

Supplementary Protocol 2: PM-FLIM-Analysis Matlab Program

1. Introduction

PM-FLIM-Analysis is an open-source Matlab program with graphical user interfaces (GUIs) that allows users to analyze the imaging data acquired with a PM-FLIM system. Specifically, only the “Intensity”, “G”, and “S” images from the PM-FLIM experiments are required; all other FLIM data, such as fluorescence lifetime images, phasor plots, and image segments based on regions of interest (ROIs) or K-means clustering, can be generated by the program. The program is easy to use for its user-friendly GUIs. Meanwhile, as the program is fully open-source and highly modularized, users with Matlab programming expertise can easily modify the code to customize existing functions or to add new functions.

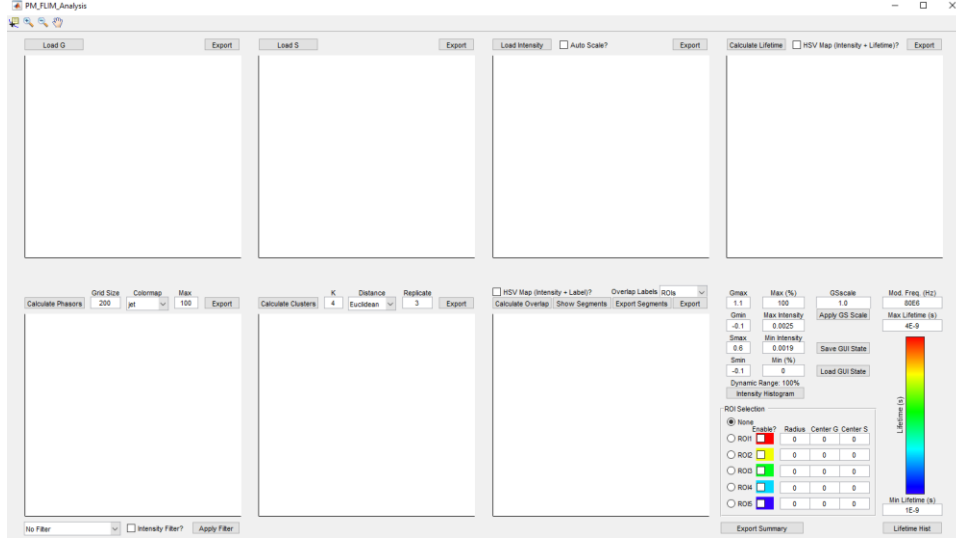
2. Installation

a. Option 1

- *This option requires a Matlab license*
- Install Matlab on your computer
- Download or clone (git clone) this GitHub repository:
<https://github.com/yzhang34/PM-FLIM-Analysis.git>
- Open the file “PM_FLIM_Analysis.m” in Matlab and RUN it

b. Option 2

- *This option does not require a Matlab license*
- Download or clone (git clone) this GitHub repository:
<https://github.com/yzhang34/PM-FLIM-Analysis.git>
- Execute "MyAppInstaller_web.exe" in the "installer" directory
- Follow the instructions to install the program
- Matlab running environment will be automatically downloaded and installed
- Once the installation is complete, the program can be started with the icon on your Desktop



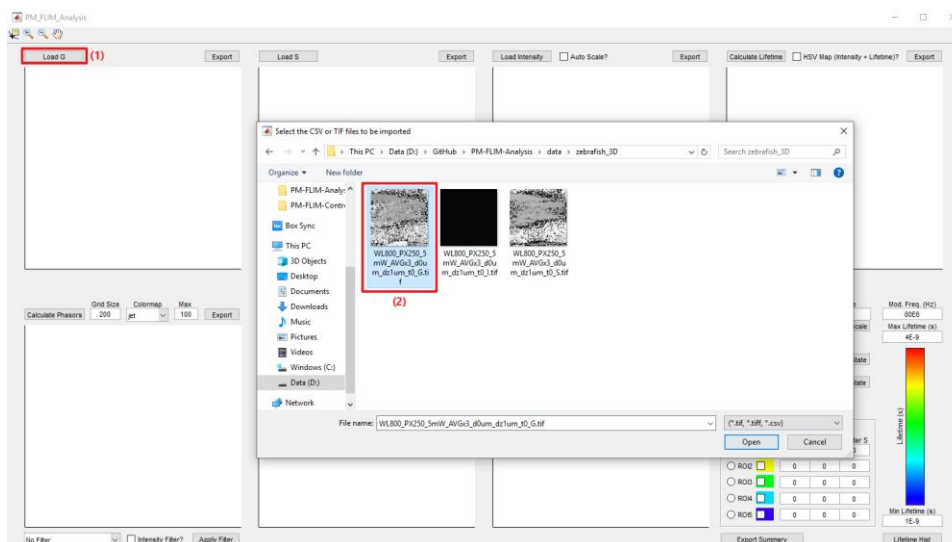
3. Data Import

The user only needs to acquire and store the “Intensity”, “G”, and “S” images from a PM-FLIM imaging experiment, as other FLIM data can be generated from these three raw images in the PM-FLIM-Analysis program.

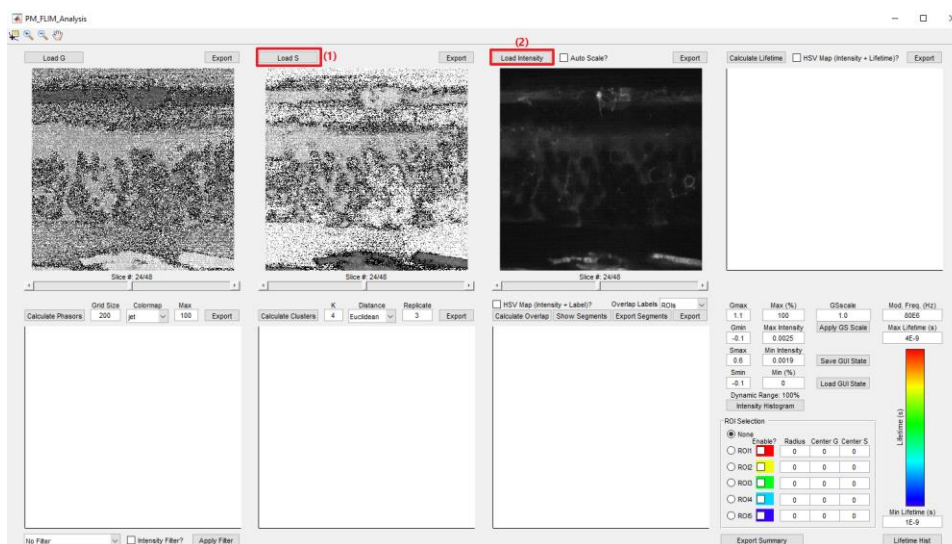
- In the “G” image, the value of each pixel is the x-coordinate (horizontal coordinate) of the phasor plot for that pixel. The G values should be between 0 to 1. Values outside of this range are caused by noise.
- In the “S” image, the value of each pixel is the y-coordinate (vertical coordinate) of the phasor plot for that pixel. The S values should be between 0 to 0.5. Values outside of this range are caused by noise.
- In the “Intensity” image, the value of each pixel is the two-photon fluorescence intensity reading, i.e., the voltage output of the detection PMT.

We have included two sets of PM-FLIM data as examples in the GitHub repository. The 2D images in “/data/BPAE_cells_2D” were acquired from fixed bovine pulmonary artery endothelial (BPAE) cells (FluoCells prepared slide #1, F36924); the 3D image stacks in “/data/zebrafish_3D” were captured from a living EGFP-labeled *Tg(sox10:megfp)* zebrafish embryo at two days post fertilization. In this protocol, we will use the 3D zebrafish data to demonstrate the functions of the program.

Import the “G” image by clicking the “Load G” button (1) and selecting the related file (2). Here, we use the “WL800_PX250_5mW_AVGx3_d0um_dz1um_t0_G.tif” file in “/data/zebrafish_3D”.



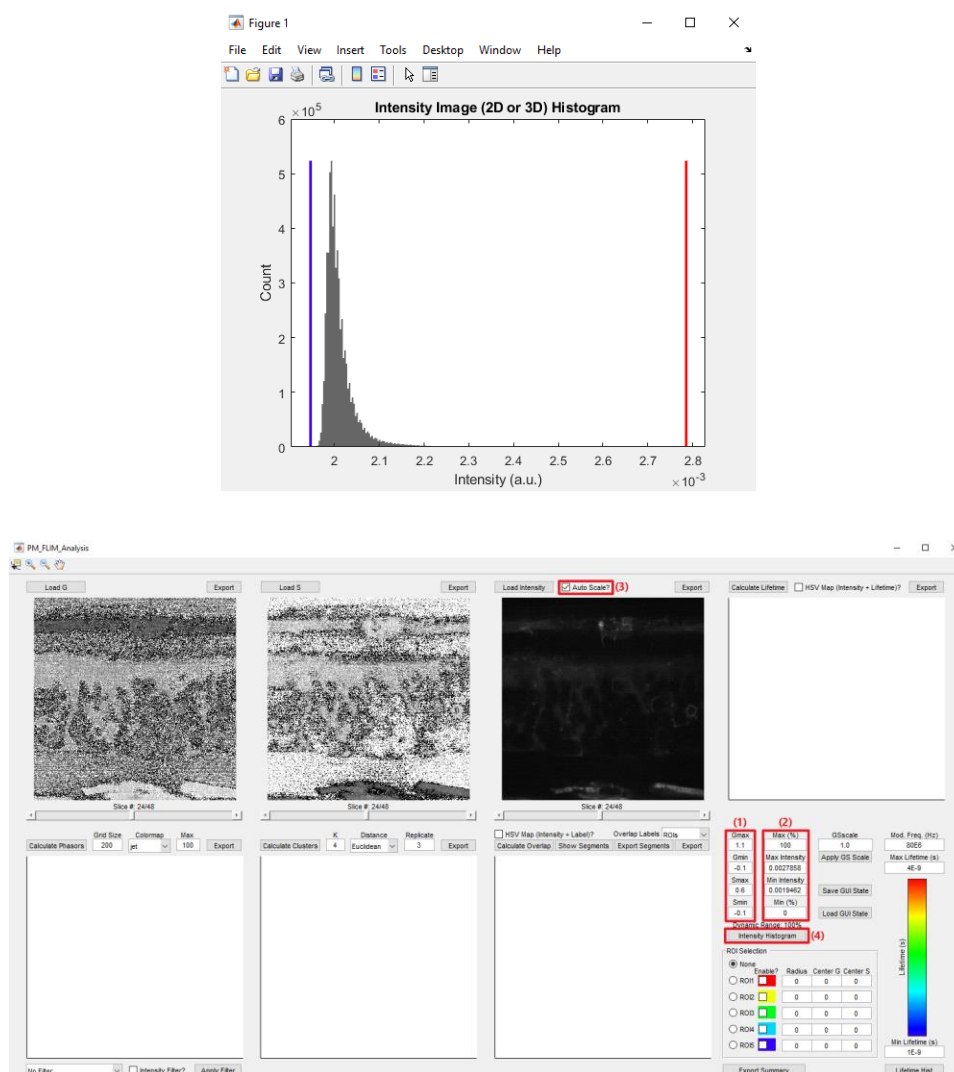
Similarly, import the “S” image, “WL800_PX250_5mW_AVGx3_d0um_dz1um_t0_S.tif”, and the “Intensity” one, “WL800_PX250_5mW_AVGx3_d0um_dz1um_t0_I.tif”, using the “Load S” (1) and “Load Intensity” (2) buttons, respectively.



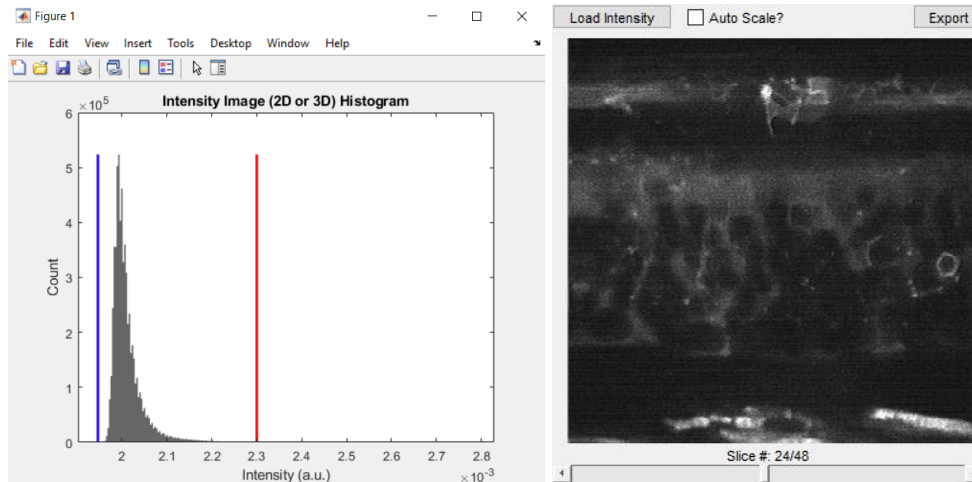
At this moment, all the raw PM-FLIM data required by the program has been imported. The sliders below the images can be used to visualize different slices in the 3D stack.

4. Data Pre-Processing

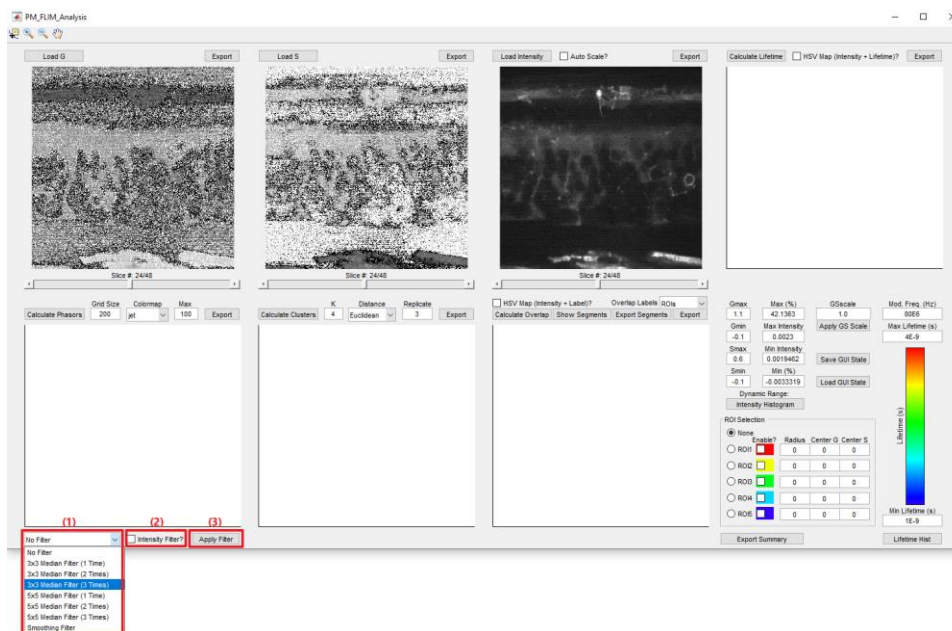
Some pre-processing steps are needed before analyzing the data. First, adjust the range of G and S by filling in the values of “Gmax”, “Gmin”, “Smax”, and “Smin” (1). The default values, “Gmax=1.1”, “Gmin=-0.1”, “Smax=0.6”, and “Smin=-0.1”, are recommended as pixels slightly perturbed by noise are included in the analysis. Next, adjust the Intensity range by either specifying the exact or percentage max and min intensity values (2) or enabling the “Auto Scale?” checkbox (3). The histogram of the intensity image can be viewed with the “Intensity Histogram” button (4), where the max and min intensities are marked with the red and blue lines, respectively:



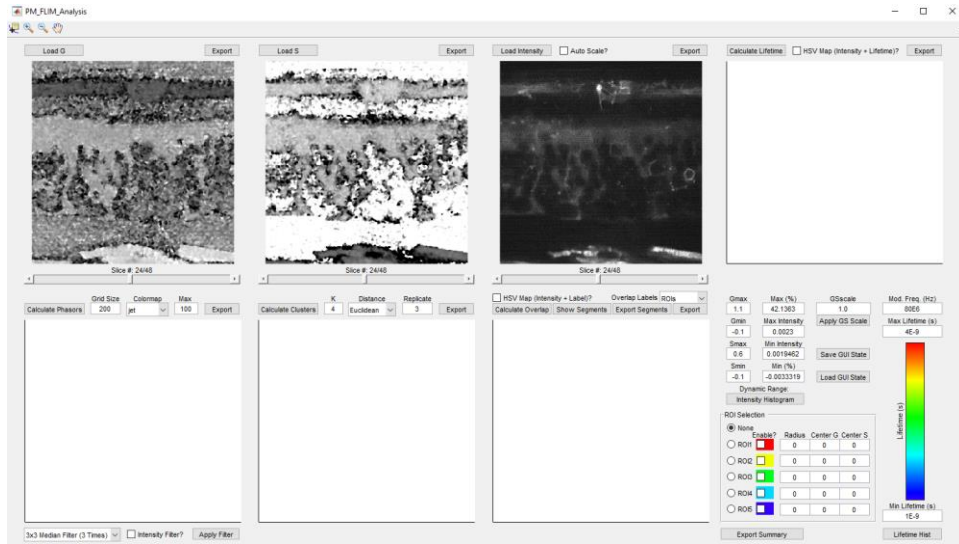
We recommend the user to adjust the max and min intensity values such that, by saturating a small percentage of pixels with high intensity, more details in the image can be visualized:



In addition, the signal-to-noise ratio (SNR) of the raw images, and consequently the SNR of generated images, can be improved by applying filters to the raw data. To do that, select a filter from the “No Filter” pop-up menu (1). If the “Intensity Filter?” checkbox (2) is enabled, the filter is also applied to the Intensity image; otherwise, only the phasor images (G and S) are filtered. Click the “Apply Filter” button (3) to perform the filtering operation. We recommend using the “3x3 Median Filter (3 Times)” or other median filters, as median filtering does not decrease the image resolution¹. The filtering operation could take some time depending on the size of the raw data. A progress window showing the filtering progress will appear during the operation.

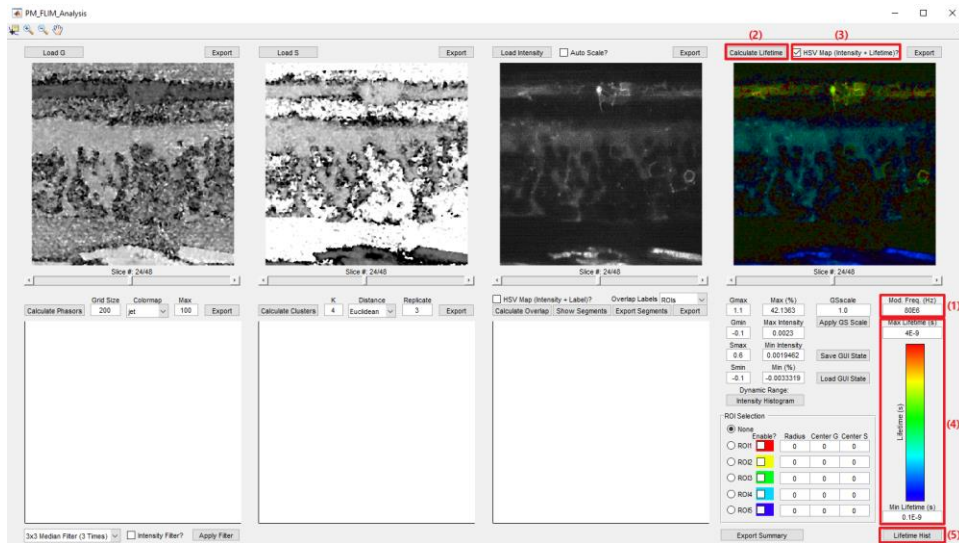
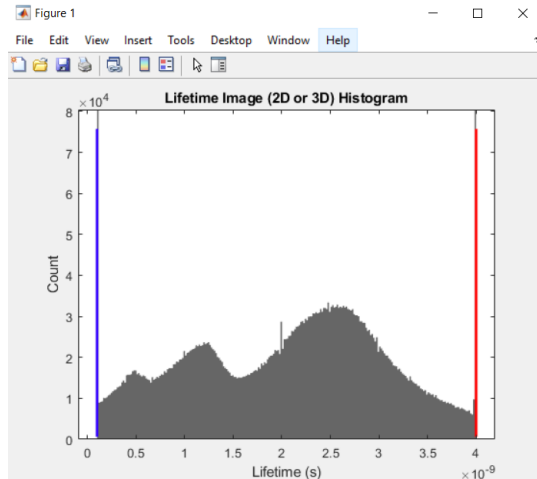


After filtering, the raw images look less noisy than before:



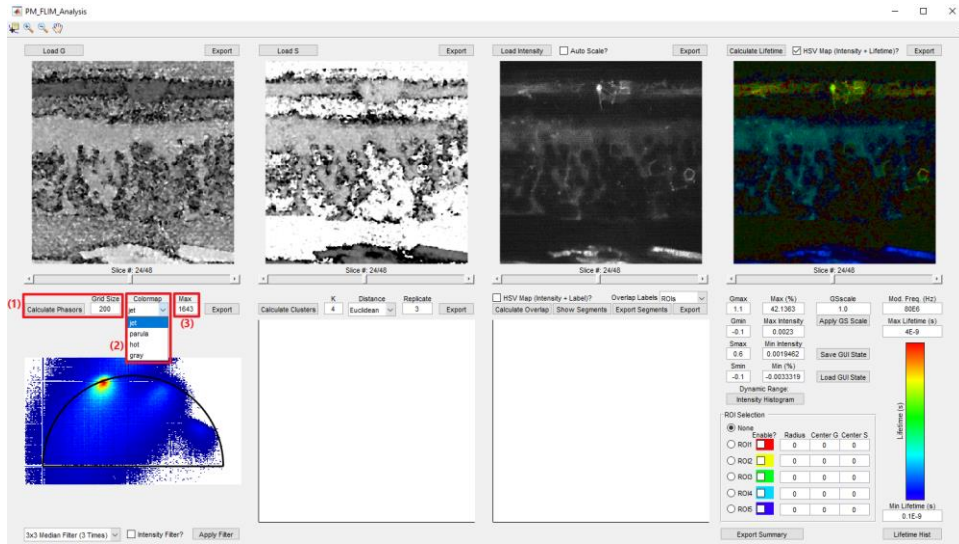
5. Fluorescence Lifetime Images

Fill in the “Mod. Freq. (Hz)” box (1) with the modulation frequency of the excitation laser during the PM-FLIM image acquisition. We use a mode-locked Ti:sapphire laser (Spectra Physics Mai Tai BB, 710-990 nm, 100 fs, 80 MHz) in our PM-FLIM system, so we write “80E6” in this box. Click the “Calculate Lifetime” button (2) to calculate fluorescence lifetimes. Note that there are two approaches to calculate fluorescence lifetimes in frequency-domain FLIM: phase-derived or modulation-derived approaches². In this work, we only consider phase-derived lifetimes for its simpler mathematical form compared to modulation-derived ones. The “HSV Map (Intensity + Lifetime)?” button (3) determines whether the lifetime image is displayed in raw or composite mode: if it is disabled, the raw lifetime image is shown in gray scale; if it is enabled, a composite lifetime image, where the image’s intensity and lifetime values are mapped to the pixels’ brightness and hue, respectively, is displayed. The range of displayed lifetime values can be adjusted with the “Max Lifetime (s)” and “Min Lifetime (s)” boxes (4). The histogram of the lifetime image can be viewed with the “Lifetime Hist” button (5), where the max and min lifetimes are marked with the red and blue lines, respectively:

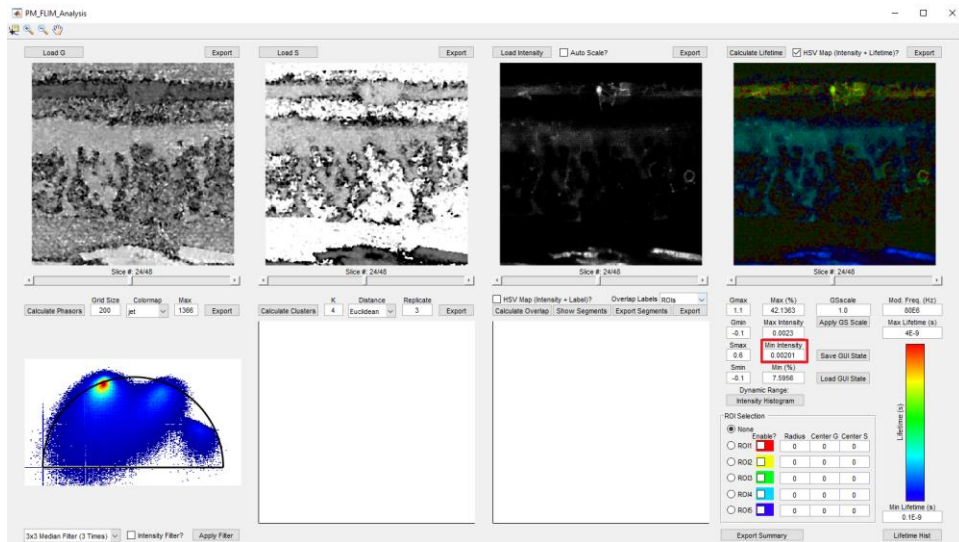


6. Phasor Plots

Phasor plots are 2D histograms of the pixels' G and S values. To generate phasor plots, specify the number of bins, or grids, in the "Grid Size" box and then click the "Calculate Phasors" button (1). A window will pop up showing the calculation progress. Once the phasor plot is generated, the user can change its colormap in the "Colormap" pop-up menu (2). The max displayed value of the 2D histogram, which is generated automatically, can also be changed in the "Max" box (3).



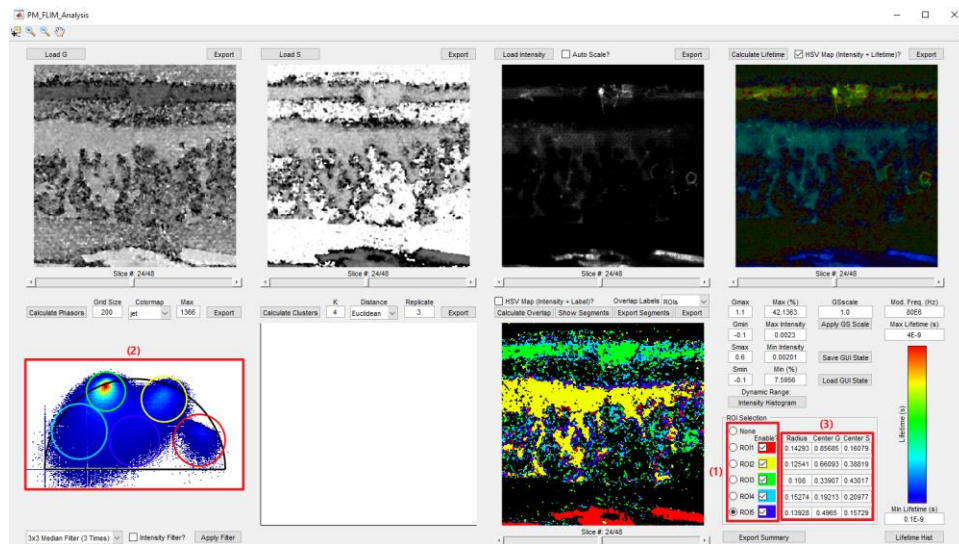
Because most of the pixels in the raw data are background noise, it is not uncommon to see these noise phasors locating outside of the semicircle. This issue can be addressed by increasing the value in the “Min Intensity” box to prevent the background noise pixels (intensity lower than the “Min Intensity” value) from being considered in calculating phasors:



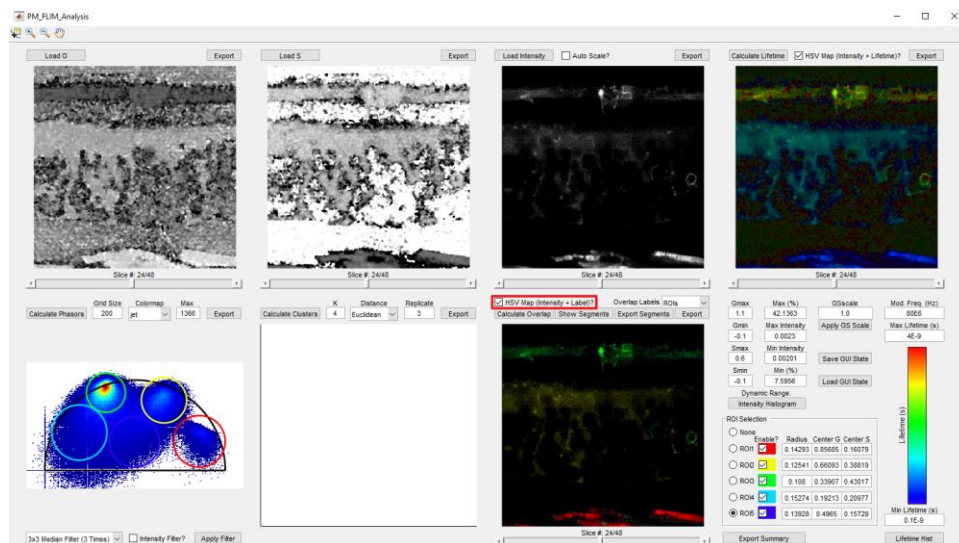
7. ROI Labeling of Phasors

The PM-FLIM-Analysis allows the user to draw ROIs on the phasor plot such that the pixels corresponding to the phasors located inside the ROIs can be identified and labeled with the ROIs' colors. By properly drawing the ROIs and labeling the pixels, the user can effectively segment the raw images based on different FLIM signatures of the image structures. Before drawing ROIs,

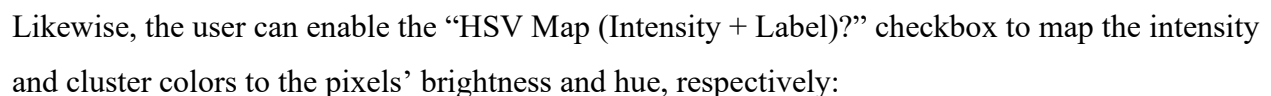
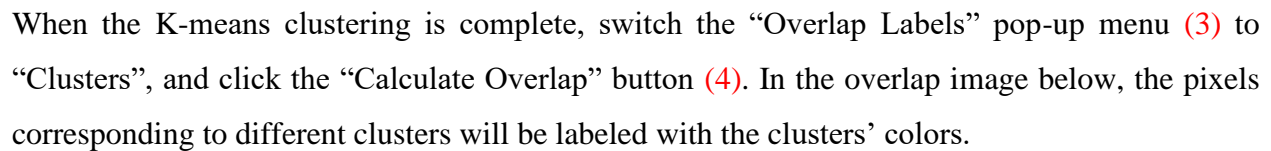
make the ROI selection with the radio buttons (1). Draw the ROI on the phasor plot (2) by clicking, holding, moving, and releasing your mouse. The corresponding pixels in the raw image will be labeled to the ROI's color whenever the ROI is drawn or updated. Up to five ROIs can be drawn simultaneously. Once drawn, the ROIs and the labeled pixels can be enabled or disabled using the “Enable?” checkboxes (1). The ROIs' radii and center G and S coordinates are shown in the “Radius”, “Center G”, and “Center S” boxes (3) when they are drawn. The user can manually modify these values to change the sizes and locations of the corresponding ROIs.

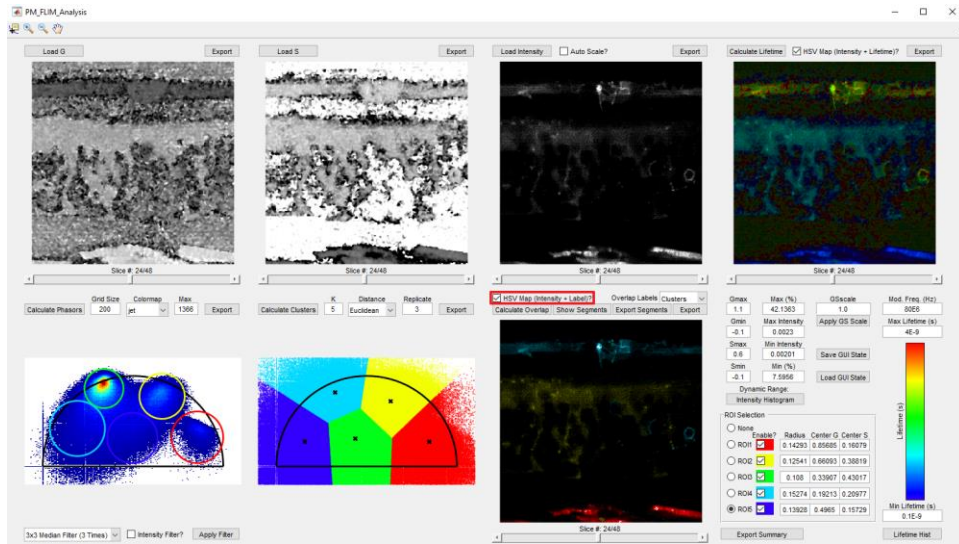


Like the composite lifetime image, the user can enable the “HSV Map (Intensity + Label)?” checkbox to map the intensity and labeled colors to the pixels' brightness and hue, respectively:



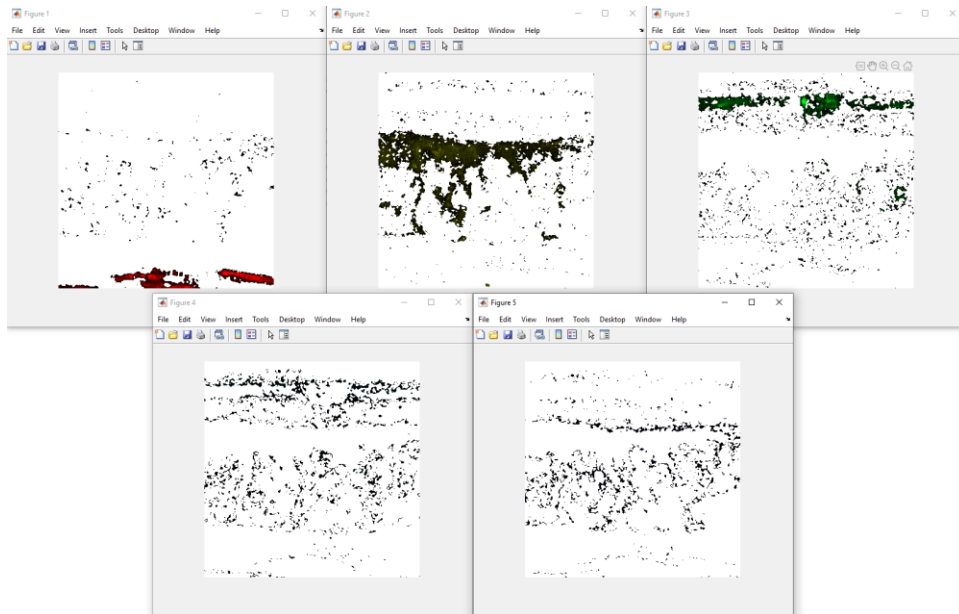
The PM-FLIM-Analysis includes an unsupervised machine learning feature, i.e., K-means clustering^{3,4}, which can be used to automate the manual ROI-based phasor labeling approach above in an unbiased way. Before performing K-means, specify the number of clusters (K), the distance metric (squared Euclidean, L1, or cosine), and the number of times to replicate the procedure (1). Start the procedure by clicking the “Calculate Clusters” button (2). The K-means clustering algorithm can be time-consuming for large images (3D or 4D). A window will pop-up showing the progress of the operation:



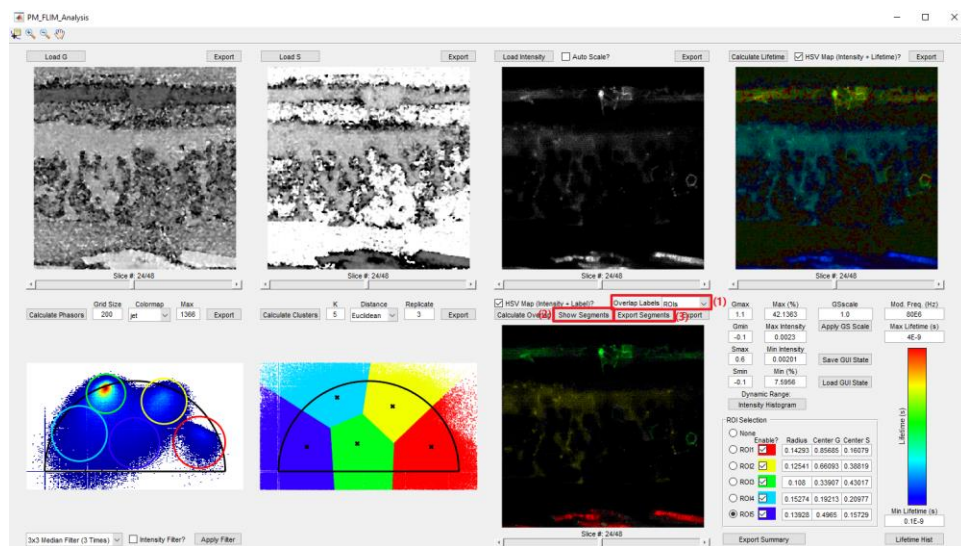
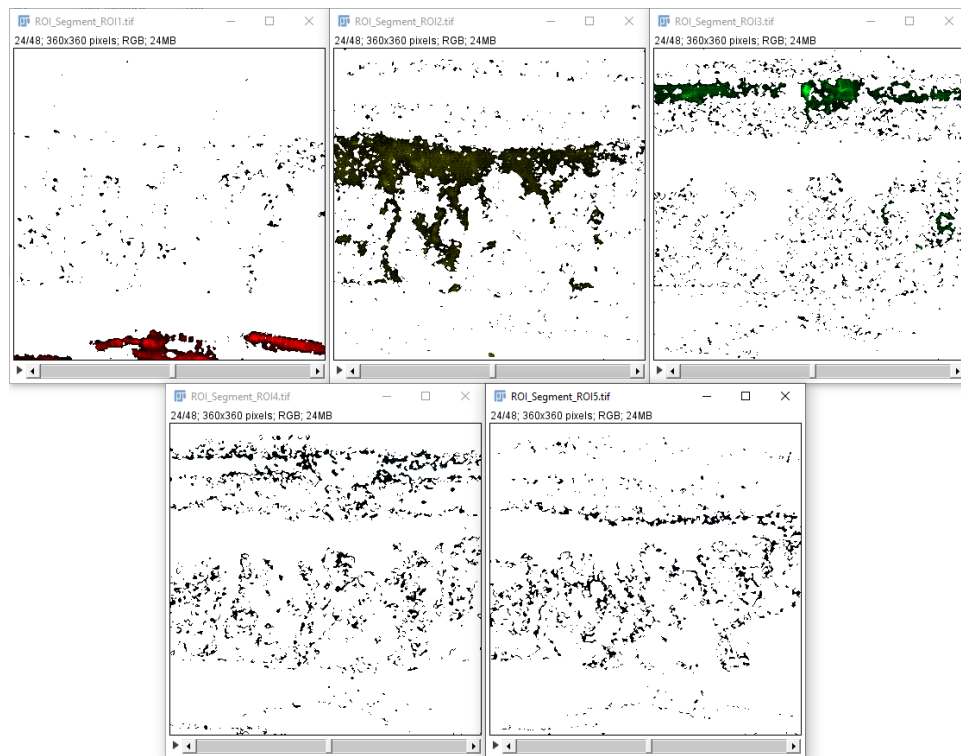


9. Image Segmentation

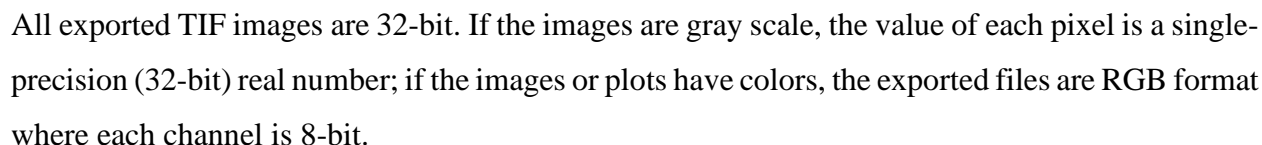
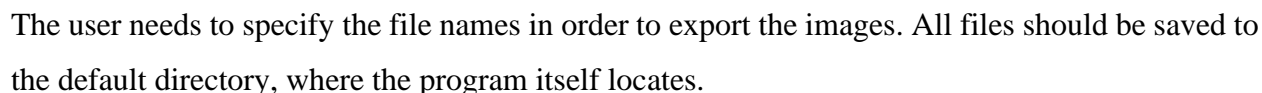
Both the ROI labeling and K-means clustering operations can be used to segment the images. For the ROI labeling and K-means clustering results, the pixels corresponding to each ROI and each cluster are grouped into a single segment, respectively. Before performing image segmentation, select whether the ROIs or clusters results are used in the “Overlap Labels” pop-up menu (1). The “Show Segments” button (2) can be used to display the segmented images as separate figures. Note that only a single frame of the stack (3D or 4D) can be displayed in this way:



The “Export Segments” button (3) can be used to export the segments into separate TIF images. In this way, the exported segmented images have the same size as the raw image (3D or 4D).

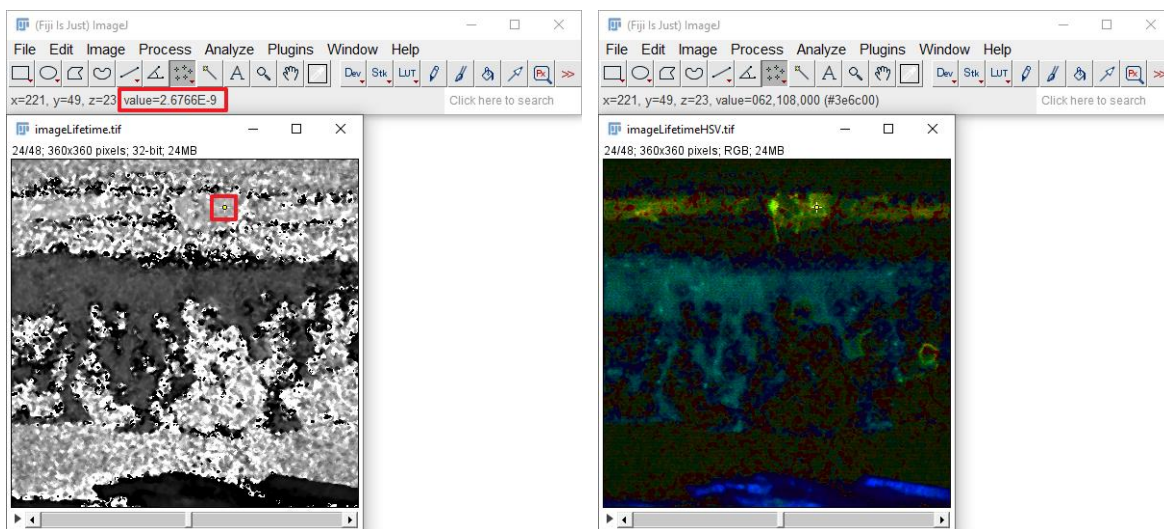


All images and plots that can be seen in the PM-FLIM-Analysis program can be exported into TIF files by using the “Export” buttons next to the images/plots. In addition, the screenshot of the program’s whole GUI can be exported using the “Export Summary” button.



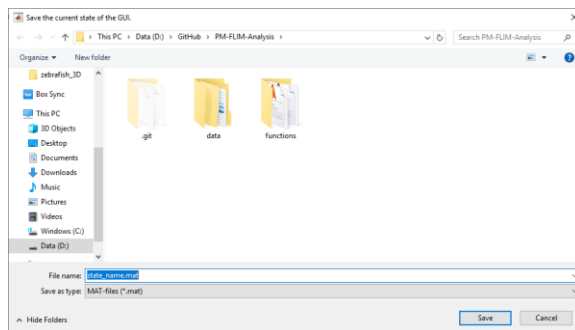
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directly viewed. For the images with colors, the information of the exact lifetime values is lost; one can refer to the lifetime colormap in the program to get this information. Also, the PM-FLIM-Analysis program will only export images to 3D stacks, even if the raw data are 4D; therefore, the user needs to manually change the exported 3D stacks to 4D by using the “Stack to Hyperstack” function in ImageJ.

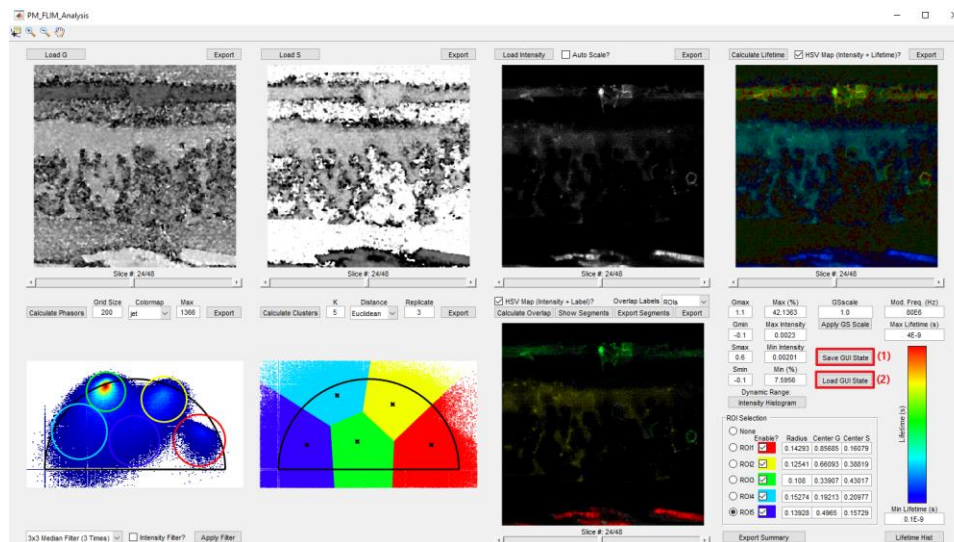


11. Save/Load Program States

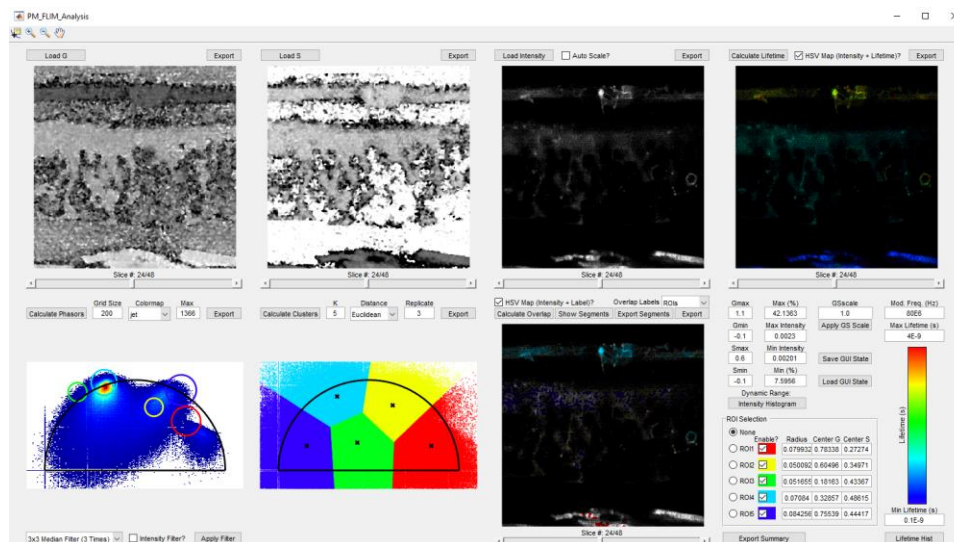
The program allows the user to save the GUIs’ current states, including the values of all edit boxes, pop-up menus, radio buttons, checkboxes, and most importantly, the radii and locations of the ROIs. To save the program states, click the “Save GUI State” button (1). A window will pop up asking the user to specify the file name. The file should be saved to the default directory, where the program itself locates.



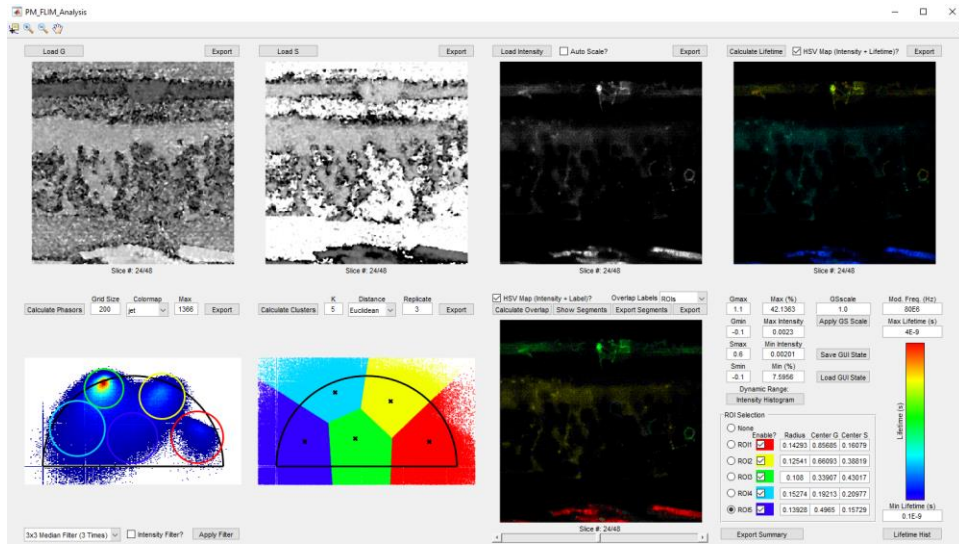
To recover the GUIs' state from a saved file, click the “Load GUI State” button (2) and select the state file (.mat) you just saved.



As an example, we modify the ROIs as follows:

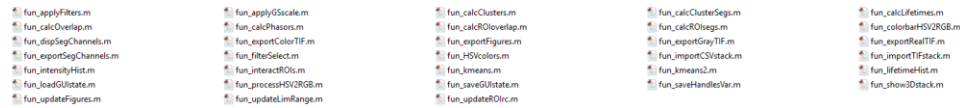


By loading the GUI state file, the ROIs, as well as other GUI elements, can be recovered to the previous states:

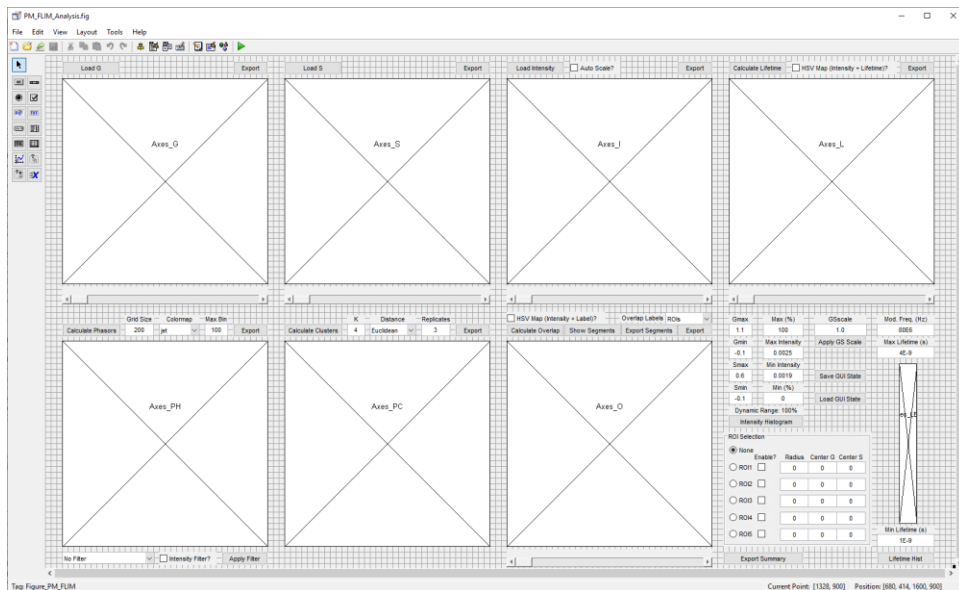


12. Add New Functions

The PM-FLIM-Analysis program is fully open-source and highly modularized into subfunctions. Users with Matlab programming expertise can easily modify the code to customize existing functions or to add new functions in the “functions” folder:



The GUI can also be changed using Matlab’s “guide” command:



References

1. Ranjit, S., Malacrida, L., Jameson, D. M. & Gratton, E. Fit-free analysis of fluorescence lifetime imaging data using the phasor approach. *Nat. Protoc.* **13**, 1979–2004 (2018).
2. Berezin, M. Y. & Achilefu, S. Fluorescence Lifetime Measurements and Biological Imaging. *Chem. Rev.* **110**, 2641–2684 (2010).
3. Jain, A. K. Data clustering: 50 years beyond K-means. *Pattern Recognit. Lett.* **31**, 651–666 (2010).
4. Zhang, Y. *et al.* Automatic segmentation of intravital fluorescence microscopy images by K-means clustering of FLIM phasors. *Opt. Lett.* **44**, 3928–3931 (2019).