Spatiotemporally controlled contraction of micropatterned skeletal muscle cells on a hydrogel sheet†

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Received 1st September 2010, Accepted 3rd November 2010
DOI: 10.1039/c0lc00364f

We have developed gel sheet-supported C_2C_{12} myotube micropatterns and combined them with a microelectrode array chip to afford a skeletal muscle cell-based bioassay system. Myotube line patterns cultured on a glass substrate were transferred with 100% efficiency to the surface of fibrin gel sheets. The contractile behavior of each myotube line pattern on the gel was individually controlled by localized electrical stimulation using microelectrode arrays that had been previously modified with electropolymerized poly(3,4-ethylenedioxythiophene) (PEDOT). We successfully demonstrated fluorescent imaging of the contraction-induced translocation of the glucose transporter, GLUT4, from intracellular vesicles to the plasma membrane of the myotubes. This device is applicable for the bioassay of contraction-induced metabolic alterations in a skeletal muscle cell.

Introduction

In vitro bioassay systems incorporating cells with physiological activity have been developed as an alternative to whole animal experiments.[1,2] Systems using skeletal muscle cells are one of the promising devices to reveal the complex mechanisms of type 2 diabetes because that disease is associated with a disorder of insulin- or contraction-induced glucose metabolism in a skeletal muscle cell *in vivo*.[3,4] Such a bioassay system could be also useful for screening candidate drugs against type 2 diabetes.

Bioassay systems fabricated by combining microelectric devices with cell-micropatterning techniques have enabled localized electrical regulation and long-term monitoring of electrophysiological responses from cells such as neural, cardiac, and skeletal muscle cells cultured on the device.[5–12] However, it is difficult to culture fully differentiated cells and to maintain their physiological activity on the solid devices. Specifically, there are few reports on devices combined with electrically excitable contractile skeletal muscle cell culture because the cells readily detached from the substrate within a few days.[13] For a stable bioassay device, it is necessary to develop an on-device culture method of fully differentiated cells, while sustaining their activity for long periods.

Recently, to overcome the difficulties associated with on-device cultivation, a manipulatable cell sheet which is composed of confluent cell monolayers and a flexible polymer sheet has been developed.[14–17] The cell sheet can be handled while sustaining the cellular structure and activity and it can be combined with the devices on demand. We have developed gel sheet-supported contractile C_2C_{12} myotube micropatterns by means of the cell transfer technique (Fig. 1(A)).[17] Micropatterned myotubes/gel sheet was prepared by transferring the myotube line patterns cultured on a glass substrate onto the surface of fibrin gel sheet with 100% efficiency. Myotubes on the gel sheet exhibited longer-term contractile activity without detachment from the gel than the cells on a conventional solid culture dish.

In the present study, we patched the myotube/fibrin gel sheet onto a microelectrode array chip to construct a skeletal muscle cell-based bioassay device (Fig. 1(B)). The Pt microelectrode arrays were previously coated with a conducting polymer, poly(3,4-ethylenedioxythiophene) (PEDOT), that has a large electroactive surface area due to its fibrous structure. This modification increases the interfacial electrical capacity of the electrodes and ensures a less invasive electrical stimulation of the cells without causing faradaic reactions and gas evolution.[18]

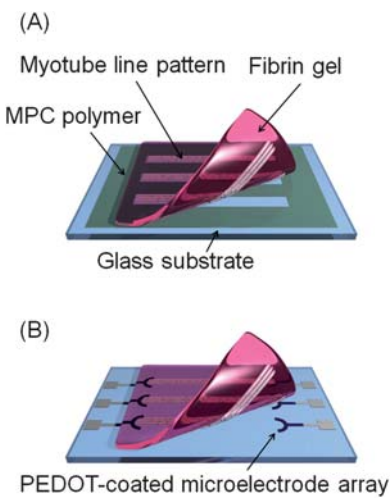

Fig. 1 Overview of the myotube/fibrin gel sheet combined with the PEDOT microelectrode array chip. (A) Cell transfer from a glass substrate to a fibrin gel. (B) Attachment of the myotube/fibrin gel onto the microelectrode arrays.

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† Electronic supplementary information (ESI) available: Movie 1 and 2. See DOI: 10.1039/c0lc00364f

The usefulness of this device for type 2 diabetes researches was demonstrated by fluorescent imaging of the contraction-induced translocation of the glucose transporter, GLUT4, from intracellular vesicles to the plasma membrane of the myotubes. GLUT4 is a principal mediator of glucose uptake in the skeletal muscle cell in response to insulin and contraction. GLUT4 distributes in the intracellular region under unstimulated state, and is transported to the plasma membrane in response to these stimuli to accelerate glucose uptake in the cells. Defection in stimuli-responsive GLUT4 translocation is closely associated with the development of type 2 diabetes, which is supported by the whole animal experiments.[3,4]

Experimental

Contractile C_2C_{12} myotube micropatterns on a fibrin gel

Wild type (WT)-C_2C_{12} myoblasts (less than six passages in age; American Type Culture Collection, Manassas, VA, USA) were cultured on a glass substrate with a line-patterned cell-resistant polymer, 2-methacryloyloxyethyl phosphorylcholine (MPC, Lipidure-CM5206E, NOF Corp., Tokyo, Japan) at 37 °C, under a 5% CO_2 atmosphere in a growth medium composed of Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemicals Industries, Ltd, Osaka, Japan) containing 10% fetal bovine serum (BioWest, Nuaille, France), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Invitrogen Corp., Carlsbad, CA, USA) until fully confluent. Myoblasts were induced to differentiate into myotubes by replacing the growth medium with a differentiation medium composed of DMEM containing 2% calf serum (Thermo Electron Corporation, Melbourne, Australia), 1 nM insulin (Sigma-Aldrich, St Louis, MO, USA), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin.

Then, the myotubes were transferred onto a fibrin gel. The fibrinogen mixture solution was prepared by dissolving 15 mg mL^{-1} fibrinogen (Sigma-Aldrich), 0.5 mg mL^{-1} aprotinin (Sigma-Aldrich), and 10 U mL^{-1} thrombin (Ito Life Science, Inc., Ibaraki, Japan) in an electrical pulse stimulation (EPS) medium composed of DMEM containing 2% calf serum, 2% MEM amino acids solution (Invitrogen Corp.), 1% MEM non-essential amino acids solution (Invitrogen Corp.), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. The mixture was poured over the cells, and the culture was left undisturbed for 2 h at 37 °C under a 5% CO_2 atmosphere to facilitate fibrin gelation and to allow the cells to adhere on the surface of the gel. Fig. 2(A) shows a photograph of the fibrin gel sheet (size, 1 cm × 1 cm; thickness, 2 mm) with myotube line patterns after detaching the gel from the substrate. The white lines seen at the center of the gel are myotube line patterns. Fig. 2(B) shows a phase-contrast micrograph of myotube line patterns on the gel. The width of each line and the gap between the lines were set at 250 μm. The side of the fibrin gel sheet with the attached myotubes was covered with an additional thin fibrin gel layer (less than 100 μm) because we found that the additional gel layer was effective for maintaining the structure of cellular micropatterns for a long term. The myotube/fibrin gel was finally placed in a carbon electrode chamber (C-Pace 100, IonOptix, Milton, MA, USA) and periodic electrical pulses (amplitude, 0.7 V mm^{-1}; frequency, 1 Hz; duration, 2 ms) were applied for 4 days in the EPS medium to endow the cells with contractile activity.[17]

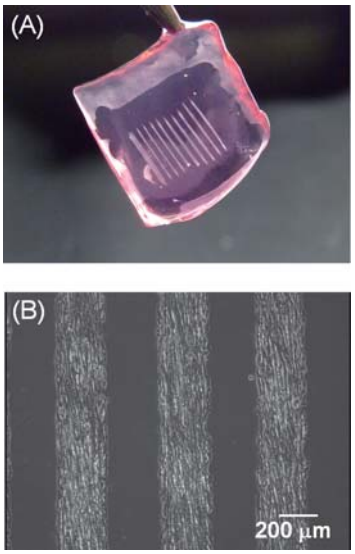

Fig. 2 (A) Photograph of a fibrin gel with myotube line patterns. (B) Phase-contrast micrograph of myotube line patterns on the gel.

Conducting polymer-coated Pt microelectrode arrays

Pt microelectrode arrays were fabricated on a glass substrate using conventional photolithography and lift-off techniques. Electropolymerization of poly(3,4-ethylenedioxythiophene) (PEDOT) on the microelectrode surfaces was carried out for 1 min in an aqueous solution containing 50 mM 3,4-ethylenedioxythiophene (Sigma-Aldrich) as a monomer and 100 mM KNO_3 as a dopant under potentiostatic conditions at +1.0 V *vs.* Ag/AgCl. Thickness of the PEDOT layer on the electrode was 467 ± 22 nm ($n = 5$) measured using a surface profiler (P-10, KLA-Tencor, CA, USA).

The contractile myotube/fibrin gel sheet was placed on the PEDOT microelectrode arrays so that the side of the gel sheet with the attached myotubes was in contact with the electrode surface. The myotube line patterns were carefully aligned with the microelectrode patterns using tweezers under microscopic observation. Periodic electrical pulses were applied between the desired microelectrode pairs to induce myotube contraction in the EPS medium.

GLUT4 translocation assay by immunostaining

Transfected C_2C_{12} myotube stably expressing rat GLUT4, with a c-myc epitope tag in the first extracellular loop and an enhanced cyan fluorescent protein (ECFP) at the carboxyl terminus, was used to detect membrane-bound GLUT4 using an anti-c-myc antibody.[19,20] WT- and transfected myoblasts mixed at a 1 : 1 ratio were grown and differentiated on a MPC polymer patterned substrate[19,20] and transferred onto a fibrin gel as described above. The myotubes were electrically stimulated using the carbon electrode chamber for 4 days to endow the cells with contractile activity. Then, the gel sheet was placed on the PEDOT microelectrode arrays for localized electrical stimulation of arbitral myotube line pattern for 3.5 h (amplitude, 2 V; duration, 3 ms; frequency, 10 Hz; train, 1 s; interval, 10 s) in the EPS medium. After that, the cells were fixed with 1% *p*-formaldehyde (Electron Microscopy Sciences, Fort Washington, PA,

USA) in PBS(−). The blocking step was carried out using 5% calf serum in PBS(−) to avoid non-specific adsorption of the antibody and reduce the background signal. In fact, there was no significant non-specific binding of mouse IgG antibody against myotubes assessed by the enzyme-linked immunosorbent assay. The cells were immunostained using the mouse monoclonal anti-c-myc first antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Then, Alexa Fluor 594-conjugated anti-mouse IgG second antibody (Invitrogen Corp.) was bound to the first antibody for 1 h at room temperature. The immunostained gel sheet was mounted on a cover glass with Vectashield (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were observed using a LSM 700 laser scanning microscope and the associated LSM software, ZEN 2009 (Carl Zeiss MicroImaging Co., Ltd, Tokyo, Japan).

Results and discussion

To achieve long-term minimally invasive stimulation of muscle cells, the electrodes were coated with PEDOT. Fig. 3 shows the micrographs of bare (A and B) and PEDOT-coated Pt microelectrodes (C and D). It is clear that the white shiny bare Pt microelectrode surface (A) was changed to black by the PEDOT modification (C). After the electrical pulse application (amplitude, 2 V; frequency, 1 Hz; duration, 10 ms) for 15 h in the EPS medium, the color of the bare Pt microelectrode changed to brown (B). This discoloration would be attributed to the electrochemical corrosion of the Pt layer, which means oxidation of the Pt layer at a high potential pulse cycle, followed by dissolution as the Pt ion into the medium.[21] On the other hand, the PEDOT-coated microelectrode showed no apparent morphological change even after 15 h of pulse application (Fig. 3(D)), and remained intact for more than 3 days pulse application. The PEDOT film of the electrode (D) was removed by aqueous sodium hypochlorite which causes over-oxidation and degradation of the PEDOT film,[22] which revealed that the underlying Pt microelectrodes remained intact (Fig. 3(E)). In addition to corrosion of the Pt layer, the faradaic reactions at the Pt electrode/medium interface cause drastic changes in culture conditions such as pH, which would injure the cells. In fact, we observed gas evolution, which was associated with water electrolysis at the bare Pt electrode, resulted in damaging the cells on the electrodes.[18] When using the PEDOT-coated microelectrode with a large surface electric capacity, the non-faradaic charging current will suppress unfavorable faradaic reactions and ensure stable long-term electric stimulation.

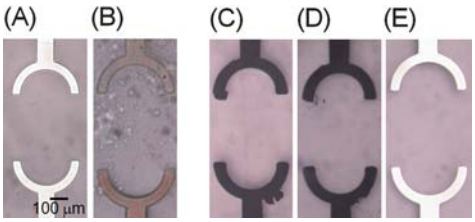

Fig. 3 Micrographs of bare (A and B) and PEDOT-coated Pt microelectrodes (C and D) before (A and C) and after (B and D) electrical pulse application (amplitude, 2 V; frequency, 1 Hz; duration, 10 ms) for 15 h in the EPS medium. (E) Pt microelectrode after detachment of PEDOT layer from the electrode D.

The electric field distribution on the microelectrode array was simulated by the finite element method using FEMLAB software (COMSOL 3.1, COMSOL AB, Sweden). The three-dimensional model geometry, including microelectrode arrays, mimicked those of the experimental setup. The simulated model microelectrode arrays did not include the electrode material properties. Fig. 4(A) shows a micrograph of the PEDOT microelectrode arrays and Fig. 4(B) shows the simulated electric field distribution for a model microelectrode array when a voltage of 2 V was applied between the central microelectrode pair. The electric field was concentrated between the active electrodes and did not spread out to the neighbouring regions. Fig. 4(C) shows the electric field distribution along the cross-section a–b in Fig. 4(B). The vertical axis in Fig. 4(C) represents the distance from the microelectrode surface. The simulated electric field rapidly decreased with increasing distance from the microelectrode surface. These data suggested that the electric field generated by the microelectrodes was effective only in the region near the microelectrode surface. Assuming the vertical axis in Fig. 4(C) to be the thickness of the gel, for effective electrical stimulation the myotubes/fibrin gel should be attached to the microelectrode array chip so that the side of the gel sheet with the transferred myotubes was in contact with the electrode surface.

Fig. 5(A) shows a phase-contrast micrograph of WT-C_2C_{12} myotube line patterns on the fibrin gel aligned with the PEDOT microelectrode arrays. During the alignment, the lined structure of the myotubes was maintained without detachment from the gel, allowing arbitrary and repetitive changes of the localized electrical stimulation site on the myotube line patterns. As can be seen in Fig. 5(A), the focus on the cells was matched with that of on the microelectrodes, suggesting the cells are in close vicinity of the electrode surface. The distance between the cells and the PEDOT layer was less than 100 μm, as assessed from the defocusing Z distance using a confocal microscopy. Fig. 5(B) shows the time courses of contractile displacements of myotube line patterns on the PEDOT microelectrode arrays when stimulated with periodic electrical pulses (amplitude, 2 V; frequency, 1 Hz; duration, 10 ms). Contractile displacement was determined from the real-time movie of myotube contraction shown in Movie S1, ESI†.

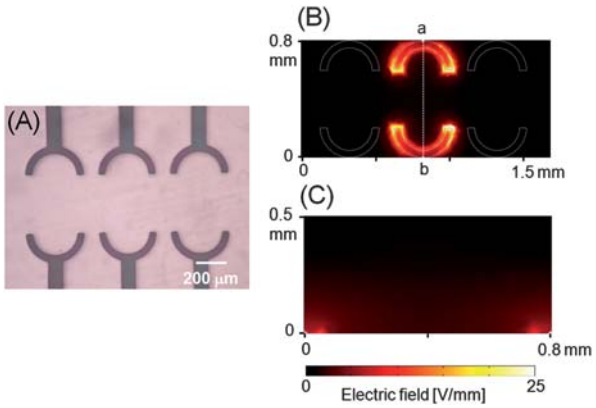

Fig. 4 (A) Micrograph of the PEDOT-coated microelectrode arrays. (B) Simulated electric field distribution for a model microelectrode array where a voltage of 2 V was applied between the central microelectrode pair. (C) Simulated electric field distribution along the cross-section a–b in (B).

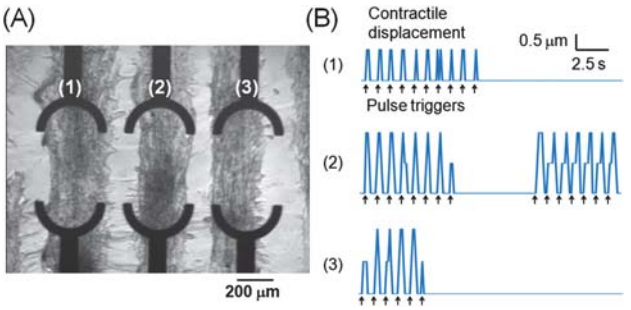

Fig. 5 (A) Phase-contrast micrograph of WT-C_2C_{12} myotube line patterns aligned with the microelectrode arrays. (B) Time course of myotube line patterns contraction stimulated with periodic electrical pulses (amplitude, 2 V; frequency, 1 Hz; duration, 10 ms). The arrows below the time course represent the pulse triggers.

Pulse triggers are indicated as arrows below each time course. The numbers (1) to (3) in Fig. 5(B) represent the contractile behavior of myotube line patterns labeled (1) to (3) in Fig. 5(A). At first, all the myotube line patterns were stimulated, followed by resting patterns (3) to (1) in order, and finally we selectively stimulated just pattern (2). Each myotube line pattern exhibited independent contractile behaviour synchronized with the electric pulse supplied through the aligned microelectrodes. These results suggested that contractile activity of each myotube line pattern could be arbitrarily controlled using the microelectrode arrays.

Fig. 6 depicts the expression assay of GLUT4 by the selective stimulation of the transfected myotube line patterns. Myotube line pattern on the left side was stimulated for 3.5 h at 10 Hz (amplitude, 2 V; duration, 3 ms; train, 1 s; interval, 10 s) and the right side pattern was rested, as shown in Movie S2, ESI†. The myotubes were immunostained with the anti-c-myc first antibody and the Alexa Fluor 594-conjugated anti-mouse IgG second antibody after the electrical stimulation and cell fixation. The antibodies used are impermeable to the cell membrane, which makes it enable to stain only the GLUT4 expressed on the cell surface.[19] As can be seen in Fig. 6(B) and (C), electrically stimulated myotubes displayed an obvious increase in fluorescent intensity above that of unstimulated cells by about 4-fold. This result suggests that the contraction-induced GLUT4 translocation occurred from intracellular vesicles to the plasma membrane, in agreement with our previous study using the western blot analysis of GLUT4 translocation in a random-cultured myotube monolayer.[19,20]

In a conventional random-cultured myotube monolayer, it was difficult to match the target myotubes with their GLUT4 translocation activity after the fixation process because it is hard to seek the "contracted target myotubes" on the culture dish during the electrical stimulation. Therefore, the averaged GLUT4 translocation activity of all cultured cells was detected by the western blot analysis, which would underestimate the true activity of the contractile myotubes.

Our device made it possible to easily identify the GLUT4 translocation activity of the contracting myotubes for the first time, by artificially patterning and locally targeted stimulation of the myotubes on the gel sheet. Furthermore, by patterning myotube lines subjected to different stimulation conditions next to each other, high-contrast imaging of the contraction effect on

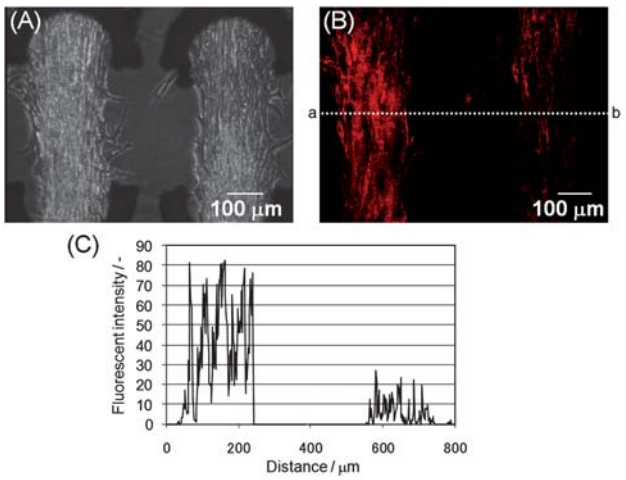

Fig. 6 (A) Phase-contrast micrograph of the transfected myotube line patterns locally stimulated with the PEDOT microelectrode arrays (amplitude, 2 V; duration, 3 ms; frequency, 10 Hz; train, 1 s; interval, 10 s). (B) Fluorescent image of the myotube line patterns immunostained with anti-c-myc first antibody and Alexa Fluor 594-conjugated anti-mouse IgG second antibody. (C) Fluorescent intensity of each immunostained myotube line pattern along the cross-section a–b in (B).

GLUT4 translocation in myotubes was achieved. This device would be applicable for quantitative bioassays of various contraction-induced metabolic alterations in myotubes.

Conclusions

We have developed a micropatterned C_2C_{12} myotubes/fibrin gel culture system integrated with a PEDOT microelectrode array chip for skeletal muscle cell-based bioassay. Arbitrary control of micropatterned myotubes contraction with localized electrical stimulation enabled high-contrast imaging of contraction-induced GLUT4 translocation phenomena in myotubes. This device would easily permit focusing the stimulation site on a desired specific tissue construct, such as a neuromuscular junction formed in a neuron–skeletal muscle cell co-culture. Such applications would be reported in future studies.

Acknowledgements

This work was supported by a Core Research for Evolutional Science and Technology grant from the Japan Science and Technology Agency, partly by Special Coordination Funds for Promoting Science and Technology, Formation of the Innovation Center for Fusion of Advanced Technologies from the Japan Science and Technology Agency, by the Research Grant Program of Asahi Glass Foundation, and by Grants-in-Aid for Scientific Research B (17310080) from the Ministry of Education, Science, and Culture, Japan.

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