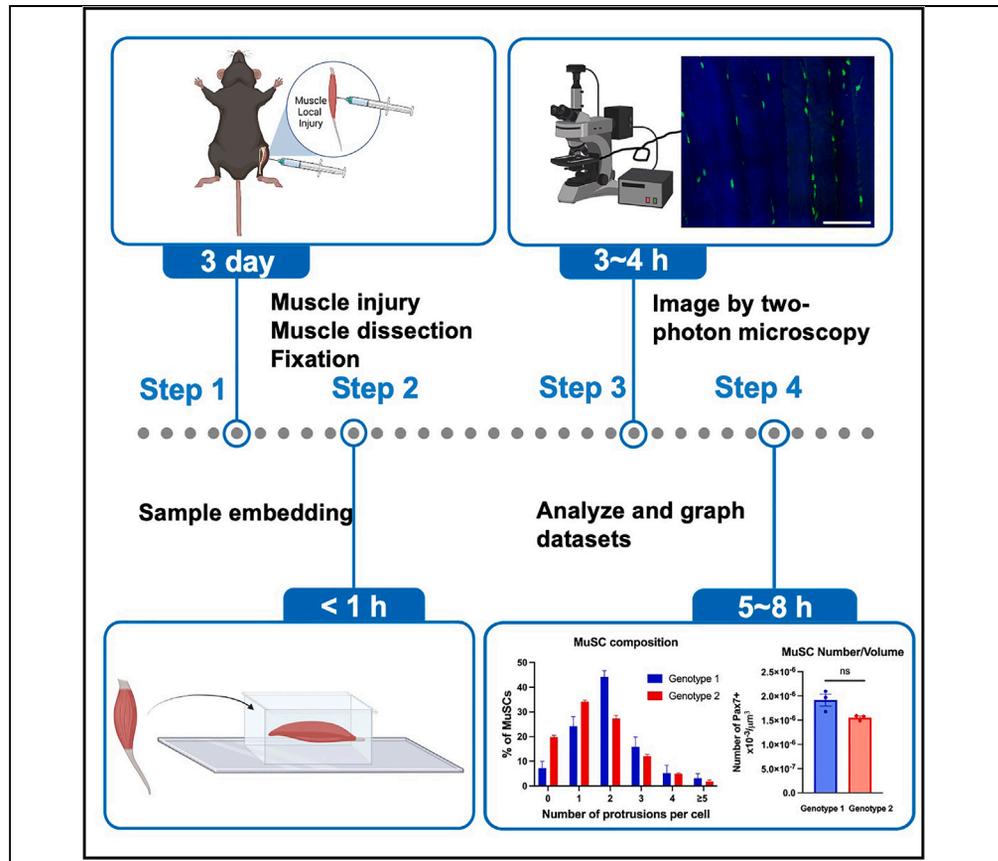


## Protocol

# Ex vivo two-photon imaging of whole-mount skeletal muscles to visualize stem cell behavior



Quiescent skeletal muscle stem cells (MuSCs) are morphologically and functionally heterogeneous and exhibit different lengths of cellular extensions, which we call protrusions. Here, we present a protocol for *ex vivo* two-photon imaging of MuSCs in their native environment. We describe steps for muscle dissection, fixation, embedding, imaging, and analysis of datasets. This protocol allows the examination of MuSC morphology and protrusions at the single-cell level as well as stem cell numbers.

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

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### Highlights

Whole-mount skeletal muscle tissue for two-photon imaging

Workflow to evaluate muscle stem cell morphology and analysis of cellular protrusions

This protocol can be adapted to other muscle-resident cells

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## Protocol

# Ex vivo two-photon imaging of whole-mount skeletal muscles to visualize stem cell behavior

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<https://doi.org/10.1016/j.xpro.2023.102772>**SUMMARY**

Quiescent skeletal muscle stem cells (MuSCs) are morphologically and functionally heterogeneous and exhibit different lengths of cellular extensions, which we call protrusions. Here, we present a protocol for ex vivo two-photon imaging of MuSCs in their native environment. We describe steps for muscle dissection, fixation, embedding, imaging, and analysis of datasets. This protocol allows the examination of MuSC morphology and protrusions at the single-cell level as well as stem cell numbers.

For complete details on the use and execution of this protocol, please refer to Ma et al. (2022).<sup>1</sup>

**BEFORE YOU BEGIN**

Skeletal muscles constitute about half the body weight and serve many essential roles, such as movement, breathing, and thermogenesis.<sup>2</sup> Skeletal muscle also has a high capacity to regenerate, governed by the microenvironment and resident stem cells called muscle stem cells (MuSCs, also called satellite cells).<sup>3</sup> MuSCs are located within the basal lamina and sarcolemma and have the ability to self-renew, proliferate, differentiate, and regenerate myofibers following injury.<sup>4</sup> Therefore, studying MuSCs is essential to understand muscle regeneration and design therapies for myopathies. It has been shown that MuSCs are functionally heterogeneous,<sup>5–7</sup> and exhibit distinct transcriptomes.<sup>8–13</sup> However, the majority of the MuSC studies utilize histological sections of skeletal muscles,<sup>14–16</sup> isolated MuSCs,<sup>8–14</sup> or isolated single fibers ex vivo.<sup>15</sup> Yet, these methods disrupt muscle integrity. Imaging whole muscles ex vivo by two-photon microscopy provides advantages for three-dimensional and deep tissue imaging and allows the examination of MuSC morphology and function in their natural environment. Previously, we demonstrated that MuSCs exhibit different patterns of cellular protrusions and are morphologically and functionally heterogeneous, allowing the categorization into three functionally different stem cell states.<sup>1</sup>

Since Pax7 is the most utilized and universal marker of adult MuSC,<sup>4</sup> to visualize MuSCs ex vivo, this protocol uses the Pax7EGFP mouse model, which has been shown to monitor dynamic behavior of stem cells,<sup>14</sup> but any other mouse model that labels MuSCs (i.e., Pax7ERT2Cre/Ai9<sup>FL/FL</sup>) can be utilized for the same purpose. The protocol below describes the specific steps for imaging and analysis of MuSCs on whole-mount tibialis anterior muscles but applies to any other skeletal muscle of the lower leg. This protocol is useful to visualize and compare MuSCs independently of age or disease



condition. For example, in muscle dystrophy, a mutation in the *dystrophin* gene leads to fragile myofibers, and cycles of muscle degeneration and regeneration. It was previously shown that although the disease starts because of the structural defect, it develops progressively into a stem cell disease due to impaired MuSC function.<sup>17–19</sup> This impairment of MuSCs is also illustrated by changes of MuSC morphology and subtype distribution, which could be studied by using this protocol, as examined in Ma et al.<sup>1</sup> This protocol can also be used to quantify the number of MuSCs per total tissue volume. Most studies in the literature utilize prospective cell isolation methods, which rely on enzymatic digestion efficiency and fluorescence-activated cell sorting (FACS) based on cell surface markers<sup>14,17,18,20–23</sup> or *ex vivo* methods to isolate single muscle fibers, in combination with Pax7 antibody staining.<sup>24</sup> Both these methods often result in cell loss due to either incomplete tissue digestion and/or cell death during these long experimental procedures and are likely exacerbated in diseased muscles that are inherently more fibrotic and fragile. Additionally, only the intact/non-broken fibers are used for quantification and therefore single fiber isolation selects for MuSC measurements in somewhat “healthier” fibers. To overcome these limitations, the method described here, reliably allows for MuSC visualization by two-photon microscopy and permits evaluation of MuSC numbers per muscle volume in their native environment on all imaged muscles regardless of disease state.

The sensitivity of fluorescence imaging in thick tissues is limited by out-of-focus background signal. This introduces a diffuse background fluorescence that does not encode any spatial information and minimizes the imaging contrast. When confocal microscope is utilized, a pinhole rejects the out-of-focus background and produces an unblurred image. However, this greatly reduces the limitation of extended imaging. Two-photon excitation microscopy is an alternative to confocal microscopy that provides clear advantages for 3D imaging. Compared to other more complex and costly super-resolution microscopy, two-photon microscopy is more appropriate to visualize MuSCs morphology and protrusions in whole-mount muscle. Other methods, such as Stimulated emission depletion (STED) microscopy allows cell imaging with 50 nm resolution,<sup>25</sup> while Stochastic Optical Reconstruction Microscopy (STORM) activate only a small percentage of large number of individual fluorophores at one time that together form a super-resolved image, allowing images with as little as 5 nm resolution<sup>26</sup> However, the average length of protrusion is 10 μm, and too fine resolution is not necessary. Moreover, a major advantage of two-photon microscopy is that it allows visualization of a large area of the tissue, giving us the opportunity to image many MuSCs at the same time. Finally, structure illumination microscopy (SIM) is difficult on thick samples,<sup>27</sup> while two-photon microscopy could provide signals as deep as 350 μm. Overall, two-photon microscopy is a reliable and accurate method to evaluate large number of MuSCs with high resolution analysis and covering substantial muscle volume. The principal advantages of two-photon microscopy are reduced phototoxicity, increased imaging depth, and the ability to initiate highly localized photochemistry in thick samples.

### Institutional permissions

This protocol utilizes skeletal muscle tissue post-mortem from research animals. Their treatment and care were approved by the University of Pennsylvania according to Institutional Animal Care and Use Committee guidelines. Similar usage of animals needs permission from relevant institutions.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
4% Paraformaldehyde in PBS	Thermo Scientific	Cat# J19943-K2
Phosphate-buffered saline (PBS)	Thermo Scientific	Cat# 14040-133
Notexin	Latoxan	Cat# L8104-100UG
Isoflurane	Abbott Laboratories	Cat# 05260-05

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: Pax7EGFP, 2- to 6-month-old males	Tichy et al. <sup>14</sup>	<a href="https://pubmed.ncbi.nlm.nih.gov/30139374/">https://pubmed.ncbi.nlm.nih.gov/30139374/</a>
Software and algorithms		
Fiji	Open source	<a href="https://fiji.sc/">https://fiji.sc/</a>
Fiji plugin - Neuroanatomy plugin	Open source	<a href="https://imagej.net/imagej-wiki-static/Neuroanatomy">https://imagej.net/imagej-wiki-static/Neuroanatomy</a>
Leica software	Licensed software	<a href="https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/downloads/">https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/downloads/</a>
Microsoft Excel	Licensed software	<a href="https://www.office.com">https://www.office.com</a>
GraphPad Prism v. 9.1.2	Licensed software	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
Other		
Anesthesia induction chamber	Conduct Science	N/A
Isoflurane vaporizer	VetFlo	N/A
Anesthesia breathing circuit and nose cone	VetEquip	N/A
Carbon dioxide delivery system	Quietek	N/A
Electric razor	Amazon	N/A
Forceps	Fine Science Tools	Cat# 91150-20 Cat# 11051-10
Dissection scissors	Fine Science Tools	Cat# 14060-11 Cat# 14002-12 Cat# 14028-10 Cat# 15000-08
Superfrost Plus microscope slides	Fisher Scientific	Cat# 12-550-15
Cover glass 22 × 22 mm	Amazon	N/A
Elmer's no-wrinkle rubber cement	Amazon	
Blotting paper	Thomas Scientific	Cat# 2228-1515
Transfer pipets	Fisher	Cat# 13-711-7
BD alcohol swabs	Amazon	N/A
Insulin syringes with BD Ultra-Fine needle 31G 3/10 mL 6 mm	Amazon	N/A
Leica SP8 confocal/multiphoton microscope system	Leica Microsystems	N/A

## MATERIALS AND EQUIPMENT

### Two-photon microscopy

- A Leica SP8 Multiphoton Microscope system (with Scientifica motorized stage with interchangeable variable height platform) equipped with Chameleon Vision II Sapphire laser (tuning range 680–1080 nm) is used.
- The laser is tuned to an excitation wavelength of 910 nm.
- An HCX APO 20× 1.00 NA water immersion lens is used for imaging steps.
- Bandpass filter: 525/20, 460/50, 610/75 for the emission of green, blue (second harmonics: SHG), and red fluorescence.

### Custom-made polycarbonate chamber (3D printed)

- Length: 25 mm,
- Width: 25 mm,
- Height: 3 mm or 3.5 mm,
- Width between inner and outer square: 5 mm,
- Material: clear impact-resistant polycarbonate sheets.

## STEP-BY-STEP METHOD DETAILS

### Muscle injury, isolation, and fixation

⌚ Timing: 3 days

This section describes steps to perform point injury to muscle and isolate skeletal muscle samples for later whole-mount imaging. 2–6-month-old male Pax7EGFP mice are used in this protocol. Skeletal muscle injury is induced by intramuscular injection of notexin (NTX), a snake venom toxin that causes myotoxic damage.<sup>28</sup> If MuSC number and cell morphology in steady state conditions is desired, then the muscle injury can be omitted.

**Optional:** Inject notexin (NTX) to Pax7EGFP mice for skeletal muscle injury.

1. Anesthetize Pax7EGFP mice with isoflurane in an anesthesia induction chamber.
2. After the mouse is fully sedated, transfer the mouse to a flat surface and position the nose cone around the snout and mouth of the mouse to keep it anesthetized.
3. Shave the hair off the hindlimb with an Electric Razor.
4. Wipe the hindlimbs of the mice with ethanol wipes.
5. Inject 10  $\mu$ L (10  $\mu$ g/mL) of NTX diluted in PBS intramuscularly into the tibialis anterior muscle at one site per muscle (10  $\mu$ L per muscle). The needle for injection should first poke through the fascia but then stay at the top of the muscle.

**△ CRITICAL:** The needle can be inserted at approximately 45°. The injury should be close to the top of the muscle for easier visualization by two-photon microscopy. Otherwise, the signal intensity of cells within the injury and second harmonics of the fiber start to disappear at about 400  $\mu$ m.

6. Allow the mice to recover post-injury depending on the desired time frame after injury.
7. Following IACUC guidelines, euthanize Pax7EGFP mice.
8. Collect tibialis anterior (TA) muscle from uninjured and injured Pax7EGFP mice.
  - a. Position the mouse spine on a Styrofoam dissection board, secure limbs with a 21G needle, and spray the mouse with 70% (vol/vol) ethanol.
  - b. Use forceps to pinch the center of the mouse belly skin and cut an opening horizontally. Peel off the leg skin by pulling the opening in opposite directions.
  - c. Carefully remove the fascia and use sharp forceps to expose the underneath TA muscle.
  - d. Run the sharp tip of the forceps underneath the TA muscle to detach the muscle from the tibia.
  - e. Cut the distal TA tendon, and while holding it, use a dissection scissor to cut the muscle along the longitudinal line of the TA muscle. Carefully remove TA at its proximal attachment.
9. Place the muscle in a 6 cm dish containing 4% paraformaldehyde/1  $\times$  phosphate-buffered saline (PBS) buffer on ice.
10. Wrap the 6 cm dish containing TA muscles with aluminum foil.

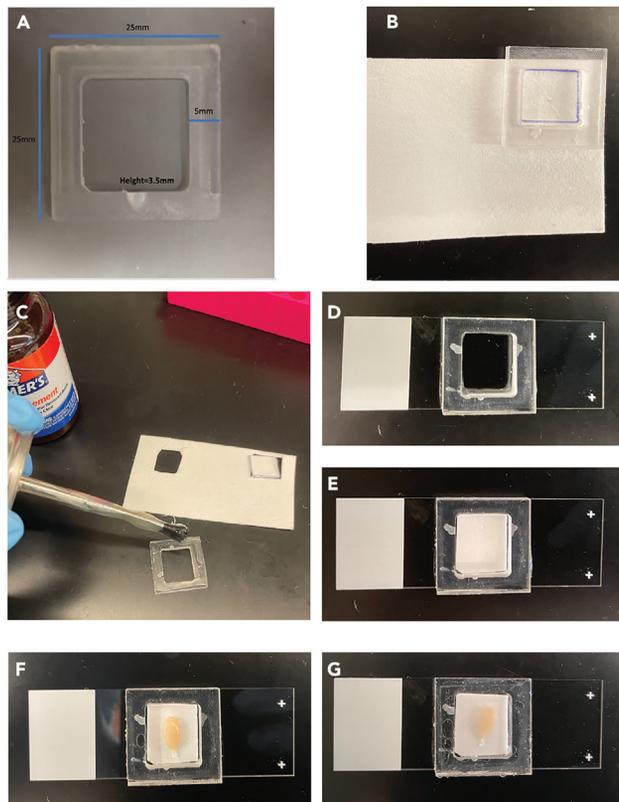
**Note:** The aluminum foil is used to protect samples containing internal fluorescence from too much light exposure before imaging.

11. Place the 6 cm dish in an orbital shaker in the cold room (4°). Leave the TA samples in their current condition for 2 h to fix the muscle samples.

**Note:** Fixation at 4 degree is recommended to better preserve fluorescence intensity and reduce background fluorescence. Too long fixation time (longer than 4 h for TA muscle) will quench the green fluorescent protein signal.

12. After fixation, aspirate 4% paraformaldehyde from the 6 cm dish and wash the muscles with 3 mL of 1  $\times$  phosphate-buffered saline (PBS) for 3 times.
13. Preserve the muscles in 3 mL of 1  $\times$  phosphate-buffered saline (PBS).

**▯▯ Pause point:** The muscles can be preserved in PBS in the cold room for weeks before imaging.



**Figure 1. The process of tibias anterior (TA) muscle embedding for two-photon microscopy**  
(A) Dimensions of custom-made polycarbonate chamber.  
(B–E) Key steps for creating chamber.  
(F and G) Steps of TA muscle embedding.

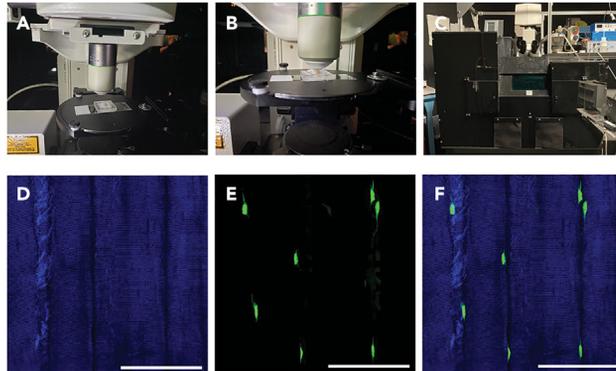
**Note:** Too long cold room storage will cause increased autofluorescence. It is recommended to image the tissue as soon as possible and within 1–3 weeks after fixation to minimize the potential differences in fluorescence intensity.

### Imaging chamber preparation

⌚ Timing: <1 h

Fixed muscles were mounted on a custom-made polycarbonate chamber and immersed in  $1 \times$  PBS, and a coverslip was lowered from the top to close the chamber.

14. Create a polycarbonate window with the following dimensions: Length: 25 mm, Width: 25 mm, Height: 3 mm or 3.5 mm, width between inner and outer square: 5 mm (Figure 1A). [troubleshooting 1](#).
15. Cut a piece of filter paper based on the size of the inner square (Figure 1B).
16. Apply Elmer's rubber cement to the back of the polycarbonate window (Figure 1C) and attach the window to the plus slide of the Superfrost plus microscopy slide to create a polycarbonate chamber (Figure 1D).
17. Place the obtained filter paper inside the chamber (Figure 1E) to decrease the movement of muscle samples relative to the chamber.
18. Use forceps to place one TA muscle inside the chamber carefully (Figure 1F).



**Figure 2. Two-photon imaging of tibialis anterior (transverse) of Pax7EGFP mouse *ex vivo***

(A) Polycarbonate chamber positioned underneath the lens.  
 (B) Focused position and water deposited on top of coverslip for water-immersion lens.  
 (C) Microscope lens covered by blackout chamber to avoid intense light from surrounding to enter the microscope.  
 (D–F) Images of muscle under two-photon microscopy (D). Second harmonics (SHG) of muscle fibers (E). Bright GFP signals appeared at the periphery of muscle fibers (F). Merged image. Scale bar = 100  $\mu\text{m}$ .

19. Apply Elmer's rubber cement to the top of the polycarbonate window to glue the coverslip to the window.
20. Use transfer pipets to fill the polycarbonate chamber with 1  $\times$  PBS.
21. Carefully lower the cover glass on top of the window to close the chamber (Figure 1G).
22. Use the sharp tip of the forceps to push gently on top of the cover glass and use Kim Wipe to remove excess mounting media (1  $\times$  PBS). The whole setup is finished and ready for two-photon imaging.

**Note:** The current imaging chamber setup is used for upright microscope. If inverted microscope is used, the imaging chamber setup would be a little different: keep steps 14, 16, 18, 19, 20, and 21, and skip steps 15 and 17. For step 18, place the TA muscle upside down, so that the anterior of the muscle faced down, and use Elmer's rubber cement to attach the muscle to the Superfrost plus microscope slide to decrease the movement of muscle samples relative to the polycarbonate chamber.

### Two-photon *ex vivo* imaging

⌚ Timing: 3–4 h per sample

Perform two-photon imaging of whole-mount uninjured or injured TA muscle and visualize the structure of interest. This should be specific to each research group. In our case, we image muscle fiber with second harmonics (SHG) and muscle stem cells expressing green fluorescent proteins in Pax7EGFP mice.

23. Turn on the microscope controller, confocal unit, Leica software (LAS X), PC, and tunable multi-photon (MP) laser for imaging.
24. Move the motorized stage from the preparation area to the imaging area. Place the chamber containing the muscle sample on the motorized stage. Adjust the XY position of the microscope motorized platform so that the muscle sample is directly underneath the lens and secure the chamber by tightening the screw (Figure 2A).
25. Deposit distilled water on top of the coverslip for the water-immersion lens.
26. Adjust the height of the lens to find focus (Figure 2B).
27. Turn off room lights, turn on wide-field epifluorescence, and use the eyepiece to find focus. [troubleshooting 2](#).

**Note:** To avoid bleaching, keep the epifluorescence at the lowest power.

28. Turn off the epifluorescence shutter and cover the microscope with a blackout chamber or blackout cloth (Figure 2C).

**Note:** Avoid intense light from surrounding to enter the microscope.

29. Turn on the MP laser shutter. Set the laser wavelength at 910 nm for green fluorophore and SHG.
30. Select the imaging area and conditions. [troubleshooting 3](#), [troubleshooting 4](#).
  - a. Set the laser power at 13% for a start.
  - b. Set the detector gain to 100%. If the signal remains low, slowly increase the laser power.
  - c. Turn on tilescan and z-stack and adjust XYZ position to find the field of interest where muscle stem cells are visible and lower than the collagen deposition layer.
31. Set scan settings of LAS X (Figures 2C–2E).
  - a. Format 1024 × 1024.
  - b. Speed: 400 Hz.
  - c. Bidirectional: ON Phase X: –33.04.
  - d. Zoom factor: 1.5.
  - e. Image size: 369.05 μm × 369.05 μm, Pixel Size 360.75 μm × 360.75 μm.
  - f. Line Average: 2.
  - g. Tilescan field size: 3 × 4; merge images by advanced statistical stitching.
32. Set the upper limits of a z-stack, ensuring the upper limits are lower than the collagen deposition layer. Set lower limits of a z-stack before the muscle cell signal disappears. Start the z-stack acquisition with the 1 or 1.5 μm step size.

**Note:** It usually takes two hours to finish scanning for an image with a z-stack about 100 μm in-depth and tilescan with field size 3 × 4.

33. Save the original file (lif format) to a hard drive for further data analysis.

⏸ **Pause point:** Image analysis can be conducted at any time after the imaging.

### Image analysis using Fiji software

⌚ **Timing:** 5–8 h per image

Acquired optical sections from two-photon microscopy are analyzed in Fiji software. To define the muscle stem cell (MuSC) number in the TA muscle, acquired optical sections are stacked in Fiji, and MuSCs are counted and normalized to the volume stacked. To quantify the length and number of the protrusions of MuSC, minimum 100 MuSCs per image should be analyzed using the Simple Neurite Tracer (SNT) plugin in Fiji. MuSC circularity/aspect ratio, circularity, and orientation ratio are measured using set measurements and the angle tool in Fiji. Cells are defined as misaligned with myofiber if the angle between the cell body and myofiber is greater than a 15-degree angle, and the ratio is calculated as the number of cells misaligned divided by the total number of cells per cell type.

34. Load the original image into Fiji software (Figure 3A) using File > Open (choose split channels) and save the image as tiff format using File > Save as > tiff.
35. Mark individual cells for analysis using the "multi-point" function (Figure 3B).
  - a. Click to show the label on all slices.
  - b. Only select cells if all details are visible.
  - c. Save the numbered image and the numbering system as a selection (.roi file).
36. Create maximum projection of the image (Figure 3C).
  - a. Go to Image > Stacks > Z project.



**Figure 3. Fiji software analysis of muscle stem cells (MuSCs) parameters**

(A) Panel of Fiji.

(B) Selected MuSCs labeled with numbers.

(C) Maximum projection of (B).

(D) Measurement of Cell area and circularity.

(E) Measurement of protrusion length of two selected cells. Primary protrusion paths are highlighted in green, and secondary protrusion is highlighted in blue color.

- b. Choose Max intensity.
- c. Save the Max projection as a tiff file.
- d. Load saved number selection by dragging the ROI file to Fiji software.
37. Setup the measurement parameter by clicking Analyze > set measurement > click area and shape descriptors.
38. Manually draw a circle encompassing the cell body of the muscle stem cell to obtain cell area and circularity (Figure 3D). [troubleshooting 5](#).
  - a. Use the freehand selection tool to circle the cell body.
  - b. Click Analyze > Measure to get area and circularity results.
  - c. Export data to copy into Excel to the corresponding cell number.
39. To assess whether the cells are aligned with the myofiber, use the angle tool to measure the angle between the cell body and the myofiber. If the angle is larger than a 15-degree, the cell is considered as misaligned with the fiber. The misaligned ratio is calculated as the number of cells misaligned divided by the total number of cells per cell type.
40. Download the Neuroanatomy plugin to Fiji software for measurement of protrusion length by update function.
41. Open the Neuroanatomy plugin and load the numbered tiff file by:
  - a. Plugins > Neuroanatomy >SNT > select numbered tiff file as the image file
  - b. Select only XY view in the user interface.
42. Select the path of protrusions and obtain individual protrusion protrusions by:
  - a. Move along the Z direction and click at the beginning and the end of each protrusion to create a protrusion path.

- b. In the Path Manager, click on the path and obtain protrusion by Tag > Morphometry > Length.
- c. Export data to copy into Excel to the corresponding cell number.

**Note:** In quiescence, MuSCs could have 0–9 protrusions. We have described in detail the morphological and functional properties for each MuSC subtype in Ma et al.<sup>1</sup> Based on the starting position of the protrusions, this imaging protocol allows for further characterization as follows:

- i. Primary protrusions: extruding from the cell body.
  - ii. Secondary protrusions: branching from Primary protrusions.
  - iii. Tertiary protrusions: branching from secondary protrusions.
  - iv. Quaternary protrusions: branching from tertiary protrusions.  
Secondary, Tertiary, and Quaternary protrusions are called branches. Branches should be marked in the Excel file.
43. To calculate the number of MuSCs per volume, find a cuboid volume of the image that is filled with muscle fibers, calculate the volume and make a maximum projection, and count the total number of MuSCs in the cuboid, and calculate the final number of MuSCs per volume.
- a. Find an area full of fibers and use the rectangle tool to select and crop the region of interest.
  - b. Obtain dimensions by selecting Image > Show info > Width, Height, Z-step size (Figure 4A).
  - c. Select the Z sections of muscle and create maximum projection for the volume of interest (Figure 4B).
  - d. Obtain the depth of the volume of interest by multiplying Z-step size with the number of Z sections used to create volume of interest.
  - e. Count all the MuSCs on the maximum projection image by Plugins > Analyze > Cell Counter (Figure 4C).
  - f. Export data and copy it into Excel.
  - g. The MuSC numbers were normalized to 1  $\mu\text{m}^3$  volume of muscle fibers.
44. Analyze the data in Excel and graph datasets as desired in GraphPad Prism. Data can be grouped as number of cells per volume, MuSC composition based on the number of protrusions, MuSC misalignment ratio, MuSC protrusion length, MuSC area, and MuSC circularity.

### EXPECTED OUTCOMES

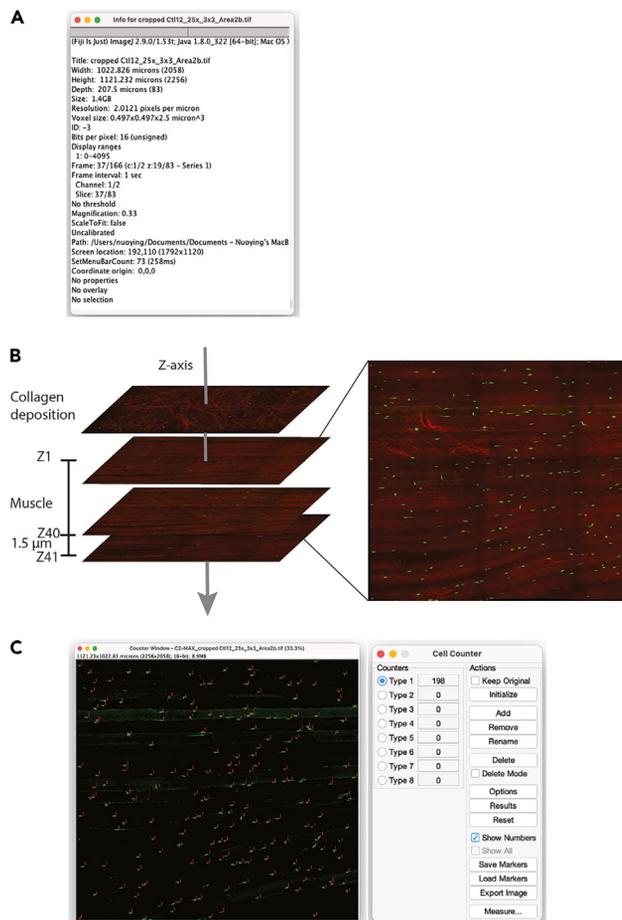
This protocol demonstrates the methodology to visualize muscle stem cell morphology and protrusions in their native environment as well as to quantify the number of stem cells per muscle volume. We utilized Pax7EGFP to label muscle stem cells; however, Ai9 could be used to track muscle stem cells if the Ai9<sup>F<sup>l</sup>/F<sup>l</sup></sup> mice (The Jackson Laboratory, stock no. 007909) are crossed with Pax7ERT2Cre (The Jackson Laboratory, stock no. 017763). These mice (Pax7ERT2Cre/Ai9<sup>F<sup>l</sup>/F<sup>l</sup></sup>) will label MuSCs in red (tdTomato) upon tamoxifen injection, as we described previously.<sup>1</sup> This protocol can likely be applied to additional resident skeletal muscle populations in transgenic mice lines where fluorescent protein is coupled to appropriate cellular markers. Here, we utilized data from two different genotypes of mice to demonstrate different ways to represent the data (Figure 5).

### QUANTIFICATION AND STATISTICAL ANALYSIS

Data comparisons between genotypes are conducted using unpaired Student's *t*-tests with Welch's correction or two-way analysis of variance (ANOVA) using GraphPad Prism 7 software. Cells from 3–4 mice or samples per experimental condition should be examined, with at least 100 cells imaged per sample. Data are presented as means  $\pm$  SEM. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001; n.s., not significant.

### LIMITATIONS

Two-photon microscopy—especially when combined with powerful mouse genetic models—can provide critical insight into the mechanisms that govern stem cell activity, by enabling the visualization



**Figure 4. Fiji software analysis of number of cells per volume**

(A) Panel of information showing the width, height, and depth of the figure.

(B) Illustration of muscle maximum projection creation by using Z1 to Z41.

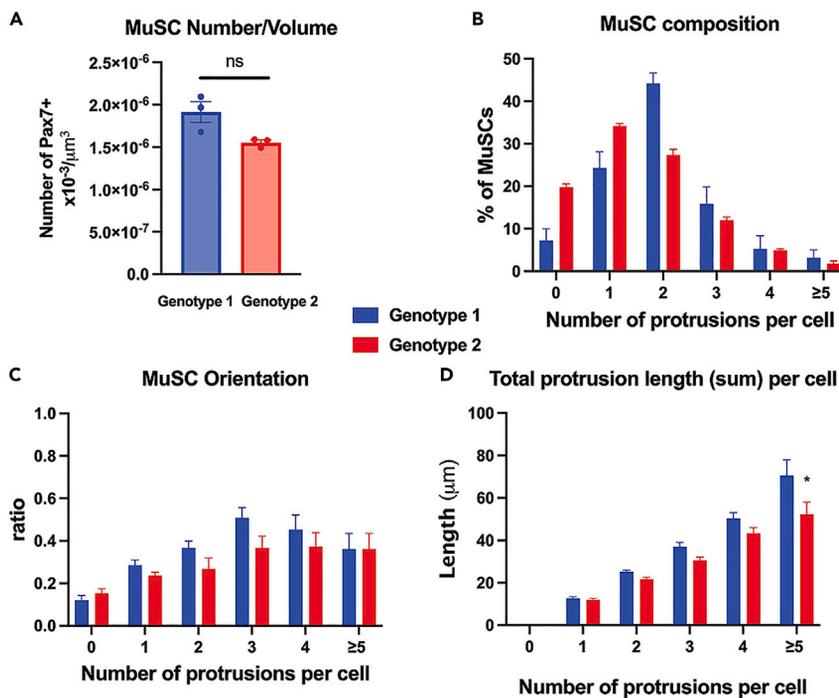
(C) Counting the total number of cells in the maximum projection image using Cell Counter.

of stem cells within their native environment in skeletal muscles. Yet, depending on the size of the particular skeletal muscle, there is a limitation of the tissue volume that can be imaged. In our experience, imaging of smaller muscles such as Extensor Digitorum Longus (EDL) can cover >80% of tissue volume but larger muscles such as gastrocnemius, there imaged volume cannot exceed more than 1/3 of total volume. The current protocol describes a robust two-photon imaging procedure to visualize muscle stem cells in whole muscle. This protocol utilizes the internal fluorescence protein to identify MuSCs and measure protrusion length. Since measurements of protrusion length are based on fluorescent intensities, it is critical to keep the MP laser power and detector gain at the same level across samples in the same project, and samples should be imaged in as short a time window as possible. Besides, if transgenic mice are not used, extra work is required to clear muscle samples using tissue-clearing methods<sup>29,30</sup> and stain MuSCs and their protrusions with antibodies before imaging. Overall, two photon microscopy is a powerful relatively new technique for imaging stem cells and as the method further develops, it might bring future opportunities for overcoming some of the current limitations.

## TROUBLESHOOTING

### Problem 1

The muscle sample does not fit in the polycarbonate chamber.



**Figure 5. The comparisons of MuSCs parameters between genotype 1 and genotype 2 by GraphPad Prism**

(A) The number of MuSCs per volume.  
 (B) The fraction of MuSCs with different numbers of protrusions.  
 (C) Orientation analysis of MuSCs show the ratio of MuSCs misaligned with muscle fibers.  
 (D) Total sum protrusion length.  
 Data are represented as means  $\pm$  SEM. \* $P < 0.05$ , ns = not statistically significant.

### Potential solution

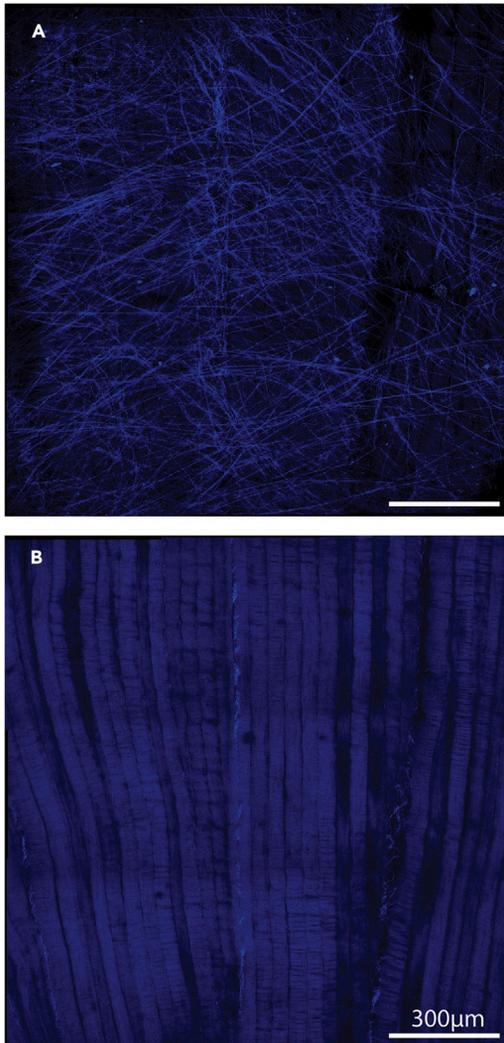
The height of the polycarbonate chamber should be just higher than the depth of the muscle so that when the cover glass is lowered on the polycarbonate window, it does not have direct contact with the polycarbonate window. It is recommended to make two polycarbonate windows, with heights of 3 mm and 3.5 mm, respectively. The TA muscle dissected from adult C57BL/6J mice should fit with the 3 mm-height-chamber. However, the hypertrophied TA muscle from mdx4cv mice (The Jackson Laboratory, stock no. 002378) should fit with a 3.5 mm height chamber. If larger muscles are used (i.e., gastrocnemius), the chamber should be modified accordingly.

### Problem 2

Cannot find focus using epifluorescence.

### Potential solution

- Move the laser beam of epifluorescence to the edge of the muscle sample.
- First, lower the lens as close to the surface of the cover glass of the polycarbonate chamber as possible.
- Then, slowly raise the lens. At the edge of the muscle sample, through the eyepiece, half of the field should be blank, and at the other half, muscle fibers should appear as a set of stripes or parallel lines.
- Carefully move the motorized stage so that the laser focuses on the middle of the muscle and slowly raises the lens. The parallel lines representing the myofibers should be visualized in the whole field. Muscle stem cells expressing green fluorescent protein should appear as small bright spots.



**Figure 6. Representative images of collagen deposition layer and muscle fibers**

Comparison between collagen deposition layer (A) and muscle fibers (B). Scale bar = 300  $\mu\text{m}$ .

### Problem 3

Find a dark circle in the fiber.

### Potential solution

This area is bleached by the epifluorescence laser. Avoid this area when selecting the region of interest. Turn the laser power of the epifluorescence laser to the lowest possible level when finding focus.

### Problem 4

Cannot distinguish between collagen deposition layer and muscle fibers.

### Potential solution

The muscle fibers are aligned parallelly with the striated appearance on each muscle fiber. Collagen in the collagen deposition layer is anisotropic. Representative images demonstrating collagen deposition layer (Figure 6A) and muscle fibers (Figure 6B) are included.

### Problem 5

An error appears in Fiji software stating "Type ... to delete points", and the "freehand selection" tool cannot be used to draw the circle encompassing the cell.

### Potential solution

The reason is that each image only allows one item to be selected at a time (either numbers from the "multi-point" tool or area from the "freehand selection" tool). To enable the "freehand selection" tool, the numbers first need to be overlaid to the maximum projection image, which could be achieved by

- Image > Overlay > add selection.
- Image > Overlay > flatten.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Foteini Mourkioti ([fmour@penmedicine.upenn.edu](mailto:fmour@penmedicine.upenn.edu)).

#### Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Nuoying Ma ([nuoying@seas.upenn.edu](mailto:nuoying@seas.upenn.edu)), and Foteini Mourkioti ([fmour@penmedicine.upenn.edu](mailto:fmour@penmedicine.upenn.edu)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

This study did not generate code. Any data supporting this study's findings are available from the corresponding author upon reasonable request.

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### AUTHOR CONTRIBUTIONS

N.M. acquired two-photon images, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. F.M. conceived the original idea, designed and supervised all the experiments, directed the project, interpreted the data, and edited the manuscript. Both authors revised the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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