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Biological chip technology to quickly batch select optimum cryopreservation procedure

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Abstract In the practices of cryobiology, selection of an optimum freeze/thawing program and an idealistic cryo-protective agent often requires rather tedious, time consuming and repetitive tests. Integrating the functions of sample preparation and viability detection, the concept of biochip technology was introduced to the field of cryopreservation, aiming at quickly finding an optimum freezing and thawing program. Prototype devices were fabricated and corresponding experimental tests were performed. It was shown that microflow-channel chip could not offer a high quality solution distribution. As an alternative, the spot-dropping chip proved to be an excellent way to load the sample quickly and reliably. Infrared thermal mapping on such a chip showed that it had a rather uniform heat transfer boundary. Applying the spot-dropping chip combined with the thermoelectric cooling device, the final output of cryopreservation of multiple samples was tested, and the optimal freeze/thawing program as well as the potentially best concentration of the cryo-protective agent was found by analyzing the results. Further, application of this technique to measure the thermo-physical properties of the cryo-protective agent was also investigated. The study demonstrated that a biochip with integrated automatic loading and inspection units opens the possibility of a massive optimization of the complex cryopreservation program in a quicker and more economical way.

Keywords biological chip, cryobiology, cryopreservation, viability evaluation, freeze injury, biological material, micro total analytical system

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1 Introduction

Cryopreservation has been an important way to achieve long-term preservation of biomaterials. It is widely applied in tissue engineering and organ transplantation. Although the biomaterial can be preserved in a low enough temperature, it is prone to be destroyed during freezing or thawing as well as the variation of infiltration pressure across the cell membrane thus induced [1–8]. According to the “Two Factor” theory of Mazur, there exists an optimum freezing rate for each kind of biomaterial, at which a minimum injury can be obtained [6,9]. On the other hand, although the loading of cryo-preservation agent (CPA) to biomaterials has successfully reduced injury during freezing, inappropriate concentration of CPA is, however, useless or even harmful to the cell [4,10]. Therefore, the establishment of the best cryopreservation procedure with an optimum CPA is the key to the successful preservation of the target biomaterial.

Up to the present, tremendous efforts have been made to tackle the above serious issues. However, most of the previous works were carried out mainly by routinely following certain programs by intentionally varying the freezing parameters on a specific biomaterial loaded with CPA. Clearly, conclusions drawn from such results lack generality. To find an optimum cryopreservation procedure and the best CPA concentration, tremendous experiments, although tedious, expansive and repetitive, are necessary [11,12]. Therefore, if all these complex procedures could be integrated into a single device to develop a quick, economic and effective method to select the optimum cryopreservation procedure for the biomaterial, it will be of significant value for the practice of cryobiology. Hence, the concept of the biochip is introduced here for cryobiology research, aiming at finding an easy way to batch select the best cryopreservation procedure [13]. For this purpose, two types of biochip devices were constructed and tested. Typical results were reported and discussed.

2 Fluid handling by microflow channel biochip

To distribute the sample, water, and CPA at a desired specific ratio quickly, a biochip with micro-flow channels was

designed. Figure 1(a) schematically shows its four-branch structure, while Fig. 1(c) gives an actually made three-stage prototype for flow channel. The fluid can be divided into several parts through flowing in such a channel. The micro-valves are installed at the entrance and the outlet of the microflow channel to control the loading and dispelling of the fluid. Sample container array is set at the end of the microflow channel, in which the samples and CPA are mixed together. Although such chip configuration has been popularly used in many previous biochemical researches [14], experiments in this paper indicate that its flow channel as made in Fig. 1(c) cannot achieve a uniform distribution easily. The reason can be attributed to the significant differences among flow resistances in each channel. As an alternative, another better structure, the spot-dropping method, is adopted to realize the biochip, which can help handle the sample distribution quickly.

3 Experiments on spot-dropping biochip

3.1 Principle and method

Referring to some sample handling approaches developed in the past in biochip technologies, a micro-hole container array

is designed, and the spot-dropping method is adopted to handle a uniform distribution of the sample and CPA using a micro-injector. To evaluate the viability of the tested biomaterial, a thermocouple for temperature measurement is placed at the center of each hole container. The freezing curve-based monitoring method is applied to find the optimum CPA concentration and cryopreservation procedure [15]. Because the freezing point of the biological sample relies heavily on the structure, length of the chain, and saturation of cell membrane lipids, there is an evident shift in the freezing curve between the intact sample and the one already injured due to freezing. In this way, a quantitative evaluation on cryo-injury or viability of the biological sample can be performed. All the procedures are simple to handle, and the results do not depend on the operator, which guarantees an objective evaluation. For such reason, the present method can be regarded as highly quantitative and suitable for testing a wide variety of biological samples.

Schematic structure of the spot-dropping chip is illustrated in Fig. 2(a). The device can accomplish the tasks like spot-dropping, freezing, thawing, and evaluation of the biological sample and CPA. In this system, the micro-hole container array is placed to contain the mixture of the sample and CPA. A thermoelectric cooling device (TCD) is set below the container as shown in Fig. 2(b). Silicone oil with high heat

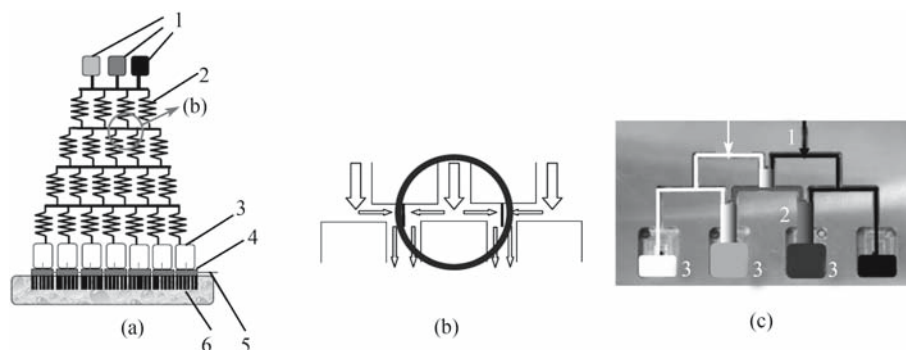


Fig. 1 Schematic structure of microflow channel analytical chip (a) Profile; (b) channel detail; (c) prototype

1. Solution inlet; 2. Microflow channel; 3. Sample container array; 4. Temperature sensor; 5. Thermoelectric cooling device; 6. Fin heat Dissipater

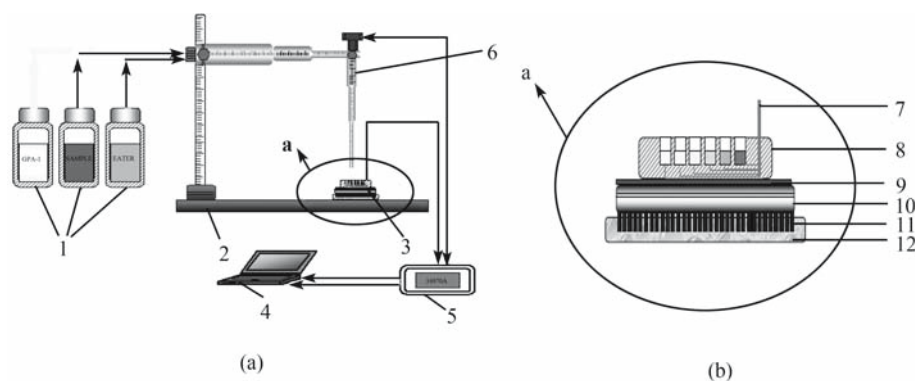


Fig. 2 Schematic of spot-dropping analytical chip system (a) Profile; (b) analytical chip

1. Container; 2. Spot dropping machine; 3. Analytical chip; 4. Computer; 5. Data acquisition system; 6. Automatic spot dropping device; 7. Temperature sensor; 8. Micro hole container array; 9. Copper plate; 10. Thermoelectric cooling device; 11. Heat dissipater; 12. Cooling water

transfer coefficient is filled between the TCD and the sample container array to reduce the heat transfer resistance. In this experiment, a uniform freezing of the samples at different containers has been achieved. That is to say, the temperature decreasing rate of each container is guaranteed to be as similar as possible. Different cooling capacity of the TCD can be realized by varying the current. In this way, a uniform boundary for the sample in each container during freezing and thawing processes can be obtained.

In the experiment the substrate of the sample container is made of stainless steel with six cylindrical holes drilled inside. The diameter of each hole is 6 mm, with a depth of 3 mm. Flatness of the side wall and the bottom of the cylindrical holes should be ensured to achieve a uniform freezing. Figure 2 gives the schematic diagram of the cylindrical array. The temperature sensor is T-type thermocouple. All the sensors have been calibrated in the ice-water mixture so that the measurement error can be controlled within ± 0.1 °C. The thermocouple is placed in the center of the cylindrical hole, without contacting with the bottom. The head of the thermocouple is inserted into the sample to test its temperature change during the experimental process. The temperature transient is recorded by a 48-channel 34 970 data acquisition system made by Agilent Inc., USA.

When doing the experiment, the biological sample solution and the CPA to be selected are added into each bottle of containers. According to the type and the concentration of the CPA, various bottles can be used to make sure that a different type of CPA is held in a different bottle. The containers are connected to the spot-dropping device via pipes.

Then, a sequence to be followed can be made like this: determining the sample type and the concentration of the CPA; calculating the spot volume of the spot-dropping device; turning on the flow channel of sample, CPA, and water; and accomplishing the spot-dropping process according to the desired spot volume, respectively. After that, the solution was left alone for a period of time. The solutions could be shaken, if necessary, to enhance a good mixture between the sample and the CPA. Then the TCD below the micro-hole container array was started, and the freezing and thawing of solutions in the micro-containers was performed by controlling the input

power. After that, data acquisition of the temperature sensor array was initiated. The freezing process was performed again after finishing the freezing and thawing process of the sample. The temperature transient during the freezing process was recorded. Finally, the results were compared with the freezing curves of intact biological sample solution, and the cryopreservation procedure having a minimum difference between the freezing curves of intact sample and that of the injured one was regarded as the optimum one.

3.2 Infrared temperature mapping of sample container array

Figure 3(a) is the sample container array and Fig. 3(b) is the infrared temperature mapping after 2 min's cooling by TCD under 3 A current. It can be observed that the bottom temperatures of the sample containers are approximately the same everywhere. For spatial temperature measurement resolution of the present infrared thermometer is 0.06 °C, this confirmed the uniform freezing of the sample containers during the freezing process. Similarly, Fig. 3(c) is the infrared temperature mapping of the sample containers array after the sample containers array was frozen by TCD with 3 A current and then thawed for 1 min in the air. It can be seen that the bottom temperature distribution of the sample containers array also appears rather uniform during the thawing process. This guaranteed the uniform thermal boundary during freezing and thawing, which eliminated the uncertainty due to the difference of the sample container boundary during the process of selecting the optimum cryopreservation procedure.

3.3 Experiments for selecting optimum CPA

To test the feasibility of the present method and quickly select the best cryopreservation procedure by using the biochip, fresh egg white was chosen as the test sample and the experiment was performed to select an optimum CPA concentration under a specific freezing procedure. The mixture of dimethylsulfoxide (DMSO) and water was adopted as the CPA. It was made as follows: add a certain amount of DMSO and water into the containers as illustrated in Fig. 3(a), and connect

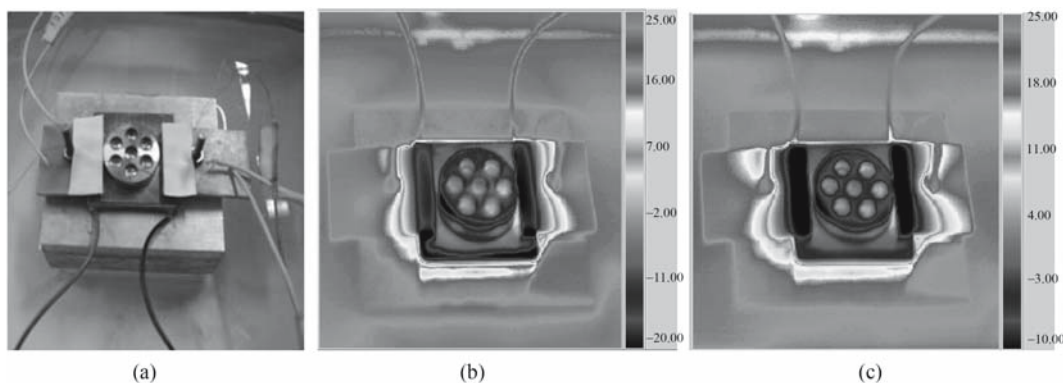


Fig. 3 Micro-hole container array and characterization on its temperature uniformity (a) Device; (b) infrared temperature mapping during freezing; (c) infrared temperature mapping during rewarming

these containers with the spot-dropping device via the pipes. To simplify the experiment, only three typical conditions of CPA concentration were investigated. Three micro-holes were symmetrically selected with each spaced by an empty hole container. The DMSO solutions with a quantity of 7 μL , 14 μL , and 1 μL in each hole was dropped respectively to the container using the spot-dropping device, and the water volume for the above corresponding holes were chosen as 21 μL , 14 μL , and 7 μL , respectively. In this way, 28 μL of mixed water-DMSO solutions with their volumetric concentrations as 25%, 50%, and 75% was obtained separately. After that, 28 μL of the egg white was added and shaken to fully mix the sample and the CPA, and the mixture was left alone for 5 min. Then the data acquisition system was turned on to record the temperature transient. After 1 min, the cooling device was switched on with the current of 3 A supplied, and kept refrigerated for 3 min. After turning off the cooling device, the samples were thawed in the air. Repeat the whole process after finishing all these procedures, and record the temperature at the same time. The experimental results can then be used to evaluate the freezing process.

According to the research of Liu and Zhou [15], there are two steps to evaluate the biological tissue viability by using the freezing curve-based monitoring method. The first step is to freeze the fresh sample, and repeat the freezing process under the same condition as described above. The second one is to compare the curves of the two freezing process, and evaluate the cryoinjury in the first freezing and thawing process. Through analyzing the freezing curves of the solutions of the biological sample and the CPA with different concentration, the mixed solution could be found to have a super cooling degree during the freezing process. For example, when the temperature reached the maximum super cooling degree, the CPA solution would have a jump in the temperature curve because of the heat release during phase change. When the sample was injured, the internal structures of the sample would be changed, which led to the variation of the thermophysical properties. When evaluating the change in the freezing curve, the jumping time of phase change of the injured sample could be found to appear quite different, compared with that of the intact one. Such difference revealed the degree of cryoinjury.

From Fig. 4(a), it can be observed that when the CPA concentration is 25%, the temperature jump of the two freezing cycles of the sample occurs at 106.5 and 112 s, respectively, and the difference between them is 5.5 s. On the other hand, from Fig. 4(b), it can be observed that when the CPA concentration is 50% (the results are not given in the paper for brevity) and 75%, the difference between the time when the temperature jump occurs in the two freezing curves is 10.5 and 24.5 s, respectively. It can be concluded that when CPA concentration is 25%, the difference between the time when the temperature jump occurs in the two freezing curves appears the smallest. When CPA concentration is 75%, such time difference is the largest. According to the principle of biological viability evaluation, it can be concluded that under

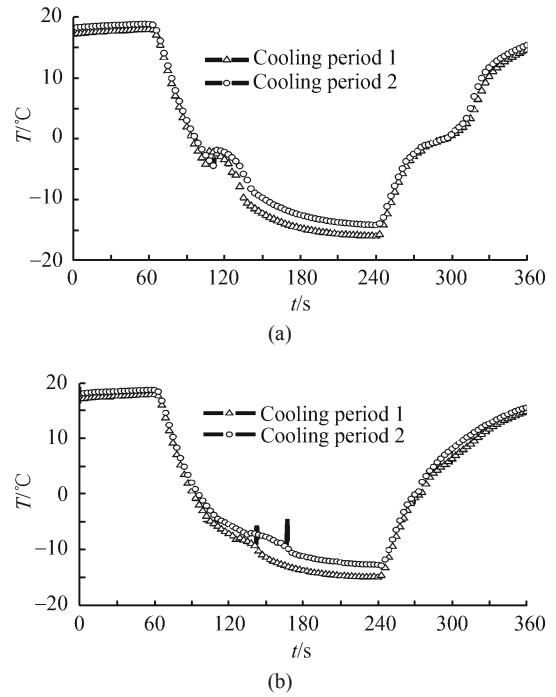


Fig. 4 Temperature decreasing curve of the solution mixture between biological sample and CPA (a) At a concentration of 25%; (b) at a concentration of 75%

the above cooling rate (about 10 $^{\circ}\text{C}/\text{min}$), the sample with the CPA concentration of 25% suffers less cryoinjury, and the viability of the sample will be relatively high.

Further, additional experiments can still be performed using the same method. In each test, more different concentrations of CPA can be compared to select the optimum concentration of the CPA. It should also be pointed out that the sensitivity of the present method depends on the particular response of the sample to the CPA. The resolution for this method to distinguish the effects of the CPA concentration on the cryopreservation efficiency needs further research in the near future.

3.4 Experimental results and analysis of super-cooling of CPA

The spot-dropping freezing device as mentioned above could not only be used to quickly distribute CPA with desired concentration, but also be applied to test the viability of the biological sample under different CPA concentrations within a short period of time. In addition, this method is rather useful in measuring the thermophysical property of the CPA with different concentrations, which has also been an important basic issue in cryobiology. To illustrate such application, additional experiments were performed, in which three CPA solutions with different concentrations were tested. The experimental process will be explained and preliminary results for their future application will be discussed.

Similar experimental approach as mentioned above is followed. Three micro-holes symmetrically distributed were chosen with an interval between each other. The DMSO with

the volumetric concentration as 50% by a volume of 7, 14, and 28 μL was dropped into the holes separately, and water with volume of 21, 14, and 7 μL was added to the above three holes correspondingly. Therefore, a mixed solution 35 μL for water and DMSO could be obtained with their volumetric concentrations as 10%, 20%, and 40%, respectively. After that, the device was shaken to mix the sample and the CPA completely. Then the mixture was left alone for 5 min. The data acquisition system was turned on to record the temperature. After 0.5 min, the TCD was turned on with a current of 3 A supplied, and refrigerated for 3 min. Then the power of the TCD was switched off, so that the samples could be left to thaw in the air. After finishing the whole process, the TCD and data acquisition system were turned off.

As illustrated in Fig. 5, there exist super-cooling degrees in DMSO solutions with different concentrations during their freezing processes. At the largest super-cooling point, the temperature curve of the CPA solution jumps because of the heat release during phase change. For DMSO with different concentrations, the magnitude of super-cooling and the time for it to appear are quite different. For example, when the CPA concentration is 10%, the largest super-cooling degree is $-6.1\text{ }^\circ\text{C}$, but when the concentration is 20% and 40%, such value is $-11.5\text{ }^\circ\text{C}$ and $-13.7\text{ }^\circ\text{C}$, respectively. Therefore, it can be seen that the super-cooling degree increases with the increase of DMSO concentration. This phenomenon has also been confirmed by some previous researches. The super-cooling reflects the freezing condition of the CPA, and directly determines the property of the CPA as well as the cryopreservation output of a biological material. If the thermophysical property of the CPA is not available, it generally needs massively repeated experimental work. By using the spot-dropping cooling device, a quick distribution of the CPA solution with several components can be achieved and the super-cooling degree and other important parameters can be measured conveniently.

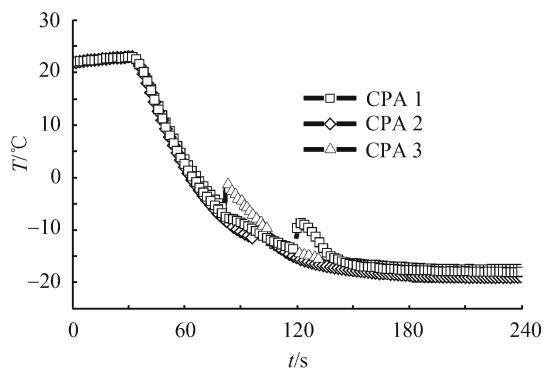


Fig. 5 Temperature decreasing curve of water solution loaded with cryoprotective agent DMSO at different concentrations

4 Discussion

In the present experiments, the thermocouple is placed at the bottom of the sample container array. It is close to, but does

not contact with, the bottom. Although the position of the thermocouple in the container is fixed, difference due to its position uncertainty also contributes to the temperature measurement. Liu and Zhou [15] have provided experimental curves that indicate the effect of the thermocouple position to the measurement of water drops. Clearly, this would have obvious effect on the accuracy of the whole system. Attention should be paid to such phenomenon in future practices. It is very important that the thermocouple be installed precisely. Meanwhile, the thermocouple in each container should be calibrated in advance to correct any possible errors caused by different positions of the temperature sensors.

The present cooling device can still be improved by adopting a specific programmed cooling device so as to accurately control the cooling rate, or by installing the heat block and protecting shell to avoid disturbance from the heat radiation and convection. In this way, a better testing precision can still be possible. It should be mentioned that the sample to be tested in the cryopreservation process may not necessarily be in liquid phase. The solid sample is also suitable for testing by cutting or crushing it into small pieces. Further, the method for evaluating the biological viability can be optional. For example, in addition to the freezing curve-based monitoring method, the electrical impedance detection as proposed before can also be used. This is realized by innovating the present device, such as installing electrodes in the testing containers. Thus, simultaneous testing on temperature and impedance can be performed, which guarantees a comprehensive and accurate evaluation.

It should be pointed out that in this paper, the CPA solution had been prepared in the first step, and then the biological sample was added. The samples in each container were kept in the CPA solution for the same period of time. In reality, by adding the sample firstly and then controlling the loading event and order, different diffusion time can be achieved for various CPA so that the results it caused to the cryopreservation could be varied. The difference of the preservation output under various diffusion times could be compared. Of course, the cooling process could also be controlled and investigated, and the CPA could be added during the cooling process to test the effect of the temperature at which the CPA is loaded to the samples. Such work still need further research in the near future.

5 Conclusion

Through integration and miniaturization of the devices for sample distribution and viability evaluation, the biological chip technology to quickly batch select the optimum cryopreservation procedure was established for the first time in this paper. The experimental results show that the micro-flow channel chip does not have a good distribution performance for handling the sample, while loading of samples by spot-dropping chip is quick and reliable. Furthermore, the bottom surfaces of the sample containers have gained a uniform

temperature distribution during the freezing and thawing process. Therefore, by using the spot-dropping cooling unit, an optimum CPA concentration can be selected for the cryopreservation process. In addition, the chip can also be used to measure the thermophysical properties of the CPA solution or to investigate the effect of CPA loading condition to the cryopreservation result of the biological sample. In conclusion, the chip analysis method as proposed in this paper proves to be a simple, economic, and quick way to batch select the optimum cryopreservation procedure.

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