Background Removal

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1 Theory

Autofluorescence of parts other than the cells is unavoidable, for example, it can be caused by the cell dishes. Therefore, the images need to be corrected for this unwanted autofluorescence. Images with water are taken to determine the background signal. The resulting smoothed image set $B(k,i)$ is subtracted from all images.

1.1 Imaging protocol

For hyperspectral imaging, it is recommended to avoid grided polymer dishes in combination with the Olympus immersion oil to eliminate chemical reactions. Using the same amount of liquid for all files (water file, calibration file, and cells) is advised. 4 ml of liquid are suggested to avoid surface tensions.

For measuring ungrided dishes, a scratch mark needs to be added and any splintered glass is to be removed. After focusing the microscope on the scratch mark, the grid is moved to an unscratched area in the centre and the objective is lifted 10 µm to ensure measuring in liquid. As focusing on a self-made scratch is challenging, multiple files for each water and calibration file are suggested.

1.2 Reference image and reference spectra

To calibrate the image and obtain quantitative values, a calibration mechanism is applied.

In this procedure, hyperspectral images of a calibration fluid are taken. This calibration fluid is optimized for having a non-zero response upon all available hyperspectral channels. Therefore, it consists of 30 µM NADH and 18 µM FAD. The resulting, smoothed image stack $C_{raw}(k,i)$ is related to the normalized spectrum of the calibration fluid $f(k)$. This reference spectrum is measured with the Fluoromax-4 spectrofluorometer (Horiba, Japan).

Often, preprepared stock solutions (1 mM NADH, 1 mM FAD) are used to prepare for preparing the daily measured calibration fluid. Be aware that with time this stock fluid (especially NADH) loses its autofluorescence. In such a case, calibration values below the water file may be measured. Such fluids/values **cannot** be used since it would result in a division by negative values.

1.3 Relating the hyperspectral images to quantitative values

Applying the above-mentioned correction methods results in the following formula and a flattened image $y_f(k,i)$:

$$y_f(k,i) = \frac{f(k) \times \left(y_{raw}(k,i) - B_s(k,i)\right)}{C_s(k,i) - B_s(k,i)} \tag{1.1}$$

where $f(k)$ equals the reference values of the fluoromax and $y_{raw}(k,i)$ is the cell file. $B_s(k,i)$ represents the smoothed water image and $C_s(k,i)$ the smoothed calibration image.

1.4 Image smoothing

A combination of removing outlier values (cosmic ray removal) and wavelet smoothing can be applied for image smoothing.

1.4.1 Outlier removal

The implemented algorithm to remove outliers (cosmic rays) is inspired by ”*A Fast Algorithm for Cosmic Rays Removal from Single Images*” by W. Pych [1]. It consists of the following steps:

1. View the intensity distribution in a histogram.
2. Compute the mode of the histogram, i.e., the peak indicating the most frequent intensities.
3. Find gaps in the histogram, in other words w_{gap} neighboring intervals without any values:

$$w_{gap} = \frac{th * \sigma}{w_c} \tag{1.2}$$

   where th is the threshold parameter (arbitrary, usually 3), σ indicates the standard deviation of the image, and w_c denotes the width of a class interval.
   Only continue if such a gap is found.
4. Flag pixels with intensity values above the first occurring gap as outliers.
5. Replace the flagged pixels with the median of the surrounding pixels. The surroundings are defined as the window around the pixel of interest of side length $2 * window\ size + 1$ (see Figure 1.1)

Outlier removal is conducted on each raw image and after applying equation (3.1).

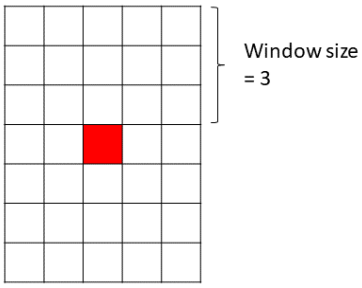

Figure 1.1: Selected window around outlier vaue, e.g. window size = 3

1.4.2 Wavelet filtering

Image smoothing is achieved via wavelet transformation with a hard threshold. The applied biorthogonal wavelet is Cohen–Daubechies–Feauveau 9/7 (also used for lossy compression of JPEG images).

Hard thresholding removes noise from images according to equation (1.3).

$$\Phi_T^0(x)_i = \begin{cases} x_i & if\ |x_i| > T \\ 0 & otherwise \end{cases} \tag{1.3}$$

In the GUI, the selected threshold (between 0 and 1000) is converted into a percentage value and computed individually for each image with regards to the maximum signal. Smoothing for cell images $y_{raw}(k,i)$ is optional and conducted after applying equation (3.1). The water $B(k,i)$ and calibration file $C_{raw}(k,i)$ require good smoothing. In the following, smoothed images are presented with a subscript s.

2 GUI Segmentation

- Version 2.1, Matlab R2022b -

The segmentation GUI (Figure 2.1) is used for localizing cells and background areas in hyperspectral images. Generally, segmentation is conducted on the DIC image (final image in image stack), and hyperspectral images can be used for verification. Three tools can be used for segmentation: free hand, ellipse, and polygon. Currently, the position/size of a drawn shape **must NOT** be changed (not saved).

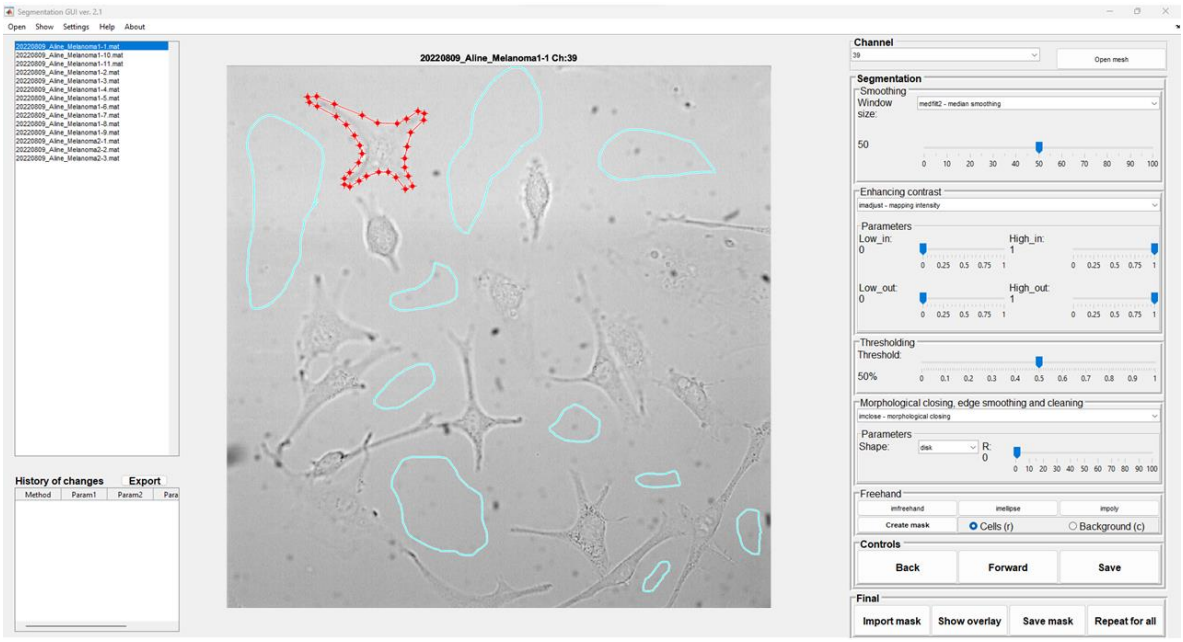

Figure 2.1: Segmentation GUI

The following steps are required:

1) Open → select folder to choose files from (program will list all .mat files in this folder)

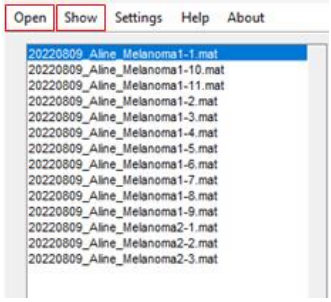

Figure 2.2: Open folder and show selected image

2) Select image from list of files and click “Show” for it to appear.
3) Segmentation is generally conducted on DIC images. However, other channels can be selected to check the behaviour. If required, apply smoothing or choose enhancement options to improve appearance.

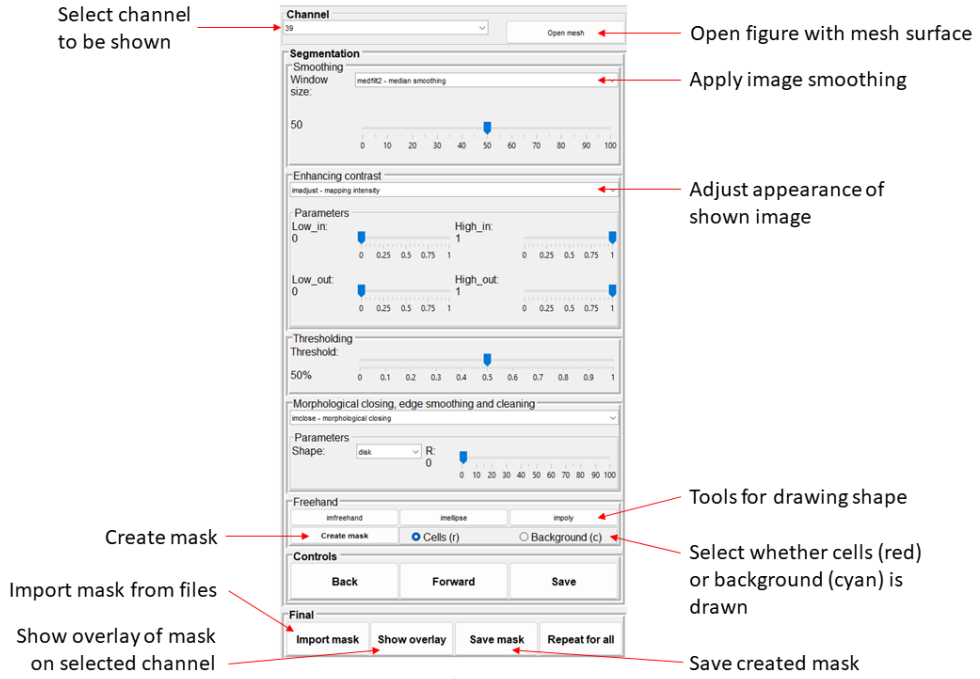

Figure 2.3: Steps in segmentation

4) Select radiobutton “Cells (r)” for segmenting cells. The outline of the shape will appear in red. Choose “Background (c)” for specifying background areas. Both options can be drawn at once, each selection will later be saved in a separate folder (see Figure 2.1).
5) There are three options of drawing shapes: freehand, ellipse, and polygon. Once a shape is drawn, **do not** adjust its position or shape! Any changes will not be saved. If required, delete the shape (right click, delete) and redraw the shape.
6) After having segmented all desired areas (both background and cells), click create mask (afterwards no areas can be added).
7) Click “Save mask” to save the two created masks. The folder “mask” is created for cell masks, folder “maskB” for background masks. If the folder already exists, files with the same name in this folder will be overwritten.
8) Use “Show overlay” to check your results. The mask will be overlayed over the selected channel. Automatically, the previously created mask will appear. You can also overlay a saved mask: import it via “Import mask” before using “Show overlay”.

2.1 Known bugs

- Update masks when drawn shapes are adjusted (position/size/…)
- Buttons “Back”, “Forward”, “Save”, “Repeat for all” may not functional (not checked in this revision)

3 GUI Preprocessing

- Version 5.1, Matlab R2022b -

GUI Preprocessing is used for background removal and smoothing. Cosmic rays removal and smoothing of images are highly recommended.

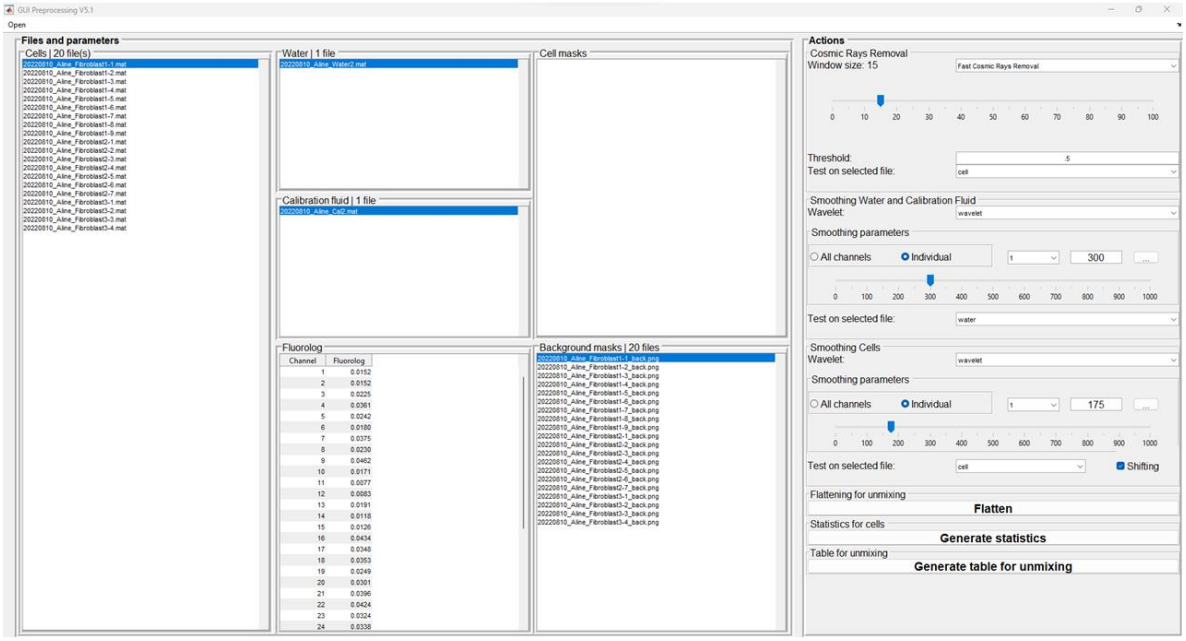

Figure 3.1: GUI Preprocessing

Using the GUI requires the following steps.

1) Load files

   "Open" allows the user load data into the GUI (see Figure 3.2). In addition, shortcuts for each option have been implemented.

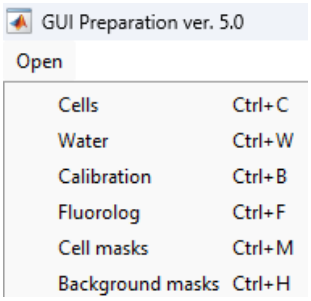

Figure 3.2: Menu "Open"

   For flattening, the following files are required: Cells, Water, Calibration, Fluorolog. When using the shifting option, background masks (suffix _back) are also necessary. Using "Generate statistics" requires cell masks (same name as input file) and images that are prepared and smoothed to be in the same folder as the cells (suffix _prepared_smoothed).

2) Cosmic Rays Removal

   The selected threshold is used according to equation (1.2). The window size specifies the surrounding pixels used as an average value to replace the outlier. The threshold defines whether a vale is considered an outlier. The selection can be tested by choosing a test file (cell, water, calibration) and entering the channel number to be analyzed.

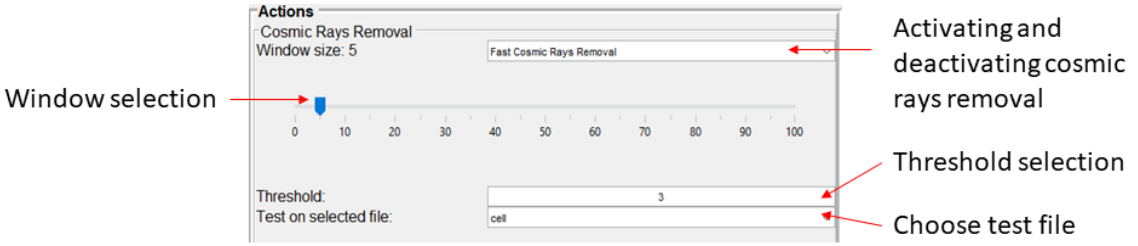

Figure 3.3: Cosmic Rays Removal

3) Smoothing of water and calibration file

For a good performance of the algorithm, it is essential to have a smooth water and calibration file! The available soothing options are presented in Figure 3.4.
The parameters can either be set equal for all channels ("All channels") or selected individually ("Individual"). The value may be adjusted with the edit text field or the slider.
For individual selection, the channels (same as in fluoromax), can be chosen and the assigned value for each channel can be adjusted one after another.
Testing the smoothing parameters can be conducted by choosing a test object (water or calibration) and entering the channel number in the popup window.
Switching back to "All channels" will assign the current slider/edit value to all channels.
When loading a new fluoromax file, the "All channels" is activated.
Running the flattening program will save the selected parameters under the name of the water file with the following suffices attached to each other:

-_SmoothingP_
- Date & time (YYYY-MM-DD-HH-SS)
- _noCRR OR _CRR
- optional: _w+window size
  _t+ threshold (decimal point replaced by comma)

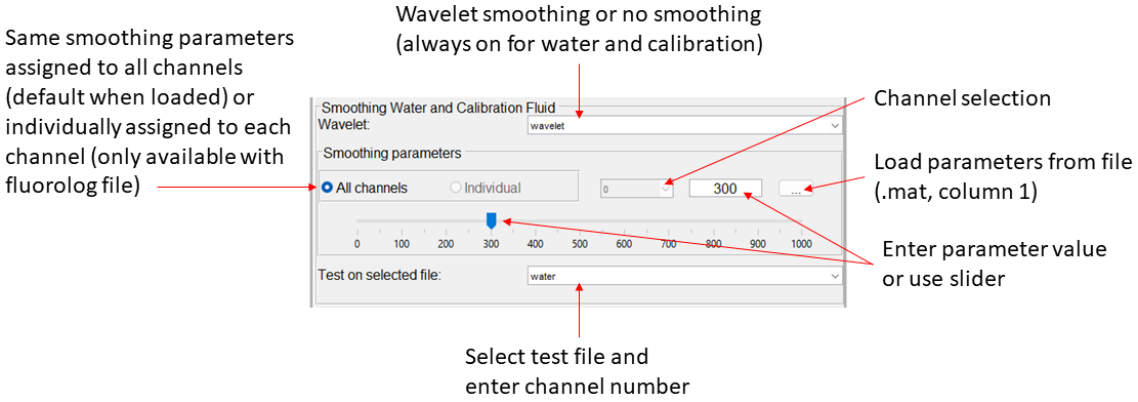

Figure 3.4: Select smoothing parameters for water and calibration file

4) Smoothing of cell file

Parameters for smoothing the cell file are set similarly to the water and calibration file. In addition, there is the option to activate and deactivate shifting (see Figure 3.5).
The program performs flattening according to equation (1.1) resulting in the flattened image $y_f(k,i)$.
The shifting algorithm requires background masks (same file name as selected cells plus suffix _back). When it is activated, the median of the assigned background pixels (k_{back}, i_{back}) is computed on the flattened image $y_f(k,i)$. The analyzed image is shifted down by this specific value (for negative medians, this results in an upwards shift).

$$myMedian = median\left(y_f(k_{back}, i_{back})\right) \tag{3.1}$$

$$y_{f,c}(k,i) = y_f(k,i) - myMedian \tag{3.2}$$

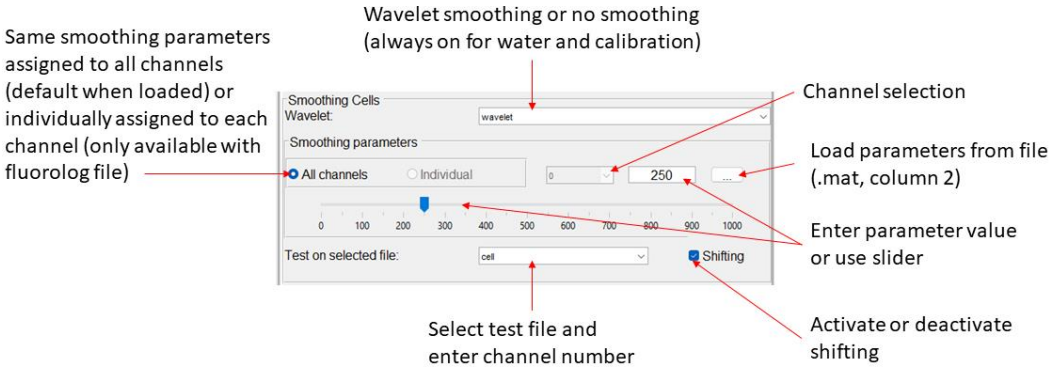

Figure 3.5: Smoothing parameters for cell images

5) Flatten
   Flattening applies the equation presented in (3.1) including the optional shifting mechanism explained in the previous step.
6) Generate statistics
   Generate statistics takes the cell files from "Cells" and looks for the smoothed version of them in the same folder (suffix "_prepared_smoothed"). In addition, cell masks are required for all cells (same name as cell file). The option "Generate statistic" will create an excel table ("stats") including the mean intensity for each marked cell.

3.1 Known bugs

- "Generate table for unmixing" has not been tested
- Issues might arise for old files (the old structure was not considered when implementing the changes)

4 References

[1] W. Pych, "A Fast Algorithm for Cosmic-Ray Removal from Single Images," *Publications of the Astronomical Society of the Pacific*, vol. 116, no. 816, p. 148, 2003/12/29 2004, doi: 10.1086/381786.