Orientation of Cells Cultured in Vortex Flow with Swinging Plate in Vitro

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ABSTRACT

An effect of flow on cell culture has been studied *in vitro*. A silicone disk was placed in the center of culture dish of 52 mm internal diameter to make a doughnut-shaped canal. The dish was placed on a tilted plate, which rotates to make a vortex flow around the silicone disk with a swing motion. Variations were made on the diameter (20 mm, 30 mm, and 40 mm) of the silicone disk and the rotational speed (2.1 rad/sec, 5.2 rad/sec) of the swinging plate, which tilts with 0.1 rad from the horizontal plane. Five kinds of cells were cultured in the vortex flow of Dulbecco's Modified Eagle's Medium for seven days: C2C12 (mouse myoblast), L6 (rat skeletal muscle cell), A7r5 (rat aortic smooth muscle cell), CS-2P2-C75 (primary normal porcine aortic endothelial cell), and L929 (mouse fibroblast). The experiments show the following results. The orientation of cells depends on flow and on kinds of cells. A7r5 and CS-2P2-C75 line along the streamline of the flow. C2C12 and L6 adhere along the direction of the flow in the first stage, and tilt to the perpendicular direction to the flow differentiating to myotubes with fusion in the second stage.

Keywords: Biomedical Engineering, Muscle Cells, Endothelial Cells, Cell Culture, Flow and Orientation

1. INTRODUCTION

Cell culture technique has been progressed and several methods have been studied to arrange cell orientation to make engineered tissue. Myocytes can be cultured and can be differentiated to myotubes *in vitro*. The repetitive contractive movement can be controlled with electric pulses. The efficiency of contraction depends on the orientation of myotubes. Acceleration technique for orientation of cells has been studied to make muscle tissue *in vivo* and *in vitro* [1-3]. Control methodology for orientation of cells would be applied to regenerative tissue technology. For example, myoblasts have been clinically applied to ischaemic cardiomyopathy in the field of regenerative medicine.

Behavior of biological cells depends on various environmental factors, such as electric [4, 5], magnetic [6] and mechanical fields [2, 3, 7]. The cells might tilt to the direction of these fields. Erythrocytes, for example, orient under the blood flow [8-11].

Several systems for the medium circulation have been designed to apply flow stimulation on the cell culture [9]. Most of them use pumps and tubes, which might cause an extra effect of materials of the flow path.

In the present study, a novel simple culture methodology with a vortex flow of the medium has been developed to make orientation of cells *in vitro*.

2. METHODS

Culture system with vortex flow

A cell culture system has been designed to apply a vortex flow on cells *in vitro*. A polystyrene culture dish without collagen coating (Iwaki, 3010-060, Asahi Glass Co., Ltd, Tokyo) was used. A silicone rubber disk of 3 mm thick (K-125, Togawa Rubber Co., Ltd., Osaka) is attached on the inner bottom at the center of the culture dish of 52 mm internal diameter to restrict the space for the flow of the medium (Fig. 1). Variation was made on the disk's diameter between 20 mm and 40 mm. The silicone rubber disk is stuck on the bottom of the dish with affinity between their surfaces without bond.

The culture dish is placed on the plate, which inclines at 0.1 rad of the horizontal plane (Fig. 2). The plate rotates to generate a swing motion (WAVE-SI, Taitec, Co., Ltd., Koshigaya). Variation was made on the rotating speed of the plate between 20 and 50 revolutions per minute (rpm). The motion produces a one-way clockwise vortex flow in the medium around the silicone rubber disk in the dish. Fifteen dishes can be simultaneously placed on the plate, which generates the same vortex flow in the medium in each dish.

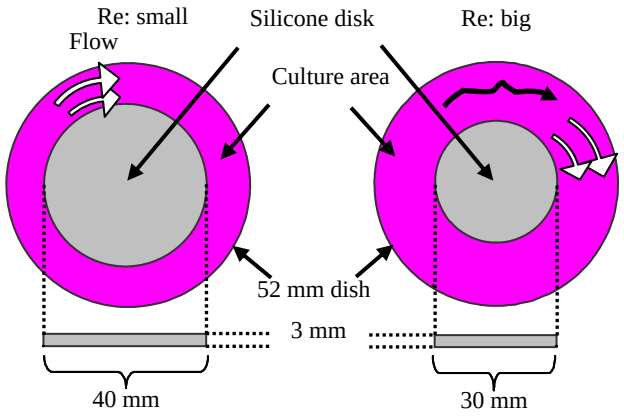

Fig. 1: Culture dish with silicone disk. The doughnut-shaped area is filled with medium.

Fig. 2: Culture dishes on swinging plate in incubator.

The continuously swinging plate is placed in an incubator, where temperature of 37 degrees Celsius and carbon dioxide partial pressure of 5 percent are maintained.

Cell Culture

Variations were made on cells: C2C12 (Mouse myoblast cell line originated with cross-striated muscle of C3H mouse), L6 (rat skeletal muscle cell), A7r5 (rat aortic smooth muscle cell), CS-2P2-C75 (primary normal porcine aortic endothelial cell), and L929 (mouse fibroblast). Each kind of cells was suspended in the Dulbecco's Modified Eagle's Medium (D-MEM) with density of 1.0×10^6 cells per mL. Fetal bovine serum (FBS) was added to the medium with the volume rate in 10 percent of FBS and 90 percent of D-MEM. The suspension was poured into the dish and cultured in the incubator for seven days, while the plate was continuously rotating to make a steady vortex flow around the silicone rubber disk in the medium.

The volume of the suspension is 2 mL for silicone disk of 40 mm diameter, and 3 mL for that of 30 mm diameter and 20 mm diameter, respectively. The volume of the medium is adjusted to cover whole surface of the bottom of the culture dish around the silicone disk, and not to flow over the superior surface of the silicone disk during the swing motion of the plate. The cells were cultured in the vortex flow of the medium, while the plate was continuously rotating at 37 degrees Celsius in the incubator. The medium was exchanged every two days.

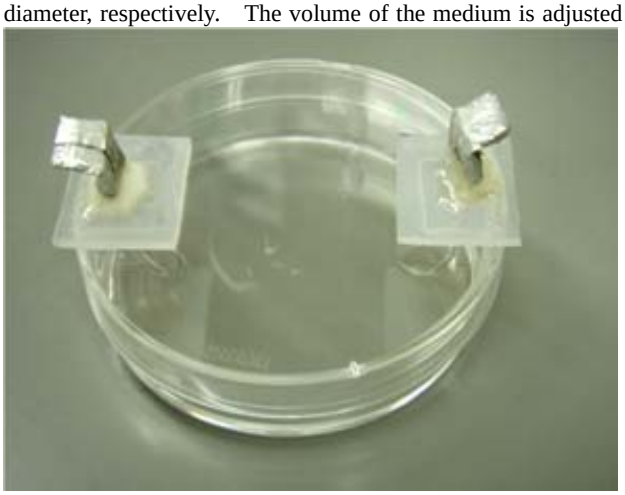

Fig. 3: A pair of electrodes attached on the cover of the culture dish.

The directions of orientation of cells were observed with an inverted phase-contrast microscope. On the external surface of the bottom plate of the culture dish, lines were attached to mark the radial direction.

In the test of muscle cells, the electric stimulation was applied to the culture medium, and the contractive movement was observed under the microscope. The electric pulses (the pulse width of one millisecond, the period of one second, and the amplitude of 70 V) were applied through a pair of electrodes of platinum wire, which are attached on the cover of the culture dish (Fig. 3).

The number of cells adhered on the bottom of the culture dish was counted after cultivation with the flow. The cells were exfoliated with an enzymatic treatment with the medium, and the suspension was introduced into the interstitial space between the slide glasses to be counted under the microscope. The results were compared to those of cultivation without the flow.

3. RESULTS

The experiments with C2C12 both at 2.1 rad/sec and 5.2 rad/sec show that cells adhere to the bottom of the culture dish adjacent to the silicone disk in 24 hours (Fig. 4), and that no adhered cell is observed in the circumferential area of the dish. The muscle cells are arranged along the circumferential streamline around the silicone rubber disk (Fig. 4). The cells proliferate and fuse to become myotubes in the vortex flow of the medium. The array of myotubes grows around the silicone disk day by day, and the alignment curves to the radial direction (Figs. 5, 6). The results show that fused cells elongate to the radial direction around the silicone disk, and that the major axes orient to the direction perpendicular to the flow on the third day of culture (Fig. 5). The experiment shows that cells proliferate to form confluent monolayer (Fig. 6).

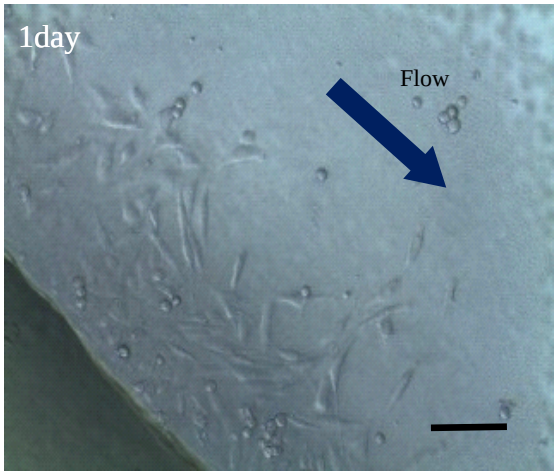

Fig. 4: C2C12, 5.2 rad/sec, 1 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 30 mm.

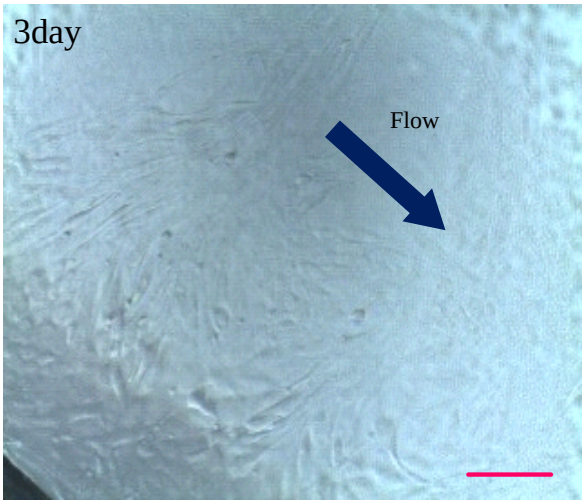

Fig. 5: C2C12, 5.2 rad/sec, 3 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 30 mm.

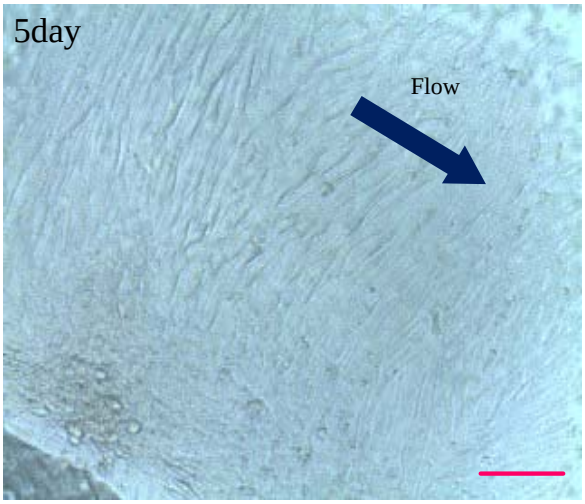

Fig. 6: C2C12, 5.2 rad/sec, 5 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 30 mm.

The experiment also shows that the elongated fused cells (Fig. 7) differentiate to myotubes to repeat contraction synchronously with the electric pulses applied to the medium on the seventh day of culture.

Only around the silicone disk of 20 mm diameter at 5.2 rad/sec, fused cells of C2C12 did not elongate to radial direction. The experimental results show that cells adhere and orient to the circumferential direction only adjacent to the silicone disk of 20 mm diameter at 5.2 rad/sec.

The experiment with L6 at 2.1 rad/sec shows that cells adhere to the bottom of the culture dish adjacent to the silicone disk in 24 hours, and that no adhered cell is observed in the circumferential area of the dish (Fig. 8). The results show that cells elongate to the radial direction around the silicone disk,

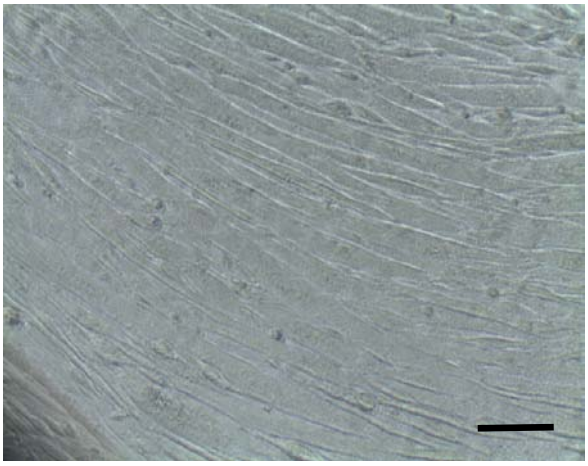

Fig. 7: C2C12, 5.2 rad/sec, 30 mm, 7 day. The bar shows 0.1 mm. Myotubes contract with electric pulses through electrodes (Fig. 3).

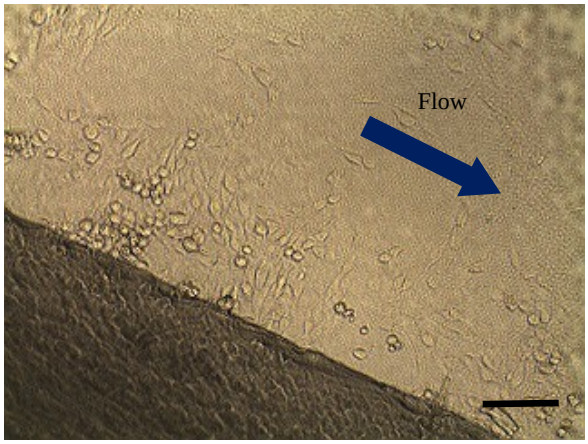

Fig. 8: L6, 2.1 rad/sec, 1 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 40 mm.

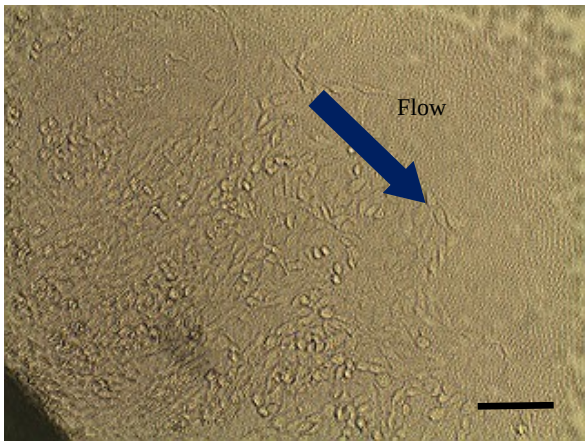

Fig. 9: L6, 2.1 rad/sec, 3 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 40 mm.

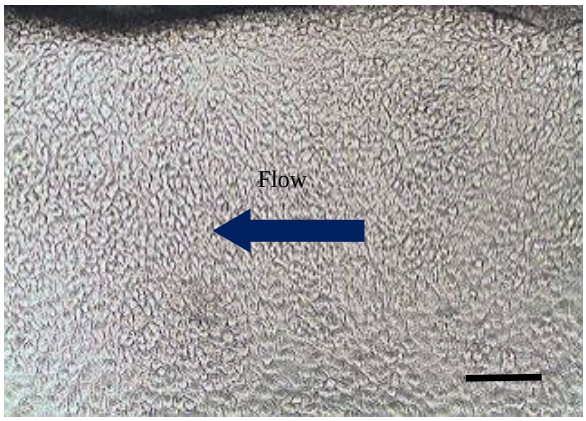

Fig. 10: L6, 2.1 rad/sec, 5 day. The bar shows 0.1 mm. Above: silicone disk of 40 mm.

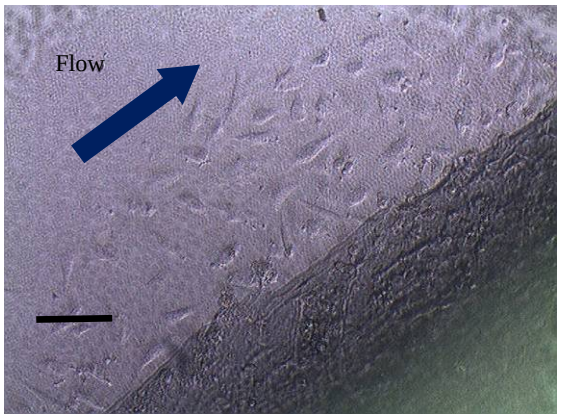

Fig. 11: A7r5, 2.1 rad/sec, 1 day. The bar shows 0.1 mm. Bottom-right: silicone disk of 30 mm.

and that the major axes are oriented to the direction perpendicular to the flow on the third day of culture (Fig. 9). The results are similar around a silicone disk of every diameter at every rotational speed of the swinging plate (Fig. 10).

The experiment with A7r5 at 2.1 rad/sec shows that cells adhere to the bottom of the culture dish adjacent to the silicone disk in 24 hours (Fig. 11). The experiment shows that the area of adherence extends to the radial direction, and that cells elongate to the spindle shape of which long axis tilts to the circumferential flow direction after the third day of culture (Fig. 12).

The experiment with CS-2P2-C75 at 2.1 rad/sec shows that cells adhere to the bottom of the culture dish adjacent to the silicone disk in 24 hours (Fig. 13). The experiment shows that the area of cells adherence extends to the radial direction, and that cells elongate to the spindle shape of which long axis tilts to the circumferential flow direction on the third day of culture (Fig. 14).

The experiment with L929 at 2.1 rad/sec shows that cells adhere to the bottom of the culture dish adjacent to the silicone disk in 24 hours, and that no adhered cell is observed in the circumferential area of the dish. The results show that cells elongate to the radial direction around the silicone disk, and that the major axes are oriented to the direction perpendicular to the flow after the third day of culture (Fig. 15). The results are

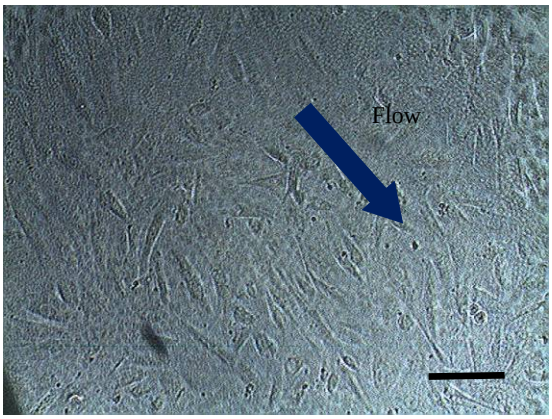

Fig. 12: A7r5, 2.1 rad/sec, 5 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 30 mm.

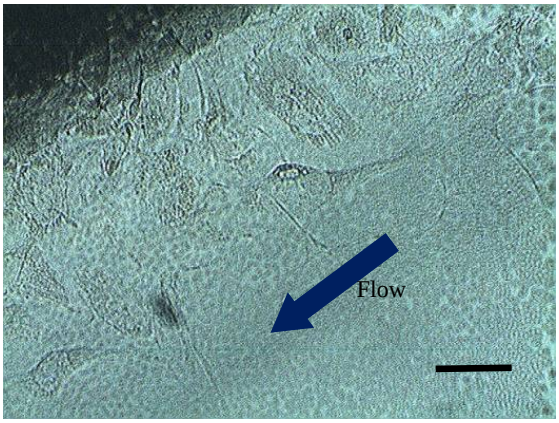

Fig. 13: CS-2P2-C75, 2.1 rad/sec, 1 day. The bar shows 0.1 mm. Above-left: silicone disk of 30 mm.

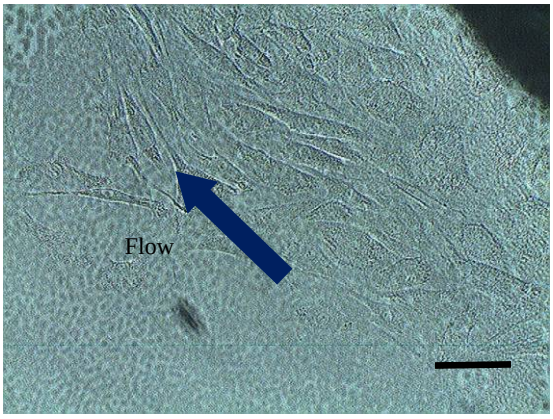

Fig. 14: CS-2P2-C75, 2.1 rad/sec, 3 day. The bar shows 0.1 mm. Above-right: silicone disk of 30 mm.

similar around a silicone disk of every diameter.

The experiment with L929 at 5.2 rad/sec shows that cells do not adhere to the bottom of the culture dish around the silicone disk in seven days, and that cells are floating in the medium. The results of successive experiment show that cells adhere to the bottom of the culture dish and that proliferate to form confluent monolayer in successive seven days after the flow stops.

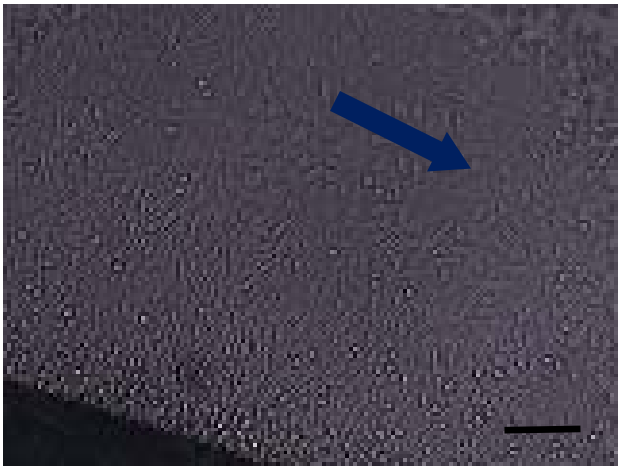

Fig. 15: L929, 2.1 rad/sec, 5 day. The bar shows 0.1 mm. Bottom-left: silicone disk of 30 mm. The arrow shows direction of flow.

The medium flow around the silicone disk synchronously with the movement of the swinging plate was observed with the movement of a tracer particle.

The experimental results show that cells arrays extend to the area, where cells have not adhered yet. The number of cells adhered on the bottom of the culture dish is smaller with the flow than without the flow.

4. DISCUSSION

The previous study shows that electric stimulation enhances differentiation of muscle cells [4, 5]. Another study shows mechanical stimulation improves tissue-engineered human skeletal muscle [3].

The previous study shows that cells orient along grooves [11]. In the previous study, micro patterning technique has been applied to the experiment to investigate relation between morphology of the surface and orientation of cells [12, 13]. Oriented fibers have been applied to the scaffold of cells [14]. The mechanical property of the scaffold should be adjusted that of myotubes, when the combined unit works as an actuator. Orientation of myotubes without scaffold might have an advantage for a simple design of a tissue-engineered actuator [15, 16].

The orientation of cells in the present system depends on several factors (Fig. 16(a)): (1) shear stress under flow, (2) gravitational force with slope, (3) centrifugal force with rotation, (4) interactive force between cell and scaffolds (adhered chemicals or wall of dish), (5) interactive force between cells (repulsion or fusion). The following is estimation on the process of orientation of cells in the present study. Before adhesion of cells, adhesion of protein might occur on the bottom of the dish (Fig. 16(b)). The medium flow might orient molecules of protein, which prepare the scaffold for cells. Cells adhere in the inner circular area where wall shear stress is low. Adjacent to the aligned cells, following cells adhere along the stream line but tilt to the radial direction according to repulsion between cells (Fig. 16(c)). Muscle cell bridges between oriented proteins (Fig. 16(d)). Muscle cells fuse

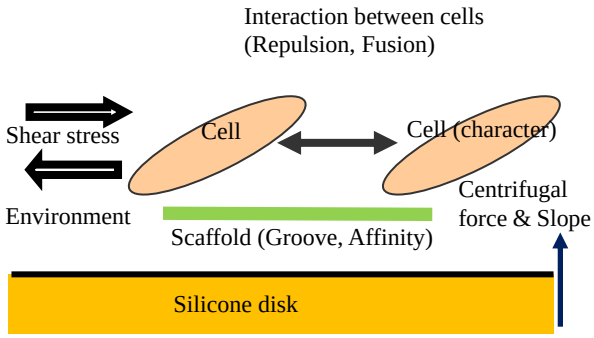

Fig. 16(a): The orientation of cells in the present system depends on several factors.

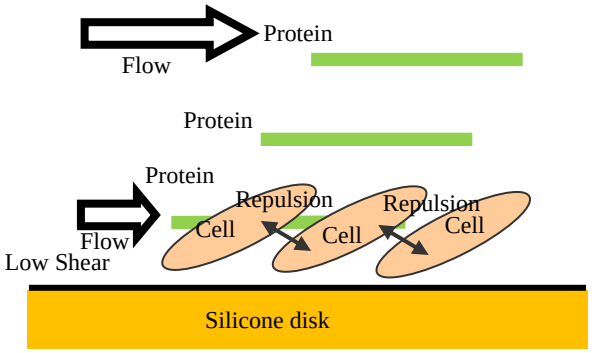

Fig. 16(b): Model on orientation of cells in the flow in the first stage.

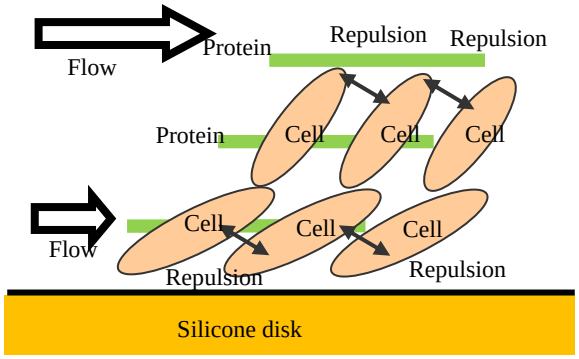

Fig. 16(c): Model on orientation of cells in the flow in the second stage.

along longitudinal direction. During these process, the direction of the orientation of myotubes curves to the direction perpendicular to the stream line (Fig. 16(e)). Endothelial cells, on the other hand, adhere and tilt to the stream line, which makes orientation parallel to the stream lines.

It is rather mysterious that muscle cells orient not along the circumferential direction but along the radial direction, which is perpendicular to the flow direction. The muscle cells might fuse each other along the longitudinal direction to make myotubes. Interaction between cells might affect the orientation, too. The inner part is easy for cells to adhere to the bottom surface, because of the low shear stress in the flow. The area of cells adhesion extends from the rim of the silicone

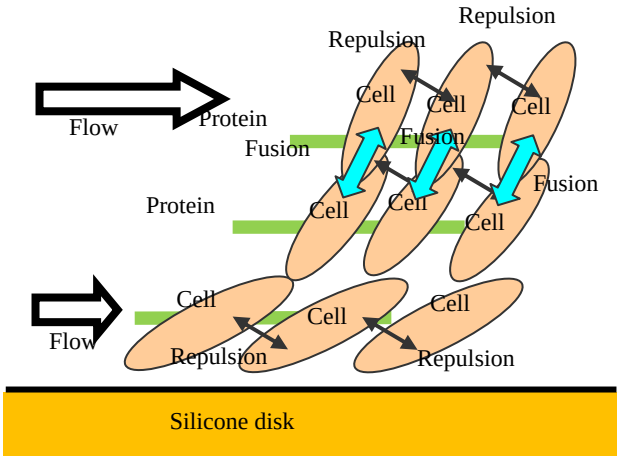

Fig. 16(d): Fusion and repulsion of cells in the flow.

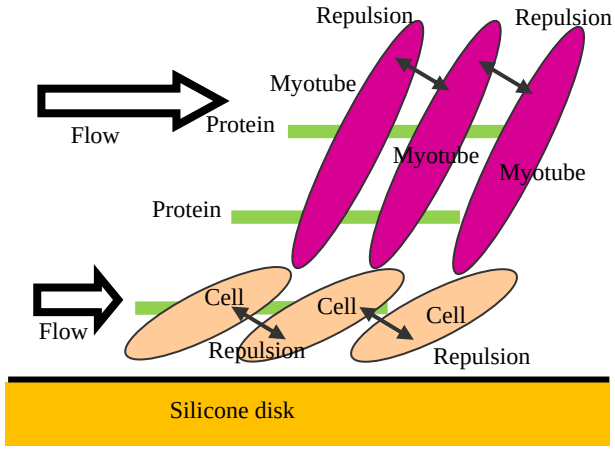

Fig. 16(e): Model on orientation of myotubes in the flow.

disk to the rim of the dish: from inside to out side along the radial direction. Cells cannot adhere in the area of high wall shear stress. Cells cannot adhere in the area of turbulent flow, either. Turbulent flow tends to occur in the fringe area, at the high rotational speed, and in the wide canal between disk and fringe, where Reynolds number is high. Reynolds number (Re) is calculated by Eq. (1).

$$Re = (D\ V\ X) / E \quad (1)$$

Estimated Re is the value between 240 and 1100, where D is density of the fluid (0.001 kg/m3), V is the velocity of the flow (0.04 - 0.1 m/s), X is the width of the flow path (0.006 – 0.011 m) and E is the viscosity of the fluid (0.001 Pa s).

The silicone disk placed in the center of the dish might support the circumferential steady flow, which enhances orientation of cells. The experimental results show that regular arrays of myotubes are grown up in the vortex flow around the silicone disk, while the directions of myotubes are random without the silicone disk [1]. The vortex flow in the dish without the center disk is unsteady and turbulent. Turbulent flow might disturb to orient cells in a flow of higher velocity at higher rotational speed, and in a wider space between the silicone disk and the fringe of the dish (Fig. 1).

The cells cannot orient with flow stimulation after cells proliferation progress. To make orientation of myotubes, continuous flow stimulation is necessary throughout of the process between adhesion of cells and differentiation to myotubes, while the adhesion of cells is decelerated by the flow. The cells might be stabilized in the direction, where the stress on cells is minimized. The previous study shows that smooth muscle cells orient to minimize internal mechanical stress [17]. The cells might receive stress being stretched to the flow direction. The effect of the steady flow might be different from that of pulsatile flow [9].

It is not easy to estimate wall shear stress value on the bottom surface of the dish in the flow of medium, because of the free counter surface of the medium. A test with a flow between parallel plates has advantage to estimate wall shear stress [18]. Endothelial cells tilt to the direction of flow in the present study. Because the endothelial cells orient in the same condition of flow, the shear stress might be in the range of several Pa, which is estimated in the previous studies [8, 10, 19].

In the previous studies, horse serum has been added to initiate differentiation from myocyte to myotube in the cell culture [1]. The myotubes have formed in the present study without horse serum. The flow might stimulate differentiation from myocyte to myotube.

The present study shows that the effect of the flow on orientation of cells depends on the kind of cells, because the effect both of mechanical stress and of environment relate to the property of cells. The muscle cells might have a higher affinity to stick to the bottom of the culture dish.

5. CONCLUSION

The orientation over square millimeter level has been achieved on the myotubes in the present study. The orientation of cells depends on flow and kinds of cells. A7r5 and C75 line along the streamline of the medium flow. C2C12 and L6 adhere along the direction of the medium flow in the first stage, and tilt to the perpendicular direction to the medium flow differentiating to myotubes with fusion in the second stage.

6. ACKNOWLEDGMENT

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