Analysis of time-domain lifetime data

Kirstin A. Walther

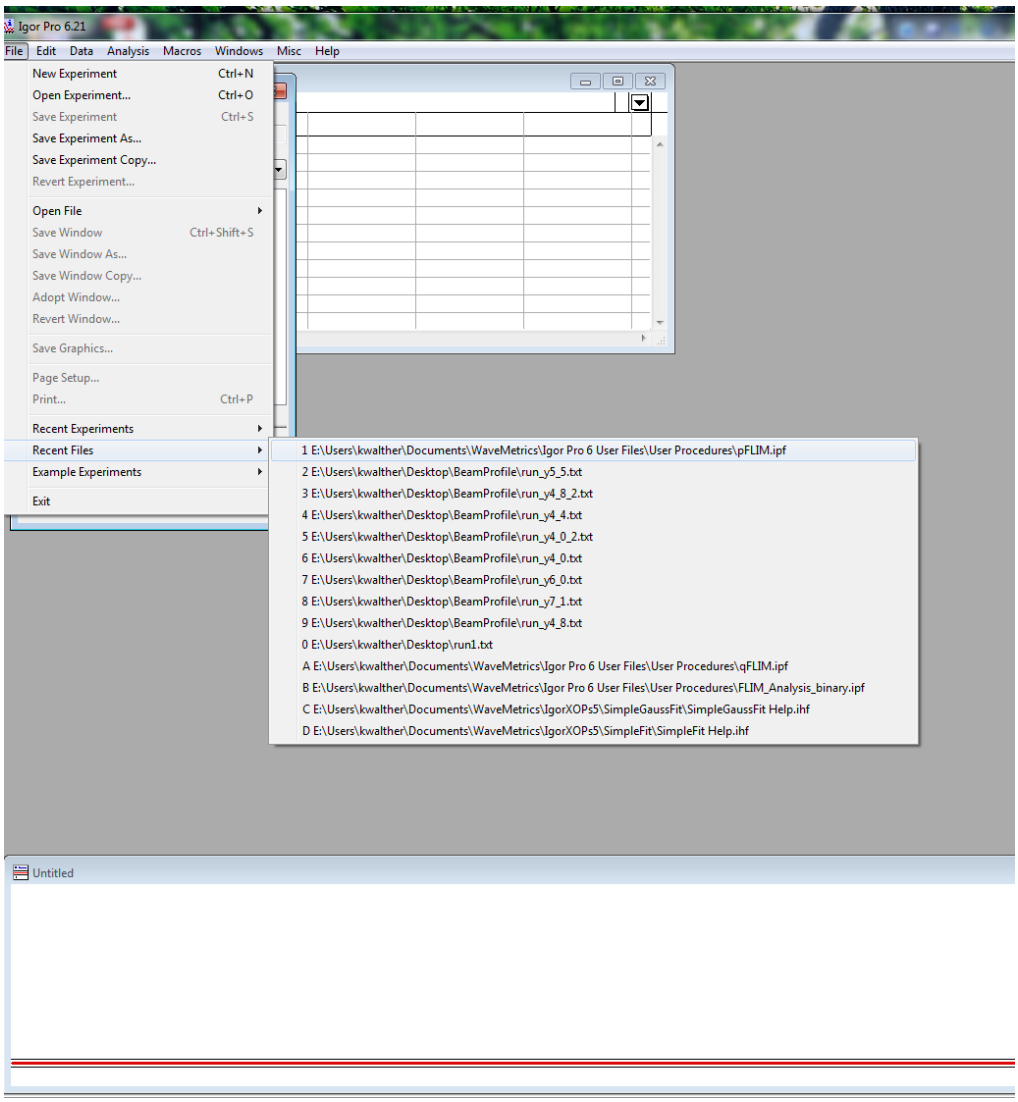
03.03.2011

1. Installation

a) Make sure you have installed and updated Igor to the latest version (6.20B02 on Mac or 6.2.1 on Windows).
b) Download pFLIM.zip as well as TabControl (http://www.igorexchange.com/project/ACL_tabcontrol) and multiopenfiles (http://www.igorexchange.com/project/multiopenfiles).
c) "pFLIM.ipf", "ACL_TabUtilities.ipf", and "ACL_Tab Control Utilities.ihf" should be saved in
   - My Documents/WaveMetrics/Igor Pro 6 User Files/User Procedures
d) "pFLIM.xop", "multiopenfiles.xop", and "multiopenfile Help.ihf" should be saved in
   - My Documents/WaveMetrics/Igor Pro 6 User Files/Igor Extensions

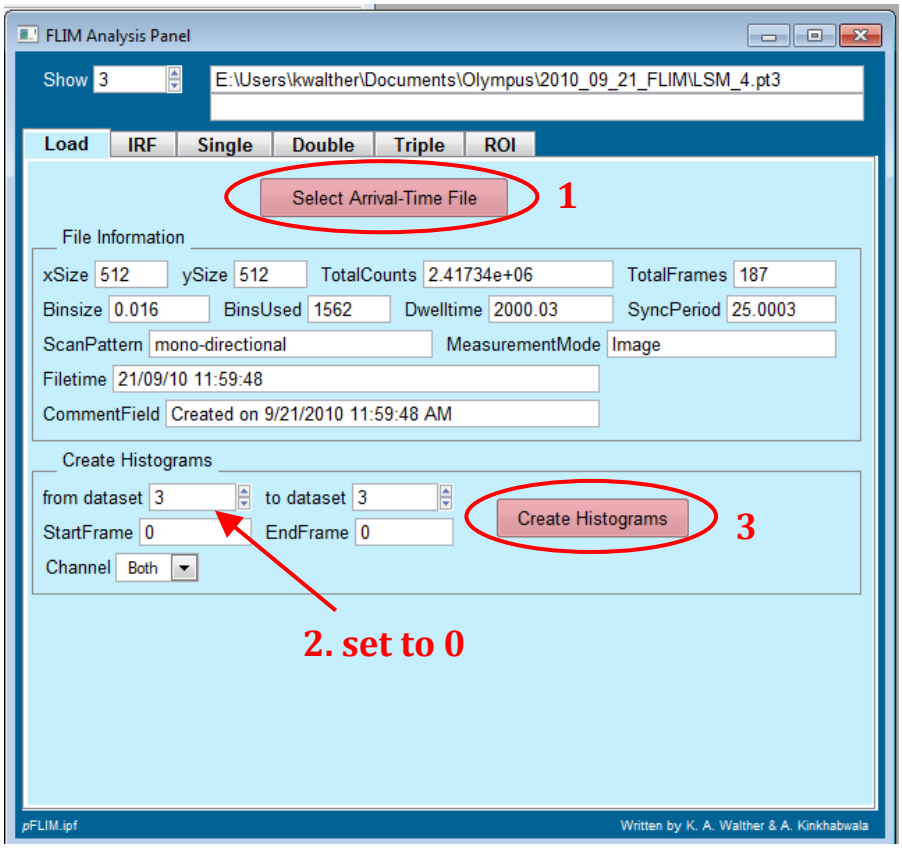
2. Running the program

a) If Igor is already open, exit Igor.
b) Either
   – Double-click pFLIM.ipf, which automatically starts Igor, or
   – Start Igor by clicking on its icon, then go to the File pulldown menu:
     (1) Open File -> Procedure, or
     (2) Recent Files, if you had opened pFLIM.ipf previously.
c) Macros -> Compile.
d) A new menu item, "FLIM" will appear.
e) FLIM -> Initialize Analysis. This will hide the .ipf file and will open the User Panel.



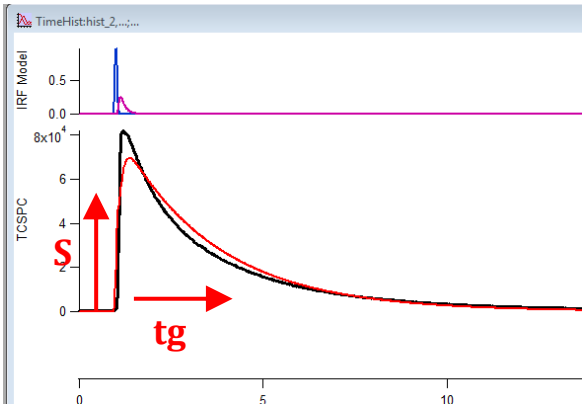
3. Loading Data

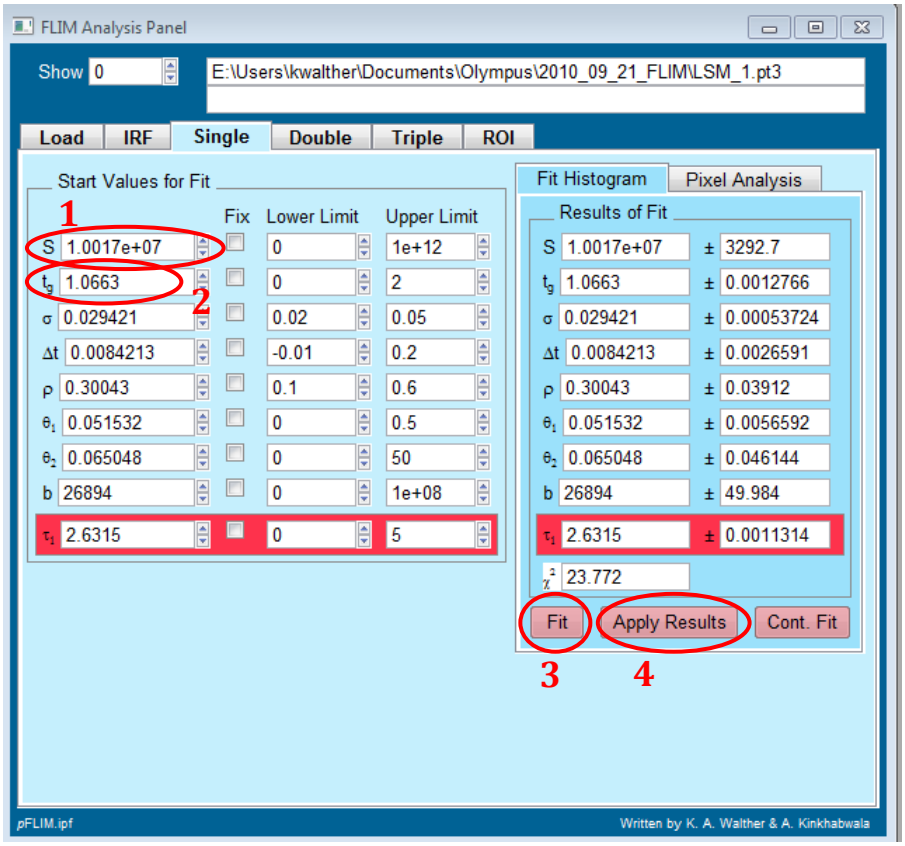
a) Make sure that all folders in the pathname to your .pt3 files as well as the actual filenames **do not contain: "+" or "–" signs or other unusual characters (keep it to letters, numbers and "_") and are not too long.** If the program crashes this is likely the source of the problem.
b) On the User panel click on Select Arrival-Time File. In the following dialogue select the .pt3 files you want to analyze. Once you click "ok", you have to wait a bit. In this time, the program scans through the .pt3 file and retrieves general file information, like the size of the image, the total counts, etc. This will then be displayed in the fields on the Load tab of the User panel. **Also make sure that the correct file path and name is displayed in the window at the top right of the panel.** If parts are missing there was a problem with the naming of the folders and files (see 3a).
c) Now is a good time to save the experiment. **If there is any problem with the file names, the program will crash in the next step!**
d) You can view all the files names you selected:
   - Open the data browser: Data -> Data Browser.
   - Double-click root:Data:FileTable.
e) Now go to the latest dataset you loaded (increase the number in Show with the arrows or use the up key until you reach the end). Be aware, if you loaded 10 datasets, Show will only go to 9 because it starts at 0.
f) Now change the number displayed in from dataset to 0 (Create Histograms box on the Load tab.
g) Save the experiment.
h) Click Create Histograms button. The program now scans through the .pt3 file to create the time histogram.
i) **Make sure to never move the .pt3 files!** The Igor experiment has its locations stored and needs to access the .pt3 file during the image processing. So if you move the files, Igor won't be able to find them any more! (If you really need to move your files you can replace the file location in the FileTable wave).
j) Once Igor is done calculating the histograms, save the experiment.
k) Now you can either continue by analyzing the histogram (continue with (**4**)) or by creating the Intensity image (if you want to select ROIs to select certain cells and recalculate the histogram) (continue with (**5**)).

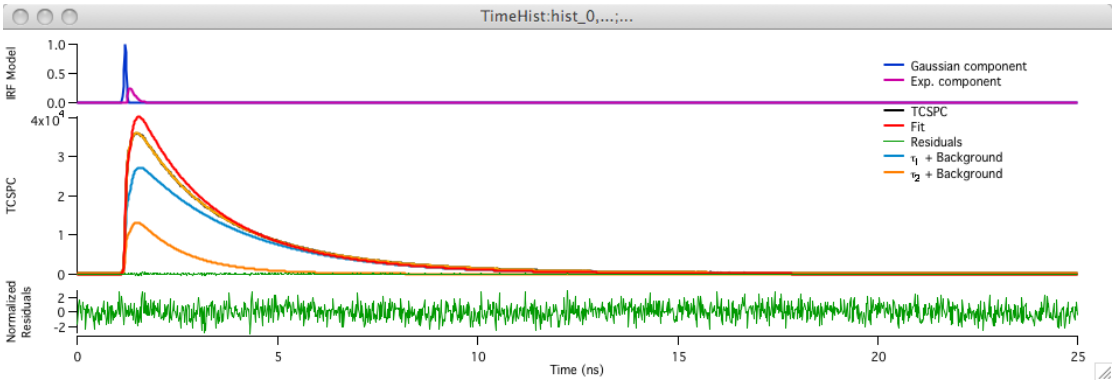

FLIM Analysis Panel
Show 3
E:\Users\kwalther\Documents\Olympus\2010_09_21_FLIM\LSM_4.pt3
Load
IRF
Single
Double
Triple
ROI
Select Arrival-Time File
1
File Information
xSize 512
ySize 512
TotalCounts 2.41734e+06
TotalFrames 187
Binsize 0.016
BinsUsed 1562
Dwelltime 2000.03
SyncPeriod 25.0003
ScanPattern mono-directional
MeasurementMode Image
Filetime 21/09/10 11:59:48
CommentField Created on 9/21/2010 11:59:48 AM
Create Histograms
from dataset 3
to dataset 3
StartFrame 0
EndFrame 0
Channel Both
Create Histograms
3
2. set to 0
pFLIM.ipf
Written by K. A. Walther & A. Kinkhabwala

4. Analyzing the histograms

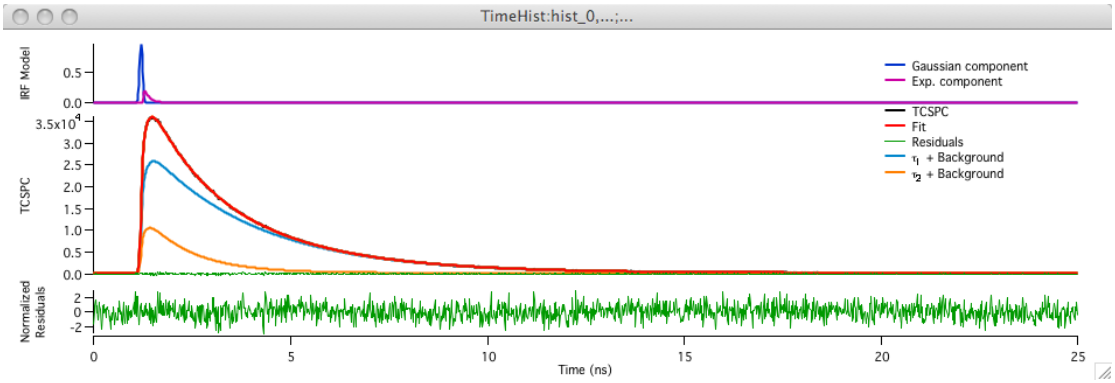
a) For a donor sample, you probably want to use a single-exponential, so go to the Single tab.
b) For a donor + acceptor sample, go to the Double tab.
c) If your donor is already double-exponential you might want to try to fit the donor + acceptor data with a triple-exponential fit. However, you can't do any image analysis with triple-exponentials yet.
d) Now you want to adjust the starting values for the Igor fit:
   - **S** defines the height of the red curve (your "Start Values for Fit") and should be close to the number of photons in the histogram (see the **Load** tab). This value is automatically transferred when you created the histogram. Meaning, you shouldn't have to change it at all.
   - Adjust $\mathbf{t_g}$ until the initial rise of the histogram of the black and the red curves roughly correspond.
e) With these starting values Igor should be able to fit the curve automatically, so click Fit (on the Single, Double, or Triple tab) in the User panel. An orange curve showing the result of the fit will appear in the graph.
f) If the fit was successful, click the Apply Fit Results button. This will copy the values from the fit to the "Start Values for Fit" part of the User panel. You will also see that the red curve will now overlap the orange curve (so you don't see the orange curve any more).
g) If the fit was not successful (you don't like it or even got an error message) **DON'T** click the Apply Fit Results button. Just change the "Start Values for Fit" and try again.
h) It CAN happen that the limits I put on the parameters are too narrow. If you see that a fit results in one of the parameters reaching its limit (upper or lower) try increase or decreasing this limit and repeat the fit. At some point the fit will converge (the button Cont. Fit keeps fitting the data until χ^2 does not significantly decrease any longer).
i) **Make sure that the IRF always stays reasonable.** You can see its components in the top part of the TimeHist graph. If you don't know how an IRF should look, you can contact me.
j) Again, I recommend you first analyze all histograms before continuing with the single-pixel analysis.







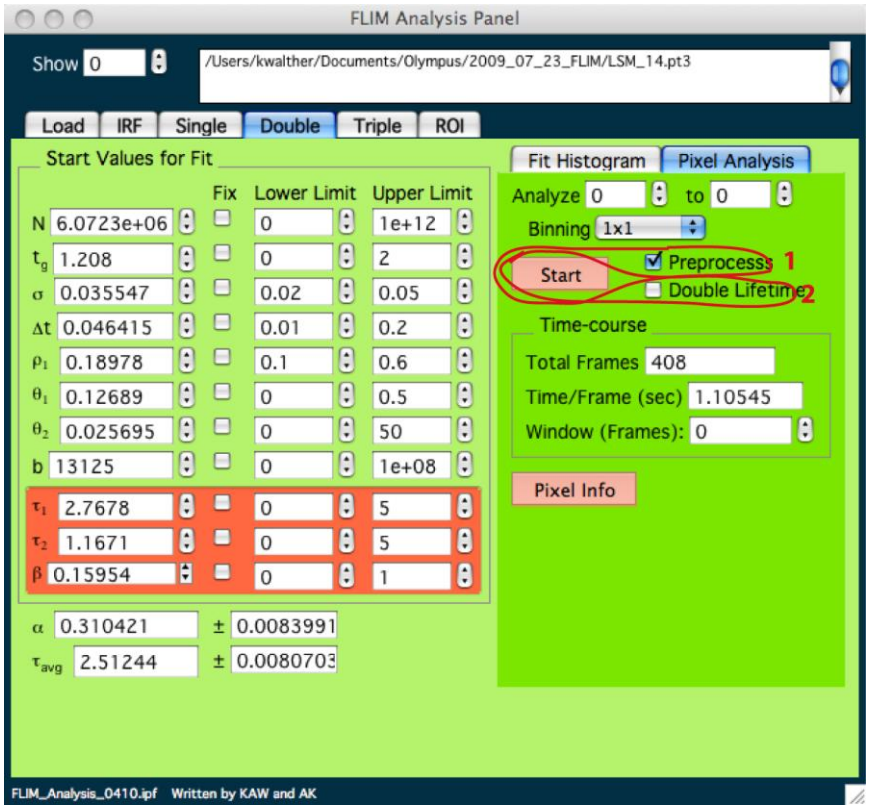
Graph after clicking Fit Double



Graph after clicking Apply Results

5. Single-pixel Analysis

a) This is done in the sub-tab Pixel Analysis (only available for single- and double-exponential fits).
b) First we want to create the Intensity image.
c) This is done by checking Preprocess (and unchecking Single Lifetime on the Single tab or Double Lifetime on the Double tab).
d) If you want to bin the image (e.g. 2x2 or 4x4) change this accordingly.
e) Again, select all datasets by setting the value of Analyze dataset to 0 and to to the last dataset loaded.
f) **Save the experiment.**
g) Click the Start button.
h) This will again take some time, quite some time. The program sorts the .pt3 file by pixel x, pixel y and the relative arrival time, taking into account any binning. The result is saved as a binary .bin file. Finally, the intensity image is created.
i) Once this is accomplished, you can continue the single-pixel analysis.
j) **For the single-pixel analysis, the values shown in the Start values for Fit part of the panel will be used.** That's why it's important to remember to press the Apply Fit Results button after a successful fit.
k) Uncheck Preprocess and check Single Lifetime or Double Lifetime.



l) Again click the Start button.
m) In general, you can do the Preprocessing and the Single/Double lifetime analysis in one go. For this, just check both boxes. This is useful if you will be away from the computer for a while.

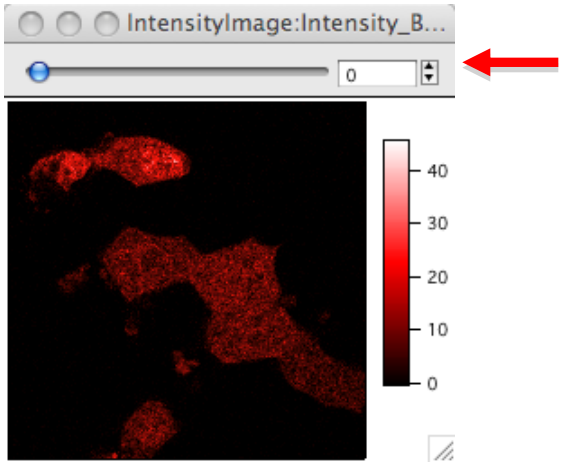
n) The program now creates and displays the following images for double exponential lifetime analysis:
   - **Alpha image**: this is probably what you are interested in as everyone always talks about α. $\alpha = (\beta/\tau_2) / ((1-\beta)/\tau_1 + \beta/\tau_2)$, where β tells you what fraction of the signal photons came from the second lifetime (e.g. a value of 0.2 means that 20% of the signal photons came from τ_2 while 80% came from τ_1).
   - **Average tau** image: Avg $\tau = (1-\beta) * \tau_1 + \beta * \tau_2$
   - In addition you get these images weighted by the signal image.
o) In addition the program creates but does not by default display the following image:
   - Signal counts image: how many of the photons in each pixel came from the actual fluorophores (as opposed to background).
p) You can choose which images to display by clicking Display Options under the FLIM menu. Then check or uncheck the appropriate boxes.
q) In addition, you can create the following images through the FLIM menu:
   - Background counts image: how many of the photons in each pixel came from background (if you add the signal counts image to this you would get the intensity image).
   - Beta image.

6. Movies

a) If you want, you can split up the data to make frames in a movie (e.g. you took data over 30 min but want to make a movie with 5 min frames). This will create sequences of images, which you can look at using the slider that will automatically appear above each image (e.g. each individual image would cover 5 min, and since you took data for 30 min, there will be a sequence of 6 images).

b) In the Time-course section of the Pixel Analysis sub-panel you can see how many frames you took when acquiring the data. You can also see how long it took to scan through each frame (this was set by you through the scanning speed). By changing the variable Window (Frames) you can choose how many frames to analyze together. If the value is 0 (default) the program will analyze the whole data set together as one frame.

c) Note: if there are 10 frames (0 to 9) and you choose to analyze 3 frames together (e.g. 0-2, 3-5, 6-8) there will be one frame (9) left over at the end. This frame is therefore not included in the movie!

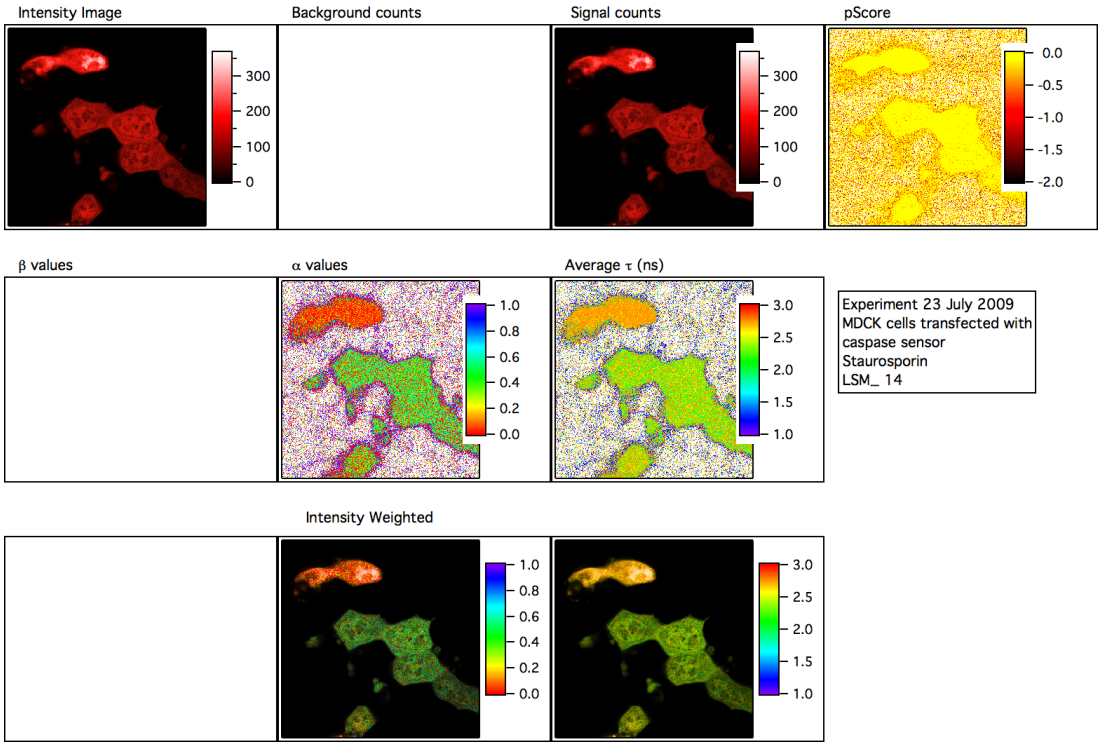


7. ROIs

a) For the single-pixel analysis ROIs are irrelevant since you anyway look at each pixel separately. The time-histogram on the other hand by default includes ALL photons that arrived anywhere in the image. So this you might want to change if, for example, you wish to exclude a bright dead cell that would otherwise "contaminate" your total arrival histogram.
b) There are 2 ways to create an ROI:
   - Manually drawing an ROI
     (1) Go to the ROI tab.
     (2) Click Create ROI. This will open a new small panel.
     (3) Click on the graph you want to create the ROI on (probably the Intensity image).
     (4) Click Start ROI Draw in the new panel.
     (5) On the chosen image, there will be tools appearing on the left side. Choose which type of shape you want to create (you can combine several) and start drawing.
     (6) When you are done, click Finish ROI. The ROI(s) will now be shown in green.
     (7) Click Save ROI Copy.
     (8) Click Erase ROI, which erases the ROI from the image (it's saved though).
   - Creating an ROI by thresholding an image
     (1) Select the Intensity or Signal image
     (2) Go to the menu Image, then Threshold
     (3) In the new window that opened choose the range between min and max that you want to include in your ROI (e.g 20 to Inf).
     (4) Choose When done: New Image
     (5) Done
     (6) A new image with your ROI appears, just close it
     (7) In the ROI tab, click Copy threshold ROI
c) In the list of ROIs on the user panel, a new ROI has appeared.
d) If you want, you can now create another new ROI, just follow the instructions above.
e) To display your saved ROIs, just select them in the list on the User panel. It will be display on the intensity graph, the white parts will be excluded, the transparent parts included.
f) To recalculate the histogram from the selected parts of the image, just click Recalculate Histogram. This again takes a moment (not too long).
g) Now you can re-fit the new histogram (the old one is overwritten!!) and see if you can get a better fit.

8. Pretty Layout

If you want to print or externally save (as pdf…) the results for a dataset, you can use the PrettyLayout function listed under FLIM. This assumes that you currently display all necessary graphs (open Display Options) created with the double-exp analysis (otherwise there will be empty spots on the layout). The source code (Windows -> Procedure Windows -> pFLIM.ipf, then search for PrettyLayout) can also serve as a template for your own layouts.



9. Location of relevant waves and images within the Igor experiment (as seen in the Data Browser)

- **root**
  - **Data**
    - FileInfo_Variables — information displayed on the Load tab of the user panel**
    - FileInfo_Strings
    - FileTable — complete pathname and filename of .pt3 files you selected
    - hist_0 — Time histograms
    - hist_1
    - ...
  - **Analysis**
    - **Manual**
      - TrialValuesIRF** — start values for the IRF fit
      - TrialValuesSingle** — start values for the Single fit
      - TrialValuesDouble** — start values for the Double fit
      - TrialValuesTriple** — start values for the Triple fit
      - **IRF**
        - IRFInit_0 — waves shown in TimeHist graph
        - IRFInit_1 — corresponding to the values displayed in the
        - ... — Start Values section of the IRF tab
      - **Single**
        - SingleInit_0 — waves shown in TimeHist graph
        - SingleInit_1 — corresponding to the values displayed in the
        - ... — Start Values section of the Single tab
      - **Double**
        - DoubleInit_0 — waves shown in TimeHist graph
        - DoubleInit_1 — corresponding to the values displayed in the
        - ... — Start Values section of the Double tab
      - Triple
        - TripleInit_0 — waves shown in TimeHist graph
        - TripleInit_1 — corresponding to the values displayed in the
        - ... — Start Values section of the Triple tab
    - **Fits**
      - FitValuesIRF** — results of the fits to the time
      - FitValuesSingle** — histograms
      - FitValuesDouble**
      - FitValuesTriple
      - **IRF**
        - IRFFit_0 — waves shown in TimeHist graph
        - IRFFit_1 — corresponding to the result of the IRF fit for
        - ... — each data set
      - **Single**
        - SingleFit_0 — waves shown in TimeHist graph
        - SingleFit_1 — corresponding to the result of the Single fit
        - ... — for each data set
      - **Double**
        - DoubleFit_0 — waves shown in TimeHist graph

DoubleFit_1	corresponding to the result of the Double fit
...	for each data set

- **Triple**

TripleFit_0	waves shown in TimeHist graph
TripleFit_1	corresponding to the result of the Triple fit
...	for each data set

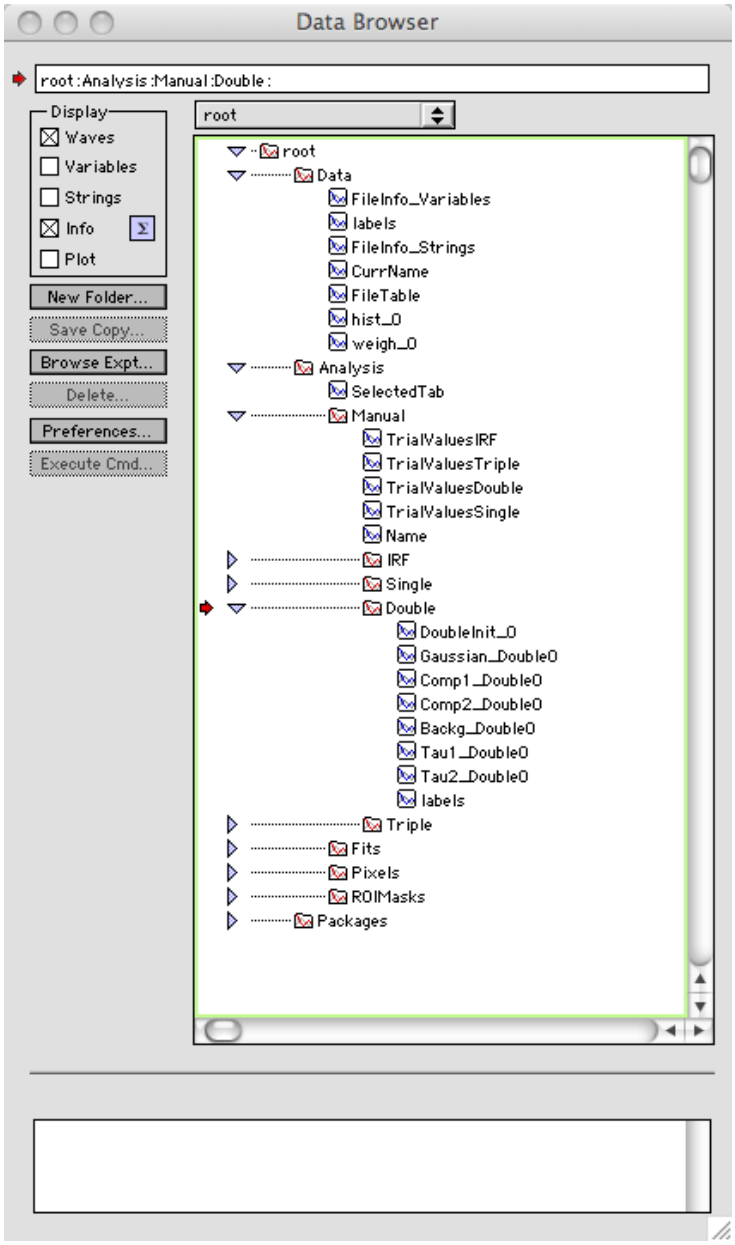
- **Pixels** all images created by the single-pixel analysis

Intensity_B1_0	Intensity image of dataset 0 binned 1x1
Signal_B1_0	Signal image of dataset 0 binned 1x1
pScore_B1_0	pScore image of dataset 0 binned 1x1
AvgTau_B1_0	τ_{avg} image of dataset 0 binned 1x1
Alpha_B1_0	α image of dataset 0 binned 1x1
AvgTau_B1_W_0	τ_{avg} image of dataset 0 binned 1x1, weighted by signal image
Alpha_B1_W_0	α image of dataset 0 binned 1x1, weighted by signal image
Intensity_B2_0	Intensity image of dataset 0 binned 2x2
Intensity_B1_1	Intensity image of dataset 1 binned 1x1
...	

- **ROIMask** everything related to the ROIs

ROIMask1_0	1st ROI Mask created for dataset 0
ROIMask2_0	2nd ROI Mask created for dataset 0
ROIMask1_1	1st ROI Mask created for dataset 1

** Double-click these waves to display them in a table, then go to Table -> Horizontal Index -> Dimension Labels. In the table this changes the display of the horizontal point number (0, 1, 2, …) to a label of the column that will tell you what value is displayed in each column. The rows stand for each dataset (e.g. the values of dataset 0 are shown in row 0, and so on).


Data Browser
root:Analysis:Manual:Double:
Display
Waves
Variables
Strings
Info
Plot
New Folder...
Save Copy...
Browse Expt...
Delete...
Preferences...
Execute Cmd...
root
root
Data
FileInfo_Variables
labels
FileInfo_Strings
CurrName
FileTable
hist_0
weigh_0
Analysis
SelectedTab
Manual
TrialValuesIRF
TrialValuesTriple
TrialValuesDouble
TrialValuesSingle
Name
IRF
Single
Double
DoubleInit_0
Gaussian_Double0
Comp1_Double0
Comp2_Double0
Backg_Double0
Tau1_Double0
Tau2_Double0
labels
Triple
Fits
Pixels
ROIMasks
Packages