General

The Fiji macro is used for single cell fluorescence characterization from microscopy images. It will extract single cell information regarding the size, position, and fluorescence signal in all channels. Additionally, it can measure fluorescence colocalization and count foci. The macro is compatible with any image extension but has only been tested on .lif, .ims, and .tiff files. The macro is also compatible with XY and XYZ images. When using a Z stack, the macro does not work in 3D but can work on projections, on all Z planes individually, or identify a focal plane for specified measurements.

To run an automated analysis, a few parameters need to be specified by the user. This requires some manual testing and visual inspection/validation of the selected parameters. A test function is available to test how certain combination of parameters affects the image analysis. Important parameters in this regard are how to properly segment nuclei, how to threshold positive signal for colocalization analysis, and how to threshold foci detection.

Installation

To use the macro, you need to first make sure you run the latest Fiji version (Help/Update ImageJ…) (last tested version was v1.54c) and download the macro (MMMEA.ijm). The macro can be called in Fiji (Plugins/Macros/Run…).

The macro uses pre-existing plugins in some of its function which need to be installed. The specific functions are described in later sections.

- To open images, the macro uses Bio-formats which needs to be added to the list of update sites of Fiji (Help/Update…/Manage update sites).
- If ROI segmentation is done via Stardist, Stardist also needs to be added to the list of update sites.
- To measure colocalization, the macro uses the EZcolocalization plugin. The plugin can be downloaded from https://github.com/DrHanLim/EzColocalization and moved to the plugin folder of Fiji.
- If additional channels need to be added and aligned to the original images, the macro uses the turboreg and hyperstackreg tools that can be downloaded from http://bigwww.epfl.ch/thevenaz/turboreg/
  and https://github.com/ved-sharma/HyperStackReg and moved to the plugin folder of Fiji.

Workflow

When started (Plugins/Macros/Run…), the macro will prompt a series of successive dialog boxes to specify parameters. These boxes and all the options are described below. The macro will then look in the specified folder for images with the specified extension. It will then process all images in batch.

On all images, the macro will:

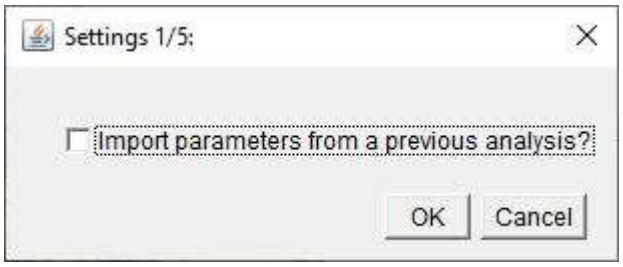
1. Identify the focal plane from a Z stack if the option is selected or make a Z projection if the option is selected.
2. Add and align additional channels if the option is selected.

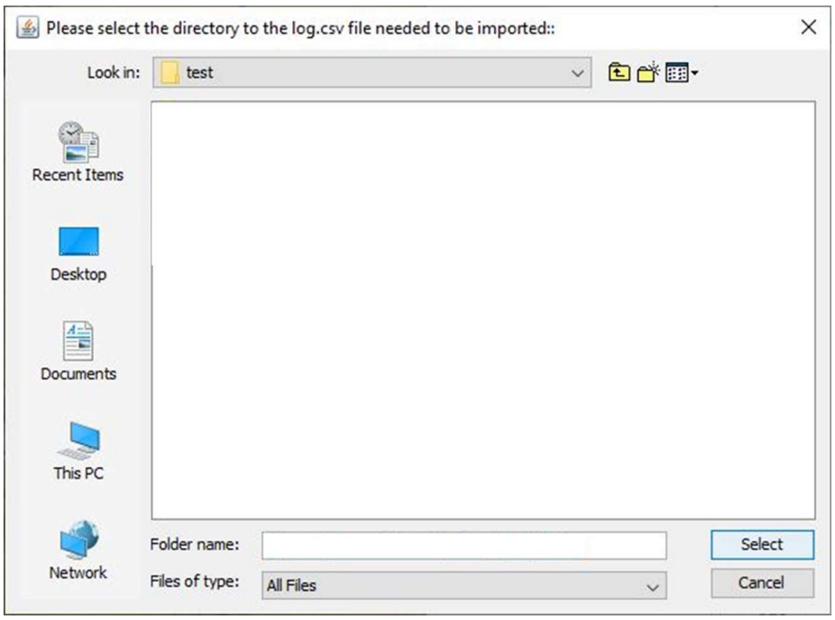
3. Remove images that have too high intensity pixels if the option is selected.
4. Subtract background noise from all channel/Z plane by the method selected.
5. Identify ROIs by thresholding or by Stardist segmentation.
6. Filter unwanted ROIs based on size, fluorescence signal or filter out-of-focus ROIs.
7. If the option is selected, measure colocalization in all ROIs by using the EZcolocalization plugin combined with the Coste's method of thresholding, Fiji built-in thresholding methods or the Find Maxima function.
8. Measure position, size, and fluorescence of all ROIs.
9. If the option is selected, count the number of foci in specified channel(s) of all ROIs.
10. Save results in a comma-separated values file for analysis, the ROI manager of each image, and a log file with all user inputs for traceability of analysis.
11. The macro can also save an adjusted .Tiff file, a .png montage, a .tiff file of the channels used in EZcolocalization, and an image of the ROIs before and after applying filters.

User input dialog boxes

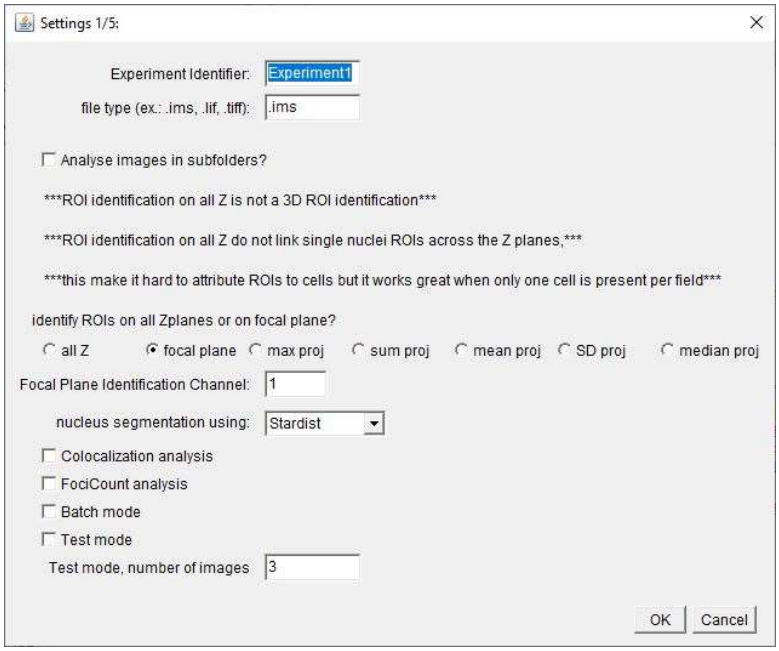
Use parameters from a previous analysis

The first dialog box asks the user if she/he wants to import user inputs from a previous analysis. If selected, this function will prompt the next window to ask the user to identify the folder that contain the "log.csv" file of a previous analysis. Once identified, the macro opens the "log.csv" file and load all user inputs from that analysis. This allows to quickly starts a new analysis on a new set of images with previously established parameters without the need to re-enter/define them again. If this is not desired, simply click on "OK" and the macro will prompt the next dialog box.





General settings



In the next dialog box, the user needs to specify general information/settings of the experiments.

The first row asks for an experiment identifier. This is needed to identify and create a result subfolder.

The next row asks for the file type of the images to be analysed. When the analysis starts, the macro will analyse all images in the selected folder that have this extension. This means that only one type of image can be analysed in a single analysis. .lif files contain several images as "series. When ".lif" is selected as a file type, all series will be automatically analyzed individually.

The next checkbox asks if you want to analyze images also in subfolders. This depends on your data structure. If selected, the macro will search in subfolders for images of the same filetype but will ignore analysis subfolders that were created in previous analysis.

The next parameter to enter defines how the macro should handle Z stacks. The macro can work on all Z planes, on a Z projection or on a single focal plane.

- When working with a XY image (Single plane, no Z stack), select the "all Z" option.
- When working on all Z of a Z stack, the macro does not allow 3D image analysis. It will identify ROIs on all Z planes individually and then perform measurements in all the ROIs on their respective Z planes. Thus, it does not identify ROIs through the Z stack as being part of a 3D ROI. As such, it is not recommended to use this option except if the user can link ROIs together in post-processing steps using their X and Y coordinates. This is usually doable when only a few ROIs are present in the image (commonly used on images with 1 to 8 ROIs).
- When working on a single focal plane, the macro will select the focal plane identification channel (identified in the next line of the dialog box) and loop through the Z stack to identify the highest variance Z plane. The highest variance plane is typically the focal plane of the image. It will be the plane showing the highest heterogeneity in pixel intensities and will have the majority of the ROIs in focus. In the case of cells grown in monolayer, and by using a DNA staining channel to identify the focal plane, this will have all the nuclei in focus, centered in the middle of the nuclei in the Z plane.

The next parameter to enter is only relevant if selecting the "focal plane" option above. If using a different option, just ignore this box. If using "focal plane", this parameter identifies which channel to use to identify the focal plane. Please enter the number of the channel to be used. The number refers

to its position in the order of the channels when opened in Fiji. Typically, any channel with specific signal works but a channel with nuclear DNA staining such as DAPI is typically used.

The next option to select is the ROI segmentation method to be used. Segmentation can be used by one of the Fiji built-in thresholding method or by Stardist.

- Fiji built-in Thresholding method is the standard way of segmenting and works well on well defined, well separated ROIs without the need for additional resources.
- The star-convex object detection neural network Stardist can provide improved segmentation, especially for hard to segment ovoid ROIs. To work, it needs a segmentation model that has been trained on manually curated nuclei segmentation images. For best performance, the model should be trained on manually segmented ROIs acquired under identical microscopy setup. Without such a model in hand, it is possible to test publicly available models and assess the appropriateness of that model. For more info, https://github.com/stardist/stardist).

The next parameters select the type of additional (optional) analysis such as colocalization and foci count.

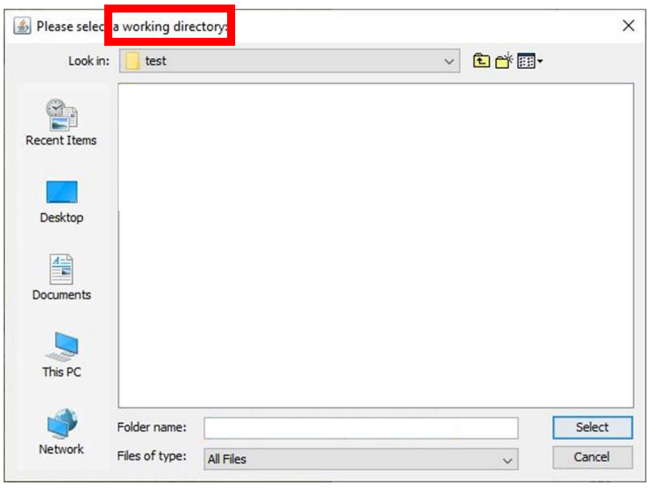
A Batch mode can be selected to process images without having Fiji opening the image windows.

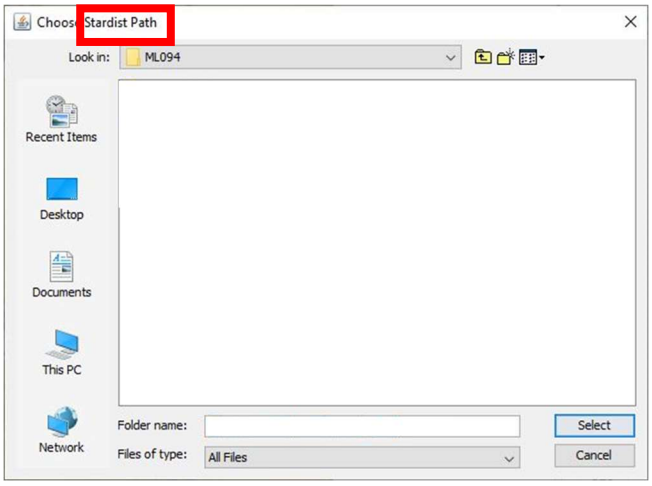
A test mode is available and can be selected to test an analysis and its combination of parameters on a limited number of images of the dataset.

Selecting the working folder

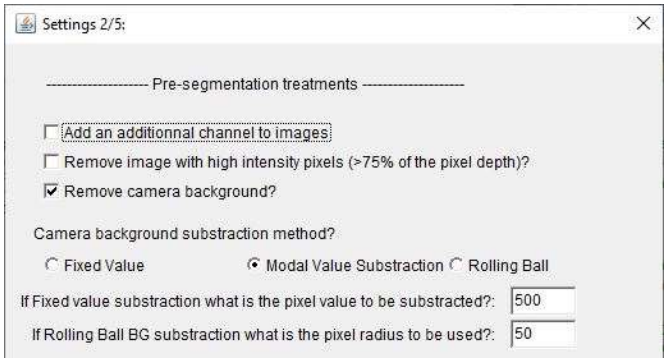
Once the general settings are established, the user is prompted to identify the folder in which the images to be analysed are located. The macro will then check if any images of the selected file extension are present in that folder and select all of them for analysis. The macro will also check if any spaces are present in the file paths or in the image names and, if present, replace the spaces with underscore as spaces in paths can cause problems.

Additionally, if Stardist has been selected as the method for ROI segmentation, the user will also be prompted to identify the folder that contain the Stardist model to be used.





Pre segmentation



Before segmentation, a few options are proposed.

First, an option for adding additional channels is available. This option can be used to manually add an additional channel obtained from an independent microscopy acquisition run of your experiment. The additional channel(s) will be added after the existing channels and one of the added channel can be selected as the segmenting channel for ROI identification by using the number it will have once added (for example, if two channels are added to an image containing three channels, they will be the channel 4 and 5). Information about this feature is describe later.

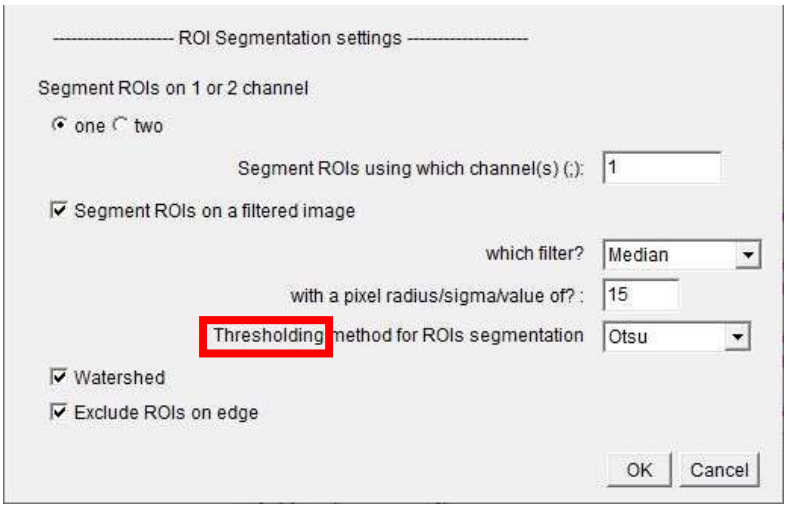
Another option proposed is to remove high intensity pixel images. The threshold defining a high intensity pixel is arbitrary fixed at 75% of the maximal pixel depth (maximal pixel intensity value defined by the number of bits of the camera used). The purpose of this option is to remove some acquisition artifacts that create false “hot” pixels. Such artefacts can skew automatic thresholding of signals, making only the few high-intensity pixels being considered as positive. In such case, it is sometimes better to just remove the image from the automated analysis.

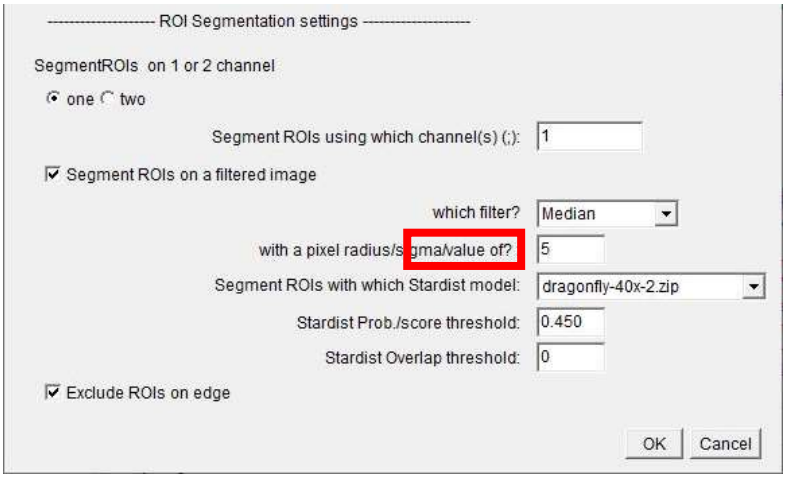
Lastly, three methods are proposed to perform background subtraction:

- It is possible to remove a fixed value. To establish which value to use, it is possible to image an empty well and measure the modal/mean/median value and use it as the value of background signal to be removed. It is also possible to open an image and draw an ROI where there is no cell/signal and then measure the background signal intensity.
- It is also possible to measure on all Z plane/channel/image the modal value and subtract that value as a measurement of the background signal. In our hands, the pixel intensity mode always represents the background signal intensity as there are always more background pixels than positive pixels and background pixels share the same value in contrast to positive pixel which display a larger intensity heterogeneity, independent of the cell confluency.
- Lastly, it is possible to use the rolling ball algorithm to subtract background, which evaluates the local background (https://imagej.net/plugins/rolling-ball-background-subtraction). When using this option, the radius in pixel of the largest object that is not part of the background should be given to the macro. This must be measured prior to the analysis if this option is selected.

Segmentation – Thresholding

For segmentation options, the window is different depending on which method has been chosen in the general settings.





First, in both cases, an option to segment on one or two channel is proposed and the user must enter the number of the channel(s) to be used for segmentation. The standard way is to segment on one channel (DNA staining channel). The option to segment on two channels is useful in specific cases and, if selected, the user must enter the number of the two channels to be used for segmentation, separated by a semi-colon.

For example, in the case of images without a DNA staining channel, we could successfully segment nuclei using the filtered signal from an RNAPIIpS2 antibody showing a strong nuclear staining. However, EdU-stained regions of the nuclear periphery were located outside of the RNAPIIpS2-segmented nuclei. In that case, better nuclei segmentation was achieved by creating an artificial channel by adding the pixel values of the RNAPIIpS2 channel to the pixel values of the EdU channel,.

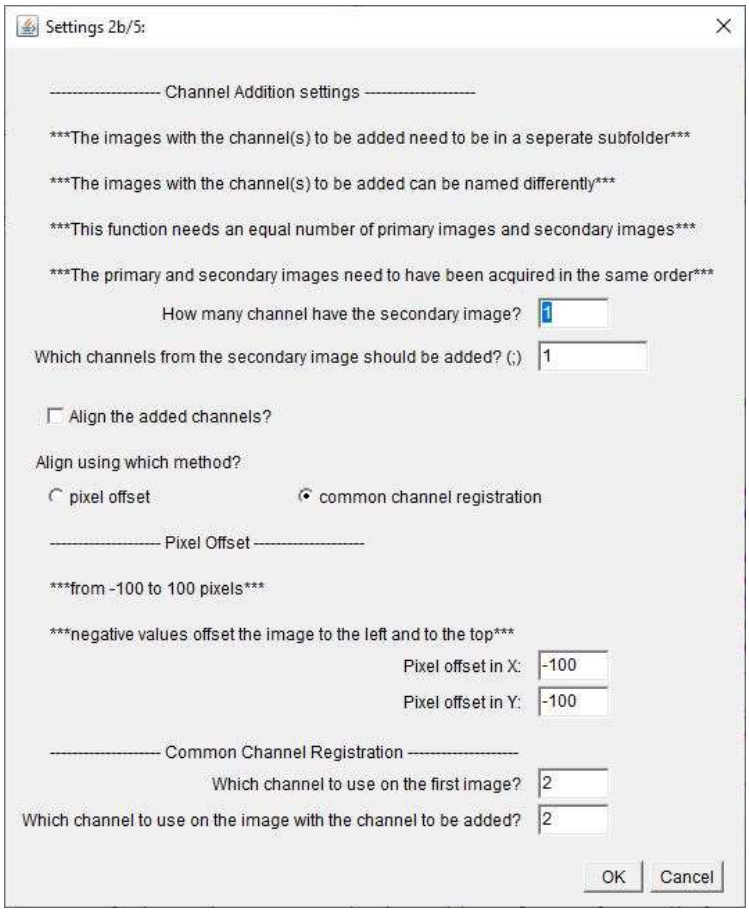
Then, an option to segment on a filtered image (median filter, gaussian blur, gamma filter) can be selected. This allows to “blur” the nuclei signal and, in some cases, allows a better segmentation.

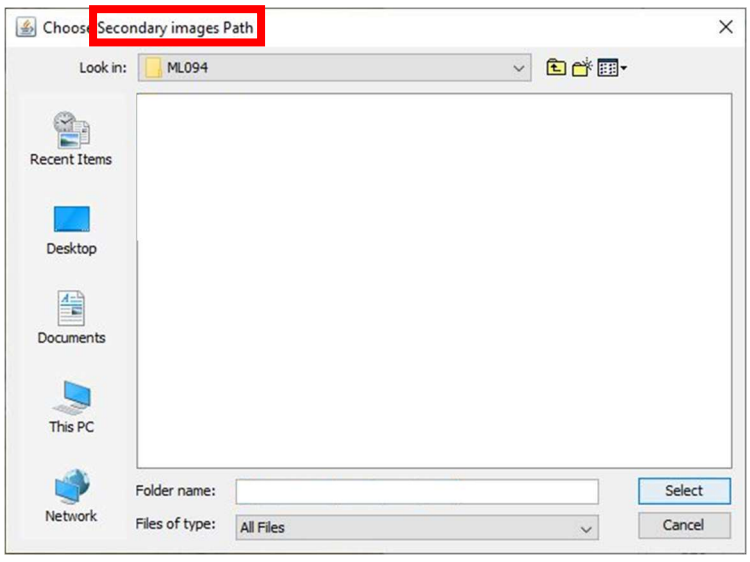
Once the choice of using filters, the choice of which filter and the related pixel radius to be used by the filter is chosen (if you don’t want to use a filter, ignore the filter related boxes), the user must select which Fiji built-in thresholding method to be used to segment ROIs.

You can test manually the effect of the filters, of the pixel radius used by the filter (Process/Filters…), and of the choice of thresholding method (Image/Adjust/Threshold…) before the analysis to test the appropriateness of the combination of parameters selected for segmentation. In addition, it is possible to use the test mode to test the effect of different combination on the quality of segmentation.

An option to remove ROIs that touch the border of the image can be selected (recommended) and an option to use a watershed can also be selected. Watershed allows to separate touching ROIs. If possible, maintain the confluency of the cells to a level that you don’t need to use it because sometimes it can split an ROI representing a proper nucleus.

If Stardist was chosen as a segmentation method, most of the options are the same, except that the choice of the thresholding method is replaced by the choice of Stardist trained model. The user should have provided the path to the folder containing Stardist models previously and the user can here now select the trained model needed that will be used for segmentation. Additionally, Stardist specific parameters should be specified, the Probability/Score Threshold (typically at 0.45) and the Overlap Threshold (tolerance threshold for overlap – typically at 0). In most cases, we found that it is not needed to change or test those parameters.





Channel Addition Option

If the option to add a channel was selected, an additional dialog box will be opened. This option, as stated before, relates to adding channels of the same fields to circumvent certain acquisition problems. To be useful, a few criteria must be met.

First, the exact fields of view in terms of XYZ imaged in the first acquisition must be reproduced in the second acquisition. This will allow segmenting ROIs in a channel of one of the two acquisition and measuring in channels of the second acquisition.

For the macro to be able to add channels, the two acquisitions must be in different folders. To add the correct secondary acquisition channels to the correct primary acquisition, the macro needs the number of images in both primary and secondary acquisitions to be the same and it will then sort them alphabetically and add the first secondary image to the first primary image and go through the list in order. This affects the way that images should be named. Typically, acquired images are numbered in order of acquisition and in such way, acquiring the same fields in the same order allow adding the correct secondary images to the correct primary images.

If these rules for acquiring secondary images of the same fields are followed, adding channels from one acquisition to the other is simple. The user needs to specify how many channels have been acquired in the secondary image and which channel should be extracted from this image and added to the primary image.

If there is a small drift in X and/or Y, it is possible to align the images to correct for this artefact by selecting the option for alignment. Two methods are proposed in the dialog box.

The first method allows a systematic XY drift correction between the two images by applying a XY pixel offset. To know the exact XY correction needed to apply, the user will have to manually quantify the offset needed for alignment by testing it either in the test mode or manually on typical images (Image/Transform/Translate…). This tool is limited to an offset of up to 100 pixels in either direction. By entering positive pixel offset value, the secondary image will be moved either towards the bottom or towards the right. By entering negative values, the secondary image will be moved towards the top or towards the left.

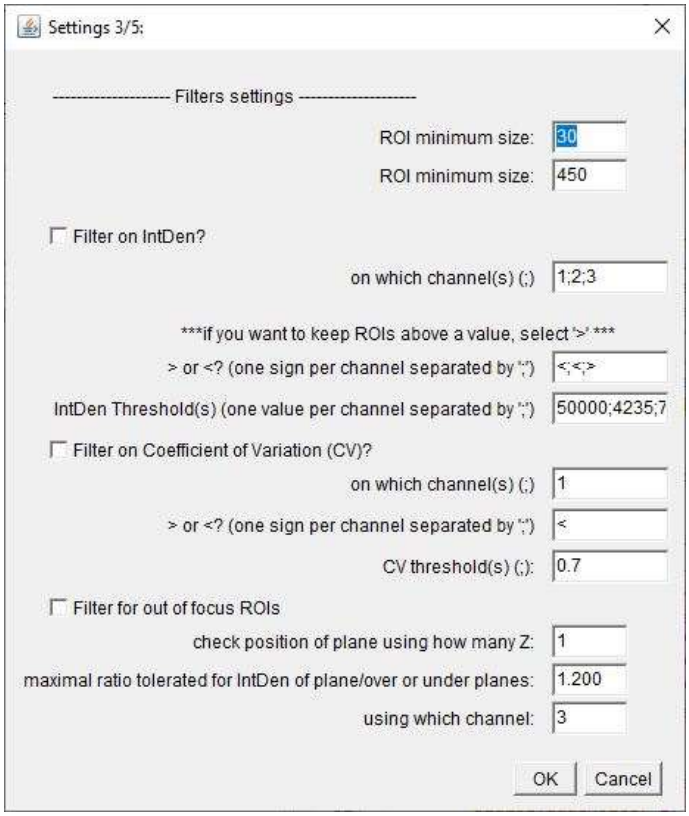
The second method of alignment is by common channel registration which will measure and automatically align the offset between the two images and correct it. There is a special requirement regarding the use of this method. The secondary acquisition should reacquire an existing channel in the primary acquisition. This common channel will then be used to identify “landmarks” in that channel and align them between the acquisitions. To allow accurate alignment, this common channel should be a channel with consistent and abundant signal. Indeed, trying to align an image using faint, rare landmarks or using the background noise will create insufficient alignment. When selecting common channel registration as a method for alignment, the macro needs to know which channel number in the primary and secondary images is the common channel to be used for alignment. If the alignment was unsuccessful by using this method, the image will be removed from analysis and the name of the image will be added to the log.csv file for traceability.

ROI Filtering

Once ROIs have been segmented, it is possible to remove bad quality ROIs from the analysis by applying different filters. To properly use filters, the user needs to manually establish filtering threshold values before the analysis or not use them and filter after the analysis. The only filter that is required is a size filter, as a ROI minimum and maximum size parameters are required in subsequent steps of the macro. However, to avoid size filtering, it is possible to work around this requirement by putting very low and high values. Indeed, in principle, no filter step is required as it is fully possible to filter ROIs on size and fluorescence after the analysis is completed.

The size filter needs a minimal and a maximal ROI size. If used properly, it allows the removal of cell debris, fragmented cells, fused ROIs, badly segmented ROIs, and other segmentation artefacts. The values to put

as minimal and maximal size should be in the units given in the images metadata (if the images are given in µm, size filter parameters should be in µm. If there is no distance unit provided, the parameters should be in pixels).



Additional filters can be applied on any channels based on fluorescence signal (IntDen) or on heterogeneity/ homogeneity of the signal (CV). The IntDen filter allows to remove ROIs that have a fluorescence signal in any channels above or below a certain threshold while the CV filter allows to remove ROIs that have homogeneous or heterogeneous signal in any channel in term of pixel intensities. The CV filter has been used, for example, to remove ROIs that had a false-positive high signal in a specific channel due to the signal of an adjacent ROI bleeding into the neighboring ROIs. In that case, these ROIs would have a high CV due to the presence of mainly low intensity (negative) pixels and high intensity pixels from the neighboring ROI.

To use those filters, the appropriate filter must first be selected in the dialog box. Then, three parameters must be entered. First, each parameter can be applied to multiple channels that need to be specified. In the case of multiple channels to be filtered, the number of all the appropriate channels must be entered separated by a semi-colon (;). The second and third parameters follow the same rules as the channel selection. If multiple channels are selected in the first parameter, the second and third parameters need the specific information replated to the selected channels in the same order. The second parameter regards in which direction the filters are applied ("<" or ">") and the third parameter regards the threshold value of the filters. For example, in the "Settings 3/5" dialog box above, if the "Filter on IntDen?" box would be ticked, every ROI with an IntDen below 50000 in channel 1 would be removed; every ROI with an IntDen below 4235 in channel 2 would be removed; and every ROI with an ROI above 7000 would be removed. Additionally, if the "Filter on CV?" would be ticked, every ROI with a channel 1 CV above 0.7 would be removed from the analysis.

Lastly, it is possible to filter out-of-focus ROIs. This filter is only relevant if the images contain Z stacks, and the analysis is done on a single focal plane. It is also less relevant if the ROIs are all on the same focal plane. One type of ROI where this can be useful is on the analysis of nuclei of mESCs which grow in 3D colonies. In that case, on the selected focal plane, some ROIs are positioned centered on the focal plane, but some others are centered on a higher or lower Z plane. This creates the situation that some cells would be analysed in the center of their nuclei while others would be analyzed in their nuclear peripheric regions. To remove the latter, the macro can remove out-of-focus nuclei by measuring the signal in a selected channel above and below the focal plane using the information of the Z stack, and then compare it to the signal on the focal plane to estimate for each ROI if it is in focus or not. To use this filter, it must

first be selected in the dialog box. Then, the macro needs the information about how many Z above and below of the focal plane measurements should be done to determine if ROIs are in focus. The next parameter is the threshold ratio of signal (IntDen) between the planes above or below and the focal plane that establish what is an out-of-focus ROI. The last parameter is the channel number that these measurements should be done on. For example, in the "Settings 3/5" dialog box above, if the "Filter for out of focus ROIs" box would be ticked, every ROI displaying an IntDen 1.2x bigger as measured in the third channel, 1 plane above or below the focal plane, would be removed.

Measurements

All ROIs will be measured in terms of their location, shape, and size. Additionally, fluorescence measurement will be done in all channels. The typical list of measurements for images containing 4 channels will be as such:

"ExperimentID,Image,ROIname,Z,ROI,area,Xcentroid,Ycentroid,XcenterOfMass,YcenterOfMass,Perimeter,XBoundingRectangle,YBoundingRectangle,FitEllipse_Width,FitEllipse_Height,FeretDiameter,Roundness,Solidity,c1_mean,c1_SD,c1_median,c1_mode,c1_min,c1_max,c1_IntDen,c1_skewness,c1_kurtosis,c2_mean,c2_SD,c2_median,c2_mode,c2_min,c2_max,c2_IntDen,c2_skewness,c2_kurtosis,c3_mean,c3_SD,c3_median,c3_mode,c3_min,c3_max,c3_IntDen,c3_skewness,c3_kurtosis,c4_mean,c4_SD,c4_median,c4_mode,c4_min,c4_max,c4_IntDen,c4_skewness,c4_kurtosis".

For information on each parameter, please consult https://imagej.nih.gov/ij/docs/menus/analyze.html.

Colocalization measurement

In addition to standard measurements, it is possible to measure colocalization between 2 channels. This option uses the EZcolocalization plugin which must be installed (see the installation section above) (https://github.com/DrHanLim/EzColocalization). The macro allows you to measure colocalization between 2 different pairs of channels and each channel number in each pair of colocalization measurement needs to be specified in the "channels to measure" boxes (separated by semi-colon).

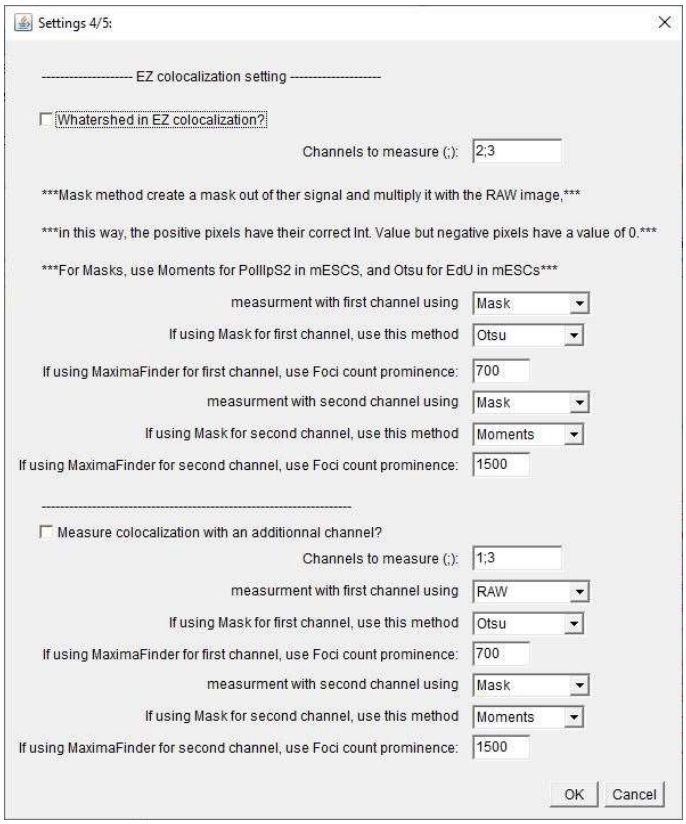
The macro will use the previously segmented ROIs to measure colocalization in all of them but EZcolocalization takes the ROIs, makes a mask out of the ROIs, and segments the mask image itself to establish ROIs. As such, if watershed was used in the segmenting step, it is recommended to ask EZcolocalization to watershed also in this "resegmenting" of ROIs. If so, the "Watershed in EZcolocalization?" box needs to be selected.

Several different mathematical quantifications can be done to quantify colocalization. EZcolocalization provides the options of the Threshold Overlap Score (TOS), Pearson Correlation Coefficient (PCC), Spearmen's Rank Correlation Coefficient (SRCC), Intensity Correlation Quotient (ICQ), and Mander's Correlation Coefficient (MCC or M1 and M2). The MCC gives you the reciprocal colocalization measurement, M1 and M2 (how much channel 1 colocalizes with channel 2 and how much channel 2 colocalizes with channel 1). In our hands, The M2 value is not always accurate. To circumvent this, each pair of colocalization measurement is measured twice, alternating the channel orders. This allows the measurement of two M1 which represents reciprocal measurements. The typical list of measurements for measuring the colocalization, for example between channel 2 and 3, will be as such:

“c2_c3_TOS(linear),c2_c3_TOS(log2),c2_c3_PCC,c2_c3_SRCC,c2_c3_ICQ,c2_c3_M1,c2_c3_M2,c3_c2_TOS(linear),c3_c2_TOS(log2),c3_c2_PCC,c3_c2_SRCC,c3_c2_ICQ,c3_c2_M1,c3_c2_M2”.

To learn which coefficient is more appropriate to each use, please read: “Kenneth W. Dunn, Malgorzata M. Kamocka, and John H. McDonald, A practical guide to evaluating colocalization in biological microscopy, Am J Physiol Cell Physiol. 2011, DOI:10.1152/ajpcell.00462.2010”.

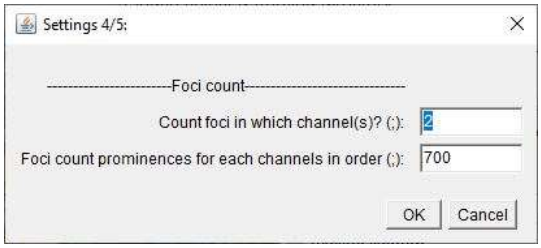
Lastly, the EZcolocalization plugin uses the Coste’s method to identify the thresholds between positive pixels and negative pixels (https://doi.org/10.1529/biophysj.103.038422). The Coste’s method, in our hands, can cause some thresholding artefacts. When there is high intensity signal present in the image, it often leads to low intensity pixels being thresholded as negative pixels. For that reason, the macro gives you options to circumvent Coste’s method problems.



In this dialog box, the user must enter for each channel which thresholding method to use (“measurement with first/second channel using”).

- By selecting the “RAW” option, non-modified images will be provided to EZcolocalization and the Coste’s method will be used.
- By selecting the “Mask” option, the macro will threshold the channels using one of the Fiji built-in thresholding methods. The desired method should then be specified, and it is recommended to manually test and assess the appropriateness of the thresholding. By thresholding in this way, the macro will create a mask image based on the thresholding result and use it to artificially make all negative pixels of the channel have an intensity of zero while positive pixels will keep their original values. A potential artefact of this method occurs when there is little to no positive signal. In that case, the built-in thresholding methods tend to consider negative pixels as positive.
- By selecting the “Maxima Finder” option, the macro will identify foci using the “Find Maxima…” feature (Process/Find Maxima…) and use the result to create a mask as in the previous option. This option is available but not recommended as the result of the Find Maxima option is a mask where only a single pixel for each focus is considered positive. To use this option, the user needs to specify which prominence should be used by the tool to identify foci. The prominence is used to define the stringency of foci identification and should be manually tested prior to the analysis.

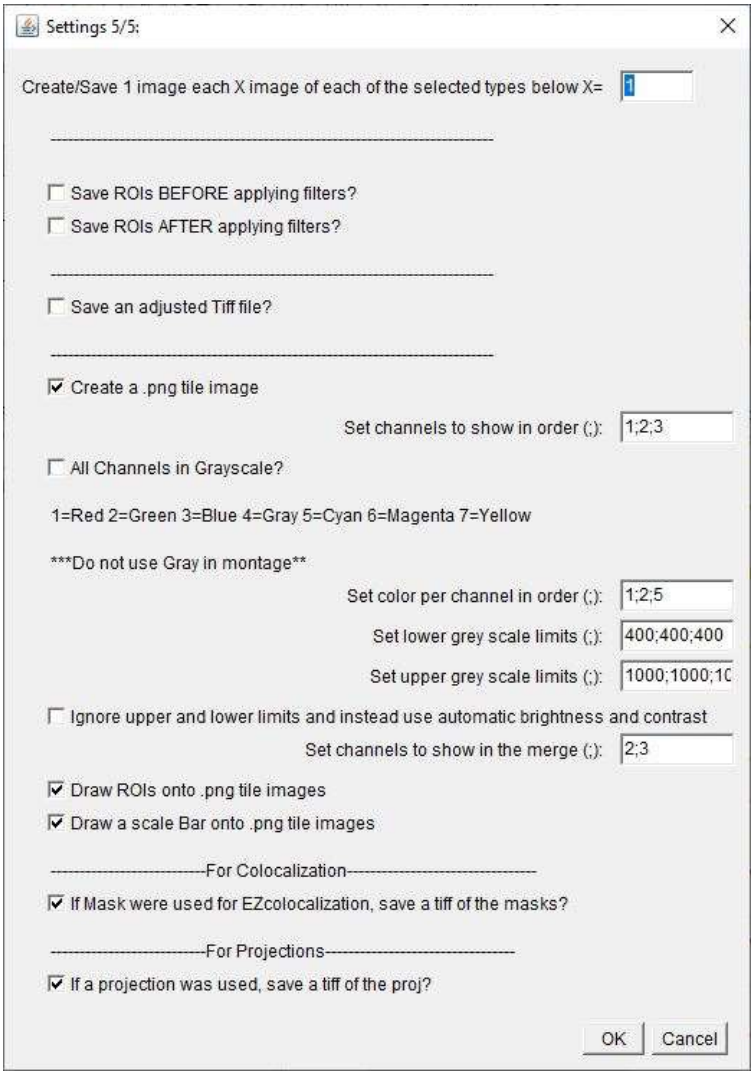
Foci count



In addition to standard measurements, it is possible to quantify the number of foci in any channels. To do so, the user must specify the number of the channel(s) in which he wants to count foci (separated by semi-colon). For each channel, the user must define the prominence to use to define foci (in order, separated by semi-colon). The prominence defines the stringency of foci identification.

The typical list of measurements for measuring the foci count, for example in channel 2, will be as such:

"c2_FociCount ".



Saving options

The standard output documents saved are:

- a result .csv document with all measurements for all analysed ROI for all images;
- the Fiji ROI manager .zip files for all images to allow to link measurement to their original ROI;
- a log.csv file that contains all user-inputs and information about which images have been removed from analysis to allow data traceability and transparency of analysis.

Several additional saving options are available. To reduce the size and number of files created, it is possible to save the selected optional files for only one image each X.

- It is possible to save an image with all ROIs before and after applying filters to verify the usefulness of filters.
- It is possible to save an adjusted (background subtracted and auto-contrast) .tiff image.
- It is possible to create a .png tile montage displaying all the individual channels with the ROIs borders and an overlay panel for rapid visualization of the images. When selecting this option, several parameters must be specified.
    - First, the channels to show (in order and separated by semi-colon) should be specified.
    - Then, the user has the possibility to show the channels in grayscale ("All Channels in Grayscale?" box) or in color. If using color, the user must specify the display color of each channel in order using the numerical code shown in the dialog box. Please, do not use

grey (4) as a color outside of the “All channels in Grayscale” option as it will create errors for unknown reasons.

- For visualization, the user must specify if the channels should be displayed using automatic brightness and contrast, or if he wants to use fixed values to allow direct comparison of signals intensities between images. If so, the lower and upper grey scale limits should be specified in order and separated by semi-colon. In the dialog box example, the channels 1,2 and 3 would be shown in red, green, and cyan respectively with their grey scale limits being set at 400-1000 for channel 1, 400-1000 for channel 2, and 400-1000 for channel 3.
- Then, a merge panel will be added with the channels specified in the “Set channels to show in the merge” box.
- Lastly, an option to show ROIs borders in the images and an option to show a scale bar is available.

- It is possible to make a .tiff file with only the two channels used for EZcolocalization. This is useful to assess the appropriateness of the thresholds applied to the channels to identify positive pixels.
- If a projection has been used, it is possible to save the projection as a .tiff file.