Fixation of Biological Samples for Electron Microscopy and Microanalysis: Conventional Chemical Artefact or Life-Like Cryoimmobilisation? A tutorial.

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Abstract

This paper looks briefly at the problems associated with preparing biological specimens using chemical fixatives, the problems being artefacts both in the structure and chemistry of the preserved specimen. The alternative approach is to use a physical method of preservation, namely rapid freezing. The main objective is to transform specimen water into an amorphous vitrified form without any crystallization, thereby avoiding ultrastructural alteration and translocation or loss of chemical constituents, the latter causes problems for x-ray microanalysis and immunochemistry. Water can form three types of ice at ambient pressure which are identifiable in the electron microscope. The dynamics of freezing, the nucleation of water, crystal growth and latent heat effects are important factors, as is subsequent processing which may induce phase changes in the frozen specimen. The application of high pressure during freezing has advantageous effects in that the melting point and the homogeneous nucleation temperature of water are depressed to -22 and -92 °C respectively. There are many current freezing methods which all entail their own practical considerations which affect the freezing process. The review finishes by suggesting suitable methods for particular types of specimen.

Keywords: fixation, artefacts, cryofixation, freezing, electron microscopy, ultrastructure, x-ray microanalysis, immunochemistry.

Introduction

The purpose of this paper is to consider, in the first instance, the effects of chemical fixatives used to preserve biological specimens. The alternative to a chemical fixative is a physical fixative, the paper then deals with very rapidly applied low temperature, or cryoimmobilisation. A major feature of biological specimens is their water content and it is this which causes problems in freezing. This is because its properties are such that during freezing, it can rewarm and even thaw the specimen temporarily through the release of latent heat. The dynamics of freezing and ice nucleation are confronted by various freezing methods which all attempt to solidify the specimen without ice crystal formation. This paper brings together many theoretical and practical considerations from both the literature and personal experimentation in an attempt to make a coherent set of points which will be helpful to those undertaking rapid freezing. After considering the serious problems associated with chemical fixation, it examines the properties of water and how these bear on the freezing process. The methods of freezing are then examined, followed by discussion of important practical factors such as specimen size, coolant properties and others which will affect the results. It concludes with recommendations as to which method is best suited to a particular type of specimen.

Electron microscopy offers extremely high resolution images of our world but where bio-medical specimens are concerned, the question arises as to how much of what we see is real? Preparing specimens for electron microscopy purposes is fraught with problems. Consider first a question - how would a fresh, living sample of tissue or a small organism react to being immersed in a chemical fixative?

Clearly, a fixative is a poison that immobilises and kills the specimen, producing adverse structural and extractive effects; there will be leaching of soluble or ionic...
components (Morgan 1980, Bone et al. 1982, Coetzee and van der Merwe 1984). The solvents used for subsequent dehydration also produce further shrinkage and extraction of components (Glauer 1975). Critical point drying of soft specimens for scanning electron microscopy (SEM) can cause even greater shrinkage and distortion (Boyd et al. 1977).

Many other changes which result from fixation methods for transmission electron microscopy (TEM) regarding specimen volume, post-mortem changes and other artefacts have long been documented and were collated by Hayat (1981a, b). It is often necessary to use cryo-fixatives to obtain sections suitable for TEM. The cryo-fixative fixes the specimen by rapid freezing and dehydration and sometimes also by the formation of ice crystals. This is achieved by placing the specimen in a cryo-fixative solution, which is typically a mixture of acetone and methanol, followed by rapid freezing in liquid nitrogen. After the specimen is dried, it is ready for sectioning and examination under the TEM. The cryo-fixative helps to preserve the ultrastructural details of the specimen, allowing for high-resolution imaging.

The objective of cryofixation

The primary objective, as far as the cell water is concerned, is to immobilise it more or less instantaneously before the molecules have time to migrate and organise into ice crystals. Robards (1984) calculated that cryoimmobilisation of Abutilon (Malvaceae) trichrome tip cells with a diameter of 15 μm could be completed in less than a millisecond, which is a million times faster than the ten minutes necessary for glutaraldehyde fixation.

The nature of water

Water constitutes approximately 80% of many cells and is the main and probably the most problematical.
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Figure 1. (a) The “flickering cluster” model of the structure of liquid water in diagrammatic form. The oxygen atoms are at the center of a tetrahedron of two positive and two negative charges. The O-H distance is 0.1 nm and the closest approach of two molecules is 0.28 nm as governed by Van der Waals forces. Redrawn from Nemethy and Scheraga (1961). (b) Diagram of water bonding showing 3, 4, 5 and 6-membered rings. Redrawn from Angell (1982).

It has been estimated that if melting is simply a breaking of hydrogen bonds, then comparison of the latent heats of fusion (333 J/g) and of vaporisation (2255 J/g) suggests just 15% of H-bonds are broken in the liquid at any one moment (Dubochet et al. 1988). It was pointed out by Baid (1987a) that latent heat release during cryofixation may be much reduced or even negligible if no crystallization takes place.

Cell water

Cell water exists in several states: (i) some water in the cell is bound to macromolecules in such a way that it is part of the structure of the molecule, if it were removed then the molecule would collapse, (ii) water molecules are highly polar and are affected by electrostatic forces and those adjacent to the bound molecules are said to be ‘perturbed’. This means that they are involved in hydration shells although there is conjecture as to how many layers of water molecules are ‘ordered’ in this manner (Kellenberger 1987). (iii) The remaining water can be described as free, bulk or ‘unperturbed’ water. Kistler (1936), described the measurement of ‘bound’ water by the freezing method, citing work from 1906 onwards. He concluded that the amount of unfrozen water in a specimen depends partly on the freezing mode.

Important temperatures

The freezing point of water is not fixed, whereas the melting point (T_m) is constant at 0 °C. In other words, as water is cooled it can remain liquid at temperatures far below 0 °C (at ambient pressure), depending on thermodynamics and its purity. If pure water is cooled, it can be supercooled and remain liquid down to approximately -40 °C, below which it will freeze. This point is the homogeneous nucleation temperature. In cells, various constituents may act as impurities in the water and form nucleation sites for clusters of water molecules on which ice crystals will begin to grow at some temperature higher than -40 °C, this being the heterogeneous nucleation temperature. The nucleation temperature is kinetic in nature and is a function of the cooling rate.

In order to convert the specimen water to a solid, amorphous, uncrystallized form, very rapid cooling is necessary down to near liquid nitrogen temperature. When this can be achieved, the resulting specimen is said to be ‘vitrified’ because the water is in a state which is analogous to a glass. If the specimen is then rewarmed slowly, it will reach a point at which the solidified amorphous water converts into crystalline ice; this is the devitrification temperature and occurs at -117 to -133 °C. The variation seems to depend on the rates of cooling and re-warming: Pryde and Jones (1952) showed that it always occurred in pure water during calorimetry experiments at -129 °C. Bachmann and
Mayer (1987) implied that only below this temperature can molecular mobility in water be permanently suspended and hence structural and ionic migration be totally inhibited; although the molecules may rotate, they may not migrate.

When systems containing cryoprotectants are cooled, a temperature is reached in the region of -113 to -133 °C, when internal molecular relaxation times $\tau_{\text{out}}$ (see 'Dynamics of freezing' section below) reach hundreds of seconds: at this point the liquid system becomes an uncrystallized glass, this is the region of the glass transition temperature. During the rewarming of vitrified pure water, differential scanning calorimetry gave findings that suggested that the beginning of devitrification occurred at -137 °C, this occurred when rewarming at 30 °C/min (Mayer 1988). When rewarming at 10 °C/min, the change was noted at -150 °C, this underlines the thermodynamic and kinetic nature of these phenomena.

The properties of water can be changed by the application of high pressure. The optimum pressure is 2.1 kbar, when the melting point is lowered to -22 °C and undercooling is possible down to -92 °C (Kanno et al. 1975). This means that the critical cooling range may only be 38 °C (-92 to -130 °C) compared to the normal 0 to -139 °C (to the devitrification temperature). Further details in 'High pressure freezing' section below.

**Nucleation of ice**

Water molecules have been described above as forming transient bonded clusters. When cooling down to about 0 °C takes place, these clusters become temporarily more stable, but energy considerations drive instantaneous dissolution so that the liquid state is maintained until some critical temperature is reached at which molecular dynamics and perhaps cell constituents dictate that a solid form will be maintained: this being a small ice nucleus. At -10 °C, 16000 molecules are necessary to form a critical sized cluster (Echlin 1992b); considering that a water molecule occupies a sphere of radius 0.28 nm, it can be seen that the diameter of a cluster is 10 nm. At about 40 °C, the energetics are lower and only 200 molecules are needed to form a critical sized nucleus, with a diameter of about 1.5 nm.

The effects of supercooling and intracellular particles were modelled by Toscano et al. (1975). They showed that, regarding homogeneous nucleation, with cooling rates of up to 10,000 °C/min. supercooling down to -63 °C was possible although faster cooling rates immediately brought this back to about -38 °C. With regard to heterogeneous nucleation, they showed that the presence of very small particles, acting as catalysts, significantly increases the nucleation temperature during rapid cooling to the region of -10 °C, depending on the size of the particles.

**Figure 2. Internal relaxation time ($\tau_{\text{in}}$) and escape time ($\tau_{\text{out}}$) for the homogeneous and heterogeneous nucleation of water. $\tau_{\text{in}}$ is the relaxation time of water molecules within the supercooled liquid. $\tau_{\text{out}}$ is the escape or relaxation time out of the liquid state into the frozen solid state. Modified after Angell & Choi (1986).**

**Dynamics of freezing**

As stated above, liquid water can be cooled to below its freezing point, when it is said to be supercooled: in this state it is metastable with hexagonal ice. The process involves two opposing time scales: $\tau_{\text{in}}$ and $\tau_{\text{out}}$ as shown in Fig. 2 (Angell and Choi 1986). $\tau_{\text{in}}$ is a measure of the internal relaxation time of molecules within the supercooled metastable state: it scales inversely with viscosity, at room temperature these are $10^{12}$ s and $10^2$ poise. At the glass transition temperature, $\tau_{\text{in}}$ is $10^3$ s and viscosity is $10^{13}$ poise. $\tau_{\text{out}}$ is the escape time, this being the time for relaxation out of the metastable liquid state to the stable crystalline state of a given volume e.g. 50%. As the water cools, $\tau_{\text{out}}$ initially decreases as the force for crystallization increases. In the region of -70 °C for homogeneous nucleation and -41 °C for heterogeneous nucleation, the curve for $\tau_{\text{out}}$ forms a 'nose' and increases again as temperature falls further and viscosity increases (Angell 1983). The 'nose' results from the competition between
Figure 3. (a) Stereo pair of a 0.1 μm-thick resin section of a red blood cell after freezing in Freon 22 followed by freeze substitution. Note the branching ice cavity which is probably continuous. Field width 100 μm. (b) Semi-thin (0.5 μm) of a red blood cell. This image shows possible intrusion through the cell membranes at the left side, from which crystals have spread along the cell. This suggests that crystallization occurs first in the extracellular medium and may then invade the cell. (c) Stereo pair of a carrot parenchyma cell in a 0.5 mm³ specimen which was frozen on a copper block and then freeze fractured and freeze dried in the cryoSEM. Note that the empty space left by the ice forms a continuous cavity between the matrix of cell constituents which were deposited by phase segregation between the ice crystal branches. Field width 2 μm.

the competing forces for crystallization and dissolution, i.e. molecular mobility: the latter decreases with falling temperature and increasing viscosity.

The significance of $\tau_{\text{tot}}$ is that it represents the time for embryonic nuclei to form "seed" crystals: these result from chance, statistical fluctuations in the molecular configurations of liquid water combined with the time necessary for these to grow spontaneously until the chosen fraction of water is crystallized. Essentially, the area above the reflex curve for $\tau_{\text{tot}}$ represents a window of opportunity in time for crystallization to occur because the times are long enough to enable e.g. 50% crystallization "out" of the liquid. In other words, waiting long enough in metastable conditions will in principle always allow escape (Austen Angell, personal communication). The time taken to crystallize just 0.01% of the water will be less. The smallest possible extent of crystallization is when there are just critical nuclei formed, then
Figure 4. Temperature-pressure-phase diagram of water. Liquid water occurs above the curve and solidified water below it. The dotted boundaries indicate areas of uncertainty. Ice type I can occur in three forms: hexagonal, cubic and amorphous vitreous. At 2.1 kbar, the freezing point is depressed to -22°C: this is made use of in high pressure freezing. Modified after Walley et al. (1968) and Duboclet et al. (1988).

the whole curve has shifted to the 'nucleation curve' which has its minimum at lower temperatures (Senapati et al. 1991). Cryofixation must cool the specimen fast enough to avoid the water remaining long enough at a particular temperature and hence the T° dependence on ice crystals to nucleate and grow. This is all on a severe sub-second time scale. It can be noted that for homogeneous nucleation, this both begins and forms a plateau or 'nose' at lower temperatures than for heterogeneous nucleation.

Crystal growth and latent heat

Crystallization proceeds rapidly once freezing is triggered (Stephenson 1956). Water is a large reservoir of thermal energy in the cell and once the molecules bond to the nearest crystal, they release this energy as the latent heat of fusion of bulk ice. This is 334 J/g at the melting point of water. If the conversion occurs at e.g. -38.3 °C, calculated as a 'heat of fusion of ice nuclei' by Rasmussen (1982), the heat release is only 125.5 J/g.

Freezing near the melting point of water releases the equivalent of 80 calories which equates with a potential local temperature rise of 80 °C. This is quite astonishing and presents a major problem in cryofixation, not only must the cooling method cool the specimen from ambient temperature, it must also cope with tremendous potential heat formation during the process! This was modelled by Stephenson (1956) and illustrated from a cooling experiment by Ryan (1992), where the thermal record was seen to reverse direction and temporarily rise above 0 °C due to warming of the thermocouple by latent heat which was released while the hydrated specimen was plunging slowly through a 10 cm deep cold gas layer into liquid propane.

Ice crystals, once initiated, can grow during cryofixation at velocities possibly up to 30 cm/sec (Bald 1987a) and form a highly branched, dendritic structure in the cell. The appearance of many holes in thin sections is misleading because they are probably profiles of
a single entity, or at least a single entity within any membrane-bound compartment of the cell. This was indicated by electron diffraction (Dubochet and McDowall, 1984) and is strongly indicated in Fig. 3. The diameter of a crystal branch is probably a reflection of local water concentration and the local cooling rate; this means the cooling rate within that compartment because latent heat release during crystallization within that compartment will affect the cooling rate probably at a very localized level.

Types of ice

Ice is polymorphic, with a tenth form reported by Polian and Grimaditch (1984), most of the polymorphs of ice occur at high pressures (Fig. 4). The three forms of ice associated with EM cryomethods can be identified with certainty in the cryo-TEM by electron diffraction (Dubochet et al. 1982, Michel et al. 1991).

When ice forms in nature, it is by slow cooling and is normally hexagonal in structure. This form is often found in cryo-fixed specimens because cooling is not rapid enough. The description is derived from the hexagonal arrangement of the oxygen atoms, when viewed from the appropriate direction. The thermal conductivity of hexagonal ice near the melting point is 2.2 W/m°C which is approximately four times that of water at the same temperature. The conductivity increases inversely as temperature is lowered until at liquid nitrogen temperature (Fig. 5): it is approximately five times greater than at 0 °C (Echlin 1992a). This property is of little interest in cryofixation because once this ice is formed, the objective of cryofixation has been thwarted. However, it may assist centre-line-cryofixation, this is where good freezing can occur in the centre of otherwise crystallized specimens which were cooled from two sides or more (Ryan et al. 1990, Ryan 1992). Hexagonal ice is the main stumbling block in cryofixation because its formation disrupts ultrastructure, due to its ramifying dendritic branching form. When damage is severe, the ice dendrites can be several micrometers in diameter. The crystals are composed of pure water, which means that any chemical constituents which may be the goal of analytical methods must be translocated, and thereby concentrated, by a distance which is at least the radius of the dendritic branch of the ice crystal. This can render quantitative x-ray microanalysis meaningless under some conditions.

Cubic ice is a metastable form with a crystal diameter of 30-100 nm which exists between −80 and −150 °C (Dubochet et al. 1988, Echlin 1992b). It is formed when vitrified ice is irradiated and/or warmed in the electron microscope. It also occurs in small bulk specimens between the vitrified surface layer and the deeper hexagonal ice region (Dubochet et al. 1987). It is not normally formed in bulk during rapid cooling, although Angell and Choi (1986) showed it to be expected in their Fig. 1a because this would not be a surprising development when cooling from strongly super-cooled water. The reason given is that this follows the Oswald step rule: when a metastable system breaks down, it moves to the nearest free energy minimum rather than the lowest, because the nearest free energy minimum can be reached with the smallest activation energy.

Amorphous or vitreous ice is the goal of cryofixation and was first reported by Burton and Oliver (1935) and was also shown under experimental conditions by Bruggeller and Mayer (1980): it is achieved when the specimen water is solidified without having time to form any microcrystalline structure. To complicate matters, there are perhaps several forms of amorphous ice: the normal form which is the goal of rapid cooling has a density of 0.91 g/cc but a high pressure, high density form with a density of 1.31 g/cc can be formed by subjecting hexagonal ice to pressures in excess of 10 kbar, although this is of little interest in cryofixation. Echlin (1992b) has reviewed the subject of ice regarding low temperature microscopy.

In specimens which are later freeze-substituted or freeze-dried, the types of ice are partly irrelevant because these processes are performed above the devitrification temperature so that amorphous ice converts to cubic. Further, if specimens are not cryo-dehydrated by freeze-substitution or freeze-drying, when the temperature rises to about −70 °C, cubic ice converts to hexagonal ice (Mayer and Hallbrucker 1987). This may also be irrelevant in freeze-drying because of the rapid sublimation of ice in vacuum at that temperature. The most likely ice artefact to be encountered in the practice of cryomethods is the holes created in specimens by hexagonal ice - either in hydrated or cryo-dehydrated specimens i.e. those which have been freeze-dried or freeze-substituted.

Phase transitions and recrystallization

Ice is essentially metastable, dependent on temperature and pressure conditions. If cooling has produced amorphous, vitrified water then at some temperature in the region of −130 °C, cubic ice will be formed (Dubochet et al. 1988). Cubic ice will convert to hexagonal ice, starting at about −130 °C, but a period of about one week is necessary to induce the cubic-hexagonal conversion at this temperature. The conversion occurs after about 30 minutes at −70°C (Dowell and Ranfret 1960), although in the electron microscope, sublimation would remove the ice from a specimen; this highlights again the dynamic nature of some cryo-events. Hexagonal and cubic ice can be "vitrified" by electron irradiation in the electron microscope at temperatures below −203 °C; this ice revealed a peculiarity in that the vitreous ice derived from hexagonal ice de-vitrified back into that form again rather than into cubic ice (Lepault et al. 1983). A recent report relates the
amorphization in the cryoTEM by electron radiation of ice III or ice IX which was obtained by high pressure freezing (Sartori et al. 1996).

Polarised light cryomicroscopy was used by Luyet (1960) to make a valuable finding. He described 'irruptive recrystallization' which occurred when apparently crystal-free films of different solutions were warmed until they became opaque due to crystal formation. This occurred over a range of temperatures which related to the molecular weight of the solute, extremes of solute concentration altered this by only one or two degrees. The recrystallization temperatures given were: -129 °C if the surroundings consisted mostly of pure water, -80 °C formolin, -65 °C glycerol, -31 °C sucrose, -12 °C gelatin and -6 °C soluble starch. These findings are very relevant to the storage of frozen specimens. Obviously, any specimen with amorphpous vitrified compartments will defrivr at about -130°C, but it is likely that other specimens can endure much higher subzero temperatures without undue effect on the ultrastructure seen after freeze-substitution. An example was the spleen tissue stored for 48 h at -60 °C prior to normal freeze-substitution (Ryan 1992, Fig 19). Previous examples of this were described by Woolley (1974) and Barlow and Sleigh (1979) who freeze substituted specimens at -50 °C; also, Steinbrecht (1985) who exposed cryofixed sensory hairs to -43°C for 45 minutes without damage, although just two minutes at -24 °C produced obvious secondary damage.

Post-freeze ice crystal growth

Hexagonal ice is the lowest energy state of frozen water but it is not stable. If freezing has produced hexagonal ice, then the crystal dendrites will have a certain diameter. If the temperature is raised slowly certain transformations will take place: molecular relaxation will occur and the ice crystals will grow. This is secondary ice crystal damage and has long been known to occur during storage, the development of large ice crystals in stored ice cream is a common example. Luyet (1960) made another valuable finding which he termed 'irruptive recrystallization'. This was when large ice crystals grew during rewarming at the expense of nearby smaller crystals - again, the dynamics of water molecule migration in frozen specimens are impressive. A frozen system of 10% glycerol showed the phenomenon after 15 min at -20 °C although a frozen system of 10% gelatin showed no change after 36 h (this supported Luyet's concept of irruptive recrystallization being dependent on molecular weight).

An important study by MacKenzie (1981) using differential thermal analysis of a range of gels extended understanding in this field. He found that, during rewarming at 2 °C/min, frozen specimens were thermally stable up to remarkably high subzero temperatures. At between -35 and -10 °C, they acquired a new translational motion (termed 'antemelting') and between -30 and -6 °C, they exhibited exotherms and grain growth, from previously unfrozen structural water in the 'frozen' specimens. The exotherms, in effect, were manifestations of further release of latent heat of fusion of ice. As with the findings of Luyet (1960), the temperatures were dependent on the molecular weight of the solute. It is likely that this phenomenon features in the 'collapse' of freeze-dried specimens. It would be interesting to freeze-dry up to these temperatures and then vent the specimen to dried gas, to terminate the vacuum drying process, before completing the temperature rise to room temperature, as a measure against 'collapse'.

Factors which may facilitate vitrification

A central tenet in cryofixation is that vitrification of specimen water to the amorphous, vitreous state is possible (McDowall et al. 1983). This implies many things, some of which appear to contradict various statements regarding nucleation and the effect of natural nucleating agents in the cell. For the purposes of the following discussion, it should be borne in mind that much of what is said above with regard to important temperatures, nucleation of ice, bound water, crystal growth and latent heat release during ultra-rapid cooling is somewhat conjectural in the context of biological specimens, much information having been derived from pure water, solutions of salts and cryoprotectants or mathematical modelling. Also, as previously stated, many of the thermal properties are dynamic in nature - the important temperatures are not set points, many thermal events occur over a range of temperatures and are dependent on the rate of cooling or rewarming.

Firstly, cell water comprises about 80% of the cell, also approximately 15% of that water is probably 'bound' (Robards and Sleytr, 1985). This means that only some 68% of the cell is water that needs to be vitrified: Toscano et al. (1975) modelled cooling in red blood cells using 63% water. It is well established that vitrification is routine with thin aqueous films. When water is vitrified then no crystallization takes place and therefore there must be considerable undercooling with no nucleation and no release of latent heat of fusion. The necessary cooling rate for vitrifying a 1 mm-thick water layer has been calculated as being in excess of 3 M °C's (Bald 1986). The necessary cooling range is generally from ambient to below the devitrification temperature, i.e. +20 to -130 °C, some 150 °C. At the calculated cooling rate, this would take 50 μs.

If the \( \tau_{\text{out}} \) curves are now considered, in effect, the area on or above the curve leads to nucleation because there is time for the event to occur (Austen Angell, personal communication). The main strategy for avoiding crystallization during cryofixation when faced with very short \( \tau_{\text{out}} \) times (<1 μs) is to reduce the time that the
specimen dwells in the vicinity of \( \tau_{\text{out}} \) by increasing the cooling rate: other strategies are emulsification, cryoprotection, increased pressure or use of a smaller sample (Angell and Choi. 1986). The minimum time (\( \tau_{\text{cool}} \)) for heterogeneous nucleation to occur appears to be in the region of 0.5 \( \mu \)s at about -40 °C; this would require a much faster cooling rate - it would require approximately 300 M \( ^\circ \)C/s. The \( \tau_{\text{out}} \) curve for homogeneous nucleation indicates a minimum time in the region of 1 ms at about -80 °C; this could be captured in a freezingtime of 50 \( \mu \)s. This suggests that it may be easier to suppress homogeneous nucleation than heterogeneous nucleation by rapid cooling. The basic question remains as to what is the real cooling rate for vitrification?

A further consideration is that cells contain salts and that these depress the freezing point by about three degrees and that blood cells may supercool to about -63 °C (Toscano et al. 1975); this may correlate with the notion that ultra-rapid cooling will probably of itself produce pronounced supercooling and thereby facilitate transformation to amorphous ice. Finally, as summarised by Rebars and Sleytr (1985) in their Fig. 2.4, the recrystallization temperature of frozen cells is raised to about -93 °C (or higher. Steinbrecht 1985). Ryan 1992). This means that in cells without highly hydrated domains, the critical temperature range for cryopreservation may be only some 30 °C (-63 to -93 °C) and not the 90 °C for water films cooling from their homogeneous nucleation point to their devitrification temperature, or the approximately 120 °C from some heterogeneous nucleation temperature.

**Cooling methods**

**Plunge freezing**

Plunge cooling involves immersing the specimen, preferably at speed, into a cold liquid cryogen. It is perhaps the simplest method to use without specialised equipment because home-made devices can be made easily. The main requirement is an insulated container to hold liquid nitrogen (LN\(_2\)) and a support within it to house a container for the secondary coolant. Liquid nitrogen is not used to freeze specimens because, while with a standing temperature of -196 °C and being the coldest liquid easily available, it is particularly ineffective for cooling something very quickly. The reason for this is that it is a boiling liquid at -196 °C (it freezes at -210 °C) and as soon as a warm specimen enters the LN\(_2\), if it is not plunging at very high velocity, the LN\(_2\) in contact with the specimen boils due to turbulent natural convection and forms a film of gaseous nitrogen around the specimen, this insulates the specimen for some time until the film boiling collapses and nucleate boiling takes over. Eventually, this diminishes and the final cooling is by laminar natural convection (Bald 1984). LN\(_2\) can be pumped under vacuum to increase the boiling temporarily and render the nitrogen into a semisolid 'slush' (MacKenzie 1969); this can be used to freeze cryoSEM specimens. A cooling bath which subcooled the nitrogen by means of a surrounding LN\(_2\) jacket under vacuum was described by Umbrath (1974).

An early use of plunge freezing in liquid air was performed by Richards et al. (1943) who froze nerve axoplasm on grids and freeze-dried it for examination by TEM.

There are several factors which influence the effectiveness of plunge cooling: these include the particular coolant, its temperature, the size of the specimen, the specimen/support arrangement, the plunge velocity, the depth of the coolant and potentially critical, the cold gas layer above the coolant. Generally, these factors interrelate: (i) an efficient coolant is required, (ii) the specimen should be as small as possible, at least in one dimension, (iii) if it is ideally small then the specimen support should not be so massive as to function as a heat reservoir and thereby impede cooling, also it should be streamlined to facilitate coolant flow over the specimen, (iv) the velocity through the coolant needs to be fast so as to maintain the specimen surface at the temperature of the coolant, thereby maximising the thermal gradient between the sample and the coolant (v) the coolant should be deep enough to maintain forced convective cooling over the critical cooling range, generally taken to be below the devitrification temperature but possibly not as argued above, depending on the domains of hydration within the specimen, (vi) if the specimen is ideally small then it will be thermally very sensitive and if the support is ideal then the specimen could freeze in the cold gas above the coolant if its depth is not minimised (Ryan and Purse 1984, Chang and Baust 1991). An exception to this could be a carefully arranged 'stagnation point' cooling set-up where the specimen is placed on a curved leading edge; this is derived from heat transfer studies in aerodynamics (Elder et al. 1982, Bald 1987c).

The main coolants for cryopreservation are propane and ethane. Ethane is more efficient but more expensive and needs control valves which operate at higher pressure; ethane is necessary when frozen films are destined for cryoTEM because the coolant will evaporate in the vacuum of the microscope much more readily; it is also necessary for cryosectioning vitrified specimens because it can be more easily evaporated in the cryoultramicroscope (Sitte. 1996). These gases are easy to condense but they are explosive and care must be taken in their use (Ryan and Liddicoat 1987, Sitte et al. 1987).

A wide range of liquid coolants has been tested: namely liquid air, liquid helium, liquid nitrogen (as a boiling liquid, subcooled liquid, melting solid or 'slush', supercritical above 33 bar and under high pressure at 2.1 kbar), iso-pentane, hexane, butane, propane, ethane, ethanol, acetone, ethylene glycol and various Freons.
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including at least one new Freon-replacement (Müller et al. 1993). They have been used in a variety of cooling devices; the coolants and their use were reviewed by Ryan (1992). Their description will not be repeated here, except to note again that the most efficient for cryofixation are propane and ethane. The main reason for their efficiency is that while they solidify at near LN2 temperature, their boiling points are much higher although many other factors play a part in cooling efficiency such as density, specific heat and thermal conductivity (Table 1).

during crystallization; this was shown in Fig. 1 of Ryan et al. (1987) where similar sized epoxy and hydrated specimens were compared. Also, thermocouples cannot be made small enough at present to occupy a very small area in a specimen, this means that thermocouple results reflect a generalisation whereas cooling rates vary throughout a specimen from the surface to its centre and even vary perhaps within cells and their compartments, depending on local water content.

A modelling approach was taken by Bald (1984) where many coolants were analysed, based on experi-mental results reported by Silvesier et al. (1982): it was found that the most efficient coolant is liquid nitrogen when used above its critical pressure of 33.5 bar. A different approach was to freeze substitute specimens and analyse the resulting ice crystal damage. This was done to varying degrees by Van Yenrooj et al. (1975), Schr"{o}webe and Terracio (1980), Escag (1982), Elder et al. (1982) and Handley et al. (1981). A more systematic study involved plunging gelatin or blood cells in different coolants and at different velocities and then quantifying ice crystal damage, the experiments were also modelled with results which were in good agreement with the experimental findings (Ryan et al. 1990). The

<table>
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<tr>
<th>Coolant</th>
<th>T_{\text{MP}}</th>
<th>T_{\text{BP}}</th>
<th>T_{\text{sub}}</th>
<th>density (g/cc)</th>
<th>specific heat (J/g K)</th>
<th>thermal conductivity (W/cm K)</th>
<th>viscosity (h_{\text{bar}}(T_0-T_{\text{MP}}))</th>
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<td>Nitrogen</td>
<td>-210.1</td>
<td>-195.9</td>
<td>14.2</td>
<td>0.869</td>
<td>2.0</td>
<td>0.00153</td>
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Table 1. Thermophysical properties of cryogenic fluids near their melting points, where available, sorted by h_{\text{bar}}(T_0-T_{\text{MP}}). Data taken largely from Table 1 of Bald (1984). T_{\text{MP}} - melting point, T_{\text{BP}} - boiling point, T_{\text{sub}} - available subcooling below the boiling point. Nitrogen and methane (E) exhibit excessive vapour to be useful as coolants at ambient pressure. The viscosity value for Freon 22 was interpolated from values for ethane. The value h_{\text{bar}}(T_0-T_{\text{MP}}) is the calculated surface heat transfer coefficient (h_{\text{bar}}) while plunging through a coolant at constant velocity, being the summation of forced convective and discrete nucleate boiling, acted on by the mean temperature difference between the sample surface and fluid during plunging (T_0-T_{\text{MP}}), before coming to rest. T_0 was based on the surface temperature of a modelled spherical, exposed sample. The heat fluxes for ethane and propane agree closely in ratio with thermocouple results from exposed gelatin specimens (Ryan et al. 1987).
Cryofixation of biological specimens

<table>
<thead>
<tr>
<th>Coolant</th>
<th>TMP °C</th>
<th>T °C</th>
<th>Cooling rate °C/sec</th>
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<td>Ethane</td>
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<td>-185</td>
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<tr>
<td>Propane</td>
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<td>Propane + Pentane</td>
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<td>-165</td>
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<tr>
<td>Nitrogen (boiling)</td>
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<td>-196</td>
<td>731</td>
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</table>

Table 2. Cooling rates from a bare thermocouple measured between 0 and -100 °C while plunging at a velocity of 2.25 m/sec into a 13 cm depth of coolant. The melting point (TMP) and temperatures (T) of the coolants tested in the experiment are shown. Note that ethane and propane could be slightly supercooled. The result for liquid nitrogen was extrapolated from the cooling curve because the thermocouple did not reach -100 °C during the moving part of the plunge. Note the order of coolants in terms of efficiency; also that the colder the coolant, the more efficient was the cooling. Table adapted from Ryan et al. (1987).

A much gentler approach was described by Boyne (1979) who used a glycerine-damped mechanism to ensure gentle contact which eliminated recoil, so that the very early stage of heat transfer was not momentarily interrupted. The recoil suppression system was found by oscilloscope monitoring to suppress all bounce for up to 50 ms, this being sufficient to facilitate good freezing. A falling copper block filled with LN2 was described by Edelmann (1989) for capturing dynamic muscle experiments. Livesey et al. (1989) described a similar device to that of Escaig except that the copper block was gold coated and the system was microprocessor-controlled for automatic cycling. Maintenance of contact between the specimen and the block after initial touching onto the block is extremely important; in some systems, this is achieved by a holding magnet (Cryopress), pneumatic pressure (LifeCell) or a spring, airbag and non-return clutch system (Leica-Reichert MMB80).

Cryoblock materials were modelled by Bald (1983), where the thermophysical properties were considered at both liquid helium temperature (about -269 °C) and LN2 temperature (-196 °C). Important properties in the process were the density, specific heat, thermal conductivity and thermal diffusivity. Besides tabulating these, he listed the modelled interface temperature between the specimen and block on first contact as well as other relevant combinations of parameters. The interesting point to come out of this work is that the properties of candidate metals are so variable with changing temperatures (Fig. 6). The most efficient material would be tin if it were maintained at -270 °C, followed by aluminium then silver maintained at -257.4 °C; however, the moment that a specimen contacts the cryoblock, the metal surface jumps to some higher temperature and thermophysical properties immediately change to different values. Copper was the most efficient metal at LN2 temperature. Metal blocks cooled to LHe temperature will actually cool specimens more slowly than if used at their optimum temperatures which he presented in his Table 3. Several useful comments were made regarding the potential of tin, sapphire and ultra-pure aluminium.
Cryo-fixation of biological specimens

A slightly different device was described by von Zglinicki et al. (1986), this featured a needle cooled in liquid propane which was stabbed manually into a specimen. These devices were designed for in situ freezing, where specimens were minimally perturbed prior to freezing. A more recent device is the pneumatic cryo-puncher described by Zierold (1993a). This system is an automated sampling method: the punch moves vertically from storage in LN₂, then horizontally to above the specimen to be sampled, plunges down at some 3.5 m/s, penetrating 2-5 mm in the specimen, resting for some 50 ms. The movement is then reversed with the whole process taking about 1 second.

Jet freezing

This method involves squirting coolant, conventionally propane, onto the specimen from a pressurised reservoir using compressed gas. Sometimes it is used with a single jet on bulk specimens but more routinely with thin specimens in a sandwich of metal foils which are then placed between two jets so that cooling is from both sides simultaneously. The sandwich planellettes were introduced originally for plunge cooling specimens destined for freeze-fracture, their advantage being that they possess a high surface area/volume ratio and thus cool very efficiently (Gulik-Krzywicki and Costello, 1978). One advantage of jet freezing is that coolant can be moved over the specimen surface faster than with plunge cooling, thereby effecting faster cooling. The technique was described by Burstein and Maurice (1978) with a single jet to freeze eye corneas and small bulk botanical samples. Müller et al. (1980b) described the double jet for sandwiched specimens: this device arose from a test system for double jet development in the high pressure freezer (Martin Müller, personal communication).

Various arrangements of jets and substrates have been used. High conductivity foils are normally used, but an insulating layer of Thermostar, bearing cultured cells, has also been utilised (Frisch et al. 1981. Espevik and Elgsæter 1981, Knoll et al. 1982, Plattner and Knoll 1984). Jet freezing was adopted by Van Venet et al. (1981) to preserve different temperature phases of lipids. This was accomplished by means of a temperature controlled housing for specimens from which they were moved into the path of the photoelectrically-activated propane jet. Improvements in double-jet design and operation have been described by Haggis (1986), who described a simple test for checking synchronicity of the jets by placing a thin metal foil in the place of the specimen. He also described modifications regarding the amount and temperature of propane delivered during operation and a lower thermal mass specimen support. Gilkey and Stachelin (1986) and Müller et al. (1993)

Figure 6. (a) Thermal conductivity and (b) thermal diffusivity curves of candidate metals for cryoblock freezing. Thermal conductivity describes the heat energy transfer in steady state situations where thermal conditions are in equilibrium; the rate of transfer depending on the thermal gradient and the conductivity of the material. Thermal diffusivity is the property relevant to rapid freezing situations where transient thermal conditions exist. It is derived by dividing the thermal conductivity by the product of the density and specific heat capacity of the material. Note how conductivity deteriorates as temperature falls towards absolute zero, whereas diffusivity improves in these materials. Redrawn from Bald (1987d).

Cryo-needle freezing

Cryo-needle freezing is effectively a variation of the impact/slaming method: the specimen is frozen by contact with a cold solid. Chang et al. (1980) described a pistol that shot a liquid nitrogen-cooled biopsy needle into a specimen and automatically retracted the sample.
describe further improvements to currently available instruments from RMC and Balzers respectively.

An improved cryo-jet for bulk specimens was described by Greene and Walsh (1992, 1994). This is capable of delivering up to fifteen doses of liquid propane in controllable amounts, an important aspect was the pre-conditioning of the jet prior to freezing specimens. An innovative form of propane jet was described by Zierold and Schäfer (1988) and Zierold (1991) who, in effect, used a shower of liquid propane to immobilise a single *Amoeba* in a defined physiological state on a microscope slide under a dissecting microscope for SEM and x-ray microanalysis.

With the latest JFD 030 commercial device from Balzers, Wunderlich et al. (in prep.) found improved cooling by discarding the deflection shields which are meant to help synchronise the jets. They seemed to cause a pre-cooling of the specimen (this is the opposite to the finding reported by Müller et al., 1993), which illustrates how problematical cryofixation can be. Their measured cooling rates of similar specimens cooled by propane showed three times faster cooling by jet cooling compared to plunge cooling.

**Spray freezing**

This method is essentially a specialisation whereby microdroplet specimens are plunge cooled by spraying them as an aerosol into a liquid coolant. The advantage of small droplets is their high surface area to volume ratio which enables very rapid cooling. Spray freezing was introduced into electron microscopy by Williams (1954) for freeze drying viruses. The microdroplets were impinged at high velocity onto a collodion film stretched over a cold copper block.

The method was introduced for freeze fracture by Bachmann and Schmitt (1971). They sprayed specimens into a small cavity in a cold copper block which contained liquid propane, the cavity shape was designed to prevent blowing-away of the coolant when specimens were sprayed into it. The cryogen was subsequently pumped away at a raised temperature (-85 °C) and replaced by cold butyl-benzene as a cryoglu to hold the frozen specimen droplets together for freeze fracture. Further descriptions of the original technique were given by Bachmann and Schmitt-Fumian (1973a,b) and Plattner et al. (1973).

A somewhat different approach was described by Knoll et al. (1991, 1992), where uncell microspecimens were shot into liquid propane through a fine sieve plate in the form of fine jets, rather than microdroplets. The method was devised for resolving short interval steps in membrane fusion processes (see general review by Ryan and Knoll 1994).

An approach where specimens are frozen for freeze substitution in KR’s laboratory is seen in Fig. 7.

**Figure 7.** Spray freezing using an artist’s air brush. Note that the microdroplets are sprayed into the cryogen which contains a cryotube with frozen freeze substitution medium for automatic collection of the frozen droplets. The tube is later removed, the bulk of the cryogen can be wicked away if desired before further processing. A cardboard shield with a hole in it prevents the build up of droplets around the edge of the cryogen container where they might freeze less efficiently, it also minimises loss of cryogen due to the action of the air brush.

The advantage of the technique is that the specimens are frozen in the cryogen and collected automatically by falling into a cryotube which contains frozen substitution medium. All that needs to be done after freezing is to transfer the tubes into the freeze substitution system.

**High pressure freezing**

This method took over 20 years to develop into a routine technique; the principle was described by Riehle (1968) and Riehle and Holecil (1973) and a practical device was described by Moor and Riehle (1968). This is essentially a double jet method using liquid nitrogen to cool thin sandwiched specimens, not only above its critical pressure (33.5 bar) where no...
vapour will form and thus realise the cooling potential of the LN2 (Bald 1984), but at a far higher pressure of 2.1 kbar or more, at which the thermophysical properties of water are changed (Fig. 8). The melting point of water is reduced to -22 °C and supercooling may occur down to -92 °C (Kanno et al. 1975). Normally, freezing increases the volume of water but high pressure hinders this expansion and may also impede crystallization. Moor (1987) gave an account of the accumulated wisdom at that time regarding the practicalities of the method using the Balzers HPM 010.

Important advances were made when 1-hexadecene, a paraffin oil, was found to be a suitable medium to use with specimens. It is non-toxic, non-queous, hydrophobic, with low viscosity and low surface tension (Studer et al. 1989, Michel et al. 1991). A second commercial device (Leica EM HPF) was used by Studer et al. (1995) to vitrify 150 μm-thick slices of cartilage.

High pressure freezing was originally designed for use with freeze fracture and freeze etching (Moor and Riehle 1968). However, frozen specimens can be detached from the specimen sandwiches and cryosectioned using a cryoultramicrotome (Michel et al. 1991, Richter et al. 1991). Also, cell suspensions or fine needle biopsies can be frozen within 40 s of excision and then freeze substituted and resin sectioned (Hohenberg et al. 1994, 1996).

### Thermocouple results

Cryofixation equipment and coolants can be characterised by thermocouple experiment. This can test the order of efficiency of different coolants, their best operating temperature (usually the coldest temperature for liquids or some optimum temperature for solids), the speed of delivery of the coolant (velocity in plunge, jetting and slamming), the duration of cooling (depth of plunge, volume of coolant in jetting), the maintenance of coolant contact (especially in slamming). Many aspects of these considerations can be found through the previous reviews by Ryan (1992) and Ryan and Knoll (1994) and will not be repeated here: Table 1 of Ryan (1992) lists earlier reviews with comments on their content.

The important comment regarding thermocouple experiments is that they do not indicate actual specimen cooling rates. Cooling in the cell occurs almost on a molecule-by-molecule basis, because as one part of a cell freezes, if it crystallizes then the release of latent heat will influence the cooling of adjacent areas - this is probably reflected in the different sizes of ice dendrite in neighbouring membrane-bound compartments, although local water content in a compartment will also play a part in the process. No thermocouple can be made at present fine enough to probe the finer points of the very dynamic cooling/freezing process. In fact, the finest indicator of cooling/freezing rate is probably the diameter of the ice crystal dendrites themselves, but this cannot be interpreted without detailed knowledge of the specimen composition and properties: then modelling can give a value closer to the real cooling rate.

### Cryofixation and time-resolved freezing

Freezing times of 80 to 160 ms were recorded by Ryan et al. (1990) in 415 μm-thick gelatin specimens sandwiched between 100 μm-thick copper planchettes. These were deliberately large specimens used to model cooling and not very realistic when considering proper sandwich specimens, which are generally much thinner. In other words, real (thinner) specimens probably freeze within 10 to 15 ms, also this means the whole specimen - the freezing time at any one point, especially when vitrification is achieved, could be in the order of 50 μs as discussed earlier. Jones (1984) modelled freezing at a depth of 10 μm in specimens described in the literature and concluded that freezing at a particular location could occur over a temperature range of 10 °C and took from 50-250 μs. The uncertainties regarding the temperature at which freezing actually occurs and any delay in precise timing of contact with the coolant to the time of freezing could introduce another error of 0.5 ms. This can be regarded as the worst case in time-resolved experiments - events occurring in shorter times may not be captured by cryofixation. In freeze fracture studies, the...
variation in the plane of fracture may add a further 0.5 ms to the error.

Many biomedical/physiological processes can be arrested at short notice following a stimulus. There have been many applications of time-resolved freezing following electrical or chemical stimulation, electrophoresis, chemical relaxation after a temperature jump, electroporation (which is analogous to relaxation after applying a radio frequency electrical field) and flash photolysis methods. The subject was reviewed by Ryan and Knoll (1994).

Practical factors affecting cryofixation

1. Specimen size

Of all the factors influencing the quality of freezing, the most important one is probably the size of the specimen. If the specimen is a large piece of tissue, then only a narrow zone at the surface will freeze without damage; when plunging or jetting with ethane or propane or with impact freezing, this is often confined to <1 to 5 μm in depth. Far better results can be obtained when the specimen is small in at least one dimension. For example, a 0.25 mm cube can be frozen without apparent damage after freeze substitution. However, these specimens are difficult to prepare and undergo physiological changes prior to freezing. Double-jet freezing usually involves specimens which occupy the space between the bars of EM grids which are used as spacers between metal foils, thus providing specimens which are suitably small. If a specimen can be sliced and mounted on a foil support (similar to a tennis racket) then cooling can occur from both sides during plunging and converge with improved results in the centre line (Ryan et al. 1990): such slices are also ideal for cryoblock cooling. Ideally-sized specimens are the microdroplets formed during aerosol spray-f Freezing. In high pressure freezing, specimen size is still a factor because highly hydrated specimens will not vitrify to the full depth of a 500 μm-thick specimen.

2. Coolant

The most effective liquid coolant tested to date is ethane, certainly for plunge and microdroplet spray-freezing and seemingly also for jet freezing (Müller et al. 1993). There is no point, however, in using ethane if propane will vitrify thin specimens satisfactorily although ethane is recommended for thin film specimens for cryoTEM because it will evaporate more easily in the microscope, similarly for cryo-ultramicrotomy at the lowest temperatures. Sucrose-embedded specimens for the Tokuyasu method can be frozen in LN₂ because of the heavy cryoprotection afforded by the 2.3 molar sugar embedding medium (Tokuyasu 1973); although if lower concentrations of sucrose are used then better cryogens are needed. The most suitable solid cryogen appears to be copper at LN₂ temperature, although tin, silver, sapphire and aluminium at various lower (and hard-to-maintain) temperatures might be more effective (Bald 1987d). The most effective liquid coolant is potentially LN₂ above its critical pressure, this is used in high pressure freezing.

3. Coolant temperature

Liquid coolants are more effective at their lowest temperatures, this is true also when they are supercooled (see Table 2). A practical problem can be in maintaining these temperatures because most coolants will solidify as they cool down towards LN₂ temperature. They either need a small heat input to prevent this or mixing with some other coolant to depress the coolant freezing point and thereby possibly degrade their cooling performance. Coolant supercooling is possible using a magnetic stirrer. Solid coolants operate less at a particular optimum temperature (Bald 1983, 1987d).

4. Specimen/coolant velocity and depth/volume

Specimen/coolant velocity means the relative velocity of one compared to the other: in plunging it means the plunge velocity of the specimen into the coolant, similarly with spray freezing it means microdroplet velocity. In the ease of jet freezing, it means coolant velocity over the specimen. In all cases the parameter should be maximised as far as is practicable because an important objective in cryofixation is to instantly reduce the surface temperature of the specimen to that of the coolant and then maintain that condition as long as is necessary for the cooling to take effect. Depth/volume means that the coolant in plunge (or spray) freezing should be deep enough to accommodate the necessary specimen travel and in jet freezing it means that the volume of coolant in the reservoir should be enough to last for the required duration of freezing. The required volume derived by modelling of e.g. a copper block tends to be remarkably small: in order to successfully freeze a 5 mm square slice of kidney 500 μm thick, a block just 1 cm in diameter and 1.6 cm long would suffice (Bald 1987d).

5. Specimen support and coolant contact

The shape and bulk of the specimen support can be an important factor in cooling efficiency. If the specimen is suitably small for efficient cryofixation but is mounted on a massive metal support then the heat of the support will interfere with efficient cooling, although the “stagnation point” cooling mode may be important if the specimen/support relationship is conducive to this mode of cooling (Elder et al. 1982, Bald 1987c). Equally important is the consideration of streamlining of the specimen support, if this is impaired then coolant flow over the specimen surface will be impaired thereby affecting coolant contact with the specimen and cooling efficiency. Both of the considerations of low thermal
mass and streamlining were described by Ryan and Purse (1984, 1985) with the design of aluminium foil arch supports, these can be later strengthened for cryosectioning by in-filling the arch with a cryogel, such as those described by Karp et al. (1982), Steinbrecht and Zierold (1984), Michel et al. (1991) and Richter et al. (1991). In slam cooling, it is common to have some insulating material to back-up the specimen, although earlier methods used sliced liver for the purpose. In jet cooling, Haggis (1986) commented on the fact that a modification of the paired planchettes probably gave better cooling.

6. Cold gas layers

The importance of avoiding exposure to cold gas layers during plunge cooling was highlighted by Ryan and Purse (1984) when ideally-small tissue blocks mounted on foil supports were found to freeze prematurely, and slowly, while travelling through a deep cold gas layer en route to liquid propane. Under such circumstances, everything possible must be done to avoid exposing the specimen for too long to cold gas. The effect of cold gas in cryoblock cooling was investigated by Van Harreveld et al. (1974); they estimated that slices of brain tissue pre-chilled by 8.4 °C while travelling 2 cm in cold helium gas en route to the LN2-cooled silver block. To a certain extent, specimens for cryoblock freezing tend to be bulkier than for other methods and therefore they probably do not suffer quite so much damaging precooling. It is possible that a certain amount of precooling may be beneficial to the cooling process, although comparison of cooling rates of thermocouple/gelatin specimens cooled from 4 and 20 °C showed no difference in the cooling slopes (Ryan, unpublished results).

The effect of the cold gas layer was modelled by Chang and Beust (1991). Their Fig. 5 indicates that a 5 μm-diameter microdroplet specimen travelling at 10 m/s should not pass through more than 1 mm depth of cold gas (of secondary cryogen), and that at 1 m/s the critical height of cold gas is only 0.1 mm. Considering a 50 μm specimen, the critical heights are 10 mm at 1 m/s and 40 mm at 10 m/s.

The problem of cold gas layers has long been recognised in jet freezing (Haggis 1986). In this context, it is the blast of propane gas from the device that can precede the jet of liquid coolant that can do the damage: to prevent this it is necessary to condition the jet prior to freezing a specimen (Greene and Walsh 1992, 1994). Precrcooing was one of the major problems in high pressure freezing; it was overcome by introducing 2 ml of iso-propyl alcohol at the moment of initiating the freezing cycle, it delays LN2 cooling by about 15 ms while the pressure builds to above 2.1 kbar. If this were not done then the specimen would freeze before the high pressure was generated, resulting in poor cryosectioning.

Discussion

Freezing method suitability

This section borrows heavily from one of the sessions in Prof. Sitt`s workshops (see Acknowledgements).

1. Plunge freezing

Ethane should be used for the bare grid method, this is where small specimens such as macromolecules, viruses etc. are contained in a water film destined for cryoTEM; the specimen in this type of preparation is less than 1 μm thick (Adrian et al. 1984, Lepault et al. 1991). There is no alternative because only this coolant will evaporate rapidly from the grid in the vacuum of the microscope. Ethane should also be used for small specimens destined for cryosectioning where the amorphous vitrified state is required, again the evaporation of the ethane in the cryoultramicrotome due to its high vapour pressure will reduce `wetting` of the knife by the specimen. Plunge cooling is also useful for small droplets of viscous suspensions destined particularly for cryosectioning, it is not suited to aqueous droplets because they distort or disappear on impact with the liquid. Plunge freezing is also suited to bulk samples for light microscopy where ice damage may not be visible.

2. Jet freezing

(a) Single-sided - for in situ surface cooling of bulk specimens e.g. exposed spinal cord in physiological x-ray microanalysis studies where there is probably no real alternative (Mazzone et al. 1979, Greene and Walsh 1992, 1994). This method has been used for freezing cell cultures on Thermorox discs (Pscheid et al., 1981).

(b) Double-sided - for liquid samples between sandwiched foils which are normally spaced apart by an EM specimen support grid; there is no real alternative method, particularly for freeze fracture/freeze etch studies (Müller et al. 1989). The cooling rates are probably faster than by plunge cooling because of the large amount of coolant that passes over the specimen in a short time period. The method does not lend itself easily to follow-up cryosectioning.

3. Spray freezing

An ideal method for aqueous suspensions of unicells or fine emissions which can be sprayed from a nebuliser or air brush to form an aerosol, the droplets of which are in effect plunge frozen in a liquid coolant (Bachmann 1987). The method has also been applied to specimens which are squirted as a fine jet into the coolant (Knoll et al. 1991, 1992). Specimens can be collected easily over frozen media for freeze substitution or processed into a
cryogluue for freeze fracture and probably also for cryosectioning. This method is probably the least used of those in the literature and yet it has great potential with small specimens because they are ideally sized and receive omni-directional cooling. The highest cooling rates occur at the center of the droplets where the cooling fronts converge.

4. **Metal mirror freezing**

This method is best suited for large pieces of tissue, preferably thinly sliced, for which there is probably no alternative method. It gives single-sided cooling with higher surface cooling rates than plunge freezing, although follow-up pressure is necessary to maintain the initial contact with the cryoblock because any momentary bouncing of the specimen away from the cooling surface is highly disruptive to the cooling process. The method can deform tissue on a macro scale although the smaller micro-elements can be excellently preserved (Sitte 1996). The method can also be used for cell suspensions: these are pipetted into a tapered plastic ring which is cut from a plastic embedding mould so that the ring tapers away from the block and the suspension forms a slightly convex meniscus. Frozen specimens can be ‘dissected’ or rendered into smaller pieces and then mounted on cryoultramicrotome pins by placing the frozen piece on the mirror and carefully lowering the pin bearing a small drop of soft, liquid soap onto it and holding this onto the block until the soap solidifies. This does not impair the quality of the frozen specimen if done carefully. A practical drawback with metal mirror freezing is the entrapment of air bubbles on the front surface which will impair freezing. The specimens should not be allowed to desiccate and are best prepared in a humidity chamber or glove box (Sitte et al. 1987).

5. **High pressure freezing**

This is probably the only method for freezing **botanical specimens** because of their high water content, these specimens are particularly difficult to freeze at ambient pressure by other methods. The introduction of 1-hexadecene to replace aqueous media in the cooling sandwich and also to replace interstitial gas in spongy tissue has made the method even more successful (Michel et al. 1991). It is also successful with harder tissues which can be easily divided, such as cartilage (Studer et al. 1995). Recently, the method has been adapted for freezing **cell suspensions** by allowing short lengths of dialysis tube to fill by capillarity prior to freezing (Hohenberg et al. 1994). Also, **biopsy needle samples** have also been frozen within only a few seconds of sampling (Hohenberg et al. 1996). The yield of well frozen depth in the specimen is far greater in high pressure freezing than with any other method (Santori et al. 1993).

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**Figure 9** Flow chart of cryomethods for specimens after cryofixation. The dotted lines indicate pathways for cryosection and the boxed area indicates subsequent handling at ambient temperatures. The embedding step after freeze-drying can also be carried out at ambient temperature.

The description of the above freezing methods implies interference with the specimen during its preparation for freezing. The specimen may be stressed by handling, anaesthesia, dissection/excision trauma, cutting of the blood supply, loss of hydrostatic pressure, anoxia, osmotic change, ion flux and enzymatic imbalance. These are considerations also against conventional chemical fixation, except that in the majority of cryofixation situations, these effects are often much shorter lived. Certainly, cryofixation can be greatly more informative.

A recent study which compared slam freezing and high pressure freezing found that both methods vitrified liquid crystalline solutions of DNA containing up to 85% water, although both methods induced molecular reorganisation. The slam freezing method preserved the cholesteric structure but induced periodic changes due to mechanical compression. The high pressure method failed to preserve the structure of the liquid crystal in that the long range stratification was lost, possibly due to the relatively low cooling rate high pressure freezing. This work shows that vitrification, considered up to now to be the main criterion for good preservation, can damage specimens and that high pressure freezing must be used with caution as to its effects on specimens which are fragile or pressure-sensitive (Leferestier et al. 1996).

Perhaps the ultimate benefits can be seen in situations where dynamic cellular events or experiments are arrested by rapid freezing on a chosen sub-second time scale (reviewed by Ryan and Knoll 1994). The other important approach is in situ freezing which has been done with falling cryoglobs (Edelman 1989, Severs et al. 1993, 1995) and coolant jets (Greene and...
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This has been done more often with biopsies, where the specimen is frozen simultaneously with sampling (Hagler et al. 1983, 1989; Zglitziicki et al. 1986, Zierold 1993a,b). This approach is used for x-ray microanalysis but might also be contemplated for use in immunocytochemistry. A variety of processing pathways are available after the initial freezing of the specimen (Fig. 9); the specimen can be maintained at low temperature for observation or returned by various routes to ambient temperature for examination by electron microscopy.

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References


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