Tracings of Orientation of Cell on Scaffold with Micro Striped Pattern after Stimulation of Vertical Excess Gravity

Takuya TAMURA, Shigehiro HASHIMOTO, Haruka HINO, Tsubasa ITO, Yuji ENDO

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

ABSTRACT

The orientation of a single cell on the scaffold with the micro striped pattern after stimulation of the vertical excess gravity has been traced *in vitro*. The micro striped pattern (3 μm width, 3 μm interval, and 0.2 μm < height < 0.4 μm) was made on the PDMS (polydimethylsiloxane) plate of the scaffold by the photolithography technique. C2C12 (mouse myoblast cell line) was used in the test. To apply the normal mechanical force field (< 100 G) to the cells adhered on the scaffold of the plate, the plate was set at the bottom of the culture dish in the swing rotor in a conventional centrifugal machine placed in an incubator (310 K, 5% CO_2). After the centrifugation for several (1, 2, or 5) hours, the behavior of each cell was traced at the time lapse images for 12 hours. For the control study, cells were cultured on the scaffold of PDMS with the same micro pattern. The experiments show that the orientation along the micro striped pattern is strengthened by the vertical excess gravity and the effect is maintained for several hours after stopping of the excess gravity.

Keywords: Biomedical Engineering, Cell Culture, Excess Gravity, Micro Pattern and C2C12.

1. INTRODUCTION

A biological cell shows passive and active behaviors in an environment. The cell is sensitive to the mechanical stimulation, and shows several responses: deformation, and migration. While the shear stress deforms the cell, the cell deforms by itself to adapt to the shear field. The cell has compliance, and is deformed by force. The cell deforms, on the other hand, to minimize the intra force. The cell is moved by the force. The cell moves in response to the force.

While the strong stimulation above the threshold damages the cell, the stimulation below the threshold might remain in the cell as a memory for the response in the next step. The muscle tissue might decrease in the micro gravitational field [1-6]. The muscle tissue might increase, on the other hand, in the hypergravity [7-17]. The previous study shows that the excess gravitational field thickens the myotubes *in vitro* [13].

The acceleration technique for orientation, proliferation and differentiation of cells has been studied to make tissue *in vivo* or *in vitro*. Control methodology for orientation, proliferation and differentiation of cells would be applied to the regenerative tissue technology [5-29].

In the present study, the effect of mechanical field on orientation and deformation of cells has been studied by centrifuge *in vitro*, and the orientation of each cell on the scaffold with the micro striped pattern after stimulation of the vertical excess gravity has been traced *in vitro*.

2. METHODS

Micro Striped Pattern

The micro striped pattern has been made in three partial areas (1 mm square each) on the PDMS (polydimethylsiloxane) plate of the scaffold by photolithography technique (Fig. 1). Both the width (*W*) and the interval (*I*) of the rectangular ridges are 3 μm. Variation has been made on the height (*H*) of the ridges. Each area has its own constant height: 0.2 μm, 0.3 μm, or 0.4 μm.

Mold for Micro Pattern

The borosilicate glass (Tempax) disk was used for the base of the mold through micromachining process (Fig. 2). The diameter and the thickness of the disk are 50 mm and 1.1 mm, respectively. To remove micro particles on the surface of the glass, the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1, Samco Inc., Kyoto, Japan).

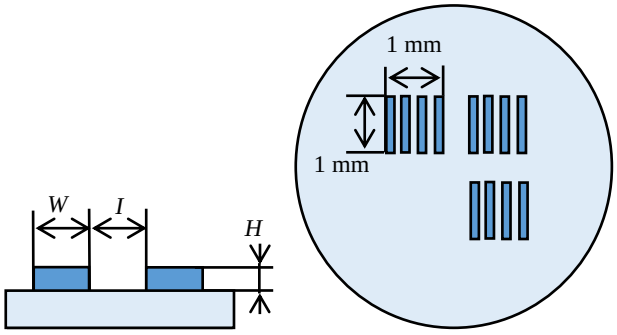

Fig. 1: Dimension of micro pattern (at cross section, left) in three areas on scaffold (right).

To improve affinity to photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the glass plate at 3000 rpm for 30 s with a spin coater. The positive photoresist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the glass with the spin coater (at 3000 rpm for 20 s). The photoresist was baked in the oven (DX401, Yamato Scientific Co., Ltd) at 373 K for ninety seconds.

The pattern for the slit was drawn on the mold with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). The pattern was baked in the oven at 373 K for five minutes.

The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for two minute, rinsed with the ultrapure water for three minutes twice, and dried by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan).

The glass plate with the photoresist material was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan). For etching, the gas of CF_4 (30 cm^3/min at 1013 hPa) was applied at 100 W at 2 Pa. The duration time was varied between four and nine minutes, according to the depth of the micro groove. Each area was separately etched by covering the other area by the glass plate.

To remove the residual OFPR-800LB on the surface of the glass, the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1).centrifuge

After the mold of the glass disk 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 338 K for one hour in an oven (DX401). The baked plate of PDMS (1 mm thickness) was exfoliated from the mold of the slide glass plate. The PDMS plate was cut to make a disk of 25 mm diameter, and stacked on the bottom of the culture dish (without coating of collagen) of 35 mm diameter. The surface of the PDMS plate was exposed to the oxygen gas (0.1 Pa, 30 cm^3/min) in the reactive ion etching system (FA-1: oxygen plasma ashing, 50 W) for thirty seconds just before cell culture.

Hyper Gravitational Force Field

The hyper-gravitational force was applied vertically on the scaffold plane with the micro striped pattern by the centrifugal force (Fig. 3). A swing rotor has been used to apply the vertical force to cultured cells (Fig. 4). The normal force at the bottom surface of the culture dish was applied by the centrifuge. The centrifugal force (F_c) is calculated by Eq. 1.

$$F_c = m\, r\, \omega^2 \quad (1)$$

In Eq. 1, m is mass, r is radius of the rotation, and ω is angular velocity. In the gravitational field, gravitational force (F_g) is calculated by Eq. 2, where g is gravitational acceleration.

$$F_g = m\, g \quad (2)$$

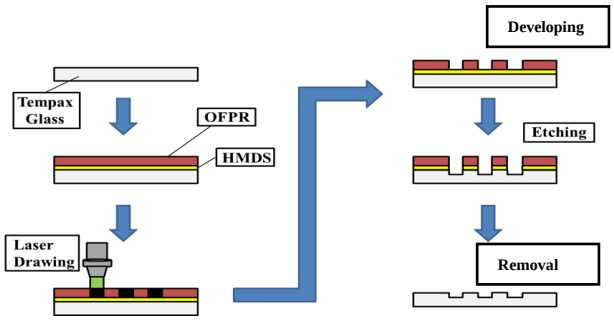

Fig. 2: Photolithography process for mold.

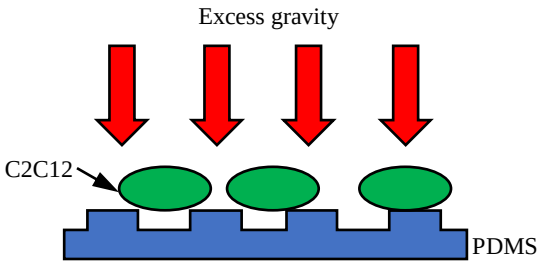

Fig. 3: Excess gravity applied to cell at micro pattern.

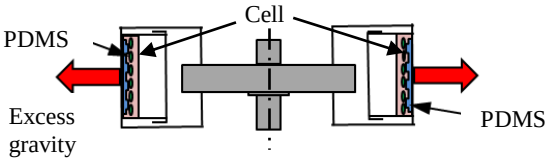

Fig. 4: Swing rotor in centrifugal machine.

Fig. 5: Centrifugal machine (left) in incubator (right).

In the present study, the centrifugal acceleration (< 100 G: 1 G is equal to the gravitational acceleration) is generated with the centrifugal machine (CN-1040, Matsuuraseisakusyo. Ltd, Tokyo, Japan) (Fig. 5). Asymmetrical position of the micro-stripe areas are used for the marker to trace each cell behavior (Fig. 1).

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the experiment. C2C12 of the passage between five and ten was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. The cells were seeded on the micro pattern at the density between 2000 cells/cm^2 and 10000 cells/cm^2.

After the cells were cultured in the incubator (CO_2 5%, 310 K)

for one hour in the static state, excess gravitational stimulation (50 G, or 100 G) was applied for several hours (1, 2, or 5 hours) by centrifuge in the incubator.

To keep the content of carbon dioxide of 5 % at 310 K, the cells were incubated in an incubator through the entire experimental term including the term of exposure to the excess gravitational field. Namely, the centrifugal machine was placed in the incubator during the centrifugation (Fig. 5). In the control group (normal force of 1 G), the cells were cultured without centrifuge on the PDMS plate, which was placed in the polystyrene dish (35 mm diameter) with collagen coating.

Image Analysis

After stimulation of the excess gravity, cells were subsequently cultured in the dish placed in the incubator combined with an inverted phase-contrast microscope (LCV110-SK, Olympus Co., Ltd., Tokyo). The behavior of each cell was analyzed on the time lapse image captured every five minutes for 12 hours after stimulation of the excess gravity. "Image J" was applied to analyze the behavior of each cell. On the microscopic image, the outline of each cell was traced, and the contour of each cell was approximated to ellipsoid (Fig. 6). As to the ellipsoid, the length of the major axis (a), and the minor axis (b) were measured. The ratio of axes is calculated as the shape index (S) by Eq. 3.

$$S = 1 - b / a \quad (3)$$

At the circle, S = 0. As the ellipsoid is elongated, S approaches to unity. The number of cells (Ns), of which S is higher than 0.5, was counted, and the shape index ratio (Rs) is calculated by Eq. 4.

$$Rs = Ns / Nt \quad (4)$$

In Eq. 4, Nt is the total number of cells.

The cell, of which S is higher than 0.5, was selected, and the angle (θ) of the major axis of the cell was measured related to the longitudinal direction of the micro ridge (Fig. 7). When the major axis of the cell aligned to the longitudinal direction of the micro ridge, the angle (θ) is defined as 90 degrees. The number of the oriented cells (No) was counted as the cell, of which the angle (θ) is between –75 degrees and +105 degrees. In the area of each micro-pattern, the orientation ratio (Ro) was calculated by Eq. 5.

$$Ro = No / Nt \quad (5)$$

In Eq. 5, Nt is the total number of cells.

3. RESULTS

Fig. 8a shows the manufactured mold on the borosilicate glass disk, which has micro pattern at the center part of the surface. Fig. 8b exemplifies cells on the micro pattern, which has vertical stripes in Fig. 8b.

In Figs. 9-16, data at zero hour shows the value immediately after stopping the stimulation of the centrifuge. In Figs. 9-11, each bar shows the mean value of nine samples.

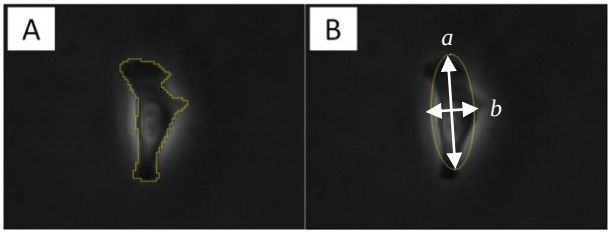

Fig. 6: Outline of each single cell is traced (A), and approximated to ellipsoid (B).

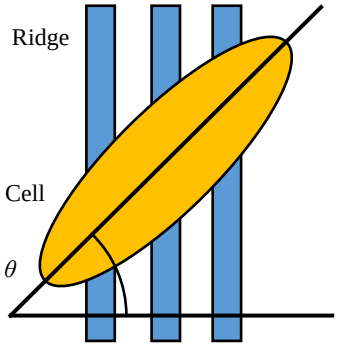

Fig. 7: Angle (θ) of major axis of cell related to longitudinal direction of micro ridge.

Fig. 9 shows the orientation ratio after stopping the excess gravity of 100 G for 1 hour. The orientation ratio is higher in the stimulation group than in the control group (without the centrifuge). Especially at the zero hour, the ratio is approximately 80 % in the stimulation group, while the ratio is zero in the control group. The ratio scatters in the control group during 12 hours after the stimulation of the gravity. Fig. 10 shows the shape index ratio after stopping the excess gravity of 100 G for 1 hour. The ratio is high in the stimulation group at the zero hour. The high ratio is maintained even after 8 hours. Fig. 11 shows the shape index ratio after stopping the excess gravity of 100 G for five hours. The ratio is rather low compared with Figs. 9 and 10. The cells are rounded after five hours of the stimulation of the excess gravity of 100 G.

Fig.12 exemplifies the tracings of the angle (θ) of the major axis of each cell related to the longitudinal direction of the micro ridge in the control group. The value scatters, and asymptotically approaches to 90 degrees after 6 hour. Fig.13 exemplifies the tracings of the angle (θ) of the major axis of each cell related to the longitudinal direction of the micro ridge in the stimulation group of 100 G. After five hours of the stimulation of the excess gravity, the value asymptotically approaches to 90 degrees. Each cell makes orientation along the longitudinal direction of the ridge faster in the stimulation group than in the control group.

Fig.14 exemplifies the tracings of the angle (θ) of the major axis of each cell related to the longitudinal direction of the micro ridge in the stimulation group: after two hours of the stimulation of the excess gravity of 50 G. Variation was made on the height (H) of the ridge in each area: 0.4 μm (a), 0.3 μm (b), or 0.2 μm (c). The tendency for each cell to align to the

longitudinal direction of the ridge decreases on the low ridge (Fig. 14c). Fig.15 exemplifies the tracings of the angle (θ) of the major axis of each cell related to the longitudinal direction of the micro ridge in the control group of 1 G. Variation was made on the height (H) of the ridge in each area: 0.4 μm (a), 0.3 μm (b), or 0.2 μm (c). The tendency for each cell to align to the longitudinal direction of the ridge decreases on the low ridge (Fig. 15c). Fig.16 exemplifies the tracings of the angle (θ) of the major axis of each cell on the surface without the micro pattern in the control group of 1 G. The angle is measured between 0 degree and 180 degree related to the voluntary defined line of direction. The angle of each cell varies at random with rotational migration of the cell on the plane surface.

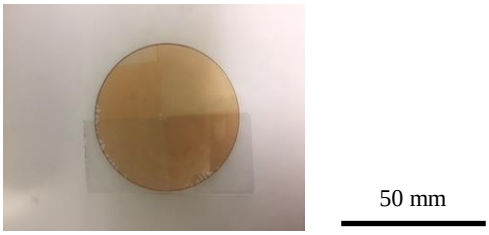

Fig. 8a: Micro pattern (center) on surface of mold: diameter, 50 mm

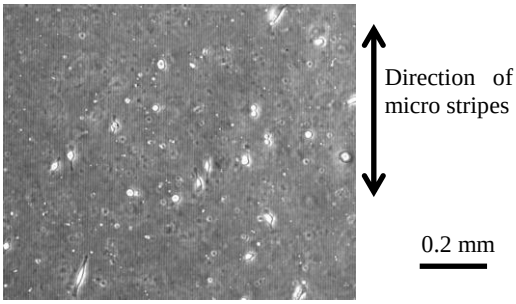

Fig. 8b: Cells on micro pattern (vertical stripes): dimension from left to right is 1 mm.

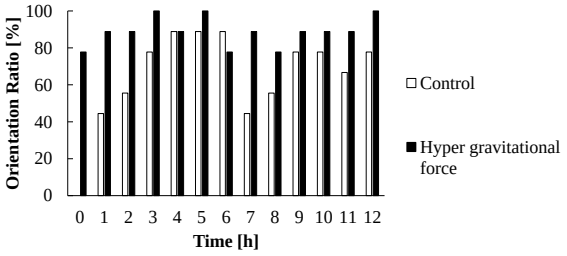

Fig. 9: Orientation ratio (*Ro* [%]) after stopping excess gravity (100 G for 1 hour).

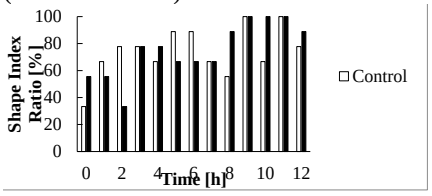

Fig. 10: Shape index ratio (*Rs* [%]) after stopping excess gravity (100 G for 1 hour).

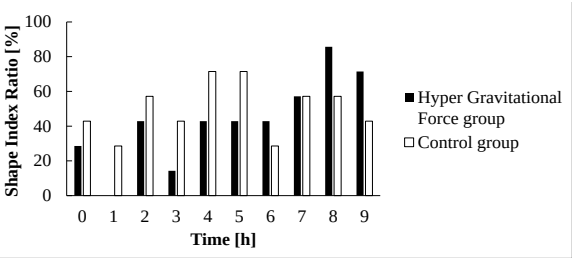

Fig. 11: Shape index ratio (*Rs* [%]) after stopping excess gravity (100 G for 5 hours).

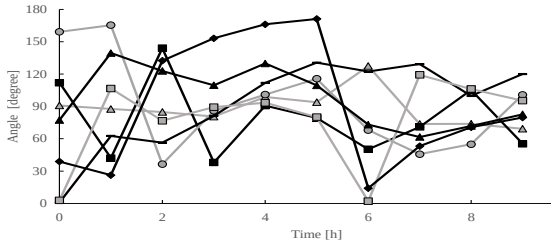

Fig. 12: Angle (θ) tracings (control, 1 G).

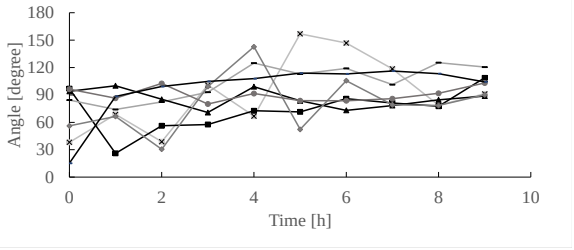

Fig. 13: Angle (θ) tracings after stopping excess gravity (100 G for 5 hours).

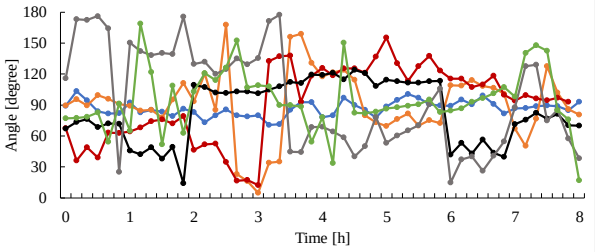

Fig. 14a: Angle (θ) tracings after stopping excess gravity (50 G for 2 hours, 0.4 μm).

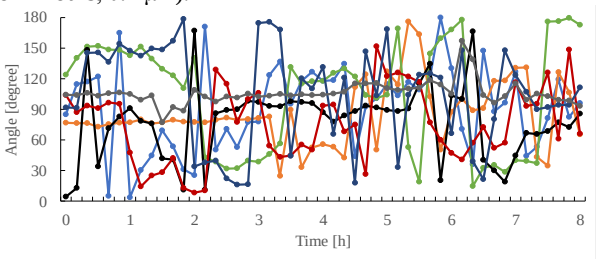

Fig. 14b: Angle (θ) tracings after stopping excess gravity (50 G for 2 hours, 0.3 μm).

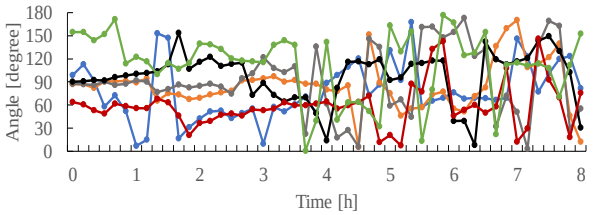

Fig. 14c: Angle (θ) tracings after stopping excess gravity (50 G for 2 hours, 0.2 μm).

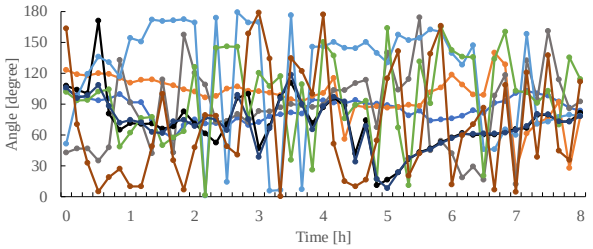

Fig. 15a: Angle (θ) tracings (control 1G, 0.4 μm).

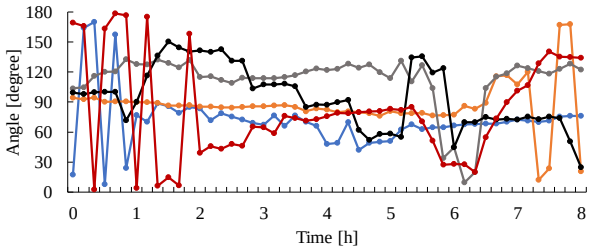

Fig. 15b: Angle (θ) tracings (control 1G, 0.3 μm).

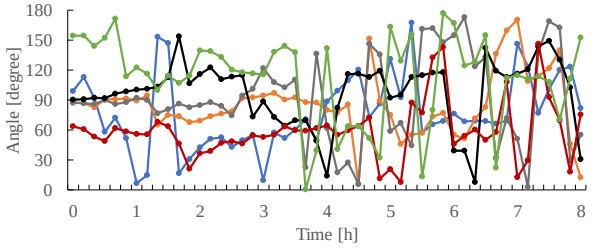

Fig. 15c: Angle (θ) tracings (control 1G, 0.2 μm).

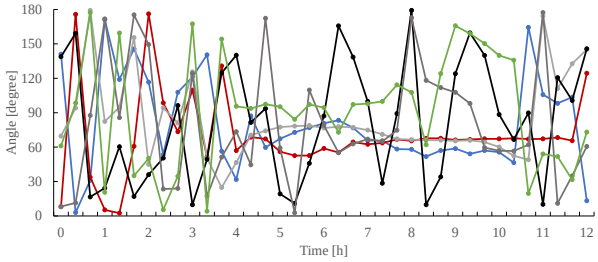

Fig. 16: Angle (θ) tracings on flat surface (control 1G).

4. DISCUSSION

To apply continuous uniform mechanical stimulation to the cells, centrifugal force is used in the present study. The effect of mechanical field on orientation and deformation of several kinds of cells was studied using centrifuge *in vitro* in the previous studies [5-17]. The differentiation of cells was accelerated by the mechanical stimulation by centrifuge in the previous study [11, 14]. The response of biological system to the microgravity field has been studied at a space satellite. The cell cycle might extend in the space [6]. C2C12 differentiation might be accelerated and the myotube might align to the direction perpendicular to the centrifugal field [8].

At the surface, the object is exposed to the force with two kinds of directions: normal, and tangential. The object is pressed by the normal force. The object is, on the other hand, sheared on the surface with the tangential force. Immediately after the centrifugation, cell might start to show active response to the mechanical stimulation. The cell shows adaptation against stimulation. The stimulation leaves hysteresis in the cell. The gravitational stimulation governs the behavior of the cell after stimulation.

Migration of a cell on the scaffold plate was observed in previous studies [26]. The time lapse image of every ten minutes is effective to trace the movement of each cell. The orientation of the single cell depends on the morphology of the scaffold surface [24-29]. The height of the micro ridge of 0.4 μm is too law for C2C12 to make orientation at 1 G [29]. The normal force by the excess gravity applied to the cell might emphasize the orientation of the cell along to the striped micro pattern. The variation of the height of micro pattern in three areas realizes the simultaneous observation of the behavior of each cell (Figs. 14 & 15).

During centrifugation, the direction of the force field tilts from horizontal direction because of the gravity (1 G) of the earth. In the case of centrifuge of 50 G, the shift of the angle is 1 degree. The gravity in the fluid is reduced by the buoyancy. The density of the cell of C2C12 is 1.065×10^3 kg m^{-3} measured by the centrifugal method: each separator adjusted to various density of the fluid of phthalate-ester is compared with the density of the cell in the glass capillary by centrifuge [30]. The density of medium of DMEM, on the other hand, is 1.0055×10^3 kg m^{-3}. The difference between two densities is 0.0595×10^3 kg m^{-3}. The net effect of the centrifugal force on the cell in the medium is 6 G at centrifuge of 100 G.

5. CONCLUSION

The orientation of a single cell on the scaffold with the micro striped pattern after stimulation of the vertical excess gravity has been traced *in vitro*. The micro striped pattern (3 μm width, 3 μm interval, and 0.2 μm < height < 0.4 μm) was made on the PDMS (polydimethylsiloxane) plate of the scaffold by the photolithography technique. After the vertical force of the excess gravity (< 100 G) was applied to C2C12 (mouse myoblast cell line) for several hours by the swing rotor in a conventional centrifugal machine placed in an incubator, the orientation of each cell was traced at the time lapse images for 12 hours. The experiments show that the orientation along the micro striped pattern is strengthened by the vertical excess gravity and the tendency is maintained for several hours after stopping of the excess gravity.

6. ACKNOWLEDGMENT

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