Micro Back-markers on Thin Film of Scaffold to Measure Repetitive Local Contraction of Myotubes In Vitro

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ABSTRACT[1]

The repetitive contraction of the myotube has been measured by the local movement of micro-markers on the thin film of the scaffold during the electric stimulation *in vitro*. The scaffold is made of the thin polydimethylsiloxane film (6 μm thickness), of which the back side has an arrangement of micro-protrusions (4 μm diameter, 2 μm height, and 30 μm interval) made using the photolithography technique. Mouse myoblasts (C2C12) were seeded on the film at the counter surface to the protrusions at the density of 50000 cells/cm^2. The cells differentiated on the scaffold into myotubes over 12 days in a medium containing 10% FBS (fetal bovine serum) and 1% penicillin/ streptomycin in the incubator. The electric pulses (30 V amplitude, 1 s pulse cycle, and 1 ms pulse width) were applied between electrodes of titanium wire dipped in the medium. The contraction of the myotube was observed by a microscope through the transparent scaffold. The experimental results show that the amplitude of the cyclic variation of the distance between micro-markers relates to the distance and the alignment. The designed scaffold can be applied to analyze the local contractile movement of the layer of myotubes *in vitro*.

Keywords: Biomedical Engineering, Cell Culture, Myotube, 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 regenerative medicine. Both intra- and inter-cellular forces have been tried to be measured by several preparations [2-11]: the laser technique, the atomic force microscope, and the fluorescence technique. Only at the end of test, are both the scanning electron microscope and the stain technique available.

The photolithography technique realizes the micro topography for local markers on the scaffold [2-6]. When the myotube on the scaffold contracts, the scaffold should contract synchronously to detect the contractile force of the myotube by the deformation. The myotube should firmly adhere to the scaffold, which has both enough deformability and traceability with markers.

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

2. METHODS

Micro-Pattern-Markers

The scaffold of the transparent film with micro-pattern markers has been designed to measure the contractile movement of myotube under the electric stimulation *in vitro* (Fig. 1). The scaffold is made of a thin film, of which the backside has the arrangement of micro-protrusions. The protrusions on the polydimethylsiloxane (PDMS) film were made by the photolithography technique.

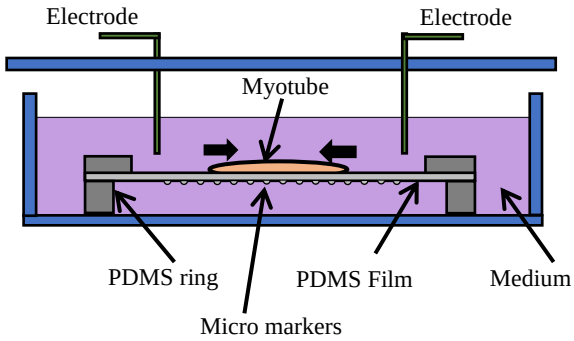

Fig. 1: Myotube contraction on scaffold film with micro-pattern markers by electric-pulses stimulation.

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Each protrusion has the hemisphere shape (4 μm diameter, and 2 μm height). The distance between adjacent protrusions is 0.03 mm. The thickness of the base film of PDMS is 6.4 μm.

The front side without protrusions is used for the scaffold of the cell culture. The protrusions play a role of the position marker on the film. The array of protrusions is made in the square area of 3 mm × 3 mm at the center part of the film.

The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the titanium coated glass disk at 1000 rpm for 30 s with the spin coater. The pattern for micro-protrusions was drawn on the photoresist with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan).

The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan). The surface of titanium with photoresist material was etched with the plasma gas using the reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan). OFPR-800LB was removed by acetone, after confirmation of the pattern of etching. The dimension of the micro-pattern of the mask was confirmed with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). The height along the selected line on the micro pattern was traced.

PDMS Ring

The supporter of the cell culture film is made of the donut-ring of PDMS. 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.

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

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. PDMS ring, which was hydrophilized by the plasma ashing (50 W, for 30 s) of the oxygen (0.1 Pa, 30 cm^3/min) 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 the residual part of the PDMS film outside of the PDMS ring was cut off, the PDMS film with the PDMS ring was carefully peeled off from the mold using acetone [12]. After rinsed by the pure water, the PDMS film was sandwiched by another PDMS ring, and dried in the oven at 333 K for one hour.

The cell-culture surface of PDMS was exposed to the oxygen gas (0.1 Pa, 30 cm^3/min) for thirty seconds with the power of 50 W in the reactive ion etching system to be characterized as hydrophilic. The surface was preserved in the ultrapure water to keep the hydrophilic property before the cell culture.

Cell Culture

Myoblasts (C2C12: mouse myoblast cell line originated with cross-striated muscle of C3H mouse, the passage from fourth to ninth) were used in the test. D-MEM (Dulbecco's Modified Eagle's Medium) containing 10% decomplemented FBS (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 (the 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 the 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-shaped 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. The electric pulse (the amplitude of 30 V, the pulse width of 1 ms, and the period of 1 s) was generated with the electric stimulator (SEN5201, Nihon Kohden Corporation, Tokyo, Japan). The stimulator was connected to the electrodes.

When the movement of the myotube was microscopically observed, the synchronous movement of the adjacent micro-pattern markers was recorded by a

movie camera. The length (x) between micro-markers was measured (Fig. 2). The strain (ε) of x was calculated by Eq. (1).

$$\varepsilon = (x / x_{mean}) - 1 \quad (1)$$

In Eq. (1), x_{mean} is the mean value of x during the cyclic movement of the myotube. The angle (θ) between the longitudinal direction of the adjacent contracting myotube and x was measured (Fig. 2). To obtain variations of x and θ, several combinations of markers were selected at the microscopic image (Fig. 3b).

3. RESULTS

The myoblasts were cultured on the film of the scaffold where they differentiated into myotubes (Fig. 3a). The markers on the rear surface of the film were observed by adjusting the focus on the microscope. Fig. 3b exemplifies several combinations of markers with the variation of parameters: x, θ and d. The repetitive contraction of myotubes synchronous to the period of the electric pulses was able to be observed through the transparent scaffold at the microscope.

Fig. 4 exemplifies the tracings of the strain (ε) of the distance between micro-markers. The amplitude A was measured by the difference between the maximum value and the minimum value at the cyclic variation of ε. Fig. 5 shows the relationships between the amplitude (A) and the length (x). The amplitude tends to decrease with the length. The dotted line shows the regression line ($A = -0.011\,x + 1.6$). The correlation coefficient (r^2) is 0.31.

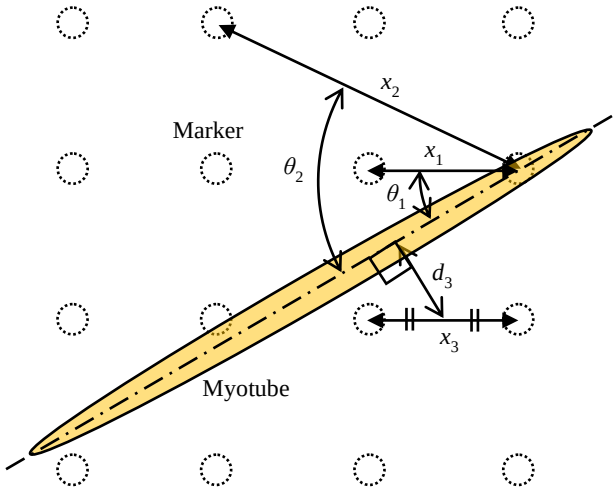

Fig. 2: Length (x) between markers: angle (θ) between longitudinal direction of adjacent contracting myotube and x: distance (d) of middle point of x from longitudinal axis of myotube.

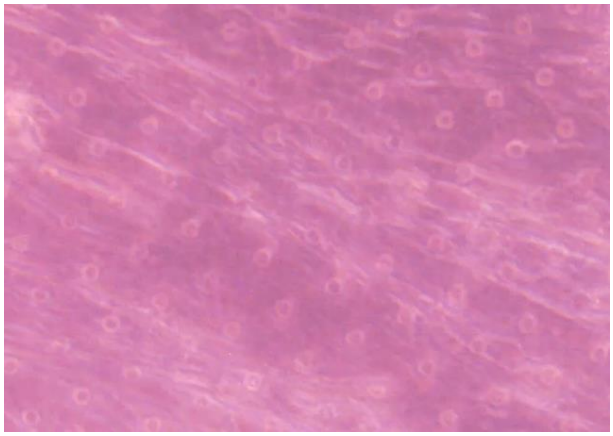

Fig. 3a: Micro-markers on back side of film are observed through myotubes by microscope.

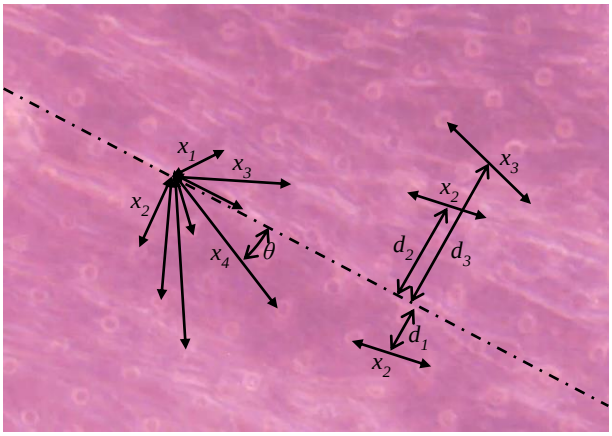

Fig. 3b: Several combinations of markers for variations; x (length), θ (angle), and d (distance): related to the longitudinal direction of myotube (chain line).

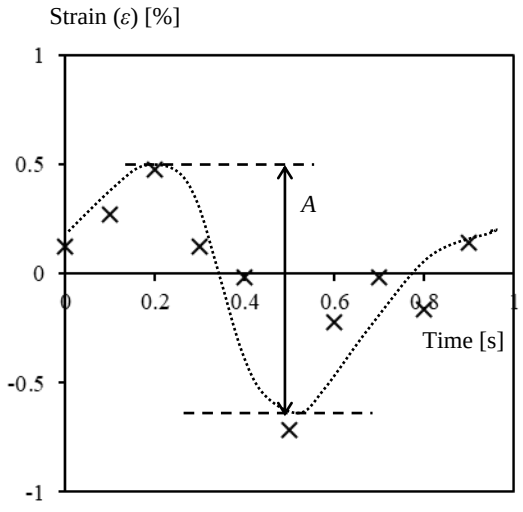

Fig. 4: Tracings of strain (ε) of length (x) between micro-markers with cyclic contraction of myotube: A, amplitude.

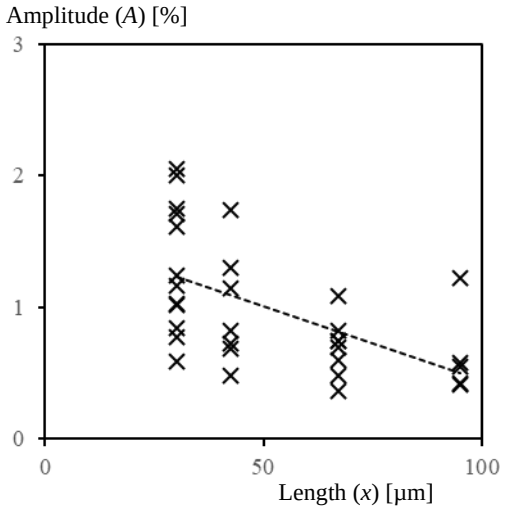

Fig. 5: Amplitude (A) vs. length (x): $A = -0.011\ x + 1.6$, $r^2 = 0.31$.

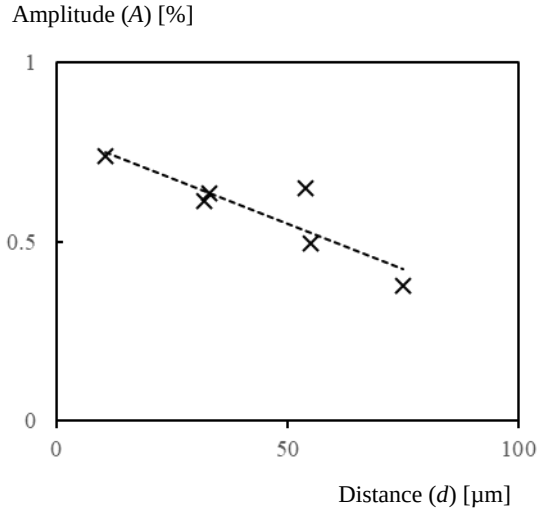

Fig. 6: Amplitude (A) vs. distance (d): $A = -0.005\ d + 0.8$, $r^2 = 0.78$.

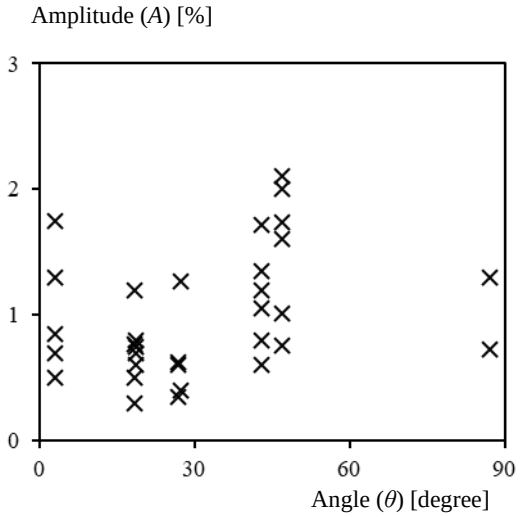

Fig. 7: Amplitude (A) vs. angle (θ).

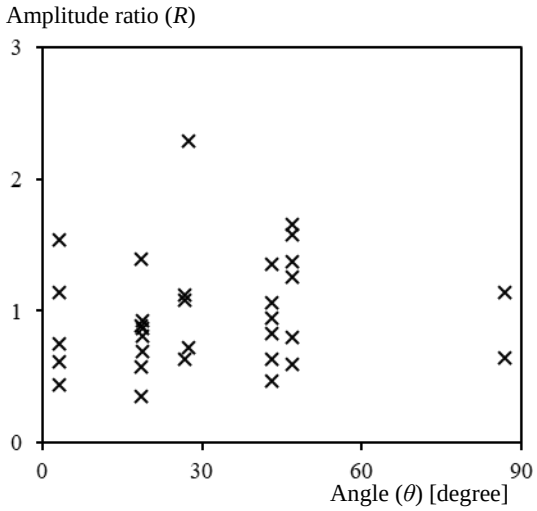

Fig. 8: Amplitude ratio (R) vs. angle (θ).

Fig. 6 shows the relationship between the amplitude (A) and the distance (d). The amplitude (A) decreases with the distance (d). The dotted line shows the regression line ($A = -0.005\ d + 0.8$). The correlation coefficient (r^2) is 0.78.

Fig. 7 shows the relationships between the amplitude (A) and the angle (θ). The amplitude is big at 45 degree. The length was shortest (x_1 in Fig. 3b) at 45 degree.

The shorter length (x) tends to make the larger amplitude (A) (Fig. 5). The amplitude (A) is corrected about the distance (x) using the regression line of Fig. 5. Fig. 8 shows relationship between the amplitude ratio (R) and the angle (θ). The amplitude ratio R is calculated by Eq. (2).

$$R = A / (-0.011\ x + 1.6) \quad (2)$$

The amplitude ratio is constant regardless of the angle (Fig. 8).

4. DISCUSSION

The contractile movement is not uniform: neither over the layer of the myotubes, nor within the single myotube. The contraction ratio of each single myotube depends on the position. The directions of myotubes are not parallel to each other throughout the bigger tissue. The contractile movement of the tissue depends on the alignment of each myotube. To analyze the local movement of the myotube in the tissue, the local markers are necessary [2]. To measure the local movement of myotubes, the short distance (30 μm) between markers is effective (Fig. 5). The alignment of each myotube is distributed, although the alignment of each myotube depends on that of the neighbor myotube. The arrangement of markers in the present study can detect

the local movement of myotubes, even if the alignment of myotubes scatters.

The contraction of the specimen of muscle tissue was measured by laser preparation in the previous study [7]. The local movement should be observed to analyze the local force of the contraction at the myotube. The local movement of the marker has been observed at the microscope synchronously with the contraction of the myotube in the present study.

In the previous study, cells were cultured on the micro pillars [6]. The deformability of the micro pillar depends on its height. The behavior of myoblasts, however, depends on the microtopography of the surface of the scaffold [13-15]. To separate the effect of microtopography on the behavior of myoblast from the contraction of myotubes, the micro-protrusions are used as the markers on the rear surface of the scaffold in the present study. The previous study showed that the orientation of the myoblast depends on the height of the micro ridges [15]. In the present study, cells are cultured on the flat thin sheet with the micro-protrusions on the rear surface, so that each cell can adhere on the sheet at any direction regardless of the position of the micro-protrusions.

The photolithography technique can be applied to control deformability of the surface [6, 16-19]. The harder scaffold accelerated differentiation of cells in the previous study. The sheet lined with micro-protrusions array has distribution of local micro deformability. The behavior of the 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 the scaffold, the medium was filled into the rear-side space of the film through the gap between U-shaped parts of the base ring. The optical transparency through the film was also improved by the filling medium in the rear space of the film.

In the previous study, the optical scattering by micro pillars on the scaffold disturbed optical microscopic observation at cells on the scaffold [3, 13]. To minimize the scattering, the height of each micro-pillar 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 the stimulation of electric pulses [20-22]. The cells have been cultured on the scaffold for 12 days in the medium in the present study.

The film of the scaffold should be deformed synchronously with the myotube to detect the contraction force of the myotube. The surface of the scaffold is hydrophilized by the oxygen plasma 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 the contraction of the myotube [23-26]. The scaffold should have enough deformability to follow the contraction with the small force [27-29]. The thickness of the PDMS film [30] of the scaffold has been made as thin as possible in the present study.

5. CONCLUSION

The repetitive contraction of myotubes has been measured by the local movement of micro-markers on the thin film of the scaffold under the electric stimulation *in vitro*. The scaffold is made of a thin polydimethylsiloxane film (thickness of 6 μm), of which the back side has an arrangement of micro-protrusions (4 μm diameter, 2 μm height, and 30 μm interval) made by the photolithography technique. C2C12 (mouse myoblast) was cultured to be differentiated into myotubes on the scaffold for 12 days. The contraction of myotubes by the electric pulses was observed through the transparent scaffold at the microscope. The experimental results show that the amplitude of the cyclic variation of the distance between micro-markers depends on distance and on alignment. The designed scaffold has potential to be applied for the analysis of the local contractile movement of the layer of myotubes *in vitro*.

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