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Microfluidic cell culture system with on-chip hypoxic conditioning*

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Abstract—**We have demonstrated a portable microfluidic cell culture system with multi-gas (CO_2 and O_2) incubation which we can cultivate under hypoxia without bulky peripheral apparatus such as gas tanks, regulators, and flow controllers. The system contains a chip of 26 mm × 48 mm which is capable to diffuse CO_2 and absorb O_2 through a gas-permeable wall of nested media reservoir. The media was water-jacketed with aqueous solution containing 0.8 M sodium bicarbonate as CO_2 supply and 1M sodium ascorbate as oxygen scavenger. The partial CO_2 pressure (pCO_2) in media reservoir stabilized at least 10.2% ± 0.11% for at least 72 hours. The partial O_2 pressure (pO_2) in the media reservoir decreased to 4.2%. Portable on-chip hypoxic culture of SV40-T2 cells for 72 h was also demonstrated.**

I. Introduction

Oxygen (O_2) has a critical role in many cellular responses involving homeostatic balance in order to maintain growth, proliferation, and controlled cell death[1]. Particularly, oxygen tension around stem cells affects all aspect of behaviors of stem cells[2], such as hypoxia-induced differentiation mediated by Hypoxia- Inducible Factors (HIFs) [3]. Thus, controlling oxygen conditions is one of useful methods for studying the nature of stem cells.

Recent studies reveal that microfluidic platforms are powerful tools to manipulate the oxygen condition in microenvironment [4-7]. Many microfluidic devices producing hypoxic conditions to date [4-7], however, mainly focused on precise and micro-scaled control of oxygen tension intended for generation of oxygen gradients on-chip, while portability of the entire system including gas supply and concentration control has received little attention. These devices would often require large apparatus such as gas tanks/regulators, and frequent calibration processes to ensure desired concentration profiles for actual use. These requirements may render running experiments under a variety of O_2 concentration practically difficult. Moreover, since monitoring of stem cells may take over a week to observe the evidence of differentiation[2], generating gradients within microchannels could be disadvantageous in terms of the temporal stability of concentration profiles.

We previously developed a method to maintain an elevated range of CO_2 levels within a pocket-sized microfluidic cell culture chip [8]. This microfluidic chip can maintain the cell culture media at a physiological pH and osmolality for over two weeks. The cell culture medium stored in this microfluidic chip is jacketed with an aqueous solution of carbonate/ bicarbonate source, which produces appropriate gas-phase CO_2 by heating the chip at the certain temperature (typically 37 °C). This on-chip incubation system does not require any bulky peripheral apparatus such as incubator, external gas-chamber and regulators.

Here, we have successfully controlled the O_2 concentration of the cell culture media in a microfluidic long-term cell culture chip lower than the atmospheric level in addition to the CO_2 level. We have modified the aqueous solution with which the medium was jacketed by adding the sodium salt of ascorbate (sodium ascorbate: Asc-Na) as an O_2 reducer. Ascorbate easily oxidizes to dehydroascorbic acid and is readily available as O_2 scavenger, dietary supplements, and food additives [9]. Therefore, the resulted multi-gas on-chip regulation system had high portability, cost performance, safety and disposability.

II. Materials and Methods

A. Microfluidic cell culture chip with on-chip incubation

Fig.1 shows the structural views of the microfluidic cell culture chip with on-chip incubation. As shown in Fig.1A the chip has two nested reservoirs partitioned with 1 mm-thick PDMS. Cell culture media are stored in the inner reservoir ("media reservoir"), and aqueous solutions of carbonate source and oxygen scavenger are introduced to the outer reservoir ("jacket reservoir"). Since CO_2, O_2 and water molecules easily diffuse into PDMS, gas/moisture exchange occurs between the media reservoir and the jacket reservoir. Two identical microfluidic loops are placed below the nested reservoir. The cell culture medium is introduced from the inlet of each loop placed at the bottom of the media reservoir, flows through the channel, then reached back to the media reservoir. To settle cells in the channels, one 300 μm-deep cell culture well is placed in-line on each microfluidic loop. The cell culture wells were formed by punching small holes on the PDMS slab. The jacket reservoir, the upper part of the media reservoir, and the fingerplate to fit to a Braille cell are made of plastics, whereas the lower part of the media reservoir is made of PDMS slab with microchannels.

B. Fabrication of the Microfluidic Chip

The assembly processes of microfluidic chip are shown in Fig.1B. First, a PDMS slab with microchannel features was fabricated by a soft-lithographic method as previously described[8]. Two φ1mm holes for cell culture wells were punched in the microchannel features (Fig.1A). A 300μm-thick PDMS membrane with four holes was also formed. The PDMS membrane and slab are plasma-bonded

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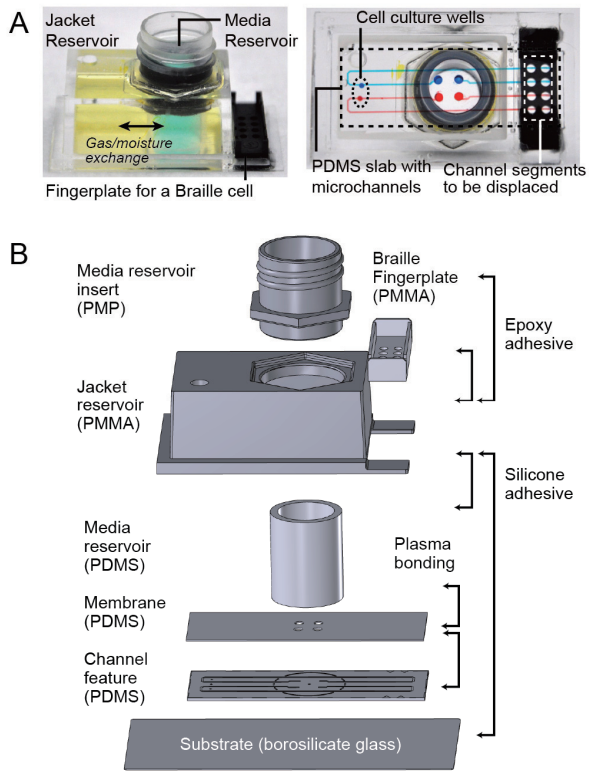

Figure 1. Microfluidic chip for long-term cell culture with on-chip multigas incubation. A) Assembled chip. The reservoir for cell culture media is filled with green-tinted water; the reservoir for water jacket with yellow-tinted solution. Both reservoirs are partitioned with a tubular wall made of poly(dimethylsiloxane) (PDMS) to facilitate gas/moisture exchange. A PDMS-made slab containing two identical microfluidic circuits with in-line cell culture wells are under the reservoirs. The inlets/outlets of both microfluidic circuits are placed at the bottom of the media reservoir. The PDMS slab with microchannels are partially covered with the media reservoir. The exposed region of the PDMS slab is covered by a fingerplate picked from a Braille cell. B) Exploded view of illustrated parts that consistutes the microfluidic chip. The PDMS-made membrane, which is bonded to the recessed channel features, also acts as diaphragms of pumps when displaced by pins of a Braille cell.

together with aligning the ends of the channel feature with holes on the membrane. The resulted PDMS slab with microchannels was bonded to a coverglass for mechanical support and forming the bottom of the cell culture well. A PDMS tube (10mm-ID, 12mm-OD, 10mm-height) formed by injection molding was then plasma-bonded to the membrane side of the PDMS slab. The assembly of a microfluidic chip with a jacket reservoir is also shown in Fig.1B. A polymethylmethacrylate (PMMA) part for a jacket reservoir was fabricated by conventional injection molding. The PMMA part was bonded by silicone adhesive applied to the surface of the coverglass with the PDMS slab, and the top surface of the PDMS tube. A media reservoir insert and a fingerplate dismounted from a Braille cell (SC11, KGS, Saitama, Japan) were then glued to the PMMA part. The whole chip was cured at room temperature for 24h. The media reservoir was closed with a screw cap seal. A Braille cell was re-attached to the fingerplate to generate peristaltic flows by pin movement as previously described[8].

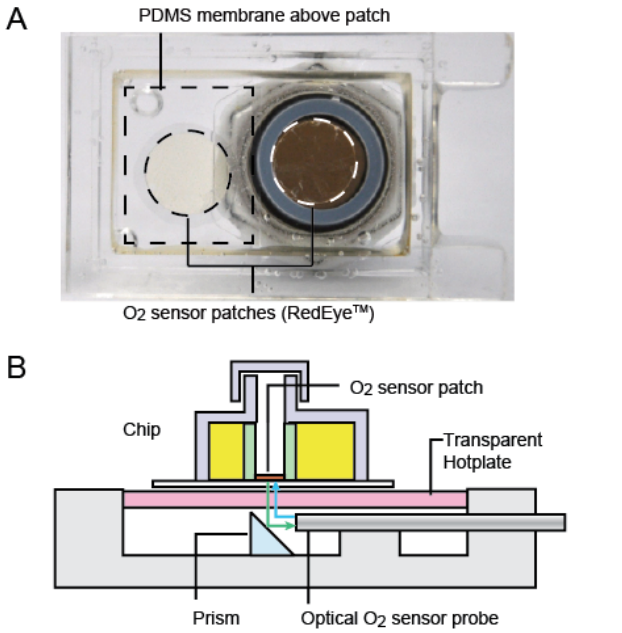

Figure 2. Setup for noninvasive sensing of O_2 in the microfluidic chips using a fluorescence-based optical sensor system. A) Fluorescent O_2 sensor patches applied to a chip mock, which does not include PDMS membrane and channel feature (Fig.1B). The patch placed at the bottom of the jacket reservoir (left) was covered with a PDMS membrane to simulate the O_2 level in the microfluidic channels., whereas the other patch was bonded on the bottom of the media reservoir and was uncovered. B) Fixture of the O_2 sensor system. The optical probe is a part of fluorescence O_2 sensing system (NeoFox™) and was fixed below a transparent hotplate. A prism was used to bend light toward the chip under test placed on the transparent heater

C. Evaluation of multi-gas (CO_2 and O_2) incubation on chip

The jacket reservoir of the microfluidic chip was filled with 4ml of aqueous solution ("jacket solution"). To prepare jacket solution, sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), and sodium ascorbate (Asc-Na) were dissolved in deionized water at room temperature. The concentration of $NaHCO_3$ and Na_2CO_3 was fixed to 0.8 M and 65 mM, respectively. For evaluation of partial CO_2 pressure (pCO_2), the media reservoir was filled with 1.5ml of 10mM $NaHCO_3$ in deionized water. The chip with filled reservoirs was incubated on a hotplate at 37°C for 72 h. The pH and temperature in the media reservoir were measured every 24h by a pH meter (TPX 999i, Toko). pCO_2 was computed from measured pH and temperature as previously described[8].The partial O_2 pressure (pO_2) in the media reservoir and the jacket reservoir were monitored noninvasively using an optical oxygen sensing system (NeoFox™, Ocean Optics) (Fig.2). To measure pO_2 in the media reservoir, thin patches containing ruthenium complex (RedEye™) were placed at the bottom of the reservoir (Fig.2A). The bottom of the microfluidic chip was heated at 37 °C during monitoring using the setup shown in Fig.2B. To reduce the side effect of the blue light emitted from the NeoFox probe on oxidation of ascorbate, the duration of each measurement was limited to 1s with an interval of 10 min.

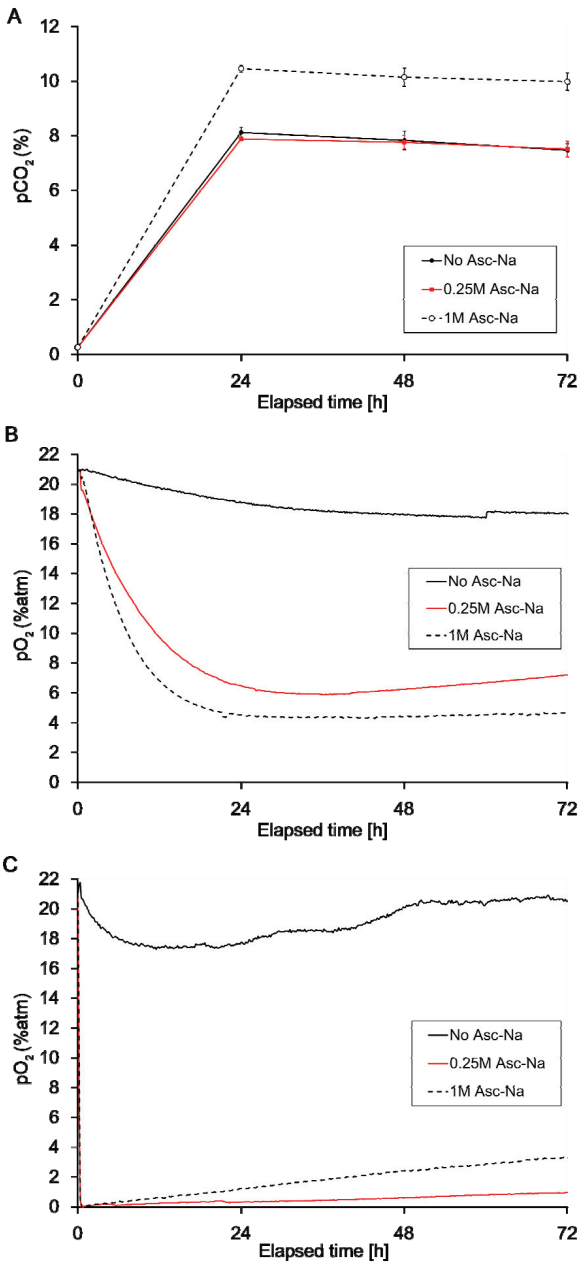

Figure 3. The effect of sodium ascorbate (Asc-Na) contained in the jacket reservoir on the partial CO_2 and O_2 pressure (pCO_2 and pO_2) in the chip. A) pCO_2 in the media reservoir (mean ± *SD*, $N = 3$). B) pO_2 in the jacket reservoir. C) pO_2 in the media reservoir. For all measurements the amount of $NaHCO_3$ and Na_2CO_3 are 0.8 M and 65 mM, respectively.

D. Cell culture

SV40-large T antigen-transfected rat alveolar type2 epithelial (SV40-T2) cells were cultured in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells to be seeded into chips were trypsinized and resuspended in fresh medium at 10^5 cells/ml. The microfluidic channels were treated with 100 µg/ml fibronectin solution at room temperature for 3h prior to seeding. 1 µl drops of suspension on the inlet were placed at the bottom of the media reservoir. The seeded cells were transferred into the cell culture wells using a peristaltic action of the Braille cell attached to the chip. After the jacket reservoir was then filled with a jacket solution, the bottom of the chip was heated at 37°C on a transparent hotplate. Cells cultured on chip were photographed using a phase-contrast microscope (DMIL LED, Leica) with a CCD camera (Retiga 2000R, QImaging) during heating the chip. Each microfluidic loop was pumped with 4-stranded peristaltic action of the Braille pins. The refresh rate of the pins was 1 s. At the end of each cultivation on chip, the cells were stained with LIVE&DEAD™ cell viability assay kit (Invitrogen) and observed with a fluorescent microscope (EVOS fl, AMG).

III. Results and Discussion

A. Partial CO_2 pressure (pCO_2)

First, the effect of Asc-Na in the jacket solution on pCO_2 of the media was evaluated. As shown in Fig.3A, the pCO_2 level of the jacket solution with 0.25 M Asc-Na reached to 8.0 % at 24h and slightly decreased to about 7.5% over two days, and had no significant difference from the case of the solution without Asc-Na. However, the pCO_2 level of 1M Asc-Na increased to 10.5% and approached to 10.0% over time. This result suggests that the certain amount of Asc-Na accelerate the dissociation of bicarbonate to gas phase CO_2. The generation of carbonic acid can be explained by the oxidation of Asc-Na and subsequent gaining proton by HCO_3^-. A HCO_3^- accepts a proton eventually generates gas phase CO_2: $HCO_3^- + H+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2(g)$. The difference in the pCO_2 levels between 0.25M and 1M Asc-Na also suggests that the ratio $[Na_2CO_3]/[\text{Asc-Na}]$ could determine the increase of pCO_2, because CO_3^{2-} can accept one more proton compared to HCO_3^-.

B. Partial O_2 pressure (pO_2)

The ability of Asc-Na to remove oxygen from the media reservoir (Fig.3B) and from the jacket reservoir (Fig.3C) was examined. As shown in Fig. 3B, the pO_2 in the media reservoir without Asc-Na (i.e. it contains only $NaHCO_3$ and Na_2CO_3) even decreased gradually from 20.9% to 18%, due to generation and diffusion of CO_2 into the media reservoir. The O_2 scavenging effect was observed for both 0.25M and 1M Asc-Na in the media reservoir. The pO_2 level was decreased to approximately 6% and 4%, respectively. The pO_2 level for 0.25M Asc-Na decreased slowly than that for 1M Asc-Na. Fig.3C shows the pO_2 level in the jacket reservoir. The drift in the pO_2 level of the jacket solution without Asc-Na may be due to purging of O_2 by generated CO_2. However, pO_2 level recovered to 20.9% after 24h later. It may be resulted from the diffusion of O_2 from the media reservoir through PDMS. The pCO_2 levels of the jacket reservoir that contains 0.25M and 1M Asc-Na show sharp drops to near 0% and mild linear increase possibly because of the diffusion of CO_2 gas through the PDMS layers. The result also shows the successful scavenging of O_2 by Asc-Na and diffusion of O_2 through the PDMS wall between both reservoirs.

C. On-chip cell culture with hypoxic conditions

The hypoxic conditioning of the cell culture well on the microfluidic channel (Fig.1A) was examined by cell culture and viability assay. Fig.4 shows the time-lapse recording of

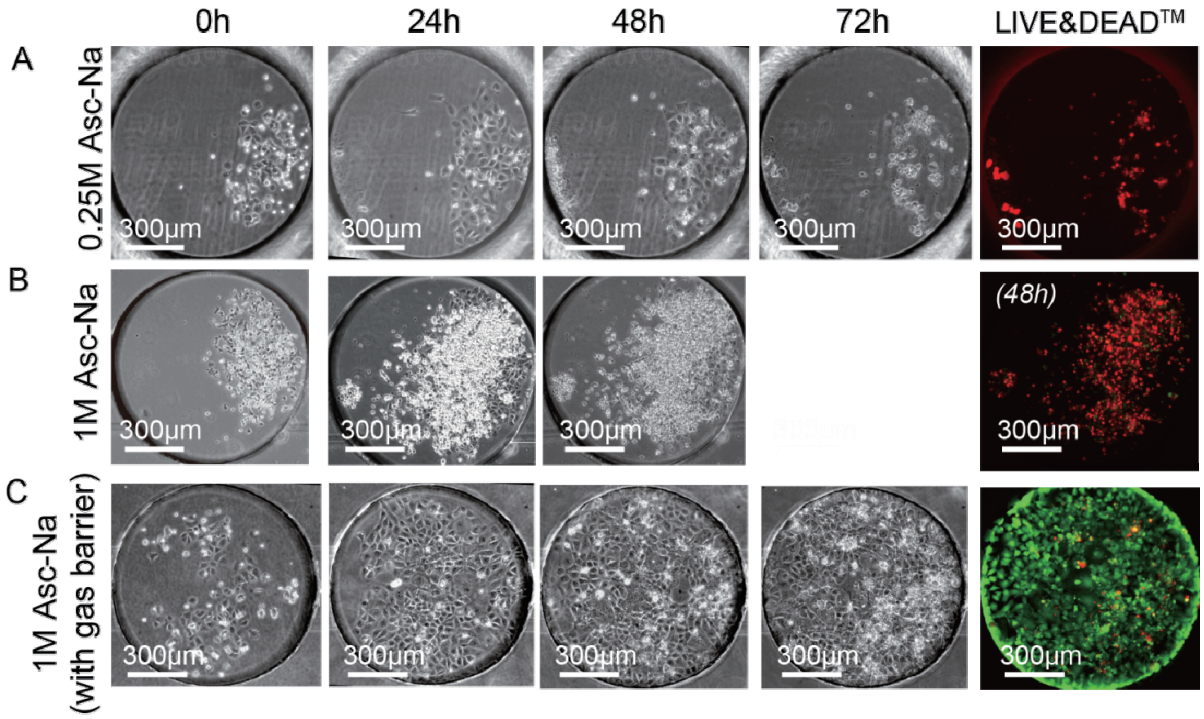

Figure 4. Cell culture of SV40-T2 cells on the microfluidic chip with various sodium ascorbate (Asc-Na) concentration in the jacket solution. A) Cells incubated with jacket solution containing 0.25 M Asc-Na. B) Cells incubated with jacket solution containing 1 M Asc-Na. At 48h all cells died and the experiment was stopped. C) Cells incubated with jacket solution containing 1 M Asc-Na and existence of gas barrier between the PDMS membrane and jacket solution (Fig. 1B). For all results of LIVE&DEAD™ stain, red regions indicate dead cells, and green regions live cells. For all measurements the amount of $NaHCO_3$ and Na_2CO_3 are 0.8 M and 65 mM, respectively.

SV40-T2 cells incubated on-chip with several conditions of O_2 scavenging. Figs. 4A and B show that seeded cells (at 0 h) were attached the bottom of cell culture well, and were alive at 24h. However, all cells seemed to shrunk at 48 h. The cells with 1M Asc-Na were detached. The cells of 0.25M Asc-Na died at 72 h. The result suggest that the pO_2 level in the microfluidic channels was closer to that of the jacket reservoir (Fig.3C) than that of the media reservoir (Fig.3C). It can be explained by the extensive absorption of O_2 through the thin PDMS membrane intended for Braille-driven pumps (Fig.1B).

To confirm the effect of O_2 scavenging through the PDMS membrane, the PDMS membrane was covered with a plastic wrap to prevent the diffusion of O_2 from the microchannels to the jacket solution. Fig.4C shows that the cells surrounded with 1 M Asc-Na-containing jacket solution were healthy and proliferated even at 72h when the PDMS membrane was covered with gas barrier. The result shows that the pO_2 level in the microfluidic channels was close to approximately 4.2%, which is that of the media reservoir (Fig.3C), indicating successful hypoxic conditioning to 4~5 % pO_2.

IV. Conclusion

We have developed a system for on-chip cell culture under hypoxic conditioning for three days. Addition of Asc-Na to the jacket solution successfully maintained the level of pCO_2 and pO_2 in the media reservoir to a constant value for up to seven days. We have also found that covering the PDMS membrane with plastic wrap was effective to prevent excess drop of pO_2, which was necessary to culture cells on-chip successfully.

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