CaliFLIM 4.4 manual

2017-04-26

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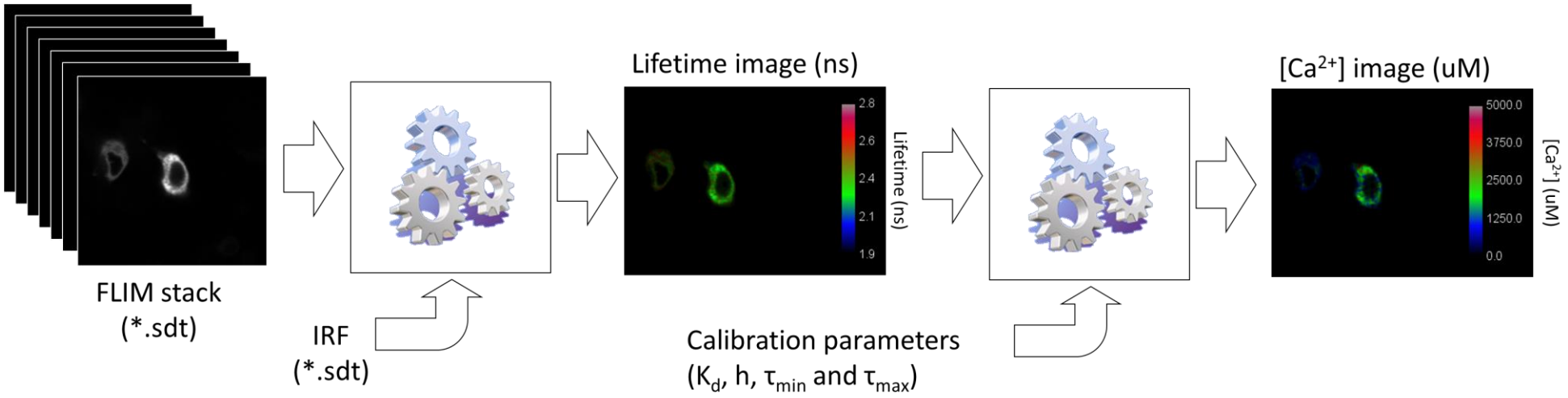
Table of content

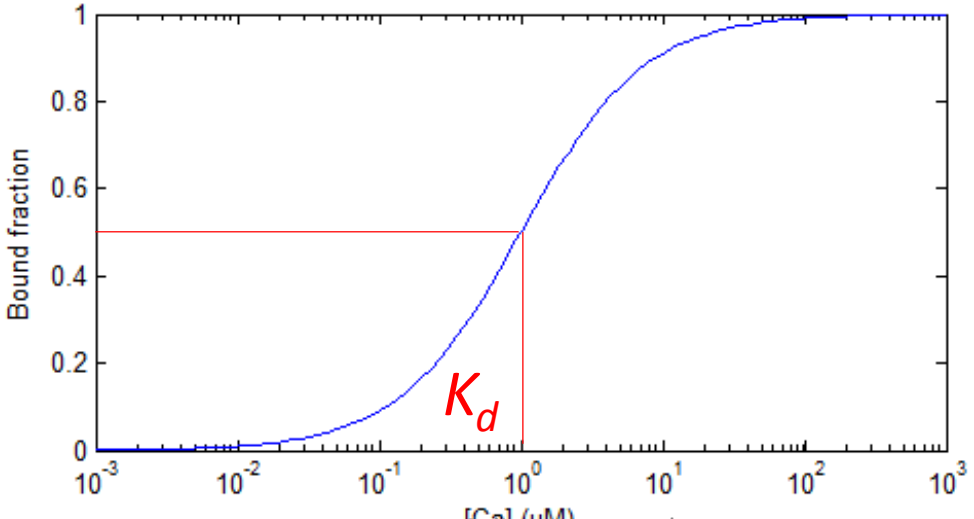
Basic features of CaliFLIM (1/3)

- It is based on ImageJ macro language (Java based), and completely open source
- It uses the raw TCSPC files (.sdt) from B&H acquisition as input
- It is based on a non-fitting approach called Centre of Mass Method (CMM), and therefore much faster than fitting approach (such as SPCImage from B&H).
- It allows the computation of FLIM images (providing the IRF) and absolute Calcium concentration images providing the calibration parameters.



Basic features of CaliFLIM (2/3)

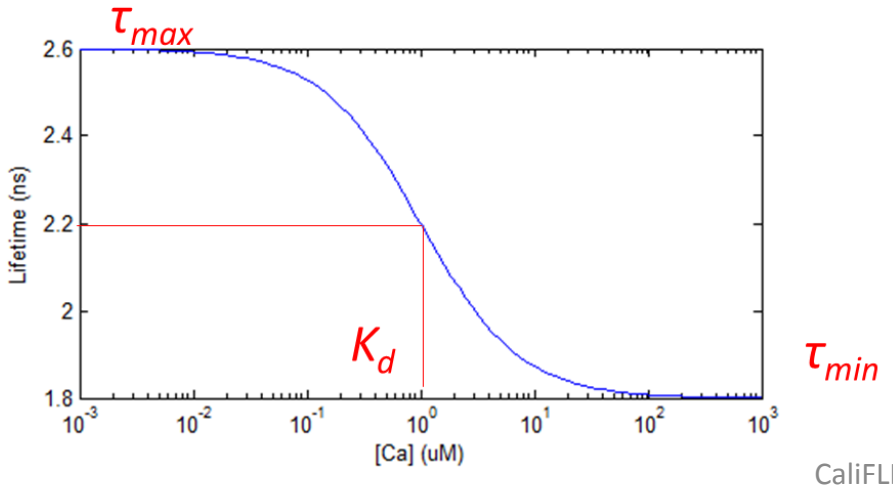
- Obtaining the absolute lifetime image requires providing an IRF dataset. This image is comparable to what would be normally obtained with other methods.
- The calibration requires the knowledge of the dissociation constant (K_d) and the Hill's coefficient (h) as well as the lifetime in virtually no calcium and saturating calcium.



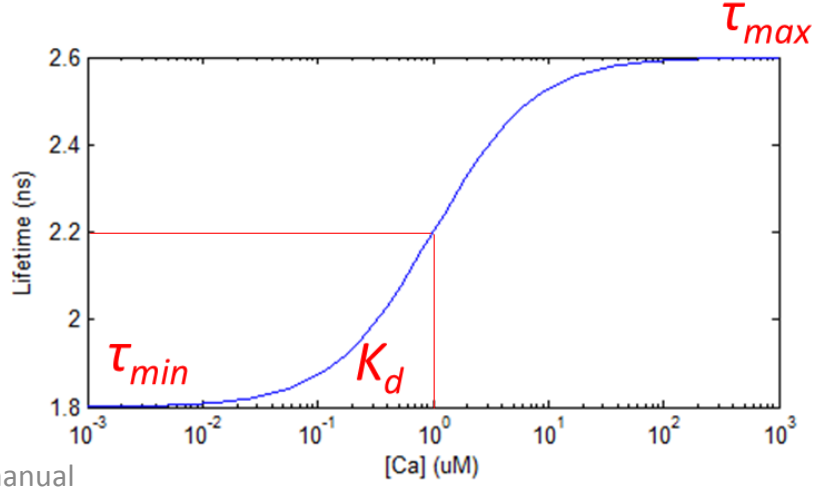
Basic features of CaliFLIM (3/3)

- The calibration can be used for both **FRET sensor** and **direct lifetime sensor**.
- For FRET sensors, the lifetime decreases with the increasing Calcium (due to FRET) so, in virtually no calcium the lifetime is maximum τ_{max} and minimum at saturating calcium τ_{min}.
- For Direct lifetime sensors, the trend is the opposite. This is common for intensity Calcium dyes which increase its brightness with Calcium concentration. In this case the increase in quantum yield is directly correlated to an increase in fluorescence lifetime.

FRET sensor



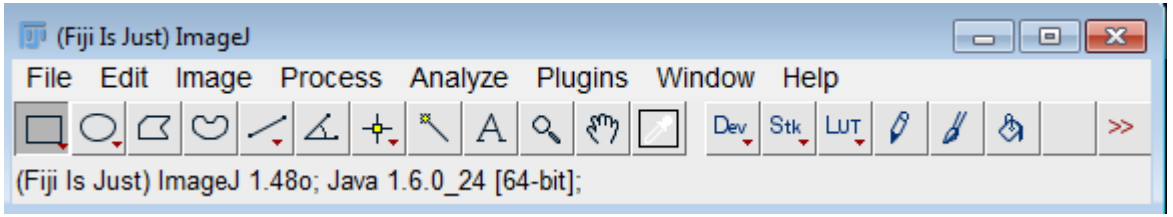
Direct lifetime sensor



Getting Fiji

- Fiji stands for “Fiji is just ImageJ”. It is a version of ImageJ with all the importer for Bio-formats such as the Becker & Hickl .sdt format.
- Fiji is freely available for download on http://fiji.sc/Fiji

(please use the life-line version as it is more stable)



Installing CaliFLIM

- CaliFLIM can be simply installed by copying the file *CaliFLIM x.xx_.ijm* in a folder in the following directory

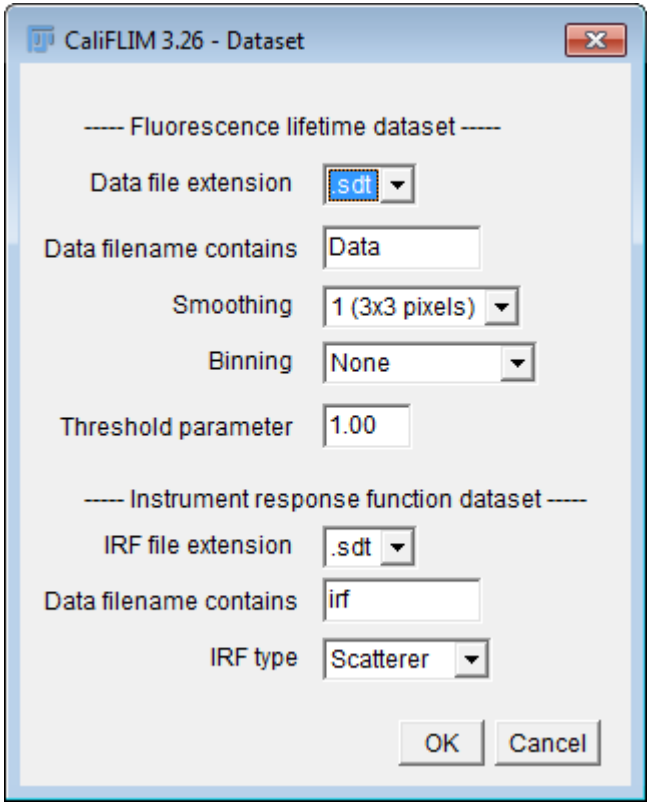
\Fiji.app\plugins\Scripts\Plugins\

- The folder can for instance be called FLIM utilities (in which case the file can be found in \Fiji.app\plugins\Scripts\Plugins\FLIM utilities\CaliFLIM x.xx_.ijm)
- CaliFLIM can now be found and run in Plugins → FLIM utilities → CaliFLIM x.xx

Before running CaliFLIM

- CaliFLIM can batch-process all your data providing the following:
- All the dataset to be processed are named consistently (the file name contains a token name that is consistent across the datasets).
- All the data to be processed are in the same folder along with the IRF and the calibration datasets (high / low calcium datasets) if they are used.

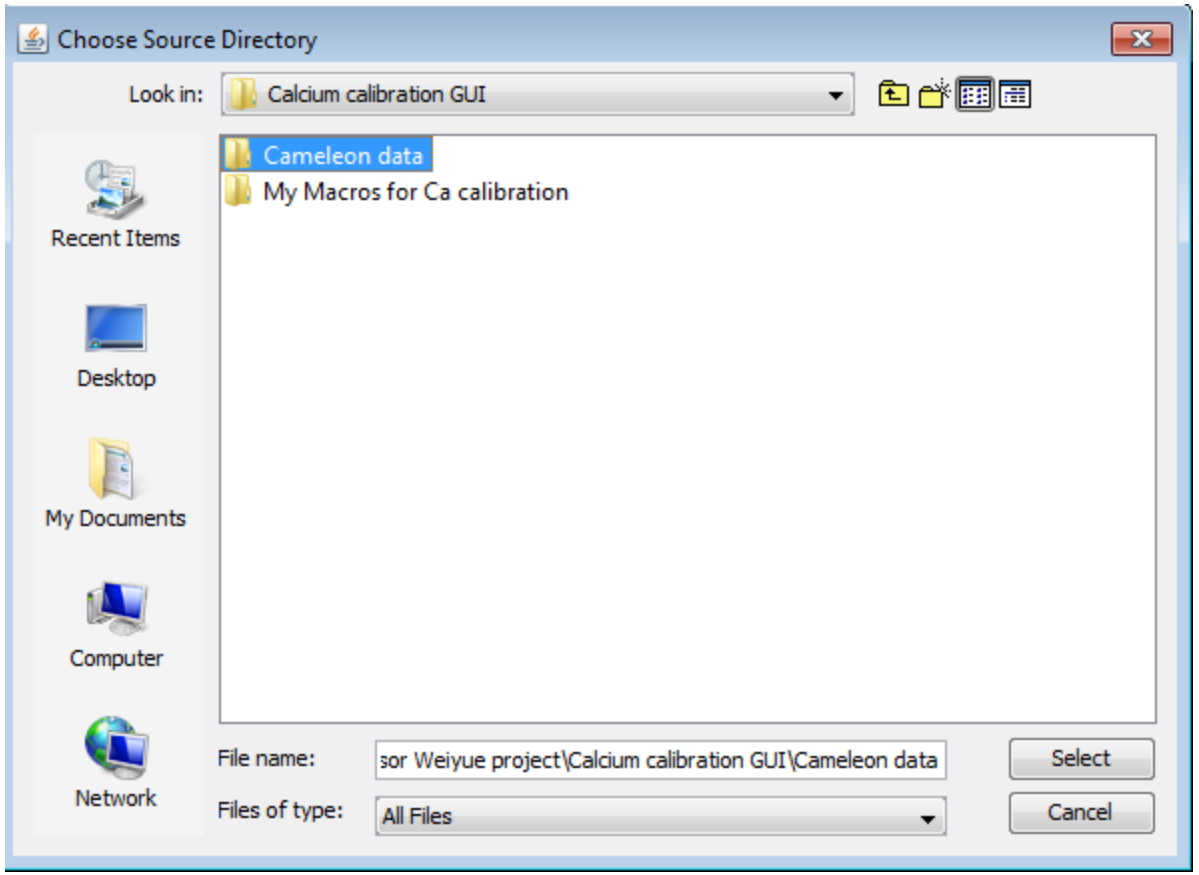
FLIM dataset window



- Choose the type of file: either .sdt (from Becker&Hickl acquisition software) or from .tif if simulated or saved as tiff stack.
- Enter the token name that the dataset file names contain
- Choose a smoothing level (this smoothing is identical to B&H “binning” method) (Default: 3x3 pixels)
- Choose a binning level (Note: binning decreases the size of the image by binning adjacent pixels. Default: None)
- Choose a threshold parameter. (Increasing the threshold parameter removes dimmer pixels from the analysis. Default: 1)
- Choose the type of file for the IRF (.sdt or .tif)
- Enter the token name that the IRF file name contains
- Choose the IRF type. (Scatterer or reference dye such as Rhodamine B or Rhodamine 6G, or “none” if no IRF are available)

- Press OK

Choose a directory

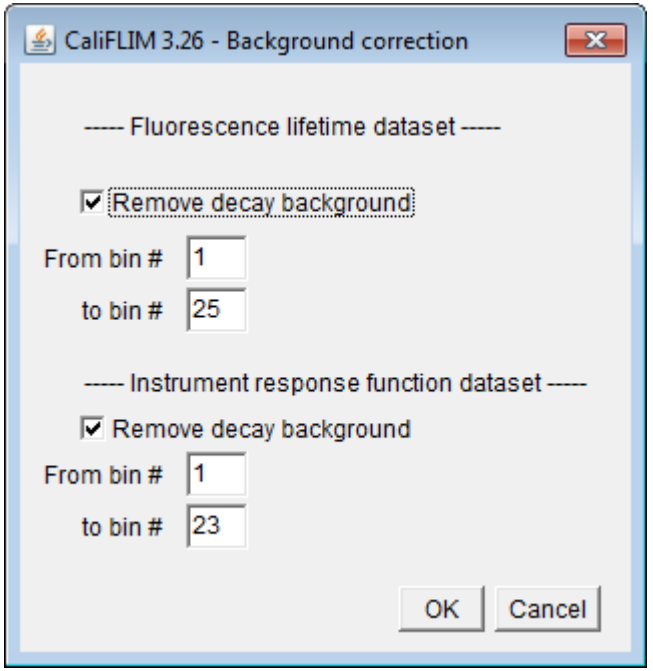


- Select the folder where the dataset is contained
- Press Select

Background correction settings

- Choose whether to remove the decay background or not. If the background must be removed, the bin positions used to calculate the background level must be given. The default values for the bin positions ("from" and "to") are automatically set from the dataset by detecting the flat region before the rising edge.
- If an IRF is available (scatterer or reference), the user is additionally asked to choose to remove its background too.

- Press OK

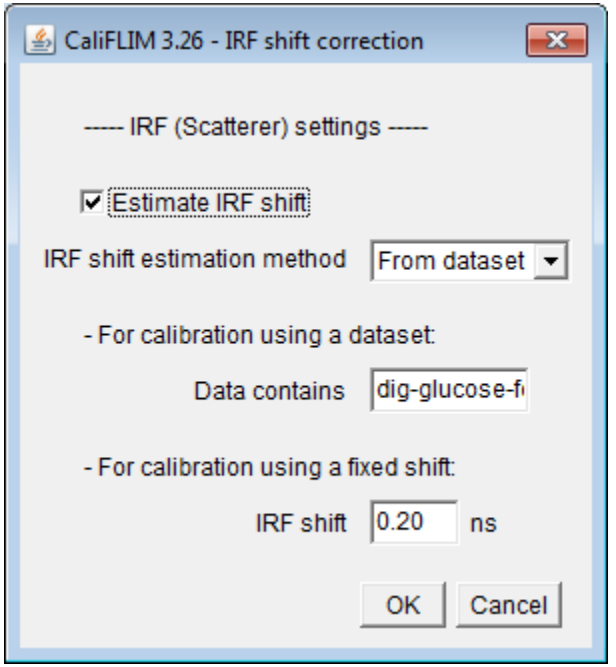


IRF settings

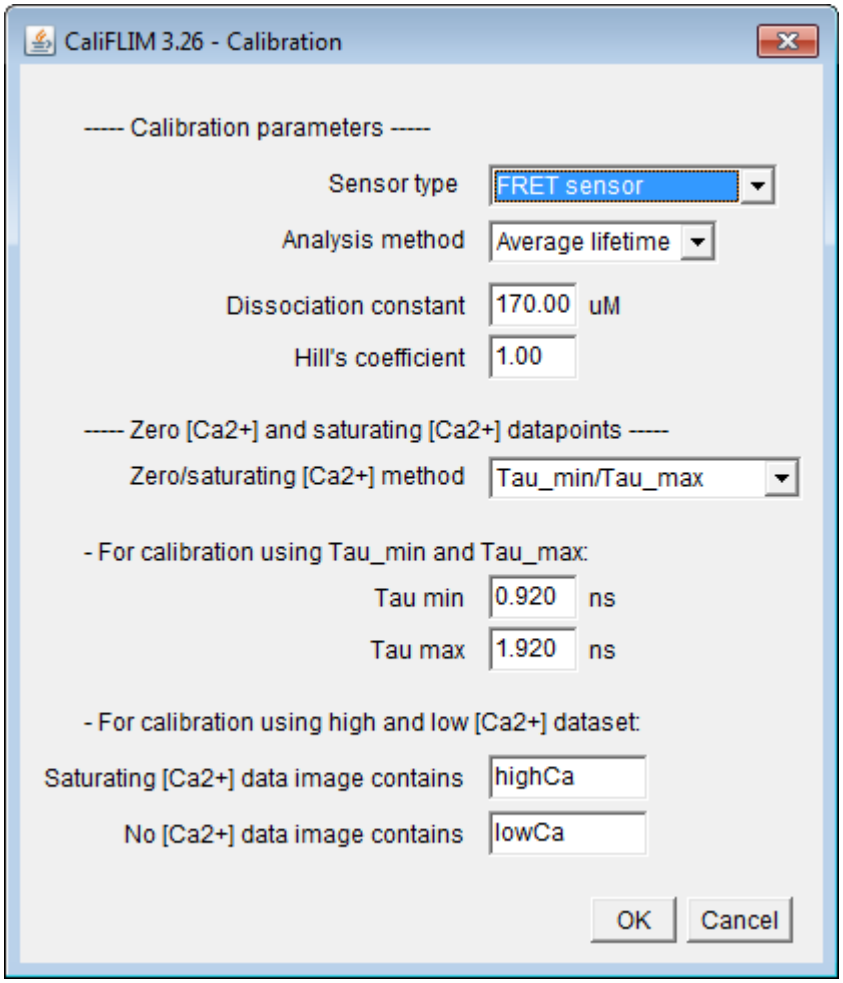
- If an IRF is available (scatterer or reference), it is possible to estimate the IRF shift (temporal shift between IRF measurement and decay). It can be estimated from a single dataset (for instance you can choose any of you typical dataset from the same day), or it can be user set with a fixed value. The default dataset name is the first dataset in the list in the folder.

- If a reference IRF is chosen, the user is additionally asked to set the reference lifetime

- Press OK

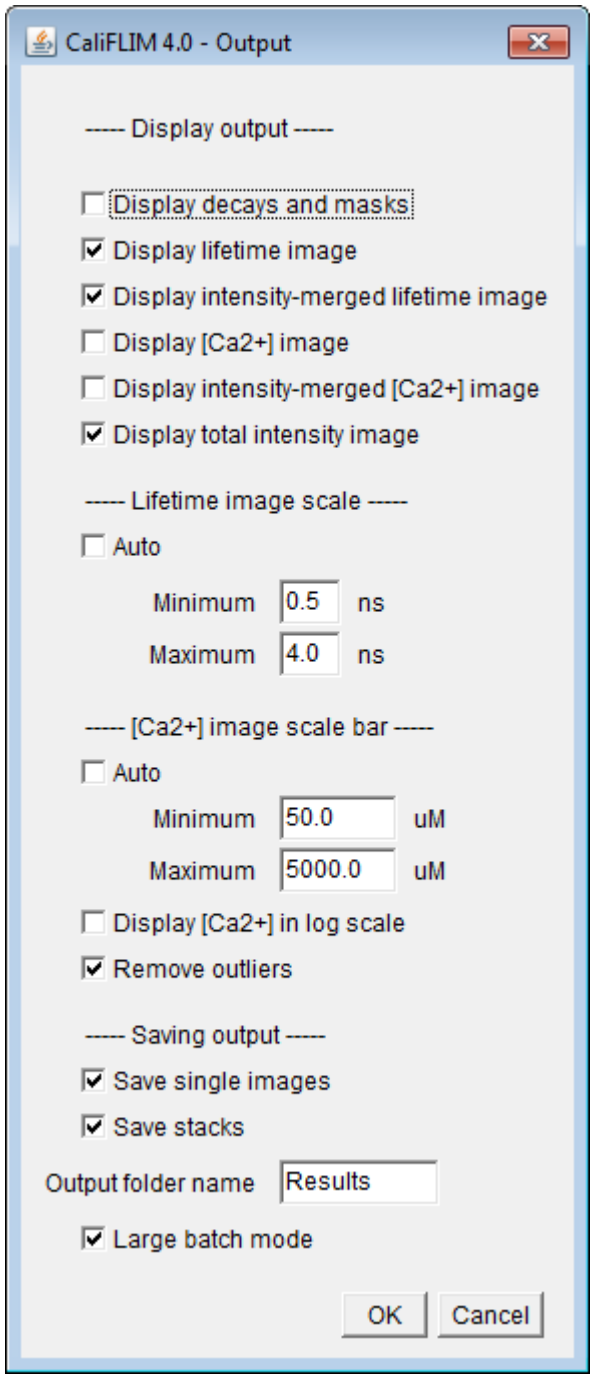


Calibration window



- Choose the sensor type (FRET sensor or Direct lifetime sensor)
- Choose the calibration method. (The “Fractions” method is more accurate but requires high quality data and background removal. Default: Average lifetime)
- Enter dissociation constant and Hill’s slope values for the sensor used
- Choose the input method for τ_{min} and τ_{max}. The “Tau_min/Tau_max” method uses the lifetime values given below. The “high/low [Ca] dataset” method accepts calculates τ_{min} and τ_{max} from the dataset described below (the IRF of those corresponding dataset must be given, but it can be the same as the one for the dataset).

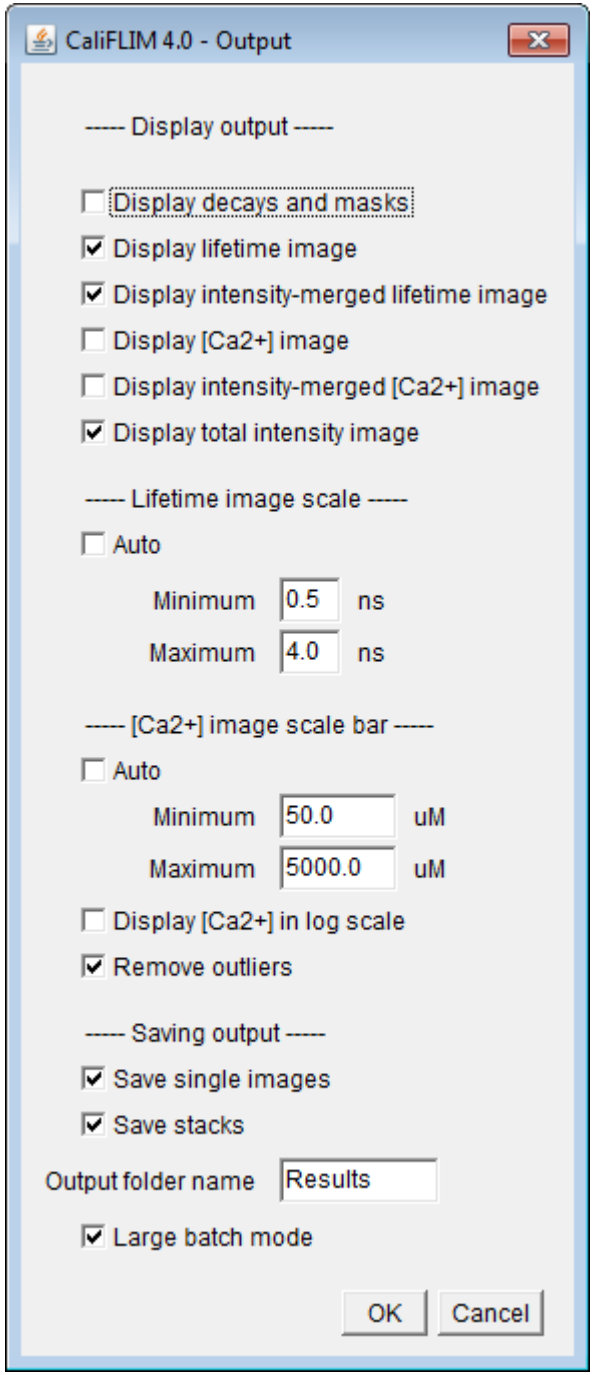
- Press OK



Output window (1/2)

- Choose which images will be displayed. The “Display decays and masks” will display the decays obtained from binning all the pixels above the threshold in the image, the mask used in the analysis (corresponding to the chosen threshold). The FLIM stack is also shown.

- Choose the images to auto-scale for each image by ticking “Auto” or enter the values for minimum and maximum for the false colour scale bars. The Calcium concentration can also be displayed in log scale
- Choose to remove outliers in the Calcium concentration image or not (noise on the lifetime image may cause the Calcium concentration to take very large values, removing outliers in the Calcium concentration image reduces the effect of this noise).



Output window (2/2)

- Choose whether the output images are saved and if so, what name folder should be used (the output folder is created in the same folder as where the dataset). The user can choose to save the results for each dataset as individual images or as a stack, or both.
- The “Large batch mode” is useful to analyse large dataset. The images are NOT displayed in this mode but the analyses can be done in the background without using lots of computer memory and can be processed faster. The output can be visualized in the saved images (if “Save displayed output” is selected)

- Press OK

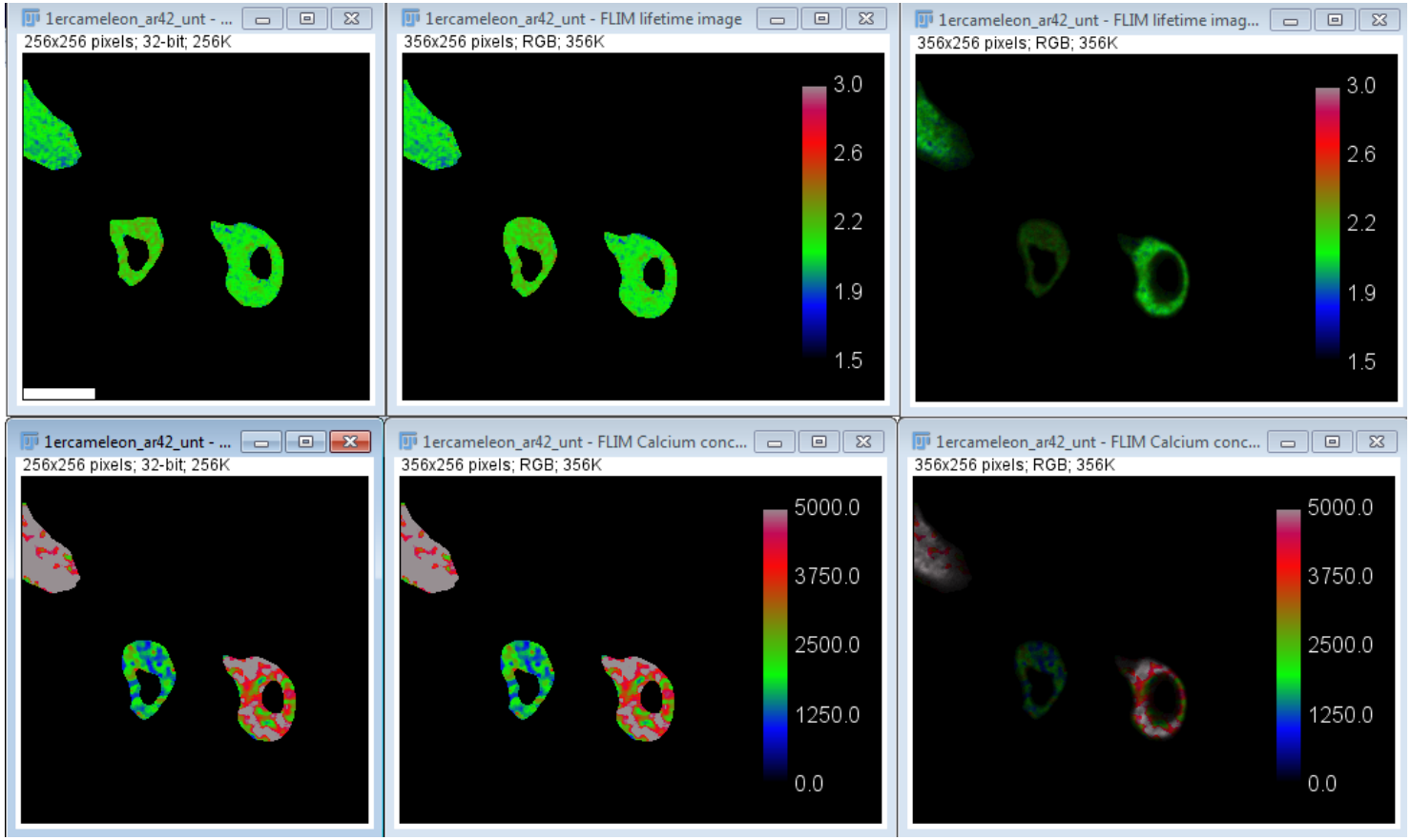
- After pressing OK, the data will be processed. All images selected as output will be shown (and saved if selected). The process may take up to ~2s per image depending on the specifications of the computer used for analysis.

Output images (1/2)

Raw image

Image

Merged image



Lifetime image

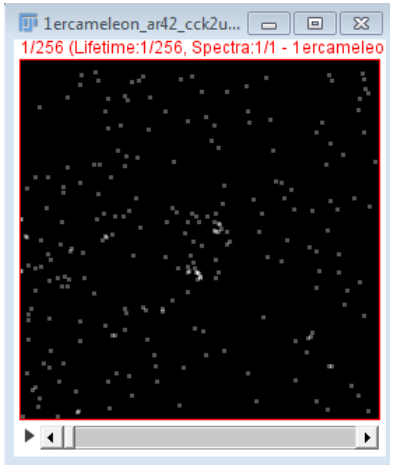
[Ca] image

Output images (2/2)

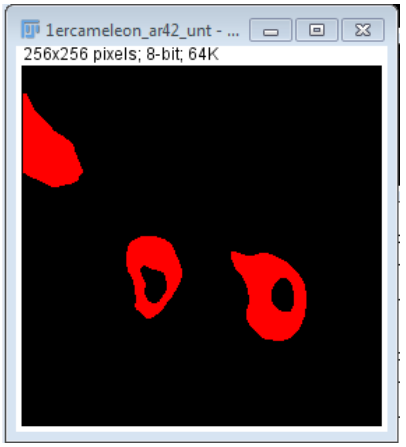
Shown when “Display decays and mask” is ticked.

Total intensity image

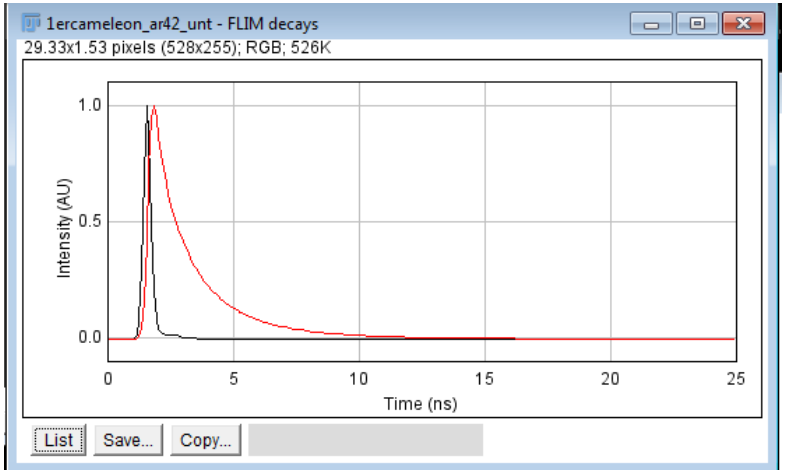
FLIM stack



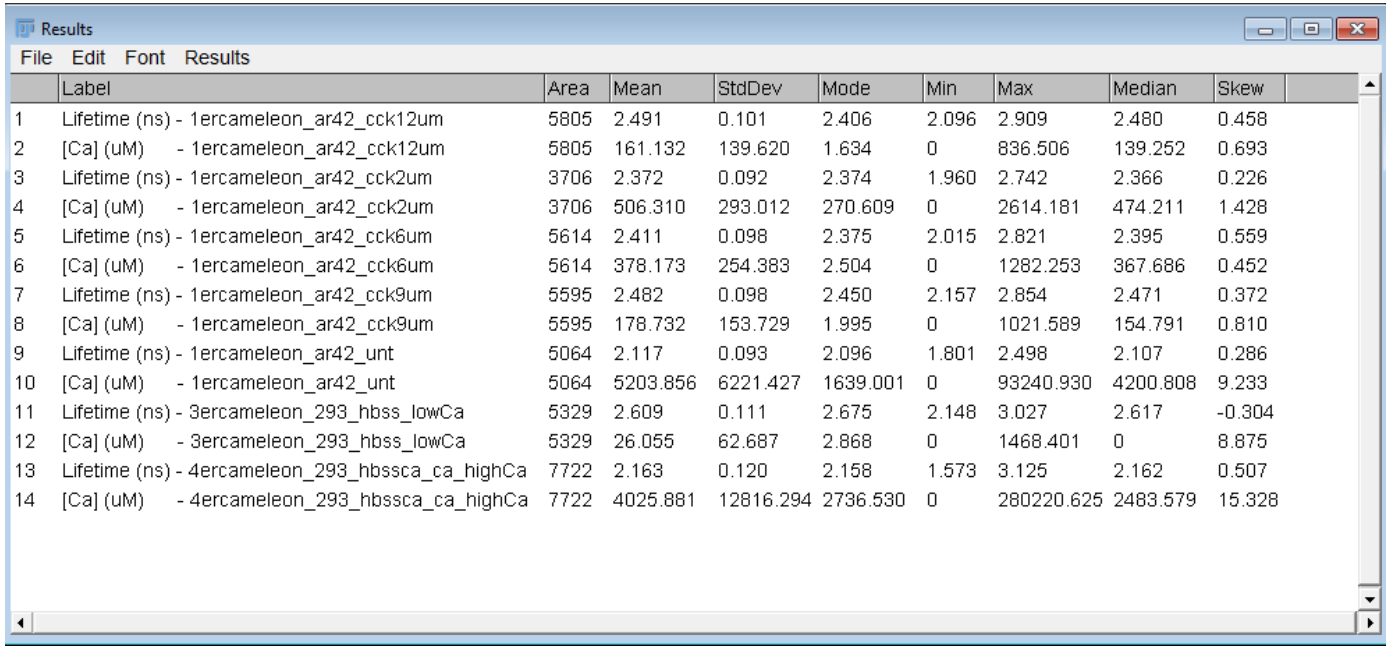
Mask



Fluorescence decay



Output window - Results



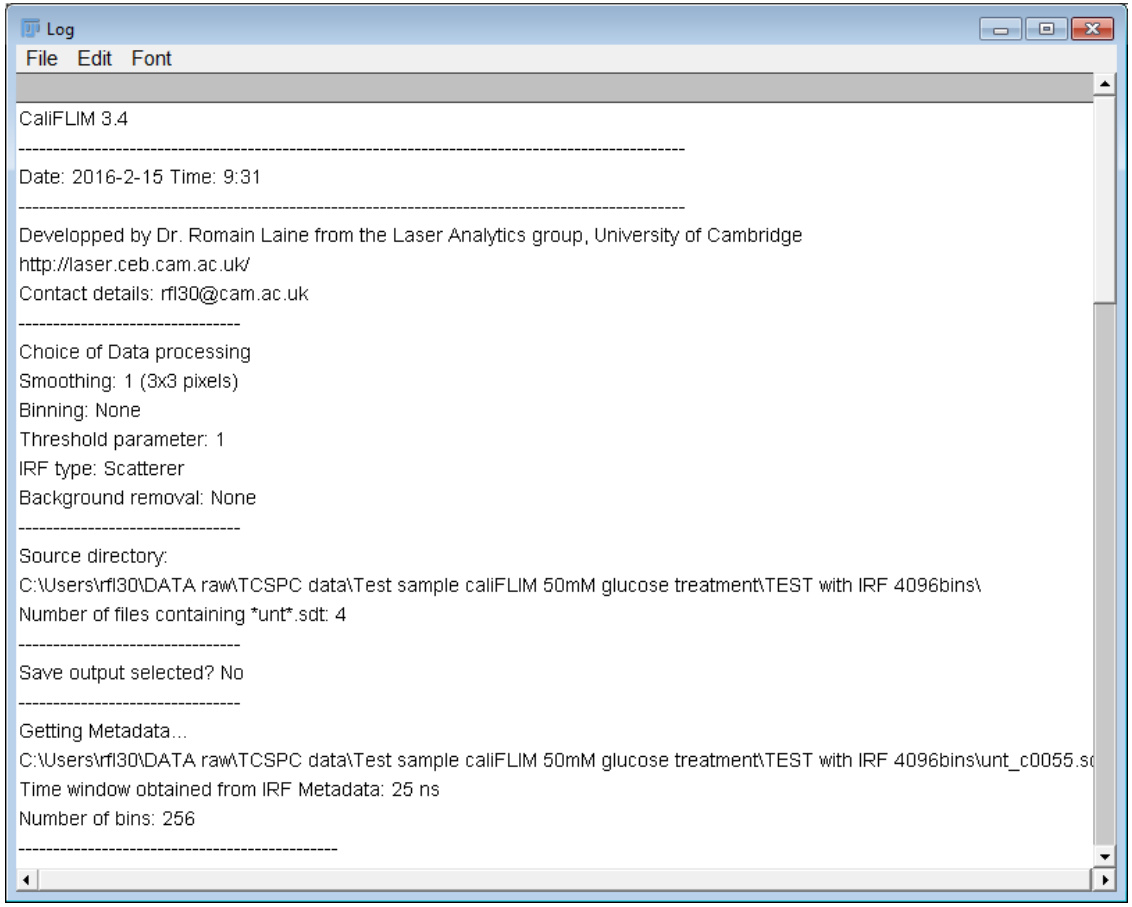
Results

File Edit Font Results

	Label	Area	Mean	StdDev	Mode	Min	Max	Median	Skew
1	Lifetime (ns) - 1ercameleon_ar42_cck12um	5805	2.491	0.101	2.406	2.096	2.909	2.480	0.458
2	[Ca] (uM) - 1ercameleon_ar42_cck12um	5805	161.132	139.620	1.634	0	836.506	139.252	0.693
3	Lifetime (ns) - 1ercameleon_ar42_cck2um	3706	2.372	0.092	2.374	1.960	2.742	2.366	0.226
4	[Ca] (uM) - 1ercameleon_ar42_cck2um	3706	506.310	293.012	270.609	0	2614.181	474.211	1.428
5	Lifetime (ns) - 1ercameleon_ar42_cck6um	5614	2.411	0.098	2.375	2.015	2.821	2.395	0.559
6	[Ca] (uM) - 1ercameleon_ar42_cck6um	5614	378.173	254.383	2.504	0	1282.253	367.686	0.452
7	Lifetime (ns) - 1ercameleon_ar42_cck9um	5595	2.482	0.098	2.450	2.157	2.854	2.471	0.372
8	[Ca] (uM) - 1ercameleon_ar42_cck9um	5595	178.732	153.729	1.995	0	1021.589	154.791	0.810
9	Lifetime (ns) - 1ercameleon_ar42_unt	5064	2.117	0.093	2.096	1.801	2.498	2.107	0.286
10	[Ca] (uM) - 1ercameleon_ar42_unt	5064	5203.856	6221.427	1639.001	0	93240.930	4200.808	9.233
11	Lifetime (ns) - 3ercameleon_293_hbss_lowCa	5329	2.609	0.111	2.675	2.148	3.027	2.617	-0.304
12	[Ca] (uM) - 3ercameleon_293_hbss_lowCa	5329	26.055	62.687	2.868	0	1468.401	0	8.875
13	Lifetime (ns) - 4ercameleon_293_hbssca_ca_highCa	7722	2.163	0.120	2.158	1.573	3.125	2.162	0.507
14	[Ca] (uM) - 4ercameleon_293_hbssca_ca_highCa	7722	4025.881	12816.294	2736.530	0	280220.625	2483.579	15.328

The “Results” window shows the measurements obtained from the raw lifetime and Calcium concentration images. In particular the mean and standard deviation of the values

Output window - Log

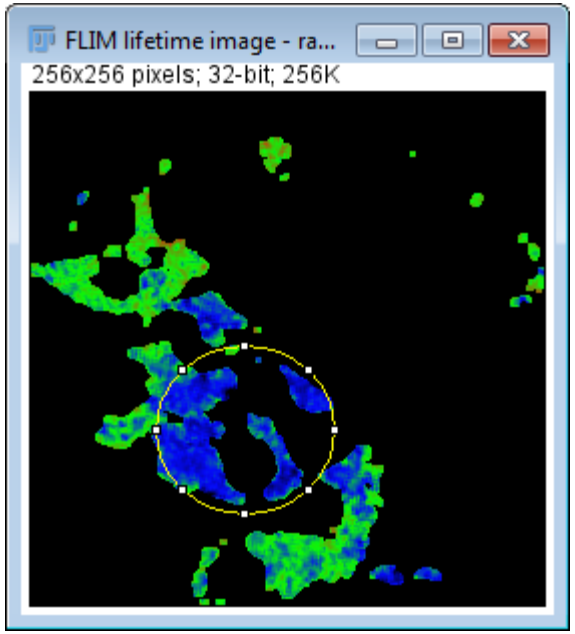


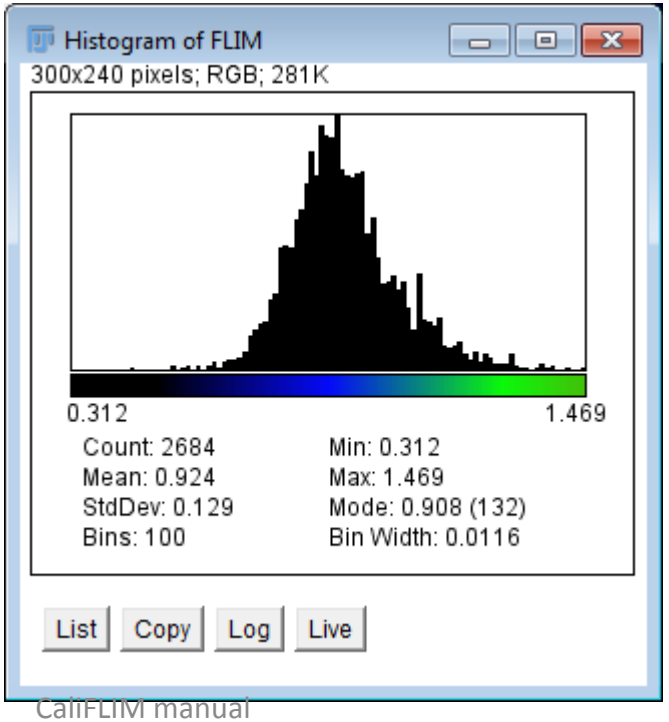
The "Log" window shows various information about the data processing, such as the chosen processing parameters (smoothing, etc.), the calibration parameters (K_d, h, etc.).
It is useful to check which lifetimes were used for the analysis (τ_{min} and τ_{max}), what dataset were used as IRF and calibration dataset (the path is indicated).
This window also shows the progress of the analysis:
See "Open file number: x/x".

The total time elapsed time to process all the datasets.

Further analysis

- Further analysis can be performed on the raw images, the raw lifetime images or raw concentration images.
- Here's an example about using the raw lifetime image to extract a histogram of lifetimes from a specific region of the image, defined by the ROI

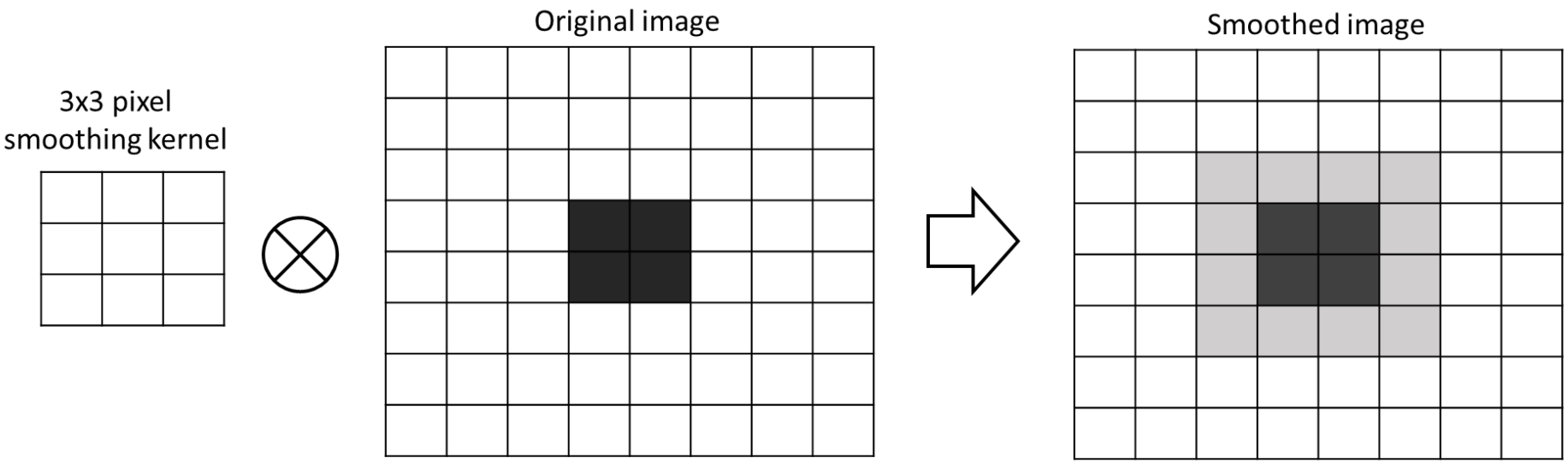




Smoothing

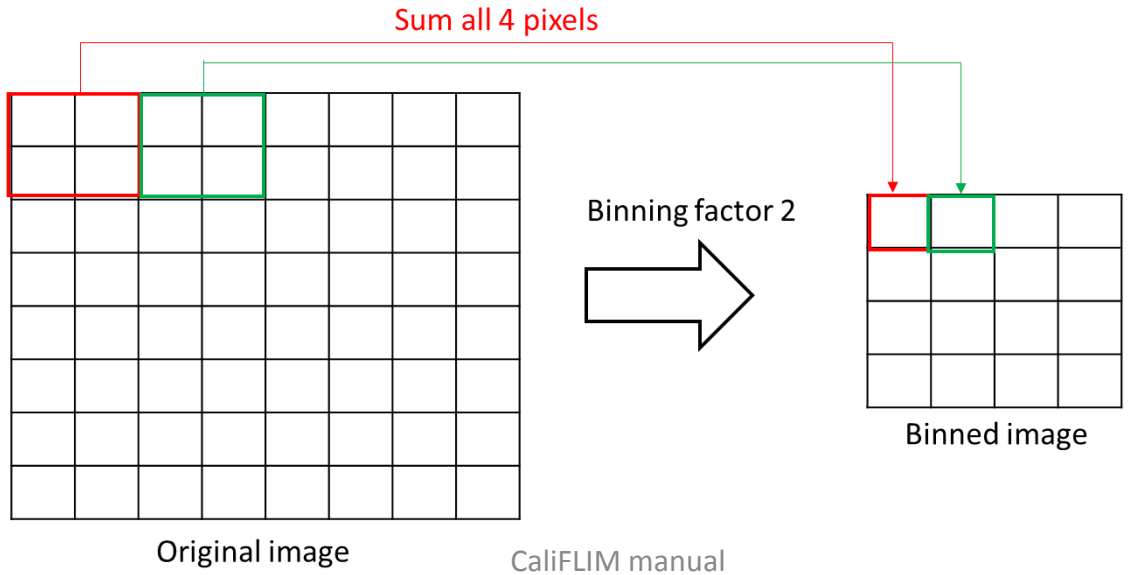
- Smoothing is applied by convolving each image of the FLIM stack with a square kernel of predefined size (3x3, 5x5, etc.).
- The spatial resolution of the lifetime image is reduced by the pixel-to-pixel noise is also reduced.
- A typical smoothing is 3x3. It retains good spatial resolution while significantly improving the signal to noise ratio.
- This method is identical to the “binning” method of SPCImage.

NB: The total intensity image is also affected by smoothing.



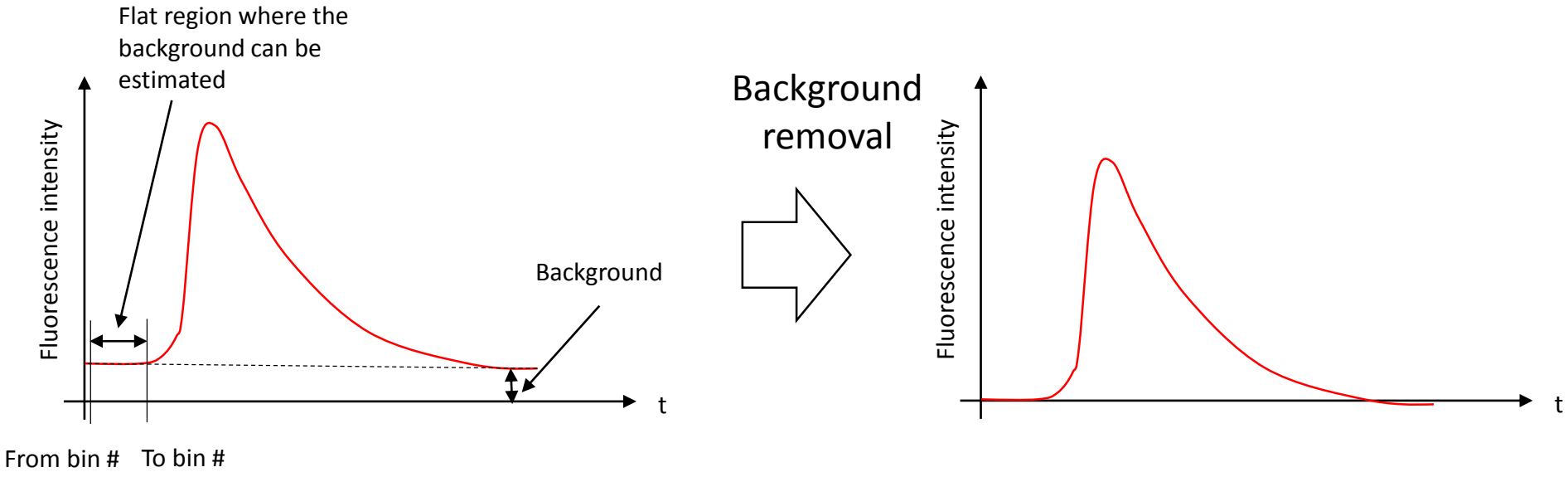
Binning

- Binning improves the signal to noise ratio by adding together adjacent pixels.
- A binning of 2 bins every group of 2x2 pixels into one single pixel. It therefore reduces the size of the image by a factor 2, but potentially increases the number of photons in each pixels by a factor up to 4x.



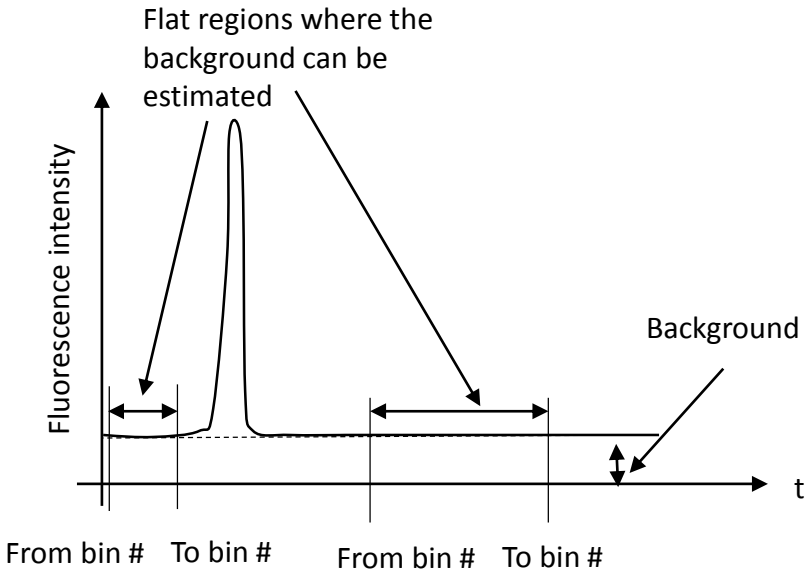
Decay background (1/3)

Some TCSPC data may be affected by the presence of a background (stray light or afterpulsing). In this case, it is important to remove the background from the decay for the results to be accurate.



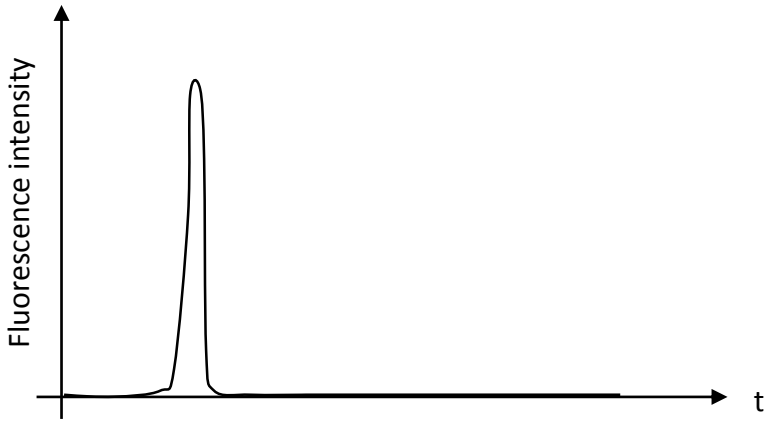
Decay background (2/3)

Similarly, the IRF decay may also be affected by the presence of background.



IRF decay

Background removal

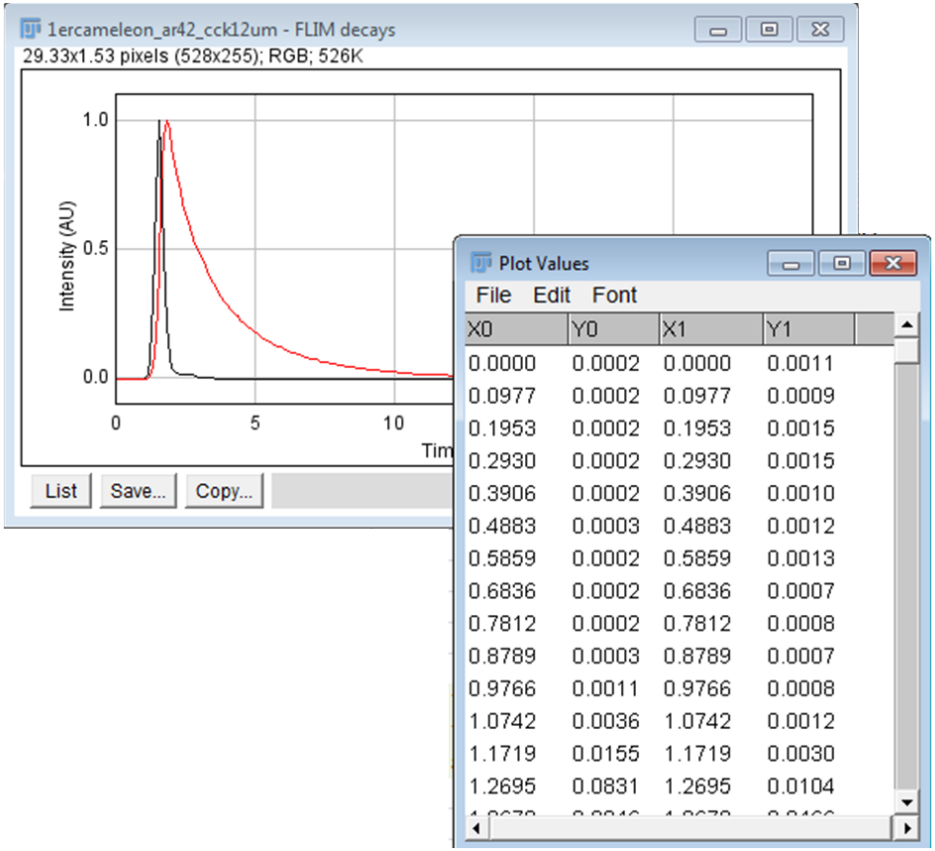


IRF decay

Decay background (3/3)

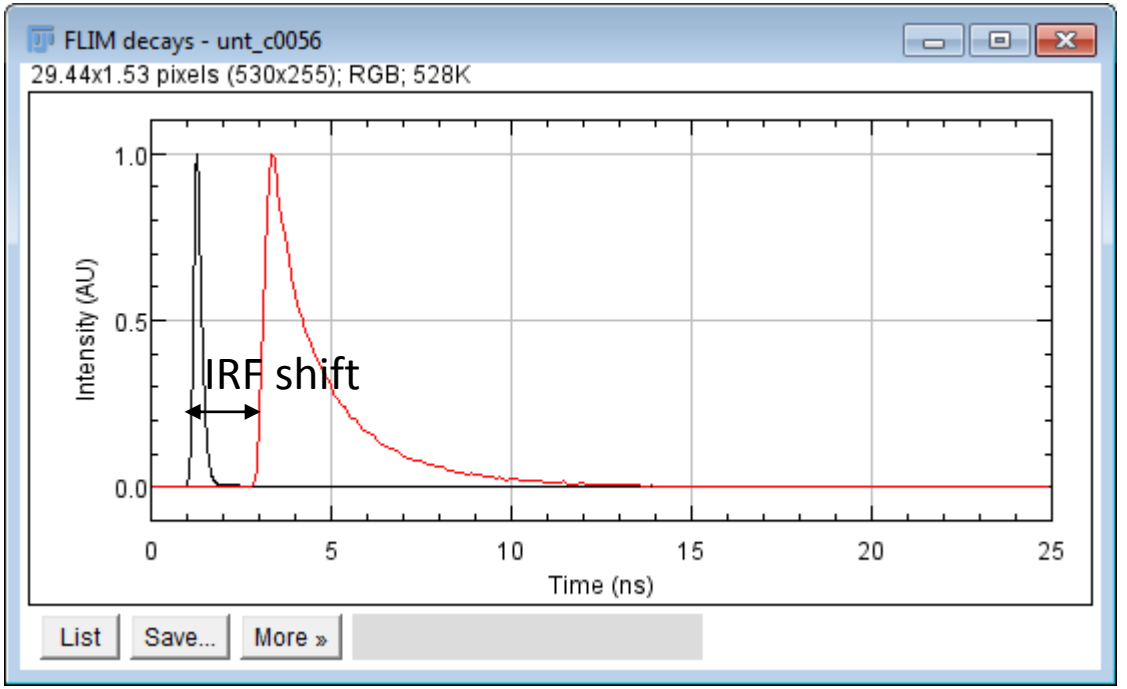
The fluorescence decays and IRF decays can be shown by ticking “Display decays and masks”.
The actual data can be seen, saved or copied by using the buttons “List”, “Save” or “Copy”.

Those data can then be visualised using data plotting software such as MS Excel, Origin etc.
The appropriate positions of the bins (From bin # / To bin #) to use for the background estimation.



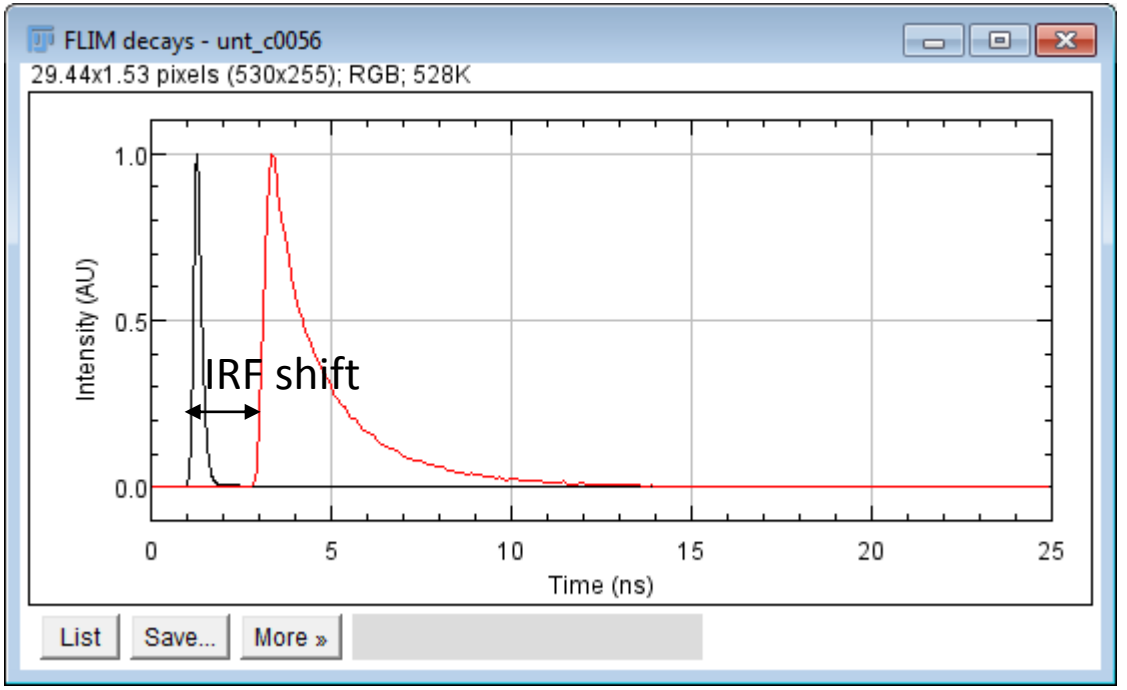
IRF shift (1/2)

- The IRF shift is the delay between the start of the IRF and that of the data decay as illustrated.
- It is important to correct for that when estimating the absolute lifetime or using the “Fraction” method of concentration estimation.
- It is due to the fact that the IRF may be measured in a different spectral channel as the data and in a different plane in the sample.



IRF shift (2/2)

- It can be defined by the user (“User set”) or estimated from a dataset.
- The dataset estimation is performed by computing the difference between the temporal position of the IRF and that of the decay at 20% of the maximum. Any dataset can be chosen for this.



Threshold parameter and mask (1/2)

The threshold used in CaliFLIM uses the Auto Threshold method on the Total intensity image to define a mask.

The method called Li's Minimum Cross Entropy (from Fiji). For more information about Li's Auto threshold: http://fiji.sc/Auto_Threshold#Li

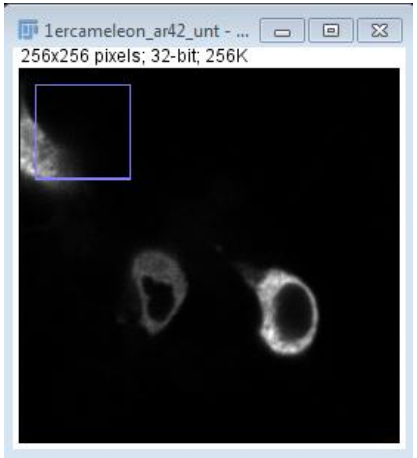
The threshold parameter used by CaliFLIM adjusts the threshold value found by the Li method by being multiplied to it.

For instance, if Li's Auto threshold method found an optimal threshold of 100 (photons), if the threshold parameter is set to 1, the image will be thresholded at 100.
A threshold parameter of 2 will use 200 as a threshold, and so on.

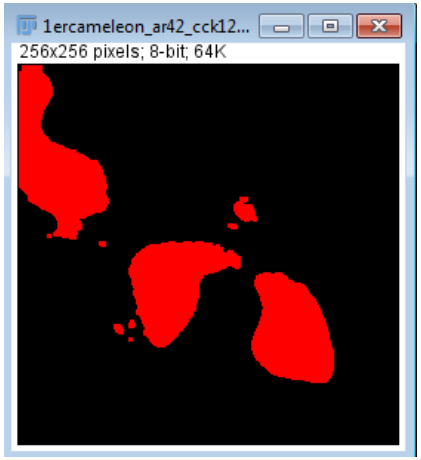
Therefore, keeping the threshold parameter at 1 uses directly the Li's Auto threshold. Increasing the threshold parameter will threshold more pixel (resulting in less pixels in the image), and decreasing the threshold parameter will result in more pixels kept in the image.

Threshold parameter and mask (2/2)

Total intensity image

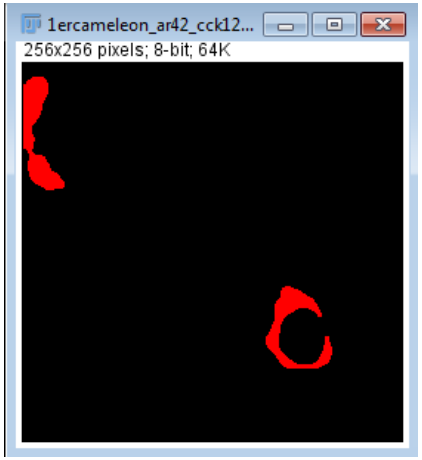


Threshold parameter = 0.2



Threshold parameter = 1

Threshold parameter = 5



IRF type (Scatterer vs. Reference)

- The IRF scaterrer is usually acquired by placing a reflecting surface in the sample plane and measuring some scattered light on the detector. This will describe the IRF well but is often acquired in a different spectral channel from the dataset. This often means that there is a temporal shift between the data and the IRF. For quantitative lifetime analysis, it is important to correct for that (use IRF shift correction).
- For reference lifetime IRF, a known sample can be used with single exponential lifetime and known fluorescence lifetime (this can be measured by fitting method for instance). This approach allows for the measurement to be performed in the exact same conditions from the dataset, theoretically with no shift (in this case the shift can be often ignored).

Calibration parameter method (Tau_min/Tau_max vs. high/low [Ca] dataset)

The calibration parameters τ_{min} and τ_{max} can be input in two ways:

- The values of τ_{min} and τ_{max} can be set as input directly by selecting "Tau_min/Tau_max". Those values can be typically obtained by alternative methods such as fitting methods. A better approach is to load them as dataset in CaliFLIM and obtain the lifetimes given by CaliFLIM, either the average over the whole image, or in Global Binning or by selecting an area within the image to determine the local average lifetime (within certain cellular compartments etc.), see "Further analysis" section for details on how to perform this.
- The two datasets acquired in high and low [Ca^{2+}] can be given as input for CaliFLIM to calculate τ_{min} and τ_{max}. If this option is selected, the datasets are loaded and thresholded identically to the dataset (see Threshold parameter and Mask for details) and the average dataset of the whole image is used as τ_{min} and τ_{max}.

The 2 analysis methods of CaliFLIM

Two methods are available:

- **Average lifetime**
- **Fractions**

- The Fractions method assumes is accurate under our assumptions. However it requires that τ_{min} and τ_{max} are known absolutely and therefore the IRF file needs to be of good quality for this method to work accurately.
- Generally speaking, the Fraction method requires a larger number of photons (Total photon > 1000 for each pixel)

Theoretical background – Average lifetime (1/3)

Hill's equation describes the fraction of bound molecules as a function of the free Calcium concentration.

$$p = \frac{[Ca^{2+}]^h}{[Ca^{2+}]^h + K_d}$$

Where *p* is the fraction of bound ligand, $[Ca^{2+}]$ is the concentration of free calcium, K_d is the apparent dissociation constant and *h* is the Hill coefficient

Theoretical background – Average lifetime (2/3)

In this method, we assumed that the average lifetime <τ> vary with the same trend as the fraction and therefore

$$\langle\tau\rangle \approx \tau_{max} - (\tau_{max} - \tau_{min}) \frac{[Ca^{2+}]^h}{[Ca^{2+}]^h + K_d}$$

And then

$$[Ca^{2+}] \approx \left(K_d \frac{\tau_{max} - \langle\tau\rangle}{\langle\tau\rangle - \tau_{min}} \right)^{\frac{1}{h}}$$

Theoretical background – Average lifetime (3/3)

Assuming that the dissociation constant and the Hill coefficient are known, the average lifetime <τ> and the limiting lifetimes (τ_{min} and τ_{max}) can be experimentally obtained.

$$\langle\tau\rangle = \frac{\sum F(t_i)t_i}{\sum F(t_i)} - \frac{\sum IRF(t_i)t_i}{\sum IRF(t_i)}$$

Assuming that the measured fluorescence decay F(t) is given by

$$F(t) = IRF(t) \otimes I_0 e^{-\frac{t}{\langle\tau\rangle}}$$

Theoretical background – Fractions (1/3)

This methods takes into account the two-component system of FRETing and non-FRETing donors

$$F(t) = IRF(t) \otimes I_0 \left(p_1 e^{-\frac{t}{\tau_1}} + p_2 e^{-\frac{t}{\tau_2}} \right)$$

Which leads to

$$\langle \tau \rangle = \frac{p_1 \tau_1^2 + p_2 \tau_2^2}{p_1 \tau_1 + p_2 \tau_2}$$

With $p_2 = 1 - p_1$

Theoretical background – Fractions (2/3)

Therefore p_1 can be obtained using

$$p_1 = \frac{\tau_2(\tau_2 - \langle\tau\rangle)}{(\tau_2 - \tau_1)(\tau_2 + \tau_1 - \langle\tau\rangle)}$$

However

$$p = \frac{[Ca^{2+}]^h}{[Ca^{2+}]^h + K_d}$$

And therefore

$$[Ca^{2+}] = \left(K_d \frac{p}{1-p}\right)^{\frac{1}{h}}$$

Theoretical background – Fractions (3/3)

And finally

$$[Ca^{2+}] = \left(K_d \frac{\tau_{max}}{\tau_{min}} \frac{(\tau_{max} - \langle\tau\rangle)}{(\langle\tau\rangle - \tau_{min})} \right)^{\frac{1}{h}}$$

With <τ> given by (same as in Method 1)

$$\langle\tau\rangle = \frac{\sum F(t_i)t_i}{\sum F(t_i)} - \frac{\sum IRF(t_i)t_i}{\sum IRF(t_i)}$$