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Isochoric supercooling cryomicroscopy

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ABSTRACT

We introduce an isochoric (constant-volume) supercooling cryomicroscope (ISCM), enabling the ice-free study of biological systems and biochemical reactions at subzero temperatures at atmospheric pressure absent ice. This technology draws from thermodynamic findings on the behavior of water in isochoric systems at subfreezing temperatures. A description of the design of the ISCM and a demonstration of the stability of the supercooled solution in the ISCM is followed by an illustration of the possible use of the ISCM in the preservation of biological matter research. A comparison was made between the survival of HeLa cells in the University of Wisconsin (UW) solution in the ISCM at +4 °C under conventional atmospheric conditions and at −5 °C under isochoric supercooled conditions. Continuous real-time monitoring at cryopreservation temperature via fluorescence microscopy showed that after three days of isochoric supercooling storage, the percentage of compromised cells remained similar to fresh controls, while storage at +4 °C yielded approximately three times the mortality rate of cells preserved at −5 °C.

1. Introduction

This project aimed to develop a device for the study of organic matter in aqueous solutions, under a microscope, at what are subfreezing temperatures at atmospheric pressure, but in the absence the ice. This can be achieved by maintaining the water in the cryomicroscope chamber in a thermodynamically supercooled state. However, the supercooled state is a thermodynamically metastable condition that has thus far not proven conducive to long-term or highly repeatable experiments of the sorts required by a laboratory research platform [1–3]. The technology introduced in this paper leverages emergent research on isochoric (constant-volume) thermodynamics to address this issue, functioning to enhance the stability of supercooling at subfreezing temperatures to the point of full experimental reliability [4–7].

Briefly, the physical premise of ice formation within an isochoric system is as follows: when water or an aqueous solution is confined in a closed volume absent air or other compressible elements, the formation and growth of lower-density ice-Ih will yield an increase in hydrostatic pressure, which in turn decreases the thermodynamic driving forces for further growth. This growth and self-pressurization process forces the thermodynamic path of the system to follow the water-ice liquidus line and will proceed until a stable two-phase ice-water thermodynamic equilibrium is reached at some subzero temperature and some elevated pressure. Thermodynamic analysis and experiments show that when frozen along this path, the frozen portion of an aqueous isochoric system will increase gradually to approximately 60% of the total volume at approximately −22 °C and 210 MPa [8,9], and it has been demonstrated that the portion remaining unfrozen may be used to preserve sensitive biological matter in an ice-free state [10,11].

Due to this same self-pressurizing mechanism, theoretical and experimental studies have shown that isochoric conditions restrict not only bulk ice growth but also the initial nucleation of ice nuclei, thereby enhancing the supercoolability of water in isochoric systems [5,7,12]. Isochoric conditions have repeatedly been demonstrated to enhance aqueous supercooling relative to conventional isobaric (constant pressure, typically atmospheric) conditions, even in the face of

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heterogeneous environments and external perturbations [4,5]. Importantly, recent experimental and theoretical works suggest that mechanism(s) of action in isochoric supercooling may be volumetric in nature [13]. In a simplified single-nucleus analysis of microscale systems, it was shown that the random formation of a sub-critical ice crystal with a smaller density than water anywhere in the system must yield a nigh-instantaneous increase in local pressure, which impedes the further growth of the ice crystal due to Le Chatelier's effect and prevents it from reaching critical nucleus size [14]. Furthermore, recent experimental work has demonstrated that in identical chambers with nigh-identical interfacial surface conditions, isochoric conditions significantly increase supercooling stability, further suggesting a volumetric mechanism of action that transcends surface effects [12]. Recently, we have also shown that supercooling in isochoric chambers is sufficiently stable for the long-term preservation of engineered human cardiac microtissues in a microfluidic device, which has an abundance of possible heterogeneous nucleation sites [15].

It is well known that hyperbaric freezing is another cryopreservation method developed according to Le Chatelier's principle. Hyperbaric pressures generated by mechanical means have been used in cryobiology and food preservation as a way to avoid the detrimental effects of ice formation at subzero centigrade temperatures. A study on the effects of elevated pressure on vitrification and ice nucleation can be found in Refs. [16,17]. The effect of hyperbaric pressures on liver cryopreservation was described in Refs. [18–20]. Hyperbaric pressures have become an accepted technology for the preservation of food, without the deleterious effects of ice [21,22]. However, the use of hyperbaric pressures in cryopreservation and for food storage has several disadvantages relative to isochoric freezing and isochoric supercooling. One disadvantage is related to the technical requirements. Hyperbaric preservation requires mechanical devices to generate pressure. In contrast, in isochoric systems, the pressure (if generated at all) is self-generated by the process of freezing [23]. Furthermore, it was shown that elevated pressures damage living biological matter [18–20]. In fact, pressures higher than those which depress the freezing temperature below −2 °C, already begin to affect the viability of living cells [18–20]. Isochoric freezing self regulates the pressure to yield minimal pressure at any subfreezing temperature because it follows the liquidus phase transition line [23], and isochoric supercooling avoids the confounding effects of pressure all together.

One of the most important experimental tools for the study of biological matter at subfreezing temperatures is cryomicroscopy. A comprehensive review of the use of microscopy for the study of biological matter at subfreezing temperatures, which lists important contributions in the field since the work of Julius von Sachs, was given by Diller [14]. Our thermodynamic findings on isochoric supercooling led us to the concept of the isochoric supercooling cryomicroscope (ISCM) presented in this paper. In such a cryomicroscope organic matter can be observed for long periods at subfreezing temperatures submerged in an aqueous phase, at atmospheric pressure. Hyperbaric microscopy, such as systems described in Refs. [24–26], can also be used to study biological materials in a single aqueous phase at subzero centigrade temperatures. However, hyperbaric microscopy requires means to generate pressure mechanically. The main drawback of hyperbaric microscopy at subzero centigrade temperature is that they employ elevated pressures. Elevated pressure also affects membrane integrity or cell viability [18–20], and therefore a hyperbaric microscope cannot separate between the effects of temperature and pressure on the biological material under observation. Our goal here is to develop a technology that will allow us to study only the effects of low temperatures on cells. Indeed, it may be interesting to compare cells' survival at subzero centigrade temperatures in isochoric and hyperbaric cryomicroscopy to elucidate the effect of pressure on cell survival in future studies. It should be emphasized that an isochoric aqueous system implies that the entire constant volume is filled with the solution, and the presence of air or any other gaseous phase is not allowed because such phases have substantially higher compressibility than that of water [27].

Our work herein is described in three parts. First, we provide the design of the ISCM. Then, we perform a series of experiments to verify the stability of pure water and a supercooled aqueous solution in the ISCM. Last, we illustrate the use of the ISCM in a 3-day long study with fluorescence microscopy in which we continuously monitor the survival of HeLa cells in a University of Wisconsin (UW) preservation solution in an isobaric configuration of the ISCM at +4 °C (standard preservation condition) in comparison with HeLa cell in UW in an isochoric configuration of the ISCM at −5 °C. In the study, we demonstrate that the supercooled solution in the isochoric cryomicroscope is extremely stable. We found by continuous monitoring of cells at cryopreservation temperature under the microscope that the percentage of damaged cells (determined by real-time in situ sodium iodide staining) after three days of isochoric storage at −5 °C in ISCM was the same as in fresh controls, while the percentage of damaged cells in isobaric storage at +4 °C was three times higher than at −5 °C in ISCM.

2. Materials and Methods

2.1. Design of the isochoric supercooling microscopy (ISCM) chamber

Following is a brief description of the design of the isochoric cryomicroscope chamber. The ISCM consists of (1) Main body, (2) Screwing cap, (3) Top observation sapphire window, (4) Bottom observation window sapphire, (5) Isochoric chamber, (6) O-ring of the chamber, (7) Coolant inlet, (8) Coolant outlet, (9) Constant low-temperature jacket, (10) Top glass cover, (11) Bottom glass cover, (Fig. 1a and b). The outer diameter of the low-temperature jacket is 80 mm. The height of the ISCM device is 35 mm. A 5 mm height and a 5 mm diameter isochoric chamber forms between the top and bottom sapphire observation windows and the stainless-steel walls of the main body. The experiments are carried out in this isochoric chamber. To avoid damaging the ISCM by random uncontrolled ice nucleation, which in an isochoric system can elevate the pressure to 210 MPa [8,9,28], the chamber sapphire windows and the main body were designed to withstand pressures of up to 210 MPa. Therefore, the ISCM can work at a range temperature from room temperature to −22 °C (0–210 MPa). The ISCM is made of 316 stainless steel. The system is maintained at a constant desired temperature by a continuous flow through the constant low-temperature jacket. The cooling fluid is a mixture of 50% ethylene glycol in water pumped continuously through the system by a refrigerator bath (Serial: 1807–02616, PP15R-40-A11B, PolyScience INC., USA). The temperature is set on the controls of the refrigerator bath and verified by measuring the inlet and outlet temperatures of the coolant with a thermocouple. During the cryopreservation process, the whole system is insolated with insulation materials to ensure uniform temperature across the sample at steady state. Top and bottom glass covers were used to avoid condensation on the sapphire glass windows.

2.2. Isochoric and isobaric experimental protocol

In many of the experiments performed for this study, we compare isochoric and isobaric behavior in the same ISCM chamber. The general experimental protocol is as follows:1) the main body and the bottom sapphire chamber are assembled. 2)the isochoric chamber (5) is filled with solution and biological materials. 3) the top observation sapphire window (4) is set to seal the isochoric chamber carefully to avoid any entrapment of air. (It should be strongly emphasized that avoiding air in the isochoric chamber is important as the presence of air, which has different compressibility than water, is the main cause for isochoric experiment failure [27]. 4) the upper glass (10) and lower glass (11) windows are attached to the ISCM with glue after the delivery of dry air (Cleaning Duster, Office Depot Inc., China) as a jet into the space between the sapphire windows and where the glass windows are set. This ensures that there is no moisture inside the ISCM and avoids

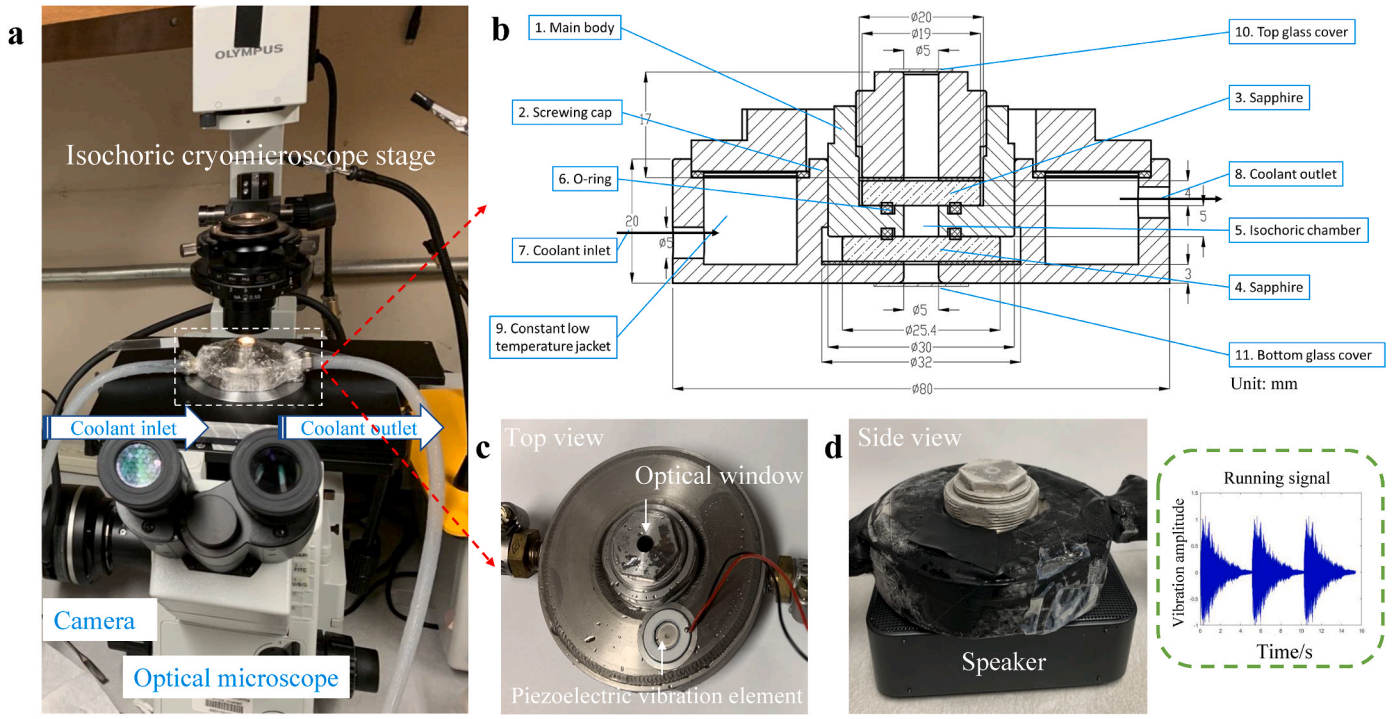

Fig. 1. The isochoric supercooling cryomicroscopy (ISCM) system. **a)** The setup of the ISCM system. **b)** The schematic of the ISCM chamber. **c)** Top view of the ISCM chamber with piezoelectric vibration element. **d)** Side view of the exterior excitation when speaker under the ISCM chamber.

condensation at low temperatures. 5) At the start of the experiment, the entire system, including the coolant delivery tubes and the ISCM, is insulated. Our measurements show that there is no change in temperature between the inlet and outlet of the cold jacket. 6) The device was used with two types of microscopes, an OLYMPUS IX71 and a Nikon ECLIPSE TE300. The objective was used in air, the numerical aperture is 0.3, and the working distance is 16 mm (Nikon plan fluor 10*/0.30, WD 16.0). The ISCM was set on the microscope stage, and the experiments consisted of recording the events in the low-temperature isochoric and isobaric chambers under the microscope (Fig. 1a).

For isochoric experiments, both the top (3) and bottom (4) sapphire observation windows were set in place, as shown in Fig. 1b. The assembly method was such that it allows for continuous displacement of liquid until the sapphire mates with the steel surface. This ensures that no air bubbles are in the system. In isobaric experiments, the top sapphire observation window (3) is not used, while all the other steps are the same as in the isochoric experiment.

The ISCM system is maintained at a constant desired temperature by a continuous flow through the constant low-temperature jacket. The cooling fluid is a mixture of 50% ethylene glycol in water pumped continuously through the system by a refrigerator bath. The temperature is set on the controls of the refrigerator bath and verified by measuring the inlet and outlet temperatures of the coolant with a thermocouple. During the cryopreservation process, the whole system is insolated with insulation materials to make sure the uniform temperature across the sample.

It should be noted that we were unable to continue meaningful observation of the interior of the isochoric supercooling crymicroscope after the onset of freezing. Freezing was observed to occur at −7 °C ~ −10 °C, but because of the high thermal conductivity of the sapphire lens relative to the 316SS chamber walls, ice grows preferentially across the sapphire surface and interferes with further visual observations of the chamber interior. As such, ice formation can be very clearly visually detected, but further observation of the interior post-nucleation cannot be achieved, and likewise the extent of the limited isochoric ice growth cannot be verified. Future iterations of this device should incorporate alternative chamber materials with higher thermal conductivity to force ice growth to proceed radially from the walls.

2.3. Tests to verify the stability of the supercooled solution in the ISCM chamber

An important part of this study is the tests to evaluate the ability of the ISCM to maintain an aqueous solution in a supercooled state for long periods when exposed to external excitations.

2.4. Means to deliver external excitations

The stability of supercooled solutions in the ISCM was examined by applying external excitations to the device and detection of ice nucleation through the microscope ocular. Two types of external excitations were used in this study. One is a piezoelectric element (WHDTS 20 mm 113 kHz driven by 6 V, part number 43224–14239; WHDTS Co., Ltd) which was placed on the isochoric chamber as shown in Fig. 1c. The second type of external excitation is a loudspeaker, with the excitation delivered in two ways. One way is to place the JBL GO2 speaker (Output power 2.5 W, JBL Co., Ltd., California, USA) on the microscope table. The sound is generated by a self-coded MATLAB program, which simulates the sound of a gong. A second way is to remove the ISCM from the microscope table and place the ISCM on the JBL GO2 speaker, as shown in Fig. 1d. The loudspeaker was set to the maximal volume with a frequency of 0.2 Hz, and the vibrations were delivered for an hour, after which the ISCM was put back on the microscope stage and the presence or absence of ice recorded.

2.5. A study of the stability of supercooled pure water in the isochoric and isobaric chambers

The first stability study used deionized water (type II, Fisher Science Education, with a conductivity of 2.3×10^{-4} S/m). The stability of supercooled water in an isochoric ISCM system was evaluated in comparison with a similar ISCM isobaric setting and was performed using

the external excitation devices described above. The sample of water was injected into the ISCM chamber, and the chamber was prepared to be in either the isochoric or isobaric configuration. The temperature of the ISCM with deionized water in an isochoric and isobaric setting was reduced slowly from room temperature to −5 °C. At this temperature, both isochoric and isobaric systems are supercooled. Experiments were done in an isochoric system configuration and in an isobaric system configuration as described earlier. In the first part of the study, we delivered an external excitation with a piezoelectric element. The element was placed on the isochoric chamber, as shown in Fig. 1c, and activated for 30 s. This experiment was repeated three times.

A loudspeaker was used to generate external excitations of a larger magnitude than the piezoelectric element vibration. The exterior excitations delivered by the loudspeaker were applied to the same volume of water in an ISCM in an isochoric setting and in an isobaric setting, at −5 °C. In one type of experiment, we applied 1 h of excitation by the JBL Go2 speaker to the microscope stage when the ISCM chamber was on the microscope stage, and system was monitored for ice formation through the microscope ocular in the isobaric configuration and in the isochoric configuration. In another type of experiment, as illustrated in Fig. 1d, the ISCM was removed from the microscope stage, exposed to 1 h of the much larger excitation delivered directly by the speaker, then returned to the microscope to observe the presence or absence of ice. These experiments were repeated ten times for each protocol, all at −5 °C, and under both isochoric and isobaric conditions.

Upon the nucleation of ice, we observed that the ice grows in a dendritic form across the sapphire lens, darkening the image. A typical initial ice formation process (which proved indistinguishable between isochoric and isobaric configurations) is shown in Fig. 2a. At -5 °C, ice formed only in the isobaric chamber open to the atmosphere and not in the isochoric chamber. Freezing occurred in isochoric chambers at −7 °C ~ −10 °C, and the appearance of freezing was identical to Fig. 2a.

2.6. A study on the stability of a supercooled solution with cells in the isochoric and isobaric chambers

Another set of experiments on the stability of a supercooled aqueous solution in the ISCM chamber in the isochoric and isobaric configuration was performed with cells to evaluate how the presence of cells affects the stability of a supercooled aqueous solution in the ISCM chamber.

2.7. Cell handling protocol

HeLa cells (epithelial cell from cervical carcinoma, with ATCC number of CCL-2, obtained from Cell culture facility at UC Berkeley) were used in this experiment. The protocol for the preparation of the cells in the solution is as follows: Fresh HeLa cells cultured in a flask (T-25 tissue culture flask, Corning Inc.) stored in a +37 °C incubator were taken out and washed with PBS (Thermo Fisher Scientific Inc., USA), two times. Then 300 μl trypsin-EDTA (Thermo Fisher Scientific Inc., USA) was added to the flask and kept for 1 min. Next, the flask was washed with PBS another two times. Another 300 μl trypsin-EDTA was added, and the flask was stored in the incubator for 4 min. Then, cells were taken out and centrifuged for 5 min (1000 rpm/min, Centrifuge model: Biochrom Corp mini) to form a supernatant. Then, the supernatant was poured out to get a cells suspension left in the flask. This cells suspension was used for the experiment below: 1) stability of supercooled solutions with cells, 2) the study on the temporal effect of the preservation temperature on cell survival.

2.8. Stability of supercooled solutions with cells

For the study on the stability of an aqueous solution with cells, experiments were performed with HeLa cells in a cell growth medium (10% FBS + 90% DMEM). The cells suspension obtained after centrifuging was dropped in cell growth medium (10% FBS + 90% DMEM, Thermo Fisher Scientific Inc., USA) in a test tube (15 ml Conical

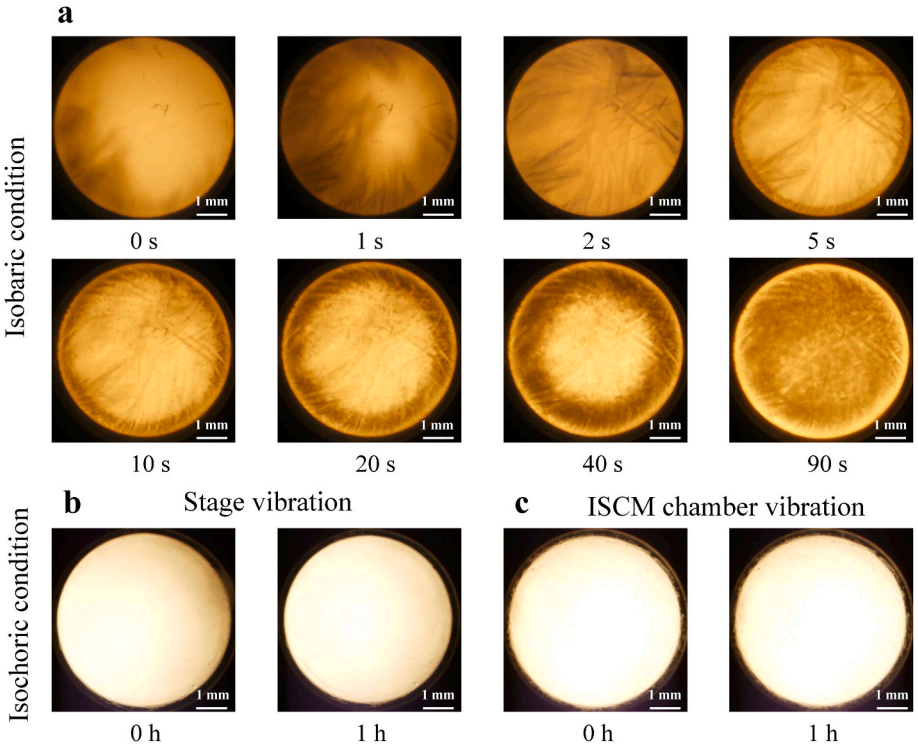

Fig. 2. The stability of the DI water at isobaric condition compared with isochoric condition. **a)** Process of ice formation and growth in the chamber at isobaric condition from 0 s to 90 s. **b, c)** No ice was observed in isochoric supercooled system after 1 h of excitation **(b)** neither the microscope stage vibrated **(c)** nor the ISCM chamber directly vibrated.

centrifuge tube, Fisher Scientific Inc. USA) and mixed. The samples were removed from the test tube with a syringe and injected into the ISCM chamber. The ISCM chamber with HeLa cells in cell growth solution was exposed to a strong external excitation (JBL GO2 speaker) at −5 °C in two different configurations, isochoric and isobaric. The ISCM filled with cell growth solution in the two different configurations was placed on the external excitation source for three days, and the speaker was turned on three times per day, 20 min per time. The experiment set is illustrated in Fig. 1d. During the three days experiment, after the external excitations were delivered, the ISCM was placed under the microscope to determine the presence of the ice crystal inside the chamber.

2.9. Illustration of the possible use of ISCM: A study on the temporal effect of the preservation temperature on cell survival

To illustrate the use of the ISCM, we performed a study in which we observed HeLa cells in the ISCM in a supercooled solution, over time, for three days. Experiments were performed with HeLa cells in the University of Wisconsin solution (UW solution) (Belzer UW Cold Storage Solution, Bridge to Life, USA), which has an intracellular composition and is commonly used for cells and organ preservation at low hypothermic temperatures. To further take advantage of the capabilities that the ISCM provides, we also added propidium iodide (PI) to the solution. It has been established that PI can be used to monitor cell membrane integrity [6] and cell viability in real-time for dynamic viability assays [29]. Propidium iodide is a fluorescent stain that is a cell membrane impermeant and stains cells with disintegrating membranes by entering the cell and binding to nucleic acids. PI is commonly used in cell membrane electroporation experiments to evaluate the integrity of the cell membrane [6].

The cells suspension obtained after centrifuging was dropped in the UW solution in a tests tube (1.5 ml Microcentrifuge tubes, USA SCIENTIFIC Inc.) and mixed. Propidium iodide (Propidium Iodide, SIGMA-ALDRICH, initial concentration is 1.0 g/ml) was added to the cell preservation solution medium to form a mixture containing 2 μl PI dye per 1 ml cell solution. A sample was removed from the test tube with a syringe and injected into the ISCM chamber.

To illustrate the use of the ISCM to monitor the temporal effect of the storage conditions on the preserved cells, we performed a set of experiments in which HeLa cells in the ISCM isobaric configuration at +4 °C and in the ISCM isochoric configuration at −5 °C were monitored continuously for three days under the microscope, and photographs of bright field and fluorescence images were taken in time, under the microscope. Three repeats were performed for each of the experimental conditions described here, and the cells were monitored continuously throughout these three days with bright field and fluorescence microscopy at supercooled temperatures. The ratio of the total number of cells and stained cells was calculated. To determine if the different temperatures at which the fluorescence measurements were performed in the isobaric and isochoric chambers had an effect on the results, at the end of the three days experiments, the isochoric and isobaric chambers were brought to room temperature for half an hour and the ratio stained cells/total cells was also measured at that temperature. For the −5 °C isochoric preservation experiment, we first cool the cell from +37 °C to +4 °C in the refrigerator and then decrease the temperature from +4 °C to −5 °C with an average cooling rate of 0.14 °C/min. For the +4 °C cryopreservation experiment, the cooling procedure is the same as for the −5 °C isochoric experiment in the process from +37 °C to +4 °C. Every experimental condition was repeated three times.

2.10. Cell counting method

To analyze the effect of temperature, the total cells were counted using a bright-field optical image, and the PI-stained cells were counted using fluorescence imaging. The ratio value of PI stained cells to total cells was recorded. Three different view areas of the sample in each group of the experiment were observed for both bright field and fluorescence imaging, and the average value is calculated. The *software ImageJ* was used for cell counting. A least significant difference (LSD) test was performed for all experimental groups ($p < 0.05$).

3. Results and discussion

3.1. Design of isochoric supercooling cryomicroscope chamber

This paper provides a description of the design of the microscope chamber to facilitate reproduction by other groups in the field. In brief, the interior of the isochoric chamber was designed to maintain a constant volume and to be observed under a microscope through two sapphire windows that form the upper and bottom walls of the chamber. The chamber and the sapphire windows were designed to withstand the increase in pressure that can occur if the supercooled solution becomes unstable and ice nucleation begins (up to 210 MPa).

An isochoric supercooling cryomicroscope was built in this paper. It has the ability to maintain macroscopic volumes of aqueous solutions with the biological matter in an isochoric (constant volume) chamber, in a supercooled state without ice, at precisely controlled subfreezing temperatures, in solutions with precisely controlled compositions, for extended periods of time, under a microscope ocular. The ISCM has the potential to become a valuable tool for the study of biological matter at subfreezing temperature absent ice. Potential applications include developing a fundamental understanding of biological processes in a range of subfreezing temperatures, albeit the absence of ice, under conditions in which they were not studied before, developing new technologies for cryopreservation, studying chemical reactions at subfreezing temperatures, studying living organisms at subfreezing temperatures and many others.

3.2. Stability of supercooled aqueous solutions in the ISCM

The principal motivation in deploying an isochoric cryomicroscope chamber is the significant evidence that large volumes of aqueous solution are stable when supercooled in an isochoric system, e.g. Refs. [4,5,12]. Therefore, an important part of this study is verifying the ability of the ISCM to maintain an aqueous solution in a supercooled state for long periods of time while exposed to the external excitations that can cause ice nucleation in isobaric systems [12].

Experiments were performed on supercooled water at −5 °C to compare the stability of supercooled water in an ISCM in an isochoric configuration and in an isobaric configuration when exposed to severe external excitations. Briefly, the ISCM with deionized water in an isochoric and isobaric configuration was set under the ocular of a microscope, and the temperature reduced slowly from room temperature to −5 °C. At this temperature, both isochoric and isobaric configurations were supercooled (no ice formation was observed under the microscope). Exterior perturbations were applied when the water in both the isochoric and isobaric chambers was in a supercooled liquid state at −5 °C. In the first series of experiments, vibrations were applied to the isochoric chamber via a piezoelectric vibrating element (WHDTS, USA). After activating for 30 s and performing three repeats, it was found that the supercooled water in the isochoric chamber remains liquid. For a stronger external excitation, we used a JBL Go2 loudspeaker. We performed a total of 10 independent repeats, each with the isochoric and isobaric systems, and applied two different types of exterior excitation, as described in the **Materials and Methods** section. These excitations include 1 h of excitation by: I) the JBL Go2 speaker with both the ISCM and speaker side-by-side on the microscope stage, II) the isochoric chamber sitting atop the JBL Go2 speaker, as illustrated in Fig. 1d. As a result of these external excitations, the liquid in the isobaric chamber froze in all experiments upon the first application of the excitation (10/10). In contrast, no occurrence of nucleation (0/10) was observed within

the isochoric chamber from any of the exterior excitations. All the experiments were monitored under the microscope and recorded.

Fig. 2 illustrates the findings in these experiments. Fig. 2a shows the process of ice formation in the isobaric ISCM chamber exposed to a perturbation as Fig. 1c. The image taken through the microscope ocular shows that the external excitations caused ice formation, which began at the chamber wall and propagated inward into the chamber. This is typical of heterogeneous nucleation induced by the walls of the chamber. We will discuss the significance of the fact that ice nucleation develops from the stainless steel walls later. The ice formation process for pure water in the isobaric chamber under the microscope at −5 °C was videotaped. We observed that once ice nucleation occurs, the ice grows in a dendritic form across the bottom sapphire wall. When ice forms, the image becomes opaque. Fig. 2a shows the typical ice formation process. We used the temperature of −5 °C in the experiments reported here because we have never observed nucleation at this temperature in the isochoric configuration. Nucleation and ice formation in an isochoric chamber were observed at temperatures of −7 °C ~ −10 °C. It should be emphasized that at −5 °C, ice formed only in the isobaric chamber open to the atmosphere and not in the isochoric chamber. Freezing occurred in isochoric chambers at approximately −7 °C ~ −10 °C, and the appearance of freezing was identical to Fig. 2a.

Fig. 2b and c provide optical microscopy evidence that there was no ice nucleation in the supercooled liquid in the isochoric chamber during and after 1 h of excitation by the JBL Go2 speaker, neither when the isochoric chamber was on the microscope stage (Fig. 2b) nor when the isochoric chamber was on the loudspeaker directly (Fig. 2c).

After validating the stability of isochoric supercooling with deionized water, we also verified that the addition of cells and a cellular growth media would not corrupt this stability. Fig. 3 shows a typical appearance of the solutions with cells in the ISCM, in isobaric and isochoric configurations after external excitations. In all the experiments with cells in the isobaric configuration, there was ice nucleation as soon as the first exterior excitation was applied. In contrast, for the solution in isochoric configuration, no ice nucleation happened in the three days excitation experiments. Evidently, isochoric conditions provided enhanced stability to metastable supercooled water, even in the presence of heterogeneous nucleation sites, consistent with previous findings [13,12].

In addition to demonstrating the stability of the supercooled solution in the ISCM, this part of the study suggests that the ISCM could be used to determine the threshold for ice nucleation for different solution compositions, and temperatures, as a function of time and the intensity of external perturbations.

3.3. *Observation of supercooled cells in ISCM under fluorescence microscopy*

Low temperatures reduce metabolism and are therefore commonly used for preservation. One of the most important applications of low-temperature biology is the long-term preservation of biological materials. Preservation of biological matter at low hypothermic temperatures is commonly done using solutions that mimic the intracellular composition. Organs such as the liver, kidney, and hearts used for transplantation are routinely preserved at a temperature of about +4 °C [30]. Data from hypothermia studies at above freezing temperatures show that a decade reduction of temperature decreases the metabolism by a factor of about two to three [31]. However, to the best of our knowledge,

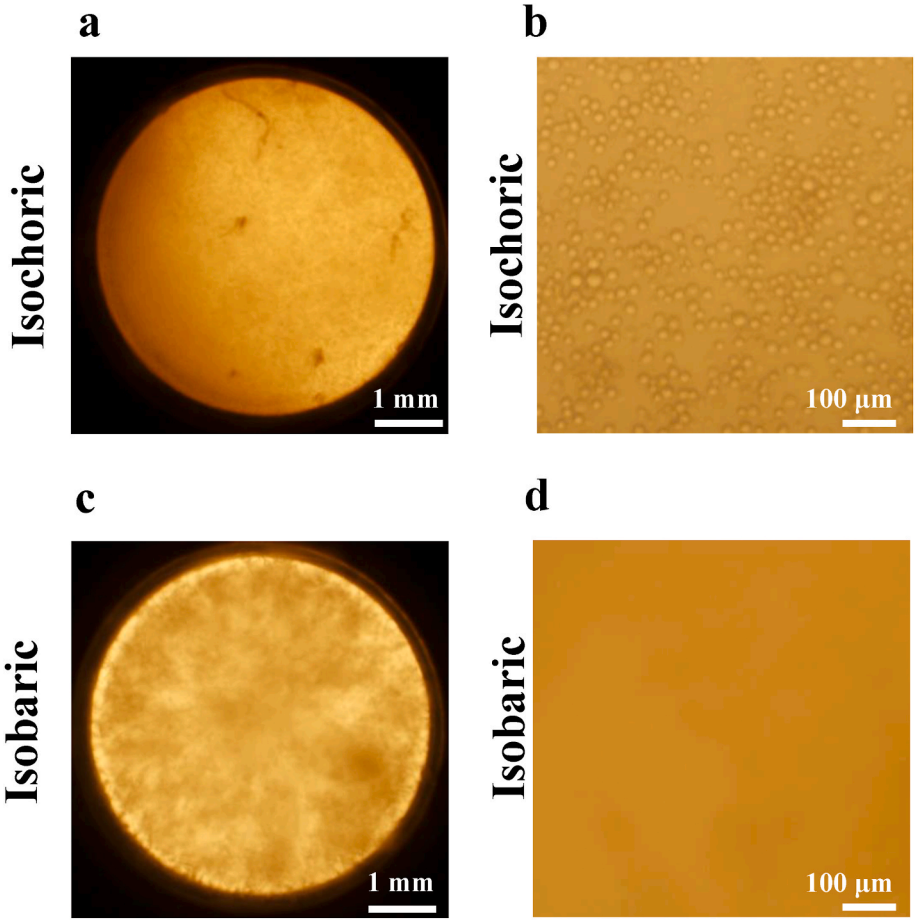

Fig. 3. The typical microscope images of the HeLa cells suspended in DMEM with 10% FBS at −5 °C under isochoric supercooled condition **(a, b)** and isobaric frozen condition **(c, d)**. Panel **b** and **d** is the enlarged view of panel **a** and **c**, respectively.

until now, there was no means to compare long-term biological materials preservation at above and below freezing temperatures in the absence of ice under a microscope. The ISCM provides this ability.

To illustrate the potential use of the ISCM, we monitored HeLa cells in University of Wisconsin (UW) preservation solution under bright field and fluorescence microscopy, continuously, for three days at the conventional preservation temperature of +4 °C in the ISCM in the isobaric configuration and in a supercooled state at −5 °C in the ISCM in the isochoric configuration. To further take advantage of the capabilities that the ISCM provides, we also added propidium iodide to the preservation solution (2 μl/ml). An illustration of the images used for the analysis of the results is shown in Fig. 4. The Figure illustrates typical images taken on days one, two, and three at cryopreservation temperatures. The figures also show the appearance of the cells when after three days of storage, they were brought to room temperature for half an hour. An image analysis software (*ImageJ*) was used to analyze the optical data. The results of the analysis of the experiments are given in Fig. 5, which shows a comparison of the percentage of stained HeLa cells across experiments. The figures give the average and the standard deviation from three repeats for each experimental condition and nine sites of measurements.

We observe that the ratio of stained (compromised) cells to total cells in isochoric storage at −5 °C remains almost unchanged from that in fresh cells throughout the three days of the experiment, while the ratio for storage at +4 °C increases substantially with the duration of preservation. After three days, the percentage of cells stained at +4 °C is a factor of almost three larger than at −5 °C. This demonstrates the value of biological materials storage at lower subfreezing temperatures in an isochoric supercooled state.

It should be emphasized that the observation that supercooling aids in preservation is not new. For example, it was previously shown that supercooling with antifreeze proteins as well as with a combination of antifreeze proteins and cryoprotectants improves survival for both the liver and the heart, e.g. Refs. [32,33]. A recent series of studies has shown that stabilizing the supercooled state by coating the air-water interface with an immiscible layer can produce phenomenal results [34–37]. The main goal of the experiments in the third part of this paper was to illustrate the potential use of an isochoric supercooling cryomicroscope. Here we show that the preserved cells can be continuously monitored under the microscope and the events recorded in time. We believe that the ability to monitor cells in a supercooled solution under a microscope will find additional applications, in particular when used with various fluorescence stains.

Supercooling cryomicroscopes similar to the one described in this study can be designed using arbitrary other techniques for stabilizing the supercooled state, including incorporation of antifreeze proteins to supercool the solution or use of an immiscible layer to cover the air-water interface. We chose to use the isochoric supercooling technique for several reasons. Firstly, the effective nucleation point depression with antifreeze proteins alone is relatively small. Secondly, concerning coating the air-liquid interface with an immiscible layer, we have previously performed several experiments in which we compared the

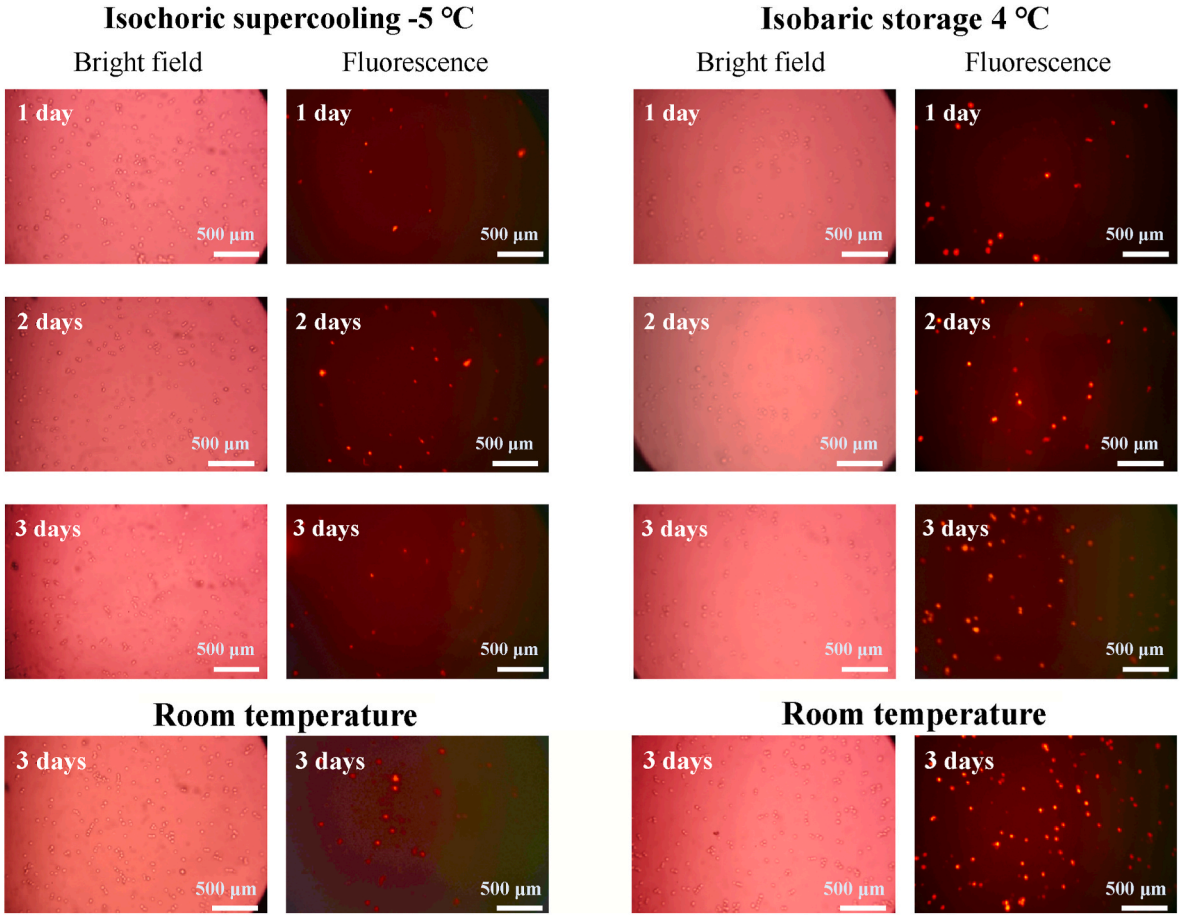

Fig. 4. Comparison of the membrane integrity of HeLa cells in UW solution at −5 °C isochoric system (left panels) and 4 °C isobaric system (right panels) under continuous real-time monitoring at cryopreservation temperatures via fluorescence microscopy during three days of storage. The propidium iodide was added to monitor cells' membrane integrity in before cryopreservation. More cells were stained red means more cells lose their membrane integrity. The cells were warmed to room temperature after three days preservation (bottom row). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

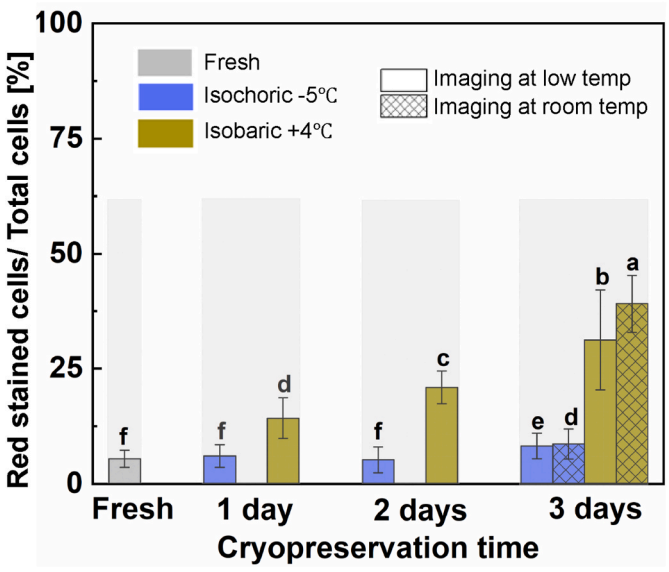

Fig. 5. Quantitative comparison of membrane integrity of HeLa cells in UW solution stored in −5 °C isochoric supercooled system and in 4 °C isobaric system under continuous real-time monitoring via microscopy during storage by calculating the ratio of the number of cells stained red to the total number of cells in the microscopic field of view. Different letters from a to f indicate significant difference ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stability of isochoric supercooled solutions with supercooled solutions in which the air-liquid interface was coated with an immiscible layer, and found isochoric conditions to yield superior stability and depth of supercooling. In Ref. [12], we found that isochoric supercooled solutions are more stable to external excitations than a supercooled solution in which the air-water interface is covered with an immiscible layer. This is of importance in an environment with external perturbations, such as in practical microscope experiments or during transportation. Furthermore, the stability afforded by an isochoric system appears volumetric in nature [13,12], while that afforded by the addition of an immiscible solution on the air-water interface affects that interface only. Ice nucleation can be induced by the volumetric phenomenon of cavitation [5] or when an object with nucleation sites is inserted in the supercooled solution [3,15,27], and isochoric supercooling has been shown to demonstrate some immunity to both of these mechanisms [13,12]. Furthermore, we recently performed a study comparing the maximum degree of supercooling possible in a chamber in which the air-water interface was small relative to the other surfaces of that chamber [13]. In that study, we compared the nucleation threshold in the chamber under isochoric conditions, isobaric conditions, and isobaric conditions with an immiscible layer coating the air-water interface, using identical chambers with or without a hydrophobic petroleum wall coating. In all cases, isochoric conditions substantially lowered the nucleation temperature, while the isobaric conditions (with or without the immiscible layer) yielded similar nucleation temperatures across tests, all of which were higher than the equivalent isochoric nucleation temperatures. In light of these observations, we elected to use isochoric supercooling in the design of the supercooling cryomicroscope presented herein.

In conclusion, in this paper, we describe and demonstrate the performance of an isochoric supercooling microscopy chamber designed to study the behavior of biological matter at subfreezing temperatures in a supercooled state. The device may become of value in studying isochoric supercooling preservation of biological matter, and we expect that substantial fundamental and practical studies will be possible with this device. We have also shown that isochoric supercooling is stable for long periods of time and can be used to glean interesting findings in a temperature range that was not accessible to microscopic research in the past.

Code availability

ImageJ software was used to count cells in images in this work. The software is available from the National Institutes of Health (NIH).

Author contributions

BR and CL conceived the work. YHZ and LL performed the experiment. YHZ, and BR wrote the manuscript. CL, MPP, BR edited the manuscript.

Declaration of competing interest

The authors Boris Rubinsky and Matthew J. Powell-Palm are leading an early stage start-up in the field of isochoric preservation named BioChoric Inc., which could be perceived as potential competing interests. The other authors have no known competing financial interests.

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