ProMoIJ User's Manual

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1. Installation.

ProMoIJ is a pack of ImageJ macros developed to work with the image software Fiji (downloadable from: https://fiji.sc/#download). The version of Fiji used to create ProMoIJ may be also downloaded from http://www.achucarro.org/downloads (FIJI versions). We cannot warrant that ProMoIJ would run properly with other versions.

The following ImageJ plugins should be installed in Fiji to run ProMoIJ macros 2 and 3:

- TurboReg (http://bigwww.epfl.ch/thevenaz/turboreg/) (Macro 2)
- MultiStackReg (http://bradbusse.net/downloads.html) (Macro 2)
- Correct 3D drift (http://imagej.net/Correct_3D_Drift) (Macro 2)
- Bleach Correction (http://imagej.net/Bleach_Correction) (Macro 3)

To install plugins download and save the corresponding files to the Fiji plugins folder. The version of these ImageJ plugins used during testing may be downloaded from http://www.achucarro.org/downloads (Required Plugins). Please, note that the version of Fiji provided with ProMoIJ already includes the imageJ plugins Correct 3D drift and Bleach Correction.

Finally, to install ProMoIJ download it from http://www.achucarro.org/downloads (ProMoIJ), unzip, and save the ProMoIJ folder to the Fiji plugins folder.
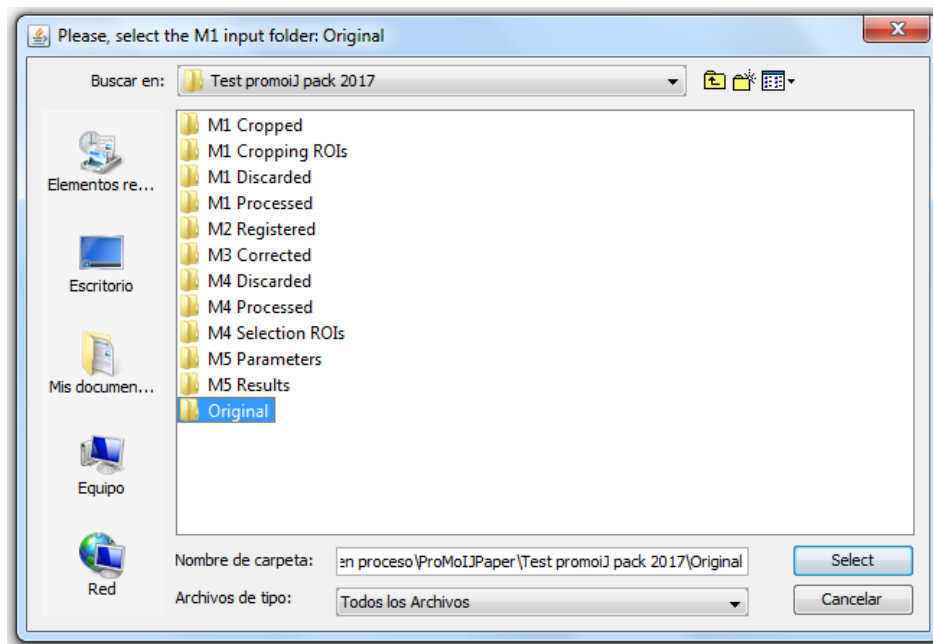
2. Folders

A series of input and output folders is required to run ProMoIJ, to save processed images, parameters, and other data. As ProMoIJ sequentially runs 5 macros, the processed images saved in the output folder of one macro are generally the input images for the next macro. A schematic drawing of the process is shown here:
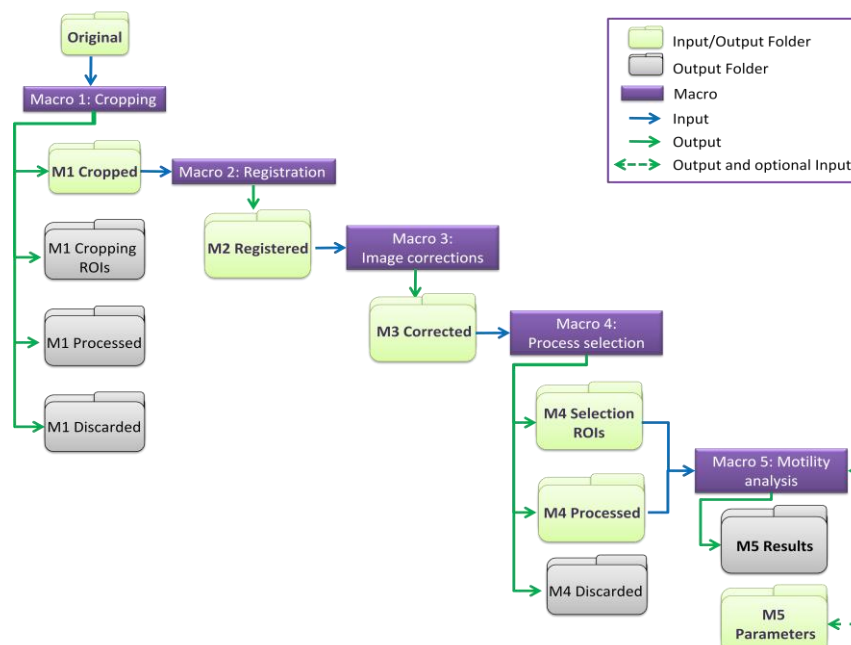
- Input folders: All macros require that users create a folder containing only the images to be processed or analyzed. There are no specific requirements for this folder's name but it is recommended to use short names (e.g. Original).

- Output folders: All macros require folders to save different file types: images, ROIs, parameters, and results. These folders may be created in advance or while running the macro (when the user is asked to). Again, it is recommended to use short names for the folders. All the required empty folders may be downloaded from http://www.achucarro.org/downloads (Folders Example).

Important note: During the execution of Macro 4, ROI (Region Of Interest) files of process selections and orientation vector will be created and saved in a folder (e.g. M4 Selection ROIs). This folder is required to run Macro 5. The name of the ROI files allows the association between selections and images during analysis with Macro 5, thus it is absolutely required to preserve this folder and the name given by Macro 4 to the files.

3. **Menu**

ProMoIJ Menu is accessed by clicking on the Plugins menu and then on the ProMoIJ submenu (Plugins ▷ ProMoIJ ▷ Menu ProMoIJ ↓). From this Menu you can execute any ProMoIJ macro. You may also execute ProMoIJ macros by clicking on the name of the corresponding macro in the ProMoIJ submenu of Fiji Plugins menu.
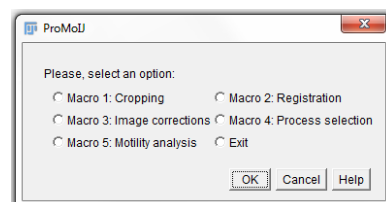

4. **Macro 1: Cropping.**

This macro allows separating the channel that will be used to analyze process motility, which is mandatory for further processing and analysis. In addition, this macro allows reducing the number of Z-slices and/or time-frames, and cropping cells to generate smaller images for processing, which is recommended for large images (1Gb or more).

4.1. <u>Folders selection</u>. The user is asked to select the following folders:

    4.1.1. **M1 input folder (e.g. Original):** selected folder must contain only the images to be processed.

    4.1.2. **M1 output folder for processed images (e.g. M1 Processed):** images contained in the folder selected in the step 4.1.1. are transferred to the folder selected here after processing.

    4.1.3. **M1 output folder for discarded images (e.g. M1 Discarded):** any image with no ROIs associated (not used) is transferred to this folder.

    4.1.4. **M1 output folder for ROIs (e.g. M1 Cropping ROIs):** the ROIs used to select the cells are saved in this folder.

    4.1.5. **M1 output folder for cropped images (e.g. M1 Cropped)**: cropped images are saved in this folder. These images will only contain the area of the original image occupied by the bounding box of each single cell ROI, and the channel, Z-slices and time frames selected. The name of these images is the name of the original image ending in "_Crop.tif".

4.2. <u>Parameters menu:</u>
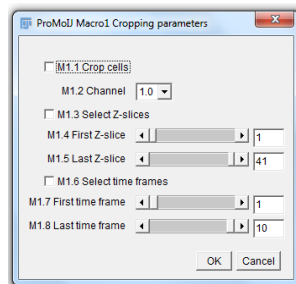
ID	Name	Possible values	Recommended	Description
M1.1	Crop cells	□/√	√	Activates cell cropping
M1.2	Channel	1 - ∞	N/A	Channel used to analyze cells
M1.3	Select Z-slices	□/√	N/A	Activates selection of Z-slices
M1.4	First Z-slice	1 - ∞	N/A	Selects the first Z-slice to be used
M1.5	Last Z-slice	1 - ∞	N/A	Selects the last Z-slice to be used
M1.6	Select time frames	□/√	N/A	Activates selections of time frames
M1.7	First time frame	1 - ∞	N/A	Selects the first time frame to be used
M1.8	Last time frame	1 - ∞	N/A	Selects the last time frame to be used

4.3. <u>ROIs design:</u>

    4.3.1. A maximum projection of the image with color-coded time frames (Red/Green) is
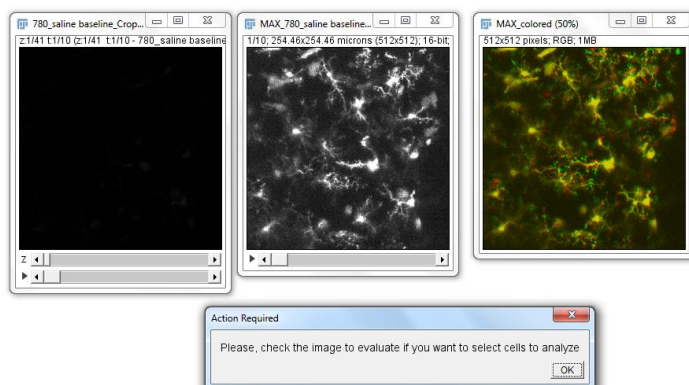
shown. Click OK after checking the image.

4.3.2. Macro 1 asks whether you will select any cell of the image:

4.3.3. By clicking "No" Macro 1 saves this image to the "M1 output folder for discarded images" and proceeds with the next image.

4.3.4. By clicking "Yes" Macro 1 allows you to select cells by drawing ROIs around them and adding them to the ROI Manager by using the button "Add" or by pressing the letter "t". It is recommended to draw a ROI as small as possible. Once you are done press OK.
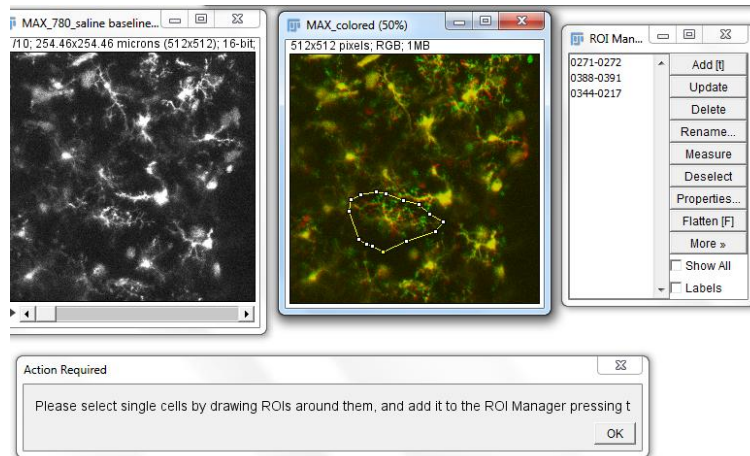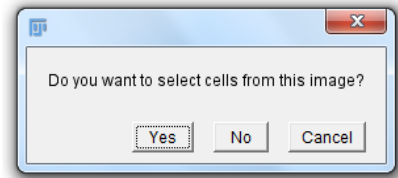
4.3.5. Macro 1 shows all the ROIs you drew and asks whether you want to select another cell. By pressing "Yes" you are allowed to modify previous ROIs, delete them, or add new ones by using the ROI Manager. When ready, press "No" and Macro1 will save the ROIs and the cropped images to the corresponding folders.
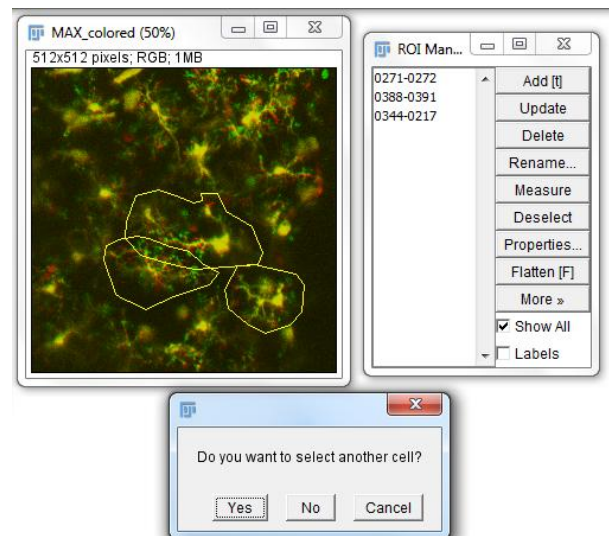
5. **Macro 2: Registration.**

Macro 2 performs batch registration of images using two ImageJ registration plugins: "MultistackReg v_1.45" and "Correct 3D drift"

5.1. Folders selection. The user is asked to select the following folders:

5.1.1. **M2 input images folder (e.g. M1 Cropped)**: selected folder must contain only the images to be registered, and these images must contain only one channel.

5.1.2. **M2 output folder (e.g. M2 Registered)**: registered images are saved in this folder with the name of the original image followed by "_Reg.tif".

5.2. Parameters menu:
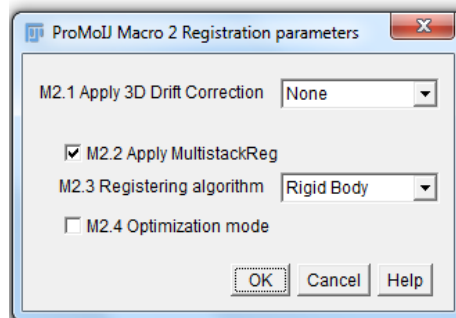
| ID	Name	Possible values	Recommended	Description
M2.1	Apply 3D Drift Correction	None Before After Before & After MultistackReg	After	Activates 3D registration with the plugin "Correct 3D drift"
M2.2	Apply MultistackReg	□/√	√	Activates registration with the plugin "MultistackReg"
M2.3	Registration algorithm	Translation Rigid Body Affine Scaled Rotation	Rigid Body	Selects the algorithm used during registration
M2.4	Optimization mode	□/√	√	Optimization and analysis

5.3. Optimization mode:

5.3.1. By checking "Optimization mode" in the main menu two maximum projection images are shown:
- the original image (MAX_Original)
- the registered image (MAX_Registered)

Please, do not close any image as the macro will close them for you.

5.3.2. Click OK in the Optimization window.

5.3.3. Images are closed and an Optimization Menu (Optimization mode window) appears.
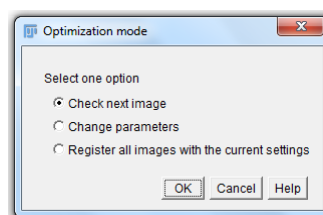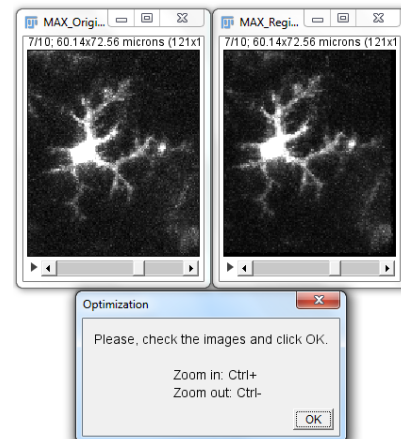
Options	Description
Check next image	Proceeds with the registration of the next image using the optimization mode
Change parameters	Allows changing parameters in Macro 2 Registration parameters menu
Register all images with the current settings	Exits optimization mode, registers all images using the current settings, and saves them

*An appropriate registration is achieved when the soma of microglial cells remains almost static through all time-frames.

6. **Macro 3: Image corrections**

Macro 3 performs batch photo-bleaching correction using the ImageJ plugin "CorrectBleach_V2.0.2" and applying its "Histogram Matching" method. In addition, Macro 3 uses a Difference of Gaussians filter to reduce noise and background.

6.1. Folders selection. The user is asked to select the following folders:

6.1.1. **M3 input images folder (e.g. M2 Registered)**: selected folder must contain only the images to be corrected.

6.1.2. **M3 output images folder (e.g. M3 Corrected)**: corrected images will be saved in this folder with the name of the original image followed by "_Sub.tif".

6.2. Parameters menu:
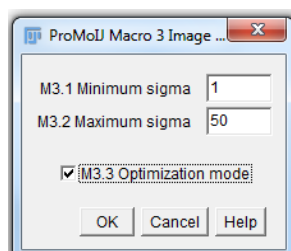
ID	Name	Possible values	Recommended	Description
M3.1	Minimum sigma	1- ∞	2	Image smoothing (higher values = smoother image)
M3.2	Maximum sigma	1- ∞	50	Local background subtraction, (lower values = more subtraction)
M3.3	Optimization mode	□/√	√	Optimization and analysis

*It is recommended to use the smallest minimum sigma (do not go over 3) and the highest maximum sigma that adequately subtract noise and background.

6.3. Optimization mode:

6.3.1. By checking "Optimization mode" in the main menu two maximum projection images are shown:
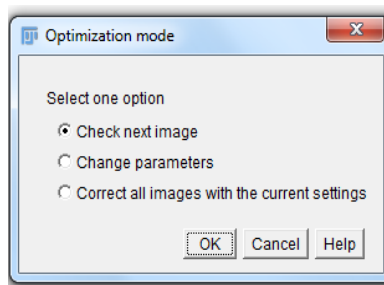- the original image (MAX_Original)
- the corrected image (MAX_Corrected)
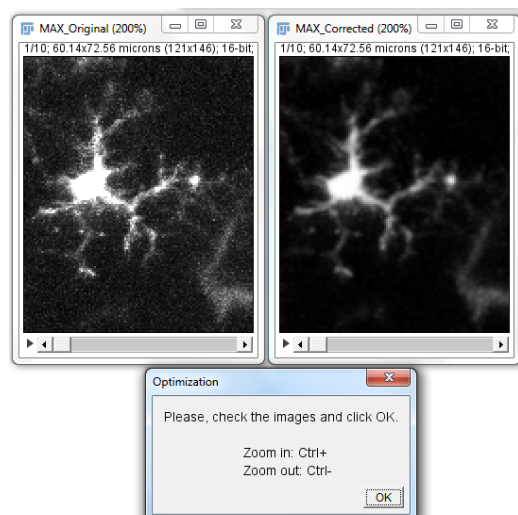
Please, do not close any image as the macro will close them for you.

6.3.2. Click OK in the Optimization window.

6.3.3. Images are closed and an Optimization Menu (Optimization mode window) appears.

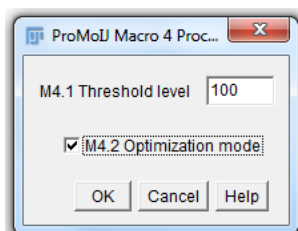| Options	Description
Check next image	Proceeds with the correction of the next image using the optimization mode
Change parameters	Allows changing parameters in Macro 3 Image corrections menu
Correct all images with the current settings	Exits optimization mode, corrects all images using the current settings, and saves them

7. **Macro 4: Process selection**

Macro 4 facilitates the selection of the process and indication of its orientation (orientation vector).

7.1. Folders selection. The user is asked to select the following folders:

7.1.1. **M4 input images folder (e.g. M3 Corrected)**: selected folder must contain only the images to be used.

7.1.2. **M4 output folder for processed images (e.g. M4 Processed)**: images contained in the folder selected in the step 7.1.1 are transferred to this folder after processing.

7.1.3. **M4 output folder for discarded images (e.g. M4 Discarded)**: any image with no ROIs associated (not used) is transferred to this folder.

7.1.4. **M4 output folder for ROIs (e.g. M4 Selection ROIs)**: process selections and orientation vectors are saved to this folder.

7.2. Parameters menu:

ID	Name	Possible values	Recommended	Description
M4.1	Threshold level	0 - ∞	N/A	Intensity threshold used to identify static regions (should be higher than the threshold used for analysis, M5.4)
M4.2	Optimization mode	□/√	√	Optimization and analysis

It is recommended to check the images to determine the "Optimization mode" which allows checking the threshold using the tool "Threshold" of ImageJ (Image ▷ Adjust menu ▷ Threshold... or Ctrl+Shift+t). More instructions about how to establish a good threshold can be found below.

7.3. ROIs design:

7.3.1. Macro 4 highlights in red the pixels that maintain an intensity value above the user-defined threshold level (parameter M4.1) through all time frames. Click OK to continue.
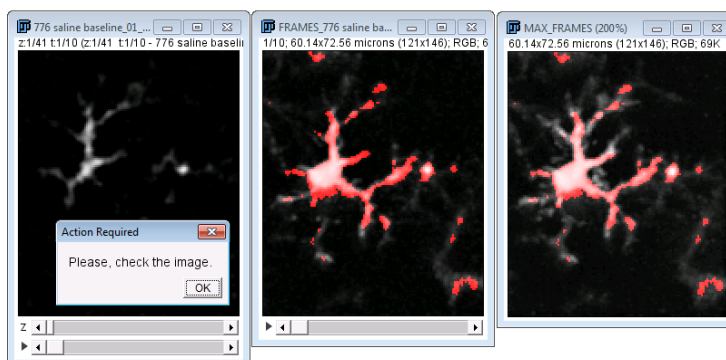

7.3.2. Macro 4 asks whether you want to analyze any process in this image:

7.3.3. By clicking "No" Macro 4 saves this image to the "M4 output folder for discarded images" and proceeds with the next image.
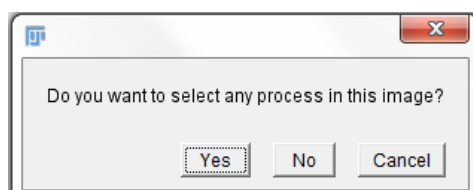

7.3.4. By clicking "Yes" Macro 4 allows you to select processes by drawing ROIs around them and adding them to the ROI Manager by using the button "Add" or by pressing the letter "t". It is recommended to draw the ROI as small as possible. Selected process should be confined within the XYZT stack of images and include
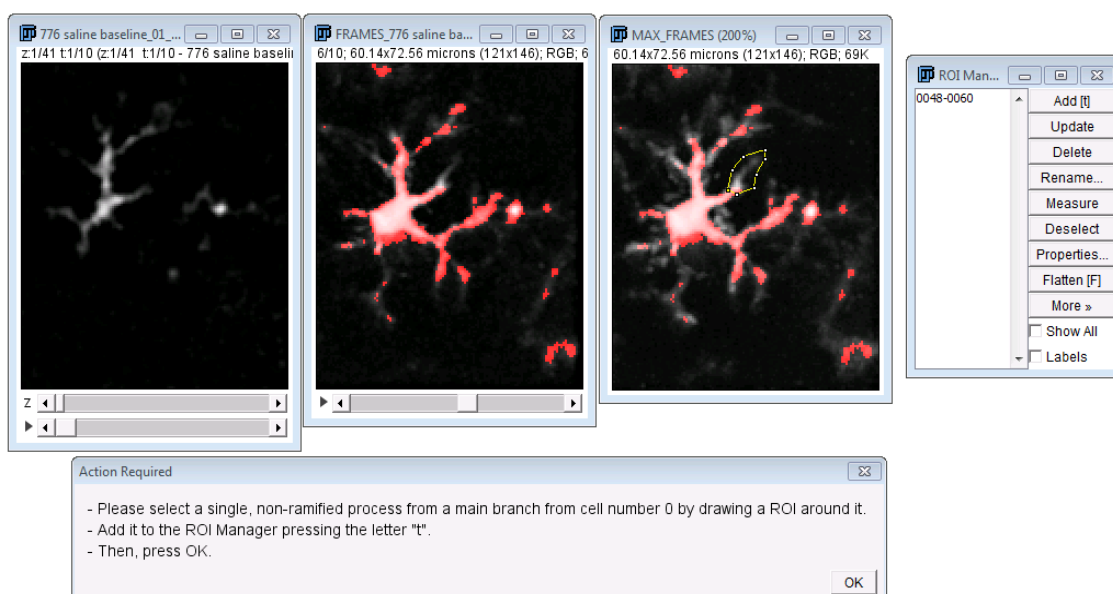
a constant region (highlighted in red).

7.3.5. In addition, the investigator must indicate the orientation of the process by drawing an arrow pointing **from a constant basal region of the process to its tip** (orientation vector). Drawing may be done in any of the images shown. The basal constant region is the constant region of the process (highlighted in red) included in the selection that is farthest away from its tip. Finally the macro saves all the selections and vectors for further analysis in the "ROIs folder".
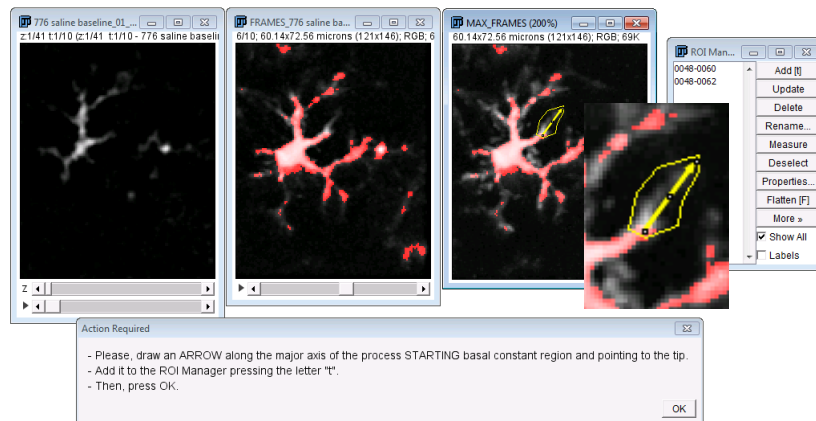
7.3.6. Macro 4 shows the ROIs in the image and asks whether you want to select more processes from the same cell.
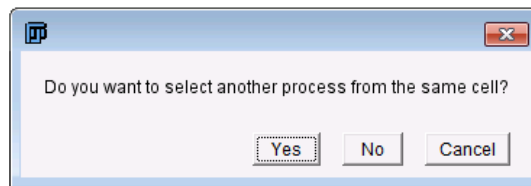
7.3.7. Click "Yes" and repeat steps from 7.3.4. to select more processes.

7.3.8. By clicking "No" Macro 4 saves all the ROIs with the name of the image and a number identifying the cell.

7.3.9. Macro asks whether you want to select processes from another cell in the current image.

7.3.10. By clicking "Yes" the macro allows drawing new ROIs repeating steps from 7.3.4.

7.3.11. By clicking "No" the macro closes the current image and load the next one.

7.4. Optimization mode:

7.4.1. By checking "Optimization mode" in the main menu you will be able to set an intensity threshold level using the tool "Threshold" of ImageJ (Image▷Adjust menu▷ Threshold... or Ctrl+Shift+t).
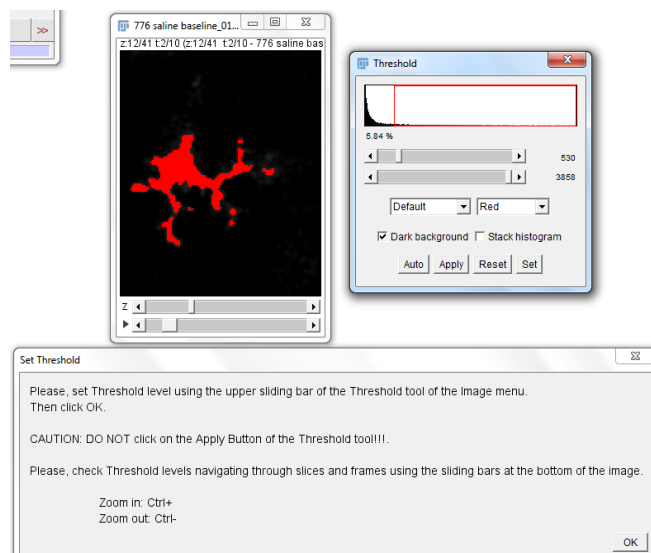
7.4.2. Select the threshold level using the upper sliding bar of the Threshold window while maintaining the lower sliding bar on its right limit. Please, do not click on the Apply button. The threshold should be selected to only include pixels that clearly correspond to cellular elements. It is preferable to select a highly restrictive threshold than a permissive one, even if it excludes some cellular elements.

7.4.3. Click OK in the Set Threshold window.

7.4.4. Macro 4 highlights in red the pixels that maintain an intensity value above the user-defined threshold level through all time frames.
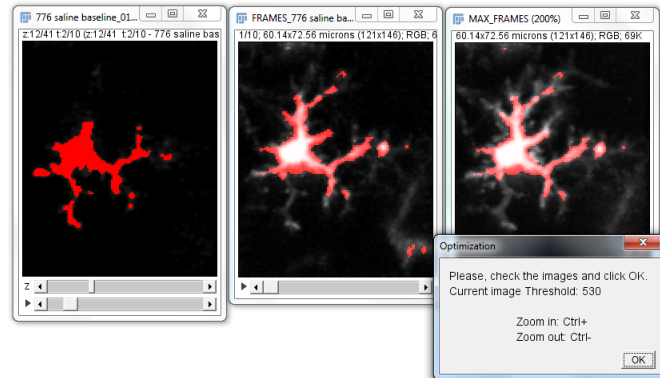
7.4.5. Click OK in the Optimization window to continue. Images are closed and an Optimization Menu (Optimization mode window) appears.
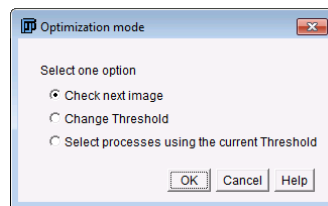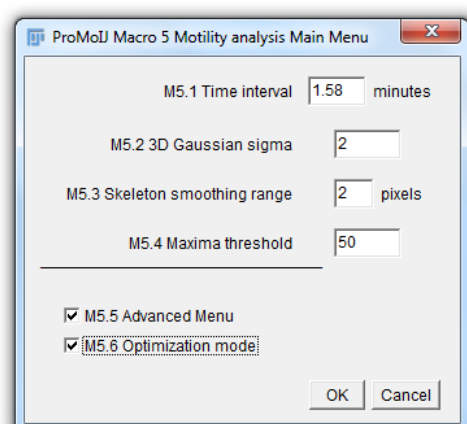

Options	Description
Check next image	Shows next image highlighting in red pixels that maintain through all time-frames an intensity level above a previously defined threshold
Change Threshold	Allows changing threshold level
Select processes using the current Threshold	Exits optimization mode, and allows process selection using current threshold level

8. **Macro 5: Motility analysis.**

   8.1. Motility analysis is performed by estimating process length and tip position in each time frame. ProMoIJ Macro 5 automates this analysis by reconstructing the 3D skeleton of the process. Once the 3D skeleton is reconstructed, the length and position of the skeleton tip in each time frame are analyzed to estimate process velocity (changes in length, μm/min, including protraction and retraction) and tip velocity (changes in the position of the tip, μm/min)

   8.2. Folders selection. The user is asked to select the following folders:

8.2.1. **M5 input images folder (e.g. M4 Processed)**: selected folder must contain only the images to be analyzed.

8.2.2. **M5 input ROIs folder (e.g. M4 Selection ROIs)**: selected folder must contain process selections and orientation vectors ROI files generated with Macro 4. The name of these ROI files allows association between selections and images, thus it is very important to preserve the name given by Macro 4.

8.2.3. **Input/output parameters folder (e.g. M5 Parameters)**: parameters used are saved and loaded from this folder.

8.2.4. **Output results folder (M5 Results):** result tables are saved in this folder.

8.3. Main menu (basic parameters):

ID	Name	Possible values	Default values	Description
M5.1*	Time interval	0 - ∞	1.58	Time interval between frames, in minutes
M5.2*	3D Gaussian sigma	0 - ∞	1	3D smoothing (higher values = smoother image)
M5.3*	Skeleton smoothing range	0 - ∞	2	Range (in pixels) for 3D skeleton smoothing, (higher values = smoother skeleton)
M5.4*	Maxima threshold	0 - ∞	50	Intensity threshold used to identify a process
M5.5	Advanced menu	□/√	□	Advanced parameters (Suppl. Table 1)
M5.6	Optimization mode	□/√	□	Optimization and parameters saving

It is recommended to use the Optimization mode to check 3D skeleton reconstruction with the current parameters and change them, if necessary, to get an appropriate match between reconstructed skeletons and selected processes.

Advanced parameters are set to default values that may be modified in the Advanced Parameters Menu, accessed by checking the corresponding option in the Main Menu (M5.5).

8.4. Advanced parameters:

Nº	Name	Possible values	Default value	Description
M5.7	Orientation vector direction	Base-to-Tip Tip-to-Base	Base-to-Tip	Indicates the direction of the orientation vector
M5.8	Y step	1 - ∞	1	Distance in pixels between Y profile lines.
M5.9	Tolerance	1 - ∞	Equal to M5.4 value	Minimum difference between peaks intensities to define a maxima
M5.10	Relative values to find borders	□/√	□	Maximum intensity of the border is defined as certain ratio of the profile maxima
M5.11	Border/maximum ratio	0 - ∞	N/A	Ratio of profile maxima used to calculate maximum intensity of the border
M5.12	Border inflexion amplitude	0 - ∞	5	Minimum amplitude of concave inflexions to separate two processes
M5.13	Activate pre-detection step	□/√	√	Pre-detection step
M5.14	Percentage of ROI	0 - 100	75	Minimum percentage of the process selection ROI that should be reached by the process skeleton
M5.15	Process comparison range	0 - ∞	2	Y range (in pixels) to search for the process in the previous time frame when branching is encountered

M5.16	Comparison height	0 - ∞	20	Range of XZ coordinates of the previous time-frame to obtain the mean XZ coordinate that will be used to calculate distances when branching is encountered.
M5.17	Size of gaps	0 - ∞	2	Size (in pixels) of allowed process gaps
M5.18	X gap extension	0 - ∞	0.5	Number of pixels used to expand X limits of previous XZ plane per Y position when a gap is encountered
M5.19	Z gap extension	0 - ∞	1	Number of pixels used to expand Z limits of previous XZ plane per Y position when a gap is encountered
M5.20	Skeleton smoothing filter	Gaussian Mean	Gaussian	Type of skeleton smoothing

8.5. Optimization mode:

> The "Optimization mode" in the main menu allows the comparison of the reconstructed 3D skeleton and the original image, to optimize the parameters utilized.

8.5.1. Four different images will be shown (please, do not close any image as the macro will close them for you):
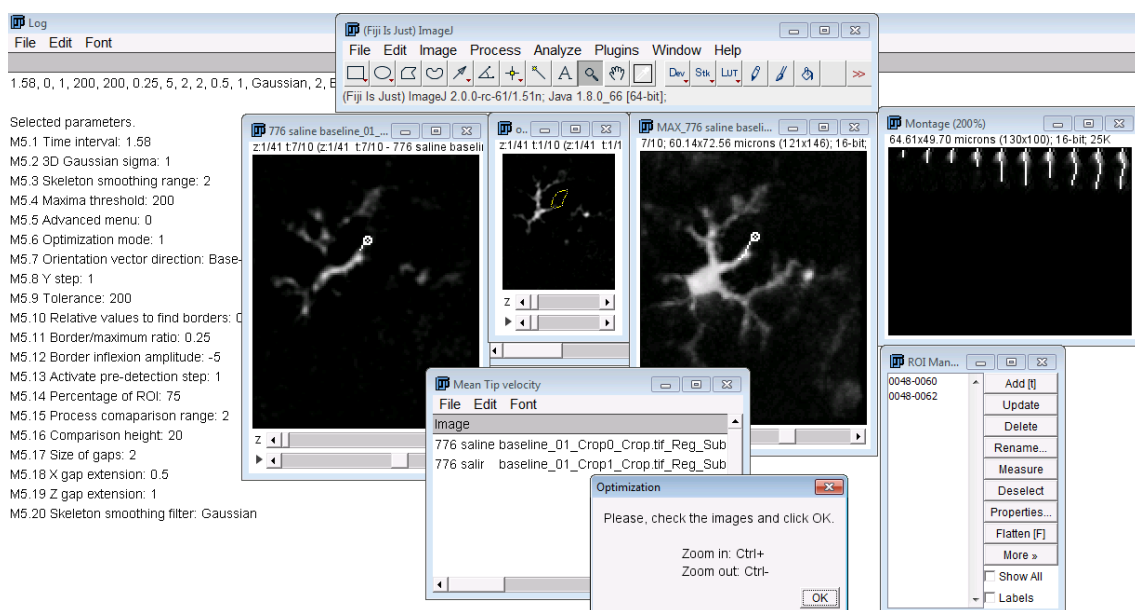

> - Original image: with the reconstructed process in white color.
> - Original: a copy of the original image showing the ROI of the process.
> - MAX_name of original image: a Z-slice maximum projection of the original image with the reconstructed process.
> - Montage: an image showing Z-slices maximum projections including a white line that represents the skeleton of the process.

8.5.1.1.   Result tables.

8.5.1.2.   Log: parameters used during current analysis.

8.5.2. Click OK in the "Optimization" window to continue

8.5.3. Images are closed and an Optimization Menu appears:
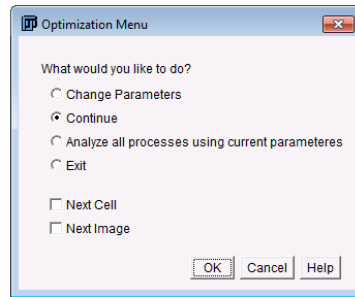
Options	Description
Change parameters	Allows changing parameters in Macro 5 Main Menu
Continue	Proceeds with the analysis of the next process
Analyze all processes using current parameters	Exits optimization mode and proceeds with the analysis using current parameters.
Exit	Saves current parameters to the "Parameters folder" and finish (results tables will not be saved)
Next Cell	When continue is selected, proceeds with the analysis of processes in the next cell
Next Image	When continue is selected, proceeds with the analysis of processes in the next image

It is recommended to check the match between reconstructed 3D skeletons and processes in several processes (5-10) from different images. If there is not adequate match, first try changing parameters from M5.2 to M5.4 (M5.2 and M5.3 parameters should never be above 2 and 4, respectively). If the problem persists (no match between the reconstructed skeleton and the original process) try changing advanced parameters.