

Introduction and general recommendations

For every analysis with the Stripenator please consider that the analysis can only be as good as the quality of your experiments and images. This is dependent on cell seeding, staining, microscope setup and finally image quality. The Stripenator works with many image file formats but needs multi-channel image files like ZVI, CZI, LIF, AF or TIFF. Raw data format should be used, because using jpgs or other compressed formats can distort the results. The raw format is based on your microscope camera. Usually these images possess more than 8-bit grey scale per channel (Fluorescence images are usually generated with a grey scale camera and have “no colour” in the raw format.) Furthermore, we recommend structuring the folders by for example cell lines or trial before producing the images and to only save images into the folders; consequently, the analysis will run more smoothly without further reorganization of your files.

In the following Step-by-Step manual, ImageJ *operations* and *windows* are formatted in *Italic*.

Cell seeding

The seeding is based on many factors like cell proliferation rate, test dish/well size and surface, as well as experimental conditions including treatment and time points. Optimize the conditions to achieve a cell monolayer of about 70 to 80% cell confluency at the time of imaging. You might reduce the seeding if you plan longer time points after laser irradiation. Be aware of cells moving e.g. out of the irradiated frame. We would recommend point or square irradiation for long time points. Nevertheless, the Stripenator can still analyse cells with stripes which are turned in different directions in one image as a result of cell motility over time.

Installation and initialization of the Stripenator

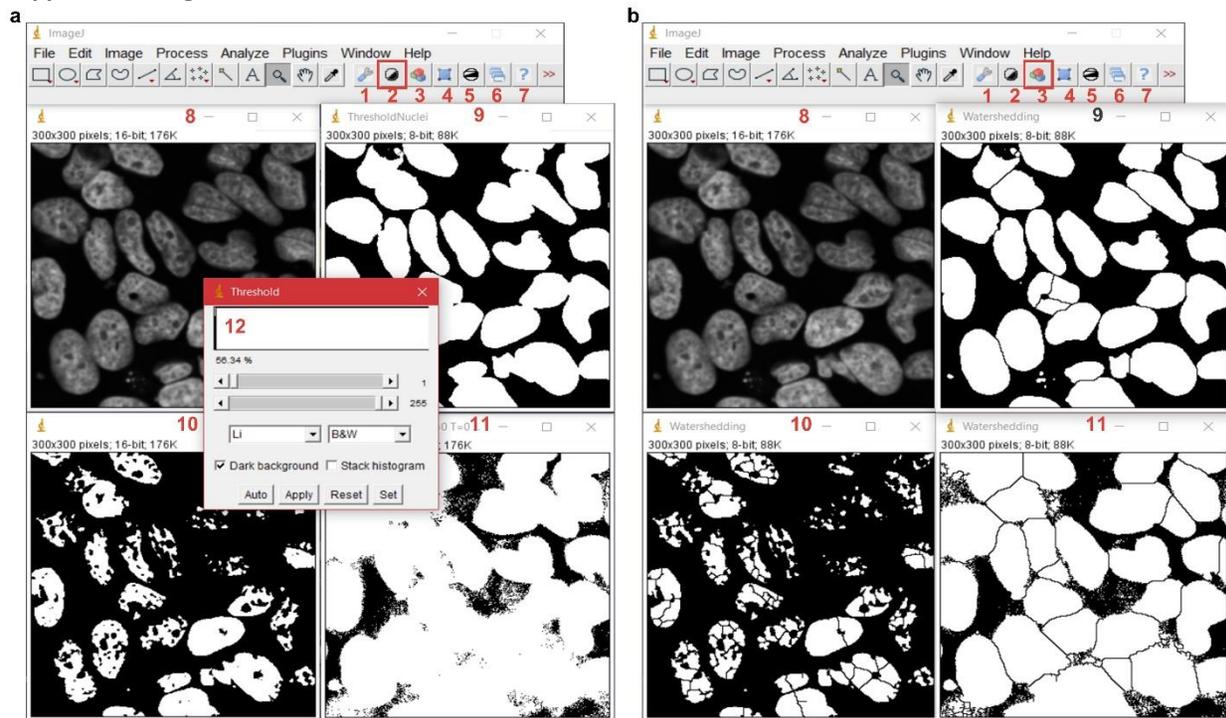
1. Install ImageJ bundled with Java 1.8.0 or higher. You can install different versions of ImageJ with different sets of Startup macros. (recommended folder: C:\ImageJ) (<https://imagej.nih.gov/ij/download.html>).
2. Install Bio-Formats Importer plugin (<https://www.openmicroscopy.org/bio-formats/>)
3. Open ImageJ and Open the *Startup macro window* by clicking on *Plugins/Macros/Startup Macros....*
4. Install the Stripenator macro by copying the whole text of the Stripenator.txt file into the open text editor window of the *Startup Macro window* in ImageJ. Click on *File/Save*.
5. Close ImageJ and relaunch. If the Stripenator was correctly installed a new set of buttons should appear as shown in Fig. 1a (1-7).

Adjusting the threshold and separation of cells/nuclei

1. Click on the *wrench button* (Fig. 1a 1) to open the *Stripenator Option window* (Fig. 1a 9)
2. Adjust the parameters for thresholding, size and channel order to fit your cells and images. If you are unsure of these parameters, we suggest trying the pre-set parameters and check the box *open new file* and click *ok*. Please either check automated thresholding or uncheck and type in lower or upper thresholding values.
3. A new window will appear to select a file. Select a representative file for first parameter adjustment and testing.

- The selected Image will be automatically opened with the Bio-Formats Importer. Remember the order in which the channels are opened by the Bio-Formats importer (Fig. 1a 8). The front channel is the channel on the top of the stack after opening the image with Bio-Formats. This is usually the channel with the highest number (Ch 3 or 4).
- Select the DAPI or other nuclear staining channel (Supplemental Fig. 1a 8) for testing and adjusting the threshold. For DAPI/Hoechst images with a normal signal/background ratio, the *Li* or *Huang* settings often do a good job. A good thresholding should keep the shapes of the cells/nucleus visible and distinguishable (Supplemental Fig. 1a 9), not produce holes in the cells (Supplemental Fig. 1a 10) or make the nuclei too large or artificially overlapping (Supplemental Fig. 1a 11).
- Click on the *contrast button* (Supplemental Fig. 1a 2) to apply the preselected thresholding method and to create a duplicate with the potential thresholded mask (Supplemental Fig. 1a 9).

Supplemental Figure 1.

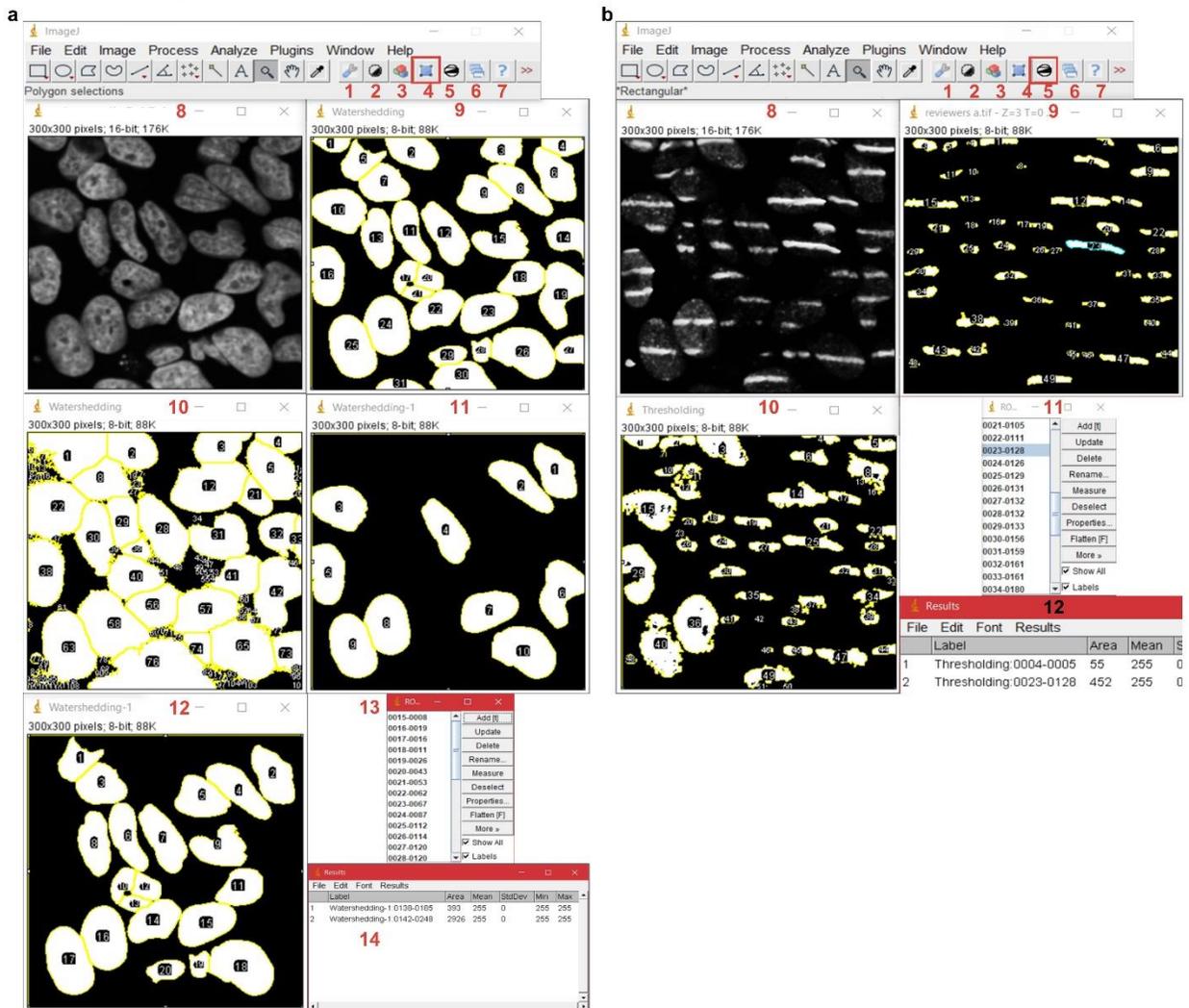


- If the thresholding was not successful, repeat step 5-7 with adjusted parameters. You can also test the different threshold methods on a duplicated DAPI picture (*Image/Duplicate*). Use the *Threshold window (Image/Adjust/Threshold)* to test different auto threshold methods or manual values. Make sure *Dark background* is activated. Transfer your tested settings in the *Stripenator Option window* (Fig. 1a 9) and test again (step 6).
- If nuclei are overlapping watershedding can be helpful. To test watershedding, select the image with the best thresholding result (Supplemental Fig. 1a 9) and click on the *brick stone button* (Supplemental Fig. 1b 3). A good example for watershedding is shown in Supplemental Fig. 1b 9. Supplemental Fig. 1b 10,11 show bad examples for watershedding based on a flawed thresholding.

Selecting cells/nuclei and damaged areas

1. To test the size criteria of the cell/nucleus selection, click on either the thresholded image or the thresholded and watershedded image and click on the *blue selection rectangle button* (Supplemental Fig. 2a 4). A selection of the cells is being performed based on your size criteria in *Stripenator Option window* (Supplemental Fig. 3a 8). These regions of interest (ROIs) will be marked in your thresholded image (Supplemental Fig. 2a 9) and listed in the *ROI-Manager window* (Supplemental Fig. 2a 13). If incorrect size criteria are chosen, debris, partial cells or even background might be detected as ROI (Supplemental Fig. 2a 10) or smaller ROIs might be omitted (Supplemental Fig. 2a 11). If *exclude cell on image edge* was checked in the parameters, cells bordering the image will be ignored (Supplemental Fig. 2a 12).
2. You might determine the size of a big cell and a small cell to get an idea of the size using your thresholded image by using the *ROI-manager* and *Measure*. The *Result window* will show the size of the selected ROIs (Supplemental Fig. 2a 14). In case of the background being selected instead of the ROIs, please try the option *invert* in the *Stripenator Option window*.

Supplemental Figure 2.



3. A similar approach can be applied for the detection of damaged areas e.g. stripes by choosing the threshold method and the size range of selected areas. The parameters for the damage detection settings can be also adjusted in the *Stripenator Option window (wrench button)*.
4. To apply and test the thresholding and selection parameters, choose the damage reference channel (Supplemental Fig. 2b 8) and click on the *contrasted rectangle button* (Supplemental Fig. 2b 5). A duplicate of the damage reference channel will appear based on the threshold and size settings (Supplemental Fig. 2b 9) and the damage reference ROIs are saved in the *ROI-Manager* (Supplemental Fig. 2b 11). If the damage area selection step was not fitting (Supplemental Fig. 2b 10), try other thresholding or size parameters similar done for the nuclei.

Running the automated analysis

1. When all analysis parameters are determined close all images and channels. If using multiple picture analysis, the images should be similar regarding staining quality and microscope acquisition. To run the automated analysis, open the *Stripenator Option window* with the *wrench button* and apply the determined settings for the cell/nucleus and damage area detection (Supplemental Fig. 3a). Please remember to choose the correct order of the channels: One *Cell/nucleus channel* needs to be chosen, such as a DAPI staining, one *Damage reference channel*, such as γ H2A.X, one analysis *Test channel A* and if a second channel for analysis is present this channel should be determined as *Test channel B*; if not present, the fourth channel must be *inactivated*.

Supplemental Figure 3.

a

b

	Label	Area	Mean	StdDev	Min	Max
1						
2	1 DamageReference0.Stripe0		55	27315.98	6238.158	18098
3	2 DamageReference0.Stripe1		98	33143.66	8333.235	18106
4	3 DamageReference0.Stripe2		99	30541.22	7787.955	18496
5	4 DamageReference0.damage summary		252	30849.36	7982.346	18098
6	5 DamageReference0.overall cell/nucleus		1230	7976.4	10534.29	946
7	6 DamageReference0.background without damage		1067	4182.169	3084.989	946
8	7 TestChannelA+0.Stripe0		55	10318.24	2419.926	4309
9	8 TestChannelA+0.Stripe1		98	12203.32	3767.97	4719
10	9 TestChannelA+0.Stripe2		99	8710.838	2333.707	5256
11	10 TestChannelA+0.damage summary		252	10419.85	3356.953	4719
12	11 TestChannelA+0.overall cell/nucleus		1230	6850.603	3289.985	1660
13	12 TestChannelA+0.background without damage		1067	6260.136	2781.143	1660
14	13 CellNucleusChannel0.Stripe0		55	6428.891	1086.991	4276
15	14 CellNucleusChannel0.Stripe1		98	9909.469	3987.817	4055
16	15 CellNucleusChannel0.Stripe2		99	17406.04	2344.306	12002
17	16 CellNucleusChannel0.damage summary		252	12094.81	5343.56	4055
18	17 CellNucleusChannel0.overall cell/nucleus		1230	15925.35	4788.018	6899
19	18 CellNucleusChannel0.background without damage		1067	16014.99	4926.305	6899
20	19 TestChannelB+0.Stripe0		55	26142.64	3706.116	16198
21	20 TestChannelB+0.Stripe1		98	30450.43	7162.398	12657
22	21 TestChannelB+0.Stripe2		99	27890.7	5262.63	18017
23	22 TestChannelB+0.damage summary		252	28504.63	6036.792	12657
24	23 TestChannelB+0.overall cell/nucleus		1230	32127.33	7133.866	10146
25	24 TestChannelB+0.background without damage		1067	32365.21	7303.514	10146
26	25 DamageReference1.Stripe0		155	35048.19	8437.128	18246
27	26 DamageReference1.Stripe1		318	33395.27	7965.459	18150
28	27 DamageReference1.damage summary		473	35247.71	8537.763	18150
29	28 DamageReference1.overall cell/nucleus		2062	9386.395	10858.14	856

In the case of double channel selection or the channel number not fitting the actual channel number, an error message will appear after opening the image and starting the automated run. The user will have to correct

accordingly. If only the order was chosen wrongly, no error will appear. Please pay attention to the correct order in which the Bio-Format importer opens the channels. If multiple images in one folder should be analysed with the same parameters, check *multiple pictures*. Check *open new file* and click *ok* to have a new window appear to select the image file or the first image file in a whole folder to be analysed. The example images can be tested with parameters shown in Supplemental Fig. 3a.

2. After the image is opened click on the *Run All button* (Supplement Fig. 3a 6) and observe the Stripenator performing the analysis by automatedly selecting channels, duplicating, thresholding, cropping, and closing images. Please wait until you are notified that the analysis is finished to obtain the results. If you detect wrong parameters, the analysis can be aborted with the “escape” key or by closing one of the windows.

Data extraction and interpretation

1. After the automatic run, the result sheets for each image can be found in the output folder located in the same folder as the analysed images. There you will find a .zip file, a ROI mask that can be opened in the *ROI-Manager* to see which cells/nuclei were detected for each image, a .txt file with the chosen parameters and an .xls sheet with the output data.
2. To analyse the data, open the output .xls sheet and save as a new file with the .xlsx extension.
3. The output file shows the area, mean intensity, standard deviation of the mean intensity, min. and max. intensity per cell/nucleus, damage area and channel. For big files and easier handling, the labels can be copied, and files can be restructured with excel’s “find and replace” and “sorting and filtering” command or “text filters” (Supplement Fig. 3 b). Each row is named first with the channel name, then each damage area detected in a cell beginning with 0 or all damage area summarized in the “damage summary”, the “overall cell/nucleus” including damage area and background or only the “background without damage” per cell, followed by the cell number beginning with 0. If no damage was detected in the reference channel of the nucleus no damage summary and no damage area values are exported.
4. To obtain the mean intensities, for example for the *Test channel A*, results could be sorted for *Test Channel A* and damage summary and background without damage and the values copied. Another approach would be to calculate a damaged area/background intensity ratio for each cell to normalize the damage intensity values to their own background. Consequently, if the damaged and background area would have similar intensities because of no change, as for DAPI expected, a value of 1 would be obtained versus higher values if damage was more intensely stained than the background. Depending on the experimental setup other values such as the area or the intensity of each single damage area can also be analysed.