

QuaSI user guide

Installation	1
<i>From downloaded .ijm file</i>	<i>1</i>
Macro execution.....	2
1. <i>Visualization</i>	<i>3</i>
2. <i>ROI mask generation/ ROI definition.....</i>	<i>5</i>
3. <i>ROI quantitation.....</i>	<i>7</i>
4. <i>Foci analysis.....</i>	<i>7</i>
5. <i>ROI inspection.....</i>	<i>10</i>
Output and Results.....	11
<i>General.....</i>	<i>11</i>
<i>Visualization module.....</i>	<i>12</i>
<i>ROI definition module</i>	<i>12</i>
<i>ROI quantitation module.....</i>	<i>13</i>
<i>Foci detection module.....</i>	<i>13</i>
<i>ROI inspection module.....</i>	<i>14</i>
FAQ	15

Installation

For installation of Fiji/ImageJ, see <https://imagej.net/Fiji/Downloads>

From downloaded .ijm file

Install in the menu:

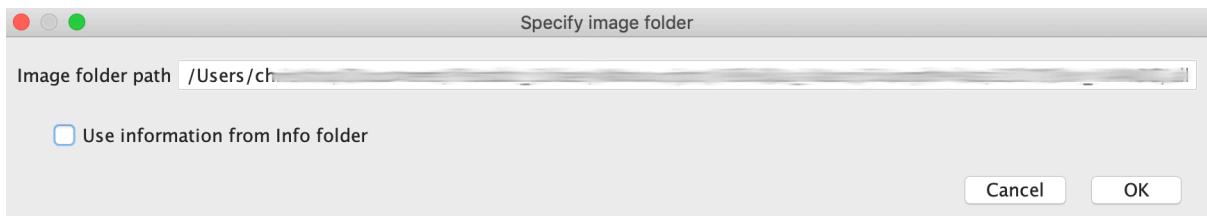
- in Fiji menu, go to Plugins > Install
- select the .ijm file, click ‘open’
- save in plugins/Macros folder, click ‘save’
- restart Fiji
- the QuaSI macro is found under Plugins > Macros and can be started from there

Alternatively:

- drag&drop the .ijm file into the Fiji toolbar
- the macro code will open; start the Macro by clicking ‘Run’

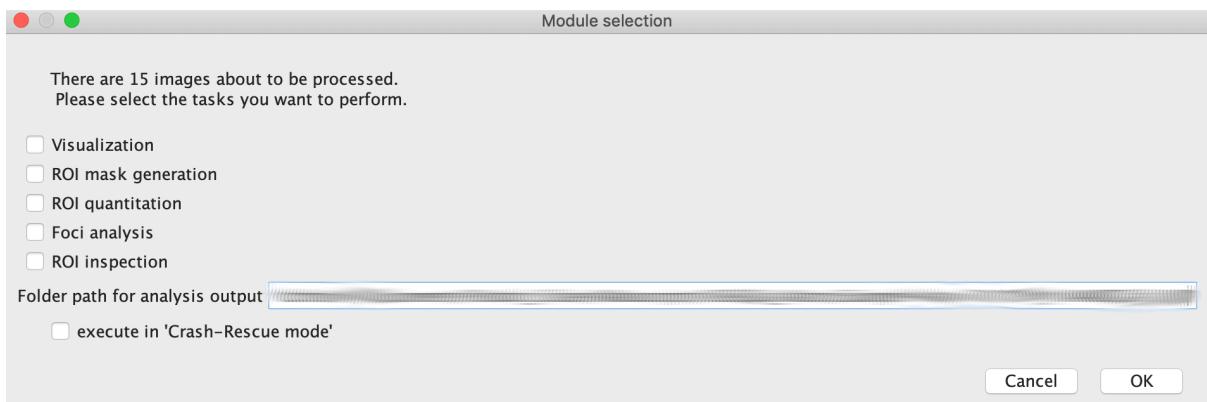
Macro execution

By default, the macro operates on all open images. If no images are open upon start of the macro, the user is prompted to either open the images he or she wants to process, or to specify a folder that contains these images. Please make sure that this folder contains **only the images to process**, no other files or subfolders



By default, the images in the specified folder are opened to retrieve the metadata. When working with many images or big image stacks, this can be time-consuming and memory-intensive. If the same set of images has been processed with QuaSI before, the checkbox 'Use information from Info folder' can be used to avoid image opening and load the metadata from the Info folder directly.

In the 'Module selection' window, you can check the boxes of the modules you want to execute. Within each module, all images are loaded and processed one after the other. If in a previous run with the same image set, the macro crashed or had to be cancelled, the option 'execute in Crash-Rescue mode' will pick up the workflow after the last successfully processed image.



In general, modules are independent from one another and can be run separately. The ROI quantitation and ROI inspection module, however, depend on a previously defined ROI mask. Nevertheless, it is possible to run the ROI mask generation module 'stand-alone', and import the ROI mask (or information from the Visualization module) in a later execution of QuaSI.

Most of QuaSI's functions are performed on 2D-images. For this reason, Z-stacks have to be projected onto one plane – a function that is integrated in every module. If your images have only one plane to begin with, always choose the 'mid section', 'keep stack', or 'all slices separately' option.

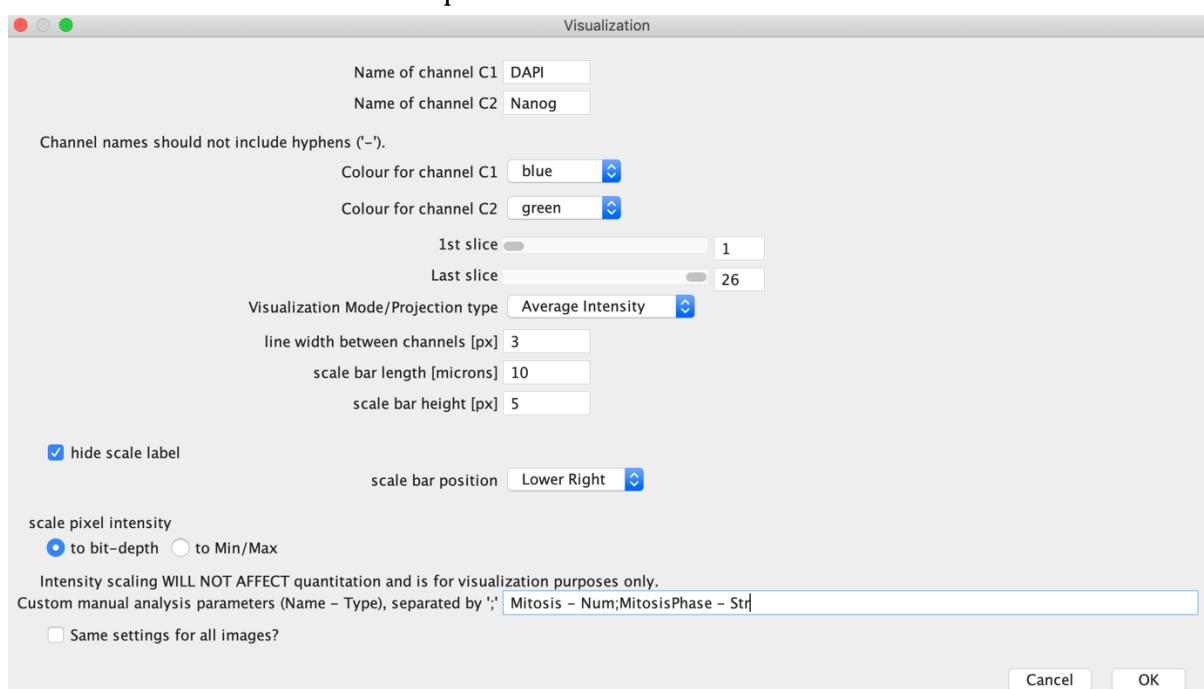
The macro output (see also [Output and Results](#)) will be saved in a folder called "Analysis/" under the specified path. If QuaSI is performed on open images, a copy of each

image or stack will also be saved in this folder (under Analysis/Temp/), but can be deleted after completion of the macro.

1. Visualization

The Visualization module provides you with several options to display your images. In general, grayscale images of different channels are shown side-by-side with a merged image. False colors, projection type (for Z-stacks), and scale bar option can be set in the central Visualization dialog. For Z-stacks, slices at the beginning and end of the stack can be cropped off with 2 sliders.

Pixel intensity values can be scaled in 2 ways: to the bit-depth of the image (this depends on the acquisition settings and equipment) and to the minimum and maximum intensity of the respective channel. Any intensity scaling here is linear and for visual purposes only and will have no effect on later quantitation.

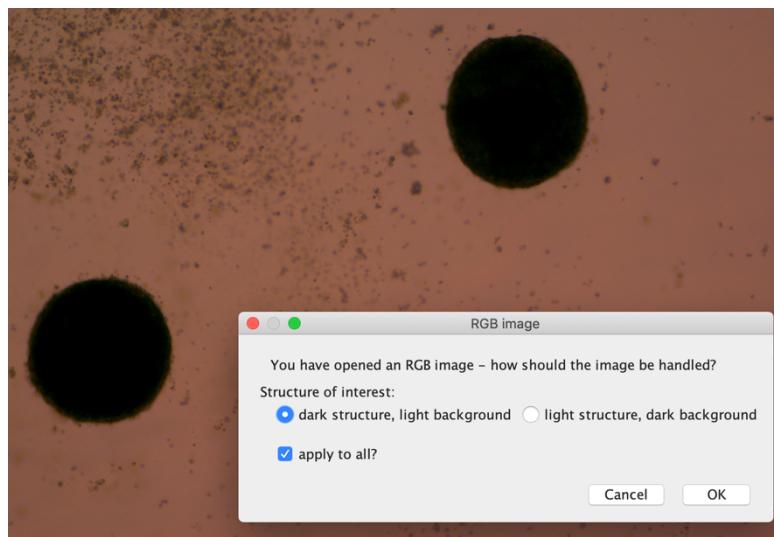


The 'Custom manual analysis parameters' can be entered by the user and consist of 2 parts: a freely chosen name (e.g. 'Mitosis') and a data type (Str or Num for string/character data or numbers, respectively). Different analysis parameters need to be separated by semicolons (;). If you do not want to perform image annotations, simply delete the default entry from the text field.

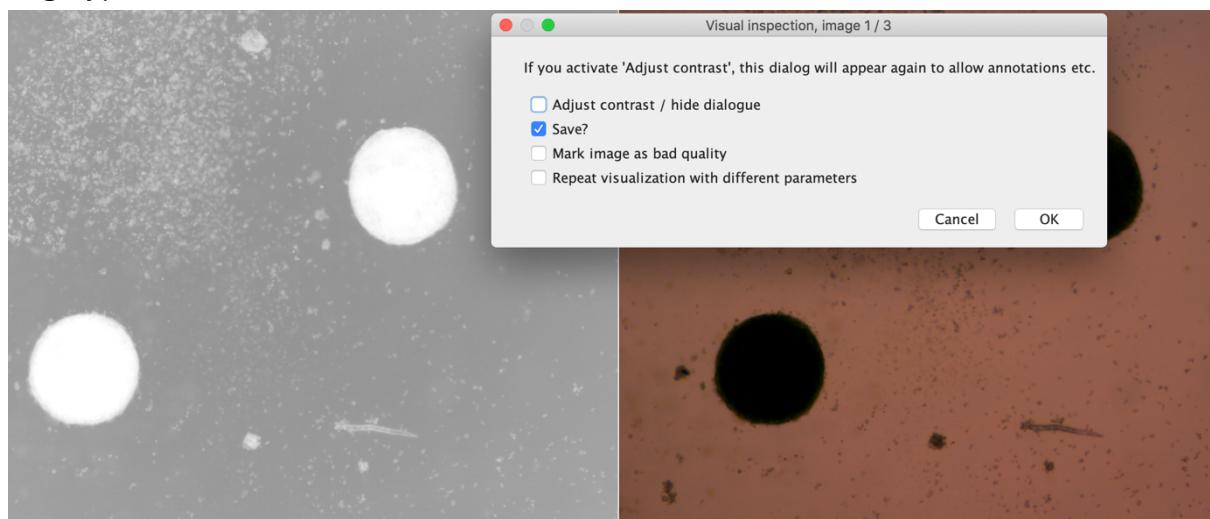
It is advisable to set and readjust the visualization settings until you are satisfied with the results and the visualization reflects best what you want to observe. For a nuclear stain like DAPI, for example, blue is a popular false color chosen in many publications. Blue on black is very poor contrast, however, and if you want to see finer nuclear structure, a different color is recommended.

For every image, the Visualization dialog will reappear with the last used settings. Checking 'Same settings for all images?' will skip the dialog and go to the Visualization output directly.

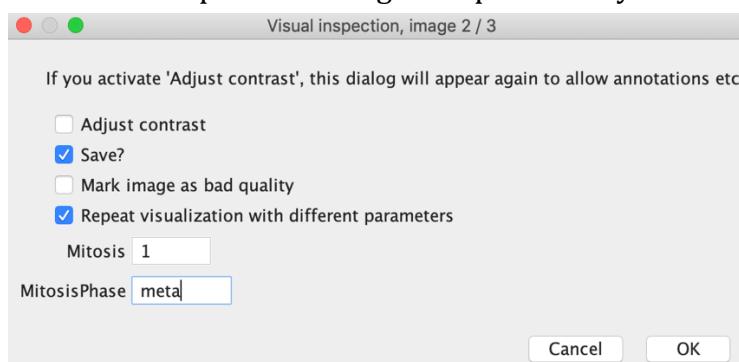
If you work with RGB images, you will be asked what your structure of interest looks like.



RGB images will be converted greyscale before processing in all modules; therefore, you need to specify if you are interested in dark or light structures. The greyscale image will be displayed side-by-side with the original image. In analogy to fluorescent images, your structure of interest will always be displayed in white, whereas the background appears in grey/black.



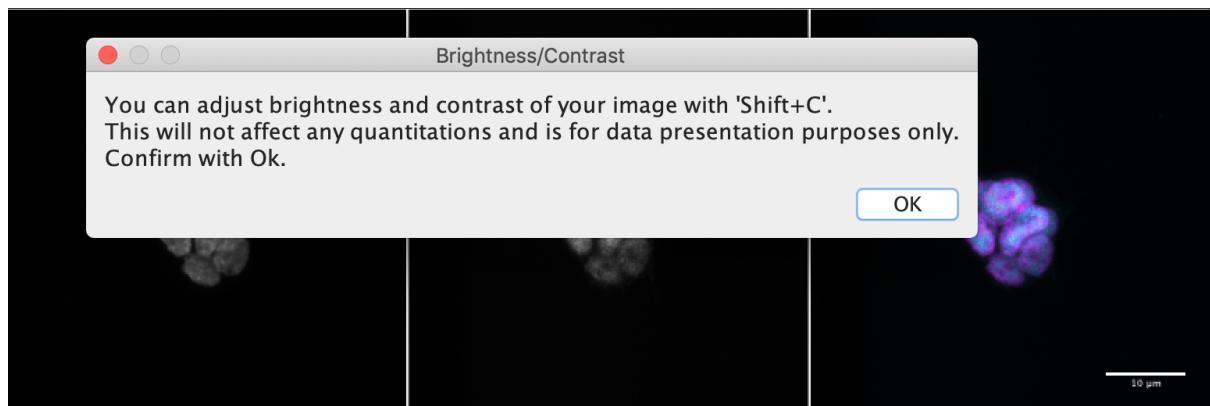
The Visual inspection dialog will open side-by-side with the assembled image:



You can enter your image annotations here, save the image assembly or mark bad quality images. Checking 'Repeat visualization with different parameters' will reopen the

Visualization dialog (see previous page). The ‘Save?’ checkbox refers to the image assembly only, and not to the image annotations. The latter will be saved automatically once you click OK.

Please note that you cannot alter images or use the ImageJ toolbar while dialog windows are displayed. By checking ‘adjust contrast’, you can hide the dialog window, and alter the image display settings manually, e.g. by adjusting brightness.

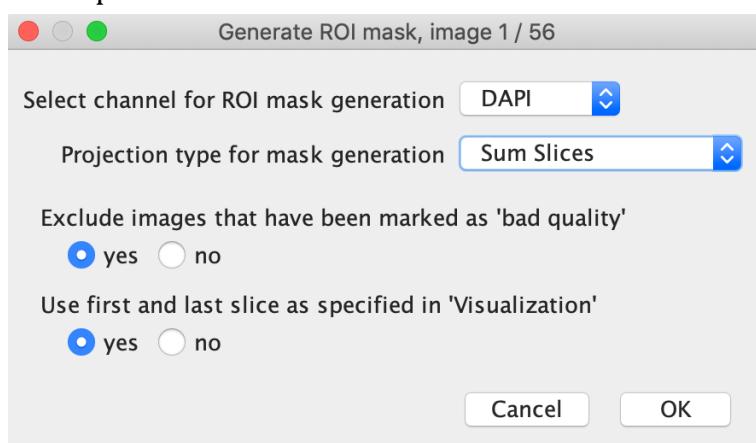


Do not click OK until you are done. Clicking OK will return you to the Visual inspection dialog.

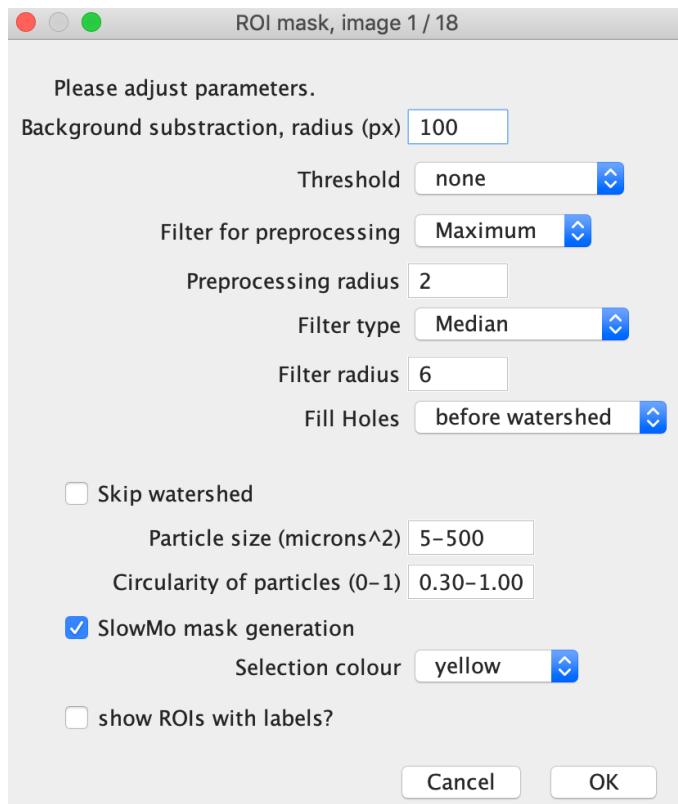
Confirming with OK in the Visual inspection window will complete the Visualization module for this particular image and the next image of the dataset will be loaded.

2. ROI mask generation/ ROI definition

Definition of ROIs (regions of interest) is key to most image analyses. QuaSI bundles several ImageJ functionalities in order to allow automated ROI recognition, but also allows manual curation. The first dialog of the ROI definition module is used to select the channel on which the ROI mask is generated (e.g. DAPI for nuclear ROIs) and the projection type in case of stacks. If you have performed the Visualization module (either in the same QuaSI run or previously), you can import some information into the ROI definition: You can automatically skip images marked as ‘bad quality’ and you can apply user-defined Z-borders. If you did not run the Visualization module, simply select ‘no’ for both options.

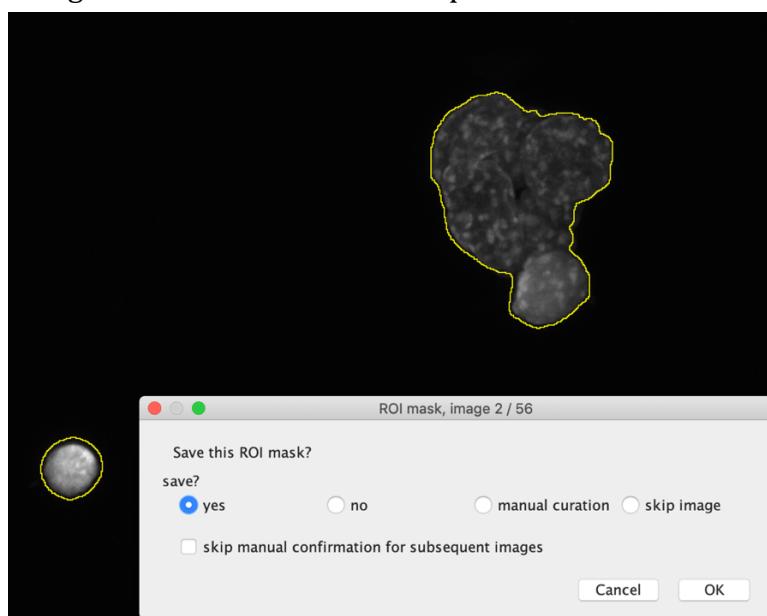


After the initial dialog, you can set the parameters for ROI recognition. Details about the individual options can be found on the ImageJ help pages (<https://imagej.net/docs/guide/146.html>).

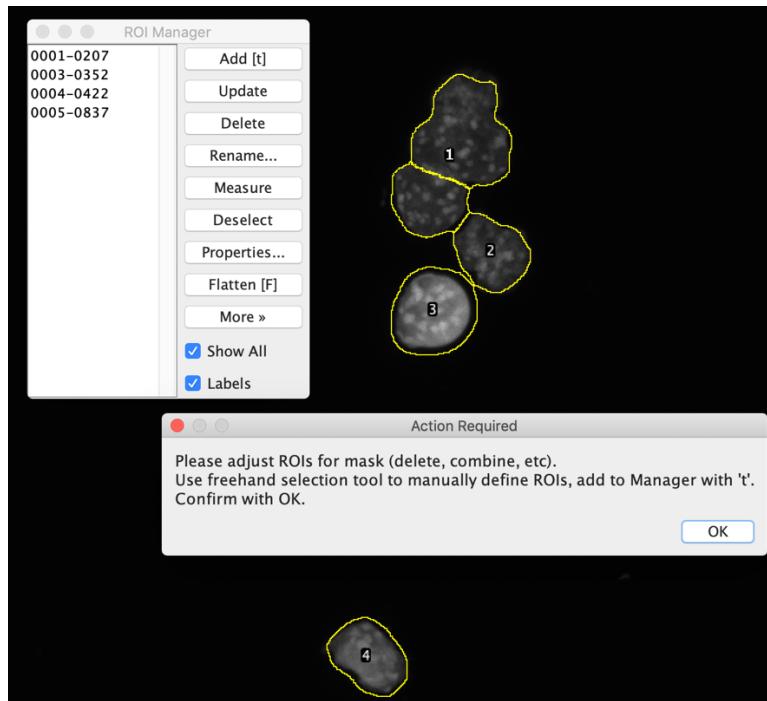


In the beginning, setting the parameters will involve a lot of ‘playing around’ and trial-and-error. The ‘SlowMo mask generation’ option will animate each step of the image transformation as a visual feedback to the user. Based on this, parameters can be readjusted. These animations come at the cost of processing speed, though, and we recommend disabling the ‘SlowMo’ option after you are confident about your settings.

The generated ROI mask will be presented to the user for confirmation:



'yes' will lead to loading of the next image, 'no' will go back to the 'Generate ROI mask' dialog, and 'manual curation' will allow you to manually delete, combine, or draw ROIs. 'Skip image' will load the next image without saving a ROI mask.



Upon selection of 'manual curation', you will be able to access the ROIs in the ROI Manager and e.g. remove unwanted ROIs, or combine ROIs. In the example above, two ROIs that have been falsely separated by the watershed algorithm have been combined to one (see ROI No. 1). Please note that if you combine two ROIs (right click > COMBINE), you will have to delete the second ROI in order to avoid duplications. With the freehand selection tool (automatically activated upon entrance to 'manual curation' mode), you can select structures that have not been recognized automatically. Click 'Add [t]' or hit 't' on your keyboard to add these selections to the ROI Manager. Once your manual adjustments are done, click OK in the 'Action Required' window. This will return you to the 'Save ROI mask?' dialog.

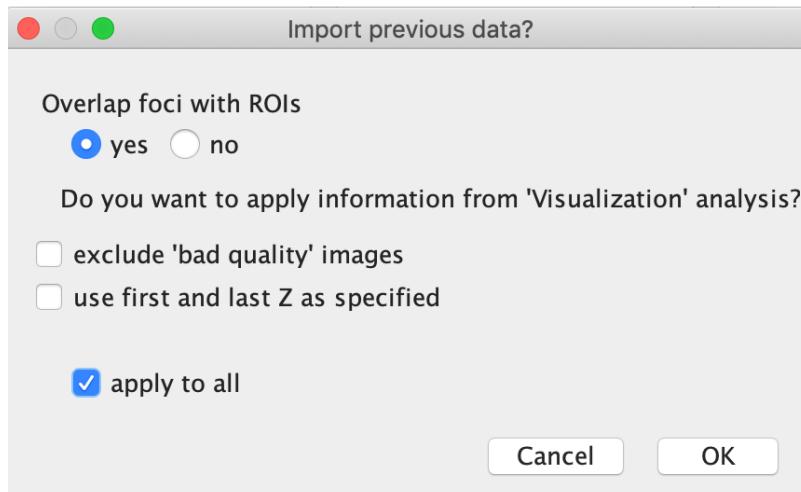
3. ROI quantitation

The ROI quantitation automatically loads the generated ROI masks and quantifies all ROIs on the image for each channel separately. Additionally, the whole image is quantified, which can later be used for calculations of background signal (see section [Output and Results](#)). ROI quantitation is fully automated and is executed in 'batch mode' to increase speed, i.e. the user cannot see the loaded images. The only input required is the projection type to work on.

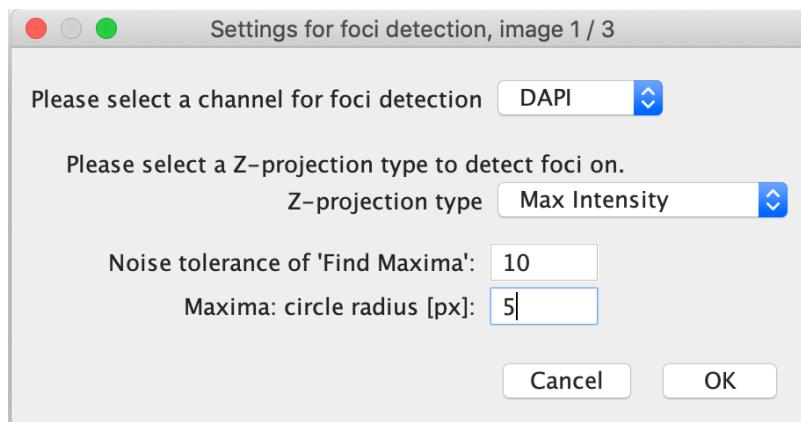
4. Foci analysis

The initial dialog allows to specify the cross-talk of the Foci analysis module with the Visualization and ROI definition modules. If you want to run the foci analysis as a stand-

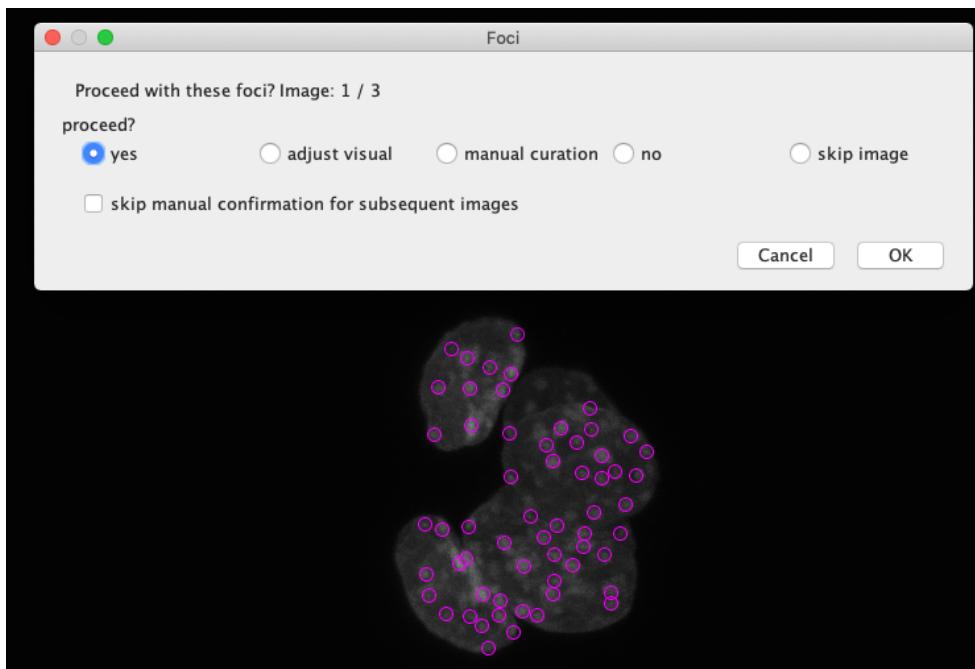
alone application, please select “no” for the ‘Overlap with ROIs’ option and uncheck the boxes for import of information from the ‘Visualization’ module.



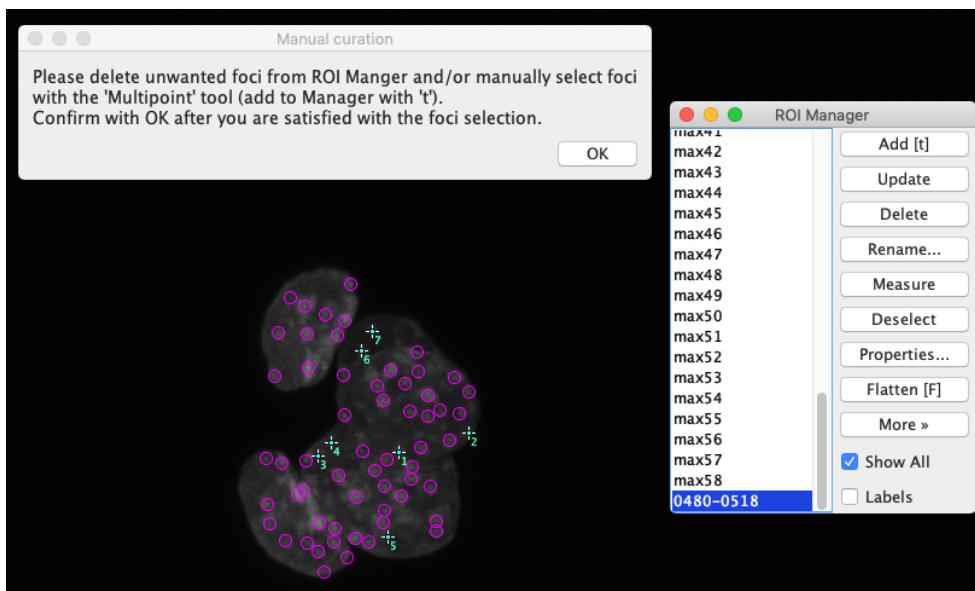
Foci are detected with ImageJ’s “Find Maxima...” algorithm. You can select the channel and projection type to perform foci detection on, and specify the noise tolerance used by the “Find Maxima...” algorithm. A circular selection will be drawn around each focus – the radius of this circle can be set by the user dependent on the structure of the foci of interest.



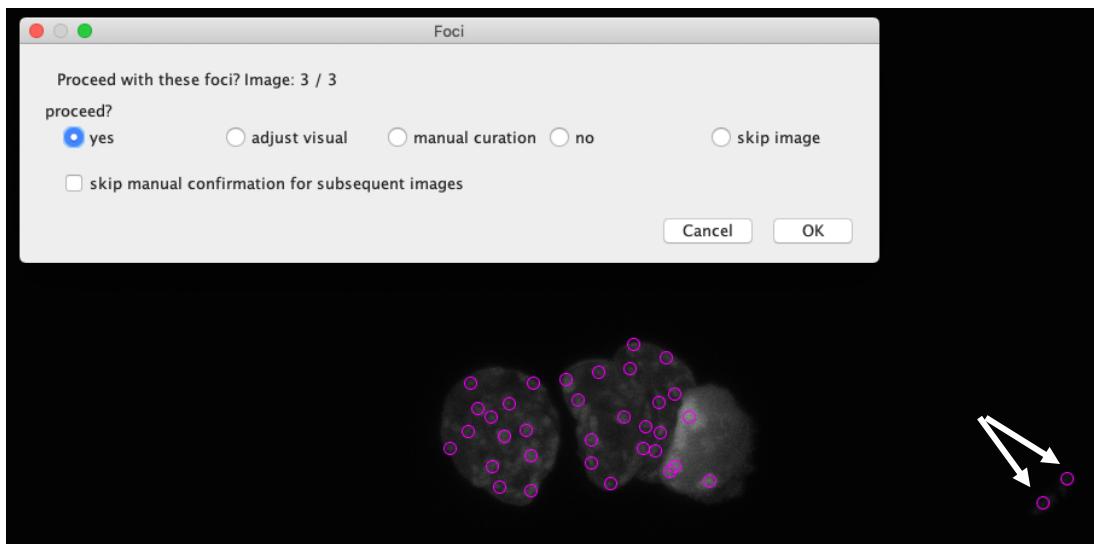
Like in the ‘ROI definition’ module, the generated foci mask will be presented to the user and can be edited manually. The ‘adjust visual’ option allows the user to adjust brightness/contrast settings or hide the foci mask via the ROI Manager. This might be useful to check if the detected maxima accurately reflect the foci apparent to the eye. If not, selecting “no” will allow you to adjust the parameters of foci detection, in particular the noise parameter for the “Find Maxima...” function.



Upon selection of 'manual curation', you will be able to delete foci via the ROI Manager and add foci with the Multipoint selection tool (automatically activated). Just click on all the foci you want to add; a crosshair will appear with each click. After you are done selecting, hit 't' once and the foci will be added as a multipoint selection to the ROI manager (see screenshot below). If you confirm with 'OK', the multipoint selection will be automatically converted to single circular selections by QuaSI, and will be presented to you for final confirmation. Entering 'manual selection' mode again will allow you to delete unwanted or accidentally added foci.



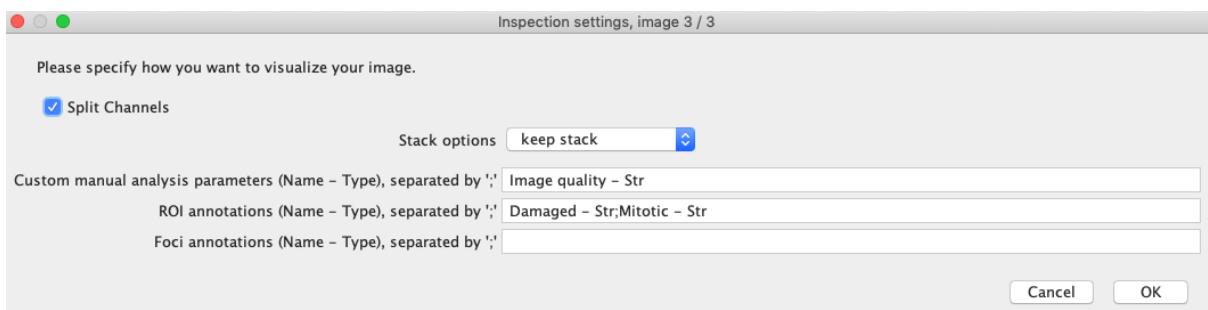
After confirmation of the foci mask, the foci will be quantified and associated with previously defined ROIs, if applicable. Foci outside of cellular structures as in the example below will also be quantified and annotated as "out".



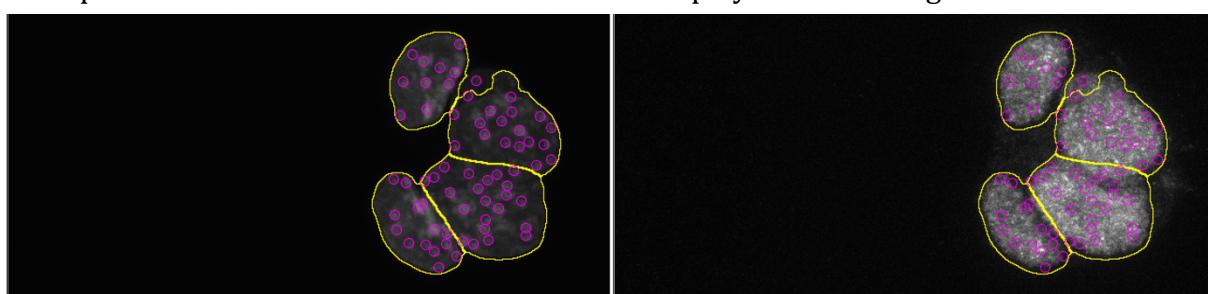
5. ROI inspection

The ROI inspection module is designed to manually inspect the generated ROI and foci masks, and (if applicable), to annotate them on-the-fly. Unlike the previous modules, which work on Z-projections of image stacks, the ‘ROI inspection’ module offers the choice to keep the stack and go through the image slice by slice. In case of images with only one plane, please select the ‘keep stack’ option.

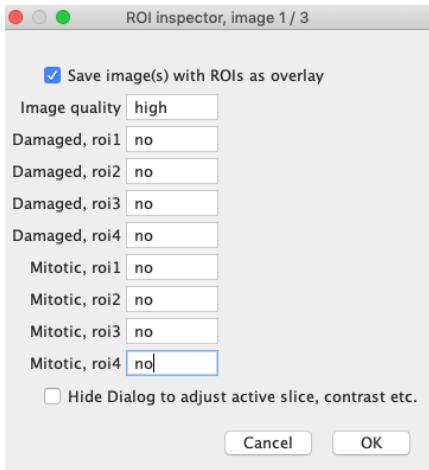
The ROI inspection module provides three types of annotation (see the last three lines in the dialog window below): General annotations about the image (comparable to the annotations in the ‘Visualization’ module), annotations of individual ROIs, and annotations of individual foci. Please note that the latter options might not be feasible if the number of ROIs/foci is too large. If you do not wish to use a particular annotation type, delete the default entry from the field, as exemplified for ‘Foci annotations’ in the screenshot.



You can choose to keep the channels in one image window or split the channels as in the example below. The ROI and foci masks will be displayed in the images.



The user-specified annotation fields will be displayed side-by-side with the image. The ‘Hide dialog ...’ option can be used to adjust brightness/contrast, go through the stack, or hide ROI labels/outlines. Once you click OK (and ‘Hide Dialog’ is unchecked), the annotations will be saved.



Output and Results

General

Upon start of QuaSI, a folder with the default name “Analysis” will be created. It will contain up to 7 subfolders:

- Info
- Visualization
- ROIs
- Quant
- Foci
- ROI-inspection
- Temp

The Info-folder contains a txt-file with image names and relevant metadata, e.g. pixel size, number of channels, etc. (see screenshot). If QuaSI is completed without crashes or cancellations, these data are found in a file named “Info-Data_all.txt”. If the macro terminated prematurely, a file called “Image-Data.txt” is generated, which is used by the ‘Crash-Rescue mode’.

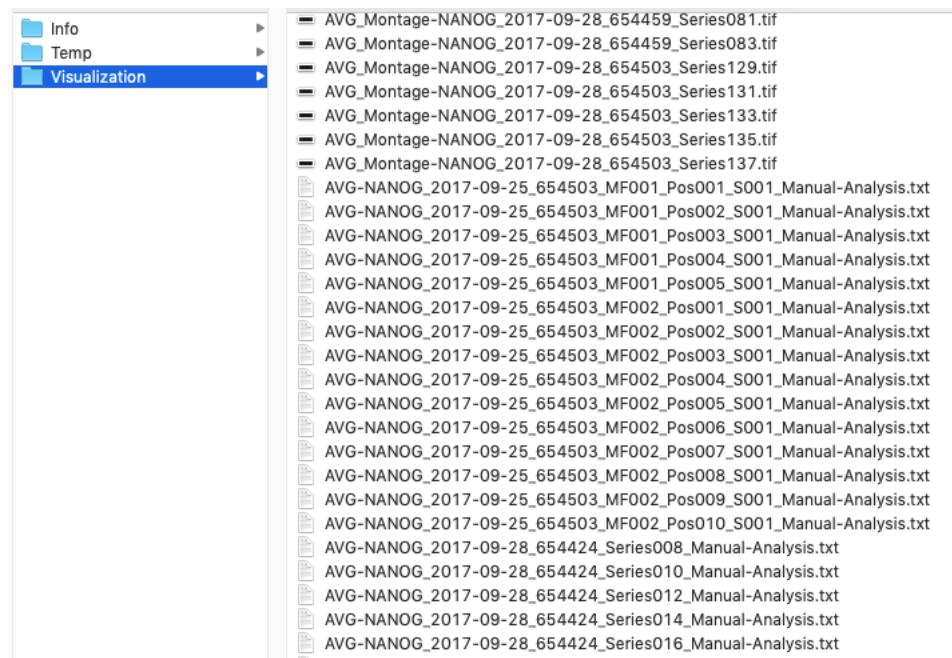
seriesNA original name	seriesA processed name	widthA image width	heightA image height	channelA # channels	sliceA # slices	frameA # frames	XsizeA pixel width	YsizeA pixel height	ZsizeA pixel depth	unitA pixel size unit
NANO_G_2017-09-25_654503_MF002_Pos001_S001.tif	NANO_G_2017-09-25_654503_MF002_Pos001_S001	1024	1024	2	33	1	0.096	0.096	0.797	microns
NANO_G_2017-09-25_654503_MF002_Pos002_S001.tif	NANO_G_2017-09-25_654503_MF002_Pos002_S001	1024	1024	2	34	1	0.096	0.096	0.797	microns
NANO_G_2017-09-25_654503_MF002_Pos003_S001.tif	NANO_G_2017-09-25_654503_MF002_Pos003_S001	1024	1024	2	35	1	0.096	0.096	0.797	microns

If the macro is executed on open images (and not on images in a specified folder), a copy of each image is saved as a tif-file in the Temp-folder. Each QuaSI module will load and process the images from the Temp-folder. This ensures stability of the software and frees up the RAM (especially when working with many images that have to be imported from a microscope-specific image type), but obviously comes at the cost of disc space. For this reason, the user is asked whether he or she wants to delete the Temp-folder after successful macro execution.

Visualization module

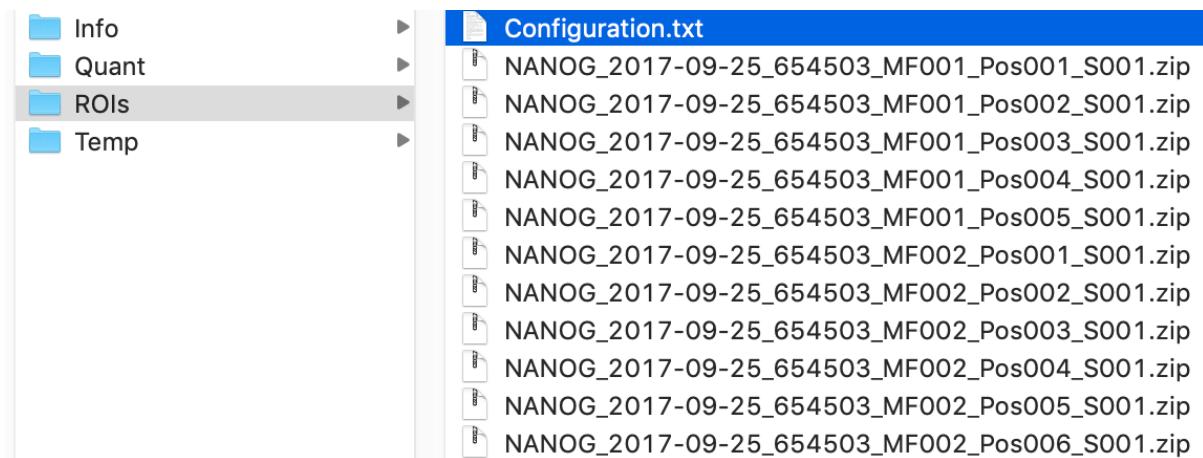
The Visualization-folder will always contain a txt-file ("Image-Data_Visualization.txt") with information about how the image panels have been generated, e.g. which projection type and which intensity scaling has been used. Additionally, the assembled image panels will be saved as tif-files if the user has checked the 'Save?' option. The scale bar is saved as Overlay and is not directly included in the image.

If image annotations have been performed, they are saved as a separate txt-file for each image. This prevents loss of data if QuaSI crashes halfway during execution. To facilitate downstream analysis of the annotation data, an R script for combining these separate files into one table is provided via github (<https://github.com/BauerCU/QuaSI>).



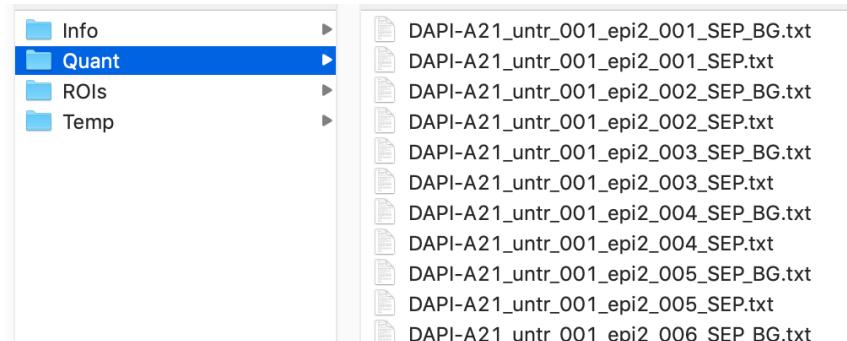
ROI definition module

The ROI masks generated by the ROI definition module are saved as zip-files in the ROIs subfolder. The configuration txt-file contains information about the image transformations that were used to define the ROI mask. Zip-files can be opened with the ROI Manager from within ImageJ/Fiji or loaded automatically with QuaSI's ROI inspection module.



ROI quantitation module

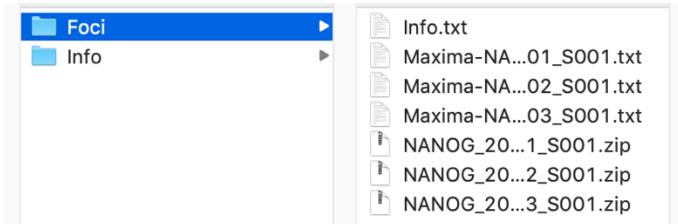
The Quantitation output folder will contain a parameters file with all image names, the projection type the quantitation was performed on, etc. Additionally, there will be at least two txt-files for each image – one that contains the ROI quantitations, and one that contains quantitation of the entire image for assessment of background signal (indicated by the “_BG” suffix). Quantitations are saved as separate txt-files for all channels, indicated by a prefix with the user-specified channel name (“DAPI-” in the example below). The naming convention will be the following: “ChN-ImageName_PTY(_BG).txt” with ChN being the channel name und PTY being the projection type (in the example: “SEP” for all slices separately).



Each txt-file contains the full exported ImageJ-Results table with information on fluorescence intensities, ROI location, size and shape, etc. Information about the individual columns can be found [here](#). The provided R import script will aggregate the data and also extract information from the file names.

Foci detection module

In the Foci-folder, there will be an Info-file with the used parameters (noise threshold, etc.), a txt-file for each image with the foci-quantitations (“Maxima-...”), and the foci-masks as zip-files that can be imported into the ROI Manager.

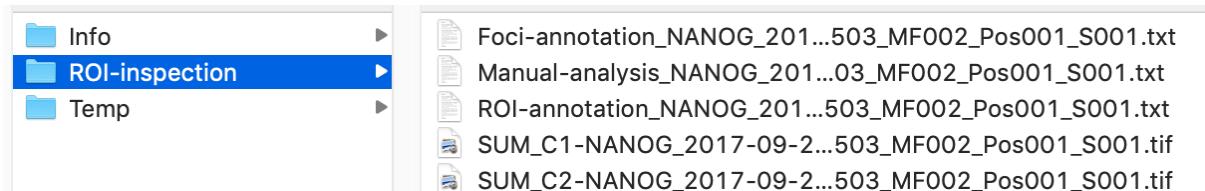


Each Maxima-txt-file contains information about the detected foci and their association with ROIs if selected by the user. Please be aware that the provided intensity values (mean, median, integrated density, standard deviation, but not the maximum intensity) are influenced by the user-defined selection radius. It is therefore highly recommended to keep circle size stable between images if you want to work with foci intensities.

	maxNameA	maximaNA	maximaXA	maximaYA	meanA	maxIntA	stdevA	medianA	intdenA
	Foci ID	associated ROI	X coordinate	Y coordinate	mean focus intensity	max. focus intensity	focus intensity std deviation	median focus intensity	focus integrated density
3	max1	roi4	437.999	529.999	97.188	142	21.446	98	71.953
4	max2	roi1	446.999	424.999	89.25	128	22.082	95	66.077
5	max3	roi4	455.999	552.999	99.938	125	12.452	102	73.989
6	max4	roi1	476.999	385.999	78.812	116	17.992	77	58.349
7	max5	roi4	442.999	525.999	88.338	134	14.487	88	65.401
8	max6	roi1	445.999	396.999	75.125	101	12.626	76	55.619
9	max7	roi3	485.999	565.999	69.8	98	11.536	71	51.677
0	max8	roi2	514.999	426.999	68.35	98	17.828	76	50.603
1	max9	roi1	470.999	397.999	73.225	98	12.735	75	54.213
2	max10	roi3	486.999	531.999	81.275	96	7.559	82	60.172
3	max11	roi1	443.999	373.999	75.725	96	7.27	76	56.063
4	max12	roi4	468.999	557.999	69.662	95	12.744	69	51.575
5	max13	roi1	481.999	355.999	56.425	95	12.483	59	41.775
6	max14	roi4	467.999	568.999	60.275	92	12.785	59	44.625
7	max15	roi3	523.999	530.999	65.9	92	12.008	68	48.789
8	max16	roi4	424.999	503.999	60.975	92	13.897	60	45.143

ROI inspection module

Dependent on the user settings, the ROI inspection module may contain up to four different file types per image: txt-files for general image annotations ("Manual-analysis_..."), ROI- and foci-annotations, and the image with ROI and foci masks saved as overlay. In the screen shot, the channels have been split, which means two images have been saved separately ("C1" and "C2", respectively).



FAQ

In the Visualization module, I am asked if I want to overwrite previous data. What does this mean?

This dialog refers to your image annotations. If you choose not to overwrite (e.g. because you have simply changed the color settings), the image annotations from the previous visualization are kept. If you choose 'yes', your current annotations (including empty fields) will be saved instead.

My images only have one plane. Which projection type do I use?

Select the option '**mid section**' – this will essentially skip the Z-projection step for images with only one slice. In the ROI Inspection module, use '**keep stack**', in the ROI quantitation module, select '**all slices separately**'.

The Maxima-txt-files generated by the 'Foci analysis' module contain duplicated values, i.e. the same focus as identified by the XY-position is listed several times.

What can I do?

If you use the 'manual curation' option and manually add foci with the Multipoint Selection tool, please make sure to **add the selection to the ROI Manager only once after selecting all desired foci**. The multipoint selection will initially appear as one ROI in the ROI Manager, but will be automatically resolved into separate selections by the QuaSI macro. These selections will be presented to you for final confirmation or further editing.

I have added a scale bar to my image assemblies in the 'Visualization' module, but I don't see it any more. What happened?

Scale bars are saved as Overlay. If you open the image panels with a program other than ImageJ/Fiji, you won't see them. If you want to include an image assembly with scale bar in e.g. a presentation, open the respective panel in ImageJ/Fiji, click Image>Overlay>Flatten. A copy will be created with the scale bar drawn onto your image. Save this in an image format of your choice and include it in your presentation.

I don't see the ROI masks on the images saved with the 'ROI inspection' module. What can I do?

ROI and foci masks are saved as Overlay. If you open the image panels with a program other than ImageJ/Fiji, you won't see them. If you need an image with the ROIs displayed on it, open the respective panel in ImageJ/Fiji, click Image>Overlay>Flatten. A copy will be created with the ROIs drawn onto your image. Save this in an image format of your choice.