**User Guide for ImageJ/Fiji Macro for measurement of micro-irradiated stripe region and background intensity**

**Introduction**

The macro consists of 3 sub-macros that should be run in order. The first macro (**process\_nd2\_and\_drawROIs**) allows the user to select nuclei that are appropriate for measurement. The second macro (**clip\_and\_align\_nuclei**) performs image alignment/registration using the plugin “MultiStackReg” for all selected nuclei. The third macro (**measure\_stripe**) interactively allows the user to select appropriately aligned nuclei and adjust the stripe area (micro-irradiated region) for measurement. For each selected nucleus, the mean intensity of the stripe and background region for each time point is output to a table. All macro functions that are interactive will save the users’ progress at any time and allow for continuation/reload of previous work.

**Initial Set up:**

Step 1: Install TurboReg and MultiStackReg plugins for ImageJ/Fiji.

Go to **Help > Update** and click on **Manage Update Sites**. Check **BIG-EPFL** and **MultiStackReg** from the list and click **Close**. Then click **Apply Changes** and restart ImageJ/Fiji.

Step 2: Load all macros in ImageJ/Fiji.

Go to **Plugins > Macros > Install** and select the macro file (**Clip\_and\_Align\_Nuclei.ijm**). This will load all three macros to the **Plugins > Macros** menu.

**Macro Workflow:**

Step 1: Draw ROIs around selected nuclei

Go to **Plugins > Macros > process\_nd2\_anddrawROIs.** The macro will prompt for a directory where sub-folders containing nd2 movie files and binary tif files with the initial stripe region are located. The stripe region file format expected is a binary file (1/0) data type where the value 1 is set for the stripe region and 0 for background (this file can be exported from the microscopy software). The macro will expect the stripe region file to have the same name as the nd2 file with the suffix “\_ROI.tif”, for example: img001.nd2 & img001\_ROI.tif.

The macro will perform the following for each nd2 file in the sub-folders of the selected directory:

* Open nd2 file and perform a Z-Project (max intensity)
* Open tif file and draw the stripe regions over the max-intensity image
* Open the ROI Manager and prompt user to select appropriate nuclei for analysis. Selection tools can be a rectangle or polygon ROI. Add each ROI to the ROI Manager. When finished, select OK. The macro will save the ROIs as a zip file. It will also save the max-intensity image and convert the nd2 movie to a tif movie for later processing.
  + *Tips for selecting nuclei*: Choose nuclei overlapping a single stripe region. Do not choose nuclei where it is not possible to draw an ROI without including other nuclei or background artefacts. This will interfere with the image registration process.

Note: The macro can be cancelled at any time. When restarted, the macro will load the max intensity image and ROIs that have been selected in previous run. The user can then continue with ROI selection or move to the next movie.

Step 2: Clip and align nuclei (runs without user interaction)

Go to **Plugins > Macros > clip\_and\_align\_nuclei.** Select the same directory as chosen in Step 1 above. The macro will clip each ROI from the original tif movie over all time steps. A contract enhancement and median filter is applied to each frame of the movie and the plugin **MultiStackReg** is invoked to perform image alignment/registration for movement of the nuclei over the time frame of the movie. The plugin will use the altered image for calculation of alignment parameters but will align the original image (later used for intensity measurements).

The macro runs in “batch mode” so no windows will be displayed. A text message will show progress and the user will be alerted when the macro is finished.

Step 3: Accept/reject nuclei, adjust stripe regions and measure intensity

Go to **Plugins > Macros > measure\_stripe.** Select the same directory as chosen in Step 1&2 above. For each nucleus displayed, perform the following steps:

1. Scroll over the time frames of the movie and decide if the alignment succeeded. Sometimes, **MultiStackReg** is not able to properly register the movie if the nucleus has rotated or shifted too much from one frame to the next. Often this may occur towards the end of the movie and most frames are correct. If the nuclei should be completely discarded due to bad alignment, uncheck the box. If most of the movie is okay but the intensity measurement should not be taken after a certain time point due to bad alignment towards the end of the movie, then enter the starting failure frame and keep the box checked.
2. The region where the stripe intensity measurement will be taken is shown as a rectangular ROI. Adjust the ROI size/position as needed to cover the actual stripe (scroll through the movie to determine best position). If the width of the ROI is consistently too small, this can be adjusted automatically for the next nucleus image by changing the “Expand by” text box to a larger width.
3. Click OK when finished and the macro will measure the stripe and background intensity for the nucleus. The background intensity will be determined by performing an automatic threshold on each frame. First, the image is contrast enhanced and a median filter is applied. Then, a threshold is applied (Otsu) to produce a mask, and Fill Holes is executed to fill any small holes in the mask. This mask region should cover the entire nucleus. The stripe region is subtracted from the nuclear mask and the mean intensity over this background region is recorded for each frame. The mean intensity of the stripe region restricted to the area of the nuclear mask is also recorded. The macro will display plots of (1) background (2) stripe and (3) stripe/background ratio intensities. It will also display the outline of the ROI used to measure the background intensity. Check that the image mask and the intensity measurements look reasonable and correct throughout the time frames of the movie (Figure 1). Use the dialog box to indicate whether the measurements for this nucleus should be added to the output file (a new failure frame can also be indicated here, if necessary). Click OK to continue. \*\*\*

\*\*\* Note: We have observed some issues with this step when running on a PC (works correctly on Mac OS). If the output plots look incorrect (stripe region is not being measured), please open an Issue in github and we will try to help troubleshoot the problem.

The intensity information for each nucleus will be saved to a comma-delimited csv file that can be opened in Microsoft Excel in the relevant sub-folder (Figure 2): <folder\_name>\_<date\_and\_time>\_measurements.csv. The column labeled “RatioSaturated” indicates the ratio of pixels at the image saturation level (max value of a 12-bit image (4095)).

Note: Click Cancel at any time to stop the macro. Re-starting the macro will allow for continuation from the last nucleus processed.

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| Graphical user interface, application  Description automatically generated |
| Figure 1: Intensity measurement results. |

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| Graphical user interface, application, table, Excel  Description automatically generated |
| Figure 2: CSV file output format |