

Muscle-powered Cantilever for Microtweezers with an Artificial Micro Skeleton and Rat Primary Myotubes*

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Abstract

A technique to attach muscle cells to artificial micro mechanical systems was studied using dog body hair and rat primary skeletal muscle. Muscle-powered cantilever for microtweezers were fabricated which consisted of a single strand of hair for the skeleton and differentiated myotubes for the actuator. The three-dimensional mechanical part of the microtweezers was fabricated using a focused ion beam-induced ion milling technique. The micro hair skeleton was used as a scaffold for the muscular cells and the mechanical structure. Electrical stimulation induced related contraction of the myotubes and displacement of the muscle-powered cantilever of the microtweezers, although the displacement was not yet enough for useful microtools.

Key words: Bio Actuator, Myotube, Skeleton, Tweezers, Muscle

1. Introduction

Attempts have been made to construct autonomous and self-sustainable systems using a bio hybrid micro mechanical system, *i.e.* a mechanobionic system. The mechanobionic system stands between the bottom-up and top-down fabrication methodologies to construct autonomous and self-sustainable systems based on a hybrid system between living biological cells and artificial micro parts fabricated by a micromachining technique. The living cells use glucose supplied from an external environment as an energy source through the glycolytic cycle, and they produce energy which can be converted to effective work within themselves. In particular, myocytes have a high physical capability, *i.e.* strong contractions due to myosin-kinesin interactions. The control of movements and sensory information is provided from the cyclic reaction pathway of proteins and amino acids inside and outside of the cells as information transmission. Hence, cells can work as self-sustainable mechanical systems with signal processing, control, and sensation of the dynamic external environment. The mechanobionic system should employ such highly functional mechanisms of biological cells to enhance its mobility and self-sustainability.

This concept was demonstrated in previous studies using myocardiocytes as bio actuators in bio hybrid systems⁽¹⁻⁶⁾. These studies provided new techniques to replace the actuators with cells and bio molecules *in vitro*, suggesting a possibility of achieving high functionality by fusing biological and artificial components. These bio hybrid systems with muscular tissue were flexible and had an elastic thin filmy configuration when

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fabricated with cell sheet ^(1,2,3), hydro gel ⁽⁴⁾, or PDMS thin film ^(5,6). Such mechanical bio components could be the parts of biomimetic soft mechanics such as a mollusk. Flexible mechanics achieved with biological tissue could have an advantage for avoiding destruction of the structure; however, sufficient stiffness would be necessary to achieve accurate positioning. A rigid skeleton and a flexible joint, such as in endoskeleton or exoskeleton animals, are important for engineered muscular robots to determine a precise posture of movement. Das *et al.* ⁽⁷⁾ plated myoblasts on silicon microcantilevers to investigate differentiation of myoblasts, although the cantilevers displayed no deformation. The issues for realizing high-performance muscular robots are precise positioning of muscle cells on the micro skeleton with joints and contradictory combination of rigid skeleton and flexible joints in a micro space. In particular, it is difficult to define the position of myoblasts or myocytes in the cell adhesiveness pattern on 3-D structures.

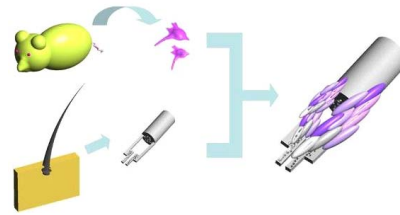


Fig. 1 Bio hybrid muscle-powered microtweezers with rat primary myocytes and dog body hair. Myoblasts fused to form a multinuclear myotube on the micro fabricated hair skeleton.

We have focused on how to define the movements of the engineered muscle as robots and our research topics have been the adhesion position and alignment of the myotubes, functional stimulations, and the micro mechanics. In this study, we proposed that anisotropic stiffness of a skeleton could define the movements of the muscular contraction. We assumed that providing an anisotropic stiffness hinge mimicking a joint and skeleton for engineered muscle would allow easy controlling of skeleton movement direction; *i.e.*, isotropic contraction of engineered muscle could be converted to anisotropic deformation on the artificial anisotropic skeleton and hinge. We found that the micro skeletal structure could be provided for the engineered muscular micro device. We fabricated the microtweezers with the hinge for an engineered skeletal muscular device using focused ion beam- (FIB-) induced ion milling to determine the moving direction of the hinge. Demonstration of this mechanobionic system with skeletal muscle cells was carried out as a muscle-powered cantilever of microtweezers driven by rat skeletal muscle cells with an artificial micro skeletal system made of dog hair (figure 1).

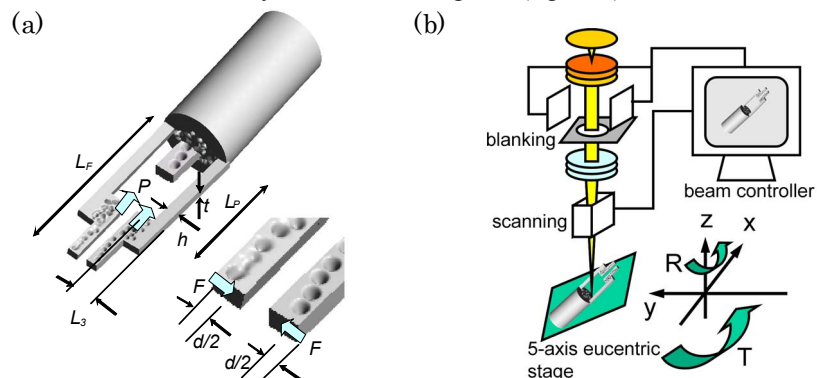


Fig. 2 Microtweezers skeleton. (a) Design of the microtweezers skeleton. The skeleton was fabricated on a section of hair shaft. The beams of the skeleton were bent to the medial side by muscular contraction force P applied at their mid-point. Parameters are listed in Table 1. (b) A Schematic of 3-D rapid prototyping method using FIB-induced ion milling. Beam scanning and the 5-axis eucentric stage were simultaneously controlled to fabricate the 3-D prototype product from biological material.

Table 1: Dimension of the microtweezers in figure 2 and 3

Beam height h [μm]	Beam width t [μm]	Beam length L _p [μm]	Overall length L _F [μm]	Moment arm length L ₃ [μm]	Displacement d [μm]
5	5	50	100	15	1

2. Experimental

2.1 Structural materials

We decided to fabricate a micro skeleton from dog body hair to satisfy the following requirements: the structural materials for the artificial skeleton of the muscle-powered microcantilevers should have no cytotoxicity; they should have sufficient stiffness and strength for the skeleton; and they should be workable into a microstructure. Body hair fibers mainly consist of keratin protein, and they have multilayer structures in their cross section. Their diameter is normally less than 100 μm which is a suitable dimension for the muscle-powered microcantilevers in this study. Hair medullae are porous fibrous structures several micrometers in diameter in the center of the hair shaft. In a previous study, Yasuda *et al.* ⁽⁸⁾ found that a large surface area increased the membrane adherence of a cardiomyocyte on a rough carbon surface; therefore, the porous structure of the hair would be a suitable scaffold for the myocytes of the muscle-powered microcantilevers.

Human hair has a Young's modulus E of 4 to 6 GPa ^(9, 10) which is 3% of the E for silicon, but similar to the E of Nylon 66 (E = 2.3 GPa). A higher stiffness would result in the width of the flexible hinge of the microtweezers being thinner than the minimum dimension of fabrication, and use of a more flexible material, such as polydimethylsiloxane (PDMS, E = 2 MPa ⁽¹¹⁾) would not provide enough stiffness for self-standing. Therefore the moderate stiffness of hair fiber would be suitable for the myocyte-driven tweezers.

2.2 Mechanical design of the muscle-powered microcantilever for microtweezers

Our muscle-powered microtweezers were designed with the following specifications: d = 1 μm for the range of tips movement desired; F = 1 μN for maximum gripping force of the tips; and P = 2 μN for contraction force of the myotubes on the tweezers. The skeleton of the muscle-powered microtweezers had one pair of microcantilevers trimmed from the cross section of a hair fiber, as shown in figure 2. The beams of the cantilevers were trimmed from the homogeneous cortex of the hair fiber. An adhesion site for myocytes as an input force of the forceps was placed on the hair medulla at the middle of the tweezers. The myoblasts and myotubes aligned along the hair contracted in the axial direction of the fiber. Thus, the beams were bent, and then the forceps closed by the contraction force of the myotubes. The designed displacement at the tips d was summation of deflection d_p in bending at beam L_p and tilting i of beam L_F-L_p (equation (1)). The beam deflection d_p and tilting angle i was calculated from equation (2) derived from Castigliano's theorem.

Table 1 shows the calculation conditions and the results obtained, based on Young's modulus E = 4 GPa.

$$d = 2\{d_p + (L_F - L_p)\tan i\} \quad (1)$$

$$= 2\left\{\frac{PL_p^2L_3}{2EI} + (L_F - L_p)\tan\frac{PL_pL_3}{EI}\right\} \quad (2)$$

2.3 Fabrication of the microcantilevers skeleton

The microcantilevers skeleton was fabricated by 3-D rapid prototyping using the FIB-induced ion milling technique (figure 2). The FIB apparatus (SII NT, SMI-9800) could focus a Ga ion beam to 7 nm at half maximum full-width. The specimen atoms were

sputtered by ion bombardment; in this way, the region of thermal heating and the work-affected layer were less than for other thermal or chemical processes. Unlike a conventional MEMS fabrication process, this technique did not employ any organic solvent which would cause albuminoid degeneration; therefore it was appropriate for biological materials. Cytotoxicity of Ga implanted into specimens has not been reported and the micro structure fabricated by FIB-induced ion milling had no toxicity *in vitro* ⁽¹²⁾. The FIB-induced ion milling fabrication process offers much flexibility for shapes and materials, thus we applied it to the rapid prototyping for our micro mechanobionic system.

A strand of body hair (30 to 50 μm in diameter) from a dog was immersed more than one day in ethanol to degrease it. It was inserted into a pulled glass capillary (Narishige, PC-10, GD-1) and the blunt end was glued (Shin-Etsu Chemical, RE-42) to fix the hair into position. After sputter coating (Sanyu Electron, SC-701) with a 500 \AA thick Au film, the hair was trimmed into the forceps shape with the FIB apparatus. The outline of the skeleton was formed multi directionally from the hair using a eucentric stage with 5-axis motorization at a beam current of 28.5 nA μm^{-2} .

2.4 Skeletal muscular cells

Isolating and culturing of rat primary myoblasts were done based on the literature ^(13, 14, 15). Primary myoblasts were isolated from neonatal rat (Sankyo Lab, Wister, 1-day old) extremities.

Multiple myoblasts fuse to form a multinuclear myotubes. Rat primary myotubes, 10-30 μm in diameter and 200-1000 μm in length, are easily formed from rat extremity tissue *in vitro* and can be controlled via the electrical potential of a neuron or an electrical stimulator. Therefore we decided to employ them as actuators of the microtweezers (figure 1). The multinucleated myotubes began to contract spontaneously or by electrical stimulation several days after beginning differentiation. The contraction force was approximated as 1 to 2 $\mu\text{N}/\text{cell}$ using the contraction force data of engineered muscle tissue ⁽¹⁶⁾. Thus primary myotubes would be appropriate actuators for the bio hybrid muscle-powered microtweezers.

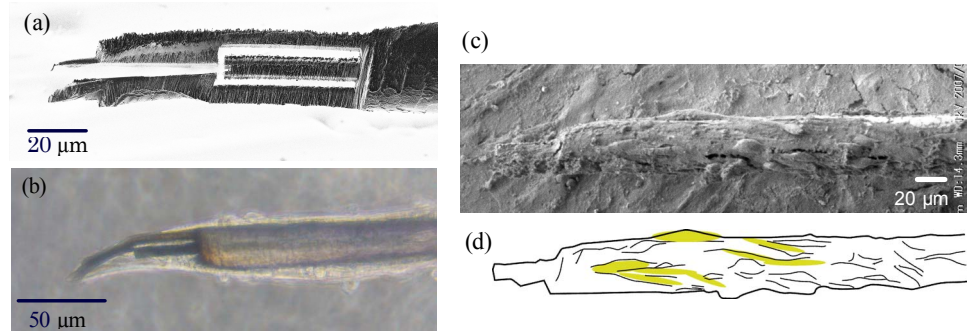


Fig. 3 Muscle-powered cantilever on the tip of a dog hair. (a) Scanning ion image of microcantilevers skeleton. (b) Cultured rat primary myotubes adhered on the skeleton. One side of the cantilever was broken just before this experiment due to meniscus force. Myoblasts completely covered the skeleton. (c) Scanning micrograph of the tip of the microcantilever. Myoblasts and myotubes were aligned along the axial direction of the hair shaft. The ends of the microcantilever was broken at the time of the fixation process. The structure was completely covered by cells. (d) The sketch shows adhesion cells and myotube like cells (gray areas) on a strand of hair.

2.5 Construction of muscle-powered microcantilever and cell immobilization

The fabricated microcantilevers were then attached 1 mm above the bottom of the dish. Cell adhesive material was coated on the tip of the skeleton using a drop of PBS solution with 25 $\mu\text{g mL}^{-1}$ fibronectin and cured at 37 $^{\circ}\text{C}$ for 1 h. The drop of myoblast suspension was stationary-cultured on the tip for 1 h at 37 $^{\circ}\text{C}$ after rinsing with PBS. Finally, growth medium was added to cover the tip of the skeleton and culturing was done at 37 $^{\circ}\text{C}$ in 5%

CO₂. The medium was replaced with differentiated medium (Dulbecco's modified Eagle medium with 7% horse serum and 100 U mL⁻¹ of penicillin/ streptomycin) on day 4 after seeding.

2.6 Morphology and behavior

The morphology and differentiation of myoblasts on the muscle-powered microcantilevers and neighboring myotubes were observed every 24 h with an inversed phase-contrast microscope (Olympus, IX-71).

The electrical stimulation (Nihon Kohden, SEN-3301) for myotubes contraction was done with a unipolar rectangular pulse (1 Hz, 50 V amplitude, 10-400 ms duration). The displacement of the tip of the muscle-powered microcantilever was recorded and analyzed by video with 30 frames per second and 0.1 $\mu\text{m pixel}^{-1}$ resolution. The displacement of time-series was analyzed from the first moment of the cross-correlation function between each frame.

After the stimulation, the muscle-powered cantilever were fixed using paraformaldehyde and freeze dried for scanning electron micrography (SEM) (Keyence, VE-8800).

3. Results

The skeleton of the muscle-powered microcantilevers was fabricated by FIB, as shown in figure 3 (a). Deformation that occurred during the ion beam bombardment was recovered due to immersion of the deformed skeletal in the medium because of its moistening effect on the hair. The fabrication process required 5 to 7 hours to trim off.

The myoblasts were adhered on the hair skeleton immediately after a droplet of a suspension of the isolated myoblasts was dropped onto it. The myoblasts spread and covered the entire hair skeleton at 4 days after seeding and were oriented along the axial direction of the hair shaft. The tip of the muscle-powered cantilever was driven by muscular contraction due to electrical stimulation on day 7 (figure 4), although the displacement was not only on the tip but also the shaft of the hair (see figure 4 (a)). The displacement was 0.6 μm at a maximum on the tip when stimulated with 10 ms duration pulse. The displacement occurred simultaneously with 1 Hz stimulation.

Morphology of the muscular cells was observed by SEM after fixation and freeze-drying. The SEM image shown in Figure3 (c) suggested the myotubes and myoblasts were aligned along the longitudinal axis of the hair fiber, and they were adhered over the whole area.

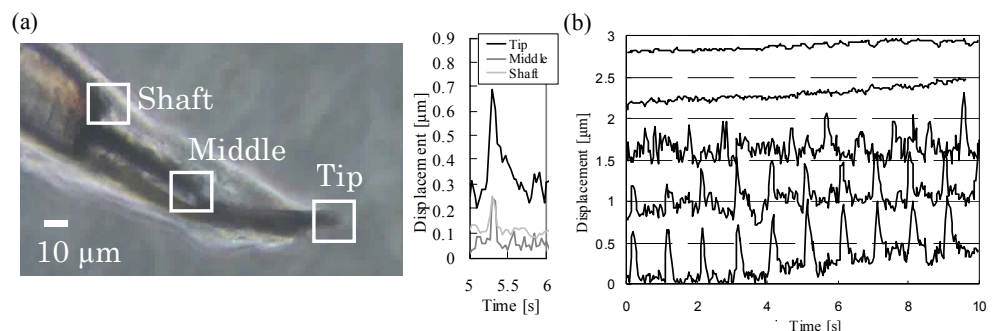


Fig. 4 Displacements of the tweezers. (a) Displacements of three points were calculated using binary video images. (b) Displacements of the tip for various duration times of pulse stimulation. Electric stimulation was applied as a unipolar square voltage: pulse width, 10 to 400 ms; amplitude, 50 V; frequency, 1 Hz. The curves from bottom to top are in the order 10, 20, 30, 300 and 400 ms.

4. Discussion

The FIB technique does not require toxic organic solvents for the 3-D fabrication

process, so that proteins would experience less damage. The hair skeleton of the muscle-powered microcantilevers fabricated by FIB had no cytotoxicity, and became a cell scaffold where good cell adhesion and differentiation occurred, which suggested that products processed by FIB could be employed for bio hybrid structures as biocompatible materials.

The surface of the hair skeleton developed a spiky surface due to ion bombardment during beam irradiation. That could reduce the strength of the hair skeleton structure; when rinsing the hair skeleton structure and changing medium one beam of the hair skeleton was broken.

Myoblasts adhered on the hair skeleton and were aligned autonomously along the axial direction of the hair fiber due to use of fibronectin and the high concentration of the suspension of rat primary myoblasts. From the SEM observation, myotubes were also differentiated along the axial direction, although the mechanism of the alignment was not investigated. Similar alignment phenomenon of cell line myoblasts has been reported on parallel polymer strings⁽¹⁷⁾. Thus the aspect ratio of a structure skeleton would be one of the controllable factors of muscular devices.

Electrical stimulation induced contraction of all the myotubes on the hair skeleton; thus the displacement appeared to be due to bending from anisotropic stiffness with co-contraction of the overall hair shaft. The displacement was not enough for a demonstration of micro operation, therefore, the anisotropic design of the cantilevers should be optimized for more effective contraction of myotubes by utilizing alignments and differentiation of myotubes, such as myotube growth on a geometric patterned surface.

The effect of the duration of the electrical pulse stimulation was examined (figure 4 (b)). The displacements decreased with longer pulse stimulation durations. To develop more advanced muscle-powered microtweezers, as the next step for study, the frequency and amplitude induced response on electrical stimulation should be investigated to demonstrate functional manipulations. Also, partial stimulation and cellular positioning on the skeletal structure should be investigated using a neuromuscular junction which connects skeletal muscle and peripheral neuron. Co-culturing of a neuromuscular unit should realize high functional controlling of this muscle-powered microcantilevers and mechanobionic systems.

5. Conclusion

We constructed a mechanobionic muscle-powered microcantilever for microcantilevers with a micro skeleton made of dog hair and differentiated aligned myotubes. Good cell adhesion and differentiation on the hair micro skeleton suggested that products processed by FIB could be employed for bio hybrid structures as biocompatible materials. The designed skeletal structure for the muscle-powered cantilever was fabricated by FIB-induced ion milling. Biocompatibility of FIB-fabricated micro parts and cellular alignment on the hair skeleton were confirmed. Differentiated myotubes on the skeleton had enough contraction force due to electrical stimulation to bend the hair. Our demonstration of the bio hybrid micro mechanism is preliminary work for construction of a highly functional and self-subsisting micro system.

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References

- (1) Tanaka,Y, Morishima,K, Shimizu,T, Kikuchi,A, Yamato,M, Okano,T and Kitamori,T, An actuated pump on-chip powered by cultured cardiomyocytes, *.Lab on a chip*, Vol.6, No.3,(2006),pp.362-8.
- (2) Tanaka,Y, Morishima,K, Shimizu,T, Kikuchi,A, Yamato,M, Okano,T and Kitamori,T, Demonstration of a PDMS-based bio-microactuator using cultured cardiomyocytes to drive polymer micropillars, *.Lab on a chip*, Vol.6, No.2,(2006),pp.230-5.
- (3) Xi,J, Schmidt,JJ and Montemagno,CD, Self-assembled microdevices driven by muscle, *.Nature materials*, Vol.4, No.2,(2005),pp.180-4.
- (4) Zimmermann,W, Schneiderbanger,K, Schubert,P, Didie,M, Munzel,F, Heubach,J, Kostin,S, Neuhuber,W and Eschenhagen,T, Tissue Engineering of a Differentiated Cardiac Muscle Construct, *.Circ Res*, Vol.90, No.2,(2002),pp.223-230.
- (5) Feinberg,AW, Feigel,A, Shevkoplyas,SS, Sheehy,S, Whitesides,GM and Parker,KK, Muscular Thin Films for Building Actuators and Powering Devices, *.Science*, Vol.317, No.5843,(2007),pp.1366-1370.
- (6) Kim,J, Park,J, Yang,S, Baek,J, Kim,B, Lee,SH, Yoon,E, Chun,K and Park,S, Establishment of a fabrication method for a long-term actuated hybrid cell robot, *.Lab on a Chip*, Vol.7, No.11,(2007),pp.1504-1508.
- (7) Das,M, Gregory,CA, Molnar,P, Riedel,LM, Wilson,K and Hickman,JJ, A defined system to allow skeletal muscle differentiation and subsequent integration with silicon microstructures, *.Biomaterials*, Vol.27, No.24,(2006),pp.4374-4380.
- (8) Yasuda,SI, Sugiura,S, Kobayakawa,N, Fujita,H, Yamashita,H, Katoh,K, Saeki,Y, Kaneko,H, Suda,Y, Nagai,R and Sugi,H, A novel method to study contraction characteristics of a single cardiac myocyte using carbon fibers, *.American journal of physiology. Heart and circulatory physiology*, Vol.281, No.3,(2001),pp.H1442-6.
- (9) Bonser,R and Purslow,P, The Young's modulus of feather keratin, *.J Exp Biol*, Vol.198, No.Pt 4,(1995),pp.1029-33.
- (10) Wei,G and Bhushan,B, Nanotribological and nanomechanical characterization of human hair using a nanoscratch technique, *.Ultramicroscopy*, Vol.106, No.8-9,(2006),pp.742-754.
- (11) Olah,A, Hillborg,H and Vancso,GJ, Hydrophobic recovery of UV/ozone treated poly(dimethylsiloxane): adhesion studies by contact mechanics and mechanism of surface modification, *.Applied Surface Science*, Vol.239, No.3-4,(2005),pp.410-423.
- (12) Hoshino,T, Ozasa,A, Kometani,R, Suzuki,T, Matsui,S and Mabuchi,K, Development of a regeneration-type neural interface: A microtube guide for axon growth of neuronal cells fabricated using focused-ion-beam chemical vapor deposition, *.Journal of Vacuum Science & Technology B: Microelectronics and Nanometer Structures*, Vol.24, No.6,(2006),pp.2538-2543.
- (13) Cognard,C, Constantin,B, Rivet-Bastide,M, Imbert,N, Besse,C and Raymond,G, Appearance and evolution of calcium currents and contraction during the early post-fusional stages of rat skeletal muscle cells developing in primary culture, *.Development*, Vol.117, No.3,(1993),pp.1153-1161.
- (14) Dennis,RG, Kosnik,PE, Gilbert,ME and Faulkner,JA, Excitability and contractility of skeletal muscle engineered from primary cultures and cell lines, *.Am J Physiol Cell Physiol*, Vol.280, No.2,(2001),pp.C288-295.
- (15) Dennis,RG and Kosnik,PE, Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro, *.In vitro cellular & developmental biology. Animal*, Vol.36, No.5,(2000),pp.327-35.
- (16) Huang,Y, Dennis,RG, Larkin,L and Baar,K, Rapid formation of functional muscle in vitro using fibrin gels, *.J Appl Physiol*, Vol.98, No.2,(2005),pp.706-713.
- (17) Neumann,T, Hauschka,SD and Sanders,JE, Tissue Engineering of Skeletal Muscle Using Polymer Fiber Arrays, *.Tissue Engineering*, Vol.9, No.5,(2003),pp.995-1003.