Observation of Biological Cells in Rhombus Parallelepiped Flow Channel

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ABSTRACT

A flow channel has been designed to observe the behavior of cells in a shear flow *in vitro*. A thin sheet of silicone rubber is sandwiched by two transparent walls to form a parallelepiped flow channel of 20 mm length × 0.1 mm depth. Variation is made on the width of the channel (from 1 mm to 3 mm) with a rhombus shape to vary the wall shear stress. Behavior of cells on the wall is observed under a flow with an inverted phase contrast microscope. The shear stress on the wall is calculated with an estimated parabolic velocity profile between the parallel disks. After several cells adhered to the wall, the shear stress was applied on the cells with the flow of the medium by a syringe pump. The constant flow rate of 5×10^{-9} m^3 s^{-1} produces the wall shear stress between 1 Pa and 3 Pa. Three kinds of cells were examined with the methodology: L929 (fibroblast-like, mouse connective tissue), HUVEC (Normal Human Umbilical Vein Endothelial Cells) and C2C12 (Mouse myoblast cell line originated with cross-striated muscle of C3H mouse). The experimental results show that the response of cells can be observed under stimulation by the fluid at the controlled wall shear stress in the flow channel.

Keywords: Biomedical Engineering, Flow Channel, L929, HUVEC, C2C12, and Shear Stress.

1. INTRODUCTION

Mechanical stress is one of the interested points in the environment of cells, because they receive mechanical forces *in vivo*. Several methods have been designed to apply the mechanical stress to cells [1-11].

A transmission point of the stress to a specimen is important. In many studies, the stress is applied to a scaffold [1, 2]. When the fixation between the cell and the scaffold is not enough, the stress is not transmitted to the cell.

Every cell receives the stress when it is exposed to the field, such as gravitational field [3] or flow. A flow can be used to apply the stress to a specimen [4-11]. The specimen directly receives the shear stress in the shear flow.

A flow chamber with a laminar flow is effective to study the responses of cells to the shear stress quantitatively [5]. In the present study, responses of cells to the fluid shear stress have been studied with a parallelepiped channel *in vitro*.

2. METHODS

Rhombus Flow Channel

A one-way flow system has been designed to observe the behavior of cells in a shear flow *in vitro*. The system consists of a flow channel, a syringe pump, and tubes (Fig. 1). The micro-syringe-pump (Fusion200, CXF1020, ISIS Co., Ltd., Osaka) was used for the syringe pump. A plastic tube of 3 mm internal diameter and of 5 mm external diameter was used for a connector to the flow channel.

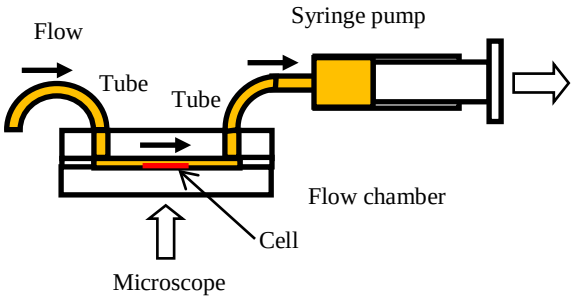

Fig. 1: Flow to syringe pump through flow channel.

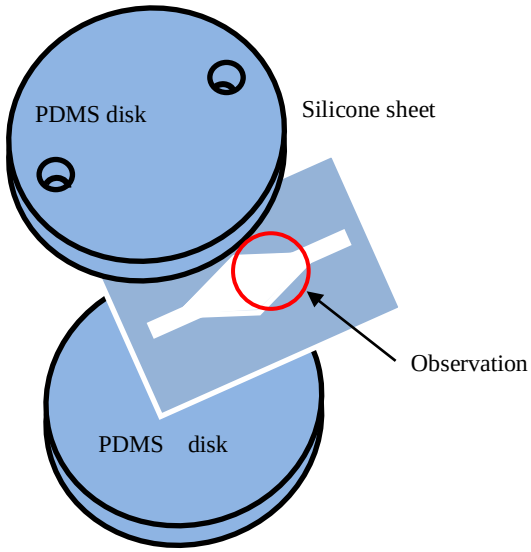

Fig. 2: Thin silicone rubber sheet with rhombus open space is sandwiched by two transparent polydimethylsiloxane (PDMS) disks.

The flow channel consists of two transparent disks and a thin sheet of silicone rubber (Fig. 2). The upper disk is made of polydimethylsiloxane (PDMS). The lower disk is made of PDMS or polystyrene (a culture dish).

A silicon wafer was used for a surface mold for the PDMS disk. The diameter and the thickness of the wafer are 50 mm and 0.30 mm, respectively. The surface of the wafer was cleaned with the isopropyl alcohol, and coated with 0.001 mm thickness of parylene in the parylene coater (PDS-2010, Speciality Coating Systems, Indianapolis). After the wafer was enclosed with a peripheral wall of polyimide, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corporation) was poured with the curing agent (Dow Corning Corporation) on the wafer. After degassing, PDMS was baked at 383 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd).

The diameter of two PDMS disks is 50 mm. The thicknesses of the upper and the lower disks are 10 mm and 2 mm, respectively.

A thin sheet (0.1 mm thick) of silicone rubber, which has a rhombus void space of 3 mm × 20 mm (Fig. 3), is sandwiched between the disks. The void space forms a channel of 20 mm length × 0.1 mm depth, where the width varies from 1 mm to 3 mm.

The three plates stick together with their surface affinity without adhesives. At the upper disk, two holes of 5 mm diameter (Fig. 4) are machined by a punching tool. The silicone tube is stuck at the holes without adhesives (Fig. 4). The inner surface of the chamber was exposed to the oxygen gas in a reactive ion etching system (RIE-10NR, Samco Inc., Kyoto) to be characterized as hydrophilic (oxygen plasma ashing), before being assembled.

Immediately after the characterization, the flow path of the chamber was rinsed with a saline solution, and the suspension of cells was introduced, successively.

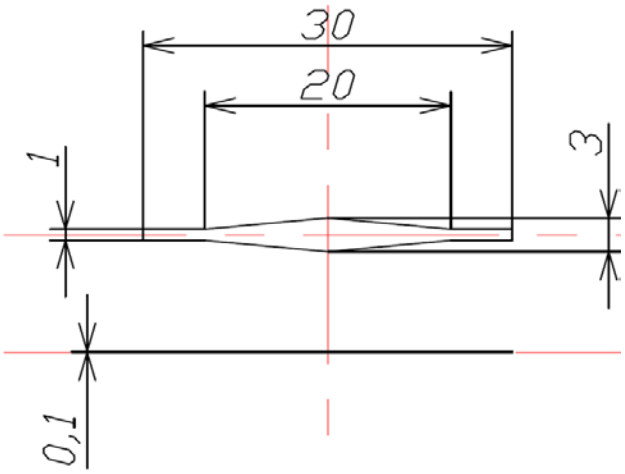

Fig. 3: Dimension (mm) of flow path.

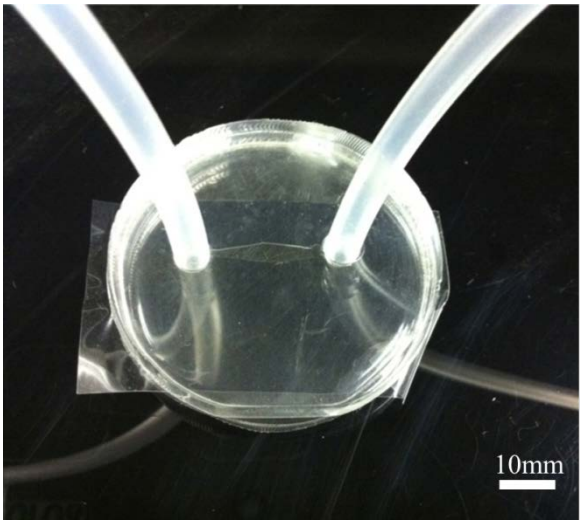

Fig. 4: Flow channel consists of two transparent polydimethylsiloxane (PDMS) disks and thin silicone rubber sheet, and connected to plastic tubes.

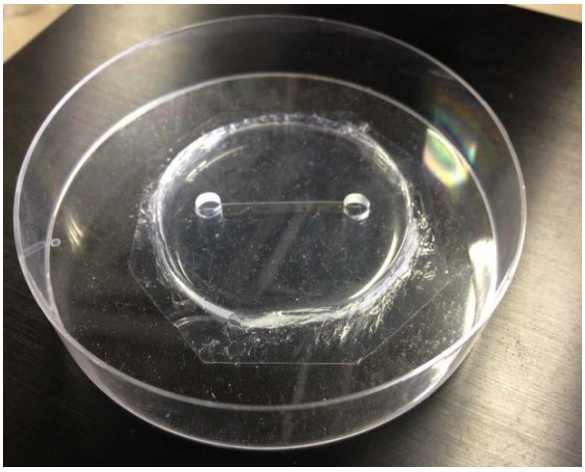

Fig. 5: The flow chamber consists of thin silicone rubber sheet sandwiched between transparent polydimethylsiloxane (PDMS) disk and culture dish.

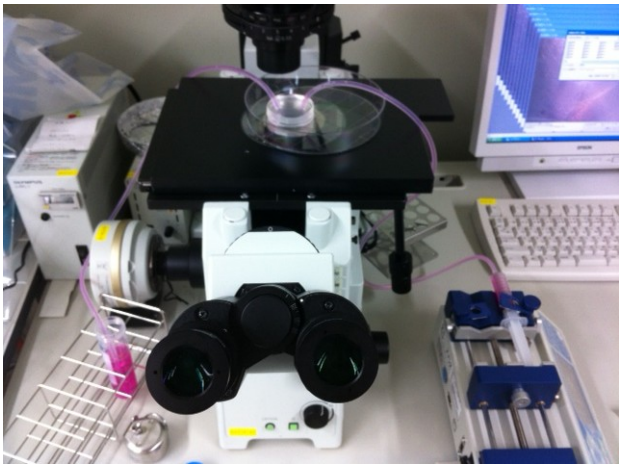

Fig. 6: Observation system under flow: flow channel and microscope (middle), syringe pump (right).

One of the tubes is connected to the plastic syringe pump (Fig. 1). The room temperature was maintained at 25 degrees Celsius. The chamber is placed on the inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo).

Cell Culture

Three kinds of cells were alternatively used in the experiment: L929 (fibroblast-like, mouse connective tissue, RCB1422, Riken Bio Resource Center, Tsukuba, passages 3–10), HUVEC (Normal Human Umbilical Vein Endothelial Cells, passages 2–5), or C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse, passages 2–10). The medium was refreshed every two days.

L929 was cultured on a dish with the E-MEM (Eagle's minimal essential medium) containing 10% FBS (fetal bovine serum) in the incubator for one week. Before the flow test, the cells were exfoliated from the plate of the culture dish with trypsin, and suspended in the E-MEM.

HUVEC was cultured on a dish with the EGM-2 (Endothelial Cell Growth Medium) in the incubator for one week. Before the flow test, the cells were exfoliated from the plate of the culture dish with trypsin, and suspended in the EGM-2.

C2C12 was cultured on a dish with the D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS in the incubator for one week. Before the flow test, the cells were exfoliated from the plate of the culture dish with trypsin, and suspended in the D-MEM.

In the case of HUVEC and C2C12, the lower PDMS disk was changed to the culture dish of polystyrene (Fig. 5).

The suspension was introduced to the channel and kept in the incubator for 24 hours to make cells adhere to the plate of the channel before the flow test. In some tests with C2C12, the cells were cultured in the incubator only for 3 hours before the flow test (Fig. 15).

Flow Test

When the cell is observed under flow, the channel was set on the microscope out of the incubator (Fig. 6). The constant flow of the medium was applied to the adhered cells with the syringe pump (Fig. 1).

In the first test with L929, the flow rate increases every minute step by step from 3×10^{-11} m^3 s^{-1} to 6×10^{-8} m^3 s^{-1}. The wall shear stress increases gradually, proportional to the stepwise increase of the flow rate. The flow path was carefully examined to avoid mixing of air bubbles, which might stir the medium in the flow chamber and induce exfoliation of cells. The behavior of cells on the plate of the chamber was observed with the microscope. The photos of cells were taken during the flow test intermittently.

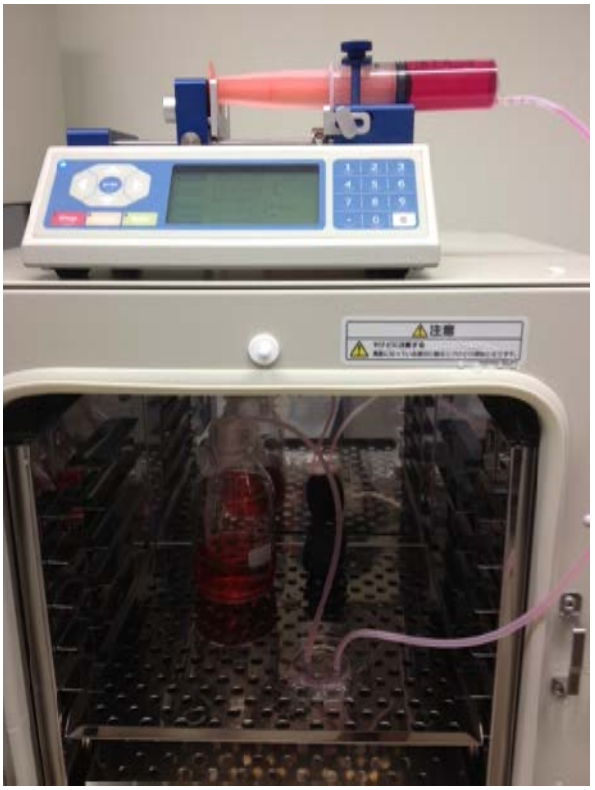

Fig. 7: Cell culture under flow: flow channel in incubator, and pump outside of incubator.

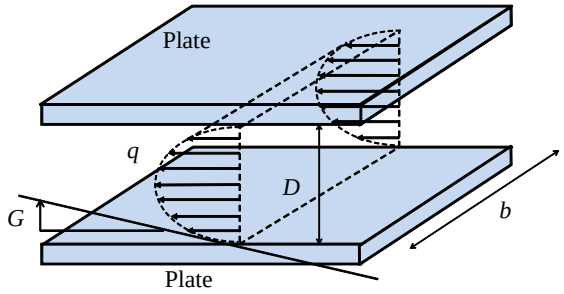

Fig. 8: Parabolic velocity profile between parallel plates.

In the test of cell culture under flow, the channel is kept in the incubator, and the medium is pumped with the syringe pump. The syringe pump is located outside of the incubator, and connected to the channel with the tube (Fig. 7).

Shear Stress on Wall

The shear rate (G, [s^{-1}]) on the wall of the plate is calculated by Eq. 1, in which a parabolic velocity profile between parallel plates is hypothesized (Fig. 8).

$$G = 6\,q\,/\,(b\,D^2) \qquad (1)$$

In Eq. 1, q is the flow rate [$m^3\ s^{-1}$], b is the width of the channel [m] and D is the distance [m] between two parallel walls. In the present study, D is 0.1 mm. Because the rhombus channel has a variation on the width, b ranges from 1 mm to 3 mm (Fig. 3). The width at the inlet of the channel is 1 mm, and increases from 1 mm to 3 mm in proportion to the distance from the inlet. At the middle point, the width is 3 mm, and then decreases from 3 mm to 1 mm. The width is 1 mm at the outlet. The variation of the width makes variation on the wall shear stress in the channel, so that cells at variety of wall shear stresses can be simultaneously observed in the constant flow rate.

The shear stress T [Pa] is the product of viscosity N [Pa s] of the fluid and the shear rate G [s^{-1}] of the flow (Eq. 2).

$$T = N\,G \qquad (2)$$

The viscosity of the medium was measured with the cone and plate type of viscometer (TVE-22L, Toki-Sangyo Co., Ltd. Tokyo).

3. RESULTS

The result of measurement with the viscometer shows that the viscosity of the medium at the shear rate of 600 s^{-1} is 0.0010 Pa s at 298 K, and that the viscosity is 0.0008 Pa s at 310 K. At the flow rate of 5×10^{-9} $m^3\ s^{-1}$, the calculated shear rate on the PDMS wall of the flow chamber by Eq. 1 varies from 3000 s^{-1} to 1000 s^{-1}, when the width of the flow path varies from 1 mm to 3 mm. The calculated shear stress, thus, varies from 3 Pa to 1 Pa for viscosity of 0.001 Pa s, when the shear rate varies from 3000 s^{-1} to 1000 s^{-1}.

The distance from left to right is 2.0 mm in Figs. 9-15. The medium flows from left to right in Figs. 9-15. The outlet of the chamber is located near the right end of Figs. 9-12, and the width of the flow path linearly decreases from 1.4 mm to 1.0 mm in the figures. As the decrease of the width of the flow path, the wall shear stress increases.

Fig. 9 shows L929 on PDMS in the channel before flow stimulation. Fig. 10 shows L929 under the flow of 6×10^{-8} $m^3\ s^{-1}$, which generates the wall shear stress on PDMS between 12 Pa and 37 Pa estimated by Eqs. 1 & 2. Some cells elongate to the downstream along the streamline of the flow in 41 min (A in Figs. 9 & 10). Some cells (B in Figs. 9 & 10) exfoliate from PDMS, and flow to the downstream in 41 min.

Fig. 11 shows L929 cultured on PDMS under the flow (1.4×10^{-10} $m^3\ s^{-1}$) for four days. The result shows that the number of L929 increases to the sub-confluent state on PDMS in the flow channel under flow stimulation.

Fig. 12 shows HUVEC on the dish (Fig. 5) after flow stimulation with the wall shear stress between 1 Pa and 3 Pa. The results show migration and exfoliation of cells. HUVEC moves on the wall and is exfoliated at the lower shear stress than L929 (A, B in Fig. 12).

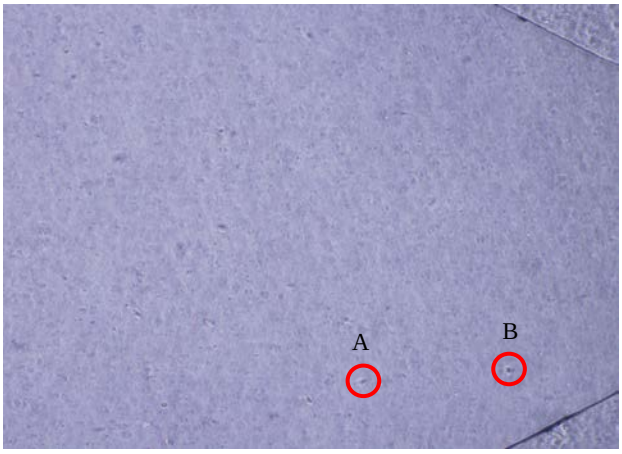

Fig. 9: L929 before flow stimulation. Dimension from left to right is 2.0 mm.

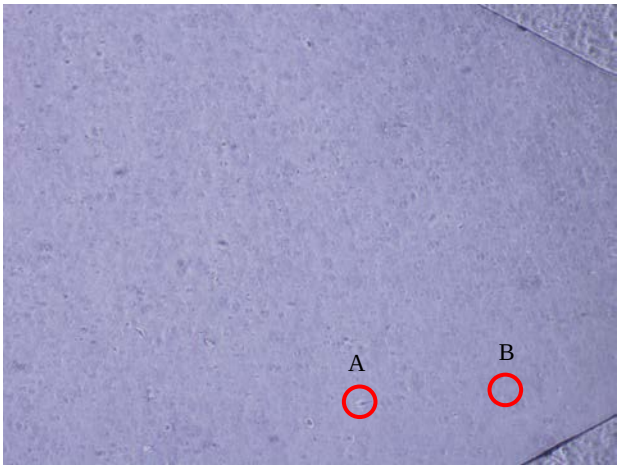

Fig. 10: L929 after flow stimulation for 41 min. A: elongate. B: disappear.

Fig. 11: L929 cultured under flow for four days. Dimension from left to right is 2.0 mm.

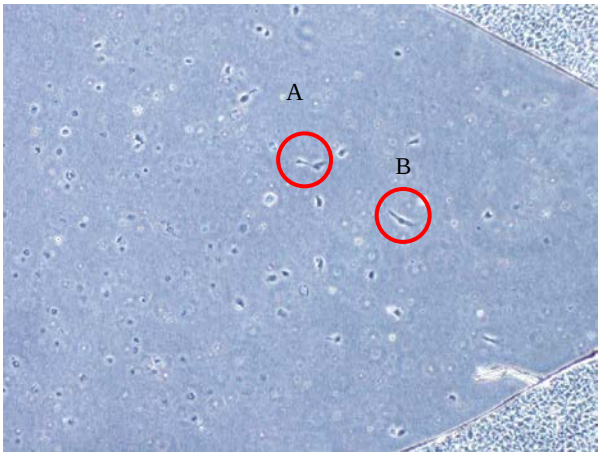

Fig. 12: HUVEC after flow stimulation (wall shear stress of 1 Pa -3 Pa). To be exfoliated (A, B).

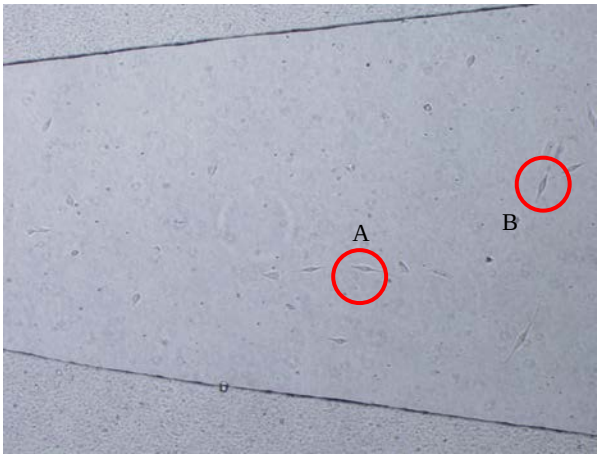

Fig. 13: C2C12 after flow stimulation for 1 day (wall shear stress of 1 Pa -3 Pa). To be exfoliated (A, B).

Figs. 13 and 14 show C2C12 on the dish after flow stimulation with the wall shear stress between 1 Pa and 3 Pa for one day and two days, respectively. Before flow stimulation, C2C12 was cultured without flow in the incubator for 24 hours for adhesion of C2C12 to the bottom of the dish. The longitudinal axes of some cells are perpendicular to the flow direction (B in Fig. 13). Many cells exfoliated in two days of culture (A, B in Fig. 13).

Fig. 15 shows C2C12 after flow stimulation for 2 day with the wall shear stress between 0.1 Pa and 0.3 Pa. Before flow stimulation, C2C12 was cultured without flow in the incubator for 3 hours for adhesion of C2C12 to the bottom of the dish. Some cells exfoliate from the bottom of the dish, and flow to the downstream.

4. DISCUSSION

The cells can be cultured on the oxygenated PDMS disk, although adhesiveness of cells is higher on the polystyrene dish than on the PDMS disk.

The fresh medium should be supplied into the flow channel, because the medium in the channel does not face to the oxygen gas and because the volume of the medium in the cannel is very small. The results show that the flow rate of the medium is above the minimum value to maintain cell culture.

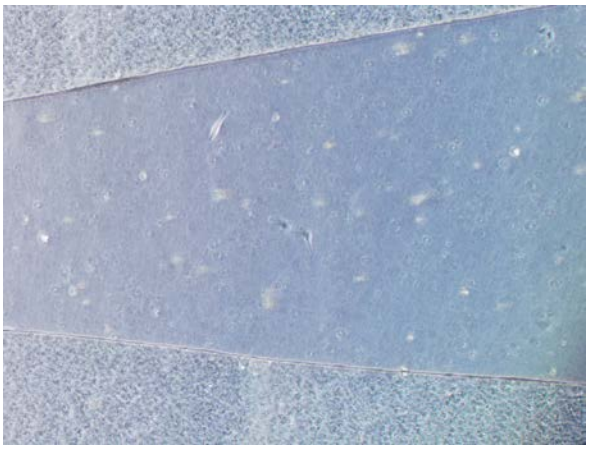

Fig. 14: C2C12 after flow stimulation for 2 day (wall shear stress of 1 Pa -3 Pa).

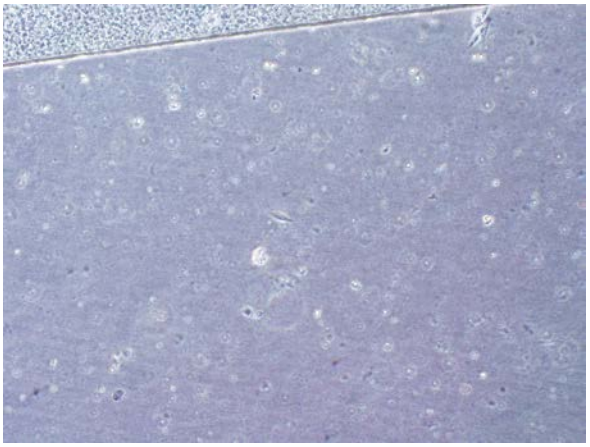

Fig. 15: C2C12 after flow stimulation for 2 day (wall shear stress of 0.1 Pa -0.3 Pa).

The area for observation is selected mainly near the outlet area, because the flow might be more stable near the outlet than near the inlet. The flow separation might occur near the inlet area, where the width of the flow path increases.

To estimate the wall shear rate in the chamber, a parabolic velocity profile is hypothesized as a laminar flow in the present experiment. Reynolds number (*Re*) is a useful index for estimation of the laminar flow.

$$Re = d\,D\,v\,/\,N \tag{3}$$

In Eq. 3, d is density [kg m^{-3}] of the fluid, D is distance [m] between two parallel walls, N is viscosity [Pa s] of the fluid, and v is the mean velocity of the flow [m s^{-1}]. The mean velocity is calculated by Eq. 4.

$$v = q\,/\,(b\,D) \tag{4}$$

In Eq. 4, q is the flow rate [m^3 s^{-1}], and b is the width [m] of the canal.

Re is in the range between 1.7 and 5, calculated with d (10^3 kg m^{-3}), N (10^{-3} Pa s), D (10^{-4} m), q (5×10^{-9} m^3 s^{-1}), and b (ranges from 1 mm to 3 mm) in the present experiment. The number is small enough to estimate the laminar flow.

The parallelepiped rhombus chamber designed in the present study realizes the wall shear stress field, where the shear stress varies linearly along the stream line. The chamber is convenient to observe the response of cells simultaneously with the variation of the wall shear stress, while the wall shear stress is constant in the parallelepiped rectangular chamber of the previous study [5].

Both acceleration of proliferation and orientation of cells are important targets in the research field of regenerative medicine on the cultured biological tissue. Another study shows mechanical stimulation improves a tissue-engineered human skeletal muscle [1].

The previous studies show that a mechanical field, on the other hand, governs behavior of cells [12, 13]. The shear flow governs the orientation of endothelial cells [6-11]. Vascular cells are subject to mechanical forces by the bood flow [2]. The shear stress affects the orientation of the smooth muscle cells in the biological tissue [2, 4].

5. CONCLUSION

The parallelepiped flow channel has been designed to observe the response of cells to the fluid shear stress under a microscope. The experimental results show that both deformation and exfoliation of cells can be observed under stimulation with the fluid at the controlled wall shear stress in the rhombus flow channel.

6. ACKNOWLEDGMENT

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REFERENCES

[1] C.A. Powell, B.L. Smiley, J. Mills and H.H. Vandenburgh, “Mechanical Stimulation Improves Tissue-engineered Human Skeletal Muscle”, **American Journal of Physiology - Cell Physiology**, Vol. 283, No. 5, 2002, pp. C1557–C1565.

[2] J. Goldman, L. Zhong and S.Q. Liu, “Degradation of α-actin Filaments in Venous Smooth Muscle Cells in Response to Mechanical Stretch”, **American Journal of Physiology- Heart and Circulatory Physiology**, Vol. 284, 2003, pp. H1839-H1847.

[3] S. Motoda, S. Hashimoto, T. Iwagawa and A. Nakajima, “Effect of Excess Gravitational Force on Cultured Myotubes in Vitro”, **Proc. 15th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2011, pp. 118-123.

[4] H. Kang, Y. Fan and X. Deng, “Vascular Smooth Muscle Cell Glycocalyx Modulates Shear-induced Proliferation, Migration, and NO Production Responses”, **American Journal of Physiology - Heart and Circulatory Physiology**, Vol. 300, 2011, pp. H76–H83.

[5] F. Sato, S. Hashimoto, K. Oya and H. Fujie, “Responses of Cells to Fluid Shear Stress in Vitro”, **Proc. 16th World Multi-Conference on Systemics Cybernetics and Informatics**, Vol. 2, 2012, pp. 97-102.

[6] S. Hashimoto and M. Okada, “Orientation of Cells Cultured in Vortex Flow with Swinging Plate in Vitro”, **Journal of Systemics Cybernetics and Informatics**, Vol. 9, No. 3, 2011, pp. 1-7.

[7] R.H.W. Lam, Y. Sun, W. Chen and J. Fu, “Elastomeric Microposts Integrated into Microfluidics for Flow-mediated Endothelial Mechanotransduction Analysis”, **Lab on a Chip**, Vol. 12, 2012, pp. 1865-1873.

[8] S. Hsu, R. Thakar, D. Liepmann and S. Li, “Effects of Shear Stress on Endothelial Cell Haptotaxis on Micropatterned Surfaces”, **Biochemical and Biophysical Research Communications**, Vol. 337, No. 1, 2005, pp. 401-409.

[9] M.L.C. Albuquerque, C.M. Waters, U. Savla, H.W. Schnaper and A.S. Flozak, “Shear Stress Enhances Human Endothelial Cell Wound Closure in Vitro”, **American Journal of Physiology - Heart and Circulatory Physiology**, Vol. 279, pp. H293-H302, 2000.

[10] P. Uttayarat, M. Chen, M. Li, F.D. Allen, R.J. Composto and P.I. Lelkes, “Microtopography and Flow Modulate the Direction of Endothelial Cell Migration”, **American Journal of Physiology - Heart and Circulatory Physiology**, Vol. 294, 2008, pp. H1027-H1035.

[11] P.P. Hsu, S. Li, Y.S. Li, S. Usami, A. Ratcliffe, X. Wang and S. Chien, “Effects of Flow Patterns on Endothelial Cell Migration into a Zone of Mechanical Denudation”, **Biochemical and Biophysical Research Communications**, Vol. 285, 2001, pp. 751-759.

[12] D.A. Lauffenburger and A.F. Horwitz, “Cell Migration: A Physically Integrated Molecular Process’’, **Cell**, Vol. 84, 1996, pp. 359–369.

[13] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons and A.R. Horwitz, “Cell Migration: Integrating Signals from Front to Back’’, **Science**, Vol. 302, 2003, pp. 1704-1709.