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Optimization of Single Myofiber Isolation Protocol from the Flexor Digitorum Brevis Muscle to Study Satellite Cells

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Abstract

Background: **Satellite cells, which are located between the basal lamina and sarcolemma of the myofiber, have a tremendous ability to repair damaged skeletal muscle. Although they remain quiescent in their niche, they activate rapidly following injury; understanding the mechanisms underlying this activation process has the potential to open new avenues for muscle regenerative therapies. The flexor digitorum brevis muscle "FDB" is one of the best candidates to study satellite cell physiology in the unperturbed myofiber, as it is composed of relatively short myofibers attached to the muscle tendon in an oblique manner, enabling a high rate of intact fiber isolation compared to muscles with longer fibers.** ***Aim:*** **To optimize the preparation process to avoid maldigestion that renders broken and contracted myofibers not suitable for studying quiescent satellite cells and the subsequent activation processes.** ***Material and Methods:*** **FDB muscles from both sides from C57BL/6 male mice were used. The muscles were digested with collagenase digestion solution then cultured in myofiber culture medium in low CO_2 incubators at 37°C.** ***Results:*** **This study revealed that a short incubation period in the digestion solution of two hours is optimum for harvesting the largest number of viable myofiber. Dishes coated with laminin were used to allow the attachment of the single myofibers and further facilitate the immunofluorescence staining process.** ***Conclusion:*** **The used method provides a better tool to isolate the single myofibers from the FDB muscle and hence better analysis of satellite cell activation.**

Keywords: Satellite cells, FDB, single myofiber, activation

Introduction

Skeletal muscles are composed of several bundles of tightly backed fibers separated by interstitial septa, each bundle is formed of numerous functional contractile multinucleated myofibers. During regular day activities, these myofibers undergo multiple wear and tear; a physiologic process that is more vigorous in the context of skeletal muscle injury[(1)]. The recovery after muscle damage is carried out by satellite cells, which are myogenic progenitors, and play a crucial role in

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supporting various processes related to muscle development and repair. These cells are situated between the basal lamina and the plasmalemma of the myofiber[2,3]. In the developmental stage, certain satellite cells actively multiply and contribute myonuclei to the growing myofibers. In contrast, during the maintenance phase (homeostasis), most satellite cells in adult muscles remain inactive. However, in response to conditions such as increased muscle use or muscle injury, satellite cells can be stimulated to enter the cell cycle. This activation leads to the production of progeny that either fuse with existing myofibers or form new myofibers[4]. Initially, satellite cells were identified using electron microscopy because of their location under the myofiber basal lamina[3,5]. More recently, advances in light microscopy and immunostaining have made it possible to monitor satellite cells based on the expression of specific markers, such as the Pax7 transcription factor[6]. Pax7 is uniquely expressed by satellite cells, and using antibodies for immunodetection allows the reliable identification of satellite cells in their natural position across various species [7]. Additionally, genetically manipulated reporter mice with fluorescent tags enable direct detection of satellite cells[8]. The progression of satellite cells through the myogenic program is believed to be regulated by the up- or down-regulation of Pax7 and myogenic regulatory factors (MRFs)[9]. When muscle damage occurs, satellite cells are activated, exiting their dormant phase and rapidly re-entering the cell cycle to proliferate in response to external signals. This process is known as satellite cell activation[10]. MyoD is the earliest marker for activated satellite cells, and during the G1 phase, MyoD directly controls the expression of Cdc6, a gene that plays a role in making chromatin accessible for DNA replication, facilitating cell cycle entry[11]. Satellite cell progeny can be distinguished from their quiescent counterparts based on their distinct gene expression patterns[12,13]. In vitro studies have shown that the up-regulation of Myf5 marks the earliest phase of myogenic commitment, followed by concurrent expression of MyoD, which characterizes the majority of newly activated satellite cells. Subsequently, there is a down-regulation of Pax7[14]. However, if Pax7 expression remains elevated after proliferation, satellite cells do not undergo terminal differentiation and instead return to a quiescent state, promoting self-renewal and maintaining the pool of basal satellite cells[9,15]. Satellite cells reside in a specialized niche consisting of the extracellular matrix (ECM), vascular and neural networks and various surrounding cell types[16]. These niche components interact dynamically with satellite cells through cell-cell interactions and autocrine or paracrine signaling, regulating satellite cell quiescence, activation, self-renewal, proliferation, and differentiation[1]. Understanding these interactions is crucial for comprehending the mechanics of skeletal muscle diseases and developing a reservoir of transplantable cells to treat muscle dystrophies. A common in vitro approach used to study satellite cells involves isolating myofibers while keeping the satellite cells in their natural position underneath the myofiber basal lamina, essentially maintaining an "in situ" niche[17]. Such protocols aim to investigate satellite cell behavior during the early stages of activation and proliferation. Properly preparing the single myofiber and its culture is essential for isolating myofibers in good

condition for further processing. Hence, this study was conducted to optimize the preparation process for isolating single myofibers from the flexor digitorum brevis (FDB) muscle, resulting in better myofiber health and excellent immunofluorescent staining outcomes.

Material and Methods

Mice

In this study, all animal experiments were carried out at the cancer and cardiovascular research building, University of Minnesota medical school, Minneapolis, MN, USA. We followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee at the University of Minnesota, under protocol number 2009-38488A. The experimental procedures were conducted using four male mice of the C57BL/6 strain, aged between 3 to 4 months, which were obtained from Jackson Laboratories (strain code: 000664). Throughout the study, the mice were kept in a controlled environment with regulated temperature and lighting conditions. They were provided with unrestricted access to food and water. At the end of the experiments, the mice were humanely euthanized via cervical dislocation.

FDB dissection

Careful dissection of the FDB muscle is critical to prevent damage to the muscle (the muscle is surrounded by an area of dense connective tissue that should be removed carefully before cutting the muscle origin and insertion). First and in both sides, the toes were hanging by a silk thread, after visualizing the muscle the tendon of origin is sharply dissected at the heal, hung with a fine forceps, then with a small sassier under a magnifying lens the muscle was separated from the underlying connective tissue until the four tendons of insertion were noticed (figure 1). Then the four tendons were cut at once.

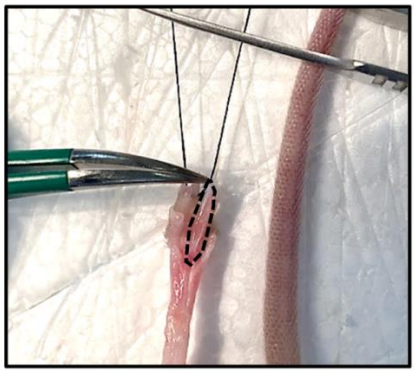

Figure 1: FDB muscle dissection

The toes were hung by a silk thread, the muscle-tendon was cut and held by fine forceps which holds the muscle to expose the tendinous insertion (outlined)

Myofiber isolation

After the flexor digitorum brevis (FDB) muscles were dissected, both muscles were placed in fresh culture media consisting of DMEM with glutamine, 2% sterile filtered Fetal Bovine Serum (FBS), and 0.1% penicillin-streptomycin. Additionally, 0.01 g of

collagenase A from Roche (product number: 11088793001) was added to the media. Each mouse's right FDB muscle was incubated for 2 hours, while the left FDB muscle was incubated for 3 hours at a temperature of 37 °C in an environment with 5% CO2, following previously established methods[18, 19]. After the incubation period, the FDB muscles were washed with 2 mL of culture media without collagenase. To release the fibers from the muscle bundles, the FDB muscles were gently triturated against the wall of a dish previously coated with horse serum, using the cut end of a P1000 pipette tip (figure 2). The isolated myofibers were collected and cultured adherently on glass bottom coverslips that were coated with laminin from Millipore (product number: #08110, ECL Cell attachment matrix). The culture medium used for the myofibers consisted of DMEM with glutamine, 20% sterile filtered FBS, 0.1% penicillin-streptomycin, and 0.1% GlutaMAX. The myofibers were then returned to a controlled environment at 37 °C with 5% CO2 for a period of 72 hours and subsequently processed for staining.

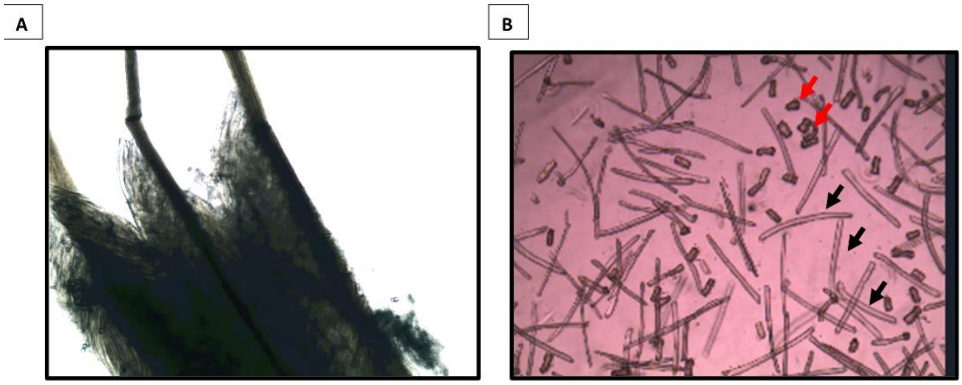

Figure 2: FDB myofiber isolation

A. Phase contrast image for the FDB muscle during the digestion process
B. Isolated myofibers after the trituration of the muscle in the Pasteur tips, the black arrows point at the viable and healthy myofibers while the red ones point at the contracted and broken fibers magnification was at 100X.

FDB myofiber immunostaining

For the immunostaining process, the FDB muscle fibers were first fixed with 4% paraformaldehyde (PFA) from Santa Cruz Biotechnology (product number: #sc-253236) in PBS for a duration of 10 minutes. Subsequently, the fibers were incubated in a blocking solution consisting of 3% bovine serum albumin (BSA) from Fisher Bioreagents (product number: #BP1605-100) and 0.3% Triton X-100 from Sigma (product number: #X100) for 30 minutes at room temperature. Next, the muscle fibers were subjected to immunostaining using a mouse anti-Pax7 antibody obtained from the Developmental Study Hybridoma Bank (DSHB) with catalog number PAX7 (RRID: AB_528428) from Iowa City. The Pax7 antibody was used at a dilution of 1:100. Additionally, a rabbit anti-MyoD antibody from Santacruz was utilized at a dilution of 1:500. The incubation with these primary antibodies was carried out overnight at a temperature of 4°C. On the following day, the coverslips containing the fibers were stained with goat anti-mouse Alexa 488-conjugated and goat anti-rabbit Alexa 555-conjugated secondary antibodies from Life

Technologies for a duration of 1 hour at room temperature. To detect nuclei, a DAPI counterstain from Life Technologies with product number D3571 was used. Finally, the coverslips were mounted using Immu-Mount from Thermo Scientific with catalog number 9990402 before imaging. The images were captured at 200X magnification using a Zeiss Axio Imager M1 upright microscope equipped with an AxioCam HRc camera and ZEN software from Zeiss[19].

Morphometric analysis

Collected images were analyzed by counting the number of Pax7+ nuclei "satellite cell clusters" and the number of MyoD+/Pax7+ in each myofiber, Analysis on 50 myofiber from each muscle in each digestion period was done.

Statistical analysis

Data analysis was conducted using Student two-tailed t-tests, and the results were presented as means along with standard errors. Statistical significance was determined at a p-value <0.05. GraphPad Prism 8.0 software from GraphPad Software Inc. in San Diego, CA, United States, was utilized to perform all statistical testing.

Results

Pax7 Immunostaining of the FDB myofiber after 72 hours in culture

Satellite cells that residue beneath the basal lamina of an intact myofibers retain their stemness for longer period compared to satellite cells harvested by enzymatic digestion[9], this was evident by the persistent Pax7 expression throughout the 72 hours period of culture, we found that the satellite cells and their progeny underneath the basal lamina still show a strong Pax7 expression after 72 hours in culture (Figure 3).

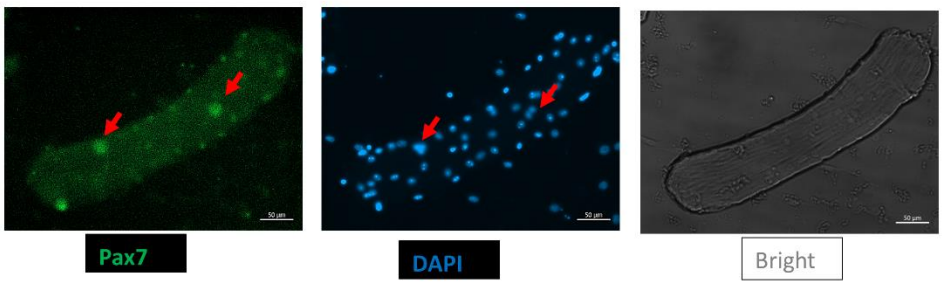

Figure 3: Pax7 staining of the FDB myofiber
Pax7+ nuclei of the satellite cells and their progeny (arrows)

Pax7 and MyoD co-staining of FDB myofibers

To study the activation and further proliferation of the satellite cells in ex vivo conditions, we stained the 72-hour cultures obtained from the 2 digestion periods (2 vs. 3 hours) with Pax7 and MyoD and we counted the number of MyoD+ nuclei among the Pax7+ ones. First, we found slightly and nonsignificant higher numbers of Pax7+ cells per clusters in the 2 hours incubated fibers when compared to the 3 hours which may highlight better digestion condition and healthier myofibers. However, the number of MyoD+/Pax7+ cells was quite similar when comparing the 2 extraction protocols about 100% (figure 4 A-C).

Discussion

This study showed the benefits of the FDB muscle as a potential tool for the assessment and investigation of ex vivo skeletal muscle stem cell biology. Our findings validated the FDB muscle single Myofiber isolation has inherent experimental advantages[17], our results agree with the previous study done by Tarpey et al in which they used the FDB muscle in various aspects of studying the skeletal muscle physiology in the context of injury, regeneration and contractility.

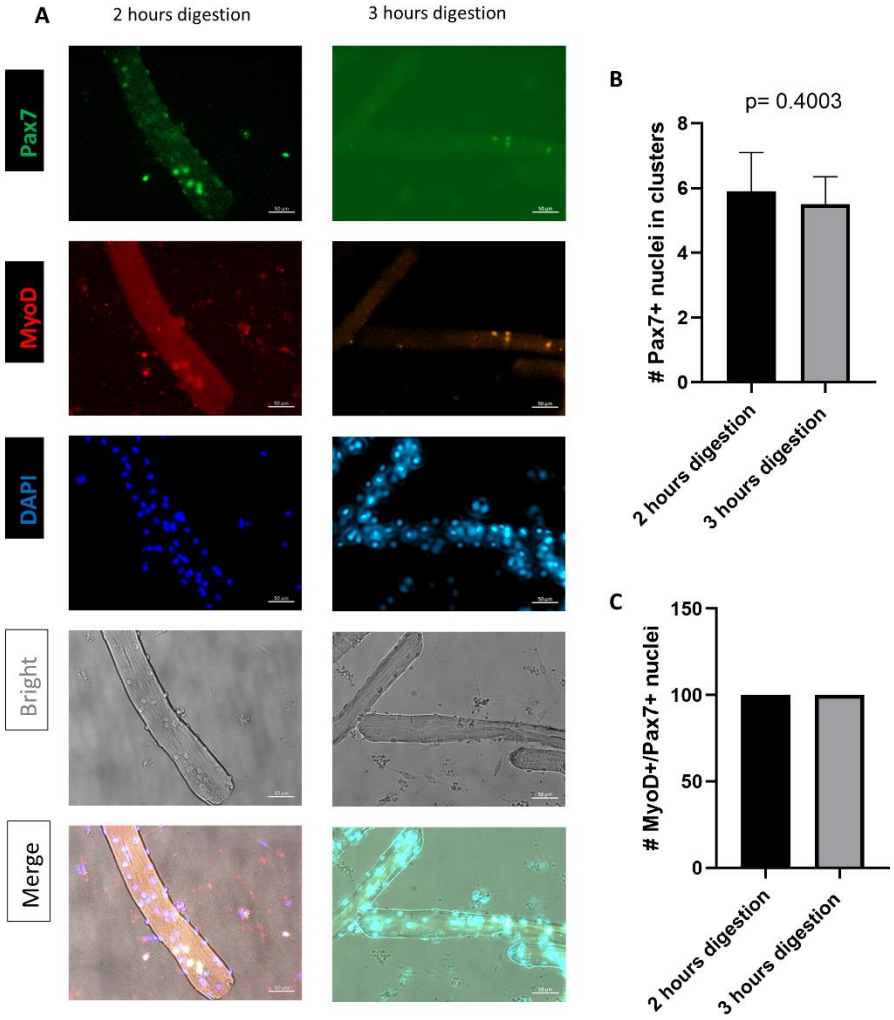

Figure 4: Pax7 and MyoD co-staining

A. Representative immunofluorescence-stained images for the FDB.
B. Analysis of the number of nuclei per cluster.
C. Analysis of the numbers of MyoD+ in the Pax7+ nuclei.

The accessible location of the FDB, directly under the skin, and its relatively small size facilitate its accurately dissect and isolate the muscle for further assessment compared to the other methods using the Extensor digitorum longus (EDL) as a

common method for studying the single myofiber ex vivo[19,20]. Having smaller and more numerous myofibers, the FDB muscle became the preference of the researchers investigating mechanisms underlying satellite cell activation and early proliferation[20-22], our results agree with multiple studies that used the isolated myofiber from both the FDB and EDL to study the homeostasis in the quiescent and later the early activation stages[19,20]. In this study, it was observed that satellite cells remained on the surface of the parent myofiber during their proliferation and differentiation processes. These satellite cells exhibited a limited number of proliferative cycles and underwent rapid differentiation without fusing with the parent myofiber. The location of satellite cells underneath the basal lamina of the intact myofiber was consistent with the findings of previous studies[19]. Furthermore, other studies have utilized live imaging of single cultured fibers to gain insights into the migration pattern of satellite cells (reference 24). This live imaging approach allowed researchers to observe and study how satellite cells move and interact during their activities[23]. In our study we provided the analysis of the satellite cells proliferation in the unperturbed basal lamina, we have seen that after 72 hours in culture the satellite cells performed around 2-3 cell divisions[15] with an average of 5-6 cells per cluster, we compared between 2 of the published protocols of the FDB single myofiber extraction and we found that with relatively shorter duration, the enzymatic digestion wasn't robust and yet produced more healthier myofibers suitable for further processing and staining[17,19]. The longer duration of digestion and hence the vigorous digestion process, may be causing minor breaks in the basal lamina and causing the loss of more fibers either by being broken or hypercontracted which affect the purpose of single myofiber studies in which the integrity of the single myofibers is fundamental to study the satellite cells. In the ex vivo culture setting, satellite cells exhibit a retention of their stemness and remain in their quiescent state underneath the basal lamina for the initial 24-hour period[24]. This suggests that the culture conditions maintain the satellite cells in a dormant and undifferentiated state during this early phase. The myofiber culture system has proven to be a valuable tool for studying the behavior of satellite cells in various contexts. It has provided significant insights into the biology of the satellite cell population, allowing researchers to explore both intrinsic factors (properties inherent to the satellite cells themselves) and extrinsic factors (influences from the surrounding environment). By culturing individual myofibers, researchers have been able to investigate satellite cell heterogeneity concerning factors such as myofiber type, muscle type, or regenerative potential. This approach allows for a detailed examination of how satellite cells behave differently based on specific features of the muscle fibers they interact with, shedding light on the diverse characteristics and functions of satellite cells in muscle regeneration and maintenance[25,26]. Orchestrated by single Pax7 gene expression, later they begin to upregulate other myogenic regulatory factors (MRFs) particularly the MyoD which in turn regulate the progression of the cell cycle from the quiescence to the actively proliferating state, by the third day in culture we found that almost all the satellite cells irrespective to the preparation protocol begin to take the committed path and

upregulate the MyoD to become fully active and ready for subsequent cascades of cell proliferations and yet differentiation to rescue the damaged skeletal muscle, our results agree with the previous studies which stated the MyoD expression was upregulated later in the satellite cells of the single myofiber compared to the satellite cells harvested from the muscle digest[10,25].

Conclusion

The preparation and culture of FDB muscles in vitro can serve as a valuable model to study the behavior of satellite cells within intact muscle fibers during growth and routine muscle processing. By employing this in vitro approach, researchers can mimic and observe satellite cell behavior under controlled conditions, providing valuable insights into their activation and cell cycle entry processes. To effectively study satellite cell activation and cell cycle entry, it is recommended to maintain FDB muscles for short-term digestion periods. This ensures that the muscle retains its structural integrity and allows for the examination of satellite cell responses to specific stimuli or conditions. Short-term digestion periods are particularly useful in capturing the initial phases of satellite cell activation and proliferation, providing important information about the early events in muscle regeneration.

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