High-throughput culture and embedment of spheroid array using droplet contact-based spheroid transfer

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Spheroids are one of the most representative models of 3D cell culture, which can be easily formed using conventional hanging drop method. However, medium change and spheroid transferring process are the bottlenecks that reduce the throughput of the entire process in the hanging drop culture. In addition, the embedment of spheroid into hydrogel still depends on the individual pipetting process. To overcome these issues, we present poly(dimethylsiloxane) (PDMS)-based simple devices which can exploit droplet contact-based spheroid transfer using a drop array chip (DAC) having an array of well structures and peripheral rims. When the upper spheroid-containing drops were in contact with the lower liquid drops, the air–liquid interface disappeared at the merged surface and the spheroids settled down due to gravitational force. This method was applied to repetitive medium change and live/dead staining of spheroids cultured with the hanging drop method. To simultaneously embed the spheroids into the corresponding collagen hydrogel drops, a PDMS-based pillar array chip (PAC) was contacted in advance with the spheroid-containing DAC. The contacted PAC then contained the spheroids trapped in small drops of liquid reduced in volume to around 0.5 μl . Consequently, the spheroids were embedded into the collagen drops at once by contacting the spheroid-containing PAC with the collagen-loaded DAC. The embedded spheroids using the DAC–PAC contacting method showed a reliable invasion behavior compared to the embedded spheroids using conventional manual pipetting. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5039965>

INTRODUCTION

Since the cells grown in three-dimensional (3D) culture models show more *in vivo*-like behaviors than those grown in two-dimensional (2D) monolayer, there have been great advances in the study of 3D cell culture models.^{1–5} In particular, spheroid, a spherical cell cluster, is one of the most representative models of 3D cell culture due to its compactness and the wide range of applicable cell types. It has a high similarity to the *in vivo* tissue environment, as it is the aggregate of cells in a foreign material-free environment where cell–cell interaction becomes dominant.^{6–9}

A hanging drop method is a facile method used in spheroid formation because the only thing it needs is a drop depositing site that can be used for hanging. Nonetheless, there are some disadvantages, such as the vulnerability to spillover and the unsuitability for long-term culture.¹⁰ The latter is due to the smaller volume of the medium than other methods, which leads to frequent medium changes. In addition, the medium change process needs to be performed carefully without touching the spheroid, resulting in low throughput. The process of individual medium change also causes the time difference between the first and the last, and the spheroid culturing devices are exposed to the external environment for a longer time as the number of well increases.

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As one way of solving these problems, microfluidic hanging drop devices^{11–14} have been presented, which link all hanging drop arrays to microchannels and connect them to fluidic pumps. However, because these methods have been applied to the spheroid array network that has been designed to use a multi-spheroid interaction, the spheroids in the microfluidic hanging drop devices may interfere with each other due to the interconnected microchannel structure. Therefore, the medium change of the separated hanging drop array without the use of additional equipment remains a challenge.

Meanwhile, as an attachment-free condition of the spheroid limits its biological relevance, the spheroids embedded in a hydrogel have been studied because the hydrogel provides mechanical support and cell–matrix interactions.¹⁵ In addition, several spheroid invasion assays have been investigated using the phenomenon that the embedded spheroid consisting of metastatic cells invades the hydrogel matrix to mimic and study the metastasis of tumors in the body.^{16–18} Although the spheroid-in-hydrogel models have been widely used, the embedment of spheroid into hydrogel is still performed by manipulating the spheroid one-by-one using a pipette in most studies. This process is time-consuming and labor-intensive since it requires delicate pipetting and should be performed under magnifying equipment such as a stereoscope for successful spheroid embedment. There are several studies that have been reported to overcome this problem using diffusion-mediated alginate¹⁹ or collagen–alginate²⁰ encapsulation, or micro-injection of cell–polymer suspensions directly into the collagen matrix.²¹ However, it is difficult to apply the embedment of spheroids into collagen alone,^{19,20} or to rely on automated equipment.²¹ A different method needs to be considered to embed multiple spheroids into the hydrogel consisting of natural extracellular matrix (ECM) proteins such as collagen, fibrinogen, or laminin, without external equipment.

Here, we have focused on the feature of the hanging drop array system, which has a high accessibility to spheroids since they are formed at the bottom of liquid drops. Several studies have demonstrated the high-throughput transfer of spheroids in the hanging drops to the low aspect ratio wells of the transfer and imaging plate,²² the hydrophilic spot arrays,²³ and the cell culture dish²⁴ by contacting the drops with them. However, their purpose of the transfer is different from the simultaneous medium change as the transfer method can be used once at their devices. The embedment of spheroid into hydrogel is also difficult to apply, because the volume of the drops should be reduced so as not to significantly change the concentration and distribution of contacted hydrogel drops.

To expand the droplet contact-based spheroid transfer method to high-throughput medium change and embedment of spheroid into ECM, we present poly(dimethylsiloxane) (PDMS)-based drop/pillar array chips that can be used for droplet contact-based spheroid transfer. A drop array chip (DAC) is designed to allow repetitive spheroid transfer by contacting two drops on each side. It can be applied to medium change, which can facilitate long-term cultivation of the spheroid in the hanging drop. To embed the spheroids into the collagen drops, a pillar array chip (PAC) is introduced to contact with the spheroid-containing DAC for reducing the volume of the spheroid-containing drop in advance. The contacted PAC then traps the spheroids in small drops of liquid whose volume is significantly reduced compared to the contacted drops in DAC. Consequently, the spheroids are simultaneously embedded in the collagen drops by contacting the spheroid-containing PAC with the collagen-loaded DAC, without changing the concentration and distribution of the spheroid-embedded hydrogel.

MATERIALS AND METHODS

Fabrication of a PDMS-based drop array chip (DAC) and a pillar array chip (PAC)

DAC and PAC were fabricated using the polymethyl methacrylate (PMMA) plates (YM Tech, Daejeon, Korea) as molds. The PMMA molds were fabricated by a CO₂ laser cutter (C40-60W; Coryart, Anyang, Korea). To make the mold for the DAC, a PMMA wafer (circular disk with a thickness of 3 mm and with a diameter of 12 cm) was etched by laser to make an array of rim structures. After etching, the PMMA pieces attached with a double-sided adhesive tape (Kyodo Giken Chemical, Saitama, Japan) were adhered to the inner circle of each rim

structure. In the case of the PAC mold, a PMMA wafer was etched four times by laser to make an array of pillars with 1 mm height. The detailed procedures for preparing the PDMS-based array chips and their molds are shown in Fig. S1 ([supplementary material](#)).

To fabricate the PDMS-based DAC and PAC, a PDMS monomer and a curing agent (Dow Corning, MI, USA) were mixed at a ratio of 10:1 and poured on the patterned PMMA molds. Since the air bubbles in the PDMS mixture make the PDMS devices to have an uneven surface, the PDMS mixture poured on PMMA molds passed through a degassing process in a vacuum chamber for 20 min. After baking for 30 min at 90 °C on a hot plate, the PDMS devices were peeled off from the master mold. The PDMS-based DAC was finally obtained by combining the cured PDMS with a glass slide. Meanwhile, the PDMS-based PAC requires an additional PDMS stamping step to make the pillar surface smoothen. An uncured PDMS mixture of 10:1 ratio was spin-coated (500 rpm, 1 min) on a silicon wafer. The top surface of the PDMS pillars was coated by stamping with uncured PDMS, thereby making the PDMS pillar surface evenly. Then, the PDMS-based PAC was obtained after curing in an oven at 65 °C for 2 h. As a final step, all the fabricated PDMS-based DAC and PAC were autoclaved and passed through 5 min ultraviolet (UV) sterilization before use.

Determination of the volume and concentration of transferred liquid drop

To quantify the volume and concentration of transferred liquid drop after contacting, phosphate-buffered saline (PBS)-based 1 mM erioglaucine solution was used as a sample solution.²⁵ 0.5–10 μ l of the erioglaucine solution were loaded into 100 μ l PBS. Then, the calibration curves according to the concentration of erioglaucine were obtained by setting the spectrometer wavelength to 406 nm and measuring the peak absorbance of the erioglaucine solution.

To measure the volume after contacting two DACs, 1 mM erioglaucine solution was loaded into each well of the both DACs. After the contact, the whole drop of the bottom DAC was aspirated by the pipette and mixed with 100 μ l PBS for the peak absorbance measurement. The volume of the bottom drop after DAC–DAC contacting was calculated from the calibration curve. For determining the transferred liquid volume after contacting DAC with PAC, 1 mM erioglaucine solution was loaded to each well of the DAC and contact with PAC. The transferred liquid drop on the PAC was aspirated by the pipette and mixed with 100 μ l PBS, and the volume of the transferred liquid drop was calculated by passing through the same steps with DAC–DAC contacting.

To measure the concentration after contacting the DAC–DAC, 1 mM erioglaucine solution was loaded to the upper DAC and PBS was loaded to the bottom DAC. After contacting two DACs, the drop of the bottom DAC was gently mixed with a pipette, and 5 μ l of the drop was moved and mixed with 100 μ l PBS. The concentration of the bottom drop after DAC–DAC contacting was calculated from the calibration curve.

The effect of contacting duration on the volume and the relative concentration of the bottom drop after contacting the DAC–DAC was evaluated by changing the duration to 2 s, 4 s, 8 s, 16 s, and 32 s. The initial volume of both the upper and bottom drops was set to 7 μ l.

2D and 3D cell culture

MCF-7 cells were maintained in a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The medium was changed every 24 h. The cells were cultured in a humidified incubator of 37 °C, 5% CO₂. For spheroid formation, the cell suspensions were obtained from the cell monolayers by a trypsinization process. The cells were counted and diluted with additional media to the desired seeding concentration (1000/3000/5000/10 000 cells per drop). To verify the effect of media additives to the spheroid formation, neutralized collagen type I solution or methylcellulose solution were mixed with the cell suspension for the desired concentration (25/50/200 μ g/ml for collagen, 0.12/0.24/0.48/1.2/2.4 mg/ml for methylcellulose). After mixing the cell suspension gently with a pipette, 8 μ l of the suspension was loaded to each well of the DAC. The medium was replaced every 24 h by contacting the spheroid-containing drops in DAC with the

7 μ l drops of fresh media in another DAC. To prevent the drop evaporation, the DAC was placed on the two PMMA columns with a height of 1 cm, and the culture dish was filled with 10 ml PBS. The growth of the spheroids was monitored every day with a microscope (IX72; Olympus, Tokyo, Japan) and a charge-coupled device (CCD) camera (DP72; Olympus).

Cell labeling and imaging

The spheroids in DAC were transferred to another DAC by contacting two drops, which had 7 μ l drop array of a PBS containing 20 μ M calcein-AM and 10 μ M ethidium homodimer-1. The spheroids were incubated under 5% CO₂ at 37 °C condition for 15 min. Bright field images and fluorescent images were captured using a CCD camera on a fluorescence microscope, and the viability of the cells was evaluated qualitatively.

Embedment of spheroid into collagen drop

A neutralized collagen solution was diluted to 3 mg/ml with PBS, and 4 μ l of the solution was loaded into each well in another DAC. While the DAC was placed on an ice to maintain the loaded collagen drops unpolymerized, the spheroid-containing DAC was contacted with the PAC to transfer the spheroid with a small amount of medium. After transferring the spheroids, the PAC was contacted to the collagen-loaded DAC for 1 min to settle down the spheroid. The spheroid-embedded collagen drops were polymerized in an incubator at 37 °C for 20–30 min, and the whole DAC was immersed in the cell culture medium.

To compare the invasion of embedded spheroids in DAC with the conventional method, a one DAC had of a set of spheroid-embedded collagen drops by which the spheroids were transferred to collagen drops with manual pipetting. All the embedded spheroids were observed and imaged every 24 h for 7 days.

Image analysis

All the captured images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA, <http://imagej.nih.gov/ij/>) unless otherwise specified. The area to be analyzed was selected by adjusting the color threshold and measured after setting the scale to convert a pixel unit to a micrometer. For the measurement of the spheroid diameter, it was assumed as a sphere and the mean diameter (d_s) was obtained from the measured area (A_s) by the equation $d_s = 2\sqrt{A_s/\pi}$.

RESULTS AND DISCUSSION

Design and working principle

DAC and PAC are designed to utilize droplet contact-based transfer for culturing and staining the spheroids, and embedding them into collagen drops to make a spheroid–collagen drop array. First, a DAC is used as a hanging drop platform for the spheroid culture [Fig. 1(a)]. The dimensions of the well array are fitted to a 384-well plate, and each well structure has a peripheral rim to prevent the liquid drop spilling over. At the outside of the well array, a spacer exists to determine the height limit and to help alignment of two DACs at the DAC–DAC contacting step. The PDMS chip is adhered with a glass slide to obtain mechanical strength that the DAC cannot be bent during the hanging drop culture or handling. A PAC is used as an intermediate to reduce the drop volume at the spheroid embedment process [Fig. 1(b)]. Like DAC, the dimensions of the pillar array are fitted to the 384-well plate, and the spacer exists at the outside of the pillar array. To develop the spheroid to have more *in vivo*-like features, long-term cultivation (over 6 days) is performed with the DAC by exchanging the medium daily with DAC–DAC contacting [Fig. 1(c)]. The developed spheroids are placed on a PAC with small media drops which have a significantly reduced volume by DAC–PAC contacting [Fig. 1(d)]. Next, the spheroid-containing PAC is in contact with a collagen-loaded DAC to embed the

FIG. 1. Schematic illustrations of the PDMS-based array chips and the droplet contact-based transfer methods. (a) A schematic of drop array chip (DAC) in a 3D view; the inset depicts a cross-sectional view of a single spot of the DAC. (b) A schematic of pillar array chip (PAC) in a 3D view; the inset depicts a cross-sectional view of a single spot of the PAC. (c) A schematic illustration of the DAC–DAC contacting method for the repetitive change of the spheroid medium. (d) A schematic illustration of the DAC–PAC contacting method for the embedment of spheroid into the collagen matrix.

spheroids into the collagen drops. After collagen polymerization, the spheroid–collagen DAC is immersed in the culture medium to observe the invasion of the spheroid.

Characterization of the DAC and DAC–DAC contacting

As the physical characterization about the droplet contact-based spheroid transfer has not been studied to date, we first characterized it with the DACs. For this purpose, the liquid drops in the DACs are designed to have a constant wetting area even after contacting the DAC–DAC by adding the rim structure which confines the wetting area to the edge of the rim. It has also been reported that the rims around the drops prevent them from overflowing in other hanging drop devices.^{11,26} As a result, when the same volume of liquid was loaded in each well, all the drops had a similar shape and their positions were well aligned [Fig. 2(a)].

Next, a pair of eriochlorine solution-loaded DACs were used to characterize the volumetric change of the drops after the droplet contact-based transfer. The working volume of the upper

FIG. 2. A fabricated DAC and the characterization of DAC–DAC contacting. (a) A picture of the fabricated DAC. 1 mM erioglaucone solution was loaded in each well. Scale bar = 1 cm (scale bar of the inset = 1 mm). (b) The volume and relative concentration of bottom drop after the DAC–DAC contacting. The initial volume of the bottom drop was set to 7 μL . The relative concentration was defined as the erioglaucone concentration of the bottom drop divided by the concentration of the initial upper drop.

DAC was set up to 8 μL , since some drops were spilled over the chip during the experiment when the loading volume was more than 9 μL (data not shown). For the same reason, the working volume of the bottom DAC was set to 7 μL so as not to exceed the upper volume limit after contacting two DACs. When the two drops were in contact, they formed a capillary bridge which had a form of a catenary curve. As the height of the upper chip from the bottom chip became higher, the width of the bridge became smaller, and the bridge splits into two drops in a certain time. In the ideal condition (when gravity is neglected), the volume of separated drops is the same. However, when considering Bond number of the PBS drop at the DAC ($\text{Bo} = \rho g L^2 / \gamma \sim 0.1$, where ρ is density of the liquid, g is gravitational acceleration, L is characteristic length of the drop, and γ is surface tension of the liquid), it is expected that the volume of drop can also be affected by gravity which has a smaller order than the capillary force.

While the two drops are in contact, there should be liquid exchange from the upper one to the bottom one and vice versa, since the convection results from the merging of the two drops and the diffusion of the molecules. This leads to a concentration change of the two drops when different liquid drops are in contact. To characterize the concentration change of the drops after the droplet contact-based transfer, we used the erioglaucone solution-loaded DAC for the upper one and the PBS-loaded DAC for the bottom one.

First, we checked the effect of the contacting duration on the concentration change of the bottom drop, which may be an important factor for affecting the relative concentration of the bottom drop (Fig. S2, [supplementary material](#)). Because the mixing of two drops is also affected by diffusion, the longer duration makes a higher mixing efficiency while not affecting the volume of the drop. To reduce the concentration change of the bottom drop, we chose less than 2 s for the contacting duration.

Next, the volume and concentration change of the bottom drop were characterized according to the volume of the initial upper drop [Fig. 2(b)]. In all working volume ranges, an exchange of two liquids occurs during the contact, but the concentration change of the bottom drop does not exceed 20%. For long-term cultivation of spheroid using the DAC–DAC contacting method, the drop volume should be maintained regardless of the culturing day. When we set the initial volume of the cell suspension drop (the drop of the upper chip) to 8 μl and the volume of the fresh medium drop (the drop of the bottom chip) to 7 μl , we could expect the long-term cultivation of the spheroid using DAC–DAC contacting to be possible.

MCF-7 spheroid formation and growth in the DAC

To find an optimal composition of the MCF-7 cell suspension for spheroid formation, various concentrations of collagen or methylcellulose solutions were mixed with the cell suspension and loaded to the DAC (Fig. S3, [supplementary material](#)). When no hydrogels were added, they formed one large spheroid at the center of the drop and multiple satellite spheroids near the side of the well at the seeding number over 3000 cells/drop. In the case of collagen addition, regardless of its concentration, the cells formed multiple spheroids near the wall. It can be explained that the collagen has binding sites to which the cells can be attached. When the cell suspension was mixed with collagen solution, they attached to different binding sites of collagen and started aggregation. As the collagen fibers can be attached to the side of the well, the collagen solution was regarded as an unsuitable additive in our device.

Unlike collagen, methylcellulose does not give binding sites to cells.²⁷ The addition of methylcellulose facilitated the MCF-7 cells to form one single spheroid at the seeding concentrations more than 0.24 mg/ml. However, the higher concentration of methylcellulose made the drop more viscous, making it difficult to transfer the spheroid in the drop to another drop when two drops were in contact (data not shown). Therefore, 0.24 mg/ml methylcellulose solution was used in our experiment and we confirmed that the spheroid was successfully formed.

With the optimal composition of the spheroid-forming medium, the MCF-7 cell suspension was loaded to the DAC with a different seeding concentration and monitored for 6 days (Fig. 3). During the spheroid culture of the whole experiment, the environment of the spheroid was changed by contacting the spheroid-containing drop and another drop (Video S1, [supplementary material](#)). When the drops are in contact, the merged part of two drops has been turned from the air/liquid interface to the liquid/liquid interface. We could expect that the surface tension at the merged part becomes negligible compared to the gravitational force exerted on the spheroid, because the difference in the surface tension between media or PBS is significantly lower than the air/liquid interface. Consequently, the spheroids moved downward by gravity, while the two drops mostly maintain their initial composition due to the short contact time [Fig. 2(b)]. After spheroid transfer, the DACs were separated and the spheroid-containing DAC was inverted to make a hanging drop state. The spheroids were aligned at the center of the drops as they settled down along the surface of the drops.

A single spheroid was generated in each drop at all the seeding concentrations, and the spheroid diameter and the viability were changed as the culturing time increased [Fig. 3(a)]. At day 5, we observed the ring composed of dead cells, which regarded as a necrotic core. The diameter of the spheroid was also directly proportional to the seeding concentration [Fig. 3(b)]. However, when the seeding concentration was 10 000 cells/drop, the spheroid diameter was not increased as the culturing time increased. This result supposed that the upper limit of the cell number might exist because the volume of the medium drop was limited. In the following experiments, we used the spheroids seeded with a concentration of 5000 cells/drop as they were larger than 500 μm , the criterion for the formation of a necrotic core inside the spheroids.^{7,28,29}

FIG. 3. The growth of MCF-7 spheroids according to culturing time. The culture media of spheroids were changed using the DAC–DAC contacting method. (a) The bright-field images and live/dead fluorescence images of the spheroids with an initial seeding number of 5000 cells/drop. Scale bar = 200 μm . (b) A graph of spheroid diameter versus culture time. MCF-7 cells were seeded in four different conditions (1000, 3000, 5000, and 10 000 cells/drop).

Characterization of the PAC and DAC-PAC contacting

After peeling off the PAC from the master mold, the top of the pillars in the chip had a rough grooved surface due to laser etching in the fabrication process (Fig. S4, [supplementary material](#)). These grooved structures allowed the drops to easily spread and flow down along the sides of the pillars, as the rough surface gave a larger contact area compared to the flat surface. When the top surface of the pillars was coated with a thin PDMS layer by the PDMS stamping, the pillars had a smooth surface and the transferred drops were stably positioned on them [Fig. 4(a)]. The diameter of the top surface was about 0.3 mm smaller than the set value, because the intensity of the laser decreased as the distance from the focal plane increased.

Next, the volume of the drops transferred on the pillars with different diameters was measured to characterize the DAC–PAC contacting method [Fig. 4(b)]. The liquid drop with a volume of 8 μl was in contact with the top surface of the PDMS pillar. Then, the liquid drop formed a capillary bridge between the rim edge of the DAC and the pillar edge of the PAC. As the distance between the two chips increased, the waist of the capillary bridge became smaller and broke off into the two drops. Unlike the DAC–DAC contacting, the transferred liquid was under the action of hydrophobic PDMS surface, while that in DAC was under the action of the liquid filled in the well. On that account, the transferred volume did not exceed half of the initial volume of the upper drop. In addition, the volume of the transferred drop was proportional

FIG. 4. A fabricated PAC and the characterization of DAC–PAC contacting. (a) A picture of fabricated PAC. 1 mM erio-glucine solution was transferred on the top surface of each pillar after contacting the DAC–PAC. Inset shows an enlarged image of the single pillar of the PAC. Scale bar = 1 cm (scale bar of the inset = 1 mm). (b) A graph of the volume of transferred drop on PAC versus the diameter of the pillar of PAC. The contact was performed until the whole flat surface of the pillar is in contact with the liquid drop of the DAC.

to the diameter of the pillar, because the wetting area of the pillar surface was confined to the pillar edge.

There were two conditions to select the pillar diameter range for spheroid embedment into the collagen drop: (i) the drop should be small to minimize the composition change in the collagen which will be in contact, and (ii) the transferred drop should not be dried until it contacts with the collagen drop to avoid the spheroid damage. As the smaller drop has a higher surface area to volume ratio, the pillars whose diameters are smaller than 1.4 mm were difficult to apply at the spheroid transfer due to the drop evaporation. For spheroid embedment by DAC–PAC contacting, we used the PAC consisting of the pillars with a diameter of 1.8 mm.

Spheroid embedment into collagen drop using PAC and invasion of the spheroid

The spheroids grown in DACs for more than 6 days were simultaneously transferred to the collagen drops using the PACs optimized from the previous part [Fig. 5(a)]. First, the spheroid-containing DAC was in contact with the PAC. The spheroids and the small amount of medium were simultaneously transferred to the PAC. Next, the PAC was inverted to place the spheroids on the bottom of the drops and in contact with the collagen drops of another DAC. During contacting time, all the spheroids sank to the bottom of the well. Because the volume of the upper drop was reduced up to 0.5 μL and the volume of the collagen solution in the bottom well was set to 4 μL , the volume of the transferred liquid was reduced and the spheroids could be encapsulated by the collagen solution. After collagen polymerization, the spheroid-embedded collagen drops were formed and the position of the spheroids was fixed. Since the polymerized drops were inside the well structure, they were not detached from the DAC even after immersion in the cell culture medium.

We also tested the DAC–DAC contacting with a medium drop and a collagen drop to transfer the spheroid into the collagen drop. However, a significant difference in the color of

FIG. 5. Spheroid embedment into collagen gel by DAC-PAC contacting and invasion of embedded spheroid. (a) The embedment of spheroid into collagen gel by DAC-PAC contacting. Spheroids were transferred from the hanging drop array (left) to the pillar array (middle) and then collagen drop array (right). The spheroids embedded in the collagen drops were imaged under the dark field to show the translucent collagen which was polymerized. Scale bar = 2 mm. (b) Invasion of spheroid embedded in collagen gel. Scale bar = 500 μm . (c) The graph of relative invasion ratio versus culture time. The invasion ratio was defined as an invaded area of the spheroid divided by the size of the spheroid at day 0.

the polymerized collagen was observed compared to the collagen drop that did not come into contact (Fig. S5, [supplementary material](#)). It seemed that the transferred liquid drop dilutes or pushes out the collagen located at the center of the well, since the volume of transferred liquid would be large when the large liquid drop was in contact with hydrogel drop.

The invasion of MCF-7 cells was observed from the time the spheroids were embedded in the collagen drops, and the area occupied by invaded cells increased over time [Fig. 5(b)]. This invasive behavior was different from the spheroids in the liquid drop, since the collagen matrix enabled the cells to bind and migrate. To verify the reliability of the spheroid embedment method by DAC-PAC contacting, it was compared to the conventional method (spheroid embedment by manual pipetting) by observing the invasion of spheroids. We measured the relative invasion ratio of the spheroids, defined as the occupied area of the invading cells divided by the initial spheroid size, to normalize the deviation resulting from the difference in the initial spheroid size. As a result, the spheroids embedded by the DAC-PAC contacting showed a similar invasion rate compared to the manual pipetting method [Fig. 5(c)]. Consequently, the DAC-PAC contacting method can be said to make a spheroid-hydrogel drop array in a high-throughput manner while keeping the model reliability the same as the conventional method.

CONCLUSIONS

In this study, the culture and embedment of spheroid array in a high-throughput manner were demonstrated using droplet contact-based spheroid transfer. For the spheroids cultured in the hanging drop method, the media were easily changed to fresh media or the staining solution

by contacting spheroid-containing DAC with the other liquid-loaded DAC. After the contact, the concentration of the spheroid-containing drop remained above 80% of its initial concentration. With the DAC–DAC contacting method, we were able to culture MCF-7 spheroid array in the DAC over 6 days. In addition, the spheroid array was immediately embedded in the collagen drop array when using a DAC–PAC contacting method. The spheroid-containing DAC was first in contact with the PAC to transfer the spheroid array to the PAC with a reduced liquid volume of around 0.5 μ l. Next, the spheroids were embedded in the collagen drops at once by contacting the spheroid-containing PAC with the collagen-loaded DAC, and this can be contrasted with one-by-one embedment of the spheroid by manual pipetting. It is expected that the DACs and PACs for droplet contact-based spheroid transfer can solve the throughput issue of the hanging drop culture and the spheroid embedment process. The throughput of the devices can be improved further when the number of arrays in the chips increases. At this time, the machine which shows x , y , z linear movement can be integrated with the devices to reduce the deviations that come from the handling by hand. Also, these devices can be used with a liquid handling machine for automated liquid loading during spheroid culturing and embedment, because the proposed method prevents the pipette tips of the machine from touching any spheroids during the experiment. Besides the culture and embedment of the spheroids, we expect that various multi-step assays for the spheroid arrays will be possible in a high-throughput manner with the droplet contact-based spheroid transfer chips.

SUPPLEMENTARY MATERIAL

See [supplemental material](#) for a detailed description of the fabrication process of the PDMS-based array chips and their PMMA molds (Fig. S1); the graph of the volume and the relative concentration of bottom drop according to the contacting duration (Fig. S2); the microscopic images of the MCF-7 spheroids (Fig. S3); comparison of the contacting scheme between the drop and the pillar surface (Fig. S4); the images of polymerized collagen drops after DAC–DAC contacting (Fig. S5), and a video demonstrating the spheroid transferring process from the upper spheroid-containing drops to the bottom media-loaded drops.

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