Development of Stabilizers for Live Cell Preservation at Room Temperature

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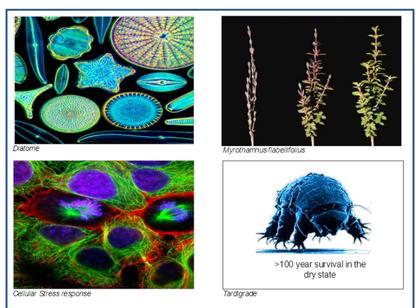
Abstract

We report that we are able to achieve mammalian cell survival at ambient temperature maintaining normal cell signaling capability and metabolism. These results provide a roadmap for achieving room temperature logistics of mammalian cells in a dry state.
This technology con eventually be applied to ambient storage of eukaryotic cell stocks for biomedical research, toxicology screens, cell-based therapeutics and blood supply logistics.

Introduction

Cryopreservation at ultra-low temperatures using freezing mediums with DMSO is the current best practice for long term storage of mammalian cells. Such conditions can cause devastating losses of cells due to common failures of cold storage systems. Such losses can also result from the sensitivity of valuable cell types, both primary and engineered, to the freezing and thawing process. Hence, recovery of viable cells from the frozen state is challenging and, as is typical for many cell types, only a fraction of the cells survive with the fully desired phenotype. Based on the studies of extremophiles, such as tardigrades, rotifers and brine shrimp, which can survive for many years in the dry state, we hypothesized that techniques and protocols could be developed that mimic this molecular phenomena in eukaryotic cell cultures. Many candidate approaches have failed over the past 15 years to preserve cells under these conditions in short term studies, and no method to date has been successful at preserving cells without cryopreservation for more than a few hours. We applied a combinatorial screening approach to identify formulations containing biostabilizers that preserve air-dried eukaryotic cells at ambient temperature. The use of specific biostabilizers in defined formulations protect cells from the stress of dehydration, dry-storage, and rehydration while preventing the execution of apoptosis programs. We report here that we are able to achieve statistically significant improvements in cell survival rates and preservation times as compared to previously published dry down methods. These results provide a roadmap for achieving room temperature storage of mammalian cells in a dry state. The technology can be further developed for products that extend cell survival at ambient temperatures. This technology could eventually be applied to ambient storage of eukaryotic cell stocks for biomedical research, toxicology screens, cell-based therapeutics and blood supply logistics.



Many examples exist in nature where whole, multi-cellular organisms can survive extreme stress conditions. Nature has developed chemical and molecular mechanisms that protect the organism against cell death and degradation.

Development of more facile cell preservation strategies, particularly those used in cryopreservation of the last 20 years have only seen marginal improvement or as in the case of hypothermal storage, been largely unsuccessful. Nonetheless, the goal of Ambient Biopreservation of live cells has will have a profound impact on cell-based assays and therapeutics. Biomatrica has developed technology and products that are currently used world wide to protect molecular analytes such as DNA, RNA, Proteins in liquid and the dry state under ambient storage conditions

Materials and Methods

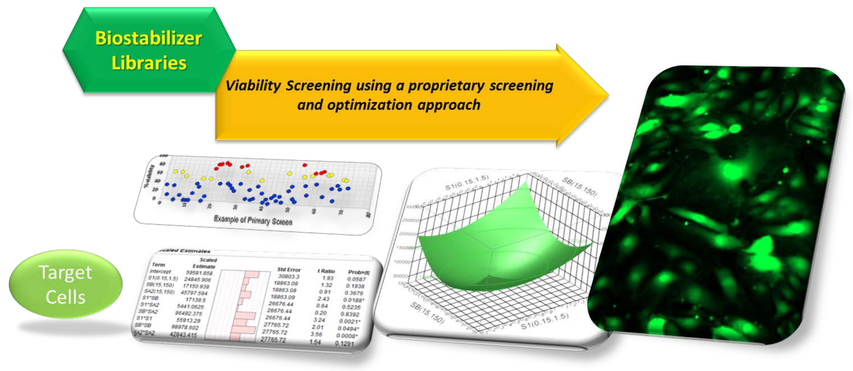
Cell preparation for dry down and recovery of fibroblasts.
Human neonatal fibroblasts and mesenchymal stem cells (MSCs), were obtained from commercial sources and cultured in fully constituted Cascade Media 106 media or MesenPro® (Life Technologies). Once expanded the cells were seeded into white or clear tissue culture treated 96-well plates at a density of 5000 cells per well and allowed to grow overnight at 37C, 5% CO_2 and 95% RH. Oxygen levels were ambient. The cells were then treated with biostabilizers and dried down by a proprietary process. Once dried, the cells were stored for 1 day to 4 weeks and sealed in a foil barrier pouch at room temperatures. After the storage period, the cells were retrieved and exposed to revitalization by a proprietary process, and the cells were allowed to recover for 1-24 hours prior to use.

Cell preparation for dry down and recovery of common laboratory cell lines.
The common laboratory cell lines, HeLa (cervical carcinoma), H4 (glioblastoma), the NF-kB luciferase reporter lines HEK293.NF-kB.LUC & HEK293T.NF-kB.LUC, and a rat insulinoma line were prepared and treated as above, albeit in appropriate tissue culture media.

Cell proliferation, and metabolic assays

Cells were plated at 1000 or 5000 cells/test in white or clear 96-well tissue culture treated plates and used in proliferation and/or metabolic assays with luminescent or colorimetric readouts. The cells were subjected to the stabilization process and revitalized as described above. Several assays were utilized to measure cell viability & proliferation. Proliferation assays of fibroblasts were plated in white 96-well plates at 1000 cells/test and were treated with Celltiter-Glo® (Promega) at time 0 hours and 48 hours post plating. Metabolism was assessed using two different assays, the Celltiter 96® Aqueous One MTS assay (Promega) and a novel NanoLuc® profurimazine-based real-time bioluminescent cell viability assay. Fibroblast standards were prepared as mitomycin C treated cultures capable of metabolic functions in a growth arrested state. All assays were conducted according the instructions provided by the manufacturer.

Biostabilizer Formulations
Biostabilizer formulations were discovered by way of a screening strategy that combinatorially employs Biomatrica's extensive stability libraries and proprietary algorithms. After several iterations and refinements, a class of formulations was arrived upon capable of sheltering cells from the structural and biochemical stress of desiccation and allowing cells to be stored at ambient temperature.



Formulation development begins with assay development for the target cells and is conducted by combinatorial screening and through the use of proprietary algorithms.

Results

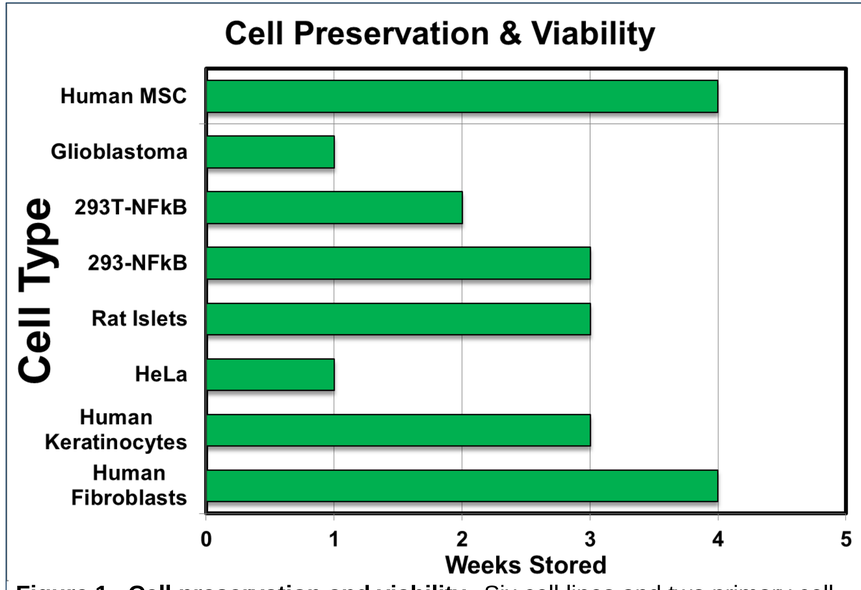


Figure 1. Cell preservation and viability. Six cell lines and two primary cell preparations were cultured in 96-well plates treated with stabilizers and dried down. The cells were stored for various times and revitalized. The cells were allowed to recover for 24 hours and analyzed for ATP content. Survival criterion was established as 70% survival relative to growth arrested controls. Longer time points are being analyzed

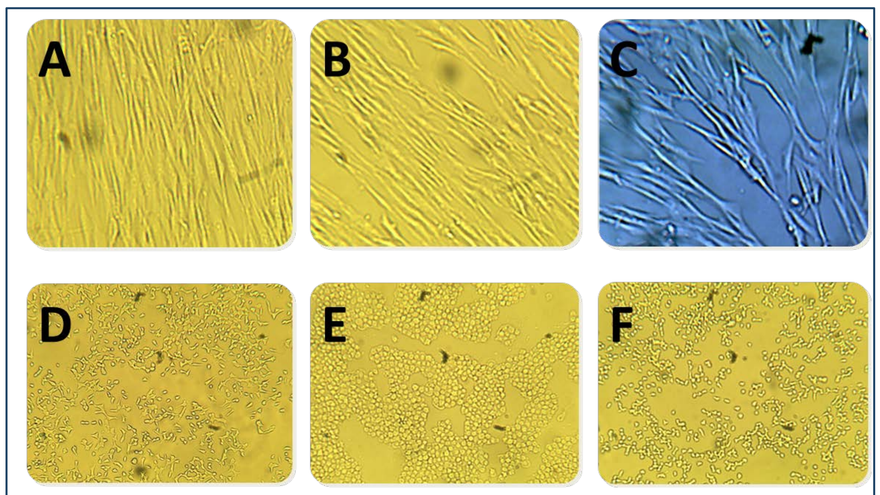


Figure 2. Fibroblasts and rat insulinoma morphology during the preservation and revitalization process. Human neonatal fibroblasts (A-C) and rat insulinoma cells (D-F) were initialized into the stabilization process (A, D), dried down and stored for 5 days (B, E) and revitalized (C, F). Throughout the process, the general morphology of the cells was maintained with a return to pre-dry down morphology after revitalization (compare A to C, and D to F).

Figure 3. Performance of Revitalized Cells in the Promega NanoLUC-Live Assay. Human fibroblasts were cultured in Cascade Biologics Medium 106, containing low serum growth supplement and seeded in white, TC treated 96-well plates at 20K cells per test in a randomized pattern to avoid plate bias (Treated, Blue; untreated formulation only, Red). The cells were allowed to recover for 48 hours before conducting he NanoLUC-LIVE assay.

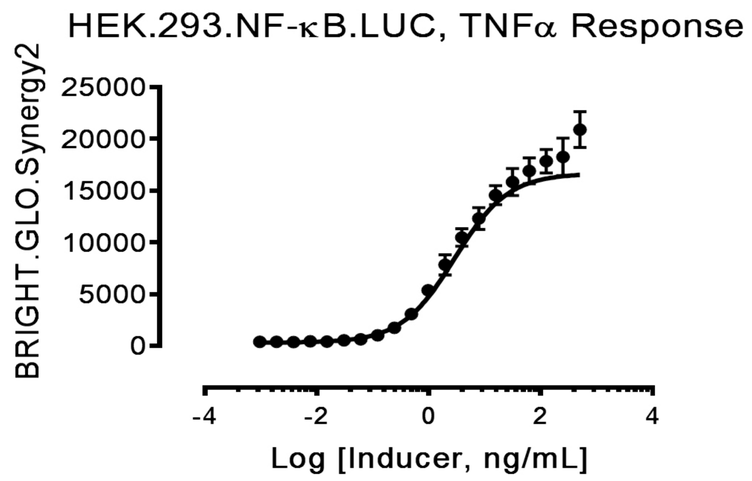


Figure 4. Reporter activity is maintained post revitalization in the HEK293.NF-kB.LUC reporter cell line. HEK.293.NF-kB .LUC cells were plated at 10K cells per well in a white 96-well plate and preserved for 72 hours. The cells were revitalized and allowed to recover for 48 hours in normal tissue culture media under typical conditions. The cells were then exposed to increasing doses of TNFα and incubated overnight. TNFα induced NF-kB mediated luciferase expression was monitored by the addition of Bright-Glo® on a Biotek Synergy2 multimode plate reader in luminescence mode. Induction can clearly be observed with increasing TNFα concentration indicating the signaling pathway remains intact.

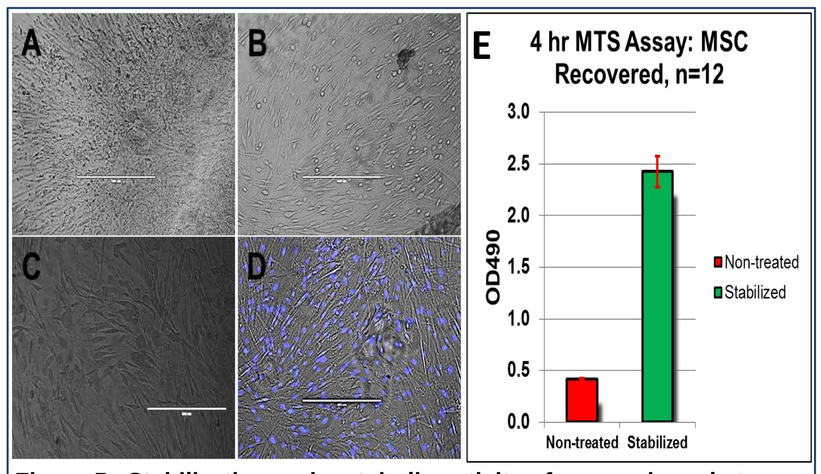


Figure 5. Stabilization and metabolic activity of mesenchymal stem cells. MSCs were cultivated on clear 96-well plates at 10K cells per well for 24 hours. The cells were then preserved and revitalized for 4 hours after 5 days of storage at room temperature in a foil barrier pouch. After 4 hours of revitalization, the media was changed and Celltiter 96 Aqueous One reagent (MTS assay) was added to the control (A) and test wells (B), and the plate was immediately read. Four hours later, the plate was read again and the optical density at 490 nm was monitored on a Biotek Synergy 2 multimode plate reader. Results are reported in (E). (A) unprotected cells after dry down and revitalization, (B) stabilized cells after revitalization undergoing the MTS assay, (C) preserved cells prior to the addition of the revitalization solution, (D) revitalized cells stained with Hoechst 3442 to visualize the nuclear morphology.

Summary

- **Dry stabilization of a number of cell lines and primary cells can be achieved and maintained for periods approaching one month (Figure 1).**
- **Stabilized cells maintain their morphology after revitalization (Figure 2).**
- **Revitalized cells are metabolically active as demonstrated by a novel NanoLuc® profurimazine-based real-time bioluminescent cell viability assay. Comparison of the signals to growth arrested cells serving as a reference for cell numbers demonstrate that the cells are also proliferating shortly after revitalization. (Figure 3).**
- **Reporter gene expression remains unaltered after storage and revitalization suggesting that naturally occurring signaling pathways are also intact (Figure 4).**
- **Mesenchymal stem cells can be stabilized for long periods of time in the dry state (Figure 1) and show a robust metabolism and phenotype after revitalization (Figure 5).**