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Advanced technologies for the preservation of mammalian biospecimens

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Abstract

The three classical core technologies for the preservation of live mammalian biospecimens — slow-freezing, vitrification, and hypothermic storage — limit the biospecimens' biomedical applications. In this Review, we summarize the principles and procedures of these three technologies, highlight how their limitations are being addressed via the combination of microfabrication and nanofabrication, materials science and thermal-fluid engineering, and discuss the remaining challenges.

Mammalian biospecimens — cells, tissues and organs — are widely used in scientific research and in clinical applications[1]. They are procured either from animal or human donors via direct isolation, or via in vitro fabrication and modification, and typically used at later time points and at different geographical locations[2,3]. By maintaining and extending the life and functions of biospecimens outside their native environment and conditions, biopreservation bridges the spaciotemporal gap between the sources and acquisition times of biospecimens and their destinations and times of use, enabling their widespread distribution, transportation and application[4] (Fig. 1). For instance, the preservation of reproductive

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Competing interests

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cells and tissues (such as oocytes, spermatozoa, ovaries and embryos) is essential for assisted-reproductive technology, whose global market value is forecasted to reach over US $45.4 billion by 2025 (ref.[5]). Moreover, the preservation of tissues and organs for transplantation and replacement saves or improves millions of lives every year[6]. In 2019, there were 11,3000 patients on the waiting list for lifesaving organ transplants in the United States alone[7], and the true need for transplantation is estimated to be more than ten times that number[8]. Biopreservation also aids the provision of reliable, accessible and abundant mammalian-cell sources, especially stem cells, blood cells and genetically engineered cells, which are indispensable for cell-based medicine[9,10].

Biopreservation involves keeping biospecimens at low temperatures, so as to reduce or suspend their physiochemical and metabolic activities, and then recovering them back to physiological states at a desired future time. For long-term preservations (more than one week for suspended cells), the classical technology is cryopreservation — that is, the storage of biospecimens at cryogenic temperatures (below −60 °C) to completely arrest their bioactivities. To attenuate adverse effects during cryopreservation (such as ice formation), one or more cryoprotective agents (CPAs; such as dimethyl sulfoxide) are added to the biospecimens. There are two common methods of cryopreservation: slow freezing, and vitrification. Slow freezing is commonly used for the preservation of mammalian cells and microtissues[11,12], either in an insulated container at –80 °C with cooling rates of about 1 °C min^{-1}, or in a programmable freezer with controllable cooling rates, followed by storage in a −80 °C freezer or in a −196 °C liquid-nitrogen tank[13]. Vitrification directly transforms biospecimens from a liquid state into a glassy state through non-equilibrium cooling to minimize or eliminate ice formation. Hence, vitrification is generally considered to be superior to slow freezing for banking stress-sensitive biospecimens, such as oocytes, stem cells and some tissues[14–16]. Vitrification employs either a high concentration (6–8 M) of CPA (conventional vitrification)[17], or an increasing cooling rate with a reduced CPA concentration (low-CPA vitrification)[18].

For short-term preservations (typically less than 12 hours, in particular for human livers), hypothermia or static cold storage are typically used. These methods involve the storage of biospecimens at subnormothermic temperatures (usually 0–4 °C) to reduce their metabolic and degradation rates and to avoid ice formation. Also, because hypothermic storage doesn’t trigger substantial thermal stresses, nor needs cytotoxic CPAs, it is preferably used to preserve large-volume tissues and organs with complex and delicate structures (such as microcapillaries) that are highly susceptible to such ‘cryoinjuries’[6]. Hypothermia has been widely adopted for the preservation of solid tissues and organs for transplantation (in particular, corneas[19], livers[20], kidneys[21] and pancreases[22]).

In this Review, we examine the processes of these classical biopreservation methods, the associated mechanisms of cryoinjury, and limitations in the applicability of the methods, and highlight recent advances for overcoming these limitations. In particular, we focus on recent progress in preservation strategies for the reduction of cellular injuries and for improving the outcomes of biopreservation. The optimization of the compositions and concentrations of CPAs and of solutions for biopreservation have been summarized eleswhere[23,24].

Preservation processes

Depending on the temperature and concentration of solutes (including CPAs), biospecimens can be liquid or vitrified, which are stable states, or supercooled or supersaturated, which are thermodynamically unstable phases (Fig. 2a). For preservation, biospecimens are typically kept in one of the stable regions: either the vitrified state for long-term cryopreservation, or the liquid state for short-term hypothermic storage. The thermodynamic paths of biopreservation processes are depicted in Figs. 2b,c. During slow freezing, biospecimens follow the thermodynamic path A→C→E→F→G→I→L→Z. Ice crystals initiate at E and then propagate and grow (E→F→G), a process known as 'freeze concentration', as it elevates extracellular osmolality (or solute concentration), driving cell dehydration and deformation. Conventional vitrification follows the path A→D→II→M→Z; high-concentration CPAs are loaded at non-freezing temperatures, and a rapid and non-equilibrium cooling process leads to vitrification. The higher the CPA concentration, the lower the cooling rate required to inhibit ice formation. Therefore, low-CPA vitrification, which should follow the path A→C→III→N→Z, uses such a high cooling rate that the required CPA concentration is comparable to that of slow freezing[18,25]. Hypothermic storage typically follows the path A→B→IV→O→Y, with a storage temperature usually above the equilibrium freezing line. Supercooling storage follows the path A→B→V→P→X, with a storage temperature below the equilibrium freezing point, so as to extend preservation time. Notwithstanding their simplified diagrams of phase transition, thermal and osmotic time courses provide a comprehensive view of the procedures of biopreservation, and illustrate their risks and limitations.

Current preservation technology

All the classical preservation approaches have intrinsic limitations. During slow freezing, the biospecimens are subject to ice formation, freeze concentration and morphological deformation (E→F→G in Fig. 2). Extracellular ice crystals can damage the plasma membrane as a result of 'crushing', and any appreciable formation of intracellular ice is almost always fatal to the cells, as it disrupts subcellular organelles and their cellular membrane as well as the cytoskeleton. Freeze concentration causes significant osmotic stress, leading to cell dehydration and deformation under a high concentration of solutes (point G in Fig. 2a,c). A classical two-factor theory of such cryoinjuries that considers solute effects under slow cooling and intracellular ice formation under fast cooling[26] suggests an optimal cooling rate for each specific type of cells that minimizes both types of injury, and indicates a reversed U-shape relationship curve between cell survival and cooling rate[27]. The optimal cooling rate for cells depends on the cells' characteristics, especially cell size and membrane-transport properties[28–31].

Conventional vitrification uses a high concentration of CPA so as to increase the biospecimen's glass-transition temperature and to decrease its diffusion coefficient of water molecules (and thus increasing the biospecimen's viscosity). However, because highly concentrated CPA at high temperatures (path A→D in Fig. 2) can be highly toxic to mammalian cells, the exposure time is often minimized and the temperature decreased[32]. Therefore, it is almost mandatory to use a multistep protocol to load CPAs before

preservation (path A→D in Fig. 2c) and to unload them after preservation (path M→Z in Fig. 2c). This is a time-consuming, tedious and stressful process[33,34]. However, low-CPA vitrification relies on such a high cooling rate that water molecules do not have time to relocate, realign and reorganize to form ice crystals before they are immobilized in a glassy state (which is akin to the liquid state in microstructure). Moreover, to prevent devitrification and ice recrystallization, the required heat-transfer rate is higher during warming than during cooling[35,36]. This is probably due to the fact that the warming rate would be slowed down, owing to the absorbance of latent heat from −15 °C to 0 °C (path N→Z in Fig. 2b), and to the presence of myriad tiny ice embryos and interfaces in vitrified biospecimens, which can drive the nucleation of large ice crystals[37]. Hence, high surface-to-volume ratios and small sample volumes are required to obtain high cooling rates and warming rates (as indicated by the high slopes for the paths C→ III and N→Z in Fig. 2b). This is achieved through conventional heat transfer via conduction and convection in liquid nitrogen or in a water bath[25].

During conventional hypothermic storage (path A→B→IV→O→Y in Fig. 2) above the equilibrium freezing temperatures, biospecimens undergo substantial physiochemical and metabolic activities under suboptimal conditions, consuming nutrients and oxygen, depleting energy, and producing noxious metabolites (such as tumour necrosis factor alpha and nitric oxide)[38–41]. In addition, the cut-off from circulation systems and the lack of nutrient and metabolite transport aggravate ischaemic injuries[42]. Furthermore, when hypothermic tissues or organs are rewarmed to physiological temperatures on reperfusion, the suddenly available oxygen produces overwhelming oxygen-free radicals beyond cellular-scavenge capacity, inducing pro-apoptotic signal transduction and concomitant damages (ischaemia-reperfusion injury)[43]. Therefore, biospecimens in hypothermic storage usually last for short periods (path IV→O in Fig. 2b), from several hours (4–6 hours for hearts and lungs) to few days (1 day for human kidneys and 3 days for rat kidneys)[44–46]. Hence, the shortcomings of the classical approaches of biopreservation demand innovation and optimization.

Advances in slow freezing

Slow freezing involves cell-specific cooling rates so as to avoid excessive solute effects and intracellular ice formation (Fig. 3a). Such cryoinjuries depend on the quality and quantity of ice crystals, and on the cells' characteristics. For many cell types and for tissues with more than one cell type, optimal cooling rates remain elusive. A number of methods and devices are being used to investigate and address these issues.

Dynamic measurement of membrane transport.

Understanding the osmotic behaviour of cells and quantifying the transport properties of water and CPAs across cell membranes (in particular the osmotically inactive volume, the cell-membrane permeability coefficient of water, and the associated activation energy) are prerequisites for the development of optimal slow-freezing procedures. Methods based on Coulter Counters have been used to determine cell volumes and osmotic responses by measuring the impedance of cell suspensions, but these methods cannot be used to study the osmotic responses of specific cells, and are influenced by the presence of debris and

agglomerates[47–50]. However, polydimethylsiloxane or glass microfluidic devices bearing microchannels and perfusion chambers for trapping the cells (Fig. 3b) can measure these properties at single-cell-resolution. These devices also enable the real-time monitoring of cell volumes on changes in the extracellular solutions for a range of cell types (as has been demonstrated for rat basophilic leukaemia cells[51], human umbilical vein endothelial cells[52] and mouse dendritic cells[53]). Also, because these methods use monolayers of immobilized individual cells, they avoid the measurement ambiguities associated with cells cultured on conventional Petri dishes, as they often overlap and move out of the imaging focus plane. And with the assistance of cryostages[54] or thermocouples[55], these systems can quantify the transport properties of cell membranes at supra-zero and sub-zero temperatures. This enabled the discovery of lipid phase transitions occurring in the cell membrane at temperatures of 0–12 °C, as indicated by a discontinuity in the activation energy[54]. Furthermore, accompanied by hydrodynamic switching, on-chip perfusion can be used to change the extracellular solution at a controllable rate[56]. Therefore, these microfluidic devices allow for the optimization of the choice of CPAs, their concentrations and the cooling rate, to reduce osmotic stresses, control water and CPA transport, and minimize intracellular ice formation[52] (that is, they modify the path E→F→G in Fig. 2).

On-chip cryopreservation.

Microfluidic chips can also be used for improved on-chip cryopreservation efficiency, accuracy and safety. For instance, microfluidic channels have been used to freeze, thaw, culture and analyse suspensions of cells in a streamlined manner to save time, labour and costs[57,58], and glass slides with etched two-dimensional arrays of picometric wells have been designed to hold individual cells in each well (Fig. 3c). In such ‘cryo-chips’, the cells retain their positions during the freeze–thaw cycle, allowing for comparisons of individual cells before and after cryopreservation. Cryo-chips enable the investigation of correlations between cell characteristics and cryopreservation outcomes, and hence provide insights than can’t be obtained via conventional bulk cryopreservation (because of the inherent heterogeneity of cell populations)[59]. Cryo-chips also allow for multiparametric analyses of the cryopreserved cells (such as apoptotic rate, mitochondrial membrane potential, and intracellular metabolism[60]). For example, single-cell arrays have revealed that primary hepatocytes with a high mitochondrial membrane potential before freezing are significantly less likely to survive the freeze–thaw cycle[61]. Also, on-chip cryopreservation provides precise control of the cooling and warming procedures. For instance, microchannels allowed for the cryopreservation of human spermatozoa without the need for CPAs, because of the microchannels’ high heat-transfer rate[62]. And the insertion of microheaters into microfluidic devices, to temporarily hold them at −25 °C for 3 mins before plunging them into liquid nitrogen, improved yeast-cell viability after cryopreservation[63]. Moreover, fluid flow over a monolayer of adherent cells on microfluidic devices can induce shear stress on the cells, increasing the number of focal-point adhesions between cells and the surface, enhancing cellular survival during cryopreservation[64]. Therefore, microfluidic devices can offer higher levels of manoeuvrability for sample preparation and analysis, of controllability of the cooling and warming procedures, and of the optimization of the cryopreservation conditions.

Magnetic cryopreservation.

The formation of intracellular ice, especially of large intracellular crystals, is almost always fatal to mammalian cells[65,66]. The application of a magnetic field to biospecimens can alter the kinetics of ice nucleation and propagation to improve slow freezing. For instance, by imposing an external magnetic field, human periodontal ligament cells and pulp tissues were preserved via slow freezing with a significantly reduced CPA concentration, and then transplanted into patients to heal normal periodontal ligaments[67–69]. These studies suggest that biospecimens can be preserved by maintaining them under a magnetic field of 0.01 mT and at a temperature of −5 °C for 15 min, and then by slowly cooling the biospecimens to approximately −30 °C before plunging them into liquid nitrogen (Fig. 3d). It has been shown that cells or tissues exposed to a magnetic field have, after cryopreservation, improved viability, adhesion and proliferation, osteogenic and adipogenic differentiation, and histological integrity[67,68,70,71]. These findings imply that slow freezing with a magnetic field is a feasible strategy for the banking and transplantation of teeth. Although the precise working mechanisms remain to be fully understood[72], it appears that magnetic fields can act directly on water molecules by re-orientating, vibrating or spinning them, preventing them from clustering and nucleating and facilitating supercooling or intracellular vitrification (which would correspond to a decrease in the temperature of point E in Fig. 2a). Hence, subjecting biospecimens to a magnetic field of adequate strength during slow freezing can reduce the usage of CPAs and enhance cryopreservation outcomes, especially for dental stem cells and dental tissues[71].

Advances in vitrification

In principle, vitrification avoids all cryoinjuries associated with ice formation during slow freezing. However, vitrification requires a high concentration of cytotoxic CPAs to prevent ice formation. Traditionally, CPAs are loaded and unloaded via tedious and time-consuming multistep procedures. In addition, the use of CPAs restricts sample sizes to be in the order of 100 μl so as to obtain sufficiently high cooling and warming rates (Fig. 4a). These shortcomings have been alleviated by innovations in biomedical devices, biomaterials and thermal-fluid engineering.

Automated CPA loading and unloading.

Various types of microfluidic device have been developed to facilitate and improve the outcomes of multistep CPA loading and unloading processes during vitrification. The devices can automatically add and remove CPAs (that is, they can modify the paths A→D and M→Z in Fig. 2c) with smoother osmotic-change profiles than conventional step-wise methods[73,74]. By capitalizing on laminar flow and on the steady molecular diffusion occurring in the microchannels, these microfluidic devices can reduce CPA loading time and cell-exposure time to cytotoxic CPAs at non-freezing temperatures, and decrease both the rate of cell-volume change and dehydration overshooting to reduce osmotic stress[74] (Fig. 4b). As a result, cell viability and cell functionalities after cryopreservation have been improved, and the manoeuvrability, reliability and fidelity of the experiments have been enhanced[75]. Specifically, a microfluidic device has been designed to steadily and slowly load CPA into bovine and murine oocytes and zygotes, and can decrease cell-shrinkage rate,

osmotic stress and sublethal damage to the cells[76]. Similar microfluidic strategies have been used in the context of assisted reproductive technologies[77]. Overall, the use of microfluidic technology for automated CPA loading and unloading during vitrification effectively eases the tedious and stressful procedures of CPA loading and unloading, reduces osmotic stresses and the rates of cell-volume changes, and enhances the cryopreservation outcomes[78].

Hydrogel microencapsulation.

Besides the use of high CPA concentration and small sample volumes, vitrification can be carried out by changing the biophysical and biochemical properties of the biospecimens to be preserved (that is, by modifying the general phase diagram depicted in Fig. 2a for a particular biospecimen). For instance, water confined in a calcium alginate hydrogel matrix can vitrify more readily than free bulk water[79]. In fact, ice formation owing to devitrification and ice recrystallization during warming are major obstacles to preservation by vitrification[36] (Fig. 4a). Calcium-alginate hydrogels possess a remarkable capacity to inhibit devitrification and recrystallization, and therefore can be used to create localized nanolitre volumes of ice-free microenvironments[80] (Fig. 4c). Embryonic stem cells and mesenchymal stem cells encapsulated in microcapsules of calcium alginate hydrogel via droplet-based microfluidics for cryopreservation via ‘local’ vitrification reduced the needed CPA concentration 4 times and increased the maximum sample volumes 100 times[80]. This method has also been used to preserve ‘ready-to-use’ cell–biomaterial constructs[81] and individual pancreatic islets[82]. Also, the alginate hydrogels enhance the metabolic activity and proliferative capacity of the cells after cryopreservation[83]. Supramolecular gels have also been used to minimize cryoinjuries during vitrification[84]. Besides protecting encapsulated cells by confining the nucleation and growth of ice crystals and decreasing osmotic stresses during cryopreservation, hydrogels can also have excellent biocompatibility (particularly, natural polymer hydrogels), controllability (synthetic polymer hydrogels), and versatility (supramolecular hydrogels), and hence their wider use in cell and tissue engineering, transfusion and transplantation applications[85].

Droplet vitrification.

Small sample volumes and low throughputs limit the use of low-CPA vitrification[86]. For low CPA concentrations, vitrification can be achieved at high cooling rates and high throughputs by continuously ‘shooting’ cell-laden microdroplets directly into liquid nitrogen via droplet generators (Fig. 4d). This method has been used to preserve hepatocytes, fibroblasts, cardiomyocytes, embryonic stem cells and mouse embryos[87–89]. A similar strategy preserved mouse oocytes in microdroplets using low CPA concentrations[90], as well as red blood cells[91]. In droplet-mediated vitrification, the optimal droplet size has been determined via theoretical and numerical analyses of heat transfer and ice formation, which take into account the effects of a gas bubble that forms around the microdroplet on contact with liquid nitrogen (because of the Leidenfrost effect)[92]. Vitrification of macroscopic cell-laden droplets (3–5 mm in diameter) can also preserve cells, as shown for primary hepatocytes at cooling rates of the order of 10^3 °C min^{-1} (ref.[93]). However, this droplet-vitrification method exposes the cells directly to liquid nitrogen, which can cause pathogenic contamination and cell loss[94–96]. However, cell-laden droplets and liquid nitrogen can be

segregated via cell printing on a silver film[97]. Incidentally, droplet vitrification is widely used to preserve plant shoot tips and germplasms[98–100].

Nanoscale warming.

To effectively suppress devitrification and ice recrystallization, the critical warming rate must exceed the critical cooling rate by at least one order of magnitude[35,36]. However, warming rates achievable by convective heating in a 37 °C water bath are only 10–100 °C min^{-1}, even for small samples (about 1 ml). Laser beams and a laser absorber material such as carbon black have been used to increase the warming rate[101], which allowed the vitrification of mouse oocytes in a CPA solution diluted 3-fold with nearly 100% survival of the cells[101]. Warming rates of 1.4×10^7 °C min^{-1} have been achieved for zebrafish embryos at a low concentration of CPA (2 M of propylene glycol) by microinjecting gold nanorods coated with carbon black and exciting them with pulsed laser beams[102] (Fig. 4e). Moreover, the uniformity of the warming, which reduces the formation of thermal stresses and mechanical cracks, can be improved via the application of electromagnetic fields[103].

The warming rates of large-volume (1–80 ml) biospecimens (in particular, human dermal fibroblast cells, porcine arteries, and porcine aortic heart-valve-leaflet tissues) preserved in standard vitrification solution (VS55) were elevated to 130 °C min^{-1} or higher by coupling magnetic fields and radiofrequency-excited iron oxide nanoparticles coated with mesoporous silica[104]. Such nanoscale-heating approach improved the viability of the cells and tissues as well as their biomechanical properties[104]. The vitrification of mesenchymal stem cells from human umbilical cord has also been enhanced by a similar strategy combining magnetic-induction heating and superparamagnetic Fe_3O_4 nanoparticles[105]. The combination of nanoparticles and electromagnetic heating can thus significantly increase the warming rates of cryopreserved biospecimens (the slope of the path M→Z in Fig. 2b), inhibiting the formation of ice owing to devitrification and recrystallization, and hence decreasing the required concentrations of CPAs.

Advances in hypothermic storage

Slow freezing and vitrification have been used to preserve cells and simple or small tissues[106,107]. Yet most large or complex tissues and organs cannot tolerate the extensive ice formation and deformation triggered by slow freezing, nor the highly concentrated CPA and osmotic stresses of vitrification. Therefore, large tissues are mostly preserved in liquid states, especially hypothermia (Fig. 5a). However, biospecimens in hypothermia can degrade rapidly and undergo ischaemic injuries (such as ischaemia-reperfusion injury). A few strategies can be used to alleviate or avoid these issues.

Temperature-controlled machine perfusion.

Ischaemic injuries can be alleviated by supplying nutrients and removing wastes from the preserved tissue via machine perfusion, with different tissues and organs requiring different perfusates and perfusing temperatures (Fig. 5b). Differently from conventional static cold storage, normothermic machine perfusion (at 37 °C) can reinstitute physiological-like conditions for tissues and organs and circumvent cold injuries, ischaemic injuries and

reperfusion injuries, and it allows for the close monitoring of viability and functions[108]. Therefore, it has been widely used to preserve livers[109], hearts[110] and lungs[111] for transplantation. Normothermic machine perfusion is also effective to assess tissue and organ conditions before transplantation[112,113], and can even be used to recondition or restore questionable or high-risk organs[114]. It maintains preserved organs in a physiological state, avoiding cooling and recovering procedures and therefore significantly decreasing the possibility of graft injury and extending the preservation time of human livers[115]. Oxygenated sanguineous normothermic machine perfusion has also been used to recover the metabolism and functions of livers after extended warm ischaemia and simple static cold storage[116,117]. And in combination with the management of glucose, oxygen and waste products, it has been used to preserve the viability and functions of human livers that had been rejected for clinical transplantation for 7 days[118], raising the possibility of restoring marginal organs to expand the organ-donor pool.

Alternatively, subnormothermic machine perfusion (at 20–30 °C) has been used to repair damages induced by warm ischaemia in rat liver for orthotopic transplantations[119,120], and to maintain human liver function post-ischemia with minimal injury[121,122]. And hypothermic machine perfusion (at 4–8 °C) can significantly decrease metabolic rates for extended storage[123]. It has been used to improve transplantation outcomes for human livers[124], kidneys[125,126], and even ‘orphan’ organs from extended-criteria donors[20]. Moreover, perfusion via controlled oxygenated rewarming (at 8–20 °C) — that is, the slow rising of temperature and oxygen concentration (which would alter the path O→Y in Fig. 2b,c)[127,128] — can gently restore cellular conditions and functions to minimize ischaemia-reperfusion injury, which is mainly caused by abrupt changes in oxygen concentration and temperature[127,129]. Machine perfusion can also be used to gradually load CPAs (path A→B in Fig. 2c) to solid organs before static cold storage and to gradually unload the CPAs after it[123,130] (path O→Y in Fig. 2c).

Supercooling and deep supercooling.

The rates of biophysical and biochemical activities decrease as the storage temperature decreases[131] (as per the Arrhenius equation). In conventional hypothermia at 0–4 °C, the metabolic rates of biospecimens are decreased by 90% or more (with respect to the physiological rates at a temperature of 37 °C; refs.[132–134]). Therefore, hypothermic storage is effective for the short-term preservation of mammalian cell suspensions[135,136], two-dimensional and three-dimensional cell aggregates[137], and cell–biomaterial constructs[138,139]. It is in fact the gold-standard method for the preservation of large tissues and organs for clinical transfusion and transplantation. For example, donated human blood is routinely stored at 4 °C in a blood bank for a maximum of 42 days before transfusion or disposal. Excised livers, pancreases and kidneys are usually flushed with a hypothermic storage solution and stored at hypothermic temperatures (0–4 °C) for preservation and shipping before transplantation[140,141]. Because the limit of hypothermia is largely determined by tissue degradation, decreasing storage temperatures and thus degradation rates extend the duration of preservation via hypothermia. However, decreases in storage temperature that push the sample to the unstable supercooled phase (point H in Fig. 2) risks the viability of the biospecimens, owing to spontaneous ice formation. By

staying at high sub-zero temperatures (higher than −6 °C), the risk of freezing is low and supercooling can preserve cells and organs for as long as 4 days[39,123,142,143] (path V→P in Fig. 2b,c). The lower the temperature, the higher the probability of ice formation[39] and thus of biospecimens in the supercooled state to be affected by stochastic freezing events. However, theoretical, numerical and experimental studies have identified that the primary freezing mechanism for supercooled water and aqueous solutions involves heterogeneous ice nucleation at the water surface[144–147]. Therefore, sealing the water surface with an immiscible oil phase can 'remove' these primary ice-nucleation sites, and stabilize the supercooled state (Fig. 5c). For pure water, such 'deep supercooling' state can be maintained at temperatures as low as −20°C for at least 100 days[144]. Deep supercooling has been used to preserve human red blood cells at −13 °C and −16 °C for 100 days[144], as well as adipose-derived stem cells for 7 days at −16 °C, with high cell viability and functionalities maintained[148]. Deep supercooling of tissues and organs could significantly extend their hypothermic preservation and maintain their quality after storage, for later use in clinical assessment, allocation and transplantation.

Outlook

For small biospecimens, current preservation methods usually handle a million or more cells. Yet the preservation outcomes of individual cells can vary significantly, even for the same protocols, owing to intrinsic heterogeneities in the characteristics of the cells, in the properties of their microenvironment, and in physiochemical conditions[149,150]. For 'precision preservation', it is therefore necessary to scale down methods for biospecimen preservation to the level of single cells. This is particularly important for small quantities of precious biospecimens, such as oocytes, zygotes and embryos. Currently, the actual success rates of cryopreservation for these reproductive cells and tissues are dismal (in particular, the birth rate of cryopreserved human oocytes is less than 5%; refs.[151,152]). Precision preservation would allow the manipulation and monitoring of the cells at single-cell resolution, with enhanced handling accuracy and preservation efficiency.

A large quantity of cell suspensions, cell–biomaterial constructs, tissues and organs are needed for clinical transfusions and transplantations (for example, billions of chimaeric antigen-receptor T cells are required in immunotherapy for the treatment of acute leukaemia[153]). Therefore, it is imperative to scale-up preservation methods for large-volume biospecimens. For instance, the vitrification of tissues and organs for long-term preservation can benefit from the optimization of CPA mixtures and the incorporation of nanoscale heating[154]. And supercooling and deep supercooling can be scaled up to preserve a large quantity of cells suspensions, tissues and organs for extended periods by modulating ice-nucleation mechanisms. Because supercooling approaches do not use cytotoxic CPA and do not trigger the formation of ice crystals, they do not need CPA removal and cell recovery, which is clinically advantageous.

In clinical contexts, the safety of preserved biospecimens has to be assured. Insufficient assessments of safety can lead to graft rejection, graft-versus-host diseases, and teratoma formation (for pluripotent stem cells) after transfusion and transplantation[155]. Freeze–thaw cycles in cryopreservation can cause massive losses in cell viability and in cellular functions,

owing to apoptosis and necrosis (which can be alleviated via inhibitors of caspases, proteases and kinases[156,157]). Moreover, cytotoxic CPAs such as dimethyl sulfoxide can sacrifice cell viabilities and alter the cell cycle and cell genetics (for instance, they can cause chromosomal instabilities and aberrations, eventually leading to cell-function alterations and tumorigenesis[158]). Therefore, the cytogenetic status of preserved biospecimens has to be determined through karyotyping before use in any clinical applications[159]. Furthermore, CPAs can cause DNA methylation and histone modifications, and thus induce epigenetic changes to cellular functions (such as uncontrollable cell proliferation, cell differentiation and tumour formation[160,161]). Because epigenetic changes cannot be detected via karyotyping, the tumourigenic potential of preserved biospecimens should be evaluated by tumourigenic assessments of DNA damage and via the expression levels of tumour suppressor genes and oncogenes[10]. Although non-toxic CPAs (sugars, proteins, peptides, amino acids, and their derivatives[162,163]) are available, these are often impermeable to the plasma membrane of mammalian cells, and hence their use has to rely on strategies, such as nanoparticle-mediated intracellular delivery, for CPA delivery into mammalian cells[164,165]. Furthermore, pathogenic contaminations (for example, via *Aspergillus sp.* and the Hepatitis B virus in liquid nitrogen[94–96]) from preservation materials and devices in close contact with the biospecimens should be avoided; in fact, biopreservation systems must be sterilized (by ultraviolet radiation or via filtration[166]) and operated in sterile hoods.

The development of preservation technologies for biospecimens should be driven by the intended applications, most commonly cell therapies and assisted reproduction[13,167] in the case of cells, and transplantation in the case of large tissues and organs. For instance, thorough monitoring of the conditions of preserved organs is crucial for the prevention of early graft failure after transplantation[45]. Also, because the condition and quality of organs donated after brain death differ from those donated after circulatory death (which experience longer warm ischaemia before preservation), preservation protocols should be tailored accordingly[115,122,168]. Preservation methods should indeed be adjusted for the specific condition, settings, and application purposes of the biospecimen. Also, the choice of animal models for research should be most representative of human physiology. Naturally, the use of pig livers rather than mouse livers in preservation studies bears higher relevance to the preservation of human livers[169]. However, the choice of a most appropriate animal model is not always obvious, and is organ-specific[170]. Furthermore, guidance on good manufacturing practices for the collection, processing, preservation, and assessment of each type of biospecimen is urgently needed to ensure the safety, feasibility, reliability and fidelity of the preservation procedures[171,172].

The development and implementation of biopreservation are closely related to socioeconomic factors. On the one hand, the costs associated with the clinical use of preserved biospecimens (for stem cell therapy, oocyte cryopreservation and organ transplantation, in particular) is often prohibitively high, especially in developing countries without robust healthcare systems[173]. The total discounted costs of liver transplantation per patient in the United States approached US $1.5 million in 2014, and are predicted to increase to over US $2 million in 2034 (ref.[174]); yet household median income is around 55,000 (figure for 2018; ref.[175]). Also, 25% of the organ transplantations are carried out in the United States, which has 4% of the world’s population; only 0.5% of the organ

transplants were carried out in Africa[6], which has 16% of the world's population. Hence, the wider accessibility and affordability of biopreservation methods for clinical applications remain a significant unmet need. On the other hand, regulatory and governance challenges do also bear significant influence on the clinical application of preserved biospecimens. For instance, oocyte cryopreservation is illegal for unmarried women in China[176], and few stem cell therapies have received market approvals[177,178]. Widespread clinical application of biospecimen preservation won't thus be possible unless technology development seriously considers affordability, accessibility and regulatory challenges.

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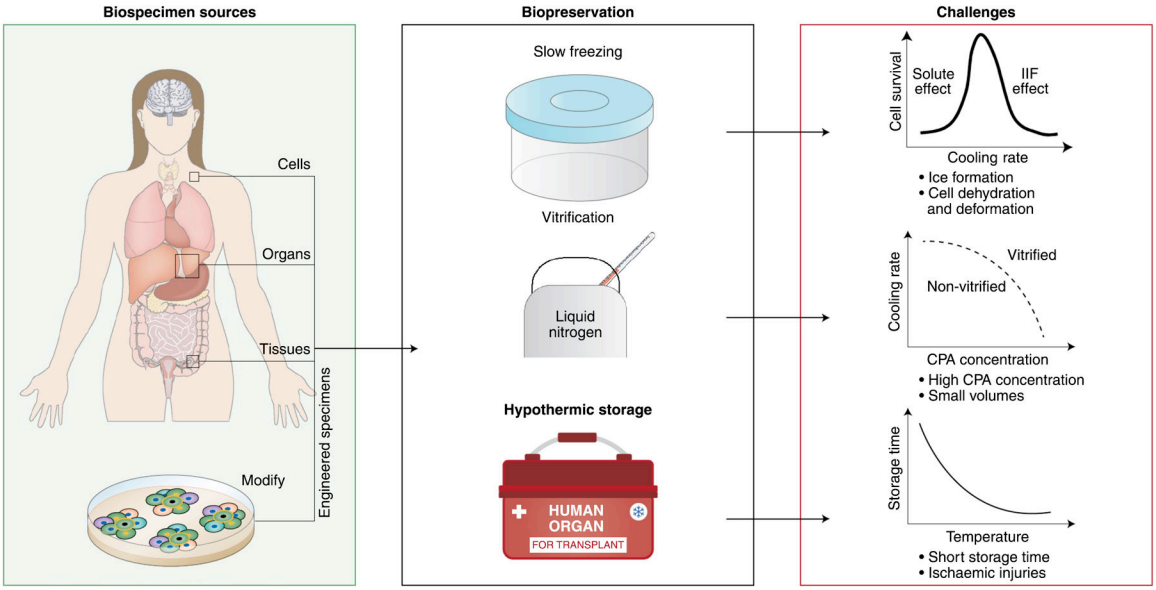

Fig. 1 |. Preservation of mammalian biospecimens.

The preservation of cells, tissues and organs from human donors, and of engineered specimens from them, involves slow freezing, vitrification or hypothermic storage (with machine perfusion). Preserved biospecimens enable a wide range of biomedical applications. CPA, cryoprotective agent.

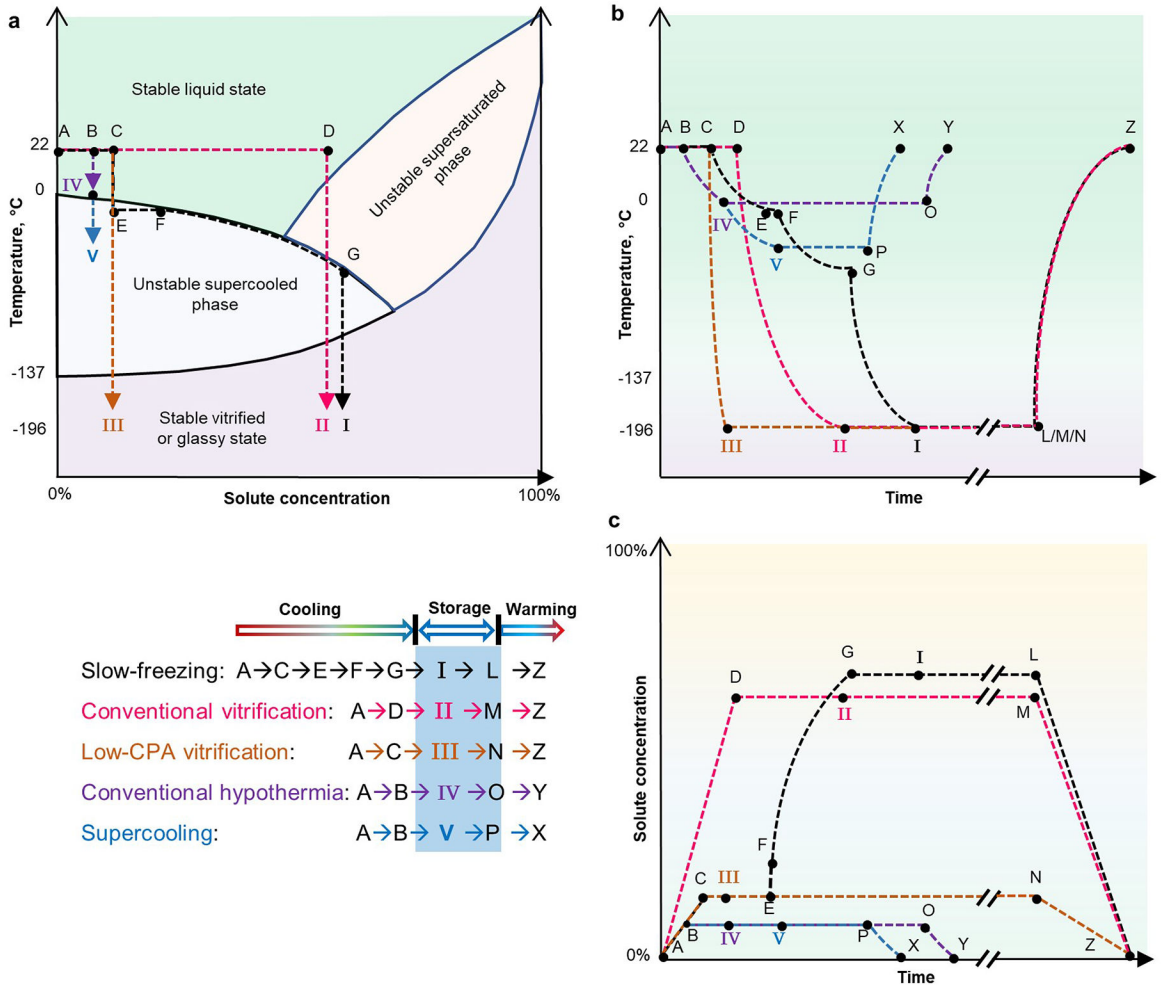

Fig. 2 |. Phase diagrams, and thermal and osmotic time courses, for five main biospecimen-preservation methods.

For each preservation method — I, slow freezing; II, conventional vitrification; III, low-CPA vitrification; IV, conventional hypothermia; and V, supercooling — the thermodynamic paths involved in the cooling, storage and warming steps are indicated. **a**, Diagram of the phases of matter of biospecimens processed with different preservation methods. Arrows indicate the direction of the preservation process. **b**, Thermal time course. **c**, Time course of the solute concentration (or osmolality). Axes not drawn to scale. The rates of temperature change and of osmolality change are indicated by the slopes of the curves. Long-term storage is indicated by '//'. For simplicity, panel **a** doesn't show the steps of storage and warming, and panels **a** and **b** do not show temperature fluctuations arising from the release (during cooling) and absorption (during heating) of latent heat associated with phase changes. It is assumed that there is no solute precipitation during freeze concentration (path E→F→G) and during plunging into liquid nitrogen (path G→I). Temperature profiles during cooling are approximated as convex curves (in paths C→I, D→II, C→III, B→IV, and B→V) because cooling rates decrease as the temperature of the biospecimen drops, and as concave curves (in paths L/M/N→Z, O→Y, and P→X) during warming because the warming rate decreases as the biospecimen temperature increases[179].

The solute-concentration paths in **c** are drawn as straight lines because CPA loading and unloading can be controlled arbitrarily before and after preservation.

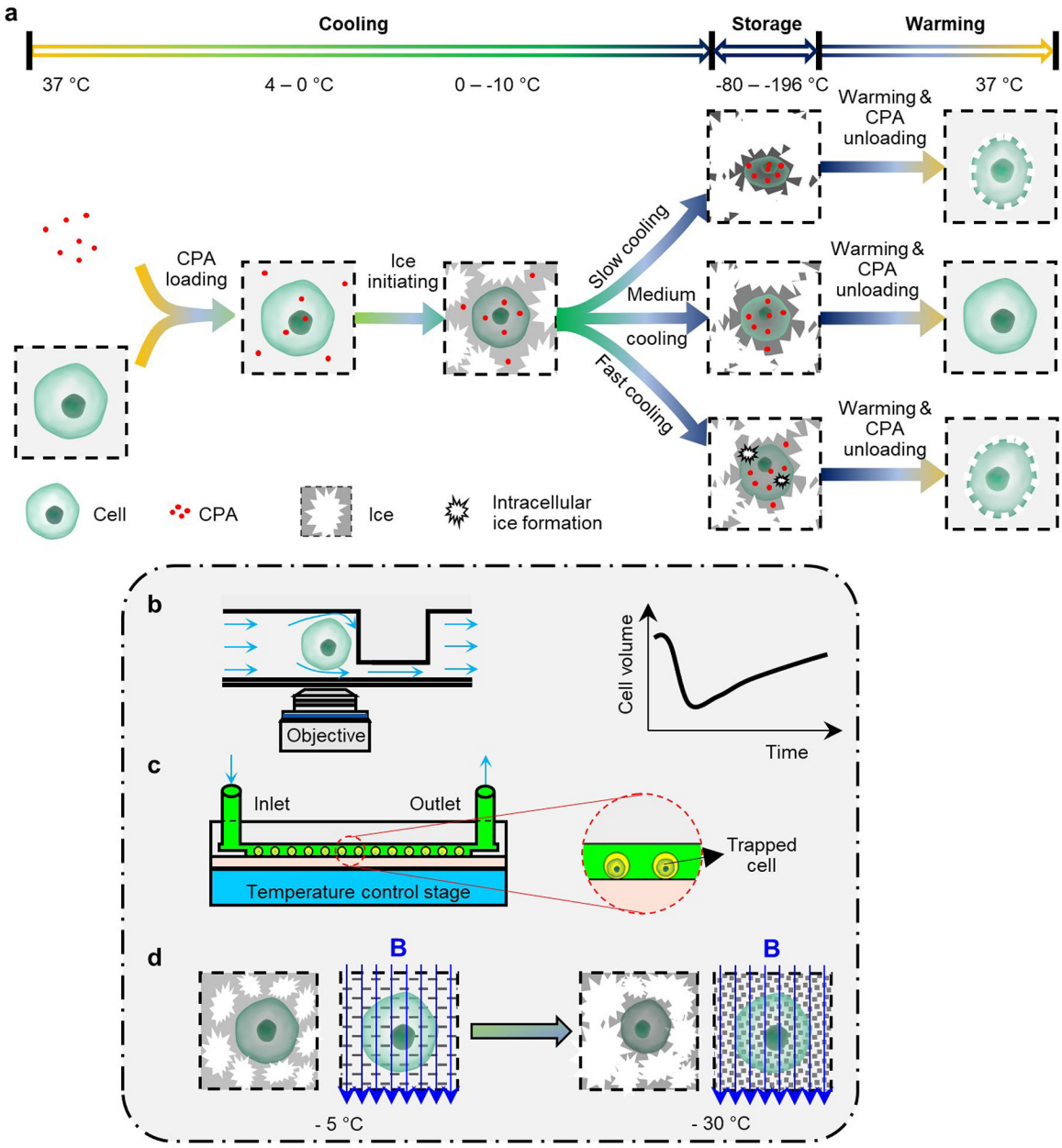

Fig. 3 |. Slow freezing.

a, Steps in slow freezing, and mechanisms of the cryoinjuries that it can cause. For specific types of cell, there are optimal cooling rates that minimize cell injury, owing to excessive cell dehydration and intracellular ice formation. **b**, Microfluidic perfusion for the investigation of the cell-transport properties of water and of the cryoprotectant agents (CPAs). **c**, On-chip cryopreservation allowing for the visualization and study of cell responses at the single-cell level. **d**, Magnetic cryopreservation for the modulation of the kinetics of ice formation. Magnetic fields can drive the formation of finer ice crystals and thus less cellular damage.

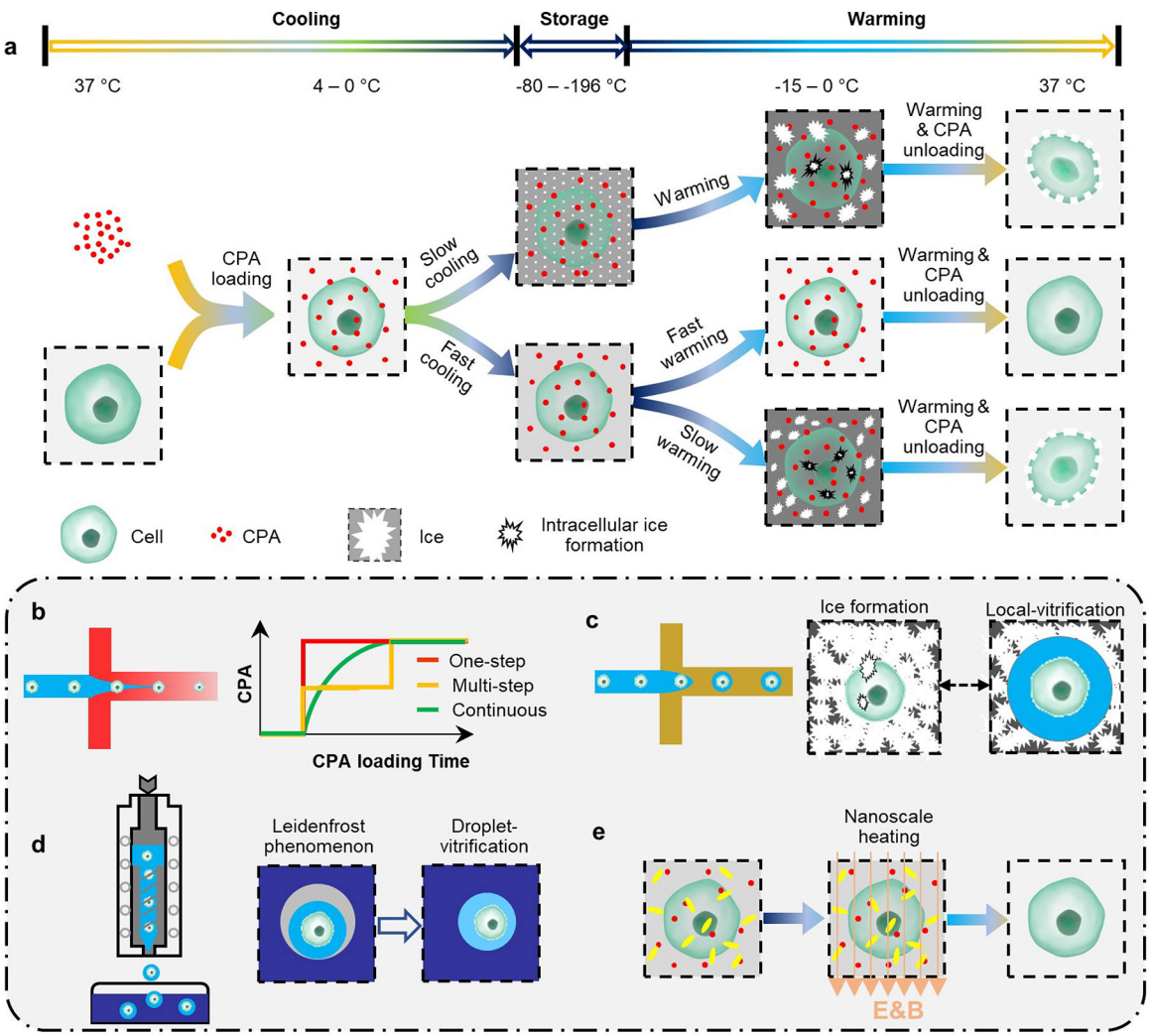

Fig. 4 |. Vitrification.

a, Steps in vitrification, and the mechanisms of the cryoinjuries that it can cause. **b**, Automated loading of cryoprotectant agents (CPAs) to minimize loading time, cell exposure to the agents, and osmotic stresses. **c**, Local vitrification via hydrogel microencapsulation. Hydrogel microcapsules inhibit devitrification and ice recrystallization during warming, and create an ice-free microenvironment for the encapsulated biospecimens. **d**, Droplet vitrification by 'shooting' cell-laden microdroplets into liquid nitrogen. During rapid cooling, a gas bubble (grey) can form around the microdroplets (Leidenfrost effect), causing reduced cooling rates. **e**, Nanoscale heating, which combines nanoparticles (yellow) and electromagnetic fields, can significantly increase warming rates, and thus alleviate cryoinjuries and the volume constraints of vitrification.

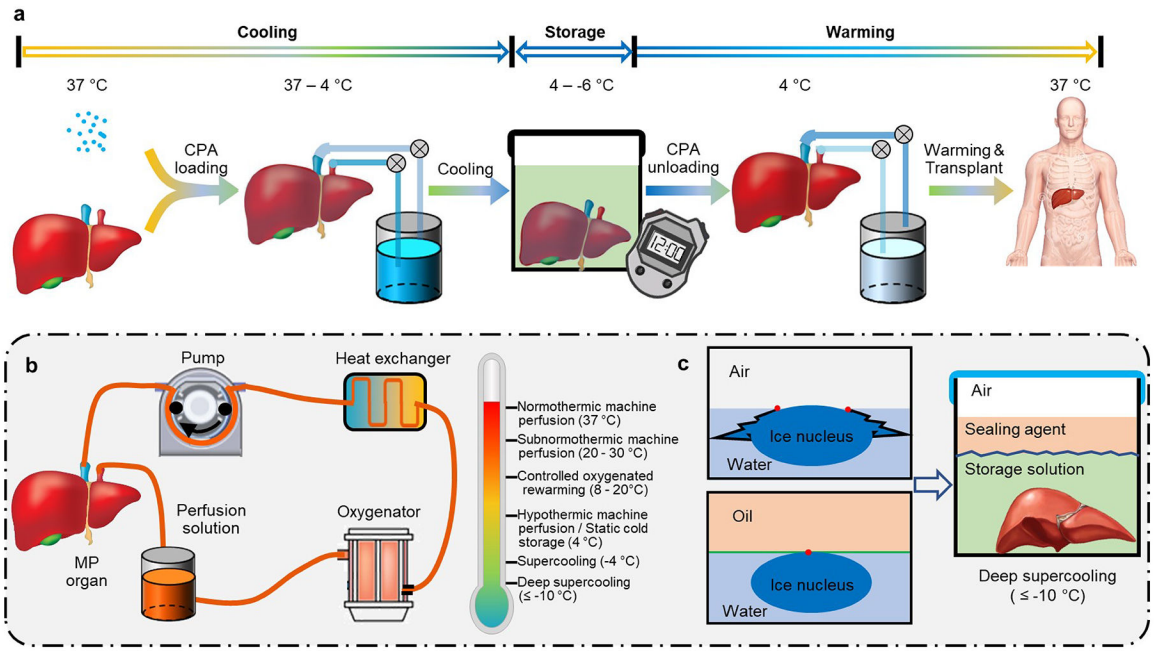

Fig. 5 |. Hypothermic storage.

a, Steps in the hypothermic storage of tissues and organs. Owing to their metabolic activities and to fast ‘decaying’ cellular processes, tissues and organs can only be stored for much shorter durations than slow-frozen or vitrified biospecimens. **b**, In hypothermic storage, oxygenated machine perfusion alleviates ischaemic injuries and reconditions tissues and organs. The temperature range depends on the characteristics and conditions of the tissues and organs to be preserved. **c**, Deep supercooling (at temperatures lower than −10 °C) extends the duration of hypothermic storage, because it eliminates primary ice-nucleation sites at the water–air interface by ‘sealing’ it with water-immiscible agents.