

zSTACK FLUORESCENCE QUANTIFICATION V.2.0 — MACRO DOCUMENTATION

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Feb. 2020

I Table of Contents

II Introduction

This macro designed for ImageJ/Fiji and written by Augustin Walter aimed to quantify a Signal Of Interest (SOI) from zStacks images.

Briefly, this script measures the mean and the integrated density inside one or more Regions Of Interest (ROIs) as well as some shape descriptors of this/these ROIs. It allows the user to draw one or more ROIs to measure the SOI inside them or, if no ROI is specified, the SOI inside the whole field is measured.

The macro performs two different types of pixels intensities quantification, one by directly measuring intensities inside ROIs and the other one by measuring intensities within a threshold. These two methods will be developed later in the section IV.

II.1 Requirements

This macro requires at least ImageJ version 1.48 and the plugin Bio-Format installed. It is recommended to use Fiji distribution instead of ImageJ alone.

II.2 Creative Common Liscence

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II.3 Changelog

Version 2.0 changelog:

- Add a new way to measure pixels intensities: by using a threshold and by measuring intensities inside this threshold,
- Add an option that allow user to draw multiples ROIs directly inside the macro before the analysis,
- Bug correction during zStack size calculation,
- Other bugs fix.

III Macro functioning

The macro processes all supported images files found into the directory specified by the user(Fig. 1). Depending on the options selected by the user (see subsection III.3), the user will be first asked to draw ROIs for each acquisition into the selected directory. For each acquisition, one file containing all the ROIs is save in the same folder than the images and entitled 'name_of_the_image_file_[ROIs].zip'. Then the Signal Of Interest (SOI) is measured inside each ROI and all the results are saved in a single Excel file located in the output directory created inside the parent directory.

III.1 Supported file types

At the beginning the user is prompted to select a directory that contains the image acquisition to analyze. All the supported files inside the directory are analyzed.

III.1.1 ImageJ and Bio-Format supported files

There are three type of image acquisition that are supported: MetaMorph image acquisition ('.nd' file with the corresponding '.tif' files), Leica Image File ('.lif' files) and '.merge' files (see subsection III.1.2).

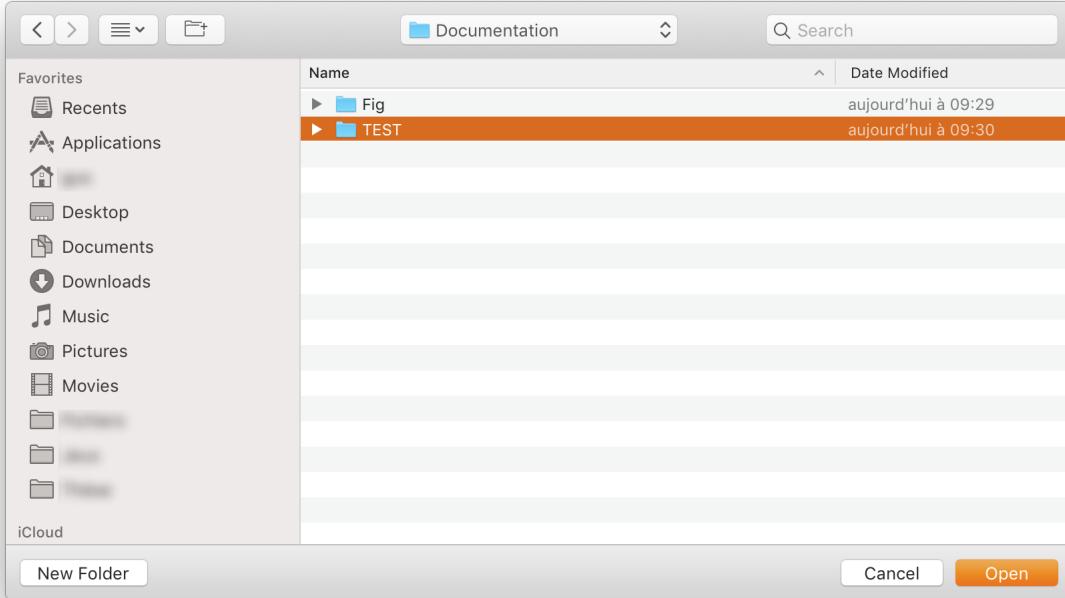


Figure 1: **Select directory prompt.**

At the beginning, the user is prompted to select the directory that contains image acquisitions to be analyzed. All the supported image acquisition files inside the selected directory are analyzed.

- MetaMorph '.nd': These files require the Bio-Format plugin to be opened. During the analysis, if the 'Draw ROIs' option is selected, a '.zip' file is created with the name of the '.nd' file. A tiff that corresponds to the zProjection where the ROIs are represented as an overlay is also saved,
- Leca Image File '.lif': The script analyses all the series inside the LIF file. If the LIF file contains more than one series, the ROIs drawn by the user will be saved as '.zip' file and one file is created for each LIF series.
NB: series are image acquisitions stored inside a same LIF file.
- Merge files '.merge': Merge files are used in some ImageJ/Fiji macro/plugins created by A. Walter. These files are similar to the MetaMorph ND files and are more described in the subsection III.1.2).

It is possible to set first slice from which each zStack is processed, for that user needs to create a text file that contains the number of the first slice (integer) followed by '-' and then save the file with the same name than the '.nd', '.lif' or '.merge' replacing the file extension by '.stack' (see subsection III.2.2).

III.1.2 Merge files, a file type designed used by macros created by A. Walter

NB: MERGE files can be used from version 3.0 of the macro.

Merge files work similarly to the MetaMorph ND files. This format is useful to assemble files if the image acquisitions are saved as series of tiff files. So one acquisition is composed by:

- One zStack saved as tiff file for each wavelength channel,
- For all the tiff files, one MERGE file.

A MERGE file is a simple text file where the '.txt' extension is replaced by '.merge'. This file must have the following structure:

```

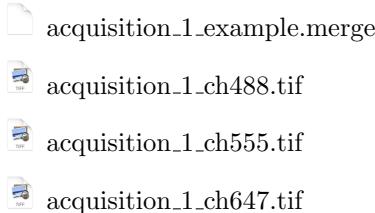
channels=n
channel_1=name_of_the_first_channel_stack.tif
channel_2=name_of_the_second_channel_stack.tif
...
channel_n=name_of_the_n_channel_stack.tif
slices=1-m

```

In the previous lines, *n* correspond to the number of channels in the acquisition and *m* to the number of slices of the zStacks. If do not want to modify the number of slices, use *0* as slices value.

Example:

Image acquisition files in the parent directory:



Content of the MERGE file:

```

channel=3
channel_1=acquisition_1_ch488.tif
channel_2=acquisition_1_ch555.tif
channel_3=acquisition_1_ch647.tif
slice=0

```

III.2 Macro settings

When the macro is started, after the main directory selection, a new window entitled 'General Settings' appears (Fig. 2). This window is divided in two parts:

- The general analysis settings,
- And the settings concerning z-Stack size and the output folder.

III.2.1 General analysis settings

In the upper region of the window, two checkboxes are displayed. The first one is entitled 'Draw ROIs before analysis' and allow user to draw one or more Regions Of Interest on each image file found inside the main directory before the analysis: see subsection III.3. If this box is not checked, the analysis is performed on the entire field of the images.

The second checkbox is entitled 'Use a threshold to measure signal intensity' and will change the way the SOI is measured. In fact, if this box is checked, the SOI is measured using a custom threshold defined by the user. All the informations about the SOI and this threshold are provided by the user in a second setting window that appears directly after the 'General Settings' one (see subsection III.2.3).

III.2.2 Smaller zStack of the analysis

The second part of the 'General Settings' window is about the z-Stacks size. In fact, the SOI analysis is made on z-projection of the z-Stacks using the 'sum slices' parameter. This means that all the pixel values of all slices will be add together for each X,Y coordinates. According to this, it is obvious that one must have the same number of slices in each z-stack if one wants to compare the pixel intensities in these stacks. That is why the macro automatically determines the smaller z-stack inside the main directory and this value is displayed in the box 'Number of slices' of the 'Smaller stack found' section. The name of the smaller z-Stack is also displayed. The user can decrease this number but increasing this number is depreciated.

If one wants to reslice (crop) one or more z-Stacks in the main directory, it is possible to create text file that has the same name of the ND, LIF or MERGE file, replacing the '.txt' by '.stack'. Inside this file the first slice of each series is specified. One specifies the first slice as an integer of the series to modify. Only the series that the user wants to reslice are specified in the STACK file.

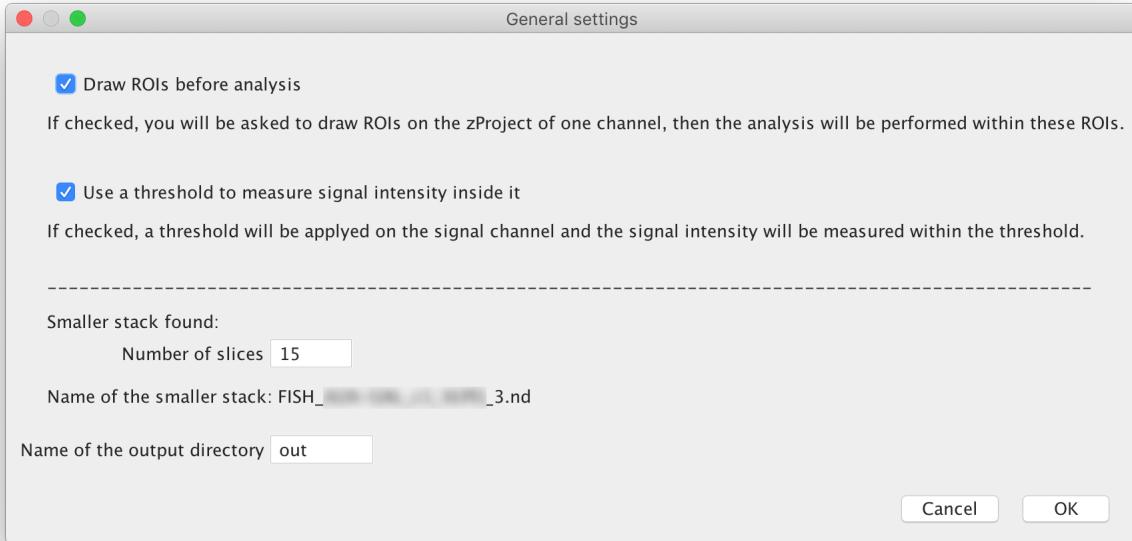


Figure 2: General Settings window.

When the macro is started, after the main directory selection, a new window entitled 'General Settings' appears. This window is divided in two parts, one concerning the general settings of the analysis and the other concerning the z-Stacks parameters and the output folder.

Example for a LIF file:

A LIF file contains 5 series that are independent image acquisitions of a same experiment and series 1, 2 and 5 must be resliced. The STACK file that has the same name of the LIF file (replacing the '.lif' by '.stack') and contains the following lines:

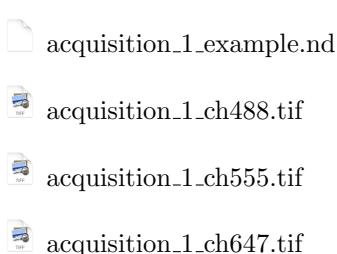
```
series_1=5-
series_2=7-
series_5=2-
```

The number of series is one-based. To reslice a series, write a line starting with 'series_i' (i is the number of the series to reslice) followed by '=' and the first slice as an integer. The number of the first slice must be followed by '-'.

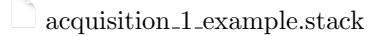
NB: the number of slice is 1-based, i.e. the number of the first slice is 1.

Example for a ND file that contains 1 series:

Image acquisition files in the parent directory:



The user can create a STACK file:



That contains:

```
series_1=3-
```

So the script will analyse the z-Stack from the slice 3 and the slices 1 and 2 will be ignored.

When the macro looks for the smaller z-Stack, the reslice induced by a STACK file is taken in account. So if the smaller z-Stack has a size of 12 and an other z-Stack of 15 slices is resliced from slice 5 to 15, then the smaller z-Stack size found will be 10 slices instead of 12.

III.2.3 Signal of interest and Threshold analysis

In the 2.0 version, there are two analysis modes of the Signal Of Interest: one by simply measuring pixels intensities on the SOI channel within ROIs and the other one by thresholding the SOI channel to create a mask of the signal and then by measuring pixels intensities within ROIs and within the SOI mask. The selection is made by checking or not checking the 'Use a threshold to measure signal intensity inside it' checkbox in the 'General Settings' window (Fig. III.2.1).

These two method of signal analysis will be described in the section IV.

If the previous checkbox is leaved unchecked, a setting window concerning the SOI settings is displayed (Fig. 3). In this window the user selects the channel index corresponding to the signal of interest (one-based) and defines a name for the SOI (this name will be used by the macro to refer to the SOI).

If the previous checkbox is checked, then the same setting window is displayed so the users has to set the SOI channel index and the name of the SOI moreover in the second part of the window, the user sets settings that refer to threshold used before the pixels intensities quantification (see subsection IV.2). In this part of the window, one has to set the filter used to remove noise on the SOI channel and the radius of the filter. Then one has to set the threshold method used to threshold the SOI image. NB: this threshold is used to create a mask of the SOI and then the pixels intensities will be measured inside this mask for all ROIs previously defined by the user (see subsections III.3 and IV.2).

To select the appropriate filter and threshold method, one must try different filters (median or Gaussian blur) and threshold method on some images of the experiment. The sequence for testing filter and threshold is:

1. Open the image corresponding to the SOI channel,
2. Run subtract background (*Process > Subtract Background...*),
3. Duplicate image (*Image > Duplicate...*) and try filters with different radius,
4. When some good filter and parameters are found, duplicate the image again and try different thresholds (*Image > Adjust > Threshold...*).

Finally write down the optimal parameters and use them into the script processing.

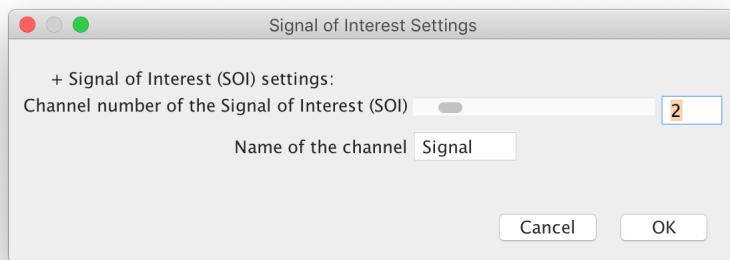


Figure 3: **Signal of Interest Settings window (without threshold).**

Settings window for the SOI, the user indicates the index of the SOI channel (one-based) and the name for the signal measured (this name will be used by the macro to refer to the SOI).

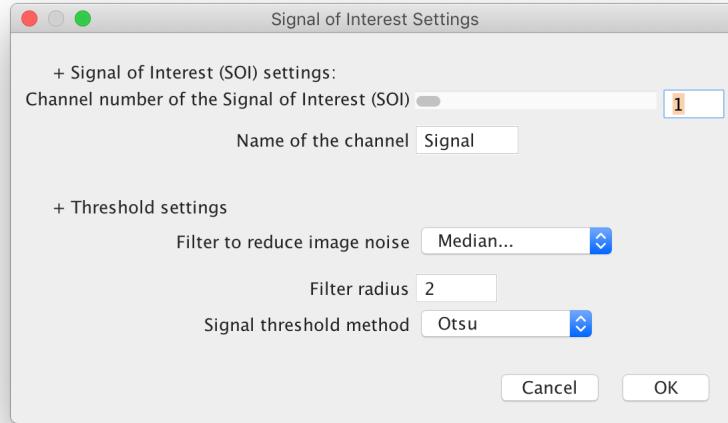


Figure 4: **Signal of Interest Settings window (with threshold window).**

Settings window for the SOI, the user indicates the index of the SOI channel (one-based) and the name for the signal measured (this name will be used by the macro to refer to the SOI). The second part of the window refers to the settings of the threshold. Before thresholding the SOI a filter is applied to remove noise, the user may have to chose the filter and the radius. Finally the threshold method is selected.

III.3 Drawing ROIs

If the checkbox 'Draw ROIs before analysis' is checked in the 'General Settings' window, the macro will ask user to draw ROIs for each image inside the main directory and for each series (for LIF files) and thus before the analysis.

For each image file and for each series, the zStack is opened and a Max Intensity zProjection is displayed on the right of the zStack (Fig. 5). A small dialog window prompt the user to use a selection tool and draw the first ROI on the zProject image, note that it is also possible to draw ROI on the zProject. When the first ROI is drawn, the user click the 'Ok' button, then the ROI is displayed and labeled on the zProject image and a new window appears asking the user if he/she wants to draw a new ROI (Fig. 6). If yes, a second ROI can be drawn and so on...

When its done, the ROIs of the current image/series is saved in the main directory as a '.zip' file with the same name of the analysed image followed by '_[ROIs_s1]'. Example: if the image is entitled '*acquisition_121220_1.LIF*' then the file containing ROIs is entitled '*acquisition_121220_1-[ROIs_s1].zip*'. The number that follow '*s*' refers to the index of the series (for LIF files).

III.4 Opening and modifying a previous analysis

IV Signal analysis

IV.1 Direct pixels intensities quantification

IV.2 Threshold pixels intensities quantification

V Figures

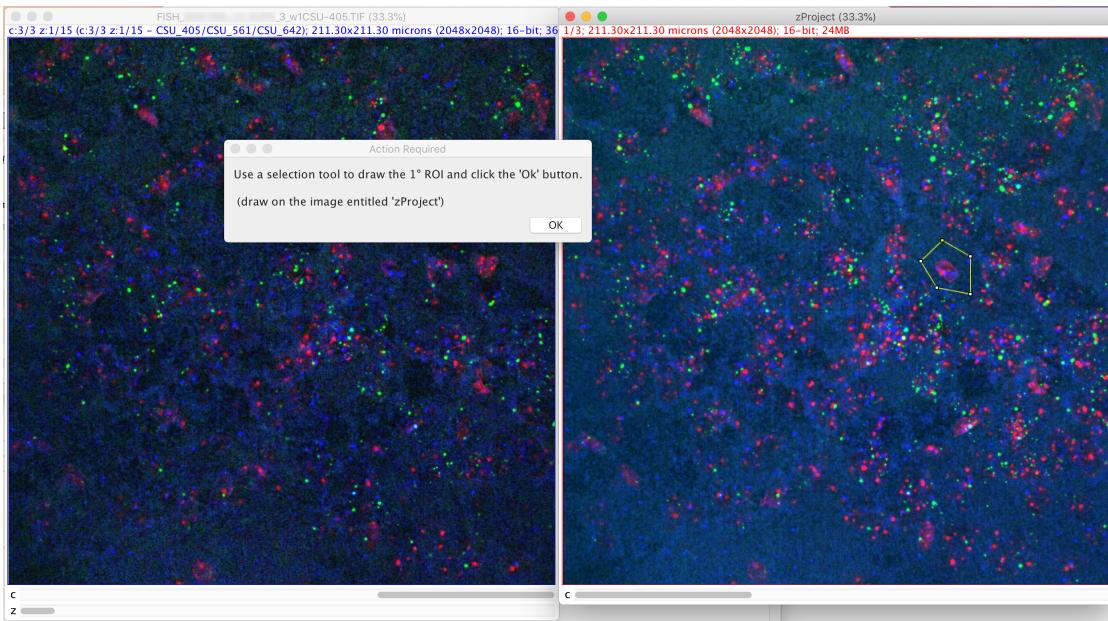


Figure 5: Draw ROI dialog window.

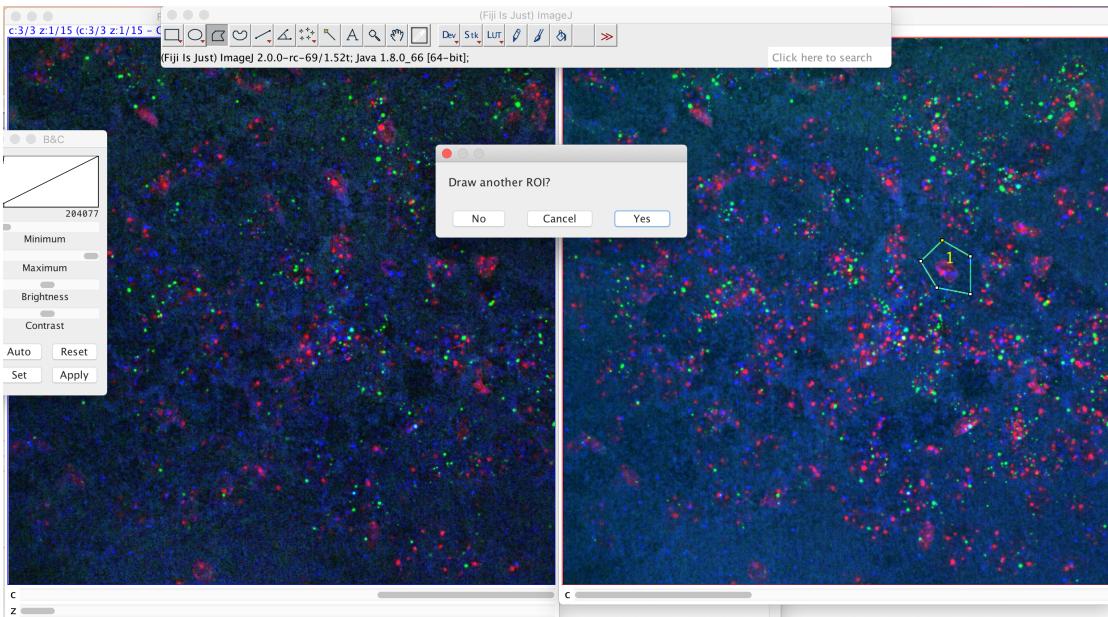


Figure 6: Draw another ROI dialog window.

Figure 7: Positionnement des cerveaux sur le socle de sucre

Figure 8: Paramètres du Microtome