MiNA

AN IMAGEJ MACRO TOOL FOR MITOCHONDRIAL NETWORK MORPHOLOGY RESEARCH

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INTRODUCTION TO MINA

The Mitochondrial Network Analysis (MiNA) macro tools were developed for ImageJ to provide biologists studying mitochondrial network morphology and dynamics with a simple, effective method of quantifying mitochondrial network morphology in images of cultured adherent animal cells. Mitochondrial network morphology is a dynamic characteristic of cells that is related to efficiency of oxidative phosphorylation, cell cycle phase, apoptotic cell death, and other important aspects of normal and aberrant cell physiology. MiNA provides a simple solution to the problem of quantifying the extent of mitochondrial fusion

MiNA uses existing ImageJ plugins and additional processing to provide a rapid method for measuring parameters related to mitochondrial network morphology. This documentation describes the measurements made using MiNA, how the measurements are made, and the limitations of the measurements. The basic algorithm used by the plugin for preprocessing the images and analyzing the mitochondrial network morphology is described in detail and the reasoning for each step explained.

BEFORE USING THE MACRO

MiNA was produced for use in ImageJ and FIJI software platforms. If using FIJI, all necessary plugins are present for using the tool and MiNA may simply be installed and used. The ImageJ and FIJI packages can be downloaded at their respective links below.

- **FIJI**
- **ImageJ**

If you choose to install the ImageJ platform, you will need to install a few additional plugins. Keep in mind that, as the plugins are actively maintained, they are subject to change. The software presented here was tested with the most current plugin versions available at the date this was written. These plugins are listed below and linked to their homepages

1. **AnalyzeSkeleton**
2. **Bio-Formats**
3. **Enhance Local Contrast (CLAHE)**

INSTALLING THE SOFTWARE

The following protocol is the **recommended** method of using the macro and has been tested using various hardware (PC, Mac) and operating systems (Windows, Mac OSX, Linux) with success. It can also be used with ImageJ alone, provided the necessary plugins have been installed. Instructions for using the macros under the latter installation method are not provided.

1. Download and install FIJI according to the documentation on the software webpage. The package is available **here**.
2. Download the MiNA macros at: https://github.com/ScienceToolkit/MiNA.
3. Install the macro tool by opening FIJI, then clicking **Plugins -> Macros -> Install** and selecting the macro file (ending in ".ijm") downloaded from the GitHub repository.

IMAGE PROCESSING ALGORITHM

TERMINOLOGY

The terminology for mitochondrial morphology used here is adopted and modified from Leonard [1]. Leonard describes mitochondria as belonging to one of four morphological categories: puncta, rods, networks, and large/round. MiNA is specifically designed to determine the extent of fragmentation into small, punctate or linear structures versus the extent of fusion into larger, more highly branched structures. It therefore combines puncta, rods, and large/round structures into a single category – 'individuals'. Individuals are contrasted with 'networks', where network refers to any structure with at least three branches. A branch is a rod-shaped structure of any length connecting two junction pixels, end-point pixels, or a junction pixel to an end-point pixel. The terms used are well summarized by figure 1 (adopted from Valente *et al.* – in review).

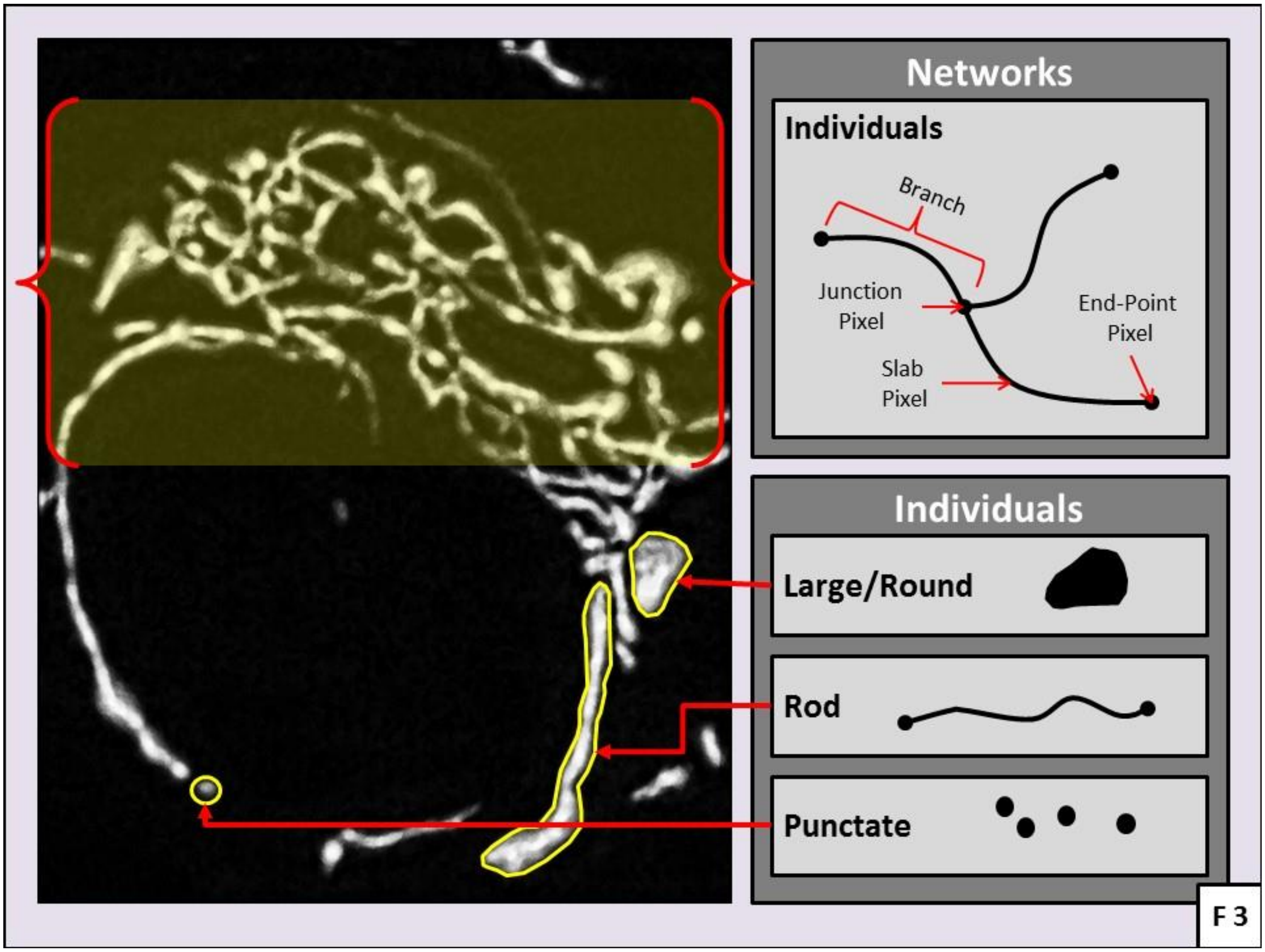
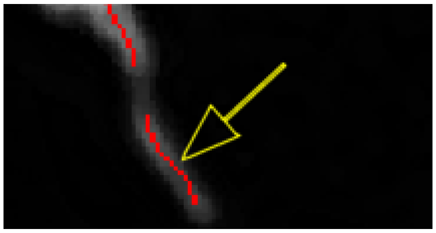
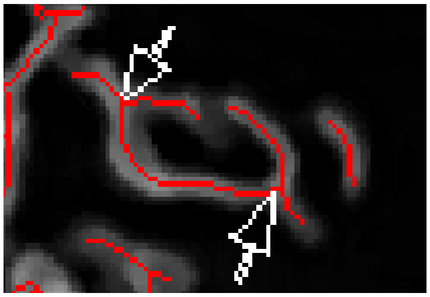
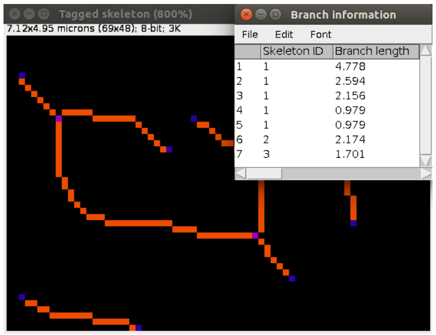
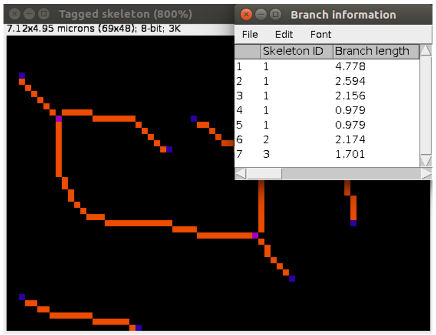


Figure 1: Visual description of terms used in this documentation.

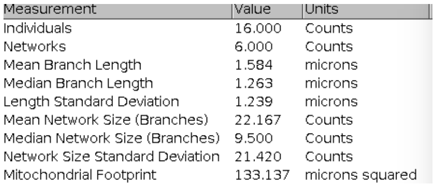
REPORTED VARIABLES

The macro tools calculate a number of variables that are reported back to the user in a table. These variables are described in the table below.

Variable*	Description	Example
filepath**	This is the complete filepath for the image processed by the Batch Analysis macro.	C://JohnDoe/Pictures/Mitochondria.czi
Individuals *individuals*	This is the number of objects in the image that do not contain a junction pixel. The individual in the example is indicated by the yellow arrow.	
Networks *networks*	This is the number of objects in the image that contain at least 1 junction pixel. In the example the object contains two junction pixels as indicated by the white arrows.	
Mean Branch Length *meanLength*	The average length of all branches (distances between connected end point or junction pixels). This is computed as: $\bar{l} = \frac{1}{n}\sum_{i=0}^{i=n} l_i$, where li is the length of branch i and n is the total number of branches.	 7.12x4.95 microns (69x48); 8-bit; 3K Skeleton ID Branch length 1 1 4.778 2 1 2.594 3 1 2.156 4 1 0.979 5 1 0.979 6 2 2.174 7 3 1.701 In this skeleton, there are 7 branches of lengths 4.778, 2.594, 2.156, 0.978, 0.979, 2.174 and 1.701 microns. The mean branch length is the sum divided by 7, giving 1.951 microns as the mean.
Median Branch Length *medianLength*	The median branch length is the middlemost value of all branch lengths when sorted or the mean of the two	Using the same skeleton as in the Mean Branch Length example, we find that the medial value in this set of

Variable*	Description	Example
	most medial values if there are an even number of branches.	number is 2.156 microns. This is then the Median Branch Length.
Length Standard Deviation *lengthStandardDeviation*	This is the standard deviation of all branches lengths treated as a <u>population</u>. The formula for the population standard deviation is: $sd = \sqrt{\frac{(l_i - \bar{l})^2}{n}}$, with the variables as defined earlier.	Using the above example once again, we fined the standard deviation to be 1.218 microns.
Mean Network Size (Branches) *meanNetworkSize*	This is the mean number of branches per network. It is calculated as: $\bar{s} = \frac{1}{n}\sum_{i=0}^{i=n} s_i$where si is the size of the network i in branches and n is the total number of branches.	 Tagged skeleton (800%) Branch information File Edit Font 7.12x4.95 microns (69x48); 8-bit; 3K Skeleton ID Branch length 1 1 4.778 2 1 2.594 3 1 2.156 4 1 0.979 5 1 0.979 6 2 2.174 7 3 1.701 In this image there are 2 individuals and one network. The network contains 5 branches. Since this is the only network the mean must be 5.
Median Network Size (Branches) *medianNetworkSize*	The median network size is the middlemost value of all branch counts per network when sorted or the mean of the two most medial values if there are an even number of networks.	The median example for a set of 4 networks with 7, 4, 3, and 2 branches respectively would be 3.5 branches.
Network Size Standard Deviation *networkSizeStandardDeviation*	This is the standard deviation of the number of branches per network as a <u>population</u>. The formula for the population standard deviation is: $sd = \sqrt{\frac{(s_i - \bar{s})^2}{n}}$, with the variables as defined earlier.	Using the example set of branches from the prior example, the network size standard deviation would be 1.87 branches.

<table>
<tr><th>Variable*</th><th>Description</th><th>Example</th></tr>
<tr><td>Mitochondrial Footprint

<em>mitochondrialFootprint</em></td><td>This is the total area in the image consumed by signal after being separated from the background. It is the number of pixels in the binary image containing signal multiplied by the area of a pixel if the calibration information is present.</td><td>
<table>
<tr><th>Measurement</th><th>Value</th><th>Units</th></tr>
<tr><td>Individuals</td><td>16.000</td><td>Counts</td></tr>
<tr><td>Networks</td><td>6.000</td><td>Counts</td></tr>
<tr><td>Mean Branch Length</td><td>1.584</td><td>microns</td></tr>
<tr><td>Median Branch Length</td><td>1.263</td><td>microns</td></tr>
<tr><td>Length Standard Deviation</td><td>1.239</td><td>microns</td></tr>
<tr><td>Mean Network Size (Branches)</td><td>22.167</td><td>Counts</td></tr>
<tr><td>Median Network Size (Branches)</td><td>9.500</td><td>Counts</td></tr>
<tr><td>Network Size Standard Deviation</td><td>21.420</td><td>Counts</td></tr>
<tr><td>Mitochondrial Footprint</td><td>133.137</td><td>microns squared</td></tr>
</table>
For the complete cell used for the above examples, the mitochondria produced a 133.137 micron^2 footprint.</td></tr>
</table>

*Variable names as shown in the **Single Image** macro are shown in standard font first, while the equivalent in the output table of the **Batch Analysis** macro are shown in italics underneath. The variation in naming convention is to remove spaces so the variable names may be used as headers for that macro.

filepath is only a variable in the **Batch Analysis macro

OPTIONAL PREPROCESSING STEPS

UNSHARP MASK

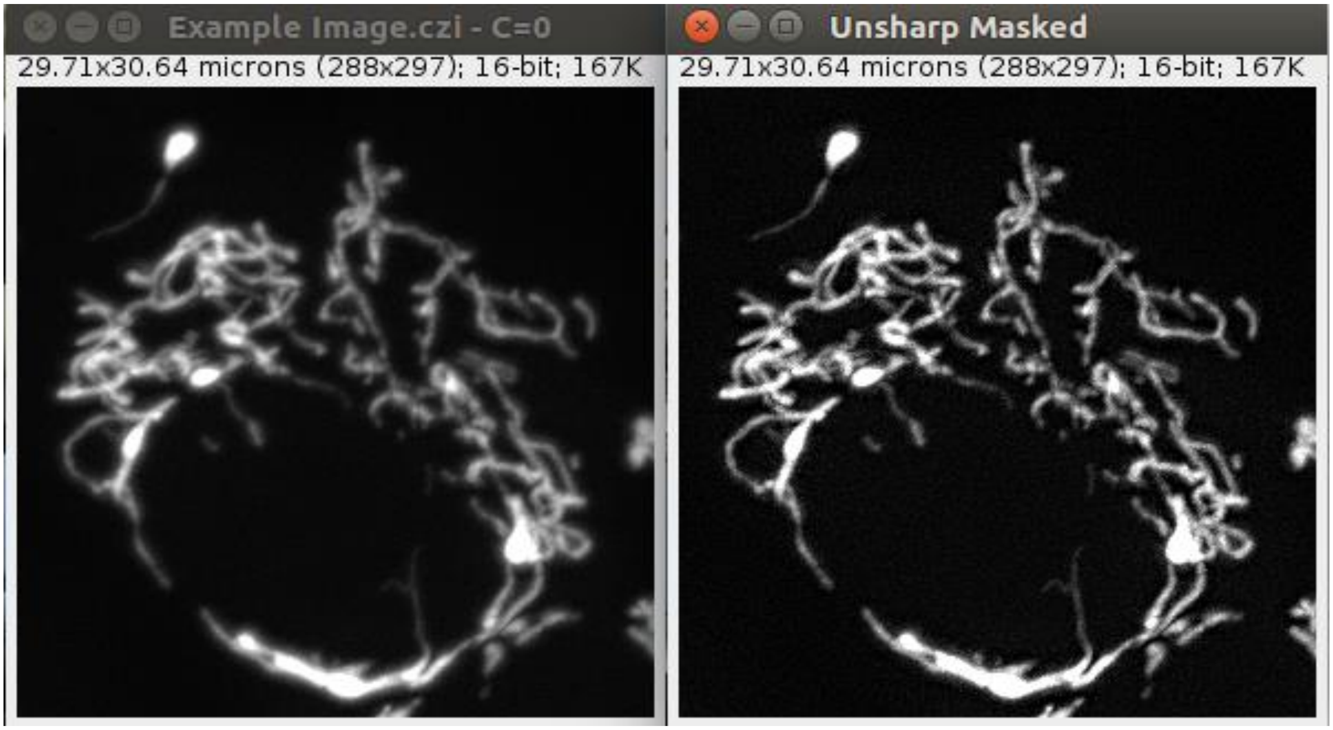

Example Image.czi - C=0
29.71x30.64 microns (288x297); 16-bit; 167K
Unsharp Masked
29.71x30.64 microns (288x297); 16-bit; 167K

Figure 2: Effect of unsharp mask on a fluorescent image of a mitochondrial network.

An unsharp mask subtracts a copy of the image that is blurred with a Gaussian function of radius r [3]. This has the effect of enhancing high frequency information to produce a sharper image. The masking produces a visual effect similar to deconvolution, but is much faster in execution time. The results of applying an unsharp mask is demonstrated in figure 2.

CONTRAST LIMITED ADAPTIVE HISTOGRAM EQUALIZATION

Images may have some regions with greater brightness than others. To improve the contrast between all features and the background CLAHE can be employed [4]. The employment of the process aims to equalize the histogram about the image without exaggerating features too aggressively. CLAHE is demonstrated on the previously deconvoluted image and shown in figure 3.

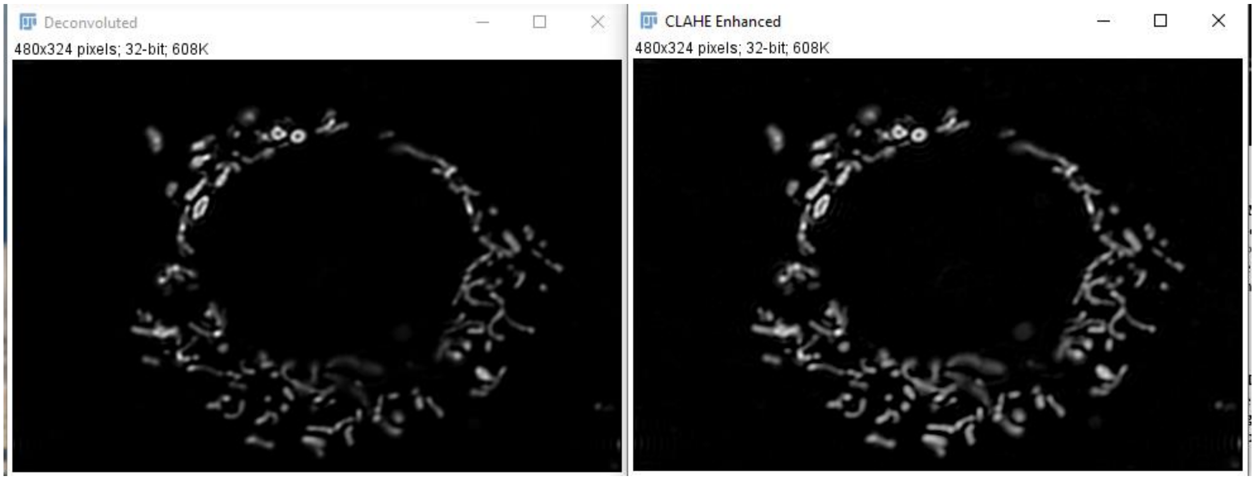


Figure 3: Effect of CLAHE on a deconvolved image.

MEDIAN FILTERING

Using a 2-pixel radius, a median filter is applied to the image. This filter replaces the central pixel of the neighbourhood with the median of the surrounding pixels in a 2-pixel radius. This aids in ensuring spurious details such as salt and pepper noise are removed, which may otherwise have negative effects on the analysis. This filtering method's effect is demonstrated in figure 4.

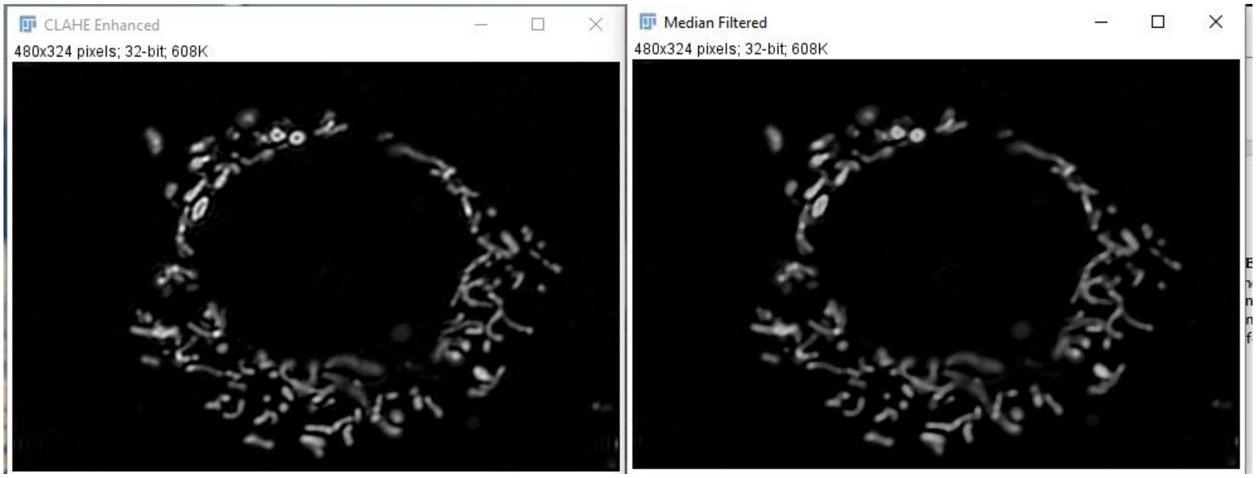


Figure 4: Effect of median filtering on a deconvolved image.

IMAGE ANALYSIS

The mitochondrial morphology by producing a skeleton which is then analysed. The steps automated by the macro tools are outlined here in brief.

THRESHOLDING AND CONVERSION TO BINARY IMAGE

The image is first step is to produce a threshold image using ImageJ's default algorithm. The default picks the threshold value by first removing outliers from the histogram, then iteratively smoothing the histogram with a 4-element averaging window until only 2 peaks exist. The midrange between these is the threshold value. Pixels with values greater than this are set to a maximum, and those below it to a minimum. This is then converted to a binary image. This makes mitochondrial signal 255 and everywhere else 0. The result is shown in figure 5.

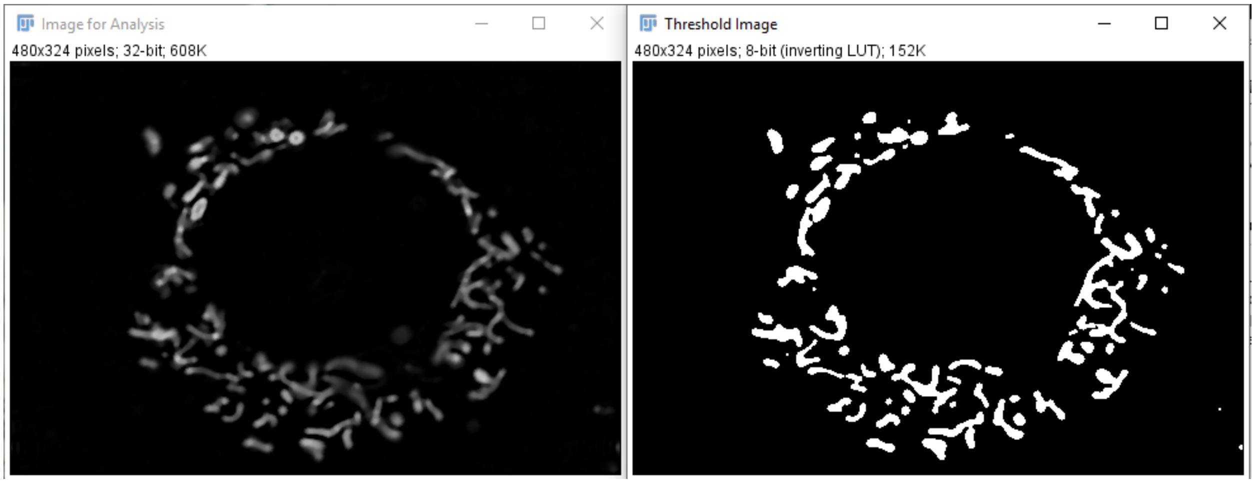


Image for Analysis
480x324 pixels; 32-bit; 608K
Threshold Image
480x324 pixels; 8-bit (inverting LUT); 152K

Figure 5: Binary Image produced by thresholding with default algorithm.

SKELETONIZING THE BINARY IMAGE

A skeleton is then produced of the binary image using the skeletonize routine found under the tab for binary operations. This iteratively removes external pixels until everything is represented by lines 1 pixel in width. The skeleton is shown overlaying the original image in figure 6.

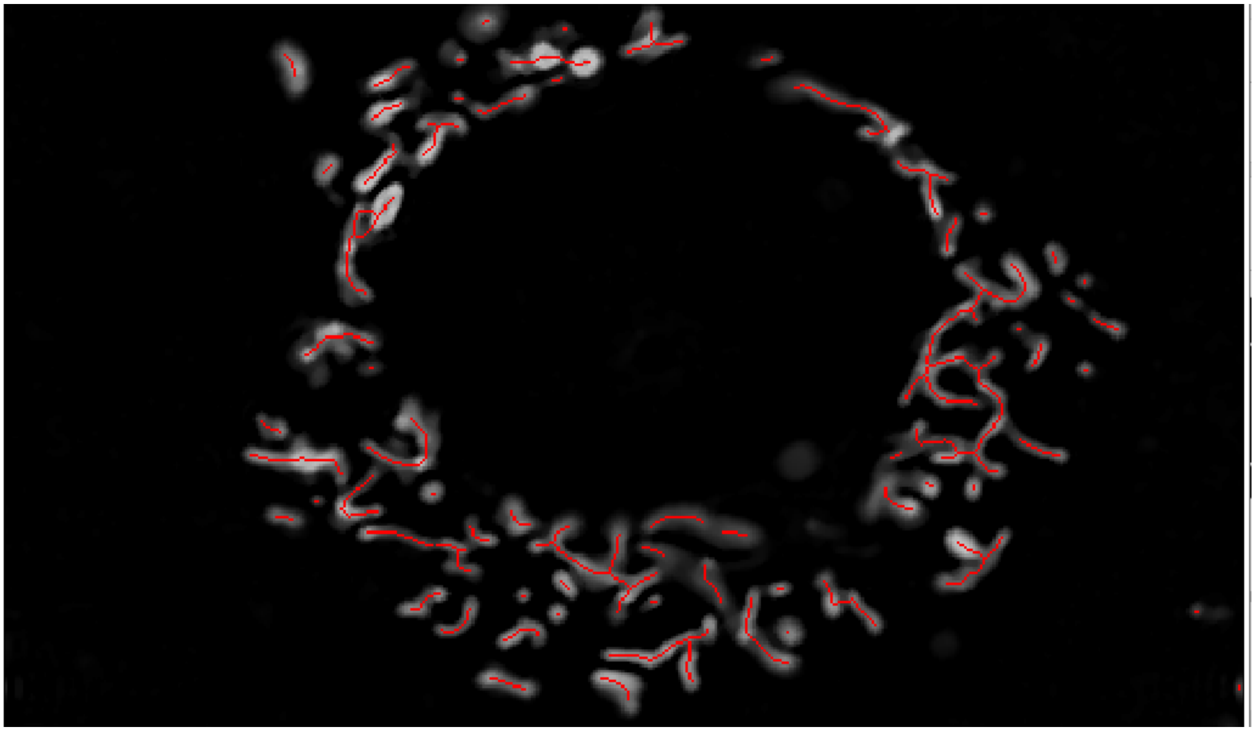


Figure 6: Skeleton overlaid on top of the binary image.

ANALYZING THE SKELETON

The skeleton is analyzed using a plugin included in the FIJI package produced byIgnacio Arganda-Carreras [2]. MiNA uses the output of this plugin to determine the various characteristics of the cell's mitochondrial network architecture and prints out a summary. This summary is in a tabular form so that it may be saved in a format convenient for further interpretation.

USING THE MACRO TOOLS

The program makes use of native micrograph file formats, which can be opened using FIJI, or ImageJ with the addition of the BioFormats plugin. These formats contain information regarding the working resolution, or pixel dimensions, of the image and the units of this measurement. These are used for analysis. The macros are intended for processing single images or a folder (or nested folders) of images. The plugin can be operated on an image in a standard format, such as TIFF, JPEG, or PNG. Note that the units will be in pixels if an uncalibrated format is used!

With the macro file installed, you will see two additional icons in the ImageJ toolbar. One is labelled with an S (for **S**ingle images), the other a B (for **B**atch processing). The procedure is essentially the same for processing one image as a bunch using the batch tool.

1. To use the single image tool, the image you wish to process must be opened first. For the batch mode, no images should be open to start.

2. With this set up complete, simply click the appropriate tool, select which preprocessing steps you wish to apply using the GUI (you may adjust the parameters for each with the sliders if the defaults do not work for your purposes).

3. Clicking OK begins the processing.

4. For each image, the macro will overlay the produced skeleton on the original preprocessed image and ask the user if the fit is suitable.

5. If it is, the information from the skeleton will be collected and added to the output.

LIMITATIONS

Currently there are some limitations to the software. For example, it can only handle only two-dimensional information. This limits its usage to flat cell types. The three-dimensional skeletons typically fail due to poor resolution in the Z plane causing discontinuities. This may be corrected using interpolation, but that has not been implemented in this version of MiNA. It is also important to note that the skeletons produced are imperfect. They are an approximation and errors do occur. It is important to look at the overlaid skeleton and determine whether the fit is accurate enough for analysis purposes.

REFERENCED MATERIAL

[1] A. P. Leonard *et al.*, “Quantitative analysis of mitochondrial morphology and membrane potential in living cells using high-content imaging, machine learning, and morphological binning,” *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1853, no. 2, pp. 348–360, 2015.

[2] I. Arganda-Carreras, “AnalyzeSkeleton,” *ImageJ*, 2016. [Online]. Available: http://imagej.net/AnalyzeSkeleton.

[3] T. Ferreira and W. Rasband, “ImageJ User Guide.” p. 198, 2012.

[4] S. Saalfeld, “Enhance Local Contrast (CLAHE),” *ImageJ*, 2010. [Online]. Available: http://imagej.net/Enhance_Local_Contrast_(CLAHE).