

Submission ID #: 69732

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Project Page Link: https://review.jove.com/files_upload.php?src=21268308

Title: Methods for In Situ Quantification of Mitochondrial Morphology in Muscle and Terminal Schwann Cells of Mice

Authors and Affiliations:

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera?

No

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

Olympus, SZ61

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

SCOPE shots: 2.3.2, 3.3.1, 3.3.3

Videographer: Please film the above-mentioned shots using the scope kit.

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? **20-30 feet**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 19

Number of Shots: 43

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**. Answers are slightly edited in accordance with journal guidelines.

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Aaron Morton:** Our research investigates mitochondrial morphology of terminal Schwann cells at the neuromuscular junction for the first time.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the research contexts or systems in which this protocol can be applied.**~~

- 1.2. **Aaron Morton:** Our approach enables quantification of mitochondrial morphology in otherwise hard-to-obtain tissues, such as capillary and lymphatic beds.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.3. **Aaron Morton:** This protocol allows researchers to study and quantify mitochondrial morphology in skeletal muscle and terminal Schwann cells.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**How can future studies build on this work? Describe the questions or research directions that can be investigated next.**~~

- 1.4. **Aaron Morton:** Future studies can adapt this approach to investigate mitochondrial morphology in microcirculation and lymphatic vessels
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparation of Tibialis Anterior (TA) Muscle Samples

Demonstrators: Aaron Morton and Shadi Golpasandi

- 2.1. To begin, obtain a wild type and a D2.mdx (*D2-m-d-x*) mouse [1]. Insert the needle at the distal tendon toward the right knee of the anesthetized mouse [2-TXT]. Inject 50 microliters of 2 micromolar Far-red fluorescent mitochondrial stain on the tibialis anterior or the TA muscle while withdrawing the needle along the injection path and incubate for 1 hour [3].
 - 2.1.1. WIDE: Talent with wild-type and D2.mdx mice on table.
 - 2.1.2. CU: Shot of inserting needle at the distal tendon near the right knee of anesthetized mouse. **TXT: Anesthesia: 2% Isoflurane**
 - 2.1.3. CU: Shot of injecting mitochondrial stain into the right TA muscle while withdrawing the needle.
- 2.2. Using scissors and forceps, dissect and isolate the TA muscle before euthanasia [1].
 - 2.2.1. Talent dissecting the TA muscle.
- 2.3. Place the isolated TA muscle in 4% paraformaldehyde at room temperature for 15 minutes [1]. Then, under a stereomicroscope, gently separate the fibers by teasing [2].
 - 2.3.1. Talent placing the isolated TA muscle in 4% paraformaldehyde.
 - 2.3.2. SCOPE: The fibers are being separated.
- 2.4. Add cell-permeant nucleic acid stain to the 4% paraformaldehyde, to obtain a final concentration of 5 to 10 microgram per milliliter for nuclear labeling [1].
 - 2.4.1. Talent adding cell-permeant nucleic acid stain into the 4% paraformaldehyde.
- 2.5. Then, wash the TA muscle 3 times in PBS for 5 minutes each to remove excess dye [1].
 - 2.5.1. CU: Talent adding PBS in the sample to remove excess dye.
- 2.6. Dehydrate the muscle sequentially in 50%, 75%, and 100% ethanol for 5 minutes each [1].
 - 2.6.1. CU: Sample being transferred between ethanol solutions labeled 50%, with 75%, and 100% ethanol in the background.
- 2.7. Then, incubate the sample sequentially in tissue clearing solutions I (*one*) and II (*two*) for 30 minutes each, ensuring they are fully submerged [1].

- 2.7.1. Talent submerging the sample in clearing solution.
- 2.8. Now, place the muscle on a coverslip [1]. Use a 2.5 by 2 by 1 centimeter glass block weighing 7.8 grams to gently flatten it and transfer the muscle to clearing solution II - ~~to prevent dehydration and photobleaching until imaging [2].~~
- 2.8.1. CU: Shot of sample being placed on a coverslip.
- 2.8.2. Shot of glass block flattening the sample placed on the coverslip. ~~TXT: Glass block: 2.5 cm x 2 cm x 1 cm; mass 7.8 g~~
- 2.8.3. ~~Talent transferring the sample to clearing solution II.~~ **NOTE: Not filmed, VO merged in concise format**

3. Preparation of Gluteus Maximus (GM) Muscle Samples

- 3.1. Obtain an S100 β (*S-one-hundred-beta*) mouse [1] and make a small skin incision above the gluteus maximus or GM muscle [2]. Inject 75 microliters of 2 micromolar far-red fluorescent mitochondrial stain beneath the GM muscle [3].
 - 3.1.1. WIDE: Talent with S100 β mice on table.
 - 3.1.2. CU: Shot of making small incision above the GM muscle in S100 β mice.
 - 3.1.3. CU: Shot of injecting far-red fluorescent mitochondrial stain beneath the GM muscle.
- 3.2. Close the wound [1] and let the dye incubate for 45 minutes to selectively label Terminal Schwann cells [2].
 - 3.2.1. CU: Shot of closing the wound.
 - 3.2.2. Shot of mouse lying undisturbed.
- 3.3. Then, dissect the GM muscle within 10 minutes [1] and immediately place it in chilled 0.9% sterile saline. Only if using D2.mdx mice, add fluorescent acetylcholine receptor dye at a 1 to 500 dilution to the microdissection bath and incubate the tissue for 30 minutes [2]. Trim the excess fat and connective tissue [3] and transfer the GM muscle onto a coverslip with the ventral side facing down for inverted microscopy [4].
 - 3.3.1. CU: Shot of dissecting the GM muscle. ~~TXT: Limit dissection and cleaning to <10 min~~
 - 3.3.2. Talent placing the GM muscle in saline. ~~TXT: For D2.mdx: Add fluorescent AChR dye (1:500) in bath; incubate 30 min~~
 - 3.3.3. CU: Shot of trimming excess fat and connective tissue from the GM muscle placed in saline.
 - 3.3.4. Talent transferring the GM muscle onto a coverslip. ~~TXT: Ventral side facing down for inverted microscopy~~

- 3.4. Add 2 microliters of general mounting medium onto the dorsal surface of the GM [1] and flatten the tissue using glass block before imaging [2].

- 3.4.1. Talent adding 2 μ L of general mounting medium onto the dorsal surface of the GM.
- 3.4.2. Shot of glass block flattening the tissue.

4. Confocal Imaging of Muscle Samples

- 4.1. In the software, set the excitation wavelength to 646 nanometers [1].
 - 4.1.1. SCREEN: 69732_screenshot_1_4.1.1_REC.mp4 00:20-00:28.
- 4.2. After adding 2 drops of clearing solution 2, select the 63x objective lens to image the TA muscle [1] and set the Z stack depth close to 100 micrometers [2]. Then, select deconvolution mode and apply system-optimized parameters [3]. Set the step size to one micrometer and acquire images [4].
 - 4.2.1. Talent selecting the 63x objective lens. ~~TXT: Add 2 drops of clearing solution II before imaging~~
 - 4.2.2. SCREEN: 69732_screenshot_2_4.2.2_REC (1).mp4 00:15-00:23 *Video editor: Please highlight the box "Z Size [μ m] with its value"*
 - 4.2.3. SCREEN: 69732_screenshot_3_4.2.3_REC-(1).mp4. 00:15-00:21
 - 4.2.4. SCREEN: 69732_screenshot_4_4.2.4_REC-(1).mp4. 00:02-00:08
- 4.3. To acquire terminal Schwann cells image, select the 20 times magnification objective lens [1], set the Z stack depth to approximately 17 micrometers and acquire images [2]. Then, select the 63 times magnification objective lens [3] and acquire images of individual terminal Schwann cells [4].
 - 4.3.1. Talent selecting the 20x objective lens.
 - 4.3.2. SCREEN: 69732_screenshot_5_4.3.2_REC-(1).mp4 00:22-00:28. *Video editor: Please highlight the box "Z Size [μ m] with its value"*
 - 4.3.3. SCREEN: 69732_screenshot_6_4.3.4_REC.mp4 00:10-00:20

5. Quantification of Mitochondrial Morphology

- 5.1. Import individual Z slices to train the software to recognize mitochondrial signal [1] and conduct quantitative analysis using AI driven software [2]. Develop image masks for investigator review [3].
 - 5.1.1. SCREEN: 69732_screenshot_7_5.1.1_REC.mp4 00:02-00:15.
 - 5.1.2. SCREEN: 69732_screenshot_8_5.1.2_REC.mp4 00:05-00:22.
 - 5.1.3. SCREEN: 69732_screenshot_9_5.1.3_REC.mp4.
- 5.2. Separate 3D (*Three-D*) image stacks into individual optical sections following algorithm optimization [1].
 - 5.2.1. SCREEN: 69732_screenshot_10_5.2.1_REC-(1).mp4 00:05-00:17.
- 5.3. Select every fifth Z-slice from each 3D image and calculate the average per sample [1].

- 5.3.1. SCREEN: 69732_screenshot_11_5.3.1_REC-(1).mp4 00:00-00:10.
- 5.4. Standardize imaging parameters across all samples and quantify mitochondrial morphology [1]. Perform immunohistochemistry and statistical analysis to assess group differences and correlations [2].
- ~~5.4.1. SCREEN: Shot of cursor adjusting the intensity thresholds, edge detection settings, and voxel size calibration for 1 sample. NOTE: 5.4.1 and 5.4.2 Not filmed~~
- 5.4.2. Talent viewing the quantified mitochondrial morphology.
- ~~5.4.3. Representative shot of talent adding stain to the slide.~~
- 5.4.4. Talent at the computer performing statistical analysis.

Results

6. Results

- 6.1. Mitochondrial connectivity was reduced in mdx muscle [1] compared to the wild type mice [2].
 - 6.1.1. LAB MEDIA: Figure 2A-D. *Video editor: emphasize B and D as well as the area indicated with yellow arrow.*
 - 6.1.2. LAB MEDIA: Figure 2A-D. *Video editor: emphasize A and C as well as the area indicated with yellow arrow*
- 6.2. A greater number of small mitochondrial fragments [1] and a reduced number of large interconnected mitochondrial networks were observed in mdx mice [2].
 - 6.2.1. LAB MEDIA: Figure 3B. *Video editor: Emphasize the red bar at "<100" point on x-axis.*
 - 6.2.2. LAB MEDIA: Figure 3B. *Video editor: Emphasize the red bar at "1000" point on x-axis.*
- 6.3. In wild-type mice, mitochondria in terminal Schwann cells exhibited interconnected loop structures near bifurcations [1]. In contrast, in mdx mice, mitochondria appeared fragmented and lacked loop formations near bifurcations [2].
 - 6.3.1. LAB MEDIA: Figure 4D and 4F. *Video editor: In 4D, emphasize the area indicated with yellow arrows.*
 - 6.3.2. LAB MEDIA: Figure 4D and 4F. *Video editor: In 4F, emphasize the area indicated with yellow arrows.*
- 6.4. Confocal and electron microscopy confirmed similar doughnut shaped mitochondrial structures in terminal Schwann cells [1].
 - 6.4.1. LAB MEDIA: Figure 5A-D. *Video editor: Emphasize the area indicated with yellow arrows.*
- 6.5. Postsynaptic analysis showed subsarcolemmal mitochondria clustered around acetylcholine receptors [1], lacking the characteristic pretzel like structure [2].
 - 6.5.1. LAB MEDIA: Figure 5H. *Video editor: If possible, emphasize the teal colored structure.*
 - 6.5.2. LAB MEDIA: Figure 5H. *Video editor: If possible, emphasize the red colored structure.*

- 6.6. Image analysis revealed that terminal Schwann cell mitochondria in mdx muscle showed increased area [1] and fragmentation compared to wild type muscle [2], with no difference in relative particle size [3].
 - 6.6.1. LAB MEDIA: Figure 6C. *Video editor: Emphasize the grey bar for mdx.*
 - 6.6.2. LAB MEDIA: Figure 6D. *Video editor: Emphasize the grey bar for mdx.*
 - 6.6.3. LAB MEDIA: Figure 6E. *Video editor: Emphasize both bars.*
- 6.7. Muscle force measurements showed reduced twitch [1] with greater variability [2] and decreased peak force in mdx muscle [3].
 - 6.7.1. LAB MEDIA: Figure 7A. *Video editor: Emphasize the red bar for mdx.*
 - 6.7.2. LAB MEDIA: Figure 7A. *Video editor: Emphasize the black dotted lines over red bar for mdx.*
 - 6.7.3. LAB MEDIA: Figure 7B. *Video editor: Emphasize the red bar for mdx.*
- 6.8. Force production across increasing stimulation frequencies was also significantly reduced in mdx muscles [1].
 - 6.8.1. LAB MEDIA: Figure 7C. *Video editor: Emphasize the red line for mdx.*
- 6.9. The mdx muscle showed a leftward shift in fiber size [1], indicating approximately 60% smaller myofibers compared to wild type [2].
 - 6.9.1. LAB MEDIA: Figure 7F. *Video editor: Emphasize the red bars for mdx.*
 - 6.9.2. LAB MEDIA: Figure 7E. *Video editor: Emphasize the red bar for mdx.*