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Single myofiber isolation and culture from a murine model of Emery-Dreifuss muscular dystrophy in early post-natal development.

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Editorial comments:

During this time, we have a change in policy. Once the manuscript is accepted, it is being published without the video. Hence publication without full figures is not acceptable. I am returning your submission to you for holding on to it. Once you have everything in place, you can send this back to me and we can work on acceptance of this submission.

We completed our revision adding the lacking pictures.

We also added a sentence highlighted in red in the ACKNOWLEDGMENTS section of the last version of the manuscript (61516_R1_RE, already edited for the editorial requirement) to thank a person that help us with the images.

TITLE:

Single Myofiber Isolation and Culture from a Murine Model of Emery-Dreifuss Muscular Dystrophy in Early Post-Natal Development

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KEYWORDS:

Emery-Dreifuss muscular dystrophy, mouse model, myofiber, immunofluorescence, satellite muscle stem cells, differentiation, muscle regeneration

SUMMARY:

Here, we propose a method to efficiently obtain single muscle fibers at early post-natal developmental stages from homozygous mutant *Lamin Δ8-11* mouse model, a very severe model for Emery-Dreifuss muscular dystrophy (EDMD).

ABSTRACT:

Emery-Dreifuss muscular dystrophy (EDMD) is caused by mutations in the *LMNA* gene, which encodes the A-type nuclear lamins, intermediate filament proteins that sustain the nuclear envelope and the components of the nucleoplasm. We recently reported that muscle wasting in EDMD can be ascribed to intrinsic epigenetic dysfunctions affecting muscle (satellite) stem cells regenerative capacity. Isolation and culture of single myofibers is one of the most physiological ex-vivo approaches to monitor satellite cells behavior within their niche, as they remain between the basal lamina surrounding the fiber and the sarcolemma. Therefore, it represents an invaluable experimental paradigm to study satellite cells from a variety of murine models. Here, we describe a re-adapted method to isolate intact and viable single myofibers from post-natal hindlimb muscles (*Tibialis Anterior, Extensor Digitorum Longus, Gastrocnemius* and *Soleus*). Following this protocol, we were able to study satellite cells from *Lamin Δ8-11* -/- mice, a severe EDMD murine model, at only 19 days after birth.

We detail the isolation procedure, as well as the culture conditions for obtaining a good amount of myofibers and their associated satellite-cells-derived progeny. When cultured in growth-factors rich medium, satellite cells derived from wild type mice activate, proliferate, and eventually differentiate or undergo self-renewal. In homozygous *Lamin* Δ8-11 -/- mutant mice these capabilities are severely impaired.

This technique, if strictly followed, allows to study all processes linked to the myofiber-associated satellite cell even in early post-natal developmental stages and in fragile muscles.

INTRODUCTION:

Skeletal muscle is a differentiated tissue with one of the most extended ability to regenerate after exercise or trauma[1]. This characteristics is mainly observed due to the presence of stem cells, called satellite cells because of their peripheral position between the basal lamina and the plasmalemma of the myofiber[2]. During post-natal development, satellite cells proliferate and progressively differentiate, thus contributing to skeletal muscle growth. Once in the adulthood, satellite cells enter a reversible quiescent state, and upon physiological or pathological trauma, they activate, proliferate and differentiate in order to repair the damaged muscles[3]. Defects in the capacity of satellite cells to properly transit through these different regenerative phases and to undergo self-renewal have been firmly linked to muscle wasting, either during physiological aging[4–6] or in muscle degenerative diseases, such as muscular dystrophies[7–10].

Two main culture approaches exist to study satellite cells ex vivo: primary myogenic cultures from mononucleated cells, mechanically and chemically dissociated from whole muscle[11,12]; or culture of isolated myofibers[13–20]. In the first case, the process of satellite cells isolation involves the trituration of whole muscles extracted from the mouse, a chemical digestion, filtration and fluorescent activated cell sorting (FACS)[21]. This procedure, although effective in isolating satellite cells from a variety of models, entails several variables that expose satellite cells to stress and disrupts their physiological niche[22,23]. By contrast, myofiber isolation involves a gentler digestion of muscle tissue with matrix degrading enzymes and a mechanical shredding that causes reduced trauma to stem cells[20]. This second approach allows a much more efficient retrieval of viable satellite cells, that remain physically attached to their myofiber between the basal lamina and the sarcolemma, thus allowing analysis within their physiological niche[19,20].

Many different protocols have been proposed during the past years to properly and efficiently isolate single myofibers from skeletal muscles. Already in 1986 Bischoff proposed a protocol to isolate fibers from the *Flexor Digitorum Brevis*[13] and later, in 1995, Rosenblatt et al. modified the protocol to obtain a more efficient separation of myofibers[14]. Since then, many other authors proposed adjusted procedures on other muscles, such as *Extensor Digitorum Longus* (EDL) and *Tibialis Anterior* (TA)[15–20], that are longer, even if more fragile, muscles[14]. Isolated myofibers can then be cultivated both in adhesion, to allow for the expansion of satellite cells-derived myoblasts, or in floating conditions, until 96 hours, to follow the progeny derived from single satellite cells[19] (**Figure 1**). Variable concentrations of serum within the culture medium are used to trigger satellite cells activation, proliferation and/or differentiation, to study the capacity of these cells to properly transit through these different phases[1].

We recently described the epigenetic mechanism behind the exhaustion of the satellite stem cell pool in the mouse model of EDMD, the *Lamin* Δ8-11 -/- mouse[7]. Since these mice usually die between 4-8 weeks of age[24], due to severe muscle loss, an attempt was made to capture the molecular defects underlying the early onset of the disease by focusing our analysis on post-natal muscle development. Floating single myofibers were isolated and cultured from wild type and *Lamin Δ8-11* -/- mutant[7] 19 days-old mice. At this stage, muscle defects are already evident, but mice are still viable. However, since all the above-mentioned protocols for single myofibers extraction were optimized for skeletal muscles of adult mice, we needed to adapt them to our purposes: very small mice in term of age and size, and very fragile myofibers. Thus, we describe here our re-adaptation of the protocol proposed by the Rudnicki laboratory[19] to obtain a significant number of single viable myofibers from mice during post-natal development and from severe dystrophic muscles, such as those derived from *Lamin Δ8-11* -/- micel[24]. The final goal of this approach is to provide a standardized procedure to allow for the study of myofibers-associated muscle stem cells in any other mouse model when the early stages of post-natal development are of interest, or in the case of mouse models carrying any specific disease that makes myofibers more susceptible to mechanical stress.

PROTOCOL:

All the experimental procedures were performed under the ethical approval of the Italian Ministry of Health and the Institutional Animal Care and Use Committee (authorization n. 83/2019-PR). The animals were maintained in an authorized facility at San Raffaele Hospital, Milan, Italy (authorization n. N. 127/2012-A).

1. Muscle dissection and myofiber culture

1.1. Equipment preparation.

1.1.1. Before starting, prepare all the necessary solutions as described in **Table 1**. These solutions need to be freshly prepared.

1.1.2. Clean all the surfaces and tools with 70% ethanol that will be used during the procedure.

1.1.3. Before starting with mice sacrifice, perform coating of 100 mm and 35 mm Petri dishes using Horse serum (HS). Coat all dishes to prevent myofibers from attaching to the plastic. Consider using one 100 mm dish and four 35 mm dishes per mouse.

1.1.4. After removing the excess of HS, store coated dishes in an incubator at 37 °C for 30 min. Then fill it with washing solution or culture medium (two 35 mm dishes per mouse).

NOTE: Alternatively, a solution of 10% HS in Dulbecco's modified Eagle's medium (DMEM) can be used to coat dishes. Always use coated dishes. It is possible to use culture dishes of different size, but small dimension Petri dishes are recommended.

1.1.5. For fibers isolation, prepare sterile Pasteur pipettes as shown in **Figure 2**. For every different mouse prepare one large hole bore pipette for muscle handling and mechanical disaggregation (**Figure 2A**) and one small hole pipette for fibers selection (**Figure 2B**). Cut each glass pipette, possibly using a diamond pen, to the desired length and smooth pipette's edges on a flame.

1.1.6. Coat each pipette by briefly wetting it with HS before use.

1.2. Mouse sacrifice and muscle dissection

1.2.1. Pre-warm the digestion solution at room temperature for 10 min before starting the dissection procedure. Polypropylene FACS round-bottom tubes are the most suitable containers for the purpose.

1.2.2. Immediately before the beginning of muscle retrieval, sacrifice the mouse by CO_2 asphyxiation or according to proper national IACUC recommendation.

1.2.3. Wet its lower body with 70% ethanol before cutting the skin to make the removal of the hair easier.

1.2.4. Put the mouse in a prone position on a support of polystyrene covered with aluminum paper and cut the skin starting from the middle of the back longitudinally and in the direction of the legs.

1.2.5. Carefully remove the skin without touching the muscle and tendons. It is possible to rip off all the skin.

1.2.6. Cut the two legs of the mouse and rapidly proceed with the dissection.

NOTE: If it is more comfortable, it is possible to continue the dissection on the entire mouse but working on the leg allows more mobility and precision in the later cuts.

1.2.7. Fix the leg on the support at the level of the foot using a pin and start isolating skeletal muscles of interest in this order: TA, EDL, *Gastrocnemius* and *Soleus*.

1.2.8. Lift the lower tendon of TA with a sharp tweezer at ankle height and cut it, then cut with fine scissor all around the TA muscle to the other tendon at the level of the patella (**Figure 3A**). Transfer into the digestion solution.

1.2.9. Lift the lower tendon of EDL and separate it from other muscle by gently pulling it upward up to the other tendon. Cut and place it into the digestion solution.

NOTE: Since EDL may be extremely small to be cut separately from TA, they can be dissected together (**Figure 3B**). Then, if the entire muscle is too big, cut it in 2-3 pieces starting from the tendon and following the fibers in a longitudinal direction (**Figure 3C**).

1.2.10. Rotate the leg showing the back muscles and fix the foot using the pin. Lift the Achille's tendon, *Gastrocnemius* will automatically separate from other muscles. The upper tendon is up in the back of the patella. Cut it and add the muscle to digestion.

1.2.11. Lift the external tendon of the leg (with respect to the body) and obtain the *Soleus.* Gently separate it from the other muscles by scrolling under with the tweezer.

1.2.12. Do the same for the other leg (steps from 1.2.6. to 1.2.10.).

1.3. Muscle digestion

1.3.1. Incubate the digestion solution containing all the muscles in a water bath at 37 °C for about 45-50 min. During the digestion time, regularly check the muscle to avoid over-digestion. Every 10 min invert the digestion tubes 10x with an energetic movement (avoid vortexing).

1.3.2. Stop the digestion when muscles start to loosen up and myofibers are visible.

1.3.3. At the very end of the digestion time shake the samples.

1.3.4. To stop the digestion, carefully transfer the digestion suspension to a pre-warmed 100 mm Petri dish with 10 mL of washing solution.

NOTE: Avoid muscle over-digestion as this will inevitably result in the isolation of hyper contracted myofibers. Usually with this protocol, muscles of homozygous mutant mice take 45 min to be digested, while muscles of wild type mice take 50-55 min. Digestion time needs to be experimentally validated.

1.4. Single myofibers isolation

1.4.1. First isolate the fibers already dissociated under a dissecting microscope by picking them individually with a coated P200 pipette or small hole Pasteur and transfer them from the 100 mm Petri into a new 35 mm Petri dish with 5 mL of pre-warmed washing solution.

1.4.2. To release further myofibers, pipette the muscle up and down using a large hole bore glass pipette with warm medium, until fibers are mechanically released. Do not be too persistent as this will result in damaging fibers.

1.4.3. Continue releasing myofibers from the muscle until the dish contains a desirable amount. If the Petri dish is kept at room temperature for more than 8 min, stop and perform a minimum of 5 min incubation at 37 °C, 5% CO_2 to re-equilibrate the medium.

1.4.4. Before transferring the single myofibers to the culture medium, leave them at 37 °C, 5% CO_2 in wash dish for at least 1 h. This helps myofibers to adjust to the in vitro condition.

NOTE: Adult myofibers are less susceptible to stress and can be washed ~ 2-3x. However, in this condition (at 19 days post-natal age) it is better to perform only one washing step to prevent myofiber damage. Therefore, always pay attention to keep selected myofibers sufficiently clean by not carrying debris or hypercontracted fibers.

1.5. Single myofiber culture

1.5.1. Transfer individual myofibers to a new prewarmed dish with the appropriate culture medium (high serum medium to allow satellite cells activation, see **Figure 1**).

1.5.2. Change the medium to the isolated myofibers, by transferring them to a new coated dish with new culture medium, only after 48-72 h of culture to avoid any stress that will lead to myofibers hypercontraction and disruption.

2. Downstream applications: Myofibers crosslinking and immunofluorescence

NOTE: The myofibers-associated satellite cells can be visualized by immunofluorescence (IF) at the time of interest. Since most of the published protocols are optimized to perform IF on adult myofibers, here a detailed protocol is presented to obtain reliable results also on myofibers isolated from post-natal muscles.

2.1. Myofiber crosslinking

2.1.1. Before start, prepare all the necessary solutions as described in **Table 2**.

2.1.2. Precoat with HS as many 1.5 mL microcentrifuge tubes as the number of samples. Be sure to remove all HS before proceeding. Since crosslinked fibers are tougher than the living ones and more difficult to pipette, be sure to crosslink about 200-300 fibers separately per tube.

2.1.3. Under a dissecting microscope, collect all the fibers that can be considered healthy, transfer them to the microcentrifuge tube and leave the tube vertical for 5 min inside the incubator to allow fibers to settle.

2.1.4. Remove the supernatant very slowly from the tube.

2.1.5. Crosslink fibers by adding 1 mL of 4% paraformaldehyde (PFA) at RT to the tube. Do it gently to avoid fibers distress.

2.1.6. To prevent fibers interweaving during the crosslinking, keep the tube in very gentle agitation for 10 min.

2.1.7. Keep the tube in a vertical position for 5 min at RT to allow fibers to settle, then discard the supernatant ensuring to remove the majority of PFA volume.

2.1.8. Add 1 mL of phosphate buffered saline (PBS) and keep the tubes vertical for 5 min at RT to let the fibers to sediment.

2.1.9. Remove the supernatant and repeat the washing procedure (step 2.1.7.) twice again.

2.1.10. Keep crosslinked samples at 4 °C.

NOTE: It is possible to keep crosslinked myofibers at 4 °C for a week. If the fibers remain more than a week in this condition, this will inevitably result in myofibers intertwinement.

2.2. Immunofluorescence

2.2.1. Keep the tubes containing the fibers standing at RT for at least 5 min to allow for fiber sedimentation.

2.2.2. Remove the supernatant, leaving just a small volume to be sure not to remove any fiber.

2.2.3. Add 1 mL of 0.5% Triton X-100 in PBS and incubate for 5 min with gentle agitation.

2.2.4. Put tubes in vertical position for 5 min, then remove supernatant.

2.2.5. Add 1.5 mL of PBS and incubate with gentle agitation for 5 min.

2.2.6. Keep tubes in vertical position for 5 min, then remove the supernatant.

2.2.7. Add 1 mL of blocking solution and incubate for 1 h at RT with gentle agitation.

2.2.8. Keep tubes 5 min in vertical position, then remove the supernatant.

2.2.9. Dilute primary antibodies in blocking solution and incubate over-night at 4 °C in gentle agitation (for suggested concentrations see **Table of Materials**).

NOTE: Alternatively, the primary antibody can be incubated for 3 h at RT. However, overnight incubation gave optimal staining. The incubation volume for both primary and secondary antibodies should be of 300 µL when sedimented fibers reach the 100 µL notch of the 1.5 mL microcentrifuge tube. When fibers are less abundant, 100-200 µL of the solution is recommended.

2.2.10. Leave the tubes in vertical position for 5 min and then remove the supernatant.

2.2.11. Perform 3 washes in 1 mL of 0.25% Tween-20 in PBS, incubating for 5 min in gentle agitation and then leaving the tubes standing for 5 min each time.

NOTE: From here on, perform all the steps in the dark, to avoid fluorochromes bleaching.

2.2.12. Dilute secondary antibodies in blocking solution and incubate for 1 h at RT with gentle agitation (for concentrations see **Table of Materials**).

2.2.13. Wash twice in 1 mL of 0.1% Tween-20 in PBS, incubating for 5 min with gentle agitation and then leaving the tubes in standing position for 5 min.

2.2.14. Remove the supernatant, add 1 mL of DAPI solution and incubate for 5 min with gentle agitation.

2.2.15. Let the tubes stand vertically in a rack for 5 min, then remove the supernatant.

2.2.16. Wash with 1 mL of PBS, incubating for 5 min with gentle agitation and then leaving the tubes stand in a rack for 5 min.

2.2.17. Remove the supernatant, leaving a volume of about 50 µL.

2.3. Mounting of fluorescently labeled myofibers on microscope slides

2.3.1. Cut a P200 pipette tip and coat it with blocking solution

NOTE: It is enough to pipette the solution up and down several times before picking the fibers, this will avoid fibers to stick to the tip wall.

2.3.2. After immunostaining, collect the fibers from the tube and spread them on a microscope glass slide.

2.3.3. Under a dissecting microscope, using only the natural light reflected by the mirror, use a new (not cut) P200 pipette tip to spread the fibers and to remove the excess liquid solution.

2.3.4. Leave the slides to air dry in the dark for about 10-15 min, until very low amount of solution remains.

2.3.5. Add the mounting medium on the slide (the proper amount of mounting medium should be calibrated on the dimension of the coverslip: for a 24 x 40 mm coverslip, 20 µL is enough) and then slowly lay a cover glass on the area containing the fibers. Be careful not to create bubbles between the glasses.

2.3.6. Press the coverslip so that the fibers will lay on a single horizontal plan.

2.3.7. Fix the coverslip using nail polish.

2.3.8. Store the samples at 4 °C for up to 4 weeks.

2.3.9. Acquire the desired images of fluorescently labeled myofibers with a confocal microscope.

REPRESENTATIVE RESULTS:

We typically digest four different muscles (TA, EDL, *Soleus* and *Gastrocnemius*) to retrieve a good amount of long and viable fibers that could survive 96 h in growth-factors rich medium (**Figure 4A,B**). Only the most intact fibers will be transferred in culture medium, as they will survive; all the others, that are easy to discriminate and select, need to be discarded.

When myofibers are maintained in a growth-factors rich medium satellite cells derived from wild type mice start to activate and proliferate, see **Figure 1**. Upon 48 h of culture, in healthy condition (*Lamin Δ8-11* +/+), satellite cells upregulate MyoD and undergo their first division. Activated Pax7+/MyoD+ satellite cells then proliferate and by 72 h in culture, they generate cell aggregates bound to the myofiber, even more visible at 96 h (**Figure 5**). During these divisions, some of them can repress MyoD expression, undergoing self-renewal to repopulate the stem cells pool, while those that maintain MyoD become committed to differentiation by downregulating Pax7 expression. After 96 h, this culminates with satellite cells clusters in which MyoG+ committed cells become clearly visible, enabling the cells to differentiate into new myofibers (**Figure 1** and **Figure 6**). Notably, with this experiment, we described a delayed dynamics of satellite cell differentiation in homozygous mutant *Lamin Δ8-11* mice (-/-) as compared to their wild type counterparts (+/+), see **Figure 6**.

The final outcome of each single experiment let us think that the protocol developed for single myofiber isolation and culture from this model of severe muscle dystrophy is well developed and ensure good quality myofibers for all the further applications.

FIGURE AND TABLE LEGENDS:

Figure 1: Graphical representation of satellite cells' regenerative phases modelled in floating myofibers. Upon 48 h of culture in growth-factors rich medium, Pax7+ cells get activated and undergo the first division, giving rise to a doublet of Pax7+/MyoD+ cells. MyoD positive cells then proliferate and expand, giving rise, in 72 hof culture, to a cluster of several cells which are the progeny of a single satellite cell. Upon 96 h of culture Pax7+/MyoD+ cells become differentiating Pax7-/MyoG+ cells. During the expansion phase, a subset of Pax7+/MyoD+ cells downregulate MyoD expression undergoing self-renewal into quiescence.

Figure 2: Preparation of bore Pasteur pipettes. (**A**) Longitudinal and frontal view of how the big hole bore pipette must appear. (**B**) Longitudinal and frontal view of how the small hole bore pipette must finally appear.

Figure 3: Representative pictures of single muscle dissection. (**A**) Isolation of the TA muscle. Avoiding the removal of the thin layer covering the muscle protected the myofibers inside. (**B**) TA and EDL muscles isolated together still attached to their upper tendon at the level of the patella. (**C**) Division of TA and EDL after isolation by cutting them along the longitudinal axis.

Figure 4: Examples *o*f healthy and viable myofibers. Representative phase contrast images of viable myofiber in suspension. (**A**) The red arrow indicates a myofiber with visible sarcomere organization and a satellite cell on its side; the orange arrows indicate some pieces of broken myofibers, and some debris present in the first dish before the final selection for culture. Scale bar 100 µm. (**B**) More complete view of other myofibers under a smaller magnification. Scale bar 500 µm.

Figure 5: Differences in the dimension of stem cell clusters in wt and mutant mice. Immunofluorescence staining of myofibers extracted from 19 days *Lamin Δ8-11* mice (+/+ and -/-) after 96 h of culture. Pax7+ satellite cells were observed. The dimension of the cell cluster in most of the cases, was significantly bigger in *Lamin Δ8-11 +/+* than in *Lamin Δ8-11 -/-*, both in terms of spatial occupancy and in the number of cells present. Scale bar 10 µm.

Figure 6: Representative immunofluorescence experiment. Immunofluorescence experiment performed, after 96 h of culture, on myofibers extracted from 19 days *Lamin Δ8-11* mice (+/+ and -/-). Pax7+/MyoG+, Pax7+/MyoG- and Pax7-/MyoG+ cells were observed. Images obtained with a confocal microscope. Scale bar 25 µm.

Table 1: Recipes for solutions used in section 1.

Table 2: Recipes for solutions used in section 2.

DISCUSSION:

Isolation of intact single myofibers is an essential method in the field of myogenesis when the main objective is to characterize cell-autonomous regenerative capacities of muscle stem cells within their niche, in healthy and pathological conditions. However, whenever more satellite cells are needed to carry out, for example, biochemical or genomic studies, FACS-isolated satellite cells might be the best option to collect a sufficient amount of material.

Single myofibers isolation allows to follow ex-vivo, but in the most physiological way, the dynamics of all the steps single satellite cells undergo during muscle regeneration, that are: activation, cell division (asymmetric and symmetric), proliferation, differentiation and return to quiescence by self-renewal. Once myofibers are grown in floating conditions, the single satellite cells activate and expand forming a cluster of cells, all deriving from the same satellite cell. Immunofluorescence analysis for proliferation, differentiation, activation or stemness markers is then optimal to quantify the proportion between proliferating, differentiating or self-renewing satellite cells.

The most critical step in our protocol, is the key to obtaining viable and intact myofibers. Use 19

± 2 days-old mice for a rapid but gentle muscle dissection, tendon-to-tendon isolation to avoid any muscle damage. Our advice is to use only sharp scissors and small sharp tweezers and to limit the entire muscle dissection procedure to ten minutes. When it is difficult to isolate very small muscles (i.e., EDL and TA), it is possible to cut them together and to later divide them by using fine scissors cutting along the longitudinal plan following the fibers. This strategy will eventually give less intact myofibers, but viability will not be compromised. The same must be performed on big muscles like *Gastrocnemius* to facilitate digestion. Optimization of digestion time, which needs to be empirically validated, and minimal manipulation of isolated fibers are also two crucial aspects for the positive outcome of subsequent analysis.

The advantage of the protocol reported here is that it can be applied on very small mice (in age and dimension), even when their muscles are extremely fragile. Even if not mentioned above, it is possible to follow this protocol of dissection to then culture viable myofibers for longer period using basement membrane-coated dishes[18,19]. It is important to consider that this situation is completely different from floating condition, where adhesion stimuli and proximity stimuli are absent.

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DISCLOSURES:

No competing interests.

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Figure 1

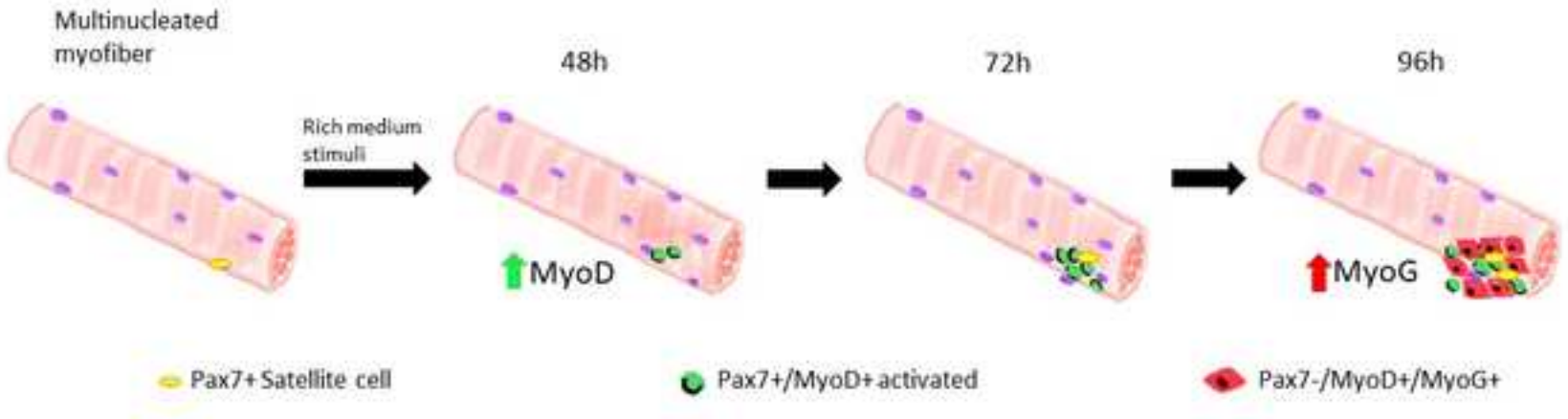

Figure 2

Click here to access/download;Figure;figure 2 JoVE.jpg

Figure 3

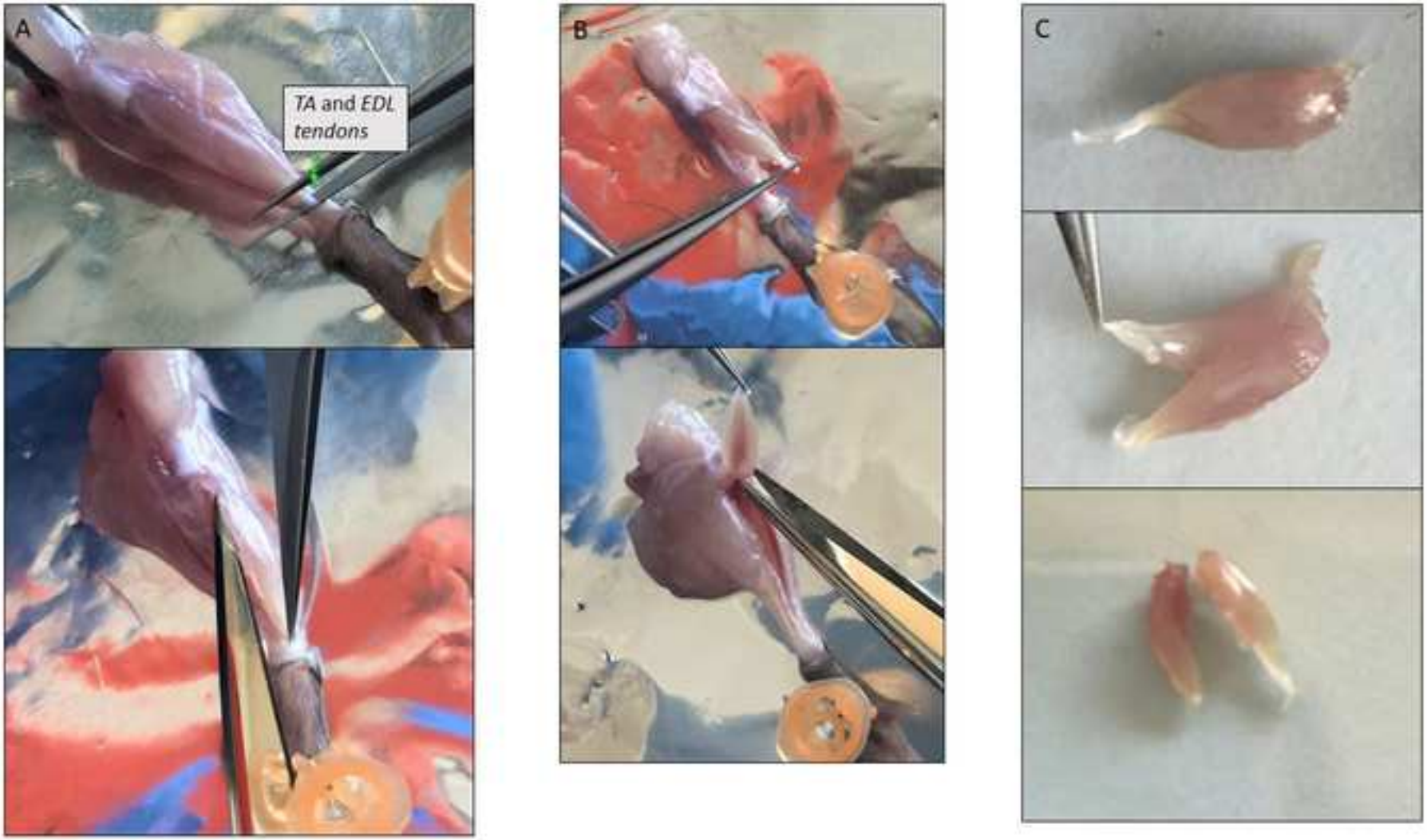

Figure 4

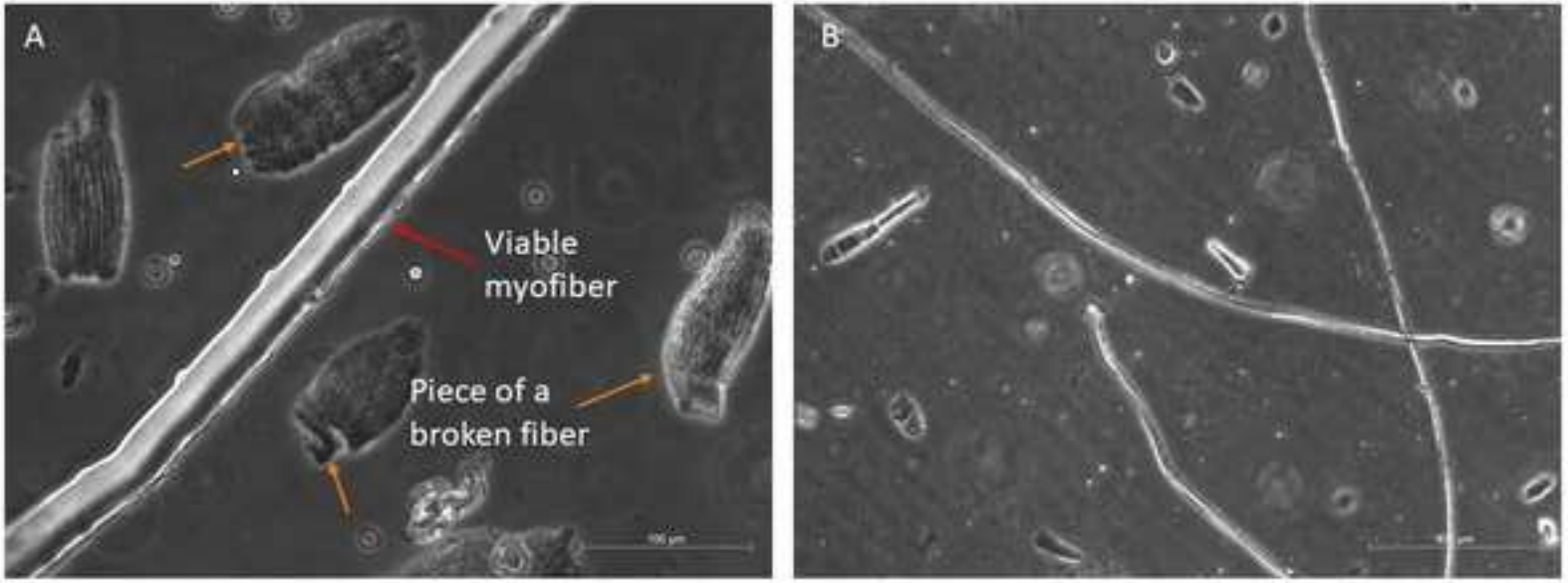

Figure 5

Click here to access/download;Figure;figure 5R JOVE.jpg

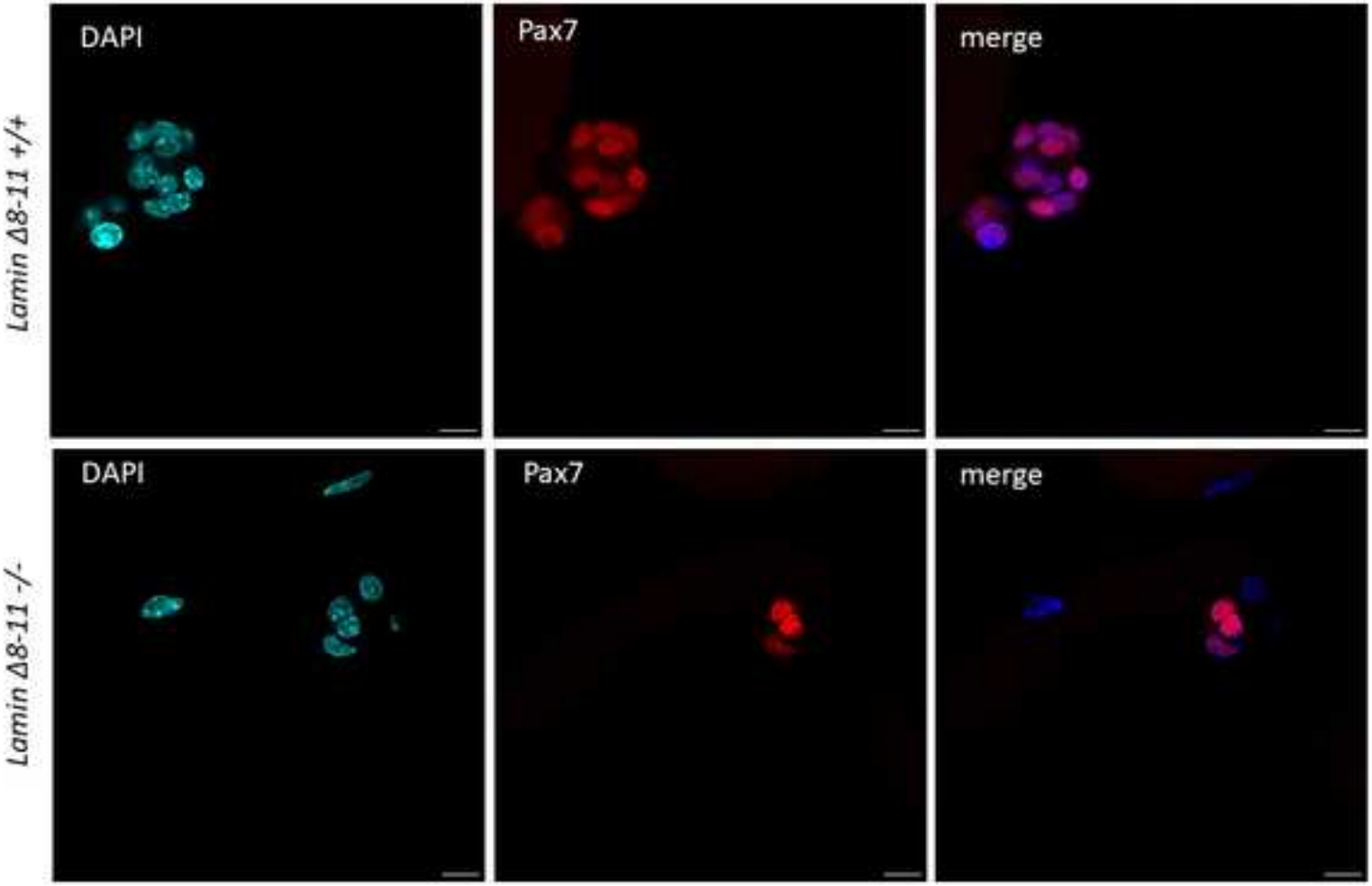

Figure 6

Click here to access/download;Figure;figure 6R JOVE.jpg

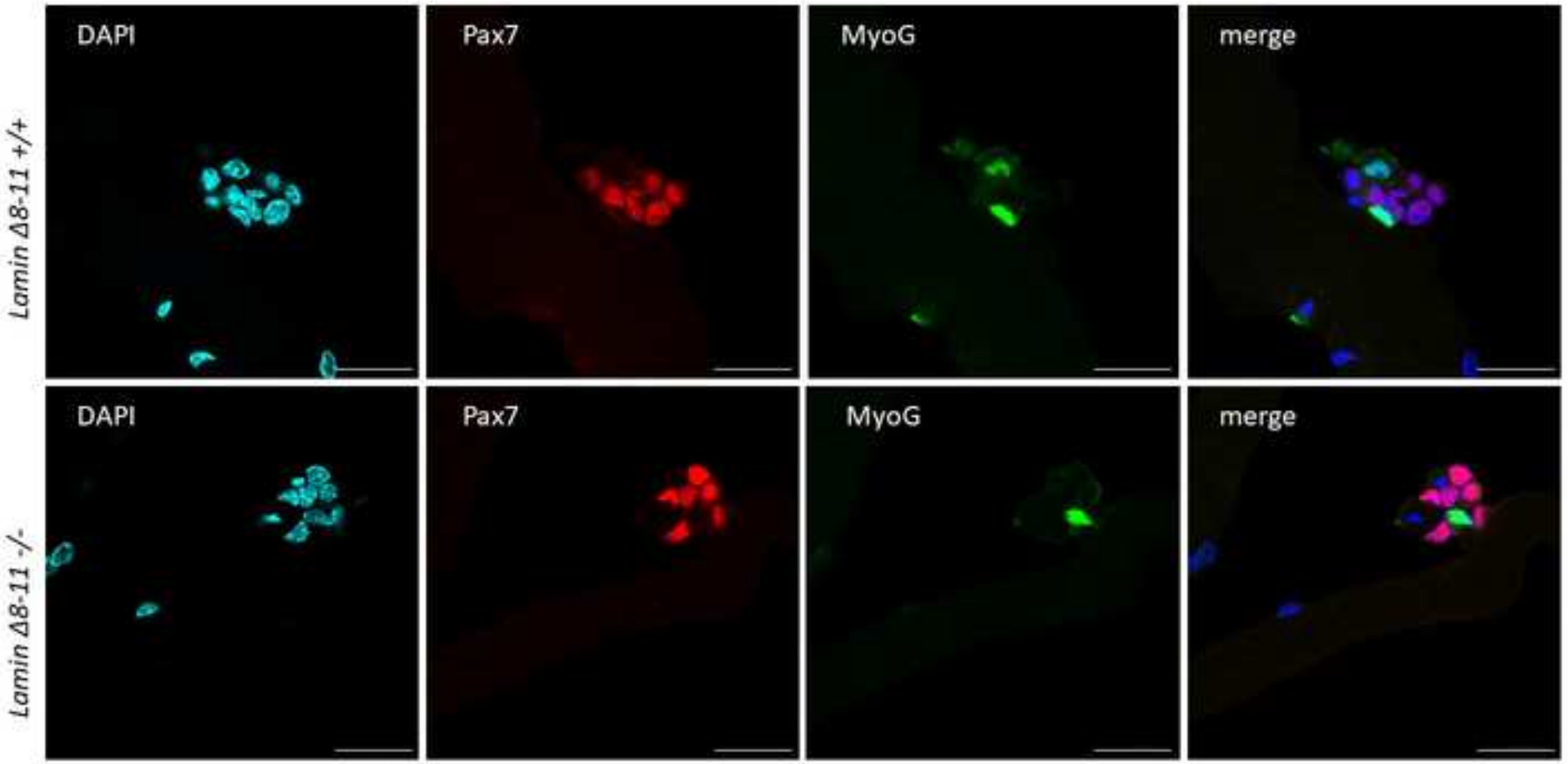

Table 1

Name of the solution in the text	component	percentage
washing solution	DMEM high glucose	90%
	Horse serum (HS)	10%
digestion solution	DMEM high glucose	9.80%
	Collagenase I	0.20%
culture medium	DMEM high glucose	78%
	Fetal bovine serum (FBS)	20%
	Chicken embryo extract (CE	1%
	Penicillin-Streptomycin (P/S	1%

suggested final volume/sample	notes
4 mL	Keep sterile, keep at 4°C until usage
20 mL	Extremely harmful powder. Filter solution with 0.22µm filter and then
10 mL	Keep sterile, keep at 4°C until usage

Table 2

Name of the solution in the text	component	percentage	suggested final volume/sample
4% PFA	Paraformaldehyde	4%	2-3 mL
	PBS	96%	
0.5% Triton X-100	Triton X-100	0.50%	2-3 mL
	PBS	99.50%	
0.25% Tween-20	Tween-20	0.25%	10 mL
	PBS	99.75%	
0.1% Tween-20	Tween-20	0.10%	10 mL
	PBS	99.90%	
blocking solution	Fetal bovine serum	10%	10 mL
	PBS	90%	
DAPI	DAPI	0.10%	2-3 mL
	PBS	99.90%	

notes
Powder and then solution are extremely harmful
Triton X-100 is extremely viscous, preferentially cut the tip of the pipette to aliquot it
Tween-20 is extremely viscous, preferentially cut the tip of the pipette to aliquot it
Tween-20 is extremely viscous, preferentially cut the tip of the pipette to aliquot it
Prepare fresh solution and store at 4°C for no longer than 3 weeks (always check clearness before usage)
Keep in the dark

Reagents	Manufacturer
4′,6-diamidino-2-phenylindole	Sigma
Chicken embryo extract	Seralab
Collagenase type I	SIGMA
Donkey anti-Rabbit 488 antibody	Thermofisher
Dulbecco's modified Eagle's medium	Gibco
Fetal bovine serum	Corning
Horse serum	Gibco
MyoG antibody	Millipore
Paraformaldehyde	SIGMA
Pax7 antibody	Developmental studies Hybridoma bank
Penicillin and Streptomycin	Euroclone
Phosphate saline buffer	Euroclone
Prolong gold antifade mountant	Thermofisher
Triton X-100	SIGMA
Tween-20	SIGMA

Lab equipment	Manufacturer
Bunsen burner	
Confocal microscope	Leica
Diamond pen	bio-optica
Dissection pins	
FACS polypropylene tubes	Falcon
Falcon tubes (50 and 15 mL)	Falcon
Glass coverslips	Thermofisher
Glass Pasteur pipettes (22cm)	VWR
Glass slides	Thermofisher
Micro dissecting scissors	
Micropipette (1 mL and 200 µL)	Gilson
Micropipette tips	Corning
Petri dishes (100 and 35 mm diameter)	Thermofisher
Plastic pipettes (5 and 10 mL)	VWR
Rubber pipette bulbs	VWR
Sharp tweezers	
Stereo dissection microscope with transı	Leica
Tissue culture hood or lamina flow cabinet	
Tissue culture incubator (humidified, 37°C, 5% CO2)	
Water bath at 37°C	VWR

Catalog Number	Comments
D9542	
CE650-DL	
C0130-500MG	
R37118	to be used 1:200
10569-010	
35-015-CV	
26050-088	
219998	to be used 1:100
P6148	
	to be used 1:20
ECB3001D	
ECB4004L	
P36930	
T8787	
P1379	

We would like to thank very much all the reviewers for the precise and useful comments and for the general appreciation of our manuscript. Please find below a point-by-point rebuttal for the specific comments and raised concerns.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In "Single myofiber isolation and culture from a murine model of Emery-Dreifuss muscular dystrophy in early post-natal development", Pegoli et al present a refinement of methods for isolating and culturing adult myofibers that has been adapted for early post-natal developmental stages. This is valuable to the research community not just for studying earlier developmental stages, but because the conditions used to isolate adult myofibers are too harsh for muscle that has been damaged or weaker such as in models of muscular dystrophies. They have used this approach successfuly to evaluate differences in development of muscle from a severe Emery-Dreifuss muscular dystrophy mouse model.

Major Concerns:
The methods presented make perfect sense to me, having myself isolated EDL muscle in the past. Moreover, the notes are extremely useful to identify the critical steps and explain detailed aspects of the isolation method. I particularly like the explanations of why these subtle aspects of the protocol are important such as limiting agitation to prevent the myofibers from aggregating. The preparation for microscopy section is also useful. I have no particular criticisms of the protocol and just a few minor suggestions for the presentation.

We are really grateful to this reviewer for his/her kind comments.

Minor Concerns:
Line 115: slightly unusual usage. I would suggest altering to "adapted what was present in the literature to our needs: very small mice in term...."

We agree with the reviewers' comment, however to comply with editorial requirements, we have now removed this specific sentence.

We report here the amended text: "*Thus, we describe here our re-adaptation of the protocol proposed by the Rudnicki laboratory[19] to obtain a significant number of single viable myofibers from mice during post-natal development and from severe dystrophic muscles, such as those derived from Lamin Δ8-11 -/- mice[23]*"

Lines 135, 138, 209, and 267: used "need" when grammatically should be "needs".

Sorry about that, we did not notice and we thank you very much for correcting it. As stated above, lines 135, 138 and 267 have been removed now because of some editorial requirements, however we have corrected the use of "need" vs "needs" whenever still present.

Line 162: usage is odd for "we take into consideration". Perhaps better to say "This protocol was optimised for muscle taken at 17-21 days post-natal". Also note in this section that the description of supporting the mouse during the dissection is not clear. it would be good to have an image of the setup and perhaps to likewise show in an image where it is good to pin the legs to not interfere with the dissection whilst stabilizing them sufficiently to support the dissection. As the figure images are reportedly not available until after the current lockdown situation, it is hard to assess what may or may not need to be added to the

figure, but I would note that for people doing this the first time this is the most critical step to make visually clear. Digestion and other steps are generally clear enough from the descriptions as they are just putting things in tubes. However, when I first did this I was immensely aided by someone who had posted a video of it and things like exactly where to cut and how to grab the tip of the muscle before cutting to hold and stabilize it etc are not easy to follow from a description so all this here should be added to the figure to show visually exactly what is meant.

We totally agree with the reviewer about the fact that this is key for the positive outcome of the procedure. However, this part will be specifically filmed in the video and we will pay particular attention to explain it in detail. On the other hand, we have tried to describe much more details in the Protocol text and we think that this, together with the image (now figure 2) and the video will be sufficient to help scientists performing this key procedure.

Line 155: in describing the wide bore pipettes etc it would be helpful to a reader who has not done this before to give actual dimensions and show images. The myofibers are quite large and so what is meant by wide-bore here is much bigger than some people reading the protocol might imagine.

We thank the reviewer very much for this useful suggestion. Indeed, we have decided to add a figure (Figure 1 in the revised version of the manuscript) showing some pictures of the Pasteur pipettes necessary for proper muscle disaggregation and handling. The real images will be taken once the laboratory will re-open after the lockdown imposed by the Covid19 emergency.

Line 340: it would be helpful to give the reader an expected time frame so that they do not walk away and completely dry out their sample. Perhaps also more a description of what it looks like at the right stage of drying or even adding an image as this is one step where people often go too far.

We thank the reviewer and we agree with his/her indications. We have now added in the text an indicative amount of time (10 minutes). Also, we have now decided to include this part as one of those that will be filmed in the final video, to show in detail how the slide should look like.

Line 366: odd usage. Perhaps better to say "clearly visible, enabling the cells to differentiate into new myofibers"

We have corrected the sentence following reviewer's advice as: *"After 96 hours, this culminates with satellite cells clusters in which MyoG+ committed cells become clearly visible, enabling the cells to differentiate into new myofibers (figure 4 and 6)."*.

Reviewer #2:

Manuscript Summary:

In this report Pegoli et al., described a novel method to isolate satellite stem cell from homozygous mutant Lamin Δ8-11 mouse model to study detailed insights associated with mammalian myogenesis. The critical point in this study lies in their solid demonstration of obtaining satellite stem cells from very small quantity of muscle tissues, thus making the study of muscle physiology arising from small quantity of muscle, possible.

The manuscript is well written and the experiments are nicely done. The results represent successful demonstration of the strength of their protocol. In addition, this protocol is well adapted and applicable to condition where the quantity of muscle is limited, such as in early stages of post-natal development etc.

I believe that the community would benefit from this new method. Thus, I strongly suggest in favor of accepting this manuscript for a publication in JoVE.

Major Concerns:
None

Minor Concerns:
None

We are grateful to this reviewer for his/her appreciation of our manuscript. We thank you very much.

Reviewer #3:
Manuscript Summary:
The authors present an optimization of protocol to obtain isolated fibers from young and/or fragile muscle. Iμn this context they used a model presenting deletion mutation in the lamin gene associated with severe dystrophy.
All the protocol steps and products are well detailed. The picture of the figure 1 are missing, but the authors will send them after the end of Covid embargo.
They also follow the activation of satellite cells using known markers as Pax7, MyoD and Myogenin.

We would like to thank the reviewer for his/her nice comments and for the general positive evaluation of our manuscript.

Minor Concerns:
In the figure 4 : would it be possible to add a phase contrast picture to locate satellite cells on the fibers

We thank the reviewer for his/her the good advice. However, we did not take phase contrast pictures of the fibers showed in Figure 4. To do so we will have to perform new sacrifices and stainings on new myofibers. This might be difficult to achieve now, because of the lockdown imposed by the Covid19 emergency.

In the discussion :
1- You only talk about fixed fibers in the protocol and the discussion. Would it be possible to follow fibers in live imaging ? and to add this part in the discussion.

This is an interesting question, because it is not reported in literature. We only found a paper on adherent myofibers, but differentiated, starting from myoblasts, and the live imaging was performed for a really short amount of time using phase contrast to monitor the innate ability of myofibers to contract (Pimentel et al. JoVE 2017). Another paper (Ratnayake et al. Bioessays 2017) performed live imaging following the differentiation of single satellite cells.
In our experience it is not possible to perform live imaging on floating myofibers, even if using microscopes adapted with incubator chamber, because it might be difficult to optimize the focus on floating fibers for long period of time.

2- What is the maximum time to follow fibers and associated satellite cells? Indeed you only present 96h fixed fibers with myogenin staining. However if you want to follow later differentiation : the question is how long can you keep your fibers and associated satellite cells in culture? And do they fuse together and with the fiber? It would be interesting to discuss this.

As reported by Pasut et al. 2013, it is possible to cultivate isolated myofibers on Matrigel-coated dishes and they will survive for longer period of culture than 96h, but this situation is completely different from floating condition, where adhesion stimuli and proximity stimuli are absent. In our experiment after 96h of culture floating myofibers start to be hypercontracted and die.
In light of your constructive comments, we have now decided to include a short section in the discussion paragraph about the possibility to culture myofibers for longer time on matrigel-coated dishes.