

Electric Stimulation for Acceleration of Cultivation of Myoblast on Micro Titanium Coil Spring

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ABSTRACT

The electric stimulation has been applied on myoblasts to accelerate cultivation on a micro coil spring *in vitro*. A micro coil spring made of the titanium wire of 0.085 mm was used for the scaffold for the cell culture. The coil spring has the dimension as follows: 0.65 mm diameter, 0.15 mm pitch, 5 mm length. For the counter electrode, the titanium film (100 nm thickness) was coated on the glass. C2C12 (mouse myoblast cell line) was seeded and cultured in Dulbecco's Modified Eagle Medium with 10 percent of fetal bovine serum. The electric pulses were applied between the coil spring and the titanium film for thirty minutes per day: the period of 1 s, the pulse width of 0.001 s, and the voltage amplitude of 0.5 V (the current amplitude of 0.5 mA). The cells around the coil spring were observed with an inverted phase contrast microscope for ten days. The experimental results show that the movement of cell depends on the local electric field, and that the electric stimulation tends to accelerate differentiation of myoblasts to myotubes around the micro coil spring.

Keywords: Biomedical Engineering, Micro Coil Spring, Electric Pulses, C2C12, Movement, and Differentiation.

1. INTRODUCTION

Biological cells are exposed to electric fields *in vivo*. The cells are sensitive to the electric stimulation. In the previous study, the cells showed accelerated adhesion to the scaffold by electric stimulation [1]. Cell culture technique has been progressed recently. Acceleration technique for making tissue from cells *in vitro* might be applied to regenerative medicine [2]. Micro machining technique enables the design of the electrode around cell culture. The movement of cell depends on the local electric field [3, 4]. Dielectrophoresis is a phenomenon, in which a cell moves by the local electric field distortion [5, 6].

In the previous study, myoblasts were cultured on a micro coil spring *in vitro* [7, 8]. In the previous experiment, the coil spring was inserted in the electric circuit and the electric current flowed along the coil spring [8]. If the electric field is effective to accelerate adhesion of cells to the micro spring, additional electrodes should be equipped around the spring to apply electric pulses.

If the myotubes cultured on the micro coil spring, the force generated among the tissue might make deformation of the

spring. The force can be calculated from the deformation of the spring [7]. The balance between forces by myotubes and by the spring can form a micro actuator. In the present study, the electric pulses have been applied to myoblast to accelerate adhesion of cells to the scaffold of the titanium micro coil spring.

2. METHODS

Micro Coil Spring

A micro coil spring (Hi-Lex Corp., Takarazuka, Japan) made of the pure titanium (JIS class 2) wire of 0.085 mm was used for the scaffold. The coil spring has the dimension as follows: 0.65 mm diameter, 0.15 mm pitch, 5 mm length.

Titanium Surface Electrode

The titanium film was machined for the surface electrode (Fig. 1). Before the coating of titanium, the surface of the glass was hydrophilized by the oxygen (> 0.1 Pa) plasma ashing for ten minutes at 100 W by RIE (FA-1, Samco International, Kyoto, Japan). Titanium was coated on the surface of the borosilicate glass (Tempax) disk (50 mm diameter, 1.1 mm thickness) or slide glass in the electron beam vapor deposition apparatus (JBS-Z0501EVC JEOL Ltd., Tokyo, Japan). The thickness of coating is 100 nm.

The pattern of the coating is controlled by the film type of mask. A carbon disk (0.5 mm of thickness) was used for the mask for the deposition of titanium surface electrode (Fig. 2). The rectangular hole (5 mm × 20 mm) was machined at the mask by the ultrashort pulse laser (IFRIT, Cyber Laser Inc., Tokyo, Japan).

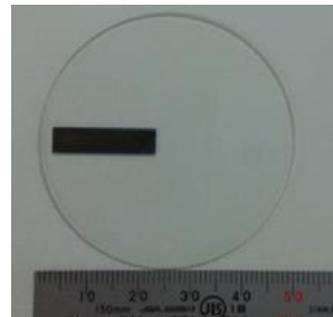


Fig. 1: Surface electrode of titanium on glass.

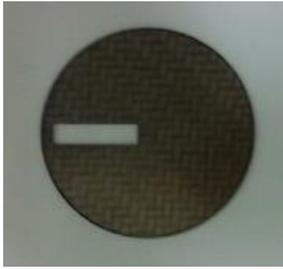


Fig. 2: Carbon disk of mask for pattern of surface electrode.

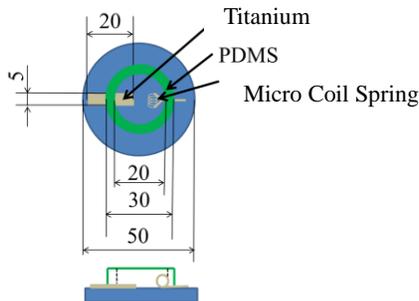


Fig. 3a: Design of electrodes of titanium film and spring.



Fig. 3b: Manufactured electrodes of titanium film and spring.

PDMS Ring

To control the scattering of the cell, the space around the coil spring is limited within a center hole (diameter of 10 mm) of the polydimethylsiloxane (PDMS) ring, which is placed on the bottom of the dish (Fig. 3).

The wall of culture dish was made of the ring of Polydimethylsiloxane (PDMS) (Fig. 3). 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, 6 cm³ of PDMS was poured on the glass disk. After degassing again, PDMS was baked at 373 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd). The baked PDMS was machined by the punch to make the ring: the internal diameter is 20 mm for the micro coil spring, or 10 mm for the titanium wire.

The micro coil spring was fixed at the ring (Fig. 3). A titanium wire of 0.5 mm diameter was used to compare with the titanium coil spring (Fig. 4).

Titanium Surface Electrode Covered with SU-8 10

For the comparison, variation was made on the surface of the

titanium electrode: the naked titanium surface electrode (Fig. 1), and the titanium surface electrode covered with the negative photoresist material of high viscosity (SU-8 10: Micro Chem Corp., MA, USA) (Fig. 5). The glass slide (25 mm × 75 mm × 1 mm) coated with titanium was hydrophilized by the oxygen (>0.1 Pa) plasma ashing for ten minutes at 100 W by RIE. SU-8 10 was coated on the surface at 5000 rpm for one minute with a spin coater. After baked in the oven at 373 K for six minutes, the photoresist was exposed to the ultraviolet light in the mask aligner (M-1S, Mikasa Co. Ltd., Japan) at 10 V for 250 s. The photoresist was baked in the oven at 373 K for ten minutes. The photoresist was developed with SU8 developer (Nippon Kayaku Co., Ltd, Tokyo, Japan) for five minutes, and rinsed with the ultrapure water.

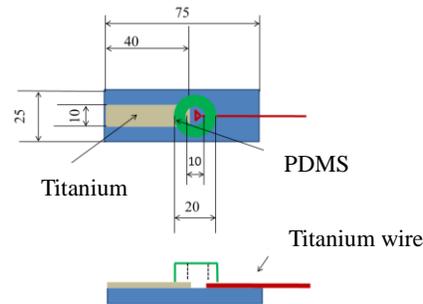


Fig. 4a: Design of electrodes of covered titanium film and wire.



Fig. 4b: Manufactured electrodes of covered titanium film and wire.

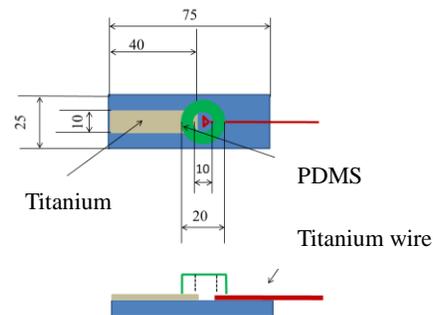


Fig. 5a: Design of electrodes of titanium film and wire.



Fig. 5b: Manufactured electrodes of titanium film and wire.

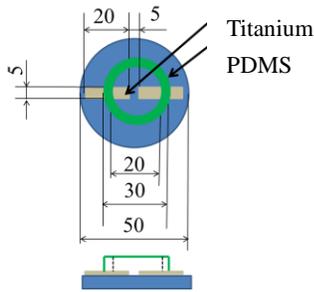


Fig. 6: Design of electrodes of titanium films.

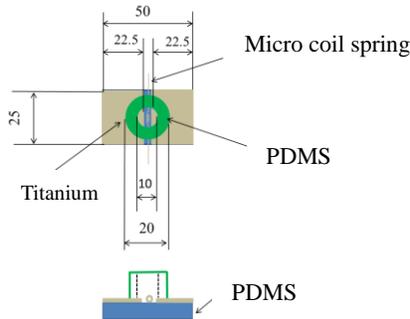


Fig. 7a: Design of spring between electrodes of titanium film.

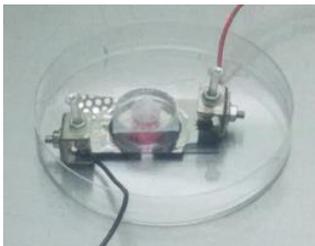


Fig. 7b: Scaffold of spring between electrodes of titanium film placed in dish.

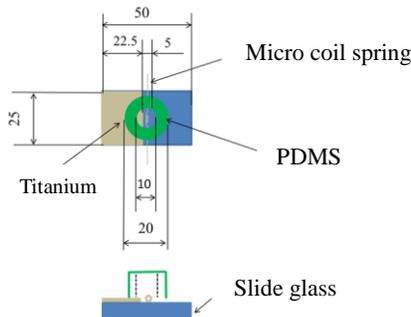


Fig. 8a: Design of electrodes of titanium film and spring.

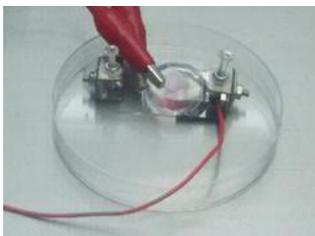


Fig. 8b: Manufactured electrodes of titanium film and spring.

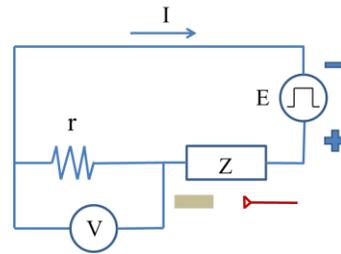


Fig. 9: Circuit for electric stimulation.

Four kinds of setting of couple of electrodes were designed: the surface film and the surface film (Fig. 6), the surface film and the coil spring (Fig. 3), the surface film and the titanium wire (Fig. 4), the surface film coated with SU8-10 and the wire (Fig. 5).

In the setting A, the coil spring is placed in the middle position between two surface electrodes (Fig. 7). In the setting B, the coil spring is placed as one of the electrode: the counter electrode is the surface electrode (Fig. 8). The distance between electrodes is 5 mm.

Electric Stimulation

The electric pulse E was generated with an electric stimulator (SEN5201, Nihon Kohden Corporation, Tokyo, Japan). The stimulator was connected to the electrodes (Fig. 9). An electric resistance r of 1 k Ω is serially inserted between the micro coil spring and the stimulator. The voltages (V) were monitored by an oscilloscope during application of electric pulse to the coil spring. 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.

Cell

C2C12 (Mouse myoblast cell line originated with cross-striated muscle of C3H mouse) was seeded in the area inside of the PDMS ring. The cells were cultured in the D-MEM (Dulbecco's Modified Eagle Medium). The medium contains FBS (decomplemented fetal bovine serum) with the volume percent of ten. The medium also contains penicillin and streptomycin with the volume percent of one. The surface electrodes and the micro coil spring were dipped in the medium.

Cell Movement

At the first trial, C2C12 was seeded in the area inside of the PDMS ring at the concentration of 1000 cells per cm^2 . The electric pulses were applied between the surface electrode and the micro coil spring: the period of 0.00001 s, the pulse width of 0.000005 s, and the voltage amplitude of 1 V. The cells were observed with an inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo) for eight hours. The time-lapse microscopic image was recorded every five minutes.

At the second trial, C2C12 was seeded at the concentration of 10000 cells per cm^2 . The electric pulses were applied between the surface electrode and the micro coil spring: the period of 0.00001 s, the pulse width of 0.000005 s, and the voltage amplitude of 10 V. The time-lapse microscopic image was

recorded every thirty seconds for thirty minutes, and every two minutes for six hours.

At the third trial, C2C12 was seeded at the concentration of 10000 cells per cm^2 . The electric pulses were applied between the surface electrode covered with SU8-10 and the titanium wire: the period of 0.00001 s, the pulse width of 0.000005 s, and the voltage amplitude of 10 V. The time-lapse microscopic image was recorded every thirty seconds for thirty minutes.

At the fourth trial, C2C12 was seeded at the concentration of 10000 cells per cm^2 . The electric pulses were applied between the surface electrode and the titanium wire: the period of 0.00001 s, the pulse width of 0.000005 s, and the voltage amplitude of 10 V. The time-lapse microscopic image was recorded every thirty seconds for thirty minutes.

During the microscopic observation, the temperature and the content of the carbon dioxide gas were maintained at 310 K and 5%, respectively.

Cell Culture

C2C12 was seeded in the area inside of the PDMS ring at the concentration of 10000 cells per cm^2 . After cultivation of 24 hours, the electric pulses were applied to the medium for thirty minutes per day (Fig. 10): the period of 1 s, the pulse width of 0.001 s, and the voltage amplitude of 0.5 V (the current amplitude of 0.5 mA). The cells around the coil spring were observed with the inverted phase contrast microscope for ten days. The cell culture experiments are divided into two groups: using the setting A (Fig. 7), or B (Fig. 8).

At the end of the culture, the morphology of the surface of the micro coil spring was observed by a scanning electron microscope (SEM, JSM6360LA, JEOL Ltd., Tokyo, Japan).



Fig. 10: Cell culture with electric stimulation.

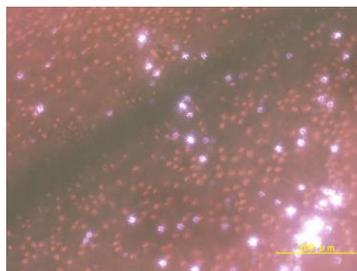


Fig. 11: C2C12 near covered surface electrode and wire in 2 min. Dimension from left to right is 0.9 mm.



Fig. 12a: C2C12 near surface electrode and wire before electric stimulation. Dimension from left to right is 0.9 mm.



Fig. 12b: C2C12 near surface electrode and wire electric stimulation for 8 min. Dimension from left to right is 0.9 mm.

3. RESULTS

The results of cell movement with the electrode of the micro coil spring show the following movement of cell. The cells show vibration under electric stimulation between the surface electrode covered with SU8-10 and the titanium wire (Fig. 11). The cells approach to the titanium wire under electric stimulation between the surface electrode and the titanium wire. Fig. 12b shows the cells out of focus because of the movement.

In the successive cultivation, cells differentiate into myotubes in ten days, and cells make tissue layer around the micro coil spring in sixteen days (Fig. 13). Cells survive under continuous application of cyclic electric pulses for six hours (Fig. 14).

Fig. 15 shows the tracing of the electric pulse which is applied between the electrodes: amplitude of 0.5 V, and width of 0.001 s.

Although the image of cells on the coil spring is not very clear, several cells adhere on the coil spring in several days of culture: 7 days on the coil spring between the surface electrodes, and 5 days on the coil spring as one of the electrodes (Fig. 16), respectively.

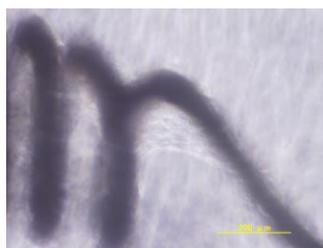


Fig. 13: Cell culture on coil spring on day 16. Dimension from left to right is 0.9 mm.

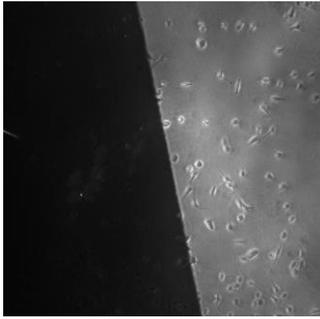


Fig. 14: C2C12 near coil spring under electric stimulation for 6 hours. Dimension from left to right is 0.9 mm.

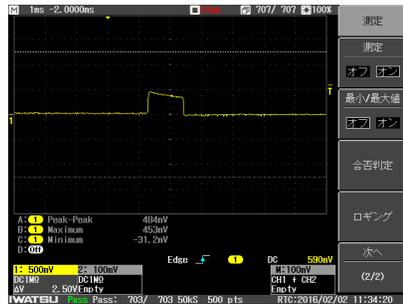


Fig. 15: Voltage at resistance during application of electric pulse between film and spring.

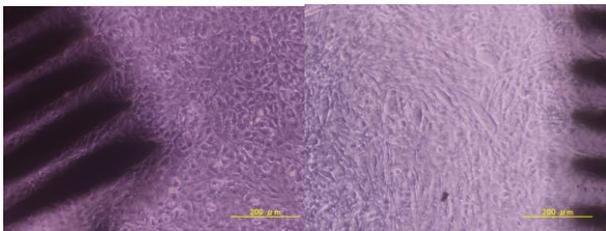


Fig. 16a: Cell culture on day 6; surface electrode (left), spring electrode (right). Dimension from left to right is 0.9 mm, each.

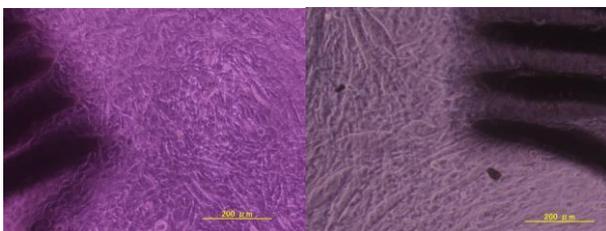


Fig. 16b: Cell culture on day 10; surface electrode (left), spring electrode (right). Dimension from left to right is 0.9 mm, each.

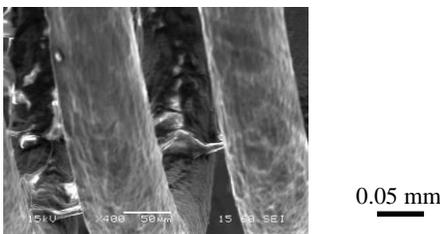


Fig. 17: SEM image of cells on coil spring.

The microscopic image shows that myoblast cultured on the glass differentiates into myotubes. Between two surface electrodes, myotubes are observed in 10 days of culture (Fig. 16b). Between the surface electrode and the coil spring electrode, on the other hand, myotubes are observed in 6 days (Fig. 16a).

Fig. 17 exemplifies SEM image of the coil spring at the end of the cell culture. The figure shows that the cells and the extracellular matrix make bridges between pitch of the spring.

4. DISCUSSION

Electrolysis does not occur in the present experiment. To prevent the medium from electrolysis, electric pulses are applied to the medium.

The movement synchronous to the electric pulse applied between the electrodes was observed at the cells between electrodes. The movement of the cell might depend on the distance between electrodes. The movement of cell is not observed between electrodes with the distance of 5 mm, but observed between electrodes with the distance of 3 mm in the present experiment.

Most of cells adhere and differentiate to myotubes not on the micro coil spring, but on the glass plate in the present study. In the previous study, more cells adhered to the micro coil spring on the culture dish [7, 8]. The behavior of cells might depend on affinity between cell and the scaffold. The differentiation of myoblast seems to be accelerated at the setting B in the present study, in which the coil spring is placed as one of the electrode.

The electric stimulation has been applied to human body in the clinical treatment [9]. The cell culture technique was applied to make a bio-actuator combined with micro-machine technique in the previous studies [10, 11]. In the previous studies, the several kinds of acceleration technique to make orientation of cells were tried *in vitro*: with the shear flow [12, 13], with the gravitational force [14], with the electric field [1, 15], with the magnetic field [16], or with the micro pattern of the surface [17-19].

The micro coil spring gives a good scaffold for cell culture. The spiral morphology of the coil spring might make the spiral orientation of myotubes [8]. When cells make tissue, a space for supplying medium is necessary around cells. A coil spring has a spiral space along the wire: the interval of 0.085 mm between the wires. The space might give a path for the medium to approach to the cells. The coil spring deforms in proportion to the force. The force generated in the muscle tissue cultured on the coil spring might be estimated by the displacement of the coil spring [7].

The movement of cultured myotubes is able to be controlled with electric pulses supplied to the medium. The laser system has been applied to measure the cyclic movement in the biological system [20].

Titanium is one of the materials, which has been used for biological application [21, 22]. Titanium has been implanted to human body as a strut of valves, a root of teeth, pins in orthopedic treatment, and a part of joint. C2C12 is able to

adhere and proliferate on the surface of the micro coil spring of titanium. The cells are also able to differentiate into myotubes around the coil spring [7, 8].

Under the electric stimulation of pulses, the proliferation of cells might decrease [1]. The electric pulses, on the other hand, might control the migration to the micro coil spring, and accelerate differentiation to myotubes.

5. CONCLUSION

The electric stimulation has been applied on myoblasts to accelerate cultivation on a micro coil spring *in vitro*. A micro coil spring made of the titanium wire of 0.085 mm was used for the scaffold for the cell culture. The electric pulses were applied between the coil spring and the titanium film coated on the glass for thirty minutes per day: the period of 1 s, the pulse width of 0.001 s, and the voltage amplitude of 0.5 V. The experimental results show that the movement of cell depends on the local electric field, and that the electric stimulation tends to accelerate differentiation of myoblasts to myotubes around the micro coil spring.

6. ACKNOWLEDGMENT

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