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High ice nucleation temperature of zebrafish embryos: slow-freezing is not an option☆

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Abstract

Although fish embryos have been used in a number of slow-freezing cryopreservation experiments, they have never been successfully cryopreserved. In part this is because little is known about whether ice forms within the embryo during the slow-freezing dehydration process. Therefore, we examined the temperature of intraembryonic ice formation (T_{IIF}) and the temperature of extraembryonic ice formation (T_{EIF}), using a cryomicroscope. We used both unmodified zebrafish embryos and those with water channels (aquaporin-3 or AQP3) inserted into their membranes to increase permeability to water and cryoprotectants, examined at 100% epiboly to the 6-somite stage. In these experiments we examined: (1) the spontaneous freezing of (external) solutions; (2) the spontaneous freezing of solutions containing embryos; (3) the effect of preloading the embryos with cryoprotectants on T_{IIF}; (4) whether preloading the embryos with cryoprotectant helps in survival after nucleating events in the solution; and (5) the damaging effects of extracellular nucleation events versus solution toxicity on the embryos. The solutes alone (embryo medium—EM, sucrose culture medium, 1 M propylene glycol in EM, and 1 M propylene glycol in a sucrose culture medium) froze at -14.9 ± 1.1, -17.0 ± 0.3, -17.8 ± 1.0, and -17.7 ± 1.4, respectively. There was no difference amongst these means ($P > 0.05$), thus adding cryoprotectant did not significantly lower the nucleation point. Adding embryos (preloaded with cryoprotectant or not) did not change the basic freezing characteristics of these solutes. In all these experiments, (T_{EIF}) equaled (T_{IIF}), and there was no difference in the freezing point of the solutions with or without the embryos ($P > 0.05$). Additionally, there was no difference in the freezing characteristics of embryos with and without aquaporins ($P > 0.05$). The formation of intraembryonic ice was lethal to the zebrafish embryos in all cases. But this lethal outcome was not related to solution injury effects, because 88–98% of embryos survived when exposed to a higher solute concentration with no ice

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present. Taken together, these data suggest that slow-freezing is not a suitable option for zebrafish embryos. The mechanism of this high temperature nucleation event in zebrafish embryos is still unknown.
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Although investigators have examined fish cryobiology for over 50 years, successful cryopreservation of fish embryos has not been achieved. Successful cryopreservation of fish embryos would benefit conservation, aquaculture, and medical research because of the importance of the zebrafish as a genetic and developmental vertebrate model. Generally, fish embryos are larger than most mammalian embryos that have been successfully cryopreserved, and exhibit different cryobiological parameters. For example, zebrafish embryos exhibit very low permeability to water and cryoprotectants. This has made them poor candidates for traditional cryopreservation techniques involving cryoprotectant loading and osmotic removal of water during slow cooling [8]. To address this low permeability challenge, we created modified zebrafish embryos that have aquaporin-3 (AQP3) fused with a green fluorescent protein (GFP) expressed in their membranes [9]. These membrane channels allow water and cryoprotectants to cross their membranes at higher rates than normal (e.g., 5× to 10× higher). With the enhanced membrane permeability of the AQP3-expressing embryos, we renewed our search for a successful slow-freezing protocol for the zebrafish embryos. The objective of a slow-freezing protocol is to remove intracellular water before it freezes. It has been estimated that 90% of intracellular water must be removed to avoid the formation of intracellular ice [15,21]. If intracellular ice formation (IIF) occurs, it is generally lethal [16]. In the absence of cryoprotectants, IIF typically occurs about 10 °C below the external ice formation temperature, T_{EIF}. However, with the addition of cryoprotectants, the temperature of intraembryonic ice freezing (T_{IIF}) usually drops to −20 to −30 °C, which is much lower than the colligative freezing point depression of the cryoprotectants and typically much lower than T_{EIF} [22,23]. For example, in the absence of cryoprotectants, mouse embryos freeze intracellularly at around −10 to −15 °C. However, if the mouse embryo is equilibrated in a cryoprotectant, no intracellular ice forms, even though there are ice crystals in the external medium, until the temperature falls to around −40 °C [23]. There is also some dependence of T_{IIF} on cooling rate [18,23].

It is the large drop in T_{IIF} below T_{EIF} that is essential to successful cryopreservation using a slow cooling protocol. During slow cooling, extracellular ice forms, pulling pure water out of the external solution and concentrating it, which leads to osmotic dehydration of the cells as they supercool. If 90% of the water can be removed from the cells before IIF occurs, lethal ice formation can be avoided. Otherwise, cell death is likely.

A number of marine invertebrate embryos, such as oysters, sea urchins, and polychaete worms, have been successfully frozen [3,7,19,20]. However, not all aquatic organisms have been found to be good candidates for slow-freezing cryopreservation. For example, starfish oocytes were found to form intracellular ice at relatively high temperatures very close to the temperature of extracellular ice formation [11]. Liu et al. [12] examined the intraembryonic ice formation in zebrafish embryos immersed in oil using differential scanning calorimetry. They found that embryos with a chorion surrounded by perivitelline fluid had a higher T_{IIF} than those without a chorion. They argued that this higher T_{IIF} occurred as a consequence of the seeding of intraembryonic ice by external ice in the perivitelline fluid. Because the dechorionated zebrafish embryo is the most likely candidate for future cryopreservation and the intraembryonic ice formation temperature for dechorionated zebrafish embryo had not been previously measured in the presence of external media, we were interested in examining whether fish embryos behaved like most other cells in regards to their ice nucleation characteristics. Additionally, we were

interested in understanding whether the presence of the water channels in the membranes would alter their freezing characteristics. To understand how the embryos might withstand freezing, a number of experiments were conducted with control and AQP3-modified embryos. These experiments examined: (1) the spontaneous freezing of (external) solutions; (2) the spontaneous freezing of solutions containing embryos; (3) the effect of preloading the embryos with cryoprotectants on T_{IIF}; (4) whether preloading the embryos with cryoprotectant helps in survival after extracellular nucleating events in the solution; and (5) the damaging effects of extracellular nucleation events versus solution toxicity on the embryos.

Methods

Animal care

Zebrafish adults, *Danio rerio*, were maintained in five liter aquaria (temp = 28.5 °C; pH 7.0) with a 14/10 h light/dark illumination cycle. Each aquarium contained 12–16 fish of both sexes that were fed dry pellets (Tropical Micropellets, Hikare, Japan) twice a day, supplemented with live brine shrimp. To collect embryos, a glass container with mesh lid and attached plastic aquarium plants was placed into three to four aquaria at night. The adults spawned at the onset of the light cycle. Twenty minutes after the start of the light cycle, the containers were removed and embryos retrieved. All embryos were placed in a fine mesh tea-strainer, rinsed with a dilute commercial bleach solution (0.075% in aquarium water) for 5–10 s, and then rinsed three times with 400 ml of aquarium water/rinse. After this bleach treatment, all embryos were dechorionated as previously described [28]. Embryos were microinjected with 800 pg of either AQP3-GFP or GFP cRNA at the 1- to 4-cell stage, as described in [9], or not injected at all (controls), and then cultured at 28.5 °C to epiboly stage in agar-coated 35 mm Petri dishes with 10–20 embryos/dish. Embryos were screened for the GFP marker using a fluorescent microscope. Only those fluorescent, expressing embryos were used in the experiments. We constructed the AQP3-GFP from donated constructs, and made the cRNA as described in [9].

Rearing solutions were either embryo culture medium (EM) which is a 10% Hanks' buffer [28] or sucrose culture medium (SUC CM) which is EM containing 0.3 M sucrose and 1% (w/v) ficoll. When experiments called for cryoprotectant, the cryoprotectant was always added to the specific culture medium in which the embryos were raised. We used both types of culture media in these experiments because both supported the growth of the normal and AQP3-modified embryos. The SUC CM was chosen to provide a growth medium for the embryos which approximates the intracellular osmolality of the embryos. All care and welfare for the animals met NIH animal care standards.

Spontaneous nucleation of solutions

To keep the freezing medium simple, we analyzed the spontaneous nucleation of EM, 1 M PG made up in EM, and 1 M PG made up in SUC CM. These solutions ($N = 5$/solution; volume = 250 μl) were loaded into a small quartz holding vessel and placed onto a Linkam Cryostage (VTO 232 and BCS 196, Linkam, Surrey, UK) on an Olympus BX-41 microscope with a video attachment (Sony Exwave HAD) and monitor. The solutions were cooled at 2 °C per minute, and the nucleation point was identified by ice-crystal growth in the solution. Temperatures reported are those of the Linkam stage thermocouple.

Spontaneous nucleation of solutions with embryos

The solutions (EM, SUC CM, 1 M PG in EM, and 1 M PG in SUC CM) were prepared and analyzed as above, but either a control, unmodified embryo ($N = 5$) or an AQP3-modified embryo ($N = 5$) were removed from their raising medium, added to the test solution, and immediately cooled at 2 °C per minute. The T_{EIF} and T_{IIF} points were identified by ice-crystal growth in the solution and embryo, respectively. In the embryo, this was often visualized as a blackening of all or part of the embryo.

Seeded solutions

Slow-freezing protocols typically use a seeding step to initiate extracellular ice formation at a relatively high temperature. Our goal here was to use a simple, slow-freezing protocol to examine T_{EIF} and T_{IIF} with cryomicroscopy during the seeding and freezing process. The embryos (control and AQP3-modified) were equilibrated in a 2 M PG solution in EM or in SUC CM for 1 h before cooling. A 2 M PG solution was used for preloading because our biophysical modeling suggested that the AQP3-modified embryos would have an intraembryonic concentration of ca. 1 M PG after being immersed in 2 M PG solution for 1 h [9]. No appreciable change in size was noticed during the loading procedure, however, no detailed measurements were made at this time. The preloaded embryos were then cooled to −8 °C in 2 M PG and the external solution was nucleated with cold forceps. This solution was held at −8 °C for 5 min and then cooled at 2 °C/ min to −40 °C. These experiments were repeated 8–9 times per treatment and T_{EIF} and T_{IIF} determined. The seeding temperature of −8 °C was chosen because the thermodynamic or equilibrium freezing/melting point of the most concentrated solution used in these experiments involving ice formation (2 M PG in SUC CM) is about −5 °C. A margin needs to be provided to ensure seeding. We chose −8 °C.

Solution damage

Nucleation events can be damaging due either to ice-crystal formation or to the increase in solution strength accompanying ice-crystal formation. To investigate this, unmodified and AQP3-modified embryos were placed into a solution of 2 M PG in EM for 1 h to preload them with cryoprotectant, then into a solution of 3.26 M PG in double strength EM, cooled to −8 °C, and held for 5 min. This is the concentration of PG and EM that the embryos would experience in a 2 M PG in EM solution that has been frozen at −8 °C. These numbers were computed by assuming ideal solutions in which EM is just NaCl, molality = osmolality for PG, the osmolalities of EM and PG are additive, and the freezing point depression is given by 1.86 °C/osmol. In parallel experiments, control and AQP3-modified embryos were placed in 2 M PG in EM for 1 h at 22 °C to preload them with cryoprotectant, then this solution was cooled to −8 °C, nucleated with cold forceps and held for 5 min. All embryos were rewarmed to 22 °C, suspended in EM, placed in a water bath at 28 °C, and their development monitored. The embryos that were tested for intracellular ice were observed for 24 h post-treatment. The embryos were tested at approximately 100% epiboly when the blastoderm cells cover the yolk. If they continued to develop to the 24 h stage (during the pharyngula period) they were considered alive. At this stage, any stimuli elicits muscle contractions and tail movements. Generally, if intracellular ice formed, the embryos fell apart within minutes. The experiments were repeated five times for control and AQP3-modified embryos with 5 embryos/ trial.

Statistical analysis

Statistics were computed using Instat (Graphpad Software, San Diego, CA). A paired t test was used to determine whether there was a difference between two means, and a one-way ANOVA for the differences amongst means. Results were presented as means ± SEMs, and probability values $P < 0.05$ were chosen as the level of significance.

Results

The test solutions spontaneously froze at −14.9 °C for EM, at −17.0 °C for SUC CM, at −17.8 °C for 1 M PG in EM, and a −17.7 °C for 1 m PG in SUC CM (Table 1). Thus, the addition of the cryoprotectant depressed the T_{EIF} by about 3 °C, but this depression was not statistically significant ($P > 0.05$). The introduction of embryos into the test solutions had no effect on the T_{EIF} of the solutions ($P > 0.05$). The most important observation we made was that intraembryonic ice forms as soon as the ice front reaches the embryo. In these spontaneously nucleating solutions the ice front reached the embryo in less than a second. As the embryos spontaneously froze, they went

Table 1
Spontaneous nucleation temperatures: T_{EIF} and T_{IIF} (cooling rate = 2 °C/min, no seeding)

Test solutions	Solutions alone T_{EIF} (°C)	Control embryos T_{EIF} (°C) and T_{IIF} (°C)	AQP3-modified embryos T_{EIF} (°C) and T_{IIF} (°C)
EM	−14.9 ± 1.1	−14.2 ± 1.0	−13.2 ± 1.0
SUC CM in EM	−17.0 ± 0.3	−16.2 ± 0.5	−18.0 ± 1.5
1 M PG in EM	−17.8 ± 1.0	−18.2 ± 1.9	−15.1 ± 1.3
1 M PG in SUC CM	−17.7 ± 1.4	−16.8 ± 0.7	−17.5 ± 0.8

Values are mean (±SEM); $N = 5$–9. T_{EIF}, temperature of extracellular ice formation; T_{IIF}, temperature of intraembryonic ice formation. All solutions made up in EM. The calculated freezing point depression (FPD) of the test solutions, from top to bottom, are ~−0.07, −0.55, −1.9, and −2.45 °C, respectively. T_{EIF} is lower than the FPD because of supercooling, as expected.

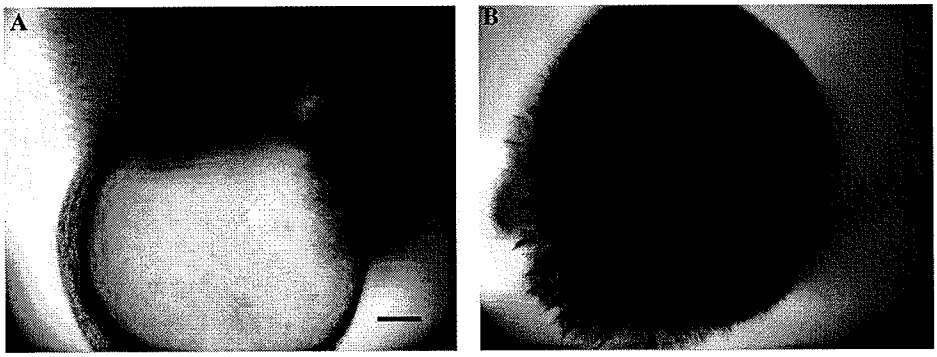

Fig. 1. When solutions spontaneously nucleate, the ice front touches the embryo and the entire embryo blackens in less than 1 s. (A) The ice front as it begins to pass through the embryo. (B) What the embryo looks like as the freezing front exits the embryo. Bar = 100 μm.

through rapid black flashing that started as the ice front reached the embryo (Fig. 1). In short, T_{EIF} equaled T_{IIF} regardless of the treatment ($P > 0.05$; Table 1).

Similarly, when the external solution was seeded, T_{EIF} still equaled T_{IIF} (i.e., −8 °C) regardless of the treatment ($P > 0.05$). Specifically, control embryos raised in EM ($N = 7$) and AQP3-modified embryos raised in EM ($N = 9$) and SUC CM ($N = 8$), equilibrated in 2 M PG for 1 h, cooled to −8 °C and seeded with cooled forceps formed intraembryonic ice within 30 s (after the ice front reached the embryo) while being held at −8 °C. This type of freezing was different than the type of freezing observed for the spontaneous nucleation events described above. It was slower taking anywhere from 5 to 30 s for the embryos to turn fully black. The embryos seemed to freeze from the outside inwards with dark waves moving across the embryo until it appeared solidly black (Fig. 2). The embryos did not survive this treatment.

We examined whether the embryo damage observed above was due to intraembryonic ice formation or to solution effects injury (Table 2). Control and AQP3-modified embryos were pretreated in 2 M PG for 1 h, as above. The first groups of pretreated control and AQP3-modified embryos were put into a 2 M PG solution, cooled to −8 °C, and seeded. These embryos froze intraembryonically and had no survival after 24 h (Table 2). Actually, none remained intact for more than 10 min after thawing. In comparison, another group of control and AQP3-modified embryos were cooled to −8 °C in 3.26 M PG in double strength EM, held for 5 min with no seeding, and returned to room temperature. After culturing them for 24 h, the controls had 98% survival and the AQP3-modified

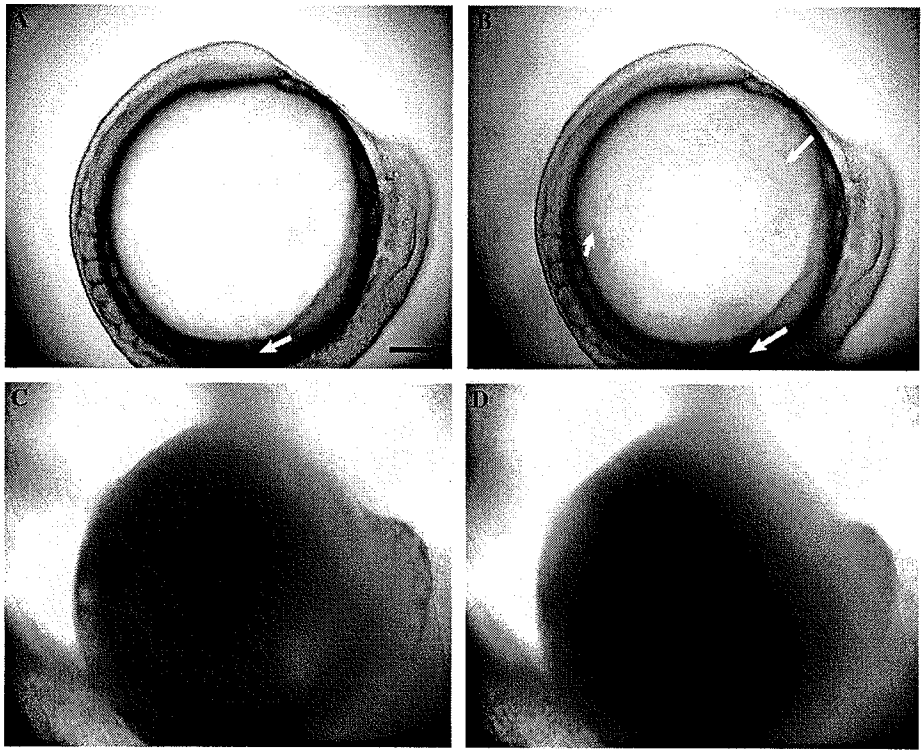

Fig. 2. An AQP3-modified embryo was held at −8 °C in 1 M PG in EM for 1 min, then the solution was seeded outside the image field with cold forceps. (A) Within 2 min the ice front approached the embryo from below, causing a slight darkening of the blastoderm (arrow). (B) Nucleation continues in the blastoderm, appearing as several gray areas in the blastoderm (arrows). (C) Most of the embryo is frozen. (D) The embryo is completely frozen and dark black (ice crystals appear as lighter areas in the image). Images were taken at 0.5 s intervals, bar = 100 μm.

Table 2
Embryo survival

Embryo type	Test condition	Mean % survival at 24 h	*N*
Control	Cooled to −8 °C in 2 M PG in EM and seeded	0	25
AQP3	Cooled to −8 °C in 2 M PG in EM and seeded	0	30
Control	Cooled to −8 °C in 3.26 M PG in 2× EM; held 5 min	98 ± 2	45
AQP3	Cooled to −8 °C in 3.26 M PG in 2× EM; held 5 min	88 ± 5	44

After testing, embryos were warmed to room temperature, suspended in EM, and cultured at 28 °C for 24 h.

embryos had 88% survival (Table 2). We concluded that it was the formation of intraembryonic ice that was lethal, not solution damage.

Discussion

Zebrafish form intraembryonic ice within seconds after the appearance of external ice in the extracellular solution near the embryo. This intraembryonic ice formation is lethal, while the higher osmotic strength of the solution that results from ice formation is not detrimental to the embryo. Additionally, the presence of water channels in these embryos does not alter these observations. These factors together suggest that zebrafish would not be good candidates for slow-freezing. This is because the ability to survive slow-freezing depends upon the embryo remaining unfrozen and supercooled in the presence of ice formation in the

external medium, so that the embryo undergoes dehydration during cooling.

Liu et al. [12] used differential scanning calorimetry to analyze the freezing point of intraembryonic water in zebrafish embryos immersed in oil. They found that embryos with a chorion, surrounded by perivitelline fluid, had T_{IIF}'s of −11.9 to −20.5 °C, depending on stage. This agrees well with our T_{IIF} of −13.2 to −14.2 °C for dechorionated embryos in EM at 100% to 6-somite stage used in this paper. Apparently, the perivitelline fluid in their experiments played the same role as the embryo medium in our dechorionated embryos. Namely, ice formation in the extracellular fluid, whether it is in the perivitelline space or external medium, triggers intraembryonic ice formation. Consistent with this view, Liu et al. found that dechorionated embryos in oil (where external aqueous fluid is absent) froze at temperatures of 3.1–11.6 °C lower than those immersed in perivitelline fluid.

Tanghe et al. [24] found that the presence of aquaporins in the membranes of yeast cells improved their ability to survive freezing. That is also the case for mouse oocytes that were later fertilized and cryopreserved [5,6]. In our experiments, the addition of aquaporins to the membranes of the fish embryos did not change their freezing response. They behaved exactly like the control embryos in terms of their spontaneous and intraembryonic ice-freezing temperatures.

Taylor and Pegg [25] examined the role of solution effects injury on smooth muscle. They first calculated, from phase diagrams, the rise in cryoprotectant and salt concentration that would occur after the test solution was frozen to a specific temperature. They suspended muscle tissue in that higher concentration solution and cooled it to the specific temperature. No ice was present. They then compared its functional survival after warming to the functional survival of tissue that had been suspended in the normal low concentration test solution and frozen to that same temperature. They found that the former (exposure to the high concentration of cryoprotectant/salt in the absence of ice) was relatively innocuous, whereas the latter (exposure to that same high concentration of cryoprotectant/salt in the presence of ice) was lethal. These observations parallel our results for the zebrafish embryo in which 88–98% survived after exposure to higher solute concentrations in the absence of ice, but 0% survived after extracellular and intraembryonic ice formation occurred.

Köseoglu et al. [11] investigated starfish oocyte cryopreservation and they found that intracellular ice formed at very high subzero temperatures that were very close to T_{EIF}. They speculate that this high subzero T_{IIF} in the starfish oocyte could be due to (1) the presence of large pores in the membrane [13]; (2) the sensitivity of the oocyte membrane to damage by ice [2,17]; or (3) the ability of the membrane to induce heterogeneous intracellular nucleation [26]. It is clear for both the starfish oocyte and the zebrafish embryo that the formation of extracellular ice invariably leads to the formation of intracellular ice within a few degrees of T_{EIF}. In the zebrafish embryo, the formation of extracellular ice next to the embryo precipitated the formation of intraembryonic ice within 30 s or less. The presence of cryoprotectants within the AQP3-modified embryos did not change this pattern.

We can think of three possibilities to explain the fact that intraembryonic ice formation occurs at the same temperature at which external ice forms and appears to be initiated immediately when the external ice comes in contact with the embryo surface.

1. The first possibility is that one or more gaps or pores exist in the blastoderm surface of sufficient diameter to permit the growth of external ice into the embryos at temperatures at least as high as −8 °C. Mazur [13,14] has argued that because the melting point of ice crystals with Ångström-sized radii (at their tips) of curvatures is reduced substantially below 0 °C, the temperature at which ice can grow through pores of Ångström range diameters is also substantially reduced below 0 °C. He further suggested that the suppression of the melting point and the limiting pore diameter can be estimated from the Kelvin equation: $\Delta T = 2vT^{\circ}\sigma SL\cos(\theta)/aL_{f}$, where v is the molar volume of ice, T° is the melting point of planar ice, σSL is the interfacial tension between the liquid and ice, θ is the contact angle between ice

and the wall of the pore, a is the radius of the pore, and L_f is the latent heat of fusion.

Acker et al. [1] have experimentally tested the nucleation temperature of tissue culture cells that possess gap junctions in their membranes and found that these temperatures are consistent with the Kelvin equation. If θ is assigned a value of 75°, they calculated that in order for external ice to grow through a surface membrane at −10 °C, the pore or gap in the surface would have to be ~12 Å in radius. That diameter is roughly consistent with the known radii of gap junctions [4,10].

We should emphasize that our microscope observations do not tell us with certainty that the observed intraembryonic ice is in fact located within the blastodermal cells. It is quite possible that external ice grows through one more gaps between the blastodermal cells and then propagates through intercellular space in the blastoderm and then into the yolk.

2. A second possibility is that when external ice makes contact with the embryo surface, it causes physical deformation of that surface and that deformation either forms gaps in the surface or enlarges pre-exiting gaps or pores. We emphasize a physical deformation because our experiments dictate against chemical damage arising from the increased solute concentration brought about by external freezing. The same argument would dictate against membrane damage arising from osmotic water efflux arising from that higher solute concentration as proposed by Muldrew and McGann [17].

3. A third possibility is that proposed by Toner and colleagues [26,27]; namely, that when external ice makes contact with the outer membrane, it induces changes in membrane structure that cause the inner surface of the membrane to become an effective ice nucleating agent of the internal water.

At the moment, these three possibilities remain just that—possibilities. Our experiments provide no direct evidence to choose among them. However, our results strongly suggest for zebrafish that any type of ice crystal present in the external medium can and will cause damage. Consequently, a vitrification process completely free from any ice-crystal formation will be necessary to successfully freeze these embryos.

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