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On-chip Incubation System for Long-term Microfluidic Cell Culture

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Abstract—**We demonstrate the use of a microfluidic cell culture chip with Braille pin-driven pumping, capable of on-chip CO_2 incubation that does not require an external chamber or gas supply. The proposed chip consists of a poly(dimethylsiloxane)(PDMS)-made microfluidic chip, flip-mounted on a glass slide, that contains a nested pair of cell culture media reservoirs and water-jacket, insulated by a permeable PDMS wall. By using 0.8 M sodium bicarbonate with 65 mM sodium carbonate as the water-jacket and placing on a 37 °C surface, the chip maintained osmolality shift and the pCO_2 in the media reservoir stabilized within < 3 mmol/kg and 5.0% ± 0.2% over at least 24 hours. The incubation capabilities were demonstrated through microfluidic culture of CV-1 epithelial cells under an inverted microscope for at least 12 days.**

I. Introduction

Miniaturization and automation of whole cell culture systems at low cost give a new opportunity for non-expert practitioners to use cell-based assay for medical diagnoses and environmental monitoring on site without cell culture labs and expertise on cell culture. Cell culture in microfluidic systems does not only provide solutions for the miniaturization leading to a reduction of cell volume and culture media to incubated during culture, but also offers promising technology to mimic *in vivo* environment [1]. However, many microfluidic cell culture devices [2-6] that have been developed so far still require at least one the following: gas-phase environmental control in principle unchanged from CO_2 incubators [7], continuous perfusion that requires non-micro scale amount of media, and large and complex peripheral control of microfluids. The fact shows that miniaturizing a system that maintains physicochemical properties of cell culture media such as pH is more difficult than the miniaturization of cell culture itself. Although gas exchange through poly(dimethylsiloxane) (PDMS) enables control of gas concentration for cell culture[8] and microorganism cultures[9], the need of flow-through gas phase CO_2 may compromise the portability and the cost due to the weight and safety issue of gas reservoirs.

We have developed a low-cost microfluidic chip with nested reservoirs partitioned with PDMS, enabling heat-activated CO_2 incubation functionality that is fully enclosed in the chip. The osmolality and the pH of the cell culture medium in the inner reservoir are maintained at physiological levels when an aqueous solution with bicarbonate source in the outer reservoir is heated to activate the mass diffusion of CO_2 and moisture into the medium through the PDMS partition.

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II. Methodology

A. Microfluidic Channel Features

Fig.1A shows the microchannels contained in the microfluidic chip. A cover glass (substrate) coated with 30-μm SU-8 3035 photoresist was first exposed to collimated UV (365nm) from the photoresist side, and then diffused UV from a transilluminator to the glass side through two pre-aligned photoplotted films. The feature patterned with the exposure from the transilluminator formed the rounded sidewall that could be pumped[10], while rectangular-sidewall channels and alignment marks were patterned with the collimated i-line. Another SU-8 was spun and patterned to form two in-channel cell culture wells (W300μm × L600μm × H100μm). PDMS (Shin-etsu KE106) prepolymer was poured into the substrate placed in a container to make a 1-mm PDMS layer, cured at 110 °C in an oven, released, and cut to make a channel feature layer.

B. Microfluidic chip

Fig.1B shows a fabricated microfluidic chip, and Fig.1C the construction processes. A 400μm-thick membrane of PDMS was fabricated by spinning the prepolymer on an silicon wafer. Four holes for the inlets and outlets of the microchannels were punched in the membrane. A PDMS tube (10mm-ID, 12mm-OD, 10mm-height) was formed by conventional casting .The channel layer, the punched PDMS membrane, and the PDMS tube were exposed to air plasma and immediately bonded together PDMS layers were bonded to a glass slide by air plasma. The PDMS tube defined the sidewall of the inner reservoir. Then plastic parts (outer reservoir, inner reservoir insert with an o-ring, and fingerplates) were bonded to the chip with silicone adhesive. As shown in Fig.1C, all components were placed on the topside of the chip to enable easy optical access from the bottom using an inverted microscope. The whole chip was cured at room temperature for 24h. The inner chamber could be sealed by fitting a Poly Crimp seal. A Braille cell (SC11,

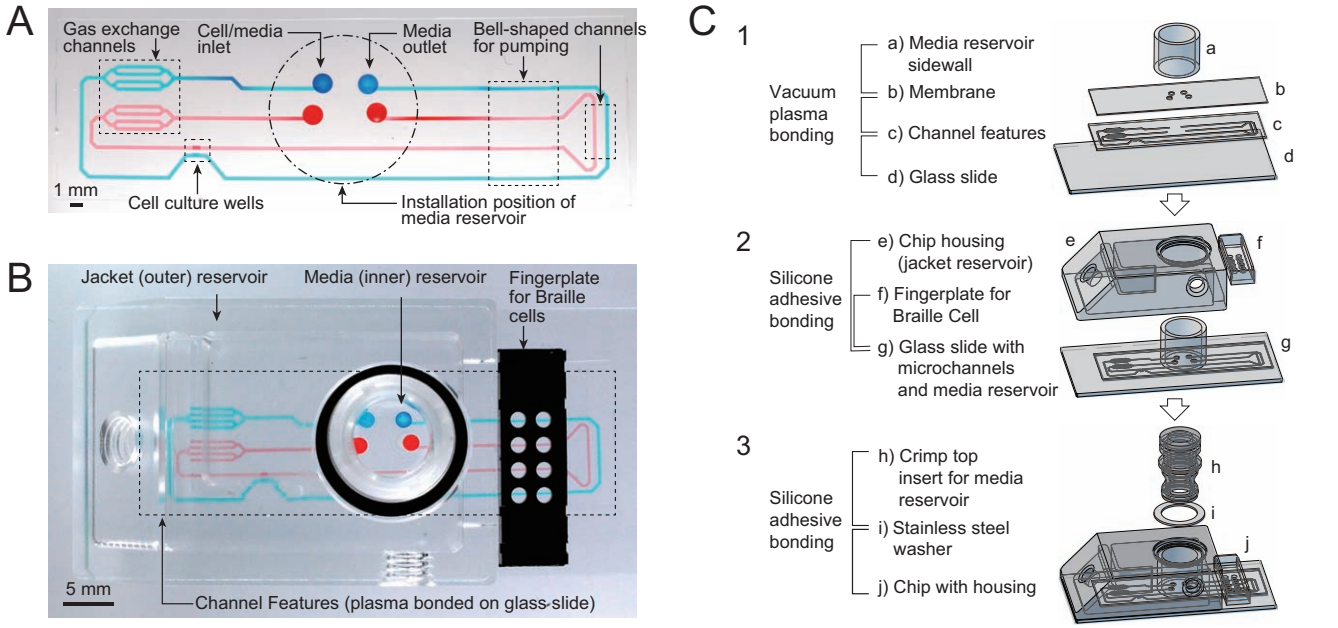

Fig.1. Microfluidic chip for on-chip CO_2 incubation. A) Layout of the microchannels. Displacing the fluid in the channel with Bell-shaped cross-section either by a Braille cell or a hand tool generates flow for cell seeding and media circulation. Channels between the inlets and the wells were designed to allow gas exchange and observation of two wells at once. B) Upper view of the chip. Each inlet/outlet is individually accessible after emptying the media reservoir. The jacket reservoir covers most segments of channels. A Braille cell is attached to the chip when operating. C) Assembly processes of the whole chip including channels and reservoirs. The crimp top insert(h) facilitates easy opening and closing the media reservoir.

KGS Corp.) was attached to the fingerplate to generate peristaltic flows by pin movement as previously described [2].

C. Osmolality / pCO_2 Evaluation

The jacket (outer) reservoir of the microfluidic chips was filled with 4.5 ml $NaHCO_3$ solution in deionized water supplemented with Na_2CO_3 for all experiments. The concentration of $NaHCO_3$ was determined to maximize the pH sensitivity against pCO_2[11]. 400 µl of 290 mmol/kg osmolality standard solution, 10 mM $NaHCO_3$ solution was filled to the media reservoir for evaluation of osmolality and pCO_2, respectively. The chip with filled reservoirs was incubated on a transparent hotplate set to 37°C. During the incubation each microfluidic loop was pumped with 4-stranded peristaltic action of the Braille pins. The refresh rate of the pins was 1 second.

The osmolality of the 10 µl solution sampled from the inner chamber were measured by an osmometer after cooling the solution to room temperature and opening the septum with a dermal punch. The pH and temperature in the inner chamber were directly measured by inserting the pH electrode in the media reservoir. The pCO_2 of the solution was calculated from the pH using the Henderson-Hasselbalch equation[11]:

$$pCO_2 = A_{Na} / (K_H \bullet 10^{\,pH\,-\,pK1}),$$

where A_{Na} is the concentration of $NaHCO_3$, K_H the Henry's law constant for CO_2 (gas phase) in water, and pK_1 the first composite acidic constant of carbonic acid (H_2CO_3). The values of K_H and pK_1 were obtained from the temperature-dependent empirical expressions described elsewhere[12].

D. Cell culture

African green monkey kidney (CV-1) cells were maintained in Dulbecco Modified Eagle Medium supplemented with 5% fetal bovine serum. The microfluidic channels in the chip were treated with 100 µg/ml fibronectin solution at 37°C for 16h prior to seeding. Cells were trypsinized and resuspended in fresh medium at 10^5 cells/ml. 0.2-µl drops of suspension on the holes were placed at the bottom of the media reservoir. The seeded cells were transferred into the cell culture wells using a peristaltic action by the Braille cell. The jacket reservoir was filled with $NaHCO_3$/ Na_2CO_3 solution, and the chip was incubated the same way as osmolality/pCO_2 evaluation. For microscopy, the chip with the Braille cell attached was removed from the hotplate for up to 10 minutes.

III. Results and Discussions

A. Osmolality

Osmolality of the standard solution (290 mmol/kg) that was incubated on-chip at 37°C for 24h is shown in Fig.2 (white bars). A conventional PDMS microfluidic chip ("No Jacket") could not maintain the osmolality within the physiological levels, even for one day. This was because of high vapor permeability of PDMS and the evaporation rate increased by higher temperature. Meanwhile, when approximately 2/3 of the PDMS surface of chip was water-jacketed ("With Jacket"),

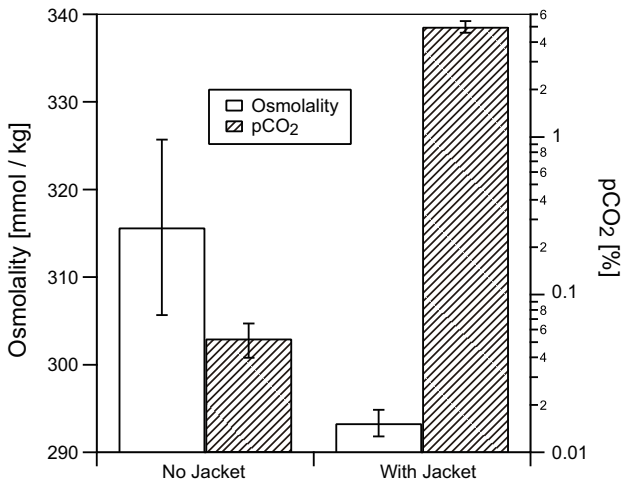

Fig.2. Changes in osmolality of 290 mmol/kg standard solution in the media reservoir and pCO_2 in the media reservoir after incubation on-chip at 37 °C for 24 h ($N = 3$; mean ± *SD*). Comparisons were made in the presence of the water jacket (i.e. "No Jacket" or "With Jacket").

the rise in osmolality was limited within physiological levels. Since a Braille-driven microfluidic cell culture chip requires both low cytotoxicity and high elasticity, PDMS is still the best material for the chip despite its high permeability. The on-chip crimp-top media reservoir overcame the permeability of PDMS, and promises to be a simple and effective method to support both cell culture and displacement pumping by external Braille devices.

B. Partial CO_2 pressure (pCO_2)

Fig.2 also shows the pCO_2 in the media reservoir after incubation at 37°C for 24h. The pCO_2 of a conventional PDMS chip ("No Jacket") after 1-day incubation was close to atmospheric levels (~0.03%) due to the high CO_2 permeability of PDMS. Covering the media reservoir with $NaHCO_3$ solution ("With Jacket") maintained the pCO_2 level close to 5%, which is needed to prevent media from pH rise.

Fig.3 shows the pCO_2 controlled by the concentrations of $NaHCO_3$ and Na_2CO_3 in the water jacket. When solutions that contained only $NaHCO_3$ were used for the water jacket, the pCO_2 overshot the desired level in approximately 12 h, then fell. This overshooting of pCO_2 can be suppressed by adding Na_2CO_3. The formation of CO_3^{2-} ions effectively buffers the thermal decomposition of HCO_3-, which eventually generates gas-phase CO_2[13]. Fig.3A shows the result of weak buffering by 10 mM Na_2CO_3, a minimum buffering that can suppress the overshoot of pCO_2. This result showed that about 0.6 M or higher concentrations of $NaHCO_3$ are needed to maintain the pCO_2 around 5%, without overshooting. Fig.3B shows the stable pCO_2 levels that depend on buffering with Na_2CO_3. This result shows that the pCO_2 of the inner reservoir can be controlled to a specified level within 4 - 7 % using 0.8M $NaHCO_3$ and Na_2CO_3 adjusted within 20 - 80 mM. The result suggests that the

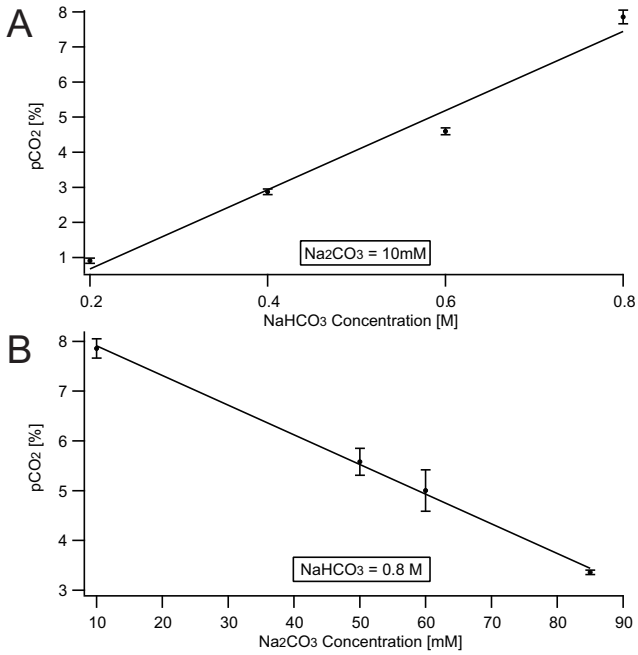

Fig.3. pCO_2 in the media reservoir water-jacketed with solutions of various formulations. A) Effect of $NaHCO_3$ in the water jacket as a source of CO_2 ($N = 3$; mean ± *SD*). The jacket was buffered with 10 mM Na_2CO_3. B) Effect of Na_2CO_3 buffering ($N = 3$; mean ± *SD*). The concentration of $NaHCO_3$ was fixed to 0.8 M.

amount of the reagents for a desired pCO_2 level within 1 - 10% is predictable from fitted curves in Fig.3. Since long-term drifting of pCO_2 due to water-jacketing was small enough compared to the buffering capacity of conventional cell culture media, the water jacket formulation determined from Fig.3 maintained the media within physiological levels for longer than one day. However, to allow pH drops due to proliferated cells on chip, the target pCO_2 level for the formulation prediction can be adjusted to slightly (e.g. 0.2%) lower level than the conventionally adopted one.

C. Long-term cell culture

Fig.4 shows the time-lapse recording of CV-1 cells incubated on-chip in the atmosphere. The pCO_2 level predicted from Fig.3B was intentionally adjusted to about 4.8%. The seeded cells attached to the well proliferated normally with a doubling time of less than 48h. In Day 4, the cells in the channels looked healthy, with good contact-inhibition. The cells proliferated toward upstream (i.e. left to right). Some cells climbed the 100μm-height walls of the well and migrated to the 30μm-height microchannel. The cells growing upstream were similar to the result demonstrated previously with Braille microfluidics [2]. The cells that migrated outside the well had a similar morphology as the cells grown in the wells. We also found, by comparing the micrographs between time frames, that these cells were migrating on the surface of the well. In Day 8, the cells reached confluent, and some cells formed colonies,

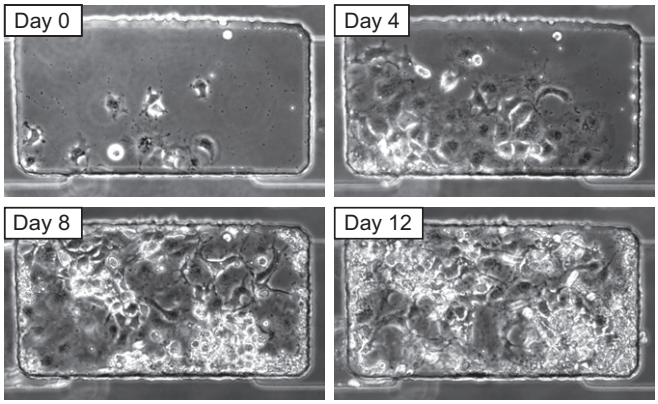

Fig.4. Time-lapse recording of CV-1 cells cultured into the well placed in the microfluidic channel. The water jacket contained 0.8 M $NaHCO_3$ and 65 mM Na_2CO_3; about 4.8% CO_2 (see Fig.3B). The size of the well (the rectangular outline) is 600μm × 300μm.

which are less contact inhibited than that of CV-1 cultures using conventional methods. The partial loss of contact inhibition can be explained by recirculation of the medium in the microfluidic channels. Since the medium in the channels has high velocity for its low volume flow rate, the medium around the cells is quickly exchanged even when the effect of conditioning the medium persists. The fact suggests that cell cultures under microfluidic recirculation will require some adjustments of media supplements (such as lower serum or growth factors) from the established conventional formulations. In Day 12, detached cells were present while the cells at the bottom were still alive. Then the medium in the chip was so acidic and depleted that we had to replace the whole medium of the media reservoir to fresh one to continue the culture. At this stage, the cells proliferated on the channel and on the bottom of the media reservoir seem to have contributed to the depletion rather than conditioning. Therefore, unlike one-way perfusion systems, cells reside upstream of wells would be rather unnecessary for cell culture with medium recirculation.

The long-term culture of CV-1 cells with on-chip incubation, shown in Fig.4, indicates that the proposed chip can incubate cell culture medium at physiological pH and osmolality for at least 12 days. In addition, the cell culture wells connected in-line to the microfluidic channels traps the cells in suspension at the bottom, but they did not hinder proliferation or migration of the cells inside. In addition, the proposed on-chip CO_2 incubation system maintains a larger volume of cell culture media in a more stable condition and for a longer duration than the microfluidic CO_2 incubator, which consists of a microheater and microsized bicarbonate chambers[14]. This is because the proposed incubation system is almost hermetically sealed, and provides CO_2 and humidity to cell culture media inside the chip both through the walls of whole PDMS-made microfluidic channels and through the macrofluidic media reservoir.

IV. Conclusion

We successfully achieved CO_2 incubation using the on-chip $NaHCO_3/Na_2CO_3$ solution-jacketing of PDMS-made airtight-closable media reservoir and partially covering a Braille-driven PDMS-membrane microfluidic chip. Braille pin-pumping and time-lapse recording were also achieved without compromising portability and imaging setups. In combination with the Braille-based integrated microfluidic platform, this chip configuration could be used to design portable microfluidic devices that maintain not only animal cells, but also other materials that are sensitive to physicochemical conditions over a long period.

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