A Pumpless Perfusion Cell Culture Cap with Two Parallel Channel Layers Keeping the Flow Rate Constant

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This article presents a novel pumpless perfusion cell culture cap, the gravity-driven flow rate of which is kept constant by the height difference of two parallel channel layers. Previous pumpless perfusion cell culture systems create a gravity-driven flow by means of the hydraulic head difference (Δh*) between the source reservoir and the drain reservoir. As more media passes from the source reservoir to the drain reservoir, the source media level decreases and the drain media level increases. Thus, previous works based on a gravity-driven flow were unable to supply a constant flow rate for the perfusion cell culture. However, the proposed perfusion cell culture cap can supply a constant flow rate, because the media level remains unchanged as the media moves laterally through each channel having same media level. In experiments, using the different fluidic resistances, the perfusion cap generated constant flow rates of 871* $\pm$ *27* $\mu L\ h^{-1}$ *and 446* $\pm$ *11* $\mu L\ h^{-1}$*. The 871 and 446* $\mu L\ h^{-1}$ *flow rates replace the whole 20 mL medium in the petridish with a fresh medium for days 1 and 2, respectively. In the perfusion cell (A549 cell line) culture with the 871* $\mu L\ h^{-1}$ *flow rate, the proposed cap can maintain a lactate concentration of about 2200 nmol* mL^{-1} *and an ammonia concentration of about 3200 nmol* mL^{-1}*. Moreover, although the static cell culture maintains cell viability for 5 days, the perfusion cell culture with the 871* $\mu L\ h^{-1}$ *flow rate can maintain cell viability for 9 days.* © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 1466–1471, 2012
Keywords: perfusion cell culture, continuous cell culture, bioreactor, A549 cell line

Introduction

Cell metabolism is affected by the environmental conditions such as dissolved oxygen, nutrient, and metabolite concentration.[1] Perfusion cell culture technology provides more well-defined and homogenous cell culture environment by continuously supplying nutrient and removing waste from cell metabolism. Most conventional cell cultures (static cell cultures) supply culture medium in a noncontinuous manner. The manual and periodic replacement of a medium risks contamination and, more importantly, results in a fluctuating culture environment.[2,3] However, a perfusion cell culture keeps the culture environment stable and provides a well-defined condition for high accurate cell-based assay and high efficiency cell bioreactors by continually providing nutrients and removing waste.[3–8] Moreover, a stable culture environment is essential for increasing protein expression,[8,9] cell-to-cell communication,[10] and cell metabolism.[11] Those factors increase accuracy of the cell based assay as well as the productivity of mammalian cell culture bioreactor.

There are two main types of perfusion cell culture systems: pump systems and pumpless systems. External pumps such as syringe pumps and peristaltic pumps[12–14] are used to supply fresh medium and remove waste. However, some of these pumps are bulky and expensive; and they have many tubes and power unit that take up large amounts of space. So, it is complex and labor-intensive to handle a perfusion system within conventional equipment, such as an incubation chamber or a petridish.

In pumpless systems,[15–19] fresh media is supplied by means of the hydraulic head difference (Δh) between a vertical source reservoir and a drain reservoir. However, the media levels of the source and drain reservoirs change when a medium passes from the source reservoir to the drain reservoir. A decrease and increase in the media levels of the source and drain reservoirs reduce the hydraulic head difference (Δh), which consequently decreases the flow rate. Thus, existing perfusion systems[15–19] must either make the Δh value very high as a means of ignoring any changes in the media level of the reservoirs or pack the cell culture area[15,19] to maintain the media levels.

In this article, we propose a new design for the source reservoir and the drain reservoir to prevent the media level from changing and to generate a constant flow rate. In our proposed perfusion device, the source and drain reservoirs are made with two parallel channel layers. The two layers ensure a constant hydraulic head difference (Δh) because the

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media moves laterally through each channel having same media level and the media level remains unchanged. In a practical application, we applied the proposed method to the cap of a petridish which is one of conventional cell culture tools. So, the proposed method is easy for a general user to handle and culture cell with general bioexperiment protocol or equipments such as incubation chamber. The proposed perfusion cap is able to perform perfusion cell culture by just putting cap on a dish in the conventional incubation chamber without any tubes and power cables.

Materials and Methods

Cell preparation

The A549 cell lines of nonsmall cell lung cancer were used to demonstrate the feasibility of the proposed perfusion cap. The A549 cell lines, which were obtained from ATCC (#CCL-185), were cultured a media of Roswell Park Memorial Institute (RPMI) medium 1640 (CellGro, #10-040-CV) supplemented with 10% (v/v) fetal bovine serum (CellGro, #35-010-CV) and 1% (v/v) penicillin-streptomycin (Invitrogen, #15140-122). We used a 5% CO_2 incubator (Sheldon Mfg), and the cell was routinely passaged at a confluence level of 80% at every 2–3 days. For the collection of cells, we used a washing and detaching process with a solution of 0.25% trypsin-EDTA (Invitrogen, #25300-062) which was neutralized with 10% RPMI-1640. The solution was centrifuged at 2,000 rpm for 3 min and then resuspended in a fresh media. The density of cells are calculated by the automatic cell counting kit of AccuChip (Digital Bio) and make target cell density. For the cell passage, 5×10^5 cells of the detached cells seeded in a 100-mm petridish (Corning, #430167); the dish was filled with 20 mL of the media for cell culture. The passage number of the cells used in the experiments is about 20–30.

Principle and design of the perfusion cap

The proposed perfusion cap (Figure 1) consists of two channel layers which act as the source reservoir (the second channel layer) and the drain reservoir (the first channel layer). The cross-sectional area of each channel is 4 mm × 4 mm. So, when a medium enters or exits the channel, it moves laterally through the channel. Figure 1 shows the working principle of the present device. A fresh medium fills the second channel layer (the source reservoir). Gravity causes the fresh medium in the second channel layer to flow into the petridish. Because the first and second channel are connected, when the fresh medium in the second channel layer moves laterally to the right direction through the second channel, the air filling the first channel layer also move to the left direction through the first channel. As the air moves to the left direction in the first channel, the old medium in the petridish is sucked up to the first channel layer and moves laterally to the left direction through the first channel. As a portion of the fresh medium flows out from the first channel layer, the same portion of the old medium is sucked up into first channel layer. Regardless of how much of the fresh medium flows out, the height difference between the first and second channel layers (the hydraulic head difference, Δh) remains constant, because we design that the old and the fresh media move laterally through only the first and second channel layer, respectively. Thus, the constant hydraulic head difference (Δh) produces a constant

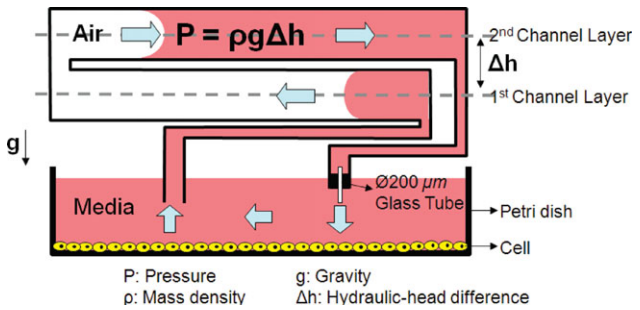

Figure 1. A pumpless perfusion cell culture cap that changes medium with a constant flow rate.

flow rate. The proposed perfusion cap is placed on a petridish. The gap between the cap and petridish is 300 μm for CO_2 gas exchange. So, new media flowing in the petridish could be exposed to CO_2 gas which maintains pH 7.4 for cell culture.

Figure 2 shows the design of the pumpless perfusion cell culture cap for a petridish. To generate a constant flow rate, we attached source and drain reservoirs to the cap of the petridish. Thus, the general user can easily use it with conventional cell culture equipment, such as a petridish and an incubation chamber. To prevent any changes in the media level, we made sure that the first channel layer (the drain reservoir) and the second channel layer (the source reservoir) were sufficiently narrow for a medium to move laterally in each channel layer. As long as the fresh medium and the sucked medium move laterally in the second and first channel layers respectively, the hydraulic head difference (Δh) remains constant. As shown in Figure 2a, a single channel is bent at each channel layer, and hole ① connects the two channel layers. Hole ② is the source outlet, and hole ③ is the drain inlet. A fresh medium flows through hole ② from the second channel layer (the source reservoir) to the petridish and waste is sucked up through hole ③ from the petridish into the first channel layer (the drain reservoir). The channel is 4-mm wide and 4-mm high. And the total volume of each channel layer is about 30 mL. A precise glass capillary is connected at the end of hole ②. The flow rate depends on the diameter and length of the glass capillary.

Figure 3 shows how the proposed device is filled with a fresh medium. After dipping the glass capillary into a fresh medium, the fresh medium is sucked by a syringe that is connected to the end of hole ③ as shown in Figure 3a. Only the second channel layer must be filled with the fresh media for media to flow through hole ② not through hole ①. To warm up the media and air of the first channel, the cap is placed in the incubation chamber for 10 min. After that, we put the cap on the petridish for culture cells in the incubation chamber without any interconnecting tubes. As Figure 3b, when the fresh media in 2nd channel layer flow into the petridish, old media which are same amount of fresh media suck up and fills 1st channel layer. Finally, the fresh media stop flowing at the media level of 1st channel when all inflowing fresh media flow into the petridish (Figure 3c). Figure 4 shows the machined perfusion cap placed on the petridish.

Fabrication of the perfusion cap

The fabrication process of the present perfusion cap is composed of two steps: (1) cutting and drilling plastic for making channels and holes, (2) bonding three parts (1st

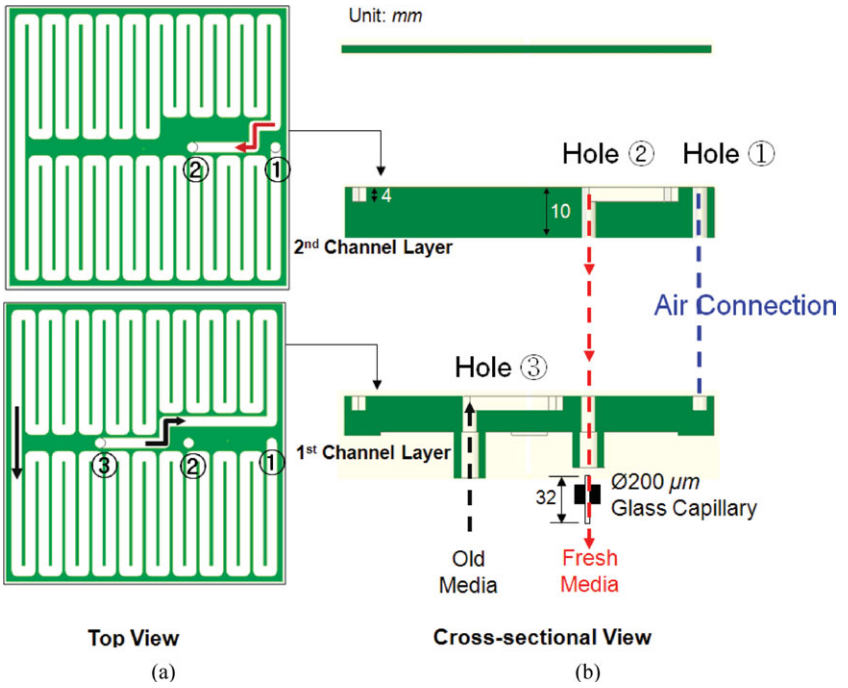

Figure 2. Design of a pumpless perfusion cap with the two channel layers: (a) top view; (b) cross-sectional view.

channel layer, 2nd channel layer, and cover plate) with epoxy. The plastic material is a cyclic olefin copolymer (COC). The cyclic olefin copolymer (COC) has a transparency and an excellent biocompatibility as well as has enough stiffness for the physical machining. Computer numerical control (CNC) machine tools are used for three parts (1st channel layer, 2nd channel layer, and cover plate) by cutting and drilling the cyclic olefin copolymer (COC). To bonding three parts, the thin epoxy is evenly put on the surface of the plastics and three parts are combined for 30 min until the epoxy is stiffened. Before using the perfusion cap, the caps are dipping into 70% ethanol for 30 min to sterilize caps.

Analysis of the flow rate and shear stress

The flow rate (Q), which is a function of the pressure drop (ΔP) and the fluidic resistance (R), can be expressed as follows:

$$Q = \frac{P}{R} \tag{1}$$

When two parallel channel layers are used, the hydraulic head difference remains constant and the hydraulic pressure drops as follows:

$$\Delta P = \rho g \Delta h, \tag{2}$$

where ρ is the fluid density, g is the gravity acceleration, and Δh is the hydraulic head difference. If we keep Δh constantly, the pressure drop is also constant. In this work, Δh is the height difference between two channel layers which remains constantly. When the pressure drop is constant, the constant flow rate is in inverse proportion to the fluidic resistance. The fluidic resistance is calculated in terms of the channel width (w), height (h), and length (l) and the shape of the cross section.[20]

Thus, the fluidic resistance for a circular cross section of the channel:

$$R = \frac{8\mu l}{\pi R^4}. \tag{3}$$

And the fluidic resistance for a rectangular cross section of the channel:

$$R = \frac{12\mu l}{wh^3}\left[1 - \frac{h}{w}\left\{\frac{192}{\pi^5}\sum_{i=1,3,5,\ldots}^{\infty}\frac{1}{i^5}\tanh\left(\frac{\pi w i}{2h}\right)\right\}\right]^{-1}. \tag{4}$$

In our device, the media passes through the channels of the two channel layers and a very narrow glass capillary.

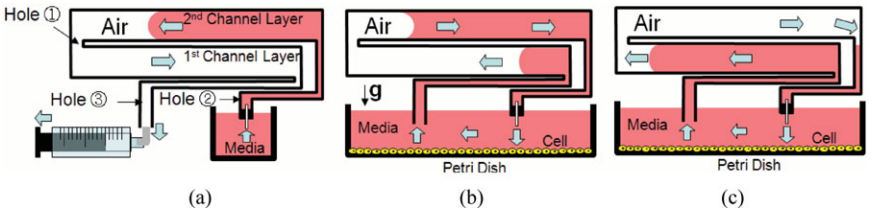

Figure 3. Experimental procedure and setup: (a) the second channel layer is filled with a fresh medium; (b) the cap is placed on a petridish for starting perfusion. The fresh media in 2nd channel layer flow in the petridish. Same amount of old media in the petridish suck up and fill 1st channel layer as the fresh media flowing out; (c) All fresh media in 2nd channel layer flow out and stop flowing at media level of the 1st channel layer.

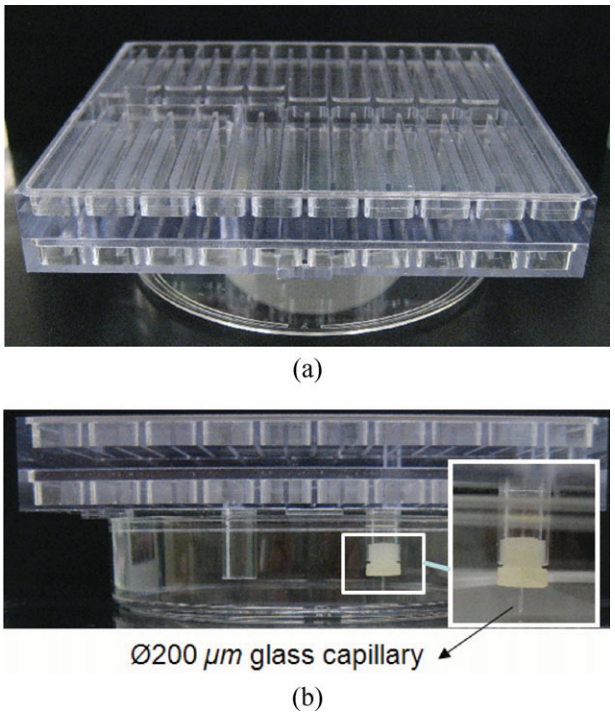

Figure 4. Machined pumpless perfusion cap: (a) top view; (b) sectional view.

There are two kinds of fluidic resistance: one kind is from the channel layer (R_C); the other kind is from the glass capillary (R_G). The value of R_C was designed to be much lower than the value of R_G. With this difference, the flow rate can be changed conveniently by varying the dimensions of the glass capillary. The channel is 4-mm wide and 4-mm high. Each of these dimensions is 200 times greater than the diameter of the glass capillary. The value of R_C (for a height of 4 mm, a width of 4 mm, and a length of 1,866 mm) is 0.087 Pa $s^{-1}\cdot\mu L^{-1}$. In contrast, the value of R_G (for a diameter of 200 μm and a length of 32 mm) is 815.3 Pa $s^{-1}\cdot\mu L^{-1}$. The fact that the value of R_C is 9,320 times smaller than that of R_G means that R_C can be ignored. In our experiments, the required flow rates are about 865 and 432 μL h^{-1}. These rates ensure that 20 mL of the culture medium in the petridish is replaced on day 1 and 2, respectively. On the theoretical basis of (1), (2), and (3), flow rates of 865 and 432 μL h^{-1} can be generated if the glass capillaries have a diameter of 200 μm and lengths of 16 and 32 mm and the hydraulic head difference (Δh) is 10 mm.

The flow rate is proportional to the cross sectional area of the capillary and inversely proportional to the length of the glass capillary. In this article, the flow rate could be easily changed by the length of the same diameter glass capillaries. To verify the uniformity and variability of the flow rates for glass capillaries (Drummond Microcaps #1-000-0005, 200 μm diameter) with lengths of 32, 24, 16, and 8 mm, we start to measure flow rates after 8-h incubation. We measured the length of the channel of the second channel layer during 2 h. And then, the volume of media could be calculated by dimension of channel (4-mm wide, 4-mm high, and measured length media pass). All the data were measured three times and we used a fresh medium in the CO_2 incubator at 37°C.

To verify if shear stress due to high flow rate is effect cell detachment or cell growth, the shear stress in the proposed cap is analyzed by SOMSOL ver.4.2 software. Figure 5

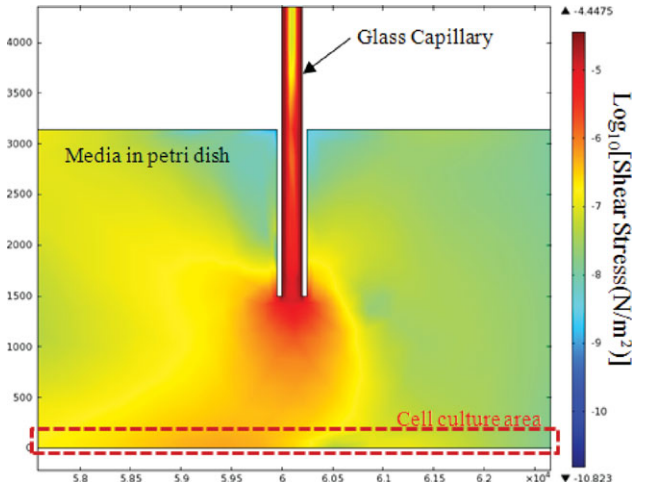

Figure 5. Numerical analysis for the distribution of shear stress near glass capillary and on the bottom of petridish (cell culture area) at 871 μL h^{-1} flow rate. The shear stress on the bottom of petridish is about 1×10^{-7} N m^{-2} (yellow region in Figure).

shows the shear stress at 871 μL h^{-1} flow rate. The highest shear stress (about 1×10^{-5} N m^{-2}) is generated near glass capillary in the proposed cap due to the narrow diameter (200 μm) of glass capillary. However, the bottom of the petridish (culture area) is far away from glass capillary (about 1 mm) and has low shear stress (about 1×10^{-7} N m^{-2}). Previous research has shown that cells culture well when the shear stress is $<1 \times 10^{-5}$ N m^{-2} shear stress.[21]

Lactate and ammonia and cell proliferation assay

For concentration measurement of lactate and ammonia, a BioVision lactate assay kit (#K607-100) and a BioVision ammonia assay kit (#K370-100) are used, respectively. All the data were measured twice. We extract 1 mL media from the petridish and centrifuge at 13,000*g* for 10 min to remove insoluble material. To test all samples for 9 days at once, we store samples in -20°C. Viable Cells are counted using CCK-8 assay kit (Dojindo Molecular Technologies, Kumamoto, Japan). Viable cells are proportional to absorbance of CCK-8 solution.

Statistical analysis

To analyze the difference of by-products and cell viability between static and perfusion cell culture, P values are calculated by one-way ANOVA (analysis of variance). Statistically difference was considered when $P < 0.05$. "*" is that P value is <0.05, which mean the data is statistically different from the data of the static cell culture.

Results and Discussion

Measurement of the flow rate

Figure 6 shows flow rates according to 1/length of the glass capillary. The measured flow rates are 446 ± 11 μL h^{-1} for the 32 mm length, 640 ± 17μL h^{-1} for the 24 mm length, 871 ± 27 μL h^{-1} for the 16 mm length, and 1,583 ± 104 μL h^{-1} for the 8 mm length. The standard deviations of the measured flow rates were <7%. The measured flow rate closely matches the theoretical number derived from (3). R^2

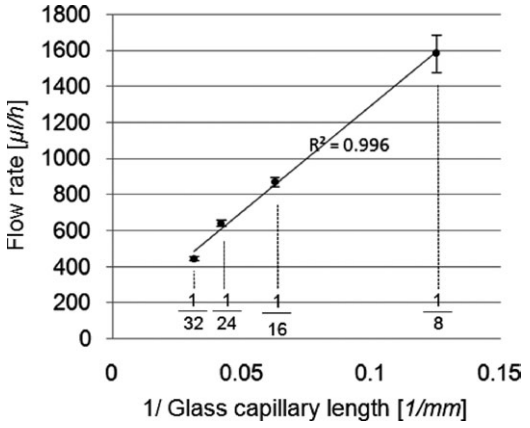

Figure 6. The measured flow rates over time.

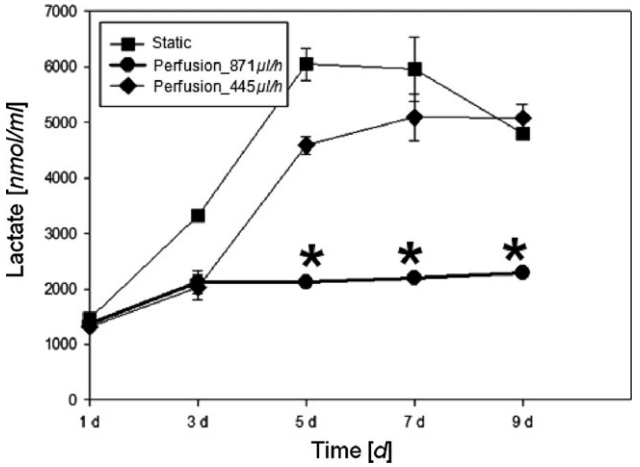

Figure 7. Comparison of the lactate concentration of the static and perfusion cell cultures over time (*P value < 0.05 compared to static cell culture, two-sided paired t test).

values after liner regression are 0.996, indicating that there is a good correlation between the length of the glass capillary and the flow rate. Thus, the results confirm that the proposed method can supply a medium at a constant flow rate and that the flow rate can be easily changed by using a glass capillary of different dimensions.

Measurement of by-products in cell cultures

In a cell culture, by-products such as lactate and ammonia accumulate in the culture medium over time. The best perfusion effect in a cell culture is the fact that the culture environment is kept stable by the supply of nutrients and the removal of waste. In the perfusion cell cultures, a culture medium that is supplied at a slow (446 μL h^{-1}) flow rate or a fast (871 μL h^{-1}) flow rate change the whole 20 mL medium in the petridish on days 2 and 1, respectively. To demonstrate the perfusion effect, we measured the lactate concentration (Figure 7) and the ammonia concentration (Figure 8) over time for a static and perfusion cell cultures. In the case of the fast (871 μL h^{-1}) flow rate perfusion cell culture, the lactate and ammonia concentrations were stable and constant for 9 days, though the static cell culture shows an increase in the concentration of both the lactate and the ammonia. However, the slow (446 μL h^{-1}) flow rate perfusion cell culture fails to replace enough by-products with a fresh cell culture medium. This failure is what causes the increase of the lactate and ammonia concentrations in the static cell culture. In case of lactate in Figure 7, the fast (871 μL h^{-1}) flow rate perfusion cell culture shows significant smaller lactate concentration from 3 days than static cell culture. In case of ammonia in Figure 8, the fast (871 μL h^{-1}) flow rate perfusion cell culture shows significant smaller ammonia concentration from 7 days than static cell culture.

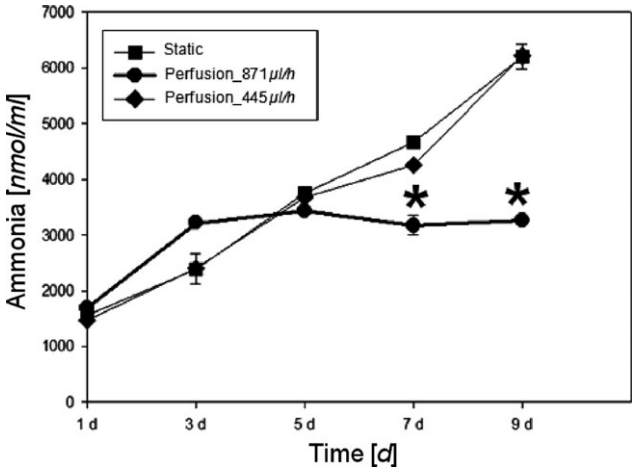

Figure 8. Comparison of the ammonia concentration of the static and perfusion cell cultures over time (*P value < 0.05 compared to static cell culture, two-sided paired t test).

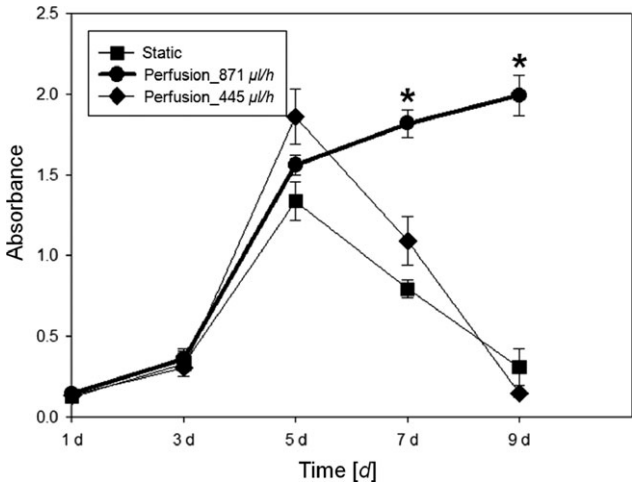

Figure 9. Comparison of absorbance of static and perfusion cell cultures. Absorbance of CCK-8 is proportion to the number of viable cells. (*P value < 0.05 compared to static cell culture, two-sided paired t test).

Measurement of cell growth

The proposed perfusion cap ensures long time cell culture because a stable condition maintains the cells for a long time. To check the behavior of the proposed perfusion cap in a prolonged cell culture, we measured the viability of cells in static and perfusion cell cultures over time. Figure 9 shows the cell viabilities for one static and two perfusion cell cultures. All the data were measured twice. For 5 days,

Table 1. Doubling Times of the A549 Cell Line According to the Cell Culture Types

Cell Culture Types	Static	Perfusion	
		High Flow Rate (871 μL h^{-1})	Low Flow Rate (446 μL h^{-1})
Doubling time (h)	26.41	26.46	26.35

the cell viability levels of the perfusion cell culture were similar to those of the static cell culture. The perfusion cell cultures with the slow (446 μL h^{-1}) flow rate and the fast (871 μL h^{-1}) flow rate looked similar for 5 days. Table 1 shows the similarity of the doubling times of the static and two perfusion cell cultures. These results were calculated between days 1 and 5. However, after 5 days, the viabilities of fast (871 μL h^{-1}) flow rate perfusion cell culture are significantly different from the viability of the static cell culture and slow (446 μL h^{-1}) flow rate perfusion cell culture. While the cell viability of the static cell culture dramatically decreased, the perfusion culture with the fast (871 μL h^{-1}) flow rate showed a slight increase in cell viability. The perfusion cell culture with the slow (446 μL h^{-1}) flow rate displayed cell viability for 7 days; however, after that time, the viability level diminished. As shown in Figures 7 and 8, the perfusion cell culture with the slow (446 μL h^{-1}) flow rate has a similar increase in lactate and ammonia by-products as the static cell culture. Thus, the environment of the perfusion cell culture with the slow (446 μL h^{-1}) flow rate is unstable because all by-products are not sufficiently replaced with fresh nutrients due to slow flow rate.

Conclusion

Our novel pumpless perfusion cell culture device uses the height difference between two parallel channel layers to produce a constant gravity-driven flow rate. In the proposed perfusion cap, we used glass capillaries with a diameter of 200 μm and lengths of 16 and 32 mm. The cap successfully supplies a fresh medium and removes the by-products. The cap with the 16-mm long glass capillary has a flow rate of 871 $\pm$ 27 μL h^{-1}, and the cap with the 32-mm long glass capillary has a flow rate of 446 $\pm$ 11 μL h^{-1}. For 9 days, we subjected a A549 cell line to a static cell culture and a perfusion cell culture; we also analyzed the by-products and cell viability levels. The experimental results confirm that the perfusion cell culture with the fast flow rate (871 μL h^{-1}) maintained a stable environment and cell viability for 9 days. Thus, our experiment demonstrates that the perfusion cell cap can create a stable environment by changing the medium at a constant rate without using any additional equipment. By extension, if the proposed perfusion cap is successfully miniaturized, it could be applied to well plates such as 24- or 48-well plates.

Acknowledgments

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