

# **2DFRETIMGPROC**

## Two-Dimensional FRET Biosensor Image Processing for Single Cell Analysis

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### **Introduction:**

The 2dfretimgproc package is a software for use within the MATLAB environment to perform two-dimensional image processing for single-chain or dual-chain designs of fluorescent biosensors. The package is comprised of multiple GUIs (graphical user interfaces) for simple and intuitive applications of various standard image processing tasks. The package is designed for use as a 'pick-and-choose' environment of performing individually desired processing tasks, as well as a connected interface to perform a standardized processing workflow. The various GUIs available for use are outlined and explained in detail below. **This package outlines the standard processing guidelines and workflow used by the Klaus Hahn Lab at the University of North Carolina at Chapel Hill.**

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### **Standardized Processing Workflow:**

Before initiating the standardized processing package, it is important to know the order of processing steps and how to proceed through the workflow. The general processing workflow is laid out as such:

Selection of Options for Processing →

- Processing Options
- Image Selection

Bleedthrough Correction (for dual-chain biosensors only) →

- Can be accessed within selection of options or prior
- Calculate Bleedthrough Correction Coefficients (alpha and beta)

Camera Alignment (for two-camera systems only) →

- Can be accessed within selection of options or prior
- Transformation Calculation

#### Image Initialization →

- Shade Correction
- Dark Current Correction
- Application of Camera Transformation

#### Background Subtraction →

- Region of Interest
- Background Region

#### Thresholding & Masking →

- Setting Threshold
- Applying Mask

#### Registration →

- Cross-correlation Analysis
- Shift Registration in Horizontal/Vertical Axes

#### Photobleach Correction →

#### Ratio Calculation →

- Bleedthrough Correction

#### Filter Application →

- Gaussian Filter
- Median Filter
- Hybrid-Median Filter

## Prerequisites for Standardized Processing Workflow:

To use for standardized processing workflow, there are a few requirements that the user must be aware of in order to avoid incorrect processing. The requirements are described as follows:

- 1) The standard channels for a dual-chain biosensor are the donor emission channel, the acceptor emission channel, and the FRET (fluorescence resonant energy transfer) emission channel. For a single-chain biosensor design, the acceptor channel is typically not captured. The images are required to be in a specific format before processing can occur. **Each respective channel MUST be in images of the TIF file format and be the same dimensions in x, y, and time.** The TIF file format supports multi-frame images contained to a single file. Typically, images acquired from time-lapse experiments will be in individual files for each time point. This software requires that for each channel, all of the **time points must be “stacked” into respective, single TIF files.** For example, if your

FRET channel has 100 individual time points as separate images, these 100 images must be “stacked” into a single image file that contains all 100 time points. If there are a different number of images in the respective TIF files for each channel, then processing cannot be done (must be same dimension in time).

- 2) The second requirement for processing is that **the images for each emission channel must be in separate files**. In two-camera microscopy systems, images are sometimes produced in a “stitched” format. For example, the donor and FRET images for a specific time point may be output by the software on the microscope as a single image file containing both the images for donor (i.e. left half of image) and FRET (i.e. right half of image). These **images are required to be “split” and put into separate TIF files**.
- 3) The third requirement is to **provide images of standard illumination in the microscope’s field of view for each respective channel**. These images are highly imperative for processing as oftentimes the illumination across the field of view is not equal. These images are known as **“shade correction” images** and will be applied in the first step of the standardized processing workflow. These images must be the same size (x and y dimensions) as the images to be processed. **(Note: if dark current corrections are going to be applied, these images are also required to be of the same size in the x and y dimensions, although there only needs to be two of these images supplied in the workflow – one for each camera, donor camera and acceptor camera).**

## Pre-Processing:

To help quicken the process of meeting the requirements (1) and (2), there are two GUIs in the package, under the folder “0\_Pre-Processing Tools”, named “image\_splitterGUI” and “tif\_stackerGUI”. The image splitter GUI is available to split “stitched” images as described above into separate files. The TIF stacker GUI is available to take a directory housing individual time series images and save them into a single TIF file containing all of the time series images. The details of these pre-processing GUIs are explained in detail below.

## Standard Processing Protocol:

Depending on the options selected in the first step, most of the overall processing steps will be self-contained into a respective GUI. Again, the explicit details of each of these GUIs is described below. **In order to move on to the next step of the processing, the current working GUI must be closed**. Processing will fail in the standardized workflow if the previous step was not completed properly. In order to complete each step properly, **the proper images must be explicitly saved before closing the current GUI**. This is to ensure the ability to use an encapsulating processing workflow as well as to use the GUIs individually. The requirements to move on to the next step in the standardized processing workflow are detailed here:

### Selection of Options for Processing →

- Once the desired options are selected, press the button labeled “Save Options and Continue” in the lower left-hand corner. This will move on to manually selecting images to process. First, the user will select the working directory – this is the directory that the new images will be saved in and where file selection prompts will begin. For each necessary image, the required image to select will be displayed at the top of the corresponding window for Windows and Linux systems. Because Mac OS systems do not allow text to be displayed in these windows, these prompts are also displayed in the MATLAB environment’s command line on all systems. Once all necessary images have been selected, two files will be formed in the working directory – “run\_opts.mat” and “img\_data.mat”. These two files contain data pertaining to the options selected and the images chosen, respectively. More details on these configuration files are discussed below in the description of the options GUI.

### Bleedthrough Correction (for dual-chain biosensors only) →

- If the user is using a dual-chain biosensor design, bleedthrough correction coefficients must be calculated to correct for bleedthrough of fluorescence into the FRET channel. A GUI system to calculate these coefficients can be opened via the options selection GUI or before processing occurs (bleedthrough correction GUI is located in the folder “O\_Pre-Processing Tools” folder).

### Camera Alignment (for two-camera systems only) →

- If using images coming from a two-camera system, it is often standard procedure to apply a registration transformation to one of the images to account for any warping/distortion/misalignment due to unaligned fields of view between the two cameras. Here, there is a GUI incorporated to use images of micro-beads within in the field of view to calculate a transformation matrix to be applied to one of the channels. This GUI is incorporated from a large software package (Biosensors) developed by the Danuser. Details regarding this GUI can be found within its corresponding folder inside the 2dfretimgproc package, or on the Danuser Lab website and will not be explained within this document. However, for use in the standardized processing workflow, **the transformation matrix must be applied to the donor channel. Be sure you are calculating the transformation correctly and for the correct channel as minimalizing registration artifacts is imperative to ratio analysis.** The corresponding GUI can be accessed via the options selection GUI during the standardized processing workflow or before processing occurs (transformation creation GUI is located in the folder “O\_Pre-Processing Tools” folder). If you choose to access the GUI via the options selection GUI, be sure to still select your transformation matrix inside the options selection GUI once the transformation matrix has been created and saved as a “.mat” file. Link to Danuser Biosensors package:

<http://www.utsouthwestern.edu/labs/danuser/software/#biosensor-anchor>

### Image Initialization →

- After the options and images are selected, an initialization script will run to apply shade correction, dark current correction, and the camera transformation. There is no GUI for these processes as these are basic mathematical operations. The updated images are saved as new TIF files in the working directory.

### Background Subtraction →

- After initializing the images, the user is prompted with the background subtraction GUI. First, the user will typically import one of the shade corrected images (file name ends with “\_sc.tif”) by clicking on “File” in the upper left-hand corner and selecting to import an image. Once the user is finished with the GUI they will need to save the information collected before continuing. Again, under “File”, the user will select to **save the region box information with the filename “region\_boxes.txt”** in the working directory. Once this file is saved, close the GUI to continue.

### Thresholding & Masking→

- Upon closing the previous GUI, the thresholding task will open. This GUI is adapted from a previously made GUI called “MovThresh” developed by Dr. Denis Tsygankov. First, the user must import an image to establish the mask (image with the suffix in the filename of “\_scbg\_roi.tif”). After finding a sufficient threshold, the user must select “File” in the upper left-hand corner and choose to save the mask produced by the threshold. **The mask must be saved for the specific channel name (i.e. “maskfor\_donor”, “maskfor\_fret”, or “maskfor\_acceptor”). Repeat this for each channel. If the user selected to use a single mask, the filename for the one mask must be “maskfor\_”.** There is also an option to manually draw masks instead of using a threshold. This will open a different GUI described below, but the saving procedures remain the same. Once all necessary masks have been saved, close the GUI to continue.

### Registration →

- If the user chose to perform cross-correlation registration, they will be prompted with a registration GUI if they chose to manually perform the shifting. If automatic registration was chosen, there is an algorithm in place that will calculate and save the necessary images for the next step and no GUI will be displayed. If using the manual registration GUI, the user must import a base image, or the image to register to (typically the fret or acceptor channel), and a register image, or the image to have registration applied to it (typically the donor channel). **The images from the masking step will be saved as “donor\_masked.tif”, “fret\_masked.tif”, and acceptor\_masked.tif”. These are the images that should be imported into the processing.** Once manual registration is complete, **the user must save the registered donor image with the filename “donor\_reg”.** Once the registered donor image is saved, close the GUI to continue.

### Photobleach Correction →

- After the previous processing step, photobleach correction will occur if the user selected the option. If there are more than four time frames in the time series of images, the user will be prompted with a question box asking if they would like to include exclusion criteria on the photobleach correction curves. Otherwise, the processing will skip photobleach correction. If the user chooses to do so, they will be prompted with a GUI where they can **import the latest image from the workflow process for each channel ("fret\_masked.tif", "acceptor\_masked.tif", and "donor\_masked.tif"/"donor\_reg.tif")**. **After selecting the photobleach curve, the photobleach corrected images must be saved as "donor\_pbc", "fret\_pbc", and "acceptor\_pbc", respectively**. Once finished with the GUI, the user can close it to continue. If the user did not choose to choose exclusion criteria for the photobleach correction curve, a script will automatically apply the correction and save the images as needed for the next step of processing.

### Ratio Calculation →

- An automatic script is run at this step of the processing workflow to calculate and save the ratio images based on the last images from each channel. This script also calculates and saves the bleedthrough corrected FRET image if using a dual-chain biosensor design. If filter application was not selected in the options, the processing workflow will finish and exit.

### Filter Application →

- If selected in the options, after ratio calculations are complete, the user will be prompted with a GUI that can be used to view any of the images from the workflow as well as apply smoothing filters to the images. Once the user is finished, the user can close the GUI to finish and exit the workflow process.

## Outline of GUIs in Package:

- 0\_Pre-Processing Tools
  - transformationCreationGUI
  - bleedthrough\_correction
  - image\_splitterGUI
  - tif\_stackGUI
- 1\_Initialize, Shade & Dark Current Correction
  - optionsGUI
- 2\_Background Subtraction
  - background\_subtractionGUI

- 3\_Thresholding & Masking
  - MovThresh
  - freehand\_thresh
- 4\_Registration
  - registrationGUI
- 5\_Photobleach Correction
  - photobleachGUI
- 6\_Ratio Images
- 7\_Post-Processing Tools
  - filterGUI
  - ratio\_mask

## In-Depth Explanation of GUIs:

### transformationCreationGUI

This GUI is used to calculate a transformation matrix to align images that were acquired via different cameras (typically a camera for the donor emission and a camera for the FRET/acceptor emission). To use this GUI and to build an appropriate transformation matrix for camera alignment registration, bead images of auto-fluorescing beads for either channel is required as input into the GUI. For more in-depth details on use and functionality, please see the link to Danuser's Biosensors package:

<http://www.utsouthwestern.edu/labs/danuser/software/#biosensor-anchor>

### bleedthrough\_correction

The bleedthrough correction GUI is designed to calculate standard bleedthrough correction coefficients, commonly depicted under the terms alpha for and beta. Alpha and beta are representative of a relative amount of bleedthrough, or spectral overlap artifacts. The donor and emission channels both capture a specific wavelength of light. Depending on the fluorophores used, the emission spectra given off by a specific excitation wavelength may overlap into the spectral capture range of the other detection channel. For example, a short-wavelength fluorophore, such as CFP, has an emission spectra that overlaps the excitation spectra of a higher-wavelength fluorophore, such as YFP. Thus, when capturing FRET, the FRET channel may be capturing re-fluorescence caused by the CFP emission exciting the YFP fluorophore in addition to the amount of real FRET signal. On the other hand, the excitation spectra of YFP extends back small amount into the wavelength of light used to excite CFP. Because of this, whenever the light to excite CFP is emitted, some YFP is directly excited and gets added into the FRET signal. The alpha coefficient corrects for the first scenario while the

beta coefficient accounts for the second scenario. These two coefficients are then used to calculate a corrected FRET image that subtracts out any potential bleedthrough. In equation form, this looks like:

$$\text{Bleedthrough corrected FRET} = \text{FRET} - \alpha \cdot \text{donor} - \beta \cdot \text{acceptor}$$

To calculate the bleedthrough correction coefficients using this GUI, control images must be acquired during the time of the experiment. For alpha, one or multiple sets of donor and FRET images must be acquired while only emitting the excitation wavelength of the donor fluorophore onto a sample only expressing the donor fluorophore. For beta, one or multiple sets of acceptor and FRET images must be acquired while only emitting the excitation wavelength of the donor fluorophore onto a sample only expressing the acceptor fluorophore.

The GUI initially starts with a window to select the number of image sets for calculating both alpha and beta. Each image must be a separate TIF file and registration via a camera transformation matrix must be applied prior to calculations if needed. The user can also select to apply shade correction images or dark current correction images to their control images. Once the user is satisfied with their options, they can select to continue. The user will be prompted to select the control images. For alpha, the user must select images in the order of: donor, FRET, donor, FRET, etc. Similarly, for beta, the user must select images in the order of: acceptor, FRET, acceptor, FRET, etc. Once all of the images have been selected, calculations will run in the background to create graphs comparing intensity values on a pixel-by-pixel basis. These plots will be displayed one at a time in a second window. Alpha values will be calculated one-by-one first before beta values.

When the first plot appears, the data will be presented with the fret intensity values for each pixel on the y-axis. The x-axis of the plot will either display the intensity of the corresponding pixel of the donor or acceptor image depending on which coefficient is currently being calculated. On the right-hand side of the window, both images for the current coefficient calculation will be displayed and labeled, with a slider bar to the right of the images to change the range of the grayscale colormap of the images. There are three slider bars surrounding the plot. These are for displaying lines on the plot to exclude points from the coefficient calculation, which is determined as the slope of the line best fitting the points on the plot and intersecting the y-axis at (0,0). The slider bar to the right of the plot draws a line that will exclude points BELOW the line. The slider bar above the plot draws a line that will exclude points TO THE RIGHT of the line. The slider bar to the left of the plot draws a line that has the same magnitude slope as the initially calculated best-fit line, but with a negative value. Points on the lower left-hand side of this line will be excluded for calculations. Once these lines have been set to exclude any points, the best-fit lines can be recalculated to acquire a new coefficient value. If the user is satisfied with this value, there is a button to continue to the next image set to calculate a new coefficient value.

Once all image sets have had bleedthrough coefficients calculated, a final window will appear displaying all of the calculated alpha and beta values. The respective values are averaged and



displayed on the right-hand side of the window. The user can select to remove outliers from the averaged coefficient values by highlighting them in the table on the right (the table on the left constantly shows all values) and selecting the button to exclude values at the bottom of the window. This will update the average values displayed on the right-hand side. The user can choose to save these values into a text file, exit the GUI (which will return the values in a 1x2 array), or re-start the whole GUI.

### **image\_splitterGUI**

The purpose of this GUI is to aid in the process of formatting images properly to use the standardized processing workflow. The intent of this GUI is to take “stitched” image files containing two images on either half, and split them into individual image files. This GUI can handle single frame TIF images as well as multi-frame TIF images. The user is given the option to split a single image file or every image file within a specified directory. The user must select an image file if the former option is chosen. If the latter option is chosen, the user must specify the directory where the images are located. The user can do this by clicking the button in the upper right-hand side of the GUI window that states “Search for Image” or “Search for Directory”. The user also has the option to save both sides of the selected image(s) or to toss either side. The user can specify the names of the newly saved files by entering either a filename or a prefix to a filename – the default is adding the prefix “left\_” or “right\_” to the original filename when saving the split images. The user can select a name of a new directory where the new images will be saved. This new directory will be saved within the directory containing the image(s) the user selected to split. If a directory already exists with the given name, the user will be asked if they want to save the images inside the pre-existing directory or to select a new directory name. If they choose to save inside the pre-existing directory, the user must be aware that if their newly saved images share a filename with a pre-existing image inside the directory, the old images will be overwritten. If the user would like to save the split images inside the same directory as the selected image(s) to split, the user can simply type “.” into the textbox. It is possible to create a new directory for storing these images anywhere by specifying the typical pathname notations (i.e. using “.” and “/” where appropriate). Once the user has selected all of their options, the user can press the button in the lower right-hand corner of the GUI window to begin splitting images. Before this happens, the user is prompted with a window on how to format the new images’ filenames. The user is given three straightforward options: allowing for enumerated filenames, adding a prefix to the original filename, or just using the input specified previously. The user will not have the third option available if they are splitting more than one image file because the newly saved images must have different names.

### **tif\_stackGUI**

This GUI is to aid in the process of formatting images properly to use the standardized processing workflow. This GUI is intended to take multiple single frame TIF images (denoting a time series) and “stack” them into a single multi-frame TIF file. The user is able to select a directory in which images exist. All of the images within that directory are displayed in a list

(sorted lexicographically) on the right-hand side of the GUI window. The order of files in this list depict the order in which they will be stacked. For a time-series of images, make sure the files are in the proper order. The user also has the option to query the list by a keyword to filter out images not wanted to be put into a TIF stack that are also within the specified directory. The number of images in the list is displayed for the user to see. If the files are enumerated with a number, the sorting of the filenames can be out of order. For example, if you have multiple files to be stacked with the names "file1", "file2", ..., "file10", etc., in lexicographical order these files are displayed as "file1", "file10", "file11", ..., "file2", "file20", etc. To accommodate for this ordering, there is a checkbox located underneath the list to apply "natural" sorting. This should sort the filenames in the appropriate order. Once the user is satisfied with the images in the list, the user can press the "Create TIF Stack" button in the lower left-hand corner of the GUI window. Once the button is pressed, a window will appear to save the newly stacked TIF image wherever the user would like, and with whatever name.

### **optionsGUI**

The options GUI is the first window that appears once the masterScript is run to begin the standardized processing workflow. This GUI serves no other purpose than to select the options the user wishes to use during processing. By running the masterScript file, the options GUI will save the selected options into a file named "run\_opts.mat" that will be saved in the current directory. If the masterScript is called and "run\_opts.mat" is located anywhere in the set path in MATLAB, the user will be prompted with a window to select the options saved within that file. Otherwise, the options GUI will open as normal. Within the options GUI the user has several different options to choose from that are separated into different panels in the GUI window and are detailed as follows:

#### **Biosensor Design Options:**

- Single Chain Biosensor
  - o The user will not be able to apply bleedthrough correction.
  - o There is no option to process acceptor images, only donor and FRET images.
- Dual Chain Biosensor
  - o The user will be required to input bleedthrough correction coefficients (default to zero for alpha and beta, i.e. no bleedthrough correction).
  - o The user will be required to input acceptor images along with donor and FRET images.
- Bleedthrough Coefficients
  - o The user can type in bleedthrough correction coefficients for alpha and beta in the respective text boxes.
  - o The user also has an option to "Calculate" these values, which launches the "bleedthrough\_correction" GUI located in the pre-processing tools, and will return the calculated values into the text boxes in the options GUI.

#### **Masking & Registration\* Options:**

- Use Single Mask Across All Channels

- If this is selected options is selected, the user will be required to only save one mask after thresholding has been calculated. This single mask image file must be named as “maskfor\_” for the processing to run correctly. If this option is selected, the user loses the ability to perform automatic registration\* without opening the registration GUI. This is because automatic registration cannot be calculated if each image is masked using the same mask (remaining active pixels in each image channel will be the same, so registration will calculate to zero shift).
- Use Registration GUI for Manual Registration\*
  - Selecting the top option in this options panel will result in a GUI opening to perform manual registration. The GUI will be described in detail below, but the user must be aware that selecting this option will still allow them to use automatic or manual registration\*, but the user will have to save the registered image manually as “donor\_reg”. Because the donor emission is captured using a different channel than acceptor and FRET (acceptor and FRET are in the same channel), the donor channel must be registered to the FRET/acceptor channel to save the number of calculations to be performed (one image being registered to the other two instead of two images being registered to the remaining one image).
- No Registration\*
  - Registration\* will not be calculated or applied to the images.
- Automatically Perform Registration\*
  - This option requires the user to use individually masked channels which automatic registration will use for calculations. The user must name these masks “maskfor\_donor”, “maskfor\_fret”, and “maskfor\_acceptor”, respectively. This will output a registered donor image named “donor\_reg”.

\*Registration in this context is in reference to a cross-correlation analysis being used to calculate x- and y-offsets of the corresponding channel images to perform a shift transformation in order to better align the images, since co-localization of the images is imperative for ratio analysis. This registration is termed separately from the camera alignment transformation (also a form of registration) in order to lessen disambiguation of the two registration processes.

Select Ratio Images to Calculate and Save:

- FRET/Donor
  - This option will calculate and save the ratio image of the FRET channel divided by the donor channel, pixel-by-pixel.
- Donor/FRET
  - This option will calculate and save the ratio image of the donor channel divided by the FRET channel, pixel-by-pixel.
- FRET/Acceptor
  - This option will calculate and save the ratio image of the FRET channel divided by the acceptor channel, pixel-by-pixel.
  - This option is only available for dual chain biosensor analysis.

- Acceptor/FRET
  - This option will calculate and save the ratio image of the acceptor channel divided by the FRET channel, pixel-by-pixel.
  - This option is only available for dual chain biosensor analysis.

#### Other Options:

- Align Cameras w/ a Transformation Matrix
  - This option should only be selected if the user acquired images with a two-camera setup.
  - The user has the option to “Calculate” a transformation matrix for camera alignment registration if not done so prior to running the options GUI. Pressing this button will open the “transformationCreationGUI”.
  - If this option is selected, the user must select the desired transformation matrix file (“.mat” format) to use in the workflow.
- Perform Dark Current Correction
  - If the user selects this option, after the options GUI is finished, the user must input a dark current image for both the donor channel and the FRET/acceptor channel. These images must be the same size as the images selected to process in the x and y dimensions.
- Perform Photobleach Correction
  - If the user selects this option, when the workflow reaches the point to apply photobleach correction to the images, the user will be prompted with a window asking to perform automatic photobleach correction, or to open the photobleach correction GUI which allows the user to view the photobleach curves and exclude points on the graph when fitting a curve.
- Apply Filter to Ratio Image
  - Selecting this option will open an extra GUI after the ratio image(s) are calculated. This filtering GUI will allow the user to import any image from the workflow and apply a filtering/smoothing procedure.
- Information about using this package:
  - There is a block of text within this options panel briefly describing some of the image prerequisites and the configuration files “run\_opts.mat” and “img\_data.mat”, as stated above.
  - The text reads as follows:
    - “To use this processing package for FRET biosensor ratio analysis, the input images must be in a certain format. This package only allows for TIF images to be processed. Each channel of images (i.e. FRET, donor, and acceptor) must be in separate image files and of the same size in the dimensions of x, y, and time. A time series of a channel must be in a single, multiframe TIF file. There are several tools provided in the folder labeled '0\_Pre-Processing Tools' for splitting images with concatenated channels and for saving multiple images of a time series into a single, multiframe TIF file.

Upon clicking 'Save Options and Continue', a MATLAB file of the name 'run\_opts.mat' will be saved in your working directory. This file will contain the information of the options you selected. If a 'run\_opts.mat' file is in the MATLAB search path, its contents can be used for quick batch-like processing. Similarly, there is another configuration file called 'img\_data.mat' that stores the names of the image files initially provided. If you elect to use the 'img\_data.mat' file, be sure there are no identically named files of the images within the MATLAB search path."

Lastly, there is a button in the bottom right-hand corner of the options GUI labeled "Save Options and Continue". Pressing this button will close the options GUI and return the selected options (including the alpha and beta values input and the file name of the camera alignment transformation matrix selected) to the masterScript which will produce the "run\_opts.mat" file discussed above. Next, the user will be prompted to select all of the necessary images based on the options selected. The order of the images to select are as follows (this list assumes all options were selected that require importing images – if the user did not select the necessary options that require any of the following images, then the user will not be prompted to select the image):

- Donor image file
- FRET image file
- Donor shade correction image
- FRET shade correction image
- Acceptor image file – dual chain biosensor option
- Acceptor shade correction image – dual chain biosensor option
- Dark current correction image for donor channel – dark current correction option
- Dark current correction image for FRET/acceptor channel – dark current correction option
- 

Once all of the necessary images have been selected, the filenames for the respective images will be saved into a second configuration file named "img\_data.mat". Like the "run\_opts.mat" file, if a file of the name "img\_data.mat" is located in the current set path in MATLAB, the user will be prompted with a box upon closing the options GUI asking if they would like to select new images for processing (which will create a new "img\_data.mat" in the current directory – if the old "img\_data.mat" file is in the current working directory also, it will be deleted) or if they would like to use the images listed in the "img\_data.mat" file.

**WARNING:** If the user selects to use the existing "img\_data.mat" file, MATLAB will search the whole set path to find files with the specified file name within the configuration file. If multiple files within the set path share the same filename as listed in the configuration file, then MATLAB may grab the image data from the wrong file. Please make sure only the files the user wishes to process are within the set path.

### **background subtractionGUI**

The background subtraction GUI is meant to assist in applying background subtraction calculations for the images of each channel. To start, the user will import the desired image into the GUI by electing “File” in the upper left-hand corner of the GUI window and choosing the option to import an image. Once the image loads into the GUI the user can use the slider bars to the right of the image to change the dynamic range of the colormap. Once the user can clearly visualize the sample in the image, they can select a region of interest (cropping mechanism) and a background region (region denoting only the background in the sample across all time frames) by pressing “Tools” in the upper left-hand corner of the GUI window and selecting “Apply Region Box.” A region box object will appear in the top-left corner of the image and the part of the image within this region box will be displayed in the lower right-hand corner of the GUI window. The user can resize and drag this region box object to a location in the image that encapsulates the entirety of the sample of interest across all time frames. The slider bar below the image can be used to view the other time frames within the image to ensure the sample remains within the region box. Once the user is satisfied with the location and size of the region box, they can press the button “Update Region of Interest” in the configuration panel to the right of the image. Above this button is a list of four values denoting the region of interest (default is entire image) in the following fashion:

[(x coordinate of top left corner of region box) (y coordinate of top left corner of region box)  
(width of region box) (height of region box)]

By pressing the “Update Region of Interest” button, the values in this list will be updated to represent the location and size of the region box at its current location. Likewise, for the background region, the user can resize and drag the region box to a location that the sample of interest never crosses into throughout the time frames. The goal here is to define a region that only consists of background area where each pixel value within the region will be averaged to designate the background intensity of the image. This value will then be subtracted from every pixel intensity value in the image. Once the user is satisfied with the location of the region box to denote the background region, the user can press the “Update Background Region” button update the list of values to current position of the region box to designate the background region. Once the region of interest and background region have been selected, the user can press “File” in the upper left-hand corner and select “Save Regions”. This will prompt the user with a window to save a text file that contains the list values of the two regions specified. In order for the standard processing workflow to read this text file, this text file must be named “region\_boxes”. After saving the region boxes text file appropriately, the user can simply close the GUI window to continue. It is imperative to note that these regions will be applied to each channel individually to calculate respective background subtraction values. Each channel’s images will be cropped and saved using the region of interest values.

Other options within this GUI include a dropdown menu to select a different colormap to use for the images, a movie button to play through the entire time series of images, a movie pause time text box to input the pause between each frame (in milliseconds) when the movie button

is pressed, standard zoom in/out and image pan tools, and a coordinate value display that shows the intensity value and the coordinate value of the image where the mouse pointer is located.

### **MovThresh**

Developed by Denis Tsygankov.

The purpose of the MovThresh GUI is to generate masks based off of static threshold values for each time frame in an image file. This GUI will only generate a mask for the largest sample (single cell processing) within each time frame of the image. The details and instructions for MovThresh can be found within the accompanying text file named “How to use MovThresh”. There exists a button in the lower right-hand corner of the GUI window labeled “Manually Draw Masks”. Clicking this button will ask the user if they would like to continue on to a separate GUI with the stipulation of not being able to return. This other GUI is the “freehand\_thresh” GUI described below.

The proper use of this GUI is contingent on the masking options the user selected in the options GUI. If the user selected to use a single mask in processing, a mask can only be generated from one channel’s images (ideally the channel with the highest intensity values, making it the easiest to threshold). Once the threshold is selected, the user must save the single channel mask as “maskfor\_” by pressing “File” in the upper left-hand corner of the GUI window and selecting to save the mask. If the user selected to use separate masks for each channel, the user must import each channel’s images one at a time, settle on a threshold selection, and save the respective mask as “maskfor\_donor”, “maskfor\_fret”, or “maskfor\_acceptor”. Once all necessary masks have been saved, the GUI can simply be closed to continue the standard processing workflow.

The specific options available in MovThresh are detailed in the aforementioned text file.

### **freehand\_thresh**

This GUI was an add-on to MovThresh that was developed in order to increase the robustness of the software for more use cases in generating masks for images. Specifically, this GUI allows for freehand drawing of masks for specified time frames of the image sequence. The user must import the necessary channel images based on the masking options selected in the options GUI (single mask or mask for each channel individually). The specific details for saving masks and which images to import are described above in the details for “MovThresh”. When the GUI is initiated and an image file is imported, the user will be prompted with a window requiring them to enter numbers that represent the index of the time frames of the image that they wish to draw masks for. If drawing masks for each individual channel, be sure to input the same values for each imported image when prompted.

Once the time frames have been selected, the user can toggle through the time frames for which they have not yet drawn masks by clicking the time frame index in the list to the right of the image. The total number of frames the user chose to draw masks for is displayed above the list of indices. The user can also switch time points through any of the previously selected time frames by using the slider bar under the image. If a mask is drawn for a time frame, it will no longer appear in the list to the right of the image. If the user wishes to redraw a mask, the user must use the slider bar under the image to return to that time frame.

To draw a mask, when on the desired time frame, the user may press “Draw Mask” to initiate the drawing tool. The mouse pointer will turn into a crosshair that denotes where the drawing will take place. The drawing tool in MATLAB requires that the user draw a closed loop object in one fell swoop. The beginning point and the end point of the drawing will be connected via a straight line to ensure the loop is closed. Once the object is drawn, it can be dragged around the image to better fit the boundaries if needed. Once the loop is drawn, the user can press “Save Mask” to save the region enclosed by the loop and the current time frame will be removed from the list. Once a mask has been drawn for each time frame, the user can save the masks by pressing “Save All Masks and Continue” or by pressing “File” and selecting “Save All Masks”. Once finished, simply close the GUI to continue processing.

Other options in this GUI include slider bars to the right of the image to adjust the dynamic range of the colormap of the image, a dropdown box to change the colormap of the image, a movie button to play through all the specified time points, a movie pause text box to enter the pause time (in milliseconds) between changing each time frame when the movie button is pressed, and standard zoom in/out and image pan tools.

**WARNING:** Unfortunately, MATLAB has an inherent bug with the object used to create the region enclosed by the loop. This bug has several consequences. If the drawing is messed up and needs to be redone, the user can delete it by right clicking inside the loop and selecting “Delete”. The user will then have to press the button “Save Mask” and re-press “Draw Mask” to draw a new region. If the user selects “Save Mask” without drawing a mask or after deleting a mask, the time frame will not be removed from the list of remaining time frames.

**WARNING:** All subsequent processing steps will only use the time frames specified for use within this GUI. The time frames specified will be saved into the “run\_opts.mat” file for the current processing workflow.

### **registrationGUI**

The registration GUI is intended for visualization of and manual manipulation of a cross-correlational analysis in the x and y dimensions of two image sets from two different channels (typically acquired on two different cameras). The intent is to apply a shift to one image to better align it with the other image for high co-localization, which is imperative for ratio analysis. To use this GUI, begin by pressing “File” in the upper left-hand corner of the GUI



window and selecting the option to import images. The user will be required to import two different image files as follows:

- 1<sup>st</sup> - Base Image: the base image will be the image that the register image gets aligned to. The shift will not be applied to this image (typically the FRET or acceptor channel images).
- 2<sup>nd</sup> - Register Image: the register image will be the image that gets aligned to the base image. The shift will be applied to this image (typically the donor channel image).

For compatibility with the standard processing workflow, the user should choose the FRET or acceptor channel image as the base image and the donor channel image as the register image. Once these images are imported, the GUI will perform a cross-correlation analysis on the images before displaying them. This step may take a few minutes depending on how many time frames are within the image stack. The base image will be displayed in the upper left-hand side of the GUI window, the register image will be displayed in the upper right-hand side of the GUI window, and an overlay of the two images will be displayed in the lower left-hand corner of the GUI window.

The overlay image is a composite image of the base and register images (where either image is assigned a specific color) with the designated offsets in x and y applied to the register images. Initially, the offsets will be set to automatically calculated values that algorithmically maximize colocalization between the two images (via the cross-correlational analysis). These offsets are displayed in the configuration panel in the lower right-hand corner of the GUI window. Inside this configuration panel is an option to use manual registration. If this option is selected, the user can use the slider bars directly below and to the right of the overlay image to manually shift the register image around in the x and y axes, respectively. The user is given a shift window of +/-10 pixels in both the x or y directions. The overlay image will automatically update with the manually given offset values, which are also displayed in the configuration panel. Once the user has settled on offset values, they may save the register image with the shift (designated by the selected offset values) applied. For the standard processing workflow, the shifted donor channel image must be saved with the name "donor\_reg". Once finished, simply close the GUI to continue processing.

A unique feature of this GUI is the implementation of a "mirror pointer" in the displayed base and register images. If the mouse pointer is hovering over either the base or register image, a small cyan box is displayed at the location of the mouse pointer that indicates where the mouse is in the coordinate plane of either image (top left corner of cyan box). This is to help observe if there is a subtle misalignment between the two images.

Other options in this GUI include a channel/overlay colormap dropdown box to select the colors used by the base and register images (and what the overlay color merges to), slider bars to the right of the base image and register image to adjust the dynamic range of a set grayscale colormap, a movie button to play through the entire time series of images, a movie pause time

text box to input the pause between each frame (in milliseconds) when the movie button is pressed, standard zoom in/out and image pan tools, and a coordinate value display that shows the intensity value and the coordinate value of the both the base image and register image where the mouse pointer is located.

### **photobleachGUI**

The photobleach GUI is used to observe and apply photobleach correction to time series images. In the standard processing workflow, this subsequent process must be applied to each channel used in processing.

Begin by pressing “File” in the upper left-hand corner of the GUI window and selecting to import images. Select the images you wish to correct for photobleaching. After selecting the images, the GUI will calculate an average intensity curve for the time series if the time series has more than four time points. This is because the average intensity values for each time point are fit with a double-exponential curve which requires at least four time points to achieve a fit. These average intensity points and the resulting double-exponential fit curve will be displayed in the graph. A key for the colors represented in the graph are displayed in configuration panel at the bottom of the GUI window, along with information regarding the fit curve.

In this GUI, the user has the option to select points in the graph to exclude when calculating the fit. To achieve this, press “Tools” in the upper left-hand corner of the GUI window and select “Exclusion/Inclusion Box”. Doing so will display a region selection box onto the graph. This region box can be dragged and resized to surround points to perform an action on. Once the desired points are within the region box, the user can press “Exclude Points” or “Include Points” in the configuration panel to perform the desired action. Once the desired points have been included/excluded, press “Recalculate Curve” to calculate and display the new fit. Once the user is satisfied with the curve, press “File” and select to save images. This will apply the photobleach correction curve to the time series data and save the output image. Repeat this process for each channel in the current processing. In the standard processing workflow, the output image must be named “donor\_pbc”, “fret\_pbc”, or “acceptor\_pbc” with respect to the imported image. Once all necessary photobleach corrected images have been saved, simply close the GUI to continue the standard processing workflow.

This GUI also allows for the option of zooming in/out and panning around the graph if needed.

### **filterGUI**

The filter GUI is used to apply filtering or smoothing onto an image set. The purpose of filtering is to minimize random noise in the intensity signal of an image, but at the cost of potential artifacts. This action is oftentimes arbitrary, and thus it is placed within the “Post-Processing Tools” folder within the package. After launching the filter GUI, import an image by pressing “File” in the upper left-hand corner of the GUI window and selecting to import an image file. Once the image is displayed, the user will have four filtering options to choose from. These

options can be selected in the configuration panel in the bottom half of the GUI window. The filtering options are as follows:

- Hybrid-median filter
- Median filter
- Mean filter
- Gaussian Smoothing

Selecting one of the first three options will require the user to input a dimension size ( $N$ ) for the filtering box ( $N \times N$ ) when calculating the filtered image. The input must be an odd integer that is greater than or equal to 3 (box of size  $1 \times 1$  is equivalent to no filtering and an even number input will result in a filtering box that has a shift bias in one direction, i.e. must use a filtering box of a size that includes a span of pixels that is equal in every direction from the pixel currently being filtered). The Gaussian smoothing selection requires the input of a sigma value, which denotes the standard deviation used in the Gaussian calculation when computing the filtered image. Once the filter option is selected and the respective parameter specified, the user can press the “Apply Filter” button to begin calculating the filtered image. Once this image is computed, it will be displayed to the right of the original image. The slider bars between the two images adjust the dynamic range of the colormap applied to both images, filtered and unfiltered. If the user is satisfied with the filtered image, they can save the image by pressing “File” and selecting to save the filtered image. The save box will be filled in with the text “\_hmc”, “\_medianf”, “\_meanf”, or “\_gauss\_smooth” depending on which filter option was used to generate the filtered image. This text can be changed, but it is intended to act as a suggested tag to append to the desired filename.

Other options in the GUI include a dropdown box to select the colormap to be applied to both displayed images, a movie button to play through the entire time series of images, a movie pause time text box to input the pause between each frame (in milliseconds) when the movie button is pressed, standard zoom in/out and image pan tools, and a coordinate value display that shows the intensity value and the coordinate value of both the unfiltered image and filtered image where the mouse pointer is located.

### **ratio\_mask**

The ratio masking GUI is used for placing an internal mask on an already masked ratio image to shroud noisy or unnecessary regions. This operation is performed arbitrarily after processing and thus the GUI is placed within the “Post-Processing Tools” folder. To begin, press “File” in the upper left-hand corner of the GUI window and select to import images. The user will be asked to import two images. The second image to import is the masked ratio image. The first image to import is an image from the processing workflow of the same sample (must be the same size as the masked ratio image in x, y, and time dimensions) that can be masked or unmasked and from any channel. Once these images are imported, the first image, or “masking” image is on the left side of the GUI window and the ratio image is on the right side of the GUI window. The masked ratio image will have a line surrounding the masked boundary of the sample within the image. In the upper left-hand corner of the configuration panel (located below the images) is a slider bar that ranges from 0 to the maximum intensity value in the

masking image that was imported. The user may use this slider bar or the text box above the slider bar to manually select a threshold value for the masking image. There will be a boundary line in the masking image representing the closed threshold boundary given by the selected threshold value that will automatically update as the user changes the threshold value. The user can select a threshold on this image that cuts out noisy signal areas or areas in the sample that have relatively low intensity values. Once the user has selected a mask on the masking image, the “Apply Mask” button can be pressed to apply this mask onto the masked ratio image. The intent is to black out the noisy regions or regions with low intensity values (in the masking image) in the ratio image. Having a masking threshold on the masking image that is larger in size than the already masked ratio image will do nothing as the new mask will include values in the ratio image that are within the closed loops of the new mask. If the user wants to remove the mask from the ratio image, the “Remove Mask” button can be pressed to return the masked ratio image to its original form. Once the user is satisfied with the new mask, they may save the newly masked ratio image by pressing “File” and selecting to save the image.

Other options in this GUI include a dropdown box to select the colormap to be applied to both displayed images, a dropdown box to select the color of the boundary lines in both images, slider bars to the right of either the masking image and ratio image to adjust the dynamic range of the set colormap, a movie button to play through the entire time series of images, a movie pause time text box to input the pause between each frame (in milliseconds) when the movie button is pressed, standard zoom in/out and image pan tools, and a coordinate value display that shows the intensity value and the coordinate value of the both the unfiltered image and filtered image where the mouse pointer is located.