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# Evaluation of a bioreactor with stacked sheet shaped organoids of primary hepatocytes

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**Hepatocyte organoids have an *in vivo*-like cell morphology and maintain cell viability and function *in vitro*. On the other hand, the oxygen supply to hepatocytes is sometimes limited in the core of organoids that are more than 100 μm in thickness. In this study, we designed and examined a new bioreactor using sheet-shaped organoids (organoid-sheets) in which the thickness was controlled to prevent hepatocyte death in the core of organoid due to limitation of oxygen supply. The cell culture space consisted of stacked organoid formation spaces and medium flow channels. Each space was separated by flat porous polycarbonate membranes, and the organoid thickness was controlled at 100 μm with a stainless steel spacer. Freshly isolated hepatocytes ($7.0\times10^7$) were immobilized in the bioreactor, yielding a cell density of $4.5\times10^7$ cells/cm$^3$-bioreactor. Of the five flow rates tested (1.0, 5.0, 10, 20, and 50 mL/min), the bioreactor with the 10 mL/min had the highest ammonia removal and albumin secretion activities for at least 14 days. In conclusion, a new bioreactor controlling organoid thickness is useful for achieving high cell density culture and the maintenance of hepatocyte function to avoid cell death in the core of the organoids due to limitation of oxygen supply. The bioreactor may be useful for the development of various applications using cultured hepatocytes.**



[**Key words:** Primary hepatocyte culture; Three dimensional culture; Tissue engineering; Organoid; Bioreactor; Bioartificial liver; Oxygen transfer]

Hepatocyte culture methods have been developed to study the complex liver functions, which include metabolism, secretion, excretion, detoxification, and storage. Therefore, cultured hepatocytes have been widely used for many applications, such as *in vitro* pharmacological, toxicological and metabolic studies. In addition, a bioartificial liver (BAL) using cultured hepatocytes is hoped to provide temporary liver support for a bridge to liver transplantation or reversible liver failure. For development of such applications, cultured hepatocytes have to maintain their viability and function over long term *in vitro* culture.

The *in vivo*-like hepatocyte morphology plays an especially important role in the maintenance of their viability and function. In the usual two-dimensional (2D) monolayer culture, for example, hepatocytes rapidly decrease their functions within a few days, thereby limiting their applications (1). In three-dimensional (3D) multicellular aggregates (organoids) culture methods, in contrast, hepatocytes maintain their viability and function *in vitro* for several weeks (2, 3). The reason why 3D organoids maintain their viability and function longer than 2D monolayers is not entirely clear. Recently, spherical organoids (spheroids), a well-known organoid type, were reported to reconstruct *in vivo*-like hepatocyte morphology with abundant cytoplasmic organelles, bile canaliculi, and close cell–cell contact with tight junctions and gap junctions (4, 5).

In our previous study, we developed a unique 3D organoid culture method to promote multicellular aggregation via loading centrifugal force (2, 3). The use of hollow fibers (HFs) for hepatocyte culture and suitable centrifugation achieved high cell density culture and formation of cylindrical organoids (cylindroids) in the inner spaces of the HFs. It was reported that cultured hepatocytes forming cylindroids were able to maintain their functions for a long-term *in vitro* (6, 7).

On the other hand, the oxygen supply sometimes becomes limited for hepatocytes in the core of organoids with greater than 100 μm thickness, because oxygen is transferred by diffusion from the organoid surface into the core. As in our previous approaches, we controlled organoid thickness to prevent the death of hepatocytes in the core of organoid due to limitation of oxygen supply. This was achieved specifically by using elliptic HFs (<150 μm minor axis), which formed elliptic cylindroids with a 100 μm minor thickness. These elliptic cylindroids were able to maintain their viability and function longer than cylindroids that often have dead hepatocytes in their core (8). Moreover, we developed a useful 3D organoid culture method to control their thickness by using flat porous membranes (9) with spacers (100 μm thickness) between the flat porous membranes that controlled the organoid thickness. The resultant sheet-shaped

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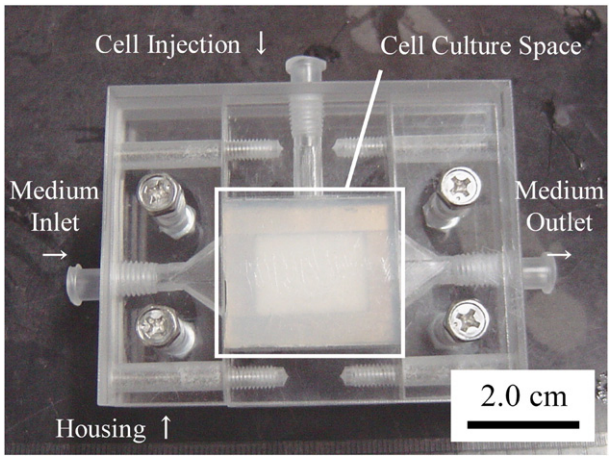


FIG. 1. Schematic diagram of bioreactor for hepatocyte culture. The bioreactor consisted of a housing and a cell culture space. The housing had medium inlet/outlet ports and a cell injection port. Scale bar: 2.0 cm.

organoids (organoid-sheets) had thickness of less than 100 μm and maintained their viability and function, similar to elliptic cylindroids. Therefore, our approach shows that organoid thickness control is reasonable in the formation of 3D organoids to prevent hepatocyte death in the core of organoid due to limitation of oxygen supply.

To demonstrate an application of the organoid-sheet, we designed a new bioreactor and examined culture system conditions, such as cell immobilization, culture circuit, and medium flow rate. The goal was to evaluate the usefulness of the organoid-sheet in the maintenance of hepatocyte function.

## MATERIALS AND METHODS

**Hepatocyte isolation and culture** Hepatocytes were isolated from Wistar rats (male, 7–8 weeks old, 220–230 g) (Kyudo, Kumamoto, Japan) by the two-step *in situ* liver perfusion method with collagenase (0.05%) (Wako Pure Chemical Industries, Osaka, Japan) (10). Cell viability was determined by the trypan blue exclusion method, and hepatocytes with over 80% cell viability were used for the culture.

Hepatocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with EGF (50 ng/mL) (Funakoshi, Tokyo, Japan), insulin (10 μg/mL), proline (60 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA), hydrocortisone (7.5 μg/mL) (Wako), $CuSO_4 \cdot 5\ H_2O$ (0.1 μmol/L), $H_2SeO_3$ (3 ng/mL), $ZnSO_4 \cdot 7\ H_2O$ (50 pmol/L), linoleic acid (50 μg/mL), penicillin (58.8 μg/mL) (Wako), and streptomycin (100 μg/mL) (Meiji Seika Kaisha, Tokyo, Japan).

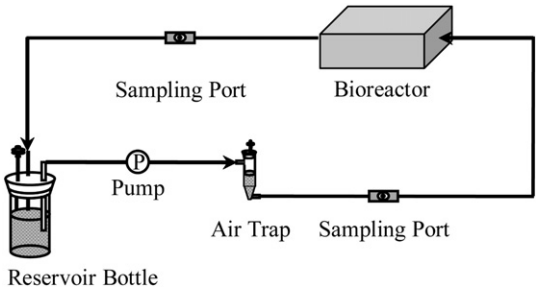


FIG. 3. Schematic diagram of culture system. The culture circuit contained an air trap, a medium reservoir, a peristaltic pump, and sampling ports. After cell immobilization, the bioreactor was joined to the culture circuit.

**Bioreactor** The bioreactor consisted of a housing and a cell culture space (Fig. 1). The housing had medium inlet/outlet ports and a cell injection port. The cell culture space consisted of stacked organoid formation spaces and medium flow channels, one on top of the other (Fig. 2). Each space was separated with flat porous polycarbonate membranes (1.5 × 2.0 cm, 5.0 μm pore size) (Millipore, Billerica, MA, USA). The thickness of the culture space for hepatocyte organoid formation and medium flow was controlled at 100 μm with stainless steel spacers (100 μm thickness) (Nilaco, Tokyo, Japan).

**Culture system** Freshly isolated hepatocytes ($7.0 \times 10^7$) were injected through the cell injection port of the bioreactor. Then the bioreactor was centrifuged at 60 ×*g* for 90 s to promote organoid-sheet formation. After centrifugation, the bioreactor was joined to a culture circuit containing an air trap, a medium reservoir, a peristaltic pump, and sampling ports (Fig. 3).

To evaluate the medium flow rate, hepatocytes in the bioreactor were cultured under five flow rates: 1, 5, 10, 20, and 50 mL/min. After a 4 h incubation, the culture medium was exchanged with fresh culture medium and then daily for 14 days.

**Hepatocyte function** To evaluate the ammonia removal activity of hepatocytes, the culture medium was exchange with fresh culture medium supplemented with $NH_4Cl$ (1.0 mmol/L) (Wako). The changes in ammonia concentration in the culture medium over 24 h were measured by a commercial kit (Ammonia Test Wako) (Wako).

Similarly, the albumin concentration in the culture medium over 24 h was measured by an enzyme-linked immunosorbent assay (ELISA) with purified rat albumin, peroxidase-conjugated antibody (MP Biomedicals, Morgan Irvine, CA, USA), and a commercialized kit (Protein Detectot ELISA Kit) (Kirkegaad and Perry Laboratolies, Gaithersburg, MD, USA).

The ammonia removal activity was evaluated daily for 7 days and on days 10 and 14. The albumin secretion activity was evaluated on days 0, 1, 3, 5, 7, and 14. Each activity was normalized by the initial immobilized cell number in the bioreactor.

**LDH release** To evaluate cell death and injury in the bioreactor, the culture medium was exchanged with fresh culture medium. The released lactase dehydrogenase

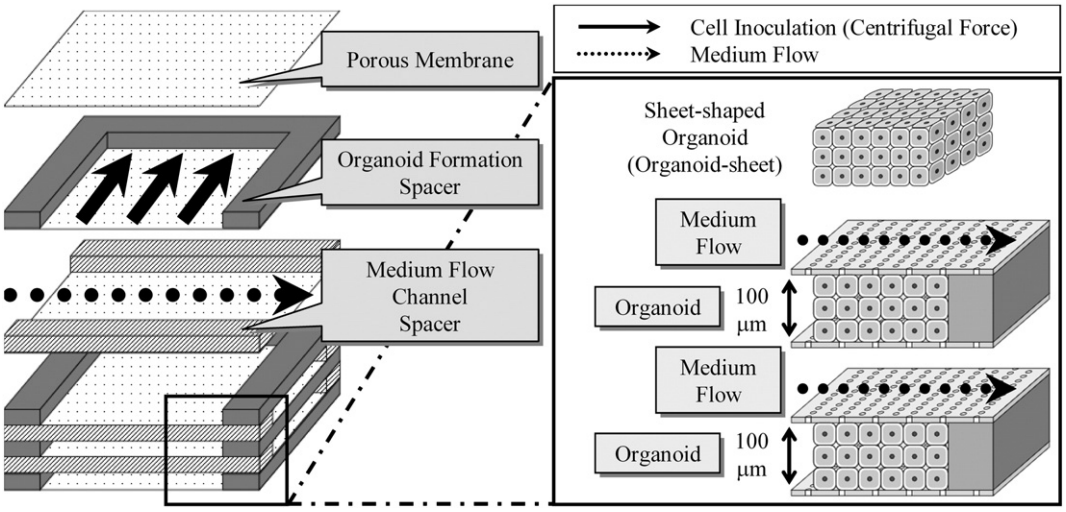


FIG. 2. Schematic diagram of cell culture space in the bioreactor. The space consisted of stacked organoid formation spaces and medium flow channel, one on top of the other. Each space was separated with flat porous membranes. The thickness of the culture space for hepatocyte organoid formation and medium flow was controlled at 100 μm with spacer.