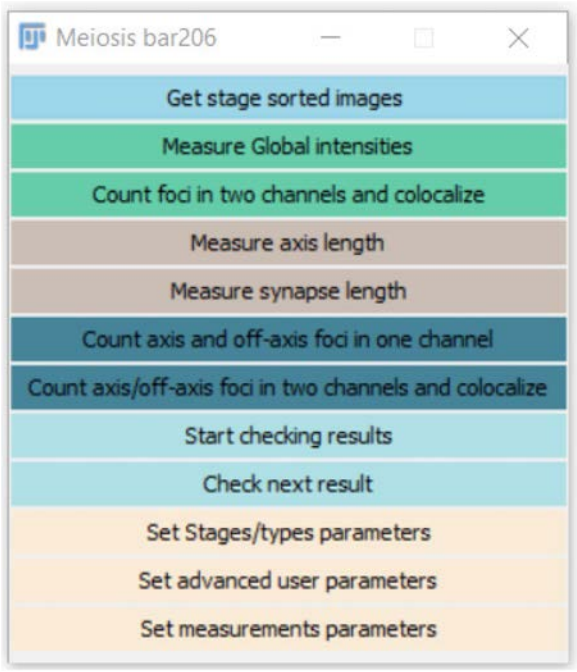
MeïQUANT



VERSION 2.06

MAY, 10TH 2023

The macro uses the Actionbar and skeletonize 2D/3D plugins. These are available here: https://imagej.nih.gov/ij/plugins/index.html

- Fiji comes with skeletonize plugin included. Otherwise, download it from https://imagej.net/plugins/skeletonize3d. Version 2.1.1 was used.
- https://biii.eu/actionbar-imagej

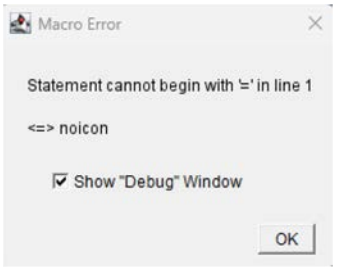
The macro was tested using ImageJ 1.53t, Java 1.8.0_172 and a 64-bits windows OS.

It is recommended to steer clear of using mind spaces or unusual characters when naming input folders, parent folders, and file names.

Any questions or concerns, please do not hesitate to contact julien.cau@biocampus.cnrs.fr

KNOWN ISSUES

1- MeiQUANT main window (Meiosis bar) is not displayed in color and the following error window is displayed



➔ try installing this version of Action bar (https://figshare.com/ndownloader/files/26750024)

TABLE OF CONTENTS

This extensive manual offers a detailed explanation of each MeiQUANT tool. You can easily navigate to the specific chapter dedicated to the tool you are interested in without having to go through the entire manual. The manual is thoughtfully organized so that you can access a complete description of any tool without the need to go through all the other tools.

For each tool, the steps to follow are described and numbered, with corresponding figures illustrating each step and highlighting what to do with stickers that have the same number. The nomenclature used in the manual is as follows:

The nomenclature used in the manual is as follows (unless an error is left):

- Command: an ImageJ command (such as Image>Properties),
- Built-in function: a command that can only be run within a macro (ie. a macro language specific command)
- Tool: unless *tool* is enclosed in quotation marks, *tool* refers one of the MeïQUANT algorithm behind each described button

SHORT MEIQUANT DESCRIPTION

The aim of the MeiQUANT macro collection is to measure meiosis-associated features, including axes and recombination foci. The process involves the following main tools:

A. MEIOSIS STAGE IDENTIFICATION: since an image may contain multiple meiotic stage nuclei, the user is prompted to draw the outlines of each nucleus and crop/save it based on its meiotic stage.

B. Analysis:

B.1 GLOBAL INTENSITIES MEASUREMENTS: this routine measures the global intensities of a set of channels of interest. This can be utilized for analyses that are not related to meiosis.

B.2 FOCI IDENTIFICATION & COLOCALISATION. This routine is a simpler version of routine B6. It identifies any foci within a Region of Interest (ROI) (and does not discriminate axis/off-axis foci position). It has the potential to be utilized for analyses unrelated to meiosis.

B.3. AXIS LENGTH MEASUREMENTS: this routine measures the total length of the identified axes with various processing options available.

B.4. SYNAPSED/NON-SYNAPSED AND WHOLE AXIS LENGTH MEASUREMENTS: this routine is used to identify the whole axis and quantify synapsis. The intensities of a channel of interest on both axis types (whole and synapsed/non-synapsed) can be measured.

B.5 FOCI IDENTIFICATION: This routine identifies and categorizes foci based on their proximity to the axes, whose total length can be calculated (option).

B.6. TWO COLOR FOCI COLOCALISATION: This last routine allows identification of axes foci and measurement of colocalization.

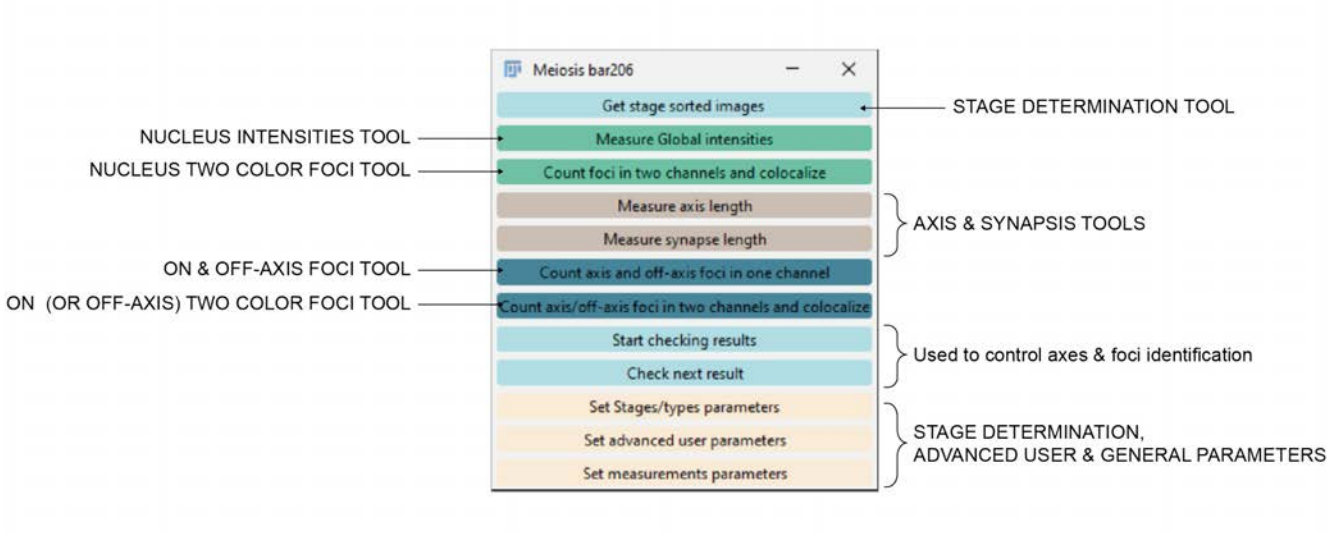


Figure 0.1. The main Meiosis bar window of the MeiQUANT tools collection.

HOW TO INSTALL MeïQUANT.

If you are unfamiliar with the ActionBar plugin, please follow these instructions below.

After installing the ActionBar plugin, locate the ActionBar folder in the main ImageJ/Fiji's plugins directory. You have two options:

a. Create a "plugins/actionBar/Meiosis" subfolder and place the "meiosis_bar206.ijm" file there.

b. Modify the macro's code to specify a different location within the /plugins/actionBar folder. Follow STEP1 to STEP3.

STEP 1. Copy the "meiosis_bar206.ijm" file to a location within the ImageJ/Fiji folder. Copy the file's path, starting from the parent folder of ImageJ/Fiji (highlighted in blue in the top panel of Figure 0.2). For example, on a PC, you would need to copy "\plugins\actionBar\Meiosis".

STEP 2. In Fiji, create a new script (File > New > Script). Then, in the new.ijm window (lower left panel of Figure 0.2), go to File > Open and select the location of the file. Alternatively, drag and drop the meiosis_bar206.ijm file on the main ImageJ/Fiji window. This opens the macro in the classical macro editor if using ImageJ and the script editor in Fiji.

STEP 3. The "meiosis_bar206" script will open. Modify the location on line 2 while ensuring that it remains within parentheses (the text should appear in purple if using the script editor, lower right panel figure 0.2).

After successfully installing the macro, you can execute it by navigating to plugins > ActionBar > Meiosis > meiosis_bar206 (or any other specified path within the parent plugins > ActionBar if option b is used).

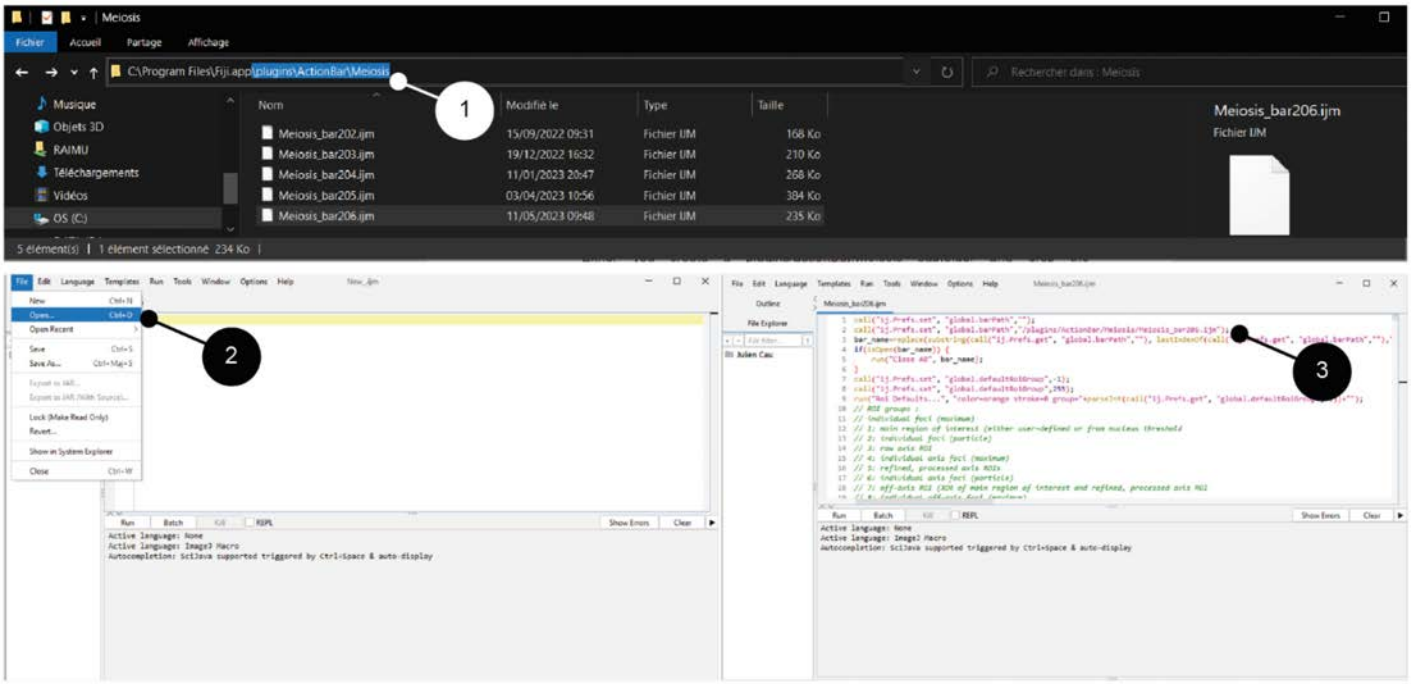


Figure 0.2. MeiQUANT installation.

HOW TO GET BACK TO RESULTS OF PREVIOUS MeiQUANT ANALYSES.

Please note that running the same tool on the same dataset will overwrite the previous results, including result files, ROI .zip files, and any other generated files.

If you want to review the results of a previous analysis, ensure that the stage/types prefixes used before match the current stage/prefixes list. In case you have modified the default analysis prefixes and want to check the results with a new instance of MeïQUANT (launching the plugin for a second time, or the next day), click on the "Set Stages/Types parameters" button (refer to figure 0). This action will trigger the appearance of the following two windows (refer to figure 0.3).

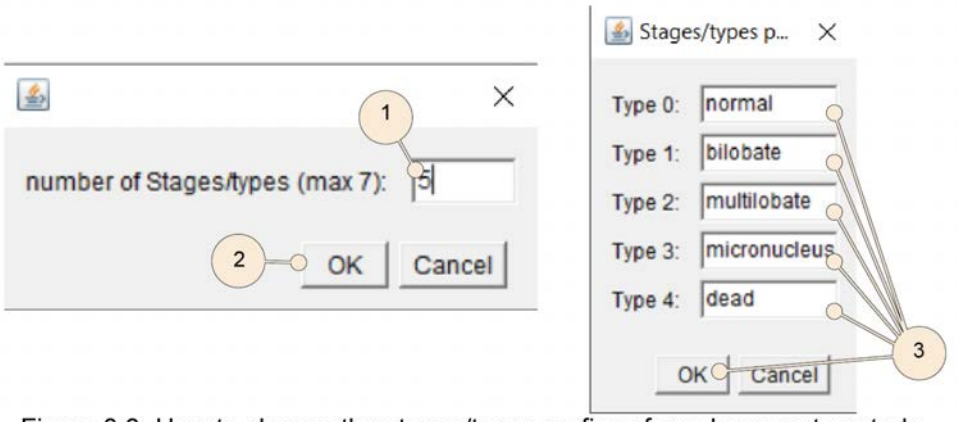


Figure 0.3. How to change the stages/types prefixes for subsequent controls

STEP 1.Provide the number of stages.

STEP 2. Press the OK button.

STEP 3. Enter the prefixes in the "type n" fields and click OK. You can now proceed to the control by clicking on “Start checking results”. Follow the tools descriptions below for the first window configuration.

HOW TO MODIFY MeïQUANT.

Using the script editor in Fiji is highly recommended for this task. The macro follows the structure outlined below.

- The tools within the meiosis bar share common preferences, where shared variables are stored. These variables are initialized with "" for string preferences, false for boolean preferences, and -1 for numeric preferences. Default values are then passed into the preferences. Therefore, when referencing the preference, the required default value would be either "", -1, or false. Note that numeric preferences are stored as strings by ImageJ and need to be converted back to numeric values using the ParseInt() built-in macro language function.
- Functions shared across multiple tools/buttons are enclosed between <codeLibrary> and <\codeLibrary> tags. Specific functions used only in one tool are located after the main tool/button macro code, separated by a short line of ////////////////////.
- Each button is separated by a long //////////////////// code block, starting with <line><button> and ending with </macro></line>. The "Get stage sorted images" button triggers a custom actionBar that is executed through the run("Action Bar", bar);

command, where "bar" is a string. Modifying this string should be limited to experienced users.

All macros for meiosis-specific tools follow the same scheme:

- The macro begins with dialog menu items, including tools' parameters and the image folder.
- Result table(s) are created, and their columns are initialized.
- A do/while loop is used to analyze all relevant (stage) images within the image folder:
    - Stage images are identified using the findStageFiles() function.
    - Image format verifications are performed. Once all requirements are met, the reference ROI (Region of Interest) is identified using the getReferenceROI() function. If the reference ROI is not created, the macro skips to the next image.
    - If necessary, the intensity within the reference ROI is measured using the measureReferenceIntensities() function.
    - The rawAxis is then identified through the getRawAxisRoi() function and processed (pruned) using the refineRawAxisRoi() function. This process is performed twice with different function parameters to detect synapsed and non-synapsed axes. The refineRawAxisRoi() function also triggers any requested measurements within the identified axis selections, such as skeleton lengths and intensities.

  For foci tools only:

    - The countFoci() function enables foci identification in one channel within a specified ROI. If necessary, the off-Axis ROI is obtained using the getOffAxisRoi() function. This step also generates Euclidean distance to foci maps , which are used for requested colocalization analyses. Both functions create overall maxima selections, such as total_foci (maxima), axis_foci (maxima), or off-axis_foci (maxima).
    - If colocalization analysis of foci is requested, it utilizes the findColoc() function with a colocalization area derived from Euclidean distance to foci of one channel maps and the overall foci maxima selection in the other channel. All random foci distribution colocalization analyses are performed using the generateRandomColocValue() function.
    - If particle analysis is requested, it goes through the analyseParticle() function, which generates overall foci particles as well as individual particles associated with foci.
- At the end of the loop, the parameters used for the analysis are burnt in the main result table.

MEÏOSIS STAGE IDENTIFICATION: GET STAGE SORTED IMAGES

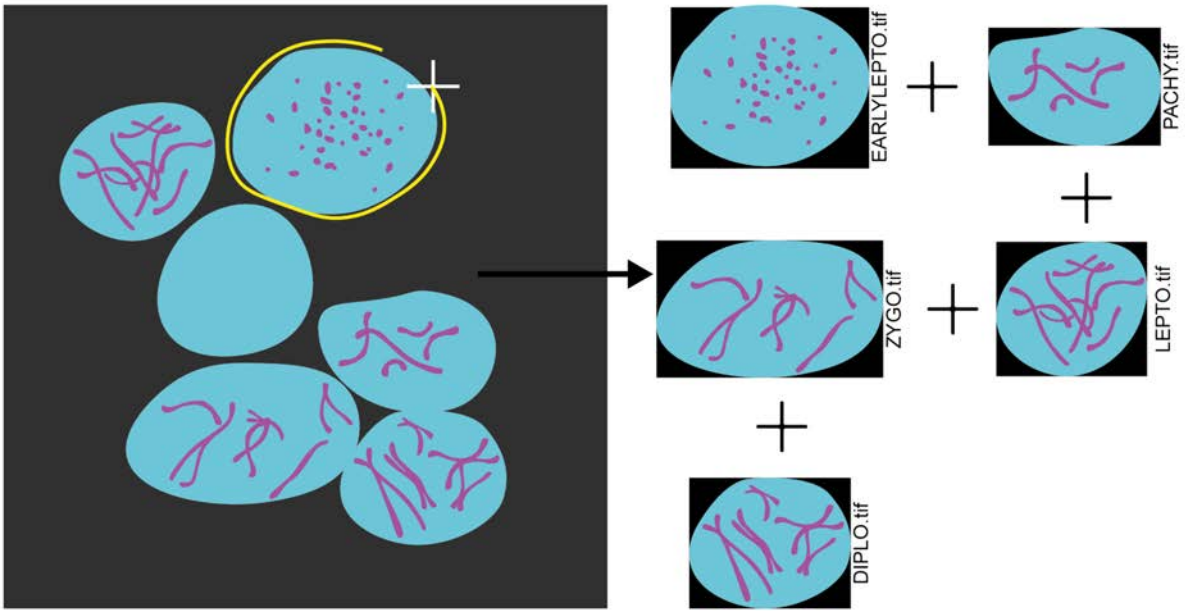


Figure 1.0. Visual Summary of the tool.

This procedure involves creating individual nucleus images and saving them in a subfolder with a stage prefix and the original image's name. As shown above (figure 1.0), while the user-defined nucleus selection appears circular (left panel, yellow line upper right nucleus), the actual images are rectangular in shape (upper left “EARLYLEPTO.tif image, right panel). To prevent contamination of a nucleus image with portions of other nuclei (as illustrated in the lower right nucleus), pixels outside the user-defined selection are set to black.

Click on the "Get stage sorted images" button before starting the analysis. This will open the "File parameters" menu (Figure 1.1). Follow these steps to complete the process:

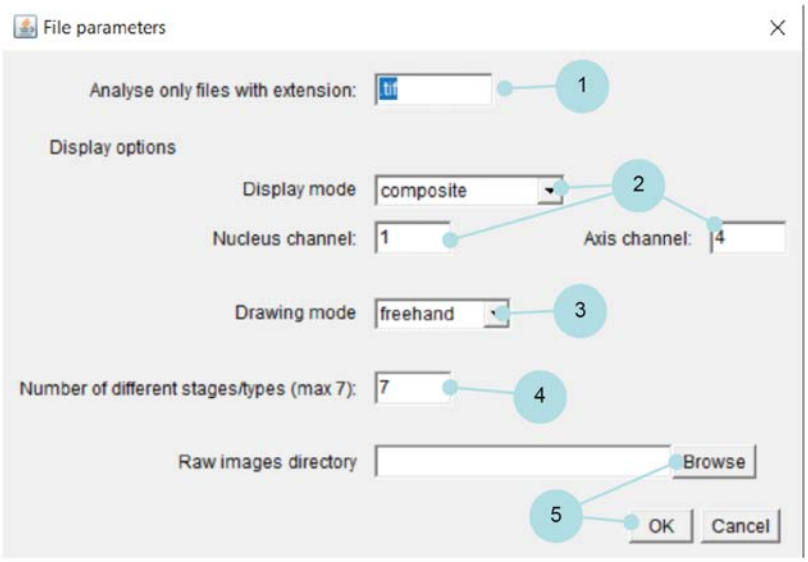


Figure 1.1 the get stage sorted images

STEP1. Select the "Analyse only files with extension" option to find the image files you want to open. This is useful for discarding unwanted files such as non-image files. The extension must

be compatible with the bioformats plugin. If you change the extension, the new default value will be displayed/saved the next time you hit the button. However, if you restart ImageJ or the toolbar, the default value will be back to ".tif". The routine uses ImageJ's preferences, and the default value can be changed in the routine's script on line #135 [call("ij.Prefs.set", "global.extension",".tif");].

STEP2. Choose a display mode. If "composite" is selected, the stack will be displayed as a composite. If a grayscale mode is used, the corresponding channel (nucleus/axis) will be displayed in grayscale mode. If the fields are left with -1, the first channel (#1) will be shown.

STEP3. Select a drawing mode. This will set the selection command to either freehand (you can manually crop the nucleus) or magicwand “tool” (then the magic wand is used).

STEP4. Fill in the "Number of different stages/types" field with the corresponding number of buttons needed to create a crop bar (see STEP 6).

STEP5. If the "Raw Image directory" field is left blank or you wish to switch to a different dataset, click on the "Browse" button. Please note that any files in subfolders will not be analyzed. The path to this folder will be utilized in subsequent routines and referred to as input. Once you have made any necessary changes, click "OK".

STEP6. The following menu (see figure 1.2) is utilized to specify the names of the different stages. These prefixes will be used to save the cropped nucleus. Fill in the fields but avoid using spaces, as this may cause issues with file identification. You can use underscores if needed. Note that if you change the default prefixes and have already analyzed images before (i.e., after quitting ImageJ once and then returning to cropped image analysis), you may need to modify the default prefixes again by using the "Set Stages/Types Parameters" button. Click on OK.

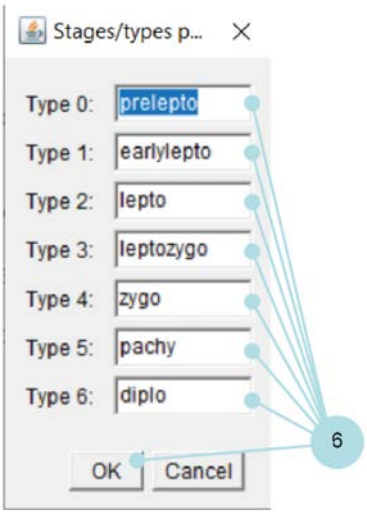


Figure 1.2. the stage/type parameter menu

Figure 1.3. the crop stages bar

STEP7. The custom crop stages bar (shown in figure 1.3) will pop-up. You can use the freehand/magicwand selection “tool” to draw around a nucleus of interest, and then add the selection to the ROI Manager. You can do the same for any other nuclei of interest in the image that belong to the same stage. Then, you can click on the corresponding stage button (prelepto, earlylepto, lepto, leptozygo, zygo, pachy, or diplo). This will duplicate the image using the selection and set pixels outside the selection to black for each channel. The processed image will then be saved in a "processedData" subfolder as a .tif file, with a meiosis stage prefix and an identification suffix (_0, for example). The user-defined ROI will also be saved in the same folder as a .roi file. The custom bar has no name, so it cannot be automatically closed. If you want to create a new custom bar, you need to close the current one manually first.

STEP8. To access the subsequent file, click on the "Next file to crop" button.

GLOBAL INTENSITIES MEASUREMENTS

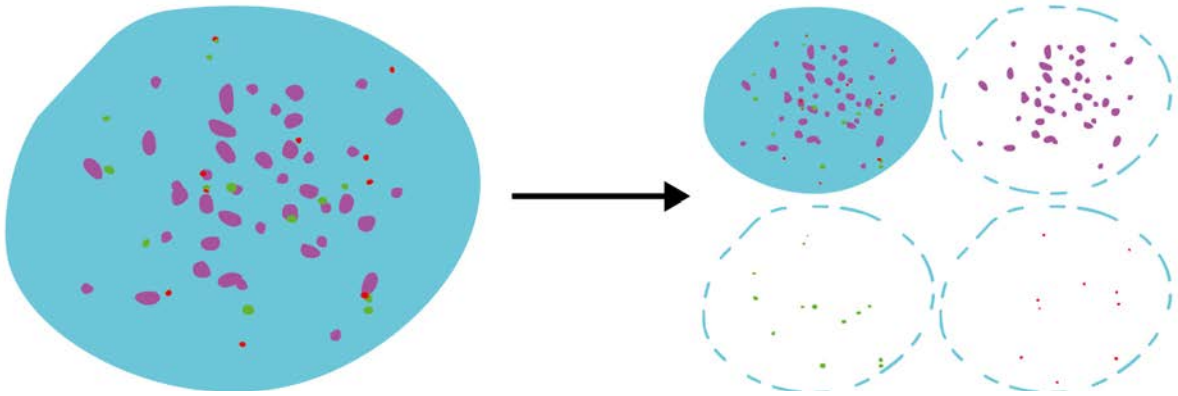


Figure 2.0. Visual summary of the tool.

This tool allows overall intensities measurements in the reference selection. Prior to clicking on the "Measure global intensities" button in the main meiosis bar, it is recommended to configure additional parameters (such as expanded threshold list or intensity measurements types) through an OPTIONAL STEP A.

When you click on the tool's button, the appearance of the menu will depend on whether you previously used the "Start cropping images" or any other analysis routine, as shown in figures 2.1 or 2.2. If you had previously set the input folder or a processedData folder using the same instance of the bar, the menu will appear as in figure 2.1. However, if the cropped image directory is not found, you should click the browse button (fig 2.2), choose the directory, and click OK. Then, the first found image will be opened, and you will be prompted to select the channels to measure (fig. 2.2).

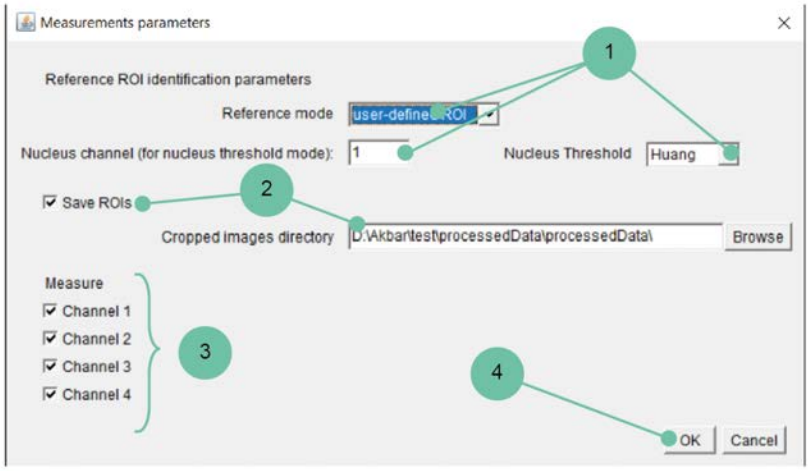


Figure 2.1. The Measure Global intensities menu (when some previous routine was used).

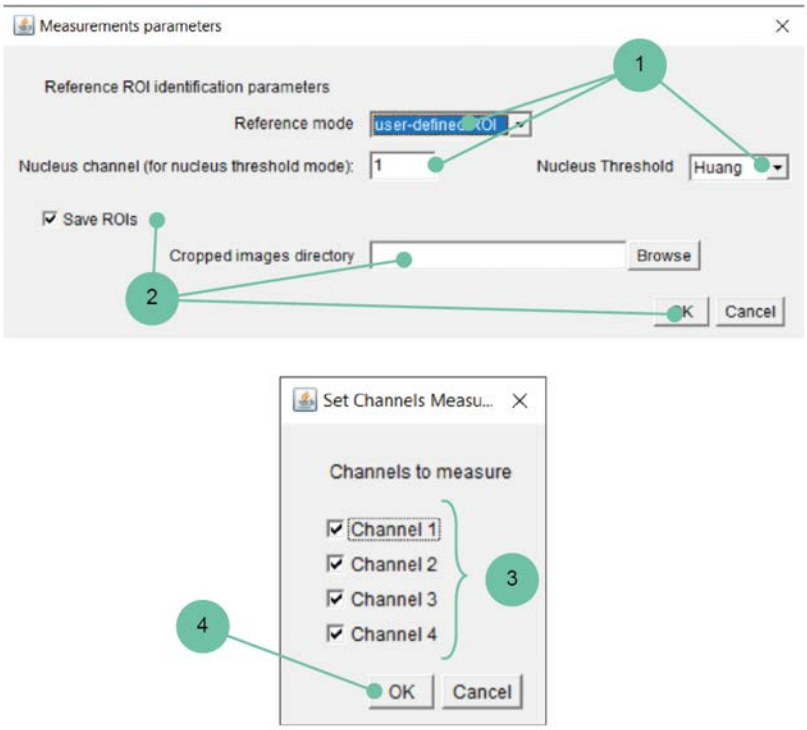


Figure 2.2. The Measure Global intensities menu (if no input folder was previously set).

STEP1. To set the ROI for intensity measurements, there are two options available. The first one is to use the "user-defined ROI" which corresponds to the ROI saved during the stage-cropping step. If this ROI is selected but not found, an error message will be displayed. When opting for this reference mode, you can proceed directly to STEP2 without the need to fill in the nucleus channel and threshold fields. The other option is to use a nucleus threshold, which is typically based on a DAPI staining channel. To use this mode, choose the channel to use for the threshold (the first channel is channel 1) and select the appropriate threshold algorithm. A variety of automatic threshold methods can be accessed by clicking on the "set measurements parameters" button (see fig. 2.4, see OPTIONAL STEP A). The parameters should be set as shown in either figure 2.1 or 2.2 left. Figure 2.1 will be displayed if the stage-sorted images location was known before starting the routine, otherwise a simplified figure 2.2 left is shown.
It should be noted that this algorithm was primarily designed for analyzing images with multiple nuclei. If there is only one nucleus and no threshold can be used to set the ROI, the entire cropped image can be selected as the ROI by pressing Ctrl A when using the stage buttons.

STEP2. Set the input and output parameters. If using MeiQuant for the first time, click OK so that the first image within the folder selected at this STEP is checked and its number of channels found.

STEP3. This step allows you to choose which image channels you want to measure. If you had previously set the location of the stage-sorted images, then a menu will appear (fig 2.1) where you can select the channels. If you had not set the location or changed it, a prompt will ask you to enter the number of channels (fig 2.2 middle), and then select the channels to be measured. If the routine does not find the correct number of channels, the measurement for that channel will be skipped and "not found" will be displayed in the corresponding column of the intensities.xls result file.

STEP4. Click on OK. The software measures each channel that the user selects for analysis. The resulting IntDen values are saved in an intensities.xls file that is located in the same folder as the stage-sorted images. It's important to note that the "intDen" values are the raw (uncalibrated) values measured by ImageJ. One can also get the mean values by checking the "Show mean values" checkbox displayed in Figure 2.4. During the analysis, the images are not displayed. However, you can choose to display them by selecting the "show images while processing files" option in the "set Measurements Parameters" of the main meiosis bar (fig. 2.4).

Type	Image name	nucleus ch. intDen (nucleus)	Ch.2 intDen (nucleus)	Ch.3 intDen (nucleus)	Ch.4 intDen (nucleus)	Comment	Parameter	Value
zygo	zygo_1	6717308.057	1417927.985	2861879.823	4623256.536		Smooth original images	no
zygo	zygo_2	7792437.241	1892989.352	3729226.687	4760835.193		ROI chosen	nucleus
zygo	zygo_3	8782333.185	2404293.225	4556193.087	7634535.123		nucleus channel	1
zygo	zygo_4	9832096.932	2045905.941	3611008.811	7089418.125		nucleus threshold type	Huang
zygo	zygo_5	7619641.210	1703393.281	3353657.368	4054338.536		Channel 1 analysed	yes
zygo	zygo_6	6511154.996	2142011.809	2812246.058	4478057.751		Channel 2 analysed	yes
zygo	zygo_7	6874684.812	1890202.902	3506897.255	5551677.560		Channel 3 analysed	yes
zygo	zygo_8	7731258.809	5093977.581	8584730.823	6190172.090		Channel 4 analysed	yes
zygo	zygo_9	7995019.567	2925961.879	5773618.256	4870472.109		Images from	D:\...
zygo	zygo_10	8204919.782	3293139.528	6349503.306	5026638.855		ROI saved	yes
zygo	zygo_11	7882653.716	2430646.431	3656478.985	4158824.952		ROI and any other output folder	D:\...
zygo	zygo_12	6655304.467	4288352.401	7504413.360	5482073.772		options:	
zygo	zygo_13	8334075.189	1425619.845	3610398.772	5036697.209		Show warning messages	no
zygo	zygo_14	8167993.055	997611.905	2150308.746	3304041.558		Show images	no
							Get integrated density intensity values	yes
							Get mean intensity values	no
							Include reference Roi when measuring intensities	yes
							Meiosis bar tool	Measure Global intensities
							Meiosis bar version	v2.05
							ImageJ version	1.53t99

Figure 2.3. a result file of the Measure Global intensities menu.

OPTIONAL STEP A. This allows users to configure additional parameters (that are shared between all MeiQUANT tools). To do so, first click on the "Set Measurements parameters" button in the meiosis bar window before running the tool. This will display the "Measurements parameters" window, as illustrated in figure 2.4.

OPT. STEP A1: If you wish to expand the list of displayed nucleus threshold methods to include all possible automatic methods found in ImageJ, select the "display the full list of threshold methods" checkbox.

OPT. STEP A2: You can also use the "Show IntDen/mean values" checkboxes to customize intensity measurement types.

OPT. STEP A3: Ignore any other parameters that are not relevant, click OK, and proceed to run the "Measure Global Intensities" tool by clicking on its button in the meiosis bar window.

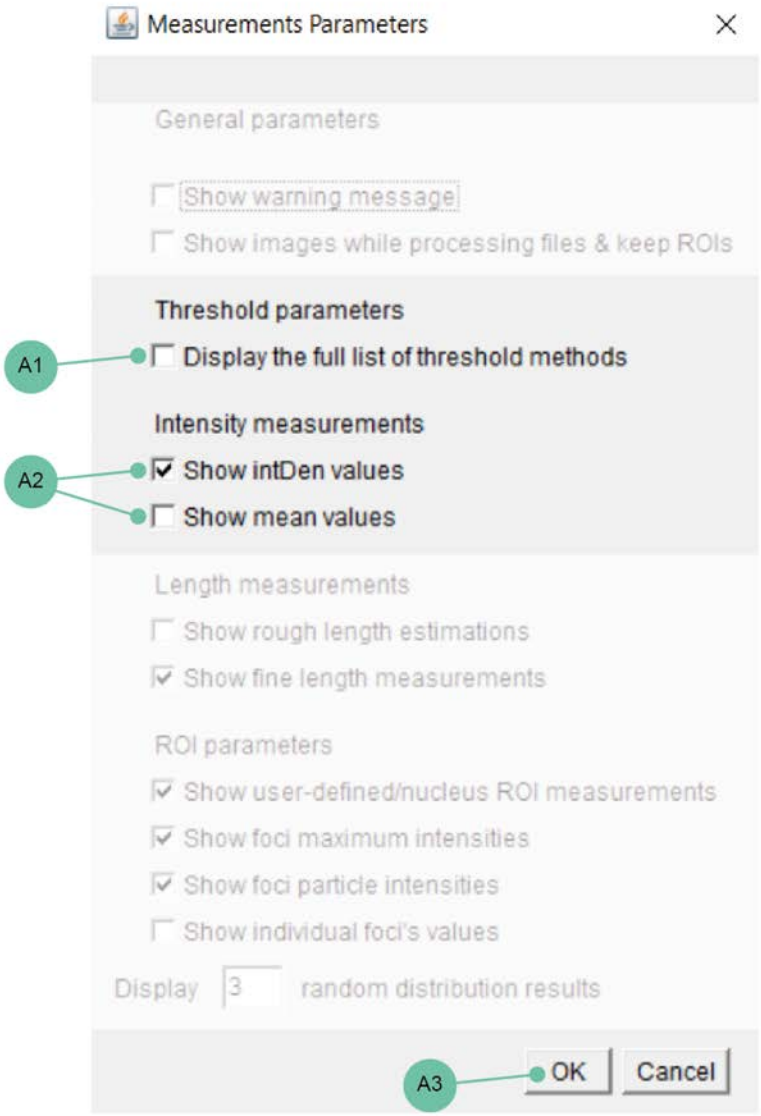


Figure 2.4. The general parameters window.

LIMITATIONS. The intensity values are measured without subtracting any noise, offset, or background. When the full dynamic range is used in most widefield cases (e.g., 0-65535 gray levels for 16-bit images), camera/system noise and offset values are negligible. However, when low-intensity, reduced dynamic range images are used, rough correction of intDen/mean values is necessary, which involves measuring an empty area and using the above formulas. Additionally, the impact of background signal should be considered, as it changes across the image. Therefore, it is recommended to process the images before using the macro to account for any changes in background signal.

$$mean_{corr} = mean_{measured} - mean_{empty}$$

$$intDen_{corr} = \left(mean_{measured} - mean_{empty}\right) * \frac{intDen_{measured}}{mean_{measured}}$$

FOCI IDENTIFICATION & COLOCALISATION

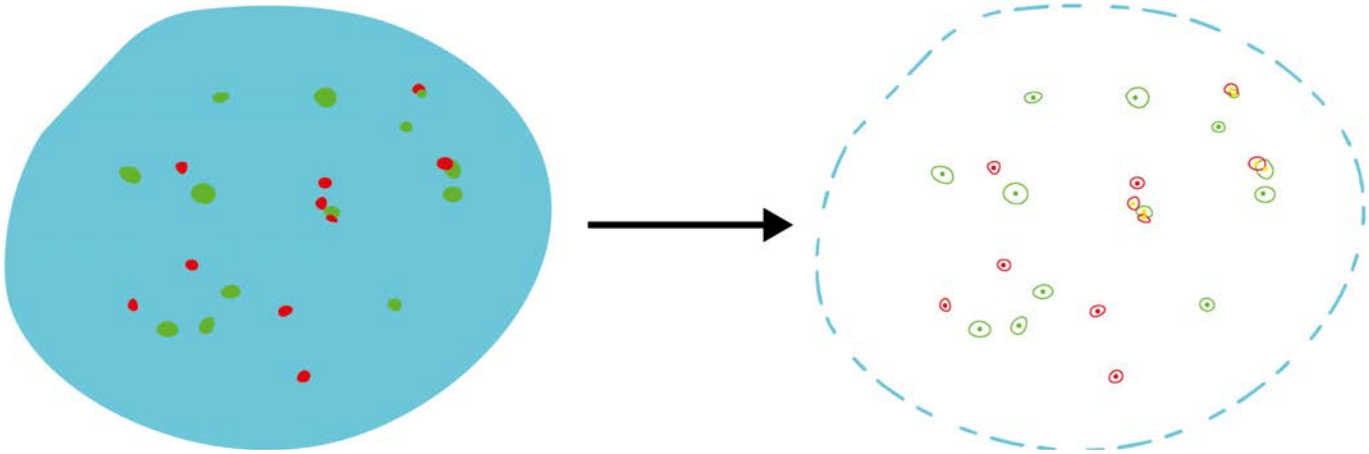


Figure 3.0. Visual summary of the tool.

The algorithm called "count foci in two channels and colocalize" has multiple functions. Firstly, it identifies the foci present in two channels. Secondly, it measures colocalization in the way described in the Lachmanovich 2003 Journal of Microscopy Vol. 212, Pt 2 November 2003, pp. 122–131 article. Moreover, the macro is capable of creating random images and checking whether the number of observed colocalized foci could be randomly obtained. Prior to clicking on the "Count foci in two channels and colocalize" button in the main meiosis bar, it is recommended to configure additional parameters (threshold methods list, foci ROI types to be kept/or measured, intensity measurements types, etc…) through an ➔ OPTIONAL STEP A.

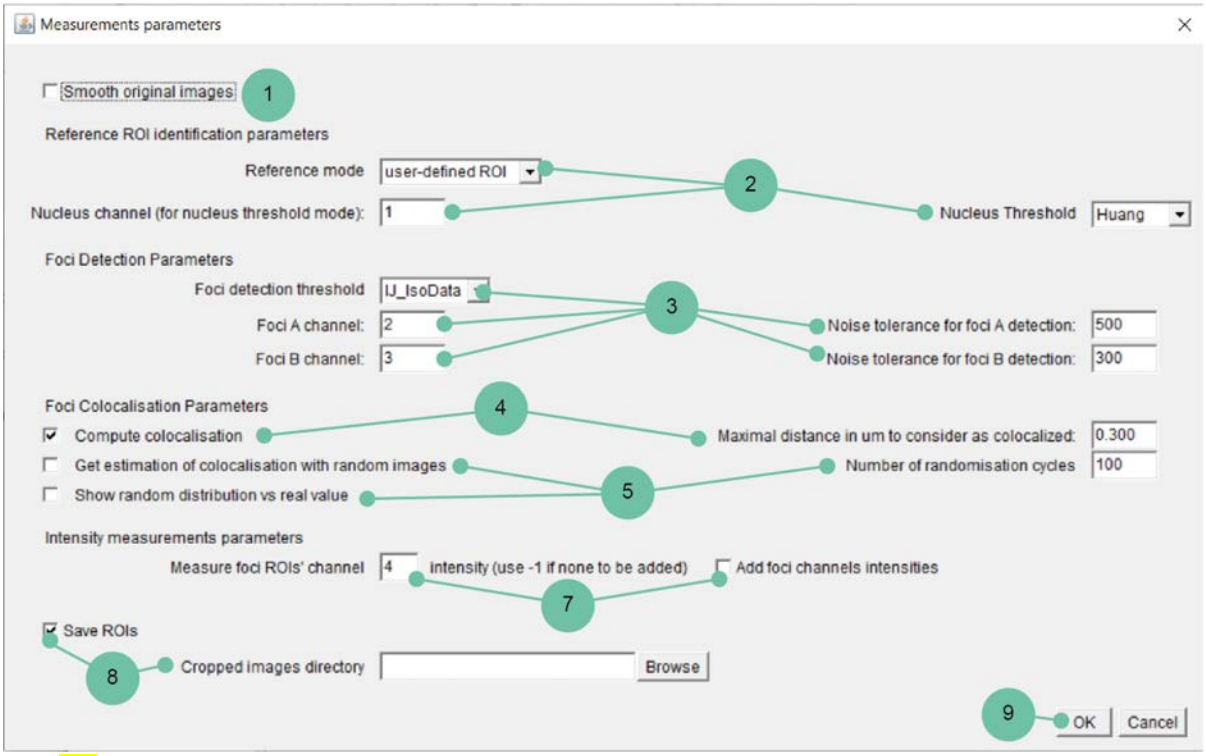


Fig. 3.1. The “Count foci in two channels and colocalize” menu. User intervention is not necessary for step 6.

➔ OPTIONAL STEP A. First, set the options for output measurements (refer to the optional step description below if needed).

STEP 1. If the images are noisy, check the "smooth original images" box.

STEP2. Choose the region of interest (ROI) to be used for measurements. You can either select a user-defined ROI (created by cropping the image). When opting for this reference mode, you can proceed directly to STEP3 without the need to fill in the nucleus channel and threshold fields. The other option is to use a thresholded nucleus staining (fig. 3.1). Fill in the appropriate fields. These ROIs will be referred to as the "reference ROI" below.

STEP 3. Set the parameters for detecting foci. Choose the foci channels A and B. ImageJ's "find maxima" command will be used to detect the foci of interest. Enter the prominence value (which was formerly referred to as noise) in the "noise tolerance for foci detection" box. Note that the algorithm may detect same-color twin foci as single foci. Use the "strict" option of the "Find maxima" command, which is only available from ImageJ 1.52. The macro takes advantage of all the possibilities offered by this version of Maxima Finder plugin.
The macro offers two modes, all of which use the nucleus or user-defined ROI as the input ROI:

- The algorithm identifies maxima without any threshold by setting the foci detection threshold to "none." Foci that have a prominence value above the specified value are detected as maxima. The corresponding particles are then identified using the "maxima within tolerance" output option of the Find Maxima command.
- Maxima can be detected by applying either a Huang or an IJ_IsoData automatic threshold to the image (this list can be expanded). The foci detection is then restricted to the thresholded area, and only the foci within this area and above the prominence value are detected as maxima. This uses the "above lower threshold" option of the Find Maxima command. The corresponding particles are identified using the "segmented particles" output option, which is described in STEP 6).
  Foci are identified within the reference ROI. These foci are referred to as "total_foci (maxima)". If a colocalisation analysis is not needed, the ROIs are assigned to ROIGroups based on Figure 3.2.

RoiGroup	ROI
1	reference ROI
17	fociA maxima
23	fociB maxima
16	individual fociA maximum
18	individual fociA particle
32	individual fociB maximum
34	individual fociB particle
107	fociA particles
113	fociB particles

Figure 3.2. ROIGroups associated with ROI generated by the macro (no colocalisation option).

STEP 4. If you choose to use the colocalisation option (by selecting the compute colocalisation checkbox as shown in fig. 3.1), a mask of the total foci is created (assuming some maxima were found in the reference ROI). This mask is then used to generate a 32-bit euclidean distance map, which can be created manually using the Process>FindMaxima command and selecting the single points output, followed by process>Binary>Options and choosing 32-bits as EDM (Euclidean Distance Map) output, and then running process>Binary>Distance Map.

After generating the distance map, a threshold is applied to it using a converted colocalisation distance. The maximum distance in micrometers to be considered as colocalised is converted into pixels and used as a threshold. A selection is drawn using the thresholded area and stored in the RoiManager as "colocalised_area_foci[letter]". The complementary ROI is drawn for not colocalised fociA/B areas. If the default colocalisation distance value of -1 is used no colocalised area ROI is generated.

If a foci maximum from the other channel falls within the colocalised area selection (e.g., A for colocalised_area_fociB ROI), it is considered colocalised. This is done by selecting total_fociA (maxima) and colocalised_area_fociB selections and using RoiManager>More> “AND”. It is recommended to use the minimal resolution distance (typically 230-250nm for a 1.4 NA lens and a widefield setup) as the colocalisation distance threshold[1]. This is because if foci A and B were from the same channel and separated by a distance below the minimal resolution distance, they would not be seen as distinct foci. Theoretical minimal resolution distances can be found elsewhere[2] or calculated using, for example, MetroloJQC plugin[3]. Colocalized foci are counted (as shown in fig. 3.3).

After the selections are made, they are assigned to a ROIgroup for future analysis, as illustrated in Figure 3.4.

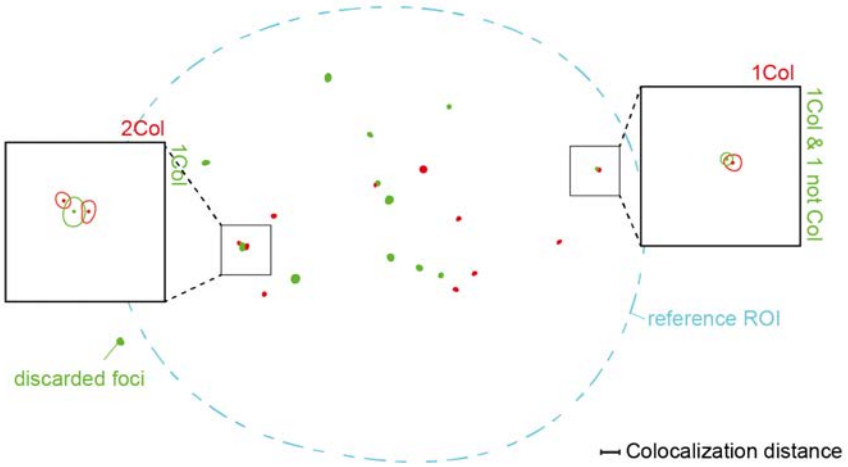


Figure 3.3. An example of colocalized foci. Left inset shows that the number of colocalized foci in one channel can be different in the other channel.

RoiGroup	ROI
1	reference ROI
17	fociA maxima
23	fociB maxima
29	colocalised area fociA (maxima)
35	colocalised area fociB (maxima)
41	not colocalised area fociA (maxima)
47	not colocalised area fociB (maxima)
53	random foci A
59	random foci B
65	fociA maxima colocalised with fociB

71	fociB maxima colocalised with fociA
77	fociA maxima not colocalised with fociB
80	individual fociA maximum colocalised with fociB
82	individual fociA particle colocalised with a fociB
83	fociB maxima not colocalised with fociA
89	random fociA maxima colocalised with fociB
95	random fociB maxima colocalised with fociA
96	individual fociB maximum colocalised with a fociA
98	individual fociB particle colocalised with a fociA
119	fociA particles colocalised with a fociB
125	fociB particles colocalised with a fociA
131	fociA particles not colocalised with a fociB
137	fociB particles not colocalised with a fociA
144	individual fociA maximum not colocalised with a fociB
146	individual fociA particle not colocalised with a fociB
160	individual fociB maximum not colocalised with a fociA
162	individual fociB particle not colocalised with a fociA

Figure 3.4. ROIGroups associated with ROI generated by the macro (colocalisation option selected)

STEP 5. You may want to consider verifying whether the observed colocalised pixels in both channels could have occurred randomly. To do this, you can select the "get colocalisation with random images" option. Please note that this option will not be available if the "compute colocalisation" checkbox is not selected (fig. 3.1).
The process involves shuffling the observed foci number in one channel within the entire reference ROI. The resulting random foci distributions (see fig. 3.5 for examples) are saved in the ROI Manager. You can change the number of random distributions saved in the ROI Manager by using the general parameters menu (see OPTIONAL STEP A).

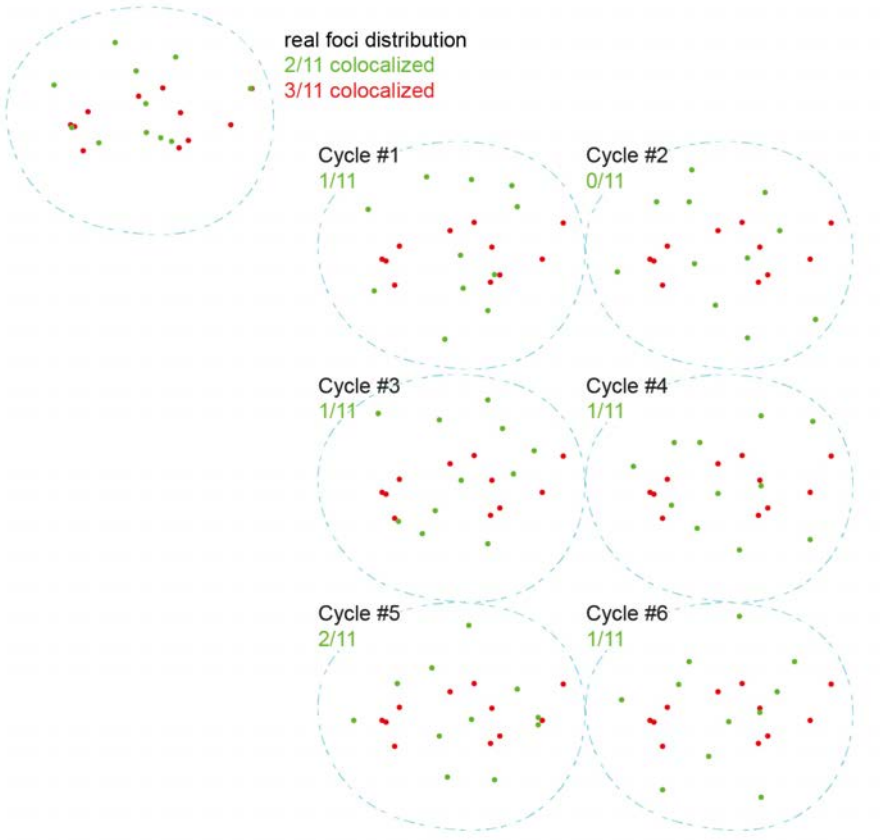


Figure 3.5. Examples of images obtained through 6 randomization cycles of the location of the green foci. The number of green foci randomly colocalized with real, observed red foci is indicated.

Next, each random foci distribution is compared to the observed foci localisation (fig. 3.5) in the other channel using the corresponding euclidean distance map ROI (for example, random fociA_0 (maxima) with colocalised area fociB). You can specify the number of random images to be generated in the "number of randomisation cycles" field (fig. 3.1). It is recommended to use a number of cycles greater than 30 to increase the likelihood of obtaining a normal distribution of randomly colocalised foci.

If the average number of randomly colocalized foci is zero, no further analysis is performed. Otherwise, the mean number of randomly colocalized foci observed for each random image is calculated. If the “Show random distribution vs real value” checkbox gets selected, the distribution of the number of randomly colocalized foci for all random images is plotted (as shown in fig 3.6). The plot is saved as a .tif file in a parent controlData folder and can be opened using ImageJ. You can extract its coordinates (by clicking on the list button, fig. 3.6).
The frequency of the number of randomly colocalised foci is indicated by grey hollow dots. A Gaussian fit is applied (blue line), and the actually observed, real number of colocalised foci is indicated by a red line.

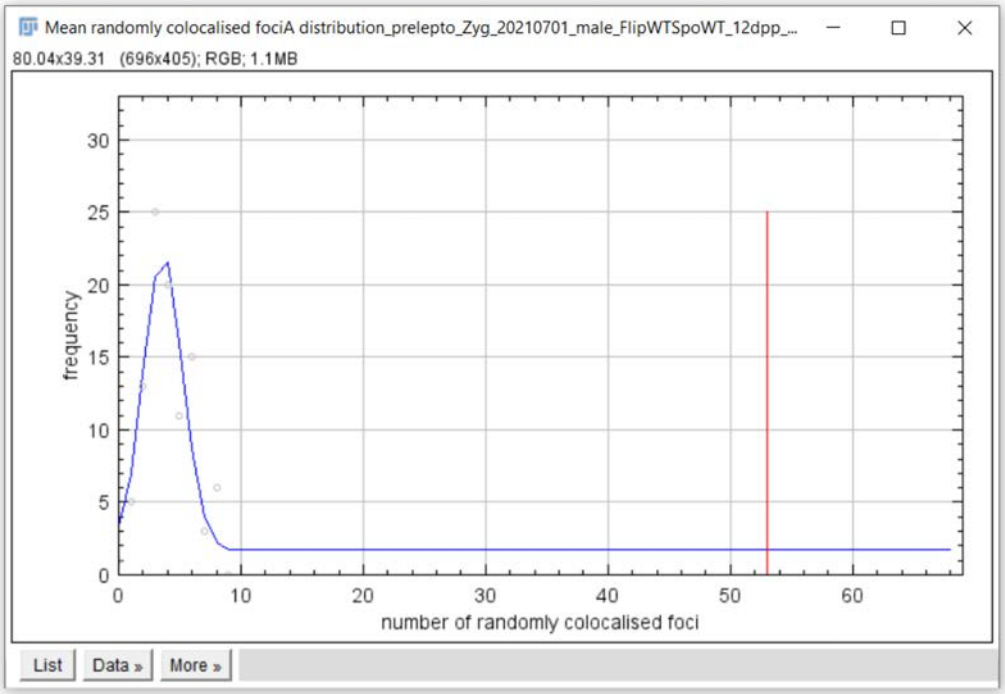


Fig. 3.6. randomly colocalised foci number distribution across all randomly generated images

Assuming a normal distribution, using the 68–95–99.7 statistical rule (also known as the empirical rule) the probability function P states that the probability to have all random values in between μ ± 3σ is 99.7% (here, μ represents the mean of the distribution, and σ represents its standard deviation)

$$P(\mu - 3\sigma \leq X \leq \mu + 3\sigma) \approx 99.73\,\%$$

When the blue Gaussian is clipped, which is depicted in figure 3.7 or the left panel of figure 3.8 (μ - 3σ < 0), the p value is marked with an asterisk. Additionally, a comment "*: take p value with caution" is shown in the "comment" column of the 2foci.xls result file.

The p value is given by the following formula and uses the error function (erf). To calculate the error function, Horner's method is utilized.

$$pValue = \frac{1 + \text{erf}\left(\frac{\text{realColocFoci} - \text{mean}}{\sqrt{2} * \text{stDev}}\right)}{2}$$

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} * \int_0^x e^{-t^2}\, dt$$

The p-value reflects the proportion of the Gaussian curve below the observed colocalized foci number. If the p-value is close to 1, it indicates that obtaining the observed colocalized foci number through random foci distribution within the reference ROI is unlikely (as shown in the middle panel of figure 3.8). In cases where the distribution is clipped or the p-value is not 1 (as illustrated in the right and left panels of figure 3.8), it is recommended to use alternative statistical analysis. To access the random distribution raw values, click on the "List" button as explained earlier and run these other statistical test using these values.

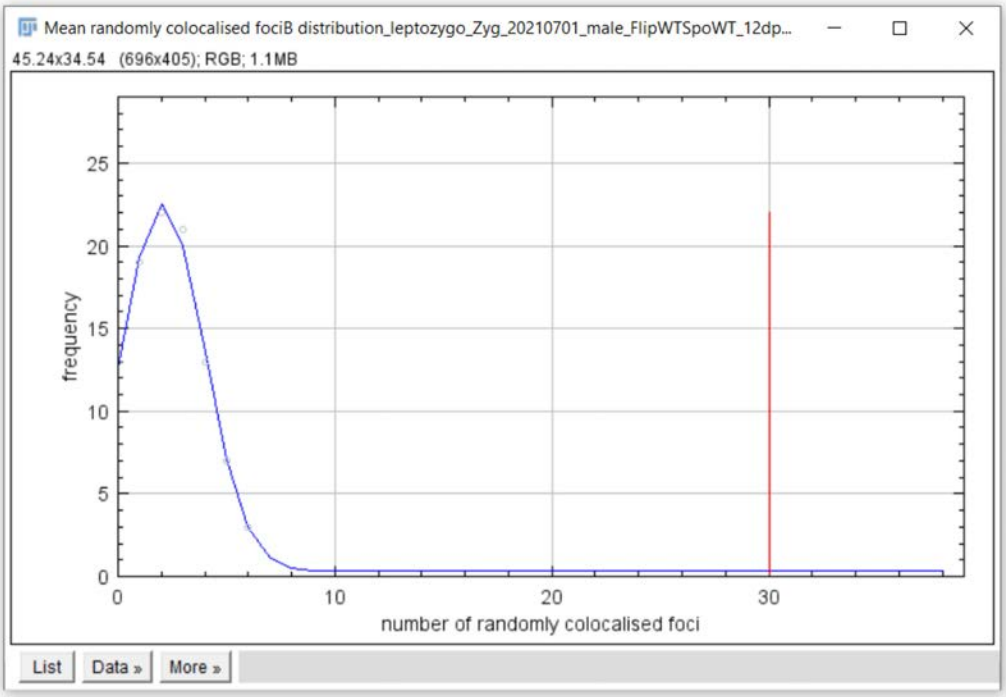


Fig. 3.7 “clipped” randomly colocalised foci number distribution across all randomly generated images

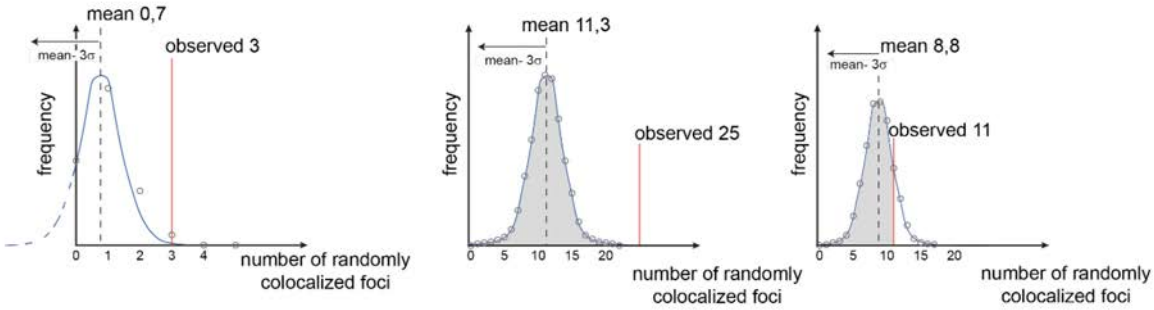


Fig. 3.8. The different possible types of distributions. Left and middle panel : p value=1, right panel pvalue=0,75

STEP 6. Particles are detected by utilizing the Find Maxima options in the reference ROI. The newly created total particle ROI is then split into individual particles, and each individual particle is compared to the total_foci[letter] maxima ROI. This step can be time-consuming. To save time, the "show foci particle intensities" option in the general parameters menu can be deselected (as described in OPTIONAL STEP A). If a split particle contains a maximum, it is assigned a new name, such as fociA_ID (particle).

If both "show individual foci's value" and "show foci maximum intensities" option are enabled in the measurements parameters menu (OPTIONAL STEP A, fig. 3.9) a corresponding maximum point ROI is generated (as shown in the example fociA_ID (maximum)). Both the maximum and the particle share the same ID.

If the "compute colocalisation option" is selected (STEP 4, fig. 3.1) the macro checks for a triple overlap between i) the individual ROI, ii) the corresponding total_foci, and iii) the colocalized area created at STEP 4 using the total maxima of the other channel. If such an overlap exists, the particle (and its associated individual maximum, if generated following the OPTIONAL STEP A configuration) are marked as colocalized using the corresponding ROIGroup (as illustrated in figure 3.4). If no overlap is detected, and the particle overlaps with a maximum ROI (in this case, total_fociA (maxima)), the particle is marked as not colocalized.

If a particle does not contain any maximum, it will be removed from the process (ie selection left with default ROI group 255). The macro will group together particles that are either colocalised or not colocalised to create a ROI of all colocalised or not-colocalised particles.

STEP 7. You can select additional channels to measure intensities in ROIs. You can also choose to measure foci channels A and B, and add another channel for intensity measurements within ROI. If you do not want to do any measurements, leave the numeric field at -1 and uncheck the "add foci channel intensity" box. You can also remove nucleus/user-defined ROI measurements, or show/hide mean or IntDen or particle/maximum intensities by following the OPTIONAL STEP A.

For global particle measurements (as displayed in the 2Foci.xls table), the macro will use the corresponding ROI (e.g. colocalised total_fociA (particles)), and for global maxima calculation, it will measure the corresponding ROI (e.g. total_fociA (maxima)) using the getRawStatistics macro language built-in function. The number of maxima will be multiplied by mean to calculate the IntDen value.

STEP 8. Choose the desired output options. In case the "Save ROIs" option is chosen, all regions of interest (including nucleus and user-defined regions, foci in both channels, and if applicable, colocalized foci in both channels and random foci) will be stored in a related "2foci_RoiSet_[name].zip" file, which will be placed in a parent ControlData folder. The results will be saved in a "2foci.xls" file (which will be located in the same folder where the input images are saved). The result spreadsheet contains all measurements as well as the parameters used, as illustrated in Figure 3.10. Additionally, individual values for foci (if requested) will be saved in a separate "individual2Foci.xls" file.

STEP 9. After clicking OK, the analysis will begin running. If you wish to view the images while processing, you can follow OPTIONAL STEP A.

OPTIONAL STEP A. First click on the "Set measurements parameters" button (fig. 0) before using the "Count foci in two channels and colocalize" tool. This will bring up the Parameters menu (as shown in figure 3.9).

PARAM A1. You can choose whether or not to display warning messages. It is recommended to keep them hidden to avoid blocking the batch processing of the macro.

PARAM A2. By default, the images will be hidden during the analysis. However, if you prefer to view them, you can select the "Show images while processing files" option in the Parameters window (fig. 3.9).

PARAM A3. The default list of thresholds for nucleus, axis and or foci is limited to preset thresholds that have been found to be suitable for test images. If you would like to expand the list to the full range of ImageJ's automatic threshold methods, you can select the "Display the full list of threshold methods" option.

PARAM A4. Foci ROIs can be measured in both maxima/individual maximum or particles/individual particle, and the corresponding tickboxes should be used to restrict the analysis.

PARAM A5. In case of a random colocalisation analysis request, enter the number of random distributions to be displayed/saved in the ROI manager ("display [] random distribution"). This includes the random (axis or off-axis) foci distribution ROI, as well as its corresponding colocalised random foci ROI (ie. the random foci that may colocalise with the real foci distribution in the other channel).

PARAM A6. By default, the intensity measurements type that is measured is IntDen (raw, uncalibrated integrated density). If you would like to add Mean values or remove IntDen, you can use the "Show IntDen values" and "Show Mean values" options.

PARAM A7. In this tool, intensities are calculated in various regions of interest (ROIs). By default, the reference ROI for intensity measurement is triggered, which can either be a user-defined ROI or the nucleus ROI. To remove the user-defined nucleus ROI measurements, uncheck the "show user-defined nucleus ROI measurements" option.

PARAM A8. Individual foci can be measured individually. Select the “Show individual foci’s values” if needed. This will generate an "individual2Foci.xls" file at STEP8.

Note that the length measurements options apply to other tools and can be ignored in this case. Click on OK (A9).

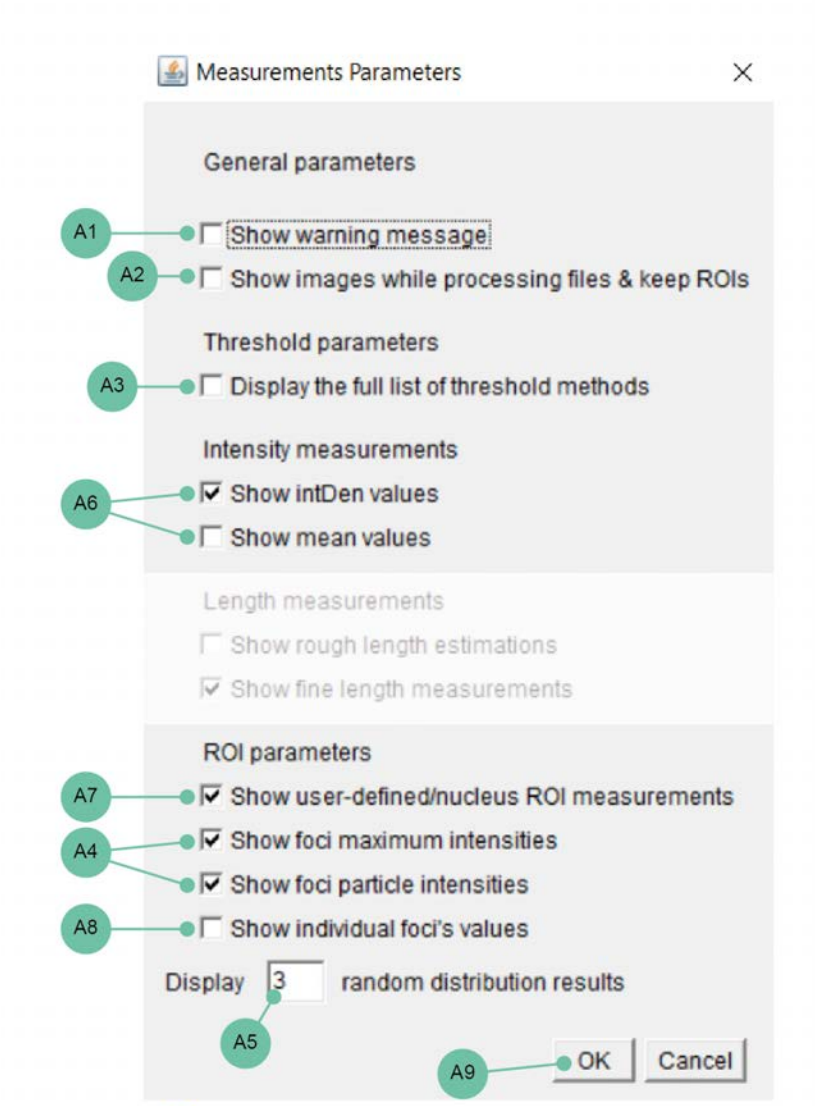


Figure 3.9. The (general) measurements parameters window.

Type	Image name	fociA	fociB	colocalised fociA	colocalised fociB	Mean randomly colocalised fociA	Mean randomly colocalised fociB	Ch.4 intDen (colocalised fociA maxima)	Ch.4 intDen (colocalised fociA particles)	Ch.4 intDen (colocalised fociB maxima)	Ch.4 intDen (colocalised fociB particles)	Ch.4 intDen (not colocalised fociA maxima)	Ch.4 intDen (not colocalised fociA particles)	Ch.4 intDen (not colocalised fociB maxima)	Ch.4 intDen (not colocalised fociB particles)	Ch.4 intDen (not (user-defined ROI)	Comment	Parameter	Value
diplo	diplo_1	72	84	61	61	3.23 (pValue: 1*)	3.29 (pValue: 1*)	5482.130	76742.231	5684.732	124584.128	896.874	8546.085	963.033	13306.032	4024561.850	* take pValue with caution	Smooth original images	no
earlylepto	earlylepto_1	44	70	34	34	3.64 (pValue: 1*)	3.94 (pValue: 1*)	7314.626	99889.117	7903.119	191014.889	2025.060	22345.831	4674.928	78840.708	3240803.007	* take pValue with caution	ROI chosen	user-defined
earlylepto	earlylepto2	18	24	15	15	2.03 (pValue: 1*)	2.26 (pValue: 1*)	2510.761	33849.000	2771.822	46058.852	394.401	2211.098	566.286	11248.254	867725.657	* take pValue with caution	Foci Detection Threshold	IJ_IsoData
leptozygo	leptozygo_1	125	38	30	30	2.5 (pValue: 1*)	2.63 (pValue: 1*)	6228.318	96208.377	6589.464	28918.050	16354.771	188753.558	1082.481	5444.835	8553644.579	* take pValue with caution	Images from	D:\...
leptozygo	leptozygo_2	60	8	2	2	0.25 (pValue: 0.9996*)	0.33 (pValue: 0.9983*)	373.811	5022.456	373.811	1027.725	9475.503	80558.089	840.702	20178.823	5223316.525	* take pValue with caution	ROI saved	yes
lepto	lepto_1	29	40	22	22	3.19 (pValue: 1*)	3.29 (pValue: 1*)	4587.197	68436.181	5020.407	112531.649	955.251	8718.917	3153.293	40732.332	2101302.977	* take pValue with caution	ROI and any other output folder	D:\....IData\
pachy	pachy_1	49	78	38	38	3.95 (pValue: 1*)	3.65 (pValue: 1*)	4056.354	46709.645	4438.437	65570.896	1737.521	13446.102	1576.623	18620.255	2935605.802	* take pValue with caution	options:	
prelepto	prelepto_1	130	61	53	53	4.22 (pValue: 1*)	4.49 (pValue: 1*)	10848.710	165638.214	11523.852	62045.803	12722.623	129137.456	954.055	5689.253	8605528.436	* take pValue with caution	Show warning messages	no
zygo	zygo_1	12	13	9	9	0.35 (pValue: 1*)	0.33 (pValue: 1*)	1992.150	21372.942	2261.714	45349.868	376.853	2848.857	303.338	5040.541	1153467.438	* take pValue with caution	Show images	no
																		Get integrated density intensity values	yes
																		Get mean intensity values	no
																		Include reference Roi when measuring intensities	yes
																		Show foci's Maximum intensities	yes
																		Show foci's particle intensities	yes
																		Meiosis bar tool	Count foci in two channels and colocalize
																		Meiosis bar version	v2.06
																		ImageJ version	1.53t99

Fig. 3.10. a result file of the Count axis foci in two channel and colocalize macro

Type	Image name	focus' ID	focus' channel	Ch.4 intDen (focus' maximum)	Ch.4 intDen (focus' particle)	particleROIIndex	maximumROIIndex	focus' type	focus' colocalisation status
diplo	nuc1	0	A	29.461	547.087	218	294		yes
diplo	nuc1	1	A	95.375	1192.510	219	295		yes
diplo	nuc1	2	A	79.697	477.204	220	296		yes
diplo	nuc1	3	A	104.619	1534.216	221	297		yes
diplo	nuc1	4	A	1.924	795.118	222	298		yes
diplo	nuc1	5	A	78.805	839.638	223	299		yes
diplo	nuc1	6	A	92.002	1502.395	224	300		yes
diplo	nuc1	7	A	54.679	511.685	225	301		yes
diplo	nuc1	8	A	70.698	758.024	226	302		no
diplo	nuc1	9	A	90.971	689.876	227	303		yes
diplo	nuc1	10	A	50.113	1371.468	228	304		no
diplo	nuc1	11	A	67.182	517.431	229	305		yes
diplo	nuc1	12	A	44.274	552.846	230	306		yes
diplo	nuc1	13	A	21.390	1139.601	232	307		yes
diplo	nuc1	14	A	92.193	593.483	233	308		no
diplo	nuc1	15	A	130.857	809.956	234	309		no
diplo	nuc1	16	A	128.372	1143.928	235	310		yes
diplo	nuc1	17	A	51.507	350.188	236	311		yes
diplo	nuc1	18	A	62.437	673.466	237	312		yes
diplo	nuc1	19	A	66.671	710.285	238	313		yes
plo	nuc1	20	A	51.266	341.845	239	314		yes
diplo	nuc1	21	A	41.304	709.890	240	315		yes
diplo	nuc1	22	A	63.869	557.879	241	316		yes

Fig. 3.11. a individual particle result file individual2Foci.xls file of the Count foci in two channel and colocalize macro

STEP 10. In order to control the precision of the analysis, if the "save ROIs" option was selected, the "Start checking results" option should be utilized from the main meiosis bar (fig. 0). This will trigger the appearance of the "Check results" window (as shown in figure 3.12). If you have just executed the macro, then the default folder and method should suffice. However, if you wish to scrutinize the macro's results at a later stage, make sure to specify both the right folder and the "Count axis/off-axis foci in two channels and colocalize" method.

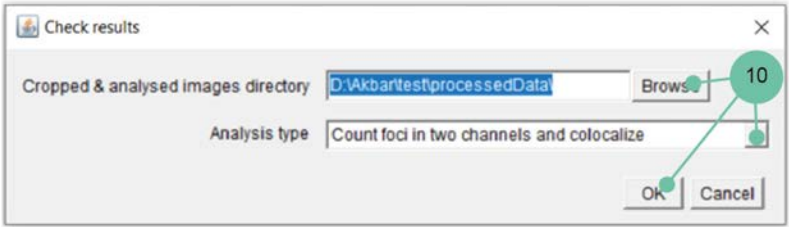


Figure 3.12. The check results menu

Upon opening the first image to be analyzed, the corresponding 2foci_RoiSet_[name].zip file ROI set will also be loaded, as illustrated in figure 3.13. The ROI Manager will display various ROIs, including:

- The reference ROI that was set in STEP 2 (here user-defined ROI). This is the ROI used for further identifications (ie. axes).
- The total foci (maxima) in channel A and B (STEP 3).
- The colocalised area for both channel's maxima, which was obtained in STEP 4.
- Real, colocalised foci for both channels, as obtained in STEP 4.
- If the "get estimation of colocalisation with random images" option is selected, the result of the first three randomisation cycle ("randomFociA/B", STEP 5).
- Depending of the configuration, the individual particles obtained in STEP 6, along with the respective, corresponding maxima. The total particles of interest are also displayed.

To proceed to the analysis of the next image, simply click on the "check next results" button (fig. 0) of the main Meiosis bar.

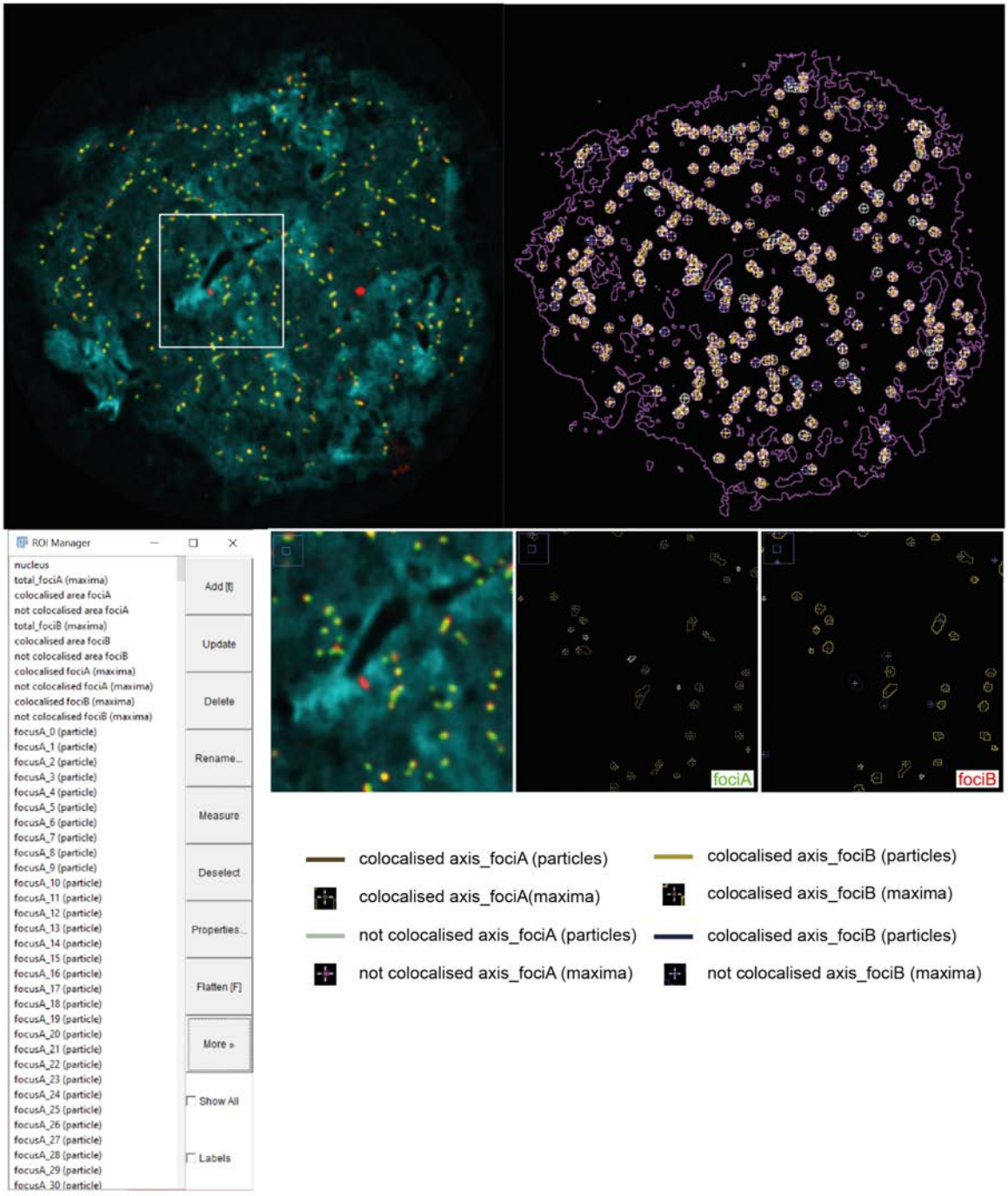


Figure 3.13. Control ROIs. Foci are displayed in both channels, as well as colocalised foci in both channels.

MEASURE AXIS LENGTH

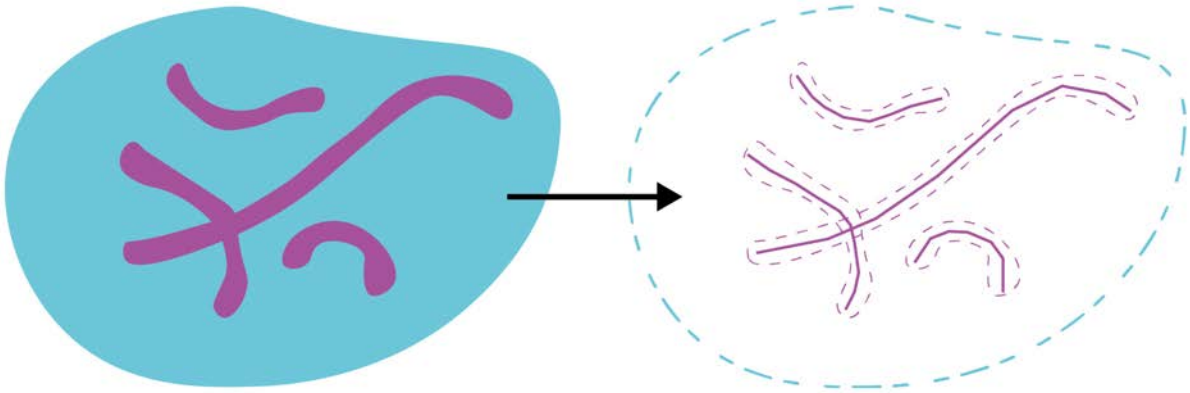


Figure 4.0. Visual summary of the tool.

To measure the length of the axis, you can use the "Measure axis length" tool which identifies the axis and calculates its length. It is advisable to configure additional parameters (such as expanded threshold list or intensity/length measurements types) through an ➔ OPTIONAL STEP A before using the tool. In case you need to prune the axis (see figure 4.2), you can investigate the suitable pruning parameters using the "Set advanced user parameters" tool, as shown in figure 0. Once you select the advanced user mode in the “Measure axis length” tool, you can use these parameters for further analysis.

The algorithm has four steps. First, it will use axis complex staining to locate the axis. Then, it will create a binary mask from the located axis. After that, the mask will be processed to eliminate any unwanted axis branches, which is referred to as the pruning process. Finally, the algorithm will measure the total length of the axis.

From version 2.06, there is no possibility anymore to test various pruning modes. These can be compared using the “set advanced user parameters” tool (fig. 0).

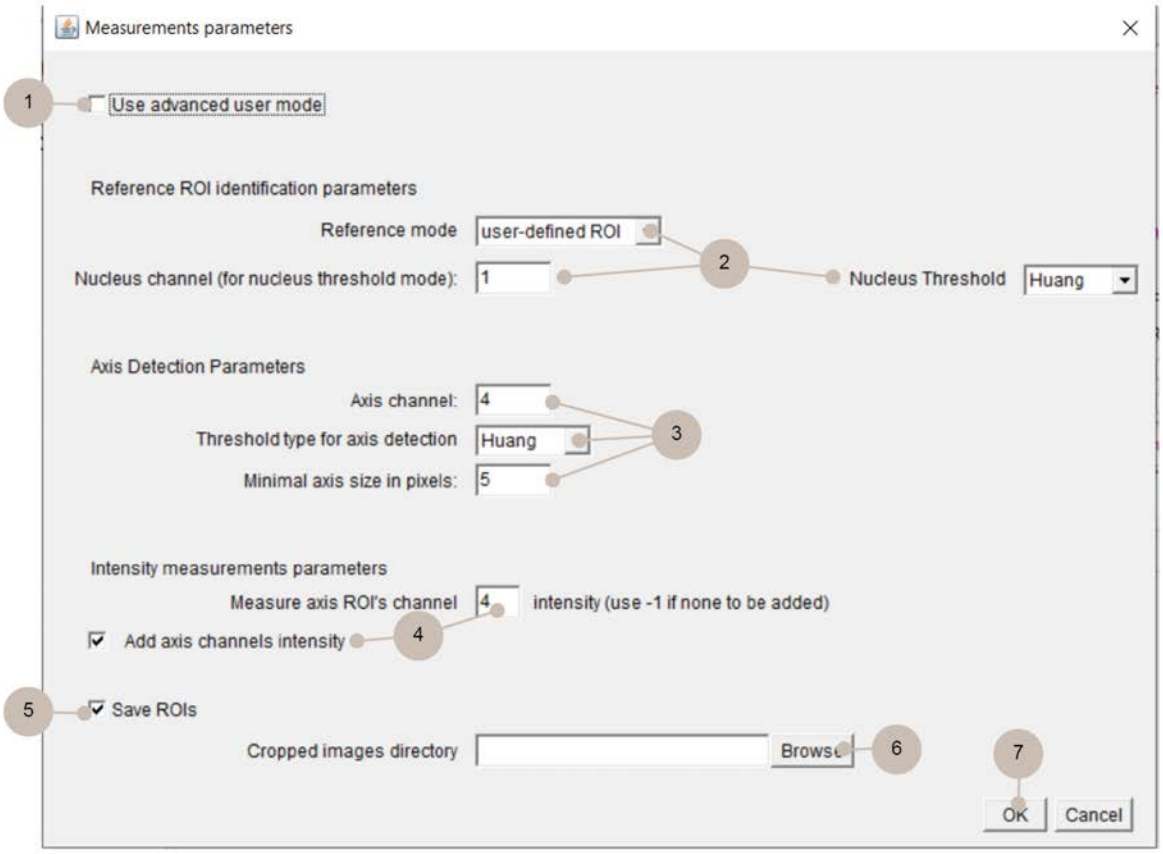


Figure 4.1. The Measure Axis Length menu

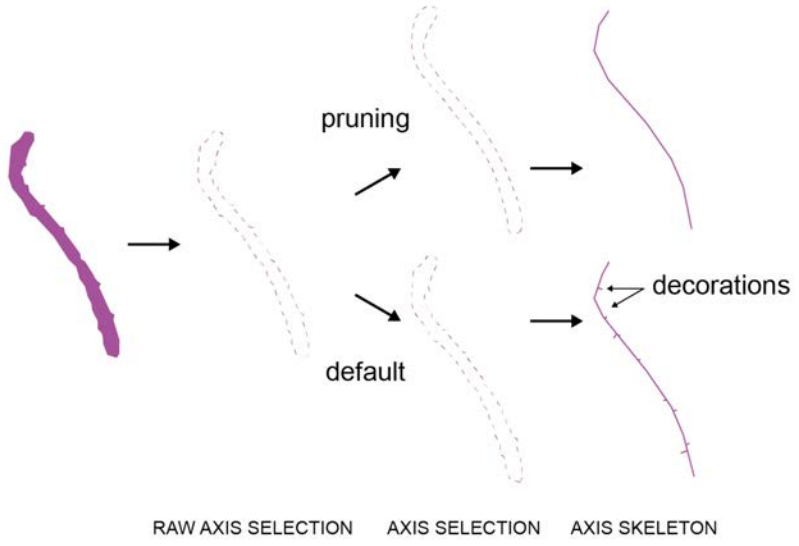


Figure 4.2. Pruning the axis. The analysis of the axis skeleton assumes that its outlines are smooth enough to avoid the detection of unwanted decorations. If the raw axis ROI has a rough, spiky perimeter and pruning is not performed (default, bottom), the resulting axis selection includes decorations, which can bias the skeleton's length measurements. Although the example shown here is relatively mild, decorations can present a significant challenge.

STEP 1. It is advisable to use the advanced user mode when analyzing noisy images, such as those captured using STED setups. To activate this mode (and trigger ➔ OPTIONAL STEP

B), select the "smooth original images" option in the "Advanced user parameters" menu that appears after clicking OK in STEP 7.

STEP 2. Select the region of interest (ROI) to be used for further measurements. If the "user-defined ROI" option is chosen, the companion .roi file generated during stage-cropping will be utilized. If this file is not found, an error message will be displayed. When opting for this reference mode, you can proceed directly to STEP3 without the need to fill in the nucleus channel and threshold fields. Alternatively, an intensity threshold can be employed. The macro uses the term "nucleus threshold," as the channel employed for thresholding is typically based on some (DAPI) nuclear staining. However, this can be applied to any other staining. If the "nucleus threshold" mode is employed, indicate the channel used for thresholding (the first channel is channel 1), then select the appropriate threshold algorithm. To view all the available ImageJ automatic threshold methods, follow OPTIONAL STEP A.
The user-defined or nucleus ROI is referred to as the "reference ROI" below.
Please note that the algorithm suite was initially designed to analyse multiple images containing nuclei. If no threshold can be used to define the ROI and the image features a single cropped nucleus, select the entire image (Ctrl A) as the ROI when employing the cropping buttons.

STEP 3. Configure the parameters for axis detection. First indicate the axis channel number (keeping in mind that the first channel is labeled as channel #1). Set the appropriate threshold value to be utilized. The algorithm uses this threshold to detect any pixel above the threshold value. If no pixel above the threshold is detected, the analysis is terminated, and the message "no axis detected" will appear in the final table. The threshold generates a temporary "axis threshold" selection in the ROI Manager. The axis elements are then detected utilizing the "Analyze Particle" command and the nucleus/user-defined ROI.

Specify the minimum pixel size for thresholded axis elements. Use this value to exclude unwanted, small items. However, increasing this value too high may not be appropriate for early stages. All particles that satisfy the criteria are identified and added to the ROI Manager (additional criteria may be included in advanced user mode, see OPTIONAL STEP B, see figure 4.3). These particles are then merged together (using the combine ROI Manager option) in a temporary ROI. In instances where overlapping axes produce a loop, the axes are not detected correctly, and the space within the loop is considered as part of the axis. To correct this, apply the "AND" option of the ROI Manager to the "axis threshold" ROI and the temporary ROI (figure 4.4). Both ROIs are subsequently deleted, and a "raw whole axis" ROI is included in the manager. If the advanced user mode is not selected, the raw axis is retained, and an axis ROI similar to the raw axis is created. If a pruning method is employed in OPTIONAL STEP B, the raw axis mask is modified. The skeleton analysis is performed utilizing the "skeletonize 2D/3D" plugin and the modified processed mask (note that the plugin must be installed if not using Fiji).

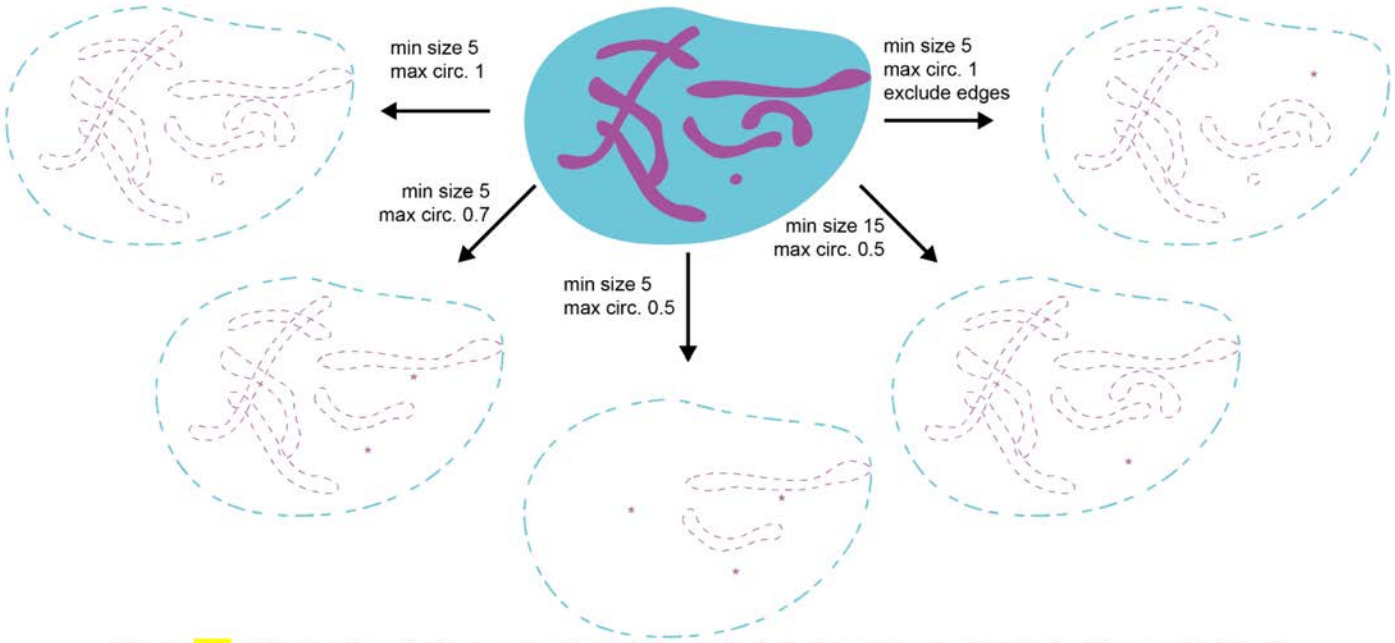


Figure 4.3. Effects of exclusion parameters. The excluded elements are denoted with an asterisk. By default, the minimum size in pixels can be increased to get rid of unwanted elements, as shown in the comparison between the left and lower-right drawings. In addition, advanced user parameters allow for further exclusion criteria. For instance, the maximum circularity can be used to remove round-shaped objects, as seen in the comparison between the left and lower drawings, but this may also result in the removal of clusters of axes (as depicted in the lower-left drawing). The use of edge exclusion (as shown in the right drawing) offers additional possibilities for exclusion.

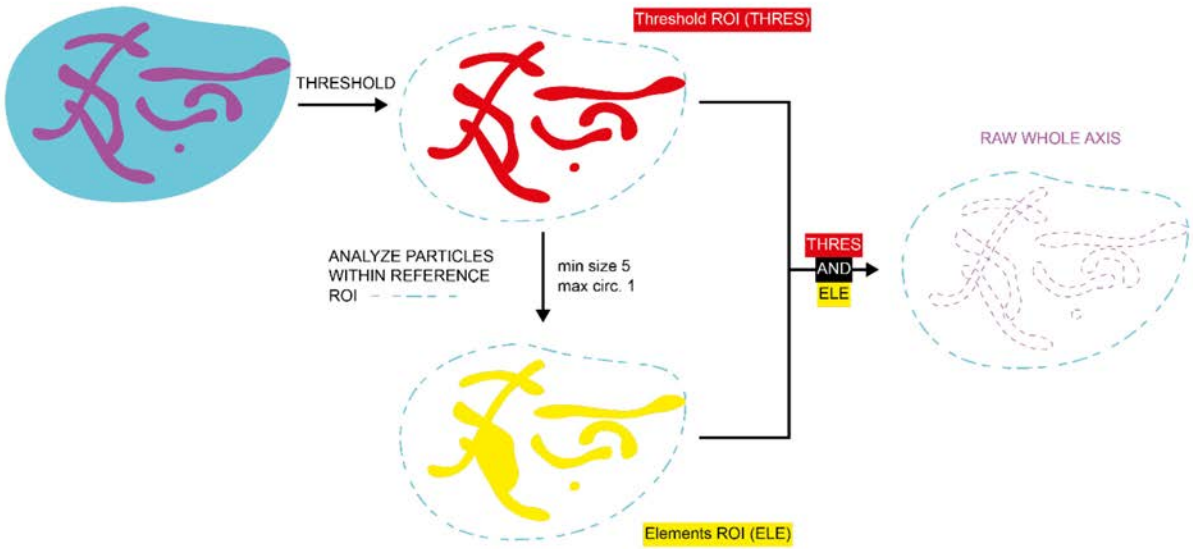


Figure 4.4. Process of axis elements detection. The staining of the axis is first thresholded, and the "analyze particles" command is used to identify axis elements. However, to exclude axis loops (such as the three overlapping axes in the lower left corner of the nucleus), additional processing is required. This involves combining temporary threshold ROI and fused elements selection.

➔ OPTIONAL STEP B: To improve the detection of the axis in your analysis, you can use the "advanced user mode option". When you select this option and click "OK" at STEP 7, a new menu will appear.

STEP 4. You can set additional intensity measurements channels or regions of interest (ROIs). By default, the program will user reference and axis ROIs. You can measure the intensities in both ROIs and for selected channels. If you want to measure the axis channel, select "add axis channels intensity". If you want to add another channel for intensity measurements within the ROIs, enter a value in the numeric field "Measure axis ROI's channel intensity". If you leave the field with -1 and do not select the "add axis channels intensity" checkbox, no measurements will be done. If you want to remove the nucleus or user-defined ROI measurements, you can follow an OPTIONAL STEP A.

STEP 5. Set the output items. If the "saveROIs option is ticked, all ROIs generated during the analysis will be saved in a companion .zip file. The file will be located in a parent controlData folder. Each ROI set will be saved using the image's name and a "lengths-RoiSet" prefix. Whether you use this option or not, all measurements will be found in a lengths.xls file saved in the folder you selected in STEP 7 (an example of such a file is shown in figure 4.10).

STEP 6. Change or fill in the cropped-images directory using the browse button.

STEP 7. Click "OK" to start the analysis. If you selected the "Use advanced user parameters" option in STEP 1, an advanced user parameter window will pop up (described in OPTIONAL STEP B). Otherwise, the analysis will start. The program runs in batch mode, but if you want to display the images while processing, you can follow an OPTIONAL STEP A.

OPTIONAL STEP A. Before using the "Measure axis length" tool, you may set the measurements parameters options by clicking on the "Set measurements parameters" button (fig. 0). This will bring up the Parameters menu (Figure 4.5).

PARAM A1. You can choose whether to display warning messages during the analysis. It's best to hide them as they can block the batch processing of the macro.

PARAM A2. By default, the program will hide the images during the analysis (BatchMode), but you can select the "show images while processing files" option in the parameters window to display them.
PARAM A3. The default list of threshold methods (for nucleus and axis) is limited to preset methods that are suitable for test images. If you want to expand the list to include the full ImageJ's automatic thresholds list, select the "Display the full list of threshold methods" option.

PARAM A4. You need to select the lengths measurement type. There are two different routines available (fig. 4.6):

- The first (rough) one takes advantage of the 1 pixel thickness of the skeleton. It measures the total area in pixels of the ROI and converts it to micrometers using the image's calibration. This may result in weird results because a diagonal pixel will be considered as 1 (e.g., 0.25um if the pixel width is 0.25um) although it should be considered as the square root of 2 (i.e., approximately 0.35um).
- The second (fine) one is a raw measurement of the skeleton (and uses Volker Baecker's macro based on some described algorithm[4]). It assumes a pixel aspect ratio of one (same pixelWidth and pixelHeight values). Lengths are calculated from the middle of one pixel to the middle of another pixel.

You can remove fine or rough length measurements by unselecting "show rough length estimation" or "show fine length measurements," respectively.

PARAM A5. The default intensity measurement (type) is set to Integrated Density (raw IntDen). You can add Mean values or remove IntDen by using the "Show IntDen values" and "show Mean values" options.

PARAM A6. Measurements of intensities are taken in various regions of interest (ROIs). By default, the reference ROI (which can either be user-defined or the nucleus ROI) is used to measure intensity. To remove this, deselect "show user-defined nucleus ROI measurements". Ignore the other option that apply to foci tools.

Once all parameters are set, click on OK (A7).

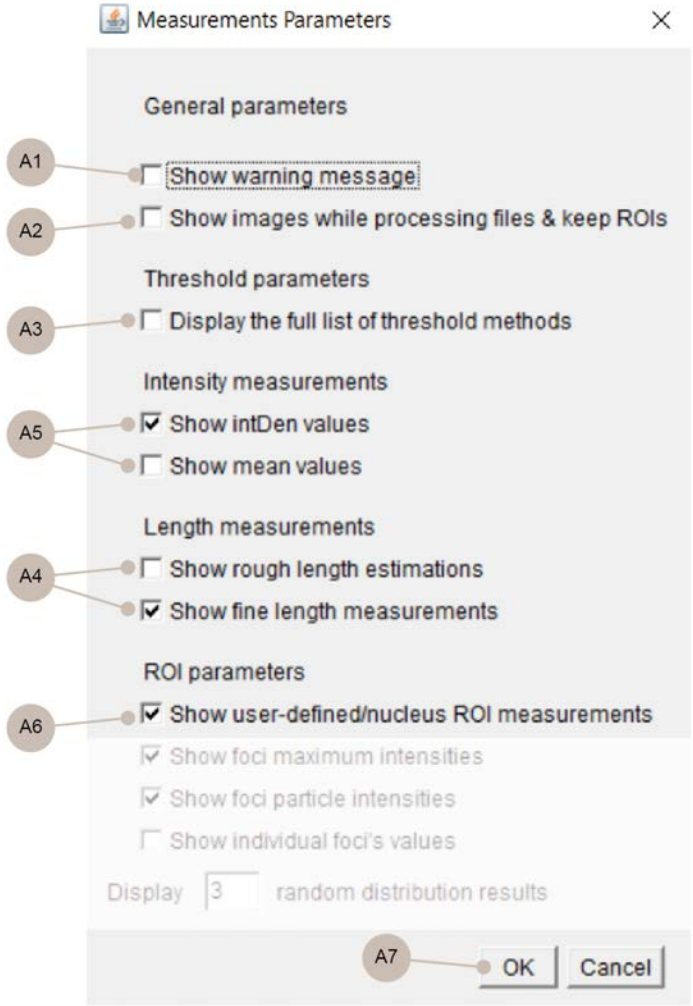


Figure 4.5. The (general) parameters window.

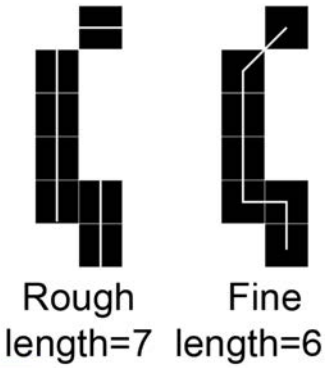


Figure 4.6. Rough and fine measurements modes.

OPTIONAL STEP B. Advanced user options can be used to improve the accuracy of detecting the axis in images. This is especially helpful for images that have a lot of noise or those that are super-resolution. If you choose to use the advanced user options, a window called "Advanced user parameters" will appear at STEP 7 (as shown in Figure 4.7).

OPT. STEP B1. A checkbox labeled "smooth original images" that can be ticked to remove noise from the image. This filter replaces each pixel with the average of its 3x3 neighbourhood and applies to all color channels.

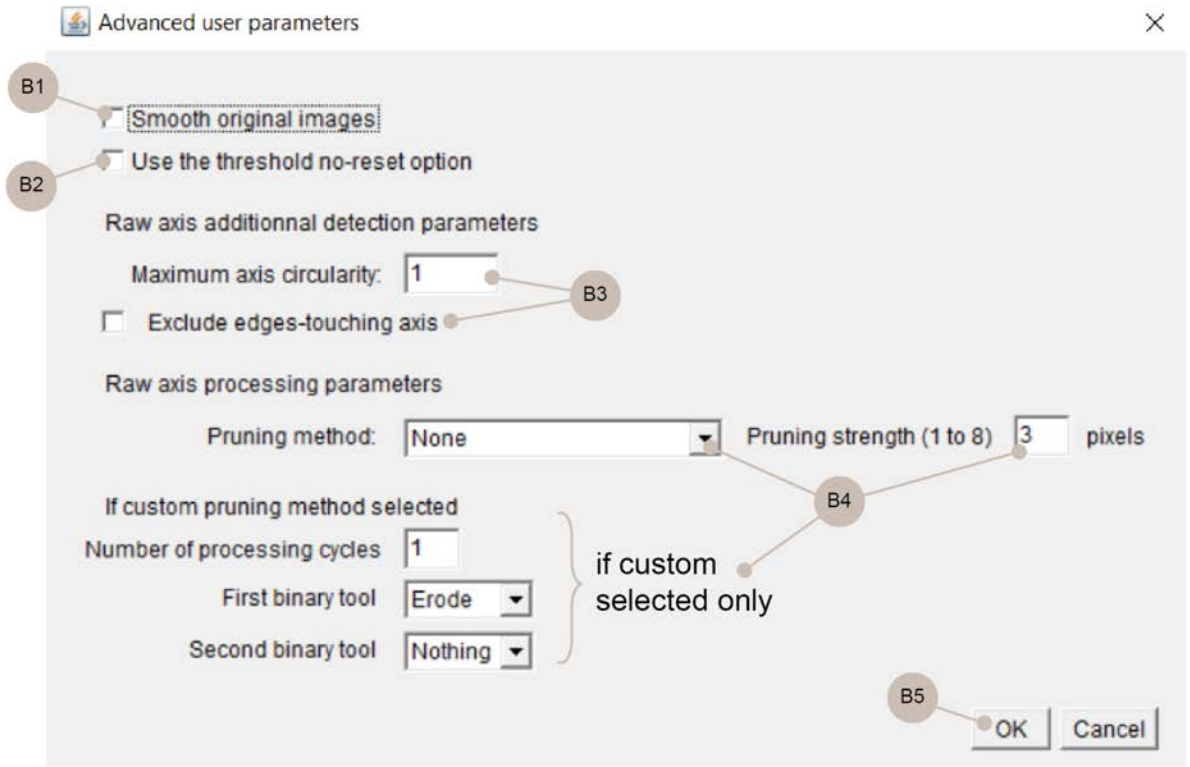


Figure 4.7. The Advanced user parameters menu

OPT. STEP B2. Detecting axes in images with a high offset value, such as Abberior .msr files, can pose challenges. The presence of extremely high background intensity pixels and excluded pixels with intensities set to 0 during cropping can introduce bias in the automatic

thresholding process, as depicted in figure 4.8. To address this issue, the "use the threshold no reset option" is provided.
Different versions of ImageJ, ranging from 1.52e to 1.53s, have gone through various implementations, retractions, and re-implementations of autothreshold options. The later versions have introduced a stable threshold mode called "don't reset range" in the Image>Adjust Threshold command. Thresholding problems may arise when working with images that have more than 8 bits because the automatic threshold methods use an 8-bit histogram of the image. To do this, the range of the 16-bit image (0-65535) is converted to an 8-bit range (0-256), and then the threshold is calculated based on the 8-bit histogram.
Let's consider an example of a 16-bit .msr file displayed using the Image>Adjust>Brightness&Contrast auto display range (figure 4.8). In this case, the black intensity value is 32767 (representing the minimal intensity in the ROI, with the background spanning from 32767 to 32769), and the white intensity value is 32790 (representing the maximal intensity in the ROI). The axis signal is between 32770 and 32790. When the "reset" option (the default mode in Adjust Threshold, left hand side of figure 4.8) is selected, the range extremes are converted to a threshold range of 0 and 256. Consequently, the automatic threshold encounters two intensity clusters: the clipped area and the rest (background+signal). It finds a threshold value that fails to distinguish the background from the signal within the reference ROI, as indicated by the red area in the histogram displayed in the middle left panel of figure 4.8. As a result, the entire reference ROI is thresholded, and the axis ROI erroneously resembles the reference ROI.
On the other hand, when the "don't reset" option is chosen, the displayed extremes are converted to a threshold range of 32767 to 0 and 32790 to 256. The (discrete) 8-bit histogram is then computed (middle right panel of figure 4.8), and automatic threshold values are calculated within the red range on the histogram. Consequently, the axis is correctly thresholded, and the axis ROI accurately represents the axis.

OPT. STEP B3. To improve the axis detection, you may need to adjust some parameters and apply a pruning method (see figure 4.3).

- You can set the maximal circularity, which controls the roundness of the detected objects (of the Analyze>Analyze particle command of ImageJ). A value close to 1 means more circular, while a value close to 0 means more elongated. Lowering the circularity can help remove non-axis artifacts, but if axes overlap, you may want to keep some round aggregates by setting the maximum circularity to 1.
- Another option is to “exclude edge-touching axis”, which removes axis signals that touch the border of the image or a user-defined region of interest (ROI). This option can have side-effects when dealing with cropped nucleus images with many overlapping axes.

OPT. STEP B4. After adjusting these parameters, you can apply a pruning method to remove unwanted decorations from the raw axis outlines (see figure 4.2). This is particularly important for superresolution images, where small details can affect the accuracy of the measurements. By default, no pruning is applied (“None”). Four pre-set pruning options are available, including, erosion (shrinking), dilation (enlarging), or a combination of erosion and dilation to smooth or fill holes. Alternatively, you can specify custom pruning parameters. The pruning method generates a binary mask based on the raw axis ROI and applies the selected operations to it. The macro uses the process>binary>options command.
In Figure 4.9, the parameters used for 2x Erosion and a strength of 3 are displayed. The count value in the options command represents the number of adjacent background or foreground pixels required for erosion (=a pixel is removed from the edge) and dilation operations (before

a pixel is added to the edge), respectively. If a count value less than 1 or greater than 8 is entered, it is automatically overridden and a count value of 3 is used instead. Additionally, the pad edges when eroding and black background options are utilized (from version 2.06).
If you select the Custom pruning method, you can set the number of cycles and choose the “binary tool” to be used. For example, the "2x Erosion then 2x Dilation" method applies two erosions followed by two dilations. To find the best parameters for the custom pruning method, you can use the "set advanced user parameters" button (fig. 0). Finally, a "whole axis" ROI is drawn using the processed mask with the pruning method in parentheses.

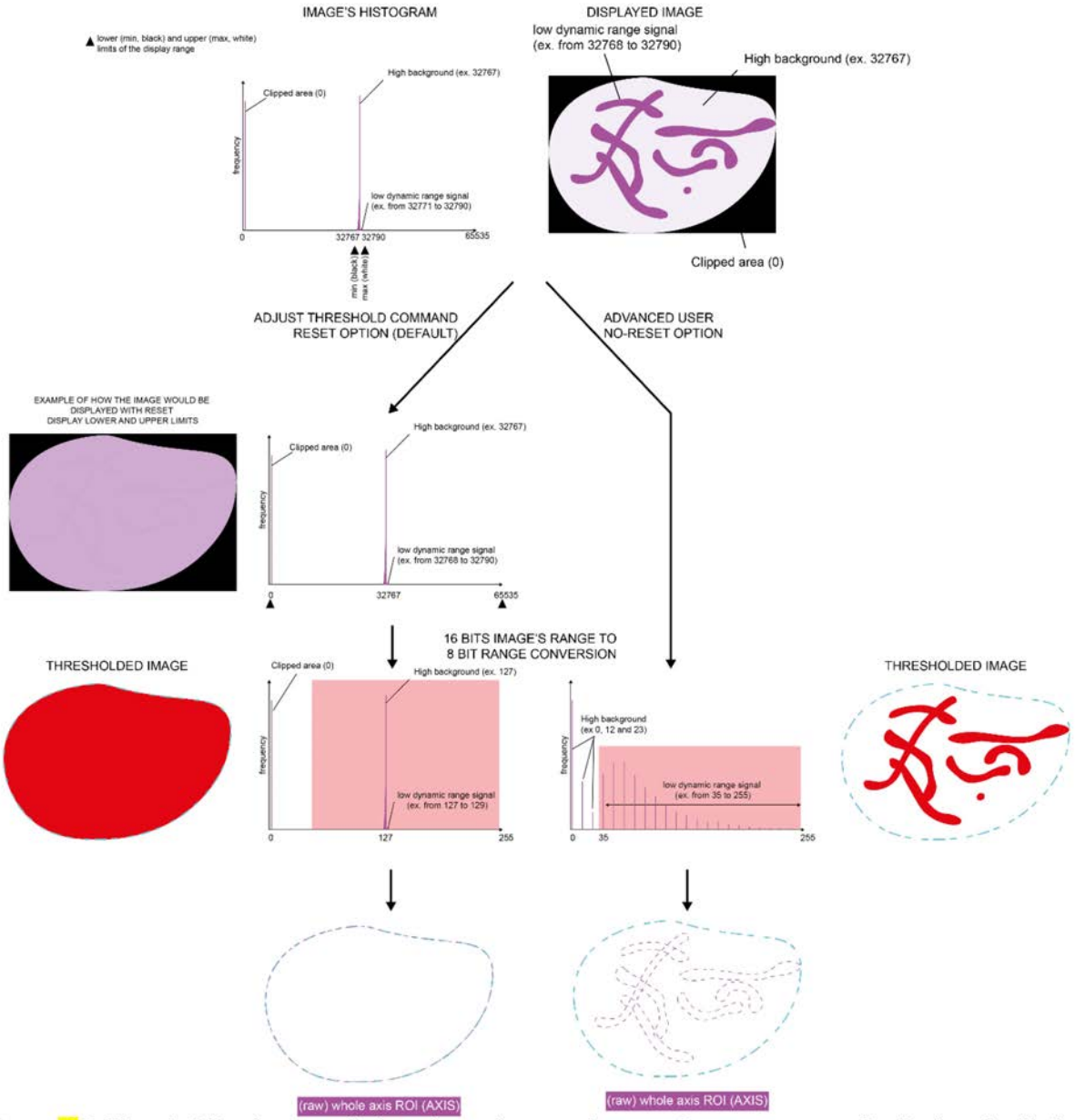


Figure 4.8. Thresholding issues with low dynamic range images. Images are usually displayed with the "auto" min/max display option (Image>Adjust>Brightness&Contrast). When these images are cropped, the clipped-out area outside the reference ROI (set to intensity 0) can interfere with axis detection. This interference occurs because the automatic thresholding process utilizes a default reset range option (shown on the left). The original histogram considered for thresholding is a full-range histogram spanning from 0 to 65535 (if using 16-bit images). An example demonstrates how the image would appear with such display parameters (displayed in the middle left of the figure). When this original histogram gets converted into a 8 bits histogram, pixels in the clipped area with a value of 0 are taken into consideration in the calculation of the automatic threshold. If the dynamic range of the signal, in comparison to the neighbouring background, has similar values that differ significantly from 0, the

entire reference ROI is incorrectly thresholded, resulting in the axis ROI being similar to the reference ROI. However, by utilizing the "no-reset" option, the displayed histogram “is employed as input” for the conversion from 16 bits to 8 bits, which is performed before the threshold calculation. This approach enables accurate axis detection.

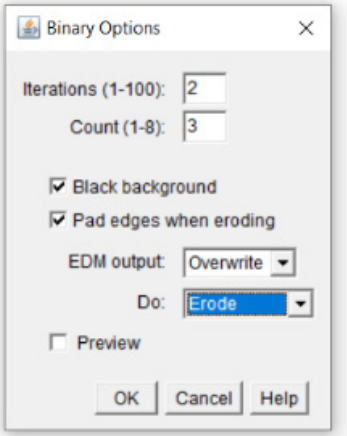


Figure 4.9. An example of how the pruning method “2x Erosion” translates in ImageJ.

OPT. STEP B5. Click on OK to proceed to the analysis.

LIMITATIONS. Intensity values are typically measured without taking into account any noise, offset, or background. When using the full dynamic range of the camera (for example, 0-65535 gray levels for 16-bit images) in widefield cases, the impact of camera/system noise and offset values is usually negligible. However, when working with low intensity or reduced dynamic range images, some level of correction for intDen/mean values is necessary, which involves measuring an empty area and using specific formulas (see below). It's important to consider the impact of background signal as well, as it can vary across the image. To address this, it is recommended to process the images before applying the macro (e.g. use a deconvolution suite).

$$mean_{corr} = mean_{measured} - mean_{empty}$$

$$intDen_{corr} = \left(mean_{measured} - mean_{empty}\right) * \frac{intDen_{measured}}{mean_{measured}}$$

Type	Image name	fine whole axis length (um)	whole axis ch. intDen (whole axis)	Ch.2 intDen (whole axis)	whole axis ch. intDen (user-defined ROI)	Ch.2 intDen (user-defined ROI)	Comment	Parameter	Value
zygo	zygo_1	325.894	4407702.530	901446.647	5515314.368	1710177.183	(pruning Mode: 2x [Erosion & Dilation])	Smooth original images	no
zygo	zygo_2	440.169	4485457.609	1419242.416	5120993.754	2070002.423	(pruning Mode: 2x [Erosion & Dilation])	ROI chosen	user-defined
zygo	zygo_3	333.267	6862647.033	1607897.090	8600599.392	2792088.185	(pruning Mode: 2x [Erosion & Dilation])	Axis channel	4
zygo	zygo_4	384.826	6318011.906	1388608.317	7527318.495	2292151.888	(pruning Mode: 2x [Erosion & Dilation])	Axis Threshold	Huang
zygo	zygo_5	369.970	3585851.032	1189319.729	4375776.640	1894090.157	(pruning Mode: 2x [Erosion & Dilation])	Axis Min size	5
zygo	zygo_6	350.582	3912245.646	1352920.585	4889160.614	2423823.104	(pruning Mode: 2x [Erosion & Dilation])	Axis Max Circ.	1.0
zygo	zygo_7	346.770	4858535.493	1191250.951	6062264.827	2167817.374	(pruning Mode: 2x [Erosion & Dilation])	Exclude edge-touching axis	no
zygo	zygo_8	406.972	6040271.254	4273980.159	6890604.551	5618196.062	(pruning Mode: 2x [Erosion & Dilation])	Measure axis length	yes
zygo	zygo_9	455.253	4581903.253	2421729.987	5118501.665	3062661.525	(pruning Mode: 2x [Erosion & Dilation])	Measure axis' ROI intensity	Ch. 2 & axis channel (Ch.4)
zygo	zygo_10	407.866	4664706.207	2671697.646	5344615.224	3551410.511	(pruning Mode: 2x [Erosion & Dilation])	Pruning Mode(s) tested	2x [Erosion & Dilation]
zygo	zygo_11	294.242	4023362.562	2017148.465	4716818.410	2817523.158	(pruning Mode: 2x [Erosion & Dilation])	Pruning Strength (when used)	3
zygo	zygo_12	485.111	5060483.390	3144322.184	5947987.696	4741530.098	(pruning Mode: 2x [Erosion & Dilation])	Images from	D:\...
zygo	zygo_13	367.896	4723597.662	984761.731	5331780.460	1486174.922	(pruning Mode: 2x [Erosion & Dilation])	ROI saved	yes
zygo	zygo_14	330.581	2827688.455	605504.449	3437457.734	1057289.988	(pruning Mode: 2x [Erosion & Dilation])	ROI and any other output folder	D:\...
								options:	
								Show warning messages	no
								Show images	no
								Get integrated density intensity values	yes
								Get mean intensity values	no
								Get rough length values	no
								Get fine length values	yes
								Include reference Roi when measuring intensities	yes
								Meiosis bar tool	Measure axis length
								Meiosis bar version	v2.05
								ImageJ version	1.53t99

Figure 4.10. a result file of the Measure axis length macro

STEP 8. In order to ensure precise analysis, when opting to save ROIs, you can utilize the "Start checking results" feature in the main meiosis bar (fig. 0). This will bring up the "Check results" menu (refer to figure 4.11). If you ran the macro, both the right images folder and tool are automatically displayed. However, if you wish to oversee the results of the macro later on, you should adjust both the folder and select the "Measure axis length" analysis type.

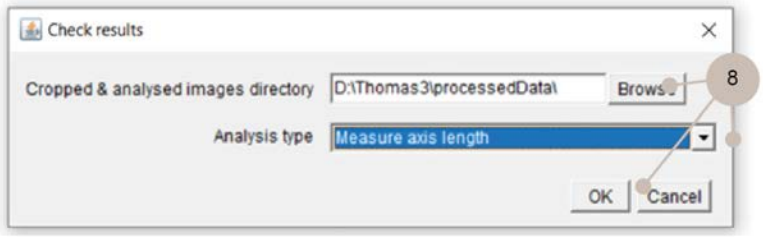


Figure 4.11. The check results menu

The first image analysed will be opened together with its companion ROI set (lengths_RoiSet_[name].zip), as in fig. 4.12. There are a few different ROIs in the ROI Manager that are important to control:

- The "reference ROI" is the one you chose in STEP 2, and it's used as a basis for identifying other important parts of the image (like axes).
- The "raw whole axis" is the original ROI before any extra processing (OPTIONAL STEP B).
- If a pruning method is set (OPTIONAL STEP B), then the raw whole axis and whole axis will be different. In the example image (fig. 4.12), the method called "2x Erosion" was used.
- The corresponding skeleton ROI for the whole axis

To move on to the next picture, just click the "check next results" button on the main Meiosis menu (fig. 0).

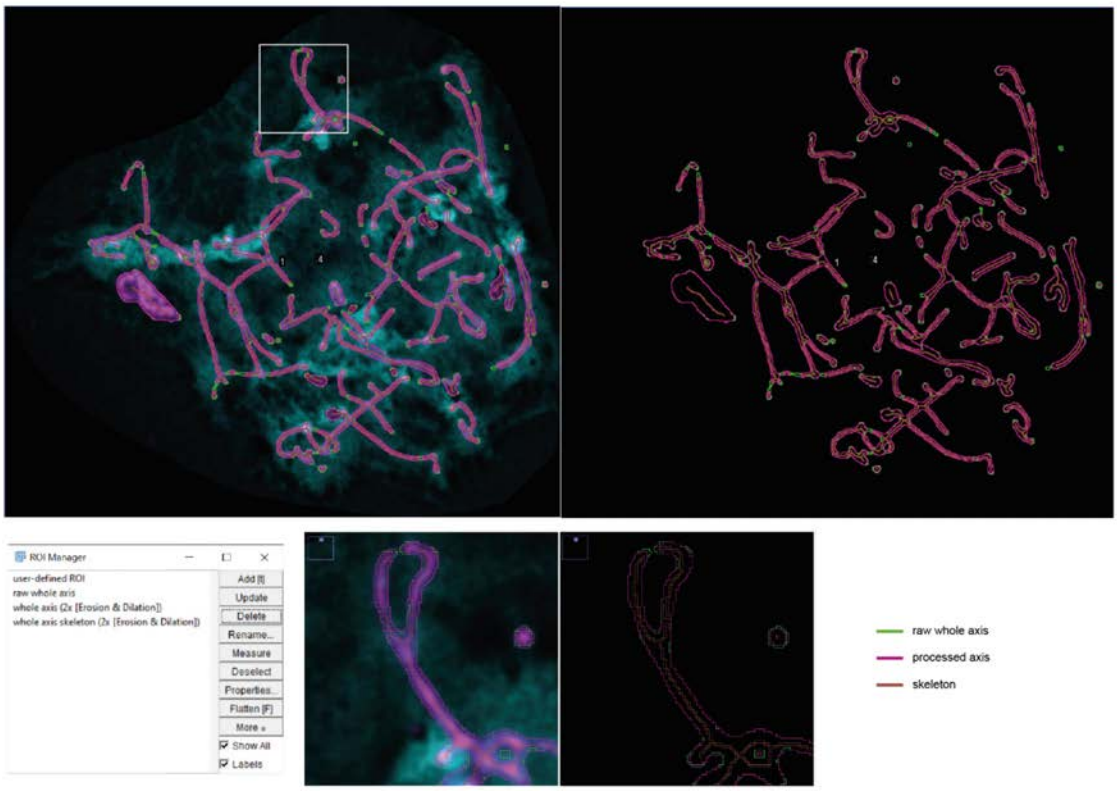


Fig. 4.12. The resulting ROIs produced by the Measure Axis Length macro.

MEASURE SYNAPSE LENGTH

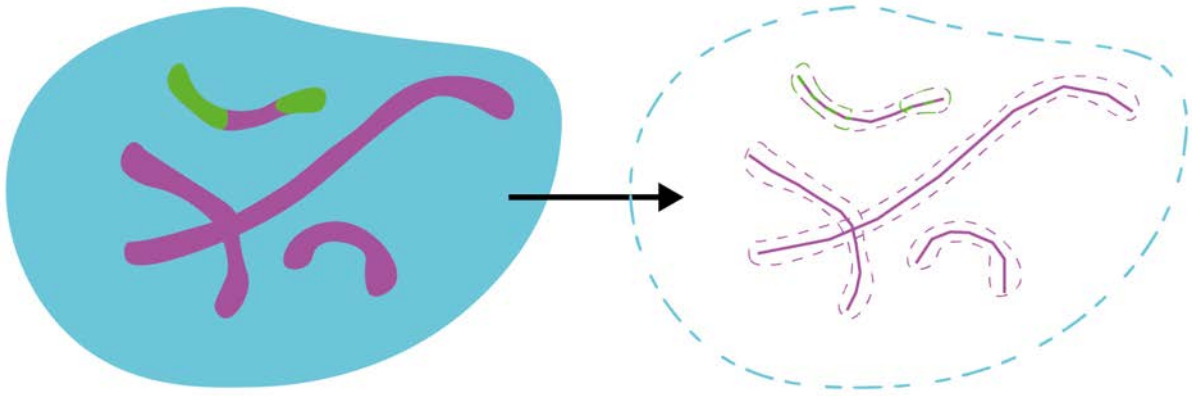


Figure 5.0. Visual summary of the tool.

The tool, that identifies the axis and its synapsed or non-synapsed sections, can be further customized by configuring additional parameters (such as expanded threshold list or intensity/length measurements types) through an ➔ OPTIONAL STEP A. To do so, first click the “Set Measurements Parameters” button (as shown in figure 0). If you need to prune the whole axis, as well as the synapsed and non-synapsed sections (as depicted in figure 5.1), you can explore the appropriate pruning parameters using the "Set advanced user parameters" tool, as illustrated in figure 0. Once you select the advanced user mode in the “Measure synapse length” tool, you can apply these parameters for further analysis.

The “Measure synapse length” process involves several steps: first, the algorithm will use axis complex staining to locate the raw whole axis. It will then create a binary mask from the axis and use a pruning process to remove unwanted "branches" from the mask (advanced user mode). After this, the total length of the axis will be measured. These steps will repeated to distinguish between synapsed and non-synapsed axes. Additional intensity measurements are available depending on the type of axis (whole, synapsed, or unsynapsed).

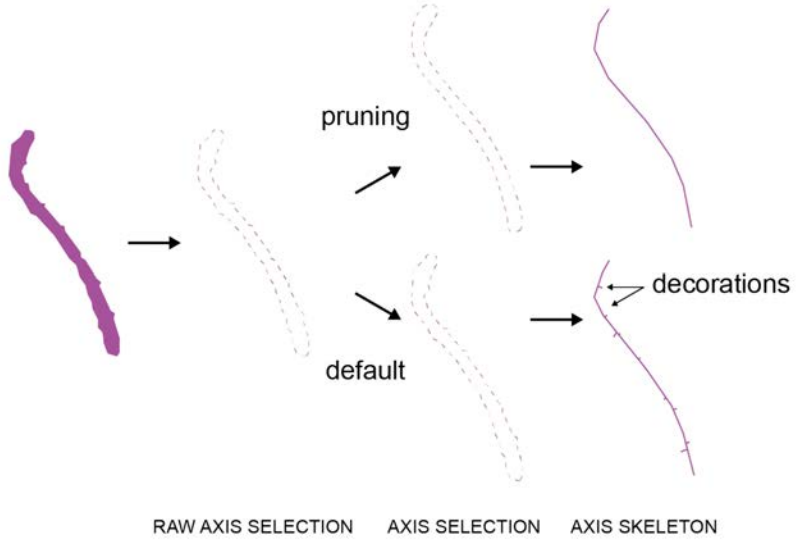


Figure 5.1. Pruning the axis. The analysis of the axis skeleton assumes that its outlines are smooth enough to avoid the detection of unwanted decorations. If the raw axis ROI has a rough, spiky perimeter and pruning is not performed (default, bottom), the resulting axis selection includes decorations, which can bias the skeleton's length measurements. Although the example shown here is relatively mild, decorations can present a significant challenge.

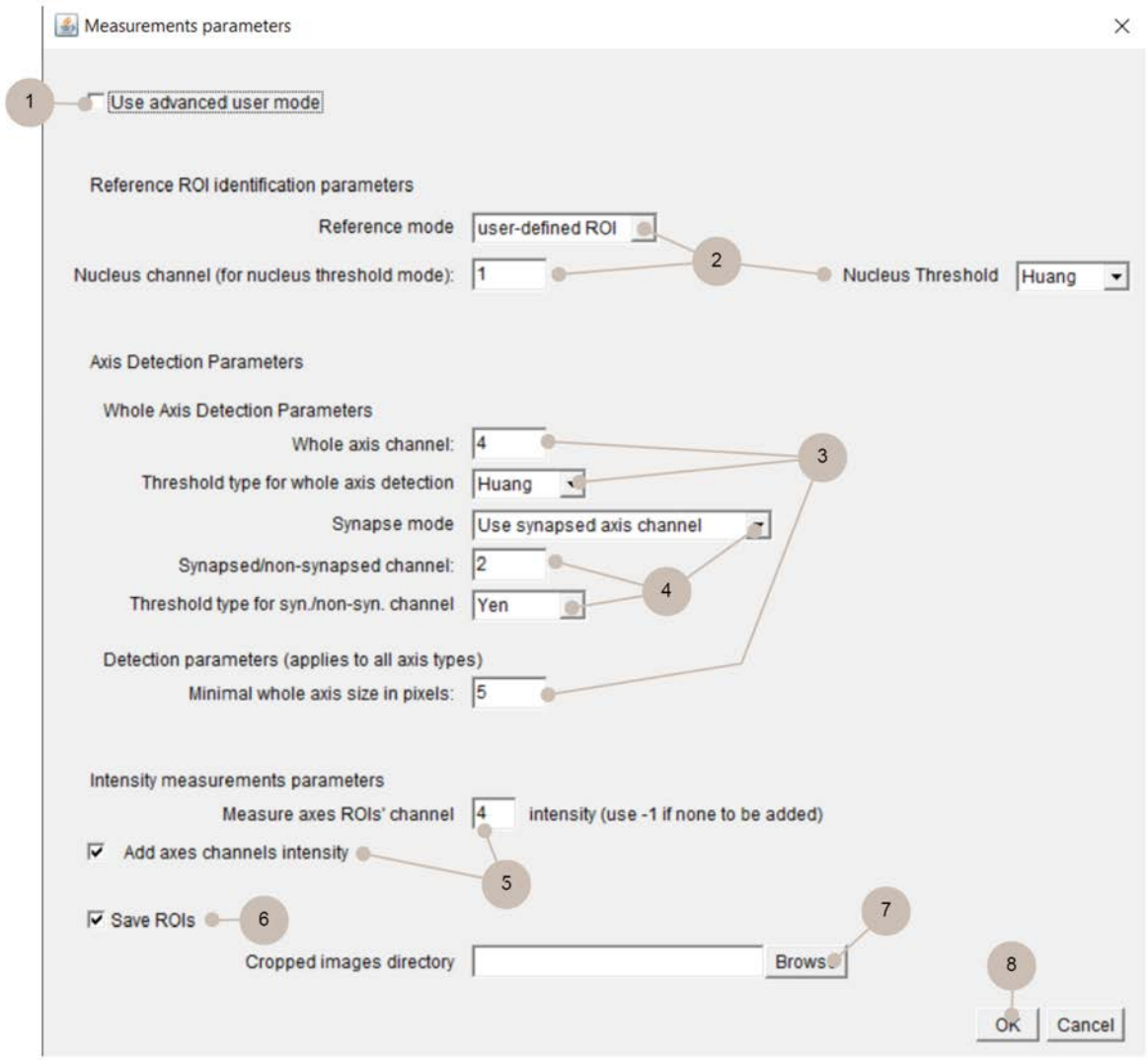


Figure 5.2. The Measure Synapse Length menu.

STEP 1. If working with noisy images, it's recommended to use the advanced user mode (figure 5.2, this option involves ➔ OPTIONAL STEP B, see below for a detailed description of this mode) and tick the "smooth original images" option in the "Advanced user parameters" menu that appears after clicking OK at STEP 8 (fig. 5.7). Select this checkbox if pruning is needed (see figure 5.1).

STEP 2. Next, set the ROI that will be used for further measurements. If "user-defined ROI" is selected, the companion .roi file generated during stage-cropping will be used. If the file is not found, an error message will be displayed. When opting for this reference mode, you can proceed directly to STEP3 without the need to fill in the nucleus channel and threshold fields. Alternatively, an intensity threshold can be used. The macro refers to this as the "nucleus" threshold, as the channel used for the threshold is often based on some nucleus staining, but it can be used for any other staining. If using this mode, set the channel used for threshold (the first channel is channel 1), and select the appropriate threshold algorithm from the list of available ImageJ built-in automatic thresholds. To display the full list of threshold methods

options, follow OPTIONAL STEP A. The user-defined or nucleus ROIs are referred to as the "reference ROI" below.

Note that the algorithm was initially designed to analyze multiple nucleus-containing images. If there is a single, cropped nucleus in the image and no threshold can be used to set the ROI (for instance no nuclear staining), set the whole image (Ctrl A) as the ROI when using the cropping buttons.

STEP 3. To set the axes detection parameters, first enter the whole axis channel, being mindful that the first channel is channel #1. Set the appropriate threshold to be used, and optionally add additional automatic threshold methods using OPTIONAL STEP A. The algorithm applies the respective threshold and displays "no axis detected" in the final table if no pixel above the corresponding threshold is detected. The threshold generates a temporary selection in the ROI Manager named "axis threshold".

Next, the algorithm detects valid axis elements using the Analyze>Analyze Particle command, starting from the nucleus/user-defined ROI (for whole axis element validation). Enter the minimum pixel size of thresholded axes bits to discard unwanted, small items, though be mindful that raising this value too high might not be appropriate for early stages (figure 5.3). The algorithm for detection ensures that any holes contained within loops formed by overlapping axes are not detected, as shown in Figure 5.4.

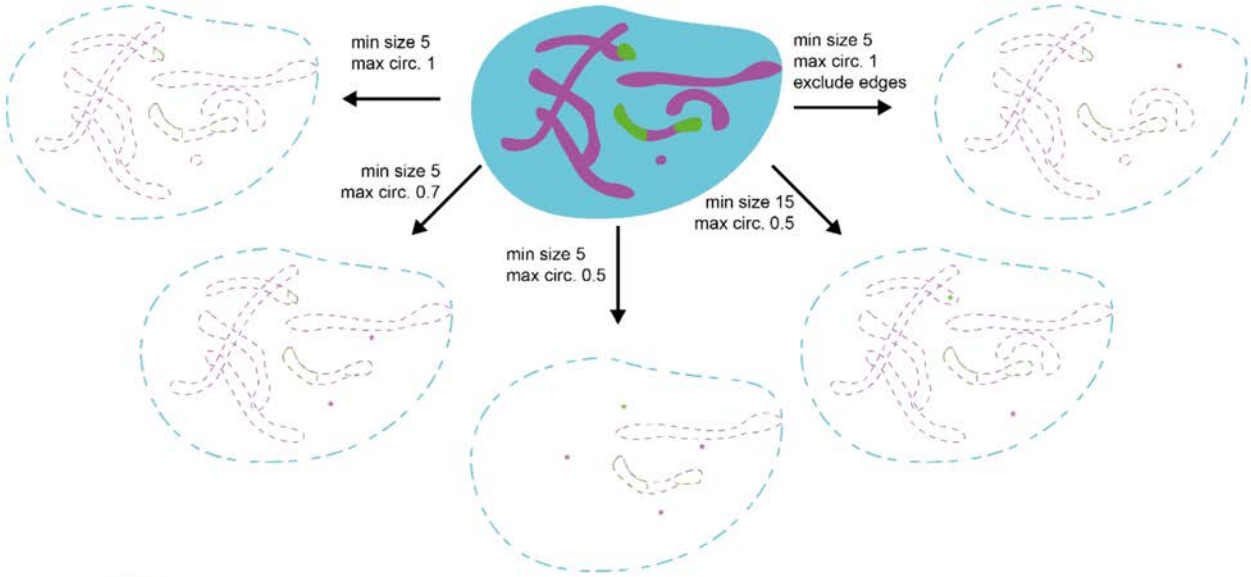


Figure 5.3. Effects of exclusion parameters. The excluded elements are denoted with an asterisk. By default, the minimum size in pixels can be increased to get rid of unwanted elements, as shown in the comparison between the left and lower-right drawings. However, this can remove synapsed/non-synapsed elements (lower right drawing). In addition, advanced user parameters allow for further exclusion criteria. For instance, the maximum circularity can be used to remove round-shaped objects, as seen in the comparison between the left and lower drawings, but this may also result in the removal of clusters of axes (as depicted in the lower-left drawing). Note that this shape criterion can have an effect of synapsed/non-synapsed sections of the axis. The use of edge exclusion (as shown in the right drawing) offers additional possibilities for exclusion.

STEP 4. Next, choose whether the other channel is a marker of a synapsed or non-synapsed complex, though this choice has no consequences in subsequent steps. Select the corresponding channel and threshold.

The synapsed/non-synapsed axis detection uses the same axis threshold parameter and minimum pixel size exclusion criterion. The threshold generates a temporary selection in the ROI Manager named “synapsed/non-synapsed axis threshold". Next, the algorithm detects valid axis elements using the Analyze>Analyze Particle command within the previously detected whole axis (for synapsed/non-synapsed axis element validation). Enter the minimum pixel size of thresholded axes bits to discard unwanted, small items, though be mindful that raising this value too high might not be appropriate as well when small sections of the whole axis are synapsed/non-synapsed (figure 5.3).

Additional detection parameters that are set at OPTIONAL STEP B also apply to synapsed/non-synapsed axes.

For both types (whole and synapsed/non-synapsed axis), all identified elements that meet the specifications are validated and added to the ROI Manager, then combined in a temporary ROI. If overlapping axes create a loop, the algorithm considers the hole within the loop to be part of the axis (as shown in figure 5.4). To correct this, the AND option of the ROI Manager is applied to:

- the "axis threshold" ROI and the temporary ROI for raw whole axis ROI creation
- the "synapsed/non-synapsed axis threshold" ROI, the temporary ROI, and the previously identified raw whole axis ROI for raw synapsed/non-synapsed axis ROI creation.

The created raw axes ROI are added to the manager.

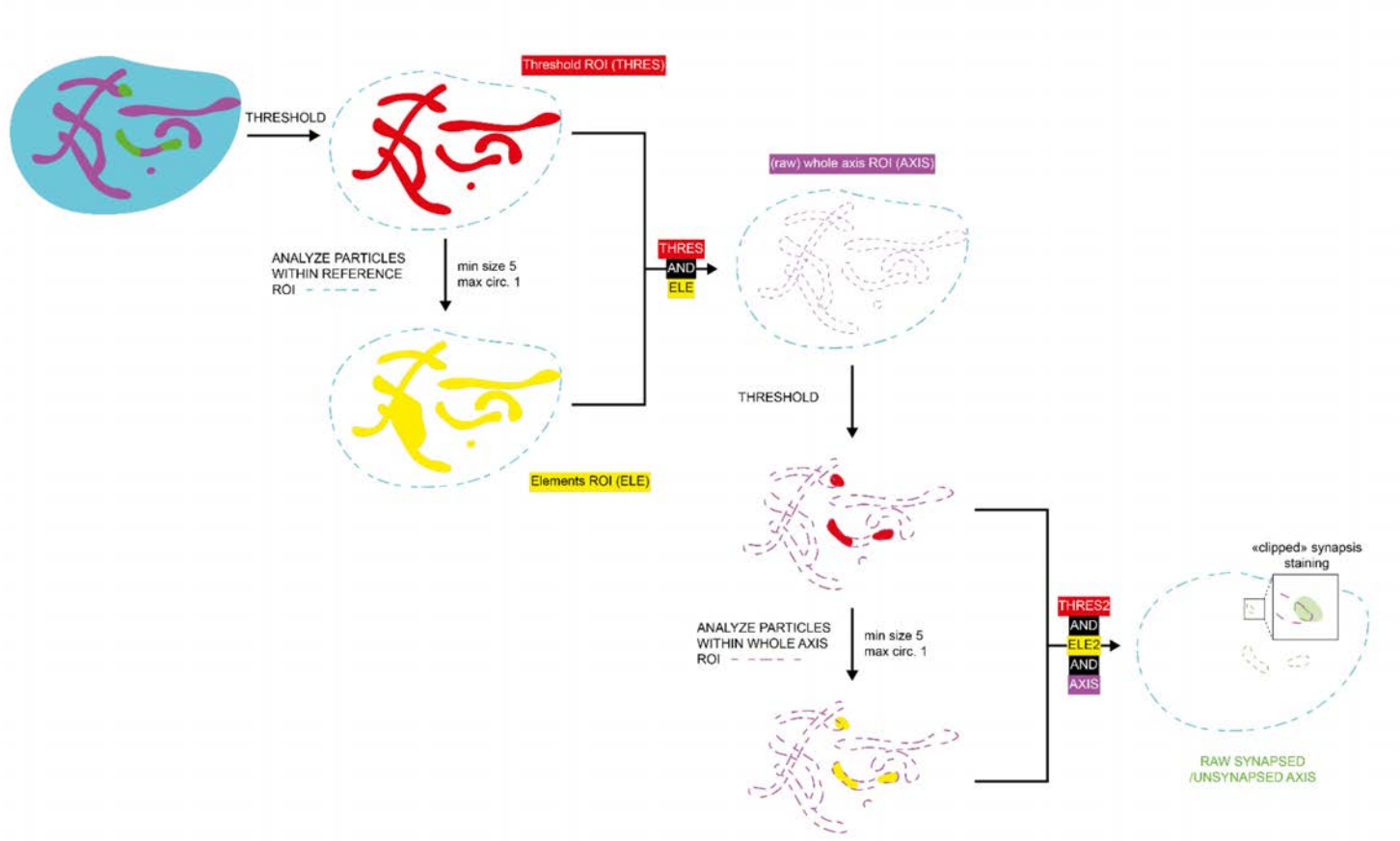


Figure 5.4. Process of whole and synapsed/non-synapsed axis elements detection. The staining of the axis is first thresholded, and the "analyze particles" command is used to identify axis elements. However, to exclude axis loops (such as the three overlapping axes in the lower left corner of the

nucleus), additional processing is required. This involves combining temporary threshold ROI and fused elements selection. The process is identical for synapsed/non-synapsed sections. However, the selection combination is extended to the whole axis ROI. As a consequence, a synapsed/non-synapsed element may get truncated, as shown in the inset.

If a pruning method is introduced at OPTIONAL STEP B, the raw axis masks are modified through binary operations. These processed final axes ROI (either whole axis or synapsed/unsynapsed axis ROIs) are use for skeleton>skeletonize 2D/3D plugin analysis.

➔ OPTIONAL STEP B. To improve the detection of the whole axis and synapsed/non-synapsed axis, you can use the advanced user mode option (fig. 5.2, STEP 1). After selecting this option, a new menu will appear when you click OK in STEP 8.

STEP 5. Set additional intensity measurements channels or ROI. The macro can measure intensities in the nucleus/user-defined ROI and the axes ROIs (whole and synapsed/non-synapsed). You can also select which channels you want to measure intensities in, and choose the measurement type (mean, integrated density). If you don't want to measure intensities in a particular channel, leave the field with -1 and do not select "add axis channels intensity". To remove measurements or change the intensity measurement types, follow OPTIONAL STEP A.

STEP 6. Set the output items. If you select the "saveROIs" option, all generated ROIs will be saved in a .zip file in a parent controlData folder. The ROIs will be named using the image's name and a "synapse-RoiSet" prefix. If you choose not to use this option, you can not control the accuracy of the axes and synapsed/non-synapsed axis detection & skeleton analysis. All measurements will still be saved in a synapse.xls file in the folder you selected in STEP7.

STEP 7. Change/fill-in the cropped-images directory using the browse button.

STEP 8. Clicking OK will start the analysis, unless you selected the “Use advanced user parameters” option at STEP 1, in which case the Advanced user parameter window will pop up (see OPTIONAL STEP B). If you want to see the images while they are being processed, you can follow OPTIONAL STEP A.

OPTIONAL STEP A. Set the measurements parameters options. Before using the “Measure synapse length” tool, you must first set the measurement parameters by clicking on the Set measurements parameters button. This will bring up the Parameters menu (figure 5.5).

PARAM A1. In the Parameters menu, you can choose whether to display warning messages or not. If you keep them hidden, they will not block the batch processing of the macro.

PARAM A2. By default, the images are hidden during the analysis, but you can choose to display them by selecting the “show images while processing files” option.

PARAM A3. The default list of threshold (for nucleus, axis, synapsis) is limited to preset thresholds that have been found to work well with test images. If you want to expand the list to the full ImageJ’s automatic thresholds list, you can select the “Display the full list of threshold methods” option.

PARAM A4. You can select the lengths measurement types. There are two different routines available: the first (rough) one takes into account the 1-pixel thickness of the skeleton, while

the second (fine) one is a raw measurement of the skeleton that takes into account “horizontal, vertical and diagonal pixels” (see fig. 5.6). To remove the fine or rough length measurements, you can unselect “show rough length estimation” or “show fine length measurements” respectively. To remove fine or rough length measurements, unselect “show rough length estimation” or “show fine length measurements” respectively.
PARAM A5. The default intensity measurements for reference ROI, axis, synapse/non-synapsed axis are IntDen. If you want to add Mean values or remove IntDen, you can use the “Show IntDen values” and “show Mean values” options.

PARAM A6. The macro measures intensities in different regions of interest (ROIs). By default, the macro will measure the intensity in the reference ROI, which can be either user-defined or the nucleus ROI. If you want to exclude this measurement, you can unselect the “show user-defined nucleus ROI measurements” option.

Note that the other options are specific to the foci tools and do not apply here. Proceed to analysis and click OK (A7).

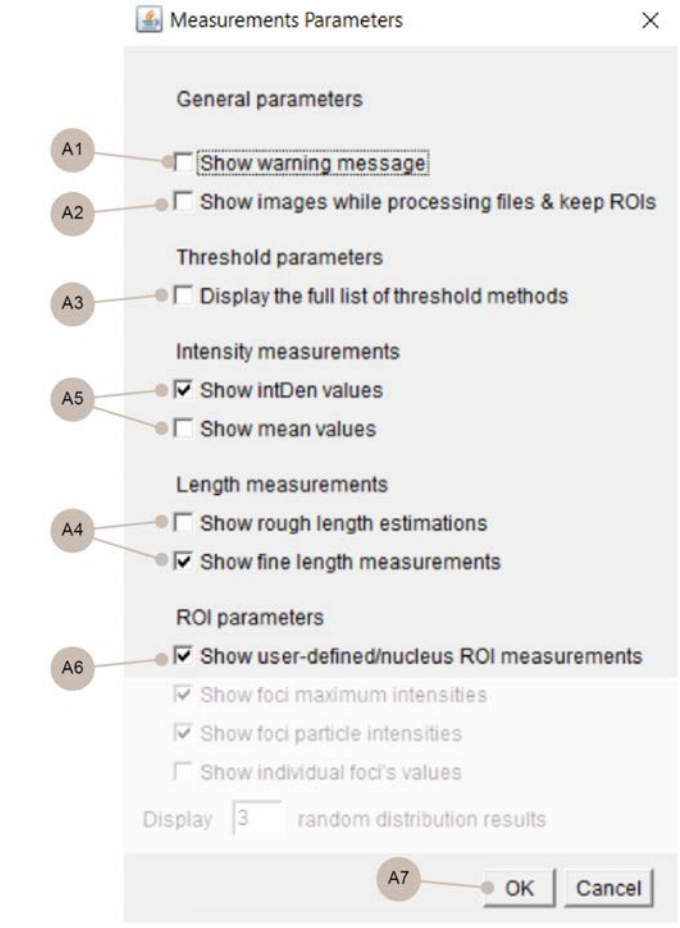


Figure 5.5. The (general) parameters window.

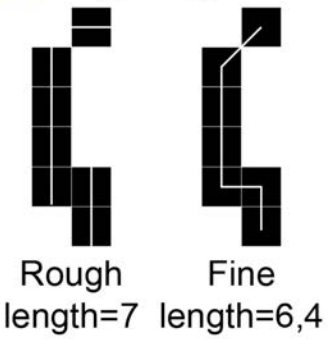


Fig. 5.6. Rough and Fine length measurements.

OPTIONAL STEP B. Advanced user options can be used to get accurate axis detection. This is very useful for either noisy images and/or superresolution images. Whenever the “Use advanced user option” is chosen, the “Advanced user parameters” window will pop up at STEP 7 (figure 5.7).

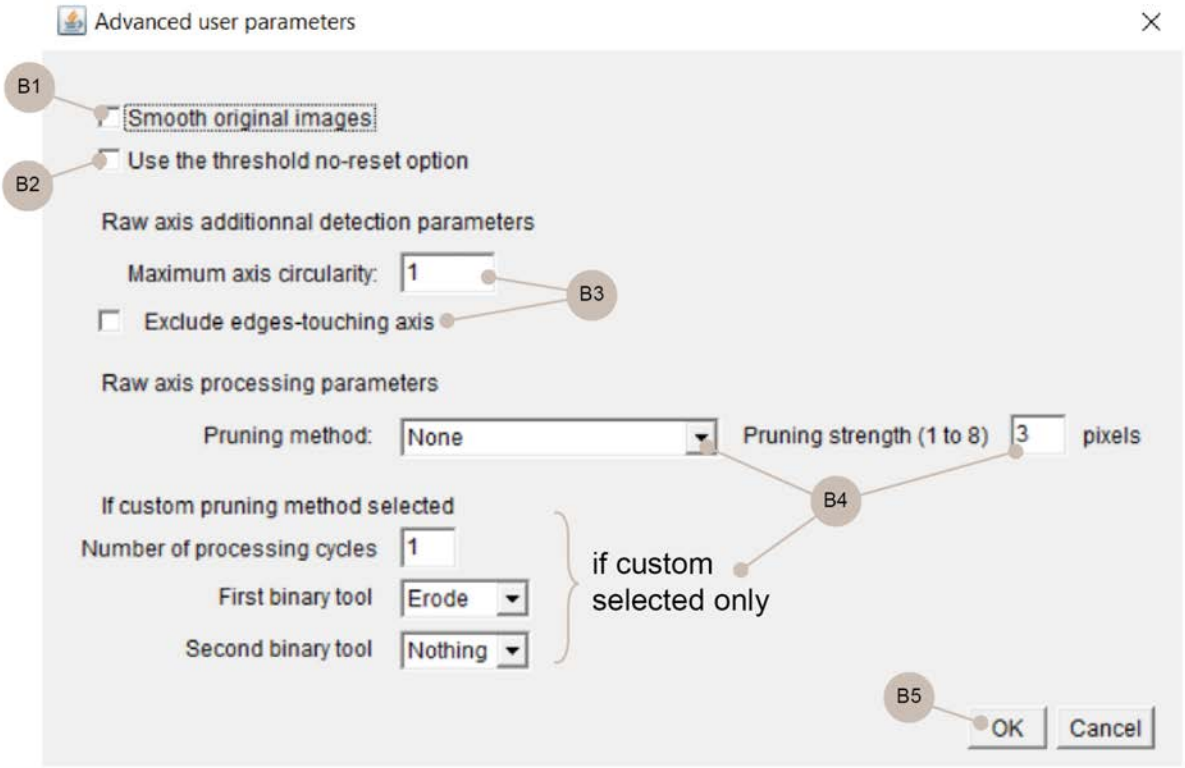


Figure 5.7. The Advanced user parameters menu

OPT. STEP B1. Tick the smooth original images checkbox to remove noise (such as photon shot noise). This filter replaces each pixel with the average of its 3x3 neighbourhood and applies to all channels.

OPT. STEP B2. Detecting axes in images with a high offset value, such as Abberior .msr files, can present challenges. These challenges arise due to the presence of extremely high background intensity pixels and pixels excluded from analysis (with intensities set to 0 during cropping), which can introduce bias during the automatic thresholding process, as shown in figure 5.8. To overcome this issue, the "use the threshold no reset option" is provided.
Various versions of ImageJ, ranging from 1.52e to 1.53s, have undergone multiple implementations, retractions, and re-implementations of autothreshold options. The later versions have introduced a stable threshold mode known as "don't reset range" in the Image>Adjust Threshold command. When working with images that have more than 8 bits, thresholding problems may arise because the automatic threshold methods utilize an 8-bit histogram of the image. To facilitate this, the range of the 16-bit image (0-65535) is converted to an 8-bit range (0-256), and the threshold is calculated based on the 8-bit histogram.
Consider an example of a 16-bit .msr file displayed using the Image>Adjust>Brightness&Contrast auto display range (figure 5.8). In this scenario, the black intensity value is 32767 (representing the minimum intensity in the ROI, with the background

ranging from 32767 to 32769), and the white intensity value is 32790 (representing the maximum intensity in the ROI). The axis signal exists between 32770 and 32790. When the "reset" option (default mode in Adjust Threshold, left-hand side of figure 5.8) is selected, the range extremes are converted to a threshold range of 0 and 256. Consequently, the automatic threshold encounters two intensity clusters: the clipped area and the rest (background+signal). It finds a threshold value that fails to differentiate the background from the signal within the reference ROI, as indicated by the red area in the histogram displayed in the middle left panel of figure 5.8. As a result, the entire reference ROI is thresholded, leading to the axis ROI erroneously resembling the reference ROI.
In contrast, when the "don't reset" option is chosen, the displayed extremes are converted to a threshold range of 32767 to 0 and 32790 to 256. The (discrete) 8-bit histogram is then computed (middle right panel of figure 5.8), and automatic threshold values are calculated within the red range on the histogram. Consequently, the axis is correctly thresholded, and the axis ROI accurately represents the axis.

OPT. STEP B3. If needed enter additional raw axis detection parameters:

- The maximal circularity. The ImageJ Circ. parameter is $4\pi * (\text{area}/\text{perimeter}^2)$. A circularity value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon. If axes are quite separated, then consider lowering the circ. value to get rid of non-axis round artefacts. However, if axes are overlapping, set the maximum circ. value to 1 to avoid getting rid of round aggregates of overlapping axes.
- Tick the “exclude touching edges” option if the reference ROI of STEP 2 (either user-defined ROI or the threshold ROI) has cut some axis signal in a way that it touches the edges of the image/ROI. When using cropped nucleus images with plenty overlapping axes, ticking the option will remove most of the axes.

OPT. STEP B4. Set the pruning method & options. Pruning is a way to modify the raw axis outlines and get rid of unwanted axis decorations (for accurate axis length measurement). This is mandatory when using superresolution images. The raw whole axis ROI is used to generate an 8 bits mask, using fill/clear commands (this can be reproduced manually by using the ROI, then the Edit>Fill and Edit >Clear Outside menus). The binary mask is then processed. The options are:

- The default method is “None”: the mask is not processed.
- 2x Erosion: two erosions are used. Erosion removes pixels from the edges of objects. Use this if you want to shrink the initial raw axis detected.
- 2x Dilation: two dilation are used. Dilation adds pixels to the edges of objects. Use this if you want to enlarge the initial raw axis detected.
- 2x Erosion then 2x Dilation: (process>binary>erode twice then process>binary>dilate twice) or 2x[erosion/dilation] : two erosion/dilation cycles (process>binary>Open) are done on the mask. Use this if you want to smooth/fill holes.
- Custom: this method can be used to apply custom pruning parameters.

The macro uses the process>binary>options command. Figure 5.9 shows the parameters used for 2x Erosion and a strength of 3. The pruning strength value (“count” in the options command is the number of adjacent background pixels necessary before a pixel is removed from the edge of an object during erosion and the number of adjacent foreground pixels necessary before a pixel is added to the edge of an object during dilation. Whenever a value lower than 1 or higher than 8 is entered, the entered value is overridden and 3 pixels are used. The pad edges when eroding option is used as well as the black background option.

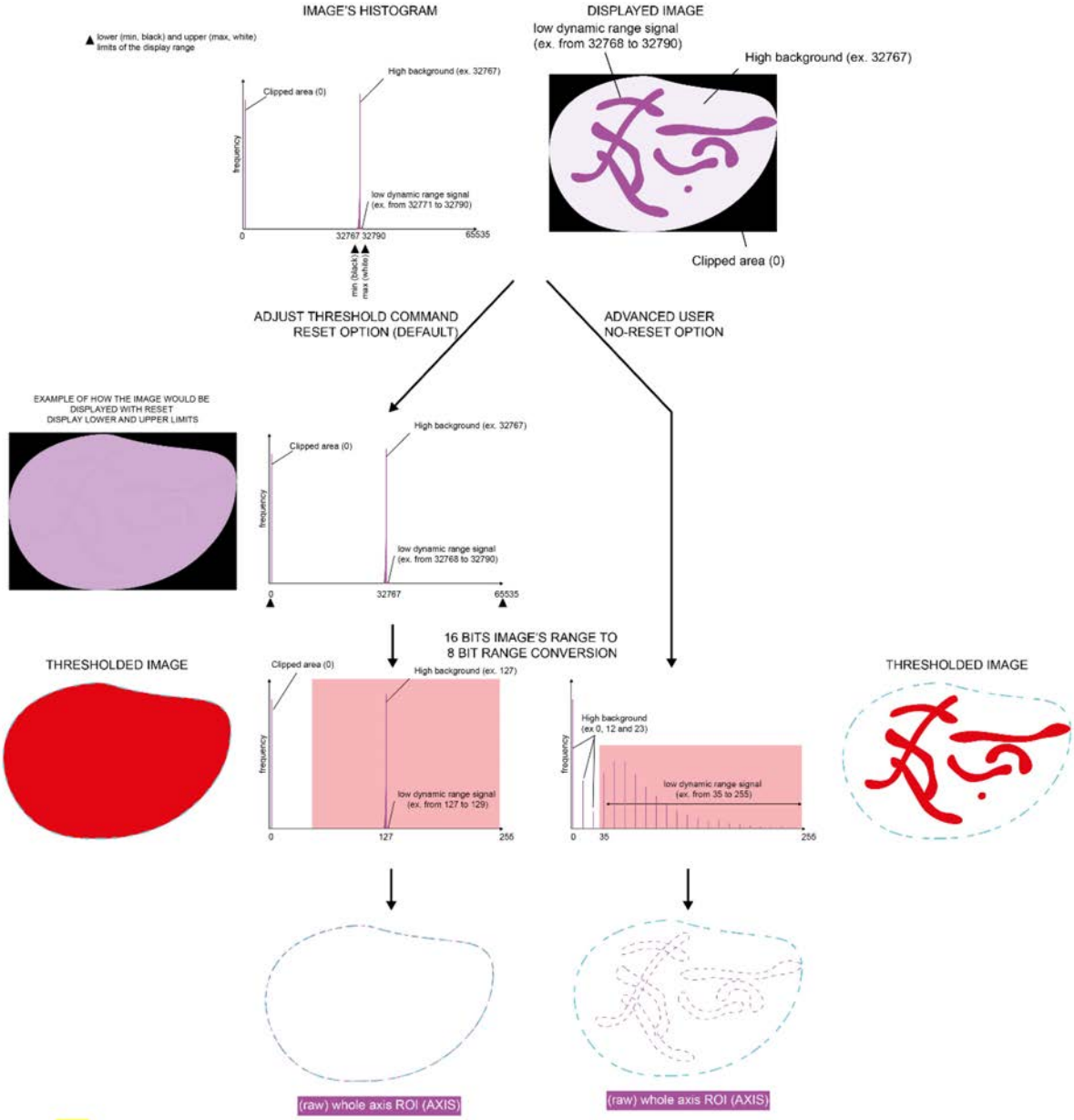


Figure 5.8. Thresholding issues can arise when working with low dynamic range images. Typically, these images are displayed using the "auto" min/max display option (Image>Adjust>Brightness&Contrast), and the limited dynamic range may not be immediately noticeable. However, when such images are cropped, the area outside the reference region of interest (ROI) that is clipped out and set to an intensity of 0 can interfere with the detection of axes. This interference occurs because the automatic thresholding process relies on a default reset range option (illustrated on the left in the figure). The original histogram considered for thresholding encompasses the entire range of intensities, from 0 to 65535 (if working with 16-bit images). To illustrate the impact of these display parameters, an example is provided, demonstrating how the image would appear when displayed with such settings (shown in the middle left panel of the figure). During the conversion of the original histogram into an 8-bit histogram, pixels in the clipped-out area with a value of 0 are taken into account in the calculation of the automatic threshold. If the dynamic range of the signal, in comparison to the neighboring background, has similar values that significantly differ from 0, the entire reference ROI is incorrectly thresholded, resulting in the axis ROI resembling the reference ROI. However, by utilizing the "no-reset" option, the displayed histogram serves as the input for the conversion from 16 bits to 8 bits, which occurs before the threshold calculation. This approach enables accurate axis detection.

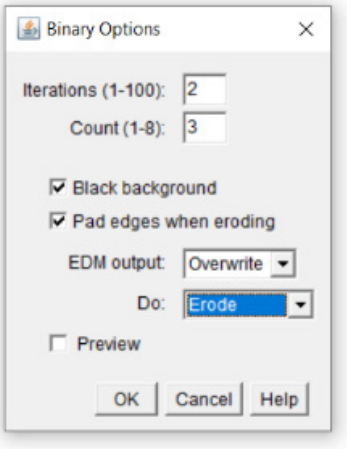


Figure 5.9. An example of how the pruning method “2x Erosion” translates in ImageJ.

If “Custom” pruning method is selected, set the number of cycles (=iterations). Select the first “binary tool” to be used. If another round of processing cycles is to be applied, select the second “binary tool”. For instance “2x Erosion then 2x Dilation” first sets the process>binary>options command to Iterations 2 and Do: Erode. Two erosions are first applied. Then the process>binary>options command is further used with iterations 2 and Do: Dilate. To set the best custom pruning method parameters, use the set advanced user parameters button first.

A “whole axis” or “synapsed/non-synapsed axis” ROI is then drawn using the processed mask (with the pruning method within brackets).

Type	Image name	fine whole axis length (um)	fine synapsed axis length (um)	whole axis ch. intDen (whole axis)	synapsed axis ch. intDen (whole axis)	Ch.4 intDen (whole axis)	whole axis ch. intDen (synapsed axis)	synapsed axis ch. intDen (synapsed axis)	Ch.4 intDen (synapsed axis)	whole axis ch. intDen (user-defined ROI)	synapsed axis ch. intDen (user-defined ROI)	Ch.4 intDen (user-defined ROI)	Comment	Parameter	Value
pachy	pachy1	117.626	6.614	57582650.106	6255842.523	55126650.852	3365296.462	1118328.491	1032764.386	62747825.796	33467231.554	60112991.516		Smooth original images	no
pachy	pachy2	145.190	7.847	71564980.075	6772080.061	66256901.317	4847144.148	1147022.566	951573.227	76955926.516	31062390.479	71151580.239		ROI chosen	user-defined
pachy	pachy3	95.484	7.428	48236129.001	4659889.711	44060602.574	4423283.201	1039752.625	1034131.876	51545299.227	20311606.868	47146221.195		Axis channel	3
pachy	pachy4	173.466	6.252	84894874.468	9247865.244	85016249.406	3868831.832	1306836.229	1511963.318	90574505.473	37466243.553	90928711.422		Axis Threshold	Huang
pachy	pachy5	157.344	3.208	135449873.659	5483432.435	111569374.136	2956425.577	921632.850	785785.567	143070802.827	33769345.493	121394188.528		Axis Min size	5
pachy	pachy6	161.541	14.927	114692421.989	6348649.255	90881126.529	6783877.443	1956793.156	1404609.634	121854871.335	26387664.148	97988200.719		Axis Max Circ.	1.0
pachy	pachy7	193.732	8.397	134870596.984	6943196.423	118371199.121	3997840.245	1520061.125	670055.493	142840336.570	31158651.274	126485809.360		Exclude edge-touching axis	no
pachy	pachy8	134.934	4.906	101221184.060	8920326.142	69463798.234	4266373.378	1385616.420	933618.602	109644577.160	37480160.310	74700286.916		Measure axis length	yes
pachy	pachy9	125.663	8.118	95659968.998	6610361.537	68582314.070	6519630.371	1446821.867	1517883.705	103272205.865	30136458.696	73476021.423		Synapsis reference	synapsed
pachy	pachy10	205.822	15.520	117438153.813	8154133.529	81321993.299	8075035.278	2885652.748	332512.953	123698658.725	32839952.594	87971170.287		synapsed axis channel	2
pachy	pachy11	173.107	3.328	107219487.544	6653708.977	75725472.438	1529373.044	838808.606	224347.564	112174036.845	27977968.356	83040090.621		Measure axis' and synapsed axis' ROIs intensities	Ch. 4 & axis and synapsed axis channels (Ch.3and Ch.2)
pachy	pachy11	152.753	6.998	78631213.866	8864239.195	92598182.167	2866129.032	1207594.863	2017689.940	84686990.664	42008799.649	99031121.860		Pruning Mode(s) tested	2x Erosion
zygo	zygo1	158.875	1.552	52869930.479	11334760.933	34862652.913	939701.897	851820.888	398522.294	57131926.018	23392355.961	36725345.498		Pruning Strength (when used)	3
zygo	zygo2	281.305	3.687	100797105.848	16451507.470	66214106.536	1924323.849	1390159.656	555562.172	110540001.467	48206986.197	69765555.500		Images from	D:\...
zygo	zygo3	116.337	8.934	50106338.444	6632943.039	42773343.378	2887296.303	1478407.398	1709333.978	53989208.262	19949726.843	45491886.037		ROI saved	yes
zygo	zygo4	251.284	10.013	82763230.331	46528244.030	23092220.384	3353629.693	6739086.688	146435.108	87150845.611	74976289.706	28404952.496		ROI and any other output folder	D:\...
														options:	
														Show warning messages	no
														Show images	no
														Get integrated density intensity values	yes
														Get mean intensity values	no
														Get rough length values	no
														Get fine length values	yes
														Include reference Roi when measuring intensities	yes
														Meiosis bar tool	Measure synapse length
														Meiosis bar version	v2.05
														ImageJ version	1.53t99

Figure 5.10. a result file of the synapse length macro

STEP 9. To control the analysis accuracy, whenever the save ROIs option was selected, use the “Start checking results” in the main meiosis bar. The “Check results” menu pops up (figure 5.11). If you just run the macro, then the default folder and method are OK. If you control afterwards the macro’s results, set both folder and “Measure synapse length”.

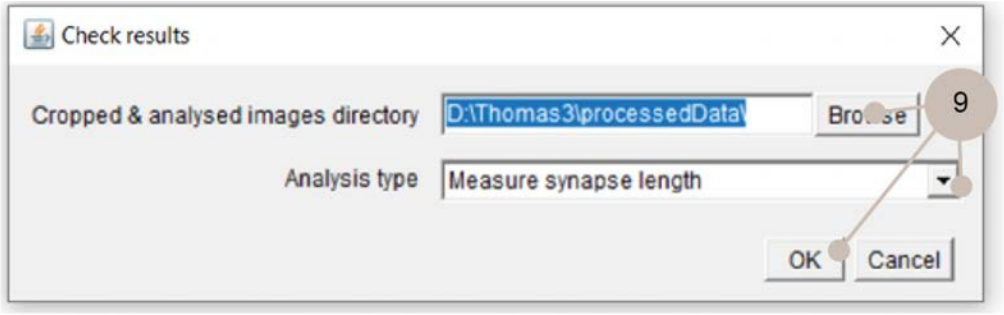


Figure 5.11. The check results menu

The first image analysed will be opened together with its companion synapse_RoiSet_[name].zip file ROI set, as in fig. 5.12. The ROI Manager contains several ROIs:

- The reference ROI, as set in STEP2 (here user-defined ROI). This is the ROI used for further identifications (ie. axes). Its index should be 0 and ROIGroup is 1.
- For whole and synapsed/non-synapsed axes, the raw axis is the initial ROI (as given by STEP3 and STEP4) before pruning processing. Both whole and synapsed/non-synapsed raw axes belong to ROIGroup3.
- If the advanced user option is left unselected and was not used previously, then no raw axes processing is done and raw [type] axis and [type] axis are identical. Processed whole axis or synapsed/non-synapsed axis belong to ROIGroup5.
- The generated skeletons belong to ROIGroup9.

To control the next analysed image, click on “check next results” button on the main Meiosis bar

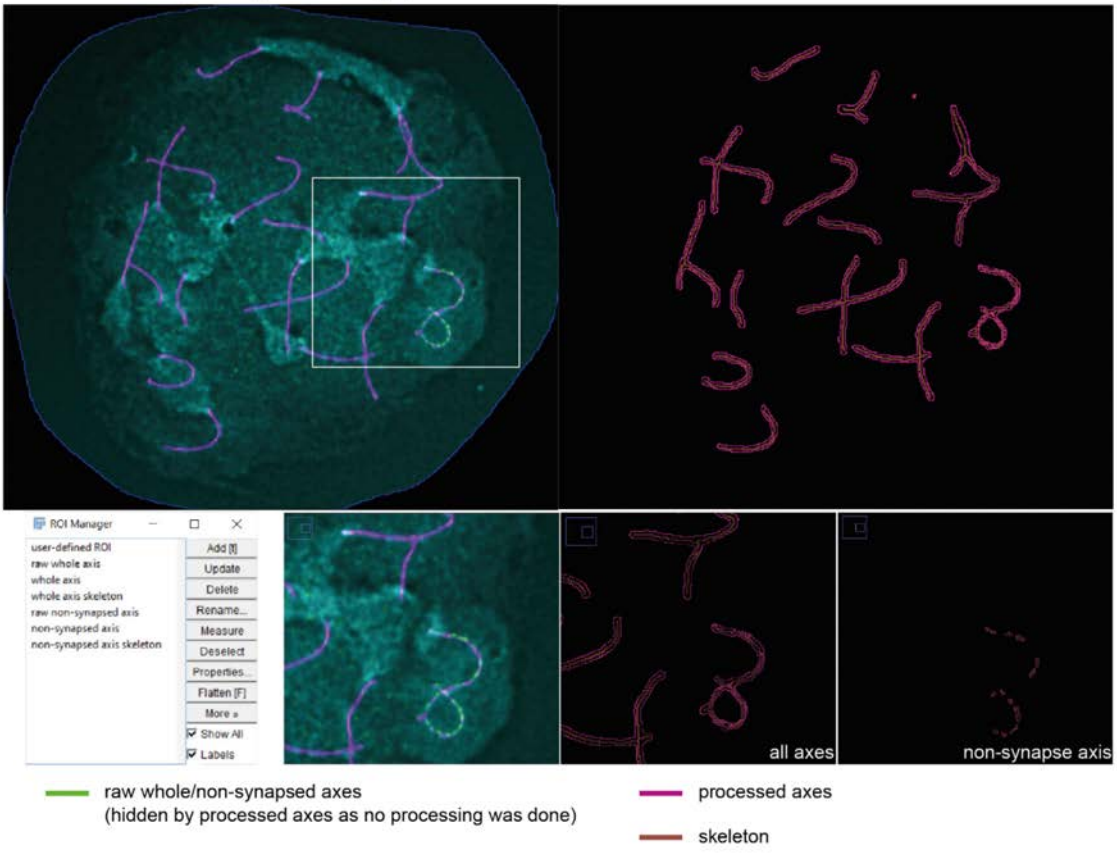


Fig. 5.12. The resulting ROIs produced by the Measure Synapse Length macro.

COUNT AXIS & OFF-AXIS FOCI

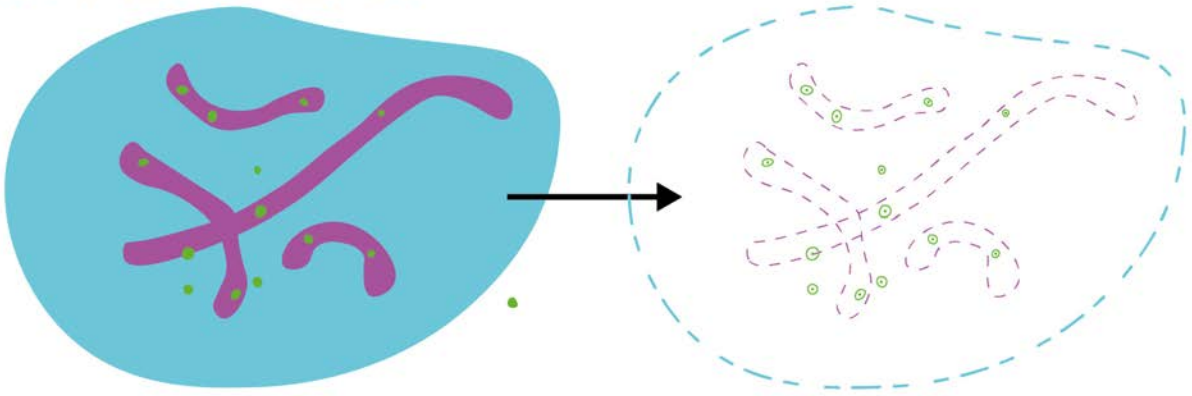


Figure 6.0. Visual summary of the tool.

The algorithm for "count axis and off-axis foci in one channel" has the following steps: i) detection of the axis (based on axis complex staining), ii) identification of foci, iii) counting the number of off-axis and on-axis foci (referred to as "axis" foci). Moreover, the macro can evaluate the total length of the axis. To optimize the results, it is recommended to set some measurement options through the description of ➔ OPTIONAL STEP A.

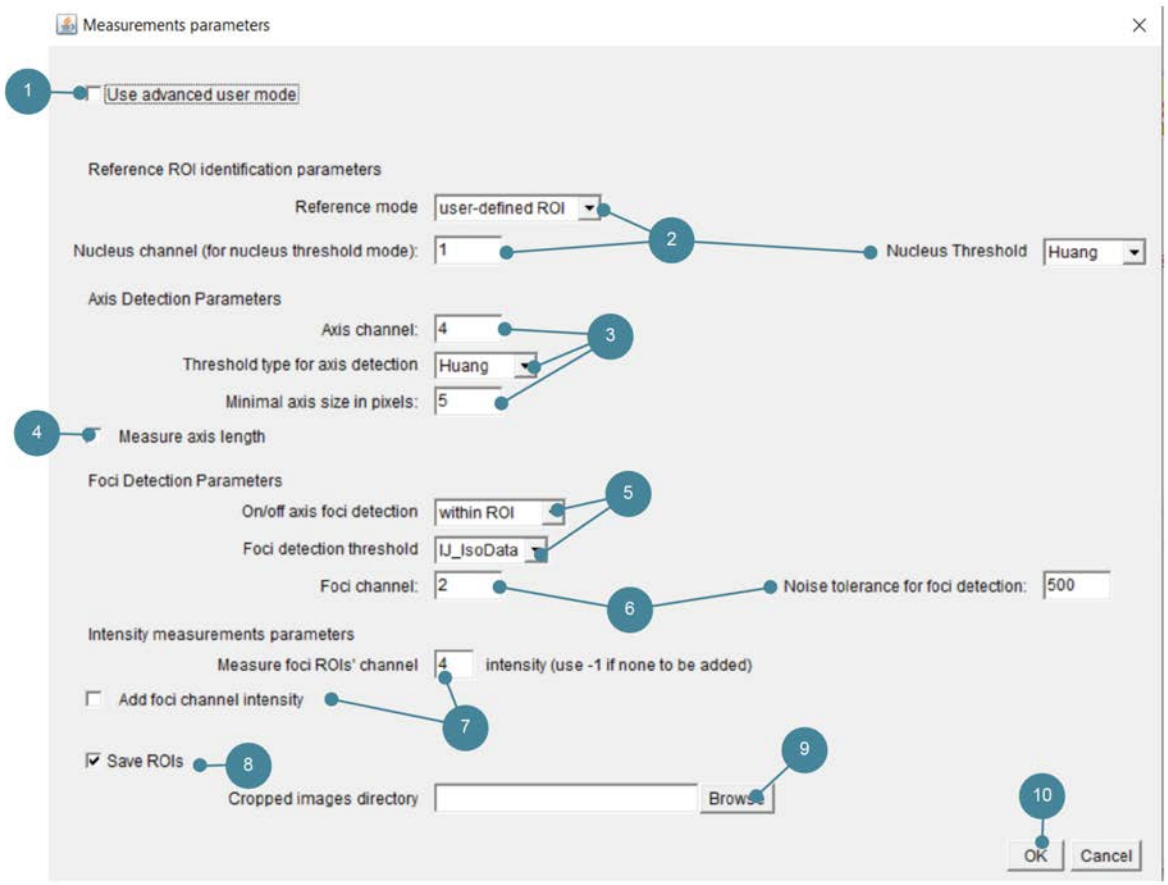


Fig. 6.1. The “count axis and off-axis foci in one channel menu

STEP 1. For images with high levels of noise, like those acquired from STED setups, it is recommended to choose the advanced user mode checkbox, which will prompt an ➔ OPTIONAL STEP B. After completing STEP 10, the advanced parameters window will appear,

where you can choose the "smooth original images" option in the following "Advanced user parameters" menu. This smoothing procedure is applied to all channels and eases both the axis and foci detection. OPTIONAL STEP B includes further parameters that can aid the detection of axis elements during STEP 3.

STEP 2. Choose the region of interest (ROI) that you want to use for measurements. You can either select the ROI you previously defined by cropping the image, or use a thresholded staining of the nucleus. When opting for the “user-defined” reference mode, you can proceed directly to STEP3 without the need to fill in the nucleus channel and threshold fields. If using “nucleus threshold”, fill in the nucleus channel and nucleus thresholds fields.
This ROI will be referred to as the "reference ROI" in the following steps.

STEP 3. Configure the axis detection parameters. Specify the channel that contains the axis (note that the first channel is channel #1). Set the appropriate threshold to be used for the detection. The algorithm will start by applying this threshold. If no pixels above the threshold are detected, the analysis will stop and the final table will display "no axis detected". The threshold generates a temporary "axis threshold" selection in the ROI Manager. The algorithm will then detect the axis elements using the "Analyze Particle" command and the reference ROI.
Specify the minimum pixel size for thresholded axis elements to exclude small and unwanted items. Be careful not to raise this value too high in the early stages. You can use OPTIONAL STEP B to set additional exclusion parameters. The effects of the different exclusion parameters are presented in figure 6.2. The algorithm Identifies and add all particles within the specifications to the ROI Manager. It first combine these particles in a temporary ROI using the "combine ROI Manager" option. However, overlapping axes that form a loop can cause the hole within the loop to be considered part of the axis. To correct this, it uses the AND option of the ROI Manager on the "axis threshold" ROI and the temporary ROI (as shown in figure 6.3) to create a "raw whole axis" ROI to the manager.

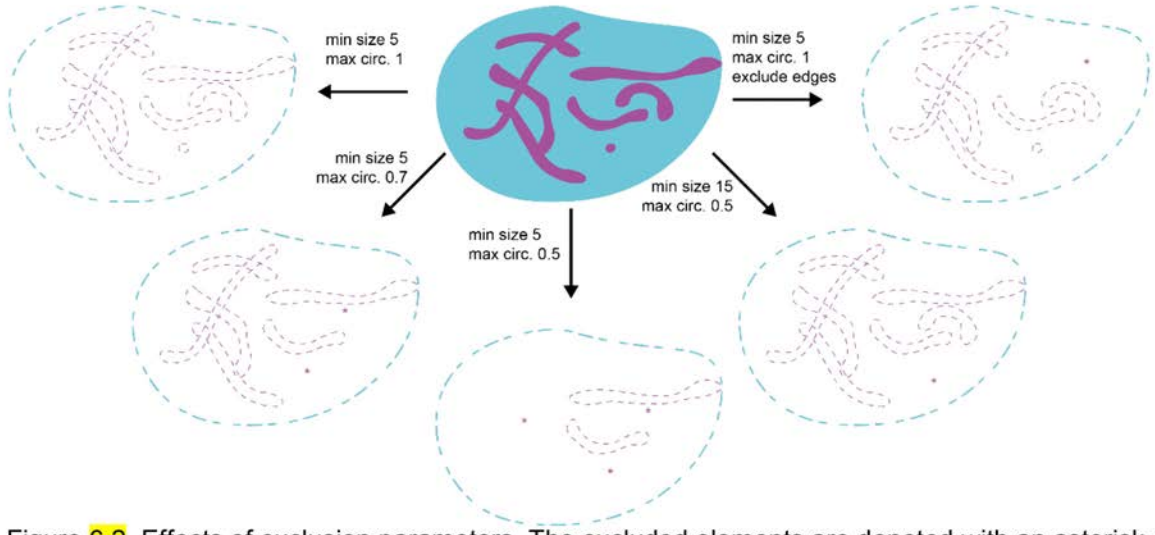


Figure 6.2. Effects of exclusion parameters. The excluded elements are denoted with an asterisk. By default, the minimum size in pixels can be increased to get rid of unwanted elements, as shown in the comparison between the left and lower-right drawings. In addition, advanced user parameters allow for further exclusion criteria. For instance, the maximum circularity can be used to remove round-shaped objects, as seen in the comparison between the left and lower drawings, but this may also result in the removal of clusters of axes (as depicted in the lower-left drawing). The use of edge exclusion (as shown in the right drawing) offers additional possibilities for exclusion.

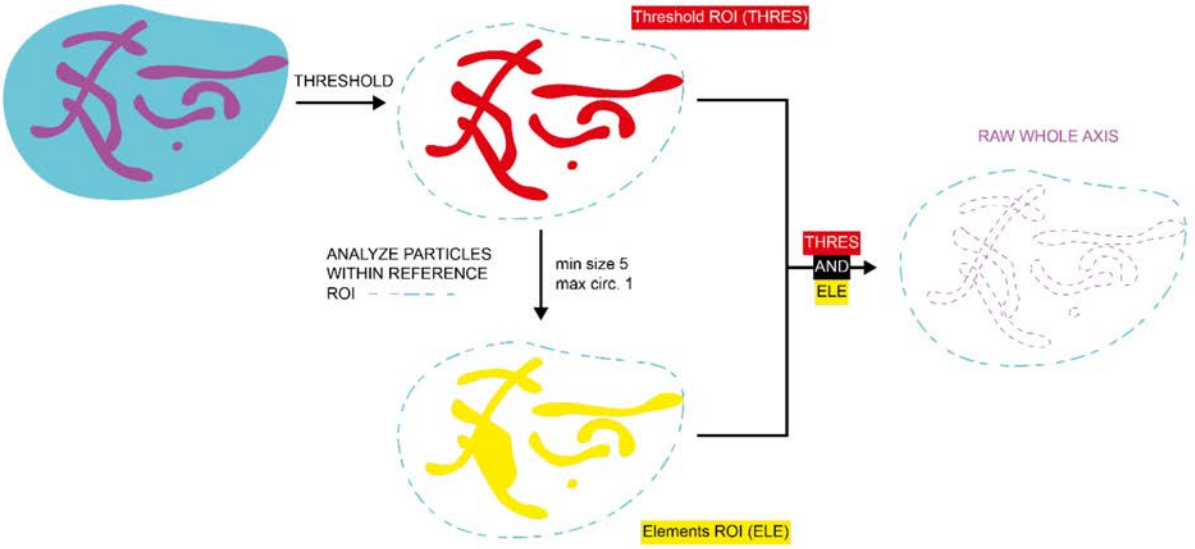


Figure 6.3. Process of axis elements detection. The staining of the axis is first thresholded, and the "analyze particles" command is used to identify axis elements. However, to exclude axis loops (such as the three overlapping axes in the lower left corner of the nucleus), additional processing is required. This involves combining temporary threshold ROI and fused elements selection.

STEP 4. If you choose to tick the "Measure axis length" checkbox, the modified processed mask will undergo a skeleton analysis using the skeleton>skeletonize 2D/3D plugin. By default, the raw (whole) axis selection is duplicated into a whole axis ROI, used to create a binary mask. Then the skeleton analysis is run. Note that you must have the plugin installed to proceed if you use ImageJ. Following the analysis, a skeleton ROI is added to the ROI Manager, and various length measurement options can be established with OPTIONAL STEP A. For superresolution images or those that have been affected by noise, it is advisable to use smoothing pruning (refer to OPTIONAL STEP B). Pruning is only necessary when measuring axis length and not when identifying the axis. It is important to maintain the same pruning method to compare results between different conditions since it alters the outlines of the axis.

STEP 5. Enter general foci detection parameters. A focus is defined by its intensity maximum and optional outlines (referred to as particle, see top panel figure 6.5). For the purpose of axis/off-axis discrimination, two modes are proposed (figure 6.4). A crucial aspect of the algorithm is that the position of a focus is determined by the location of its maximum, which is identified using the Find Maxima command (see lower inset of top panel of figure 6.5).

- The legacy "using masks" method (<v2.01) is based on a mask analysis. For on-axis and off-axis foci detection, the foci channel is duplicated twice:
  - The first duplicate image is used to fill in black (=set to 0) the out-of-axis pixels. The Axis ROI is selected, and the Edit>clear outside command is run. Then the find maxima algorithm is run using the user-defined noise value. This gives the on-axis foci maxima.
  - The second duplicate image is used to fill in black the on-axis pixels. The combination of the reference ROI [XOR] axis ROI is selected, and the Edit>clear outside command is run. The same find maxima operation is made as with the first duplicate. This gives off-axis foci.

  The use of this method may result in bias.. If the background signal in the image is high and small axis fragments are detected, the find maxima algorithm may detect background in the foci channel as a focus (when background is more than the prominence value in the vicinity of a large area with intensity 0 (the other side of the

selection set to black, As illustrated in the lower inset of the bottom left panel of figure 6.4, background can be wrongly detected as a focus. This can also occur when the file format's "zero" (camera offset, vendor's choice) is high. To address this issue, either increase the min axis pixel size to discard small axis fragments or use the "within ROI" option. Another issue can arise when a focus is located at the edge of the detected axis, causing it to be split into two parts (one in the axis mask and the other "half" in the off-axis mask), and may be detected as two foci (one off-axis and one on-axis focus) (see figure 6.4, right-hand side focus).

- The "within ROI" (from v2.01) detects foci within the reference ROI (referred to as total_foci (maxima), ROIGroup 11) and then keep maxima that are located within the axis ROI (axis_foci (maxima), ROIGroup 13). This method is implemented in a more time-consuming way but is more elegant and is now the default method.

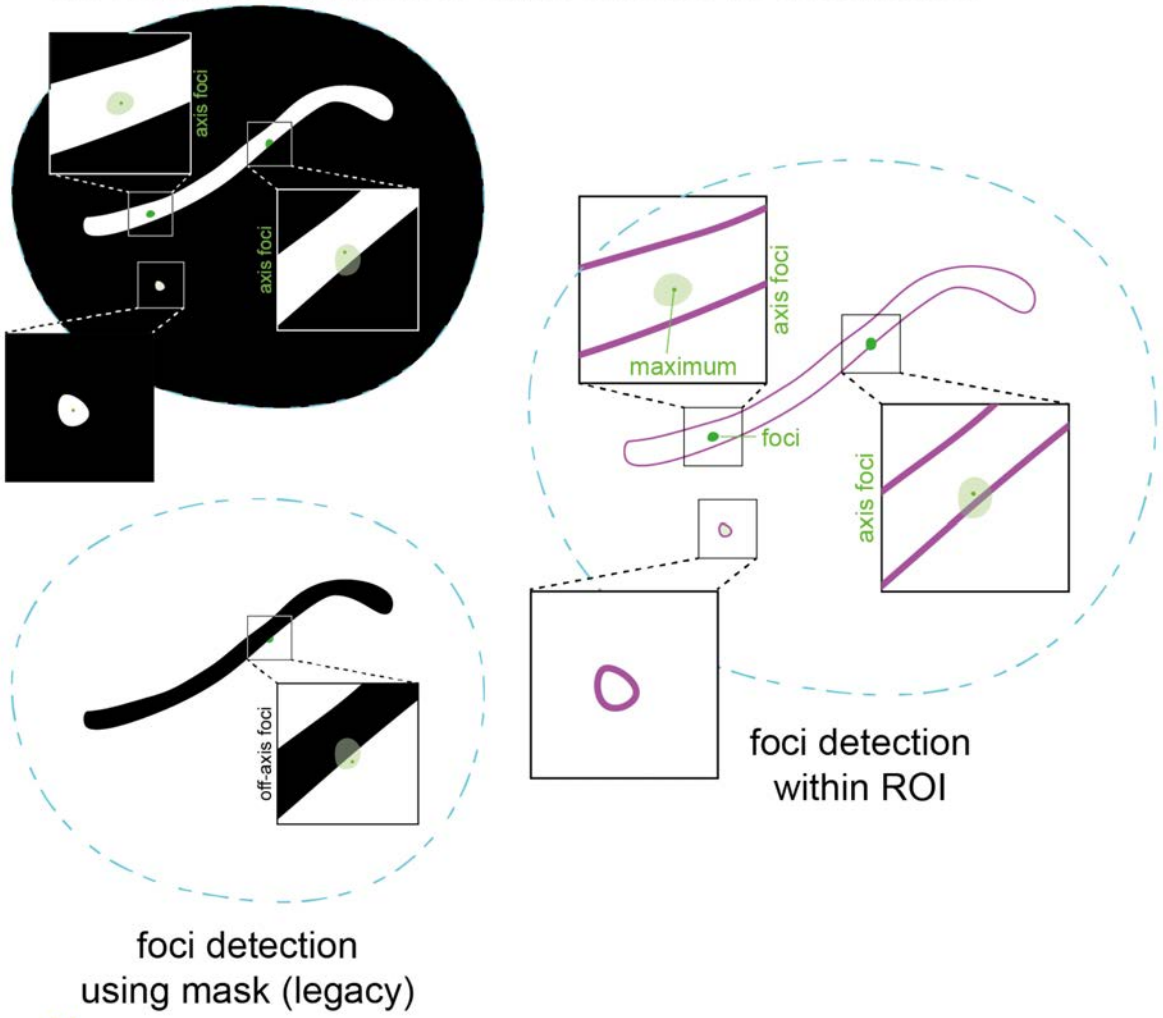


Figure 6.4. axis/off-axis foci detection methods. Once the axis ROI is drawn, the using mask method sets set the pixels outside the ROI to 0, which allows for the detection of axis foci (shown in the top left panel). On the other hand, pixels inside the ROI are set to 0 to detect off-axis foci (shown in the bottom left panel). The right-hand side insets in the upper left and lower left panels illustrate the problem of foci duplication, while the bottom inset in the upper left panel demonstrates how the background can be detected as a maximum. The right panel shows the within ROI method.

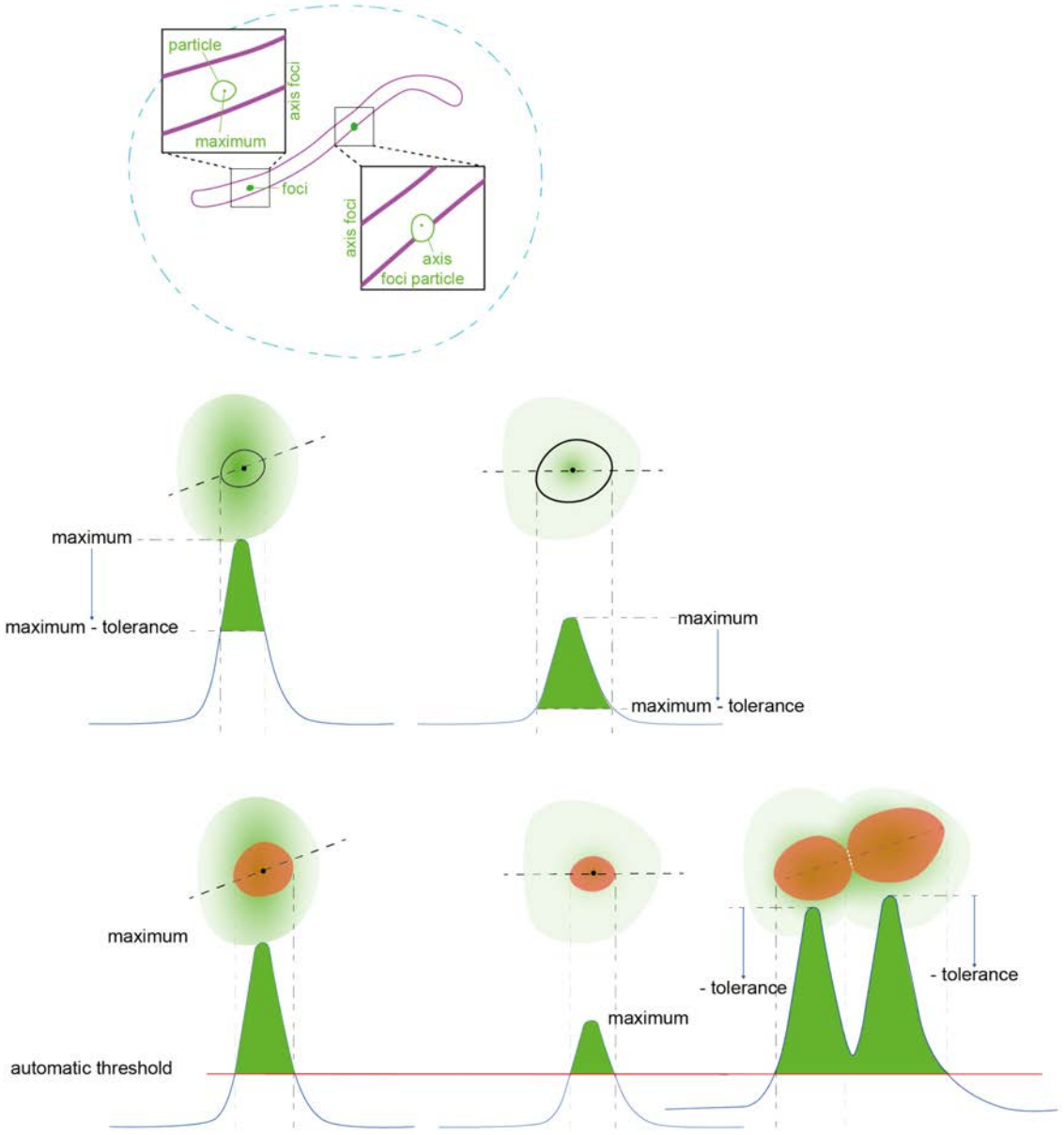


Figure 6.5. The focus' particles detection. In the top panel, the concept of focus' maximum and particle is illustrated. When the foci detection threshold is set to "None," particle's outlines are defined based on the focus' maximum value minus the tolerance/prominence determined by the user. Consequently, the bottom intensities of particles can vary across a nucleus, as shown in the middle panel. However, if an automatic threshold is used instead, all particles are defined using the same intensity (threshold), and particle definition is independent of the find Maxima command (however maxima identification is restricted to the thresholded area). Hence, a particle can be without any identified maximum and must be removed from the list of foci's particles, as foci are primarily defined by a maximum. When two maxima are found within the same thresholded area (as shown in the right case of the bottom panel), the area is segmented using a classical watershed analysis method implemented in the Find Maxima command.

Foci are characterized by their maximum intensity, detected using the "Find Maxima" command, and their boundaries, referred to as "particles" (as shown in the top panel of Figure 6.5). If the maximum intensity of a focus is within the axis ROI, it will be classified as an axis focus, even if its particle extends into the off-axis ROI.

Two approaches are suggested for identifying foci within the reference ROI (foci detection threshold parameter):

- In the first approach, foci are identified without applying any threshold (referred to as foci detection threshold “None"). Pixels with a prominence value above their neighbouring pixels are detected as maxima. The corresponding particles are identified using the "maxima within tolerance" output option of the Find Maxima command (middle panel figure 6.5). Unlike the second approach, this method does not rely on a fixed intensity threshold but rather uses the maximum intensity of the focus. Consequently, this can result in foci with different particle sizes, which may seem counterintuitive. For example, the right focus in middle panel of figure 6.5 may have a lower maximum intensity but a larger size compared to the left focus. Note that with this mode, the particles of two foci cannot come into contact with each other.

- In the second approach, maxima can be identified using either an automatic threshold (default are Huang and IJ_IsoData methods). Foci detection is limited to the thresholded area, and only pixels within this area and with an intensity above a certain prominence value compared to the neighboring pixels are detected as maxima. This is achieved by using the "above lower threshold" option of the Find Maxima command. The corresponding particles are identified using the "segmented particles" output option. If necessary, the list of automatic thresholds can be expanded to include the full ImageJ's list using OPTIONAL STEP A. The threshold is applied to the histogram of the axis ROI, and the "don't reset range" option of the Image>Adjust>Threshold menu is selected. If required, this can be modified as described in OPTIONAL STEP B.

STEP 6. Specify the foci channel and prominence value for the Find Maxima command in the "noise tolerance for foci detection" field. Previously, the term "noise" was used instead of "prominence," although there are some differences between the versions of the Find Maxima command before and after ImageJ 1.52. It's important to note that twin foci of the same color may be identified as a single focus. The macro utilizes the "strict" option of the "Find maxima" command, which is only available from ImageJ 1.52 onwards. This ensures that the macro takes full advantage of all the features provided by this version of the Find Maxima command.

Foci maxima are detected and categorized as either axis or off-axis foci using masks or within the axis ROI. Subsequently, particles are identified starting from the nucleus/user-defined ROI, utilizing the Find Maxima options mentioned earlier (referred to as total particles, ROIGroup 101- ROIGroups are summarized in figure 6.7). This particular substep is time-consuming. To bypass this step, you can unselect the "show foci particle intensities" option in the general parameters menu (see OPTIONAL STEP A).
The total particles ROI is then split, and each individual particle is compared to the corresponding axis or off-axis maxima selection. If a split particle contains an axis maximum, it is renamed as "axis_focus_ID (particle)" (ROIGroup 6). If the measurement parameters (see OPTIONAL STEP A) "show foci maximum intensities" and "show individual foci's value" options are selected, a maximum point ROI called "axis_focus_ID (maximum)" (ROIGroup 4) is created, which shares the same ID as the particle.

If the particle does not contain any axis maximum, it is then compared to off-axis maxima. If an off-axis_foci maximum fits within the particle, the particle is renamed as "off-axis_focus_ID (particle)" (ROIGroup 10), and if applicable, the associated "off-axis_focus_ID (maximum)" ROI is associated with ROIGroup 8. If the particle does not contain any maximum, it remains unassigned to any ROI group (default value of 255), and it is removed from the ROI manager at the end of the process.
For the sake of clarity, the removal of these individual particles from the manager can be done using the "remove individual foci's ROI from RoiManager" general option (OPTIONAL STEP A, figure 6.8).

RoiGroup	ROI
1	reference ROI
3	raw whole axis
4	individual axis foci maximum
5	whole axis
6	individual axis foci particle
7	off-axis
8	individual off-axis foci maximum
9	whole axis skeleton
10	individual off-axis foci particle
11	total foci maxima
13	axis foci maxima
15	off-axis foci maxima
101	total particles
103	axis foci particles
105	off-axis foci particles

Figure 6.7. ROIGroups used for the different types of ROI.

STEP 7. Configure additional intensity measurement channels or regions of interest (ROIs). The tool will identify the nucleus/user-defined ROI, as well as the axis and off-axis foci, as maxima and particles. Intensity measurements can be performed within these ROIs and for selected channels. If you want to include intensity measurements for the foci channel, check the "add foci channel intensity" option. If you wish to add another channel for intensity measurements within an ROI, enter the corresponding channel number in the "Measure foci ROI's channel intensity" field. If the field is left as -1 and the "add foci channel intensity" checkbox is not selected, no measurements will be conducted for foci's associated ROIs (particles and maxima).

To show or hide either the maxima or particle foci ROIs, check or uncheck the "show foci maximum intensities" and "show foci particle intensities" checkboxes in the general parameters menu (see OPTIONAL STEP A). For overall particle measurements displayed in the foci.xls table, all individual particles of interest are merged, creating a fused ROI (e.g., axis_foci (particles), ROIGroup 103). The corresponding values (such as IntDen or Mean) are calculated across the entire fused ROI. For global maxima calculations, the corresponding ROI (e.g., axis-foci (maxima)) is measured using the getRawStatistics(nPixels, mean, min, max, std, histogram) macro language built-in function. The number of maxima (nPixels) is multiplied by the mean value to obtain the IntDen value.

STEP 8. Select the control output options. If the “Save ROIs” option is selected, all ROIs (nucleus/user-defined, axis +/- skeleton, axis-foci and off-axis foci) will be saved in a companion foci_RoiSet_[name].zip file located in a parent ControlData folder. Results are saved in a foci.xls file (located in the same folder where the input images are stored) (see figure 6.14 for an example). Individual foci values are stored in a individualFoci.xls file (see figure 6.15). Measurement of individual foci’s values can be skipped (see OPTIONAL STEP A).

STEP 9. Change/fill-in the cropped-images directory using the browse button.

STEP 10. Click "OK" to start the analysis. If you selected the "Use advanced user parameters" option in STEP 1, an advanced user parameter window will pop up (described in OPTIONAL STEP B). Otherwise, the analysis will start. The program runs in batch mode, but if you want to display the images while processing, you can follow an OPTIONAL STEP A.

OPTIONAL STEP A. Configure the measurement parameters options. Prior to using the "Count axis and off-axis foci in one channel" tool, click on the "Set measurements parameters" button (see figure 0), which will open the Parameters menu (refer to figure 6.8).

PARAM A1. Decide whether to display warning messages or not. It is recommended to keep them hidden as they can interfere with the batch processing of the macro.

PARAM A2. By default, the images are hidden during the analysis. If you prefer to have the images displayed, select the "show images while processing files" option.

PARAM A3. The default list of methods for nucleus, axis, or foci thresholds is limited to preset automatic threshold methods that are suitable for test images and structures. If you want to expand the list to include the full range of ImageJ's automatic thresholds, select the "Display the full list of threshold methods" option.

PARAM A4. If you choose the "Measure axis length" option in STEP 4, you can modify the length measurement type. Two routines are available:

- The first (rough) routine takes advantage of the 1-pixel thickness of the skeleton. It measures the total area of the ROI in pixels and converts it to micrometers using the image's calibration. However, this method may produce unusual results due to diagonal pixels being treated as 1-pixel length (e.g., a diagonal pixel of 0.25um width may be considered as 0.35um instead of the correct √2 value).
- The second (fine) routine is a direct measurement of the skeleton and considers "horizontal, vertical, and diagonal pixels" in its calculations. However, it is measuring distances between pixels centers.

PARAM A5. The default intensity measurement is IntDen. If you want to include Mean values or remove IntDen measurements, use the "Show IntDen values" and "show Mean values" options.

PARAM A6. By default, the parameters will measure the intensity within the reference ROI (either user-defined or nucleus ROI, STEP2). To exclude the measurement of the user-defined nucleus ROI, deselect the "show user-defined nucleus ROI measurements" option.

PARAM A7: Foci ROIs can be measured for both maxima/individual maximum and particles/individual particle. Use the corresponding checkboxes to restrict the analysis to the desired measurement.

PARAM A8: During the process of defining total axis/off-axis foci particles, individual particles and their corresponding maxima are identified and measured. To skip this step (i.e., remove individual selections from the list of measured ROIs), uncheck the "Show individual foci's values" option.

Ignore the last option, which applies to two-color foci only. Click OK (A9) and proceed to "Count axis and off-axis foci" (refer to figure 0).

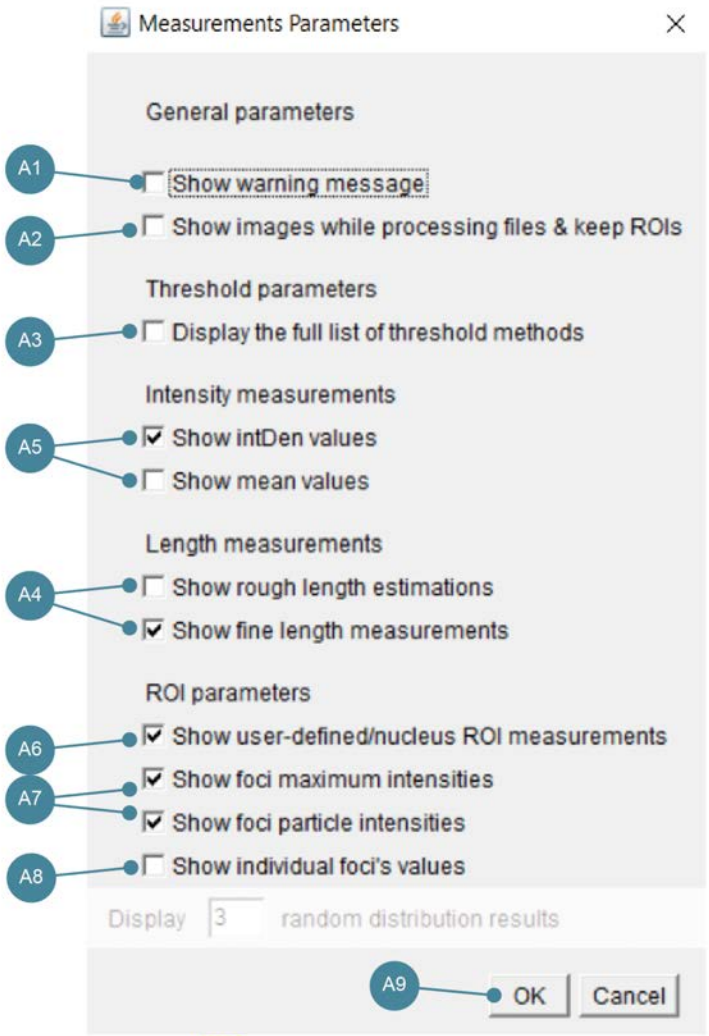


Figure 6.8. The (general) parameters window.

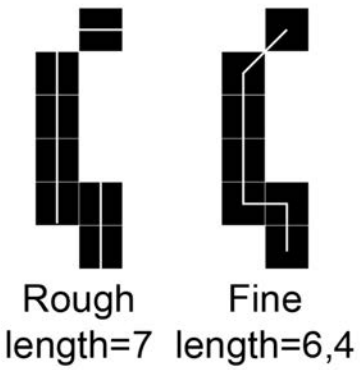


Figure 6.9. Rough and fine measurements modes.

OPTIONAL STEP B. For precise foci and axis detection, advanced user options are available. These options prove particularly beneficial for images with high noise levels or super-resolution images. When selecting the "Use advanced user option" checkbox, the "Advanced user parameters" window will appear during STEP 7 (refer to figure 6.10).

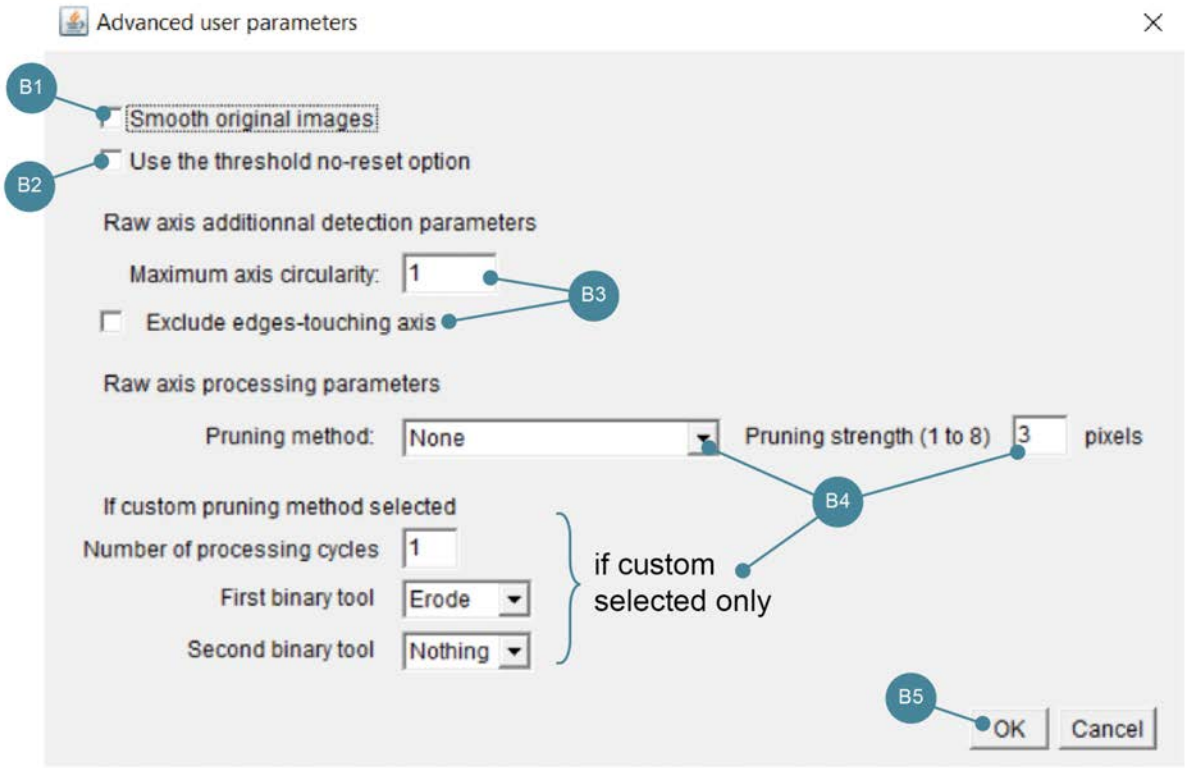


Figure 6.10. The Advanced user parameters menu

OPT. STEP B1. Enable the "smooth original images" checkbox to eliminate noise, such as photon shot noise, from the images. This filter replaces each pixel with the average value of its 3x3 neighborhood and applies to all channels. It serves two purposes: i) smoothing the axis to facilitate accurate detection, and ii) smoothing the foci, resulting in fewer errors and noise-associated foci.

OPT. STEP B2. Axis detection can be challenging when working with images that have a very high offset value, such as Abberior .msr files. In such cases, the presence of extremely high background intensity pixels, along with pixels excluded from analysis (having intensities set to 0 during the cropping step), can bias the automatic thresholding process, as shown in figure 6.11. To overcome this issue, a solution is provided through the "use the threshold no reset option".

Different versions of ImageJ, ranging from 1.52e to 1.53s, have undergone various implementations, retractions, and re-implementations of autothreshold options. The later versions have introduced a stable "don't reset range" threshold mode of the Image>Adjust Threshold command. In summary, when working with images that have more than 8 bits (say 16 bits), certain thresholding issues can arise. This is because the thresholding process relies

on utilizing an 8-bit histogram derived from the converted 16 bits image/histogram. To enable this, the range of the original 16-bit image (0-65535) is first transformed into an 8-bit range (0-256), and subsequently, the threshold is calculated based on this 8-bit histogram.
Let's consider an example of a 16-bit .msr file displayed using the Image>Adjust>Brightness&Contrast auto display range. In this case, the black intensity value is 32767 (representing the minimal intensity in the ROI, with the background spanning from 32767 to 32769), and the white intensity value is 32790 (representing the maximal intensity in the ROI). The axis signal lies between 32770 and 32790. When the "reset" option (the default mode in Adjust Threshold) is applied, the range extremes are converted to the threshold range extremes of 0 and 256. Consequently, the automatic threshold encounters two intensity clusters: the clipped area and the rest (background+signal). It finds a threshold value that fails to differentiate the background from the signal within the reference ROI, as indicated by the red area in the histogram shown in the middle left panel of figure 6.11. As a result, the entire reference ROI is thresholded, and the axis ROI erroneously resembles the reference ROI.
In contrast, when the "don't reset" option is selected, the displayed extremes are converted to the threshold range extremes of 32767 to 0 and 32790 to 256. The (discrete) 8-bit histogram is then computed (middle right panel of figure 6.11), and automatic threshold values are calculated within the red range on the histogram. Consequently, the axis is properly thresholded, and the axis ROI accurately represents the axis.

Due to the implementation, retraction, and re-implementation cycles of the reset/no-reset option (it got different names through versions) in the Adjust Threshold command across different ImageJ versions, MeiQuant may have provided correct axis/foci segmentation without utilizing the "no-reset" advanced user option. If you have upgraded ImageJ/Fiji, it is possible that the segmentation is no longer accurate unless the "Use the threshold no-reset option" checkbox is selected.

OPT. STEP B3. If necessary, you can use additional parameters for raw axis detection (see figure 6.2):

- Maximal circularity: The circularity value, calculated as 4π * (area/perimeter^2) in ImageJ, indicates the shape of an object. A value of 1.0 represents a perfect circle, while lower values indicate increasingly elongated polygons. If there are non-axis round artifacts present, consider lowering the circularity value. On the other hand, if axes are overlapping, set the maximum circularity value to 1 to avoid removing round aggregates of overlapping axes.
- Tick the "exclude touching edges" option if the reference ROI from STEP 2 (either a user-defined ROI or the threshold ROI) has cut off some axis signals that touch the edges of the image/ROI. When working with cropped nucleus images containing numerous overlapping axes, ticking this option will remove most of the axes.

OPT. STEP B4. If using the “measure axis length option at STEP4 and get unwanted decorations in axis skeleton, configure the pruning method and options (see figure 6.12). Pruning is necessary to modify the outlines of the raw axes and eliminate unwanted axis decorations, ensuring accurate axis length measurement. This step is particularly important when working with superresolution images. The process involves using the raw axis ROI to generate an 8-bit mask using the fill/clear commands (which can be manually replicated by using the ROI, followed by Edit > Fill and Edit > Clear Outside menus). The binary mask is then processed using the following options:

- The default method is "None," where the mask is not processed.
- "2x Erosion" performs two erosion operations, which remove pixels from the edges of objects. This is useful if you want to shrink the initially detected raw axes.

- "2x Dilation" involves two dilation operations, adding pixels to the edges of objects. Use this if you want to enlarge the initially detected raw axes.
- "2x Erosion then 2x Dilation" or "2x [erosion/dilation]" applies two cycles of erosion/dilation (process > binary > open) on the mask. This option is suitable for smoothing or filling holes in the axes.
- "custom" allows you to apply custom pruning parameters.

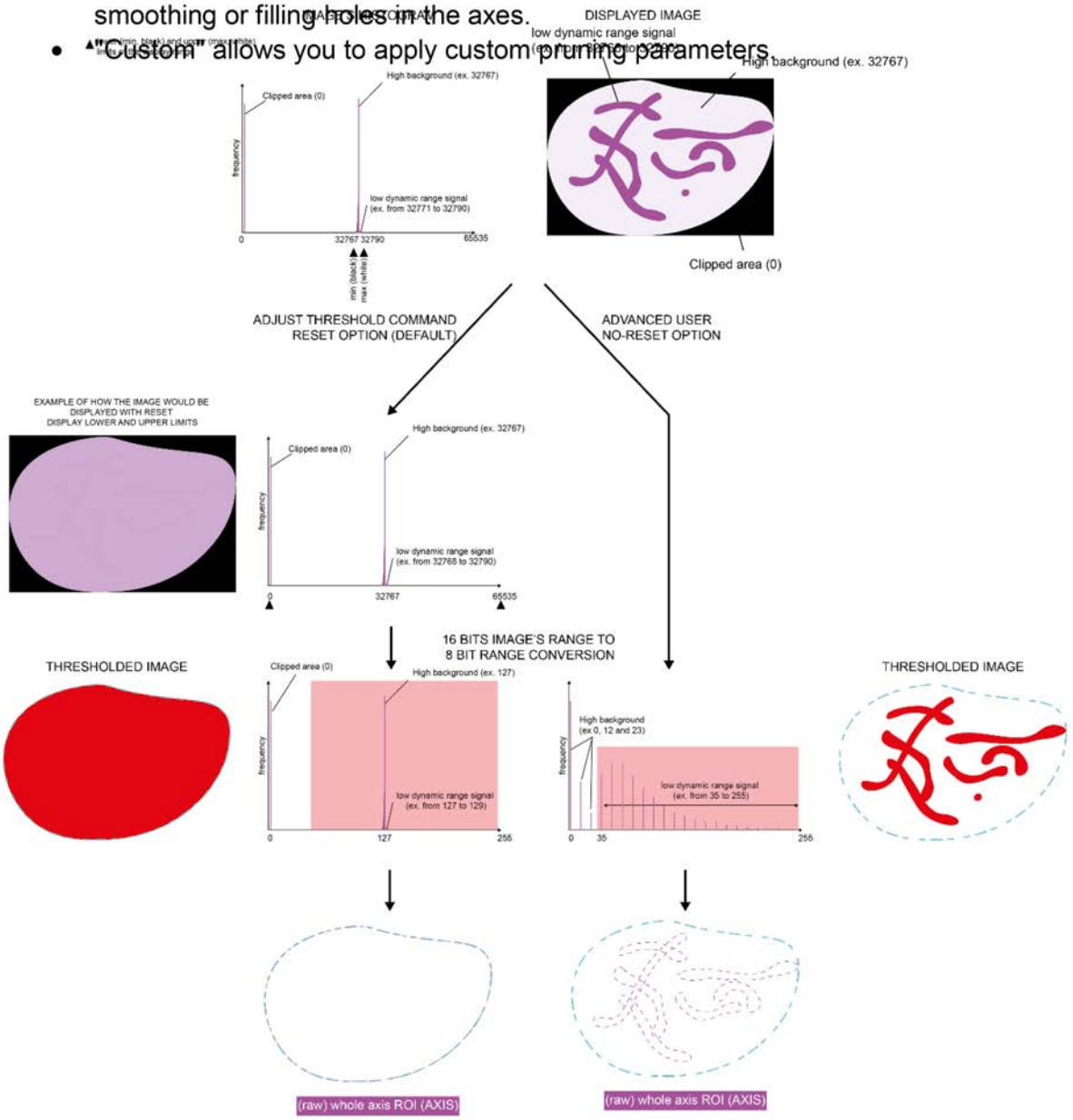


Figure 6.11 illustrates the issue that arises when low dynamic range images are displayed with the "auto" min/max display option (Image>Adjust>Brightness&Contrast). When these images are cropped, the clipped out area outside the reference ROI (set to intensity 0) can interfere with axis detection. This interference occurs because the automatic thresholding process utilizes a default reset range option (shown on the left). The histogram considered for thresholding is a full-range histogram spanning from 0 to 65535 (if using 16-bit images). An example demonstrates how the image would appear with such display parameters (displayed in the middle left of the figure). Consequently, pixels in the clipped area with a value of 0 are included in the calculation of the automatic threshold. If the dynamic range of the signal, in comparison to the neighbouring background, has similar values that differ significantly from 0, the entire reference ROI is incorrectly thresholded, resulting in the axis ROI being similar to the reference ROI. However, by utilizing the "no-reset" option, the displayed histogram “is employed as input” for the conversion from 16 bits to 8 bits, which is performed before the threshold calculation. This approach enables accurate axis detection.

The macro utilizes the process > binary > options command. Figure 6.13 illustrates the parameters used for 2x Erosion with a strength of 3. The pruning strength value ("count" in the options command) determines the number of adjacent background pixels required for removing a pixel from the edge of an object during erosion and the number of adjacent foreground pixels necessary for adding a pixel to the edge of an object during dilation. If a value lower than 1 or higher than 8 is entered, it is overridden, and the default value of 3 pixels is used.

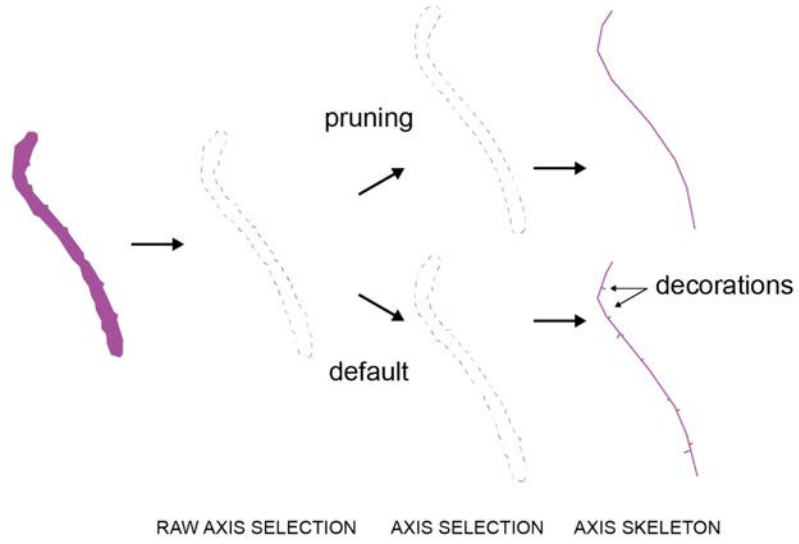


Figure 6.12. The pruning process.

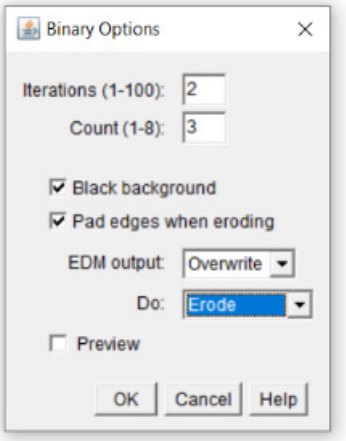


Figure 6.13. An example of how the pruning method “2x Erosion” translates in ImageJ.

When choosing the "Custom" pruning method, specify the number of cycles or iterations to be performed. Select the first “binary tool” to be used for processing. If additional rounds of processing cycles are required, choose the second “binary tool”. For example, in the case of "2x Erosion then 2x Dilation," set the process > binary > options command to have 2 iterations and select "Erode" as the operation. This applies two erosions in the first round. Then, continue with the process > binary > options command using 2 iterations and select "Dilate" as the operation for the second round. To determine the best, optimal parameters for the custom

pruning method, click the "Set advanced user parameters" button before running the “count axis & off-axis foci in one channel” tool (see figure 0).

Subsequently, a "whole axis" ROI is drawn based on the processed mask, where the pruning method is indicated within brackets.

OPT. STEP B5. Click on OK to proceed to the analysis.

Type	Image name	axis_foci	off-axis_foci	foci ch. intDen (axis foci's maxima)	Ch.3 intDen (axis foci's maxima)	foci ch. intDen (axis foci's particles)	Ch.3 intDen (axis foci's particles)	foci ch. intDen (off-axis foci's maxima)	Ch.3 intDen (off-axis foci's maxima)	foci ch. intDen (off-axis foci's particles)	Ch.3 intDen (off-axis foci's particles)	foci ch. intDen (user-defined ROI)	Ch.3 intDen (user-defined ROI)	Comment	Parameter	Value
zygo	zygo1	71	11	727.936	491.691	393648.513	332369.757	742.379	384.840	70400.369	45482.010	1710177.183	3743054.562		Smooth original images	no
zygo	zygo2	94	5	1004.151	636.168	700668.147	523825.392	1535.575	743.963	68017.272	38848.597	2070002.423	4480294.599		ROI chosen	user-defined
zygo	zygo3	115	15	953.729	626.509	910211.516	682804.636	1052.900	692.201	127120.277	100526.256	2792088.185	6132077.321		Axis channel	4
zygo	zygo4	108	9	1127.700	521.360	777994.894	423891.566	1146.153	407.169	86714.790	36628.230	2292151.888	4246052.450		Axis Threshold	Huang
zygo	zygo5	94	4	911.281	626.024	661251.392	534429.836	906.821	380.542	27790.651	14491.635	1894090.157	3901607.854		Axis Min size	5
zygo	zygo6	92	9	856.116	496.653	684442.040	454828.632	1081.494	517.962	106840.149	63915.258	2423823.104	3403844.487		Axis Max Circ.	1.0
zygo	zygo7	86	13	839.525	507.731	596887.743	421260.716	913.229	469.041	109172.060	68074.917	2167817.374	4231547.352		Exclude edge-touching axis	no
zygo	zygo8	246	31	1350.080	1223.345	2781216.989	3102732.888	1232.832	1085.747	285187.477	292699.057	5618196.062	9924710.298		Measure axis length	no
zygo	zygo9	163	9	986.097	919.721	1387078.125	1538053.754	1082.489	846.571	97389.948	86442.540	3062661.525	6182691.664		Pruning Mode(s) tested	2x [Erosion & Dilation]
zygo	zygo10	180	10	1058.978	1016.572	1688313.762	1842845.447	980.541	685.907	108696.049	104255.067	3551410.511	6949225.961		Pruning Strength (when used)	3
zygo	zygo11	122	6	851.237	681.980	1035158.049	1010017.006	705.839	522.205	37523.354	34285.284	2817523.158	4402000.521		Foci detection method	within ROI
zygo	zygo12	203	31	1077.845	1011.560	2020879.936	2194065.746	1097.999	899.990	292389.208	303813.951	4741530.098	8495336.351		Foci Detection Threshold	IJ_IsoData
zygo	zygo13	26	4	687.893	542.643	212249.923	196441.736	668.834	790.683	28900.352	31669.851	1486174.922	3814675.056		Foci channel	2
zygo	zygo14	20	3	737.638	514.118	148991.143	129174.831	1194.380	387.426	37860.053	16026.288	1057289.988	2362999.489		Foci Detection noise	500
															Measure foci's ROI intensity	Ch. 3 & foci channel (Ch.2)
															Images from	D:...
															ROI saved	yes
															ROI and any other output folder	D:...
															options:	
															Show warning messages	no
															Show images	no
															Get integrated density intensity values	yes
															Get mean intensity values	no
															Include reference Roi when measuring intensities	yes
															Show foci's Maximum intensities	yes
															Show foci's particle intensities	yes
															Meiosis bar tool	Count axis and off-axis foci in one channel
															Meiosis bar version	v2.05
															ImageJ version	1.53t99

Figure 6.14. a result file of the Count axis and off-axis foci in one channel macro

Type	Image name	focus' ID	focus' type	foci ch. intDen (focus' maximum)	Ch.3 intDen (focus' maximum)	foci ch. intDen (focus' particle)	Ch.3 intDen (focus' particle)
zygo	zygo_1	0	axis	784.264	526.352	5774.938	5188.350
zygo	zygo_1	1	axis	1528.574	1520.421	16037.986	18616.515
zygo	zygo_1	2	axis	979.639	510.958	6495.192	4222.296
zygo	zygo_1	3	axis	993.997	758.438	7369.694	8067.895
zygo	zygo_1	4	axis	726.272	844.818	4493.471	6539.847
zygo	zygo_1	5	axis	694.539	595.216	5493.474	5489.934
zygo	zygo_1	6	off-axis	667.832	237.172	6779.067	3401.884
zygo	zygo_1	7	axis	638.938	523.821	4188.838	4105.132
zygo	zygo_1	8	axis	933.438	358.350	7399.016	3890.055
zygo	zygo_1	9	axis	603.035	593.817	3644.920	4178.916
zygo	zygo_1	10	axis	1056.602	408.563	7969.261	3939.526
zygo	zygo_1	11	axis	540.571	458.152	4100.407	4023.072
zygo	zygo_1	12	axis	716.920	203.056	5848.626	2292.725
zygo	zygo_1	13	axis	688.282	365.996	3869.819	2570.163
zygo	zygo_1	14	axis	504.959	294.896	2704.968	1886.442
zygo	zygo_1	15	axis	561.569	470.462	4040.711	4363.471
zygo	zygo_1	16	axis	581.107	192.163	3589.147	1461.591
zygo	zygo_1	17	axis	1083.613	732.478	6733.437	5663.609
zygo	zygo_1	18	axis	1109.484	814.243	8130.835	8590.143
zygo	zygo_1	19	axis	604.666	580.411	4019.524	4413.765
zygo	zygo_1	20	axis	655.609	639.270	4028.233	4988.512
zygo	zygo_1	21	axis	521.602	251.570	2997.448	1704.353
zygo	zygo_1	22	axis	565.957	278.891	3869.762	2907.523
zygo	zygo_1	23	axis	595.992	216.394	3365.593	1438.912
zygo	zygo_1	24	axis	529.473	314.894	2748.691	1874.632
zygo	zygo_1	25	axis	778.389	645.905	5442.465	6751.882
zygo	zygo_1	26	axis	672.856	650.349	7948.026	6914.172
zygo	zygo_1	27	axis	797.137	399.788	4935.755	3639.333
zygo	zygo_1	28	off-axis	1221.978	465.532	12159.803	5674.368
zygo	zygo_1	29	off-axis	813.496	497.607	10318.646	7714.892
zygo	zygo_1	30	axis	833.896	979.954	11921.057	13253.844
zygo	zygo_1	31	axis	956.436	569.561	6522.359	5231.928
zygo	zygo_1	32	axis	627.530	658.266	3836.690	5477.008
zygo	zygo_1	33	axis	576.031	496.810	5870.496	5902.579
zygo	zygo_1	34	axis	701.589	431.405	4022.129	2918.146
zygo	zygo_1	35	axis	722.578	345.404	5513.679	3123.805
zygo	zygo_1	36	off-axis	945.133	588.602	9335.999	7803.831
zygo	zygo_1	37	axis	520.517	569.329	2753.011	3919.839
zygo	zygo_1	38	axis	531.948	465.697	3145.202	3084.667
zygo	zygo_1	39	axis	1055.763	397.473	8817.204	4590.553
zygo	zygo_1	40	axis	940.845	440.587	8448.379	5456.377
zygo	zygo_1	41	axis	863.692	688.996	6519.652	6212.844
zygo	zygo_1	42	off-axis	736.248	437.916	5293.254	3570.807
zygo	zygo_1	43	axis	857.551	725.505	7055.309	6700.137
zygo	zygo_1	44	axis	1040.529	633.182	8511.808	6954.748
zygo	zygo_1	45	axis	550.979	600.701	5217.849	6248.061
zygo	zygo_1	46	off-axis	717.717	447.685	5564.025	4342.260
zygo	zygo_1	47	axis	526.841	448.663	2526.658	2532.560

Figure 6.15. a subset of an individualFoci result file of the “Count axis and off-axis foci in one channel”

STEP11. To ensure precise analysis control, when selecting the save ROIs option, utilize the "Start checking results" tool in the main meiosis bar. This will prompt the appearance of the "check results" window (refer to figure 6.17). If you simply run the macro without any modifications, the default folder and method will be sufficient. However, if you wish to monitor and manage the macro's results, specify both the folder and the option for "Count axis and off-axis foci in one channel".

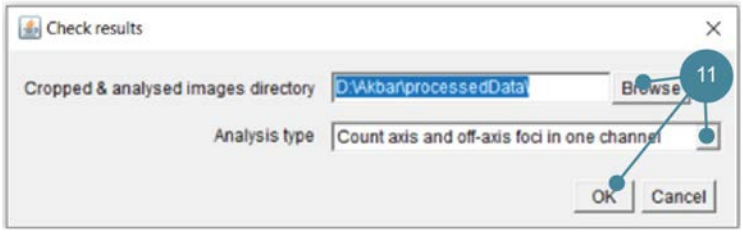


Figure 6.16. The check results menu

The first image analysed will be opened together with its companion foci_RoiSet_[name].zip file ROI set, as in fig. 6.17. The ROI Manager contains several ROIs:

- The reference ROI, as set in STEP2 (here nucleus). This is the ROI used for further identifications (ie. axes).
- The raw whole axis is the initial ROI, that is further processed in whole axis (2x Erosion) (as the preset 2x Erosion pruning method was used in OPTIONAL STEP B). From this is derived the Off-Axis ROI (XOR of reference ROI and whole axis ROI).
- The total_foci maxima (as the within ROI mode was used at STEP 5) There would be no total foci maxima ROI if the within mask mode was used. These are further split into axis_foci maxima and off-axis_foci maxima.
- Foci particles are identified and sorted according to whether they sit within the whole axis ROI or not into axis_foci (particles) ROI and off-axis_foci (particles) ROI.
- Individual foci particles (and corresponding maxima) are included whenever the “show individual foci’s values” option is used together with the “show foci particle intensities”.

All ROIs are attributed a ROIGroup (see figure 6.7 the groups list),To control the next analysed image, click on “check next results” button on the main Meiosis bar (figure 0).

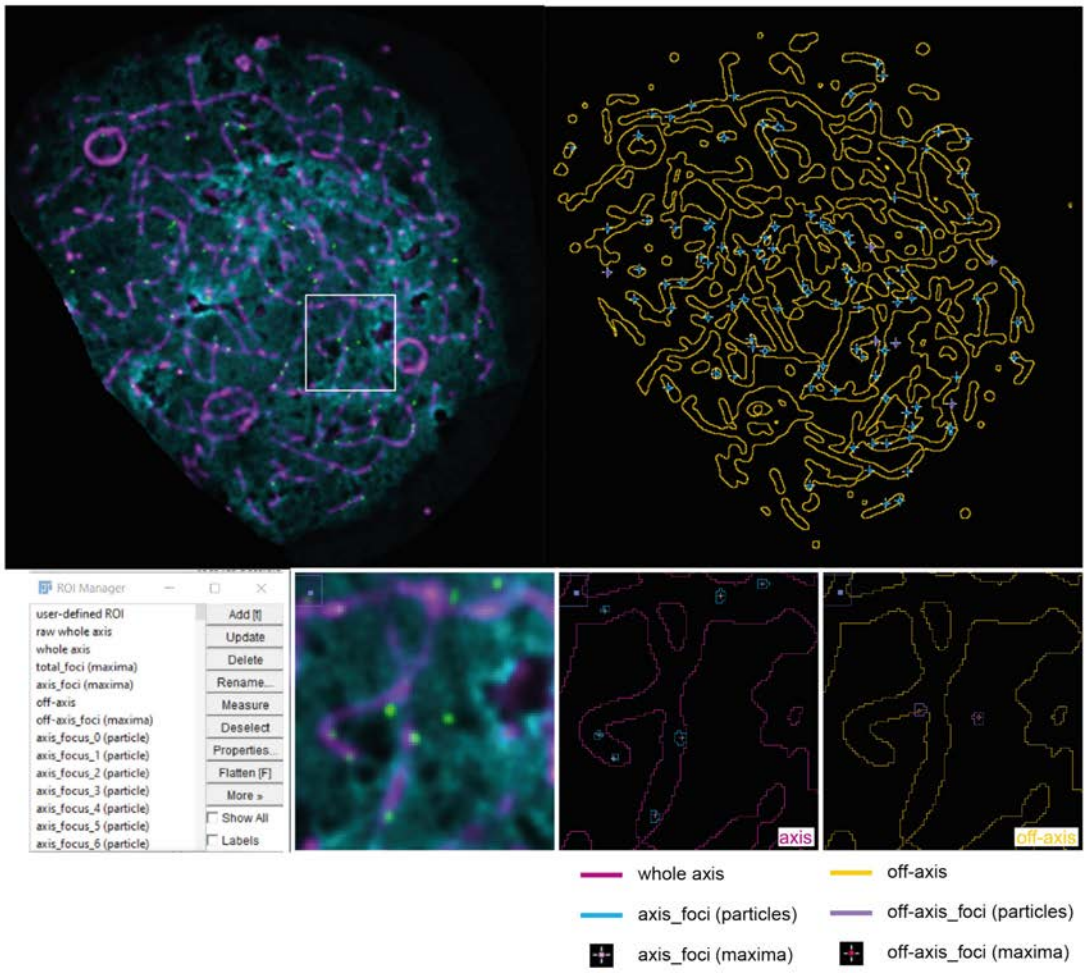


Figure 6.17. Control ROIs as generated by the Count axis and off-axis foci in one channel macro.

COUNT AXIS/OFF-AXIS FOCI & COLOCALIZE

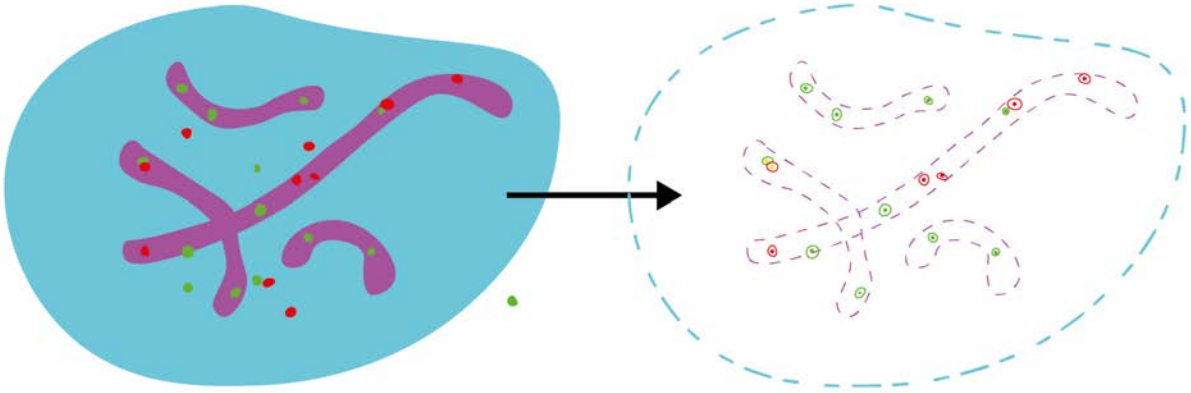


Figure 7.0. Visual description of the tool.

The tool is designed to detect the axis and foci from two different stains. To customize the step-by-step process, you have the option to configure additional parameters through an ➔ OPTIONAL STEP A. By clicking the "Set Measurements Parameters" button (as shown in Figure 0), you can modify parameters such as an expanded threshold list, intensity/length measurement types, and ROI parameters.

The algorithm "count axis/off-axis foci in two channels and colocalize" performs the following tasks: i) Locates the axis based on staining specific to the axis complex, ii) Identifies axis foci in two channels (or off-axis foci, noting that the tool cannot detect both types of foci simultaneously), iii) Measures colocalization, following the methodology described in the Lachmanovich 2003 Journal of Microscopy, Vol. 212, Pt 2, November 2003, pp. 122-131.

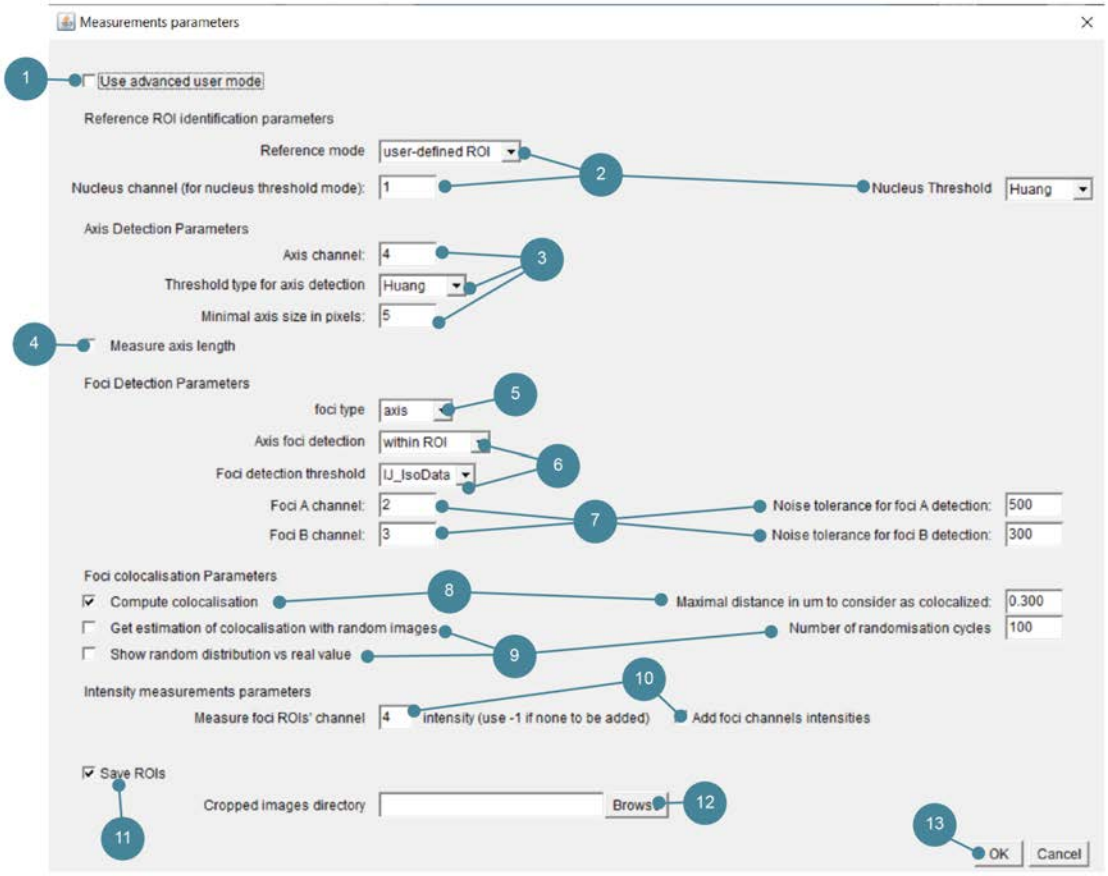


Fig. 7.1. The “count axis/off-axis foci in two channels and colocalize” menu

STEP 1. When working with noisy images, particularly those obtained from STED setups, it is advisable to utilize the advanced user mode. This option introduces an additional ➔ OPTIONAL STEP B.. By selecting the "use advanced user mode" option, you gain access to further capabilities, such as advanced criteria for axis elements validation (see below) or the ability to prune the axis and remove undesired axis decorations. Once this option is chosen, the "Advanced user parameters" menu will be presented after completing STEP 12.

STEP2. Select how the reference ROI is defined (ie. the ROI that will restrict all further measurements). There are two options (aka reference mode) available (as shown in Figure 7.1):

- "User-defined ROI": This corresponds to the ROI that was saved during the stage-cropping step. If you select this option but the ROI is not found, an error message will be displayed. When opting for this reference mode, you can proceed directly to STEP3 without the need to fill in the nucleus channel and threshold fields.
- "Nucleus threshold": In this mode, a threshold based on a DAPI staining channel is utilized. To use this mode, you need to select the channel to be used for the threshold (the first channel is referred to as channel 1) and choose the appropriate threshold algorithm. Additional automatic threshold methods are accessible for a wider range of options (refer to OPTIONAL STEP A for more details).

STEP3. Configure the parameters for axis detection as follows:

- Specify the axis channel (remember that the first channel is labeled as channel #1).
- Set the appropriate threshold to be applied. First, the algorithm uses this threshold. If no pixels above the threshold are detected within the reference ROI, the analysis will be aborted, and the final table will display "no axis detected."

The threshold generates a temporary "axis threshold" selection in the ROI Manager. Using the Analyze>Analyze Particle command, axis elements (ie bits of axis) are detected. The next step is used to exclude unwanted (small) elements.

- Enter the minimum pixel size for the thresholded axis elements. Note that setting this value too high may not be suitable for early stages. Additional axis detection parameters can be utilized (refer to OPTIONAL STEP B for more details and figure 7.2).

Next, the elements that meet the specifications are identified and added to the ROI Manager. These elements are then combined into a temporary ROI using the "Combine" option in the ROI Manager. In cases where overlapping axes create a loop, resulting in improper identification, the hole within the loop is considered part of the axis. This is rectified by applying the "AND" option of the ROI Manager to the "axis threshold" ROI and the temporary ROI (see figure 7.3). Subsequently, both ROIs are deleted, and a "raw whole axis" ROI is added to the manager.

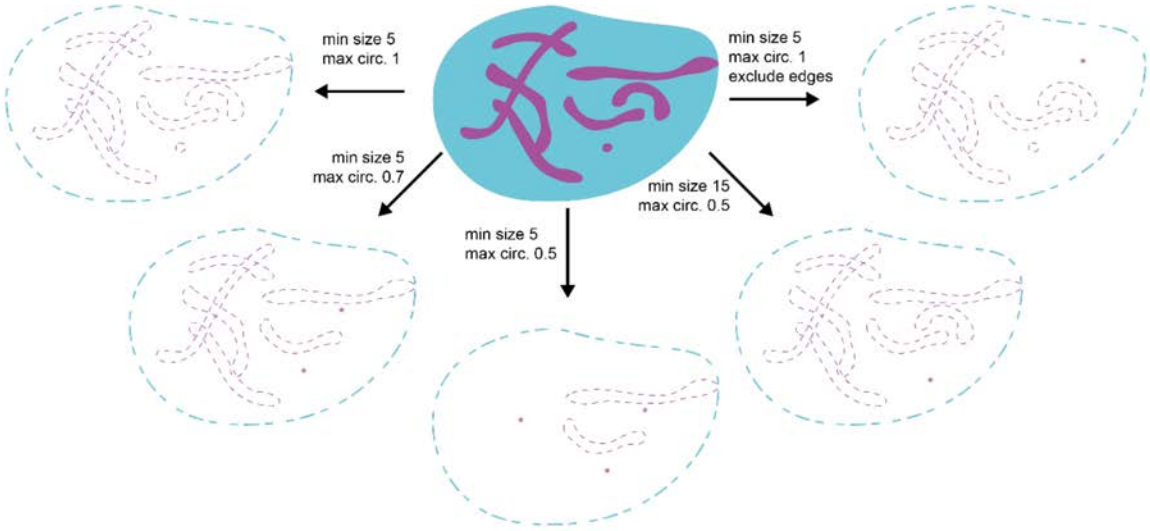


Figure 7.2 illustrates the impact of exclusion parameters, with excluded elements marked by an asterisk. By default, increasing the minimum size in pixels enables the elimination of undesirable elements, as demonstrated by the comparison between the left and lower-right illustrations. Furthermore, advanced user parameters provide additional exclusion criteria. For example, maximum circularity can be employed to remove round-shaped objects, as observed in the comparison between the left and lower drawings. However, it's important to note that this may also result in the removal of clusters of axes, as depicted in the lower-left drawing. The use of edge exclusion, as displayed in the right drawing, offers further possibilities for exclusion

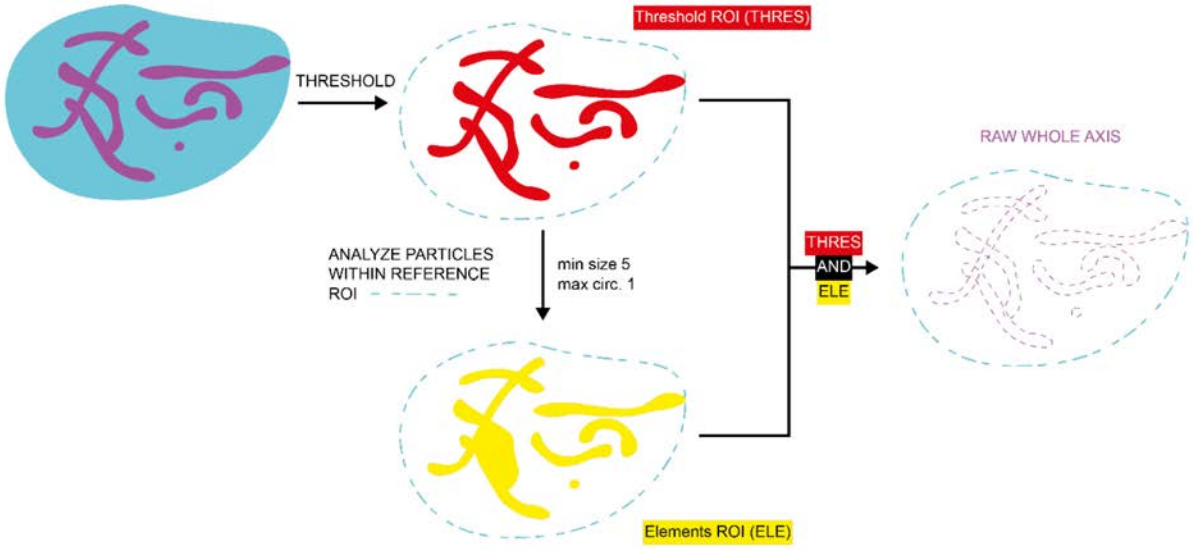


Figure 7.3. The process of detecting axis elements involves several steps. Initially, the staining of the axis is subjected to thresholding, and the "analyze particles" command is utilized to identify the individual axis elements. However, to handle cases of axis loops, where multiple axes overlap (as seen in the lower left corner of the nucleus), further processing is necessary. This includes combining a temporary threshold ROI with the fused elements selection to address this situation.

STEP4. Optionally, you have the choice to measure the overall length of the axis. When the "Measure axis length" checkbox is selected, a skeleton analysis is conducted using the "Skeletonize 2D/3D" plugin. By default, the raw axis ROI is duplicated to create the (final) "whole axis" ROI, which serves as input for this analysis. Prior to the skeleton analysis, the raw axis ROI can undergo additional processing or refinement using the pruning method described in OPTIONAL STEP B (refer to figure 7.4). The resulting refined axis is represented by the "whole axis" ROI, with any applied pruning method indicated within brackets, if applicable. Subsequently, the modified processed mask is utilized as input for the analysis.

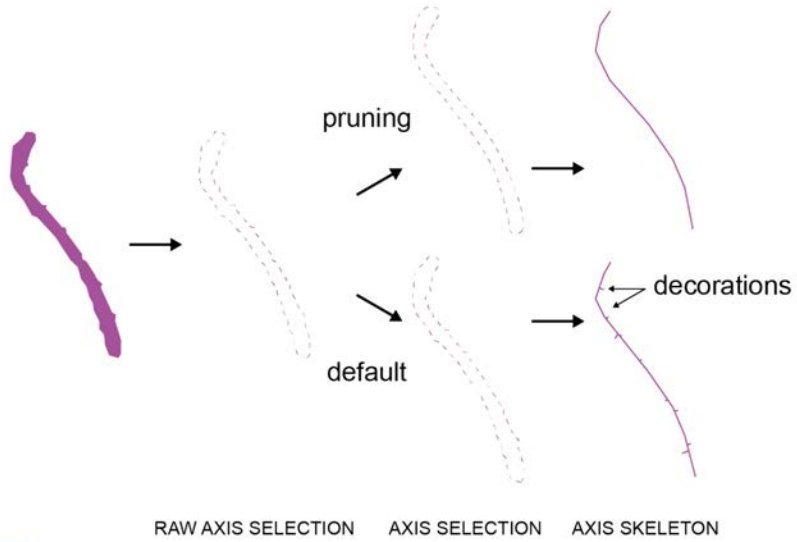


Figure 7.4 illustrates the process of pruning the axis. The analysis of the axis skeleton relies on smooth outlines to ensure the exclusion of unwanted decorations. In the bottom part of the figure (default), the raw axis ROI with a rough and spiky perimeter is displayed without pruning. Consequently, the resulting axis selection includes decorations, which can introduce bias to the length measurements of the skeleton. Although the example depicted here demonstrates a relatively mild case, decorations can pose a substantial challenge.

The following three steps are used to detect foci in both foci channels. A focus is primarily identified by its maximum intensity (as shown in figure 7.5). The tool also has the ability to analyze the particles associated with the foci.

STEP 5. Choose the type of foci you want to consider, whether they are foci along the axis or off-axis foci. The foci of the other type will be excluded from the analysis and not added to the ROI Manager. It's important to note that the position of a focus in relation to the axis is determined by its maximum intensity. If a focus's particle overlaps both the axis and off-axis regions, and its maximum intensity is within the axis region, the particle selection will be referred to as an "axis focus (particle)" (as shown in the lower inset of figure 7.5).

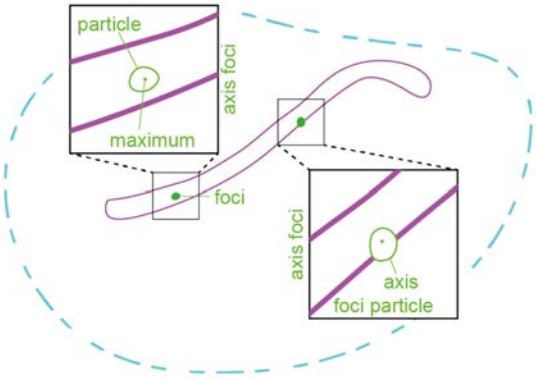


Figure 7.5. Foci are defined by their maxima and outlines (=particles).

STEP 6. The macro utilizes the Find Maxima command to its fullest potential. Two Axis foci detection modes are proposed for the purpose of distinguishing between on-axis and off-axis foci.

- The legacy "using masks" method (<v2.01) involves duplicating the foci channel image twice. The first duplicate image is used to mask out the off-axis pixels by setting them to black (figure 7.6, upper left panel). The Axis ROI is selected, and the Edit > Clear Outside command is applied. Then the Find Maxima command is run with the user-defined noise/prominence value, which identifies the on-axis foci maxima. The second duplicate image is used to mask out the on-axis pixels (figure 7.6, lower left panel). The combination of the reference ROI [XOR] axis ROI is selected, and the Edit > Clear Outside command is applied. The same Find Maxima operation is performed as with the first duplicate, resulting in the detection of off-axis foci. This method has a few biases. If the background signal in the image is high and small pieces of the axis are detected, the Find Maxima command may detect background foci channel signals above 0 (see figure 7.6, lower inset of the upper left panel). This is also true when the file format has a high "zero" value (camera offset determined by the vendor's choice). To address this, you can discard small axis pieces by increasing the minimum axis pixel size or use the "within ROI" option. Moreover, whenever a focus lies at the edge of the detected axis, it may be split into two parts (one in the axis mask and the other "half" in the off-axis mask), leading to the detection of two foci (one off-axis and one axis focus, as shown in the right-hand side insets of the left panels in figure 7.6).
- The "within ROI" option (from v2.01, figure 7.6 right panel) is used to detect foci within the reference ROI (referred to as total_foci (maxima)), and then filter out the maxima located outside the axis ROI to obtain the axis_foci (maxima). This method is implemented in a more time-consuming manner but is considered more elegant and is now the default method. Maxima that are not within the axis ROI are discarded.

Two modes are provided for the detection of foci based on their intensity values, considering that a focus cannot be adequately summarized by a single pixel's intensity value. Both modes utilize the reference ROI as the input ROI, meaning that foci outside the reference ROI will be excluded from the analysis. The foci detection threshold modes are as follows:

- "None": In this mode, maxima are identified without applying any threshold. Foci with intensities above a certain prominence value are detected as maxima. The corresponding particles are identified using the "maxima within tolerance" output option of the Find Maxima command (refer to figure 7.7, top panel). With this mode, it is impossible for particles of two foci to come into contact with each other.
- Automatic threshold (IJ_IsoData or Huang): In this mode, maxima are identified using either the Huang or IJ_IsoData automatic thresholding methods. It's important to note that foci detection is limited to the thresholded area, and only foci above a specified

prominence value within this area are detected as maxima (figure 7.7 bottom panel). This can be manually reproduced using the "above lower threshold" option of the Find Maxima command. The corresponding particles are identified using the "segmented particles" output option. If two maxima are located within the same thresholded area, the area will be split into two separate zones.

Foci maxima are detected and stored in the RoiManager (e.g axis_fociA (maxima)). The different ROIs generated are associated with various ROIGroups (figure 7.8 and 7.9) for the purpose of further, downstream, analysis by the user.
Foci particles are detected starting from the nucleus or user-defined ROI using the Find Maxima command options mentioned earlier (specifically, the segmented particles output). The ROI encompassing all particles is split, and each individual particle is compared to the corresponding axis/off-axis maxima selection.
This particular substep can be time-consuming. To expedite the process, you can deselect the "show foci particle intensities" option in the general parameters menu (refer to OPTIONAL STEP A). If a split particle contains an axis/off-axis maximum, it is given a new name, such as "axis_focusA_ID (particle)". If the measurement parameters (as specified in OPTIONAL STEP A) include "show foci maximum intensities" and "show individual foci's value", a maximum point ROI is created for the corresponding focus' particle (following the previous example, it would be "axis_focusA_ID (maximum)"). Both the maximum and particle share the same ID.
In cases where the individual particle does not contain any axis maximum, no ROI group is assigned (remaining at the default value of 255), and the particle is removed from the ROI manager at the end of the process.
Individual axis/off-axis particles are merged to create a [type] foci (particles) ROI if the "compute colocalisation" option is not selected.

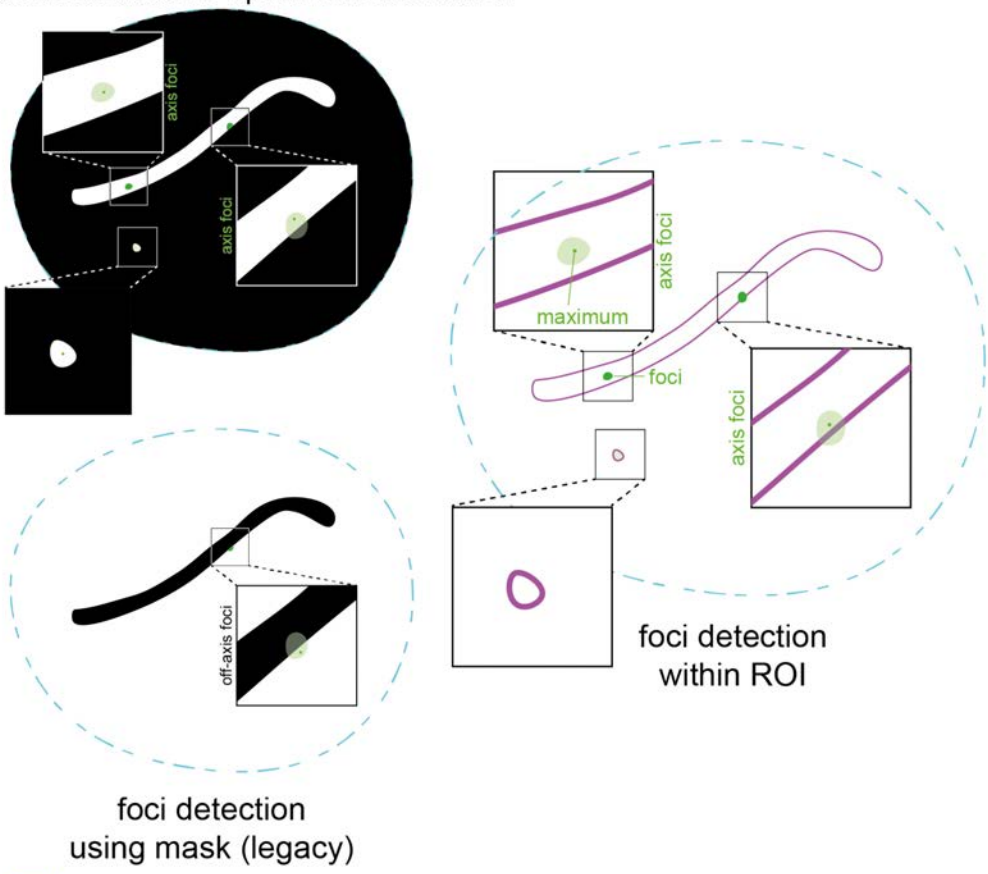


Figure 7.6 illustrates the axis/off-axis foci detection methods. After drawing the axis ROI, the "using mask" method sets the pixels outside the ROI to 0, enabling the detection of axis foci (depicted in the

top left panel). Conversely, the pixels inside the ROI are set to 0 to detect off-axis foci (shown in the bottom left panel). The right-hand side insets in the upper left and lower left panels demonstrate the issue of foci duplication, while the bottom inset in the upper left panel showcases how the background can be detected as a maximum. The right panel displays the "within ROI" method.

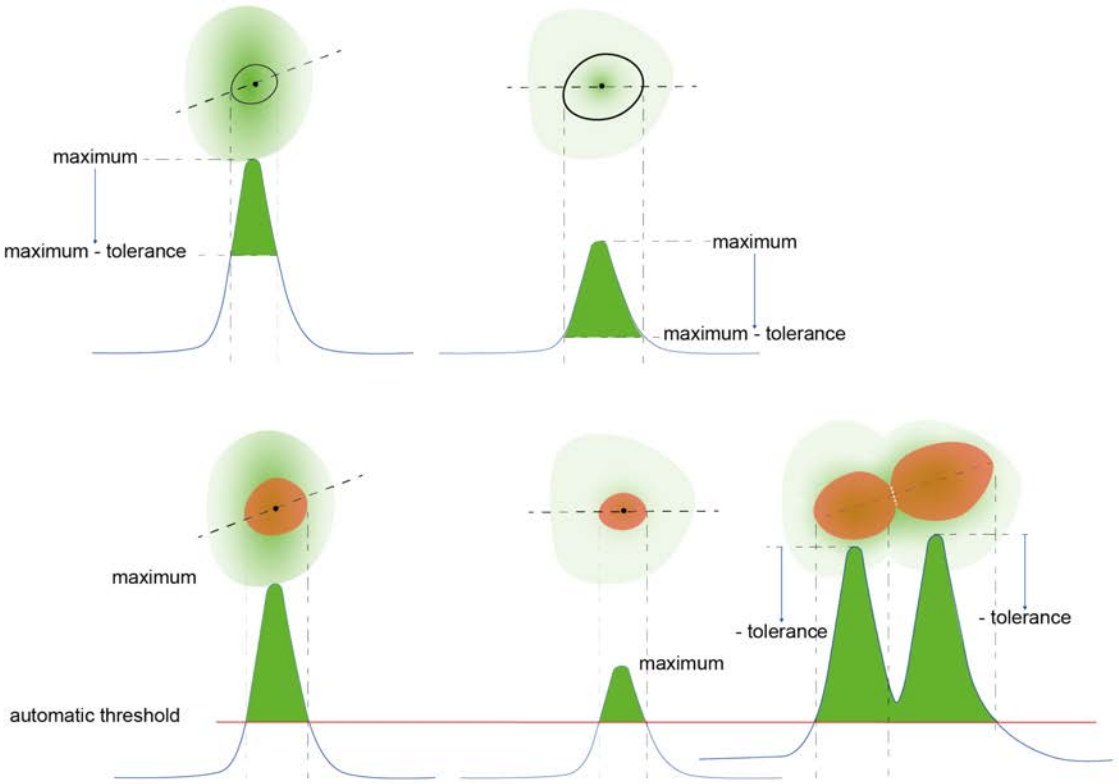


Figure 7.7 Maximum and particle detection methods. When the foci detection threshold is set to "None," particle outlines are determined based on the focus's maximum value minus the user-defined tolerance or prominence (top panel). As a result, the bottom intensities of particles can vary across a nucleus (compare the particles sizes of left and right foci of the top panel). If an automatic threshold is utilized, all particles are defined using the same intensity (threshold), and the particle definition is independent of the Find Maxima command (although maxima identification is limited to the thresholded area, bottom panel). Consequently, a particle may exist without any identified maximum and should be excluded from the list of focus particles, since foci are primarily defined by a maximum. In situations where two maxima are found within the same thresholded area (as shown in the right case of the bottom panel), a classical watershed analysis method implemented in the Find Maxima command is employed to segment the area (dashed white line).

RoiGroup	ROI
1	reference ROI
3	raw whole axis
5	whole axis
7	off-axis
9	whole axis skeleton
19	axis fociA maxima
25	axis fociB maxima
31	colocalised area axis fociA (maxima)
37	colocalised area axis fociB (maxima)
43	not colocalised area axis fociA (maxima)
49	not colocalised area axis fociB (maxima)
55	random axis foci A
61	random axis foci B
67	axis fociA maxima colocalised with axis fociB
73	axis fociB maxima colocalised with axis fociA
79	axis fociA maxima not colocalised with axis fociB
84	individual axis fociA maximum colocalised with an axis fociB
85	axis fociB maxima not colocalised with axis fociA
86	individual axis fociA particle colocalised with an axis fociB
91	random axis fociA maxima colocalised with axis fociB
97	random axis fociB maxima colocalised with axis fociA
100	individual axis fociB maximum colocalised with an axis fociA
102	individual axis fociB particle colocalised with an axis fociA
121	axis fociA particles colocalised with an axis fociB
127	axis fociB particles colocalised with an axis fociA
133	axis fociA particles not colocalised with an axis fociB
139	axis fociB particles not colocalised with an axis fociA
148	individual axis fociA maximum not colocalised with an axis fociB
150	individual axis fociA particle not colocalised with an axis fociB
164	individual axis fociB maximum not colocalised with an axis fociA
166	individual axis fociB particle not colocalised with an axis fociA

Figure 7.8. ROIGroups associated with ROI generated by the macro in the axis mode.

RoiGroup	ROI
1	reference ROI
3	raw whole axis
5	whole axis
7	off-axis
9	whole axis skeleton
21	off-axis fociA maxima
27	off-axis fociB maxima
33	colocalised area off-axis fociA (maxima)
39	colocalised area off-axis fociB (maxima)
45	not colocalised area off-axis fociA (maxima)
51	not colocalised area off-axis fociB (maxima)
57	random off-axis foci A
63	random off-axis foci B
69	off-axis fociA maxima colocalised with off-axis fociB
75	off-axis fociB maxima colocalised with axis off-fociA
81	off-axis fociA maxima not colocalised with off-axis fociB
87	off-axis fociB maxima not colocalised with off-axis fociA
88	individual off-axis fociA maximum colocalised with an off-axis fociB
90	individual off-axis fociA particle colocalised with an off-axis fociB
93	random off-axis fociA maxima colocalised with off-axis fociB
99	random off-axis fociB maxima colocalised with off-axis fociA
104	individual off-axis fociB maximum colocalised with an off-axis fociA
106	individual off-axis fociB particle colocalised with an off-axis fociA
123	off-axis fociA particles colocalised with an off-axis fociB
129	off-axis fociB particles colocalised with an off-axis fociA
135	off-axis fociA particles not colocalised with an off-axis fociB
141	off-axis fociB particles not colocalised with an off-axis fociA
152	individual off-axis fociA maximum not colocalised with an off-axis fociB
154	individual off-axis fociA particle not colocalised with an off-axis fociB
168	individual off-axis fociB maximum not colocalised with an off-axis fociA
170	individual off-axis fociB particle not colocalised with an off-axis fociA

Figure 7.9. ROIGroups associated with ROI generated by the macro in the off-axis mode.

STEP 8. If the colocalization option is enabled (by selecting the "compute colocalisation" checkbox), a mask is generated for axis type foci (either axis or off-axis foci as set at STEP5) in both channels, provided that some maxima were found in the corresponding channels and within the axis type (axis or off-axis) ROI. The mask consisting of single points is then used to calculate a 32-bit Euclidean distance map. You can manually achieve this by using the Process>FindMaxima command and selecting the single points output. Ensure that the mask is displayed with an inverted lookup table (LUT) as indicated in the top banner of the mask window. Then, set the output options by going to Process>Binary>Options and select 32-bits as the EDM (Euclidean Distance Map) type. Run the process by selecting Process>Binary>Distance Map.
Next, a threshold is applied to the EDM Map using the converted colocalization distance. The "maximal distance in µm to be considered as colocalized" value is converted into pixels, and the converted value is used as the threshold. A selection is drawn based on the thresholded

area and saved in the ROI Manager as "colocalised_area_[type]_foci[letter]". A complementary ROI is also created for the non-colocalized foci areas, labeled as not colocalised fociA/B areas (refer to figure 7.8 and 7.9 for ROIGroups).
When a specific foci maximum (as identified in STEP 7) from the other channel (e.g., A if coloc area B) falls within this ROI, it is considered as colocalized. If the default value of -1 is kept for the "maximal distance in µm to be considered as colocalized," no colocalized area ROI will be generated.
As described in Lachmanovich 2003, it is recommended to use the minimal resolution distance (typically 230-250nm for a 1.4 NA lens and a widefield setup) as the colocalization distance threshold. You can refer to the book "Handbook of Biological Confocal Microscopy" by Pawley JB, Springer 2006 for more information on other microscopy setups. The rationale behind using the minimal resolution distance value as a threshold is straightforward: if foci A and B are from the same channel, and their separation distance is below the minimal resolution distance, they would not be distinguishable. Hence, they are considered as colocalized.

When foci of interest are detected in both channels A and B, the maxima of the foci in a specific channel (let's say axis-fociA (maxima) ROI) and the corresponding coloc area ROI (colocalised_area_axis_fociB in this case) are compared using the RoiManager>More>"AND" operation. The maxima that fall within the colocalised_area ROI are counted and used to generate a new ROI, specifically the colocalised axis_fociA (maxima) ROI (in this case colocalised axis_fociA (maxima)). When particle analysis is conducted, individual particles containing a focus's maximum are identified, and corresponding maximum and particle ROIs are generated. The position of this maximum ROI in relation to the colocalised area selection of the other channel is examined. RoiGroups are employed to indicate the colocalisation status of the maximum. The associated particle is assigned the same colocalisation status as its maximum.

Whenever individual particles analysis is done (see the end of STEP 7 description), when an individual axis/off-axis particle is detected, the tool examines whether there is a triple overlap between the individual particle ROI, the corresponding axis/off-axis foci (maxima) selection, and the colocalised area generated using the axis/off-axis maxima from the other channel. If such an overlap exists, the particle (and its associated individual maximum, if generated as per the OPTIONAL STEP A configuration) is labeled as colocalised using the corresponding ROIGroup (refer to figures 7.8 and 7.9). If no overlap is observed, as the particle was previously shown overlapping with a maximum ROI (following this example axis_fociA (maxima)), it means that the particle is not colocalised and is tagged accordingly. All individual colocalised or not-colocalised particles selections are merged to generate selections consisting of all colocalised or not-colocalised particles respectively.

STEP9. You can check whether the observed colocalised pixels in both channels are likely to occur randomly by selecting the "get colocalisation with random images" option. This option will only work if the main "compute colocalisation" is selected.
Here's how it works:

- The foci (=single points maxima) of one channel are randomly shuffled within the entire axis ROI. The randomly localised foci/maxima from the first set of random images (for both channel randomisations) are saved in the ROI Manager. The number of random images can be adjusted using the general parameters menu (see OPTIONAL STEP A). Examples of random images are colocalisation results are shown in figure 7.10.
- Next, the distribution of random foci of each random image generated is compared with the observed foci localisation in the other channel using its corresponding Euclidean

distance Map ROI (e.g., random axis_fociA_0 (maxima) with colocalised area axis_fociB). You can enter the number of randomisation cycles to be generated.

The average number of randomly colocalised foci is calculated for both combinations. It is recommended to use a high number of cycles (>30) to obtain a normal distribution of randomly colocalised foci. If the average number of random colocalised foci is 0, no further analysis is performed.

- You can investigate whether the difference between the mean number of randomly colocalised foci and what was observed is significant. Select the “Show random distribution versus real values” option. A plot is generated showing the distribution of random colocalised foci numbers for all random images (see fig 7.11 for an example) The plot is saved as a .tif file in a parent controlData folder, but it can be opened using ImageJ, and the list of values can be extracted by clicking on "list". The frequency of the number of randomly colocalised foci is represented as grey hollow dots, and a Gaussian fit is applied (shown as a blue line). The actual observed number of colocalised foci is indicated by a red line.

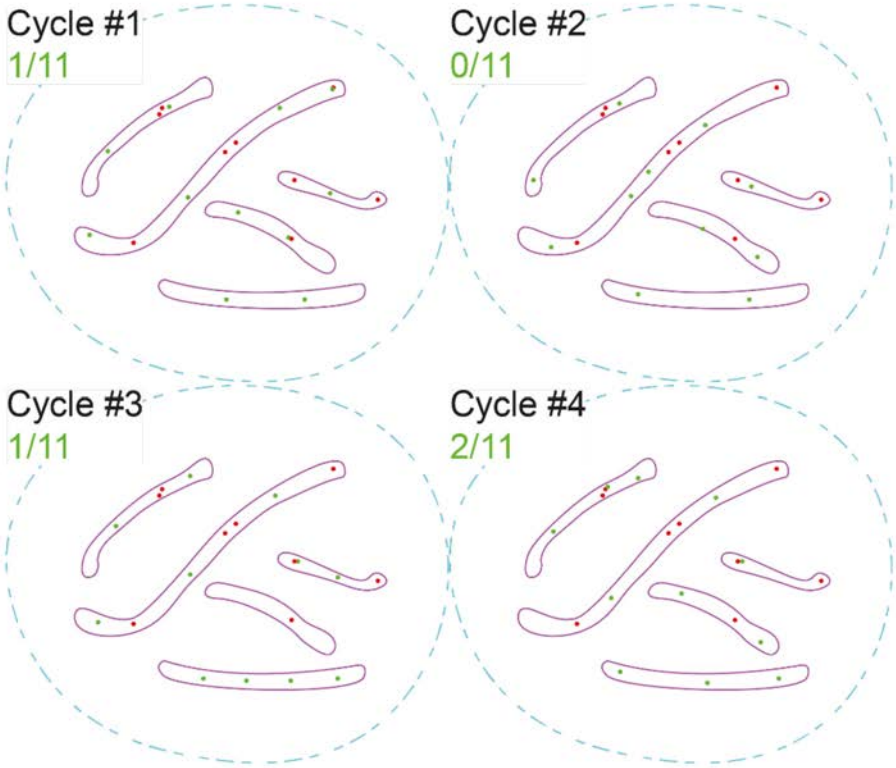


Figure 7.10. Four random distributions of green (axis) foci are compared to the actual red foci. The number of colocalised random green foci, compared to the total number of green foci (11), is indicated below each cycle.

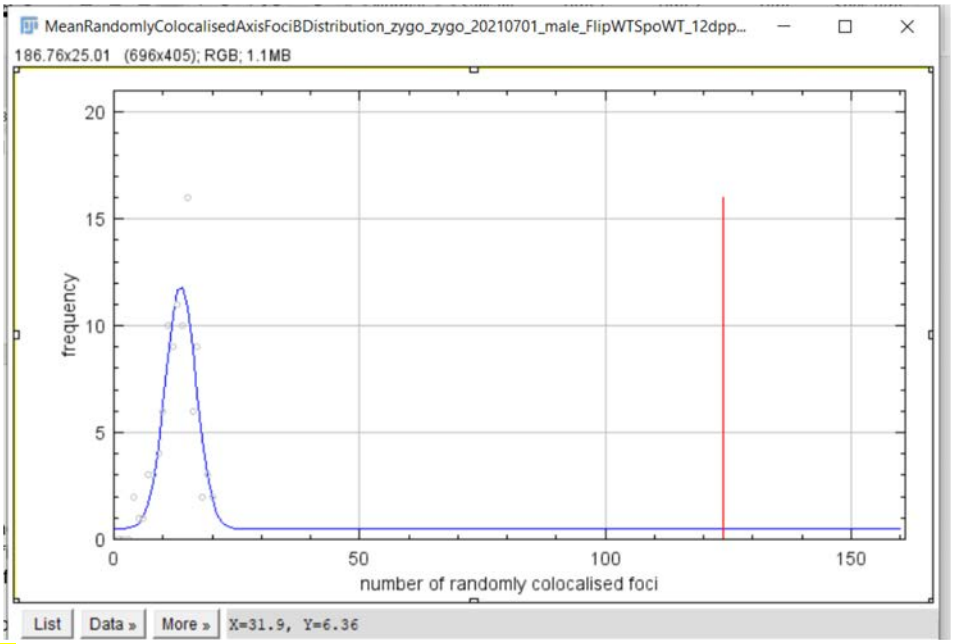


Figure 7.11 illustrates the distribution of the number of randomly colocalised foci across all generated images.

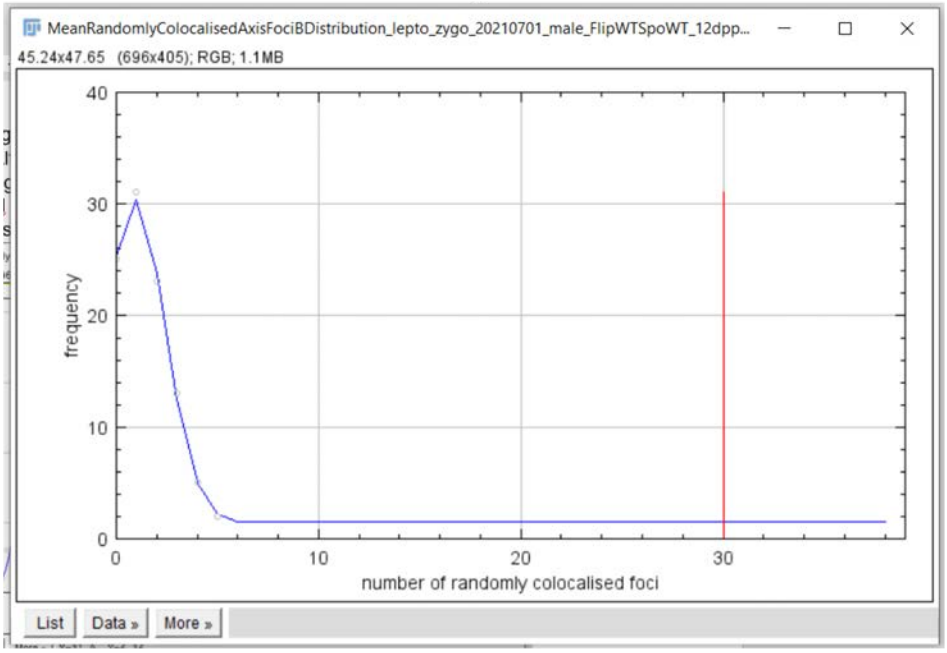


Fig. 7.12 “clipped” randomly colocalised foci number distribution across all randomly generated images

If μ is the mean number of randomly colocalised foci, and σ its standard deviation, according to the 68–95–99.7 statistical rule (also known as the empirical rule), assuming a normal distribution, the probability function P indicates that there is a 99.7% chance that all random values fall within the range of μ ± 3σ.

$$P(\mu - 3\sigma \leq X \leq \mu + 3\sigma) \approx 99.73\,\%$$

If the blue Gaussian distribution is clipped, as shown in Figure 7.12 or the left panel of Figure 7.13 (where μ - 3σ < 0), the p value is indicated with an asterisk. Furthermore, in the "comment" column of the 2[type]foci.xls result file, a comment "*: take p value with caution" is included.

The p value is determined using the following formula and involves the use of the error function (erf). Horner's method is employed to calculate the error function.

$$pValue = \frac{1 + \text{erf}(\frac{\text{realColocFoci} - \text{mean}}{\sqrt{2} * \text{stDev}})}{2}$$

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} * \int_0^x e^{-t^2}\, dt$$

The p-value represents the portion of the Gaussian curve below the observed number of colocalized foci. A p-value close to 1 suggests that the likelihood of obtaining the observed colocalized foci number through random foci distribution within the reference ROI is low (as depicted in the middle panel of Figure 7.13). However, if the distribution is clipped or the p-value is not 1 (as shown in the right and left panels of Figure 7.13), it is advisable to utilize alternative statistical analyses. You can access the raw values of the random distribution by clicking on the "List" button, as explained earlier (eg. click the bottom left list button of figure 7.13), and perform other statistical tests using these values.

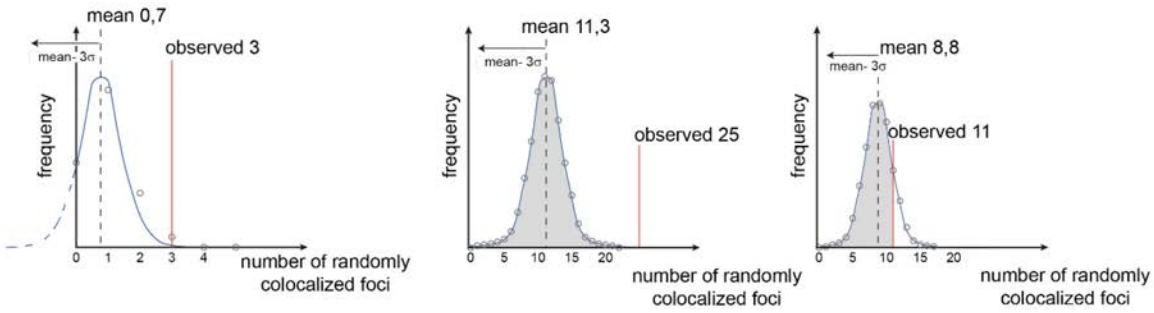


Fig. 7.13. The different possible types of randomly colocalised foci number distributions. Left and middle panel : p value=1, right panel pvalue=0,75

STEP 10. Configure additional intensity measurements for channels or ROIs. The tool has already identified the reference ROI, the axis ROI (and off-axis ROI if the type set at STEP5 is off-axis), as well as the overall foci's maxima of interest (axis or off-axis) for both channels, the colocalised and not colocalised maxima, and, based on the OPTIONAL STEP A configuration, individual focus' particles and their associated maxima with their colocalisation status, as well as the overall respective foci's particles.

Please note that axis/off-axis ROIs are not included in the list of possible measurement ROIs. However, you can use the "Measure axis length" tool if necessary.

At OPTIONAL STEP A, you have the following options to add/remove measurements to/from the ROI list:

a) Include the reference ROI.

b) Include foci maxima selections, which encompass overall foci maxima and individual focus maxima.

c) Include foci particles, which include overall foci particles or individual focus particles.

d) Include individual focus selections, specifically maxima if option b is selected and particles if option c is selected. If you choose this option, the values will be displayed in a separate result file.

If you remove all options, no measurements will be performed.

If you require intensity measurements for the foci channels A and B, select the "add foci channel intensity" option (refer to figure 7.1). To add another channel for intensity measurements within the ROIs, enter the corresponding numeric value in the "Measure foci ROI's channel intensity" field. If the field is left as -1 and the "add foci channel intensity" checkbox is not selected, no measurements will be performed.

You can also set the measurement type (mean or IntDen values) at OPTIONAL STEP A.

For global particle measurements displayed in the 2Foci.xls table, the corresponding ROI is utilized, such as "colocalised axis_FociA (particles)". For global maximum calculations, the corresponding ROI (e.g., "axis-foci (maxima)") is measured using the getRawStatistics(nPixels, mean, min, max, std, histogram) built-in function of the macro language. The number of maxima (nPixels) is multiplied by the mean value to obtain the IntDen value.

STEP 11. Select the control output options. If the “Save ROIs” option is selected, all ROIs (nucleus/user-defined, axis, axis foci in both channels, if relevant colocalized axis foci in both channels and random foci) will be saved in a companion 2axisFoci_RoiSet_[name].zip or 2off-axisFoci_RoiSet_[name].zip file located in a parent ControlData folder.

Results are saved in a 2axisFoci.xls or 2off-axisFoci.xls file (located in the same folder where the input images are stored) (see figure 7.19). Individual foci values are stored in an individual2(off-)axisFoci.xls file (figure 7.20).

STEP12. Change/fill-in the cropped-images directory using the browse button.

STEP 13. Click OK. If the “Use advanced user parameters” option was selected at STEP 1, the Advanced user parameter window will pop-up (see OPTIONAL STEP B). Otherwise analysis starts. The macro runs in batchMode, to display the images while processing, follow OPTIONAL STEP A.

OPTIONAL STEP A. Configure the measurement parameter options. Before using the "Count axis/off-axis foci in two channels and colocalise" tool, click on the "Set measurement parameters" button (see figure 0), which will open the Parameters menu (refer to figure 7.14).

PARAM A1. Choose whether to display warning messages or keep them hidden. It is recommended to keep them hidden to avoid interference with batch processing of the macro.

PARAM A2. By default, the images are hidden during the analysis. If you prefer to have the images displayed, select the "show images while processing files" option.

PARAM A3. The default list of methods for nucleus, axis, or foci thresholds is limited to preset automatic threshold methods suitable for test images and structures. If you want to expand the list to include the full range of ImageJ's automatic thresholds, select the "Display the full list of threshold methods" option.

PARAM A4. If you choose the "Measure axis length" option in STEP 4, you can modify the length measurement type. Two routines are available (see figure 7.15):

- The first (rough) routine takes advantage of the 1-pixel thickness of the skeleton. It measures the total area of the ROI in pixels and converts it to micrometers using the image's calibration. However, this method may produce unusual results due to diagonal pixels being treated as 1-pixel length.
- The second (fine) routine is a direct measurement of the skeleton and considers "horizontal, vertical, and diagonal pixels" in its calculations. However, it measures distances between pixel centers.

PARAM A5. The default intensity measurement is IntDen. If you want to include Mean values or remove IntDen measurements, use the "Show IntDen values" and "show Mean values" options.

PARAM A6. By default, the parameters will measure the intensity within the reference ROI (either user-defined or nucleus ROI, STEP2). To exclude the measurement of the user-defined nucleus ROI, deselect the "show user-defined nucleus ROI measurements" option.

PARAM A7. Foci ROIs can be measured for both maxima/individual maximum and particles/individual particle. Use the corresponding checkboxes to restrict the analysis to the desired measurement.

PARAM A8. During the process of defining total axis/off-axis foci particles, individual particles and their corresponding maxima are identified and measured. To skip this step (i.e., remove individual selections from the list of measured ROIs), uncheck the "Show individual foci's values" option.

PARAM A9. If you have chosen the "compute colocalisation" option in STEP8, you can specify the number of random distributions of fociA and fociB that should be retained in the ROIManager.

Click on OK (A10) and launch the “Count axis/off-axis foci in two channels and colocalise” tool (figure 0).

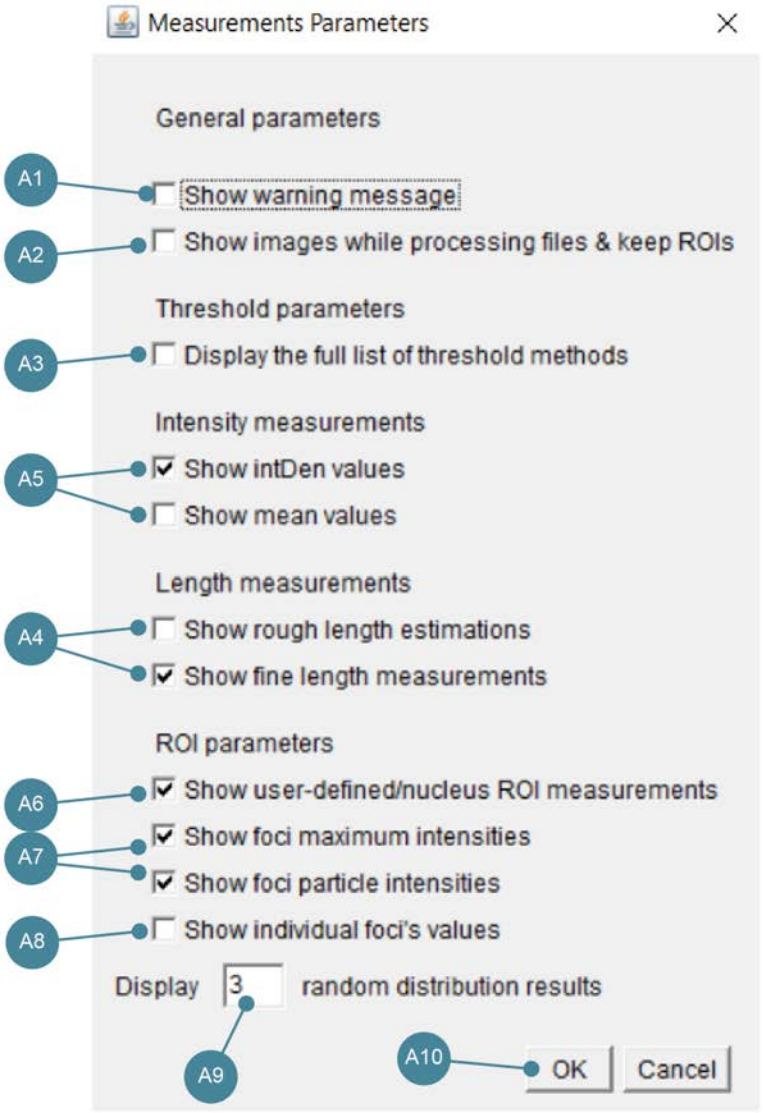


Figure 7.14. The (general) parameters window.

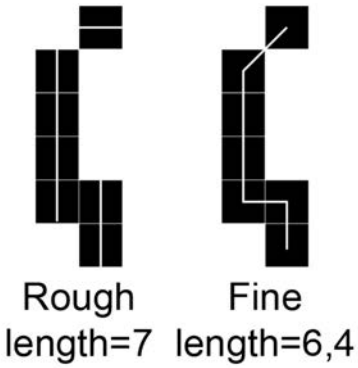


Figure 7.15. Rough and fine measurements modes.

OPTIONAL STEP B. For precise foci and axis detection, advanced user options are available. These options are especially useful for images with high levels of noise or super-resolution images. If you check the "Use advanced user option" checkbox, the "Advanced user parameters" window will be displayed during STEP13 (see figure 7.16).

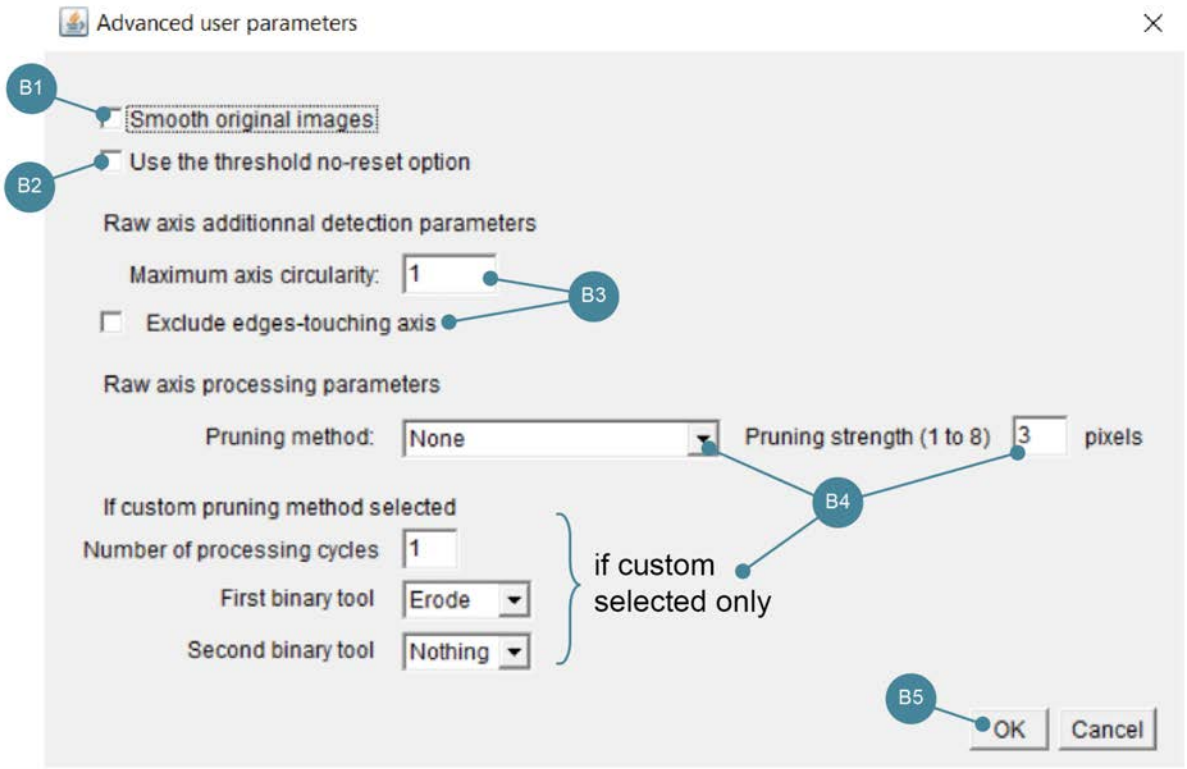


Figure 7.16. The Advanced user parameters menu

OPT. STEP B1. To eliminate noise, such as photon shot noise, from the images and improve the accuracy of detection, you can enable the "smooth original images" checkbox. This filter replaces each pixel with the average value of its 3x3 neighborhood and is applied to all channels. It serves two purposes: i) smoothing the axis for better detection, and ii) reducing errors and noise-related foci by smoothing the foci.

OPT. STEP B2. Detecting the axis can be challenging when working with images that have a high offset value, such as Abberior .msr files. In such cases, the automatic thresholding process can be biased due to extremely high background intensity pixels and excluded pixels with intensities set to 0 during the cropping step (as shown in figure 7.17). To overcome this issue, you can use the "use the threshold no reset option".
Different versions of ImageJ (ranging from 1.52e to 1.53s) have implemented, retracted, and re-implemented various autothreshold options. The later versions have introduced a stable "don't reset range" threshold mode in the Image>Adjust Threshold command. When working with images with more than 8 bits (such as 16-bit images), certain thresholding issues may arise. This is because the thresholding process relies on an 8-bit histogram derived from the converted 16-bit image/histogram. To enable this, the range of the original 16-bit image (0-65535) is first transformed into an 8-bit range (0-256), and then the threshold is calculated based on this 8-bit histogram.
Let us consider an example of a 16-bit .msr file displayed using the Image>Adjust>Brightness&Contrast auto display range. In this case, the black intensity value is 32767 (representing the minimal intensity in the ROI, with the background spanning from 32767 to 32769), and the white intensity value is 32790 (representing the maximal intensity in the ROI). The axis signal lies between 32770 and 32790. When the "reset" option (the default mode in Adjust Threshold) is applied, the range extremes are converted to the threshold range extremes of 0 and 256. Consequently, the automatic threshold encounters two intensity

clusters: the clipped area and the rest (background+signal). It finds a threshold value that fails to differentiate the background from the signal within the reference ROI, as indicated by the red area in the histogram shown in the middle left panel of figure 7.17. As a result, the entire reference ROI is thresholded, and the axis ROI erroneously resembles the reference ROI.
In contrast, when the "don't reset" option is selected, the displayed extremes are converted to the threshold range extremes of 32767 to 0 and 32790 to 256. The (discrete) 8-bit histogram is then computed (middle right panel of figure 6.11), and automatic threshold values are calculated within the red range on the histogram. Consequently, the axis is properly thresholded, and the axis ROI accurately represents the axis.
Due to the implementation, retraction, and re-implementation cycles of the reset/no-reset option in the Adjust Threshold command across different ImageJ versions, MeiQuant may have provided correct axis/foci segmentation without utilizing the "no-reset" advanced user option. If you have upgraded ImageJ/Fiji, it is possible that the segmentation is no longer accurate unless the "Use the threshold no-reset option" checkbox is selected.

OPT. STEP B3. If needed, you have the option to utilize additional parameters for the detection of raw axis:

- Maximal circularity: The circularity value, calculated as 4π * (area/perimeter²) in ImageJ, provides information about the shape of an object. A value of 1.0 represents a perfect circle, while lower values indicate increasingly elongated polygons. If there are non-axis round artifacts present, you can decrease the circularity value. Conversely, if there are overlapping axes, setting the maximum circularity value to 1 will prevent the removal of round aggregates formed by overlapping axes.
- To address situations where the reference ROI from STEP 2 (either a user-defined ROI or the threshold ROI) cuts off some axis signals that touch the edges of the image/ROI, you can select the "exclude touching edges" option. When working with cropped nucleus images that contain numerous overlapping axes, leaving this option checked may result in the removal of a significant number of axes.

OPT. STEP B4. If you have selected the "measure axis length" option at STEP4 and encounter unwanted decorations in the axis skeleton, you can configure the pruning method and options to address this issue (refer to figure 7.4). Pruning is necessary to modify the outlines of the raw axes and eliminate any undesired axis decorations, ensuring accurate measurement of axis length. This step is particularly crucial when working with superresolution images.

The process involves using the raw axis ROI to generate an 8-bit mask using the fill and clear commands. Alternatively, you can manually replicate this process by using the ROI, followed by the Edit > Fill and Edit > Clear Outside menus. The resulting binary mask is then processed using the following options:

- The default method is "None," which means no further processing is performed on the mask.
- "2x Erosion" applies two erosion operations, removing pixels from the edges of objects. This can be useful if you want to shrink the initially detected raw axes.
- "2x Dilation" involves two dilation operations, adding pixels to the edges of objects. This option is suitable if you want to enlarge the initially detected raw axes.
- "2x Erosion then 2x Dilation" or "2x [erosion/dilation]" applies two cycles of erosion and dilation (process > binary > open) on the mask. This option is helpful for smoothing or filling holes in the axes.
- The "Custom" option allows you to apply custom pruning parameters, giving you more control over the pruning process.

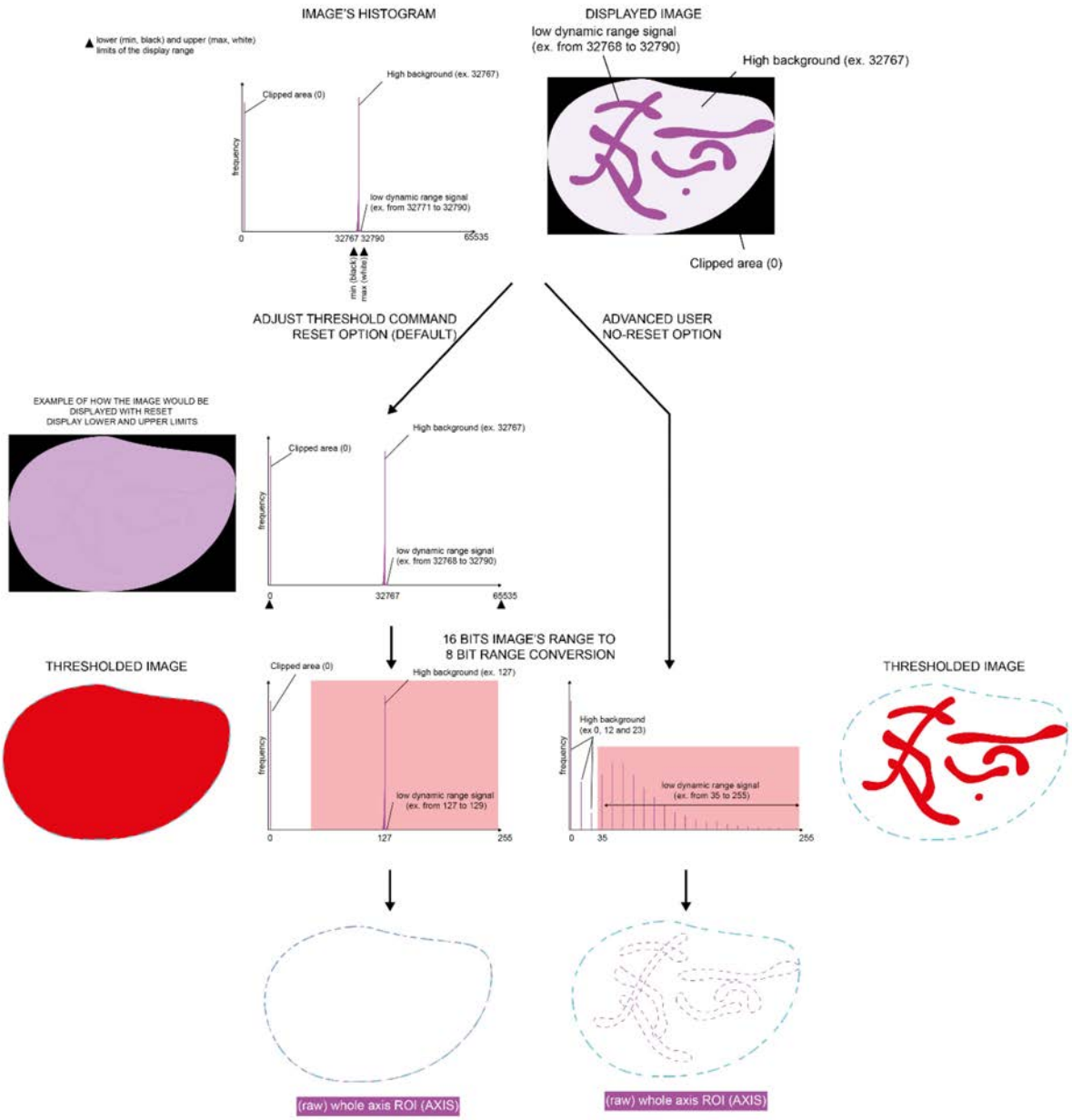


Figure 7.17 demonstrates an issue that arises when displaying low dynamic range images with the "auto" min/max display option (Image>Adjust>Brightness&Contrast). When these images are cropped, the area outside the reference ROI, which is set to intensity 0, can cause interference with axis detection. This interference occurs because the automatic thresholding process uses a default reset range option (shown on the left side of the figure). The histogram considered for thresholding is a full-range histogram spanning from 0 to 65535 (for 16-bit images). The figure illustrates an example of how the image would appear with these display parameters (shown in the middle left of the figure). As a result, pixels in the clipped area with a value of 0 are included in the calculation of the automatic threshold. If the dynamic range of the signal, compared to the neighboring background, has similar values that significantly differ from 0, the entire reference ROI is incorrectly thresholded. This leads to the axis ROI resembling the reference ROI. However, by using the "no-reset" option, the displayed histogram is utilized as input for the conversion from 16 bits to 8 bits, which is performed before the threshold calculation. This approach ensures accurate axis detection and eliminates the issues caused by the clipped area with intensity 0..

The macro makes use of the process > binary > options command. Figure 7.18 showcases the parameters employed for 2x Erosion with a strength of 3. The pruning strength value, referred to as "count" in the options command, determines the minimum number of adjacent background pixels needed to remove a pixel from the object's edge during erosion, as well as the minimum number of adjacent foreground pixels required to add a pixel to the object's edge during dilation. If a value below 1 or above 8 is inputted, it is disregarded, and the default value of 3 pixels is applied.

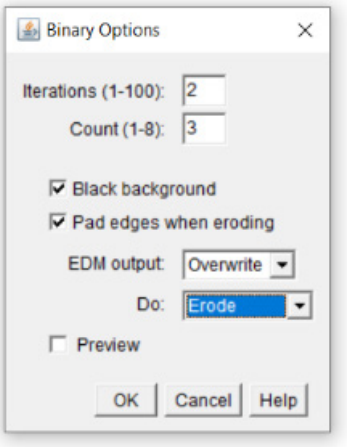


Figure 7.18. An example of how the pruning method “2x Erosion” translates in ImageJ.

When opting for the "Custom" pruning method, you need to specify the number of cycles or iterations to be executed. Start by selecting the first “binary tool” for processing. If additional rounds of processing cycles are required, choose the second “binary tool”. For instance, in the scenario of "2x Erosion then 2x Dilation," set the process > binary > options command with 2 iterations and select "Erode" as the operation for the first round. This applies two erosions during this stage. Then, continue with the process > binary > options command, using 2 iterations once again, but this time select "Dilate" as the operation for the second round. To determine the most suitable and optimal parameters for the custom pruning method, click on the "Set advanced user parameters" button prior to executing the "count axis & off-axis foci in one channel" tool (refer to figure 0).

Consequently, a "whole axis" ROI is drawn based on the processed mask, and the pruning method is indicated within brackets.

OPT. STEP B5. Click on OK to proceed to the analysis.

axis_fociA	axis_fociB	colocalised axis_fociA	colocalised axis_fociB	fociA ch. intDen (axis fociA's maxima)	fociB ch. intDen (axis fociA's maxima)	whole axis ch. intDen (axis fociA's	fociA ch. intDen (axis fociA's particles)	fociB ch. intDen (axis fociA's particles)	whole axis ch. intDen (axis fociA's	fociA ch. intDen (axis fociB's maxima)	fociB ch. intDen (axis fociB's maxima)	whole axis ch. intDen (axis fociB's	fociA ch. intDen (axis fociB's particles)	fociB ch. intDen (axis fociB's particles)	whole axis ch. intDen (axis fociB's particles)	Comment		Parameter	Value
71	100	59	59	728	492	129	393649	332370	120291	523	557	134	535173	758644	253852			Smooth original images	No
94	12	10	10	1004	636	102	700668	523825	95933	1135	2228	102	42847	297881	10136			ROI chosen	user-defined
115	59	48	48	954	627	199	910212	682805	284705	933	1063	235	280537	339051	80772			Axis channel	4
108	5	2	2	1128	521	167	777995	423892	158488	835	3131	234	12111	195827	19377			Axis Threshold	Huang
94	120	80	80	911	626	109	661251	534430	113555	617	660	111	728323	886060	229591			Axis Min size	5
92	91	71	71	856	497	93	684442	454829	108779	705	567	93	644277	589578	144170			Axis Max Circ,	1
86	6	1	1	840	508	112	596888	421261	118860	400	3680	230	9205	308252	29358			Exclude edge-touching axis	No
246	262	226	224	1350	1223	127	2781217	3102733	409607	1006	1526	142	2801094	4558915	608165			Measure axis length	No
163	190	145	145	986	920	117	1387078	1538054	255480	696	1099	128	1341042	2178575	356270			Pruning Mode(s) tested	2x [Erosion & Dilation]
180	204	160	160	1059	1017	118	1688314	1842845	288850	817	1193	129	1747108	2637619	406118			Pruning Strength (when used)	3
122	144	103	103	851	682	134	1035158	1010017	258336	666	736	136	1168565	1470268	405186			Foci detection method	within ROI
203	222	176	176	1078	1012	88	2020880	2194066	237748	772	1390	105	1742800	3161912	325447			Foci Detection Threshold	IJ_IsoData
26	75	20	20	688	543	119	212250	196442	62864	301	659	94	330594	745501	214604			Foci type	Axis
20	16	8	8	738	514	73	148991	129175	25889	479	1164	75	40641	159736	10370			Foci channel A	2
																		Foci Detection noise A	500
																		Foci channel B	3
																		Foci Detection noise B	300
																		Compute Colocalisation	Yes
																		Max, distance for colocalisation	0,3
																		Generate random foci & colocalise	No
																		Measure foci's ROI intensity	Ch, 4 & foci channels (Ch,2 and Ch,3)
																		Images from	D:\Akbar\processedData\
																		ROI saved	Yes
																		ROI and any other output folder	D:\Akbar\controlData\
																		options:	
																		Show warning messages	No
																		Show images	No
																		Get integrated density intensity values	Yes
																		Get mean intensity values	No
																		Include reference Roi when measuring intensities	No
																		Show foci's Maximum intensities	Yes
																		Show foci's particle intensities	Yes
																		Meiosis bar tool	Count foci in two channels and colocalize
																		Meiosis bar version	v2,05
																		ImageJ version	1,53t99

Fig. 7.19. a result file of the Count axis foci in two channel and colocalize macro

Type	Image name	focus' ID	focus' channel	focus' type	focus' colocalisation status	fociA ch. intDen (focus' maximum)	fociB ch. intDen (focus' maximum)	whole axis ch. intDen (focus' maximum)	fociA ch. intDen (focus' particle)	fociB ch. intDen (focus' particle)	whole axis ch. intDen (focus' particle)
zygo	zygo_1	0	A	axis	yes	784.264	526.352	64.338	5774.938	5188.350	1092.848
zygo	zygo_1	1	A	axis	yes	1528.574	1520.421	355.084	16037.986	18616.515	7124.363
zygo	zygo_1	2	A	axis	yes	979.639	510.958	61.854	6495.192	4222.296	777.631
zygo	zygo_1	3	A	axis	yes	993.997	758.438	202.636	7369.694	8067.895	3529.542
zygo	zygo_1	4	A	axis	yes	726.272	844.818	50.117	4493.471	6539.847	593.165
zygo	zygo_1	5	A	axis	no	694.539	595.216	65.905	5493.474	5489.934	1140.638
zygo	zygo_1	6	A	axis	yes	638.938	523.821	88.187	4188.838	4105.132	1242.875
zygo	zygo_1	7	A	axis	yes	933.438	358.350	86.179	7399.016	3890.055	1424.992
zygo	zygo_1	8	A	axis	yes	603.035	593.817	118.145	3644.920	4178.916	1252.072
zygo	zygo_1	9	A	axis	yes	1056.602	408.563	183.706	7969.261	3939.526	2485.369
zygo	zygo_1	10	A	axis	yes	540.571	458.152	241.695	4100.407	4023.072	2397.566
zygo	zygo_1	11	A	axis	no	716.920	203.056	403.802	5848.626	2292.725	4577.700
zygo	zygo_1	12	A	axis	yes	688.282	365.996	194.282	3869.819	2570.163	1665.387
zygo	zygo_1	13	A	axis	no	504.959	294.896	146.353	2704.968	1886.442	1054.791
zygo	zygo_1	14	A	axis	yes	561.569	470.462	55.837	4040.711	4363.471	587.043
zygo	zygo_1	15	A	axis	no	581.107	192.163	128.877	3589.147	1461.591	1133.850
zygo	zygo_1	16	A	axis	yes	1083.613	732.478	89.382	6733.437	5663.609	999.445
zygo	zygo_1	17	A	axis	yes	1109.484	814.243	60.854	8130.835	8590.143	1111.265
zygo	zygo_1	18	A	axis	yes	604.666	580.411	171.410	4019.524	4413.765	1643.501
zygo	zygo_1	19	A	axis	yes	655.609	639.270	145.207	4028.233	4988.512	1295.619
zygo	zygo_1	20	A	axis	no	521.602	251.570	115.173	2997.448	1704.353	1000.336
zygo	zygo_1	21	A	axis	yes	565.957	278.891	107.369	3869.762	2907.523	1183.492
zygo	zygo_1	22	A	axis	no	595.992	216.394	75.119	3365.593	1438.912	688.495

Fig. 7.20. a individual particle result file individual2Foci.xls file of the Count axis foci in two channel and colocalize macro

STEP 14. To control the analysis accuracy, when the option to save ROIs was chosen, utilize the "Start checking results" feature located in the main meiosis bar. This action will prompt the appearance of the "Check results" window (refer to figure 7.21). If you have recently executed the macro, the default folder and method are suitable. However, if you wish to review and monitor the macro's results later on, you should specify both the folder and the "Count axis/off-axis foci in two channels and colocalize" method.

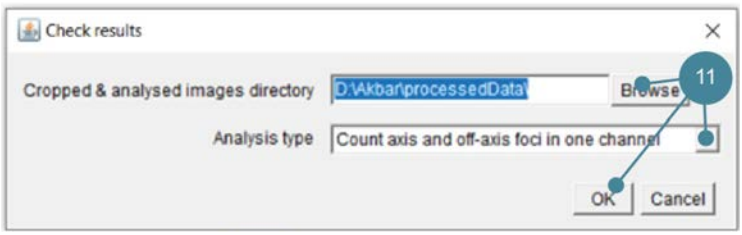


Figure 7.21. The check results menu

The first image will be opened along with its corresponding companion file named "2foci_RoiSet_[name].zip" in the ROI set, as shown in figure 7.22. The ROI Manager will contain multiple ROIs, including:

- The reference ROI, which was defined in STEP 2 (in this case, a user-defined ROI). This ROI is used for further identifications, such as axes.
- The raw axis ROI and the processed axis ROI (obtained from STEP 3 and OPTIONAL STEPB). If the advanced user mode was not selected (STEP1), the raw whole axis and whole axis will be identical. If the "measure length" option was selected (STEP4), the skeleton will also be included.
- The axis/off-axis foci (maxima) in channels A and B (from STEPs 5-7). If the "within ROI" option was chosen, the total_foci [letter] (maxima) ROI will remain.
- The colocalized area for the maxima in both channels, as obtained in STEP 8.
- Colocalized foci obtained using the actual localization of identified axis foci A and B in STEP 8.

- If the option to "get estimation of colocalization with random images" is selected, the result of the first randomization cycle ("randomFociA/B" from STEP 9).

Depending on the configuration, the individual particles obtained in STEP 7, along with their corresponding maxima, and the total particles of interest.

To control the next analysed image, click on “check next results” button on the main Meiosis bar (figure 0).

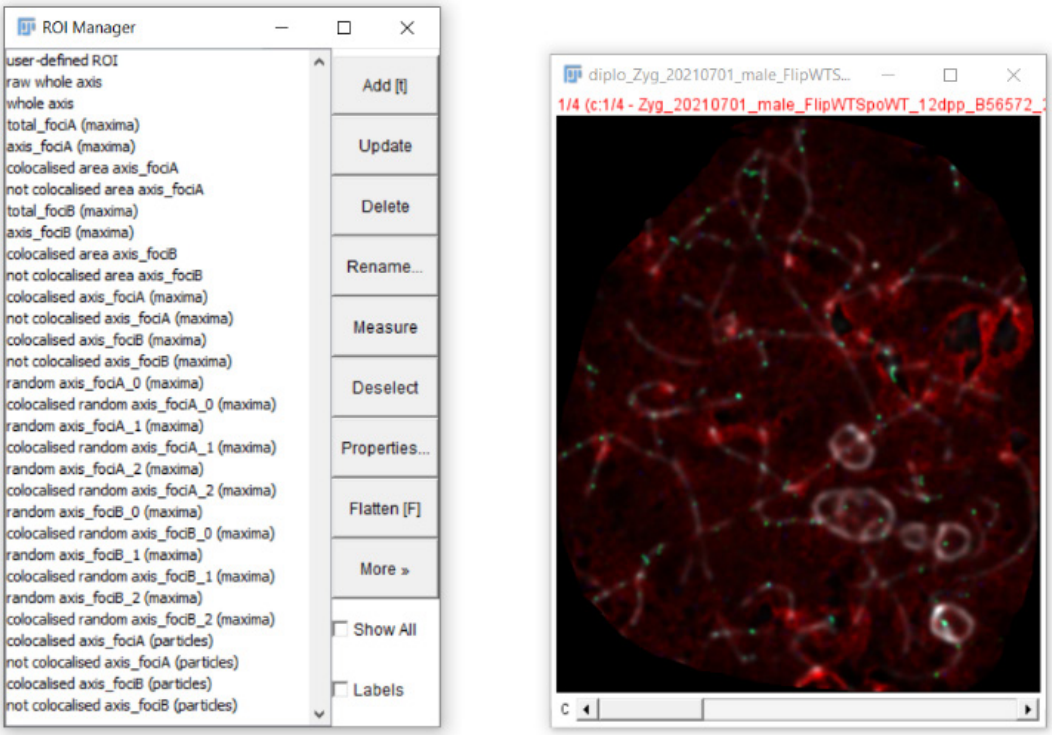


Figure 7.22. Control ROIs. Axis foci are displayed in both channels, as well as colocalised foci in both channels. When randomisation is used, an example of random distribution in both channel is shown. In this figure, the “show individual Foci’s values” was not selected.

SET ADVANCED USER PARAMETERS

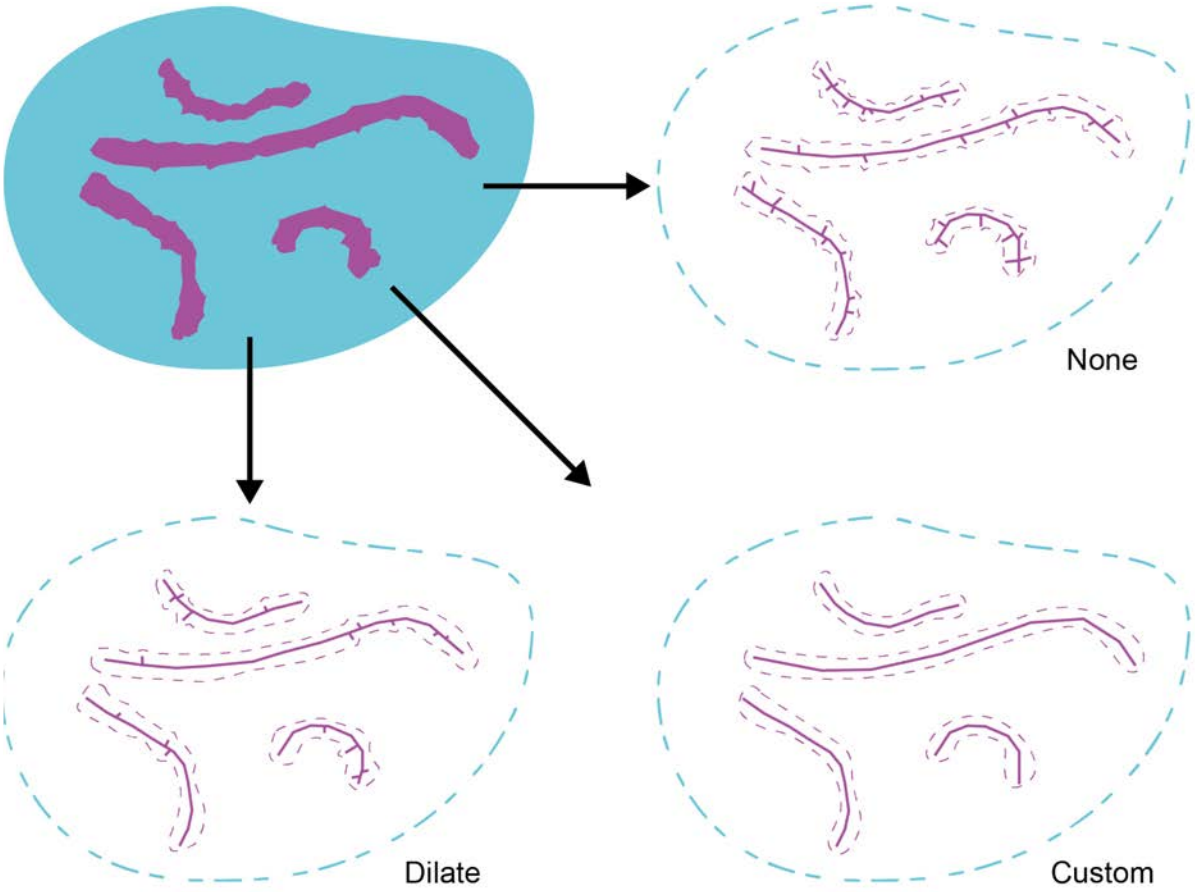


Figure 8.0. Visual summary of the tool

The MeïQUANT tools should provide an adequate set of parameters for segmenting axes and foci from regular widefield images. It is advisable to preprocess these images using a deconvolution suite.

However, axis and foci detection can be challenging with certain datasets. To address this issue, the "Set Advanced user parameters" tool (shown in figure 0) is designed to determine the optimal detection parameters before conducting lengthy analyses, as inaccurate feature detection can compromise the results. The main feature of the tool is to identify what are the best pruning parameters. Mind the tool will focus on axes (and does not help, for instance, to see how smoothing eases foci detection.

Here are some general guidelines for improving identification:

HOW TO IMPROVE FOCI IDENTIFICATION?

- In cases where noisy images result in false positives, it is recommended to use the advanced user parameters option (STEP B1 of OPTIONAL STEP B of the foci tools) and enable image smoothing.
- If foci are detected in small axis elements that only contain background foci staining, switching the axis foci detection mode to "within ROI" can help. If you are already using this mode, consider changing the automatic threshold method used (from “None” to some automatic threshold) or try a different automatic threshold method.

HOW TO IMPROVE AXIS IDENTIFICATION?

- If the chosen raw axis threshold does not yield satisfactory results, alternative methods can be considered. You can switch from the preselected threshold methods to ImageJ's full list of automatic threshold methods in ➔ OPTIONAL STEP A. Additionally, if there is a significant difference in background signal between the reference ROI and the cropped-out areas set to 0, using the "no-reset" option described in STEP 2 can be beneficial.
- It is possible that non-specific axis elements are also being detected. In such cases, employing the minimum element size exclusion criterion (STEP 4) or the shape exclusion criterion (STEP 5) can help eliminate them. Although the pruning process was not primarily designed for element removal, experimenting with various pruning methods, such as the erosion binary tool, to eliminate unwanted objects and then restore the elements to their initial shape (with dilate “binary tool” for instance), may also prove useful.images.

The "Set advanced user parameters" tool enables users to finely adjust the parameters for axis identification. By clicking on the "Set advanced user parameters" button (shown in figure 0), the "Advanced user parameters" menu will appear (as depicted in fig. 8.1).

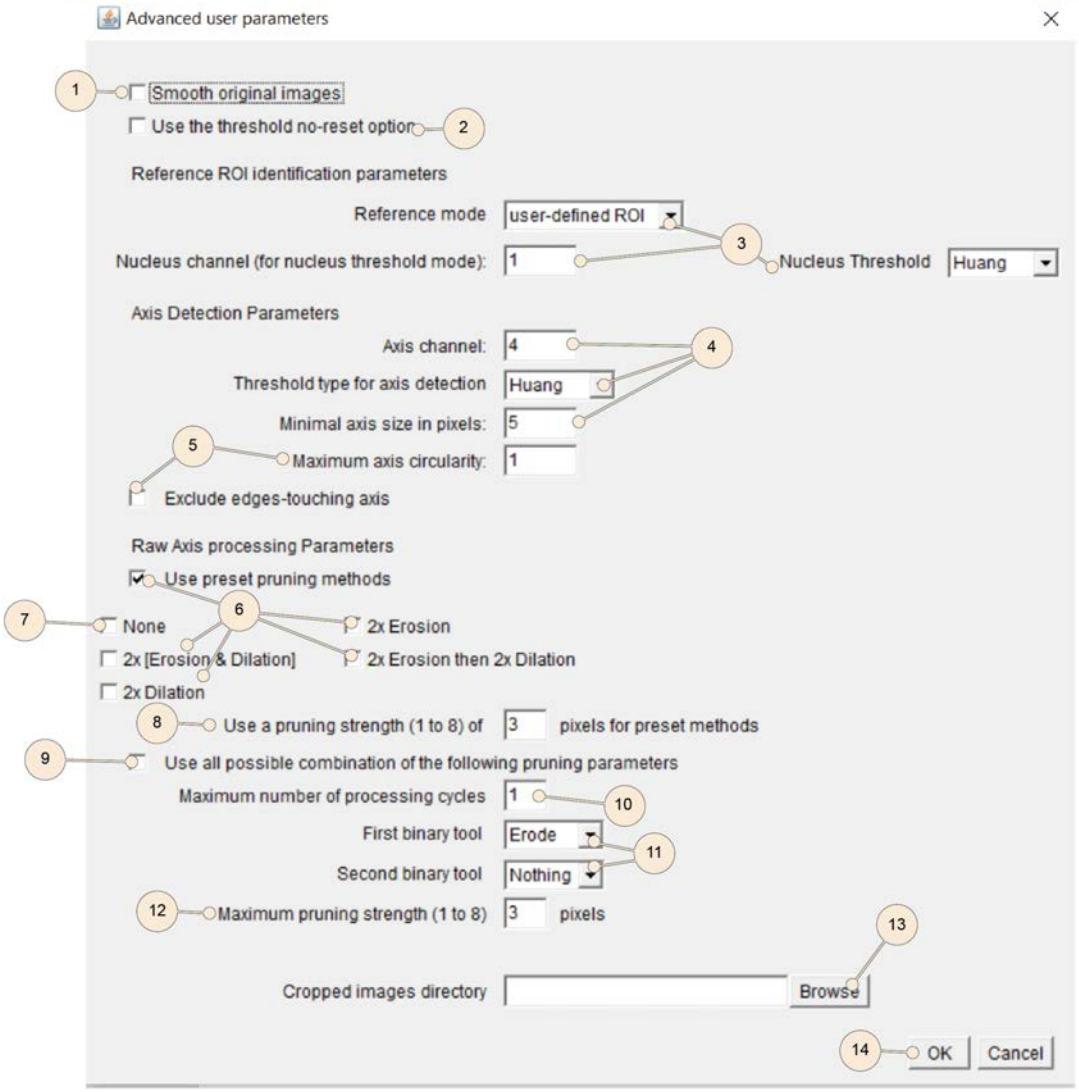


Figure 8.1. The advanced user parameters tool

STEP 1. To eliminate noise, such as photon shot noise, from the images and try a first way to smooth the outlines of axes, you can enable the "smooth original images" checkbox. This filter replaces each pixel with the average value of its 3x3 neighborhood and is applied to all channels.

STEP 2.When working with images that have a high offset value, such as Abberior .msr files, the automatic thresholding process can be biased in such cases due to the presence of extremely high background intensity pixels and the pixels with intensities set to 0 during the cropping step (as illustrated in figure 8.2, left panel). To address this issue, you can utilize the "use the threshold no reset option."
Different versions of ImageJ, ranging from 1.52e to 1.53s, have implemented, retracted, and re-implemented various autothreshold options. Therefore, MeiQuant may have provided correct axis segmentation without utilizing the "no-reset" advanced user option before, and after you upgraded ImageJ/Fiji, it is possible that the segmentation is no longer accurate unless the "Use the threshold no-reset option" checkbox is selected. In recent ImageJ versions, a stable "don't reset range" threshold mode has been introduced in the Image>Adjust Threshold command.
Why is the axis so grossly misdetected? When working with images that have more than 8 bits (such as 16-bit images), the thresholding process relies on an 8-bit histogram derived from the converted 16-bit image/histogram. The range of the original 16-bit image (0-65535) is first transformed into an 8-bit range (0-256), and then the threshold is calculated based on this 8-bit histogram. To better understand, let's consider an example of a 16-bit .msr file displayed using the Image>Adjust>Brightness&Contrast auto display range (top panel figure 8.2). In this case, the black intensity value is 32767 (representing the minimal intensity in the ROI, with the background spanning from 32767 to 32769), and the white intensity value is 32790 (representing the maximal intensity in the ROI). The axis signal lies between 32770 and 32790. When the "reset" option (the default mode in Adjust Threshold) is applied, the range extremes are converted to the threshold range extremes of 0 and 256 (lower left histogram figure 8.2). Consequently, the automatic threshold encounters two intensity clusters: the clipped area and the rest (background+signal). It finds a threshold value that fails to differentiate the background from the signal within the reference ROI, as indicated by the red area in the histogram. As a result, the entire reference ROI is thresholded, and the axis ROI erroneously resembles the reference ROI. Despite trying all available automatic threshold methods provided by ImageJ, none of them may successfully achieve precise segmentation of the axis.

In contrast, when the "don't reset" option is selected, the displayed extremes are converted to the threshold range extremes of 32767 to 0 and 32790 to 256. The (discrete) 8-bit histogram is then computed (middle right panel of figure 6.11), and automatic threshold values are calculated within the red range on the histogram. Consequently, the axis is properly thresholded, and the axis ROI accurately represents the axis.

STEP 3. You need to specify the region of interest (ROI) that will be utilized for further measurements. There are two options available:

- User-defined ROI: If you select this option, the companion .roi file generated during the stage-cropping process will be used. In case the file is not found, an error message will be displayed. By choosing this reference mode, you can proceed directly to STEP 4 without filling in the fields for the nucleus channel and threshold.
- Intensity threshold: Alternatively, you can use an intensity threshold to determine the ROI. In this mode, referred to as the "nucleus" threshold, the channel used for thresholding is typically associated with nucleus staining, although it can be applied

to any other staining as well. If you opt for this mode, specify the channel to be used for thresholding (the first channel is labeled as channel 1) and select the suitable threshold algorithm from the list of available built-in automatic thresholds provided by ImageJ. If you want to explore the full range of threshold methods, you can refer to OPTIONAL STEP A for additional options.

The user-defined or nucleus ROIs are referred to as the "reference ROI" in the subsequent steps. The algorithm was initially designed to analyze multiple images containing nuclei. However, if your image consists of a single cropped nucleus and there is no specific staining to use available to define the ROI (for example, due to the absence of nuclear staining), you can set the entire image as the ROI by selecting the whole image (Ctrl A) when using the cropping buttons.

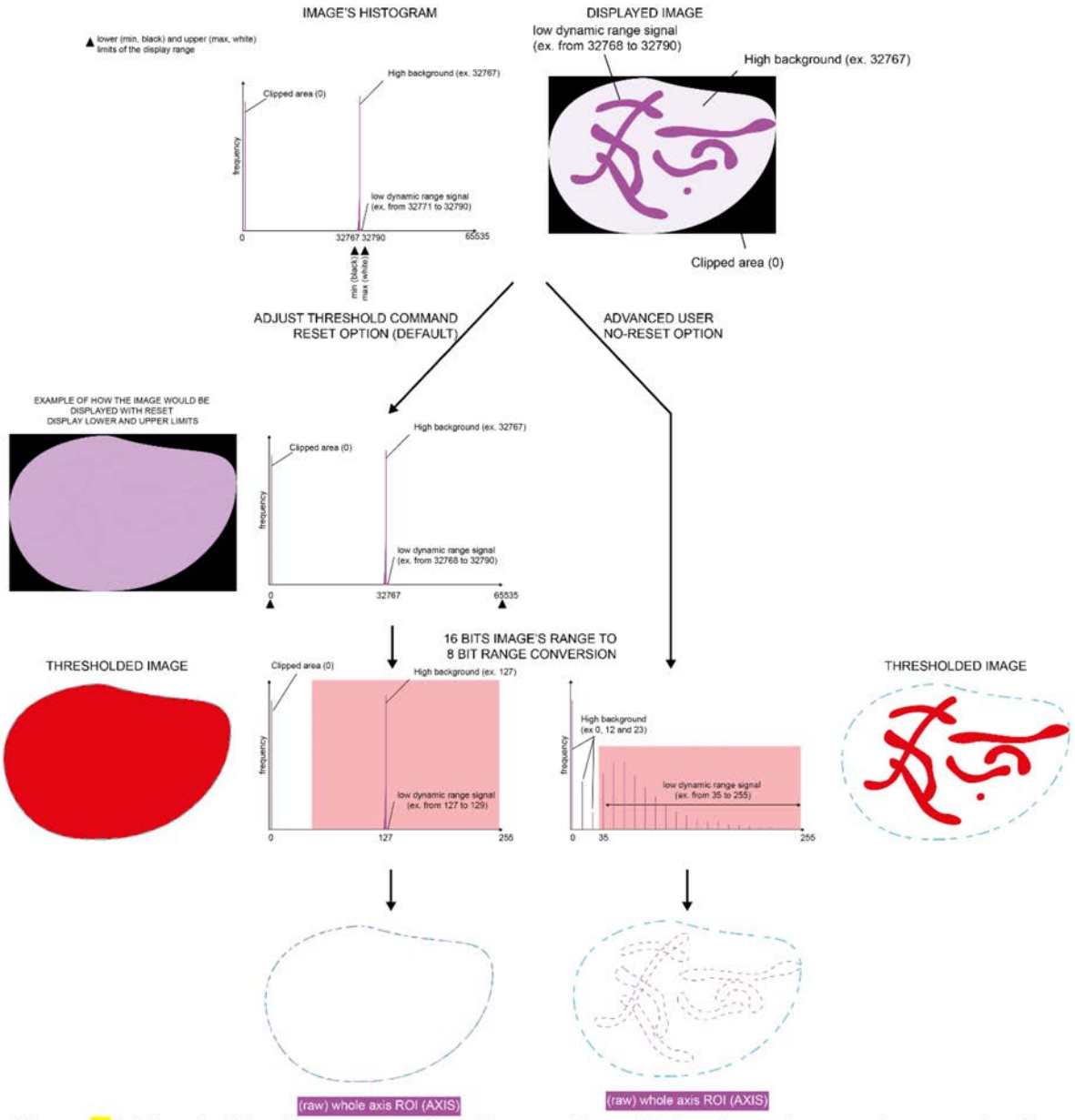


Figure 8.2. Thresholding issues can arise when working with low dynamic range images, despite having the correctly displayed using the "auto" min/max display option in ImageJ's Image>Adjust>Brightness & Contrast scommand. These issues become apparent when the images are cropped, resulting in a clipped-out area outside the reference ROI with an intensity value of 0. The problem arises because the automatic thresholding process utilizes a default reset range option, which considers the original full-range histogram spanning from 0 to 65535 (for 16-bit images). To illustrate this, consider an example where the image is displayed with the full-range display parameters (middle

left panel). When this original histogram is converted into an 8-bit histogram, the pixels in the clipped area with a value of 0 are included in the calculation of the automatic threshold. This inclusion can lead to incorrect thresholding of the entire reference ROI if the dynamic range of the signal, because signal and background show tiny intensity differences while their intensities values are significantly different from 0. As a result, the axis ROI ends up resembling the reference ROI. However, using the "no-reset" option addresses this issue. With this option selected, the displayed histogram is directly used as input for the conversion from 16 bits to 8 bits, which occurs before the threshold calculation. By employing this approach, accurate axis detection can be achieved, as the thresholding process takes into account the correct histogram information.

STEP 4. Configure the parameters for axis detection by following these steps:

- Specify the channel that contains the axis. Keep in mind that channels are numbered starting from 1, with the first channel being channel #1.
- Set the appropriate threshold for axis detection. This threshold will be applied initially. If no pixels above the threshold are detected, the analysis will stop, and the final table will indicate "no axis detected." The threshold operation creates a temporary selection called "axis threshold" in the ROI Manager (as shown on figure 8.3).
- The algorithm proceeds to detect the axis elements using the "Analyze Particle" command in conjunction with the reference ROI. Specify the minimum pixel size for thresholded axis elements (refer to figure 8.4 caption). This parameter helps exclude small and unwanted items from consideration. However, it is important not to set this value too high in the early stages of analysis.

STEP 5. If necessary, you have the choice to incorporate additional parameters for the detection of the raw axis. These parameters include:

- Maximal circularity: The circularity value is calculated as 4π * (area/perimeter²) in ImageJ and provides insights into the shape of an object. A value of 1.0 represents a perfect circle, while lower values indicate increasingly elongated polygons. By decreasing the circularity value, you can account for non-axis round artifacts. On the other hand, if there are overlapping axes, setting the maximum circularity value to 1 will prevent the removal of round aggregates formed by overlapping axes.
- The "exclude touching edges" option: This option addresses situations where the reference ROI from STEP 3, whether it's a user-defined ROI or the threshold ROI, truncates some axis signals that touch the edges of the image/ROI. By selecting this option, you can ensure axis elements that touch the edges of the image/ROI are discarded. In the case of cropped nucleus images with numerous overlapping axes, leaving this option checked may result in the unintended removal of a significant number of axes.

The algorithm identifies and adds all particles that meet the specified criteria to the ROI Manager. Initially, these particles are combined into a temporary ROI using the "combine ROI Manager" option (refer to figure 8.3). However, in cases where overlapping axes form a loop, the hole within the loop may be incorrectly identified as part of the axis. To address this issue, the algorithm utilizes the "AND" option in the ROI Manager. It performs an "AND" operation between the "axis threshold" ROI and the temporary ROI. This operation creates a "raw whole axis" ROI in the ROI Manager that should yield to accurate axis outlines.

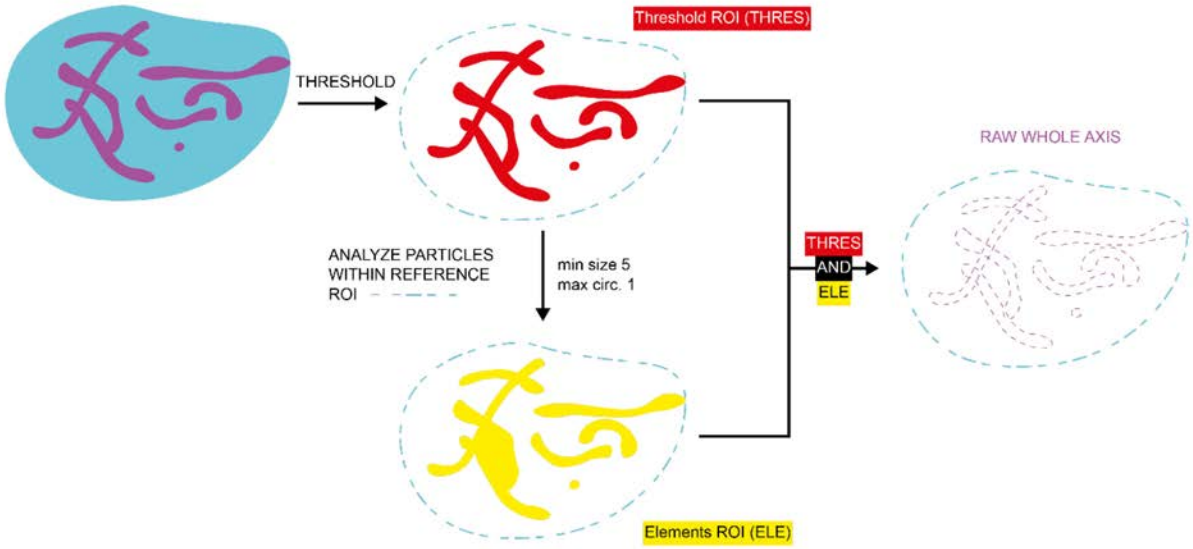


Figure 8.3. Process of axis elements detection and validation. Initially, the staining of the axis is subjected to thresholding (red area), and the "analyze particles" command is utilized to identify these raw elements (yellow area). However, to ensure the exclusion of axis loops, such as the three overlapping axes observed in the lower left corner of the nucleus, additional processing steps are necessary. This involves combining the temporary threshold ROI (THRES, red) with the Elements ROI (ELE, yellow).

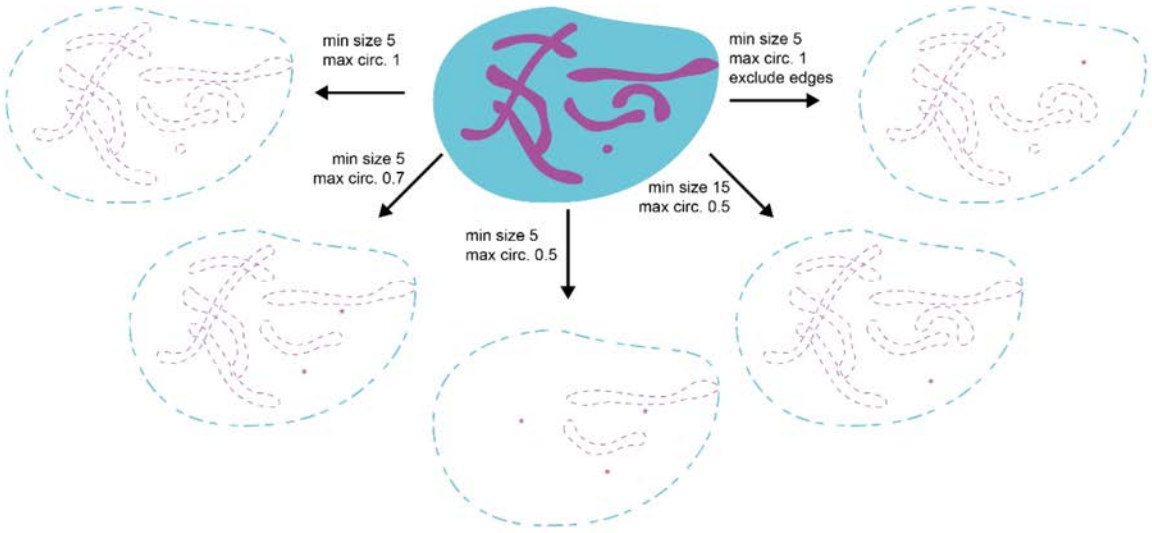


Figure 8.4. illustrates the effects of exclusion parameters on the analysis. The excluded elements are marked with an asterisk. By default, increasing the minimum size in pixels helps eliminate unwanted elements, as seen in the comparison between the left and lower-right drawings. Advanced user parameters (STEP4) provide additional exclusion criteria. For example, setting a maximum circularity can remove round-shaped objects, as shown in the comparison between the left and lower drawings. However, it is important to note that this may also result in the removal of clusters of axes, as depicted in the lower-left drawing. Another option for exclusion is the use of edge exclusion, as demonstrated in the right drawing, which offers further possibilities for excluding undesired elements.

Note that an important feature of the raw axis is that it is detected within the reference ROI.

To remove unwanted skeleton decorations, two pruning modes are suggested. In both modes, the raw axis ROI is utilized to create a binary mask, which is then processed further using “binary tools”. The creation of the mask involves using fill and clear commands (this can also be done manually by applying the ROI and using the Edit>Fill and Edit>Clear Outside commands). The process>binary>options command (refer to figure 8.5) is employed, with the selection of "pad edges when eroding" and "black background" options.
The pruning strength values mentioned in STEP 8 and 12 correspond to the "count" parameter in the options window (figure 8.5). The count represents the minimum number of adjacent background pixels required for a pixel to be eliminated from the edge of an object during erosion, and the minimum number of adjacent foreground pixels needed for a pixel to be added to the edge of an object during dilation.

It is possible to use both pruning modes simultaneously, as one mode does not exclude the other. However, it's important to note that they are not cumulative. For example, if 2x Erosion is selected in STEP 6 and a custom pruning method is used in STEP 9, the raw axis will first undergo the preset 2x Erosion method. Subsequently, the same original raw axis will be pruned using the custom parameters. In other words, the product of the 2x Erosion of the raw axis will not be pruned again using the custom method.

You can choose to utilize preset methods, or you can proceed directly to STEP 9.

STEP 6. If you wish to explore methods that have proven effective with certain image datasets, check the "Use preset pruning methods" box. Then, select the desired preset methods to be applied. Refer to figure 8.6 for an illustration of how the preset methods correspond to the options in process>binary>options command.

STEP 7. To compare the effects of pruning versus not pruning, choose "None" from the list of preset methods.

STEP 8. Specify the pruning strength to be applied with the selected preset methods. If a value below 1 or above 8 is entered, it will be disregarded and a default value of 3 pixels will be used instead. Note that the tool will only apply the user-defined strength value (as compared to custom pruning below).

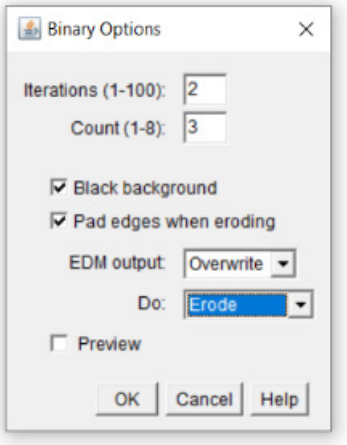


Figure 8.5. An example of how the pruning method “2x Erosion” translates in ImageJ.

Preset method	Cycles (iterations)	Strength (Count)	“Binary tool” 1 (Do:)	“Binary tool” 2 (Do:)
2x Erosion	2	User-defined	Erode	-
2x Dilation	2	User-defined	Dilate	-
2x [Erosion & Dilation]	2	User-defined	Open	-
2x Erosion then 2x Dilation	2	User-defined	Erode	Dilate

Figure 8.6. Preset methods built-in parameters

STEP 9. Alternatively, you can choose to use all possible combinations of the following custom pruning parameters. If you do not require custom pruning analysis, leave the checkbox unchecked and proceed directly to STEP 13.

STEP 10. Enter the maximum number of processing cycles (limited to 100, as in the option command). For example, if you enter 4, the tool will attempt all cycle values ranging from 1 to 4.

STEP 11. Specify the first “binary tool” to be employed among the list. These are the traditional binary methods (Erode, Dilate, Open) used in the initial round of processing. Open consists of one erosion followed by one dilation. If necessary, you can set a second “binary tool”. The method will apply the “binary tools” sequentially according to the specified cycles. For instance, if 2 cycles are executed and the first “binary too”l is erosion while the second “binary tool” is dilation, this will result in 2x erosion followed by 2x dilation.

STEP 12. Set the maximum pruning strength. If the maximum strength is 3, the tool will attempt all strength values ranging from 1 to 3. This is combined with the maximum cycles values. Therefore, if you set the maximum cycles value to 4 and the maximum strength to 3, the tool will prune the raw axis a total of 12 times. Assuming the cycles value is represented by 'c' and the strength value is represented by 's', and only one “binary tool” (erosion) is utilized, the combinations will be as follows:

• One (c=1) erosion with a count/strength value of s=1

• One (c=1) erosion with a count/strength value of s=2

• One (c=1) erosion with a count/strength value of s=3

• c=2 erosions with a count/strength value of s=1

• c=2 erosions with a count/strength value of s=2

• c=2 erosions with a count/strength value of s=3

• c=3 erosions with a count/strength value of s=1

• c=3 erosions with a count/strength value of s=2

• c=3 erosions with a count/strength value of s=3

• c=4 erosions with a count/strength value of s=1

• c=4 erosions with a count/strength value of s=2

• c=4 erosions with a count/strength value of s=3

Note: If you do not select the preset methods or a custom method, select "Use preset pruning methods" and "None".

STEP 13. Modify or fill in the directory for the cropped images using the browse button.

STEP 14. Proceed with the analysis by clicking OK. The tool will generate the raw axis ROI and whole axis ROI, which will be drawn using the processed mask (with the pruning method specified in brackets). Skeleton analysis will be conducted using the "skeletonize 2D/3D" plugin and each modified processed mask (note that the plugin must be installed if not using Fiji). The tool does not produce any file. Wait until the “All advanced parameters tested on input images” message is displayed.

STEP 15. Check the segmentation results using the Start Checking results button (figure 0). This action will prompt the appearance of the "Check results" window (refer to figure 8.9). Scroll the RoiManager list to identify the best pruning parameters. Click on “Check next result” button (figure 0) to proceed to the next analysed image.

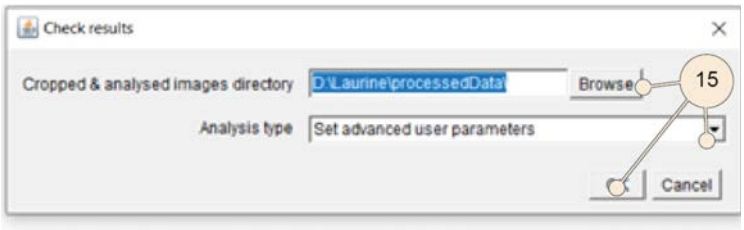


Figure 8.9. The check results window

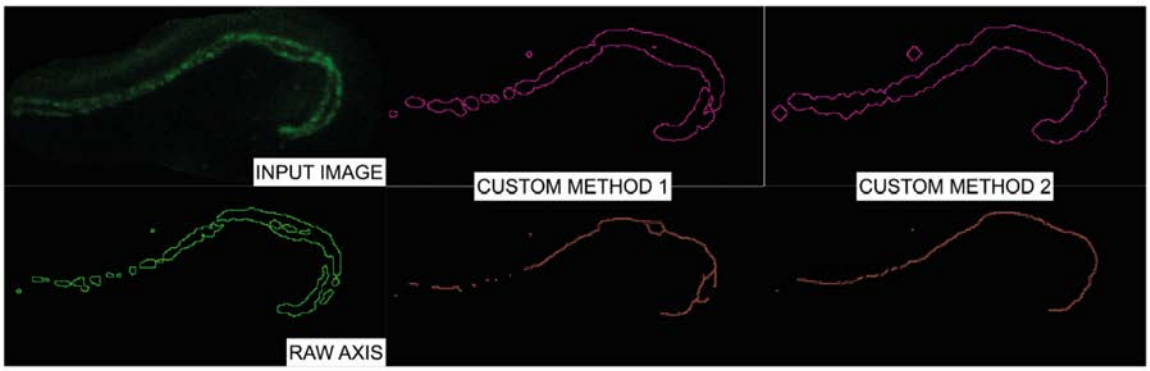


Figure 8.10. Control ROIs. Processed axis ROI are displayed in purple while axis skeleton are displayed in brown. Raw axis ROI is displayed in green.

VERSIONS

Crop tools (from start cropping images to diplo button)

V1.01	Creation
V2.01	• Single window menu: the input (cropped) image folder is directly available from the Start Cropping images main menu window
V2.02	• Pachy button correction (typo removal) • More meiotic sub-stages added (prelepto, earlylepto and lepto/zygo)
V2.04	• Correction of a ROI error when generating .roi files (ROI were copied from the original image rather than the cropped image).
V2.05	• Correction of an unwanted cut/paste error for the early lepto button
V2.06	• The crop buttons are all gathered in a single Get stage sorted images button.

Measure Global intensities:

V1.01	creation
V2.01	• Single window menu: the input (cropped) image folder is directly available from the Start Cropping images main menu window
V2.04	• The algorithm parameters are stored using a unified function, shared with the other buttons
V2.07	• Correction of a bug affecting user-defined ROI measurements

Count Foci in two channels and colocalize

V2.06	Creation
V2.07	• Correction of a bug affecting user-defined ROI measurements

Measure axis length:

V2.01	creation
V2.01	• Noisy (electronic or shot noise only) images can be pre-smoothed before axis detection. • The possibility to process the (raw) axis ROI is implemented (pruning mode).
V2.03	• The possibility to measure intensities of a channel of interest within the whole axis' ROI is introduced
V2.04	• Improved algorithm's robustness (when no axis is detected or when the processed mask is empty after pruning). • A bug is corrected (raw axis and process axis are identical) • The algorithm parameters are stored using a unified function, shared with the other buttons. • The axis channel within the whole axis' ROI can be measured together with another channel of interest.

V2.05	• The different pruning modes (refered to as pruning methods) are removed from the tool. The default pruning mode is “None” (ie. no pruning). Smooth option and pruning options can be set selecting the “use advanced user parameters”. Note that only a single method is used from this version. Different parameters can be tested using the “set advanced user parameters” button of the main bar.
V2.06	• Using different pruning options is not available anymore (see the “set advanced user parameters”. Changing the pruning method is possible through the set advanced user parameters tickbox of the main “measure axis length” menu. • The “black background” setting of ImageJ’s binary tools options is selected so that erosion now shrinks the mask and dilation enlarges it.
V2.07	• Correction of a bug affecting user-defined ROI measurements

Measure synapse length:

V2.02	creation
V2.03	• The possibility to measure intensities of a channel of interest within the axes’ ROIs is introduced
V2.04	• Improved algorithm’s robustness (when no axis/synapsed axis is detected or when the processed mask is empty after pruning). • The algorithm parameters are stored using a unified function, shared with the other buttons • The user can set general parameters in a separate “set measurements parameters” button (reference ROI measurements, Integrated density & mean values computation, rough/fine axis length measurements). Warning message can be hidden as can the images be displayed upon request. • The axis/synapsed or non-synapsed channels within the axes’ ROIs can be measured together with another channel of interest.
V2.07	• Correction of a bug affecting user-defined ROI measurements

Count axis and off-axis foci in one channel

V1.01	creation
V2.01	• The “within ROI” option for foci detection is introduced
V2.02	• Correction of error messages arising when no foci is detected (“the image does not have an active selection”) • Whenever the image is duplicated, all selection are deselected to avoid unwanted channel shift introduction. • Maxima Finder version (process>FindMaxima) changed to 1.52 and more. Tolerance changed to prominence and strict option implemented. • Code simplification so that FindMaxima is not run twice.

V2.04	• Robustness is improved (cases where the reference ROI is not found, the axis is not found are taken into account). • The algorithm parameters are stored using a unified function, shared with the other buttons • The user can set general parameters in a separate “set measurements parameters” button (reference ROI measurements, Integrated density & mean values computation, rough/fine axis length measurements). Warning message can be hidden as can the images be displayed upon request.
V2.05	• Foci are identified as maxima and associated particle. Implementation of a foci detection threshold. If none is selected, particles are identified as pixels within tolerance/prominence of the maximum. Otherwise, particles are identified using the selected threshold using the Find Maxima threshold. • Particles and maxima intensities are calculated individually in a separate spreadsheet file.
V2.06	• Subtle modification of how multipoint maxima’s Integrated Density is measured.

Count axis foci in two channels and colocalize

V1.01	creation
V1.02	• Correction of a bug (noise value for foci B detection was noise A value).
V2.01	• The “within ROI” option for foci detection is introduced
V2.02	• Correction of error messages arising when no foci is detected (“the image does not have an active selection”) • Whenever the image is duplicated, all selection are deselected to avoid unwanted channel shift introduction. • Maxima Finder version (process>FindMaxima) changed to 1.52 and more. Tolerance changed to prominence and strict option implemented. • Code simplification so that FindMaxima is not run twice. • Correction of a bug (random foci where located at the edges of the axis)
V2.03	• The user has the possibility to measure either on-axis foci or off-axis foci.
V2.04	• p values below satisfaction criterion are now calculated and a warning message is added in the comment column. • The algorithm’s robustness is improved (when no foci are detected in either channel A or B). • The user can set general parameters in a separate “set measurements parameters” button (reference ROI measurements, Integrated density & mean values computation, rough/fine axis length measurements). Warning message can be hidden as can the images be displayed upon request.
V2.05	• The user can set general parameters in a separate “set measurements parameters” button (Integrated density &

	mean values computation, whether foci's maxima and/or particles should be considered for ROI measurements). • Correction of some display bug in the 2foci spreadsheet. • Robustness is improved (cases where the reference ROI is not found, the axis is not found are taken into account).
V 2.06	• When colocalisation option is used and intensities within foci are measured, colocalised and not colocalised foci are measured separately. • Axis mask processing options are only displayed when "use advanced user parameters" option is selected
V2.07	• Correction of a bug affecting user-defined ROI measurements

Options

V2.05	• Options are introduced, within a "set measurements parameters button in the main bar", as to control warning messages, display of processed images, lengths/intensity measurements parameters.
V 2.06	• Axis mask processing parameters can be changed separately from the main analysis tools in a "set advanced user parameters" button • Following introduction of a variable crop stages bar, prefixes used for analysis can be changed in a "set stages/types parameters" button • Stages/types prefixes is not case sensitive anymore
V2.07	• Correction of a bug affecting user-defined ROI measurements