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RESEARCH PAPER

On-chip CO_2 incubation for pocket-sized microfluidic cell culture

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Abstract We have developed an on-chip CO_2 incubation system based on mass/heat transfer from aqueous solutions of bicarbonate source to cell culture media through a permeable poly(dimethylsiloxane) (PDMS) wall. Heating a carbonate-buffered bicarbonate solution successfully regulated CO_2 generation without any feedback control. Because a microfluidic cell culture chip with the incubation system does not require an external chamber or gas supply, the entire microfluidic cell culture setup becomes pocket sized. Using 5 ml of 0.8 M sodium bicarbonate with 65 mM sodium carbonate as the water jacket, the chip maintained the temperature, osmolality, and pH of 750 µl cell culture medium within physiological levels when the chip was placed on a 37°C surface. The osmolality shift and pCO_2 of the media reservoir stabilized within <5 mmol/kg and $5.0 \pm 1.0\%$ over at least 9 days. The incubation capabilities were demonstrated through microfluidic culture of COS-7 epithelial cells under an inverted microscope for 17 days.

Keywords Cell culture · Braille device · Microfluidic · On-chip CO_2 incubation

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1 Introduction

Miniaturization of cell culture systems that maintains the temperature, pH, and tonicity of cell culture medium in a physiological range (i.e., mimicking serum) offers multiple advantages that have been under-explored to date. If cells were shippable in a small box containing a life support system for cell viability, the end user both on the site and at a central lab would receive the cells ready-to-use, thereby saving time, as experiments could be performed immediately. This means that pocket-sized cell culture systems could offer alternative solutions to medical diagnosis, combining advantages of quick turnaround time of on-site sampling and various analyses provided by central labs. This on-chip cell culture system could also be applied to environmental monitoring. It could be a promising tool to detect an unlimited range of noxious substances on-site without relying on experimental animals.

Culture of cells in microfluidic devices is an effective approach to minimize the size of cell culture systems, thereby minimizing the volume of cells, reagents (e.g., cell culture media), etc. Many microfluidic devices successfully maintain cells over long-term periods within a small chip and reduced the amount of cell culture media, such as microfluidic perfusion systems with micro-wells (Korin et al. 2009; Kane et al. 2006), digital microfluidics-based complete cell culture systems (Barbulovic-Nad et al. 2010), or integrated cell culture chips with microsized-magnetic stirrers (Kimura et al. 2008). In addition, portable microfluidic systems using osmosis pumps (Xu et al. 2010), a refreshable Braille display (Gu et al. 2004), screw (Hulme et al. 2009), and solenoids (Addae-Mensah et al. 2010; Hulme et al. 2009) can be successfully used for handheld cell culture. However, these devices still require a CO_2 incubator, a humidified incubator that supplies

gas-phase CO_2 to balance carbonate–bicarbonate buffering system (Ham and Puck 1962) to maintain the cell culture media in physiological conditions. Providing gas-phase CO_2 through microchannels (Polinkovsky et al. 2009; Forry and Locascio 2011) still requires a gas supply, which may compromise the portability and cost because of weight and safety issues. Blending cell culture media to improve the buffering capacity (Futai et al. 2006) is often undesirable, as it alters well-established protocols and is not applicable to cell cultures using proprietary media.

We have developed a portable microfluidic system that allows cell culture in the atmosphere, and thereby cell imaging using conventional microscopes. The system consists of a microfluidic chip (size: 3 × 5 × 2 cm) containing poly(dimethylsiloxane) (PDMS) microfluidic channels, an 8-pin Braille cell, and a 37°C hotplate. To prevent the evaporative loss of cell culture media due to the permeability of PDMS (Heo et al. 2007), we covered the PDMS channels with an outer reservoir, or "jacket reservoir". We found that the water molecules in the jacket reservoir were diffused through the PDMS wall into the cell culture medium. These water molecules prevent the shift of the media osmolality, a measurable quantity related to the tonicity (Waymouth 1970). We also found that by filling the jacket reservoir with a bicarbonate/carbonate buffer solution at 37°C successfully maintains the pH of standard cell culture media at physiological levels without any external CO_2 supply or feedback control.

2 Materials and methods

2.1 Microfluidic channels

Figure 1a shows a top view of the microfluidic channels installed in the chip. The chip has two PDMS microfluidic loops in which cell culture medium circulates under the peristaltic actions of Braille pins. Four Braille pins positioned on each microfluidic loop went through the following six states at constant intervals: ●○○○ → ●●○○ → ○●●○ → ○○●● → ○○○● → ●○○●, where ● is an actuated pin (i.e., channel occluded above the pin); ○ retracted pin (i.e., channel open), to pump the fluid toward the right. The refresh rate (duration of each state of pins) was ranged from 0.2 to 1 s to control the flow rate. The spacing of long sides of the channel was set to 2.4 mm, the pitch of the pins that constitutes a Braille character. Each loop has two 1.5-mm diameter inlet/outlet holes connected to the media reservoir, two segments that have a bell-shaped cross-section suitable for occlusion by Braille pins, and one 1-mm diameter in-line cell culture well into which cells settle by gravity. The distance between the cell culture well and the edge of the media reservoir (shown as a dashed line circle in Fig. 1a) was longer than 9 mm to avoid distortion of illumination from a condenser through the chip. The required distance (9 mm) was obtained from the equation $h \times n_{max} \times NA_{max}$, where h is the height of the chip housing (12 mm), n_{max} the maximum refractive indices of materials used (~1.5), and NA_{max} the maximum numerical aperture of commonly used condensers (~0.5). As shown in Fig. 1c, we performed simulations of the movement of cells entering the well and the flow velocity at the well bottom, when the maximum inflow velocity generated by a Braille pin was continuously exerted. The simulation results confirmed that the size of the well is appropriate for cells to settle under Braille pumping. The results of particle tracing showed that the cells travel approximately 200 μm forward in 1 s and sank to a distance of 50–100 μm from the bottom, with only 1–2 min required for complete settling. Flow velocity in the well is slow enough (ca. 20 μm/s max. at the bottom) to keep cells on the bottom of the well.

To fabricate a mold for the channel features, a 30-μm thick layer of negative photoresist (SU-8 3035, Nippon Kayaku, Tokyo, Japan) was first formed on a dry cover glass (C050701, Matsunami, Osaka, Japan) by spin-coating at 4,500 rpm. To pattern channels with rectangular cross-sections, the photoresist layer was exposed to 300 mJ/cm^2 collimated 365 nm UV through a 12,700 dpi photoplotted film (Unno Giken, Tokyo). Then, to pattern channels with bell-shaped cross sections, the photoresist layer was exposed to 120 mJ/cm^2 diffused 365 nm UV through a photoplotted film and a cover glass (i.e., from the back surface). A transilluminator (TFL-40V, UVP, Upland, CA, USA) was used for exposure to diffused UV. Release agent (HD-1100, Harves, Saitama, Japan) was then spun at 3,000 rpm on the surface that included the patterned photoresist. The mold was washed with deionized water to remove excess release agent.

2.2 Microfluidic chip

Figure 1b is a top view of the microfluidic chip. The PDMS part shown in Fig. 1a was covered with a transparent plastic housing on the top and a cover glass at the bottom. The space defined by the housing and the cover glass creates the jacket reservoir. The PDMS tube placed inside of the plastic housing and a plastic insert with a screw top create the media reservoir. The plastic insert was then bonded to both the plastic housing and the PDMS tube to ensure easy access to the media reservoir and no leakage of liquid. Finally, a removable fingerplate of a Braille cell was fixed on the exposed region of the PDMS. The cells were cultured on the cover glass at the bottom of the chip and observed from the bottom, while the Braille cell is attached to the top surface of the chip.

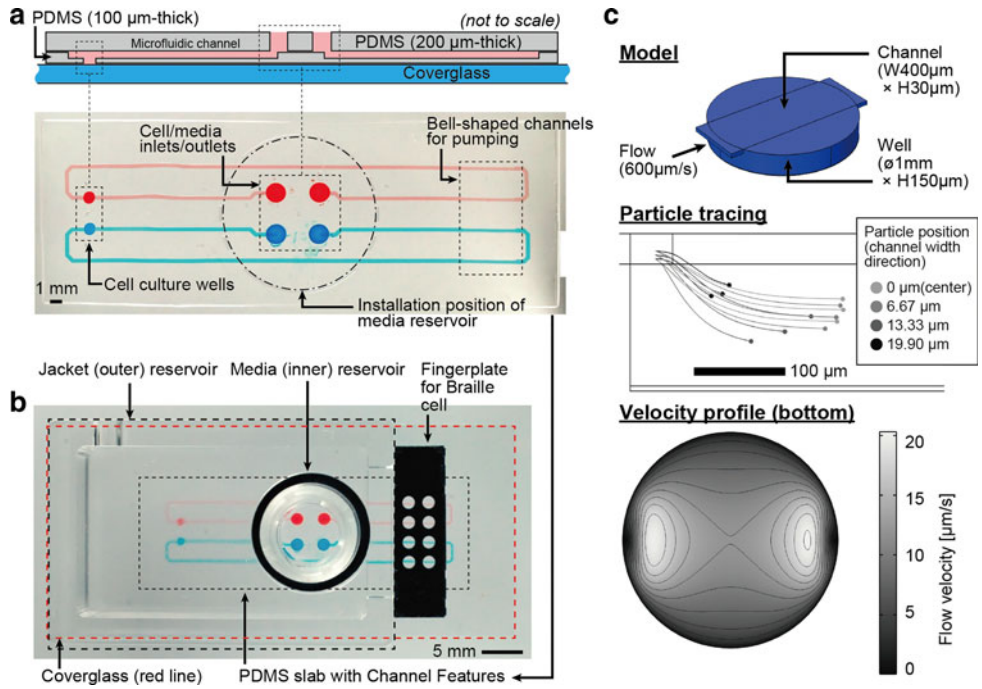

Fig. 1 Microfluidic chip enabling on-chip incubation and Braille pumping on-stage. **a** The upper view and the schematic cross-sectional view of a PDMS microfluidic channels installed on the chip. Two microfluidic loops have the same functionality of cell culture. The cell culture wells and the inlet/outlet holes were formed by punching in a PDMS channel feature and membrane, respectively, with a hole-puncher (see Fig. 2, Step 1). Features at the right with "bell-shaped" cross sections are needed for pumping by Braille pins. **b** Image of the fabricated chips with and without the water-jacket reservoir. A Braille cell is fit onto the fingerplate when operating. **c** Simulation of the movement of cells entering the cell culture well (particle tracing) and flow velocity profiles at 1 μm above the bottom of the well. The velocity at the inlet was set to 600 μm/s. In "particle tracing", 3 particles each were released at the center of the channel in the direction perpendicular to this paper, 6.67 μm away the center, 13.33 μm from the center, and 19.90 μm (near the edge of the channel)

Figure 2 illustrates the fabrication steps of the chip. In Step 1, PDMS parts (a–c) and the washed cover glass (d) were bonded immediately after vacuum plasma treatment (SC-708, Sanyu Electron, Tokyo; Conditions: 20 mA, 20 Pa, 30 s). All PDMS parts were formed from a PDMS prepolymer (KE-106, Shin-etsu, Tokyo) and cured at 110°C in an oven. The sidewall of the media reservoir (a) was a 10-mm ID, 12-mm OD, 10-mm height PDMS tube formed by conventional casting of the prepolymer. A 200-μm thick membrane (b) was fabricated by spinning the prepolymer on an untreated silicon wafer at 500 rpm for 30 s. The cured membrane was peeled off the wafer, transferred to a polyethylene bag, and four 2-mm diameter holes were punched using a biopsy punch. A 150-μm thick layer with the channel features (c) was fabricated by spin-coating the prepolymer on the mold. Two 1-mm diameter holes, as the cell culture wells, were then punched on the channel layer. A cover glass (d) (26 mm × 54 mm, Matsunami) was washed prior to plasma bonding by immersion in 1% v/v detergent (TMSC, Tama Chemicals, Kanagawa, Japan) in deionized water at 65°C for 10 min.

As shown in the Step 2 of Fig. 2, a poly(methylmethacrylate) (PMMA) chip housing (e) and a fingerplate of a Braille cell were bonded with silicone adhesive (AX-041, Cemedine, Tokyo) to the glass surface of the chip (g). The chip housing was fabricated by conventional machining of a cast PMMA. Cast PMMA was selected due to its low birefringence to enable differential interference contrast (DIC) microscopy of the chip. In addition, the roughness of the inner surfaces of the chip housing was lowered by solvent vapor polishing to obtain good DIC or phase-contrast images.

Step 3 in Fig. 2 shows the process for adding a re-sealable media reservoir. A poly(methylpentene) (PMP) screw-top insert (h) and a metal washer (i) were inserted into the media reservoir and bonded together with silicone adhesive. The entire chip was cured at room temperature for 24 h. The screw-top insert was fabricated by additional machining on a PMP screw-top vial (95096, Grace, Deerfield, IL, USA). The media reservoir was capped with an open-hole cap with PTFE/Silicone septa (95324, Grace, IL, USA). Finally, a Braille cell (SC11, KGS, Saitama, Japan)

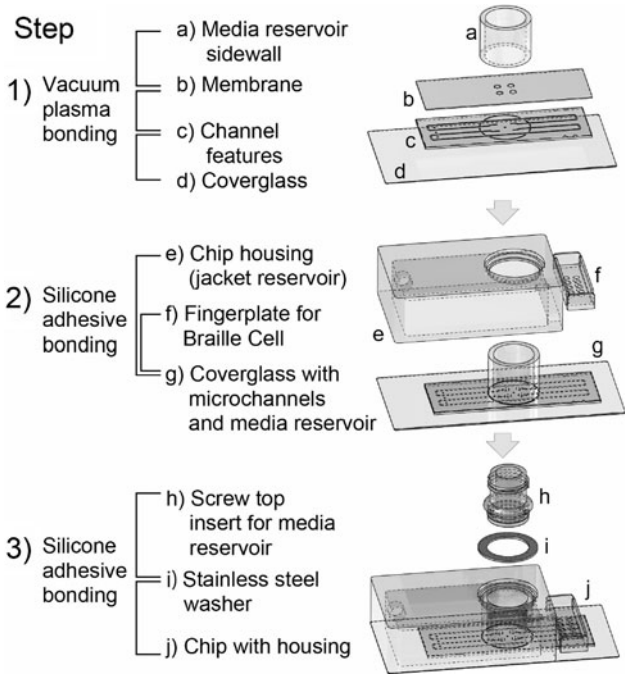

Fig. 2 Construction of the microfluidic chip. Parts *a*, *b* and *c* were fabricated by casting of PDMS prepolymer. Other parts were fabricated by conventional machining or used as provided. The assembly requires plasma bonding (Step 1) and conventional gluing processes using silicone adhesive (Steps 2 and 3)

was installed onto the fingerplate attached to the exposed microfluidic channels.

2.3 Evaluation of osmolality

To evaluate osmolality, the media reservoir was filled with 750 µl of 290 mmol/kg osmolality standard solution (OA-029, Wescor, Logan, UT, USA). The jacket reservoir was filled with 5 ml $NaHCO_3$/Na_2CO_3 solution in deionized water. The solution was injected into the jacket reservoir through a hole at the side of the chip housing. The hole was then covered with polyimide tape. The chip was then placed on a hotplate (MATS-55SF, Tokai Hit, Shizuoka, Japan) set at 37°C. Each microfluidic loop was pumped simultaneously with the four-stranded peristaltic action of Braille pins. The refresh rate was 1 s. To measure the osmolality of the solution in the media reservoir, the chip was removed from the hotplate and cooled to room temperature. Then, 10 µl of solution from the media reservoir was sampled by pipetting and the osmolality was measured using a vapor pressure-type osmometer (5100C, Wescor).

2.4 Evaluation of partial CO_2 pressure (pCO_2)

We calculated the pCO_2 value of the media reservoir from the measured pH value of a dilute bicarbonate solution, a method founded on the pCO_2 electrode (Severinghaus and Bradley 1958). The media reservoir was filled with 750 µl of 10 mM $NaHCO_3$ in deionized water. The concentration of $NaHCO_3$ was set to 10 mM to maximize the pH sensitivity against pCO_2 (Severinghaus and Bradley 1958). The jacket reservoir was filled with 5 ml solution of $NaHCO_3$ and Na_2CO_3 in deionized water. $NaHCO_3$ is a source of CO_2 as the thermal decomposition of HCO_3^- ions from $NaHCO_3$ generates gas-phase CO_2. Na_2CO_3 was added because the pCO_2 level can be adjusted by changing the concentration of Na_2CO_3; the formation of CO_3^{2-} ions from Na_2CO_3 effectively buffers the thermal decomposition of HCO_3^- (Filley and Kindig 1985). The chip was then incubated on a 37°C hotplate. The pH and temperature of the media reservoir were measured with a pH electrode (9669-10D, Horiba, Kyoto, Japan) inserted in the media reservoir through a hole punched in the septum. The gap between the electrode and the hole was sealed with paraffin film. The pCO_2 of the solution was then calculated from the measured pH by using the following equation:

$$\begin{aligned} \mathrm{pCO_2} &= \frac{c_0}{K_H} \\ c_0 &= \frac{C_\mathrm{T}}{1+\frac{K_1}{10^{-\mathrm{pH}}}+\frac{K_1K_2}{10^{-2\mathrm{pH}}}} \sim \frac{A_\mathrm{Na}}{\frac{K_1}{10^{-\mathrm{pH}}}+\frac{K_1K_2}{10^{-2\mathrm{pH}}}} \end{aligned} \quad (1)$$

where c_0 is the concentration of CO_2 in the solution ($[H_2CO_3^*]$), C_T the dissolved inorganic carbon ($[H_2CO_3^*] + [HCO_3^-] + [CO_3^{2-}]$), A_Na the concentration of total sodium ions (in the case of pCO_2 measurement, $A_\mathrm{Na} = 10$ mM: the concentration of $NaHCO_3$), K_H Henry's law constant for CO_2 (gas phase), and K_1 and K_2 the first and second composite dissociation constant of carbonic acid.

We estimated the pCO_2 value around the media reservoir, pCO_2(est), as a function of the concentration of Na_2CO_3, by (1) assuming that pCO_2 around the media reservoir is equal to the volume fraction of the absorbed gas-phase CO_2 in PDMS:

$$\frac{\mathrm{pCO_2(est)}}{1[\mathrm{atm}]} \sim \frac{V_{\mathrm{CO_2(inPDMS)}}}{V_\mathrm{PDMS}} = S \times \frac{c_{0(\text{in jacket solution})}}{K_\mathrm{H}} \quad (2)$$

where S is the solubility of CO_2 in PDMS, and (2) making the following assumptions for the C_T and pH in the jacket solution:

$$C_\mathrm{T} \sim A_{Na} = N + x, \quad 10^{-\mathrm{pH}} \sim \frac{K_2 N}{x} \quad (3)$$

where N and x is the concentration of $NaHCO_3$ and Na_2CO_3, respectively. Assigning (1) and (3) to (2) gives the following equation of the estimated pCO_2:

$$\mathrm{pCO_2(est)} = \frac{SK_2N^2}{K_\mathrm{H}K_1}\frac{1}{x} \quad (4)$$

The values of K_H, K_1, and K_2 used in (1) and (4) were calculated using temperature-dependent empirical expressions (Plummer and Busenberg 1982):

$$-\log K_1 \sim -356.3094 - 0.06091964T + \frac{21834.37}{T} + 126.8339 \log T - \frac{1684915}{T^2},$$

$$-\log K_2 \sim -108.3865 - 0.03252849T + \frac{5151.79}{T} + 38.92561 \log T - \frac{563713.9}{T^2},$$

$$\log K_H \sim 108.3865 + 0.01985076T - \frac{6919.53}{T} - 40.4515 \log T + \frac{669365}{T^2}.$$

where T is the temperature. The value of S used in (4) was 1.29 [cm^3(STP)/cm^3/atm] (Merkel et al. 2000).

2.5 Cell culture

To promote cell adhesion to the bottom of the cell culture wells, the cell culture wells and the microfluidic channels in the chip were filled with 100 μg/ml solution of human fibronectin (F5749, Sigma, St. Louis, MO, USA) and let it stand at 37°C for 16 h prior to cell seeding. African green monkey kidney cells (COS-7) were maintained in Dulbecco's modified Eagle's medium (DMEM; 12800, Invitrogen, Carlsbad, CA, USA) supplemented with 5% v/v fetal bovine serum (FBS; Moregate, QLD, Australia), 1% v/v penicillin/streptomycin (P1433, Sigma), and 3.7 g/l $NaHCO_3$ (S5761, Sigma). To seed cells into the chip, trypsinized cells were pelleted by centrifugation at 100×g and resuspended in fresh medium at a density of 10^6 cells/ml. 0.5-μl drops of suspension were placed on the inlets at the bottom of the media reservoir. The cells in the inlet were fed into the cell culture wells by the same four-stranded peristaltic action sequence. The refresh rate was 300 ms. The number of cells in the wells was monitored under an inverted microscope (DMIL LED, Leica, Wetzlar, Germany). We stopped the peristaltic action when the desired amount of cells reached the bottom of the culture wells. Then the cells remaining in the channel were pushed back to the inlet with some reversed peristaltic actions. The cells that remained at the bottom of the inlet (i.e., in the media reservoir) were flushed with medium. The typical time required to seed the cells was about 3 min. The required seeding time increases as the number of seeding cells increases. After seeding, the media reservoir was filled with medium, the jacket reservoir was filled with 5 ml 0.8 M $NaHCO_3$/65 mM Na_2CO_3 solution, and the chip was incubated on a 37°C hotplate. The solution in the jacket reservoir and the medium in the media reservoir were not exchanged during culture.

2.6 Imaging

For live-cell imaging, the chip with Braille cell was temporarily removed from the hotplate and placed on a plain stage of the inverted microscope. The microchannels in this system were easier to observe than the previous Braille-based system (Futai et al. 2006) because the new chip had a completely flat bottom surface.

Phase-contrast images were acquired using a CCD camera (Retiga-2000R, QImaging, BC, Canada). The duration of image acquisition was <10 min. Cell counting was performed on the phase-contrast images. Doubling time was calculated using a dedicated website (Roth 2006). To assess cell viability, the media reservoir was replaced with phosphate-buffered saline and then stained with LIVE/DEAD Viability/Cytotoxicity Kit (L-3224, Invitrogen) according to the manufacturer's instruction. A confocal laser-scanning microscope (FV10i-DOC, Olympus, Tokyo) was used to acquire fluorescent images.

2.7 Simulation

Both fluidic and thermal simulations were conducted using the COMSOL Multiphysics V4.2 with Microfluidics Module (COMSOL, Stockholm, Sweden). Preset material properties of air and water were used for the following simulations.

A fluidic simulation was performed to estimate the flow velocities at the bottom of the cell culture well. As shown in Fig. 1c, a three-dimensional solid model of a cell culture well with inlet/outlet was built as a union of a 1-mm diameter and 150-μm height cylinder (well) and a 1.1 mm × 400 μm × 30 μm block (channel). The boundary conditions at the end of the channel were uniform velocity (600 μm/s; determined by feeding microbeads into the channel with Braille pumping) at the inlet with no pressure at the outlet. The physical model used was incompressive laminar flow without inertial term (Stokes flow). A surface that lays 1 μm above the bottom of the cylinder was used as the dataset for plots of flow velocities. For particle tracing, a sedimentation velocity due to gravity was taken into account. The value of the sedimentation velocity used was 1.736 μm/s, calculated using an approximate relation: $v \sim r^2/4$ (Miller and Phillips 1969), and a typical value of detached cell radius: 5 μm.

A thermal simulation was performed to estimate the power requirement of heating element placed at the bottom of the chip, when the chip assembly was enclosed by a box made of expanded polystyrene. As shown in Fig. S2a, a three-dimensional model of a chip, a heating element, and the air in the box was built as a union of a 70 mm × 70 mm × 30 mm block (material: air) and a 50 mm × 26 mm × 12 mm block (material: water). For simplicity,

the water block represented a chip. The boundary condition at the air surface was inward heat flux with the external temperature of 10°C and the heat transfer coefficient of 2 W/(m^2 K), the quotient of 0.04 W/(m K) (thermal conductivity of expanded polystyrene), and 0.02 m (wall thickness). The bottom surface of the water block was set as a boundary heat source. The total boundary power that heats the heat source to 37.0°C was obtained by simple iteration of the solving processes.

3 Results and discussion

3.1 Osmolality

We examined the suppression of evaporation of the on-chip CO_2 incubation system. Figure 3 shows the osmolality shift of the osmolality standard solution (290 mmol/kg) placed in the media reservoir with and without on-chip incubation. A microfluidic chip with an empty jacket reservoir (i.e., without on-chip incubation) did not prevent evaporation. The osmolality shift was approximately 6 mmol/kg/day. The result shows that with an empty jacket reservoir, the allowed consistency of the osmolality of cell culture media, which requires the change of osmolality to remain within ±10 mmol/kg (Freshney 2005), cannot be achieved for more than a day. In contrast, when the jacket reservoir is filled with aqueous solution (0.8 M $NaHCO_3$), the rise in osmolality is suppressed to within about 1 mmol/kg/day.

These results indicate that the amount of water that diffuses through the PDMS was enough to prevent evaporation through the microfluidic channel features that are exposed to the atmosphere for pumping. The results suggest that it is not necessary to cover the exposed microfluidic channels. In fact, covering all PDMS surfaces with a CO_2 source might increase the risk of generating super-hypoxic conditions.

3.2 Partial CO_2 pressure (pCO_2)

We evaluated the relationship between formulations of water-jacket solutions and the pCO_2 level in the chip. Figure 4 shows the pCO_2 in the media reservoir incubated at 37°C for 3 days with jacket solutions of various Na_2CO_3 concentrations (the concentration of $NaHCO_3$, the main source of CO_2, was fixed to 0.8 M). The pCO_2 of the media reservoir was controlled to a specified level within 4–8%, an appropriate range for buffering standard cell culture media, using 0.8 M $NaHCO_3$ and Na_2CO_3 adjusted within

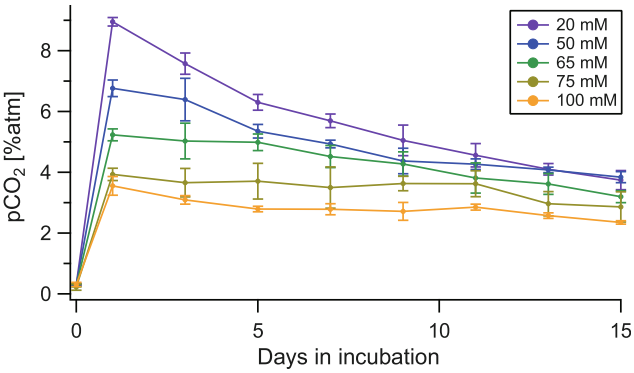

Fig. 4 Temporal change of partial CO_2 pressure (pCO_2) in the media reservoir of the chip, when the chip bottom was incubated at 37°C for 3 days ($N = 3$; mean ± SD). Comparisons were made between different concentrations of Na_2CO_3. For all cases, the initial pCO_2 was the atmospheric level, and the water jacket contained 0.8 M $NaHCO_3$

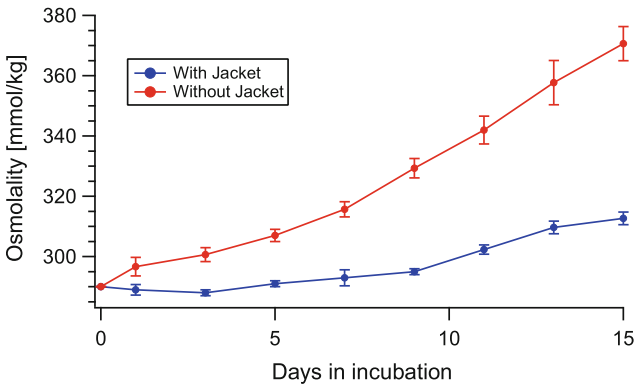

Fig. 3 Temporal change of the osmolality of the standard solution (290 mmol/kg) placed in the media reservoir of the microfluidic cell culture chip at day 0 while the chip bottom was incubated at 37°C for 15 days ($N = 3$; mean ± SD). Comparisons were made between the chip with water jacket (0.8 M $NaHCO_3$ + 65 mM Na_2CO_3) in the jacket reservoir and the chip with an empty jacket reservoir

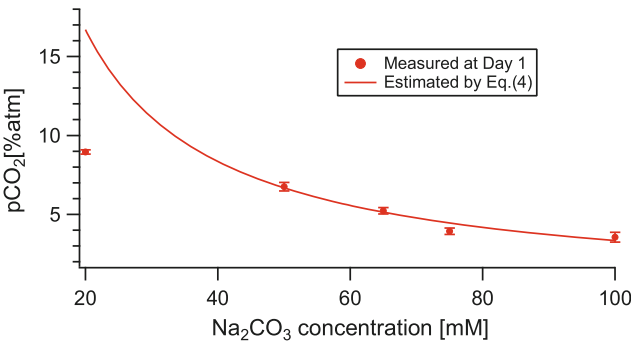

Fig. 5 Comparison of the theoretically-estimated pCO_2 to the measured pCO_2 levels (Fig. 4) at day 1 with various Na_2CO_3 concentrations ($N = 3$; mean ± SD). The calculation of pCO_2 was done by using Eq. (4), a function of Na_2CO_3 concentration. The temperature and the concentration of $NaHCO_3$ were fixed to 33.0°C (typical temperature in the media reservoir at incubation) and 0.8 M, respectively

20–100 mM. For Na_2CO_3 concentration = 20 mM, the pCO_2 tended to overshoot and then fall. In contrast, larger Na_2CO_3 concentrations (≥75 mM) caused a slower response. The result shows that adjusting the Na_2CO_3 buffering of the jacket solution is important not only for setting the level of pCO_2 but also for ensuring the long-term stability of pCO_2 levels.

In Fig. 5, we compared the measured pCO_2 values at day 1 and the estimated pCO_2 values from the Na_2CO_3 concentration: Eq. (4). We selected the pCO_2 values of day 1 as peak levels of pCO_2. The trend of the estimated pCO_2 was consistent with the measured peaks of pCO_2 values for high Na_2CO_3 concentrations (>50 mM). For Na_2CO_3 concentrations = 20 mM, measured pCO_2 value at day 1 was inconsistent with the estimation. This discrepancy can be explained by taking into consideration the rapid increase of the pCO_2 level as shown in Fig.S2. In other words, a small overshoot in the transient pCO_2 for higher Na_2CO_3 buffering might reduce the discrepancy between the long-term pCO_2 level and Eq. (4). Therefore, we infer that the combination of Na_2CO_3 buffer and the pH rise occurring after transient CO_2 emission contributed to the predictability and the controllability of the pCO_2 levels.

3.3 Cell culture

We evaluated the growth rate of the cells cultured on the microfluidic chips with on-chip CO_2 incubation. When on-chip CO_2 incubation was not used, the pH of the medium in the media reservoir (DMEM with 5% v/v FBS) increased rapidly (in typically 1 h) over pH 8.2, exceeding the acceptable pH for cell culture. Figure 6 shows the variation in growth rates of COS-7 cells cultured on-chip with initial count of cells seeded into the wells. For the initial cell counts within 30–170, the growth rates ranged from 0.012 to 0.024/h. These growth rates were comparable to that of typical conventional cultures, 0.014–0.020/h, converted from a published range of doubling times: ca. 35–48 h (DSMZ 2011). Initial cell count within 80–120 (corresponding to a recommended range of initial seeding density of COS-7 cells) gave a growth rate of approximately 0.02/h (doubling time: 35 h). The results shown in Fig. 6 suggest that the characteristics of cell growth on the developed cell culture chip are comparable to that of conventional culture.

Using the developed on-chip CO_2 incubation system, which successfully maintained appropriate osmolality, pCO_2, and cell growth rate, we next evaluated the ability of long-term microfluidic culture of cells proliferating from low density to very high density. Figure 7 shows the time-lapse recording of COS-7 cells cultured with the on-chip incubation and the medium circulation with a Braille cell. At day 0, the seeded cells attached normally to the bottom of the cell culture well and proliferated normally to day 5

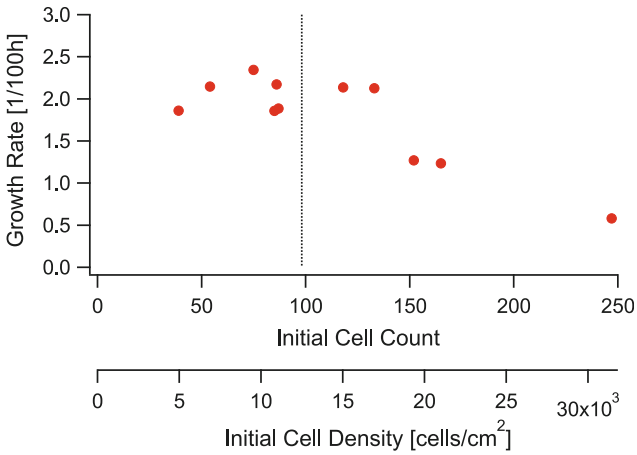

Fig. 6 Effect of the initial cell count in a cell culture well to the growth rate of the COS-7 cells cultured on cell culture wells on the microfluidic chip with on-chip CO_2 incubation. The formulation of water jacket was 0.8 M $NaHCO_3$ and 65 mM Na_2CO_3 in water. Growth rates at log phases were calculated from the cell numbers counted between days 0 and 3. The *dotted line* denotes a typical seeding density of COS-7 cells (12.5×10^3 cells/cm^2). Initial cell count of 250 is within a typical range of harvest densities of COS-7 cells ($25–35 \times 10^3$ cells/cm^2) and results in a low growth rate (0.006/h)

with a doubling time of 37 h. On day 5, the cells in the channels looked healthy, with good contact inhibition. Some cells near the wall of the well had climbed up the vertical wall of the well and migrated out of the well toward the 30-μm height microchannel. On day 17, the cells were over-confluent and less contact inhibited than those of COS-7 cultures using conventional methods. However, staining the cells with calcein (green fluorescence) showed that most of the cells were alive in the over-confluent condition.

The maintenance of over-confluent cells can be explained by forced recirculation of the medium by Braille pumping. Because the flow is a maximum of 20 μm/s at the bottom of the well (Fig. 1c), the medium around the cells is refreshed while conditioning of the medium still persists. The medium circulating in the wells seems to have enough capacity to feed cells on day 17 as cells have not only migrated upstream (i.e., a sign of media depletion in a microfluidic cell culture) but also in both directions. Although we see no problems with depletion of nutrients or oxygen in the medium flowing over the cells, the over-confluency could be prevented or delayed using media with reduced serum, glucose, or any growth factors.

The cell culture with on-chip CO_2 incubation, shown in Fig. 7, indicates that the developed microfluidic chip can incubate cell culture medium at physiological pH and osmolality for over 2 weeks. In addition, the developed 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

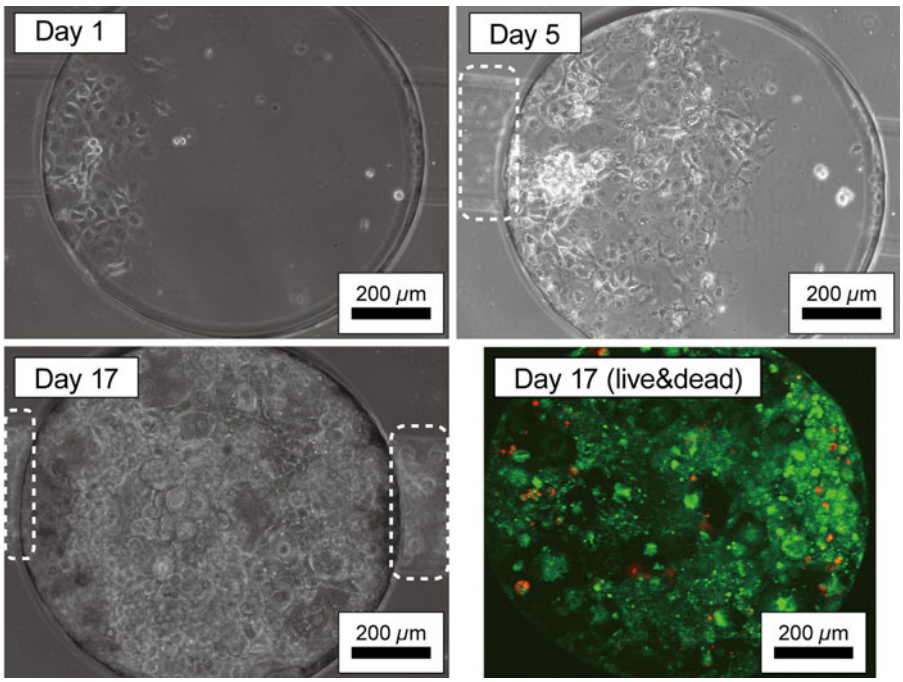

Fig. 7 Time-lapse recording of COS-7 cells seeded in the well of the microfluidic channel and incubated on-chip in the atmosphere. The formulation of water jacket used was 0.8 M $NaHCO_3$ and 65 mM Na_2CO_3 in water. The flow direction was left to right at seeding and right to left at cultivation. *Boxes* with *white dashed line* indicate the channels with cells that migrated from the cell culture well. The initial cell count introduced in the well was 39. In the image of *Day 17 (live&dead)*, live cells are labeled with green fluorescence of calcein and dead cells with red fluorescence of ethidium dimer

consists of a microheater and microsized bicarbonate chambers (Choi et al. 2006). This is because the developed incubation system is almost hermetically sealed, and provides CO_2 and humidity to cell culture media inside the chip both through the PDMS membrane covering the microfluidic channels and through the PDMS wall of the media reservoir.

3.4 Portability

Overall, the chip, the Braille cell, and the control circuit board with cables weigh 50 g. The size of the chip with a Braille cell is 70 mm × 70 mm × 20 mm, and that of the circuit board is 48 mm × 25 mm × 15 mm. Therefore, the portability of the entire cell culture system depends mostly on the miniaturization of the heating element and of the required battery size. We believe that the transparent hotplate we used can be easily replaced by a small self-regulating (Positive Temperature Coefficient: PTC) sheet heater that has a heating area equivalent to the chip bottom (26 mm × 50 mm) and a thickness of ~2 mm. The power requirement of the circuitry and of the heater remains a problem, particularly if we want the system to be operating while it is being packed, shipped and in transit.

We estimated the power requirement of the chip heater using computational thermal simulation while considering the heat dissipation through the chip and the packing material. As shown in Fig. S3a, the model of a chip with a heater was placed in the air volume of 70 mm × 70 mm × 30 mm. The clearance between the chip bottom acting as a boundary heat source and the air boundary was 2 mm. The simulation showed that the power required to maintain the temperature of chip bottom at 37°C is 249 mW, when the volume element is enclosed by an expanded polystyrene box with a wall thickness of 2 cm (Fig. S3b). In addition, the current drawn by the circuit board in operation was 50 mA @ 5V. Thus, the total power consumption of a potential package of cell culture system was estimated to be 500 mW. In other words, one LR20 battery (typically 12,000 mAh and 140 g typ.) should hold the system for 1.5 days.

In summary, the above estimation suggests that the developed cell culture system can be truly pocket-sized in a moderate external temperature (~20°C) and for short-term use (~1 day). For shipping with 1-week transportation time, the size and the weight of a package must be increased to ~100-mm square and ~1 kg, but this is still a reasonable size for a package to be shipped. The power and space requirement could be further reduced if we optimize the layout of the microfluidic channels to minimize the footprint of the chip-Braille assembly, and if we also optimize the high-voltage converter circuit to minimize the power consumption when the Braille pins are not moving.

4 Conclusion

We found that contacting cell culture media and a $NaHCO_3/Na_2CO_3$ solution through a permeable wall made of PDMS successfully prevented evaporation and maintained the pCO_2 around the media to a stable level for a prolonged time without any feedback control system.

We also confirmed the stabilization of the pCO_2 by Na_2CO_3 buffering, which can be explained by an equilibrium shift of carbonate species. Also, the result of long-term culture showed that microfluidic culture in the cell culture wells with on-chip CO_2 incubation did not hinder proliferation or migration of the cells inside.

This heat-activated CO_2 incubation functionality based on mass/heat transfer between aqueous solutions is suitable for building a pocket-sized platform for cell culture in combination with Braille-driven microfluidic systems. The pocket-sized platform could help design a compact and automated system not only for long-term maintenance of substances that are sensitive to environmental conditions, but also for control of environmental factors in a miniature device with limited energy, cost, and space.

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