Lambert

Toggel

Fluorescence lifetime imaging camera

Software version 1.3.0

Contents

1. Toggel

A next-generation fluorescence lifetime imaging microscopy (FLIM) camera. Toggel simplifies FLIM for researchers and imaging centers by combining excellent light sensitivity with easy image acquisition and data analysis.

Toggel features a unique image sensor that combines excellent light sensitivity with advanced fluorescence lifetime imaging capabilities. This image sensor was designed and optimized specifically for fluorescence lifetime imaging applications and enables lifetime imaging at unprecedented frame rates with the single-image fluorescence lifetime imaging microscopy (siFLIM) method.

This manual describes the hardware and software of the Toggel. The software version described in this manual is LIFA 1.3.0.

Please read this manual carefully before using Toggel. If you have any questions that are not answered by this manual, then please don't hesitate to contact Lambert Instruments.

Lambert ▰▰▰▰▰

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2. Hardware

2.1 Hardware interfaces

Figure 2.1: Toggel hardware interfaces.

- *Power*: Power supply. Only use the power supply that shipped with the Toggel.

- *Aux*: Auxiliary port.

- *Mod out*: Modulation out signal 0 - 5 V 50 Ω.

- *Mod in*: Modulation in.

- *Trig in*: Trigger in.

- *Data*: Camera Link interface for camera communication and image data transfer.

2.2 Chiller

The Toggel can optionally be temperature stabilized with a water-based temperature controller system. The connectors at the sides of the camera (see figure 2.1) can be used to connect your chiller to the Toggel.

> **Warning!**
>
> The water temperature must be above dew point! Setting the water temperature below dew point may result in condensation on the camera electronics.

3. Software installation

3.1 Installing LIFA on a new computer

To install the Lambert Instruments FLIM Attachment (LIFA) software on a new computer, please follow the steps below:

1. Install the Camera Link frame grabber card in the computer.

2. Install *vc_redist.x64.exe* which is located in the folder *1. Microsoft Visual C++ 2017 Redistributable (x64)* on the USB drive that shipped with the Toggel.

3. Install *Sapera_LT_8.20_SDK.exe* from the folder *2. Sapera SDK*. When presented with the choice between Camera Link and Gig-E during installation, choose only the Camera Link frame grabber option.

4. Run *setup.exe* from the folder *3. Xcelera-CL_LX1_101* and follow the installation instructions.

5. In the Windows Start menu, select *Teledyne DALSA Sapera LT → Sapera Configuration*.

6. In *Sapera Configuration*, in the *Serial Port Configuration* panel, select the following settings:

   - Physical port: Xcelera-CL_LX1_1_Serial_0
   - COM port mapping: COM27
   - Teledyne DALSA camera detection: Genicam GenCP for CameraLink
   - Baudrate: 115200

7. After applying the settings in *Sapera Configuration* you may need to restart the computer for the settings to be applied.

8. Run *setup.exe* from the folder *4. LIFA-1.2* and follow the installation instructions.

3.2 Installing software updates

Before installing a software update, please first uninstall the Lambert Instruments LIFA software:

1. Right click the Windows logo in the bottom left corner of the screen and select *Apps and Features* in the menu that appears.

2. Scroll down in the list until you see *LIFA* by Lambert Instruments in the list and left click on it.

3. Click the button *Uninstall* that has appeared and follow the instructions in the screen that appears.

Now you can install the new version of the software by double clicking the file *setup.exe*. The LIFA Setup Wizard will appear to guide you through the steps required to install LIFA on your computer. Click *Next* to proceed to the selection of the installation folder. After choosing your preferred installation folder, click *Next* twice to start the installation process. Once the installation process has completed, please click *Close* to finish. You can now start LIFA by clicking the shortcut on your desktop.

3.3 Frame grabber drivers

If you need to reinstall the frame grabber drivers then please refer to steps 3-7 in section 3.1 of this manual.

3.3.1 Known issue: Recovering from standby

The frame grabber drivers sometimes cause the computer to become unresponsive after the computer has been in standby mode. When this happens, Windows may show an error screen and reboot. For this reason, it is recommended to disable automatic standby mode:

1. Right click the Windows logo in the bottom left corner of the screen and select *Power Options*.

2. In the section *Sleep*, select the option *Never*.
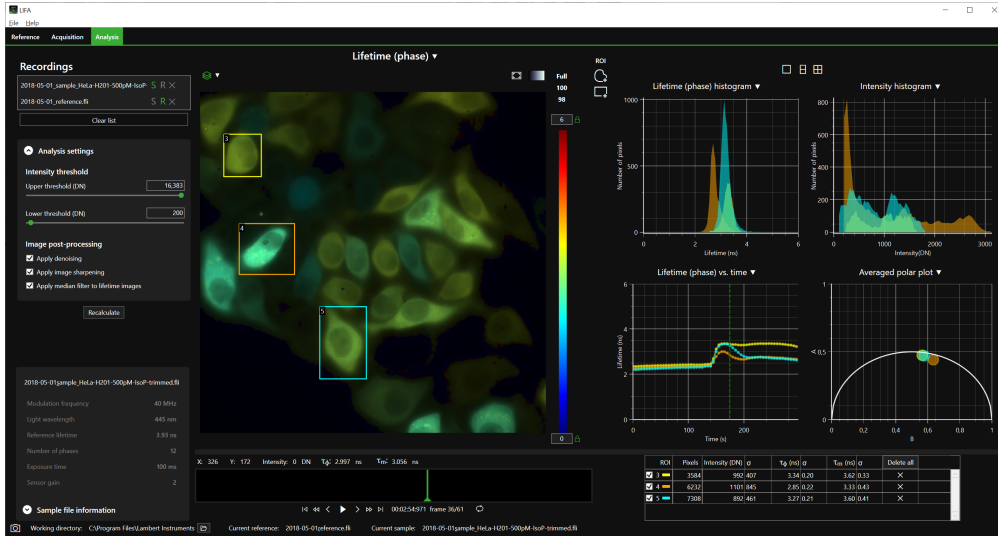
4. Software overview

Figure 4.1: Overview of the LIFA software.

4.1 Hardware communication

When the software is started, it will initiate communication with the frame grabber and the camera. The status of this communication is shown in the bottom left corner of the screen (see figure 4.2). Initially, the camera icon will be gray. Once the camera is connected, the camera icon will turn green to indicate that it is ready to start recording.
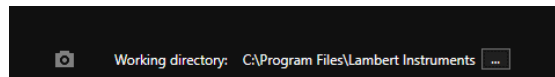

Figure 4.2: Camera status (left) and current working directory (right) at the bottom of the screen.

4.2 Working directory

Your current working directory is shown at the bottom of the screen. If you have autosave enabled (see section 4.6), then all files you recorded will automatically be saved in this directory. If you save a file using the menu *File -> Save sample* or *Save reference*, then the dialog box will start in the current working directory.

You can change the working directory by clicking the button ... next to the working directory at the bottom of the screen (see figure 4.2).

4.3 Reference

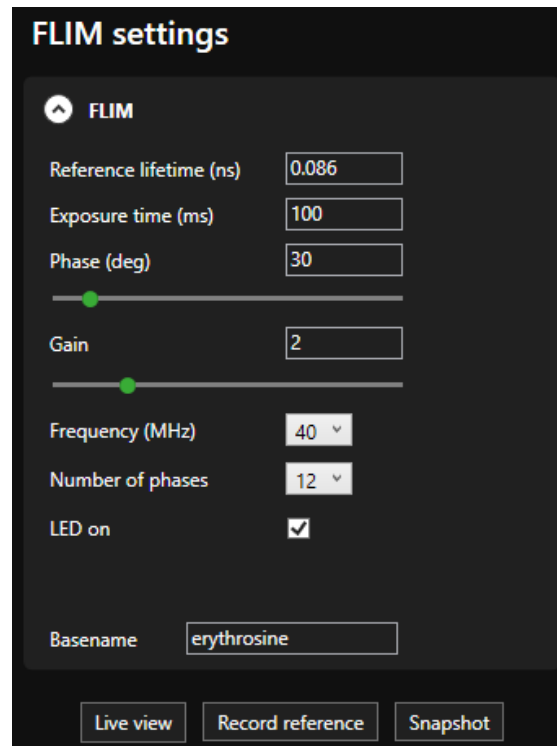In the Reference tab you can record references and review the recorded image data.

Figure 4.3: FLIM settings.

4.3.1 Camera settings

On the left side of the screen you can adjust the camera settings and FLIM parameters:

- *Reference lifetime (ns)*

- *Exposure time (ms)*: Please note that the minimum exposure time is 34 ms.

- *Phase (deg)*: The phase shift in units of degrees between the pixel toggling and the modulation output signal in the range 0 - 345 degrees in steps of 15 degrees. You can either enter the desired phase in the textbox or use the slider below it to adjust the phase. The selected phase is applied immediately.

- *Gain*: CCD gain applied during the conversion from electrons to digital numbers (DN). Available values are 1, 2, 4, 8 and 16.

- *Frequency (MHz)*: The toggling frequency of the pixels. You can enter the desired value in the textbox. Currrently, the pixel toggling only functions properly at 20 MHz and 40 MHz.

- *Number of phases*: Total number of phases that will be recorded during image acquisition.

- *LED on*: You can use this checkbox to manually switch the modulation out signal on or off. This can be useful when you want to look at your sample through the eyepiece of the microscope.

- *Basename*: This is where you can enter a couple of words that describe your recording. The basename will be inserted into the filename along with any options you have selected in the automatic filename format preferences (see section 4.6).

4.3.2 Live view

You can start or stop the live view by clicking the *Live view* button (see figure 4.3). You can use the scroll wheel on your mouse to zoom in or out on the image. To restore the zoom level to the default setting, please click the squared circle at the top right corner of the image.

4.3.3 Record reference

Records a reference image stack with the selected settings (see figure 4.3).

4.3.4 Snapshot

Records a single image with the selected settings (see figure 4.3). Please note that snapshots are not saved automatically, even when *Automatically save recordings* is enabled in the preferences screen. Snapshots also don't show up in the file list on the *Analysis* tab. To save a snapshot, you can right click the image and select *Save image* in the context menu that appears. You can also select the option *Save snapshot as .fli file* from the *File* menu to save the snapshot as a .fli file.

4.3.5 Saving FLIM data

If you have enabled *Automatically save recordings* (see section 4.6), then your data will be saved automatically to the working directory you have selected. If *Automatically save recordings* is not enabled, then you can save a recording by opening the *File* menu and selecting *Save Sample* or *Save Reference*.

4.4 Acquisition

In the Acquisition tab you can record and review samples, time-lapses and siFLIM time-lapses.

4.4.1 Camera settings

The camera settings on the Acquisition tab are synchronized with the camera settings on the Reference tab. So if you change the exposure time on the Acquisition tab, then that setting will be applied to the Reference tab as well.

4.4.2 Live view

You can start or stop the live view by clicking the *Live view* button.

4.4.3 Record sample

Records a sample image stack with the selected settings.

4.4.4 Snapshot

Records a snapshot with the selected camera settings. Note that snapshots are not saved automatically. To save a snapshot, you can right click the snapshot and choose between saving the image as it appears on the screen or saving the raw light intensity values.

4.4.5 Time-lapse

You can let the software acquire FLIM image data with a specified interval between acquisitions by recording a time-lapse.
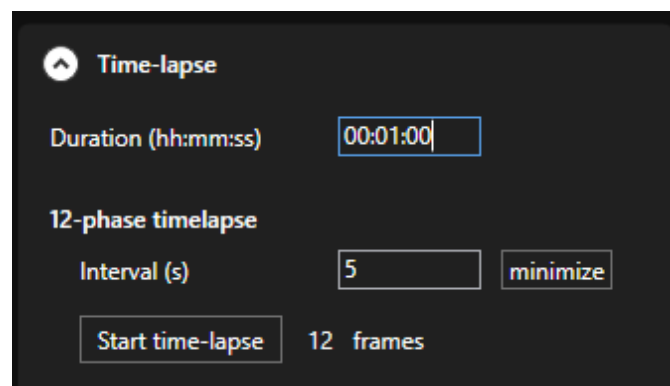

Figure 4.4: Toggel time-lapse settings.

- Duration (hh:mm:ss): The total duration of the time-lapse. For time-lapses longer than 24 hours, you can enter the duration in the format dd:hh:mm:ss. The software will automatically calculate the number of image stacks that will be recorded based on the interval you entered. You can also enter the duration in units of seconds. For example: If you enter 300 in the *Duration* textbox, then the software will convert it to 00:05:00 for you.

- Interval (s): The interval between the start of two image stack acquisitions. This interval includes the time it takes to record the image stack. So if you set the time-lapse interval to 10 seconds, then the acquisition of the each image stack starts 10 seconds after the acquisition of the previous image stack.

- Minimize: You can click the *minimize* button next to the interval textbox to let the software estimate the time it takes to record the image stack and enter that value as the interval.

4.4.6 siFLIM acquisition

siFLIM acquisition settings are available in the *Time-lapse* panel on the left side of the screen in the *Acquisition* tab. During a siFLIM time-lapse, the camera will continuously record dual images without an interval between the images. You can adjust the number of frames in the siFLIM time-lapse by setting the duration of the time-lapse in the *Duration (hh:mm:ss)* field.[1]
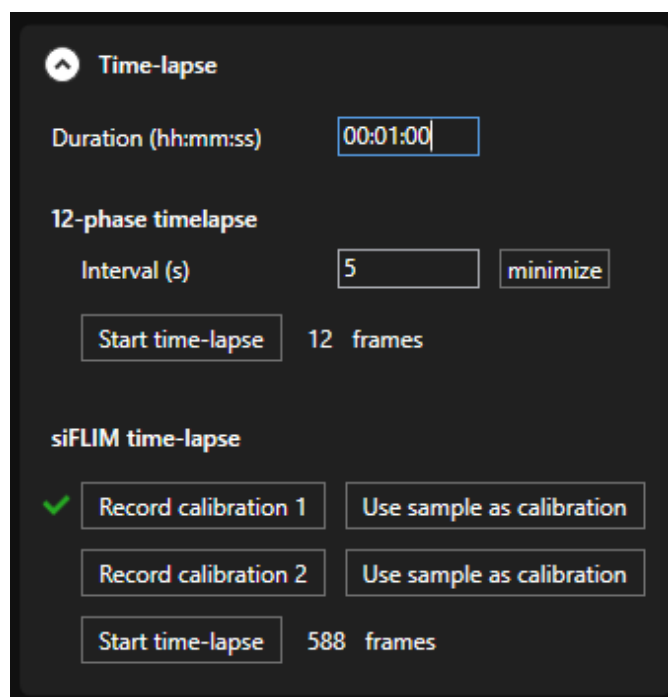
Figure 4.5: siFLIM settings and calibration files. The green check mark indicates that the calibration file has been loaded successfully.

Before starting a siFLIM time-lapse, you need to have loaded or recorded a reference file and two calibration files. You can follow the steps below to record the required files:

1. Choose a reference with a known fluorescence lifetime. Erythrosine has a fluorescence lifetime of 0.086 ns and is a good reference because it has excellent modulation.

2. Place the reference on the microscope.

3. In the LIFA software, go to the reference tab, enter the reference lifetime, optimize the camera settings and click *Record reference*.

4. Choose two calibration samples. The fluorescence lifetime of your calibration samples should be in the same range as your siFLIM sample. That is, their lifetime should ideally not differ more than 1.5 to 2 ns from the lifetime of your siFLIM sample. But the lifetime of your calibration samples should also not be identical.

   Example: If your siFLIM sample has a lifetime of approximately 2.5 ns, then you can use fluorescein (with a lifetime of 4.0 ns) and coumarin (2.6 ns). Alternatively you can use a fluorescent plastic that shipped with the Toggel as a calibration sample. However, these plastics usually have a multi-exponential decay which can lead to unexpected lifetime results. If you decide to use a plastic as a calibration sample, then keep in mind its lifetime can show some excitation wavelength dependency. That's why we recommend

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[1]You can also enter the duration in units of seconds. For example: if you enter 300 in the *Duration* textbox, then the software will convert it to 00:05:00 for you.

measuring the plastic sample lifetime with an erythrosine reference to make sure its lifetime is in the right range with the excitation light source you plan to use.

5. Place your first calibration sample on the microscope. The fluorescence lifetime of your calibration samples does not have to be known and you don't need to manually enter the fluorescence lifetime of your calibration samples in the software.

6. In the *Acquisition* tab of the LIFA software, optimize the camera settings and then click *Record calibration 1*. After recording the calibration measurement, a checkmark will appear next to the *Record calibration 1* button.

7. Repeat steps 5 and 6 for the second calibration sample but this time click the button *Record calibration 2*.

8. Place the siFLIM sample on the microscope, optimize the camera settings, adjust the time-lapse duration and then press *Start siFLIM time-lapse*. The software will automatically calculate the optimal siFLIM phase and will use that phase during siFLIM acquisition. If you want to manually set the siFLIM phase, then you can disable the automatic optimal phase calculation in the acquisition tab of the preferences screen (see section 4.6.2).

You can also set the recording that is currently selected as sample to be used as one of your calibration files by clicking *Use sample as calibration*.

If you have previously recorded files available that you would like to use as calibration files, then start by opening the desired file by opening the *File* menu and selecting *Open → Open Sample*. Once you have opened the file, you can set it to be a siFLIM calibration file by clicking *Use sample as calibration*.

Please note that the modulation frequency and number of phases of the calibration files should match the modulation frequency and number of phases of the reference file. If the calibration files match the reference file, then a green check mark will appear next to the *Record calibration 1/2* buttons.

To see which file is being used as a calibration file, hover your mouse over the *Record calibration 1/2* or *Use sample as calibration* buttons to make a tooltip with the selected filename appear.

When both calibration files have received a green check mark, you are ready to start recording a siFLIM time-lapse. During the siFLIM time-lapse, the *Intensity* image, the *Lifetime (phase)* image and graphs on the *Analysis* tab will be updated live.

4.5 Analysis

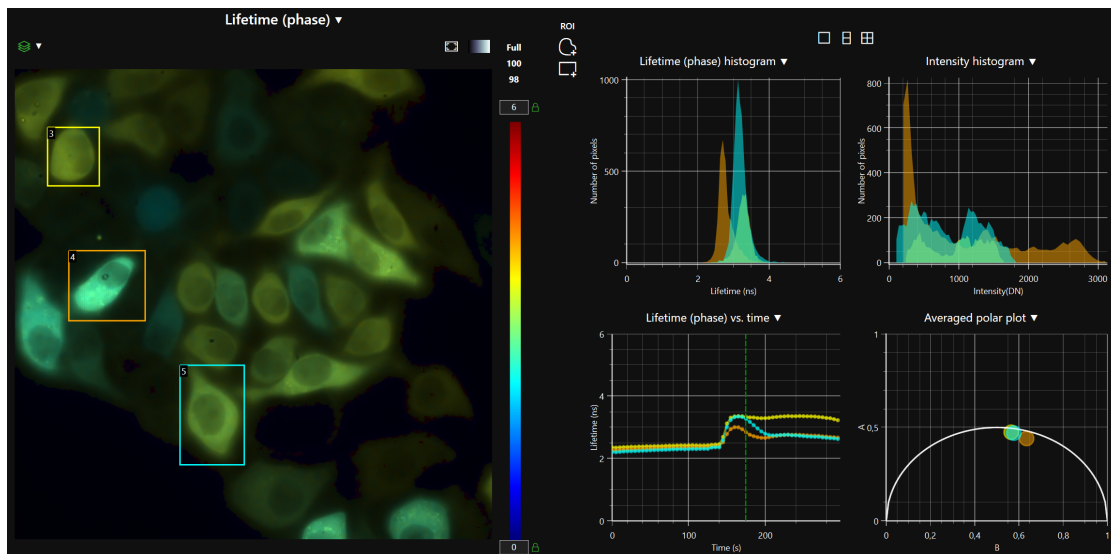In the analysis tab you can further analyze the image data you captured with the Toggel.

Figure 4.6: Fluorescence lifetime image and corresponding graphs.

4.5.1 Opening files

You can open a file that's stored on your computer by opening the *File* menu and choosing *Open → Open Reference* or *Open Sample*. These options can also be accessed by the keyboard shortcuts Ctrl + R (open reference) and Ctrl + S (open sample). For more information about keyboard shortcuts, please refer to section 4.7.

When opening a sample, you can also select multiple files in the selection dialog window and open them simultaneously. The files will then all be added to the file list.

To open a siFLIM time-lapse, start by opening a reference file. Next, open the *File* menu and select *Open Sample*. Navigate to the folder that contains your siFLIM time-lapse and open the file that contains the siFLIM data. You can recognize these files by the text *siflim data* in the filename and their larger file size. Opening a siFLIM file this way will automatically load the calibration files and perform the required lifetime calculations.

4.5.2 File list

The file list shows all the files that you have recorded or opened during the current session. You can select a file from the file list as your current reference file by clicking the *R* next to the filename. By clicking the *S* next to the filename, you can set the selected file as your current sample. This will show its data in the rest of the analysis tab.

To remove a file from the file list, click the × icon. This will remove the file form the files list, but the file will not be removed from your computer. You can also clear the entire file list by clicking the *Clear list* button.
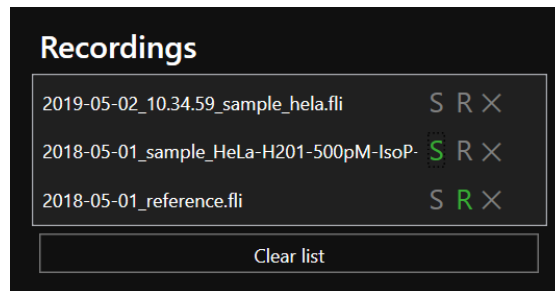
Figure 4.7: Currently selected files are indicated with green letters.

4.5.3 Image viewer
The image viewer can show the following images for your current sample:

- *Intensity*: Average light intensity of the images in the phase stack.

- *Lifetime (phase)*: Lifetime calculated from phase. You can switch between grayscale and pseudocolor by clicking the gradient button in the top right corner of the image. The lower and upper limits of the color scale can be adjusted by entering the desired values in the text boxes at the top and bottom of the gradient on the right side of the image.

  If you want to lock the limits of the color scale, then you can do so by clicking the lock icon next to the text box. When locked, the icon will change to a closed lock, its color will change to green and the lock values will also be applied to all relevant graphs. When unlocked, the icon will change to an open lock and its color will change to white.

- *Lifetime (modulation)*: Lifetime calculated from modulation.

- *Phase stack*: Full phase stack that was recorded. You can use the scroll bar below the image to scroll through the phases.

- *Background*: Raw background image.

Pseudocolor overlay
The image viewer can overlay the fluorescence lifetime from phase or modulation as a pseudocolor on the light intensity image. When you select *Lifetime (phase)* or *Lifetime (modulation)* in the image viewer, an icon will appear in the top left corner of the image viewer. When you click this icon, the fluorescence lifetime will be shown as a pseudocolor overlay on the light intensity image. After clicking the overlay icon, a panel will appear that lets you select the minimum and maximum light intensity for which to show a pseudocolor overlay. The overlay panel also contains the *Full*, *100* and *98* preset buttons to quickly pick a minimum and maximum light intensity for the overlay.
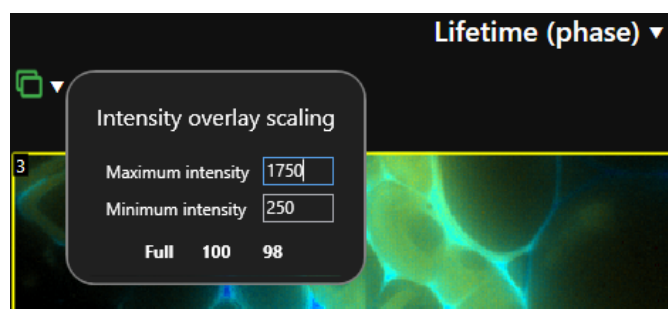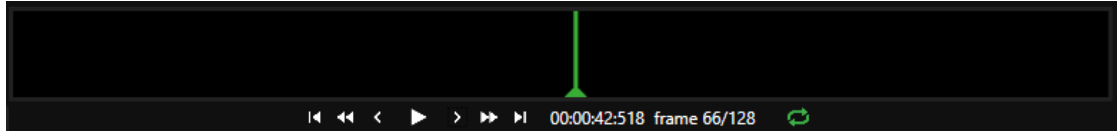

Figure 4.8: Pseudocolor overlay settings panel.

4.5.4 Timeline

When you are recording or analyzing a time-lapse, a timeline will appear below the image viewer.

Figure 4.9: Timeline of a time-lapse recording with a label showing the current frame information and playback controls.

You can use the repeat button to enable or disable repeat playback of the time-lapse. The other playback controls (from left to right in figure 4.9)

- Go back to the beginning of the time-lapse

- Go back 10% of the total duration of the time-lapse

- Go back 1 frame

- Play or pause the time-lapse

- Go to the next frame

- Skip forward 10% of the total duration of the time-lapse

- Skip to the end of the time-lapse

When recording a time-lapse, the green indicator will move right whenever a new stack has finished recording. You can click and drag this indicator to see the previous images in your time-lapse. While scrolling through a time-lapse this way, the indicator will not automatically update whenever a new phase stack has been recorded. When you drag the indicator all the way to the right, it will automatically move right again when a new phase stack has finished recording.

4.5.5 Region of interest (ROI) tools and statistics

To draw a region of interest (ROI), click one of the ROI tools at the top of the screen between the image viewer and the graphs area. There are three types of ROIs available:

- Freehand: Click and drag to draw a ROI.

- Rectangular: Click and drag to set the size of the rectangle.

- Polygon: Click in several locations in the image and the points will be connected automatically to create a ROI. To finish the ROI, click the first point again.
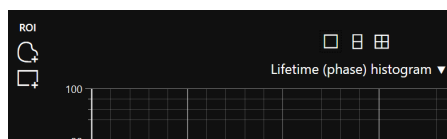

Figure 4.10: ROI and graph tools.

4.5.6 ROI statistics

The ROI statistics are shown in a table in the bottom right part of the analysis tab. It is not possible to move a ROI to a different part of the image. Please note that if a threshold is specified for the lifetime calculation, that the fluorescence lifetime will only be calculated for the pixels that have a light intensity that is between the upper and lower limits of the threshold.

ROI	Pixels	Intensity (DN)	σ	τ_ϕ (ns)	σ	τ_m (ns)	σ	Delete all
☑ 1 ▬	5544	1151	820	2.75	0.20	3.28	0.44	✕
☑ 2 ▬	7144	844	445	3.19	0.25	3.55	0.49	✕
☑ 3 ▬	4140	910	421	3.31	0.22	3.62	0.36	✕

Figure 4.11: ROI statistics table.

Removing a ROI

To remove a ROI, click the trash can icon in the ROI table in the bottom right part of the analysis tab.

> **Attention!**
>
> You can click and drag in the ROI statistics table to select statistics and then copy them to the clipboard by pressing Ctrl + C. You can then paste them into other applications like Excel to Notepad by pressing Ctrl + V.

4.5.7 Graphs

Several analysis graphs are available to visualize the recorded image data. When no ROI is selected, the graphs will show the information for the entire image. When a ROI is selected, the graphs will show the information for that ROI.

You can choose the number of graphs that are displayed at the right side of the screen in the analysis tab. To do so, click one of the three icons in the top left corner of the graphs area to select 1, 2 or 4 graphs (see figure 4.10).

Inside the graphs, you can click any point to display a label with more information about that data point.

Attention!

- You can zoom in and out in all graphs by using the scroll wheel on your mouse.

- You can export the data points that are shown in a graph by right-clicking the graph and choosing *Export data*. This will export the data shown in the graph to a .CSV file.

- Double click inside the graphs to go back to the original zoom level.

- You can show a specific part of a graph by holding down the Alt key and right-clicking and dragging to select an area of the graph.

The following types of graphs are available:

Sine fit
Displays the sine fit in any pixel you click in the image viewer.
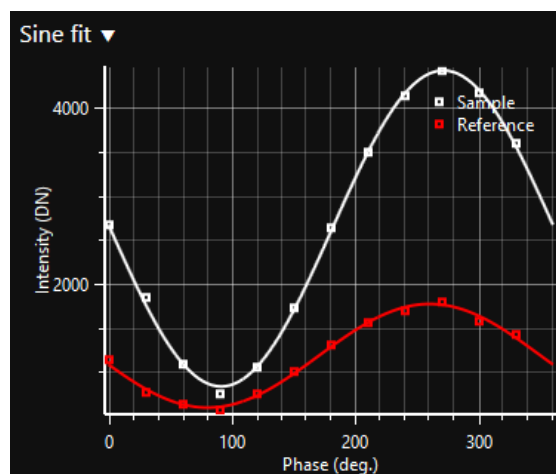

Figure 4.12: Sine fit through reference and sample data in the selected pixel.

Polar plot

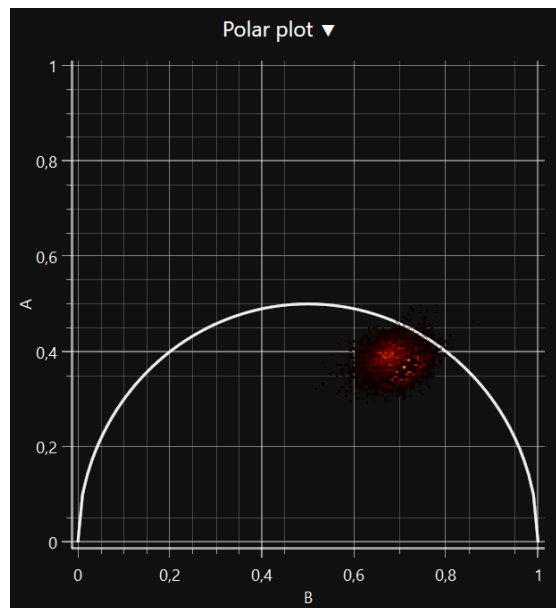Displays a phasor plot[2] of the pixels in the selected ROIs.

Figure 4.13: Polar plot representation of the pixels in the selected ROIs.

Averaged polar plot

Displays a phasor plot of the pixels in the selected ROIs, but each ROI is represented by a circle at the average position of all ROI pixels in the phasor plot. The outline and fill color of the ROI correspond to the accent color of the ROI.
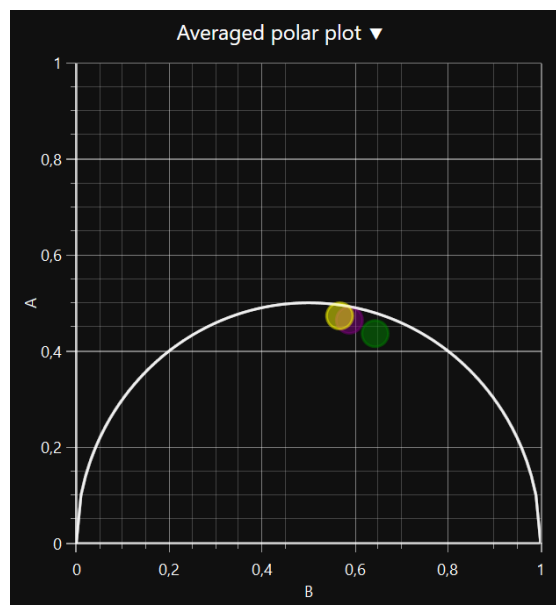

Figure 4.14: Averaged polar plot representation of the pixels in the selected ROIs.

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[2]Redford and Clegg, *Polar Plot Representation for Frequency-Domain Analysis of Fluorescence Lifetimes*, Journal of Fluorescence, Vol 15, No 5 (2005)

Scatter plot

The following scatter plots are available:

- *Intensity vs Lifetime (phase)*

- *Intensity vs Lifetime (modulation)*

- *Lifetime (phase) vs time*: For time-lapse recordings, this graph will show the average life-time from phase in the selected ROIs as a function of time.

- *Lifetime (modulation) vs time*: For time-lapse recordings, this graph will show the aver-age lifetime from modulation in the selected ROIs as a function of time.

- *Intensity vs time*: For time-lapse recordings, this graph will show the average intensity from modulation in the selected ROIs as a function of time.
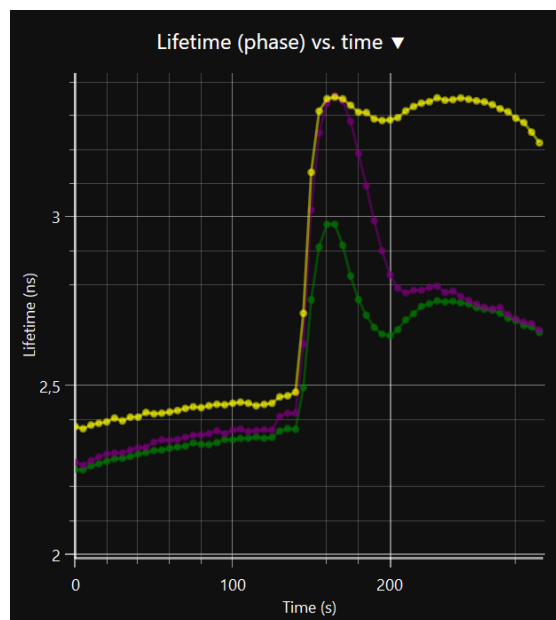
Figure 4.15: Scatter plot of phase lifetime as a function of elapsed time in a time-lapse recording.
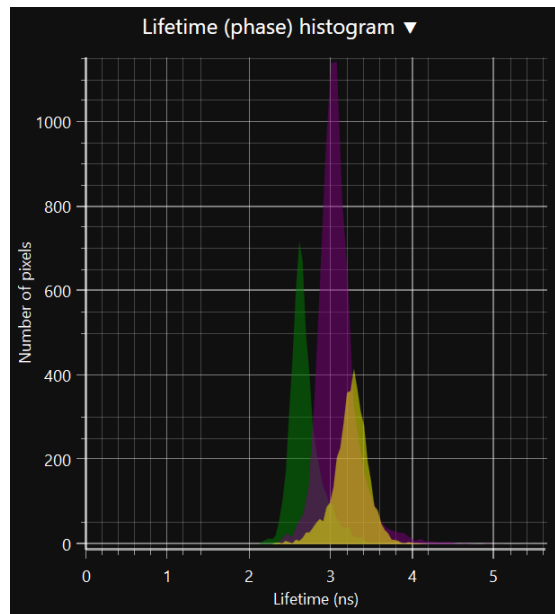
Figure 4.16: Histogram of the phase lifetime in the selected ROIs.

Histogram
The following histograms are available:

- *Intensity*: Histogram of the light intensity in the pixels of the selected ROI.

- *Lifetime (phase)*: Histogram of the lifetime from phase in the pixels of the selected ROI.

- *Lifetime (modulation)*: Histogram of the lifetime from modulation in the pixels of the selected ROI.

4.6 Preferences

You can adjust the software preferences by selecting *Preferences* from the *File* menu.

4.6.1 General

Automatically save recordings

When enabled, new recordings will be saved to disk automatically. The filename will be generated based on your selection in the *Automatic filename format* section.
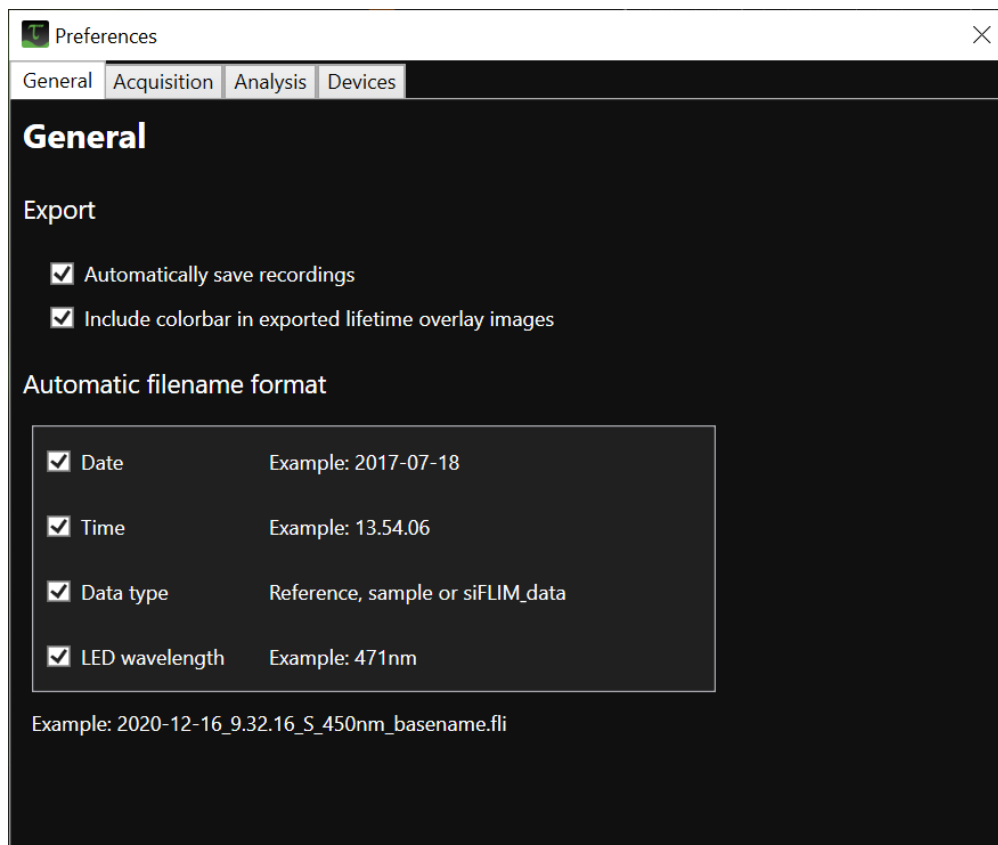

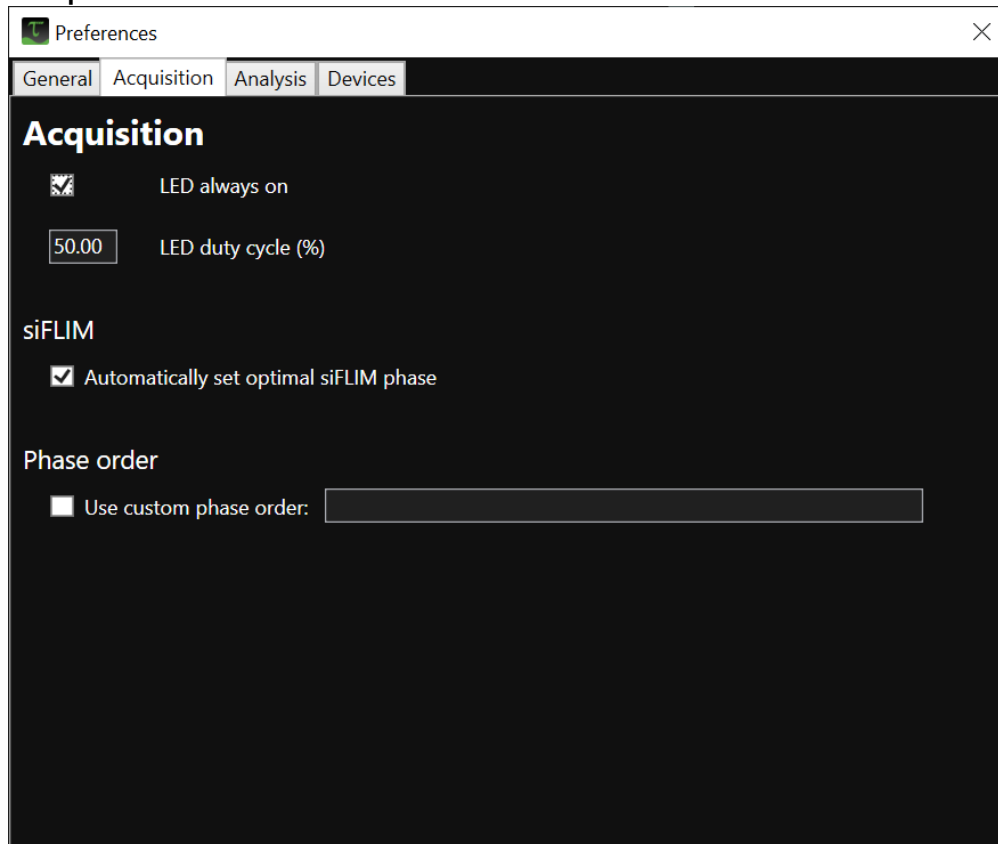**Figure 4.17:** General preferences screen in the LIFA software.

Automatic filename format

In this section you can select which filename elements you would like to use in the automatically generated filenames:

- *Date*: The current date in the format YYYY-MM-DD.

- *Time*: The current time in the format HH.MM.SS.

- *Data type*: The data type of the recording. This can be either reference or sample, depending on the tab you are in when you record the data (*Reference* tab for reference files, *Acquisition* tab for sample files).

- *LED wavelength*: If you are using a Lambert Instruments Multi-LED, then the wavelength of the LED you are using can be added to the filename.

An example of the automatic filename is shown below the list of filename elements.

4.6.2 Acquisition

Figure 4.18: Acquisition preferences screen in the LIFA software.

LED always on

When enabled, the LED modulation signal will remain active, even when the camera is not recording. Keeping the LED on will improve the temperature stability of the LED. The Lambert Instruments Multi-LED will automatically close its shutter when the Toggel is not recording. That way, the LED can stay on without bleaching your sample.

LED duty cycle

Percentage of each modulation cycle that the LED is on. The duty cycle setting can be adjusted in steps of $\frac{1}{24} \approx 4.17\%$. Please note that the software will automatically round your input to the closest possible value for the duty cycle.

Automatically set optimal siFLIM phase

The optimal siFLIM phase will automatically be calculated and applied when this checkbox is checked. If you prefer setting the siFLIM phase manually, then you can uncheck this box and the currently selected value of the phase slider in the FLIM settings panel will be used during siFLIM acquisition.

Phase order

When *Use pseudo random phase order* is enabled, the software will record the images in a pseudo random phase order to minimize the effects of photobleaching. When this setting is disabled, the images will be recorded by incrementing the phase by $\frac{360}{N}$ after each phase image, where N is the number of phases that is selected in the FLIM settings panel.
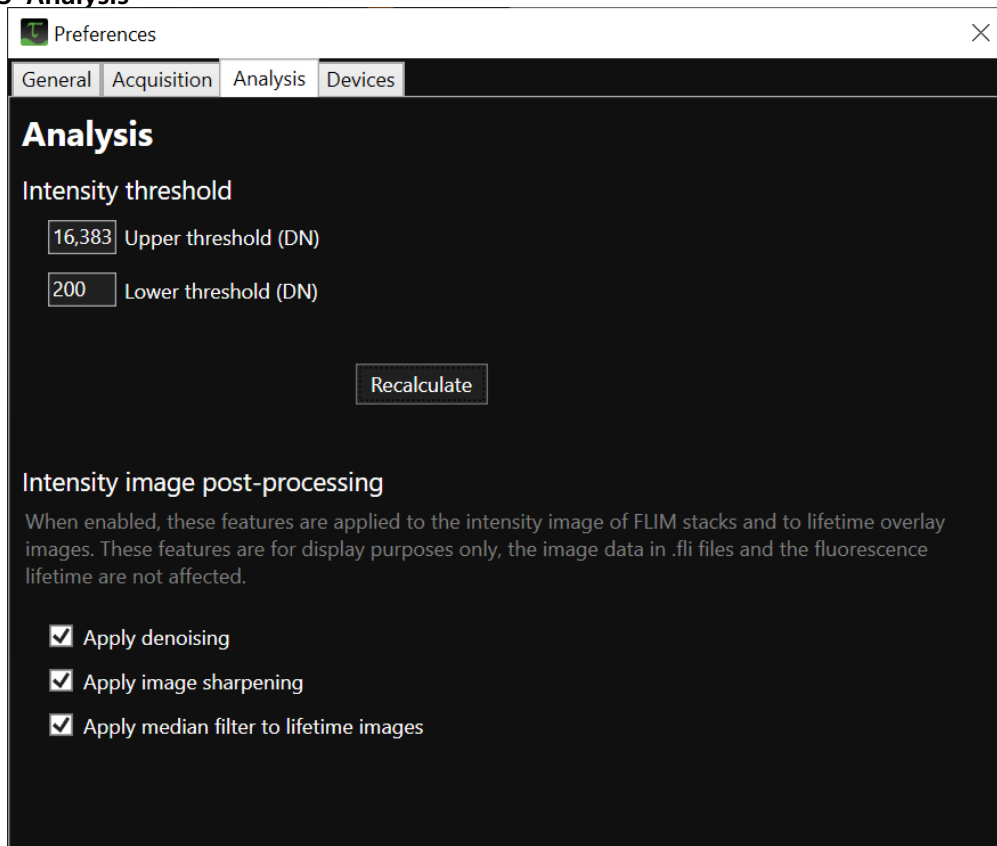
4.6.3 Analysis

Figure 4.19: Analysis preferences screen in the LIFA software.

Intensity threshold

Pixels that have a light intensity below the lower threshold or above the upper threshold will be ignored during lifetime calculation.

- *Relative threshold*: Lets you specify the percentage of all possible light intensities that should be used as the lower and upper threshold. 100% light intensity corresponds to overexposure and 0% light intensity corresponds to no light detected.

- *Absolute threshold*: Lets you specify the light intensity in digital numbers (DN) for the lower and upper threshold.

Recalculate

After changing the intensity threshold or the reference filter size, you can press the *Recalculate* button to recalculate the fluorescence lifetime with the new threshold values.

4.6.4 Intensity image post-processing

Optional post-processing can be applied to the intensity image of FLIM stacks and lifetime overlay images. These options are disabled by default. When LIFA is started, the previous state of these options will be restored.

Denoising

When enabled, non-local means denoising will be applied to the intensity image. The standard deviation of the background image will be used as the filter strength parameter for the denoising algorithm. As a result, the effect of denoising will be stronger for images with a lower signal-to-noise ratio.

Sharpening
When enabled, the intensity images will be sharpened by applying unsharp masking.

Lifetime overlay smoothing
When enabled, a median filter will be applied to the fluorescence lifetimes to smoothen the appearance of the pseudo-color lifetime overlay.
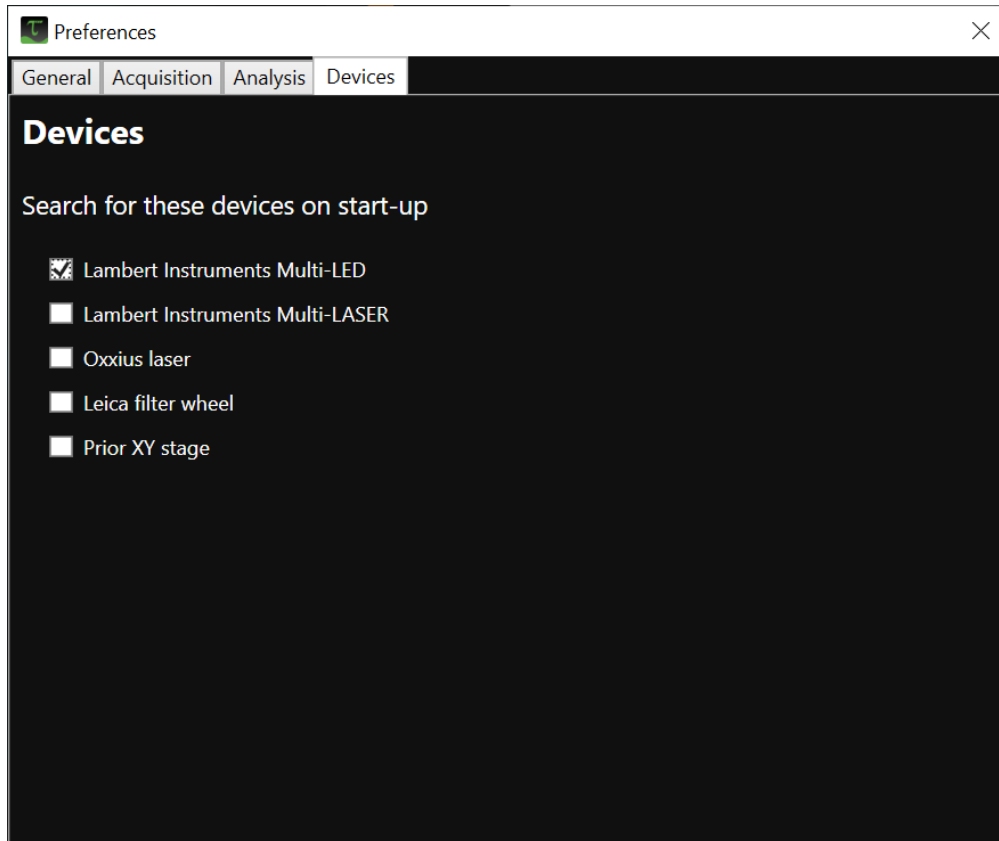
4.6.5 Devices

Figure 4.20: Device preferences screen in the LIFA software.

Search for devices on start-up
This tab lets you select which devices the software should look for on startup. The software will always look for a Toggel camera, but the other devices in this list can be disabled.

4.7 Keyboard shortcuts

The following keyboard shortcuts are available.

Functionality	Keyboard shortcut
Open reference file	Ctrl + R
Open sample file	Ctrl + S
Open preferences screen	Ctrl + P
Recalculate fluorescence lifetime	F5

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