NeuronMetrics User Manual

NeuronMetrics™ (Narro et al., 2007) is a semi-automated software tool that computes a skeletonized representation of the neurite arbor, the cell body **region of interest** (ROI), and the neuron's bounding convex polygon. From these features it calculates total neurite length, Polarity Index (PI), branch number, and the area and perimeter of the polygon. NeuronMetrics is implemented as Java plugins for NIH's ImageJ image analysis software, which was developed by Wayne Rasband and is in the public domain.

NOTE: In this PDF file, use the bookmarks to go directly to different sections of this manual. If you are viewing this pdf with Adobe Reader, when you follow a link, note that there are unlabeled "Forward" and "Back" navigation arrows at the bottom of Adobe Reader (the arrows with circles around them). The blue diamond bullets (◆) indicate steps the user performs.

To use NeuronMetrics, you must first download and install ImageJ and all necessary plugins as described under the Installation section. Also note the file naming rules.

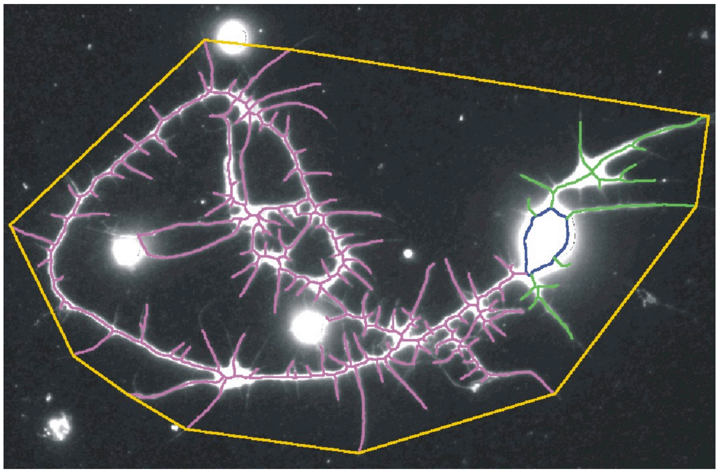
The NeuronMetrics Overview describes what the software does. Additional information is published in Narro et al., 2007. The Quick Reference Instructions provide both a handy reference for experienced users and an overview of the processing steps in the NeuronMetrics pipeline. The Detailed Instructions provide new users with step-by-step instructions to using NeuronMetrics. New users are advised to read the NeuronMetrics Overview and Quick Reference Instructions to gain an understanding of the software before trying to use it with the aid of the Detailed Instructions.

Copyright Notice and Disclaimer

NeuronMetrics Overview

For a published description of NeuronMetrics, including its biological context, algorithms used, validation, and comparison with other image-analysis approaches, see Narro et al. (2007).

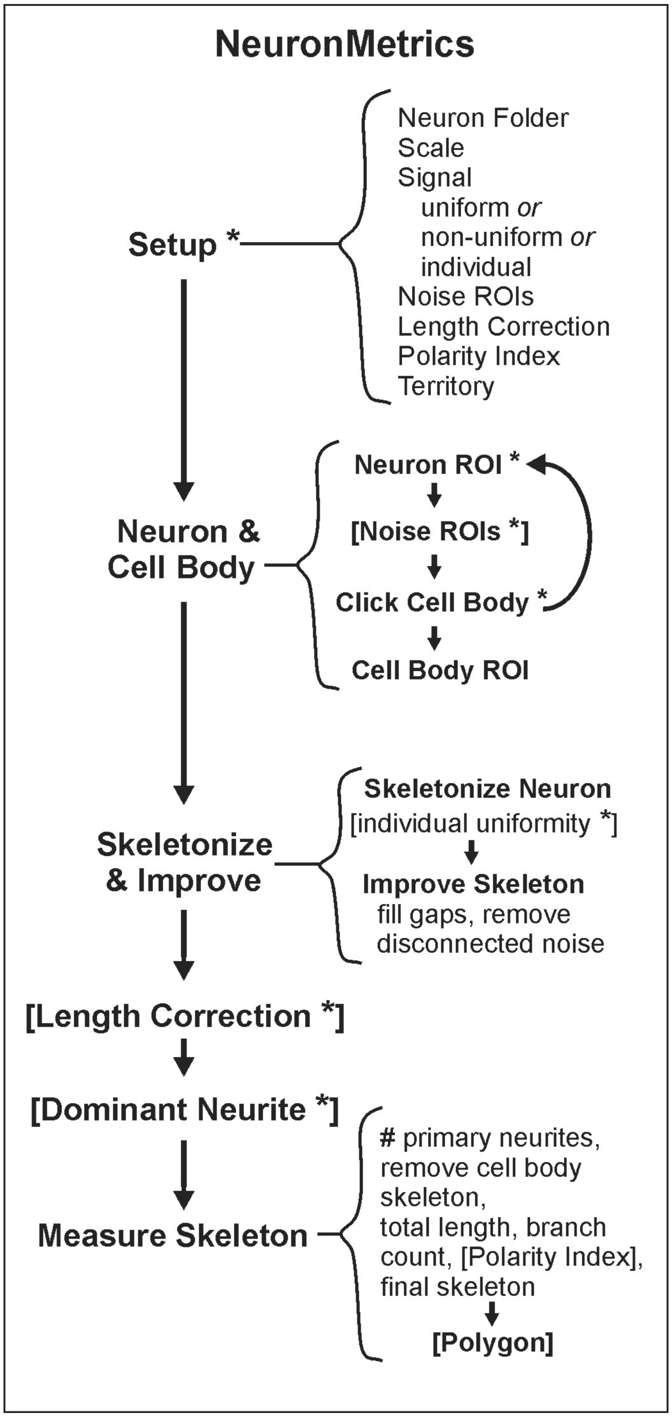
Optimal processing with NeuronMetrics requires high-contrast images of neurons as input. NeuronMetrics computes a skeletonized representation of the neurite arbor, the cell body **region of interest** (ROI), and the neuron's bounding convex polygon.



The neurite skeleton (dominant primary neurite in magenta, remainder in green) provides an excellent representation of neuronal morphology, cell body region of interest (dark blue) and the neuron territory (orange polygon). All have been thickened to 3 pixels to improve visualization. (Adapted from Fig. 1 of Narro et al., 2007.)

These features are used to quantify the length of the arbor, branch number estimate, primary neurite number, and territory. All numeric data are output to a single tab-delimited text file that may be imported into other software for further analysis.

Schematic Overview



The schematic overview (adapted from Fig. 2 of Narro et al., 2007) shows NeuronMetrics' image processing steps. The main modules are Setup, Neuron & Cell Body, Skeletonize & Improve, Length Correction (optional), Dominant Neurite (optional), and Measure Skeleton. In Setup, a dialog box is used to indicate the location of the input images, their scale, which skeletonization mode to use for the input image set (see Uniform Versus Non-uniform Neuron Signal), and the optional steps to be performed. *[continued on next page]*

Processing begins with the Neuron & Cell Body module which runs a suite of four modules. If a secondary label is also imaged, this image will be displayed alongside the primary image. The Neuron ROI, NoiseROIs (optional), and Click Cell Body modules run sequentially on each displayed neuron image, allowing the user to circle the neuron of interest, circle noise to eliminate it (optional), and click to indicate the location of the cell body. Afterwards, the folder of images is automatically run through the Cell Body ROI module. The user runs the Skeletonize & Improve module to skeletonize the neuron (Skeletonize Neuron module), fill gaps in the skeleton and remove disconnected noise (Improve Skeleton module). These steps are fully automated unless the skeletonization mode for the neuron images varies. In this case, the module pauses with the image displayed and prompts the user to indicate which skeletonization mode to use.

If the neuron contains regions of self-fasciculation, the Length Correction module may be run, and if the Polarity Index is to be computed, the Dominant Neurite module is used to isolate the dominant primary neurite. Last, the Measure Skeleton module is run to automatically compute all morphometric parameters. This includes running the Polygon module which computes the territory.

To expedite processing of a batch of neuron images from a selected input folder, input files are automatically opened, processed through automated steps, and closed. Output files and folders are automatically created, named, and saved. If the module includes manual intervention, the tool that will be used is pre-selected, and the user proceeds to the next step by pressing the space bar or clicking a button. These features greatly reduce the time spent on file management and maneuvering about the graphical interface.

Notes

Running Modes

Most plugins are designed to run in both single and batch mode. To run in single mode, open an image before running the plugin. To run in batch mode, first use the Setup plugin to indicate the input folder, then run the plugin.

Do Not Multi-task

To avoid problems, do not use any other applications while running NeuronMetrics. Do not move the mouse around except to provide user input that NeuronMetrics needs. This means do not read e-mail, read electronic journal articles, or work on some document. NeuronMetrics seems to get confused about which window is active.

Memory Limitations

The plugin that uses the most memory is the Dominant Neurite plugin. If images are large, ImageJ may run out of memory if more than one preview image is open at a time. Problems can occur with a 5.2 MB image and 300 MB of memory allocated for ImageJ. However with 500 MB of memory for ImageJ and a 5.9 MB image, all preview images can be open at the same time. Allocating memory for ImageJ is discussed under Installation.

Output

In either single or batch mode, all output folders go to the parent of the folder containing the input image. We recommend creating an outermost folder to contain both the input and output. In other words, create a folder (e.g., Experiment 1) and put your folder of input images in it. Then all output folders will go to folder Experiment 1.

Secondary Images

If two types of images were acquired, each type must be in a separate folder. In other words, the images of the primary label must be in one folder and the images of the secondary label must be in a different folder (e.g., named myExper_Bgal). A plugin automates moving the secondary images if they happen to be in the same folder as the primary images.

.txt files

If you re-run a plugin that generates a text file, you must manually update the .txt files.

Overwriting files

None of the plugins over-write .txt files. Plugins do over-write .tiff and .roi files (e.g., skeletons, cell of interest ROIs).

File Naming Rules

To implement automatic naming of output folders and files, a naming scheme was developed. It imposes the following constraints:

1) image files: the last character in the name MUST be a digit.

    OK: image1, 133-2-36

    NOT OK: image1hormone, 133-2-36a

2) secondary image folder: If there are secondary images, they must be in a folder named myExper_Bgal (case sensitive).

3) secondary image files: If there are secondary images, their names must be the same as the corresponding primary image with "-x" appended.

    OK: image1 (primary image), image1-x (secondary image)

    OK: 133-2-36 (primary image), 133-2-36-x (secondary image)

The name of the image folder is pre-pended to the name of all output folders. For example, given a folder named treatmentA as input, the Skeletonize Neuron plugin outputs a folder named treatmentA_skels.

A letter, letters, or descriptive phrase is appended to the image number of output files. For example, given image1.tif as input, the Skeletonize Neuron plugin would output image1s.tif.

The names of output folders and files are described for each plugin under both the Detailed Instructions and Quick Reference Instructions.

Installation

Software Requirements

NeuronMetrics runs on PC's with Windows XP or 2000 operating systems. NeuronMetrics requires ImageJ (specifically version 1.36b), FeatureJ, and Java (specifically version 1.5.0_03), all of which are freeware. Instructions for downloading and installing all necessary software components are below.

1 - Download and Install ImageJ

If you do not have ImageJ

- Download ImageJ.
- Follow the Downloads link.
- Under the Windows section, download the version bundled with Java 1.5.
- Double-click on the ij…setup icon and follow the setup prompts.
- The ImageJ folder should now be in C:\Program Files.
- Start ImageJ. [Note: you will later substitute a specific version of ImageJ; see 2 on p. 8]
- Check the memory allocated to ImageJ under Edit > Options > Memory… It should be set to 2/3 of the RAM or 640 MB, whichever is lower.
- Close ImageJ.
- Proceed to Download and Install NeuronMetrics.

If you have a version of ImageJ installed

- Check the memory allocated to ImageJ under Edit > Options > Memory… It should be set to not more than 2/3 of your RAM. A setting of 640 MB is adequate if it does not exceed that constraint.
- The optimal version of Java is 1.5.0_03 (see Sun Microsystems).
- Proceed to Download and Install NeuronMetrics.

An alternative approach to updating Java is to completely reinstall ImageJ bundled with Java, but first you must save any plugins or macros you previously installed. To keep those plugins or macros,

- First move the old ImageJ folder out of C:\Program Files and put it somewhere (say, on the desktop).
- Follow the instructions to download ImageJ.
- Reinstall the plugins or macros you previously had by copying them from the old ImageJ folder on the desktop into the appropriate folder (plugins or macros) in the new ImageJ folder in C:\Program Files.

2 - Download and Install NeuronMetrics

- Download NeuronMetricsFiles.zip from the iBridge^{SM} Network. (From the Home page, search "NeuronMetrics".)
- Double-click on the NeuronMetricsFiles.zip icon and unzip it.
- In the NeuronMetricsFiles folder there are 3 folders, 1 .txt file, and 1 .jar file.
- Move the 3 folders (imageAnalysis, NM Tools, NeuronMetrics) into the ImageJ\plugins folder.
- Move the ExpandOrShrink.txt file into the ImageJ\macros folder. Yes, overwrite the existing copy. The version included with NeuronMetrics has been modified to get the input parameters from plugins instead of popping up a dialog box.
- Delete the existing ij.jar file from the ImageJ folder. Move the ij1.36b.jar file, which is version 1.36b of ImageJ, into the ImageJ folder, and rename it ij.jar. Yes, this will overwrite the existing copy.
- Start ImageJ.
- The version of ImageJ must be 1.36b (Check by going to Help > About ImageJ...). NeuronMetrics does not run with some later versions of ImageJ. NeuronMetrics was tested on Java 1.5.0_03. Some later versions of Java are not compatible with the Dominant Neurite module of NeuronMetrics.
- On the toolbar under Plugins you should see NM Tools and NeuronMetrics.
- NeuronMetrics will not yet run correctly. You need to download the "Other Required Plugins" (see below).
- Close ImageJ.

3 - Other Required Plugins

FeatureJ: NeuronMetrics uses Erik Meijering's FeatureJ plugin for Laplacian edge detection.

- Download both FeatureJ_.jar and imagescience.jar
- Put both FeatureJ_.jar and imagescience.jar in the ImageJ\plugins folder.
- FeatureJ will appear on the toolbar under Plugins the next time you start ImageJ.

Batch Converter: If you need to convert image files to 8-bit, this plugin (by Wayne Rasband) is required. Otherwise, it is not.

- Download Batch_Converter.class
- Put it in the ImageJ\plugins folder.
- Batch Converter will appear on the toolbar under Plugins the next time you start ImageJ.

Useful Optional Plugin

Window Closer: Closes all windows that have opened while using ImageJ.

- Download Window_Closer.class
- Put it in the ImageJ\plugins folder.
- Window Closer will appear on the toolbar under Plugins the next time you start ImageJ.

Quick Reference Instructions

NeuronMetrics Processing Steps

In ImageJ, the plugins are under Plugins > NeuronMetrics, or Plugins > NM Tools.
Output goes to the parent folder of the folder of neuron images. To avoid problems, do not multi-task.

Stitch Images

(if necessary)

- We use PanaVue software.
- If necessary, convert to 8-bit images (use ImageJ Plugins > Batch Converter) prior to stitching.
- Both the primary and any secondary images (e.g., anti-HRP and anti-βgal, respectively) need to be stitched.

Move Secondary-Label Images

(if necessary)
Move the images of the secondary label out of the folder with the primary labeled neurons.

- Create a destination folder named myExper_Bgal.
- Use NM Tools > File Mover.

1 - Save Scale plugin

(if necessary to measure, set, and save the scale)

- In ImageJ, open an image containing a micrometer or scale bar.
- Use the 'line selection' tool to draw a line.
- Analyze > SetScale...
- Leave the image open.
- Run the 1 Save Scale plugin.
- Close the image.

Note: This scale is used only by NeuronMetrics plugins, not by other ImageJ functions.
detailed instructions

2 - Setup plugin

View or change the settings.

Run:

- Set the input Neuron Folder.
- Check the optional features you will compute.
- We recommend saving the first 3 overlays:
  - All Computed Features
  - Cell Body ROI on Neuron
  - Improved Skeleton on Neuron
- Click OK

detailed instructions

3 - Neuron and Cell Body plugin

Runs 4 plugins: Neuron ROI, Noise ROIs (optional), Click Cell Body, and Cell Body ROI.

Input: (batch) neuron image folder.

Also uses: corresponding secondary-labeled image (if any).

Run:

◆ Draw ROI around cell of interest
◆ Draw ROIs around noise enclosed by the neuron (optional).
◆ Click on the cell body.
◆ After finishing the steps above for all images in the folder, the cell body ROI automatically is created for all images in the folder.
◆ Check the cell body ROI overlays.

Output:

- folder myExper_cellROIs containing files image#roi.roi
- folder myExper_noiseROIs containing files image#nz.roi (optional)
- folder myExper_cellBodyPoints containing file myExper_ cellBodyPoints.txt
- folder myExper_cellBodyROIs containing files image#cb.roi
- folder myExper_cellBodyROI_overlay containing files image#cbn.tif (recommended)

Troubleshooting

- For cell body ROIs that are not satisfactory or skipped images (failures in the Cell Body ROI plugin), run the Cell Body Roi Manually plugin under NM Tools to manually outline the cell body.
- For other failures, run the appropriate plugin individually (Neuron ROI, Noise ROIs, Click Cell Body, Cell Body ROI).
- Do NOT run images that need to be stitched, but have not been. Their names violate the naming rules which will cause failure in later plugins.

4 - Skeletonize and Improve plugin

- Runs 2 plugins: Skeletonize Neuron, and Improve Skeleton.
- If you have changed your mind about the uniformity of the neuron signal, use the Setup plugin to change the 'Neuron Signal' setting now.
- Creates skeleton, then fills gaps, eliminates non-connected noise.

Input: (batch) neuron image folder

Also uses: corresponding Cell of Interest ROIs, Cell Body ROIs, Noise ROIs (if any).

Run:

◆ Fully automatic unless Neuron Signal is set to "set individually" in Setup, in which case, the plugin pauses on each image to let the user indicate whether the signal is uniform or non-uniform.
◆ Check improved skeleton overlays.

Output:

- folder myExper_skels containing files image#s.tif
- folder myExper_improvedSkels containing files image#_Improved_Skeleton.tif
- folder myExper_improvedSkels_overlays containing files image#_Improved_Skeleton_Overlay.tif (recommended)

Troubleshooting:
Large segment of neurite is missing from the improved skeleton due to failure to fill a gap in the preliminary skeleton. Fill the gap manually in the initial skeleton using ImageJ's 'Straight Line Selection' tool:

- Open the initial skeleton & zoom in on the gap until you can see individual pixels.
- If necessary, set the foreground color to white (Edit > Options > Colors, or double-click the eye dropper tool).
- Using the 'Straight Line Selection' tool draw a straight line between the 2 pixels at the ends of the gaps.
- Draw the line (Edit > Draw).
- It should be 1 pixel wide. Only the Straight Line tool draws 1 pixel wide, so do not use any other tool.
- Save the changes to the image.
- Re-run the edited initial skeleton through Improve Skeleton.

5 - Length Correction plugin (optional)

Use the 'Help' button in the plugin, or see the detailed instructions.

6 - Dominant Neurite plugin (optional)

Use the 'Help' button in the plugin, or see the detailed instructions.

7 - Measure Skeleton plugin

- Runs 2 plugins: Measure Skeleton, and Polygon (optional).
- computes: total length, polarity index (optional), # primary processes, total branch count, and polygon data (optional)
- finalizes skeleton: erases primary neurites that are too short (see Setup > Advanced... > Primary Neurites > Length Threshold) and removing the skeleton inside the cell body ROI.

Input: (batch) improved skeleton folder

Also uses: cell body points (.txt), cell ROI, cell body ROI, cell body skeleton, neuron (if saving overlay).

Run:

- ◆ Fully automatic.
- ◆ Check overlay of all features computed.

Output:

- folder myExper_finalSkels containing files image#fs.tif,
- folder myExper_skelDat containing myExper_skelData.txt and (if polygon was run) myExper_allData.txt
- folder myExper_ overlaysAll containing files image#_overlayAll.tif (recommended)

If polygon was run,

- folder myExper_polys containing files < imageID >p.tif
- folder myExper_polyDat containing file myExper_polyData.txt

Detailed Instructions

Save Scale plugin

Purpose

(if necessary)
Determines the number of pixels per unit distance in an image. If you already know the scale of the images in pixels/myUnits, just enter it in the Setup dialog box. The scale is used by NeuronMetrics plugins to convert the length and area data to meaningful units before output. The scale is saved and will appear under 'Scale' in the Setup dialog box.
Note: This scale is used only by NeuronMetrics plugins, not by other ImageJ functions.

Input

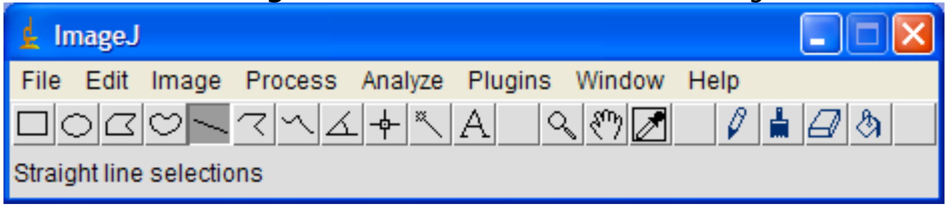
An image containing a scale bar or micrometer.

Output

Sets and saves the scale used by NeuronMetrics plugins. View it in the Setup dialog box.

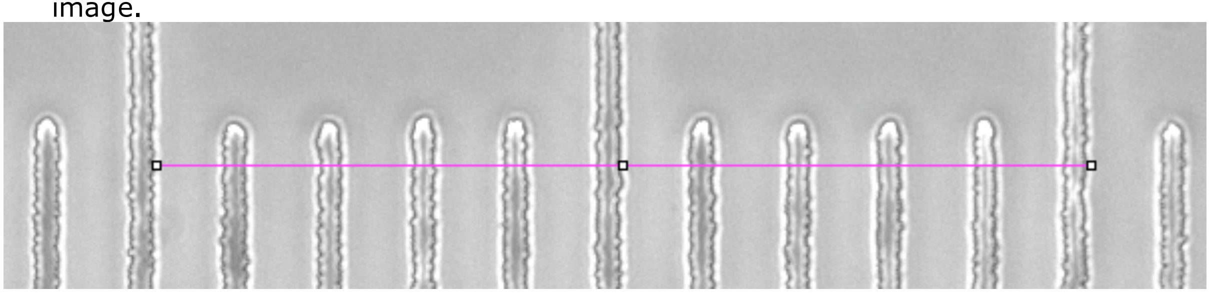
How To Use

- In ImageJ, open an image containing a micrometer or scale bar.
- Select the **Straight line selections** tool in the ImageJ toolbar.

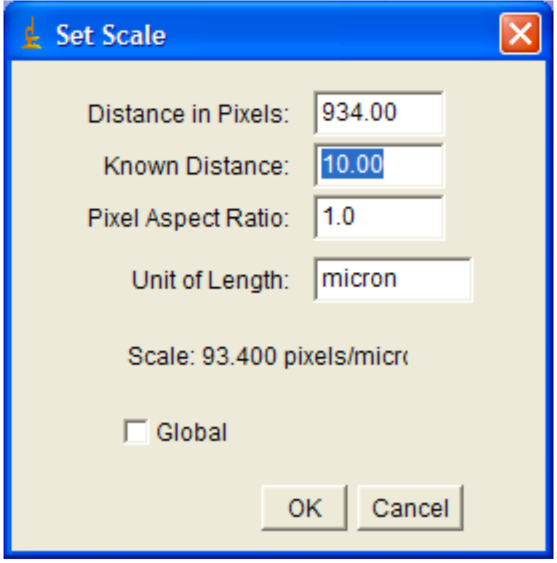


Straight line selections

- Manually draw a straight line selection (magenta) on the micrometer or scale bar in the image.



- Set the scale under Analyze > SetScale...



Distance in Pixels: 934.00

Known Distance: 10.00

Pixel Aspect Ratio: 1.0

Unit of Length: micron

Scale: 93.400 pixels/micr

☐ Global

Distance in Pixels: automatically filled based on the length of the straight line selection.

Known Distance: enter the appropriate value.

Pixel Aspect Ratio: use the default value of 1.0.

Unit of Length: enter the appropriate unit.

Scale: automatically reflects the values entered in the editable boxes above.

Global: do not check.

Click OK.

- Leave the micrometer or scale bar image open.
- Run the 1 Save Scale plugin.
  The scale should appear in the Setup dialog box (see next section) under Scale.
- Close the image.

Setup Plugin

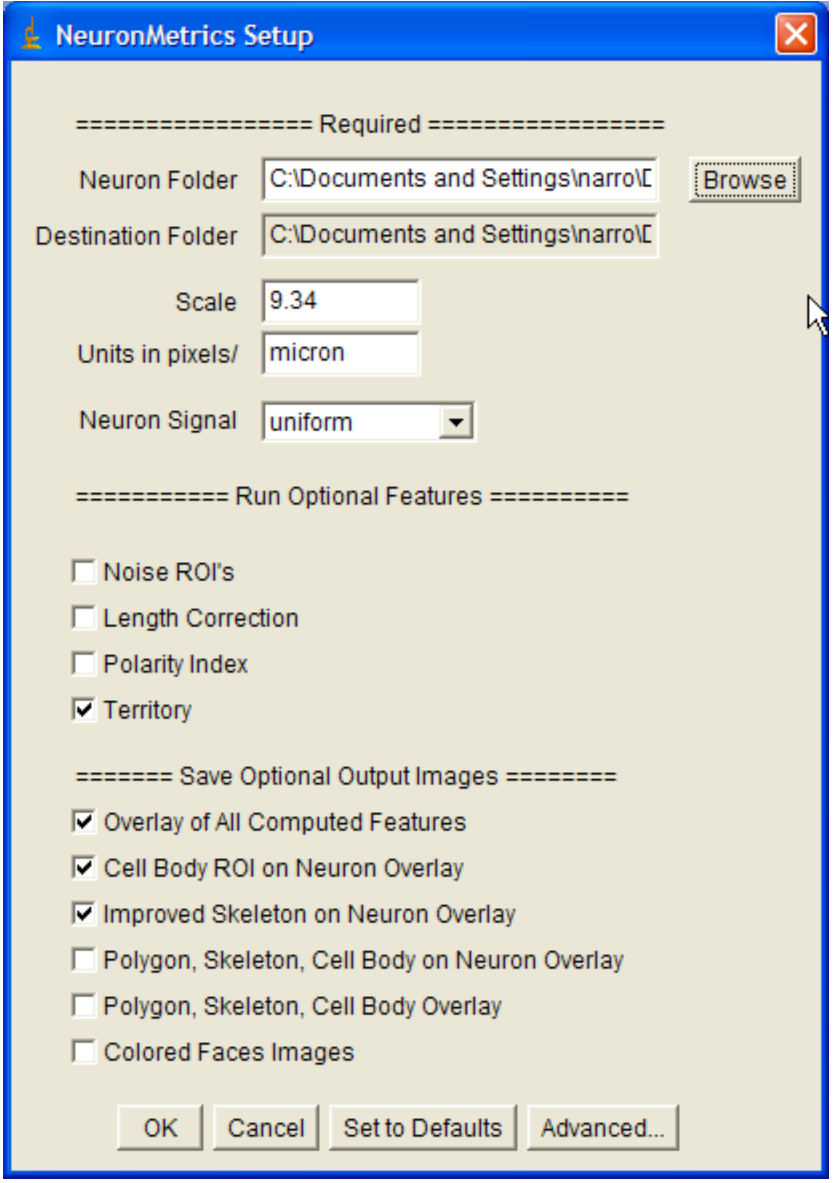
Purpose

Sets running parameters for NeuronMetrics.

How To Use

- Run by going to Plugins > NeuronMetrics > Setup.
  - Displays the Setup dialog box.

Setup dialog box



NeuronMetrics Setup

================ Required ================

Neuron Folder: C:\Documents and Settings\narro\D

Destination Folder: C:\Documents and Settings\narro\D

Scale: 9.34

Units in pixels/: micron

Neuron Signal: uniform

========== Run Optional Features ==========

□ Noise ROI's
□ Length Correction
□ Polarity Index
☑ Territory

======= Save Optional Output Images ========

☑ Overlay of All Computed Features
☑ Cell Body ROI on Neuron Overlay
☑ Improved Skeleton on Neuron Overlay
□ Polygon, Skeleton, Cell Body on Neuron Overlay
□ Polygon, Skeleton, Cell Body Overlay
□ Colored Faces Images

Neuron Folder should be set to the location of the folder containing the images to be processed. Use the Browse button to select a folder.

Destination Folder is the location of the folder where all output goes. It automatically sets to the parent folder of the Neuron Folder and is not editable.

Scale is used for the conversion from pixels to meaningful units. This scale is used only by NeuronMetrics plugins, not by other ImageJ functions.

Units in pixels/ are the aforementioned meaningful units.

Neuron Signal sets the skeletonization mode. The best setting depends on how uniform the signal is within a labeled neuron.

- **Uniform** is used for neurons with high, relatively uniform signal throughout.
- **Non-uniform** is used for neurons in which the signal varies.
- **Set individually** is used when some neurons in a folder have uniform signal and others have non-uniform signal. When running the Skeletonization plugin, a dialog box appears asking the user whether the displayed image should be processed in uniform or non-uniform mode.

Noise ROIs causes this plugin to be run when the multi-step 3 Cell Body & Neuron plugin is run.

Length Correction indicates that the user will manually run the Length Correction plugin. The data it generates will be used by the Polarity Index plugin (if run) and Measure Skeleton plugin.

Polarity Index indicates the user will manually run the Polarity Index plugin. The data it generates will be used by the Measure Skeleton plugin.

Territory causes the Polygon plugin to be run when the Measure Skeleton plugin is run.

Checking any of the **Save Optional Output Images** boxes causes the appropriate plugin to run and automatically save the output image. The most useful images for monitoring the quality of image processing are the first three which are checked by default.

Overlay of All Computed Features displays the skeleton, cell body ROI, and, if computed, also displays the dominant neuron and polygon. Each feature is a different color.

Cell Body ROI on Neuron Overlay displays the cell body ROI on the neuron and is useful for catching and correcting problems in computed cell body ROI prior to continuing processing.

Improved Skeleton on Neuron Overlay displays the improved skeleton (after gap filling and removal of disconnected noise) on the neuron. Useful for catching and correcting problems prior to further processing.

Polygon, Skeleton, Cell Body Overlay displays the final skeleton (gaps filled, disconnected noise and cell body skeleton removed), cell body ROI, and, if computed, the polygon on the neuron.

Colored Faces Images displays the faces found as part of the branch correction. Used for software testing or for creating colorful Miro-esque representations of otherwise annoying, complex neurons.

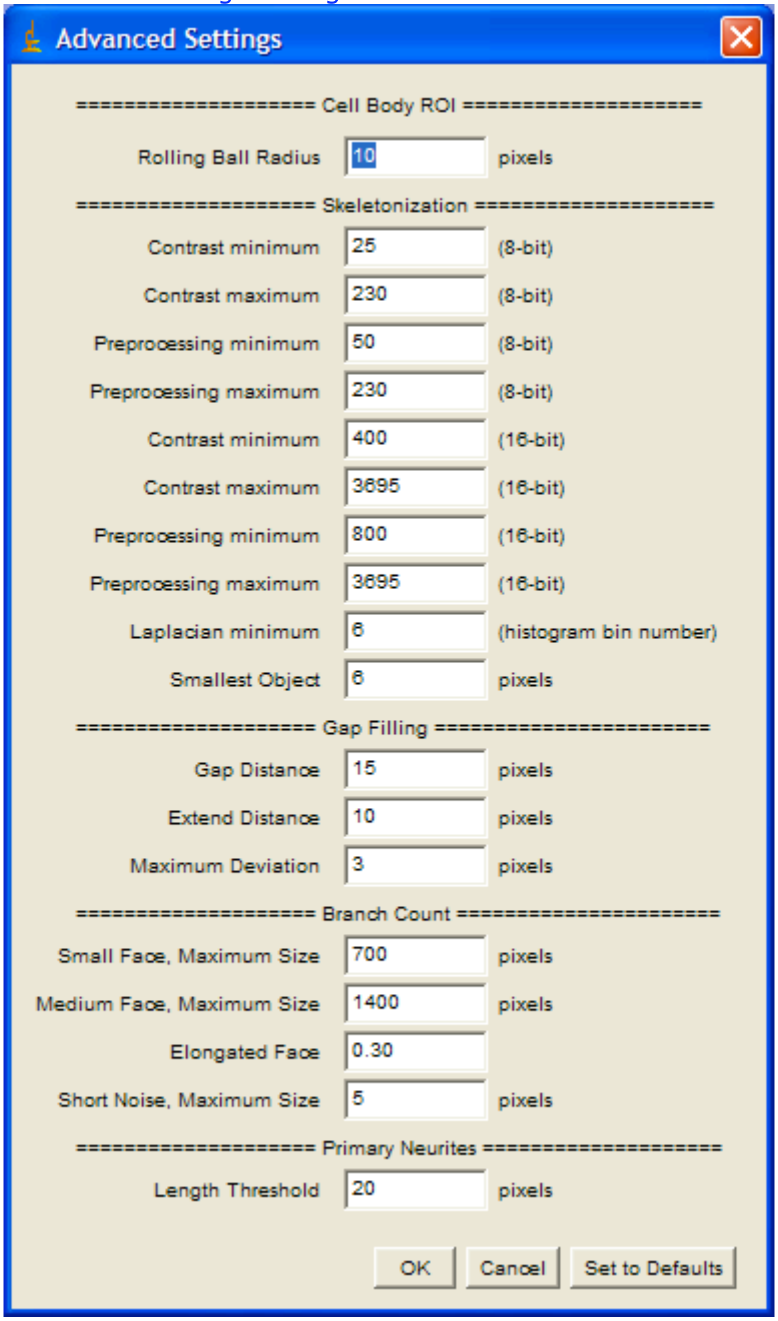
OK closes the dialog box and sets/resets the parameters.

Cancel closes the dialog box without resetting the parameters.

Set to Defaults resets the parameters under Run Optional Features and Save Optional Output Images to the NeuronMetrics default settings. The only Required parameter that is reset is Neuron Signal. If for some reason you want to run with no scale, manually set Scale to 1 and Units to “pixels” or leave it blank.

Advanced… button displays the Advanced Settings dialog box (see next page).

Advanced settings Dialog box.



==================== Cell Body ROI ====================

Rolling Ball Radius 10 pixels

==================== Skeletonization ====================

Contrast minimum 25 (8-bit)

Contrast maximum 230 (8-bit)

Preprocessing minimum 50 (8-bit)

Preprocessing maximum 230 (8-bit)

Contrast minimum 400 (16-bit)

Contrast maximum 3695 (16-bit)

Preprocessing minimum 800 (16-bit)

Preprocessing maximum 3695 (16-bit)

Laplacian minimum 6 (histogram bin number)

Smallest Object 6 pixels

==================== Gap Filling ====================

Gap Distance 15 pixels

Extend Distance 10 pixels

Maximum Deviation 3 pixels

==================== Branch Count ====================

Small Face, Maximum Size 700 pixels

Medium Face, Maximum Size 1400 pixels

Elongated Face 0.30

Short Noise, Maximum Size 5 pixels

==================== Primary Neurites ====================

Length Threshold 20 pixels

For additional information on the parameters in Advanced Settings, see Narro et al. (2007), in particular online Supplement 1.

Cell Body ROI

Rolling Ball Radius is used to establish the cell body ROI. Imagine a small ball or circle rolling around in the cell body (soma) of the neuron image. Decreasing the radius of the ball allows it to roll closer to the perimeter of the soma and farther out into the primary neurites.

Skeletonization parameters are difficult to adjust effectively unless you have background in image processing. If necessary, adjust them until you find settings that improve skeletonization for your images.

Smallest Object removes noise from the preliminary skeleton by eliminating all connected skeletonized objects this size or smaller. Setting this value too high may result in loss of short, disconnected tips of neurites.

Gap Filling parameters are used in the Improve Skeleton plugin to fill gaps between endpoints on segments of the preliminary skeleton.

Gap Distance is the maximum distance that the algorithm will attempt to fill.

Extend Distance affects how closely the two skeleton fragments must be aligned for the gap between them to be filled. A higher value increases the alignment stringency.

Maximum Deviation affects how closely the two skeleton fragments must be aligned for the gap between them to be filled. A lower value increases the alignment stringency.

Branch Count parameters regarding faces affect the branch count correction. The noise parameter sets a length threshold for what is counted as a branch. It is imperative to read the paper and understand these parameters before attempting to adjust them.

Small Face, Maximum Size is used to limit over-correction of the branch count in regions of self-fasciculation. Use ImageJ’s wand tool to measure the area of faces in self-fasciculating regions of the improved skeleton, then adjust this parameter accordingly.

Medium Face, Maximum Size is used to limit over-correction of the branch count in regions of self-fasciculation. Use ImageJ’s wand tool to measure the area of faces in self-fasciculating regions of the improved skeleton, then adjust this parameter accordingly.

Elongated Face is used to limit over-correction of the branch count in regions of self-fasciculation. Use ImageJ’s wand tool prior to measuring the Feret’s Diameter of faces in self-fasciculating regions of the improved skeleton, then adjust this parameter accordingly.

Short Noise, Maximum Size sets the upper limit on what is considered noise rather than a true neurite (what is too short to be a neurite). Increasing the value will reduce the branch count.

Primary Neurites parameters are used to reduce the time required to find primary neurites and to set a length threshold for them.

Length Threshold is used to exclude skeletonized neurites emanating from the cell body that are deemed to be too short to be true primary neurites. Increasing the value will reduce the primary neurite count.

Neuron ROI Plugin

Purpose

Indicates the neuron of interest and separates it from any adjacent neurites or noise.

Input

batch mode: neuron image folder via the Setup dialog box.
single mode: Open a neuron image.
also uses: secondary label image, if any.

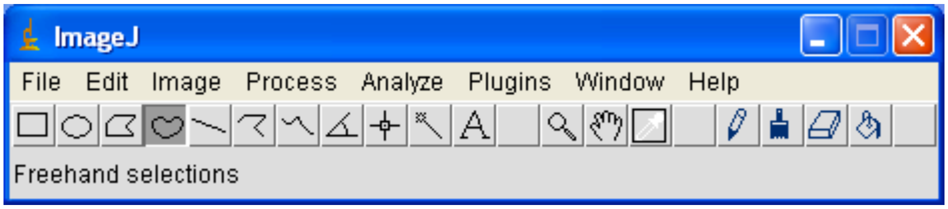
Output

neuron ROI
folder myExper_neuronROIs containing files image#roi.roi

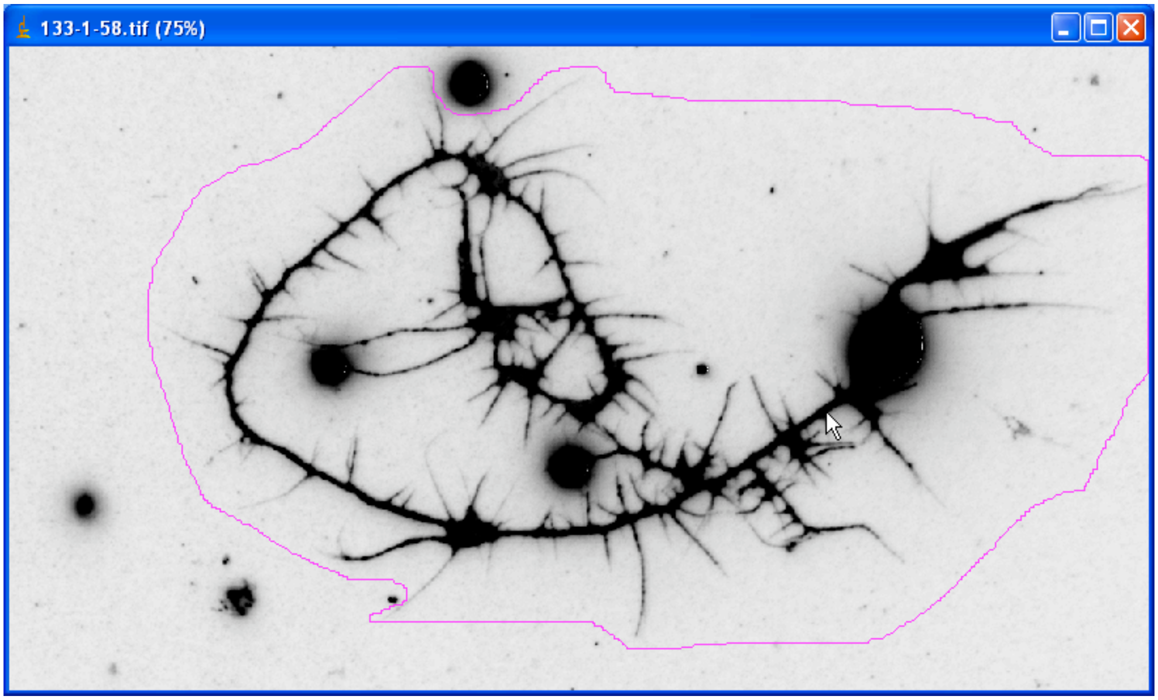
How To Use

◆ Run by going to Plugins > NeuronMetrics > Neuron ROI.

- The plugin automatically inverts the neuron image and opens the secondary image if there is one.
- Use ImageJ's Freehand Selections tool, which is automatically activated, to 'circle' the neuron of interest.



Freehand selections



Example of the neuron of interest circled in magenta.

- Press the spacebar.

  - The ROI is automatically saved.

  - In batch mode, the plugin will proceed to the next image.

Noise ROIs Plugin

Purpose

Indicates noise that contacts the neuron and needs to be removed to prevent it from appearing as part of the skeletonized neuron. This tool is particularly useful for removing noise that is enclosed by neurites and therefore cannot be excluded by ‘circling’ the neuron when defining the Neuron ROI.

Input

batch mode: neuron image folder via the Setup dialog box.
single mode: Open a neuron image.
also uses: secondary label image, if any.

Output

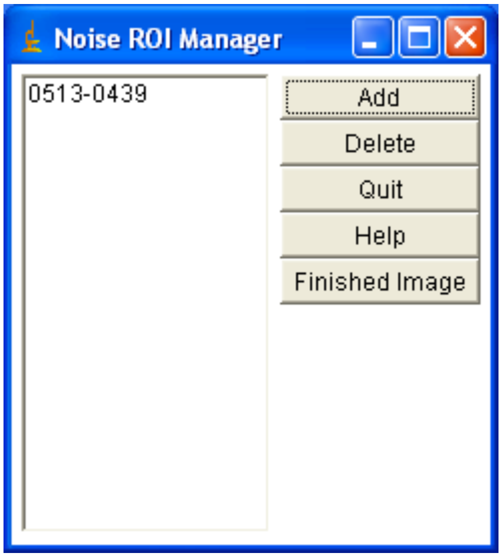
noise ROIs
folder myExper_noiseROIs containing files image#nz.roi

How To Use

◆ Run by going to Plugins > NeuronMetrics > Noise ROIs.

- The plugin automatically displays the neuron image and opens the secondary image if there is one.

- The Noise ROI Manager window appears.



Add: After ‘circling’ some noise, which creates a ROI, click Add to place an ID number for the ROI on the list.

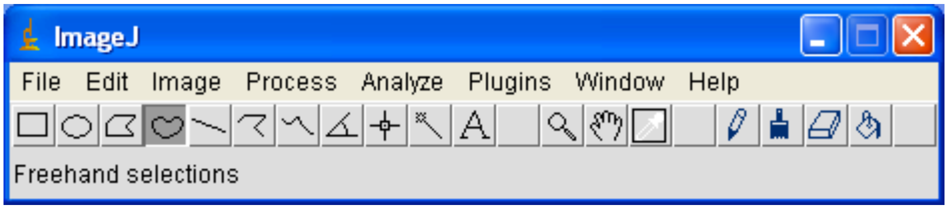
Delete: Select an item on the list. Note that the corresponding ROI is displayed on the neuron image. Click Delete to remove the ROI from the list.

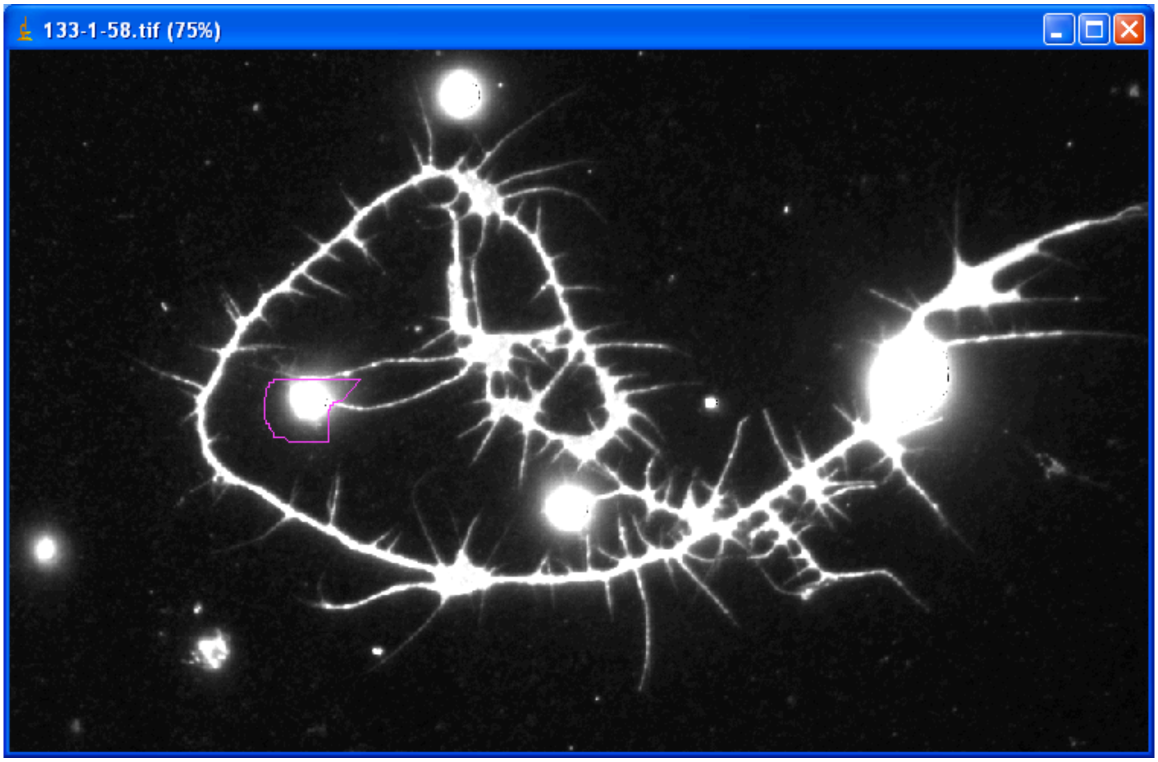
Quit: Quits the tool and closes all associated windows.

Help: Displays instructions on how to use the tool.

Finished Image: After drawing 0 or more noise ROIs, click Finished Image to save any ROIs and, in batch mode, proceed to the next image.

- Use ImageJ's **Freehand Selections** tool, which is automatically activated, to 'circle' some noise.





One ROI (magenta) circling noise.

- Click the **Finished Image** button

  • Automatically saves the ROI(s)

  • In batch mode, proceeds to the next image.

Click Cell Body Plugin

Purpose

Indicates the location of the neuron's cell body (soma). The location is used to automatically create the cell body ROI and in the Measure Skeleton plugin.

Input

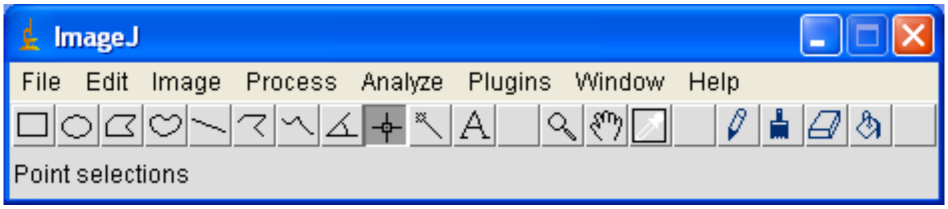
batch mode: neuron image folder via the Setup dialog box.
single mode: Open a neuron image.
also uses: secondary label image, if any.

Output

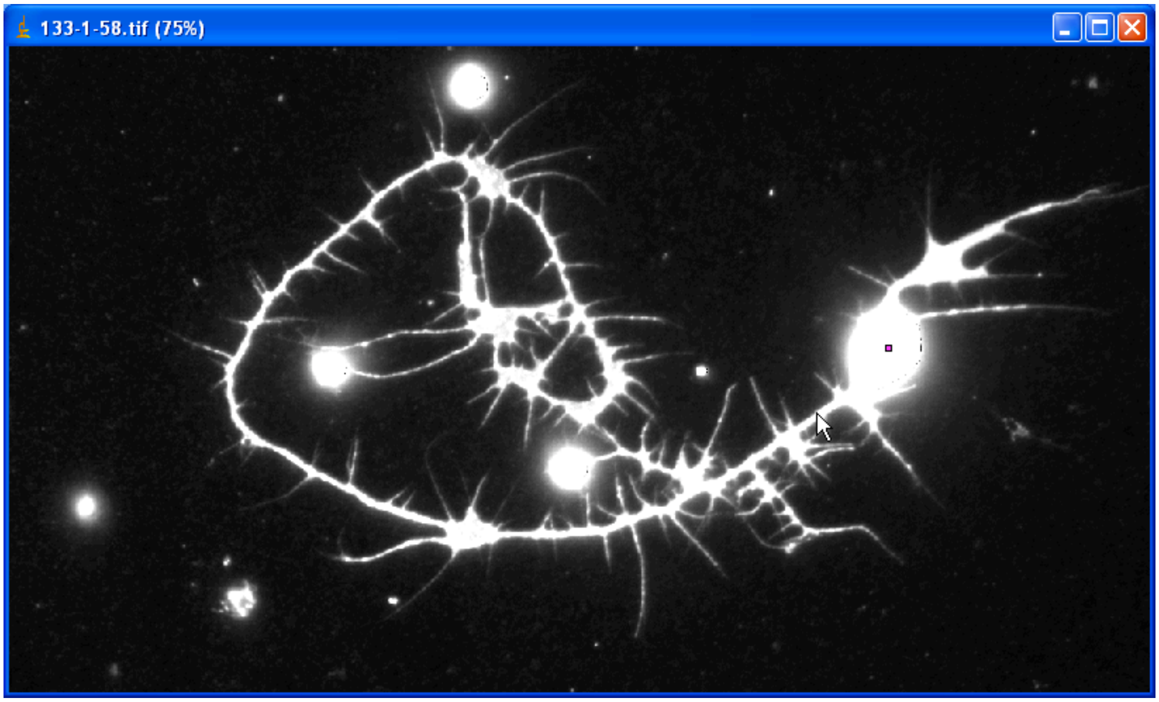
a point on the cell body
folder myExper_cellBodyPoints containing file myExper_cellBodyPoints.txt

How To Use

- Run by going to Plugins > NeuronMetrics > Click Cell Body.
  - The plugin automatically displays the neuron image and opens the secondary image if there is one.
- Click on the cell body (soma) of the neuron using ImageJ's **Point Selections** tool, which is automatically selected.



Point selections



A small point selection (magenta) marks the cell body.

- Press the spacebar.

  • Adds the point to the Results table.

  • In batch mode, proceeds to the next image.

  • Automatically saves the data in the Results table at the end of the batch or single image.

Cell Body ROI Plugin

Purpose

Automatically creates the cell body ROI which is used to locate the base of primary neurites so they may be counted. The ROI is also used to clear the cell body skeleton from the final skeleton so it is not included in the total length measurement.

Input

batch mode: neuron image folder via the Setup dialog box.
single mode: Open a neuron image.
also uses: cell body points, neuron ROI, noise ROIs (if any).

Output

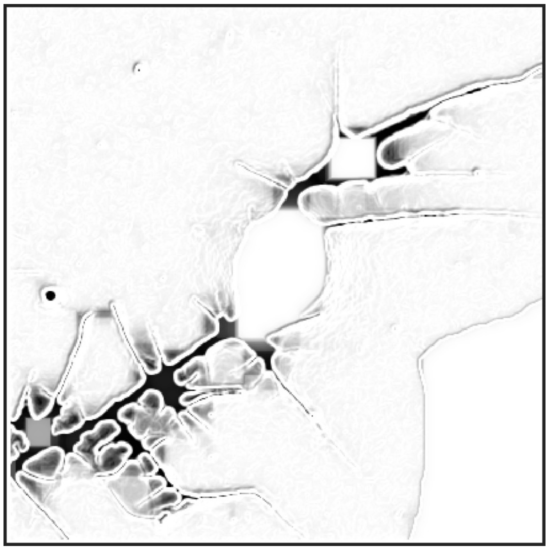
cell body ROI

- folder myExper_cellBodyROIs containing files image#cb.roi
- folder myExper_cellBodyROI_overlay containing files image#cbn.tif (recommended)

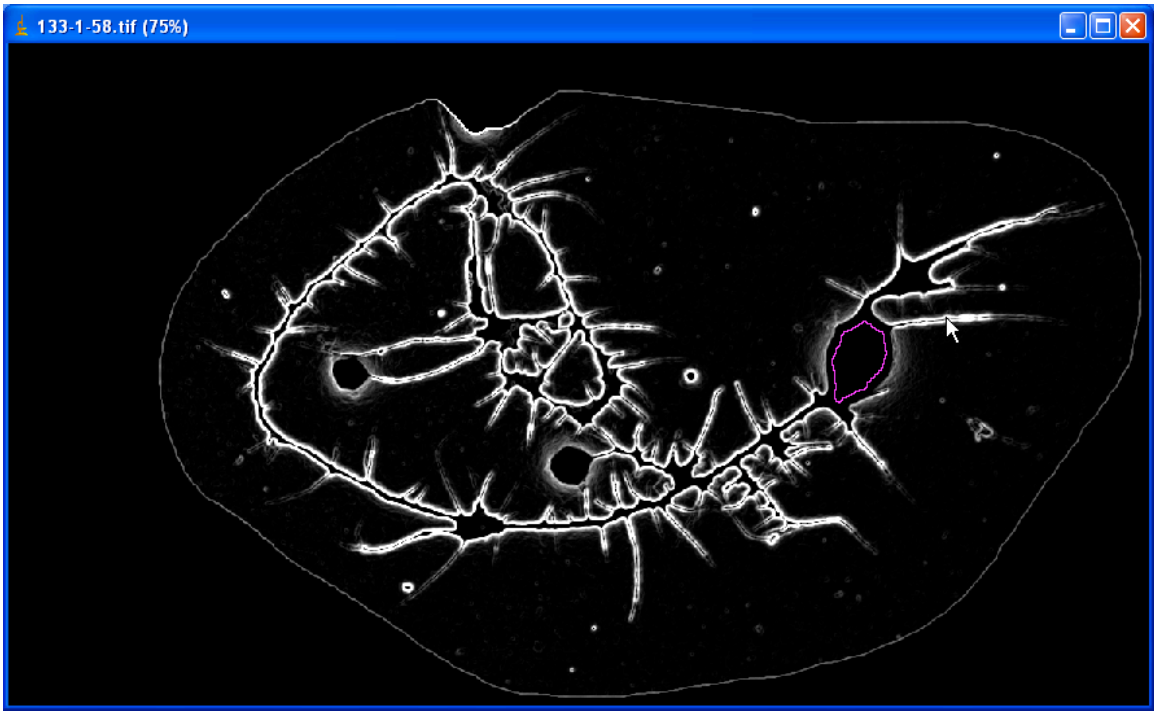
How To Use

◆ Run by going to Plugins > NeuronMetrics > Cell Body ROI.

- The plugin is fully automatic.
- Various stages of image processing will be displayed briefly including:



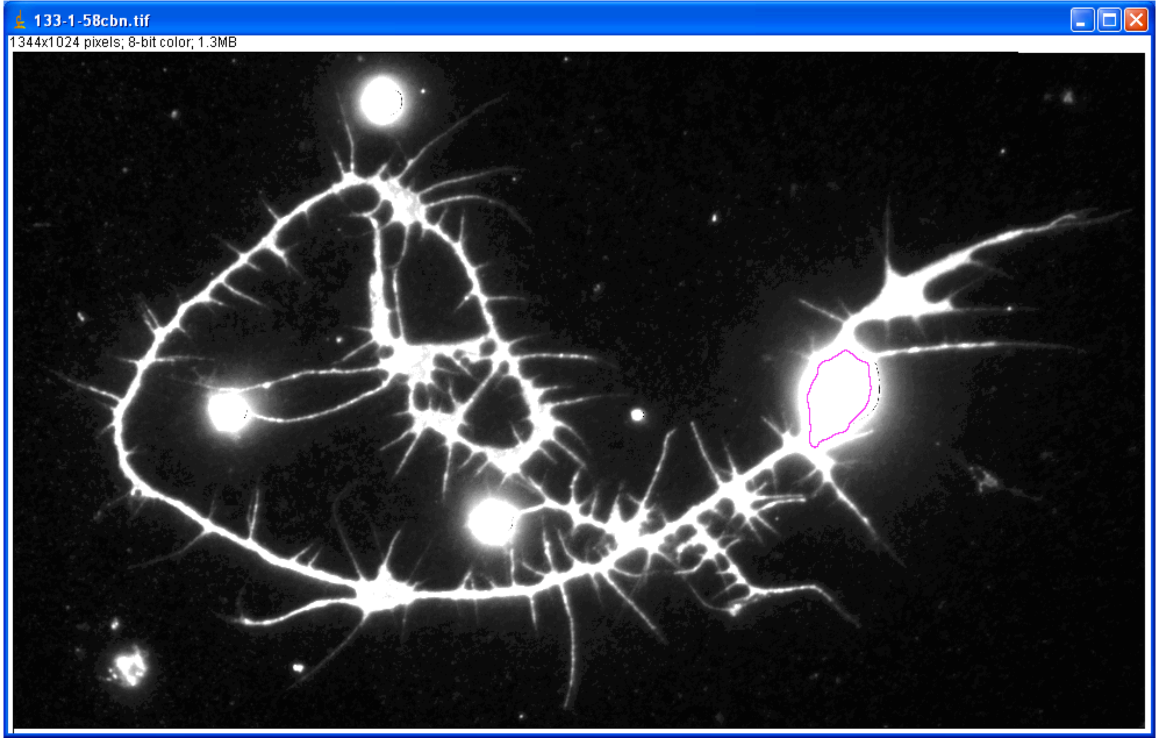
Edge detection and rolling ball background subtraction to enhance the cell body region.



The image with background cleared outside the neuron ROI, edge detection, rolling-ball processing, and the cell body ROI (magenta) created by ImageJ's Wand tool.

• Automatically saves the cell body ROI.

- After the batch or single neuron processing is complete, view the overlay(s) of the cell body ROI on the neuron to check for problems.



Cell body ROI overlaid on neuron.

Troubleshooting

- If a cell body ROI is not satisfactory, or an image is skipped (a message will appear in the Log window), or the Cell Body ROI plugin fails on an image in some other way, run the Cell Body Roi Manually plugin under Plugins > NM Tools to manually draw the cell body ROI.

- If you re-ran the Click Cell Body plugin, did you remember to manually update the .txt file containing the cell body point data?

Cell Body Roi Manually Plugin

(Optional)

Purpose

Facilitates manually creating the cell body ROI which is used to locate the base of primary neurites so they may be counted. The ROI is also used to clear the cell body skeleton from the final skeleton so it is not included in the total length measurement.

Input

batch mode: neuron image folder via the Setup dialog box.
single mode: Open a neuron image.
also uses: secondary image (if any).

Output

cell body ROI

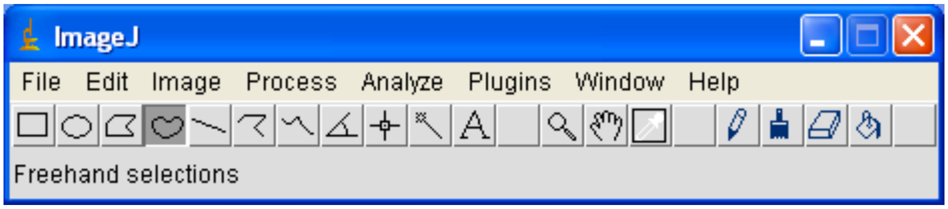
- folder myExper_cellBodyROIs containing files image#cb.roi
- folder myExper_cellBodyROI_overlay containing files image#cbn.tif (recommended)

How To Use

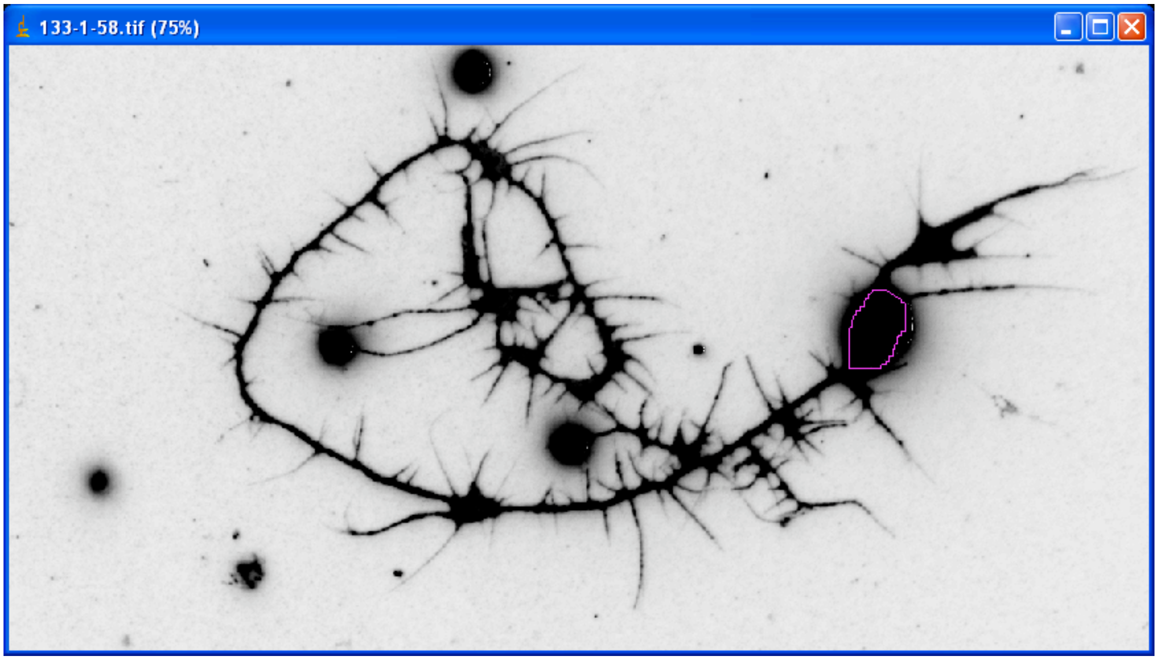
◆ Run by going to Plugins > NM Tools > Cell Body Roi Manually.

- The plugin automatically opens the neuron image and opens the secondary image if there is one.

◆ Draw a ROI slightly within the cell body (see example below) using ImageJ's Freehand Selections tool, which is automatically selected.



Freehand selections



Manually drawn cell body ROI.

- Press the spacebar.

  - Automatically saves the cell body ROI.

Skeletonize Neuron Plugin

Purpose

Creates a preliminary skeleton representing the neuron. This preliminary skeleton image is further processed by the Improve Skeleton plugin.

Input

batch mode: neuron image folder via the Setup dialog box.
single mode: Open a neuron image.
also uses: neuron ROI, noise ROIs (if any).

Output

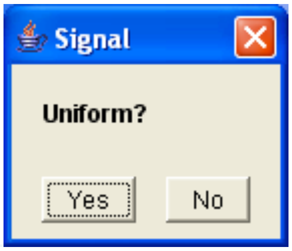
preliminary skeleton
- folder myExper_skels containing files image#s.tif

How To Use

◆ Set the Neuron Signal using the Setup dialog box if you have not already done so.

◆ Run by going to Plugins > NeuronMetrics > Skeletonize Neuron.

- If all images in the folder are being processed using the same skeletonization mode (all neurons have either uniform signal or all have non-uniform signal), the plugin requires no user input. It just runs.
- If some images in the folder need to be processed in uniform mode and some in non-uniform mode, the Neuron Signal should be set to “set individually” in the Setup dialog box. In this mode, a dialog Signal dialog box will appear during the processing of each image to let the user indicate the skeletonization mode to use.



- Various stages of image processing will be displayed briefly.
- The skeletonized image is automatically save.

Improve Skeleton

Purpose

Improves the preliminary skeleton by filling gaps and removing disconnected noise. This skeleton image is further processed by the Measure Skeleton plugin.

Input

batch mode: The preliminary skeleton image folder is automatically found based on the
Neuron Folder setting in the Setup dialog box.
single mode: Open a preliminary skeleton image.
also uses: corresponding Cell Body ROIs, neuron (if saving overlay).

Output

improved skeleton
folder myExper_improvedSkels containing files image#is.tif

How To Use

- Run by going to Plugins > NeuronMetrics > Improve Skeleton.

  - The plugin is fully automatic.
  - For several seconds nothing appears to be happening, but processing is occurring.
  - Various stages of image processing will be displayed briefly.
  - The improved skeletonized image is automatically save.

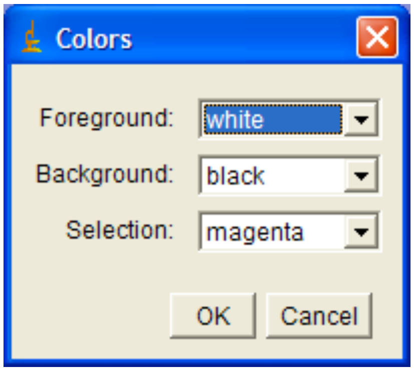
- Check the output from the overlay of the improved skeleton on the neuron (recommended).

Troubleshooting

If the skeleton is missing a significant portion of a neurite due to failure to fill one or more gaps, you may manually fill the gaps:

- Set the **foreground color** in ImageJ to **white** so the new pixels drawn to fill the gap will be white.

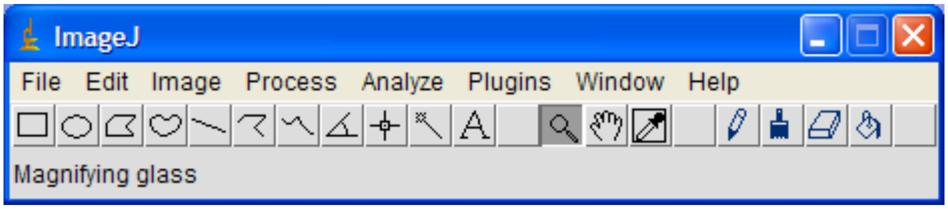
  Edit > Options > Colors...

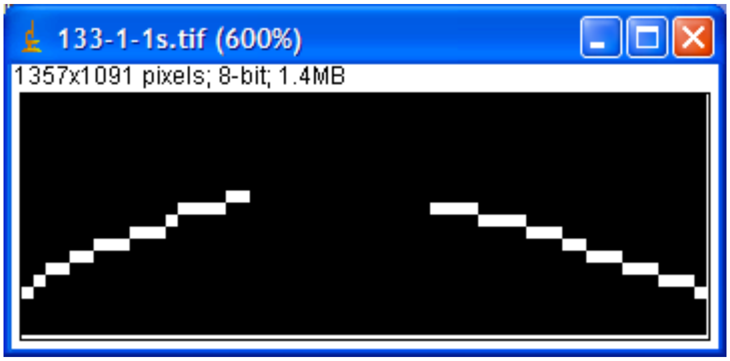


Foreground: white
Background: black
Selection: magenta

- Open the corresponding **preliminary skeleton** image in ImageJ.

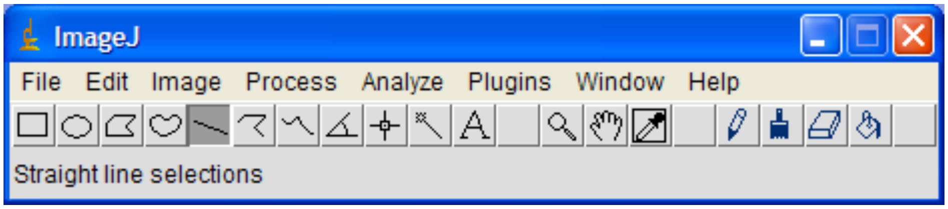
- Zoom in, using the magnifying glass tool, until the pixels are large enough for you to accurately draw a line from one pixel in the skeleton to another.

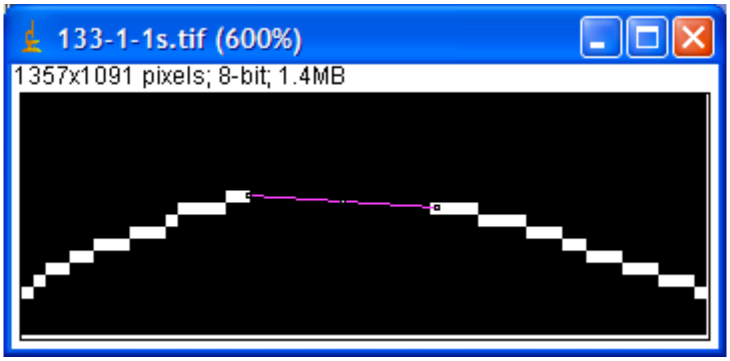




Zoomed in on the gap in the skeleton.

- Manually draw a straight line selection between two pixels to bridge the gap using the **Straight line selections** tool.

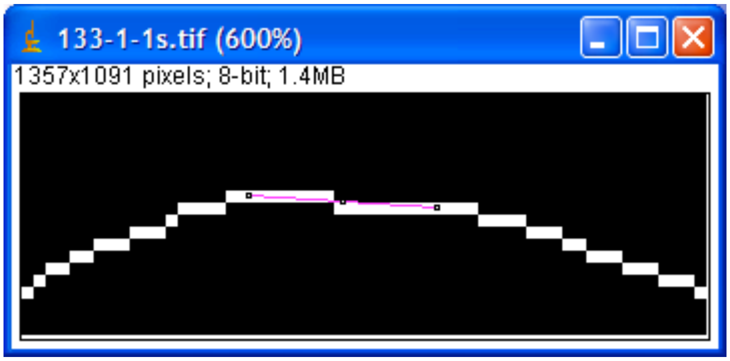




Straight line selection in magenta.

- Draw the selection to fill the gap with pixels.

  Edit > Draw



After drawing in white, the gap is filled.

Note: "Drawing" a straight line selection results in a 1-pixel-wide skeleton, as desired. Using other selection tools may not. If you use a different selection tool, you may have to re-skeletonize the image.

- Save the edited preliminary skeleton image, File > Save As > Tiff…

- Yes, replace the existing image.

- Run the edited preliminary skeleton image through the Improve Skeleton plugin in single mode.

  - Open the image.

  - Plugins > Neuron Metrics > Improve Skeleton

Length Correction Plugin
(Optional)

Purpose
Corrects the length in a region of self-fasciculation by drawing a free-form curve to indicate the length that needs to be added. The curve may be weighted. The length correction is used by the Dominant Neurite and Measure Skeleton Plugins.

Input
batch mode: The improved skeleton image folder is automatically found based on the
Neuron Folder setting in the Setup dialog box.
single mode: Open an improved skeleton image.
also uses: neuron images

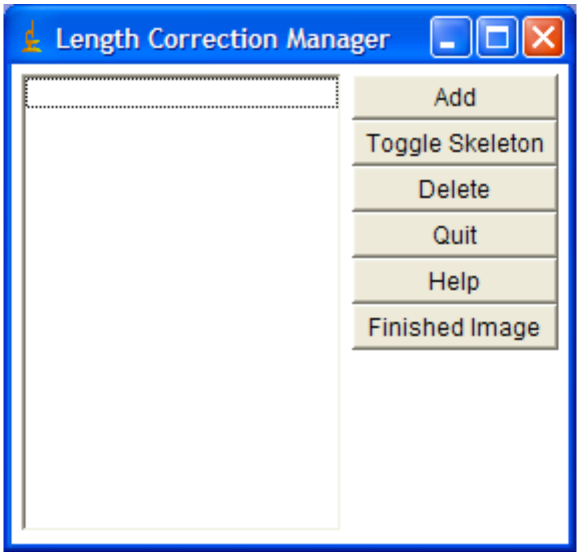
Output
Length correction data and images of the free-form lines that were drawn.

- folder myExper_lenCorrData containing myExper _lenCorrData.txt with the numeric length correction data.
- folder myExper_lenCorr containing image#lenCorr.tif (for each input image, one output image showing a skeletonized representation of the freeline objects the user drew).

How To Use

◆ Run by going to Plugins > NeuronMetrics > Length Correction.

The plugin displays the neuron image that corresponds to the input improved skeleton image and the Length Correction Manager window.



Add: After indicating a section of the neuron that needs length correction, which creates a selection object, click Add to place an ID number for the selection on the list.

Toggle Skeleton: alternately displays the neuron with or without the improved skeleton superimposed.

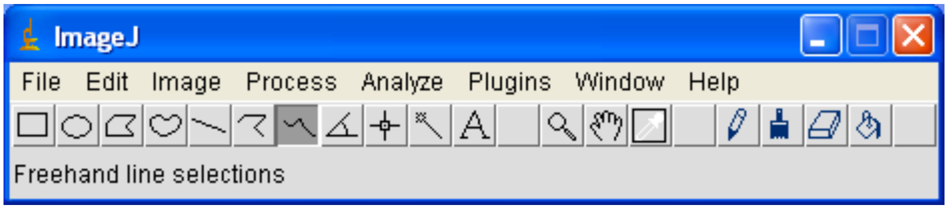
Delete: Select an item on the list. Note that the corresponding selection object is displayed on the neuron image. Click Delete to remove the item from the list.

Quit: Saves the data in the Length Correction Data window. Quits the tool and closes all associated windows. The selection images from images that were completely processed (the Finished Image button was clicked) before quitting have been saved already.

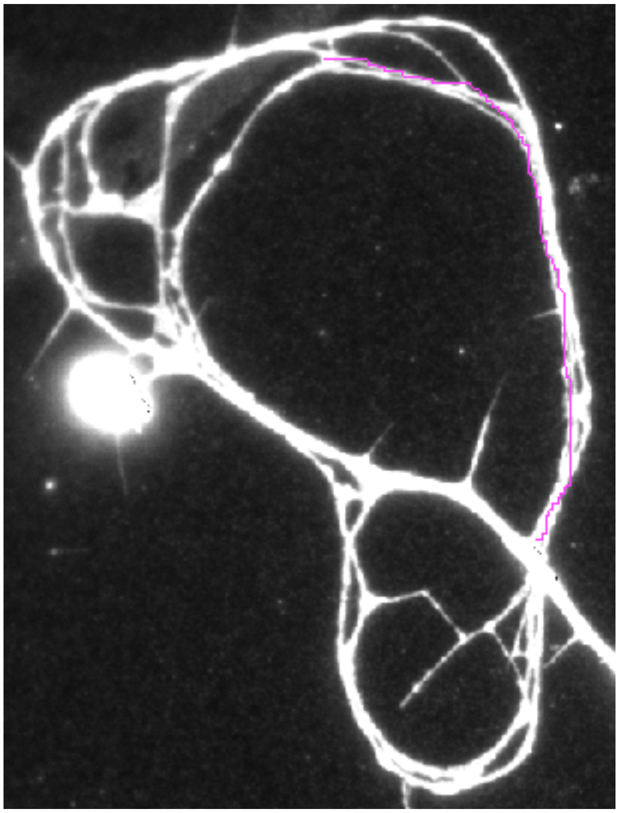
Help: Displays instructions on how to use the tool.

Finished Image: After drawing 0 or more selections, click Finished Image to save any selections, and to compute and write the length correction data to the data table. In batch mode, proceeds to the next image.

- Toggle the skeleton to see how the neuron was skeletonized.
- Draw a freehand line selection, the tool is automatically activated, to indicate the section of neurite for which length should be increased.

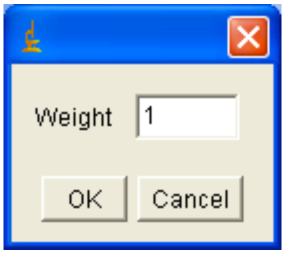


Freehand line selections

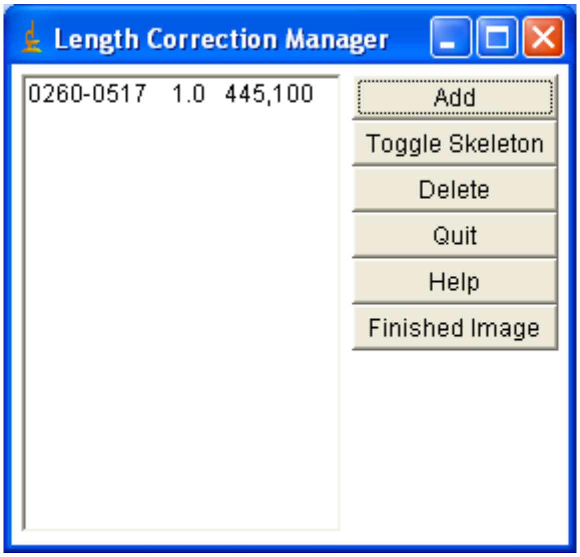


Freehand line selection (magenta)

- Click the Add button and a dialog box for weighting the line appears. Enter the weight. Fractional values may be used.


Weight 1

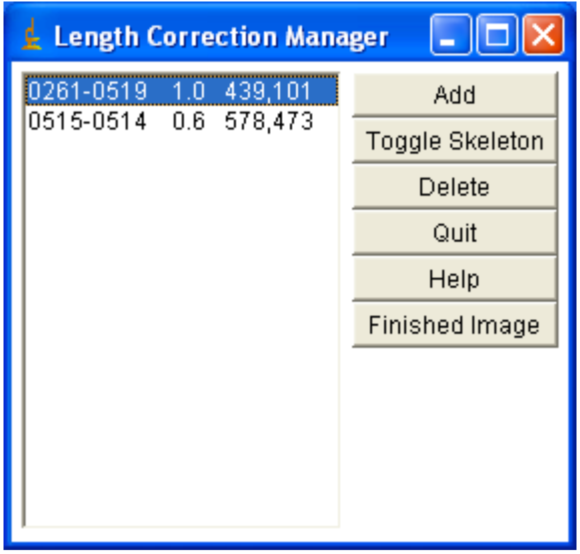
- When OK is clicked, the data appear in the list on the Length Correction Manager window.



```
0260-0517   1.0  445,100
```

Multiple selections may be made on a single neuron or a single, long selection, appropriately weighted, may be made.

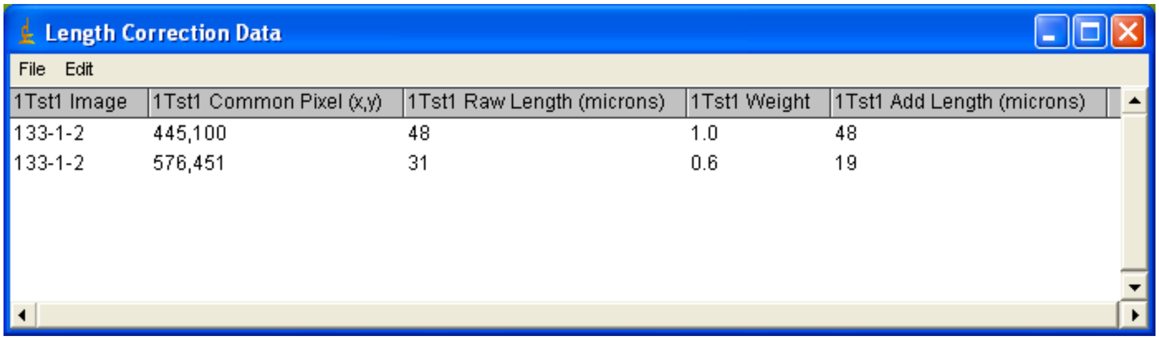
- To delete an item, select it in the list and click the Delete button.



```
0261-0519   1.0  439,101
0515-0514   0.6  578,473
```

- Click the Finished Image button when all corrections for the image have been made.

- The data are written to the Length Correction Data Window.



1Tst1 Image	1Tst1 Common Pixel (x,y)	1Tst1 Raw Length (microns)	1Tst1 Weight	1Tst1 Add Length (microns)
133-1-2	445,100	48	1.0	48
133-1-2	576,451	31	0.6	19

- In batch mode, proceeds to the next image.
- Data in this window are automatically saved as a .txt file at the end of a batch or single image.

See Narro et al. (2007) for additional information on Length Correction.

Dominant Neurite Plugin

(Optional)

Purpose

Identifies and isolates the dominant primary neurite in the improved skeleton image so its length may be computed. The length is used to calculate the Polarity Index when the Measure Skeleton plugin is run. If the skeletonized dominant neurite does not contact other neurites, it just needs to be identified. If it contacts other neurites, the skeleton needs to be disconnected in one or more locations to isolate the dominant neurite. If it is not obvious which neurite is dominant, multiple neurites may be identified and isolated. The plugin will determine which is longest.

Input

batch mode: The improved skeleton image folder is automatically found based on the Neuron Folder setting in the Setup dialog box.
single mode: Open an improved skeleton image.
also uses: neuron images, cell body ROI.

Output

Dominant neurite length data and images of the dominant neurite and edited skeleton.

- folder myExper_editedSkels containing edited skeleton images (image#es.tif) and a color version (image#ecs.tif).
- folder myExper_domProcSkels containing dominant neurite skeleton images (image#ds.tif) and a colored dominant neurite image (image#drs.tif).
- folder myExper_domProcData containing myExper_domLengths.txt.

How To Use

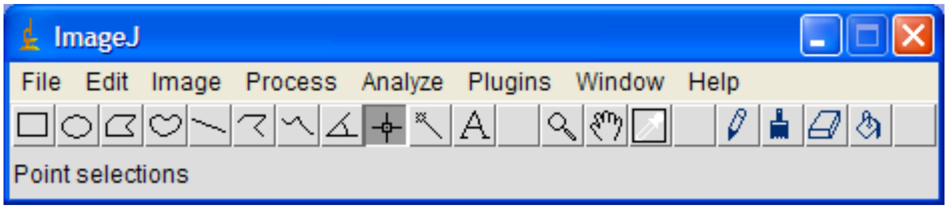
The Basic Steps are:

1) 'Indicate' a neurite that is, or may be, the dominant one.
2) Make breaks in the skeleton if that process contacts other processes.
3) Preview your work to verify that the process is isolated.
4) Repeat for as many process as necessary.
5) Click 'Finished Image' when you are finished with the image.

Details are provided below. The breaks in a process are not finalized until you either indicate another process or click the 'Finished Image' button. After 'Finished Image' is clicked, the length of the dominant neurite is computed and written to a data window, and skeleton images are saved. The length data are saved to a .txt file at the end of a batch (or image if in single mode).

Making Breaks

With the cross-hair tool (point selections) selected,



click NEAR the skeleton where you want to break it. The tool will find the NEAREST skeleton pixel and make the break there. The clicks are marked on the image and their x,y coordinates are added to the list in the Skeleton Breaker window. There are 3 types of breaks:

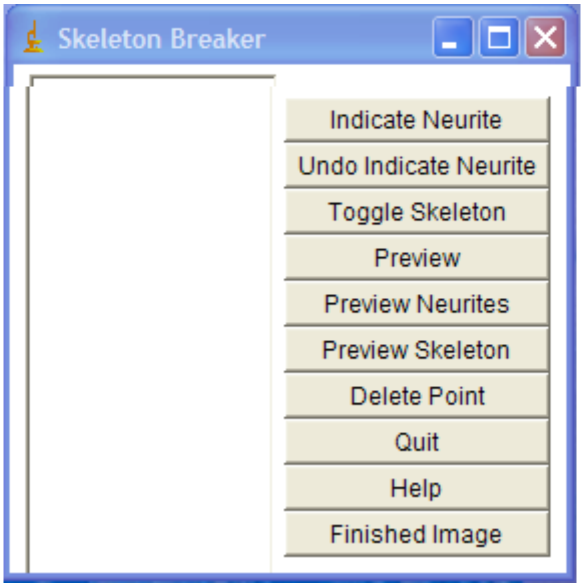
Simple (click): Clicking near the skeleton results in a 'simple' break of 3 pixels centered on the skeleton pixel closest to the click point. A simple break is appropriate when two branch tips touch each other.

Trimmed (t + click): Clicking while holding down the ' t ' key results in a 'trimmed' break in which skeleton pixels are removed from the click site to the nearest branch point in a manner that ensures no small branch stub remains. A trimmed break is appropriate when a tip touches somewhere along another process.

Crossover (c + click): Like a trim, but used when there is a crossover. The difference is that the pixels are NOT permanently erased thereby allowing the user to edit the other process involved in the crossover if necessary.

- Run by going to Plugins > NeuronMetrics > Dominant Neurite.

  The plugin displays the Skeleton Breaker window and the neuron image that corresponds to the input improved skeleton image.



Indicate Neurite: Each time you want to indicate that a primary neurite is or may be the longest, press the 'Indicate Neurite' button. Click on the desired primary neurite. Its skeleton will appear. If the neurite of interest contacts other neurites, the skeleton extends into those neurites and you need to click to indicate break points (see Making Breaks above). When you have isolated the process, either click 'Indicate Neurite' again if there are additional candidates for dominant neurite, or click the 'Finished Image' button.

Undo Indicate Neurite: Used in the event of a mistake.

Toggle Skeleton: Causes skeleton to disappear/reappear to provide an unobstructed view of the neuron. The skeleton must be visible when clicking to indicate break points.

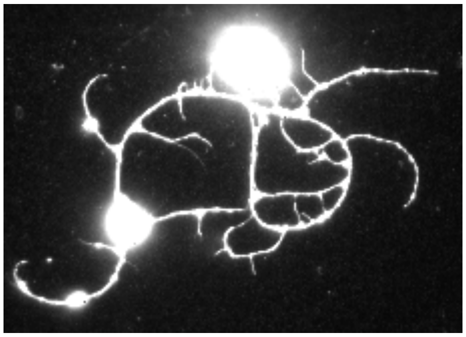
Preview: Displays the skeleton for the current process taking the breaks into account. Useful for determining if the process is isolated.
Preview Neurites: Displays the skeleton for all processes worked on thus far, taking breaks into account. Each process is a different color.
Preview Skeleton: Similar to 'Preview Neurites' but also displays the unprocessed parts of the skeleton.

Delete Point: Used in the event of a mistake. Removes a break point from the break list. In the list, select (highlight) the point(s) to be removed, then click 'Delete Point'. In the image, deleted points are re-colored gray. If necessary, determine the x,y coordinates of the point to be deleted by mousing over it in the image. Use the x,y coordinates to locate the point in the list.

Quit: Quits a batch. Data for the current image are NOT saved. Other images and length data are saved.

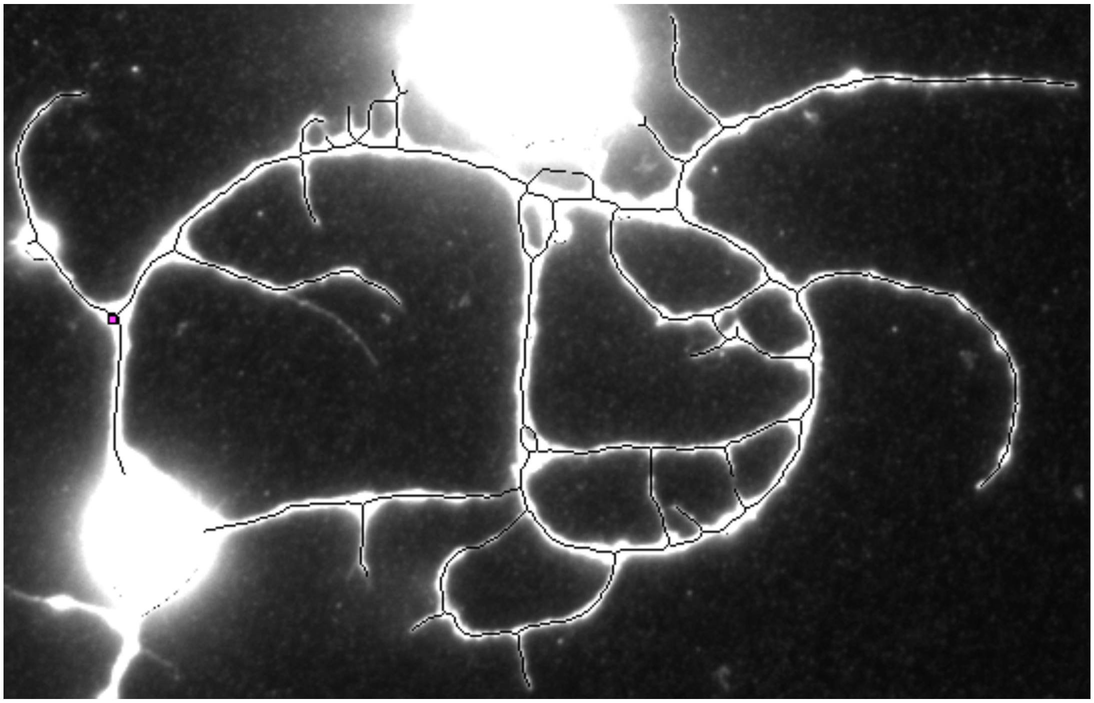
Help: Displays this set of instructions.

Finished Image: Finalizes the breaks in the last neurite edited, computes the length of the longest neurite. The length of the longest neurite is written to the data window and skeleton images are saved.



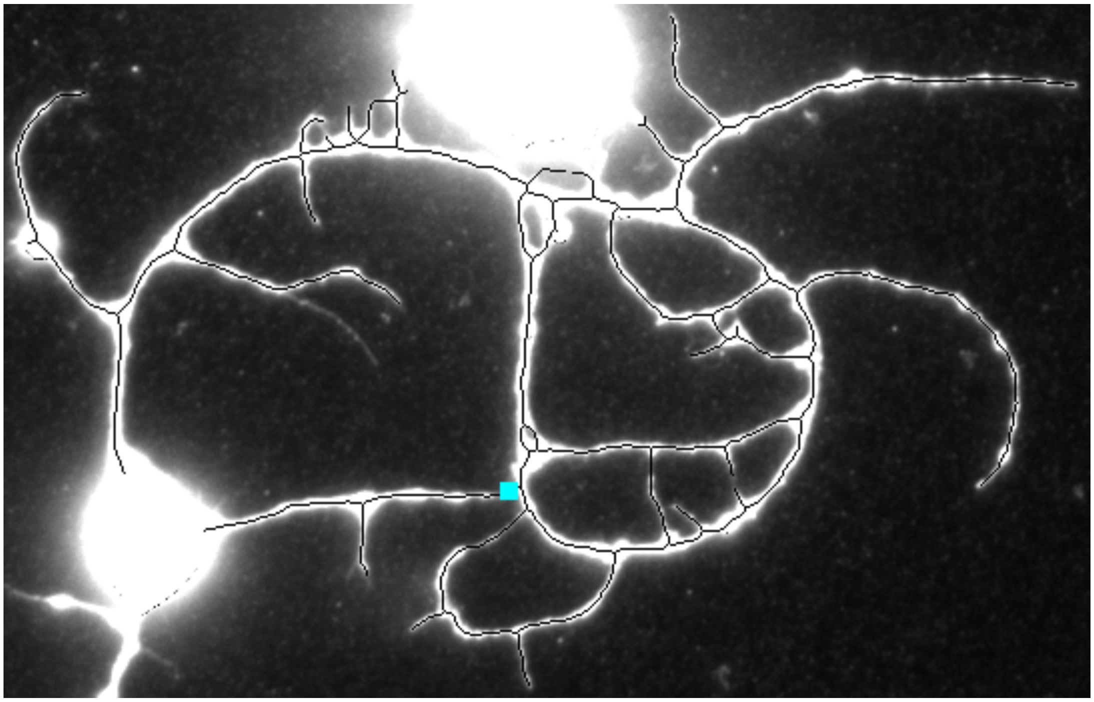
The neuron image.

- Press the Indicate Neurite button.

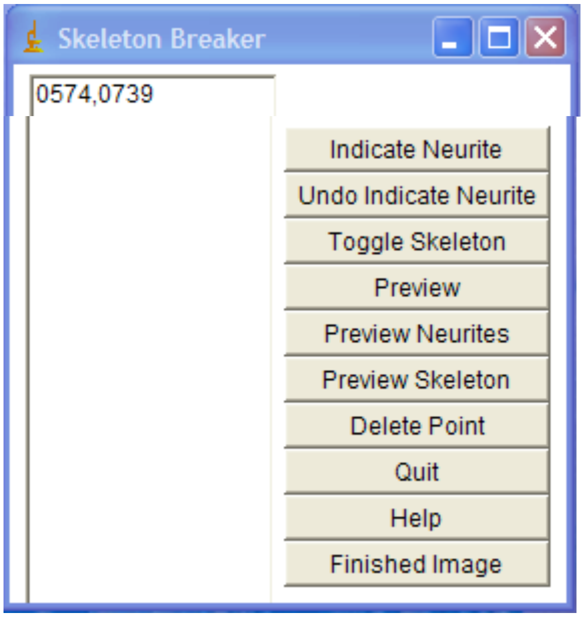


After pressing Indicate Neurite and clicking on dominant primary neurite (small magenta square), the skeleton appears.

- Toggle the skeleton, if necessary, to see an unobstructed view of the neuron.
- Click near a location where the skeleton needs to be broken.



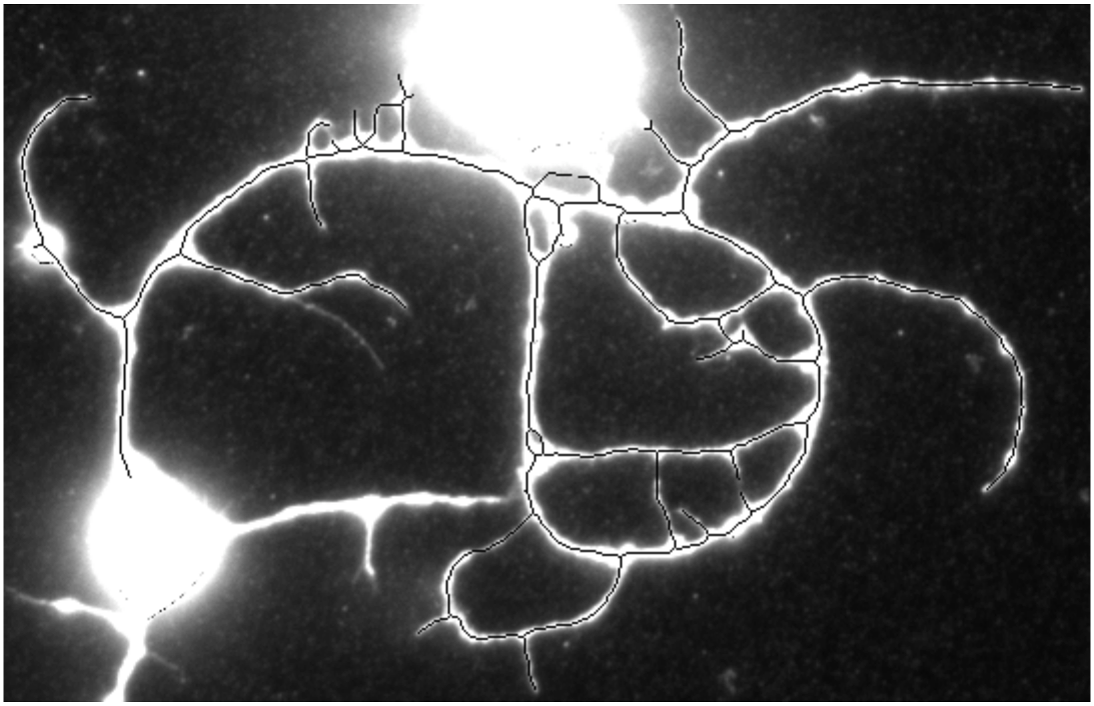
A cyan square marks the location of the click.



0574,0739

The break point’s x,y coordinates are added to the list.

- Press the Preview button to see if the neurite is now isolated.

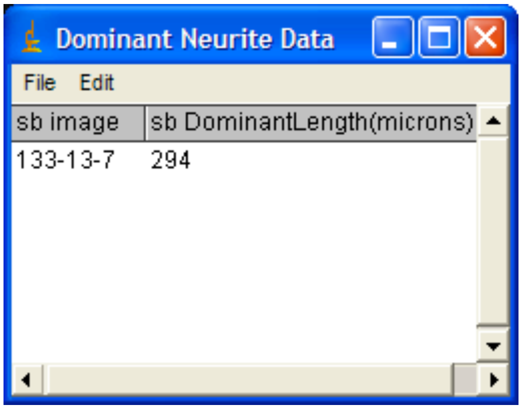


It is clear this dominant neurite has been isolated.

- If the neurite is not yet isolated, **close the preview window** and continue making breaks and checking the preview image until the neurite is isolated.

- Press the Finished Image button.

- The length of the dominant neurite is written to the data window.



sb image	sb DominantLength(microns)
133-13-7	294

- Images of the dominant neurite and edited skeleton are saved.

- In batch mode, proceeds to the next image.

- The data in the Dominant Neurite Data window are automatically saved at the end of a batch or single image.

Troubleshooting

- If the plugin dies in the middle of a batch, you must MANUALLY SAVE the length data to a .txt file using 'File > Save As' in the data window.

- Pay attention to which window is active, the image window or the 'Skeleton Breaker' window. The spacebar repeats the action of the last button clicked as long as the Skeleton Breaker window is selected (instead of the image window). This is a built-in feature that could not be disabled, not a bug.

- If images are large, ImageJ may run out of memory if more than one preview image is open at a time. (Problems can occur with a 5.2-MB image and 300 MB of memory allocated for ImageJ. However with 500 MB of memory for ImageJ and a 5.9-MB image, all preview images can be open at the same time.) Allocating memory for ImageJ is discussed under Installation.

Measure Skeleton Plugin

Purpose

Finalizes the skeleton and uses it to compute the quantitative data. If computing the territory is checked in the Setup dialog box, the Polygon plugin is also run. The skeleton is finalized by erasing primary neurites that are too short (see Setup > Advanced... > Primary Neurites > Length Threshold) and removing the skeleton inside the cell body ROI. The data computed are total length, polarity index (optional), # primary processes, total branch count, and polygon data (optional).

Input

batch mode: The improved skeleton image folder is automatically found based on the Neuron Folder setting in the Setup dialog box.
single mode: Open an improved skeleton image.
also uses: cell body points (.txt),corresponding Cell Body ROIs (if present), neuron (if saving overlay).

Output

Skeleton data (
Polygon data (area and perimeter) and images of the polygons

- folder myExper_finalSkels containing files image#fs.tif,
- folder myExper_dat containing myExper_skelData.txt and, if polygon was run, myExper_allData.txt which combines the data from Polygon and Measure Skeleton into one .txt file.
- folder myExper_ overlaysAll containing files image#_overlayAll.tif (recommended)

How To Use

◆ Run by going to Plugins > NeuronMetrics > Polygon.
  - Fully automatic.
  - Various stages of image processing will be displayed briefly.
  - The data and images are automatically saved.
  - Check overlay of all features computed.

Polygon Plugin

Purpose

Computes the convex hull polygon which represents the territory occupied by the skeletonized neuron and the cell body ROI. Also computes the area and perimeter of the polygon.

Input

batch mode: The final skeleton image folder is automatically found based on the Neuron Folder setting in the Setup dialog box.
single mode: Open a final skeleton image.
also uses: corresponding Cell Body ROIs (if present), neuron (if saving overlay).

Output

Polygon data (area and perimeter) and images of the polygons

- folder myExper_polys containing files image#p.tif
- folder myExper_dat containing file myExper_polyData.txt

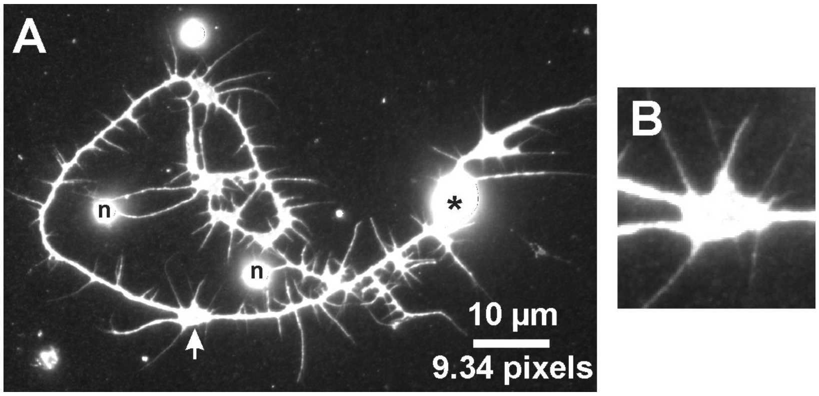
How To Use

◆ Run by going to Plugins > NeuronMetrics > Polygon.
  - Fully automatic.
  - Various stages of image processing will be displayed briefly.
  - The data and polygon images are automatically saved.

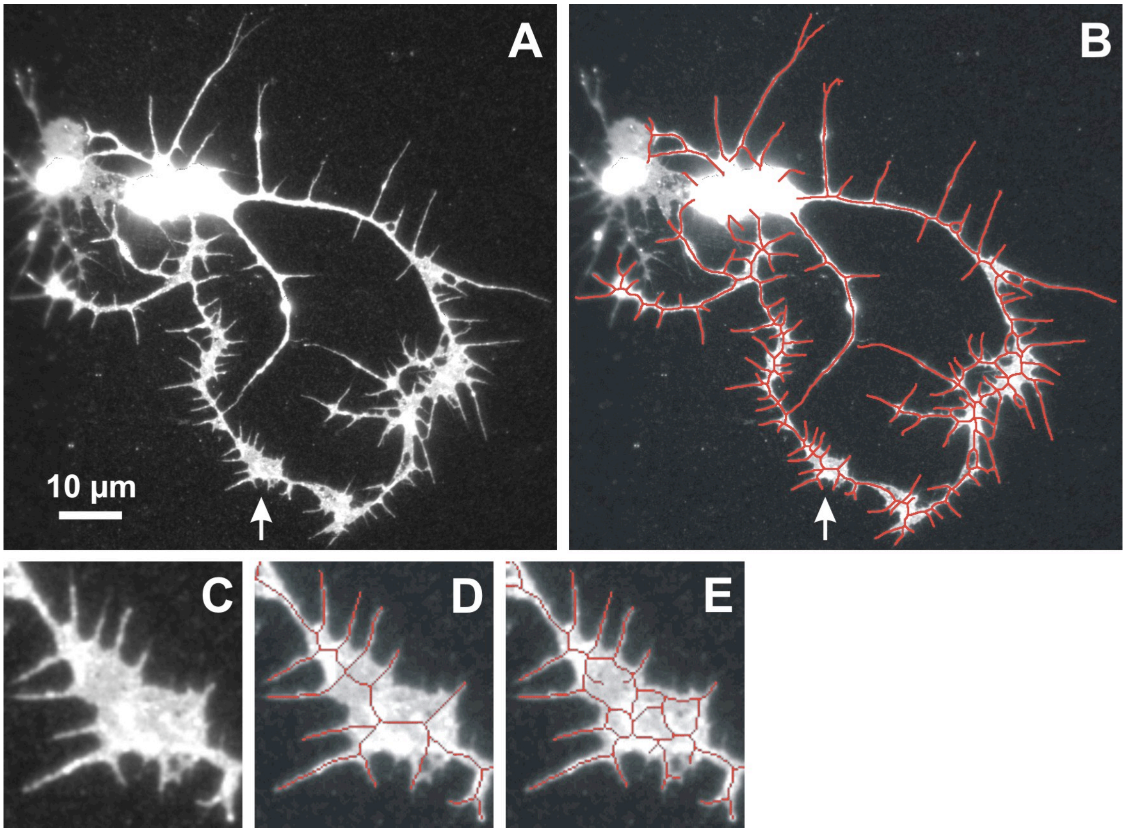
Explanations

Uniform Versus Non-uniform Neuron Signal

Skeletonization may be done in one of two modes depending on whether the signal in the neuron is uniform throughout or is non-uniform. Examples are show below.



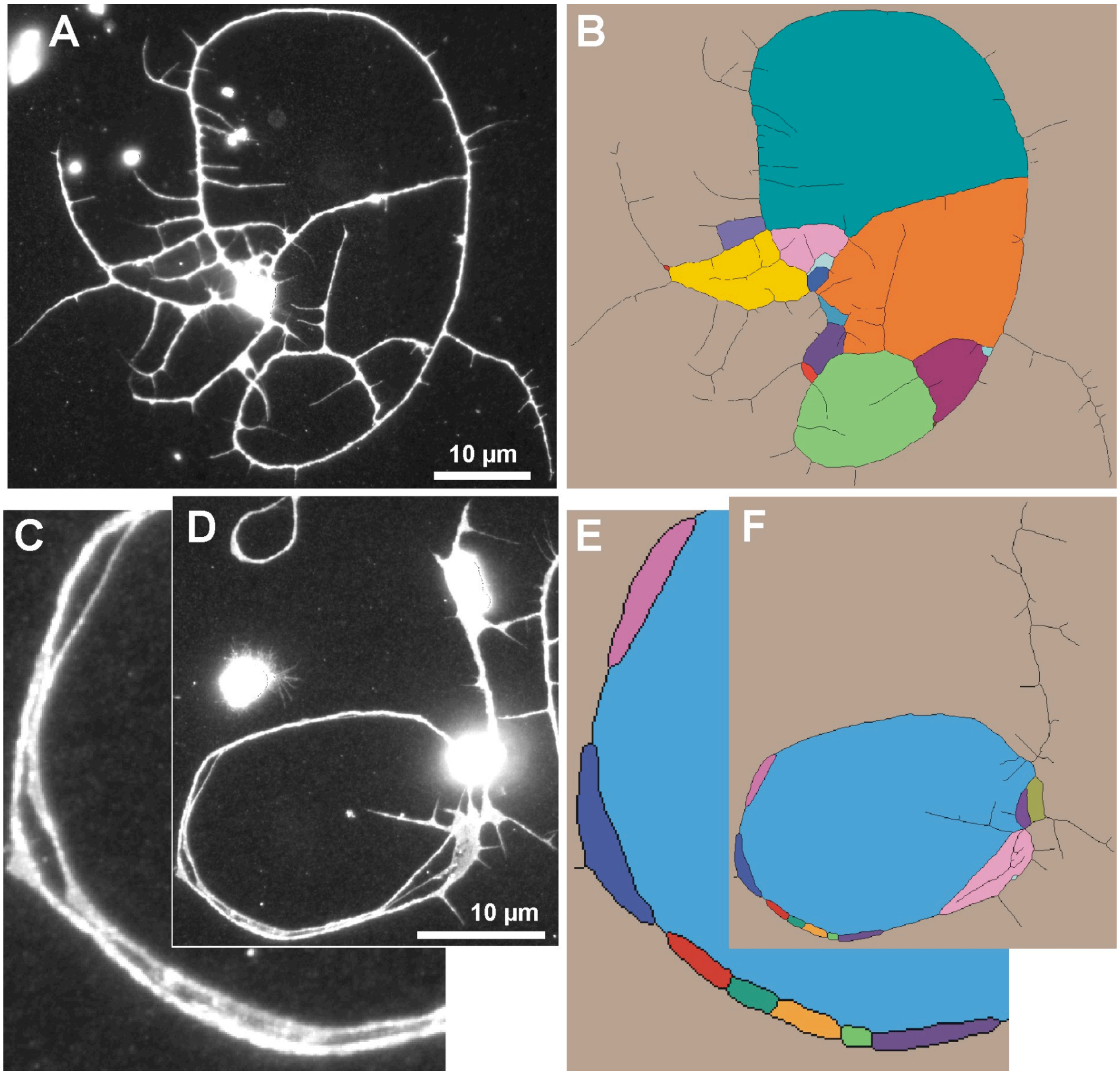
Example of uniform signal. (A) Neuron with high, uniform signal throughout. (B) Enlargement of a broad region with uniform signal (see arrow in A). Adapted from Fig. 1 of Narro et al. (2007).



Example of non-uniform signal. (A) Neuron with non-uniform signal. (B) Skeleton, created by processing in non-uniform mode, overlaid on neuron. (C) Enlargement of broad region with non-uniform signal. (D) Skeleton created by processing in non-uniform mode. (E) Skeleton created by *erroneously* skeletonizing in uniform mode is a poor representation of the region. Adapted from Fig. 5 of Narro et al. (2007).

Faces

Faces are the regions of the image that are enclosed when neurites contact one another. For an explanation of how they are used to correct the branch count, see Narro et al. (2007).



Examples of neurons with numerous inter-neurite contacts and the corresponding skeletonized images in which each face region is differentially colored. Reproduced from Fig. 6 of Narro et al., 2007.

Polarity Index

The polarity index is defined as the percentage of total length contributed by the primary neurite with the greatest combined length of its trunk and arbor (Kraft et al., 1998).

Tips

How to View a Set of Images

There are software tools that enable you to view a set of images. Such tools have forward and backward arrows to facilitate rapid viewing of all images in the folder.

On a Windows computer use:
- Microsoft Office Picture Manager
- Windows Picture and Fax Viewer

Update .txt files

- If you re-run a plugin that generates a text file, the .txt files are auto-incremented by appending $1, $2, etc. to the end of the file name so data are not lost. However, plugins use the text file with a name that does NOT have $# appended to it (i.e., the first file created). Thus, if you re-run a plugin that generates a .txt file, you <u>must</u> edit the FIRST file created to add updated data to it, <u>or</u> you <u>must</u> rename the correct text file by deleting the $# from its name. If you do not, downstream plugins that depend on the data in the .txt file will skip or fail on the images for which data cannot be found.

- NEVER open a .txt file in Microsoft Word. Word inserts all sorts of hidden things in files and you may have trouble getting rid of them. Such hidden delights may cause problems when a NeuronMetrics plugin tries to read the .txt file.

- Use a text editor such as WordPad to open, edit, and save a .txt file.

Do Not Multi-task

To avoid problems, do not use any other applications while running NeuronMetrics. Do not move the mouse around except to provide user input that NeuronMetrics needs. This means do not read e-mail, read electronic journal articles, or work on some document. NeuronMetrics seems to get confused about which window is active.

About .roi Files

The file extension .roi is used for files that store information about a region of interest (ROI), also referred to as a "selection". In ImageJ, if such a file is opened directly, the ROI will be visible. Its shape will be informative, but not its location. If instead an image file is open first, say the image on which the ROI was created, and then the .roi file is opened, the ROI will display in the location on which it was created. A .roi file can be opened on any image having the same dimensions as the image on which the ROI was created. In other words, a ROI that was created on a neuron image and saved, can be opened on the corresponding skeleton file.

References

Kraft R, RB Levine, and LL Restifo (1998). The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. *J Neurosci* 18:8886-8899.

Narro ML, F Yang, R Kraft, C Wenk, A Efrat and LL Restifo (2007). NeuronMetrics: software for semi-automated processing of cultured-neuron images. *Brain Research* 1138:57-75.