Measurement of Contractile Force of Myotube on Scaffold of Thin Film with Micro-pattern-markers

Yusuke TAKAHASHI, Shigehiro HASHIMOTO, Kenta SUGIMOTO, Daiki WATANABE, Haruka HINO

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

ABSTRACT

A scaffold of a transparent film with micro pattern markers has been designed to measure the contractile force of myotube under electric stimulation *in vitro*. The scaffold is made of a thin film (thickness 0.006 mm), of which the back side has arrangement of polydimethylsiloxane micro-protrusions (0.004 mm diameter, 0.002 mm height, 0.003 mm interval) made by the photolithography technique. C2C12 (mouse myoblast) was seeded on the film at the counter surface to the protrusions at the density of 50000 cells/cm^2. The cells were cultured on the scaffold for 12 days in the medium containing 10% FBS (fetal bovine serum) and 1% penicillin/ streptomycin at 310 K with 5% of CO_2 content. The electric pulses (amplitude of 30 V (0.06 A) ; pulse cycle of 1 s; pulse width of 1 ms) were applied between electrodes of the titanium wire dipped in the medium. The contraction of myotubes is able to be observed through the transparent scaffold at the microscope. The contractile force of myotube at the electric stimulation estimated by the deformation of the film was 10^{-4} N. The designed scaffold has a potential for the measurement of the local contractile force of the myotube microscopically *in vitro*.

Keywords: Biomedical Engineering, Cell Culture, C2C12, Micro Protrusion and Photolithography.

1. INTRODUCTION

The biological tissue can be made by the cell culture technique *in vitro* [1]. The tissue should have enough strength for the clinical application in the regenerative medicine. Intra- and inter-cellular forces has been tried to be measured by several preparations [2-10]: the laser technique, the atomic force microscope, and the fluorescence technique. Only at the end of test, the scanning electron microscope, and the stain technique are available.

The photolithography technique realizes the micro morphology for local markers on the scaffold [3-5]. In the case of the myotube contraction on the scaffold, the scaffold should contract synchronously to detect the contractile force by the deformation of the scaffold. The myotube should adhere to the scaffold, which has both enough compliance and traceable markers.

The scaffold of the transparent thin film, of which backside has micro-protrusions array, has been designed to estimate the local contractile force of myotube under the electric stimulation *in vitro* in the present study.

2. METHODS

Micro-Pattern-Markers
A scaffold of the transparent film with micro pattern markers has been designed to measure the contractile force of myotube under electric stimulation *in vitro* (Fig. 1). The scaffold is made of a thin film, of which the back side has arrangement of micro-protrusions. The protrusions on polydimethylsiloxane (PDMS) film were made by the photolithography technique. Each protrusion has the hemisphere shape (0.004 mm diameter, 0.002 mm height). The pitch between adjacent protrusions is 0.03 mm. The thickness of the base film of PDMS is 0.0064 mm. The side without protrusions is used for the scaffold of the cell culture. The protrusions play a roll of the position marker. The array of protrusions is made in the square area of 3 mm × 3 mm at the center of the film.

Photomask for Micro Pattern
A photomask is made for the micro pattern (Fig. 2). The surface of the glass was hydrophilized by the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing (100 W, for five minutes) in a reactive ion etching system (FA-1, Samco International, Kyoto, Japan).

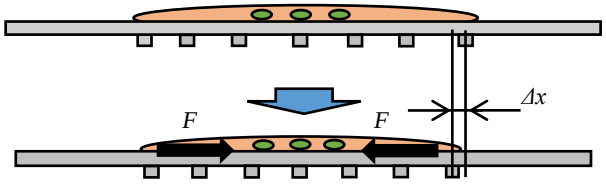

Fig. 1a: Deformation (Δx) of scaffold film with protrusions by force (F) of contraction (from upper to lower) of myotube.

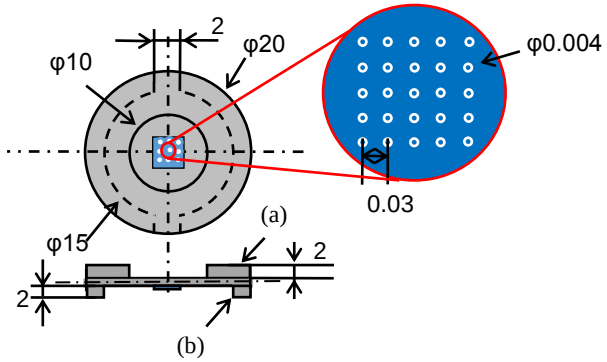

Fig. 1b: Scaffold film with micro pattern markers: unit, mm.

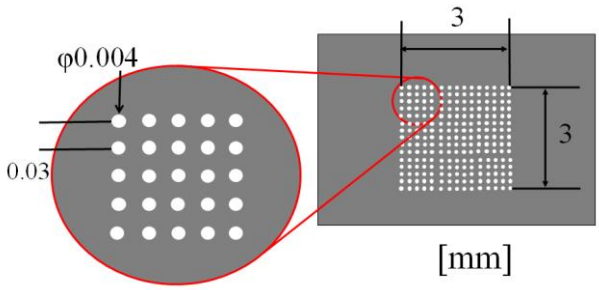

Fig. 2: Photomask for micro pattern.

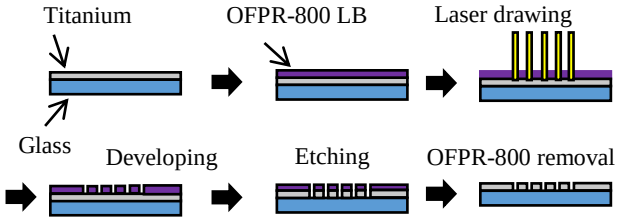

Fig. 3: Photolithography process for photomask.

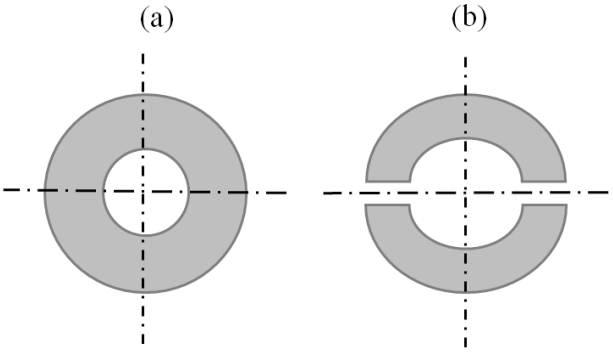

Fig. 4: Supporter ring of PDMS for scaffold film: a and b corresponds to those in Fig. 1b.

Titanium was deposited on the surface of the glass plate (76 mm × 26 mm × 1 mm, Matsunami Glass Ind., Ltd., Japan) in the electron beam vapor deposition apparatus (JBS-Z0501EVC, JEOL Ltd., Tokyo, Japan) by the rate of 0.5 nm/s for 200 s (Fig. 3). The thickness of coating is 200 nm. The oxygen (0.1 Pa, 30 cm^3/min) ashing was applied on the surface of the titanium in the reactive ion etching system (100 W, for five minutes, FA-1). To improve the affinity between the glass and the photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated on the disk at 3000 rpm for 30 s with a spin coater.

The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the disk at 1000 rpm for 30 s with the spin coater. The photoresist was baked in the oven (DX401, Yamato Scientific Co., Ltd) at 368 K for five minutes. The pattern for micro- protrusions was drawn on the photoresist with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the pattern on the mold with the laser drawing system, the parameters were selected as follows: the voltage of 2.8 V, the velocity of 0.02 mm/s, the acceleration of 0.5 mm/s^2.

The pattern was baked on the heated plate at 368 K for five minutes. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for twenty minutes. The pattern was rinsed by the pure water, and dried by the spin-dryer (300 rpm 30 s; 1100 rpm, 30 s with N_2: SF-250, Japan Create Co., Ltd., Tokorozawa, Japan).

The surface of titanium with photoresist material was etched with the plasma gas using reactive ion etching system (RIE-10NR, Samco Inc., Kyoto, Japan). For etching, the gas of SF_6 (50 cm^3/min at 1013 hPa) with Ar (50 cm^3/min at 1013 hPa) was applied at 100 W at 4 Pa for six minutes. OFPR-800LB was removed by acetone, after confirmation of pattern of etching.

The dimension of the micro-pattern of the mask was measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan) (Fig. 12). The height along the cross sectional line of micro pattern was traced.

PDMS Ring

The supporter of the cell culture film was made of the donut-ring of PDMS (Fig. 4). PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was mixed with the curing agent (Sylgard 184 Silicone Elastomer Curing Agent, Dow Corning Corp., MI, USA). The volume ratio of PDMS to curing agent is ten to one. After degassing, PDMS in a dish was baked at 333 K for one hour in an oven. The baked PDMS was machined by the punch to make the donut-ring. Two kinds of ring were made. One has the outer diameter of 20 mm, the inner diameter of 10 mm and the thickness of 2 mm (Fig. 4a). The other one has the outer diameter of 20 mm, the inner diameter of 15 mm and the thickness of 2 mm (Fig. 4b). The latter one is divided into two parts to be two U-shape elements.

Mold for Thin film of Scaffold with Micro Pattern

The mold for the thin film of the scaffold with micro protrusions array was made on the surface of the glass plate by the photolithography technique (Fig. 5). The surface of the glass was hydrophilized 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 International, Kyoto, Japan). To improve affinity between glass and photoresist material, HMDS was coated on the disk at 3000 rpm for 30 s with a spin coater. The positive photoresist material of OFPR-800LB was coated on the disk at 3000 rpm for 30 s with the spin coater. The photoresist was baked in the oven at 368 K for five minutes.

The photomask was mounted on the surface of OFPR-800LB, and the photoresist was exposed to the UV light through the mask in the mask aligner (M-1S, Mikasa Co. Ltd., Japan) at 15 mW/cm^2 for 10 s. The photoresist was baked in the oven at 393 K for five minutes. The photoresist was developed with NMD-3 for twenty minutes. The pattern was rinsed by the pure water, and dried by the spin-dryer (300 rpm 30 s; 1100 rpm, 30 s with N_2: SF-250).

The morphology of the surface of the mold was measured with a stylus profiler (Dektak XT-E, Bruker Corporation) (Fig. 6). The height of the step of the surface of the mold was measured to estimate the thickness of the film (Fig. 13). The height along the cross sectional line of micro pattern was traced (Fig. 14).

Thin film of Scaffold with Micro Pattern

PDMS was mixed with the curing agent. The volume ratio of PDMS to curing agent is ten to one. After degassing, PDMS was poured on the mold, which was placed on the spin coater (at 7000 rpm for 30 s), to make the thin film of scaffold with the micro pattern (Fig. 7). PDMS ring, which was hydrophilized by the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing (50 W, for 30 s) in the reactive ion etching system (FA-1), was placed on the thin film of PDMS. The film was baked with the ring in the oven at 368 K for thirty minutes. After residual PDMS film outside of PDMS ring was cut off, PDMS film with PDMS ring was carefully peeled off from the mold using acetone [11]. After rinsed by the pure water, PDMS film was sandwiched by another PDMS ring, and dried in the oven at 333 K for one hour (Fig. 8).

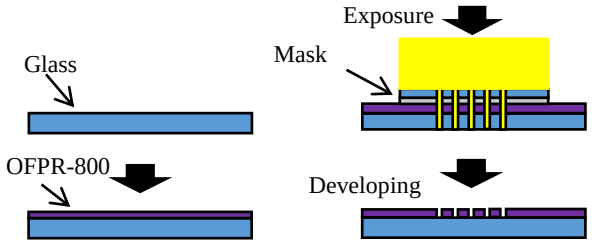

Fig. 5: Photolithography process for mold.

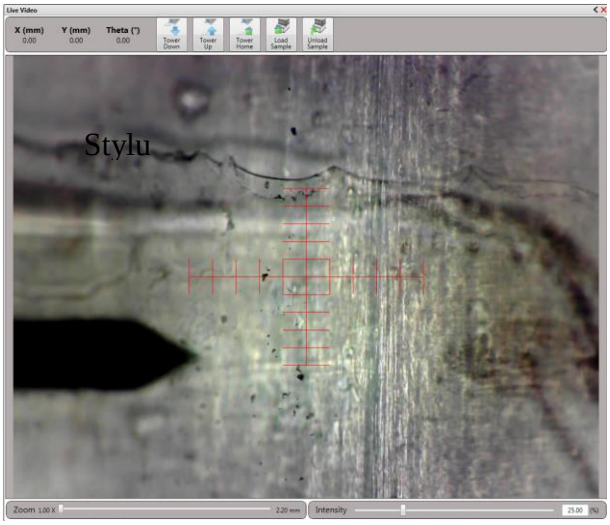

Fig. 6: Measurement of surface morphology of mold with stylus (left) profiler

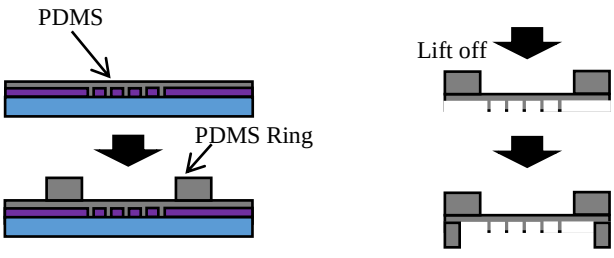

Fig. 7: Film made on mold and lifted with ring.

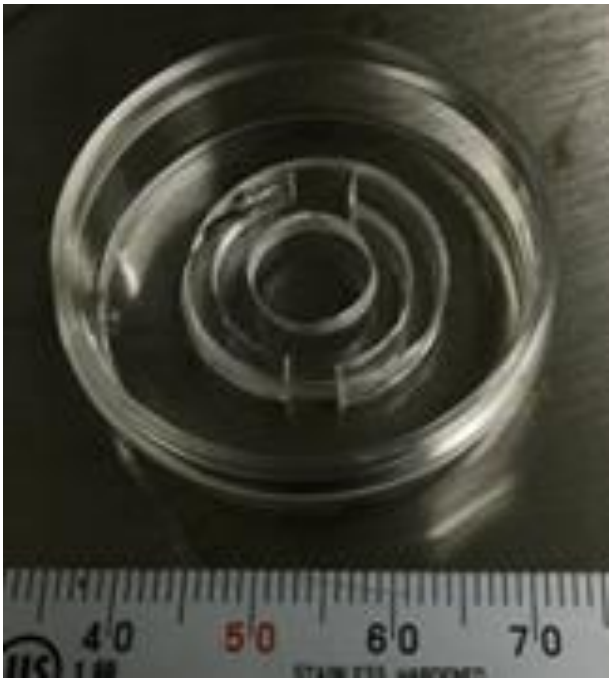

Fig. 8: Scaffold film in dish.

The morphology of the protrusions array on the rear surface of the film was observed by a scanning electron microscope (SEM, JSM6380LD, JEOL Ltd., Tokyo, Japan) (Fig. 15). The culture dish of PDMS was exposed to the oxygen gas (0.1 Pa, 30 cm^3/min) at power of 50 W for thirty seconds in the reactive ion etching system (FA-1) 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.

Cell Culture

Myoblasts were used in the test: C2C12 (mouse myoblast cell line originated with cross-striated muscle of C3H mouse), passage from fourth to ninth. 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 cells. C2C12 was seeded on the sheet at the counter surface (back side) to the protrusions at the density of 50000 cells/cm^2. The culture dish was kept in the incubator to maintain both the temperature of 310 K and the carbon dioxide partial pressure of 5 percent. The cells were observed with a phase contrast microscope (IX71, Olympus, Tokyo) during the cell culture for twelve days. The medium was changed every two days.

Electric Stimulation

A pair of the U-shape electrodes was made of titanium wires (diameter of 0.50 mm, TI-451385, The Nilaco Corporation), which were fixed on the cap of the culture dish with extra paste of PDMS (Fig. 9). The electric pulse V_0 (amplitude of 30 V, pulse width of 1 ms, period of 1 s or 0.5 s) was generated with an electric stimulator (SEN5201, Nihon Kohden Corporation, Tokyo, Japan).

The stimulator was connected to the electrodes (Fig. 10). An electric resistance (r = 51 Ω) is serially inserted between the electrode and the stimulator (Fig. 11). The voltages (V) were

monitored by an oscilloscope during application of the electric pulse to the electrode (Fig. 17). The electric current I, which flows through Z (medium), is calculated by Eq. 1.

$$I = V / r \quad (1)$$

In Eq. 1, V is the voltage between terminals of the electric resistance. When the movement of the myotube was observed, the synchronous movement of the adjacent micro pattern markers was recorded by a movie camera and the image was analyzed by "Image J". The force F (Fig. 1a) generated in myotube by the electric stimulation is estimated at the deformation of the scaffold film by Eq. 2.

$$F = E \varepsilon A \quad (2)$$

In Eq. 2, E is the elastic modulus of the film of the scaffold, and A is the cross sectional area of the film. The local strain of the film (ε) is estimated by the distance between micro pattern markers on the film of the scaffold.

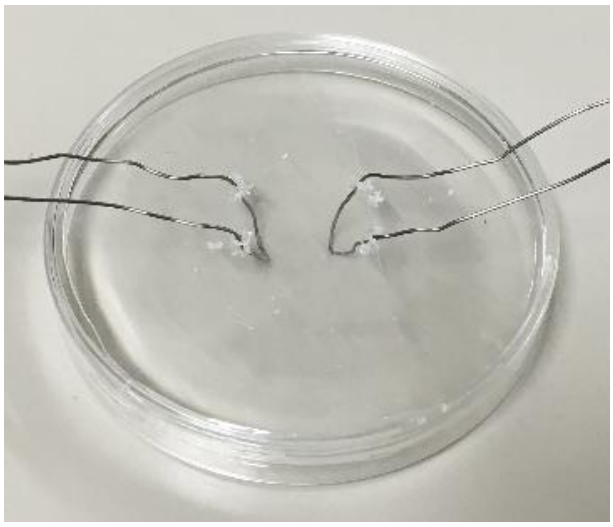

Fig. 9: Electrodes on cap of dish.

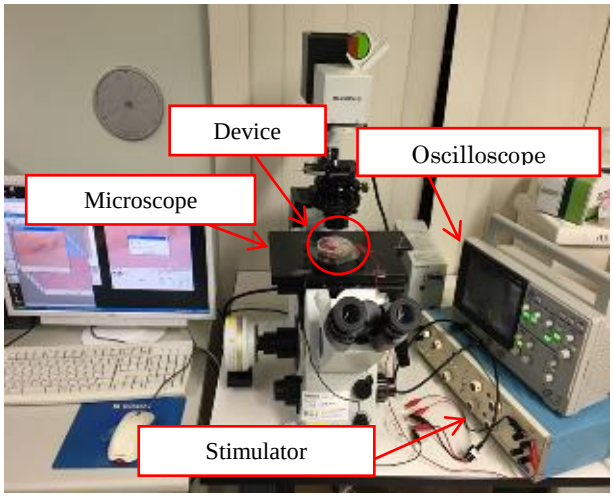

Fig. 10: Experimental system.

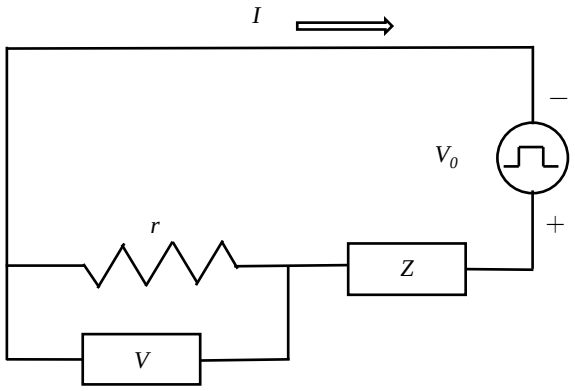

Fig. 11: Electric circuit: Z, impedance of the medium.

3. RESULTS

Fig. 12 shows the laser microscope image of the photomask. The diameter of each hole is 0.004 mm, and the pitch between adjacent holes is 0.03 mm. The measurement by the stylus shows that the dimension of the step of the mold is 0.0064 mm, which corresponds to the thickness of the film of the scaffold (Fig. 13). The measurement by the stylus on the mold shows that the depth of the hole is 0.002 mm, which corresponds to the height of protrusions on the film (Fig. 14).

Fig. 15 shows the scanning electron microscope image of micro protrusions array on the film. Each protrusion has the hemisphere shape, and the pitch between adjacent protrusions is 0.03 mm. The myoblasts are able to be cultured on the film of the scaffold to be differentiated into myotubes (Fig. 16). The markers on the counter side of the film are able to be observed by adjusting the focus on the microscope (Fig. 16b).

When the electric pulse (amplitude of 30 V) was applied between electrodes of titanium wire dipped in the medium, the amplitude of the voltage between the terminals of 51 Ω was 3.3 V (Fig. 17). The amplitude 0.06 A of the electric current is calculated from these values. The repetitive contractions of myotubes synchronous to the period of the electric pulses were able to be observed through the transparent scaffold at the microscope.

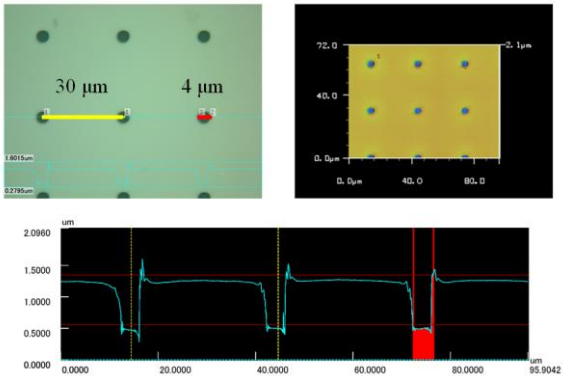

Fig. 12: Photomask measured with laser microscope.

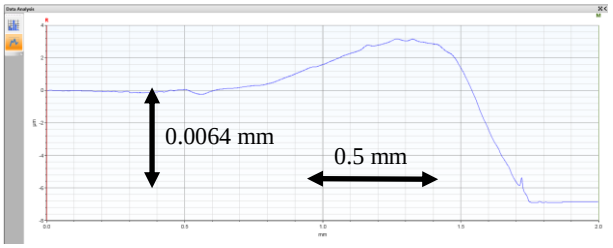

Fig. 13: Tracings of height of step (correspond to thickness of film) on mold.

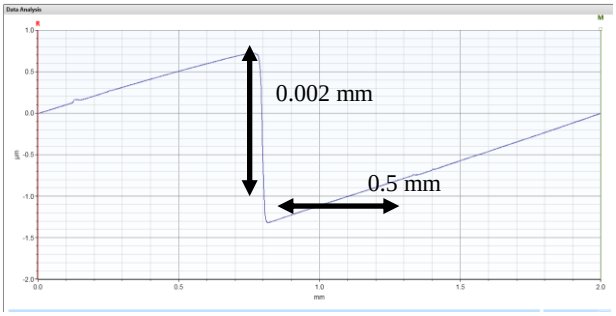

Fig. 14: Tracings of depth of hole on mold.

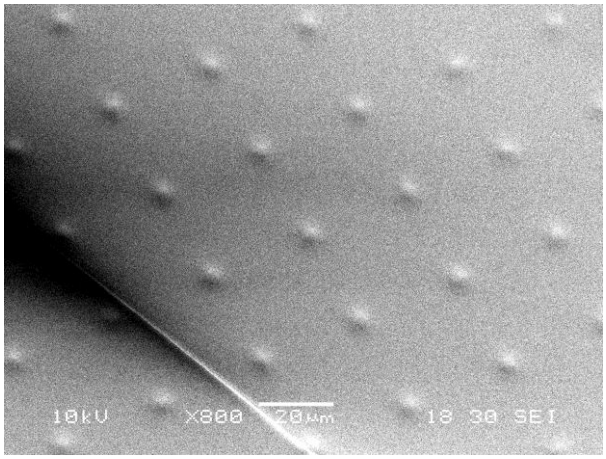

Fig. 15: SEM image of micro protrusions array on film: dimension from left to right is 0.15 mm.

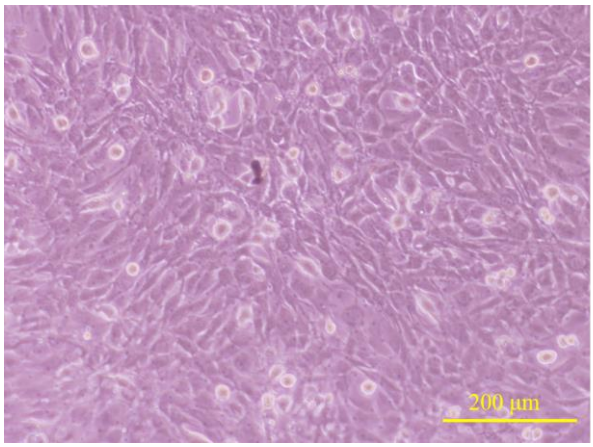

Fig. 16a: C2C12: day 1: dimension from left to right is 0.9 mm.

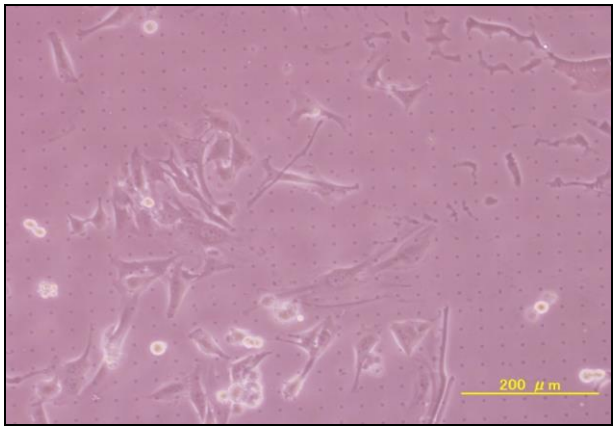

Fig. 16b: C2C12: day 2 with arrangement of markers: dimension from left to right is 0.9 mm.

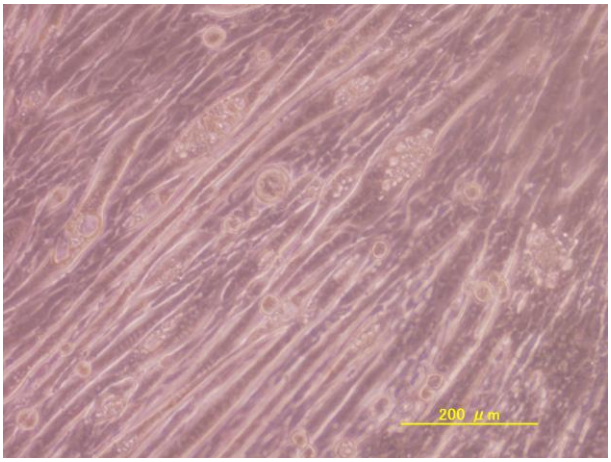

Fig. 16c: Myotubes: day 12: dimension from left to right is 0.9 mm.

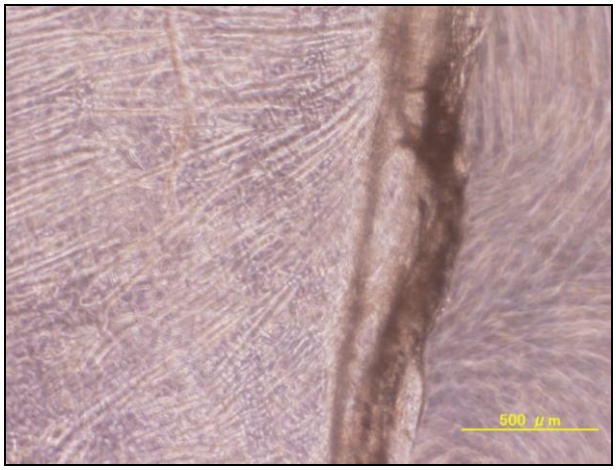

Fig. 16d: Myotubes: day 9: dimension from left to right is 2.3 mm.

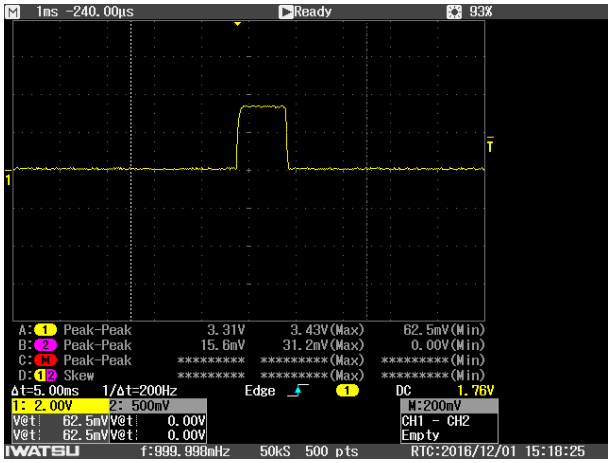

Fig. 17: Voltage between terminals of resistance.

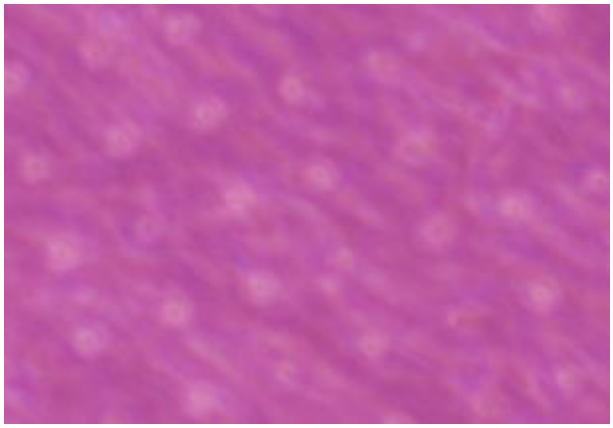

Fig. 18a: Myotubes and markers: dimension from left to right is 0.21 mm.

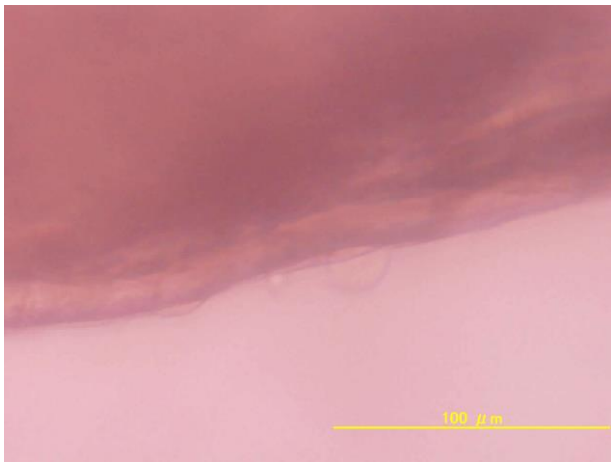

Fig. 18b: Free end of film with myotubes (right): dimension from left to right is 0.21 mm.

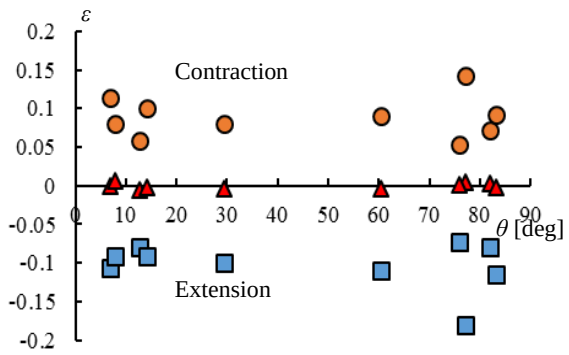

Fig. 19: local strain of the film (ε) vs. angle between longitudinal axis of myotube and deformation measurement direction (θ) [degree]: mean (△), max (○), min (□).

The repetitive movement by electric pulse of 30 V at 1 Hz (Fig. 18) was measured every 0.3 s on the five movies (for 15 s, each). In Fig. 19, each data shows the mean value of 30 samples, which are the distances between adjacent micro pattern markers on the film of the scaffold. The maximum and the minimum data show that the local strain amplitudes (ε) is around 0.1. The cyclic strain occurs in the parallel (θ = 0 deg) and the perpendicular (θ = 90 deg) direction to the longitudinal axis of the myotube. In the case with the ruptured film, the amplitude of the movement (Δx) was 0.0016 mm on the free end of the torn film (Fig. 18 b).

4. DISCUSSION

The contraction of the specimen of muscle tissue was measured by laser preparation in the previous study [2]. The local movement should be observed to analyze the local force of contraction at the myotube.

In the previous study, cells were cultured on the micro pillars [10]. The deformability of the micro pillar depends on its height. The behavior of myoblasts, however, depends on the micromorphology of the surface of the scaffold [12-14]. To separate the effect of micromorphology on the behavior of myoblast from the contraction of myotubes, the micro-protrusions are used as the markers on the counter side of the scaffold in the present study.

The previous study show that the orientation of myoblast depends on the height of the micro ridges [14]. In the present study, cells are cultured on the flat thin sheet with the micro pillars on the rear side, so that the cell can adhere on the sheet at any direction regardless of the position of the micro pillar.

The photolithography technique can be applied to control compliance of the surface [10, 15-17]. The harder scaffold accelerated differentiation of cells in the previous study. The sheet lined with micro- protrusions array has distribution about local micro compliance. The behavior of cell might depend on the local micro deformability of the scaffold.

The myotubes do not make orientation on the scaffold film in the present study. If the contractions were measured at the oriented myotubes, the total force might be stronger.

To observe the entire scaffold plane at the same level of the focus, the bend of the film is minimized with the base ring of PDMS. To keep the position of the film of scaffold, the medium was filled on the rear side of the film through the space between U-shaped bases.

In the previous study, the optical scattering by micro pillars on the scaffold disturbed optical microscopic observation at cells on the scaffold [3, 12]. To minimize the scattering, the height of the micro-pillars is shortened in the present study.

C2C12 (mouse myoblast) was seeded on the film at the counter surface to the protrusions at the density of 50000 cells/cm^2. After myoblasts were cultured for ten days in the culture dish as the control study, the differentiated myotube showed contraction synchronized with stimulation of electric pulses [18-21]. The cells were cultured on the scaffold for 12 days in the medium in the present study.

The film of the scaffold should deform synchronously with the myotube to detect the contraction force of the myotube. The surface of the scaffold is hydrogenised by oxygen ashing in the present study. The surface of the thin film of the scaffold should have enough affinity to the myotube to keep adhesion during contraction of the myotube [22-25].

The scaffold should have enough compliance to follow the contraction with the small force [26-29]. The thickness of the PDMS film of the scaffold is made as thin as possible in the present study.

At the torn film (Fig. 18 right), the force F generated in myotube by the electric stimulation is calculated at the deformation of the scaffold film by Eq. 2. The strain ε (3×10^{-4}) is calculated by the ratio ($\varepsilon = \Delta x / x$) between the shortening amplitude of the film Δx (= 0.0016 mm) and the total length of the film of the scaffold (x = 5 mm). The cross sectional area of the film A is calculated by the product of the diameter of the film (10 mm) and the thickness of the film (0.0064 mm). The contractile force of myotube at the electric stimulation estimated by the movement of the markers of the film is 10^{-4} N, when $E = 2.0\times10^6$ Pa [10], $\varepsilon = 10^{-3}$, and $A = 6\times10^{-8}$ m^2.

The force of the single myotube cannot be distinguished in the present study. Because the measurement direction of the strain may not parallel to the direction of the contraction of the myotube, the movement does not show the maximum value. The movement of myotube should be measured along the longitudinal direction of the myotube using the arrangement of micro markers.

5. CONCLUSION

The scaffold thin film (0.006 mm thickness, 10 mm diameter) with micro-markers has been successfully fabricated by photolithography technique, and applied to culture of C2C12. Myotubes is able to be observed through the transparent scaffold film with micro-markers at the microscope. When the electric pulses (amplitude of 30 V, 0.06 A, cycle of 1 s, pulse width of 1 ms) were applied between electrodes of titanium wire dipped in the medium, the repetitive contraction of myotubes is able to be observed through the transparent scaffold. The contractile force of myotube at the electric stimulation estimated by the deformation of the film was 10^{-4} N. The designed scaffold has a potential for the measurement of the local contractile force of myotube *in vitro*.

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