Skeletal Muscle Fiber Typing & Cross-Sectional Area Analysis using Immunofluorescence Staining for Mice

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1 Objective

The objective of this SOP is to provide a step-by-step protocol for staining and analyzing skeletal muscle fibers from any muscle group in any mouse model. Skeletal muscle fibers are classified into four major types based on their fatigability, contractile properties, and metabolic profiles: Type I, IIA, IIB, and IIX.

Type I fibers, also known as slow-twitch or oxidative fibers, are highly fatigue-resistant and contain abundant mitochondria and capillaries. In contrast, Type II fibers, also known as fast- twitch, contain fewer mitochondria and capillaries and therefore require more ATP to support their high contractile activity. The proportion of each fiber type varies depending on the specific muscle and environmental conditions such as pathology or exercise.

Fiber-type composition can be quantified using immunofluorescence staining to detect the myosin heavy chain (MyHC) isoforms expressed in each fiber. Commonly used primary antibodies include MyHC-I (slow-twitch, oxidative), MyHC-IIA (fast-twitch, oxidative-glycolytic) MyHC-IIB (very fast-twitch, glycolytic), and laminin for cross-sectional area (CSA) measurement.

To assess changes in muscle fiber size in contexts such as disease or exercise, the CSA of individual muscle fibers in a tissue section can be measured. Fiber size distributions are typically visualized by staining with a primary antibody against laminin or dystrophin, followed by plotting the resulting measurements as a histogram on any statistical software.

2 Applicability/Scope

This SOP is intended for simultaneous qualitative identification of skeletal muscle fiber types (Type I, IIa, IIx, IIb) based on myosin heavy chain (MHC) isoform expression analysis using immunofluorescence. It is specifically applicable to freshly frozen, cryosectioned skeletal muscle tissue obtained from **mice** that has been cryosectioned (typically 10 µm thickness) and mounted onto charged microscope slides. This procedure is applicable for the quantitative measurement of individual muscle fiber CSA. This method is designed to resolve the technical challenge of high background staining inherent in using **mouse-derived primary antibodies** on **mouse tissue sections** through the utilization of the Vector M.O.M. (Mouse on Mouse) Fluorescein Kit. This protocol is not applicable for tissues derived from other species or for primary antibodies raised in non-mouse hosts. Successful execution requires a fluorescence microscope equipped with appropriate filters for detecting fluorescein (green) signals.

3 Summary of the Procedure

1. Tissue Preparation and Fixation:
   a. **Slide Preparation:** Details on typical cryostat settings (-20°C), section thickness (10 µm), and handling of charged microscope slides (25 x 75 x 1.0 mm). Fresh-frozen mouse

skeletal muscle cryosections (typically 10 µm thick) are transferred onto charged slides, air-dried, and briefly fixed (paraformaldehyde) to preserve morphology and immobilize antigens. Slides are then washed in phosphate-buffered saline (PBS).

2. Endogenous Mouse IgG Blocking (M.O.M. Specific Step):
   a. To prevent non-specific background staining caused by the secondary antibody binding to endogenous immunoglobulins within the mouse tissue, sections are incubated with the proprietary **Vector M.O.M. Mouse Ig Blocking Reagent**.
3. Primary Antibody Incubation (Fiber Typing):
   a. Following a wash step to remove excess blocker, sections are incubated with specific **mouse-derived primary antibodies** raised against different myosin heavy chain (MHC) isoforms (e.g., anti-MHC I, anti-MHC IIa, anti-MHC IIx). This step is typically performed overnight at 4°C to ensure optimal antigen-antibody binding.
4. Secondary Detection (M.O.M. Specific Step):
   a. Slides are washed to remove unbound primary antibodies. Sections are then incubated with the **M.O.M. Biotinylated Anti-Mouse Ig Reagent**. This specialized secondary antibody is designed to bind specifically to the mouse primary antibodies applied in Step 3 with minimal cross-reactivity to the endogenous mouse IgG blocked in Step 2.
5. Fluorescent Visualization:
   a. After subsequent washes, the biotinylated secondary antibody is detected by applying **Fluorescein Avidin DCS**. The strong affinity of avidin for biotin localizes the fluorescein fluorophore to the target MHC proteins, resulting in a green fluorescent signal under excitation.
6. Mounting and Imaging:
   a. Slides are washed a final time and mounted using an aqueous, anti-fade mounting medium to preserve fluorescence prior to the adding of a microscope cover (glass- 24x 50 mm). The sections are imaged using a fluorescence microscope equipped with appropriate filters for fluorescein (FITC/GFP).
7. Image Analysis (Fiber Typing & CSA):
   a. Captured images are analyzed using image processing software (e.g., ImageJ/Fiji). Muscle fibers are identified by type based on their positive staining for specific MHC isoforms. The individual cross-sectional area (CSA) of these identified fibers is then manually or automatically traced and quantified.

4 Definitions

- **Skeletal Muscle Fiber Typing:** The qualitative categorization of individual muscle fibers based on the specific isoform of Myosin Heavy Chain (MHC) protein they express. In mouse skeletal muscle, these are typically categorized as Type I (slow-twitch, oxidative), Type IIa (fast-twitch, oxidative-glycolytic), Type IIx (fast-twitch, glycolytic), and Type IIb (very fast-twitch, glycolytic).

- **Freezing Artifacts:** If the muscle is frozen too slowly, the water inside the muscle fibers forms large ice crystals. These crystals expand and physically tear the protein structure, creating a Swiss cheese appearance under the microscope.

- **Cross-Sectional Area (CSA) Analysis:** A quantitative measurement determining the two-dimensional surface area of individual muscle fibers when cut perpendicularly to their longitudinal axis. CSA is a critical indicator of muscle morphology, used to assess hypertrophy (excessive growth), atrophy (wastage), or response to therapeutic intervention.

- **Immunofluorescence (IF):** A laboratory technique that uses antibodies chemically conjugated with fluorescent dyes (fluorophores) to detect and visualize specific proteins (antigens) within biological tissue samples under a fluorescence microscope.

- **Myosin Heavy Chain (MHC):** The primary contractile protein of the thick filament in muscle fibers. The specific isoform of MHC present dictates the fiber's contractile speed and is the specific antigen targeted by the primary antibodies in this protocol.

- **Vector M.O.M. (Mouse on Mouse) Fluorescein Kit:** A specialized immunodetection system specifically designed to overcome high background staining when using a primary antibody raised in a **mouse** on tissue sections also derived from a **mouse**. Standard anti-mouse secondary antibodies cannot distinguish between the exogenous mouse primary antibody, intended to detect MHC, and the endogenous mouse immunoglobulins (IgG) naturally abundant in the tissue section, leading to high non-specific background. The M.O.M. kit utilizes a proprietary Mouse IgG Blocking Reagent to neutralize endogenous tissue immunoglobulins, followed by a specialized biotinylated anti-mouse secondary antibody that specifically targets the primary antibody.

- **Fluorescein Avidin DCS:** The visualization component of the M.O.M. kit. It consists of the protein Avidin conjugated with Fluorescein (a green fluorophore). Avidin binds with extremely high affinity to the biotinylated secondary antibody, providing an amplified, bright green, fluorescent signal (excitation ~495nm; emission ~515nm) at the site of the target antigen.

- **Region Of Interest (ROI):** Specific areas selected during image analysis for quantification of fiber areas.

- **Fiji:** A distribution of ImageJ software tailored for biological image analysis.

5 Materials and Equipment Required

Reagent	Company	Catalogue number
M.O.M. Kit	Vector Kit	BMK-2202
Phosphate Buffered Saline		
MyHC-I- Primary- BA-D5-supernatant (mouse IgG2b) Dilution Factor: 1/100	Developmental Studies Hybridoma Bank	BA-D5, RRID: AB_2235587)
MyHC-I- Secondary- Goat anti-mouse IgG2b CF405S Dilution Factor: 1/200 in 50% Glycerol	MilliporeSigma	SAB4600477
MyHC-IIA-Primary- mouse IgG1 Dilution Factor: 1/50, 50% Glycerol	Developmental Studies Hybridoma Bank	SC-71, RRID: AB_2147165
MyHC-IIA-Secondary- Goat anti-mouse IgG1 A488 Dilution Factor: 1/200, 50% Glycerol	Thermo Fisher Scientific	A-21121, RRID: AB_2535764
MyHC-IIB-Primary- purified (mouse IgM) Dilution Factor: 1/100	Developmental Studies Hybridoma Bank	BF-F3, RRID: AB_2266724
MyHC-IIB-Secondary- Goat anti-mouse IgM A594 Dilution Factor: 1/200, 50% Glycerol	Thermo Fisher Scientific	A-21044, RRID: AB_2535713
Laminin-Primary- anti-laminin (rabbit) Dilution Factor: 1/100	Sigma-Aldrich	L9393, RRID: AB_477163
Laminin-Secondary- Goat anti-rabbit IgG A647 Dilution Factor: 1/200, 50% Glycerol	Thermo Fisher Scientific	A-21244, RRID: AB_2535812
FluorSave mounting medium	Calbiolchem (without DAPI)	

6 Step-by-Step Procedure

6.1 Experiment

1. This protocol can be conducted at a non-sterile lab bench.

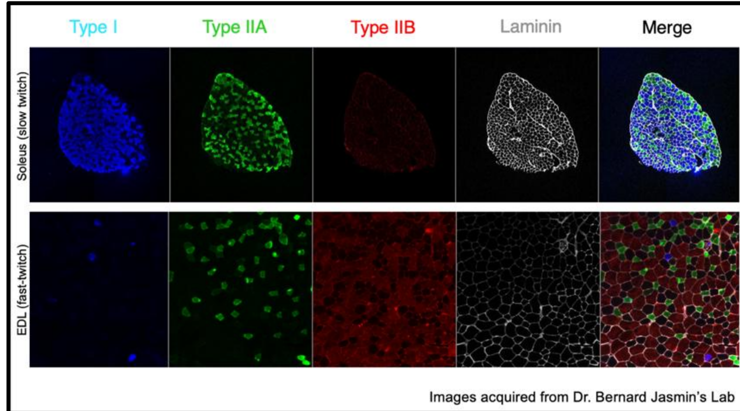
Prepare Reagents

Solution		
A	M.O.M. Mouse Ig blocking Reagent	Add 1 drop (45 µl) of stock solution to 1.25 ml of PBS
B	M.O.M. Diluent	Add 80 µl of protein concentrate stock solution to 1 ml of PBS

2. Take out charged microscope slides (size 25 x 75 x 1.0 mm) containing pre-cut muscle from -80°C freezer.
   a. Any muscle of interest can be used as long as the thickness of the cut is 10 µm.
3. Air dry frozen/freshly cut sections for 10-15 min at RT [no fixation is needed!].
   a. Fixation often creates strong chemical cross-links between proteins. While this is great for preserving general tissue architecture, it freezes the proteins into a rigid network. As a result, fixation can hide the specific epitope the antibody is trying to find and change the conformation of the MHC protein. Thus, the fiber-typing antibodies won't work, or the staining will be very weak and non-specific.
4. Incubate sections for **1h** in working solution of **M.O.M. Mouse Ig blocking Reagent (A)**.
5. Wash sections **2 x 5 min** in PBS with no agitation (at least 500 µl).
6. Incubate for **5 min** in working solution of **M.O.M. Diluent (B)**.
7. Pouroff excess of M.O.M. Diluent from sections.
8. Dilute primary antibodies in **M.O.M. Diluent (B)** to the appropriate concentration [at least 200 µl per slide].
9. Incubate sections inside staining dish with lid with primary antibodies for **45-60 min in any non-sterile incubator at 37 °C.**
   a. Sections should be placed flat inside the staining dish and incubated without rocking.
10. Wash sections **3 x 5 min** in PBS (at least 500 µl) with no agitation.
11. Dilute secondary antibodies in **M.O.M. Diluent (B)** to the appropriate concentration [200 µl per slide].
12. Incubate sections inside staining dish with lid with secondary antibodies for **30 min in any non-sterile incubator at 37 °C.**
   a. Sections should be placed flat inside the staining dish and incubated without rocking.
13. Wash sections **3 x 5 min** in PBS (at least 500 µl) with no agitation.
14. Mount section with FluorSave [Calbiolchem] mounting medium without DAPI.
   a. If nuclei visualization is required, add DAPI (1 ug/mL in PBS) after the 3rd wash with PBS in step 13, leave on for 5 mins. Perform a 4th wash with PBS, remove any excess liquid prior to adding the Fluorsave.
15. As soon as the FluorSave is applied, add glass coverslip (24 x 50 mm) and seal with nail polish. Washing before mounting is not required.
16. Wait until all of the nail polish has dried before storing in a 4°C fridge (for short-term usage) or until needed for imaging (can image as soon as the nail polish has dried, although waiting 24 hours is recommended).
   a. Store @-20°C for long term.

6.2 Imaging

- Using a fluorescent microscope, images can be taken at 20X or at 40x magnification.
- Images for each channel highlighting the different fiber types should look like the image below:

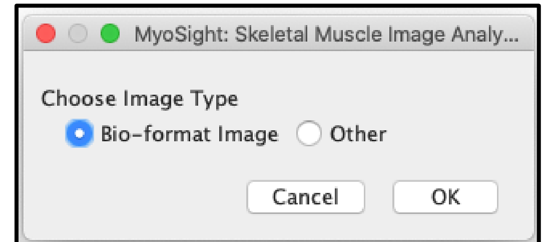


- To perform analysis, the merged image will be utilized.

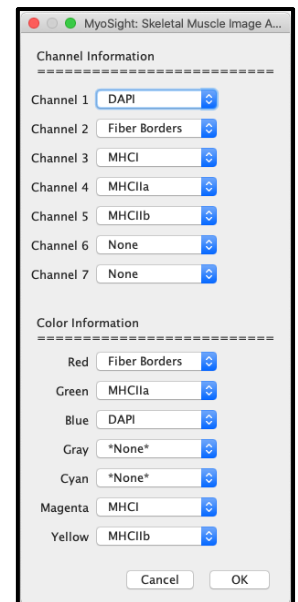
6.3 Analysis

- Download Fiji.
- Reference for analysis: steps (3A-15) were obtained verbatim from the Myosight Instruction Manual(3).

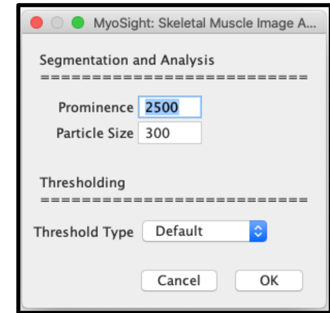
1. Save Images in a folder on Desktop.
2. Download the Myosight script into the folder containing the images to be analyzed.
3. Open Fiji software -> Click on Plugins -> Macros -> Run the Myosight.ijm script -> click on Bio-informat Image option -> click 'ok'.
    a. The program will automatically begin by prompting the user to set up for the analysis. First, a window will appear and prompt the user to choose "Bio-Format Image" or "Other". For best results, use images saved in the format given by the microscopes software, and choose "Bio-Format Image". If TIFF or JPEG formats are used, select "Other".



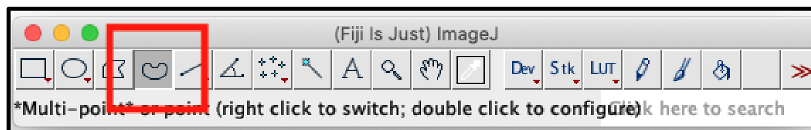
4. If "Bio-Format Image" is selected, the next dialog box will appear where the user enters channel and color information.
    a. If you are unsure which channels were used for each stain, open up the original image file to be analyzed in FIJI and choose the "split channels" option. Each channel will be given its own window titled "C0", "C1", or "C2", etc., for channel 1,2,3, respectively. When filling out the color information, ensure the color listed on the left matches the color/channel of the stain listed on the right. Then click 'ok'.



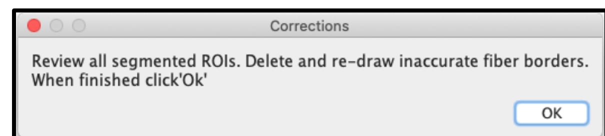
5. MyoSight will then prompt the user to choose a folder to save all output data and will create a new folder titled "Results". Choose a folder and click 'ok'.
    a. We recommended choosing a different folder to save results in for every image. If the same folder is chosen, MyoSight will overwrite the previous results folder. It is best to create a folder for every image so that the original image file and results folder are in the same folder.



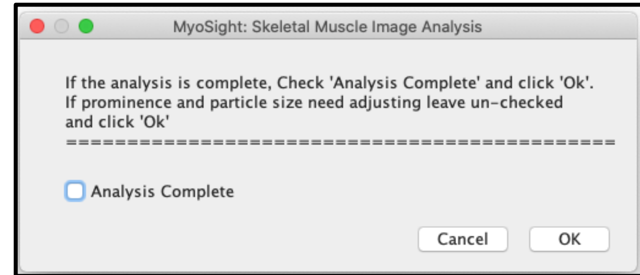
6. Next, select the image file you wish to analyze. Click 'ok'.
7. MyoSight will then open the image and prompt the user to enter prominence, particle size values, and threshold type.
    a. 'Prominence' is how sensitive the segmentation of the image will be; too high and not all ROIs will be recognized, too low and the program will create too many segments. Prominence is pre-set at 2500, but should be optimized for each image.
    b. 'Particle Size' is the minimum size required to be recognized as an ROI and is preset at 300µm. You may wish to set this lower if you have smaller fiber sizes.
    c. Threshold type refers to the method of thresholding used and is preset at "Default". Some methods will consider almost any pixel with color as threshold, some only threshold the bright test colors. This setting may need to be optimized depending on the quality of the laminin stain.
    d. If TIFF or JPEG formats are used, scaling information will also have to be entered. This information comes from measuring a scale bar on one of the images using FIJI.
8. Once these values are set, click 'ok' and MyoSight will proceed with analyzing myofiber borders. After the initial analysis, the user will be prompted to make corrections by deleting inaccurate analyses or drawing-in missing fibers (image to the right). Do not click 'ok' until all changes have been made.
    a. **Figure 2** from the MYOSIGHT manuscript illustrates common inaccuracies that must be manually corrected for greatest accuracy.
        i. To delete ROIs, place the cursor over the ROI label (which will be a number) and press 'delete' on the keyboard.
        ii. To draw in missing ROIs, or re-draw inaccurate ROIs, select the "Freehand Selections" icon on the FIJI tool bar (image below) and draw the missing ROI. After drawing, the user must select "Add" on the ROI manager (or press 't' on the keyboard) for the new ROI to be included in the rest of the analysis.



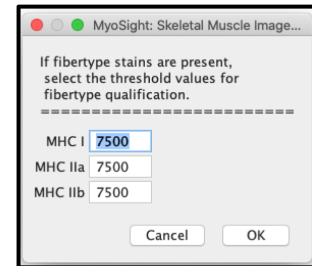
        iii. Please refer to FIJI's operating instructions for further assistance.
9. After all CSA corrections are made, or if you wish to reanalyze with new input, click 'ok' on the pop-up window.



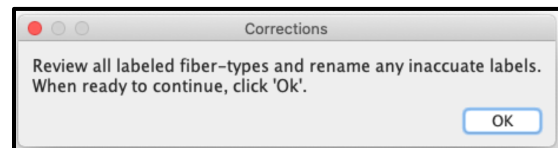
10. Another pop-up window will appear and gives the user the option to adjust the prominence, particle size and threshold type and re-analyze the image, or to continue to the fiber type analysis. If the analysis is complete, check 'Analysis Complete' and click 'ok'. If you wish to reanalyze, click 'ok' and repeat the process.

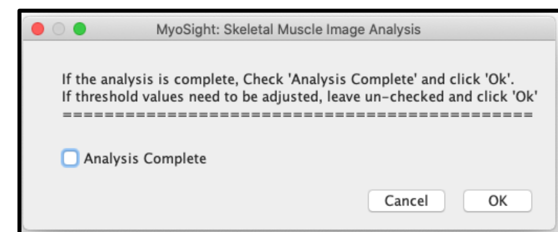


11. MyoSight will then prompt the user to enter threshold values used to determine fiber type.
    a. The threshold value entered for each fiber type determines the program's sensitivity to each fiber type stain; too high and MyoSight will not recognize the stain, too low and background 'noise' or autofluorescence could give a false positive.
    b. These values are pre-set at '7500', but should also be optimized for each image.

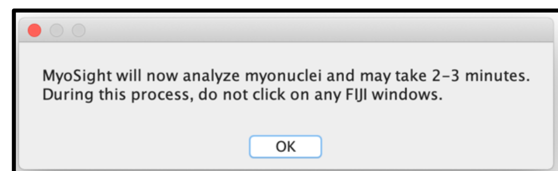


12. Once these values are set, click 'ok' and MyoSight will proceed with analyzing fiber type. After the initial fiber-type analysis is complete, the user will again be prompted to make corrections (Step 10).
    a. For an inaccurate fiber type label (labelled incorrectly due to the software mislabelling the multiple staining present), select the individual fiber by placing the cursor over the ROI label (which will be a fiber type) and select the ROI. Then, on the ROI manager, select "Rename" and give the fiber the correct fiber type label.
13. After all fiber type corrections are made or if you wish to complete the analysis, click 'ok' on the pop-up window. MyoSight also gives the user the option to adjust these values if they are unsatisfied with the analysis. Check 'Analysis Complete' if you are satisfied with the analysis. If threshold values need adjusting, leave unchecked, click 'ok', and repeat the process.





14. If nuclei are to be analyzed, the window below will then appear to alert the user that MyoSight will be analyzing nuclei even though nothing may appear to be happening. Once the user clicks 'ok' on the window below, MyoSight will then proceed with central and perinuclei analysis. These analyses do not require input from the user. Do not click on any FIJI windows once this process starts or it may be interrupted.

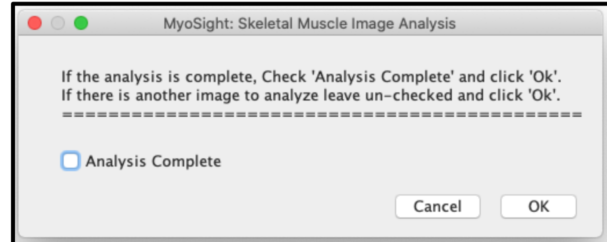


15. After all analyses are complete, MyoSight will automatically save all data in the designated folder, close all windows, and finally prompt the user to analyze another image, or to end the analysis.

Output files include images of all analyses taken and a results.txt file listing CSA, fiber type, central nuclei and perinuclei data.

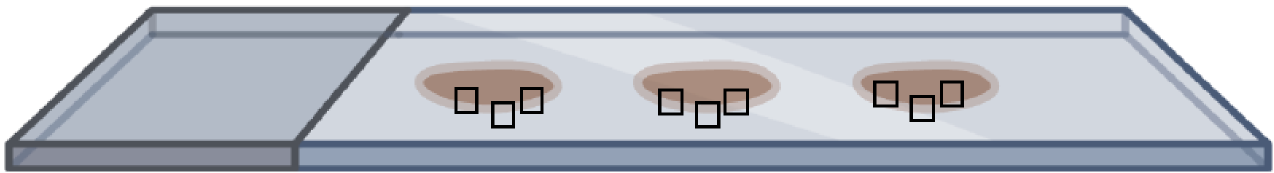
a. If the user wishes to analyze another image, leave the box unchecked and click 'Ok'. MyoSight will then prompt the user to select another folder to save the new results in (Step 5), creates another folder titled "Results", and prompts the user to select another image file.

b. Once an image file is selected MyoSight begins the new analysis with the same channel and color information as what was entered last. This way if there are multiple images taken the same way, the user does not have to re-enter this information for every image. Once the analysis is finished, check "Analysis Complete" and click 'ok' and the program will close.



7 Quality Assurance and Quality Control

- For technical replicates, perform 3–4 cropped images per tissue section. These multiple cropped regions act as technical replicates to ensure that measurements are not biased by local variability within a single section.
- For biological replicates, analyze tissue from multiple animals (often at least three per condition) to account for biological variability.
- In addition, including positive controls (e.g., samples known to exhibit hypertrophy) and negative controls (such as sections processed without primary antibodies) is advised to validate that the staining and analysis are performing as expected.
- Example of slide with muscle tissues; Positive control = 1 mouse will have 3 cross-sections and 3 images taken for each section (see squares):



- **Avoid Freezing Artifacts!** Freezing artifacts will make the CSA analysis inaccurate, compromise the fiber type staining, and increase non-specific binding of the antibodies. See example of freezing artifacts below:

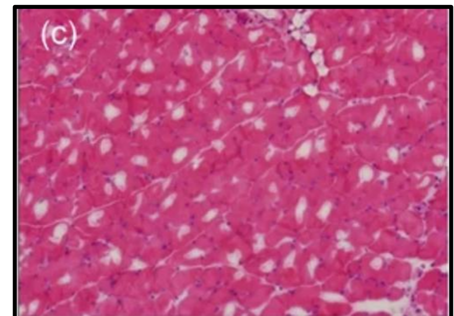
  

  Image from: Dapic et al., *Mass Spectrom Rev.,* 2019

  Tip: To avoid freezing artifacts, never place muscle directly into a -80°C freezer or onto dry ice. Always use liquid nitrogen-cooled isopentane. This snap-freezes the tissue in a very fast manner, turning the water into a glass-like state (vitrification) rather than forming destructive ice crystals.

These quality assurance and quality control measures help ensure the reliability, accuracy, and consistency of the procedure.

8 Safety Precautions

Use normal laboratory precautions.

9 References

1. Lumen Learning. (n.d.). *Muscular Levels of Organization*. Anatomy and Physiology I. Retrieved February 21, 2022, from https://courses.lumenlearning.com/cuny-csi-ap-1/chapter/muscular-levels-of-organization/
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3. Babcock LW, Hanna AD, Agha NH, Hamilton SL. MyoSight-semi-automated image analysis of skeletal muscle cross sections. Skelet Muscle. 2020 Nov 16;10(1):33. doi: 10.1186/s13395-020-00250-5. PMID: 33198807; PMCID: PMC7667765.
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10 Attachments/Checklists

10.1 Procedure Checklist

Sample Preparation

- ❑ Retrieve tissue sections from -80°C storage
- ❑ Air dry sections at room temperature for 10–15 minutes (do not fix)
- ❑ Label each slide clearly

Reagent Preparation

- ❑ Prepare M.O.M. Mouse Ig Blocking Reagent (add 1 drop [45 µl] to 1.25 ml PBS)
- ❑ Prepare M.O.M. Diluent (add 80 µl Protein Concentrate stock to 1 ml PBS)
- ❑ Dilute primary antibodies (MyHC-I, MyHC-IIA, MyHC-IIB, and laminin) in M.O.M Diluent at specified concentrations
- ❑ Dilute secondary antibodies in M.O.M. Diluent at specified concentrations
- ❑ Ensure FluorSave mounting medium is ready (without DAPI)

Immunostaining Steps

- ❑ Incubate slides with blocking reagent for 1 hour

- ❑ Wash slides twice (5 minutes each in PBS)
- ❑ Incubate with M.O.M. Diluent for 5 minutes and remove excess
- ❑ Incubate with diluted primary antibodies (200 µl per slide) for 45–60 minutes at 37°C
- ❑ Wash slides three times (5 minutes each in PBS)
- ❑ Incubate with diluted secondary antibodies (200 µl per slide) for 30 minutes at 37°C
- ❑ Wash slides three times (5 minutes each in PBS)
- ❑ Mount with FluorSave and store slides at -20°C until imaging

Imaging & Analysis

- ❑ Capture images using a fluorescent microscope (20x or 40x)
- ❑ Save images on the desktop
- ❑ Use Fiji software to crop images (3–4 crops per image) and generate ROIs according to the mentioned steps above.
- ❑ Analyze images using Fiji according to the mentioned steps above.
- ❑ Export segmentation results (CSV file) to Excel for further analysis

10.2 Quality Assurance / Quality Control Checklist

Technical Replicates

- ❑ Obtain 3–4 cropped images per tissue section to serve as technical replicates

Biological Replicates

- ❑ Process tissue sections from at least 3 independent biological samples (animals or conditions)

Software & Automation

- ❑ Utilize Fiji for standardized image cropping and ROI generation

Controls

- ❑ Positive Controls: Include samples known to exhibit hypertrophy (or expected staining patterns)
- ❑ Negative Controls: Include sections processed without primary antibodies to check for non-specific binding
- ❑ Validate image calibration by confirming scale (microns) before analysis

11 Examples & Tips

Tips:

- o Image dimensions must be in **microns** for CSA measurements in Fiji!
- ○ Scale from pixels/inches to microns in Fiji