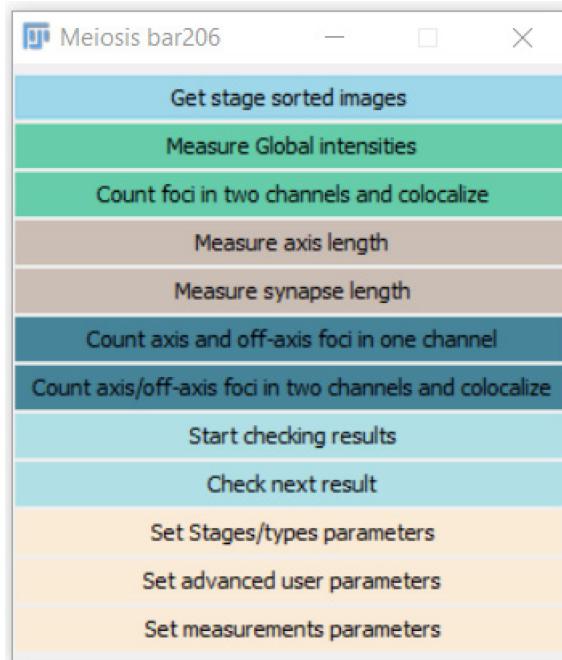


THE MEIOSIS BAR



VERSION 2.06

APRIL, 7TH 2023



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

- <https://imagej.net/plugins/skeletonize3d> Skeletonize3D_-2.1.1 version 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.

Mind spaces or fancy characters should be avoided (input folders and parents folders, file names).

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This collection of macros is intended to measure synaptonemal complexes-associated features (such as synaptonemal axes or recombination foci). The process uses the following main routines:

A. MEIOSIS STAGE IDENTIFICATION: as an image may contain several meiotic stages nuclei, the user is prompted to draw the outlines of each nucleus and crop/save it according to its meiotic stage.

B. Analysis:

B.1 GLOBAL INTENSITIES MEASUREMENTS: this first routine is intended to measure global intensities of a set of channels of interest.

B.2 FOCI IDENTIFICATION & COLOCALISATION. This routine is a simpler version of routine B6. It is detecting any foci within a ROI of interest (eg. does not discriminate axis/off-axis foci position)

B.3. AXIS LENGTH MEASUREMENTS: this routine is used to measure the total length of the identified synaptonemal axes. Some processing option are available.

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

B.5 FOCI IDENTIFICATION: foci are identified and categorized, with respect to their proximity to the synaptonemal axes. The total length of the identified axes can be calculated (using a single processing option).

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

MEIOSIS STAGE IDENTIFICATION: GET STAGE SORTED IMAGES

This step will produce single nucleus images and save them with the original image's name and a stage prefix in a subfolder.

STEP1. Before starting the analysis, first click “Get stage sorted images” button. The next menu appears.

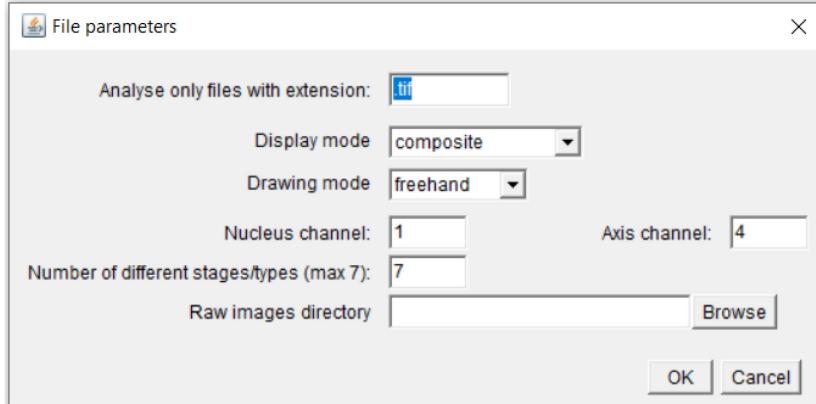


Figure 1. the get stage sorted images

- Analyse only files with extension: this option is used to find the image files to be opened. This is useful not to discard any unwanted file (such as non-image file for instance). The extension has to be compatible with the bioformats plugin. Upon change, the next time the button is hit, the new default extension will be displayed/saved. However, if one reruns ImageJ or the toolbar, the default value is back to “.ics”. The routine uses ImageJ’s preferences. The default value may be changed in the routine’s script on line #3 [call("ij.Prefs.set", "global.extension",".tif");].
- Choose a display mode. If “composite” is used, 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 (#1) will be shown.
- The drawing mode will set the selection tool to either freehand (you can manually crop nucleus) or magicwand tool (then the magic wand is used).
- Fill in the number of different stages/types (max. 7) field. This will create a crop bar with the corresponding number of buttons.
- The macro uses bioformats for opening the images. If the Raw Image directory field is empty, or you want to change dataset, click the browse button. Mind files within subfolders will not be analysed. This folder’s path is then used in the next routines and referred to input.
- Click on OK

STEP2. The next menu (figure 2) is used to set the stages names. These prefixes are going to be used to save the cropped nucleus. Fill-in the fields. Avoid spaces (as this may block the file identification. If necessary use underscores. Mind that if the default (shown below) prefixes are changed and images are analysed beforehand (ie. after you quit imageJ once and then go back to cropped images analysis), you may have to change the default prefixes again (using the set Stages/types parameters button).

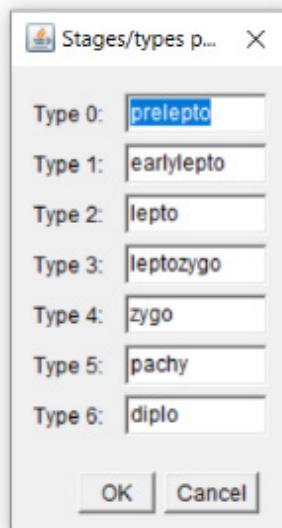


Figure 2. the stage/type parameter menu



Figure 3. the crop stages bar

STEP3. The custom crop stages bar (see figure 3) will pop-up. Once the file is opened and bar displayed, use the freehand/magicwand selection tool to draw the outlines of a nucleus of interest. Add the selection to the ROI Manager. Any other same-stage nucleus of interest from the image can be drawn and its selection added to the ROI Manager.

Then hit the corresponding stage button (lepto, zygo, pachy or diplo). Briefly, the image is duplicated using the selection. For each channel, pixels outside the selection are set to black. The file is then saved in a “processedData” subfolder as a .tif file. A meiosis stage prefix is added as well as a identification suffix (_0 for instance). The user-defined ROI is saved as well within the same folder as a .roi file.

STEP4. To open the next file, hit the “next file to crop” button.

GLOBAL INTENSITIES MEASUREMENTS

The “measure global intensities” algorithm can be used to measure whole intensities. Depending on whether the “Start cropping images” or any other analysis routine was used beforehand, the menu will appear as in figure 2.1 or 2.2. Whenever the input folder (ie. where the original full frame images are saved) was set or some processedData folder (ie. where the stage-sorted cropped images were generated) was previously set using the same instance of the bar, then the menu appears as in figure 2.1. If the cropped image directory was not found, then click the browse button (fig 2.2), select the directory and click OK. The first found image will be opened and the user will be prompted to select the channels to measure (fig. 2.2).

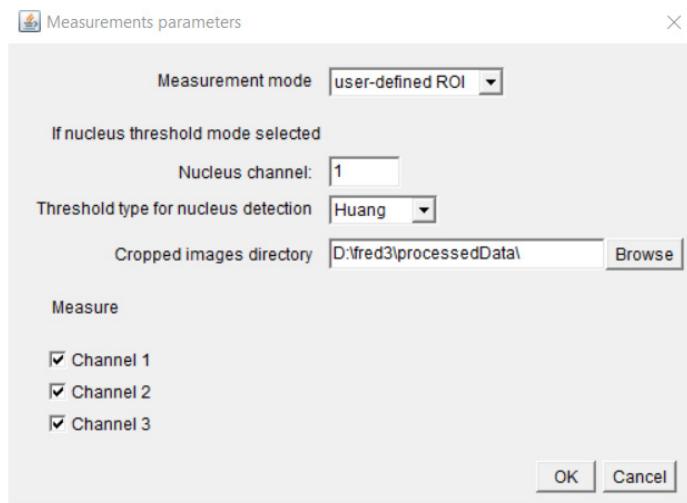


Figure 2.1. The Measure Global intensities menu (when some previous routine was used).

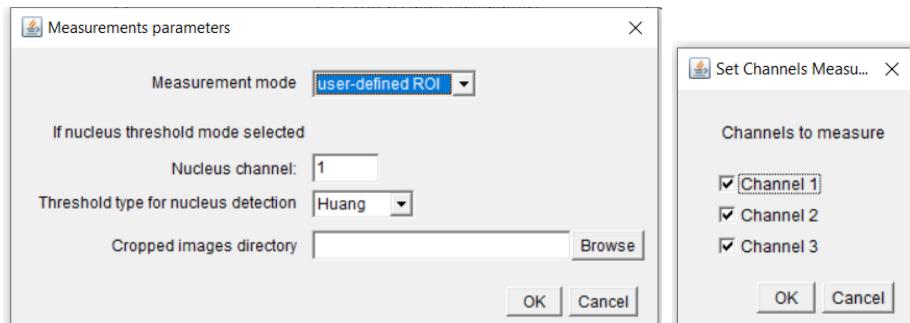


Figure 2.2. The Measure Global intensities menu (if no input folder was previously set).

STEP1. Set the ROI that will be used for intensity measurements. If the “user-defined ROI” is selected, then the companion .roi file generated during the stage-cropping step (“start cropping images, next file to crop and lepto to diplo buttons) will be used. If this file is not found, some error message is displayed. The other possibility is to use some intensity threshold. The macro refers to “nucleus” threshold as most of the time, the channel used for the threshold is based on some nucleus staining. This can be however used for any other staining. If using this “nucleus threshold” mode, set the channel used for threshold (the first channel is channel 1), then select the adequate threshold algorithm. A broader range of automatic thresholds methods can be displayed using the “set measurements parameters” button (see fig. 2.4). Set these parameters as in 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.

Note: Mind that the algorithm suite was initially designed to analyse multiple nucleus-containing images. If no threshold can be used to set the ROI and the image contains a single, cropped nucleus, then set the whole image (Ctrl A) as the ROI when using the lepto/zego/pachy/diplo buttons.

STEP2. Select the channels to be measured. If the stage-sorted images location was previously set, then the menu will appear as in fig 2.1. Select the channels to be analysed. If the stage-sorted images location was unknown or was changed (whenever the option “change images folder” was selected in menu of figure 2.1), the user is prompted to enter the number of channels (figure 2.2 middle) and then to select the channels to be measured. If the routine does not find the right number of channels, the measurement is skipped (and “not found” is displayed in the corresponding column of the intensities.xls file).

STEP3. Each selected channels are measure. The raw IntDen value, along with the mean intensity, is saved in an intensities.xls file located in the same folder where stage-sorted images are located. The “intDen” values are the raw (uncalibrated) intDen values as measured by ImageJ.

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.

Images are not shown during the analysis. To have them displayed, use the set Measurements Parameters of the main meiosis bar. In the parameters window (fig. 2.4), select “show images while processing files” option. The default settings only display (raw) integrated densities values. It is possible to change this in the parameters window (intensity measurements).

WARNING. Intensity values are measured without any subtraction of noise/offset/background. In most widefield cases, whenever the full dynamic range is used (eg. 0-65535 gray levels for 16 bits images), camera/system noise and offset values are negligible. This is not true whenever low intensity, reduced dynamic range images are used. Rough correction of intDen/mean values supposes measurement of an empty area and the above formulas. Mind intDen correction supposes both mean and intDen values should be measured.

The impact of background signal is to be considered as well. As background signal changes across the image, it is recommended to process the images before using the macro.

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

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

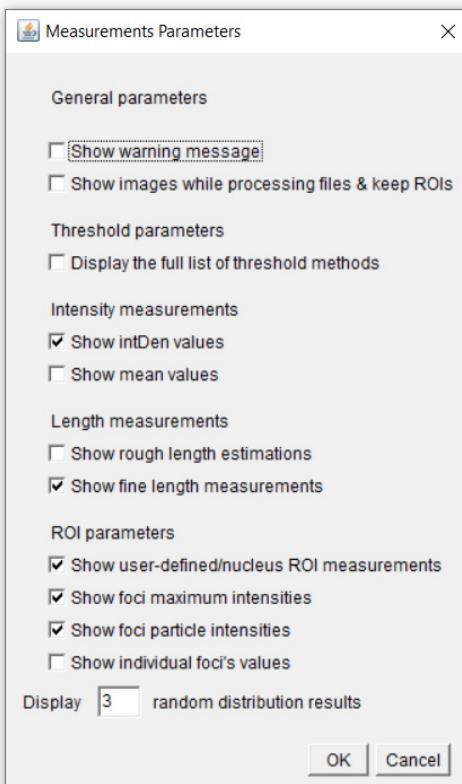


Figure 2.4. The general parameters window.

FOCI IDENTIFICATION & COLOCALISATION

The “count foci in two channels and colocalize” algorithm will i) identify axis foci in two channels, ii) measure colocalisation as described in Lachmanovich 2003 Journal of Microscopy, Vol. 212, Pt 2 November 2003, pp. 122–131. Additionally, the macro can create random equivalent images and check whether the observed colocalised foci number could be randomly obtained.

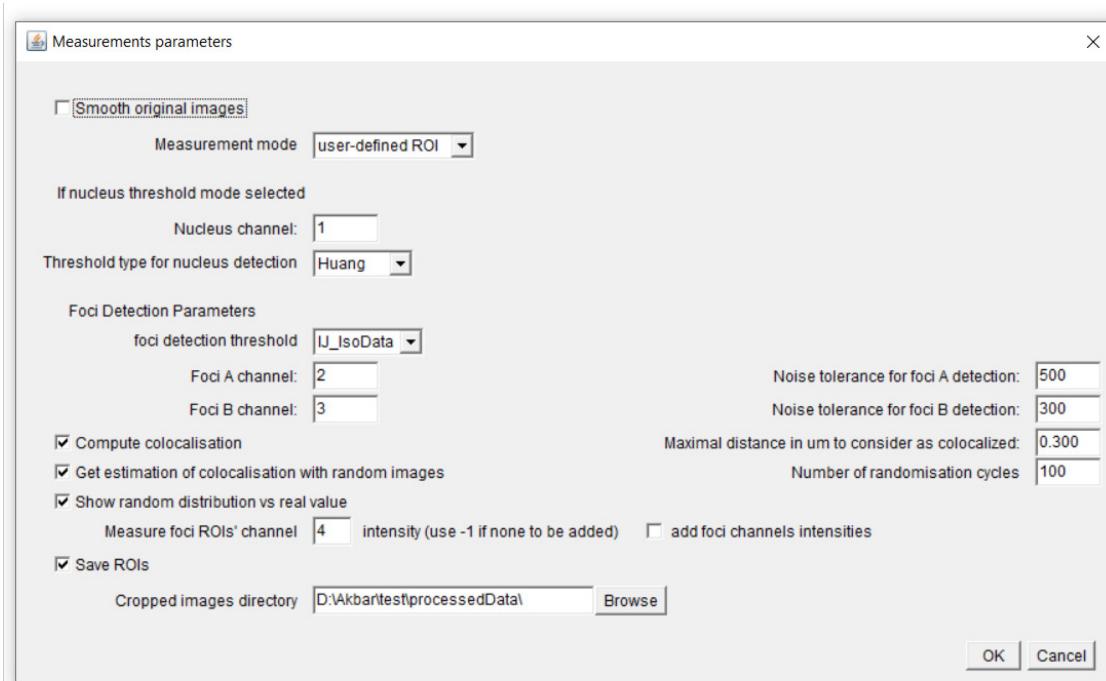


Fig. 3.1. The “Count foci in two channels and colocalize menu

→ **OPTIONAL STEP A.** Set the output measurements options (see optional step description below).

STEP 1. Whenever images are noisy, tick the “smooth original images”.

STEP2. Select the original region of interest to be used for the measurements. As in the previous routines, the user may either select the user-defined ROI (ie. the .roi that was generated upon image cropping), or use a thresholded nucleus staining (figure 3.1). This user-defined or nucleus ROIs are referred as “reference ROI” below.

STEP 3. Enter foci detection parameters. The foci channels A and B are selected and foci of interest are detected using the find maxima algorithm. The prominence value used is entered in the “noise tolerance for foci detection” box of the menu. Prominence was formerly referred to as noise (although there are differences with Maxima Finder menu- aka. “Find maxima” function- versions before and after ImageJ 1.52). Mind that same-color twin foci may be detected as single foci. The “strict” option of the “Find maxima” function is used (only available from ImageJ 1.52). The macro takes full advantage of all possibilities offered by this version of Maxima Finder.

3 modes are proposed, that all use the nucleus/user-defined ROI as input ROI:

- Maxima are identified without any threshold (foci detection threshold “none”). Foci above prominence value are detected as maxima. The corresponding particles are identified using the “maxima within tolerance” output option of the Find Maxima tool.

- Maxima are identified using either a Huang or an IJ_IsoData automatic threshold. Foci detection is restricted within the thresholded area and foci, within this area, above prominence value are detected as maxima (with “above lower threshold option” of the Find Maxima tool used). The corresponding particles are identified using the “segmented particles” output option (see **STEP 6**).

Foci are detected within the reference ROI (referred as total_foci (maxima)). If the colocalisation analysis is not requested, ROI are attributed ROIGroups according to 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 using the colocalisation option (compute colocalisation checkbox selected), a mask of the total foci is generated (provided some maxima were found in the reference ROI) which is further used to compute a 32-bits euclidean distance map. This can be manually done using Process>FindMaxima tool and the single points output, then process>Binary>Options and select 32-bits as EDM (Euclidean Distance Map) output and then run process>Binary>Distance Map. A threshold is then applied to the EDM Map using a converted colocalisation distance. The “maximal distance in um to be considered as colocalised” is converted into pixels and the converted value used as a threshold. A selection is drawn using the thresholded area and stored in the RoiManager as “colocalised_area_foci[letter]” (see figure **3.3** for respective ROIGroups). The complementary ROI is drawn for not colocalised fociA/B areas. When a given foci maximum (as identified in **STEP 3**) of the other channel (say A if coloc area B) is within this ROI, it will be considered as colocalised. If the default -1 value is kept in the “maximal distance in um to be considered as colocalised”, no colocalised area ROI is generated. As in Lachmanovich 2003, it is recommended to use as colocalisation distance threshold the minimal resolution distance (typically 230-250nm for a 1.4 NA lens and a widefield setup). See Pawley JB Handbook of Biological Confocal Microscopy, Springer 2006 for instance for other configurations). The rationale of using a minimal resolution distance value as a threshold is quite straightforward: would foci A and B be from the same channel, if their separating distance is below the minimal resolution distance, they could not be seen as different.

Whenever some foci of interest are found in both channels A and B, maxima of foci of interest of a given channel (say fociA (maxima) ROI) and the corresponding coloc area ROI (colocalised_area_fociB in this case) are compared (using the RoiManager>More> “AND”). Maxima that fit into the coloc area ROI are counted and used to create a new ROI (in this case colocalised fociA (maxima)).

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

STEP 5. Consider checking whether the observed colocalised pixels in both channels could be randomly obtained. Select “get colocalisation with random images”. This option will not run if the main “comput colocalisation” is not selected. Briefly, the observed foci number in one channel are shuffled within the whole reference ROI. randomly localised foci of the first random images (for both channel randomisations) are saved within the ROI Manager. The number of random distributions saved in the ROI manager can be changed using the general parameters menu (see **OPTIONAL STEP A**).

Each random foci distribution within the reference ROI is then compared with the observed foci localisation in the other channel using the corresponding Euclidean distance Map ROI (eg. random fociA_0 (maxima) with colocalised area fociB). Enter the number of random images to be generated in “number of randomisation cycles”. The average number of randomly colocalised foci is calculated for both combinations. It is recommended using a number of cycles >30 so that chances are high a normal distribution of randomly colocalised foci is obtained.

When the average random colocalised foci is 0, no further analysis is performed. Otherwise, the distribution of random colocalised foci number for all random images is plotted. This plot can be saved (see fig 3.4 for an example) if the “show random distribution versus real value option is selected.” Although this plot is saved as a .tif file in a parent controlData folder, they can be opened using imageJ and the list can be extracted (click on list). The frequency of number of randomly colocalised foci are indicated as grey hollow dots. A Gaussian fit is applied (blue line). The actually observed, real number of colocalised foci is indicated with a red line.

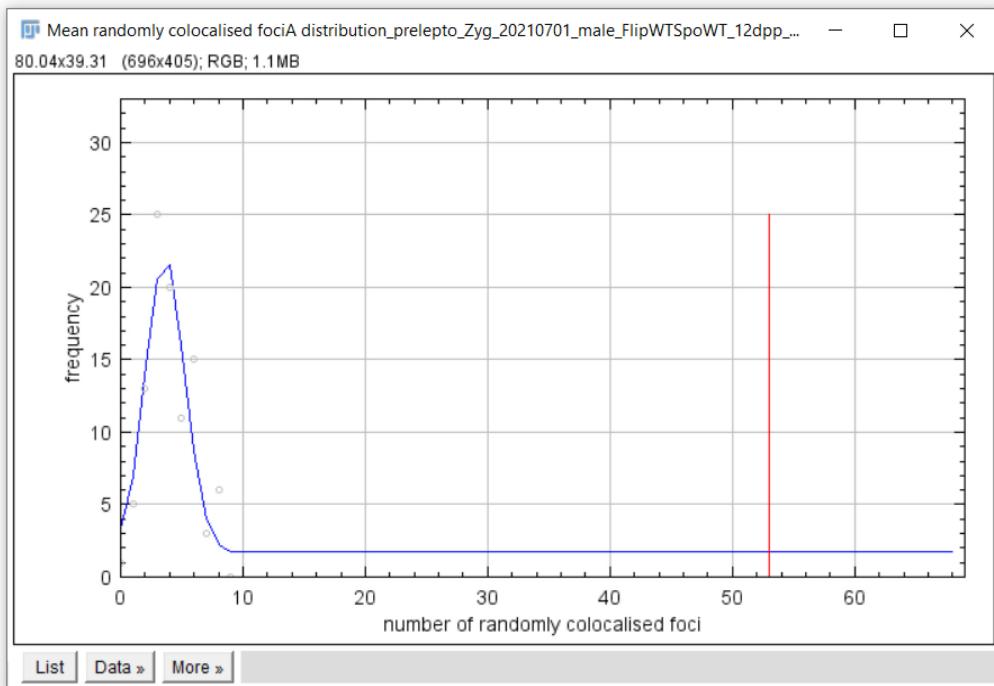


Fig. 3.4. randomly colocalised foci number distribution across all randomly generated images

The mean and standard deviation values of the randomly colocalised foci number are used for p value analysis. If $\text{mean} - 3 * \text{stdDev} < 0$ (ie. the blue Gaussian is clipped, as in figure 3.5), the p value is displayed with an asterisk (*) and a comment “*: take p value with caution” is displayed in the “comment” column of the 2foci.xls result file).

The p value is:

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

Where erf is the Gaussian error function:

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

For the purpose of erf calculation, the Horner's method is used.

The pvalue indicates the proportion of the Gaussian below the observed, real colocalised foci number. When close to 1, this means the observed colocalised foci number is unlikely to be obtained with random foci distribution within the reference ROI.

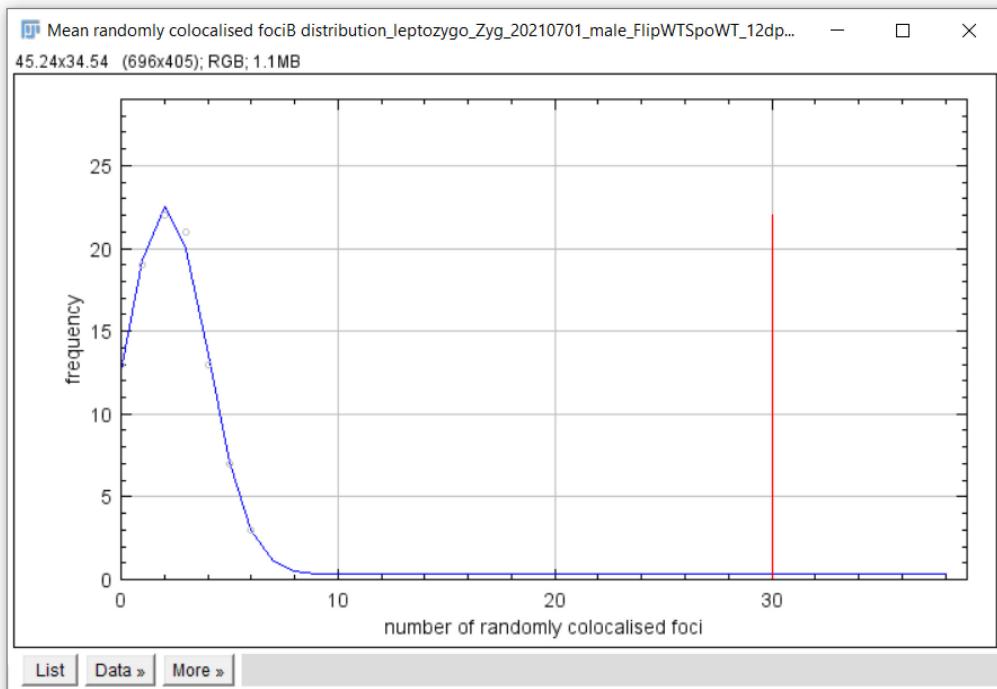


Fig. 3.5 “clipped” randomly colocalised foci number distribution across all randomly generated images

STEP 6. Then, particles are identified starting from the nucleus/user-defined ROI and using the above-mentioned Find Maxima options. Total particles ROI is split and each individual particle is compared to the total_foci[letter] maxima ROI.

This substep is quite time consuming. To skip this, deselect the “show foci particle intensities” option of the general parameters menu (see **OPTIONAL STEP A**). Whenever a split particle contains a maxima, then it is renamed (for instance as an fociA_ID (particle)). If measurements parameters (see **OPTIONAL STEP A**) “show foci maximum intensities” is used together with “show individual foci’s value”, then the corresponding maximum point ROI is created (following the previous example as fociA_ID (maximum)). Both maximum and particle share the same ID. If the “compute colocalisation option was selected”, the macro checks whether there is a triple overlap of the individual ROI, the corresponding total_foci and the colocalised area generated using total maxima of the other channel. If any, the particle (and its associated individual maximum – if any was generated following **OPTIONAL STEP A** configuration) are tagged as colocalised using the corresponding ROIGroup (see figures 3.3). If no overlap is observed, as the particle was overlapping with a maximum ROI (following this example total_fociA (maxima)), this means the particle is not colocalised and tagged as such.

If the individual particle does not contain any maximum, then no ROI group is attributed (ie. left to default 255 value) and the particle is purged from the ROI manager at the end of the process. Individual, colocalised or not-colocalised particles are fused together to create a ROI of all colocalised or not-colocalised particles.

STEP 7. Set additional intensity measurements channels or ROI. The macro will identify nucleus/user-defined ROI and the foci of interest (axis or off-axis) for both channels as maxima and particles. Intensities can be measured in these ROIs and for a selection of channels. If the foci channels A and B should be measured as well, select “add foci channel intensity”. Would you like to add another channel for intensity measurements within ROI, fill-in the numeric field

“Measure foci ROI’s channel intensity. If the field is left with -1 and the “add foci channel intensity” checkbox is not thicked, no measurements will be done.

To remove nucleus/user-defined ROI measurements or show/hide mean or IntDen or particle/maximum intensities, see **OPTIONAL STEP A**. For global particle measurements (as displayed in the 2Foci.xls table), the corresponding ROI is used (for instance colocalised total_fociA (particles)). For global maxima calculation, the corresponding ROI (say total_fociA (maxima)) is measured using the getRawStatistics(nPixels, mean, min, max, std, histogram) command. The number of maxima (nPixels) is multiplied by mean to derive the IntDen value.

STEP 8. Select the control output options. If the “Save ROIs” option is selected, all ROIs (nucleus/user-defined, foci in both channels, if relevant colocalized foci in both channels and random foci) will be saved in a companion “2foci_RoiSet_[name].zip” file located in a parent ControlData folder. Results are saved in a “2foci.xls” file (located in the same folder where the input images are stored) (see figure **3.7**). Individual foci values (if requested) are saved in an “individual2Foci.xls” file.

STEP 9. Click OK. The analysis starts. The macro runs in batchMode, to display the images while processing, follow **OPTIONAL STEP A**.

OPTIONAL STEP A. Set the measurements parameters options. Before using the “Count foci in two channel and colocalize” tool, click on the “Set measurements parameters button”. The Parameters menu (figure **3.6**) will pop-up.

- Choose whether to display warning messages or not. As those block the batch processing of the macro, consider keeping them hidden.
- The default parameters hide the images during the analysis. To have them displayed, use the set Measurements Parameters of the main meiosis bar. In the parameters window (fig. **3.6**), select “show images while processing files” option.
- The default list of threshold (for nucleus, axis, synapsis or foci) is restricted to preset threshold that were found to be adapted to test images. Would you like to expand the list to the full ImageJ’s automatic thresholds list, select the “Display the full list of threshold methods” options.
- The default intensity measurements is IntDen. Would you like to add Mean values or remove IntDen, use the “Show IntDen values” and “show Mean values” options.
- Ignore the length measurements options that applies to other tools.

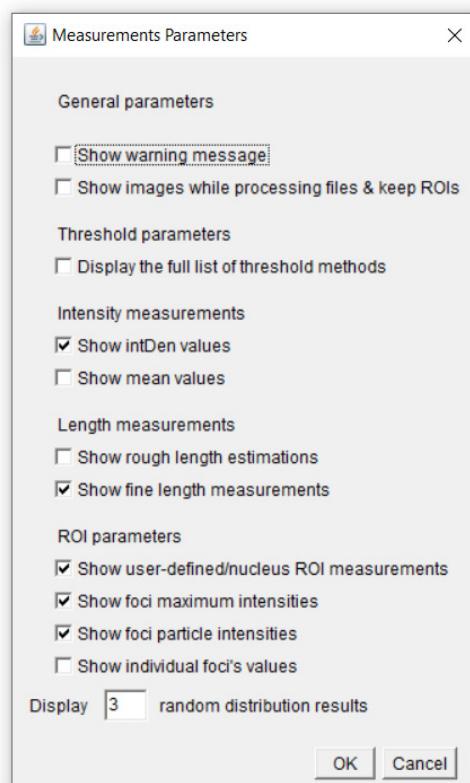


Figure 3.6. The (general) parameters window.

- Intensities is measured in the different ROIs. The default parameters will trigger reference ROI (either user-defined or nucleus ROI) intensity measurements. Unselect “show user-defined nucleus ROI measurements” to get rid of this. Foci ROIs can be measured both in maxima/individual maximum or particles/individual particle. Use the corresponding tickboxes to restrict analysis.
- Whenever random colocalisation analysis is requested, the “display [] random distribution results will leave in the ROIManager the requested random axis/off-axis foci ROI as well as the corresponding random foci that may colocalise with the real foci distribution in the other channel.

Type	Image name															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	Ch.4 intDen (not colocalised particles)	4024561.850		
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		
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		
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		
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		
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		
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		
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		
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		
																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	1.53199	

Fig. 3.7. 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' particle)	Ch.4 intDen (focus' maximum)	particleROInd ex	maximumROIInd ndex	focus' colocalisation status	focus type
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. 3.8. a individual particle result file individual2Foci.xls file of the Count foci in two channel and colocalize macro

STEP 11. 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” window will pop up (figure 3.9). If you just ran the macro, then the default folder and method are OK. If you control afterwards the macro’s results, set both folder and “Count axis/off-axis foci in two channels and colocalize” method.

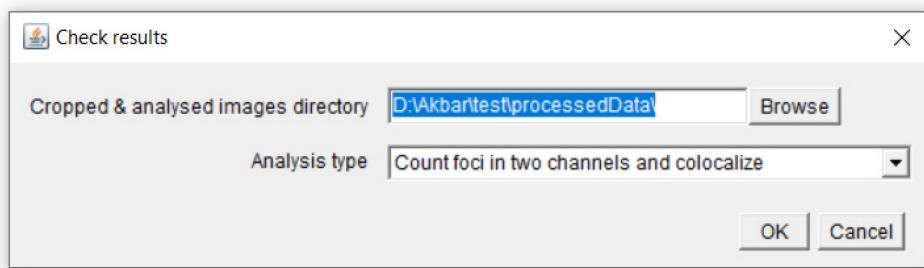


Figure 3.9. The check results menu

The first image analysed will be opened together with its companion 2foci_RoiSet_[name].zip file ROI set, as in fig. 3.10. The ROI Manager contains several ROIs:

- The reference ROI, as 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 as obtained in **STEP 4**.
- Colocalised foci obtained using the real localisation of identified axis foci A and B (= those obtained from **STEP 3**) as obtained in **STEP 4**.
- If the “get estimation of colocalisation with random images” option is selected, the result of the first randomisation cycle (“randomFociA/B”, **STEP 5**).
- Depending of the configuration, the individual particles as obtained in **STEP 6**, together with the 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

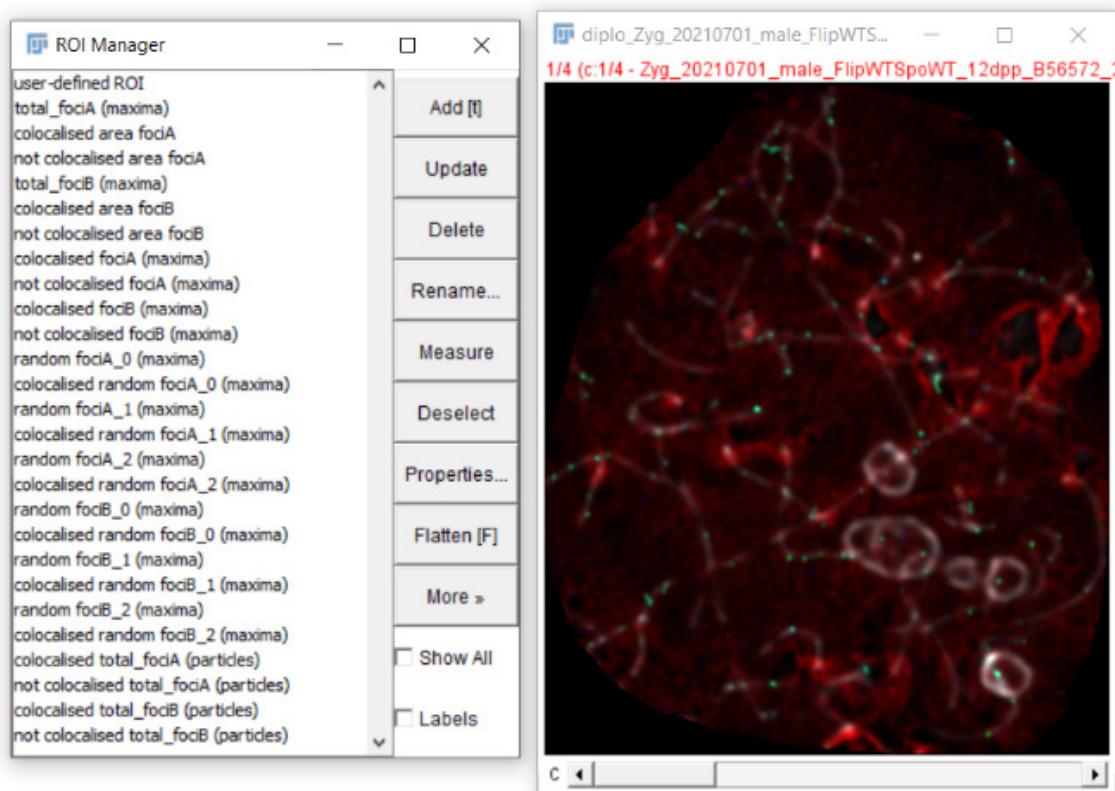


Figure 3.10. Control ROIs. Foci are displayed in both channels, as well as colocalised foci in both channels. When randomisation is used, an example of random distribution in both channel is shown. In this figure, the “show individual Foci’s values” was not selected.

MEASURE AXIS LENGTH

The algorithm will i) locate the axis (based on some synaptonemal complex staining), ii) produce a binary mask out of it, iii) process the mask to reduce the number of unwanted axis “branches” (referred as pruning process), iv) measure the total length of the axis. The algorithm is to be used to test different pruning modes before using the “Count axis and off-axis foci in one channel”. The macro generates ROIs associated with ROIGroups.

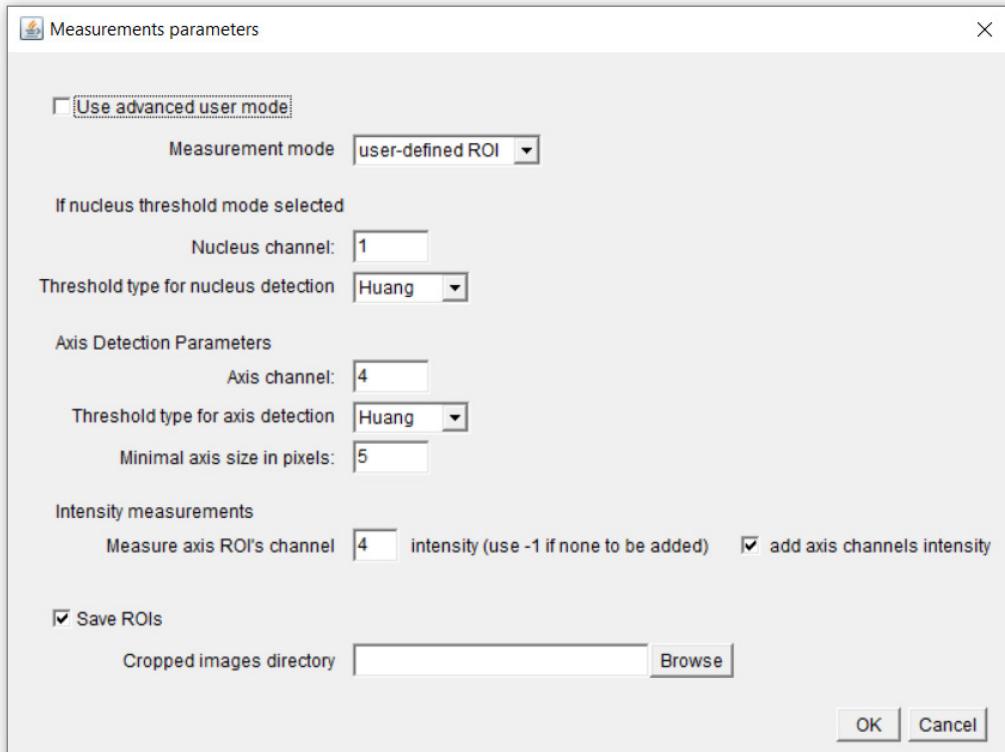


Figure 4.1. The Measure Axis Length menu

→ **OPTIONAL STEP A.** Set the output measurements options (see optional step description below).

STEP 1. It is recommended to use the advanced user mode whenever noisy images are used (such as those from STED setups). Tick the “smooth original images” option in the next “Advanced user parameters” menu that pops up upon clicking OK at **STEP 7**.

STEP 2. Set the ROI that will be used for further measurements. If the “user-defined ROI” is selected, then the companion .roi file generated during the stage-cropping step will be used. If this file is not found, some error message is displayed. The other possibility is to use an intensity threshold. The macro refers to “nucleus” threshold as most of the time, the channel used for the threshold is based on some nucleus staining. This can be however used for any other staining. If using this “nucleus threshold” mode, set the channel used for threshold (the first channel is channel 1), then select the adequate threshold algorithm. The list can be expanded to all available ImageJ built-in automatic thresholds by clicking on “Set measurements parameters” and tick the display the full list of threshold methods options” (figure 4.3). This user-defined or nucleus ROIs is referred as “reference ROI” below.

Note: Mind that the algorithm suite was initially designed to analyse multiple nucleus-containing images. If no threshold can be used to set the ROI and the image contains a single,

cropped nucleus, then set the whole image (Ctrl A) as the ROI when using the cropping buttons.

STEP 3. Set the axis detection parameters. Enter the axis channel (mind the first channel is channel #1). Set the appropriate threshold to be used. The algorithm will first use this threshold. If no pixel above threshold is detected, then the analysis aborts and “no axis detected” will be displayed in the final table. The threshold is used to generate a temporary “axis threshold” selection in the ROI Manager. Then axis elements are detected using the Analyze Particle items and the nucleus/user-defined ROI.

Enter the minimal pixel size of thresholded axis elements. Use this to discard unwanted, small items. Mind raising this value too high might not be appropriate for early stages. All within specs particles are identified and added to the ROI Manager (additional criteria can be added in the advanced user mode). These particles are then fused (using the combine ROI Manager option) altogether in a temporary ROI. Whenever overlapping axes overlap in such a way that they create a loop, the axes are not identified properly and the hole within the loop is considered as being part of the axis. This is further corrected using the AND option of the ROI Manager, applied to the “axis threshold” ROI and the temporary ROI. Both ROIs are then deleted and a “raw whole axis” ROI is added to the manager. If the advanced user mode is not selected, the raw axis is left untouched and an axis ROI is created, similar to raw axis. If a pruning method is introduced at optional STEP B, then the raw axis mask will be modified. A skeleton analysis is done, using the skeleton>skeletonize 2D/3D plugin and the input modified processed mask (mind the plugin needs to be installed).

→ **OPTIONAL STEP B:** Use the **ADVANCED USER MODE OPTION** to get better axis detection. Whenever the option is selected the next menu will pop up upon clicking OK at **STEP 7**.

STEP 4. Set additional intensity measurements channels or ROIs. The macro will identify nucleus/user-defined ROI and the axis. Intensities can be measured in both ROIs and for a selection of channels. If the axis channel is to be measured, select “add axis channels intensity”. Would you like to add another channel for intensity measurements within ROI, fill-in the numeric field “Measure axis ROI’s channel intensity”. If the field is left with -1 and the “add axis channels intensity” checkbox is not thicked, no measurements will be done. To remove nucleus/user-defined ROI measurements, follow **OPTIONAL STEP A** before.

STEP 5. Set the output items. If the “saveROIs option is ticked, all ROIs generated during the macro will be saved in a companion .zip file. The file is located in a parent controlData folder. Each ROI set is saved using the image’s name and a “lengths-RoiSet” prefix. Whether the option is used or not, all measurements will be found in a lengths.xls file saved in the folder selected in STEP7.

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

STEP 7. 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. Set the measurements parameters options. Before using the “Measure axis length” tool, click on the Set measurements parameters button to set how the skeletons will be measured. The Parameters menu (figure **4.2**) will pop-up.

- Choose whether to display warning messages or not. As those block the batch processing of the macro, consider keeping them hidden.
- The default parameters hide the images during the analysis. To have them displayed, use the set Measurements Parameters of the main meiosis bar. In the parameters window (fig. 4.2), select “show images while processing files” option.
- The default list of threshold (for nucleus and axis) is restricted to preset threshold that were found to be adapted to test images. Would you like to expand the list to the full ImageJ’s automatic thresholds list, select the “Display the full list of threshold methods” options.
- The default intensity measurements (in reference ROI and axis/off-axis ROIs) is IntDen. Would you like to add Mean values or remove IntDen, use the “Show IntDen values” and “show Mean values” options.
- Select the lengths measurement types. Two different routines are available:
 - The first (rough) one takes advantage of the 1 pixel thickness of the skeleton. The total area in pixel of the ROI is measured and converted in um using the image’s calibration. This may yield weird results (as a diagonal pixel will account for 1 – say 0.25um if the pixel width is 0.25um - although it should be considered as $\sqrt{2}$ – and 0.35um).
 - The second (fine) one is a raw measurement of the skeleton. It uses the geometry of the skeletonRoi and takes into account “horizontal, vertical and diagonal pixels”.

To remove fine or rough length measurements, unselect “show rough length estimation” or “show fine length measurements” respectively.

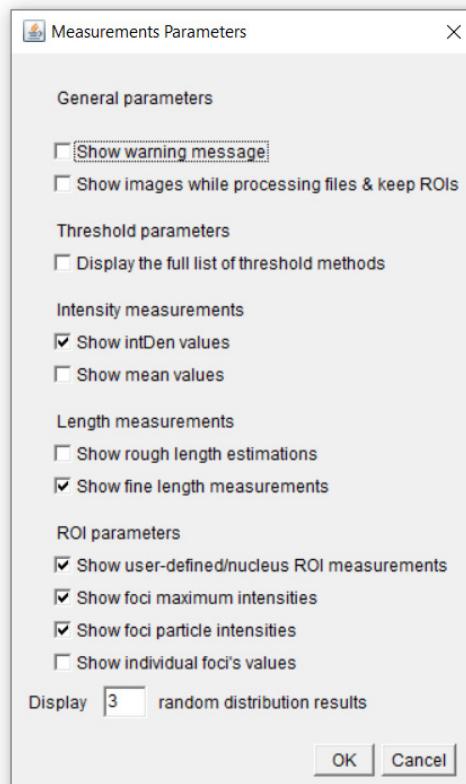


Figure 4.2. The (general) parameters window.

- Intensities is measured in the different ROIs. The default parameters will trigger reference ROI (either user-defined or nucleus ROI) intensity measurements. Unselect “show user-defined nucleus ROI measurements” to get rid of this. Foci ROIs can be measured both in maxima/individual maximum or particles/individual particle. Use the corresponding tickboxes to restrict analysis.
- Ignore the other options (that apply to foci detection).

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 4.3).

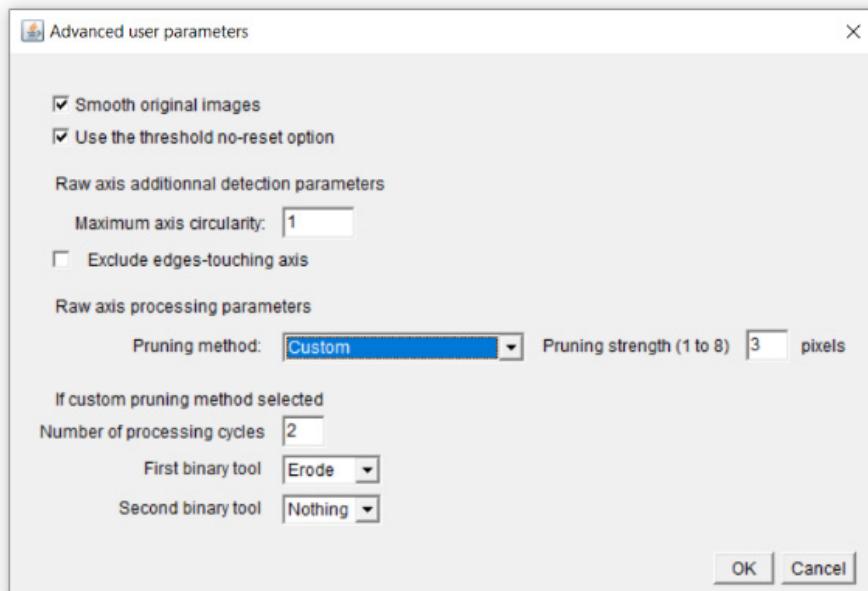


Figure 4.3. The Advanced user parameters menu

- 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.
- When using images with a very high offset value (such as the Abberior .msr files), those very high background intensity pixels together with pixels excluded from analysis (whose intensities are set to 0 in the cropping step), may bias the automatic threshold. From ImageJ 1.52e version to 1.53s, different autothreshold option were implemented/retracted/re-implemented. Later version allow a stable “don’t reset range” threshold mode. Briefly, more than 8 bits images are thresholded using the 8bits histogram automatic threshold methods. To allow this, the 16 bits 0-65535 range is converted to an 8 bits range (0-256), then the histogram is calculated. Let us consider now a 16 bits .msr file displayed using an Image>Adjust>Brightness&Contrast auto display range (say black is 32768, the ROI’s minimal intensity and white is 32784, the ROI’s maximal intensity). Reset option (the Adjust threshold’s default mode) will convert range extrema to range extrema (0 to 0 and 65535 to 256). Don’t reset will convert the displayed extrema to range extrema (32768 to 0 and 32784 to 256). Then the 8 bit histogram are computed and automatic threshold values calculated. As a result of implementation/retraction/re-implementation cycles, it may be that previous meiosis bar versions were yielding correct axis/foci segmentation before and are no longer correct until this “Use the threshold no-reset option” option is ticked.

- 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 1 (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, not unticking the option will remove most of the axes.

- 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 tools (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 tool. Figure 4.4 shows the parameters used for 2x Erosion and a strength of 3. The pruning strength value (“count” in the options tool 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.

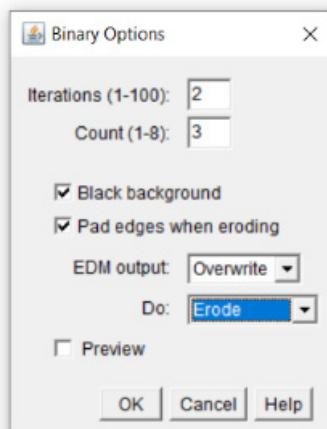


Figure 4.4. An example of how the pruning method “2x Erosion” translates in ImageJ.

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 tool to Iterations 2 and Do: Erode. Two erosions are first applied. Then the process>binary>options tool 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” ROI is then drawn using the processed mask (with the pruning method within brackets).

LIMITATIONS. Intensity values are measured without any subtraction of noise/offset/background. In most widefield cases, whenever the full dynamic range is used (eg. 0-65535 gray levels for 16 bits images), camera/system noise and offset values are negligible. This is not true whenever low intensity, reduced dynamic range images are used. Rough correction of intDen/mean values supposes measurement of an empty area and the above formulas. Mind intDen correction supposes both mean and intDen values should be measured.

The impact of background signal is to be considered as well. As background signal changes across the image, it is recommended to process the images before using the macro.

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

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

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

Figure 4.4. a result file of the Measure axis length macro

STEP 8. 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 4.5). 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 axis length”.

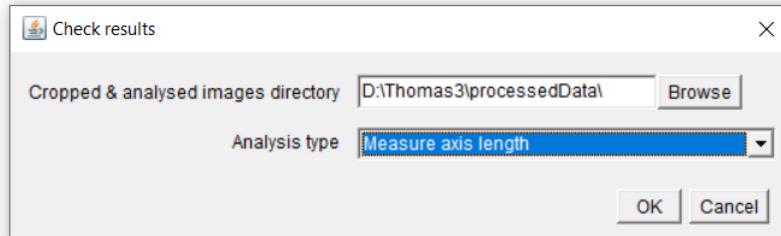


Figure 4.5. The check results menu

The first image analysed will be opened together with its companion lengths_RoiSet_[name].zip file ROI set, as in fig. 4.6. The ROI Manager contains several ROIs, which are associated with ROI groups if further analyses should be performed by the user.

- The reference ROI, as set in **STEP 2**. This is the ROI used for further identifications (ie. axes). The ROI index and group of the reference ROI should be 0 and 1 respectively.
- The raw whole axis (as obtained in **STEP 3**) is the initial ROI before pruning processing. Its ROI group is 3.
- The default pruning method is “None”, if this is not changed in the advanced user parameters menu (**OPTIONAL STEP B**), raw whole axis and whole axis are identical. In figure **4.6**, the preset “2x Erosion” pruning method was chosen. Its ROI group is 5.
- The corresponding whole axis skeleton ROI (ROI group 9).

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

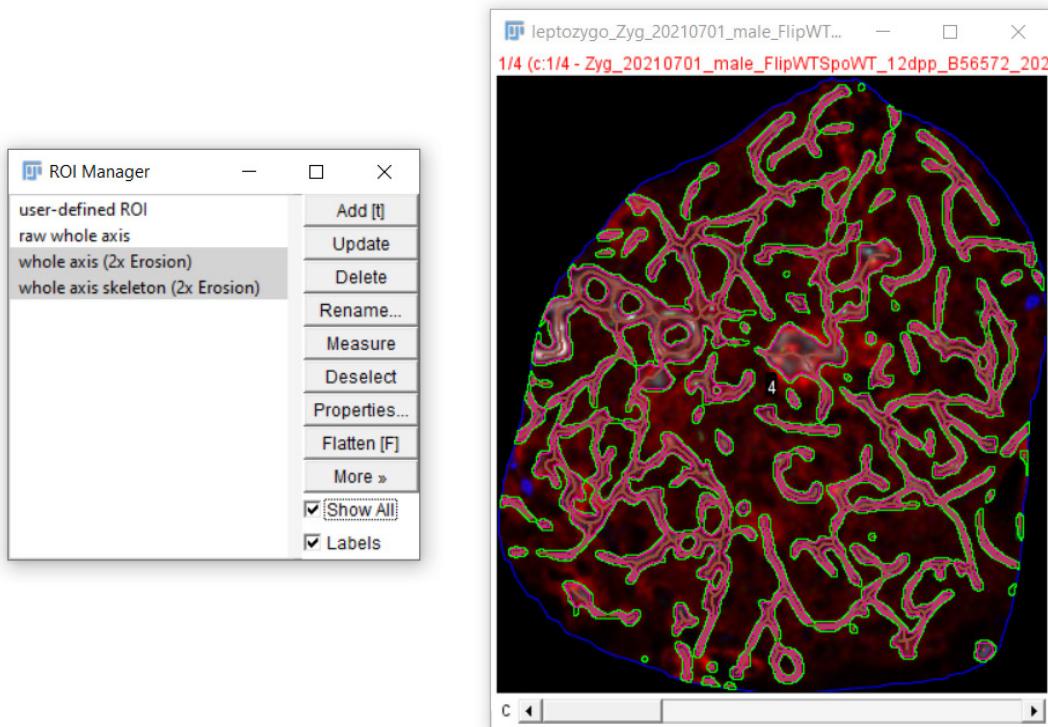


Fig. 4.6. The resulting ROIs produced by the Measure Axis Length macro.

MEASURE SYNAPSE LENGTH

The algorithm will i) locate the whole axis (based on some synaptonemal complex staining), ii) produce a binary mask out of it, iii) process the mask to reduce the number of unwanted axis “branches” (referred as pruning process), iv) measure the total length of the axis. Then these steps will be used to identify the synapsed/non-synapsed axis. Some additional intensity measurements are available, with respect to the axis type (whole, synapsed/unsynapsed). The macro generates ROIs associated with ROIGroups.

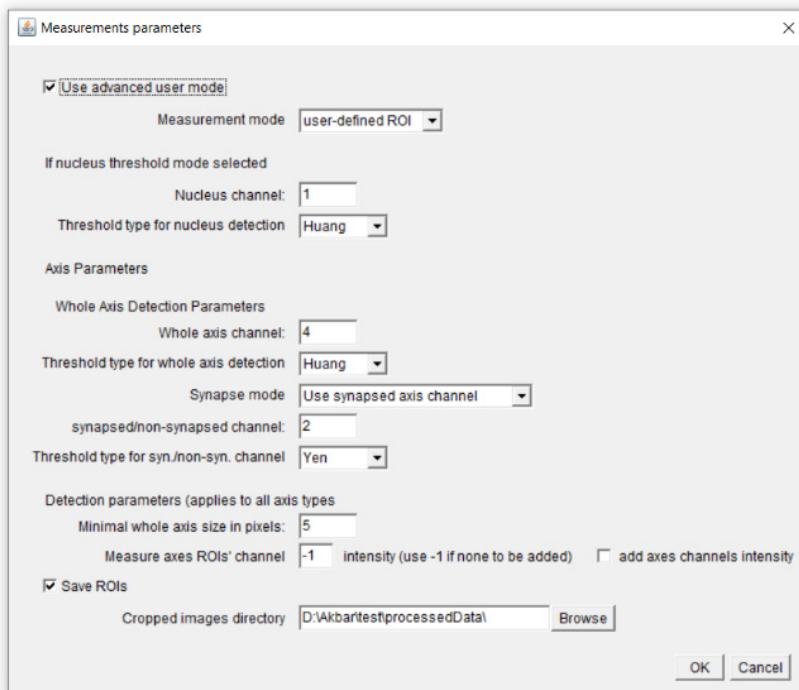


Figure 5.1. The Measure Synapse Length menu.

→ **OPTIONAL STEP A.** Set the output measurements options (see optional step description below).

STEP 1. It is recommended to use the advanced user mode whenever noisy images are used (such as those from STED setups). Tick the “smooth original images” option in the next “Advanced user parameters” menu that pops up upon clicking OK at **STEP 7**.

STEP 2. Set the ROI that will be used for further measurements. If the “user-defined ROI” is selected, then the companion .roi file generated during the stage-cropping step will be used. If this file is not found, some error message is displayed. The other possibility is to use an intensity threshold. The macro refers to “nucleus” threshold as most of the time, the channel used for the threshold is based on some nucleus staining. This can be however used for any other staining. If using this “nucleus threshold” mode, set the channel used for threshold (the first channel is channel 1), then select the adequate threshold algorithm. The list can be expanded to all available ImageJ built-in automatic thresholds by clicking on “Set measurements parameters” and tick the display the full list of threshold methods options (figure 5.3). The user-defined or nucleus ROIs is referred as “reference ROI” below.

Note: Mind that the algorithm suite was initially designed to analyse multiple nucleus-containing images. If no threshold can be used to set the ROI and the image contains a single, cropped nucleus, then set the whole image (Ctrl A) as the ROI when using the cropping buttons.

STEP 3. Set the axes detection parameters. Enter the whole axis channel (mind the first channel is channel #1). Set the appropriate threshold to be used. Additional automatic threshold methods can be added following **OPTIONAL STEP A**. Choose whether the other channel is a marker of a synapsed complex or a non-synapsed complex. This has no consequence in any further step. Choose the corresponding channel and threshold.

For both whole axis and synapsed/non-synapsed axis, the algorithm will first use the respective threshold. If no pixel above threshold is detected, then the analysis aborts and “no axis detected” or no “synapsed/non-synapsed axis” will be displayed in the final table. The threshold is used to generate a temporary “axis threshold” or “synapsed/non-synapsed axis threshold” selection in the ROI Manager.

Then axis elements are detected using the Analyze Particle items and either the nucleus/user-defined ROI (whole axis detection) or the previously detected whole axis for synapsed/.non synapsed axis.

Enter the minimal pixel size of thresholded axis elements. Use this to discard unwanted, small items. Mind raising this value too high might not be appropriate for early stages. Additional raw whole or synapsed/non-synapsed axis detection parameters can be set at **OPTIONAL STEP B**. All within specs particles are identified and added to the ROI Manager. These are then fused (using the combine ROI Manager option) altogether in a temporary ROI. Whenever overlapping axes overlap in such a way that they create a loop, the axes are not identified properly and the hole within the loop is considered as being part of the axis. For whole axis, this is further corrected using the AND option of the ROI Manager, applied to the “axis threshold” ROI and the temporary ROI. Both ROIs are then deleted and a “raw whole axis” ROI is added to the manager. For synapsed/non-synapsed axis, this correction step is used to restrict the raw synapsed axis to the whole axis. Hence, the AND option of the ROI Manager is applied to the “synapsed/non-synapsed axis threshold” ROI, the temporary ROI and the previously identified raw whole axis ROI. A “raw synapsed axis/non-synapsed axis” is added to the manager.

If a pruning method is introduced at optional STEP B, then the raw axis masks will be modified. A skeleton analysis is done, using the skeleton>skeltonize 2D/3D plugin and the input modified processed mask (mind the plugin needs to be installed).

→ **OPTIONAL STEP B:** Use the **ADVANCED USER MODE OPTION** to get better whole axis and synapsed/non-synapsed axis detection. Whenever the option is selected the next menu will pop up upon clicking OK at **STEP 7**.

STEP 4. Set additional intensity measurements channels or ROI. The macro will identify nucleus/user-defined ROI and the axes (whole and synapsed or non-synapsed). Intensities can be measured in these ROIs and for a selection of channels. If the axis/“synapse” channels are to be measured, select “add axis channels intensity”. Would you like to add another channel for intensity measurements within ROI, fill-in the numeric field “Measure axis ROI’s channel intensity). If the field is left with -1 and the “add axis channels intensity” checkbox is not thicked, no measurements will be done. To remove nucleus/user-defined ROI measurements or set intensity measurements types (mean, integrated density) follow **OPTIONAL STEP A**.

STEP5. Set the output items. If the “saveROIs option is ticked, all ROIs generated during the macro will be saved in a companion .zip file. The file is located in a parent controlData folder. Each ROI set is saved using the image’s name and a “synapse-RoiSet” prefix. Whether the

option is used or not, all measurements will be found in a synapse.xls file saved in the folder selected in STEP7.

STEP6. Change/fill-in the cropped-images directory using the browse button.

STEP 7. 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. Set the measurements parameters options. Before using the “Measure synapse length” tool, click on the Set measurements parameters button to set how the skeletons will be measured. The Parameters menu (figure **5.2**) will pop-up.

- Choose whether to display warning messages or not. As those block the batch processing of the macro, consider keeping them hidden.
- The default parameters hide the images during the analysis. To have them displayed, use the set Measurements Parameters of the main meiosis bar. In the parameters window (fig. **5.2**), select “show images while processing files” option.
- The default list of threshold (for nucleus, axis, synapsis) is restricted to preset threshold that were found to be adapted to test images. Would you like to expand the list to the full ImageJ’s automatic thresholds list, select the “Display the full list of threshold methods” options.
- The default (reference ROI, axis, synapse/non-synapsed axis) intensity measurements is IntDen. Would you like to add Mean values or remove IntDen, use the “Show IntDen values” and “show Mean values” options.
- Select the lengths measurement types. Two different routines are available:
 - The first (rough) one takes advantage of the 1 pixel thickness of the skeleton. The total area in pixel of the ROI is measured and converted in um using the image’s calibration. This may yield weird results (as a diagonal pixel will account for 1 – say 0.25um if the pixel width is 0.25um - although it should be considered as $\sqrt{2}$ – and 0.35um).
 - The second (fine) one is a raw measurement of the skeleton. It uses the geometry of the skeletonRoi and takes into account “horizontal, vertical and diagonal pixels”.

To remove fine or rough length measurements, unselect “show rough length estimation” or “show fine length measurements” respectively.

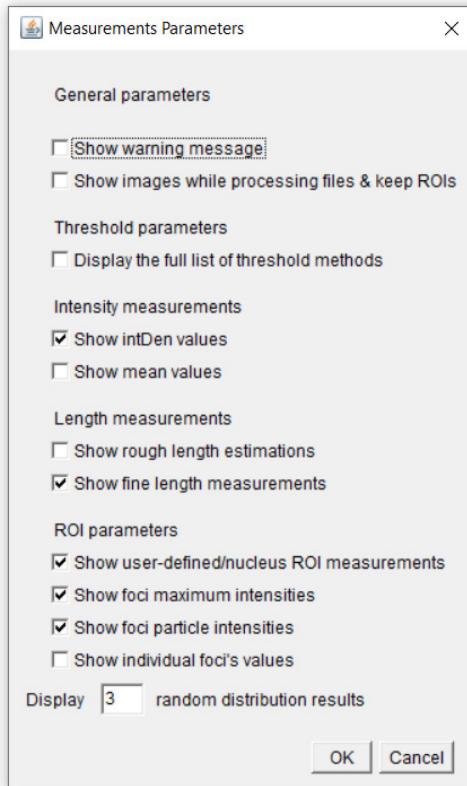


Figure 5.2. The (general) parameters window.

- Intensities is measured in the different ROIs. The default parameters will trigger reference ROI (either user-defined or nucleus ROI) intensity measurements. Unselect “show user-defined nucleus ROI measurements” to get rid of this.
- Ignore the other option (they apply to foci tools).

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 5.3).

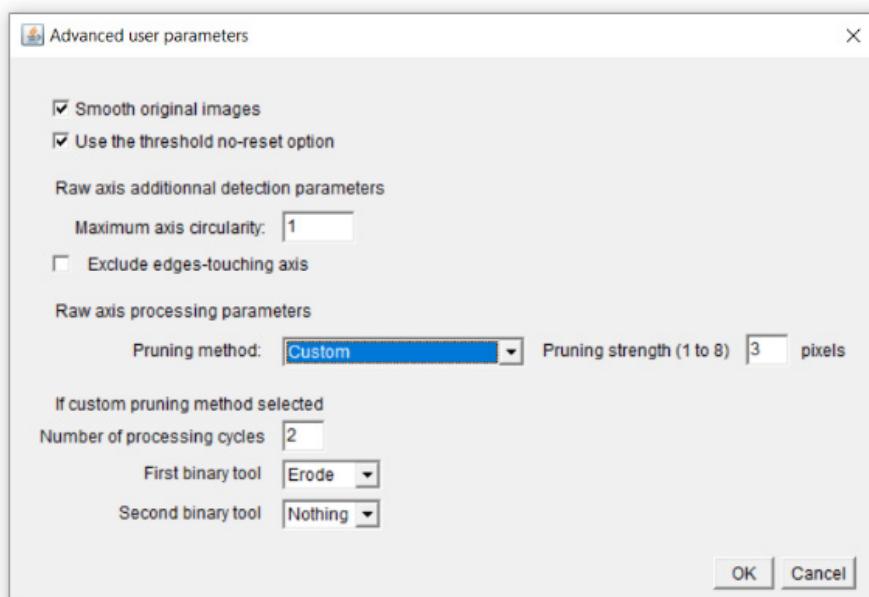


Figure 5.3. The Advanced user parameters menu

- 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.
- When using images with a very high offset value (such as the Abberior .msr files), those very high background intensity pixels together with pixels excluded from analysis (whose intensities are set to 0 in the cropping step), may bias the automatic threshold. From ImageJ 1.52e version to 1.53s, different autothreshold option were implemented/retracted/re-implemented. Later version allow a stable “don’t reset range” threshold mode. Briefly, more than 8 bits images are thresholded using the 8bits histogram automatic threshold methods. To allow this, the 16 bits 0-65535 range is converted to an 8 bits range (0-256), then the histogram is calculated. Let us consider now a 16 bits .msr file displayed using an Image>Adjust>Brightness&Contrast auto display range (say black is 32768, the ROI’s minimal intensity and white is 32784, the ROI’s maximal intensity). Reset option (the Adjust threshold’s default mode) will convert range extrema to range extrema (0 to 0 and 65535 to 256). Don’t reset will convert the displayed extrema to range extrema (32768 to 0 and 32784 to 256). Then the 8 bit histogram are computed and automatic threshold values calculated.

As a result of implementation/retraction/re-implementation cycles, it may be that previous meiosis bar versions were yielding correct axis/foci segmentation before and are no longer correct until this “Use the threshold no-reset option” option is ticked.

- 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 1 (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, not unticking the option will remove most of the axes.

- 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 tools (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 tool. Figure 5.4 shows the parameters used for 2x Erosion and a strength of 3. The pruning strength value (“count” in the options tool 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.

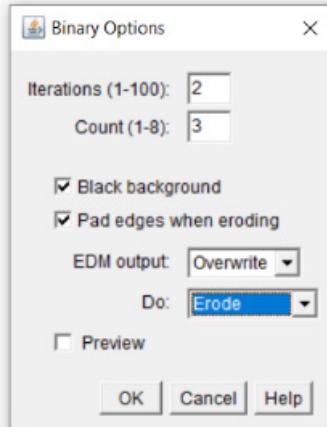


Figure 5.4. An example of how the pruning method “2x Erosion” translates in ImageJ.

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 tool to Iterations 2 and Do: Erode. Two erosions are first applied. Then the process>binary>options tool 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” ROI is then drawn using the processed mask (with the pruning method within brackets).

Type	Image name	Parameter	Value
pachy	pachy1	Smooth original images	no
pachy	pachy2	ROI chosen	user-defined
pachy	pachy3	Axis channel	3
pachy	pachy4	Axis Threshold	Huang
pachy	pachy5	Axis Min size	5
pachy	pachy6	Axis Max Circ.	1.0
pachy	pachy7	Exclude edge-touching axis	no
pachy	pachy8	Measure axis length	yes
pachy	pachy9	Synapsis reference	synapsed
pachy	pachy10	synapsed axis channel	2
pachy	pachy11	Measure axis' and synapsed axis' ROIs intensities	Ch. 4 & axis and synapsed axis channels (Ch.3and Ch.2)
pachy	pachy11	Pruning Mode(s) tested	2x Erosion
zygo	zygo1	Pruning Strength (when used)	3
zygo	zygo2	Images from	D:\...
zygo	zygo3	ROI saved	yes
zygo	zygo4	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 5.5. a result file of the synapse length macro

STEP8. 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 5.6). 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”.

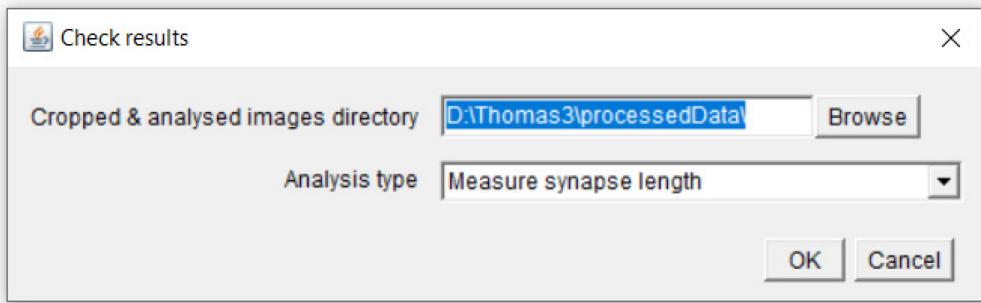


Figure 5.6. The check results menu

The first image analysed will be opened together with its companion `synapse_RoiSet_[name].zip` file ROI set, as in fig. 5.7. 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) 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

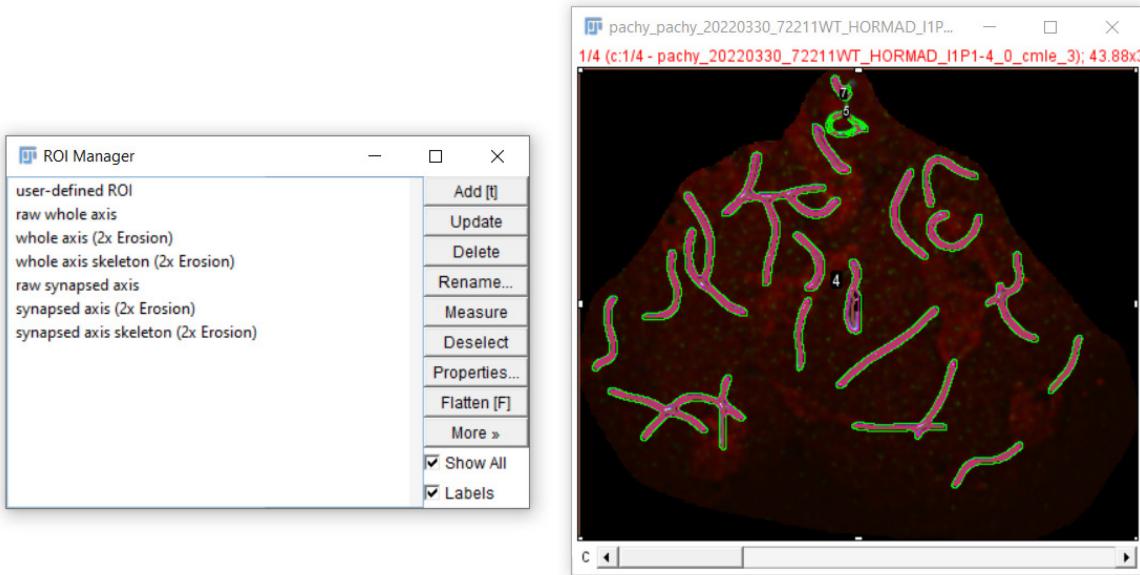


Fig. 5.7. The resulting ROIs produced by the Measure Synapse Length macro.

COUNT AXIS & OFF-AXIS FOCI

The “count axis and off-axis foci in one channel” algorithm will i) locate the axis (based on some synaptonemal complex staining), ii) identify foci, iii) count the number of off-axis and on-axis (referred as “axis”) foci. Additionally, the total length of the axis can be evaluated. The macro generates ROIs that are associated with several ROIGroups.

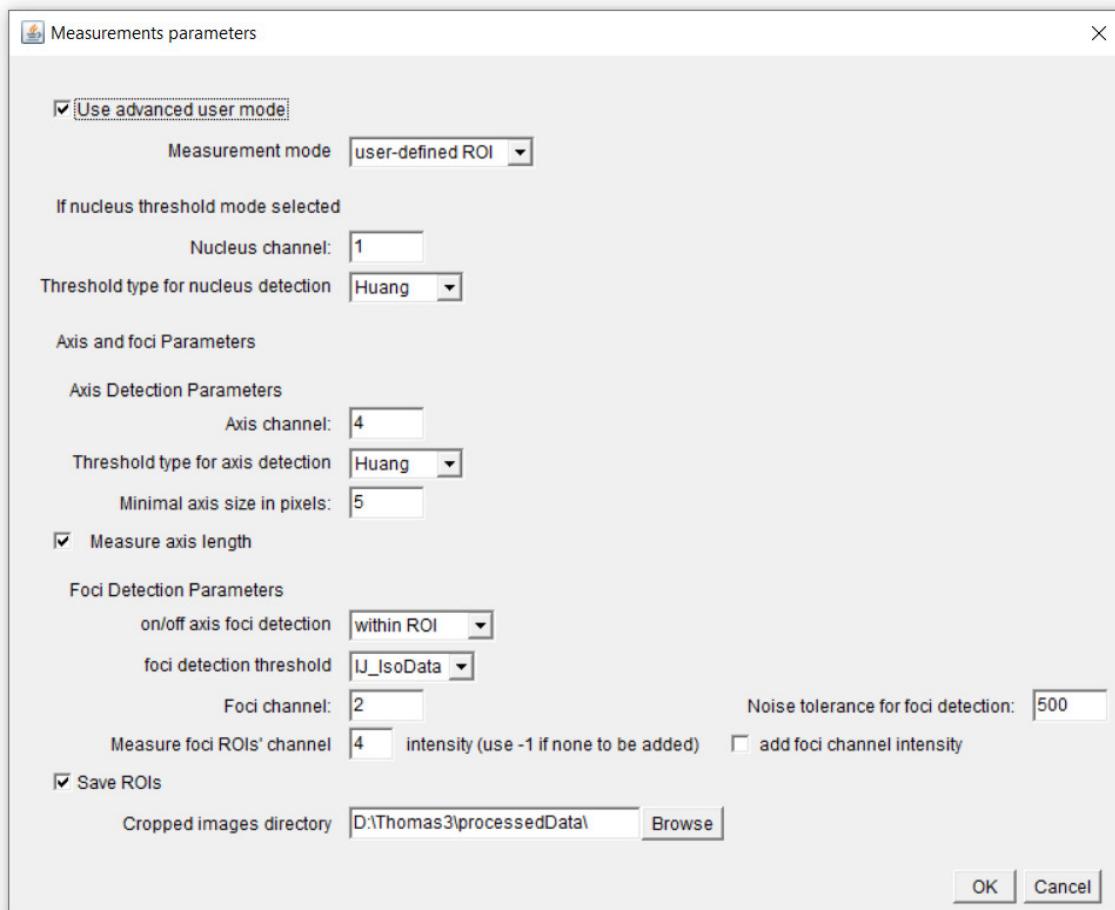


Fig. 6.1. The “count axis and off-axis foci in one channel menu

→ **OPTIONAL STEP A.** Set the output measurements options (see optional step description below).

STEP 1. It is recommended to use the advanced user mode whenever noisy images are used (such as those from STED setups). Tick the “smooth original images” option in the next “Advanced user parameters” menu that pops up upon clicking OK at **STEP 7**.

STEP 2. Select the original region of interest to be used for the measurements. As in the previous routines, the user may either select the user-defined ROI (ie. the .roi that was generated upon image cropping), or use a thresholded nucleus staining. This user-defined or nucleus ROIs is referred as “reference ROI” below.

STEP 3. Set the axis detection parameters. Enter the axis channel (mind the first channel is channel #1). Set the appropriate threshold to be used. The algorithm will first use this threshold. If no pixel above threshold is detected, then the analysis aborts and “no axis detected” will be displayed in the final table. The threshold is used to generate a temporary “axis threshold” selection in the ROI Manager. Then axis elements are detected using the Analyze Particle items and the nucleus/user-defined ROI.

Enter the minimal pixel size of thresholded axis elements. Use this to discard unwanted, small items. Mind raising this value too high might not be appropriate for early stages. Use **OPTIONAL STEP B** to set additional exclusion parameters. All within specs particles are identified and added to the ROI Manager. These are then fused (using the combine ROI Manager option) altogether in a temporary ROI. Whenever overlapping axes overlap in such a way that they create a loop, the axes are not identified properly and the hole within the loop is considered as being part of the axis. This is further corrected using the AND option of the ROI Manager, applied to the “axis threshold” ROI and the temporary ROI. Both ROIs are then deleted and a “raw whole axis” ROI is added to the manager. The raw axis ROI can be further processed/refined using **OPTIONAL STEP B**’s pruning method. A “whole axis” ROI is then drawn (with the pruning method within brackets if any).

Whenever “Measure axis length” checkbox is ticked, a skeleton analysis is done, using the skeleton>skeletonize 2D/3D plugin and the input modified processed mask. Mind the plugin has to be installed. A skeleton ROI is added to the manager. Different length measurements options can be set using **OPTIONAL STEP A**.

STEP 4. Enter foci detection parameters. The foci channel is selected and foci are detected using the find maxima algorithm. The prominence value used is entered in the “noise tolerance for foci detection” box of the menu. Prominence was formerly referred to as noise, although there are differences with Maxima Finder menu- aka. “Find maxima” function- versions before and after ImageJ 1.52. Mind that same-color twin foci may be detected as single foci. The “strict” option of the “Find maxima” function is used (only available from ImageJ 1.52). The macro takes full advantage of all possibilities offered by this version of Maxima Finder.

Three modes are proposed, that all use the nucleus/user-defined ROI as input ROI:

- Maxima are identified without any threshold (foci detection threshold “None”). Foci above prominence value are detected as maxima. The corresponding particles are identified using the “maxima within tolerance” output option of the Find Maxima tool.
- Maxima are identified using either a Huang or an IJ_IsoData automatic threshold. Foci detection is restricted within the thresholded area and foci, within this area, above prominence value are detected as maxima (with “above lower threshold option” of the Find Maxima tool used). The corresponding particles are identified using the “segmented particles” output option. If needed this two automatic thresholds list can be further expanded to 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).

For the purpose of axis/off-axis discrimination, two modes are proposed.

- 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 function 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 function is run. The same find maxima operations is made as with the first duplicate. This gives off-axis foci.

This method has the following bias:

- If the image’s background signal is high and small pieces of axis are detected, background foci channel signal over 0 may be detected with the find maxima algorithm.

This is also true whenever the file format "zero" (camera offset, vendor's choice) is high. Discard small axis pieces by raising the min axis pixel size or use the "within ROI" option.

- Whenever a foci lies at the very edge of the detected axis, it will be split into two parts (one in the axis mask, the other "half" in the off-axis mask) and may be detected as two foci (one off-axis and one on-axis foci)

- The "within ROI" (from v2.01):

Another option is to detect foci within the reference ROI (referred as total_foci (maxima), ROIGroup 11) 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. Maxima that are not within the axis ROI are off-axis_foci (maxima) and belong to ROIGroup15.

Foci maxima are detected and sorted as axis/off-axis foci with either modes (using masks or within ROI).

Then, particles are identified starting from the nucleus/user-defined ROI and using the above-mentioned Find Maxima options (referred to as total particles, ROIGroup 101).

Note: an axis foci will have its foci maximum within the axis ROI, while its associated foci particle may spread in the off-axis ROI as well.

This substep is quite time consuming. To skip this, deselect the "show foci particle intensities" option of the general parameters menu (see **OPTIONAL STEP A**). Total particles ROI is split and each individual particle is compared to the corresponding axis/off-axis maxima. Whenever a split particle contains an axis maxima, then it is renamed as an axis_foci_ID (particle) (ROIGroup 6). If measurements parameters (see **OPTIONAL STEP A**) "show foci maximum intensities" is used together with "show individual foci's value ", then the corresponding maximum point ROI is created (axis_foci_ID (maximum), ROIGroup 4). Both maximum and particle share the same ID. If the particle does not contain any axis maximum, it is then compared to off-axis maxima. If a maximum of the off-axis_foci (maxima) ROI fits into the particle, then the particle is renamed as a off-axis_foci_ID (particle) (ROIGroup 10) and, if created, the associated off-axis_foci_ID (maximum) ROI is associated to ROIGroup 8. If the particle does not contain any maximum (this happens), then no ROI group is attributed (ie. left to default 255 value) and the particle is purged from the ROI manager at the end of the process. These can be purged from the manager using the "remove individual foci's ROI from RoiManager" general option (fig **6.3**).

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 6.2. ROIGroups used for the different types of ROI.

STEP 5. Set additional intensity measurements channels or ROI. The macro will identify nucleus/user-defined ROI and the foci (axis and off-axis) as maxima and particles. Intensities can be measured in these ROIs and for a selection of channels. If the foci channel should be measured as well, select “add foci channel intensity”. Would you like to add another channel for intensity measurements within ROI, fill-in the numeric field “Measure foci ROI’s channel intensity). If the field is left with -1 and the “add foci channel intensity” checkbox is not thicked, no measurements will be done.

To show/hide either the maxima or particle foci ROI, select/unselect the “show foci maximum intensities” and “show foci particle intensities” checkboxes of the general parameters menu (see [OPTIONAL STEP A](#)). For global particle measurements (as displayed in the foci.xls table), all individual particles of interest are fused together and a ROI is created (for instance axis_foci (particles), ROIGroup 103), then the corresponding values (ie. IntDen or Mean) are calculated across the whole fused ROI. For global maxima calculation, the corresponding ROI (say axis-foci (maxima)) is measured using the getRawStatistics(nPixels, mean, min, max, std, histogram) command. The number of maxima (nPixels) is multiplied by mean to derive the IntDen value.

STEP 6. 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 [6.6](#)). Individual foci values are stored in a individualFoci.xls file (see figure [6.7](#)). If individual foci/off-axis foci maxima or particles should be removed, select the “remove individual foci’s ROI from ROI Manager” checkbox in the general parameters window (see [OPTIONAL STEP A](#)).

OPTIONAL STEP A. Set the measurements parameters options. Before using the “Count axis/off-axis foci in two channel and colocalize” tool, click on the Set measurements

parameters button to set how the skeletons will be measured. The Parameters menu (figure 6.3) will pop-up.

- Choose whether to display warning messages or not. As those block the batch processing of the macro, consider keeping them hidden.
- The default parameters hide the images during the analysis. To have them displayed, use the set Measurements Parameters of the main meiosis bar. In the parameters window (fig. 6.3), select “show images while processing files” option.
- The default list of threshold (for nucleus, axis, synapsis or foci) is restricted to preset threshold that were found to be adapted to test images. Would you like to expand the list to the full ImageJ’s automatic thresholds list, select the “Display the full list of threshold methods” options.
- The default intensity measurements is IntDen. Would you like to add Mean values or remove IntDen, use the “Show IntDen values” and “show Mean values” options.
- Whenever the “Measure axis length” option is selected, select the lengths measurement types. Two different routines are available:
 - The first (rough) one takes advantage of the 1 pixel thickness of the skeleton. The total area in pixel of the ROI is measured and converted in um using the image’s calibration. This may yield weird results (as a diagonal pixel will account for 1 – say 0.25um if the pixel width is 0.25um - although it should be considered as $\sqrt{2}$ – and 0.35um).
 - The second (fine) one is a raw measurement of the skeleton. It uses the geometry of the skeletonRoi and takes into account “horizontal, vertical and diagonal pixels”.

To remove fine or rough length measurements, unselect “show rough length estimation” or “show fine length measurements” respectively.

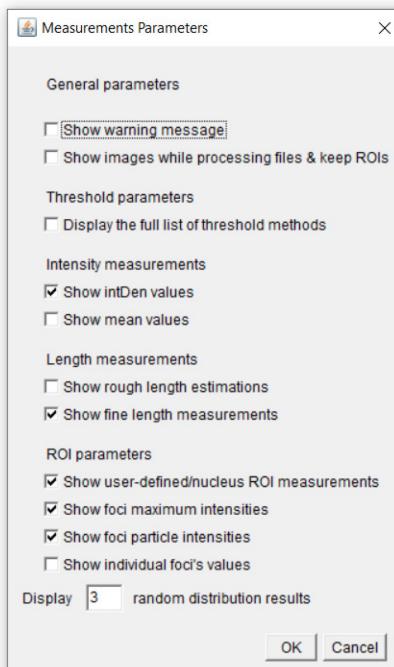


Figure 6.3. The (general) parameters window.

- Intensities is measured in the different ROIs. The default parameters will trigger reference ROI (either user-defined or nucleus ROI) intensity measurements. Unselect “show user-defined nucleus ROI measurements” to get rid of this. Foci ROIs can be measured both in maxima/individual maximum or particles/individual particle. Use the corresponding tickboxes to restrict analysis.
- Ignore the last option that applies to two color foci only.

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 6.4).

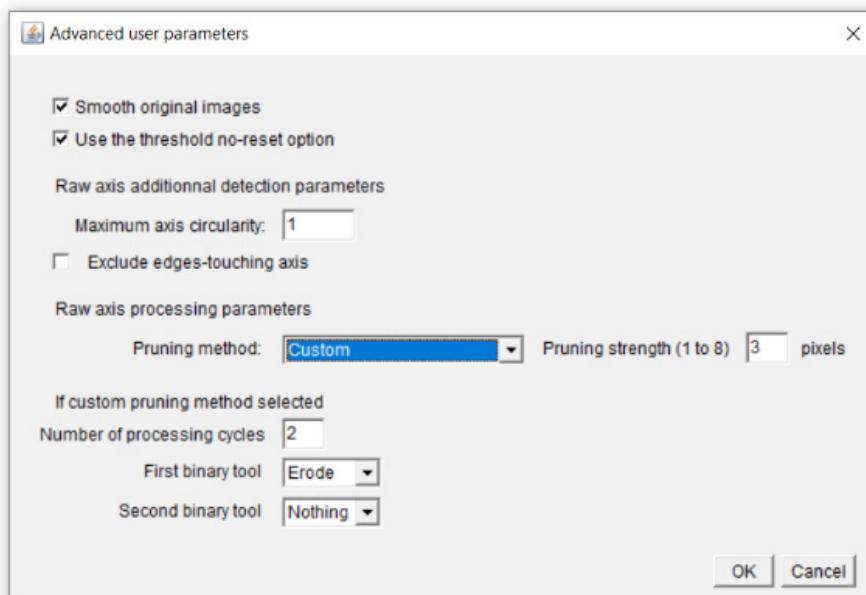


Figure 6.4. The Advanced user parameters menu

- 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.
- When using images with a very high offset value (such as the Abberior .msr files), those very high background intensity pixels together with pixels excluded from analysis (whose intensities are set to 0 in the cropping step), may bias the automatic threshold. From ImageJ 1.52e version to 1.53s, different autothreshold option were implemented/retracted/re-implemented. Later version allow a stable “don’t reset range” threshold mode. Briefly, more than 8 bits images are thresholded using the 8bits histogram automatic threshold methods. To allow this, the 16 bits 0-65535 range is converted to an 8 bits range (0-256), then the histogram is calculated. Let us consider now a 16 bits .msr file displayed using an Image>Adjust>Brightness&Contrast auto display range (say black is 32768, the ROI’s minimal intensity and white is 32784, the ROI’s maximal intensity). Reset option (the Adjust threshold’s default mode) will convert range extrema to range extrema (0 to 0 and 65535 to 256). Don’t reset will convert the displayed extrema to range extrema (32768 to 0 and 32784 to 256). Then the 8 bit histogram are computed and automatic threshold values calculated. As a result of implementation/retraction/re-implementation cycles, it may be that previous meiosis bar versions were yielding correct axis/foci segmentation before and are no longer correct until this “Use the threshold no-reset option” option is ticked.

- 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 1 (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, not unticking the option will remove most of the axes.

- 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 tools (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 tool. Figure 6.5 shows the parameters used for 2x Erosion and a strength of 3. The pruning strength value (“count” in the options tool 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.

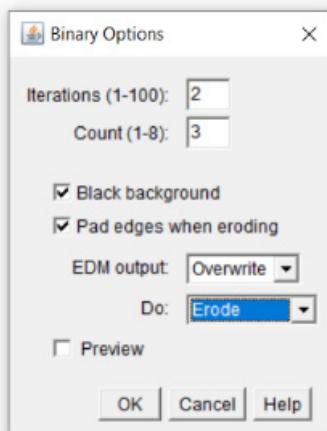


Figure 6.5. An example of how the pruning method “2x Erosion” translates in ImageJ.

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 tool to Iterations 2 and Do: Erode. Two erosions are first applied. Then the process>binary>options tool 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” ROI is then drawn using the processed mask (with the pruning method within brackets).

Type	Image name	Parameter	Value
zygo	zygo1	Smooth original images	no
zygo	zygo2	ROI chosen	user-defined
zygo	zygo3	Axis channel	4
zygo	zygo4	Axis Threshold	Huang
zygo	zygo5	Axis Min size	5
zygo	zygo6	Axis Max Circ.	1.0
zygo	zygo7	Exclude edge-touching axis	no
zygo	zygo8	Measure axis length	no
zygo	zygo9	Pruning Mode(s) tested	[Erosion & Dilation]
zygo	zygo10	Pruning Strength (when used)	3
zygo	zygo11	Foci detection method	within ROI
zygo	zygo12	Foci Detection Threshold	IJ_IsoData
zygo	zygo13	Foci channel	2
zygo	zygo14	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	1.5399

Figure 6.6. 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 (foci's maximum)	Ch.3 intDen (foci's 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 6.7. a subset of an individualFoci result file of the Count axis and off-axis foci in one channel macro

STEP7. 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” window pops up (figure 6.8). 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 “Count axis and off-axis foci in one channel”.

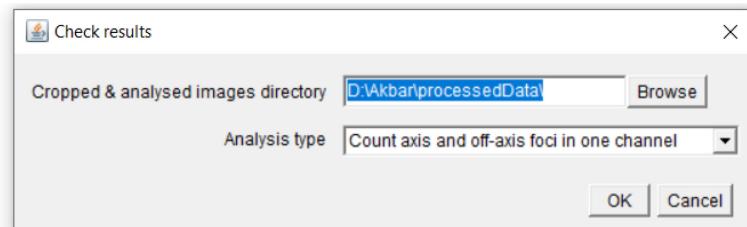


Figure 6.8. The check results menu

The first image analysed will be opened together with its companion foci_RoiSet_[name].zip file ROI set, as in fig. 6.9. 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). It's ROIGroup is 1 and index should be 0.
- The raw whole axis is the initial ROI (ROIGroup 3), that is further processed in whole axis (2x Erosion) (as the preset 2x Erosion pruning method was used). It belongs to ROIGroup5. From this is derived the Off-Axis ROI (XOR of reference ROI and whole axis ROI). The off-axis ROI belongs to ROIGroup7. The axis skeleton ROI is from ROIGroup9.
- The total_foci maxima (as the within ROI mode was used at STEP 4 (ROIGroup 11) There would be no total foci maxima ROI if the within mask mode was used. These are further split into axis_foci maxima (RoiGroup 13) and off-axis_foci maxima (RoiGroup 15).
- Foci particles are identified and sorted according to whether they sit within the whole axis ROI or not into axis_foci (particles) ROI (RoiGroup 113) and off-axis_foci (particles) ROI (RoiGroup 115).
- Individual foci particles (and corresponding maxima) are shown (see figure 6.2 for ROIGroups), as in figure 6.9b, whenever the “show individual foci’s values” option is used together with the “show foci particle intensities”.

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

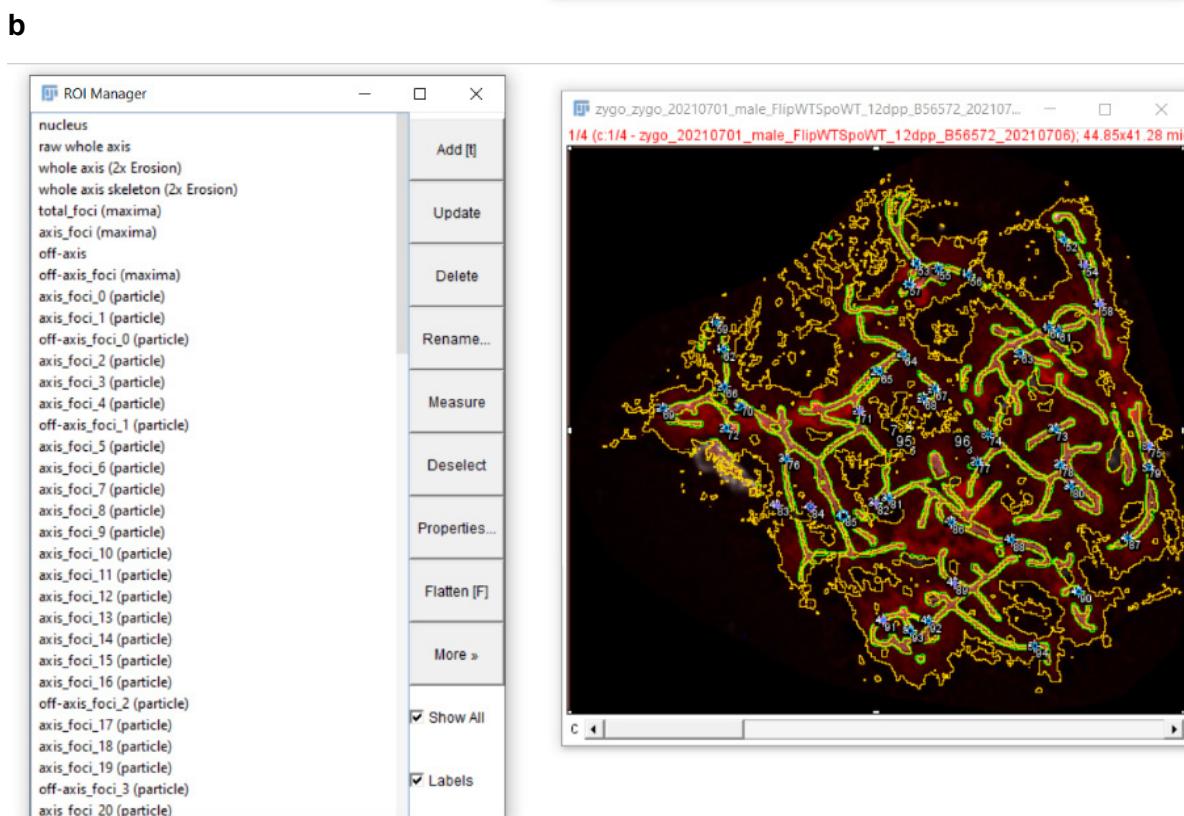
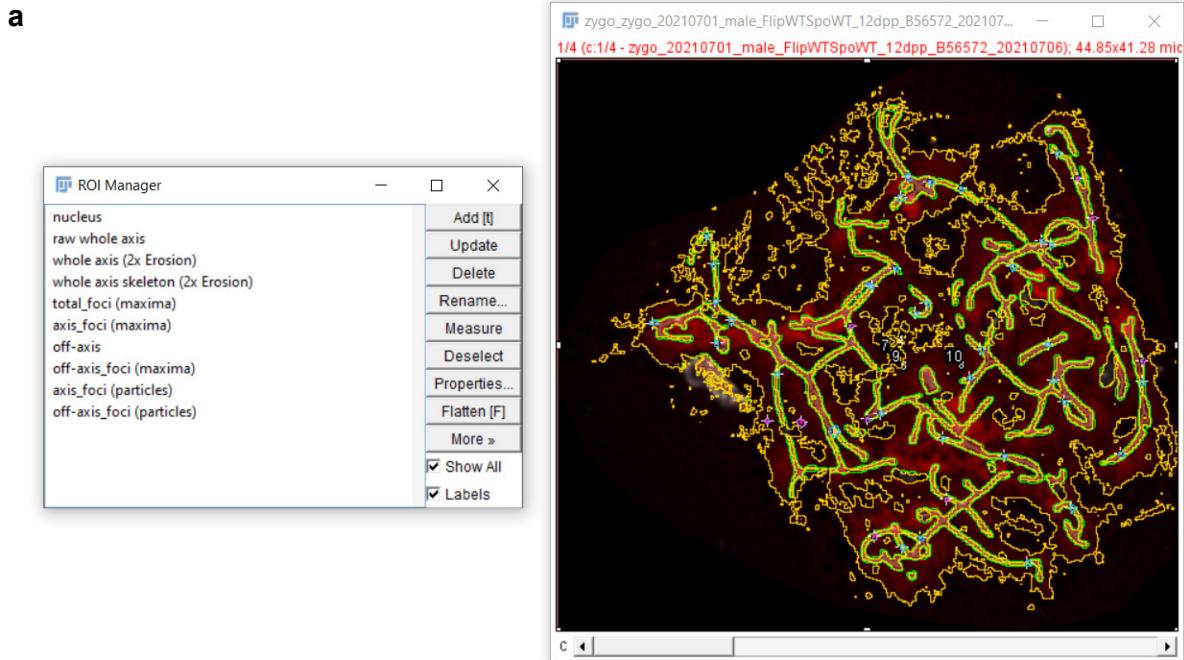


Figure 6.9. Control ROIs as generated by the Count axis and off-axis foci in one channel macro. a: show individual foci's values and show foci particle intensities options not selected. b:both options selected.

COUNT AXIS/OFF-AXIS FOCI & COLOCALIZE

The “count axis/off-axis foci in two channels and colocalize” algorithm will i) locate the axis (based on some synaptonemal complex staining), ii) identify axis foci in two channels, iii) measure colocalisation as described in Lachmanovich 2003 Journal of Microscopy, Vol. 212, Pt 2 November 2003, pp. 122–131. Additionally, the macro can create random equivalent images and check whether the observed colocalised foci number could be randomly obtained.

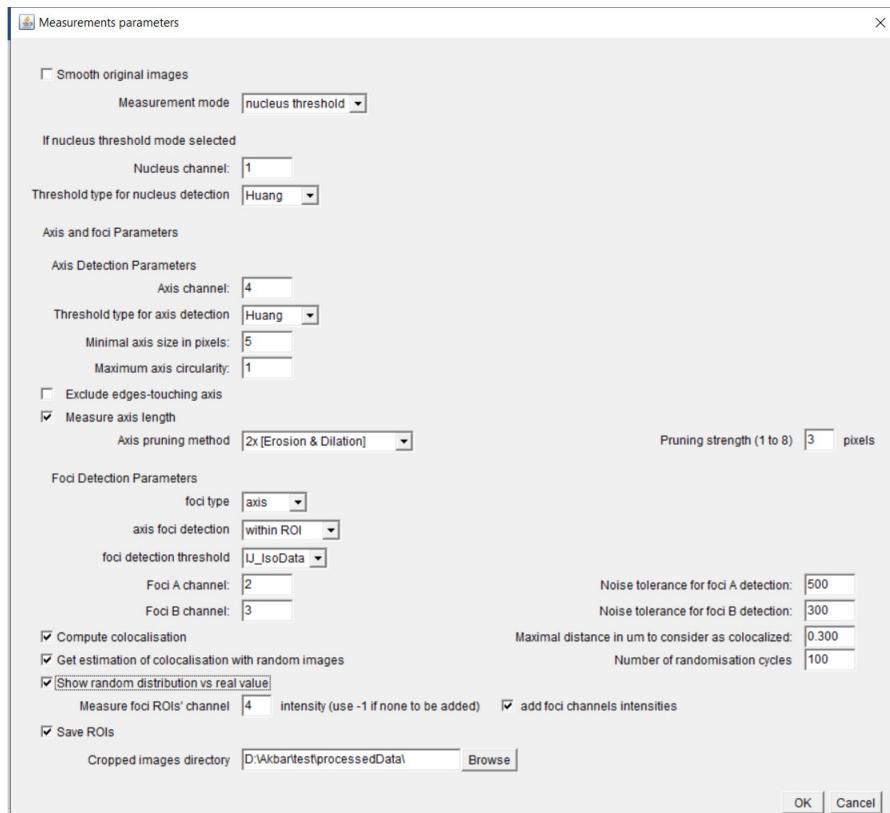


Fig. 7.1. The “count axis/off-axis foci in two channels and colocalize” menu

→ **OPTIONAL STEP A.** Set the output measurements options (see optional step description below).

STEP 1. It is recommended to use the advanced user mode whenever noisy images are used (such as those from STED setups). Tick the “smooth original images” option in the next “Advanced user parameters” menu that pops up upon clicking OK at **STEP 10**.

STEP2. Select the original region of interest to be used for the measurements. As in the previous routines, the user may either select the user-defined ROI (ie. the .roi that was generated upon image cropping), or use a thresholded nucleus staining (figure 7.1). This user-defined or nucleus ROIs are referred as “reference ROI” below.

STEP3. Set the axis detection parameters. Enter the axis channel (mind the first channel is channel #1). Set the appropriate threshold to be used. The algorithm will first use this threshold. If no pixel above threshold is detected, then the analysis aborts and “no axis detected” will be displayed in the final table. The threshold is used to generate a temporary “axis threshold” selection in the ROI Manager. Then axis elements are detected using the Analyze Particle item. Enter the minimal pixel size of thresholded axis elements. Use this to discard unwanted, small items. Mind raising this value too high might not be appropriate for early stages. Additional detection parameters may be used (see **OPTIONAL STEP B**). All within specs

particles are identified and added to the ROI Manager. These are then fused (using the combine ROI Manager option) altogether in a temporary ROI. Whenever overlapping axes overlap in such a way that they create a loop, the axes are not identified properly and the hole within the loop is considered as being part of the axis. This is further corrected using the AND option of the ROI Manager, applied to the “axis threshold” ROI and the temporary ROI. Both ROIs are then deleted and an “axis” ROI is added to the manager. The raw axis ROI can be further processed/refined using **OPTIONAL STEP B**’s pruning method. A “whole axis” ROI is then drawn (with the pruning method within brackets if any).

Whenever “Measure axis length” checkbox is ticked, a skeleton analysis is done, using the skeleton>keletonize 2D/3D plugin and the input modified processed mask. Mind the plugin has to be installed. A skeleton ROI is added to the manager. Different length measurements options can be set using **OPTIONAL STEP A**.

STEP 4. Enter foci detection parameters. The foci channels A and B are selected and foci of interest are detected using the find maxima algorithm. The prominence value used is entered in the “noise tolerance for foci detection” box of the menu. Prominence was formerly referred to as noise (although there are differences with Maxima Finder menu- aka. “Find maxima” function- versions before and after ImageJ 1.52). Mind that same-color twin foci may be detected as single foci. The “strict” option of the “Find maxima” function is used (only available from ImageJ 1.52). The macro takes full advantage of all possibilities offered by this version of Maxima Finder.

3 modes are proposed, that all use the nucleus/user-defined ROI as input ROI:

- Maxima are identified without any threshold (foci detection threshold “none”). Foci above prominence value are detected as maxima. The corresponding particles are identified using the “maxima within tolerance” output option of the Find Maxima tool.
- Maxima are identified using either a Huang or an IJ_IsoData automatic threshold. Foci detection is restricted within the thresholded area and foci, within this area, above prominence value are detected as maxima (with “above lower threshold option” of the Find Maxima tool used). The corresponding particles are identified using the “segmented particles” output option.

For the purpose of axis/off-axis discrimination, two modes are proposed.

- 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 function 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 function is run. The same find maxima operations is made as with the first duplicate. This gives off-axis foci.

This method has the following bias:

- If the image’s background signal is high and small pieces of axis are detected, background foci channel signal over 0 may be detected with the find maxima algorithm. This is also true whenever the file format “zero” (camera offset, vendor’s choice) is high. Discard small axis pieces by raising the min axis pixel size or use the “within ROI” option.

- Whenever a foci lies at the very edge of the detected axis, it will be split into two parts (one in the axis mask, the other “half” in the off-axis mask) and may be detected as two foci (one off-axis and one on-axis foci)

- The “within ROI” (from v2.01):

Another option is to detect foci within the reference ROI (referred as total_foci (maxima)) then keep maxima that are located within the axis ROI (axis_foci (maxima)). This method is implemented in a more time-consuming way but is more elegant and is now the default method. Maxima that are not within the axis ROI are off-axis_foci (maxima).

- Set the foci type. Only foci of interest (as indicated in the foci type field figure 7.1, ie. either axis or off-axis) will be kept (and those of the other type will be discarded from analysis/roiManager). Foci maxima are detected and stored in the RoiManager (e.g axis_fociA (maxima)). The different ROIs generated are associated with various ROIGroups (figure 7.2 and 7.3) for the purpose of further, downstream, analysis by the user.

STEP 5. If using the colocalisation option (compute colocalisation checkbox selected), a mask of the axis/off-axis foci is generated (provided some maxima in the corresponding channel and axis/off-axis ROI were found) which is further used to compute a 32-bits euclidean distance map. This can be manually done using Process>FindMaxima tool and the single points output, then process>Binary>Options and select 32-bits as EDM (Euclidean Distance Map) output and then run process>Binary>Distance Map. A threshold is then applied to the EDM Map using a converted colocalisation distance. The “maximal distance in um to be considered as colocalised” is converted into pixels and the converted value used as a threshold. A selection is drawn using the thresholded area and stored in the RoiManager as “colocalised_area_[type]_foci[letter]” (ROIGroups 31 and 33 for axis and off-axis fociA respectively and 37 and 39 for axis and off-axis fociB respectively). The complementary ROI is drawn for not colocalised fociA/B areas (see figure 7.2 and 7.3 for ROIGroups). When a given foci maximum (as identified in **STEP 4**) of the other channel (say A if coloc area B) is within this ROI, it will be considered as colocalised. If the default -1 value is kept in the “maximal distance in um to be considered as colocalised”, no colocalised area ROI is generated. As in Lachmanovich 2003, it is recommended to use as colocalisation distance threshold the minimal resolution distance (typically 230-250nm for a 1.4 NA lens and a widefield setup). See Pawley JB Handbook of Biological Confocal Microscopy, Springer 2006 for instance for other configurations). The rationale of using a minimal resolution distance value as a threshold is quite straightforward: would foci A and B be from the same channel, if their separating distance is below the minimal resolution distance, they could not be seen as different.

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 7.2. 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 7.3. ROIGroups associated with ROI generated by the macro in the off-axis mode.

Whenever some foci of interest are found in both channels A and B, maxima of foci of interest of a given channel (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”). Maxima that fit into the coloc area ROI are counted and used to create a new ROI (in this case colocalised_axis_fociA (maxima)).

STEP6. Consider checking whether the observed colocalised pixels in both channels could be randomly obtained. Select “get colocalisation with random images”. This option will not run if the main “comput colocalisation” is not selected. Briefly, the observed foci number in one channel are shuffled within the whole axis ROI. randomly localised foci of the first random images (for both channel randomisations) are saved within the ROI Manager. The number of random images can be changed using the general parameters menu (see **OPTIONAL STEP A**).

Each random foci distribution within the axis ROI is then compared with the observed foci localisation in the other channel using the corresponding Euclidean distance Map ROI (eg. random_axis_fociA_0 (maxima) with colocalised area axis_fociB. Enter the number of random images to be generated in “number of randomisation cycles”. The average number of randomly colocalised foci is calculated for both combinations. It is recommended using a number of cycles >30 so that chances are high a normal distribution of randomly colocalised foci is obtained.

When the average random colocalised foci is 0, no further analysis is performed. Otherwise, the distribution of random colocalised foci number for all random images is plotted. This plot can be saved (see fig 7.4 for an example) if the “show random distribution versus real value option is selected.” Although this plot is saved as a .tif file in a parent controlData folder, they can be opened using imageJ and the list can be extracted (click on list). The frequency of number of randomly colocalised foci are indicated as grey hollow dots. A Gaussian fit is applied (blue line). The actually observed, real number of colocalised foci is indicated with a red line.

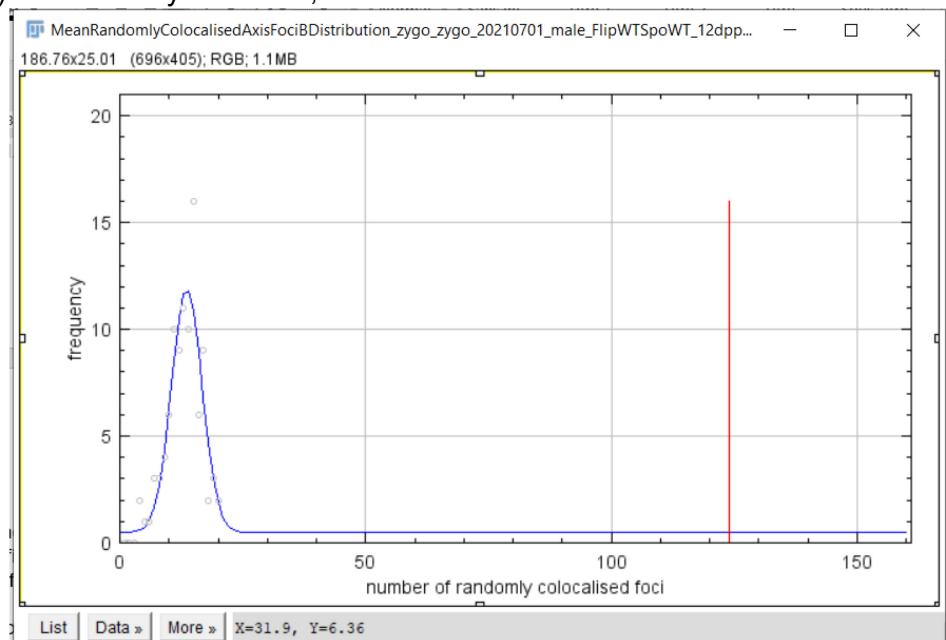


Fig. 7.4. randomly colocalised foci number distribution across all randomly generated images

The mean and standard deviation values of the randomly colocalised foci number are used for p value analysis. If $\text{mean} - 3 * \text{stdDev} < 0$ (ie. the blue Gaussian is clipped, as in figure 7.5), the p value is displayed with an asterisk (and a comment “*: take p value with caution is displayed in the “comment” column of the 2foci.xls result file).

The p value is:

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

Where erf is the Gaussian error function:

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

For the purpose of erf calculation, the Horner's method is used.

The pvalue indicates the proportion of the Gaussian below the observed, real colocalised foci number. When close to 1, this means the observed colocalised foci number is unlikely to be obtained with random foci distribution within the ROI of interest (axis or off-axis).

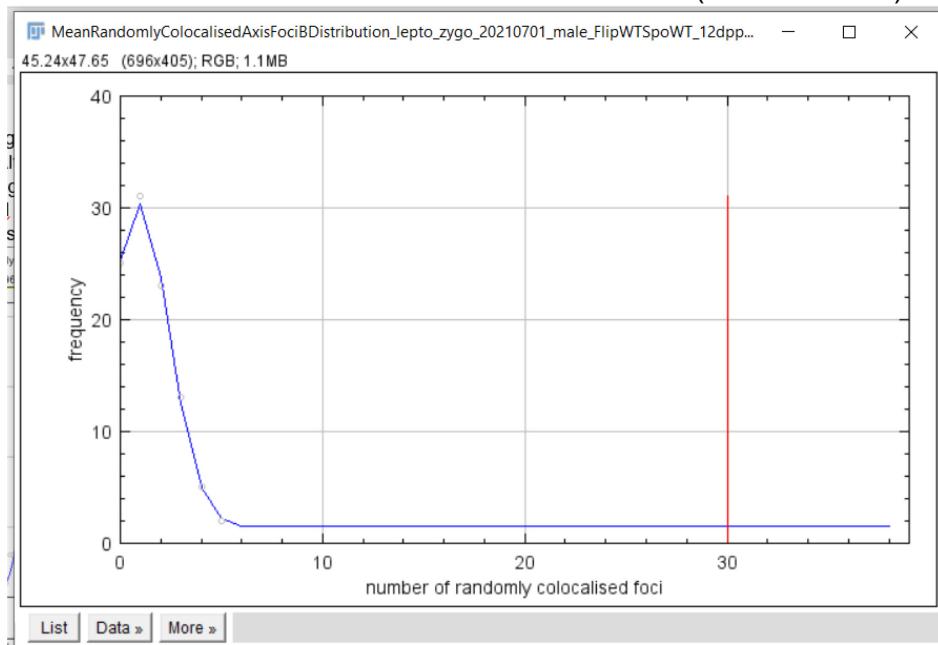


Fig. 7.5 “clipped” randomly colocalised foci number distribution across all randomly generated images

STEP 7. Then, particles are identified starting from the nucleus/user-defined ROI and using the above-mentioned Find Maxima options. Total particles ROI is split and each individual particle is compared to the corresponding axis/off-axis maxima.

Note: an axis foci (or off-axis foci) will have its foci maximum within the axis ROI (or off-axis), while its associated foci particle may spread in the off-axis ROI (or axis ROI) as well.

This substep is quite time consuming. To skip this, deselect the “show foci particle intensities” option of the general parameters menu (see **OPTIONAL STEP A**). Whenever a split particle contains an axis/off-axis maxima, then it is renamed(for instance as an axis_fociA_ID (particle)). If measurements parameters (see **OPTIONAL STEP A**) “show foci maximum intensities” is used together with “show individual foci’s value”, then the corresponding maximum point ROI is created (following the previous example as axis_fociA_ID (maximum)). Both maximum and particle share the same ID. If the “compute colocalisation option was selected”, the macro checks whether there is a triple overlap of the individual ROI, the corresponding axis/off-axis foci and the colocalised area generated using the axis/off-axis maxima of the other channel. If any, the particle (and its associated individual maximum – if any was generated following **OPTIONAL STEP A** configuration) are tagged as colocalised using the corresponding ROIGroup (see figures **7.2** and **7.3**). If no overlap is observed, as the particle was overlapping with a maximum ROI (following this example axis_fociA (maxima)), this means the particle is not colocalised and tagged as such.

If the individual particle does not contain any axis maximum, then no ROI group is attributed (ie. left to default 255 value) and the particle is purged from the ROI manager at the end of the process. Individual, colocalised or not-colocalised particles are fused together to create a ROI of all colocalised or not-colocalised particles.

STEP 8. Set additional intensity measurements channels or ROI. The macro will identify nucleus/user-defined ROI and the foci of interest (axis or off-axis) for both channels as maxima

and particles. Intensities can be measured in these ROIs and for a selection of channels. If the foci channels A and B should be measured as well, select “add foci channel intensity”. Would you like to add another channel for intensity measurements within ROI, fill-in the numeric field “Measure foci ROI’s channel intensity. If the field is left with -1 and the “add foci channel intensity” checkbox is not thicked, no measurements will be done.

To remove nucleus/user-defined ROI measurements or show/hide mean or IntDen or particle/maximum intensities, see **OPTIONAL STEP A**. For global particle measurements (as displayed in the 2Foci.xls table), the corresponding ROI is used (for instance colocalised axis_FociA (particles)). For global maxima calculation, the corresponding ROI (say axis-foci (maxima)) is measured using the getRawStatistics(nPixels, mean, min, max, std, histogram) command. The number of maxima (nPixels) is multiplied by mean to derive the IntDen value. .

STEP 9. 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 **7.6**). Individual foci values are stored in an individual2(off-)axisFoci.xls file.

STEP 10. 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. Set the measurements parameters options. Before using the “Count axis/off-axis foci in two channel and colocalize” tool, click on the Set measurements parameters button to set how the skeletons will be measured. The Parameters menu (figure **7.6**) will pop-up.

- Choose whether to display warning messages or not. As those block the batch processing of the macro, consider keeping them hidden.
- The default parameters hide the images during the analysis. To have them displayed, use the set Measurements Parameters of the main meiosis bar. In the parameters window (fig. **7.6**), select “show images while processing files” option.
- The default list of threshold (for nucleus, axis, synapsis or foci) is restricted to preset threshold that were found to be adapted to test images. Would you like to expand the list to the full ImageJ’s automatic thresholds list, select the “Display the full list of threshold methods” options.
- The default intensity measurements is IntDen. Would you like to add Mean values or remove IntDen, use the “Show IntDen values” and “show Mean values” options.
- Whenever the “Measure axis length” option is selected, select the lengths measurement types. Two different routines are available:
 - The first (rough) one takes advantage of the 1 pixel thickness of the skeleton. The total area in pixel of the ROI is measured and converted in um using the image’s calibration. This may yield weird results (as a diagonal pixel will account for 1 – say 0.25um if the pixel width is 0.25um - although it should be considered as $\sqrt{2}$ – and 0.35um).

- The second (fine) one is a raw measurement of the skeleton. It uses the geometry of the skeletonRoi and takes into account “horizontal, vertical and diagonal pixels”.

To remove fine or rough length measurements, unselect “show rough length estimation” or “show fine length measurements” respectively.

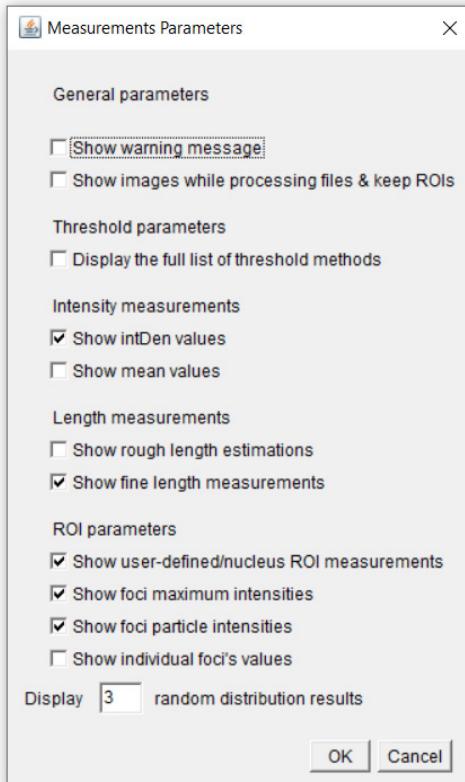


Figure 7.6. The (general) parameters window.

- Intensities is measured in the different ROIs. The default parameters will trigger reference ROI (either user-defined or nucleus ROI) intensity measurements. Unselect “show user-defined nucleus ROI measurements” to get rid of this. Foci ROIs can be measured both in maxima/individual maximum or particles/individual particle. Use the corresponding tickboxes to restrict analysis.
- Whenever random colocalisation analysis is requested, the “display [] random distribution results will leave in the ROIManager the requested random axis/off-axis foci ROI as well as the corresponding random foci that may colocalise with the real foci distribution in the other channel.

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 10** (figure 7.7).

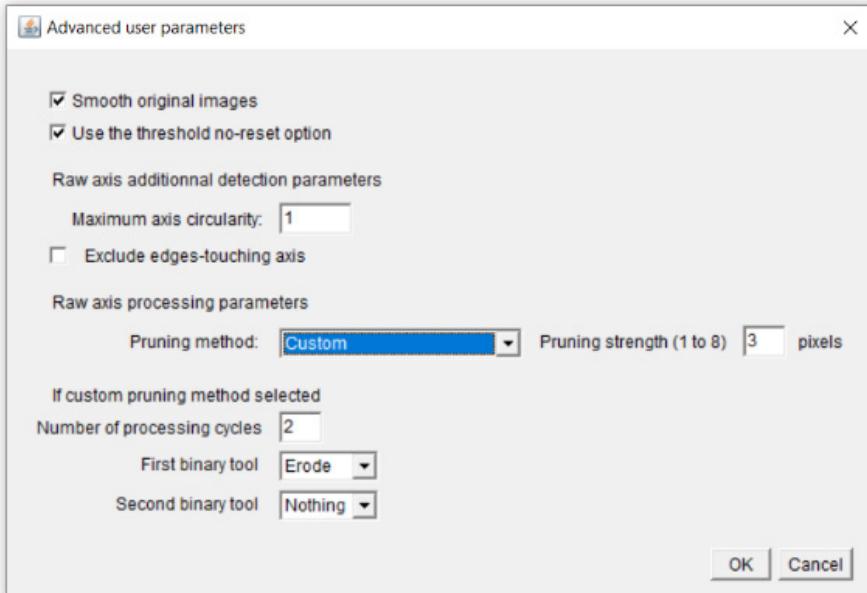


Figure 7.7. The Advanced user parameters menu

- 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.
- When using images with a very high offset value (such as the Abberior .msr files), those very high background intensity pixels together with pixels excluded from analysis (whose intensities are set to 0 in the cropping step), may bias the automatic threshold. From ImageJ 1.52e version to 1.53s, different autothreshold option were implemented/retracted/re-implemented. Later version allow a stable “don’t reset range” threshold mode. Briefly, more than 8 bits images are thresholded using the 8bits histogram automatic threshold methods. To allow this, the 16 bits 0-65535 range is converted to an 8 bits range (0-256), then the histogram is calculated. Let us consider now a 16 bits .msr file displayed using an Image>Adjust>Brightness&Contrast auto display range (say black is 32768, the ROI’s minimal intensity and white is 32784, the ROI’s maximal intensity). Reset option (the Adjust threshold’s default mode) will convert range extrema to range extrema (0 to 0 and 65535 to 256). Don’t reset will convert the displayed extrema to range extrema (32768 to 0 and 32784 to 256). Then the 8 bit histogram are computed and automatic threshold values calculated.
As a result of implementation/retraction/re-implementation cycles, it may be that previous meiosis bar versions were yielding correct axis/foci segmentation before and are no longer correct until this “Use the threshold no-reset option” option is ticked.
- 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 1** (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, not unticking the option will remove most of the axes.

- 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 tools (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 tool. Figure 7.8 shows the parameters used for 2x Erosion and a strength of 3. The pruning strength value (“count” in the options tool 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.

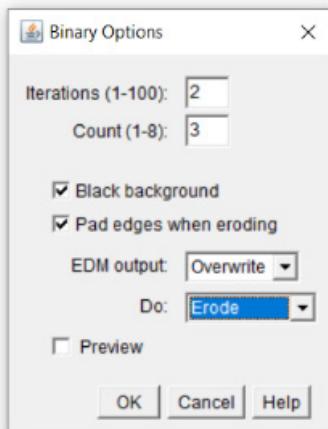


Figure 7.8. An example of how the pruning method “2x Erosion” translates in ImageJ.

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 tool to Iterations 2 and Do: Erode. Two erosions are first applied. Then the process>binary>options tool 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” ROI is then drawn using the processed mask (with the pruning method within brackets).

Parameter	Value
Smooth original images	No
ROI chosen	user-defined
Axis channel	4
Axis Threshold	Huang
Axis Min size	5
Axis Max Circ,	1
Exclude edge-touching axis	No
Measure axis length	No
Pruning Mode(s) tested	2x [Erosion & Dilation]
Pruning Strength (when used)	3
Foci detection method	within ROI
Foci Detection Threshold	IJ_IsoData
Foci type	Axis
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	1,5399

Fig. 7.9. a result file of the Count axis foci in two channel and colocalize macro

Type	Image name	focus' ID	focus' channel	focus' type	focus' localisation 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. 7.10. a individual particle result file individual2Foci.xls file of the Count axis foci in two channel and colocalize macro

STEP 11. 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” window will pop up (figure 7.11). If you just ran the macro, then the default folder and method are OK. If you control afterwards the macro’s results, set both folder and “Count axis/off-axis foci in two channels and colocalize” method.

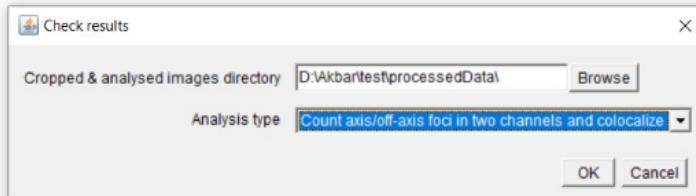


Figure 7.11. The check results menu

The first image analysed will be opened together with its companion 2foci_RoiSet_[name].zip file ROI set, as in fig. 7.12. The ROI Manager contains several ROIs:

- The reference ROI, as set in **STEP 2** (here user-defined ROI). This is the ROI used for further identifications (ie. axes).
- The raw axis ROI is displayed as well as the (processed) axis ROI (as given by **STEP 3**). If the advanced user mode was not selected (**OPTIONAL STEP B**), the raw whole axis and whole axis are identical. The skeleton is added if “measure length” option was selected.
- The axis/off-axis foci (maxima) in channel A and B (**STEP 4**). If the within Roi option was chosen, the total_foci [letter] (maxima) ROI is left.
- The colocalised area for both channel’s maxima as obtained in **STEP 5**.
- Colocalised foci obtained using the real localisation of identified axis foci A and B (= those obtained from **STEP 4**) as obtained in **STEP 5**.

- If the “get estimation of colocalisation with random images” option is selected, the result of the first randomisation cycle (“randomFociA/B”, STEP6).
- Depending of the configuration, the individual particles as obtained in **STEP 7**, together with the 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

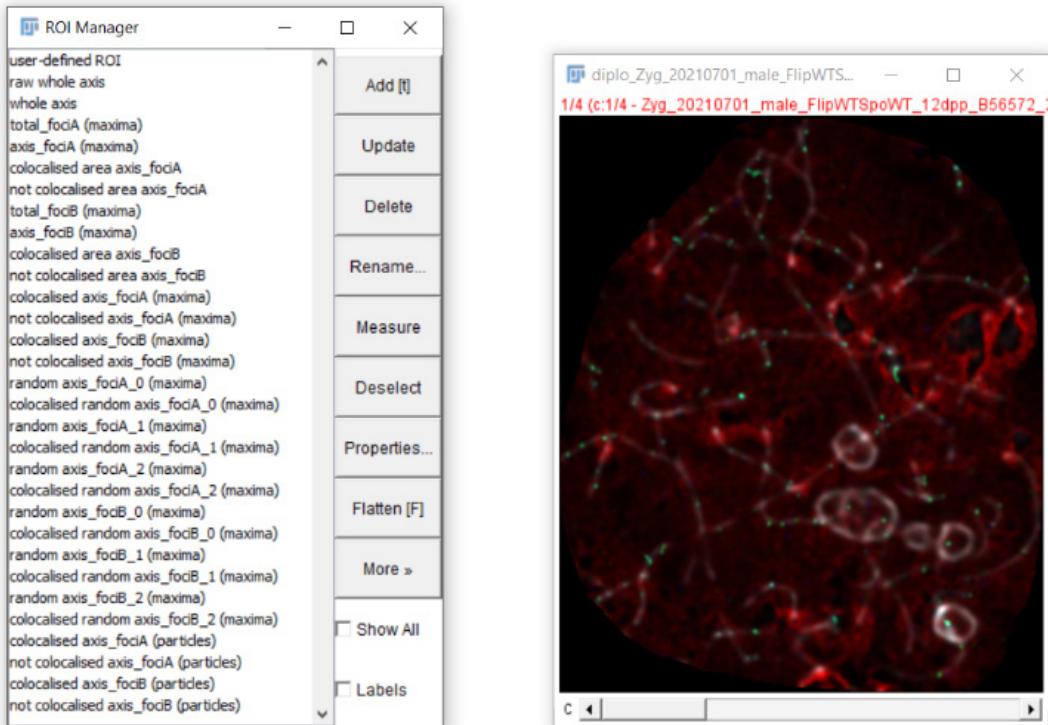


Figure 7.12. Control ROIs. Axis foci are displayed in both channels, as well as colocalised foci in both channels. When randomisation is used, an example of random distribution in both channel is shown.

In this figure, the “show individual Foci’s values” was not selected.

SET ADVANCED USER PARAMETERS

The tools associated with the meiosis bar should include enough parameters to segment axis and foci from widefield images. It is recommended to preprocess those images with a deconvolution suite.

Foci identification is based on a robust find maxima algorithm. Whenever noisy images yield false positive, it is recommended to use advanced user parameters option (referred to as **OPTIONAL STEP B** above) and select image smoothing. This filter replaces each pixel with the average of its 3x3 neighbourhood and applies to all channels.

Whenever raw axis segmentation is inaccurate, advanced parameters should be used. The meiosis bar provide raw axis processing tools. Click “Set advanced user parameters” button. The “Advanced user parameters” menu pops-up (figure 8.1).

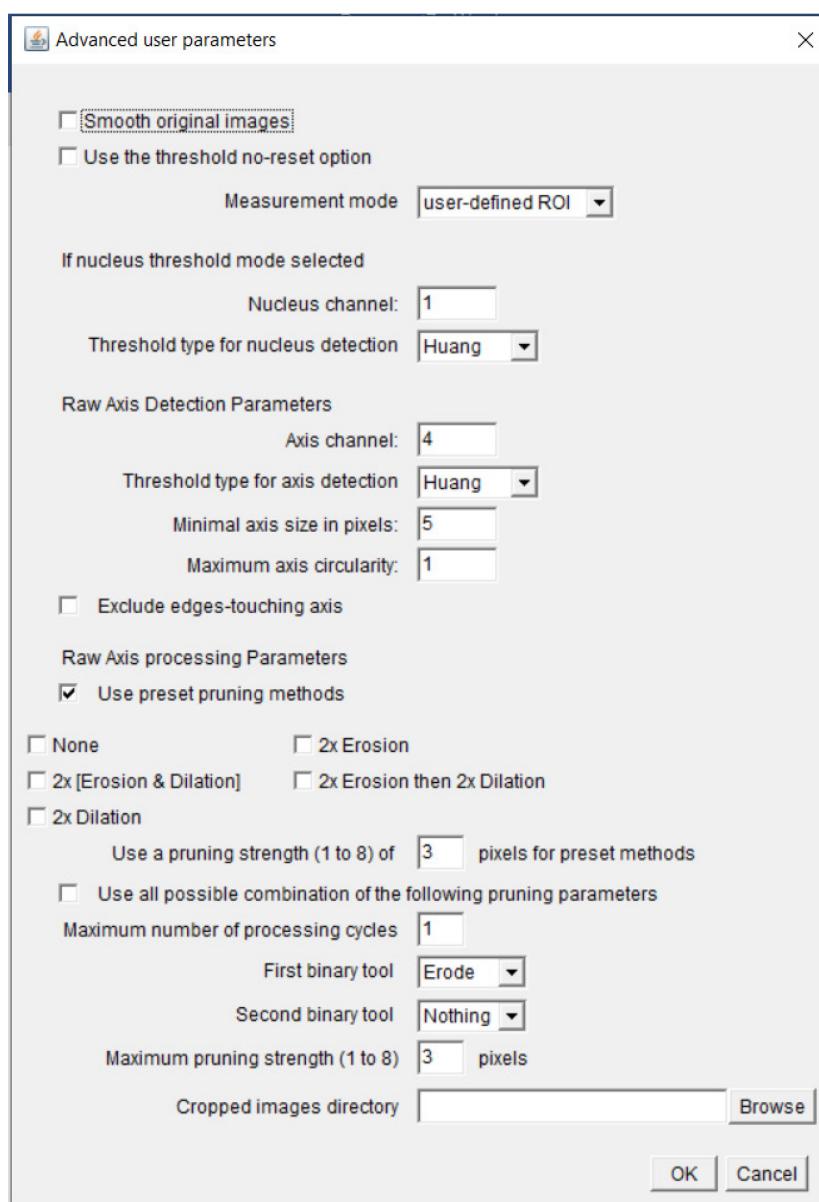


Figure 8.1. The advanced user parameters tool

STEP 1. Tick the smooth original images checkbox to remove noise (such as photon shot noise).

STEP 2. When using images with a very high offset value (such as the Abberior .msr files), those very high background intensity pixels together with pixels excluded from analysis (whose intensities are set to 0 in the cropping step), may bias the automatic threshold. From ImageJ 1.52e version to 1.53s, different autothreshold option were implemented/retracted/re-implemented. Later version allow a stable “don’t reset range” threshold mode. Briefly, more than 8 bits images are thresholded using the 8bits histogram automatic threshold methods. To allow this, the 16 bits 0-65535 range is converted to an 8 bits range (0-256), then the histogram is calculated. Let us consider now a 16 bits .msr file displayed using an Image>Adjust>Brightness&Contrast auto display range (say black is 32768, the ROI’s minimal intensity and white is 32784, the ROI’s maximal intensity). “Reset” option (the Adjust threshold’s default mode) will convert range extrema to range extrema (0 to 0 and 65535 to 256). “Don’t reset” will convert the displayed extrema to range extrema (32768 to 0 and 32784 to 256). Then the 8 bit histogram are computed and automatic threshold values calculated.

As a result of implementation/retraction/re-implementation cycles, it may be that previous meiosis bar versions were yielding correct axis/foci segmentation before and are no longer correct until this “Use the threshold no-reset option” option is ticked.

STEP 3. Enter all raw axis detection parameters as used in the other tools. Raw axis is detected within the reference ROI. Set how this ROI should be chosen (either the user-defined ROI that was drawn upon cropping the nucleus or a nucleus ROI). Whenever the nucleus threshold option is used, set the nucleus channel and automatic nucleus threshold method. Then set the regular axis detection parameters (axis channel, automatic axis threshold method and minimal axis size).

STEP 4. Set advanced user 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 cuts some axis signal in a way that it touches the edges of the image. When using cropped nucleus images with plenty overlapping axes, not unticking the option will remove most of the axes.

STEP 5. Set the pruning methods to be tested. Pruning is a way to modify the raw axis outlines and get rid of unwanted axis decorations (for accurate axis length measurement). Briefly, the raw axis ROI is used to create a binary mask that is then further processed using binary tools. The mask is creating using fill/clear tools (this can be reproduced manually by using the ROI, then the Edit>Fill and Edit >Clear Outside menus). The process>binary>options tool is used (figure 8.2), with the pad edges when eroding option & black background option selected. The pruning strength value (“count” in the options tool 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. Pruning is quite mandatory when using superresolution images. Two types of methods can be tried :

STEP 5a. Tick the “Use preset pruning methods” if you would like to try methods that were found adequate with some image dataset. Select all preset methods to be tried and set the pruning strength used with those preset methods (whenever a value lower than 1 or higher than 8 is entered, the entered value is overridden and 3 pixels are used). See figure 8.3 to see how the preset methods translate with the process>binary>options.

STEP 5b. Alternatively, select the “Use all possible combinations of the following parameters” pruning parameters. Maximum number of processing cycles is the maximum number of iterations. The First binary tool is the binary method (Erode, Dilate, Open) used in the first round of processing. If a second binary tool is entered, after all iterations of the first tool, the mask is subjected to another round of processing iterations using the second binary tool. The maximum pruning strength is the maximum count number of the process>binary>option tool.

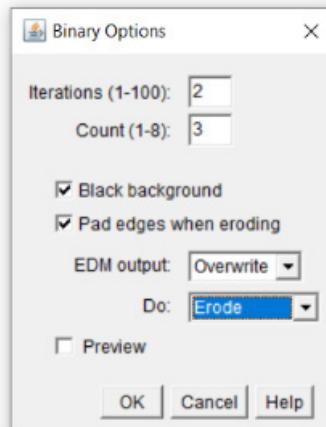


Figure 8.2. An example of how the pruning method “2x Erosion” translates in ImageJ.

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 8.3. Preset methods built-in parameters

“whole axis” ROI are then drawn using the processed mask (with the pruning method within brackets).

STEP 6. Check the segmentation results using the Start Checking results button. The “Check results” window pops up (figure 8.4).

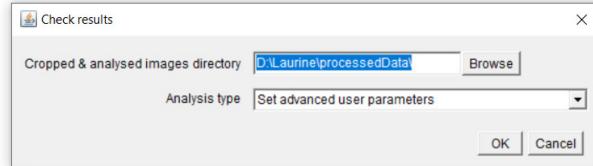


Figure 8.4. The check results window

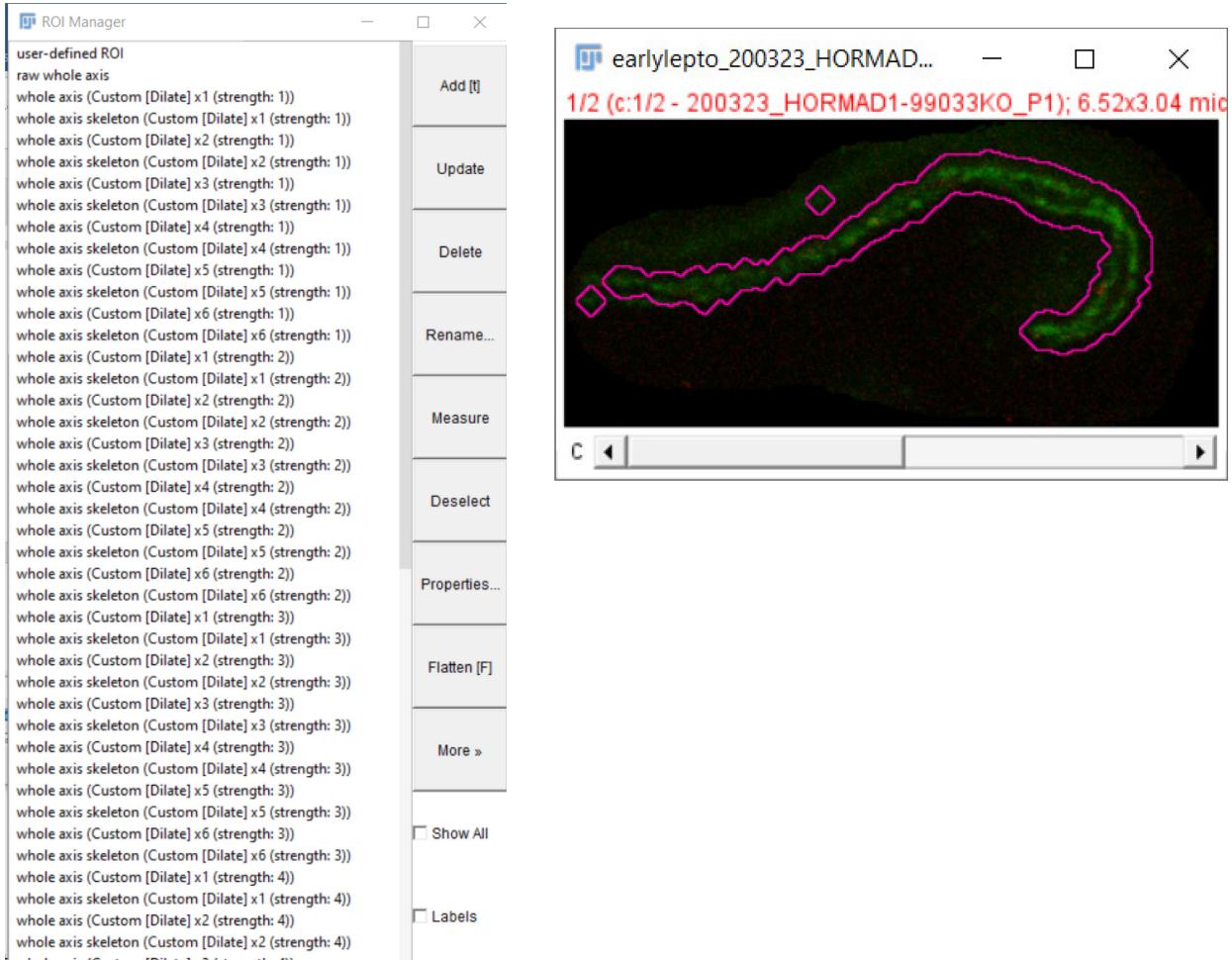


Figure 8.5. Control ROIs. Axis ROI are displayed in purple while axis skeleton are displayed in brown (not visible here)

VERSIONS

Crop tools (from start cropping images to diplo button)

V1.01	Creation
V2.01	<ul style="list-style-type: none"> Single window menu: the input (cropped) image folder is directly available from the Start Cropping images main menu window
V2.02	<ul style="list-style-type: none"> Pachy button correction (typo removal) More meiotic sub-stages added (prelepto, earlylepto and lepto/zygo)
V2.04	<ul style="list-style-type: none"> Correction of a ROI error when generating .roi files (ROI were copied from the original image rather than the cropped image).
V2.05	<ul style="list-style-type: none"> Correction of an unwanted cut/paste error for the early lepto button
V2.06	<ul style="list-style-type: none"> The crop buttons are all gathered in a single Get stage sorted images button.

Measure Global intensities:

V1.01	creation
V2.01	<ul style="list-style-type: none"> Single window menu: the input (cropped) image folder is directly available from the Start Cropping images main menu window
V2.04	<ul style="list-style-type: none"> The algorithm parameters are stored using a unified function, shared with the other buttons

Count Foci in two channels and colocalize

V2.06	creation
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Measure axis length:

V2.01	creation
V2.01	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> The possibility to measure intensities of a channel of interest within the whole axis' ROI is introduced
V2.04	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> The different pruning modes (referred 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	<ul style="list-style-type: none"> 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.

Measure synapse length:

V2.02	creation
V2.03	<ul style="list-style-type: none"> The possibility to measure intensities of a channel of interest within the axes’ ROIs is introduced
V2.04	<ul style="list-style-type: none"> 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.

Count axis and off-axis foci in one channel

V1.01	creation
V2.01	<ul style="list-style-type: none"> The “within ROI” option for foci detection is introduced
V2.02	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Subtle modification of how multipoint maxima's Integrated Density is measured.

Count axis foci in two channels and colocalize

V1.01	creation
V1.02	<ul style="list-style-type: none"> Correction of a bug (noise value for foci B detection was noise A value).
V2.01	<ul style="list-style-type: none"> The “within ROI” option for foci detection is introduced
V2.02	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> The user has the possibility to measure either on-axis foci or off-axis foci.
V2.04	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> 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).
V2.06	<ul style="list-style-type: none"> 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

Options

V2.05	<ul style="list-style-type: none">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	<ul style="list-style-type: none">Axis mask processing parameters can be changed separately from the main analysis tools in a “set advanced user parameters” buttonFollowing introduction of a variable crop stages bar, prefixes used for analysis can be changed in a “set stages/types parameters” button