MICROFLUIDIC EXPERIMENTAL PLATFORM USING MICRO-ROTATION FLOW FOR PRODUCING MULTIPLE SIZE-CONTROLLED THREE-DIMENSIONAL SPHEROIDS

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ABSTRACT

We propose a microfluidic experimental platform for producing size-controlled spheroids. Cells are collected to form a spheroid in chambers by micro-rotational flow in two minutes. The developed array could control the size of three-dimensional spheroids hydrodynamically with the standard deviations less than 19 % by varying the cell density of the medium without altering the device geometry. Using the developed perfusion system we experimentally observed that the sizes of formed spheroids remained constant for two days and that the detoxification enzyme, CYP1A1, activities increased.

KEYWORDS: spheroid, micro-rotation, hepatocyte, microfluidics

INTRODUCTION

Spheroids have been extensively studied that are aggregates of cells in culture and retain three-dimensional architecture and tissue-specific functions. To date various spheroid-forming devices have been developed [1][2]. Spheroid formation platforms that can measure physiologically active substances need to satisfy four requirements: (1) They can form spheroids with good size controllability; (2) have spaces for spheroids to grow; (3) allow formed spheroids to be cultured for a long time (i.e., longer than one day) and permit their morphologies to be observed at any time; (4) allow reagents that dye or chemically stimulate cells to be supplied to the system.

In our chamber, cells were attracted to the center, where they aggregated and formed a spheroid [3]. Our spheroid-forming chamber was superior to other microfluidic devices in that (1) the spheroid size could be controlled by varying the cell density of the medium without altering the device geometry and (2) the chamber provided a space for spheroids that were supported in the center hydrodynamically. (3) The chamber was made of PDMS, which allowed us to observe spheroids at any time. (4) Furthermore, developed original perfusion system composed of a reservoir, pump and dumpener can stain cells with fluorescent dyes *in situ*. Therefore, the our platform satisfied four requirement for spheroid formation platforms.

In this study, we designed an array composed of five sets of chambers coupled in series; each set of chambers consisted of three chambers connected in parallel. This enabled us to create 11 spheroids a test with a comparable size controllability to the previous work. We also developed the platform to enable a spheroid with a specified diameter to be cultured over two days with high yields and to allow the spheroid to be observed at any time. Using the developed platform, we investigated the functions of a detoxification enzyme, cytochrome P450, in the formed spheroid by measuring ethoxyresorufin-O-deethylase (EROD) activity when reagents were supplied to the system. The results of the study demonstrate the potential of the developed array for biological research.

CHAMBER DESIGN AND PERFUSION SYSTEM SET UP

A designed micro chamber has a circular cylindrical shape with two inlet channels tangent to the circle at the bottom and two outlet channels at the top, as shown in Fig. 1. The micro-rotation flow is generated by flowing fluids from two inlet channels at the bottom [3]. To enhance the throughput of spheroid formation, we developed an array that consists of several sets of chambers coupled in series; each set consists of several chambers connected in parallel (Fig. 1(a)). The chamber was composed of poly-dimethylsiloxan (PDMS) formed by a photolithographic process.

We developed perfusion system composed of reservoir, peristaltic pump, shredder channels, filter and filtration cycle that could form and culture spheroids for a long term based on a perfusion system reported in in previous work [3]. In the previous system when we cultured cells for a long time (i.e. longer than one day), the velocity vector distribution in the chamber was sometimes observed to change. This was because excess cells that were not used to form spheroids and continued to circulate in the system and aggregated in undesired sites. In this study, we developed a filtration cycle to collect these excess cells from the medium and a filter to prevent cell aggregation entering the channels tangent to the chamber effectively, as shown in Fig. 3. As a result, we achieved long-term culture of spheroids.

EXPERIMENTAL

Hepatocyte (HepG2) cultured in a 75 cm^2 culture flask were detached. Temperature and pH of the medium circulating in the system were maintained by a thermostatic bath and circulating CO_2 gas during experiments. Firstly, the media containing cells were introduced into the chamber at a volumetric flow rate more than 4.4 ml/min, when the cells rotated at in the entire area of the chamber stably. Secondly, we decreased the flow rate down to 1.2 ml/min gradually over 2 min, when the cells near the center of the chamber were attracted towards the center to form a spheroid. Using the developed

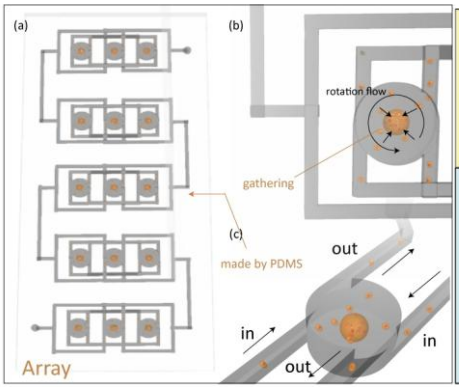

Fig. 1 (a) Schematic diagram of spheroid formation array.

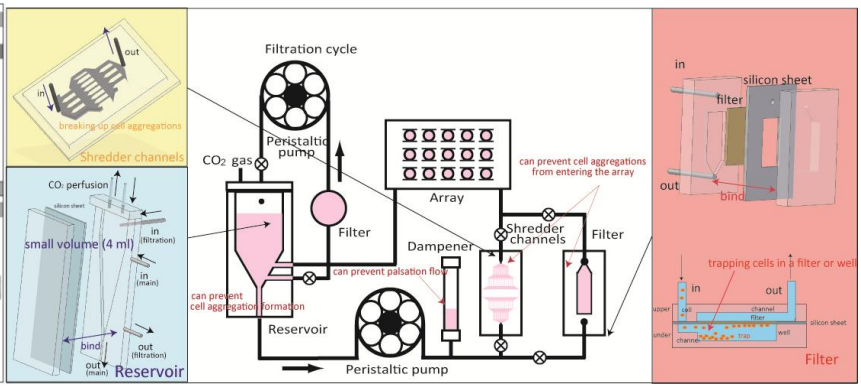

Fig. 2 Spheroid formation device consisting of an array and a perfusion system with a reservoir, shredder channels, a dampener, a filtration system, a peristaltic pump and a filter.

perfusion system, we cultured spheroid for two days while measuring the spheroid sizes. In addition, two days after the spheroids formation, we investigate cell viability by a live/dead assay using Calceing AM (4 μM) and EthD (8 μM) and CYP1A1 activation by EROD methods. Stationary and fluorescence two-dimensional images were captured using a CCD camera (Cool SNAP-cf, Nippon Roper Co., Ltd and EOS Kiss X3, Canon).

RESULTS AND DISCUSSION

Experiments were conducted with 3-mm-diameter chambers and cell densities of 200×10^4, 500×10^4 and 1300×10^4 cells/mL (Fig. 3). The mean size of the spheroids in one array could be controlled to be 134, 180, 237 μm with the standard deviations of 25 (18.7%), 30 (16.6%), 40 μm (16.9%), respectively. Previously, a single-chamber device formed hepatic spheroids with diameters in the range of 130-430 μm with a standard deviation of approximately 15%. These results demonstrate that an array with 10 chambers enables good controllability of the spheroid size.

Developed system enabled more than 80% of the formed spheroids to gently rotate and to be cultured in the chambers for more than two days. Fig. 4(a)–(c) show an optical image and fluorescence images of a formed spheroid stained with Calcein AM and EthD-1 after 48 h. No fluorescence was observed from the EthD-1 in the cells, but fluorescence was observed from Calcein (compare Fig. 4(b) and (c)). These images verified that cells in the spheroid lived in the chamber for 48 h. Fig. 5 shows the change in the spheroid size over 48 h. The sizes of all spheroids remained constant over 48 h. The maximum relative change in the spheroid sizes over two days was 1.5% (see Fig. 5).

Fig. 6 shows that the metabolic function (as indicated by CYP1A1 activity) of the formed spheroids increased over time. Hepatic function activation was assessed by analyzing fluorescence images obtained using EROD [4]. In this analysis, the change in the fluorescence intensity could depended on the spheroid size. Hence, the sizes at 0 h of the three groups were identified and used to normalize the increase in the EROD activity. The EROD activity of spheroids formed by our array increased after 1 day (see Fig. 6). Fig. 5 shows that the sizes of all three types of spheroids remained constant over 1 day. Thus, it is not necessary to consider the effect of their sizes. In addition, it is highly unlikely that the number of cells in a spheroid increases by a factor of three or four without a change in the spheroid size [5]. Thus, it is reasonable to assume that the detoxification activity of the spheroids increased with time.

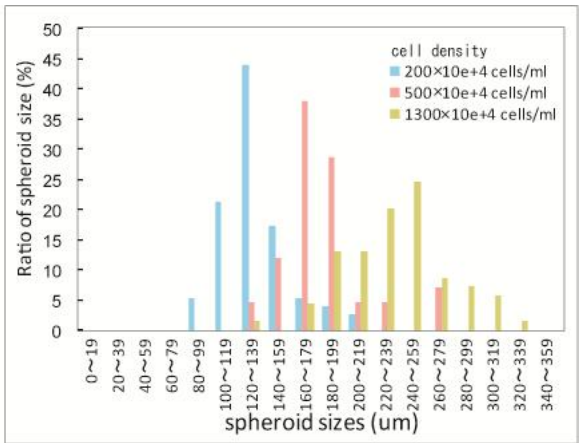

Fig. 3 Spheroid size distribution in array.

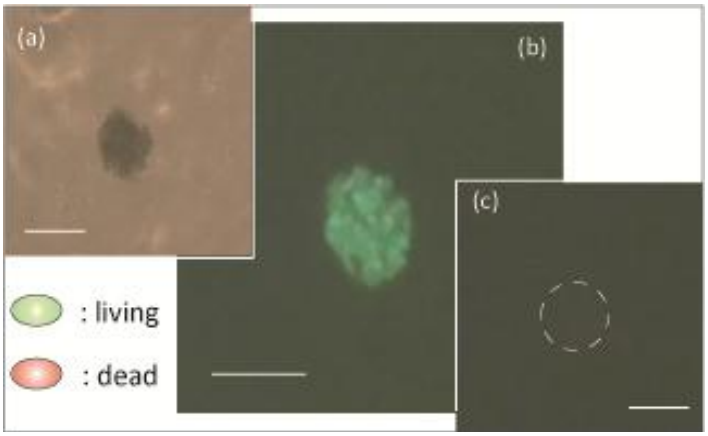

Fig. 4 Spheroid stained by calcein AM and EthD-1 after culturing for 2 days to verify the viability of the cells. (a) Optical image; (b) and (c) fluorescence images using calcein AM and EthD-1, respectively.

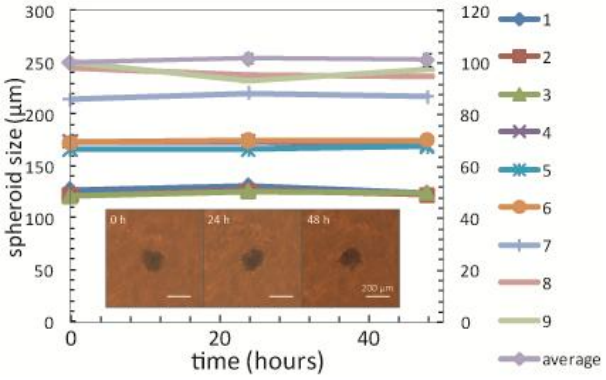

Fig. 5 Morphology of aggregations at 0, 24 and 48 h during spheroid formation. Change in cell aggregation diameter. Numbers in the right legend show numbers of spheroids. Left axis represents sample size. Averages in the legend mean average of ratio of all spheroid sizes when the size in 0 h is as a standard. Right axis corresponds to the ratio.

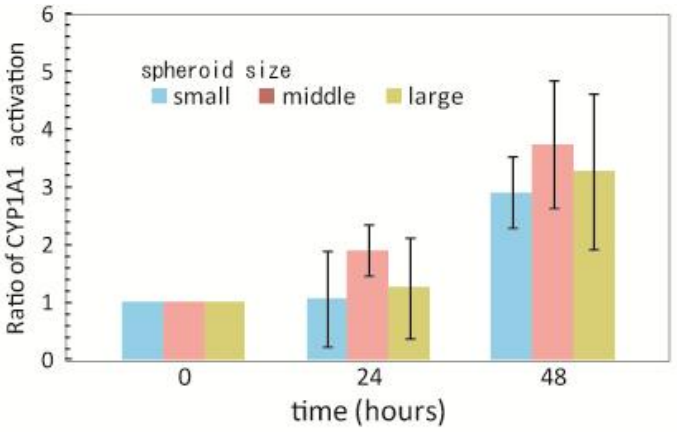

Fig. 6 CYP 1A1 activities of HepG2 at 0, 1 and 2 day. Small, middle and large mean size group of the spheroids with diameter in the ranges 110-140 μm, from 160-180 μm and from 200-24 μm.

CONCLUSION

In this study, we demonstrated the effectiveness of our spheroid forming platform that is composed of the perfusion system and micro chamber arrays in a size control and a long term culture. Using the arrays, the spheroid diameters could be controlled in the range 130-237 μm with standard deviations less than 19% with a good throughput of 1000%. A long-term culture longer than 2 days were conducted and revealed that the sizes of the formed spheroids remained constant and that their CYP1A1 activities increased over time. This array will be useful in drug screening and biological research as a bridge between cell research and tissue research.

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