Stopping the biological clock
Merging biology and cryogenics in applied cryobiology

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Abstract. The ability to stop all cellular activity for a prolonged period of time, yet still be able to deliver functional recovery on demand, is the practical output of the science of cryobiology. This cessation of biological time allows cells to be preserved for months or years in medicine and biotechnology. This review discusses the historical development of cryobiology, the basic scientific principles underpinning cryopreservation, and how the technologies are currently used.

1. Introduction
The impact of low temperatures on cells and tissues has held the fascination of natural scientists over many centuries. For example, Boyle, in his 17th century treatise recorded many observations on the mechano-physical effects of ice formation and freezing in natural systems [1]. In the same era Spallanzi [2] commented on the effects of cold to stop biological activity (motility) in spermatozoa of several species, using newly available microscopes and a century later Mantegazza [3] observed sperm cooled and frozen below -10°C. He made the visionary comment that freezing temperatures might be used in some future time to set up sperm banks to facilitate animal breeding programmes in different countries. Microscopy techniques allowed plant scientists to gain further insight into freezing and biology. For example in the 1890s Molisch [4] developed a unique microscope system to observe the freezing process in plant tissues which immediately highlighted one of the central problems with ice formation: once ice starts to form, pure water was captured by the growing ice front and the cells became extremely dehydrated because the solutes in the original solution were concentrated in the remaining unfrozen liquid which progressively reduced in volume as lower and lower temperatures were reached. Immediately on thawing, multiple and extreme changes to cell structure were evident. Maximov [5] demonstrated that in order to tolerate seasonal ice formation in nature, plants cells often required a ‘hardening’ step ahead of freezing which was associated with production of high concentrations of sugars and other solutes in the tissues. Later understanding (see below) would elucidate that this accumulation of sugars can counteract the damaging ‘freeze dehydration’ associated with ice formation. It was also an early indication that such similar solutes acted against osmotic injury as ‘osmolytes’ to protect either freezing injury or drought conditions in some plant species. If they could be provided in other cases where cells were to be preserved by freezing, these ‘osmolytes’ might be used as natural ‘antifreezes’ or ‘cryoprotectants’ – literally solutes which would enhance...
biological survival in the presence of ice. In the early 20th century, this combined background knowledge base led to interest in applications of cryoprotectants (CPA) to provide successful cryogenic storage of cells, culminating in the seminal work of Polge, Smith & Parkes (1949). For the first time repeatable recovery of living cells from temperatures down to -79°C was demonstrated. This will be further discussed below.

2. The role of fundamental cryobiology to cell and tissue cryopreservation

2.1 Historical Aspects

The term ‘cryobiology’ first appeared in the 1960s, with establishment of the International Society for Cryobiology in 1963. The term cryopreservation is now widely used to signify storage of living cells at deep cryogenic temperatures (in the region of -170°C to -196°C). Our understanding of the biophysical processes involved has developed in the intervening time but is still as yet incomplete. It has also become clear that attainment of the cryogenic state sufficiently stable to permit recovery of viable cells is possible by applying different approaches which achieve the same end point. These can be broadly described as controlled rate slow cooling (CRSC) or fast cooling rate vitrification (VT) and these will be discussed below. The term cryopreservation encompasses both. Polge and colleagues [6] in fact applied CRSC to preserve fowl sperm using glycerol and sugars as CPA – although they understood that this was something different to ‘freezing’, because they actually used the term ‘vitrification’ in the title of their work. It was only after another 20 years of research that it was understood that their approach was not VT as we know and apply it today.

2.2 The importance of the water-ice phase transition in cryobiology

Whilst low temperatures can disturb cell functions at many different levels, the injuries can take many hours or even days to become irreversible (for example red blood cells can be routinely stored for >20 days in the chilled liquid state around +4°C); however the formation of ice even after cooling to only high sub-zero temperatures causes almost immediate cell disruption [7]. Early evidence suggested that no mechanical damage was produced by ice formation under these conditions, whereas the reduction of free water in the system following ice nucleation (ice forms only from water and excludes any dissolved solutes into the residual aqueous volume) leads to a significant hypertonic stress on the cells [7, 8] which was the key injury. More detailed discussions on these topics can be found in later reviews [9, 10].

Once ice nucleation commences water molecules join ice crystals as spatially-defined arrays yielding the commonly observed hexagonal ice. In any CRSC protocol, ice preferentially nucleates in the external medium because cell membranes, to some extent, act as a barrier to ice crystal growth. The phase transition also results in the easily-detectable release of energy as the latent heat of ice formation, and can be a useful indicator of the underlying important physical processes in cryopreservation (see discussion on ‘ice seeding’ below). The proportion of ice continues to grow from within the residual aqueous solution as colder temperatures are reached with a parallel progressive increase in percentage of water removed as ice, such that by about -6°C, some 80% of original water content has been removed as ice, starting from the isotonic solutions in which mammalian cells exist [7].

This might be considered to be an irreversibly damaging consequence of growing ice formation as deeper cryogenic temperatures are reached; however, at some point the whole matrix (ice, the tiny fraction of residual water, solute content and entrapped cells) undergoes another transition (see below) to a ‘pseudo-glassy’ state. This ‘locks’ the system into a format which can remain stable over many years. There are multiple biological targets for this extreme dehydration, including destabilisation of cell membranes and a change in the intracellular milieu (including pH, protein damage and disruption of essential organelles such as mitochondria and cytoskeleton), all of which can result individually or in combination in cell death [9, 10].
It will be clear that optimal cryogenic dehydration depends on the kinetics of water movement across the cell membranes, i.e. it is rate dependent. The outcome can be dictated by the rate of cooling, and if this rate is too high, there may be a sufficient residual cytoplasmic water fraction capable of nucleating ice in intracellular spaces. Nucleation of ice and progressive intracellular ice formation (IIF) have been found in many cases to almost invariably result in lethal cell injury. Using mammalian embryos or oocytes as model systems in cryobiology, many of these events have been identified using direct observation of the freezing processes by cryomicroscopy [11–13]. In fact, due to their large size these cells have been used to reveal many of the mechanistic effects of cryopreservation. Cryomicroscopy also provided evidence that that one mode of action of the added protective solutes (or cryoprotectant additives – CPA) included inhibiting IIF during cryogenic cooling [14].

3. Cryoprotectants – the essential compatible solutes which protect against freezing injury
A variety of biocompatible osmolytes were identified which impact favourably on cryopreservation processes, provided they can be delivered safely ahead of the cooling process. These became known collectively as cryoprotectants (CPA). Polge’s early work focused on the use of glycerol as a CPA [6], and studies indicated that solutes with a high propensity to form hydrogen bonds with water, such as sugars, other polyols, or in some cases polymers, were, in turn, often useful as CPA [15, 16]. It was suggested that these are solutes which can favourably impact on the nature and volume fraction of ice crystal growth during cooling. Protection is assured on a kinetic basis until cooling has progressed to sufficiently low temperatures that total solidification of the aqueous matrix occurs (which ‘locks-in’ the stabilised biological components). Progression to lower cryogenic temperatures is then without significant biological impact. Cryogenic cell preservation is often performed on a pragmatic basis to temperatures below -170°C, which are provided by the vapour phase or liquid phase of liquid nitrogen (LN₂), because this has become the widely-accessible, relatively inexpensive cryogen of choice. However, living cells can be recovered from even lower temperatures such as -269°C [17].

Most cells of interest for cryopreservation are suspended in isotonic (and dilute) aqueous salt solutions with effectively a very high water content. The chemico-physical properties of ice formation in such systems dictate that there is a large increase in salt concentrations (by more than x10 fold) in the residual aqueous fraction even at relatively high subzero temperatures such as -5 C [18]. An early explanation of CPA action was formulated on the depression of freezing point which follows from the addition of significant concentrations of an additional (biocompatible) solute, which in turn is a function of the molar concentration of the added CPA. This became known as the colligative action of CPAs [19] and explains why such high concentrations of CPA (often > 1M) are needed in cryopreservation.

Once there was a basic understanding of CPA action, other compatible solutes were sought. Lovelock and Bishop identified the agent dimethyl sulphoxide [DMSO] in the 1960s [8] as a potential CPA and subsequently this has become one of the most widely recognised CPAs. The term ‘cryoprotectant’ itself not was formally recognised until a decade later [20]. In reality only a small number of other hydrophilic solutes acting as primary CPAs have been shown to be effective. For example in some areas (such as embryo cryopreservation) another polyol, propylene glycol (1,2 propane diol, PrOH) is now widely used in CRSC, whilst ethylene glycol has been used in some instances for tissue cryopreservation [21, 22].

In order to protect intracellular compartments, CPAs must cross cell membranes. Effective CPAs such as DMSO and PrOH cross cell membranes to provide intracellular protection. Other agents modifying the water-ice phase transition (e.g. sugars or polymers) provide some CPA effects but less effectively [23]. CPAs can thus be pragmatically described as either cell-permeating (primary) or non-permeating (secondary) agents [16, 17] (see Table 1). Non-permeating CPAs modulate ice crystal growth in the extra-cellular environment, but they cannot normally provide primary cryoprotection [23, 24]. Sugars such as trehalose and sucrose are often included in CPA mixes but at lower
concentrations (between 0.1-0.3M). Polymers such as polyethylene glycol have been used at between 2 -5% w/v [25], whilst hydroxethyl starch was found to be a beneficial additive to supplement DMSO [26]. In many cases adding secondary CPAs permits a reduction in the concentration of primary CPAs such as DMSO [26, 27] which helps to minimise CPA toxicities. In VT protocols ficoll has been used as a polymer additive [28]. Such secondary CPA possess non-ideal solution properties and thus restrict the mobility of free water to join the growing ice fronts on a kinetic basis during cooling.

Any agents which modify cell water relationships can exert wide-ranging toxicities in certain situations, and this is also true for CPA [16, 20]. Also, application of CPAs at concentrations of 1M or greater will have direct osmotic impact on cells [29–31], both during addition and equally during removal post-cryopreservation[32, 33]. Thus, CPA exposure protocols prior to cryopreservation must be optimised. Chemical CPA toxicities may be mitigated by using lower exposure temperatures, but this reduces rates of intracellular diffusion [34]. For these reasons, step-wise, time and temperature CPA-controlled protocols have to be developed to minimise these risks. Currently such interactions can only be partially modelled [31], so prospective empirical studies are also often needed.

Transmembrane CPA permeation is largely a simple physico-chemical process. When the kinetics are considered, the temperature is critical. For example, at ambient temperature, CPA permeation into mammalian oocytes requires around 10 minutes to approach equilibrium [35]. This process is faster at higher temperatures (e.g. 37°C), but this also exacerbates any chemical toxicities associated with the CPA.

| Commonly used Cryoprotectants (CPA) |
|--------------------------------------|
| Cell permeating agents (mostly rapid permeating solutes) | Sugars (which may permeate cells to a degree depending on molecular size) | Amines (slowly or only partially permeating) | Polymers (remain in the extracellular environment) |
| Dimethyl sulfoxide*** | Sucrose*** | Proline* | Polyethylene glycol (PEG)*** |
| Ethylene glycol*** | Trehalose*** | Beta-nure* | Hydroxy ethyl starch*** |
| Propylene glycol*** | Raffinose** | Glutamine | Polyvinylpyrolidone (PVP)** |
| Glycerol** | Mannitol** | | Ficoll** |
| Methanol* | Glucose* | Serum proteins (mixture)** |
| Ethanol* | Galactose* | Milk proteins (mixture)? |
| Acetamide* (often in mixtures) | Sorbitol* | Peptides (mixtures) |
| Butylene glycol* (less frequent - in mixtures) | | |

Table 1. Commonly used cryoprotectants identified by choice of application as widespread***, moderate** or infrequently-used* (e.g. for specific cell types) [36]
4. The importance of cooling and warming rates, and temperature stability during cryo-storage.

For successful cryopreservation, rates of cooling play an important role because these dictate the sustainable optimal cryo-dehydration which can be achieved during the transit through the high subzero temperature range down to the region where true long term cryo-stability can be assured. A number of studies have now demonstrated this stability to be below -100°C [37, 38]. Cryobiology research has documented that a physico-chemical ‘glassy’ state exists at about -120°C with the aqueous mixtures commonly encountered in cryopreservation, which has been termed the Tg (glass transition). Both CRSC and VT depend on control of temperature change but each with different optimal rates to achieve Tg whilst avoiding injury.

4.1 Kinetics of optimal temperature change during CRSC

The term ‘slow cooling’ is relative but early work in embryo cryopreservation indicated that success could be achieved using rates of about -0.3°C min⁻¹ [17, 39]. Mazur and his colleagues [39] developed a ‘2-factor hypothesis’ to describe two different injury scenarios when cooling proceeded at rates either side of optimum (see figure 1). In particular, cooling rates must allow optimal cryo-dehydration to an extreme degree where there is no possibility for intracellular free water to form intracellular ice crystals (IIF), an event which is almost invariably lethal [40]. During CRSC, the essential dehydrative force is ice formation itself, removing intracellular free water on a kinetic basis by osmosis [13]. Sub-optimal rapid cooling has been correlated with IIF, whilst suboptimal slow cooling was believed to result from over-long exposures to extreme cell dehydration before reaching Tg, with membrane bilayer disruption as one possible target [41]. Further details can be found elsewhere [42, 43] and go some way to explain why cells cannot be successfully preserved long-term unless deep cryogenic temperatures are reached. However, once this pathway has been successfully negotiated, cell functionality can be maintained for many years without change [44]. Needs for reliable control of cooling for CRSC have driven the development of automated cryo-coolers using liquid nitrogen (LN₂) vapour as the cryogen [45, 46] or Stirling engine cooling [26]. The temperature range for Tg for commonly-used CPA (around -120°C) also explains why long storage in liquid or vapour phase N₂ (i.e. below -150°C) has been so successful [37, 38] because this is well below this critical Tg and provides a degree of safety against small storage temperature fluctuations having a negative impact on outcomes.

As discussed, the achievement of optimal cryo-dehydration depends on intracellular free water moving across the cell membrane in osmotic response to the extracellular ice fraction which increases during cooling. This can be facilitated by ensuring extracellular ice forms as close to the equilibrium melting (or pragmatically, the equilibrium freezing) temperature of the mixture, in a process known as ‘ice seeding’ in the sample, which can be achieved practically in a number of ways [43, 47].

4.2 Vitrification – the alternative approach for cryopreservation

Vitrification (VT) also depends on extreme cell cryo-dehydration, ultra-low temperatures and passage through Tg, but these are achieved in a different approach. Early work in cryobiology demonstrated that water molecules could be manipulated by various physical approaches at ultra-low temperatures to prevent ice nucleation and reach a ‘glassy state’, but only by cooling extremely rapidly [48, 49] under conditions which could not easily be transferred to routine cell cryopreservation. The seminal work from Rall and Fahy [50] later demonstrated that use of very high concentrations of CPA (e.g > 50% w/w) could inhibit ice crystallisation using practically achievable cooling rates (in the range at or about -200°C min⁻¹ and still achieve Tg; using this approach mouse embryos were successfully cryopreserved by VT.

However, such high CPA concentrations raised concerns about toxicity, and to mitigate this CPA mixtures were introduced whereby each component was added at concentrations below their toxic levels whilst maintaining the total combined CPA concentration [51]. VT is now applied routinely in areas such as human embryo cryopreservation [19, 50, 51] where low sample volumes (<10ul) can be cooled in special containers (such as pulled straws or cryoloops) with suitably fast cooling. Evidence
suggests that applied optimally, VT may yield more success in embryo cryopreservation than CRSC [52–54].

![Figure 1. A simplified version of Mazur’s 2-factor hypothesis. A cell treated with CPA protection subjected to cooling at different rates. Maximum functional recovery (lower bell shaped curve) is achieved with an optimal cooling rate providing reversible cryo-dehydration occurring over the high subzero temperature range. If cooling is too slow, (left side of image) irreversible slow and excessive dehydration can take place which injures cell membranes and organelles. If cooling is too fast (right side of figure), cells do not have time to optimally dehydrate, and residual intracellular water can form ice, which is again injurious and can compromise structure of organelles. For many nucleated mammalian cells, ‘optimal’ cooling equates to rates of around -0.5°C to -1°C per minute down to -100°C and below. Reproduced from [47] with permission.](image)

4.3 The importance of robust storage temperatures for cell cryopreservation

True long-term storage can only be assured at temperatures safely below the $T_g$ for the commonly-used CPA. Storage at higher subzero temperatures can result in slow but measurable ongoing attrition to recoverable cell function [55], but may yield useful safe periods if these are carefully defined; for example, storage periods up to 10 years have been approved by FDA (Food & Drug Administration in USA) for cryopreserved red blood cells [56]. Storage in liquid or vapour phases of liquid N2 ($<-170°C$) provides such essential reliability. However, it must be remembered that accessing cryo-banks, or routine filling with cryogen, can cause temperature fluctuations which could prove harmful to cell viability. For example, experiments showed that significant temperature upshift (e.g. from -135°C to -60°C) could occur within a few minutes of lifting storage racks out from a cryo-container [57]. Repeated temperature cycling through the $T_g$ range led to a decrease in cell functional recoveries [57].

The choice of storage (in either liquid or vapour phase N2) for cell samples also needs consideration. There is a possibility to transfer infectious agents found in commercially produced LN2 which is not a sterile product [58]. For some VT applications, such as embryo cryopreservation, direct exposure to cryogen improves rapid cooling by direct plunging in ‘open devices. This has prompted
the proposal to use closed embryo containers [59]. On the contrary, samples may be cooled and stored in vapour phase N\textsubscript{2} but the relatively poor heat capacity of the vapour phase reduces achievable fast cooling rates and increases the risk of rapid and large temperature fluctuations when opening cryo-storage dewars. Temperature stability can be improved by simple measures such as adding a copper rod [60] as a thermal ‘cold conductor’ up from the cryogen reservoir into the body of the storage dewar. In addition, ensuring temperature stability during transport of cryopreserved samples can be essential to avoid loss of function[61]. Best practice dictates adherence to written protocols and maintenance of proper temperature records in all parts of the pathways for storage and distribution.

4.4 Importance of warming protocols for successful cryopreservation
Successful recovery of embryos from cryopreservation requires the same focused attention to scientific principles as required during cooling. Biophysical events are dictated by the increasing temperatures, and there are risks to embryo survival if warming is not optimised.

As soon as cryopreserved cells warm above the T\textsubscript{g} range (around -120°C), high solute damage can accumulate. Water molecules become mobile above -110°C, even in samples cooled under vitrification conditions [62], whilst slow frozen cells have a significant (extracellular) ice burden in the sample. During warming, existing ice crystals may undergo ice crystal growth and re-organisation (known as Ostwald ripening), which may impart further injury depending upon the kinetics of the process [63]. Even with vitrification, small intracellular ice nucleation centres may have established during the cooling process, which did not grow to become injurious ice crystals once T\textsubscript{g} was safely passed. However, once mobile water becomes available during warming, these nucleation centres may catalyse new or ongoing ice crystal growth, such that ‘freezing during thawing’ can be conceptualised [64].

Whilst fast warming has traditionally been favoured for embryo rewarming protocols [65], the scientific principles for rewarming have yet to be fully understood. Historically it has was argued that slow warming may be beneficial to allow time for osmotic re-equilibration processes to take place [17] when the highly shrunken cells start to encounter more liquid water as the ice matrix melts at high subzero temperatures. However, it has since been shown that slow warming could injure cells such as blastocyst embryos [66].

Rapid warming rates have been shown to be very important for rewarming vitrified animal and human embryos (60, 61). The risk of osmotic injury continues progressively even after the last ice crystal has melted, and during the essential CPA dilution phase, because there will initially be high CPA concentrations in the intracellular environs. Return of the cells to normal culture conditions for assessment and selection can yield reverse osmosis and harmful cell swelling unless CPA dilution steps are carefully controlled. These are the reasons that slow, step-wise CPA dilution protocols have been developed over many years [11, 68]. Also, inclusion of osmotic buffers such as sucrose during the dilution phase have been found to be helpful in mitigating injury [69].

Currently fundamental knowledge on the cryobiology of warming and CPA dilution is incomplete, and protocols have been developed largely on an empirical basis. As a technical note, terms such as ‘fast’ or ‘slow’ warming are relative and should be defined and measured in any clinical protocol to ensure robust repeatability. For example, different holding devices (e.g. vials, straws, loops) have different thermal characteristics which will impact on cell warming rates, irrespective of the temperatures of the warming baths used.

5. Summary
The technologies and understanding of cell and tissue cryopreservation have developed rapidly over the past 40 years. There is widespread application of cryobiology in many areas including reproductive medicine, haematopoietic stem cell treatments in cancer, targeted immune cell therapies such as CAR T – cells and animal and plant gene banks [70–73]. Combining the evidence for the various events during cryopreservation, for example during CRSC, allows a schematic of the overall
process to be proposed (see figure 2) However our understanding of cell survival during cryogenic preservation remains incomplete, with many outcomes being acceptable rather than optimal. Further research is needed to improve post-thaw recoveries and understand molecular impacts of cryopreservation on short and long-term outcomes for the recovered cells.

Figure 2. A schematic for a CRSC protocol. A cell is shown moving through the process. In region I (top left) the cell is exposed to the CPA and cooled to a hypothermic temperature (+10°C) for a period of time to allow CPA to permeate into the cell. In region II, cooling is then continued to the predicted temperature for the first ice nucleation to occur (often around -5°C). Steps may be taken to encourage ice nucleation by ‘ice seeding’. Ice nucleation can be detected both visually or by release of latent heat. Time is allowed (often about 10 minutes) to complete latent heat release before continuing slow cooling (in this case -1°C/min). Progressive cell cryo-dehydration occurs as the cell is cooled down to a deep low temperature (-80°C), close to the glass transition range of the mixture. After that, in region III, the cell can be safely cooled more rapidly down to the storage temperature (-196°C). In region IV, rewarming phase, the cell is warmed rapidly and osmotic balance is restored as free water is liberated from the ice fronts. In region V, the cell is carefully diluted out from the CPA solution and returned to normal environment. Reproduced from [43] with permission.

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