Cell death along single microfluidic channel after freeze-thaw treatments

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Cryotherapy is a prospective green method for malignant tumor treatment. At low temperature, the cell viability relates with the cooling rate, temperature threshold, freezing interface, as well as ice formation. In clinical applications, the growth of ice ball must reach a suitable size as cells could not be all killed at the ice periphery. The cell death ratio at the ice periphery is important for the control of the freezing destruction. The mechanisms of cryoinjury around the ice periphery need thorough understanding. In this paper, a primary freeze-thaw control was carried out in a cell culture microchip. A series of directional freezing processes and cell responses was tested and discussed. The temperature in the microchip was manipulated by a thermoelectric cooler. The necrotic and apoptotic cells under different cryotreatment (duration of the freezing process, freeze-thaw cycle, postculture, etc.) were stained and distinguished by propidium iodide and fluorescein isothiocyanate (FITC)-Annexin V. The location of the ice front was recorded and a cell death boundary which was different from the ice front was observed. By controlling the cooling process in a microfluidic channel, it is possible to recreate a sketch of biological effect during the process of simulated cryosurgery. © 2010 American Institute of Physics. [doi:10.1063/1.3324869]

I. INTRODUCTION

Cryotherapy has been commercialized on kinds of tumor treatment.^{1–3} One of the key functions of cryotherapy is to define a destructive cell margin within the tumor while inducing minimally damage to adjacent healthy cells. However, in surgical applications, it is hard to control or predict the destructive boundary. This has brought numerous researches on the freeze-thaw process and cell injury, either experimentally or numerically.^{4–11} Two urgent needs for surgical applications are to explore mechanisms of cell injury that can maximize cell death within the tumor and minimize the damage to boundary cells which might be dead or live; or develop mature tools to control the cooling-thawing procedures that weaken the dependence of surgeons' experiences. Also deep investigations of cell destruction near the ice periphery can provide more information to evaluate the efficiency of cryotherapy and design feasible clinical tools.

The mechanisms of cell or tissue destruction after cryosurgery have been studied for several decades.^{12,13} Ice crystals formed inside or outside the cells, named intracellular ice formation and extracellular ice formation, could rupture cell membrane and induce necrotic death.^{6,14} The high concentration solution near the ice front was believed lethal to cells as osmotic stress drew water from the inside of the cell and induced cell shrinkage. Gene regulated death (apoptosis) was reported usually occurring at the ice front, where the temperature was not low enough to kill all the cells at one moment.^{15–18} Smith and Fraser¹⁹ took tissue sections through a region of rat liver frozen *in vivo* and found a well defined line between live and dead cells after postincubation for

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48 h. Yang *et al.*^{20,21} investigated the speed of ice growth and temperature history at the ice front. MBT-2 tumor cells were cultured and then subjected to simulated cryosurgery. The surviving cells were stained with crystal violet and the cell death boundary was detected by image processing. Cells were mostly alive beyond the death boundary, and mostly dead within it. They found that the growth speed of ice affected the distance between the cell death boundary and the stable ice front. The relationship of cell death and temperature history was also tested. Care must be taken to optimize the cell killing by freezing or other mechanical or chemical treatment around the boundary of the ice ball.

Numerous *in vitro* studies have been carried out at different cooling rates. However, traditional cell culture in Petri dish could not efficiently mimic a physiological micro- or macroenvironment *in vitro*. In this study, a microfluidic cell culture platform was fabricated; cells were immobilized on the bottom surface of the flow channels.²² By confining cells in polydimethylsiloxane (PDMS) fabricated microchannel, the setup had benefits as follows. (1) The *in situ* observation of ice growth from the coolest location to the stable ice boundary can be achieved. (2) The biochemical test of cell activity could be synchronously implemented and the relationship between cell injury and the freezing process could be established and modeled. (3) The flexibility of PDMS chip design could simulate complex microenvironment such as blood flow or three-dimensional culturing.

In this work, a preliminary study of the freezing effects on cells was carried out in a one-dimensional cell model in a PDMS based microchannel. The freezing periphery, i.e., the ice front, was tracked. The distribution of cell necrotic death, apoptosis, was analyzed, and a “death boundary” was observed a certain distance from the ice front. The effects of the repetition of freezing-thaw cycle, duration time, and culturing time after cryotreatment were tested.

II. MATERIALS AND METHODS

A. Microchip fabrication

Microfluidic devices were fabricated in PDMS using standard soft lithography method and replica molding.²³ Microscale patterns were first created using computer-aided design and then printed out on high-resolution transparencies. Transfer of the printed patterns to silicon wafer was carried out in a 10 000 class clean room (Public Micro-Nano Machining Shop, Peking University). SU-8 2050, a negative photoresist (Microchem, Newton, MA), was used as a mold. The patterns of channels in the photomask were replicated photolithographically. The PDMS prepolymer mixture [Sylgard 184, Dow Corning (Shanghai) Mgmt. Co., Ltd., China] was prepared with a mass ratio of A:B=10:1 and poured over a photoresist/silicon wafer master in a Petri dish. A set of stiff plastic refills (D=3 mm) was cut as 8 mm tall which served as the mold of the fluidic connection ports. The mixture was degassed for 30 min before cured at 80 °C for 30 min in an oven (DHG9023-A, Yashilin Testing Equip. Co., Ltd., Beijing, China). The port mold was removed and the devices were cut by a razor blade. The devices were then sealed to 1 mm thick, 25 × 75 mm² glass slides by oxygen plasma treatment (PlasmaTherm Etcher, Diener Electronic Co., Ltd., Germany) on the glass surface and the bottom of the slab. The PDMS chips were sterilized by exposing to UV light around 4 h in a safety cabinet (MSC-Advantage, Thermo Fisher Scientific, Inc., MA, USA) before experiments.

B. Freezing-thawing system and operation

The subzero temperature was achieved by two bonded thermoelectric chips (TECs) (Jingjie Industry and Trading Co., Ltd., Tianjin, China). The setup is illustrated in Fig. 1. The parameters of the coolers are listed in Table I. Each TEC has a cooling side and a heating side. The heating side was cooled by a designed copper heat exchanger where room temperature water (~27 °C) continuously flew through and carried the heat out. To achieve a higher cooling rate, TEC chips were bonded side to side, as illustrated in Fig. 1. Heat conductive glue was carefully spread between all contacting faces to achieve favorable conductivities. The temperature was measured at specific locations by K-type thermocouples. The temperature profile was recorded through a data

FIG. 1. Schematic diagram of cooling system for one-dimensional freezing in a PDMS microchip. The cooler was a set of TECs. The cooling side of the bottom TEC was attached to the top surface of the PDMS chip and the cooling effect was conducted through the PDMS slab to the channel. The image on the right was a phase contrast picture of seeded CaSki cells in the microchannel.

acquisition system (UTL/D-08LS1V0N, UTOP Electronic Co., Ltd., Guangzhou, China). The power was supplied by a dc-stabilized source (WYK-5030, Huatai Electronics Co., Ltd., Yangzhou, China).

When the experiment started, room temperature water was forced flowing through the copper heat exchanger, then a proper voltage (TEC1-12714 15 V, TEC1-12710 2 V) was applied to the TECs to induce freezing in the microchannel. It was found that the ice front appeared at the beginning of the microchannel at 1–2 min after the voltage was applied. Then it kept spreading downstream for 7–8 min. After it came to a stop, the ice front was not absolutely stabilized but mildly oscillating back and forth. Then after 5 min the TECs were moved away and the thawing process started. The most distant position within this 5 min was defined as the stabilized ice front. In the following cycles based on the same chip, the ice front was maintained at same position as the first cycle by slightly adjusting the voltage of the cooler if necessary. The lowest temperature achieved at the TEC cooling side was around -40°C , water in reservoir around -20°C , and ice front about 0°C . The thawing process was carried out at room temperature. Repetitions of freeze-thaw cycle could be carried out based on the purpose of the test.

C. General cell culture

Cervical cancer CaSki cell line was cultured in plastic culture Petri dish at 37°C , under 5% CO_2 in Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT), penicillin (100 u/ml, Hyclone, Logan, UT). CaSki cultures were maintained in 60 mm Petri dish (Corning) in a CO_2 incubator (Forma Series II, Thermo Fisher Scientific, Inc., MA, USA) at 37°C and humidified atmosphere with 5% CO_2 . Cells were diluted at a ratio of 1:5 every 3 days to maintain them in the exponential growth phase ($\sim 1 \times 10^6$ cells/ml). Cells were harvested by adding 0.25% trypsin-EDTA (Hyclone, Logan, UT).

TABLE I. The parameters of the TEC.

TH= 27°C							
	Maximum current	Maximum temperature differential	Maximum voltage	Maximum power	Outside dimension (mm)		
Type	(A)	($^{\circ}\text{C}$)	(V)	(W)	L	W	H
TEC1-12710	10	67	15.4	89	50	50	4.4
TEC1-12714	14	67	15.4	120	62	62	4.6

to the culture and centrifuged at 300 g for 10 min to remove the supernatant. The cells were then resuspended in the culture medium at a concentration of 1×10^6 cells/ml before seeding in the microfluidic device.

D. Cell seeding and culture in the microchip

The channels were coated with fibronectin from bovine plasma (Sigma, St. Louis, MO) to facilitate cell adhesion. Fibronectin is a glycoprotein which interacts with the cell surface receptor or extracellular matrix to mediate cell adhesion. Fibronectin was prepared at a concentration of 50 $\mu\text{g/ml}$ in phosphate-buffered saline (PBS) (Beijing Chemicals Co., Ltd., China) before each experiment, and then flowed into the microfluidic device and incubated at 37 °C for 30 min. Prepared CaSki cells were suspended in the culture media at around 1×10^6 cells/ml, injected into the sample reservoirs of the microfluidic device, and allowed 30 min to spread and attach. The cells in the media readily flowed into the central channel by gravity due to the liquid level difference in the end reservoirs. After adding extra culture media in both reservoirs, the device with seeded cells was incubated in a 37 °C, 5% CO₂ incubator (Forma Series II, Thermo Fisher Scientific, Inc., MA, USA) for 12 h before cryoexperiments. We found that renewing the media in the reservoirs every 3–4 h provided enough media to support cell growth inside the device.

E. Sample treatment

To test the apoptosis and necrosis, double staining of Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) apoptosis detection kit (KeyGen Biotech. Co., Ltd., Nanjing, China) was performed in a microfluidic cell culture system as follows. Apoptotic cells translocate the membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the outside, which can be easily detected by staining with a fluorescent conjugate of Annexin V, which has a high affinity for PS. PI is a membrane impenetrable and usually used to identify necrotic cells or late apoptotic cells whose membrane was not intact. With the combination of two chemicals, intact cells, initial apoptosis, middle or late apoptosis, and necrosis could be distinguished. The reagent was diluted by binding the buffer to a final concentration suggested by the supplier. After the freeze-thaw treatment, the culture medium was removed and the channel was washed twice with fresh PBS buffer. A drop of prepared reagent (around 50 μl) was added to the reservoir and filled the culture channel. The chip was incubated in the CO₂ incubator at 37 °C for 10 min before testing the cell viability. Test experiments were made to confirm that the death distribution was not related to the concentration distribution of the dyes. For the same chip, the dyes were added to the beginning reservoir and the ending reservoir, respectively, and there was not obvious difference between the two sets of images, which indicated the dyes were fully diffused along the whole channel.

F. Fluorescence microscopy

Fluorescent and phase contrast images were taken on an inverted fluorescence microscopy (TI-U INVERTED, Nikon, Japan) with a charge coupled device (CCD) camera (Monochrome Cooled Digital Camera Head DS-Qi1Mc, Nikon, Japan). Great care was exercised to decrease photobleaching due to excessive exposure. The settings for the CCD camera and the software were kept identical from one experiment to another when comparison between experiments was desired. In this experiment, Annexin V-FITC (465–495 nm) was excited by blue light and emitted green fluorescence, while PI (535/617 nm) was excited by green light and emitted red fluorescence.

III. RESULTS AND DISCUSSIONS

Three times of freeze-thaw treatment were performed on the same single channel chip. The first freeze-thaw cycle, i.e., cycle I, was defined as freezing for 15 min (about 10 min ice pro-

(a)

(b)

FIG. 2. The images of ice spreading in the microchannel: (a) The moving ice front (around 2000 μm from the beginning reservoir) where the cooling rate was high; (b) the stabilized ice front (around 4100 μm from the beginning reservoir).

ceeding and 5 min stabilization). The second one, i.e., cycle II, was a repeat of cycle I on the same channel. Cycle III was the same as cycles I and II except that the freezing duration was increased to 40 min (about 10 min ice proceeding and 30 min stabilization).

A. Direct observation of ice crystal spreading in the microfluidic channel

The TEC chip cooler was placed right on top of the beginning reservoir, as shown in Fig. 1, and one-dimensional freezing process was started. In our observations, the ice crystals were usually sharp and advancing fast when they were not far away from the cooler, such that the cooling rate (here defined as temperature drop per unit time) was high, as shown in Fig. 2(a) (around 2000 μm away from the beginning reservoir). As the ice front was further away from the cooler, the spreading speed declined, and the ice crystals and ice front became smoother, as shown in Fig. 2(b). Figure 2(b) is also the image when the ice front came to stabilize. The stabilized ice front was recorded and marked in the following figures. In our experiment, the ice front usually got stabilized when it was about 4100 μm away from the beginning reservoir. It is worth noting

FIG. 3. Phase contrast and fluorescent images of cells (a) before and [(b) and (c)] after 15 min freezing (region located 1600–2000 μm from the beginning reservoir).

that the stabilized position of the ice front was not absolutely stable. Slow oscillations, at most $\pm 200 \mu\text{m}$, could occur within the stabilization period of 5 min. Within the error due to the oscillations, the ice front was maintained almost the same position for all the experiments for comparison. Future work is needed to reduce the oscillations. It is also worth noting that there were evidences indicating that the ice near the ice front might not fill up the whole channel section, therefore some cells on the slide might not really be in ice even though the ice front was seen spreading over. Further work has to be done to give accurate measurement of ice shape in the microchannel.

B. Distribution of cell viability after the first freeze-thaw cycle

The physiological state of the CaSki cells after the first freeze-thaw cycle (cycle I) was tested by fluorescent labeling. After freezing for 15 min (10 min ice proceeding and 5 min stabilization), the chip was thawed in the safety cabinet at room temperature for around 5 min before testing. The green fluorescence was emitted from the membrane where Annexin V-FITC entered and conjugated with PS on the inner side of the membrane. The red fluorescence was emitted from nucleus when the membrane was not intact and PI entered.

Figure 3 showed the cells located about 1600–2000 μm from the reservoir, i.e., the region in white dash box in (a). Figures 3(b) and 3(c) are the same regions after the freezing treatment. After 15 min freezing, significant cell shrinkages were observed by comparing phase contrast images, Figs. 3(a) and 3(b). Cells with severe membrane rupture were identified by the strong red fluorescence from the nucleus since PI could not enter the intact cells.

Further downstream, Figs. 4(a) and 4(b), are a phase contrast and a fluorescent mapping of the cells along the channel, respectively. (Due to the confinement of the microscopy view field, a seamless connection for images has been conducted.) Cells located upstream (before 2000 μm mark on the ruler) were all dead which was not shown here. It is seen that less cells were stained in the downstream direction which indicated that more cells survived. A gray dotted line is used to

FIG. 4. The mapping of (a) phase contrast images and (b) fluorescent images along the channel after freezing for 15 min and thawing for 5 min (cycle I, region located between 2000 and 4100 μm). Upstream cells were all dead so the images were not shown (within 2000 μm). The red dotted line represented the death boundary where dead cells only occurred in the upstream of the line. The gray dotted line was the stabilized ice front. The region between two lines was defined as zero-loss region as no cells were dead in this position. The arrows in (a) indicated the position of stabilized ice front by comparing with Fig. 2(b).

mark the location of the ice front after stabilization. One can compare Fig. 4(a) with Fig. 2(b) to get the information about the stabilized ice front. On the left of the gray dotted line, ice crystals spread over the channel when stationary state was achieved.

A red dotted line is marked on the nearest necrotic cell in the upstream of the stabilized ice front. Cells in the downstream of this line were seen mostly alive. From the fluorescent image it is observed that between the two lines (red line and gray line) there was a region without any necrotic death, despite that ice crystals had spread over. We temporarily defined the position of the red dot line, where the last necrotic dead cell was located, as death boundary, and the region between it and the stabilized ice front as “zero-loss region.” To be noted, living cells undergo apoptosis even without any inciting events. In comparing experiments without freezing, there existed cells that were stained with green fluorescence, but very few emitted both green and red. So we defined the death boundary by the location of the last necrotic cell along the channel.

Near the death boundary, apoptotic cell death was proposed and discussed in a few literatures.^{18,24,25} Apoptotic death might not occur during or soon after the freezing treatment, but after some time it could be detected. Figures 5(a) and 5(b) are the same cells located 1300 μm upstream of the death boundary measured after 10 min in safety cabinet and 120 min in CO_2 incubator, respectively. The apoptotic cells were marked by short arrows. In Fig. 5(a), the marked cells were at the beginning of the apoptosis. Their membranes were intact and only emitted weak green fluorescence by Annexin V-FITC. PI could not cross the intact membrane so the nucleus was not labeled. After incubation for 120 min, as shown in Fig. 5(b), both membrane and nucleus of marked cells were labeled which confirmed the apoptotic death. On the contrary, no obvious change was recorded in the zero-loss region as a comparison of Figs. 5(c) and 5(d), which means there is no obvious apoptosis occurring. To test the apoptosis of cells without freezing, cells without freezing treatment were labeled after incubation of 10 and 120 min, respectively, and the results were almost the same. It confirmed that this fast apoptosis was due to the freezing treatment rather than the 120 min of incubation.

C. Distribution of cell viability after more freezing cycles and longer freezing duration

The repetition of freeze-thaw cycle and duration of freezing have been shown to produce a larger area of necrosis in an animal model.^{26,27} To test the effects of more freeze cycles and longer duration, we performed another two freeze-thaw cycles (named cycles II and III) based on cycle I. The stabilized ice front has always remained at about the same location as cycle I, with a deviation of at most 200 μm .

FIG. 5. The fluorescence images of the cells after incubation for different period after the freeze-thaw cycle I on the same chip. (a) The upstream ($\sim 1300 \mu\text{m}$ away from death boundary) of the death boundary after 10 min incubation. (b) The same position as (a) after incubation for 120 min. The white arrow indicated the apoptotic cells. Marked cells in (a) were primarily apoptotic as only the membrane was fluorescently labeled. In (b) both nucleus and membrane were labeled for the same marked cells which illustrated that the late apoptosis occurred after incubation. (c) Near the death boundary after 10 min incubation. (d) Near the death boundary after 120 min incubation.

After one more freeze-thaw cycle, by comparing cycle II in Fig. 6(a) with cycle I in Fig. 4, it is seen that the death boundary and the zero-loss region were almost unchanged, except that there is one more death which made the death boundary put downstream by about $100 \mu\text{m}$. After further one more cycle, with freezing duration extended to 40 min (10 min ice proceeding and 30 min stabilization), by comparing cycle III and cycle II, it is seen that there was not any change in zero-loss region. The results implied that more cycles and duration time did not have significant

FIG. 6. The overlay of fluorescence and phase contrast images of the cells for different cooling duration: (a) Duration 15 min for cycle II and (b) duration 40 min for cycle III.

FIG. 7. Full view of cell viability along the whole channel (0–4100 μm) after cycle III.

influence on the zero-loss region. On the contrary, for the region upstream out of the zero-loss region, as the arrow marked in Fig. 6, it is seen that more cells were damaged in cycle III than cycle II.

After cycle III, a complete mapping of the cell viability along the whole channel was shown in Fig. 7. The trend of cell viability from upstream to downstream and the partitions by the boundaries can be easily captured.

IV. CONCLUSION

In this study a series of directional freezing processes and cell responses in a microchannel was experimentally investigated. The results showed the trend of cell viability from the upstream to downstream, and implied that there was a narrow zero-loss region near the ice front, in which the cells were not killed by the ice. More freeze-thaw cycles and longer duration time did not have significant impact on the zero-loss region, as long as the freezing process was kept the same and the ice front remained at the same location. On the contrary, in the upstream out of the zero-loss region, more damages were observed. Future work will focus on the mechanisms of the slow freezing on cell injury (near the ice front) and controlling the area around zero-loss region by optimizing the freeze-thaw techniques. The three-dimensional shape of the ice front in the microchannel still needs more accurate measurement to help understand the effects of the ice on the cells.

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