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Interface-engineered Caco-2 cell culture on a collagen-coated liquid-liquid interface in a microfluidic device

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Abstract

Epithelial tissues form selective barriers essential for physiological homeostasis. Conventional in vitro models rely on solid substrate, which limit the physicochemical flexibility of the cellular microenvironment. Here, we introduce a microfluidic platform in which a collagen-coated liquid-liquid interface formed between perfluorocarbon (FC-43) and culture medium serves as a substrate for epithelial cell adhesion. By culturing Caco-2 cells in the device, we show that the liquid interface supports cell attachment and the formation of near-confluent monolayers. Immunofluorescence observation reveals the development of tight junctions and organized actin cytoskeletons, indicating early-stage epithelial maturation. Our new microfluidic system enables the formation of stable liquid-liquid interfaces that serve as viable and flexible substrates

for epithelial cell culture, offering new opportunities for multiphase microfluidic models of epithelial barriers.

Keywords

gut-on-a-chip; liquid-liquid interface; collagen; Caco-2; FC-43

Introduction

Epithelial tissues form selective barriers that regulate the transport of molecules, ions, and gases, play an important role in physiological homeostasis [1]. In vitro models of epithelial barriers, particularly culturing Caco-2 cells, have been widely used to study the function of the epithelial tissues [2,3,4]. The microfluidic culture system known as gut-on-a-chip enables the cultivation of Caco-2 cells under conditions that more closely recapitulate the in vivo intestinal environment. By precisely controlling fluid flow and inducing mechanical stimuli, this platform provides physiologically relevant mechanical cues that are difficult to reproduce in conventional static culture systems [5,6,7]. Gut-on-a-chip platforms recapitulate the apical-basolateral compartmentalization of the human intestinal epithelium by dividing upper and lower microchannels with a solid porous membrane, upon which intestinal epithelial cells are cultured to establish a polarized epithelial barrier. Although porous membranes enable compartmentalization, they may also impose non-physiological diffusion barriers and mechanical constraints.

Liquid–liquid interfaces provide a distinct platform for cell culture compared with conventional solid substrates [8, 9, 10], offering a scaffold-free and mechanically tunable environment that eliminates the rigidity and pore-related constraints inherent to synthetic solid membranes. Moreover, when functionalized with extracellular matrix proteins, such interfaces can support uniform epithelial maturation and stable

monolayer formation, demonstrating their potential as engineered bioactive substrates [10]. Owing to their dynamic responsiveness to chemical and physical stimuli, such interfaces can also serve as reservoirs or delivery media for gases and hydrophobic molecules [11]. Incorporating a liquid–liquid interfacial strategy into microfluidic cell culture systems may therefore offer an alternative to porous membrane–based designs and mitigate the inherent limitations of synthetic solid membranes in gut-on-a-chip platforms. Among candidate materials, perfluorocarbon liquids such as FC-43 are particularly attractive due to their chemical inertness, immiscibility with aqueous media, and high gas solubility [11], making them well suited for multiphase microfluidic applications.

In this study, we propose a novel microfluidic cell culture system by utilizing collagen-coated liquid-liquid interface serves as a cell-adhesion substrate for epithelial cells (Fig. 1A). The rectangular cuboid shaped microfluidic device allows stable formation of the liquid-liquid interface for cell culture by sequential flow manipulation (Fig. 1B). Using Caco-2 cells, a well-known epithelial cell line, we demonstrate that cells can adhere and form near-confluent monolayers on the liquid-liquid interface. Immunofluorescence images reveal that the formation of tight-junction and organized actin cytoskeletons, indicating epithelial early-stage maturation. Our results establish liquid-liquid interface as viable and functional substrates for epithelial cell culture and highlight their potential for the development of flexible, multiphase microfluidic models of epithelial barrier.

Results and Discussion

Liquid-Liquid interface formation in the microfluidic device

The liquid-liquid interface was formed by first filling the microchannel with the perfluorocarbon liquid FC-43, followed by the introduction of an aqueous solution containing collagen (Fig. 1 B-ii). The effect of channel dimensions on the formation of the liquid-liquid interface was evaluated using the rectangular cuboid shaped microfluidic device.

First, the channel height and length were fixed at 7 mm and 10 mm, respectively, while the channel width was varied (1, 1.5, 2.0, 5.0 and 10 mm). Liquid-liquid interfaces were subsequently formed in each channel by introducing collagen solution at 50 μL/min. The rectangularity of the aqueous layer was introduced as an intuitive and easily interpretable indicator, and the rectangularity is calculated by

$$Rectangularity(\%) = \frac{The\ area\ of\ the\ collagen\ solution\ region}{The\ area\ of\ the\ minimum\ bounding\ rectangle\ of\ the\ collagen\ solution\ region} \times 100 \quad (1)$$

where the collagen solution region is observed as a surface defined by the channel height × channel length (see Fig. 2 or Fig. S1-4). The rectangularity for each channel size is shown in Fig. 2. When the channel width ranged from 1 to 2 mm, the rectangularity of the aqueous layer was 94% or higher, however, when the channel width was 5 or 10 mm, the rectangularity decreased to 88% (Fig. 2). These results suggest that channel width affects the formation of the liquid–liquid interface. To analyze this effect, the Bond number (B_o) a dimensionless parameter defined as

$$B_o = \frac{\rho g L^2}{\sigma} \quad (2)$$

was introduced to evaluate whether gravitational forces or interfacial tension dominate when two fluids of different densities are in contact. Here, ρ denotes the density difference between FC-43 and the collagen solution, g is the gravitational acceleration, L is the channel width, and σ is the interfacial tension coefficient. In this experiment, as the channel width L was varied, its effect on the Bond number was directly assessed. Taking the Bond number for a channel width of 1 mm as a reference, the Bond numbers for channel widths of 1.5, 2, 5, and 10 mm were 2.25, 4, 25, and 100 times greater, respectively. For channel widths of 5 and 10 mm, the Bond number increased by one to two orders of magnitude. As the Bond number increases, gravitational forces increasingly dominate over interfacial tension. Thus, for channel widths of 5 and 10 mm, the gravitational effect on the aqueous layer exceeds that of surface tension, resulting in a pronounced meniscus and a reduction in rectangularity. In this study, a high rectangularity was interpreted as indicating an interface nearly parallel to the channel bottom, which is favorable for microscopic observation; accordingly, devices exhibiting high rectangularity were selected for the demonstration. For a practical application, the device with a width of 2 mm was selected for the cell culture experiments because it offered advantages in operation, including easier bubble removal.

In addition, the effect of channel height on the formation of the liquid-liquid interface was evaluated (Fig. 3). The channel height was varied at 7, 5, and 4 mm. When the height was 4 mm, the FC-43 layer was almost completely displaced upon the introduction of the collagen solution, preventing interface formation. At a height of 5 mm, the liquid–liquid interface was formed, but the rectangularity decreased to

approximately 87%, comparable to the rectangularity observed for aqueous layers in channels 5 and 10 mm wide at a height of 7 mm. These results indicate that channel height is also a critical parameter for the formation of the liquid–liquid interface.

Rectangularity was evaluated by varying both the channel size and the flow rate of the collagen solution (Fig. S1-4). Our results suggest that interface formation is largely insensitive to the flow rate of the collagen solution, whereas the channel size significantly governs the interfacial shape.

Caco-2 cell culture on the liquid-liquid interface

The feasibility of adhesive cell culture on the liquid-liquid interface with a collagen layer was demonstrated by culturing Caco-2 cells (a human colorectal adenocarcinoma cell line). After forming liquid-liquid interface using FC43 and collagen-containing serum-free DMEM, the cell suspension was introduced and seeded at a density of 1.5×10^5 cells/cm^2. The growth of Caco-2 cells on the liquid-liquid interface is shown in Fig. 4. At 2 days post-seeding, most Caco-2 cells had adhered to the interface, resulting in the formation of a monolayer. This monolayer was maintained for 7 days. Previous studies have suggested that Caco-2 cells begin to exhibit early functional characteristics of the intestinal epithelium, such as tight junction formation, after approximately 7 days of culture [12]. In the present study, we therefore chose 7 days of culture period to assess the early-stage maturation of Caco-2 monolayers from a proof-of-concept perspective.

To evaluate the effect of collagen coating on the liquid-liquid interface, the behavior of Caco-2 cells seeded onto a liquid-liquid interface without collagen coating is shown in Fig. S5. Caco-2 cells did not adhere on the liquid-liquid interface to form a monolayer; instead, they aggregated with each other and were observed floating on the liquid-liquid interface. These results indicate that bringing the collagen solution into

contact with FC-43 results in the formation of a collagen layer on the liquid-liquid interface that is sufficiently robust to support cell adhesion.

Caco-2 cells monolayer maturation

Immunofluorescence staining was performed to observe the characteristics of cultured Caco-2 cells as an intestinal epithelial model. Figure 5 shows a comparison of immunofluorescence images of ZO-1, F-actin and nuclei in Caco-2 cells cultured in the microfluidic device and in 96-well plate. In both conditions, nuclei were distributed, indicating the formation of a confluent cell layer. Although autofluorescence from FC-43 was observed in the images of the microfluidic devices, ZO-1 localization at cell-cell junction was confirmed, suggesting the formation of epithelial barrier properties in Caco-2 cells cultured in the microfluidic device. F-actin localization was observed under both conditions, suggesting that the cytoskeletal organization supporting epithelial structure was maintained in Caco-2 cells cultured in the microfluidic device. Taken together, these results suggest that Caco-2 cells cultured in the microfluidic device formed a confluent epithelial layer with tight junction and supporting cytoskeletal organization, recapitulating intestinal epithelial characteristics. Caco-2 cell culture and immunostaining within the microfluidic device were conducted in triplicate (n=3), and representative results are shown in this study.

Conclusion

We propose a unique rectangular cuboid shaped microfluidic device that enables Caco-2 cell culture on a liquid-liquid interface. The collagen coated liquid-liquid interface was formed by sequentially perfusing collagen-containing aqueous solution into the microfluidic channel filled with perfluorocarbon FC-43. Caco-2 cells were successfully cultured on the interface by forming a near-confluent monolayer, and

immunofluorescence imaging confirmed tight-junction formation and organized actin cytoskeletons, indicating early-stage epithelial maturation. Unlike conventional systems that rely on plastic or silicone rubber substrates for cell adhesion, our system enables direct cell culture on a liquid–liquid interface, offering a novel platform with potential for advanced drug transport studies and co-culture experiments with other cell types. This approach is expected to open new avenues for drug permeability studies and co-culture experiments with other cell types.

Experimental

Device materials, reagents and Caco-2 cell

The materials for device fabrication and assembly were obtained from the following suppliers: PDMS (Silpot 184W/C, Dow Corning Toray, Tokyo, Japan), cover glass (MICRO COVER GLASS, MATSUNAMI GLASS IND., LTD., Osaka, Japan). Regents for experiments were obtained from the following suppliers: Formaldehyde (FA) (Formaldehyde, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), TritonX-100 (Triton™X-100, Sigma-Aldrich, St. Louis, MO, USA), ZO-1 antibody (ZO-1 Monoclonal Antibody [ZO1-1A12], Alexa Fluor™ 488, Thermo Fisher Scientific Inc., Waltham, MA, USA), Phalloidin (Phalloidin, Red Fluorescent Dye Conjugate, Acti-stain 555, Cytoskeleton, Inc., CO, USA), Hoechst33342 (Hoecst 33342 solution, Dojindo Laboratories, Kumamoto, Japan), PBS (D-PBS(-) (10x), Nacalai Tesque, Inc., Kyoto, Japan), DMEM (DMEM(1x)+GlutaMAX™-I, Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), FBS (Fetal Bovine Serum, Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), Collagen type I-C (Cellmatrix® Type I-C, Nitta Gelatin Inc., Osaka, Japan) and FC-43 (3M, St. Paul, MN, USA).

Device fabrication

The microfluidic device is fabricated by following soft lithography. The polyacetal plate was milled to fabricate the molds which was designed by three-dimensional computer-aided design software. The fabrication process is shown in Fig. S6. Briefly, PDMS was casted on molds and cured at 85℃ for 5h (Fig. S6 i). The cured PDMS was removed from the mold and holes with diameters of 1 mm and 1.5 mm were punched for the port and reservoir, respectively (Fig. S6 ii). The port and reservoir were attached on to the microfluidic device (Fig. S6 iii). A thin layer of uncured PDMS was applied to the bottom of the device, attached to a coverslip and cured at 85℃ for 2h (Fig. S6 iv).

Caco-2 cell culture

The procedure of the Caco-2 cell culture in the microfluidic device is shown in Fig. S7. First, the device was filled with FC-43 (Fig. S7 i). Next, collagen-containing serum-free DMEM (60 μg/mL) was introduced into the channel at a flow rate of 50 μL/min using a syringe pump (KDS, kd Scientific, MA, USA) from the port (Fig. S7 ii). In this study, the collagen concentration was selected as the optimal condition based on preliminary experiments (data not shown). In breif, excessively low collagen concentrations impede cell adhesion, while excessively high concentrations lead to gelation, making fluid handling impossible. The microfluidic device is put in the refrigerator (4℃, 2h) (Fig. S7 iii). Thereafter, culture medium was introduced into the channel at a flow rate of 10 μL/min for 10 min to replace the existing liquid layer (Fig. S7 iv). The human large intestinal cancer cell line, Caco-2 cells (CACO-2, KAC Co., Ltd., Kyoto, Japan) were harvested and resuspended in a culture medium (DMEM : FBS = 9 : 1) (Fig. S7 v). A portion of the cell suspension was aspirated using a pipette, and the pipette tip was mounted onto the device reservoir. The cell suspension was then introduced into the channel from the port using a syringe pomp at a flow rate of

20 μL/min for 1 min and Caco-2 cells were seeded onto the liquid-liquid interface at a density of 1.5 × 10^5 cells/cm^2. The microfluidic device was then put in an incubator (37℃, 5% CO_2). The culture medium was replaced daily by flowing fresh medium through the channel at a flow rate of 10 μL/min for 10 min. For control experiments, Caco-2 cells were seeded into 96-well plates at a density of 1.5 × 10^5 cells/cm^2, and the culture medium was replaced daily. The microfluidic device and the 96-well plate were put in the incubator (37℃, 5% CO_2).

Immunostaining procedure for cultured Caco-2 cells

The immunostaining procedure for Caco-2 cells cultured in the channel device and 96-well plate cultured for 7 days were fixed with 4% FA/PBS for 20 min and permeabilized with 0.5% Triton X-100/PBS at room temperature for 20 min, respectively (Fig. S8 ii, iv). Blocking was performed with 1% BSA/PBS at 4℃ for overnight (Fig. S8 vi). The samples were then incubated at room temperature for 3h in a solution containing ZO-1 antibody (5 μg/mL), Phalloidin (0.1 μM), and Hoechst33342 (2 μg/mL). Between each procedure, PBS was filled to wash the channel or wells (Fig. S8 i, iii, v, vii).

Image acquisition and quantification

The appearance of the aqueous layer was captured using a smartphone (Google Pixel 9a, Google LLC, CA, USA). Bright-field images of the cells were acquired using an inverted microscope (Leica DMil, Leica Microsystems, Wetzlar, Germany) equipped with a FLEXACAM camera. Fluorescence images were acquired using an all-in-one fluorescence microscope (BZ-X700, Keyence Corporation, Osaka, Japan). The fluorescence images were saved in 14-bit TiFF format, and the images were processed using the ImageJ software (NIH).

Supporting Information

Supporting Information File 1: Additional figures

File Name: Supporting information for “Interface-engineered Caco-2 cell culture on a collagen-coated liquid-liquid interface in a microfluidic device”

File Format: PDF

Data Availability Statement

All data generated or analyzed during this study are included in this manuscript and its Supporting Information file.

Author contributions

Satoru Kuriu: conceived, designed and performed the experiments. Satoru Kuriu and Soo Hyeon Kim analyzed the data and wrote the main manuscript and figures. All authors have reviewed the manuscript.

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Figure Legends

Figure 1: Illustration for the liquid-liquid interface formation. (A) Concept of the microfluidic device. (B) Fluid operation description. (i) FC-43 filling. (ii) Collagen solution filling. (iii) Culture medium filling. (iv) Caco-2 cells introduction. (C) Fabricated microfluidic device. The dash line indicates the boundary of the liquid-liquid interface.

Figure 2: Rectangularity and Bond number as a function of channel width (1.0, 1.5, 2.0, 5.0 and 10 mm). Representative interface images are shown above each bar. The images of the collagen solution region were captured from the direction indicated by the arrows. Scale bars are 5 mm. n=3.

Figure 3: Rectangularity as a function of channel height (7, 5, and 4 mm). Representative interface images are shown above each bar. For a channel height of 4 mm, almost all of the FC-43 was replaced by the collagen solution; therefore, the rectangularity was not calculated, and it is labeled as “No interface formation”. Scale bars are 5 mm. n=3.

Figure 4: Time-lapse images of the progression of Caco-2 cells at the liquid-liquid interface. Images indicate Day1, Day2 and Day7, respectively. Region with a lower number of cells is magnified to show that Caco-2 cells grow on the interface.

Figure 5: Immunofluorescence images of Caco-2 cells cultured in a microfluidic device and 96-well plates. Nuclei, ZO-1, F-actin and Merge images are displayed.

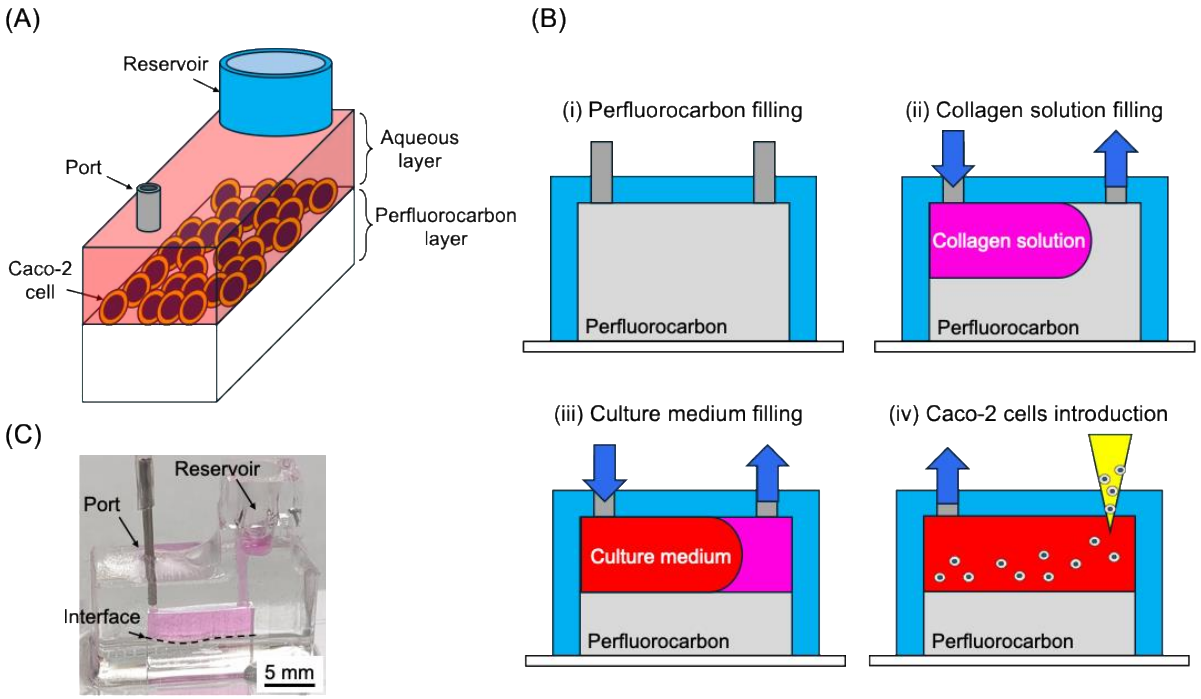
(A)
Reservoir
Port
Aqueous layer
Perfluorocarbon layer
Caco-2 cell
(B)
(i) Perfluorocarbon filling
Perfluorocarbon
(ii) Collagen solution filling
Collagen solution
Perfluorocarbon
(iii) Culture medium filling
Culture medium
Perfluorocarbon
(iv) Caco-2 cells introduction
Perfluorocarbon
(C)
Reservoir
Port
Interface
5 mm

Fig.1

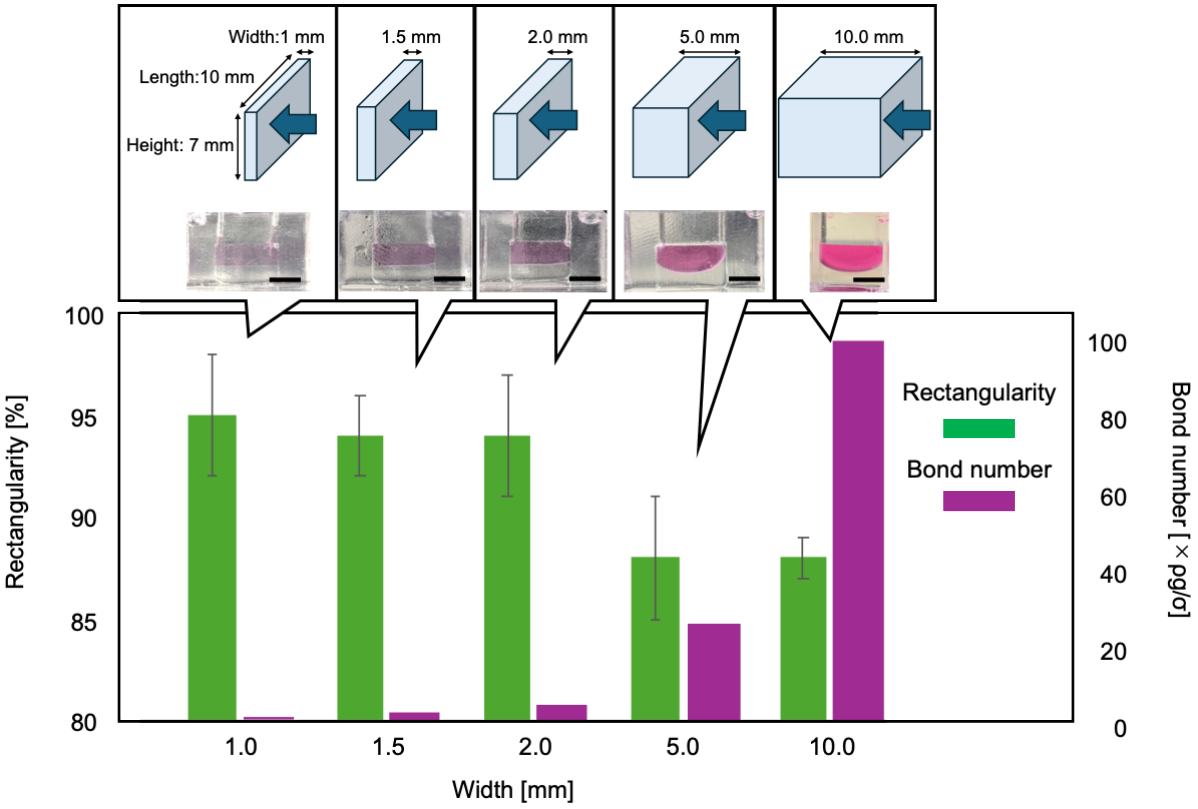
Width:1 mm
Length:10 mm
Height: 7 mm
1.5 mm
2.0 mm
5.0 mm
10.0 mm
Rectangularity [%]
Bond number [× ρg/σ]
Rectangularity
Bond number
Width [mm]
1.0
1.5
2.0
5.0
10.0
100
95
90
85
80
100
80
60
40
20
0

Fig. 2

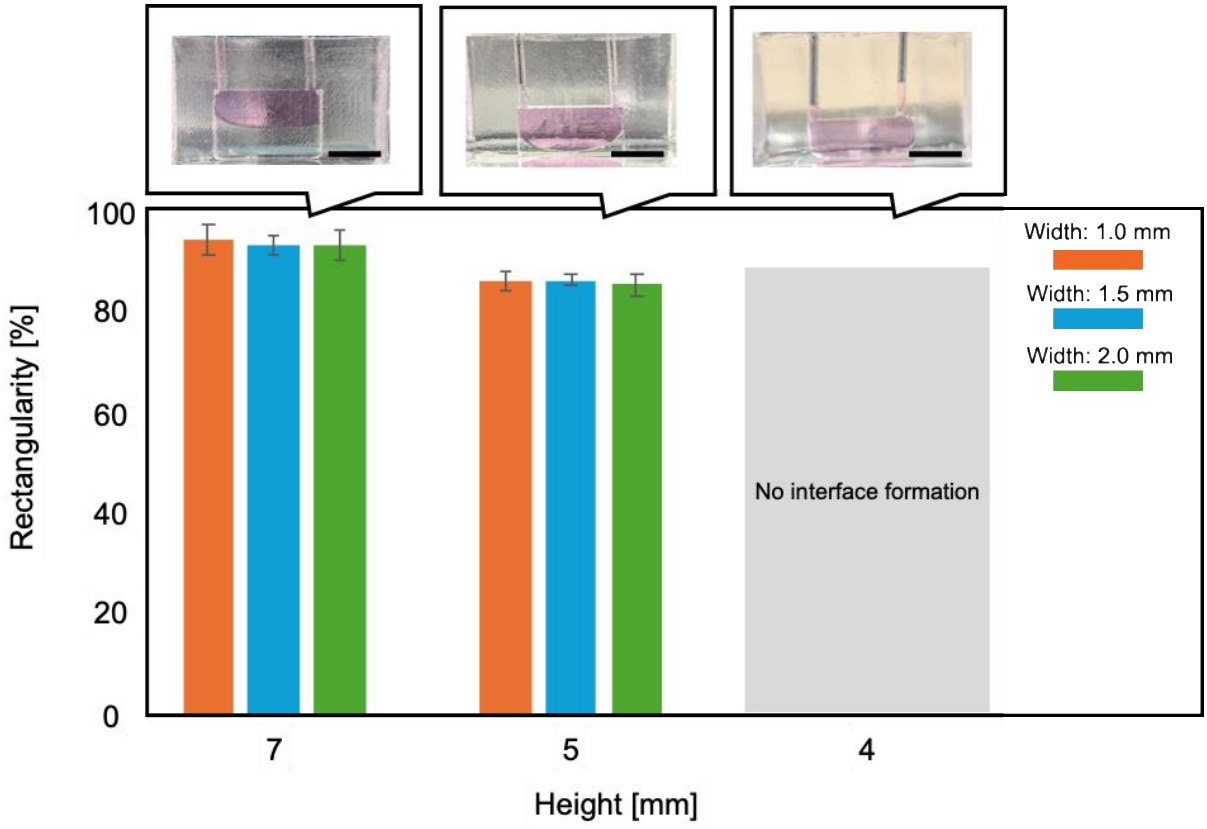
Rectangularity [%]
100
80
60
40
20
0
7
5
4
Height [mm]
No interface formation
Width: 1.0 mm
Width: 1.5 mm
Width: 2.0 mm

Fig.3

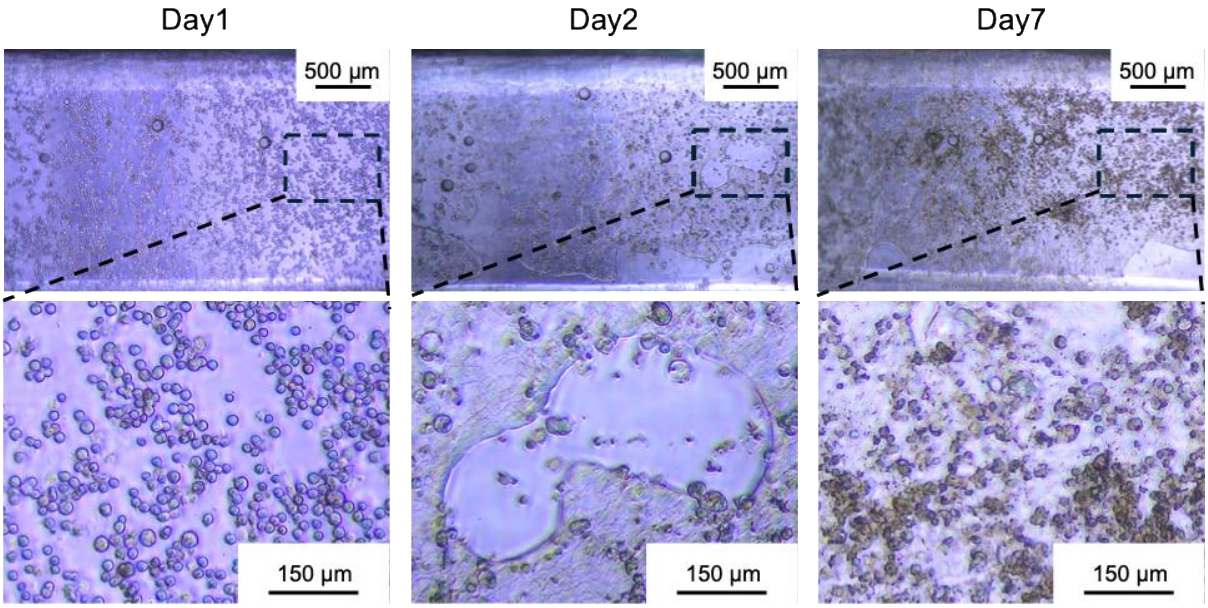

Fig.4

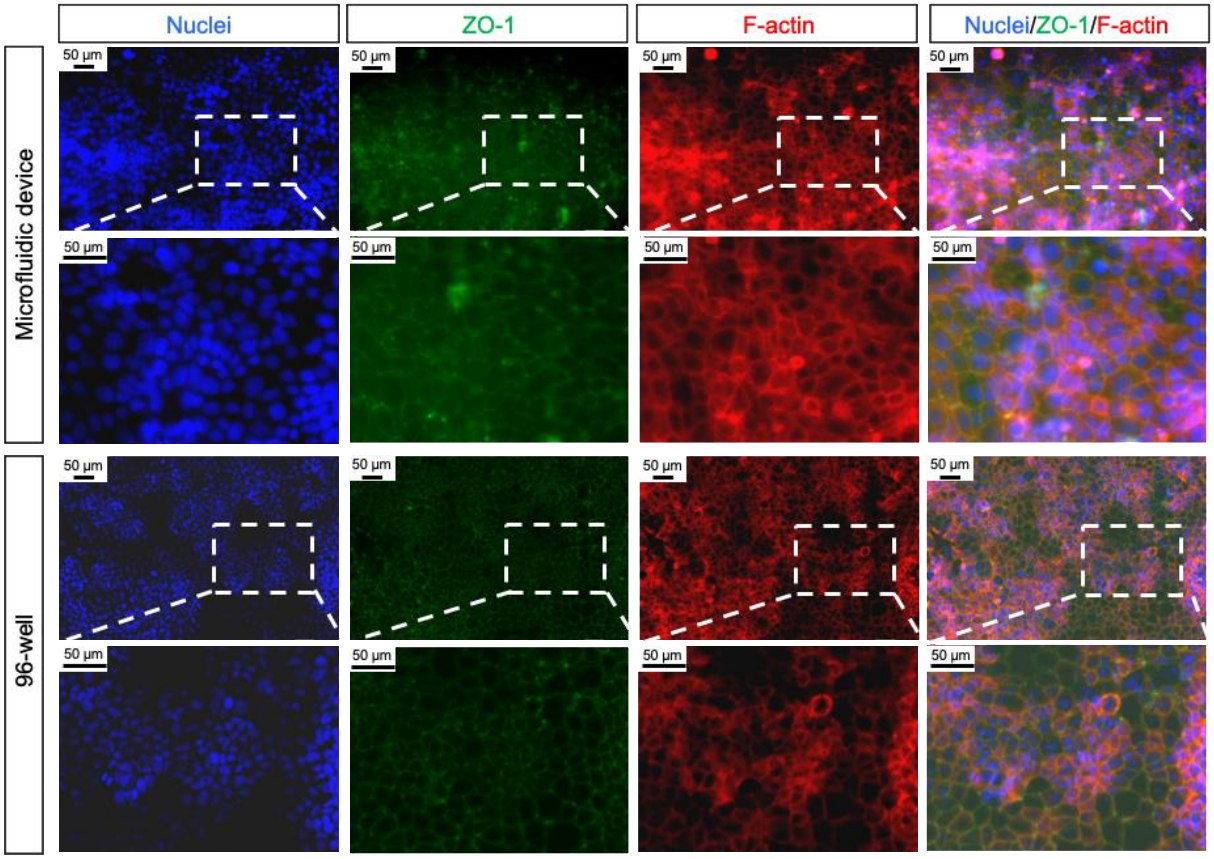
Nuclei
ZO-1
F-actin
Nuclei/ZO-1/F-actin
Microfluidic device
96-well
50 μm

Fig.5