BRET Analyzer plugin for Fiji

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Contents

1 Setup **2**

2 Buttons description **2**

3 Image naming convention **3**

4 Buttons use **4**
4.1 Clean 4
4.2 Crop 5
4.3 Divide 6
4.4 Analyse 8

5 Output files structure **11**

1 Setup

Move BRET_Analyzer-1.0.8.jar file into the folder Fiji.app/plugins/ (on MacOS X, right click Fiji.app > Show content folder).

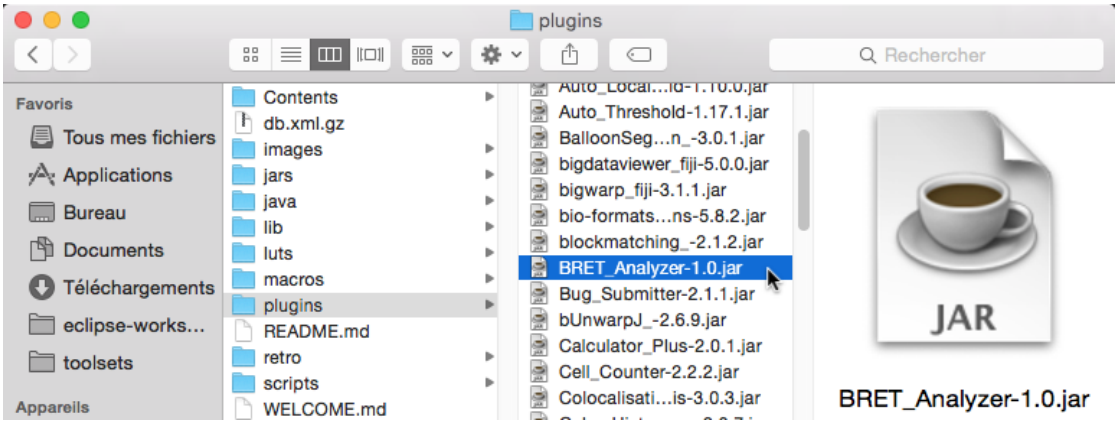


Figure 1 – Location to put .jar file.

After the file has been placed, (re)start Fiji. "BRET Analyzer" should appear in the menu "Plugins". Selecting it will open a new window with the plugin's methods.

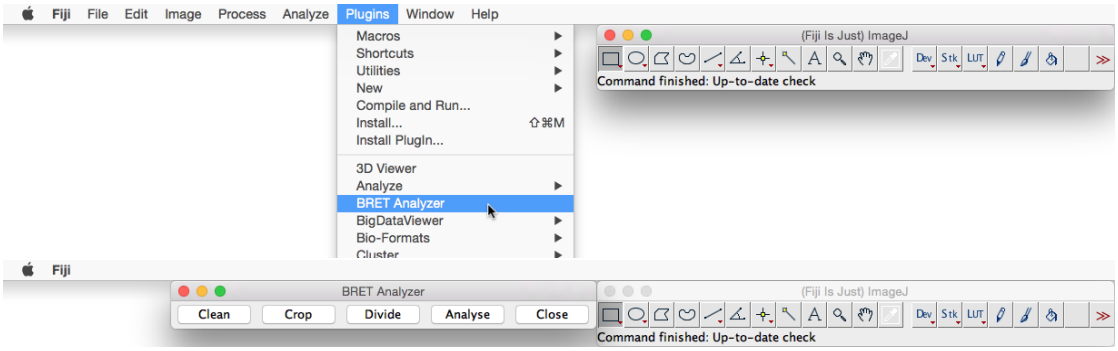


Figure 2 – Starting the plugin.

2 Buttons description

2.1 Clean button

Apply a median filter of radius 1.

Remove the background signal by subtracting the median value of a region that contains only noise. Optionally subtract a background image (dark frame) to remove inhomogeneous background.

Optionally align the stacks to correct small xy drift due to the multipositioning of the microscope by computing the translations coordinates for a stack of images and applying it to both.

2.2 Crop button

Extract sub regions to analyse them separately with different thresholds (should be used only on last resort if no thresholding method works well for the whole image).

2.3 Divide button

Set to 0 all points below a computed dynamic threshold on the clean donor stack of images. The threshold can be obtained using different methods. Automatic Threshold methods which compute the threshold level based on the whole image (or stack). Automatic Local Threshold methods compute threshold for each pixel based on the surrounding pixels in a given radius. Manual methods: selecting an area and calculating its median or mean * coefficient. In addition to one of the previous methods, an overal *Minimum threshold* can be set.

Divide pixel by pixel the acceptor by the donor, to obtain ratiometric images.

Set the image to 16 colors and adjust the range of values to visualize it in pseudo-colors.

2.4 Analyse button

Select regions of interest and measure the mean and standard deviation on each slice.

Plot data for each regions.

2.5 Close button

Close Results window, Log window and all images.

3 Image naming convention

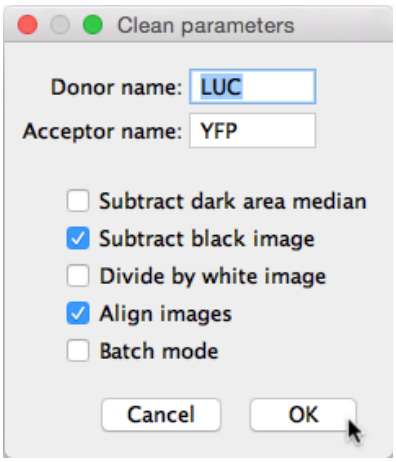
It is suggested to name raw image files xxxDonorName.tif and xxxAcceptorName.tif, where xxx can be anything as long as it's the same for donor and acceptor images. DonorName and AcceptorName can be anything as well, but they have to be entered into the fields "Donor" and "Acceptor" when used. This way, when choosing the donor, acceptor will be automatically found and both will be opened (this also works if DonorName and AcceptorName are in the middle of the image names). This is a necessary step to do batch processing.

It is possible to use any name, but both donor and acceptor will have to be browsed. If "DonorName" is not found in the raw file name, the subfolder will be the donor name without .tif extension. The DonorName and AcceptorName will be added in the names of the clean images.

4 Buttons use

4.1 Clean

1. Select parameters then click OK.



- *Donor and Acceptor names*: see Image naming convention.
- Tick *Subtract dark area median* to remove the median of an area corresponding to background. This step is recommended if no black image (camera offset) has been taken, but shouldn't be used otherwise.
- Tick *Subtract black image* to subtract a dark frame to account for the offset of the camera and the noise offset of potential photon pollution. This image should be taken with the same conditions and camera acquisition parameters as donor and acceptor images (one for each), but with no light source (no light excitation: laser, LED... or emission: fluorescence, bioluminescence...). Ideally it should be a stack of as many frames as possible to minimise noise.
- Tick *Divide by white image* to enable the flat fielding correction. It requires imaging an homogeneous sample to determine the response of each pixel to a given photon flux. Ideally, as for the black image, it should be a stack of as many frames as possible to minimise noise. Note that *Subtract black image* needs to be ticked as well as the offset is required to compute the pixel sensitivity. The effective computation is given by the following equation:

$$Shading\_corrected = \frac{RAW - Black}{White - Black} \tag{1}$$

- Tick *Align images* to correct potential xy drift. As it is time consuming, use it only if necessary.

- Tick *Batch mode* to change the behavior from the selection of a couple of images to the selection of a folder in which all couples of images respecting the image naming convention will be processed without intervention (automatic selection of background area).

2. Select raw donor image (and raw acceptor image if necessary). If *Batch mode* was ticked, this step is replaced by the selection of a folder containing the raw images.

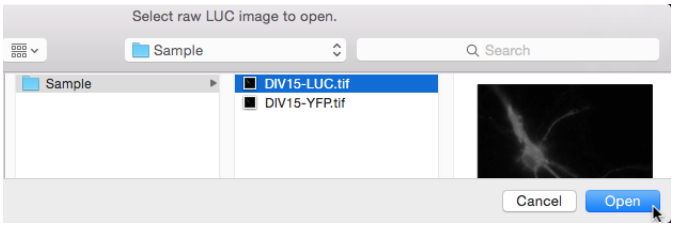


Figure 3 – Window to select raw donor image.

3. If *Subtract background image* is ticked in the parameters, select donor then acceptor background images.

4. If *Subtract dark area median* is ticked in the parameters, a 32*32 pixels area minimizing mean value over the stack is automatically selected. If this area is not representative of the background, select another one. Click "OK". If *Batch mode* was ticked, the automatic region is used without prompting the user to choose.

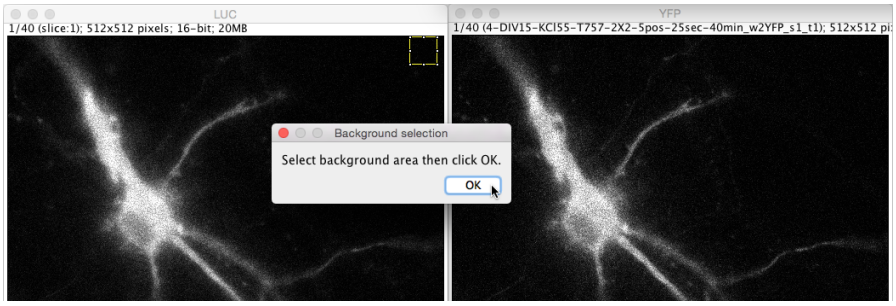


Figure 4 – Background area selection.

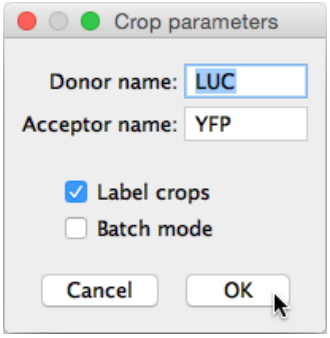
5. Images are processed then saved in a subfolder.

Note: TurboReg, the plugin used to register the movement is not part of the Fiji distribution anymore. If it is missing, you will have to download from http://bigwww.epfl.ch/thevenaz/turboreg/ the package for your distribution. Unpack it, place the file TurboReg_.jar into Fiji.app/plugins/ and restart Fiji.

Image alignment might fail in some cases, for example if there is a low contrast. Also, a drift may appear on long stacks.

4.2 Crop

1. Select parameters then click OK.



- *Donor and Acceptor names*: see Image naming convention.
- Tick *Label crops* to assign a name to each crop and save the list of names in a file.
- Tick *Batch mode* to change the behavior from the selection of a couple of images to the selection of a folder in which all couples of images respecting the image naming convention will be processed.

2. If images are not already open, select donor image (and acceptor image if necessary). If *Batch mode* was ticked, this step is replaced by the selection of a folder containing the raw images and all the following steps will be applied to each of the couples of images found.

3. Add regions to be cropped to the ROI manager by tracing a region then clicking on *Add* in the ROI Manager window or doing *Ctrl+T*. Click on *Show All* to display all the regions. Once all the regions have been added to the ROI Manager, click OK.

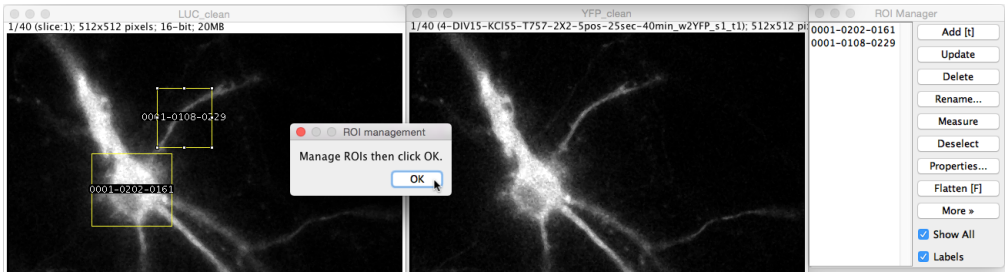


Figure 5 – Areas to crop added to the ROI Manager.

4. If *Label crops* was ticked, enter a label for each crop then click OK.

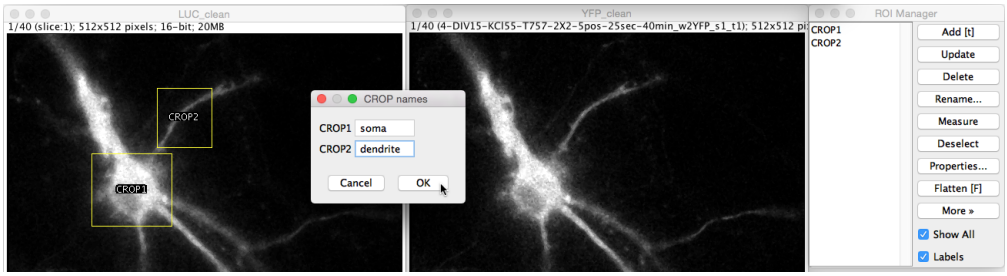
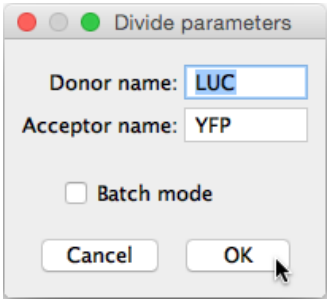


Figure 6 – Give a label to each crop.

5. Images are saved then closed.

4.3 Divide

1. Select parameters then click OK.



- *Donor and Acceptor names*: see Image naming convention.
- Tick *Batch mode* to change the behavior from the selection of a couple of images to the selection of a folder in which all couples of images respecting the image naming convention will be processed. The folder chosen can be either the one containing raw images or the one containing clean images.

2. If images are not already open, select donor image (and acceptor image if necessary). If *Batch mode* was ticked, this step is replaced by the selection of a folder containing the raw or clean images.

3. Select the parameters for the division then click OK.

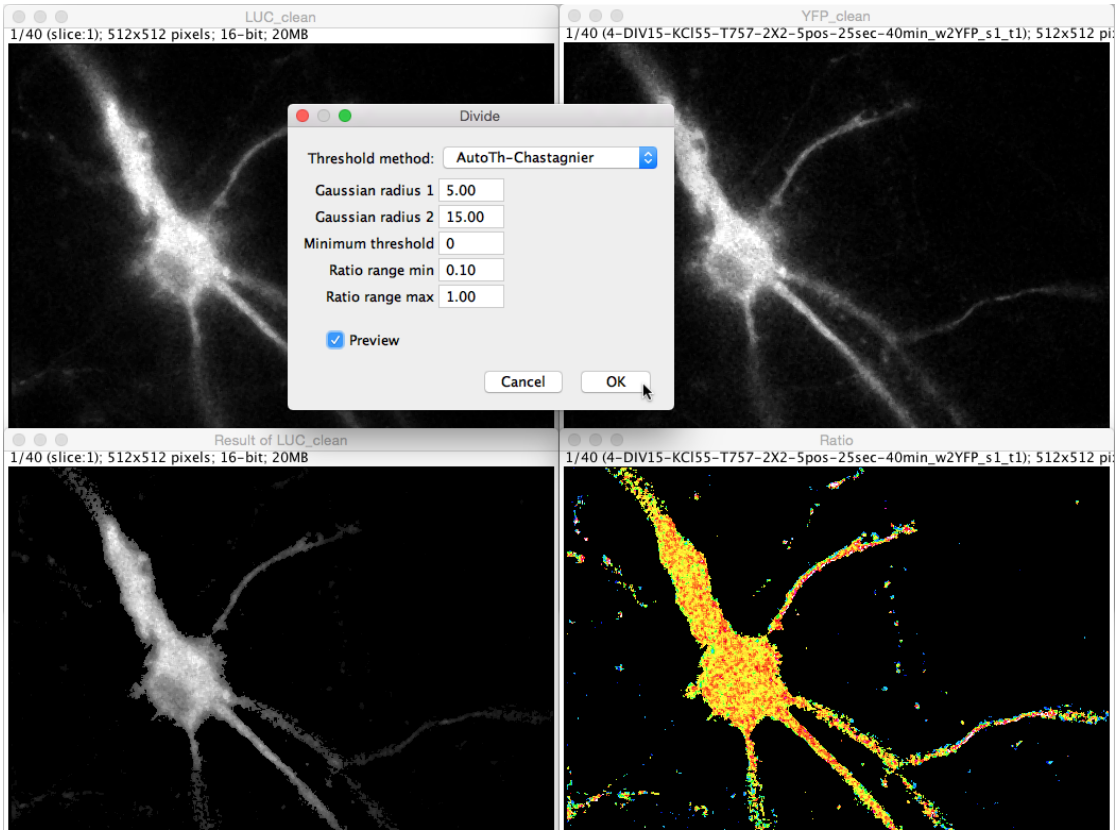
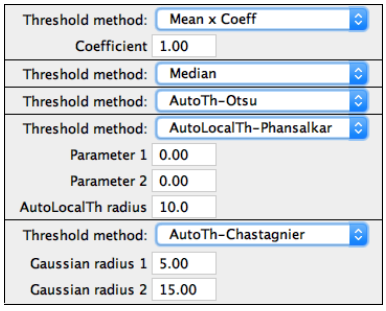


Figure 7 – Divide operation with preview enabled. Top left: donor clean. Top right: acceptor clean. Bottom left: donor clean thresholded using a combination of the *Threshold method* with its specific parameters, here *Gaussian radius 1 and 2*, and a *Minimum threshold* for which every pixel with a value below is set to 0. Bottom right: acceptor clean divided by donor clean thresholded, displayed in pseudocolors scaled between *Ratio range min and max*.



The way the different *Threshold methods* work is described below. For all of them, a local or global threshold is computed and values below it are set to 0. This threshold is computed for each slice independantly.

- *Mean x Coeff*: a global threshold is computed by taking the mean of a region traced by the user and multiplied by the coefficient entered in the parameters.

- *Median*: a global threshold is computed by taking the median of a region traced by the user. It means half the pixels of the region are kept and half are set to 0.

- *AutoTh*: Automatic Thresholds are described at https://imagej.net/Auto_Threshold, except for *AutoTh-Chastagnier* which is specific to the plugin.

- *AutoLocalTh*: Automatic Local Thresholds are described at https://imagej.net/Auto_Local_Threshold. *Parameter 1 and 2* have different usage depending on the method selected. A value of 0 keeps the default value of the method. *AutoLocalTh radius* defines the area used to compute the threshold.

- *AutoTh-Chastagnier*: this method is described in the article https://www.frontiersin.org/articles/10.3389/fncom.2017.00118/full, figure 3. *Gaussian radius 1 and 2* change the effect of the gaussian blurs used in the method. Their default value of 5 and 15 are adapted for neuronal cultures imaged with a 40x objective.

4.4 Analyse

Analyse button opens an *Analyse* window together with the *ROI Manager* window. *Analyse* window is composed of buttons and parameter fields on the left, the paths list of opened images on right top, and the list of ROI tags on right bottom.

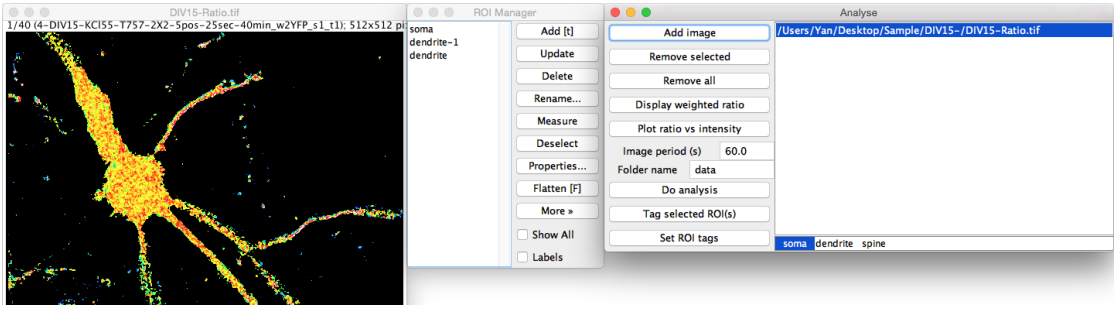


Figure 8 – Analyse. Left: an example of ratio image opened. Middle: the ROI Manager with three regions. Right: the *Analyse* window itself.

1. *Add image* opens a dialog window asking the user to choose a ratio image. Image is opened and its path is added to the list. If ROIs exist for the image they are added into the *ROI Manager*. If an image was previously displayed, it is replaced by the new one, and its ROI(s) are saved.

2. *Remove selected* removes the currently selected path from the list and closes the image after saving its ROI(s).

3. *Remove all* empties the list of paths and closes all images, saving ROI(s) of currently displayed one.

4. *Display weighted ratio* opens the donor clean image corresponding to the ratio image currently displayed and the preview of the ratio color brightness weighted by the intensity level of the donor. A dialog allows to adjust the range of the brightness. The *Low threshold* sets the percentage of pixels that will be black (the ones with the lowest intensity on donor image), while the *High threshold* sets the percentage of pixels that will have maximum brightness (the ones with the highest intensity on the donor image). The brightness is scaled linearly for the remaining pixels, based on their donor intensity value. *Weight on image histogram* use the thresholds on each image independently, saturating the same proportion of pixels on each image, showing relatively intense parts of an image. *Weight on stack histogram* use the thresholds for the whole stack, highlighting the variations of donor intensity along the stack.

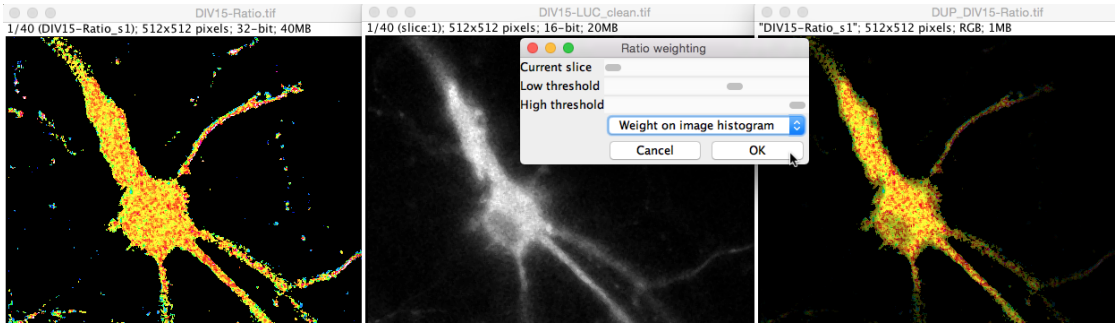


Figure 9 – Weighting process. Left image: original ratio. Middle image: weight (donor intensity). Right image: weighted preview of current slice. The dialog allows preview of current parameters. *Current slice* sets the slice to preview. *Low threshold* sets the percentage of black pixels. *High threshold* sets the percentage of pixels with full brightness. Pixels with values between the two thresholds are displayed with brightness linearly proportional to their weight values. *Weight on image histogram* computes thresholds on each image, while *Weight on stack histogram* computes thresholds for the whole stack.

5. *Plot ratio vs intensity* opens a dialog asking the user to choose between plotting the data versus intensity or intensity ratio. If versus intensity ratio is chosen, two intensity windows are opened in the following instead of one and all must be applied to both. The user is asked to choose an intensity image (this image is automatically found if the process has already been done). The region is automatically copied on first slice (or on the slice previously used). Depending on acquisition of images, eventually adjust the slice and the location of the region. Press *Escape* to change intensity image. Repeat the operation for all regions of all the images in the path list.

   The resulting displayed output depends on the nature of the ratio images. If all of them consist of a single slice, a 2D plot is displayed with intensity [ratio] as x axis and BRET ratio values as y axis. If at least one of them is an image stack (multiple time points), an interactive 3D plot is displayed with timepoints as x axis, intensity [ratio] normalized as y axis (minimal value is set to 0 and maximal to 255), and BRET ratio values as z axis.

   The results are also saved in a text file named *vsInt.csv* or *vsIntRatio.csv* in the same folder as the results from *Do analysis*. The first column corresponds to the intensity [ratio], and the next columns to the different timepoints.

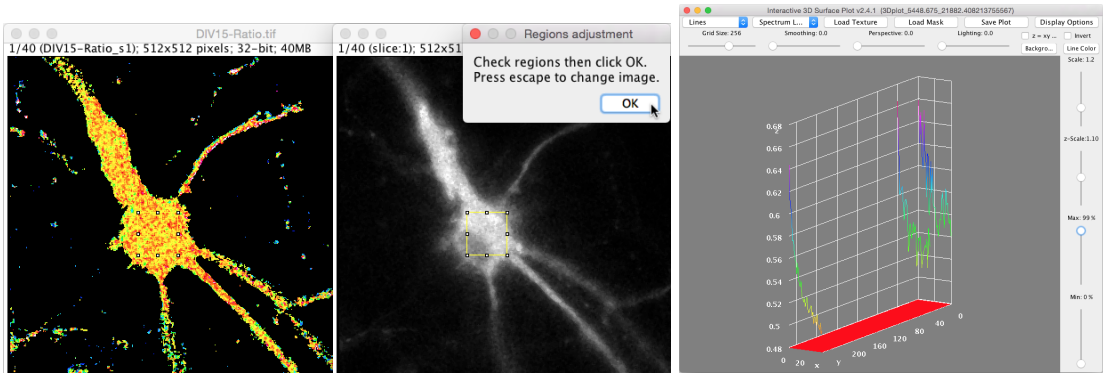


Figure 10 – Plot ratio vs intensity. Left: BRET ratio image with a region outlined. Middle top: dialog to validate region. Middle bottom: intensity image, for example a fluorescence image indicating the amount of fluorescent proteins. Right: resulting 3D plot. The three lines correspond to three regions across time. Lines, Spectrum LUT and a Smoothing of 0 should be selected if they are not by default. Hold left click and drag the plot to rotate it.

6. *Image period (s)* sets the time between two images. The timing for each image is saved in minutes within the results, starting at 0. If *Image period (s)* is set to 0, the user will be asked to choose a text file containing one timing in seconds per line, when clicking *Do analysis*.

7. *Folder name* sets the name of the folder in which the results are saved, including the result of *Plot ratio vs intensity*. This folder is placed in the common ancestor folder of all images in the paths list. If it already exists, clicking on *Do analysis* will pop up a dialog telling the user to either keep the folder name and overwrite existing data, or change the folder name to save it in a new one. The full path is displayed in the log window when results files are saved.

8. *Do analysis* measures the means and standard deviations of each ROI of each image present in the paths list. Those measures are displayed in the table *Analyse results*, Figure 11.

   The results are also saved in one or several files. In the first file, which is named after the folder (here: data, hence data.csv), all the results are saved (the full content of the *Analyse results* table). If regions of interest were tagged, another file is created for each used tag, containing only the data for regions with this tag.

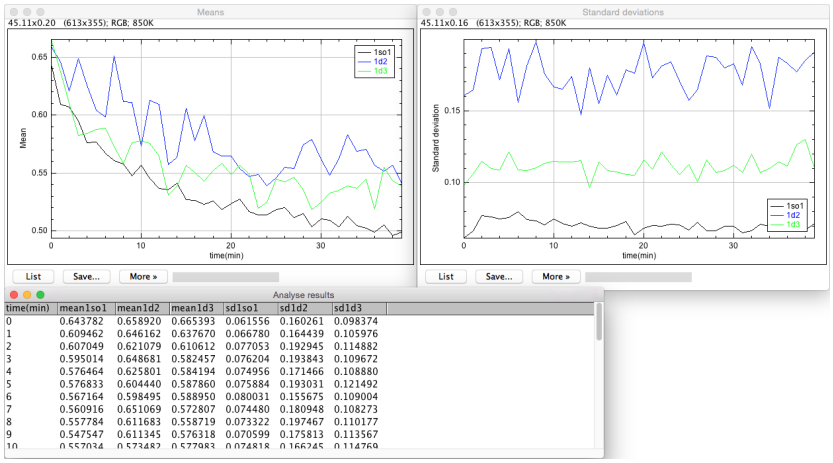


Figure 11 – Top left: traces of the mean value of regions across time. Top right: traces of the standard deviation value of region across time. Same colors are used for both graphs. Bottom: Analyse results table. First column is the time in minutes. Next columns are the mean values. Last columns are standard deviation values. The title of the columns is composed of 4 parts: mean or sd, the image number in the list, the shortened tag of the ROI (replaced by _ if the ROI is not tagged), the number of the ROI for that image. The shortened tags are the shortest beginning of the tags with no possible confusion. For example, with the three tags we use here: soma, dendrite and spine, shortened tags are: so, d and sp. Two letters are used for soma and spine to distinguish them. If the windows *Means* and *Standard deviations* are moved on the screen for better visualization, manually close them to save their position. This way, they will be opened at the same location when doing futur *Do analysis*. Redoing the analysis or using the close button will ignore any movement of those windows.

9. *Tag current ROI* will assign currently selected tag from the tag list to the ROI(s) selected in the ROI Manager.

10. *Set ROI tags* opens a new dialog where the user can enter a list of ROI tags separated with ; as seen in Figure 12.

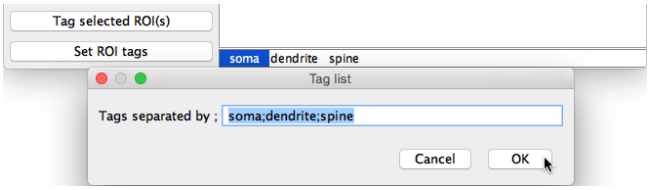


Figure 12 – Setting ROI tags. The purpose of the tags is to set the regions once, use *Do analysis* once and generate automatically data separated in files for each tag.

5 Output files structure

Following the naming convention, raw images are named xxxDonor.tif and xxxAcceptor.tif. Where xxx is any character string, Donor and Acceptor are character strings from the parameters'

fields Donor and Acceptor.

Clean process will create a subfolder xxx in which everything will be put, starting with clean images: xxxDonor_clean.tif and xxxAcceptor_clean.tif.

Crop process will extract a sub area of the clean images and save them as xxxCROP<i>_Donor_clean.tif and xxxCROP<i>_Acceptor_clean.tif, where <i> is crop's number. It will also save regions in cropAREAs.zip, and the crop's number with a label in file CROPs.txt, if *Label CROPs* is ticked in parameters.

Divide process will create the ratiometric image xxxRatio.tif. The threshold used and associated parameters are saved in the text file xxxRatio_thUsed.txt. If CROP images are processed, the image will be named xxxCROP<i>_Ratio.tif and text file xxxCROP<i>_Ratio_thUsed.txt, where <i> is the crop's number.

Analyse process will use the character string entered in parameters' field *Folder name*, which will be referred as yyy in the following. A text file yyy_paths.txt is created containing the paths of images used in *Do analysis*. It allows the user to select it instead of an image during execution of *Analyse* to quickly load all the images contained in the file and their corresponding regions of interest. A sub-folder yyy is created which will contain the following files: spreadsheets yyy_MeanOfRatios.csv and yyy_MeanOfRatios.xls containing means and standard deviations of all pixels in regions of interest from the ratio image; yyy_RatioOfMeans.csv and yyy_RatioOfMeans.xls containing ratio of mean of acceptor pixels over mean of donor pixels for each region of interest followed by the number of pixels used (only pixels kept for the division are used so the number might change for a given region); same spreadsheets but including only one tag, e.g. yyy_tag_MeanOfRatios.csv; images Image<i>.png showing the regions selected on each ratiometric image with <i> the image number in the list. ROIs for each image are saved as xxxRatio_ROIs.zip.

In addition to those files created with *Do analysis*, *Plot ratio vs intensity* also generates files: a text file xxxRatio_vsInt.txt or xxxRatio_vsIntRatio.txt containing informations to automatically open intensity image and regions for each image; a text file yyy/vsInt.csv or yyy/vsIntRatio.csv summarizing the results for all images in paths list.

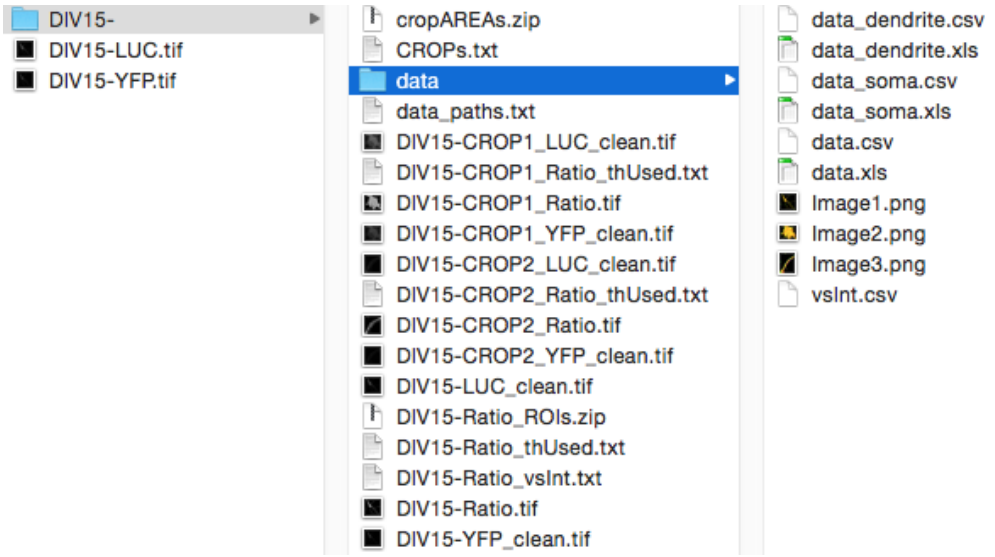


Figure 13 – Files structure. Here xxx is DIV15-, Donor is LUC, Acceptor is YFP, yyy is data.