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ORIGINAL ARTICLE

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# Control of myotube contraction using electrical pulse stimulation for bio-actuator

**Abstract** The contractility of tissue-engineered muscle on the application of electrical signals is required for the development of bio-actuators and for muscle tissue regeneration. Investigations have already reported on the contraction of myotubes differentiated from myoblasts and the construction of tissue-engineered skeletal muscle using electrical pulses. However, the relationship between myotube contraction and electrical pulses has not been quantitatively evaluated. We quantitatively investigated the effect of electrical pulse frequency on the excitability of myotubes and developed bio-actuators made of tissue-engineered skeletal muscle. C2C12 cells were seeded on a collagen-coated dish and in collagen gel and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. When the cells reached confluence or after 2 days in culture, the medium was shifted to DMEM containing 7% horse serum to allow them to differentiate to C2C12 myotubes. We electrically stimulated the myotubes and tissue-engineered skeletal muscle, and contractions were observed under a microscope. The myotubes contracted synchronously with electrical pulses between 0.5 and 5 Hz and unfused tetanus was generated at 10 Hz. The contractile performance of tissue-engineered skeletal muscle made of collagen gel and C2C12 was similar to that of the myotubes. Both the rheobase and chronaxie of the myotubes were lowest when the electric field was applied parallel to the myotube axis, and the values were 8.33 ± 2.78 mA and 1.19 ± 0.38 ms, respectively. The motion of C2C12 myotube contraction depended on the pulse frequency and showed anisotropy in the electric field. These results suggest that a tissue-engineered bio-actuator may be controlled using electrical signals.

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## Introduction

There have been many attempts at developing micromechanical and chemical systems such as the "lab on a chip."[1] It is believed that downsizing and integration of the driving system is necessary for development of these microsystems. Muscle cells, myofibers, are microscale linear actuators driven by the activation of actin–myosin molecular motors and transform biochemical energy into mechanical energy.[2] Therefore, muscle remains an attractive method for building actuators and powering devices from the micro- to macroscale. A muscle-derived actuator, termed a bio-actuator, has the potential of being flexible and highly efficient on the microscale. The development of bio-actuators with cardiomyocytes has already been reported,[3–5] and some of them generated flow without the application of electrical or mechanical energy.[5] However, it is necessary to kill animals to isolate highly differentiated cardiomyocytes. In addition, controllability is obtained by diminishing the involuntary movement of cardiomyocytes. We focused on the development of tissue-engineered skeletal muscle from an established cell line as a bio-actuator since it does not require the killing of additional animals and may have higher controllability than that of cardiomyocytes.

One of the goals of this study was to develop tissue-engineered skeletal muscle consisting of myofibers. There have been several reports published concerning tissue-engineered skeletal muscles with or without[6] scaffolds made of collagen[7] or fibrin gel.[8] However, the tissue-engineered skeletal muscle did not differentiate to myofibers, so it is difficult to differentiate multinucleated myotubes (fused myoblasts) to myofibers in both two- and three-dimensional cultures. We investigated myotube contraction as the first step for controlling tissue-engineered skeletal muscle with myofibers.

The dynamic state of skeletal muscle tissue depends on the frequency of applied electrical pulses, and the contrac-

tile force depends on not only frequency but also the number of excited myofibers. Furthermore, the electrical properties of skeletal muscle tissue are highly anisotropic,[9] and myotube contraction should be controlled by the direction of the electric field. Therefore, for controlling the contraction of tissue-engineered skeletal muscle by electrical pulses, investigating the frequency characteristics, excitation threshold, and anisotropy of myotubes is important. The electrical properties of myotubes have been well investigated.[10–13] According to previous studies, the contraction of myotubes is induced using outward current pulses[10] and depends on the frequency.[13] However, the frequency characteristics, excitation threshold, and anisotropy of myotubes have not been extensively evaluated. The aim of this study was to investigate the frequency characteristics, excitation threshold, and anisotropy in electric fields of myotubes for controlling and developing bio-actuators. Furthermore, we developed a tissue-engineered skeletal muscle made of collagen gel and myoblasts and compared the frequency characteristics of the tissue-engineered skeletal muscle with those of myotubes.

## Materials and methods

### Cell culture

C2C12 cells (ECACC, Salisbury, UK) from normal adult C3H mouse muscle[14] were used in this study. The cells were seeded on a 100-mm collagen-coated dish (AGC Techno Glass, Chiba, Japan) and cultured until subconfluent in a growth medium (GM) that consisted of high-glucose Dulbecco's modified Eagle's medium (DMEM) solution (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO) and 1% antibiotics (10 000 units/ml penicillin G, 10 mg/ml streptomycin sulfate, and 25 μg/ml amphotericin B: Sigma-Aldrich, St. Louis, MO, USA). The cultured cells were trypsinized in a 0.25%-trypsin/1-mM-ethylenediamine-tetraacetic acid (EDTA) solution (Nacalai Tesque, Kyoto, Japan) and resuspended in the GM. The cell suspension was added to 60-mm collagen-coated dishes (AGC Techno Glass) yielding a final density of $1.0 \times 10^4$ cells/cm$^2$. The seeded cells were cultured in the GM until confluence and then the GM was shifted to a differential medium (DM) consisting of high-glucose DMEM solution supplemented with 7% horse serum (StemCell Technologies, Vancouver, Canada) and antibiotics to enhance differentiation of the cells to multinucleated myotubes. The culture remained in the DM for 6 days. The cell morphology was observed with a phase-contrast microscope (TE-300, Nikon, Tokyo, Japan) every 2 days during medium replacement.

### Three-dimensional culture of C2C12 cells

Type-I collagen gel (Cellmatrix, Nitta Gelatin, Osaka, Japan) was used as a scaffold for the C2C12 cells. The cells were embedded in a cold type-I collagen gel solution at a density of $1.0 \times 10^7$ cells/ml. The cell suspension (100 μl) was placed between two stainless steel pins connected 13 mm apart to a polycarbonate plate by a micropipette. After gelation of the cell suspension at 37°C, the construct was put into the GM[15] and cultured to allow the trypsinized cells to repair and proliferate. After 2 days in culture, the GM was shifted to DM and cultures were maintained in the medium for 12 days. The culture was performed at 37°C in a 5% $CO_2$ atmosphere.

### Electrical stimulation system

Our proposed electrical stimulation system is shown in Fig. 1. The electrodes were made of platinum plate (3 mm × 18 mm) and were attached to a polycarbonate jig. An electrical pulse generator (WF1974: maximum output voltage 10 Vp-p, range of duty ratio 0.01%–99.99%: NF, Yokohama, Japan) was connected to the electrodes and a resistor was inserted in series with the electrical circuit to facilitate monitoring of the current. An amplified (As-904: gain × 15, maximum output voltage 150 V: NF) electrical signal was applied to the medium through the electrodes. The waveforms of the applied voltage and current were monitored with an oscilloscope (54810A, Agilent, Santa Clara, CA, USA).

The electric potential distribution in the medium without cells was measured to clarify the direction of the electric field for inducing myotube contraction. An electrical pulse at an amplitude of 10 V for 20 ms and a DC offset of 0 V was applied to the medium in the culture dish without cells. A platinum pin electrode was placed on the positioning stage (Shotomaster 3, Musashi, Tokyo, Japan) and placed in the medium 2 mm from the bottom of the dish and then the voltage was measured with the oscilloscope.

### Frequency properties of myotubes

The myotubes were randomly oriented on the dish 6 days after differentiation induction. Myotubes oriented longitu-

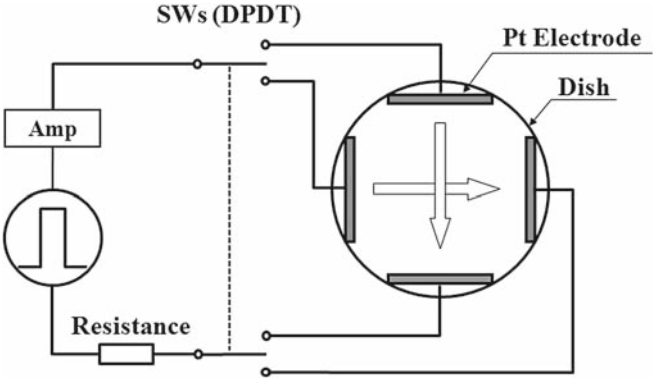



**Fig. 1.** Schematic of electrical pulse stimulation system. *Arrows* show the direction of the electric field. *SWs*, switches, which change the direction of the electric field; *DPDT*, double pole double throw

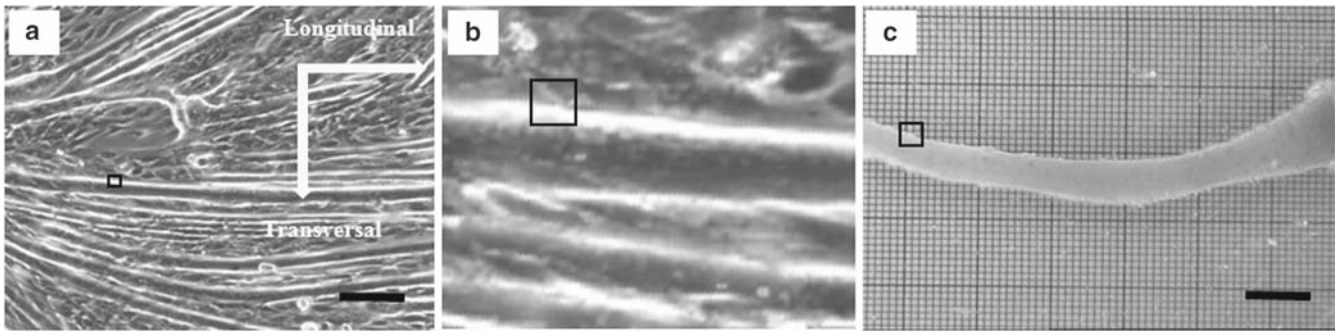



**Fig. 2a–c.** Photographs of the myotubes and tissue-engineered skeletal muscle for image analysis. **a** Microphotograph of myotubes. *Arrows* show direction of the electric field: *bar* 100 μm. **b** Magnified microphotograph of myotubes shown in part a. **c** Macro photograph of tissue-engineered skeletal muscle: *bar* 1 mm. The *box* is the area in which the mean value of gray scale is calculated

dinally and transverse to the direction of the electric field, termed the L and T groups, respectively, were focused on for evaluating the anisotropy with respect to the electric field during myotube contraction. The myotubes were stimulated using electric pulses at an amplitude of 20 V for 10 ms at frequencies in the range 0.5–10 Hz. The contraction was observed under a phase-contrast microscope and the digitized images were recorded with a computer using a CMOS camera (Moticam 2000: maximum pixels 1600 × 1200, maximum frame rate 40 frames/s: Shimadzu, Kyoto, Japan) attached to the microscope.

Image analysis was performed to quantitatively evaluate the relationships between myotube contraction and electrical pulse frequency and the direction of the electric field (Fig. 2a). The recorded video was converted to bitmapped images, and these images were converted to a gray scale with 256 values (white: 255, black: 0). After being converted to the gray scale, the mean value of the gray scale inside the box (10 × 10 pixels) (Fig. 2b) was calculated. A change of the gray scale inside the box just arround the contracting cells corresponds with the cell movement. The calculated data was divided by the frame rate of the video (16 frames/s) to investigate the relationship between the gray scale and time. Finally, the obtained data were transformed into the frequency domain using Fourier transforms for comparison with the frequency of electrical stimulation.

### Frequency properties of tissue-engineered skeletal muscle

Twelve days after differentiation induction, the frequency properties of the tissue-engineered skeletal muscle were evaluated. The tissue-engineered skeletal muscle was placed in the center of the dish shown in Fig. 1 and stimulated using electric pulses at an amplitude of 10 V for 20 ms at a frequency in the range 0.5–10 Hz. The contraction was observed under a microscope (KH-2700R, HIROX, Tokyo, Japan: 984 × 850 pixels).

Image analysis was performed according to the method described above. The mean value of the gray scale inside the box (10 × 10 pixels) (Fig. 2c) was calculated and the calculated data were divided by the frame rate of the video (30 frames/s).

### Measurement of myotube excitability

Evaluating myotube excitability is important for controlling bio-actuators. The excitation threshold of a tissue can be evaluated from the relationship between current and pulse width, which is known as a strength–duration curve (S-D curve).[16] While the myotubes were stimulated with electric pulses, the amplitude was decreased from 45 to 1 V with the pulse duration varying from 0.1 to 500 ms at each amplitude to evaluate myotube excitability. The pulse duration when the myotubes began contracting was plotted, and then the current flowing in the electrical circuit was measured. Throughout the experiment, the frequency was kept at 1 Hz. The plotted data were estimated as follows using the least-squares method:

$$I = a + b/d, \tag{1}$$

where $I$ is the current, $a$ is the rheobase, which is defined as a long-duration asymptote, $d$ is the pulse duration, and $b$ is a constant. The rheobase is the lowest current that induces cell contraction. The chronaxie is the pulse width that induces cell contraction with minimum electrical energy at which the current is twice the rheobase. The chronaxie was calculated from the estimated curve by putting $I = 2a$ and solving $d = b/a$. The medium used in the stimulation experiments was GM with 25 mM HEPES {2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid} and the experiments were performed in room air at 37°C. Differences in mean values were compared within the groups (L and T), and significant differences were determined using Student's $t$ test. The level of significance was set at $P < 0.05$.

## Results

The electric potential distribution in the culture medium is shown in Fig. 3. The stimulating electrodes were located on the left (anode) and right (cathode) rim, as shown in Fig. 3. The voltage amplitude decreased with an increase in the distance from the anode. We focused on myotubes located around the center of the dish (6 × 6 mm) since the direction and intensity of the electric field were constant.

The cultured cells reached confluence after 4 days (Fig. 4a). Both the mononuclear myoblasts and the multinuclear myotubes were visible after 2 days of the differentiation induction (Fig. 4b). At 6 days of the differentiation induction, the myotubes were about 30% times thicker than those at 2 days and a few myotubes spontaneously contracted (Fig. 4c). The number of myotubes decreased with cultivation after 8 days of differentiation induction due to detachment from the dish, and the mononuclear myoblasts remained on the culture dish.

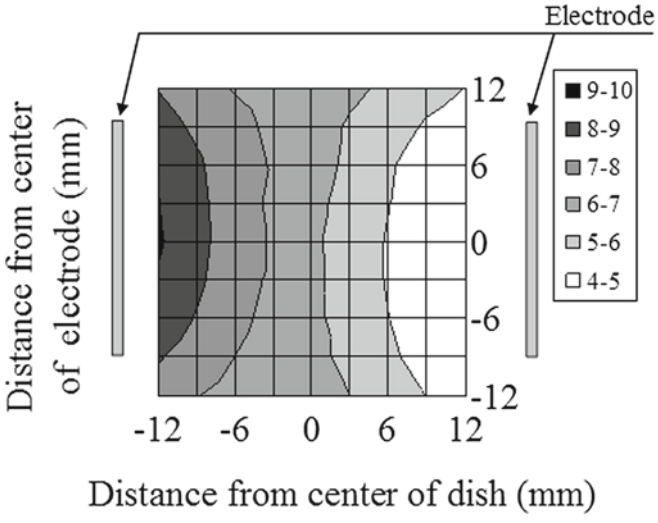



**Fig. 3.** Electric potential distribution in the medium. The key indicates the potential in volts

Most of the myotubes oriented in the longitudinal direction of the electric field contracted rhythmically with electrical pulses of between 0.5 and 5 Hz, and no rhythmical contraction was visible at 10 Hz. Figure 5 shows the results of the converted gray scale of the myotubes. The mean value of the gray scale varied with electrical pulses between 0.5 and 5 Hz in the L group. At 10 Hz, the mean value of the gray scale rose at the start of electrical stimulation and the value was constant during stimulation. In the T group, no rhythmical change in gray scale was observed for electrical pulses of any frequency.

The variety in gray scale, shown in Fig. 5, was converted to the frequency domain using Fourier transforms and the obtained frequency spectrum is shown in Fig. 6. The peak frequency spectra of the gray scale were at the same frequencies as the electrical pulses between 0.5 and 5 Hz in the L group. In contrast, there was no specific frequency spectrum observed for any frequency in the T group. Conversion to a frequency spectrum at 10 Hz was not possible because the frame rate of the recorded video was about 16 frames/s.

The length and diameter of the tissue-engineered skeletal muscle were approximately 13.5 mm and 450 μm, respectively. The tissue-engineered skeletal muscle contracted on electrical pulse stimulation and the contraction was visible with the naked eye. Figure 7 shows the result of image

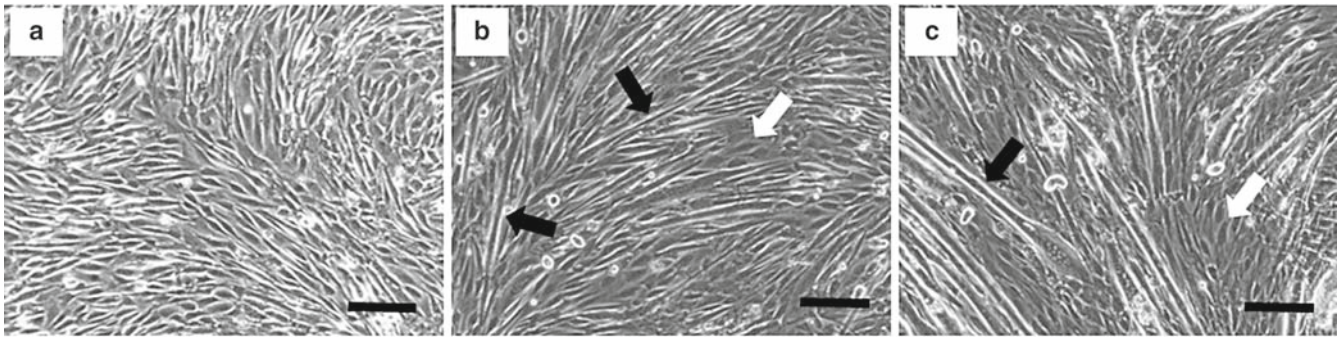
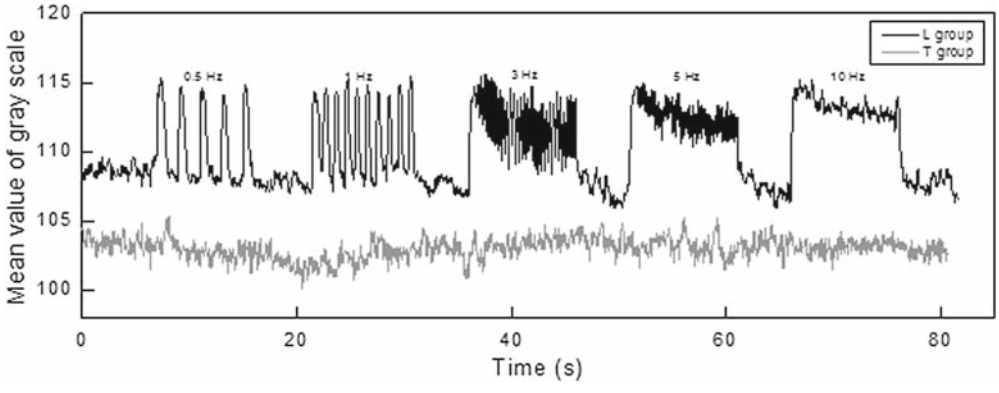



**Fig. 4a–c.** Phase-contrast microscopic images of C2C12 cells and myotubes. **a** Confluent, **b** 2 days after differentiation induction, **c** 6 days after differentiation induction. *Black arrows* indicate myotubes and *white arrows* indicate myoblasts: *bars* 100 μm

**Fig. 5.** Time-dependent changes of mean value of the gray scale. The frequencies of the applied electrical pulses are indicated on the graph. *L*, lateral orientation; *T*, transverse orientation



analysis of the tissue-engineered skeletal muscle. The variation of the mean value of gray scale and the frequency spectrum were similar to that of the myotubes shown in Fig. 5.

The shape of the S-D curve of each group was essentially hyperbolic, and the curve of the L group was below that of the T group (Fig. 8). The current required for excitation of the myotubes increased with decreasing pulse duration for both groups. The calculated results of the rheobase and chronaxie are shown in Fig. 9. The rheobase values of the L ($n = 9$) and T groups ($n = 4$) were 8.3 ± 2.8 mA and 17.1 ± 4.5 mA, respectively, and the value of the L group was significantly lower than that of the T group (Fig. 9a), $P < 0.01$. The chronaxies of the L ($n = 9$) and T groups ($n = 4$) were 1.2 ± 0.4 ms and 1.7 ± 0.4 ms, respectively, and the value of the L group tended to be lower than that of the T group (Fig. 9b), although the difference was not significant.

## Discussion

Mature skeletal muscle fibers have no ability to regenerate, but mononuclear satellite cells, which are found between mature muscle fibers and their associated basal lamina, fuse together to form myotubes following a muscle injury. Subsequently, mature skeletal muscle regenerates by growing

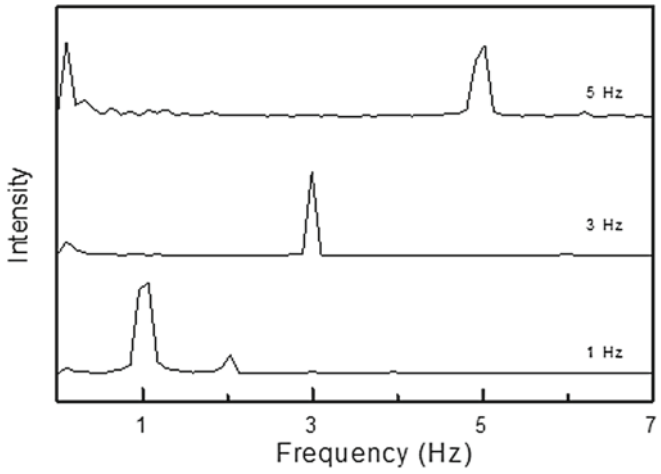



**Fig. 6.** Frequency spectra of myotube contraction. *Numbers* on the lines indicate the frequency of applied electrical pulses

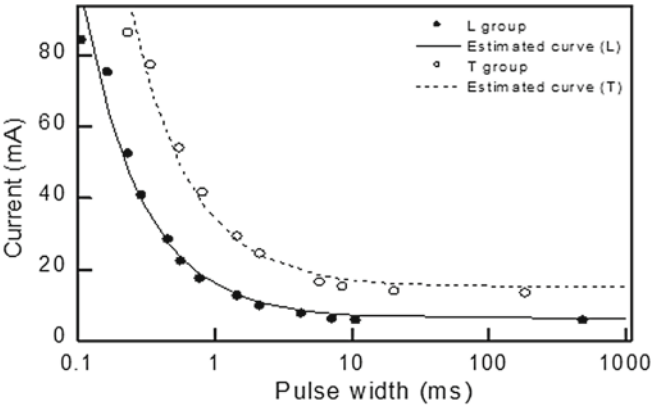
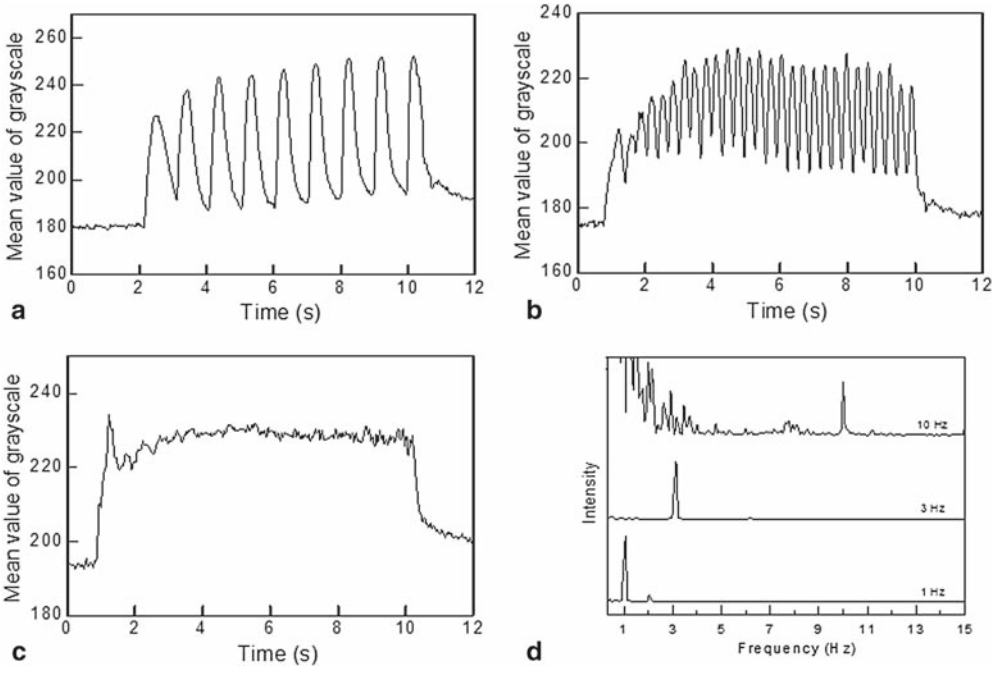



**Fig. 8.** Strength–duration curve of myotubes at 1 Hz. Plots indicate the points at which myotubes began to contract. The *lines* are estimated curves using the least-squares method

**Fig. 7.** Results of image analysis of the tissue-engineered skeletal muscle contraction by electrical pulses of **a** 1 Hz, **b** 3 Hz, **c** 10 Hz. **d** Frequency spectra



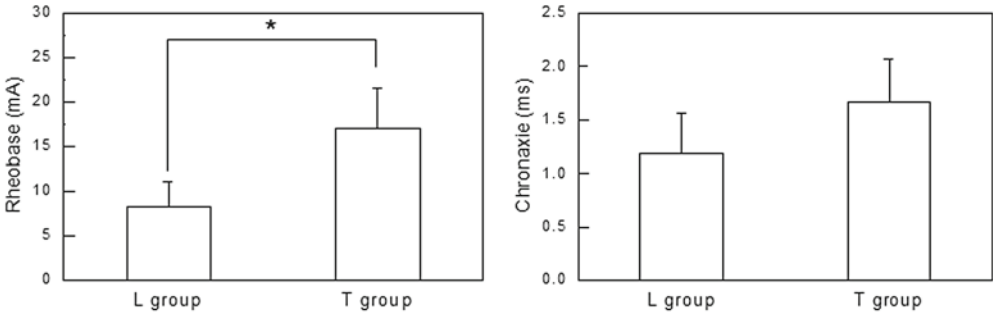



**Fig. 9.** Rheobase and chronaxie of myotubes for pulses of 1 Hz. *Columns* show the mean values and *bars* shows SDs. (L group: $n = 9$, T group: $n = 4$, $^*P < 0.01$)

myofiber from myotubes. In this study, C2C12 myoblasts fused and formed myotubes after 2 days of differentiation induction. However, myotube contraction was not detected when an electrical pulse was applied at that time. After 8 days of differentiation induction, the myotubes started to detach from the substrate, and the number of myotubes after 12 days of differentiation induction was lower than that at 8 days. Cooper et al.[17] also reported that myotubes detached from the dish at 5–6 days after differentiation and concluded that this detachment was due to extensive sarcomeric development and the onset of spontaneous contraction. Therefore, electrical stimulation was performed after 6 days of differentiation induction in this study. Although branching myotubes were observed throughout the experiment, only nonbranching myotubes aligned in the direction of the electric field were examined for induction of myotube contraction.

Muscle fiber generates an action potential by acetylcholine isolation from the synaptic end bulb. The action potential propagates along the sarcolemma and transverse tubules, and subsequently calcium ions are released from the sarcoplasmic reticulum (SR), and then the skeletal muscle contracts. In this study, the myotubes contracted on electrical pulse stimulation without nerve impulses. Saito[10] reported that cultured chick myotubes contracted by using calcium ions that were released mainly from the SR when an outward current pulse flowed through the cell membrane. It can be assumed that the action potential was generated due to the difference between extracellular fluid (culture medium) resistance and intracellular fluid resistance, and then calcium ions were released from the SR when the electric field was applied.

The frequency characteristics of myotubes in an electric field have not been thoroughly described. Marrota[13] reported that myotubes contracted rhythmically with electrical pulses of between 1 and 5 Hz, and myotube contraction could not be observed at 10 Hz under a phase-contrast microscope. However, the variation in myotube contractions with electrical pulses has not been quantitatively evaluated. In our study, a gray scale was used as an index of myotube movement. The mean value of gray scale between 1 and 5 Hz changed rhythmically, and the peak spectrum obtained from Fourier transformation was at the same frequency as the electrical pulse stimulus. This result suggests that myotubes contract synchronously with electrical pulses of between 1 and 5 Hz. Although a previous study reported that myotube contraction was not observed at 10 Hz,[10] the mean value of the gray scale increased at the start of stimulation, maintained a constant value, and decreased at the end of stimulation. In native skeletal muscle tissue, individual twitch contractions occur when low-frequency electrical stimulation is applied. As the frequency increases, repeated stimulation before relaxation of skeletal muscle produces additional activation of the contractile elements.[2] This is called unfused tetanus. Therefore, unfused tetanus occurred at 10 Hz.

An S-D curve gives useful information about the excitation of a tissue. Although many studies on the excitability of muscle tissue have been carried out,[18] there has not been sufficient evaluation of that of myotubes. Rheobase and chronaxie values estimated from voltage–duration measurements differ from the proper values determined from current–duration measurements since the complex impedance of the electrode–medium interface varies with both the pulse width and the applied voltage.[19] Therefore, we measured the relationship between current flow in the electrical circuit and pulse width. In this study, the plotted curve, shown in Fig. 8, was hyperbolic and was similar to that of muscle tissue. The trajectory of the curve in the L group differed to that of the T group. This result indicates that the myotubes may exhibit anisotropy in their excitation response to an electrical pulse. To quantitatively evaluate the excitation of the myotube, the chronaxie and rheobase were calculated from the estimated curve. According to the results shown in Fig. 9, the rheobase in the L group was significantly lower than that in the T group, and the chronaxie in the L group tended to be lower than that in the T group. These results suggest that myotubes may have the ability to contract with low electrical energy if the electric field is applied parallel to the longitudinal direction. The anisotropy in the conduction velocity of cardiac muscle is well known. Additionally, anisotropy in the electrical properties of skeletal muscle has been investigated.[9] Furthermore, Klee and Plonsey[20] suggested that cell shape plays an important role in electrical behavior. Therefore, the anisotropy of myotube contraction with respect to the electric field may be due to the differences in electrical conduction properties and the cell shape.

The contraction of tissue-engineered skeletal muscle is similar to that of myotubes (Figs. 5 and 7). This result indicated that the tissue-engineered skeletal muscle contraction strongly depended on myotube contraction. The chronaxie

of the myotube was approximately 1.2 ms in the L group, and it was longer than that of the skeletal muscle tissue, which is reported generically as 0.1–0.3 ms. Thus, the myotubes may not have been mature enough to differentiate to myofibers in this study. There have been some studies addressing the design of the culture environment with electrical pulse stimulation[21] and optimizing substrates[17,22] to enhance the differentiation and maturation of myoblasts to myotubes. In particular, the elastic property of substrates may play an important role in enhancing the maturation of myotubes.[22] Therefore, it is necessary to design an optimal culture environment for the development of bio-actuators made of tissue-engineered skeletal muscle since the contractile force of myofiber is higher than that of myotubes.[6]

This is the first report on measuring the rheobase and chronaxie of myotubes without electrodes inserted. Our proposed method can be used to easily measure the responsiveness and excitability of myotubes in two-dimensional culture. It may be necessary to evaluate the variation of myotube excitation with cultivation period to investigate the effects on myotube structure.

Our results reveal that myotube contraction may be controllable by adjusting the frequency, duration, and direction of electrical pulses. Morishima et al.[23] reported that a micropillar made of polydimethylsiloxane could be driven by cardiomyocytes and its displacement and force was approximately 6 μm and $8 \times 10^{-8}$ N. We expected that the myotube might displace microstructural objects at the micrometer scale and tissue-engineered skeletal muscle generated a higher force than that of myotubes. Furthermore, since myotubes and tissue-engineered skeletal muscle contract due to the electric field without the insertion of electrodes, a micromachine with bio-actuators may be controllable from outside the body without any direct physical connection. There is much further work required for developing and enhancing bio-actuators as a driving source of micromachines and for use in regenerative medicine. We will design an optimal culture environment and scaffold in future work.

## Conclusions

Our results quantitatively indicated that myotube contraction was controllable by changing the frequency, duration, and direction of electrical pulses, and that myotubes contracted at low electrical energy by applying a parallel electric field. Furthermore, the contractile performance of tissue-engineered skeletal muscle made of collagen gel and C2C12 cells is similar to that of myotubes. These results suggest that a bio-actuator could be developed and controlled using electrical pulses. In addition, our proposed method is useful for evaluating myotube excitability in two-dimensional culture.

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