Documentation for FPALM Analysis GUI

1. Run "gui_cp.m". This program opens the user interface that runs all other programs associated with FPALM analysis.
2. The **Path To Any Image In Series** may be either manually entered or selected with a dialog box using the **Browse** button. An image series with each image saved as individual files must use the naming convention, <*base name*><*frame #*>.<*image type*>. The **Precision** box must be set to match the number of figures used in <*frame #*> in this case (e.g. <*base name*>*_001.tif* … <*base name*>*_100.tif* would require a precision of 3). For multilayer tiff files leave **Precision** blank.
3. All analysis output generated by the GUI is saved in the **Analysis Output Directory** which may be entered manually or selected with a dialog box using the **Browse** button.
4. The **Custom Frame Range** can be enabled to limit the FPALM analysis to frames **From** through **To**. Note that if a custom frame range is desired, it must be set before generating a background subtraction profile. If the custom frame range is not enabled all frames in an image series of a given <*base name*> will be analyzed.
5. The **Plot Histogram of Image** button is used to generate a histogram of the pixel values in an image in the series selected for analysis. If **Custom Frame Range** is enabled, the histogram will be displayed for the frame designated by **From**, otherwise a histogram of the first image in the series will be displayed by default.
6. The **Preview** tab contains the following options concerning the displaying of the FPALM analysis figure. Note that to display the analysis figure the **Show Preview: Update FPALM rendering every <#> frames** option must be enabled. **<#>** determines the number of frames between updating the FPALM rendering of localized molecules.
    6.1. **Frame Delay** can be enabled set a user determined delay (in ms) between analysis of each image in a series. During analysis the **Frame Delay** can be changed using the keyboard shortcut: *shift* + *d* while the FPALM analysis figure is highlighted.
    6.2. **Manual Scale Limit** can be enabled to manually set the upper limit (in pixel value) for the display of each frame of the localization routine. If not enabled, each frame is relatively scaled by Matlab's *imagesc* function. During analysis the **Manual Scale Limit** can be changed using the keyboard shortcut: *shift* + *s* while the FPALM analysis figure is highlighted.
    6.3. Enabling **Show Colorbar** display a grayscale colorbar in the FPALM analysis figure. During analysis the **Show Colorbar** can be enabled/disabled using the keyboard shortcut: *shift* + *b* while the FPALM analysis figure is highlighted.
7. The **Optics** tab is used to set the following parameters of the experimental setup.
    7.1. **Camera Pixel Size** is where the effective pixel size (in μm) of the camera (actual size/total magnification) used to acquire images.

7.2. The **Pixel/Photon Conversion Factor** (in pixel value/photon) is used to convert image pixel values to number of detected photons per pixel where applicable. Note that this parameter is dependent on the gain settings of the camera.

7.3. **Emission Wavelength** is used to set the peak emission wavelength (in nm) of the fluorescent probe used in the experiment. The **Emission Wavelength** is used when calculating the $1/e^2$ radius of the image of a single molecule.

7.4. The **Numerical Aperture** (NA) of the microscope objective lens is used when calculating the $1/e^2$ radius of the image of a single molecule.

7.5. **PSF Radius Scale Factor** is used to scale the radius of the theoretical point spread function (PSF) of a single molecule since "real" objectives usually have slightly larger PSF than predicted by theory (Hess and Webb, *Biophys. J.* 2002).

7.6. **$1/e^2$ Radius Of PSF** displays the theoretically calculated single molecule PSF (in μm and pixles) based on the **Camera Pixel Size**, **Emission Wavelength**, **Numerical Aperture** parameters. The value scaled according to the **PSF Radius Scale Factor** is also displayed and is the value that is used during analysis (2D Gaussian fits and spot size in FPALM rendering). Note that a scaled value of less than ~1.5 pixels will result in a significant number of failed localizations which will result in "pixelization" in the FPALM rendering.

8. The **ROI** tab is used to select an ROI for analysis from the full frame images of a series. A custom ROI speeds up the analysis process and reduces the size of analysis output files.

8.1. **Custom ROI** must be enabled to define an ROI. If not enabled full frames will be analyzed. **X Offset** and **Y Offset** define the upper left corner of the ROI relative to the full frame. The ROI is **X Size** in width and **Y Size** in height. Note that the custom ROI parameters are saved in the .mat file generated by the **Compute WF & Mean Background** button and are then loaded from this file when needed during other phases of analysis.

8.2. The **Preview** button is used to display the ROI defined by the custom ROI parameters.

8.3. The **Select Region** button is used to manually select a custom ROI using the mouse pointer in a display of either the first frame of the image series or the frame designated by **From** if **Custom Frame Range** is enabled.

9. The **Background** tab is used to setup the background subtraction for FPALM analysis. Options are available to use either a weighted summed (time-integrated) widefield profile or a rolling ball algorithm.

9.1. The summed-widefield method (Hess et al., *PNAS* **104**:17370-17375, 2007) is used when the **Use Rolling Ball Background Subtraction** check box is *not* enabled.

9.1.1. **Percentage of Mean Background** determines the absolute weighing of the time-integrated widefield sum. The default value is 95%.

9.1.2. **Zero Level** is used to subtract a baseline offset (in pixel value) from every image in the series. The **Auto** button is used to determine the zero level from a histogram of either the first frame of the image series or the frame designated by **From** if **Custom Frame Range** is enabled. The zero level is set as the peak value of the histogram.

9.1.3. The **Compute WF & Mean Background** button generates a .mat file that contains the background subtraction profile used during the localization routine.

9.2. A rolling ball algorithm (Sternberg, *IEEE Computer*, 1983) is used when the **Use Rolling Ball Background Subtraction** check box is enabled. With this method, background subtraction is performed independently for each frame in the data series.

9.2.1. The **Rolling Ball Radius** is the radius (in pixels) of the sphere that is "rolled" along the underside of the surface generated by the pixel values of an image to form the background subtraction profile. This radius must be set to be at least as large as the radius of the image of a single molecule.

9.2.2. Prior to forming the background profile, each image is smoothed by a Gaussian of full width half-maximum (FWHM) set by **FWHM Of Gaussian Smoothing**. The default FWHM is 1 pixel.

9.3. **Background Noise** (in photons) is used to calculate the localization precision. The **Auto** button is used to calculate the background noise (in photons) as the standard deviation of a user selected region in a display of either the first frame of the image series or the frame designated by **From** if **Custom Frame Range** is enabled.

10. The **Localization** tab is where the parameters associated with localizing and fitting 2D Gaussians to single molecule images are set.

10.1. **Use Photon Thresholds** can be enabled to enter thresholds in units of photons per pixel. Note that the **Pixel/Photon Conversion Factor** must already be set in the **Optics** tab. If not enabled the thresholds will be in units of pixel values.

10.2. **rbox** sets the radius (half width) of a the "box" used to enclose the image of a single molecule in order for it to be fitted to a 2D Gaussian. **rbox** is dependent on the **$1/e^2$ radius of PSF** and must be large enough to enclose the entire image of the single molecule but no so large as to enclose a significant number of pixels that contain only signal from background noise.

10.3. When **Advanced Thresholding** is enabled the following thresholds are set to determine the criteria for image intensity peaks to be considered single molecules. Note that with this option, r_0 is fixed according to the (scaled) calculated value (see above) during 2D Gaussian fits.

10.3.1. **Minimum Intensity Threshold** determines the minimum value of any pixel to be further considered for analysis as a single molecule. Each (background subtracted) frame of an image series is initially scanned for

pixels with values above this threshold. Pixels with values exceeding this threshold are marked for further analysis as potential single molecules.

10.3.2. **Threshold 2** ensures that an intensity peak in an image is not too small to be a single molecule.

10.3.3. **Threshold 3** ensures that an intensity peak in an image is not too large to be a single molecule.

10.3.4. **At Least __ Pixels Above Threshold 2** determines the minimum number of pixels in an intensity peak that are must exceed **Threshold 2** for the intensity peak not to be too small to be the image of a single molecule.

10.3.5. **No More Than __ Pixels Above Threshold 3** determines the maximum number of pixels in an intensity peak that are allowed to exceed **Threshold 3** before the considered too large to be the image of a single molecule.

10.4. When **Advanced Thresholding** is not enabled only **Minimum Intensity Threshold** needs to be specified and any pixels in the image that have an intensity above or equal to this value will be fit (including r_0 as a fitting parameter) to a 2D Gaussian. Further analysis of fitting parameters and quality of fit must later be used to distinguish single molecules from spurious pixel intensities.

10.5. **Box Overlap Factor** determines how close (in pixels) the centers of the images of any two molecules can be without being excluded from analysis. Note that when the images of multiple single molecules overlap they can not be reliably localized.

11. The **Render** tab is used to display the FPALM analysis results as either an **FPALM Rendering**, an **Expanded Widefield Sum**, or a **FPALM Density Plot**.

11.1. **Write To File** can be enabled to save rendered images. Note that renderings are saved as 8 bit tiff images. This option can be disabled to preview the renderings without saving.

11.2. **Sub-Region Of ROI To Render** provides the option to render only a sub-region of the previously determined **Custom ROI**. **X Shift** and **Y Shift** define the upper left corner of the sub-region relative to the **Custom ROI**. The sub-region is **X Width** in width and **Y Width** in height. Note that the sub-region specified applies to all rendering buttons and **X Shift**, **Y Shift**, **X Width**, and **Y Width** must all be set to zero to render the full **Custom ROI**.

11.3. The **Set Tolerances** button opens a window to specify tolerances on fitting parameters (from 2D Gaussian fits) to be used in the FPALM Rendering. Molecules will only be rendered whose parameters lie in the range specified by **Min** and **Max**. Tolerances are specified for $\mathbf{r_0}$ (when **Advanced Thresholding** is not enabled), either the localization precision (**Loc. Prec.**) or number of detected photons per molecule (**N**) (depending on whether **Use Loc. Prec.** is enabled). A maximum fractional uncertainty (**Max. Frac. Unc.**, specified for $\mathbf{r_0}$ and **N**) and a maximum uncertainty (**Max. Unc.**) for the x and y coordinates of each localized molecule are also specified (calculated using the error estimates

obtained from the 2D Gaussian fitting routine). The **OK** button returns to the **Render** tab.

11.4. The **FPALM Rendering** button generates a plot of the positions of localized molecules as a 2D Gaussian spot of size proportional to the calculated localization precision and weighted according to number of detected photons.

11.4.1. **Expansion Factor** determines the level of zoom of the rendering relative to **Camera Pixel Size** (the original pixel size). This parameter is necessary in order for the rendered spots to be larger than one pixel.

11.4.2. **Intensity Weighting Factor** provides a global weighting factor for all rendered molecules to avoid saturation of the rendered image in area where large numbers of molecules have been localized.

11.4.3. **Size Factor** provides a global scaling factor for the size of rendered molecules, i.e. molecules are plotted as 2D Gaussian with size **Size Factor** x localization precision (σ) (e.g. a **Size Factor** of 2 plots the spots with size 2σ).

11.4.4. **Show Scale Bar @** can be enabled to include a 1 μm scale bar in the rendered images at the coordinates (x, y) specified in the text box.

11.5. The **Expanded Widefield Sum** button generates a widefield sum (sum of raw images from a series) expanded by a factor of **Expansion Factor** to allow for direct comparison of the conventional widefield fluorescence image to the **FPALM Rendering**. Note that the frame range summed is the same as that specified by **Custom Frame Range** before using the **Compute WF & Mean Background** button.

11.6. The **FPALM Density Plot** button generates a plot where pixel values are determined by the number of localized molecules located within each pixel.

11.6.1. The color range of the density plot ranges from 0 to **Colormap Max**.

11.6.2. The positions of localized molecules are binned into pixels of size determined by **μm/pixel** to generate the density plot.

12. The **Localize Molecules** button initiates the routing to localize molecules (starts the einzelreader.m script) using the parameters set in the **Localization** tab. Note that the .mat file generated by this routine uses the naming convention: <*base name*>_<***From***>-<***To***>_*t*<***Minimum Intensity Threshold***>-<***Threshold 2***>-<***Threshold 3***>_*npt*<**At Least __ *Pixels Above Threshold 2***>-< **No More Than __ *Pixels Above Threshold 3***>*.mat* when **Advanced Thresholding** is enabled, or <*base name*>_<***From***>-<***To***>_*t*<***Minimum Intensity Threshold***>*.mat* when **Advanced Thresholding** is not enabled.

12.1. When **Use Saved 'WF & Mean Bkg'** is enabled, a dialog box will appear that allows user specification of a particular <*base name*>*_wf_sum.mat* file to be used during the analysis. If an appropriate <*base name*>*_wf_sum.mat* file does not already exist, one will now be generated based on the parameters set in the **Background** tab.

12.2. **Show Preview** can be enabled to display a figure depicting the frame by frame analysis as it occurs. This figure displays the full frame widefield sum, the raw image of the current frame, the background subtracted current frame with color-coded boxes around molecules (red = too dim/too small, green = localized molecule, yellow = too bright/too large), and the **FPALM Rendering** of all molecules localized as of the current frame. The **FPALM Rendering** is updated how ever many frames specified in the text box associated with the **Show Preview** option.

12.3. On completion of the localization routine the following options are available.

12.3.1. **Save (Full Frame) Widefield Sum** can be enabled to write a full frame (no expansion) widefield sum to file as an 8 bit tiff.

12.3.2. **Show Loc. Prec. Histogram** can be enabled to display a histogram of the calculated localization precision of each localized molecule.

13. Menu Bar

13.1. **File**

13.1.1. **Load Preferences** allows the user to load saved preferences (all fields in the user interface) for analysis of a particular data set. Preferences can also be loaded using the keyboard shortcut Ctrl+L.

13.1.2. **Save Preferences** allows the user to save the preferences (all fields in the user interface) used for analysis of a particular data set. Preferences can also be saved using the keyboard shortcut Ctrl+S.

13.1.3. **Save As Default Preferences** sets the current settings to be loaded by default when the user interface is first opened.

13.1.4. **Exit** closes the user interface. The keyboard shortcut Ctrl+X can also be used to exit.

13.2. **Image**

13.2.1. **Combine Image Series** will concatenate separate (multilayer) tiff files into one file for analysis. This option can also be accessed using the keyboard shortcut Ctrl+Z.

13.2.2. **Render FPALM Image** will generate an **FPALM Rendering** from a previously generated .mat of localized molecular positions. Note that options that are currently entered in the **Optics** and **Render** tabs will be used to create the rendering.

13.3. **Help**

13.3.1. **About** lists the date of last modification of the user interface and the contact information for the authors of this software.

13.3.2. **Documentation** loads the help file associated with the user interface.