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Original Article

Continuous Oxygen Supply in Pump-less Micro-Bioreactor Based on Microfluidics

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Abstract This research aims a continuous and uniform oxygen tensions and oxygen gradients supply in microfluidic cell culture chip based micro-bioreactor without any external pumps by modifying the existing siphon based perfusion strategy using conventional tools to control constant hydrostatic pressure for constant fluid flow rate. In this study, the microfluidic based micro-bioreactor is fabricated using a polydimethylsiloxane (PDMS) replication process. The micro-bioreactor chip is composed of simple oxygen perfusion setup connecting to capillary-like branched microchannels and a cell culture and support loading area. The function of the new pump-less fluid perfusion in microfluidic cell culture chip and maintains constant oxygen supply in the cell culture area has been experimentally evaluated. And the results demonstrate that this proposed microfluidic flow system in a micro-bioreactor is proved to provide a linear distribution of oxygen throughout the cell culture area without any external and internal interference. Also, oxygen diffusion into the culture area through a microscale channel has been successfully established by the constant hydraulic pressure controlled by siphoning effect. Such flow system was used in a PDMS based microfluidic micro-bioreactor design to provide the high-throughput oxygen diffusion and perfusion, and the uniform oxygen/nutrient distribution continuously supplied for dermal fibroblast cell culture. This could be a potential and effective model to be incorporated into tissue regeneration studies, drug screening model, and in cancer tissue model studies for understanding angiogenesis, where oxygen tension and perfusion cultures play important roles.

Keywords: Micro-bioreactor, Dermal fibroblast cell, Pump-less perfusion, Oxygen diffusion, Hydrostatic pressure

Introduction

For the great advancement of micro-environment technology, microfluidic based cell culture model study has the great potential to replace *in-vivo* animal models for cosmetic product analysis, wound healing applications and other drug screening applications. This cell culture model acts as an *in-vitro* skin model for the study of skin biology, and therapeutic drug analysis by pharmacological and toxicological assessments. So, the cell growing with a micro-environment mimics the physiological real tissues. During the cell culture in a micro-environment, the microfluidic based nutrition perfusion along with oxygen generation and diffusion of cell culture are the prime challenges in tissue regeneration.

Microfluidic perfusion bioreactor systems also possess enormous potential in other view, and therefore, have been extensively researched by chemists and cellular biologists. For instance, the application of flow micro-bioreactor technology in chemistry has attained noteworthy success on account of its greater surface-to-volume ratios in the exploration of highly exother-

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mic and rapid reactions[1-4]. Research relating to biological applications of continuous-flow microfluidic bioreactors is wide in the fields of cell sorting[5], cell transfection[6], PCR reactions[7], and high throughput screening[8]. Importantly, microfluidic based integrated lab-on-a-chip for cell culture under perfusion for growing mammalian cells are of enormous interests[9].

Prominently, and in addition to perfusion culture, generation of oxygen diffusion for cell culture in tissue regeneration study[10], various researchers have attempted to conquer this challenge through the utilization of oxygen rich fluids like perfluorocarbons[11] and silicone oils[12]. In addition, a number of elastomeric micro-bioreactor devices have been developed to investigate the oxygen gradient generation for cell culture[13-16]. Recently, a microfluidic device has been studied for oxygen gradient production, through chemical reactions which take place across the microfluidic channel for cell culture, without direct contact of chemicals with cultured cells[17]. Though above literature were of significant attempts for improvising the microfluidic device to generate oxygen gradient for cell culture, still there remain numerous challenges which limit their practical applicability. The ultimate goal of the oxygen gradient generation is to supply uniform oxygen tensions and oxygen gradients for the cultured cells thoroughly. But most of the devices face difficulty in uniform distribution of oxygen concentration throughout the cell culture area[15,16]. Another important limitation of these devices is the complex microfluidic design with a need for external fluidic pumps, such as syringe and/or peristaltic pumps, to control the gas flow or chemical reactions for an oxygen gradient generation, a process which is further complicated in cases where the whole setup in the incubator must remain suitable for cell culture. Consequently, modern microfluidic researchers aim to develop passively-driven perfusion microfluidic devices, which have wider applicability due to their simplicity in fabrication, operation and portability, and are less expensive. Most of these passive-driven perfusion devices utilize the gravity based principle for fluid flow; however, this gravity-driven principle is not ideally suited to maintaining a steady flow rate, as the changing fluid level in the reservoir affects hydraulic pressure. For instance, a pump-less constant flow rate perfusion system was developed using a balanced droplet dispensing method[18]. Another microfluidic device with two parallel channels were fabricated to obtain constant hydraulic head difference, therefore attains the constant flow rate perfusion[19]. Though these devices rectified the problem of maintaining the constant hydraulic head difference, the volume of reservoir needs refilling every few minutes to hours, that makes inconvenient for long term cell culture and allows the high possibility of contamination. Recently, the present group engineered a microfluidic device for pump-less perfusion of culture medium at constant flow rates, where the volume replenishment of the reservoir was sorted out to everyday atleast[20]. The principle is based on a modified siphon, which helps to maintain a steady flow of fluids for extended periods by delivering a controlled hydrostatic pressure.

By incorporating this concept from the previous article, the present paper details the development of an extremely simple three dimensional microfluidic device, for pump-less perfusion of culture medium and oxygen producing compound on and across a cell culture area. An aqueous solution of the biocompatible and implantable oxygen releasing biomaterial, sodium percorbonate[21], was perfused in a microchannel which is patterned at a specific micron-scale distance across the mirofluidic cell culture area. This energy efficient siphoning principle of perfusion of oxygen releasing material on the pump-less microfluidic device facilitates the gradual permeation of liberated oxygen through gas permeable PDMS chip for the cultivation of cells without direct contact of chemicals to the cells/culture medium. Subsequently, perfusion of the culture medium onto the cell culture channel helps in the uniform distribution of oxygen throughout the cell culture area. Moreover, the microchannels for the culture medium were designed in such a way as to mimic the intact branches of blood vessels and capillaries which supply oxygen and nutrients for tissue formation *in vivo*.

Results and Discussion

The measured fluorescence intensities for four different flow rates of oxygen producing material; sodium percarbonate (SPC) (1 μL/min, 10 μL/min, 100 μL/min and 200 μL/min) with respect to various time intervals were utilized to calculate the dissolved oxygen concentration in the cell culture area using the Stern-Volmer equation[22].

$$I_0 / I = 1 + K_q[O_2] \quad (1)$$

Where I_0 is the dye intensity at 0% oxygen, I is the dye intensity at a given dissolved oxygen concentration $[O_2]$, and K_q is the Stern Volmer quenching constant. Since the collisional process of RTBP dye quenching by oxygen has been described by the Stern-Volmer equation, the present work used a K_q value of 2.7 $\times 10^{-3}$ μM^{-1} based on the previous report[13,22]. Parenthetically, the oxygen concentrations at different time

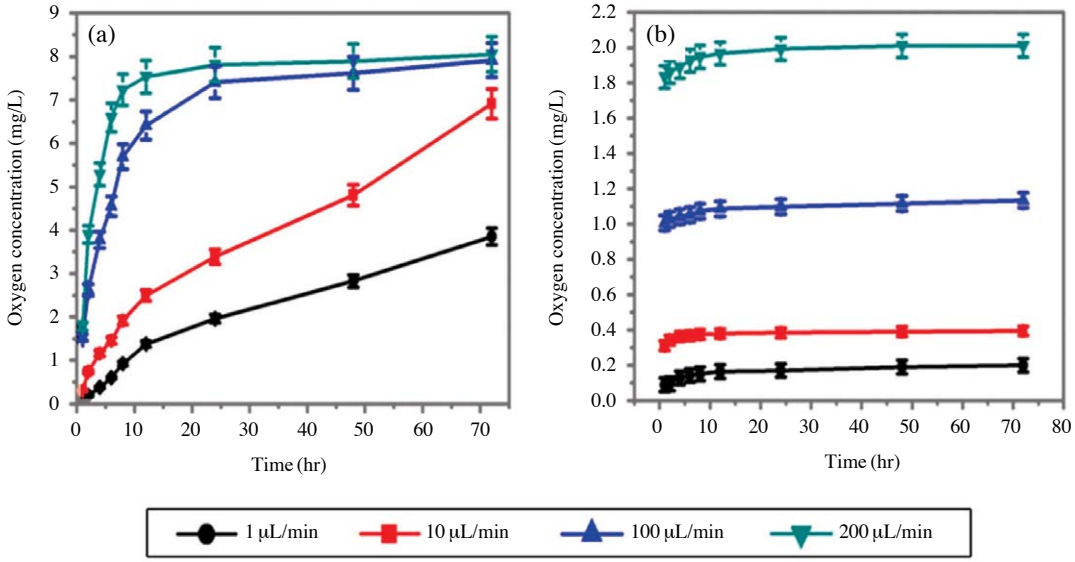

Figure 1. RTBP dye fluorescence quenching due to oxygen diffusion into the cell culture area of oxygen side channel perfused with aqueous SPC at the rate of 1 μL/min, 10 μL/min, 100 μL/min and 200 μL/min, and the oxygen diffusion and distribution were highly uniform at different flow rates with their respective concentrations. (a) The concentration of oxygen diffused into the cell culture area (at position only 5 mm closer to the oxygen side channel) with respect to SPC perfusion rates, which is calculated by Stern-Volmer equation at different time intervals. (b) Different oxygen concentrations at different positions (5, 10, 15, 20, 25 mm) within the cell culture area is averaged at different time intervals and plotted for every flow rates of aqueous SPC perfusion in the oxygen side channel, while the RTBP dye perfusion in culture channel.

intervals diffused into the cell culture area of the oxygen side channel under SPC perfusion rate of 1 μL/min, 10 μL/min, 100 μL/min and 200 μL/min were determined using eqn (1), and were found to be in the range of 0.08 to 3.85 mg/L (1-66 mm Hg), 0.30 to 6.92 mg/L (5-118 mm Hg), 1.52 to 7.91 mg/L (26-136 mm Hg) and 1.78 to 8.05 mg/L (31-138 mm Hg), respectively (Figure 1a). It is worth mentioning that in physiological *in-vivo* condition, the oxygen tension in various parts of the tissues of animal models was found to be in the range of 20 mm Hg to 100 mm Hg (1-8 mg/L or 17-137 mm Hg of oxygen in the blood vessels of diameters 20-300 micron)[23]. Thus, the oxygen diffusion ranges observed in this present work are comparable to values for mammalian cells *in vivo*. However, it is clear that the flow rate of 1 μL/min of SPC is not enough for sufficient oxygen diffusion when compared to other flow rates. On the other hand, the flow rate of 100 and 200 μL/min showed the rapid diffusion of oxygen, though a flow rate of 10 μL/min revealed a steady and constant diffusion of oxygen. The phenomenon of distribution of diffused oxygen throughout the cell culture area was attained by slow perfusion of RTBP dye (approximately 0.1 to 10 μL/min) in the capillary-like branched microchannels through cell culture area, while perfusion of SPC in the oxygen side channel. In order to investigate the successful oxygen distribution within the culture area, fluorescence intensities at different positions of the cell culture area (5, 10, 15, 20 and 25 mm) were measured and were averaged for various time intervals. The averaged intensities were then used to calculate the oxygen concentration distributed throughout the culture area with respect to the SPC flow rate (Figure 1b). The linear distributions of oxygen concentration of 0.14, 0.36, 1.07 and 1.93 (mg/L)/mm were observed in the SPC flow rates of 1, 10, 100 and 200 μL/min, respectively. It has been widely studied that at low oxygen tensions around 1% (0.4 mg/L), skin fibroblasts proliferate rapidly and have long viability and functionality for wound healing[24]. In this research, an oxygen concentration distribution of 0.36 (mg/mL)/mm at a flow rate of 10 μL/min has an oxygen tension of approximately 1%, which is found to be an appropriate flow to accelerate the diffusion of sufficient oxygen into the culture area for skin fibroblast cell culture, based on previous literature[25]. Since a 1 μL/min flow rate has low oxygen tension (0.14 (mg/L)/mm=~0.5% O_2), cultured cells may be subjected to hypoxia conditions[24]. Conversely, 100 μL/min (1.07 (mg/L)/mm=~2.5% O_2) and 200 μL/min (1.93 (mg/L)/mm=~5% O_2) has high oxygen tension, which may cause skin fibroblasts to experience hyperoxia[25].

To support this, a WST-1 cell proliferation assay

was carried out on 3, 7, 14 and 21 day cultures of dermal fibroblasts in the cell culture area of the microfluidic chip, under perfusion of culture medium in the culture channel and SPC perfusion in the oxygen side channel (Figure 2a). The results showed that the absorbance was decreased with increasing the SPC flow and oxygen tension of cultured area sequentially from 1, 10, 100 and 200 μL/min respectively. Compared to static and various perfusion culture's system; the 1 and 10 μL/min SPC flow in the cultured cells shows the high proliferation rate of fibroblasts, but the proliferation of fibroblasts was reduced significantly ($p = 0.037$) for cultures exposed to 100 and 200 μL/min SPC perfusion. The absorbance of static, 1 and 10 μL/min SPC flow in the cultured cells was higher than 100 and 200 μL/min SPC flow, and clearly it indicates that the 1 and 10 μL/min SPC flow in the cultured area is cell friendly and biocompatible that could serve as a good application; which incorporate this suitable perfusion into the co-cultures system in a bioreactor that can use for the developing tissue. This result indicated that the high oxygen tension induced hyperoxic condition can cause fibroblast cells to undergo apoptosis, which is thought to have resulted in the reduced cell number. Indeed, live/dead staining (Figure 2b) confirmed the results of the proliferation assay, where 100 and 200 μL/min showed a comparatively high number of dead cells (red) on both 3 and 21 day cultures. Though, the proliferation of cells under both static and perfusion cultures with 1 and 10 μL/min SPC flow showed similar growth until day 7. Cells are more compatible and well adhered in the 1 and 10 μL/min SPC perfusion culture's system. Previous studies reported that dermal fibroblasts have their own antioxidant defense mechanism to fight against exposure of high oxygen concentration than required[26,27]. We believe that the present work's results on similarities in cell proliferation observed in static and perfusion cultures (1 and 10 μL/min SPC flow) until day 7 should be due to the cellular antioxidant defense against oxidative stress produced by ambient oxygen concentration in static culture. Thereby, cells in static culture showed a similarity in growth profile with perfusion cultures. Also, the studies revealed that the cells cultured with and without low oxygen tension began to show slight differences in their growth profile only after 5th day of cultures[24,25]. According to present work, the cell viability of cultures under perfusion at 1 and 10 μL/min started to increase significantly (p value of 0.021) on day 14 and 21 compared to static cultures, which is due to the generation of low oxygen tension as documented by previous literature[24,25]. These results were further supported by live/dead staining at day 3 and day 21 cultures (Figure 2b) where, 1 and 10 μL/min perfusion cultures showed a higher number of live cells (green) than the static cultures. Indeed,

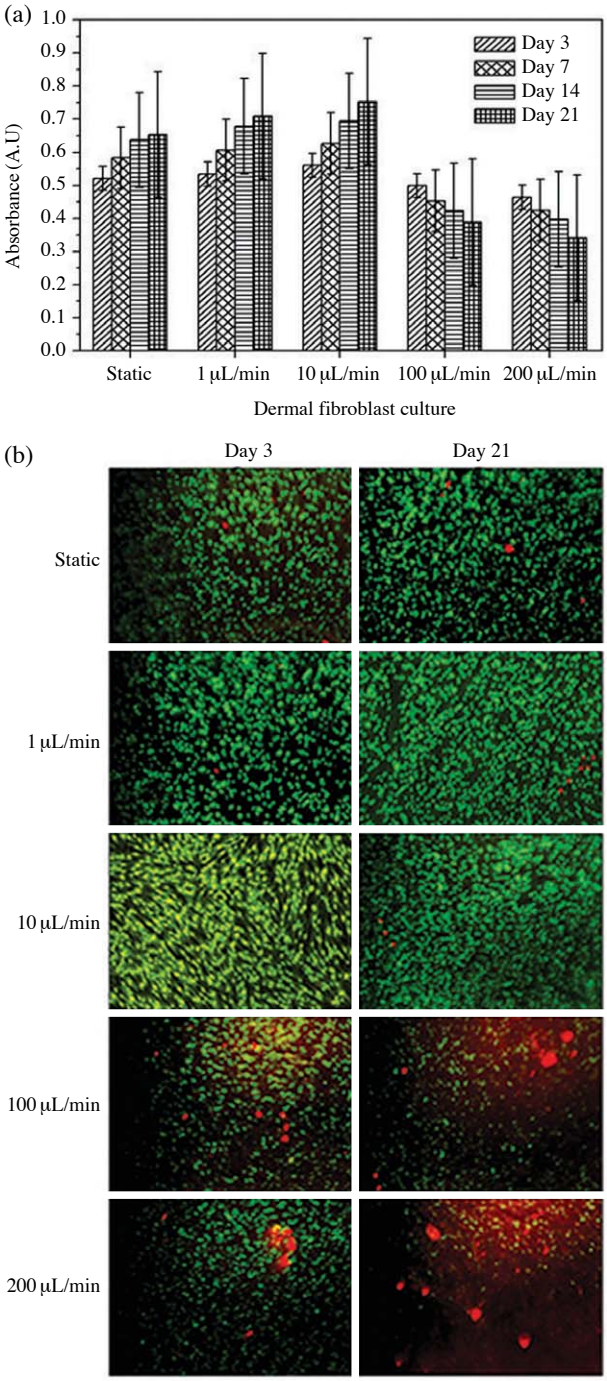

Figure 2. WST-1 cell proliferation assay (a) and live/dead (green/red) cell staining (b) of dermal fibroblast cells grown for 21 days in the cell culture area of microfluidic chip under culture medium perfusion (0.1 to 10 μL/min) in culture channel and SPC perfusion at various flow rates (1, 10, 100 and 200 μL/min) in the oxygen side channel.

cultures under a perfusion rate of 10 μL/min showed higher cell viability than static as well as other perfusion cultures on 21 day culture, owing to the oxygen tension of 1% being appropriate for dermal fibroblasts[24].

Conclusions

In conclusion, the present research developed a simple PDMS microfluidic pump-less perfusion bioreactor, which generates oxygen tension for skin cell culture by diffusion of oxygen from the oxygen side channel. The oxygen side channel was designed 200 μm apart from the cell culture area of the microfluidic chip. It was perfused with oxygen producing biomaterial, sodium percarbonate, at various flow rates to optimize the degree of oxygen diffusion in the cell culture area, without direct contact of chemicals with cells. The flow of SPC in the oxygen side channel was driven by modified siphoning principle for steady and constant pump-less perfusion for a longer period of time. The diffused oxygen was distributed linearly throughout the cell culture area through integrated perfusion in the culture channel, which is one of the main targets of microfluidic bioreactor researchers. The design of the culture channel mimics the physiological branched capillary, which is an added advantage of this microfluidic chip for cell culture. Taken together, the developed pump-less microfluidic perfusion chip works is a simple and cost-effective way for the generation of oxygen and diffusion into the specific cell culture area, by pump-less perfusion of an oxygen producing material. These phenomena have a high potential to be incorporated into tissue regeneration studies, in *in vitro* tissue model development for drug screening studies, and in cancer tissue model studies for understanding angiogenesis, where oxygen tension and perfusion cultures play important roles. So, the new microfluidic channel based bioreactor device could fulfil all the characteristics which are absolutely important for skin cell co-culture. Because it shall provides various features, including hydrophilic surface for supporting the cells to adhere and proliferate, continuous perfusion flow of culture medium with oxygen gradient through this capillary network for human dermal fibroblast cell culture within matrix allows the development of bio-mimetic dermal construct, and it can able extent the layer arrangements for 2nd and 3rd layer cells to grow under immersion and emersion conditions by simply adding and removing the culture medium respectively, without lifting or disturbing the skin construct.

Materials and Methods

Fabrication of microfluidic perfusion bioreactor

PDMS based bio-mimetic branched blood capillary-like microchannels were fabricated by standard soft lithographic technique. For the pump-less perfusion of oxygen producing biomaterial, the oxygen side channel (Figure 3), with a diameter of 400 μm, was set aside from the cell culture area (3 cm in length and 1.5 cm in width) at a distance of 200 μm, were fabricated by following the previous literature[28]. For the fabrication of capillary like branched culture channels, the silicon wafer mold was fabricated using a negative photoresist (SU-8 2050; MicroChem). Followed by, the master mold was taken in a Petri dish, the glass pipette having an outer diameter of 400 μm was fixed on the anchors at the distance of 200 μm from the cell culture area with the help of optical microscope as shown in Figure 3. Then the polydimethylsiloxane (PDMS) (Dow Corning) pre-polymer solution (10 : 1 ratio of base compound and curing agent) was poured above on the Petri dish with mold and pipette setup after removing bubbles and cured on the hot plate for 30 min at 70˚C. After curing the PDMS with glass pipette attached was peeled off the master mold from the Petri dish. Then the glass pipette was pulled out carefully using ethanol as lubricant, thereby the 400 μm diameter oxygen side channel was fabricated on

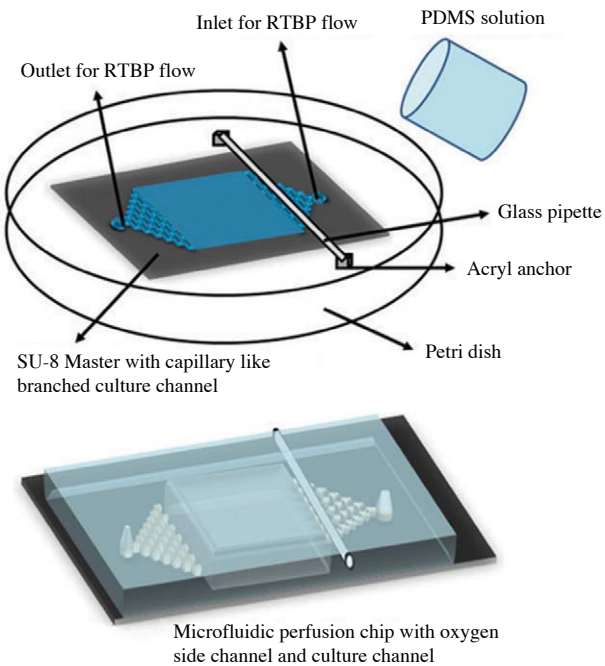

Figure 3. Fabrication of microfluidic perfusion bioreactor chip.

top of capillary-like branched culture channel three dimensionally. Inlet and outlet ports of the capillary-like microchannels were made using PDMS punch. Finally, after oxygen plasma treatment PDMS layer was irreversibly bound with the glass slide to prevent the fluid leakage. Either openings of the oxygen side channel were connected with silicon tubings.

Principle of microfluidic perfusion setup

In order to automatically maintain a constant hydrostatic pressure, and therefore, a steady flow rate perfusion, modified siphoning phenomenon has been adopted from our previous literature with slight alteration[20]. Basically, the siphoning principle drives the fluid flow from the upper reservoir to the lower reservoir, even if the height of tube connecting each reservoir is higher than the heights of both reservoirs[29]. The gravity required to drag the liquid from the short length tube near the upper reservoir is much lower than the gravity driven pulling down of liquid from the top of the tube to the lower reservoir as the tube is longer in length. The fluid is sucked from the upper reservoir continuously due to the high gravity exerted on the fluid at the longest column of the tube to the lower reservoir, which results in a continuous fluid flow.

However, this phenomenon has the drawback of having to manually maintain the constant hydrostatic pressure to keep a steady flow rate, as it changes with respect to the changing liquid level in the upper reservoir. The schematic representation of the pump-less perfusion principle in this work is shown in Figure 4. The flow of aqueous sodium percarbonate, from the higher reservoir at the height of H_1, through a tube connected to the oxygen side channel of the microfluidic chip, ends up in the lower reservoir at the height of H_2, as shown in Figure 4a. As the first step, the siphoned flow is initiated by manual withdrawal using a syringe. The release of withdrawal leads to continuous fluid flow due to the pressure difference between the fluid level in the upper reservoir and the end of the tube (Supporting video). The pressure at upper reservoir is low, whereas, the pressure at the drain point of the tube is lower, since it is at the lowest position. This pressure difference is responsible for the pump-less continuous perfusion of oxygen producing material. Indeed, the elevation head $\Delta H = H_1 - H_2$ in this step 1 determines the velocity and flow rate of oxygen producing material through the oxygen side channel.

$$V_{Step1} = (2g\Delta H)^{1/2} = [2g\,(H_1 - H_2)]^{1/2} \quad (2)$$

$$Q_{Step1} = A * V_{Step1} \quad (3)$$

However, the elevation head in step 1 is variable, which results in a varying flow rate. In step 2, as shown in Figure 4b, the tubing from upper reservoir that is exposed to atmospheric pressure is connected to the tubing from IV set (supporting video). By doing this, the atmospheric pressure at the surface of the upper reservoir has been transferred to the surface of the liquid in the drip chamber of the IV set, and consequently to the air vent. In general, the level of air drops in the tubing and the flow of fluid from the vented IV set is controlled by the pressure exerted by the blood

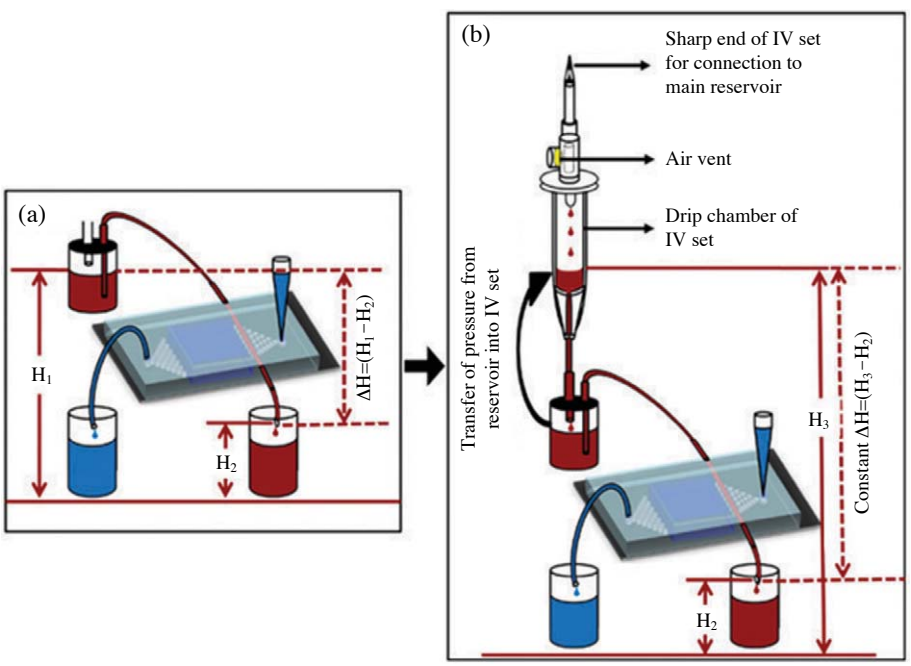

Figure 4. Schematic of working principle of pump-less fluid perfusion in microfluidic cell culture chip. (a) Step 1 of pump-less perfusion of fluid in oxygen side channel. (b) Step 2 of steady perfusion by constant hydraulic pressure controlled by siphoning effect.

on the walls of the veins[30]. Here, pressure build up during the flow of liquid through the microfluidic system initiated by siphon principle acts as the pressure exerted by the blood on the walls of the veins. This pressure develops an air expansion at the surface of upper reservoir and consequently in the drip chamber. This negative pressure in the drip chamber causes the air to flow in and fluid to drip as a droplet from the main reservoir to the drip chamber. The volume of liquid draining out from the chip is equally replenished as a droplet from the main reservoir into the drip chamber, thereby constantly maintaining the elevation head[20]. Therefore the liquid in the drip chamber of the IV set is constant due to the controlled pressure build up, which is powered by the elevation head. Certainly, for step 2, the invariable elevation head $\Delta H = H_3 - H_2$ helps to determine the steady fluid velocity and flow rate as follows.

$$V_{Step2} = (2g\Delta H)^{1/2} = [2g(H_3 - H_2)]^{1/2} \quad (4)$$

$$Q_{Step2} = A * V_{Step2} \quad (5)$$

Principle of oxygen diffusion into culture area

Optical microscopic images of the microfluidic chip, with oxygen side channel and capillary-like branched microchannels and pump-less perfusion setup, are shown in Figure 5 and 6. Since PDMS is well known and widely studied in cell culture for its property of gas permeability[31], the present work investigated the flow of oxygen producing material, sodium percarbonate (SPC) (Sigma aldrich), in the oxygen side channel in the chip, thereby accelerating the diffusion of oxygen into the cell culture area. By fixing various elevation heads, four different flow rates such as 1, 10, 100 and 200 μL/min of oxygen producing material has been studied (Figure 6c) to explore the appropriate flow rate for linear diffusion of oxygen into the cell culture area throughout the culture period. This SPC, a commonly used chemical for oxygen generation, is

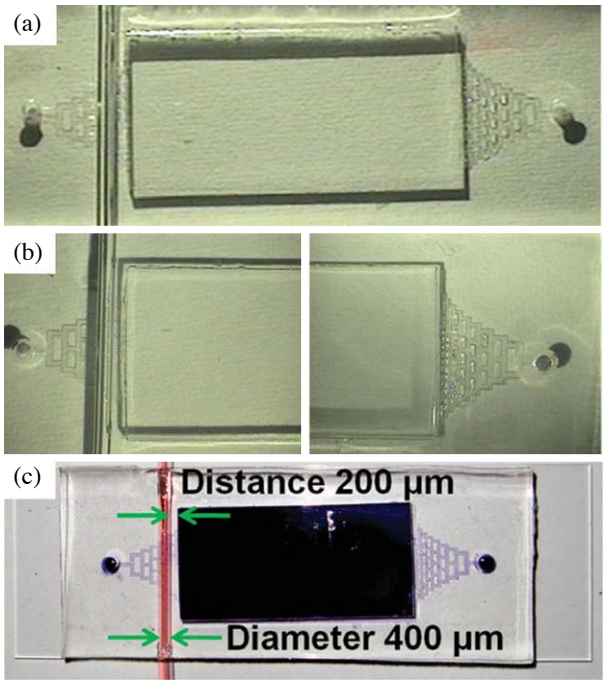

Figure 5. Optical microscopic images with different magnifications ((a) 10 X (b) 20 X) and (c) photo of food dye filled microfluidic chip with oxygen side channel, capillary-like branched channels and culture area.

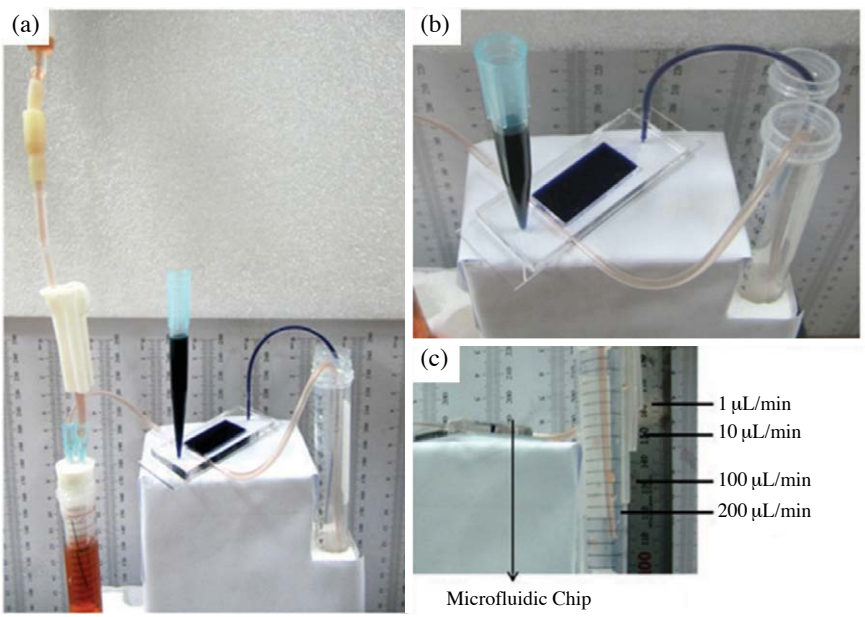

Figure 6. (a & b) Pump-less perfusion setup showing fluid flow in culture channel and oxygen side channel. (c) Adjustable flow rate through the oxygen side channel is powered by controlling the elevation head via simply changing the height of the drain tube.

an adduct of hydrogen peroxide and sodium bicarbonate, which liberates oxygen spontaneously upon contact with water, based on the chemical equation below[28].

$$[Na_2CO_3]_2 \bullet 3H_2O_2 \rightarrow 2Na^+ + 2CO_3^{2-} + 3H_2O_2 \quad (6)$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (7)$$

It has been calculated using the ideal gas law and stoichiometry of the equation that 1 gram of SPC can generate about 120 mL of oxygen[28]. Hence, perfusion of this aqueous SPC on oxygen side channel accelerates the diffusion of liberated oxygen through the gas permeable PDMS chip into the cell culture area. In order to assess the oxygen diffusion, a fluorescent probe sensitive to oxygen, Ruthenium-tris (2,2′-bipyridyl) dichloride hexahydrate (RTBP) (1 mg/mL) (Sigma aldrich), for which the fluorescence intensity quenches in the presence of oxygen, was introduced into the cell culture area. Prior to the introduction of RTBP, the channels were flushed with nitrogen gas in order to eliminate the oxygen. For control, fluorescence intensity of 1 mg/mL of RTBP in deionized water was analyzed as ~0% oxygen. The successful oxygen diffusion was confirmed by analyzing the sequential quenching of fluorescence intensity of RTBP dye in the cell culture area (at the position closer (5 mm) to the oxygen sidechannel) from the control. Likewise, the successful oxygen distribution throughout the cell culture area was confirmed by quenching analysis at different positions (5, 10, 15, 20, 25 mm) within the cell culture area. Intensity was measured at different time intervals (1 to 72 hr) with respect to the four different SPC flow rates (1, 10, 100 and 200 μL/min).

Dermal fibroblast culture in micro-bioreactor

Human dermal fibroblast cells (HDFn) were cultured in supplemented basal medium 106 (Invitrogen) with 1% penicillin-streptomycin antibiotic. At 70% cell confluency, cells were detached from the culture vessel using trypsin/EDTA (Sigma aldrich), washed with PBS and counted using haemocytometer (Marienfeld laboratory glassware). The microfluidic perfusion chip was disinfected with 70% ethanol overnight and UV sterilized for 30 min prior to cell culture. HDFn cell density of 10^5 cells/mL was cultured in the cell culture area of microfluidic chip and incubated at 37 ˚C with 5% CO_2 and humidified environment in a controlled incubator. After 24 hr, SPC perfusion and culture medium perfusion were initiated. On 3, 7, 14 and 21 day cultures, cell proliferation efficiency on microfluidic bioreactor was elucidated using a WST-1 cell proliferation kit (Roche) by following the manufacturer's protocol. Experiments in triplicate were carried out and the statistical significance ($p < 0.05$) was analyzed using one way analysis of variance ANOVA.

On 3 and 21 day cultures, cells in the chip were stained with live/dead cell staining dye kit (Biovision) following the manufacturer's procedure. From the kit, mixture A (live-dye, a cell-permeable green fluorescent dye, Ex/Em=488/518 nm) stains live cells and mixture B (propidium iodide, a cell non-permeable red fluorescent dye, Ex/Em=488/615 nm) stains dead cells specifically. Finally, the stained cells were examined under a fluorescence microscope by using FITC and rhodamine filter.

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