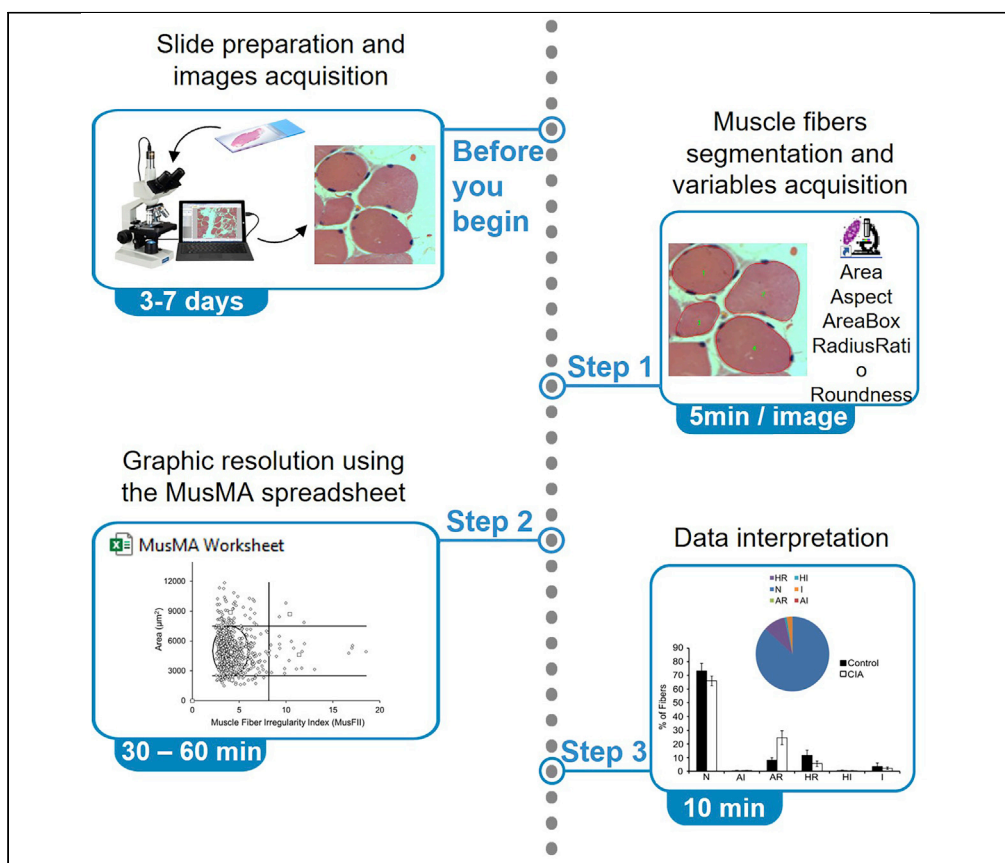


Protocol

Protocol to segment and characterize the morphometry of individual cross-sectioned striated muscle fibers from adult murine models



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Highlights

MusMA is a protocol to segment cross-sectioned muscle fibers

MusMA provides an overview of the morphometry of muscle fibers

MusMA data correlate with muscle function and cardiovascular risk

The muscle fiber morphometric analysis (MusMA) is a protocol to segment and characterize the morphometry of individual cross-sectioned striated muscle fibers. Using a semi-automated Excel spreadsheet, the protocol allows the objective measurement of muscle fibers' subpopulations, aiming to characterize physiopathological conditions related to muscle tissue. The main limitation of MusMA is the need for high-quality tissue slides and images and control samples to set up the analyses.

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

Bartikoski et al., STAR
Protocols 3, 101855
December 16, 2022 © 2022
The Authors.
<https://doi.org/10.1016/j.xpro.2022.101855>



Protocol

Protocol to segment and characterize the morphometry of individual cross-sectioned striated muscle fibers from adult murine models

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SUMMARY

The muscle fiber morphometric analysis (MusMA) is a protocol to segment and characterize the morphometry of individual cross-sectioned striated muscle fibers. Using a semi-automated Excel spreadsheet, the protocol allows the objective measurement of muscle fibers' subpopulations, aiming to characterize physiopathological conditions related to muscle tissue. The main limitation of MusMA is the need for high-quality tissue slides and images and control samples to set up the analyses.

BEFORE YOU BEGIN

The protocol below describes specific steps for using cardiac striated muscle tissue or skeletal striated muscle tissue from Sprague Dawley rats or DBA/1J mice, respectively, stained with hematoxylin and eosin (H&E). However, we also used this protocol for other tissue staining including Congo red, Gomori trichrome, Sterase and ATPase pH 9.4 (Figure 3).

Institutional permissions

All experimental procedures involving animals were approved by the Ethics Committee for the Use of Animals of the HCPA (protocols: 17-0021 for – for striated skeletal muscle tissue; 18-0367 – for



striated cardiac muscle tissue) and performed in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort.

Please, be sure to acquire all permissions from your institution.

Tissue preparation and fixation

⌚ Timing: 1 week

1. After the euthanasia of animals, dissect the skeletal striated muscle (SSM) or the cardiac striated muscle (CSM) samples.
2. Immerse the tissue in 10% buffered formalin for fixation for up to 3 days.
3. Dehydrate and embed the tissue in paraffin blocks to further cutting using a standard microtome.

Tissue staining and images acquisition

⌚ Timing: 1 week

4. Make 4 μm slices of paraffin-embedded tissue.
5. Arrange the slices on microscope slides.
6. Perform H&E staining (e.g., [Fischer et al., 2008](#)) or alternative staining for muscle tissue.

Alternatives: You can also stain your samples with other staining methods like Congo red staining, Goromi trichrome stain, Sterase stain, or ATPase pH: 9.4 stain ([Figure 3](#)).

7. Acquire at least 5 images per sample in an optical microscope at 200 \times or 400 \times magnification, looking for regions containing cross-sectioned muscle fibers. From these images you will perform the segmentation of muscle fibers. All pictures need to be acquired using similar conditions (i.e., magnification, light intensity, staining, and saved file format).
8. Install Image Pro Plus 6.0 software (or similar software, to perform muscle fiber segmentation);
9. Download the Excel MusMA Worksheet.

Spatial calibration (to convert the unit of area from pixels to μm^2)

⌚ Timing: 10 min

10. Photograph your microscope's millimeter calibration ruler.
11. In the Image Pro-Plus software, access 'Measure' \rightarrow 'Calibration' \rightarrow Spatial.
12. Select 'New', then open the image generated at step 10 to obtain the unit/pixel factor to convert pixels to μm^2 .

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Software and algorithms</i>		
Image pro plus 6.0*	Media Cybernetics	https://www.mediacy.com/78-products/image-pro-plus
Statistical Package for Social Sciences version 18.0 (SPSS 18.0 Inc, Chicago, IL, USA)	IBM	https://www.ibm.com/products/spss-statistics
Excel spreadsheet (excel file)	Microsoft	Available as supplemental information 1
Qcapture Pro	QImaging	https://software.com.br/p/qcapture-pro

(Continued on next page)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Continued</i>		
Other		
Olympus BX51 microscope**	Olympus	https://www.olympus-lifescience.com/pt/microscope-resource/primer/java/lightpaths/bx51fluorescence/
Glass blades**	Precision	N/A
Hematoxilin	Laborclin	https://www.laborclin.com.br/?s=hematoxilina&submit=Pesquisar
Eosin	Sigma-Aldrich	https://www.sigmaaldrich.com/BR/pt/product/sial/e4009
Microm HM 360 Automated Microtome**	Marshall Scientific	https://www.marshallscientific.com/Microm-HM-360-Automated-Microtome-p/m-hm360.htm
Animals for skeletal striated muscle sampling: DBA/1J male mice, wild-type, 60 days old.	Obtained from the animal experimentation unit of the Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil	N/A
Animals for cardiac striated muscle sampling: Sprague Dawley male rats, wild-type, 60 days old.	Obtained from the animal experimentation unit of the Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil	N/A

* Other software, including original software or open packages, could be used to that once it provides the same variables of size and shape used in the MusMA.

**The reagent or resource may be replaced by the user for similar products.

MATERIALS AND EQUIPMENT

Microscope information

For protocol development, we used Olympus BX51 microscope, and the QCapture X64 program. However, other microscopes or camera can be used.

Alternatives: Microscopes other than the one mentioned here can be used. Likewise, other brands of glass slides or dyes can be used.

Software information

The protocol for segmentation and variables acquisition was developed using the IPP6 software (Media Cybernetics, Bethesda, MD, USA; available for purchase). Other software, including original software or open packages, could be used to that once it provides the same variables of size and shape used in the MusMA. Statistical analyzes were performed using the Statistical Package for Social Sciences version 18.0 (SPSS Inc, Chicago, IL, USA). The MusMA spreadsheet was developed using the Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

STEP-BY-STEP METHOD DETAILS

The step-by-step protocol helps you to segment and acquire muscle fiber morphometric data using the IPP6 software. In the second subsection, the Excel spreadsheet used to quantify the subpopulations of muscle fibers is detailed. The excel spreadsheet is available as the [Data S1](#) (Excel spreadsheet designed for the analysis of primary morphometric measurements obtained from fibers segmentation, related to steps 19 and 20). Finally, we exemplify the alternatives to demonstrate the morphometric data generated by MusMA ([supplemental information 2 – Figure S1](#)).

Muscle fiber segmentation and variables acquisition

⌚ Timing: 5 min per image; about 25 min for each sample

In this step, you perform the segmentation of individual muscle fibers (that is, the delimitation of their contour), followed by the acquisition of the variables of size (Area) and shape that characterize the morphometry of each muscle fiber.

The following variables of shape are extracted: Aspect (reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object, as determined by Major Axis/Minor Axis; Area/box (reports the ratio between the area of each object, and the area of its imaginary bounding box); Radius Ratio (reports the ratio between Max Radius and Min Radius for each object); Roundness (roundness of the fiber, as determined by the following formula: $\text{perimeter}^2 / (4 * \pi * \text{area})$). These four measurements are used to calculate the Muscle Fiber Irregularity Index (MusFill), as detailed in the step 19, item c.

We suggest using at least 50 muscle fibers from 5 different fields from each sample.

1. Open H&E image in the IPP6 (Figure 1A).
2. Click on the 'irregular AOI' icon (Figure 1B).
3. A small window opens (Figure 1C – yellow box) and the mouse pointer changes to a magic wand.
4. Adjust the 'magic wand' range – refers to the number of gray levels (we suggest values from 10 to 30 to start the segmentation);
5. Adjust the 'magic wand' smooth – refers to post-filtering of the outline (we suggest values from 3 to 5; the same value need to be used to all muscle fibers) (Figure 1C).
6. To define the outline of the first fiber, left click inside the fiber. A red outline delimiting the object appears.
7. You may slightly alter the position of the left click inside the muscle fiber until the outline fits the fiber properly (Figure 1D).

△ CRITICAL: the adequate marking of the limit of the muscular fibers is essential for the acquisition of accurate measurements of area and shape.

8. When the fiber outline is adequate, press *ctrl* in the keyboard and left click inside the next fiber to be analyzed. At this point, the contour of the first selected fiber will change to green (Figure 1E).

△ CRITICAL: do not keep the ctrl button pressed while you are only adjusting the correct outline of the fiber. Unfortunately, the Image Pro Plus 6.0 does not allow you to undo the errors if you have made a mistake in the segmentation, so you will have to restart the segmentation if any fiber is incorrectly segmented.

9. Repeat the previous step until all fibers from the image are outlined in green.
10. Right click in any place of the image when all fibers were segmented. Since the segmentation is based on the difference in intensity of the muscle fiber pixels and the image background, not all image fibers can be segmented.
11. Click on the count/size icon (Figure 2A). Then, click on Measure → Select measurements (Figure 2B).
12. Select the following measurements: Area, Area/box, Aspect, Radius ratio and Roundness. Then, click ok.
13. At the count/size window: Edit → Convert AOI(s) to object(s) (Figure 2C).
14. At this point, the contour of all segmented fibers will become red, and each fiber will be numbered, from the top to the bottom of the image (Figure 2C – right image).
15. In the count/size window: file → data to clipboard (Figure 2D). Paste data from the first image in an Excel spreadsheet (Figure 2E).
16. Repeat steps 1–15 for all images of all participants (animals) and experimental groups.
17. Save this spreadsheet as 'MusMA raw data'; create a tab to each condition to facilitate pasting your data to the spreadsheet of analysis (described in the next section).
18. In the 'Measure' menu → Snap measurements to save the image with the segmented fibers.

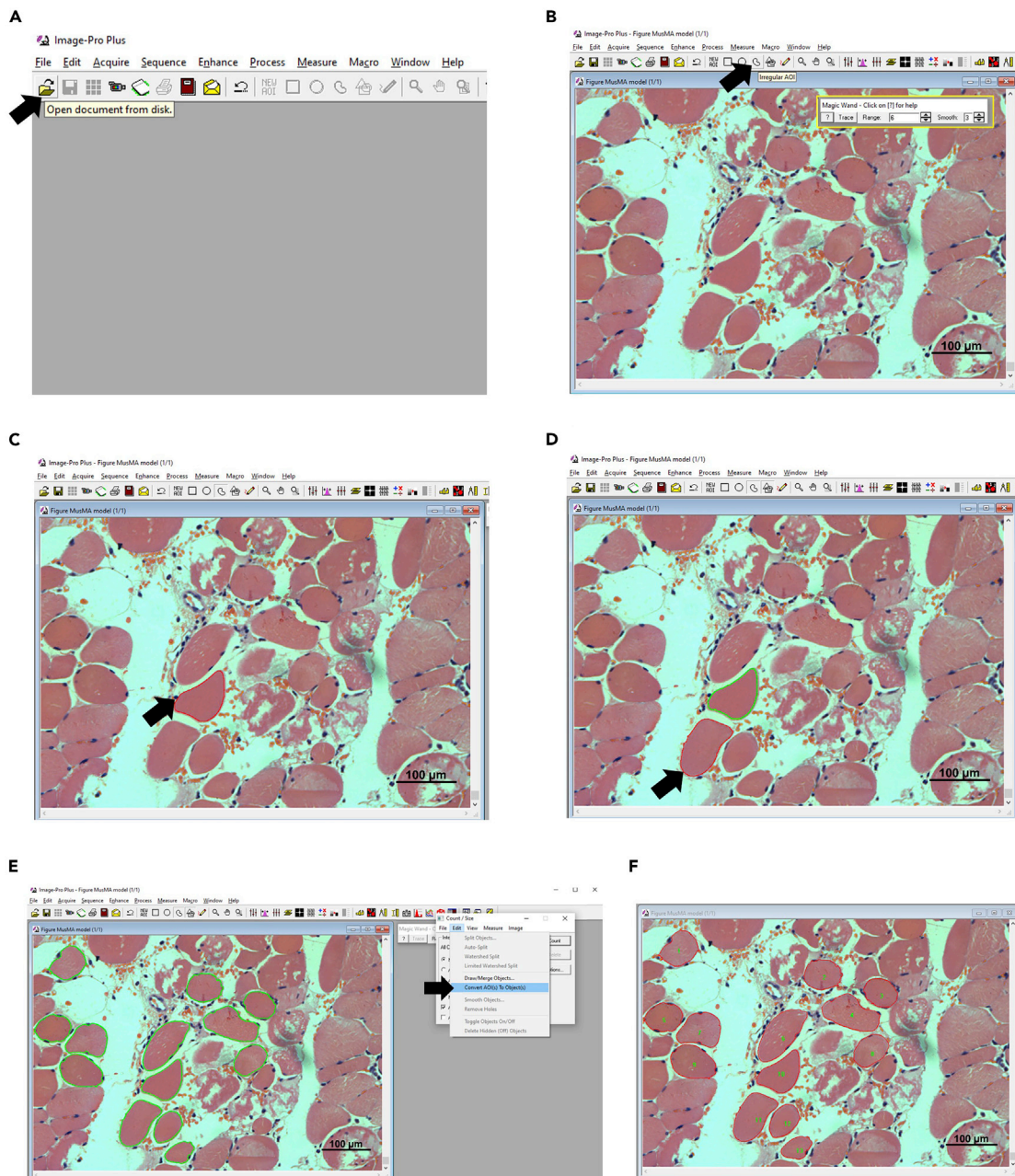


Figure 1. Interface of IPP6 and muscle fiber segmentation protocol

(A) The arrow indicates the icon to open images.

(B) Select the 'Irregular Area of Interest' tool, as indicated by the arrow. After selecting AOI, the cursor changes to a magic wand symbol. A little box of options opens (yellow bow), with two adjustable variables, to know: 'range' and 'smooth'.

(C) The magical wand allows the segmentation of the fibers, as indicated by the red line in the fiber pointed by the black arrow.

(D) After properly segmenting the first muscle fiber, press the ctrl key while left clicking on the next fiber to be segmented. This will fix the fiber just segmented, and the outline of the cell turns into green.

(E) A representative image with various fixed cells is shown in the detail.

(F) By clicking on 'Convert AOI(s) to Objects', segmented fibers are converted to numbered objects.

Using MusMA to analyze the raw data

© Timing: 30 min to 1 h

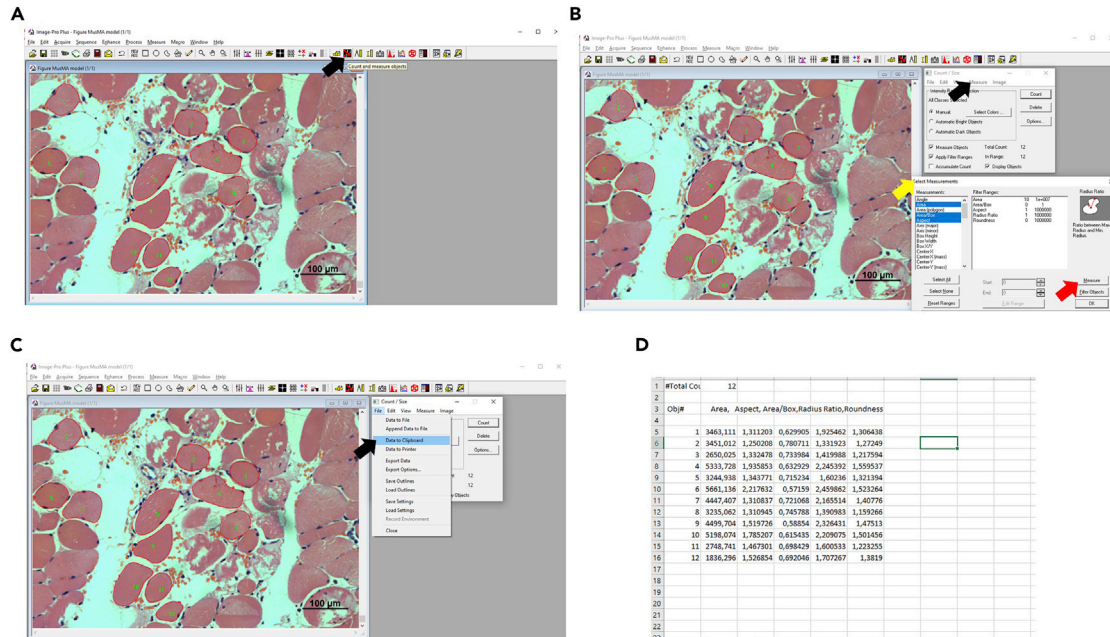


Figure 2. Starting the variable acquisition

(A) The arrow indicates the 'count/measure' tool.

(B) After clicking on 'measure' (black arrow), a new window opens, allowing you to choose the measurements of interest, as indicated by yellow arrow (Area, Area/box, Aspect, Radius Ratio and Roundness). The measure button (red arrow) then calculate all variables to each muscle fiber.

(C) In count/size' window, click on File, then 'data to clipboard'.

(D) Representative data pasting raw data from IPP6 in an excel file.

These steps describe how to analyze raw data using a specific Excel spreadsheet designed for MusMA (Data S1). The first worksheet is named 'normal fibers and settings', and is used to set the parameters of normal (or 'regular') fibers as well as the thresholds that define the different quadrants according to fibers' morphometry. The other two worksheets (named 'Condition 1' and 'Condition 2') are identical and intended for the analysis of the morphometry of the muscle fibers from different groups (or conditions). You may generate as many 'condition' worksheets as necessary considering the number of experimental groups.

19. Setting the ellipse of normal muscle fibers and the thresholds to separate the subpopulations of muscle fibers.
 - a. Download and open the 'MusMA clear.xls' excel file (Figure S1).
 - b. In the 'normal fibers and settings' worksheet, paste data of at least 50 normal fibers (from the control condition) in the yellow marked cells (beginning in C11 through G11; Figure 4A).
 - c. The MusFII, in dark yellow cells (cell H11 down), is automatically generated (Figure 4A, arrow).
 - d. Cells from H11 down (Figure 4A, orange cells on the left) are marked with an 'X' and have the mathematical formula to automatically calculate MusFII.
 - e. After pasting raw data, delete the formula (i.e., delete the "X") in those lines that do not contain any data. Attention: this step needs to be done for all occurrences in cells marked with an "X", both in "normal fibers and settings" and all "Conditions" datasheets.

△ CRITICAL: The deletion of the "X" in cells where there is no muscle fiber data is essential for the correct plotting of the graphs.

- f. Adjust the number of standard deviations related to the average of fibers' area (P2 cell) and MusFII (P3 cell) to set the ellipse surrounding 'normal fibers' (Figure 4B, arrow).

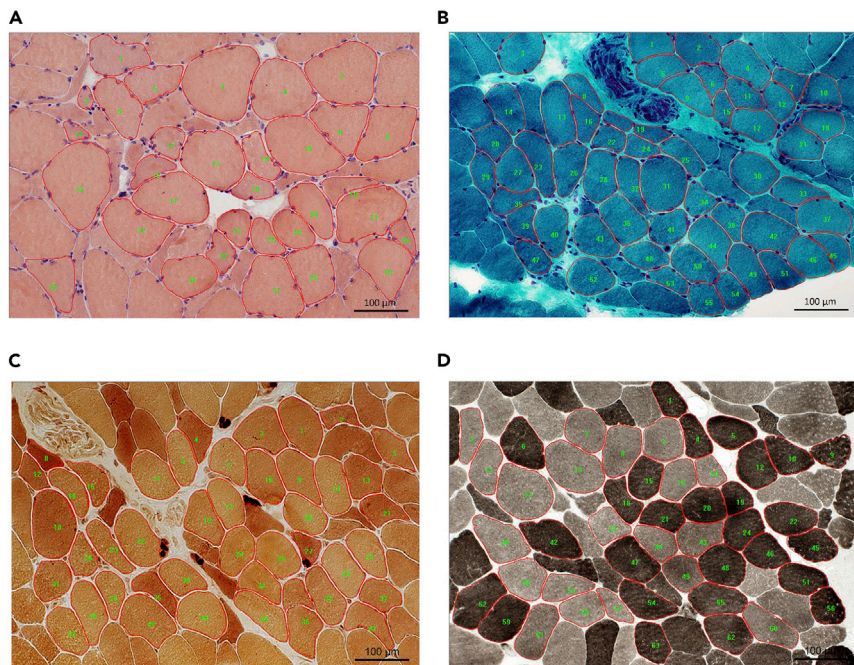


Figure 3. MusMA differentiates muscle fibers in 4 staining conditions

(A) Congo red staining.

(B) Gömöri trichrome stain.

(C and D) (C) Sterase stain; and (D) ATPase pH: 9.4 stain. Images were obtained from the public bank of neuromuscular images of the WUSTL (<https://neuromuscular.wustl.edu/>).

△ **CRITICAL:** to set the normal ellipse (as shown in Figure 4B), adjust the number of standard deviations so that the ellipse fits as many normal fibers as possible, while leaving the smallest possible empty area inside. Avoid ellipses that are over-area (i.e., too much white area due to excessive number of standard deviations) or too small (i.e., too many control fibers outside the ellipse because of too low number of deviations).

- g. To adjust the thresholds that set the quadrants in the MusMA plot, paste data from all experimental conditions in the light-yellow cells below 'setting the thresholds' (from Z11 through AD11) (Figure 4C, black arrow).
- h. The MusFI is automatically generated in the AE11 down (Figure 4C).
- i. Through altering the number of standard deviations in S4, S6 and S8 cells (Figure 4C, blue arrow), adjust the thresholds that define each quadrant in the right graph.
- j. All parameters used to set the normal population and the thresholds are automatically applied to all the conditions' worksheets. Please, do not change any cells in the spreadsheet, except for those whose font color is marked in red (cells: P2, P3, S4 S6 and S8).

20. Data analysis and interpretation.

- a. Duplicate the "Condition" tab to generate a tab for each condition or experimental group.
- b. In each 'Condition' worksheet, paste raw data in the yellow box (B13 through G13 cells down) (Figure 4E, arrow). Column A is specific to each experiment / animal / patient. We estimated 500 fibers per experiment / animal / patient, for 10 independent experiment / animal / patient.
- c. MusFI is automatically calculated in H11 down (Figure 4E).
- d. After pasting data from morphometric measurements, the MusMA Excel spreadsheet automatically generates an Area x MusFI plot and calculates the percentage of muscle fibers in each subpopulation (i.e., normal, irregular, atrophic, or hypertrophic).

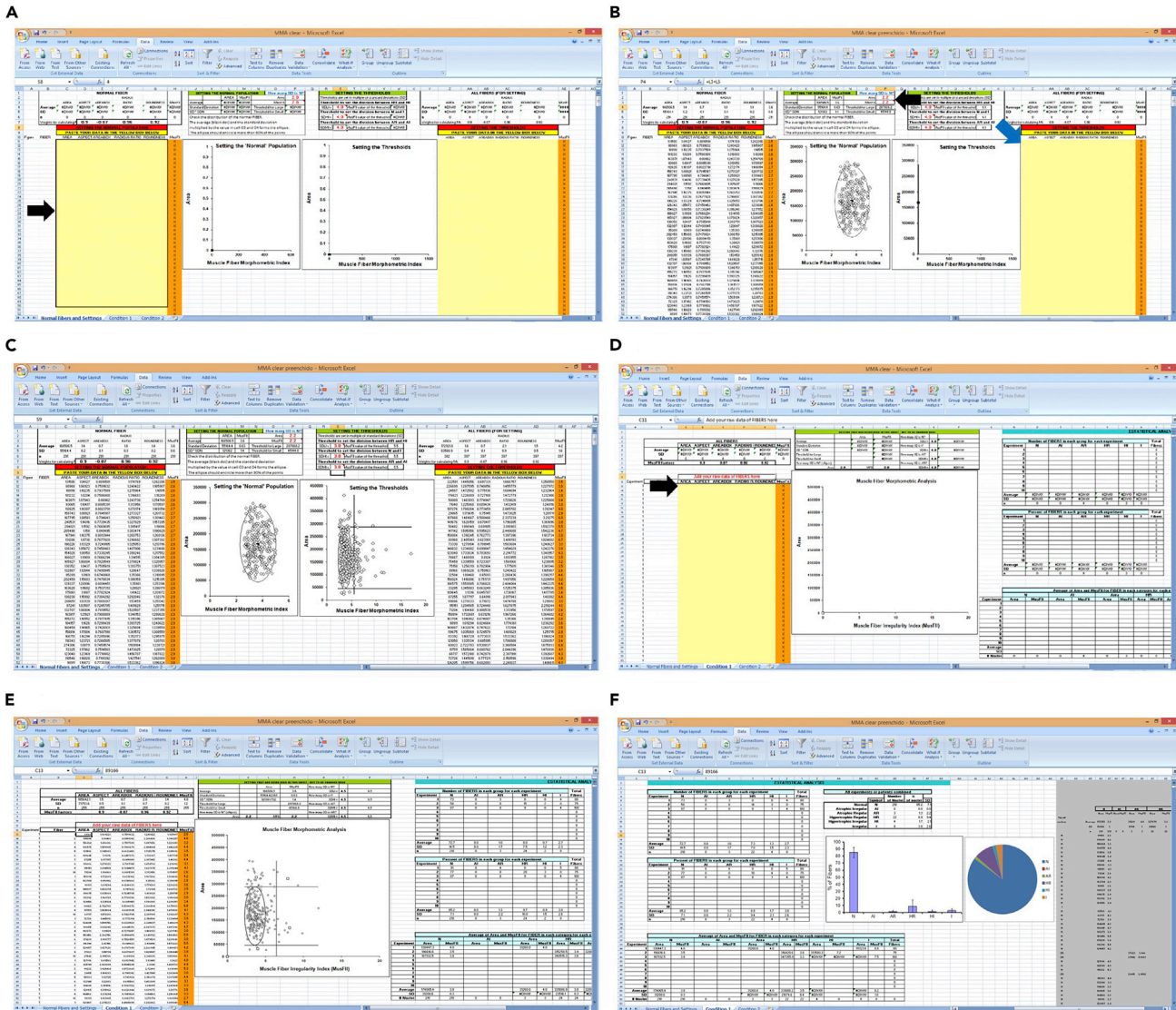


Figure 4. Graphical resolution of MusMA

Starting the analysis.

(A) Layout of MusMA clear.xls file. The arrow indicates the location where you may paste raw data to set the normal ellipse (beginning in C13 to G13).

(B) Setting the normal ellipse. Set the number of standard deviations related to the average of Area and MusFIL (black arrow), to adjust the normal ellipse. Then, adjust the thresholds to define the quadrants. Paste raw data of all conditions in the box pointed by the blue arrow. Then, alter the number standard deviations (red cells in S column) to define the quadrants that separate the subpopulations of fibers.

(C) The image shows the final settings of the normal ellipse (left graph) and the thresholds (right graph). (D) 'Condition' worksheets. 'Condition 1' worksheet refers to an experimental condition or group. Paste raw data in the C13 cell, as indicated by the black arrow. Column A indicates different experiments (or animals or individuals). Per condition, we estimated 500 fibers to each experiment / animal / individual (column B), and 10 independent experiments / animals / individuals.

(E) The Area x MusFIL dot blot of Area x MusFIL is automatically generated. You must generate, from the duplication of the 'Condition 2' tab, as many 'condition' tabs as necessary considering the number of experimental conditions.

(F) Bar and pie charts for the percentage of muscle fibers with each phenotype.

- e. Results and graphs are also automatically done. Main graph is a scatter plot (x, y) of area and MusFIL of individual fibers (Figure 4F; Figure S1B). Two additional graphs are generated: a bar graph corresponding to the average \pm standard deviation (and a pie chart).

EXPECTED OUTCOMES

This protocol aims to provide an objective tool to assess the morphometry of muscle fibers from histopathological images. The MusMA technique provides an accurate screening of muscle tissue samples, allowing the screening of muscle health based on the morphometry of muscle fibers.

The semiautomated excel spreadsheet automatically generates different graphs to help you with the comprehension of data. The Area x MusFII graph generated at “condition” worksheets separated muscle fibers according to the threshold defined at the “Normal fibers and settings” worksheet; the Area x MusFII graph separate muscle fibers in 6 different states: Normal fibers, Hypertrophic regular, Hypertrophic irregular, Irregular, Atrophic Irregular, Atrophic Regular. Thus, MusFII provide an overview about the health of muscle tissue. The percentage of fibers in each quadrant and the standard deviation to each condition is shown in AE6 and AF6 cells down. Comparisons between Area x MusFII graphs from different conditions allow seeing meaningful changes in muscle fibers as well as cluster of fibers changing the state during treatment or disease (Figure S1). To complement Area x MusFII graph, the spreadsheet generated 2 graphs: “% of fibers x fibers state” (Figure S1C) and a pie chart showing the averaged percentage of fibers with each morphometry. In addition, is possible to choose compare the average of area and MusFII between the experimental conditions (Figure S1D). Nevertheless, the MusMA spreadsheet provides in every worksheet a table with information about fibers state and the variables collected in IPP6 software.

In conclusion, MusMA is an objective technique we believe can be used both in experimental sciences and complementing the analysis of pathologists in clinical decision making. In the latter case, MusMA could be employed combined with clinical parameters in several human musculoskeletal conditions, both in diagnosis and to predict the risk of muscle failure or loss of function.

LIMITATIONS

The quality of the MusMA outputs are dependent on the muscle preparation and staining that are used. Therefore, results will depend on:

The quality of histological section and staining. Histological sections with tissue fragmentation, torn fibers, or other technical artifacts make segmentation unfeasible and impair the protocol’s execution.

The quality of acquired images. If the margins of the muscle fibers are poorly defined, segmentation will not be correct.

The protocol’s success requires a setting performed from control or healthy condition. From the segmentation of this tissue, the regions of the MusMA graph are defined. Without control or normal population, it is impossible to perform the analysis.

TROUBLESHOOTING

Problem 1

Poor quality muscle tissue (e.g., poorly fixed) ([before you begin](#)).

Potential solution

Re-acquire muscle tissue, taking care of everything from the tissue acquisition to fixation and histological preparation.

Problem 2

Poor staining of histological sections ([before you begin](#)).

Potential solution

The quality of the staining is fundamental for the segmentation of the fibers. Recolor the muscle tissue. Be sure the staining solutions are suitable for use. If necessary, remake the solutions required for staining, including the dyes.

Problem 3

Few muscle fibers in cross section in the images to be segmented ([before you begin](#)).

Potential solution

Capture more images from the muscle tissue sheets, looking for regions enriched in cross-cut muscle fibers.

Problem 4

Marking of cardiac striated muscle fibers in a region other than the central region of the fiber (steps 6–8).

Potential solution

Perform segmentation in the central region of the muscle fiber. For this, they must segment only those fibers in which the nucleus of the muscle fiber is observed.

Problem 5

Not properly setting the normal ellipse and thresholds that separate different muscle fiber phenotypes (steps 19a–f).

Potential solution

Adequately adjust the number of standard deviations in the excel sheet, so that both the normal ellipse and the thresholds between populations are correctly delimited.

RESOURCE AVAILABILITY

Lead contact

Requests for reagents will be fulfilled by the Lead contact: Eduardo Cremonese Filippi-Chiela (eduardochiela@gmail.com).

Materials availability

We provide an Excel datasheet in which you must perform the analysis of the primary data generated after the segmentation of muscle fibers. This file is available as [supplemental information 1](#).

Data and code availability

Original data have been deposited to Mendeley Data: <https://doi.org/10.17632/pfs8vpt9zs.1>.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101855>.

ACKNOWLEDGMENTS

The authors would like to thank the Research Incentive Fund of the Hospital de Clínicas de Porto Alegre, CNPq (National Counsel of Technological and Scientific Development) and PNPd/CAPES (Coordination for the Improvement of Higher Education Personnel) Program for financial support.

AUTHOR CONTRIBUTIONS

Conceptualization, B.J.B., L.L., and E.C.F.C.; methodology, E.C.F.C., V.E.G.d.S., F.S., and C.U.C.; investigation, B.J.B., L.L., and E.C.F.C.; writing – original draft, B.J.B. and L.L.; writing – review & editing, B.J.B., L.L., E.C.F.C., R.M.X., and M.R.A.d.S.; resources, E.C.F.C.; supervision, G.L., R.M.X., M.R.A.d.S., and E.C.F.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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