Paper:

# Parallel Formation of Three-Dimensional Spheroid Using Microrotational Flow

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**We propose three-dimensional (3D) spheroid formation involving perfusion and a lab-on-a-chip containing spheroid-forming chamber arrays. Cells are collected forming a spheroid in the chamber in microrotation. We previously reported a single chamber form hepatic spheroids 130 to 430 $\mu$m in diameter, controlling size by varying chamber diameter and cell density. Here, we scaled the system up by a factor of 10 while maintaining size control of 180±30 $\mu$m in diameter. Results were comparable to those using a single-chamber device. Long-term culture confirmed that cells in the spheroid maintained viability and diameters did not change after 24 hours. The system is readily applicable for creating size-controlled spheroids ensuring reliable, predictable in vitro data for drug screening and biological research.**

**Keywords:** lab-on-a-chip, spheroid, microrotation flow, hepatocyte

## 1. Introduction

Microfluidics enable cells to be manipulated precisely on a micron scale [1–7]. Since several cell species exhibit far greater functions when aggregating and forming a spheroid [8, 9], related cell manipulation based on these techniques have been extensively studied, using a micropatterned surface with polyethylene glycol hydrogel [4] or an extracellular matrix [5], microcontainers that trap cells [6], microfluidic hydrodynamic cellular patterning of embryonic stem cells [10], and hydrodynamic force [11]. Crucial criteria on these devices control size, positioning, and high concentration of spheroids in the device, yielding a highly efficient experimental platform for cell biology and good substitution for human organs containing different cells at high density.

We previously reported a three-dimensional (3D) hepatic spheroid-forming chamber able to control spheroid size using a microrotational flow [12]. Perfusion media containing hepatocytes were introduced into a microchamber in which a microrotational flow was generated. Cells are attracted to the microchamber center by the fluidically confined region and aggregate there to form a spheroid. Hepatocytes are responsible for metabolic and detoxification processes in the liver and they acquire over 500 liver-specific functions when forming spheroids, depending on their size [11, 13, 14]. Hepatic spheroids resemble tumor masses in which oxygen diffusion is limited to 150–180 $\mu$m [15, 16], so size must be smaller than 180 $\mu$m to prevent necrosis. The system could accurately create spheroids with diameters from 130 to 430 $\mu$m with good accuracy. This chamber is superior to other microfluidic devices in that spheroid size is controlled by altering cell density medium without changing device geometry. The device provides space for spheroids to grow, since they are concentrated at the chamber center by hydrodynamics. The device chamber forms individual spheroids independently in the closed space, enabling the device to stimulate spheroids independently and measure function by *in situ* observation. Given that the device has only a single spheroid-forming chamber, the system created only one spheroid in each experiment. Given that this device is taken advantage of for biological experiments or drug screenings, single-chamber device throughput is insufficient, since several three-dimensional spheroids, at least, should be produced in one assay. Here, we have attempted to scale the system up without losing size control. We designed and manufactured a spheroid-forming lab-on-a-chip with multiple spheroid-forming chambers. Throughput was augmented by a factor of 10 from the previous single-chamber device. We also refined perfusion to increase successful long-term culture. We demonstrated multiple spheroid formation using our proposal and studied the viability and size variation of spheroids related to culture time.

## 2. Experiments

### 2.1. Array Concept and Design

Microchamber design is an array component reported previously. Briefly, the microchamber, as shown in **Fig. 1**, is a circular cylinder with two inlet channels tangential to the cylinder at the bottom and two outlet channels at the top. The microrotational flow is generated by fluids flowing from the two inlet channels. To enhance spheroid formation throughput, we developed an array consisting of several microchambers forming spheroids with the same features, as shown in **Fig. 1**.

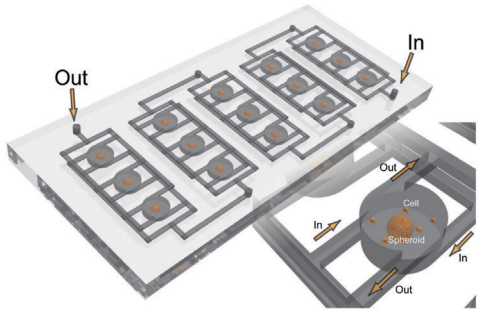


**Fig. 1.** Spheroid formation array. Hepatocytes are assembled by microrotation flow and form a spheroid in the chamber center.

The more chambers that are coupled in a series, the higher the pressure from the pump required to generate microrotation flow in chambers. Pressure that is too high destroys bonding between device layers. The number of chambers thus coupled is limited by bonding strength and the volume of the medium with high cell density. In this study, based on experiment results, bonding was not destroyed when 5 chambers were coupled in one array.

To realize higher throughput, several chambers were connected in parallel. Given that the volumetric flow at which spheroids are formed is 0.4±0.05 ml/min in a single chamber device [12], surplus water pressure loss caused in the chamber at the end of one set must be limited to within 25% of water pressure loss in the chamber at the center. We calculated water pressure loss in chambers connected in parallel before design, with channels divided into three parts – (1) an inlet channel tangential to the chamber cylinder (A), (2) rectangular parts in a channel (B), and (3) straight parts in a channel that distribute media to the chamber at the edge of the set (C), as shown in **Fig. 2**. Water pressure losses in A and C are as follows [17]:

$$\Delta p = \lambda \frac{(h+W)L}{4hW}\left(\frac{\rho U^2}{2}\right) \quad \ldots\ldots\ldots \quad (1)$$

$h$ is height, $W$ width, $L$ length, $U$ flow speed, $\rho$ density. $\lambda$, the friction coefficient of the oblong channel, is as follows:

$$\lambda = \frac{64}{Re}kc \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Factor $kc$ is determined by the channel aspect ratio. $Re$ is the Reynolds number. Water pressure loss in B is as follows [18]:

$$\triangle p = \xi\left(\frac{'U^2}{2g}\right) \quad \ldots\ldots\ldots\ldots\ldots \quad (3)$$

$\xi$ is

$$\xi = 0.946\sin^2\frac{\theta}{2g} + 2.05\sin^2\frac{\theta}{2g} \quad \ldots\ldots \quad (4)$$

$\xi$, coefficient of loss, is determined by the angle of curved channels. Loss of water pressure in A is caused in both chambers at the edges and center, since each chamber

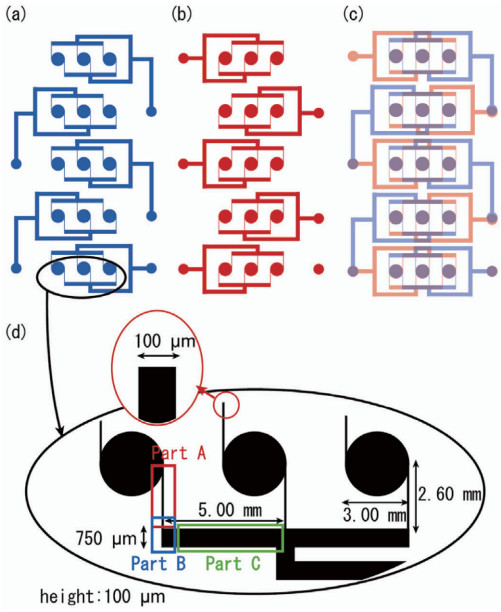


**Fig. 2.** (a) Middle and (b) upper array design. (c) Flow channel connection. (d) Magnified view of array.

have its own kind of channels (100 $\mu$m × 100 $\mu$m). Water pressure loss at B and C is caused only in chambers at the edge, which may induce a difference in flow velocity between chambers at the edge and center. We set each parameter in the set consisting of 3 chambers connected in parallel as shown in **Fig. 2 (d)**. In A, $kc$ was 0.90, $Re$ 1.2 $\times 10^3$, $h$ 100 $\mu$m, $W$ 100 $\mu$m, and $L$ 2.6 mm. In C, $Re$ was 3.1 $\times 10^2$, $h$ 100 $\mu$m, $W$ 750 $\mu$m, and $L$ 5.0 mm. Assuming that $v$ (kinetic viscosity) and $\rho$ of the medium were water, water pressure loss of A and C were 17 $U^2$ and 1.9 $U^2$. Considering angles in B ($\theta$ was 90°), water pressure loss in B was 0.5 $U^2$. These results show water pressure loss in B and C to be 14% of that in A. Even if $v$ of the medium is higher than water due to containing bovine serum or glucose, the increase in water pressure loss occurs in A and C equally, and water pressure loss in B and C becomes less than 14% of that in A, so we used the design in **Fig. 2** as the array.

## 2.2. Fabrication

The array of polydimethylsiloxane (PDMS) (Silpot 184 W/C, Dow Corning Corp.) was formed photolithographically as follows: Negative photoresist SU-8 (SU-8 10, MicroChem Corp.) was patterned to produce circular cylinders and channels with array geometries on a clean glass slide using two types of geometries as molds for upper and middle layers as shown in **Fig. 3(a)**. Degassed liquid PDMS was poured over the SU-8 mold to form a chamber and two channels, as shown in **Fig. 3(b)**. PDMS structures were cured on a hotplate at 65°C for 6 hours and peeled from the mold as shown in **Fig. 3(c)**. Upper layer and inlets and outlets, the chamber and throughhole, from the upper layer to the middle layer in the middle layer were made with a punch as shown in **Fig. 3(d)**. After PDMS bonding surface structures were exposed to

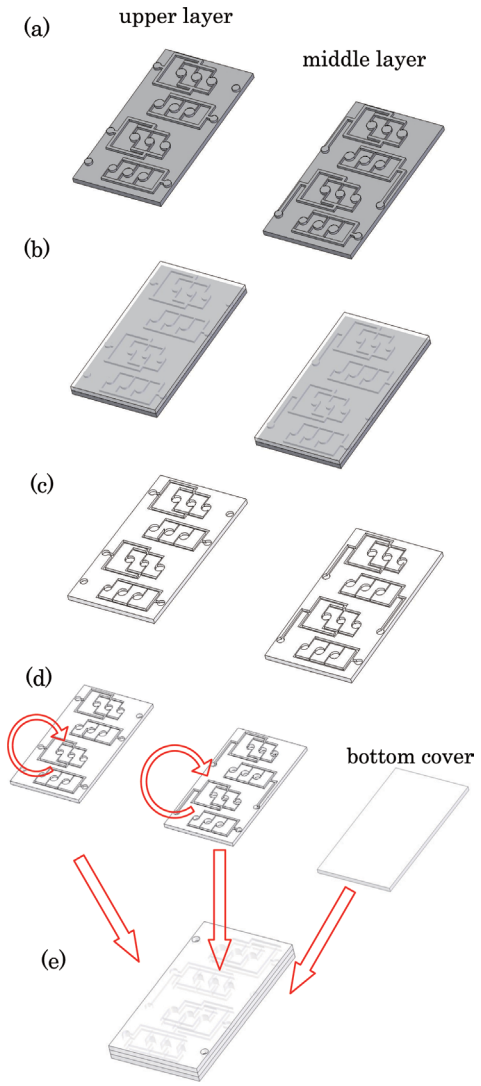


**Fig. 3.** Fabrication.

oxygen plasma, the upper and middle layers and bottom cover were manually aligned under microscopic observation and bonded as shown in **Fig. 3(e)**. Channels with the chamber were 100 $\mu$m wide and 100 $\mu$m high. The chamber was 2 mm high and 3 mm in diameter

## 2.3. Cell Culture

Human hepatocellular liver carcinoma cell line HepG2 (DS Pharma Biomedical Co., Ltd.) was grown in Minimum Essential Medium (MEM) supplemented with 10% FBS (DS Pharma Biomedical Co., Ltd.) and 2 mM glutamic acid, 2 mM pyruvic acid, and 1% nonessential amino acid (DS Pharma Biomedical Co., Ltd.) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

## 2.4. Perfusion Setup

The three-dimensional spheroid-forming device must be able to provide the rotating spheroid with a steady cell suspension flow for sufficiently long enough because spheroids require 24 hours to enhance functions [4]. In

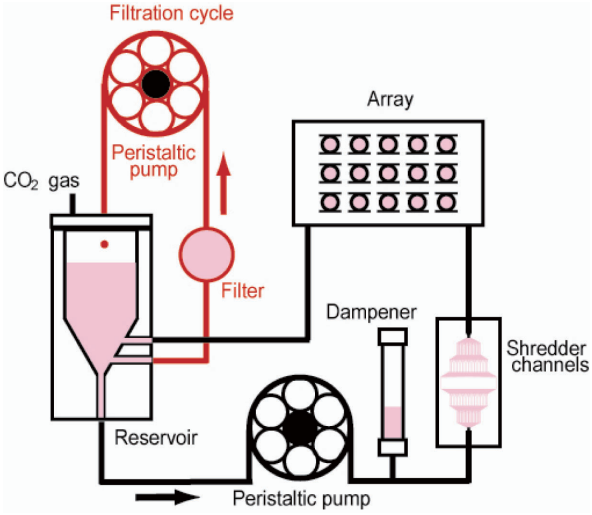


**Fig. 4.** Spheroid formation device consisting of an array and perfusion with a reservoir, a shredder channel, dampener, filtration cycle, and peristaltic pump.

previous work, we reported original perfusion consisting of a peristaltic pump, reservoir, dampener, and shredder channel providing a steady cell culture medium flow containing HepG2 to the rotating spheroid [12]. When we cultured cells in the previous system for a long time, however, the velocity vector distribution in the chamber often changed due to large cell aggregations clogging channels and making the microrotation flow unstable.

To prevent cells from aggregating in undesirable areas, we here constructed a filtration cycle connected with a new refined reservoir. When the medium containing cells circulated in a filtration cycle, extra cells in the medium not used for spheroid formation were collected in the filter as shown in **Fig. 4**. The decrease of extra cells in the medium prevented undesired cell aggregation.

## 2.5. Reservoir

We developed a small-volume 4 ml tank to ensure that perfusion was compact as shown in **Fig. 5**. Cells and culture medium are stored in the reservoir. Reagents that dye or chemically stimulate cells are supplied from the reservoir, which has an outlet at its base to prevent cells from residing too long in the reservoir and aggregating to form undesired spheroids in the reservoir. In this study, we added one more inlet and outlet for the filtration cycle.

## 2.6. Filtration Cycle

Cells sometimes formed undesired aggregations and clogged channels in long-term cultures due to extra cells not used to form spheroids that continued circulating and aggregated at the site. The filtration cycle we developed to collect these extra cells from the media consisted of a peristaltic pump and Terufusion®Final Filter PS (Terumo) having a filter with 0.2 $\mu$m pores. When media containing cells circulates in the filtration cycle, extra cells in the medium are collected in the filter and the medium is sterilized by collecting bacteria and trash.

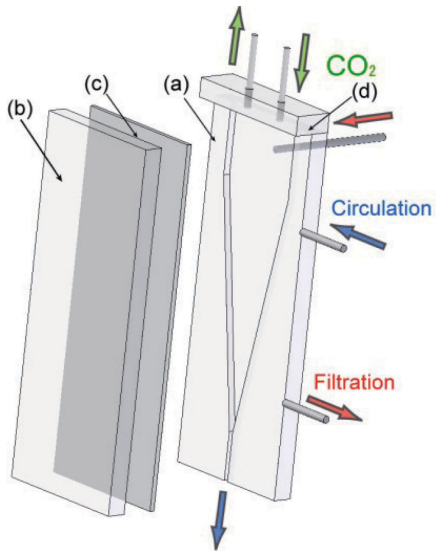


**Fig. 5.** The reservoir consisted of (a) a channel, (b) cover, (c) silicon sheet, and (d) top cover. The silicon sheet increased adhesion between (a) and (b).

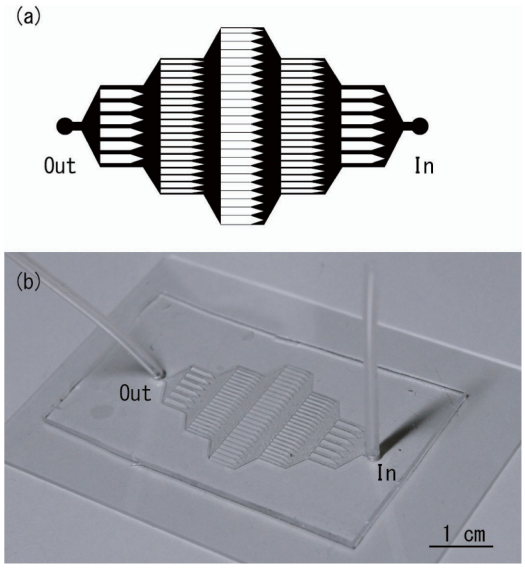


**Fig. 6.** (a) Shredder channel design. The narrowest channel was 50 $\mu$m in the middle. Large aggregations of cells are broken up by shear stress and/or trapped in the channel when aggregations of cells flow in the channel. (b) Shredder channel fabricated photolithographically.

### 2.7. Pump

The peristaltic pump (Peri-Star$^{TM}$ PRO, World Precision Instruments) generated more stable flow and determined the volumetric flow of circulating media more precisely by digital control. Cells might be injured by a peristaltic pump in the system, so we clenched a bolt which could adjust constriction of the tube loosely and created spaces in the tube in order to prevent compression of flowing cells.

### 2.8. Dampener

We connected a pulsation dampener to the peristaltic pump to prevent the pump's pulsatile flow [12] using an air pocket trapped in the dampener to absorb pump pulsation.

### 2.9. Shredder Channels

We previously reported shredder channels [12] in which cells aggregated and formed spheroids in the tubes and reservoir that clogged channels and destabilized perfusion flow. We used shredder channels to prevent large cell aggregates from entering the chamber as shown in **Fig. 6**. The narrowest channel was 50 $\mu$m in central section. Even if cell aggregation clogged one of the shredder channels, flow velocity changed little because channels had many alternative channels for moving the medium.

### 2.10. Cameras

Stationary and fluorescence images were obtained by a CCD camera (Cool SNAP-cf, Nippon Roper Co., Ltd., and EOS Kiss X3, Canon).

### 2.11. Live/Dead Cell Viability Assay

Cells in spheroids were stained using 4 $\mu$M calcein-acetoxymethyl ester (AM) and 8_methidium homodimer-1 (EthD-1) to detect living cells within 24 hours. We exchanged the circulating medium with a medium containing these two dyes and incubated cells for 20 min before measurement, then stationary and fluorescent images were obtained by a CCD camera.

### 2.12. Cell Experiments

We previously reported how cells aggregated and formed spheroids. Briefly, cells were cultured and detached by ethylenediaminetetraacetic acid (EDTA) (DS Pharma Biomedical Co., Ltd.) and trypsin (DS Pharma Biomedical Co., Ltd.). To prevent undesired cell aggregations, they were then cultured for 30 min in a medium containing 1000 PU/ml dispase (Sanko Junyaku Co., Ltd.). After removing dispase by centrifuging the medium, we adjusted cell density to $500\times10^4$ cells/ml. The temperature and pH of the circulating medium were maintained by inserting the reservoir in a thermostatic bath and circulating 5% $CO_2$ gas constantly in the reservoir.

Media containing cells were introduced into the array at a volumetric flow of 3.3 ml/min until the cell flow around the entire array is stable. We then gradually reduced the flow to 1.2 ml/min, causing cells near the array center to be attracted toward the center, forming a spheroid. Stationary and fluorescent images were obtained using a CCD camera.

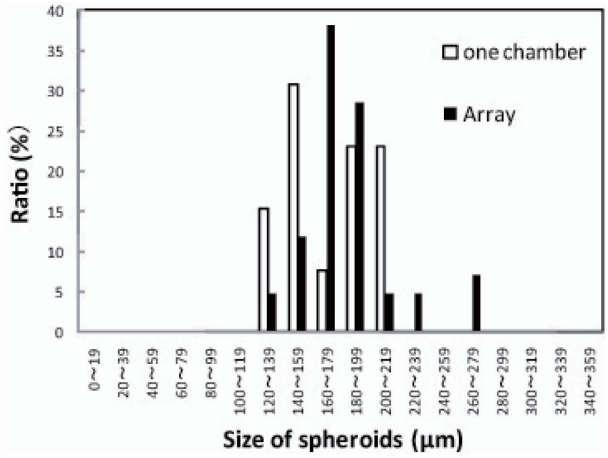


**Fig. 7.** Distribution of spheroid sizes in one chamber and array ($N = 40$).

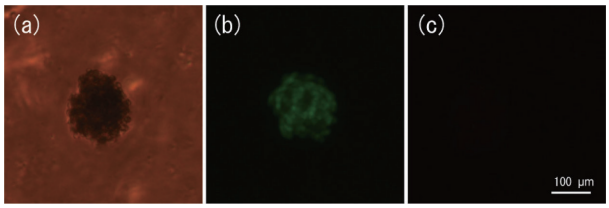


**Fig. 8.** The spheroid was stained by calcein AM and EthD-1 after culture for 24 hours to verify cell viability. (a) Optical image. (b) Fluorescent images of calcein AM and (c) EthD-1.

## 3. Results and Discussion

### 3.1. Spheroid Size Distribution in Chamber and Array

Aggregating cells and forming spheroids in the array were similar to doing so in a single-chamber as reported [12]. When the medium was introduced into the chamber at a volumetric flow of 3.3 ml/min, microrotational flow was observed throughout the chamber. As the volumetric flow dropped, cell collision frequency increased. Cells gradually aggregated and formed spheroids within 120 s. The volumetric flow of the circulating medium at which spheroids stayed inside the chamber and rotated was 1.2 ml/min. A volumetric flow at which a spheroid was formed and stay inside the chamber was three times that in previous work [12] because each set consisted of 3 connected parallel chambers. For more chambers to be connected in parallel to form spheroids in one set, more volumetric flow will be required.

When we studied formed spheroid sizes with the single-chamber 3 mm in diameter and the cell density of $500 \times 10^4$ cells/ml, mean spheroid size was $170 \pm 27$ $\mu$m and standard deviation was 16.0% [12]. Mean spheroid size in one array was $180 \pm 30$ $\mu$m and standard deviation was 16.6% as shown in **Fig. 7**. Results were comparable to those with the single-chamber device. Standard spheroid deviation size in chambers connected in parallel was 14.8%, so the difference in the water pressure loss may affect spheroid formation less than manufacturing error.

Having formed spheroids in 40 of 60 chambers – 10 per attempt on average – in the array, we successfully increased throughput by a factor of 10. Fukuda et al. reported hepatic spheroid culture arrays using microfabrication and collagen/polyethylene glycol microcontact printing [19]. Their arrays created 200 spheroids using one chip. The spheroid diameter on the Col/PEG SM chip exhibited a uniform distribution – $155 \pm 8$ $\mu$m – and its standard deviation was 5%. The array controls spheroid size more precisely than ours, but our array provides space for spheroids to grow, since they concentrate at the chamber center thanks to hydrodynamics. Spheroids formed from stem cells such as Embryo-Stem (ES) cells and induced pluripotent stem cells are expected to be used for biological research and drug screening [10]. Spheroid formation chambers must have space for spheroid growth, since spheroids formed from stem cells grow bigger [20].

### 3.2. Live/Dead Assay After 24 Hours

The perfusion system we developed provided a stable flow to the chamber and produced a long-lived culture (>24 hours) with high success. **Figs. 8 (a)–(c)** show optical and fluorescence images of spheroids stained with calcein AM and EthD-1 by exchanging circulating medium with medium containing these two dyes after 24 hours. No fluorescence was observed from EthD-1 in cells, but fluorescence was observed from calcein AM – compare **Figs. 8 (b)** and **(c)**. These images show that cells in the spheroid lived in the chamber for at least 24 hours. The device exchanged circulating medium and spheroid stained by fluorescent dyes was observed *in situ*. This way, we clarified spheroid and protein functions in more detail by measuring fluorescent intensity in images.

### 3.3. Time-Dependent Change in Spheroid Size

**Figure 9** shows time-dependent changes of hepatic spheroid size for one day = 24 hours, compared to size just after spheroid formation. Maximum change in spheroid size in 24 hours was 1.5%., so spheroid size did not change for 24 hours. Lin et al. reported aggregation from HepG2 packed into a compact aggregation during spheroid formation [3], and they exhibited continuous increasing shrinkage over time and showing rapid shrinkage in the first 12 hours. The difference in spheroid size between their and our results may be due to the difference in the way spheroids were formed. The hanging-drop culture is the way to culture and aggregate cells in a drop hanging on the lid of a culture dish. The cells settle out and aggregate by the microgravity in each drop. That means microgravity drag cells to the bottom part of the drop and force spheroid to shrink. In previous work, we found that a spheroid formed in our chamber confined to the chamber center by hydrodynamics for 24 hours was not directed toward the chamber center [12], so spheroids formed in

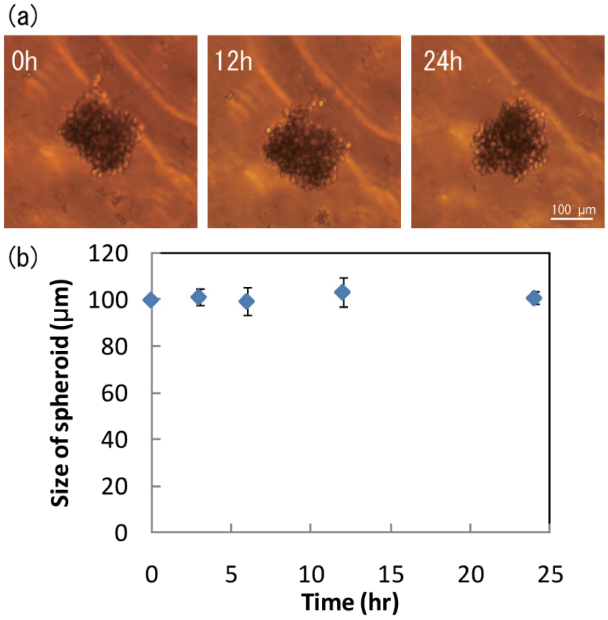


**Fig. 9.** (a) HepG2 aggregation morphology at 0, 12, and 24 hours during spheroid formation. (b) Change in cell aggregation diameter ($N = 8$).

our array may not change size within 24 hours. It may be that cells stopped accumulating into compact aggregations and connected loosely. Given that cells were not separated from the spheroid and remained in spheroids, cells may interact with one another constantly and secrete an extracellular matrix. Thus cells may connect one another tightly.

## 4. Conclusions

We have succeeded in controlling spheroids 180 $\mu$m in diameter reproducibly 16.6% in spheroid formation chambers and in enhancing size-controlled spheroid formation throughput by a factor of 10 using microchamber arrays. The device we developed consisted of a microchamber, PDMS channels, and perfusion consisting of a reservoir, pulsation dampener, shredder channel, filtration cycle, and peristaltic pump. This enabled long-term culture of spheroids for over 24 hours with a high rate of success. Live/dead viability assay verified that almost all cells in the spheroid lived more than 24 hours. The array controlled a hepatic spheroid diameter of $180\pm30$ $\mu$m and maintained spheroid sizes for over 24 hours. If we change cell density in the media, as with the single-chamber device, we can form spheroids of desired sizes. This array is the biological potential for technological innovation by forming a bridge between cell and tissue research.

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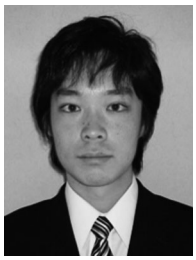

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