SynJ Documentation

Introduction

Overview

SynJ (as *Synapse ImageJ*) is an ImageJ toolbox for the analysis of different characteristics of isolated neurons, with particular focus on accurate measurements of neuronal morphology (neurite arborization), synapse detection, and fluorescence intensity measurements. The toolbox is meant as an improvement over existing methods to analyze confocal microscopy pictures of isolated neurons, like SynD (Schmitz et al., 2011).

Installation

To SynJ archive must be copied in the *ImageJ* > *Macro* > *Toolbox* directory, to have the source file *SynJ.ijm* and its complementary directory *SynJ* in the same directory. Besides, the toolbox uses two external plugins: "Ridge detection" and "MorphoLibJ". It is recommended to use FIJI instead if the basic distribution of ImageJ.

When SynJ is installed, it can be accessed from the main FIJI interface by selecting the double greater sign (≫) at the far right of the interface. As a result, the right side of FIJI will update to show the SynJ tools. From left to right, the bottoms are for "Detect Soma"; "Detect Neurite"; "Detect Synapses"; "Mark synapses"; "Save Menu" and "Options" (Fig 1 A-C).

Basic workflow

The detection is based on the intensity of the different channels in a multichannel confocal image. The "Morphology channel" refers to soma and neurite (dendrites/axon); it is the channel with a space filler, a MAP2 (dendritic) staining, SMI312 or tau (axonal) staining, or β3-tubulin microtubule staining. The "Synapse channel" refers to the primary channel to identify synapses, or any other staining that shows a punctuate pattern. The "Other channel" refers to the third channel, where

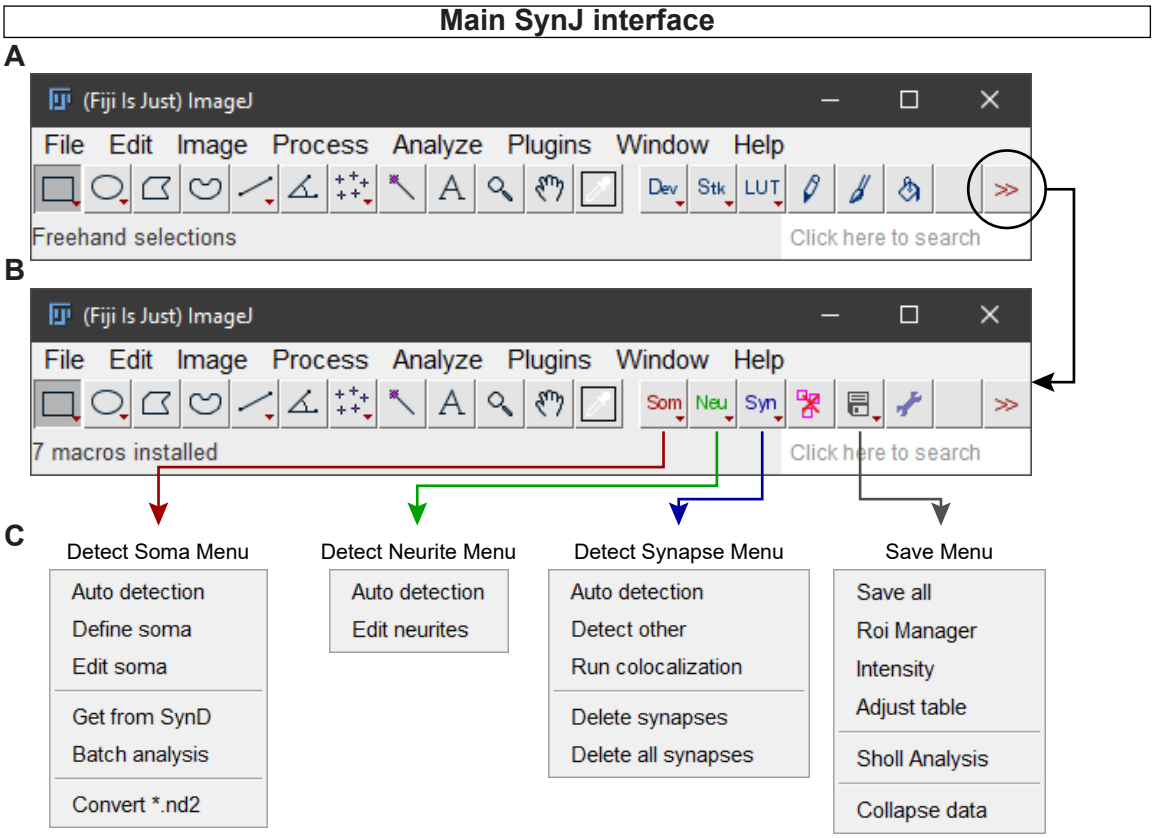
there is another protein of interest. If it shows a punctuate pattern, this channel could be used for colocalization analysis.

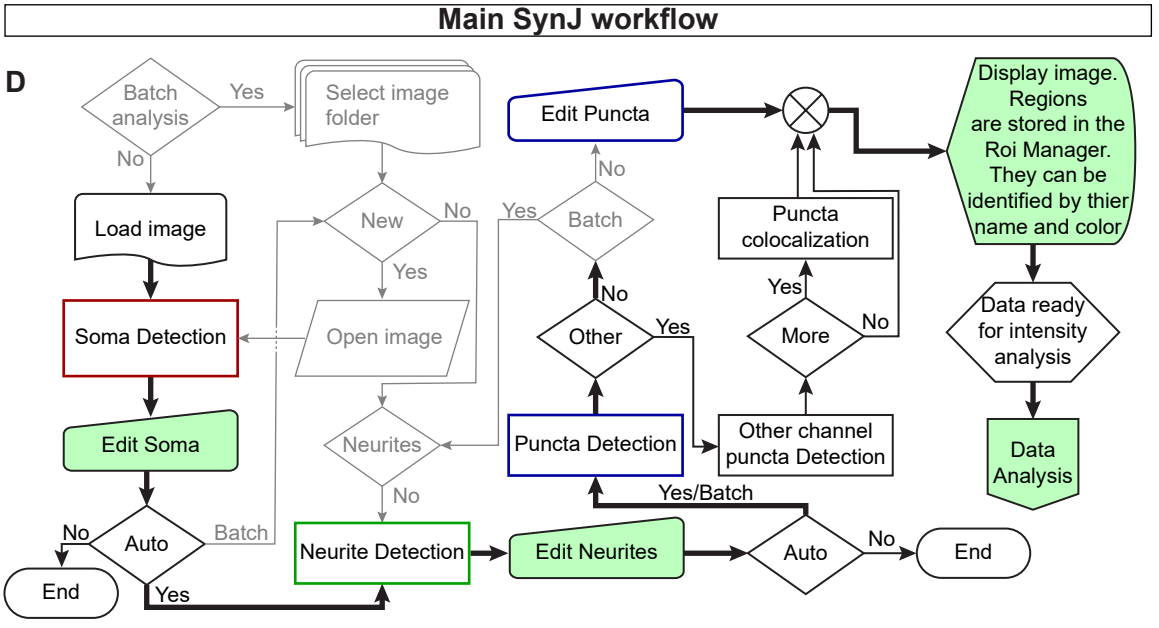
Once the channels are defined, there are three main ways to use SynJ: Semi automatically, batch processing, and importing from SynD (Fig 1D). The basic workflow for the semi-automatic processing consist of loading an image, detect and editing the soma mask, detect the neurites and adjust the mask to include undetected neurites or to delete background detections, detect the synapses and review the detected synapses to include or exclude puncta. If selected, there will be an additional detection for the other channel, and then a colocalization analysis. After the detection is done, the user can extract the intensity of the three channels in Sholl analysis or as mean intensity inside the soma, along with the neurite mask or inside individual detected puncta.

The batch processing workflow is useful when the user wants to get a quick overview of the data. The first step in the batch processing is to have all the images in the same folder. After selecting that folder in the “Batch Analysis” submenu, the user is asked to draw the soma mask per each individual image. When this is done, the program will continue detecting the neurites and the synapses, save the masks and the intensity and then move on with the next image. The disadvantage of this method is that the quality of the detection is highly variable depending of the signal-to-noise ratio in the individual images, so it is recommended to empirically adjust different detection parameters to be as inclusive as possible.

Besides, SynJ allows converting neurites masks previously generated in SynD. This could be useful for different reasons, for example, lack of MATLAB license, meaning that the user cannot keep on working with SynD; the user would like to have better control over the individual masks and detection output; more image analysis implementations, and many other cases. To import SynD masks into SynJ, the user should indicate the folder where the images are located and the folder where the output of SynD is located. SynJ will then load the images and try to match them automatically with their respective neurite mask. Then, the program will continue with synapses detection and intensity analysis.

Appendix - SynJ
Figure 1 - Main interface and workflow





Usage

The option menu

To give the user the best control over the detection depending on their need, SynJ offers different options for the different detection. The main categories are channel options, soma detection, neurite detection, synapse detection, and saving options (Fig 2A). The subcategories are visual options, colocalization (Fig 2B), and advance options (Fig 2C).

Channel options: the user will set the channels of a 3 color image to map the morphology, synapses, and another channel, where “other” mean another channel that the user would like to calculate intensity, and that can be used for colocalization analysis.

Soma detection: allows the user to adjust the parameter for the automatic detection of the soma. Briefly, the minimum soma radius and the maximum soma radius indicate the expected size, in pixels, of the soma.

Neurite detection: here, the user can adjust the parameter to improve the neurite mask. The “Gaussian blur sigma” is used to remove the scatter noise from the image. The “Estimate line width” is the approximate neurite width in pixels, while the “Minimum neurite length” will filter detected lines shorten than the indicated value, in pixels.

Synapse detection: the users can reduce the scatter noise and increase the contrast of each puncta using the “Tophat radius” (in pixel), while with the “Synapse radius” the user will set the desired particle size. The “Synapse smooth” option is used to smooth the edges of the detected puncta to fit the actual image better. The user can filter synapses based on their relative intensity with “Synapse threshold (std)”. When synapses are detected, the standard deviation of the intensity of the signal along the neurite mask is calculated and detected puncta are considered positive if their intensity values are greater than the mean + n * StdDev of the neurite intensity, where “n” is the value input by the user.

Saving options: here, the user can set the desired folder where to save the mask and the result table, as well as save it with the full name or with the coverslip and cell ID. For the full name options, the result files with be saved as “Result_MyImageName.csv”, “Sholl_MyImageName.csv” and “RoiSet_MyImageName.zip”. For the coverslip and cell ID, the image file must have a name with the format YYMMDD_condition_csID_cellID, where YYMMDD is a date in the format “year/month/day”, for example, 191128 for the 28th November 2019, and csID is the coverslip ID and cellID the cell ID. The result files will then be saved as “Result_csID_cellID.csv”, “Sholl_csID_cellID.csv” and “RoiSet_csID_cellID.zip”. It is recommended to use the full name option.

By checking the other three options, the other three dialogs will appear.

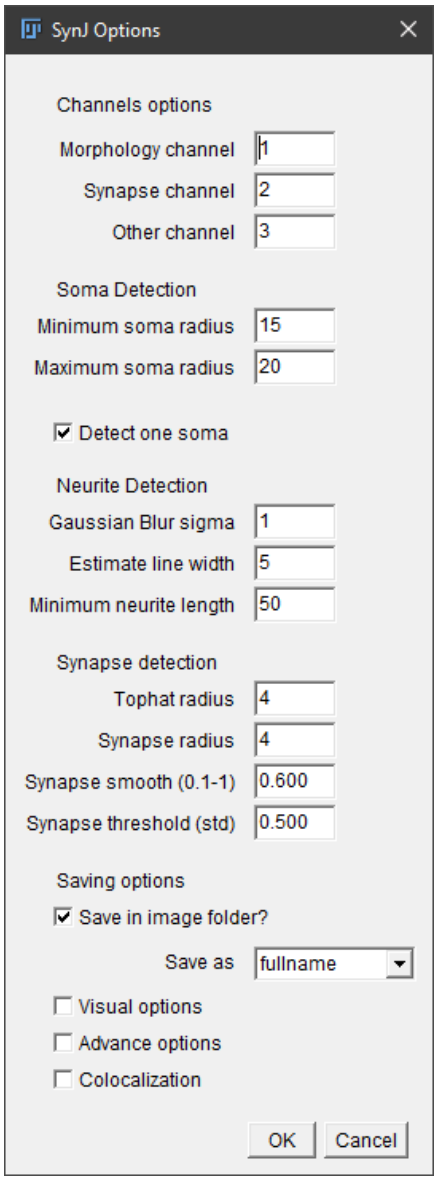
Visual options: gives the user the possibility of changing the color of the selection for the morphology mask, for the synapses that will be included in the intensity analysis, “keep synapses”, and for the one that will be excluded, “delete synapses”. In addition, there is the possibility to change the color for the second detected channel (the name “postS” refers to postsynapse), as well as the colocalization color.

Advanced options: in the advance options, the user can fine-tune the detection of neurites by indicating the percentage of saturated pixels in the image, and the “High” and “Low contrast”, which represent the relative value of the signal were to calculate the mask. By changing the number of “Closing interaction”, the user can correct for small empty spaces in the mask. For synapses, the user can choose to detect puncta with a wider neurite mask with the “Neurite padding” option or to detect puncta inside the soma.

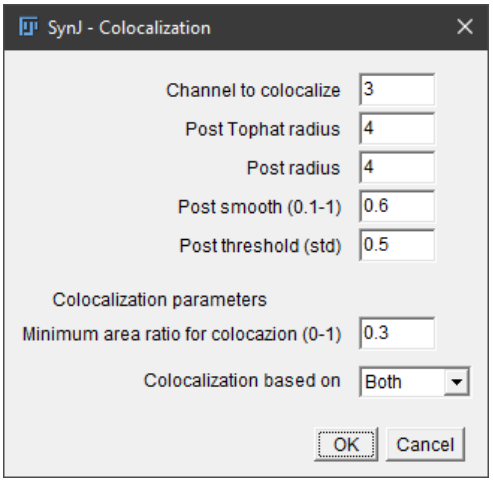
Colocalization options: in case the “Other channel” present a punctate pattern, the user can decide to calculate the object-based colocalization between the two channels. The user must then select in which channel to perform the detection that will follow the same principles of the **Synapse detection** option (see above). In addition, the user can select the “Minimum area-ratio for colocalization”, which is the ratio between the area of the overlapping region to the area of the

Appendix - SynJ
Figure 2 - Menu options

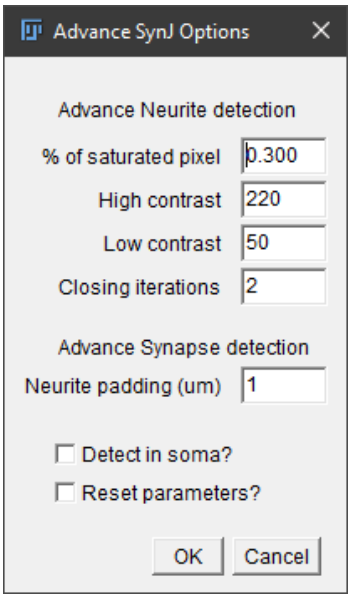
A



B



C



synapse/postsynapse. Finally, the user can choose to base the colocalization based on the synaptic mask, the “other” mask, or both (more stringent).

Detection

Soma detection

The “*Detect Soma Menu*” allows the user to select an **Automatic detection**, where SynJ will start by detection the soma, input the user to adjust the mask manually, then it automatically moves on with the neurites and synapses detection.

If the user selects **Define soma,** SynJ will select the polygon tool of ImageJ and ask the user the manually draw the soma, when done it will ask the user to edit the soma in case the user would like to adjust the tracing. The option **Edit soma** will prompt the user to adjust the soma mask with the brush tool of ImageJ. The maximum soma radius parameter in the options defines the brush tool size.

The automatic detection of the soma is based on the threshold difference between the neurites and the background. First, a morphological opening of the maximum soma radius will be applied to the image and subtract to the original image. Then a morphologically closing function, with the minimum soma radius, is applied to the resulting image to identify the area with the biggest difference. The soma will be defined with a Minimum error threshold inside this area.

Both **Get from SynD** option, and the **Batch analysis** will analyze multiple image files present in one folder.

The **Convert *.nd2** will prompt the user for batch converting Nikon *nd2* images into tiff images.

Neurite detection

The “*Detect Neurite Menu*” allows the user to select an **Automatic detection**. In this case, SynJ will first smooth the image to remove the scatter noise around the neurites, then with the use of

the *Ridge detection plugin,* it will identify and select the neurites. Depending on the intensity of the background, it can be that SynJ will detect false positive neurites. Therefore it is suggested to inspect the image after detection manually and, in case, adjust the high and low contrast parameters (in 8-bit values) in the Advance SynJ Option menu (Fig 2C). Alternatively, increasing the minimum neurite length will decrease the number of false-positive detection, but it will also reduce the overall detection of the neurite mask.

After the automatic detection, the user can adjust the neurite mask via the **Edit neurites**. SynJ will select the brush tool and set the brush size to the estimated line width parameter. A popup window will explain the usage of the brush. Briefly, the user can press the **SHIFT** key to add a new selection and **ALT** (**OPTION** for Mac) to delete part of a selection. By pressing "OK" on the popup window, the adjusted mask will be stored in the Roi Manager.

Synapses detection

The "*Detect Synapses Menu*" allows the user to detect puncta automatically inside the neurite mask with the **Auto detection**. SynJ will then smooth the signal from the synapse channel with a Gaussian blur filter of radius "*Synapse radius"*, and then performs a LoG (Laplacian of Gaussian) operation with the MorphoLibJ plugin of size "*Tophat radius*". The resulted image is smoothed again with a Gaussian blur filter of radius "*synapse smooth*". An automatic local-threshold is then applied to the image to identify the potential synapses. The detected regions are then filtered based on intensity. The synaptic mean intensity value, and its standard deviation, are calculated based on the neurite mask. Regions are considered synapses if:

$$I_{ROI} \geq I_{Neurite} + std \times \sigma_{Neurite}$$

Where I_{ROI} ≡ Intensity of the putative synapse; $I_{Neurite}$ ≡ Intensity of the synapse channel inside the neurite mask; *std* ≡ value of the "Synapse threshold" option; $\sigma_{Neurite}$ ≡ Standard deviation of the synapse channel intensity inside the neurite mask. Positive regions are marked with the "*Keep synapse color*", the other will consider as negative and marked with the "*Delete synapse color*". If

149 the user opted for the "*auto detection"* then SynJ will automatically delete the negative regions.
150 Otherwise, the user can delete unwanted regions via the "*Delete synapses*" option, while the
151 "*Delete all synapses*" will delete all synapses regardless of how they are marked, resulting with
152 only the soma and neurite mask.
153 If the option for colocalization was selected, SynJ will automatically repeat the synapse detection
154 on the third channel, using the parameters of the "*Colocalization*" submenu (Fig 2B). Otherwise,
155 the user can run the detection manually via the **Detect other**. Once both pre- and postsynapses
156 are detected, SynJ will perform an object-based colocalization between the two sets of regions
157 via the **Run colocalization**. For every region in the "synapse" set, SynJ will calculate the closest
158 region in the "postsynapse" set. If the two regions overlap, then SynJ will calculate ratios between
159 the area of the colocalized region with the area of the synapse and of the postsynapse. Finally,
160 based on the "*Minimum area ratio for colocalization*" and the "*Colocalization based on*", SynJ will
161 mark the synapse-postsynapse pair as colocalizing and save the colocalizing region in the
162 RoiManager. The "*Colocalization based on*" option can be set to "Synapses", "Postsynapses",
163 and "both". The latest being the more stringent since it depends on both $ratio_{C\text{-}S}$ and the $ratio_{C\text{-}P}$
164 to be bigger than the minimum area ratio for colocalization. SynJ will then report the colocalization
165 analysis in a result table (Fig 3D) discussed below.
166

Analysis

167 Once the tracing per cell is complete, the user can save the Roi Manager as well as perform
168 several intensity-based analyses (Fig 1C). The **Intensity** analysis will measure the area and the
169 intensity of the three channels per every region in the Roi Manager (Fig 3A). The **Save all** option
170 will save the Roi Manager and the Result intensity automatically.
171 The **Adjust table** option will either add new features for different versions of SynJ, neurite ends,
172 for example, or recalculate the intensity value if the channel map is changed. The **Collapse data**

option will collect all the *.csv files in a single folder. Then it will calculate the average intensity and area of the synapses per individual file and store the new values in a new table. It is recommended to have the entire dataset in such folder to make use also of the condition label automatically generated by SynJ. The result table will then report the name of the files used for the analysis in the CellID column. If the filename is expressed as "YYMMDD_experimentID_coverslipID_cellID" SynJ will extract the coverslip and experiment identifiers, and additionally, add a unique group identifier. The intensity values are grouped based on the different masks, soma, neurites, and synaptic. Finally, SynJ will calculate the synapse density as the total number of synapses divided by the neurite length in µm (Fig 3B).

SynJ offers the possibility to calculate the intensities of the three channels as a function of the distance from the soma (**Sholl analysis**). The user will define the step increase and the mask (neurites or synapses) were to calculate the intensities of the three channels. The results will then be displayed as a table with the distance (in px) from the soma and the intensity of the three channels per mask, additionally, SynJ will calculate the number of dendrites and synapses in the specific shell (Fig 3C).

For the colocalization, SynJ will report the synapse-postsynapse pairs, their distances, in px, the total overlapping area, in px, and the fraction of the area of the colocalization to the area of the presynapse and postsynapse. Finally, the last column determines if SynJ will consider that pair as a positive colocalization (1) or not colocalizing particles (0) (Fig 3D).

Summary

In summary, SynJ is an ImageJ toolbar that is designed for the deep intensity analysis of single neurons, giving information on the soma, neurite, and synapses. It is flexible, and it can be used for different cell types that present complex morphology, like astrocytes or microglia, and that are stained for punctuate structures. Moreover, SynJ takes advantage of the ImageJ platform, first being free and open-source, and implemented periodically by the image analysis community.

Appendix - SynJ
Figure 3 - Result tables

A

Result “image_name”.tif:	
Region	Name of the selected ROI. For the neurites reports the number of ends in brackets.
Area	Area of the region in μm^2. For neurites, length of the region in μm
Morphology	Intensity of the morphology channel inside the region
Synapses	Intensity of the synase channel inside the region
Other	Intensity of the other channel inside the region

B

Results_”experiment”:	
CellID	Name of the image (YYMMDD_expID_csID_cellID)
Coverslip ID	Second to last argument of the title (csID above)
Experiment ID	Second argument of the title (expID above)
Group ID	Increasing number based on conditions
Soma Area	Area of the soma region in μm^2.
Soma Morphology	Intensity of the morphology channel inside the soma
Soma Synaptic	Intensity of the synapse channel inside the soma
Soma Other	Intensity of the other channel inside the soma
Neurite Length	Length of the neurite region in μm.
Neurite Ends	Number of ends in the neurite mask
Neurite Morphology	Intensity of the morphology channel in the neurites
Neurite Synaptic	Intensity of the synapse channel in the neurites
Neurite Other	Intensity of the other channel in the neurites
Synaptic Area	Area of the synapse region in μm^2.
Synaptic Morphology	Intensity of the morphology channel in the synapse
Synaptic Synaptic	Intensity of the synapse channel in the synapse
Synaptic Other	Intensity of the other channel in the synapse
Synapses per μm	The total number of synapses divided by the neurite length

C

Result Sholl:	
Distance	Length of the radius from the soma (in px)
Morphology(N)	Intensity of the morphology channel inside the neurite mask
Synases(N)	Intensity of the synapse channel inside the neurite mask
Other(N)	Intensity of the other channel inside the neurite mask
Dendrite #	Number of dendritic region in the Sholl radius
Morphology(S)	Intensity of the morphology channel inside the neurite mask
Synases(S)	Intensity of the synapse channel inside the neurite mask
Other(N)	Intensity of the other channel inside the neurite mask
Synapse #	Number of dendritic region in the Sholl radius

D

Result Colocalization:	
Region	Synapse_ID
ClosestRegion	Post_ID
CenterDistance	Distance between the two regions (in px)
Overlap	Area of the region (in px)
PreOverlap	Ratio Area(Colocalize) / Area(Synapse)
PostOverlap	Ratio Area(Colocalize) / Area(Post)
Colocalize	1(true)-0(false) based on the region

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Reference

Schmitz, S.K., Hjorth, J.J., Joemai, R.M., Wijntjes, R., Eijgenraam, S., de Bruijn, P., Georgiou, C., de Jong, A.P., van Ooyen, A., Verhage, M.*, et al.* (2011). Automated analysis of neuronal morphology, synapse number and synaptic recruitment. J Neurosci Methods *195*, 185-193.