From Eye to Insight

FALCON FLIM

Manual

20200110_EN

FLIM

Tips

＊Check box for Gating function is not available for FLIM, but the values apply to the FLIM.

＊PMT is not available for FLIM

＊Notch filter supported Pulse laser is required

(Notch filter not-supported pulsed laser could available for enough bright sample)

＊LAS X FLIM/FCS can be opened in the following LAS X wizard using the FLIM button and is available for FLIM experiment there:

- FCS: In the Setup Imaging operating step
- FRET AB, FRET SE: in the Setup operating step in the Workflow or Acquisition tab
- Live Data Mode: In the Acquire operating step
- Electrophysiology: In the Acquire operating step

＊Click the 

「LAS_X_SingleMoleculeDetection.exe shortcut」 open the An evaluation in LAS X FLIM/FCS. LAS X Small is not available for the analysis

＊STED 77 nm and WLL pulse timing are synchronized.

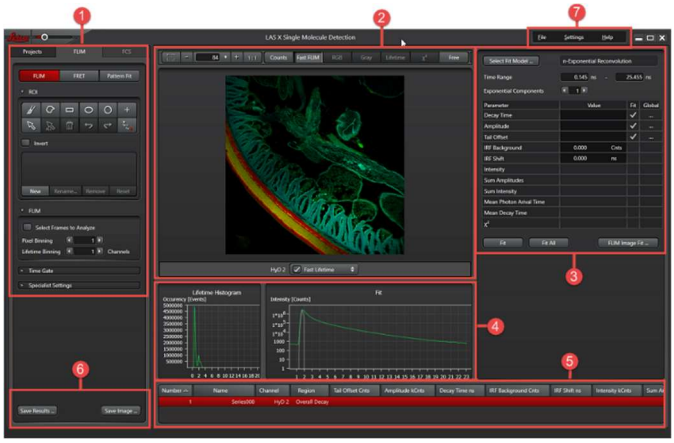
WLL pulse picker is not available only when STED 775 nm is ON

STED 775 nm is only available when WLL is 80 MHz

FLIM Calling up

Click the **FLIM** button.　The LAS X FLIM/FCS opens on the second monitor.

In second monitor, the FLIM setting dialog field is displayed.

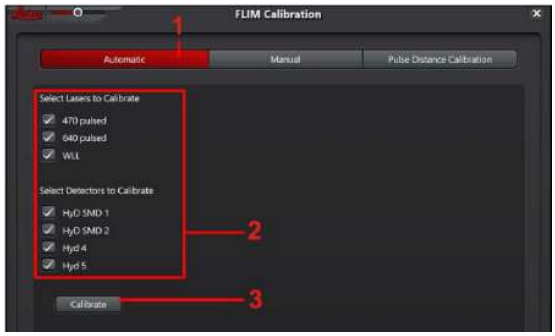


Calibration of laser and detectors

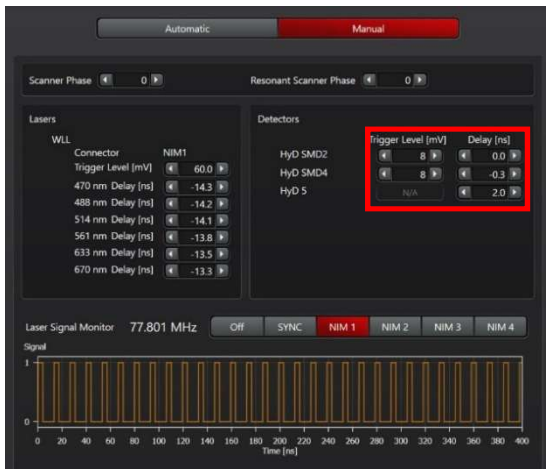
Click the ⑦ Setting 

open the FLIM Calibration dialog

＊WLL：Automatic/Calibrate

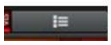


*Trigger level is available for background level adjustment (Only SMD-HyD)



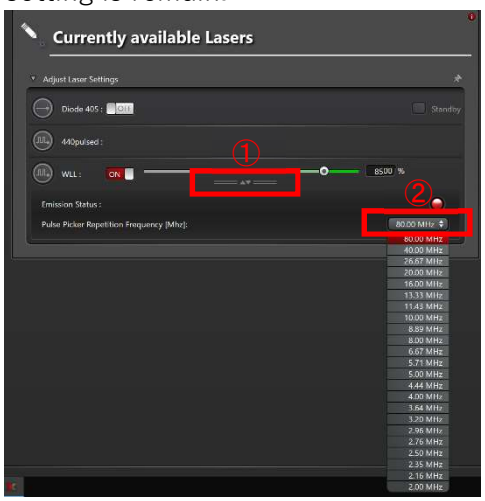
*Sequential setting is disappear after calibration

＊WLL with pulse picker, you can define the repetition rate the dialog.

The dialog open from the button  in Acquire menu. Click the red frame, open the pulldown menu. Then, you can choose the frequency 80, 40, 20 and 10 MHz.

The last used setting frequency is remain

＊The dialog open in Hardware Configurator/Laser Config. Click the ① and ② red button, open the pulldown menu. Then you can choose the frequency. The last used setting is remain.



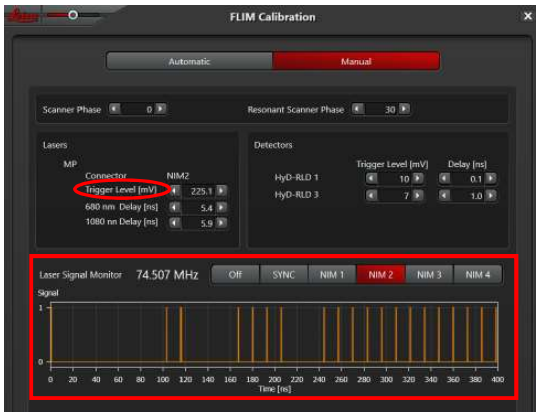
＊Check the Ref. Line [nm] in red frame is same as Ex laser

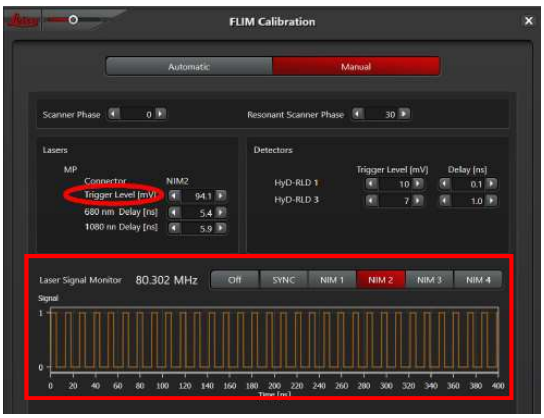
＊STED 77 nm and WLL pulse timing are synchronized.

WLL pulse picker is not available only when STED 775 nm is ON

STED 775 nm is only available when WLL is 80 MHz

＊MP：Manual/NIM2-4（The number depend on the system）Adjust the pulse width with Trigger level





F LIM Settings (Main monitor)

Filter Supported WLL-Lines：

All WLL lines supported by a filter system are represented by a button and are enabled when you click the button.

Line Repetitions	Define the number of scans per line when acquiring a FLIM image. The resulting acquisition time for the FLIM image is automatically displayed under Duration of each image. Line accumulation for intensity image.
Frame Duration	Define the duration for acquisition of a single FLIM image
Frame Repetitions	Define the number of scans per frame when acquiring a FLIM image. Frame average for intensity image
Acquire until max … photons/pixel	Until a certain number of photons have been detected in the brightest pixel within a FLIM display window Frame average for intensity image
Sum of all Channels	All channels
Brightest Channel	Brightest Channel
Dimmest Channel	Darkest Channel

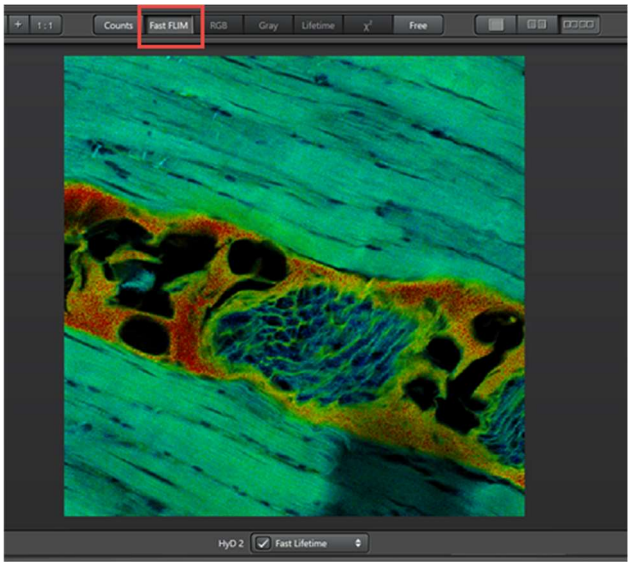
Specialist Settings : To increase the speed of image acquisition, detector channels for data acquisition can be combined manually into FLIM display channels.

If this function is activated, new dialog field (in the Fig. left), Manual FLIM Channel Assignment, opens.

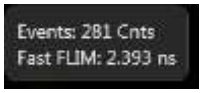
Click the first drop down menu. The following dialog (in the Fig. left) opens for assigning the channels.

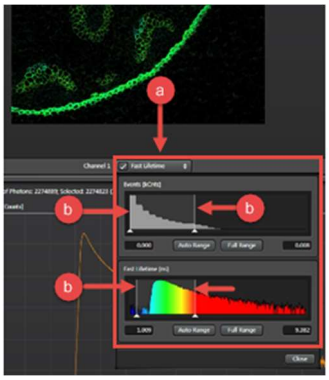
Single Detector	Assignment of exactly one detection channel. All activated FLIM detectors are listed in this area. By clicking on an entry, you can precisely assign one detection channel to selected display channel.
Multiple Detector	Assignment of several detection channels. To increase the image-capturing speed, you can assign multiple detection channels to the selected display channel in this field. Each time + is clicked, another drop down menu is added. Then, select each desired detection channel from the drop down menus. Clicking X removes the individual drop down menu again.
All Detectors	Assignment of all active detection channels. Clicking the button assigns all active detection channels to display channel.

A Fast FLIM image is displayed in the sub-monitor for each detection channel. For each pixel, an averaging of all fluorescence lifetimes and fluorescence intensities that occur in the specimen is displayed in ta false color image. The contrast for lifetime and intensity is set automatically, but can be adjusted.



Right click on the image, and choose Show Data Cursor.
Then, shown this dialog.





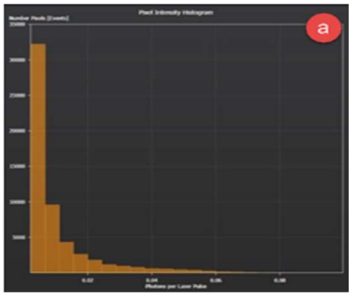
If necessary, change the contrast for lifetime and intensity. To open the dialog for adjusting the contrast, click the control element next to the channel designation below the FLIM image display window. You can change the contrast by using the mouse to drag the white margin lines to desired position.

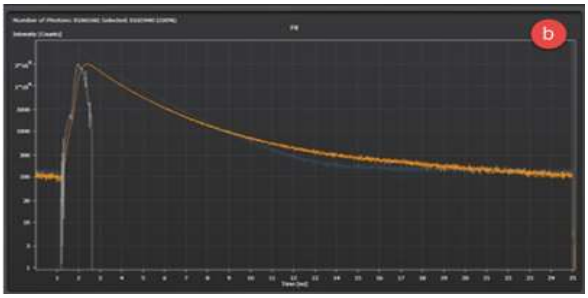
Underneath the image view, ⓐ : Pixel Intensity Histogram, the pile-up control histogram, ⓑ : Lifetime Decay Curve are displayed live

Pixel Intensity Histogram : Display the Events over the photon/Laser pulse

Use the pixel intensity histogram to adjust the laser intensity live, so that a maximum of 0.5 photons/laser for HyD and 1.0 photons/laser for SMD-HyD are measured.

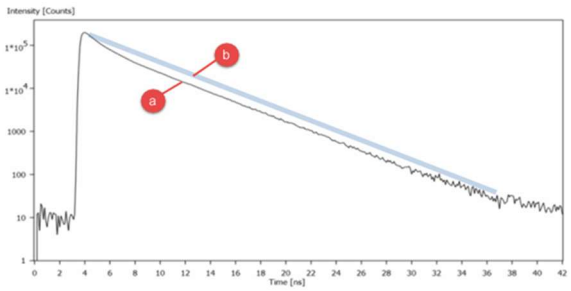
The color depends on the false color





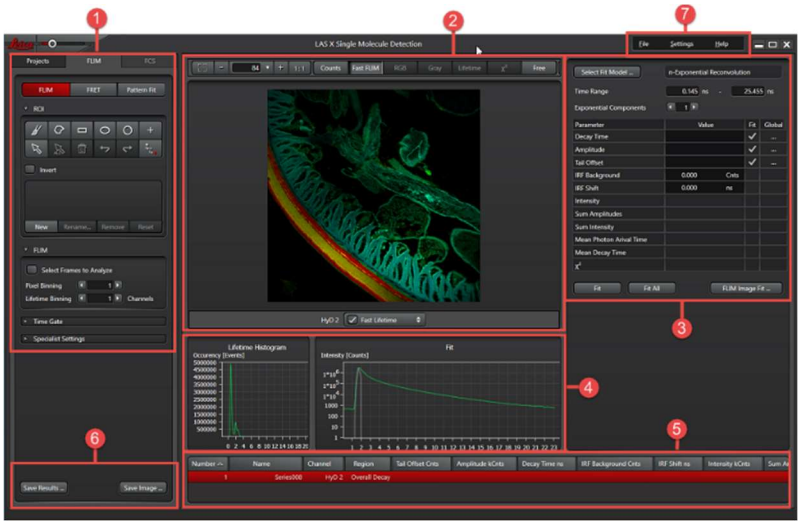
＊A pulse pile-up is the name of for the case when emitted photons are not taken into account for a high count rate due to the dead time of the detector during the counting.
This has the following effects, which ditort the result of the FLIM measurement
- The measurement lifetime becomes shorter
- A mono-exponential fading on the lifetime (1 component) appear to be double exponential with a second component a shorter lifetime.

ⓐ : Curve progression with pulse pile-up effect
ⓑ : Ideal curve progression without pulse pile-up effect



＊Required photon number for Fitting
1 component: 100,　2 components: 1000,　3 components:10000

FLIM



8-① Setting for analysis

8-② Display window for FLIM image. During FLIM test and FLIM measurements, a fast FLIM image is already being displayed live. Below the display window, there are controls for adjusting the contrast of the individual channels.

8-③ Parameter setting are displayed for the fit on the right edge of the screen

8-④ In the diagram area, histogram is displayed in left and the lifetime decay curve is displayed on the right

8-⑤ A navigation table is displayed below the diagram in which is a row entry for each image, each ROI and each FLIM display channel.

8-⑥ In this dialog, you can save the FLIM image

8-⑦ In this dialog, you can export FLIM data

Adjust Photon/Pulse

18-①

Using ROIs you can limit the region of interest of the fast FLIM image.　For this purposes, various tools are available in this dialog

From the upper left

①Brush

②Polygon

③Rectangle

④Ellipse

⑤Circle

⑥Individual pixel

⑦Selecting a ROI

⑧Select all ROIs

⑨Delete selected ROI(s)

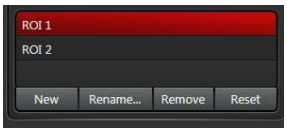
⑩Undo last operation

⑪Restore last operation

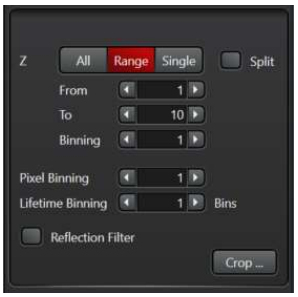
⑫Automatically form a ROI

Invert：Reverse the color display in the FLIM image and shows the image in inverted colors.

Additional function for handling ROIs



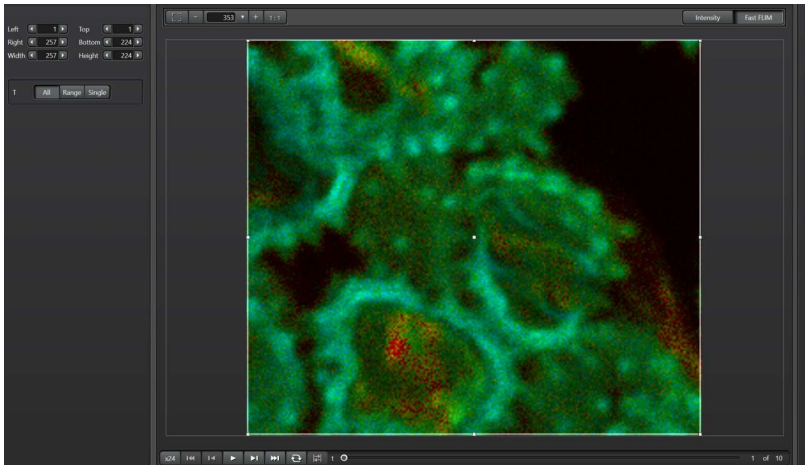
New	Creates an entry for ROI.　Afterwards, you can draw the ROI.
Rename	Enables you to enter a designation for the ROI entry
Remove	Deletes selected ROIs
Reset	Deletes all ROIs

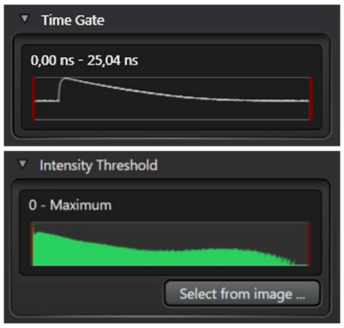


From、To	Define a range for the images to be analyzed from the repetitions of the scan. For this purpose, the From and To input fields are available.
Pixel Binning	Set the number of pixels compiled during image analysis.
Z/T	The dimension of the current image series is displayed
All	All single images are analyzed
Range	You can define a range for the single images of the series to be analyzed. For this purpose, the From and To input field are available.
Single	You can select a single image under Index to be used for analysis
Split	Only for Z or T. Each single image od series is represented separately with a row in the results table and a curve in the lifetime decay or phasor and can be analyzed indivually
Lifetime Binning	Set the number of time frames are compiled in the lifetime decay curve. This setting only affected the display of the curve (it became smoother). It is not included in the analysis.
Reflection Filter	If this function is enabled, the values generated by reflections when WLL are cut from the lifetime curve.

Crop

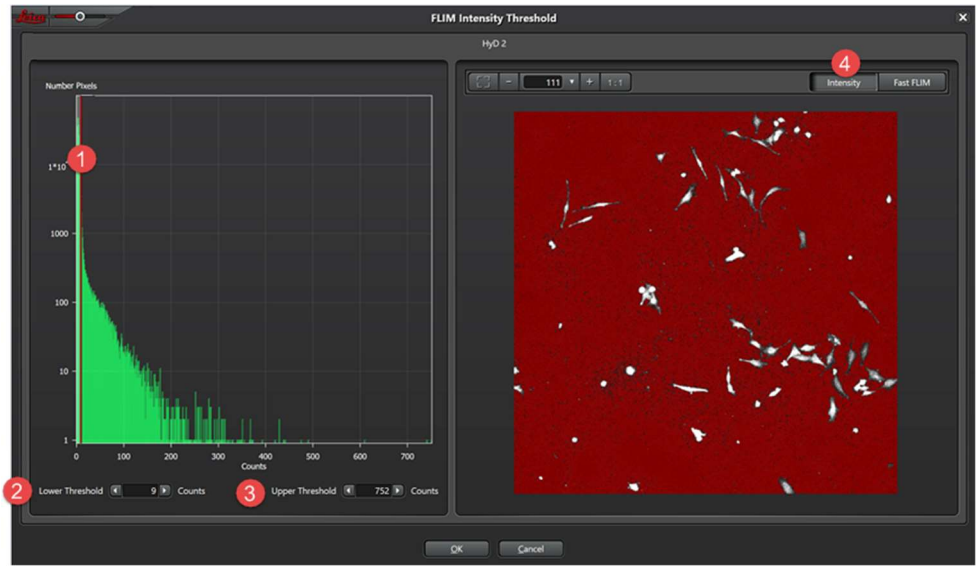
It is possible to crop lifetime series in time and space and to bin in time as in Z.





In this dialog, you can define a time gate, starting the time of the excitation pulse, in which the analysis of your FLIM image data is carried out. The time gate is applied to each frame.

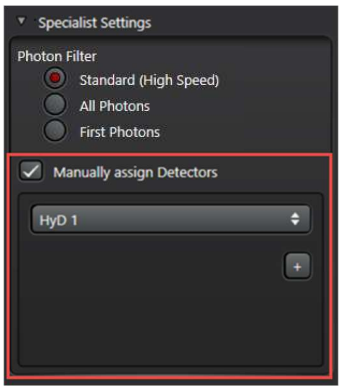
In this dialog, you can define an intensity range for which the analysis of your FLIM image data is carry out. The intensity range is applied to all further analyses and treated as an ROI. By moving the red lines with the mouse



①By moving the red line or entering the ②lower and ③upper value. At the same time, in the right area of the display window you can see which areas have been selected in the image.

④Here, you can define the threshold value in the left window are by moving red line① or entering the lower② and upper values③. At the same time, in the right area of the display window you can see which areas have been selected in the image.

Intensity	All hidden areas not included in the evaluation are displayed red
FastFLIM	All hidden areas not included in the evaluation are displayed gray

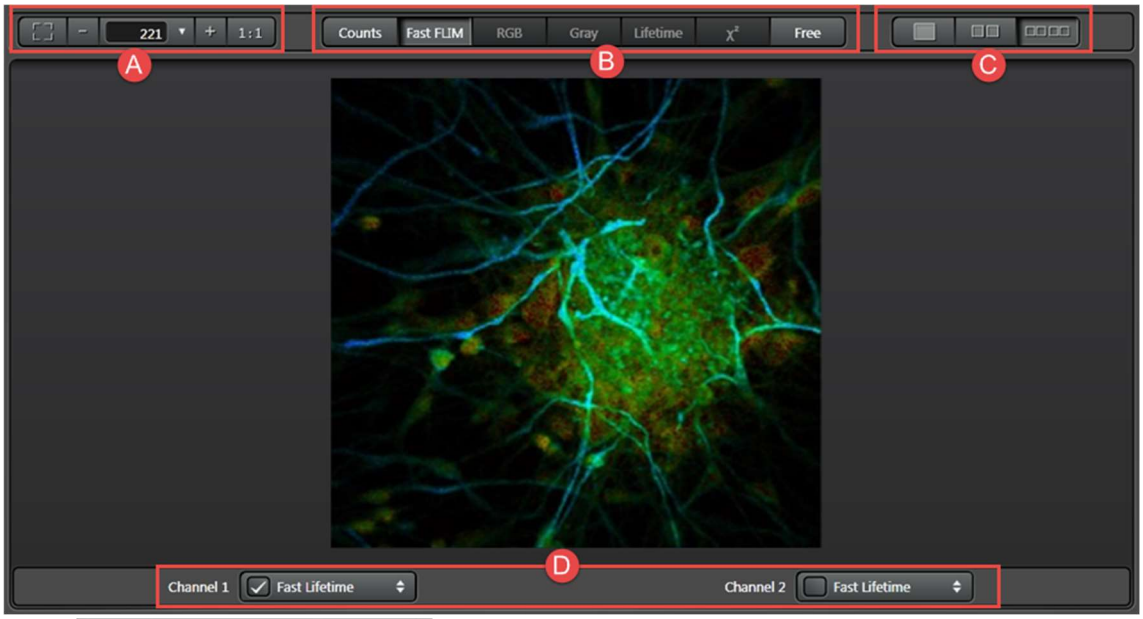


Narrow down the time gate by dragging the red lines with the mouse.

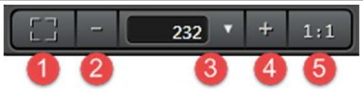
The setting is already applied directly to the image display window (Fast FLIM and Counts) as well as the decay curves. The raw data is unchanged by this setting.

Standard (High Speed)	For the lifetime decay curve time intervals between two laser pulses are evaluated only if exactly one photon has been emitted in them. For the FLIM image fit, the dead time correction is applied. HyD deadtime is approximately 1.5 nsec.
All Photons	All emitted photons are detected (Raw data)
First Photon	Occasionally, 2 or more photons are emitted after a laser pulse. In each case, however, only the first emitted photon is counted. This ultimately leads to a falsification of the results known as the pile-up effect.
Manual assign Detectors	To increase the speed of image acquisition, detector channels for data acquisition can be combined manually into FLIM display channels.

8-②



Ⓐ



1：Reset the zoom for the display window

2：Reduce the zoom for the display window

3：Enter the zoom factor for the display window (%)

4：Increase the zoom for the display window

5：Sets the zoom for the display window to the 100%

Ⓑ

During FLIM test and the FLIM measurement, you can choose the following views

Intensity	For each pixel, show the number of the counted photons in gray-scale
Fast FLIM	For each pixel, show an average of the all fluorescence lifetimes and intensities in false color
χ^2	For each pixel, show the intensity of the error or the fit precision
Free	You can freely select the parameters for intensity and color using a dropdown menu in the contrast setting

After the FLIM Image Fit（Ref. page 27）, display options that shown in next page are available.

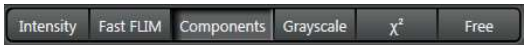
Display option for FLIM

Components	For each channel, the special distribution of the intensities is displayed in a separate RGB color
Grayscale	Same as RGB, but the intensities are represented as grayscale
Lifetime	The special distribution of the lifetime for each component is displayed in false colors (Rainbow colors) and the amplitudes are displayed as an intensity.

Display option for FRET

Efficiency	The average Apparent FRET efficiency is shown in image form as a false color
FRET	The average FLIM FRET efficiency is shown in image form as a false color
Binding	Proportion of the bound donor molecules to the total number of donor molecules in % is displayed using a false color image
Distance	The distance of the donor and acceptor are displayed using a false color image

Display option for Pattern Fit



Components	For each channel, the special distribution of the intensities is displayed in a separate RGB color
Grayscale	Same as RGB, but the intensities are represented as grayscale

©：Display Options for the Channels

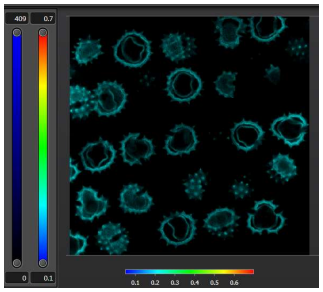
①All channels are displayed superimposed in one image

②All detection channels are displayed side-by-side

③All lifetime channels and detection channel are displayed side-by-side

④Displays bars for intensity range and lifetime range for manual setting in left of window

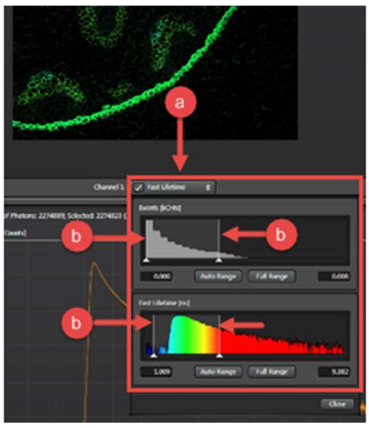
⑤Show a lifetime scale below the display window



Ⓓ : Contrast Setting

If you disable the checkbox on the button, no image is shown for the corresponding channel.

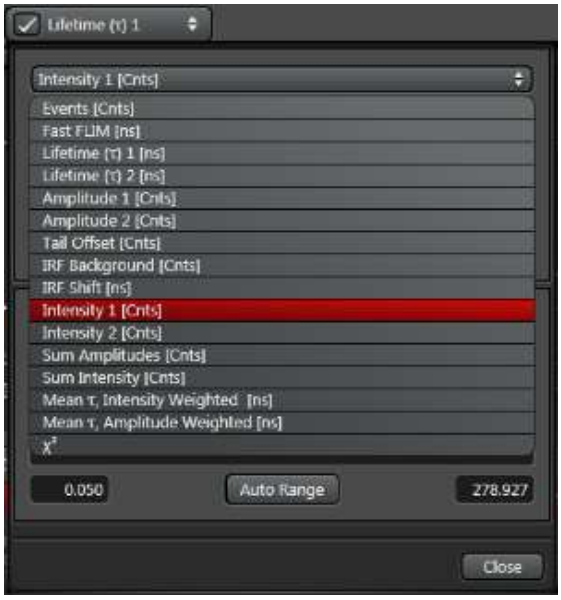
Clicking the button opens a dialog window that you can use to set the contrast for the current image display.



Auto Range	Limits the range automatically to optimal imaging
Full Range	Uses the entire are for displaying the imaging

Narrow down the lifetime scale by dragging the △ by mouse

Ⓑ Clicking the button opens a dialog window (in the below Fig.) that you can choose parameters.



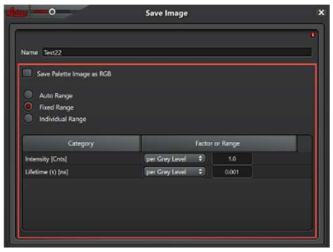
8-⑥This dialog allows you to save your analysis results or FLIM images

Save Results…	The current status of all analysis is stored, in the project directory. The data can be called up again in LAS X FLIM/FCS and further analyzed.
Save Image…	The colored Fast FLIM or FLIM image is created and stored as a normal intensity image in the .lif format as a 3 channel image (RGB) in the project directory and can be further processed as such. An evaluation in LAS X FLIM/FCS is no longer possible with this format.

You can enter a designation under Name

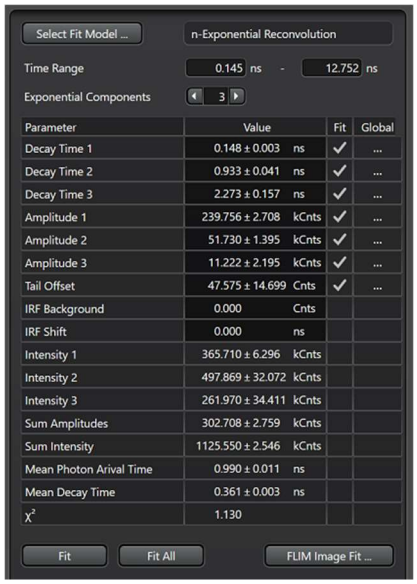
Save Pallet Image as RGB

If this function is enabled, the colored Fast-FLIM or FLIM image is created and stored as a normal intensity image in the .lif format as a 3-channel image (RGB) in the project directory and can be further processed as such.



Auto Range		Scales the image automatically between minimum and maximum so that the image is displayed properly, but no values are quantified.
Fixed Range		You can select the scaling range for each display Category separately. The values are quantifiable.
	Per Grey Level	Scaling by gray-scale value. Here, under Factor or Range, enter a factor with which the corresponding grayscale value is weighted. The values are quantifiable.
	Range	Scaling by intensity. Here, select a range under Factor or Range by entering values for minimum and maximum. The values are quantifiable.
Individual Range		Here, you can define the scaling ranges for each component individually, just like you can for Fixed Range. The values are quantifiable.

8-③ Fit Settings: FLIM



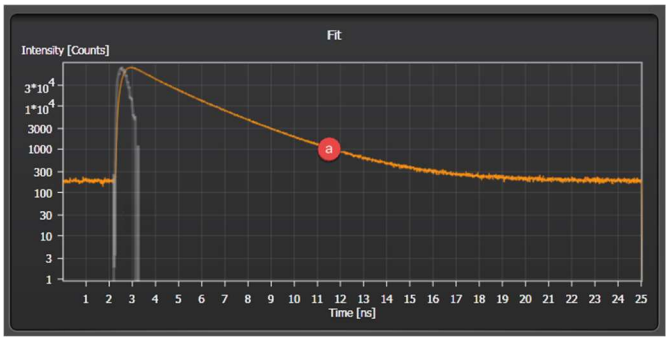
Fit	18-⑤Clicking this button calculates the curve fitting with the setting listed above for all curves a selected in navigation table
Fit All	18-⑤Clicking this button calculates the curve fitting with the settings listed for all curves of the selected detected channel
FLIM Image Fit…	Open the FLIM image Fit dialog, in which you can configure setting for the pixel-to-pixel curve fitting of the curves selected in the navigation table and start the pixel-to-pixel curve fitting.

Exponential Components	The number of fluorescent components (To determine it, Ref. Next page)
Select Fit Model…	For FLIM experiments, 2 models are available for calculating the curve fitting
n-Exponential Reconvolution with IRF	The decay curve is fitted taking into the IRF. This also enable using the beginning of the decay curve for analysis. This improves the statistics of the data and thus enable a correct estimate of relative amplitudes from the sum of the exponential decay curves.
n-Exponential Tail Fit	Only the rear area that is not affected by the IRF is used for the analysis. This attains correct for the lifetimes as the lifetimes are significantly greater than the IRF width. The disadvantage of this model is that no good curve fitting can be calculated for components with short curve fitting. ＊It is not available for the sample that contain lifetime shorter than IRF (e.g. 500 nsec).
Decay Time	Lifetime (nsec)
Amplitude	Intensity fluorescence
Tail Offset	Background intensity
IRF Background	Background intensity when acquiring IRF (Meaningful if a measured IRF is used)
IRF Shift	Chronological offset of the IRF (Meaningful if a measured IRF is used)
Intensity	The total intensity of the lifetime
Sum Amplitude	Total of the amplitude

Mean Photon Arrival Time	Mean photon detected time
Mean Decay Time	Mean lifetime (nsec)
χ^2	Fit error or the fit precision for pixel

Fit of the Lifetime Decay Curve: Determining the Number of Exponential Components

In order to determine the number and distribution of individual fluorescent components in the image using the mean lifetime decay curve, the experimentally obtained decay curve must initially be calibrated with a theoretical exponential curve.

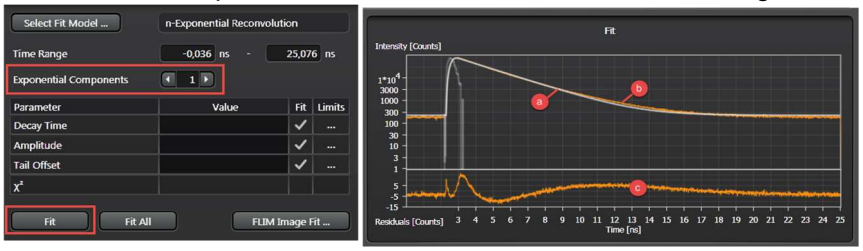


During the FLIM image acquisition, the Fit dialog shows the decay curve of the fluorescence intensity (Intensity) or the number of measured photons in the image (all pixels) over time excitation pulse (Time) ⓐ :Average value of the measured photons over time after the excitation pulse

Select Fit Model and start Exponential Components value 1, click Fit.

The calculation is carried out and the results are shown as follows in the Fit dialog

In addition to the measured decay curve ⓐ, the diagram shows the theoretical exponential curve with a componentⓑ and the deviation in the lower rangeⓒ



If the following criteria are met, this indicates that the correct number of components have been measured:

- Curves ⓐ and ⓑ lie nicely on top of each other
- Curve ⓒ exhibits only minor fluctuations
- The FLIM image after the Fit is no noisy
- The value for χ^2 which indicates the Fit precision, attenuates only slightly (minimum =1) when the number of the components for the components for the fit is increased

In the above case, all three criteria negate the assumption that these is only a single exponential component

The Time gate and Intensity Threshold (Ref. page 10) are effective for χ^2

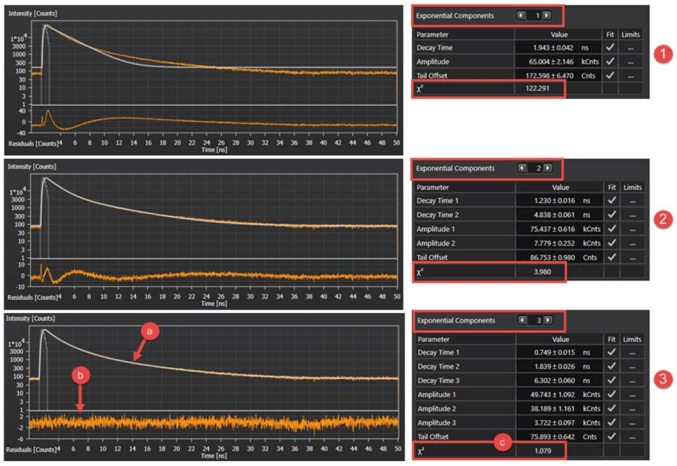
Example

In the following example, the fit has been carried out with 1, 2 and 3 exponential components. The result allows you to assume that these 3 exponential components here, since the following are true for an Exponential Component value of 3:

ⓐ : The experimental and the theoretical lifetime decay curve lie nicely on top of each other.

ⓑ : The deviation curve exhibits little fluctuation

ⓒ : The value for χ^2 is close to 1



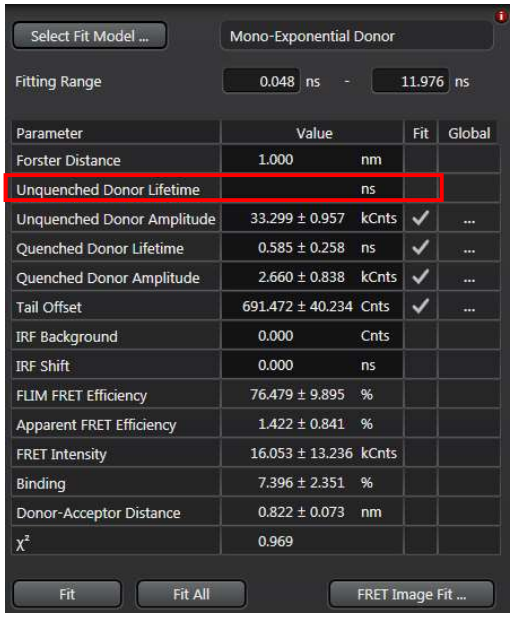
8-③ Fit Settings: FRET

Fit	18-⑤Clicking this button calculates the curve fitting with the setting listed above for all curves a selected in navigation table
Fit All	18-⑤Clicking this button calculates the curve fitting with the settings listed for all curves of the selected detected channel
FLIM Image Fit…	Open the FLIM image Fit dialog, in which you can configure setting for the pixel-to-pixel curve fitting of the curves selected in the navigation table and start the pixel-to-pixel curve fitting.

Förster Distance	The distance at which the energy transfer efficiency is 50%
Unquenched Donor Lifetime	The lifetime of Donor without FRET
Unquenched Donor Amplitude	The intensity of Donor without FRET
Quenched Donor Lifetime	The lifetime of Donor with FRET
Quenched Donor Amplitude	The intensity of Donor with FRET
Tail Offset	Background intensity
IRF Background	Background intensity when acquiring IRF (Meaningful if a measured IRF is used)
IRF Shift	Chronological offset of the IRF (Meaningful if a measured IRF is used)
FLIM FRET Efficiency	This is calculated only from the donor molecules in the pixel at which FRET actually occurs
Apparent FRET Efficiency	The average Apparent FRET efficiency of all molecules
FRET Intensity	Intensity of the interacting donor molecules
Binding	The binding strength between donor and acceptor (Proportion of the bonded donor molecules to the total number of the donor molecules %)
Donor-Acceptor Distance	Average distance between donor and acceptor（nm）
χ^2	Fit error or the fit precision for pixel

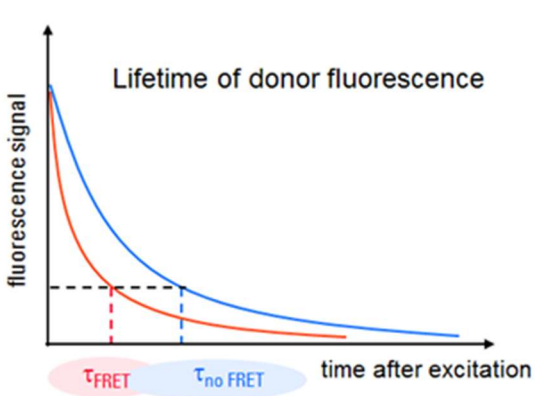
Calculating FLIM FRET efficiency

Create a Fast FLIM images using donor only sample and FRET sample. In donor only sample, copy Unquenched Donor Lifetime (Mono-Exponential Donor) or Mean Photon Arrival Time (Multi-Exponential Donor). In FRET sample curve fitting, disable the Fit in Unquenched Donor Lifetime and paste. Then, carry out the curve fitting by clicking on Fit. Next, carry out pixel-for pixel fit for the image



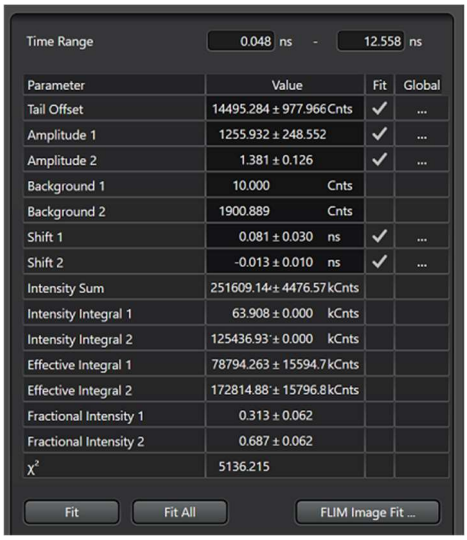
- Mono-Exponential Donor: This calculation model is applied if the fluorescence lifetime of the donor has a mono-exponential curve in the absence of the acceptor
- Multi-Exponential Donor: This calculation model is applied if the fluorescence lifetime of the donor has multi-exponential curve in the absence of the acceptor

In the presence of the acceptor, the lifetime of the donor decreases. The FRET efficiency ($FRET_{eff}$) as a measurement for the molecular bonding is calculated according to the formula below.



$$FRET_{eff} = \frac{\tau_{no\ FRET} - \tau_{FRET}}{\tau_{no\ FRET}}$$

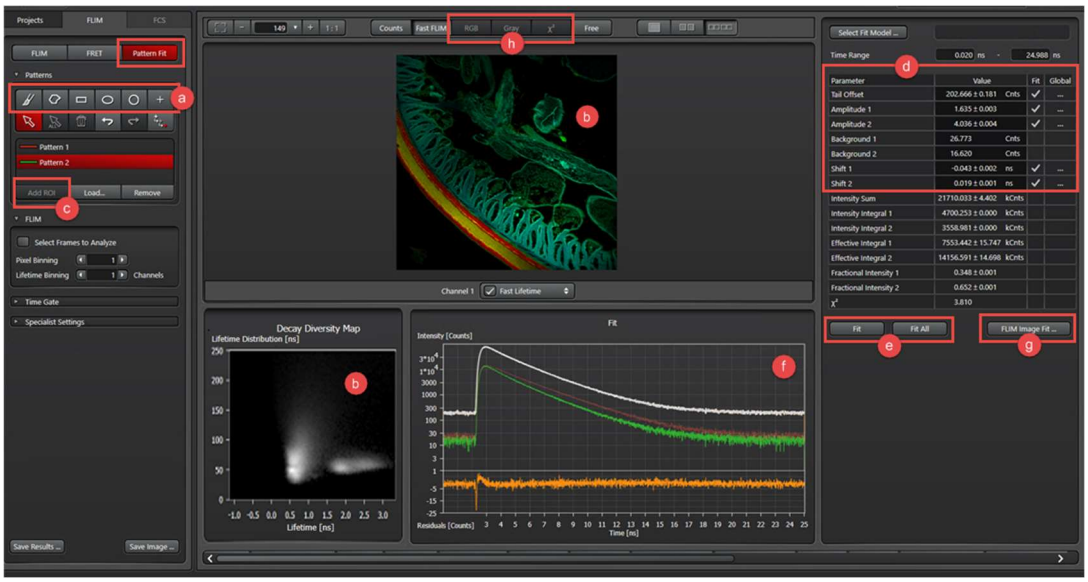
8-③ Fit Settings: Pattern Fit



Fit	18-⑤Clicking this button calculates the curve fitting with the setting listed above for all curves a selected in navigation table
Fit All	18-⑤Clicking this button calculates the curve fitting with the settings listed for all curves of the selected detected channel
FLIM Image Fit…	Open the FLIM image Fit dialog, in which you can configure setting for the pixel-to-pixel curve fitting of the curves selected in the navigation table and start the pixel-to-pixel curve fitting.

Tail Offset	Background intensity
Amplitude	Intensity of fluorescence
Background	Background
Shift	Reset the pattern in the fit through a slight time offset
Intensity Sum	Total intensity amount
Intensity Integral	The integrated intensity is the number of photons in the pattern you have selected in the decay histogram. The number of parameter depends only the size and brightness of the selected ROI.
Effective Integral	The effective intensity is the intensity if the component in your sample. It integrates all photons of all pixels for this component. If you want to know how much of this component is included in your samples, the effective intensity specifies the number of photons.
Fractional Intensity	This parameter specifies the proportion/fraction of the photons of this component. All proportion should add up to 1.
χ^2	Fit error or the fit precision for pixel

Separating Components with FLIM Pattern Matching



ⓐ,ⓑ,ⓒ: Define reference samples

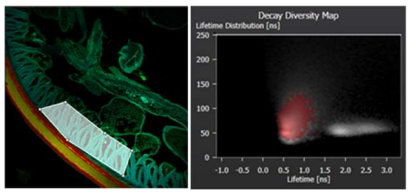
You can define reference patterns for the components in 2 ways: By plotting POIs in the decay diversity map or in the Fast FLIM image.

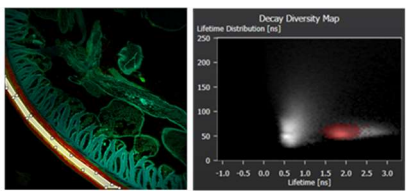
1. Switch to the Pattern Fit tab

2. In the Patterns dialog field, select a drawing tool and draw a ROI in a range you can define clearly

In Fast FLIM image: Color differentiation

In the decay diversity map: Definition of the lifetime distribution

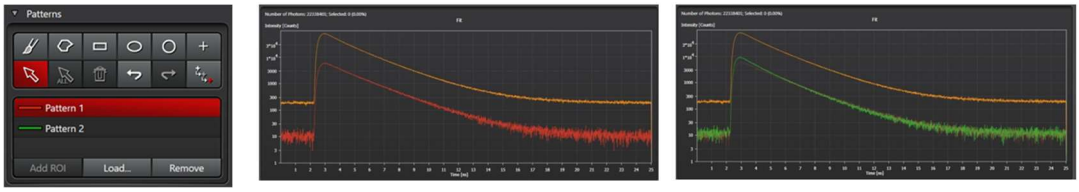




3. In the Patterns dialog, for each plotted pattern, click the Add ROI button to add the reference pattern

The entry is created and color allocated to the pattern. At the same time, a lifetime decay curve for the pixels allocated to the selected ROI in the same color is displayed under Fit (The Orange curve shows the decay curve over the entire image).

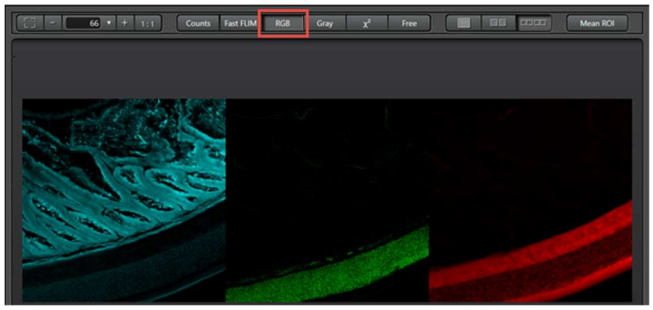
4. Repeat step 2 and 3 for each additional ROI that you would like to delineate as a component.



ⓖ： Carry out fit for decay curve

1. Set the remaining parameter to Fit for adjustment or fix to their own values (Fit) or link them globally (Global).

2. Carry out the curve fitting by clicking on Fit in the area of the parameter setting for the selected channels or Fit All for all channels.



After the pixel-for pixel fit for the image, the resulting images displayed in RGB mode.

、ⓓ、ⓔ、ⓕ

8-③ Fit Settings: Mean ROI

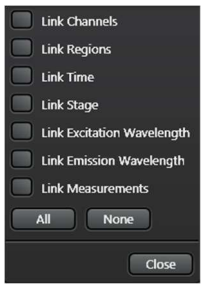
Only available for time lapse image

1. Turn on the Mean ROI
2. Select FLIM, FRET or Pattern Fit
3. select a drawing tool and draw ROIs

The lifetime changes within the ROIs are recorded.

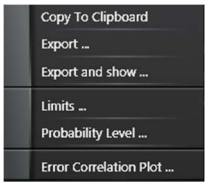
Below the viewer, the Mean ROI dialog box display the ③average photon arrival time and ④the intensities for each ROI. For each ROI there is a curve in the color of the ROI

8-③ Fit Setting

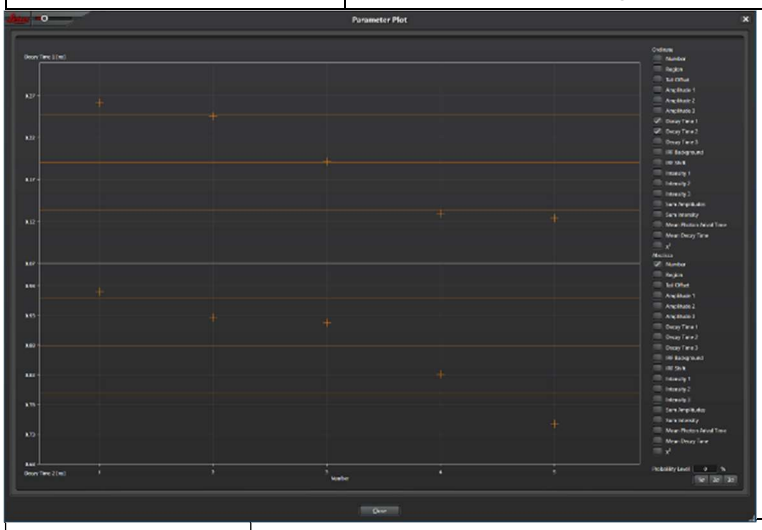


Fit	If the option is enabled, the value will be determined from the theoretical curve during curve fitting.
Global	If you have selected multiple data records, this gives you ability to select the same parameter value for all data records.

Right click, display the dialog



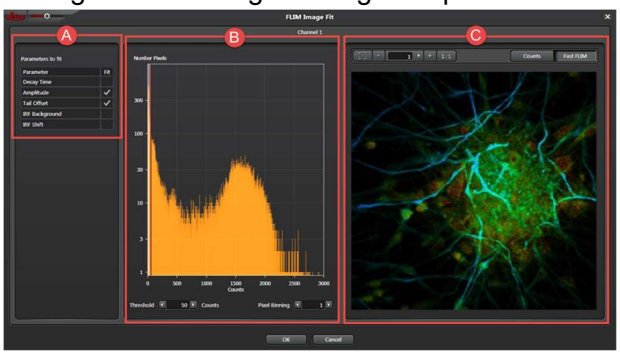
Copy to Clipboard	Store the Fit parameters
Export…	Save the Fit parameters as an Excel
Export and show…	Save the Fit parameters and open it as Excel table
Limits…	Open the limit dialog, in which you can select configure upper and lower limit for the respective parameter.
Probability Level…	You can enter a self-defined vale the accuracy for all parameters. The specification in the Fit Setting is adapted based on the value. （σ1:68.27%、σ2:95.45%、σ3:99.73%）
Error Correlation plot…	Display the data of a bootstrap analysis for the fit parameters in graphic form



Ordinate	Select the parameters for display on the Y-axis
Abscissa	Select the parameters for display on the X-axis
Probability Level	You can enter a self-defined percentage for the confidence interval, you can also define a multiple of the standard deviation σ.

FLIM Image Fit…

Configure the setting for image adaptation for the selected detection channel in this dialog



Ⓐ Parameter to fit: Calculates the pixel-to-pixel image adaptation automatically.

FLIM:

Decay Time	Lifetime
Amplitude	Intensity
Tail offset	Background intensity
IRF Background	Background intensity when acquiring IRF (meaningful if a measured IRF is used)
IRF Shift	Chronological offset of the IRF (meaningful if a measured IRF is used)

FRET:

Unquenched Donor Lifetime	Lifetime of the donor fluorescence without FRET
Unquenched Donor Amplitude	Intensity of the donor fluorescence without FRET
Quenched Donor Lifetime	Lifetime of the donor fluorescence with FRET
Quenched Donor Amplitude	Intensity of the donor fluorescence with FRET
Tail Offset	Background intensity

Pattern Fit:

Tail Offset	Background intensity
Amplitude	Intensity of fluorescence

Ⓑ Histogram: Distribution of pixels over the number of counts

Threshold	You can define the threshold value the left window area by the red line. At the same time. in the right area of the display window you can se which areas have been selected in the image. If you have selected Intensity for the view, all hidden areas not included in the evaluation are displayed transparently in the Fast FLIM and in red in the Counts.
Pixel Binning	Configure the number of pixels complied for calculating the curve fit If the intensity is too weak.

Ⓒ In addition to the histogram, a preview image is displayed

①Reset the zoom for the display window

②Reduces the zoom for the display window

③Allows you to enter the zoom factor the display window manually and adjust it using slider

④Increase the zoom for the display window

⑤Sets the zoom for the display window

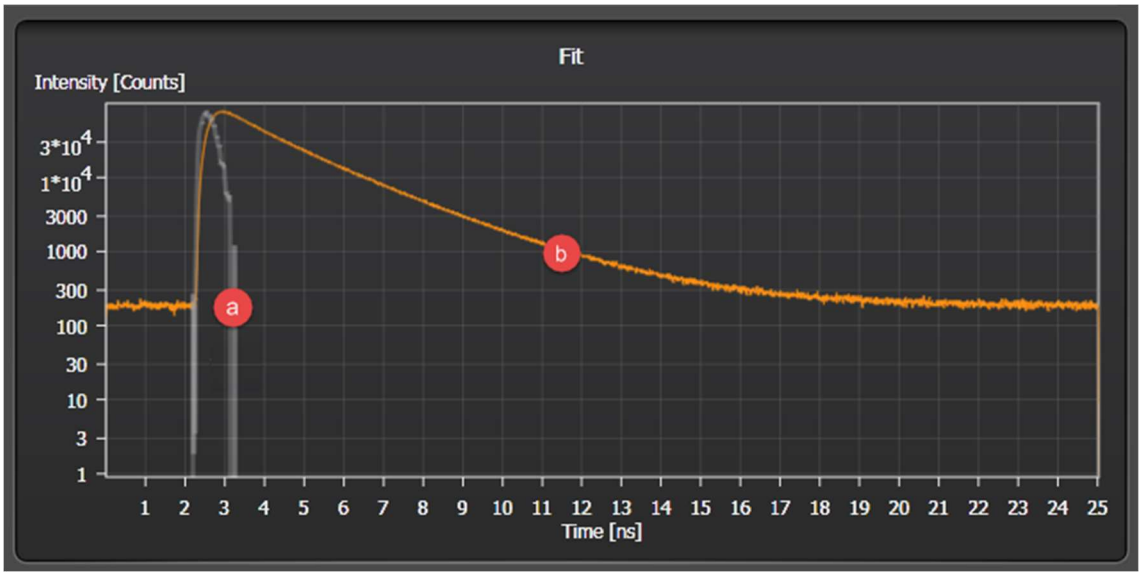
⑥Show the number of counted photons in gray-scale value

⑦Shows the distribution of lifetimes in the selected false color coding

Ⓓ OK

8-④ Fit

The decay curve of the fluorescence intensities (Intensity) over time after the excitation pulse (Time)



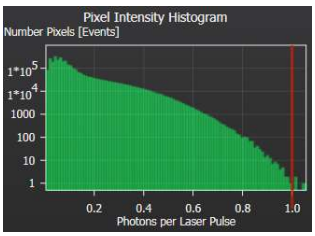
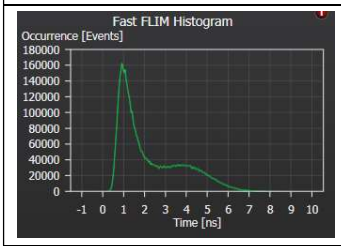
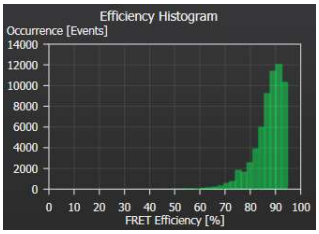
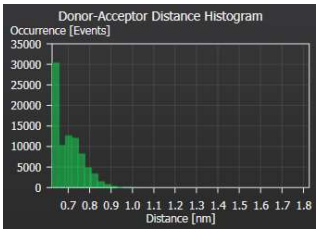
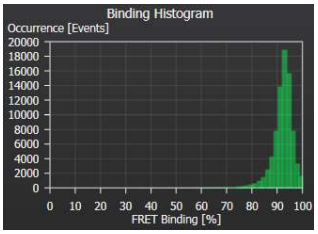
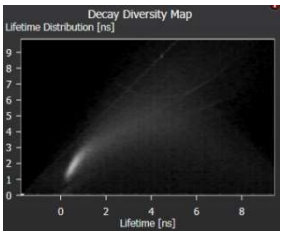
ⓐ: IRF（*I*nstrument *R*esponse *F*unction） of the excitation laser pulse

ⓑ: Sum of the fluorescence intensities over time after excitation pulse

You can select an area by moving the left and right border edge of the diagram using the mouse

Rescale	Zoom in and out on the curve using the mouse wheel
Show Legend	Show the legend in the diagram
Show Data Cursor	Show the value for time and intensity for each point
Intensity logarithmic	Show the intensity value in a logarithmic or linear view
Show number Photons	Show the total number of the counted photons in the selected area
Export diagram data…	Export the diagram data to the excel table
Export diagram data and show	Export the diagram data to the excel table and open the excel table
Copy diagram data clipboard	Store the diagram data in the clipboard
Export Image	Store the diagram as TIFF, JPEG, PNG, BMP, GIF

Histogram

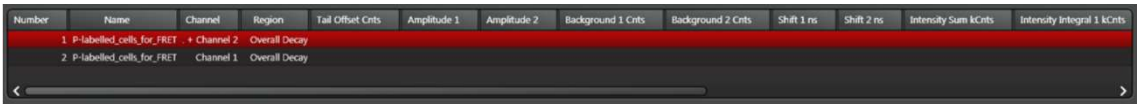
	Pixel Intensity Histogram	Display the Events over the photon per Laser pulse for each pixel
	Fast FLIM Histogram	Display the Events over the photon arrival time for each pixel
	Efficiency Histogram	Display the Events over the average Apparent FRET efficiency of all molecules for each pixel
	Donor-Acceptor Distance Histogram	Display the Events over the distances between Donor and Acceptor for each pixel (The distances are specified in nm and are reliable a long as the Forster distance has been defined correctly)
	Binding Histogram	Display the Event over the FRET binding for each pixel (The bond strength between donor and acceptor: Proportion of the bound donor molecules to the total number of donor molecules in %)
	Decay Diversity Map	Display the standard deviation of the photon arrival times over the average lifetime for each pixel

Right click on the histogram, display the diagram.

Rescale	Reset the scaling to the default setting
Show Legend	Show the legend in the diagram
Show Data Cursor	Display the values for time and intensity for each point on the curve as a tool tip when hovering over them
Intensity logarithmic	Shows the events in a logarithmic view. Otherwise, it is linear.
Show number Photons	Open the Lifetime Histogram dialog, in which you can enter the number of data points to be displayed under Number Data point.
Export diagram data…	Export of the result data
Export diagram data and show	Export of the result data and show the data in an Excel table
Copy diagram data clipboard	Store the result data in the clipboard
Export Image	Store the lifetime histogram as an image TIFF, JPEG, PNG, BMP, GIF

8-⑤ Result table

Before the measurement: There are one entry for each FLIM display channel



Number	Name	Channel	Region	Tail Offset Cnts	Amplitude 1	Amplitude 2	Background 1 Cnts	Background 2 Cnts	Shift 1 ns	Shift 2 ns	Intensity Sum kCnts	Intensity Integral 1 kCnts
1	P-labelled_cells_for_FRET	+ Channel 2	Overall Decay									
2	P-labelled_cells_for_FRET	Channel 1	Overall Decay									

After the measurement: One row is created for each image, each ROI and FLIM display channel.

Number	Name	Channel	Region	Tail Offset Cnts	Amplitude kCnts	Decay Time ns	IRF Background Cnts	IRF Shift ns	Intensity kCnts	Sum Amplitudes kCnts	Sum Intensity kCnts	Mean I
1	P-labelled_cells_for_FRET	Channel 1	Overall Decay	32,790	56,616	3,041	0,000	0,000	10760,212	56,616	10760,212	
2	P-labelled_cells_for_FRET	Channel 1	ROI 1	3,341	6,411	3,073	0,000	0,000	1231,565	6,411	1231,565	
3	P-labelled_cells_for_FRET	Channel 2	Overall Decay	3,112	3,354	2,782	0,000	0,000	583,290	3,354	583,290	
4	P-labelled_cells_for_FRET	Channel 2	ROI 1	0,321	0,424	2,841	0,000	0,000	75,373	0,424	75,373	

Right click on the table open the window

Select Columns…	Open a new dialog in which you can select which columns (parameters) are shown in the result table
Copy table content	Copies the entire table contents to the clipboard
Copy selected Rows	Copies the content of selected rows to the clipboard
Copy all	Copies the values of all parameters to the clipboard, including columns not selected under select columns…
Export table content…	The table contents can be exported in Excel or CSV format
Export selected rows …	Selected contents can be exported in Excel or CSV format
Export all…	Entire table contents can be exported in Excel or CSV format
Export table content and show…	The table contents can be exported and show this in Excel
Export selected rows and show…	Selected contents can be exported and show this in Excel
Export all and show…	Entire table contents can be exported and show this in Excel
Parameter Plot…	You can carry out the data analysis for multiple data records and graphically display it for selected parameters (Ref. Next page)

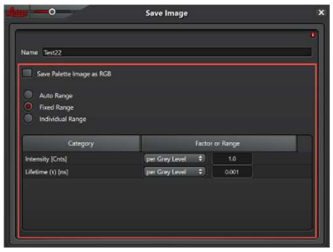
8-⑥This dialog allows you to save your analysis results or FLIM images

Save Results…	The current status of all analysis is stored, in the project directory. The data can be called up again in LAS X FLIM/FCS and further analyzed.
Save Image…	The colored Fast FLIM or FLIM image is created and stored as a normal intensity image in the .lif format as a 3 channel image (RGB) in the project directory and can be further processed as such. An evaluation in LAS X FLIM/FCS is no longer possible with this format.

You can enter a designation under Name

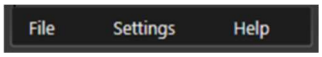
Save Pallet Image as RGB

If this function is enabled, the colored Fast-FLIM or FLIM image is created and stored as a normal intensity image in the .lif format as a 3-channel image (RGB) in the project directory and can be further processed as such.



Auto Range		Scales the image automatically between minimum and maximum so that the image is displayed properly, but no values are quantified.
Fixed Range		You can select the scaling range for each display Category separately.
	Per Grey Level	Scaling by gray-scale value. Here, under Factor or Range, enter a factor with which the corresponding grayscale value is weighted. The values are quantifiable.
	Range	Scaling by intensity. Here, select a range under Factor or Range by entering values for minimum and maximum. The values are quantifiable.
Individual Range		Here, you can define the scaling ranges for each component individually, just like you can for Fixed Range. The values are quantifiable.

8-⑦

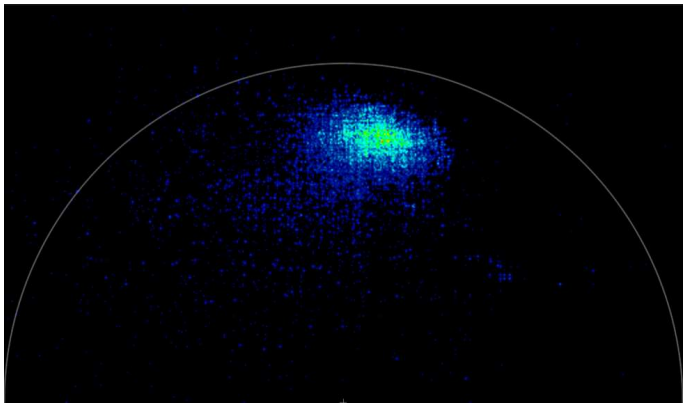


File

New Experiment	Create a new experiment in the project directory
Open Experiment…	Open a previously saved experiment and load into the project directory
Import…	Open an image file in the.ptu format for importing into the project directory
Export…	Open an image file in the .ptu format for exporting
Export Raw Data…	Open an image file in the .ptu format for exporting
Save	Save the current project or experiment in the .lif dormat
Save as…	Save the current project or experiment in the .lif dormat
Close	Close the selected experiments in the project directory
Close All	Close all opened experiments in the project directory
Exit	Close the LAS X FLIM/FCS

Phasor Plot

A Phasor plot is a graphic visualization of the raw data of a FLIM acquisition in a vector space. Each pixel in a FLIM image is transformed into a point of the phaser diagram. The position depends on the pixel's average lifetime. This analysis is fast and returns a graphic display of the measuring curve



Zero lifetime is located at (1, 0) and infinite lifetime at (0, 0); the radius is 0.5. By changing lifetime from zero to infinity, the phase point moves along a semicircle (universal circle) from (1, 0) to (0, 0).

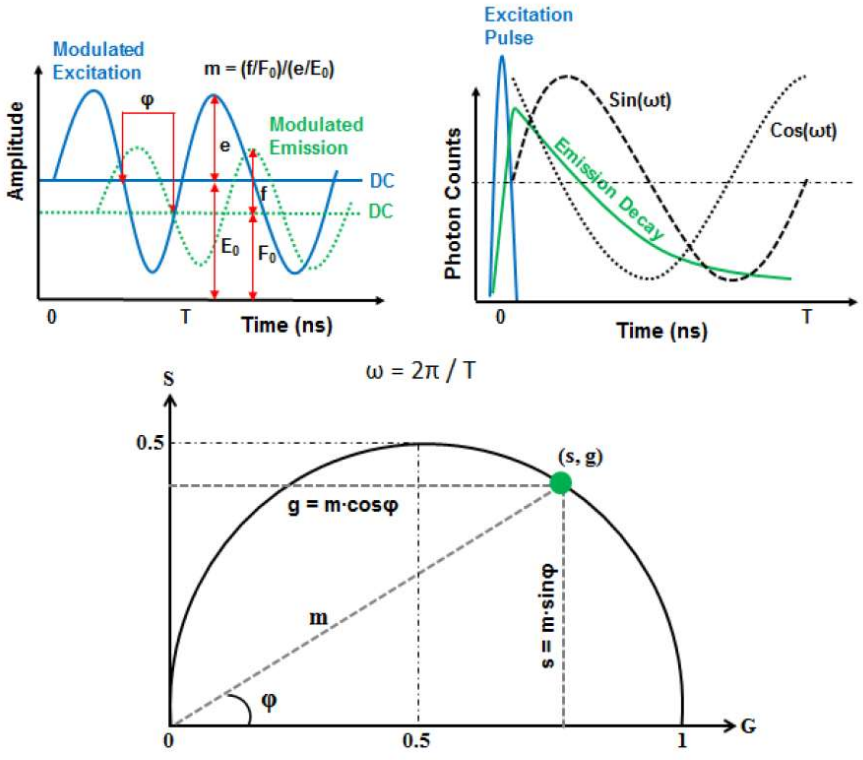


Figure 1: Mapping FD and TCSPC FLIM data to the phasor plot.

Ref: ISS, FLIM Analysis using the Phasor Plots

$$\hat{I}(\mathrm{t}) = \hat{I}(0)\sum_i^N \alpha_i e^{-t/\tau_i}$$ Eq. (1)

$*\hat{I}(\mathrm{t})$:The number of the instantly emitted photons at the time t. α_i、τ_i :The amplitude and fluorescence decay time of the i-th component o the mixture, respectively.

Due to the finite response of a system, the measured decay signal is a convolution form of the intrinsic decay and *IRF* plus the noise *n(t)*, as shown in Eq. (2).

$$I(\mathrm{t}) = IRF \otimes \hat{I}(\mathrm{t}) + n(t) = \mathrm{IRF} \otimes \left\{\hat{I}(0)\sum_{i=1}^N \alpha_i e^{-t/\tau_i}\right\} + n(t)$$ Eq. (2)

Each decay trace can be plotted as a single point in the Phasor plot by applying sine and cosine transforms to the measured decay data, as shown by Eq. (3). This is equivalent to the real and imaginary components of the Fourier transform of the decay data.

$$\mathrm{g}_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\cos(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt} \qquad \mathrm{s}_{i,j}(\omega) = \frac{\int_0^\infty I_{i,j}(t)\sin(\omega t)dt}{\int_0^\infty I_{i,j}(t)dt}$$ Eq. (3)

ω: The laser repetition angular frequency and calculated by multiplying the laser repetition rate with 2 Π. By taking Eq. (1) into Eq. (3) and solving the integrals, we can then derive the following relationships between the phasor and the lifetime, given by Eq. (4)

$$\mathrm{g} = \sum_{i=1}^N \frac{f_i}{1+\omega^2\tau_i{}^2} \qquad s = \sum_{i=1}^N \frac{f_i\omega\tau_i}{1+\omega^2\tau_i{}^2}$$ Eq. (4)

$*N$ is the number of the fluorescent species. f_i is the fractional contribution of the i-th species of the fluorescence lifetime τ_i to the total intensity.

For a single-lifetime species (*N* = 1), Eq. (4) is reduced to Eq. (5) and the lifetime can be directly determined by the coordinate value of a phasor.

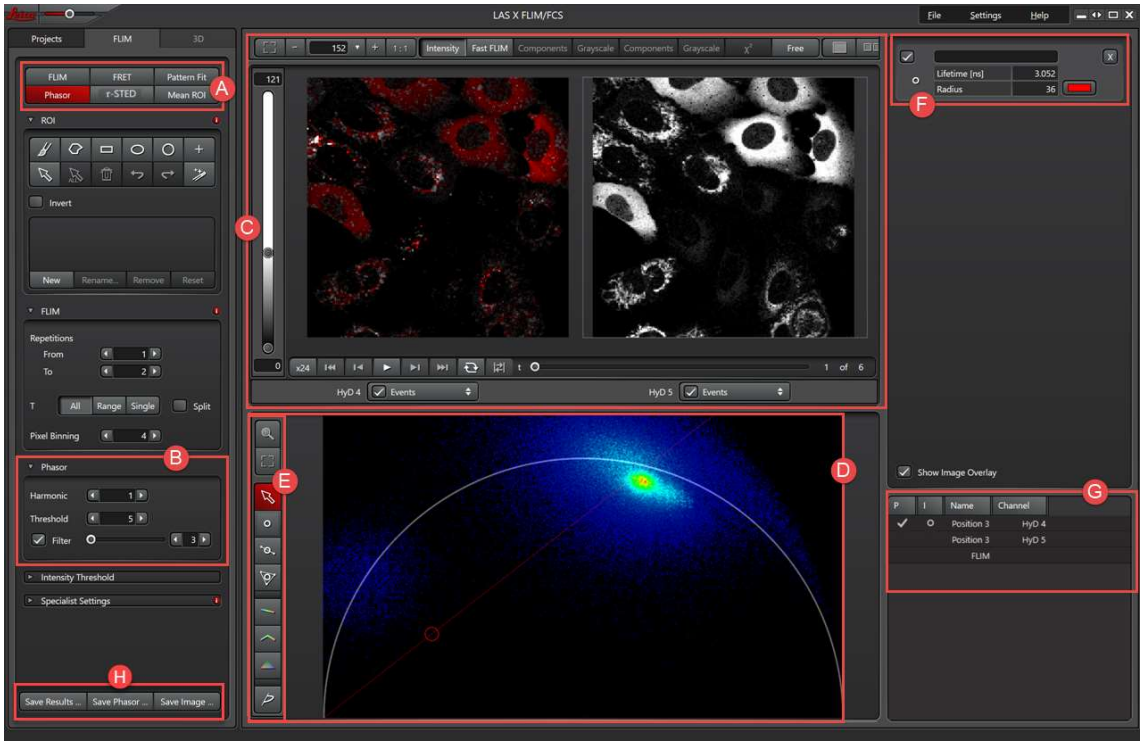
$$\mathrm{g} = \frac{1}{1+\omega^2\tau^2} \qquad \mathrm{s} = \frac{\omega\tau}{1+\omega^2\tau^2} \qquad \tau = \frac{1}{\omega}\left(\frac{s}{g}\right)$$ Eq. (5)

For a multiple-lifetime species (*N* > 1), Eq. (4) can be re-written by Eq. (6).

$$\mathrm{g} = \sum_{i=1}^N (f_i g_i) \qquad \mathrm{s} = \sum_{i=1}^N (f_i g_i)$$ Eq. (6)

$$\mathrm{g} = \frac{1}{1+\omega^2\tau_i{}^2} \qquad \mathrm{s} = \frac{\omega\tau}{1+\omega^2\tau_i{}^2}$$

Ref: ISS, FLIM Analysis using the Phasor Plots



Ⓐ Selection field for the various FLIM methods. Here, you select the Phasor method.

Ⓑ Phasor dialog for settings of the display in the display window.

Ⓒ Display window for the FLIM image. Intensity mode is the default setting for the phasor plot method.

Ⓓ Phasor display window.

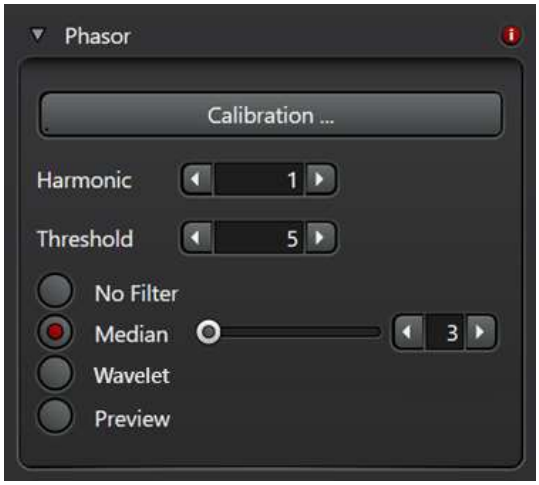
Ⓔ Toolbar for the phasor plot.

Ⓕ Data display of the components for the each selected tool for the phasor plot.

Ⓖ Display of the loaded image data records.

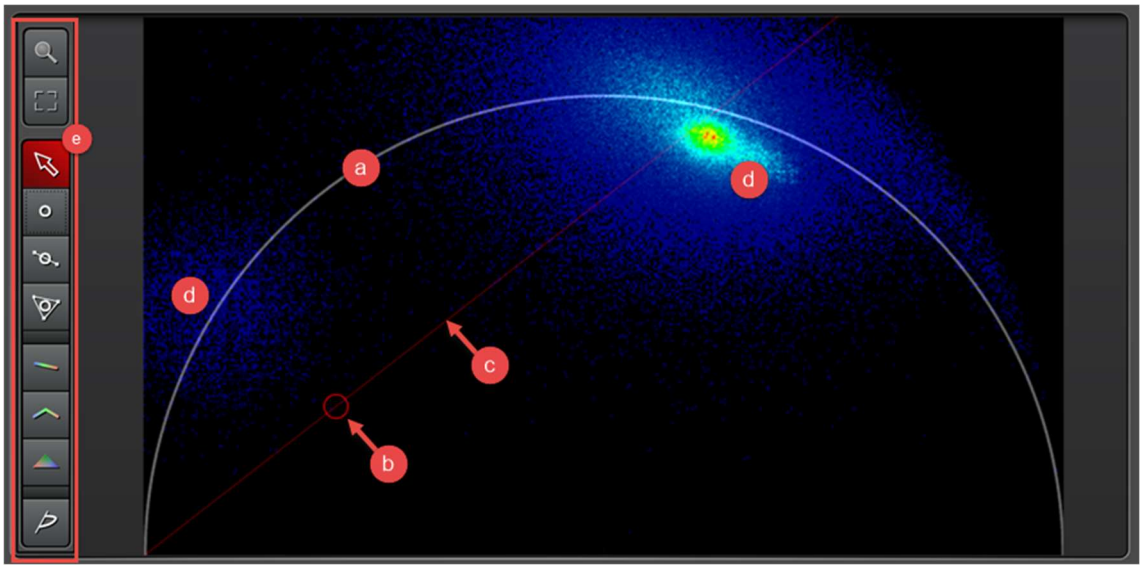
Ⓗ Buttons for various save options e.g. you can save the Phasor Plot as an RGB image.

36-Ⓑ



Harmonic	This parameter indicates the number of the harmonic wave of the Fourier transformation that the respective lifetime is multiplied by. It corresponds to the fundamental wave. For very short lifetime, the interesting regions in the Phasor plot are located in the lower range at a small phase angle. By increasing this value (to max. 9), you can shift the visualization of the interesting regions further to the center to a greater phase angle.
Threshold	Specifying a threshold value filters artifacts out of the Phasor plot. A value of 5 is present as default.
No Filter	No Filter is applied.
Median	This is a median filter that smoothes the image while accentuating and contouring structures in the image. This enhances the visibility of details. When the checkbox is enabled, you can adjust the filter using the slider or by entering a value. This filter is to be used for analysis purposes. This filter is to be used, for analysis purpose.
Wavelet	This advanced filter use a wavelet transform to reduce noise and preserves intensity edges in the image. For this, it is necessary to know the noise level, which is determined by the detection in counting mode. This filter is used for e.g. optimizing STED acquisitions using a Phasor plot.
Preview	The filter is suitable for the live mode to prepare the experiment. If this check box is checked, you can use the slider or enter a value to set the number of photon from the neighborhood over which the average is taken.

36-Ⓓ Phasor Diagram



ⓐ Universal Circle

Ⓑ Circle cursor with phase line (ⓒ). In the display window, those pixels that are located within the cursor are displayed in color in the intensity image.

ⓒ Phase line. It represents the angle of the phase shift.

Ⓓ Cluster of lifetimes. They refer to the individual lifetime components. Monoexponential components are located on the semicircle, multiexponential components below the semicircle.

ⓔ Toolbar. Depending on which select, youcan adjust the appearance and perform analyses.

	Zoom Open a rectangle using the mouse in order to enlarge a section.
	Reset Resets the zoom to the default setting.
	Select With this, you can select draw elements (cursor).
	Draw Cursor Draw a second cursor with associated phase line. In the start setting for the phasor method, a cursor is displayed.（Ref. p40）
	Draw Ratio Cursor for two Components With this, you can draw a connecting line between line between two components.（Ref. p42）
	Draw Ratio Cursor for three Components

	With this, you can draw a connecting line between line between three components. （Ref. p43）
	Draw Connected Color Coding Line With this, you can draw a line with a color scaling the phasor plot. （Ref. p41）
	Draw Connected Color Coding Lines With this, you can draw multiple connected line with a color scaling the phasor plot. （Ref. p41）
	Draw Connected Color Coding Triangle With this, you can draw triangle with a color scaling the phasor plot. （Ref. p41）
	Draw FRET Trajectory With this, you can draw a path for the unquenched donor relative to FRET efficiency. （Ref. p44）


Draw Cursor
選択後、蛍光寿命の中心をクリック

ⓐ You can show and hide the cursor and the phase line foet his component using the checkbox.

Ⓑ The associated tool is displayed with its symbol.

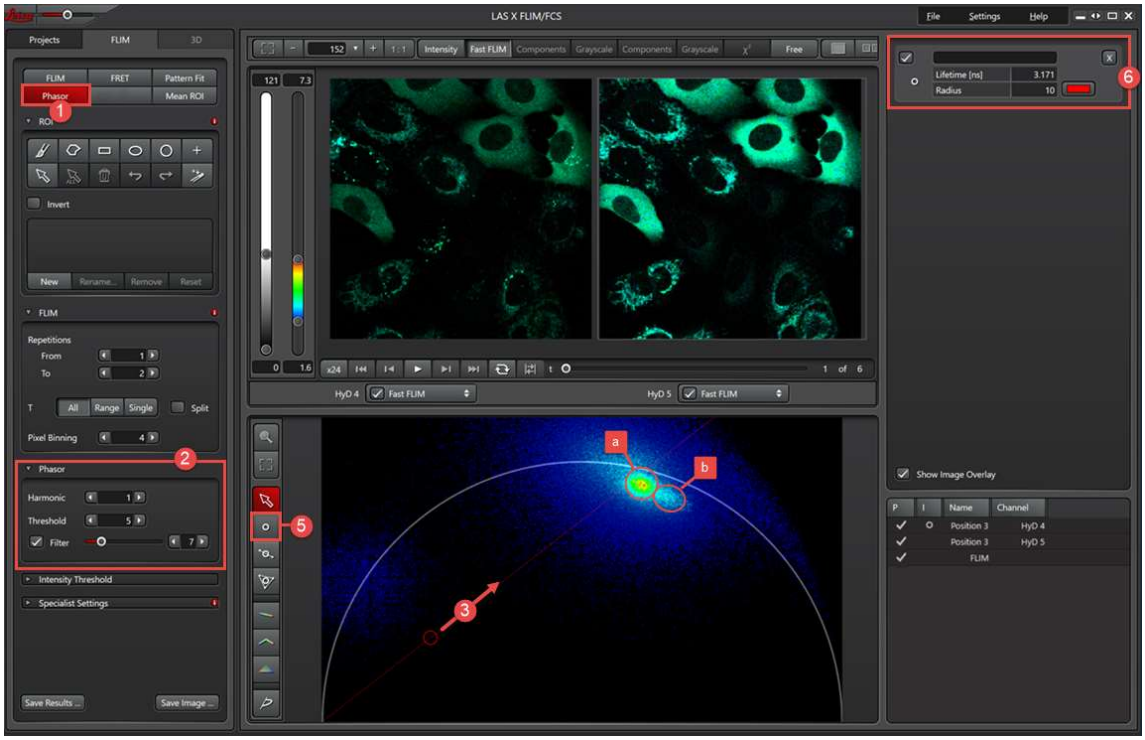
ⓒ Using the color field, you can adjust the color of the cursor and phase line.

Ⓓ By clicking **X**, you disable the selected tool.

Lifetime [ns]	The lifetime of component
Radius	The size of cursor

P	If the check mark is set, this data is displayed in the phasor plot.
I	Indicates whether the image is displayed
Name	Designation of the data record in the project directory. For each image of a data record, a separate row is created.
Channel	Designation of the acquisition channel.

Determining and Visualizing the Lifetime using the Phasor Method

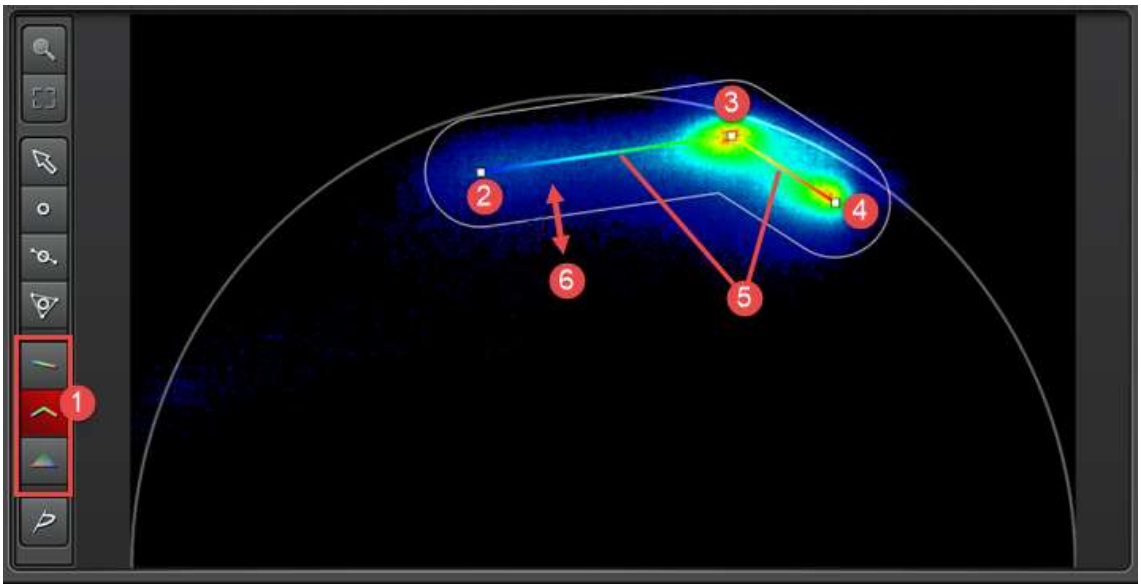


1. Select the Phasor method (①). The Phasor diagram (②) is displayed. Two pixel clusters can be discerned, which points to the presence of two components: a strong one (b) and a weak one (b). In the following, you define the strong components and determine the associated lifetime.
2. Adapt the setting in the Phasor dialog (②) in such a way that the visualization of the pixel clusters in the phasor diagram is clearly recognizable.
3. Move the cluster over the first pixel cluster (③) with the mouse button pressed down.
4. Adjust the cursor size to the pixel cluster using the mouse wheel.
5. At the top right edge of the screen (⑥), a field is displayed for the cursor where you can read off the lifetime for the defined component.

Lifetime	Lifetime of the component
Radius	Size of the Cursor

6. In order to define the second component, select the Draw Cursor tool from the toolbar (⑤). A second cursor with phase line is displayed.
7. Repeat steps 3 to 4 dor the second component.

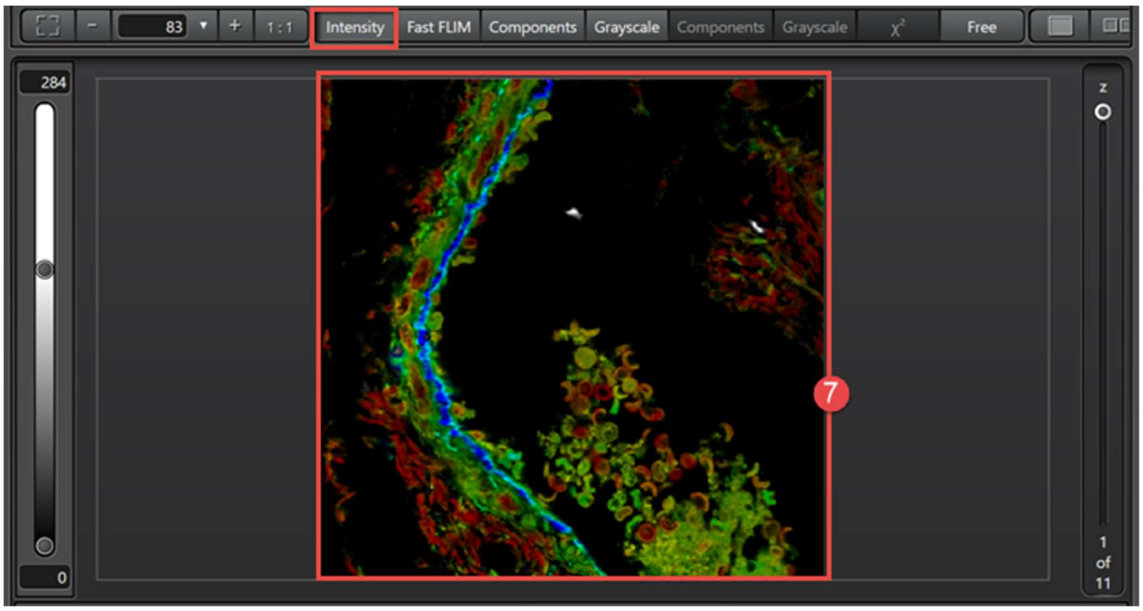
Adding Color Coding in the Phasor Diagram



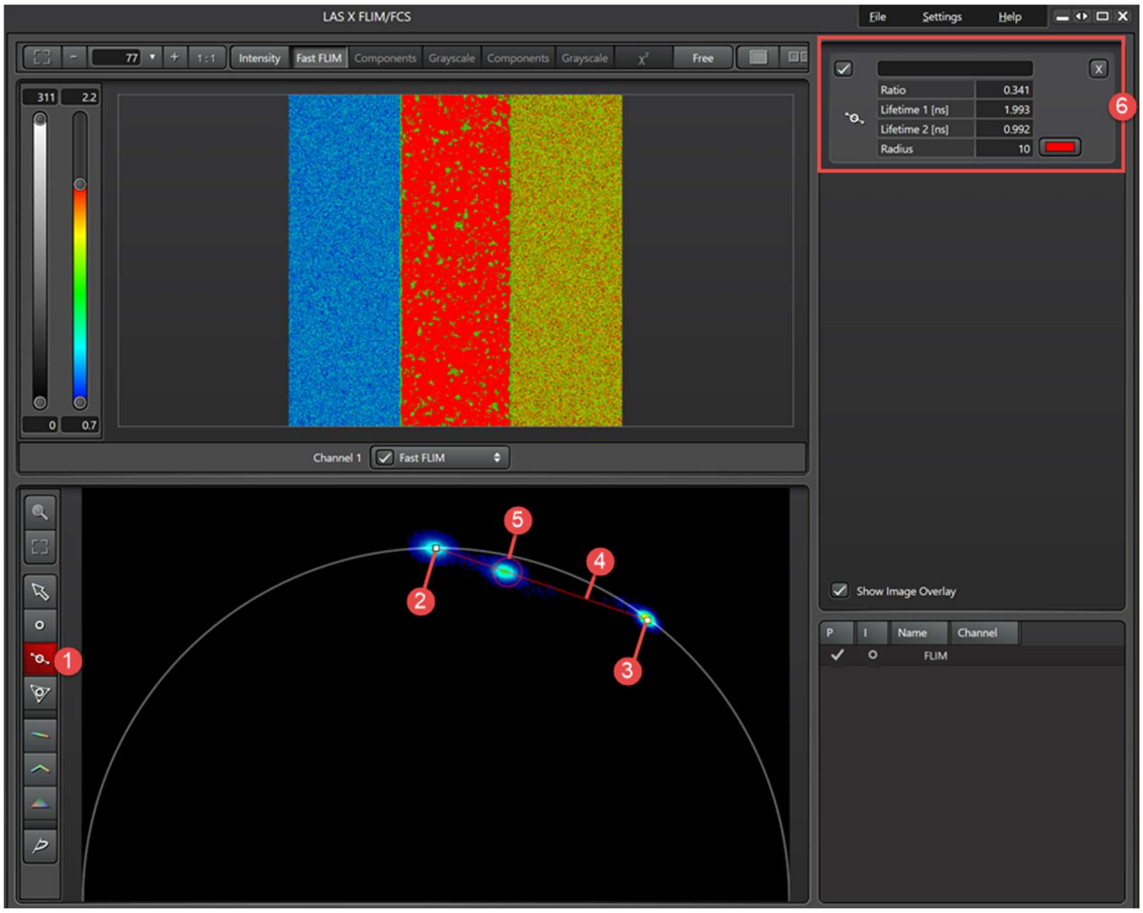
1. Click on the desired tool from the toolbar to select it (①).
2. Draw a line, connecting line or triangle by clicking the starting point (②), holding down mouse button, drawing the line to the next point and releasing the mouse button there (③). For all further points, repeat the operation (③ and ④).

Depending the selected tools, the lines are plotted with a color gradient from red to blue (). In addition, a white frame marks the range (⑥) to which the color coding is to be applied. All pixels outside this range are displayed in white in the intensity image.

3. You can adjust the range by clicking the white line and dragging it to the desired distance (⑥). In this window, the intensity image is displayed within the defined range with color coding (⑦).



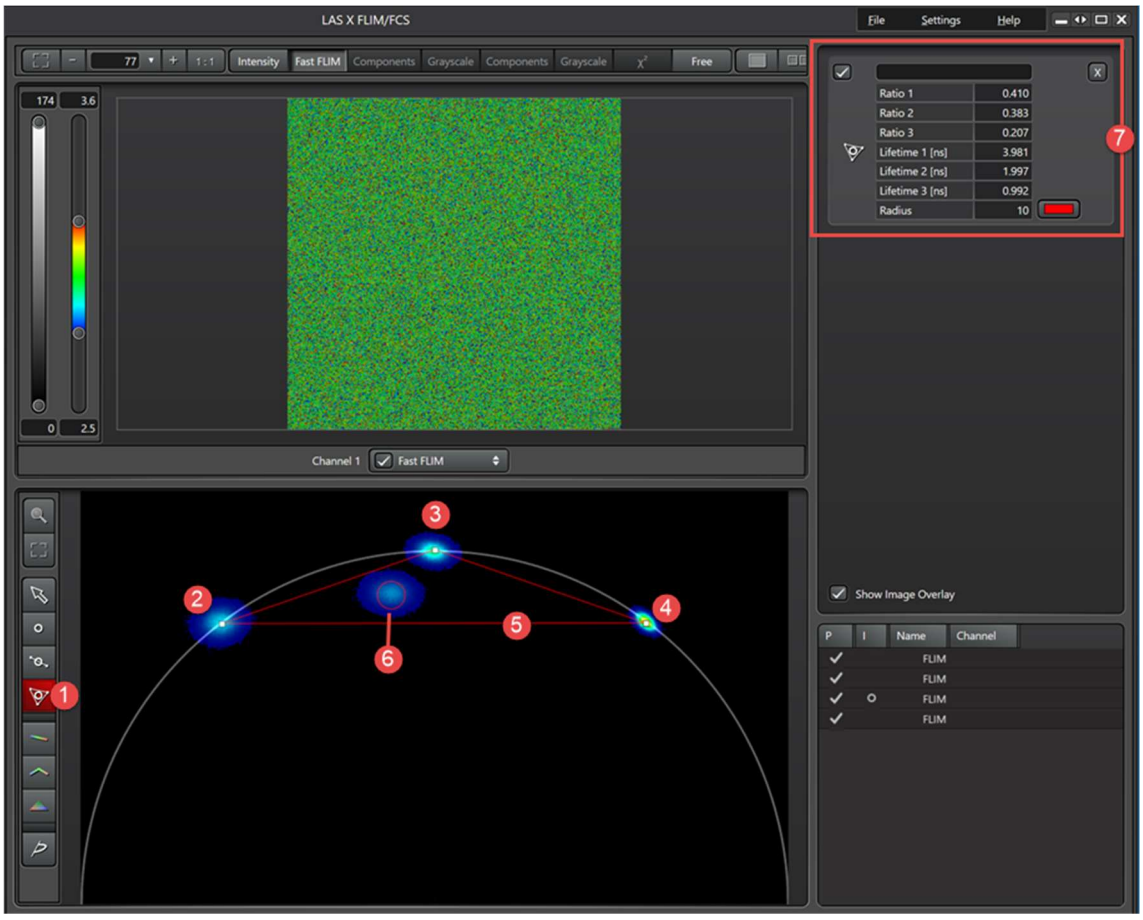
Determining the Ration of Two Components in the Phasor Diagram



1. Select the Draw Ration Cursor for two Components tool by clicking it on the toolbar (①).
2. In the Phasor plot, click the center of the area that represents 2 components (②, ③).
3. Finish the graphic by clicking. The connecting line is drawn using the cursor (④)
4. Hold the cursor, hold the mouse button pressed and move the cursor back and forth along the connecting line and adjust its size (⑤). This component contains two lifetimes and has its position below the universal circle.
5. In the respective position, the associated data is displayed in the top right area (⑥).

Ratio	Ratio of the two components (②, ③)
Lifetime 1, Lifetime 2	Lifetime of the components (②, ③)
Radius	Size of the cursor (⑤)

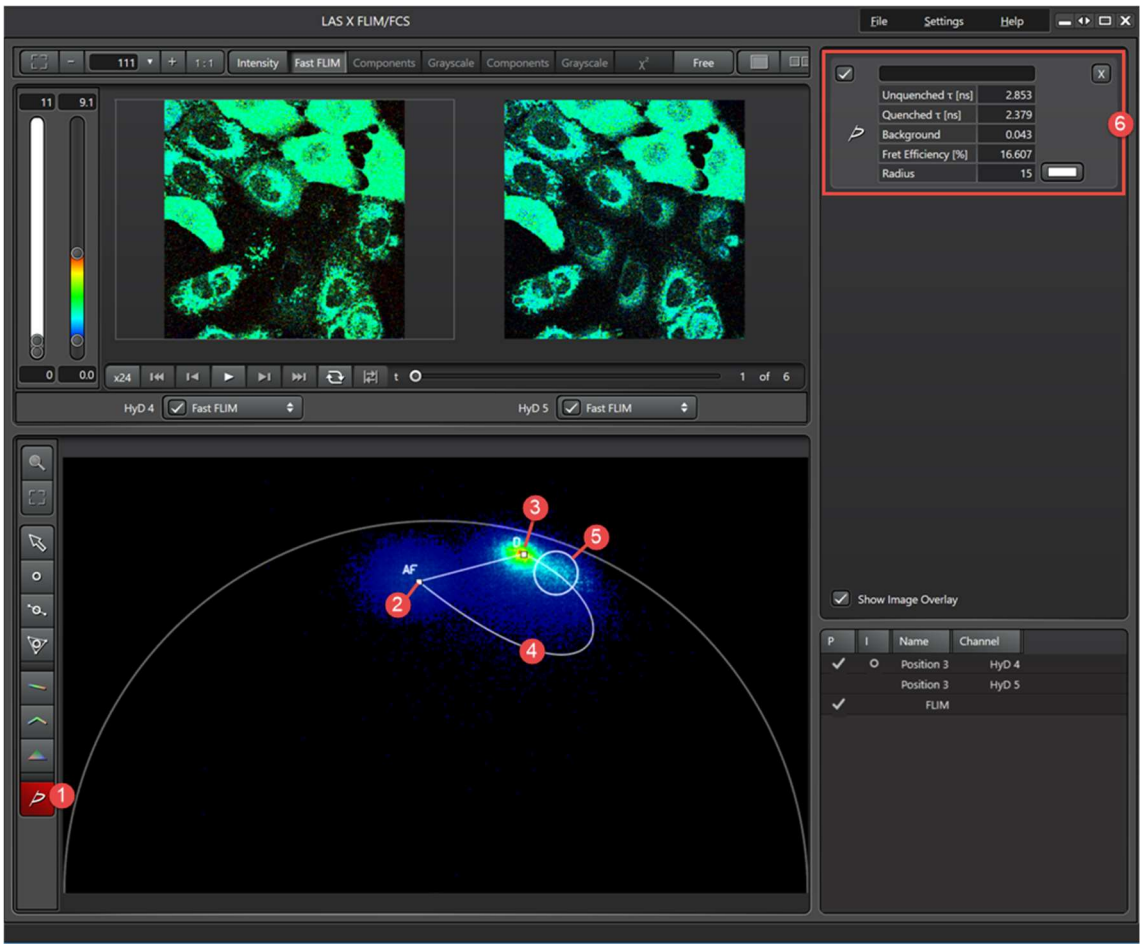
Determining the Ration of Three Components in the Phasor Diagram



1. Select the Draw Ratio Cursor for three Components tool by clicking it on the toolbar (①)
2. In the Phasor plot, click the center of the area that represents 2 components (②, ③, ④).
3. Finish the graphic by clicking. The connecting line is drawn using the cursor (⑤).
4. Hold the cursor, hold the mouse button pressed and move the cursor back and forth along the connecting line and adjust its size (⑥). This component contains two lifetimes and has its position below the universal circle.
5. In the respective position, the associated data is displayed in the top right area (⑦).

Ratio	Ratio of the three components (②, ③, ④). Total = 1
Lifetime 1, Lifetime 2, Lifetime 3	Lifetime of the components (②, ③, ④)
Radius	Size of the cursor (⑥)

Drawing a FRET Trajectory in the Phasor Diagram



1. Select the Draw FRET Trajectory tool by clicking it on the toolbar (①).
2. In the Phasor plot, click the center of the region that corresponds to autofluorescence (②)
3. Then, press and hold the mouse button to draw a line the component that corresponds to the unquenched donor (③)
4. Finalize the graphic by double-clicking. The FRET Trajectory is draw together with this (④).
5. A Cursor for the analysis of the quenched donor (FRET) is displayed. Click the cursor and move It back and forth along the trajectory with the mouse button pressed (⑤).
6. In the respective position, the associated data is displayed in the top right area (⑥).

Unquenched τ	Lifetime of the unquenched donor
Quenched τ	Lifetime of the quenched donor (FRET)
Background	Ratio of the background noise
FRET Efficiency	FLIM-FRET efficiency.

Images from Phasor Method

画像上で右クリックし、Export Raw Image を選択すると、下記画像が表示されます。

Save Phasor Mask	Save as an image of the Phasor mask that can be used for further analysis and calculations.　The Phasor mask describe the region of the Phasor plot that represented in the Fast FLIM image.
Apply Phasor Mask to Image	Applies the Phasor mask to the images.　This particularly suitable for STED images where all longer lifetimes with the excitation ring, are truncated.
Save Phasor GS	Save the values for the g and s coordinates of the Phasor plot (2 images).　The X-axis of the Phasor plot represents the real component (g) and the Y-axis represents the imaginary components (s).

H

Save Image

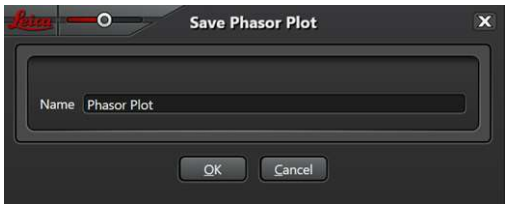
(Ref. page 33)

Save Result

The current status of all analysis result is stored, including all settings, in the project directory. The data can be called up again in LAS X and further analyzed.

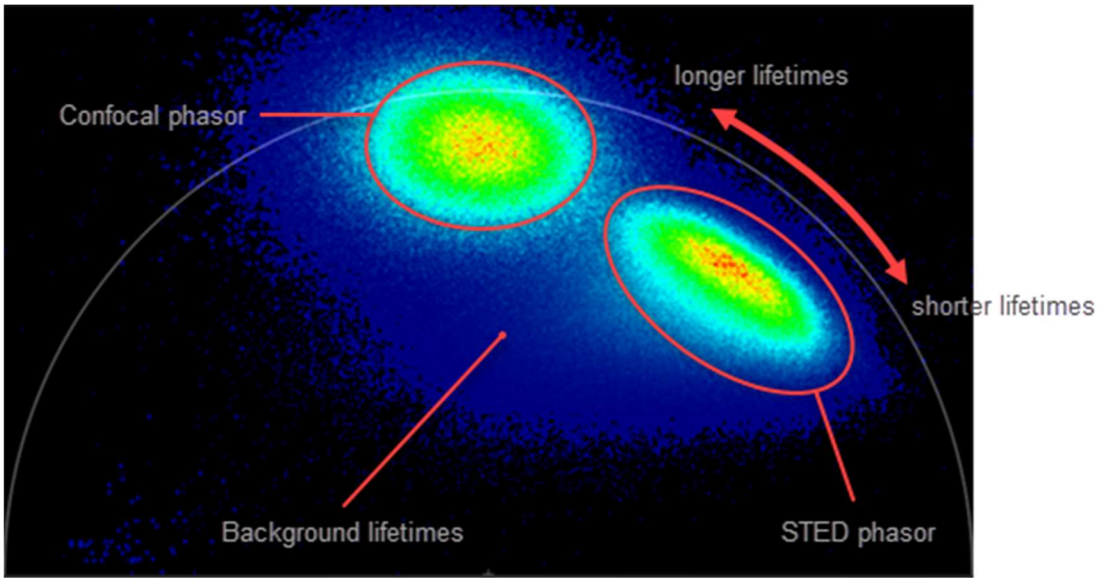
Save Phasor

You can save the Phasor plot as an RGB image in the project directory. Select Split (Ref. page 10), then Z Stack or Time series data are saved as video in the format.

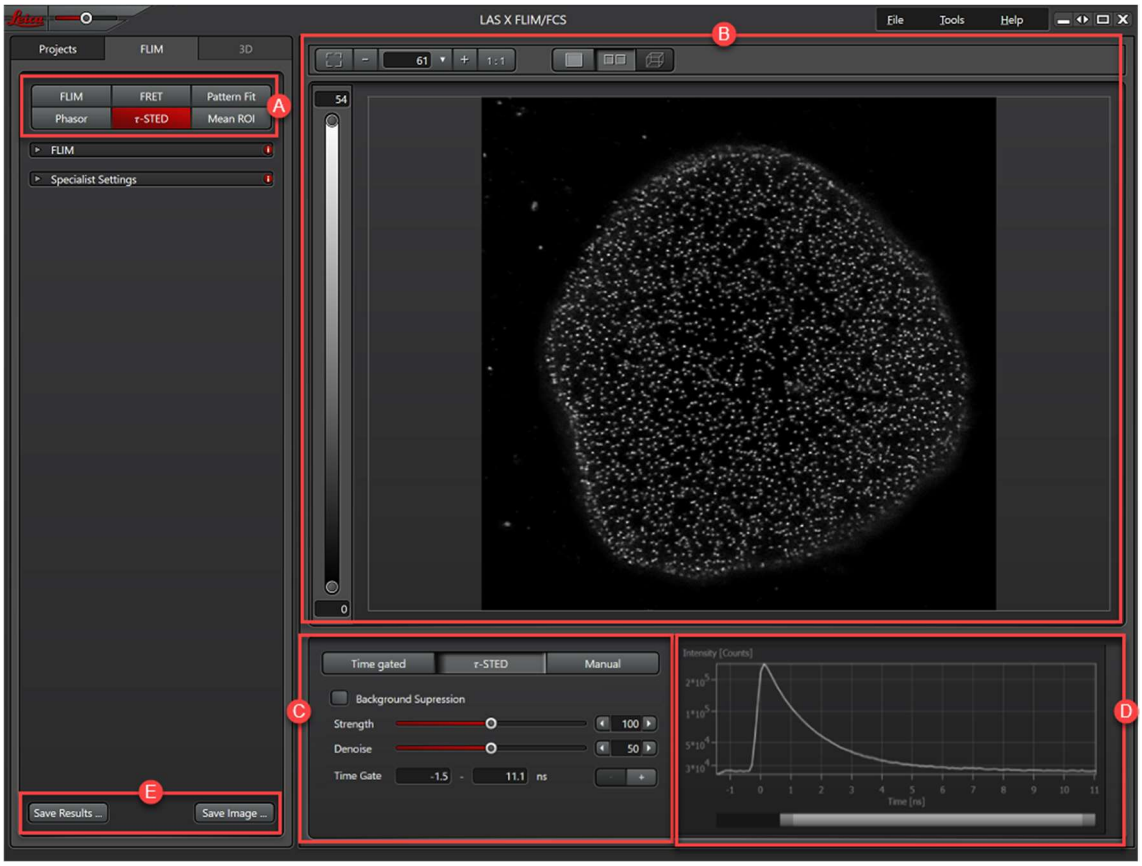


τSTED: What Is This ?

The τSTED method exploits the difference in the lifetimes by applying the component separation from the Phasor plot to STED images. In STED images, shorter lifetimes are mostly located in the range of the de-excitation donut, whereas the longer lifetimes primarily occur in the central excitation range. All longer lifetimes that are still present in the range of the de-excitation ring (donut) and the image background are filtered out, which increases the lifetime contrast between the central excitation range and de-excitation ring. This allows for improving the S/N and the resolution.



τSTED: The User Interface



Ⓐ : Selection field for the various STED-visualization method. Here, you select the τSTED method.

Ⓑ : Display window for the FLIM image. An intensity image is displayed for the τSTED method.

Ⓒ : Setting range for the various STED method. The τSTED is default. Time Gate serves as a comparison with the conventional methods.

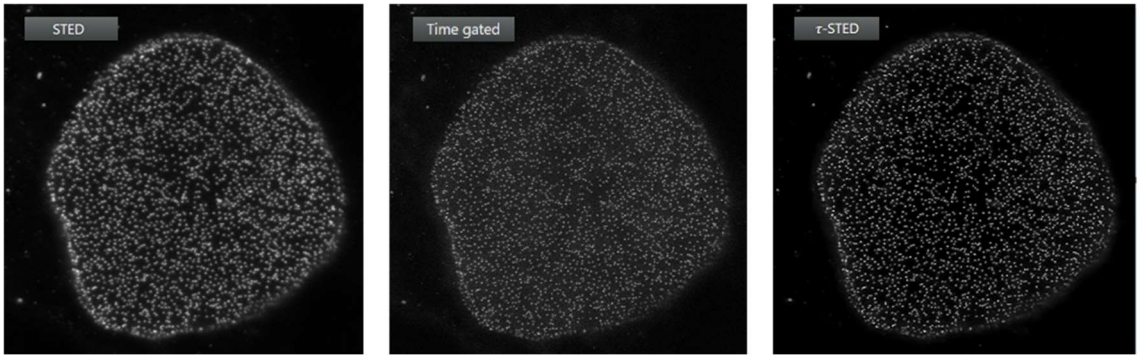
Ⓓ : The lifetime Decay Curve of the FLIM image is displayed here.

Ⓔ : Buttons for various save options, Save Image/Result

Display Methods: Time gated and τSTED

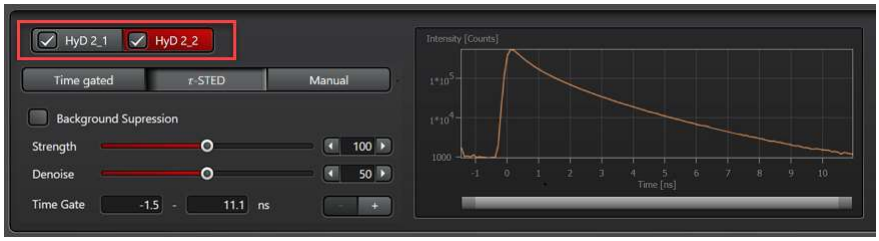
The various methods are described for the range FLIM to τSTED with their adjustment options and results. You can adjust phasor mask itself under Manual.

The following figure shows the result of the three methods side-by-side. The highest resolution can be achieved with the τSTED.

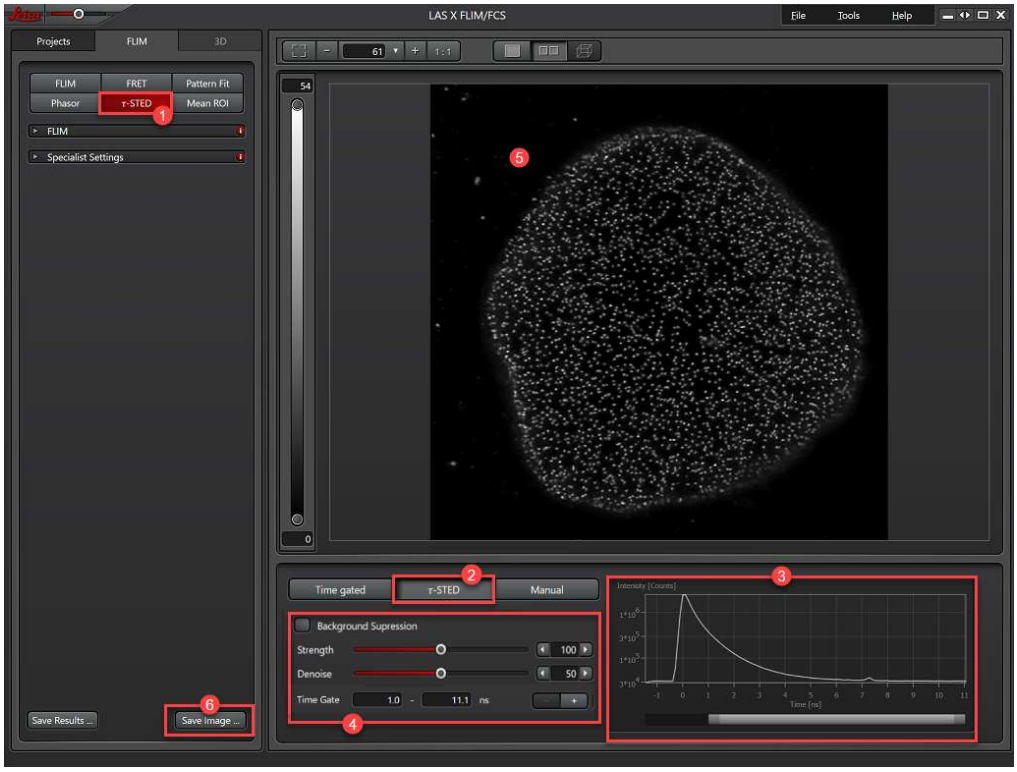


Multi-channel Acquisitions

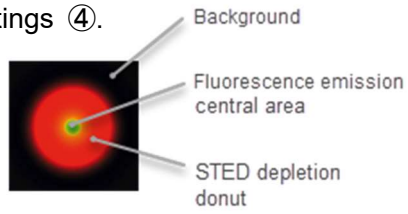
τSTED is also possible for multi-channel acquisitions. A button for channel selection is displayed for any channel. It is also possible for images without STED to be in one channel and images with STED to be in another channel.



τSTED: Automatically Optimizing STED Analyses Using the FLIM Phasor Method



Select the τSTED①, then the τSTED method② has already been preselected. The lifetime decay curve of the fast FLIM image is displayed in the area on the right③. Adjust the settings ④.

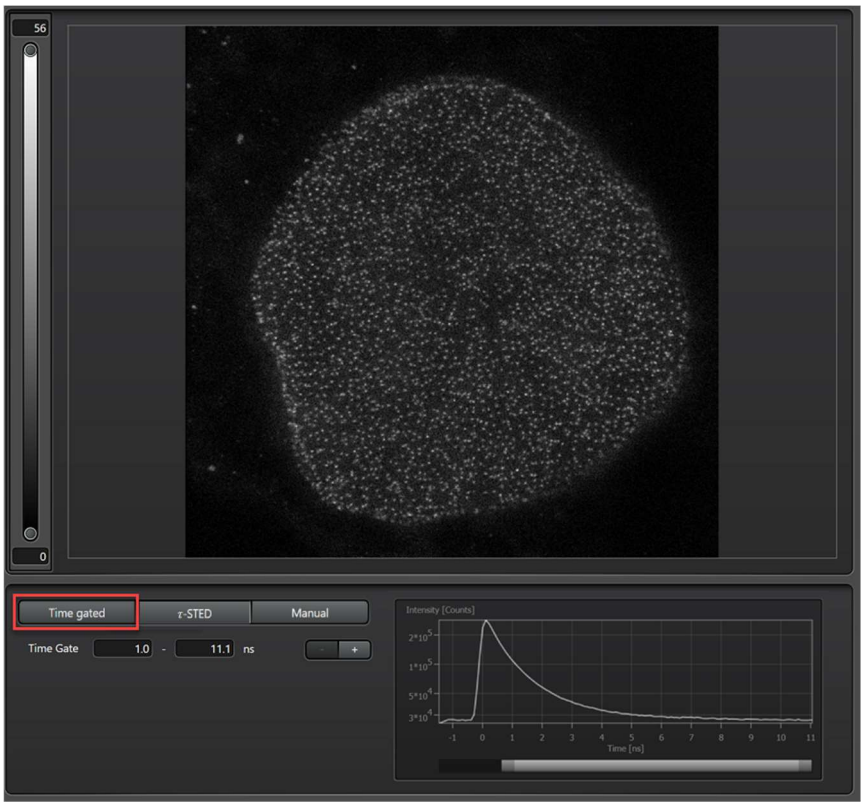


Background Suppression	Removes the background using its lifetime properties.
Strength	Set the strength of the suppression of the pixels located in the range of the de-excitation ring(donut) and in the background. If the value is set too high, then pixels from the central range will also be suppressed. A value of 100 is preset.
Denoise	Reduces the noise. A value of 50 is preset.
Time Gate	Define the beginning and end of time gates for the detection here. You can add/remove time gates by clicking the +/-. For the set time gate, a slider is displayed under the lifetime decay curve and can also be used for setting the time gate.

Save the Image by clicking the Save Image button⑥.

Time gated

You can set a time gate for the detection here. You can improve the resolution by doing this, but with a lower signal strength.

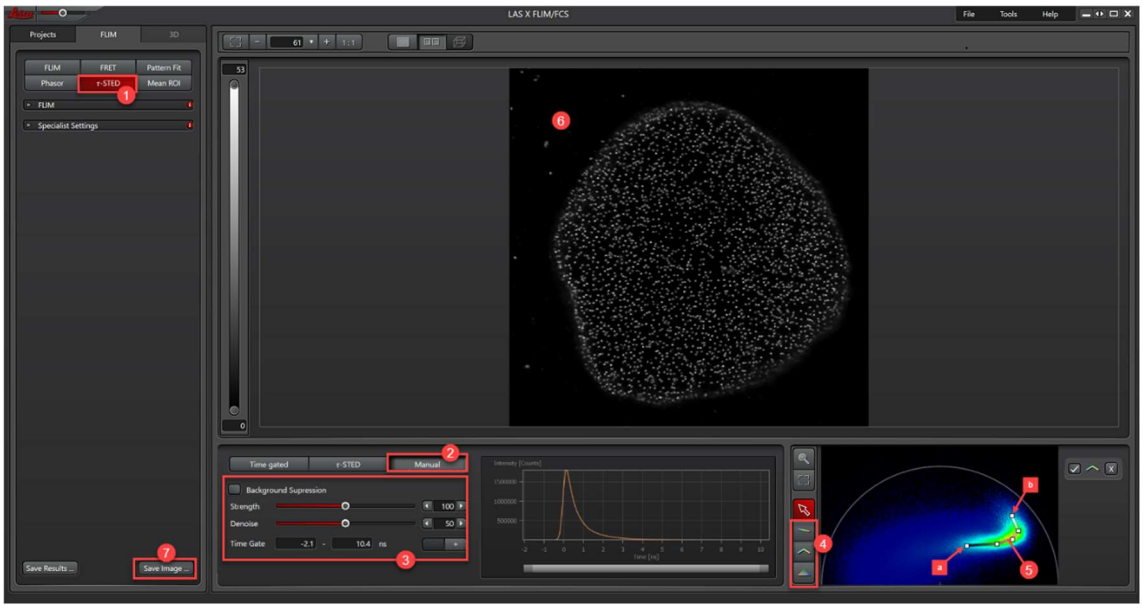


Time Gate : You can define the beginning and end of the time gate here. You can add/remove time gate by clicking the +/-. sing. For the set time gate, a slider is displayed under curve and can also be used setting the time gate.

Save the Image by clicking the Save Image button.

Manual

You have the option here of manually configuring the Phasor mask.



Select the ①τSTED and ②Manual method. The area on the right shows the Phasor Plot of FLIM image. The line corresponds to the basis for calculating τSTED.

Configure the desired settings③.

Add black and white color coding to the FLIM image to show the intensities. To do so, select one of the tools from the toolbar④. A black and white LUT is stored for the tools.

Draw a connected line into the desired are of lifetime component⑤.

As the beginning point (Black), select a point with short lifetimes in the border area of the Phasor (a). Since longer lifetimes also occur in the background, the Phasor shows curvature to the left. Therefore, set your beginning point in the background area. Then draw a line along the phasor and, as the end point (White), select a point with long lifetimes in the border area of the Phasor(b).

The display window will show the τSTED as an intensity image in grayscale, in other words, the longer lifetime, the brighter the corresponding pixels⑥.

Save the Image by clicking the Save Image button⑥.