GETTING STARTED

Safety Precautions

1. The LED head emits visible or invisible radiation when the EasyLife X is turned ON and the LED head is connected to the main unit via BNC and CPC cables. Do not look directly inside the LED unit when it is powered!
2. Always switch OFF the EasyLife X before removing or installing the LED head.
3. The sample compartment has a mechanical safety shutter, which blocks the excitation light when the sample compartment lid is open. Always wear safety glasses appropriate for the wavelength range emitted by the LED if you are going to defeat the safety shutter.
4. Never attempt to use LED heads other than those supplied by OBB.
5. Do not disassemble the EasyLife X or the LED head – there is no maintenance required for either unit. Unauthorized attempts to access and interfere with internal components may cause electrical and radiation hazards and may void the warranty.

Basic Operation

Warning: Do not disconnect the USB-to-Serial adapter cable while the computer is powered ON. This will cause Windows to increment the number of COM ports and change the one used for the EasyLife X. You will then have to reconfigure the COM port used by the Delay gate Generator.

A. You will need the following items to carry out a fluorescence lifetime measurement:

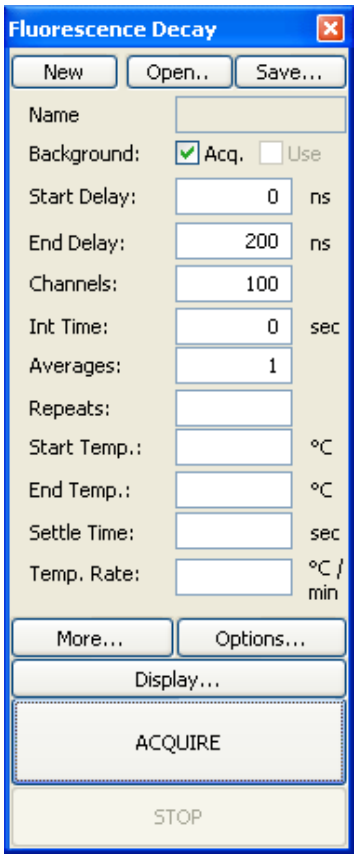
1. Fluorescent sample, which can be one of the following:
   - Solution in a 10 mm x 10 mm cuvette
   - Solution in a microcuvette, which is adaptable to 10 mm x 10 mm cuvette holder
   - Solid sample (e.g. slide) mounted on an EL-1000 solid sample holder (optional accessory)
   - Sample placed in a K-158 liquid nitrogen dewar (optional accessory)
2. Scatterer solution (to acquire the instrument response function, IRF), which can be one of the following:
   - Colloidal silica (available as Ludox from Aldrich) highly diluted in water
   - Glycogen in water
   - In an emergency situation, powdered non-dairy creamer in water can also be used
3. Emission filter (typically a cut-off type) to cut off the excitation light
4. K-136 set of neutral density filters (optional, but helpful accessory)

B. To prepare the instrument for the measurement:

1. Turn the EasyLife X power switch ON. The red LED power indicator on the EasyLife X front panel should be lit.
2. Turn the computer ON.
3. Make sure the emission slit is partly open, e.g. start with a 3-mm opening (2 full turns = 1 mm).
4. Place the sample in the sample holder
5. Place the emission filter in the filter holder

C. The next step is to set the acquisition parameters in EasyLife X. The following example is based on a 1 nM fluorescein solution in 0.1 N NaOH.

1. For a basic experiment, **Configure, Hardware,** choose **none** from the **Temperature Controller** choice list. **OK**.
2. Click **Acquire** on the menu bar and select **Fluorescence Decay** on the drop down menu. This brings up the **Fluorescence Decay** dialog box. Set the required parameters:



- **Background:** check the Acq. box.
- **Start Delay** (ns). Enter a value, which is about 3 to 5 ns in front of the excitation pulse. If you don't know where the excitation pulse is, enter 0.
- **End Delay** (ns). Add about 5 to 8 times your sample lifetime to the **Start Delay** and enter here. If you have no idea what the lifetime of your sample is, enter a relatively large value, e.g. 200 ns.
- **Channels**. This is the number of data points defining the decay curve. Enter a desired value, e.g. 200 (use less channels to speed up the experiment and more channels to improve accuracy of the measurement).
- **Int Time** (s). This controls the time over which the output signal is averaged at each time delay step. Enter 0 s for a quick scan. Use a lower value to speed up the experiment or a larger value to improve signal to noise.
- **Averages**. Number of scans to be acquired and averaged. Enter 1 for a quick scan, use more averages to improve the signal to noise.
- Leave the other text boxes blank.

3. The **Fluorescence Decay** dialog box has three buttons **More…, Display…,** and **Options…** which lead to further dialog boxes. See **Common Acquisition Controls** and **Fluorescence Decay** for more information.

D. In order to acquire the fluorescence decay:

1. Click **ACQUIRE (PREP)** and then **START**, which acquires the background, and then continues to acquire the decay curve. **Note**: The green Trigger LED on the front panel of the EasyLife X should be lit now. The **Background Acq** checkbox will then dim, and the **Use** box will be checked.

After the scan is completed, you will likely see a decay trace, which will look like one of 3 traces depicted in figure 9.

First of all, it's obvious that the time window is much too large. We deliberately selected a broad time range in order to locate the signal. Now we can narrow the time window to a meaningful range. In this case we go back to **Acquire** menu and change **Start Delay** to 65 ns and **End Delay** to 95 ns. A rule of thumb is to use the total time range (i.e. range = **End Delay - Start Delay**) of approx. 5 to 8 times the lifetime value. The lifetime can be approximated as the full width of the decay trace at half maximum. The **Start Delay** should be a few nanoseconds in front of the excitation pulse, so the pre-pulse baseline could be determined.

If the intensity is too high, the decay will look like trace A, i.e. it will have a truncated top part. If this is the case, reduce the slit width and repeat the measurement (Step D) with the new **Start Delay** and **End Delay** settings. Alternatively, you may use a neutral density filter (optional) to reduce the intensity. Keep in mind that a filter with optical density (OD) of 0.3 will reduce the intensity approximately 2 times.

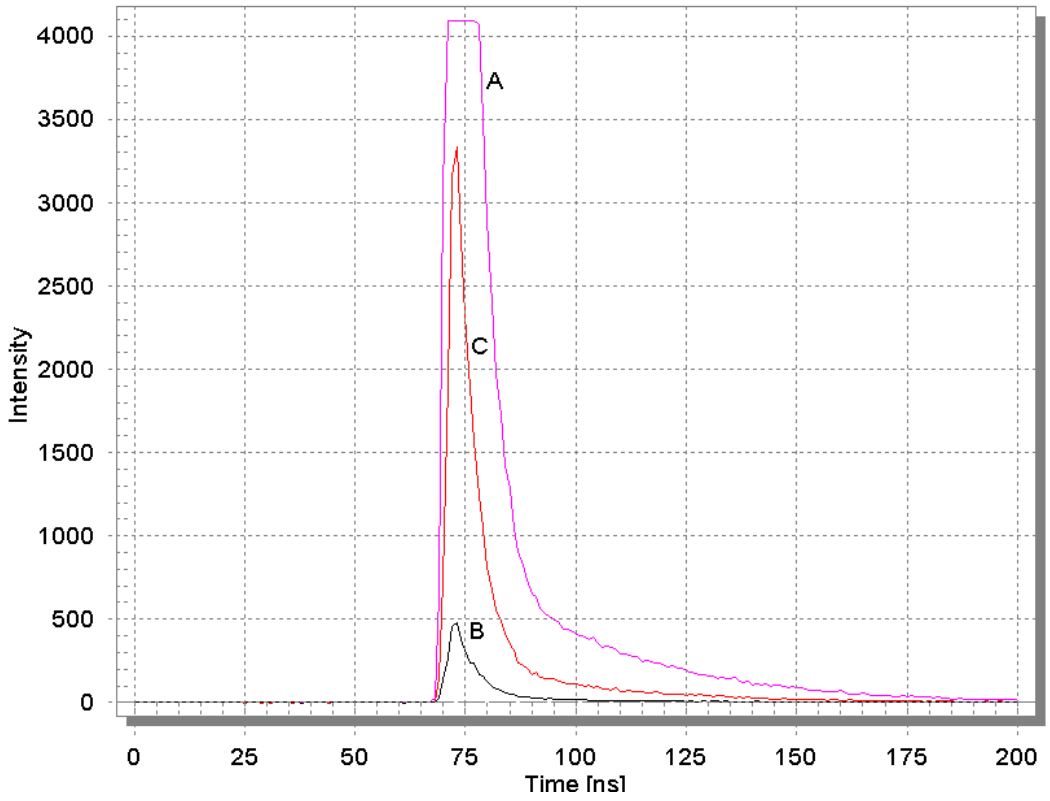


Figure 9. Three different outcomes of decay curve measurement: A – intensity too high; B – intensity too low; C – good level of intensity. The time range is too broad for all 3 traces.

If the intensity is too low (i.e. figure 9 trace B), you may want to increase the slit opening to admit more light to the detector. Open the slit wider and repeat the measurement (Step D) with the new **Start Delay** and **End Delay** settings. If the slit is fully open (6 mm) and the intensity is still low, you can decrease the gain setting in the Hardware Configuration, ADC Setup dialog to allow a small signal to fill the allowed range. The way to improve the signal to noise is to increase the integration time, use more averages, or both.

If your decay looks like trace C in figure 1, the intensity is adequate, so you can just repeat the measurement (Step D) with the new **Start Delay** and **End Delay** settings. The final decay should look like trace A in figure 10.

E. The next step is to record the Instrument Response Function (IRF) using the scatterer solution. To record the IRF do the following:

   1. Remove the sample and place the cuvette with scatterer in the sample holder.
   2. Remove the emission filter.
   3. **DO NOT CHANGE** such parameters as **Channels** or **Start Delay** and **End Delay**.

You may change **Integration Time** or **Averages** if required.

4. Click **ACQUIRE (PREP)** and then **START** to measure the IRF.

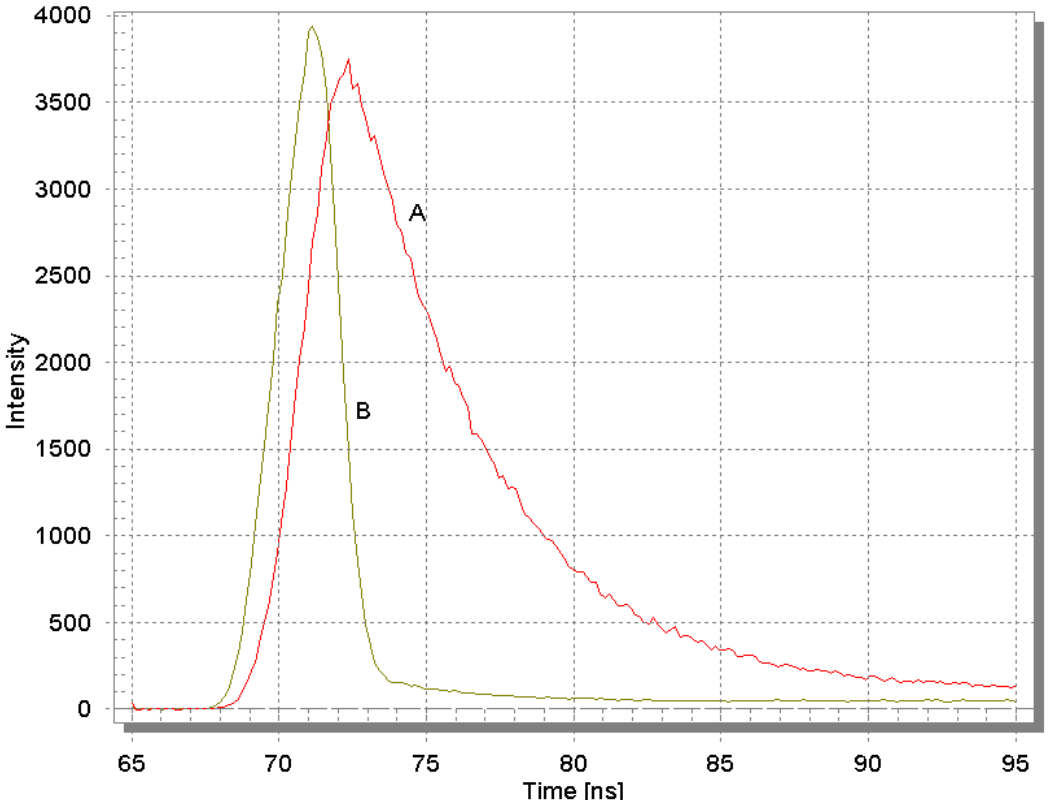


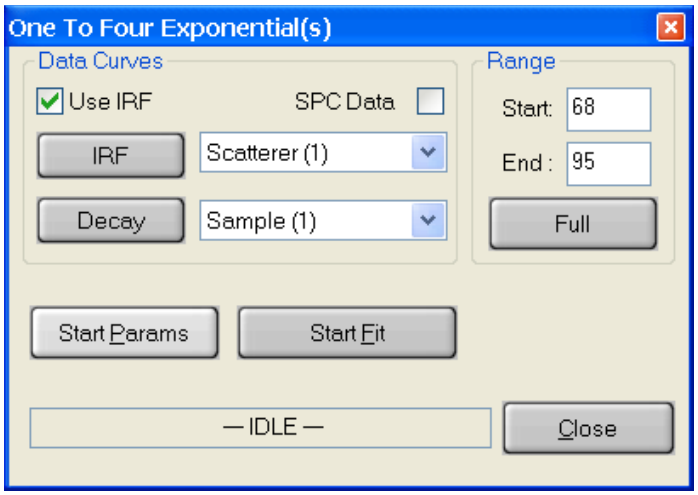
Figure 10. A typical outcome of correctly conducted fluorescence decay measurement. A – fluorescence decay; B – instrument response function (IRF).

If the intensity is too high (truncated top), lower the concentration of the scatterer or place a neutral density filter in the excitation or emission filter holder and repeat the measurement. Avoid changing the emission slit opening in order to adjust the intensity of the scatterer, as it may change the size of the illuminated spot on the PMT photocathode leading to potential undesirable artifacts.

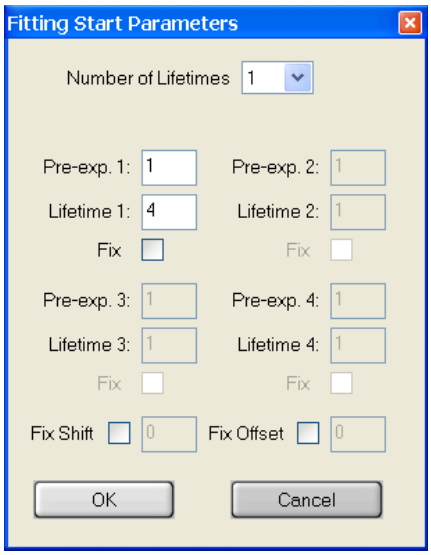
If the intensity is too low, increase the concentration of the scatterer and repeat the measurement. As stated above, avoid changing the emission slit opening to adjust the intensity of the scatterer.

F. Once the fluorescence decay and the IRF have been measured (figure 10), the fluorescence lifetime can be determined with the use of analysis software.

   1. Click **Math** on the menu bar, then **Data Analysis** on the drop down menu and across to highlight **1 To 4 Exp. Lifetime** on the second drop down menu. Click on this to bring up the **One To Four Exponential(s)** dialog box shown below. Make the following selections:



- **Use IRF** – controls whether the instrument response function will be used in the analysis. In most cases the IRF is used except when the lifetime of the sample is very long compared to the temporal width of the LED pulse. Toggle this option on ☑ or off ☐ by checking/unchecking the box.
- **SPC Data** – Used only when single photon counting data has been imported for analysis. Toggle this option on ☑ or off ☐ by checking/un-checking the box. Leave it unchecked for GX-EL data.
- **IRF** - Select the scatterer decay curve by clicking on the name of the appropriate curve in the legend. Click on the IRF button. The name of the curve will appear in the window.
- **Decay** - Select the sample decay curve by clicking on the name of the appropriate curve in the legend. Click on the decay button. The name of the curve will appear in the window.
- Click **Start Parameters** to enter the fitting parameters.



- **Number of Lifetimes -** The number of different lifetimes used to analyze the decay curve. Select a number between 1 and 4.
- **Pre-exp., Lifetime, Fix** - For each of the lifetimes to be used in the fit an initial guess for the lifetime and the pre-exponential factor **(Pre-exp)** must be given. Only the relative values of the pre-exponential factors are relevant here so that for a single exponential fit the value 1 is normally used. Each of the lifetimes chosen for the analysis may be fixed at the input value or allowed to float in the fit. Toggle this option on ☑ or off ☐ by clicking on it. Occasionally, the fit will not succeed if the starting values are too far off. If this occurs, try changing the starting values.
- **Fix Shift** – The shift parameter accounts for some instrumental artifacts, such as the 'color shift' effect or potential instrumental instabilities. It corrects for a zero-time shift of the excitation pulse relative to the decay (see the Data Analysis chapter for details). The shift parameter may be fixed at the input

value (in nanoseconds) or allowed to float in the fit. Toggle this option on ☑ or off ☐ by clicking on it. A value of 0.0 is usually a good initial guess.

- **Fix Offset** – Since fluorescence decays are obtained with an analog measurement, there will always be a small offset over imposed on the decay signal. This offset may be included as a parameter in the analysis. The offset parameter may be fixed at the input value or allowed to float in the fit. Toggle this option on ☑ or off ☐ by clicking on it.
- Click **OK** to accept fitting parameters and exit the dialog.

- **Start, End** – Position the mouse pointer at the desired start delay, click and hold down the left mouse button, drag the mouse to the desired end delay and release the button. The selected range will be shown on the screen in a different color. Alternatively, the start delay and end delay for the portion of the sample decay that is to be analyzed may be entered from the keyboard.
- Click **Full** if you want to analyze the decay over the entire time range.
- Click **Start fit** to initiate lifetime analysis.

The progress of the analysis is shown in the box marked -IDLE- in the above illustration. The fit may be stopped at any time by clicking the **STOP** button.

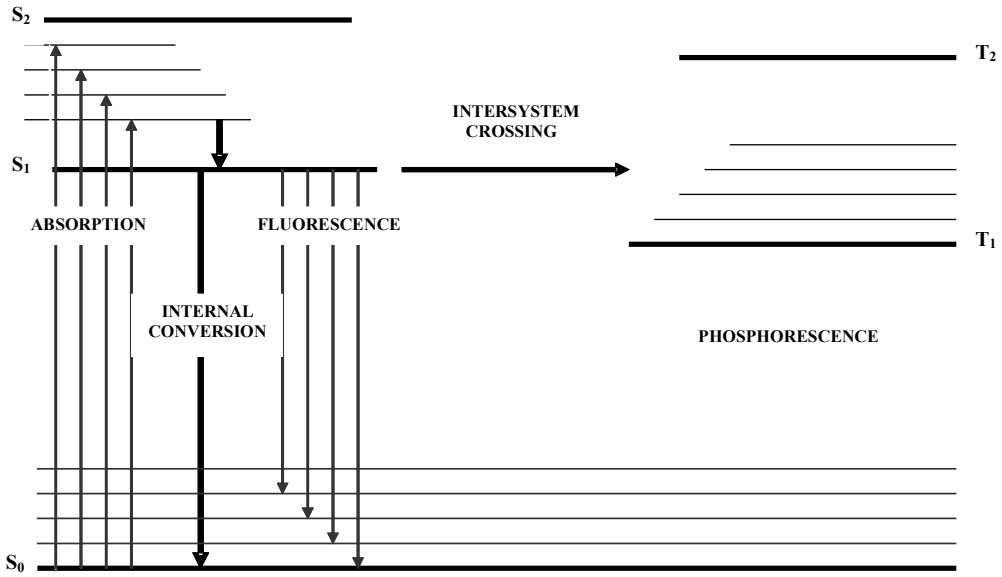
The results of the fit are shown in two different ways. The fitted curve, the weighted residuals, the autocorrelation function and the deconvoluted decay (i.e. D(t)) appear in the workspace. The values of the parameters found in the fit and some statistical information on the fit are entered into a text editor, which pops up in a window at the end of the fit.

Click **Close** to exit this analysis mode.

Simple Theory of Fluorescence Decay

Fluorescence is the emission of light from a molecule resulting from a transition from one electronic state to a lower electronic state of the same multiplicity. The most commonly observed fluorescence from organic molecules is caused by a transition from an excited singlet state to the ground singlet state. A highly simplified ***Jablonski diagram*** illustrates the processes involved.

The various processes that occur in the excited state of a molecule include:



- Emission of a photon - this results in fluorescence if the transition is between states of the same multiplicity and in phosphorescence if the transition is between states of different multiplicities.
- Internal conversion (IC) - the non-radiative loss of excitation energy through conversion of an excited state to a highly excited vibrational level of a lower state of the same multiplicity. The excitation energy in this case dissipates as heat.
- Inter-system crossing (ISC) - the non-radiative transition from a singlet state to a triplet state (or vice versa).
- Quenching - the non-radiative loss of excitation energy via encounter with another molecule (quencher).

Each of these processes has a characteristic time in which it occurs. Emission and internal conversion typically occur on the picosecond through hundreds of nanoseconds time scale. Inter-system crossing also occurs in this time range when it is an effective mechanism for de-excitation of the excited state. Quenching is concentration-dependent, but commonly will compete with emission at millimolar and higher concentrations for good quenchers, as the quenching process is a diffusion-controlled process. Lastly, the molecule may undergo a photochemical conversion such as dissociation.

The Kinetic Scheme

It is possible to describe the de-excitation of a simple fluorescent molecule A by a first order kinetic scheme.

PROCESS	RATE CONSTANT	DESCRIPTION
$^1A^* \rightarrow A$	k_r	radiative emission
$^1A^* \rightarrow A$	k_{ic}	internal conversion
$^1A^* \rightarrow {}^3A^*$	k_{isc}	inter-system crossing
$^1A^* \rightarrow B + C$	k_d	dissociation
$^1A^* + Q \rightarrow A$	k_q	quenching

Scheme 1

The rate of loss of the excited singlet state may thus be given by

$$\frac{-d\left[{}^1A^*\right]}{dt} = \left[{}^1A^*\right]\left\{k_r + k_{ic} + k_{isc} + k_d + k_q[Q]\right\} \qquad \text{Eq. 1}$$

This equation may be solved to yield an exponential decay law:

$$\left[{}^1A^*\right] = \left[{}^1A_0^*\right]\exp\left(\frac{-t}{\tau}\right) \qquad \text{Eq. 2}$$

where $[^1A^*_0]$ is the initial excited state concentration and the lifetime τ is given by:

$$\tau = \frac{1}{\left\{k_r + k_{ic} + k_{isc} + k_d + k_q[Q]\right\}} \qquad \text{Eq. 3}$$

If other processes such as energy transfer or excimer formation occur, this kinetic scheme becomes more complex and the decay law may become non-exponential.

Convolution

A fluorescence decay D(t) of a single fluorophore in homogeneous solution can be usually described as a single-exponential function in accordance with the 1st order kinetic equation 2:

$$D(t) = a \exp\left(-\frac{t}{\tau}\right) \qquad \text{Eq. 4}$$

where τ is the lifetime defined in Eq. 3.

If the sample contains more than one fluorophore or the fluorophore is contained in a heterogeneous environment, the fluorescence decay can be better described as a sum of exponential functions:

$$D(t) = \sum_{i=1}^{n} a_i \exp\left(-\frac{t}{\tau_i}\right) \qquad \text{Eq. 5}$$

where τ_i are fluorescence lifetimes of various fluorescent forms and a_i are corresponding pre-exponential factors. The interpretation of a_i depends on the nature of the sample: if the emission comes from a single fluorophore in different conformational forms or environments, the a_i factors are proportional to respective populations. If multiple lifetimes result from the presence of several fluorophores in the sample, the a_i pre-exponential factors will depend not only on their populations, but also on radiative probability constants and molar extinction coefficients of respective fluorophores.

The decay curves described by equations 4 and 5 will only be observed if the sample is excited by an infinitely narrow pulse. In most cases the temporal width of the excitation pulse cannot be neglected and the observed decay $D_{obs}(t)$ will be distorted by convolution with an instrument response function L(t) in accordance with the following equation:

$$D_{obs}(t) = \int_0^t L(t-s)D(s)ds \qquad \text{Eq. 6}$$

The instrument response function (IRF) L(t) can be determined experimentally by using a scatterer solution instead of a sample. The IRF accounts for the shape of the excitation pulse and for the temporal response of the detection system. Figure 11 shows an example of the traces obtained in a typical fluorescence decay experiment.

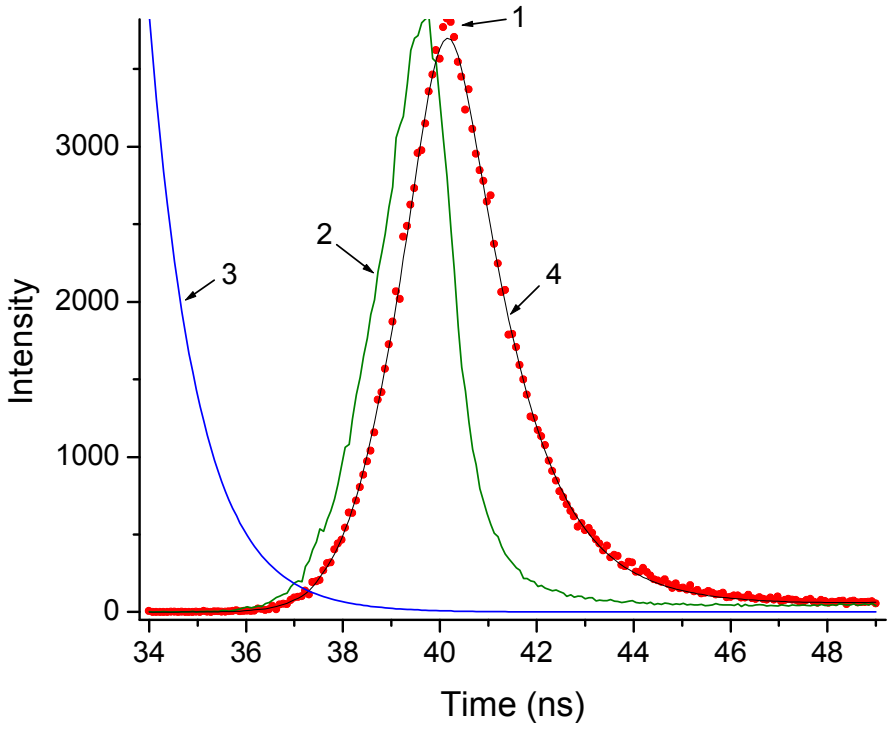


Figure 11. Illustration of the convolution effect as described in Eq. 6: 1 - experimental decay $D_{obs}(t)$; 2 - instrument response function L(t); 3 – undistorted exponential decay D(t); 4 – the best fit to experimental decay, i.e. convolution of curves 2 and 3.

Once the $D_{obs}(t)$ and L(t) have been measured, the analysis software performs iterative reconvolution according to Eq. 6 varying the fit parameters a_i and τ_i until the best fit to the experimental decay is obtained.