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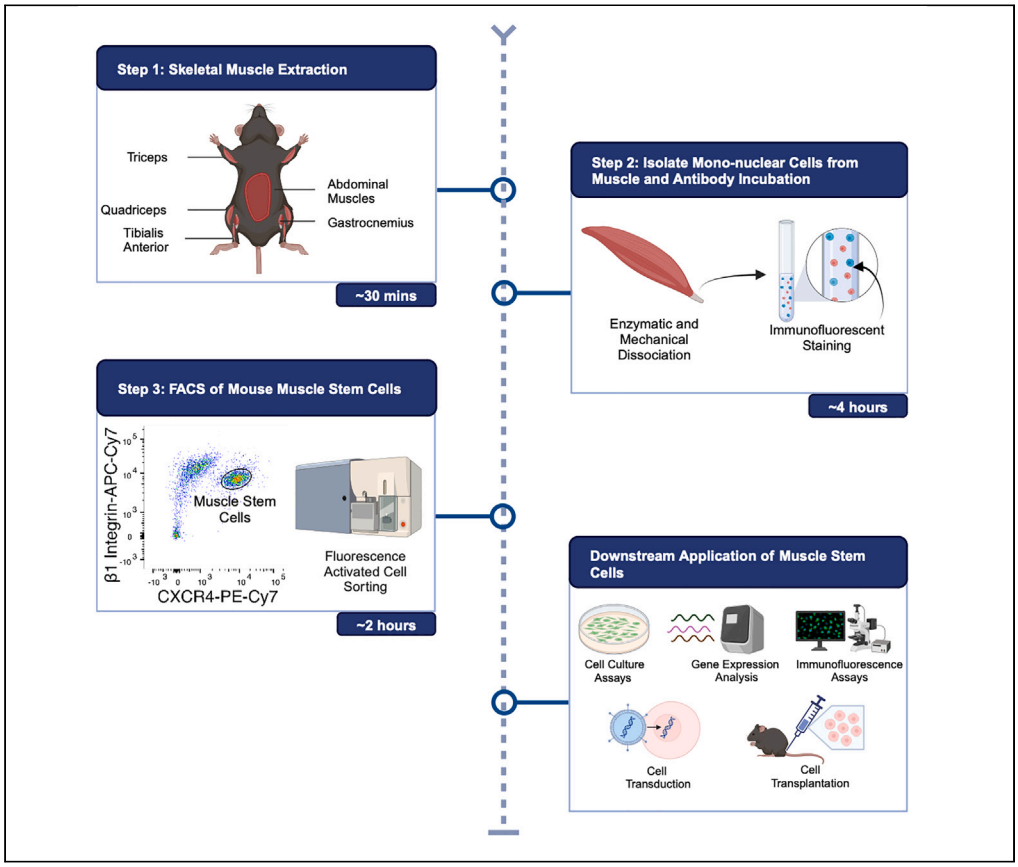
Protocol for the isolation of mouse muscle stem cells using fluorescence-activated cell sorting



Muscle stem cells (MuSCs) are the building blocks for regenerating skeletal muscle after trauma. If we intend to maximize the therapeutic potential of MuSCs, we must further study their molecular and functional properties. Here, we present a protocol for the isolation of mouse MuSCs via a two-step enzymatic and mechanical dissociation of skeletal muscle coupled with fluorescence-activated cell sorting (FACS). FACS-isolated MuSCs can be used for various downstream applications including cell culture, cell transduction, immunofluorescence, and gene expression assays.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

H1: Optimized technique to isolate mouse MuSCs by FACS

H2: *Ex vivo* culturing approaches to study MuSC fate decisions

H3: Immunofluorescence and RNA analysis of MuSCs

H4: Approaches for transduction and transplantation of MuSCs

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Protocol for the isolation of mouse muscle stem cells using fluorescence-activated cell sorting

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SUMMARY

Muscle stem cells (MuSCs) are the building blocks for regenerating skeletal muscle after trauma. If we intend to maximize the therapeutic potential of MuSCs, we must further study their molecular and functional properties. Here, we present a protocol for the isolation of mouse MuSCs via a two-step enzymatic and mechanical dissociation of skeletal muscle coupled with fluorescence-activated cell sorting (FACS). FACS-isolated MuSCs can be used for various downstream applications including cell culture, cell transduction, immunofluorescence, and gene expression assays.

For complete details on the use and execution of this protocol, please refer to Almada et al. (2021).[1]

BEFORE YOU BEGIN

Preparing plates for cell culture

Timing: Prepared 24 h prior to the procedure, duration: 10 min

Pre-coat wells of a multi-well culture plate with enough Coating Media (See materials and equipment) to cover the entire bottom of the well and incubate the plates for ~24 h at 37°C.

Institutional permissions

All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California. Others who plan to replicate this protocol should receive appropriate approval from their institution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-mouse CD11b (Mac-1) (1:200)	BioLegend	Cat# 101212; RRID: AB_312795
APC anti-mouse CD31 (1:200)	BioLegend	Cat# 102510; RRID: AB_312917

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
APC anti-mouse CD45 (1:200)	BioLegend	Cat# 103112; RRID: AB_312977
APC anti-mouse Ly-6A/E (Sca-1) (1:200)	BioLegend	Cat# 122512; RRID: AB_756197
APC anti-mouse TER-119 (1:200)	BioLegend	Cat# 116212; RRID: AB_313713
APC-Cy7 anti-mouse CD29 (β_1 integrin) (1:200)	BioLegend	Cat# 102226 RRID: AB_2128076
Biotin anti-mouse CD184 (CXCR4) (1:100)	BioLegend	Cat# 146516; RRID: AB_2650787
PE-Cy7 streptavidin (1:200)	BioLegend	Cat# 405206
Goat anti-mouse-IgG-AF555 (1:500)	Invitrogen	Cat# A28180; RRID: AB_2536164
Mouse anti-mouse myogenin (1:100)	BD Biosciences	Cat# 556358; RRID: AB_396383
Mouse anti-mouse PAX7 (1:20)	DSHB	RRID: AB_528428
Chemicals, peptides, and recombinant proteins		
ACK lysing buffer	Gibco	Cat# A1049201
Human basic fibroblast growth factor (bFGF)	Sigma-Aldrich	Cat# F0291-25UG
Calcein blue, AM	Invitrogen	Cat# C1429
Barium chloride	Sigma-Aldrich	Cat# 202738-5G
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A9418-50G
Cardiotoxin (CTX)	Latoxan	Cat# L8102-1MG
Collagen I, rat tail	Gibco	Cat# A1048301
Collagenase type II, powder	Gibco	Cat# 17101015
Dispase II, powder	Gibco	Cat# 17105041
Donor bovine serum (DBS)	Gibco	Cat# 16030074
Dulbecco's modified Eagle's medium (DMEM), high glucose	Gibco	Cat# 11965118
0.5 M EDTA, pH 8.0	VWR Chemicals	Cat# E177-100ML
Fetal bovine serum (FBS)	Avantor	Cat# 89510-196
GlutaMAX supplement	Gibco	Cat# 35050061
Normal goat serum	Jackson Immuno Research Laboratories	Cat# 005-000-121
Ham's F-10 nutrient mix (F10)	Gibco	Cat# 11550043
Hank's balanced salt solution (HBSS), with calcium and magnesium, no phenol red	Gibco	Cat# 14025134
Hoechst 33342 solution, 20 mM	Thermo Scientific	Cat# 62249
Laminin mouse protein, natural	Gibco	Cat# 23017015
Phosphate-buffered saline (PBS), without calcium and magnesium	Corning	Cat# 21-040-CV
Propidium iodide (PI)	Sigma-Aldrich	Cat# 537060
Triton X-100	Thermo Scientific	Cat# J66624.AP
TRIzol LS reagent	Invitrogen	Cat# 10296010
Tween 20	Sigma-Aldrich	Cat# P1379
Paraformaldehyde solution, 32%	Electron Microscopy Sciences	Cat# 15714-S
Penicillin-Streptomycin, 10,000 U/mL	Gibco	Cat# 15140122
Polybrene	Sigma-Aldrich	Cat# TR-1003-G
PEG-it virus precipitation solution	System Biosciences	Cat# LV810A-1
Experimental models: Organisms/strains		
Mouse: C57BL/6J, male, approximately 7 weeks old	The Jackson Laboratory	Cat# 000664; RRID:IMSR_JAX:000664
Human: 293T/17 (HEK 293T/17)	ATCC	Cat# CRL-11268
Software and algorithms		
Fiji (ImageJ)	https://fiji.sc	N/A
FlowJo	https://www.flowjo.com	10.9.0

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
40 µm cell strainers, sterile	Corning	Cat# 352340
70 µm cell strainers, sterile	Corning	Cat# 352350
Beckman Coulter Avanti J-15R centrifuge	Beckman Coulter	Cat# B99517
Beckman Coulter Microfuge 20R centrifuge	Beckman Coulter	Cat# B31612
Glass Pasteur pipets	VWR International	Cat# 14673-043
Hemocytometer	Hausser Scientific	Cat# 3110V
Insulin syringe with 31 gauge needle	BD Biosciences	Cat# 328438
Latex bulbs	Avantor	Cat# 82024-550
Nalgene filter units, 0.2 µm	Thermo Scientific	Cat# 157-0020
Polypropylene conical tube, 15 mL	Falcon	Cat# 352097
Polypropylene conical tube, 50 mL	Falcon	Cat# 352098
Polystyrene round-bottom tube, 5 mL	Falcon	Cat# 352052
Shaking water bath	Thermo Scientific	Cat# TSSWB27

MATERIALS AND EQUIPMENT

- Digest 1 Mix: DMEM with 0.2% Collagenase Type II.

[Keep on ice].

- Digest 1 Stop Solution: F10 with 20% FBS or DBS.

[Keep on ice].

Digest 2 Mix	
Reagent	Final concentration
Collagenase Type II (285 U/mg)	0.0125%
Dispase II (1.81 U/mg)	0.05%
F10	To final volume

⚠ CRITICAL: Warm to 37°C starting at step 24.

- Staining Media (SM): HBSS with 2% FBS or DBS.

[Keep on ice].

Coating Media	
Reagent	Final concentration
Collagen Type I	1 µg/mL
Laminin	10 µg/mL
PBS	To final volume
[We use coating media stored at 4°C for up to 2 weeks after preparation.]	

Growth Media (GM)	
Reagent	Final concentration
Donor Horse Serum (DHS)	20%
Penicillin/Streptomycin	1%

(*Continued on next page*)

Continued	
Reagent	Final concentration
GlutaMAX	1%
F10	To final volume
[We use GM stored at 4°C for up to 2 weeks after preparation.]	

Differentiation Media (DM)	
Reagent	Final concentration
Donor Horse Serum (DHS)	2.5%
Penicillin/Streptomycin	1%
Dulbecco's modified Eagle's medium (DMEM)	To final volume
[We use DM stored at 4°C for up to 2 weeks after preparation.]	

STEP-BY-STEP METHOD DETAILS

Preparation of muscle digestion media

Timing: ~10 min

Enzymatic digestion of skeletal muscle is the essential first step. Ensure that Digest mix 1 is stored on ice prior to step 8.

1. Prepare Digest 1 mix containing 0.2% Collagenase Type II (285 U/mg) dissolved in DMEM.
   a. Pass digest 1 solution through a filtered tissue culture bottle (Thermo Scientific, Cat# 157-0020) and keep on ice until step 8.

Extraction of mice skeletal muscle

Timing: ~10–15 min per mouse

This step details our strategy for extracting skeletal muscle from mice. Maximizing your skeletal muscle amount at the beginning will increase your yield of FACS isolated muscle stem cells at the end of the protocol.

2. Prepare a de-contaminated dissection field on a benchtop by cleaning it with 70% ethanol.
3. Transfer 20 mL of Digest 1 mix into a tube labeled Digest 1. Prepare a tube for each replicate mouse. Keep the tubes on ice.
4. Euthanize mice according to your institution's approved IACUC protocol.
5. Spray down mouse with 70% ethanol, remove skin, and extract hindlimb (Figures 1A and 1B), triceps (Figure 1C), and abdominal (Figure 1D) skeletal muscles and place muscles in tube containing ice-cold Digest 1 mix.
6. Seal the tube containing Digest 1 and muscle with parafilm. Keep the tube on ice until step 8.
7. Repeat steps 4–6 for each additional replicate mouse. Disinfect dissection tools and dissection platform between each mouse with 70% ethanol to avoid cross-contamination of samples.

⚠ CRITICAL: It may be necessary to wash skeletal muscle in a petri dish filled with PBS prior to step 6 to remove excess blood from the dissection.

Enzymatic and mechanical dissociation of skeletal muscle to release mono-nuclear cells associated with skeletal muscle

Timing: ~2 h for 1 mouse. Add 15–30 min for each additional mouse.

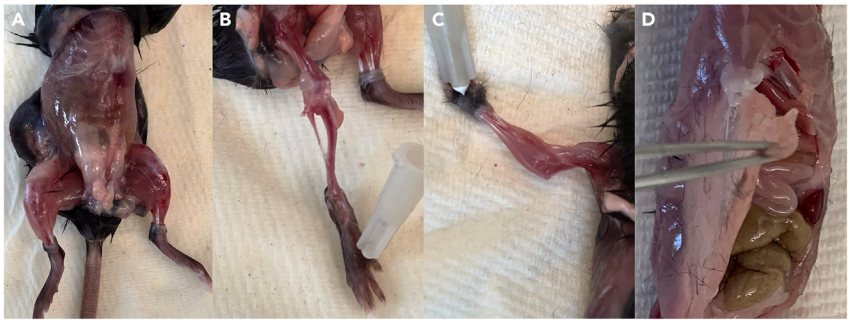

Figure 1. Extraction of hindlimb, triceps, and abdominal skeletal muscles from adult mice

Mouse skeletal muscle dissection.

(A) Create a transverse incision along the waist and peel away skin and underlying fascia to access (B) hindlimb, (C) forelimb, and (D) abdominal skeletal muscles.

Enzymatic digestion of skeletal muscle loosens the connections between bulk skeletal muscle fibers and mechanical dissociation releases single fibers. Gravitational sedimentation is performed to separate interstitial cells from single fibers containing MuSCs. Lastly, a second digestion step releases MuSCs from underneath the basal lamina of myofibers prior to FACS.

8. Disinfect tubes with 70% ethanol and incubate tube(s) containing Digest 1 and muscle at 37°C in a shaking (55 RPM) water bath for 90 min.
    a. Use weighted rings to keep 50 mL conical tubes secured to the bottom of the shaking bath.
9. Prepare the following during Digest 1 incubation:
    a. Prepare glass tips (1 per mouse) for trituration by safely breaking off the thin barrel of a glass Pasteur pipet. Attach a latex bulb over the broken opening of the pipet (Figure 2).
    b. Label 2 × 50 mL conical tubes per mouse.
    c. Prepare 5 mL of Digest 1 stop solution (20% FBS or DBS in F10) for each mouse.
10. After Digest 1 incubation is complete, remove floating debris from Digest 1 tubes by carefully decanting each tube.
11. Inactivate Digest 1 by adding 5 mL of Digest 1 stop solution to each tube.
12. Gently swirl the tube(s) to mix.
13. Carefully add 25 mL of PBS to each Digest 1 tube by pipetting to the wall of the tube.
    a. Slowly decant the supernatant, leaving only a little liquid covering the muscle.
14. Repeat Step 13 two more times to remove residual Digest 1 Mix.
    a. The supernatant should become clearer with each wash.
15. After the third wash, pour all muscles into a petri dish that contains 25 mL of PBS to start the trituration process (Figure 3A).
16. Gently triturate the largest muscles (i.e., quadriceps and gastrocnemius) first, mincing several times for each muscle. See Methods video S1 for an example of optimal trituration cadence and technique (Methods video S1).
    a. During the first round of trituration, the PBS solution should turn very cloudy from myofibers that are released into solution (Figure 3B).
17. Collect all the solution (including released myofibers which contain MuSCs under their basal lamina) from the petri dish using a serological pipet. Split the collected solution equally into two 50 mL collection tubes for each mouse.

    Note: After each successive round of trituration, there will be a gradual decline in the cloudiness of the supernatant in the petri dish.

18. Add 25 mL of PBS into the petri dish for a second round of mincing. Triturate smaller muscles (i.e., triceps, tibialis anterior, and abdominal muscles). Repeat step 16 (Figure 3C).

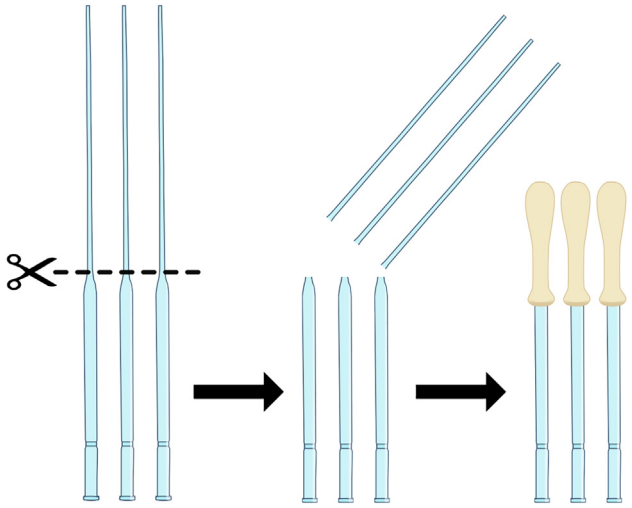

Figure 2. Preparing glass Pasteur pipets for trituration

Safely cut Pasteur pipets at the dotted line and place a bulb on the cut end of the pipet. We note that the users can fire-polish the uncut end to create different size diameters which may help with the mincing step.

19. Add 25 mL of PBS into each dish for a third round of mincing. Triturate all muscles. Repeat step 16. (Figure 3D).
20. Fill up each sedimentation collection tube to 50 mL with PBS. Place all tubes upright in a stationary 37°C incubator for 25 min for gravitational sedimentation to occur.

⚠ CRITICAL: The sedimentation step separates interstitial cells (low density) from myofibers (high density). Tubes should be minimally perturbed through steps 19–26. Rough handling at this stage will lead to myofibers being resuspended in the supernatant, which will decrease the final MuSC yield.

21. During the first sedimentation step, label one 50 mL conical tube per mouse and keep the tube on ice.

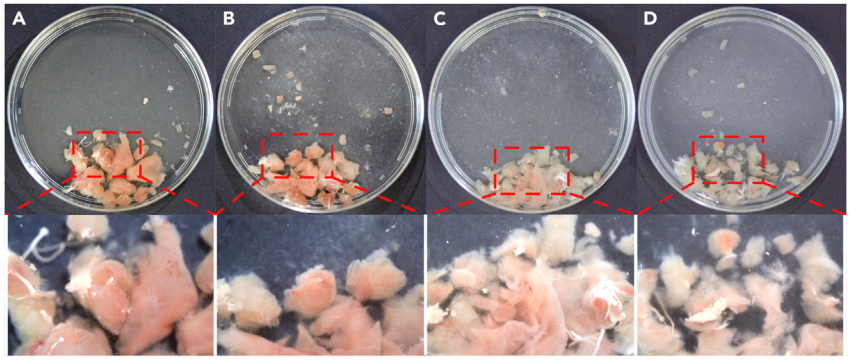

Figure 3. Mechanical dissociation of skeletal muscle to release single fibers

Trituration of skeletal muscle releases myofibers. (A) Digested muscle is poured into a dish containing PBS and subsequently triturated.

(B) A gentle round of trituration releases myofibers, with (C) a second round of trituration liberating additional myofibers from muscle.

(D) If optimally performed, the third round of trituration results in muscles appearing paler and less opaque because of releasing most of the myofibers. In a subsequent digestion step, MuSCs can be released from the basal lamina of myofibers.

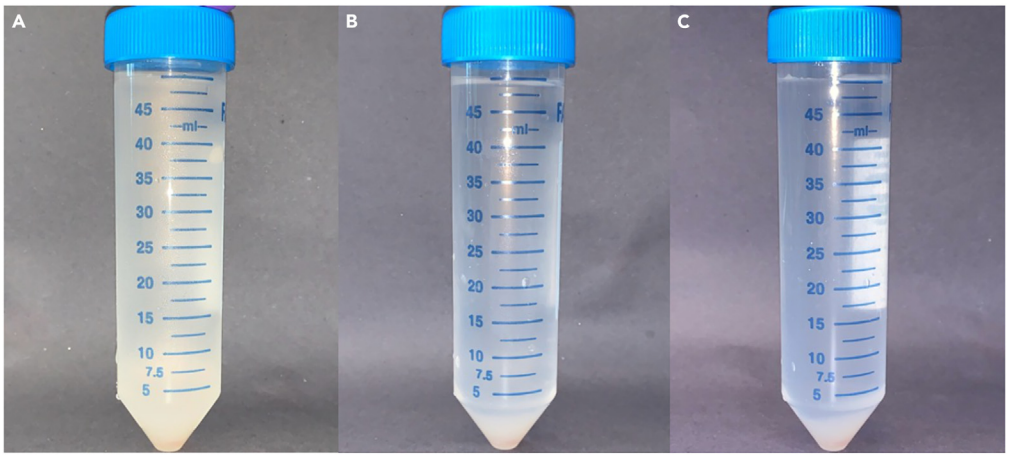

Figure 4. Gravitational sedimentation of myofibers

Phases of gravitational sedimentation.

(A) Interstitial cells (ICs) are dispersed in the supernatant after the first round of sedimentation.

(B) By the second round, the supernatant becomes clearer, as IC cells are progressively depleted from the supernatant.

(C) By the third round of gravitational sedimentation, the supernatant is visibly clear and myofibers are enriched at the bottom of the tube.

a. Collect the interstitial cells (ICs) (i.e., supernatant in collection tubes), including MuSCs liberated early from myofibers after Digest 1, and use them for single-color and fluorescent-minus-one (FMOs) controls.

22. After the first sedimentation step is complete, carefully remove the collection tubes from the incubator.
    a. Collect 15 mL of supernatant from each tube and deposit the solution into the ICs tube(s) and leave on ice.
    b. Subsequently, add 15 mL of fresh PBS to each sedimentation tube by pipetting onto the side of the wall for each tube. Be careful to not disturb the myofiber sediment at the bottom of the tube.
23. Incubate the collection tubes for 15 min at 37°C (Figure 4B).
24. During the second sedimentation step, prepare Digest 2 mix (0.0125% Collagenase Type II (285 U/mg)/0.05% Dispase II (1.81 U/mg) in F10). Keep Digest 2 mix at 37°C until needed in step 27.
    a. Filter Digest 2 mix through a filtered tissue culture bottle (Thermo Scientific, Cat# 157-0020).
25. Carefully remove 20 mL of supernatant from each sedimentation tube. Gently add 20 mL of fresh PBS into the tube without disturbing sediment at the bottom of the tube.
26. Incubate the sedimentation tubes for 10 min at 37°C (Figure 4C).
27. Gently remove supernatant to the 15 mL mark for each tube and immediately add 15 mL of pre-warmed Digest 2 mix to each tube.
28. Seal each tube with parafilm and incubate tubes at 37°C in a shaking (55 RPM) water bath for 30 min.

⚠ CRITICAL: Do not digest myofibers for longer than 30 minutes. Over-digestion leads to excessive cleavage of cell surface markers that are necessary for FACS-based isolation of MuSCs. Thus, longer times in Digest 2 may decrease MuSC yield during FACS-isolation.

29. After 30 min, immediately inactivate Digest II by adding 2 mL of Fetal Bovine Serum or Donor Bovine Serum (DBS) to each tube and gently swirl the tube to mix.
30. Release MuSCs from digested myofibers by quickly pipetting the digested fibers up and down 10x times with a 25 mL serological pipet.

31. Centrifuge tubes for 15 s (40 × *g*) at 4°C to sediment leftover debris.
32. Collect the supernatant from each tube and filter it through a 70 μm cell strainer placed in a new 50 mL tube labeled according to each mouse replicate.
33. Centrifuge (400 × *g*) each tube for 5 min at 4°C.
    a. Remove supernatant and resuspend mono-nuclear cells in 0.5–1 mL of SM to count cells on a hemocytometer.
    b. Count ICs for the single-color/Fluorescent minus one (FMO) control samples at this step.

Note: If too many red blood cells are seen in the pellet, perform an Ammonium Chloride Potassium (ACK lysis) treatment to lyse them according to the manufacturer's protocol https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/red-blood-cell-lysis-using-ack-lysing-buffer.html.

Antibody staining of mono-nuclear cells associated with skeletal muscle

Timing: ~2–2.5 h, depending on the number of mice

In this step you will use antibody staining to negatively exclude fibro-adipogenic progenitors (FAPS), endothelial cells, and blood cells while enriching for PAX7-positive MuSCs. In this protocol, we use positive selection cell surface markers, CD29 (β1 Integrin) and CXCR4, which has previously been shown to overlap with Vcam1 and Alpha-7 integrin/CD34 on quiescent or early activated MuSCs.[2]

34. Label 5 mL FACS tubes for unstained, single-color controls (SSC), and fluorescence-minus-one (FMO) controls listed below:
    a. Unstained.
    b. SSC-Propidium Iodine (PI).
    c. SSC-Calcein Blue (CB).
    d. SSC-APC.
    e. SSC-APC-Cy7.
    f. SSC-PE-Cy7.
    g. FMO-APC.
    h. FMO-APC-Cy7.
    i. FMO-PE-Cy7.
35. Label FACS tubes for each experimental sample.

⚠ CRITICAL: Keep a ratio of $1e^6$ cells per 100 μL of SM. We have carefully titrated our antibodies to work optimally at this ratio.

36. Transfer $4e^6$ cells to their respective FACS tubes in a final volume of 400 μL of SM.
37. Stain cells in control and experimental samples with fluorescently conjugated antibodies according to our staining scheme to detect target antigens (Table 1).
    a. Shield stained cells from light and keep tubes partially immersed in ice to prevent cells from internalizing antibodies.
38. Incubate stained cells on ice for 30 min.
39. Wash stained cells by adding 3 mL of SM to each FACS tube. Centrifuge (360 × *g*) tubes for 5 min at 4°C. Decant the supernatant and resuspend in 400 μL of SM.
40. Incubate stained cells with PE-Cy7 Streptavidin antibody for 20 min and keep on ice.
41. Wash stained cells by adding 3 mL of SM to each tube. Centrifuge (360 × *g*) for 5 min at 4°C. Remove the supernatant and repeat the wash step one additional time, for 2 washes in total.
42. Filter cells through a 40 μm cell strainer and into freshly labeled FACS tubes to prepare for FACS analysis. Keep stained cells in tubes immersed on ice.
43. 15 min prior to FACS analysis, filter cells through a 40 μm cell strainer.
44. Add PI and Calcein Blue to corresponding tubes 15 min prior to FACS (Table 1).

Table 1. FACS primary antibody scheme

	Antibody	APC anti-mouse Ly-6A/E (Sca-1)	APC anti-mouse CD31	APC anti-mouse TER-119	APC anti-mouse CD45	APC anti-mouse CD11b (Mac-1)	APC-Cy7 anti-mouse CD29 (B1 integrin)	Biotin anti-mouse CD184 (CXCR4)	Streptavid-in-PE-Cy7	Calcein blue (CB)	Propidium iodine (PI)
	Dilution	1:200	1:200	1:200	1:200	1:200	1:200	1:100	1:200	1:1,000	1:1,000
	Unstained	-	-	-	-	-	-	-	-	-	-
	PI	-	-	-	-	-	-	-	-	-	+
	CB	-	-	-	-	-	-	-	-	+	-
Single Color Control (SCC)	APC SCC	+	+	+	+	+	-	-	-	-	-
	APC-Cy7 SCC	-	-	-	-	-	+	-	-	-	-
	PE-Cy7 SCC	-	-	-	-	-	-	+	+	-	-
Fluorescence Minus One (FMO)	APC FMO	-	-	-	-	-	+	+	+	+	+
	APC-Cy7 FMO	+	+	+	+	+	-	+	+	+	+
	PE-Cy7 FMO	+	+	+	+	+	+	-	+	+	+
Experimental Sample(s)		+	+	+	+	+	+	+	+	+	+

Primary Antibody Scheme of FACS Controls and Experimental Samples. '+' indicates the addition of antibody; SCC = Single Color Control; FMO = Fluorescence Minus One.

Flow cytometry and FACS-based purification of MuSCs

Timing: ~2–4 h, depending on the number of mice

Using the appropriate single color and fluorescent-minus-one (FMO) controls, we discuss the key steps and gating strategy for FACS-purifying MuSCs (Figure 5).

45. Set up the BD FACS Aria III cytometer as specified by the manufacturer.
    a. Use a 70 μm nozzle with a flow rate of 2.0–4.0.
46. Run your unstained control to set the background voltage for each channel.
47. Run each single-color control (SCC) sample and set voltage for positive cells and record events for the top 25% of expressing events for fluorophore compensations.
48. After compensation for all channels, run FMO samples to assist with drawing negative gates for APC (CD45, CD11b, TER119, SCA1, CD31) and positive gates for APC-Cy7 (β1 Integrin) and PE-Cy7 (CXCR4) (Figure 5).
49. Sort $CD45^-$; $Cd11b^-$; $TER119^-$; $SCA1^-$; $CD31^-$; β1 $Integrin^+$; $CXCR4^+$ MuSCs for further analysis.
    a. Freshly sorted MuSCs will appear as small, rounded cells that divide over the next 48–72 h as they exit quiescence.

Note: This protocol can easily be used with other positive enriching cell surface markers including Vcam1 and α7-Integrin/CD34, which we previously showed enriches for a largely overlapping PAX7-positive cell population when extracted from uninjured skeletal muscle.[2]

Downstream *ex vivo* and *in vivo* analysis of sorted MuSCs

In this section we discuss downstream applications including cell culture, immunofluorescence (IF), cell transduction, gene expression analysis, and transplantation of MuSCs.

50. Culturing and Passaging MuSCs (Time: 30–60 min to passage).
    a. Pre-coat wells with coating media (See materials and equipment) for 24 h prior to FACS.
    b. Freshly isolated MuSCs can be sorted directly into pre-coated wells that contain GM.
        i. Supplement cultured MuSCs with daily addition of 5 ng/mL bFGF and incubate at 37°C with 5% CO_2.
        ii. Fresh MuSCs FACS-isolated from uninjured muscle will start to divide in 48–72 h.

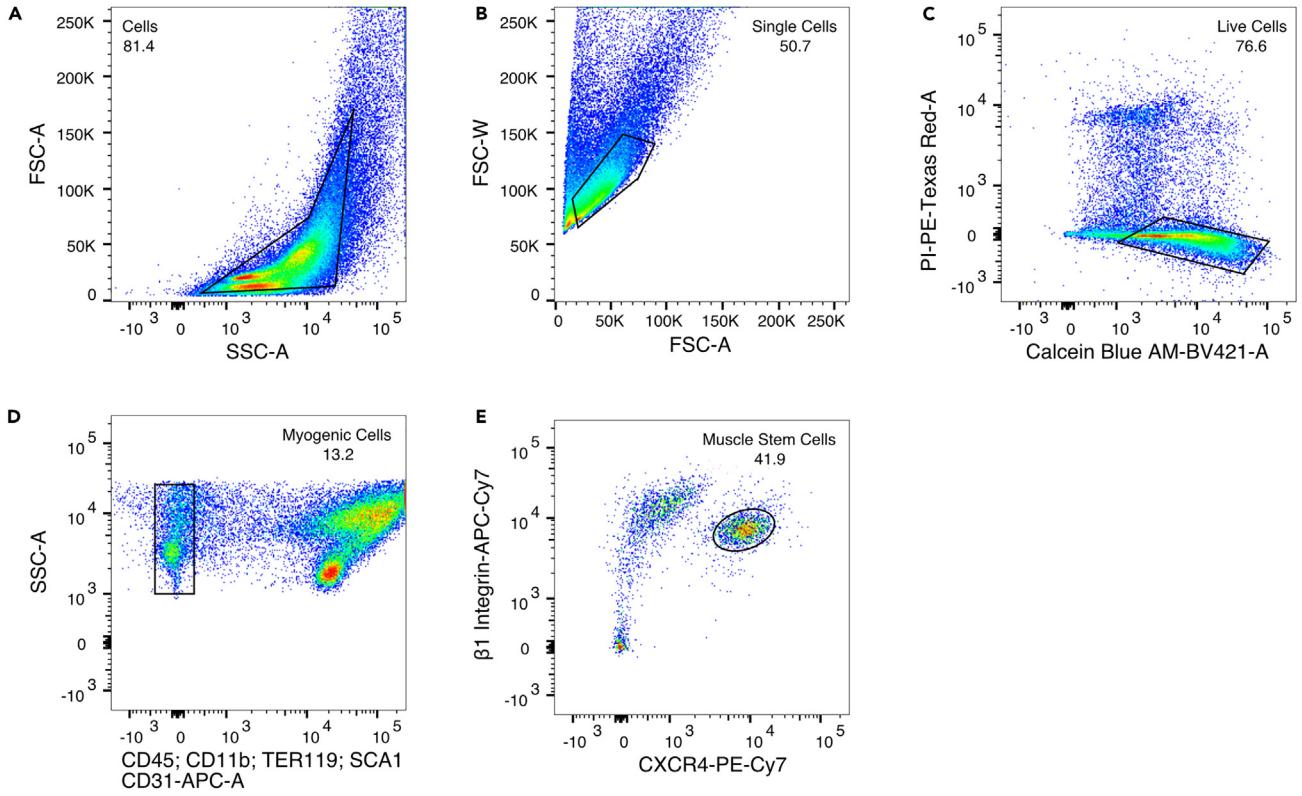

Figure 5. FACS gating plots of β1-integrin and CXCR4+ MuSCs

FACS gating strategy for sorting prospective MuSCs.

(A) Debris are removed.

(B) Single cells are isolated.

(C) PI-negative and Calcein blue-positive cells (live) are gated.

(D) Muscle-resident FAPs (Sca1$^+$), blood cells (CD45$^+$, CD11b$^+$, TER119$^+$) and endothelial cells (CD31$^+$) are excluded by negative selection markers.

(E) MuSCs are identified as double-positive for β_1-integrin and CXCR4.

c. After 3 days of culturing, we remove $^1/_2$ the volume of GM and replaced $^1/_2$ the volume with fresh GM. We repeat this 3 times.

⚠ CRITICAL: Do not remove media completely within the first few days since MuSCs have low adherence in the first 48 hours after FACS isolation.

d. After 5 days of culturing, we passage the progeny of MuSCs (i.e., myoblasts) onto new tissue culture plates by performing the following:
   i. Detach MuSCs from plates by removing $^1/_2$ the volume of GM and replacing it with an equal volume of warmed 5 mM EDTA and incubate plates for 20 min at 37°C.
   ii. Confirm that >90% of cells have been detached from the plate under a microscope and then use a pipet to wash the bottom of each plate to gently push cells into a cell suspension.
   iii. Transfer the cell suspension to a labeled 15 mL conical tube and centrifuge (360 × *g*) for 5 min at 4°C.
   iv. Resuspend the resulting pellet in GM to count cells in the suspension.
   v. Seed cells at the appropriate density and continue culturing in GM.
   vi. Supplement with daily additions of bFGF.

Note: We passage our cell cultures at 75%–80% confluent to prevent terminal differentiation. Re-fresh the media every 2–3 days, as in step 50c.

51. Differentiating MuSC-derived myoblasts into myotubes in culture (Time: 48–72 h).
    a. Seed MuSCs into pre-coated wells containing differentiation media (DM).
        i. We perform differentiation assays in 96-well plates and thus seed ~8,000 cells into 96-wells containing DM and without bFGF.
    b. To differentiate myoblasts into fully formed myotubes, culture the cells in DM for ~48–72 h.
52. Total RNA Extraction from freshly sorted MuSCs for analyzing Gene Expression (Time: 15–30 min).
    a. Prepare an RNase-free environment on the lab bench and label 1.5 mL microcentrifuge tubes.
    b. Aliquot 500 μL of TRIzol LS (Invitrogen, Cat #10296010) into each tube prior to going to the FACS facility.
    c. Sort 1–50,000 MuSCs into 500 μL of TRIzol LS, immediately vortex tube(s), and then add 250 μL of TRIzol LS to bring volume to 750 μL.
    d. Incubate tubes with RNA/TRIzol for 5 min at 25°C.
    e. Store RNA in TRIzol LS at −80°C for up to 2–3 months.
    f. Perform extraction of RNA according to the manufacturer's protocol (TRIzol LS) https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Ftrizol_ls_reagent.pdf.
53. Immunofluorescence staining of fixed MuSCs (Time: 2 Days).
    a. Remove $^1/_2$ the volume of GM from each well. Add back the same volume with 8% paraformaldehyde (PFA) (4% PFA final concentration) and incubate MuSCs at RT for 20 min.
    b. Stop fixation by washing cells with Phosphate Buffered Saline (PBS) with 0.1% Tween 20 (PBST) 3 times for 5 min.
    c. Permeabilize cells with 0.3% Triton-X for 20 min at 25°C.
    d. Wash cells with PBST 3 times for 5 min.
    e. Add blocking buffer (5% Normal Goat Serum, 3% BSA, 0.3% Triton-X in PBS) for 2 h at RT.
    f. Without washing, add primary antibody in blocking buffer to each well and incubate for ~12 h at 4°C.
    g. Remove primary antibody solution and wash cells 4 times for 15 min with PBST at 25°C.
    h. Add secondary antibody in blocking buffer to wells and incubate for 1 h at 25°C.
    i. Remove the secondary antibody from solution by washing cells with PBST 4 times for 15 min at 25°C.
    j. Add Hoechst stain (1 μg/mL Hoechst in PBS) to cells and incubate for 15 min at 25°C. Alternatively, you can add Hoechst into the third wash in step 53i.
    k. Remove Hoechst stain solution and wash cells with PBST for 5 min.
54. Transduction of MuSCs with Lentivirus (Time: 2.5 h for transduction).
    a. We titrate our virus to determine the optimal density that gives maximal transduction and minimal toxicity (anywhere from 1:100-1:500) as determined by a GFP fluorescence and/or antibiotic selection.
    b. We make virus in 293T cells, concentrate virus with PEG-it viral precipitation reagent (System Biosciences, Cat# LV810A-1), and then resuspend in GM supplemented with 25 mM HEPES.
    c. Sort 3,000 MuSCs into coated wells of a 96 well plate containing 200 μL of GM.
    d. In a viral-only Biohazard class II biosafety cabinet, prepare diluted lentiviral stock by diluting lentivirus solution with 8 μg/mL Polybrene (Sigma-Aldrich, Cat. TR-1003-G) at the empirically tested viral ratio.
    e. Remove $^1/_2$ the volume of GM from each well and add back the same volume with diluted viral media to get the desired ratio. Carefully, pipet each well 5x times with a P200 pipet and incubate the plate for 15 min at 37°C.
    f. Quickly parafilm plates shut to prevent spilling of virus while spinning cells and centrifuge (930 × *g*) plates for 90 min at 32°C.
    g. Wash out virus by removing $^1/_2$ volume (100 μL) and replacing $^1/_2$ volume (100 μL) with fresh GM 8 times.

h. After the last wash, add bFGF to a final concentration of 5 ng/mL before returning to 37°C to each well daily.

Note: Depending on your viral construct (i.e., constitutive versus inducible) your protein of interest can be expressed within 24–48 h.[3]

55. Transplantation of MuSCs into pre-injured mice (Time: 2 h).
    a. Following your institution's IACUC protocol, 24 h prior to transplantation, inject a chemical agent into the Tibialis Anterior (TA) muscle of mice to induce injury.

Note: We readily use 25 μL of 10 μM cardiotoxin (CTX) or 50 μL of 1.2% Barium Chloride ($BaCl_2$).

    b. Sort MuSCs at desired numbers into 500 μL of staining media (SM) in Eppendorf tubes and keep on ice.
    c. Pellet cells by spinning them down at 400 × *g* for 5 min, remove supernatant, and resuspend cells in ~25 μL of saline.

Note: When transplanting a low number of cells (less than 10K) you will likely not see a pellet.

    d. To prevent cells from sticking to the plastic walls of the syringe, pre-coat the BD Biosciences syringe with FBS.
    e. Using a 31-gauge insulin syringe, take in 25 μL of cells (in saline) into the syringe.
    f. Inject the needle at a 25° angle for a few cm, then reduce angle to 0° (parallel with the TA) for a few more cm such that the tip of the needle is the mid belly of the TA.
    g. Inject the entire volume and slowly remove the needle from the TA and dispose in a sharps bin.

⚠ CRITICAL: Removing the needle too quickly leads to leakage of cell volume from TA, and thus, compromising your transplantation experiment.

EXPECTED OUTCOMES

After following our detailed protocol, you will negatively select for muscle-resident blood cells, fibro-adipogenic progenitor cells (FAPS), and endothelial cells and positively select for FACS-purified MuSCs. For example, after performing all single-color controls and appropriate fluorophore compensations, in the subsequent gating you will (1) removed debris (Figure 5A); (2) select for single cells (Figure 5B); (3) select for live cells that are negative for PI and positive for Calcein Blue (Figure 5C); (4) negatively selected for FAPS, blood cells, and endothelial cells (Figure 5D); and (5) then positively enriched for MuSCs based on co-expression of β_1-integrin and CXCR4 (Figure 5E). Using this two-step enzymatic and mechanical dissociation of skeletal muscle coupled with FACS protocol, we typically obtain 60,000–80,000 MuSCs on the first sort from healthy adult mice (8–12 weeks old). We and others have shown that FACS isolated $PAX7^+$ MuSCs will actively proliferate in culture after several days (Figure 6A) and readily differentiate into myotubes when switched to low-serum differentiation media (Figure 6B).

LIMITATIONS

The two-step enzymatic and mechanical dissociation of skeletal muscle coupled with FACS protocol described here is a derivative of the first FACS-based method to purify MuSCs that was developed nearly 18 years ago,[4,5] which several labs continue to use in their research. This protocol takes ~6–8 h to go from live mice to stained mono-nuclear cells that are ready for FACS, which is several hours longer and yields 2–3 fold less cells than several recently developed shorter FACS-based protocols

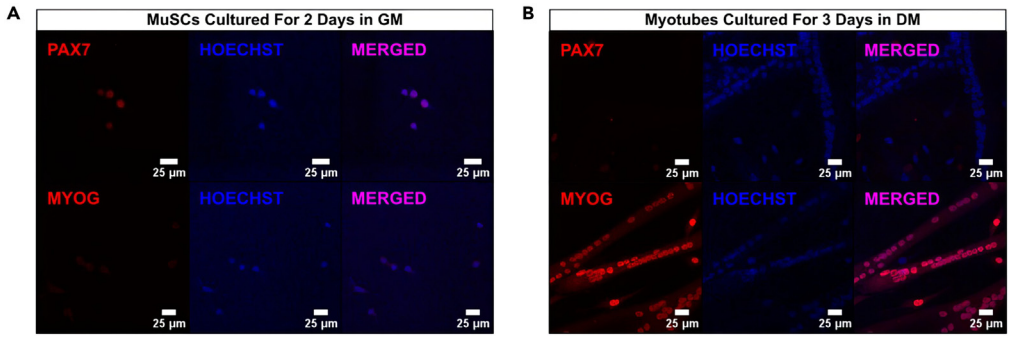

Figure 6. Immunofluorescence staining of cultured primary muscle progenitor cells and myotubes

(A) 3,000 MuSCs were cultured for 2 days in growth media supplemented with bFGF and stained for PAX7 and Myogenin (MYOG). MuSCs grown in GM are positive for PAX7 but negative for MYOG.

(B) 10,000 MuSC-derived myoblasts were cultured for 3 days in differentiation media and stained for PAX7 and MYOG. Myotubes express no PAX7 but high levels of MYOG. Staining was performed with anti-PAX7 (1:20, DSHB) and anti-MYOG (1:100, BD Biosciences).

for isolating MuSCs.[6] We also note that while β_1-integrin/CXCR4 has been previously shown to isolate the majority of PAX7-expressing MuSC populations from uninjured muscle, whether this is also true when isolating MuSCs from pre-injured muscle (i.e., via cardiotoxin, $BaCl_2$ injection) still needs further study. Lastly, we detail this method here to enhance reproducibility of data between labs, including our previously published work linked to this protocol which used this two-step FACS-based method.[1] We suspect that MuSCs isolated from the two-step method versus the one-step method likely yields cells at different activation states which may enrich for different subsets within the MuSCs pool.

TROUBLESHOOTING

Problem 1

Too many blood cells in the final myofiber-associated cell pellet for FACS.

Potential solution

- Perform an Ammonium Chloride Potassium (ACK lysis) treatment to remove red blood cells from your sample at step 33. This will lyse cells and prevent red blood cells from being taken into the FACS analysis, and thus, removing them from all FACS events and increasing your target cell population.

Problem 2

Poor separation between β1-integrin and CXCR4 in the final gating step during FACS.

Potential solution

- Make sure you count your cells to ensure you stain 1,000,000 cells per 100 µL of SM at the appropriate dilutions (1:100 for CXCR4). If too many cells are added, each antigen will not be saturated with antibody, and thus, the separation between positive and negative populations will be reduced. Additionally, make sure to perform at least a second wash (but potentially a 3rd wash) after incubating cells with the PE-Cy7 Streptavidin (for CXCR4) antibody (step 41), since this will increase the separation between CXCR4$^+$ and CXCR4$^-$ cells in the final gating step (Figure 5E).

Problem 3

Low yield of FACS-sorted MuSCs.

Potential solution

- Increase the quantity of extracted skeletal muscle.
- Ensure complete dissection of gastrocnemius, soleus, quadriceps, hamstrings, triceps, and abdominal muscles.
- Isolate MuSCs from younger mice. To maximize MuSC yield, extract muscles from mice that are between 8-12 weeks old. We have isolated 100,000 MuSCs from mice in the 4–6-week range.
- Avoid over-digestion of adult skeletal muscle. Incubating muscle with Digest 1 and 2 for longer than specified times may cleave off cell surface antigens on adult MuSCs that are targeted for antibody staining.
- Over trituration during mechanical dissociation leads to excessive debris that carries over into FACS. This will decrease your MuSC yield since your target cells become rarer events, meaning that you'll have to sort for a longer period. If FACS time is limited, you will not have enough time to sort out all the MuSCs. We recommend you follow the suggested trituration technique we present in Methods video S1.

Problem 4

FACS-isolated MuSCs are easily detached from culturing plates during routine media changes and passaging.

Potential solution

- Whenever media changes are needed in the first week in culture, make sure to remove half the media and put back half the media 3 times. We do this for routine passaging, media changes, as well as during the fixation step for Immunohistochemistry (IHC). Removing media with an aspirator within the first week of culturing will lead to many cells being removed from the well (Steps 50, 53, 54).

Problem 5

The majority of virally infected MuSCs die within 72 h after infection.

Potential solution

- Titrate your virus and use the minimal amount that gives you maximal transduction measure by a fluorescent reporter or drug-selection marker.
- Ensure that you perform 8 washes (step 54 *g*) prior to placing back in the 37°C incubator.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Albert E. Almada (aealmada@usc.edu).

Materials availability

This protocol did not produce new materials.

Data and code availability

This protocol did not produce nor analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102656.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.E., A.Z.M., and A.E.A.; writing, G.E. and A.Z.M.; reviewing and editing, G.E., A.Z.M., and A.E.A.; funding acquisition, A.E.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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