

Construction of Multiple Hepatic Lobule like 3D Vascular Networks by Manipulating Magnetic Tweezers toward Tissue Engineering

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Abstract— In this paper, we have constructed actively perfusable multiple hepatic lobule-like vascular networks in a 3D cellular structure by using magnetic tweezers. Without well-organized channel networks, cells in a large 3D tissue cannot receive nutrients and oxygen from the channel, and therefore, the cells will be dead after few days. To construct well-organized channel networks, we fabricated a hepatic lobule like vascular networks by using magnetic fields in our previous works [1, 2]. However, the size of the hepatic lobule like vascular network was more than five times larger than real hepatic tissue. To improve the previous research, we have proposed several things. First, we have constructed the vascular network having similar size of the real thing in this step. Second, we have cultured the constructed structure for a long-time (more than two weeks) to verify the biocompatible condition. Third, we assemble the constructed hepatic tissues to make a large size of organ, liver. Finally, an actively perfusable system have been adopted to implement a bioreactor system by adding micro pump.

I. INTRODUCTION

Micro/nano robotics have a variety of biological applications, such as cell manipulation, assembly of cells and vascular network, cell sorting, cell analysis, and cloning. Among these applications, assembly of cells and channels are important research for regenerative medicine [3]. In spite of advance in medical science, the organ transplantation still has problems. The gap between waiting patient and donor numbers keep increasing. In addition, organ transplantation donor has a risk of rejection response. Therefore, in vitro 3D tissues are required to realize regenerative medicine.

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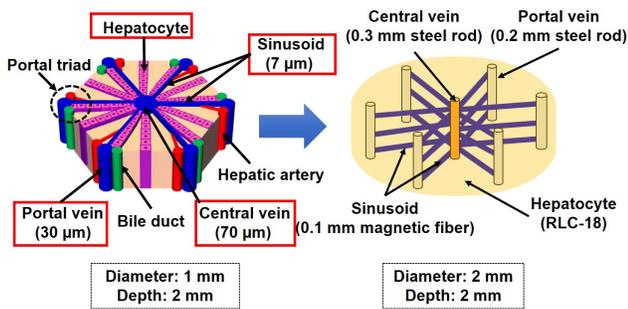
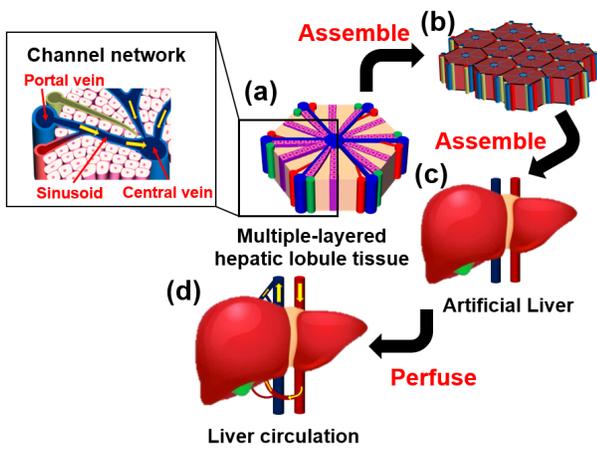
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For constructing 3D cellular structure, many approaches have been proposed. 3D bio printing is a represent method to build tissues thanks to its highly precise positioning and resolution [4, 5]. However, in this case, the assembly speed depends on the size of the constructed tissue and the underlying layers tend to collapse as additional layers are printed on the top of them. Another method is cell sheet engineering [6, 7]. It can assembly a large size of tissues at high speed. Stereolithography also can assemble 3D cellular structure [8]. However, the cell density is too low. Especially, it is difficult to supply nutrients and oxygen to 3D cellular structure thicker than 200 μm because they assembled only cells without channel network [9]. To maintain cells embedded in large-sized 3D cellular structures in living condition, well-organized vascular networks are required.

The final goal of this research is to construct liver by assembling hepatic lobule like structure. Liver is an important organ because of its many functions such as detoxification of blood, helping fighting infection, and aiding in digestion. In addition, it is an ideal cellular model for drug test and screening, and cell behavior study. For this, as the first step, many researchers have constructed hepatic lobule like structure. D. R. Albrecht et al. patterned cells and microspheres by dielectrophoresis (DEP) manipulation [10]. C. T. Ho et al. patterned hepatic and endothelial cells via an enhanced field-induced dielectrophoresis (DEP) trap [11]. G. S. Jeong, et al. culture cells using honeycomb structures made by natural extracellular matrix (ECM) [12]. However, they constructed only two-dimensional structure due to the lack of spatial organization techniques. To realize similar models with hepatic lobule, Fabrication of 3D hepatic lobule like structure with high similarity is necessary. 3D hepatic lobule like structure have been constructed by using several approaches. V. L. Tsang, et al. fabricated 3D cellular structure by multilayered photo patterning platform for embedding cells in hydrogels of complex structure [13]. X. Ma, et al. made 3D hydrogel-based triculture model by 3D printing [14]. C. Ma, et al. culture cell-laden hydrogel by pneumatic-aided micro-molding [15]. Although they construct 3D hepatic tissue, the depth of the structure is less than 200 μm . To construct large sized hepatic lobule tissue, perfusable vascular network to supply nutrients and oxygen is required.

Recently, assembly of hepatic lobule-shaped microtissue containing liver cells for constructing multiple layered tissues have been proposed. Z. Liu, et al. fabricated hepatic lobule tissues based on Ca-alginate cell sheets and assembly them by using glass micro-pipette [16]. J. Cui et al. utilized a gelatin



methacryloyl (GelMA) hydrogel as a matrix to construct 3D lobule-like microtissues for co-culture and assembled them using micro bubbles [17]. Although they achieved multi-layered structure, the vascular networks are too simple compare to real things.

In our previous works, we constructed hepatic lobule like vascular network in 3D cellular structure by using magnetic fibers [1, 2]. The magnetic fibers were controlled by magnetic tweezer. However, the size of the constructed hepatic lobule like structure was five times larger than real hepatic tissue. In this case, we cannot transfer nutrients and oxygen to the cells far from the vascular networks. In this paper, we construct the similar size of hepatic lobule like structure to realize similar morphology with real hepatic tissues. In addition, we assemble the constructed hepatic lobule tissues for making an artificial liver.

Fig. 1 explain the final goal of our research. First, we fabricate multi-layered hepatic lobule tissue with 3D vascular network as in Fig. 1 (a). Second, the fabricated tissues are assembled like Fig. 1 (b), and therefore, an artificial liver will be fabricated (Fig. 1 (c)). Finally, the culture medium will be perfused to make liver circulation. In this step, we focus on fabricating a multi-layered hepatic lobule structure with high similarity and assemble the multi-layered tissues. In addition, we inject the dyed solution to verify the connection of the constructed tissues.

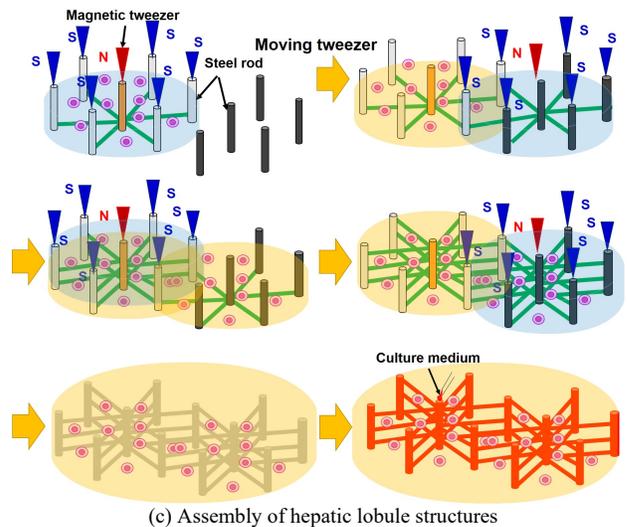
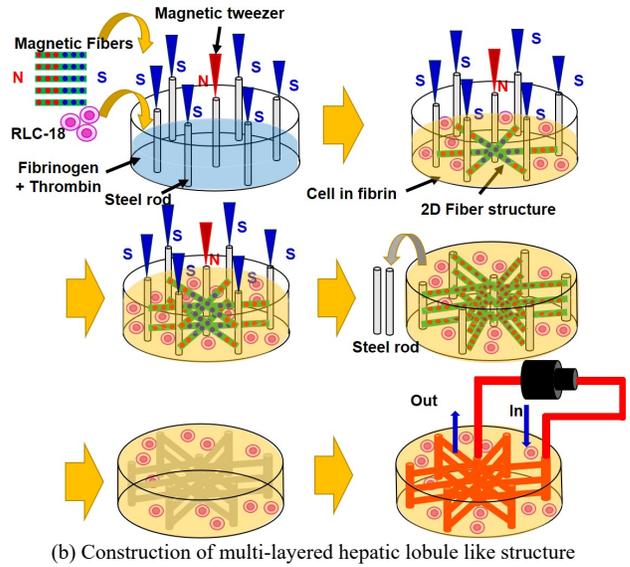
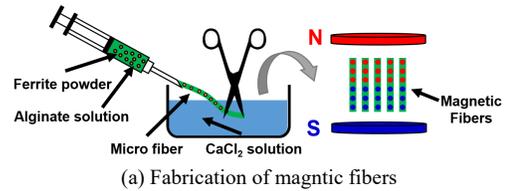


Fig. 3 Construction procedure of multiple hepatic lobule like structure

II. CONSTRUCTION METHOD OF HEPATIC LOBULE TISSUE

In this section, we will explain construction process of a multi-layered hepatic lobule-like vascular networks. To realize hepatic tissue with high similarity, we investigate the morphology of the real hepatic tissue. The hepatic lobule has 1 – 1.5 mm of diameter and 1.5 – 2 mm of depth [20]. Moreover, it has several veins like Fig. 2 (a). In this case, we focus on a blood flow from portal vein to central vein through sinusoids. The veins have different size of diameters as shown in Fig. 2 (a). To imitate the vascular network, we employ two types of steel rods and magnetic fibers. To reduce the size of the hepatic tissue, the steel rods and fibers are changed from previous works [1, 2]. Steel rods having 1.5 mm and 1 mm of diameters are replaced 0.3 mm and 0.2 mm of diameters.

Magnetic fibers are redesigned from 0.4 mm of diameter to 0.15 mm of diameter by changing tip of the needle like Fig. 2 (b). As the result, we can construct a hepatic tissue having similar size of real things.

Fig. 3 displays the construction process of multiple hepatic lobule like vascular network in 3D cellular structure. As a first step, magnetic fibers were fabricated (Fig. 3 (a)). We mixed 0.1g of ferrite particles (SF-H470) into 1 mL of 1.0 % w/v alginate solution. The mixed solution was loaded into a 1 mL of syringe having 32G needle (0.15 mm of diameter). To inject the solution as a constant speed (0.7 mL/min), we used a syringe pump. The solution was injected to 2.0 % w/v calcium chloride solution and then 0.15 mm alginate gel fibers was fabricated. The fabricated fibers are trimmed as the same length by employing scissors. Subsequently, the trimmed fibers were arranged in vertical to magnetize them by magnetizer (IMC-1050, IMS Co., Ltd.) at 3T like Fig. 3 (a). As a result, we completed magnetic fiber having different magnetic poles at the end of fibers. After then, we washed the magnetic fibers with 0.9 % of NaCl several times.

As a second step, a multi-layered hepatic lobule like structure was constructed (Fig. 3 (b)). We mounted seven steel rods on 35 mm of petri dish to create a hexagonal structure and then magnetized them using magnetic tweezers. The steel rod located in center of the dish was magnetized to N magnetic pole, whereas the steel rods placed on the edge of the structure were magnetized to S magnetic pole. Next, 0.2 mL of thrombin (10 units/mL), aprotinin solution, and rat liver cells (RLC-18, 1.8×10^7) were deposited into the dish. The magnetic fibers were dropped into the dish and then the steel rods attracted the end of fibers. After the fibers were attached to the steel rods stably, we poured the 2 mL of fibrinogen solution (10 mg/mL fibrinogen, 20 mM HEPES, and 0.9% w/v NaCl were dissolved in 10% PBS). After 30-50 minutes in an incubator (5% CO₂ at 37°C), the fibrinogen solution were transformed to fibrin gel and then the first layered structure was constructed. By repeating these steps several times, we can construct a multi-layered hepatic lobule structure.

After constructing desired multi-layered structure, the steel rods were removed from the fibrin gel. The inner alginate fibers were dissolved by injecting 2 mL of sodium citrate solution (55-mM, 0.45 % NaCl) and it would be channel. Finally, we installed actively perfusable system to supply nutrients and oxygen to the cells via 3D vascular networks.

Lastly, we assemble the multi-layered structures to construct multiple hepatic lobule like structures (Fig.3 (c)). For assembling the second hepatic tissue, five steel rods were arranged beside the first hepatic tissue. After then, we moved the tweezer to magnetize the steel rods for second structure and deposited fibers and rat liver cells. Subsequently, the fibers were attached to the steel rods. After 30-50 min, the fibrinogen solution was solidified and then we remove steel rods. Finally, we injected dyed solution into the center of the first structure to verify the channel network between two hepatic tissues.

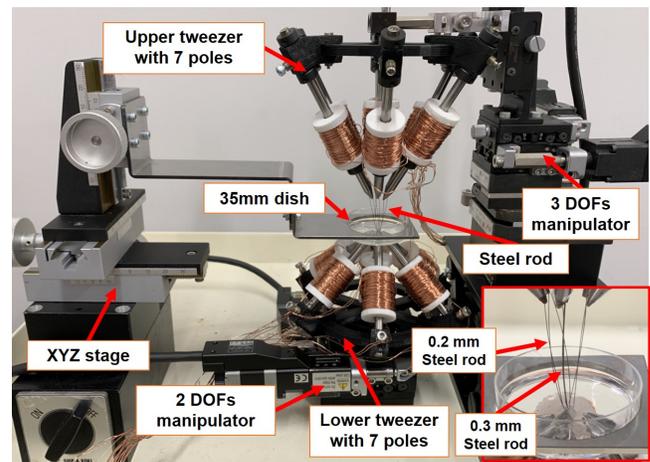


Fig. 4 Magnetic tweezer with lower and upper manipulators

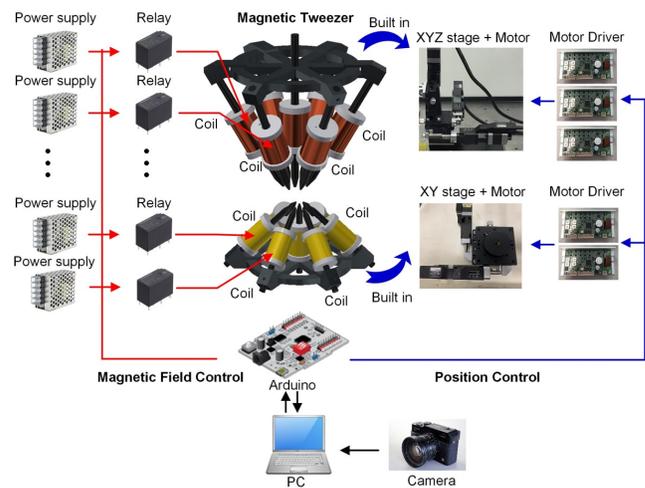


Fig. 5 System of magnetic tweezer

III. DESIGN OF MAGNETIC TWEezer

A. System of magnetic tweezer

Magnetic tweezer has several advantages for constructing 3D vascular network. First, it can increase the construction speed regardless of the target size. Second, it exerts force at a distance without damage to the target materials. Third, it can maintain the biocompatible condition of the cells. Thanks to these merits, magnetic fields have been applied to the biological applications [19-24]. However, the applications were limited such as measuring mechanical properties and manipulating a single particle. In this paper, we utilize magnetic tweezer to manipulate magnetic fibers and then construct multi-layered hepatic tissues. For this, there are several necessary conditions. First, the tweezer should generate enough magnetic forces that can attract magnetic fibers to the steel rods. In previous work, we already analyzed the magnetic fields using 2D [1] and 3D simulator [2]. Second, seven poles that can make hexagonal structure is indispensable. Third, micro-scaled position control is required to make mm-sized structure. Especially, manipulators to control lower and upper tweezers at the same time are required. Based on several necessary condition, we designed a magnetic tweezer.

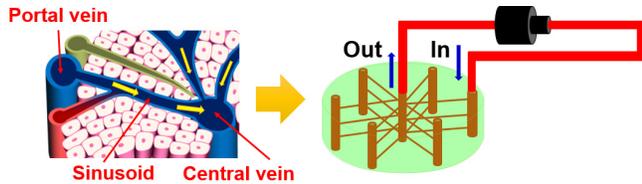


Fig. 6 Realize 3D perfusable vascular network by adding micropump

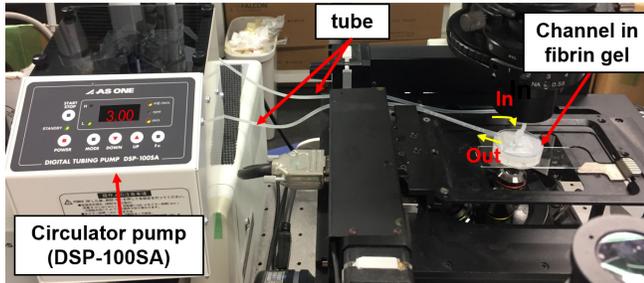


Fig.7 Experimental setup for 3D perfusable vascular network

Fig. 4 shows the magnetic tweezer with lower and upper manipulator. To construct hepatic lobule like structure that has similar size of real thing. The system is similar to previous paper [2]. An additional thing is to add a lower manipulator to make a movement of lower tweezers. In this system, we used lower and upper tweezer to generate enough magnetic force on the steel rods. For assembling hepatic tissues like Fig. 1, precise control is required. For this, XY stage (TSD-602) with two stepping motors and XYZ stage (TSD-405SL) with three stepping motors manipulate the lower tweezers and the upper tweezers, respectively. In Fig. 5, a system of the magnetic tweezers is described. The microcontroller controls the manipulators that manipulate lower and upper tweezers, simultaneously. In addition, we used 0.3 mm and 0.2 mm of steel rods to make the diameter of the central and portal veins, respectively.

B. Actively perfusable channel network

To verify the efficiency of the vascular network in cellular structure, we demonstrated that the perfused vascular channels by adding micro pump. The hepatic lobule like channel network is described in left side of Fig. 6. The blood is transferred from the portal vein to the central vein through sinusoids. For making that kind of channel network, we connected micro pump like right side of Fig. 6. To realize the concept of the circulation, we designed the experimental system as in Fig. 7. A circulator pump (DSP-100SA) make a continuous flow as 3mL/min of flow rate through 2 mm diameter of micro tube. The flow rate was decided from the research data in [25].

IV. RESULT AND DISCUSSION

A. Hepatic lobule like vascular network in fibrin gel

In Fig. 8 and Fig. 9, we constructed hepatic lobule structure with high similarity by using 0.3 mm, 0.2 mm of steel rods, and 0.15 mm of magnetic fibers. Fig. 8 displays the experimental results of the fabricated structure. We fabricated

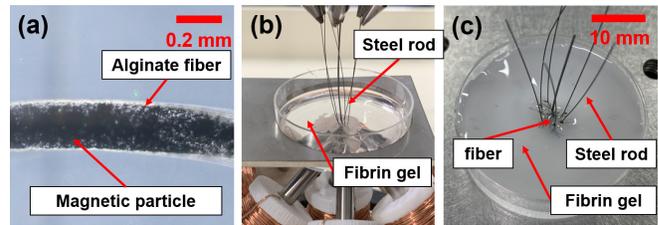


Fig. 8 Experiment result of 3D vascular network in fibrin gel, (a) fabricated alginate gel fiber embedding magnetic particles, (b) constructing multi-layered structure using tweezers, (c) bird view of the structure before removing steel rods

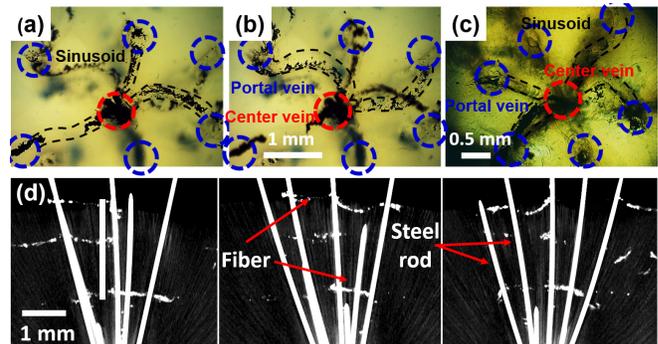


Fig. 9 Constructed 3D vascular network in fibrin gel, (a) top view – third layer of vascular network, (b) top view – second layer of vascular network, (c) bottom view of the structure – first layered vascular network, (d) side view by taking X-ray inspection

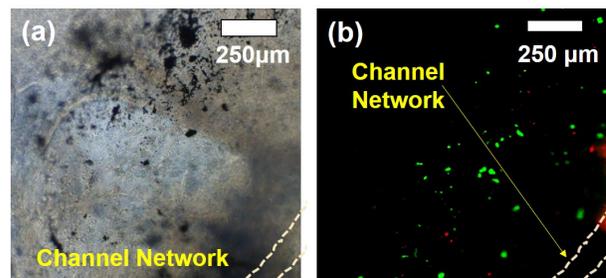


Fig. 10 Images of 3D cellular structure with channel network after two weeks, (a) Bright field image of the cells in the constructed structure, (b) Fluorescent image after the live/dead staining of cells in the constructed structure

0.15 mm of magnetic fiber like Fig. 8 (a) by applying 32 Gauge needle. Fig. 8 (c) indicates the bird view of the multilayered structure before removing steel rods. From Fig. 9, we can verify the shape of the structure. Fig. 9 (a) and (b) show the top view of the third and second layer of structure, respectively. The first layer can be seen in Fig. 9 (c) as a bottom view. From Fig. 9 (d), side view of the structure, we can verify the three-layered vascular network. These views were obtained by X-ray inspection from different angles (μ B3500-LCTX, Matsusada Precision). As a result, we constructed hepatic lobule like vascular network in fibrin gel with high similarity that has 2.5 mm of diameter and 2.3 mm of depth.

B. Cell viability in 3D cellular structure with channels

Fig. 10 illustrates the images of 3D cellular structure with channel network after two weeks. Fig. 10 (a) show the bright field images of the constructed structure. Fig. 10 (b) indicate

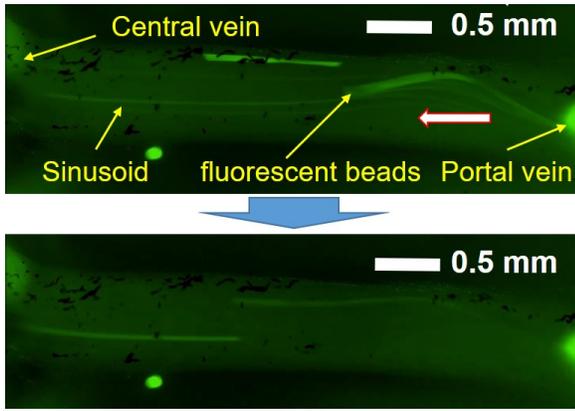


Fig. 11 Actively perfusable channel network

the fluorescent images after the live/dead staining of cells in the constructed structure. The process is as follows; after constructing multilayered structures, we cultured them in an incubator for two weeks. During these periods, we refreshed the culture medium and aprotinin every day. After two weeks, the cells in the structure were stained with Calcein-AM and Propidium iodide (PI). The green things indicate the living cells, while the red things point out the dead cells.

In previous works [1, 2], we verified that the cells close to the culture medium show a higher survival rate than the cells far from the culture medium. To be specific, cells located away from the culture medium (contact – 750 μm) show high viability as 83% of cell viability, whereas cell viability was decreased to 63% at the area (1400 - 1800 μm). In Fig. 10, every cells were located within area (channel – 750 μm from the channel). The viability of cells in the area was 81%.

More important thing is that fabricated structure having 2.5 mm of diameter can supply the nutrients through the vascular network to whole of the cells in structure. The cell farthest from the vascular network is approximately 620 μm , and therefore, every cell in hepatic lobule like structure smaller than the structure will be located within the area that shows high viability as 81 % (channel – 750 μm from the channel, same as Fig. 10).

Fig. 11 demonstrates the actively perfusable channel network by adding fluorescent beads. From these figures, we can verify that the channel network can transfer the nutrients or oxygen from the portal vein to the central vein via sinusoids.

C. Multiple Hepatic Lobule like Vascular Network

At the previous section, we verified the efficiency of the proposed hepatic lobule like vascular network by analyzing cell viability. In this section, we assemble two hepatic lobule structures by moving magnetic tweezers. Fig. 12 shows the experiment result of two hepatic lobule structure in fibrin gel. By moving magnetic tweezers from the first target to the second target, we can easily magnetize the steel rods and then assemble multiple structure described in Fig. 3 (c). Fig. 13 display the constructed multiple hepatic structures. To verify the connection between two hepatic lobule structures, we

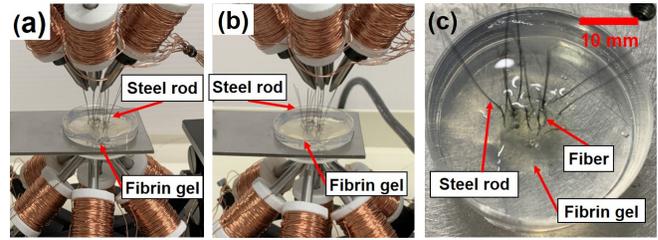


Fig. 12 Experiment result of multiple hepatic lobule structures in fibrin gel, (a) constructing the first structure, (b) constructing the second structure by moving tweezers, (c) bird view of the structure before removing steel rods

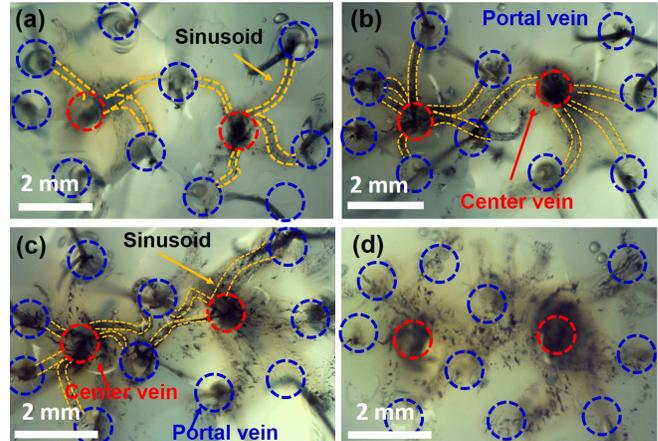


Fig. 13 Constructed multiple hepatic lobule structures in fibrin gel (top view), (a) first layer of vascular network, (b) second layer of vascular network, (c) third layered vascular network before removing steel rods, (d) vascular network after removing steel rods

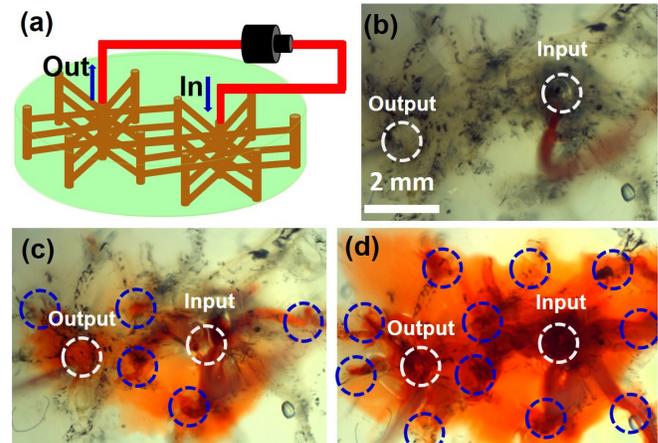


Fig. 14 Inject dyed solution into the center of the second structure (a) injection method using micro pump (b-d) verification of multiple vascular network by injecting dyed solution

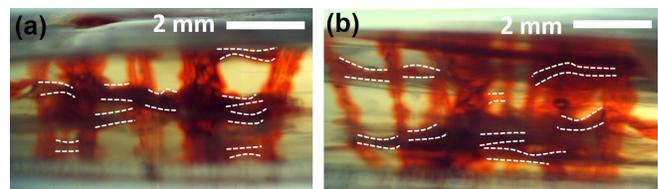


Fig. 15 Side view of the constructed structure at the different angles

connect the micropump like Fig. 14 (a). The dyed solution as a red color was injected into the center of the second structure as a constant flow rate (30 $\mu\text{L}/\text{min}$) and was withdrawn from

the center of the first structure at the same flow rate. In Fig. 14 (a), we injected the solution into the white circle (input), and then the solution was dispersed to the blue circle like Fig. 14 (d). Fig. 15 illustrate the side view of the constructed structure at the different angles. From these figures, we can verify the connection between two structures.

In the future, we will assemble hepatic tissues to construct organ, liver and then verify that the circulation of the multiple hepatic lobule can improve the liver metabolism and liver function by culturing then for 2 weeks. Finally, we will test the constructed channel network in vivo using mouse.

V. CONCLUSION

In this paper, we constructed 3D hepatic lobule in cellular structure like structure having high similarity. In order to construct similar sized hepatic tissue with real things, the steel rods and magnetic fibers were changed from 1.5 mm and 1 mm of steel rods, and 0.4 mm of magnetic fibers to 0.4 mm and 0.2 mm of steel rods, and 0.15 mm of magnetic fibers. As a result, we constructed hepatic lobule like vascular network with 2.5 mm of diameter and 2.3 mm of depth. In addition, we cultured the structure for two weeks and verified that the constructed structure can keep the high viability more than 81%. The actively perfusable channel network was also proposed to make a circulation like a real thing and then we demonstrated the channel network by adding fluorescent beads. Moreover, we succeed in constructing two hepatic lobule like structure by moving lower and upper magnetic tweezer. For this, we add manipulator that can control lower tweezer. In the future, we will increase the number of assembly structure and then construct an organ, liver.

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