3D Microfluidic Perfusion Cell Culture System for Concentration Gradient and Air Bubble Trapping Functions

Dong Hyeok Park[1], Xuan Don Nguyen[1], Moon Jeong Kim[1], Karl Morten[2] and Jeung Sang Go[1]
[1]*School of Mechanical Engineering, Pusan National University, Busandaehak road 63-2, Busan, Republic of Korea*
[2]*Nuffeild Department of Obstetrics & Gynaecology, University of Oxford, Oxford, U.K.*

Keywords: Cell Culture, Three Dimensional Culture, Perfusion, Concentration Gradient, Microchannel Mixer, Air Bubble Trap.

Abstract: This paper presents a cell culture well-plate for three dimensional perfusion cell cultures. A concentration gradient generator, a microchannel system, is embedded in the plate bottom for not only the perfusion culture but transfer of reagents with linear concentration gradient to 4 wells of the plate. The concentration uniformity of gradient generated is guaranteed by adding microchannel mixers at the end of generator. Sudden expansion reservoirs, air bubble traps, make perfusion cell cultures plate long-term culture without interruption of perfusion flow caused by injection of air bubbles in the microchannels. The performance of the developed 3D microfluidic perfusion cell culture system is examined experimentally and compared with analytical results. Then, it is applied to test the cytotoxicity of cells infected with Ewing's sarcoma. Cell death is observed for different concentrations of H_2O_2. Finally, the 3D perfusion cell culture well-plate is presented with not only similar structure to conventional 3×4 well-plate but expansion of concentration range from a 4 fold of dilution in 4 wells to a 100 fold of dilution in 7 wells.

1 INTRODUCTION

In vitro cell culture has been used for detecting biomarkers from cell growth with reagents. Incubating conditions like 37 ℃ of temperature, 5-10 % of CO_2 control to keep pH condition for media, relative humidity and sterilization are arranged for *in vitro* culture (Schumacher and Strehl, 2002; Caicedo-Carvajal and Liu, 2011). Even though the conditions are satisfied, the 2D culture, which cultures cells onto plates with media and nutrient factors without circulation of them, could be understood individual cellular phenomena but is difficult to capture the physiological behaviour of cells *in vivo* (Baker and Chen, 2012).

Differences of the cell morphologies, protein expressions and cell proliferations are reported between 2D culture and 3D perfusion culture, which mimics *in vivo* environment containing transportation of cellular growing factors like oxygen and nutrients, emission of cellular wastes (Baker and Chen, 2012; Li and Valadez, 2012; Caicedo-Carvajal and Liu, 2012).

For these reasons, 3D perfusion systems have been suggested to address closer environment with in vivo environment (Li and Valadez, 2012; Ong and Zhang, 2008; Yi and Lin, 2017), but they are needed to address numerous experiments with varying concentration of their target reagents or drugs and a significant gap exists between the producers of microfluidic technologies (mainly engineer) and end-user (expert in life sciences) for manipulation of microfluidic devices (Langelier and Livak-Dahl, 2011).

We present a 3D perfusion cell culture plate based on microfluidic design with a structure similar to conventional 2D well-plate for end-user's favor. It not only transfers injecting samples like reagents, media or drugs into cells, but also generates their concentration gradient for high-throughput experiments.

Sudden expansion reservoirs arranged in ahead of microchannel system prevent air bubbles, which disturb designed microfluidic functions (Sung and Shuler, 2009), from introduction into the microchannel system for keeping stable injection of samples and long-term culture.

Park, D., Nguyen, X., Kim, M., Morten, K. and Go, J.
3D Microfluidic Perfusion Cell Culture System for Concentration Gradient and Air Bubble Trapping Functions.
DOI: 10.5220/0007472001990206
In *Proceedings of the 12th International Joint Conference on Biomedical Engineering Systems and Technologies (BIOSTEC 2019)*, pages 199-206
ISBN: 978-989-758-353-7

2 DESIGN OF THE 3D MICROFLUIDIC PERFUSION CELL CULTURE SYSTEM

Figure 1 shows the schematic drawing of the 3D microfluidic perfusion cell culture plate developed in this study. It consists of two plates, the cell culture plate and the perfusion channel plate. In the cell culture plate, the four culture wells are designed with the same size as the wells in a general 2D 3×4 well-plate and the microfluidic system integrated with a concentration gradient generator, microchannel mixers and sudden expansion reservoirs for trapping injecting air bubbles embedded in the bottom of the culture plate.

2.1 Concentration Gradient Generator

The concentration gradient generator was designed for high-throughput sample screening. Concentration of samples like drugs or reagents could be obtained by mixing two different liquids with a linear volume flow rate ratio.

Figure 1(b) shows the linear concentration gradient generator. The microchannel is bifurcated into four branch microchannels. To distribute the volumetric perfusion flow rate, the flow resistance was considered. Based on the Poiseuille's law, the flow resistance can be obtained by dividing the pressure drop with the perfusion flow rate, analogous to Kirchhoff's circuit law in electricity, and it is expressed in Eq. 1.

$$R_f = \frac{\Delta P}{Q} = \frac{128\mu L}{\pi {D_h}^4} \quad (1)$$

where R_f is the flow resistance, ΔP is the pressure drop, Q is the perfusion flow rate, D_h is the hydraulic diameter, μ is the dynamic viscosity of a fluid, and L is the channel length. The flow resistance is proportional to the length and reversely proportional to the fourth power of the hydraulic diameter of the branch microchannel. Thus, in the design of the linear concentration gradient generator, the length of the branch microchannel was varied to control the volumetric perfusion flow rate because a small variation in the diameter as a result of the fabrication accuracy sensitively affects a large change in the flow resistance.

In the design, four different concentrations of 40%, 30%, 20% and 10% were considered for the four perfusion culture wells, which were the linear concentration gradient.

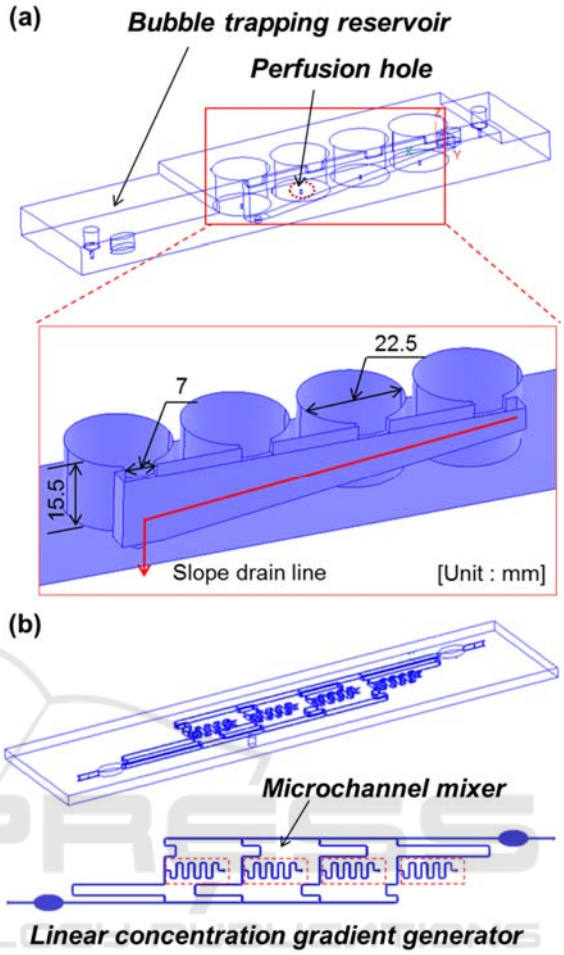

Figure 1: Proposed 3D microfluidic perfusion cell culture system. (a) Upper cell culture well plate, (b) bottom perfusion channel plate.

When a solute and a dilute are introduced through two inlets, the solute is divided into the four branch microchannels with a ratio of 40%, 30%, 20% and 10% of the perfusion flow rate and the dilute is divided with a ratio of 10%, 20%, 30% and 40%. Then, they meet with the ratios of the volumetric flow rates of 4:1, 3:2, 2:3 and 1:4, respectively.

2.2 Microchannel Mixer

When the solute and dilute are ejected from the concentration gradient generator, they are in a laminar flow region with low Reynolds number caused by the small hydraulic diameter of the microchannel. Thus, they are required to be mixed well to obtain a uniform concentration in the perfusion culture wells.

Due to the difficulty of using turbulent mixing as well as ultrasonic or magnetic stirring in a laminar flow, the mixing is mainly depended on diffusion of the solute molecules into the dilute through the interface of two laminated fluid flows and the diffusion is occurred along the microchannel. The channel length, L_{mixing}, to complete the mixing of the two laminated fluids can be calculated by multiplying the diffusion time, τ and the fluid velocity, u and expressed in Eq. 2 (Nguyen and Wereley, 2006).

$$L_{mixing} = u\tau = u\frac{w^2}{2D} \quad (2)$$

where w is the microchannel width and D is diffusive coefficient. The velocity of the fluid flow, u is also obtained by dividing the perfusion flow rate with the cross-sectional area.

Additionally, to improve the mixing efficiency and size compactness, the meandered microchannel mixer was designed and numerically simulated. In the analysis, diffusion of methanol solution by referring rhodamine-110, a fluorescent substance, into water, was considered. Injecting flow rate was 200 μl/min through each inlet. The width and depth of the microchannel mixer was 0.5 and 2 mm, respectively. The theoretical length to complete the diffusion mixing was calculated as 60 mm from Eq. 2. The mixing ratio was evaluated by calculating the coefficient of variation (hereafter, C.V.) of the solute concentration on the cross-sectional plane along the microchannel mixer. The C.V. is defined in Eq. 3.

$$C.V. = \frac{Standard\ deviation}{Mean\ concentration} \times 100\ (\%) \quad (3)$$

To determine the uniform mixing on the cross-sectional plane, the mixing was evaluated when the C.V. was less than 5%. Finally, the concentration gradient generator was simulated by connecting the branch microchannels and the meandered microchannel mixers with a length of 60 mm. Figure 2 shows the linear concentration gradient generator with ratios of 39.7%, 29.9%, 20.0% and 10.4%. The error was evaluated to be less than 4% compared with the design values.

2.3 Sudden Expansion Reservoir

When air bubbles adhere onto the surface of the microchannels, the designed flow resistance in the concentration gradient generator couldn't be kept their designed value. The sudden expansion reservoir is a cylindrical shape's chamber collecting or trapping air bubbles before their entering into the microchannel. When air bubbles existed in the samples injected through the inlet and reached into

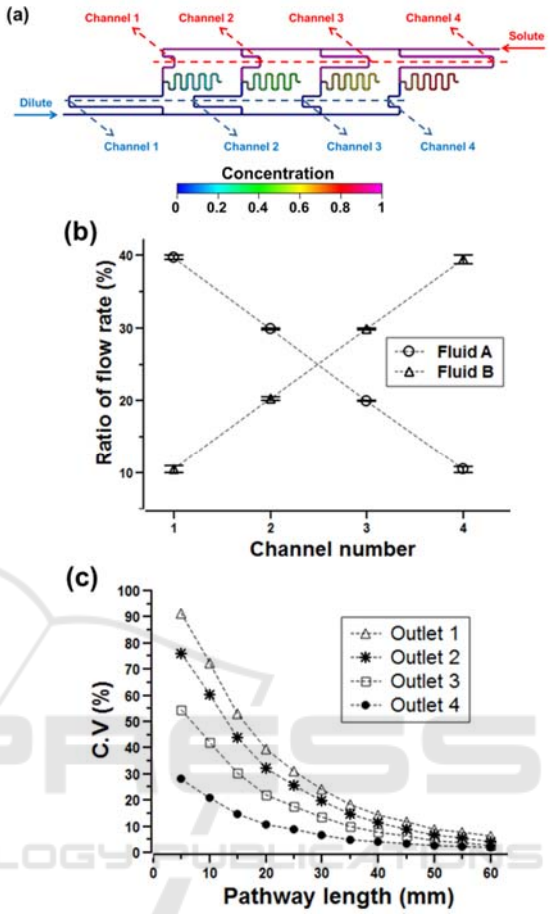

Figure 2: Numerical analysis of the perfusion microfluidic channel. (a) The concentration gradient generator integrated with microchannel mixers, (b) linear distribution of flow rates, (c) coefficient of variations.

the reservoir, they rise due to buoyancy resulting from the difference in density. To trap air bubbles in the reservoir, the rising velocity must be higher than perfusion velocity. The rising velocity of an air bubble can be calculated from the balance between the buoyancy force and the drag force acting on the air bubble expressed in Eq. 4. (Zheng and Yapa, 2000) and the perfusion velocity is also obtained by dividing the perfusion flow rate with the cross-sectional area of the channel.

$$v_{rising} = \frac{g\Delta\rho d_{bubble}}{18\mu} \quad (4)$$

where, v_{rising} is the rising velocity of a bubble, $\Delta\rho$ is a difference of density between the surrounding fluid and the bubble, d_{bubble} is a diameter of the bubble and μ is the dynamic

viscosity of a fluid. From the inequation between the rising velocity and perfusion velocity, the diameter of a rising bubble, d_{bubble} can be expressed in Eq. 5.

$$d_{bubble} = \frac{18\mu Q}{g\Delta\rho A} \quad (5)$$

where, A is an area of the cross-sectional plane. In the perfusion flow condition used for numerical analysis of the concentration gradient generator, the minimum diameter of rising bubbles was 8 μm and bubbles with below 8 μm of diameter is negligible for clogging microchannel with 0.5 mm of width and 2 mm of depth. Figure 3 shows the behaviour of air in the sudden expansion reservoir was simulated in 2-dimensional structure. The transient analysis showed that the air bubbles in the reservoir collected as much as approximately 55.7 mm^2, and the reservoir capacity was 60 mm^2. This implies that the trapping capacity of air bubbles is almost equal to the volume of the sudden expansion reservoir.

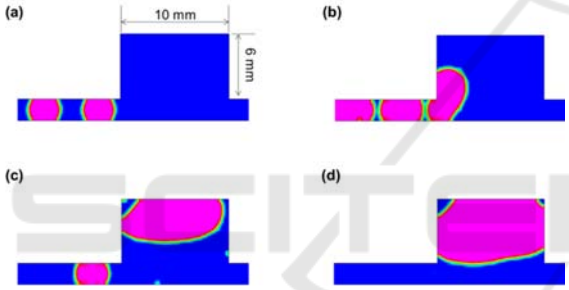

Figure 3: CFD simulation of behaviour of air bubbles in the 2D sudden expansion reservoir.

The reservoir was an elliptic shape with a major length, minor length and height of 10, 5.5 and 6 mm, respectively. The trapping capacity of the reservoir was 260 μl.

3 FABRICATION OF THE 3D MICROFLUIDIC PERFUSION CELL CULTURE

The 3D microfluidic perfusion cell culture well plate was fabricated by using a milling machine and bonding two plates of biocompatible and transparent PMMA. The four cell culture wells were fabricated with the same size as those in the general 2D 3×4 well-plate. Additionally, the size and shape of the wells were fitted to the scaffolds for the 3D culture of cells. At the centre of the bottom of each well, a hole was bored to supply media continuously. The open drain channel was machined at top side of wells with an inclined angle of 7° to guide the overflown media from the perfusion culture wells by gravity.

The linear concentration gradient generator connected with the meandered microchannel mixers was machined in the bottom perfusion channel plate. To bond the well plate and the channel plate, a transparent and UV curable adhesive (MP-4102, CALO®) was coated onto the bottom of the upper well plate. After the outlets of the microchannel mixers were aligned with the holes of the culture wells and the two plates were bonded physically, UV was irradiated onto the edges for 5 seconds at 9 Watt and onto the whole surface for 30 minutes at 9 Watt.

Figure 4 shows the fabricated 3D microfluidic perfusion cell culture plate. To evaluate the bonding quality, the blockage of the microchannels due to adhesive filling and the leakage of the unbound parts were examined. When water was introduced with a large flow rate of 500 μl/min, no local blockage was observed in the microchannels.

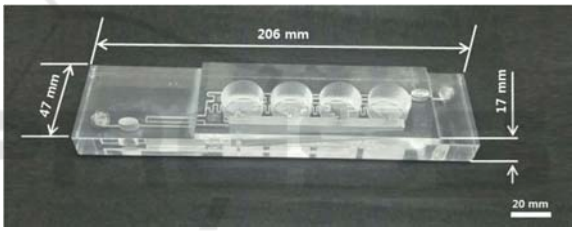

Figure 4: The 3D perfusion cell culture plate fabricated from PMMA.

4 EXPERIMENTS

4.1 Performance Evaluation of the Microfluidic System

4.1.1 Concentration Gradient Generation

The perfusion flow rate injecting into the 3D microfluidic perfusion cell culture plate was controlled by a syringe pump (PHD 2000, Harvard).

First, the concentration gradient generator was examined. Deionized (DI) water dissolved with rhodamine-110 molecules was injected into one inlet while only DI water was injected into the other inlet. To obtain a reference in fluorescence intensity versus concentration, rhodamine-110 with a controlled concentration ranging from 0.0 to 18.7 μg/ml was prepared as shown in figure 5. The fluorescence intensity of Rhodamine-110 was measured for the different concentrations with a

microplate reader (FLUOstar, OPTIMA). The calibration curve was fitted with an R^2 of 0.9820 shown in figure 5. The inlet flow rate of two samples was 80 µl/min. The syringes were refilled every 6 hours and three times. During the refilling process of the water, the air bubbles moving into the perfusion microchannel were observed. The samples with volume of 150 µl in each well were taken every 30 minutes. Then the fluorescence intensity of the samples was measured. Figure 6(a) shows that the linear concentration gradient of 40%, 30%, 20% and 10% in the four perfusion wells was generated with an error of 9.7%. The C.V. was calculated to be 1.9% on average by taking samples several times shown in figure 6(a). Moreover, the performance of the linear concentration gradient generator was tested without the air bubble trapping reservoirs. As soon as air bubbles were introduced into the branch microchannels, the flow entering the linear concentration gradient generator was seriously disturbed and detoured into the rest of the branch microchannels. As a result, the linear concentration gradient was no longer consistently maintained shown in figure 6(b).

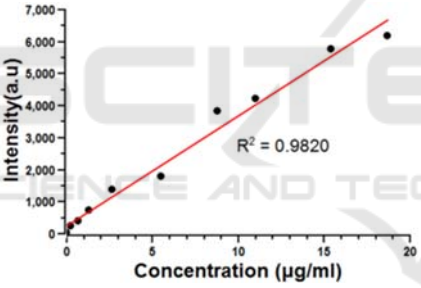

Figure 5: Calibration of rhodamine-110 concentration to fluorescent intensity.

4.1.2 Air Bubble Trap

The trapping capacity of the air bubble trap reservoir was tested. Initially, the 3D microfluidic perfusion cell culture plate was filled with blue coloured water for visualization. Then, air in a syringe was injected with a flow rate of 50 µl/min. The trapping of the air bubbles in the reservoir was monitored over time.

Figure 7 shows that the air in the reservoir was trapped due to buoyancy. The trapping capacity of the reservoir was measured when the air bubbles started to enter into the microchannel. It was measured to be 268 µl, which agrees well with the designed capacity. The small discrepancy was caused by the extra volume as a result of the meniscus of the air bubble.

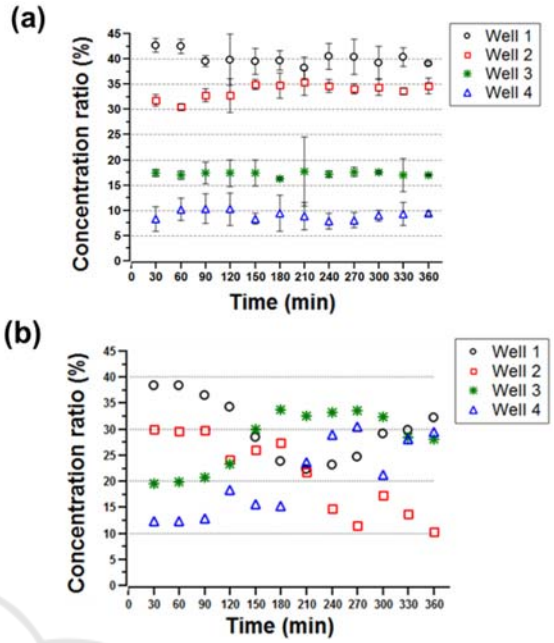

Figure 6: Concentration gradient of rhdamine-110 generated in 3D microfluidic perfusion cell culture plate. (a) Perfusion test with sudden expansion reservoir, (b) without reservoir.

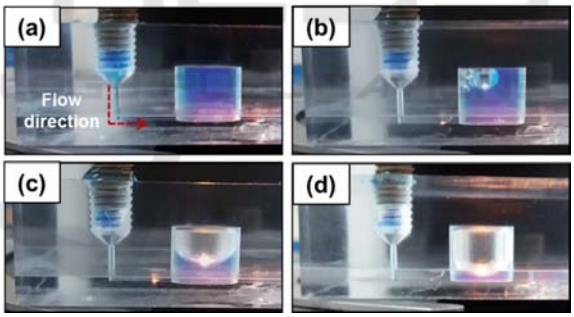

Figure 7: Visualization of trapping air bubbles in the sudden expansion reservoir. (a) The reservoir filled with blue coloured water, (b)-(d) injecting air, (d) limitation of trapping air bubbles.

4.2 Cell Cytotoxicity Test

The 3D microfluidic perfusion cell culture plate was used for a cytotoxicity test of Ewing's sarcoma cells, A-673 (ATCC® CRL-1598™), which are rare tumour cells and mostly expressed in the bones or tissues of children. It is not clearly known what causes Ewing's sarcoma. However, experimental reports have shown that the concentration of hydrogen peroxide, H_2O_2, has a key role in cancer development (Lopez-Lazaro, 2007). Thus, the

toxicity test of the cells used different concentrations of H_2O_2 as a cytotoxic drug.

For the 3D cell perfusion cell culture, scaffolds were inserted into the four perfusion wells, and the Ewing's sarcoma cells were seeded onto the scaffolds. Then, to mimic an in vivo environment, the media were warmed in a water bath, and the temperature was maintained 36 ºC. Moreover, the 3D perfusion cell culture plate was installed in a warm chamber to acclimate it to a temperature of 36 ºC. By using a peristaltic pump (MNI PULS 3, Glison®), the nutrient medium and a mixture of the medium and H_2O_2 were introduced into the two inlets, respectively, and the perfusion flow rate was 40 µl/min for each. Specifically, the perfusion flow rate was selected by considering the designed linear concentration gradient generator in the four culture wells and the previous work using a perfusion cell culture with a perfusion flow rate ranging from 0.1 to 1 ml/min (Cartmell and Porter, 2003)

The perfusion cytotoxicity was assayed for 4 hours. During this time, 100 µl of drained media were collected every 30 minutes and mixed with 10 µl of propidium iodide (PI). Then, the fluorescence intensity was analysed. The excitation and emission wavelengths were 540 and 620 nm, respectively. The gain value was set as 1800. The intensity of the PI relates the degree of cell death. The degree of cell death was measured for the four different concentrations of H_2O_2. Figure 8 shows that cell death increased continuously until up to 240 minutes, while the specific threshold concentration of the cell death was not found. Moreover, more cells were killed at the higher concentration. A higher PI signal was observed at a higher concentration of H_2O_2, and there were less cell proteins on the scaffold. To conduct long-term culture assays using the perfusion cell culture system, the 3D microfluidic perfusion cell culture plate needs to increase its capacity for air bubble trapping, after which, it needs to be compared with a 2D static cell culture of the same cells.

5 APPLICATION OF THE 3D MICROFLUIDIC PERFUSION SYSTEM

In this study the 3D microfluidic perfusion cell culture plate with a 4 fold dilution of concentration gradient is presented. The designed 4 fold dilution of screening shows the feasibility of our design and application to cell culture processes. Even though the well size is similar to the general 3×4 well-plate's one, the structure is quite different size with the general 3×4 well-plate. Most microplate reading tools are for fixed to general well-plate size, so the well-plates can be inserted on the reading tools directly. Thus, the structure is improved to have similar size with the general 3×4 well-plate for simplifying cell culture processes, increasing high-throughput rate of sample screening as well as compatibilities with general measuring tools as shown in figure 9.

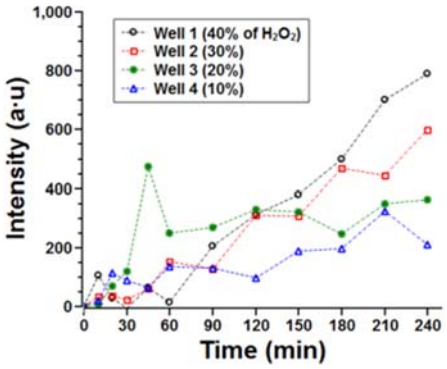

Figure 8: Result of PI stained DNAs from Ewing's sarcoma cells.

The concentration range is expanded from a 4 fold dilution of 10-40% to a 100 fold dilution of 1-100% based on the design. Figure 10 shows the detail concentration gradient for 7 wells with a hundred fold dilution and figure 11 shows a performance of generating concentration gradient by injecting rhodamine-110 into the improved 3D microfluidic perfusion well-plate. From the result of perfusion of rhodamine-110 in the improved well-plate, after 30 minutes of perfusion the intensity corresponding to concentration of rhodamine-110 is close to reference samples, which are concentration fabricated manually to comparison with perfusion sample.

The system performance is expected to be stable by connecting three sudden expansion reservoirs in a row at each inlet side.

Polycarbonate is chosen for fabrication of the improved 3D perfusion well-plate considering the biocompatibility and feasibility of autoclave sterilizations.

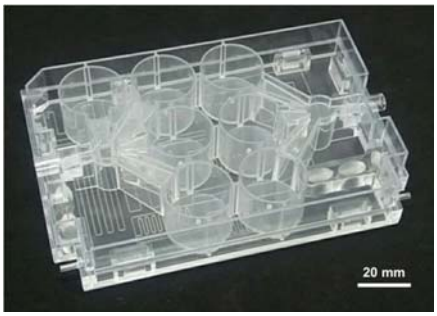

Figure 9: Improved 3D microfluidic perfusion well-plate.

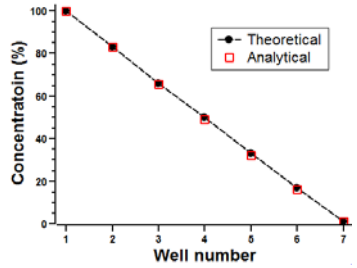

Figure 10: Result of numerical analysis of expanded concentration gradient range.

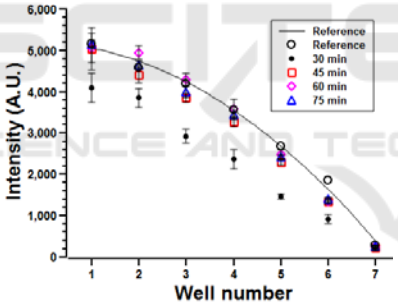

Figure 11: Fluorescent intensity with a concentration of rhodamine-110.

6 CONCLUSIONS

A 3D microfluidic perfusion cell culture system consisting of concentration gradient generators to provide a linear concentration integrated meandered micromixers for nutrients or drugs and air bubble trapping reservoirs was developed in this study to examine cell cultures with a closer relevance to in vivo microenvironments.

The performance of the concentration gradient generator connected with the microchannel mixer was designed by considering flow resistances of microchannels and examined by measuring the fluorescence intensity of rhodamine-110 in the perfusion cell culture wells. The linear concentration in the wells had a 4 fold of dilution. Additionally, the trapping capacity of the sudden expansion reservoir for air bubbles was determined by transient numerical analysis and visualization. Both methods showed that the incoming air bubbles float upward in the sudden expansion trapping reservoir due to the buoyancy force and gathered until the reservoir was full. The maximum trapping capacity of the reservoir was determined to be the same volume as the sudden expansion reservoir. In addition, the linear gradient concentration in the wells was stably maintained for a long-term cell culture with the air bubble trapping reservoir, while the concentration in the wells without it was seriously disturbed.

Finally, the 3D microfluidic perfusion well-plate was improved for compatibilities with measuring tools like microplate-readers increase of high throughput rate by expanded concentration range from a 4 fold of dilution to a 100 fold dilution. Also, the improved well-plate was fabricated from polycarbonates for the biocompatibility and processing the autoclave sterilization.

The suggested 3D microfluidic perfusion cell culture plate is potentially applicable to high throughput screening of drugs, nutrients, and growth factors. For future works, its comparison with a 2D static cell culture well plate should be done for a specific targeted cell.

ACKNOWLEDGEMENT

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (No. 2017R1A2B2006264).

REFERENCES

Schumacher, K., Strehl, R., 2012. Advanced Technique for Long Term Culture of Epithelia in a Continous Luminal-Basal Medium Gradient. *Biomaterials*.

Caicedo-Carvajal, C., Liu, Q., 2011. Cancer Tissue Engineering: A Novel 3D Polystyrene Scaffold for In Vitro Isolation and Amplification of Lymphoma Cancer Cells from Heterogeneous Cell Mixtures. *Journal of Tissue Engineering*.

Baker, B., Chen, C., 2012. Deconstructing the Third Dimension – How 3D Culture Microenvironments Alter Cellular Cues. *Journal of Cell Science*.

Li, X., Valadez, A., 2012. Microfluidic 3D Cell Culture: Potential Application for Tissue-Based Bioassays. *Bioanalysis.*

Caicedo-Carvajal, C., Liu, Q., 2012. Using a Novel 3D Perfusion **b**ioreactor to Culture β-actin-RFP Reporter Osteosarcoma. *Biowire Spring.*

Ong, S., Zhang, C., 2008. A Gel-Free 3D Microfluidic Cell Culture System. *Biomaterials.*

Yi, L., Lin, J., 2017. Development and Applications of Microfluidic Devices for Cell Culture in Cell Biology. *Molecular Biology.*

Langelier, S., Livak-Dahl, E., 2011. Flexible Casting of Modular Self-Aligning Microfluidic Assembly Blocks. *Lab on a Chip.*

Sung, J., Shuler, M., 2009. Prevention of Air Bubble Formation in a Microfluidic Perfusion Cell Culture System Using a Microscale Bubble Trap. *Lab on a Chip.*

Nguyen, N., Wereley, S., 2006. *Fundamentals and Applications of Microfluidics*, ARTECH HOUSE. Boston, London, 2nd edition.

Zheng, L., Yapa, P., 2000. Buyant Velocity of Spherical and Non-Spherical Bubbles/ Droplets. *Journal of Hydraulic Engineering.*

Lopez-Lazaro, M., 2007. Dual Role of Hydrogen Peroxide in Cancer: Possible Relevance to Cancer Chemoprevention and Therapy. *Cancer Letters.*

Cartmell, S., Porter, B., 2003. Effects on Medium Perfusion Rate on Cell-Seeded Three-Dimensional Bone Constructs *in Vitro. Tissue Engineering.*