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Measurement of the size of intracellular ice crystals in mouse oocytes using a melting point depression method and the influence of intracellular solute concentrations

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Abstract

Characterization of intracellular ice formed during the cooling procedures of cells significantly benefits the development and optimization design of cryopreservation or cryosurgery techniques. In this study, we investigated the influence of the concentration of extracellular non-permeable and permeable solutes on the melting points of the intracellular ice in mouse oocytes using cryomicroscopy. The results showed that the melting points of the intracellular ice are always lower than the extracellular ice. Based on this observation and the Gibbs-Thomson relation, we established a physical model to calculate the size of intracellular ice crystals and described its relationship with the concentrations of intracellular permeating solutes and macromolecules. This model predicts that the increased concentration of macromolecules in cells, by increasing the extracellular non-permeating solute concentration, can significantly lower the required concentration of permeable solutes for intracellular vitrification. The prediction was tested through the cryomicroscopic observation of the co-existence of intracellular vitrification and extracellular crystallization during cooling at 100°C/min when the extracellular solutions contain 5 molal (*m*) ethylene glycol and 0.3 to 0.6 *m* NaCl.

Keywords

cryomicroscopy; Gibbs-Thomson relation; intracellular ice crystal size; melting point depression

Introduction

Investigating the factors influencing the characteristics of intracellular ice formation (IIF) is of critical importance for cryopreservation and cryosurgery techniques. For example, during an equilibrium freezing procedure for cell cryopreservation, to prevent cell damage caused by IIF or exposure to solutions with high solute concentrations (so-called “solution effects” injury) [10,17,18,28], an optimal cooling rate is required for each cell type to maintain appropriate intracellular water content during cooling [17,18]. For the vitrification cryopreservation procedures, the designed cooling rates should exceed a specific cooling rate threshold called “the critical cooling rate” to minimize the ice volume fraction to the order of 10^{-6} during cooling [3,20,21]. Cryosurgery techniques, on the contrary, are developed to form sufficient IIF to kill unhealthy or harmful cells primarily through the resulting mechanical damage [8,

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9]. Therefore, to design and optimize these procedures, it is indispensable to acquire an accurate knowledge of the size, morphology and amount of the intracellular ice formed with different protocols.

Recently, novel cryomicroscopic approaches have been developed to investigate the mechanisms of IIF during cooling [25] and intracellular recrystallization during warming [23]. However, for the detection of the size of intracellular ice crystals, there exist serious technical and theoretical difficulties. For example, electron microscopy has been used to visualize the ice crystals in frozen cells in a few studies [24]. The complicated sample preparation processes for electron microscopy, *esp.* the freeze-substitution process, as well as the temperature change involved, may change the size of intracellular ice crystals formed during cooling, resulting in inaccuracies of the measurement. Electron microscopy is also laborious and costly. Therefore, development of a facile method to estimate the size of intracellular ice crystals is of both theoretical and practical importance. The Gibbs-Thomson relation has been widely used to calculate or estimate the size of micro-particles by measuring the depression of their melting points [4,15,27]. Since the size of intracellular ice crystals generally ranges from several nanometers to less than 0.1 μm [12,13,24,32], the melting point depression of intracellular ice would be predicted to be approximately 1 to 10°C. Although these facts indicate that the melting depression method may be a promising approach, the current thermal analytical instruments cannot be used for such measurement due to the small volume of intracellular solutions. For example, using the traditional differential scanning calorimeter (DSC) or differential thermal analyses (DTA), the sample volume required is approximately several microliters, which requires a use of several thousand mouse oocytes or cells of other types with similar sizes. It is also physically difficult to separate cells from their extracellular solutions, whose presence will affect the accuracy using those thermal methods. The newly developed nanocalorimeters [31], however, cannot achieve the temperatures low enough to be in the range of cryopreservation or cryosurgery procedures. To overcome these difficulties, in this study, we established an optical method using cryomicroscopy to calculate the size of intracellular ice crystals by measuring the melting point depression of intracellular solution of relatively large cells, *e.g.* mouse oocytes.

In current models used to predict the probability, size, and amount of IIF, intracellular solutions are generally treated as dilute or “ideal” solutions. Mouse oocytes contain approximately 25ng proteins per cell [30], as well as an unknown amount of other types of macromolecules, *e.g.* RNAs and saccharides. Aqueous solutions of proteins and saccharides have higher vitrification temperatures and viscosities than typical permeating solute solutions [3,7,20]. It has also been demonstrated that the ice growth in aqueous solutions containing macromolecules is considerably slowed [1] and ice morphology is changed [19]. Consequently, the vitrification tendency of intracellular solutions, when the concentration of the macromolecules is high enough, is likely different from the extracellular solutions. Therefore, it is important to investigate the effects of the concentration of macromolecules in the intracellular solutions on the characteristics of intracellular ice. In this study, we established a simple model to quantitatively analyze the influence of these macromolecules on the size of intracellular ice crystals.

Methods and Materials

All chemicals were obtained from Sigma Aldrich chemical company (St Louis, MO, USA) unless otherwise stated.

Source of oocytes

The collection of mouse oocytes was performed as previously described [21]. Superovulation of CD-1 female mice was performed by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG; Calbiochem, La Jolla, CA), and was followed 48 hrs later by injection of human chorionic gonadotropin (hCG, Calbiochem, La Jolla, CA). Cumulus masses were collected from oviducts 13–14 hr after hCG injection and treated with ~100 IU/ml hyaluronidase dissolved in the Flushing and Holding Medium (FHM; [16]) for 3–5 min to remove cumulus cells. Oocytes were then washed in FHM and incubated in 5% CO₂ at 37°C. All FHM and phosphate-buffered saline (PBS) solutions used in this study contained 4 mg/ml bovine serum albumin (BSA, Fraction V) and were maintained at 35–37°C during the oocyte isolation and handling procedures. All procedures using animals were approved by our institutional animal care committee and conducted in accordance with standards as described in the Guide for the care and use of laboratory animals (National Research Council, Washington DC).

Cryomicroscopy

A standard cryomicroscope (Linkam, UK) was used for the measurements. To minimize the influence of the extracellular ice formation on the observation of intracellular ice, two pieces of commercial tape, with approximately 100µm in thickness, were sandwiched between two quartz chips (Linkam, UK) as shown in Fig. 1. a. In this way, when mouse oocytes were loaded between the chips, the distance between their plasma membranes and the chips was minimized to several micrometers and the zona pellucidae were slightly and visibly transformed but the shape of the oocytes themselves was not altered.

In the extracellular solute, ethylene glycol (EG) was chosen as the permeating solute (concentrations: 0, 1, 2, 3, 4 *m*) and NaCl as the non-permeating solute (concentrations: 0.15, 0.30, 0.45, 0.6 *m*). The combination of these EG and NaCl concentrations yields totally 5×4=20 solutions. To minimize osmotic damage during addition of EG, when the concentration of EG was higher than 2 *m*, the cells were first equilibrated with lower EG concentration solutions with the same NaCl concentrations. For example, for the 4 *m* EG and 0.3 *m* NaCl solutions, the cells in FHM medium were drawn into a fine pulled glass capillary, expelled into a ~1ml solution containing 1 *m* EG, 0.3*m* NaCl and 4 mg/ml BSA in a petri dish, and equilibrated for approximately 10 mins. With the similar procedure, the EG concentration was gradually increased to 2, 3 and then 4 *m*. We used an optical microscope (Optiphot CE, Nikon, Japan) to monitor the procedure and the completion of equilibrium process was considered to be achieved when all of cells fully swelled. At the end, the cells were drawn into a new capillary, expelled into a drop (~10µl) of solution containing 4 *m* EG and 0.3 *m* NaCl (no BSA) in the center of a quartz chip, and sandwiched by another chip as shown in Fig. 1. a. Similar procedures were performed for other solutions containing higher than 2 *m* EG.

The sandwiched samples were mounted on the platform of the cryomicroscope with their edge covering the “cold point” beside the platform. To prevent the loss of intracellular water during freezing, the cooling rate was chosen as the maximum value available for this type of cryomicroscope, 100°C/min, and the samples were cooled directly from the room temperature to –80°C (the “cold point” provided the seeding points). Due to the low water permeability of the mouse oocyte membrane at subzero temperatures, the loss of intracellular water during an equilibrium freezing process with 100 °C/min as the cooling rate is approximately 1% [29]. Therefore, the loss of intracellular water during the cooling process was considered to be negligible. Time-lapse photographs of the cells were captured using a digital video system (SPOT RT camera and software, Diagnostic Instruments, Inc. Sterling Heights, MI, USA). The warming rate was chosen as 0.1°C/s (6°C/min). The values of temperatures were directly read

from the temperature control panel of the cryomicroscope. The melting point of intracellular solutions was calculated as the temperature at which all the intracellular ice crystals melt and the cytoplasm became transparent. Similarly, the melting points of extracellular solutions were also measured by observing the extracellular ice melting. To prevent the influence of recrystallization during warming on the size of intracellular ice crystals [23], the results from the cells partially freeze during cooling are not included. Figure 1. c shows an illustration of the cryomicroscopic observation of the melting processes as stated above. The experiments were repeated for 5–6 times for each solution, with approximately 4–6 oocytes each time.

Physical Models

The size-dependent melting point depression of an ice crystal in solutions is governed by the Gibbs-Thomson relation (please see Table 1 for symbol definitions):

$$\frac{\Delta T}{T_m^\infty} = \frac{T_m^\infty - T_m}{T_m^\infty} = \frac{2\sigma}{H_f \rho_s r}, \quad (1)$$

where ΔT is the depression of the melting point of the ice crystal (T_m) from the melting point of plane ice in the solutions (T_m^∞), H_f is the fusion heat of ice, ρ_s is the density of ice, r is the curvature of the ice surface and σ is the ice-solution interface energy or surface tension. For intracellular solutions, there exists no plane or “bulk” ice in them but ice crystals at the level of several nm, and it is physically impossible to use cryomicroscopy to measure the values of $T_{m_i}^\infty$ directly. According to our experimental design, the intra- and extracellular solutions are in an osmotic equilibrium before the cooling procedures for cryomicroscopy are implemented, so they have the same values of T_m^∞ , i.e. $T_{m_i}^\infty = T_{m_e}^\infty$; during the cooling procedures, the relatively high cooling rate prevents the water permeation from mouse oocytes to extracellular solutions, and as a result, the values of $T_{m_i}^\infty$ will not change. Therefore, the values of $T_{m_i}^\infty$ can be substituted by those of $T_{m_e}^\infty$. Furthermore, the size of ice crystals in the extracellular solutions is at the level of 10^{-4} to 10^{-3} m, and the difference between T_{m_e} and $T_{m_e}^\infty$ is also negligible. Based on the analyses above, Eq.1 can be modified for intracellular solutions as:

$$\frac{\Delta T_i}{T_{m_i}^\infty} = \frac{T_{m_i}^\infty - T_{m_i}}{T_{m_i}^\infty} = \frac{T_{m_e}^\infty - T_{m_i}}{T_{m_e}^\infty} = \frac{T_{m_e} - T_{m_i}}{T_{m_e}} = \frac{\Delta T^*}{T_{m_e}} = \frac{2\sigma}{H_f \rho_s r},$$

where ΔT^* is the difference between the measured melting points of extra- and intracellular ice crystals. The value of σ is theoretically determined by

$$\sigma = \frac{\sigma_\infty}{1 + \frac{2\delta}{r}}, \quad (2)$$

where σ_∞ is the value of σ on a plane ice and δ is the Tolman's length in the solutions [2]. To simplify the analytic procedure, intracellular ice crystals are assumed to be spherical and r is their average radius. Although the values of σ_∞ , H_f and ρ_s change slightly when the temperature changes [2], the melting points of the extracellular solutions used in this study ranged only from approximately -0.5°C to -10°C [14], so the temperature dependence of σ_∞ , H_f and ρ_s was considered to be negligible and their values are calculated as those at 0°C [7]. According to previous publications, the values of δ are on the order of magnitude of 10^{-10} m and r ranges from several nm to less than $0.1\mu\text{m}$, which is a difference of at least one order of magnitude,

so the influence of the intracellular ice crystal size on the surface tension was ignored. With these simplifications, the value of r can be calculated as:

$$r = K \cdot \frac{T_{m_e}}{\Delta T^*}; \quad (3)$$

where $K = \frac{2\sigma_\infty}{H_f \rho_s} \equiv 0.2 \text{ nm}$. Using Eq.3, the size of intracellular ice crystals can be calculated from the measured melting points of intra- and extracellular crystals.

Since the same cooling rate (100°C/min) was used in all of our experiments, the major factor influencing the size of intracellular ice crystals is the intracellular solute concentration in cells. Although complicated physical models have been established to correlate the size of intracellular ice crystals with freezable water contents and solution viscosities in cells [12,13, 32], it is technically difficult to determine these values due to the non-ideality of intracellular solutions generated by the existence of macromolecules. To simplify the analytic procedure and reveal the influence of these macromolecules, we created a simple empirical equation based on our experimental results: the size of intracellular ice crystals decreases exponentially as the molalities of intracellular macromolecules and permeating solutes increase, *i.e.*,

$$r = C \exp(-A \cdot m_i - B \cdot n_i), \quad (4)$$

where m_i is the molality of intracellular permeating solutes, n_i is the molality of intracellular macromolecules, and A , B and C are positive constants to be determined. Because the cooling rate is high enough to prevent intracellular water from permeating out of the cells, the values of m_i and n_i before IIF starts are the same as their initial values. Therefore, these initial concentrations determine the situation for the primary crystal formation (please see detailed explanations in the Discussion section). In this case, EG is the only permeating solute, so $m_i = m_e$, where m_e is the initial extracellular EG molality. It is technically difficult to determine n_i , but its value is related to the initial extracellular NaCl molality, n_s , by:

$$n_i = \frac{M_i}{w_i} = \frac{M_i}{N_i} \cdot 2n_s, \quad (5)$$

where M_i is the total number of moles of intracellular macromolecules, w_i is the mass of intracellular water, and N_i is the total number of moles of osmotically active non-permeating solutes and ions in one cell. Because both N_i and M_i are constants, n_i is proportional to n_s with

a constant coefficient, $\frac{2M_i}{N_i}$. Based on these analyses and combining the constants, Eq.4 can be rearranged as:

$$\begin{aligned} r &= \exp(a \cdot m_e - b \cdot n_s - c) \text{ or} \\ \log(r) &= a \cdot m_e - b \cdot n_s - c. \end{aligned} \quad (6)$$

This analysis predicts the logarithm of r is a bi-linear function of initial extracellular EG and NaCl molalities. With the calculated values of r from the experimental results using Eq.3, the constants a , b and c can be calculated through simple curve fitting.

Results

For all the oocytes, the values of T_{mi} were always lower than those of T_{me} . For example, Fig. 2 illustrates a significant difference between the melting temperatures when the initial extracellular solution contains 0.15 *m* NaCl and 3 *m* EG. The difference between the values of T_{mi} of different cells with the same EG and NaCl concentrations in their extracellular solutions was less than 1°C, and the average values of T_{mi} were calculated for all the cells across all the experiments for a particular combination of the EG and NaCl concentrations. The difference between the values of T_{me} for the same solutions was less than 0.5°C. Figure 3 shows the comparison between the average values of the T_{mi} and T_{me} in the whole initial NaCl and EG concentration range, and the values of ΔT^* are listed in Table 2.

Substituting the values of ΔT^* into Eq.3, the values of r are calculated and the results are listed in Table 3. Figure 4 demonstrates the bi-linear function predicted by Eq.6. The fitting parameters are, $a = -0.22$, $b = -2.3$ and $c = 3.68$. The R^2 value is 0.99.

Discussion

To test the accuracy of using cryomicroscopy to measure the solution melting points, the measured values of extracellular melting points were compared to the values of previous studies [7,14]. For the solutions containing no EG, the measured melting points of the NaCl solutions are only 0.1–0.2°C different from the published NaCl/water phase diagram [7]. For the solutions containing 1 and 2 *m* EG, the difference between measured melting points and synthetic ternary phase diagram of EG/NaCl/Water [14] is less than 0.5°C, and when the EG concentration increases to 3 and 4*m*, that difference is less than 1.5 and 2°C, respectively.

As shown in Table 3, the values of r range from ~ 4 to 28 nm with different initial solute concentrations, and these are on the same order of magnitude as the results from the previous theoretical investigations or electron microscopic observations [12,24,32]. During our experiments, it was observed that there exists a probability that some cells partially freeze and recrystallize during warming. However, the probability is low, *i.e.*, less than 10% for the cells without EG and 2% for those with EG, and the results from these cells were not considered to prevent the influence of the crystal size change due to recrystallization.

There exists a distribution of the crystal size within a single cell [12,32]. When IIF starts, the crystals firstly formed are those growing in the intracellular solution with initial solute concentrations and not interacting with each other. As illustrated in Fig. 5. A, although there exists a diffusion layer with a solute concentration gradient on the crystal surface, due to the miniature diffusivity at subzero temperatures, this layer won't affect the concentration of the intracellular solution. Therefore, the size of these relatively large crystals can be directly determined by the Gibbs-Thomson relation using the values of T_{mi} and ΔT^* , and they are the so-called "primary crystals". As more and more primary crystals form, secondary or higher levels of crystallization will develop in their interstices, as schematically depicted in Fig. 5. B. The size of these secondary or higher level crystals is controlled by the both the space of the interstices and the diffusion layers of the primary crystals, and they are statistically smaller and have lower melting points than primary crystals. It is technically difficult to determine their size through cryomicroscopy due to the visual influence of primary crystals. Because the size of the primary crystals is the major factor determining the ice volume fraction and the degree of mechanical damage generated by IIF, we used the phenomenological parameter, r , as an average radius of the primary crystals, and focused on the investigation of its variation with different solute concentrations. To investigate the distribution of intracellular crystal size, it would be required for the devices such as the nanocalorimeters to be modified for the performance at subzero temperatures and used to obtain the endothermic graph of a single cell

during warming. The crystal size distribution curve can then be achieved with a similar approach applied in the determination of the pore size of capillaries by combining the Gibbs-Thomson relation and differential analyses on the endothermic curves [6,22].

Based on the calculation from Eq.6, the value of b is almost 10 times as large as a , so it is much more efficient to decrease intracellular crystal size by increasing the values of n_s than m_e . According to Eq.5, increased values of n_s proportionally increase n_i , which will significantly change the situation for intracellular ice formation. For example, when n_s is 0.15 m , approximately 1% (w/w) proteins are in the cytoplasm of mouse ovaries, and when n_s is increased to 0.3 m , the protein ratio will increase to approximately 2%. It has been demonstrated that 2% protein in water can significantly slow the ice growth and increase the solution viscosity [1,19]. There also exists an unknown amount of other macromolecules, so the effects should be even more significant. Meanwhile, the macromolecules may also decrease the amount of freezable water in cells [11,17]. All of these are also factors influencing the vitrification tendency, *i.e.* the ice volume ratio at a given cooling rate, of intracellular solutions. Therefore, it is possible to lower the required concentration of permeating solutes for the vitrification cryopreservation protocols by increasing the intracellular macromolecule concentrations.

To test this prediction, we performed the same cooling procedures on mouse oocytes pre-equilibrated with 5 m EG and 0.15 to 0.6 m NaCl solutions. When the NaCl concentration is higher than 0.3 m , the intracellular solutions can be vitrified during cooling, while the extracellular solutions crystallized. Figure 6 demonstrates the situations where intracellular vitrification is achieved in the presence of extracellular crystallization. These intracellularly vitrified cells also devitrified (became dark) during warming. These observations may confirm the rationality of the previous designs of the fast cooling protocols with relatively low permeating solute concentration and high non-permeating solute concentration [5,16,26]: the intracellular macromolecules might improve the vitrification tendency and the cells achieved intracellular vitrification in contrast with the extracellular crystallization. The established empirical equation can also be used as a rough estimation of the required extracellular non-permeating solutes for this type of design. For example, when the EG concentration is 5 m and the NaCl concentration is 0.45 m , according to Eq.6, the influence of this NaCl concentration

on the values of r is identical to $0.45 \times \frac{b}{a} \approx 4.5$ m EG, so the identical intracellular EG concentration is approximately $4.5 + 5 = 9.5$ m , which is sufficient for the achievement of vitrification during cooling at 100°C/min [3,20].

Conclusion

In this study, cryomicroscopy is used to investigate the melting point depression of intracellular solutions of mouse oocytes. Based on Gibbs-Thomson relation, the intracellular crystal size was calculated, and correlated to the solute concentrations by an empirical equation. The results validate the cell vitrification approach by increasing the extracellular non-permeating solute concentration.

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References

1. Blond G. Velocity of linear crystallization of ice in macromolecular systems. *Cryobiology* 1988;25:61–66. [PubMed: 3349812]
2. Bogdan A. Thermodynamics of the curvature effect on ice surface tension and nucleation theory. *J Chem Phys* 1997;106:1921–1929.

3. Boutron P. Comparison with the theory of the kinetics and extent of ice crystallization and of the glass-forming tendency in aqueous cryoprotective solutions. *Cryobiology* 1986;23:88–102. [PubMed: 3956232]
4. Buffat P, Borel JP. Size effect on the melting temperature of gold particles. *Phys Rev A* 1976;13:2287–2298.
5. Cho HJ, et al. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Hum Reprod* 2002;17:2419–2422. [PubMed: 12202434]
6. Cohen-Addad JP, et al. Acrylonitrile–sodium methallylsulfonate copolymer. DSC approach to membrane porosity of foam and hollow fibers. *Biomaterials* 2003;24:173–179. [PubMed: 12417191]
7. David, RL. *CRC Handbook of Chemistry and Physics*. Vol. 88. CRC Press; 2007.
8. Gage AA, Baust J. Mechanisms of tissue injury in cryosurgery. *Cryobiology* 1998;37:171–186. [PubMed: 9787063]
9. Gage AA, Baust JG. Cryosurgery—a review of recent advances and current issues. *CryoLetters* 2002;23:69–78. [PubMed: 12050774]
10. Gao DY, Critser JK. Mechanisms of cryoinjury in living cells. *ILAR J* 2000;41:187–196. [PubMed: 11123179]
11. Gao, DY.; Critser, JK. *Encyclopedia of biomaterials and biomedical engineering*. Marcel Dekker. Inc; New York: 2003. Cryopreservation of living cells.
12. Karlsson JOM, et al. Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl-sulfoxide. *Biophys J* 1993;65:2524–2536. [PubMed: 8312489]
13. Karlsson JOM, Toner M, Cravalho EG. A model of diffusion-limited ice grow inside biological cells during freezing. *J Appl Phys* 1994;75:4442–4455.
14. Kleinhans FW, Mazur P. Comparison of actual vs. synthesized ternary phase diagrams for solutes of cryobiological interest. *Cryobiology* 2007;54:212–222. [PubMed: 17350609]
15. Lai SL, et al. Size-Dependent Melting Properties of Small Tin Particles: Nanocalorimetric Measurements. *Phys Rev Lett* 1996;77:99–102. [PubMed: 10061781]
16. Lucena E, et al. Successful ongoing pregnancies after vitrification of oocytes. *Fertility and Sterility* 2006;85:108–111. [PubMed: 16412739]
17. Mazur P. Cryobiology: the freezing of biological systems. *Science* 1970;168:939–949. [PubMed: 5462399]
18. Mazur P, Leibo SP, Chu EHY. A two factor hypothesis of freezing injury: Evidence from Chinese hamster tissue-culture cells. *Exp Cell Res* 1972;71:345–355. [PubMed: 5045639]
19. Rapatz G, Luyet B. Patterns of ice formation in albumin solutions. *Biodynamics* 1972;11:125–136.
20. Ren HS, Hua TC, Yu GX, Chen XH. The crystallization kinetics and the critical cooling rate for vitrification of cryoprotective solutions. *Cryogenics* 1990;30:536–540.
21. Ren HS, Wei Y, Hua TC, Zhang J. Theoretical prediction of vitrification and devitrification tendencies for cryoprotective solutions. *Cryobiology* 1994;31:47–56.
22. Sakai K. Determination of pore size and pore size distribution. 2. Dialysis membranes. *J Membr Sci* 1994;96–91.
23. Seki S, Mazur P. Kinetics and activation energy of recrystallization of intracellular ice in mouse oocytes subjected to interrupted rapid cooling. *Cryobiology* 2008;56:171–180. [PubMed: 18359013]
24. Shimada K, Asahina E. Visualization of intracellular ice crystals formed in very rapidly frozen cells at -27°C . *Cryobiology* 1975;12:209–218. [PubMed: 1132237]
25. Stott SL, Karlsson JOM. Visualization of intracellular ice formation using high-speed video cryomicroscopy. *Cryobiology* 2009;58:84–95. [PubMed: 19041300]
26. Son WY, et al. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. *Hum Reprod* 2003;18:137–139. [PubMed: 12525454]
27. Sun J, Simon SL. The melting behavior of aluminum nanoparticles. *Thermochimica Acta* 2007;463:32–40.
28. Toner M, Cravalho EG, Karel M. Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells. *J Appl Phys* 1990;67:1582–1593.

29. Toner M, Cravalho EG, Karel M, Armant DR. Water transport and estimated transmembrane potential during freezing of mouse oocytes. *J Membrane Biol* 1990;115:261–272. [PubMed: 2374161]
30. Wassarman, PM.; Albertini, DF. The Mammalian Ovum. In: Knobil, E.; Neill, J., editors. *The Physiology of Reproduction*. New York: Raven Press; 1994. p. 79-122.
31. Xu J, et al. A microfabricated nanocalorimeter: design, characterization, and chemical calibration. *Anal Chem* 2008;80:2728–2733. [PubMed: 18351750]
32. Zhao G, Luo D, Gao D. Universal model for intracellular ice formation and its growth. *Thermodynamics* 2006;52:2596–2606.

Fig. 1.

A schematic of the cryomicroscopic observation using the sandwiched sample: **A.** Mouse oocytes are sandwiched into two quartz chips, whose distance is controlled by a two pieces of tapes of ~100μm thick; **B.** During freezing, both intracellular and extracellular solutions freeze; **C.** The melting of ice during warming.

Fig. 2.

One example (0.15*m* NaCl and 3*m* EG as initial concentrations) of the significant difference between the melting behavior of intracellular and extracellular solutions: **A.** at -40°C during warming, cells and solutions are still frozen; **B.** at -9°C , cells become transparent but the extracellular solutions are still partially frozen; **C.** at -7°C , most of the extracellular ice melts.

Fig. 3.

The comparison between the melting points of intracellular and extracellular solutions in the whole initial NaCl and EG concentration range: the upper curved plane is for the extracellular solutions and the lower one is for the intracellular solutions.

Fig. 4.
The logarithm of the size of intracellular ice crystals (r) as a bi-linear function of extracellular NaCl and EG molality.

Fig. 5. A schematic of the formation of different levels of intracellular ice crystals: **A.** the formation of primary crystals with their diffusion layers within the intact intracellular solution; **B.** the formation of secondary or higher level crystals between the primary crystals.

Fig. 6.
Examples of intracellular vitrification in the presence of the extracellular crystallization when initial EG concentration is $5m$ and the NaCl concentration is: **A.** $0.3m$; **B.** $0.45m$; **C.** $0.6m$.

Table 1

Symbol definitions and units

Symbol	Definition	Units
T	Temperature	K or $^{\circ}C$
r	Radius	m
σ	Interface energy	J/m^2
Hf	Fusion heat	J/kg_3
ρ	Density	kg/m^3
m	Molality of permeating solutes	mol/kg
n	Molality of non-permeating solutes	mol/kg
Subscript i	intracellular	
Subscript e	extracellular	

Table 2

The difference between the intracellular and extracellular melting points with different initial extracellular EG and NaCl concentrations (unit: °C).

EG molality	0 <i>m</i>	1 <i>m</i>	2 <i>m</i>	3 <i>m</i>	4 <i>m</i>
NaCl molality	2.2	2.4	2.8	3.5	5.0
0.15 <i>m</i>					
0.3 <i>m</i>	2.6	3.0	3.4	4.3	6.8
0.45 <i>m</i>	4.1	4.9	5.7	7.9	9.7
0.6 <i>m</i>	5.2	6.6	8.8	10.7	12.1

Table 3

The calculated values of the size of intracellular ice crystals formed with different initial extracellular EG and NaCl concentrations (unit: nm).

EG molality	0 m	1 m	2 m	3 m	4 m
NaCl molality	0 m	1 m	2 m	3 m	4 m
0.15 m	28.3	22.5	17.9	14.3	11.4
0.3 m	20.0	15.9	12.7	10.1	8.1
0.45 m	14.2	11.3	9.0	7.2	5.7
0.6 m	10.0	8.0	6.4	5.1	4.0