Laurdian ratiometric analysis

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Following Nature protocols paper: "Quantitative imaging of membrane lipid order in cells and organisms" , Owen et al, 2011. doi:10.1038/nprot.2011.419
https://www.nature.com/articles/nprot.2011.419
Adapting ImageJ macro of the Nature protocol (NP) paper, which is available at:
https://media.nature.com/original/nature-assets/nprot/journal/v7/n1/extref/nprot.2011.419-S1.txt

1 Overview

The tuned macro is based on published macro with the following changes:

- **GP calculation corrected** (the original GP – which I left as is - gave "all zero results"), the corrected calculation is stored in GP1 folder. Basically GP is a masked version of the PreGP image, the correction is in the implementation of masking: instead of multiplying the PreGP image by a (1bit) mask and converting to 8 bit, the mask is used to set the out-of-mask pixels to NaN to ensure they are discarded from the histogram calculation. Setting the out-of-mask values to zero is not correct as zero is a valid value for PreGP/GP

- The **masking** is **based on (Order+disorder)>2*Th** , BUT originally the disorder image was not the original file but the image saved in the disordered subfolder.
  It means that if Use-G-Factor was set to NO the it tests for (Order+disorder)>2*Th and if it is set to Yes it test for (Order+Gf*disorder)>2*Th
  I changed it to always use the original (non-scaled) disorder file for masking

- **User can select regions of interests (ROIs) of different types** (eg cells / membrane), ROIs are saved and **histograms of GP values within those regions are calculated**.
- Results folder name is fixed and does not include date/time. This way, one can use existing ROIs for repeated calculation.
- Histograms are saved for ALL (in GP1\Histograms) and for each ROI type in ROI subfolder
- Number of Bins in the histogram can be selected. Default was changed from 256 to 40. Also Histograms are printed and saved as jpeg files.
- **Pixel values are saved for each ROI in a table** (default is one table for all the ROIs of an image), for ordered channel, disordered channel, PreGP and GP1

2 Usage instructions

The macro assumes that files are saved independently for each channel of a single slice image and named with predefined suffix.

In practice, multi-slice data is acquired for the purpose of manual selection of desired slice. Several image stacks are stored in a single lif file. Thus as preliminary stage one should save the files properly in a folder or set of folders. See the instructions in the section **Error! Reference source not found.**2.1.

Once you have the files organized correctly in a folder, you can run the *Macro_Nature_Supplemental_TunedForFutermanLab.ijm* macro to calculate GP values and histograms for each image in the folder. Section 0 will guide you how to run the macro and what are the output files.

You will further need to group together the values of all the images related to specific condition, using another application (eg excel or Matlab/R script) not provided here.

Additional *CalculateGFactor.ijm* macro is provided for calculation of the G-Factor. See section 2.2

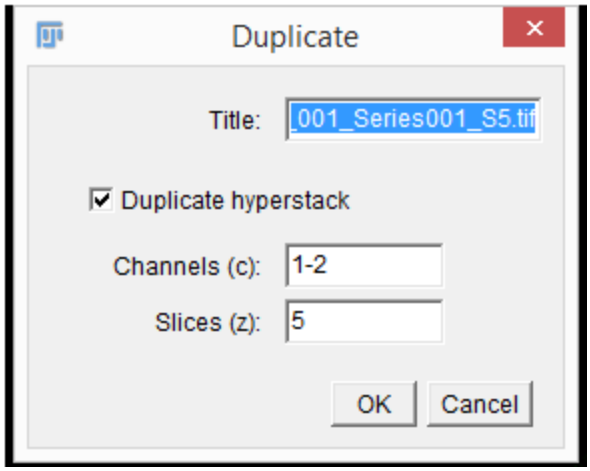
2.1 Prep Files

Several Image stacks of the two channels Files are all stored in one Lif file. As the macro works on 2D images, you need to select single slice images and export them into ImageName_sN_**ch00** and ImageName_sN_**ch01**

Either use LasX for this, OR

In Fiji:

- Export single series from the lif file:
    - Run the *ExportImagesFromComplexMicroscopyFiles.ijm* macro to separate the lif file into single stacks. The single stacks will be stored in a subfolder of the original lif location named *LIF_FILE_NAME_Tif*
- Export single slice , single channel images:
    - Open each stack,
    - Select the desired slice, Duplicate (Ctrl-Shift-D) both channels for the selected slice name it *ImageName_SN.tif*,



- Export to single channels: *File=>Save As=> Image sequence* in a subfolder (eg named SingleSlice_SingleChannel). You get files named: *ImageName_SN _{ch00/ch01}.tif*

2.2 G-Factor calculation

Prior to running G-Factor you need to prepare the ON and OFF calibration file. They need to be either 2D (single slice) images where the first in a pair is acquired with laser on, the second image should be acquired with laser off. Each of them has two channels: Ch1, Ch2.

If your images are saved in a lif file you should extract them into separate single-slice 2-channel images for ON and OFF condition.

The macro *CalculateGFactor.ijm* prompt the user to select ON and OFF pair of files and use them to calculate G-Factor based on the following equation.

- For each pair do the following
  - Measure the mean intensity of the following images:
    i. Ch1 On,
    ii. Ch1 Off
    iii. Ch2 On
    iv. Ch2 Off
  - Calculate **G=(Ch1 On - Ch1 Off) (1-0.035654) / (Ch2 On - Ch2 Off) (1+0.035654)**
  - Print out the value of G to the Log window

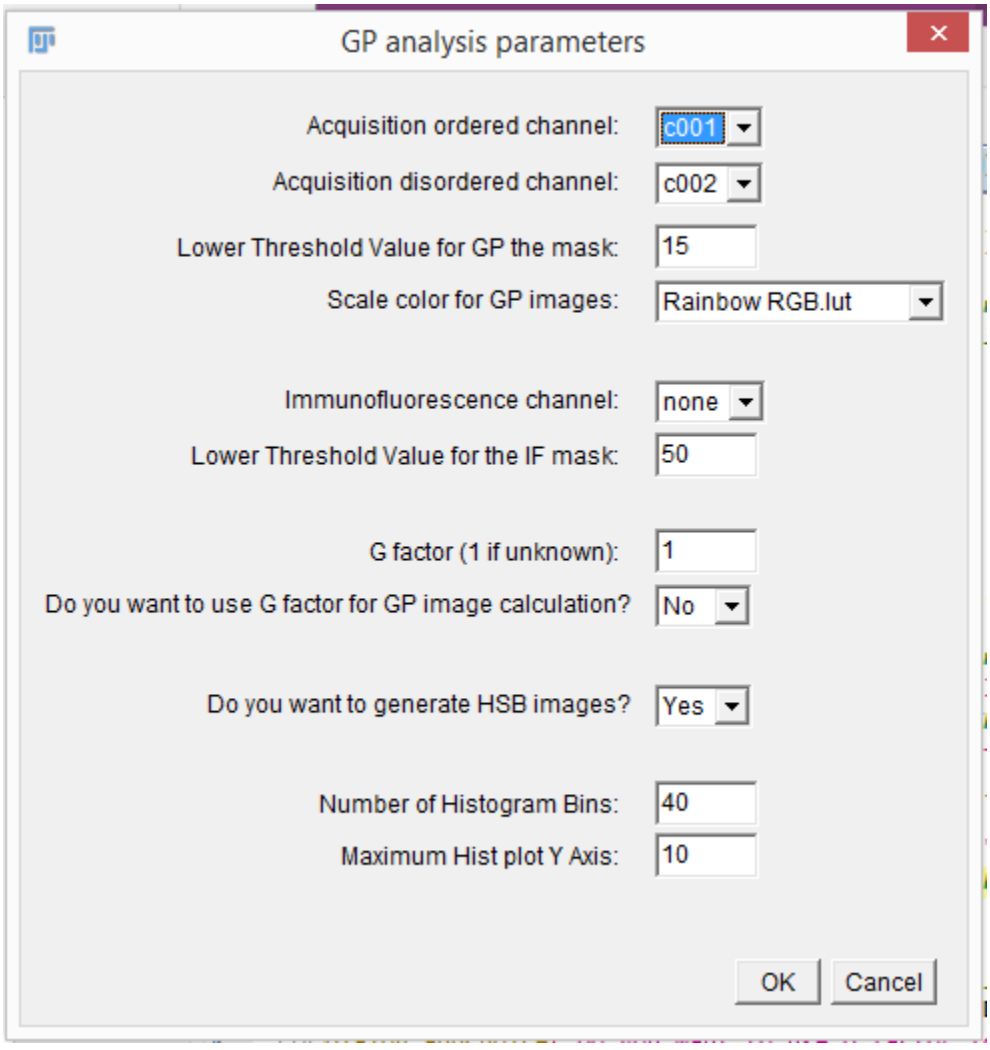
2.3 Use the GP calculation macro

Run the macro: *Macro_Nature_Supplemental_TunedForFutermanLab.ijm*

The macro will guide you to select and set parameters

1. You will be prompted to **select a folder**. The macro processes all files in a selected folder. The images should be single slice, single channel. It is assumed that the image names end with “c001” or “c002”.
   You can change this default by changing the values in the ***CHANNEL1*** and ***CHANNEL2*** array variable and *DefaultOrderedChannel*, *DefaultDisOrderedChannel* variables.

2. You can **set GP analysis parameters**



Acquisition ordered channel: default suffix for lower spectrum image

Acquisition disordered channel: default suffix for higher spectrum image

Lower Threshold Value for GP the mask: if GP values are masked using the channels used for GP calculation, then all areas where the SUM of both channels < Lower Threshold * 2 are masked out. **Make sure to check a value suitable for your dataset.**

Scale color for GP images: …

Immunofluorescence channel: if another channel is available to use for masking the GP values instead of using the ordered + disordered images

Lower Threshold Value for the IF mask

G factor (1 if unknown): You can set to different value. Default is 1, you can change the default by changing *DefaultGFactor* at the beginning of the scrip.

Do you want to use G factor for GP image calculation?

- The default was "*No"* I changed it to *"Yes"*
- ordered image is always saved as is *"Ordered Images"* subfolder, and then read to be used in actual calculation
- disordered image is saved in *"Disordered Images"* , and then read to be used in actual calculation
- when calculation is done they use (Ord-Disord) / (Ord+Disord) this is the PreGP image, which is further masked to produce the GP (or the corrected GP1) image

Yes:

  - disordered image is multiplied by G-Factor before saving
  - GP and GPc values are the same

No:

  - disordered image is NOT multiplied by G-Factor before saving
  - GPc is "corrected" for not using G-Factor in the calculation as followed

    GP[i]=((i-half_nBins)/half_nBins) // evenly spaced GP values for x-axis

    GPc[i]=-(1+GP[i]+Gf1*GP[i]-Gf1)/(-1-GP[i]+Gf1*GP[i]-Gf1)

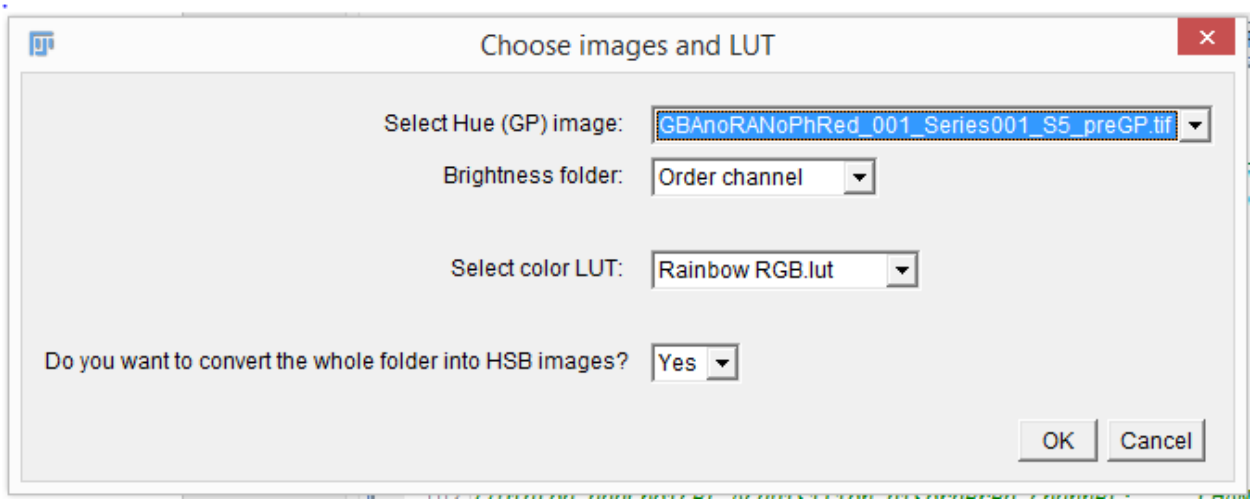
- GPc is only printed out, but not used through the calculation
- This is part of the original design of the macro
- Notes: both ordered and disordered images are converted to 8-bit before calculation. This is OK now – as you acquire images in 8-bit, but should keep this in mind if you plan to acquire in 10/12/16-bit.

Do you want to generate HSB images?

Number of Histograms Bins: default number of Bins used for histogram creation. (the original value was 256)

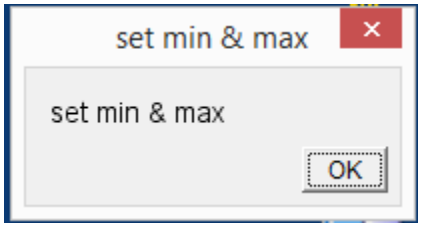
Maximum Hist Plot Y axis: The Maximal y-range in the histogram plot. Default is 10%

3. Choose Images and LUT for **creation of HSB images**



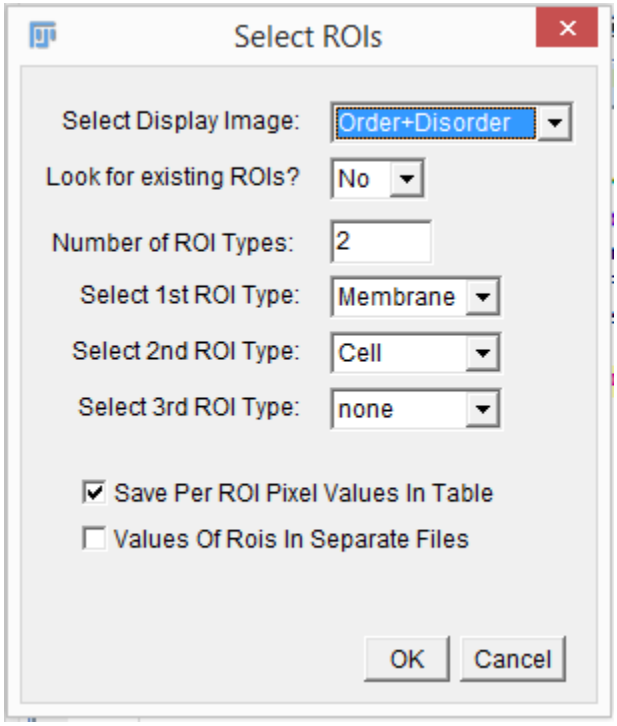
Nothing to change, just click OK

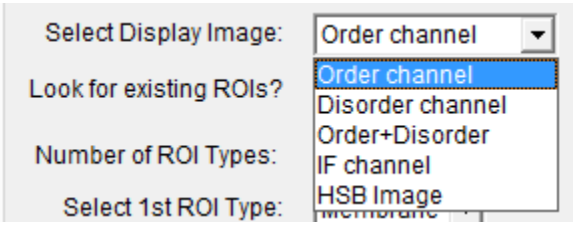
4. You will be prompted to **set the min & max values** – just click OK
   I think (?) this will control the GP / GP Corrected values – the x-axis of the histogram, keep it [-1,1]



5. ROI Selection setup
   The next step is to get GP histograms of specific areas, for this the user can draw regions of interest (ROIs). The following dialog let you select the *Display Image* = the image that will be shown to the user for selecting the regions. Currently there are 4

options as you can see below. Note that you can use the HSB image for selection, but this is will be more subjective as it will include the results, so either use IF signal if available or ordered+disordered image.





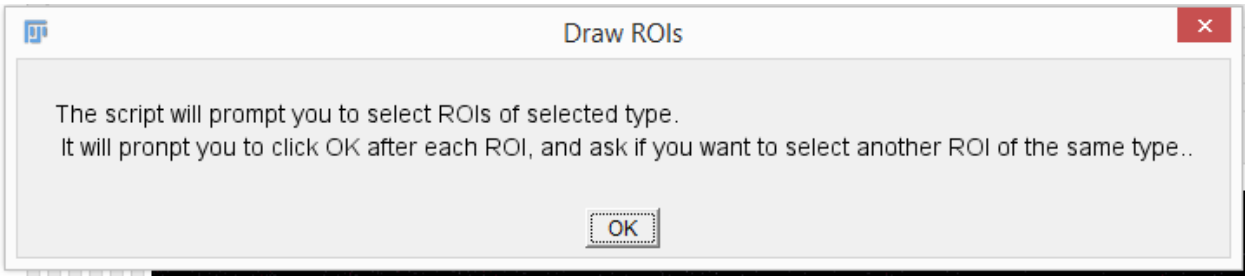
Look for existing ROIs? If you select *No* , you will be prompted to draw ROIs for number of types specified in *Number of ROI Types*. You can change number of types and select the type names from the list. These names are used when naming the selected ROIs in the ROI manager.
To change the list of names you need to change the variable *ROI_TYPES* in the macro code (inside the *ROIanalysis* function)
The selected ROIs are saved into a file named *IMAGE_NAME_**ROIs.zip*** under the *ROI images* subfolder.

If you select *Yes* .The macro will look for saved ROI file for each image, if such file is found it will use it instead of prompting you to draw new ROIs. If ROI file does NOT it will prompt you to draw new ROIs. This enable running the macro again using

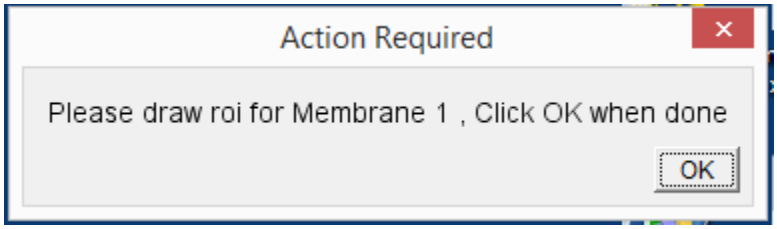
previously selected ROIs. You can create ROI file on your own but you should make sure to names of ROIs are set correctly (see description below). The easiest is to create the ROIs with the macro, and if you are not satisfied delete those of the files you are not satisfied with.
You should also be able to move ROI files between computers by simply coping the ROIs file and putting it in the right folder.

6. ROI drawing

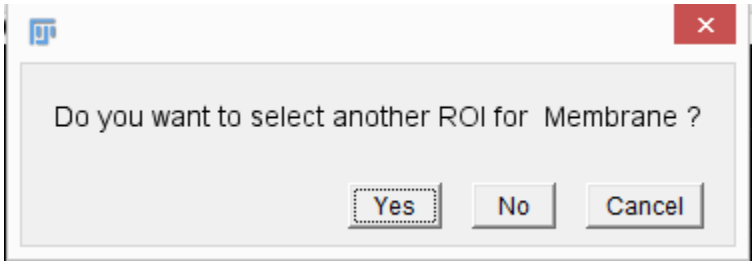
You will first get intro message – once for each image



You will then be asked to draw a closed ROI and click the OK button when done.
The default drawing tool used is the polygon tool, but you can change to any other drawing tool. (Ellipse, Rectangle or Free hand tool).



The macro will suggest you to draw additional ROIs of the same type, and when you click NO it will switch to the next type.



When drawing is done for one image, the macro will create the histograms for the the different types and save the per-ROI pixel values. This process takes a while (**BE Patient**!) and you'll see tables popping up during this process.

And again for cells
And again for the next images …

After drawing Histograms of GP values are generated for each ROI type for each image, and per-pixel values are saved for each ROI.

During this process you will see tables popping up. It may take a while, be patient !

At the end a message of *"Analysis Done !"* will be printed to the Log file.

2.4 Output files

Results are saved in subfolders under the Results folder.

Log file with date and time is saved to the Results folder

Ordered Images: original images ordered images (after conversion to 8-bit, gray scale, conversion to 32-bit)

Disordered Images: original images ordered images (after conversion to 8-bit, gray scale, conversion to 32-bit), optionally multiplied by G-Factor – only if Use G-Factor is set to YES in the first Dialog

raw GP images: non-masked GP images

GP images: originally calculated GP images, however the values are not masked correctly

GP1 images: masking is working, the same logical scheme as used in the original macro, out of mask values are set to NaN

GP1 images\Histograms: histograms of corrected GP values for each image,

HSB images: color coded GP images … GP values are used as Hue (color), selected channel (eg ordered) is used for Brightness

ROI images: include

- ROI file IMAGE_Rois.zip,
- histograms for each image for each ROI type,
- Excel file with per-ROI pixel values : IMAGE_RoiVal.xls