S. S. College, Jehanabad

Department: Zoology

Class: M.Sc. Semester II

Subject: Zoology

Topic: Cryopreservation of cells, tissues and organs - Part 2

Mode of teaching: Google classroom & WhatsApp

Date & Time: 26.08.2020 & 10:30

Teacher: Praveen Deepak

To join Department's group, students can use following link
https://chat.whatsapp.com/EHuHNfQzoAzJBMFNJvsjQx
or scan QR Code
WhatsApp No.: +91 75360 68068

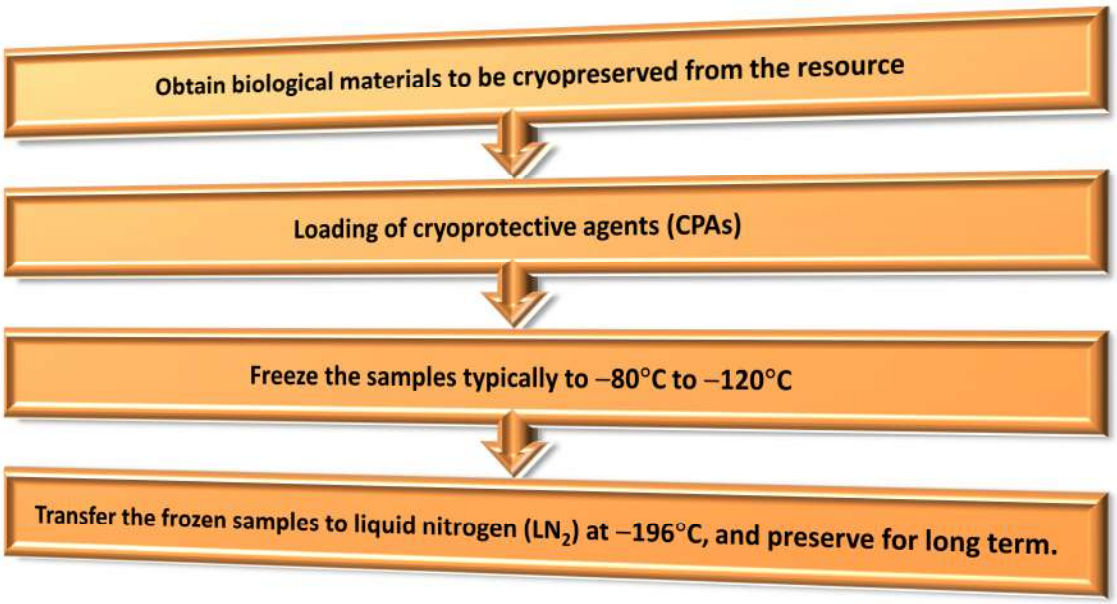
Methods of cryopreservation

As described above, cryopreservation of biological specimens can be accomplished by applying both; low freezing and vitrification methods. The major differences between the two are the concentrations of CPAs and the cooling rates used. Theoretically, if cooling is sufficiently slow, cells could efflux intracellular water rapidly enough to eliminate supercooling and thus prevent intracellular ice formation. As a result of differences in the capacity of different cells to move water across the plasma membrane, optimal cooling rates will be dependent on cell types. Slow freezing first substitutes the water within the cytoplasm with CPAs which reduces cell damage and adjusts the cooling rate in accordance with the permeability of the cell membrane. Slow-cooling protocols involve a typical cooling rate of about 1 °C/min in the presence of less than 1.0M of CPA, with use of a high-cost controlled-rate freezer or a benchtop portable freezing container. The advantages of slow freezing are that it has a low risk of contamination during the procedures and does not demand high manipulation skills. However, slow freezing has a high risk of freeze injury due to the formation of extracellular ice as is also described in first table above.

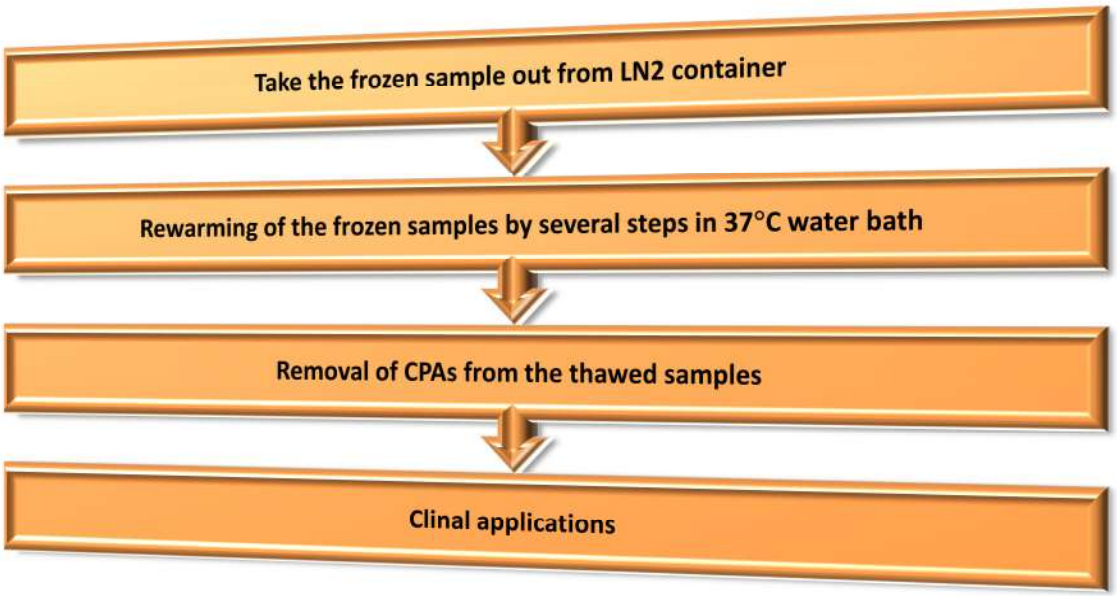
While as an alternative to the slow-freezing technique, vitrification is a process by which cell suspensions are transformed directly from the aqueous phase to a glass state after direct exposure to liquid nitrogen. The process requires cooling of the cells or tissues to deep cryogenic temperatures (i.e., with liquid nitrogen) after their exposure to high concentrations of CPA (in the ratio of 40–60%, weight/volume), with subsequent rapid cooling to avoid ice nucleation. Vitrification is largely dependent on three factors: (1) viscosity of the sample; (2) cooling and warming rates; and (3) sample volume. Thus, a delicate balance must be maintained among all the relevant factors to ensure successful vitrification. There are two methods of vitrification; equilibrium and nonequilibrium.

- **Equilibrium vitrification** requires formulation of multimolar CPA mixtures and their injection into the cell suspensions.
- **Nonequilibrium vitrification**, which is further, divided into carrier-based (including the former plastic straws, quartz microcapillaries, and cryoloops for obtaining a minimum drop volume and carrier-free systems, uses an extremely high freezing rate along with lower concentrations of the CPA mixture.

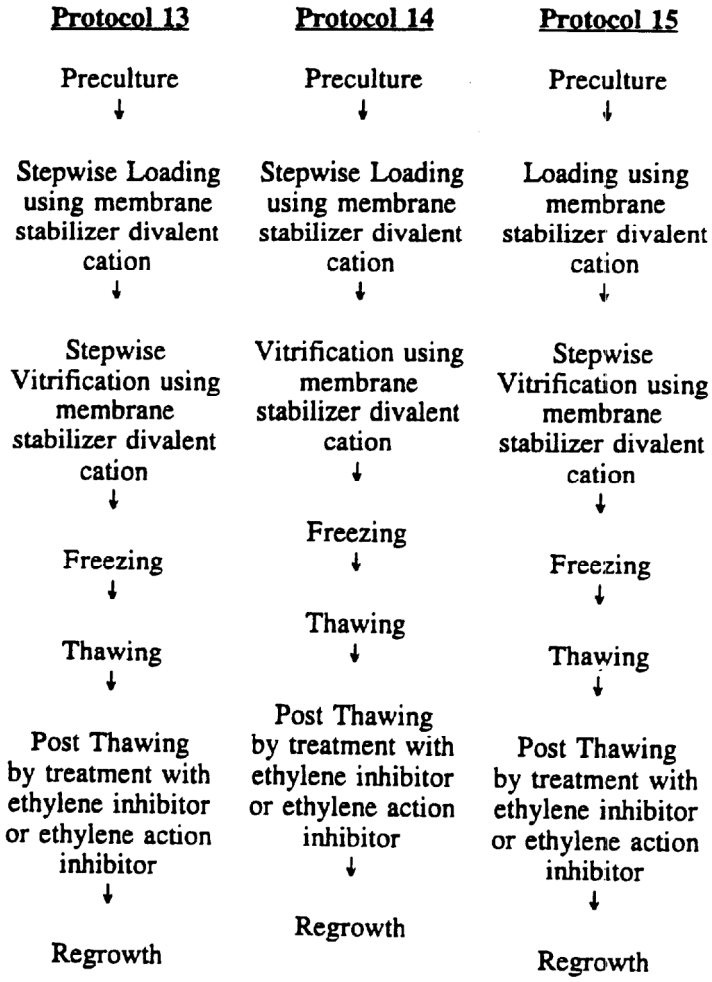
A major advantage of vitrification is the low risk of freeze injury, thereby ensuring a sufficiently high cell survival rate. However, the high potential of contamination with pathogenic agents is present, and the technique requires good manipulation skills. Both the techniques involve pre-cultures and following post-culture common steps;



After storage, biological samples are made available for clinical application or other purposes by removing the samples from LN_2 and warming with the following steps;



The whole procedure of cryopreservation, and rewarming and uses can be summarized by following flow charts as described in U.S. Patent (Patent No: US6753182B1);



Major step of cryopreservation involves the loading of cryoprotectant to the biological samples which differ from other depending upon the cell types and other physical as well as chemical parameters of the biological materials. The loading of CPAs can be categorized into following;

- Trehalose delivery method
- Physical delivery method
- Chemical delivery method
- Hydrogel based cell encapsulation method
- Emulsion method
- Extrusion method
- Microfluidic method
- Droplet based printing method

Trehalose delivery method: Trehalose is a non-permeating disaccharide, which is used as a bio-inspired CPA to protect cells or organisms against cryoinjuries. It facilitates the formation of a stable glassy matrix and promotes preferential hydration in cellular biomolecules, stabilizing their functional conformations. For intracellular cryopreservation, it is used in combination with organic solvent CPAs like glycerol or DMSO. These techniques help to achieve organic solvent-free cell cryopreservation and high post-thaw cells survival efficiency. It increases cellular membrane permeability and thus transport of non-permeable trehalose into cells. Different trehalose delivery method is given in below figure;

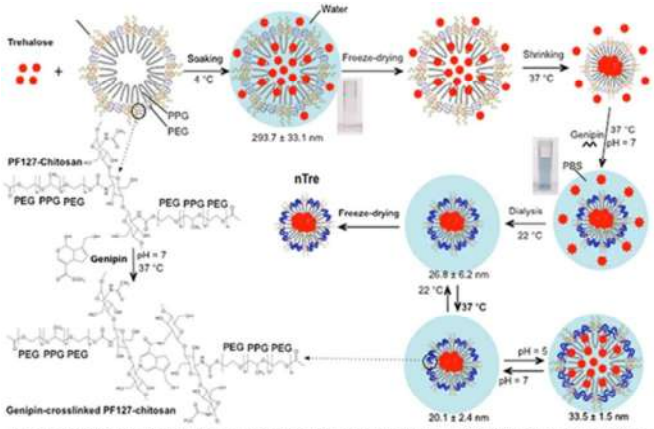
Delivery methods		Cell type	Main results		Advantages	Disadvantages
Physical delivery	Freezing-induced lipid-phase transition	Platelets	Post-thawing, 98% platelets showed/were having intact membrane	Organic solvent-free cell cryopreservation based on trehalose	1. Trehalose loading into cells easily controlled by concentration gradient 2. Reversible based on external stimuli 3. Simple processing	1. Non-specific membrane permeability 2. Membrane injury concerns induced by thermal and electric shock
	Electroporation	hADSCs	1. 84% cell survival rate post-thawing 2. Maintenance of normal cell proliferation and differentiation potential			
		UC-MSCs	61% cell survival rate post-thawing			
	PP50	Erythrocyte	1. 123 mmol/L of intracellular trehalose was achieved in erythrocytes 2. 83% post-thaw erythrocyte survival		Loading of high amounts of intracellular trehalose.	1. Non-specific membrane permeability 2. Long incubation time 3. Cytotoxicity concerns 4. Tedious washing step 5. Complex material preparation
		SAOS-2	The number of metabolically active cells at 24 h post-thaw was between 91% and 103%			
Chemical delivery	α-Hemolysin	Fibroblasts keratinocytes	Long-term post-thaw and survival rate was 80% for fibroblasts and 70% for keratinocytes		1. Reversible based on external stimuli. 2. Trehalose loading into cells easily controlled by concentration gradient	
	Genipin-cross-linked Pluronic-F127 nanoparticles (GNPs)	hADSCs	Approximately 90% cell viability and normal differentiation potentials, and distinctive markers expression was maintained		1. Utilize natural endocytosis process 2. Specific transport of high amounts of trehalose	1. Long incubation time 2. Complex material preparation
	Apatite nanoparticles	RBCs	Increasing RBC cryosurvival up to 91%, which is comparable to FDA-approved cryopreservation protocol employing glycerol			

Physical delivery method: The physical delivery methods, including freeing-induced membrane phase transition and electroporation technology, have been used to promote the permeation of trehalose into cells. These methods help to achieve organic solvent-free cell cryopreservation. The loading of trehalose can be easily controlled by manipulating the concentration gradient.

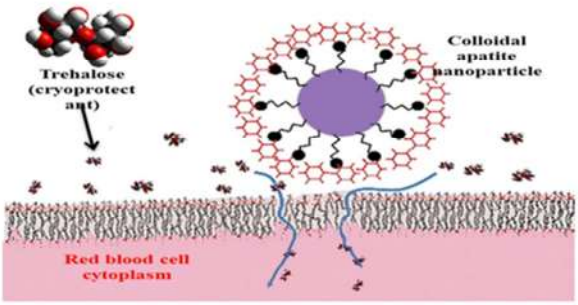
Chemical delivery method: A chemical delivery method utilizes cells membrane perforating agents like synthetic polymers, α-hemolysin, and nanoparticles to achieve intracellular delivery of trehalose. They interact with cell membrane and increase membrane permeability temporarily. This method is utilized in the cryopreservation of RBCs, fibroblasts, keratinocytes, and human mesenchymal stem cells (hMSCs).

Nanoparticles are more practiced in recent times for the cryopreservation of above biological materials. They generally utilize the natural process of endocytosis to specifically deliver trehalose into cells without any harmful effects. It has two variations; one is a pH-responsive

genipin-cross-linked Pluronic F127-chitosan nanoparticles (GNP), which efficiently encapsulated trehalose for intracellular delivery, and another is a type of biomimetic (bone-like) apatite nanoparticle, which deliver drugs and nucleic acids into various types of cells. Former method starts with incubation of cells are incubated with trehalose-loaded Genipin nanoparticles (GNPs) for 24 h and cryopreserved with culture medium containing 200mmol/l free trehalose (results in 90% more cell viability than the cells obtained with cryopreservation with DMSO). Similarly, the colloidal bioinspired biomimetic (bone-like) apatite nanoparticles are incubated with cells, like RBCs, which interacts with lipid bilayer for the trehalose translocation into the cells (shown in figure below).



The encapsulation of trehalose in genipin-cross-linked Pluronic F127–chitosan nanoparticles (GNPs) to produce nanoparticle-encapsulated trehalose (nTre)

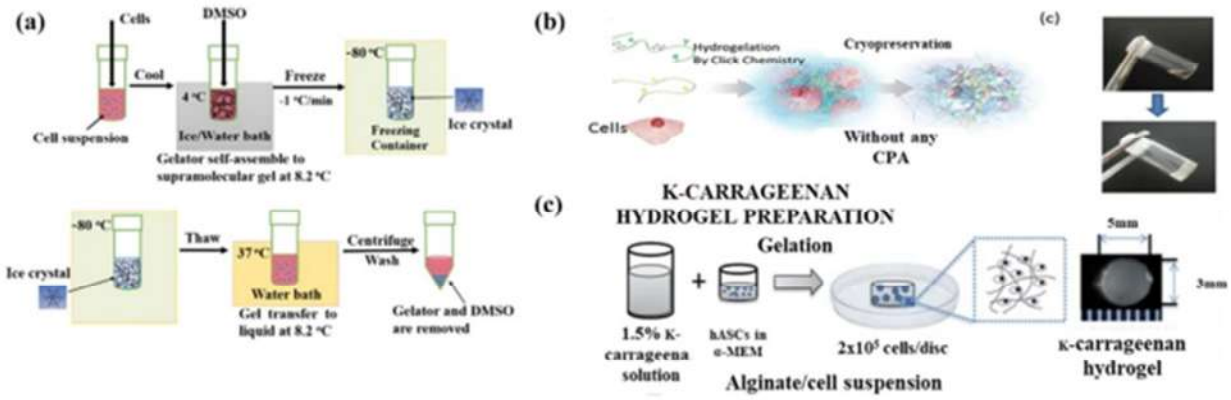


Interactions of biomimetic (bone-like) apatite nanoparticles with the lipid bilayer and enhanced delivery of trehalose into RBCs.

Source: https://link.springer.com/article/10.1007/s12209-019-00227-6

Hydrogel-based cell encapsulation method: It implies the encapsulation of living cells within semipermeable capsules using hydrogel materials. It is highly promising for various cell-based studies and applications. It provides suitable 3D microenvironment similar to the extracellular matrix, blocks the immunogenicity of encapsulated cells, and directs the differentiation of stem cells. The capsule formed not only protects the cells from mechanical and osmotic stress during the freezing and warming process, but also allow the bidirectional diffusion of nutrients, oxygen, and waste products. It is broadly divided into three categories, namely emulsion/thermal gelation, extrusion (electrostatic spray, air flow nozzle, and vibrating nozzle), and microfluidic method.

Emulsion method: In this method, pre-gel solutions are first prepared by suspending cells and gel materials. When the dispersion reaces an equilibrium state, gel formation is triggered by adding an initiator or changing the physical conditions, such as UV light. This method is very simple but gelatin used is toxic and may result in cell death and loss of functions, therefore in recent times, polyvinyl alcohol (PVA) – based hydrogel, such as PVA-gelatin cryogels and PVA-carrageenan (Car) scaffold is utilized in the cryopreservation processes.

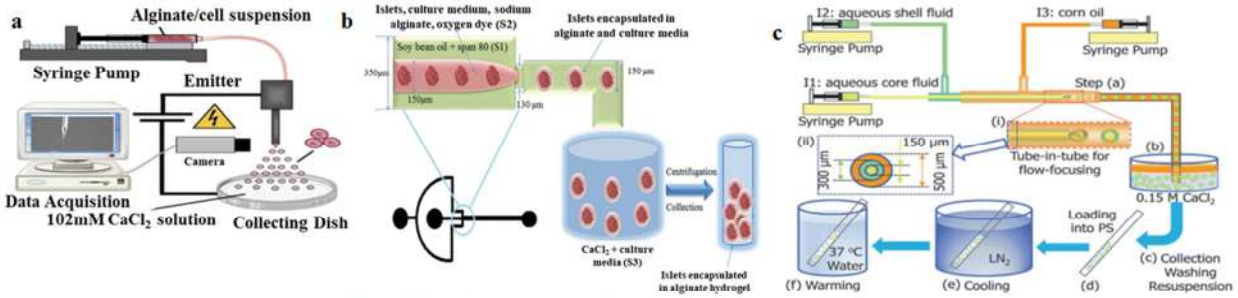


Schematic diagram emulsion method: **a** cooling and thawing process for cells encapsulated in supramolecular gel, **b** in situ hydrogelation for cell encapsulation and cryopreservation, **c** preparation and cryopreservation process for cells-carrageenan hydrogel construct. *Source:* https://link.springer.com/article/10.1007/s12209-019-00227-6

Extrusion method: It includes two methods, namely electrostatic spray and air-jet encapsulation technology. These are commonly used due to its high throughput and production of evenly sized beads. Cell encapsulation using electrostatic spray method involves generation of droplets containing cells and polymers from the nozzle, followed by spraying into a container with gelling bath to form hydrogel alginate beads. The hydrogel bead formation is assisted by the electrostatic force between the gelling bath and the nozzle. The vitrification of cell-loaded microcapsules with low concentration of DMSO maintained high post-thaw cell viability in encapsulated cells. Te Ca-alginate microcapsule provides great protection to the cells during cryopreservation.

Schematic diagram of extrusion method - a two-fluidic electrospraying method for encapsulation of cells in core–shell capsules.

Microfluidic method: In a microfluidic platform, individual rat pancreatic islets are encapsulated with FOSD functionalized hydrogel microcapsules. Here, hydrogel membrane surrounding the encapsulated islet effectively enhanced the insulin secretion after thawing. The microstructure of the hydrogel is characterized by the presence of a compact 3D porous network and considerable amount of non-freezable bound water. Large-volume low-CPA cell vitrification is achieved by microfluidic-based alginate hydrogel micro-encapsulation system, Cells in low concentration of CPA medium (2mol/l) are encapsulated into core-shell microcapsules using an elaborate microfluidic system and then loaded into 0.25ml conventional plastic straw (PS), which are then plunged into liquid nitrogen to induce vitrification. After thawing, cells liberated from the microcapsules showing no changes in viability and differential capacity. The microcapsule effectively inhibits the ice formation and further propagation during cooling and warming.



Schematic diagram of microfluidic method: **(a)** electrohydrodynamic atomization (EHDA) method for fabrication of cell-laden microcapsules with uniform size, **(b)** encapsulation of individual rat islets into alginate hydrogel using a droplet microfluidic device at room temperature, and **(c)** preparation, vitrification and warming process for cell-laden alginate-based core–shell hydrogel produced using a double emulsion flow-focusing tube-in-tube capillary microfluidic

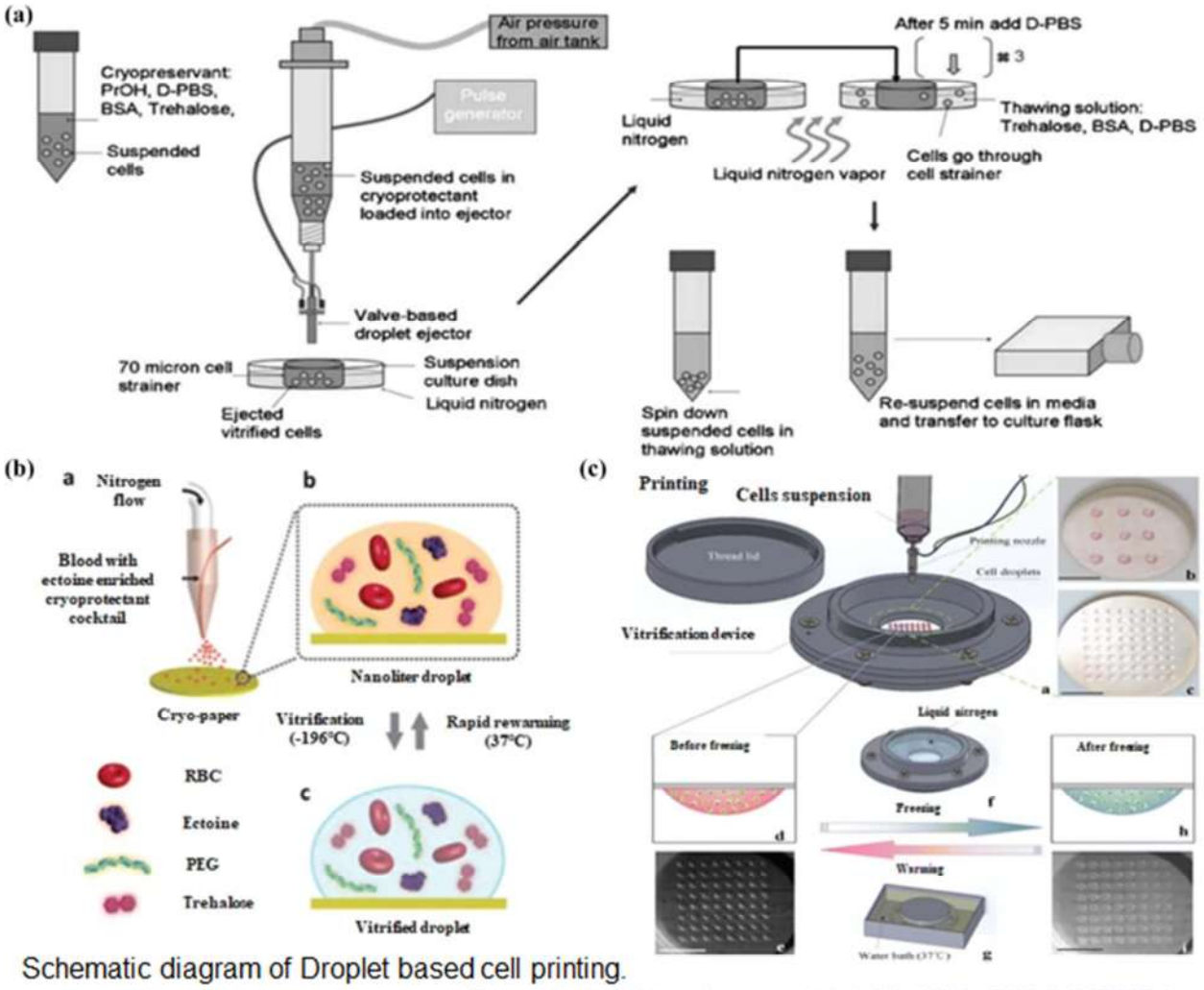
Source: https://link.springer.com/article/10.1007/s12209-019-00227-6

Droplet based cell printing: Owing to its attribute of applying even smaller volume for cryopreservation, high efficiency and low cost, it has gained acceptance in wide applications, such as inkjet printing, emulsion polymerization, and DNA arraying, and thus a series vitrification protocols based on this system has emerged which are cgaracterized by lower CPA concentration and higher cooling and warming rates. This technique not only solved the problem of high CPAs concentration compared to conventional vitrification, but also conferred significant protection to cells by reducing the time required for ice crystal formation. It is practiced in three ways, **(i)** cell-CPA solution is loaded into valve-based droplet injector, and the resulting droplets are directly injected into liquid nitrogen. The ejected cells are collected in a cell strainer and are rapidly transferred (in nitrogen vapor) to the thawing media, followed by a step-by-step thawing process. **(ii)** RBC-CPA droplets are printed onto a cryo-paper as nitrogen gas flowed through a droplet ejector, which transformed the bulk of the RBC-CPA mixture into nanoliter droplets. Vitrification is achieved by submerging the cryo-paper into liquid nitrogen. Warming process is performed by thawing the cells on a cryo-paper in phosphate-buffered saline at 37 °C, and **(iii)** cell-laden droplets are rapidly ejected onto a freezing film using a cell printer with high throughput and precise spatial controllability. Vitrification/thawing process is achieved by pouring the liquid nitrogen/warm water onto the other side via boiling heat transfer that helped to maintain high cooling/warming rate (as shown in figure below).

As discussed after incubation of cells with CPAs, the combination of both is subjected to subzero freezing by various means as described and stored the biological materials for very long time. For the application, these samples are removed from freezing device, such as ultra-deep freezer or LN_2 container and subjected to repeated controlled warming process through conventional warming method like 37°C water bath and more advanced **nonowarming**.

Conventional warming method like 37°C water bath, fails to provide sufficient warming rates and uniform warming effect with the increasing scale of biological specimen. During rewarming process, once the warming rates go below the critical warming rates, ice crystal formation (devitrification) starts. But, nanomaterials-mediated nanowarming techniques allow ultra-rapid and uniform rewarming. It involves use of some nanomaterials such as Fe_3O_4 nanoparticles Fe_3O_3 NPs) or gold nanorods (GNRs) that rapidly convert electromagnetic or light energy into

heat energy. It is significantly effective and can be used for improving cryopreservation of cells, tissues, and organs.

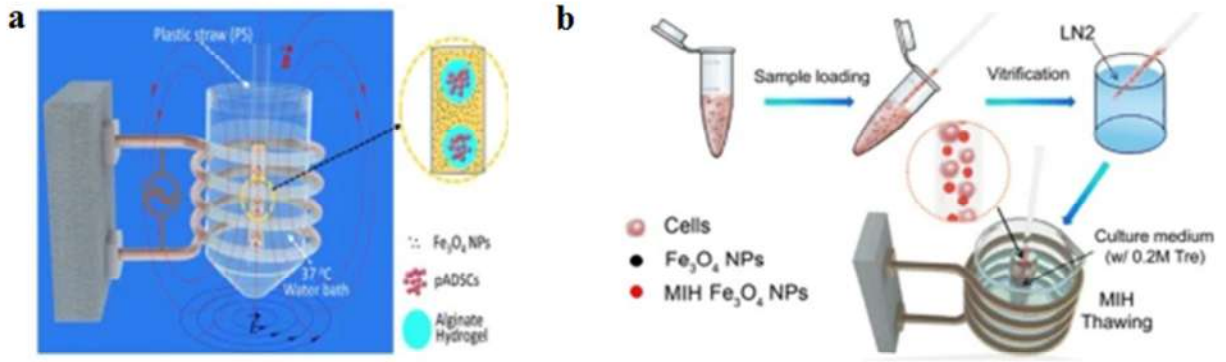


Schematic diagram of Droplet based cell printing.

Source: https://link.springer.com/article/10.1007/s12209-019-00227-6

Nanowarming is achieved by two methods, namely RF inductive warming (Radio Frequency inductive warming) and Laser radioactive warming. These are as follows;

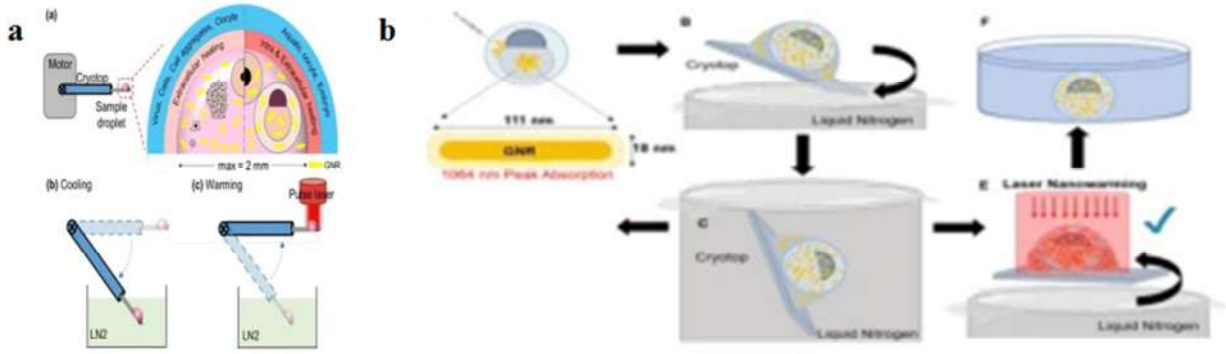
RF inductive warming process: It is a technique of induction heating using radio frequency (RF) energy that is a portion of the electromagnetic spectrum below infrared and microwave energy. Heating by this method is highly selective in the depth and along the surface, and can heat in any processing atmosphere. Heat is generated and transferred to the product via electromagnetic waves the inductor itself does not get hot and there is no product contamination. In cryopreservation, Fe_3O_4 NPs are mixed with low-CPA solution and coupled with RF heating leads to effective warming or thawing of the frozen mix. Fe_3O_4 NPs in the cryopreservation mix of biological materials acts as inductive material works for heating process (shown in figure).



Schematic diagram showing RF induction warming. **a** Nanowarming procedure for vitrified PS loaded with cell-alginate hydrogel constructs and NPs in CPA solution. **b** Vitrification and nanowarming of hUCM-MSC-laden PS with magnetic induction heating.

Source: https://link.springer.com/article/10.1007/s12209-019-00227-6

Laser radioactive warming process: In this method, gold nanoparticles are used instead of Fe_3O_4 nanoparticles and incubated with cells like human dermal fibroblast (HDF) cells. This method involves irradiation with 1064nm laser pulse for 1ms (as described in figure below). A cell viability of > 90% can be maintained in HDF cells post-laser warming. Similar warming method is used to rewarm vitrified zebrafish embryos. Before vitrification, biocompatible PEGylated GNRs are microinjected directly into Zebrafish embryos with 2.3mol/LPG, thereby helping to distribute the laser energy throughout the embryo during warming. Here, 31% viable embryos have been found with consistent structure at 1 h, 17% viable embryos continuing development at 3 h, 10% viable embryos showing movement at 24 h post-warming.



Schematic diagram showing Laser radioactive warming process. **a** Sample droplet consisting of biomaterial(s), CPA, and gold nanorods (GNRs) with a maximum volume of 1.8 µL was loaded onto a customized cryotop. For rapid cooling, cryotop was directly immersed into liquid nitrogen. Laser warming was achieved by pulsed laser irradiation yielding ultra-rapid rewarming at rates up to 2×10^7 °C/min. **d** Overview of zebrafish embryo cryopreservation and laser GNRs rewarming.

Source: https://link.springer.com/article/10.1007/s12209-019-00227-6

Two-dimensional (2D) grapheme oxide (GO) and molybdenum disulfide (MoS) nanosheets (NSs) are used to improve warming process of biological samples owing to their photothermal effects. By his method, cryopreserved human umbilical vein endothelial (HUVECs) are placed

into warming solution (37°C) under a near infrared laser field and photothermal effect is achieved at 5000mW/cm^2 for 8-10s.

Application of Cryopreservation

Cryopreservation techniques have wide application which can be categorized broadly into following areas:

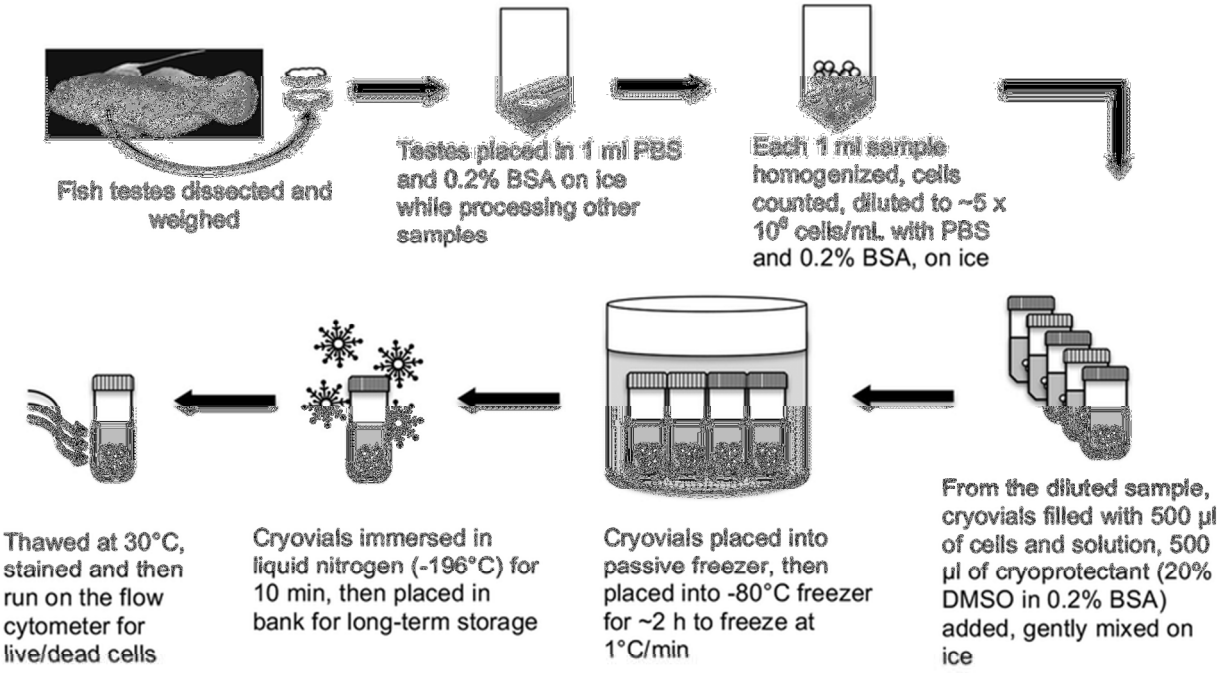
- Cryopreservation of cells or organs;
- Cryosurgery;
- Biochemistry and molecular biology;
- Food sciences;
- Ecology and plant physiology; and
- Medical applications, such as blood transfusion, bone marrow transplantation, artificial insemination, and *in vitro* fertilization (IVF).

Furthermore, it is possible for banking of cells for human leukocyte antigen typing for organ transplantation, the allowance of sufficient time for transport of cells and tissues among different medical centers, and the provision of research sources for identifying unknown transmissible diseases or pathogens. In medical field, it has wide applicability, some of which are as follows;

Oocytes and embryos preservation: It is widely used in cryopreservation of oocytes and embryos. Cryopreservation of mature oocytes is a proven technique for preserving the reproductive capacity. Results from a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle from 1986 to 2007 showed that there was no significant impact of the duration of storage on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles.The first case of embryo cryopreservation for fertility preservation took place in 1996, with the application of a natural IVF cycle prior to chemotherapy in a woman diagnosed with breast cancer.

Sperm, semen, and testicular tissue: Germ cell depletion caused by chemical or physical toxicity, disease, or genetic predisposition can occur at any age, and therefore fertility preservation is of great importance to guarantee the quality of life of patients facing chemo- and radiotherapy. In this method, sperm and semen can be used almost indefinitely after proper cryopreservation. There are new trials for cryopreserving testicular tissues in the form of cell suspensions, tubular pieces, and entire gonads but this technique is still premature. Overall, cryopreservation can be used as a first-line means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiotherapy, or surgery.

Stem cells: These are specific cells of the organisms which have the potential to differentiate into multiple types of specific cells and can be obtained from various locations other than bone marrow, including fat tissue, the periosteum, amniotic fluid, and umbilical cord blood, and therefore on this basis, they are categorized into three three types; embryonic stem cells (ESCs), mesenchymal stromal cells (MSCs), and hematopoietic stem cells (HSCs), all of which are considered as goldmines for potential application in regenerative medicine.



Cryopreservation of fish sperms

Hepatocytes: Hepatocytes are used for the study in a wide range of areas including physiological studies, investigation on liver metabolism, organ preservation and drug detoxification, and experimental and clinical transplantation. Moreover, it has gained much attention in the study in regenerative medicine and biotechnology and therefore, it has prime focus and need for cryopreservation.

Others: Other areas include the cryopreservation of neuronal cells and cardiomyocytes, which are now-a- days, routinely used in neuroscience and cardiology research. With the discovery of glucocorticoid-free immunosuppressive regimens,51 pancreatic islet transplantation may be considered as an alternative for the treatment of type 1 diabetes. For this reason, the development of islet cryopreservation methods has been ongoing, but results are still suboptimal, with a survival rate of less than 50%. Furthermore, different cell lines for research in immunology and cancer biology heavily demands the need of cryopreservation which not only ascertains easy availability of particular cell lines, but also maintains quality of cells lines for indefinite period.

Limitations of Cryopreservation

It is doubtless to say that it revolutionize many facets of human life and rerearch in various areas of science, agriculture and technology, but it has some limitations too particularly in basic as well as clinical research. Cells metabolize almost nothing at low temperatures such as −196 °C (i.e., in liquid nitrogen), which has inevitable side effects, including a genetic drift toward biological variations of cell-associated changes in lipids and proteins that could result in the impairment of cellular activity and structure. If there were no limit to the amount of CPA that could be used, cells would be preserved perfectly.

References

1. Měricka P. Cryopreservation: Cells, tissues and organs. Researchgate.
2. Taylor M. J. et. al. New approaches to cryopreservation of cells, tissues, and organs. Transfus Med Hemother 2019;46:197–215.
3. Meddahi P. et. al. Cryopreservation of cells, tissues and organs. International Publication Number – WO 2013/107797 AI.
4. Bhattacharya S. & Prajapati B. G. A review on cryoprotectant and its modern implication in cryonics. Asian J. Pharmaceutic 2016; 10(3): 1.
5. Yang J. et. al. Advanced biotechnology for cell cryopreservation. Transactions of Tianjin University 2019.
6. https://www.jsps.go.jp/j-sdialogue/2009a/data/0626_daini.pdf

ooooooooo