Lab on a Chip

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On-chip CO_2 control for microfluidic cell culture†

Samuel P. Forry* and Laurie E. Locascio

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Carbon dioxide partial pressure (P_{CO_2}) was controlled on-chip by flowing pre-equilibrated aqueous solutions through control channels across the device. Elevated P_{CO_2} (*e.g.* 0.05 atm) was modulated in neighboring stagnant channels *via* equilibration through the highly gas permeable substrate, poly (dimethylsiloxane) (PDMS). Stable gradients in P_{CO_2} were demonstrated with a pair of control lines in a source-sink configuration. P_{CO_2} equilibration was found to be sufficiently rapid (minutes) and stable (days) to enable long-term microfluidic culture of mammalian cells. The aqueous solutions flowing through the device also mitigated pervaporative losses at sustained elevated temperatures (*e.g.* 37 C), as compared to flowing humidified gas through the control lines to control P_{CO_2}. Since pervaporation (and the associated increase in osmolality) was minimized, stopped-flow cell culture became possible, wherein cell secretions can accumulate within the confined environment of the microfluidic culture system. This strategy was utilized to demonstrate long-term (> 7 days) microfluidic culture of mouse fibroblasts under stopped-flow conditions without requiring the microfluidic system to be placed inside a cell culture incubator.

Introduction

Microfluidic cell culture holds great promise for better manipulation of the cellular microenvironment,[1–4] delivering high-throughput cell-based assays,[5,6] and automating solution handling and cell passaging.[7,8] Culture methods that utilize slow or intermittent perfusion are particularly interesting because they allow cell secretions to accumulate in the soluble microenvironment and influence cell behaviour.[3,9] Although many device materials have been explored, most microfluidic culture systems today rely on poly(dimethylsiloxane) (PDMS). PDMS facilitates rapid and inexpensive fabrication of devices, allows the inclusion of valves for routing and addressing fluids, and is optically transparent, facilitating microscopic characterizations.[10] PDMS is also highly gas permeable allowing gas partial pressures to equilibrate rapidly across the device and with the external environment.[11]

Conventional mammalian cell culture protocols usually stipulate elevated carbon dioxide partial pressure ($P_{CO_2} = 0.05$ atm) and relative humidity (100%). Typically, for microfluidic cultures, these parameters are maintained by placing the whole microfluidic system inside an environmental chamber (cell culture incubator) and using gas-permeable device materials such as PDMS. To avoid this experimental constraint, some researchers have used CO_2-independent media.[6,7] Other groups have developed strategies to control the gas partial pressure on-chip for O_2[12–14] and for CO_2[15–17] by utilizing gas-filled control channels to manipulate the gas partial pressure in adjacent fluid-filled channels.

In addition to being permeable to O_2 and CO_2, PDMS is also highly permeable to water vapor.[18,19] Due to the high surface area-to-volume ratio of microfluidic systems, the volume of water lost *via* pervaporation can be significant, leading to substantial fluid displacement and changes in solute concentration.[20–22] This problem is exacerbated for cell culture applications by the elevated temperature required (*e.g.* 37 C). Although placement of microfluidic culture systems inside a high relative humidity environment (*e.g.* cell culture incubator) slows pervaporation, osmolarity shifts do still occur and negatively impact long term cell cultures.[23]

In the current work, we have developed a system that allows on-chip control of the carbon dioxide partial pressure (P_{CO_2}) in stagnant culture chambers while mitigating pervaporation. Relevant P_{CO_2} levels for mammalian cell culture were achieved rapidly (in minutes) and were stable (for days). By using fluid-filled gas control channels, pervaporation was minimized. This allowed dissolved gas composition to be manipulated without changes in osmotic pressure, even under flow-free culture conditions. This approach enabled, for the first time, long-term microfluidic culture of mammalian cells (>1 week) under intermittent perfusion without requiring a cell culture incubator or CO_2-independent media.

Biochemical Sciences Division, Material Measurement Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, Stop 6312, Gaithersburg, MD, 20899-6312, USA. E-mail: samuel.forry@nist.gov.; Tel: +301-975-5246

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Methods[24]

pH and P_{CO_2} measurements

Solution pH inside microfluidic channels was measured using a ratiometric pH sensitive dye (5-(and-6)-carboxy SNARF-1, Invitrogen, Carlsbad, CA) as described in the supporting information. Briefly, the ratio of fluorescence images acquired at two emission bands provided a metric that scaled with solution pH and was insensitive to transient changes in dye concentration (*e.g.* due to pervaporation). This rapid and robust on-chip indicator of solution pH was calibrated with known buffer solutions prepared off-chip. Changes in P_{CO_2} on-chip resulted in changes in solution pH (*via* equilibration between carbon dioxide and carbonic acid) that were readily measured. For buffer compositions where all equilibrium constants were known (*e.g.* phosphate buffers), the actual P_{CO_2} in solution over time was calculated from the measured pH using Mathematica scripts.[25] Uncertainty in the pH measurements was the greatest source of uncertainty in P_{CO_2} determination.

Microfluidic device fabrication

Multilayer microfluidic deices were fabricated in PDMS using negative relief photoresist-on-silicon molds as described previously.[26] Briefly, positive photoresist (AZ9260, Mays Chemical Company, Indianapolis, Indiana) was spin coated in two layers (100 RPM/s to 1250 RPM for 60 s) onto silicon wafers for a combined thickness of 30 um. Fluidic and control layer molds were made identically using separate transparency masks (CAD/Art Services, Inc., Bandon, OR) to pattern exposure (MA6 Mask Aligner, Suss MicroTec, Sunnyvale, CA). Photoresist was developed per manufacturer directions, dried under nitrogen and heated (120 C for 120 s) to produce features with rounded cross-sections. 20 : 1 PDMS prepolymer:curing agent was spin coated (100 RPM/s to 500 RPM for 15s; 500 RPM/s to 1500 RPM for 60s) to a thickness of 45 um on the fluidic mold while the 5 : 1 PDMS prepolymer:curing agent added to the control mold was relatively thick (≥ 5 milimeters). After partial curing (25 min at 80 C), the control layer was aligned on top of the fluidic layer and returned to the oven. After complete curing, the multilayer device was irreversibly bound to a clean glass microscope slide using a microwave oxygen plasma system.[26]

Microfluidic device operation

The microfluidic device used in cell culture experiments extended previous designs for automated microfluidic cell culture (Fig. 1).[8,27] Fluid channels were 200 microns wide with a rounded cross-section and were closed when 25 psi of pressure was applied to 200 micron wide valve lines. The valves were used to effect slow peristaltic pumping of fluid (*e.g.* media, fibronectin) through 1 mm wide culture chambers or to route solutions toward waste. A head pressure of 5 psi in the fluid channels was used to eliminate bubbles. P_{CO_2} control channels were routed near the cell culture chamber in the valve layer of the device.

The fully fabricated microfluidic device and all connecting tubing lines were sterilized by flushing with 70% ethanol solutions. Subsequently, sterility was maintained by introducing all solutions through 0.2 micron filters (SUPOR, Owens & Minor, Mechanicsville, VA) and placing the device outlet directly into an ethanol-filled waste container.

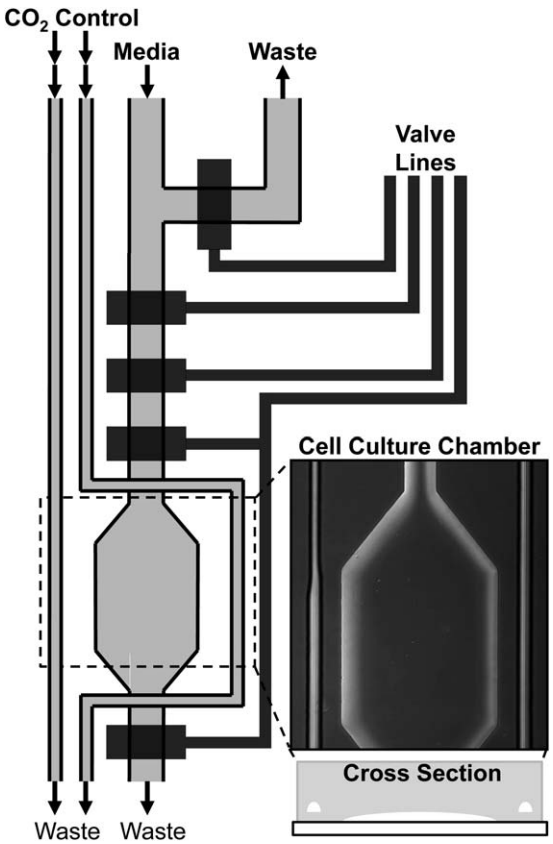

Fig. 1 Schematic depiction of the device design used for microfluidic cell culture with on-chip P_{CO_2} control. CO_2 control channels were closely positioned around the cell culture chamber to facilitate rapid equilibration with minimal imaging interference. Positive pressure valves were used to route and pump solutions through the device. The device geometry was imaged using phase contrast imaging (inset; the chamber is 1 mm wide). All chambers exhibited a rounded cross-section.

Prior to cell culture experiments, the microfluidic culture chamber was coated (25 mg L^{-1} solution for 60 min) with Fibronectin (Sigma-Aldrich, St. Louis, MO) to facilitate cell attachment.[28] The remaining fluid channels were coated (1 mg L^{-1} solution for 60 min) with pluronic (Sigma-Aldrich) to resist cell attachment. On-chip valves were used to route fluids and control how channels were coated. The device was held at 37 C (Tempcontrol 37–2, Pecon, Erbach, Germany) on a microscope stage (Cell Observer, Carl Zeiss MicroImaging, Thornwood, NY) and flushed with media (containing 5% serum) for 24 h prior to cell seeding. Cells were passaged as described elsewhere,[29] and were introduced in suspension at high densities ($\sim 10^7$ cells mL^{-1}). These cells were allowed to attach within the cell culture chamber for 2 h without flow. Subsequently, cells were cultured under stagnant conditions with hourly media exchange (one chamber volume - 45 nL - over 90 s).

Results and discussion

Pervaporation

The high gas permeability of PDMS allowes rapid equilibration of water vapor between aqueous solutions inside microfluidic

channels and the surrounding environment. When devices are heated to 37 C for typical mammalian cell culture applications this leads to rapid water loss (pevaporation). Due to the small volumes of many microfluidic systems, this can result in dramatic osmotic pressure changes inside microfluidic channels.[23] Observation of this phenomenon was facilitated by using on-chip valves to isolate solution-filled microfluidic champers and prevent flow in or out. Then, pervaporation was easily visualized as loss of volume from the system due to pervaporation resulted in complete chamber collapse (Fig. 2a). Due to the low aspect ratio geometry of these channels, the center of the chamber consistently collapsed first, though complete chamber collapse occurred in just a few hours (Time lapse movie SI 1 is included in Supporting Information). The solution ionic strength was found to affect the rate of collapse, with with higher-ionic strength solutions slowing pervaporation, presumably due to the lower water activity in these systems.[30]

Using PBS solutions to mimic the ionic strength of cell culture media, the rate of volume loss was measured for this geometry with various environments surrounding the microfluidic chamber (Fig. 2b). When warm dry gas was pumped through the adjacent control lines, chamber collapse was very rapid. This was consistent with the low relative humidity of the warmed gas driving rapid pervaporation. As expected, humidifying this gas flow, resulted in an attenuated rate of pervaporation. However, pervaporation still resulted in chamber collapse in only 6.6 h ± 2 h. Based on channel geometry, this indicated an average pervaporation rate of 5 nl h^{-1}, and a 12% increase in osmolality each hour. This mimics the conditions of previous systems for providing on-chip control of gas partial pressures.[12–17]

When pure water was pumped through the control line, pervaporation was substantially reduced and chamber collapse took nearly two days. In microfluidic cultures where fresh media is intermittently perfused, this was an acceptable rate of pervaporation and led to a calculated 1.5–3% increase in osmolality every 1–2 h (typical perfusion interval). This approach is similar to other strategies to mitigate pervaporation using on-chip water reservoirs.[27]

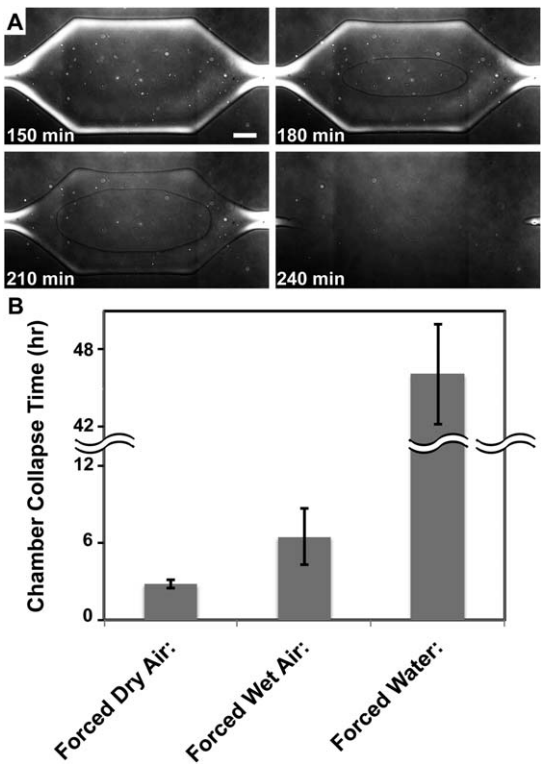

Fig. 2 Pervaporative chamber collapse. At elevated temperature (37 °C), pervaporation from stagnant solutions led to rapid volume loss. Time-lapse phase contrast images (A) showed the gradual collapse of culture chambers after entrance and exit channels were valved closed. The time required for chamber collapse (B) was measured while varying the composition of the CO_2 control channel. Pervaporation was mitigated most when water was pumped through the CO_2 control channels. The scale bar in (A) was 200 μm. Multiple images were stitched together digitally to generate this field of view. A time-lapse movie of the chamber collapse is included in the Supplemental Information.†

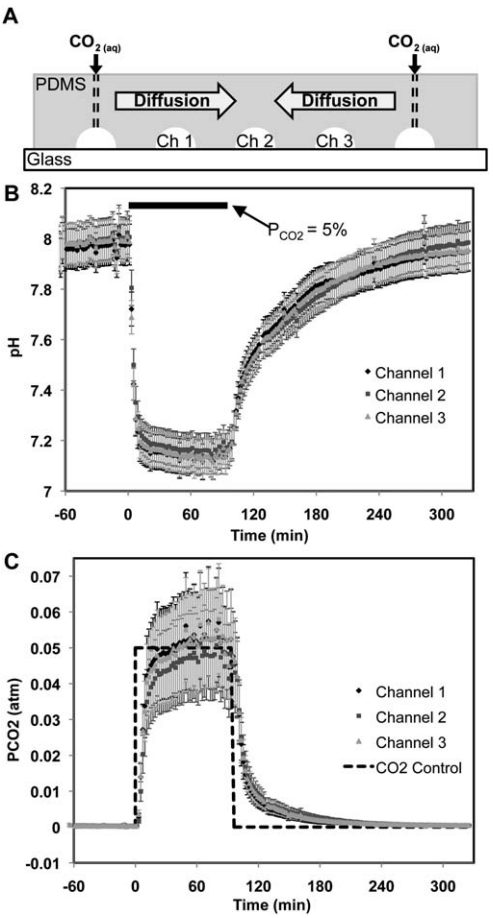

Fig. 3 On-chip modulation of P_{CO_2} using water-filled control channels. Aqueous solutions were pre-equilibrated at various P_{CO_2} setpoints and pumped through microfluidic CO_2 control channels (A), allowing P_{CO_2} to equilibrated *via* diffusion. Neighboring stagnant channels exhibited a rapid pH response to changes in P_{CO_2} setpoint (B). Using known buffer solutions, P_{CO_2} in the stagnant channels could be calculated and compared to the control channel setpoint (C). Error bars in (B) and (C) indicate the 95% confidence interval determined from the pH calibration of n = 7 different devices.

On-chip control of CO_2 partial pressure

The water pumped through control lines to mitigate pervaporation was additionally useful in controlling the partial pressures of dissolved gasses. The water was pre-equilibrated with controlled gas compositions and the control channels were routed adjacent to flow-free microfluidic chambers. Diffusion through the gas-permeable PDMS led to controllable changes in the dissolved carbon dioxide (P_{CO_2}) levels in the chambers (Fig. 3a). In solution, CO_2 was in equilibrium with its hydrated forms:

$$CO_{2(g)} \rightleftarrows CO_{2(aq)} \qquad (k'_c)^{-1} = (29.76\ \text{atm/M})^{-1}$$

$$CO_{2(aq)} + H_2O \rightleftarrows H_2CO_3 \qquad K_h = 1.70 \times 10^{-3}$$

$$H_2CO_3 \rightleftarrows HCO_3^- + H^+ \qquad Ka_1 = 2.5 \times 10^{-4}\ \text{Mol/L}$$

$$HCO_3^- \rightleftarrows CO_3^{2-} + H^+ \qquad Ka_2 = 5.61 \times 10^{-11}\ \text{Mol/L}$$

Thus, as P_{CO_2} increased (from 0 atm to 0.05 atm CO_2), solution pH decreased substantially (Fig. 3b). Then when the control channel was flushed with air-equilibrated water, the solution pH increased again. By filling the stagnant chambers with known buffer solutions, it was possible to calculate P_{CO_2} from the pH shift (Fig. 3c). An array of independent chambers (200 μm spacing) verified that the water-filled control lines were able to effectively modulate P_{CO_2} even at substantial distances (1.4 mm spacing between control lines). The temporal response using these water-filled control lines (~50 min) was substantially slower than in previous work that used gas-filled control lines. This was attributed to the significantly reduced capacity of the condensed phase for dissolved gasses and the need to equilibrate a relatively large volume of bulk PDMS to effect changes within the stagnant chambers.

Additionally, when the opposing control channels were flushed with water equilibrated at different gas compositions, a source-sink configuration was generated (Fig. 4a). The P_{CO_2} was calculated from the measured pH shift in the stagnant channels as described for Fig. 3. This configuration allowed stable gradients in P_{CO_2} to be generated across the microfluidic device (Fig. 4b), and P_{CO_2} was observed to reflect a linear gradient between the two control channels. Using this strategy, chamber P_{CO_2} was determined by its physical location within the geometry of the device. Similar strategies using complex geometries (and gas-filled control channels) have been used elsewhere to encode complex gradients along or between microfluidic channels.[14]

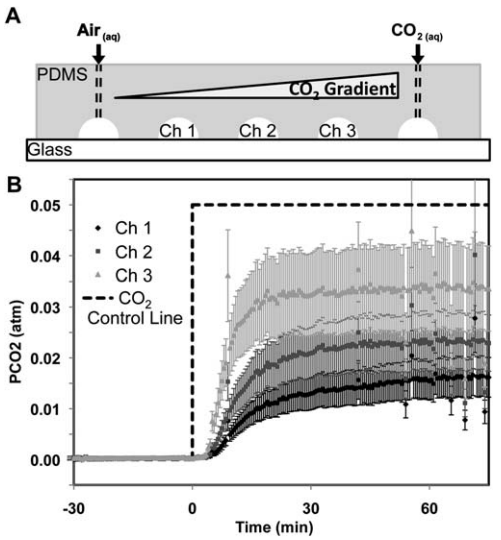

Fig. 4 Generation of gradients in P_{CO_2}. When different P_{CO_2} setpoints are used for opposing control lines in a source-sink configuration, a gradient in P_{CO_2} was expected across the device (A). P_{CO_2} measurements from chambers spaced evenly between the control channels confirmed gradient formation (B). P_{CO_2} and 95% confidence intervals were calculated from measured pH as in Fig. 2.

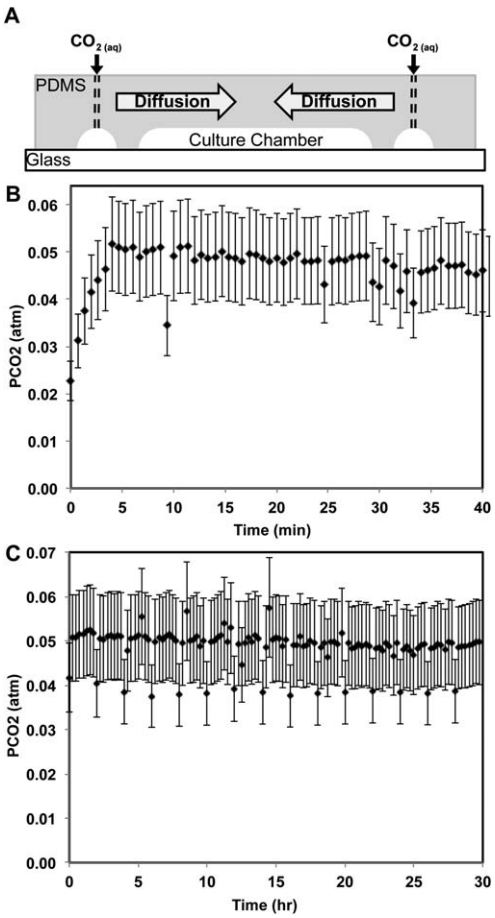

Fig. 5 P_{CO_2} control for microfluidic cell culture. On-chip control of P_{CO_2} was evaluated for the device geometry (A) and operations required to achieve long-term microfluidic cell culture. Following culture chamber perfusion with fresh air-equilibrated solution (B), equilibration to 5% CO_2 was rapid (within 5 min). At longer time scales (C), on-chip P_{CO_2} control was stable for days. In each case, P_{CO_2} and 95% confidence intervals were calculated from pH measurements as in Fig. 2; in (C), measurements were timed to capture the transient behaviour during periodic (every 2 h) perfusion with fresh solution.

Microfluidic cell culture

A particular goal of this research was to enable microfluidic cell culture without requiring a cell culture incubator. The design of the system positioned two control lines flanking a single cell culture chamber (Fig. 5a). Water pumped through the control lines reduced pervaporation, as described previously, and the composition of water pumped through these control lines provided on-chip control of P_{CO_2}. The P_{CO_2} set point was constant throughout culture experiments at 5% CO_2. Periodic perfusion of culture media was performed to maintain nutrient supply and remove accumulated metabolic waste.

When fresh, air-equilibrated solution was introduced into a device held at constant P_{CO_2}, it rapidly equilibrated on-chip (Fig. 5b). In this case, the equilibration was much more rapid ($\approx$ 5 min) than seen previously for a change in control channel set point. This was consistent with the bulk PDMS already being charged with 5% CO_2. Thus, full equilibration only had to affect the small volume of the culture chamber and could be quite rapid. Solution equilibration on-chip prior to introduction into the culture chamber was explored but was not deemed necessary. The long-term stability was evaluated by monitoring P_{CO_2} control on-chip over several days of continuous operation (Fig. 5c). Regular perfusion (every two hours here) caused a transient drop in P_{CO_2} ($\approx$ 5 min, as seen in Fig. 5b) each time fresh air-equilibrated solution was added. Otherwise, P_{CO_2} control was quite stable.

Using this strategy, microfluidic cell culture devices were operated under P_{CO_2} control at 5% CO_2 (normal culture protocol) and 0% CO_2 (Fig. 6). At 0% CO_2 (air-equilibrated control channels), cells fared poorly. Suspended cells introduced into the device (t = 0 h) exhibited minimal attachment to the fibronectin-coated surface over >2 h. Consequently, when media perfusion was initiated (at t = 2 h), most cells were displaced from the chamber. The cells that remained in the chamber exhibited an abnormal rounded morphology and minimal motility. Subsequently, cells continued to detach and were consistently displaced from the culture chamber during hourly perfusions. Overall, the culture deteriorated in <1 day, as expected since the microfluidic device was not placed inside an environmentally-controlled enclosure. Results from microfluidic culture were dramatically different when the water-filled control channels were used to maintain an elevated P_{CO_2}. At 5% CO_2, suspended cells introduced into the device (t = 0 h) attached rapidly (<40 min) and exhibited a normal spread morphology. No changes were observed when perfusion was begun after 2 h or during automated hourly perfusions thereafter.

Cells exhibited normal morphology and behavior (*e.g.* regular motility, cell division) for more than a week of culture in the microfluidic system. (Time-lapse movies of microfluidic cell culture at 0% and 5% CO_2, and for over a week of culture, are available in the Supporting Information.)

Conclusions

The strategy described here allowed on-chip modulation of carbon dioxide partial pressure while mitigating the effects of pervaporation. Pre-equilibrated aqueous solutions pumped through the microfluidic system controlled the gas partial pressure in neighbouring channels *via* diffusion through the gas-permeable PDMS substrate material. P_{CO_2} was reliably set to 5%

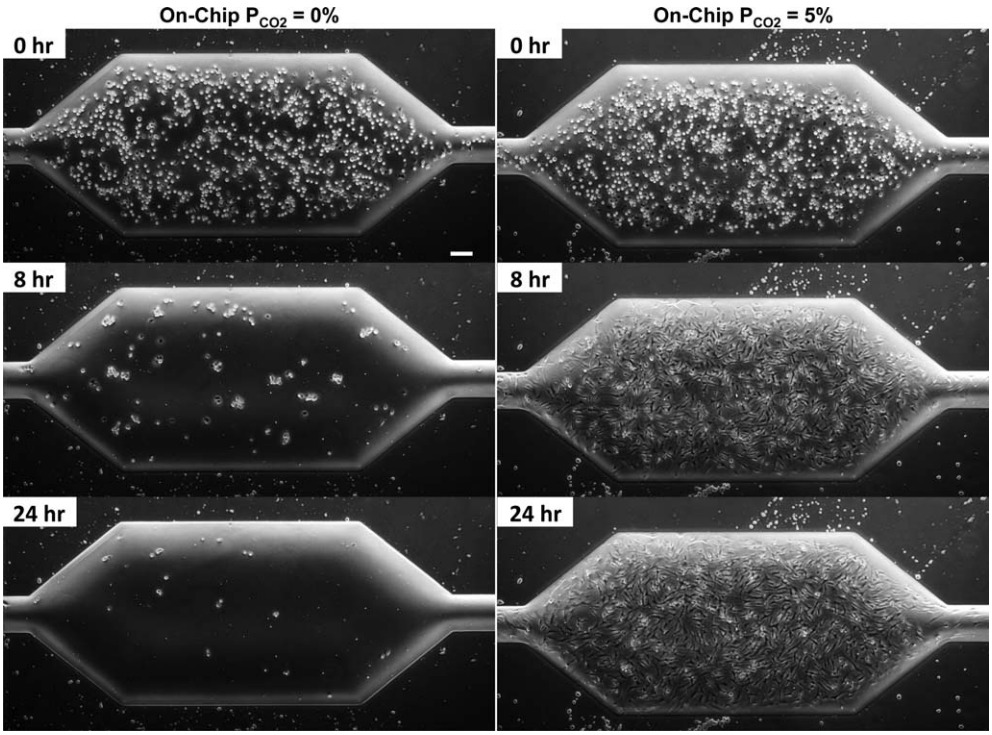

Fig. 6 On-chip microfluidic cell culture. Mouse fibroblasts were cultured in microfluidic devices on a microscope with on-chip regulation of P_{CO_2}. At 0% CO_2 (0.0 atm) the culture deteriorated within 24 h, while at 5% CO_2 (0.05 atm) the culture was maintained for 1 week. Periodic (hourly) media perfusion was initiated 2 h after introduction of suspended cells. Time-lapse movies of the cultures are available in the Supplemental Information.

(0.05 atm) across multiple stagnant chambers, and equilibration was rapid. Alternately, gradients in P_{CO_2} were generated with a source/sink control channel configuration. P_{CO_2} control was found to stable over multiple days. Additionally, the flowing control solutions also acted as water reservoirs, significantly mitigating the effects of long-term pervaporation (*e.g.* volume loss; channel collapse). This gas control strategy enabled, for the first time, microfluidic culture of mammalian cells for over a week under stopped-flow conditions without requiring a cell culture incubator or CO_2-independent media. Demonstrated here for mouse fibroblasts, this approach is general, should be applicable for any adherent cell type and opens new possibilities for portable microfluidic cell culture systems.

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