Effect of Aspect Ratio of Checkered Convexo-concave Micro-pattern on Orientation of Cultured Single Cell

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ABSTRACT

The effect of the aspect ratio of checkered convexo-concave micro-pattern on orientation of the cultured single cell has been studied *in vitro*. The checkered convexo-concave pattern has been designed with micro quadrangular prisms in the square area of 1 mm × 1 mm on a disk of glass for a scaffold by the photolithography technique. Each prism has the following dimension: 0.01 mm length, and 0.0007 mm height. Variation has been made on the width of the prism: 0.005 mm, 0.008 mm, and 0.01 mm. The variation of the width makes the variation on the aspect ratio of the top rectangular surface of the prism: 1, 1.25, and 2. C2C12 (mouse myoblast cell line) were seeded on the micro pattern, and incubated for 24 hours in the Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and 1% penicillin/ streptomycin. The cells were observed with a phase contrast microscope. The experimental results show that the orientation of myoblast can be controlled by the aspect ratio of the checkered micro convexo-concave pattern of the surface of the scaffold.

Keywords: Biomedical Engineering, Cell Culture, Checkered Micro pattern and Orientation.

1. INTRODUCTION

A biological cell adheres, migrates, rotates, and deforms on the scaffold. These behaviors of the cell depend on the micro morphology of the scaffold [1-13]. The cell might be sensitive to the morphology of the similar dimension to itself at the scaffold. The photolithography technique is available to make the micro patterns on the scaffold of the cell culture. The previous study showed that the orientation of myoblast depends on the height of the micro ridges [1].

In most of the previous studies, cells were forced to make orientation along the space between walls. In the case of parallel micro bands, cells just trace the direction of lines. To investigate the sensitivity of a biological cell against the dimension, variation has been made on the aspect ratio of checkered micro pattern of the scaffold [2].

Several methodologies have been clinically applied to regenerative medicine. The acceleration technique for proliferation, orientation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro*. The behavior of a cell depends on several factors: mechanical [14-19], electrical [20-22], and magnetic stimulations [23]. The effect of stimulations on the cell varies with the kind of cells. Control methodology for proliferation, orientation and differentiation of cells would be applied to the regenerative tissue technology.

In the present study, the effect of the aspect ratio of checkered convexo-concave micro-pattern on orientation of the cultured single cell has been studied *in vitro*.

2. METHODS

Micro Pattern

The checkered convexo-concave pattern has been designed with micro quadrangular prisms at the nine square areas (1 mm × 1 mm each) on a disk of glass for a scaffold by the lithography technique (Fig. 1). Each prism has the following dimension. The height of the prism is 0.0007 mm. The length of the top rectangular surface is 0.01 mm length. Variation has been made on the width of the top square of the prism: 0.005 mm, 0.008 mm, and 0.01 mm. The variation of the width makes the variation on the aspect ratio R of the top rectangular surface of the prism: $R = 1$, $R = 1.25$, and $R = 2$. The arctangent of the each ratio R is 45, 51, and 63 degree, respectively.

Each pattern is drawn in the square area of 0.5 mm × 0.5 mm, which is the quarter part of the square area of 1.0 mm × 1.0 mm. The square area is surrounded by a smooth surface without pattern as control. Photomasks were used to trace the micro checkered pattern on the mold.

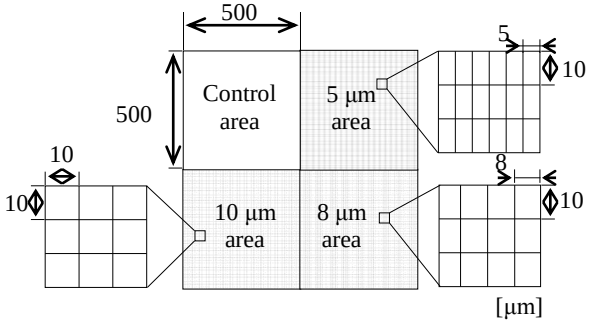

Fig. 1: Three kinds of checkered micro-pattern: aspect ratio of top rectangular surface of prism: 1 (lower left), 1.25 (lower right), 2 (upper right), and flat (upper left): mirrored pattern in Fig. 3, 5, 7, 8.

Photomask

The borosilicate glass (Tempax) disk (35 mm diameter, 1mm thickness) was used for the base of the photomask (Fig. 2). After the surface of the glass disk was cleaned by the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing (100 W, for ten minutes) in the reactive ion etching system (FA-1, Samco Inc., Kyoto, Japan), titanium was deposited on the surface with 150 nm thickness by the sputtering equipment of L-210S-FH (Canon Anelva Corporation) for eight minutes. The surface of the glass disk was cleaned again by the oxygen plasma ashing for ten minutes in the reactive ion etching system (FA-1). To improve affinity between titanium and photoresist material, HMDS (hexamethyldisilazane: Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) was coated on the disk at 3000 rpm for 30 s with a spin coater. The positive photoresist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd, Kawasaki, Japan) was coated on the titanium at 7000 rpm for 30 s with the spin coater. The photoresist was baked in the oven (DX401, Yamato Scientific Co., Ltd) at 368 K for three minutes.

The checkered pattern was drawn on the mask with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the pattern on the photomask with the laser drawing system, the parameters were selected as follows: the voltage of 2.3 V, the velocity of 0.08 mm/s, the acceleration of 0.5 mm/s^2. The photoresist was baked again in the oven at 368 K for ten minutes. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for ten minutes, rinsed with the distilled water, and dried by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan).

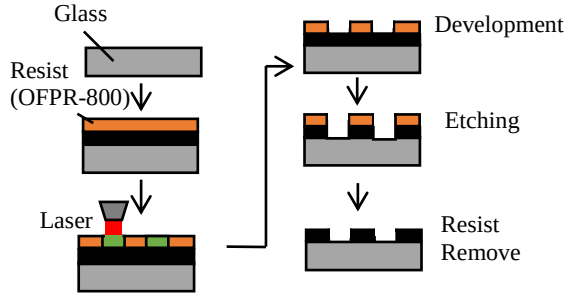

Fig. 2: Photomask by photolithography.

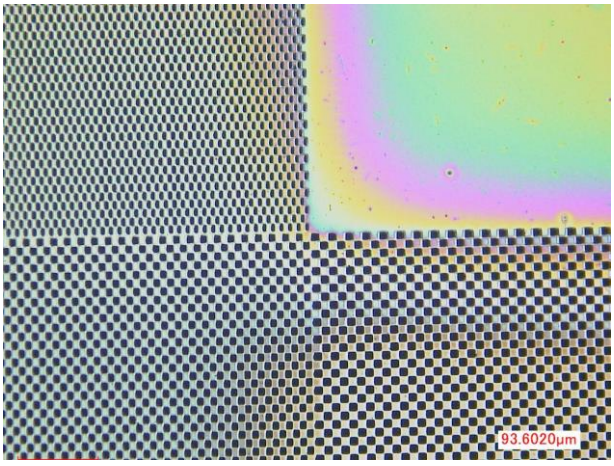

Fig. 3: Photomask after etching: dimension from left to right is 0.7 mm.

The titanium was etched with the plasma gas using a reactive ion etching system (RIE-10NR, Samuco Inc., 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. To remove the residual layer of OFPR-800LB, the mask was dipped in acetone for two minutes, rinsed with the distilled water, and dried by the spin-dryer (Fig. 3).

Mold

The borosilicate glass (Tempax) disk (35 mm diameter, 1mm thickness) was used for the base of mold for micro quadrangular prisms, after cleaning and hydrophilization (Fig. 4). The negative photoresist material of low viscosity (SU-8 2: Micro Chem Corp., MA, USA) was coated on the glass at 7000 rpm for one minute with the spin coater. The photoresist baked in the oven at 368 K for five minutes.

The photomask was adhered on the surface of SU-8 2, and the photoresist was exposed to the UV light through the photomask in the mask aligner (M-1S, Mikasa Co. Ltd., Japan) for 8 s. The photoresist baked in the oven at 368 K for ten minutes. The photoresist was developed with SU-8 developer (Nippon Kayaku Co., Ltd, Tokyo, Japan) for six minutes to make micro prisms. The glass with the micro pattern was rinsed with isopropyl alcohol for one minutes, and dried by the spin-dryer (Fig. 5).

Nine unites of the micro patterns were made at the interval of 2 mm at the bottom of the culture dish (Fig. 6). The morphology of the surface of the mold was measured by the stylus of the contact profilometer (Dektak XT-E, Bruker Corporation) (Fig. 10).

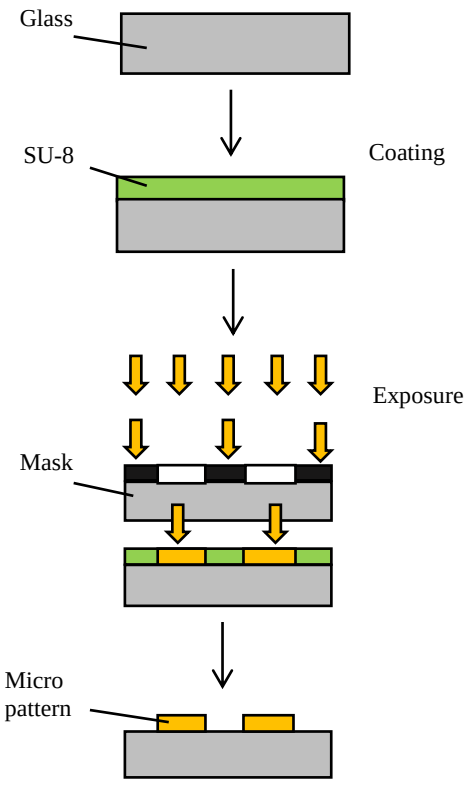

Fig. 4: Micro pattern on mold by photolithography.

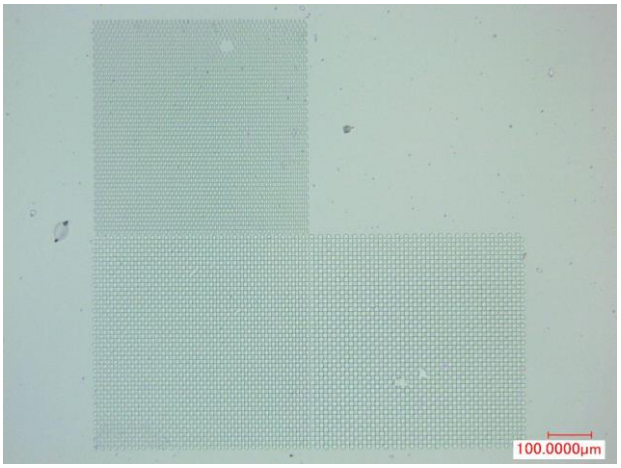

Fig. 5: Mold of micro pattern by photomask.

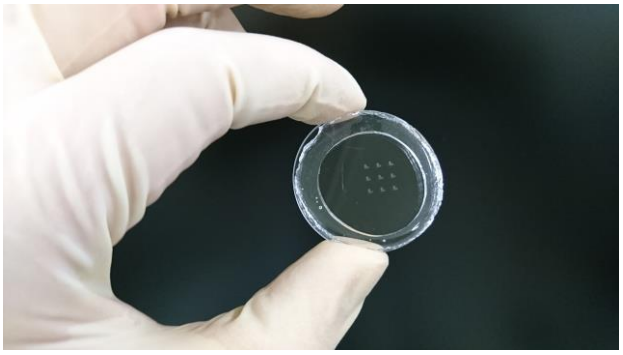

Fig. 6: Culture dish (35 mm diameter) with micro patterns.

Scaffold with Micro Pattern

After the disk of Tempax glass was enclosed with a peripheral wall of polyimide, degassed 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). The volume ratio of PDMS to curing agent is ten to one. PDMS was baked at 373 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd).

The morphology of the surface of the scaffold was observed by a scanning electron microscope (SEM, JSM6380LD, JEOL Ltd., Tokyo, Japan), after the cell culture (Fig. 11).

A disk of polydimethylsiloxane (PDMS), which has a donut shape (35 mm outer diameter, 3 mm thickness) with a hole of 20 mm diameter (culture area of 3.1 cm^2), was made for the peripheral wall of the dish (Fig. 6).

The culture plate was exposed to the oxygen gas in a reactive ion etching system (FA-1, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing).

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was seeded on the micro pattern at the density of 2000 cells/cm^2, and incubated (24 hours) in the Dulbecco's Modified Eagle Medium. The medium contains 10% fetal bovine serum and 1% penicillin/ streptomycin.

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 continuously observed with the phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture (Fig. 7).

In order to improve the contrast of the image of the contour of each cell, the cells were fixed and dried after the incubation for 24 hours. Cells were fixed with 4% paraformaldehyde for 20 minutes. In order to dehydrate the sample, the ethanol concentration was exchanged in the order of 50%, 70%, 80%, 90%, 95%, 99.5% for 3 minutes each (Fig. 8).

On the microscopic image, the contour of each cell was traced, and approximated to ellipsoid (Fig. 9). The angle (0 degree < θ < 90 degree) between the longitudinal axis of each ellipsoid and the shorter side of the square of the checkered pattern was measured. The angle of zero degree indicates direction to shorter side of the rectangle of the checkered pattern.

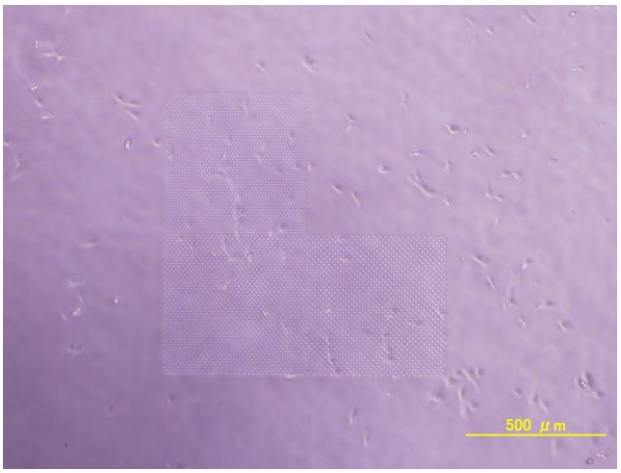

Fig. 7: C2C12 cultured on micro pattern for 24 hours: dimension from left to right is 2 mm.

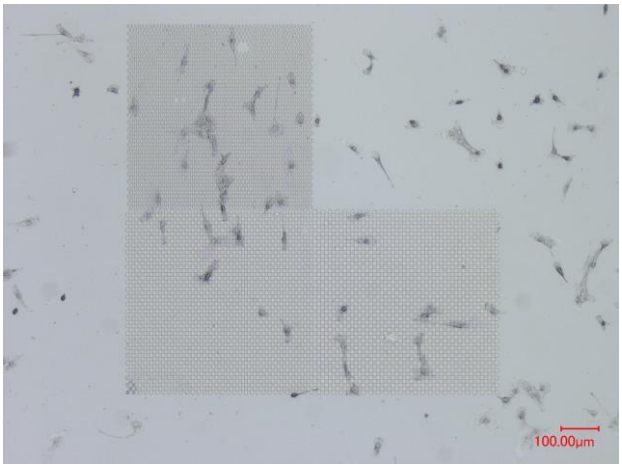

Fig. 8: C2C12 image after fixation of Fig.7: dimension from left to right is 2 mm.

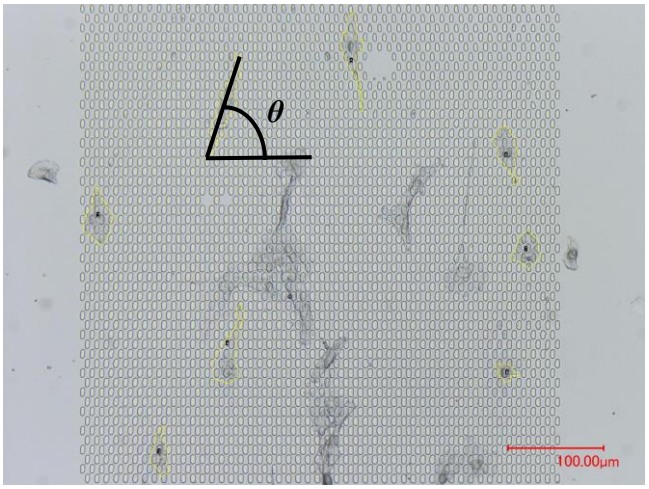

Fig. 9: Contour of each cell was traced: dimension from left to right is 0.7 mm.

3. RESULTS

Fig. 10 shows morphology of the surface of the mold. The tracings shows the height of 0.0007 mm of the micro pattern on the mold. Fig. 11 exemplifies SEM image of the surface of the scaffold. The figure shows the checkered convexo-concave pattern with micro quadrangular prisms (the top of the square of 0.01 mm × 0.01 mm). C2C12 adheres to the pattern.

Fig. 12 shows the angle θ [degree] between longitudinal axis of each cell of ellipsoid and shorter side of square of checkered pattern: R = 2 (Fig. 12a), R = 1.25 (Fig. 12b), R = 1 (Fig. 12c), and control (Fig. 12d). Data are arranged in ascending order in Fig. 12a. Data will make flat section at the large frequency. Data make straight line in Fig. 12d, which shows random distribution of angles. Data on a line of small inclination around 60 degrees (Fig.12b) show the high probability distribution around 60 degrees.

Fig. 13 shows the mean value at each area of R. Each bar shows the standard deviation. The mean value of θ is 45 degree at the random distribution between 0 degree and 90 degree. The angle shows random distribution in the area of R = 1 and control (without micro pattern). The mean value of θ approaches to the value of the arctangent of R, in the area of R = 2 and R = 1.25.

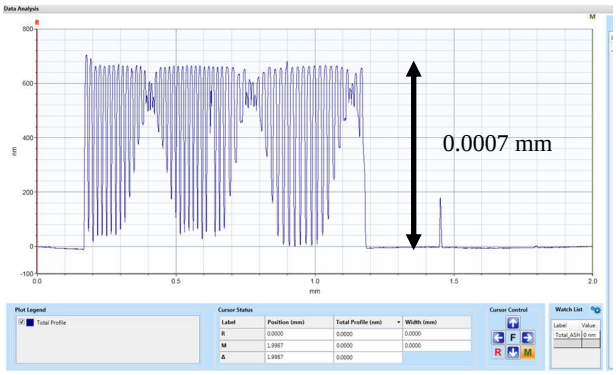

Fig. 10: Tracing (height [nm] vs. distance [mm]) measured by stylus on surface of mold.

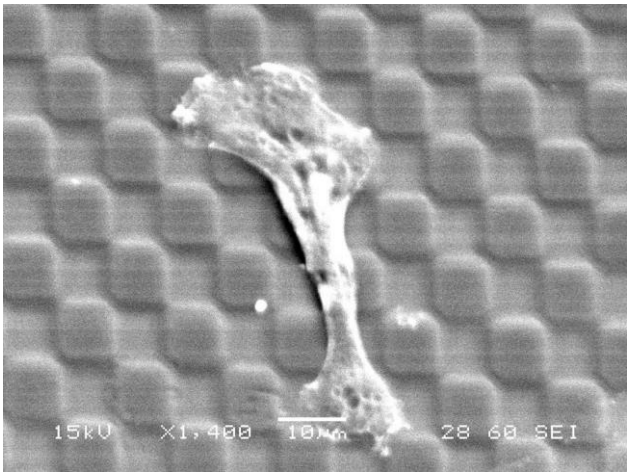

Fig. 11: SEM image of surface of scaffold with checkered micro-pattern: dimension from left to right is 0.1 mm. C2C12 adheres to pattern.

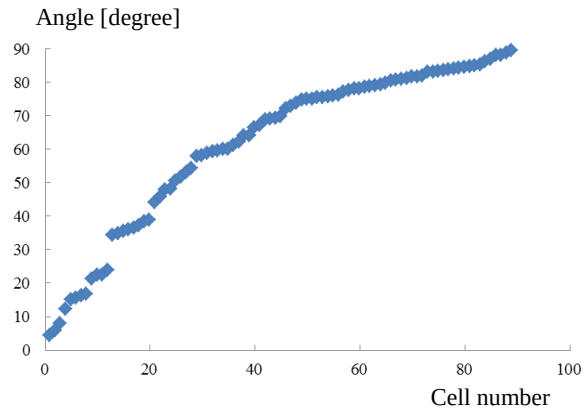

Fig. 12a: Angle [degree] between longitudinal axis of each cell of ellipsoid and shorter side of square of checkered pattern: R = 2 (88 cells).

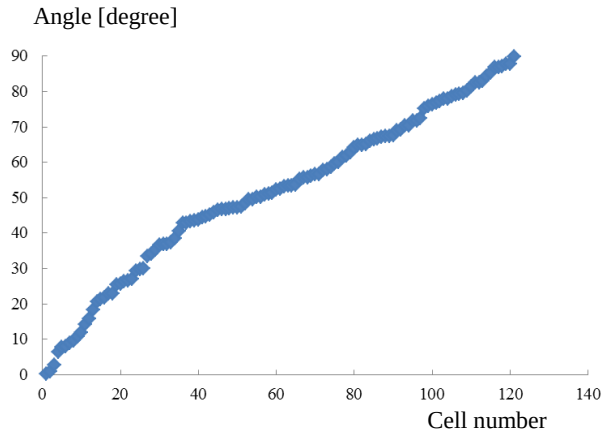

Fig. 12b: Angle [degree] between longitudinal axis of each cell of ellipsoid and shorter side of square of checkered pattern: R = 1.25 (121 cells).

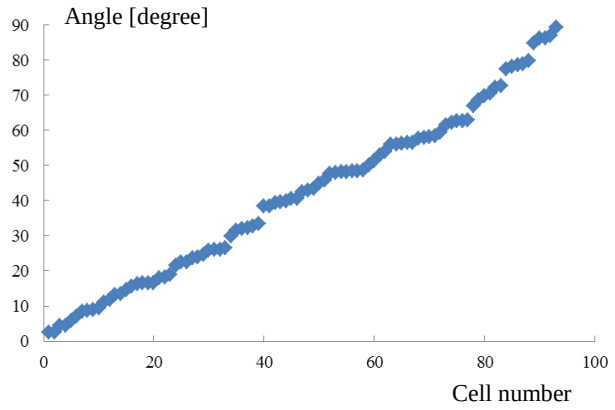

Fig. 12c: Angle [degree] between longitudinal axis of each cell of ellipsoid and shorter side of square of checkered pattern: R = 1 (93 cells).

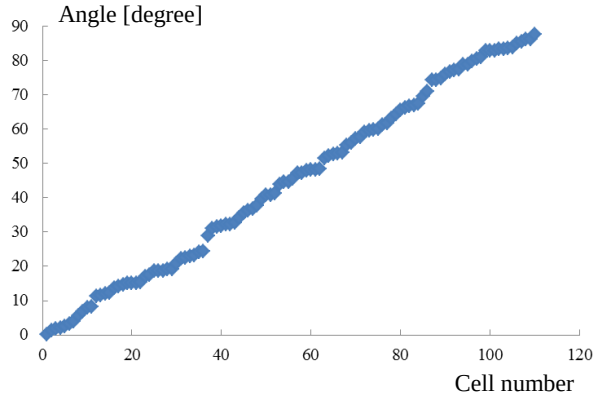

Fig. 12d: Angle [degree] between longitudinal axis of each cell of ellipsoid and shorter side of square of checkered pattern: control (without micro pattern) (110 cells).

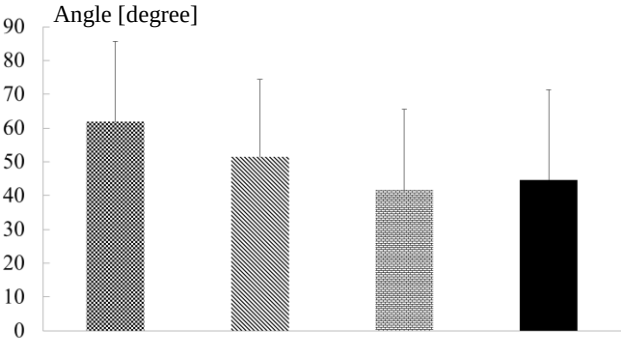

Fig. 13: Angle [degree] between longitudinal axis of each cell of ellipsoid and shorter side of square of checkered pattern. From left to right: R = 2, R = 1.25, R = 1, and control. Mean (column) ± SD (bar).

4. DISCUSSION

The control technique of orientation of cells might be applied to the alternative system to the animal experiment for tissue technology: making the engineered pseudo-environment, or the partial biological tissue *in vitro*.

The biological cell is sensitive to the property of the surface of the scaffold. The hydrophilic property of the scaffold on the scaffold in the present study is confirmed by the very small contact angle.

In the previous studies, cells were cultured on various morphological pattern of scaffold. In the conventional preparation, the morphology of the scaffold surface can be controlled with the dimension of sub-millimeter. The biological cell might be sensitive to the morphology of the scaffold surface at the dimension, which is smaller than the diameter of the cell. In the present study, the dimension of sub-micrometer at the morphology of the scaffold surface has been controlled by the photolithography technique.

The previous study shows that the orientation of myotubes can be controlled by the aspect ratio of the checkered micro convexo-concave pattern of the surface of the scaffold [2].

The contrast of the image of each cell has been improved by sample fixation. The contour of each cell can be easily traced without deformation artifact of the cell (Figs. 7&8).

The myoblast might make orientation along the longitudinal direction of the rectangular top surface of the micro quadrangular prisms. C2C12 migrates and extends along the longitudinal axis of the ridges [3].

On the pattern of 0.005 mm height, observation of cells was not easy because of the light scattering through the micro pattern in the previous study [2]. The height of the prism of 0.0007 mm has been selected as the minimum height to make orientation of a single cell in the previous study.

The effect of the height of micro ridges on the orientation of C2C12 was studied in the previous study [1]. The experimental results show that myoblasts adhere on the top of the ridge and align to the longitudinal direction of the micro ridges higher than 0.0007 mm.

The orientation in the surrounding area might follow that of the rectangular pattern. Myotube has tendency to make orientation following direction of neighbor myotube [16]. The behavior of a cell depends on that of the neighbor cell. The behavior of a single cell has been investigated in the present study. The orientation of the single cell might accelerate subsequent orientation of cells in the tissue.

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. The previous study shows that electrical stimulation enhances differentiation of muscle cells [20-22]. The previous studies show that a mechanical field, on the other hand, governs behavior of cells. The shear flow governs the orientation of endothelial cells [14-18]. Too strong mechanical stimulation damages cells. The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells. The mechanical stimulation can decrease proliferation of cells. The mechanical stress also exfoliates several cells, which makes vacancy around the adhered cell. The differentiation might be optimization of cells to the changing environment. The mechanical stress can accelerate differentiation of C2C12 into myotubes.

5. CONCLUSION

The checkered convexo-concave pattern has been designed, and the effect of the aspect ratio of each micro quadrangular prism (0.01 mm length, and 0.0007 mm height) on orientation of the cultured single cell has been studied *in vitro*. The micro pattern for the scaffold has been successfully made by the photolithography technique. Variation has been made on the width of the prism: 0.005 mm, 0.008 mm, and 0.01 mm, which makes the variation on the aspect ratio of the top rectangular surface of the prism: 1, 1.25, and 2. C2C12 (mouse myoblast cell line) were seeded on the micro pattern, and incubated for 24 hours. The experimental results show that the orientation of myoblast can be controlled by the aspect ratio of the checkered micro convexo-concave pattern of the surface of the scaffold.

6. ACKNOWLEDGMENT

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