Mitochondria Analyzer

Version 2.0.2 – Last updated July 1, 2019

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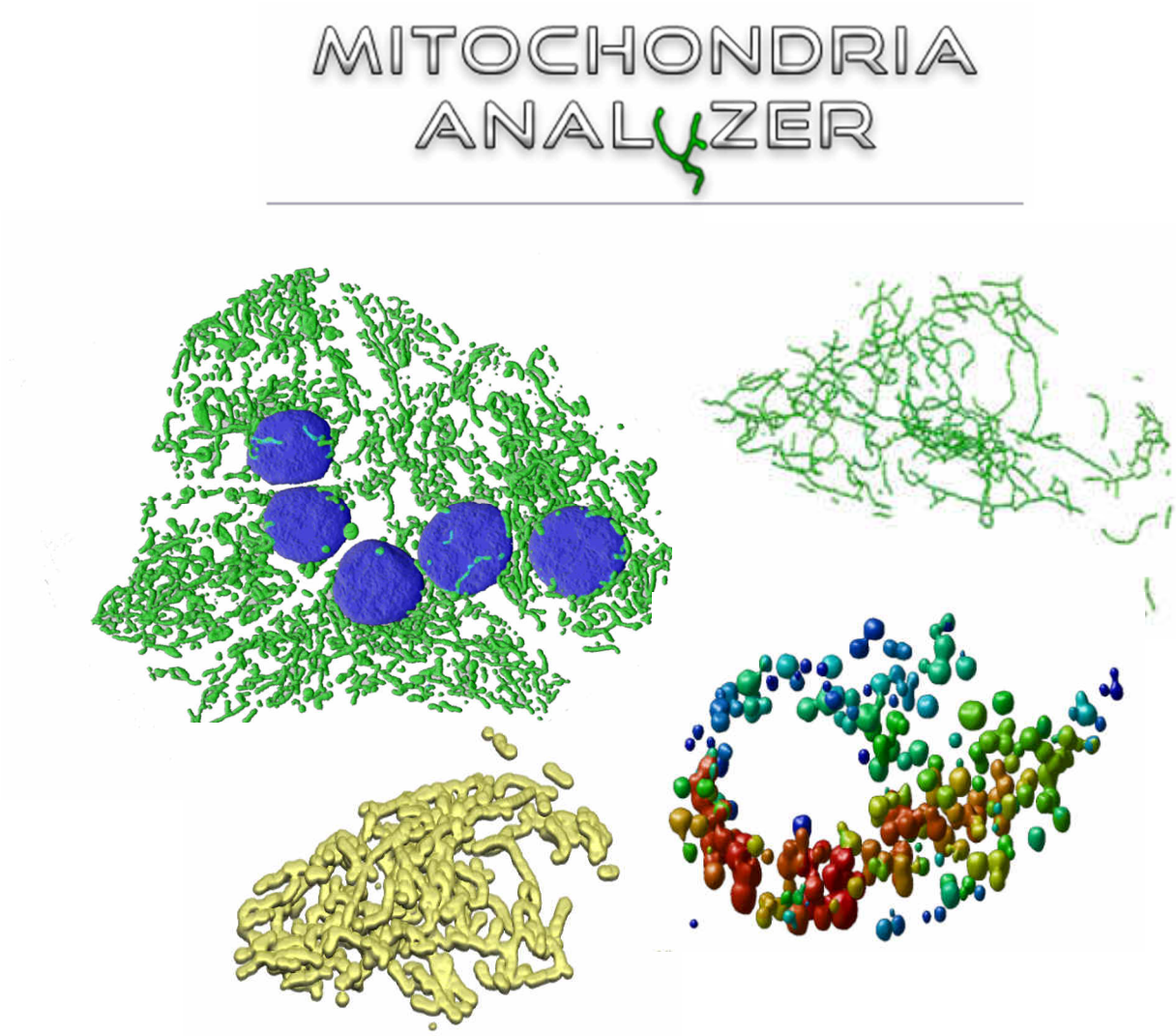


Contents

Installation

Requires Fiji and ImageJ version 1.52n or higher.
Requires Windows. No current Mac OS version available.

The following plugins are used by this program:

1) Adaptive Thresholding (Link)
2) 3D ImageJ Suite (Link)
3) MorphoLibJ (Link)
4) Sigma Filter Plus (Link)

There are 2 methods for installation.

Method 1 (Preferred)

Add the Mitochondria Analyzer Update Site to download all required files and automatically receive updates when available. To do this:

1) On the ImageJ menu, go to *Help -> Update…* to open the ImageJ Updater window
2) Select 'Manage Update Sites'
3) Select the checkboxes beside the following:
    a. 3D ImageJ Suite
    b. ImageScience
    c. IJPB-Plugins
        i. This contains MorphoLibJ
4) Press "Add Update Site' at the bottom. This will create a new entry.
    a. Under 'Name' column, type in "MitochondriaAnalyzer"
    b. Under URL column, copy and paste this: http://sites.imagej.net/ACMito/
    c. Note: this also installs Adaptive Thresholding and Sigma Filter Plus
5) Press 'Close', then press 'Apply Changes' in the ImageJ Updater window
6) Restart ImageJ, and the installation process should be complete. Check if the downloaded files have been placed in the "Updates" folder in the main Fiji.app folder, and if so, transfer the contents in the "Updates" folder to the "Plugins" folder.

Method 2

From the GitHub website (Link), download and open the MitochondriaAnalyzer.zip file and copy its contents to the ImageJ/FIJI "plugins" folder. This includes the MitochondriaAnalyzer folder as well as the dependent plugins listed above.

- Then download the opencv-windows-x86_64.jar file and paste into the plugins-> Adaptive Thresholding folder.
- The imagescience.jar file (required for the 3D ImageJ Suite) must be downloaded separately here (Link)

Once installed, the Mitochondria Analyzer folder contains the MitochondriaAnalyzer.jar file alongside a folder named “Macros”.

- These macro code files contain the source code for the functions and are easily accessible and modifiable—they can be edited as needed.

General Overview

This plugin was developed to enable semi-automated image-based analysis of fluorescently-labelled mitochondria acquired through confocal microscopy, both in 2D and 3D, for ImageJ. It also allows for simultaneous measurement of mitochondrial functional characteristics as reported by any co-stained mitochondrial probes.

The overall workflow of image-based mitochondrial analysis is summarized in Figure 1.

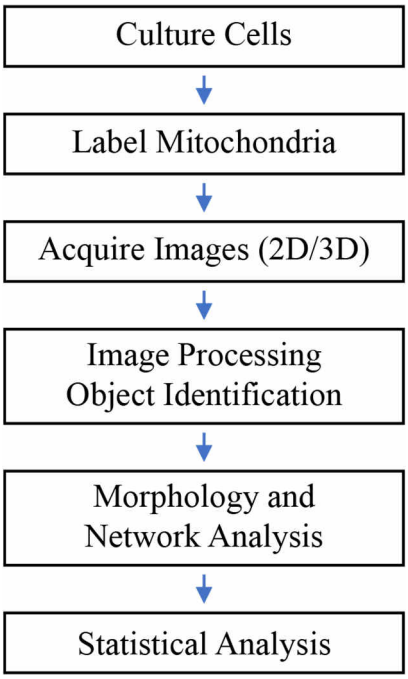


Figure 1: General workflow required for mitochondrial analysis via confocal microscopy

The general principles, rationale, methodology, and validation of the plugin are further outlined here (Paper).

In brief, the motivation for this plugin arose from the inability of methods previously found in the literature to enable accurate 3D analysis of mitochondrial morphology and networking in axially thick cells using freely available image software. These thick cells, such as pancreatic beta-cells (which were our cell type of interest) have mitochondrial networks that are dense and oriented throughout the cell and present challenges in accurately thresholding and identifying them. Therefore, we sought to develop a method to achieve superior thresholding accuracy and enable automated analysis in ImageJ/Fiji.

To achieve accurate analysis of morphology and networking the following are required:

1) High-quality and optimized image acquisition.
    a. 3D stacks should be acquired using settings matched to the optimal Nyquist rate (https://svi.nl/NyquistCalculator) and deconvolved. This is necessary to reduce axial stretching of objects (an artifact of the resolution limits of confocal microscopy) and improve the resolution of spatially close objects.

2) Accurate and faithful identification of mitochondrial objects from the images.
    a. This is accomplished through "thresholding". In our paper, we discussed the pitfalls of common thresholding techniques, such as Global thresholding.
    b. Here we have developed a rigorously tested pipeline that uses a locally based Adaptive thresholding method with several pre-processing and post-processing steps to reduce noise. It requires some optimization from the user to ensure accurate results (see Thresholding section).

Once these are achieved, ImageJ/Fiji can be used to calculate 2D and 3D morphological characteristics of the objects, and to convert them into topological skeletons to calculate network characteristics.

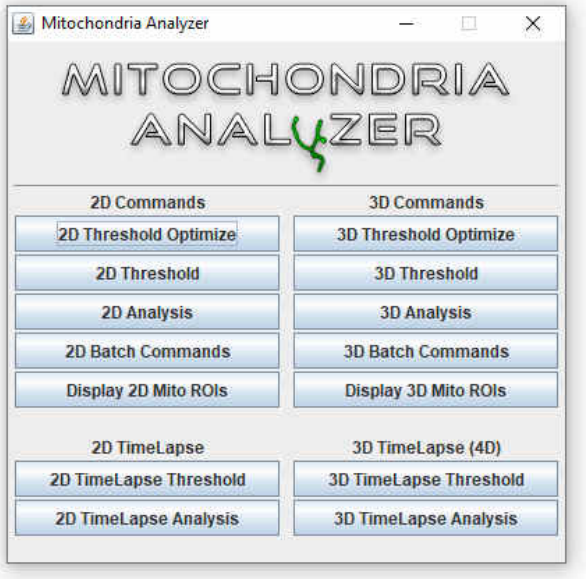
This plugin provides commands to streamline the process of thresholding and analysis and guides the user through the process. Thresholding is critical to the accuracy of quantitated results (i.e. "garbage in, garbage out"), so there are commands to help the user optimize thresholding settings and to enable the most accurate object identification.

This plugin supports 2D, 3D, and 4D (xyzt) files, as well as batch functions.

It can perform simultaneous measurement of mitochondrial morphology with other co-stained fluorescent reporter probes, such as calcium or pH probes. This allows for simultaneous morphology and functional measurements on a per-mitochondrion basis.

Overview of Commands

Access the main menu by selecting *Plugins -> Mitochondria Analyzer -> Analyzer Menu*.



Overall, the plugin should be used as follows:

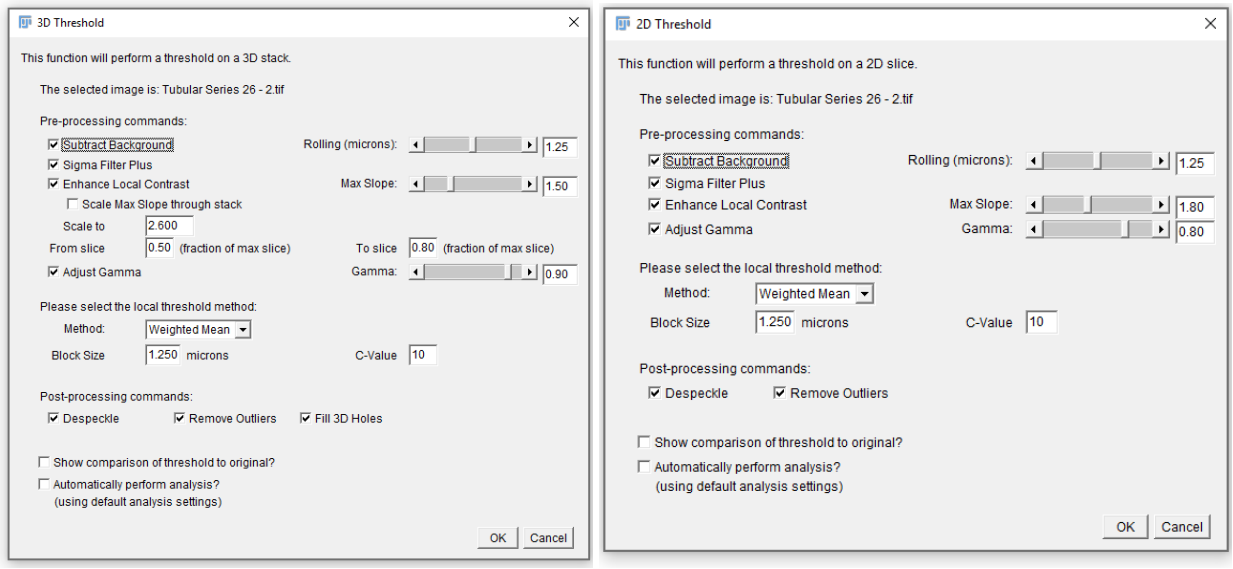
1) Acquire high-quality mitochondrial images, either 2D slices, 2D time-lapse (xyt), 3D stacks, or 4D time-lapse stacks (xyzt - 3D stacks at different time points).
2) Deconvolve 3D and 4D stacks (either with separate software, or here with ImageJ).
3) Use the 2D or 3D Optimize Threshold commands to identify the appropriate settings on test samples from each set acquired under similar imaging conditions.
4) Use the optimal settings identified in step (3) for the appropriate Threshold command on the acquired images.
5) Use the appropriate Analysis command on the thresholded images to quantitate the morphological and/or network characteristics, of the mitochondrial objects, either on a per-cell or per-mito basis.
    - The batch commands can be used for thresholding and/or analyzing multiple files in folders using the same set of thresholding settings.
6) Perform statistical analysis

Threshold Commands

The goal of thresholding is to distinguish “true” fluorescent signal from background signal via binarization, thereby grouping identified pixels into discrete objects that can be analyzed. An ideal threshold will faithfully capture the morphology of the mitochondrial signal in the original image and should avoid merging close but physically separate mitochondria or artificially splitting a contiguous mitochondrion.

The commands **2D Threshold**, **3D Threshold**, **2D TimeLapse Threshold**, and **3D TimeLapse Threshold** can be used to produce thresholds for the corresponding image type. Note: 2D TimeLapse refers to an XYT sack, and 3D TimeLapse is an XYZT stack

(i.e. 4D). As detailed in Figure 1, this plugin's threshold strategy involves a pre-processing step, a local/adaptive thresholding algorithm that needs empirical determination of its settings, and a post-processing step.



Note: Ensure original images are 8-bit and have their pixel/voxel units in microns (check by navigating to *Image - > Properties*) before proceeding.

Pre-Processing

There are 4 pre-processing steps applied to the image:

1) Subtract Background – This removes smooth continuous background from the image based on a rolling ball algorithm. The rolling ball radius ("rolling") should be at minimum the radius of the largest object of interest.
2) Sigma Filter Plus – Performs a smoothing operation on the image while preserving object edges. See this link for more details.
3) Enhance Local Contrast (CLAHE) – This method enhances the local contrast of regions in the image while avoiding over-enhancement of near-constant or noisy regions. This helps to enhance mitochondrial signal contrast from background and helps in the detection of fainter mitochondria. The degree of enhancement is determined by the Max Slope value. If this value is too high, then the risk of merging adjacent but separate mitochondrial objects increases, and if it is too low, then fainter mitochondria may not be detected in the image.
    a. Note: In z-stacks, sometimes there is a decrease in signal intensity towards the top of the sample. Therefore, in 3D/4D Threshold commands there is an option to scale the Max Slope value over a range of the stack. This range

is specified as fractions of the maximum slice number. For example, in a stack of 40 slices, a range of 0.5 to 0.8 would mean slice number 20 to 32.

4) Adjust Gamma – Gamma adjustment is a non-linear histogram adjustment, which can further improve detection of fainter objects (lower values cause increasing enhancement). In 3D stacks, this value should be higher than values used for 2D, as fainter signals usually represent out-of-focus light and would ideally not be thresholded to avoid axially stretching the resultant object.

The default settings for these steps were chosen empirically but can be tweaked by the user as needed. Additionally, they can be omitted if desired.

Thresholding Algorithm

Four different local-based thresholding algorithms are available—Mean, Median, Mid-Grey, Weighted Mean. These algorithms compute thresholds on sub-regions of an image of a given block size, and differ by how the computation is done, i.e. mean vs median pixel value of the block. This computed threshold can be further offset by a C-value.

- See the original paper (Paper) as well as here for further discussion about the theory of the different methods, and the nature of the block size and C-value.

The block size and C-value have a significant impact on the threshold and can drastically change quantification of various morphological properties downstream.

The block size relates to the size of the object of interest—given that most mitochondria have a diameter less than 1.0μM, we have set the default block size to a value slightly larger at 1.25μM. The C-value is used to remove noise and minimize background detection. Values that are too low result in excessive noise detection, and values that are too high resulting in artificially splitting contiguous mitochondria. This value reflects the Signal-to-Noise contrast in the image, as if there is low signal intensity but high noise, then high C-values would remove much of the object signal aberrantly. On the contrary, higher C-values would be more useful for a deconvolved image with a better signal to noise contrast.

The optimal settings for these values should be empirically determined by the user, for at least a few representative images from an experiment or set of images acquired under similar imaging conditions. This can be done using the 2D or 3D Threshold Optimize commands (see Threshold Optimize section for further details).

- For example, for our deconvolved 3D stacks of beta-cell mitochondria, a block size of 1.15 to 1.25 microns and a C-value of 10 or 11 is often found to be ideal, versus a value of 4 or 5 for non-deconvolved images.

Between the algorithms, we find that the Weighted Mean and Mean methods are the most useful, with the Weighted Mean being most advantageous in resolving finer structural detail of complex mitochondrial networks. Again, the choice is left for the user to judge.

Post-Processing

The post-processing commands are:

1) Despeckle – A median filter to remove salt-and-pepper noise that sometimes results after a threshold operation.
2) Remove Outliers – Another median-based filter operation to remove residual noise.
3) Fill 3D Holes – (For 3D Functions) Fills in small holes in the center of spherical objects, helping the analysis software to recognize them as spheres or punctae.

Quality Checking

As the accuracy of downstream morphological and network quantification depends on how accurately the threshold was produced, it is recommended that the final threshold result be compared against the original image (at least for a few images in a set).

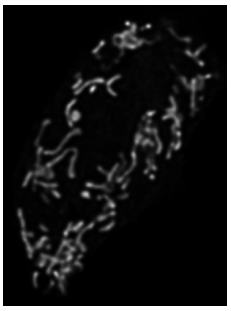
The "Show Comparison" option in the threshold commands combines the final threshold result with the original image in separate channels, allowing the user to compare how faithfully the threshold captures the structural detail of the original, and avoids incorrectly merging or splitting mitochondria. It can be difficult to determine which regions are "truly" connected or not, so there is some subjectivity involved. A degree of objectivity can be achieved using photoactivatable GFP to label truly connected networks, which can help quantify the threshold strategy's accuracy (see Paper for details).

Threshold Optimize Commands

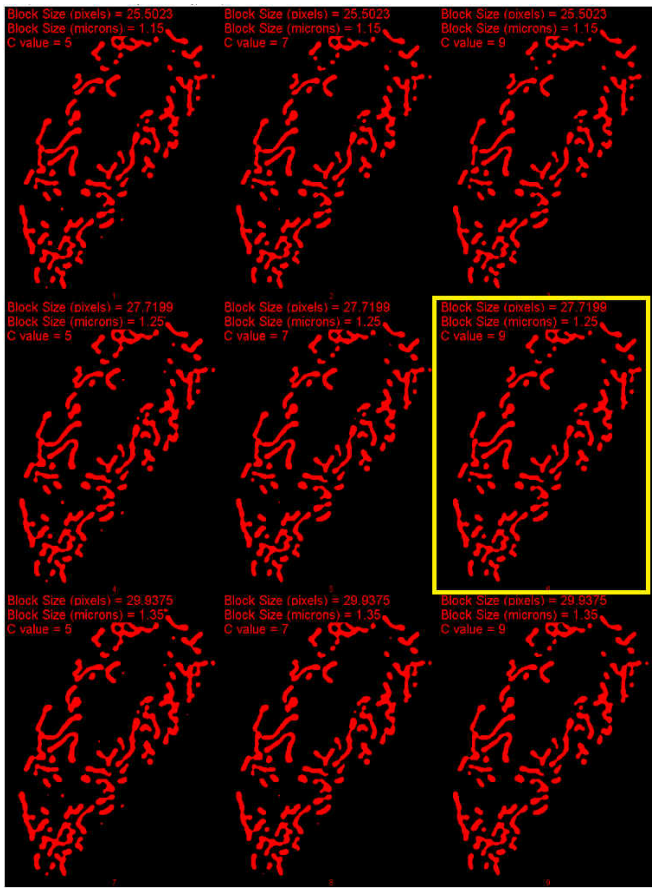
As indicated earlier, it is essential to determine the optimal block size and C-values for local thresholding. The **2D** and **3D Threshold Optimize** commands can be used to generate a montage of a combination of different block size and C-values, with the original image combined in a seperate channel. This allows the user to manually visualize the thresholded objects superimposed on the original signal and compare the results of the different settings. They can then identify the combination that yields the most satisfactory results which can be used for further images using the regular threshold commands.

The Pre-Processing and Post-Processing steps are equivalent to the options in the **Threshold** commands, and the specified settings will be used for each of the combination of block size and C-value.

See Figure 2 below for an example of 2D optimization.



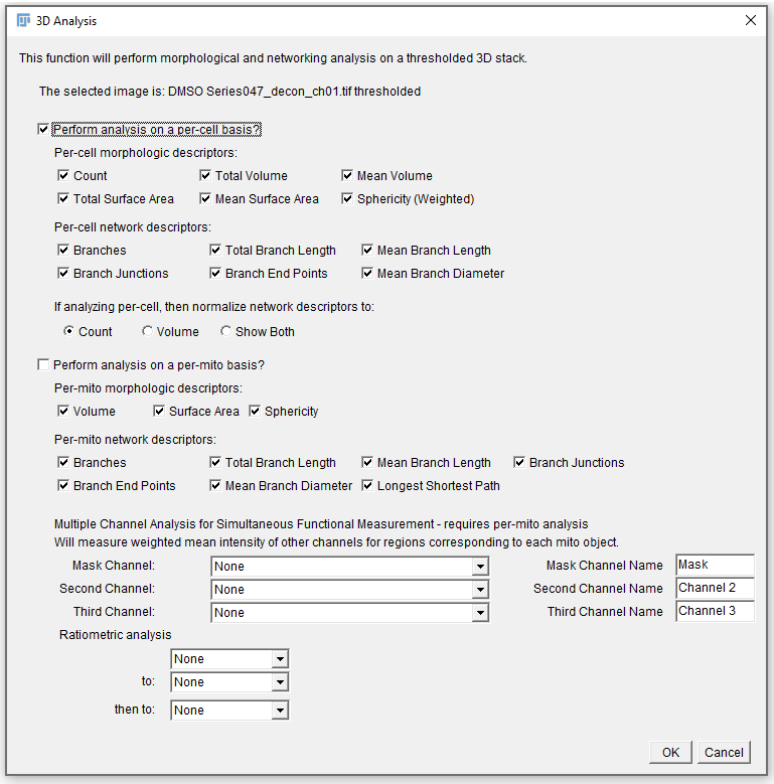
Original



3x3 Combinations of Block Size and C-values

Figure 2. Determination of optimal Block Size and C-value settings for adaptive threshold (Weighted Mean method) of a 2D slice of a MIN6 cell expressing Mito-YFP. It was judged that a block size of 1.25uM and a C-value of 9 (shown in the yellow box) seemed most faithful to the original image on the left, using the criteria discussed earlier.

Analysis Commands



Using a thresholded image of mitochondria as an input, the **2D Analysis, 3D Analysis, 2D TimeLapse Analysis,** and **3D TimeLapse Analysis** commands can be used to extract morphological and network characteristics. See Tables 1 and 2 for list of parameters for both 2D and 3D/4D.

There is an option to perform analysis on per-cell or per-mito basis. There are some differences in parameters (see Tables 1 and 2) and can be selected separately.

1) Per-cell basis – If selected, will calculate morphological and network characteristics for the whole image/cell, and display results for each image.
2) Per-mito basis – If selected, will extract each separate mitochondrial object in the image and analyze morphological and network characteristics for each unique object. Results will be displayed as values for each mitochondrion in each image.

When analyzing per-cell, the user can ask the software to automatically normalize the following network characteristics: Branches, Total Branch Length, Branch Junctions, and Branch Points to either the mitochondrial count, total volume/area, or both. This is useful as the normalized parameters are often more meaningful than the raw totals.

These values can provide meaningful information about the state of the mitochondrial networks, such as a shift to a fragmented or filamentous state. The per-mito calculations can be used for big-data analysis, to identify mitochondrial sub-populations, or for unsupervised or supervised (machine learning) classification.

Table 1. List of parameters for per-cell analysis.

Per-Cell Parameters			
2D		**3D**	
Morphological Parameters			
Count	"Number of mitochondria in image"	Count	"Number of mitochondria in image"
Total Area	"Sum of area of all mitochondria in image"	Total Volume	"Sum of volume of all mitochondria in image"
Mean Area	"Total area divided by count"	Mean Volume	"Total volume divided by count"
Total Perimeter	"Sum of perimeter of all mitochondria in image"	Total Surface Area	"Sum of SA of all mitochondria in image"
Mean Perimeter	"Total perimeter divided by count"	Mean Surface Area	"Total SA divided by count"
Mean Form Factor	"A shape measure given by: $P^2/(4\pi A)$. 1 indicates round object and increases with elongation. Expressed as mean FF of objects in image".	Sphericity (weighted)	"As objects become more spherical, the value approaches 1. expressed as a weighted mean of the image, by weighting each object's sphericity to its volume.
Mean Aspect Ratio	"A shape measure given by: d_{max}/d_{min}. Ranges from 0 (round) to 1 (elongated). Expressed as mean AR of objects in image".		
Network Parameters			
Branches	"Total number of branches in image"	Branches	"Total number of branches in image"
Total Branch Length	"Sum of length of all branches in image"	Total Branch Length	"Sum of length of all branches in image"
Mean Branch Length	"Total branch length divided by number of branches.	Mean Branch Length	"Total branch length divided by number of branches.
Branch Junctions	"Number junctions within all skeletons in image. Junctions are points where 2 or more branches meet."	Branch Junctions	"Number junctions within all skeletons in image. Junctions are points where 2 or more branches meet."
Branch End Points	"Total number of end-point, which are where branches end without connecting to another branch."	Branch End Points	"Total number of end-point, which are where branches end without connecting to another branch."
Mean Branch Diameter	"The mean diameter across all objects, using their skeletons to identify the long axis from which to measure diameter across."	Mean Branch Diameter	"The mean diameter across all objects, by computing a euclidean distance map and using their skeletons to identify the long axis from which to measure diameter across."
Note: Branches, Total Branch Length, Branch Junctions, and Branch End Points can be further expressed as a normalization to either mito count or total area.		Note: Branches, Total Branch Length, Branch Junctions, and Branch End Points can be further expressed as a normalization to either mito count or total volume.	

Table 2. List of parameters for per-mito analysis.

Per-Mito Parameters			
2D		**3D**	
Morphological Parameters			
Area	"Area of mitochondrion".	Volume	"Volume of mitochondrion"
Perimeter	"Perimeter of mitochondrion".	Surface Area	"Surface area of mitochondrion.
Form Factor	"A shape measure given by: $P^2/(4\pi A)$. 1 indicates round object and increases with elongation."	Sphericity	"A shape measure of sphericity. As objects become more spherical, the value approaches 1."
Aspect Ratio	"A shape measure given by: d_{max}/d_{min}. Ranges from 0 (round) to 1 (elongated). "		
Network Parameters			
Branches	"Number of branches in mitochondrion's skeleton."	Branches	"Total number of branches in image"
Total Branch Length	"Sum of length of all branches in mitochondrion's skeleton"	Total Branch Length	"Sum of length of all branches in image"
Mean Branch Length	"Total branch length divided by number of branches (for each mitochondrion).	Mean Branch Length	"Total branch length divided by number of branches.
Branch Junctions	"Number junctions within mitochondrion's skeleton. Junctions are points where 2 or more branches meet."	Branch Junctions	"Number junctions within all skeletons in image. Junctions are points where 2 or more branches meet."
Branch End Points	"Total number of end-point in mitochondrion's skeleton, which are where branches end without connecting to another branch."	Branch End Points	"Total number of end-point, which are where branches end without connecting to another branch."
Mean Branch Diameter	"The mean diameter across mitochondrion, using its skeleton to identify the long axis from which to measure diameter across."	Mean Branch Diameter	"The mean diameter across mitochondiron, by computing a euclidean distance map and using it skeletons to identify the long axis from which to measure diameter across."
Longest Shortest Path	"The longest shortest path, or longest graph geodesic, through mitochondrion's skeleton. Useful for differentiating large complex networks that differ in configuration"	Longest Shortest Path	"The longest shortest path, or longest graph geodesic, through mitochondrion's skeleton. Useful for differentiating large complex networks that differ in configuration"

Simultaneous Morpho-functional Measurement

A major utility of this plugin is to assess several different readouts of each mitochondrion across multiple images or treatment conditions in a (mostly) automated manner. This data can be used for mitochondrial classification, such as for sub-population analysis or machine learning. To add even greater depth, we have integrated the ability to combine morphological/networking with functional analysis through simultaneous intensiometric measurements of any co-stained mitochondrial reporter probes (for example Calcium or pH probes). This allows for "morpho-functional" measurement of mitochondria.

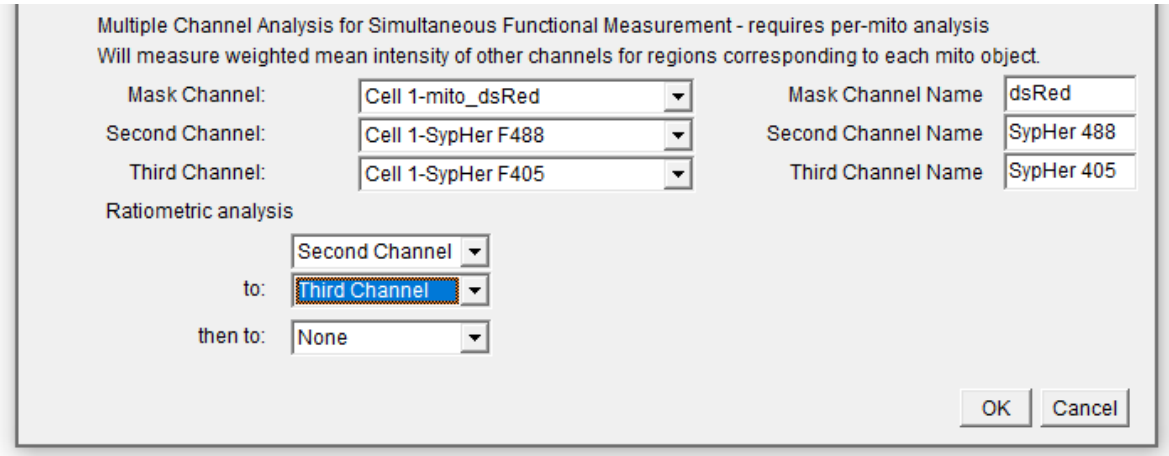
Using the **2D Analysis, 3D Analysis, 2D TimeLapse Analysis,** or **3D TimeLapse Analysis** commands, navigate to the bottom of the menu to see the multiple channel analysis settings. Note: per-mito analysis should be enabled for this to work, and channels should be opened in separate windows (this can be done with *Image -> Color -> Split Channels*).

The following is the order of actions:

1) Acquire image of mitochondria that is either stained with or expressing several fluorescent markers. One of these markers should be a structural stain such that it should not vary over treatments or samples. This will serve as the "Mask Channel" and be used to produce a threshold for identifying mitochondrial objects and doing morphological/networking analysis.
    a. In 3D, it is recommended to deconvolve the "Mask Channel" and used the deconvolved Mask for thresholding and morphology analysis.
    b. This Mask Channel can also be used to normalize the intensity of non-ratiometric probes.
2) The other channels will contain signal from separate fluorescent proteins/dyes that report mitochondrial function states, such as a green Calcium probe, or a ratiometric pH probe.
3) The image should be opened and separated into separate stacks for each channel
4) Use the Mask Channel (deconvolved if in 3D/4D) to produce a threshold
5) With either **2D Analysis, 3D Analysis, 2D TimeLapse Analysis,** or **3D TimeLapse Analysis**, navigate to the Multiple Channel Analysis section at the bottom. Up to 3 channels are supported. Select the Mask Channel (note: this will be the channel used for thresholding, not the thresholded result itself) and other functional channels. You can assign names to these channels (e.g. "Mito-dsRed", "Calcium Probe").
6) For automatic ratiometric calculation, you can specify the order of channels for which intensity measurements will be divided into, using either two or three channels. For example, if you have an image stained with a structural dye and a ratiometric probe with two channels, you can specify it as: (Second Channel) to (Third Channel) then to (None), or normalize the ratio of the first two channels to the mask channel as: (Second Channel) to (Third Channel) to (Mask Channel).

7) The software will use each separate mitochondrial object from the thresholded channel and specifically measure its morphologic, network characteristics, and use that object as a mask to measure the intensity of both the non-thresholded Mask Channel and the functional channels in the region specifically corresponding to that object.
8) When the analysis completes, you will receive a table of morphology, network, and functional intensity measurements for each mito.
   - Note in 3D/4D, the functional intensity is expressed as a weighted mean intensity of the object across its z-slices, weighted to the area of the object in that slice. This helps to prevent skewing of data by out-of-plane light.
   - Note in 2D, the functional intensity is expressed as a mean intensity, and the Std. dev of the measurement for the object is also included.

An example below of entering settings. The cell used was expressing mito-dsRed (a structural stain) that was used to produce the threshold. It was also co-transfected with mito-SypHer, a ratiometric pH probe with excitation peaks at F488 and F405. A 3D stack of this cell was taken with 3 channels (mito-dsRed, SypHer F488, SypHer 405).



TimeLapse Commands

It is important to note that the 2D TimeLapse commands requires XYT inputs, and 3D TimeLapse commands require XYZT inputs. In ImageJ, these T and Z dimensions are referred to as “frames” and “slices” respectively.

When using 2D TimeLapse commands, if you have a stack with several slices, but you wish for these slices to be interpreted as timepoints, navigate to *Image -> Properties* and switch the numbers for the *Slices* and *Frames* fields. This stack will then be read as a 2D TimeLapse (XYT) rather than a 3D stack (XYZ).

Batch Commands

Automatic batch thresholding and analysis of folders of 2D and 3D files can be done using the same set of settings for all. It is recommended that the thresholding settings be identified by using the 2D or 3D Threshold Optimize commands on at least a few of the images in each set manually to identify the optimal settings.

Start by running either **2D Batch Commands** or **3D Batch Commands**. You will be prompted to select a folder to analyze—all sub-folders contained within will also be analyzed.

You will then be prompted to select threshold settings. These are identical to the **2D** and **3D Threshold** commands, with the additional option to save the thresholded images to the same folder (as original name + " thresholded").

Next you will be prompted to select analysis settings. To enable "morpho-functional" assessment, enable the "Multiple Channel Analysis" option (otherwise all fields thereafter will be ignored).

- Note here that the multiple channel analysis for "morpho-functional" assessment varies from the **2D** and **3D Analysis** commands. Instead of requiring channels to be separated, all the channels should be combined in one file.
- Importantly, if you deconvolved one of the channels and you intend to use that deconvolved channel for thresholding, you should either replace the non-deconvolved version of that channel in the original file with it or combine it into the original file.
- Enter the channel you wish to threshold and perform morph/network analysis on by entering its Channel number ID in the file, and the ID for other channels in the Second Channel and Third Channel fields. A value of 0 will emit that field from analysis.The intensities for each object's corresponding region in both the Mask Channel, and other selected channels will be analyzed.

Results for all analyzed images are displayed on the same Table (e.g. "3D Analysis Data – per Mito") that can be saved, and/or exported to other software for data handling.

Display Mito ROI Commands

Display 2D Mito ROIs will add each mitochondrial object in a thresholded 2D slice to the ROI manager. These objects will be numbered in manner that corresponds to the results of the **2D Analysis** command, allowing the user to visualize each of the mitochondria and compare them to the corresponding results entry from the **2D Analysis**.

Display 3D Mito ROIs will add each mitochondrial object from 3D stacks (or the active frame of a 4D stack) to the 3D Manager (part of the 3D ImageJ Suite plugin). Each 3D

object will be labelled with an ROI that corresponds to its physical space throughout the stack. The 3D manager also supports viewing each mitochondrial object in the 3D Viewer.

- Note: After the command is executed, the “Live ROI” option in the 3D Manager must be enabled for the ROIs to become visible.

Deconvolution

Deconvolution can be done using specialized software such as Huygens Professional, or in ImageJ/Fiji using DeconvolutionLab2. For information on the theory of PSF and deconvolution, as well as how to generate PSFs and perform deconvolution, please visit:

1) DeconvolutionLab2 (Link)
2) PSF Generator (Link)

License & Contact

For any bugs, problems, or general question, please contact:
MitochondriaAnalyzer@gmail.com.

References

1. Legland, D., Arganda-Carreras, I. & Andrey, P. MorphoLibJ: integrated library and plugins for mathematical morphology with ImageJ. Bioinformatics 32, 3532-3534 (2016).
2. Ollion J, Cochennec J, Loll F, Escude C, Boudier T. TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization. Bioinformatics. 2013;29(14):1840-1.
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Link: https://sites.google.com/site/qingzongtseng/adaptivethreshold