Advances in Media for Cryopreservation and Hypothermic Storage

by John M. Baust

To many scientists, researchers, and technicians, the discipline of biopreservation (commonly known as *cryobiology*) remains an afterthought in product and process development. Biopreservation is a simple means to an end for storing/shipping intermediate biological materials or final products. It is, however, an extensive and involved process. Contributing to the general perception is the fact that the basic processes remain relatively unchanged, dating back over a half a century. Although countless efforts have been dedicated to the study of preservation, the tools (solutions and protocols) widely used today are for the most part based on dated principles and associated technologies. The "status quo" has led to a perception of preservation as an afterthought. And this perception has contributed to a relatively limited use of preservation (mainly the establishment of master cell banks) in the bioprocessing industry — at least until now.

The field of biopreservation study dates back to a 1949 paper by Polge, Smith, and Parks (**1**), which first reported successful cryopreservation of bovine and avian spermatozoa using glycerol and dry ice. Ten years later, Lovelock and Bishop (**2**) took the next major step in cryopreservation by identifying the cryoprotective properties of a number of agents, including dimethyl sulfoxide (DMSO). From that time, numerous investigators have worked toward the goal of understanding and improving preservation outcome.

What followed was the emergence of *colligative cryopreservation,* which is grounded in Mazur's two-factor hypothesis of cell damage (**3**). Simply stated, if cooling rates are too rapid, cells retain high levels of freezable water during the cryopreservation process, which results in lethal intracellular ice damage. Conversely, if cooling rates are too slow, cells are exposed to high (toxic) levels of penetrating cryoprotectants. So an optimal cooling rate positions cells between these two lethal conditions.

Application of the colligative-based cryopreservation strategy typically provides high levels of immediate survival (structural) but does not provide the cellular support necessary for equivalent long-term survival (>72 hours) — nor will recovered cells demonstrate completely normal physiological function. In other words, the principles underlying colligative-based cryopreservation provide only a partial solution and are not necessarily compatible with the need to recover optimally functional cells.

The two methodologies commonly used to facilitate cellular preservation are hypothermic storage (~4 °C) and cryopreservation (–80 to –196 °C). Historically, these areas have been regarded as two unique processes sharing little in common beyond the use of cold temperatures to slow biological time. Recently, however, a series of investigations on both have revealed a number of similarities between the processes at a cellular/molecular level (**4–6**). These studies have shown that cell survival (and function) can be significantly affected by process variables and that, through proper preservation solution design, preservation efficacy can be substantially improved.

Dr. Kristi Snyder retrieves cryovials from frozen inventory from a liquid nitrogen storage dewar (PHOTO COURTESY OF CELL PRESERVATION SERVICES, INC.)

Hypothermia or Cryopreservation

Hypothermic storage and cryopreservation are two very different processes for achieving

the same goal: storage of cells and tissues. The primary differences lie in their respective physical environments: storage in either a liquid or a solid/frozen state. Another difference is the storage interval (time). Hypothermic storage is typically limited to a few days to weeks, whereas cryopreservation can last from months to many years.

Control of the physical events of ice formation has been extensively studied and modeled using numerous systems, from red blood cells to spermatozoa to nucleated eukaryotic cells such as hepatocytes, keratinocytes, Chinese hamster ovary (CHO) cells, and so on (**7–12**). Studies have focused on controlling the physical–chemosmometric characteristics of cells undergoing cryopreservation using molar concentrations of chemical agents such as DMSO, glycerol, and ethylene glycol in a cell culture medium of choice containing elevated levels of animal serum or protein. Such efforts led to the development of preservation protocols that provide for structural protection and corresponding viability of cellular systems at "appropriate" levels.

Absent from traditional approaches to biopreservation is a focus on the cellular and molecular needs of cells undergoing preservation. Here, similarities between cryopreservation and hypothermic preservation can be found. Although it may appear that all biological processes halt once a system is frozen, this is not at all the case. During freezing, cells remain in a biochemically active unfrozen state while encased in a frozen ice matrix. Not until temperatures drop below the glass transition point (T_g) of the cryoprotectant–cell-solution mixture (typically below –100 °C) will cells enter a glassy state, in which biochemical and biomolecular activity cease. During freezing and subsequent thawing, when temperatures are above T_g, a relatively significant set of molecular and biochemical events occur within each cell that drastically influence its post-thaw viability and function. In this temperature range (~+15 °C to –99.9 °C) a number of similarities can be seen in cellular response mechanisms between cryopreservation and hypothermic storage.

Such events include the formation of free radicals, uncoupling of biochemical pathways, intracellular waste accumulation, ion-gradient disruption, protein denaturation and degradation, and enzyme cleavage and activation (**4, 5**). Recently, it has been shown that those and other events can activate apoptotic and necrotic cell death pathways (**13**). Such activation has been reported to lead to the phenomenon of delayed-onset cell death, which causes a disconnect between the measure of viability immediately poststorage and that of true survival 24–48 hours later (**5**).

Preservation-Induced Stress

As introduced above, the preservation process places a number of stresses on cells as a result of temperature-dependent uncoupling of metabolic and biochemical processes. These include the production of free radicals by disruption of oxidative respiration, which are detrimental to cells due to the downstream effects of lipid peroxidation, DNA and RNA damage, cytoskeleton structural component alterations, and so on. Alterations in cellular membrane structure, fluidity, and organization can also activate membrane receptors, initiating a cascade of intracellular events including stimulation of stress-response pathways and apoptosis. Disregulation of cellular ionic balance through a shutdown of membrane-bound Na^+/K^+ pumps and Ca^{2+} ion channels activates stress-response mechanisms including the release of calcium from intracellular stores, osmotic influx, and cellular swelling (**14, 15**).

A host of additional stress response mechanisms can be activated through low-temperature storage to the detriment of cells. Along with negative responses, however, concurrent activation of survival pathways can occur within cells exposed to low temperature. This represents each cell's attempt to reduce/control its stress and minimize detrimental effects. In many cases, unfortunately, such survival responses are ultimately futile and more detrimental through consumption of resources such as ATP, thereby placing cells into even greater energy deficits that further promote cell death.

The list of known cellular stresses associated with biopreservation processes is extensive and by no means complete. As investigations continue in this area and new reports emerge monthly, a common theme has surfaced in that all stress processes (both physical and biochemical) associated with preservation elicit the activation and progression of delayed apoptotic and necrotic cell death. In many cases, the latter results from a shift from apoptotic processes to secondary necrosis due to a lack of energy and a continual buildup of cell stress — to a point where apoptosis is no longer an option (**6**). Accordingly, apoptosis has been a recent focus of study in the preservation sciences.

Apoptosis

Apoptosis (gene-activated, programmed cell death) has been studied extensively in a variety of systems too numerous to comprehensively cite here (**16**). It plays an integral role in development and maintenance of complex organisms (**17–23**). Apoptosis was first reported by Kerr, et al. (**18**) in an effort to describe the physiological process of ordered cell death. Kerr referred to "shrinking necrosis," describing one salient difference between necrotic and apoptotic cell death. Since that time, research in the area has revealed a highly conserved evolutionary process that follows a structured, energy-dependent cascade of physiological and biochemical events for the deletion of damaged/unnecessary cells (**19, 22, 23**).

Cells undergoing apoptosis are often characterized by shrinkage; chromatin condensation; ordered, nonrandom DNA fragmentation; cytoplasmic blebbing; and formation of apoptotic bodies (**19, 23–25**). In contrast, cells undergoing necrosis swell, and their plasma membranes often rupture due to osmotic imbalance. Their nuclei show no dramatic morphological

changes, and ATP is not used to initiate or sustain the process. Apoptotic activation has been linked to phenomena ranging from the activation of plasma membrane cell death receptors (**26**) to mitochondrial leakage of cytochrome *c* (**27–30**). Apoptosis is a critical process driving normal cell turnover, cell death in the immune system, embryonic development, and stress-induced cell death. It has also been implicated in Alzheimer's and Huntington's diseases, ischemic cascades, and autoimmune disorders.

The past decade has seen vast interest in identifying the genes and pathways responsible for initiation and progression of the apoptotic cascade. A wealth of knowledge stems from initial studies conducted on development of the nematode *Caenorhabditis elegans* (**31–33**). Studies by Horvitz, et al. (**32**) on its development identified deletion of about 8% of the total cell number during development through activation of cell death genes (*ced-3* and *ced-4*). Following identification of those genes and pathways in *C. elegans,* multiple mammalian cell death homologs were identified (**19, 31, 33–35**). The gene encoding for interleukin-1ß–converting enzyme (ICE), a cysteine protease associated with apoptotic mechanisms in mammals, was found to be homologous to *ced-3* (**21, 36**). Subsequent studies showed that its overexpression was sufficient to trigger apoptosis. A number of similar proteases, termed *caspases* (cysteine-dependent aspartate-specific proteases, with ICE numbered caspase-1), in mammalian cells play a crucial role in the progression of apoptosis (**19–21, 25, 37–39**).

Progression of an apoptotic cascade can be characterized by three phases: initiation, execution, and termination (**19, 40, 41**). Progression requires an energy input (ATP) at each event "check point" of the process to trigger induction signals that recruit different pathways (**42–44**). Apoptotic mechanism activation results from various factors: ultraviolet radiation, toxic stress, growth factor deprivation, nutrient stress, ionic deregulation, temperature extremes (hypo- and hyperthermia), ischemia, and so on (**20, 45–50**). Following induction, a progression of apoptosis follows through execution by a series of events: cell cycle arrest, caspase activation, cytochrome *c* release from mitochondria, cell membrane phospholipid inversion, and changes in additional apoptotic-related gene products (**21, 43, 51**). Apoptosis is completed by cleavage of DNA repair enzymes, ordered DNA fragmentation, and the formation of cytoplasmic membrane blebs and apoptotic bodies yielding complete, systematic cellular disassembly (**19, 43, 48**).

The Next Generation of Solution Design

As detailed above, biopreservation has seen many new discoveries over the past few years. The area of hypothermic storage has seen an evolution in investigative focus from systemic (organ-based) physiology to addressing specific cellular needs and responses to low temperature and preservation processes. Approaches to cryopreservation have also moved beyond a primary focus on preventing ice formation to addressing molecular responses of cells to preservation processes. In both areas, a shift in investigative approach has led to a shift in preservation technology (solution) design. This shift represents an evolution in the hypothermic storage area and a paradigm change in cryopreservation science, both represented by a new focus on the fundamentals of solution design to achieve successful preservation.

For hypothermic storage, new preservation technologies have been developed by taking from, expanding, and improving upon many principles in preservation solution design from the organ transplant arena (**14,15**). For cryopreservation, the new approach represents a complete departure from traditional solution formulation while still incorporating historic principles. With identification of the events described above, preservation solution design has begun to focus on modulating many of them to prevent activation of stress pathways and subsequent cell death (**52, 53**). Although the stresses are numerous and varied, recent preservation solution development has focused on a few critical areas: maintenance of ionic and osmotic balance, inhibition of acidosis, prevention of cellular swelling, control of free radical accumulation, and staying the activation of apoptosis to increase preservation efficacy.

Ionic Composition: Ion disregulation is a critical negative event during preservation that can trigger a host of stress responses. Accordingly, new preservation solution design has focused on altering ionic concentrations away from traditional culture media (high Na^+, low K^+) to just the opposite (low Na^+, High K^+). This important aspect of solution design addresses shutdown of ion pumps during low-temperature storage. New formulations have been termed intracellular-type preservation solutions based on an ionic composition that more closely reflects that of the intracellular environment (**14, 15, 54**).

Another important feature of such solutions is that they are not designed to merely reflect intracellular ionic concentrations under normothermic conditions. This approach to solution design provides concentrations necessary to maintain a proper intracellular ionic balance at low temperature and is therefore considered transitional in ionic nature. Two commercially available solutions incorporating this concept are Viaspan brand from Barr Laboratories (www.barrlabs.com) and the HypoThermosol (HTS) family of hypothermic maintenance media from BioLife Solutions (www.biolifesolutions.com).

Buffering pH: Another critical aspect of preservation solution design is the maintenance of proper pH levels during the preservation process. Traditional cell culture media rely on phosphate and bicarbonate buffers to regulate pH. Although very effective at normothermic temperatures, such systems lose their buffering capacity at hypothermic temperatures and are

therefore rendered ineffective. Because maintaining pH is critical, new solutions have incorporated organic buffers (such as HEPES) for effective buffering throughout an extended range of hypothermic temperatures.

Osmotic Control: With disregulation of ionic gradients and alteration of intracellular concentrations, controlling osmotic swelling of cells during preservation is important. So effective hypothermic and cryopreservation solution design incorporates several components that serve as osmotic buffers, including lactobionate, sucrose, mannitol, and dextran. Such components serve several functions independently but act in conjunction with one another to control cellular swelling during preservation.

Energy Substrates: The goal of placing a biologic into preservation is to reduce cellular activity and thereby extend biological time and utility, but maintenance of cellular energy levels during and following storage remains important. Accordingly, modern preservation solutions incorporate energy substrates such as adenosine and glucose in their formulations to help maintain cellular energy reserves. These compounds serve as energy substrates during preservation, but what is more important is that they serve as readily available energy sources upon return of the cells to normothermic conditions. Thus they fulfill a critical role by providing an immediate energy reservoir for cells to use in activating repair processes and deactivation of stress and cell death pathways following preservation. Without this provision, cells emerging from preservation have inadequate energy reserves and will therefore undergo cell death either through apoptosis or secondary necrosis.

Free-Radical Scavengers: Recent studies detail the role free radicals play in inducing cell damage during and following preservation. It has been long appreciated in the area of organ preservation that including the free-radical scavenger glutathione significantly improves preservation efficacy. Although glutathione remains a mainstay in modern preservation solution design, it alone does not provide adequate protection against all categories of free radical damage. Recent advances in preservation solution design have added more potent free-radical scavengers such as vitamin E analogs to further reduce damage and subsequent activation of apoptosis (**6**).

Apoptotic Control: A new area of preservation solution design is that of targeted apoptotic control (TAC). Other principles of preservation solution design typically focus on prevention/control of global stresses and subsequent response pathway activation. TAC is a new direction in preservation solution design whereby specific inhibitors of apoptotic proteins (e.g., caspases) are incorporated into preservation solutions. This approach allows development of custom solutions designed for enhanced preservation of specialized cell systems and functions. TAC has proven effective in both hypothermic and cryopreservation applications on a research level (**13, 54, 55**).

Solution Design and Preservation Efficacy

The ultimate goal of preservation, no matter the type, is to halt biological time for a given interval, followed by on-demand return of cellular viability, structure, and function. Essentially, "cell/tissue in" should equal "cell/tissue out." Unfortunately, more often than not, attainment of the goal is far from the reality of biopreservation. Typical preservation outcomes can more accurately be characterized by retention of a high degree of cell viability as measured immediately poststorage, followed by a subsequent decline over 24–48 hours coupled with a decrease in cellular responsiveness, function, and reproductive ability. For hypothermic preservation, storage intervals are typically limited to 1–3 days for most cellular systems.

Such suboptimal characteristics have limited the application and use of preservation approaches such that biopreservation is applied only when absolutely necessary. Tissue culture remains the preferred methodology for cellular maintenance. And although effective to a degree, culture does not provide an adequately utilitarian and cost-effective means of maintaining cell and tissue systems for many

Some Useful Terminology

Apoptotic bodies are the separate vesicles that remain after a cell undergoing apoptosis has blebbed and broken apart. They represent an interim form that then undergoes phagocytosis and resultant disintegration.

Blebbing is the formation of blister-like protrusions on the surface of a cell undergoing apoptosis. Membrane blebs ultimately result in the formation of apoptotic bodies.

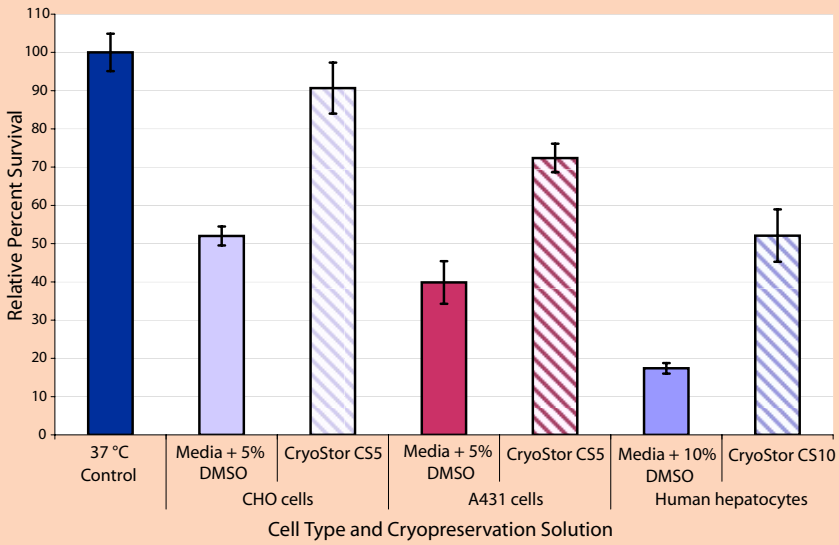
Cytochrome *c* is a small heme protein associated with the inner membrane of the mitochondria. Unlike other cytochromes, it is soluble; it can undergo oxidation and reduction, so it does not bind oxygen; and it transfers electrons in signal transduction cascades of oxidative respiration. Upon stress-activated release from the mitochondria, cytochrome *c* serves as an integral signaling molecule for initiation of the apoptotic caspase cascade.

Dimethyl sulfoxide (DMSO) is a bipolar hydroscopic molecule as well as a hydroxyl-radical-scavenger that is highly penetrable to cell membranes. DMSO is thought to enter a cell and bind free water, thereby controlling/preventing ice formation within a cell.

Free radicals are highly reactive chemicals, each atom or molecule with an unpaired electron. They result from oxidation in normal cell metabolism, but their numbers in an organism increase when it is stressed.

The **glass-transition (T_g) temperature** represents the midpoint of the temperature range in which a phase transitions from a viscous liquid state to that of a glass (amorphous solid): temperature of solution $> T_g$ = liquid; $< T_g$ = glass.

Figure 1: Comparing 24-hour postthaw viability following cryopreservation of various cell systems in two generations of cryopreservation solutions. Numerous cellular systems are used in bioprocessing, ranging from specialized cell systems to routine cell lines. Changes in solution design have led to an improvement in cell viability across the range of industrial cell systems. To illustrate, three models commonly used in biopharmaceutical settings were cryopreserved in either first-generation (culture media + DMSO) or second-generation (CryoStor) cryopreservation media to illustrate the broad-based improvement in postthaw cell viability that can be achieved through use of newly developed solutions.



applications such as cell therapy, toxicology testing, and biopharmaceutical processing. Requiring maintenance of cellular function as well as viability following preservation, scientists have moved to develop new preservation solutions based on the principles presented above. Such efforts have led to new technologies and compatible processes. For example, HypoThermosol and CryoStor brands (both from BioLife Solutions, Inc.) provide a means for more effective preservation (offering higher viabilities and functionalities) of cell and tissue systems.

Cryopreservation: Sosef, et al. (**56**) recently reported on improved hepatocyte viability and function in and after long-term culture following cryopreservation. The researchers found that a marked loss in cellular viability and reduction of function (albumin secretion, urea synthesis, and cytochrome p450a1a activity) followed traditional cryopreservation (media + serum + DMSO). This report further documented that by incorporating a new preservation solution (CryoStor CS10), they improved not only cell number and viability, but also function.

More recently, Stylianou, et. al (**57**) studied the cryopreservation of cord blood. They reported a significant increase in nucleated cell viability, in $CD34^+$ cells, and colony-forming units with a new formulation over standard approaches using a media + dextran + DMSO solution. Numerous other studies have applied new preservation solutions in cryopreservation, all of which have reported significant improvements in cell survival and function. A number of unpublished studies have used such new strategies with CHO and LNCaP cells, keratinocytes, fibroblasts, renal and peripheral blood mononuclear cells, hybridomas, and so on. Those have also found that altering preservation solution design to incorporate an intracellular-type solution provides a significant overall improvement in cryopreservation efficacy (Figure 1). In addition, a number of published studies have also detailed control and subsequent reduction of both apoptosis and necrosis realized with this biopreservation strategy (**5, 13, 58**).

Hypothermic Storage: Improvements to hypothermic storage efficacy are substantial when using new preservation solutions developed around the principles of molecularly controlling cellular responses to the preservation process. The effect of these new media on cellular preservation efficacy was recently demonstrated in a report by Snyder, et al. (**59**) for the hypothermic preservation of cardiomyocytes. Several hypothermic storage solutions were investigated — including standard culture media, Viaspan, and HTS brands — to assess their preservation efficacy (based on cell membrane integrity, metabolic activity, and contractile function) and preservation intervals (length of storage). When using a third-generation intracellular-type solution variant called HTS-FRS, maintenance of cell viability and function was observed for 72 hours of cold storage; whereas first- (media) and second-generation (Viaspan and HTS-Base brand) solutions failed to provide adequate protection after just 24 hours of storage. As Figure 2 illustrates, numerous additional reports on systems ranging from hepatocytes to renal cells to engineered and native skin and blood vessel tissue have shown similar results (**6, 49, 54, 60–62**).

In addition to those studies investigating solution applications and mechanisms of action, a recent study by Mathew, et al. (**54**) extended these principles in studies presented on the development of customized, cell-type–specific solution formulations using second- (HTS) and third-generation (HTS-FRS) solutions coupled with targeted molecular control using caspase inhibitors. This direction and theory of solution development was termed the Multisolution Hypothesis of Solution Development. Further discussed in additional articles by Snyder, et al. (**52**) and Van Buskirk, et. al. (**63**), that hypothesis could lead to the next generation of hypothermic preservation solutions.

Application and Benefits

Preservation technologies (solutions or media) developed for molecular control of cell response to the preservation process represent future options for the biopharmaceutical and cell therapy industries. Integration of preservation processes offers the chance to streamline research and development activities, further enabling expanded high-throughput screening, and

facilitates a level of control for cell based systems not only from the aspect of shipment, but also on-demand use. These solutions represent an enabling technology platform that allows for incorporation of preservation practices into many routine procedures such as

- tissue culture and preparation of plates for cell-based assays
- maintenance of engineered cells and tissues for clinical application
- cryopreservation of hybridoma cells for seeding bioreactors for monoclonal antibody production
- cell therapy.

Integrating effective preservation practices into processing applications can reduce costs of cell maintenance reagents, personnel time, and product loss (final, intermediates, and bioreactor failures). Furthermore, such preservation processes reduce necessary cell and reagent handling, thereby lowering the risks of researcher error and sterility compromise.

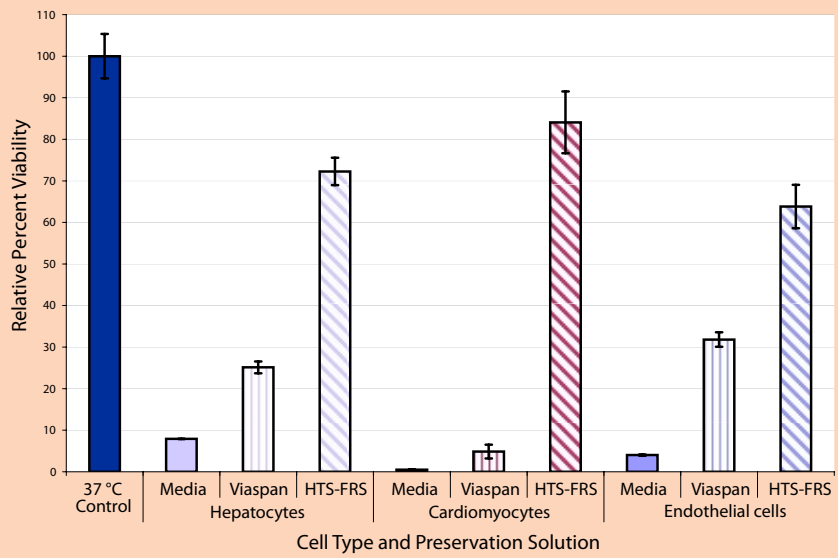
For example, in drug discovery, good preservation practices would allow for controlled bulk culturing of plated cells over days to weeks. Bulk culture (multiple plates) is typically difficult to control because of variability in growth rates and other unpredictable characteristics between plates. Integration of preservation procedures into such processes allows for the placement of ready-to-use plates into storage for a few days until needed. This approach provides further benefit when plates become ready for use at the end of a workweek and require multiple days of processing. Effective preservation allows for the placement of cells into storage over a weekend followed by on-demand use at the beginning of the following week.

The same principles apply to bioreactor culture and processing for cell or protein production applications. Effective preservation practices can serve as a key time management tool.

Biopreservation is rapidly emerging in the forefront of research and process engineers' minds as a critical step in process and product development. Without a thorough knowledge of the preservation science literature, however, it is difficult to identify and understand novel advances in the area. So acceptance of the "status quo" in preservation science tends to be the conclusion reached by many outside the field who use — or could be using — preservation procedures regularly. This is unfortunate because, as presented here, the status quo in the field of biopreservation is simply not good enough.

Over the past six years, a number of fundamental discoveries have been made in the field of biopreservation that have led to development of new approaches and technologies for preservation of biologics to yield greater cell survival and functionality than was possible before. These advances have focused, and will continue to focus, on the molecular responses of cells to preservation processes and the development and formulation of preservation media designed to modulate those responses in an effort to improve even further on the current state of the art. Such advances have opened new opportunities for research and industrial processes that rely heavily on biological components (cells and/or tissues) for product development, testing, or as a final product. Furthermore, I believe the advances and developments in new solution design and technology have placed the field of biopreservation in a position of compatibility and utility for a number of industrial segments including bioprocessing, tissue engineering, cell therapy, drug discovery, and biopharmaceutical development.

Figure 2: Comparing hypothermic storage media efficacy following three days of storage at 4 °C and one-day culture. Several generations of solution designs were evaluated for their ability to protect several model cell systems during extended cold storage. Data illustrate that use of preservation media developed based on the (third-generation) expanded principles of hypothermic solution design promotes significant improvement in cell viability (HTS-FRS). This solution design has also led to expansion of the preservation interval beyond that achievable through first- and second-generation solutions.



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