Orientation of Cultured Myotubes in Vortex Flow of Medium with Swinging Plate

Shigehiro HASHIMOTO, Masahide OKADA, Shuichi MOCHIZUKI,
Toshia FUJISATO, Masahiko YOSHIURA, Kazutomo NISHIMURA
Department of Biomedical Engineering, Osaka Institute of Technology, Osaka, 535-8585, Japan
hashimoto@bme.oit.ac.jp http://www.oit.ac.jp/bme/~hashimoto

and

Hajime OTANI
Cardiovascular Center, Kansai Medical University, Moriguchi, Japan

ABSTRACT

A novel culture methodology with the vortex flow of the medium has been developed to make myotubes array *in vitro*. A silicone plate of 3 mm thick was placed in the center of culture dish of 52 mm internal diameter. The culture dish was placed on a tilted plate, which rotates to make a vortex flow around the silicone plate with the swing motion. Variations were made on the diameter (20 mm, 30 mm, and 40 mm) of silicone plate and rotational speed (2.1 rad/sec, 5.2 rad/sec) of swinging plate, which tilts six degree (0.1 rad). C2C12 (Mouse myoblast cell line) cells are cultured in the vortex flow of Dulbecco's Modified Eagle's Medium (1.0×10^7 cells per mL) for twelve days. The volume of the medium is 2 mL for 40 mm diameter, and 3 mL for 30 mm and 20 mm diameter. The width of oriented area was measured under observation with a phase-contrast microscope. The experimental results show that cells tilt perpendicular to the flow direction in every condition and that rotational speed affects on the growth of oriented area with muscle cells.

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

1. INTRODUCTION

Cell culture technique has been progressed and myoblasts have been clinically applied to ischaemic cardiomyopathy in the field of regenerative medicine. Acceleration technique for orientation of cells has been studied to make muscle tissue *in vivo* and *in vitro* [1-4]. Control methodology for orientation of cells would be applied to regenerative tissue technology.

Behavior of biological cells depends on various environmental factors, such as electric, magnetic and mechanical fields. Erythrocytes, for example, are oriented under blood flow [5].

Several systems for medium circulation have been designed to apply flow stimulation on cell culture [6]. Most of them use pumps and tubes, which might cause contamination or materials problem.

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

2. METHODS

Vortex Flow System
A muscle cell culture system has been designed with vortex flow to orient myotubes *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) was attached on the 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 in 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 tilted by 0.1 rad to the horizontal plane (Fig. 2). The plate rotates to generate a swing motion (WAVE-SI, Taitec, Co., Ltd., Koshigaya). Variatiopn was made in 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 are simultaneously acceptable to be placed on the plate, which generates the same vortex flow in the medium in each dish. The continuously swinging plate was placed in an incubator, where temperature of 37 degrees Celsius and carbon dioxide pressure of 5 percent are maintained.

Cell Culture
C2C12 (Mouse myoblast cell line originated with cross-striated

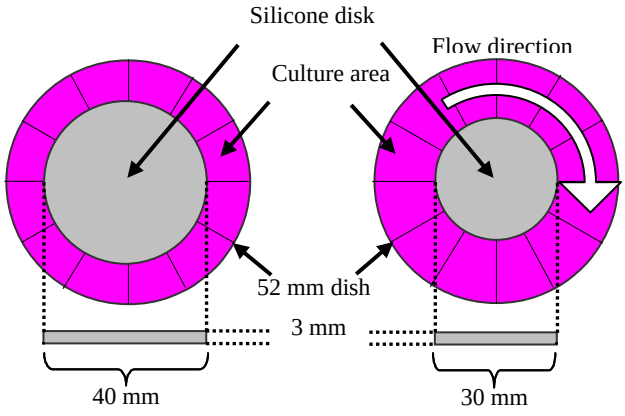

Fig. 1: Culture dish with silicone disk. Torus area is divided into twelve sectors.

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

muscle of C3H mouse) cells were suspended in the Dulbecco's Modified Eagle's Medium (D-MEM) with density of 1.0×10^7 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 an incubator for several 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. The volume of the medium was 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. C2C12 cells were cultured in the vortex flow of the medium, while the plate was continuously rotating at 37 degrees Celsius in an incubator. The medium was exchanged every two days. After four days of cultivation, ten volume percent of FBS in the medium was replaced with seven volume percent of horse serum to differentiate cells into myotubes.

For comparison, cells were cultured in the dish without a silicone rubber sheet at the counter position on the desk.

Measurement of Orientation

The directions of orientation of myotubes 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. The culture area of torus-shape between the outer fringe of the silicone disk and the inner fringe of the dish was evenly divided into 12 sectors to measure area of myotubes array (Fig. 1). Each sector has a central angle of 30 degrees (0.52 rad). The area is counted, where the longitudinal direction of each myotube is within 15 degrees (0.26 rad) declination compared to the radial direction. The area of myotubes array was calculated in each sector along the radial direction of the dish. The distance between the top of myotube array, which is oriented to radial direction, and the fringe of silicone disk was measured in every radial direction.

3. RESULTS

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

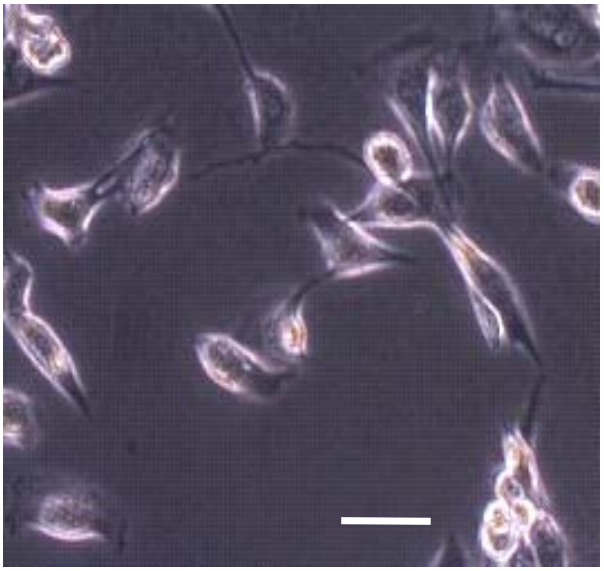

Fig. 3: Spindle-shaped C2C12 on the first day of culture. Bar indicates 0.05 mm.

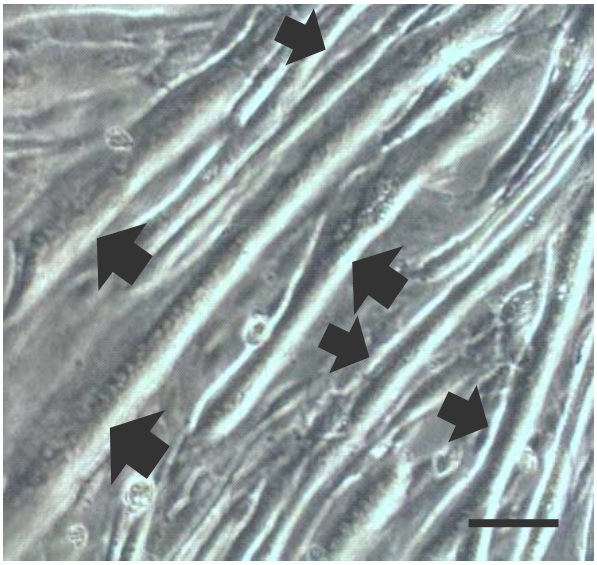

Fig. 4: Myotubes on the seventh day of culture contracted by electric stimulation. Bar indicates 0.05 mm.

Muscle cells were not oriented with a vortex flow, after cultivation for three days without a vortex flow. The adhered cells did not move and fuse to extend array. The experimental results show that cells array is extended to the area, where cells have not adhered yet.

When the diameter of the silicone disk was changed to 10 mm in the culture dish of 52 mm internal diameter, myotubes were not arrayed in the vortex flow of medium in a week of cultivation. When the internal diameter of the culture dish was changed to 85 mm with the silicone disk of 40 mm, myotubes were not arrayed in the vortex flow of medium in a week of cultivation, either. The cells were arrayed on the swinging plate, on the other hand, even when the tilting angle varies from 0.1 rad to 0.07 rad.

The muscle cells adhered to the bottom of the culture dish in two days (Fig. 3) and were arranged along the circumferential streamline around the silicone rubber disk, while the cells proliferated and fused to become myotubes in the vortex flow of the medium (Fig. 4). The experimental results show that

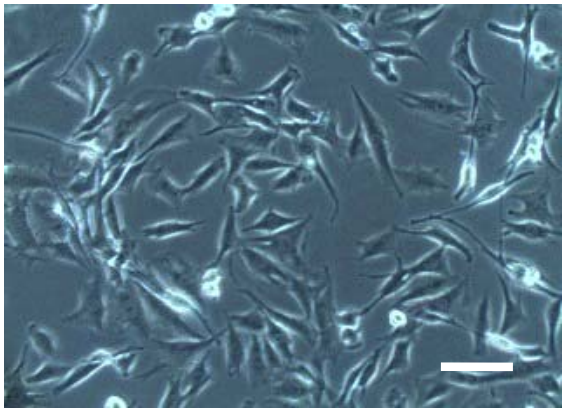

Fig. 5: C2C12 on the first day of culture in vortex flow without silicone disk. Bar indicates 0.05 mm.

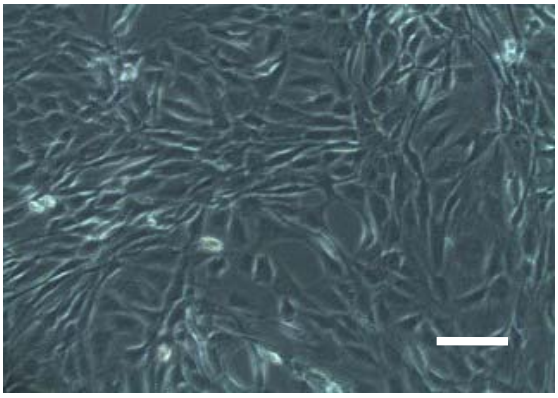

Fig. 6: C2C12 on the fifth day of culture in vortex flow without silicone disk. Bar indicates 0.05 mm.

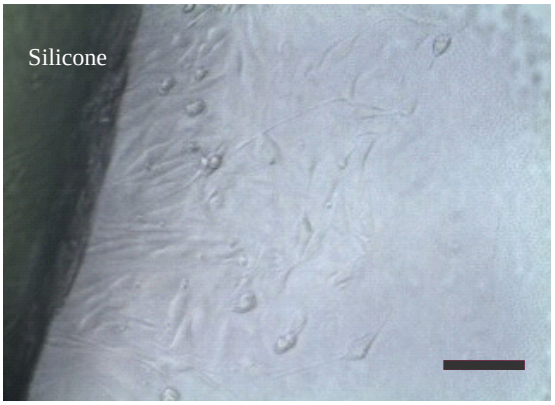

Fig. 7: C2C12 on the first day of culture in vortex flow with silicone disk of 30 mm. Bar indicates 0.05 mm.

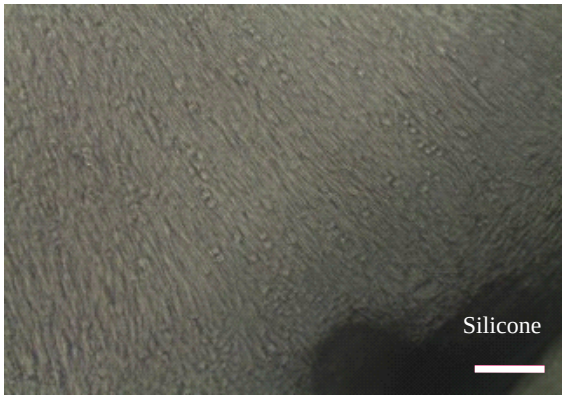

Fig. 8: C2C12 on the fifth day of culture in vortex flow with silicone disk of 30 mm. Bar indicates 0.05 mm.

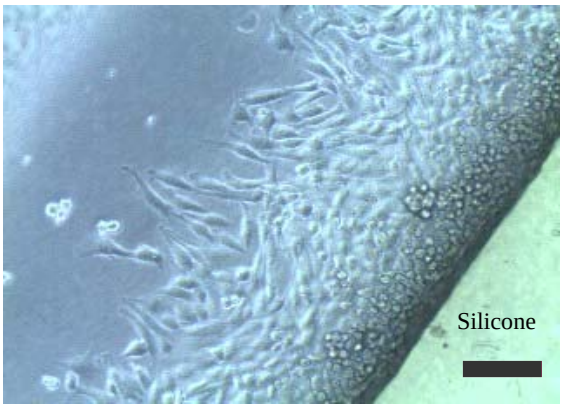

Fig. 9: C2C12 on the first day of culture in vortex flow with silicone disk of 40 mm. Bar indicates 0.05 mm.

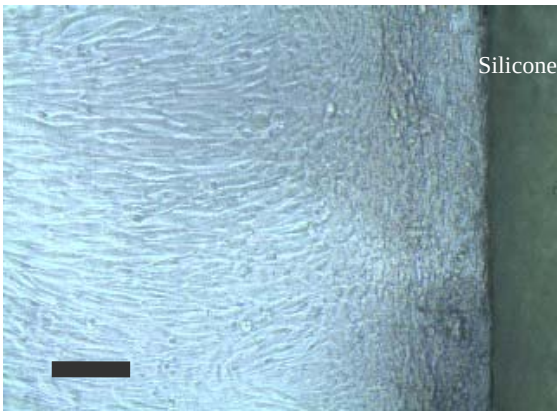

Fig. 10: C2C12 on the fifth day of culture in vortex flow with silicone disk of 40 mm. Bar indicates 0.05 mm.

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 (Figs. 5&6). The array of myotubes grew around the silicone disk day by day, and the alignment curved to the radial direction near the disk (Figs. 7-10).

Figs. 11 and 12 show the orientation of mytubes compared to the radial direction around the silicone disk, where the radial direction is indicated with a straight line. The myotube array grows near the silicone disk (Fig. 11), while the orientation decreases in the area away form the silicone disk (Fig. 12).

Fig. 13 exemplifies the array area ratio in each sector on the seventh day of culture in the vortex flow around the silicone disk. The experimental results show that myotube arrays grow to occupy one percent of culture area in every sector in a week of cultivation (Fig. 13). Figs. 14-19 show the myotube array distance from the fringe of the silicone disk at each sector. The results on two or three dishes are simultaneously displayed with differentiated marks in Figs. 14-19. The same legend symbols of data indicate data in the same dish in Figs. 14-21. The myotube arrays grow up to 1 mm distance from the fringe of the silicone disk at every sector at 20 rpm (Figs. 14-16), while the growth decreases around the disk of 20 mm diameter at 50 rpm (Fig. 17). Around the silicone disk of 40 mm diameter, myotubes array is maximized in the vortex flow at the speed of 50 rpm (Fig. 19).

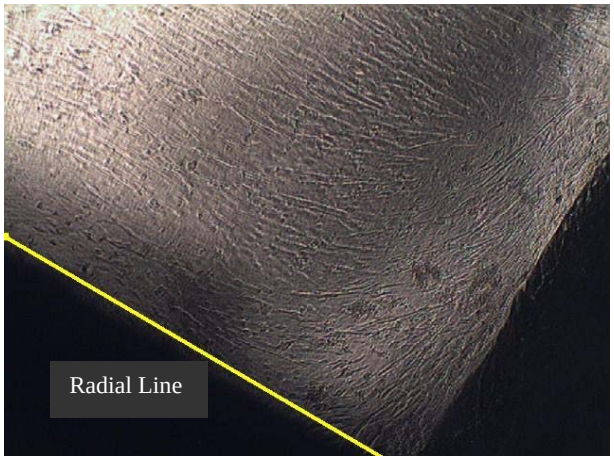

Fig. 11: C2C12 near silicone disk of 30 mm on the 12th day of culture in vortex flow. Line shows radial direction. Bar indicates 0.05 mm.

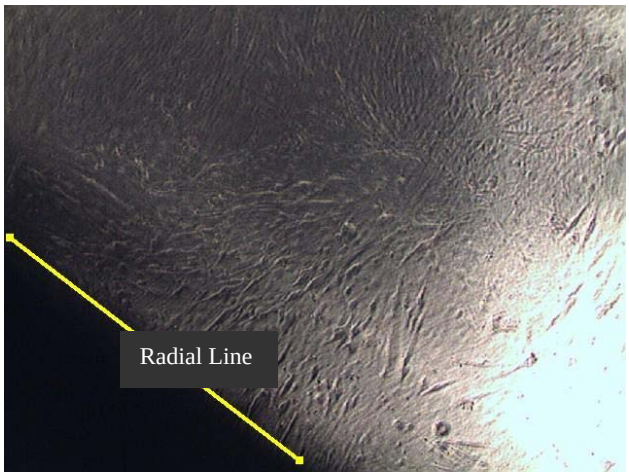

Fig. 12: C2C12 away from silicone disk of 30 mm on the 12th day of culture in vortex flow. Line shows radial direction. Bar indicates 0.05 mm.

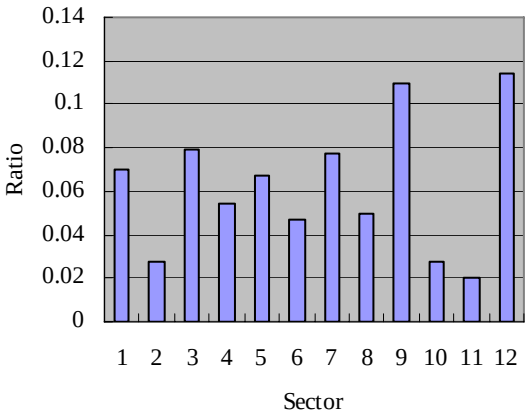

Fig. 13: Array area ratio in each sector on the seventh day of culture.

Figs. 20 and 21 exemplify the array area ratio along the radial direction between the outer fringe of the silicone disk and the inner fringe of the dish. The ratio of unity indicates that every myotube is arrayed to radial direction in Figs. 20 and 21. The results show that myotubes array to the radial direction grows in the inner side of the torus-shape flow path between the disk and the fringe of the dish. The myotubes tend to array to

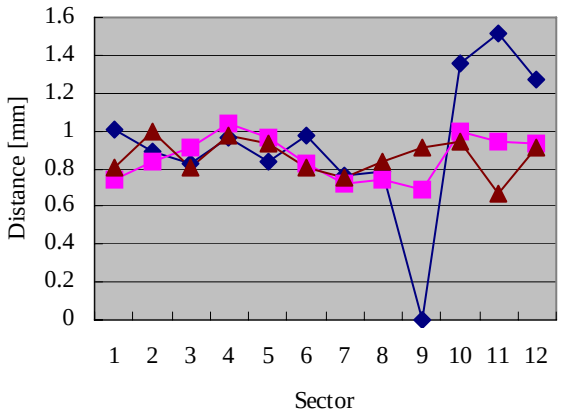

Fig. 14: Myotube array length in each sector around silicone disk of 20 mm at 20 rpm.

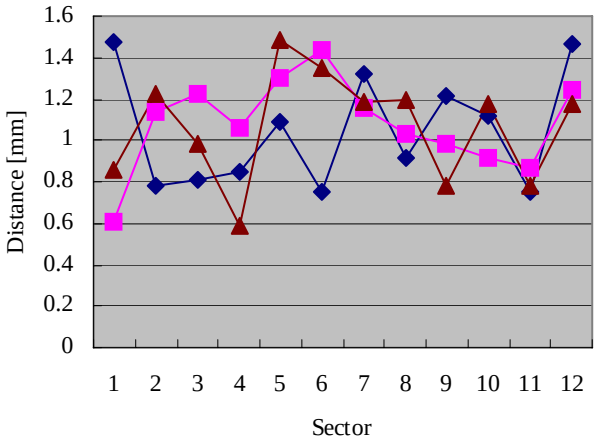

Fig. 15: Myotube array length in each sector around silicone disk of 30 mm at 20 rpm.

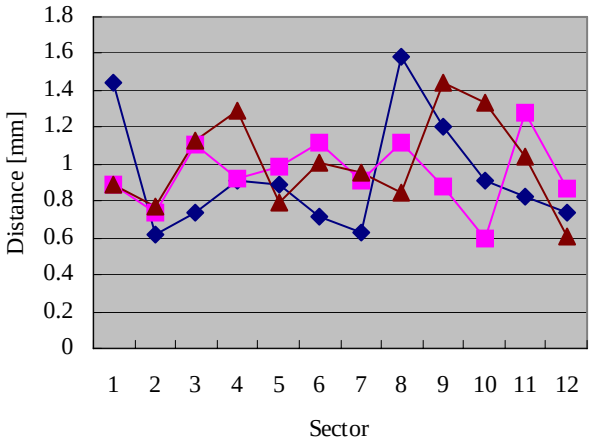

Fig. 16: Myotube array length in each sector around silicone disk of 40 mm at 20 rpm.

circumferential direction adjacent to the disk (Figs 10 & 11).

The myotubes (Fig. 4) contracted synchronously with electric pulses, which was applied to the surrounding medium at a period of 1 second through an electrode of a platinum wire. After repetitive contraction for thirty minutes with electric pulses (pulse width of 1 ms, period of 1 s, amplitude of 70 V), the myotube peeled off from the bottom of the dish and shrank.

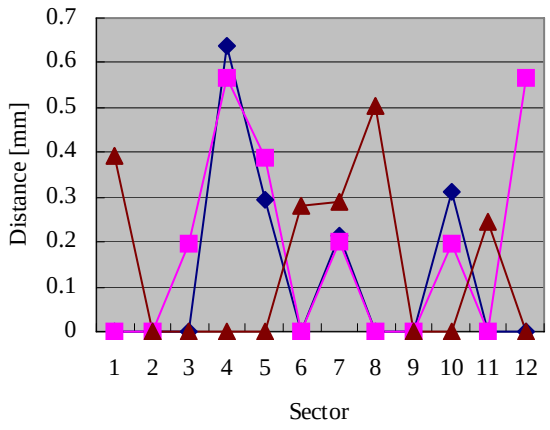

Fig. 17: Myotube array length in each sector around silicone disk of 20 mm at 50 rpm.

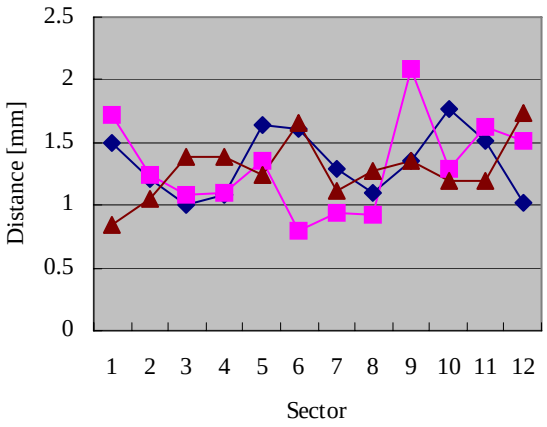

Fig. 18: Myotube array length in each sector around silicone disk of 30 mm at 50 rpm.

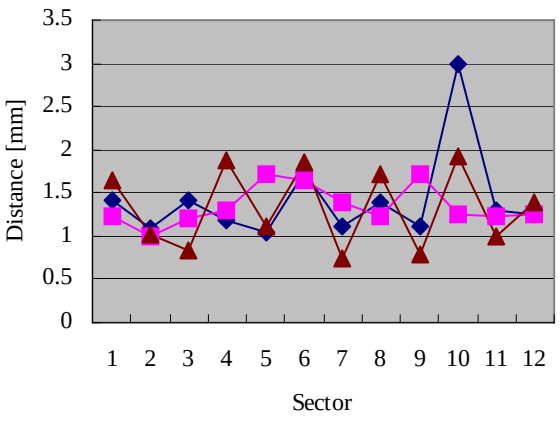

Fig. 19: Myotube array length in each sector around silicone disk of 40 mm at 50 rpm.

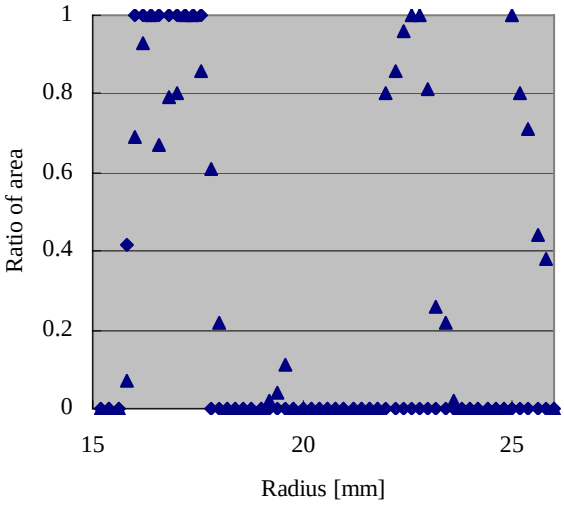

Fig. 20: Relation between array area ratio and radial position on the twelfth day of culture (30 mm disk).

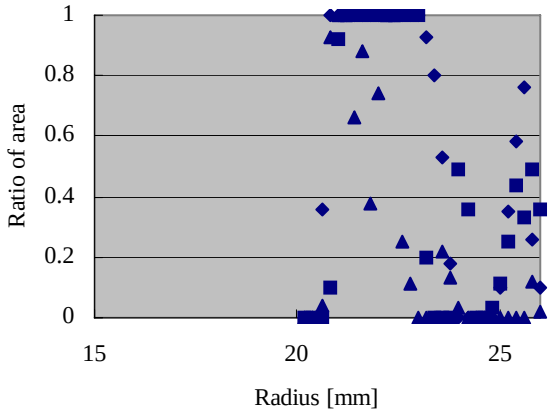

Fig. 21: Relation between array area ratio and radial position on the twelfth day of culture (40 mm disk).

4. DISCUSSION

Muscle cells were not oriented with a vortex flow, when the cells adhere to the bottom of the dish after normal cultivation for 3 days. Following the experimental results, cells were exposed to the vortex flow before adherence to the bottom of the dish.

The previous study shows that electric stimulation enhances differentiation of muscle cells [7]. Another study shows mechanical stimulation improves tissue-engineered human skeletal muscle [3].

A polystyrene culture dish without collagen coating was used in the present study, to decelerate adhesion of cells to the bottom the dish.

The experimental results show that cells tilt perpendicular to the flow direction in every condition and that rotational speed affects on the growth of oriented area with muscle cells. The cells might be stabilized in the direction, where the stress on

cells is minimized [8]. The cells, on the other hand, might accept stress being stretched to the flow direction. The slope, which is generated by the swing motion of the tilted plate, also affects to the radial direction of the myotube array.

The silicone disk placed in the center of the dish might fix the circumferential steady flow, which enhances orientation of myotubes. The vortex flow in the dish without the center disk is unsteady and turbulent. Turbulent flow might disturb to orient myotubes 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.

Orientation of myotubes might affect the effect of contraction of each myotube.

5. CONCLUSION

A novel culture methodology with the vortex flow of the medium has been developed to make myotubes array *in vitro*. The experimental results show that cells tilt perpendicular to the flow direction in every condition and that rotational speed affects on the growth of oriented area with muscle cells.

6. ACKNOWLEDGMENT

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