Design of Scaffold with Array of Micro Projections to Trace Intra- and Inter-cellular Behavior

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ABSTRACT

The transparent micro-patterned scaffold has been designed to observe the interaction between cultured cells *in vitro*. The scaffold consists of a thin cover sheet and arrangement of micro-pillars. The pillars of polydimethylsiloxane were made by the photolithography technique. Each pillar has the cylindrical shape (0.007 mm diameter, 0.002 mm height). The distance between adjacent pillars is 0.003 mm. The thickness of the base sheet of the pillars is 0.02 mm. The sheet with pillars is turned upside down, and used for the scaffold of cell culture. The pillars simultaneously play a roll of the position marker. L929 (fibroblast connective tissue of mouse), or C2C12 (mouse myoblast) was seeded on the sheet at the counter surface to the pillars at the density of 1000 cells/cm^2. The cells were cultured in the medium containing 10% FBS (fetal bovine serum) and 1% penicillin/ streptomycin. Cells are able to be traced through the transparent scaffold, but the deformation of scaffold cannot be detected. The designed scaffold has a potential to trace the intracellular and intercellular behavior microscopically *in vitro*.

Keywords: Biomedical Engineering, Cell Culture, L929, C2C12, Micro Pillar, and Photolithography.

1. INTRODUCTION

Biological tissues actively maintain the morphology by the intra- and inter-cellular forces. The morphology of each cell is kept by the interaction between skeletal proteins. Interaction between cells keeps the shape of the tissue. Interaction between the extracellular matrix and cells governs the compliance of the tissue.

The cell culture technique realizes synthesize the biological tissue *in vitro*. The tissue should have enough strength for the clinical application in regenerative medicine.

Measurement of intra- and inter-cellular forces has been tried by several preparations: using laser, atomic force microscope, and fluorescence technique. At the end of test, scanning electron microscope, and stain technique are available.

Photolithography technique realizes design of micro morphology of surface of the scaffold for cell culture [1-9]. The technique can be applied to control compliance of the surface [7, 8]. The micro morphology can be a marker on the micro location of the surface.

In the present study, the transparent micro-patterned scaffold with the micro-pillar array has been designed to observe the interaction between cultured cells *in vitro*.

2. METHODS

Micro Pattern

The transparent micro-patterned scaffold has been designed to observe the interaction between cultured cells *in vitro* (Fig. 1). The scaffold consists of a thin cover sheet and a micro-pillar array. The pillars were made of polydimethylsiloxane by the photolithography technique. Each pillar has the cylindrical shape (0.007 mm diameter, 0.002 mm height). The distance between adjacent pillars is 0.003 mm. The thickness of the base sheet of the pillars is 0.02 mm. The sheet with pillars is turned upside down, and used for the scaffold of cell culture. The pillars simultaneously play a roll of the position marker. The pattern is drawn in the square area of 0.5 mm × 0.5 mm.

Without Photomask

The borosilicate glass (Tempax) disk (50 mm diameter, 1mm thickness) was used for the base of the mold. The disk was cleaned by the oxygen plasma ashing (100 W, for ten minutes) in RIE (FA-1, Samco International, Kyoto, Japan). To improve affinity between glass and photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the disk at 3000 rpm for 30 s with a spin coater.

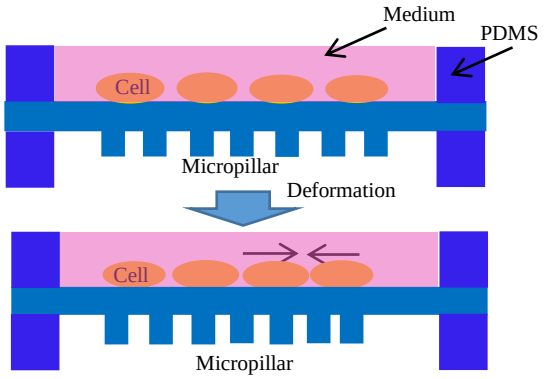

Fig. 1: Model of local deformation (lower) at scaffold sheet lined with micro-pillar array.

The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the disk at 5000 rpm for 30 s with the spin coater. The photoresist was baked in the oven at 368 K for three minutes.

The pattern for micro-pillars was drawn on the photoresist with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the pattern on the mold with the laser drawing system, the parameters were selected as follows: the voltage of 2.5 V, the velocity of 0.02 mm/s, the acceleration of 0.5 mm/s^2. The pattern was baked on the heated plate at 368 K for three minutes. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for five minutes. The pattern was rinsed, and dried by the spin-dryer.

The dimension of the micro-pillars of the mold was measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). The height along the cross sectional line of micro pillars was traced.

PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was mixed with the curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning Corp., MI, USA). The volume ratio of PDMS to curing agent is ten to one. After degassing, 0.3 cm^3 of PDMS was poured on the mold on the spin coater at 2000 rpm for 30 s, and the ring of PDMS (outer diameter of 20 mm, inner diameter of 10 mm) was put on it. PDMS was baked at 373 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd). The baked film of PDMS was removed from the mold in the acetone by lift off method (Fig. 2).

The dimension of the micro-pillars at the film was measured with a laser microscope.

With Photomask

The borosilicate glass (Tempax) disk (50 mm diameter, 1mm thickness) was used for the base of the mask. After hydrophilization by the oxygen plasma ashing by RIE (FA-1, Samco International, Kyoto, Japan), titanium was coated on the surface. To improve affinity between titanium and photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the disk. The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the disk at 2000 rpm for 30 s with the spin coater.

The pattern for micro-pillars was drawn on the mask with a laser drawing system. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan).

The titanium coating disk was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan). For etching, the gas of SF_6 (50 cm^3/min at 1013 hPa) with Ar (50 cm^3/min at 1013 hPa) was applied at 100 W at 4 Pa for five minutes. OFPR-800LB was removed by acetone, after confirmation of pattern of etching by the microscope.

The dimension of the micro-pillars at the mask was measured with the laser microscope.

The borosilicate glass (Tempax) disk (50 mm diameter, 1mm thickness) was used for the base of the mold. The positive photoresist material of OFPR-800LB was coated on the borosilicate glass disk. The surface of mask coated with titanium was mounted on the surface of OFPR-800LB, and the photoresist was exposed to the UV light through the mask in the mask aligner (M-1S, Mikasa Co. Ltd., Japan) for the following time: 5 s, 8 s, 10 s, 15 s.

The dimension of the micro-pillars of the mold was measured with a laser microscope.

After degassing, PDMS was poured on the mold on the spin coater, and the ring of PDMS (outer diameter of 20 mm, inner diameter of 10 mm) was put on it. PDMS was baked at 373 K for one hour in an oven. The baked film of PDMS was removed from the mold in the acetone by lift off method (Fig. 2).

The dimension of the micro-pillars at the film was measured with a laser microscope.

Another ring of PDMS (outer diameter of 20 mm, inner diameter of 10 mm, and height of 1 mm) was put on the back side, and baked at 373 K for one hour in an oven (Fig. 3).

The culture dish of PDMS was exposed to the oxygen gas at power of 50 W for one minute in a reactive ion etching system (FA-1, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing). The dish was preserved in the ultrapure water to keep the hydrophilic property of the surface, before the cell culture.

Cell Culture

Two kinds of cells were used in the test. C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), L929 (fibroblast connective tissue of C3H mouse).

EMEM (Eagle's Minimum Essential Medium) containing 10% FBS (decomplemented fetal bovine serum) and 1% penicillin/ streptomycin was used for the culture medium of L929. D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (decomplemented fetal bovine serum) and 1% penicillin/ streptomycin was used for the culture medium of C2C12.

L929 was seeded over the pillars at the density of 1000 cells/cm^2 (Fig. 4).

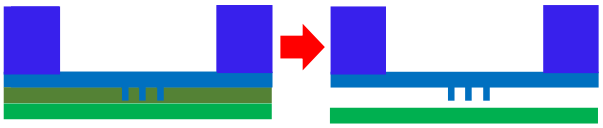

Fig. 2: Lift off.

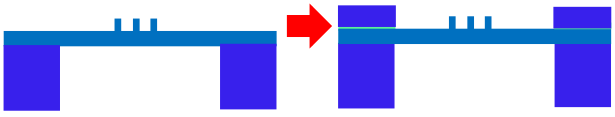

Fig. 3: Scaffold sheet lined with micro-pillar array contained on the ring of PDMS.

C2C12 was seeded on the sheet at the counter surface (back side) to the pillars at the density of 1000 cells/cm^2.

The culture dish was kept in the incubator to maintain both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent. The cells were observed with the phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture for five days. The medium was changed every two days.

3. RESULTS

In the case of "without mask", the tracing of the surface morphology across the lines of pillars on the mold (Fig. 5) measured by the laser microscope is exemplified in Fig. 6. The tracing shows the height of 0.002 mm, the diameter of 0.0078 mm, and interval of 0.0072 mm (diagonal 0.0028 mm).

In the case of "without mask", the tracing of the surface morphology across the lines of pillars on the film (Fig. 7) measured by the laser microscope is exemplified in Fig. 8. The tracing shows the height of 0.0024 mm, the diameter of 0.007 mm, and interval of 0.008 mm (diagonal 0.0036 mm).

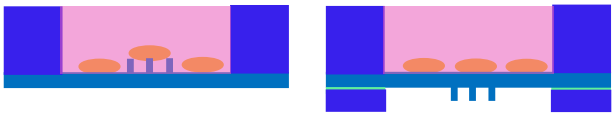

Fig. 4: L929 cultured on micro-pillar array (left), C2C12 cultured on sheet lined with micro-pillar array (right).

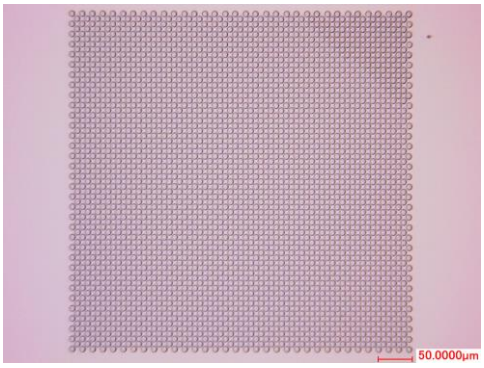

Fig. 5: Mold for micro-pillar array (without mask). Dimension from left to right is 0.7 mm.

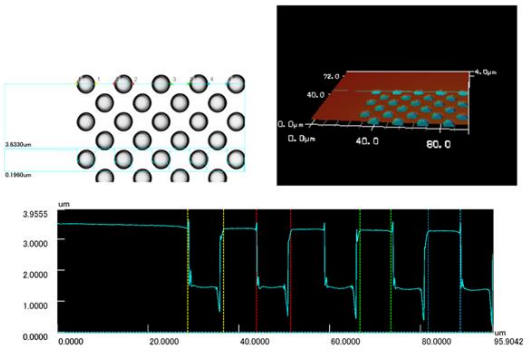

Fig. 6: Dimension of mold for micro-pillar array (without mask).

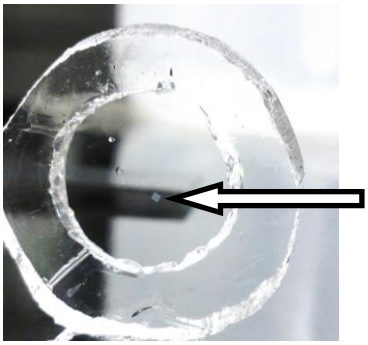

Fig. 7: Scaffold lined with micro-pillar array (without mask).

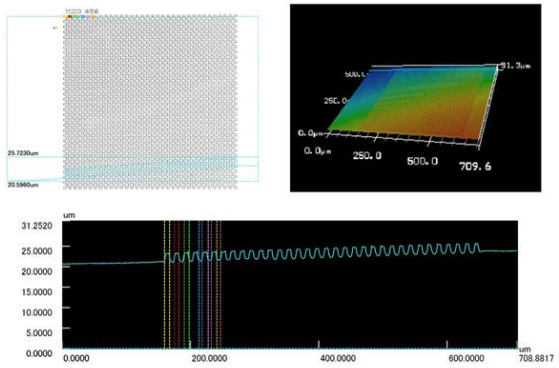

Fig. 8: Dimension of micro-pillar array (without mask).

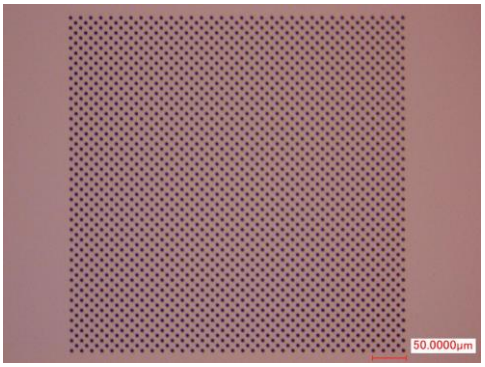

Fig. 9: Mask for micro-pillar array. Dimension from left to right is 0.7 mm.

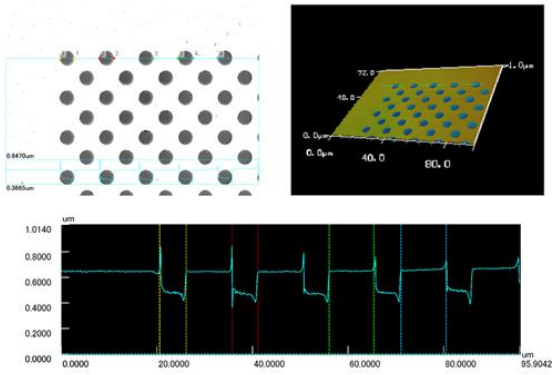

Fig. 10: Dimension of mask for micro-pillar array.

At the mask (Fig. 9), the tracing of the surface morphology across the lines of pillars on the film measured by the laser microscope is exemplified in Fig. 10. The tracing shows the diameter of 0.0055 mm, and interval of 0.0094 mm (diagonal 0.005 mm).

In the case of "without mask", the tracing of the surface morphology across the lines of pillars on the mold (Fig. 11) measured by the laser microscope is exemplified in Fig. 12. Fig. 11 shows the mold with the exposure of 8 s. The tracing shows the height of 0.0033 mm, the diameter of 0.0063 mm, and interval of 0.0081 mm (diagonal 0.0039 mm) (Fig. 12). The measurement of the molds shows underexposure (5 s (Figs. 13 &14)) and overexposure (10 s (Figs. 15 & 16) and 15 s).

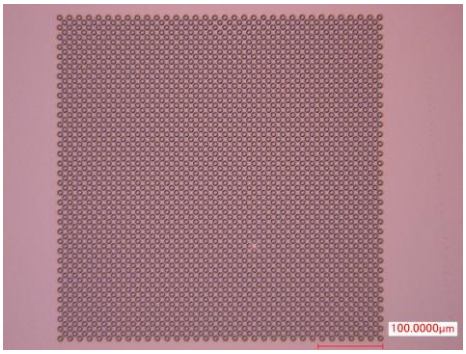

Fig. 11: Mold for micro-pillar array (8 s with mask). Dimension from left to right is 0.7 mm.

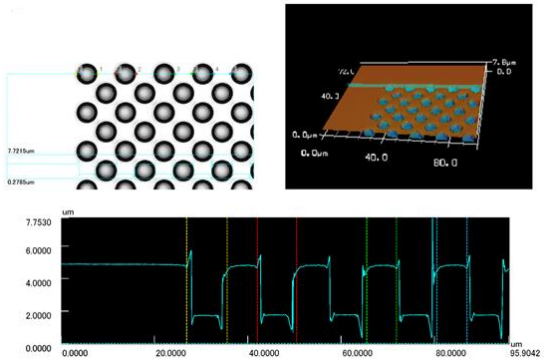

Fig. 12: Dimension of mold for micro-pillar array (8 s with mask).

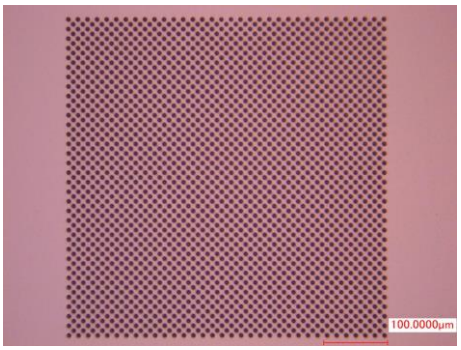

Fig. 13: Mold for micro-pillar array (5 s with mask). Dimension from left to right is 0.7 mm.

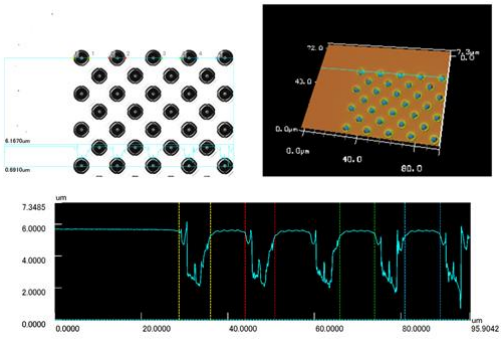

Fig. 14: Dimension of mold for micro-pillar array (5 s with mask).

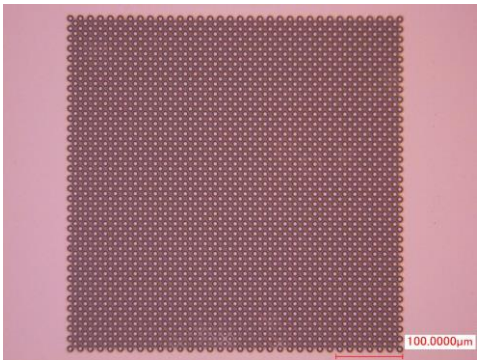

Fig. 15: Mold for micro-pillar array (10 s with mask). Dimension from left to right is 0.7 mm.

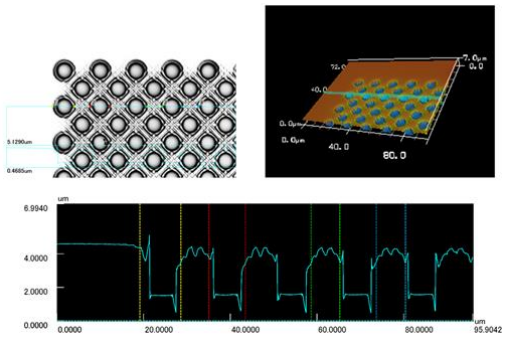

Fig. 16: Dimension of mold for micro-pillar array (10 s with mask).

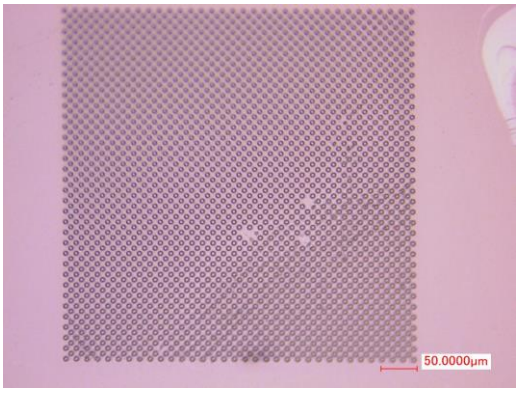

Fig. 17: Dimension of micro-pillar array (with mask). Dimension from left to right is 0.7 mm.

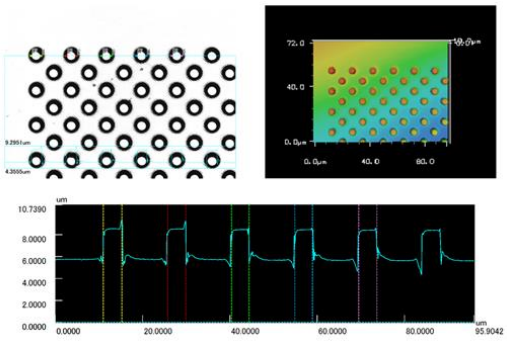

Fig. 18: Dimension of micro-pillar array (with mask).

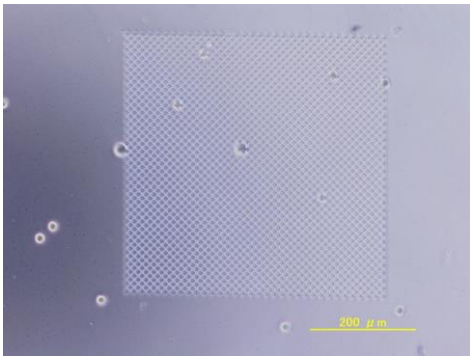

Fig. 19: L929 on micro-pillar array, day 1 (24 hours). Dimension from left to right is 1 mm.

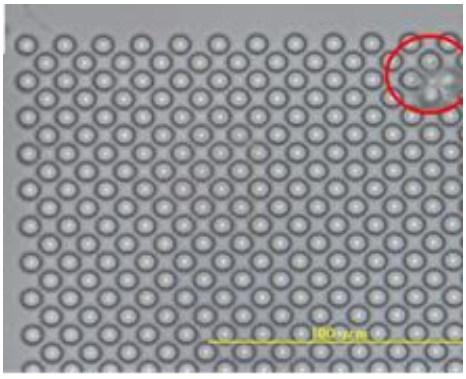

Fig. 20a: L929 on micro-pillar array, day 2. Dimension from left to right is 0.2 mm.

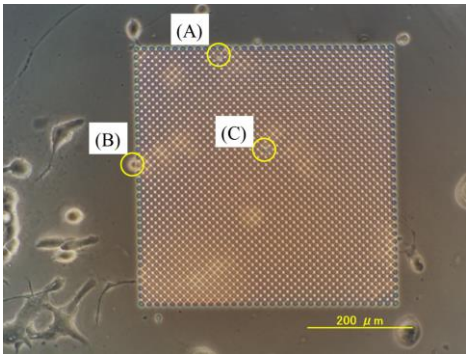

Fig. 20b: L929 on micro-pillar array, day 2. Dimension from left to right is 1 mm.

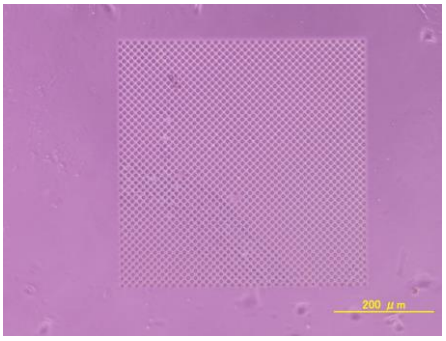

Fig. 21: C2C12 on micro-pillar array, day 1 (24 hours). Dimension from left to right is 1 mm.

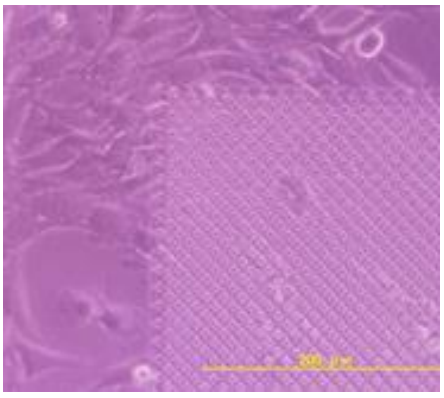

Fig. 22a: C2C12 on micro-pillar array, day 5. Dimension from left to right is 0.4 mm.

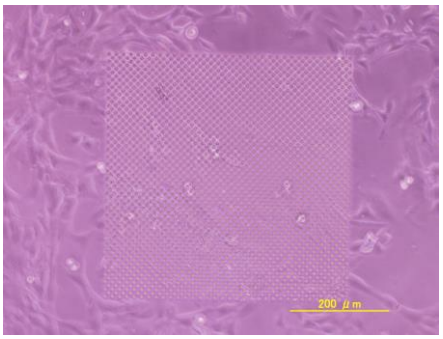

Fig. 22b: C2C12 on micro-pillar array, day 5. Dimension from left to right is 1 mm.

Fig. 17 shows the micro-pillar array made with mask. The tracing shows the height of 0.0028 mm, the diameter of 0.0041 mm, and interval of 0.010 mm (diagonal 0.0059 mm) (Fig. 18).

The behavior of L929 on the micro-pillar array is exemplified in Figs. 19 & 20.

The behavior of C2C12 on the micro-pillar array is exemplified in Figs. 21 & 22. One of the positions of the micro-pillar, where C2C12 adheres, is shifted on the day 1 (24 hours after cell seeding).

Myoblast differentiated to myotubes on the sheet in five days (Fig. 22). Most of longitudinal direction of myotubes orient

along the diagonal direction of the square of array. The closer direction between micro pillars is diagonal. Several positions of the micro-pillar are shifted on the day 5.

4. DISCUSSION

Adhesion governs the behavior of cells. When the biological tissue adheres to the scaffold, the scaffold deforms as the tissue deforms. The force can be calibrated by the compliance of the scaffold, when the local deformation of the scaffold is traced [10].

In the test of L929, cells are cultured on the pillars. The pillars of 0.007 mm diameter should be taller than 0.003 mm to be deformed with the behavior of cells. If the pillars are tall, the cells fall into the valley between the pillars [9]. The cell extends through the slit between the pillars. If the pillar is short, the compliance of the pillar is too low to make self-deformation with cells.

In the previous study [6], cells were cultured on the substrate with micro-pillar array. A biological cell adheres, migrates, rotates, and deforms on the scaffold. These behaviors of cell depend on the morphology of the scaffold. The previous study show that the orientation of myoblast depends on the height of the micro ridges [9]. In the present study, the flat thin sheet covers the micro pillars, and cells are cultured on the sheet, so that the cell can adhere on the sheet at any direction regardless of the position of the micro pillar.

The myotubes make orientation along the diagonal direction of the sheet of scaffold. The diagonal direction follows the closest pillar. The sheet lined with micro-pillar array has distribution about local micro compliance. The behavior of cell might depend on the local micro deformability of the scaffold.

The dislocation of the pillar is very small to detect deformation of the adhered cell. If myotube grows and deforms in the larger scale, deformation of the scaffold might become larger. Thickness of the sheet should be thinner than 0.02 mm to be deformed with cells to detect behavior of cells.

The optical scattering by micro pillars on the scaffold disturbs optical microscopic observation at cells on the scaffold. To minimize the scattering, the micro-pillars should be shorter.

In the present study, the control of the volume of the medium is not easy, because the volume of the medium is very small (0.2 cm^3). With the mask, time for drawing is shortened. It is easier to control the height of pillars with the mask.

5. CONCLUSION

The transparent micro-patterned scaffold has been designed to observe the interaction between cultured cells *in vitro*. The designed scaffold with micro-pillar and sheet has been successfully fabricated by photolithography technique, and applied to culture of L929 and C2C12. Cells are able to be traced through the transparent scaffold. The designed scaffold is available to trace the intracellular and intercellular deformation *in vitro*.

6. ACKNOWLEDGMENT

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