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Isolation of mouse myoblasts for differentiation and imaging of mature myofibers

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Abstract:	Skeletal muscle is composed of myofibers, the biggest cells in the mammalian body and one of the few syncytia. How the complex and evolutionary conserved structures that compose it are assembled remains under investigation. Their size and physiological features often constrain manipulation and imaging applications. The culture of immortalized cell lines is widely used but it can only replicate the early steps of differentiation. Here we describe a protocol that enables easy genetic manipulation of myofibers originated from primary mouse myoblasts. After one week of differentiation the myofibers display contractility, aligned sarcomeres and triads as well as peripheral nuclei. The entire differentiation process can be followed by live imaging or immunofluorescence. This system combines advantages of the existing ex vivo and in vitro protocols. The possibility of easy and efficient transfection as well as the ease of access to all differentiation stages broadens the potential applications. Myofibers can subsequently be used to address not only relevant developmental and cell biology questions, as well as to reproduce muscle disease phenotypes for clinical applications.
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certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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Dr. Nandita SINGH
Senior Science Editor
JoVE

Lisbon, June 16th 2016

Dear Dr. Singh,

Please find enclosed our article entitled "**Isolation of mouse myoblasts for differentiation and imaging of mature myofibers**" for consideration for publication in JoVE.

Our laboratory is interested in studying mechanisms of myofiber differentiation and how myofiber physiology is altered in different pathological conditions. We recently developed a system to differentiate myofiber in vitro and use it to study the molecular mechanisms that are altered during centronuclear myopathies and myotonic dystrophies (Falcone et al. EMBO Molecular Medicine, 2014). Therefore we believe that our report will be of interest to the broad scientific community of JoVE readers interested in studying mechanism of skeletal muscle differentiation and muscle disorders.

We thank you for your consideration.

Yours truly,

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TITLE:
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KEYWORDS:
muscle, myofiber, differentiation, *in vitro*, microscopy, development

SHORT ABSTRACT:
Muscle cells are among the most complex eukaryotic cells. We present a protocol for the *in vitro* differentiation of highly mature myofibers that allows for genetic manipulation and clear imaging during all developmental stages.

LONG ABSTRACT:
Skeletal muscle is composed of myofibers, the biggest cells in the mammalian body and one of the few syncytia. How the complex and evolutionarily-conserved structures that compose it are

assembled remains under investigation. Their size and physiological features often constrain manipulation and imaging applications. The culture of immortalized cell lines is widely used, but it can only replicate the early steps of differentiation.

Here, we describe a protocol that enables easy genetic manipulation of myofibers originating from primary mouse myoblasts. After one week of differentiation, the myofibers display contractility, aligned sarcomeres and triads, as well as peripheral nuclei. The entire differentiation process can be followed by live imaging or immunofluorescence. This system combines the advantages of the existing *ex vivo* and *in vitro* protocols. The possibility of easy and efficient transfection as well as the ease of access to all differentiation stages broadens the potential applications. Myofibers can subsequently be used not only to address relevant developmental and cell biology questions, but also to reproduce muscle disease phenotypes for clinical applications.

INTRODUCTION:

Skeletal muscle composes up to 40% of the human body weight[1]. Muscle-associated disorders represent an immense health and economic burden[2]. How this highly complex and organized tissue is formed, maintained, and regenerated constitutes an extensive and well-established research field. Depending on the specific scientific interest, the most suited approach can range from simple myotube cultures to complex *in vivo* models[3–6].

The goal of this protocol is to provide an *in vitro* system that allows for the monitoring of myogenesis through live imaging and immunofluorescence. Compared to traditional approaches, this system offers a very complete and dynamic insight into the mouse myogenic process. Cells can be followed from the myoblast stage to the mature, multinucleated myofiber displaying transversal triads and peripheral nuclei[7]. This maturation level can be achieved using regular cell culture equipment, without the need for complex stimulatory or mechanical apparatuses. Although some successful *in vitro* systems have been reported[8,9], to our knowledge, this is the only protocol generating mature mouse myofibers with T-tubules transversally paired with sarcoplasmic reticulum (SR). Thus, this *in vitro* system can be used to study the molecular mechanisms of triad formation, which are still poorly understood[10].

A further advantage of using this system is the availability of validated mouse-targeted resources, such as antibodies, drugs, and RNAi tools. The relatively simple protocol does not require laborious steps, highly-skilled manipulation, or expensive and dedicated equipment. Matured myofibers start appearing after 5 days of culture differentiation[7], displaying contractility coupled with calcium sparks (unpublished data). In one week, the different developmental stages of one the most complex cells in the mammalian body can be studied in combination with a variety of *in vitro* assays.

PROTOCOL:

Note: One mouse yields sufficient myoblasts for approximately two 35-mm dishes or two live-imaging dishes, so plan mattings, dissection, and coating (step 2.6) accordingly. Since myoblasts

are isolated through sequential centrifugations and pre-plating, the protocol should be done in batches of 5 to 10 animals.

All procedures involving animal subjects were approved by the Animal Ethics Committee of Molecular Medicine Institute.

1. Dissection of neonatal mice hind limb muscles

1.1) Prepare all solutions in advance (Materials Table) and sterilize by filtration (0.22 µm filter). Make sure all media are at 37 °C before addition to the cells, except the formulations containing basement membrane matrix (*e.g.,* Matrigel).

1.2) Sterilize the dissection material (one each of: curved scissors, straight scissors, regular forceps, and fine-tip forceps) and the work bench by wiping them with 70% ethanol.

1.3) Prepare a 100-mm petri dish with 5 mL of Dulbecco's Phosphate Buffered Saline (DPBS) for muscle collection and keep it on ice until the mincing step.

1.4) Decapitate P6-P8 mice with straight scissors and sterilize the skin with 70% ethanol.

1.5) Make an incision in the back skin and pull it gently towards the hind limbs until it is removed, completely exposing the hind-limb musculature.

1.6) Use the forceps to remove fat tissue without damaging the muscles.

1.7) To remove the tibialis anterior, extensor digitorum longus, and gastrocnemius, keep the limb stretched and bend the paw to expose the heel tendons. Use the curved scissors to separate muscle from bone, starting from the tendons, by gently sliding and cutting upwards. Excise the muscles and place them in iced DPBS.

1.8) Isolate the quadriceps by pinching the muscle with fine-tip forceps and cutting around it without damaging the femur or the knee joint.

1.9) After dissecting all animals, proceed to a sterile laminar flow cell culture hood, where all the following steps should be performed.

2. Myoblast isolation

2.1) Remove the excess of DPBS. Mince the tissue with sterilized curved scissors in order to obtain a uniform mass.

2.2) Collect the minced tissue in a 50-mL conical centrifuge tube using 5 mL of digestion mix and incubate it with agitation at 37 °C for 90 min.

2.3) Stop the digestion by adding 6 mL of dissection medium and centrifuge the suspension for 5 min at 75 x g to pellet the remaining tissue.

2.4) Carefully collect the supernatant and centrifuge it at 350 x g for 5 min; resuspend it in 5 mL of dissection medium.

2.5) Filter the cell suspension through a 40 µm cell strainer. Add 25 mL of dissection medium and pre-plate it in a 150-mm dish for 4 h in a cell culture incubator (37 °C and 5% CO_2) to allow for fibroblast adhesion.

2.6) While pre-plating, coat dishes with 500 µL of basement membrane matrix diluted 1:100 in cold IMDM for 1 h at room temperature (RT). Wash once with DPBS and plate the cells immediately (step 2.8) or leave with growth medium until plating.

2.7) After pre-plating, collect the supernatant and centrifuge it at 350 x g for 10 min.

2.8) Resuspend it in growth medium and count the cells on a hemocytometer. Adjust the volume so that between 150,000 and 250,000 cells are plated per basement membrane matrix-coated dish. Keep the cells in a cell culture incubator.

3. Myofiber differentiation

Note: After 3 days, the cells should start to fuse and form myotubes at around 70% confluency (Figure 1B).

3.1) At this point, transfect the cells, if desired, with a siRNA or DNA of interest. If the cells are not to be transfected, change directly to differentiation medium and skip to step 3.4.

3.2) Transfect with transfection reagents following the manufacturer's instructions. Incubate the cells for 5 h with siRNA-lipid complexes (20 nM + 1 µL of reagent) or DNA-lipid complexes (1 µg + 1 µL of reagent). Optimize the siRNA and DNA concentrations if necessary.

3.3) Wash them once with differentiation medium and then switch to new differentiation medium.

3.4) The following day, dilute the basement membrane matrix 1:2 in ice-cold differentiation medium. Remove the existing medium and add 200 µL of ice-cold matrix to each dish.

3.5) Incubate for 30 min in a cell culture incubator.

3.6) Supplement the differentiation medium with agrin (100 ng/mL) and carefully add 2 mL to the cells.

3.7) Carefully change half of the medium every 2 days, always supplementing with agrin to a final concentration of 100 ng/µL.

3.8) Monitor cell differentiation and viability. Depending on a variety of factors (such as fetal bovine serum and chicken embryo extract origins), the cells might take between 5 to 10 differentiation days to reach full maturation (Figure 2).

4. Immunostaining in glass-bottom dishes

4.1) For immunostaining, at any time-point of interest, wash the cells once with DPBS and fix them with 4% PFA at RT for 10 min.

4.2) Wash them 2 times with DPBS. At this point, the cells can be stored at 4 °C.

4.3) Permeabilize them with 0.5% Triton X-100 for 5 min at RT.

4.4) Wash them twice with PBS and block with blocking solution for 30 min at RT.

4.5) Incubate them with primary antibody diluted in blocking solution overnight at 4 °C.

4.6) Wash them 3 times with DPBS for 5 min at RT.

4.7) Incubate them with the secondary antibody and 0.2 µg/mL of DAPI for 1 h at RT.

4.8) Wash them 3 times with DPBS for 5 min at RT.

4.9) Add 200 µL of mounting medium and proceed to imaging.

REPRESENTATIVE RESULTS:

The extent of myofiber development is mostly determined by the purity and viability of the isolated myoblasts. The adhesion, proliferation, and fusion capacity can be used to empirically access those parameters (Figure 1 A-B). At proliferation day 2, myoblasts should have adhered and should display the typical fusiform shape. Proliferation is expected to happen extensively at this stage, leading to spontaneous myotube formation the following day (Figure 1B).

Cell confluency might need slight adjustments. It should be increased if myoblasts take more than 3 days to proliferate and fuse. It should be decreased if myofibers are not allowed to grow and elongate relatively straight due to their density. Confluency typically decreases from the center to the periphery of the dish, so the best myofibers should be found towards the outer regions.

Myotubes will quickly elongate and display multiple centrally-aligned nuclei (Figure 1C). By day 5, some cells start acquiring striations and moving their nuclei to the periphery. The number of myofibers with mature characteristics will increase with time as well as with cell thickness (Figure 1D).

The degree of differentiation can be further observed by immunofluorescence. Myofibers fixed at differentiation day 8 present transversal triads. This can be confirmed by imaging

components of the T-tubules (DPHR) and the SR (triadin), which are expected to co-localize at the triads (Figure2).

The functionality of myofibers can be addressed by live imaging. From differentiation day 3 onwards, the cells display spontaneous twitching. By transfecting a calcium sensor (*e.g.,* GCaMP6f[11]), it is possible to observe that the contractions are coupled with calcium peaks (Figure 3).

Using this system, we were able to identify a novel molecular pathway that is disrupted in centronuclear myopathies and myotonic dystrophies, which can therefore be a novel target for innovative molecular therapies[7]. We have also adapted this method to study the development of the neuromuscular junction (NMJ)[12]. Through the co-culture with rat spinal cord explants, we have described a role for dynein in NMJ formation[13].

Figure 1: Developmental stages of the myoblast culture.
A) At proliferation day 2, myoblasts have adhered and started proliferating. B) At proliferation day 3, a confluency of 60-80% is reached, and myoblasts start fusing spontaneously. C) At differentiation day 3, myotubes containing centrally-located nuclei are predominant. D) From differentiation day 5 onwards (*e.g.,* day 8), myofibers start exhibiting striations and peripheral nuclei and begin to thicken. Scale bar: 50 µm.

Figure 2: Representative confocal image of a day-8 myofiber immunostain.
A) Immunostaining for dihydropyridine receptor (DHPR, top panel) and triadin (TRDN, middle panel). An overlay of the DHPR, TRDN, and DAPI channels shows co-localization of the triad components. B) An intensity profile of the yellow line drawn in A. C) A 3D image of volume rendering of myofibers stained for α-actinin (green) and DAPI (blue). Scale bar and grid width: 5 µm.

Figure 3: Live imaging of calcium levels in myofibers with spontaneous twitching.
A) High-speed time-lapse (20 ms frames) microscopy of a calcium spark in a twitching myofiber. Calcium was detected through the expression of GCaMP6f (Addgene plasmid #40755). B) Quantification of the fluorescence intensity over time for the calcium sensor in panel A.

DISCUSSION:
The use of this protocol for the cultivation of primary myoblasts gives rise to a special niche that greatly nurtures the development of myofibers. This is partially due to other cell types that are also present in very small numbers. A balance between myoblast concentration and culture purity must be achieved. A good cell culture also depends on the quality of the products used for the medium formulation. All products derived from animal sources should be thoroughly tested. In our experience, the digestion conditions should also be monitored.

As usual for primary cultures, experimental variability can be higher than in studies with isolated fibers or immortalized myoblasts. This variability can be diminished by the standardization of medium and digestion components, mice age and size, and the time points

for culture manipulation and results collection. Nevertheless, the advantage of scrutinizing in real time the intricate mechanisms necessary for myofiber development greatly surpasses the variability drawback.

This protocol confers the advantages of *in vitro* approaches without compromising cell differentiation. Myofibers mature until triads are formed and contractions are coupled to calcium sparks. These functional outputs can be accessed in different experimental conditions. Furthermore, there can be many technical variations made to the protocol. Myoblasts can be harvested from neonatal mice with mutations of interest relating to muscle development. Cells can be lysed for biochemical analysis at different differentiation time points. Calcium indicators can be added to the culture to follow its dynamics. Optogenetic constructs can be used to enforce certain signaling pathways or to induce specific local responses. Finally, the myofibers can be co-cultured with other cells types to study their interactions.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

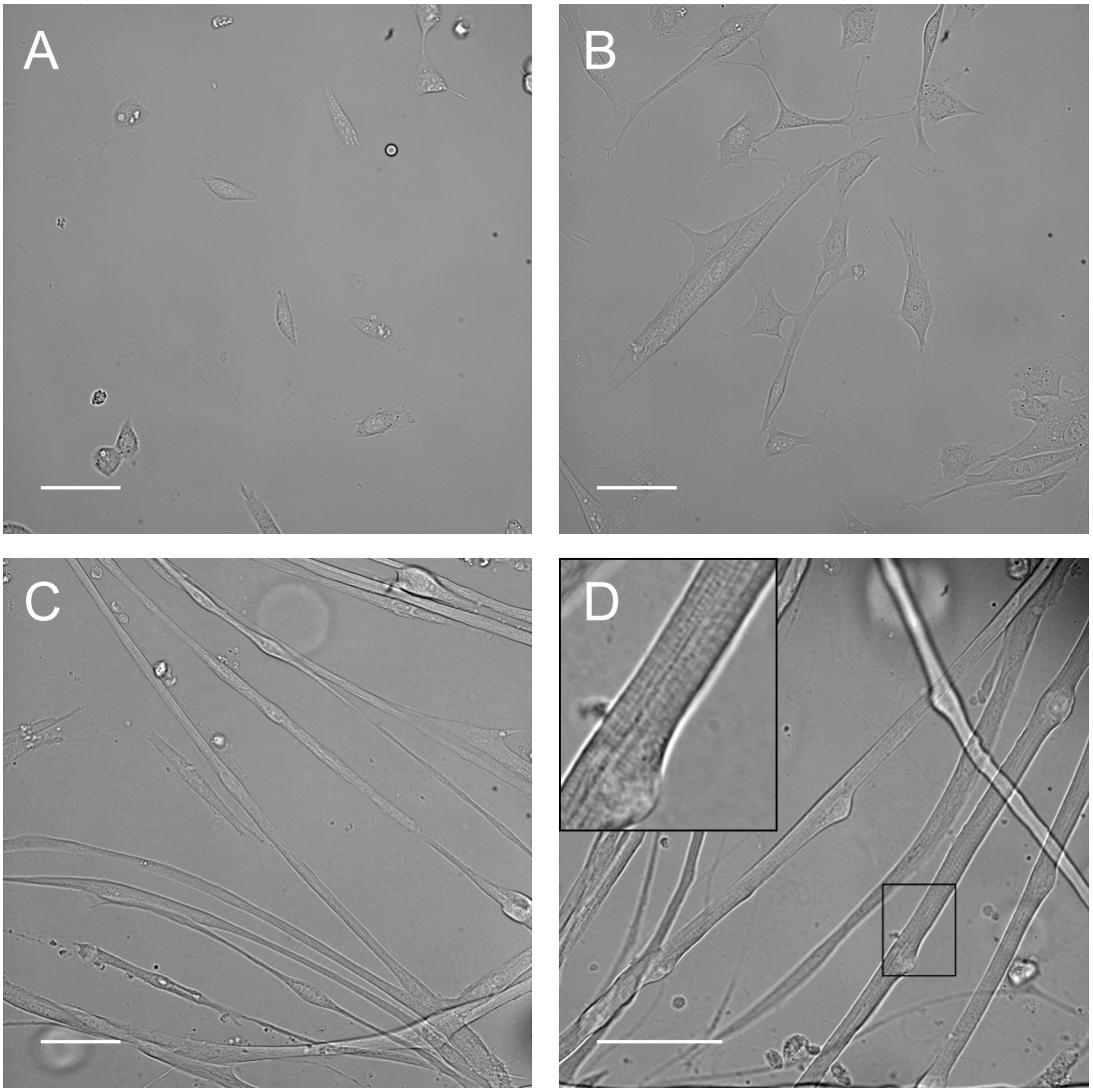


Figure 2

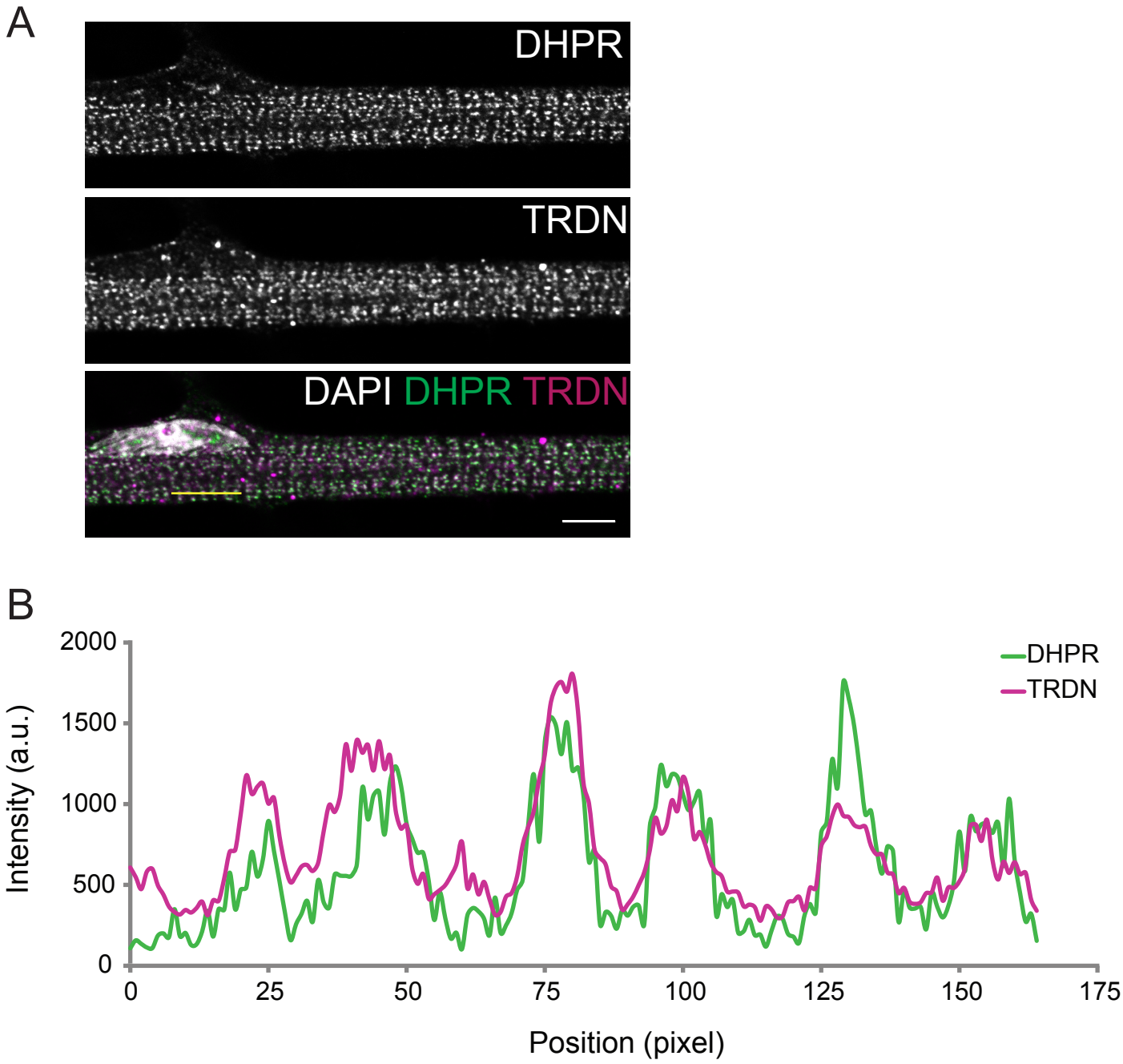
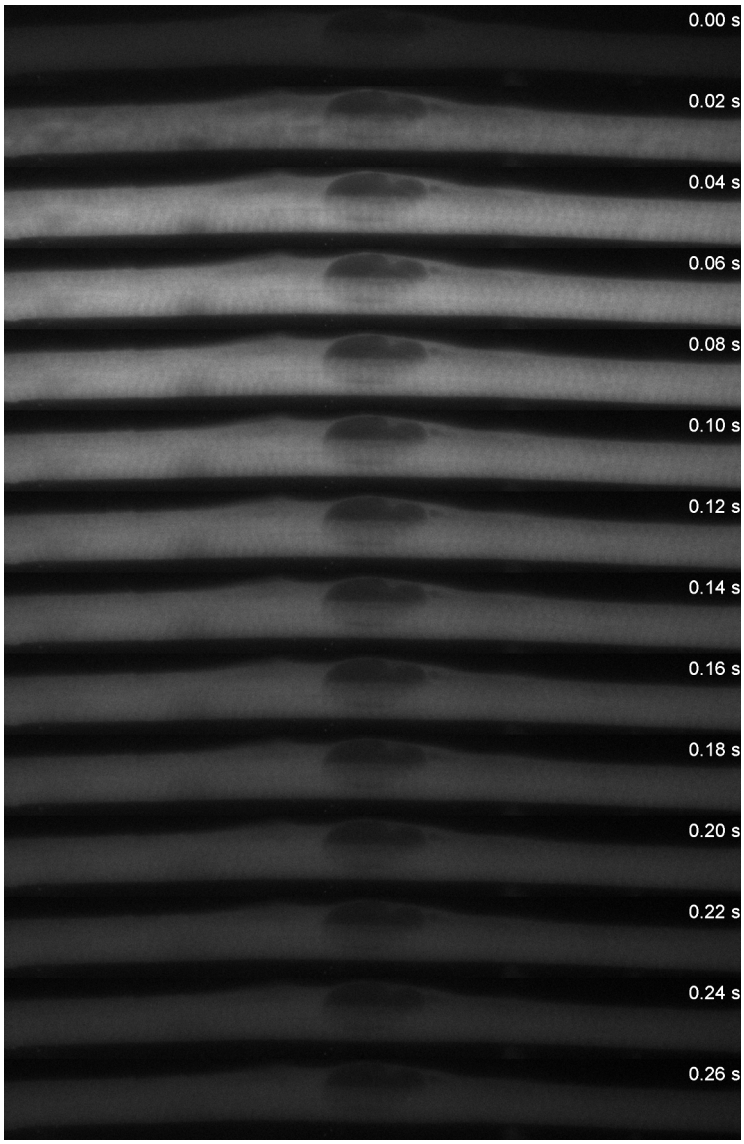
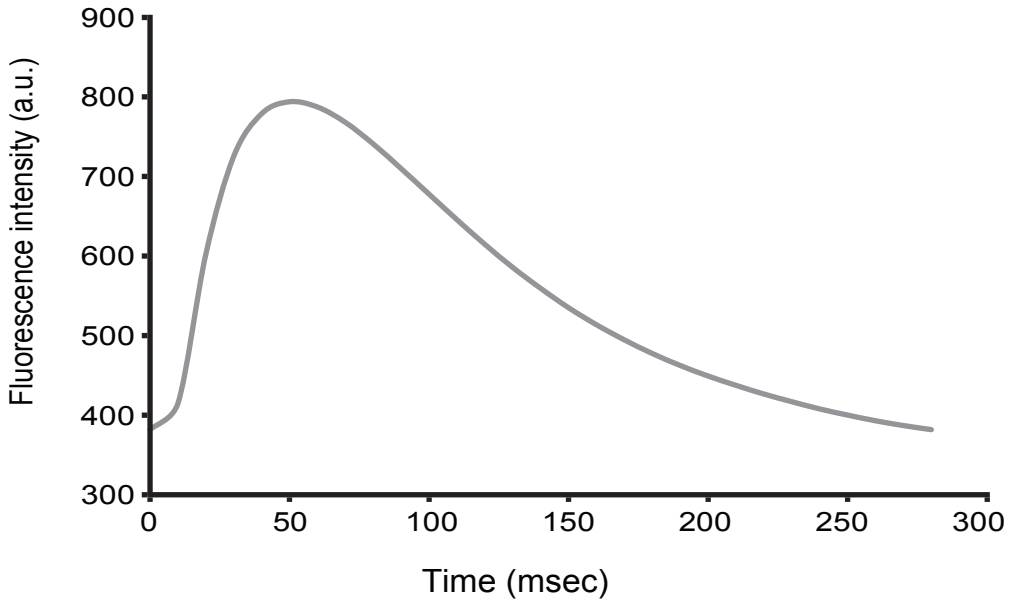


Figure 3

A



B



Excel Spreadsheet- Table of Materials/Equipment

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JoVE_Materials.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
Dispase II	Gibco	17105041
Collagenase type V	Sigma-Aldrich-Aldrich	C9263
IMDM, Glutamax supplemented	Gibco	31980022
Matrigel Growth reduced factor	Corning	354230
Chicken Embryo Extract	MP biomedical	2850145
Recombinant rat agrin	R&D systems	550-AG-100
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
Horse Serum	GE Healthcare	11581831
Lipofectamine RNAiMAX	Invitrogen	13778-150
Lipofectamine LTX	Invitrogen	15338-100
Lipofectamine 2000	Invitrogen	11668030
DPBS	Gibco	14190094
Fetal Bovine Serum	Eurobio	CVFSVF0001
Cell strainer	Corning	21008-949
Fluorodishes	World precision instruments	FD35-100
Triton X-100	Sigma-Aldrich	T9284
16% PFA (Paraformaldehyde)	Science Services GmbH	E15710
Goat Serum	Sigma-Aldrich	G9023
BSA (Bovine Serum Albumine)	Sigma-Aldrich	A7906
Saponine	Sigma-Aldrich	47036
DAPI	Sigma-Aldrich	D9542
Fluoromount-G	SouthernBiotech	0100-01

Solutions and Media

Digestion Mix	in DPBS 5 mg/ml collagenase 3.5 mg/ml dispase sterile filtered, can be stored in working aliquotes for 2 weeks at
Dissection Medium	in IMDM Glutamax supplemented 10% FBS

	1% Penicillin-Streptomycin sterile filtered
Growth Medium	in IMDM Glutamax supplemented 20% FBS 1% Chicken Embryo Extract 1% Penicillin-Streptomycin sterile filtered
Differentiation Medium	in IMDM Glutamax supplemented 2% Horse Serum 1% Penicillin-Streptomycin sterile filtered
Blocking Solution	in DPBS 10% Goat Serum 5% BSA add 0.1% saponine when incubating with primary and secondary antibodies

Comments/Description

protein concentration of the lot should be around 10mg/ml and endotoxin result should be <1.5
it is also possible to prepare in the lab (Danoviz ME, Yablonka-Reuveni Z. Methods Mol Biol (2012))

used to transfect siRNA
used to transfect DNA
used to transfect siRNA plus DNA

dishes used to cultivate cells for live imaging

use at 200ng/ul

-20 °C

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Editorial comments:

The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55141_R0_062316.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please abbreviate all journal titles.done

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.done

4. 1.1: Sterile filter how? .22 um filter?done

5. Animal care note – Decapitation without prior anesthesia might present a problem for vet reviewThe protocols described here were previously accepted by ethics committees in France and Portugal. The decapitation is done in newborns (P6-p8) as stated in the protocol

6. Formatting
-In the Author Affiliations, please use English translations for university/department names.done
-The sentence "Using this system we were able to identify a novel molecular pathway that is disrupted in centronuclear myopathies and myotonic dystrophies, and therefore can be a novel target for innovative molecular therapies7." in the Introduction is better-suited for the Results section.done
-Given that this work utilizes P6-P8 mice, a disclaimer/ethics statement noting animal work should be added at the beginning of the protocol (e.g., "All procedures involving animal subjects were approved by the Institutional Animal Care and Use Committee...")done
-Please check that all journal titles are abbreviated in the References sectiondone

7. The manuscript would benefit from copyediting for grammatical/typographical errors. A subset of these issues are below:
-1.1. Sterilize the dissection materials (one curved scissor, one straight scissor, regular and fine-tip forceps) and the work bench by wiping with 70% ethanol.done
-1.7. To remove the tibialis anterior... done
-2.3. Stop digestion by adding 6 mL of Dissection medium and centrifuge the suspension for 5 min at 75 x g to pellet the remaining tissue.done
-3.2-Wash once with Differentiation medium and switch to new Differentiation medium.done

8. Additional detail is required
-2.2-To clarify, 50 mL of what? Do you mean a 50 mL conical?done
-3.1-siRNA targeting what? Please provide additional details on the siRNA/DNA mentioned here.done (any of interest)
-3.7-What factors? done
-4.1-What time point?done (any of interest)
-4.7-What concentration of DAPI?done

9. Branding: 2.6-Glutamaxdone

10. Results: Please discuss the individual panels in Figure 1 (especially A and B) and Figure 2 (A, B, and C) in more depth. What are each of these showing? Also, elaborate on the DHPR and TRDN markers in the actual Results text.done

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a good manuscript describing in great detail the optimization of a method that indeed is well known since the eraly 80'. However this is the first of the dozens of methods available that allows complete maturation of muscle fibres in vitro. As such it is of paramount value to study muscle physiology and, possibly pathology outside of the body.

Although the study is very detailed, two issues should be dealt with while revising the ms:
1. An estimate of cell death during the various steps of dissociation should be provided (a simple Trypan Blue stianing would sufficit). Cell death assays are not feasible since the tissues are not fully dissociated during the process before the preplating step. The use of trypan blue or other similar methods would be therefore misleading due to cells still be protected in their niches. We do use trypan blue when counting cells for seeding and very rarely see positive cells.
2. We are not told whether these fibres twitch spontaneously or not. In the first case a movie would be informative. Done, in figure 3 we show a calcium spark associated to a spontaneous twitch (it is easier to represent as a figure and adds further information compared to a twitch movie).

Beyond the scope of the present work, it would be interesting to see whether a co-culture with motoneurones would show any specific effect that agrin cannot mimic.
Reference added in results

Also it would be interesting to culture human cells and also DMD cells, also in light of recent claims that iPS-derived DMD muscle cells fail to differentiate.

Major Concerns:
N/A

Minor Concerns:

N/A

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The manuscript "Isolation of mouse myoblasts for differentiation and imaging of mature myofibers" describes a new protocol for isolation and differentiation of mouse myoblasts.
The protocol is described in full details and list of reagents is complete. The protocol represents a variant of common procedures currently described in the literature to isolate mouse myoblasts. Rat agrin in the medium formulation is used to achieve optimal differentiation.
The protocol is feasible and results are convincing, at least as expression and localization of triadic protein is concerned.

Major Concerns:
N/A

Minor Concerns:
- add a short comment on the main innovation introduced by this protocol compared to traditional approaches should improved the discussion section. done
- please include a comment about the known effect of different batches of serum on the extent of muscle cell differentiation ? Do they affect also this protocol and could chemically-defined medium be used in alternative? Briefly mentioned in step 3.8) and in the discussion. We do test the impact of new batches of Fetal Bovine Serum and Chicken Embryo Extract in cell growth and differentiation. Some products from specific sources or batches have impaired the efficiency of this protocol. We have never tested chemically-defined media.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This paper nicely explains a novel approach to grow myotubes in culture that retain structural characteristics that are essential for normal function and which are normally lost in primary myotube cultures. The text is well written, the issues are nearly laid out and I am confident that this will be represent a valuable resource.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:

Personally, I'd like to see a higher power image to show the striations in the myotubes clearly in Fig 1. - the striations are visible but only just on my screen. Perhaps an inset would be useful.?done

I'd also like to see evidence of function - perhaps this will be shown in a video, but a figure that illustrates shortening or a calcium transient in response to electrical or pharmacological stimulation would add very much to demonstrate the functional differentiation of the preparation.done, figure3