Can We Turn to Nature for a Better Tissue Preservation Method?

Abstract

Research and clinical laboratories typically use two routine ways to preserve tissues: formalin-fixed paraffin-embedded preservation (FFPE) and cryopreservation. FFPE is ideal for visualizing cellular structure in the context of surrounding tissue and allows for long-term storage at room temperature, but damages DNA, RNA and protein, hindering molecular analysis. Cryopreservation preserves nucleic acids, and the bioactivity of proteins, but long-term storage is at risk if there is a technical malfunction or loss of power and is less effective for morphological studies. We developed a new preservation method inspired by the robust microorganisms, tardigrades, that can survive long periods of desiccation. We hypothesize that this method will allow for the long-term storage of tissue at room temperature.

Introduction

For decades, biospecimens have been preserved by FFPE. The benefit of this preservation method is the maintenance of cell structure at room temperature and ability to analyze samples by immunohistochemistry (IHC); unfortunately, cellular proteins and nucleic acids are damaged in the process and thus samples are incompatible with more modern analytic methods. Alternatively, flash-frozen tissues can be stored in a freezer or in liquid nitrogen, but cell structures sustain significant damage using these methods and suffer the risks of power outages or liquid nitrogen evaporation, leading to unexpected thaws.

Currently, no preservation method allows for the long-term dry storage of biospecimens while maintaining nucleic acid and protein integrity. Yet in nature, organisms like brine shrimp and tardigrades survive long periods of desiccation by cryptobiosis, a physiological state of pause. In a nutshell, organisms undergo a temporary change in response to a loss in hydration; tardigrades upregulate tardigrade-specific intrinsically disordered proteins (TDPs) in response to desiccation (Boothby et. al., 2017), while other organisms rely on disaccharides, specifically sucrose or trehalose (Erkut et. al., 2013).

We speculated that we could desiccate human diploid fibroblast cells in either the presence of trehalose or serum albumin, which contains intrinsically disordered domains. We also added ethylenediaminetetraacetic acid (EDTA), a metal ion chelator, to the samples based on preliminary results that indicated improved RNA integrity with its addition.

Results and Discussion

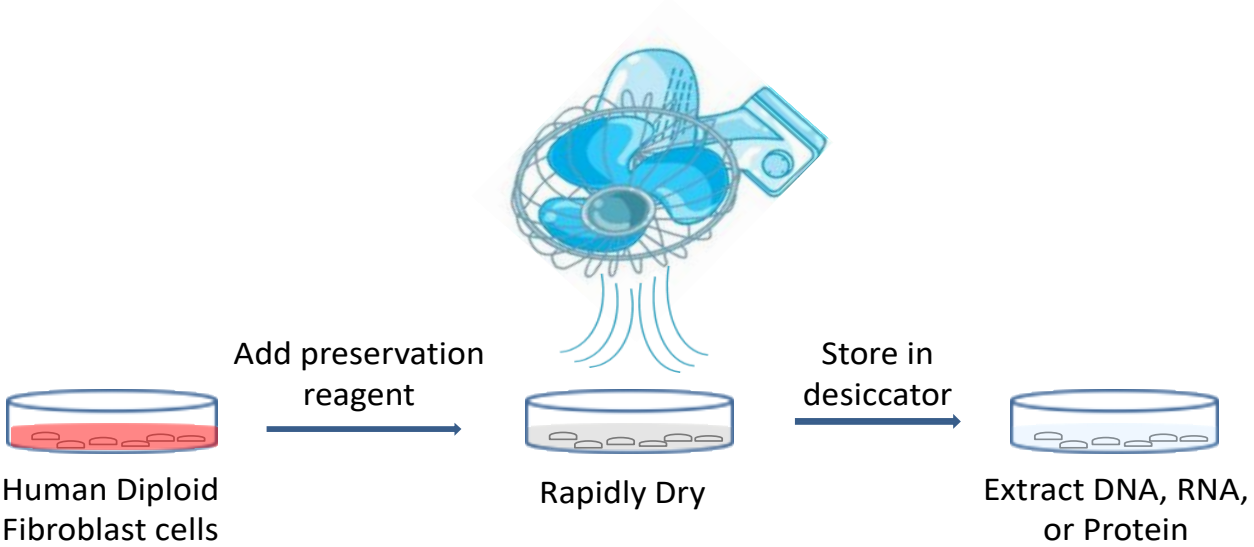

Diagram 1. General protocol to preserve cells for dry storage. Human diploid fibroblast cells are removed from an incubator, then the growth media is replaced by one of four preservation reagents. The dish is rapidly dried using a desk fan and placed in a desiccator. After a 1 week to 3 months, the dried sample is removed, and the macromolecule of choice is extracted.

The overall preservation workflow is depicted in Diagram 1. Multiple dishes with a monolayer of human diploid fibroblast cells were grown under physiological condition (5% O_2, 5% CO_2). First, the cells were washed twice with PBS to remove growth media. Next, the cells were covered with one of four preservation reagents, all of which are variations of PBS. One condition is PBS only, while the other three reagents are supplemented with EDTA, a metal ion chelator. Two of the three PBS + EDTA reagents contain additives. One is supplemented with 10 mM trehalose, a disaccharide sugar. The other contains fetal bovine serum, which is largely consisted of albumin, a protein with multiple intrinsically disordered domains. The dishes with reagent are rapidly dried in a biosafety cabinet (5 min) using a desk fan. The dry dishes were then place in a vacuum sealed pouch with silica desiccant. The pouches were stored in a desiccator with drierite and a humidity indicator. As a control, equally plated dishes were placed in the -80C freezer to be removed at 1 week to 3 months along with the dry preserved cell dishes. All were examined for DNA, RNA, and protein integrity. On the day that the cells were first preserved, equally plated cells were harvested for DNA, RNA, and protein to reflect the starting material concentration and integrity.

DNA Integrity

To determine if DNA remained in diploid fibroblasts preserved for 1 week to 3 months, we isolated genomic DNA immediately upon removal of the preserved samples from the desiccator. Our results indicate that genomic DNA can be isolated from cells that have been stored in PBS supplemented with EDTA, for up to one month (Figure 1). A dot plot with DNA concentrations segregated by preservation buffer on the X-axis and denoted by dot color to indicate length of storage shows the range of DNA concentration for all harvest time points (Figure 1A). Samples preserved for 1 week are nearly indistinguishable from frozen samples (blue dots), except for drop out samples in each condition. When visualized by agarose gel, differences in DNA integrity is pronounced (Figure 1B).

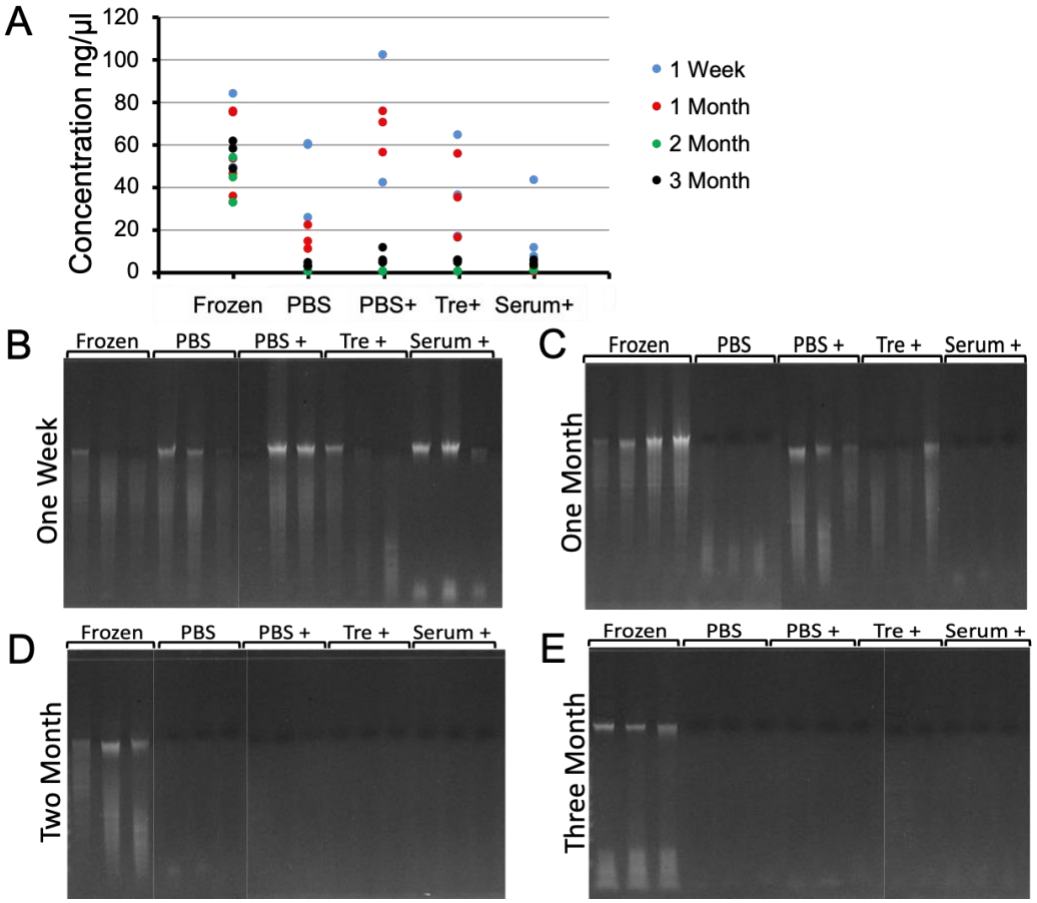

Figure 1. DNA can be desiccated for up to one month in PBS plus EDTA. Samples were stored at less than 10% humidity with PBS, PBS plus EDTA, PBS plus EDTA and Trehalose, or PBS plus EDTA and fetal bovine serum. Samples stored frozen at -80℃ for the same duration serve as controls. A) Scatter plot of DNA concentration for samples preserved for 1 week (blue dots), 1 month (red dost), 2months (green dots), and 3 months (black dots). B-E) 1% agarose gels with equal volumes of DNA. N = 3

Samples stored in PBS plus EDTA and serum have very small DNA fragments at the bottom of the gel, but very little smearing underneath the high molecular weight DNA. This is in contrast to the other sample conditions. We initially interpreted this result favorably, but by one month there is no genomic DNA remaining in samples preserved with fetal bovine serum (Figure 1C). We suspect that the serum contains more nucleases than could be inhibited by 10 mM EDTA. In future studies we will use molecular grade BSA that is compatible with PCR amplification and restriction enzyme studies.

While genomic DNA is present at 1 week in samples preserved in PBS, by one month only a trace of DNA is found in the samples preserved with PBS, suggesting that EDTA is needed to prevent nuclease activity during storage (Figure 1B and C). At one month, DNA is found in samples preserved in PBS plus EDTA with and without trehalose (Figure 1A, red dots, 1C). While it appears as though DNA integrity is compromised in samples with added trehalose, we should highlight that the trehalose samples were slow to rehydrate. Our results may not accurately reflect preservation efficiency, as we have not optimized rehydration. With time, samples preserved in trehalose were more crystal-like, consistent with a candy coating, highlighting a new experimental question to address with future experiments.

Little to no DNA is present in any dry condition at two and three months (Figure 1D and E). This coincided with a small increase in humidity level in the desiccator (10-20%). Unfortunately, we did not place humidity monitors in each sample pouch, thus it is possible that the internal humidity of the samples was even higher. Though the overall humidity was low, we speculate that it was sufficient to enable enough nuclease activity to be detrimental in the long run. We have not examined whether the nucleases were endogenous to the cells or exogenous due to low levels of microorganisms though bacterial or fungal overgrowth was not visibly obvious. In the future, an additional control plate should be included at each timepoint that will be analyzed for microbiological markers. Note, DNA isolated from freshly harvested cells is not shown due to an error in DNA extraction.

One noteworthy observation is that by 2 months, the frozen samples have visible DNA degradation (Figure 2A). Smaller DNA fragments migrate to the lower portion of the gel by month three, as indicated by large red boxes. This suggests that DNA integrity is impaired in only a few months at -80°C, an observation that has implications for long term studies. Overall concentration appears similar with time, even when DNA integrity decreases (Figure 2B). This is a valuable opportunity for future experimental questions, firstly to determine the stability of genomic DNA in frozen cells, as laboratories routinely store cell pellets at -80°C for future analysis. Secondly, this is an opportunity to examine whether our preservation reagent increases stability in a frozen state.

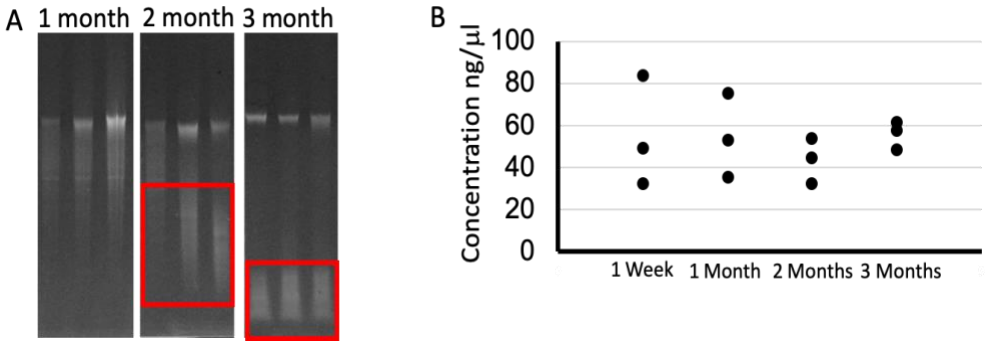

Figure 2. DNA integrity decreases without loss in overall concentration in samples frozen at -80℃ A) Genomic DNA isolated from samples preserved for 1, 2, and 3 months at -80 ℃ resolved on 1% agarose gels and stained with Syber Safe DNA gel stain. Red boxes indicate smaller DNA fragments B) Concentration in ng/ul for each flash frozen sample.

RNA Integrity

Similar to DNA samples, we isolated total RNA, a far more labile nucleic acid, from the preserved samples using Trizol. The concentration levels appear to indicate total RNA is present in preserved cells at levels comparable to frozen samples (Figure 3A). The range of RNA concentration for all harvest time points is represented as a dot plot with RNA concentrations segregated by preservation buffer on the X-axis and denoted by dot color to indicate length of storage. Unfortunately, there is little to no presence of RNA when observed by 1% agarose gel (Figure 3B-E).

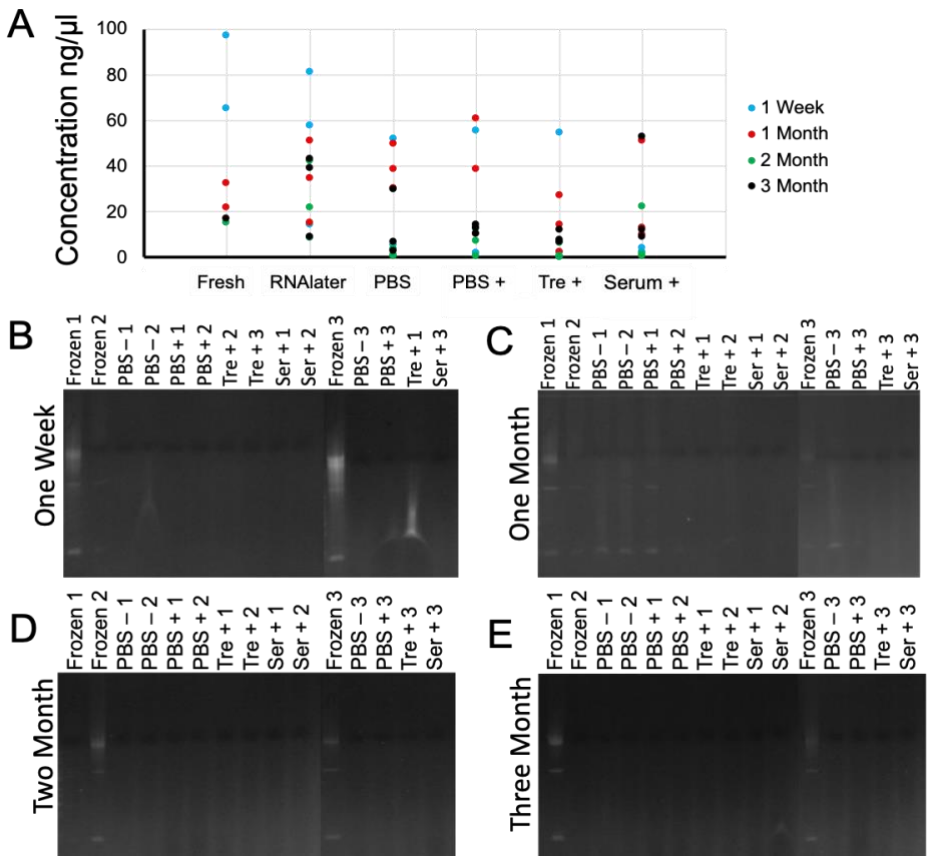

Figure 3. RNA rapidly degraded in samples at room temperature. Samples were stored in a desiccator after being dried in PBS, PBS plus EDTA, PBS plus EDTA and Trehalose, or PBS plus EDTA and fetal bovine serum. Samples stored frozen at -80°C for the same duration serve as control. A) Scatter plot of DNA concentration for samples preserved for 1 week (blue dots), 1 month (red dost), 2 months (green dots), and 3 months (black dots). B-E) 1% agarose gels with equal volumes of DNA. N = 3

The above results are in contrast to preliminary studies performed to establish storage conditions. Here, dishes of equal cell density are preserved with one of four preservation reagents described above and stored for 48 hours at low (less than 10%) or high (70%) humidity. In these conditions, high quality RNA is found in all conditions, though with varying concentrations (Figure 4). Here, we established that low humidity is more favorable to RNA storage, though in this case only for 48 hours. Given these results, it is surprising that very little RNA remained when stored for 1 week and longer (Figure 3).

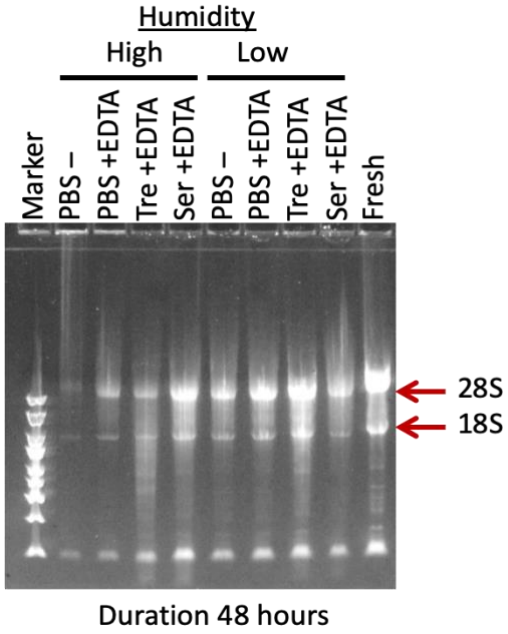

Figure 4. RNA can be isolated from samples stored dry for 48 hours. Samples were stored in 70% humidity (left side of gel) or less than 10% humidity (right side). Isolated RNA is resolved and visualized on 1% agarose gels stained with Syber Safe DNA gel stain. Red arrows indicate 28S and 48S

We wondered whether a technical error could contribute to the differences in RNA content at 48 hours as compared to 1 week and longer. One contributing factor of note is humidity. Each time the desiccator was opened, the humidity fluctuated. Additionally, each added dish of cells had a tiny amount of moisture, which likely contributed to saturation of the drierite and silica pouches. To demonstrate this possibility, we repeated the preservation process at approximately 10% and 20-30% humidity. Total RNA levels were compared to RNA freshly isolated at time zero as well as to samples preserved for 1 week at -80°C (Figure 5). RNA isolated from cells stored at 10% humidity for 1 week show visible loss of integrity when dried in the presence of PBS,

PBS with EDTA, and PBS with EDTA and BSA (Figure 5A). Note, the total concentration is similar for all samples under these conditions (Figure 5B). When humidity is increased to 20-30% for 1 week, RNA integrity and concentration levels are compromised for all reagent conditions (Figure 5C and D). This suggests that RNA is preserved in cells stored for at least 1 week in low humidity, however, even small increases in humidity compromises RNA integrity.

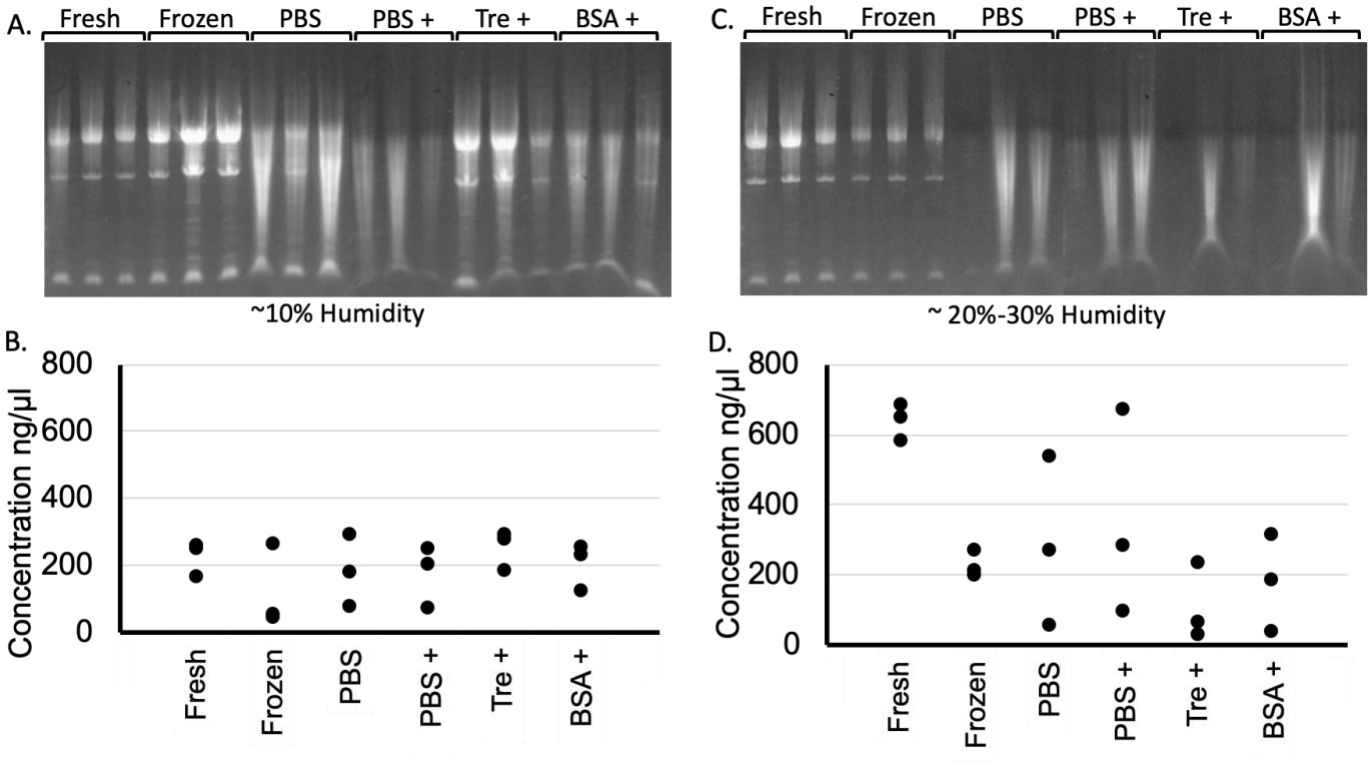

Figure 5. RNA is degraded when cells are stored dry at room temperature in ~10% to ~30% humidity for one week. A) and C) Agarose gel with RNA from freshly harvested cells and compared with RNA from cells stored frozen, or dried in PBS, PBS plus EDTA, trehalose, or BSA for 7 days. B) and D) Graph depicting RNA concentrations in ng/µl. All samples in paired gel and graph reflect three replicate plates per plating density. Y-axis is held constant. X-axis is labeled with preservation condition.

To determine how well RNA is preserved in samples frozen at -80°C, we compared samples that had been frozen for 1 week to 3 months (Figure 6). RNA integrity is similar over the duration of time examined (Figure 6A). However, there is a decrease in overall concentration, suggesting that similar to genomic DNA, RNA could be compromised during long term storage. Again, this is an important question for future assays.

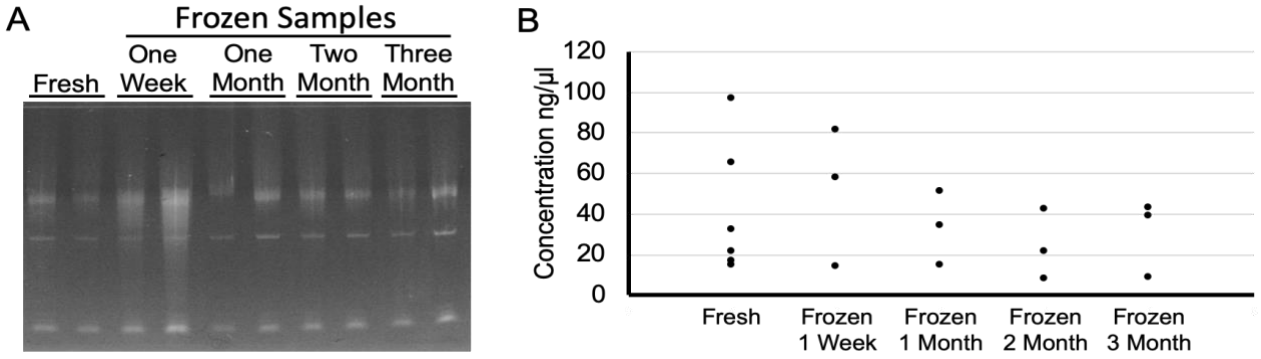

Figure 6. RNA concentration decreases without loss in overall integrity in samples frozen at -80℃ relative to RNA from freshly harvested samples of equal cell number. A) Total RNA isolated from samples fresh samples and samples preserved for 1 week, 1, 2, and 3 months at -80 ℃ resolved on 1% agarose gels and stained with Syber Safe DNA gel stain. B) Concentration in ng/ul for each flash frozen sample.

Protein Integrity

To understand how well protein is preserved in a dry state, cells preserved for 1 week to three months were lysed using standard Laemmli lysis buffer and global protein content was resolved on 4-12% polyacrylamide gels and stained with Coomassie blue (Figure 7). All samples do have several predominant bands, yet overall protein content is decreased in nearly all preservation conditions including frozen samples, as early as 1 week (Figure 7A). The exception is the sample preserved in serum as the added serum protein occludes endogenous protein levels. It was not lost on us that the serum proteins were apparently well preserved at 1 week, though even these proteins were significantly reduced by 1 month (Figure 7B). At one month, all protein content decreased, including frozen samples.

As with the DNA and RNA samples, it was not clear whether our lysis reagent was able to fully rehydrate the sample. The Laemmli lysis buffer introduced a particular challenge due to the high levels of detergent; the resulting foam hindered our ability to fully remove the lysate from each dish. Naturally, the lysis buffer is typically used on hydrated cells, which was not taken into account for this project.

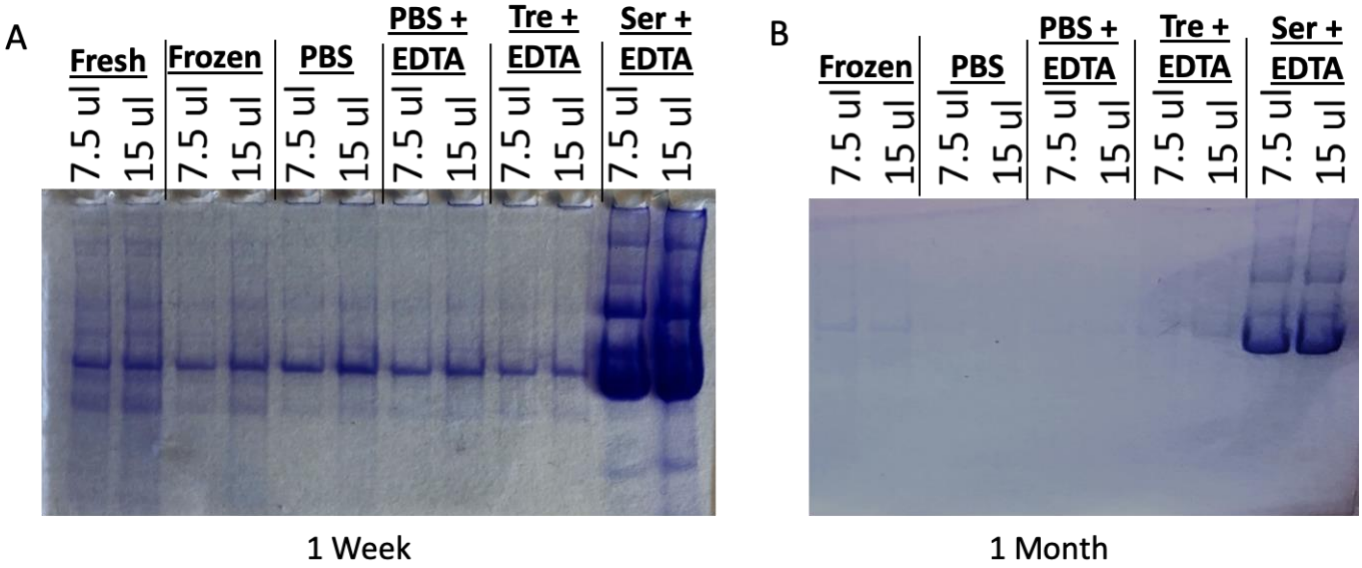

Figure 7. Global proteins decrease after 1 week preservation. Each sample lane represents 10% and 20% of total protein lysate resolved on 4%-12 % gradient denaturing polyacrylamide gel and stained with Coomassie blue. A) One week preservation B) One month preservation

Conclusions

There are several lessons to be learned from this body of work. First, we focused on a uniform preservation process, but we did not prepare for monitoring of humidity during preservation. Humidity increased to nearly 30% by month three, likely due to the numerous times the desiccator was opened to remove samples for each time point. For future analysis, silica pouches and drierite will be replaced at regular intervals to maintain more stable humidity in the desiccator. Additionally, fresh silica packets will be placed into individual sample pouches to account for variations in dryness and humidity, this will also provide some protection from external changes in humidity. As shown by the RNA, even tiny shifts in humidity can have considerable consequences in recovery of the samples.

Second, our rehydration protocol was not optimized prior to this study. By one month, the samples preserved in trehalose or serum were harder to rehydrate with lysis reagents. For our initial studies, the samples were rehydrated in the selected lysis reagent, as we did not want to risk unexpected rehydration-induced degradation. In the future, we will need to study rehydration conditions, comparing rehydration buffers, volumes, and incubation time. This will likely aid in effective sample harvest, as we speculated that the dried sample concentration was impacted by our harvest methodology.

Third, we approached this project by comparing each preserved sample to the original freshly harvested sample. In doing so, we decided to store the lysed sample while waiting to harvest each time point. This prolonged storage may have had an effect on sample recovery. In the future, we will need to empirically determine which condition is more stable, the sample lysate, or the purified macromolecule of choice.

Once we can understand and/or control humidity, rehydration, and sample stability, we can examine more complex biospecimens, such as organoids, biopsies, and tissues. We anticipate needing to further optimize the preservation reagent to accommodate multicell layers, such as adding non-ionic detergents or agents to aid permeability. Likewise, the drying protocol will need improvement to facilitate uniform treatment of multiple cell layers.

References

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