



HAL
open science

Unfractionated Bulk Culture of Mouse Skeletal Muscle to Recapitulate Niche and Stem Cell Quiescence

Louai Zaidan, Perla Geara, Matthew Borok, Léo Machado, Despoina Mademtzoglou, Philippos Mourikis, Frederic Relaix

► **To cite this version:**

Louai Zaidan, Perla Geara, Matthew Borok, Léo Machado, Despoina Mademtzoglou, et al.. Unfractionated Bulk Culture of Mouse Skeletal Muscle to Recapitulate Niche and Stem Cell Quiescence. *Journal of visualized experiments : JoVE*, 2023, 196, <10.3791/65433>. <hal-04211362>

HAL Id: hal-04211362

<https://hal.science/hal-04211362v1>

Submitted on 3 Nov 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Unfractionated Bulk Culture of Mouse Skeletal Muscle to Recapitulate Niche and Stem Cell Quiescence

Louai Zaidan^{*,1}, Perla Geara^{*,1}, Matthew J. Borok^{*,1}, Léo Machado¹, Despoina Mademtoglou¹, Philippos Mourikis¹, Frederic Relaix^{1,2,3,4}

¹Univ Paris Est Creteil, INSERM, IMRB ²Ecole nationale vétérinaire d'Alfort, IMRB ³EFS, IMRB ⁴AP-HP, Hopital Mondor, Service d'histologie

*These authors contributed equally

Corresponding Author

Frederic Relaix
frederic.relaix@inserm.fr

Citation

Zaidan, L., Geara, P., Borok, M.J., Machado, L., Mademtoglou, D., Mourikis, P., Relaix, F. Unfractionated Bulk Culture of Mouse Skeletal Muscle to Recapitulate Niche and Stem Cell Quiescence. *J. Vis. Exp.* (), e65433, doi:10.3791/65433 (2023).

Date Published

May 24, 2023

DOI

10.3791/65433

URL

jove.com/t/65433

Introduction

Movement, breathing, metabolism, body posture, and body temperature maintenance all depend on skeletal muscle, and malfunctions in the skeletal muscle can, thus, cause debilitating pathologies (i.e., myopathies, muscular dystrophies, etc.)¹. Given its essential functions and abundance, skeletal muscle has drawn the attention of research labs worldwide that strive to understand the key aspects that support normal muscle function and can serve

Abstract

Skeletal muscle is the largest tissue of the body and performs multiple functions, from locomotion to body temperature control. Its functionality and recovery from injuries depend on a multitude of cell types and on molecular signals between the core muscle cells (myofibers, muscle stem cells) and their niche. Most experimental settings do not preserve this complex physiological microenvironment, and neither do they allow the *ex vivo* study of muscle stem cells in quiescence, a cell state that is crucial for them. Here, a protocol is outlined for the *ex vivo* culture of muscle stem cells with cellular components of their niche. Through the mechanical and enzymatic breakdown of muscles, a mixture of cell types is obtained, which is put in 2D culture. Immunostaining shows that within 1 week, multiple niche cells are present in culture alongside myofibers and, importantly, Pax7-positive cells that display the characteristics of quiescent muscle stem cells. These unique properties make this protocol a powerful tool for cell amplification and the generation of quiescent-like stem cells that can be used to address fundamental and translational questions.

as therapeutic targets. In addition, skeletal muscle is a widely used model to study regeneration and stem cell function, as healthy muscle can fully self-repair after complete injury and degeneration, mostly due to its resident stem cells²; these are also called satellite cells and are localized under the basal lamina in the periphery of the muscle fibers³.

The core cells of adult skeletal muscle are the myofibers (long syncytial multinuclear cells) and the satellite cells (stem cells with myogenic potential that are quiescent until an injury activates them). The latter cells are the central cells of muscle regeneration, and this process cannot occur in their absence^{4,5,6,7}. In their immediate microenvironment, there are multiple cell types and molecular factors that signal to them. This niche is gradually established throughout development and until adulthood⁸. Adult muscle contains multiple cell types (endothelial cells, pericytes, macrophages, fibro-adipogenic progenitors-FAPs, regulatory T cells, etc.)^{9,10} and extracellular matrix components (laminins, collagens, fibronectin, fibrillins, periostin, etc.)¹¹ that interact with each other and with the satellite cells in the context of health, disease, and regeneration.

Preserving this complex niche in experimental settings is fundamental but challenging. Equally difficult is to maintain or return to quiescence, a cell state that is critical for satellite cells⁹. Several methods have been introduced to partially tackle these challenges, each with its advantages and disadvantages (detailed in the discussion section). Here, a method is presented that can partially overcome these two barriers. Muscles are initially harvested and then broken down mechanically and enzymatically before the heterogenous cell mixture is put into culture. Over the course of the culture, many cell types of the niche are detected, and satellite cells that have returned to quiescence are observed. As a last step of the protocol, the immunofluorescence steps that allow for the detection of each cell type through the use of universally accepted markers, are presented.

Protocol

All experiments complied with French and EU animal regulations at the Institut Mondor de Recherche Biomédicale

(INSERM U955), notably the directive 2010/63/UE. Animals were kept in a controlled and enriched environment at the animal facilities with certification numbers A94 028 379 and D94-028-028; they were handled only by authorized researchers and animal caretakers, and they were visually inspected by animal housing personnel for signs of discomfort during their lifetime. They were euthanized by cervical dislocation prior to dissection. No interventional procedures were performed during the animals' lifetimes; thus, acquiring approval for the procedure from an Ethics Committee and the French Ministry of Higher Education, Research and Innovation was not necessary. Indeed, no ethics clearance is required for euthanization and post-mortem dissection according to the directive 2010/63/UE. The results presented in this manuscript are from the wild-type C57BL/6NRj line (see **Table of Materials**) and the transgenic *Tg:Pax7-nGFP* line¹² (bred by our team). The protocol was applied to male and female mice aged 8-12 weeks of age.

1. Reagent and equipment preparation pre-digestion

1. Spray the dissection tools (straight and curved scissors, forceps, see the **Table of Materials**) with 70% ethanol, and dry them with paper. Coat a cork plate with aluminum foil, and keep 10 cm Petri dishes (one per animal) nearby. Have paper and 70% ethanol in reach.

NOTE: At the end of the dissection, rinse the dissection tools with water, then spray them with 70% ethanol, and dry them with paper.
2. Set a rotating water bath to 37 °C, and prepare the digestion mix (20 mL/animal) by combining DMEM with 1% penicillin-streptomycin, 0.5 U/mL collagenase, 3 U/mL dispase (see the **Table of Materials**), and 0.2% BSA in 50 mL tube(s).

3. Pass the digestion mix through a 0.22 μm filter in a cell culture hood.

NOTE: It is recommended to prepare the digestion mix fresh every time.

2. Reagent and equipment preparation post-digestion

1. Post-digestion, the mix can be frozen or cultured. For freezing, prepare 10% DMSO:90% fetal bovine serum (FBS), as well as a set of cryotubes (1 mL of cell suspension per 2 mL cryotube). For the culture, prepare culture medium (DMEM supplemented with 1% penicillin-streptomycin, 4 ng/mL bFGF, and 20% FBS) and a set of 8-well plates. The plates must be coated before plating the cells (details are provided in step 7.1).
2. For the staining, prepare 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (0.15 mL/well of the 8-well plate) and blocking solution (5% IgG-free bovine serum albumin [BSA] in PBS; 0.15 mL/well of the 8-well plate).
CAUTION: Do not breathe in the PFA powder; prepare and handle it under a chemical hood.

3. Dissection

1. Spray the euthanized animal with 70% ethanol. Make a horizontal incision (left side of the body to the right side)

with big scissors at the level of the abdomen, and cut around the waist. Pull the skin off of the hindlimbs to reveal the muscles (**Figure 1A**).

2. Place the animal on the aluminum foil-covered cork plate, and pin down the opposite forelimb and hindlimb. Quickly remove all the hindlimb muscles (front and back) into a 10 cm Petri dish placed on ice (**Figure 1B,C**). Take special care to remove the adipose tissue from the areas around the quadriceps and the posterior muscles. Fascia, nerves, and tendons can also be removed at this point if this does not compromise the overall time spent on dissection.

NOTE: An optimal dissection time for both the hindlimbs should be around 15-20 min. It is advised that the dissection time does not exceed 30 min.

3. Add drops of DMEM to the muscles occasionally to keep them moist, but not too much, as this will render chopping difficult. Repeat for the other hindlimb. Once all the muscles of one animal are in the Petri dish (**Figure 1D**), chop them finely with scissors for 7-10 min to obtain a smooth homogenate (**Figure 1E**).

NOTE: In this protocol, DMEM supplemented with L-glutamine, pyruvate, and 4.5 g/L D-glucose is used.

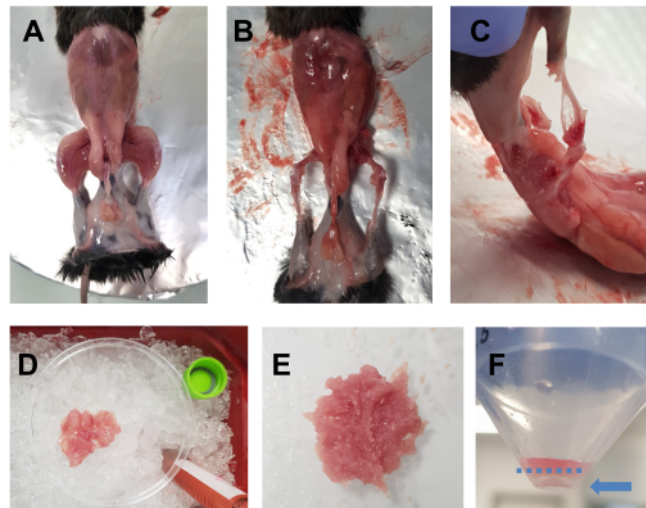


Figure 1: Pre-culture muscle preparation. (A) The skin is removed to reveal the hindlimb muscles, as described in step 3.1. (B,C) All the hindlimb muscles are harvested (B) around and (C) between the bones, as described in step 3.2. (D) The harvested muscles are placed in a 10 cm Petri dish on ice with DMEM drops to keep them moist, as described in step 3.3. (E) The muscles are finely chopped with scissors until a smooth paste is obtained with the consistency depicted in this image. (F) An image of the pellet after the final centrifugation; the blue arrow highlights the pellet, which is against the tube, under the dashed blue line. [Please click here to view a larger version of this figure.](#)

4. Digestion

NOTE: At the end of the digestion, a centrifuge at 4 °C, a bucket of ice, three cell strainers (100 μ m, 70 μ m, 40 μ m), and three 50 mL tubes (per animal) are needed for section 5.

1. Prepare and filter the digestion mix as described in step 1.2. Keep the mix on ice.
2. Once all muscles are chopped, place the homogenate in a 50 mL tube with 20 mL of digestion mix. Wrap the edges of the lid with flexible film to prevent leaking, and place the tube in a 37 °C shaking water bath at a low to medium speed (50 rpm).

3. After 1 h at 37 °C, open the lid, and mix by gently pipetting seven times up and down with a 10 mL pipette to obtain a homogenous mix. Apply new film around the lid, and place it back in the shaking water bath. After 1 h, remove the tube, and turn off the bath.

NOTE: For culture, use this incubation time to coat the plates as described in step 7.1 before moving to section 5.

5. Filtration

1. Fill the digestion tube with cold DMEM (supplemented with 1% penicillin-streptomycin) up to 50 mL. Mix by inverting the tube three times. Keep the DMEM in an ice bucket for the next steps.

2. Place a 100 μm cell strainer on a new 50 mL tube. Pass the digested mix through the cell strainer into the new tube. Centrifuge at 600 x g for 5 min at 4 °C. Pour out the supernatant into a liquid waste container.
3. Resuspend the pellet in 1 mL of cold DMEM (supplemented with 1% penicillin-streptomycin). Fill the tube up to 50 mL with the same DMEM. **NOTE:** If the centrifuging is skipped, the next pellet will be more difficult to identify and maintain.
4. Place a 70 μm cell strainer on a new 50 mL tube. Pass the centrifuged/resuspended mix through the cell strainer into the new tube. Centrifuge at 80 x g for 5 min at 4 °C. **NOTE:** This step is not mandatory but is recommended to eliminate cell debris.
5. Place a 40 μm cell strainer on a new 50 mL tube. Pass the supernatant through the cell strainer into the new tube. Centrifuge at 600 x g for 5 min at 4 °C, pour the supernatant into a liquid waste container, and resuspend the pellet in FBS under the culture hood. The pellet is very small at this step (**Figure 1F**). **NOTE:** Filtering through the 40 μm strainer removes debris, which would give a non-specific signal in the later staining of the cultures.

6. (Optional) Freezing

NOTE: Section 6 is optional. The protocol can be paused after filtering, but this can reduce the cell survival and culture success.

1. Add DMSO to obtain a 10% DMSO:90% FBS ratio, and transfer to cryotubes (1 mL of resuspended pellet per 2 mL cryotube).

2. Place the cryotube at $-80\text{ }^{\circ}\text{C}$ in a polystyrene box overnight. Move to $-150\text{ }^{\circ}\text{C}$ the next day for long-term storage.

NOTE: Short-term storage at $-80\text{ }^{\circ}\text{C}$ is also possible.

3. When starting the culture, thaw the cryotube quickly in a $37\text{ }^{\circ}\text{C}$ water bath until the cell suspension has thawed. Mix with 4 mL of DMEM under the culture hood. Spin at 600 x g for 5 min at 4 °C. Pipette out the supernatant, and continue as described in step 7.2.

7. Culturing

NOTE: Frozen or fresh cell suspensions can be expected to fill 24-32 wells of three to four 8-well plates.

1. Coat 8-well plates with the coating solution, which should be thawed at 4 °C or on ice (stock coating solution is normally kept at $-20\text{ }^{\circ}\text{C}$). Add 0.4 mL of coating solution to one well, and pipette it from well to well. After transferring the coating solution through all the wells, it can be recollected and refrozen for future cultures. Keep the coated plates at $37\text{ }^{\circ}\text{C}$ for 30 min before plating the cells.
2. Add DMEM (supplemented with 1% penicillin-streptomycin) supplemented with 4 ng/mL bFGF (see **Table of Materials**) to the FBS-cell suspension to obtain a 20% FBS:80% DMEM ratio. **NOTE:** Even though the addition of bFGF can be beneficial in primary myoblast cultures and in satellite-like cell production in bulk cultures, its addition is optional, as its omission in bulk cultures of ~ 7 days does not severely compromise the cell yields.
3. Plate 0.4 mL of the suspension per well (from step 7.2) in the coated 8-well plates.

NOTE: Calculate 30 cm² of culture per animal for frozen and fresh preparations.

4. Incubate the cultures at 37 °C with 5% CO₂ for up to 10 days, changing the medium every day after the culture begins to change to a yellowish color (usually 5-7 days).

NOTE: To quantify cells in the S phase of the cell cycle¹³, add 10 μM EdU 2 h before fixation. To capture the first S phase, add 10 μM EdU from the plating, and fix at 40 h of culture.

8. Fixation

NOTE: Sections 8-10 should be conducted at room temperature unless otherwise stated.

1. Pipette out the culture medium, and fix the cells with 4% PFA (0.15 mL/well).

CAUTION: Add PFA under a chemical hood.

NOTE: If all the wells are fixed at the same time, incubate with PFA at room temperature for 10 min. If the wells are fixed at different time points, add PFA to the wells to be fixed, and keep the plate in the incubator at 37 °C for 5 min.

2. Pipette out the PFA, and add PBS for 10 s (0.15 mL/well). Pipette out the PBS, and add fresh PBS for 5 min (0.15 mL/well).

NOTE: If all the wells are fixed at the same time, incubate with PBS at room temperature. If the wells are fixed at different time points, add PBS to the fixed wells, and keep the plate in the incubator at 37 °C for 5 min. Then, add 0.4 mL of PBS, and keep the plate in the incubator for up to 1 week.

9. Permeabilization and blocking

1. When ready to stain, pipette out the PBS, and permeabilize with 0.5% TritonX 100 in PBS (0.15 mL/well) for 8 min. Pipette out the TritonX 100, rinse with PBS for 10 s (0.15 mL/well), pipette out the PBS, and wash with PBS for 5 min (0.15 mL/well).
2. Block with 5% IgG-free BSA in PBS for 30-60 min (0.15 mL/well).

10. Staining

1. Pipette out the BSA, and add the primary antibody mix diluted in PBS (0.15 mL/well) (see the **Table of Materials**; dilutions: anti-CD31 1:100, anti-FOSB 1:200, anti-GFP 1:1,000, anti-KI67 1:1,000, anti-MyHC 1:400, anti-MYOD 1:200, anti-MYOG 1:150, anti-PAX7 1:100, anti-PDGFRa 1:50) for overnight incubation at 4 °C.

NOTE: After the antibody incubation, collect the antibody mix, add sodium azide, and keep at 4 °C or -20 °C (as per the antibody manufacturer's instructions) for future reuse.

2. Pipette out the antibody mix, rinse with PBS for 10 s (0.15 mL/well), pipette out the PBS, and wash with PBS for 5 min (0.15 mL/well).
3. Pipette out the washing PBS, add the secondary antibody mix (goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 555, goat anti-rat Alexa Fluor 647, goat anti-mouse Alexa Fluor 555, goat anti-chicken Alexa Fluor 488, all used at dilutions of 1:500-1,000) and nucleus marker (e.g., DAPI) diluted in PBS (0.15 mL/well) (see **Table of Materials**), and incubate for 1 h at room temperature, protected from light.

- Pipette out the secondary antibody mix, rinse with PBS for 10 s (0.15 mL/well), pipette out the PBS, wash with PBS for 5 min (0.15 mL/well), pipette out the PBS, and mount.

NOTE: If 8-well plates with removable separators are used, peel off the separators before mounting.

Representative Results

This protocol allows for muscle cell culture while preserving the satellite cells and most cells from their endogenous niche. **Figure 2** summarizes the main steps of the protocol, while essential parts of the dissection and digestion are presented in **Figure 1**. Dissection of the hindlimb musculature is recommended (**Figure 1A-C**), as this group of muscles is well studied and shares a developmental origin and molecular hierarchies¹⁴. Preparing all the mixes under sterile conditions is advised. In addition, lower culture contamination was noticed when the digestion mix was passed through a 0.22 μm filter under a cell culture hood.

When plating 1/30 of a bulk prep onto coated 1 cm^2 well plates, elongated cells are visible 3-4 days after the culture has started. However, this may vary depending on the cell concentration, and elongated cells may start to appear later, particularly when expanding frozen bulk preparations. Approximately 7 days later, the medium should have turned yellow, and at this point, daily medium changes are required. Additionally, at this point, the wells need to be covered with myotubes. The endothelial cells make up a minor fraction of the culture. The main indication of a successful culture is abundant PAX7+ cells, which may require fixation and staining. A convenient mouse model that could be used when possible is the *Tg:Pax7-nGFP* line¹², which allows for the visualization of PAX7 positivity in the form of GFP expression; thus, the satellite cells can be readily detected by

the reporter GFP. GFP can be observed at 20x magnification on an inverted epifluorescence microscope, thus allowing for the analysis of the culture success while the culture is ongoing. This also makes the live imaging of PAX7-GFP cells in bulk cultures possible. Cultures must not be left longer than 10 days. After that, the reserve cells begin to decline in numbers, and the myotubes may detach from the plate. Failed cultures, including based on recent experience with cultures from frozen cells, can still generate a large proportion of fibers but very few PAX7-GFP cells. When using mice without a fluorescent marker for PAX7, it is necessary to perform staining to assess the efficacy of reserve cell generation.

The following antibodies have been tested and work well with the staining protocol presented in protocol section 10: anti-PAX7 (a marker of satellite cells and activated myoblasts¹⁵; also a marker of reserve cells that emerge in this culture), anti-myosin (a marker of myotubes¹⁵), anti-CD31 (a marker of endothelial cells¹⁶), anti-GFP (if reporter mice are used), anti-MYOD (a marker of myoblasts¹⁵), anti-MYOG (a marker of differentiating myocytes¹⁵), anti-KI67 (a marker of proliferating cells¹⁷), and anti-PDGFR α (a marker of FAPs¹⁵). **Figure 3** shows myogenic and niche cells after 7 days in culture. Regarding **Figure 3A-C**, muscle bulks were cultured from wild-type mice, while for **Figure 3D-F**, muscle bulks were cultured from the aforementioned *Tg:Pax7-nGFP* line. Double staining with PAX7 and KI67 (**Figure 3A**) allowed for marking the reserve cells that appeared after 5 days in culture and had muscle stem cell characteristics, such as the exit from the cell cycle (KI67- status) and a localization as "satellites" to the formed myotubes. Double staining with MyHC and MYOG (**Figure 3B,D,E**) allowed for marking the cells that advanced through muscle differentiation and, thus, progressively expressed MYOG and then merged to form multinucleated MyHC+ myotubes. Staining with CD31 or

PDGFR α allowed for marking niche cells (**Figure 3C**), such as endothelial cells and FAPs. **Figure 3D,E** shows emerging reserve cells marked by GFP. Co-staining with GFP and MyHC allowed for evaluating the satellite cell position of the

reserve cells (**Figure 3E**), while co-staining with GFP and other activation/differentiation markers allowed for evaluating the quiescent-like nature of the reserve cells (**Figure 3F**).

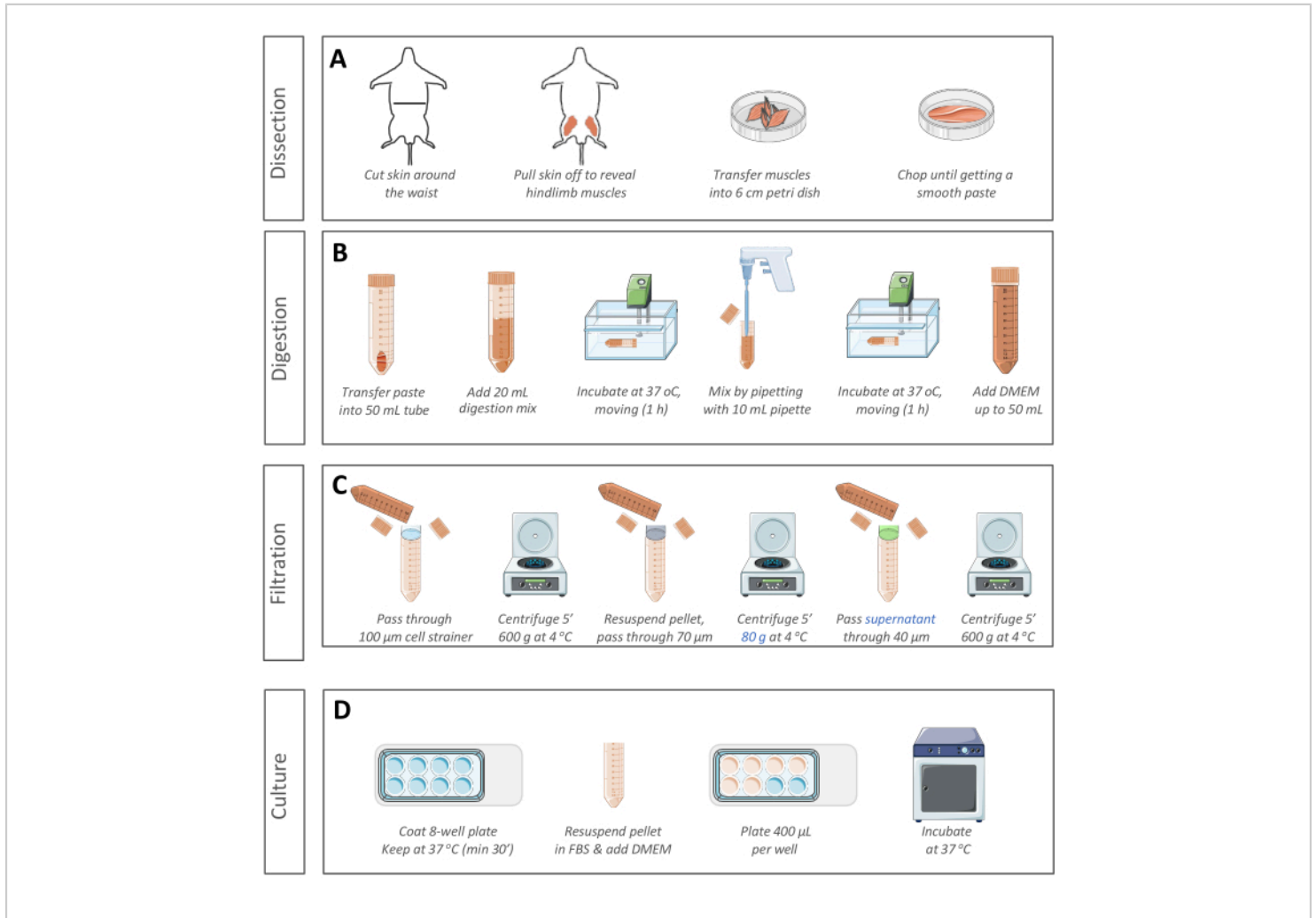


Figure 2: An overview of the protocol steps. (A–D) Sequential steps of **(A)** dissection, **(B)** digestion, **(C)** filtration, and **(D)** culture. [Please click here to view a larger version of this figure.](#)

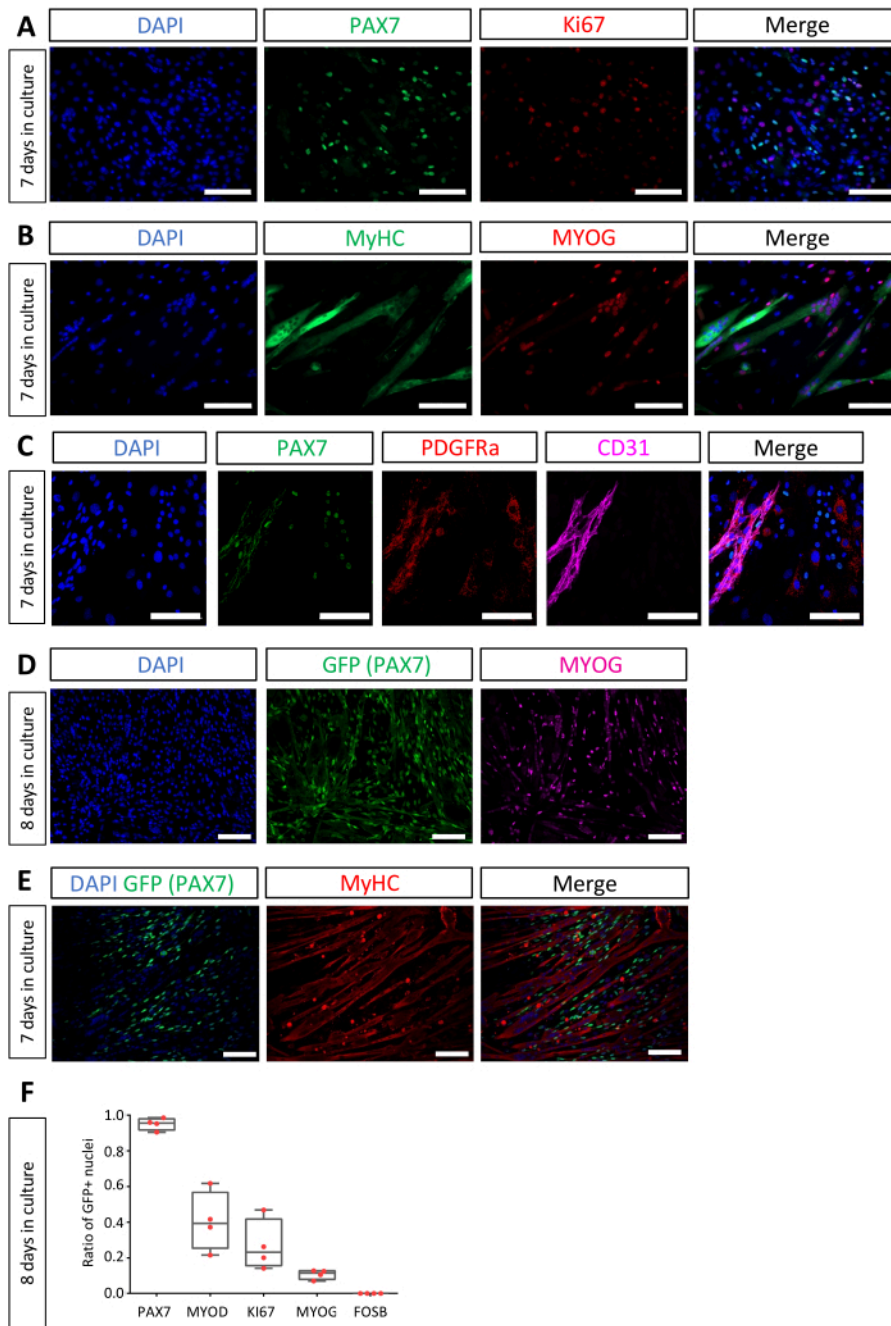


Figure 3: Immunostaining of the cell populations. (A) Immunofluorescence of myogenic (marked by PAX7; green) and cycling (marked by Ki67; red) cells. The nuclei are counterstained with DAPI (blue). The presence of non-cycling myogenic cells (Ki67-PAX7+) shows that the protocol can produce quiescent-like cells from wild-type mice that are abundant at 7 days of culture. (B) Immunofluorescence of myotubes (marked by myosin heavy chain [MyHC]; green) and myocytes (marked by MYOG; red) after 7 days of culture of cells from wild-type mice. The nuclei are counterstained with DAPI (blue). (C) Immunofluorescence of myogenic cells (marked by PAX7; green), mesenchymal fibro-adipogenic progenitors (marked by

PDGFRa; red), and endothelial cells (marked by CD31; magenta) after 7 days of culture of cells from wild-type mice. Nuclei are counterstained with DAPI (blue). **(D)** Immunofluorescence of GFP+ cells (green) and myocytes (marked by MYOG; magenta) after 8 days of culture of cells from *Tg:Pax7-nGFP* mice, in which PAX7-expressing cells are marked by nuclear GFP. **(E)** Immunofluorescence of GFP+ cells (green) and myotubes (marked by MyHC; red) after 7 days of culture of cells from *Tg:Pax7-nGFP* mice. Note that the satellite-cell-like cells appear in the culture after 7 days. **(F)** Quantification of the GFP+ cell proportion that co-expresses markers of satellite cells (PAX7), activation (FOSB), proliferation (KI67), or myogenic differentiation (MYOD, MYOG). The error bars indicate the standard deviation. Scale bar: 100 μ m. [Please click here to view a larger version of this figure.](#)

Discussion

Adult skeletal muscle function is underpinned by a finely orchestrated set of cellular interactions and molecular signals. Here, a method is presented that allows for the study of these parameters in an *ex vivo* setting that closely resembles the physiological microenvironment.

Several groups have reported *in vitro* methods to culture myogenic cells. These methods aimed to isolate satellite cells to study their myogenic progenitor properties. Two main approaches are used to isolate pure satellite cells, either from cultures of isolated fibers from soleus and/or EDL muscles¹⁸ or from FACS-isolated satellite cells, from bulk muscles, using a panel of antibodies for Pax7, CD34, and α 7-integrin for positive selection and CD45, CD31, and Sca1 for negative selection^{19,20,21}. Others have also used mechanical taps to isolate primary myoblasts from bulk cultures on gelatin-plated plates due to their fragile adhesion properties compared to other cells such as FAPs^{22,23}. While these approaches are suitable for studying the activation of satellite cells or for expanding them in culture, the cells grow in an environment deprived of the supporting cells that usually contribute to their niche. In addition, these approaches require keeping the myogenic progenitors for more than 2 weeks to establish quiescent mononucleated reserve cells with high Pax7 expression¹⁸. Co-cultures of satellite cells and other

cell types, such as macrophages, have also been reported.

This has enabled the study of the direct contribution of a specific cell to myogenic properties such as proliferation, differentiation, and fusion²⁴. Several single-cell RNA-seq studies have detailed the cellular makeup of the skeletal muscle^{25,26}. As our culture conditions favor the generation of myogenic cells, we have observed a much higher proportion of reserve cells and a lower proportion of support cells in bulk cultures compared to these *in vivo* studies.

Three critical steps have been identified in the method described here that can increase the efficiency. Firstly, fast and efficient chopping is necessary to ensure optimal muscle dissociation during the 2 h of digestion and, subsequently, a higher cell yield. Secondly, continuous rotation during digestion is very important to ensure the proper diffusion of the enzymes through the tissue. Finally, gentle mechanical dissociation is also important for optimal tissue dissociation.

There are four main limitations of this protocol. Firstly, it remains an *ex vivo* system, meaning that some physiological and biophysical cues might be lost or altered. This includes signaling to/from the muscle fibers, as the fibers are lost before plating, and the myotubes are rebuilt during culture. Secondly, it remains to be seen whether the formed myotubes have embryonic or adult characteristics and whether they represent the different fiber types in slow and fast muscles.

For reference, cultures of the C2C12 muscle cell line and human myoblasts have shown that myotubes in 2D culture are less mature than their *in vivo* counterparts^{27,28}, while primary mouse myoblast cultures seem to lead to myosin types representative of the ones found *in vivo*²⁹. Thirdly, with this method, the cells lose their *in situ* position during tissue dissociation, and the newly formed myotubes lack neuromuscular and myotendinous junctions; thus, results on spatial features should be interpreted with caution. Fourth, the presented protocol has been optimized for healthy adult muscles, but adaptations might be necessary if the starting material originates from aged or myopathic muscles that present extensive fibrosis, increased adipogenic deposition, or compromised cell activation.

Most of these limitations are common to other methods of *ex vivo* muscle studies, but this protocol has several advantages over them. It is the only protocol that allows for harvesting large numbers of quiescent-like reserve satellite cells. In addition, it represents a cheap and straightforward method that does not require the special culture conditions or expertise associated with induced pluripotent stem cells, embryonic stem cells, myosphere culture, or organoid culture. Moreover, the bulk culture does not depend on the complicated infrastructure and standardization that is associated with cultures of myogenic cells in hydrogels or other scaffolds. Compared to cultures of isolated myofibers, FACS-isolated primary cells, or preplating-enriched primary cells, the bulk culture preserves more cell populations that are present in the physiological muscle environment. These cells pass through a relatively physiological path of activation and differentiation driven by the culture medium's growth factors rather than the extreme and unusual signals from the snake venoms commonly used to study muscle regeneration *in vivo*^{30,31}. Finally, the presented cell culture is advantageous

over using C2C12 cells, which, as with any cell line, have genetic alterations compared to endogenous or primary cells.

The above advantages make this protocol a powerful tool for applications such as cell amplification and the generation of a quiescent-like satellite cell pool, which are necessary to develop cell therapies or even answer fundamental questions on quiescence and cellular/molecular regulation. In addition, the protocol can be used to modify molecular signaling *via* drugs, siRNA targeting, or transfection with vectors (plasmid, viruses) that induce the overexpression or expression of dominant negative forms of the factors of interest.

Disclosures

The authors declare no conflicts of interest.

Acknowledgments

For **Figure 2**, templates from Servier Medical Art (<https://smart.servier.com/>) were used. The FR lab is supported by the Association Française contre les Myopathies - AFM *via* TRANSLAMUSCLE (grants 19507 and 22946), the Fondation pour la Recherche Médicale - FRM (EQU202003010217, ENV202004011730, ECO201806006793), the Agence Nationale pour la Recherche - ANR (ANR-21-CE13-0006-02, ANR-19-CE13-0010, ANR-10-LABX-73), and the La Ligue Contre le Cancer (IP/SC-17130). The above funders had no role in the design, collection, analysis, interpretation, or reporting of this study or the writing of this manuscript.

References

1. Frontera, W. R., Ochala, J. Skeletal muscle: A brief review of structure and function. *Calcified Tissue International*. **96** (3), 183-195 (2015).

2. Forcina, L., Cosentino, M., Musarò, A. Mechanisms regulating muscle regeneration: Insights into the interrelated and time-dependent phases of tissue healing. *Cells*. **9** (5), 1297 (2020).
3. Mauro, A. Satellite cell of skeletal muscle fibers. *Journal of Biophysical and Biochemical Cytology*. **9** (2), 493-495 (1961).
4. Lepper, C., Partridge, T. A., Fan, C.-M. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development*. **138** (17), 3639-3646 (2011).
5. McCarthy, J. J. et al. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development*. **138** (17), 3657-3666 (2011).
6. Murphy, M. M., Lawson, J. A., Mathew, S. J., Hutcheson, D. A., Kardon, G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development*. **138** (17), 3625-3637 (2011).
7. Sambasivan, R. et al. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development*. **138** (17), 3647-3656 (2011).
8. Hicks, M. R., Pyle, A. D. The emergence of the stem cell niche. *Trends in Cell Biology*. **33** (22), 112-123 (2022).
9. Relaix, F. et al. Perspectives on skeletal muscle stem cells. *Nature Communications*. **12** (1), 692 (2021).
10. Gama, J. F. G. et al. Role of regulatory T cells in skeletal muscle regeneration: A systematic review. *Biomolecules*. **12** (6), 817 (2022).
11. Loreti, M., Sacco, A. The jam session between muscle stem cells and the extracellular matrix in the tissue microenvironment. *NPJ Regenerative Medicine*. **7** (1), 16 (2022).
12. Sambasivan, R. et al. Distinct regulatory cascades govern extraocular and pharyngeal arch muscle progenitor cell fates. *Developmental Cell*. **16** (6), 810-821 (2009).
13. Pereira, P. D. et al. Quantification of cell cycle kinetics by EdU (5-ethynyl-2'-deoxyuridine)-coupled-fluorescence-intensity analysis. *Oncotarget*. **8** (25), 40514-40532 (2017).
14. Bismuth, K., Relaix, F. Genetic regulation of skeletal muscle development. *Experimental Cell Research*. **316** (18), 3081-3086 (2010).
15. Yin, H., Price, F., Rudnicki, M. A. Satellite cells and the muscle stem cell niche. *Physiological Reviews*. **93** (1), 23-67 (2013).
16. Lertkiatmongkol, P., Liao, D., Mei, H., Hu, Y., Newman, P. J. Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). *Current Opinion in Hematology*. **23** (3), 253-259 (2016).
17. Scholzen, T., Gerdes, J. The Ki-67 protein: From the known and the unknown. *Journal of Cellular Physiology*. **182** (3), 311-322 (2000).
18. Abou-Khalil, R., Le Grand, F., Chazaud, B. Human and murine skeletal muscle reserve cells. *Stem Cell Niche*. **1035**, 165-177 (2013).
19. Pasut, A., Oleynik, P., Rudnicki, M. A. Isolation of muscle stem cells by fluorescence activated cell sorting cytometry. *Methods in Molecular Biology*. **798**, 53-64 (2011).
20. Liu, L., Cheung, T. H., Charville, G. W., Rando, T.A. Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nature Protocols*. **10** (10), 1612-1624 (2015).

21. Montarras, D. et al. Direct isolation of satellite cells for skeletal muscle regeneration. *Science*. **309** (5743), 2064-2067 (2005).
22. Qu, Y., Edwards, K., Barrow, J. Isolation, culture, and use of primary murine myoblasts in small-molecule screens. *STAR Protocols*. **4** (2), 102149 (2023).
23. Danoviz, M. E., Yablonka-Reuveni, Z. Skeletal muscle satellite cells: Background and methods for isolation and analysis in a primary culture system. *Methods in Molecular Biology*. **798**, 21-52 (2011).
24. Saclier, M., Theret, M., Mounier, R., Chazaud, B. Effects of macrophage conditioned-medium on murine and human muscle cells: analysis of proliferation, differentiation, and fusion. *Methods in Molecular Biology*. **1556**, 317-327 (2017).
25. Giordani, L. et al. High-dimensional single-cell cartography reveals novel skeletal muscle-resident cell populations. *Molecular Cell*. **74** (3), 609-621.e6 (2019).
26. Tabula Muris Consortium et al. Single-cell transcriptomics of 20 mouse organs creates a Tabula Muris. *Nature*. **562** (7727), 367-372 (2018).
27. Brunetti, J., Koenig, S., Monnier, A., Frieden, M. Nanopattern surface improves cultured human myotube maturation. *Skeletal Muscle*. **11** (1), 12 (2021).
28. Denes, L. T. et al. Culturing C2C12 myotubes on micromolded gelatin hydrogels accelerates myotube maturation. *Skeletal Muscle*. **9** (1), 17 (2019).
29. LaFramboise, W. A. et al. Effect of muscle origin and phenotype on satellite cell muscle-specific gene expression. *Journal of Molecular and Cellular Cardiology*. **35** (10), 1307-1318 (2003).
30. Azhar, M., Wardhani, B. W. K., Renesteen, E. The regenerative potential of Pax3/Pax7 on skeletal muscle injury. *Journal of Generic Engineering and Biotechnology*. **20** (1), 143 (2022).
31. Hardy, D. et al. Comparative study of injury models for studying muscle regeneration in mice. *PLoS One*. **11** (1), e0147198 (2016).