1 Instructions for Object Differentiation Matlab GUI

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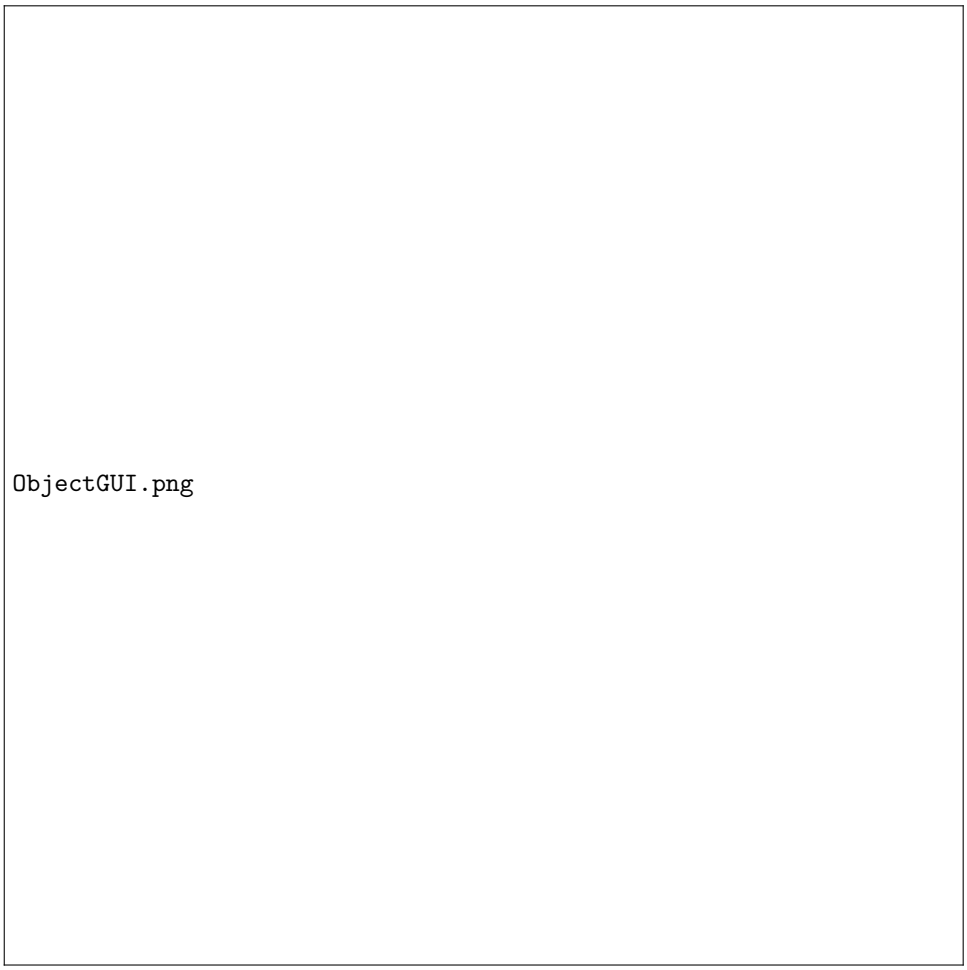
These are the instructions for using Liana Engie's Fluorescent Object Differentiation Graphical User Interface (GUI) on Matlab.

The program is designed to take a fluorescence image and quantify the intensity of brightness of the images. Background negation is done by masking out everything that is not the object of interest, finding the area of each object, and summing the brightness of individual pixels.



Input:

- A greyscale `.tif` images

Output:

- A display of the image, with the mask applied, and a list of the objects, their areas, and the brightness.
- Saved `.png` of the image with the mask applied,
- An `.xls` spreadsheet with the unmasked and masked calculated brightness of each image, and ratios of second and third channels, if applicable.

1.1 What the code does

Here I'll probably reference a paper once it's published. If you're really interested in the details, you should probably read my thesis. But, basically:

There are two levels of background negation. One (the mask) deals with the black background and nonspecific staining in the other channels, and the other level deals with non specific staining on the chomosome, or component of interest, itself. This is the case for a few reasons.

- The pixels of the black background of a fluorescence microscopy image often have a non-zero value, that can be quite substantial when the brightness of the image is summed. Thus, the brightness of the same object will change depending on how large the image is, and how much black space surrounds it. The mask negates this.
- If one of the antibodies stains lots of things quite brightly, and you only want the fluorescence in a specific area, the mask will zero in on that area (assuming the image you're using to make the mask is bright in just that area).

  For instance, a Chd1 stain may stain the nucleolus as or more brightly as the DNA, but to just get the Chd1 asociated with the DNA, use a DAPI image to make a mask and then apply the mask to the Chd1 image.
- In that area, however, there may be a large amount of background as well, depending on the specificity of the antibody. A threshold can be applied

to the second or third image that negates this. This is especially an issue for (spatially) small components with large amounts of background - differences can be hard to tell unless the background is removed.

(Not shown in the false color mask)

1.1.1 Key assumptions of the code

1. The code was developed on fluorescence images of chromosomes generated using multiple channels. It took the DAPI image, made a mask, and applied this mask to the other images to negate nonspecific staining issues. Thus, it takes the first image, *assumes that the component of interest is in the location of the brightest thing in the image*, and using a brightness threshold, makes the mask.

2. This code takes all of the `.tif` (**t**agged **i**mage **f**iles) in a folder and automatically runs through them and analyzes them. *It goes in alphabetical order, so the first alphabetically will be what is used to make the mask.*

3. The code is written for data sets with 1, 2, or 3 fluorescent channels. It assumes that each image has 0, 1, or 2 associated images, one for each additional fluorescent channel. The code runs through every `.tif` in the folder, using the number of channels as a sort of 'reading frame', so *additional or missing images will confuse it*. Essentially, if the number of `.tif` files in the folder isn't divisible by the number of channels, something is probably wrong.

   You should be able to see this when the program runs though - the false color mask will not line up with what's in the image. Also, anything that isn't a `.tif` won't be a problem at all.

1.1.2 Output outside of the GUI

After you process the images using the GUI, the program will automatically generate an `.xls` file that saves the raw calcuated data and ratios. The name of the file is generated using the Date Captured: '`dd_mm_yy Processed.xls`'. *For this reason, don't use slashes in the dates - the resulting file won't open.*

Additionally, each image, with the false color mask overlayed, is saved as a `.png` file within the folder for later access.

1.2 Opening Matlab and the GUI

This code was written in Matlab v.2007a. When opened, you should see the Command Window - probably the largest window - and possibly also the Current Directory, Command History, and maybe the Workspace. To use the GUI you only need to work with the Command Window and Current Directory.

To run the code on a set of data, put the `.tif` files to be calculated (no other `.tif`s!) into a folder with `Mask.m` and `Mask.fig`. You can do this within Matlab in the Current Directory window - just copy and paste the files into a folder together. The Current Directory must be open to the folder with the files that you want to analyze.

To open the GUI, either type

```
>> Mask
```

into the command window (case sensitive), or drag `Mask.m` from the Current Directory into the Command Window. The blank GUI should open:

1.3 Before Processing

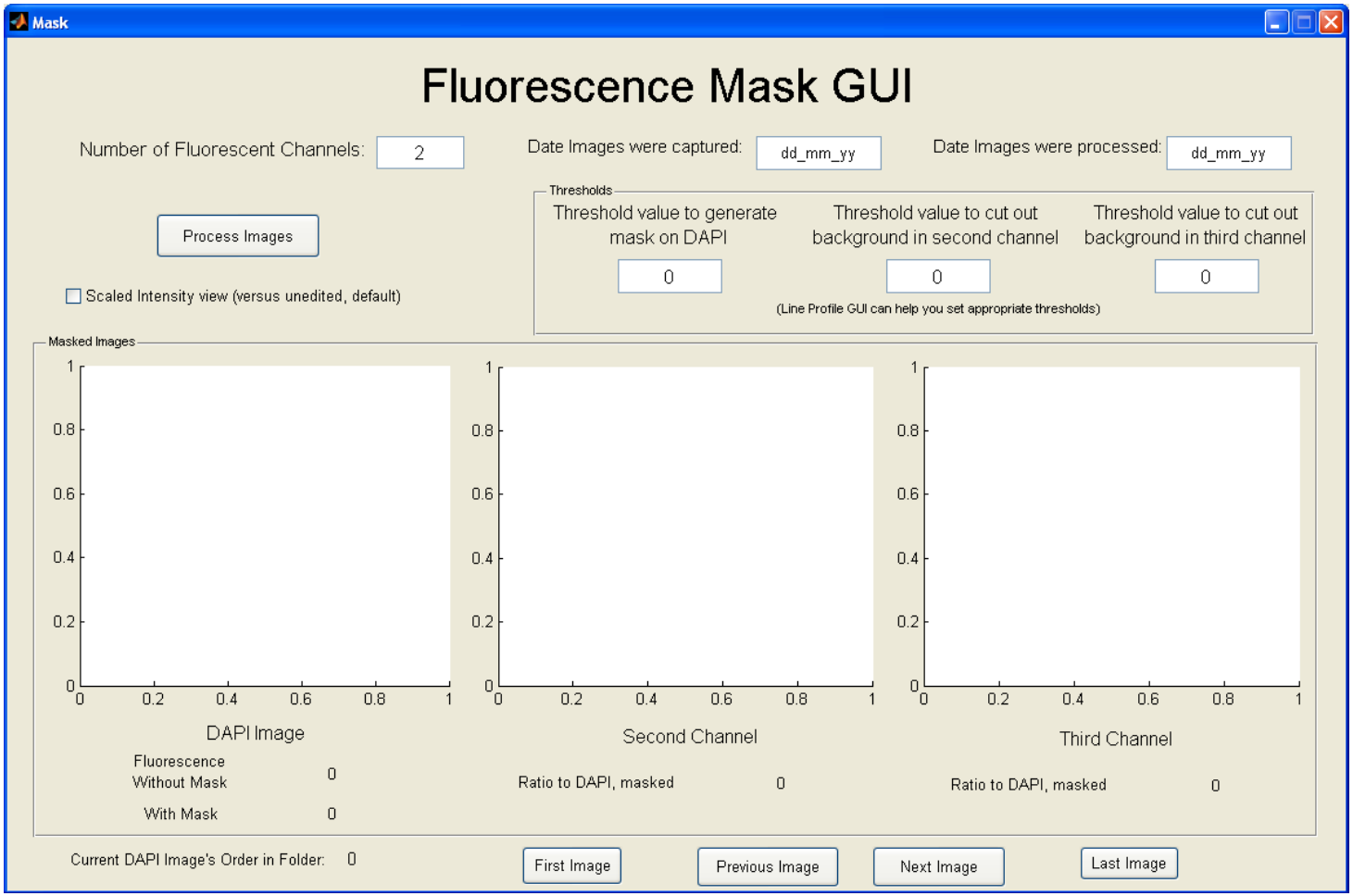
Before the images are processed, you need to set the variables.

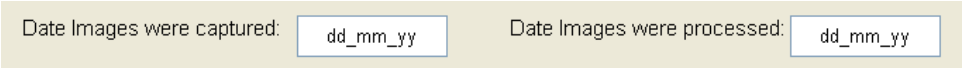
1.3.1 Setting Dates

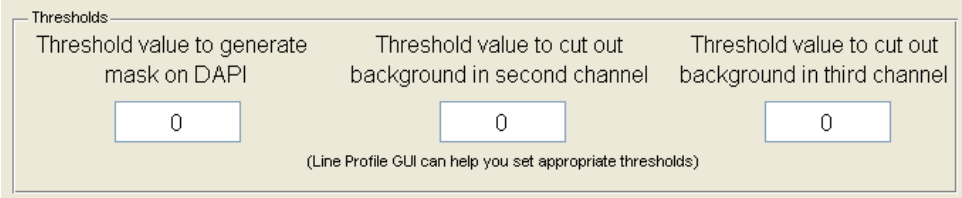
Input the dates the images were captured (will be used to make and within the `.xls` file) and the date processed (presumably, the current date - also for use in the spreadsheet). Do not use slashes or periods to separate date, month, and year, as these will confuse your computer when trying to open the spreadsheet. The default suggests using underscores, and if you do not enter anything it will remain 'dd_mm_yy'.

1.3.2 Setting Thresholds

These numbers will determine how wide the mask's coverage of the object of interest is, and control background negation within the area of the mask (see 'What the code does', above).


Mask
Fluorescence Mask GUI
Number of Fluorescent Channels:
2
Date Images were captured:
dd_mm_yy
Date Images were processed:
dd_mm_yy
Process Images
Scaled Intensity view (versus unedited, default)
Thresholds
Threshold value to generate mask on DAPI
Threshold value to cut out background in second channel
Threshold value to cut out background in third channel
0
0
0
(Line Profile GUI can help you set appropriate thresholds)
Masked Images
DAPI Image
Second Channel
Third Channel
Fluorescence Without Mask
0
With Mask
0
Ratio to DAPI, masked
0
Ratio to DAPI, masked
0
Current DAPI Image's Order in Folder:
0
First Image
Previous Image
Next Image
Last Image





There are three different thresholds. The leftmost threshold will be set upon the first image (DAPI, for instance) to create the mask. The second and third cut out nonspecific background within the mask, of the second and third images respectively (where applicable). Every pixel with a value under the threshold is cut out.

If the first threshold is 0, the mask will cover the entire image and every pixel will be counted.

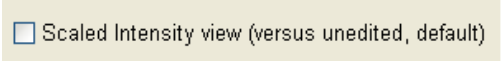
If the second and/or third thresholds are 0, the second and/or third images will not cut out anything in the area of the mask.

(If you change the threshold values while the analysis is running, or afterwards, the images will not be affected. The changed threshold will only come into effect after you press 'Process Images' again.)

The **Line Profile GUI** can help you determine what the thresholds should be.

1.3.3 'Scaled view' of images

Clicking on this checkbox will display the image with scaled range of brightness intensity. Usually, the image displays with highest possible value (depends on the image's bit depth) being absolute white, and the lowest possible value (0) being black. Scaling takes the individual image's range of values and scales the black and white colormap to it, thus making it easier to see images on the ends of the spectrum of absolute values, and differentiate features of an image.



Scaling the brightness does not change the numeric brightness of the image -

just the visual aspect.

1.3.4 Processing and force quitting

Once everything is set, press the 'Process Images' button to start the code going through every image in the folder.

The program will start going through every image in the folder, calculating their brightness values and displaying them with the mask applied. The counter on the bottom left of the window keeps track of the progression. (Note: the very last image sometimes take a few seconds longer to show up).

The numbers below the images show the raw numbers of the first image, and the ratio of the second and third's masked numbers to the first. All of the raw numbers, and the ratios, are saved into the `.xls` file.

If, while the code is processing, you want to stop it for any reason (realized that there is an extra or missing image messing up the reading frame, or that the threshold(s) need to be changed, etc) go back to the main Matlab window, click on the Command Window (the main window), and press Ctrl + C.

1.4 After Processing

1.4.1 Navigating the images

After the images are processed, the last image remains on the screen. The buttons on the bottom of the screen allow you to go back to the first, go back and forth to other images, or jump back to the last image.

Note: If your data set is especially large (several hundred images, perhaps) and you only want to view an image in the middle of the data set, it may be easier to right-click on that image's `.png` within the Current Directory and select, 'Open Outside of Matlab' rather than clicking 'Previous' or 'Next' several dozen times.

1.4.2 Recalculating data

You can recalculate the same data by just pressing the 'Process Images' button again. If you're changing thresholds and want to keep the last dataset, you need

to resave the `.xls` file under another name, as it will be rewritten (if the Date Captured remains the same).

If you want to calculate another set of data, close the GUI, go to the folder of interest within the Current Directory window, making sure Mask.m and Mask.fig are both in the folder, and reopen Mask.m.

1.5 Errors

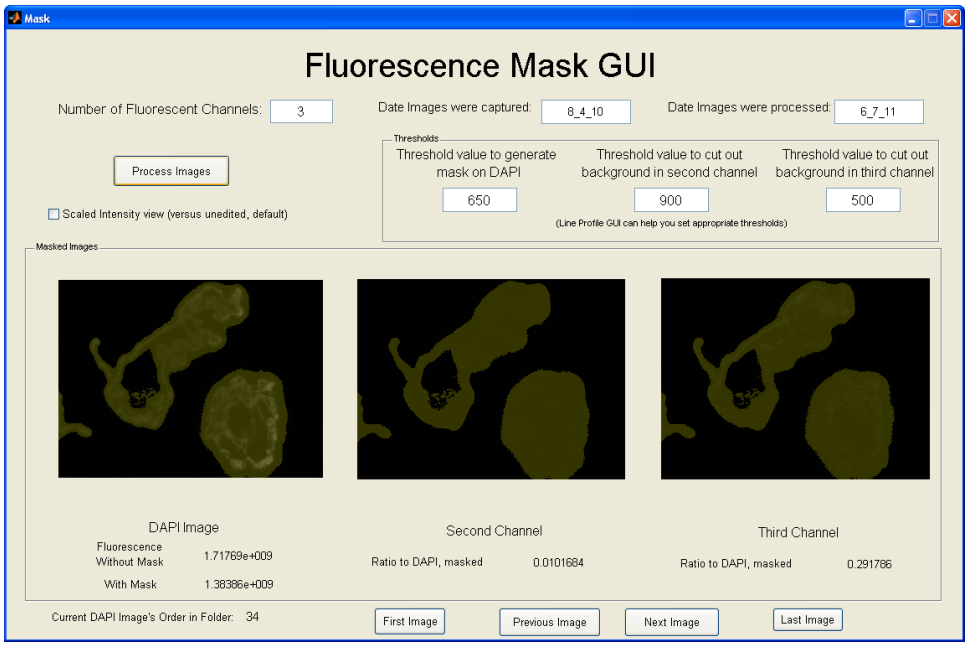


Figure 1: A three channel set of images, shown with unscaled images.

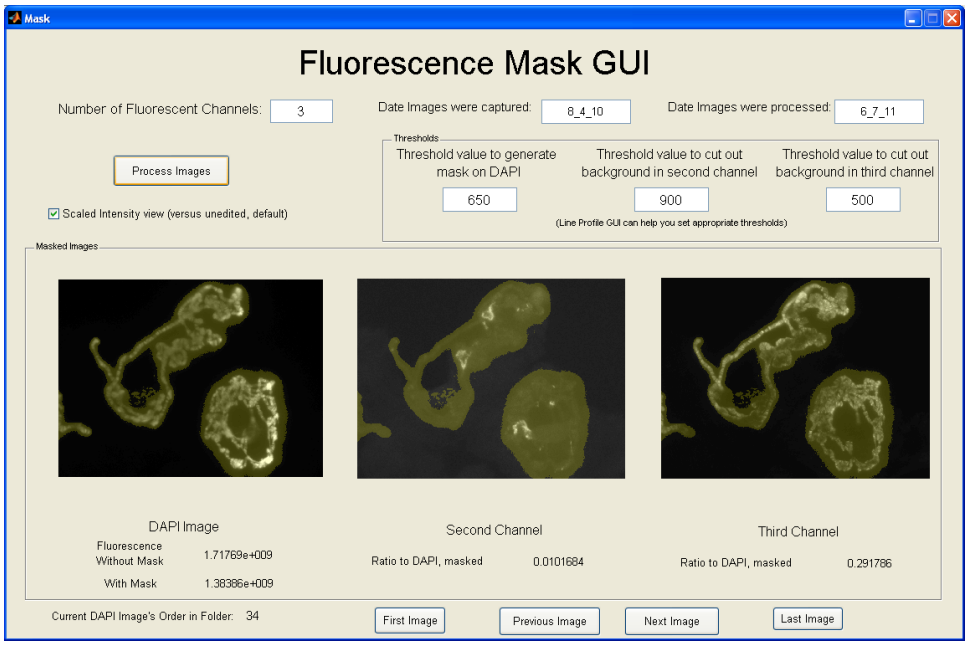
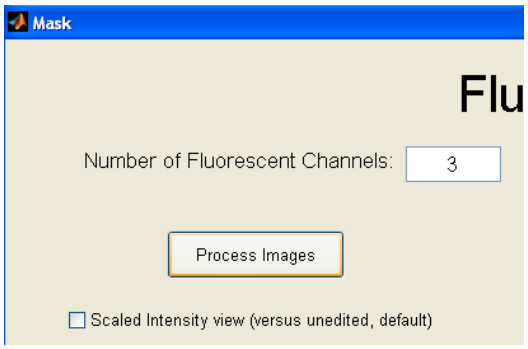


Figure 2: The same three channel set of images, with intensity scaled.



Fluorescence Without Mask	0	Ratio to DAPI, masked	0	Ratio to DAPI, masked	0
With Mask	0