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Cryoprotectant-Free Freezing of Cells Using Liquid Marbles Filled with Hydrogel

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KEYWORDS: Agarose-embedded liquid marble, Digital microfluidics, Cryopreservation, Cryoprotectant-free freezing, Agarose cracking, FBS controlled release

ABSTRACT: Cryopreservation without cryoprotectant remains a significant challenge for the reestablishment of cell culture after freeze-thaw. Thus, finding an alternative and simple cryopreservation method is necessary. Liquid marble (LM) based digital microfluidics is a promising approach for cryoprotectant-free cryopreservation. However, the use of this platform to efficiently preserve samples with low cell density and well controlled serum concentrations has not been investigated. We addressed this issue by embedding an agarose-containing fetal bovine serum (FBS) inside the LM. A low density of 500 cells/μL of murine 3T3 cells was selected for evaluating the post-cryogenic survivability. The effects on the post-thaw cell viability of the concentration of agarose, the amount of FBS inside the agarose and the volume of the LM were investigated systematically. This paper also presents an analysis on the changes in shape and crack size of post-thawed agarose. The results revealed that the embedded agarose gel serve as a controlled release mechanism of FBS and significantly improves cell viability. Post-

thaw recovery sustains major cellular features such as viability, cell adhesion, and morphology. The platform technology reported here open up new possibilities to cryopreserve rare biological samples without the toxicity risk of cryoprotectants.

1. INTRODUCTION

Cryopreservation aims to store cells, tissues and organs at cryogenic temperatures for an extended period. Although the development of cryopreservation technology has shown significant advancement to date, the technology still faces several unmet challenges.[1-2] Thus, developing efficient methods to improve the resumption of the biological functions of cryopreserved cells after their retrieval is of crucial importance. Efficient cryopreservation also plays an important role in many applications such as bio-assay development[3], tissue engineering[4], transplantation[5] and reproduction[6]. Cryopreservation is based on slowing down the physiological and biochemical processes in cells in the cryogenic state and returning back to the physiological conditions upon thawing. Cell survival and functional regrowth after cryopreservation depend on the osmotic excursions which are closely linked to both temperature changes and flux of water.[7-8] The outflow of water from the cell to the extracellular space is determined by the rate of the cooling process.[9] At a controlled cooling rate such as -1 K/min, cells respond to the exerting osmotic force and sufficiently efflux water out to the extracellular space. As cooling progresses, cells ultimately become viscous and sustain their chemical constitutes without getting frozen. In contrast, uncontrolled cooling decreases the rate of water migration.[10] As a result, water is trapped inside the cell, accelerating the formation of ice crystals. This intracellular ice formation is fatal to the cells.[11] Nevertheless, controlled cooling potentially inhibits intracellular ice crystallisation and retains unfrozen solute. Furthermore, the extremely fast cooling rate may damage cells due to excessive osmotic dehydration.[12] However, both freezing and thawing processes may induce osmotic shock to the cells and adversely affect

the function of the cell membrane. To address this issue, osmotically active additives known as cryoprotectant agents (CPAs) are added to eliminate ice formation inside the cells.[13] At the presence, the detailed mechanisms underpinning the effect of CPAs on ice crystal formation are not fully understood. The commonly used CPAs are dimethyl sulfoxide (DMSO), ethylene/propylene glycol and glycerol. CPA is usually supplied with the freezing medium and facilitates the permeation of water. However, CPA is inherently toxic, and its optimal dose varies depending on the cell type.[14] In recent new cryopreservation techniques, biomimetic polymers[15], microfluidic platforms[16] and liquid marble (LM)[17] have been used to either minimise or entirely eliminate the exposure of cells to CPA.

The liquid marble (LM) can be considered as an emerging digital microfluidics platform for liquid handling and cell culture applications.[18] LM is a "nonstick droplet" consisting of a liquid core enveloped by hydrophobic particles such as polytetrafluoroethylene (PTFE).[19] LM based digital microfluidics is still in its developing stage, but promises to be a scalable platform for the biological and chemical applications. Several research groups have employed LM for culturing cells and used this platform as the three-dimensional (3D) niche environment to study the cell-cell and cell-matrix interactions.[20-24] However, the only reported work using LM for cryopreservation has been conducted by Serrano *et al*.[17] The authors used LM to preserve murine L929 fibroblasts without the need for CPA following two different procedures: ultra-rapid freezing and slow freezing. The cell viability was investigated as a function of the droplet volume (5 µL, 10 µL and 30 µL) and cell density (50,000; 100,000 and 200,000 cells/ µL). In all cases, the reported cell viability after thawing was higher than 80%. The number of cells in each droplet of that study was relatively high, as the smallest number was 250,000 cells. Therefore, the possibility of preserving with smaller number of cells is of great importance, in particular for cryopreservation of human biological samples with lower concentration such as oocytes and sperms.

To address the low-cell-density bottleneck of LM for the freeze-thaw study, we enhanced the LM platform with an embedded agarose gel containing fetal bovine serum (FBS). This technique aims to take advantage of agarose-embedded LM to enhance heat transfer during freezing and mass transfer during thawing. This platform can also promote cell viability in the post freeze/thaw cycle. Encapsulation of cells offers physical barriers to protect them against adverse environmental conditions.[25] It is well-established that preserving samples with a small volume using LM can be beneficial. In this case, the heat transfer is assumed to benefit from the surface to volume ratio. Additionally, hydrophobic coating also plays a role in inhibiting icing during freezing,[26] as LM acts as a Leidenfrost droplet.[27-28]A Leidenfrost droplet creates a thin layer of vapour between phases acting as an insulator and minimises the heat transfer rate.[29-30] In addition, poly(L -lactic acid) (PLLA) microparticles was encapsulated inside LM for improving cell adhesion.[31] In a previous work, we encapsulated agarose gel inside LM to function as a slow-release carrier.[20] This concept also allows to load and release FBS from the agarose gel, expecting to exert positive impact upon cell viability. Ideally, FBS was used to boost the survivability in various cryopreservation methods[32]. Interestingly, the rapid thawing process eventually squeezes the agarose gel. A similar observation was reported in the literature as "freeze-squeeze" method,[33] which was used to elute DNAs from frozen agarose gel. Thus, we adopt this concept to perform the controlled release of FBS during the freeze-thaw cycle.

2. MATERIALS AND METHODS

2.1 Cell Culture

Immortalised mouse embryonic fibroblast (NIH-3T3) was obtained from the American Type Culture Collection (ATCC®, CRL-1658™, Inc., USA) and were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco®, ThermoFisher Scientific™, Inc., Australia) medium enriched with 10% FBS and 1% penicillin. Cultures were grown at 37˚C in the presence of 5% CO_2, in T25 flasks. The sub-confluent in T25 were

washed twice with Hank's Balanced Salt Solution (HBSS) (Life Technologies™, ThermoFisher Scientific™, Inc., Australia) and detached with TrypLE™ Express (Life Technologies™, ThermoFisher Scientific™, Inc., Australia) for 5 min at 37°C. The enzymatic reaction was stopped by adding 2 mL 10% FBS. The solution was subsequently centrifuged at 1000 rpm for 5 min. The cells were cultured in a humidified atmosphere with 5% CO_2 in air at 37 °C.

2.2 Preparation of Low-Melting-Point Hydrogel

Low-melting-point agarose powder (Invitrogen™) and deionised (DI) water were used to prepare the agarose solutions with different concentrations (2%, 1%, 0.5%, and 0.25% g/mL). For instance, we dissolved 0.1 g agarose into a 10-mL Falcon™ tube and dissolved the powder in 10 mL of DI water to make 1% of agarose. The solution was then heated carefully to the boiling point using a microwave oven. The solution was stirred to facilitate complete dissolution while heated. The temperature of the solution was subsequently maintained at 40°C using a hot plate to avoid gelation. The desired concentration of FBS (50%, 20% and 10) was mixed with the agarose solution to make up a final concentration.

2.3 Preparation and Freezing LM Containing Agarose Gel and Cells

The agarose sphere was generated by coating the surface of a droplet containing agarose solution loaded with a predetermined FBS concentration with the hydrophobic particles. We used PTFE powder with a nominal particle size of 1 μm (Sigma-Aldrich, product number 430935). The powder was distributed inside a 6-well plate to create an evenly layered powder bed. Then, a P10 micropipette was used to dispense a volume of 10 μL 0.5-2% low-melting-point agarose containing FBS on the hydrophobic powder bed, Figure 1A. The agarose droplet was kept inside the plate for 2 min to ensure complete gelation, Figure 1B. A 10-μL droplet containing 5×10^3 cells was dropped vertically to coalesce with the agarose gel on the powder bed, to encapsulate

the agarose gel with cells, Figure 1C. The compound droplet was then rolled on the powder bed to create an LM containing the agarose gel, Figure 1D. The LM embedded with the agarose and containing cells was then dispensed into a cryovial and subsequently placed into the freezing container (Thermo Scientific™ Mr. Frosty™) immersed in isopropanol to be frozen gradually with a rate of 1 K/min down to -80˚C inside ultra-cold freezer, Figure 1E-G. In addition, a volume of 10μL medium containing cell was dispensed into cryotube as the control experiment without LM.

2.4 Thawing the LM Embedded with Agarose Gel and Containing Cells

The cryovial containing the frozen LM was removed and placed on the hydrophobic powder bed, This facilitates a greater precision in dispensing and handling the frozen LM. Immediately, the frozen LM was dispensed into an Eppendorf tube, Figure 1H. Subsequently, the Eppendorf tube was inserted into styrofoam to ensure partial submersion of the tube bottom into the 37°C water bath, Figure 1J. The Eppendorf tube floats on a water bath, and can be swirled to facilitate the thawing process. The optimum thawing time was approximately 1-2 min. After thawing, the cells were harvested to determine the percentage of viable cells. Subsequently, the thawed agarose gel was immediately submerged into a well plate containing culture medium. The morphology was examined and imaged by Zeiss 710 LSM (Ziess, Germany) confocal microscope.

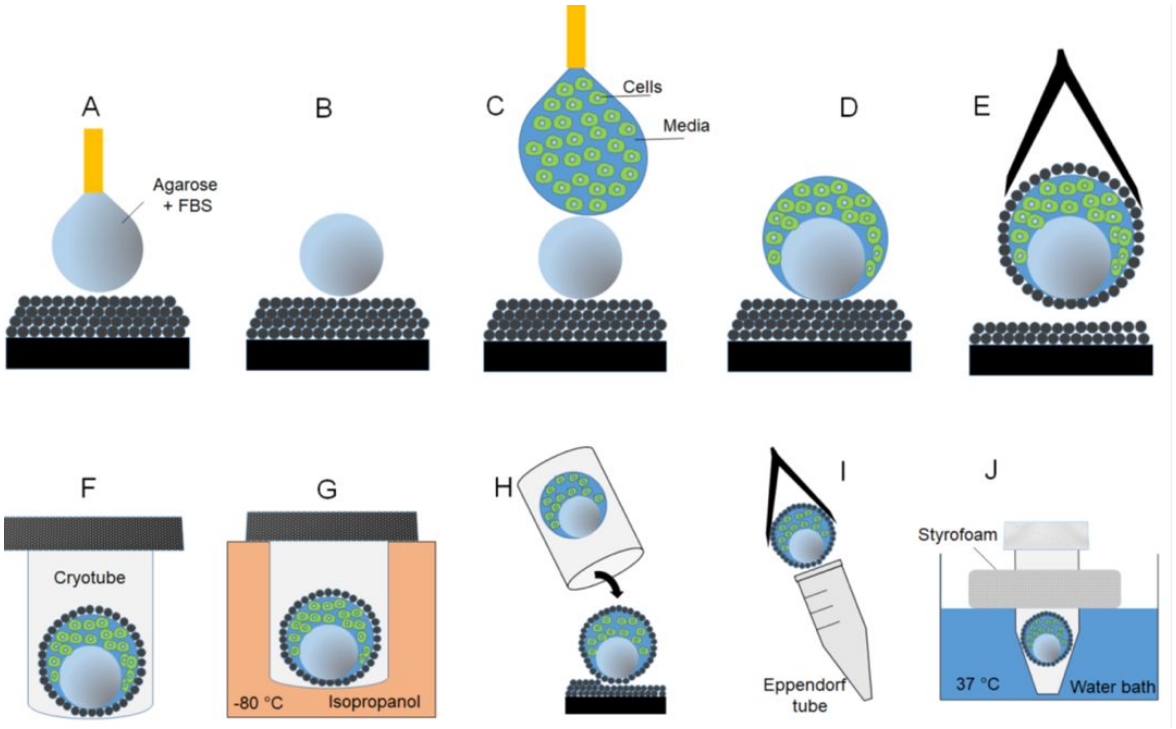

Figure 1. Preparation of liquid marble containing agarose for cryopreservation of cells: (A-B) Dispensing a hydrogel droplet containing FBS on the powder bed; (B) Impacting an aqueous medium droplet containing cells on the hydrogel; (C-D) The the agarose liquid marble is then rolled on the PTFE powder with a circular movement to ensure sufficient coating and robustness; (E) The compound marble is picked up by a tweezer and dispensed into a cryovial; (G) The cryovial containing LM was placed inside a chamber containing isopropanol to ensure controlled freezing. Procedure of thawing cryopreserved cells inside a liquid marble follows as; (H) The frozen LM was removed from cryovial and placed on the hydrophobic powder bed to ensure LM sustains shape and for dispensing made easy; (I) The frozen LM was dispensed into an Eppendorf tube by a tweezer; (J) The Eppendorf tube was inserted into a styrofoam was used to hold the Eppendorf tube, and the tube is partially submerged into 37°C water bath.

2.5 Cell Viability

We used a hemocytometer-based trypan blue dye exclusion method for the cell viability assay.[34] Prior to thawing, a 10-μL LM and cryotube (CT) containing cell suspension was added with an equal volume of 0.4% trypan blue in 0.85% saline solution, and the mixture was incubated at room temperature for 1-2 min. Subsequently, Neubauer-type hemocytometer chamber was used to count the viable (transparent) and non-viable cells (blue)

2.6 Loading of BSA into Agarose Gel

Standard bovine serum albumin (BSA), at a concentration of 1mg/ml (molecular weight = 66 kDa; Sigma-Aldrich, St. Louis, MO), was used as the model protein. The agarose solution was mixed with 0.5 mg/mL BSA (V/V) at 37 ºC. Subsequently, 10 μl of this mixture was dispensed onto the hydrophobic powder bed to cast the agarose gel. The gel was then immersed with 10 μL of deionized (DI) water to generate a compound LM for the later BSA release experiments. The LM was then frozen and thawed in the same way for cells loaded LMs as described in Figure 1.

2.7 Measurement of BSA Release from Agarose Gel

The BSA release experiments were performed during the thawing process. The amount of BSA released from the gel was collected from the loading medium surrounding the gel. After one minute of the thawing process, the liquid inside the LM was aspirated using a p10 pipette inserted into the thawed LM. Approximately 5 to 8 μL of the loading medium was collected from each LM. A total of 20 μL of the solution containing BSA was used to evaluate the amount of BSA released. To quantify the released protein, a Bradford assay was performed according to the manufacturer's protocol (Bio Rad Protein Assay; Bio Rad Laboratories).

Calibration curves for BSA standard solutions were prepared at concentrations of 0.001 to 0.5 mg/mL. Freshly prepared stock solutions were used for all experiments. After 5 minutes

incubation at room temperature, the absorption was measured for wavelengths between 450 nm to 600 nm by SpectraMax plate reader.

2.8 Cell Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was employed for evaluating the growth and proliferation of the post-thaw cells. The details of the protocol using this assay were explained in our previous work.[34] Cells were harvested from thawing LM, and seeded in a 96 well plate. The equal amount of cells from fresh culture was seeded as the positive control. Media was added to allow post-thawed cell recovery. The number of growing cells were evaluated after 24, 48 and 72 hrs. Prior to measuring cell viability, dead cells were removed by replacing the medium with a fresh medium, and MTS solution was added as recommended by the supplier. The cells were subsequently incubated at 37 °C for 4 hrs. The absorbance was measured at 490 nm using SpectraMax plate reader. Each assay was performed in triplicate. All tests were repeated three times.

2.9 Cell Morphology

Actin and nuclei were stained respectively, with ActinGreen™ 488, NucBlue™ ReadyProbes™ reagents (ThermoFisher Scientific™, Inc., Australia) and MitoTracker® CMXRos (Invitrogen) to observe the cytoskeleton reorganisation and examine the nuclei profile. Finally, we utilised an inverted fluorescent microscope (Nikon eclipse Ti2, Nikon Corporation, Tokyo, Japan) with 10X magnification to observe the cell nuclei and actin fibres.

2.10 Statistical Analysis

The results were expressed as the mean with the standard error of mean (S.E.M). All quantitative values are performed at least three times. Statistical significance was analyzed using one way ANOVA with Bonferroni multiple comparison tests. The differences were significant when $p<0.05$. Graphs were plotted using GraphPad Prism 5 software.

3. RESULTS AND DISCUSSIONS

The recent advancement in cell therapeutics and regeneration medicine leads to a demand for an efficient cryopreservable platform (particularly to eliminate the use of CPA). Although the use of LMs for cell cryopreservation has been demonstrated,[17] we aimed to further improve this platform by embedding an agarose gel inside the LM. Figure 2A depicts an agarose gel containing fluorescent beads. Figure 2E shows the structure of agarose embedded LM. We propose that the agarose gel can serve as cargo to load soluble materials and to release this load upon the freeze-thawing cycle. Additionally, the agarose gel helps to provide a thermodynamically favorable LM and also maintains its robustness. Our LM platform can release FBS from the hydrogel to yield better cell recovery in the post-thawing phase. Thus, the platform can overcome the toxicity limitation of using CPA. First, we assessed the stability of the LM during the freeze-thawing phase, following controlled and uncontrolled freezing, respectively. The LM was placed inside the vials stored in a −80 °C freezer for 24 hrs.

Controlled freezing was carried out by using a freezing container contained 100% isopropyl alcohol that provided a 1K/min cooling rate. Isopropyl alcohol was then removed to induce uncontrolled cooling, and the LM was removed from the vial and subjected to rapid thawing for approximately 1 min. We observed that LM containing agarose retains its spherical shape. In contrast, LM without agarose loses its spherical shape, Figures 2B and E. The shape transition is prominent in LM frozen without agarose gel. The freezing conditions (slow and fast)

did not show a noticeable difference in the gel shape. However, cells were found to be concentrated in the LM core. We hypothesize that the introduction of agarose gel into the liquid marbles drastically changes the kinetics of phase transition. Previously, the freezing dynamics of LM was reported to form heterogeneous nucleation at the bottom layer due to Marangoni convection.[30] This evidence supports our model, indicating that the agarose gel settling at the bottom of the LM can further slowdown the cooling rate. The agarose gel acts as a solid-liquid interface to initiate as a starting site for heterogeneous nucleation that then grows towards the surface of LM. This process will uniformly control the interfacial energy during freezing and thawing. Thus, the transition from solid to liquid phase may create less stress on the inner surface of the hydrophobic coating and maintains the robustness of the LM.

The agarose gel allows cells to accumulate in a circumferential manner at the LM bottom. Possibly, this is advantageous to delay the ice crystal formation at the bottom of the LM. As a result, the process can minimize stress on cells because the heat transfer occurs in the radial direction. In contrast, the absence of agarose gel allows the cell to cluster on the bottom of the LM. This cluster would serve as nucleation sites during the cooling process, and the growth of ice formation starts nonuniformly from the cell cluster. The nonuniform growth of ice nuclei can cause stress on the cells through cell dehydration and intracellular ice formation.[35-36] In addition to the nonuniform ice growth, nonuniform melting causes lateral stretching to the shell and leads to an inflated LM.

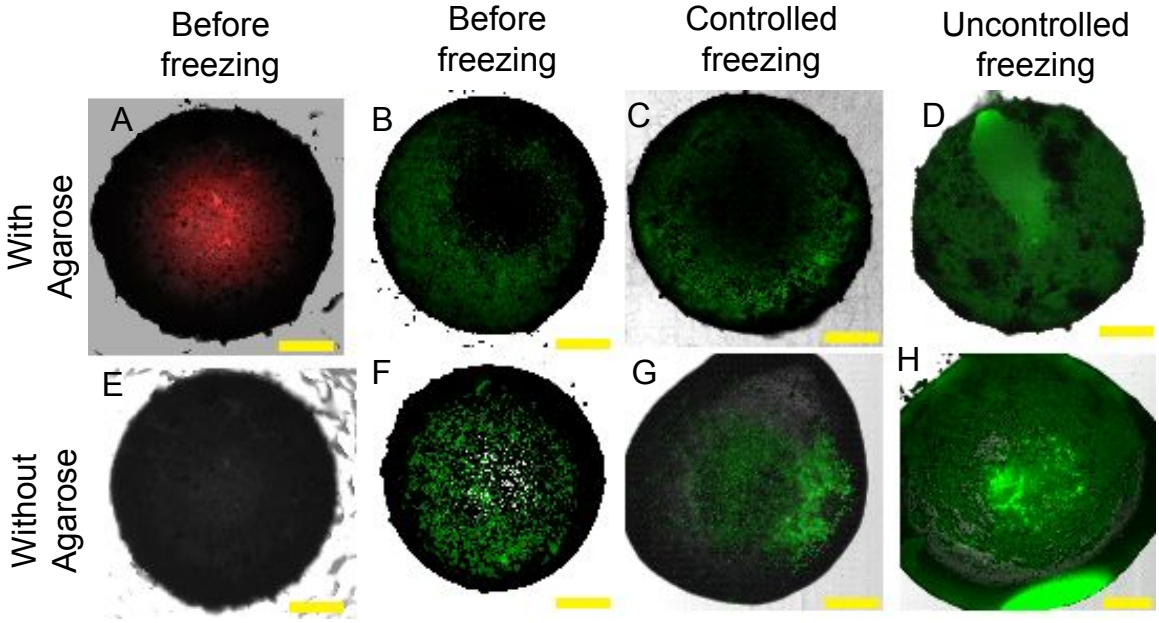

Figure 2. Liquid marbles with embedded agarose: (A) Agarose gell containing red fluorescentbeads. The cells were stained with mitotracker green before freezing: (B) Non-frozen liquid marble with agarose, the cells are distributed circumferentially surrounding the agarose gel; (C) Agarose embedded liquid marble underwent slow freezing; (D) Formation of a frozen cell cluster upon uncontrolled freezing; (E) LM without agarose; (F) cells are distributed randomly in LM without agarose; (G) Transition of LM to non-spherical shape; (H) The LM loses the spherical shape and flattens; cells are dense at the inner core. Scale bars in (A-H) are 500 μm.

Taking advantage of liquid marble with embedded agarose (LMA), we next determined its effectiveness in cryopreserving cells without a CPA. The cell viability after thawing was evaluated immediately by trypan blue dye exclusion assay. Figure 3A shows that the viability of cryopreserved cells in serum-free culture medium was approximately 30%. Surprisingly, a significant increase up to 10% of cell viability was observed in LMs containing agarose (LMA). On the other hand, the uncontrolled cooling rate severely affects cell viability, Figure 3B. We did not observe a significant change in cell viability with the advantage of using agarose gel. Subsequently, the effect of cryopreservation using these two LM conditions was compared with conventional

cryopreservation technique by adding 10% v/v DMSO to the cell suspension. The viability peaked up to 90% in both groups. Obviously, DMSO exert positive effect to cell recovery after thawing and also maintaining cell volume by altering the membrane permeability to decrease the intracellular electrolytes during freezing. Also, we noted that uncontrolled freezing with DMSO was not beneficial, Figure 3B. Although the use of DMSO has a positive outcome, this is not appreciable for the survival rate during holding time after thawing. The holding time requires storing cells under refrigerated or ambient conditions, often found in clinical applications. The exposure of DMSO for 6 hours during post-thawing holding reduced cell viability, Figure 3C. Excessive cell swelling and blebbing was observed, Figure 3D. Several studies indicate that the presence of DMSO in the surrounding media permeates the cell membrane. This causes volume changes and eventually leads to cell lysis.[37] Moreover, minimal concentration of DMSO at 7.5 -10 % has been recommended. Also, the DMSO exposure has to be limited to less than 1 hour before freezing and to 30 min after post-thawing.[38] Next, we further examined whether the presence of DMSO affects the growth of post-thawed cells. Cells derived from the LM containing 10% DMSO was incubated without eliminating the DMSO. As expected, after 24h cell attachment decreased significantly as compared to control cells. Additionally, cells shrunk and exhibited characteristic features of apoptosis (note details are provided in in Fig. S1 of Supporting Information).

In the present study, we purposely aimed to eliminate the presence of DMSO in the medium. Commonly, DMSO is removed by washing. It has been known that centrifuging right after thawing the cells is harmful and may lead to critical cell loss.[39] Moreover, our proposed protocol cannot implement the washing steps due to the relatively small volumes involved. Since LMs are potentially functional for growing three-dimensional (3D) tissues, we aim to maximize the recovery and growth of cells inside the LM. So that, LM can be used to cryopreserve cells, transport and subsequently allows culturing cells to form 3D tissues. This

objective can be achieved by modifying the LM platform to further boost the post cell recovery to a the desirable level of more than 80%.

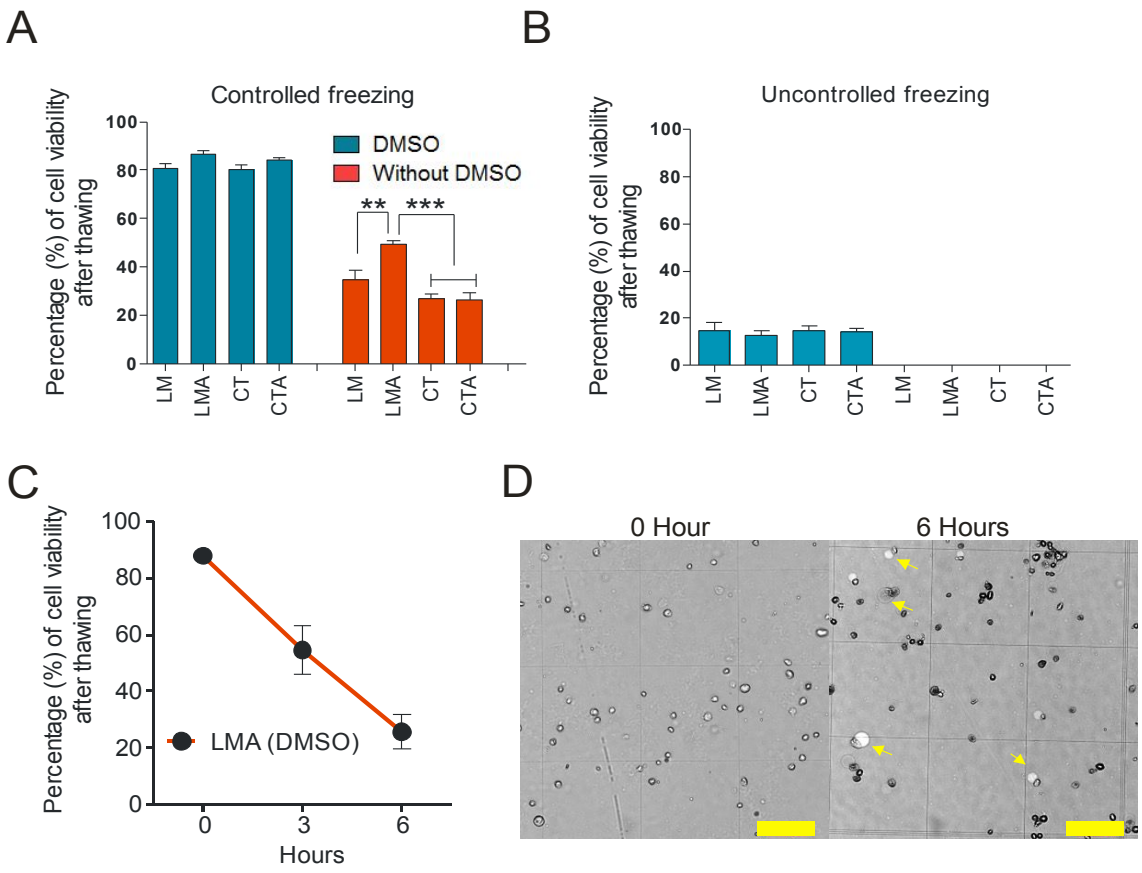

Figure 3. Cell viability after a freezing/thawing cycle in LMs (cells were suspended in culture medium without FBS): (A) Controlled freezing: LM without agarose (LM), LM containing agarose (LMA), cryotube without agarose (CT) and cryotube containing agarose (CTA). Cells were frozen with and without DMSO; (B) Uncontrolled freezing: Without DMSO, the cell viability is 0% across all cases. The percentage of agarose was fixed at 0.5%. The concentration of cell suspension and LM volume in all conditions were fixed at 5×10^3 cells in 10 µL. Statistics: ($^{**}p< 0.05$ ***p<0,001); (C) The impact of DMSO exposure on cell viability during post-thawed holding inside LM containing agarose; (D) Morphology of cells after exposure to 10% DMSO during post-thawing holding for 6 hours. Cells incubated 6 hours presenting blebs (arrow). Results of triplicate experiments are shown. Error bars represent

Many past studies reported the use of fetal bovine serum (FBS) as a cryoprotectant to improve the viability of spermatozoa[40-41], fish eggs[42] and hematopoietic stem cells.[43] Thus we selected FBS supplemented medium because the proteins and lipids present in FBS facilitate the plasma membrane permeation and prevent its destruction. We hypothesize that agarose can function as a reservoir for storing FBS and releases it upon thawing. However, It is unclear whether the presence of FBS and agarose can be synergetic, of no benefit or even can be harmful. Therefore, we next identified whether the agarose contributes to release the loaded FBS and has a positive role in cryopreservation. We designed three experimental conditions: (i) LM containing cell suspension with serum (LMS), (ii) LM containing serum with bare agarose (LMAS) and (iii) liquid marble containing serum loaded inside agarose [LM(AS)]. These conditions are described schematically in Figure 4. We tested these conditions with different percentages of FBS (10%, 20%, and 50%).

Figure 4 shows that FBS loaded agarose significantly affects the cell viability following the freeze/thaw cycle compared to other treatment groups. The viability consistently yields with the increasing FBS concentration. The results of LM(AS) and LMAS viability responding to FBS concentrations of 10% and 20% did not significantly vary. The improved viability in these groups could possibly cause by the synergy of FBS release and the effect of agarose in altering thermal properties. Also, the viability between LMAS and LMS at FBS concentrations of 20% and 50% did not differ significantly. This observation suggests that direct exposure of excess FBS with a concentration of more than 20% is detrimental to cell function and becomes toxic. Moreover, the detrimental effect can be explained by the possible presence of ice crystal during freezing-thawing with a highly concentrated FBS solution. Thus, FBS loaded agarose can release its content slowly upon thawing and minimizes the adverse effect to the cells. In general, our findings indicate that the presence of FBS inside the agarose-embedded LM positively contributes to the cryopreservation of cells. Even at the lowest concentration of FBS (i.e., 10%), the viability increases up to 57.94% ± 6.34.

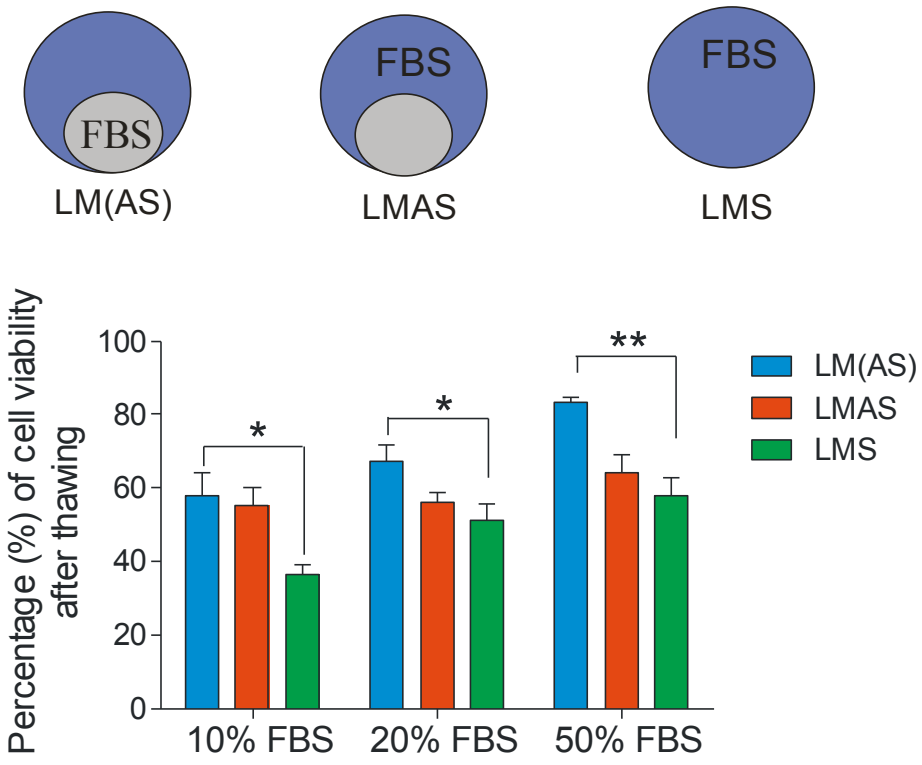

Figure 4. Cell viability evaluation of the various frozen conditions of LM after thawing. The cell suspension was fixed at 5×10^3cells per 10-µL LM containing 0.5% agarose. The LM groups are [LM(AS)] [cells and agarose loaded with FBS (10%-50%)] and (LMAS) [bare agarose with cell suspension in (10%-50%) FBS] and LMS [cell suspension in (10%-50%) FBS]. The cell viability obtained from LM(AS) groups was significantly higher in gel at 50% FBS (P**< 0.01) or in (10% and 20%) FBS (p*<0.05) compared to LMAS and LMS. Results of triplicate experiments are shown with error bar, represent mean ±S.E.M (n=6 LM).

Agarose is a gelling component of a neutral polysaccharide, which dissolves in hot water and forms a linear polymer with three-dimensional (3D) double helix networks upon cooling. The 3D helical structure is not water soluble and therefore stores large quantities of water and is useful for loading biological substances. As such, agarose can be extremely beneficial for the diffusion of solutes and soluble proteins. Loading and controlled release using agarose have been widely studied. Solute can be removed from agarose gel, either through a freeze-thaw process or by

squeezing it out with pressure. Nevertheless, to the best of our knowledge, the release of soluble proteins from agarose gel in the freezing and thawing process has never been fully studied. Freezing agarose creates ice-crystal formation and breaks its structure. During thawing the ice-crystals melt and drains out water, soluble proteins and solutes out. The release of the solvent from hydrogels is called syneresis.

The freezing and rapid thawing cycle of agarose can cause mechanical stress and exert internal osmotic stress, which impacts on the physical network of the agarose gel and forms cracks. The osmotic stress also affects the stiffness of agarose after thawing. In this study, the experimental setup did not allow us to detect any noticeable changes in the agarose structure during the initial stage of thawing. However, we investigated the morphological changes on the surface of the agarose with brightfield imaging. Figure 5 shows the morphology of the post-thawed agarose gel with a rough-textured of cracked gel. In contrast, the non-frozen agarose displays a translucent and glossy morphology. The distribution of crack size could reliably relate to the synergesis of fluid and also thought to be dependent on solute concentration. Highly hydrated gels are prone to leaving large cracks. However, agarose gel at room temperature is strongly rigid and cracks were not observed. These factors critically impede the solutes to diffuse out. Therefore, a larger and wider interconnected crack could be generated by freezing and thawing gels containing more water (0.5% and 0.25% agarose).

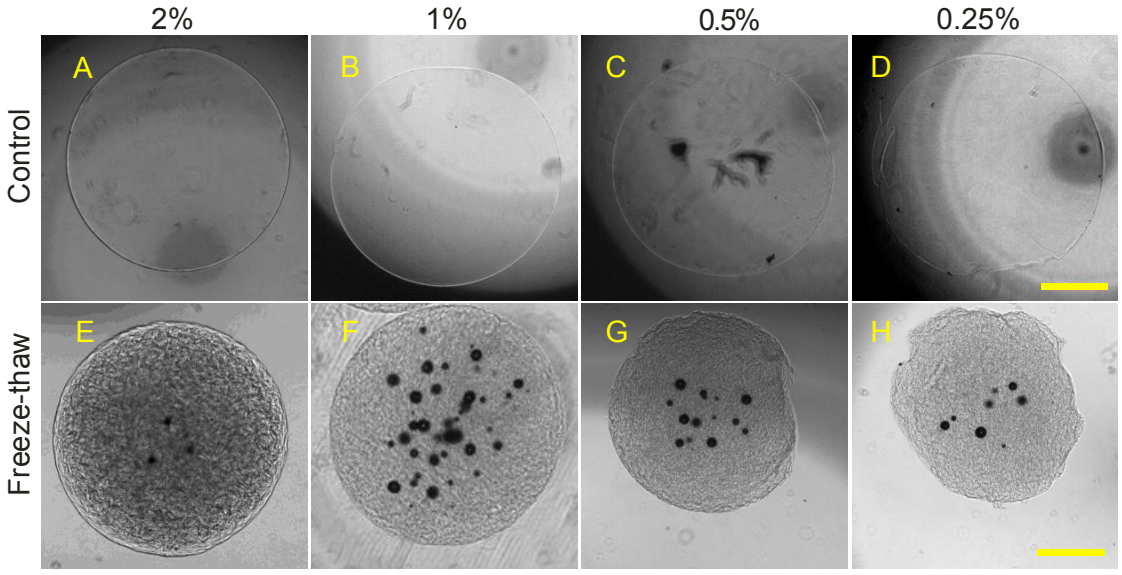

Figure 5. Brightfield images showing the agarose gels isolated from the LM (images were taken at room temperature): (A-D) Agarose at different concentrations isolated from LM and incubated at 37°C for 24 hr. The structure shows a translucent and glossy morphology; (E-H) Agarose gel isolated from post-thawed LM after 24 of freezing. The 2% agarose gel shows the rigid outer structure; (F-H) 1-0.25% gradually shows a decrease in rigidity and exhibit shrinkage. The scale bars are 500 µm.

To further confirm this phenomenon, the crack sizes at different concentration of agarose were imaged with brightfield imaging at 40× magnification, (Figure 6i-iv). Figures 6i and 6ii show that a higher concentration of agarose leads to a denser gel with smaller crack size. In contrast, the lower concentrations of agarose exhibit larger cracks, (Figure 6iii-iv). At 0.25% agarose, these cracks show a loose structure with interconnected channels, (Figure 6iv).

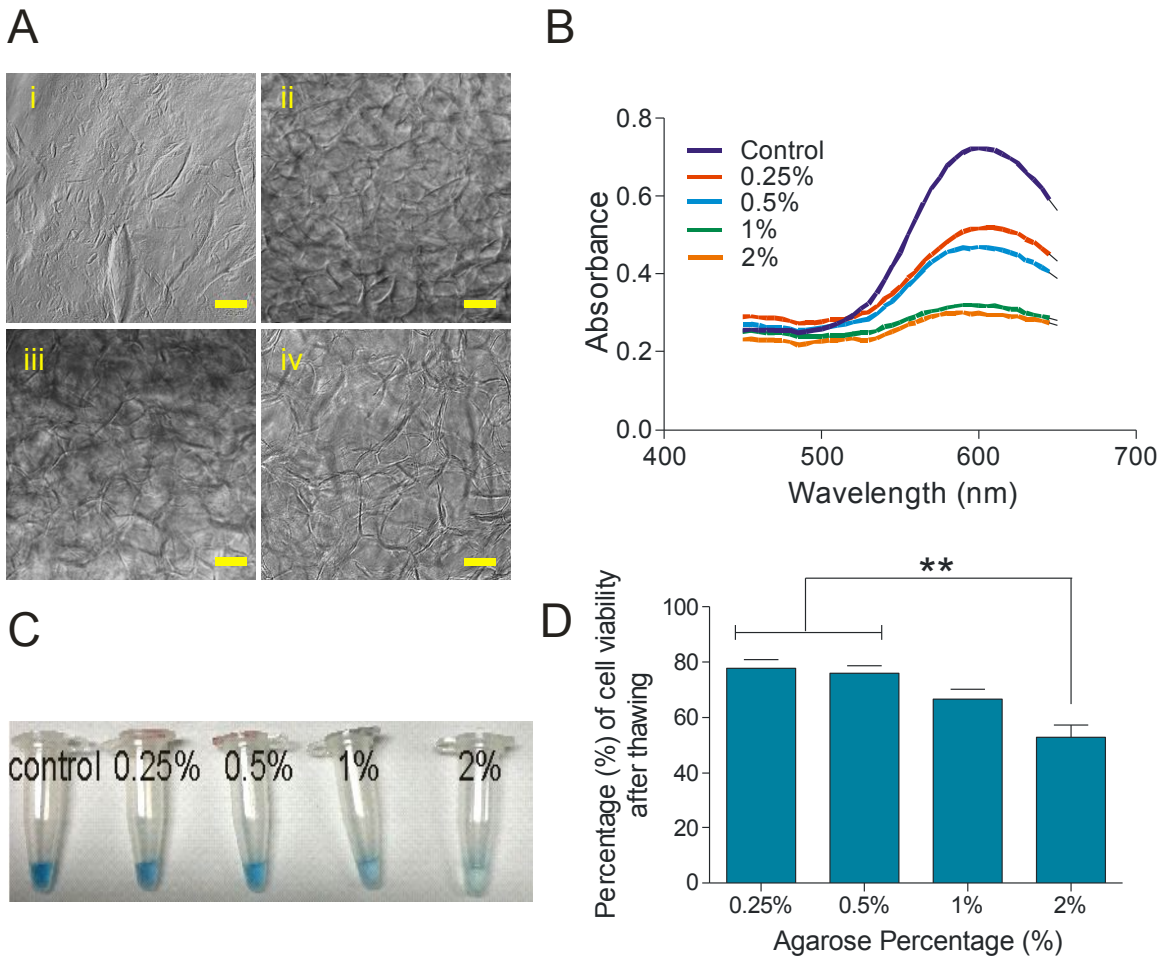

Figure 6. Effect of agarose gel concentration on protein release. (A) Images of agarose morphology with four different concentrations after thawing: (i) 2%; (ii) 1%; (iii) 0.5% and (iv) 0.25% [magnification 40×]. The crack size distribution appears to become larger as the concentration of agarose decreases. 0.25% agarose exhibits crack with a larger size. (B) Photo of the color change at different concentrations of BSA. (C)) The change in the UV-Vis absorption spectrum corresponding to a total amount of BSA released. (D) Effect of agarose concentration on cell viability after post-thawing. All agarose gels were loaded with 50% FBS. The cell number and LM volume were fixed at 5×10^3 cells in 10 µL LM. The viability is significantly higher using 0.25% and 0.5% agarose (P**< 0.01) compare to 1% and 2 % gel. Results of triplicate experiments are shown error bars, represent mean ±S.E.M (n=6 LM). The

However, the influence of freeze/thawing on the structural changes in an agarose gel and its implication on releasing soluble proteins is still hypothetical. We loaded an equal amount of BSA with agarose gel at (2%, 1%, 0.5% and, 0.25%) and evaluate the release of BSA from freeze-thawed agarose. BSA is chosen as the protein model as it is sensitive to spectrophotometric detection. Moreover, the combination of BSA with agarose did not affect the gelation process. This indicates that BSA shows the ability to be stored within the agarose polymer chains. Also, to be noted that the interaction of carboxyl groups in the protein with hydrogen bond presence on agarose polymer chain prevents protein from leaking. The size of BSA (64 Kda) is similar to the size of various proteins available in FBS (30 Kda to 70 Kda).[44] Thus, BSA can serve as a model to predict FBS release. The release was conducted at 37ºC after 1 minute. Immediately, the released medium was transferred from LM and the BSA concentration was measured using UV absorbance measurement. Interestingly, the absorbance data at 595nm showed that the diffusivity of BSA in the gel relies on the percentage of agarose. The release rate of BSA in 0.5% and 0.25% gel are higher compared to 1% and 2%. Figure 6B shows the color change relative to protein concentration. The concentration of BSA was determined from a calibration curve (details in the Supporting Information). Approximately 0.3 mg/mL of BSA was released in gels with 0.5% and 0.25% agarose. Gels at higher concentrations have a more rigid matrix. Therefore concentrated gels entrap the proteins and impede the release process. In addition, the change in release rate possibly depends on the change of surroundings temperature, which may affect the microstructure of the agarose. The UV spectrum indicates that the protein incorporated with agarose upon being exposed to freezing and thawing cycles remains stable. The rapid temperature rise during thawing causes phase transition. The crystals within the gel are converted to liquid, resulting in expansion and dissociation of agarose polymer network. This causes gels to crack and leave large gaps within the network. Thus, the less concentrated agarose gel can enhance the protein release.

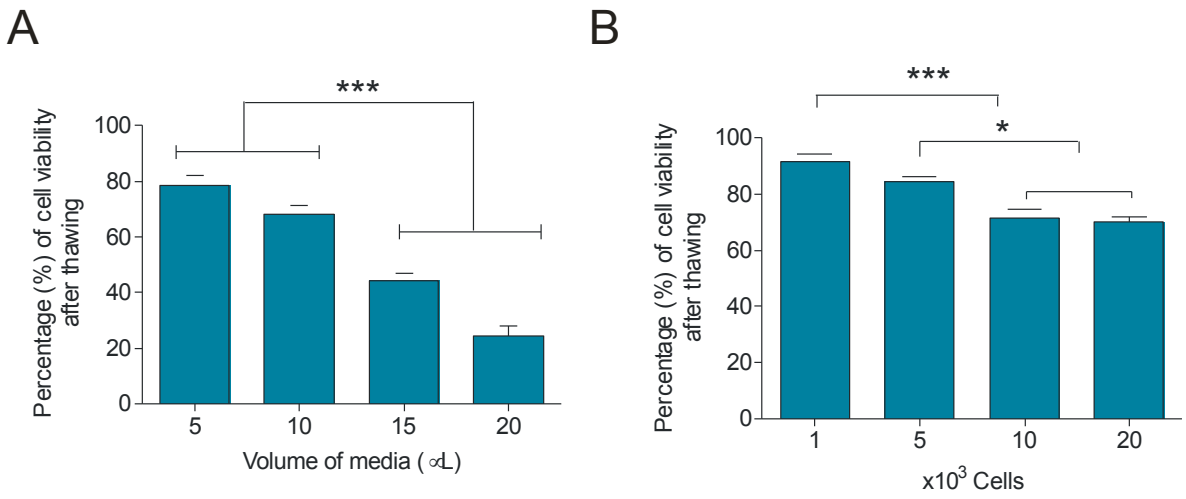

Figure 7. Effect of LM volume and cell density on post-thawing cell viability. (A) The LMs contained agarose gel at a fixed concentration of 0.5% and loaded with 50% FBS. All LMs contain 5×103 cells. Viability was significantly higher using 5 and 10 µL (***P< 0.001) comparatives to 15 and 20 µL. Results of triplicate experiments are shown Error bars represent mean ±S.E.M (n=6 LM). (B) Effect of cell density in post-thawing viability. The LMs contained agarose gel at a fixed concentration of 0.5% and loaded with 50% FBS. Results of triplicate experiments are shown Error bar, represent mean ±S.E.M (n = 5). *p < 0.05 and ***p < 0.001.

Next, we aim to elucidate whether larger cracking contributes to the release of FBS upon thawing and consequently affect the cell viability. Accordingly, we fixed the concentration of FBS at 50%, which led to the maximum cell viability at a fixed agarose concentration and systematically changed the concentration of agarose gel from 0.25% to 2%. Figure 6D indicates that agarose concentrations of 0.5 and 0.25%, significantly enhance cell viability. However, poor cell survival was observed at 1 and 2 % concentration. No meaningful differences between these two concentrations were observed. Thus, we can conclude that the existence of loosened interlinked network channels of the lower agarose concentrations attributes to the susceptibility to syneresis and volumetric changes during thawing. Thus, the larger crack quickly drains out the loaded FBS from the gel without impedance. Syneresis could not be estimated

either qualitatively or quantitatively. However, the cell viability profile is evident to conclude the agarose is functional to releasing FBS upon thawing and affects positively to recover cells.

We additionally evaluated the effect of LM volume on the efficacy of the cryopreservation process. We prepared LMs with 0.5% agarose and fixed the FBS concentration at 50% to determine the ideal volume of LM for optimal cell viability. The cell concentration was fixed at 5×10^3 cells/μL in volumes of 5 μL, 10 μL, 15 μL, and 20 μL, Figure 7A. One-way ANOVA analyses indicated that the LMs with volumes of 5 μL and 10 μL showed significantly more viable cells than those with volumes of 15 μL and 20 μL. Previous studies have shown that a small volume is more efficient for improving cryopreservation because of the higher cooling rate and the minimum effect of ice crystal formation. Thus, our study suggested that the volume of LM affects the cooling and warming rates during cryopreservation. The results indicate that the LMs with volumes of 5 μL and 10 μL were optimal for producing a maximum amount of post-thaw viable cells. Considering the negligible difference in the amount of the cell viability in these two volumes as well as the technical challenges corresponding to 5 μL LM, the volume of LM was consistently selected at 10 μL for all the experiments reported in this study. The optimal LM platform from this study was then tested to determine the effect of cell density on on recovery after thawing. Frozen LM at the cell density between 1 to 10×10^3 cells/LM was thawed to evaluate cell viability. Interestingly, cells with the density of 1 and 5×10^3 cells/LM retained the viability higher than 80%, Figure 7B.

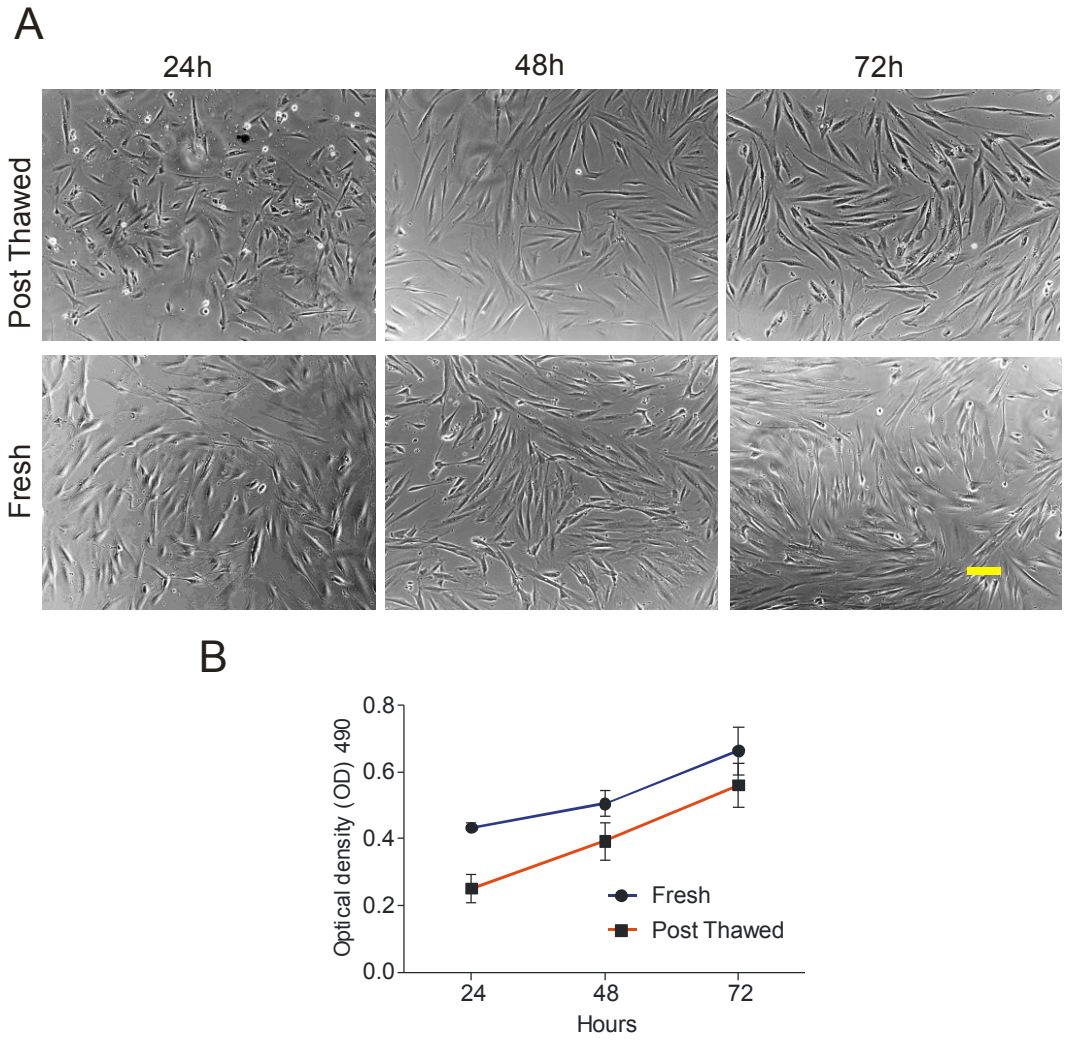

Figure 8. The proliferative capacity of the post-thawed cells compared to the fresh (non-cryopreserved) (A) The cell growth at 24hr intervals up to 72 hr (×10). (B) MTS assay was performed to evaluate cell proliferation in incubated post-thawed cells and its control cells. Cell growth was monitored at time points, including 24, 48, and 72 h. Compared to the control cells, the growth rate of cryopreserved cells showed a similar trend. Scale bar is 100 μm.

The proliferation capacity of post-thawed cells are critical for efficient cell-based therapies. Thus, we next determined whether the adverse effect of cryopreservation affects cell

proliferation. Cells were harvested from the post-thawed LM containing an agarose loaded with 50% FBS. Cells from each LM were seeded in a 96-well plate and grown in the culture media with 10% FBS for 24 hrs, 48 hrs, and 72 hrs. An equal number of cells were cultivated in a LM, which was not subjected to freezing and grown parallel as non-cryopreserved cells (control group). The cell growth was evaluated by bright fields images at intervals of 24h up to 72h, Figure 8A. The numbers of viable cells in proliferation were analyzed with the MTS viability assay. The optical density (OD) values indicate that the non-cryopreserved cells (control group) showed an increasing growth rate in 48 and 72 hrs, Figure 8B. In comparison, the growth rate of cryopreserved cells showed a similar trend, but with a lower growth rate. The reduction in growth rate could be due to loss in viability and functionality during the thawing process. Also, the subsequent thawing procedures might inactivate the enzyme systems and decrease the cellular metabolism. This could possibly affect cells with regard to cell-substrate attachment and initiate cell-cell connection response. Hence, cells lose viability or require more time for adherence and monolayer formation.

Another challenge is to sustain the cellular ultrastructure such as cytoskeleton component, which involve in preceding cell spreading and migration.[45-46] Previous studies have shown that cryopreservation altered the actin cytoskeleton in fibroblasts and also attenuated cell-cell anchoring.[47] Freezing can result in destabilization of microtubule and impairs functions of actin stress fibers. Eventually, this process may not necessarily cause cell death, but potentially leads to a change in cell phenotype. Another concern is to retain mitochondria functionality after freezing. Disruption is mitochondria function and organization of cytoskeleton negatively impact on the meiotic spindle formation during cell division. It is of interest to investigate the cellular integrity after post thawed cells were grown. We thawed cells frozen at the optimal conditions, which are 10-μL LM filled with 0.5% agarose containing 50% FBS. The cells from each LM were then harvested and plated to assess their attachment and growth. After 24 hrs growth, the

cells were stained with Mitotracker Red CMXRos and ActinGreenTM 488. The overall cell numbers of post-thaw culture were significantly reduced. Thus, we choose to analyze the morphology of the adherent cells, which has a similar distribution to fresh culture. Both post-thawed and fresh cultures did not show differences in cell morphology, Figure 9. The post-thawed cells sustained attachment and established contact with neighboring cells. Moreover, post thawed cells extensively spread their cytoplasmic projection on the leading edge of lamellipodia known as actin-dependent protrusion.[48] and cytoplasmic projections are noticeable, (Figure 9A). The freezing did not cause actin stress fiber deformation. Instead, the cells displayed prominent actin fiber formation, which may attribute to cell morphology and motility, Figures 9A. The regulation of cell metabolism is primarily dependent on proper mitochondria distribution. Thus, we assess the effect of mitochondria distribution and investigate whether cells retain mitochondrial function. Interestingly, Mitotracker Red CMXRos staining did not show significant differences in mitochondria distribution between fresh and frozen/thawed cell (Figure 9B and E). The relationship between the organization of actin cytoskeleton and distribution of mitochondria attributes to normal cell physiology.[49] We observed that mitochondria distribution was parallel to the direction of actin fibers. Overall, this study demonstrates the suitability of the model to retain cell functionality after the freeze/thaw cycle.

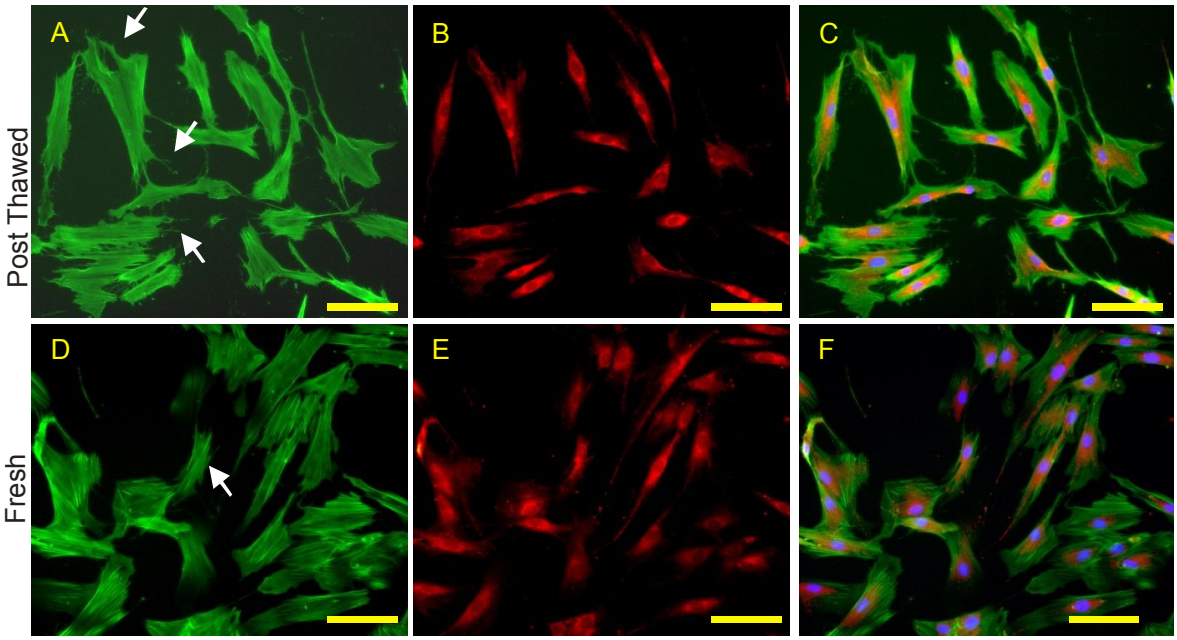

Figure 9. Cellular staining of monolayer NIH-3T3 fibroblast cells grown 24 hrs after thawing. (A) Post thawed cells were grown for 24h and stained for actin, mitochondria and nuclei. (B) For comparison, nuclei, actin, and mitochondria were stained in cells grown from the fresh cultures(A and D) The cells attach and spread for establishing connections. The spreading area is greatly enhanced by the formation of lamepodia and filophodia. (indicated by white arrow). Cells appeared to display actin stress fibers. (B and E) Mitochondrial fluorescence were normal in fresh cells as well as in thawed cells [magnification 20×] Scale bar is 100 μm.

4. CONCLUSIONS

The present study described a novel LM-based platform to provide efficient cryopreservation at low cell density without the use of a CPA. The proposed platform could systematically release FBS during the freeze-thaw cycle. The key of our method is an agarose hydrogel embedded inside the LM containing cells. We systematically investigated the effect of the volume of the LM, the concentration of agarose and the amount of FBS inside the agarose gel on the post-thaw cell viability, morphology and attachment. All experimental results indicated that the LM embedded with agarose significantly improved the preservation. Additionally, agarose loaded

with FBS offered the ability to recover the post-thawed cell. In this sense, the recovered cells are indicative of normal cellular parameters such as cell adhesion, the establishment of cell-cell contact, cell migration physiology and evidence of mitochondria functionality. We envision that future studies are required to understand how FBS affects the cell membrane integrity during freezing and thawing. Apart from that, this model can be utilized to release other types of cryoprotectant, which possibly yield an even better post-thawed cell recovery. This research can have a significant impact in addressing the challenging demands of cryotechnology. The future directions of this work can enable the researchers to scale up the platform with higher throughput, leading to automated bio-preservation for applications in agriculture and medicine.

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Author Contributions

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Notes

The authors declare no competing financial interest

Supporting Information

Effect of DMSO on post-thawed cell growth for 24 hours. DMSO exposure affects the cell morphology and also features was observed.

A standard curve is plotted by testing known concentration of BSA to calculate the total concentration of released protein from the agarose gel.

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A novel attempt to conceptualize a digital microfluidic system consisting of agarose-embedded liquid marble for cryopreservation of low cell density without using cryoprotectant