**Instructions for using Quant\_Biofilms.ijm macro** (version 6)

**About:** This macro was originally designed to find biofilm/colonies in a brightfield channel and quantify intensities in 2 fluorescence channels. The required input is a muti-series file that Bio-Formats can open, e.g. Leica LIF.

The macro can now also handle brightfield-only images, i.e. to measure area. It also has an optional “test” mode to assess segmentation results, and a subset of images can now be specified for analysis.

1. You should be able to drag-and-drop the macro onto the "status bar" of Fiji and it should open in Fiji's "Script Editor" (where you can see the actual code). I have seen the drag-and-drop open functionality broken before so if that does not work then use the menu option File>Open to open the macro.

A screenshot of a social media post

Description automatically generated

2. Run the macro using the Script Editor's "Run" button.

Text

Description automatically generated

3. A dialog is presented to set parameters and options. Note that channel numbering starts at 1, and you can set the green and/or red channel number to 0 to tell the macro that it is missing.

Parameters such as Green and Red background intensity levels and minimum colony area can be adjusted in the dialog. The “display max” values are used for output colour merge display settings for visual comparison of images.

Text, table

Description automatically generated with medium confidence

By default only a single colony at the centre of the image will be detected – if “Segment subcolonies” is selected instead all bright objects larger than the minimum size will be detected. The “Subtract Background” option may be necessary if the plate background is significant. There is an option to specify first and last images to analyse rather than all images in a large file, and a “Test segmentation” mode that shows segmentation results for selected images without carrying out further analysis.

Colocalization stats are not calculated if there are fewer than two fluorescent channels, and a tickbox allows calculation of Manders coefficients to be turned on.

4. Macro prompts you to choose the (e.g.) LIF file containing the images to analyze.

A screenshot of a cell phone

Description automatically generated

5. The macro will analyze all images in the file and write 1 row of results per image to the Results table window (this can be saved as a .csv file you can open in Excel, or you can just copy/paste into Excel!) The macro will also create a timestamped folder with a name based on the input filename that contains some "snapshot" images with identical display settings and the channels merged. The Results table is automatically saved to this folder as well. If you open the snapshot images in Fiji or ImageJ you will see an "overlay" that shows the colony area that was found as an outline.

A screenshot of a cell phone

Description automatically generated

A picture containing fruit, food, device

Description automatically generated

6. How to interpret the output:

- **colonyArea** measures the size of the colony found (calibrated units, e.g. µm2)

- **greenMean, greenMax, greenStd,** **greenTota**l are the mean (average), maximum, standard deviation and total intensity values of the green channel in the colony, respectively

- **redMean, redMax, redStd,** **redTotal**: as above for the red channel

- **greenForegroundTotal** and **redForegroundTotal** are the total intensities\*\* *of above-background pixels* in colonies in the green and red channels, respectively – these values are a good measure of total cell number and expression level

- **pctRed** and **pctGreen** are percentage of colony area above-background in the red and green channels

- **PCC** is Pearsons Correlation Coefficient for all pixels in the colony, including background

- **PCC\_obj** is “object Pearsons”, which is for above-background pixels only – it is a measure of how well red and green intensities correlate, where 1 means they vary in tandem, 0 means they are unrelated and -1 means there is negative correlation (i.e. exclusion)

- **M1\_R** is Manders coefficient M1, the proportion of red intensity located in pixels where green is above-background

- **M2\_G** is Manders coefficient M2, the proportion of green intensity located in pixels where red is above-background

\*\* Note that the *foregroundTotal* intensity values are *without* background intensity subtraction for each foreground pixel.