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UNDERSTANDING TEMPERATURE PROFILES EXPERIENCED BY BIOLOGICAL SAMPLES DURING A HYBRID VITRIFICATION TECHNIQUE

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ABSTRACT

From thermodynamic point of view, vitrification is considered as a superior preservation technique in comparison with the traditional slow-cooling cryopreservation techniques, due to formation of the glassy state in both intra and extracellular environment. While vitrification of biological samples are difficult to achieve, recently a hybrid technique involving partial desiccation of the cell samples prior to cryogenic exposure has been successfully employed to achieve vitrification. In this technique cells in monolayer attached to a substrate was suspended in a trehalose solution and then rapidly and uniformly desiccated to a low moisture content (<0.12 g of water per g of dry weight) using a spin-drying technique. The spin-dried samples were stored in liquid nitrogen (LN_2) at a vitrified state (Fig. 1). It was shown that following re-warming to room temperature and re-hydration with a fully complemented cell culture medium, 51% of the spin-dried and vitrified cells survived and demonstrated normal growth characteristics. The current study further investigates the temperature profiles experienced by the cell samples during partial desiccation and cryogenic exposure to identify possible ways to improve this novel vitrification strategy. Physical Vapor Deposition technique was employed to develop glass substrate having thermocouples (2x2x2 μm) at four radial positions across the substrate to record the thermal history of the cell samples during the entire process. Efforts are undertaken to understand the uncertainties related temperature measurement spatially and with respect to time. These temperature characterization studies are important to optimize the newly developed hybrid vitrification technique for vitrification of cellular samples.

INTRODUCTION

Vitrification is considered to be a superior to be a superior biopreservation technique to the traditional slow-cooling cryopreservation techniques. Recently a hybrid technique involving partial desiccation of the cell samples has been successfully employed to reach vitrification of biological samples. In the technique, cells attached to a glass substrate were desiccated by using a spin-drying technique and stored in liquid nitrogen (LN_2) at a vitrified state. The cells survived by re-warming to room temperature and re-hydration. This study focuses on the measurement of temperature profiles of the cells during the process to optimize the vitrification approach. Micro-thermocouples (2x2x2μm) were fabricated and calibrated on glass substrate by using Physical Vapor Deposition approach. Temperature profile of cells in different radial positions was recorded to improve the hybrid vitrification technique.

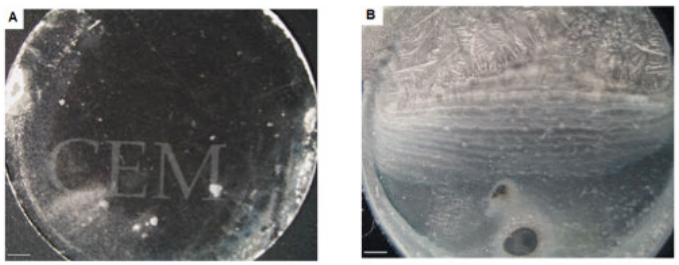

Figure 1: (A) Apparent vitrification of pre-desiccated cell samples by spin-drying following exposure to LN_2 temperatures. Transparency of the samples indicate apparent vitrification of the samples (B) Sample that was not spin-dried following exposure to cryogenic temperatures. Extensive ice crystallization renders the sample opaque.

MATERIALS AND METHODS

Design and Development of Micro-thermocouples: Micro-thermocouples were designed, fabricated and calibrated on a glass substrate by standard photolithography and lift-off techniques. Micro-thermocouples (junction area with a size of 2 μm×2 μm) were designed in different position on glass slide

(One in the center, other four 1mm away from the center and the other four 5mm away from the center).

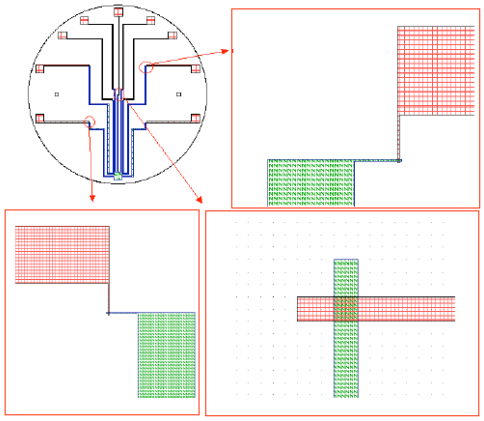

Figure 2: The design of Micro-thermocouples

The process of fabrication is shown in Figure 3.

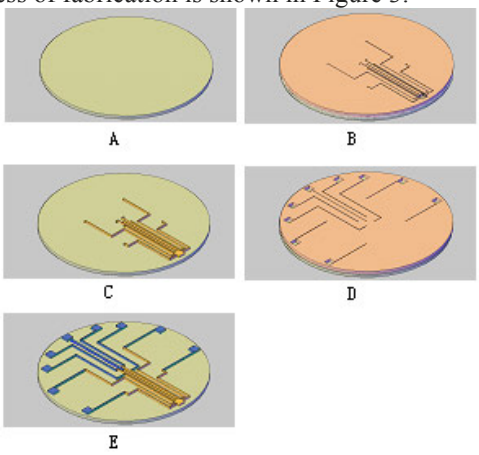

Figure 3: The fabrication of Micro-thermocouples

Ni-Cr and Nickel were selected as two lead of Micro-thermocouples. The glass substrate was cleaned with Acetone and Isopropyl Alcohol then baked on a hotplate (115 ℃) for 5 min (Figure 3.A). A layer of photoresist (SPR 220) was then spun on the glass slide. A layer of Hexamethyldisilazane (HMDS) was used to adhere photoresist to glass slide. The pattern was transferred from the mask plate to the photoresist by using the mask aligner (MJB-3) and the CEE 200XD developer (Figure 3.B). O2 plasma was done before the evaporation to clean the slide. Physical Vapor Deposition of Nickel was done by using Cooke Evaporator. The first layer (Nickel) was then obtained by lift-off with Acetone and Isopropyl Alcohol (Figure 3.C). Same process was performed to obtain Ni-Cr layer (Figure 3.D and Figure 3.E). The picture of the junction under the microscope is shown in Figure 4.

Calibration of Micro-thermocouples was done from 20℃ to -190℃. The thermocouples were bonded to wire by using conductive adhesive and then connected to Nano voltmeter (Agilent 34420A). The glass slide with thermocouples on it was cooled by liquid nitrogen (LN_2) from 20℃ to -190℃. Temperature was held every 10 ℃, and the voltage between two leads was recorded. The curve of voltage versus temperature was then obtained.

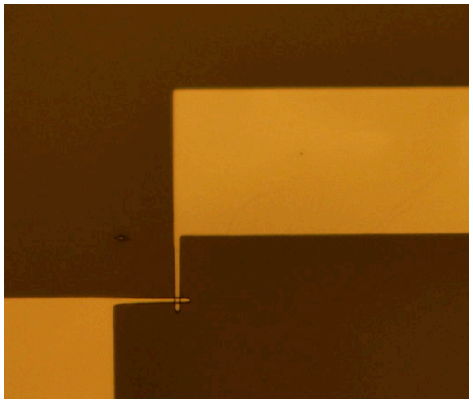

Figure 4: The junction under the microscope

Partial Desiccation using spin-drying and vitrification: Partial desiccation of the cell samples was achieved using a spin-drying technique. Spin-drying was performed using a commercially available spinning machine purchased from Brewer Science Inc. (Model Cee 200, St. Louis, MO). A genetically modified strain of HepG2 cells expressing a trehalose transporter (TRET1) was pre-incubated in 0.2 M trehalose solution and at 80% confluence, the cells were trypsinized with 0.25% trypsin/1 mM EDTA solution and placed in a tissue culture dish containing 22 mm round glass coverslips (BD BioCoat, San Jose, CA). The cells were incubated in cell culture medium and allowed to attach to the glass coverslips overnight. Before spin-drying, the cell culture media was completely removed using a Pasteur pipette and replaced with a drying solution (1.8 M trehalose, 10 mMKCl, 5 mMglucose, 20 mM HEPES, and 120 mMcholine chloride at pH 7.4). The spinning system was purged with N2 to create an inert environment and the glass coverslips were spun at 1000 rpm for 1 min.

Following partial desiccation, the cell samples were submerged in LN_2 and stored at -196^{0}C. To investigate the effect of slow freezing on viability of spin-dried samples, cells were cooled to -196^{0}C at 1^{0}C/min using a controlled rate-cooling device before being maintained at -196^{0}C. Following storage the samples were thawed and rehydrated using cell culture medium without phenol red and the membrane integrity was evaluated using Syto-13/ethydium bromide dyes.

RESULTS AND DISCUSSION

It has been demonstrated that following re-warming to room temperature and re-hydration with a fully complemented cell culture medium, 51% of the spin-dried and vitrified cells survived and demonstrated normal growth characteristics.

The curve of voltage versus temperature is shown in Figure 5. From the result, the curve in -50℃ to 20℃ is quite linear,

which indicates that it can be used to measure temperature in this range. The slope of the curve in -50℃ to 20℃ is small, which could due to the size of the thermocouple is small. There is a difference between cooling curve and re-warming curve, which is due to hysteresis. When temperature is lower than -50 ℃, the slope of the curve changes to negative, which is not the same as prior literature. Our further study will focus on finding the reason of the negative slope when temperature is lower than -50 ℃, testing the repeatability and calibrating other thermocouples to get the temperature profile for optimizing the vitrification approach.

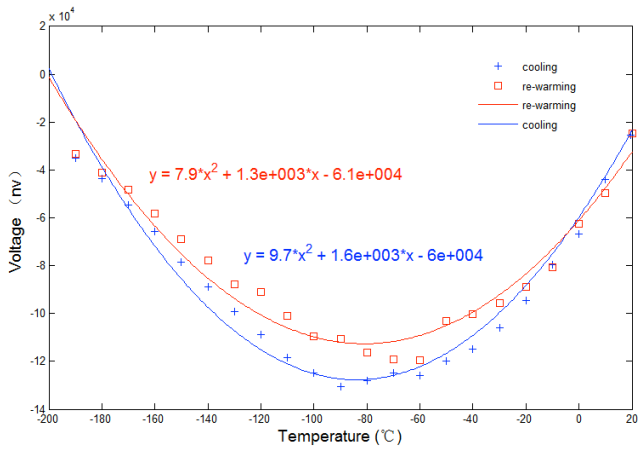

Figure 5: The curve of calibration

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CONCLUSION

The Micro-thermocouples developed here can be used to monitor temperature changes at low temperatures relevant for vitrification research. Furthermore, the thermocouple operates between 20℃ and -50℃ with excellent linearity. The reason of the slope changing to negative when temperature goes lower than -50℃ should be studied in the further research. Currently further investigation is underway to understand the temperature profiles experienced by the cell samples during partial desiccation and cryogenic exposure with a view to improve this novel vitrification strategy.

ACKNOWLEDGEMENT:

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