MuscleJ2: a rebuilding of MuscleJ with new features for high content analysis of skeletal muscle immunofluorescence slides

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Features

Fiber morphology – Centro Nuclei Fiber – Peri Nuclei Fiber - Capillary – Satellite Cell – Fiber typing – Fiber staining – ECM detection – Specific cells – Vascularization – Multi cartographies

List of Abbreviations

Cap: Capillary;
CNF: Centro Nucleated Fiber;
CSA: Cross-Sectional Area;
ECM: Extracellular Matrix;
F: Fiber;
GC: Gravity Center coordinates;
MB: Membrane;
ND: Not Defined;
ROI: Region Of Interest;
SC: Satellite Cell;
V: Vessel.

Main Data Workflow

MuscleJ2 is a multi-analysis tool allowing the morphological analysis of muscle fibers, the localization of capillaries, the peri-myonuclei and satellite cells as well as quantification of the intensity of any staining in regional sub-localizations of the fibers. It also provides quantitative analyses of fibrosis, vascularization and cell phenotype in whole muscle sections. A multi-cartography option allows users to visualize multiple results simultaneously. In the figure1 the data and analysis workflow (Panel A) has been schematized for a better understanding of the flow of data from requested input to the output produced by MuscleJ2 but also examples of function organigrams (Panel B).

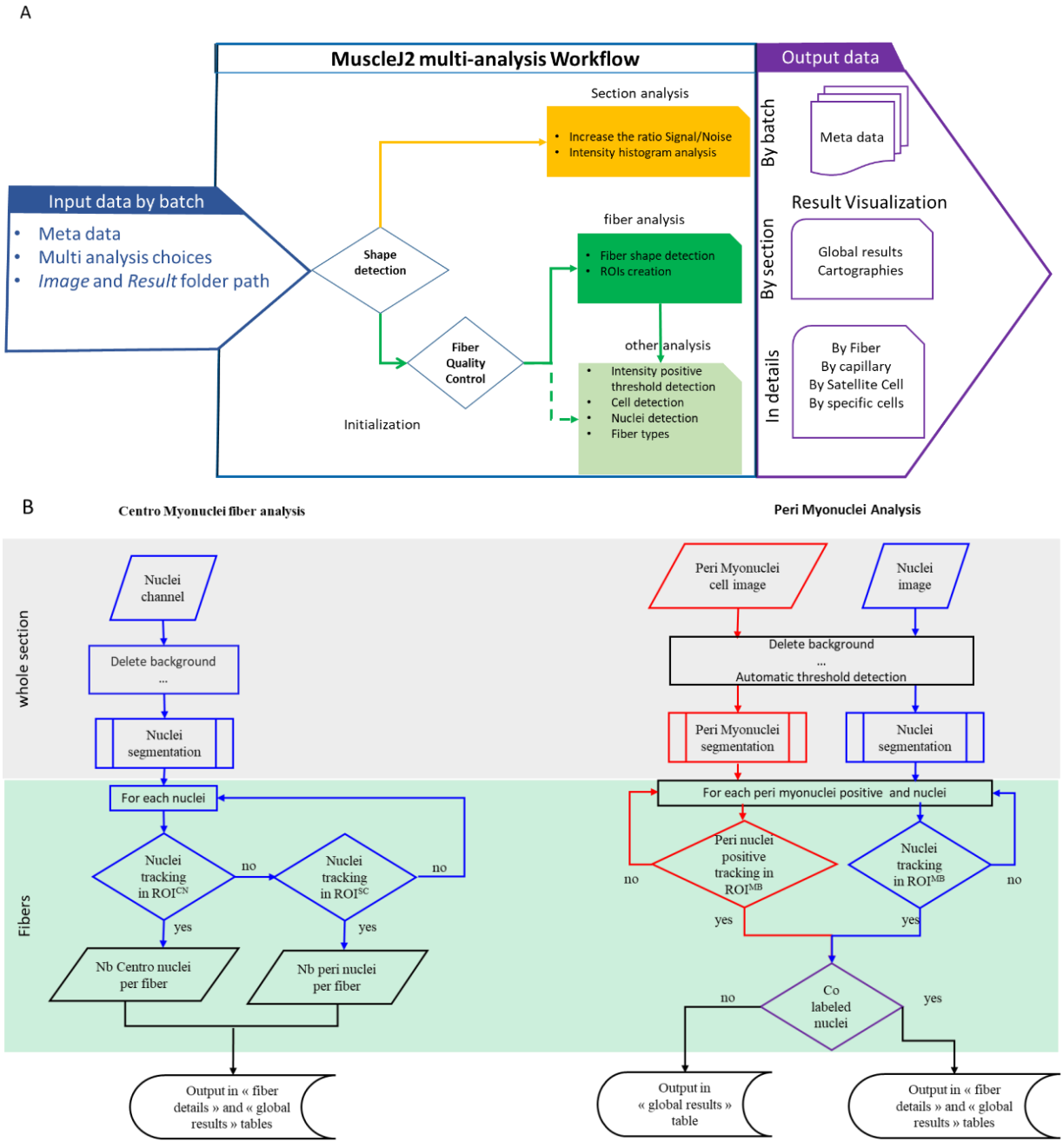


Figure 1: A. Data and analysis workflow B. Examples of function organigrams implemented in MuscleJ2 plugin.

Contents

I. MuscleJ2 in the FiJi/ ImageJ environment

A. Required environment.

FiJi

- Download FiJi from https://imagej.net/Fiji/Downloads and follow the installation instructions or update your FiJi version to the minimum required.
  The minimum version requirements are as follows:
  Fiji version from 1.51e, tested on 1.53f51
  Java version: Java 1.8.0-66 (64 bits)
  Used Plugins: Bio-Formats Plugins for Fiji (from release 5.8.2, tested on release 6.7.0)

ImageJ

- Download ImageJ from https://imagej.nih.gov/ij/download.html and follow the installation instructions or update your ImageJ version to the minimum required.
- Install *Bio-Formats* plugin in ImageJ from https://docs.openmicroscopy.org/bio-formats/6.6.0/users/imagej/installing.html or update your *Bio-Formats* plugin version to meet the minimum version requirements.
  The minimum version requirements are as follows:
  ImageJ version from 1.51e, tested on 1.52v
  Java version: Java 1.8.0-66 (64 bits)
  Used Plugins: *Bio-Formats* Plugins for ImageJ (from release 5.5.3, tested on release 6.6.0)

B. MuscleJ2 plugin installation

- Download from MuscleJ github space (https://github.com/ADanckaert/MuscleJ2/) the file named "*MuscleJ_.jar*" corresponding to the new version of MuscleJ plugin (MuscleJ2).
- Click on Plugins menu
  Select *Install…* command ➔ Open "MuscleJ_.jar"➔Save in "*Plugins*" folder.
  If you have previously installed a version of MuscleJ, it asks you if you would like to replace by the new one.
  Restart ImageJ or FiJi, and MuscleJ command should now appear in the *Plugins* menu (Fig.2).
- Finally, the amount of the computer memory devoted to ImageJ/Fiji can be increased by selecting *Edit* menu➔ *Memory & Threads* without allocating more than 75% of the computer's total memory, as ImageJ/Fiji may become slow and unstable.

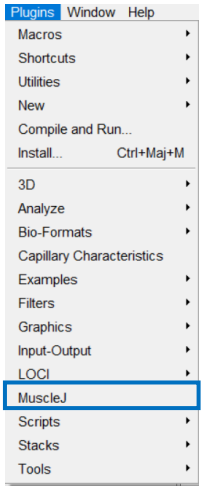


Figure 2: Plugin menu after MuscleJ2 plugin installation in ImageJ

C. Preliminary requests before starting MuscleJ2.

- Two folders, named “Image” and “Results”, must be present before starting MuscleJ2.
  The sub folders contained in the "*Results*" folder (*Artefacts*, *Cartography*, *Results_byfile*, *ROI*) will be automatically created if they do not exist.
- **The input and output folders have to be named without using spaces and symbols.**
- All images contained in the ”*Image*” input folder should have
  - the same acquisition parameters: number of channels, channel orders, staining and data acquisition (as such as Single Z or Z-Stack)
  - the same sample properties: same type of muscle and same physio-pathological state.

II. How to launch MuscleJ2

When you click on MuscleJ in the *Plugins* menu the principal dialog box appears:

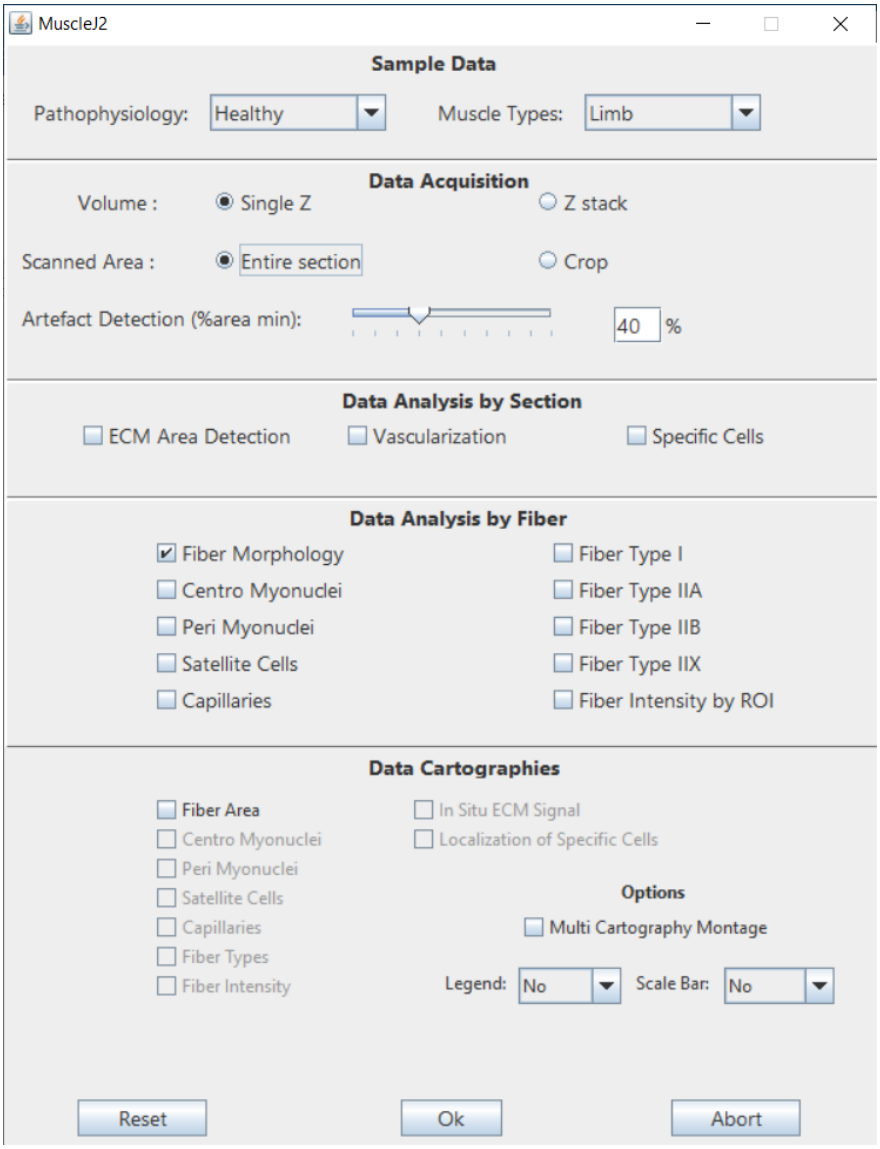


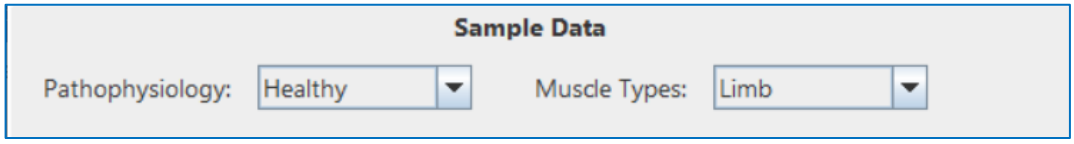
Figure 3: Principal Dialog Box allowing to start the batch run with MuscleJ2.

It is organized into 5 panels (*Sample Data panel*, *Data Acquisition panel*, *Data Analysis by Section panel*, *Data Analysis by Fiber panel* and *Data Cartographies panel*), allowing the user to enter the main metadata required by MuscleJ2 to start the analyses selected by the user.

A. Step1: MuscleJ2 Principal Dialog Box

Sample Data panel

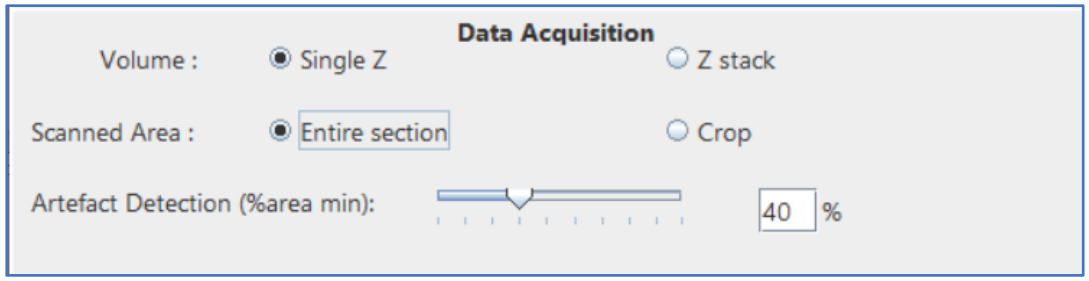
This panel allows the user to enter information on the type of muscle or pathophysiology studied on all images being analyzed.



Pathophysiology*:* This option allows users to adapt the algorithm according to their parameters of interest. When *Healthy* is selected, MuscleJ2 excludes the extreme fibers, i.e. the largest and smallest fibers. By selecting the *Damaged* option, the range of differences between the fiber CSA is much wider and all the fibers are taken into account.
Muscle Types: depending on the type of muscle, the detection of the section area detection will differ. When the "*Diaphragm"* type is selected, MuscleJ2 does not fill the holes to take account of the real surface of the tissue, unlike the "*Limb*" type.

Data Acquisition panel

MuscleJ2 uses *Bio-Format importer* in Plugins menu➔*Bio-Format* plugin to open the raw data. This plugin recognizes more than 150 different file formats (https://docs.openmicroscopy.org/bio-formats/6.6.0/supported-formats.html).
If your data format is not supported, first export your tiff-16bit image per channel from the microscope software, then create and save your multi-channel tiff-16bit images (see *Annex 1 How to create a tiff format with multiple channels*).



Volume:
"*Single Z*": click on it if you have acquired your images with the single plan of the sample.
"*Z stack":* if you choose this option, an automatic maximum projection along z axis is performed before starting analysis.

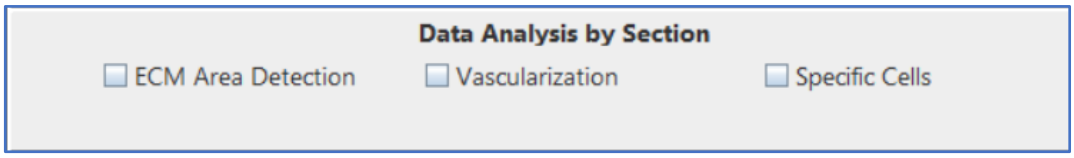
Scanned Area:
"*Entire section"*: the whole section or a large part of the tissue of interest (min. 75%) has been scanned.
"*Crop"*: if only a small area has been scanned, the analysis will be less efficient despite a series of additional filters that track the maximum intensity of the fiber contours in the laminin channel (see

Annex 2. Recommendations for cropping images of muscle sections). This option has an impact on the minimum fiber area accepted for detecting fiber shapes, and shape detection is not performed with this option. It also has an impact on the width and height of the scale bar drawn on cartographies (see Cartographies Panel below).

Artefact Detection (%area min): sets the minimum threshold for the fiber-covered area detection. This is used for the initial quality check of the fiber shape signal (relying on Laminin staining). Example: %area min=***xx***, if less than ***xx*** % of the total surface of the section does not contain segmentable fibers (area with loss of intensity, hole, deterioration of part of the muscle preventing the detection of fibers), the section will be automatically moved in the *Artifact* subdirectory of your "*results*" directory (for more information see below *C. Step3: Set path to input/output data*) and the analysis will not be performed for this section.

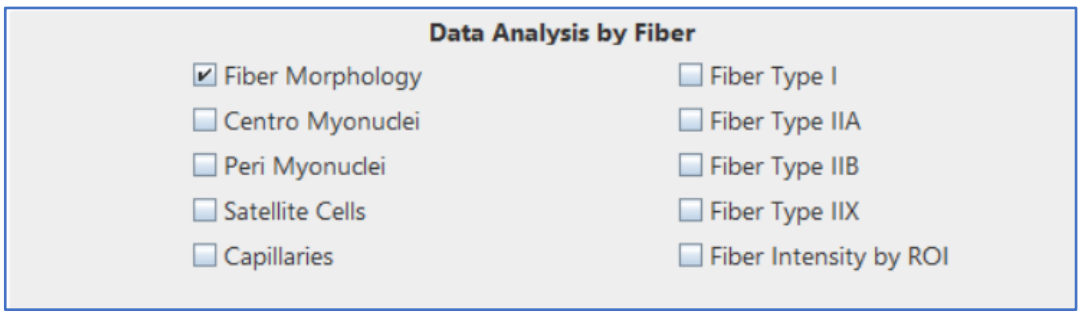
Data Analysis by Section panel

In this panel, the user can select the analyses performed on whole muscle sections or on representative parts of the image cropped manually by users.

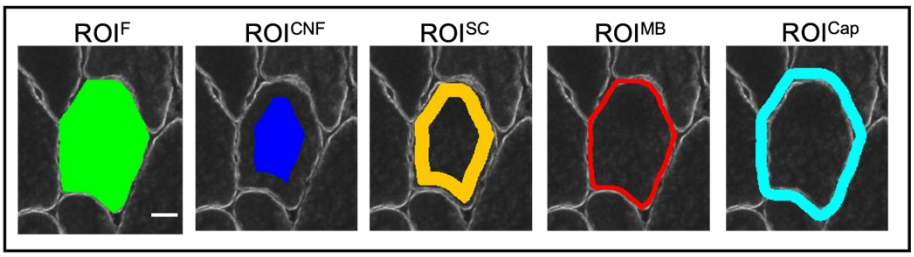


ECM area detection: select this option if you want to quantify the area covered by the ECM.
Vascularization: select this option if you want to quantify the area covered by all arteries, veins and capillaries contained in muscle section.
Specific Cells: select this option if you want to localize *Specific Cells* (positively labeled cells, either a subpart of/ or a different cell type from myofibers and vascular structures) in the entire muscle section.

Data Analysis by Fiber panel



In this panel, you can select one or more analyses to be performed. However, *Fiber Morphology* analysis is **mandatory** whenever you start a new batch run, as it is during this analysis that all ROIs are created and recorded as shown below.



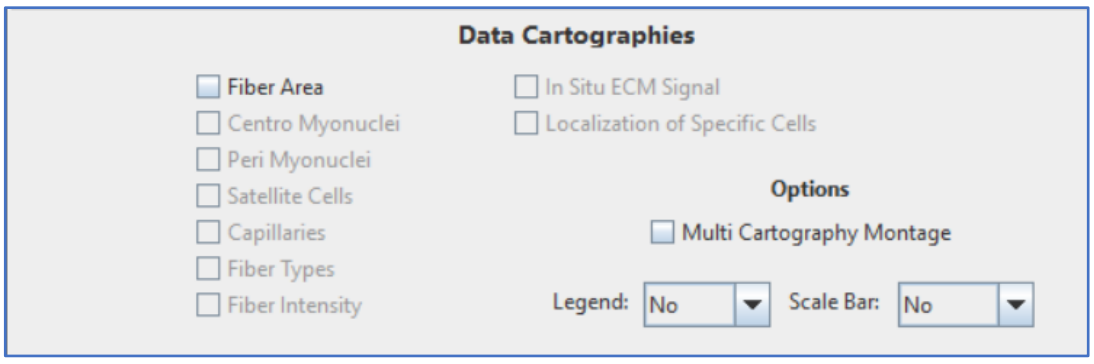
If you have already performed this analysis in a previous run, you do not need to select again it, but you have to select the "*result*" folder containing previously saved ROIs (see Annex 4. FAQ).

Fiber Morphology: select this option if you want to quantify for each muscle fiber morphological criteria, such as the cross-sectional area and the Ferret diameters in the ROI^{F}.
Centro Myonuclei: select this option if you want to quantify the percentage of centronucleated fibers and the number of centro-nuclei per fiber in the ROI^{CNF}.
Peri Myonuclei: select this option if you want to localize and quantify the number of peri-myonuclei per fiber in the ROI^{MB}.
Satellite Cells: select this option if you want to localize and quantify the number of Pax7-positive cells per fiber in the ROI^{SC}.
Capillaries: select this option if you want to quantify the number of associated-capillaries per fiber in the ROI^{Cap}
Fiber Types (I-IIA-IIB-IIX): select this option if you want to perform the fiber typing of the skeletal muscle section. This option is based on the analysis of the intensity histogram in the respective channels. A threshold resulting from the specific signal-to-noise ratio is generated and then applied to the ROI^{F} in order to discriminate positive and negative fibers. Each specific threshold is indicated in the summarizing table of results at the end of the process (see *III-B Global result table by batch*). When a fiber intensity is detected positive in two or more channels, a hybrid type is created to inform the user the ambiguities found, as such as if a fiber is positive in channel corresponding to type I and type IIA, the label associated to this fiber is Type I-IIA. If a fiber is positive in more than 2 channels, this case is considered as not defined and a new label named "ND" is created.
Fiber Intensity by ROI: select this option if you want to quantify the intensity and localization of any staining in the muscle fiber. The staining intensity is measured simultaneously in different ROIs defined previously in the "***Fiber morphology***" analysis.

Note: See section III. *Description of result files by batch* for more output providing by these section/fiber analysis panels.

Data Cartographies panel

You can perform several cartographies depending on the analysis selected in the previous panels (by section and/or by fibers).



***Multi choice check list* for data cartographies**

"*Fiber Area*[1]", "*Centro Myonuclei*", *"Peri Myonuclei"*, "*Satellite Cell*", "*Capillaries*", "*Fiber Types*" or *"Fiber Intensity"* cartographies are based on *Data analysis by fiber* panel.

"In Situ ECM Signal" or "*Localization of Specific Cells*" are based on *Data Analysis by Section* panel.

The availability of the check boxes depends on the analysis you have selected in the previous panel. Otherwise, the check box is disabled (in grey).

Options

Legend

If this option is selected, a legend will automatically be drawn on the *in situ* cartography in the top left-hand corner, in proportion to the width and height of the original image.

The legends associated to analyses performed and the fiber ROI filled with the corresponding color are reported in the following table:

Fiber Area (µm²)	Nb Centro nuclei per fiber	Nb Peri Myonuclei per fiber	Nb Satellite Cells by Fiber	Nb Capillaries per Fiber	Fiber types Pure	Fiber types Hybrid	%Intensity by fiber ROIs
ROI ^{F}	ROI ^{CN}	ROI ^{MB}	ROI ^{SC}	ROI ^{Cap}	ROI ^{F}	ROI ^{F}	ROIs $^{F, CN, MB, SC}$
<250 250-500 500-750 750-1000 1000-2000 2000-3000 3000-4000 4000-5000 5000-6000 >6000	0 1 2 3+	0 1 2 3+	0 1 2+	0-1 2-3 4-5 6-7 8-9 10+	I IIA IIB IIX Neg	I-IIA I-IIB IIA-IIB ND	0-10% 10-20% 20-30% 30-40% 40-50% 50-60% 60-70% 70-80% 80-90% 90-100%

- For the distribution by fiber surface, the range is displayed in µm^2.
- For centronuclei, myonuclei, satellite cells and capillaries, the results are displayed as the number per fiber.
- For Fiber types, two legends are drawn corresponding respectively to "*Pure Types*" and "*Hybrid Types*" (for more details on fiber types, report to section *Data Analysis by Fiber* panel-> Fiber Types option). The legend corresponding to "Pure Types" is systematically indicated. The legend corresponding to "Hybrid Types" is created using only the hybrid types present in the intensity analysis. This means that from one cartography to the next, this legend may change or not be present, depending on the number of hybrid types detected.
- For fiber intensity, different cartographies corresponding to the different ROIs are generated and results are displayed as a percentage of staining intensity.

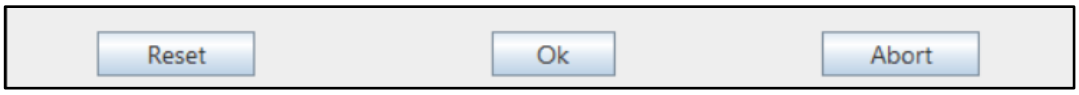
Scale Bar: in the combo box list, you can choose where the scale bar is drawn. Depending on whether the *"Entire section"* or *"Crop"* option is checked in the *Data Acquisition* panel, the scale bar indicates 300 µm and 100 µm respectively.

[1] If "Fiber Morphology" analysis has been performed during a previous run, the corresponding cartography ("Fiber Area Classes") can be chosen independently from the current analysis.

Multi cartography montage

By selecting this option, thanks to the *ImageJ* function called "*Make a montage*…", an automatic image montage is created with all the cartographies chosen in the check list with the legend and/or scale bar options previously selected. On each image of the montage, the name of the analysis carried out is written to the right of the legend, except for the first image, which corresponds to the original image entered by the user to create the cartography.

Buttons at the bottom of the main dialog box



Reset: Click on this button to clear all the options and analyses selected in the principal dialog box of MuscleJ2.
OK: Click on this button to continue.
Abort: Click on this button to abort the plugin MuscleJ2. A message will appear to confirm your choice.

B. Step2: Channel information Dialog Box

In this second dialog box the user provides the requested information such as the channel number on which the analysis will be carried out and/or a free text box to provide a label to be mentioned in the global result table as shown in the following tables.

Data Analysis per Section	Description	Channel Information	Staining examples
ECM	Network of macromolecules and smaller components that fill the extracellular space	ECM detection	Collagen, WGA
Vascularization	Artery, vein and capillaries stain	Artery, vein and capillary detection	CD31
Specific Cells	A variety of cell types outside the fibers	Specific Cell detection	CD45, PDGFRα
	Free text box: name of cells	Type of Cells	

Data Analysis per Fiber	Description	Channel Information	Staining examples
Fiber Morphology	Myofiber surrounding staining	Fiber Shape	Laminin
Centro Myonuclei	Nuclear stain	Nuclei Detection	DAPI, Hoechst
Peri Myonuclei	Co-labelled with the myonuclei marker and a fluorescent DNA stain	Peri Myonuclei detection	DAPI & eg. Anti PCM1
Satellite cells	Pax7-positive /Dapi-positive satellite cell	Nuclei detection & Satellite Cell detection	Pax7, Pax3
Capillaries	Vascular structures staining (size discriminating capillaries from larger vessels)	Vessel	CD31

Fiber types	Myofiber metabolic subtype specific staining	Fiber types	MyhI, MyhIIa, MyhIIb, MyhIIx
Fiber intensity by ROI	Channel on which the intensity of the staining is measured simultaneously in different ROIs by fiber (ROI^{FM}, ROI^{CN}, ROI^{MB}, ROI^{SC}, ROI^{Cap})	Intensity	Glycogen, Bodipy, Myh3
	Free text box: name of intensity marker	Intensity marker	Eg: Myh3

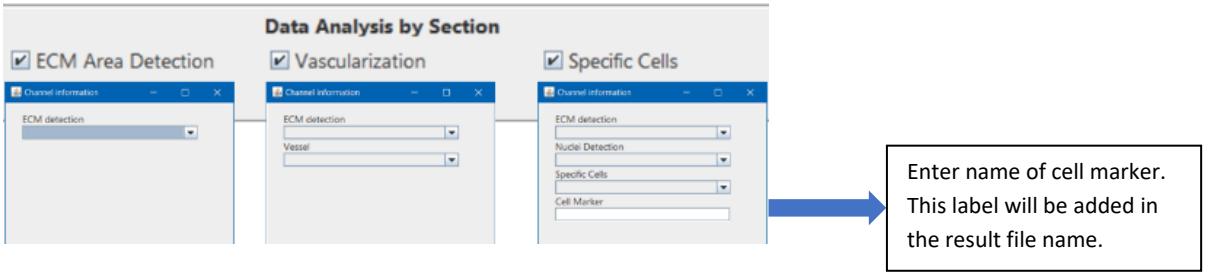
Depending on the analysis requested by the user, the order of channels must be defined. Data analysis by section and by fiber could be "mixed" depending on staining. For example, you can couple ECM Area Detection and Vascularization with Fiber morphology, Centro nuclei and Capillaries if you have corresponding channels for this multi analysis (see section *III Possible analysis combinations*).

If a cartography is selected in the next panel, a supplementary channel information is proposed to define the channel on which the cartography will be created.

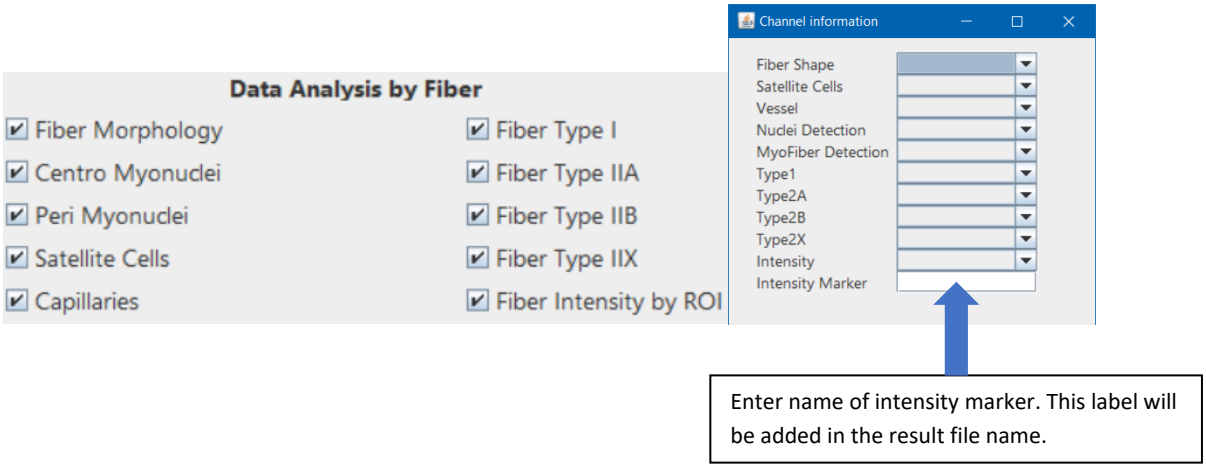
To identify the order of the channels, open one of the batch images on ImageJ with the *Bioformat Importer* plugin and observe the order in which the staining appear.

Note: all fields in this dialog box are mandatory.

- Examples of channel information associated with *Data Analysis by Section* panel.



- Examples of channel information associated with *Data Analysis by Fiber* panel.



- Buttons on dialog box bottom
  Previous: to return to the first dialog box without closing the plugin and modify your selection if necessary. Previously entered channel information will be retained.
  OK: Click on it to continue.

C. Step3: Set path to input/output data directories.

- **A new window appears to select the Image File folder** by Batch run (i.e. "*MyImages*") where you have organized your set of images to be quantified.

- **A second one appears to select a Result File folder**[2] (i.e. "*MyResults*") where you would like to save the ROIs, the results by file, the cartographies and a global result table by batch run.
  If they do not exist, a series of sub folders will be automatically created in the root of the selected result folder: "Artefacts – Cartography – Results_byfile – ROI"

You can select a result folder in which you have already performed analyses, for example, to run new analyses or cartographies.

III. Description of result files by batch

A. Data analysis nomenclature

A text file of global results per batch is created with the following nomenclature "*ImageFolderName"*_GlobalResults_"*Listofanalysisperformed"*.txt, where "*ImageFolderName*" corresponds to the name of selected image folder at the beginning of batch run and "*Listofanalysisperformed*" corresponds to the abbreviations added at the end of global result file name as described in the following tables:

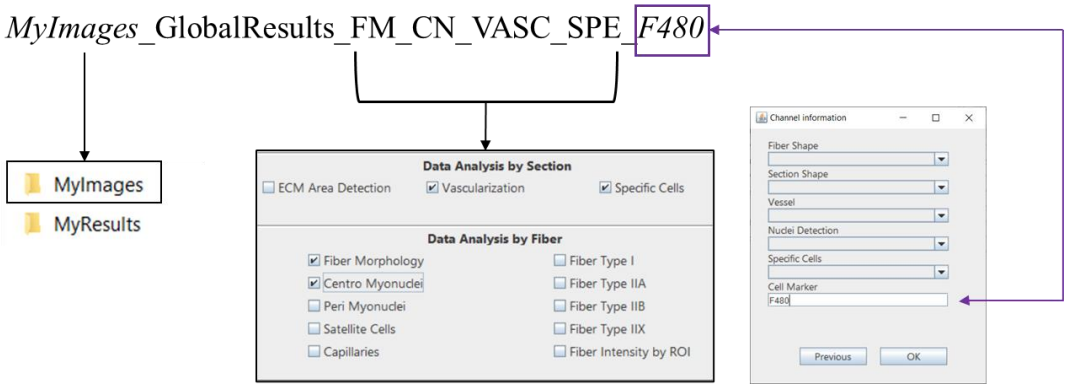
Data Analysis per Section	Abbreviation
ECM	_ECM
Vascularization	_VASC
Specific Cells	_SPE_"type of cells"

Data Analysis per Fiber	Abbreviation
Fiber Morphology	_FM
Centro Myonuclei	_CN
Peri Myonuclei	_PN
Satellite cells	_SC
Capillaries	_VC
Fiber type I	_FTI
Fiber type IIA	_FTIIA
Fiber type IIB	_FTIIB
Fiber type IIX	_FTIIX
Fiber intensity by ROI	_IbyROI_"marker"

Table 1: Abbreviations corresponding to analyses performed per section or/and per Fiber.

[2] **You have to create an empty result file folder before starting MuscleJ2 or select an existing one**.

For example, if *Vascularization, Specific Cells, Fiber Morphology* and *Centro Myonuclei* analyses are selected, the global result file is named as described in the following schema:



B. Global result table by batch

Regardless of the analyses selected, the outputs with *File Name* and *Whole Section Area* headings are listed at the beginning of each line in the table of global results per image.

Depending on analysis selected by section and by fiber, a series of output (see tables *Output by section analysis* & *Output by analysis per fiber*) are listed for each analyzed image.

For example, if *Vascularization, Fiber Morphology* and *Capillaries* analyses are selected, the global result table will have the following content:

Output for all analyses — **FM Output**

FileName	Whole Section Area	Nb Segmented Fiber	Fiber Area Mean	Fiber Feret Mean	…
xxxx	3440388.2	1797	1596.8743	57.767506	

Area distribution of segmented fibers (FM)

<250 µm2	250-500 µm2	500-750 µm2	750-1000 µm2	1000-2000 µm2	2000-3000 µm2	3000-4000 µm2	4000-5000 µm2	5000-6000 µm2	>6000 µm2	…
36	250	233	193	528	327	158	59	10	3	

VC Output — **VASC Output**

Tot Capillaries	Fibers with Capillaries	…	Vascularisation surface(%)	Vessels by mm2
3139	1598		5.263941	1024.0123334917216

Figure 4: Example of output saved in the global result table during a batch.

At the end of batch run, the results are saved in the table readable by any statistic software, facilitating an ulterior statistical analysis by batch.

ECM	VASC	SPEC
ECM Area Tot	Vascularisation surface(%)	Specific Cell Markers
% ECMArea	Vessels by mm2	Nb Specific Cells
		%Specific cell Area
		Nb Specific Cells with nuclei
		Intensity Mean
		Area Mean
		%Area of Specific cell with Nuclei

Table 2: Output by section analysis

All	FM[1]	CN	PN	Cap	SC	Types[2]	IbyROI
FileName	Nb Segmented Fiber	Nb CNF	Nb Fibers with Peri Myonuclei	Tot Capillaries		Nb Type *pure x*	Marker
Whole Section Area	Fiber Area Mean	CNF Area Mean	Nb Real Peri Myonuclei	Fibers with Capillaries		Thres. *pure x*	ROI(F) Intensity Mean
	Fiber Feret Mean	CNF Feret Mean				Nb Type Neg	ROI(F) Intensity StdDev
	<250 µm2	CNF NbCentroNuclei Mean				Nb Type *hybrid y*	ROI(MB) Intensity Mean
	250-500 µm2	<250 µm2				Nb Type ND	ROI(MB) Intensity StdDev
	500-750 µm2	250-500 µm2					ROI(SC) Intensity Mean
	750-1000 µm2	500-750 µm2					ROI(SC) Intensity StdDev
	1000-2000 µm2	750-1000 µm2					ROI(CNF) Intensity Mean
	2000-3000 µm2	1000-2000 µm2					ROI(CNF) Intensity StdDev
	3000-4000 µm2	2000-3000 µm2					
	4000-5000 µm2	3000-4000 µm2					
	5000-6000 µm2	4000-5000 µm2					
	>6000 µm2	5000-6000 µm2					
		>6000 µm2					
		1CN					
		1CN Area Mean					
		2CN					
		2CN Area Mean					
		3+CN					
		3+CN Area Mean					

Table 3: Output by analysis per fiber

C. Results in details

- Details **by fiber** depending on analysis performed.

Analysis\Output	Area (μm^2)	Max Feret	Min Feret	Nb Centro nuclei	Nb Peri Myonuclei	Nb Satellite Cells	Nb Capillaries	Intensity in appropriated channel	Fiber type
FM	x	x	x						
CN	x	x	x	x					
PN	x	x	x		x				
SC	x	x	x			x			
Cap	x	x	x				x		
Type I	x	x	x					x	x
Type IIA	x	x	x					x	x
Type IIB	x	x	x					x	x
Type IIX	x	x	x					x	x
Intensity by ROI	x	x	x					x	

Table 4: output details per fiber linked to analysis selected.

- Details **by cell** depending on analysis performed

Analysis\Output	Area (μm^2)	Max Feret	Min Feret	Cell/Nuclei GC X	Cell/Nuclei GC Y	Intensity in appropriated channel	Sharing Factor	Associated ROI
PN	x	x	x	x	x	x		ROI^{MB}
SC	x	x	x	x		x		ROI^{SC}
Cap	x	x	x	x		x	x	ROI^{Cap}

Table 5: Output details by cells inside the fiber shape.

- Sharing Factor by capillary in details

  The sharing factor indicates the number of fibers associated with individual capillaries, as represented below.

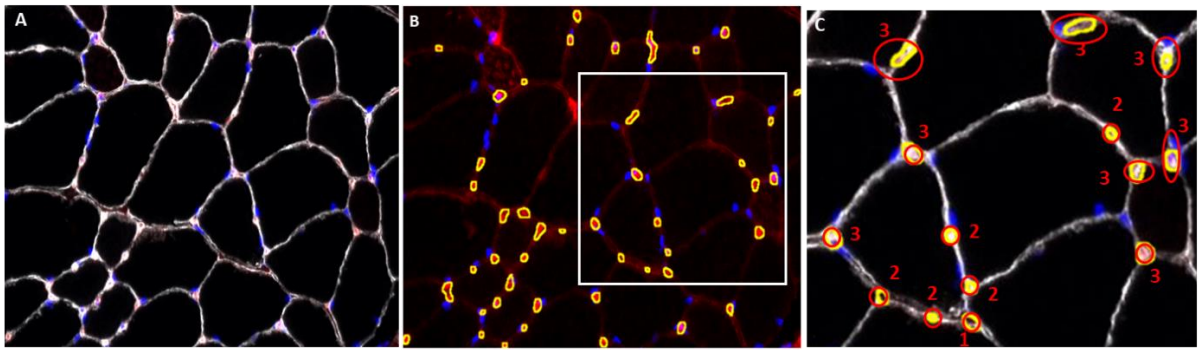


Figure 5: Sharing Factor by capillary. A. composite of image acquisition on confocal 20X (white for WGA, blue for DAPI and red for CD31). B. Capillary ROI DAPI+/CD31+ (in yellow) C. Sharing factor by capillary on a subset of image composite (SF in red) corresponding of white square in Figure 5B.

#Cap	Area	Max Feret	Min Feret	Capillary GC X	Capillary GC Y	Intensity	Sharing Factor
…							
7816	40.137527	8.474966	7.1500025	2528.5933	2414.59	193.62105	3
7836	6.7600045	3.6769567	2.6000009	5437.252	2420.6008	239.0	3
7848	21.547516	6.13209	5.2000017	4423.322	2427.171	171.29411	3
7918	31.265022	10.174113	4.727607	2864.103	2460.9448	187.45946	2
7932	49.432533	13.400098	5.850002	4507.7656	2467.1926	182.54701	3
7940	16.900011	6.5000024	4.069645	3864.4626	2470.1797	224.4	3
7947	39.292526	13.081002	4.2238417	2033.8333	2477.2034	142.53763	3
8029	10.985007	5.076664	3.2500012	1956.1757	2508.3508	179.76923	3
8070	29.152521	10.81816	3.7789562	2741.8848	2529.118	109.79710	2
8085	22.392515	6.5000024	5.055815	2898.2222	2531.4749	225.30188	2
8110	13.94251	5.2404695	3.9000013	2858.4548	2540.3486	170.54546	2
8111	8.027506	4.162032	3.1975784	2994.226	2540.4233	99.26316	1
…							

Table 6: Example of morphological criteria and Sharing Factor output associated to capillaries.

IV. Possible analysis combinations

Multiple analyses based on 4 or more channels can be combined. This table shows some examples of combined analyses:

Nb Channels	Channel combinations	FM	CN	PN	SC	Cap	Types	IntbyROI	VASC	ECM	SPE
2	DAPI/Laminin	x	x								
	Laminin/CD31	x							x		
3	DAPI/Laminin/CD31	x	x			x			x	x	
	DAPI/Laminin/one type	x	x				x				
	DAPI/Laminin/F4_80	x	x								x
	DAPI/Laminin/Dystrophin	x	x					x			
4	DAPI/Laminin/CD31/one type	x	x			x	x		x		
	DAPI/Laminin/CD31/WGA										
	DAPI/Laminin/Dystrophin/CD31	x	x			x		x			
5	DAPI/Laminine/I/IIA/IIB	x	x				I IIA IIB				

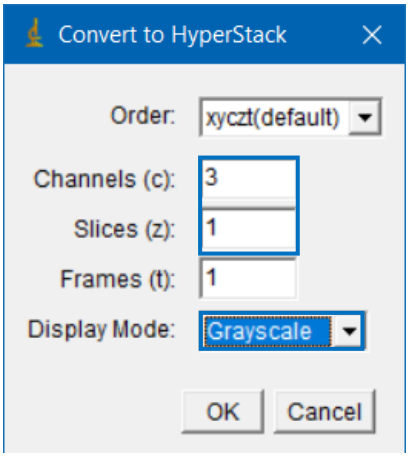
V. Recommendations / Limitations

- Images obtained from any microscope magnification (10x, 20x, 25x, 40x, 63x) can be used. The limitations are relative to the quality of image staining.
  - For optimal use of MuscleJ2, we recommend using the tool under the following conditions:
    - When gathering data, use an Apotome / Widefield microscope for single Z-slice imaging or a confocal / spinning disk microscope for Z-stack imaging.
    - When analyzing a whole section set the minimum detection threshold of 40 % to reject section artefacts
  - Use the original file format to keep the metadata associated.
  - File format that are not supported are Jpeg, Png, Tiff by channel, time series.

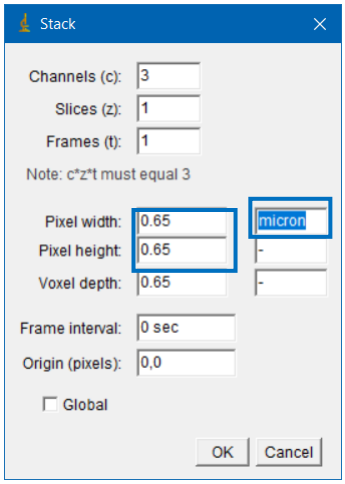
VI. Annexes

Annex 1. How to create a tiff format with multiple channels in FiJi/ImageJ

- Open your individual images by channel in FiJi/ImageJ
- Create a stack from opened images: *Image* menu->*Stacks*->*Images to Stacks*
- Convert to HyperStack: *Image* menu->*HyperStack*->*Stack to HyperStack…*
  and verify/change the text boxes (*Channels & Slices*) and *Display Mode* combo box.



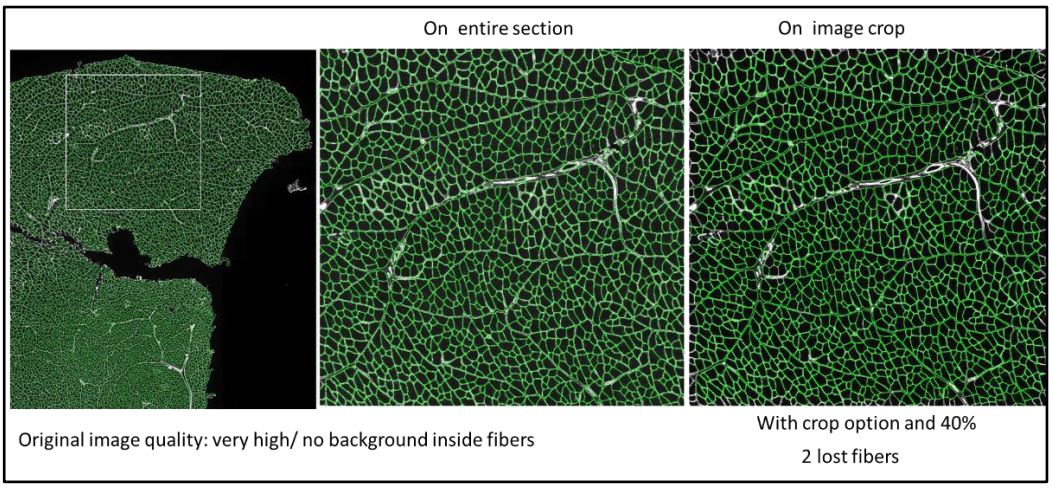
- Set properties before saving in Tiff format: *Image* menu-> *Properties…*
  Pixel width and height in microns are mandatory. These information are often provided by opening the image in the acquisition software.



Annex 2. Recommendations for cropping images of muscle sections.

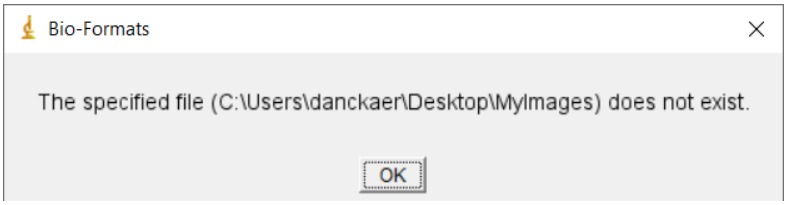
- Use original acquisition software to save your crop with the original file format or use FiJi/ImageJ ***crop*** function or ***duplicate…*** in *Image* menu to preserve the metadata associated to your sample.
- If you are acquiring a sub-section of the muscle section, select a region of interest with at least 25% of low background noise (ideally black background) as shown on the left panel of the box below. If this is not possible to acquire a large part of your section, choose a region of interest where the signal-to-noise ratio is high. As shown on the right panel of the box, few non-segmented fibers are

lost compared to the original image.



Annex 3. Troubleshooting

- MuscleJ2 stops with the following messages:
  from Bio-Formats



Diagnostic: there is probably a space or symbol in the image directory name.
You can check the *Image Folder Path* mentioned in the *Log* Window as shown below.

from Bio-Formats Importer

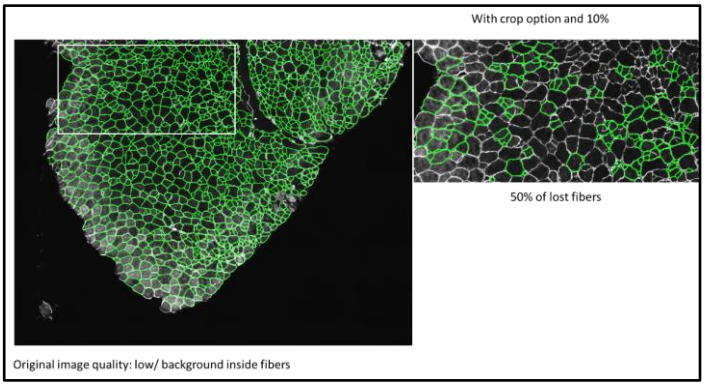
Diagnostic: the file displayed in the log box does not correspond to an image

from Image/FiJi

Diagnostics:
Image format extension is not recognized in *BioFormat Importer* plugin.

The *BioFormat Importer* plugin or your version of ImageJ/FiJi are not up to date (see prerequisites before installation).
The file name was not recognized because it contains spaces or symbols.

- **When I use the "crop" function the fibers are badly segmented**
  Diagnostic: There aren't enough surfaces without signal (black background) or the signal is low compared to background as shown in the box below.



- **Even with a minimum %area the section is rejected in the artifacts**
  solution: make a crop of the undamaged part of the muscle in the event of a hole or folding of the cut.

- **MuscleJ stops during the batch, where can I retrieve the results already obtained?**
  solution: you can find your results in the "Results" folder thanks to an updated of the global result table after each treated image as well as the results in details per fiber.

Annex 4. FAQ

- Q1: How can I reuse the morphological analysis performed with the MuscleJ macro to obtain new results with MuscleJ2's new features?
  A1: You do not need to restart the fiber morphology analysis performed previously with the MuscleJ macro but when you launch MuscleJ plugin you have to select the "Results" directory to find the ROIs saved previously by the macro in the "ROI" sub-directory.

- Q2: How can I define a different positivity threshold if the one in the global table doesn't correspond to what I want?
  A2: use data processing software (e.g. R or Excel) to sort intensities and define the threshold at which a fiber is to be considered positive by analyzing the intensity histogram of positive vs negative controls for example.

- Q3: I want to do two batch runs on the same series of images with different options. How can I keep the results of both runs?
  A3 : You can rename the directories in the selected "Results" directory if you want to keep the results of one batch. The second batch will automatically recreate the renamed directories.

Note: do not hesitate to contact corresponding authors if you encounter other troubleshooting, they will update these two previous annexes regularly.