**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