Guidance to count foci using AutoFoci

Requirements/Preparation:

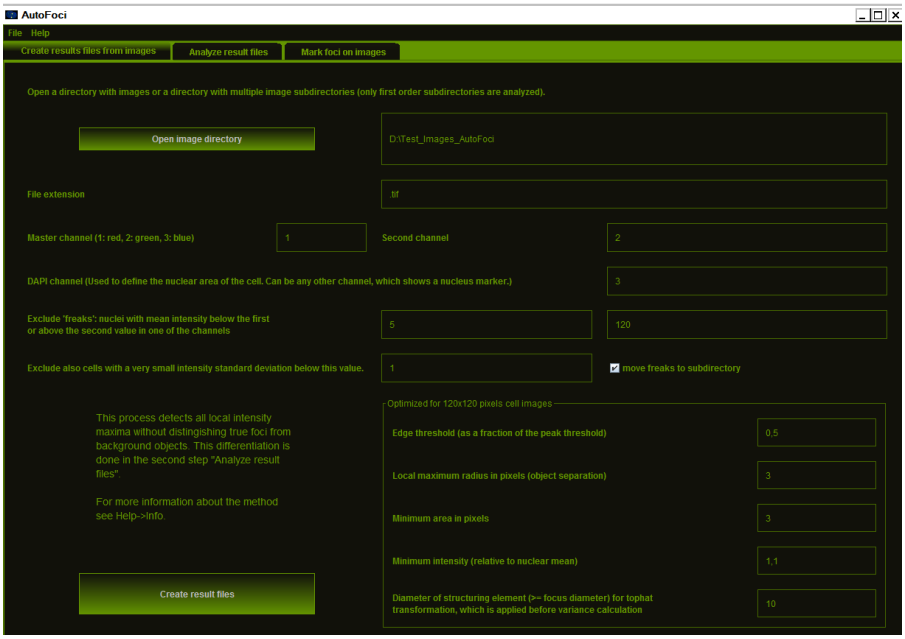
- Computer equipped with at least 8 GB RAM and a version of Java 8.
- Download the provided AutoFoci.jar file and execute it. On Linux system you might have to execute it via command line: java -jar autoFoci.jar
- Download the test images provided as "Test_Images_AutoFoci" zip file and unpack it. If you want to analyze your own images, please use the procedure described in the file "Guidance to process microscopic overview images using Cellect" (https://github.com/nleng/AutoFoci/blob/master/ImageJ/Guidance_to_process_images_using_Cellect.pdf) to generate appropriate single cell images.

Please note: AutoFoci was designed to analyze persisting foci after low radiation doses in single cell images created by using the best plane out of 5 recorded image planes for each damage marker as described in the manuscript of N. Lengert *et al.* (submitted to Scientific Reports). The selected parameters and used algorithms in AutoFoci are optimized for that condition. However, AutoFoci is able to handle single cell maximum intensity projection (MIP) images, which we recommend to use, if you aim to quantify foci after higher radiation doses and/or at earlier time points. We provide information on how to create these MIP images by using Cellect in the guideline "Guidance to process microscopic overview images using Cellect". Further adjustments of the algorithms and/or user defined parameters might be necessary depending on your needs (for detailed information about the parameter settings and the algorithms see the last section in this document and the manuscript by N. Lengert *et al.*).

Automated counting of foci:

1. Open AutoFoci and start on the first tab "Create results files from images". Enter the path of folder containing the single cell images e.g. the provided folder "Test_Images_AutoFoci" you want to analyze by clicking on the button "open image directory" (in our example D:\Test_Images_AutoFoci). Please note that it is also possible to specify a folder with multiple subfolders (containing images) as file path to enable a fast analysis of multiple samples. All images in first-level subdirectories will be analyzed.

   No parameter changes are necessary when analyzing the provided test images. If you are analyzing own samples irradiated with higher doses and/or aim to analyze earlier time points, you may want to adjust these parameters. The last section in this document will provide detailed information about the used parameters.



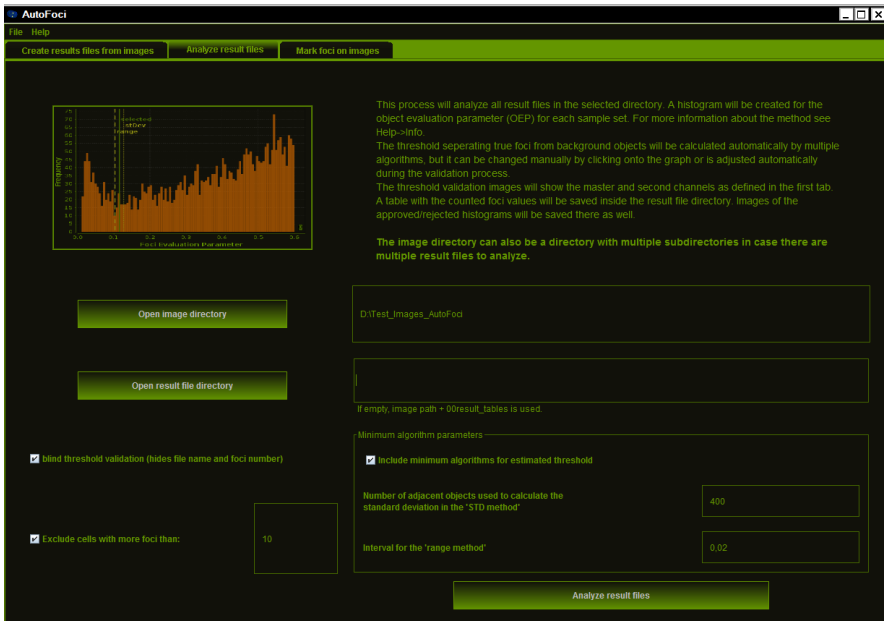
2. Proceed by clicking on the button "Create result files" in the lower left corner. This will open a "Progress window" showing you the progress of the object detection in your images. After reaching 100%, the object detection is finished and this window can be closed. The result files are stored automatically in a folder called “00result_tables” (in our example D:\Test_Images_AutoFoci\00result_tables).

   Additional folders might be created in this subdirectory if you select the option "move freaks to a subdirectory". There are several parameters to exclude images showing ‘freaky’ cells, which are defined as cells with either very low or very high signal intensity or a very low standard deviation of the signal intensity in one or both channels depicting the damage marker γH2AX or 53BP1. Depending on the reason that led to the exclusion of the cells, they will be moved to three different subdirectories called “00freaks_low”, “00freaks_high”, “00freaks_std”.

3. Go to the second tab "Analyze result files". Again, enter the file path of the folder containing the single cell images by clicking on the button "open image directory" (in our example D:\Test_Images_AutoFoci). You can either specify the file path of the result file or just leave it empty, both options will work if you have not moved the result file to another destination.

   We recommend to select "blind threshold validation", which hides the sample name (which is quite useful if more than one sample is analyzed) and the number of foci per cell during the next steps, to ensure a reliable and unbiased analysis. However, for inexperienced experimenters in foci counting it might be helpful to disable this funktion or click "Unblind" in the histogram window (in step 4) to track changes in foci number during threshold validation. It is also relevant to note that the threshold for foci detection in the OEP distributions can be manually moved into regions with clear foci or clear background signals which can help to benchmark any user’s foci rating.

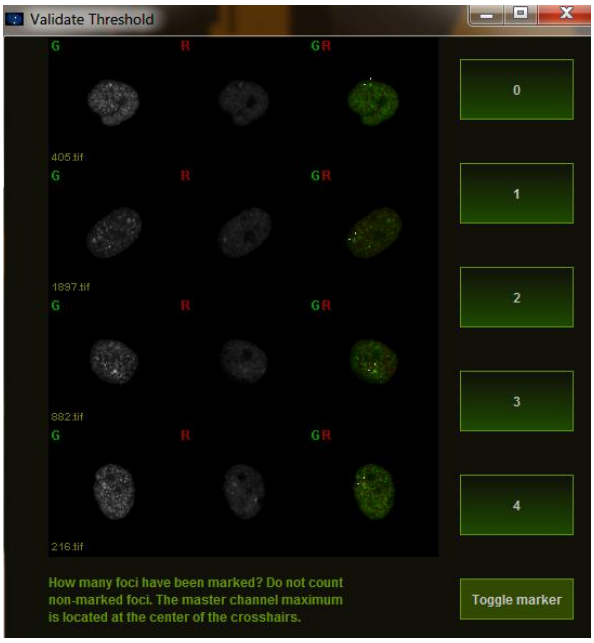
No parameter changes in the bottom right of the window are necessary when analyzing the provided test images. For further information about the input parameters, please see the last section of this guide.



4. Proceed by clicking on the button "Analyze result file" in the lower left corner. Another window will open showing histograms of the so-called Object Evaluation Parameter (OEP, defined in the manuscript by N. Lengert *et al.* submitted to Scientific Reports)). An inverse representation of the logarithmic OEP and the normal representation of the logarithmic OEP show a bimodal distribution for datasets with good staining and image quality. Please note that is it possible to zoom in the OEP histograms if high numbers of detected background signals affect the visibility of the first peak and the minimum. For this, draw a rectangle that includes the region of interest and the program will display this section enlarged. The bar chart shows the comparison of the data to an ideal Poisson distribution. Thus, these graphs provide a quick quality check before the threshold used to determine the number of foci per cell (depicted as red line in both histograms) is validated.

   The start threshold is calculated automatically as the mean derived from different algorithms (depicted as green lines) and marks the transition of true foci (first peak in the left histogram) and background signals (second peak in the left histogram), which is located around the minimum visible in the inverse histogram. For further information please see the manuscript by N. Lengert et al. (submitted to Scientific Reports). If the minimum in the OEP histograms is not pronounced when analyzing your own samples or MIP images, you may want to go back to step 3 and exclude the minimum algorithms for threshold estimation by disabling the option "include minimum algorithms". This will help to achieve a better start value for the manual threshold adjustment in the next step

5. Click on the button "Threshold Validation" to start the manual validation of the calculated threshold. A new window will open, showing you 4 cells each having one "critical" object close to the threshold, which is marked by the option "Toggle marker". Now, you have to decide how many of the marked objects are real foci according to your personal evaluation criteria. Enter the number of objects you indentify as foci by clicking the button or pressing the keys "0", "1", "2", "3" or "4" (which might be more convenient for a fast evaluation process).



The threshold is moved according to your selection and a new set of cells is displayed. This process is repeated multiple times and ends automatically when the resulting mean foci number (calculated from the last 6 thresholds) is smaller than 5% of the mean. If the validation was successful, the "Validate Threshold" window is closed automatically and the final number of foci per cell is displayed in a new window.

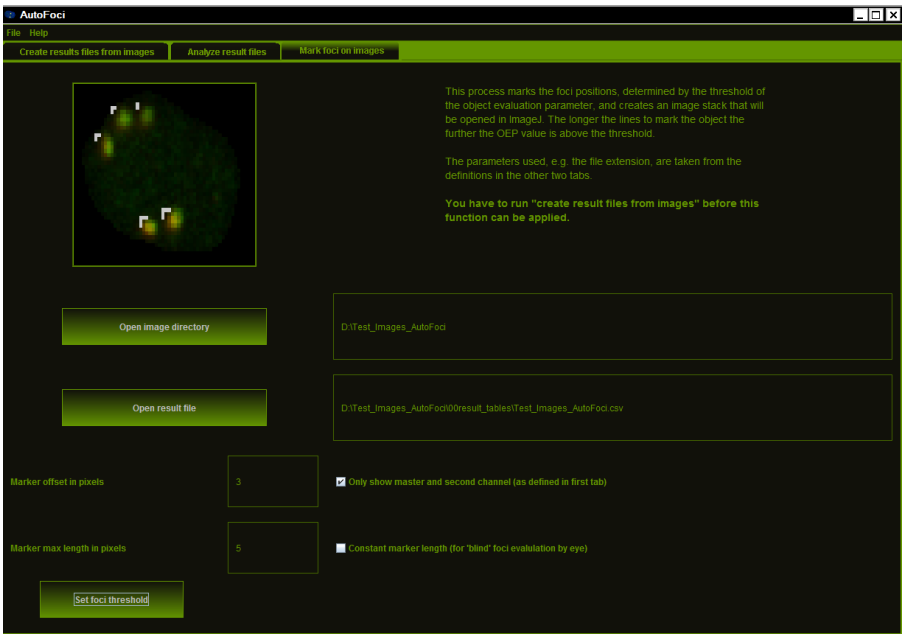
6. To save the final histograms and the number of foci per cell, click on "Approved" for a good data set or "Reject" for a data set with insufficient quality. A screen shot of the "Histogram" window can be found in the result directory. Depending on your selection, it will be stored either in a subfolder "approved" or in the folder "rejected". The sample name and the final number of foci per cell are visible in the screen shot even if you have not clicked on the button "Unblind".

Mark foci on images:

This is an additional function of AutoFoci and allows to mark the detected foci in the single cell images (after threshold validation). IMPORTANT: It is mandatory to perform step 1 and the step 2 of "Automated counting of foci" the previous method for counting foci to run this function. Then, continue with step 3 described below.

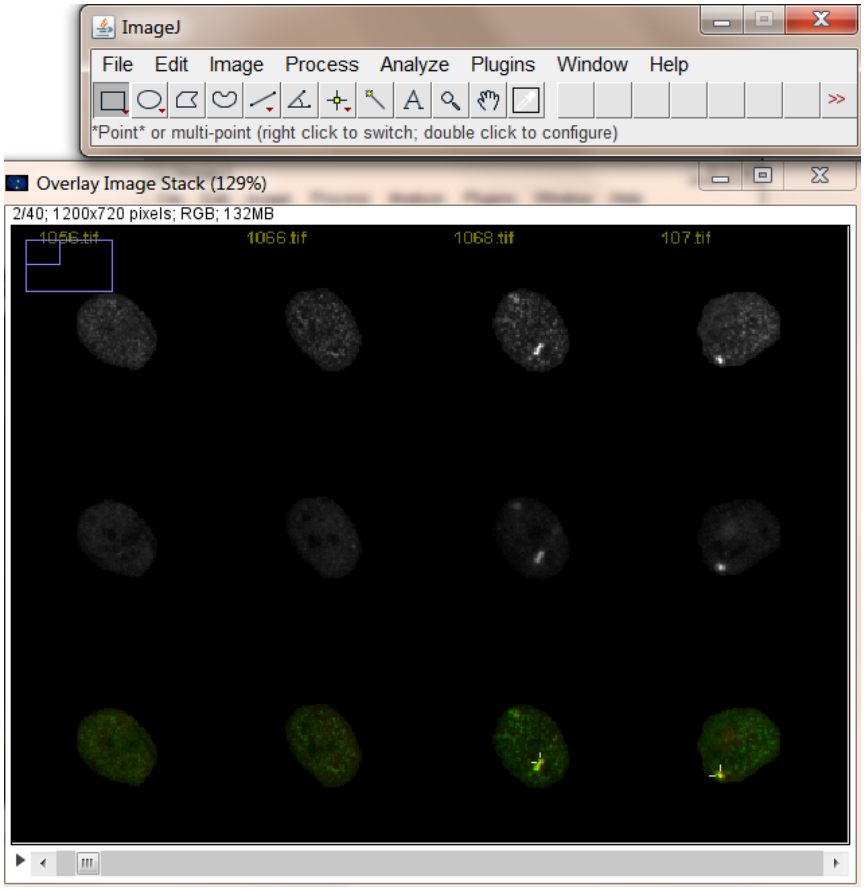
3. Enter the path of folder containing the single cell images e.g. the provided folder "Test_Images_AutoFoci" you want to analyze by clicking on the button "open image directory" (in our example D:\Test_Images_AutoFoci).

   Enter the file path of the result file generated in the second step. Here, the .csv file saved in the folder "00result_tables" is needed (in our example D:\Test_Images_ AutoFoci\00result_ tables\TestImages_AutoFoci.csv).



4. Proceed by clicking on the button "Set foci threshold". This will open a new window "Histogram". Now validate the threshold as described above in step 5 of "Automated counting of foci".

5. After you completed the validation, click on the button "Create images with markers". ImageJ will open automatically showing a gallery of single cell images, where detected foci are marked. Please save this "overlay image stack" in a desired destination.



Detailed information about the used parameters

The following parameters were optimized for images with a size of 120x120 pixels and for object detection using the first channel depicting the damage marker 53BP1, which is why this channel was defined as so-called "master channel".

Local maximum radius in pixels:
Objects are defined as pixel with an intensity value higher than any surrounding pixel (local maximum) in a given radius. The radius (set to 3 pixel by default) thus determines the minimum separation distance between two objects. This means that even if a second object with a lower intensity is present within the radius, it is not recorded separately. However, it only has a small influence on the number of detected foci as only very few foci are closer than 3 pixels to each other, especially in the performed repair studies after low doses.

Minimum intensity (relative to nuclear mean):
Objects that consist of pixels with a signal intensity lower than the mean intensity in the nucleus multiplied by a factor (factor of 1.1 by default), will be excluded from the further analysis steps. This parameter can be used to adjust the detection of background signals.

edgeThreshold:
This value determines which pixels will be included in the object area. Objects are identified by local intensity maxima in a given radius. Every adjacent pixel that shows a signal intensity of at least 50% (factor of 0.5 by default) of the local maximum and a higher signal intensity than the minimum intensity (see previous parameter) for the whole nucleus will be added to the object area.

Minimum Area:
If the object area determined by the edgeThreshold remains below a given threshold (by default 3 pixels), the object will be excluded from further analysis.

Diameter of structuring element:
Top-hat transformation requires a structuring element to perform an operation called “morphological opening”. Generally, this value should be larger than the size of big foci (set to 10 pixel by default) to ensure that smaller structures will be visible afterwards.

Parameters for minimum algorithms
The mean threshold determined by nine different algorithms is used as start threshold for the validation process. A detailed explanation of all algorithms can be found in the Supplementary Information of N. Lengert *et al.* (submitted to Scientific Reports). Two of the algorithms used to automatically estimate a threshold depend on a visible minimum in the inverse representation of the logarithmic OEP. In case the bi-modality is not pronounced enough in the histogram of a given sample set, those algorithms can be excluded from the automatic threshold calculation by disabling the option “Include minimum algorithms for estimated threshold” to still get a reasonable start threshold.

User defined settings are possible for these two algorithms detecting the minimum in the inverse representation of the logarithmic OEP. Here, the number of adjacent objects, which is used to calculate the standard deviation in the 'STD method', as well as the interval for the

'range method', in which the number of objects will be counted, can be set manually. However, usually there is no need to modify those parameters.

Poisson Quality Check

The bar plot at the right side of the "Histogram" window shows the frequency of cells with no focus, one focus, two foci, three foci, etc. within the analyzed cell population together with the corresponding Poisson distribution (calculated for the same average number of foci per cell). The deviation between both distributions is calculated as the Kullback-Leibler (KL) divergence as well as the sum of the squared residuals.

As reference values, AutoFoci displays the corresponding KL divergence and the sum of the squared residuals for an ideal experiment with a similar number of cells and a similar mean number of foci per cell in round brackets. These values were generated by simulating 100,000 ideal experiments and determining the value that was only exceeded by 5% of all simulated experiments (95% confidence interval). A clear deviation from Poisson indicates the existence of a second underlying distribution, which might have a negative influence on the quality of the results.