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Title

Investigation of medium perfusion through scaffold-free tissue constructs using endothelial cell-covered spheroids in vitro.

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Abstract

A scaffold-free tissue construct was formed by assembling endothelial cell-covered spheroids, and medium perfusion through the tissue construct was investigated using hydrostatic pressure-driven culture circuit. Primary rat hepatocyte spheroids covered by human umbilical vein endothelial cells (HUVECs) were assembled in culture chambers with a cylindrical culture space of 2 mm in diameter, and then medium was perfused through the assembled spheroids for 48 hours. The medium flow rate through the culture chamber was measured over the perfusion culture time, which decreased during the first several hours, then increased or remained low depending on the amount of spheroids in the culture chamber. Histochemical analyses showed single tissue construct formation by spheroid fusion when cultured from 2×10^5 nuclei spheroids, with the loss of boundaries between the spheroids. Moreover, a viable cell region was found at the center of the tissue construct in several locations. Poor adhesion was found between spheroids cultured from 4×10^5 nuclei spheroids. The total nuclei density in cultured tissue constructs was estimated to be about half of that in HUVEC-covered hepatocyte spheroids.

This study demonstrated the possibility of medium perfusion through scaffold-free tissue constructs by assembling endothelial cell-covered spheroids, promising for a

large tissue construct culture *in vitro*.

Key words: spheroid; perfusion culture; hepatocyte; endothelial cell; scaffold-free

1 Introduction

Scaffold-free tissue engineering is a developing culture technique which provides direct cell-cell interactions and high cell density to the tissue construct [1]. Therefore, it is promising for cell transplantation as a native tissue-like construct. Different scaffold-free culture techniques are used to study the culture conditions suitable for achieving better tissue-specific functions [2] and mechanical properties [3]. However, few types of engineered tissues have yet been able to be clinically applied as tissue grafts, namely as a sheet-shaped tissue construct [4], mainly because of the difficulty in maintaining such a large tissue construct *in vitro*.

A diffusive supply of oxygen and nutrients limits the dimension of viable tissue constructs *in vitro*, especially in those with high cell density. The blood flow in a living body enables the convectional transport of oxygen and nutrients through the blood capillaries in the tissue. In addition, blood capillaries are densely arranged to supply a sufficient amount of oxygen and nutrients from capillaries to cells in a diffusive manner [5]. An interconnected flow channel structure should be built in a tissue construct to mimic such a convectional transport system *in vitro*. A method to perfuse medium or blood through the flow channels in the tissue construct is also required.

Perfusion culture systems for tissue engineering are now being investigated in many

systems, cartilage tissue [6], blood vessel [7] and a bioreactor with tissue construct [8]. Little is known about forming flow channels within the tissue construct and perfusion culture through such flow channels. McGuigan and Sefton developed a biomimetic approach to build interconnected flow channels by assembling small endothelial cell-covered collagen modules. They used interstitial hollow spaces formed between collagen modules in the assembled construct as flow channels and demonstrated perfusion culture through these flow channels *in vitro* [9]. Assembling small tissue constructs can form a large tissue construct and interconnected flow channels within the assembled tissue construct at a same time, which is promising for maintainable large tissue constructs with a high cell density. Moreover, an endothelial cell lining of flow channels is important to mimic vascular functions, such as reduced thrombogenicity [9]. In addition, endothelial cells are also promising for obtaining improved tissue-specific functions [10] and cell viability [11].

We have previously reported scaffold-free liver tissue construct with endothelial cells regularly arranged at approximately 100 μm intervals in the construct by assembling endothelial cell-covered hepatocyte spheroids in vitro. This is expected to be a basic culture technique for establishing a vascularized tissue construct with a high cell density.[12] Perfusion culture was used to develop this scaffold-free tissue construct in

a large scale, and the medium flow through interconnected flow channels by interstitial hollow spaces between assembled spheroids was investigated. Assembled spheroids attach to each other during tissue construct formation, and this may narrow the flow channels of interstitial hollow spaces over the perfusion culture time. Perfusing culture medium at constant flow rate under this culture condition may apply excessive inner pressure in the culture circuit, and then cause rupture of the culture circuit or damage to tissue construct. Therefore, a hydrostatic pressure-driven perfusion circuit was developed to address these problems. The hydrostatic pressure applies a constant pressure to generate the medium flow, which can provide dynamic flow rate change according to a varying flow channel structure in the tissue construct during the perfusion culture. The medium flow rate depends on the flow channel structure under the constant pressure, which shows the information on flow channels in the tissue construct. We therefore measured the medium flow rate under a constant hydrostatic pressure to investigate the medium perfusion through the scaffold-free tissue construct.

2 Material and methods

1.1 Cell preparation

Human umbilical vein endothelial cells (HUVECs, Lonza Ltd, Basel, Switzerland)

were purchased and subcultured in endothelial growth medium-2 (EGM-2; Lonza) at 37°C in a humidified 5% CO_2 atmosphere. HUVECs from passage 2 after purchase were used for the experiments.

Primary rat hepatocytes were isolated from a male Wistar rat (7-8 weeks old, KYUDO. CO. LTD., Kumamoto, Japan) by the collagenase perfusion method. More than 85% viable cells were used for spheroid culture. Isolated hepatocytes were suspended in hepatocyte culture medium: D-MEM with high glucose (Invitrogen, Carlsbad, CA) supplemented with 50 μg/L Epidermal Growth Factor (EGF, BT-201; Biomedical Technologies, Inc., Stoughton, MA), 10 mg/L insulin (Sigma-Aldrich, St. Louis, MO), 60 mg/L proline (Sigma-Aldrich), 7.5 mg/L hydrocortisone (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 3.7 g/L $NaHCO_3$, 5.985 g/L HEPES, 50 μg/L linoleic acid, 0.1 μM Copper ($CuSO_4 \cdot 5H_2O$), 3 nM Selenium (H_2SeO_3), 50 pM Zinc ($ZnSO_4 \cdot 7H_2O$), and antibiotics (58.5 mg/L penicillin, 100 mg/L streptomycin).

1.2 HUVEC-covered hepatocyte spheroid formation

HUVEC-covered hepatocyte spheroids were cultured as reported previously.[12] Primary rat hepatocytes were cultured in a 6 well plate at 1.5×10^6 viable cells/well in 1.5 mL hepatocyte culture medium on a rotary shaker at 80 rpm, 37°C in a humidified

5% CO_2 atmosphere. Supernatant (1 mL per well) was replaced on day two and hepatocyte spheroids were collected on day 4. Collected spheroids were filtered using stainless wire mesh to obtain 100-150 μm sieve fractions and suspended in 1mL hepatocyte culture medium.

Cellmatrix Type I-A collagen gel (Nitta Gelatin Inc., Osaka, Japan) was reconstituted by adding 10x culture medium and reconstitution buffer according to the manufacturer's instructions. Reconstituted collagen gel (1 mL) was added to the spheroid suspension and pipetted (final concentration of collagen: 1.2 mg/mL). Collagen gel was added and the spheroid suspension was incubated at 4°C for one hour and washed by adding 10 mL cold hepatocyte culture medium and centrifugation at 40 × g, 60 s.

Collagen-coated hepatocyte spheroids and HUVECs were suspended in EGM-2. Collagen-coated hepatocyte spheroids and HUVECs were plated in 12 mL EGM-2 on 100 mm cell culture dish coated with 4% agarose. A low cell binding petri dish (Nalge Nunc International KK) was also used as a substitute for 4% agarose-coated culture dish in some experiments. Supernatant (10 mL per dish) was replaced on day two of coculture and HUVEC-covered hepatocyte spheroids were collected on day 4 of coculture. A 45-300 μm sieve fraction of HUVEC-covered hepatocyte spheroid suspension was obtained for perfusion culture experiments. To calculate cell number in

the spheroid suspension, nuclei were isolated from the spheroid suspension by adding Lysis Reagent A-100 (ChemoMetec A/S, DK-3450 Allerød, Denmark) followed by homogenization at 15000 rpm for 3 minutes using POLYRON® homogenizer (PT 1300 D; Kinematica, Inc., Bohemia, NY). The number of nuclei in the homogenized suspension was measured using the NucleoCounter (NC-100; ChemoMetec A/S) and the nuclei density of the spheroid suspension was 3.32×10^5 nuclei/mL. The average nuclei number per primary rat hepatocyte after preparation was 1.45 nuclei /cell, and the average nuclei number per subcultured HUVEC was 1.14 nuclei/cell.

1.3 Hydrostatic perfusion culture

Figure 1 illustrates the hydrostatic perfusion culture system. The culture medium was perfused from the top reservoir through the culture chamber to the bottom reservoir, then back to top reservoir by a peristaltic pump (**Fig. 1A**). The flow channel in the culture chamber was separated by a 45 µm polyester mesh filter to trap spheroids (**Fig. 1B, C, D**). Needles (18G) were placed at the end of the flow channel in the bottom reservoir to measure the flow rate of the medium (**Fig. 1E**). The culture chambers, bottom reservoir and peristaltic pump were placed in an incubator at 37°C, with a 5% CO_2 humidified atmosphere and the top reservoir was placed outside the incubator.

TYGON tubing was connected to the top reservoir to maintain the CO_2 concentration in the culture medium. The spheroid suspension was inoculated in each culture chamber (602 µL suspension for 2×10^5 nuclei/chamber, 1.204 mL suspension for 4×10^5 nuclei/chamber, no suspension for 0 nuclei/chamber) and then was cultured for 48 hours. A 1:1 mixture (v/v) of hepatocyte culture medium supplemented with EGM-2 SingleQuots (Lonza; containing Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, 2% FBS, hEGF, and GA-1000) and EGM-2 was used for the perfusion culture. The cultured tissue was isolated after the perfusion culture and frozen for histological evaluation.

1.4 Measurement of the medium flow rate

Medium drops from 18G needles in bottom reservoir were recorded by web camera (QuickCam® Orbit AF, Logitech, Fremont, CA 94555, USA) and a recording software program (Debut Video Capture Software, NCH Software, Inc., Greenwood Village, CO, 80111, USA). Movies (15 minutes) were recorded every 30 minutes. The movies were analyzed to count the medium drops using ImageJ and IGOR 6 Pro (WaveMetrics, Inc., Portland, OR 97223, USA). Medium drops were also counted manually from several original movies for the validation of movie analysis. The average medium flow rate

over 15 minutes movie was then calculated assuming the volume of a medium drop from 18G needle is 20 μL.

1.5 Immunofluorescent microscopy

Immunofluorescent microscopy was conducted as previously reported.[12] Frozen sections were obtained using a cryostat (CM1100, Leica) then fixed in acetone for 10 minutes at 4°C followed by washing in PBS (5 minutes, three changes). Sections were incubated in blocking solution (PBS containing 10% skim milk, 6% glycine) for 20 minutes. Sections were washed in PBS and incubated in a blocking solution containing primary antibodies (1:100) for one hour at room temperature. Rabbit polyclonal anti-human von Willebrand factor (vWF; Dako, Glostrup, Denmark) was used for HUVECs and goat anti-mouse albumin (BETHYL Laboratories, Inc, Montgomery, TX) was used for primary rat hepatocytes. Then sections were washed in PBS and incubated in blocking solution containing secondary antibodies (1:100) for one hour at room temperature. Swine anti-rabbit IgG FITC conjugated (Dako) and donkey anti-goat IgG rhodamine conjugated (Millipore, Billerica, MA) antibodies were used for secondary antibodies. Nuclei were also stained with Hoechst 33342. Images were captured with a fluorescent microscope (IX71, Olympus, Tokyo, Japan).

3 Results

The time course of changes in the flow rate are shown in **Figure 2**. The flow rate decreased during initial several hours of perfusion culture of a chamber assembled with 2×10^5 nuclei spheroids, and then showed an tendency to increase until the end of the culture time. On the one hand, the flow rate in the culture chamber assembled with 4×10^5 nuclei as spheroids decreased for several hours and tended to remain at a low flow rate.

Figure 3 shows the micrographs of the tissue constructs after perfusion culture. Several spheroids were found to maintain their spherical shape on the top surface of the tissue constructs. Clearance gaps were also found between the lateral surface of the tissue construct and inner wall of the culture chamber (**Fig. 3A, B**, between white arrows). The tissue constructs were frozen after perfusion culture, and sliced parallel to the medium flow direction. The sections through the center of tissue constructs were stained for histological evaluations.

The cells migrated through the polyester mesh filter to the downstream side in the culture chamber after 48 hours of perfusion culture (**Fig. 4A, C**). These cell aggregates

migrated through the polyester mesh and were carried away by the medium flow and circulated throughout the culture circuit. Small pieces of cellular aggregates were found in the top reservoir on the next day after the start of perfusion culture and this may have been responsible for the fluctuation or decrease in the flow rate of 0 nuclei (Fig. 2).

HUVECs were distributed in regular intervals of approximately 100 μm in the tissue construct (**Fig. 4B, D**). The spheroids attached to each other in cultures containing 2×10^5 nuclei, and no boundaries were observed between them (**Fig. 4E**) while several spheroids remained spherical in cultures of 4×10^5 nuclei (**Fig. 4F**). A viable cell region was found at the center of the tissue constructs in cultures containing 2×10^5 nuclei (**Fig. 4G**).

The tissue construct seemed to collapse when hepatocyte spheroids of 2×10^5 nuclei without HUVEC were cultured in similar conditions (**Fig. 5A**). Hepatocyte spheroids of 6×10^5 nuclei without HUVEC formed liver tissue constructs (**Fig. 5B**), which showed disintegrating morphology at the center of the tissue constructs (Fig. 5C). The surface area of tissue construct showed that hepatocyte spheroids were attached each other and formed a layered structure of viable hepatocytes (**Fig. 5D**). A layer of HUVEC on the hepatocyte spheroid surface might alter cell adhesion behavior of the spheroid surface, which would affect the morphological tendencies.

4 Discussion

The oxygen consumption rate of primary rat hepatocytes is approximately 0.4 nmol/s/10^6 hepatocytes [13]. Therefore, a flow rate of 0.13 mL/min of medium flow rate is required for 10^6 hepatocytes to meet the oxygen consumption rate, assuming the medium is saturated with 95% air, 5% CO_2 atmosphere. The flow rate through the tissue construct containing 2×10^5 nuclei in this study met the minimum oxygen requirement (the lowest flow rate was 0.027 mL/min at 4.5 h in constructs containing 2×10^5 nuclei ; **Fig. 2**). In contrast, the flow rate through the tissue construct containing 4×10^5 nuclei was less than that required to supply sufficient amount of oxygen to the construct.

Hepatocyte nuclei in tissue sections (Fig. 4B, D) was counted to estimate hepatocyte density. The section of HUVEC-covered hepatocyte spheroids were also counted. Albumin-overlapping nuclei in the region of tissue sections (more than 0.2 mm^2 with more than 100 albumin-overlapping nuclei) were counted, and then nuclear number per unit area was calculated. The hepatocyte nuclear density in HUVEC-covered hepatocyte spheroids was 1.1×10^3 nuclei/mm^2 and that in tissue constructs after perfusion culture were 3.9×10^2 nuclei/mm^2 (2×10^5 nuclei, Fig. 4B) and 2.9×10^2 nuclei/mm^2 (4×10^5 nuclei, Fig. 4D). Hepatocyte viability in the tissue construct containing 2×10^5 nuclei was not

maintained well during the perfusion culture despite for the adequate oxygen supply. This indicates an imbalance in the medium flow among the regions in the tissue construct, which supplies sufficient oxygen and nutrients to some regions (**Fig. 4G**), but not in other regions. The migrated cell aggregates that would circulate throughout the culture circuit and block flow channels in the tissue construct may have caused this nonuniform medium flow (**Fig. 4A, C**). The clearance gap between lateral surface of the tissue construct and inner wall of the culture chamber may be another cause (**Fig. 3A, B**, between white arrows). This clearance gap would arise from poor cell adhesion to the inner wall of the culture chamber and tissue construct shrinkage during perfusion culture.

The fusion of spheroids requires cellular activity such as cell adhesion and cell migration on the spheroid surface. HUVEC nuclei were found on the surface of spheroids in the tissue constructs (Fig. 4E, F), and HUVEC nuclei densities in tissue sections (more than 0.2 mm^2 with more than 100 vWF-overlapping nuclei in the region) were 5.1×10^2 nuclei/mm^2 (2×10^5 nuclei, Fig. 4B) and 5.8×10^2 nuclei/mm^2 (4×10^5 nuclei, Fig. 4D). Therefore, the HUVECs were thought to be alive on the spheroid surface in both tissue constructs, however, the increase in the flow rate would supply more oxygen and nutrients to the tissue constructs in cultures containing 2×10^5 nuclei

(**Fig. 2**), which may have led to better HUVEC activity in comparison to that in the tissue constructs in cultures containing 4×10^5 nuclei.

There are several investigations in underway to improve the cell viability of tissue constructs. A higher pressure load is going to be applied to increase the medium flow rate to determine whether the flow rate is increased. An oxygen carrier may also be added to the culture medium to improve the hepatocyte viability [14]. The culture circuit will be equipped with a filter to trap migrating cell aggregates to prevent the imbalanced medium flow in the tissue construct. The clearance gap will be prevented by surface treatment of the inner wall of the culture chamber with collagen to improve cell adhesion to the inner wall of the culture chamber. The three chambers were connected in parallel in the current study because handling multiple culture chambers is complicated. The culture circuits will be improved to provide multiple independent perfusion cultures to measure the time course changes of cell viability and functions. A lectin perfusion can selectively label a perfusing flow channel in order to distinguish perfusable flow channels from non-perfusable flow channels in the tissue construct [15]. Pore observation on the tissue surface would provide information on flow channel density in the tissue construct [16].

5 Conclusion

In summary, this study investigated medium perfusion through assembled endothelial cell-covered spheroids and showed the possibility of medium perfusion through the tissue construct. A scaffold-free tissue construct was formed by spheroid fusion and a viable cell region was observed at the center of the tissue constructs in some locations. Improvements in oxygen and nutrient supply by medium flow will be needed to develop a large scale scaffold-free tissue construct culture.

6 Acknowledgements

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Figure captions

Fig. 1 Hydrostatic perfusion culture system. (A) Schematic illustration. Top reservoir and bottom reservoir were connected to the atmosphere to generate hydrostatic pressure. Additional equipment (extension tube, air trap, oxygenizer) was installed in a practical experiment. Red arrows indicate the direction of medium flow. (B) Perfusion culture chamber. A silicone tube was fixed on a glass slide, then a polyester mesh filter (sieve opening: 45 μm) was inserted across the flow channel to trap spheroids. (C) Higher magnification of Fig. 1B. A cylindrical culture space with a diameter of 2 mm was formed in the culture chamber. Red arrows indicate the direction of medium flow. (D) Schematic illustration of the assembled spheroids in the culture chamber. Spheroids were trapped on the polyester mesh filter. Red arrows indicate the medium flow between the assembled spheroids. (E) Needles (18G) equipped in the bottom reservoir. Medium drops from the needle were recorded. Movies were then analyzed to calculate the medium flow rate.

Fig. 2 Time course of the changes in the medium flow rate during hydrostatic perfusion culture obtained by a movie analysis.

Fig. 3 Liver tissue construct in the culture chamber after hydrostatic perfusion culture for 48 hours. Clearance gaps were found between lateral surface of the tissue construct and inner wall of the culture chamber (between white arrows). Black arrows indicate the medium flow direction. (A) Tissue construct from a culture containing 2×10^5 nuclei spheroids. (B) Tissue construct cultured from a culture containing 4×10^5 nuclei spheroids.

Fig. 4 (A, C, G) HE staining of cultured tissue. (B, D, E, F) Immunofluorescent staining for albumin (red) (dark gray in the monochrome figure) and von Willebrand factor (green) (white in the monochrome figure). Nuclei were also stained with Hoechst 33342 (blue). White arrows indicate the medium flow direction. (A) 2×10^5 nuclei spheroids. Tissue sample shown in Fig. 3A. Cells migrated through the polyester mesh filter to the downstream side (black arrow). (B) 2×10^5 nuclei spheroids. Tissue sample shown in Fig. 3A. (C) 4×10^5 nuclei spheroids Tissue sample shown in Fig. 3B. Cells migrated through the polyester mesh filter to the downstream side (black arrows). (D) 4×10^5 nuclei spheroids. Tissue sample shown in Fig. 3B. (E) Higher magnification of Fig. 4B. Spheroids attached to each other, losing boundaries between them (white arrowheads). (F) Higher magnification of Fig. 4D. Spherical features of

HUVEC-covered hepatocyte spheroids were observed (white arrowheads). (G) Higher magnification of Fig. 4A. A viable cell region was found at the center of the tissue which had been specified based on the presence of nuclei and light pink-colored cytoplasm (between black arrows). In contrast, a dead cell region showed the absence of nuclei and dark pink-colored cytoplasm.

Fig. 5 Hepatocyte spheroids (100 – 150 μm, without HUVEC) were packed in a culture chamber and cultured under 1.2 m of hydrostatic pressure for 48 hours. White arrows indicate the medium flow direction. (A, B) Tissue in the culture chamber after hydrostatic perfusion culture for 48 hours. (A) 2×10^5 nuclei spheroids. (B) 6×10^5 nuclei spheroids. (C) HE staining of cultured tissue of 6×10^5 nuclei spheroids. Cross section through the center of the tissue sample shown in Fig. 5B (D) Higher magnification of Fig. 5C. A layered structure of the viable hepatocytes was observed (black arrows).

Fig.1

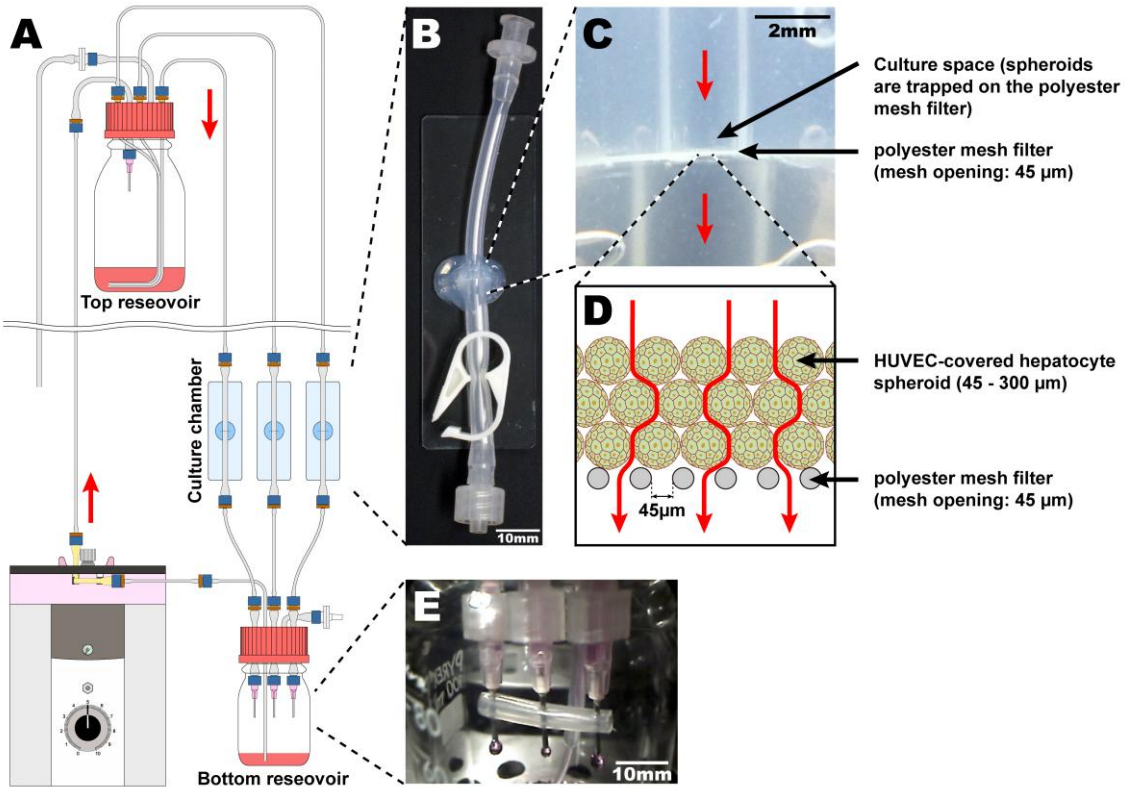

A
Top reseovoir
Culture chamber
Bottom reseovoir
B
10mm
C
2mm
Culture space (spheroids are trapped on the polyester mesh filter)
polyester mesh filter (mesh opening: 45 μm)
D
HUVEC-covered hepatocyte spheroid (45 - 300 μm)
45μm
polyester mesh filter (mesh opening: 45 μm)
E
10mm

Fig.2

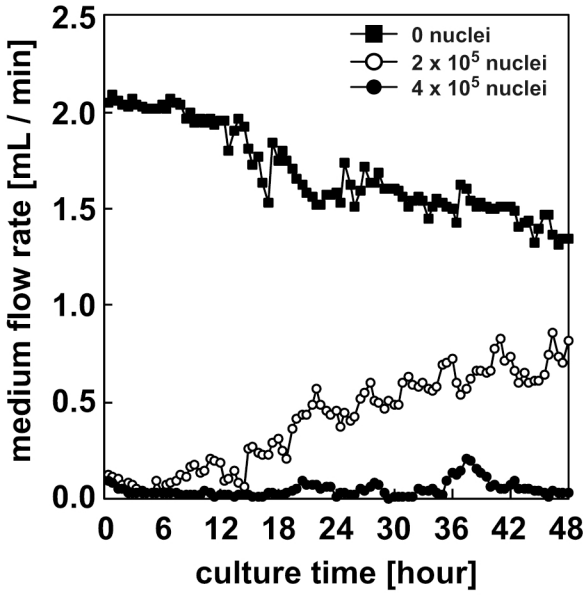

0 nuclei
2 x 10^5 nuclei
4 x 10^5 nuclei
medium flow rate [mL / min]
culture time [hour]
0.0
0.5
1.0
1.5
2.0
2.5
0
6
12
18
24
30
36
42
48

Fig.3

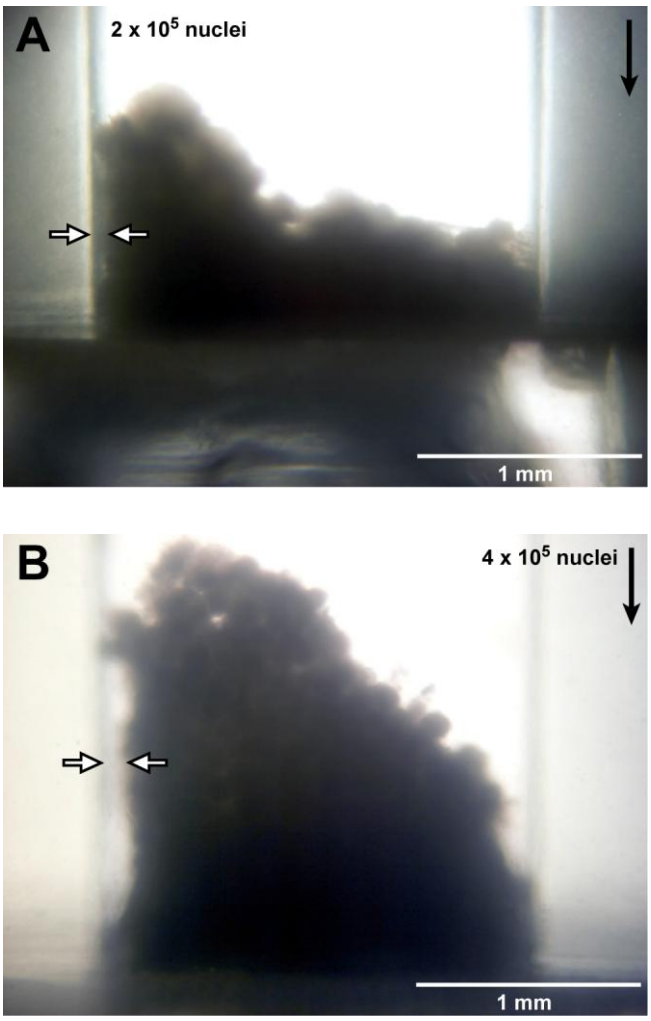


Fig.4

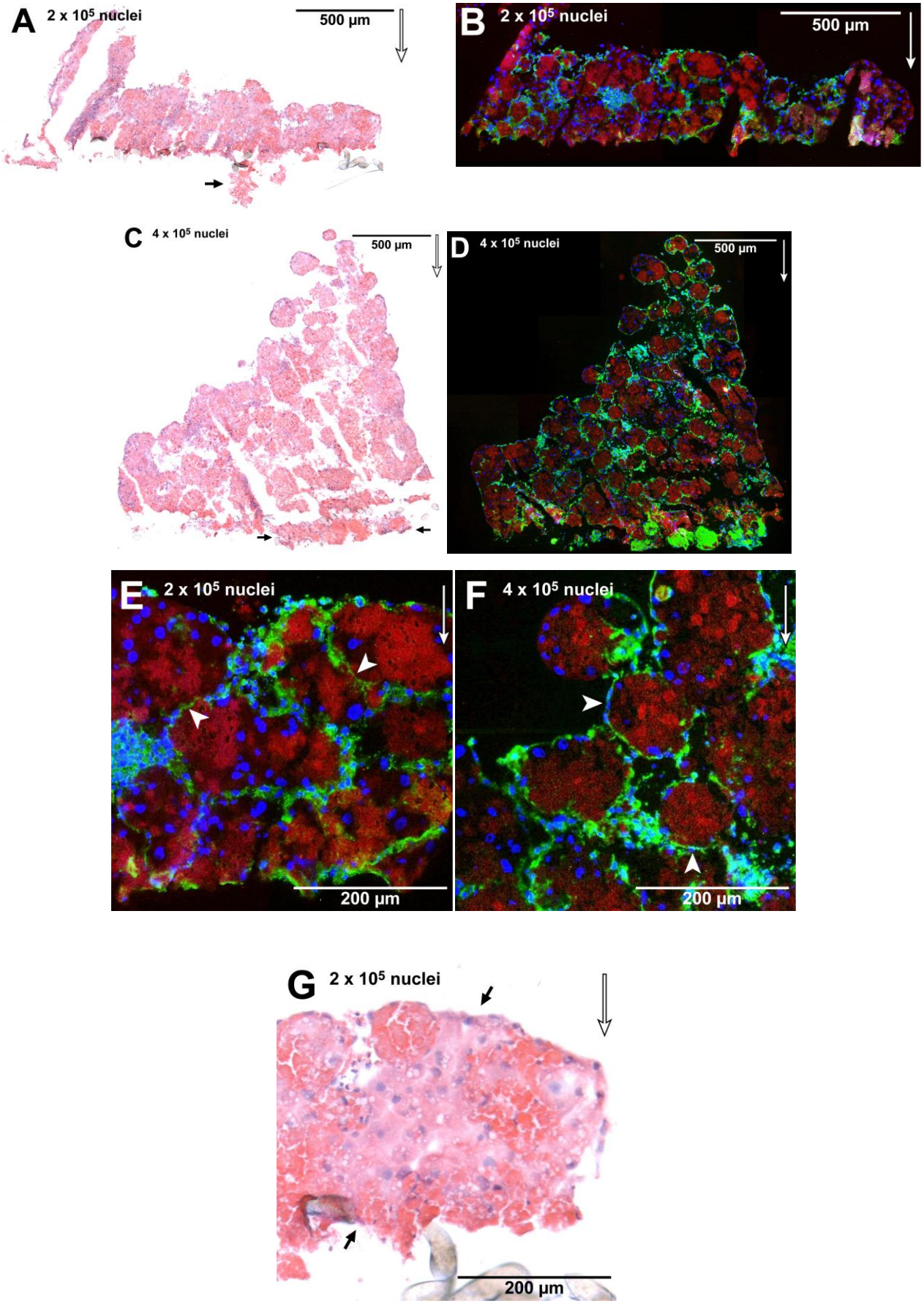
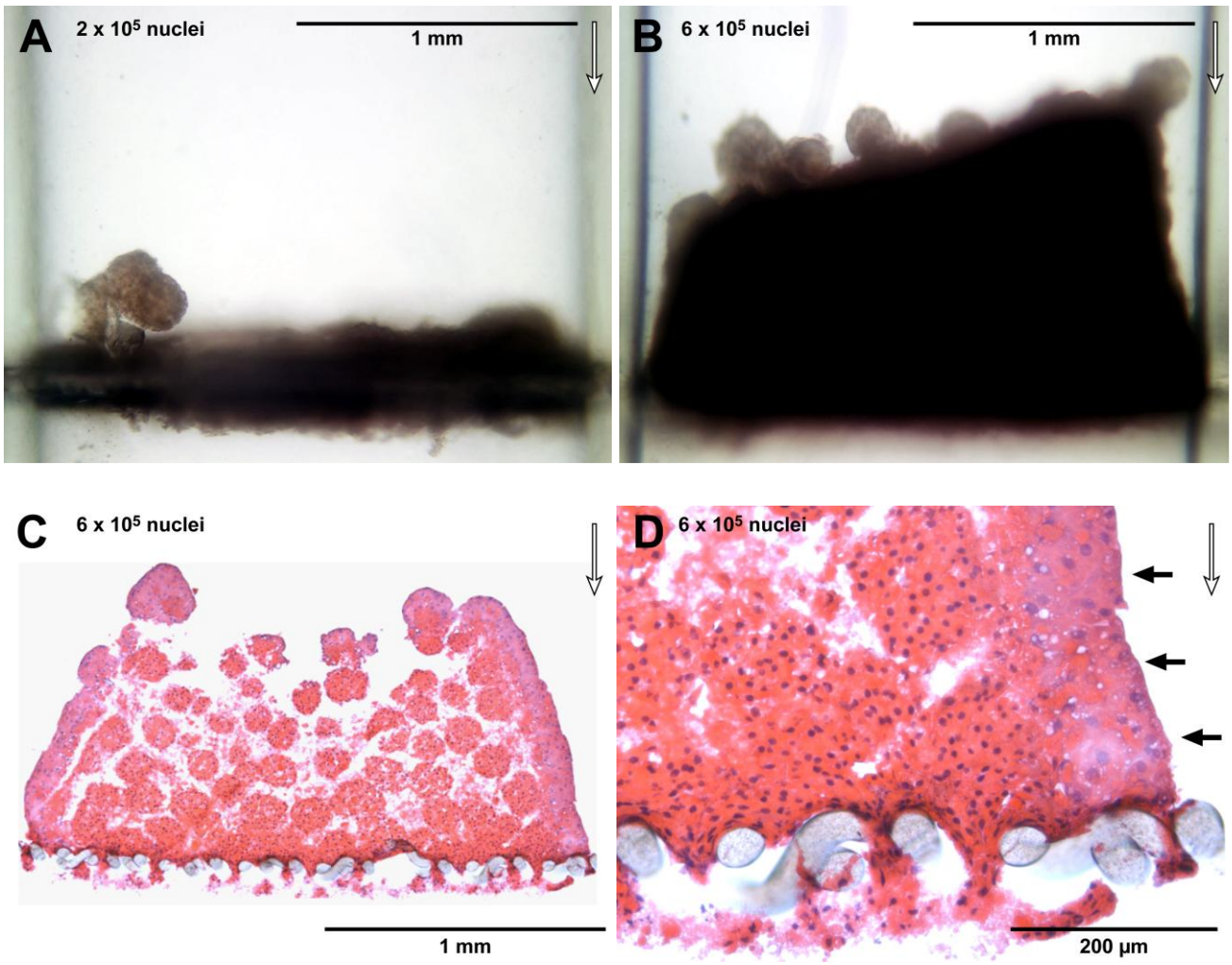

A
2 x 10⁵ nuclei
500 µm
B
2 x 10⁵ nuclei
500 µm
C
4 x 10⁵ nuclei
500 µm
D
4 x 10⁵ nuclei
500 µm
E
2 x 10⁵ nuclei
200 µm
F
4 x 10⁵ nuclei
200 µm
G
2 x 10⁵ nuclei
200 µm

Fig.5


A
2 x 10^5 nuclei
1 mm
B
6 x 10^5 nuclei
1 mm
C
6 x 10^5 nuclei
1 mm
D
6 x 10^5 nuclei
200 µm