# Behavior of Cell on Vibrating Micro Ridges

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## ABSTRACT

The effect of micro ridges on cells cultured at a vibrating scaffold has been studied *in vitro*. Several parallel lines of micro ridges have been made on a disk of transparent polydimethylsiloxane for a scaffold. To apply the vibration on the cultured cells, a piezoelectric element was attached on the outside surface of the bottom of the scaffold. The piezoelectric element was vibrated by the sinusoidal alternating voltage ($Vp$-$p$ < 16 V) at 1.0 MHz generated by a function generator. Four kinds of cells were used in the test: L929 (fibroblast connective tissue of C3H mouse), Hepa1-6 (mouse hepatoma), C2C12 (mouse myoblast), 3T3-L1 (mouse fat precursor cells). The cells were seeded on the micro pattern at the density of 2000 cells/cm$^2$ in the medium containing 10% FBS (fetal bovine serum) and 1% penicillin/ streptomycin. After the adhesion of cells in several hours, the cells are exposed to the ultrasonic vibration for several hours. The cells were observed with a phase contrast microscope. The experimental results show that the cells adhere, deform and migrate on the scaffold with micro patterns regardless of the ultrasonic vibration. The effects of the vibration and the micro pattern depend on the kind of cells.

**Keywords**: Biomedical Engineering, Cell Culture, Micro pattern and Vibration.

## 1. INTRODUCTION

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

The ultrasonic vibration has been applied to human bodies in several cases: measurements, and lithotrity [7-9]. A cell culture technique has been developed, and cells have been cultured in a controlled environment. Effects of the vibration on the cell culture were studied in previous studies [10-14]. The moderate ultrasonic vibration accelerated the proliferation of myoblasts in the previous experiment. The cells might be exfoliated from the ultrasonically vibrating scaffold. The exfoliation depends on the affinity between the cell and the scaffold. The specific character of the interaction between the cell and the scaffold might be enhanced by the vibration. The interaction might depend on the micro morphology of the surface. The behavior might depend on the kind of cells. The technology can be applied to the sorting of cells.

Several methodologies have been clinically applied to the regenerative medicine. The acceleration techniques for proliferation, orientation and differentiation of cells have been studied to make the tissue *in vivo* or *in vitro* [10, 11, 13, 15]. Control methodology for proliferation and differentiation of cells would be applied to the regenerative tissue technology. The mechanical stress is one of the interested points in the environment of cells, because they receive mechanical forces *in vivo*. The mechanical stress on cells might induce various responses: deformation, migration, proliferation, orientation, and differentiation. Several methods have been designed to apply the mechanical stress to cells [16-18].

In the present study, the effect of micro ridges on cells cultured at the vibrating scaffold 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). The height ($H$), the interval ($I$), and the length ($L$) of the rectangular ridge (Fig. 1) are around 0.001 mm, 0.003 mm, and 0.5 mm, respectively. Variation has been made on the width ($W$) of the ridge: 0.001 mm, 0.003 mm, and 0.005 mm. 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. One of the quarter areas has a smooth surface, and has no ridge (Figs. 8-11).

A silicon wafer (CZ, Type N, Matsuzaki Seisakusyo, Co., Ltd., Tokyo, Japan) was used for a surface mold for the disk in a photo lithography process [1]. The diameter and the thickness of the wafer are 50 mm and 0.30 mm, respectively. The surface of the wafer was cleaned by a spin-drier (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan).

To improve the affinity between the wafer and the photo-resist material (OFPR-800), the hydrophobic treatment was applied to the wafer. Hexamethyldisilazane (HMDS) was coated at 3000 rpm for thirty seconds with a spin coater (IH-DX2, Mikasa Co., Ltd., Tokyo, Japan). The photo-resist material of OFPR-800 (Tokyo Ohka Kogyo Co., Ltd., Tokyo, Japan) of 0.0017 mm thick was coated on the wafer at 7000 rpm (for twenty seconds) and at 3500 rpm (subsequently for five seconds) with the spin coater. The photo-resist was baked on a heated plate at 373 K for ninety seconds.

The pattern for the micro grooves was drawn on the wafer with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To save the 0.001 mm thickness of parylene coating, the dimensions of the width of the three kinds of grooves on the mold are 0.003 mm, 0.005 mm, and 0.007 mm, respectively (Fig. 1). To control the dimension of the pattern on the mold with the laser drawing system, the parameters were

selected as follows: the voltage of 3.25 V, the velocity of 0.15 mm/s, the acceleration of 0.375 mm/s$^2$. The pattern was baked on the heated plate 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 wafer was rinsed two times with the distilled water for three minutes, and dried by the spin-drier. To increase the adhesiveness of the coating, the wafer was baked at 393 K for five minutes.

The wafer 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. The gas of sulfur hexafluoride (10 cm$^3$ min$^{-1}$) with argon (20 cm$^3$ min$^{-1}$) and with oxygen (10 cm$^3$ min$^{-1}$) was applied on the wafer at 15 Pa for three minutes. To exfoliate the residual photo-resist material from the surface, the wafer was exposed to the oxygen gas of 30 milliliter per minute at the power of 100 W for five minutes using a compact etcher (FA-1, Samco Inc., Kyoto): the oxygen plasma ashing.

The dimensions of the three 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 the grooves was traced. The morphology of the micro grooves was also observed by a scanning electron microscope (SEM, JSM6360LA, JEOL Ltd., Tokyo, Japan).

The surface of the wafer with micro patterns was coated with 0.001 mm thickness of parylene in a parylene coater (PDS-2010, Speciality Coating Systems, Indianapolis, USA) (Fig. 1).

After the wafer was enclosed with a peripheral wall of polyimide, 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 wafer. The volume ratio of PDMS to curing agent is ten to one. After degassing, PDMS was baked at 383 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd). The baked disk of PDMS of 0.25 mm thickness was exfoliated from the mold, and sterilized in an autoclave.

The disk with the micro ridges was used for the bottom of the dish. Another disk of PDMS, which has a donut shape (50 mm outer diameter, 5.5 mm thickness) with a hole of 33 mm diameter, was made for the peripheral wall of the dish (Fig. 2). These two disks are contacted with the affinity between them, and make a culture area of 8.5 cm$^3$ at the inner bottom.

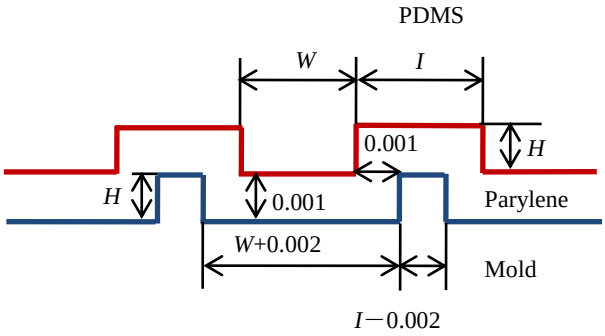


**Fig. 1:** Dimension for parylene coating on the mold for the micro ridges on PDMS. Unit of numbers is mm.

The cap for the dish of PDMS was made of PDMS. The dimension of the diameter and the thickness are 50 mm and 1 mm, respectively. The dish of PDMS was exposed to the oxygen gas for one minute at the power of 50 W 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.

The contact angles [1] were measured between the disk of PDMS and the medium (D-MEM) at 298 K by a contact angle analyzer (Phoenix-300, Meiwafosis Co., Ltd., Tokyo, Japan), before and after the oxygen plasma ashing.

**Experimental System**

The mechanical vibration was applied to cultured cells with the following experimental system. A donut shape (2 mm thick, 20 mm outer diameter, and 10 mm inner diameter) of a piezoelectric element (1Z10x20W-SYX(C-21), Fuji Ceramics Corporation, Tokyo, Japan), which has the resonance frequency of 1 MHz, is used for a vibrator.

After the piezoelectric element was placed on the silicon wafer, PDMS was poured with the curing agent over the element to make the holder of the element. The piezoelectric element mounted in the holder (total thickness of 2.2 mm) of PDMS was attached on the outside of the bottom of the dish of PDMS (Figs. 2&3). The contact between the holder and the dish is kept by affinity between them without an adhesive.

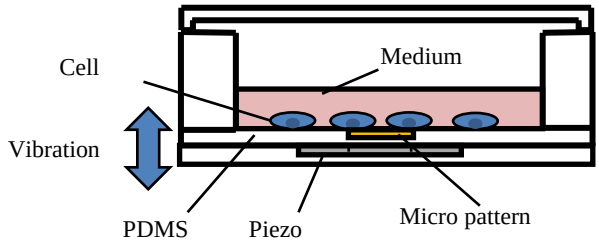


**Fig. 2:** Cell culture on the vibrating scaffold.

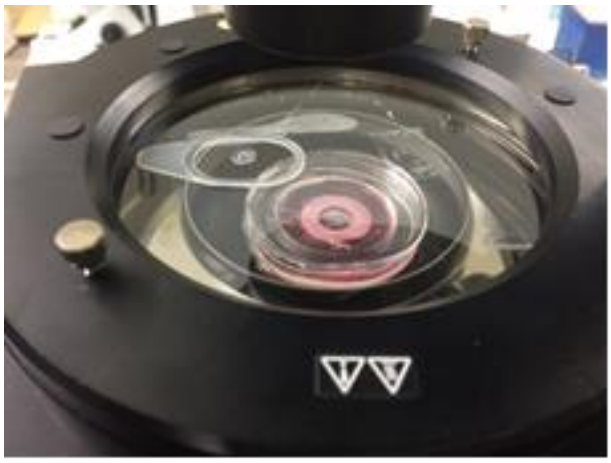

**Fig. 3:** Cell culture on the vibrating scaffold placed in the chamber of the incubator at the microscope.

To keep acoustic contact between the outside surface of the bottom of the dish of PDMS and the piezoelectric element, the gel of Vaseline is filled between them. The micro pattern is located at the center of donut ring of piezoelectric element to be observed by the microscope.

**Measurement of Vibration**

The vibration in the medium was measured with a piezo probe [19]. The piezo electric element attached on the bottom of the culture dish is vibrated by a function generator (inverse piezoelectric effect). The vibration propagates through the medium in the dish to a piezo probe, which is dipped in the center of the medium in the dish. The piezo probe translates the vibration to the electric oscillation (piezoelectric effect). The oscillation was monitored by an oscilloscope.

**Cell Culture**

Four kinds of cells were used in the test: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), 3T3-L1 (mouse fat precursor cells, cell line of 3T3 mouse), Hepa1-6 (mouse hepatoma cell line of C57L mouse), and L929 (fibroblast connective tissue of C3H mouse). The passage between the fourth and the eighth of cell line was used in the experiment with the vibration (Table 1). The passage between the third and the eighth of cell line was used in the experiment without the vibration (control).

**Table 1**: Number of passage of cell line in each test.

| Cell | Control | Vibration |
|---|---|---|
| C2C12 | 8 | 6 |
| 3T3-L1 | 7 | 8 |
| Hepa1-6 | 3 | 4 |
| L929 | 6 | 5 |

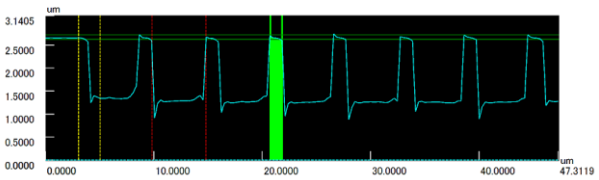


**Fig. 4:** Surface morphology of the mold measured by the laser microscope.

**Table 2**: Measured dimension of the groove on the mold.

| Group | mm | | |
|---|---|---|---|
| | Depth | Width | Interval |
| A | 0.0012 | 0.001 | 0.0028 |
| B | 0.0013 | 0.001 | 0.0049 |
| C | 0.0013 | 0.001 | 0.0069 |

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, 3T3-L1, and Hepa1-6.

Cells were seeded on the dish of PDMS at the density of 20 cells/mm$^2$. The culture dish with the piezoelectric element 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 (Fig. 3). The cells were continuously observed with the phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture.

After the culture term for adhesion of cells to the scaffold with the micro pattern, the vibration was continuously applied to the scaffold with the micro pattern. The term before the vibration was one hour for Hepa1-6. The term before the vibration was three hours for 3T3-L1, C2C12, and L929.

After the start of the vibration, the behavior of cells was successively recorded with the time-lapse microscopic pictures: pseudo extension, migration, exfoliation, and re-adhesion. The interval of the time-lapse was selected to be several minutes: between two and five minutes. The exceptional interval longer than ten minutes was selected for the long time recording for 24 hours.

## 3. RESULTS

The tracing of the surface morphology across the lines of ridges on the mold measured by the laser microscope is exemplified in Fig. 4. The tracing shows the height of 0.0013 mm, the width of 0.0010 mm, and the interval of 0.0049 mm.

Table 2 shows the mean values of the dimensions of the three kinds of the grooves on the mold, as the result of the measurement with the laser microscope.

The scanning electron microscope images of the lines of ridges are exemplified in Fig. 5. Fig. 5 shows the perspective view at the border of three kinds of ridges.

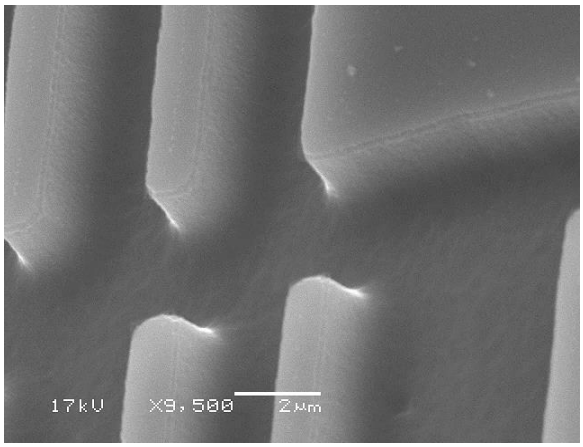


**Fig. 5:** Scanning electron microscope image of the lines of grooves on mold before parylene coating. Perspective view.

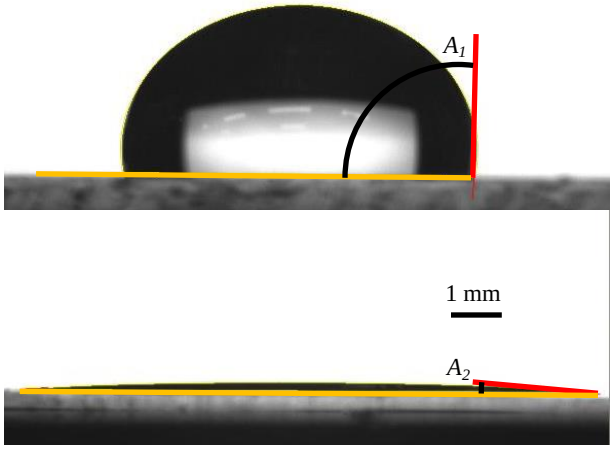


**Fig. 6:** Contact angle *A* of D-MEM on PDMS before (upper $A_1$) and after (lower $A_2$) oxygen plasma ashing.

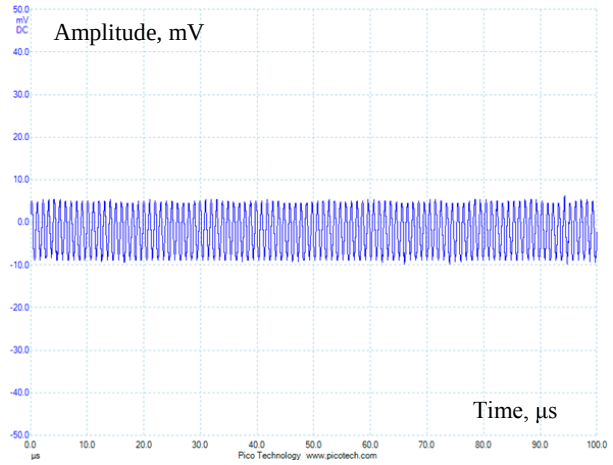


**Fig. 7:** Vibration in the medium. From left to right is 0.1 s.

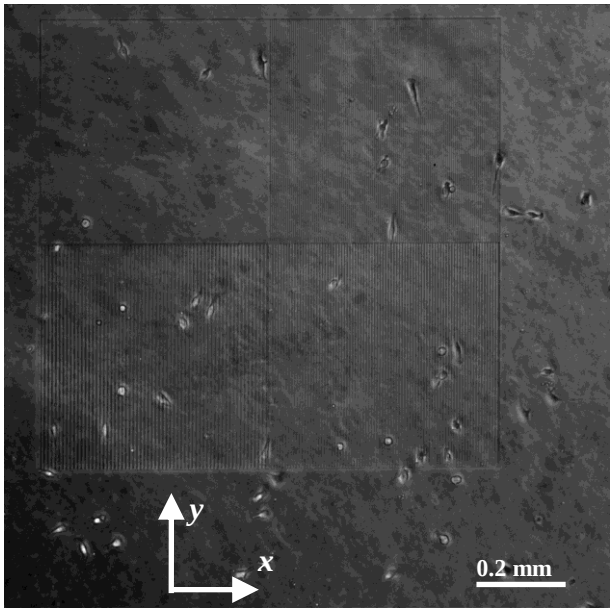


**Fig. 8:** C2C12 cultured for 3 hours with vibration.

Fig. 6 shows the contact angle of D-MEM on PDMS. The angle is 1.60 rad before the oxygen plasma ashing, and 0.065 rad after the oxygen plasma ashing. The property of the surface changed from hydrophobic (> 1.57 rad) to hydrophilic (< 1.57 rad) by the oxygen plasma ashing.

The vibration in the medium measured with the piezo probe is exemplified in Fig. 7. The wave pattern shows the frequency of 1 MHz.

The behavior of cells on the vibrating ridges is exemplified in Figs. 8-11. The arrow *y* shows the longitudinal direction of ridges in these figures. The upper left square area of 0.5 mm edge has a smooth surface, and has no ridges. The width of ridges in each quarter area among 1.0 mm edge is 0.001 mm (upper right), 0.003 mm (lower right), or 0.005 mm (lower left). Dimension from left to right is 1.3 mm in Figs 8-11.

The behavior of C2C12 on the vibrating ridges is exemplified in Fig. 8. The cells keep adhesion on the top of the ridge and migrate along the longitudinal direction of the ridges, although some cells exfoliate from the smooth surface without ridges. Migration is faster at the ridges of the narrower ridges (0.001 mm and 0.003 mm width) than at the ridges of the wider ridges (0.005 mm width). The vibration tends to accelerate migration and extension of the cells, especially on the ridges of 0.001 mm width. The repetitive elongation along the longitudinal direction of the ridges is observed on the vibrating scaffold. The elongation occurs most frequently on the ridges of 0.003 mm width.

Fig. 9 shows the behavior of 3T3-L1 on the ridge. The number of cells adhered on the scaffold is smaller than that of the other kinds of cells. The cells adhere faster on the smooth surface than on the ridges. The cells extend pseudo to every direction regardless to the longitudinal direction of the ridges. On the ridges of 0.001 mm and of 0.003 mm width, the cells make orientation along the longitudinal direction of the ridges in 24 hours of incubation, after the stop of the vibration for three hours (Table 3).

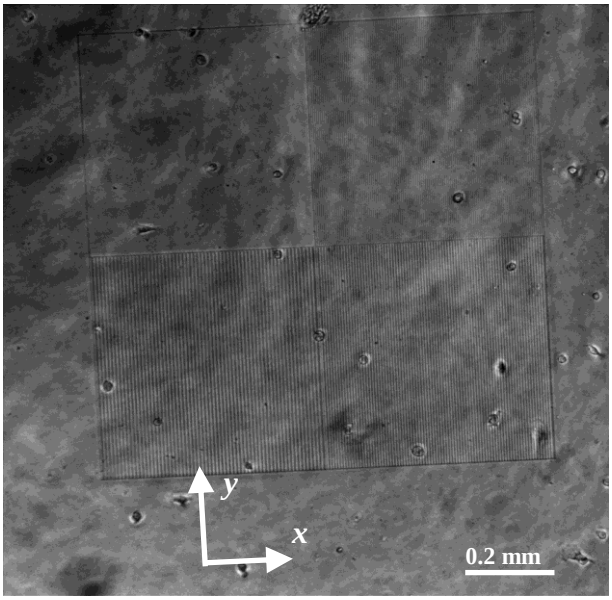


**Fig. 9:** 3T3-L1 cultured for 3hours with vibration.

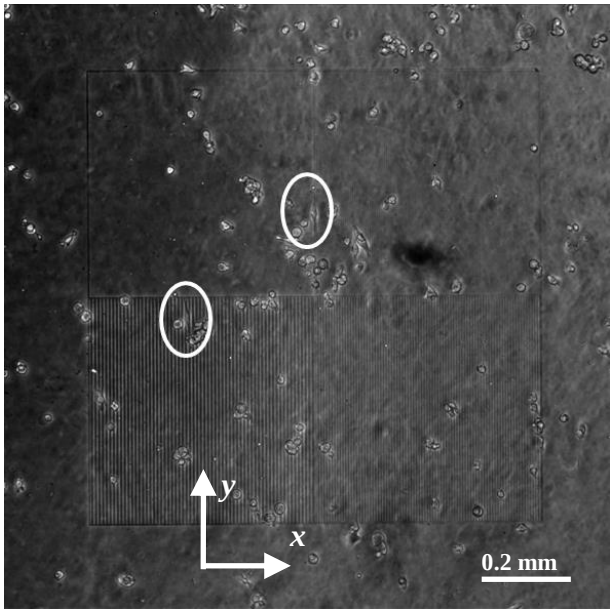


**Fig. 10:** Hepa1-6 cultured for 24 hours with vibration. Circles show oriented cells.

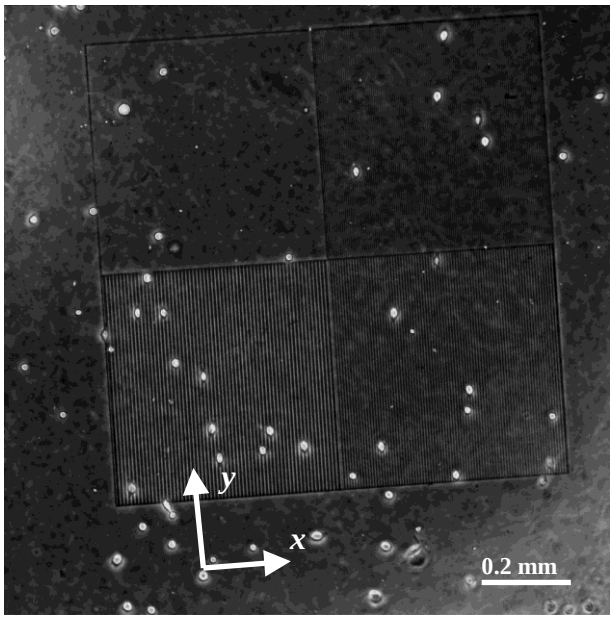


**Fig. 11:** L929 cultured for 3 hours with vibration.

Fig. 10 shows the behavior of Hepa1-6 on the ridge. The cell has round shape, so that it is difficult to distinguish deformation by adhesion. A few cells migrate along the longitudinal direction of the ridges of 0.001 mm and 0.003 mm without vibration. The vibration was started, when the cells extend pseudo. The number of adhesion of the cells decreases on the wider ridges with the vibration. The cell repeats extension and shrinkage of pseudo on the vibrating scaffold regardless of the longitudinal direction of ridges. The vibration decelerates migration of the cells. A few cells make orientation along the longitudinal direction of the ridges in 24 hours of the vibration (in the circles in Fig. 10). The exfoliated cells do not re-adhere to the scaffold during the vibration. The cells make orientation along the longitudinal direction of the ridges of 0.001 mm width in 24 hours of incubation, after the stop of the vibration for 24 hours (Table 3).

Fig. 11 shows the behavior of L929 on the ridge. The cells adhere to the scaffold in one hour. The cells slightly extend to the longitudinal direction of the ridges, but do not migrate along the ridges. The cells adhere to the scaffold more stable than the other kinds of cells, so that every cell keeps adhesion at the same place for three hours under the vibration.

Fig. 12 exemplifies migration of cells (A, C2C12 on the ridges of 0.003 mm width; B, C2C12 on the flat surface; C, L929 on the ridges of 0.001 mm width) for three hours with the vibration. Data shows the two dimensional position of every ten minutes at $x$-$y$ plane from original position (origin). The coordinates of $y$ and $x$ are longitudinal and perpendicular axis of the ridge, respectively (Figs. 8-11).

Fig. 12 shows that C2C12 migrates along the ridge, although L929 migrates regardless of the direction of the ridges. The migration of C2C12 (Fig. 12A) is larger than that of L929 (Fig. 12C). The migration of C2C12 to the random direction is confirmed on the flat surface (Fig. 12B).

Fig. 13 shows migration of the cells on the ridges of 0.003 mm width for three hours with the vibration. The figure shows the ratio of $\Delta y$ per $\Delta x$, where $\Delta y$ and $\Delta x$ are migration for every ten minutes. The ratio higher than one corresponds to the migration along the ridge. Fig. 13 shows that the ratio is high at C2C12.

Table 3 shows the number of cells of four kinds, which are oriented along the longitudinal direction of the micro ridge, and adhered on the scaffold at each timing. The number was counted in each square area of 0.5 mm edge. In Table 3, the cell is counted as the oriented cell, when the angle between the longitudinal axis of the cell and the longitudinal axis of the ridge is smaller than 0.17 rad.

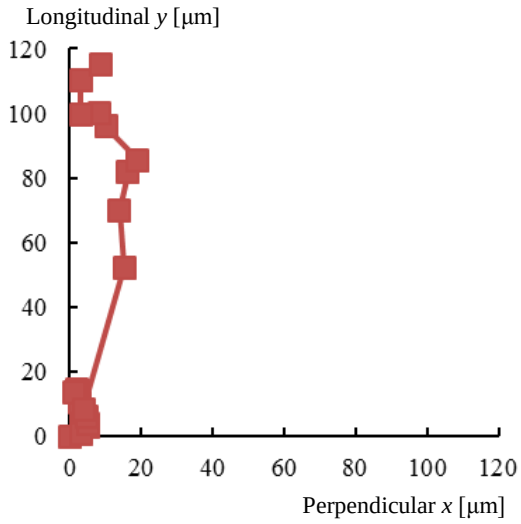


**Fig. 12A:** Migration of C2C12 on 0.003 mm width for 3 hour with vibration.

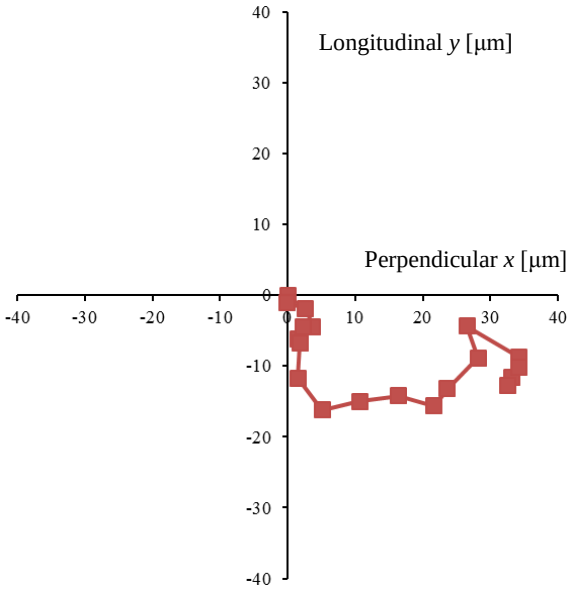


**Fig. 12B:** Migration of C2C12 on flat for 3 hour with vibration.

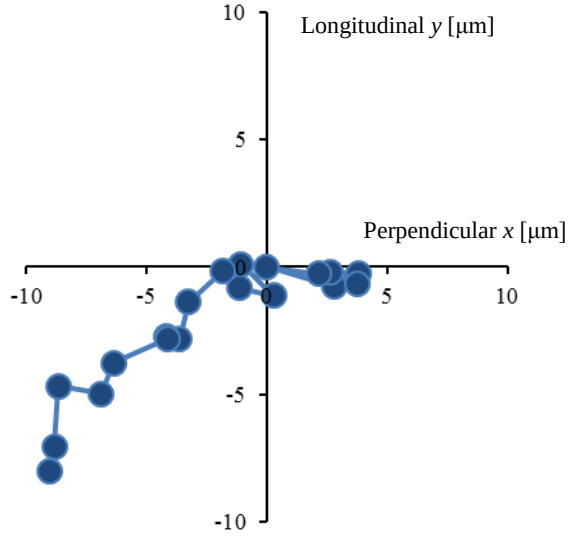


**Fig. 12C:** Migration of L929 on 0.001 mm width for 3 hour with vibration.

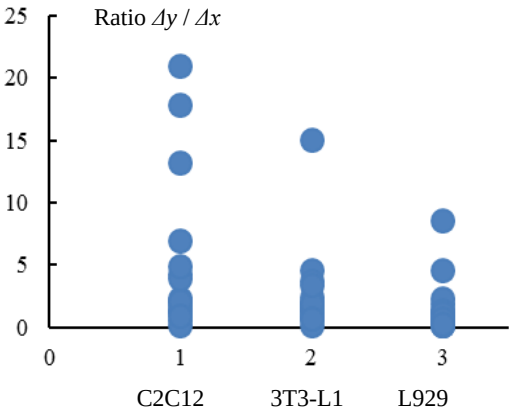


**Fig. 13:** Direction of migration of C2C12 on 0.003 mm width for 3 hour with vibration.

**Table 3**: Number of cells (Orientation/ Adhesion). vib: vibration.

| C2C12 | Flat | Width of ridge [mm] | | |
|---|---|---|---|---|
| | | 0.001 | 0.003 | 0.005 |
| 3 h | 0/3 | 1/7 | 2/9 | 0/8 |
| +3 h vib | 0/4 | 3/6 | 2/9 | 5/7 |

| 3T3-L1 | Flat | Width of ridge [mm] | | |
|---|---|---|---|---|
| | | 0.001 | 0.003 | 0.005 |
| 3 h | 0/4 | 0/4 | 0/9 | 0/5 |
| +3 h vib | 0/5 | 0/3 | 1/5 | 0/5 |
| +24 h | 2/8 | 5/8 | 3/7 | 3/12 |

| Hepa1-6 | Flat | Width of ridge [mm] | | |
|---|---|---|---|---|
| | | 0.001 | 0.003 | 0.005 |
| 1 h | 0/35 | 0/33 | 0/13 | 0/14 |
| +24 h vib | 0/19 | 1/26 | 0/20 | 1/35 |
| +24 h | 0/14 | 5/14 | 0/23 | 0/18 |

| L929 | Flat | Width of ridge [mm] | | |
|---|---|---|---|---|
| | | 0.001 | 0.003 | 0.005 |
| 3 h | 0/5 | 0/5 | 0/9 | 0/14 |
| +3 h vib | 0/4 | 0/5 | 1/10 | 0/12 |

In table 3, the number of cells in each area varies. Proliferation and migration into the area increase the number of cells in the area. Not only exfoliation from the surface of the scaffold but also migration to the other area decreases the number of cells adhered in the area.

## 4. DISCUSSION

The micro morphology, which is smaller than the diameter of cells, affects the movement of cells [20]. The morphology of the surface of the scaffold governs the orientation of cultured cells [21].

The effect of the height of micro ridges on the orientation of C2C12 was studied in the previous study. The experimental results show that myoblasts adhere on the top of the ridge and align to the longitudinal direction of the micro ridges with the height between 0.00015 mm and 0.0025 mm [1]. The height of the micro ridge is selected to be 0.001 mm in the present study.

The time-lapse is convenient not only to catch the moment of the behavior of the cell, but also to observe the movement of the cell. The migration of the cell is able to be traced, because the micro ridges play a role as markers.

In the present methodology of lithography techniques, the dimension of lines of rectangular ridges smaller than 0.001 mm is difficult to control. The dimensions of the lines of rectangular ridges of the present study are selected to save the thickness of parylene coating.

When *Vp-p* (the peak to peak value of the voltage applied to the probe) increases, the surface of the medium becomes convex and vibrates (*Vp-p* > 16 V) [11]. In the present study, *Vp-p* is selected to be 16 V, although the micro vibration might have the local stirring effect. Vibration makes agitation in the liquid.

Vibration at low frequency makes flow in the liquid. Vibration with high energy destroys structures. The surface of the medium is not vibrating macroscopically during the cell culture in the present experiment.

The acceleration technique is the important target in the research field of regenerative medicine on the cultured biological tissue: proliferation and orientation of cells. The previous study shows that electrical stimulation enhances differentiation of muscle cells [15].

The previous studies, on the other hand, show that a mechanical field governs behavior of cells. The shear flow governs the orientation of endothelial cells [17, 18].

Too strong mechanical stimulation damages cells. The mechanical stimulation can decrease proliferation of cells [16]. The mechanical stress also exfoliates several cells, which makes the vacancy around the adhered cell.

The moderate mechanical stimulation, on the other hand, might accelerate differentiation of cells [16]. The differentiation might be optimization of cells to the change of the environment. The mechanical stress can accelerate differentiation of C2C12 into myotubes.

The low-intensity ultrasonic treatment might increase mass transport. It might enhance proliferation, metabolic activity, and differentiation of C2C12 [10]. The wave length of ultrasonic vibration at frequency of 1 MHz through water is 1 mm, when the velocity of ultrasonic vibration through water is 1 km/s. The wave length is near the dimension of the aggregation of cells.

Vibration accelerates adhesion of C2C12 on the micro ridges, although vibration decelerates adhesion of 3T3-L1 on the micro ridges. C2C12 migrates and extends along the longitudinal axis of the ridges, although 3T3-L1 migrates regardless of the direction of the ridges.

## 5. CONCLUSION

The effect of micro ridges on cells cultured at the vibrating scaffold has been studied *in vitro*. Four kinds of cells were used in the test: C2C12 (mouse myoblast), 3T3-L1 (mouse fat precursor cells), Hepa1-6 (mouse hepatoma), L929 (fibroblast connective tissue of mouse). The experimental results show that the cells adhere, deform and migrate on the scaffold with the micro pattern regardless of the ultrasonic vibration. The effects of the vibration and the micro pattern depend on the kind of cells.

## 6. ACKNOWLEDGMENT

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