Tracking Local Contractile Movement of Myotubes Layer Using Matrix Pattern Markers Microfabricated on Rear Side of Scaffold Thin Film

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ABSTRACT

To measure the local repetitive contractile movement of the layer of myotubes, the movement of micro matrix pattern markers on the rear side of the scaffold thin film has been tracked under electric stimulation *in vitro*. The scaffold was made of a polydimethylsiloxane film (6 μm thickness) with micro-protrusions (4 μm diameter, 2 μm height, 30 μm 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 to be differentiated into myotubes on the scaffold for 10 days. The electric pulses (2 V amplitude, 0.5 s pulse period, 1 ms pulse width) were applied between electrodes of titanium wire dipped in the medium. Both the markers on the film and the myotubes on the counter side of the film are able to be simultaneously observed by adjusting the focus of the microscope. The experimental results show that the local cyclic movement of the layer of myotubes was able to be measured with the movement of each marker. The designed scaffold has a potential 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

Myoblasts can be differentiated into myotubes in cell culture *in vitro*. Recently, tissue technology realized engineered tissue *in vitro*. The culture condition can control tissue formation [1]. Is it possible to functionally evaluate engineered tissue prior to clinical application in the field of regenerative medicine? Intra- and inter-cellular forces has been tried to be evaluated by several preparations [2-5]: culture on the micro coil spring [6], the laser technique, the atomic force microscope [7], and the fluorescence technique. Only at the end of test, the scanning electron microscope, and the stain technique are available.

Photolithography technique provides micro topography on the surface of the scaffold [2, 3, 8, 9]. When the myotube on the scaffold contracts, the scaffold should deform synchronously to detect the contractile movement by the deformation. The myotube must adhere to the scaffold. The scaffold requires both proper deformability and traceable markers.

Polydimethylsiloxane can be applied to materials for micromachining of surface topography [10]. The scaffold of the transparent thin film, of which backside has micromachined matrix pattern markers, has been designed to track the local contractile movement of myotubes layer under the cyclic electric stimulation *in vitro* in the present study.

2. METHODS

Micro Matrix Pattern Markers

The scaffold of the transparent film with micro matrix pattern markers has been designed to track the local contractile movement of the layer of myotubes 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 (4 μm diameter, 2 μm height). The pitch between adjacent protrusions is 0.03 mm. The thickness of the base film of PDMS is 6.4 μm. The side without protrusions is used for the scaffold of cell culture. The protrusions play a role of the position marker. The matrix pattern of protrusions is made in the square area of 3 mm × 3 mm at the center of the film.

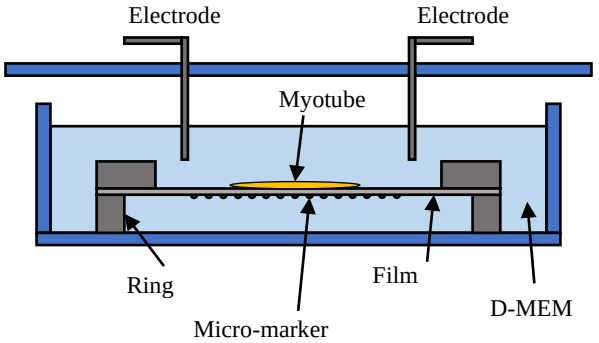

Fig. 1: Scaffold film with micro pattern markers.

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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 matrix 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 reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan). OFPR-800LB was removed by acetone, after confirmation of 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 line of micro pattern was traced.

PDMS Ring Supporter

The supporter of the cell culture film was made of the donut-ring of Polydimethylsiloxane (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 Matrix Pattern

The mold for the thin film of the scaffold with micro protrusions matrix pattern 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 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 morphology 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 Matrix 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 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.

The culture surface of PDMS was exposed to the oxygen gas (0.1 Pa, 30 cm^3/min) at power of 50 W for thirty seconds in a reactive ion etching system (FA-1) to be characterized as hydrophilic (oxygen plasma ashing). The surface was preserved in the ultrapure water to keep the hydrophilic property 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 the inverted 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 a 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 (amplitude of 2 V, pulse width of 1 ms, period of 0.5 s) was generated with an electric stimulator (SEN5201, Nihon Kohden Corporation, Tokyo, Japan). The stimulator was connected to the electrodes.

Image Analysis

When the movement of the myotube was microscopically observed, the synchronous movement of the micro pattern markers was recorded by a movie camera. At each image of the movie, the circular contour of each marker was traced (Fig. 2). Each centroid of the marker was tracked over the term of the cyclic period of the electric stimulation (Fig. 3). The two-dimensional position of each marker was defined by the coordinates of x and y: the x coordinate is to the right and the y coordinate is downward in Fig. 3.

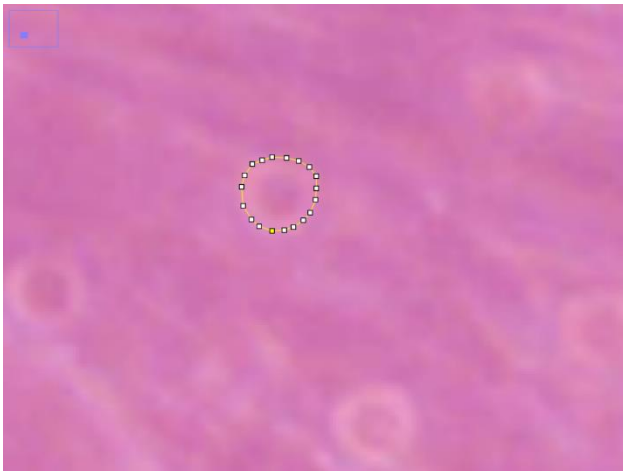

Fig. 2: Circular contour of each marker was traced: 0.07 mm from left to right.

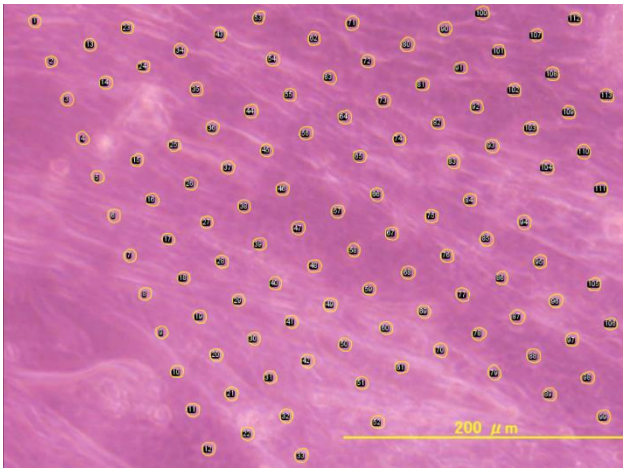

Fig. 3: Micro markers matrix on back side of film can be observed through myotubes by microscope: *x* coordinate is to right and *y* coordinate is downward: 10 days, 2 V, No. 1-113(34).

3. RESULTS

The myoblasts were able to be cultured on the film of the scaffold to be differentiated into myotubes in 10 days (Fig. 3). Both the markers on the film and the myotubes on the counter side of the film are able to be simultaneously observed by adjusting the focus of the microscope. The repetitive contraction of myotubes synchronous to the period of the electric pulse was able to be observed at the microscope.

Figs. 4-7 exemplify tracings of position of each marker. Figs. 4 and 6 illustrate the time variation of the *x*-coordinate of the marker. Figs. 5 and 7 illustrate the time variation of the *y*-coordinate of the marker. The position of each marker shows cycles with the term of 0.5 s, although data show fluctuation (Figs. 5 and 6). Fig. 7 shows larger fluctuation. The position of the marker (No. 10) is adjacent to the active myotube. The difference between movements of markers shows the local deformation of the film. Comparison these figures show the following movement. The distance between the markers is shrunk in the x direction when the distance between the markers is elongated in the y direction.

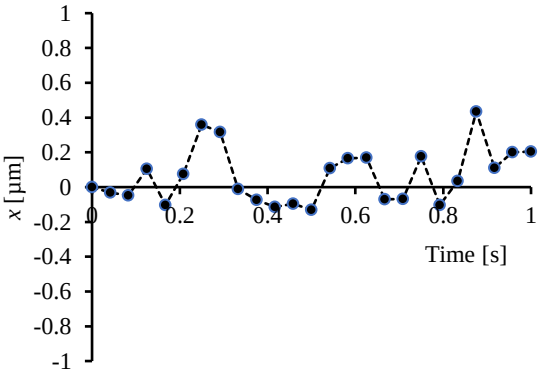

Fig. 4: Position tracking of micro marker (No. 1) in *x* direction.

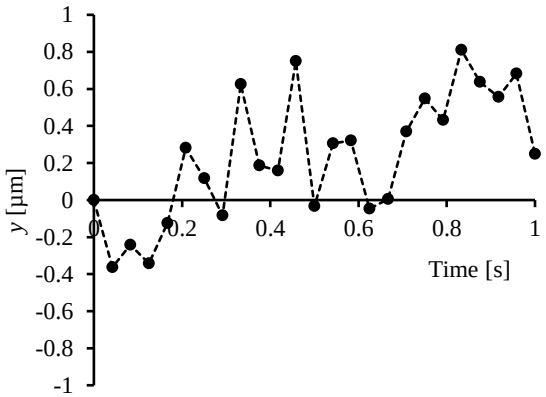

Fig. 5: Position tracking of micro marker (No. 1) in *y* direction.

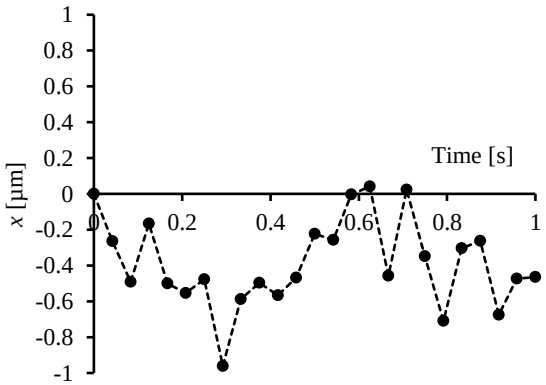

Fig. 6: Position tracking of micro marker (No. 10) in *x* direction.

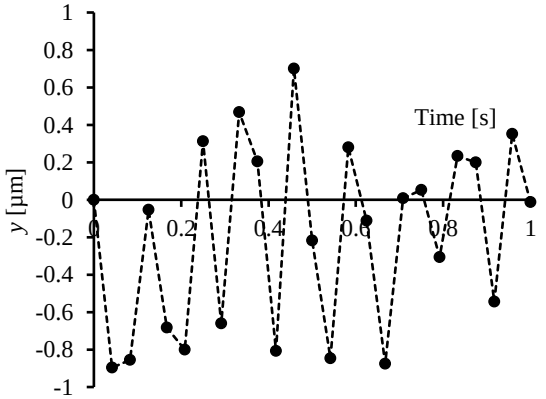

Fig. 7: Position tracking of micro marker (No. 10) in *y* direction.

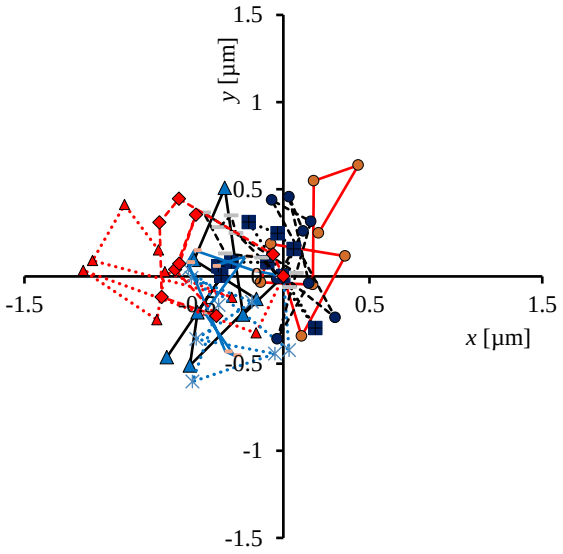

Fig. 8: Position tracking of micro markers in *x-y* direction.

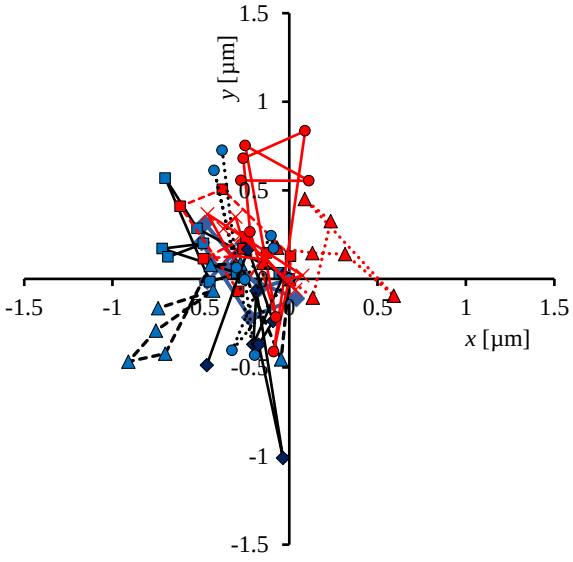

Fig. 9: Position tracking of micro markers in *x-y* direction.

Figs. 8 and 9 exemplify tracking the position of each marker in *x-y* coordinates. Each marker shows repetitive movements according to the cycle of electrical stimulation. Adjacent markers show the similar movements each other. Some markers move in the *y* direction and others move in the *x* direction.

4. DISCUSSION

The movement of every maker is not synchronous because of several factors. Each myotube makes repetitive contraction with each own rhythm even by the common electric cyclic stimulation. The timing of contraction may sometimes be delayed. The contraction of the myotube may sometimes be absent in response to electrical stimuli. The longitudinal directions of myotubes are not parallel each other. The local amplitude of contraction is not the same along each myotube.

The contractile movement of the film is not uniform. The contraction ratio of each single myotube depends on the position. The directions of myotubes are not completely parallel in the 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 [12]. To measure the local movement of myotubes, the short distance (30 µm) between markers was effective (Fig. 5). The interval of 30 µm between adjacent markers, on which repetitive movements of the myotube was calculated [13], was effective in detecting local repetitive contractile movements of the layer of myotubes. The movement of each marker depended on the distribution of the myotubes that contract repeatedly.

The alignment of each myotube distributes, although the alignment of each myotube depends on that of neighbor myotube. The arrangement of markers in the present study was able to 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 [14]. The local movement should be observed to analyze the local force of contraction at the myotube. The local movement of the marker has been observed at the microscope synchronously with the contraction of myotube in the present study.

In the previous study, cells were cultured on the micro pillars [9]. The deformability of the micro pillar itself depends on its height. The micromorphology of the surface of the scaffold, however, governs the behavior of myoblasts: migration, deformation, and differentiation [15, 16]. To separate the effect of micromorphology on the behavior of myoblast from the formation of myotubes on the scaffold, 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 direction of the micro ridges [17]. In the present study, cells are cultured on the flat thin sheet with the micro protrusions on the rear side, so that the 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 compliance of the surface [9, 18-21]. The harder scaffold accelerated differentiation of cells in the previous study. The sheet lined with micro-protrusions array on the rear side has distribution about local micro compliance of the scaffold. The behavior of cell might depend on the local micro deformability of the scaffold.

The myotubes do not make orientation related to the micro matrix pattern markers on the rear side of the scaffold film in the present study. If the contraction is measured in a tissue composed of oriented myotubes, the total force may be stronger.

To observe the entire scaffold plane at the same level of the focus, the bend of the film is minimized with the peripheral base ring of PDMS. To keep the position of the film of scaffold, the medium liquid was filled on the rear side of the film through the gap between U-shaped bases. The optical transparency also is improved by filling medium at 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 [22]. To minimize the scattering, the height of the micro-protrusions 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 [23-28]. The cells were cultured on the scaffold for 10 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 [29]. The scaffold must have the appropriate deformability to track the contraction with a small contractile force of the myotube [30-32]. If the film is too hard, it will not deform with a small force. If the film is too soft, it will remain shrunk and will not be able to contract repeatedly. The thickness of the base film of PDMS [10] is 6.4 μm in the present study.

5. CONCLUSION

To measure the local repetitive contractile movement of the layer of myotubes, the movement of micro matrix pattern markers on the rear side of the scaffold thin film has been tracked under the cyclic electric pulse stimulation *in vitro*. The scaffold was made of a thin polydimethylsiloxane film (thickness of 6 μm), of which the back side had arrangement of micro-protrusions (4 μm diameter, 2 μm height, interval 30 μm) made by the photolithography technique. C2C12 (mouse myoblast) was cultured to be differentiated into myotubes on the scaffold for 10 days. Both the markers on the film and the myotubes on the counter side of the film are able to be simultaneously observed by adjusting the focus of the microscope. The experimental results show that the local cyclic movement of the layer of myotubes was able to be measured with the movement of each marker. The designed scaffold has a potential to analyze the local contractile movement of the layer of myotubes *in vitro*. The technology can be applied to functional evaluation of engineered tissue.

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