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Technologies for Cryopreservation: Overview and Innovation

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1. Introduction

The proposed chapter investigates methods, devices and technologies for cryopreservation, explaining the most used cooling processes, as well as conventional and innovative technologies adopted. Main processes used for cryopreservation of oocytes, embryos and sperms can be reassumed in three categories:

1. slow freezing
2. vitrification
3. ultra-rapid freezing

Research is not intended to be exhaustive, but is aimed at covering most of relevant topics.

Slow freezing involves step-wise programmed decrease in temperature. The procedure is lengthy and requires the use of expensive instrumentation. The process does not exclude ice crystal formation, which can have extremely deleterious effects (Pegg, 2005).

In the vitrification process, the use of CryoProtectant Agents and the increasing of cooling rate (from 2,500 °/min to 130,000 °/min) avoid the ice crystal formation, increasing the embryos and oocytes survival. Unfortunately, common cryoprotectants are toxic and the immersion of solution directly in liquid nitrogen can be cause of contamination of embryos and oocytes with bacterium, mushroom and virus.

Ultra-rapid freezing can be considered a midway technique between slow freezing and vitrification, but its application has demonstrated lower performances than the other two processes.

2. Slow freezing

Necessary condition for slow freezing is freezing cells with a cooling rate equal or lower than 1°/min, before storaging them at -130° or lower (De Santis & Coticchio, 2011). If cell is cooled down very slowly, it will be exposed to growing concentrations of cellular solutes due to ice formation inside the solution, with a PH variation and cellular dehydration. If it is cooled down too fast, crystal nucleuses will form in the solution and inside the cell, with the destruction of cell membrane. Usually at temperature below -60°, the samples can be immersed directly in liquid nitrogen or transferred to freezer of maintenance without further loss of viability. Slow freezing generally lasts one or two hours. However, a greater amount of cells can be frozen at a time (Ha et al., 2005), and lower quantity of CPA are used than in vitrification.

2.1 Programmable freezers

Currently, programmable freezers are the most common technology for slow freezing process. Programmable freezers are based on liquid nitrogen technology, but their use is denied in areas without availability of nitrogen or during long transport. Cooling rate is controlled by a heater (Asymptote EF600, Cryologic CL8800) or by the synchronous use of two valves.

Main characteristics of the most common programmable freezers are shown in Tab. 1. [1]

	Kryo 360	Kryo 560M	Cryo-Logic 8800 + Fast CryoChamber	Thermo Scientific Forma 94741
Producer	Planer plc	Planer plc	CryoLogic	Fisher Scientific
Control range [°]	+40 to -180	+30 to -180	+40 to -120	+50 to -180
Cooling rates [°/min]	-0.01 to -50	-0.01 to -50	-0.04 to - 10 (at -40ąC)	-0.1 to -50
Heating rates [°/min]	0.01 to 10	0.01 to 10	—	0.1 to 10
Capacity [l]	1.7 or 3.3	16	11.5	17 or 48

Table 1. Programmable freezers main characteristics

2.2 Stirling engine cryocooler

The **Asymptote EF600** is the first commercially available programmable freezer which does not require liquid-nitrogen. The absence of liquid-nitrogen reduces drastically risk of contamination, and allows to freeze cells where nitrogen is not available (i.e. during transport or in other borderline applications).

Fig. 1. Asymptote EF600 (http://www.asymptote.co.uk/)

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[1] Research is not intended to be exhaustive

The Asympotote EF600's cooling source is a Stirling Engine, a closed cycle machine in which the refrigerant working fluid is contained inside the machine, and only a source of mechanical or electrical energy is required[2] in order to rich temperature below - 100°.

Studies on human spermatozoa (linear cooling at -2°/min until nucleation followed by linear cooling at -10°/min to -100°), embryonic stem cells (linear cooling at -2°/min until nucleation followed by linear cooling at -1°/min to -45°) mouse embryos (linear cooling at -2°/min until nucleation followed by linear cooling at -0.3°/min to -35° and at -10°/min to -100°) and horse semen (linear cooling at -2°/min until nucleation followed by linear cooling at -4°/min to -80°) were carried on, considering survival rate as a parameter for assessing the performances of the proposed system (Faszer et al., 2006; Morris et al., 2006).

Results show that Stirling Engine cryocooler can established the desired time-temperature profile inside the test tubes and the viability after thawing data confirm that the system can be used for slow freezing applications.

However, Stirling Engines are affected by vibrations, as stated by (Hughes et al., 2000) and (Suárez et al., 2003). Vibrations might damage cells; furthermore, manual nucleation cannot be performed at a desired temperature, since vibrations generally start the nucleation process (as reported by (De Santis et al., 2007; Edgar, 2009; Rosendahl et al., 2011)).

2.3 Pulse tube cryocooler

In order to overcome to problems connected with vibrations of Stirling Engines, a programmable freezer based on a Pulse Tube cryocooler is being developed in "Sapienza" University of Rome Laboratory of Mechanical Engineering, in collaboration with *MES - Microconsulting Energia & Software S.c.a.r.l.*[3] and *LABOR S.r.l.*[4]. Alike the Stirling Engine, the Pulse Tube machine is a closed cycle system and it does not require liquid-nitrogen. The Pulse Tube cryocooler is able to rich temperatures below -150° making the refrigerator fluid (that is generally helium or nitrogen) move oscillatory. The fluid motion is obtained using a compressor and a rotative valve. The Pulse Tube offers low vibrations, as discussed by (Ikushima et al., 2008; Riabzev et al., 2009; Suzuki et al., 2006; Wang & Hartnett, 2010).

Next to the *cold head* (the cooling part of the Pulse Tube), the refrigerator fluid absorbs heat from the test tube, cooling it. The Pulse Tube cryocooler is characterized by a higher cooling rate than the ideal one for cell freezing (0.1°/min ÷ 10°/min) in the temperature range used for cryopreservation (+30° ÷ -60°). The cooling rate is reduced in the proposed solution through a control system that can supply heat to the cryorefrigerator.

A heater is placed by the test tube older (Fig. 2). The power dissipated through the heater for Joule effect varies according to two different control systems proposed:

1. *On-Off* **regulation**. A threshold control system has been implemented: the heater is activated when the real temperature is more than 1° below the desired temperature, and it is turned off when the real temperature is more than 1° over the desired temperature. Using this control system, oscillations of ±6° around the desired temperature were obtained, as it is illustrated in Fig. 3 and Fig. 4.

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[2] The Asymptote EF600 can be connected to a conventional 240V electricity supply or to a car battery
[3] Via A. Panzini, 3 - 00137 Roma, Italy
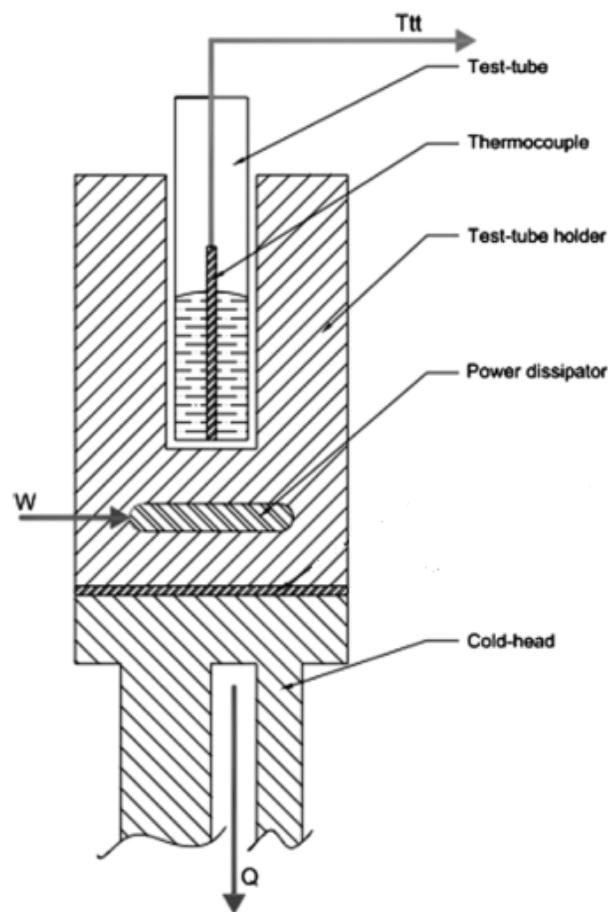[4] Tecnopolo Tiburtino, Via G. Peroni 386 - 00131 Roma, Italy

Fig. 2. Representation of the experimental apparatus. T_{tt} represents the temperature inside the test tube, measured by a thermocouple, Q is the heat absorbed by the refrigerating fluid and W is the dissipated power.

However, the oscillation might be reduced optimizing the threshold parameters. The *On-Off* regulation can be easily implemented, and it does not require the regulation of the power dissipated through Joule effect.

2. ***Predictive model* regulation**: the cooling slow-down is achived by providing an amount of heat, variable with the time, that will be able to raise the temperature of the PT cold head to the desired value (Cipri et al., 2010). The amount of heat is calculated using a predictive and adaptive model. Using this regulation modality, oscillation can be removed. However, it requires the regulation of the power dissipated through Joule effects, increasing the cost of the hardware. Moreover, more computational power is required in order to calculate the amount of heat which has to be dissipated.[5]

Results are shown in Fig. 5 and Fig. 6.

In the determination of the *Predictive model* a lot of simplifying assumptions were made (Cipri et al., 2010), and we believed that the system should have better results if the model was set in more accurate way. Further researches are fostering investigation at Sapienza Laboratory.

At this very moment, the system is not yet commercially viable.

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[5] An *On-Off* regulation is still used before the transition phase, marked by the abrupt rise of temperature typical of the subcooling
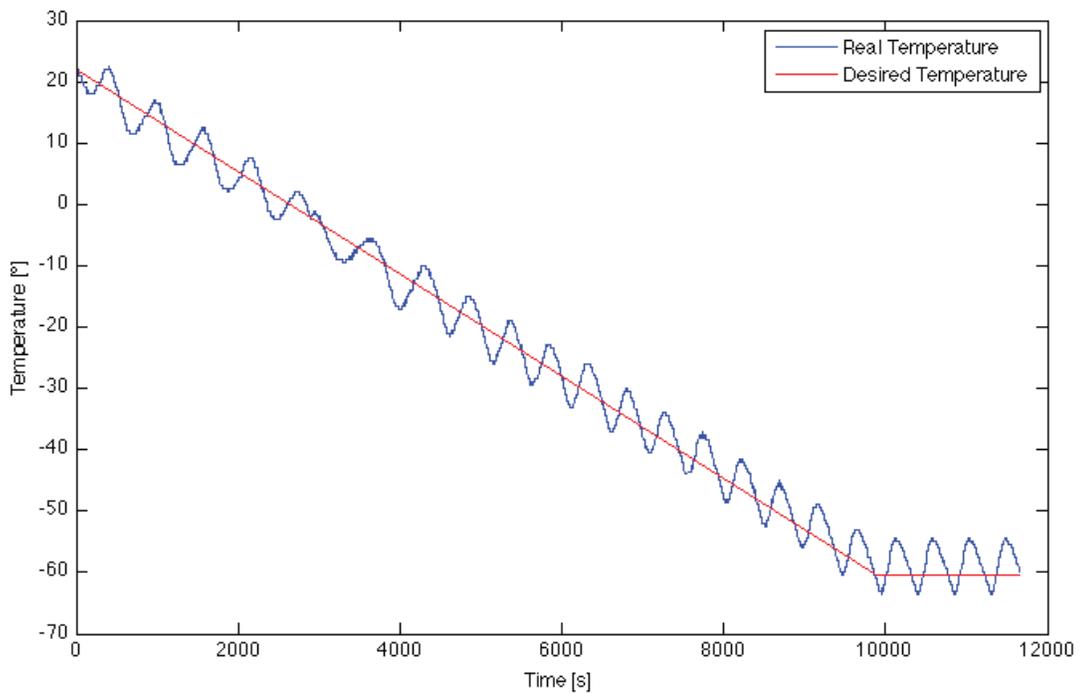
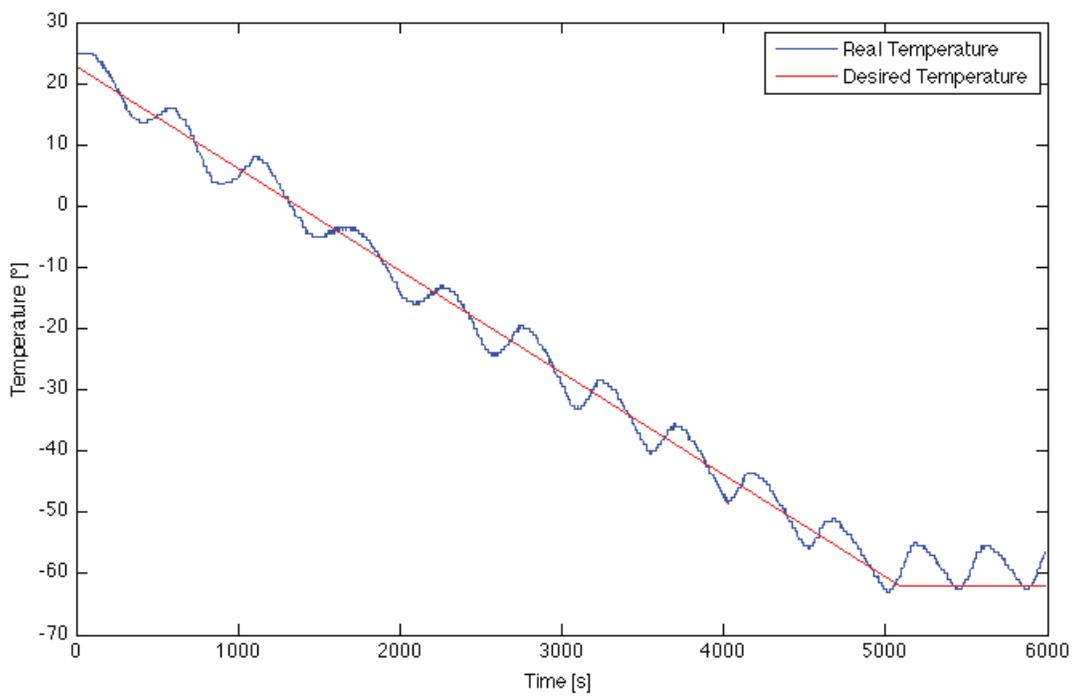Fig. 3. *On-Off* regulation - Temperature inside the test tube vs time. A desired cooling rate of -0.5°/min was selected.

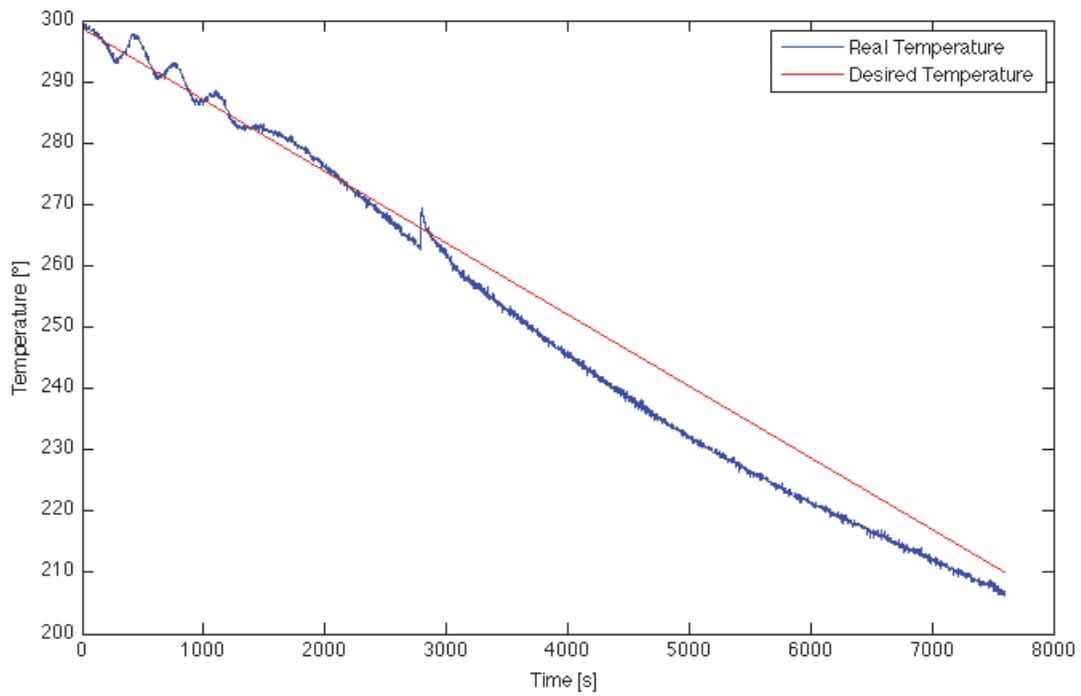Fig. 4. *On-Off* regulation - Temperature inside the test tube vs time. A desired cooling rate of -1°/min was selected.

Fig. 5. *Predictive model* regulation - Temperature inside the test tube vs time. A desired cooling rate of -0.7°/min was selected.
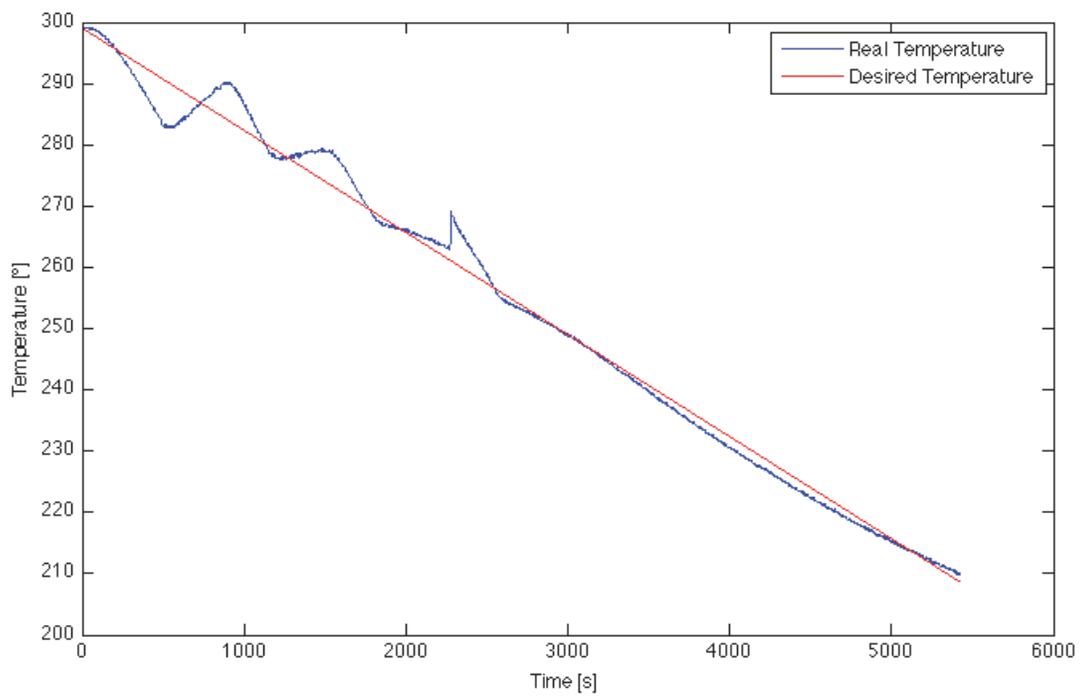

Fig. 6. *Predictive model* regulation - Temperature inside the test tube vs time. A desired cooling rate of -1°/min was selected.

3. Vitrification

A criticality of common cryopreservation methods consists in the formation of ice crystals that drastically reduces the survival of treated embryos and oocytes. Vitrification process produces a glasslike solidification of living cells which completely avoids ice crystal formation. The process is based on the principle that water, characterized by high cellular viscosity increased by the adding of CryoProtectant Agent (CPA), and frozen using a high cooling rate, is not capable of forming ice. The main limits of Vitrification process are represented by: use of potentially toxic cryoprotectant; risk of contamination of embryos and oocytes with bacterium, mushroom and virus when directly immersed in liquid nitrogen or during the storage phase. Studies have demonstrated that reduced quantity of CPA can be used if the cooling rate is increased.

A freezing rate of 2,500°/min and CPA concentration of 5-7 M is reached with the immersion of embryos and oocytes in micro-capillary straws, while in the pulled straws the cooling rate is about 20,000 °/min (Kuleshova & Lopata, 2002). Theoretically, the reaching of a cooling rate of $10^{7°}$/sec should allow to vitrify also in pure water, but this rating is not practicable at the moment. Several studies are also oriented to formulate nontoxic and more efficient Vitrification solutions, also combining different cryoprotectants such as sugars and polymers or establishing modern solutions that include non-penetrating additives.

Moreover, the implementation of *Minimum Volume methods* has allowed to reduce the concentration of cryoprotectant. EG (ethylene glycol), characterized by low toxicity, is an important component of vitrification solution, commonly combined with DMSO or PROH (propanediol). In particular, non-permeable cryoprotectans (such sucrose or PVP) can be added in the solution on order to reduce the concentration of permeable cryoprotectans and facilitate dehydration and vitrification. Researches oriented to improve the characteristics of cryoprotectans have been carrying on in order to reduce toxicity. An EG and sucrose (non-permeable cryoprotectans) solution has been tested for cryopreservation of all preimplantation stages of *in vivo* generated mouse and day-6 sheep embryos. Experiments have not shown a loss of viability in vitro or in vivo. The same solution has been proved for vitrification of human oocytes, attaining high surviveal rates using conventional straws.

Another solution used to reduce toxicity is to equilibrate the cryoprotectant using a two-step method: the pretreatment solution, named *equilibration solution*, contains 20-50% concentrations of permeating cryoprotectans. The lower concentration of permeating cryoprotectans in the equilibration solution is much less toxic than the vitrification solution. The permeating cryoprotectant enters into the cells and facilitytes the intracellular vitrification. The cells pretreatment with equilibration solution is used in oocytes vitrification: this method has been demonstrated to increase the survival rate after thawing.

Main devices, commonly use in vitrification, are *Open Supports*: *Pulled Straws, CryoLoop, CryoEM, Cryoleaf* and *CryoTop*. The risk of contamination, due to the use of *Open Supports* for vitrification, limits the use of this process for human cells and tissues, according to the European regulations. In order to reduce contamination risks, *Close Supports* have been introduced: unfortunately their use decreases the cooling rate with consequently need to improve the quantity of CPA for guaranteeing the same survival rate. Vitrification process has demonstrated high performance in term of survival after thawing, comparable to slow cooling and it has become a promising alternative in cryopreservation of mammalian embryos and especially oocytes, through application of slow-rate freezing process.

3.1 Open supports

3.1.1 Open Pulled Straw (OPS)

Open Pulled Straw (OPS) have been designed to guarantee a ultra rapid freezing without ice crystals formation. The system, ideated by G. Vajta in 1998, is based on the hypothesis that decreasing the standard straw diameter, the volume of solution to vitrify is reduced too, raising the cooling rate. This method is so characterized by a very high cooling and warming rates (over 20,000°/min) and a short contact with concentrated cryoprotective additives (less than 30 sec over -180°). This approach reduces the possibility of chilling injury and toxic and osmotic damage. Several OPS have been developed reducing the diameter of standard straws of a half, increasing the cooling rate by 10 times and reducing by 30% the concentration of CPA and the time of exposition. Common OPS are standard 0.25 mL straws with one extremity pulled and thinned by heating. This solution increases the superficies/volume rate and hastens the cooling rate of the 2 μL drop set to contain the embryo. The Open Pulled Straw produced by *MTG* are made of PVC: with a length of 93 mm, straws can have an inner tip diameter approximately of 0.65 mm for Standard OPS and of 0.3 mm for super fine OPS. Before plunging the thin straw into liquid nitrogen, embryos are treated with highly concentrated cryoprotectant (CPA) solutions of ethylene glycol (EG) and dimethyl-sulfoxide (DMSO), in variable percentage.

3.1.2 Cryoloop

Cryoloop is generally applied to investigate the contribution given by cortical areas to network interactions and cerebral functions.

The Cryoloop is manufactured from straight 23 gauge hypodermic stainless steel tubing, having external and internal diameters respectively of 0.635 mm and 0.33 mm. Methanol, drawn from a external reservoir, is pumped in a Teflon tube directly in the Cryoloop that is in contact with the brain. Before reaching the Cryoloop, tubes containing Methanol are coiled and immersed in a bath of methanol and dry-ice pellets. The mixture cools the flowing methanol at a temperature of -75°. A microthemocouple, connected to a digital thermometer allows to monitor the temperature of the Cryoloop.

The use of Cryoloop device in human oocytes vitrification is under investigation. Experiments are now focused on animal oocytes and blastocytes cryopreservation. Cryoloops used for vitrification consist of a nylon loop of 10 or 20 micron diameters mounted on a stainless steel pipe inserted into the lid of a cryovial (Fig. 7). One of the main producers is the Hampton Research Corporation.

For vitrification, blastocytes are placed on a cryoloop that has been coated with a thin film of cryoprotectant solution. Blastocytes on the cryoloop are placed into the cryovial, which is submerged and filled with liquid nitrogen and the vial is sealed. Studies demonstrate that both mouse and human blastocystes can be successfully vitrified by suspension on a nylon loop and immersing directly into nitrogen. Mouse oocytes cryopreservation has provided successful results, but this method has not been applied to human oocytes. Tests on rabbit oocytes showed a good survival rate approximately of 80% for four different protocols.

3.1.3 Cryo-electron microscopic (CryoEM)

The Cryo-electron microscopic technique involves freezing biological samples in order to view the samples with the lowest distortion and the fewest possible artifacts.
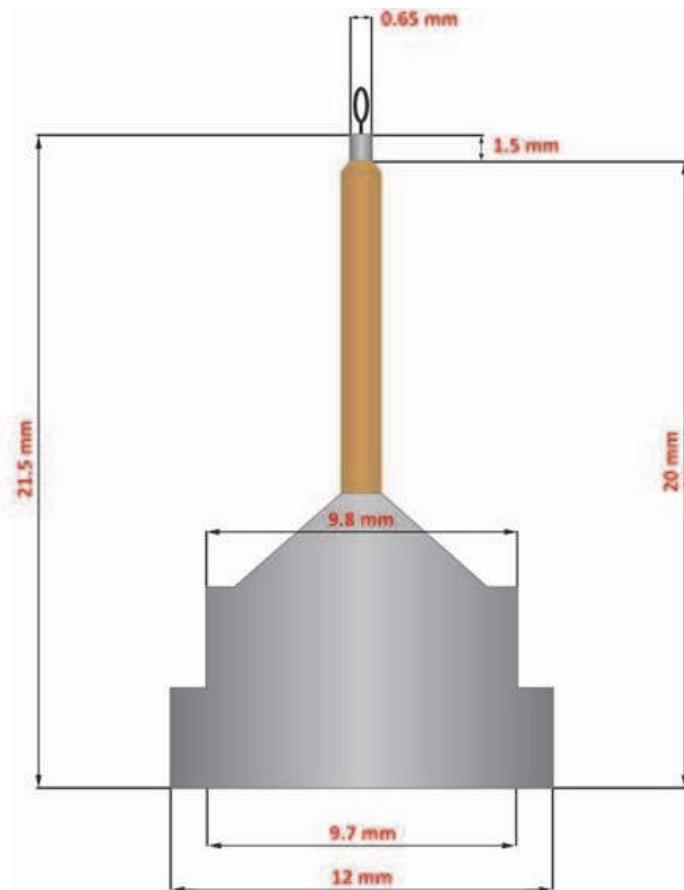
Fig. 7. CryoLoop Properly Installed in a CrystalCap Copper Magnetic - *Hampton Research Corporation*

Biological material is spread on an electron microscopy grid and is preserved in a frozen-hydrated state by rapid freezing (about $3,000°/min$), usually in ethane slush close to liquid nitrogen temperature. Specimens, maintained at liquid nitrogen temperature or colder, are contained into the high-vacuum of the electron microscope column. The frozen sample grid is then kept at liquid nitrogen temperature in the electron microscope and digital micrographs are collected with a camera. Images obtained from the cryo-electron microscopy are usually very noisy and have very low contrast. It is necessary to smooth the noise as well as enhance the contrast.

3.1.4 Cryoleaf

Cryoleaf is an open device for embryos and oocytes vitrification and storage (Fig. 8). Developed by Dr. Chian and Prof. Tan at McGill University, Montreal, the system uses PROH, EG and sucrose as cryo-protectants in the cooling phase, while in the warming procedure media contains sucrose. The recommended maximum load of the McGill Cryoleaf is 2-3 oocytes or embryos.

Oocytes or embryos are prepared for vitrification according to laboratory protocols. The outer cover of the McGill Cryoleaf is plunged into the liquid nitrogen bath, allowing the air to come out. Vitrified oocytes or embryos are quickly loaded into the McGill Cryoleaf using a suitable pipette. The excess of media, that must be less then 1 μl, has to be removed. The McGill

Fig. 8. McGill Cryoleaf Open System for Vitrification

Cryoleaf is inserted, with oocytes or embryos, directly into liquid nitrogen. Then, the Cryoleaf is blocked, sliding the protective sleeve over the tip.

3.1.5 Cryotop

Cryotop method, developed by Kuwayama in the Advanced Medical Research Institute of Kato Ladies Clinic, is based on the assumption that minimizing the volume[6] of the vitrification solution, increases both cooling and warming rates, also decreasing the chance of ice crystal nucleation/formation. Moreover the high-rate cooling decreases CPA concentration, also reducing chilling injury occurring between +15° (in human GV oocytes even +25°) and -5°, which can be minimized by passing embryos or oocytes rapidly through this temperature zone. Finally, studies have demonstrated that the use of small devices eliminates embryo fracture damages, especially in open systems.

The Cryotop tool consists of a narrow, thin film strip (0.4 mm wide, 20 mm long 0.1 mm thick) attached to a hard plastic handle for a minimum volume cooling. To protect oocytes and embryo on strip from mechanical damage and virus contamination during storage, a 3 cm long plastic tube cap is attached to cover the film part (Fig. 9).

The tool and the solutions for Vitrification and warming are market by Kitazato Co., Fujinomiya, Japan.

After a two-step equilibration in a vitrification solution containing EG, DMSO and sucrose, oocytes and embryo are loaded with a narrow glass capillary onto the top of the film strip in a volume of <0.1 ml. After loading, almost all the solution is removed so as to leave only a thin layer covering the oocytes or embryos, and the sample is quickly immersed into liquid nitrogen. Subsequently, the plastic cap is pulled over the film part of the Cryotop, and the sample is stored under liquid nitrogen (Kuwayama, 2007).

The minimal volume increases the cooling and warming rates up to 40,000°/min, contributing positively to the embryos or oocytes survival.

Cryotop vitrification method is applied successfully in various areas of animal technology and now it is indicated as the process which guarantees the highest number of babies born

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[6] According to common use, for Minimum Volume is intended less than 1 ml for direct dropping of samples into liquid nitrogen or the open pulled straw (OPS) method.

Fig. 9. Kitazato Cryotop - Kitazato Industries

after vitrification of human embryos and after cryopreservation of human oocytes worldwide. Clinical results are shown in Tab. 2

Reference	Cell	n	% Survival	% Pregnancy
Teramoto 2004	Blastocysts	197	100	57.7
Kuwayama 2005	Oocytes	64	91	41.3
Kuwayama 2005	PN Embryos D14	5881	100	-
Kuwayama 2005	Embryos D3	897	98	27
Kuwayama 2005	Blastocysts	6328	90	53
Lucena 2006	Oocytes	159	97	56.5
Antinori 2007	Oocytes	330	91	32.5
Cobo 2008	Oocytes	243	97	65.2
Cobo 2008	Oocytes	797	96	63.2

Table 2. Results achieved with Cryotop vitrification in human

3.1.6 Direct Cover Vitrification - DCV

The Direct Cover Vitrification - DCV is a new cooling method base on the minimum use of concentrated cryoprotectans and direct application of liquid nitrogen to the ovarian tissue. This way, the toxicity derived by cryoprotectants is reduced and the ice crystal injury is prevented. The ovary is immersed in a vitrification solution (0.8 ml) consisting of 15% EG, 15% DMSO and 0.5 M sucrose for 2 min.

The ovary is put in a 1.8-ml plastic standard cryovial, placed on a piece of gauze to remove the surrounding vitrification medium. Liquid nitrogen is directly applied onto the ovary for vitrification. The cap of the cryovial is closed. The lid does not have a hole. The vial is then placed into a liquid nitrogen tank.

DCV cryopreservation method, explored on mousse ovarian, has demonstrated to be highly efficient at increasing morphologically normal and viable follicles from cryopreserved ovarian tissue, compared with slow freezing and conventional vitrification.

3.1.7 Solid Surface Vitrification - SSV

The Solid Surface Vitrification - SSV has been developed at the Department of Animal Science, University of Connecticut. The method aims at defining an effective protocol to cryopreserve

bovine oocytes for research and practice of parthenogenetic activation, in vitro fertilization and nuclear transfer.

Bovine oocytes matured in vitro are transferred to a vitrification solution (35% EG, 5% polyvinyl-pyrrolidone, 0.4 M trehalose inTCM 199 and 20% FBS). A metal cube covered with aluminum foil is partially submersed into liquid nitrogen (Fig. 10): the surface reaches the temperature of -150°. Microdrops of vitrification solution, containing the oocytes, are dropped onto the cold upper surface of the metal cube and are instantaneously vitrified. The vitrified microdrops are then stored in liquid nitrogen (Dinnyés et al., 2000).
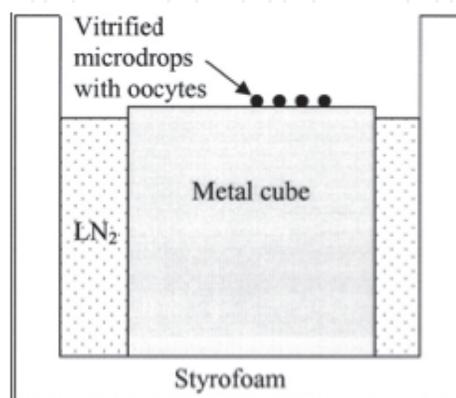

Fig. 10. The solid surface vitrification (SSV) device

3.2 Closed supports

3.2.1 Cryotip

CryoTip consists of a plastic straw with a thin part (250 μm inner diameter, 20 μm wall thickness and 3 cm length) connected to a thick part (2000 μm inner diameter and 150 μm wall thickness, 4.5 cm length) and equipped with a movable protective metal sleeve (Fig. 11) (Kuwayama, Vajta, Ieda & Kato, 2005).

Fig. 11. The CryoTip is a finely pulled straw designed for holding gametes or embryos

Embryos are loaded in approximately 1 μl solution into the narrow part of the CryoTips without any air bubbles by aspiration of medium. Subsequently, the straw is heat-sealed at both ends, the protective sleeve is pulled over the narrow part and the device is plunged into liquid nitrogen. The time required for loading, sealing, adjustment of the sleeve and plunging does not exceed 90 s. The use of the closed CryoTip system eliminates potential embryo's contamination during cryopreservation and storage without compromising survival and developmental rates in vitro and in vivo (Kuwayama, Vajta, Ieda & Kato, 2005).

3.2.2 Isachenko Method

In the *Isachenko Method* (Isachenko et al., 2005), embryos are located inside a open-pulled straws (OPS). The OPS is placed inside a sterile insemination straw (indicative size 90-mm), manufactured from standard 0.5-mL insemination straws. One end of sterile insemination straw is previously sealed using a hand-held sealer. The open end is hermetically closed by a metal ball and this container (OPS and sterile insemination straw) is plunged into liquid nitrogen ("straw in straw" vitrification). The *Isachenko Method*, applied to biopsied mouse pronuclear embryos is resulted efficient as conventional vitrification, guaranteeing a complete isolation of embryos from liquid nitrogen and avoiding potential contamination by pathogenic microorganisms.

3.3 Innovative vitrification devices

A new solution to increase the cooling rate reducing the use of cryoprotectants consists in the physical reduction of liquid nitrogen temperature, as happens in the **Vit-Master**, a new device developed at IMT, Israel. In order to avoid the vaporization of N2, the temperature of liquid nitrogen is reduced until - 210° (boiling point of nitrogen), applying a negative pressure (Arav et al., 2002). The evaporative cooling causes the nitrogen to partially solidify, thus creating a nitrogen slush. Samples immersed in nitrogen slush cool more rapidly because they come into contact with liquid nitrogen sooner than those immersed in normal liquid nitrogen (Cai et al., 2005). The *Vit Master* vitrification machine can provide a very high cooling rate (up to 135,000°/min). The cooling rate is especially enhanced in the first stage of cooling (from 20 to -10°), when it is six, four or two times higher with 0.25-ml straws, open pulled straws (OPS) or electron-microscope (EM) grids, respectively. Between -10 and -150°, the cooling rate is only about doubled by use of the *Vit Master*, but that was found to be enough to reduce the chances of devitrification and recrystallization during warming.

Research about using a **Pulse Tube** for Vitrification is ongoing at "Sapienza" - University of Rome.

4. Ultra-rapid freezing

Ultra-rapid freezing can be considered a midway technique between slow freezing and Vitrification. It is quicker than the slow-freezing technique, does not involve the use of programmable machines and requires lower concentrations of cryoprotectant agents (CPA) than those used in vitrification.

Experimental results demonstrate that this technique has lower performances than slow freezing's and vitrification's ones (AbdelHafez et al., 2010).

5. Comparison between vitrification and slow freezing

Vitrification is an attractive freezing technique: supports required are cost effective and experimental data show an high survival rate after thawing. For example, a survival rate of 99% was quoted in (*KITAZATO BioPharma Co., Ltd. - http://www.kitazato-biopharma.com/*, n.d.) using a *Cryotop* support.

However, vitrification exposes cells to a high risk of contamination, since cells are generally plunged directly into liquid-nitrogen. Risk of contamination is reported in (Bielanski et al., 2000), where cells frozen using vitrification were exposed to the bovine immunodeficiency

virus (BIV), that can be considered a model for retrovirus like the human immunodeficiency virus (HIV). Risk of Hepatitis B contamination is analyzed in (Tedder et al., 1995).

Moreover, vitrification requires a greater amount of CPA (CryoProtectant Agent) than Slow Freezing does, increasing the toxicity of the environment.

In order to reduce the risk of contamination, *closed supports* for vitrification were developed (*Cryotip* and *Isachenko Method*). However, a lower survival rate is obtained than using an *open support*.

A lower survival rate after thawing using Slow Freezing instead of Vitrification was claimed by many authors. (Fadini et al., 2009) reports a survival rate of human oocytes of 78.9% using Vitrification, while it is reduced to 57.9 % using Slow Freezing (*p-value* lower than 0.0001); similar results are shown in (Vutyavanich et al., 2010) (where survival rate of human spermatozoa is measured equal to 64.8% using Vitrification and equal to 50.4% using Slow Freezing, *p-value* equal to 0.0036). However, many authors believe that a better understanding of slow freezing principles will improve its performances (Bianchi et al., 2007; De Santis et al., 2007; Edgar, 2009; Fadini et al., 2009; Mcgrath, 2009).

Both Vitrification and Programmable Freezers (the most common machines use for Slow Freezing) require a supply of liquid-nitrogen, that is a limiting factor in many situations of inefficient or absent nitrogen distribution network, such as small industries, isolated places and during transport of cells. In order to overcome to this limitation, two alternative systems for Slow Freezing (*Asymptote EF600* and *Pulse Tube Cryocooler*) have been developed.

Stirling Engine[7] and Pulse Tube Cryocooler are closed-cycle machines, reducing risk of contamination and toxicity. A cells freezing system based upon closed-cycle machines is a viable commercial solution, especially for those markets where liquid nitrogen supply is difficult or excessively expensive, or during transport.

However, Stirling Engine exhibits high vibration, thus the nucleation process can not be inducted manually. Moreover, vibrations might damage cells. Those problems are avoided using a Pulse Tube cryocooler.

The application of a Pulse Tube Cryocooler for cells cryopreservation is under developing at "Sapienza" - University of Rome Laboratory of Mechanical Engineering. A validation of the proposed system with the assessment of cells survival rate after thawing is envisaged as next step. Future work will also focus on the development of a cost effective control system which allows the operator to set a desired cooling rate.

6. Acknowledgment

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URL: *http://www.ncbi.nlm.nih.gov/pubmed/20113959*

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[7] The Stirling Engine is used in the Asymptote EF600

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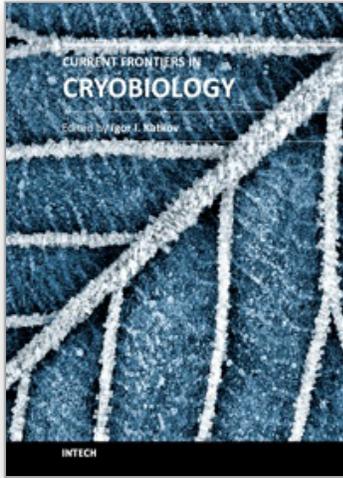
Current Frontiers in Cryobiology

Edited by Prof. Igor Katkov

Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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