MyoSight Instruction Manual

MyoSight is a semi-automated FIJI (Fiji is Just ImageJ) plugin designed to analyze skeletal muscle fiber cross cectional area (CSA), Feret’s diameter, fiber type, myonuclei and central nuclei. For best results, optimal immunofluorescent chemistry and confocal microscopy are needed. A detailed protocol for membrane, fiber-type and nuclei staining, as well as a detailed description of how to use MyoSight is described below.

To download FIJI (version 2.1.0/1.53c), visit <https://imagej.net/Fiji/Downloads>.

To download MyoSight, visit <https://github.com/LyleBabcock/MyoSight>

**Immunofluorescent Chemistry and Confocal Microscopy**

Sample Prep:

* Muscle samples imbedded in tissue-tech mounting media are frozen in liquid nitrogen-cooled isopentane and stored at -80°C
* Samples are cross sectioned at -25°C, 10μm thickness, and mounted on charged glass microscope slides
* Positive control samples for each individual stain/fluor being using should be prepared, as well as a negative control (Secondary antibody only)

Solutions / Buffers needed:

* PBS (Phosphate Buffered Saline), pH 7.2 – 7.4
  + NaCl - 137mM
  + KCl – 9.4mM
  + Na2HPO4 – 10.14mM
  + KH2PO4 – 1.76mM
* 4% Paraformaldehyde
  + 4% w/v in PBS
* Ammonium Chloride Buffer
  + 50mM Ammonium Chloride in PBS
* Permeabilization Buffer
  + 0.1% (w/v) TX-100 in PBS
* Wash Buffer
  + 0.05% (w/v) TX-100 in PBS
* Blocking buffer
  + 4% heat-inactivated goat serum (HIGS) in Wash Buffer
* Primary antibody solution, in PBS
  + Lamin (Abcam, ab11575), Rabbit IgG, 1:500 dilution
  + MHC I (DSHB, BAF8), Mouse IgG2b, 1:50 dilution
  + MHC IIa (DSHB, SC-71), Mouse IgG1, 1:50 dilution
  + MHC IIb (DSHB, BF-F3), Mouse IgM, 1:50 dilution
  + 1% Heat-inactivated Goat Serum (HIGS)
  + 0.05% (w/v) TX-100
* Secondary antibody solution, in PBS
  + Alexa Fluor 546 (ThermoFisher, A-11035), goat anti-rabbit IgG, 1:1000 dilution
  + Alexa Fluor 647 (ThermoFisher, A-21242), goat anti-mouse IgG2b, 1:200 dilution
  + Alexa Fluor 488 (ThermoFisher, A-21121), goat anti-mouse IgG1, 1:200 dilution
  + Alexa Fluor 594 (ThermoFisher, A-21044), goat anti-mouse IgM, 1:200 dilution
  + 1% HIGS
  + 0.05% (w/v) TX-100
* DAPI
  + 1:500 DAPI in PBS (Invitrogen, D3571)

Immunofluorescent Staining Procedure:

1. Fix mounted muscle slides in 4% paraformaldehyde for 5 minutes
2. Wash 5 times in PBS over 10 minutes
3. Incubate slides for 30 minutes in Ammonium Chloride buffer
4. Wash 2 times in PBS over 5 minutes
5. Incubate slides for 5 minutes in .1% TX-100 permeabilization buffer
6. Wash 2 times in wash buffer over 5 minutes
7. Incubate for 30 min with blocking buffer
8. Incubate in primary antibody solution overnight at 4°C in a humidified chamber.

------------------------------------------------------next day--------------------------------------------------------------------

1. Wash 5 times in wash buffer over 30 minutes
2. Incubate in secondary antibody solution for 2 hours at room temperature
3. Wash 5 times in wash buffer over 30 minutes
4. Incubate for 5 minutes with 1:500 DAPI in PBS
5. Wash 5 times in wash buffer over 10 minutes
6. Wash 2 times over 10 minutes in PBS to get rid of TX-100
7. Mount cover slip with anti-fade mounting media (Fluormount G), seal the edges of the coverslip with clear nail polish, and store in the dark

Confocal microscopy

* For imaging five or more channels on a Zeiss 880 confocal microscope, a lambda scan is needed, which records the emission spectra of all florofluors simultaneously. Therefore, positive controls for each fluor should be prepared on individual slides. The spectral array of each individual stain/florofluor being used should be assessed individually by the microscopes operating software first.
* Adjust the gain function to maximal brightness without any over exposure in any emissions range. This is the only digital optimization step before recording the image. Once imaging parameters are set, perform the lambda scan.
* After the lambda scan is complete, use the spectral unmixing functions in the microscopes software to separate each fluor’s emission spectra into separate channels.
* For imaging four or less channels, each channel can be optimized individually. For each channel, adjust the gain function for maximal brightness without over-exposure, and adjust the offset such that unstained areas produce no signal.
* Images are to be taken at 10-20X
* MyoSight cannot be used for Z-stacked images
* Take note of the channels used and the colors assigned to each channel. This information will be needed when using MyoSight.
* Save files in the format given by the microscope’s software. Do not convert them to TIFF or JPEG files.

**Using MyoSight**

1. Download and install FIJI and the MyoSight plug-in (links above).
2. To run MyoSight, open FIJI and go to ‘**Plugins’** 🡪 ‘**Macros’** 🡪 ‘**Run**’, then find and select the file ‘**MyoSight.ijm**’, or drag the program file into FIJI’s command bar.
3. 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”.

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1. If “Bio-Format Image” is selected, the next dialog box will appear where the user enters channel and color information
   * 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” or “C1”, “C2”, etc, for channel 1,2,3, respectively. When filling out the color information, ensure the color listed on the left matches the color/fluor of the stain listed on the right. Then click ‘Ok’.



1. 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’.
   * 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.
2. Next, select the image file you wish to analyze. Click ‘Ok’.
3. MyoSight will then open the image and prompt the user to enter prominence, particle size values, and threshold type.
   * ‘Prominence’ is how sensitive the segmentation of the image with 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.
   * ‘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.
   * Threshold type refers to the method of thresholding used and is preset at “Default”. Some methods will consider almost any pixel with color as thresholded, some only threshold the bright test colors. This setting may need to be optimized depending on the quality of the laminin stain.
   * 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.

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1. 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.
   * **Figure 2** from the manuscript illustrates common inaccuracies that must be manually corrected for greatest accuracy.
     1. To delete ROIs, place the curser over the ROI label (which will be a number) and press ‘delete’ on the keyboard.
     2. 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.

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* + 1. Please refer to FIJI’s operating instructions for further assistance.

1. **A screenshot of a cell phone

   Description automatically generated**After all CSA corrections are made, or if you wish to reanalyze with new input, click ‘Ok’ on the pop-up window.
2. 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.

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1. MyoSight will then prompt the user to enter threshold values used to determine fiber type.
   * 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.
   * These values are pre-set at ‘7500’, but should also be optimized for each image.

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1. 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).
   * For an inaccurate fiber type label, select the individual fiber by placing the curser 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.
2. 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

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1. 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.

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1. 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.
   * 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.
   * 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.

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