser (see Fig. 17a and b) at the motor driven ultramicrotome with automatic feed (speed: 0.5 mm/sec, feed: 0.5 μm). Also with such specimens this automatic operation at least is less time consuming than trimming by hand, because the risk of losing the specimen is minimised. Diamond trimming knives are superior to glass knives or the offered trimming tools of hard metal alloy, which are very brittle and therefore mostly defective. Trimming is done in four steps like "mesa-trimming": firstly two edges right and left according to Fig. 23c are trimmed, the remaining two edges after a 90°-turn of the specimen around the longitudinal axis of the specimen holder. It is not necessary to enter more than 20 μm into the block surface. That makes 40 cutting strokes each with less than 10 sec or less than 7 min per edge of the pyramid. This time should be invested for proper trimming of a block face < 0.1 x 0.1 mm². Sectioning and section transfer are performed afterwards as described above.

Mounting of high pressure frozen specimens is described in detail by Michel et al. (1991). For the small disc-shaped specimens (diameter approximately 2 mm, thickness normally below 0.2 mm) either the use of a home made special holder compatible with the object holder system of the FCS cryochamber (see
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Fig. 24a) or the adaptation of the commercial Leica-Reichert cryo vise-type-holder (see Fig. 24b) is recommended. The holder according to Fig. 24a has a slit for the insertion of the tiny samples. The width of this slit can be varied by a screw inside the cryochamber. According to Michel et al. (1991) the high pressure frozen specimen is additionally held by a cryo-glue consisting of 30 % ethanol, 30 % methanol and 40 % tap water (v/v), which is highly viscous at -135°C. At sectioning temperatures of -165°C or below this mixture solidifies. An alternative for gluing in such arrangements may be an indium sheet as described by Dubochet et al. (1988). The pure metal indium is soft even at LN sub 2 temperature and therefore well suited as an intermediate layer which adapts excellently to each specimen profile. A suitable commercial vise type holder is available as accessory for Reichert-Leica FC-systems and can be adapted by a simple counter piece according to Fig. 24b.

Some sectioning artefacts have to be expected, if fresh-frozen specimens are cut. But also these artefacts - mostly "crevasses" and section compression - can be reduced considerably if all given possibilities are used together. "Crevasses", that means clefs or fractures within the sections (see Fig. 20), occur during most cryosectioning processes. There is a considerable discussion about the generation and the specific localisation of these crevasses [see e.g., Dubochet et al. (1988), Fig. 21 on p. 164 or Richter et al. (1991), Fig. 7 on p. 26; detailed discussion in Richter (1992, 1994)], which is certainly of theoretical interest. But these fractures disturb the interpretation of the images of such frozen-hydrated sections in an extremely frustrating manner [see Chang et al. (1983) or Dubochet et al. (1988), Fig. 17, p. 161, or Fig. 25b in this review]. The most important practical question is therefore, how this phenomenon can be avoided, suppressed or at least minimised. The following recommendations may help: regular thin sectioning (the thinner the better) would probably improve the results, since in really thin cryosections (thicknesses < 50 nm) this phenomenon mostly does not occur. Like metals or resins also films of ice seem to be flexible in sufficiently thin layers. As mentioned
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**Figure 23.** Mounting with a particular orientation of slam frozen material (e.g., fraction TS' of a tissue slice TS, see Fig. 7) on a standard carrier SC ("pin", see Fig. 4b) of a cryo-ultramicrotome and subsequent trimming of a "pyramid" with a diamond trimming tool TT (see Figs 17e and 18). Mounting is easy on a Leica-Reichert KF 80/MM 80 or CPC/MM 80 system for plunge and impact freezing. The description of the procedure (a) to (c) refers to this apparatus. Further explanation in the section of "Ultrathin Sectioning...".

(a/b) After careful inspection of the slam frozen tissue slice TS according to Fig. 7 a suited part TS' is selected, which corresponds to the diameter d of the object table on SC. TS' is deposited in the centre of metal mirror MM on MB exactly below SC mounted on pin-holder PH of injection rod IR. The well frozen original contact plane is oriented downwards and in good thermal contact with metal mirror surface MM.

A droplet of a suited cryoglue CG (e.g., soft soap, but consider alternatives mentioned in the section on "Ultrathin Sectioning ...") is deposited on the table of SC, and moved with the plunge device IR/PH against TS'. It is important to work not too slow: the glue should not freeze but surround TS' to some extent (see "c"). There is no risk of recrystallisation of the vitrified border layer, which is in good thermal contact with MM.

(c) Trimming with diamond trimming tool TT according to Figs. 17 and 18 is done like "mesatrimming": after facing the first sidewall of the pyramid with corner CO2 of TT (entry depth d' ≈ 20 μm) the corresponding side wall is faced after readjustment of the tool TT (new position TT': broken line in schema) with corner CO1'. The whole procedure is repeated after a 90° rotation of SC/TS' around longitudinal axis of SC. The width w and the height h of the final cutting surface should not exceed 0.1 mm. The orientation of the new sectioning surface is exactly 90° to the longitudinal axis AA of SC: this enables a perfect adjustment and approach of the diamond knife by darkfield-(backlight)-illumination due to the shiny mirror like replication of the metal mirror MM by the slamming process. Cryosectioning is done as described in the section on "Ultrathin Sectioning...".

...several times above, homogeneous vitrification is also tremendously helpful and high pressure freezing a big advantage, if other cryofixation methods do not vitrify the specimen of interest properly. Finally, reduction of the dimension of the sectioning area parallel to the knife edge ("width" < 0.1 mm), reduction of the cutting speed and reduction of the facet and clearance angle of the knife are helpful. Of course thickness, proper freezing, reduced width, cutting speed and knife angles are not the only parameters of influence: more homogeneous specimens with a lower content of membranes are less prone to this artefact. But homogeneity cannot be influenced in most cases. Also the other artefact, the severe irreversible compression of the cryosections (Dubochet *et al.*, 1988; Richter, 1992, 1994), can be reduced considerably by several measures: most of them are identical with the measures influencing the crevasses. The perhaps most important parameter also in this case is a reduction of the width of the specimen surface to values < 0.1 mm. Contrary to 2.3 M sugar protected specimens, which are so easy to section and to stretch after thawing, fresh-frozen specimens are extremely brittle below -150°C and tend to show considerable compression. This compression usually reaches values up to 50% of the original height of the sectioning area and is irreversible, since no stretching is possible. Therefore reduction of width is a precondition for good results and trimming under the optimum preconditions described above (see Figs. 19 and 23) is an urgent need. If severe compression occurs, reduction of sectioning speed may also help in some situations, but
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Figure 24. Vise-type holders for the extremely thin disc shaped high pressure frozen specimens FS (diameter \(d = 2\) mm, thickness \(t = 0.1\) to \(0.6\) mm) must guarantee proper clamping without breaking those small fragile samples. Further explanation and comments in the sections on "High Pressure Freezing" and "Ultrathin Sectioning...". See also Figs. 12a, b and c.

(a) If a suitable workshop exists, a home-made holder (similar to Michel et al., 1991, Fig. 2, p. 6, modified) following the schematic drawings (side view above, top view below) is a suitable solution: the holder base of stainless steel consists of a cylinder part CP with V-shaped groove VG for mounting and of the lower part VI of the vise (dimensions of CP/VG identical with standard cryo-specimen holder of FCS-system). The corresponding vise clamp VC (also stainless steel) is orientated by the steel roll RO (diameter approx. 2 mm, similar to rolls used in roller bearings) and a second roll RO' inserted perpendicularly to RO between VI and VC. RO' acts as bearing for VC, which has a corresponding V-shaped groove VG'. Circular spring elements SE ("Tellerfedern") open the vise VI/VC automatically if screw SC is unlocked. With respect to the extremely thin frozen discs FS, the screw thread of SC should be as small as possible (e.g., 0.5 mm per revolution). (b) Commercial vise-type holder (accessory for Leica-Reichert cryochambers FC4/FC4D/FC4E/FCS/FCR) is a useful alternative: the cylindrical part CP/VG corresponds exactly to (a). The vise lever VL turns around axis A1 mounted in CP. The small vise clamp VC' turns around axis A2 mounted in VL. With respect to the small diameter \(d\) and thickness \(t\) of the disc shaped frozen specimen FS an adjustment piece AP (upper diagram: profile corresponds to thickness \(t\) of FS) has to be inserted to guarantee proper clamping by screw VS. The vise VC'/VL opens automatically, if VS is unlocked (spring element SE'). Instead of a loose adjustment piece AP an element AP' with the same profile is recommended. AP' can be exchanged with insert element IE hold by screw HS (compare upper and lower diagram). An Indium plate between FS and one of the vise elements VC' or AP or AP' simplifies and ensures proper clamping of FS.

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the facet angle of the cryo diamond knife used is much more important (Richter, 1994). Cryo diamonds with a facet angle of 35° produce far less compression than the usual standard cryo knives with a facet of 45°, which should not be used for fresh-frozen specimens. It is not difficult to draw the conclusion, that facet angles around 20°, which are technically possible will offer tremendous advantages in comparison to the

"low angle knife" with a facet angle of 35° or less nowadays already commercially available (Jesior, 1986, 1989). If low angle knives are used, the clearance angle \(\epsilon\) is an important additional parameter, because facet angle \(\alpha\) and clearance angle \(\epsilon\) influence compression together. It is therefore recommended to reduce also the clearance angle \(\epsilon\) to the minimum value possible.

Ultrathin Cryosections for Element Analyses

There are some differences between section
preparation for frozen-hydrated investigation including electron diffraction and preparatory work on ultrathin cryosections for subsequent element analyses, since freeze-drying is an essential step preceding most of these analyses. If we exclude frozen-hydrated EDX according to Hall and Gupta not considered in this review article (see the excellent review of Gupta and Hall, 1981) we have to notice therefore those different preconditions.

 Mostly the demanding criteria for frozen-hydrated work in the TEM/STEM with respect to vitrification and sectioning are not considered as strict guidelines for the variety of different analytical methods working on dry cryosections. Besides EDX and EELS these methods include nowadays microanalytical techniques like proton probe X-ray microanalysis, laser microprobe techniques (LAMMA), and even optical microscopy techniques concerned with ion and element localisation (see e.g., Roomans et al., 1994). In many cases (e.g., EDX, LAMMA) somewhat thicker sections may be advantageous with respect to the total number of available signals. In other cases, for example for the very promising use of EELS in combination with a high performance field emission STEM system extremely thin cryosections in thicknesses below 100 nm are needed (Leapman et al., 1994; Andrews et al., 1994): only such ultrathin cryosections exclude disturbing multiple scattering processes and offer the wanted higher sensitivity of this set up for some physiologically interesting elements with low atomic numbers like Ca. But real vitrification also under these preconditions is not regarded as a precondition and as far as EDX analyses are concerned, even hexagonal ice segregation seems often to be disregarded (e.g., Hagler and Buja, 1984; Roomans and Shelburne, 1983; Roomans et al., 1982; Shuman et al., 1976; Zierold, 1982, 1987; Zierold and Hagler, 1989). Fresh frozen specimens for EDX are mostly sectioned at temperatures considerably above the obtainable minimum temperature of cryochambers, e.g., around -120°C. This makes a remarkable difference because brittleness of the material at -120°C is considerably reduced in comparison to minimum temperatures of cryochambers ranging between -165 and -180°C. Finally sectioning is much easier if the specimen is homogeneously frozen and coarse ice segregation is excluded. Microcrystalline cubic ice or generally homogeneous nucleation does not seem to be a severe drawback in cryosectioning.

 As previously stated, element analyses on ultrathin sections by EDX with respect to the much higher electron doses needed for sufficient signals is only possible after freeze drying. Some new results in this respect have just been presented and discussed (Edelmann, 1994b). They lead to new conclusions. Formerly the considerable shrinkage of freeze dried ultrathin sections (see e.g., Sitte et al., 1994) was accepted like a physical law. There was agreement, that this shrinkage is a result of the "thermal collapse phenomena" described by Kellenberger and co-workers [see e.g., Kellenberger (1987, 1991), Kellenberger et al. (1986)] and could not be influenced to a noticeable extent. In the meantime Ludwig Edelmann of our group has clearly demonstrated, that not only can shrinkage be reduced from the range of 50% to approximately 5% by elongated drying protocols, but also, that thermal collapse phenomena due to speedy drying may provoke redistribution of ions within the frozen tissue [Edelmann (1994b), see discussion with the reviewers, pp. 76 and following): mostly the time for sufficient drying, that means for minimising artefactual shrinkage is considerably underestimated and even ultrathin cryosections in thicknesses of 100 nm and less need drying times elongated to 12 h and over in the low temperature range for proper preservation. Such preparations show only a negligible shrinkage: besides the good preservation such freeze dried sections have the advantage, that all crevasses, which disturb the images of the frozen-hydrated sections, disappear nearly completely during freeze drying as shown in Fig. 25. Cryosorption freeze drying for such long-term freeze-drying is an elegant and efficient tool, since such instruments work absolutely silent and with negligible running costs (see e.g., Sitte et al., 1994). It does not seem to be necessary to carbon coat such freeze dried cryosections, since a cryotransfer system for the transfer of the cold dry cryosections on the grids from the cryosorption freeze dryer to the loading station of a cryotransfer system is available [grid holder of the dryer, see Fig. 7, p. 53, items GB/GC in Sitte et al. (1994)].

Investigation of Frozen-Hydrated Ultrathin Sections

TEM/STEM investigation and image recording of frozen-hydrated ultrathin sections suffer from two severe drawbacks: fresh frozen biological material is extremely sensitive to electron bombardment in the scope and reveals mostly a very faint contrast in thin layers additionally blurred by the large number of electrons inelastically scattered by the ice matrix and the elements with low atomic numbers. Low dose operation of the scope is an urgent need, since the sectioned material disintegrates rapidly, as already observed and clearly stated by Dubochet and co-workers in the early days of investigations on frozen-hydrated material [see e.g., Chang et al. (1983), Dubochet and McDowall (1984), Dubochet et al.
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(1983, 1986, 1987, 1988); see also Isaacson (1977), Talmon (1987), Talmon et al. (1986). In the eighties efficient low dose systems were not available. Only screening with quite simple TV-systems (e.g., SIT-cameras) and focusing next to the preselected area ("MDF-systems" for Minimum Dose Focusing, mostly following the "rocking beam" principle) gave a possibility to reduce irradiation (electron doses). But just around 1980 some phenomena were observed, which supported the expectation that biological specimens at very low temperatures around 4K show a considerable (in favourable cases up to 300 times) higher stability under the electron beam in comparison to ambient temperature (Chiu et al., 1981; Dietrich et al., 1977, 1978, 1979, 1980; Dubochet, 1981; Dubochet et al., 1981, 1982c; Freeman et al., 1980; Knapek and Dubochet, 1980; Knapek et al., 1982; K.H. Müller et al., 1981). These data were obtained mainly on thin dry crystals of organic compounds by registration of the decay (fading) of diffraction patterns and showed a similar stabilising effect as measurements of the mass loss under the electron beam (Dubochet, 1975; Ramamurti, 1977; see also Isaacson, 1977; Salih and Cosslett, 1975; or Cosslett, 1978 and Fig. 26). That gave some hope, since such extremely low temperatures are obtainable by the use of super conducting LHe cryostat lenses in the TEM firstly designed by Fernández-Morán (1966; see also Fernández-Morán, 1985) and brought to a high degree of perfection by the Dietrich group in Munich (Dietrich et al., 1977, 1979, 1980).

With respect to the importance of such a "cryostabilisation" the exciting values obtained in the above-mentioned first run were checked by several laboratories using different instrumentation but similar samples. The results obtained in this second run by an "International Experimental Study Group" and some others could in several respects not reproducibly confirm the data collected before [see "International Experimental Study Group" Chiu et al. (1986), Downing (1983), Wade (1984), Wade and Pelissier (1982), Lamvik et al. (1983); see also Lepault et al. (1983b), Dubochet et al. (1987, 1988). They confirmed more or less previous experiments which resulted in "cryostabilisation effects" between zero and x10 [see e.g., Glaeser (1971, 1975), Glaeser and Hobbs (1975), Glaeser and Taylor (1978), Grubb and Groves (1971), Hayward and Glaeser (1979), Heide et al. (1982), Siegel (1972)]. Though in some details more material and time for additional experiments seemed to be necessary, there was at least in 1985 a general agreement on two important facts: (1) The "cryostabilisation" even at LHe temperature reaches in most studies only a factor of x3 to x10 in comparison to TEM work at room temperature, (2) There seems to be no noticeable difference between the "cryostabilisation" at LHe- and at LN2-temperature, if decay (fading) of diffraction patterns is considered. In other words: this stabilisation effect is mostly already available with LN2 cooling. The big effort for LHe-technology does not seem to be needed. Of course, most of these studies refer essentially to dry structures, which differ considerably from frozen-hydrated sections. But water-containing structures seem to behave similarly (Knapek and Dubochet, 1980). As far as a really similar "cryostabilisation" could be obtained on frozen-hydrated specimens, also the smaller stabilisation factor is highly welcome. But this kind of "cryostabilisation" was then obviously already reached and used by Dubochet's group during all investigations on frozen-hydrated specimens. They used the LN2 cold stage in the early eighties to maintain the amorphous frozen state both in ultrathin cryosections and in "bare grid" preparations of suspensions (e.g., Dubochet et al., 1988). It is important to remember that this kind of cryostabilisation could not prevent "bubbling" (immediate radiolysis effects) under the electron beam during investigation and diffraction analysis in the TEM. It is therefore of paramount importance for further work in this field to use real low dose systems, which effectively either reduce the irradiation needed for recording structures and diffraction patterns or lower the beam damage reproducibly.

As mentioned above, another parameter also influences the investigation of frozen-hydrated material in the TEM considerably: since only fresh frozen, not pre-treated and therefore unstained samples have to be investigated, there is a considerable lack in contrast. With respect to the "phase contrast transfer function" (see e.g., Dubochet et al., 1987, Fig. 3, p. 125) useful pictures with a sufficient contrast in the conventional TEM (CTEM) are mostly only obtainable considerably below the correct focus: usually the under-focus of such pictures ranges between 1 μm and 10 μm. It is difficult under those conditions to resolve small details and a big gap between the real resolving power of a modern CTEM in the atomic or molecular order (e.g., approximately 0.3 nm) and the reality in the photographic picture (e.g., 3 nm and over) results. At least partially this inconvenience can be compensated by "Electron Spectroscopic Imaging" (ESI) using an "Energy Filtering TEM" (EFTEM), since such systems allow the elimination of the relatively large amount of inelastic scattered electrons and their influence on the final picture (Bauer, 1988; Ottensmeyer and Andrew, 1980; Ottensmeyer et al., 1981; Schröder, 1992; Schröder et al., 1990). Since the ice matrix and the atoms with low atomic numbers of the
specimen embedded in the ice matrix increase inelastic scattering processes dramatically, "zero loss imaging" and image recording with an underfocus below 0.1 μm close to the "Scherzer focus" (see Schröder et al., 1990) is a considerable advantage for frozen-hydrated ultrathin sections. The combination of low dose systems with an ESI seems to be the most promising investigation technique in this field.

With respect to low dose investigation, there exists the already mentioned simple possibility to reduce the electron dose or the number of electrons needed for the formation of a photographic image by MDF-systems. This is possible by a lateral shift of the specimen or the electron beam between the first step of focusing and the subsequent recording of the image on a sensitive photographic emulsion. Similar to the general operation of the TEM usually an investigation of a frozen-hydrated specimen needs three steps. Screening, that means looking for a suitable section area for subsequent documentation is the first step: it is done at low magnification with a minimum beam intensity. Even this low intensity may be reduced by a suited image intensifying TV-system. The electron doses needed for this screening operation under these optimised conditions are negligible in comparison with the two following steps. These steps usually need considerably higher magnification and beam intensity for focusing and recording. According to experience in many laboratories, image intensifiers connected to a TV-screen are mostly not sufficient for focusing (even with special aids like Wobbler systems) and for the recording of images (Cosslett, 1978). The focusing step contributes considerably to the whole electron dose, if the selected area is used for focusing. Therefore this second step is often spatially separated from the definite third step, that means the electron exposure of the photographic emulsion: focusing takes place on an area nearby and not on the area selected for the picture. If the electron irradiation is limited to the field of the fluorescent screen observed with the light microscope (e.g., by "Köhler illumination"), the electron dose could be reduced to the small dose needed for screening and the considerable higher dose needed for a sufficient exposure of the photographic emulsion. This irradiation could not be circumvented in the past as long as only photographic emulsions were available. But also this consideration is rather theoretical: the exposure is not only affected by drift phenomena often connected with a lateral x/y-movement of the specimen or a deviation of the beam, but suffers also from the irregular profiles typical for ultrathin cryosections, which influence the focus if focusing and recording are made in different areas of such a section. Real progress in low dose investigation is therefore only possible by replacing the photographic emulsion by another tool which is more sensitive for electron irradiation, and the application of all other means of reducing beam damage like higher voltages and better vacuum conditions (see e.g., again Cosslett, 1978).

Nowadays two low dose systems fulfill the mentioned precondition, one of which (slow-scan charge-coupled devices, abbreviation: SS-CCD) is already used in many laboratories on different TEM models (see e.g., De Ruijter, 1995; Krivanek and Mooney, 1993; Tietz, 1992). The other system ("image plates" with a photo and electron stimulable phosphor layer instead of a photographic emulsion) has been in experimental laboratory use for some time in different places and scopes (Ayato et al., 1990; Burmester, 1992; Mori et al., 1988, 1990; Oikawa, 1990; Shindo et al., 1990, 1991). An image plate system of this kind for the TEM developed by Fuji has already been successfully tested in two Japanese EM-laboratories. This Fuji FDL-5000 system is just now introduced and commercially available for TEMs (1996). Besides other advantages both systems offer a real and remarkable reduction (> 10:1) of the electron dose needed for the digital recording of an electron image or an electron diffraction diagram in comparison with the most sensitive photographic emulsion available hitherto. The author of this review article has no first hand experience with SS-CCD and image plates. Therefore only second hand information from literature, colleagues and manufacturers was available.

This has to be considered, if conclusions are drawn from the comments in this section and in the following discussion. But it seems necessary to include also these recent breakthroughs in the review with respect to their paramount importance for cryowork on the extremely beam sensitive frozen-hydrated sections.

The first Slow Scan CCD camera was invented and basically developed at Bell Laboratories in USA around 1970. It was in astronomy that SS-CCD systems were firstly used in scientific research for low-light-level imaging. Scientific grade CCDs were introduced by Janesick and co-workers (Janesick et al., 1987). They are now used in different configurations for many purposes in scientific research. The first attempt for EM work was made by Mochel and Mochel (1986) using a Photometrics SS-CCD on a VG-HB5 SEM. The first purpose designed SS-CCD was built in co-operation between UCSF, Gatan and Photometrics in 1987 for a Philips EM 430. Similar systems are now commercially available from Gatan and compatible with most TEM models. In the following years a couple of SS-CCD devices were developed and described (De Ruijter, 1995). A new commercial-
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Figure 25. Freeze-drying of ultrathin cryosections is not necessarily connected with artefactual shrinking and cracks in the sections, if the drying procedure is extended over a sufficiently long period at low temperatures, as demonstrated by the electron micrographs (a) and (b). They show the same specimen at the same magnification before (b) and after freeze-drying (a). Preparation and electron micrographs by Edelmann (reproduced from Edelmann, 1994b, with permission): frog sartorius muscle, slam frozen on LN2 cooled copper mirror (Edelmann, 1989b), cryosected at -160°C on Reichert Ultracut-S/FCS with Diatome diamond knife, Simco antistatic device, cutting speed 0.4 mm/sec. Sections placed on uncoated 600-mesh copper grids. Both pictures recorded at 4400 x in Zeiss EM 902 with energy filter (doses < 500 e-/nm²). Arrows in circles: direction of sectioning. Bar: 1 µm. Further explanation in the section on "Ultrathin Section Preparation..." and the Discussion. (a) Ultrathin section freeze-dried in Leica CFD cryosorption freeze dryer according to Edelmann and Sitte (see Sitte et al., 1994) for 33 h at -100°C. (b) Frozen-hydrated section under identical conditions shows practically identical dimensions of A-bands. Ultrastructural details partially hidden by crevasses obliquely to the sectioning direction (arrows CR), which disappear during freeze-drying (see Figs 20 and 25).

SS-CCD was recently presented by Carl Zeiss, which developed a high resolution, variable speed camera for its models EM 906, EM 910 and EM 912 Omega. Finally Gatan has surpassed in 1995 the most severe limitation for SS-CCDs in electron microscopy by offering a new 2k x 2k SS-CCD "MegaScan" with a frame of approx. 40 x 40 mm² approaching towards the dimension of photographic films and image plates (Gatan, 1995). The principle of such scientific grade SS-CCDs for digital recording of TEM images or diffraction patterns according to Fig. 27 consists mostly of an electron sensitive scintillator, a fibre optic coupling plate and the CCD chip below. Both Yttrium-Aluminum Garnet (YAG)-single crystal scintillators and Gadolinium Oxy-Sulphide (GOS) powder phosphorus (e.g., P20 or P43) scintillators mounted on the top of the fibre optic plate were used successfully. Instead of the fibre optic coupling (e.g., 1:1 or with a

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Figure 26. Electron beam damage: temperature dependence of mass loss (a) and "critical dose" (b) under electron irradiation. Diagrams according to Cosslett (1978), Figs. 3 (p. 120) and 5 (p. 125), redrawn and slightly modified, with permission. Both diagrams show, that beam damage is strongly temperature dependent and that near absolute zero (e.g. at LHe temperature) a considerable increase of the "cryostabilisation effect" seems to be possible. Nevertheless previous statements about stabilisation factors above x 100 had to be revised (see Chiu et al., "International Study Group", 1986). The factor varies considerably from specimen to specimen and even from measurement to measurement (see Dubochet, 1975, Section 3 "Irreproducibility", pp. 284 ff) and remains probably generally in the range below x 10. See also further comments and additional references in the Introduction, the section on "..Frozen-Hydrated Ultrathin Sections" and the Discussion, and the review of Glaeser (1975). (a) Variations of the mass loss from a 40 nm thick film of phenylalanine with increasing electron doses at 15 kV at constant temperatures of -266°C (7°K), -223°C (50°K) and room temperature 20°C (293°K) according to preliminary data of Ramamurti (1977). The findings of Ramamurti agree very well with results of Dubochet (1975), which states after studies of the carbon loss of T4 phages and E. coli bacteria in different TEMs operated with 60 and 80 kV, that "there is no perceptible carbon loss when the irradiation is made at LHe temperature". (b) Variation of the critical irradiation dose for Coronene at 500 kV with temperature according to measurements of Salih and Cosslett (1975). The "critical dose" was measured by the disappearance of the diffraction patterns of the Coronene crystals. There occurs a considerable reduction in radiation sensitivity below LN2 temperature (-196°C). But the variations in these measurements do not allow a definite conclusion about this "stabilisation effect" at LHe temperature.

reduction 2:1) a lens-mirror coupling is possible and was already used in practice (2:1 fibre optic reduction and lens-mirror-coupling are not included in the schematic diagram of Fig. 27). Under those circumstances one 100 keV electron frees 2000 visible photons on the YAG-scintillator or 7000 visible photons on the more sensitive GOS-scintillator. The most efficient channelling of those photons is achieved with a glass fibre coupling (10 to 15 % efficiency). Approximately 30 % of the channelled photons are transferred into "well electrons" for the digital read-out. Those electrons are confined into square adjacent pixels by "potential wells" generated by a conductive "gate struc-

ture" on the top of the insulating SiOz-layer. The gate arrangement delivers typical square pixel sizes in ranges from 6 to 27 µm edge length [for technical details and additional literature see De Ruijter (1995), Section B and Figs. 1-3]. One 100 keV-electron corresponds under the mentioned preconditions to 60 (YAG) or 300 (GOS) well electrons. Since read-out noise at a read-out speed of 500 KHz ranges around 25 well electrons, one obtains a sufficient signal-to-noise ratio and a registration of single 100 keV-electrons [theoretical statistical value of detection: 0.3 electrons of 100 keV; all data and numbers according to Krivanek and Mooney (1993)].

The basic function of SS-CCD-systems is quite simple: the needed photons for the photon sensitive CCD are excited in the scintillator by the electrons of the beam and guided by the glass fibres of the fibre optic plate or collected by a lens to the polysilicon electrodes (CCD-chip; see Fig. 27a). The analogue output signals from the chip are further amplified and digitised by an intermediate electronic system and subsequently transferred to the memory of a computer. This "read-out" procedure is quite slow [approximately 2x10^4 to 2x10^6 pixels/sec according to De Ruijter (1995)] with respect to a sufficient charge transfer from relatively large areas (over 1024 x 1024 pixels) and to a low read-out noise. Read-out times are...
Table 6. Influence of pixel size, detector surface area and SS-CCD-read-out time on low dose image acquisition: according to Burmester (1992) the limited usable polysilicon detector surface of slow scan CCD cameras influences the efficiency of the system considerably in comparison with photographic plates (films) or phosphor image plates. Burmester proposes and uses a "Pixel Equivalent" in the comparison: the real pixel size is calculated using the relation between the frame size of the common photographic materials (60 mm edge length) and the size of the polysilicon electrodes (CCD-chips, see Fig. 28), since smaller magnifications are necessary to image the same specimen area on the smaller CCD-chip. Different from Burmester (1992) as size of the photographic plate or film compared to the phosphorus image plate 80 mm edge length are calculated (Fuji image plate measures 99.6 x 80.9 mm\(^2\), effective usable area 94.0 x 75.0 mm\(^2\) identical to photographic plates for most scopes). The pixel equivalent (PE) according to Burmester is given by the equation "Pixel size x 80 mm divided by detector edge length", e.g. from Gatan 512 x 512 ..... PE = 19 x 80/9.7 = 157 \(\mu\)m). One has to remember, that during CCD read-out beam blanking is necessary, which either causes delay (deflection above specimen) or additional beam damage (deflection below the specimen with respect to drift phenomena). Read-out speed and mode therefore are important parameters especially for systems with high pixel numbers and special measures (e.g. "subarea read-out" as offered for Gatan MegaScan) are really necessary for low dose work. Calculation of minimum and maximum read-out times according to De Ruijter, 1995, with lower and upper read-out values of 2 \(x\) 10\(^4\) and 2 \(x\) 10\(^6\) pixels/sec. "Subarea read-out" and "Modulation Transfer Function" (MTF, see Krivanek and Mooney, 1993; De Ruijter, 1995; Weickenmeier et al., 1995a,b) for the different SS-CCD systems are not considered in the calculation of the read-out times.

<table>
<thead>
<tr>
<th>System</th>
<th>Array (Pixels)</th>
<th>Pixel Size</th>
<th>CCD-Chip Surface (mm(^2))</th>
<th>Pixel Equivalent PE</th>
<th>Minimum Read-out Time</th>
<th>Maximum Read-out Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gatan 512 x 512</td>
<td>19 (\mu)m</td>
<td>9.7 x 9.7</td>
<td>157 (\mu)m</td>
<td>0.13 sec</td>
<td>6.5 sec</td>
<td></td>
</tr>
<tr>
<td>Gatan 1024 x 1024</td>
<td>27 (\mu)m</td>
<td>27.6 x 27.6</td>
<td>78 (\mu)m</td>
<td>0.52 sec</td>
<td>26.2 sec</td>
<td></td>
</tr>
<tr>
<td>Gatan Mega-Scan</td>
<td>2048 x 2048</td>
<td>24 (\mu)m</td>
<td>(49.2 x 49.2)</td>
<td>39 (\mu)m</td>
<td>2.10 sec</td>
<td>104.9 sec</td>
</tr>
<tr>
<td>Tietz 512 x 512</td>
<td>38 (\mu)m</td>
<td>19.5 x 19.5</td>
<td>155 (\mu)m</td>
<td>0.13 sec</td>
<td>6.5 sec</td>
<td></td>
</tr>
<tr>
<td>Tietz 1024 x 1024</td>
<td>19 (\mu)m</td>
<td>19.5 x 19.5</td>
<td>78 (\mu)m</td>
<td>0.52 sec</td>
<td>26.2 sec</td>
<td></td>
</tr>
<tr>
<td>Tietz 1024 x 1024</td>
<td>24 (\mu)m</td>
<td>24.6 x 24.6</td>
<td>78 (\mu)m</td>
<td>0.52 sec</td>
<td>26.2 sec</td>
<td></td>
</tr>
<tr>
<td>Zeiss Vario-speed 1024 x 1024</td>
<td>19 (\mu)m</td>
<td>19.5 x 19.5</td>
<td>78 (\mu)m</td>
<td>0.13 sec</td>
<td>500 sec</td>
<td></td>
</tr>
</tbody>
</table>

Most data concerning arrays, pixel sizes and CCD-chip surfaces according to Burmester (1992, Table 2.1, p. 6) dependent on the frequency of the CCD-system: they are mostly situated between 500 kHz and 2 MHz. A further increase to 5 MHz seems to be possible and advantageous. Another possibility to cut down the read-out cycles are sub-area read-out modes. Contrary to broadcast compatible CCDs the whole chip surface is used as image detector. To avoid CCD-exposure during charge read-out a shutter is needed to avoid smearing of image information. This shutter is activated by computer control using an existing beam deflector of the scope. Mostly this "beam blanking" is realised above the specimen with one of the gun-shift controls. This procedure minimises the electron dose but is not always applicable if high resolution is needed and read-out times increase above a critical level. In such cases drift of the specimen, image and/or focus sometimes results from blanking by local heating or charging. In such cases continuous irradiation of the specimen during read-out is unavoidable and the deflection has to be arranged below the specimen. Fig. 27b shows the whole set up of a TEM equipped with an SS-CCD camera. The camera itself is mounted at the bottom of the column and is exchangeable against other detectors by a special construction. During venting the column of the scope the whole CCD-camera is retracted into a housing and in this way protected from frost layers on the cold surfaces.

Several details of the design of SS-CCD
**Figure 27.** "Slow-Scan Charge-Coupled Devices" (SS-CCD systems) are one of the two promising technologies for low dose investigations on TEM/STEM-spectroscopes. Further comments and references in the section on "..Frozen-Hydrated Ultrathin Sections" and the Discussion. See also Table 6 and Figs. 28 to 30. (a) Basic principle of an SS-CCD camera for electron microscopes according to Krivanek and Mooney (1993, Fig. 1, p. 96, modified): the fast high energy electrons (e.g., 100 kV e⁻) hit the scintillator SC (e.g., YAG or GOS phosphorus) and produce visible photons (approx. 2000 to 7000 photons, dependent from the scintillator composition). Approximately 10% of these photons are "channelled" through glass fibres of the fibre plate GF to the polysilicon CCD-chip below. To reduce thermal noise the chip is cooled by the Peltier element PE. Approximately 30% of the channelled photons excite low energy "well electrons" suited as signals for digital "read-out" via pre-amplifier PA by the computer electronics EL. The read-out picture is transferred on-line to the memory ME of the computer and to the TV screen. During read-out "beam blanking" by shutter elements GS (gun-shift controls above specimen SP in side entry rod SE) or FS (fluorescent screen below object OB) is necessary to avoid continued CCD exposure and "smearing" of image information. "Autotuning" of the scope (e.g. autofocus and autostigmation) is possible by intersections with the lenses OL/PL and stigmator ST. (b) Set up of the scope with an SS-CCD system according to diagram (a): the SS-CCD camera is mounted on the base of the column CO below the fluorescent screen FS. EG = electron-gun, GP = glass plate for visual observation of picture on FS, HO = housing for SS-CCD camera retracted during venting of column. Computer control EL for read-out and autotuning according to (a). (c) The detector area of CCD-chips is limited for surfaces between 19 x 19 mm² (Standard with 1k x 1k Pixels) and 40 x 40 mm² (Gatan MegaScan with 2k x 2k Pixels). The CCD-picture of a carbon black particle shows the superb quality of such images (0.34 nm lattice planes of carbon: arrows) and represents less than 1% of the whole on-line image area of a Gatan MegaScan CCD (Specimen and electron micrograph: Gatan Inc., Pleasenton, CA, USA, with permission of Gatan-Munich).

Systems are important for practical use. The scintillator has to be covered by a thin electrical conductive gold or aluminum layer to avoid charging phenomena. The SiO₂ layer of the CCD detector with its "gates" is usually cooled to a temperature around -40°C by a Peltier element or a cryogen to reduce thermal charg-
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Figure 28. Photo- and electron-stimulable phosphor image plates (IP) are nowadays already commercially available for TEM work by Fuji (FDL 5000 system) as a substitute for photographic films. They have an identical size, similar thickness and consistency. The extremely high sensitivity to electrons of different energy levels (e.g., 50 keV to 1000 keV) make these plates suited for low dose work in different TEM/STEMscopes. Off-line read-out is accomplished by a laser beam IP-reader. Read-out information is mostly stored in DAT-cartridges. Final prints are made by Power PC and different compatible printers. After erasing the plates are re-usable. Further explanations and references in Sections 9 and 10. See also Fig. 29. Schematic diagrams according to Fuji leaflet 94.6 - SK 5-1 (AB), Mori et al. (1988, 1990) and Oikawa et al. (1990) with permission, (a) Schematic cross section diagram of an image plate IP: the IP consists essentially of the electron sensitive phosphor layer PL and a support SU. Both surfaces are covered by thin layers (PR = protection layer, BA = back layer). PL consists mainly of phosphorous with some barium halogenide compounds doped with Eu$^{2+}$ to store the energy of incident electrons. (b) "Empty" IPs are inserted into the film magazine of the TEM (see Fig. 29). After exposure by electrons, off-line read-out by laser beam scanning and registration of luminescence photons by photomultiplier. Finally, after erasing by visible light they are re-usable. (c) Comparison between the ratio of signal intensity (arbitrary units / optical density of prints) and electron doses for phosphor IPs and standard photographic emulsions (Fuji FG film) used for TEM work. Note the excellent linearity of the IPs, which is identical to SS-CCD systems (Fig. 27) in comparison to photo emulsions.

Electronic compensation of those defects by "gain normalisation" or "flat field correction" is a must, to obtain the wanted beautiful pictures. Of course: such cosmetic "corrections" or "normalisations" cannot restore the original information of the area affected by the above mentioned defects in the chain of information transfers, but they contribute probably considerably to a satisfying aesthetics and a good feeling, since easily recognisable irregularities like black dots or areas no more exist. But with respect to low dose work the sensitivity of the system and that means mainly the sensitivity of the scintillator (GOS-phosphor is 3.5x more sensitive than YAG) and the time required for all on-line operations (e.g., read-out, autotuning of the TEM) are much more important than all cosmetics and there is now a lot of possibilities to improve the situation.

A considerable experience has already been acquired in the application of those CCD cameras and some remarkable features are well known. Among these features, besides a real dose reduction >10:1 in comparison to the most sensitive photographic emulsion there is strong linearity between electron dose and signal intensity (see Fig. 28c). This feature is especially advantageous for quantitative measurements and evaluations, since complicated corrections (linearisation) are not needed, which made quantitative work based on photographic images so complicated and frustrating. A further advantage of the SS-CCD system is the wide dynamic range, which enables the registration of a diffraction pattern within only one run. Since SS-CCD systems work "on-line", they are finally elegantly suited for different modes of automatic adjustments of the whole electron optical system of a TEM. This "autotuning" includes a couple of automatic adjustments like "autofocusing" (Krivaneck, 1975 cited by Krivaneck and Mooney, 1993; Fan and Krivaneck, 1990; Krivaneck and Mooney, 1993; Dierksen et al., 1993; De Ruijter, 1995), automated electron tomography (Dierksen et al., 1993; Koster and De Ruijter, 1992) and automatic astigmatism control ("autostigmation", see Krivaneck and Mooney, 1993). Different to more complicated earlier autotuning procedures and to the "Tilt Induced Displacement" [TID, see Koster and De Ruijter (1992), Krivaneck and Fan (1992)], the "Automatic Diffractogram Analysis" [ADA, see Fan and Krivaneck (1990), Krivaneck and Mooney (1993)] needs under optimum conditions only one diffractogram (exposure 0.5 to 1.0
analysed by automatic computer routine, which takes additionally 8 sec of processing time on a Macintosh Quadra for complete autofocusing and autostigmation. According to Krivanek and Mooney (1993) this processing time will decrease again considerably, if new computer generations with increased power will be available. I suppose, that this is today (1996) certainly already reality. Gatan offers for these purposes a complete software package within its Digital Micrograph™ Model 700-0000. And this is probably just the start for further developments making the operation of a TEM more accurate and reproducible even under the extreme conditions of cryowork.

In some respects the image plate is a competitive system to the SS-CCD camera. In comparison to CCD the image plate does not need a special installation at the TEM itself, since this "plate" has the size and consistency of a photographic film and fits into the film magazine of practically any commercial TEM. Also the handling and exposure of the image plate has some similarity to the use of a photographic film. According to Fig. 28a the image plate consists basically of a flexible support film with a thickness between 0.1 and 0.2 mm. This support carries a photo- and electron-sensitive phosphorus layer in a thickness around 0.12 mm covered by a protection coat of several micrometers. The photo-stimulable phosphorus layer contains [BaFBr:Eu] which serves as detector for photons and electrons. The theory of the activation process seems to be still a matter of discussion [Burmester, 1992; Daberkow et al., 1991; Hangleiter et al., 1990 cited by Burmester (1992); von Seggern et al., 1988; Takahashi et al., 1988]. During exposure of the image plate photons or, in the TEM, electrons change charges of the detector complex (e.g., [BaFBr:Eu] → [BaFBr:Eu2+]). These charges are "stored" by the detector complexes. Digital read-out of these charges is possible by laser beam scanning, which causes an emission of visible light. After this read-out procedure any remaining TEM image on the plate is erased by visible light. After erasing the plate is usable again. The described cycle "electron exposure → reading → erasing → electron exposure" (see Fig. 28b) can be repeated several times. There is no actual experience available how often this cycle can be repeated after erasing, since delivery of the new Fuji FDL-5000 system just starts and exposure by electrons may be somewhat different to exposure by photons. Fig. 29 shows a schematic diagram of the new FDL-5000 image plate system of Fuji with all accessories necessary for TEM work. The image plates are mounted in TEM cassettes exactly identical to those for photographic films or plates and stored for use in the TEM magazine. After exposure they are inserted in the "reader" (laser scanning device) connected to a Power PC terminal. All digital data are stored on DAT cartridges for printing either by a conventional laser printer or by a very sophisticated high quality printer operated by a second PC. Of course: this periphery like the processing hardware and software
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for an SS-CCD system is a considerable investment but in this case represents also the equivalent for the compatibility with practically any modern TEM.

According to the claims of Fuji the features of the new FDL-5000 system are really interesting: in comparison to commercial SS-CCD systems the image plates offer probably a slightly higher sensitivity and an even wider dynamic range together with a similar linearity of electron dose versus signal intensity (see Fig. 28c) and a similar pixel size of 25 μm. But similar pixel sizes count only under the precondition that the same magnification is used. The striking difference relates to the considerable larger area of the image plate (effective area 94 x 75 mm²), which is identical to photographic films used for TEM work. This area surpasses CCD-frames 1k x 1k by a factor of 16 and even the even MegaScan 2k x 2k of Gatan by a factor of 4. If periodic crystalline structures of small sizes have to be recorded, at a first glance one may believe, that the area makes no important difference. But in practice this makes perhaps the point, since periodic structures in biology are exceptions and aperiodic ones are the rule. If we want to record a given surface, we must do this with the smaller detector size of the CCD-chip at a considerably smaller magnification: provided that we work at the correct focus in both cases, we lose accordingly a lot of pixels or resolution with the smaller CCD system. If we do not want to lose resolving power, we have another problem: at identical magnification, we lose a lot of surface area in our picture and therefore information about our aperiodically structured specimen. In comparison between the Fuji image plate and the largest SS-CCD detector for a TEM (Gatan MegaScan = 4k x 4k) we lose 3/4 of the picture. Vice versa the surface recorded on the image plate under comparable conditions (same resolution, same magnification, similar pixel size) is 4 x larger than the SS-CCD picture. This relates to the "Pixel Equivalent" defined by Burmester (1992, see also Table 6) and concerns certainly also future prospects of image plates and vice versa SS-CCD systems. From the technical point of view I see no severe problem, to increase the surface of image plates again above the value of 94 x 75 mm². One may for example use the whole circular field below the fluorescence screen of the scope to gain additional pixels. Read-out times do not hamper, since off-line recording is a must for image plates. In this respect the real potential of the image plate is not yet exploited. But I have the feeling, that some difficulties will arise, if the detector size of SS-CCD systems should be increased once more considerably above 40 x 40 mm². This relates mainly to the cost of manufacturing perfect CCD-chips and fibre plates in larger dimensions and to develop subarea read-out facilities working fast enough for a meaningful on-line operation. I guess that there will be no severe border of technical possibilities. But the question of the relation between manufacturing expenses and therefore prices and efficiency of the two different competitive systems will certainly be posed by financial reasons. Since read-out and autotuning facilities influence the value for low dose operation, nevertheless perfect large area SS-CCD systems will certainly gain increasing importance in our scientific field interested in frozen-hydrated cryo-sections.

To draw a conclusion from all the considerations mentioned above: since pixel size and sensitivity characteristics (linearity of electron dose versus signal intensity, see Fig. 28c) of the image plate are actually nearly identical to SS-CCD systems, the image plate seems to be superior to SS-CCDs in all respects, except one: an "on-line use" is absolutely impossible. The output of image plates is only "off-line" information. Of course this enables the reading-out of image plates exposed in different TEMs within one reader and the printing of the information of all DAT cartridges with the same equipment. But "autotuning" systems for tomography, autofocus, lens and stigmator and deflector calibration are not possible using image plates. It may be, that this severe disadvantage can perhaps be compensated by through focus series over the larger image areas offered by the more sensitive image plate. I cannot imagine this and I confess, that I for myself am in a somewhat "schizophrenic" situation and want to remind, that today (1996) all these considerations are situated in a risky field of speculation and that a clear prognosis is not possible. For sure, experience of the coming years will tell us more about the real advantages and disadvantages of both competitive systems in practical use for ultrathin frozen-hydrated cryosections, and also for sure, all scientists concerned with cryowork under extreme experimental conditions should be happy to have two fascinating alternatives for low dose operation of their scopes.

Considerable progress was also realised in "Electron Spectroscopic Imaging" (ESI) using TEM systems equipped with energy filters [see Bauer (1988) or Krivanek et al., (1995)]: the first "Energy Filter TEM" (EFTEM) was an "in column filter" built and described by Castaing and Henry already in 1962 (see also Castaing, 1975). Different from "magnetic prism spectrometers" (Shuman and Somlyo, 1982), which are mounted as "post column filters" below the fluorescent screen of the scope (see again Krivanek et al., 1995 and Fig. 30d), the "Castaing-Henry-filter" consists of two magnetic prisms and one electron mirror in-between (so called "prism-mirror-prism" or "PMP-
Figure 30. (on facing page) "Energy Filtering Transmission Electron Microscopes" (EFTEMs) with "Electron Spectroscopic Imaging" (ESI): different from "Conventional Transmission Electron Microscopes" (CTEMs) spectroscopic images in "zero loss mode" deliver better contrast of thin frozen-hydrated cryosections with underfocus values below 0.1 µm. Further explanations and references in the section on "...Frozen-Hydrated Ultrathin Sections" and the Discussion. (a) Spectroscopy with photons (visible light, upper diagram): polychromatic light PL in glass prism GP behaves similar to an electron beam with different speeds ("poly-energetic or polychromatic electron beam" PE, lower diagram) in a magnetic field ("magnetic prism" MP). White light PL is split (spectrum SP). A monochromatic fraction ML can be selected by slit SS. Electrons PE in MP describe circles, whose radii depend on their speed (R1 = faster electrons FE, R2 = slower electrons SE, R1 > R2). A "mono-energetic or monochromatic electron fraction" ME of PE is selected by slit SS similar PL → ML. This SS-selection can be used for "monochromatic imaging" (elimination of chromatic defects of electron lenses). This is possible either by "in column filters" (b) and (c) or by "post column filters" (d). (b) "Prism-Mirror-Prism-(PMP)-Filter" according to Castaing-Henry and Ottensmeyer (Zeiss EFTEM EM902): the mainly monochromatic electron beam ME of the electron gun EG (CL = condenser lens) becomes polychromatic (PE) by inelastic scattering in the object OB. Between objective OL and projective lens PL the magnetic prism MP and the electrostatic 80 kV mirror MI is installed. According to (a) the electrons describe circular pathways in the homogeneous magnetic field of MP, are rejected by MI and once more deflected by MP (opposite direction ... opposite deflection). The inelastically scattered slower electrons describe smaller radii and are eliminated by slit SS ("zero loss imaging"): for the image only unscattered and elastically scattered electrons are used, which correspond to ME. PMP-systems are limited to acceleration voltages ≤ 80 kV with respect to the electrostatic high tension mirror MI. (c) "Omega" in column filter (Zeiss EFTEM EM912): instead of PMP (b) four magnetic prisms MP1 → MP2 → MP3 → MP4 force the electron beam PE to a slope like the Greek letter Ω. Some correction lenses ("sextupoles" SP) are symmetrically arranged. In comparison to the PMP-systems (b) higher acceleration voltages are possible due to elimination of MI. Function and SS correspond closely to PMP (b). (d) "Post column" spectroscopic imaging (Shuman and Somlyo, 1982; Krivanek et al., 1994, 1995; Gubbens et al., 1995; modified diagram with permission of Gatan): the spectroscopic device uses only one magnetic prism MP and is mounted on the base of the TEM column CO. The TEM remains therefore unchanged and the spectrometer is suited as an accessory for conventional TEMs. Some correction lenses (8 quadrupoles QP and 7 sextupoles SP) are combined with MP and the selecting slit SS. Since the fluorescent screen (plane FS) cannot show the spectroscopic image, a TV system is used. After retraction of TV (arrow) on-line registration by CCD-camera is possible. Image plates (see Figs. 28 and 29) cannot be used with post-column spectrometers of this kind.

spectrometer", see Fig. 30b) incorporated in the column of the TEM between the magnetic lenses. Such PMP-arrangements allow all modes of observation, imaging and elemental mapping, but they have to be incorporated in a purpose-designed electron optical system and are not suited as an accessory for any TEMscope. After a period of complete silence Henkelmann and Ottensmeyer in 1974 had continued the developmental and application work with Castaing-Henry-filters (Henkelmann and Ottensmeyer, 1974; Ottensmeyer and Andrew, 1980; Ottensmeyer et al., 1981). They inserted the PMP-filter and the energy selecting slit (see Fig. 30b) into commercial TEM-scopes above the projector lens. They were able to demonstrate, that ESI is possible without an annoying loss of resolution and that zero loss imaging, that means elimination of the inelastic background shows increased contrast and superbly clear pictures of biological structures [see Fig. 8, p. 92 in Henkelmann and Ottensmeyer (1974)]. Once more a period of silence was inserted up to the moment that Zeiss introduced with the EM 902 a PMP-filter lens system based on the developmental work of Henry, Castaing and Ottensmeyer which immediately was very successful (Bauer, 1988; Benner et al., 1994). The main limitation of this system was the comparatively low beam voltage (80 kV or lower) given by the voltage limit of the electrostatic electron mirror. In the meantime this limit is eliminated by another "in column filter" of the Omega type working with magnetic prisms without an electron mirror [see Fig. 30c and Egerton (1986), Benner et al., (1994), Bihr et al. (1991), Krahl et al. (1990), Lario (1986), Lario et al. (1986), Rose (1978)]. This new energy filter type already allows an accelerating voltage of 120 kV and is incorporated in the Zeiss EFTEM 912 Omega. A further increase of the voltage seems to be possible. There is now a considerable experience about ESI accumulated both with PMP and with the new Omega filter lens systems (see e.g., Bauer, 1988; Benner et al., 1994; Edelmann, 1992; Michel et al., 1991, 1992; Murray, 1986; Schröder, 1992; Schröder et al., 1990), which allows the conclusion, that frozen-hydrated specimens in comparison to CTEM show both
better resolution and better contrast if ESI under "zero-loss" conditions, that means elimination of the inelastically scattered electrons is used. Actually the amount of underfocus needed for the recording of images can be reduced to minimum values below 0.1 µm far away from the need of any CTEM. The contrast of frozen-hydrated specimens available with ESI under these conditions is astonishing (see Schröder et al. (1990), see Fig. 3 on p. 32). The advantage of PMP and Omega systems is the normal use of the fluorescence screen and the possibility to work with image plates for ESI operation. According to Ottensmeyer and Andrew (1980) and Ottensmeyer et al. (1981) the resolution limit of such systems reaches values of 0.3 nm close to the CTEM. Resolutions down to 0.2 nm were demonstrated later on an experimental Omega filter by Lanio et al. (1986). Such a resolving power now really opens the entrance into molecular dimensions with EFTEMs including ESI with biological samples. And if all assumptions are correct, one can expect a general improvement of the resolving power of "Sub-Ångström Transmission Electron Microscopes" (SATEMs) down to approx. 0.6 Å (0.06 nm) by a strictly monochromatic imaging with new and more sophisticated types of magnetic filter lenses in future (Rose, 1994).

Discussion

No scientist interested in cryo-ultramicrotomy, including the author of this review article, can escape the basic question: does cryosectioning of fresh-frozen material really make sense? Or in other words: what is the gain from all this complicated work? There is no doubt, that cryosectioning according to Tokuyasu is a tremendously successful method. The same is true for other "hybrid methods" starting with a rapid cryofixation of the unpretreated fresh material and finishing with a resin block sectionable at room temperature. For example freeze substitution or freeze drying inserted between cryofixation and low temperature embedding are fully compatible with all known immuno techniques which offer in this respect much more information than frozen-hydrated sections. Both the Tokuyasu technique and the other mentioned hybrid methods open a fantastic opportunity to check, where important antigens (e.g., receptors or enzymes) are located in cells and tissues. On the other hand X-ray microanalysis, EELS, LAMMA and all the methods focused on element analysis and mapping are well introduced and give a world of new information about cells and tissues. Cell biology would be a frustrating job without this direct information on the border between microscopic and molecular level respectively morphology and biochemistry. Only the precise know-
ledge of these "hot spots" in the architecture of cells open the view on what cellular engineering really means. It is my firm opinion, that all the fascinating new statistical and biochemical techniques of molecular biology like plotting methods, high performance liquid chromatography, ultracentrifugation and sequence analyses, which deliver actually plenty of absolutely new insights in cells and cell components cannot and will not substitute the direct investigation down to the molecular level only accessible with the superb resolution of the TEM and STEM. The same is also true for confocal laser scanning and even high resolution tunneling and atomic force microscopy: a complete three dimensional study of larger tissue complexes and even cells to demonstrate the arrangement of certain proteins or nucleic acids or lipids or carbohydrates in situ, will never happen with these new methods. And one should never forget again this dimension of life-important colloids ("Die Welt der vernachlässigen Dimensionen" - "the world of the neglected dimensions") already mentioned by the famous German chemist Wolfgang Ostwald in 1915. But returning to 1996: What can we really expect of fresh-frozen work from cryofixation to the final electron micrograph? That is the first and most important question.

Perhaps a retrospective view is helpful: in the era of light microscopy between the second half of the last and the first third of this century both the limited resolving power and all difficulties in observing specimens without staining resulted in a frustrating discussion around the questions about "fact or artefact" and about all cellular structures at the border of the resolution limit of the light microscope clearly defined by Ernst Abbe. In most of the issues no real progress resulted from this often extremely aggressive discussion. A possible approach was introduced by indirect methods like polarisation microscopy or X-ray diffraction, which allowed some access to the highly ordered structures of myelin or plant cell walls. But these methods required a good knowledge of physics not often found in the community of biologists and therefore did not get the general attention needed for real success. It is interesting nowadays to read again Albert Frey-Wysslings brilliant book "Submicroscopic Morphology of Protoplasm". This second English edition was written in 1953 and includes just the first steps into electron microscopy. Nevertheless it describes clearly and correctly the painful situation in a field which missed the right tools. Some of this pain was eliminated by the invention of the phase contrast microscope by Frits Zernike (Zernike, 1945), making it possible to observe cells and tissues (at least in culture) in their living state - of course without chemical fixation, dehydration, embedding, extraction of the embedding medium, staining and all the other tortures needed for visualisation of cell and tissue structures with a light microscope. All the crazy optimists won, who always claimed with respect to stained paraffin sections, that "such beautiful pictures are probably the correct ones" after this first comparison between the living and the dead object: that was a message! Nevertheless all the unsolved problems and questions struggling on the resolution limit of the light microscope remained and neither phase contrast nor indirect methods (offering only a better "resolution" in one spatial direction for highly ordered periodic structures) could help.

If we take a look at the early days of electron microscopy (see for example Gabor, 1957 and E. Ruska, 1979) we gain the impression that most of the leading scientists in biology and medicine at that time between 1930 and 1950 had neither the confidence nor the good will to use this fascinating new tool. The strong aggression against this new technique mainly based on the feeling that most of the observed structures might be artefacts and that the electron beam probably destroys most organic matter of interest. But again: "beautiful pictures are probably the correct ones". Already in 1966 Fawcett gave a solid support for this slogan with his brilliant view of the state-of-the-art in these early days of electron microscopy with his book "The Cell: Its Organelles and Inclusions". Such crispy pictures were acting again like a tranquiliser. Finally "freeze-cleavage" together with "freeze-etching" [see e.g., Steere (1957, 1973), Moor et al. (1961), Mühlethaler (1973), or in general Benedetti and Favard (1973)] as a strictly physical method demonstrated, that the beautiful pictures obtained after a chemical fixation, dehydration, embedding, ultrathin sectioning and staining with heavy metal compounds are not so far away from the reality of the living state. The introduction of the electron microscope with all auxiliary techniques into scientific research was again a powerful demonstration of the importance of instrumental and methodological progress for new insights into the secrets of nature (P. Sitte, 1973). It is now the question in our consideration, if freeze cleavage and freeze etching enable really clear and striking statements and if the conclusions drawn on the base of their results are correct in all respects. If yes, we would be in a splendid situation and have already reached our goal. If no, we have to continue our efforts to check morphology and chemical composition of our biological specimens in a more difficult terrain. Of course the answer is "no".

According to me there are two different serious arguments to continue research at the cellular
Figure 31: Comparison between the information available by ultrathin cryo-sectioning (a) and freeze-cleavage (b) followed by Pt/C freeze-replication (EL = evaporated layer for TEM investigation). Both schematic diagrams (a) and (b) show identical areas of a eucyte cell with some typical ultrastructures: CP = cytoplasm, ER = endoplasmic reticulum with ribosomes RI, HC = heterochromatin, MI = mitochondrial plasma with ribosomes RI, NE = nuclear envelope, NP = nucleoplasm with euchromatin EU, VE = vesicle. Further comments in the Discussion. (a) The ultrathin section US gives a complete survey over all structures including the content of the different plasmaic and extraplasmic compartments of the sectioned cell (e.g., CP with RI, mitochondrion plasma MI with RI, NP with HC and EU, content of VE). It shows particles sticking on membrane surfaces (e.g. RI on rough ER). For element analysis all different components of CP, NP, MI etc. are available. Three-dimensional reconstruction and mapping is possible both by serial sectioning or by stereological single section methods. (b) freeze cleavage or fracturing is something like a "happening" since the fractures FF follow preferentially the membrane structures as "loci minoris resistentiae". Most of the plasmatic (e.g., CP, MI, NP) and extraplasmatic phases (e.g., VE) are not visible. Element analysis is not possible after replications by layer EL and dissolving of cellular structures Afterwards. Finally a meaningful three-dimensional reconstruction is impossible. For cryowork it is a severe disadvantage that identification of amorphous or crystalline freezing by diffraction patterns of $I_0$ or $I_2$ is not possible.

and molecular level with the new cryo-methods described in this review article: (1) There is a general need to check and to re-evaluate all results obtained by standard procedures on a chemical basis using purely physical methods nowadays accessible, and (2) There were some limitations clearly visible which are connected with the chemical base of our standard procedures, which exclude water as the most important and major component of our biological samples from meaningful interpretations. With regard to the first argument: already the few examples given by the few groups around Jacques Dubochet, Martin Müller, Dick Ornberg and perhaps my own group really demonstrated, that nearly each object re-investigated by pure cryo-methods offered at least interesting new aspects. For example: the ultrastructure of bacteria was in important respects different from that hitherto reported (distribution of nucleic acids, mesosornal structures; Dubochet et al., 1983). The vacuoles of plant cells offered structures never observed before (Michel et al., 1991, 1992). The extracellular matrix of cartilage was considerably different in comparison to the structures after chemical processing (Studer et al., 1995). There was finally a lot of information by high resolution diffraction patterns not observable after chemical treatment. With regard to the second argument: It was already shown by van Harreveld and co-workers, that only rapid freezing offered a possibility to preserve the distribution of rapidly moving small molecules and ions (e.g., $\mathrm{H}_2\mathrm{O}$, $\mathrm{Na}^+$, $\mathrm{K}^+$) correctly and to avoid redistribution not avoidable even after the most rapid chemically based perfusion fixation (Van Harreveld and Crowell, 1964). That means, that the volumes of extracellular and plasmatic compartments (e.g., in central nervous tissue) cannot be correctly stabilised by chemical methods. Once more van Harreveld firstly demonstrated the possibility to observe fast running processes in cells and tissues by rapid freezing (Van Harreveld et al., 1974). The most striking and "fancy" demonstration of this improvement (considerably better "time resolution" according to Plattner and Bachmann, 1982) came from John Heuser with his pictures of synaptic vesicle exocytosis [Heuser et al. (1979); see also Knoll et al. (1987, 1992), Morgenstern and Edelmann (1989), Plattner (1989), Ryan and Knoll (1994)]. Edelmann was able to demonstrate later on, that the correct analysis of ion distribution in muscle tissue depends not only on an optimum cryofixation procedure but also on the correct continuation of this purely physical approach by proper, that means, long enough, freeze drying [Edelmann (1994a,b; see also Sitte et al. (1994)]. I think, that these examples are sufficient to demonstrate the need for and the possibilities of physical procedures to solve some of the most important problems of cell and molecular biology correctly, that means the understanding of the distribution of the mixed aqueous phases in our biological samples, which probably gives us some insight into the secrets of life.

It is evident, that only purely physical processes starting with a true vitrification of the untreated sample are suited for the correct and meaning-
ful re-evaluation claimed under (1) above. It remains the question, if perhaps freeze-fracturing followed by freeze-etching and Pt/C replication according to Steere (1957) or Moor and Mühlethal er (1963) may be a suited (and simpler) alternative to cryosectioning and low dose investigation of frozen-hydrated sections in the cryo-TEM. There is no doubt that also this alternative is a sequence of purely physical steps of preparation. As mentioned above, freeze-etch images really offered the first partial confirmation, that chemically based standard procedures delivered in most respects correct information about living cells. Freeze-etching brought (completely unexpectedly) a world of new important knowledge about the molecular architecture of biomembranes (see for example the beautiful demonstration by Orci and Perrelet, 1975) and gave a solid base for the current "fluid-mosaic membrane" model of Singer and Nicolson (1972). But by two reasons freeze-etching cannot be a real alternative to cryosectioning, since it does not fulfil some of the preconditions for a correct re-evaluation: firstly the frozen specimen must be completely removed from the Pt/C-replica before investigation in the TEM is possible. It therefore gives no correct information concerning the amorphous or crystalline state of the ice, which acts as an embedding matrix of the cellular ultrastructures, by electron diffraction. Since this diffraction pattern offers the only possibility to discern between amorphous (L), cubic (Ic) or hexagonal (Ih) ice and since we know, that already microcrystalline L, segregation creates artefacts [see e.g., McDowall et al. (1984) or Studer et al. (1995)] we cannot miss the frozen-hydrated investigation at least to check the quality of our cryofixation procedure. A second reason concerns geometry (see schematic diagrams in Fig. 31): only sections (Fig. 31a) have a properly defined geometry and show us all structural details incorporated in our sample. The normal "freeze-fracture" (Fig. 31b) splits the frozen specimens completely irreproducibly mostly along the "loci minoris resistentiae" (sites of least resistance), which are the contact areas of the apolar ends of the fatty acid chains inside the biomembranes. Most of the cytoplasmic or karyoplasmic matrix is hidden by this fracturing process. Of course: we have the possibility to produce an excellent surface for subsequent Pt/C-replication in the ultramicrotome using exactly the methodology for cryosectioning at temperatures of -165°C or lower, that means using a low angle diamond knife with α < 35°, an ioniser, a cutting speed of 0.5 mm/sec, a width of the sectioned surface ≤ 0.1 mm and so on. Under these conditions we can expect a brilliant flat surface without fractured areas inside, but nobody would be happy to replicate these lousy 0.01 mm²: the whole procedure would be more complicated (cryosectioning plus replication plus preparation of the microreplica) than cryosectioning alone. Nevertheless we would miss the information about the amorphous or crystalline state of the frozen sample. The same problem occurs with element analysis by EDX: we have no possibility to analyse Na⁺ or K⁺ or any other element since they are gone. We can conclude, that only the frozen-hydrated cryosection gives us the desired answer, if true vitrification was obtained and - if "yes" - where the different fast moving ions are located in situ and in vivo. In other words: we cannot escape the cryo-ultramicrotome and the frozen-hydrated or freeze-dried investigation afterwards. Similar considerations may be necessary for a correct element analysis, since any crystallisation process during freezing without doubt is able to provoke redistribution of ions. I do not believe, that it makes a big difference for an experimental confirmation to section correctly a frozen specimen, to take a look in the cryo-TEM, to make a picture and (more important) a diffraction diagram and to move the still frozen-hydrated section on the grid by cryotransfer into a freeze dryer. Of course: freeze drying has to be realised at temperatures between -80°C and -100°C (Sitte et al., 1994). The recrystallisation to cubic ice cannot be avoided under these circumstances. But big x/y-translations of particles, fibres, membranes and probably also ions in the still frozen matrix similar to the phase change "liquid → crystalline" do not seem to be possible, as discussed later in this review (Fig. 32). All knowledge presented in the preceding argumentation concerning arguments (1) and (2) was accumulated by very laborious and exhausting experimental work from a small group of cryofreaks. Neither Alisdair McDowall and Jacques Dubochet, nor Martin Müller together with Daniel Studer, Martin Michel and Heinrich Hohenberg, nor Richard Ornborg nor our group had full access to all instruments and techniques described in this review. Nevertheless it was possible to document the specific advantages of the new cryosectioning methods for fresh-frozen material by the already mentioned serious results. But it would be a nonsense to follow the description in those papers and to repeat all "historical" mistakes: this would mean to work nowadays with instruments of a former generation and with the methods cited. It is my firm opinion, that cryosectioning of fresh-frozen samples is such a tough job, that further progress on different objects can only be expected if the really best state-of-the-art instrumentation is available and if the best suited methods are used. That includes, that the best suited cryofixation device (often a high pressure freezer), the best cryo-ultramicrotome (at the moment the Ultracut-S or better UCT together with an FCS cryo-
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Figure 32. The schematic drawings (a) to (d) concern hypothetical assumptions about amorphous solidification ("vitrification") of a liquid plasmatic phase (c) by rapid freezing "c → b" in comparison to "primary hexagonal crystallisation c → d" (GD = growing direction of hexagonal ice Ih) occurring at cooling rates below 1 million °C/sec and "secondary recrystallisation b → a" after heating a vitrified sample (b) above devitrification temperature T_d of approx. -135°C. Since even the rapidly oscillating H2O-dipoles of the plasmatic matrices MA during rapid cooling with rates above some million °C/sec have no chance to aggregate into a crystalline lattice, all included smaller molecules and especially the TEM-resolvable macromolecular components like microfilaments MF or ribosomes RI are encapsulated in vitreous ice (Iv) without noticeable x/y/z-translation during vitrification. Completely different from vitrification "c → b" during hexagonal crystallisation "c → d" the pure hexagonal crystalline phase Ih grows without incorporation of other constituents like dissolved smaller molecules, ions or larger macromolecular structures (e.g., MF or RI), since such a crystallisation is an extremely precise selective procedure: we can suppose, that both small molecules, ions and larger molecules as well as particles RI or filaments MF are rapidly shifted from the Ih crystal borders by x/y/z-translations to other places they never occupied before. They are finally included in the dense eutectic mixture of the plasmatic (or extraplasmatic) components (enriched matrix MA'). Of special interest with respect to hybrid methods (e.g., freeze-substitution or freeze-drying following a rapid vitrification) is the devitrification "b → a" of a primarily vitrified sample to a microcrystalline compound with a cubic Ic matrix observable by electron diffraction. One may speculate hypothetically upon the possibilities given in a still completely frozen solid state sample around T_d of approx. -135°C: there is certainly some probability, that during this "secondary crystallisation event b → a" x/y/z-translations of inclusions (e.g., MF, RI) and maybe even ions and small molecules are not possible. This assumption is shown in the schematic drawings (b) and (a). But this hypothesis has to be checked by comparison of cryosections in the amorphous frozen state at e.g., -170°C before increase of temperature above T_d and after devitrification to cubic Ic, e.g., by heating above -135°C. This crucial experiment should be possible. Compare further remarks and comments in the Discussion.

chamber) equipped with the needed accessories (low angle cryo diamond knife with a facet angle < 35°, cryo diamond trimming tool, high performance M3C stereomicroscope for high magnification, high power cold light source, adjustable ioniser), a suited cryotransfer system (Gatan 626-DH or Oxford CT 3500) and a cryo-TEM with a sufficient cold trap (decontamination system), one of the described low dose systems (SS-CCD or image plate) and an energy filter (EFTEM) for electron spectroscopic imaging (ESI) has to be used. If element analysis (e.g., EDX or EELS) of ultrathin or semithin sections is the goal, a freeze dryer suited for proper drying - that means minimised artificial shrinkage (e.g., cryosorption drying according to Edelmann, see Sitte et al., 1994) is necessary.

It makes no sense to start into fresh-frozen cryosectioning or element analysis without the whole chain mentioned above, since the exclusion of one of these tools reduces the efficiency considerably and makes it even more difficult to obtain useful results within a reasonable time. Of course starting without experience also makes no sense: a practical training in at least one of the leading laboratories is highly recommended to gain practice both in cryofixation, cryosectioning including cryotransfer and low dose operation of an EFTEM. If possible, this training should be made with one's own samples, since all methods need some specific adaptation on the material and it makes a big difference if a soft animal tissue or a rigid plant tissue or a cell suspension (probably the simplest case) has to be processed. Already the cryofixation as the first step is a crucial point and decides on success or failure of the whole procedure. For a first check of suitability of a special freezing method a subsequent freeze substitution and resin embedding according to Van Harreveld and Crowell (1964, see also Steinbrecht and M. Müller, 1987, or Fig. 6 in this review)
of the specimen may be the most convenient way. If fast processes (e.g., membrane fusions within exocytotic or endocytotic events) are considered, high pressure freezing may be too slow. Also this can be clarified on cryofixed, OsO₄/acetone substituted and resin embedded material. The same is true for an extracellular cryoprotection [e.g., by sugar, see Dubochet et al. (1983), McDowall et al., (1984, 1986) at least for cell suspensions. Cryo-ultramicrotomy should be started after this first check according to the rules given in the relevant preceding sections. Probably nobody will escape the somewhat time consuming process of trial and error in establishing the best individual sectioning parameters for his specific samples. And nobody will escape the painful and often frustrating transfer of the cryosections to the grid, simplified considerably by a well adjusted ioniser. As explained before, perfect vitrification is a must and already cryptocrystalline cubic ice may cause artefacts. One has to remember, that in the past many scientists were happy if severe damage by hexagonal ice could be avoided and often specimens without resolvable ice crystals were incorrectly described as "vitrified" (amorphous). Often cryptocrystalline cubic ice was considered as amorphously vitrified ice simply because it sections quite well and looks promising.

There is a lot of "laboratory folklore" around cryosectioning. Often it is claimed, that manual operation of the cryo-ultramicrotome delivers better (or even the only suitable) cryosections. That may be correct for prehistoric cryo-ultramicrotomes like MT2/FTS or OmU3/FC2. But this would be nonsense with a modern Ultracut-S/FCS-system with a perfectly adjustable cutting window, a fast return stroke and a very slow cutting speed: nobody is able to produce by manual operation of a cryo-ultramicrotome really regular serial sections below -160°C in the mostly wanted and needed thicknesses around 50 nm. Since irregularities due to manipulation forces cannot be excluded, manual operation does not work reproducibly and makes really no sense. Several steps in cryo-preparation require some experience and special attention. Some adjustments have to be found by "trial and error" for each new specimen: this relates especially to the knife and specimen geometry and adjustments (clearance angle, width of sectioning surface) as well as for the best cutting speed, cycle length and ioniser adjustment (cycle length and ioniser adjustment depend on each other). As for ambient temperature work, the setting of the feed rarely corresponds exactly with the real advance or the section thickness finally obtained: it is understandable that cryo-ultramicrotomes react much more sensitively against environmental influences and that therefore thicknesses differ much more from the advance settings in comparison to standard ultramicrotomy. Slight irregularities in section thicknesses, which can be mostly ignored at ambient temperature work, severely disturb cryosectioning, since they provoke the formation of crevasses and are multiplied by compression phenomena too. An extremely smooth operation of the cryo-ultramicrotome is therefore of the same key importance as the careful exclusion of all outside influences (air draughts, thermal influences, building vibrations and manipulation forces). And again: generally the motor driven automatic ultramicrotome delivers the best results. If useful cryosections are on the knife edge, the transfer to the grid needs some experience and pressing of the cryosections onto the grid inside the cryochamber of the ultramicrotome is an essential step, since heat extraction under the electron beam is dependent on a good thermal contact between the solid state sections, the solid state grid and grid holder. If such sufficient thermal contact is obtained, the temperature difference between the cold stage and the cryosection will remain 10°C or less. Success or failure besides this pressing procedure depends on a correct cryotransfer and a sufficient cold trap (decontamination system) around the specimen in the column of the TEM. Success also depends on the low dose operation of the microscope and all measures, which allow to minimise beam damage on the extremely sensitive frozen-hydrated material. Besides the new SS-SSD or image plate systems for digital low-dose recording of images and an EFTEM for ESI at zero loss, both accelerating voltage and vacuum conditions influence the beam damage [see e.g., Cosslett (1978), Glaeser (1971, 1975), Kobayashi and Sakaoku (1965)]. Simply: the better the vacuum and the higher the voltage the lower the beam damage. If the voltage is increased from 100 kV to 200 kV, the beam damage decreases already in the order of x0.5. In addition, an excellent vacuum seems to be helpful and will be available automatically by cryosorption, if an efficient cold trap decontamination system surrounds the specimen nearly completely.

The contradictory discussion about the "cryostabilisation effect" (misleadingly mostly termed "cryo-protective effect") of very low temperatures seems to be finished [see e.g., Chiu et al. "International Experimental Study Group" (1986), Dubochet et al. (1987, 1988), but the real effect of the lower temperatures is not absolutely understood: the big effort invested in studies on the temperature dependence of beam damage resulted mainly in the simple conclusion, that this effect depends on the specific conformation of the compounds investigated, on some geometrical and material components of the set-up and of the frozen specimen: if the defined "critical doses" of different
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compounds are compared, aliphatic compounds show a higher sensitivity under the electron bombardment than aromatic ones. Additionally, the sensitivity is dependent on the support of the sample: carbon layers reduce, collodion films enhance the sensitivity. Similarly the thickness and the geometry of the sample influence the damage under comparable conditions. And most important under the point of view of this review article: these are mostly measurements based on the fading of diffraction patterns of crystals, which are in the strict sense not comparable with our frozen-hydrated specimens encapsulated in a matrix of amorphously vitrified ice. By similar reasons the results of radiochemistry based on measurements with ESR (Electron Spin Resonance) and ENDOR (Electron Nuclear Double Resonance; see e.g., Box (1975, 1977)) are not really helpful, since the doses used for ESR and ENDOR are orders of magnitude lower than those used for imaging in the TEM. There are other indications about the benefit of low temperature on beam damage based on mass loss during electron irradiation [see e.g., Dubochet (1975), Ramamurti (1977), Cossllett (1978), and Fig. 26 in this review]: at 7°K only a very small mass loss occurs in comparison to room temperature. According to the studies of Grubb and Groves (1971) as well as Siegel (1972) there are at least two processes which are involved in beam damage and things are not so easy to reconstruct. In this context both the "caging" of fragments after chemical bond scission due to inelastic scattering of electrons, the re-unification of such fragments and cross-linking phenomena under the electron beam are discussed.

Radiolysis of H$_2$O [*"beam etching"*; see e.g., Draganic and Draganic (1971), Hochanadel (1960)] occurs also at LN$_2$ temperature and changes frozen-hydrated sections rapidly. Probably the observation of the rapid transformation of crystalline hexagonal ice (I$_h$) into amorphous I$_c$ (Lepault et al., 1983c; see also Heide, 1984; Heide and Zeitler, 1985; Talmon, 1987) is also a result of such H$_2$O radiolysis phenomena. It is not difficult from a thermodynamic point of view to expect a re-unification of the caged fragments of H$_2$O inside the frozen matrix. If this occurs, the apposition of the re-unified fragments at very low temperatures will certainly not follow the laws of crystallisation, but happen in a statistical manner similar to the production of amorphous IV on a cold substrate (Burton and Oliver, 1935a,b; see also Eisenberg and Kauzman, 1969). Lepault et al. (1983c) report, that electron doses above 2000 el/nm$^2$ cause a complete transition from I$_h$ to I$_c$. That means, that after rewarming above the devitrification temperature $T_d$ (see the section "Freezing and the Frozen State of Water ... ") recrystallisation to cubic I$_c$ occurs. After doses between 100 at 500 el/nm$^2$ Lepault and co-workers observed a change of the diffraction pattern from hexagonal I$_h$ to amorphous I$_c$. They report, that under these conditions within the ice matrix after the complete decay of the hexagonal crystalline diffraction pattern there remains even at doses up to 2000 el/nm$^2$ "some kind of memory", which starts immediately hexagonal crystallisation again after warming up above the devitrification temperature $T_d$. This fits very well in the hypothetical concept: for sure there will still persist a number of small residual domains of crystalline I$_h$, far too small to create the characteristic diffraction pattern of crystalline hexagonal ice, but absolutely sufficient for nucleation and recrystallisation in the hexagonal lattice. The results of Lepault and co-workers give some idea, how fast H$_2$O radiolysis phenomena may change any frozen-hydrated specimen under the electron beam in the microscope.

How fast beam-induced H$_2$O radiolysis acts is also visible by the "bubbling phenomenon" (e.g., Frederik et al., 1993), which starts immediately, if higher beam intensities are used for focusing or recording of images: the occurrence of gas bubbles within the frozen specimen has actually nothing to do with boiling processes, since the vapour pressure of ice below -150°C can be neglected. Also this event is based on beam-induced radiolysis phenomena, as perhaps another well known phenomenon too: it is evident and often reported (see e.g., Michel et al., 1991, Fig. 7, p. 13), that after electron irradiation the specimen contrast increases considerably. That may be a result of rapid beam etching due to H$_2$O radiolysis. At the same time both knife marks and ripples (crevasses) lose contrast and therefore lose some of their annoying character. Since crevasses form sharp edges (see Fig. 20) it seems to be feasible, that radiolytic fragments disappear easily from the pointed edges and re-unification as well as deposition of those molecules occurs to a larger extent only in the grooves on the bottom of the crevasses. The same may occur on knife marks if they have the same expected irregular appearance as observed in resin sections as a result of a dull portion of a damaged knife edge, which creates superficial curling of the embedding resin. In both cases such irregularities will disappear immediately after start of irradiation by radiolysis and a flattening of the pointed surface profile will result both by apposition and etching, as demonstrated already by Zeitler and Bahr in 1965 (see also Isaacson, 1977).

Since the discussed welcome "clearing up" phenomenon is only the start of radiolytic beam damage and since further damage results within a very short time of irradiation respectively after a very small dose, a total destruction of the sample has to be ex-
pected immediately afterwards. Low dose operation of the scope is therefore perhaps the most important challenge related with cryo-ultramicrotomy of fresh-frozen samples. In this field one is in a conflicting compromise situation with respect to the signal-to-noise ratio: if an acceptable resolution is wanted, severe beam damage has to be expected in frozen-hydrated sections, since the signals result from scattering processes or a certain electron dose needed to reduce the noise of an image. Only in the very special cases of periodic structures noisy pictures can be used for "image averaging" to obtain a fairly good resolution ["SNAP-shot method" developed for "Statistically Noisy Average Pictures" by Kuo and Glaeser (1975); see also Unwin and Henderson (1975)]. As already mentioned, such samples are the exception in our biological collection and not the normal case and there is indeed more than a formal similarity with the indirect physical methods used in "Submicroscopic Morphology" in the sense of Albert Frey-Wyssling to circumvent the limit of the resolving power of the light microscope before the TEM was introduced. Perhaps the discussed low dose systems allow the making of a series of exposures at minimum dose at rather small magnification and to execute image averaging without periodic structures, just reproducing the same field to reduce the noise component. But the real success of such a procedure is still a matter of gambling and enterprise. More serious offers result both from reproducible experience of ESI (Fig. 30) and from direct dose reduction resulting from the application of SS-CCD systems or phosphor image plates read-out by laser scanning (Figs. 27 to 29). ESI is able to reduce the noise in pictures reproducibly by a considerable reduction of the underfocus desired to establish the needed contrast (see Bauer, 1988; Murray, 1986; Schröder, 1992; Schröder et al., 1990). There is no doubt, that this improves the situation considerably and that EFTEM imaging (Fig. 30) is a powerful tool in low dose work with frozen-hydrated specimens. The observation of Michel et al. (1991) that zero-loss images of all sectioning artefacts (e.g., ripples, crevasses, knife marks) are also recorded with better contrast are no real argument against ESI, but an additional challenge to improve the performance of our cryosectioning techniques in the way discussed above (e.g., higher stability of the set up, lower $\alpha$- and $\varepsilon$-angles of the knife, reduced width of the section surfaces). At least the minimising of the underfocus under zero loss conditions will correspond to a considerable reduction of noise and dose. This is a first step forward towards the desired improvement in resolution of beam sensitive structures already realised for amorphously vitrified suspension films by Schröder et al. (1990).

Both SS-CCD systems and electron stimulable phosphor image plates described in the preceding Section (see also Figs. 27 to 29) have a direct influence on the dose needed to record a digital image. In many respects (linear signal/dose ratio, dynamic range, exposure time) the characteristics of both systems are nearly identical. But there are also a lot of differences, which today impede a clear decision to acquire one or the other system. Burmester (1992, see also Table 6) has clearly demonstrated that the resolution is directly influenced by the effective surface (frame) of the recording area. The 19 x 19 mm$^2$ frame of the 1k x 1k polysilicon detector chip of the SS-CCD systems offered in the past covers only 1/16 of the area of the image plate (effectively 94 x 75 mm$^2$) used in the Fuji FD-L-5000 system. Since the pixel size of 25 $\mu$m is nearly identical, the image plate offers much better conditions at identical resolution with respect to needed magnification. Partially this disadvantage of the CCD chip is compensated by the new MegaScan system with 2k x 2k pixels offering the fourfold efficiency. That is already a lot (see Fig. 27c). But in comparison, the image plate has still an advantage of x 4 by the larger effective detector area. In practical work also the read-out time between two subsequent slow scan operations has to be regarded as limiting parameter for low dose work. This time must increase with respect to read-out noise linear with the amount of pixels. Read-out has an 1:1 influence on the periods of beam blanking and this may create shift problems, if the beam deflection above the specimen is used. But: beam blanking below the specimen recommended in such cases does not agree with low dose and larger read-out times due to increased pixel numbers may offset the gain in surface area under these preconditions. Both efficient and reproducible subarea read-out software as already announced by Gatan and the increase of read-out frequencies (e.g., 500 KHz $\rightarrow$ 2 MHz $\rightarrow$ 5 MHz) are now important measures to reduce those inconveniences. Of course, one should not forget the most striking advantage of the on-line CCD-system: autotuning of focus and astigmatism etc. will never be possible with the off-line image plates. And just this autotuning according to Krivane and Fan (1992, see also Krivane and Mooney, 1993; Fan and Krivane, 1990) by the above mentioned "automatic diffractogram analysis (ADA)" delivers results after only one pre-exposure of about 0.5 to 1.0 sec, followed by a computer calculation, which may be shortened considerably below 8 sec with a fast modern PC. It is no question at all, that a really fast autotuning routine will reduce the needed dose considerably since time consuming do-it-your-self focusing or
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dose intensive through-focus series are not needed. But also these considerations are only valid if autofocus as the most important automatism works really reproducibly on ultrathin fresh frozen-hydrated sections at the given faint contrast conditions. Today there is no practical experience with frozen-hydrated cryosections available and further comparisons between the offered low dose systems are needed to clarify the situation. If normal ambient temperature work on heavy metal-stained resin-embedded material would be the theme, probably the image plate would have real advantages because the whole off-line read-out hardware and software can be used for different TEM models without special modifications on these scopes: the image plate just substitutes for the photographic plates and eliminates the whole darkroom efforts and all costs for co-workers, materials and environment, since image plates are re-usable. But such nice arguments do not touch low dose. Probably a combination of all systems, that means autotuning by SS-CCD with a small frame (possibly considerably below 1k x 1k or 19 x 19 mm\(^2\) detector surface with respect to extremely fast read-out and autofocus calculation), recording by large surface image plates (possibly even larger than the offered Fuji image plates of the FDL-5000 system with respect to maximum pixel numbers and minimum "Pixel Equivalents") and imaging with an energy filter at higher accelerating voltages in an ultrahigh vacuum would be the thrill - if all assumptions made are really correct and no new additional aspects arise in future.

I have described above, that in the past within an intermediate period starting after 1945 phase contrast optics in light microscopy had the important task to check, if after the chemical treatment of histoprocessing the specimens correspond correctly with the structures of the living object. After some years most of this job was done. It was possible to return to normality and to work again with (sometimes slightly improved) chemical methods which up to now remained the most important base in the wide fields of standard histology, microscopical anatomy, diagnostic histopathology and histochemistry. Only in the field of cell and tissue cultures were phase contrast and later on interference contrast indispensable tools for daily routine. Only in exceptional cases the question is again posed: do the standard procedures really deliver correct information? In such cases phase or interference contrast and nowadays confocal laser methods again serve as reference tools for checking. Cryoultramicrotomy and the related techniques of cryofixation and cryo-electron microscopy with ESI and low dose systems probably are going the same way. At the moment there is really the need to check, if and to what an extent all the beautiful pictures so easily obtainable with standard ultramicrotomy of resin blocks at ambient temperature correspond with the reality of living organisms, tissues and cells: this is the challenge of today. The re-investigation and new evaluation of the results obtained with standard ultramicrotomy in the recent decades certainly will yield an abundance of new results allowing us to improve standard techniques by comparison with the cryo-methods described in this review. "Hybrid techniques" starting with rapid cryofixation at ambient or high pressure will probably also gain additional importance by such a comparison and re-evaluation. We can expect, that their results are close to reality. This certainly is true for cryofixation, freeze substitution, freeze drying and low temperature embedding. All these procedures have been improved considerably during the last years. For example Ludwig Edelmann could demonstrate that substitution without additives like OsO\(_4\) or uranyl acetate gives excellent results in combination with Lowicryl low temperature embedding media (Edelmann, 1991a,b), if the frozen specimens are incubated for a sufficiently long time at low temperatures around -80°C and embedding in Lowicryls is performed really at temperatures of -60°C or below. Similarly freeze drying is now possible without severe artificial shrinkage ("thermal collapses") simply by long drying periods at low temperature (Edelmann, 1984a,b; Sitte et al., 1994). Also freeze drying preparation gains importance by subsequent low temperature embedding in Lowicrylys. First results indicate that ion distribution after these hybrid procedures agrees in principle with EDX results on freeze dried ultrathin sections. It is correct, that both freeze-substitution without additives like OsO\(_4\) or uranyl acetate and freeze-drying at temperatures between -80°C and -100°C are time consuming procedures, but waiting really pays! There is no doubt, that a lot of investigations including three dimensional mapping of element distributions are easier possible by sectioning resin embedded samples with a dry knife, nowadays possible with purpose designed diamond knives without frustrating charging phenomena (Helmut Gnägi, personal communication, 1996). The application of cryoultramicrotomy for fresh-frozen (vitrified) biological samples will also in future certainly remain a tricky and time consuming job. But the proof by comparing the results obtained by cryo-ultramicrotomy is needed in all these areas to draw the correct conclusions.

The most important question for the meaningful use of any of the hybrid methods (cryofixation, freeze substitution or freeze drying followed by low temperature embedding) has to be repeated in this context: is true vitrification of a specimen really need-
ed, when the amorphous sample is substituted or dried afterwards at a temperature around -80°C, where certainly devitrification and recrystallisation to cubic ice (l_6) occurs? I would say: "yes", and I want to repeat the already shortly presented opinion, that primary crystalline freezing of a liquid phase and secondary recrystallisation (e.g., devitrification to cryptocrystalline l_6) within a solid state are completely different events. Solidification of a mixed liquid phase according to Fig. 32 is a very selective process as long as sufficient time for hexagonal crystallisation is available. That means, that the crystalline l_6 formed by this "liquid → solid transformation" at ambient pressure (Fig. 32c and d) is certainly a perfect segregation into pure hexagonal crystalline l_6 and the former suspended and dissolved components. In other terms: the mixed liquid cytoplasmic phases inside the cells or the mixed extraplasmatic phases around these cells (e.g., lymphatic fluid, blood plasma or liquid phases in the big vacuoles of plant cells) during freezing segregate completely. With a very high probability in this l_6-lattice there is neither space for small ions with a hydration shell nor for macromolecular structures like globular or fibrous proteins or ribosomes. All these ions, molecules and macromolecular structures are shifted by the moving border of the growing l_6 lattice to other places (Fig. 32d). As far as l_6-lattices are built up, such movements will be extremely fast over relatively large distances. The result must be a big difference to the original liquid suspension (Fig. 32c). Real vitrification according to Fig. 32b does not allow such shifts and redistributions: here water serves as an excellent amorphous embedding medium, which surrounds ("encapsulates") all these small components of the cytoplasmic or extracellular matrix. Since even the extremely fast oscillating H_2O dipoles (see e.g., Bachmann and Mayer, 1987) have no chance to change their position, all larger particles (these are already ions with their hydration shells, see Kellenberger (1987, 1991), Kellenberger et al. (1986)) are captured by the vitrified H_2O matrix. If such a vitrified specimen is heated within a second thermodynamic event above the devitrification temperature T_d, H_2O molecules will reorganise to a cryptocrystalline cubic l_6-lattice (Fig. 32a). I cannot imagine that this could cause big movements in the still continuously solid state of the sample and I could expect the process shown in the diagrams of Fig. 32b and a, where all larger constituents of the frozen sample keep their original places and only the frozen matrix crystallises. Of course, this hypothesis has to be proven by experiments. Such proofs seem at least to be possible by freeze drying, if identical areas of a frozen specimen are shown before freeze drying in the amorphously frozen-hydrated state and after freeze drying without H_2O matrix. I suppose that results of such comparisons will support the general assumption made in Fig. 32 and that sufficient dehydration at a temperature around -80°C both by freeze drying (Edelmann, 1994a,b; Sitte et al., 1994) and by freeze substitution (Edelmann, 1991a,b; Sitte et al., 1994) can lead to quite correct pictures, which will in most respects correspond to the amorphously frozen-hydrated state. The mentioned hybrid methods will therefore probably offer the best alternatives to cryosectioning and frozen-hydrated investigation in future, if we have a solid base for meaningful conclusions by the mentioned comparisons between frozen-hydrated sections and resin sections of the same material after optimised cryodehydration (freeze-drying or freeze substitution) and optimal low temperature embedding.

Conclusion and Future Prospects

It was the goal of this review to show, that both instrumental and methodological progress in the recent years made cryosectioning of fresh-frozen samples much easier in comparison to former days. I wanted to show clearly, that these improvements count only if all the new possibilities including cryofixation (high pressure), cryo-ultramicrotomy and ESI/low dose investigation or diffraction analysis are used together and if all important accessories are at one's disposal (lowest angle cryo diamond knives, ioniser etc.). But I also wanted to point out, that "easier" does not mean "easy": cryosectioning at temperatures ≤ -165°C will always suffer from the brittleness of solid state water matrix of highly hydrated systems and will therefore remain more complicated in comparison with any hybrid procedure like freeze substitution, freeze drying or low temperature embedding, which were all also improved considerably during the last years. Cryosectioning of vitrified specimens still remains the proof, to what extent results obtainable with chemically based ambient temperature standard techniques (chemical fixation, polar solvent dehydration, resin embedding by heat polymerisation and sectioning at ambient temperature) or alternatively results obtainable with modern hybrid techniques (vitrification by rapid freezing, freeze substitution or freeze drying followed by low temperature embedding) agree with results from frozen-hydrated ultrathin sections in the cryo-TEM. I am absolutely confident that in many respects this comparison will give a clear recommendation, that standard procedures or at least hybrid methods deliver reliable results. Based on such comparative work some improvements of standard and hybrid procedures will certainly be possible. That is
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the challenge to realise this proof, which will render many new insights in the molecular structures of cells and tissues. It will stimulate and improve morphological work in many important respects. Of course there will be domains where purely physical cryopreparations will be needed also in the future: high performance cryowork of course will be needed also in the future for element analyses and for collecting pictures of our highly hydrated systems, not accessible with standard procedures. Besides these areas cryo-ultramicrotomy and frozen-hydrated work will remain the most important reference method in all cases of doubt, if the information obtained by standard or hybrid methods is really correct.

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List of Suppliers

Balzers Union AG (former Bal-Tec), Postfach 75, FL-9496 Balzers, Fürstentum Liechtenstein (High pressure freezer HPM 10, systems for cryofixation and hybrid preparation of frozen specimens)

Diatome AG, Postfach 557, CH-2501 Biel, Switzerland (Cryo diamond knives and diamond tools for cryo-ultramicrotomy including trimming tools and ioniser "Static Line II")

Fuji Photo Film Co., Ltd., 26-30 Minato-ku, Tokyo 106, Japan (FDL 5000 Image Plate System for low dose image recording)

Gatan Inc., 6678 Owens Drive, Pleasanton, CA 94588, USA (SS-CCD camera systems for low dose image recording, post column electron spectroscopic systems for EFTEM with ESI, cryotransfer systems for side entries)

Leica AG, Hernalser Hauptstraße 219, A-1171 Wien, Austria (Reichert-cryo-ultramicrotomes, high pressure freezer Leica EM HPF, automatic systems for freeze-substitution, freeze-drying and low temperature embedding, systems for ambient pressure freezing and cryopreparation)

LEO Elektronenmikroskopie GmbH, Carl Zeiss Straße 56, D-73446 Oberkochen, FRG and LEO Electron Microscopy Ltd, Clifton Road, Cambridge CB1 3QH England (former Carl Zeiss Elektronenoptik and Leica Cambridge) (In column filter-lens Cryo-TEM Omega EM 912 for EFTEM and ESI including SS-CCD recording)

LifeCell Corporation, 3606 Research Forest Drive, The Woodlands, TX 77381, USA (Slam freezer and systems for hybrid preparations of frozen specimens)

Oxford Instruments, Research Instruments, Scientific Research Division, Old Station Way, Eynsham Witney, Oxon OX8 1TL, England (Side entry cryotransfer systems, anticontamination devices for TEMscopes and cryopreparation systems for TEM/STEM/SEM)

RMC, 4400 South Santa Rita Avenue, Tucson, AZ 85714, USA (Cryo-ultramicrotomes and cryochambers, systems for cryofixation and hybrid preparations of frozen specimens)

Simco B.V., Aalsvoort 5, NL-7240 AA Lochem, Netherlands (German representative: Ziegener+Frick GmbH, Justinus-Kerner-Straße 8, D-71717 Beilstein, FRG) (Adjustable ioniser for cryoultramicrotomy)

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AMF O'Hare, pp. 131-138.


1 From 1916 on follow numerous editions of this well known book about colloid chemistry; see also from the same author: Kolloidchemie (Colloid Chemistry), 1st ed. 1909, and: Handbuch der Kolloidwissenschaft (Handbook of Colloid Science), 1924.


2The original version has the title "Gute Vorsätze" (Good Intentions) and the precise wording in German is:

"Den guten Vorsatz, sich zu bessern,
Muß mancher manchmal arg verwässern
Die so erzielte Wasserkraft
Treibt dann den Alltag fabelhaft" 

Like other poems of this category it is difficult to translate correctly, since some puns are included, for example "verwässern" ("dilute") or "Wasserkraft", which in English means "impetus" rather than "water power". Nevertheless, my friend Dr. Keith Ryan from the Marine Laboratory at Plymouth (UK) made the successful attempt to translate these four lines

"The great intent to improve oneself
must often be much diluted,
the water power thus achieved
to drive the day is aptly suited"

I want just to add, that Eugen Roth was born 100 years ago at Munich in 1895. I like him very much and I recommend all colleagues who (a) are frustrated about my tough review and (b) have some knowledge of the German language, to escape into this exciting poetry. I found also some psychological support during my exhausting literature studies about SS-CCD, ESI, and EFTEM for this review article in those extremely warm hearted short poems full of real humanity.
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Discussion with Reviewers

R. Wróblewski: I think that the most important breakthrough in cryo-ultramicrotomy was the method of tissue preparation for immunocytochemical purposes by Tokuyasu, and the way to pick up sections using a sucrose droplet in a loop. Using Tokuyasu’s methods, sections can be obtained on all cryo-ultramicrotomes, even the oldest ones. My view is supported by a number of papers produced using different cryomethods for different purposes - ranging from pure morphology through immunocytochemistry and ending with microanalysis. All other methods described by the author have some applications but are still not accepted in daily routine research.

Author: As already stated in the Abstract "The article is mainly directed to the investigation of frozen-hydrated sections in the low dose cryo-TEM and - EFTEM" and "both cryoultramicrotomy for macromolecular cytochemistry (Tokuyasu technique) and cryoultramicrotomy for element analysis are only shortly mentioned". It was not the intention to describe again well introduced techniques, which were already several times excellently reviewed (see literature cited at the begin of the section 6 "Cryosectioning According to Tokuyasu") and "accepted in daily routine research". I agree that "Tokuyasu’s work was the most important breakthrough in cryo-ultramicrotomy" and it is true that "using Tokuyasu’s method, sections can be obtained even with the oldest cryo-ultramicrotomes". But nevertheless I would not like to prepare material according to Tokuyasu protected with 2.3 M sucrose using one of the old systems like the Reichert OmU3/FC2 or the LKB-CryoKit or the Sorvall MT2/FTS: the differences in ergonomy and efficiency are too large. For frozen-hydrated work below -160°C those oldies are really not suited and the progress shown in Table 1 is really important for further studies in the field, which I wanted to stimulate.

K.P. Ryan: There is no mention of Freons as coolants - are they recommended for any aspect of rapid cool-
ing?

**Author:** According to Table 6 there is no real need to work with coolants, which will be not so easy obtainable in the future (ozone layer) and which have an inferior cooling efficiency CE in comparison with propane and ethane. This is especially true for the often used Freon 12.

**K.P. Ryan:** Electron microscopy is full of hazards which include high voltages, toxic chemicals, vacuum equipment involving high temperatures with heavy electrical currents during coating activities, and critical point drying which involves high pressures. In cryomethodology we encounter further hazards which might be worth detailing in this review - these are low temperatures which can "burn" and flammable gases which can form explosive mixtures, although these gases are normally kept below their flash point and under an inert atmosphere of gaseous nitrogen. The gaseous nitrogen is perhaps the most insidious hazard because it is unseen, has no odour and is generally not considered. Can you comment on the amount of nitrogen gas produced in a typical cryo-ultramicrotomy session?

**Author:** The question related to hazards and risks is indeed of highest importance and should not be neglected! I want again to draw the attention to our article "Safety Rules for Cryopreparation" (Sitte *et al.*, 1987b), which gives answers to all important questions. The mentioned risks were mostly overlooked or at least underestimated, especially the dangers of propane (explosions and burning) and the evaporation of LN₂ (unconsciousness and possibly death by asphyxiation). 1 liter of LN₂ develops nearly 800 liters of GN₂ (see Ryan and Liddicoat, 1987). The most severe danger results, if cryosystems with a high consumption of LN₂ run in small cabinets: this is often the case with cryo-ultramicrotomes. For example older FC-models of Reichert-Jung (FC4/FC4D/FC4E) consume up to 7 liters LN₂/h which correspond to approx. 5 m³ GN₂/h. If a cryosystem of this kind is operated in a small cabinet (often with respect to undisturbed work with closed door, switched-off air condition and in the late evening) the risk is deadly: unconsciousness results, if the O₂-content drops from a normal level of 21% to below 18%! Since unconsciousness arrives without any preceding signal like dizziness and reanimation is only possible within approximately 30 min, this is a potentially life-threatening situation. To answer in this context the question directed to the "amount of GN₂ produced in a typical cryo-ultramicrotomy session" correctly: if the session takes 2 h and work is done on one of the old FC-models 1980/90 at sectioning temperatures above -100°C, then up to 11 m³ GN₂ are produced. In a cabinet of (3 x 3 x 2) = 12 m³ volume with bad ventilation, danger for life exists obviously already within these 2 hours. Cryoultramicrotomy and similar cryowork therefore should only be done in well ventilated and larger laboratories. Risk is completely excluded if at least a second person is present in the room. Newer cryo-ultramicrotomes like Leica-Reichert FCS and FCR consume only approx. 1 to 2 liters LN₂/h - but risk still exists in the worst case discussed above.

Also splashing of liquid cryogens is really dangerous and it is often overlooked, that splashes of a "primary cryogen" like LN₂ and splashes of a "secondary cryogen" like liquefied ethane or propane behave completely different. Splashes of LN₂ are terribly dangerous, since they enter due to the superfluidity (low viscosity) of LN₂ through narrow slits easily into closed compartments like shoes (especially boots), protection gloves (often used for handling Dewar vessels) or safety spectacles: one can loose foot or hand by necessary amputation or the eyesight by LN₂ splashes. Just opposite is the risk of splashes from the viscous secondary cryogens which do not evaporate immediately and do not enter through narrow slits: here protection of skin and eyes by gloves and glasses is recommended.

I do not want to enumerate all risks of cryowork, but I recommend everybody responsible for co-workers concerned with cryo systems to refer to a paper on hazards and risks (e.g. Sitte *et al.*, 1987b) and to inform all co-workers carefully and completely about the danger. Probably it is the best, that those persons are forced to subscribe a declaration, that they have knowledge about the danger and that they will observe all safety regulations.

**G.M. Roomans:** Can you give a comment to the statements of Saubermann and co-workers in the late seventies and early eighties, who recommended cryosectioning of fresh-frozen material for element analyses at temperatures around -30 to -50°C?

**Author:** Sectioning at higher subzero temperatures in the range between -50 and -30°C is of special interest, if semithin and thicker cryosections (e.g. thicknesses between 0.5 and 2.0 μm) are cut. This is well known for sugar protected samples: higher temperatures around -80°C simplify the production of thicker sections considerably. Saubermann *et al.* (1977) analysed the cutting forces (cutting work) in thick section cryomicrotomy of fresh frozen specimens by strain gauges and found, that forces and work depend sometimes on the clearance angle of the knife and on the sectioning temperature. Based on these measurements and continued experiments they recommended cryosectioning at -
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30° for scanning electron microscopy and subsequent EDX (Saubermann et al., 1981). There was some controversial discussion about the pros and cons of section preparation at these temperatures (see e.g. Saubermann, 1981, discussion on pp. 391-396). Even if this discussion does not fit within the theme of this review article ("cryo-ultramicrotomy" with section thicknesses ≤ 0.1 µm), a re-evaluation and re-investigation of this work would be of interest in several respects: up to now it is not absolutely clear, what happens if perfectly frozen, that means correctly vitrified fresh samples, according to Fig. 32b are warmed up to higher subzero temperatures. There are investigations of Ryan (1991, see especially pp. 200-210 and Figs. 44-46) on red blood cells and spleen of the flounder, which after cryofixation were warmed up to temperatures between -80° and -10°C for some hours to several days before standard freeze substitution according to Van Harreveld and Crowell (1964). The results were really astonishing: the expected severe segregation artefacts were only observable at temperatures ≥ -20°C. Similar results were reported by Steinbrecht (1985), who "heated" fresh-frozen silk moth sensory hairs for 45 min to -43°C without visible damage (segregation of ice). These findings seem to be in good agreement with earlier experiments and considerations of Meryman (1957), Luyet (1960), Nei (1971, 1973) and MacKenzie (1981). They are of considerable importance for all hybrid methods (see Fig. 32 and Section 10 "Discussion", this review). But obviously different samples will react in a different manner if temperature is increased after rapid freezing above -80°C and a lot of experimental work will be needed to understand, what really occurs in this case.

G.M. Roomans: One should realise that most of the freezing methods recommended in this paper make it necessary to dissect the tissue (unless one works with cultured cells). The dissection procedure itself may cause artefacts: mounting of the tissue (slammer, plunging) and building up the pressure (high pressure freezing) introduce a time period during which the supply of oxygen and nutrients to the tissue is reduced, and where also drying of the tissue through evaporation of water from the outer layers is a risk. In conventional fixation, perfusion fixation is used to reduce or eliminate this problem. For X-ray microanalysis e.g., in situ freezing techniques have been proposed, even though these techniques do not give optimal results of freezing. Do you not think that in pursuing optimal freezing techniques, there is a risk of losing sight of possible artefacts occurring just before the freezing step? How should we attack this problem?

K. Ryan: You describe in several places the sizes of specimens suitable for various specimen holders or supports. Would you agree that the minimal possible size is always of utmost importance, bearing in mind the damage that can be done to the specimen during dissection or vibratoming?

Author: "Pre-freezing artefacts" are a very serious limitation of all freezing procedures, which need very small samples for efficient freezing, if studies on specific parts of larger organs or organisms are the goal and dissection is unavoidable. In this respect, already the loss of H2O from the freed surfaces is a problem: but this problem can be settled by continuous work in simple "humid chambers", which are relatively easy to realise (e.g., glove boxes with a wet tissue on the bottom develop rapidly approx. 100 % relative humidity, see also Sitte et al., 1987a, Fig. 4d4, p. 96). Much more difficult is the exclusion of pre-freezing artefacts due to dividing of larger organs by dissection or vibratoming: this is a severe limitation for high pressure, double jet or plunge freezing, which need extremely small specimens. Only direct in situ freezing of free surfaces with a propane jet (Green and Walsh, 1994) or with a slammer (e.g., an "inverted slammer": Edelmann, 1989b) enable with a minimum delay after dissection a proper freezing of such surfaces with minimum damage and changes. Often there is no other possibility, for example if deeper layers of organs like brain or kidney are of interest: they change morphology and certainly also physico-chemistry immediately after interruption of blood supply and speedy work is necessary. There already exist examples for such procedures: Bernard and Krigman (1974) have studied slam frozen deeper layers of the brain after fast slicing immediately followed by impact cryofixation. These experiments were repeated and confirmed by Van Harreveld and Fifkova (1975). They demonstrated, that the structures after this fast procedure were intact, if the delay between slicing and freezing is very small (e.g., below 10 sec). But this rapid work is only possible, if a suited cryogen-jet- or metal-mirror- or another purpose-designed-system is available, which is either not sensitive against large areas or volumes like single-jet or slamming devices (see discussion in the section on "Ambient Pressure Freezing") or excise and freeze a small fraction of the sample within a very fast event (e.g., Gatan cryo-snapper).

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