Design of Comb-shaped Surface Electrode to Measure Signal from Tissue Cultured with Electric Stimulation

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ABSTRACT

The comb-shaped surface electrode has been designed to measure the signal from the tissue cultured with electric stimulation. The pattern of the thin film of the surface electrode was made of indium tin oxide (ITO) coated on the glass by the photolithography technique. C2C12 (mouse myoblast cell line) was cultured for 13 days on the patterned scaffold. The electric pulses (period 1 s, amplitude 10 V, duration 0.002 s) were applied for 10 minutes per day. The experimental results show that myoblasts make orientation along the comb-shaped surface electrode, which can be observed through the transparent surface electrode by the optical microscope. The change of the electric resistance of the cultured tissue is able to be detected between electrodes of the experimental system *in vitro*.

Keywords: Biomedical Engineering, Surface Electrode, C2C12, Electric Pulse and ITO.

1. INTRODUCTION

The cell culture technique has been developed and several methodologies might clinically be applied to regenerative medicine [1-4]. C2C12 (mouse myoblast cell line) adheres to the scaffold, proliferates and differentiates to myotubes *in vitro* [5]. These behaviors of the cell depend on the micro property of the surface [6].

The effect of the surface property of the scaffold on cell culture has been studied in the previous studies [7]. Several micro-fabrication processes have been designed to control adhesion of biological cells *in vitro* [8, 9]. Photolithography technique enables machining for design of the surface electrode.

The muscle tissue is exposed to electric pulses *in vivo*. The movement is also controlled with the electric pulses. The biological systems have ability to optimize themselves to their environment. The optimum electric stimulation has a potential to accelerate differentiation of myoblast and growth of the muscle tissue, which might contribute to the regenerative medicine.

The electric impedance of the biological tissue was measured in the previous study to distinguish the kinds of tissue [10]. The impedance depends on the orientation of cells.

The membrane of the biological cell keeps difference of the electric potential between inside and outside. The operation of the ion channel makes change of the potential, which makes electric pulses. The electric pulses are detected, when the electrodes put on the biological tissue [11, 12].

Indium tin oxide (ITO) is an inorganic compound of indium oxide (In_2O_3) and tin oxide (SnO_2). Because the transmittance in the region of the visible light is high, the thin film of ITO is colorless and transparent. ITO is one of the useful materials, which is applied to the transparent electrode [13].

The response of biological cells might depend on the direction of electric signal [14]. Myotube contracts synchronously with the electric pulse, when electric pulse is applied on the medium through electrodes *in vitro* [15].

In the present study, the comb-shaped surface electrode of ITO has been designed to measure the signal from the muscle tissue cultured with the electric stimulation *in vitro*.

2. METHODS

Comb-shaped Surface Electrode

A comb-shaped surface electrode has been designed by the micromachining technique (Fig. 1). The comb-shaped pattern has been selected for the following reasons: to make orientation of cells, to make oriented stimulation of electric pulses, and to measure oriented signal from cells.

The indium tin oxide (ITO) film was used for the surface electrode [13]. A thin film of ITO is formed on the surface of a glass disk with the comb-shaped micro pattern (line of 0.1 mm width).

Photomask

The borosilicate glass (Tempax) disk was used for the base of the photomask (Fig. 1b). After hydrophilization by the oxygen plasma ashing for five minutes at 100 W by RIE (FA-1, Samco International, Kyoto, Japan), titanium was coated on the surface with 100 nm thickness in the sputtering system (L-332S-FH, Canon Anelva Corporation, Kawasaki, Japan). The disk was hydrophilized by the oxygen plasma ashing for ten minutes at 100 W by RIE again.

To improve affinity between titanium and photoresist material, HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co.,

Ltd., Tokyo) was coated on the disk with a spin coater. The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd, Tokyo, Japan) was coated on the disk at 5000 rpm for 20 s with the spin coater. The photoresist was baked in the oven at 368 K for three minutes.

The pattern was drawn on the mask with a laser drawing system (DDB-201K-KH, Neoark Corporation, Hachioji, Japan). To control the dimension of the pattern on the mask with the laser drawing system, the parameters were selected as follows: the voltage of 3.5 V, the velocity of 0.14 mm/s, the acceleration of 0.34 mm/s^2. The pattern was baked on the heated plate at 393 K for five minutes. The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for 3 minutes (Fig. 2). The disk was rinsed by the ultrapure water for one minute, and dried by the spin-dryer.

The titanium coating disk was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan). For etching, the gas of SF_6 with Ar was applied for six minutes. OFPR-800LB was removed by acetone (Fig. 1).

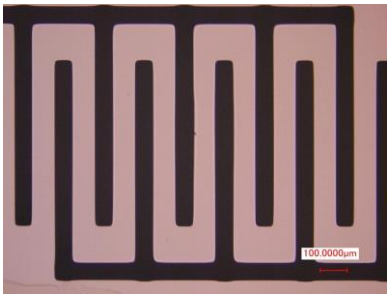

Fig. 1a: Micro pattern of mask for comb-shaped surface electrode. Dimension from left to right is 1.3 mm.

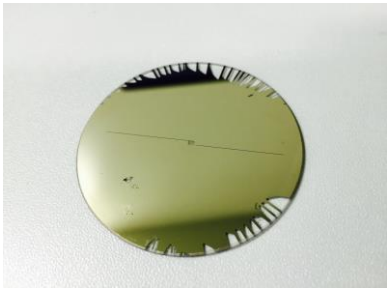

Fig. 1b: Photomask of titanium on the glass.

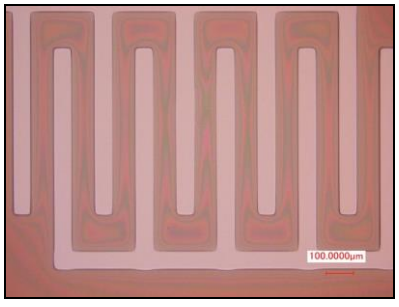

Fig. 2: Micro pattern of mask developed on the glass. Dimension from left to right is 1.3 mm.

Scaffold with Micro Pattern

The borosilicate glass (Tempax) disk (50 mm diameter, 1 mm thickness) was used for the base of the scaffold. After hydrophilization by the oxygen plasma ashing for five minutes at 100 W by RIE, the positive photo-resist material of OFPR-800 was coated on the glass with the spin coater (Fig. 3). The photo-resist was baked at 373 K for three minutes.

The surface of titanium coating mask was adhered on the surface of OFPR-800, and the photoresist was exposed to the UV light through the photomask in the single sided mask aligner (M-1S, Mikasa Co. Ltd., Japan) at 10 V for 30 s. The photoresist was baked at 393 K for five minutes.

The photoresist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan). The disk was rinsed by the ultrapure water.

ITO was coated on the surface with 0.08 mm thickness (1 mm width × 30 mm length) in the electron beam vapor deposition apparatus (JBS-Z0501EVC, JEOL Ltd., Japan).

After lift-off the ITO on OFPR-800LB by removal with acetone, the pattern of ITO was left on the glass disk (Fig. 4a). The disk was dried by the spin-dryer.

For the reference electrode, Ag/AgCl paste was baked at 393 K for five minutes (Fig. 4b). The lead wire is soldered on the each end of the film to introduce the electric current.

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

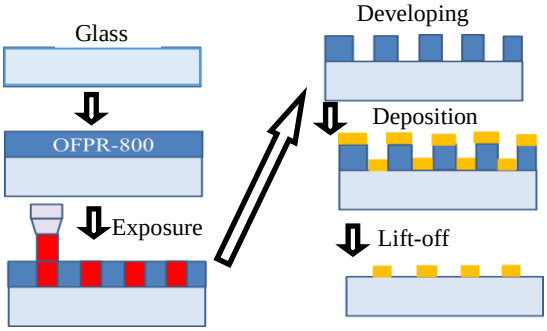

Fig. 3: Photolithography process for surface electrode.

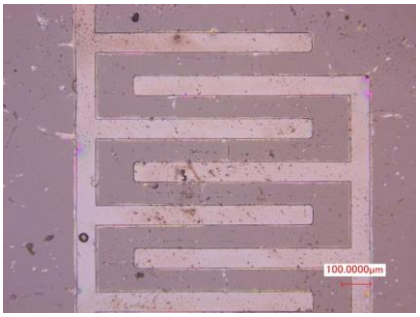

Fig. 4a: Comb-shaped surface electrode. Dimension from left to right is 1.3 mm.

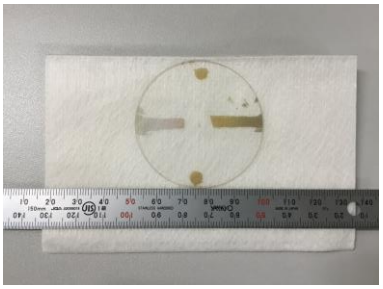

Fig. 4b: Surface electrodes on glass.

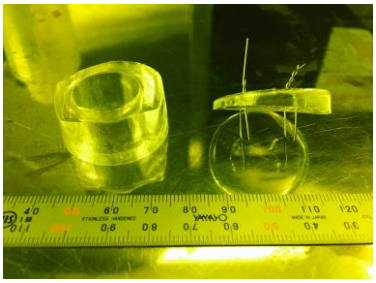

Fig. 5: PDMS ring-wall and cap with electrode wire.

PDMS Wall and Cap

A donut-ring of polydimethylsiloxane (PDMS, Dow Corning Corporation, MI, USA) is adhered with the affinity of surfaces to make the peripheral walls for the cell culture on the Tempax glass disk. The outer diameter and the inner diameter of the ring are 25 mm, and 10 mm, respectively (Fig. 5).

A pair of electrodes of titanium wire (0.5 mm diameter) for electric stimulation to observe synchronous contraction of myotubes are put through the cap of PDMS.

Both on the ITO film and on the base of the glass, the pure water contact angles were measured by the contact angle analyzer (Phoenix-300, Meiwafosis Co., Ltd., Tokyo, Japan).

Electric Pulses

The electric pulses (period 1 s, amplitude 10 V, duration 0.002 s) were generated with an electric stimulator (SEN5201, Nihon Kohden Corporation, Japan). The stimulator was connected to the ITO film, and the pulses were introduced to the scaffold of cells for ten minutes per day (Fig. 6). An electric resistance R of 51 Ω is serially inserted between the ITO film and the stimulator. The electric signals (V_1, V_2) were monitored by an oscilloscope during electric stimulation to the ITO film. V_1 and V_2 are the voltages between the terminals of resistance, and of electric stimulator, respectively. The electric stimulation was applied to the scaffold after 24 hours of culture for adhesion of the cells to the scaffold.

Cell Culture

C2C12 (Mouse myoblast cell line originated with cross-striated muscle of C3H mouse) of the ninth passage was used for the cell culture with electric stimulation. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin was used for the medium in first two days. The medium was changed to D-MEM containing 2% HS (Horse Serum) on the third day of cultivation.

The cells were seeded on the scaffold with the density of 10000 cells/cm^2. Cells were cultured in the incubator at 310 K with 5% CO_2 for 13 days. The medium were refreshed every two days. Cells were observed with an inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo) once a day. The electric potential was traced at the electrode of ITO during the pulse application once a day (Fig. 7). During the measurement, the culture dish was kept at room temperature out of the incubator.

The differentiation from myoblasts to myotubes was confirmed by contractive movement of myotubes during electric pulse application to the medium once a day, after cultivation for seven days. The cyclic electric pulses (amplitude of 10 V, duration of 0.003 s) at the period between 0.1 s and 2 s were applied to the medium through electrodes of titanium wire.

3. RESULTS

Fig. 8 shows the dimension of the micro pattern of the surface electrode. Measurement by the laser microscope shows that the comb-shaped micro pattern of surface ITO film has the dimension of 0.07 mm width, and 0.0014 mm thickness.

Fig. 9 shows the contact angle on the film of ITO (A) and on the base of the glass (B). The contact angle is smaller on the glass (0.12 rad) than on the ITO film (0.16 rad).

Fig. 10 shows C2C12 around the micro pattern of surface electrode. On the thirteenth day of culture, C2C12 differentiated to myotubes, and the myotubes orient along the longitudinal direction of the comb-shaped surface electrode.

The electric resistance r between the surface electrodes is calculated as 9 Ω from Eq. 1, when the electric resistance of 51 Ω is inserted for R (Fig. 6).

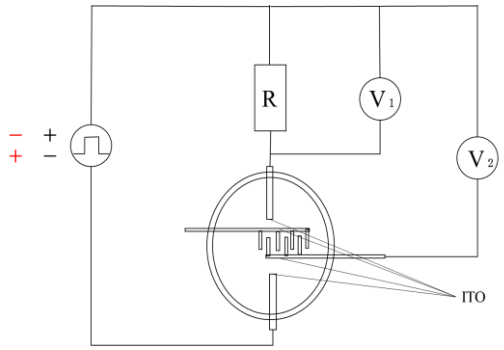

Fig. 6: Electric circuit.

Fig. 7: Measurement of electric signal on stage of microscope (left) between electrodes (right).

$$r = (V_2 - V_1) / (V_1 / R) \quad (1)$$

In Eq 1, V_1 is voltage between the terminals of resistance of R, and $(V_2 - V_1)$ is voltage between the terminals of resistance of r.

Figs. 11a and 12a show microscopic image of C2C12 on the sixth day and the tenth day of culture, respectively. Figs. 11b and 12b show the voltage monitored during application of electric pulses on the ITO film on the sixth day and the tenth day of culture, respectively. The parameters of voltage between the terminals of electric stimulator are 1 s of period and 0.002 s of duration, respectively. The amplitudes of V_2 in Fig. 11b and 12b are 1.8 V and 2.3 V, respectively. The amplitudes of V_1 in Fig. 11b and 12b are 1.0 V and 1.5 V, respectively. The ratio of V_1/V_2 increases from 0.56 to 0.65, which corresponds to decrease of the resistance between electrodes for four days of culture.

C2C12 of the same lot was cultured in the culture dish coated with collagen for the control test. The myotube shows contraction synchronous to the electric pulse stimulation after cultivation for eight days (Fig. 13). The synchronous contraction was not able to be observed, on the other hand, at myotubes cultured on the micro patterned scaffold.

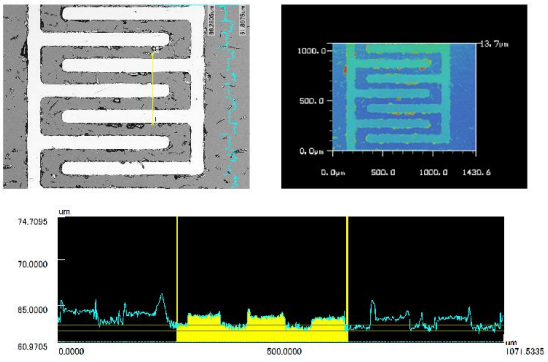

Fig. 8: Dimension of micro pattern measured with laser microscope.

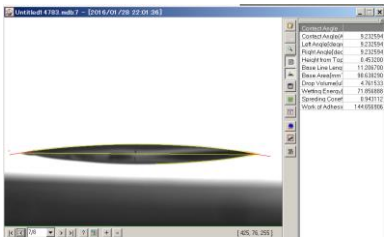

Fig. 9a: Contact angle on the film of ITO.

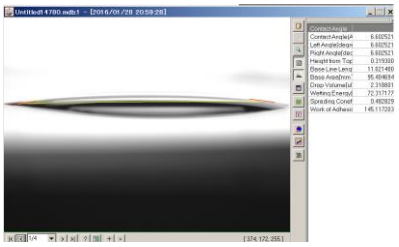

Fig. 9b: Contact angle on the base of the glass.

The cells are easily observed with the microscope through the transparent thin film of ITO on the glass surface (Figs. 10-12). The cells adhere, and proliferate both on glass and on the ITO film.

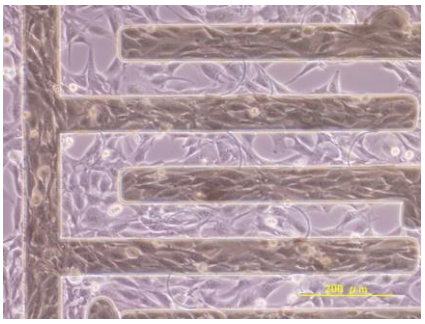

Fig. 10a: C2C12 on the second day of culture.

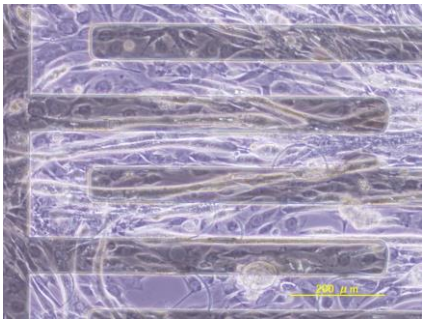

Fig. 10b: C2C12 on the thirteenth day of culture.

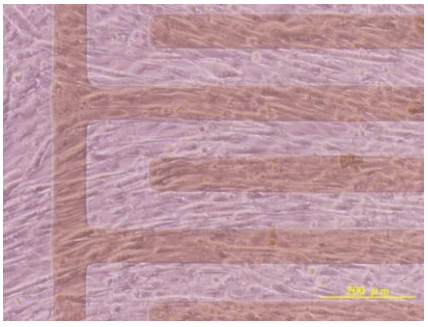

Fig. 11a: C2C12 on the sixth day of culture. Dimension from left to right is 1 mm.

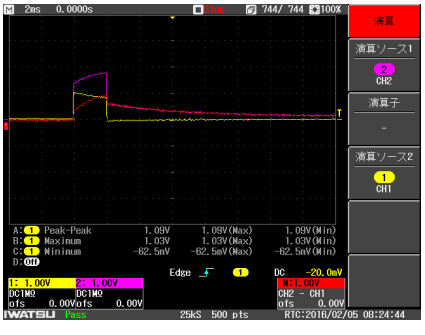

Fig. 11b: Tracings of V_1 (yellow, middle) and V_2 (purple, higher) on the sixth day of culture.

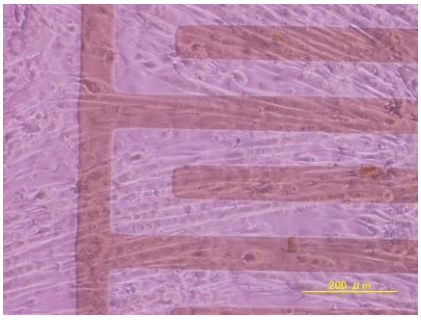

Fig. 12a: C2C12 on the tenth day of culture. Dimension from left to right is 1 mm.

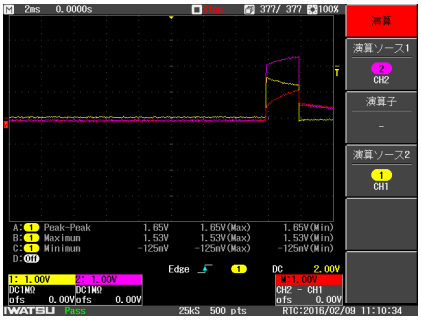

Fig. 12b: Tracings of V_1 (yellow, middle) and V_2 (purple, higher) on the tenth day of culture.

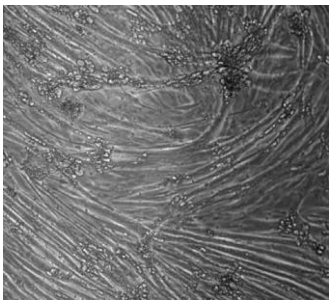

Fig. 13: C2C12 on the eighth day of culture. Dimension from left to right is 1 mm.

4. DISCUSSION

ITO has been selected as the material for the film type of the surface electrode [13] coated on the glass, because of the following properties: high conductivity, optical transparency, good biocompatibility, and chemical durability. The transparency of ITO film is good enough to observe the behavior of cells on the film. C2C12 is able to be cultured and differentiated on the ITO film. Titanium film, on the other hand, cannot be lift-off in the micro machining process to make the comb-shaped surface electrode in the present study.

The contraction synchronous to the electric pulse stimulation did not occur at the micro patterned scaffold in the present study. The movement of myotubes depends on the contacting points at the scaffold. The contact angle of the micro machined surface for cell culture is measured in the present study [13]. The strong affinity between myotube and glass might disturb the movement of myotube. The cyclic contraction of myotubes was frequently observed on the culture dish of polystyrene in the previous study [16].

The decrease of the resistance between electrodes for four days of culture might indicate organization of myotubes. Impedance also depends on the extracellular matrix [17]. The electric current of 0.03 A and electric resistance of 9 Ω makes 0.008 W of the electric power consumption between the electrodes of ITO film during the stimulation of pulse. Because the electric power consumption is very small and the electric current is pulsatile (short duration of pulse), the ITO film is not heated a lot by the electric current [13].

The behavior of cells depends on several kinds of environment: electric field [18-25], magnetic field [26], mechanical force field [27-31]. The microfluidic system has been applied to sort biological cells [26], and to trap biological cells [6]. The system also used to study local environment around the cultured cell. The micro pattern of the surface has been applied to study the surface effect of adhesion of cells [8].

5. CONCLUSION

To measure the signal from the tissue cultured with electric stimulation, the comb-shaped surface electrode has been designed and manufactured with indium tin oxide (ITO) coated on the glass. C2C12 (mouse myoblast cell line) was cultured for 13 days on the glass with intermittent stimulation of electric pulses. The experimental results show that myoblasts oriented along the comb type of the surface electrode are observed through the transparent surface electrode, and the change of the electric resistance with the change the cells is detected between electrodes *in vitro*. The designed comb-shaped surface electrode is effective to apply the asymmetric electric field on the cell culture and to measure the electric signal from the cell culture.

6. ACKNOWLEDGMENT

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