Behavior of Cell on Micro Ridge Pattern After Continuous Stimulation of Tangential Force

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ABSTRACT[1]

The behavior of each cell on the micro ridge pattern after the continuous stimulation by a tangential force has been studied *in vitro*. The stripe-pattern (0.7 μm height, 3 μm width, and 3 μm interval) was made on the surface of the scaffold plate to control the orientation of each cell during the force field stimulation. Variation was made on the angle between the longitudinal direction of the ridge and the direction of the tangential force: 0 degrees, 45 degrees, and 90 degrees. Myoblast (C2C12: mouse myoblast cell line) was used in the experiment. To apply the tangential force field to the cells, the scaffold plate was set in the tube in a conventional centrifugal machine placed in an incubator. After centrifugation for 5 hours, the behavior of each cell at the time-lapse microscope images was traced for 10 hours. The experimental results show that cells on the ridge-lines oriented perpendicular to the tangential force field have the higher activity (migration, and deformation) after stimulation.

Keywords: Biomedical Engineering, Cell Culture, Tangential Force Field, Division and C2C12.

1. INTRODUCTION

The recent cell culture technique enables observation of the behavior of each cell *in vitro*. A biological cell adheres on the scaffold and shows several active behaviors: migration, deformation, and proliferation. The cell is deformed by the force, because of its compliance. The cell deforms, on the other hand, to minimize the intra force of itself. The cell is moved by the force. The cell, on the other hand, moves by itself. C2C12 is cell line of mouse myoblast. In the previous study, both the migration and the deformation of C2C12 were restricted by the wall shear stress higher than 3 Pa in the flow *in vitro* [1]. Deformability of C2C12 was observed by the slit in the previous study *in vitro* [2, 3]. C2C12 extended along the lines of micro ridges on the scaffold surface [4]. Vibration decelerated adhesion of C2C12 on the micro ridges [5]. C2C12 migrated regardless of the direction of the vibrating micro ridges. The orientation of C2C12 was tried to be measured by the electric impedance *in vitro* [6]. Both differentiation and growth of C2C12 were delayed with electric pulses in the previous study *in vitro* [7]. The floating cells of C2C12 were tried to be sorted by the micro groove on the wall of the flow channel [8].

These behaviors might depend on the history of each cell. While the strong stimulation above the threshold damages the cell, the stimulation below the threshold remains in the cell as a memory for the response in the next step. The memory might be reset after division of the cell. To trace the hysteresis effect of the mechanical stimulation on the single cell, the time-lapse images are effective. In the previous study, the longitudinal axis of C2C12 (mouse myoblast cell line) tends to align to the direction of the excess gravity. The axis tilts to the perpendicular direction, on the other hand, after stopping of the excess gravity [9]. A single cell migrates at random on the scaffold. The cell tends to align to the longitudinal direction of the micro ridge line [10].

In the present study, the behavior of each single cell on the micro ridge pattern after the exposure to the

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tangential force field by centrifuge has been investigated *in vitro*.

2. METHODS

Micro-Ridge pattern on Scaffold Plate

To control the orientation of each cell, the micro striped pattern has been made in three partial rectangular areas of 0.4 mm × 1.6 mm on the PDMS (polydimethylsiloxane) plate of the scaffold by photolithography technique (Fig. 1). The height, the width, and the interval of the quadrangular ridges are 0.7 μm, 3 μm, and 3 μm, respectively. Each area has its own specific direction of the striped pattern. Namely, variation has been made on the angle (α) between the longitudinal direction of the ridge and the direction of the tangential force: 0 degree (parallel), 45 degree, and 90 degree (perpendicular). Three partial area was made on the same surface of the scaffold plate in parallel position, so that the behavior of cells on each area can be compared simultaneously. The pattern of each area was also used as a marker to trace each cell.

Mold for Micro Ridge Pattern

The borosilicate glass (Tempax) disk was used for the base of the mold of the micromachining process. 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 micro pattern was drawn on the mold with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for two minutes. The glass plate with the photoresist material was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan).

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. 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 plate of 15 mm × 10 mm × 1 mm, and stacked on the glass plate of 50 mm × 13 mm × 1 mm. To make the surface hydrophilic, 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 the cell culture.

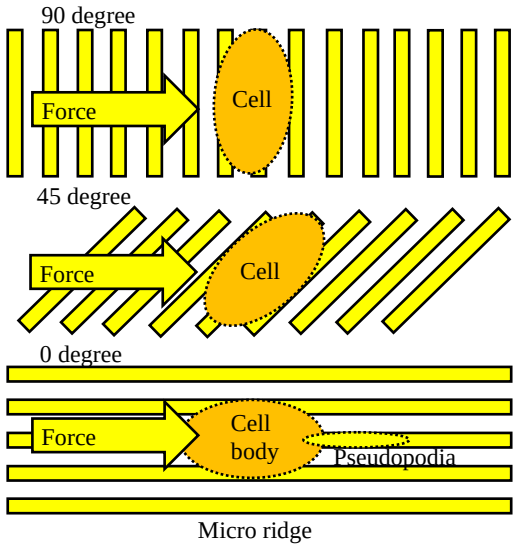

Fig. 1: Micro striped ridge pattern with three variations of direction against tangential force field: 90 degree, 45 degree, and 0 degree.

Tangential Force

The tangential force field was applied to the culture surface with the centrifugal force. The culture plate is inserted into the tube, which is contained in the rotor.

The glass plate was set so that the culture surface is parallel to the radial direction of the rotation. The centrifugal force is applied parallel to the culture surface. The centrifugal force (Fc) is calculated by Eq. (1).

$$Fc = m\, r\, \omega^2 \tag{1}$$

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

$$Fg = m\, g \tag{2}$$

In the present study, the centrifugal acceleration lower than 100 G (1 G is equal to the gravitational acceleration) ((Fc / Fg) < 100) is applied with the centrifugal machine.

Cell Culture

C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was used in the test. C2C12 of the passage four was cultured in D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (fetal bovine serum) and 1% penicillin/ streptomycin. Cells were seeded on the glass plate at the density of 3000 cells/cm^2. After the cells were cultured for 12 hours in the resting state, the excess

gravitational stimulation was applied for five hours. To apply the tangential force field to the cells, the scaffold plate was dipped in the medium in a tube in a conventional centrifugal machine (CN-1040, Matsuura seisakusyo. Ltd, Tokyo, Japan) placed in an incubator. Several tubes with the glass plate were set in the rotor of the centrifugation to cultivate cells, simultaneously. To keep the content of carbon dioxide of 5 % at 310 K, the cells were cultured in an incubator through the entire experimental term including the term of exposure to the tangential force field. Namely, the centrifugal machine was placed in the incubator during the centrifugation.

Image Analysis

After stimulation of the tangential force for 5 hours, cells on the glass plate were moved from the centrifugal tube to the culture dish. 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 images captured every ten minutes for 48 hours after stimulation of the tangential force. "Image J" was applied to analyze the behavior of each cell. On the microscopic image, the contour of each cell was traced. The contour except pseudopodia was approximated to ellipsoid. The centroid is used to trace the migration of each cell. The velocity (v [μm/min]) of the migration of each cell was calculated by Eq. (3) using the distance (Δx) between the centroids of the moving cell at the time-lapse microscopic images (at t_1 and at t_2) with the interval of ten minutes (Fig. 2).

$$v = \Delta x / (t_2 - t_1) \quad (3)$$

The area change rate (Sr [%/min]) was calculated by Eq. (4) using two-dimensional projected area of each cell at the time-lapse microscopic images with the interval of ten minutes.

$$Sr = 100 \times (S_2 - S_1) / (S_1 \times (t_2 - t_1)) \quad (4)$$

In Eq. (4), S_1 and S_2 are the areas of the tracked cell at the time t_1 [min] and at the time t_2 [min], respectively.

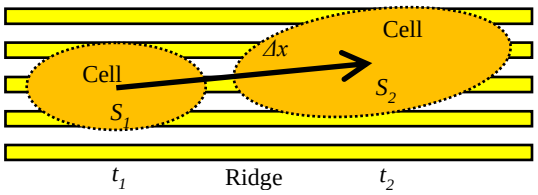

Fig. 2: Migration (Δx) and deformation (from S_1 to S_2) of cell on pattern.

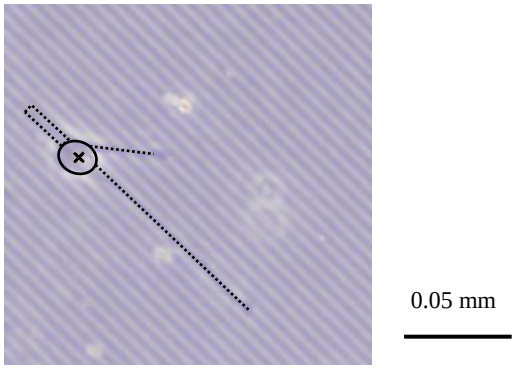

Fig. 3: Contour of cell except pseudopodia (dotted lines) was approximated to ellipsoid (solid line): centroid (cross mark).

3. RESULTS

Each cell extended pseudopodia along the longitudinal direction of the micro ridge (Fig. 3). Each cell deformed along to the longitudinal direction of the micro ridge. Each cell migrated along the longitudinal direction of the micro ridge, as the longitudinal axis of each cell was parallel to that of the micro ridge.

The following figures show the results on the surface of variations of the direction of the striped pattern: no pattern (Figs. 4a, 5a, 6a, and 7a), 0 degree (parallel to the tangential force; Figs. 4b, 5b, 6b, and 7b), 45 degrees (Figs. 4c, 5c, 6c, and 7c), and 90 degrees (perpendicular to the tangential force; Figs. 4d, 5d, 6d, and 7d), respectively. Figs. 4 and 6 show the behavior of cells for 10 hours after 5 hours exposure to the tangential force field of 50 G. Figs. 5 and 7 show the behavior of cells for 10 hours after 5 hours exposure to the tangential force field of 100 G.

Each marker corresponds to each cell tracked at the time lapse images. In Figs. 4d, 6d, and 8d, cell 1 (rhombus), cell 1.1 (square), cell 1.2 (triangle), cell 2 (cross, $S = 69\ v + 318$, $r = 0.52$), and cell 3 (circle). The cell 1 was divided into the cell 1.1 and the cell 1.2. In Figs. 4a and 6a, cell 7 (open circle), cell 8 (cross), cell 8.1 (square), cell 8.2 (closed circle), cell 8.2.1 (triangle), and cell 8.2.2 (rhombus). The cell 8 was divided into the cell 8.1 and the cell 8.2. The cell 8.2 was divided into the cell 8.2.1 and the cell 8.2.2. In some cases, the regression line of each cell tracking is displayed in the figures. In the other cases, the correlation coefficient (r) at each regression line is lower than 0.35.

Fig. 4 shows the tracing of migration velocity of each cell for 10 hours after 5 hours exposure to the tangential force field of 50 G. Fig. 5 shows the tracing of migration velocity of each cell for 10 hours after 5 hours exposure to the tangential force field of 100 G. The migration

velocity of each cell on the striped pattern of micro ridge perpendicular to the tangential force tends to increase after the tangential force stimulation (Figs. 4d and 5d). On the striped pattern of micro ridge parallel to the tangential force, on the other hand, the migration velocity is relatively lower (Figs. 4b and 5b). The tendency is remarkable after centrifuge at 50 G than 100 G. The migration velocity also increases before and after the deviation of the cell (Fig. 4a). Data of the cell on the flat surface without the striped pattern of micro ridge fluctuate. The longitudinal direction of each cell changes at random on the flat surface.

Figs. 6 and 7 shows the area change rate (*Sr*) tracing of each cell for 10 hours. The change rate of each cell contact area to the scaffold tends to be higher on the striped pattern of micro ridge perpendicular to the tangential force (Fig. 6d). On the striped pattern of micro ridge parallel to the tangential force, on the other hand, the change rate of each cell contact area to the scaffold tends to be lower (Fig. 6b). The tendency is remarkable after centrifuge at 50 G than 100 G. The higher change rate neutralized with time, after the stimulation of the tangential force field. The change rate of the cell contact area to the scaffold is high, after the division of the cell (Figs. 6a and 6d).

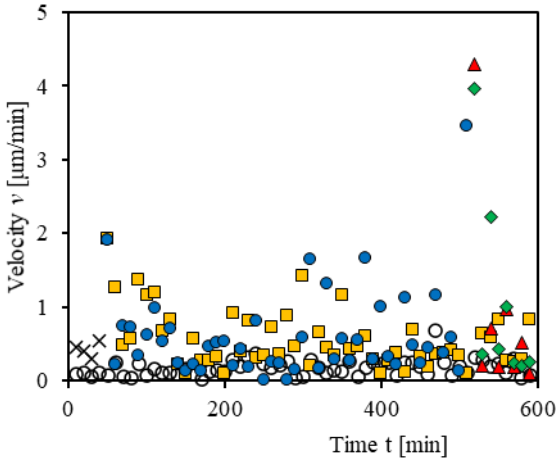

Fig. 4a: Velocity tracings of cell migration (50 G, control).

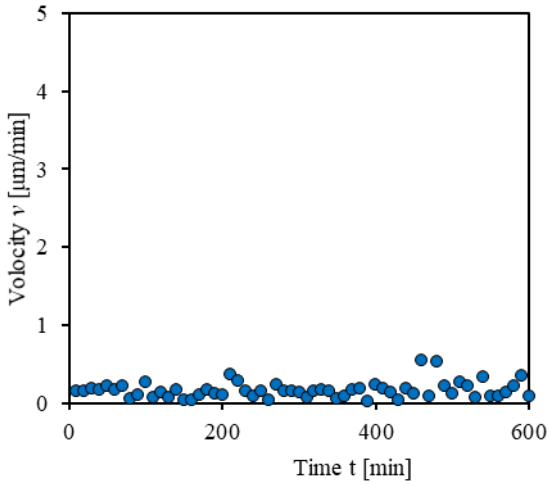

Fig. 4b: Velocity tracings of cell migration (50 G, 0 degree).

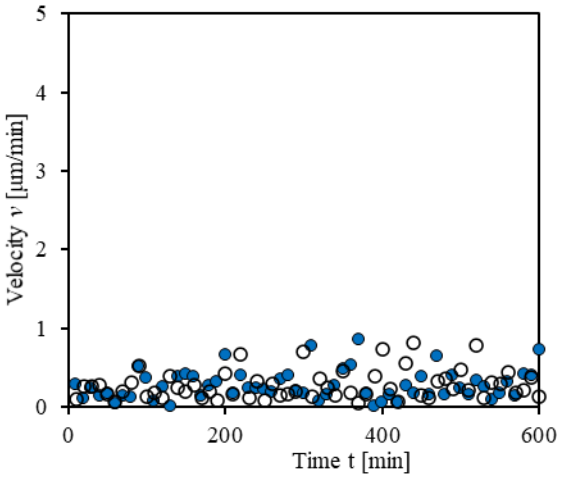

Fig. 4c: Velocity tracings of cell migration (50 G, 45 degree).

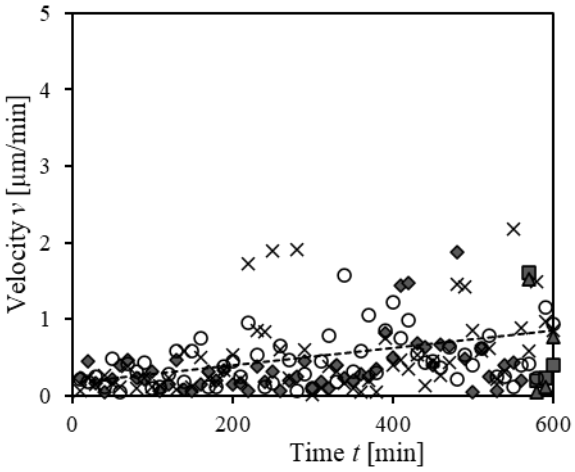

Fig. 4d: Velocity tracings of cell migration (50 G, 90 degree): cross, broken line, $v = 0.0011\ t + 0.17$, $r = 0.39$.

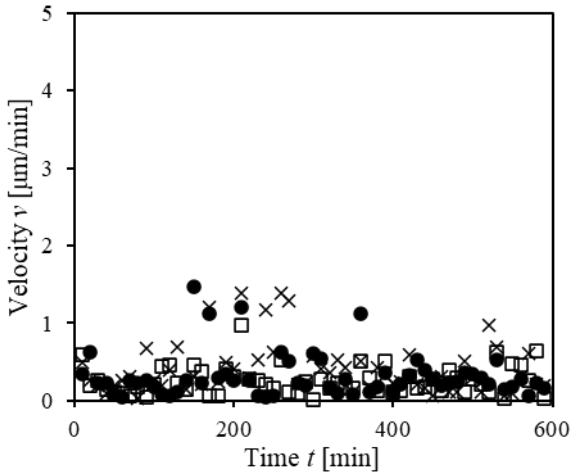

Fig. 5a: Velocity tracings of cell migration (100 G, control).

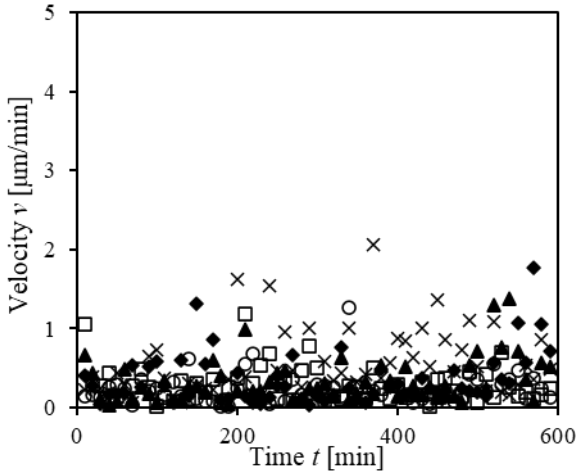

Fig. 5b: Velocity tracings of cell migration (100 G, 0 degree).

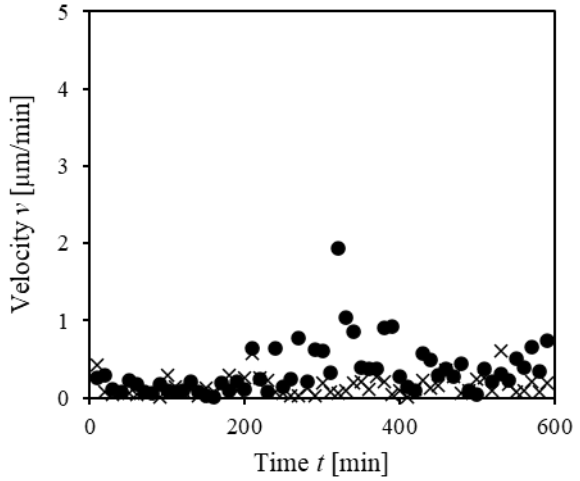

Fig. 5c: Velocity tracings of cell migration (100 G, 45 degree).

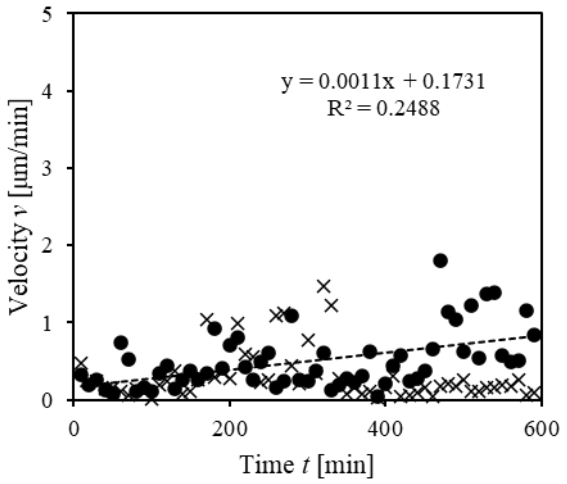

Fig. 5d: Velocity tracings of cell migration (100 G, 90 degree): circle, broken line, $v = 0.0011\ t + 0.17$, $r = 0.50$.

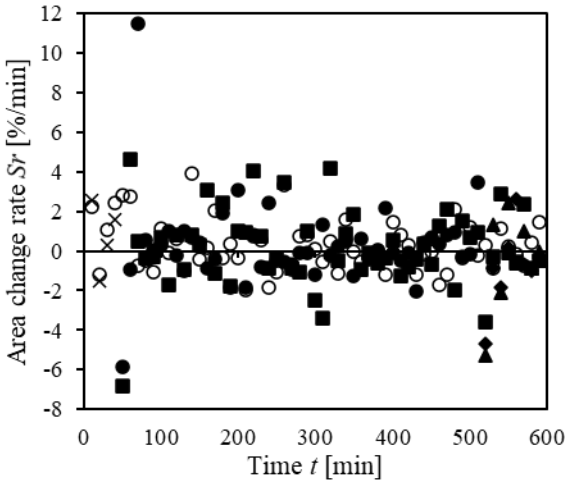

Fig. 6a: Tracings of area change rate (50 G, control).

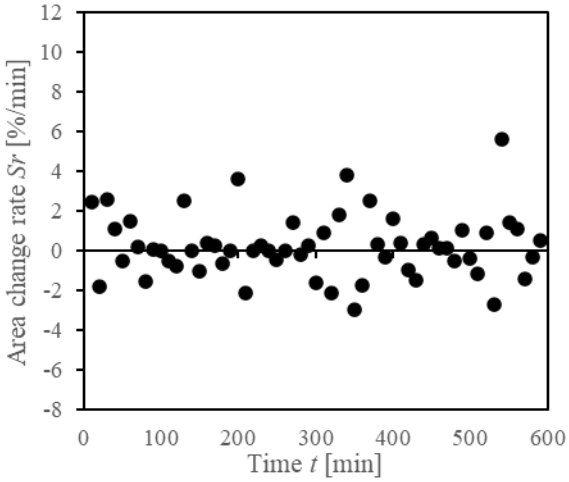

Fig. 6b: Tracings of area change rate (50 G, 0 degree).

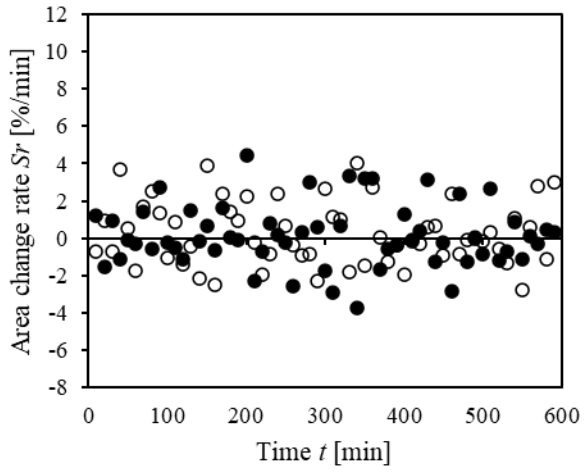

Fig. 6c: Tracings of area change rate (50 G, 45 degree).

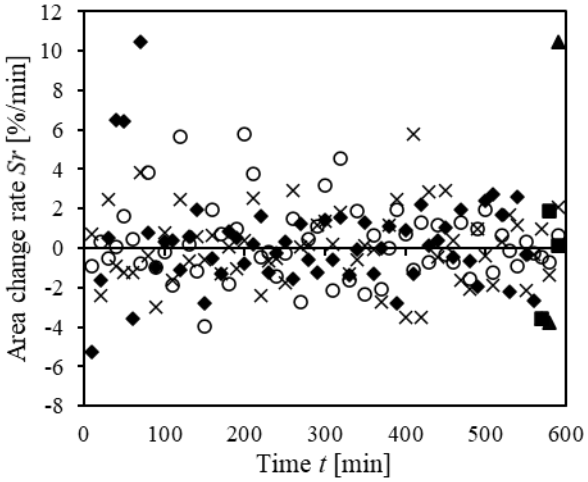

Fig. 6d: Tracings of area change rate (50 G, 90 degree).

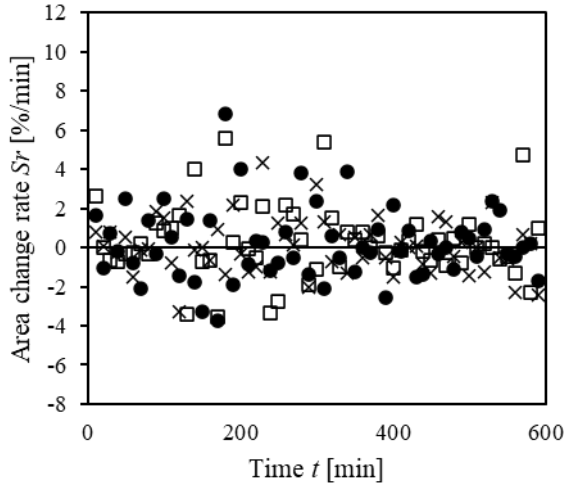

Fig. 7a: Tracings of area change rate (100 G, control).

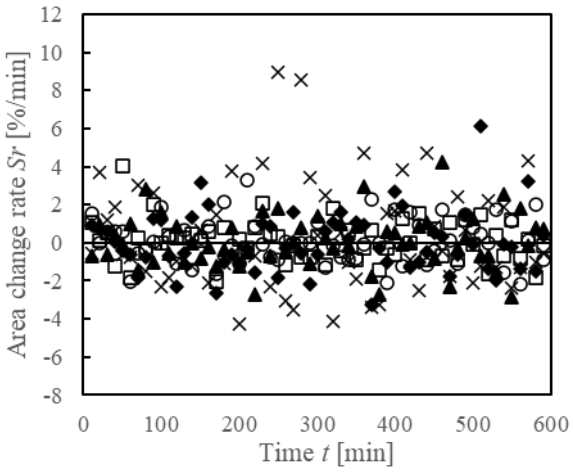

Fig. 7b: Tracings of area change rate (100 G, 0 degree).

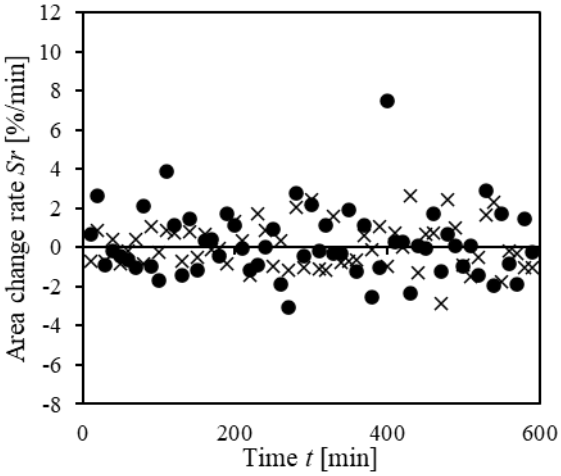

Fig. 7c: Tracings of area change rate (100 G, 45 degree).

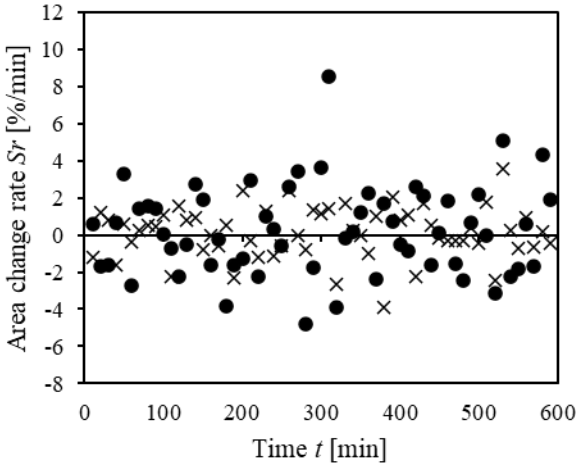

Fig. 7d: Tracings of area change rate (100 G, 90 degree).

4. DISCUSSION

The hysteresis effect of the tangential force stimulation on the activity of each cell was reset after division in the previous study. The gravity in the fluid is reduced by the buoyancy. Measurement of the density of cells by Phthalate ester method shows that the mean density of each cell is 1.07×10^3 kg/m^3. When the cells floating in the medium of the density of 1.00×10^3 kg/m^3, the effective centrifugal force ratio calculated from the difference of two density is 7 G at centrifuge of 100 G.

The apparent area of the cell contact to the scaffold was approximated to the two-dimensional projected area of the microscopic image in the present study. The three-dimensional contact area depends on the surface micro-topography. The special attention is necessary that the chemical property of the interface also affects the real contact area between the cell and the scaffold.

The height of the ridges was limited to 0.7 μm, which is minimum value to control the alignment of each cell [10]. In the present study, the striped pattern of the micro ridges on PDMS is effective to control the longitudinal direction of each cell [11].

5. CONCLUSION

The experimental results show that the activity of the cell (migration, and deformation) after stimulation is maximized when the stripe pattern is oriented perpendicular to the tangential force field.

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