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# Bulk Droplet Vitrification for Primary Hepatocyte Preservation

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**TITLE:**

# Bulk Droplet Vitrification for Primary Hepatocyte Preservation

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**KEYWORDS:**

hepatocyte, primary hepatocyte isolation, droplet vitrification, cryopreservation, cryoprotectant agent, cellular dehydration

**SUMMARY:**

This manuscript describes an ice-free cryopreservation method for large quantities of rat hepatocytes whereby primary cells are pre-incubated with cryoprotective agents at a low concentration and vitrified in large droplets.

**ABSTRACT:**

Vitrification is a promising ice-free alternative for classic slow-freezing (at approximately 1 °C/min) cryopreservation of biological samples. Slow-freezing requires extremely fast cooling rates to achieve transition of water into the glass phase while avoiding injurious ice formation. Although pre-incubation with cryoprotective agents (CPA) can reduce the critical cooling rate of biological samples, high concentrations are generally needed to enable ice-free cryopreservation by vitrification. As a result, vitrification is hampered by CPA toxicity and restricted to small samples that can be cooled quickly. It was recently demonstrated that these inherent limitations can be overcome by bulk droplet vitrification. Using this novel method, cells are first pre-

incubated with a low intracellular CPA concentration. Leveraging rapid osmotic dehydration, the intracellular CPA is concentrated directly ahead of vitrification, without the need to fully equilibrate toxic CPA concentrations. The cellular dehydration is performed in a fluidic device and integrated with continuous high throughput generation of large sized droplets that are vitrified in liquid nitrogen. This ice-free cryopreservation method with minimal CPA toxicity is suitable for large cell quantities and results in increased hepatocyte viability and metabolic function as compared to classical slow-freezing cryopreservation. This manuscript describes the methods for successful bulk droplet vitrification in detail.

**INTRODUCTION:**

Loss of cell viability and metabolic function after cryopreservation of hepatocytes is still a major issue that limits clinical applications[1–3]. The benchmark method of hepatocyte cryopreservation is slow-freezing, which is performed by pre-incubating the hepatocytes with CPA (dimethyl sulfoxide [DMSO], glucose, and albumin) and subsequent controlled rate freezing (at approximately 1 °C/min) to cryogenic temperatures[4,5]. Despite many reported optimizations, CPA toxicity together with injurious osmotic imbalances during freezing and mechanical stress of ice formation remain fundamental drawbacks of slow-freezing[6,7].

Vitrification offers an advantage over slow-freezing in that injury due to ice formation is completely avoided by a direct phase transition of water into the glass state[6]. However, to reach the glass transition temperature of pure water (-137 °C), the water must be cooled at rates in the order of one million degrees Celsius per second (i.e., the critical cooling rate) to avoid ice formation above the glass transition temperature[8]. Addition of CPAs can lower the critical cooling rate and increase the glass transition temperature of aqueous solutions[9]. However, even with high CPA concentrations (e.g., 40% v/v DMSO or higher) fast cooling rates are nonetheless required for successful vitrification[8,9].

The required cooling rates and high CPA concentrations result in two major drawbacks of vitrification. First, to enable fast cooling, the samples must have a low thermal mass. Second, to reach high CPA concentrations while avoiding osmotic injury, CPAs must be slowly introduced and fully equilibrated between the intra- and extracellular compartments[6]. This increases the exposure time of cells to toxic CPAs. Together, this makes vitrification a cumbersome process that is limited to a few small sized samples (microliters) at a time. Droplet vitrification has been proposed as a potential solution to these restrictions. By exposing miniscule cell-laden droplets (nanoliters) to liquid nitrogen the cooling rate is significantly increased, which consequently allows a considerable reduction in the CPA concentration[10–14]. Although multiple high frequency droplet-generating nozzles could potentially be used simultaneously, the extremely small droplet size limits the throughput to microliters per minute[10], which precludes efficient vitrification of large cell volumes with magnitudes higher processing rates on the order of milliliters per minute.

Recently it was demonstrated that these inherent limitations of vitrification can be overcome by bulk droplet vitrification[15]. This novel method leverages rapid osmotic dehydration to concentrate an intracellular CPA concentration of 7.5% v/v ethylene glycol and DMSO immediately preceding vitrification, eliminating the need of full equilibration of toxic CPA

concentrations. The cellular dehydration is performed in a fluidic device by a brief exposure of the hepatocytes to a high extracellular CPA concentration. Although this exposure causes rapid osmotic dehydration, it is too short for the high CPA concentration to diffuse into the cells. Immediately after dehydration, the cells are loaded in droplets that are directly vitrified in liquid nitrogen. This method eliminates the need of full intracellular uptake of high CPA concentrations while the high extracellular CPA concentration enables vitrification of large sized droplets, resulting in high throughput volumes with minimal CPA toxicity.

Droplet vitrification improves direct and long-term viability after preservation, as well as morphology and metabolic function of primary rat hepatocytes as compared to classical cryopreservation by slow-freezing[15]. This manuscript provides the methodological details for bulk droplet vitrification of primary rat hepatocytes.

**PROTOCOL:**

The primary hepatocyte isolations for this protocol were performed by the Cell Resource Core (CRC) at Massachusetts General Hospital, Boston, Massachusetts, USA. The animal protocol (#2011N000111) was approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital.

## 1. Bulk droplet vitrification

### 1.1. Prepare the vitrification solutions.

1.1.1. Add 40 mg of bovine serum albumin (BSA) to 17.0 mL of University of Wisconsin solution (UW) to make vitrification solution 1 (V1). Sterile filter the V1 solution using a 0.22 µm filter and store at 4 °C.

NOTE: UW is a commonly used organ preservation solution. It contains 50 g/L hydroxyethyl starch, 35.83 g/L lactobionic acid, 3.4 g/L potassium phosphate monobasic, 1.23 g/L magnesium sulfate heptahydrate, 17.83 g/L raffinose pentahydrate, 1.34 g/L adenosine, 0.136 g/L allopurinol, 0.992 g/L total glutathione, 5.61 g/L potassium hydroxide, and sodium hydroxide to adjust the pH to 7.4.

1.1.2. Combine 7.0 mL of UW, 6.5 mL of DMSO, 6.5 mL of ethylene glycol (EG), 40 mg of BSA, and 5.48 g of sucrose to make vitrification solution 2 (V2). Sterile filter the V2 solution using a 0.22 µm filter. Load 3.5 mL of sterile filtered V2 solution in a 3 mL syringe and store at 4 °C.

NOTE: To facilitate dissolving the CPAs, solutions are prepared in larger quantities than required.

### 1.2. Prepare the bulk droplet vitrification apparatus.

1.2.1. To prepare the mixing needle, first cut along one side of the mixing needle's outer gray sleeve, and then bend the sleeve open to remove it from the mixing needle.

1.2.2. Slide two 25 mm silicone tubing sections over the inlets of the mixing needle and secure their position with glue (**Figure 1A**).

1.2.3. Cut the needle to a length of 20 mm (i.e., the length of the inner mixing helix) as shown in **Figure 1A**.

NOTE: The mixing needle length is proportional to the volume inside the needle and therefore can be altered to control the exposure time of the hepatocytes to the high CPA concentration solution.

1.2.4. Insert the cutoff outlet of the mixing needle into a 20 G injection needle (**Figure 1B**).

NOTE: The injection needle size influences the droplet size.

1.2.5. Sterilize the mixing needle assembly by autoclaving.

1.2.6. Fill a sterile Dewar with liquid nitrogen. Sterilize the outside of the Dewar with isopropanol before placing the Dewar in a sterile laminar flow cell culture hood.

NOTE: Contamination is an important consideration during direct exposure of the droplets to liquid nitrogen. Although there was no contamination using non-sterilized liquid nitrogen in the current protocol, liquid nitrogen can be filtered or irradiated to assure sterility if needed[16].

1.2.7. Insert a funnel with the same outer diameter as the inner diameter of the liquid nitrogen Dewar into a 50 mL conical tube to create the droplet collection device (**Figure 1C**–**E**).

1.2.8. Submerge the droplet collection device in the liquid nitrogen by slowly pressing it down in its final vertical position using large forceps. Ensure that the conical tube rests in a vertical position at the bottom of the Dewar (**Figure 1D**–**E**).

NOTE: The funnel should stay inserted in and resting on the conical tube. The liquid nitrogen level should be 1 cm below the inlet height of the funnel, as shown in **Figure 1D**.

1.2.9. Mount a syringe pump in a vertical position on the wall of the cell culture hood by tying a string from the syringe pump's feet to screws protruding from the cell culture hood wall.

1.2.10. Place the liquid nitrogen Dewar with the droplet collection device under the vertically mounted syringe pump (**Figure 1E**).

1.3. Pre-incubate with CPAs.

1.3.1. After obtaining freshly isolated rat hepatocytes, count the stock concentration by Trypan blue exclusion and take 40 million viable hepatocytes.

NOTE: Gentle handling of hepatocytes in suspension is critical to prevent cell death.

1.3.2. Centrifuge at 50 x *g* for 5 min without brake.

1.3.3. Aspirate the supernatant and resuspend the hepatocytes in 3.4 mL of V1 solution.

1.3.4. Incubate on ice for 3 min.

1.3.5. Add 150 µL of DMSO and 150 µL of EG to the hepatocytes and mix gently.

1.3.6. Incubate on ice for 3 min.

1.3.7. Again, add 150 µL of DMSO and 150 µL of EG to the hepatocytes and mix gently.

1.3.8. Load 3.5 mL in a 3 mL syringe.

1.4. Perform vitrification.

1.4.1. Insert the syringe with pre-incubated hepatocytes and the V2 solution syringes onto the syringe pump adapter.

1.4.2. Attach two female Luer lock hose barb adaptors to the syringes and slide the silicone tubing of the mixing needle assembly over the barb fittings (**Figure 1F**).

1.4.3. Place the entire assembly in the syringe pump (**Figure 1E**).

1.4.4. Three min after the last addition of DMSO and EG to the hepatocytes, start the pump at 2 mL/min.

1.4.5. Stop the pump after all hepatocytes have been added to the liquid nitrogen.

**2. Cryogenic storage**

2.1. Puncture 10 small holes in the lid of a 50 mL conical tube using a 20 G needle and wrap the outside of the lid with a flexible film, as shown in **Figure 1B**. This creates a valve that enables the escape of evaporating leftover liquid nitrogen.

2.2. To collect the vitrified hepatocyte droplets, first remove the funnel from the liquid nitrogen Dewar. Next, use long forceps to take the conical tube that contains the droplets out of the liquid nitrogen and slowly pour out the excess liquid nitrogen from the conical tube.

2.3. Close the conical tube with the punctured lid and directly place the closed conical tube with vitrified hepatocyte droplets back in the liquid nitrogen Dewar.

2.4. Transfer the conical tube from the liquid nitrogen into a liquid nitrogen vapor cryotank.

**3. Rewarming of the vitrified hepatocyte droplets**

3.1. Prepare the rewarming solution.

3.1.1. Dissolve 17.13 g of sucrose in 100 mL of DMEM hepatocyte culture media to make the rewarming solution. Sterile filter the rewarming solution using a 0.22 µm filter and warm to 37 °C.

3.2. Rewarm the hepatocytes.

3.2.1. Take the conical tube with vitrified hepatocytes from the cryotank and transport it in a liquid nitrogen Dewar to the cell culture hood.

3.2.2. Lightly loosen the cap and put the conical tube back in the liquid nitrogen.

3.2.3. Add the vitrified hepatocyte droplets to an empty beaker, instantaneously add the warm (37 °C) rewarming solution, and immediately stir for 10 s.

NOTE: It is critical to work quickly to avoid freezing during rewarming**.** Depending on the study requirements, a few to all vitrified droplets can be rewarmed at once.

3.3. Unload the rewarming solution.

3.3.1. Divide the rewarming solution with hepatocytes over two 50 mL conical tubes.

3.3.2. Centrifuge the two conical tubes for 2 min at 100 x *g* at 4 °C without brake.

3.3.3. Aspirate the supernatant to leave a total volume of 12.5 mL using the conical tube's graduation mark as a reference and add 12.5 mL of ice cold DMEM per conical tube to dilute the rewarming solution to 50%.

3.3.4. Resuspend the hepatocytes and incubate on ice for 3 min.

3.3.5. Add 25 mL of ice cold DMEM per conical tube to dilute the rewarming solution to 25%.

3.3.6. Centrifuge for 5 min at 50 x *g* at 4 °C without brake.

3.3.7. Aspirate the supernatant and resuspend the hepatocytes in 2 mL of the desired culture medium for use.

NOTE: Density gradient centrifugation can be used to remove dead hepatocytes from the cell suspension if desired.

**REPRESENTATIVE RESULTS:**

Freshly isolated primary hepatocytes from five different rat livers were used for a direct comparison of bulk droplet vitrification to classic cryopreservation using the preeminent slow-freezing protocols reported in the literature[4,5]. In short, the hepatocytes were suspended in UW supplemented with BSA (2.2 mg/mL), glucose (333 mM), and DMSO (10% v/v) and frozen using a controlled rate freezer. After storage at -196 °C, the samples were thawed in a warm water bath. After all the ice melted, the DMSO was directly diluted while the glucose concentration was gradually lowered during multiple steps to reduce osmotic injury. The exact protocol can be found in detail elsewhere[15]. Preservation durations varied from 2 to 8 days and were matched between bulk droplet vitrification and freezing cryopreservation for each biological replicate to ensure equal comparison. Although shorter preservation times were tested for practical considerations, it should be noted that primary hepatocytes can be stored at -196 °C for years without loss of viability[5].

Bulk droplet vitrification results in a direct post preservation hepatocyte viability of 79%, measured by Trypan blue exclusion testing, which determines membrane integrity (**Figure 2A**). This is significantly higher than the 68% viability after classic optimized cryopreservation. The yield of bulk droplet vitrification is 56% that is 10% higher, and importantly more consistent, as compared to classic cryopreservation (**Figure 2B**).

The metabolic activity of hepatocytes, measured using a Presto Blue metabolization essay in long-term collagen sandwich cultures, was significantly higher after bulk droplet vitrification than after classical cryopreservation. Albumin synthesis, which is the most widely used parameter of synthetic function of hepatocytes, was greater by nearly two-fold after bulk droplet vitrification as compared to classic cryopreservation (**Figure 3A**). Urea production is the most widely used parameter of hepatocyte detoxification function. After a one-week culture, the urea production of bulk droplet vitrified hepatocytes was significantly higher than that of classic cryopreserved hepatocytes (**Figure 3B**).

In summary, bulk droplet vitrification improves direct post-preservation viability and long-term metabolic function of primary rat hepatocytes in collagen sandwich cultures, as compared to cryopreservation by slow-freezing.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Bulk droplet vitrification experimental setup.** (**A**) Illustration of how to prepare the mixing needle and attach two silicone tubing sections to the inlets of the mixing needle. (**B**) Illustration of the insertion of the cut mixing needle in the injection needle to complete the mixing needle assembly. (**C**) Illustration of the funnel and 50 mL conical tube that constitute the droplet collection device. (**D**) Sketch showing the position of the conical tube, the funnel, and the liquid nitrogen level in the Dewar. (**E**) Representation of the complete experimental bulk droplet vitrification setup from bottom to top: the liquid nitrogen Dewar (gray); the droplet collection device that is placed inside the liquid nitrogen Dewar (clear); the mixing needle assembly (clear-

yellow) attached to the syringes that are placed in the syringe pump (red). (**F**) Illustration of the syringes and mixing needle assembly. Two 3 mL syringes are clamped into the syringe pump adapter (blue); one syringe should be filled with pre-incubated hepatocytes in V1 solution and the other with the high CPA concentration solution (V2 solution). The mixing needle assembly is attached to the syringes by two female Luer lock hose barb adapters. (**G**) Illustration of how to create the conical tube lid for cryogenic storage which enables leftover evaporating liquid nitrogen to escape from the conical tube during storage of the vitrified hepatocyte droplets. Top: lid with punctured holes. Below: the punctured lid wrapped with flexible film. Scale bars: 1 cm.

**Figure 2: Hepatocyte viability and yield after preservation.** (**A**) Viability of fresh (gray), cryopreserved (blue), and bulk droplet vitrified (green) hepatocytes. (**B**) Preservation yield of cryopreserved and bulk droplet vitrified hepatocytes. Stars: $p < 0.05$ (Wilcoxon matched-pairs signed rank test). Whiskers: min to max.

**Figure 3: Metabolic activity in long-term collagen sandwich cultures.** (**A**) Albumin synthesis of fresh (gray), cryopreserved (blue), and bulk droplet vitrified (green) hepatocytes on culture days 3, 5, and 7. (**B**) Urea production of fresh, cryopreserved, and bulk droplet vitrified hepatocytes. Stars: $p < 0.05$ (paired one-way ANOVA followed by the Tukey correction for multiple testing). Error bars: standard deviation.

**DISCUSSION:**

Cryopreservation of hepatocytes by slow-freezing results in reduced viability and metabolic function. Vitrification offers a promising alternative for classic cryopreservation, as freezing injury is completely avoided[9]. However, pre-incubation with CPAs is required to lower the critical cooling rate[8]. Consequently, vitrification is hampered by CPA toxicity[17] and limited to small sample volumes. In efforts to overcome these limitations the bulk droplet vitrification method presented in this manuscript was developed to enable vitrification of large quantities of cells while using a low pre-incubated CPA concentration[15]. This bulk droplet vitrification leads to increased hepatocyte viability and metabolic function as compared to the most optimal slow-freezing cryopreservation method in the literature. Here, the comprehensive methods of bulk droplet vitrification and detailed insights in the practical aspects of the procedures are provided.

Multiple steps in the protocol are critical and require additional attention. Filling of the Dewar with non-sterilized liquid nitrogen potentially leads to semi-sterile conditions. Using the precautions explained in the protocol, no contamination was revealed during our long-term collagen sandwich cultures. However, additional sterilization measures can be taken if deemed necessary. Liquid nitrogen can be sterilized by radiation or filtering[16] and the system may be completely closed with a lid on the liquid nitrogen Dewar with a chimney connected to the mixing needle. Alternatively, non-contact droplet vitrification methods may be explored, although this may limit larger volume throughput[14].

Other essential parts of the protocol are the CPA pre-loading and unloading incubation durations. Longer durations could result in toxic CPA injury while shorter durations may cause osmotic injury. Also, it is important to ensure that the vitrified hepatocyte droplets always stay below the

glass transition temperature because uncontrolled macro- and microscopic ice nucleation at higher temperatures lead to severe hepatocyte injury and cell death. From a practical perspective, the most critical step in bulk droplet vitrification is the rewarming of the vitrified hepatocyte droplets. When the vitrified droplets are removed from the liquid nitrogen they can freeze within seconds if not rapidly rewarmed by instantaneously adding the warm rewarming solution.

Future optimization of bulk droplet vitrification could further improve the preservation outcomes, such as the viability, yield, and metabolic function. Both permeable and non-permeable CPAs could be tested to optimize the osmotic dehydration ahead of vitrification. This could reduce the osmotic shock during dehydration and might also allow to decrease the pre-incubated CPA concentrations. Additionally, continuous fluidic, instead of batchwise, CPA pre-incubation would theoretically allow vitrification of unlimited cell quantities. Because only general laboratory equipment is needed for bulk droplet vitrification, it is a simple and cost-effective preservation method which could potentially be used for preservation of other cell types.

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**DISCLOSURES:**

The authors declare competing financial interests. Drs. de Vries, Weng, Toner, Tessier, and Uygun have provisional patent applications relevant to this study. Dr. Uygun has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. All researcher's interests are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies.

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Figure 1



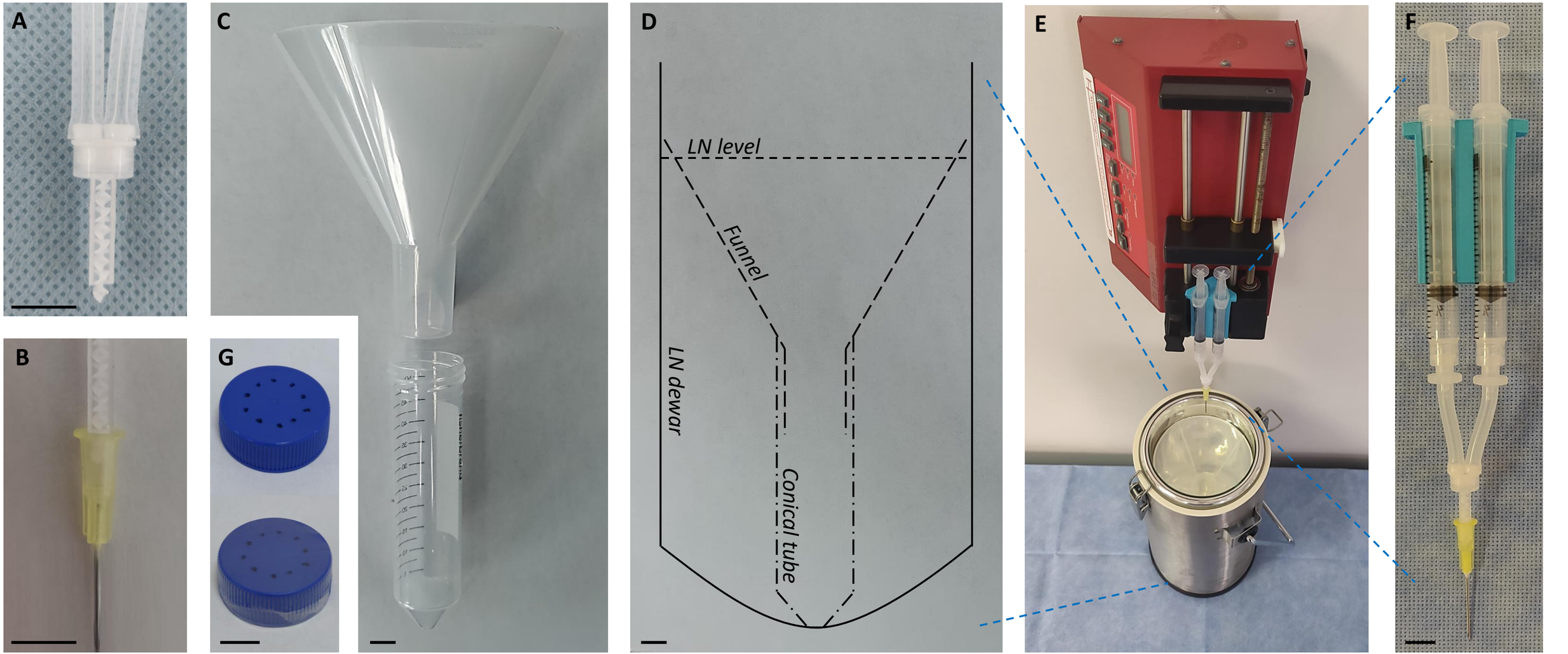


Figure 2



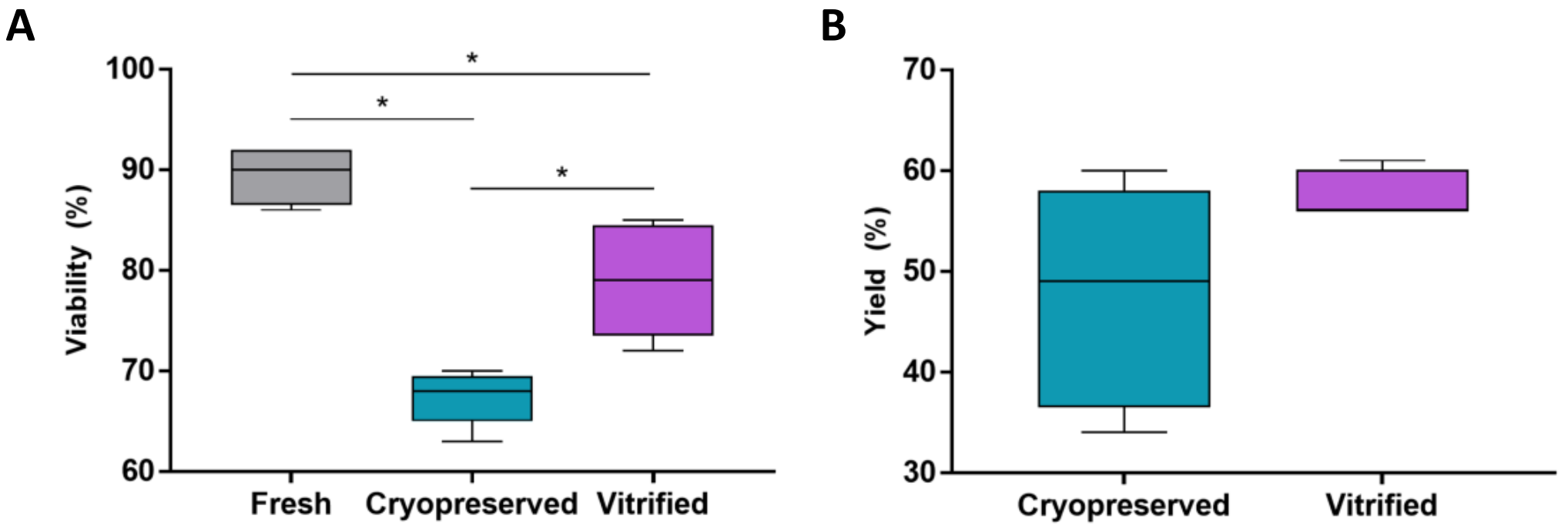
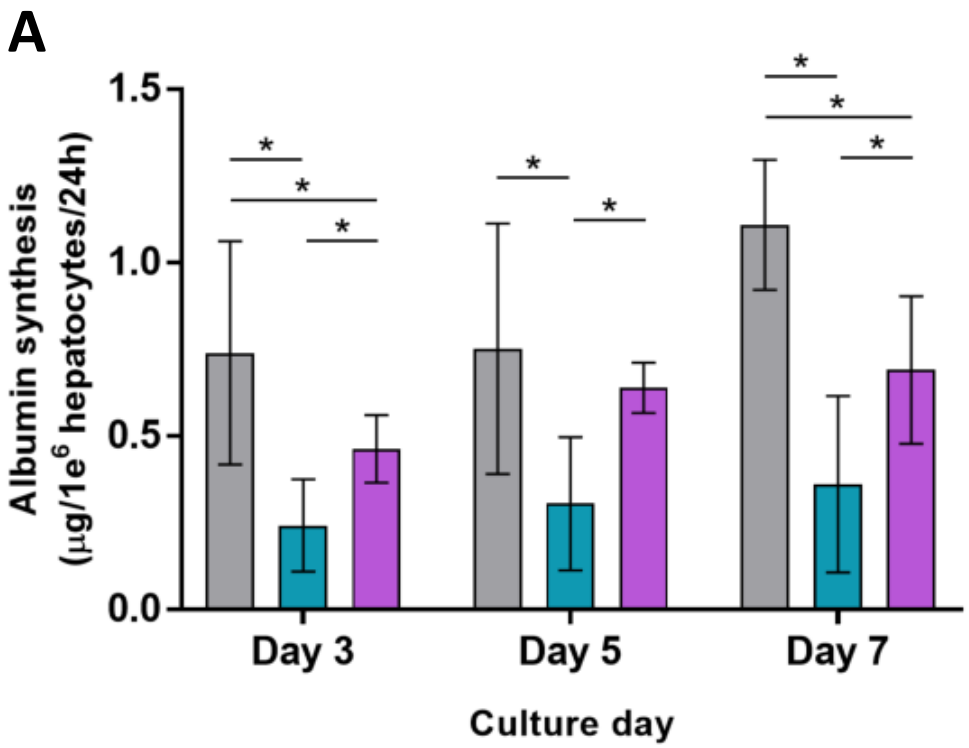
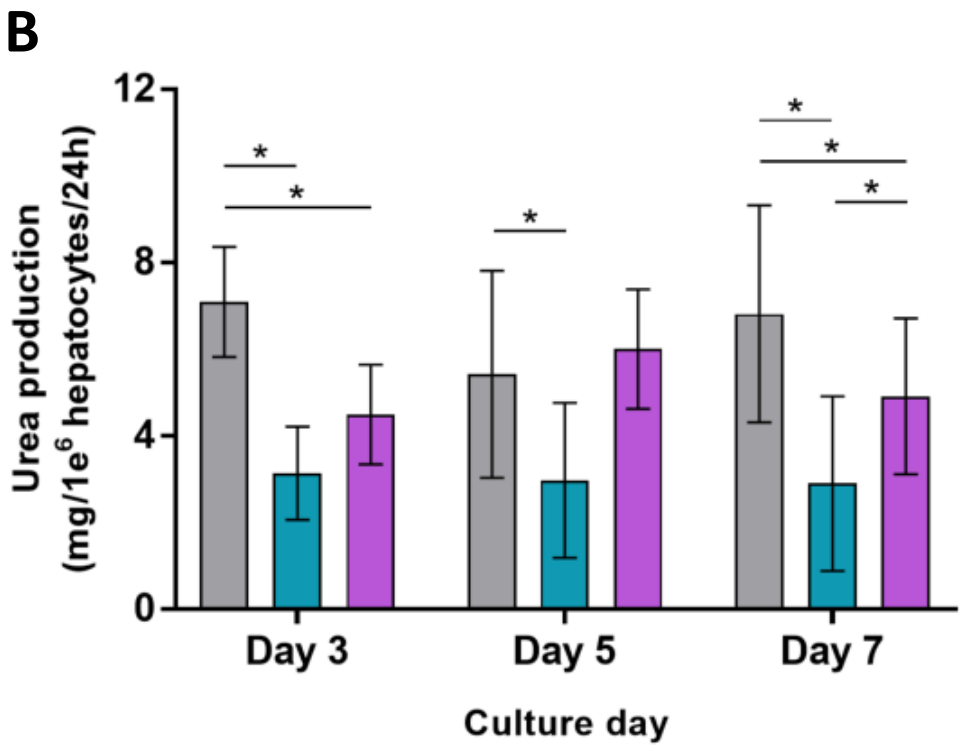


Figure 3





| Name of Material/ Equipment |
| --- |
| BD Disposable 3 mL Syringes with Luer-Lok Tips |
| Beaker |
| Belzer UW Cold Storage Solution |
| Bovine Serum Albumin |
| Cole-Parmer Female Luer to 1/16" low-profile semi-rigid tubing barb, PP |
| Cryogentic stroage tank / Cryotank |
| Dimethyl sulfoxide |
| DMEM, powder, high glucose, pyruvate |
| Ethylene Glycol |
| Extra long forceps |
| Fisherbrand Higher-Speed Easy Reader Plastic Centrifuge Tubes - Flat top closure |
| Fishing line |
| Liquid nitrogen |
| Masterflex L/S Platinum-Cured Silicone Tubing, L/S 14 |
| Mix Tips, For Use With 3HPW1 |
| Nalgene Polypropylene Powder Funnel |
| Needle 20 ga |
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| Spatula |
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| Sigma-Aldrich | CLS1000-250 | |
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| Sigma-Aldrich | A7906 | |
| Cole-Parmer | EW-45508-12 | |
| Chart Industries | MVE 800 | |
| Sigma-Aldrich | D8418 | |
| Life Technologies | 12800-082 | |
| Sigma-Aldrich | 107-21-1 | |
| Fisher Scientific | 10-316C | |
| Fisher Scientific | 06-443-18 | |
| Stren | SOFS4-15 | |
| Airgas | 7727-37-9 | |
| Cole-Parmer | EW-96410-14 | |
| Grainger | 3WRL7 | |
| ThermoFisher | 4252-0100 | |
| Becton Dickinson (BD) | 305175 | |
| Sigma-Aldrich | P7793-1EA | |
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| Fisher Scientific | SE1M179M6 | |
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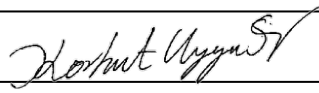
| Signature: |  |
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Dr. Phillip Steindel
Review Editor
JoVE

July 15, 2019

Dear Dr. Steindel,

Thank you for providing us with the opportunity to submit our revised manuscript to JoVE. We express our gratitude to both the Editorial Board members and the external referees for their thoughtful comments and compliments.

I am pleased to inform you that we believe we were able to address all comments and questions received. Accordingly, we are happy to resubmit our revised manuscript to be reconsidered for publication by JoVE. Please do not hesitate to contact us if you require further information. We look forward to your review of our submission.

Detailed responses to each comment are provided below.

Sincerely,

Korkut Uygun, Ph.D

**Editorial comments:**

**General:**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

We carefully reviewed are manuscript and ensured there are no spelling or grammar issues.

**2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.For example: Parafilm, Percoll**

We removed the commercial product names and symbols from the protocol and referenced them in the table of materials.

**Protocol:**

**1. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.**

To inform the reader how specific steps are performed, we added additional information for every non-trivial step in the protocol throughout the manuscript. In addition, we strongly believe the video will provide additionally clarification.

**Specific Protocol steps:**

**1. 1.1.1: What size filter?**

We used a 0.22 um filter. We added this information to the manuscript.

**2. 1.2.5: How do you sterilize?**

We autoclaved the mixing needle assembly. We added this information to the manuscript.

**3. 1.3.1: Where do hepatocytes come from? If they come directly from humans or animals, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee or its animal care guidelines.**

We added the following statement to the start of protocol: *"Ensure that the cells destined for preservation are acquired according to protocols approved by your institution's human research ethics or animal care and use committees. The primary hepatocyte isolations for this protocol were performed by the Cell Resource Core (CRC) at Massachusetts General Hospital, Boston, Massachusetts, USA. The animal protocol (#2011N000111) was approved by the Institutional Animal Care and Use Committee (IACUC) of Massachusetts General Hospital."*

Figures:

**1. Please cite Figure 1 outside of the legends section.**

We cited Figure 1 in the corresponding steps in the protocol.

**2. Figures 2 and 3: Please include the statistical test used.**

We added the used statistical tests to the caption of Figures 2 and 3.

**Table of Materials:**

**1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol. Please remove trademark (™) and registered (®) symbols from the Table and Materials.**

*We apologize for the incomplete Table of Materials. We carefully listed all equipment and materials mentioned in the protocol. Also, we removed the trademark and registered symbols as requested.*

**Comments of Reviewer #1:**

**This work proposed an approach for bulk droplet vitrification of primary hepatocyte cells. This method leverages rapid osmotic dehydration to concentrate a low pre-incubated intracellular CPA concentration in the cells. The process is performed in a fluidic device and integrated with a syringe pump, which can continuously generate large sized droplets that are vitrified in liquid nitrogen afterwards. There are several concerns from this reviewer that need to be addressed before this paper could be considered further.**

We thank the reviewer for the time and efforts in reviewing our manuscript. We value the feedback on our article and addressed each of your comments below.

**General comments:**

**1. The design is based on the syringe and syringe pump; thus the efficiency and throughput may be limited. For example, refilling, contamination...**

Droplet vitrification has been investigated in efforts to reduce the preincubated CPA concentration that is required to enable vitrification. Although sometimes referred to as "high throughput", the typical processing rates are in the range of microliters per minute. For example, Dimirci et al 2007 report 0.14nl droplets at a rate of 1000 droplets/s which results in a throughput of 8.4µl per minute[1]. This is three to four orders of magnitude lower than the 4 ml/min throughput of bulk droplet vitrification, which we regard highly more efficient as existing methods. However, this does not imply – and in that aspect we agree with the reviewer – that efficiency cannot be further improved. Our motivation behind the publication of this protocol is that we like to encourage others to use and especially improve upon bulk droplet vitrification. For example, larger syringes could be explored, two syringe pumps could be alternated for simultaneous refilling and processing, or the CPA pre-incubation step could be performed continuously using similar mixing devises as in the mixing needle.

We agree with the reviewer's comment that contamination is an important consideration when using an open method such as droplet vitrification. To prevent contamination, we used our sterile equipment in a laminar flow cell culture hood. Although the liquid nitrogen used was not sterile, we did not encounter any contamination issues which would been revealed during long term cultures that we performed after preservation. If required, liquid nitrogen can be sterilized by radiation or filtering[2]. Additionally, the system may be completely closed with a lid on the liquid nitrogen Dewar that has a chimney connected to the mixing needle. We added this important information as a note to step 1.2.6 of the protocol and to the discussion.

**2. If using a different type of cell (e.g. human hepatocytes), the viability of cells and the CPA concentration in cells in this approach is hard to be guaranteed as the short loading time…**

We agree with the reviewer that the successful use of other cell types cannot be guaranteed as suggested in step 1.3.1 of the protocol. We removed the use of primary human hepatocytes from this step and confined this protocol to the use of primary rat hepatocytes.

**3. According to the section 1.3, the CPA (e.g. DMSO) was already added into cells suspension before using fluidic device, but the authors did not mention it in the abstract and introduction.**

We completely acknowledge the reviewer's comment that pre-incubation step with CPAs – to add the relatively lower concentration of DMSO and EG into the cell – is an essential step in bulk droplet vitrification. However, this was mentioned in both the abstract as well as in the introduction. *"This novel method leverages rapid osmotic dehydration to concentrate a low pre-incubated intracellular CPA concentration directly ahead of vitrification".* To emphasize that DMSO and EG were used in this pre-incubation step we added this information to the introduction.

**4. For bulk droplet vitrification, the authors should refer the literature below. The cooling rate, collection and contamination problems have been well investigated in this paper: High-throughput non-contact vitrification of cell-laden droplets based on cell printing. Scientific reports 5 (2015): 17928.**

We thank the reviewer for pointing out that we omitted to cite and mention this important work of Shi et al. We added this reference to the introduction section where we introduce droplet vitrification approaches. Also, in the revised manuscript, we mention this reference in the discussion of potential contamination issues. This truly is an elegant approach in the way it completely overcomes potential contamination issues. However, we would like to point out that the method describes an 8 x 8 arrays of 0.2 µl to 3 x 3 arrays of 5 µl droplets that are printed on a 3 x 3 cm surface which results in a batchwise processing volume of merely 13 to 45 µl which may limit throughput in the order of milliliters.

**Specific comments:**

**1. Abstract, line 39-41, "However, pre-incubation with high concentrations of cryoprotectant agents (CPA) and extremely fast cooling rates are required to achieve glass transition while avoiding injurious ice formation." This statement is not accurate. Very high concentration CPA do not need extremely fast cooling rate. For example, VS55.**

We acknowledge the reviewer's comment that this statement was not accurate. The sentence now reads as *"However, extremely fast cooling rates are required to achieve transition of pure water into the glass state, while avoiding injurious ice formation. Although pre-incubation with cryoprotective agents (CPA) can reduce the critical cooling rate of biological samples, high CPA concentrations are generally needed to enable vitrification."*

**2. Introduction, line 64, not "glass", should be "glass state or amorphous solid".**

We changed "glass" into "glass state".

**3. Introduction, line 65, "cooled near instantaneously", not a scientific statement, please give the specific number, and it does exist in the previous literatures.**

We added the specific cooling rate as stated in the review of critical cooling rates by Hopkins et al 2012.[3]

**4. Protocol 1.1.1, line 106, please give the specification of constituents in the "University of Wisconsin solution (UW)".**

We added the exact composition of UW as a note to step 1.1.1.

**5. Protocol 3.2.3, line 175, please specify the "beads".**

We revised the word "beads" to vitrified hepatocyte droplets throughout the manuscript.

**6. Representative results, line 196, please specify the "classic cryopreservation using the most optimized slow freezing protocol reported in literature." Or give the specific reference.**

We apologize for the lack of proper context and references. This section now reads as: *"Freshly isolated primary hepatocytes of five different rat livers were used for a direct comparison of bulk droplet vitrification to classic cryopreservation using a protocol conform the most optimized slow-freezing protocols reported in literature[4, 5]. In short, the hepatocytes were suspended in UW supplemented with bovine serum albumin (2.2 mg/ml), glucose (333 mM) and DMSO (10% v/v) and frozen using a controlled rate freezer. After storage at -196°C the samples were thawed in a warm water bath. After all ice melted, the DMSO was directly diluted while the glucose concentration was gradually lowered during multiple steps to reduce osmotic injury. The exact protocol can be found in detail elsewhere*[6].

**7. Representative results, line 200, "be stored for decades without loss of viability". Where is the evidence?**

Accompanied with an appropriate reference this sentence now reads as: *"Although shorter preservation times were tested for practical considerations, it should be noted that primary hepatocytes can be stored at -196°C for years without loss of viability[4]".*

**Figures:**

**Figure 1, the scale bars are need to be added, and Figure 1A is not clear to see the details;**

We replaced the photo of Figure 1A and added scale bars. Please note that we deliberately did not include a scale bar in Figure 1E because of the photo's skewed perspective.

**Figure 2, why there is a such large range in Figure 2B on "Cryopreserved"?**

Inconsistency is one of the drawbacks of conventional hepatocyte cryopreservation by slow freezing. In hepatocyte cryopreservation literature it is common to find error bars in the range of 20 to 30 %[4, 7, 8]. Please note that the whiskers of the boxplot are min to max values with a range of 26% while most error bars in literature are often SD or SEM and which are typically smaller than min to max error bars. We believe that one important reason for this inconsistency is that during the freezing process the samples are first supercooled before the supercooled state brakes down and the samples freeze. Breakdown of the supercooled state is a stochastic event which causes inconsistency. Although the controlled rate freezing program includes a fast dip and rise in temperature intended to cause ice nucleation, the actual temperature at which this happens, as well as the time it takes for the samples to freeze varies considerably from sample to sample based on our (non-published) data.

**Figure 3, the legend bar is needed.**

The error bars are defined as standard deviation (SD) which is mentioned in the caption

**Comments of Reviewer #2:**

**Manuscript Summary:**
**Overall this manuscript is well-written, with only minor copy-edit needed. The described procedure is generally easy to follow. The procedure itself is quite an important improvement for hepatocyte preservation and is likely to be adopted by others, which makes the current manuscript a highly desirable contribution to the field.**

*Thank you for your review and kind remarks. Please find our response to each of your comments below.*

**Major Concerns:**
**1. The use of the term 'devitrification' is confusing, as it generally refers to the process of conversion of the glassy state to a crystalline phase during rewarming, a very undesirable event. The authors would be better served to simply use 'rewarming' in its place.**

We acknowledge the reviewer's comment and replaced "devitrification" with rewarming throughout the manuscript to avoid confusion.

**2. It would be helpful to have a sketch of the conical tube/ filter assembly, together with the liquid nitrogen level. Does the funnel fit snugly into the opening of the conical tube or is it much smaller and loose in the container? It's not clear how this assembly is inserted and the vessel filled without overspill. Is liq N2 added after it is inserted in place in order to fill the conical tube? The bead collection process is also a little muddling. During collection, how is the assembly lifted out and the beads collected without loss/spilling, if the liquid nitrogen level is 1 cm below the top of the funnel? A diagram would make this much simpler to follow. Is the handling all done with the user wearing Cryo gloves? Are tongs used?**

As suggested, we added a sketch of the conical tube and funnel assembly that shows the liquid nitrogen level.

Although the tip of the funnel fits loosely in the conical tube, the gap (approximately 1 mm) is small enough to keep the two components aligned. The density of these components is higher than liquid nitrogen and therefore they "sink" to the bottom of the dewar. The curvature of the bottom of the dewar keeps the conical centered. The outer edge of the funnel fits loosely (also approximately 1 mm gap) in the LN dewar which keep the assembly upright and centered.

The funnel is first inserted in the conical tube and both are slowly pressed down in the liquid filled dewar using large forceps (we added this information to the protocol). During this, the buoyancy of the air-filled conical presses it upwards and therefore keeps the funnel inserted in the conical tube. As soon as the edge of the conical tube is pushed below the LN level, the conical tube fills with LN that flows through the 1mm gap between the funnel and the conical tube's wall. When the conical tube is filled, the conical tube and funnel assembly slowly sink down until the conical tube rests on the bottom of the dewar. The conical shape of the funnel keeps everything vertical and centered during this process.

To collect the vitrified droplets, the funnel is first taken out with forceps. After removing the funnel, the conical slightly tips over but stays upright as it leans against the wall of the dewar. The conical tube is easily collected from the liquid nitrogen using the same forceps. We added this information to step 2.1.2.

We strongly believe that all these practical questions of the reviewer will be perfectly answered and demonstrated in the video. Exactly these specifics about the protocol motivated us to publish the protocol in JoVE because a short video tells more than a thousand words.

**3. Use of the engineered lid for cryogenic storage is not clear - please provide a diagram. This step is nothing more than just puncturing the lid of a conical tube and wrapping it with a flexible film.**

We changed our wording in step 2.1.1 aiming not to confuse the reader with "creating a valve". Also, we added photos to provide clarity as requested. We believe that the video will completely elucidate this step.

**Minor Concerns:**
**line 108 missing 'of'**
**line 109 capitalization inconsistent with previous usage**
**line 117 seems garbled**
**line 210 should be 'synthetic function' not 'synthetic hepatocyte' (i.e. change the word order)**

We have revised the manuscript to incorporate all the minor concerns above.

**Comments of Reviewer #3:**

**Manuscript Summary:**
**The article by de Vries et al., describes a protocol for bulk processing of samples before vitrification in liquid nitrogen.**

**Major Concerns:**
**I have no major scientific concerns. However, some sentences should be reworded. Furthermore, I do not see a reference to Figure 1 in the text and specific examples should be given for the CPAs in questions and the rates of freezing the author refers to. How slow is slow freezing? How fast is fast freezing?**

We thank the reviewers for the thoughtful comments and constructive feedback. We added appropriate values for the cooling rates in the introduction. For example, slow freezing is typically in the order of 1 °C/min while vitrification of pure water requires cooling rates in the order of one million degree Celsius per second. Also, we added references to Figure 1 in the protocol.

**Minor Concerns:**
**1) Lines 32-33: 'using low pre-incubated intracellular cryoprotectant agent concentrations'. Try not to use strings of modifiers for a given noun. You have four modifiers; a rule of thumb is limiting modifiers to two words. This sentence would read better as:' by using cryoprotectant agents at low concentrations during a preincubation step'.**

We thank the reviewer for this comment and the general advice which will improve English writing in future. We reworded the string of modifiers.

**2) Line 38: slow-freezing cryopreservation I would add a hyphen or reword to cryopreservation at rates below XXX C/min ( I think you need to define 'slow').**

We added the hyphen as suggested throughout the manuscript. Also, we defined slow freezing (see first comment)

**3) Line 57: Why multiple? Isn't DMSO enough? In case you need multiple, which ones are you referring to?**

For best results, a combination with DMSO glucose and albumin is used. We added this information to the manuscript.

**4) Line 68: Please be specific. You need to state the CPA in question. A concentration of 40% DMSO would certainly be toxic while ethylene glycol, sucrose, and 1,2-propanediol are less toxic.**

As suggested we now specifically state *"40% v/v DMSO"*.

**5) Line 86: 'low pre-incubated intracellular CPA concentration'. See #1.**

We reworded the string of modifiers.

**6) Line 91: 'loaded' not 'laden'.**

We corrected the grammatical error accordingly.

**7) Lines 95-96: 'long-term post preservation viability'. See #1. We reworded the string of modifiers.**
**8) Line 107: to a mixture OF…**

Step 1.1.1 was reworded to address comment 4 of Reviewer #1.

**9) Lines 105-244: Read carefully through the protocol part and remove capital letters from chemical names, verbs, etc. (e.g. Sucrose, Vitrification solution, 1. Bulk Droplet vitrification).**

As suggested, we replaced the capital letters from chemical names and other inappropriate words.

**10) Line 203: 'trypan blue exclusion membrane integrity testing'. See #1.**

We reworded the string of modifiers.

**11) Line 204: What is the 'most optimized protocol in literature'. Provide the CPA combination and reference.**

This issue was also raised by reviewer 1. This section now reads as: *"Freshly isolated primary hepatocytes of five different rat livers were used for a direct comparison of bulk droplet vitrification to classic cryopreservation using a protocol conform the most optimized slow-freezing protocols reported in literature*[4, 5]*. In short, the hepatocytes were suspended in UW supplemented with bovine serum albumin (2.2 mg/ml), glucose (333 mM) and DMSO (10% v/v) and frozen using a controlled rate freezer. After storage at -196°C the samples were thawed in a warm water bath. After all ice melted, the DMSO was*

*directly diluted while the glucose concentration was gradually lowered during multiple steps to reduce osmotic injury. The exact protocol can be found in detail elsewhere*[6].

**12) Lines 247-248: How do you know reduced viability was due to ice formation and not cumulative osmotic stress?**

We acknowledge the reviewer's comment and we admit that we formally don't know this. The freezing process itself also results in osmotic imbalances because solutes are excluded from the growing ice lattice. Therefore, osmotic injury and freezing injury are very hard to distinguish. To address the reviewer's comment we reworded this sentence to "Cryopreservation of hepatocytes by slow-freezing results in reduced viability and metabolic function."

**13) Line 250: how 'high' is 'high'?**

We rephrased this sentence from *"However, extremely fast cooling rates and pre-incubation with high CPA concentrations are required."* to "*However, pre-incubation with CPAs are required to lower the critical cooling rate".*

**14) Lines 251-251: do not capitalize Bulk Droplet Vitrification.**

We changed the capitals from Bulk Droplet Vitrification throughout.

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