mitoMorph:
mitochondria analysis tool for ImageJ/Fiji

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Introduction

This documentation refers to a set of tools developed to aid with the analysis of mitochondria as labelled in light microscopy images, using the program Fiji. The goal of the analysis is to classify the mitochondria in each cell according to their morphological features, and to summarize the results by cell and by image. The tools are organized into a toolset of macros which facilitate the preparation and the analysis of the data. The analysis can be done either in interactive mode or by batch processing.

The tool was developed at the Max Planck Institute of Biochemistry as a support to the research published in the article

Yim A., Koti P., Bonnard A., Duerrbaum M., Mueller C., Villaveces J., Gamal S., Cardone G., Perocchi F., Storchova Z., Habermann B.H., *mitoXplorer, a visual data mining platform to systematically analyze and visualize mitochondrial expression dynamics and mutations.* **In preparation**

NOTE: the scripts have been optimized for the images acquired specifically for that research project. Therefore the parameters could need to be adjusted for different data sets. Furthermore, currently the scripts work only on 2D images with physical units specified in microns in their header.

Toolset

A set of macros is provided with the toolset file **mitoMorph Tool.ijm**, which provide the following functionalities:

- adjust the settings for the analysis and the saving of the results;
- add and/or delete outlines (ROIs), drawn by the user, to the list of cells selected in one image;
- save images and results of both pre-processing and analysis;
- re-load the information about the cells from images previously analyzed;
- classify mitochondira in single cells or entire sets of cells from one image;
- perform batch analysis on sets of images already pre-processed (i.e. cells already outlined).

Classification of mitochondria

Mitochondria are highly dynamic organelles, displaying a variety of shapes which reflect cell activity. Commonly their shape is categorized into four categories: networked, puncta, rod, swollen. The swollen category is assigned to pathological mitochondria which are quasi-round, compact in intensity, and larger than the more common puncta. Rods are an intermediate phenotype between fragmented puncta and network of filaments. This is the categorization used in this tool.

Summary of algorithmic procedure

The analysis is performed on 2D images, which could be user-selected slices from a stack. In order to improve the accuracy of the analysis, it is generally recommended to deconvolve the images/stacks before processing. The segmentation and analysis of the mitochondria is performed on each single cell manually outlined by the user in an image. The shape of the outline is arbitrary.

Segmentation

Below is the basic workflow of the segmentation procedure, followed for each target cell:

1. crop cell from the entire image, using the user-specified outline as reference;
2. subtract the background from the cropped image, using a kernel radius of five times the average expected thickness of mitochondria (500 nm);
3. enhance the contract to cover all the dynamic range;
4. delete from the cropped image all the regions that are outside the user-given outline;
5. apply a Laplace of Gaussian (LoG) filter to the image, with the standard deviation of the Gaussian derivative kernel equal to twice the average thickness of mitochondria, to smooth out smaller features;
6. enhance the contrast locally, with a block size equal to ten times the average expected thickness of mitochondria, to account for variation of intensities in the mitochondria;
7. segment the image using the Yen thresholding approach, to separate mitochondria from background;
8. analyze and classify all the segmented objects.

Classification

The program calculates a set of features for each object segmented from the image, and use these values in combination with a set of selection criteria, to assign the object to one of the predefined morphological classes. In addition to the area, other features measured are:

- **length**: the length is measured after reducing the object to a skeleton, therefore to a combination of branches, junctions and end points, and calculating the longest shortest-path between any two end points in the skeleton;
- **minimum Feret diameter** (here indicated as **linear extension**): the minimum distance between the two parallel tangents touching the object outline in all directions. Note: in the settings window the minimum Feret diameter is called linear extension.;
- **aspect ratio**: ratio between the maximum and the minimum axes of an ellipse fitting the object;
- **circularity**: ratio between the area, multiplied by 4 times pi, and the squared perimeter of the object. A perfect circle has circularity equal to 1, while elongated shapes give values closer to 0;
- **solidity**: ratio between the area of the object and its convex area, i.e. the area of the smallest convex object enclosing it;

Objects that are too small are classified as *junk* and excluded from the analysis. The remaining objects are assigned to one of the classes of networks, puncta or swollen, based on its feature parameters. All the objects whose parameters do not fit any of those selection criteria are classified as rods, that is intermediate structures. Features and critical values used for the classification can be controlled from the **Settings** window.

Installation

Files

The software is composed of two files, the toolset **mitoMorph Tool.ijm** and the script **mask2convex_hull.groovy**.

Dependencies

This tool is an extension of Fiji and relies on the plugin FeatureJ, available after activating ImageScience in the list of update sites (see instructions in ImageScience). The tool also depends on an external routine to determine the convex area from a mask region, implemented in a separate file called **mask2convex_hull.groovy**. This script is adapted from code originally developed by Gabriel Landini and Tiago Ferreira.

Instructions

These instructions are valid for Windows, Mac and Linux systems.

1. Install Fiji, if you don't have it yet on your computer. **Windows**: it is recommended NOT to install it on the 'usual' directory (C:\Program Files), rather it is advised to create an arbitrary diretory (e.g. C:\MyPrograms) and install the software inside it.

2. Add ImageScience to the update sites (see ImageScience).

3. Assuming that the name of the base directory of Fiji is **Fiji.app**, copy the file **mitoMorph Tool.ijm** in the subdirectory **Fiji.app/macros/toolsets**. **Mac**: in order to have access to these subdirectories, you need to right-click on the **Fiji.app** directory and select *Show Package content*.

4. Create a subdirectory, if don't already have one, inside **Fiji.app/scripts/**, and copy inside the script **mask2convex_hull.groovy**.

Next time you launch Fiji, in the list of toolsets accessible by pressing the toolset switcher button (>>) in the toolbar it will appear a new selection called **mitoMorph Tool**, which provides a new set of tool buttons.

Usage

Description of the tools

The tools are loaded in Fiji by pressing the toolset switcher button (>>) in the toolbar and selecting **mitoMorph Tool**, which will provide the following tool buttons

The tool buttons are listed in order of appearance on the toolbar. The name of the tool associated with each button can be found by placing the cursor over it.

 Settings

It opens a dialog window where it is possible to specify:

- *Saving preferences*: indicate the name of the two directories where the images with all the cells outlined (default: Images) and the results from the analysis of each single cell (default: Cells) are saved. These directories are subdirectories of the one where the original image is located. **Note**: in batch processing mode the results from the analysis, both images showing the segmentation+classification and tables, are saved in a subdirectory of the one specified by the user at start. This means that, using for example the default names of the subdirectories, in interactive mode the images with the outlines and the results form the classification will be saved in /Images and /Cells, respectively, while in batch mode the input directory will be Images and the results will be stored in /Images/Cells;

- *Display preferences*: choose if the visual results from the segmentation and classification of mitochondria will display the objects rejected and classified as *junk*. Their display is generally useful for verifying the accuracy of the classification, but it can be avoided if the resulting images are intended to be used for illustration purposes;

- *Classification criteria*: control the parameter thresholds used to assign each segmented object to one of the five classes (*junk*, *filaments network*, *rod/intermediate*, *puncta/fragmented* and *swollen*). Assignment criteria are numbered to indicate that either one is sufficient for assigning an object to that class (OR condition). The features used for the classification are defined in **Introduction**.

 **Load cells from image**

Read the cell outlines in the current image and write them to the ROI Manager. It is useful when loading an image where cells had been previously selected, in order to have access to the outlines in the ROI Manager. If no cells are outlined in the current image, or if no images are open, then all selections are removed from the ROI Manager.

 **Add cell**

Shortcut function key: **F5**
Add new selection to the list of cells outlined in the image. Launch this tool after outlining a cell with a selection; the shape of the selection can be arbitrary. A new item is added to the ROI Manager.

 **Delete cell**

Shortcut function key: **F9**
Remove a previous selection from the list of cells outlined in the image. Launch this tool after selecting in the ROI Manager the item corresponding to the cell to discard.

 **Segment mitochondria from one cell**

Shortcut function key: **F6**
Segment and classify mitochondria from a single cell. The cell analyzed is the one currently selected in the ROI Manager. The script generates an image showing, side by side, the original cell and the result from the segmentation/classification. Each mitochondria is colored according to the convention illustrated in a separate legend window. Furthermore, a new line is added to the table **Mitochondria statistics**, reporting the fraction of the different mitochondria morphology measured for that cell.

 **Segment mitochondria from all cells in the image**

Shortcut function key: **F7**
Segment and classify mitochondria from all the cells outlined in the active image. The script uses the information available in the image about the cells selected, ignoring the content of the ROI Manager. For each cell the script generates an image showing, side by side, the original cell and the result from the segmentation/classification. Each mitochondria is colored according to the convention illustrated in a separate legend window. Furthermore, a new line is added to the table **Mitochondria statistics** for each cell analyzed, reporting the fraction of the different mitochondria morphology measured. Finally,

a new line is added to the table **Mitochondria summary statistics**, reporting the average fraction of the different mitochondria morphology measured in the image.

 **Segment mitochondria / batch mode**

Shortcut function key: **F8**
Segment and classify mitochondria from all the cells outlined in all the images available in a directory. All the windows at that time opened in the program are closed, and the user is asked for an input directory. The images in the directory are opened, one at a time, and all the cells outlined in each image are analyzed and the results added to the tables **Mitochondria statistics** and **Mitochondria summary statistics.** Then the images showing the classification results from each cell are saved to the subdirectory indicated in the **Settings** (default: Cells), and all images are closed before opening the successive one. At the end of the batch processing the tables contain the summary from all the images analyzed.

 **Save**

Save images and tables to predefined directories. Original images and results from analysis are saved to different directories, as specified in **Settings**. A copy of the original images is saved only if they contain cell outlines. If the original image is 2D and already in a predefined subdirectory, the program assumes that the image has been already analyzed before, and therefore it will just overwrite the image in the same directory, to avoid the generation of further subdirectories.

 **Help F keys**

Provides a list of all the keyboard shortcuts that can be used to launch some of the tools.

Scenarios

Here are outlined the recommended procedures to follow in a few common situations.

Working interactively on one image

- Open an image.
- If the image is actually a stack, move to the slice of interest.
- Draw a freehand selection around each cell of interest and add the cell to the list of interest ().
- Repeat the previous step for other cells in the image.
- If at any point you need to remove a selection, select it from the ROI Manager and delete it with  .
- Analyze a single cell by selecting it in the ROI Manager and pressing  , or analyze all the cells thus far selected by focusing on (i.e. making it active) the image and pressing  .
- During the session, or at the end of it, press  to save the image with the cells outlined and any image result generated. Select each table and go to the menu **File/Save as...** to save the contents of the tables, since they are not automatically saved.

Comparing two images from two different experimental conditions

- Open an image.
- If the image is actually a stack, move to the slice of interest.
- Draw a freehand selection around each cell of interest and add the cell to the list of interest ().
- Repeat the previous step for other cells in the image.
- Open another image and repeat all the previous steps.
- Focus on one of the two images and analyze all the cells outlined by pressing  .
- Focus on the other image and repeat the analysis.

- During the session, or at the end of it, press  to save the images with the cells outlined and any image result generated. The script will save the results from each cell in the subdirectory of the corresponding image: if unable to, it will prompt a warning and the user will need to save those results manually. Finally, select each table and go to the menu **File/Save as...** to save the contents of the tables, since they are not automatically saved.

Working on several images for different experimental conditions

- Before starting, make sure that all the images from one experiment are inside just one directory, which is different for each experiment.
- Open all the images from one experiment. If they are too many, open them in batches.
- For each image, draw a freehand selection around a cell of interest, and add it to the list of interest ().
- Repeat the previous step for all the cells of interest in all the images.
- At any moment the work in progress can be saved by pressing  . At that point the images where the cells of interest have been all added can be safely closed.
- After completing the cell selection for all the images from one experiment, close all the images and open those from the second experiment.
- Repeat all the previous steps until cells from all the experimental conditions have been selected.
- In order to start the batch processing for one experiment, press  and point to the subdirectory (default: /Images)for that experiment where all the images containing the cell selection have been saved. The program will process all the images and write the results into a subsubdirectory (default: /Images/Cells). In this case there is no need to save anything manually, because the script takes care to save both cell images and tables.