

**How to select ROIs in NovaFLIM
based on parameter - histograms.**

2

On the left-hand side one can see all the available layers. The number of layers and the type of the analysis. New image displays are created simply by dragging the layer to the main area of the window.

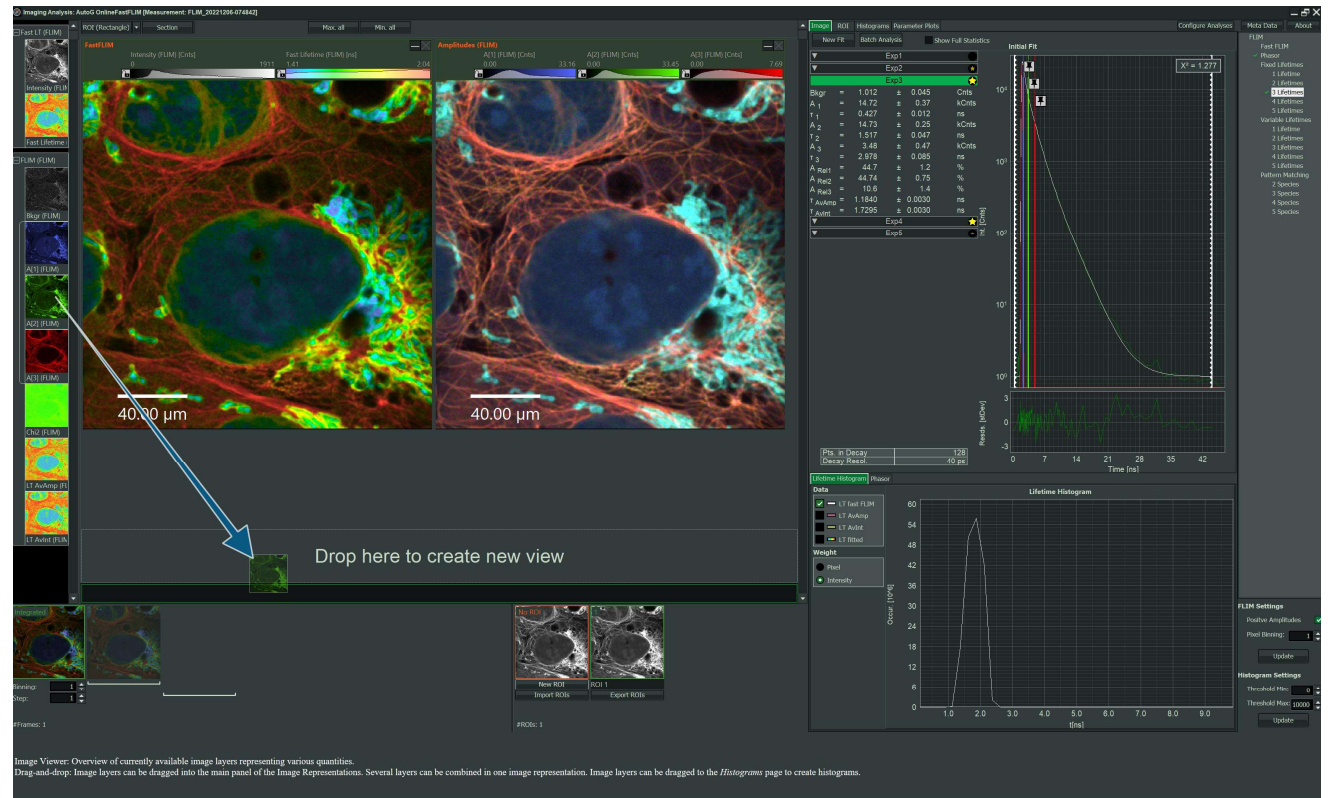


Image Viewer: Overview of currently available image layers representing various quantities. Drag-and-drop: Image layers can be dragged into the main panel of the Image Representations. Several layers can be combined in one image representation. Image layers can be dragged to the Histograms page to create histograms.

3

Add additional layer in a similar way.

The screenshot displays the Image Analysis software interface. The main window shows a multi-layered fluorescence image of cells, with a 40.00 μm scale bar. The image is composed of several layers, including FastFLIM, Intensity (FLIM) [Cnts], Amplitudes (FLIM), and various RCFs. A blue arrow points to a layer in the left sidebar with the text "Drop here to add". Below the main image, there is a section labeled "Drop here to create new view".

On the right side, there is a "Parameter Table" with the following data:

| Exp1 | Exp2 |
|--|------|
| Signal = 1.012 ± 0.045 Cnts | |
| A ₁ = 14.72 ± 0.37 kCnts | |
| τ ₁ = 0.427 ± 0.012 ns | |
| A ₂ = 14.73 ± 0.25 kCnts | |
| τ ₂ = 1.517 ± 0.047 ns | |
| A ₃ = 3.48 ± 0.47 kCnts | |
| τ ₃ = 2.978 ± 0.095 ns | |
| A _{Fast} = 44.7 ± 1.2 % | |
| A _{Rel2} = 44.74 ± 0.75 % | |
| A _{Rel3} = 10.0 ± 1.4 % | |
| T _{Match} = 1.1840 ± 0.0030 ns | |
| T _{A_{Fast}} = 1.7295 ± 0.0030 ns | |

Below the parameter table, there is a "Lifetime Histogram" plot showing the distribution of lifetimes. The x-axis is "Time (ns)" ranging from 0 to 9.0, and the y-axis is "Counts [10³]" ranging from 0 to 60. The histogram shows a peak at approximately 2.0 ns.

At the bottom right, there are "FLIM Settings" and "Histogram Settings" panels. The "FLIM Settings" panel includes "Fixed Binning" set to 1. The "Histogram Settings" panel includes "Histogram Method" set to 0 and "Threshold Min" set to 10000.

At the bottom of the interface, there is a "Data" section with a list of layers: "Fast FLIM", "LT AvAmp", "LT AvAmp", and "LT Fitted". Below this, there is a "Weights" section with "Fit" and "Intensity" options. At the bottom, there is a "Frames" section with "New RCF", "Import RCFs", and "Export RCFs" buttons.

Image Viewer: Overview of currently available image layers representing various quantities.
Drag-and-drop: Image layers can be dragged into the main panel of the Image Representations. Several layers can be combined in one image representation. Image layers can be dragged to the Histograms page to create histograms.

4

Use the scroll bar for reviewing various images.

Image Viewer: Overview of currently available image layers representing various quantities. Drag-and-drop: Image layers can be dragged into the main panel of the Image Representations. Several layers can be combined in one image representation. Image layers can be dragged to the *Histograms* page to create histograms.

5

Click and drag left or right to adjust contrast of each layer.

The screenshot displays the PicoQuant Image Analysis software interface. The main window is titled "Image Analysis: Kaski Online/FLIM [Measurement: FLIM_20211206-074547]". The interface is divided into several panels:

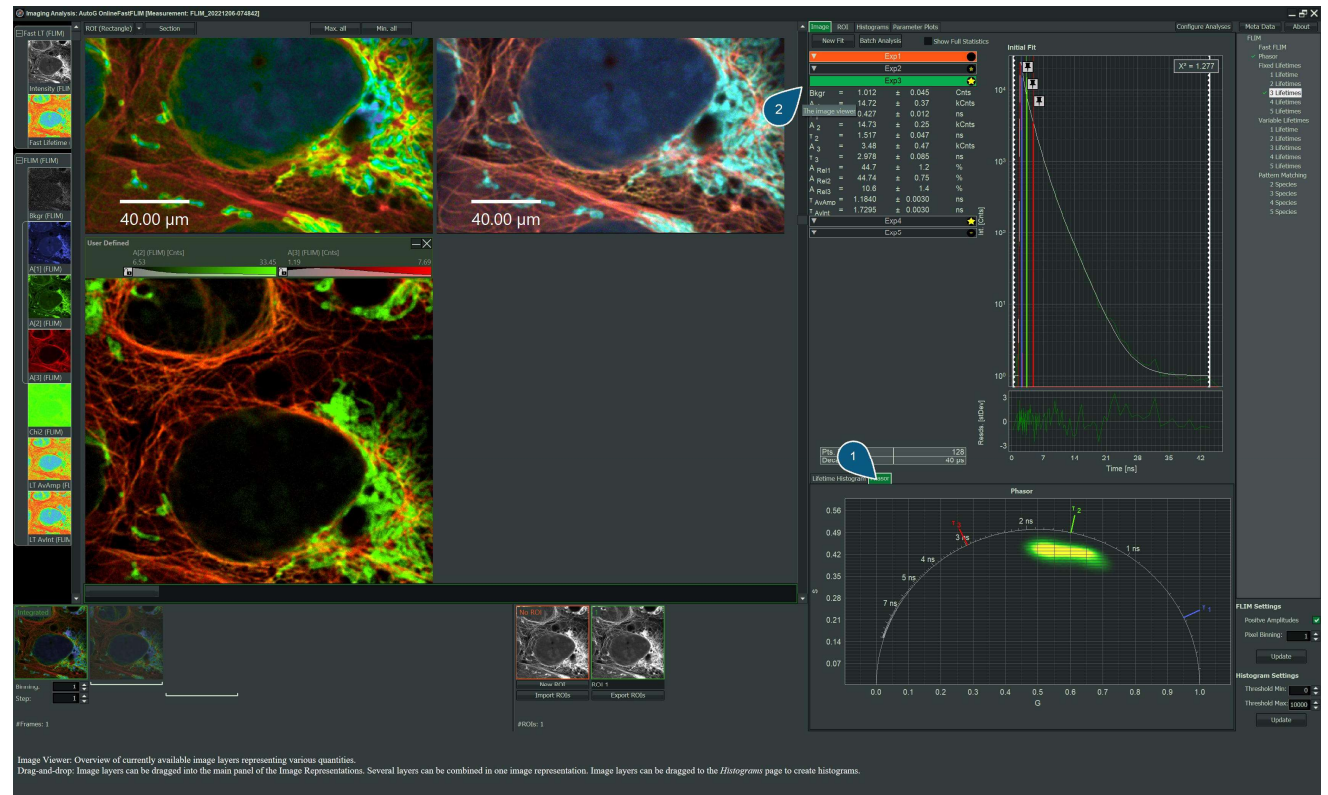
- Image Viewer:** The central area shows a large image of a cell with a blue nucleus and green cytoplasm. A blue arrow points to a "User Defined" contrast slider for the "AZI (FLIM) [Ch2]" layer, which is currently set to 33.45. Other layers include "AZI (FLIM) [Ch1]" (0.00), "AZI (FLIM) [Ch3]" (7.60), "Ch2 (FLIM)", "LT AuAmp (FL)", and "LT AuStd (FLM)".
- Image Layers:** A vertical sidebar on the left lists available layers: "Fast (FLM)", "Intensity (FLM)", "Bgr (FLM)", "AZI (FLM)", "AZI (FLM)", "AZI (FLM)", "Ch2 (FLM)", "LT AuAmp (FL)", and "LT AuStd (FLM)".
- Analysis Parameters:** A table on the right lists parameters for two experiments (Exp1 and Exp2):

| Parameter | Exp1 | Exp2 |
|------------------|----------------------|------|
| Bkg | 1.012 ± 0.046 Counts | |
| A ₁ | 14.72 ± 0.37 kCounts | |
| τ ₁ | 0.427 ± 0.012 ns | |
| A ₂ | 14.73 ± 0.28 kCounts | |
| τ ₂ | 1.517 ± 0.047 ns | |
| A ₃ | 3.48 ± 0.47 kCounts | |
| τ ₃ | 2.978 ± 0.085 ns | |
| A _{Fit} | 44.7 ± 1.2 % | |
| A _{Res} | 44.74 ± 0.75 % | |
| χ ² | 10.8 ± 1.4 % | |
| T _{Fit} | 1.1840 ± 0.0030 ns | |
| T _{Ave} | 1.7295 ± 0.0030 ns | |
- Histograms:** The bottom right shows an "Initial Fit" plot (log-linear) and a "Lifetime Histogram" (linear-linear) with a peak at approximately 2.0 ns.
- Settings:** The bottom right includes "FLIM Settings" (Positive Amplitudes checked, Pixel Binning: 1) and "Histogram Settings" (Threshold Min: 0, Threshold Max: 10000).

Image Viewer: Overview of currently available image layers representing various quantities. Drag-and-drop: Image layers can be dragged into the main panel of the Image Representations. Several layers can be combined in one image representation. Image layers can be dragged to the *Histograms* page to create histograms.

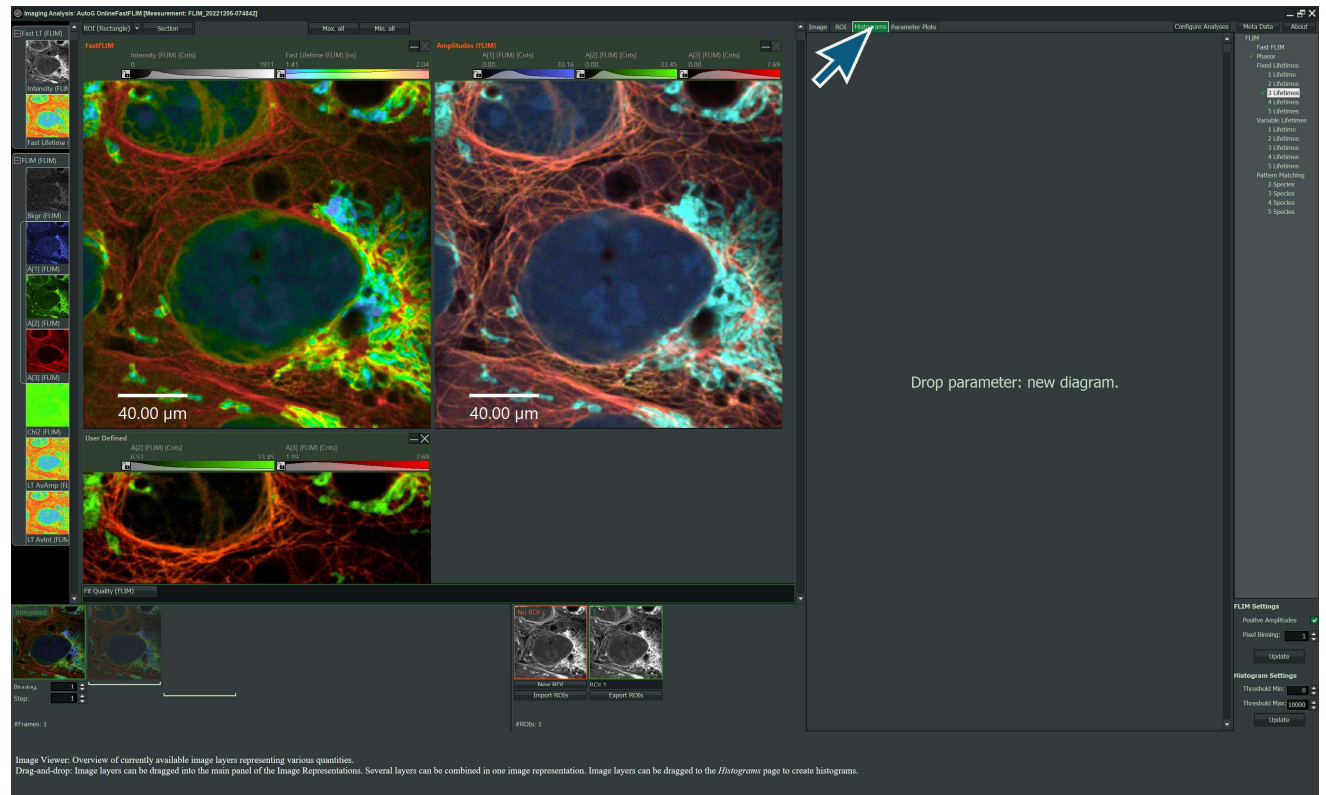
7

1. The 'Phasor' - plots are available by clicking on the tab.
2. On top there is the multi-exponential fit.



8

Switch to tab 'Histograms'. Here you can produce histograms based on the available image layers and their corresponding parameters.



9

Drag for example the Intensity layer and check how an 1-D histogram is created.

The screenshot displays the PicoQuant Image Analysis software interface. The main window is titled "Image Analysis: AutoG OnlineFastFLM [Measurement: FLM_2021026-074840]". It features a central panel with two side-by-side microscopy images of cells, each with a 40.00 µm scale bar. The left image is labeled "FastFLM" and the right "Amplitude (FLM)". A third image, "User Defined", is visible below them. A blue arrow points from the "Intensity (FLM) [Cntr]" layer in the left sidebar to a histogram window on the right. The histogram window is titled "Image: ROI: Histograms" and contains a 1-D histogram of the selected layer. A text label "Drop parameter: new diagram." is positioned above the histogram. The interface includes a sidebar on the left with a list of image layers (Intensity, Fast Lifetime, Bgr, AZ1, AZ2, AZ3, Ch2, LT AvAmpl, LT AvStd), a top menu bar with "Max. all" and "Min. all" options, and a bottom status bar with system icons and the date "10/25/2024".

Drop here to add

Drop here to create new view

Drop parameter: new diagram.

Image Viewer: Overview of currently available image layers representing various quantities.
Drag-and-drop: Image layers can be dragged into the main panel of the Image Representations. Several layers can be combined in one image representation. Image layers can be dragged to the *Histograms* page to create histograms.

10

Create new histograms by drag-and-drop function.



II

You can create 2D Histogram by drag-and-drop a layer in the y axis of an pre-existing 1D histogram.

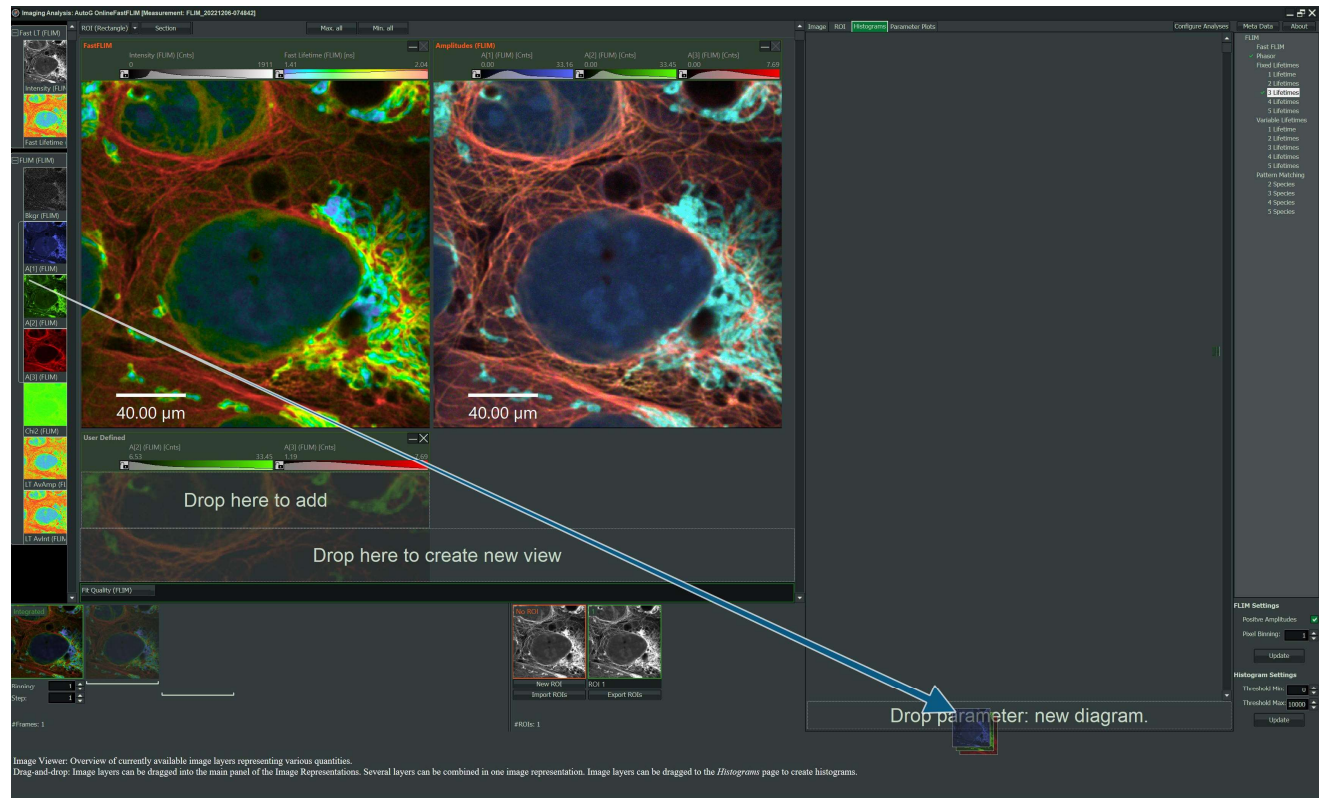
The screenshot displays the PicoQuant Image Analysis software interface. The main window is titled "Image Analysis: AxioG Observer-FastFLM [Measurement: FLIM_20211208-074842]". The interface is divided into several panels:

- Left Panel:** A vertical list of image layers including "FastFLM", "Fast Lifetime", "Rgr (FLIM)", "A2J (FLIM)", "A2I (FLIM)", "A2V (FLIM)", "Ch2 (FLIM)", "LT Ax Amp (I)", and "LT Ax Int (FLIM)".
- Main Panel:** Two side-by-side image representations of cells. The left image is labeled "FastFLM" and the right is labeled "Amplitude (FLIM)". Both images include a scale bar of "40,00 μm". Below the images are color calibration bars for "Intensity (FLIM) [Cntr]", "Fast Lifetime (FLIM) [ns]", "A2J (FLIM) [Cntr]", "A2I (FLIM) [Cntr]", "A2V (FLIM) [Cntr]", and "User Defined".
- Right Panel:** Two "Histogram" windows. The top histogram is titled "Histogram" and has a y-axis labeled "Drop: add param.". The bottom histogram is also titled "Histogram" and has a y-axis labeled "Drop: add param.". Both histograms show a distribution curve. Below the histograms are labels: "Drop: weight/ROI/Frame" and "Drop parameter: new diagram."
- Bottom Panel:** A "Histogram Settings" panel with options for "Fixed Binning" (set to 1), "Threshold Min" (set to 0), and "Threshold Max" (set to 2000). There are also "Update" buttons.

At the bottom of the software window, there is a status bar with the text: "Image Viewer: Overview of currently available image layers representing various quantities. Drag-and-drop: Image layers can be dragged into the main panel of the Image Representations. Several layers can be combined in one image representation. Image layers can be dragged to the Histograms page to create histograms."

13

One can create multiple histograms by drag-and-drop a group of layers.



14

Double-click on the area of the 2D histogram or on the area of the 1D histograms. For the case of 2D histograms one can adjust the min and max of both parameters. For the 1D case one gets the min max of 1 parameter.

Histogram Settings
 Min: 1.362 Max: 172.000
 Min: 2.213 Max: 1717.000
 Bits: 200 Bins: 386

Histogram
 Weight: none; Frames: Integrated; ROI: none
 Fast Lifetime (FLIM) [ns]
 Intensity (FLIM) [Cnts]

Histogram
 Weight: none; Frames: Integrated; ROIs: none
 Fast Lifetime (FLIM) [ns]
 Intensity (FLIM) [Cnts]

Histogram Settings
 Positive Amplitudes
 Pixel Binning: 1
 Update
 Histogram Settings
 Threshold Min: 0
 Threshold Max: 10000
 Update

Histograms: Create histograms from arbitrary image layers. To generate a 2D histogram, generate a 1D histogram, drag a second layer onto the y-axis. Double click on the histogram to change its settings. Right click to export. (Please note: Histograms are not saved.)

- Drag-and-drop: Any image layer from the Image Viewer (left) can be dragged into the Histograms panel.
- Weight/ROI/Frame: Drag-and-drop an element onto the graph to modify the histogram: Image layer: Apply as weight; ROI: Include only pixels contained in the ROI; Frames: Include only the subsection of frames.
- ROI selection in histogram: Selection options are available in histograms via right-click. Multiple ROIs are combined by an AND-operation.
- Zoom: Use the mouse wheel for zooming into images. Press the middle mouse button/wheel to move image section.

15

Adjust the limits and click OK.

The screenshot displays the PicoQuant Imaging Analysis software interface. The main window shows a multi-panel view of fluorescence lifetime images. On the left, a vertical toolbar contains various analysis tools like 'Fast FLIM', 'Biop (FLIM)', 'AZ1 (FLIM)', 'AZ2 (FLIM)', 'AZ3 (FLIM)', 'Ch2 (FLIM)', 'LT AnvImp (FLIM)', and 'LT AnvInt (FLIM)'. The central area shows two side-by-side images of a cell with a 40.00 μm scale bar. A 'Histogram Settings' dialog box is open over the right image, with a blue arrow pointing to the 'OK' button. The dialog has fields for X and Y axes with 'Min' and 'Max' values. The X-axis 'Min' is 1.362 and 'Max' is 172.000. The Y-axis 'Min' is 2.213 and 'Max' is 7717.000. The 'Bin' is set to 200. Below the images, there are 'ROI' and 'ROI2' panels with 'New ROI', 'Import ROIs', and 'Export ROIs' buttons. On the right side, a 'Histogram' panel shows a 2D histogram of Intensity [FLIM] [Cts] vs Fast Lifetime (FLIM) [ns]. Below it, three 1D histograms are shown for A[1] (FLIM) [Cts], A[2] (FLIM) [Cts], and A[3] (FLIM) [Cts]. The bottom right corner contains 'FLIM Settings' and 'Histogram Settings' panels with various checkboxes and numerical values.

Image: ROI: Histogram Parameter: FLIM

Weight: none; Frames: Integrated; ROI: none

Fast FLIM Phase

Fixed Lifetime: 1 Lifetime

Variable Lifetime: 2 Lifetime

Variable Lifetime: 1 Lifetime

Variable Lifetime: 2 Lifetime

Variable Lifetime: 3 Lifetime

Variable Lifetime: 4 Lifetime

Variable Lifetime: 5 Lifetime

Platform Matching

2 Spectra

3 Spectra

4 Spectra

5 Spectra

FLIM Settings

Positive Amplitudes:

Pixel Binning: 1

Update

Histogram Settings

Threshold Min: 0

Threshold Max: 10000

Update

Histograms: Create histograms from arbitrary image layers. To generate a 2D histogram, generate a 1D histogram, drag a second layer onto the y-axis. Double click on the histogram to change its settings. Right click to export. (Please note: Histograms are not saved.)

- Drag-and-drop: Any image layer from the Image Viewer (left) can be dragged into the Histograms panel.
- Weight/ROI/Frame: Drag-and-drop an element onto the graph to modify the histogram: image layer: Apply as weight; ROI: Include only pixels contained in the ROI; Frames: Include only the subsection of frames.
- ROI selection in histogram: Selection options are available in histograms via right-click. Multiple ROIs are combined by an AND-operation.
- Zoom: Use the mouse wheel for zooming into images. Press the middle mouse button/wheel to move image section.

17

Right click on any point of the 2D histogram. Select 'Range ROI' from the menu.

The screenshot displays the PicoQuant Imaging Analysis software interface. The main window shows a 2D histogram with a right-click context menu open over it. The menu options are: ROI Selection Off, Range ROI, Rectangle ROI, Ellipse ROI, Layer ROI, Layer ROI, ROI, ROI, and Range ROI. The histogram shows intensity (Counts) on the y-axis and Fast Lifetime (FLIM) [ns] on the x-axis. Below the histogram, there are three 1D histograms for channels A[1], A[2], and A[3]. The software interface also includes a list of layers on the left, a 'Fast FLIM' section at the top, and a 'Histogram' section on the right with various settings.

Fast FLIM
Phase
Fixed Lifetime: 1 Lifetime
2 Lifetime
3 Lifetime
Variable Lifetime: 1 Lifetime
2 Lifetime
3 Lifetime
4 Lifetime
5 Lifetime
Pattern Matching
2 Spectra
3 Spectra
4 Spectra
5 Spectra

FLIM Settings
Positive Amplitudes
Pixel Binning: 1
Update

Histogram Settings
Threshold Min: 0
Threshold Max: 10000
Update

Fast FLIM
Intensity (FLIM) [Cts]
Fast Lifetime (FLIM) [ns]
Amplitudes (FLIM)
A[1] (FLIM) [Cts]
A[2] (FLIM) [Cts]
A[3] (FLIM) [Cts]
User Defined
A[2] (FLIM) [Cts]
A[3] (FLIM) [Cts]
Fit Quality (FLIM)

Weight: none; Frames: Integrated; ROI: none
Histogram
Intensity (FLIM) [Cts]
Fast Lifetime (FLIM) [ns]

Weight: none; Frames: Integrated; ROI: none
Histogram
Intensity (FLIM) [Cts]
Fast Lifetime (FLIM) [ns]

Weight: none; Frames: Integrated; ROI: none
Histogram
Intensity (FLIM) [Cts]
Fast Lifetime (FLIM) [ns]

#Frames: 1
Histograms: Create histograms from arbitrary image layers. To generate a 2D histogram, generate a 1D histogram, drag a second layer onto the y-axis. Double click on the histogram to change its settings. Right click to export. (Please note: Histograms are not saved.)

- Drag-and-drop: Any image layer from the Image Viewer (left) can be dragged into the Histograms panel.
- Weight/ROI/Frame: Drag-and-drop an element onto the graph to modify the histogram: image layer: Apply as weight; ROI: Include only pixels contained in the ROI; Frames: Include only the subsection of frames.
- ROI selection in histogram: Selection options are available in histograms via right-click. Multiple ROIs are combined by an AND-operation.
- Zoom: Use the mouse wheel for zooming into images. Press the middle mouse button/wheel to move image section.

18

Select an area in the 2D histogram and see the changes in the active ROI. Pixels belonging to this selection of the 2D histogram get selected for the currently active ROI and are highlighted in red.

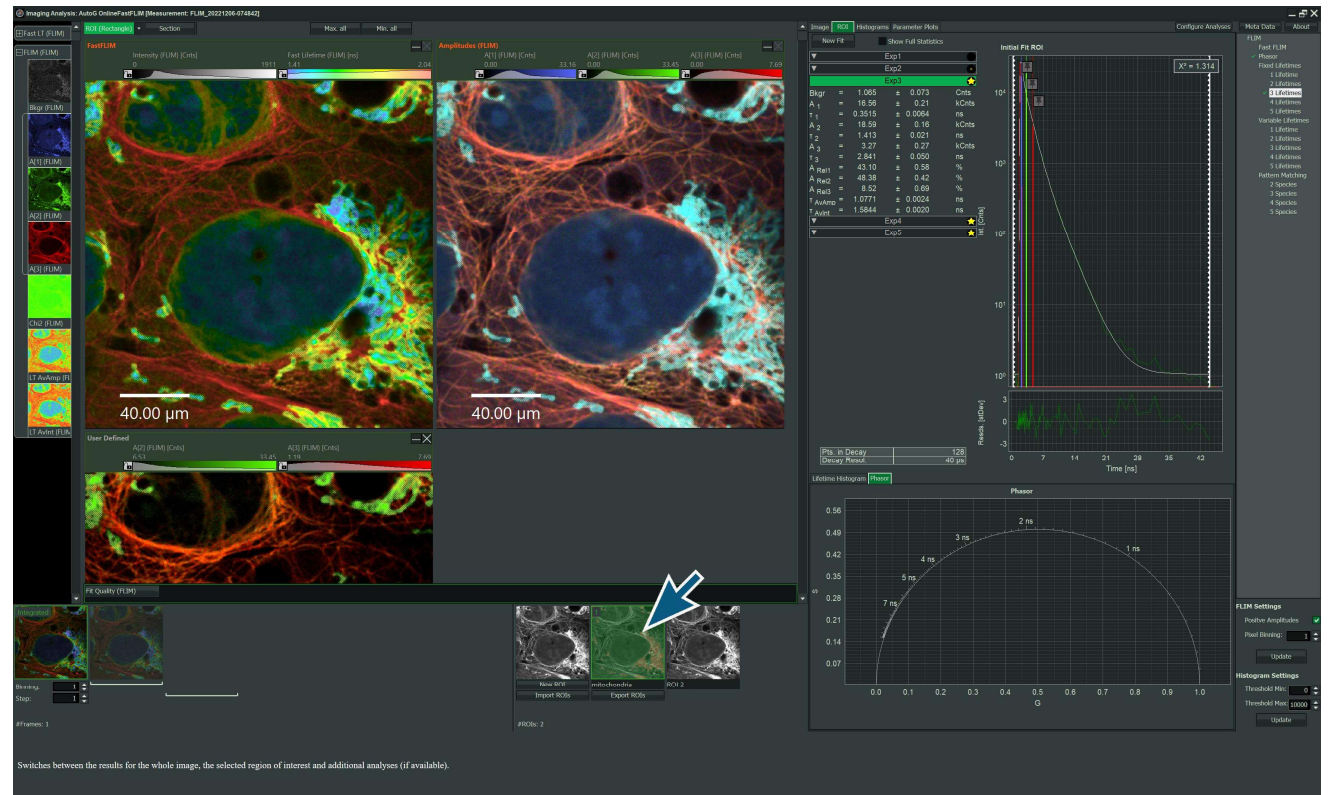
The screenshot displays the PicoQuant software interface. On the left, there are several image panels: 'FastFLIM', 'Amplitude (FLIM)', and 'User Defined'. The 'FastFLIM' panel shows a 2D histogram of Intensity (FLIM) [Cnts] vs. Fast Lifetime (FLIM) [ns]. A blue selection box is drawn on the histogram, and a red arrow points from this selection to the 'User Defined' panel, where the corresponding ROI is highlighted in red. Below the 'User Defined' panel, there are three 1D histograms labeled 'A[1] (FLIM) [Cnts]', 'A[2] (FLIM) [Cnts]', and 'A[3] (FLIM) [Cnts]'. The 'A[2]' histogram is highlighted in green, corresponding to the selection in the 2D histogram. The right side of the interface shows the 'Histogram' panel with various settings and a 'FLIM Settings' panel.

Histograms: Create histograms from arbitrary image layers. To generate a 2D histogram, generate a 1D histogram, drag a second layer onto the y-axis. Double click on the histogram to change its settings. Right click to export. (Please note: Histograms are not saved.)

- Drag-and-drop: Any image layer from the Image Viewer (left) can be dragged into the Histograms panel.
- Weight/ROI/Frame: Drag-and-drop an element onto the graph to modify the histogram: Image layer: Apply as weight; ROI: Include only pixels contained in the ROI; Frames: Include only the subselection of frames.
- ROI selection in histogram: Selection options are available in histograms via right-click. Multiple ROIs are combined by an AND-operation.
- Zoom: Use the mouse wheel for zooming into images. Press the middle mouse button/wheel to move image section.

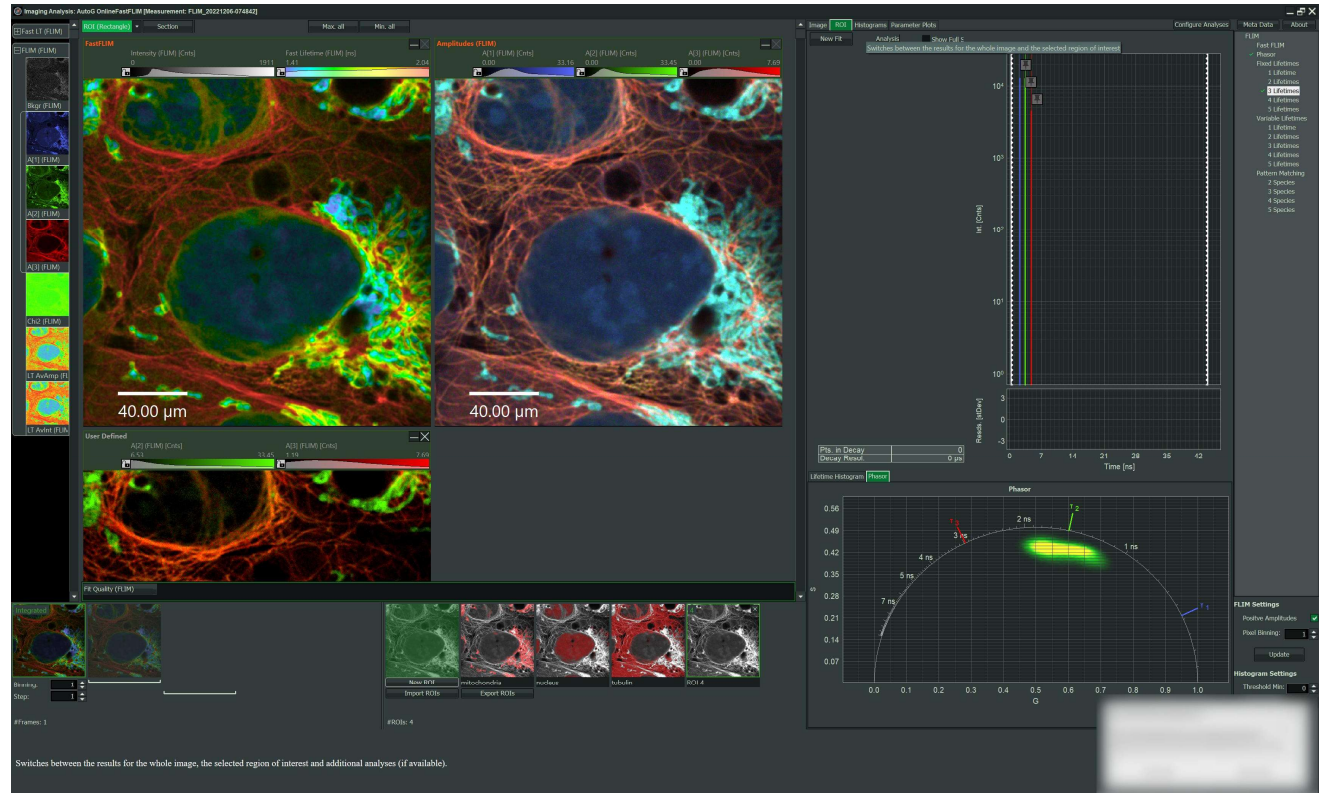
21

Double-click on the saved ROI and you will get the decay, fit and phasor plots corresponding to the ROI.



22

This process can be repeated more times.



23

By doubleclicking in any ROI , one can quickly inspect the corresponding phasor plot.

