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# Novel Approaches to Cryopreservation and Long Term Stabilization of Progenitor Cells

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It is well established that storage of cells in a liquid milieu leads to degradation processes, and to eventual loss of cell viability. At the same time, intracellular ice is lethal for the majority of cells [43]. Thus, the only stage in which cells can be stabilized would be a solid-like phase in which intracellular ice crystals are not formed, or have not grown to the "critical lethal size". Such a vitreous ("glassy") state has elevated viscosity (up to $10^{14}$ Pa×sec), so processes of chemical and physical degradation are essentially stopped for the duration of the experiments or storage. There are five basic ways to achieve vitrification; all lead to drastic decreases of water activity:

– *Equilibrium* (slow) *freezing*-out of the bulk of water to ice form, followed by storage at extremely low temperatures, usually –196°C, recently –130°C to –80°C. This is the 'mainstream' conventional cryopreservation, which in the majority of cases requires the use of permeable and impermeable cryoprotective agents (CPAs).

– Slow freezing to moderately low (around –40°C) temperatures, sublimation of the bulk of ice at very high vacuum, and secondary drying of the "cake" at elevated (up to 30°C) temperatures. This method called *lyophilization* is widely used in food production, microbiology and in the pharmaceutical industry, but has had so far very limited applications in the preservation of animal cells and higher plants.

– Ice-free vitrification of cell suspensions, tissues, and organs at very low temperatures and moderate to high rates of freezing. This method requires the use of a high concentration of vitrificants (historically but erroneously referred to as "CPAs" in analogy with slow freezing), which elevates the viscosity of the milieu and prevents the ice formation during cooling and devitrification during warming. We refer to this method as "*vitrification proper*", and it has had limited but notable success in preserving animal oocytes, embryos, and organs, as well as plant specimens.

– Ice-free vitrification of a bulk solution by very fast (abrupt) plunging into a cooling agent such as liquid nitrogen. The extremely high rate of cooling ($10^5$ to $10^6$ °C/min) and practically instant warming prevents ice formation in small samples, and does not require the use of potentially toxic high concentrations of CPAs, so we refer to this as "*CPAs-free vitrification*".

– *"High temperature" vitrification* of a highly dehydrated sample and stabilization by air/vacuum drying at temperatures >0°C.

In this paper, we discuss the mechanisms of vitrification that are common to all these methods, as well as specific aspects, advantages, and pitfalls of each one. The emphasis is put on stabilization of embryonic and adult stem cell cells by means of low and high temperature vitrification.

## 1. Vitrification of Cells: Historical Background

The idea of using freezing and drying to preserve food and other perishable materials goes back thousands of years to prehistoric times. However, long-term preservation of living objects, and particularly of cells of animals such as vertebrates, mollusks and insects, is a novel approach. A truly pioneering work on cryopreservation was performed by an Italian priest and scientist Lazzaro Spallanzani in in 1776 when he "froze" stallion sperm in snow, noting the recovery of sperm motility upon warming (published in [59]). Later, in the 19th and the first half of the 20th century scientists elucidated some aspects of the mechanisms of cold adaptation of living matter, particularly, those used by plants and fungi. The most important initial contributions in this area were made by the Father Luyet, who has been rightfully called the founder of the science of Cryobiology. From the outset, he recognized that ice damage must be avoided, and that vitrification could be a method for preservation of cell viability [40] In 1938 Luyet and Hodapp achieved survival of frog spermatozoa vitrified by plunging into liquid nitrogen [39] and later several Western European groups reported their experiences with attempts at cryo-preservation of fowl [56], human [23, 28, 48] and rabbit [23] spermatozoa with varying success. These efforts did not receive the recognition they deserved, hindered by the variability of the reported results and by the Second World War as well.

While the contributions of Father Luyet have been well recognized, the research of another pioneer in the field has not been widely known within the Western scientific community. Emmanuil Yakovlevich Graevsky. His work in 1946-1948 was not only confirmation of the Luyet's observations, but Graevsky and colleagues introduced a lot of novel techniques and

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ideas, both regarding mechanisms of vitrification and devitrification, and practical methods of vitrification. Particularly, pointed out the possibility of using vitrification as a method of long-term stabilization and preservation of cells [18, 19], and in [17, 19] he reported that following Luyet's technique, he successfully frozen frog sperm with even better than the original Luyet's results. Moreover, he was one of the first to use a small loop to effectively vitrify suspensions of bacteria [20]. Only in the past few years this method (now called "cryo-loop") has been applied to the vitrification of mammalian cells, particularly to the cryoprotectant–free vitrification of human spermatozoa, which we will discuss in more details below.

The approach to vitrification developed by Luyet and later improved by Graevsky and by Hoagland and Pincus (they also used cryo-loop in [23]) was based on the application of *relatively* fast cooling rates. Yet, recovery of the sample viability was low and very variable mostly because, as the authors recognized, sufficiently rapid cooling and warming, which must be *very* high for low concentrations of CPA's, were not achievable. One of the reasons was that very low temperature cooling agents such as liquid nitrogen and liquid oxygen were not used in their research. As a result, the mainstream of cryobiology turned to preservation methods based on slow freezing. This approach was facilitated by the discovery of the protective properties of glycerol on human and animal sperm when spermatozoa were frozen to extremely low temperature, made independently by Igor Smirnov in the USSR [57] and by Alan Parkes and colleagues in the UK [49, 58]. (Parkes also deserves mention for coining the term *cryobiology*, the study of "frosty life"). Note that the cryoprotective qualities of glycerol and other low molecular weight, mostly non-electrolytic substances had been described before. For example, Bernshtein and Petropavlovski in 1937 used 0.5 to 3.0 Mol/L glycerol and other electrolytes for freezing of bull, ram, stallion, boar and rabbit spermatozoa to a temperature of –21°C; they reported to have obtained the best results at 0.5-2 Mol/L glycerol [8]. However, only after development of stable long term cryo-preservation at very low temperatures (–196°C and lower) initiated by Smirnov's and by Parkes's groups, the true era of practical applications of cryobiology, particularly for animal breeding, began in the West and in the Soviet Union. For the next four decades, the slow-freeze method dominated the field of cryo-preservation, and it still remains the basis of the majority of cryobiological techniques in use in production facilities and research laboratories. Progressively more sophisticated understanding of the mechanisms underlying cell damage during the slow to moderate rate of freezing as well as methods to prevent it were contributed first of all by Mazur [42, 43], and also by Lovelock [38] Meryman [46], and by many others.

While 'conventional' slow freezing had given satisfactory results for many suspended cells, such as blood, sperm, and embryos, issues of ice formation and non-homogeneity of samples' thermal profiles made slow freezing and thawing a formidable task for tissues and organ. The breakthrough came when Fahy and colleagues vitrified an entire organ – a kidney [13]. The presence of high concentrations of vitrificants, particularly glycerol, allowed an ice-free bulk glassy state to be achieved both inside the organ (it was done by perfusion) and in the external milieu. By tradition, glycerol and other vitrificants were still called "cryoprotective agents" (CPAs) as for slow freezing, probably because the same chemicals were used for both applications. However, one has to remember that for ice-free vitrification, they play a completely different role (as glass-formers) than for slow freezing (as osmotic buffers and "water holders").

Vitrification eliminated many of the problems related to the slow freezing of liquid water to an ice phase, particularly extensive rehydration and osmotic damage, the increased ionic strength of concentrated eutectic solutions, and shifts in pH. However, vitrification in high concentrations of vitrificants introduced its own set of problems. Notable among them were osmotic damage during addition and removal of vitrificants, CPA toxicity, mechanical cracking of glasses, and devitrification due to inadvertent thermal cycling during storage.

Nevertheless, practical applications of vitrification as a method continued to grow, specifically after Rall and Fahy reported the successful vitrification of mouse embryos in 1985 [52]. However, more widespread use of vitrification was slowed by the inherent pitfalls of high-CPA methods, and also by the lack of under-standing of the mechanisms of vitrification and devitrification by most practitioners of the craft. By the 1980s, the fundamental work of Luyet had been largely forgotten, and the theoretical and practical contributions of Graevsky—published in Russian—had never achieved the recognition they deserved. Unfortunately, the pioneering work of the cryobiologists of the 1930s, 1940s, and 1950s is still underappreciated today.

In the 1990s, vitrification was applied to new areas such as oocytes and ovarian tissues. However, the sensitivity of germ and blood cells to cryoprotective agents made this approach prohibitive, despite the fact that vitrification can be simple, fast, and cheap. Instead, practitioners, farmers, and blood bankers committed to the slow-freezing route, with its attendant need for expensive, programmable cryo-equipment and skilled technical personnel. Vitrification was a resounding

success with non-mammalian species, particularly insect embryos and plant species [44, 51, 60, 63]. Vitrification protocols have been developed for cryopreservation of marine invertebrates [12], turbot [54], and zebrafish [65] embryos. Moreover, the recent discovery of the high ice-nucleation temperature of zebrafish embryos suggests that slow freezing is not a suitable option for zebrafish embryos, so vitrification seems to be the method of choice [22]. Greg Fahy, one of the pioneers in the use of vitrification to preserve entire organs, and his collaborator Brian Wowk recently reported progress in custom-synthesizing vitrificants, and tailoring cooling and warming profiles to fit their ice-blocking qualities [14].

The advances in methodology recounted so far improved cell survival without much reduction in the dependence on high concentrations of cryoprotective agents. A new breakthrough came in 2002 when Nawroth et al [47] vitrified human sperm *without any conventional CPAs* by using a simply constructed cryonic copper loop to present the sperm in a film, or a pellicle. The key was very rapid cooling up to hundred of thousands °C/min [24]. They had, essentially, rediscovered the method used by Luyet and Graevsky more than a half century ago. It has developed that the rate of *warming* is even more important to survival under these conditions than is cooling. Thus, it appears that intracellular vitrification can be achieved at relatively low rates of cooling without the use of CPAs and preliminary dehydration. This is presumably facilitated by the low All of the methods mentioned above rely on storage at an ultralow temperature (about –196°C in liquid nitrogen) ice-free vilification. However, independently, vitrification has long been used in food preservation and pharmaceutical industries. Here vitrification is followed b y sublimation of ice, with the objective being the achievement of long shelf-life stability. A lot of important knowledge, such as the crucial role of the glass transition temperature (Tg), stability, shelf-life, plasticizers vs. glass-formers, all have come to cell stabilization from that area. High temperature vitrification is now an interdisciplinary field, where collaboration of specialists in thermodynamics, solid matter, physical chemistry, biophysics, cell biology, and medicine has become a matter of routines. It is especially true for lyophilization and high-temperature (above 0°C) drying (vacuum or air- likewise) of mammalian cells. However, despite the claims of success in lyophilizing erythrocytes [7, 15, 35], platelets [53, 61, 64], or even spermatozoa [9, 36, 62], the clinical or commercial applications of high temperature stabilization and long term storage of mammalian cell remain an elusive goal.

The most novel approach to achieve glassy state in mammalian cells at high, even ambient temperature is vacuum or air drying at temperatures above 0°C. This is an emerging area of animal cell stabilization, in which our group has been one of the pioneers [16, 21, 31, 33, 50], however, still the data in literature are scarce [41]. We will discuss this approach and our recent results in a separate Chapter in more details.

Let us now give a brief description of basic approaches for vitrification, its achievements, and future directions.

## 2. Conventional (Slow) Freezing for Cryopreservation

Traditionally, in the cryopreservation community, slow freezing is not associated with vitrification and is often mentioned as an alternative approach. However, in fact, during growth of crystals, the bulk of water is freezing out to the ice, and remaining liquid part becomes more and more concentrated. The cell cannot survive with *big* ice crystals inside, so intracellular ice formation *in the majority of cases* kills the cells [43]. However, if freezing is performed slowly enough (the critical speed depends on the surface/volume ratio and water membrane permeability as function of temperature), than cells are loosing water practically in equilibrium with the extracellular compartment. The quantitative theory of the process was introduced by Peter Mazur [42], and developed further by other investigators. Aside from technicalities in the description processes, the major aspect remains the same: viable cells will be those ones, which are located in a solidified space between the ice crystals, and their intracellular compartments are also free of ice. Such solid state that assures survival of cell at this extremely low temperature can be only the glassy state. As we see, even for conventional slow (equilibrium) freezing *vitrification* as the final phase of the freezing process is essential for Despite that this method is widely used and has drawn a lot of attention, there are many interesting questions remaining, particularly, the central dogma that "intracellular ice always kills" recently underwent scrutiny by Dr. Locksley McGann and colleagues [1-2]. Moreover, even Peter Mazur, the author of this postulate, has recently questioned its absolute applicability to all scenarios [45].

Even though conventional equilibrium freezing has been successfully used for many type of cells, and some tissues, some cell types, such as human embryonic stem cells (hESC), are very sensitive to use of it. As we showed recently, slow freezing with 10% DMSO, which has been widely used for cryopreservation of many cell lines, including mouse ESC, led to almost complete depletion of production of a crucial marker of pluripotency, Oct-4. The native protein, under the control of the wild-type promoter, was conjugated with green fluorescent protein (the data were briefly reported in [30-32, 34] and will be published in details elsewhere). That means that even though over 50%

of the cells survived cryopreservation, the overwhelming majority of them would uncontrollably differentiate or eventually die due to senescence and apoptotic transformation, so only 10% of original stem cell population regained its 'stemness' (pluripotency) after conventional freezing (Fig. 1). Taking to the account that pace of dividing hESC much slower than "standard" cell lines (4-6 days vs. 18 to 48 hours for cells like primary fibroblasts or HeLa), the problem of optimization of cryopreservation techniques is a very challenging and important problem.

So, despite definite successes, the slow equilibrium process has its own limitations and therefore new methods have to be developed.

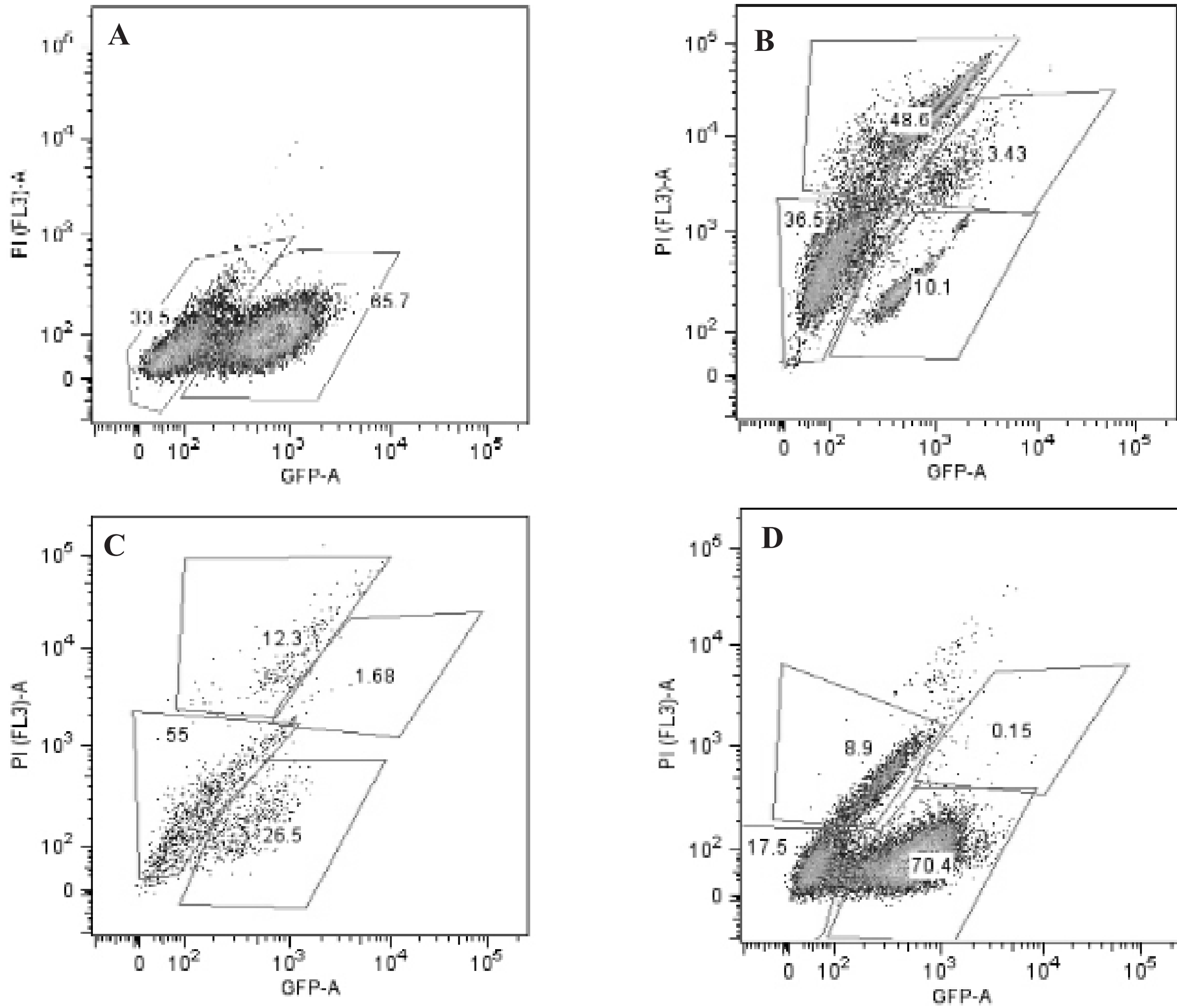
**3. "Conventional" Vitrification with High Concentrations of CPA and Moderately Fast Freezing and Warming Rate ("Fahy's approach")**

As mentioned above, there are two ways of achieving ice-free vitrification upon cooling and avoiding devitrification during warming. The first method was proposed and extensively investigated by Luyet and his colleagues in the 1930-50s and by Graevsky in 1946-48. They stated that if a specimen was cooled and warmed *very* quickly then ice would not be formed so vitrification of the bulk solution would occur. However, as Fahy pointed out [13], this approach would require special small volume techniques that were not widely available in Luyet's and Graevsky's time. As a result, this direction was essentially abandoned, and investigators focused on another approach, in which relatively low (<200 K/min) rates of cooling and warming can be used but requiring very high (about 60% (w/w) cryoprotectant concentrations. This method of vitrification was used by Fahy et al. [13] to successfully preserve living systems. They were the first to report the vitrification of a whole organ (kidney) using a combination of high concentrations of PCPs and high pressure (up to 1000 atm). For the preservation of large organs and tissues, this approach can be considered as the matter of choice due to the inherent difficulty in achieving homogeneous distribution of freezing and warming rate, which is crucial for "classical" cryobiological protocols. Report of the vitrification of mammalian embryos was soon to follow [52], and the method became a popular tool for the cryopreservation of large single cells, and cell clusters such as oocytes and embryos. However, this did not imply that the original approach proposed by Luyet for suspended small single cells was impossible in principal. The problem was simply that procedures for ultra-rapid cooling and thawing had not been sufficiently developed yet. The recently introduced carrier systems such as open pulled straws (OPS) and cryo-loops have changed the situation.

**4. Cryoprotectant-Free Vitrification by Very Fast Freezing and Instant Thawing ("Luyet-Graevsky's Approach")**

The main process involved in crystallization and conditions for ice-free can be summarized in the supplemented phase diagram proposed by Greg Fahy [13]. The larger difference between homogenous ice crystallization curve Th and the glass transition curve Tg, the higher probability that crystallization of the solution will occur during freezing. That area called Zone I and Fahy and colleagues considered probability of vitrification at the rate of freezing they used (up to thousands °C/min) very low, and probability of ice crystallization would be almost 100%. As the concentration of CPA (vitrificant) increases, the temperature of homogeneous ice crystallization drops, while Tg rises. At the point when they Th meets Tg, Zone III begins, and there is a probability of achieving vitrification during appropriately fast freezing. However, devitrification curve Td indicates that there would be a higher possibility of devitrification during warming. Td drastically increases with higher % of CPA (vitrificant) due to dramatic rise of viscosity, and when Td meets the non-homogeneous equilibrium crystallization (melting) curve Tm, Zone IV begins at the point, from which crystallization and devitrification never occur at any reasonable rate cooling and warming. Note, however, that for "*conventional*" vitrificants such as glycerol, such concentration is in the range 65-70%, which is extremely high, and basically none of known mammalian cells would withstand such enormous (tens of Osm) osmotic pressure. Thus, vitrification should be performed in a kinetic way, playing with concentration of the vitrificant and rate of freezing and warming. Fahy and colleagues defined that zone when practical vitrification is achievable, from 45% of CPA, marking this as Zone II.

The critical speed of cooling and warming, however, is reverse to the concentration of the CPA, and vise versa: as higher speed of rate of cooling/warming, as lower concentration of CPA is needed, so basically *the border* between 'non-achievable' and 'achievable' vitrification (Zones I and II) *is arbitrary*. It means that if the speed of cooling and warming are high enough, then concentration of the glass-formers can be very low (the dashed line is a new border between Zone 1 and Zone II). That has been the main direction for the last decade in vitrification cryobiology, particularly for reproductive cells. Several types of devices, such as open-pulled straws, microscopic grids, and cryoloops were explored to increase speed of cooling and thawing and, thus, to decrease the CPA concentrations to values of 25-30%. While it has been working well for oocytes and embryos, small cells like sperm and erythrocytes have been proven to be practically intolerant even to such "moderate"



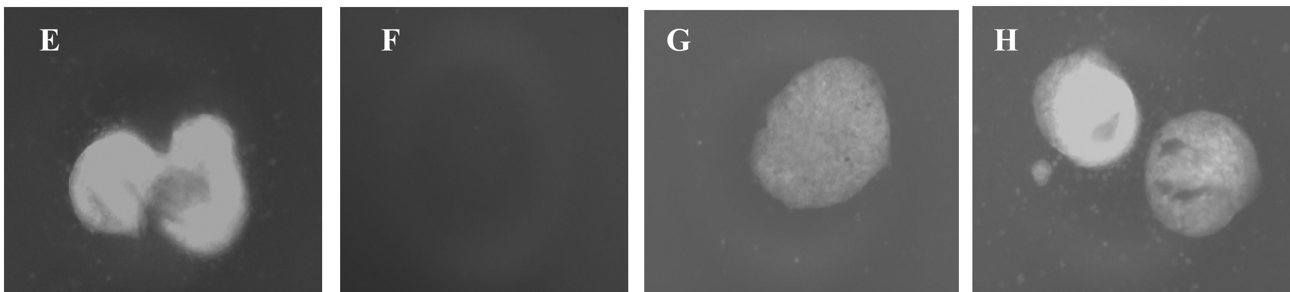




**Fig. 1.** Human Embryonic Stem Cells (hESC): Loss of a marker of pluripotency Oct-4 (transcription factor conjugated with Green Fluorescent Protein) after “standard” slow freezing and the use of 10% DMSO. Figs **A, B, C, D**: two color FACS analysis channel 1 is GFP (indicates presence of Oct-4); channel 2- PI, a stain for viability. Figs **E, F, G, H**: fluorescent microphotography of the same samples of the cells using green filter. Figs **A, E**: fresh cells (unfrozen control); 65.7% of the cells are both GFP-positive and PI-negative represent non-differentiated viable cells, while 33.5% are differentiated (GFP-negative) but viable cells. Figs **B, F**: hESC in 3 days after thawing. The four distinctive regions can be determined: 36.5% of the population are viable but differentiated cells, 48.6% are unviable differentiated cells (probably, due to apoptotic pathway), 3.43% of cell are PI-positive, but they still have a sizable of Oct-4 (probably, senescent cells), and only 10.1% of the cells are viable non-differentiated, this pool is the subpopulation, which presents valid hESC. Figs **C, G**: hESC in 7 days after thawing: amount of non-differentiated viable SC doubled due to division; substantial number of the differentiated non-viable cells from Day 3 have died out, while some viable non-differentiated on day 3 cells are gone to the differentiation pathway. Amount of senescent cells is negligible. Figs **D, H**: hESC in 14 days after thawing. Majority of the senescent cells have died out, and doubling of non-differentiated cells eventually replaced the pool of differentiated and presumably non-proliferating cells. This diagram and the general look under fluorescent microscope are almost identical to fresh control (cf. **A** *vs*. **D**, and **E** vs. **H** respectively). The data were briefly reported in [29-32] and will be published in details elsewhere.

concentrations of CPA (e.g., 30% of most widely used CPAs as glycerol, ethyleneglycol and propyleneglycol equals to 15-20 holds of the isotonic value). However, a major breakthrough came in 2001, when a group from Germany managed to successfully vitrify sperm without any conventional cryoprotectants when it was cooled and frozen in very thin films (pellicles) in copper cryoloops [47]. They basically rediscovered the original Luyet-Graevsky approach, but with all new knowledge that had been accumulated since the pioneering works in 1939-1948. We then have been engaged in collaboration with this group, and recently published several joint papers [24-27], in which we suggested that the extremely high rate of cooling (several hundreds of thousands °C/min) and almost instant warming by dissolving in a warm media made vitrification during freezing without de-vitrification during re-warming practically feasible. Taking into the account that the culture medium contained proteins (serum albumin), and the internal cellular milieu is abundant in high glass transition temperature components such as proteins, polysaccharides and nucleic, the vitrification can be achieved at very low concentrations of non-osmotically active glass formers, so no potentially toxic CPAs are needed.

**5. Vitrification by Ice Sublimation at T<0°C with Consequent Secondary Drying at Higher Temperatures (Freeze-Drying or Lyophilization)**

Another direction, in which the processes of vitrification are involved is freeze-drying or lyophilization. The latter term is sometimes mistakenly applied to ice-free high temperature vacuum drying as well. While this method of preservation of perishable items has became a method of choice in the food and pharmaceutical industry long ago, success in preservation of mammalian cells has been marginal. As there are several presentations at this Meeting regarding lyophilization, we will skip this topic. We would like to emphasize, however, that the main reason why engineers and production companies have chosen freezing-sublimation at high vacuum is the fact that at low water activity the sample becomes very viscous, and that makes evaporation of the bulk volumes of liquid practically unfeasible. In contrast, creation of the "lyophilized" sublimation cake and subsequent further rehydration during secondary drying facilitates the process.

However, it still takes days to complete freeze-drying. At the same time, if the surface/ratio of the sample can be increased drastically, such during foaming, spray drying, or drying in thin films and monolayers, it will make high temperature drying practically feasible.

**6. Vacuum and Air Drying at Temperatures Above 0°C: Application to Blood, Stem, and Adherent Cells**

As early as in 1938, Lepeshinskaya [37] observed that bacteria that were left in a foam-like solidified environment for many days retained high viability after rehydration (she called it "moisturisation"). Later in the nineteen-fifties and -sixties, Annear [3-6] dried some bacteria in a foam-like solid matrix, which he obtained by elevating temperature of the liquid (boiling). Boiling occurs when the vapor pressure on the surface of liquid exceeds the ambient pressure, so one way to achieve boiling and subsequent foam-like structure is to decrease the ambient pressure by applying moderate vacuum. For a long time, specialists working in lyophilization had tried to avoid foaming as a non-controllable process which led to lower-quality cakesRecently, this method, which has a number of advantages over lyophilization, particularly in the speed of drying and independence of time of drying from the volume of the sample (scalability), has drawn the attention of several groups, particularly Bruce Roser in UK and Viktor Bronshtein in USA [10, 11, 55]. This method has the potential to be a good alternative to lyophilization (see Fig. 2). In the future, it may be successfully applied to the stabilization of suspensions of mammalian cells, such as platelets, erythrocytes, and B-cells (oft-used for biosensors).

Yet another approach is to dry cells in monolayers, which is a natural fit for undisturbed adherent cells. Recently, we were able to dry human fibroblasts (a close biophysical analog to mesenchymal stem cells)

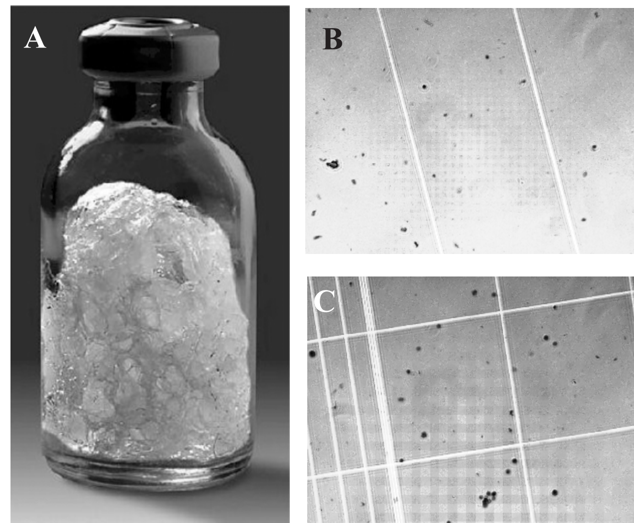



**Fig. 2**. High temperature stabilization- vitrification by drying: foamed mammalian nucleated cells. Hematopoietic stem cells vitrified by foam formation and subsequent drying at 37°C to 40°C (final product is shown in Fig. **A**). The foam can be stable for practically infinite time. Viability of cells (preliminary, non-optimized protocols) after 2 years of keeping in a fridge ad 4°C was more than 20% (staining with Trypan blue is shown on Fig. **B**). Lyophilization killed all the cells so all cells were stained after rehydration (Fig. **C**).

to water content as low as 0.47 g water /g dry weight (for comparison the water content of intact fibroblasts is in rang 5-6 g water /g dry weight). For comparison, the water content of fibroblasts in their native state is X.X g water/g dry weight. The cells maintained their viability when stored at room temperature for up to 3 weeks (manuscript in preparation). Similar success on vacuum or air-flow drying of nucleated cells have been reported by other groups as well. However, to insure high Tg of the dried sample, and therefore, long term high temperature stability, the final water content should be in the range *0.30 – 0.05* g/g dry weight. At this writing, no researchers are known to have achieved this high level of desiccation without causing irreparable damage to cells. The next few years should see further advances in this area that are as significant as those that are discussed in this review (**Fig. 3**).

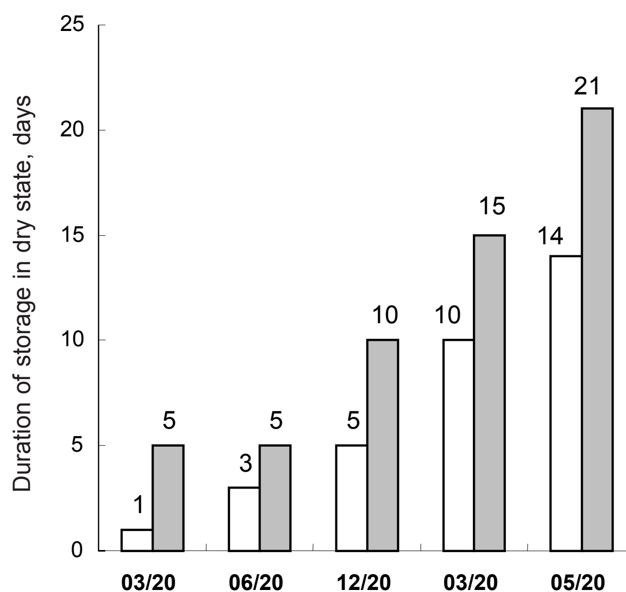



**Fig. 3.** Progress in drying of nucleated mammalian cells achieved by our Laboratory in 2002-2004. The data were briefly reported [29-33] and will be published in details elsewhere: □ – viability >50%; ■ – viability >20%/.


*Many materials reviewed in this paper were obtained in collaboration with our colleagues, and it is our great pleasure to name Fred Levine (UCSD Cancer center), Min (David) Kim (UCSD, The Burnham Institute), Vladimir and Evgenia Isachenko (Bonn University, Germany), Iskren Puhlev (UCSD Burn Center), Victor Bronshtein and Nadia Katkova (UPT, Inc), Stephanie Oppenheimer (Osiris Therapeutics, Inc), Marc Mercola, Maria Barcova, Alexey Terskikh, and Ruchi Bajpai (The Burnham Institute), Natalia Karpova (NIIH&BT, St. Petersburg, Russia), and many others.*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