Electric Measurement of Cultured Myoblast Oriented on Scaffold with Micro-pattern

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ABSTRACT

The electric impedance of cultured myoblasts oriented on the scaffold with micro pattern has been measured between surface electrodes *in vitro*. Several parallel lines of micro rectangular ridges (0.001 mm height, 0.003 mm width, and 0.003 mm interval) have been made between a pair of the surface titanium electrodes at the surface of the scaffold of glass by the photolithography technique. C2C12 (mouse myoblast cell line) was cultured for 4 days on the micro-patterned scaffold in the medium of D-MEM (Dulbecco's Modified Eagle Medium). The electric impedance between electrodes was measured once a day with the sinusoidal electric waves (frequency, 1 Hz < f < 1 MHz; amplitude, ±0.1 V < V_1 < ±5 V). The experimental result shows that electric impedance has frequency characteristic and that the impedance of the capacitance component increases with proliferation of myoblasts. The orientation of myoblasts is detected by the impedance between the surface electrodes, when the orientation is varied by the direction of micro ridges of the scaffold.

Keywords: Biomedical Engineering, Surface Electrode, Micro-ridges, C2C12, Orientation and Electric Impedance.

1. INTRODUCTION

The cell culture technique has been developed and several methodologies might clinically be applied to regenerative medicine. C2C12 (mouse myoblast cell line) adheres to the scaffold, proliferates and differentiates to myotubes *in vitro*. These behaviors of the cell depend on the micro property of the surface. The effect of the surface property of the scaffold on cell culture has been studied in the previous studies [1]. Several micro-fabrication processes have been designed to control adhesion of biological cells *in vitro* [2]. Photolithography technique enables machining for design of the micro-pattern with the surface electrode [3]. The micro stripe pattern on the scaffold plate is one of the effective methods to make orientation of cells [4]. The electric impedance of the biological tissue was measured in the previous study to distinguish the kinds of tissue [5]. The biological membrane has capacitance, and biological tissue has frequency characteristic. The impedance depends on the orientation of cells.

In the present study, the electric impedance of cultured myoblast oriented on the scaffold with micro pattern has been measured between surface electrodes *in vitro*.

2. METHODS

Scaffold

A scaffold of solid surface, which has both micro patterns and surface electrodes, has been designed to measure electric signals of oriented myoblast and myotubes (Fig. 1). Several parallel lines of micro ridges have been made in the square area of 1 mm × 1 mm at the surface of the scaffold. The height (H), the width (W), and the interval (I) of the rectangular ridge are 0.001 mm, 0.003 mm, and 0.003 mm, respectively (Fig. 2). Two types of arrangements of lines are designed at the micro ridges: parallel (A in Fig. 1), and perpendicular (B in Fig. 1) to the electric field.

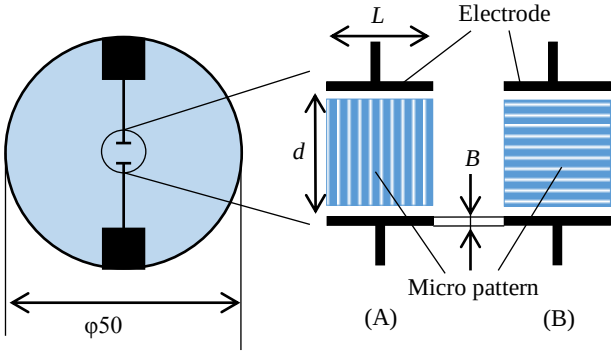

Fig. 1: Micro patterns and surface electrodes.

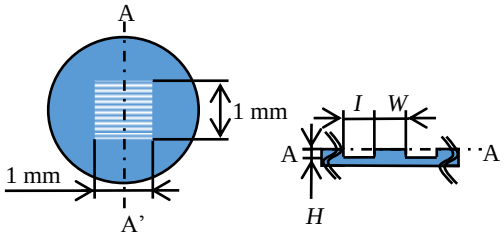

Fig. 2: Dimension of micro pattern for scaffold.

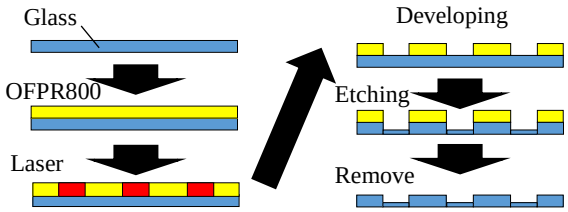

Fig. 3: Photolithography process for micro ridges.

The borosilicate glass (Tempax) disk was used for the base of the scaffold in a photolithography process (Fig. 3). The diameter and the thickness of the disk are 50 mm and 1.1 mm, respectively. To remove micro particles on the surface of the glass, the oxygen (0.1 Pa, 30 cm^3/min) plasma ashing was applied to the surface of the glass at 100 W for five minutes by a reactive ion etching system (FA-1, Samco Inc., Kyoto, Japan). To improve the affinity between the glass and the photo-resist material (OFPR-800), HMDS (hexamethyldisilazane: Tokyo Chemical Industry Co., Ltd., Tokyo) was coated at 3000 rpm for thirty seconds with a spin coater (IH-DX2, Mikasa Co., Ltd., Tokyo, Japan). The positive photoresist material of OFPR-800LB (Tokyo Ohka Kogyo Co., Ltd., Tokyo, Japan) was coated at 3000 rpm for twenty seconds with the spin coater. The photoresist was baked at the hotplate at 373 K for three minutes. The pattern for the micro grooves was drawn on the disk 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 3.2 V, the velocity of 0.14 mm/s, the acceleration of 0.34 mm/s^2. The pattern was baked at the hotplate at 373 K for three minutes. The photo-resist was developed with tetra-methyl-ammonium hydroxide (NMD-3, Tokyo Ohka Kogyo Co., Ltd., Kawasaki, Japan) for five minutes. The disk was rinsed two times with the ultrapure water for one minute, and dried by the spin-dryer. The glass was etched with the plasma gas using a reactive ion etching system (RIE-10NR, Samuco Inc., Kyoto, Japan) to make lines of the micro grooves of 0.001 mm depth. For etching, the gas of CF_4 (30 cm^3/min at 1013 hPa) was applied at 100 W at 2 Pa for thirty minutes. To exfoliate the residual photo-resist material from the surface, the disk was exposed to the oxygen gas of 30 milliliter per minute (0.1 Pa) at power of 100 W for five minutes using a reactive ion etching system (FA-1, Samco Inc., Kyoto): the oxygen plasma ashing. The dimensions of the micro grooves of the pattern were measured with a laser microscope (VK-X200, Keyence Corporation, Osaka, Japan). The morphology along the transverse lines of grooves was traced (Fig. 9).

T-Shape Surface Electrodes

A couple of T-shaped surface electrode has been designed by the micromachining technique (Fig. 1). The width of (*B*) the electrode is 0.1 mm, the length of (*L*) each electrode is 1 mm, and the distance between electrodes (*d*) is 1.2 mm. The titanium film was used for the surface electrode on the glass. The photomask B, which was made by the photomask A, was used to make the pattern of the surface electrode. The borosilicate glass (Tempax) disk (50 mm diameter, 1.1 mm thickness) was used for the base of the photomask.

Photomask A

After plasma ashing, titanium (200 nm thickness) was deposited on the surface of the glass in the electron beam vapor deposition apparatus (3.1×10^{-4} Pa, 0.5 nm/s, JBS-Z0501EVC JEOL Ltd., Tokyo, Japan). To improve affinity between titanium and photoresist material, HMDS was coated at 3000 rpm for thirty seconds with a spin coater. The positive photoresist material of OFPR-800LB was coated at 3000 rpm for twenty seconds with the spin coater. The photoresist was baked at the hotplate at 373 K for three minutes. The pattern was drawn on the mask with a laser drawing system (DDB-201K-KH). To control the dimension of the pattern on the mask with the laser drawing system, the parameters were selected as follows: the voltage of 3.2 V, the velocity of 0.14 mm/s, the acceleration of 0.34 mm/s^2. The pattern was baked at the hotplate at 373 K for three minutes. The photo-resist was developed with tetra-methyl-ammonium hydroxide (NMD-3) for five minutes. The disk was rinsed, and dried. The surface of titanium with OFPR-800LB was etched with the plasma gas using RIE-10NR (Samco International, Kyoto, Japan). For etching, the gas of SF_6 with Ar (50 cm^3/min at 1013 hPa) was applied at 100 W at 4 Pa for nine minutes. OFPR-800LB was removed by acetone (Fig. 4).

Photomask B

After plasma ashing, titanium (200 nm thickness) was deposited on the surface of the glass in the electron beam vapor deposition apparatus (3.1×10^{-4} Pa, 0.5 nm/s, JBS-Z0501EVC). The negative photoresist material of high viscosity (SU-8 10: Micro Chem Corp., MA, USA) was coated on the plate with the spin coater (at 1000 rpm for 10 s, and at 7000 rpm for 60 s). The photoresist was baked in the oven at 368 K for five minutes. The photoresist was baked at the hotplate at 368 K for three minutes. The "photomask A" was mounted on the surface of SU-8 10, 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 20 s. The photoresist was baked in the oven at 368 K for five minutes. The photoresist was developed with SU-8 Developer (Micro Chem) for five minutes. The glass surface with the micro pattern was rinsed with IPA (2-propanol, Wako Pure Chemical Industries, Ltd.) for one minute, and pure water for one minute. The surface was dried by the spin-dryer (Fig. 4).

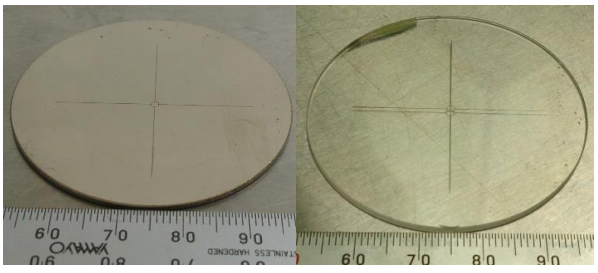

Fig. 4: Photomask A (left), B (right): titanium on the glass.

Electrode with Micro Pattern

The surface electrode was made on the borosilicate glass (Tempax) disk with micro grooves, which was made the former photolithography process (Fig. 5). After plasma ashing, Titanium (200 nm thickness) was deposited on the surface of the glass. After HMDS coating, the positive photoresist material of OFPR-800LB was coated at 3000 rpm for twenty seconds with the spin coater. The photoresist was baked at the hotplate at 373 K for three minutes. The "photomask B" 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) at 15 mW/cm^2 for 30 s. The photoresist was baked at the hotplate at 373 K for three minutes. The photo-resist was developed with tetra-methyl-ammonium hydroxide (NMD-3) for five minutes. The disk was rinsed two times with the ultrapure water for one minute, and dried by the spin-dryer. The titanium coated disk was etched with the plasma gas using RIE-10NR. For etching, the gas of SF_6 with Ar (50 cm^3/min at 1013 hPa) was applied at 100 W at 4 Pa for nine minutes. OFPR-800LB was removed by acetone (Fig. 6).

PDMS Wall

The wall of culture dish is made of the donut-ring of Polydimethylsiloxane (PDMS) (Fig. 6). After the borosilicate glass (Tempax) disk (35 mm diameter) was enclosed with a peripheral wall of polyimide tape, PDMS (Sylgard 184 Silicone Elastomer Base, Dow Corning Corp., MI, USA) was poured 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 was poured on the glass disk. After degassing again, PDMS was baked at 368 K for one hour in an oven (DX401, Yamato Scientific Co., Ltd). The baked PDMS was exfoliated from the glass disk, and machined by the punch to make the donut-ring. The outer diameter, the inner diameter and the thickness of the ring are 35 mm, 20 mm and 5 mm, respectively. The donut-ring of PDMS was adhered on the disk with the surface electrodes with extra past of PDMS. The lead wire was connected to the surface electrode with crimp style terminals.

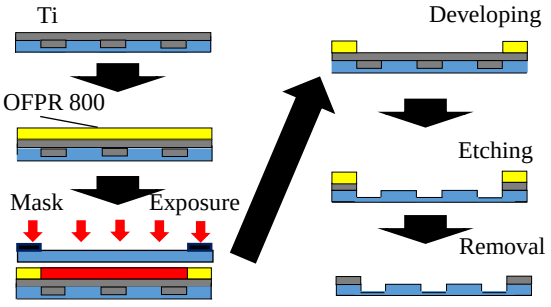

Fig. 5: Photolithography process for electrodes.

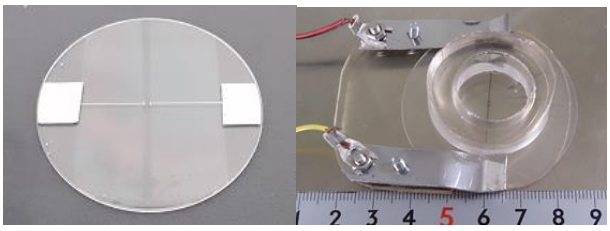

Fig. 6: Electrodes on glass (left), Culture dish with electrodes (right).

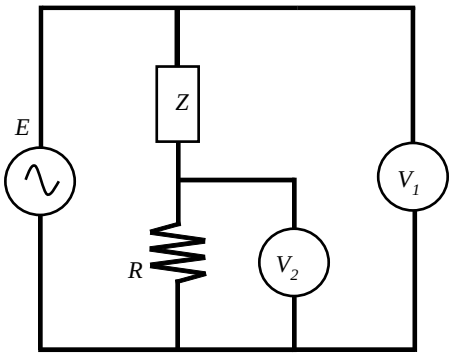

Fig. 7: Electric circuit.

Cell Culture

C2C12 (Mouse myoblast cell line originated with cross-striated muscle of C3H mouse) of the fifth passage was used for the cell culture. D-MEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Fetal Bovine Serum) and 1% penicillin/ streptomycin was used for the medium. The cells were seeded on the scaffold with the density of 5000 cells/cm^2. Cells were cultured in the incubator at 310 K with 5% CO_2 for 4 days. The medium were refreshed every two days. Cells were observed with an inverted phase-contrast microscope (IX71, Olympus Co., Ltd., Tokyo) every 24 hours.

Electric Measurement

The sinusoidal electric waves (frequency, 1 Hz < f < 1 MHz; amplitude, ±0.1 V < V_1 < ±5 V) were generated with an electric stimulator (WS8102, Toyo Corporation, Japan; 1910 Function Synthesizer, NF Corporation, Japan). The stimulator (E) was connected to the electrodes, and the sinusoidal electric waves were introduced to the medium (Z) of cells (Fig. 7). An electric resistance (R) of 10 kΩ is serially inserted between the electrode and the stimulator. The electric voltages (V_1, V_2) were monitored by an oscilloscope during measurement of impedance of the medium. V_1 and V_2 are the voltages between the terminals of the electric stimulator, and of the resistance, respectively.

Variation was made on the amount of medium to find the optimum volume to measure stable electric signal between electrodes.

3. RESULTS

Fig. 8 shows the laser microscopic image of the micro pattern on the scaffold. Measurement by the laser microscope shows that the lines of ridges on the glass has the repetitive dimension of 0.003 mm width, 0.003 mm interval, and 0.001 mm height (Fig. 9), which are close to the designed dimension. Fig. 10 shows the relationship between the voltage and the volume of the medium in the culture dish. The voltage V_2 is proportional to the current at the resistance, which depends on the impedance between electrodes. As the volume of the media increases, the impedance between electrodes decreases and the current increases. In the following measurement, the enough volume of the medium was maintained to stabilize the current between electrodes. Fig. 11 exemplifies the tracings of the voltages (V_1, and V_2) during the electric measurement.

Fig. 12 exemplifies cells after 24 hours of culture on the micro pattern A between electrodes. Each cell aligns to the lines of ridges (vertical in Fig. 12). Fig. 13 shows cells after 24 hours of culture on the micro pattern B between electrodes. Each cell aligns to the lines of ridges (horizontal in Fig. 13). Fig. 14 shows cells after 4 days of culture on the micro pattern between electrodes. Cells proliferate to make confluent layer and orientation along the lines of ridges (vertical in Fig. 14a; horizontal in Fig. 14b). Fig. 15 shows the relation between the impedance Z (between electrodes) and the frequency (f) (V_1 = ±1 V). The figure shows that the impedance is minimum at 10 kHz. The impedance varies at 10 Hz and 100 kHz during cultivation for four days. Data in Fig. 15 are rearranged to show the difference between data on the micro pattern A and B in Fig. 16. Data are similar on day 1 (Fig. 16a). Data are different each other between A and B at 10 Hz and at 100 kHz on day 4 (Fig. 16b). At 10 Hz, the impedance on the micro pattern B is higher than that on the micro pattern A.

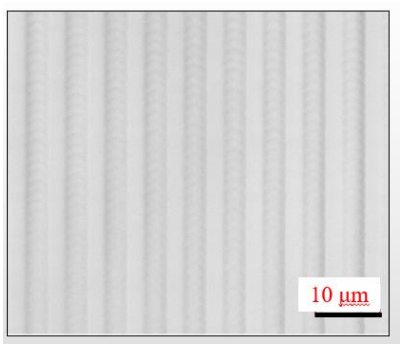

Fig. 8: Laser microscopic image of micro pattern.

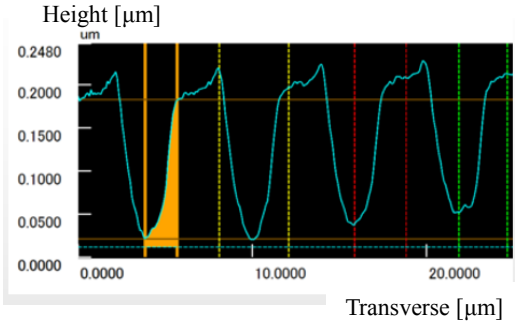

Fig. 9: Tracings of the micro pattern.

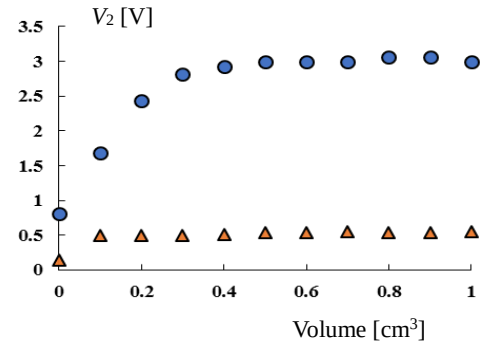

Fig. 10: V_2 (V) (Fig. 7) vs. Volume of medium (cm^3): circle (E = ±5 V), triangle (E = ±1 V).

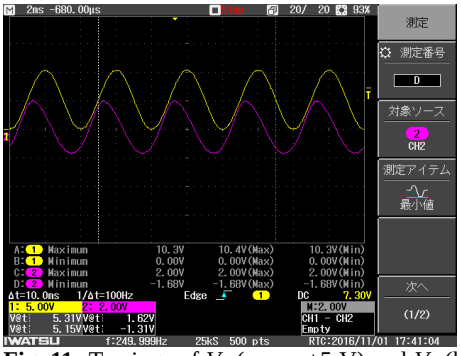

Fig. 11: Tracings of V_1 (upper ±5 V) and V_2 (lower ±1 V): 250 Hz.

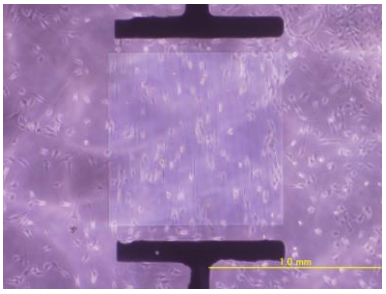

Fig. 12a: C2C12 after 24 hours of culture on micro pattern A.

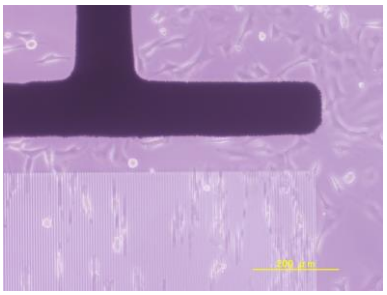

Fig. 12b: C2C12 after 24 hours of culture on micro pattern A near electrode.

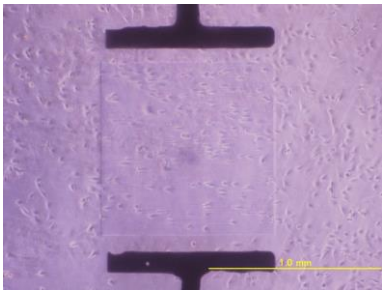

Fig. 13a: C2C12 after 24 hours of culture on micro pattern B.

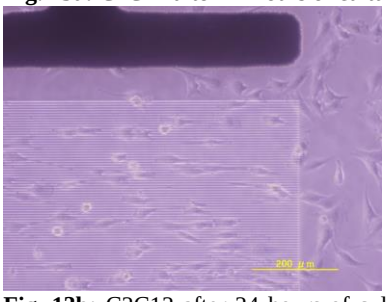

Fig. 13b: C2C12 after 24 hours of culture on micro pattern B near electrode.

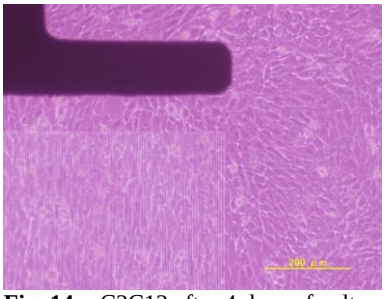

Fig. 14a: C2C12 after 4 days of culture on micro pattern A near electrode.

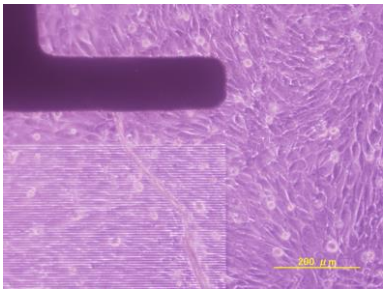

Fig. 14b: C2C12 after 4 days of culture on micro pattern B near electrode.

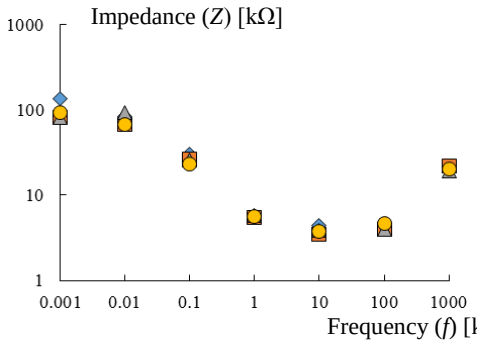

Fig. 15a: Impedance *Z* (kΩ) (cell culture on micro pattern A) vs. frequency (kHz): diagonal, day 1; square, day 2; triangle, day 3; circle, day 4.

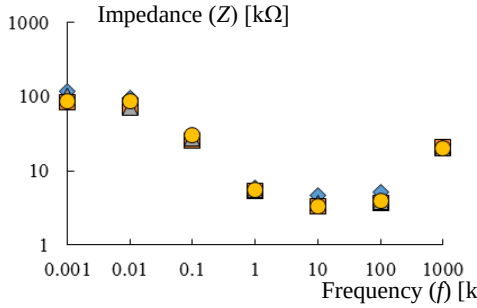

Fig. 15b: Impedance *Z* (kΩ) (cell culture on micro pattern B) vs. frequency (kHz): diagonal, day 1; square, day 2; triangle, day 3; circle, day 4.

4. DISCUSSION

To control the orientation of cultured cells *in vitro*, the photolithography technique is effective to make micro morphology on the surface of the scaffold [6, 7].

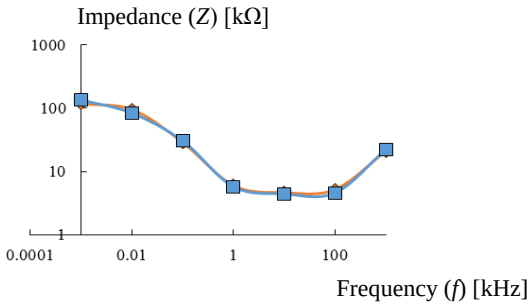

Fig. 16a: Impedance *Z* (kΩ) (cell culture) vs. frequency (kHz) on day 1: square, A; diagonal, B.

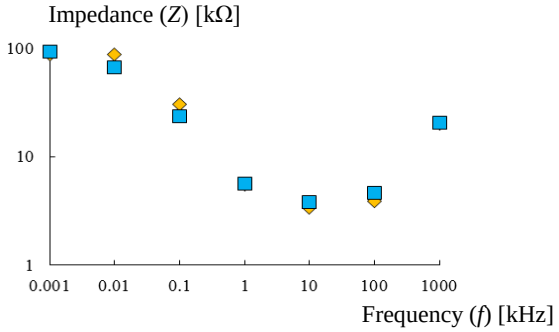

Fig. 16b: Impedance *Z* (kΩ) (cell culture) vs. frequency (kHz) on day 4: square, A; diagonal, B.

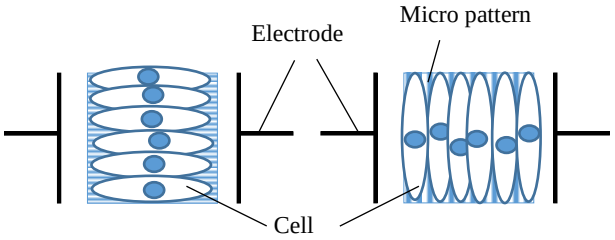

Fig. 17: Orientation of cells on micro pattern.

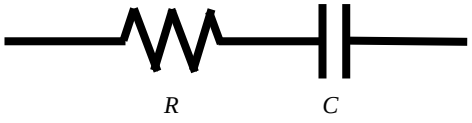

Fig. 18: Serial connection between resistance (*R*) and capacitance (*C*).

In the present study, the micro patterned scaffold has been successfully manufactured between the surface titanium electrodes on the glass by the photolithography technique. Myoblasts have been able to be cultured on the micro patterned scaffold. Orientation of myoblasts along the micro ridges has been able to be observed by an inverted phase-contrast microscope.

To detect the local electric impedance, several preparations of electrodes were designed in the previous studies [2-5, 8-11]. Those electrodes can be used for the electric stimulations on the biological cells [2-4, 11-14]. The surface electrode of the present study can also be used for the electric stimulation on the cultured cells. The response of biological cells depends on the direction of electric signal [12]. The electric stimulation

causes magnetic stimulation, simultaneously [15].

The variation of the resistance between electrodes for four days of culture might indicate the variation of the organization of myotubes in the present study. The impedance includes capacitance component, which related to the membranes of the cell. The orientation of cells might affect the capacitance component (around 10 Hz) (Fig. 16b, Fig. 17). The impedance also depends on the extracellular matrix [5].

In Fig. 11, V_1 is delayed from V_2, which indicates Z has capacitance. Using the phase difference (θ) between V_1 and V_2, the voltage between electrodes (V) is calculated by Eq. 1.

$$V^2 = V_1^2 + V_2^2 - 2 V_1 V_2 \cos\theta \quad (1)$$

When V_1 = 5 V, V_2 = 1 V, and θ is 0.94 rad, V = 4.5 V. The impedance Z is calculated by Eq. 2.

$$Z = V / (V_2 / R) \quad (2)$$

When R = 10 kΩ, Z = 45 kΩ (the electric current: V_2 / R = 0.1 mA). When the impedance Z consists of serial elements of the capacitance C and the resistance R (Fig. 18), C is calculated by Eq. 3 and Eq. 4.

$$Z^2 = R^2 + Zc^2 \quad (3)$$
$$Zc = 1 / (2 \pi f C) \quad (4)$$

When the frequency f = 250 Hz, Zc = 43 kΩ, and the capacitance $C = 2 \times 10^{-7}$ F

5. CONCLUSION

To measure the electric impedance of oriented cultured myoblasts, the micro patterned scaffold has been manufactured between the surface electrodes on the glass by the photolithography technique. The experimental result shows that electric impedance has the frequency characteristic between 1 Hz and 10 MHz and that the impedance of the capacitance component increases with proliferation of myoblasts. The orientation of myoblasts is detected by the impedance between the surface electrodes, when the orientation is varied by the direction of micro ridges of the scaffold.

6. ACKNOWLEDGMENT

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