

Effect of Surface Morphology of Scaffold with Lines of Micro Ridges on Deformation of Cells

Hiroimi SUGIMOTO, Haruka HINO, Yusuke TAKAHASHI, Shigehiro HASHIMOTO

Biomedical Engineering, Department of Mechanical Engineering,
Kogakuin University, Tokyo, 163-8677, Japan
<http://www.mech.kogakuin.ac.jp/labs/bio/>

ABSTRACT

The effect of micro ridges of the scaffold on deformation of cells has been studied *in vitro*. Several parallel lines of micro ridges have been made on a disk of transparent polydimethylsiloxane. The height, the width, and the length of the rectangular ridge are 0.001 mm, 0.003 mm, and 0.5 mm, respectively. Variation has been made on the interval of the ridge: 0.001 mm, 0.003 mm, 0.005 mm and 0.007 mm. Five kinds of cells were used in the test. C2C12 (mouse myoblast cell line), L929 (fibroblast connective tissue of mouse), Neuro-2a (a mouse neural cell line), 3T3-L1 (mouse fat precursor cells), and HUVEC (human umbilical vein endothelial cells). Cells were seeded on the micro pattern at the density of 2000 cells/cm² and cultured for 24 hours. The cells were observed with a microscope. The experimental results show that C2C12 lengthen along the line of ridge of 0.003 mm interval. The effects of the micro pattern depend on the kind of cells.

Keywords: Biomedical Engineering, Cell Culture, Micro Ridges, Photolithography, and Deformation.

1. INTRODUCTION

Multi-factors govern organization of cells *in vivo*. Because relations among the factors have not been analyzed, it is not easy to design simulator *in vitro*. Most of trials of regenerative medicine were arranged *in situ*,

A biological cell adheres, migrates, rotates, and deforms on the scaffold. These behaviors of cell depend on the micro pattern of the scaffold [1-6]. The previous study showed that the orientation of myoblast depends on the height of the micro ridges [1].

Cell culture technique has been developed, and cells have been cultured in controlled environment [1-21]. The behavior of the cell might depend on the surface micro morphology. The dependency might depend on the kind of cells. The technology can be applied to sorting of cells.

The acceleration technique for proliferation, orientation [1, 14, 20] and differentiation [3, 7] of cells has been studied to make tissue *in vivo* or *in vitro*. Several methods have been designed to stimulate cells [17-21].

In the present study, the effect of the surface morphology with lines of micro ridges of the scaffold on deformation of cells has been studied *in vitro*.

2. METHODS

Micro Pattern

Several parallel lines of micro ridges have been made at the center on a disk of transparent polydimethylsiloxane (PDMS) (Fig. 1). The height (H), the width (W), and the length (L) of the rectangular ridge (Fig. 1) are 0.001 mm, 0.003 mm, and 0.5 mm, respectively. Variation has been made on the interval (I) of the ridge: 0.001 mm, 0.003 mm, 0.005 mm and 0.007 mm. Each pattern is drawn in the square area of 0.5 mm \times 0.5 mm, which is the quarter part of the square area of 1.0 mm \times 1.0 mm. The square area is surrounded by a smooth surface without ridges as control.

The borosilicate glass (Tempax) disk was used for a surface mold for the disk in a photo lithography process (Fig. 2). The diameter and the thickness of the disk are 50 mm and 1.1 mm, respectively. The surface of the glass was cleaned by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan). To improve the affinity between the glass and the photo-resist material (OFPR-800), hydrophobic treatment was applied to the glass by the oxygen (30 cm³/min) plasma ashing at 100 W for ten minutes by a compact etcher (FA-1, Samco Inc., Kyoto). Hexamethyldisilazane (HMDS) was coated at 3000 rpm for thirty seconds with a spin coater (IH-DX2, Mikasa Co., Ltd., Tokyo, Japan).

The photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd., Tokyo, Japan) of 0.0017 mm thick was coated on the glass at 3500 rpm for five seconds with the spin coater. The photoresist was baked in the oven at 373 K for ninety seconds. The pattern for the micro grooves was drawn on the disk 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 3.2 V, the velocity of 0.151 mm/s, the acceleration of 0.350 mm/s². The pattern was baked in the oven at 393 K for five minutes.

The photo-resist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for 60 seconds. The disk was rinsed two times with the ultrapure water for three minutes, and dried by the spin-dryer.

The glass was etched with the plasma gas using a reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan) to make lines of the micro grooves of 0.001 mm depth. For etching, the gas of CF_4 (30 cm^3/min at 1013 hPa) was applied at 100 W at 2 Pa for thirty minutes. To exfoliate the residual photo-resist material from the surface, the disk was exposed to the oxygen gas of 30 milliliter per minute at power of 100 W for ten minutes using a compact etcher (FA-1, Samco Inc., Kyoto): (the oxygen plasma ashing).

The dimensions of the four kinds of the micro grooves of the mold were measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). The morphology along the transverse lines of grooves was traced (Fig. 3).

After the mold was enclosed with a peripheral wall of polyimide tape, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was poured with the curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning Corp., MI, USA) on the mold. The volume ratio of PDMS to curing agent is ten to one. After degassing, PDMS was baked at 373 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd). The baked disk of PDMS was exfoliated from the mold. The disk with the micro ridges was used as the bottom of the culture dish.

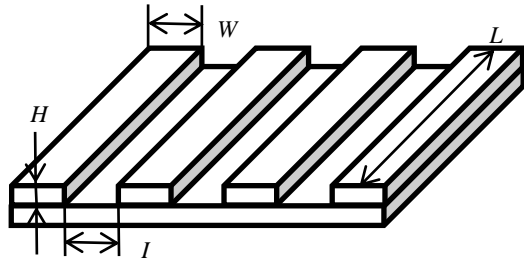


Fig. 1a: Parallel lines of micro ridges.

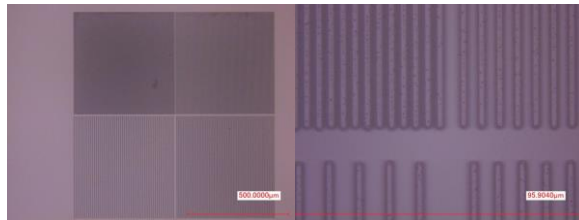


Fig. 2: Mold of micro grooves on glass plate. Interval $I = 0.001$ mm (upper left), 0.003 mm (upper right), 0.005 mm (lower right), 0.007 mm (lower left). Dimension from left to right is 1.5 mm (left), 0.1 mm (right).

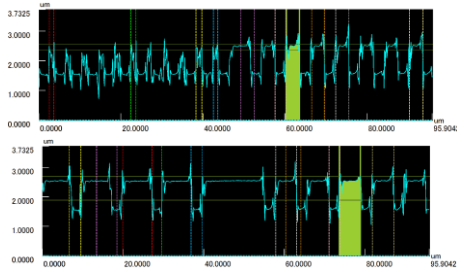


Fig. 3: Tracings of mold of micro grooves on glass plate. Interval $I = 0.001$ mm (upper left), 0.003 mm (upper right), 0.005 mm (lower right), 0.007 mm (lower left).

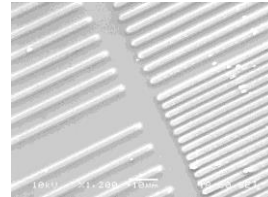


Fig. 4: Micro ridges of PDMS. Interval $I = 0.001$ mm (right), 0.003 mm (upper), 0.005 mm (left), 0.007 mm (lower). Dimension from left to right is 0.1 mm.

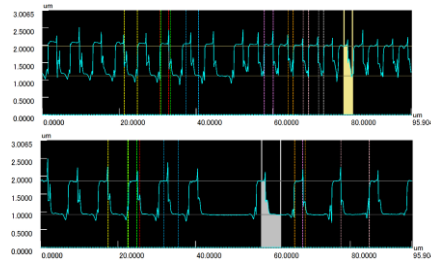


Fig. 5: Tracings of micro ridges of PDMS. Interval $I = 0.001$ mm (upper right), 0.003 mm (upper left), 0.005 mm (lower left), 0.007 mm (lower right).

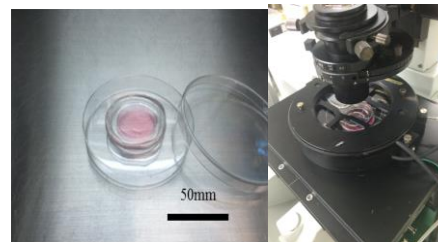


Fig. 6: Disk with micro pattern, wall and cap of PDMS (filled with medium) contained in polystyrene dish (left), placed in incubator on microscope (right).

The morphology of the micro ridges for scaffold was observed by a scanning electron microscope (SEM, JSM6360LA, JEOL Ltd., Tokyo, Japan) (Fig. 4). The dimensions of the four kinds of the micro ridges of PDMS were measured with a laser microscope. The morphology along the transverse lines of ridges was traced (Fig. 5).

Another disk of PDMS, which has a donut shape (50 mm outer diameter, 5 mm thickness) with a hole of 33 mm diameter, was made for the peripheral wall of the dish (Fig. 6). These two disks are contacted with the affinity between them, and make a culture area of 8.5 cm^3 at the bottom. The cap for the dish of PDMS was made of PDMS. The dimension of the diameter and the thickness are 50 mm and the 0.5 mm, respectively. The donut shape wall and the cap are for multiple use by sterilization in the autoclave.

The culture dish of PDMS was exposed to the oxygen gas of 30 milliliter per minute at power of 50 W for one minute in a compact etcher (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. The contact angles were measured between the disk of PDMS and the ultrapure water at 298 K by the contact angle analyzer (Phoenix-300, Meiwafofos Co., Ltd., Tokyo, Japan), before and after the oxygen plasma ashing (Fig. 7).

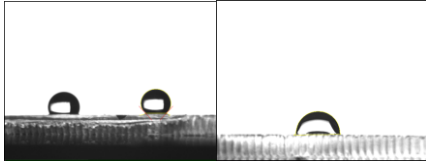


Fig. 7a: Contact angle of water on smooth surface (left), on micro pattern (middle and right), before oxygen plasma ashing. Perpendicular (left and middle) and parallel (right) view to lines of ridge.

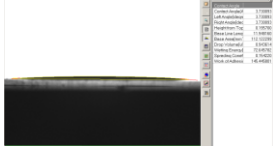


Fig. 7b: Contact angle of water on PDMS, after oxygen plasma ashing.

Cell Culture

Five 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), Neuro-2a (a mouse neural crest-derived cell line), 3T3-L1 (mouse fat precursor cells, cell line of 3T3 mouse), and HUVEC (human umbilical vein endothelial cells). The passage between the fourth and the eighth of cell line was used in the experiment.

D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (decomplemented fetal bovine serum) and 1% penicillin/ streptomycin (GIBCO, Life Technologies Japan Ltd., Tokyo, Japan) was used for the culture medium of C2C12, and 3T3-L1. HUVEC was cultured in EBM-2 (Endothelial Cell Basal Medium, Lonza) containing EGM-2 (Endothelial Cell Growth Medium, Lonza) and 2% decomplemented FBS (fetal bovine serum).

Cells were seeded on the dish of PDMS at the density of 2000 cells/cm². The culture dish was kept in the small chamber of the incubator placed on the stage of the microscope to maintain both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent. The cells were continuously observed with the phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture (Fig. 6).

The behavior of cells was successively recorded with the time-lapse microscopic pictures: deformation, pseudo extension, migration, exfoliation, re-adhesion, and proliferation. The interval of the time-lapse was selected to be two minutes for 24 hours. On the microscopic image, the outline of each cell was traced, and the contour of each cell was approximated to ellipsoid (by Image J). The length of the major axis (A), the minor axis (B), and the area (S) were measured. The ratio of axes is calculated as the shape index (X) by Eq. 1. X approaches unity at rounded cells, and zero at elongated cells. The extension is calculated as the extension ratio (E) by Eq. 2.

$$X = B / A \quad (1)$$

$$E = A / S^{1/2} \quad (2)$$

At the end of the experiment, the micro morphology of the cell with the micro ridges was also observed by a scanning electron

microscope. After fixation of cells by immersion for twenty minutes in 4% paraformaldehyde solution, cells were dehydrated by ethanol. Then, the sample was dried at critical point by JCPD-5 (JEOL Ltd., Japan).

3. RESULTS

The tracing of the surface morphology across the lines of grooves on the mold measured by the laser microscope is exemplified in Fig. 3. The tracing shows the height of 0.0009 mm, and width of 0.0028 mm. The tracing shows also the variation on the interval: 0.0010 mm, 0.0032 mm 0.0052 mm, and 0.0072 mm. The scanning electron microscope images of the lines of ridges are exemplified in Fig. 4. Fig. 4 shows the border of four kinds of ridges. The tracing of the surface morphology across the lines of ridges of PDMS measured by the laser microscope is exemplified in Fig. 5. The tracing shows the height of 0.0009 mm, and width of 0.0025 mm. The tracing shows also the variation on the interval: 0.0012 mm, 0.0033 mm 0.0052 mm, and 0.0074 mm. Fig. 7a shows the contact angle of water on PDMS before oxygen plasma ashing. The angle on the micro pattern in the direction perpendicular to the line of the ridges is 2.3 rad, which is bigger than 2.0 degree on the flat surface. The angle is 1.6 rad, on the other hand, on the micro pattern in the longitudinal direction of the line of the ridges. Fig. 7b shows the contact angle of 0.07 rad, after oxygen plasma ashing. The property of the surface changed from hydrophobic to hydrophilic by the oxygen plasma ashing.

Fig. 8 shows C2C12. Most of cells extend along the lines of ridges. Fig. 9 shows L929. L929 bridges between ridges. Fig. 10 shows Neuro-2a. The most of cell are rounded on the micro pattern, although several cells extend the neurite on the smooth surface. Fig. 11 shows 3T3-L1. Most of cells extend along the lines of ridges, although cell extends to random direction on the smooth surface. Fig. 12 shows HUVEC. HUVEC is rounded on the micro pattern at SEM image, although extension of cell observed at time lapse image. Fig. 13 shows the extension ratio E (Eq. 2) on the micro pattern. C2C12 extends at the ridges of $0.001 \text{ mm} < I < 0.004 \text{ mm}$. The extension ratios E of L929, Neuro-2a, 3T3-L1, and HUVEC do not depend on ridges. Fig. 14 shows the shape index (X). X of C2C12 decreases on the ridge of $I = 0.001 \text{ mm}$. The shape index of L929, Neuro-2a, 3T3-L1, and HUVEC do not depend on ridges.

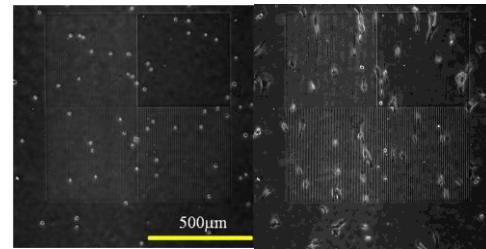
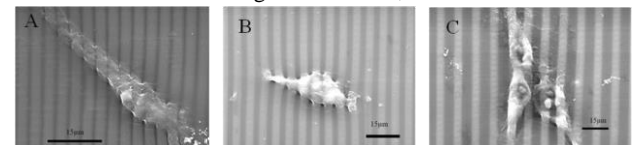


Fig. 8a: C2C12 immediately after seeding (left) and cultured for 24 hours (right): interval $I = 0.001 \text{ mm}$ (upper right), 0.003 mm (upper left), 0.005 mm (lower left), 0.007 mm (lower right). Dimension from left to right is 0.13 mm, each.



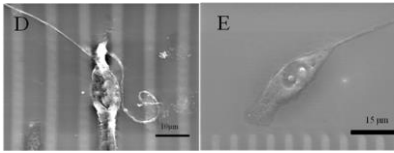


Fig. 8b: SEM image of C2C12 cultured on ridges for 24 hours: Interval $I = 0.001$ mm (A), 0.003 mm (B), 0.005 mm (C), 0.007 mm (D), smooth (E).

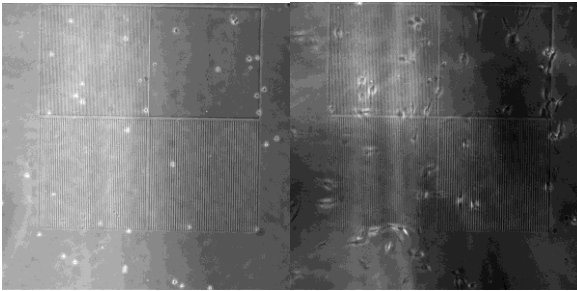


Fig. 9a: L929 immediately after seeding (left) and cultured for 24 hours (right): interval $I = 0.001$ mm (upper right), 0.003 mm (upper left), 0.005 mm (lower left), 0.007 mm (lower right), dimension from left to right is 0.13 mm, each.

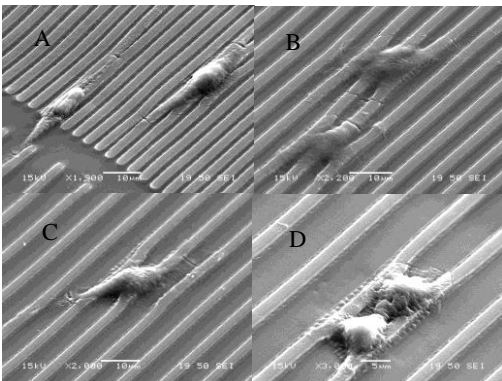


Fig. 9b: SEM image of L929 cultured on ridges for 24 hours: Interval $I = 0.001$ mm (A), 0.003 mm (B), 0.005 mm (C), 0.007 mm (D).

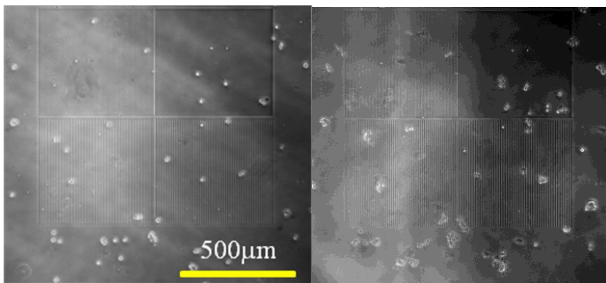


Fig. 10a: Neuro-2a immediately after seeding (left) and cultured for 24 hours (right): interval $I = 0.001$ mm (upper right), 0.003 mm (upper left), 0.005 mm (lower left), 0.007 mm (lower right). Dimension from left to right is 0.13 mm, each.

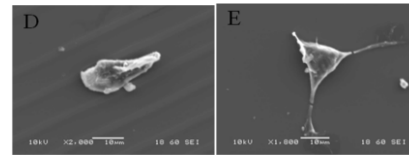
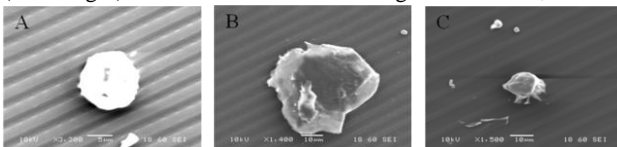


Fig. 10b: SEM image of Neuro-2a cultured on ridges for 24 hours: Interval $I = 0.001$ mm (A), 0.003 mm (B), 0.005 mm (C), 0.007 mm (D), smooth (E).

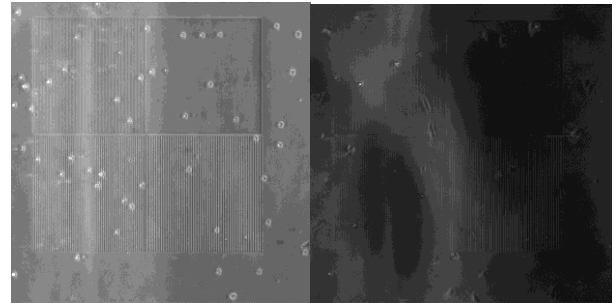


Fig. 11a: 3T3-L1 immediately after seeding (left) and cultured for 24 hours (right): interval $I = 0.001$ mm (upper right), 0.003 mm (upper left), 0.005 mm (lower left), 0.007 mm (lower right). Dimension from left to right is 0.13 mm, each.

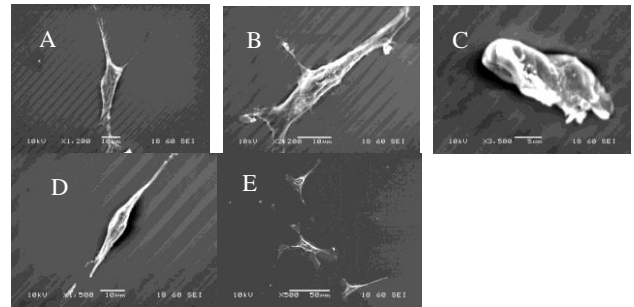


Fig. 11b: SEM image of 3T3-L1 cultured on ridges for 24 hours: Interval $I = 0.001$ mm (A), 0.003 mm (B), 0.005 mm (C), 0.007 mm (D), smooth (E).

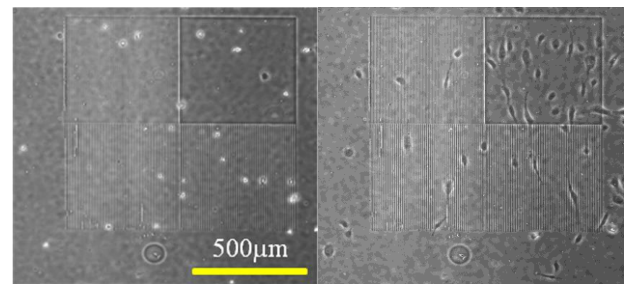
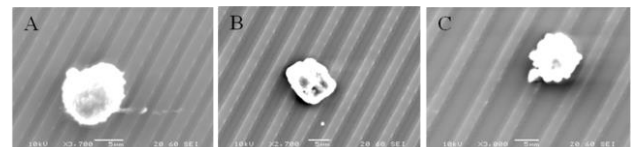


Fig. 12a: HUVEC immediately after seeding (left) and cultured for 24 hours (right): interval $I = 0.001$ mm (upper right), 0.003 mm (upper left), 0.005 mm (lower left), 0.007 mm (lower right). Dimension from left to right is 0.13 mm, each.



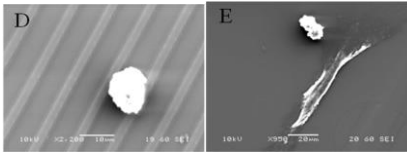


Fig. 12b: SEM image of HUVEC cultured on ridges for 24 hours: Interval $I = 0.001$ mm (A), 0.003 mm (B), 0.005 mm (C), 0.007 mm (D), smooth (E).

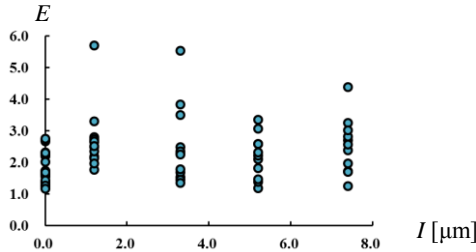


Fig. 13a: Extension ratio (E) vs. interval (I) [μm]: C2C12.

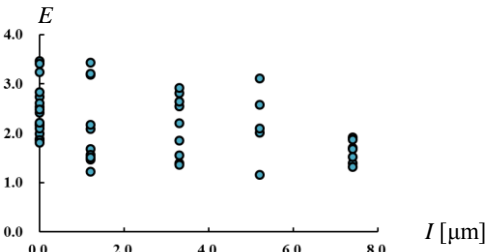


Fig. 13b: Extension ratio (E) vs. interval (I) [μm]: L929.

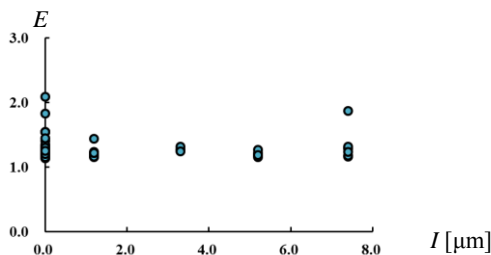


Fig. 13c: Extension ratio (E) vs. interval (I) [μm]: Neuro-2a.

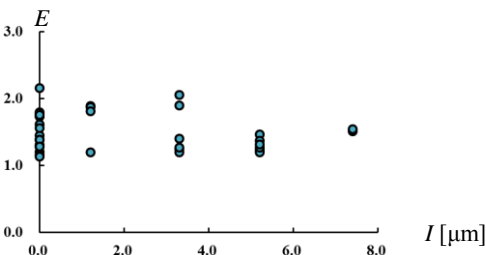


Fig. 13d: Extension ratio (E) vs. interval (I) [μm]: 3T3-L1.

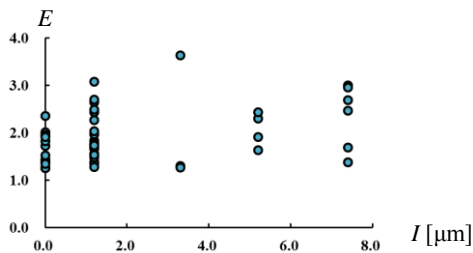


Fig. 13e: Extension ratio (E) vs. interval (I) [μm]: HUVEC.

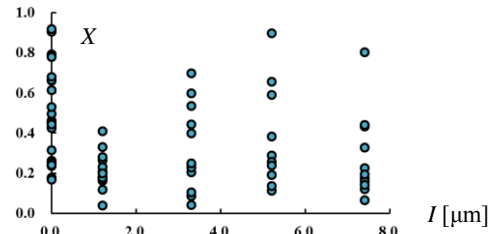


Fig. 14a: Shape index (X) vs. interval (I) [μm]: C2C12.

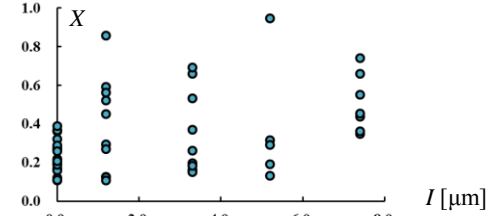


Fig. 14b: Shape index (X) vs. interval (I) [μm]: L929.

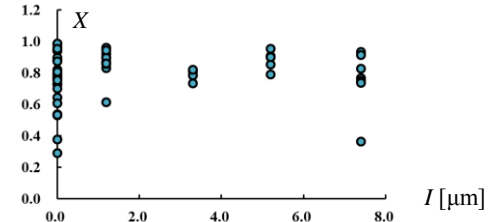


Fig. 14c: Shape index (X) vs. interval (I) [μm]: Neuro-2a.

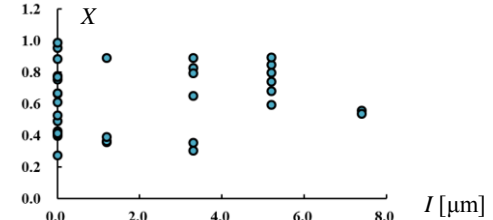


Fig. 14d: Shape index (X) vs. interval (I) [μm]: 3T3-L1.

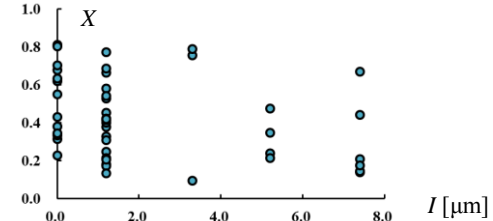


Fig. 14e: Shape index (X) vs. interval (I) [μm]: HUVEC.

4. DISCUSSION

The dimension of 0.003 mm is selected for the width of the ridge, because the value is effective to make orientation of C2C12 in the previous study [2]. The dimension of 0.001 mm is selected for the height of the ridge, because the value between 0.0005 mm and 0.002 mm is effective to make orientation of C2C12 [1]. The dimension of interval of the ridge is selected smaller value compared to the diameter of cells in the present study. The height of the ridge is 0.001 mm, which is low enough for a cell to get over. The conventional way uses pattern with the higher wall to control orientation of cells *in vitro*. If the surface micro morphology is effective to control the orientation of cells, the variation of methodology would be extended.

The behavior of a cell might depend on the adhesion of the cell to the scaffold. The adhesive property varies with the materials of the scaffold [7]. In the present study, the surface of PDMS is modified with oxygen ashing. Although the diameter of the droplet of distilled water is bigger than the interval of the lines of the ridges, the contact angle shows asymmetrical properties of the surface with ridges (Fig. 7a). Asymmetry on the hydrophilic property might govern the extension of the cell.

Some parts of the ridge are destroyed near the cell at SEM image (Figs. 8-12). The part might be destroyed by the cell, or by the treatment for microscopic observation.

5. CONCLUSION

The effect of micro ridges of the scaffold on deformation of cells has been studied *in vitro*. Several parallel lines of micro ridges have been made on a disk of transparent polydimethylsiloxane. The height, the width, and the length of the rectangular ridge are 0.001 mm, 0.003 mm, and 0.5 mm, respectively. Variation has been made on the interval of the ridge: 0.001 mm, 0.003 mm, 0.005 mm and 0.007 mm. Five kinds of cells were used in the test. C2C12 (mouse myoblast cell line), L929 (fibroblast connective tissue of mouse), Neuro-2a (a mouse neural cell line), 3T3-L1 (mouse fat precursor cells), and HUVEC (human umbilical vein endothelial cells). The experimental results show that C2C12 lengthen along the line of ridge of 0.003 mm interval. The effects of the micro pattern depend on the kind of cells.

6. ACKNOWLEDGMENT

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