Towards the Development of Optogenetically-Controlled Skeletal Muscle Actuators

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TOWARDS THE DEVELOPMENT OF OPTOGENETICALLY-CONTROLLED SKELETAL MUSCLE ACTUATORS

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ABSTRACT

Engineered skeletal muscle tissue has the potential to be used as dual use actuator and stress-bearing material providing numerous degrees of freedom and with significant active stress generation. To exploit the potential features, however, technologies must be established to generate mature muscle strips that can be controlled with high fidelity. Here, we present a method for creating mature 3-D skeletal muscle tissues that contract in response to optical activation stimuli. The muscle strips are fascicle-like, consisting of several mm-long multi-nucleate muscle cells bundled together. We have found that applying a tension to the fascicle-like muscle tissue promotes maturation of the muscle. The fascicle-like muscle tissue is controlled with high spatiotemporal resolution based on optogenetic coding. The mouse myoblasts C2C12 were transfected with Channelrhodopsin-2 to enable light (~470 nm) to control muscle contraction. The 3D muscle tissue not only twitches in response to an impulse light beam, but also exhibits a type of tetanus, a prolonged contraction of continuous stimuli, for the first time. In the following, the materials and culturing method used for 3D muscle generation is presented, followed by experimental results of muscle constructs and optogenetic control of the 3D muscle tissue.

INTRODUCTION

Live muscle tissue has the potential to be a new type of actuator, having numerous degrees of freedom, regenerative capabilities, and a wide range of sizes (5 um ~ 1 m) and force generation (~5 uN ~ 1 kN) [1]. These advantages can potentially be used for multi-DOF micro surgical tools, sub-millimeter to millimeter scale robots, and other machines that are difficult to build with traditional actuators. An ideal engineered muscle tissue requires adequate size and form factor, highly aligned multi-nucleate cell structure, and fully differentiated, mature construction [2]. In the last decade a number of research efforts have been reported on engineered muscle tissues, but conventional studies have been limited to 2D environments [3-4], muscle tissues in vivo [5], electrical stimulation [6-7], and cardiac muscle cells [8]. Also, the artificial muscle tissues are by far inferior to the natural muscle in terms of output strain, stress [9], and controllability [2]. Breakthroughs must be made to produce practically useful muscle actuators that are fully mature and functional as well as controllable.

The present work focuses on two key technologies towards the development of mature and functional skeletal muscle tissue that can be controlled with high spatiotemporal resolution. One is 3D myogenic culturing for the formation and maturation of fascicle-like skeletal muscle tissue in vitro, and the other is optogenetic coding for activating a muscle strip by projecting a light beam.

Skeletal muscle is a highly organized and hierarchical tissue. It consists of fascicles that are a bundle of skeletal muscle fibers, and research of fascicle-like structure will be necessary to scale up muscle actuators in the future. We have developed a device which can culture myoblasts into fascicle-like structures which are closer to in vivo environments. Specifically, the device allows muscle cells to grow away from contact with stiff surfaces, only anchoring at both ends. In

addition, the structure of the developed device provides slight tension to muscle tissues because the hydrogel scaffold compacts after seeding the device. With these conditions, we have already observed that the muscle tissues mature more than 2D cell culture. In this sense, we supposed that the stretching of the muscle tissue by the device might cause more mature muscle tissue to form. So, we applied additional moderate external stretching to muscle tissue for understanding a role of physical force in muscle development.

Optogenetic coding is a relatively new technology that makes excitable cells light sensitive [10]. The authors group was the first to successfully activate skeletal muscle strips with "light" by transfecting skeletal muscle cells with Channelrhodopsine 2 [11]. Unlike the traditional electric activation, which uses a pair of electrodes inserted into the muscle tissue, the optogenetic activation of muscles is non-invasive, non-contact, and wireless control and, more importantly, allows for activating densely arrayed muscle strips one by one separately or as a group in a coordinated manner. This opens up new possibilities of skeletal muscle actuators. High spatiotemporal resolution of optical, wireless control would make a bundle of muscle strips a high DOF, or even distributed DOF, actuator.

In the following, a new 3D fascicle-like skeletal muscle actuator is introduced, and its tension application mechanism is described. Experimental results confirm that stretching enhances muscle differentiation and maturation. Optogenetic control experiments are also conducted for the 3D fascicle-like muscle tissue. "Tetanus", a prolonged muscle contraction, is achieved for the first time for the light activated skeletal muscle tissues.

MATERIAL AND METHOD

Cell Culture

The C2C12 mouse myoblast cell line, used for study, was transfected with Channelrhodopsin-2 DNA to enable control and contraction via controlled light (~470 nm) using by pAAV-Cag-Chr2-GFP-2A-Puro with LipofectamineTM 2000 (Invitrogen). The myoblasts were maintained in growth media (GM), which is Dulbecco's modified Eagle medium (DMEM, ATCC) supplemented with 10% Fetal Bovine Serum (FBS, Sigma), 1% Penicillin-Streptomycin (PS, Sigma), and 0.2% Normocin (InvivoGen). The cells were passaged when 70~80% confluent. The growing media was changed every 2 days.

Fascicle-like Muscle Tissue Constructs

The myoblasts were seeded into a tubular structure together with the gel and other media. Figure 1 shows a mold made of PDMS for forming fascicle-like 3D tissues. Several days after seeding, the fascicle like 3D tissue is solidified. Both ends of the muscle tissue stick to the PDMS mold. To increase tension in the longitudinal direction, the PDMS mold is squashed with a pair of thick blocks, as shown in the figure. As a result, the fascicle-like muscle tissue is stretched in the longitudinal direction. This produces a small stress on the fascicle-like tissue.

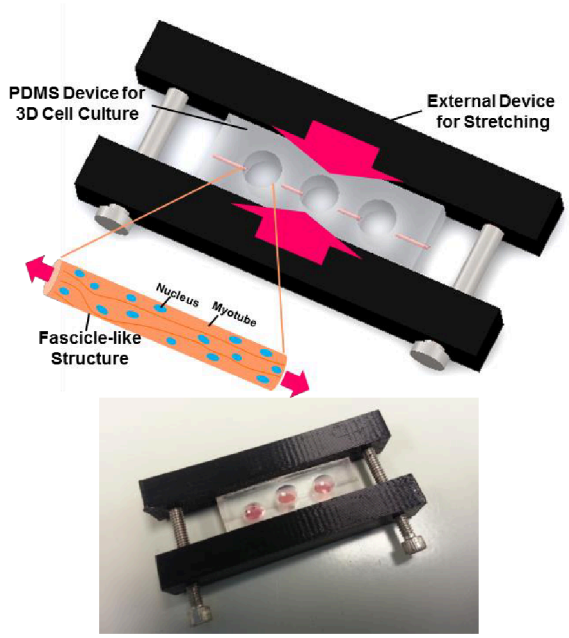


FIGURE 1. DEVICE FOR APPLYING EXTERNAL STRETCHING TO THE MUSCLE TISSUES.

For two days after seeding, the cells are cultured in GM with 1 mg/ml aminocaproic acid (AA). After that, we changed the media into differentiation media (DM) with 1 mg/ml AA. DM is the same as GM except for 10% horse serum instead of the FBS. The media was changed every day totally for 2 weeks.

The external device for stretching was constructed by 3D printer (Dimension SST 1200es) and assembled the devices to apply 10 % strain to the muscle tissues. By measuring the length changes from images, the average of actual applied strain was 13.32±1.58 %. External stretching had applied 1 hour after the seeding to reduce a possible damage during device assembly.

Quantification of the muscle tissue contraction

Muscle contraction was induced with emitting a blue light (~470 nm) to excite the cells transfected by Channelrhodopsin-2. To quantify impulse response and step response of muscle tissue, we choose two arbitrary points on the muscle tissues and measured the length between the two points. Measured tissue got external stretching from day 9 and the contraction was measured at day 14.

Immunostaining

Immunostaining technique is used to visualize actin, nucleus, and alpha-actinin, which is located in the Z band/disc and cross-links with F-actin in skeletal muscle cell. After fixation of the cell using 6% Paraformaldehyde (Sigma), the cells were treated 1% Triton-X (Invitrogen) for permiablization,

and 1% bovine serum albumin (BSA, Sigma) for nonspecific binding of antibody. Incubation with primary antibody (Invitrogen) of alpha-actinin was performed for overnight at 4°C, and second antibody (Invitrogen) was treated for 1 hour. Hoechst (Invitrogen) and rhodamine phalloidin (Invitrogen) were treated together for 30 min to visualize nucleus and actin, respectively.

RESULTS

Moderate external stretching is applied to muscle tissues to understand a role of force to muscle development. To achieve this goal, we applied a tension to the muscle tissue with approximately 10% strain in the axial direction.

Impulse and Step Responses of the Muscle Tissue

When we stimulated the muscle tissues by blue light in impulse function, emitting the light for approximately 0.35 s, muscle started to contract after 0.18 s and peak time was 0.27 s (FIGURE 2). The maximum contraction was happened after turning off the light, and the tissue was relaxed for 0.18s.This short, and sudden muscle contraction is called twitch.

Furthermore, when the muscle tissue was stimulated by the light in step function, emitting for approximately 4 s, the muscle tissue kept the contraction with the light for a relatively long time (FIGURE 3). In other words, we induced the prolonged muscle contraction that maintained for number of seconds which is called tetanus. The contraction was delayed about 0.1 s from the light. There was overshoot at the initial stage, and they kept about 60% of maximum contraction for about 2 s.

These results showed that we can control contraction of the stretch fascicle-like muscle tissue by exposure time of blue light. We induced both twitch and tetanus which means that we made the engineered muscle tissue that is more mature, and to have higher potential of muscle contraction.

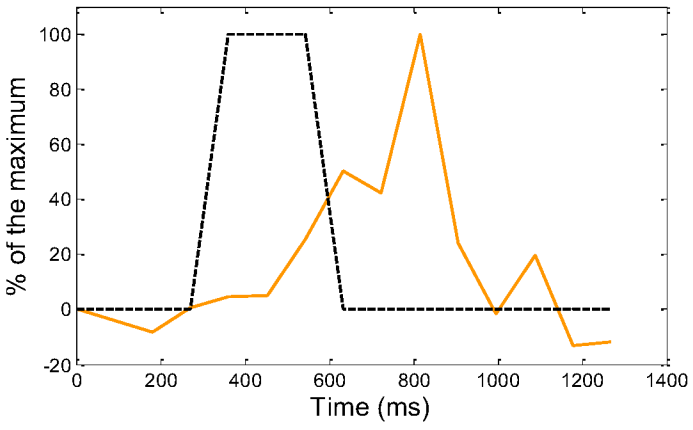


FIGURE 2. IMPULSE RESPONSE OF MUSCLE (TWITCH) WITH STIMULATING BY BLUE LIGHT (~470NM), DOTTED LINE: EMITTING OF THE BLUE LIGHT, MUSCLE LENGTH IS NORMALIZED BY INITIAL MUSCLE LENGTH.

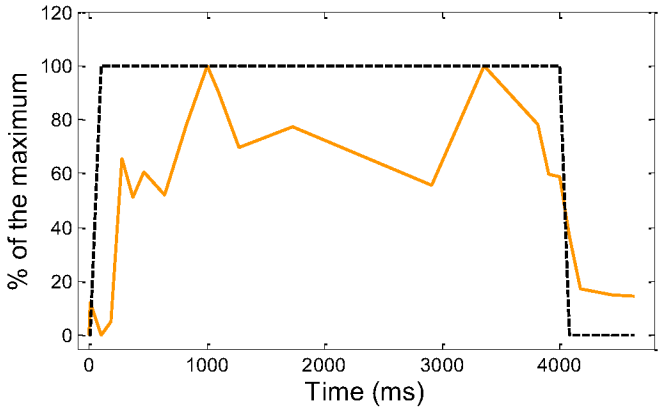


FIGURE 3. STEP RESPONSE OF MUSCLE (TETUNUS) WITH STIMULATING BY BLUE LIGHT (~470NM), DOTTED LINE: EMITTING OF THE BLUE LIGHT, MUSCLE LENGTH IS NORMALIZED BY INITIAL MUSCLE LENGTH.

Identification of Internal Structure Differences by Immunostainin ng

The fascicle-like muscle tissues were stretched from right after seeding, and fixed at day 6 and day 14. We fixed the cells at these days because the tissues usually started to contract from the day 6 and degraded after 2 weeks. We wanted to check an effect of the external stretching to muscle development in time-related manner.

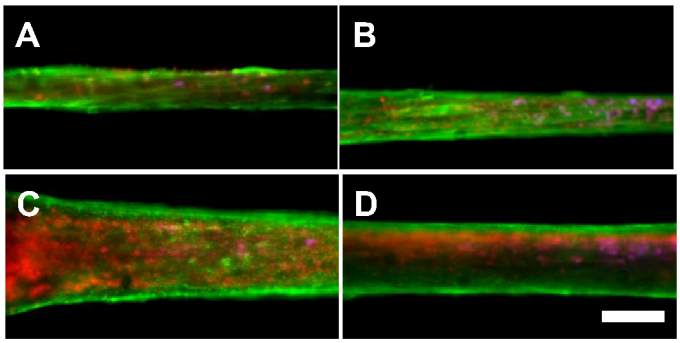


FIGURE 4. VISUALIZING OF ACTIN (RED), NUCLEUS (BLUE), AND ALPHA-ACTININ (GREEN) BY IMMUNOSTAINING, FIXED AT 6 DAYS FROM SEEDING, A, B: THE STRETCHED MUSCLE TISSUES, C, D: CONTROL, SCALE BAR = 100 UM.

When the muscle tissue got stretched for the first 6 days, they developed alpha-actinin as more filamentous and tube structures than control tissues (FIGURE 4). Alpha-actinin is a cytoskeletal actin-binding protein, and it cross-links actin filaments in order to form a lattice-like structure with forming adjacent sarcomeres. This formation with alpha-actinin helps to stabilize the muscle contractile apparatus [12, 13]. However, in this experiment, F-actin was not formed well in both conditions yet.

In this sense, we could suppose that forming filamentous structure of alpha-actinin is earlier step than actin's even though

alpha-actinin is actin binding protein. Also, external stretching could accelerate a formation of stable contraction apparatus by affecting the alpha-actinin related signaling pathways.

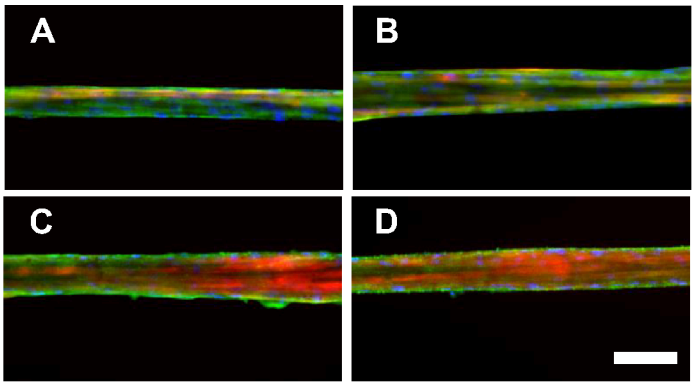


FIGURE 5. VISUALIZING OF ACTIN (RED), NUCLEUS (BLUE), AND ALPHA-ACTININ (GREEN) BY IMMUNOSTAINING, FIXED AT 14 DAYS FROM SEEDING, A, B: THE STRETCHED MUSCLE TISSUES, C, D: CONTROL, SCALE BAR = 100UM.

When the muscle tissues were stretched for 14 days, stretched tissue showed well-developed alpha-actinin in filamentous and tube structures (FIGURE 5). Also, F-actin was formed in both stretched and control tissues.

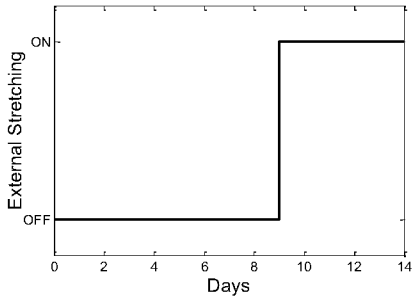
To investigate the effect of stretching in early stage of muscle development, we started to give external stretching from 9 days from seeding, which the tissues usually contract well with stimulating by the blue light (FIGURE 6). In the result, expressions of alpha-actin in the stretched and control tissues were similar, but the stretched tissue showed more striated actin, which is one of the evidences of stable striated muscle (FIGURE 6, 7). We infer from this result that applying stretching for the first few days affects to tube shape formation of alpha-actinin, and stretching at later development stage affect to formation of the mature actin structure.

CONCLUSION AND DISCUSSION

In this study, we figure out effects of moderate external stretching to the fascicle-like muscle tissues development by mechanical and biological tests.

We induced both twitch and tetanus of the stretched muscle tissues by changing stimulation time of blue light. In impulse response, the muscle tissue showed the maximum contraction after turning off the light. Channelrhodopsin-2 inside of the muscle tissue absorbs blue light (~470 nm) and open the pore in the cell membrane. Since the channelrhodopsin is nonspecific cation channels, it arrows the flow of Ca^{2+}, K^{+}, Na^{+}, and H^{+}, which affect muscle contraction. Without the light, the pore is closed, and the flow of ions is stopped, but it takes about milliseconds. Therefore, we have to consider the time delay caused from a nature of channelrhodopsin-2 when we control the muscle tissue as an actuator.

In addition, applying external stretching at early muscle development stage and at the later stage respectively affect expressions of alpha-actinin and F-actin, which play critical roles in muscle contraction. Applying the moderate stretching conditions induced alpha-actinin in tube shape and striated actin, related with stable muscle contraction apparatus.



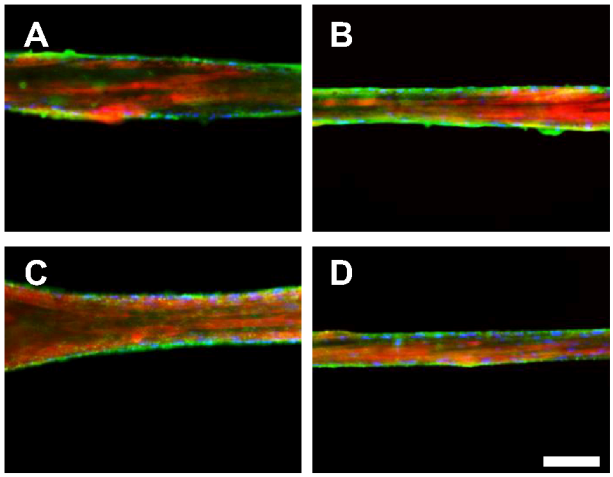


FIGURE 6. VISUALIZINGR OF ACTIN (RED), NUCLEUS (BLUE), AND ALPHA-ACTININ (GREEN) BY IMMUNO-STAINING, WE STARTED TO GIVE STRETCHING FROM 9 DAYS TO 14 DAYS FROM SEEDING, SCALE BAR = 100UM.

External stretching in the axial direction of the tissue could cause well-alignment of the muscle cells itself and intercellular structures such as cytoskeletons. The stretched muscle tissue could develop stronger cytoskeletons and binding proteins to make force equilibrium with the external stretching. Because most of these proteins are also related to muscle contraction, it could affect to signaling pathways to induce more muscle contraction and maturation.

From the results, stretching the tissue helps the muscle to have more mature intercellular structure and to have higher performance. In addition, optogenetic control of the well-developed muscle tissue enables a new type of actuator and lots of engineered muscle tissue applications in the future.

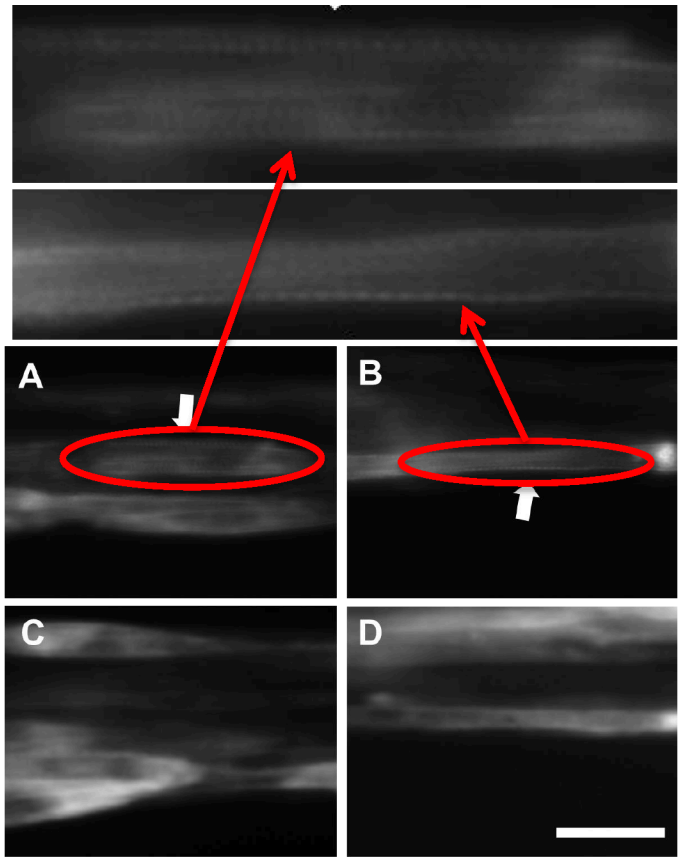


FIGURE 7. VISUALIZING OF ACTIN, WE STARTED TO GIVE STRETCHING FROM 9 DAYS AND FIXED AT 14 DAYS FROM SEEDING, WHITE ARROWS MEAN STRIATED ACTIN FIBERS, SCALE BAR = 50UM.

ACKNOWLEDGMENTS

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