AutoNeuriteJ detailed protocol

This protocol details how to use AutoNeuriteJ, and tips for parameters settings and for Results analysis with a spreasheet (e.g. Excel).

Last-updated version of AutoNeuriteJ are posted on Github: https://github.com/Grenoble-Institute-Neurosciences/AutoNeuriteJ

Installation

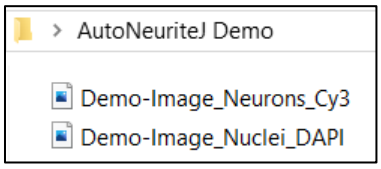
1) Download the folder from GitHub and unzip: You should get the files AutoNeuriteJ.ijm ; neurons.lut ; Demo-Image_Neurons_Cy3.tif and Demo-Image_Nuclei_DAPI.tif. Readme.md

2) Copy the text file called "neurons.lut" in the "Luts" folder of Fiji/ImageJ.

3) Copy the Image of neurons and nuclei in a single folder. The channels for neurons and nuclei have to be in two separated images. For your experiments use low density neuron cultures.

- Neuron staining (eg : Tubulin immunolabelling, membrane staining)
- Nucleus staining (eg : DAPI, Hoescht)

These images must be present in the same folder as follow :



4) Three other ImageJ/Fiji plugins are requested by the macro that can be installed using Fiji Update:

- The plugin "Analyze Skeleton 2D/3D" Developed by Ignacio Arganda-Carreras http://imagej.net/AnalyzeSkeleton (By default in Fiji)
- The plugin "MorphoLibJ" Developed by David Legland and Ignacio Arganda-Carreras https://imagej.net/MorphoLibJ (in Fiji : check "IJBP-Plugins" in the Update site window).
- The plugin "Morphology" from Gabriel Landini's website : http://www.mecourse.com/landinig/software/software.html (in Fiji : check "Morphology" in the Update site window).

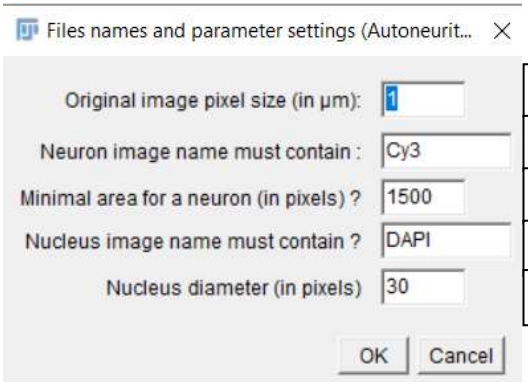
AutoNeuriteJ part I

1) Before running the macro:

- Measure the average diameter of nucleus (in pixels).
- Check the pixel size of your images (in µm).

2) Drag and Drop AutoNeuriteJ.ijm on Fiji and Start the macro

3) Run AutoNeuriteJ part I and set the parameters:

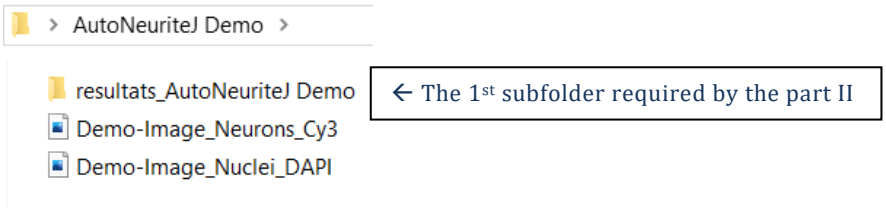


← Original image pixel size will rescale the image at 1µm/pixel
← Track the keyword "Cy3" in filenames to assign the neuron image
← Will exclude neurons if their surface is below 1500 pixels^2
← Track the keyword "DAPI" in filenames to assign nuclei image
← Used to set the size of the Gaussian filter used for segmentation

4) Set the threshold for neurons and nuclei segmentation.

For neurons we recommend to set the minimum threshold to 0 and increase it to the minimal value to avoid exclusion of thin neurites with low intensity of fluorescence. Check the continuity of neurites and record the values to set the same on other same day images.

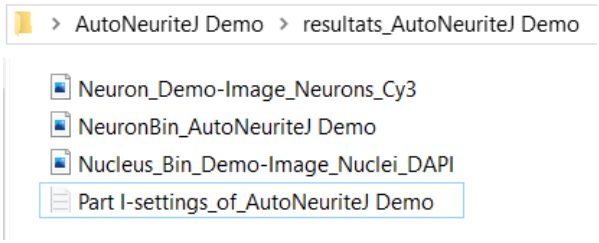
The macro creates a subfolder inside the folder containing your original images.



← The 1st subfolder required by the part II

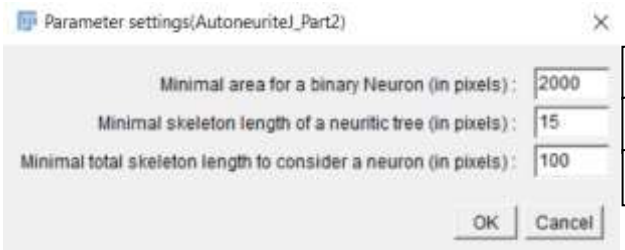
Inside this 1st subfolder you will find:

- Binary images of neurons and nuclei scaled at 1µm/pixel
- The original image of neurons rescale at 1µm/pixel
- Text file of parameters used in AutoNeuriteJ part I



AutoNeuriteJ part II

1) Run AutoNeuriteJ part II and set the parameters:



← Should be the same as part I or a bit more stringent
← Will remove very small skeletons
← Will exclude cells with too small protrusion (eg: round cells)

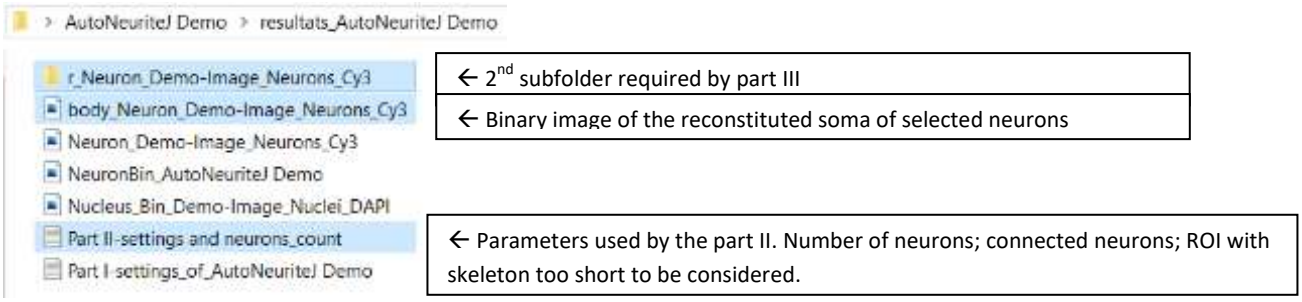
2) Declare how many conditions you want to analyze and choose the corresponding subfolders.

For parts II and III since they need less user input and are longer to process the macro will ask how many conditions you have to analyze. The number of conditions is the number of images the macro will automatically process.

AutoNeuriteJ part II runs until the Log window pops up and shows the following message:

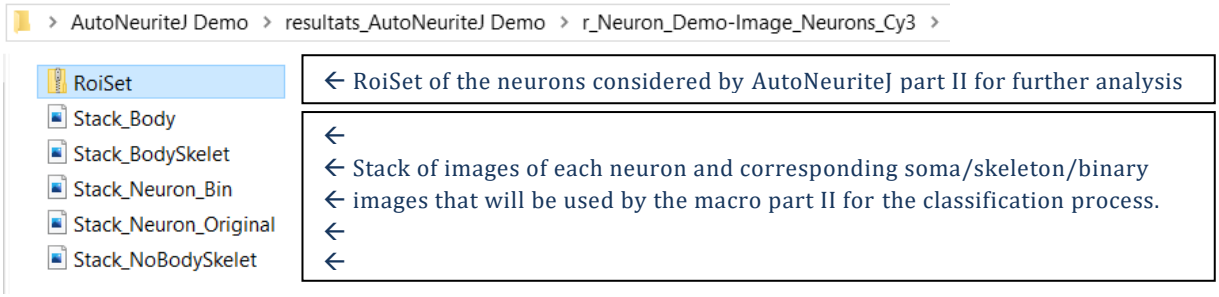
- Program ran for : “time spend by the macro part II”

Two files and a 2nd subfolder are created:



The “Part II-settings and neurons_count” text file can be used as a first control of your analysis. The most important is to check if the proportion of connected vs isolated neurons is consistent between the different conditions you want to compare. The connected neurons are excluded of AutoNeuriteJ analysis and could constitute a bias since the longest neurons are more likely to be connected to each other.

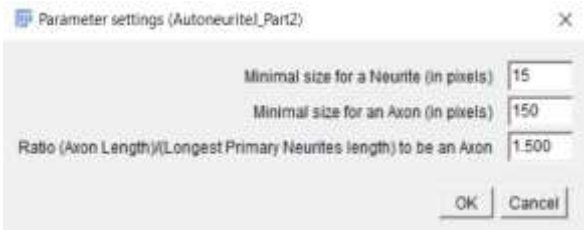
Inside the 2nd subfolder you will find:



The RoiSet can be open in ImageJ and apply to the neuron image (e.g. Neuron_Demo_Images_Neurons_Cy3) showing the neurons considered for analysis. Furthermore ROIs in the RoiSet are in the same order than individual neurons in the Stacks and Results file given by AutoNeuriteJ part III.

AutoNeuriteJ part III

1) Run the part III, set the number of conditions to analyze and set the parameters:



← Will exclude very small neurites created by the skeletonization

← Neurites shorter tahn this size will not be considered as axon

← An axon should be at least 1.5 time longer than the longest neurite.

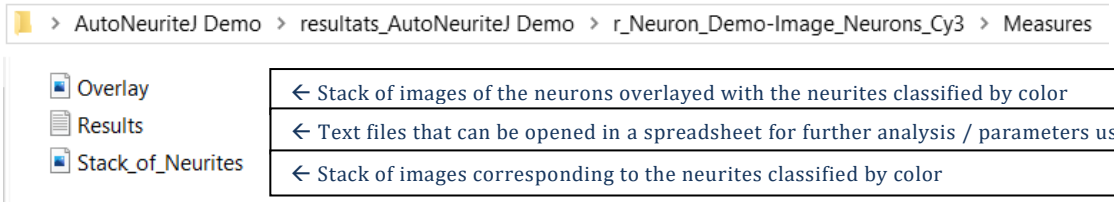
At start a "Log" window will open with the different quantification already done by the macro.

Let the macro proceed until the following message pops up:

- Program ran for : “time spend by the macro part III”

Inside the 2nd subfolder a 3rd subfolder is created:

Inside the “Measures” folder you will find the results of your analysis:



← Stack of images of the neurons overlayed with the neurites classified by color

← Text files that can be opened in a spreadsheet for further analysis / parameters used for macro part

← Stack of images corresponding to the neurites classified by color

The color code is set by the Neuron.lut file as follow:

- Red → Axon
- Green → Primary neurite
- Yellow → Secondary neurite
- Purple → Tertiary neurite and more

This overlay stack can be used for visual control of the final rendering of AutoNeuriteJ.

Analysis of results with Excel

Results files open in Excel will be as follow:

	A	B	C	D	E	F	G	H
4	AutoNeuriteJ_Part III_settings :							
5	minimal size of Axons (in pixels)=150							
6	minimal size of Neurites (in pixels)=15							
7	Axonal size ratio=1.5							
8	Neurite Order : 1=axon / 2=primary neurite / 3=secondary neurite / 4=tertiary neurite ...							
9								
10	Measures :							
11	Neuron#	Primary Neurites #	Neurite Order	Primary Neurites mean Length	Neurite Length	Longest Neurite	Axonal Tree length	Axonal Tree Branches #
12	1	2	1	152.3823	214.8234	214.8234		
13	1	2	3	152.3823	41.3848			
14	1	2	2	152.3823	89.9411			
15	1						257.6224	1
16	2	5	1	178.2612	468.1737	468.1737		
17	2	5	2	178.2612	81.8406			
18	2	5	2	178.2612	40.4558			
19	2	5	3	178.2612	55.3848			
20	2	5	3	178.2612	20.7279			
21	2	5	2	178.2612	219.1665			
22	2	5	2	178.2612	81.669			
23	2						468.1737	0

Each line corresponds to one neurite of a given neuron.

- Neuron # (neuron number)
- Primary Neurites # (number of primary neurites for a given neuron)
- Neurite Order (1=Axon ; 2=Primary; 3=Secondary etc…)
- Primary Neurites mean Length : Mean length of primary neurites except the axon (the same for every neurite of the same neuron).
- Neurite Length.
- Longest Neurite : Length of the longest neurite of the neuron. Filled at the line corresponding to the longest neurite of the neuron.
- Axonal Tree Length and Axonal Tree Branches : These columns are filled if the neuron present an axon. Thus longest neurite order should be set at 1.

Tips:

The longest neurite length is filled in the “Longest neurite Length” column. By applying filter on the column you can display only one line per neuron with the principal data. The filters can be used to get the axonal tree length or axon length.

	A	B	C	D	E	F	G	H
4	AutoNeuriteJ_Part III_settings :							
5	minimal size of Axons (in pixels)=150							
6	minimal size of Neurites (in pixels)=15							
7	Axonal size ratio=1.5							
8	Neurite Order : 1=axon / 2=primary neurite / 3=secondary neurite / 4=tertiary neurite ...							
9								
10	Measures :							
11	Neuror	Primary Neurites	Neurite Orde	Primary Neurites mean Leng	Neurite Lengt	Longest Neurite Length	Axonal Tree leng	Axonal Tree Branches
12	1	2	1	152.3823	214.8234	214.8234		
16	2	5	1	178.2612	468.1737	468.1737		
24	3	3	2	63.8839	74.7401	74.7401		
36	4	4	2	47.2487	96.397	96.397		
40	5	5	2	85.3127	129.5563	129.5563		
45	6	3	2	265.3463	323.061	323.061		
53	7	2	1	132.1899	229.5807	229.5807		
63	8	4	1	161.2879	269.6346	269.6346		
65	9	3	1	130.2826	248.1665	248.1665		
74	10	4	2	75.6734	104.0833	104.0833		
77	11	4	2	86.2726	103.9117	103.9117		
82	12	4	1	89.2401	192.6224	192.6224		

Since the images are rescale at 1µm/pixel all measures in the “Results” text file can be considered as µm.