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Crystalline Ice as a Cryoprotectant: Theoretical Calculation of Cooling Speed in Capillary Tubes

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Abstract

It is generally assumed that vitrification of both cells and the surrounding medium provides the best preservation of ultrastructure of biological material for study by electron microscopy. At the same time it is known that the cell cytoplasm may provide substantial cryoprotection for internal cell structure even when the medium crystallizes. Thus vitrification of the medium is not essential for good structural preservation. In contrast, a high cooling rate is an essential factor for good cryopreservation because it limits phase separation and movement of cellular components during freezing, thus preserving the native-like state. Here we present calculations of freezing rates that incorporate the effect of medium crystallization, using finite difference methods. We demonstrate that crystallization of the medium in capillary tubes may increase the cooling rate of suspended cells by a factor of 25-300 depending on the distance from the center. We conclude that crystallization of the medium, for example due to low cryoprotectant content, may actually improve cryopreservation of some samples in a near native state.

Keywords

cryo-microscopy; cryo-fixation; cooling rate; vitrification

Introduction

In cryo-electron microscopy of biological material cryofixation is often the most important process in sample preparation. Two major types of cryofixation are in common use: freezing at ambient pressure and high pressure freezing (HPF) (Shimoni & Müller, 1998). Ambient pressure freezing, generally by plunging into an open reservoir of liquid cryogen, is suitable for thin suspensions of isolated macromolecules and small cells. With such thin samples, typically well under 1 μm thick, very high cooling rates lead to vitrification of the liquid. Rapid contact with a metal block cooled to even lower temperatures can increase the depth of good preservation, but generally by a small factor (Escaig, 1982). Such a small improvement reflects the fact that the cooling rate is limited by heat transfer through the sample itself rather than through the cryogen. In samples over 10 μm thick, slower cooling leads to crystallization, which causes distortion and damage of the specimen. When

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subsequent stages of sample preparation involve cryosectioning or freeze substitution, amorphization of thicker parts of the sample is necessary. Freezing under high pressure inhibits crystallization by lowering the crystallization temperature to -22 °C at 210MPa (Gilkey & Staehelin, 1986). Since the high pressure does not completely stop crystallization, maintaining a high cooling rate is still important for high pressure freezing. In many cases HPF only provide amorphization of the fastest cooling part of the sample near the surface (Studer *et al.*, 1995).

When only the region of the sample near the surface freezes in the amorphous state, the geometry of the sample becomes important. It was shown earlier (Bald, 1985) that cylindrical samples amorphize better than flat, and spherical samples amorphize even better. However handling of spherical samples is difficult. In contrast, freezing in a capillary tube offers a very convenient geometry for both freezing and sectioning. In a capillary the sample freezes faster than in a slab due to collapse of the freezing front toward the center of the tube, and methods of sectioning and freeze substitution in capillaries are well developed. This method is especially convenient for freezing suspensions of small biological objects such as bacteria and yeast. At the same time it may be easily adopted to freezing of plant materials and biopsies (Shimoni & Müller, 1998).

The physical processes in freezing biological samples are well known and are strongly dependent on the cooling rate and medium composition. At low cooling rates water, the main component of any biological system, crystallizes. Solutes are excluded from crystals as they form, localizing suspended materials, including cells themselves, in a concentrated compartment that eventually either crystallizes as a eutectic or vitrifies. Growth of macroscopic crystals can cause serious mechanical deformation of cells. Exposure to concentrated medium causes osmotic loss of water from the cell. While dehydration may improve the survival rate upon thawing for some cells including some Antarctic organisms (Elnitsky *et al.*, 2008, Wharton *et al.*, 2005), spermatozoa (Koshimoto & Mazur, 2002) and blastocysts (Hochi *et al.*, 1996) for electron microscopy dehydration should be avoided as it alters the cell ultrastructure.

At higher cooling rates the limited diffusion of solutes inhibits crystallization of the water. For example, even at relatively moderate cooling rates the amount of ice formed in a solution of glycerol decreases with increased cooling rate (Morris *et al.*, 2006). Many organic materials, for example sugars, proteins and other components of cytoplasm, exhibit similar behavior. Rapid cooling also produces smaller crystals, even to the point that the crystals (and their effect on sample structure) cannot be distinguished by electron microscopy.

Cryoprotectants are often added to further reduce crystallization (Echlin *et al.*, 1977, Franks *et al.*, 1977, Le Skaer *et al.*, 1977). Like high pressure, cryoprotectants depress the crystallization temperature (Guignon *et al.*, 2009) and increase viscosity, which lowers diffusion especially at the moment of crystallization (Morris *et al.*, 2006). While low molecular weight, penetrating cryoprotectants reduce crystallization inside of the cell as well as in the medium, nonpenetrating cryoprotectants, such as dextran, only affect the medium. Dextran only provides cryoprotection within the cell by dehydrating it due to osmotic

pressure (Rowe, 1955), which may alter the native state of the cell. Still, dextran is the most widely used cryoprotectant in electron microscopy for HPF sample preparation because it provides excellent cryoprotection to the medium. In spite of wide use of dextran, there is no clear protocol for proper choice of the cryoprotectant concentration for optimal electron microscopy. Typically 20 percent of dextran is necessary to avoid crystallization of medium in capillary HPF. On the other hand it was shown that significantly lower dextran concentration samples frozen to partially crystalline ice may be successfully cryosectioned (Al-Amoudi *et al.*, 2002, Yakovlev, 2010). Thus lower dextran concentration may be used for preparation of frozen hydrated sections if the quality of cryopreservation is sufficient. While lower cryoprotectant content reduces the dehydration effect and eases some sample preparation steps (especially centrifugation) here we suggest that there is another important advantage. In this work we computationally show that reducing the amount of cryoprotectant, resulting in partial crystallization of the medium, may improve cryopreservation by producing a significant boost of the cooling rate. While such a boost will be observed in any geometry we only consider the case of a cylindrical capillary sample because of the advantages of cylindrical geometry mentioned earlier.

Rapid freezing of the sample is always non-uniform since there is a strong thermal gradient across the sample. The rate of cooling depends on the position relative to the cooling surface. In conventional high pressure freezing, the cooling rate is the only parameter that differs for different parts of the sample. The relation between the cooling rate and quality of cryopreservation has been studied in several reports. For example, the degree of cryopreservation in different parts of a slab sample was evaluated by freeze substitution followed by sectioning and electron microscopy (Studer *et al.*, 1995). The parts of the sample that are close to the surface and cool faster show good ultrastructural preservation while deeper material exhibits specific segregation patterns due to ice crystallization. At the same time there have been a number of reports in which HPF was used with capillary tubes without adding a cryoprotectant and good sample preservation was observed with no segregation pattern (Erk *et al.*, 1998). In this case faster freezing in the cylindrical geometry provides sufficient cryopreservation to avoid freezing artifacts. It is well known that HPF of water in a standard capillary does not produce amorphous ice unless some amount of cryoprotectant, equivalent to about 20 percent dextran (Al-Amoudi *et al.*, 2002), is added. One can conclude that complete vitrification of the medium is not necessary to insure good structural preservation. Ultrastructure of the cell may be well preserved when the medium crystallizes, as long as the crystals are small and the interior of the cell vitrifies. Vitrification of the cell interior in crystallized medium is possible for most bacteria and yeast that contain about 20 percent protein which acts as a cryoprotectant. However under this scenario achieving high cooling rates is necessary to avoid significant osmotic water loss by the cell.

The exact rate of cooling is not easy to determine. A number of publications have addressed the rate of cooling experimentally (Warkentin *et al.*, 2008) as well as theoretically (Kopstad & Elgsaeter, 1982). Experimental measurements are somewhat unreliable when a bulky thermocouple disturbs the cooling. The relevance of the theoretical studies to the process as it occurs in capillary freezing is limited because they either deal with the flat geometry (Jones, 1984), spherical geometry (Van Venrooij *et al.*, 1975) or ignore significant effects

from formation of ice (Shimoni & Müller, 1998). We present calculations based on solution of the heat conduction equation by finite difference methods in cylindrical coordinates that explicitly includes these effects. Our calculations demonstrate that crystallization of the medium boosts the average cooling rate in the center of the tube by up to a factor of 10 due to the higher thermal conductivity of ice. Even more important, the cooling rate as the sample passes through the freezing point may be higher by up to a factor of 300 than in the case where crystallization does not occur. We also confirm that the cylindrical geometry has the additional advantage of faster cooling due to collapse of the cooling front in the center of the sample. The results presented in this work are purely theoretical but they are very promising, and we hope that experimental confirmations of the suggested improvement in specimen preservation will follow.

Cooling rate calculations

Table 1 lists some of the parameters we use for the calculations of cooling when a capillary is plunged into liquid ethane at -170 °C. The values of water thermal diffusivity were previously reported in the range -40 °C to 60 °C (James, 1968). For ice below -40 °C we simply extrapolate the reported data. We assume a standard copper capillary with 300 micron inner diameter. Because at 0°C the thermal conductivity of copper is at least 200 times higher than that of water or ice, we simplify the problem by assuming that the copper tube cools instantaneously while the heat transfer through water limits the cooling of the sample. Further we assume that liquid ethane extracts the heat very efficiently and the temperature of the copper tube immediately drops to -170 °C upon plunging. These simplifications do not introduce significant error relative to the high uncertainty in thermal properties of rapidly and possibly partially freezing ice.

Transfer of heat in our system may be described by the heat conduction equation:

$$\frac{\partial T}{\partial t} = \alpha \frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial T}{\partial r} \right) + f \quad f = \frac{L}{C} \frac{\partial \theta}{\partial t} \quad (1)$$

where T is temperature, t = time, r = distance to center of tube, α = thermal diffusivity defined as $\alpha = k/C\rho$, C = heat capacity, ρ = density, k = thermal conductivity. The function f reflects the heat released due to crystallization of water, L = fusion heat (334 J/g) and θ is the fraction of the ice at point r at time t . For simplicity we assume that water transforms to crystalline ice exactly at zero degrees Celsius. This equation is subject to the initial condition $T_0 = 20$ °C (room temperature) everywhere in the system and the boundary condition $T = -170$ °C at the contact of copper tube and sample. This is an example of the well known Stefan problem with moving boundary conditions (Hu & Argyropoulos, 1996).

We find a solution numerically by explicit finite difference methods using two independent procedures: first, by direct modeling of freezing, and second by the enthalpy approach (Caldwell & Kwan, 2004). The essence of finite difference methods is in dividing a continuous interval into discrete intervals and finding difference quotients at each discrete interval. In order to find a solution we divide the radius of the sample into 500 intervals. To ensure stability of the algorithm the time was divided into 2,000,000 intervals. We find the

temperature distribution at each moment of time using the temperature distribution at the previous time interval and the heat flow equation, starting from the initial temperature distribution. We assume that crystallization happens at some position y between nodes m and $m+1$ (initially near the tube wall but then moving towards the center). In each node we find the heat flow as:

$$flux(j) = \alpha(j) \cdot (T(j) - T(j-1)) / dr \quad (2)$$

where dr is the distance between nodes $j-1$ and j and $\alpha(j)$ depends on temperature in the previous time point $T(j)$. The temperature is found as

$$T'(j) = T(j) + [flux(j+1) \cdot (j - 0.5) - flux(j) \cdot (j - 1.5)] \cdot dt / (j \cdot dr) \quad (3)$$

where T' is the temperature in the current moment of time and dt is the time interval. However we assume the temperature near the crystallization point y in nodes m and $m+1$ is equal to the freezing temperature. Computing all T' gives us the complete temperature distribution for the current moment of time. The position of the crystallization front has changed compared to the previous moment. To continue the iteration we find the new position of the crystallization front using the balance of heat flow:

$$y = y + [flux(m+2) \cdot \rho_i \cdot c_i - flux(m) \cdot \rho_w \cdot c_w] \cdot dt / (L \cdot \rho_w \cdot dr) \quad (4)$$

where c_i = heat capacity of ice, c_w = heat capacity of water, L = fusion heat, ρ_i = density of ice, and ρ_w = density of water.

To satisfy the boundary conditions, the temperature on the surface $j=n$ was set to -170°C . The temperature in the center $j=1$ is found as

$$T'(1) = T(1) + flux(2) \cdot 0.5 \cdot dt / dr. \quad (5)$$

At this point we can move to the next iteration and find the temperature distribution at the next moment of time. Completion of iterations for all times gives the full temperature distribution as a function of time and position.

To double check the solution obtained by heat flow modeling, we independently found a solution using a different approach called the enthalpy method. In this method we look for the solution of the equation

$$\frac{\partial H}{\partial t} = \alpha \frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial T}{\partial r} \right) \quad (6)$$

where the modified enthalpy H is a function of temperature that jumps by L/C at the fusion temperature. This method is less convenient for computation but more physically intuitive.

More details about using of the enthalpy method may be found in (Jones *et al.*, 2006). The two methods give exactly the same solutions.

Calculation of the cooling rate for completely vitrified samples was performed using the direct algorithm but setting the fusion heat to zero, replacing ice parameters such as thermal conductivity and density with the corresponding parameters for water and replacing the ice thermal diffusivity with the thermal diffusivity of water extrapolated beyond zero degrees. While these approximated parameters may slightly differ from the real thermodynamic values of amorphous ice, the difference is significantly less than the differences between the parameters of crystalline and amorphous ice. The real thermal diffusivity of vitreous ice should be close to the thermal diffusivity of water that increases very slowly as the temperature decreases. This change is smallest at high temperature and slightly higher below 100 K (Yu & Leitner, 2005). Over the full range the change is less than 20 percent, which is small compared to the four-fold jump following crystallization.

Results and discussions

Three dimensional plots of the solution for both crystallization and vitrification freezing are shown in Figure 1. For these plots the function was resampled by averaging 25 space points and 20000 time points. One can see that crystallization of the ice throughout the tube, manifested by the temperature passing zero degrees, takes less than 6 milliseconds, and the temperature drops below -160°C in less than 10 milliseconds. When crystallization is completely avoided, however, passing zero degrees takes about 15 milliseconds and complete cooling of the sample takes about 100 milliseconds.

The most important parameter to quantify the quality of the freezing is not the complete cooling time but rather the rate of temperature drop when it passes zero degrees. In the case of crystallization cooling, the rate at the beginning increases slowly and we observe a plateau followed by rapid temperature drop. For a vitreous sample the plateau is somewhat longer, and the temperature drop following is not nearly as steep. The rate of temperature drop in the center of the tube just before crystallization is $30,000^{\circ}\text{C/s}$, and $1,000,000^{\circ}\text{C/s}$ after crystallization, while the vitreous sample passes zero degrees at a constant rate of $3,000^{\circ}\text{C/s}$. In both cases, as may be expected, the rate of temperature decrease passing through zero degrees is highest at the periphery of the sample near the copper tube. Interestingly, starting from approximately half of the sample radius to the center the cooling rate as the temperature approaches zero is almost constant. At the time that the temperature drops below zero, cooling rates vary with the radius from $70,000^{\circ}\text{C/s}$ to $1,000,000^{\circ}\text{C/s}$ reaching the highest value in the center of the tube (see Fig. 1). This behavior is attributed to the cylindrical geometry where the cooling front collapses toward the center of the tube. This finding demonstrates an advantage compared to the linear geometry, allowing thicker samples to be prepared.

The freezing time we found for vitreous samples is consistent with previous calculations (Shimoni & Müller, 1998). On the other hand, freezing of the sample with ice crystallization is significantly faster, due to the high thermal conductivity of the ice. It appears that the contribution from the heat of fusion that slows down the cooling process is much less

important than the contribution from higher thermal conductivity of ice. This result leads us to the main conclusion of this work: freezing of the medium into crystalline ice may improve preservation of internal cell structure of cells and other material suspended in the medium.

Our calculations assume that the freezing sample is kept at atmospheric pressure. Increasing the pressure will certainly change the thermodynamic properties of the material and affect cooling rates. Exact calculation of this effect is difficult to perform because of uncertainty in the parameters. While the thermodynamic properties of hexagonal ice are known (Feistel & Wagner, 2006), for amorphous water between 150-273K they are difficult to determine because of instability in this range. It is known that low-density amorphous, hexagonal and cubic ices show anomalous reduction in thermal conductivity with increasing pressure. In contrast, conductivity of high density amorphous (HDA) ice increases with increasing pressure. However even at 200 MPa conductivity of HDA ice is lower than those of hexagonal and cubic ices by factors of approximately 7 and 5 respectively (Andersson & Suga, 2002). At the same time differences in density (Sotani *et al.*, 2000) and specific heat capacity (Handa & Klug, 1988, Yurtseven & Kurt, 2010) between crystalline (hexagonal or cubic) ice and HDA are much smaller. Thus the thermal diffusivity of crystalline ice is always higher than of HDA, so crystallization will always cause some increase in cooling rate. Considering other polymorphs of high pressure crystalline ice is not necessary because they form at pressures over 200 MPa (Zheligovskaya & Malenkov, 2006). While ice III has been reported to form in HPF at temperatures below -150 °C, typically it is only a minor component (Lepault *et al.*, 1997). Quantitative determination of the influence of high pressure on the cooling rate is beyond the scope of this paper.

Freezing of a real system may be slightly different from this model since several factors may affect the cooling. Crystallization at lower temperature is one of the most important factors. It has been shown (Guignon *et al.*, 2009) that both high pressure and cryoprotectant depress the crystallization temperature. We have modeled freezing behavior for freezing at -22 °C (which corresponds to 210 MPa) and found that, while it affects the total cooling time, the rate of temperature change passing -22 °C is only slightly lower. Nonuniform distribution of biological material may cause ice formation over a range of several degrees instead of at the nominal crystallization point. In the medium this effect may be expected to be low because of the low concentration of biological material. Another aspect of nonuniform supercooling may more dramatically affect sample quality. Cells consist of about 20 percent protein which may act as a cryoprotectant and depress the crystallization temperature inside the cell further than in the medium (Guignon *et al.*, 2009). Thus the cell will start to freeze after the medium has passed the crystallization point and will be cooled at a higher rate (70,000 - 1,000,000 °C/s) that will further inhibit possible diffusion and crystallization inside the cell. In order to take advantage of this fortunate circumstance one needs to make sure that the concentration of nonaqueous material, which acts as a cryoprotectant inside the cell, remains higher than in the medium even after adding cryoprotectant.

While this work focuses on theoretical analysis we want to mention results recently obtained by isochoric subcooling. The method was theoretically developed by (Szobota & Rubinsky, 2006). Later it was implemented for capillary freezing followed by freeze substitution

(Leunissen & Yi, 2009) and given the name “self-pressurized rapid freezing”. Following Leunissen & Yi we have characterized samples plunge frozen in copper capillary tubes sealed at both ends. Pressure rises in the capillary due to the formation of low density crystalline ice in the closed volume. We have demonstrated that this approach allows amorphization of 15 percent dextran solution whereas HPF requires 20 percent dextran (Yakovlev, 2010). Comparison of the pressure buildup due to ice crystallization and the strength of the tube shows that pressure in a sealed capillary is limited by the tensile strength of copper, about 200 MPa. Most HPF equipment produces similar pressures. Considering the calculations presented in this work we conclude that formation of a distinct ring of crystalline ice in the outer part of the sample during self pressure freezing allows faster cooling the inner part of the sample and causes amorphization in the center of the tube with low cryoprotectant content.

While HPF on commercial equipment uses standard capillary tubes it is clear that using tubes with smaller inner diameter would provide better freezing conditions. In the end of our work we calculated the cooling rate as a function of tube diameter. Figure 2 shows cooling rates in different size capillaries for vitreous samples and for crystallizing samples just before passing 0 °C. For crystallizing samples the cooling rate after passing zero is also shown for two locations: in the center and at half of the capillary radius. One can see that all rates increase rapidly with decreasing capillary diameter. While sectioning of smaller samples is challenging it may be implemented in the future.

Conclusions

Vitrification of the medium is often considered an essential criterion for adequate sample preservation. However, good ultrastructure preservation, as judged in freeze-substitution, is often observed under conditions where the medium crystallizes. A more useful criterion to predict preservation quality would be the rate of freezing, with higher rates limiting diffusion and other movement of cellular components. Our calculations show that the freezing rate is significantly higher when the medium does crystallize than when it vitrifies due to added cryoprotectant. One can generally assume that the cell interior is sufficiently cryoprotected to avoid crystallization, and with sufficiently rapid freezing ice crystals in the medium cannot grow large enough to cause significant distortions. Thus minimizing the amount of cryoprotectant has the potential to improve preservation of cell ultrastructure.

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References

- Al-Amoudi A, Dubochet J, Studer D. Amorphous solid water produced by cryosectioning of crystalline ice at 113 K. *Journal of Microscopy*. 2002; 207:146–153. [PubMed: 12180960]
- Andersson O, Suga H. Thermal conductivity of amorphous ices. *Physical Review B*. 2002; 65:140201.
- Bald WB. The relative merits of various cooling methods. *Journal of Microscopy*. 1985; 140:17–40.

- Caldwell J, Kwan YY. Numerical methods for one-dimensional Stefan problems. *Communications in Numerical Methods in Engineering*. 2004; 20:535–545.
- Echlin P, Le Skaer BH, Gardiner BOC. Polymeric cryoprotectants in the preservation of biological ultrastructure. II. Physiological effects. *Journal of Microscopy*. 1977; 110:239–255. [PubMed: 73592]
- Elnitsky MA, Hayward SAL, Rinehart JP, Denlinger DL, Lee RE Jr. Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica*. *Journal of Experimental Biology*. 2008; 211:524–530. [PubMed: 18245628]
- Erk I, Nicolas G, Caroff A, Lepault J. Electron microscopy of frozen biological objects: A study using cryosectioning and cryosubstitution. *Journal of Microscopy*. 1998; 189:236–248. [PubMed: 9588022]
- Escaig J. New instruments which facilitate rapid freezing at 83K and 6K. *Journal of Microscopy*. 1982; 126:221–229.
- Feistel R, Wagner W. A new equation of state for H₂O Ice Ih. *Journal of Physical and Chemical Reference Data*. 2006; 35:1021–1047.
- Franks F, Asquith MH, Hammond CC. Polymeric cryoprotectants in the preservation of biological ultrastructure. I. Low temperature states of aqueous solutions of hydrophilic polymers. *Journal of Microscopy*. 1977; 110:223–238. [PubMed: 73591]
- Gilkey JC, Staehelin LA. Advances in ultrarapid freezing for the preservation of cellular ultrastructure. *Journal of Electron Microscopy Technique*. 1986; 3:177–210.
- Guignon B, Aparicio C, Otero L, Sanz PD. Prediction of ice content in biological model solutions when frozen under high pressure. *Biotechnology Progress*. 2009; 25:454–460. [PubMed: 19294740]
- Handa YP, Klug DD. Heat capacity and glass transition behavior of amorphous ice. *The Journal of Physical Chemistry*. 1988; 92:3323–3325.
- Hochi S, Semple E, Leibo SP. Effect of cooling and warming rates during cryopreservation on survival of in vitro-produced bovine embryos. *Theriogenology*. 1996; 46:837–847. [PubMed: 16727947]
- Hu H, Argyropoulos SA. Mathematical modelling of solidification and melting: A review. *Modelling and Simulation in Materials Science and Engineering*. 1996; 4:371–396.
- James DW. The thermal diffusivity of ice and water between -40 and + 60° C. *Journal of Materials Science*. 1968; 3:540–543.
- Jones BJ, Sun D, Krishnan S, Garimella SV. Experimental and numerical study of melting in a cylinder. *International Journal of Heat and Mass Transfer*. 2006; 49:2724–2738.
- Jones GJ. On estimating freezing times during tissue rapid freezing. *Journal of Microscopy*. 1984; 136:349–360. [PubMed: 6520865]
- Kopstad G, Elgsaeter A. Theoretical analysis of specimen cooling rate during impact freezing and liquid-jet freezing of freeze-etch specimens. *Biophysical Journal*. 1982; 40:163–170. [PubMed: 7171712]
- Koshimoto C, Mazur P. Effects of cooling and warming rate to and from -70°C, and effect of further cooling from -70 to -196°C on the motility of mouse spermatozoa. *Biology of Reproduction*. 2002; 66:1477–1484. [PubMed: 11967213]
- Le Skaer BH, Franks F, Asquith MH, Echlin P. Polymeric cryoprotectants in the preservation of biological ultrastructure. III. Morphological aspects. *Journal of Microscopy*. 1977; 110:257–270. [PubMed: 73593]
- Lepault J, Bigot D, Studer D, Erk I. Freezing of aqueous specimens: an X-ray diffraction study. *Journal of Microscopy*. 1997; 187:158–166.
- Leunissen JLM, Yi H. Self-pressurized rapid freezing (SPRF): A novel cryofixation method for specimen preparation in electron microscopy. *Journal of Microscopy*. 2009; 235:25–35. [PubMed: 19566624]
- Morris GJ, Goodrich M, Acton E, Fonseca F. The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation. *Cryobiology*. 2006; 52:323–334. [PubMed: 16499898]
- Rowe DS. Colloid Osmotic Pressures of Dextran, Serum and Dextran-Serum Mixtures. *Nature*. 1955; 175:554–555. [PubMed: 14370168]

- Shimoni K, Müller M. On optimizing high-pressure freezing: From heat transfer theory to a new microbiopsy device. *Journal of Microscopy*. 1998; 192:236–247. [PubMed: 9923416]
- Sotani T, Arabas J, Kubota H, Kijima M, Asada S. Volumetric behaviour of water under high pressure at subzero temperature. *High Temperatures - High Pressures*. 2000; 32:433–440.
- Studer D, Michel M, Wohlwend M, Hunziker EB, Buschmann MD. Vitrification of articular cartilage by high-pressure freezing. *Journal of Microscopy*. 1995; 179:321–332. [PubMed: 7473694]
- Szobota SA, Rubinsky B. Analysis of isochoric subcooling. *Cryobiology*. 2006; 53:139–142. [PubMed: 16762336]
- Van Venrooij GEP, Aertsen AMHJ, Hax WMA, Vervegaert PHJT, Verhoeven JJ, Van Der Vorst HA. Freeze-etching: Freezing velocity and crystal size at different locations in samples. *Cryobiology*. 1975; 12:46–61. [PubMed: 1109872]
- Warkentin M, Stanislavskaya V, Hammes K, Thorne RE. Cryocrystallography in capillaries: Critical glycerol concentrations and cooling rates. *Journal of Applied Crystallography*. 2008; 41:791–797. [PubMed: 19529833]
- Wharton DA, Downes MF, Goodall G, Marshall CJ. Freezing and cryoprotective dehydration in an Antarctic nematode (*Panagrolaimus davidi*) visualised using a freeze substitution technique. *Cryobiology*. 2005; 50:21–28. [PubMed: 15710366]
- Yakovlev S, Luef B, Comolli LR, Downing KH. Self Pressure Freezing of *Caulobacter crescentus* for the Preparation of Frozen Hydrated Sections. *Microscopy and Microanalysis*. 2010; 16(Suppl. 2): 860–861.
- Yu X, Leitner DM. Thermal transport coefficients for liquid and glassy water computed from a harmonic aqueous glass. *Journal of Chemical Physics*. 2005; 123:1–10.
- Yurtseven H, Kurt M. Analysis of heat capacity and glass transition in amorphous ice. *Modern Physics Letters B*. 2010; 24:1915–1921.
- Zheligovskaya EA, Malenkov GG. Crystalline water ices. *Russian Chemical Reviews*. 2006; 75:57–76.

Figure 1.

Calculated three dimensional plot of temperature as a function of time and distance from the center of tube. a) calculations incorporating ice crystalization, b) calculation assuming vitrification of the water. Note different scales on the time axis.

Figure 2.

Dependence of the cooling rates on the capillary tube diameter. Black dotted line corresponds to a vitreous sample just above 0 °C; blue solid line - crystallizing sample before 0 °C; violet dot-dashed line - crystallizing sample after passing 0 °C at half radius from center; red dashed line - crystallizing sample after passing 0 °C in the center of the capillary tube.

Table 1

Values of thermal diffusivity, thermal conductivity, specific heat and density used for the calculations.

	Thermal diffusivity $\cdot 10^{-7} \text{ m}^2\text{s}^{-1}$	Thermal conductivity $\text{W}\cdot\text{m}^{-1}\cdot\text{K}^{-1}$	Specific heat $\text{J}\cdot\text{g}^{-1}\cdot\text{K}^{-1}$	Density $\text{Kg}\cdot\text{m}^{-3}$
Water	$1.35 + 0.002\cdot T$	0.6	4.2	1
Ice	$8.43 - 0.101\cdot T$	2	2.0	0.92
Copper		401		