Effect of Flow on 3T3-L1 Oriented by Stripe Pattern of Micro Ridges

Shigehiro HASHIMOTO, Haruka HINO, Shingo OKUZUMI, Yuji ENDO

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

ABSTRACT

The effect of shear flow on an oriented cell has been studied by the flow channel with the micro striped pattern of ridge lines on the surface of the scaffold *in vitro*. The lines of parallel micro quadrangular prism ridges (0.001 mm height, 0.003 mm width, and 0.003 mm interval) are made by the photolithography technique on the lower surface of the channel as the scaffold to make orientation of each cell. Variations are made about the angle between the longitudinal direction of the ridge and the direction of the flow: 0, 45, 90 degrees. The flow rate of < 24 cm^3/hour is controlled by a syringe pump to make variation of the wall shear stress of < 2 Pa. The movement of each cell of 3T3-L1 (mouse fat precursor cells) adhered on the micro pattern was analyzed at the time lapse images for 10 hours: 2 hours (before the flow stimulation), 4 hours (during flow stimulation), and 4 hours (after stopping the flow stimulation). The experimental results show that both the migration and the deformation of each cell along the micro ridges are restricted by the wall shear stress higher than 1 Pa.

Keywords: Biomedical Engineering, Flow Channel, Micro-pattern, 3T3-L1 and Shear stress.

1. INTRODUCTION

Biological cells are exposed to mechanical stimulation *in vivo*. The shear stress is one of the mechanical stimulations. The endothelial cells, for example, are exposed to the shear stress in the blood flow at the wall of the blood vessels [1-8]. Some other cells are exposed to the shear stress among the deformation of the tissue. A cell adheres on the scaffold, migrates, deforms, proliferates, and differentiates. The mechanical stress might affect the action of the cell. The biological cell takes action not only passively but also actively. The cell is moved to downstream by the flow. The cell migrates, on the other hand, to the upstream against the flow. The flow deforms the cell along the stream line. The cell changes the shape, on the other hand, to minimize the intra force. Control methodology for orientation of cells would be applied to the regenerative tissue technology. The action of cell depends on the micro morphology of the scaffold [4-12]. The cell might be sensitive to the morphology of the similar dimension to itself at the scaffold. The photolithography technique [13] is available to make the micro patterns on the surface. The previous study showed that the orientation of myoblast follows the longitudinal directions of the micro ridges [12]. Several types of flow systems have been applied to study the response of cells to the shear stress in the flow *in vitro* [5-9, 14]. In the present study, a flow channel with a micro-patterned scaffold has been designed to study quantitatively the effect of flow on the oriented cell *in vitro*.

2. METHODS

Flow Channel

A flow channel has been designed to observe behavior of cells adhered on the micro-pattern in the flow during the cell culture *in vitro*. The flow channel consists of two disks of 50 mm diameter: the upper disk and the lower disk. The borosilicate glass (Tempax) disk is used for the lower disk for the scaffold of the cell culture. The upper surface of the lower disk has the micro-pattern. The upper disk is made of polydimethylsiloxane (PDMS), of which the lower surface has concave part for the flow path.

Lower Disk with Micro-pattern

The micro-pattern is fabricated by the photolithography technique at the central part of the upper surface of the lower disk for the scaffold of the cell culture. Several parallel lines of micro grooves have been made at the lower disk (Fig. 1). The depth (D), the width (W), and the interval (I) of the rectangular groove are 1 μm, 3 μm, and 3 μm, respectively. Variation has been made on the angle (θ) between the longitudinal direction of the groove and the flow direction: 0 (parallel), 45, 90 (perpendicular) degrees. Each pattern is drawn in the rectangle area of 1.6 mm × 0.4 mm, which is in the parallel position each other with the interval of 0.05 mm.

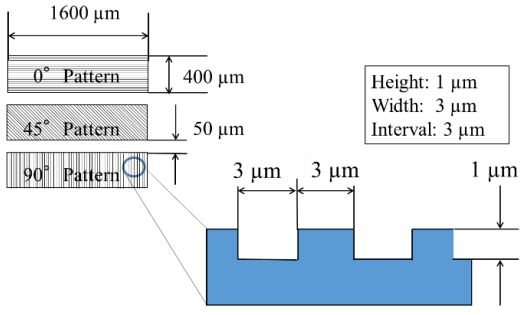


Fig. 1: Micro-pattern on lower disk of flow channel.

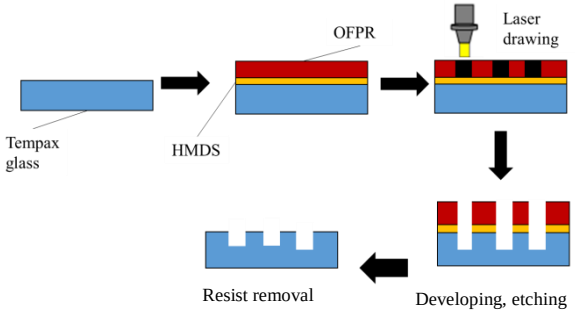


Fig. 2: Photolithography process for micro-pattern.

The borosilicate glass disk was used for the base of the lower disk in a photolithography process (Fig. 2). The diameter and the thickness of the disk are 50 mm and 1.1 mm, respectively. The surface of the glass disk was cleaned by the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing (100 W, for five minutes) in the reactive ion etching system (FA-1, Samco Inc., Kyoto, Japan). To improve affinity between glass and photoresist material, hexamethyldisilazane (HMDS: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the glass plate 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) was coated on HMDS at 3000 rpm for twenty seconds with the spin coater. The photoresist was baked in the oven (DX401, Yamato Scientific Co., Ltd) at 373 K for ninety seconds.

The pattern for the micro grooves was drawn on the photoresist material with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the pattern with the laser drawing system, the parameters were selected as follows: the voltage of 3.2 V, the velocity of 0.1 mm/s, the acceleration of 0.350 mm/s^2. The pattern was baked at the hotplate 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 two minutes. The disk was rinsed two times with the ultrapure water, and dried by the spin-dryer (SF-250, Japan Create Co., Ltd., Tokorozawa, Japan).

The glass was etched with the plasma gas using a reactive ion etching system (RIE-10NR, Samco 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) with Ar (30 cm^3/min at 1013 hPa) was applied at 100 W at 2 Pa for thirty minutes. The residual OFPR-800LB was removed by the plasma ashing (Oxygen, 0.1 Pa, 30 cm^3/min, 100 W, for five minutes) in the reactive ion etching system (FA-1).

The morphology of the micro grooves was observed with the laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). The height along the cross sectional line of the groove was traced.

Upper Disk

The mold for the upper disk (diameter; 50 mm) is made of aluminum. The mold has a convex rectangular pattern with the following dimension: the width of 4 mm, the height of 0.1 mm, and the length of 30 mm. Each end of the rectangular pattern has a hole with the female screw of M4. The mold was cleaned in the ultrasonic cleaning machine. In each hole with the female screw, one end of the bolt with the male screw was inserted. The other end of each bolt was inserted into the silicone tube (internal diameter 4 mm, external diameter 6 mm). 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. The baked disk of PDMS (5 mm thickness) with tubes was exfoliated from the mold to be used as the upper disk of the channel.

Assembly of Disks

Both the upper and the lower disks were 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). Immediately after ashing, the upper disk adheres (plasma bonding) to the lower disk to make the flow path between them. The assembly of disks was baked in the oven at 348 K for five minutes. Before cell culture, the flow channel was sterilized in an autoclave at 394 K for forty minutes. After sterilization, the channel was dried in the oven. In the clean bench, the channel was sandwiched between the transparent disks (diameter of 65 mm, thickness of 2 mm) of poly-methyl-methacrylate with six bolt/nuts as the supporter to adjust the fixation of the assembly of the flow channel.

One of the silicone tubes connects the flow channel with the syringe pump (Fig. 3). The other silicone tube connects the flow channel with the reservoir of the suspension of the cells, or with the reservoir of the medium. The channel is placed on the stage of an inverted phase contrast microscope (IX71, Olympus, Tokyo) for the observation of cells adhered on the micro pattern on the disk under the flow (Fig. 4). The CO_2 gas is blown into the reservoir of the medium to maintain the carbon dioxide partial pressure at five percent in the medium. The reservoir of the medium is placed in the thermostatic bath to maintain the temperature at 310 K (Fig. 4). The temperature of the surface of the flow chamber was measured by the infrared thermography (Fig. 6).

Wall Shear Stress

In the present experiment, the shear rate on the wall of the scaffold is estimated with a parabolic velocity profile between the parallel walls. The shear rate (γ, [s^{-1}]) on the wall of the plate is calculated by Eq. 1.

$$\gamma = 6\, q / (b\, d^{\,2}) \qquad (1)$$

In Eq. 1, q is the flow rate [$m^3\ s^{-1}$], b is the width of the channel [m] and d is distance [m] between two parallel walls. In the present study, d is 0.1 mm, and b is 4 mm. When q = 12 cm^3/hour, γ= 500 s^{-1}. The shear stress τ [Pa] is the product of the viscosity η [Pa s] of the fluid and the shear rate γ [s^{-1}] of the flow (Eq. 2).

$$\tau = \eta\, \gamma \qquad (2)$$

When γ = 500 s^{-1} and η = 0.002 Pa s (at 310 K) [11], τ = 1 Pa. Variation was made on the wall shear stress τ lower than 2 Pa by adjusting the flow rate q.

The flow adjacent to the micro grooves on the scaffold surface of the culture plate was traced by micro spheres (diameter 0.010

mm, density 2.4 g/cm^3: Thermo fisher scientific) rolling on the surface at the microscopic video image in the extra flow test.

Cell Culture with Flow

3T3-L1 (mouse fat precursor cells, a cell line derived from cells of mouse 3T3; passage between third and ninth) of the passage before tenth was used in the tests. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin was used for the medium. After the medium was prefilled in the flow channel through the inlet tube by the syringe pump, the suspension (5×10^4 cells/cm^3) of 3T3-L1 in D-MEM was injected into the channel through the inlet tube by the syringe pump (Fig. 3). After several cells adhered on the micro-pattern in the flow channel in two hours, the wall shear stress was applied on the cells by the flow pulled with the syringe pump for four hours. After the flow stimulation for four hours, the microscopic observation was continued for another successive four hours without the flow stimulation. To trace the same cell, the time-lapse pictures were taken with the constant interval of five minutes during the microscopic observation (Fig. 4).

As the control test, cells seeded on the micro pattern without flow stimulation were also observed by time-lapse microscopic images. The lower disk with micro-pattern was contained in the culture dish, and placed in the incubator on the stage of the inverted phase-contrast optical microscope.

At the microscopic image, the outline of each cell was traced by "Image J", and the contour of each cell was approximated to ellipsoid. As to the ellipsoid, the coordinates of the centroid, the length of the major axis (a), and the minor axis (b) were measured. The centroid of each ellipsoid was used to trace the migration of the cell at the microscopic time lapse images. To trace the migration of each cell by the coordinates, the x axis is corrected to the direction of the flow. The ratio of axes is calculated as the shape index (SI) by Eq. 3.

$$SI = 1 - b / a \tag{3}$$

At the circle, SI = 0. As the ellipsoid becomes flat, SI approaches to unity. The cell, of which SI is higher than 0.5, was selected, and the angle (θ) between the major axis of the cell and the longitudinal direction of the groove was measured. The number of the oriented cells (No) was counted as the cell, of which the angle (θ) is between –20 degree and 20 degree. In the area of each micro-pattern, the orientation ratio (Ro) was calculated by Eq. 4.

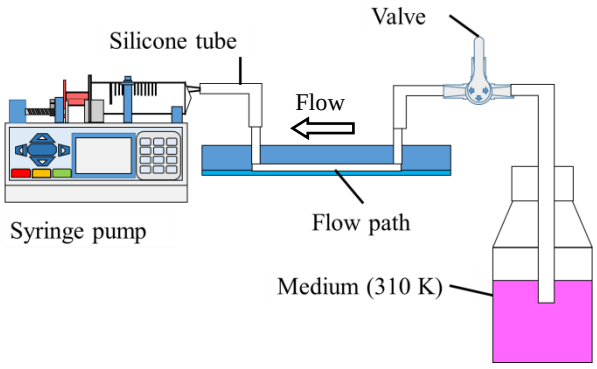


Fig. 3: Cell culture with flow: reservoir (right), flow channel (middle), and syringe pump (left).

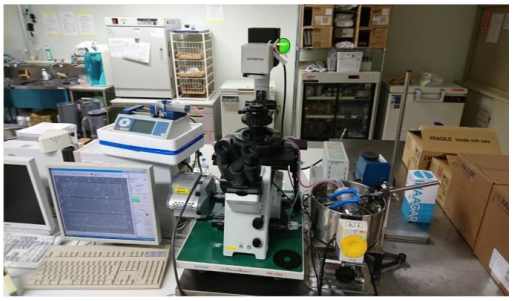


Fig. 4: Experimental system: syringe pump (left), flow channel on stage of microscope (middle), and reservoir in thermostatic bath (right).

$$Ro = No / Nt \tag{4}$$

In Eq. 4, No is the number of the oriented cells, and Nt is the total number of cells.

At the end of the test, the cell on the micro-pattern was observed by a scanning electron microscope (SEM, JSM6360LA, JEOL Ltd., Tokyo, Japan) (Fig. 9).

3. RESULTS

Fig. 5 shows the tracings of the lower disk by the laser microscope. The dimensions of the depth (D), the width (W), and the interval (I) of the rectangular groove are confirmed to be 0.001 mm, 0.003 mm, and 0.003 mm, respectively. Fig. 6 shows the thermographic image of the flow chamber. The thermography shows the distribution of temperature around the flow chamber is maintained at 310 K. In Fig. 7, the red lines shows the tracings of micro spheres rolling on the surface with the micro grooves on the culture plate for 0.2 s in the extra flow test. The micro spheres rolling over the micro grooves (the angle between the longitudinal direction of groove and the flow is 45 degrees) shows the parallel steady stream lines. Fig. 8 shows the inverted-phase-contrast microscopic image of cells on the micro-pattern. Several cells adhere in two hours. Fig. 9 shows the scanning electron microscope images of cells on the ridge between grooves at the end of the test. Some cells adhere along the ridge line, while another cell makes a bridge between ridges.

Fig. 10 shows the number of cells adhered on the micro pattern. The number of cells increases without exposure to the shear stress (Fig. 10A). Although the initial number of cells is scattered, the number of cells increases after stopping (6 h) the exposure to the shear stress. Most of cells are exfoliated by the exposure to the shear stress of 1.5 Pa for four hours (Fig. 10D). Most of cells were exfoliated immediately after exposure to the shear stress of 2.0 Pa. Fig. 11 shows the oriented ratio (%) of cells. Most of cells keep orientation along the ridge between grooves. The orientation ratio at 90 degrees tends to decrease by the exposure to the shear stress. Fig. 12 exemplifies the tracings (every hour) of the migration of each cell. The migration along the striped micro ridge between grooves (including migration to the upstream) decreases under exposure to the shear stress of 1 Pa (Fig. 12c). Fig. 13 exemplifies the tracings (every hour) of the shape index of each cell. Cells are exposed to the wall shear stress of 0.5 Pa for four hours (from 2 h to 6 h). The cell tends to be

rounded (decrease of *SI*), when the cell is exposed to the wall shear stress (from 2 h to 6 h). After stopping exposure to the shear stress, the cell tends to be elongated except on the micro pattern of 90 degrees (Fig. 13c).

4. DISCUSSION

In the present experimental device, the depth of the groove is 1 μm, which is minimum value for making orientation of cells [12]. The effect of the shear flow on myoblast was investigated in the previous study [9]. The effect of the shear flow on fibroblast [15] has been investigated in the present study.

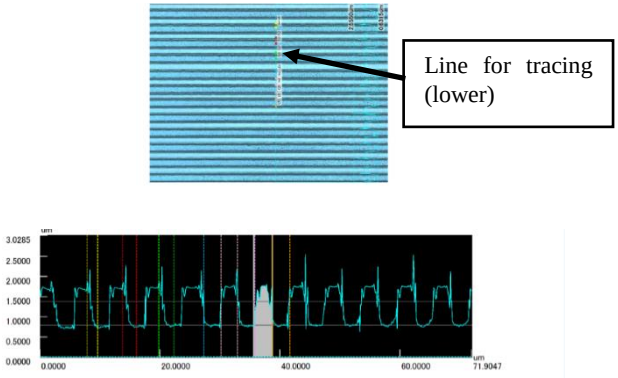


Fig. 5: Tracings (lower) of surface of mold along the line at laser image (upper) for upper plate; unit, μm.

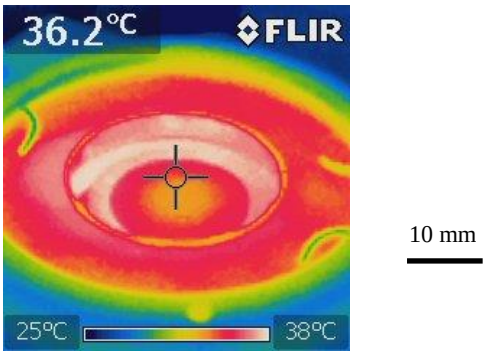


Fig. 6: Thermographic image of flow chamber.

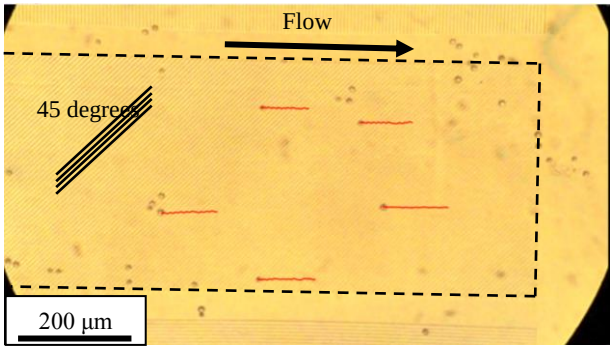


Fig. 7: Tracings of micro spheres rolling on the surface with the micro grooves (45 degrees) on the culture plate in the extra flow test: red line shows tracing for 0.2 s.

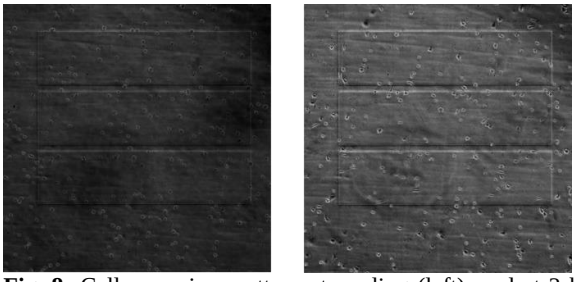


Fig. 8: Cells on micro-pattern at seeding (left), and at 2 hours without flow after seeding (right).

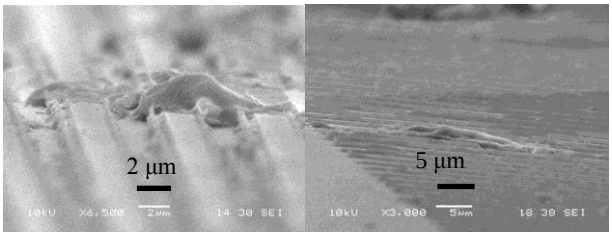


Fig. 9: SEM image of cell on micro ridge between grooves.

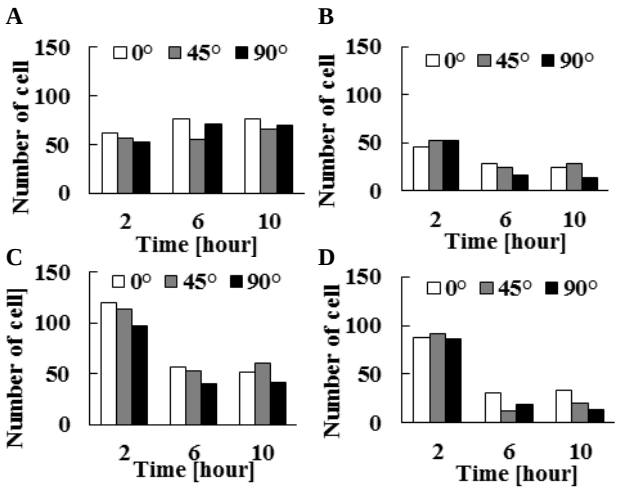


Fig. 10: Number of cells adhered on each micro-pattern vs. time: control (A), 0.5 Pa (B), 1 Pa (C), 1.5 Pa (D).

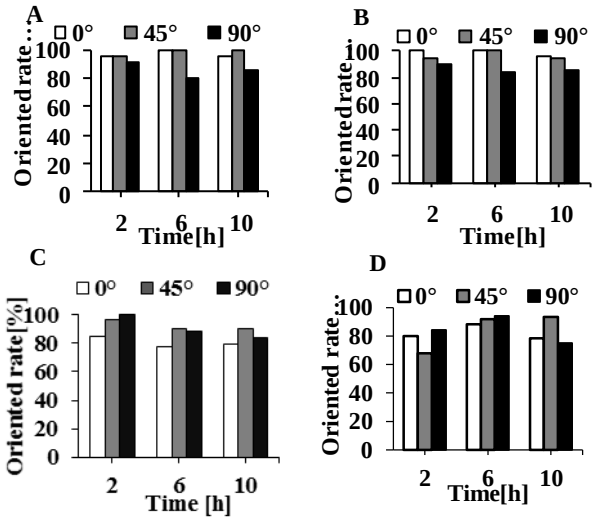


Fig. 11: Oriented ratio (%) of cells vs. time on each micro-pattern: control (A), 0.5 Pa (B), 1 Pa (C), 1.5 Pa (D).

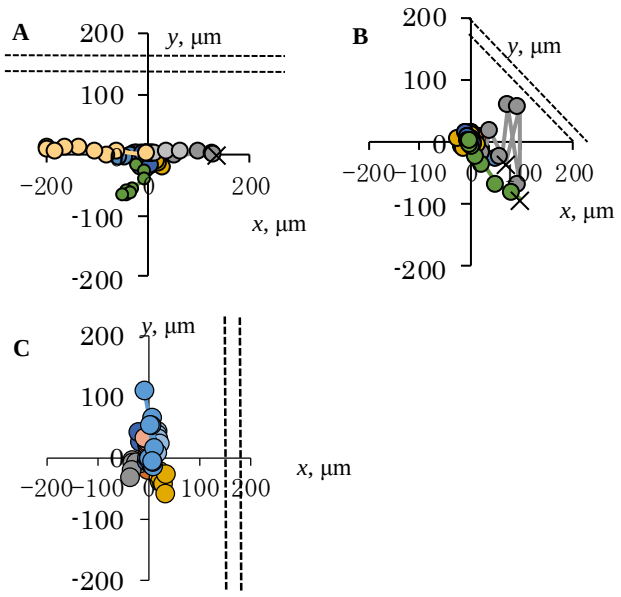


Fig. 12a: Migration of cell on micro pattern without flow: 0 degree (A), 45 degrees (B), and 90 degrees (C): unit μm.

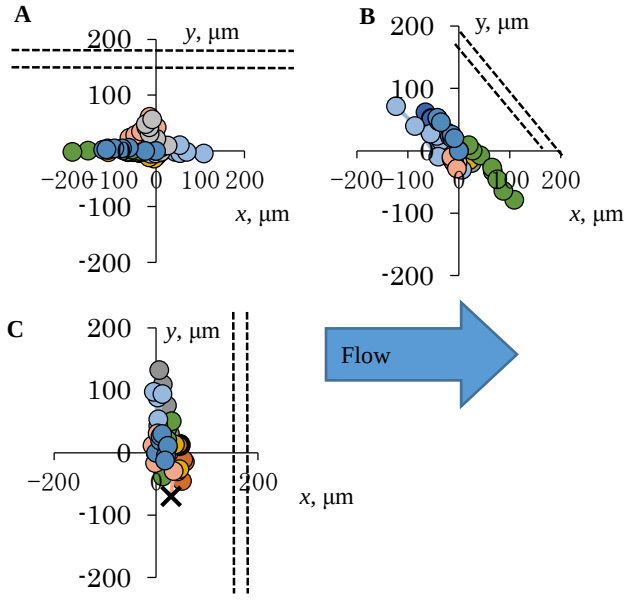
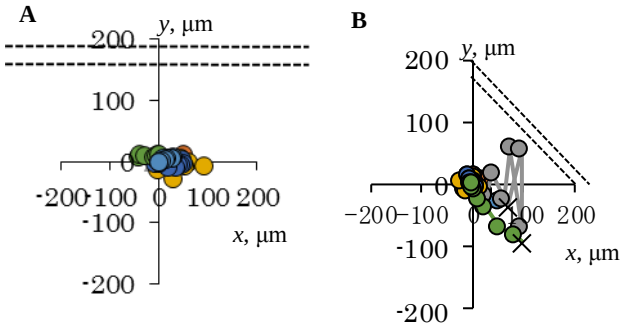


Fig. 12b: Migration of cell on micro pattern at 0.5 Pa: 0 degree (A), 45 degree (B), and 90 degree (C): unit μm.



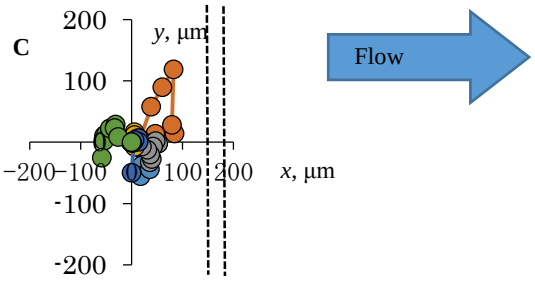


Fig. 12c: Migration of cell on micro pattern at 1 Pa: 0 degree (A), 45 degrees (B), and 90 degrees (C): unit μm.

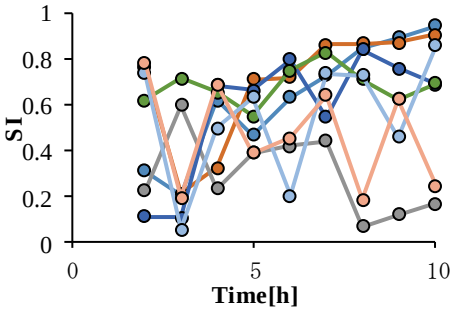


Fig. 13a: shape index (*SI*) of each cell (0 degree): wall shear stress of 0.5 Pa from 2 h to 6 h.

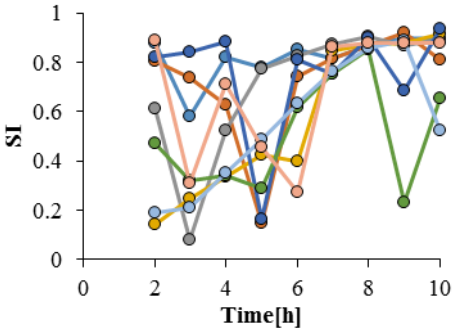


Fig. 13b: shape index (*SI*) of each cell (45 degrees): wall shear stress of 0.5 Pa from 2 h to 6 h.

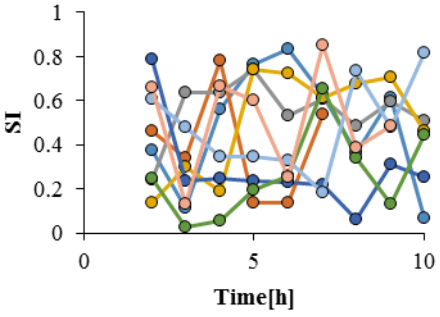


Fig. 13c: shape index (*SI*) of each cell (90 degrees): wall shear stress of 0.5 Pa from 2 h to 6 h.

The flow is one of the stimuli on cells in the experimental systems: the donut type canal [3], the cylindrical pipe, the channel between parallel walls [9], and Couette type flow using rotating disk. For quantitative study, Couette type flow or Poiseuille type flow has advantage.

On the scaffold, the direction of each cell randomly scatters. To control the direction of adhesion of the cell, the micro pattern of the surface of the scaffold is available. Both the migration and the deformation of the cell depend on the micro morphology of the surface of the scaffold. The photolithography technique can be applied to make the micro morphology on the surface [13]. In the present study, the lines of micro ridges of minimum height [12] are designed on the scaffold to make orientation of cell. The dimension of the width of the micro groove is smaller than the diameter of the cell, so that each cell does not fall into the groove. The wall shear stress around 1 Pa is selected in the present study as the typical value, which affected cells in the previous study [9]. Three types of direction (parallel, diagonal, and perpendicular) has been tested for the orientation of cells in the flow. The micro pattern on the scaffold can also be used as markers to trace the cell. Cells keep the active action (deformation, migration, and proliferation) in the flow stimulation at the lower shear stress (< 3 Pa). The cell also migrates to the counter direction of the flow. The higher wall shear stress (> 3 Pa) reduces the migration of cells. A cell deforms to a round shape at the high wall shear stress. The wall shear stress of 1 Pa enhanced the migration of C2C12 along the ridge in the previous study [9]. Cells restart migration after stopping the wall shear stress of 3 Pa within four hours, which shows that the cells are not damaged. The reaction might depend on the kinds of cell. C2C12 made perpendicular orientation of myotubes to the flow direction in the previous study [3].

5. CONCLUSION

The effect of shear flow on an oriented cell has been studied by the flow channel with the micro striped pattern of ridge lines on the wall of the scaffold *in vitro*. The lines of parallel micro quadrangular prism ridges (0.001 mm height, 0.003 mm width, and 0.003 mm interval) are made by the photolithography technique on the lower surface of the channel as the scaffold to make orientation of each cell. Variation is made about the angle between the longitudinal direction of the ridge and the direction of the flow: 0, 45, 90 degrees. The experimental results show that both the migration and the deformation of each cell of 3T3-L1 (mouse fat precursor cells) along the micro ridge are restricted by the wall shear stress higher than 1 Pa. The flow channel system with the micro pattern of ridge lines *in vitro* is effective to evaluate the effect of flow on oriented cells quantitatively.

6. ACKNOWLEDGMENT

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