FLIO Data Acquisition and Analysis

The Road to Success

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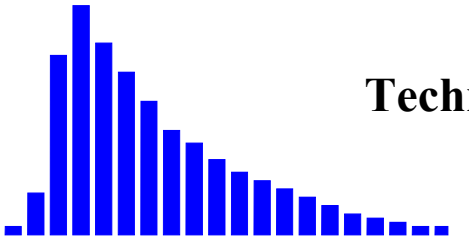
Refers to:

SPCIMage Version 7.4 and later



Technology Leader in Time-Correlated Single Photon Counting

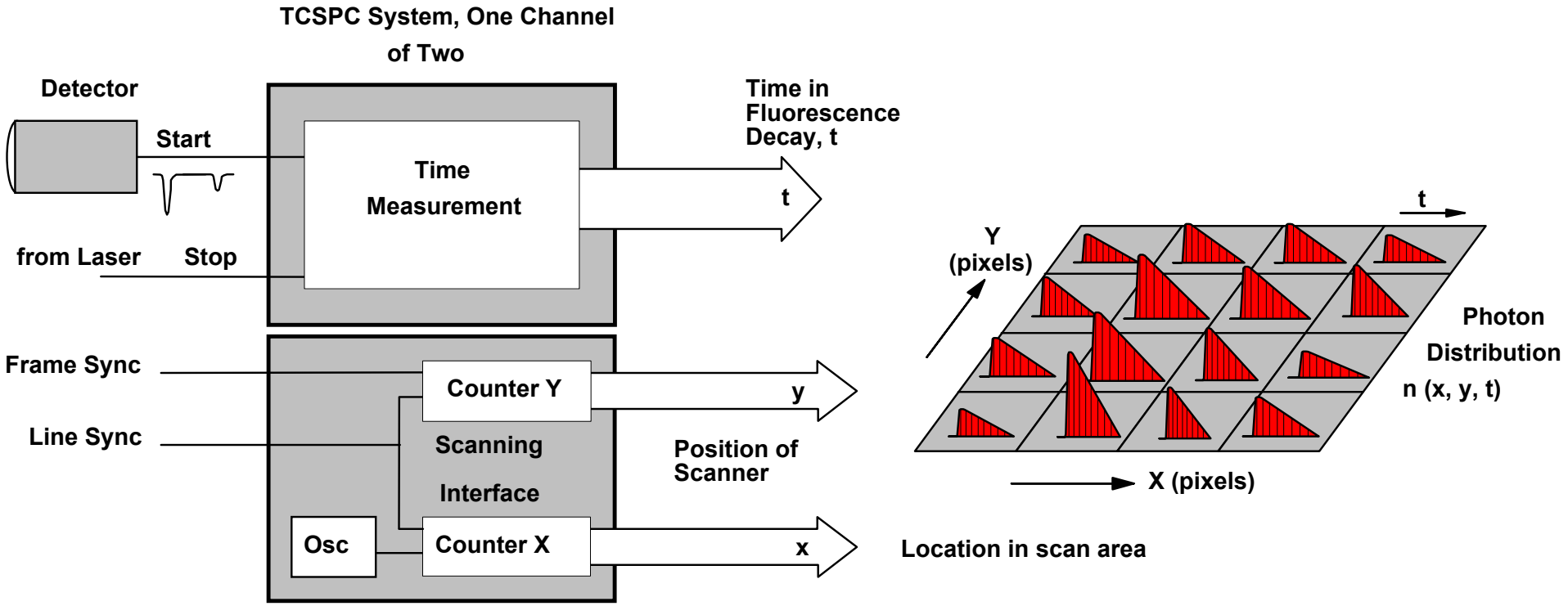
1. Understanding FLIO Data Recording

1.1 FLIO Recording is Single-Photon Recording

The FLIO scans the fundus with a high-frequency pulsed laser beam and detects single photons of the fundus fluorescence.

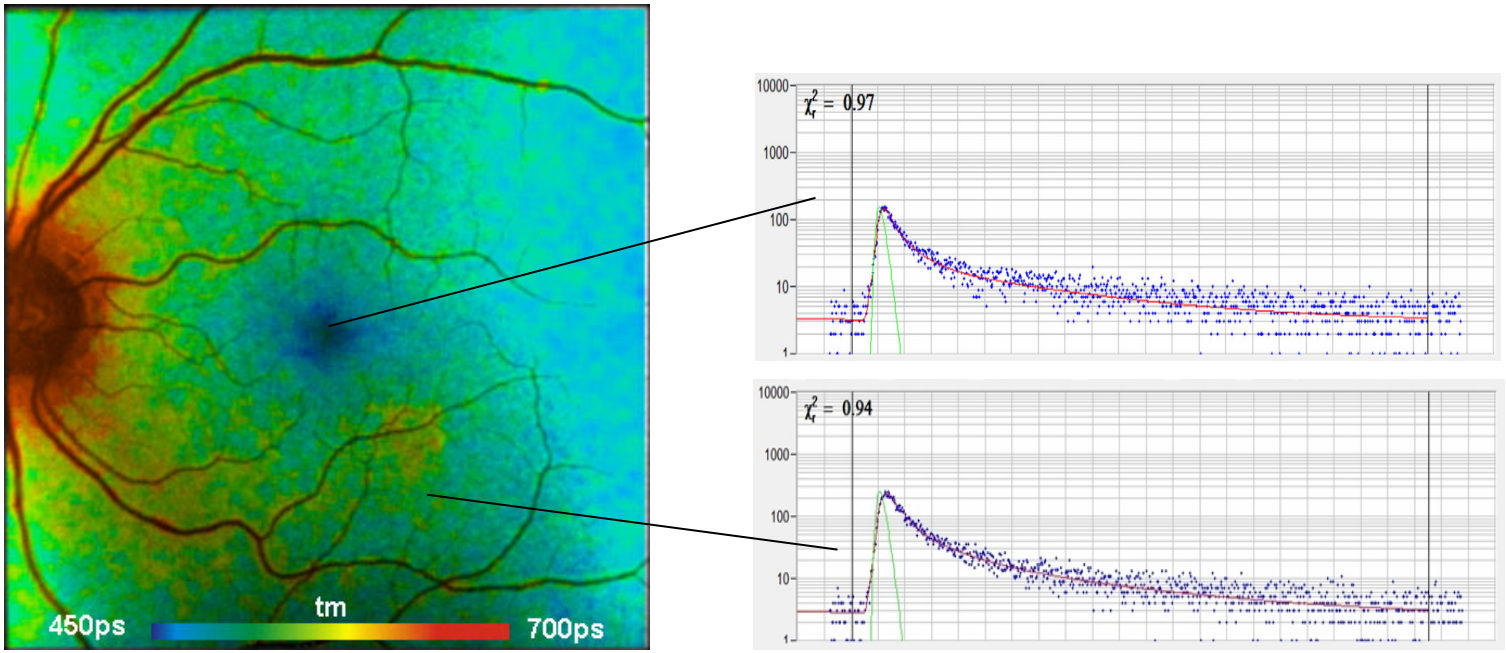
Every photon is characterised by its time after the laser pulse and the location of the laser beam at the fundus at the time of detection.

The recording system builds up a photon distribution over the coordinates x,y, in the scan area and the time of the photons after the laser pulse, t.



1.2 FLIO Data are Photon Data

Every pixel of a FIO Image contains a fluorescence decay curve, consisting of photon numbers in consecutive time channels.



Consequences:

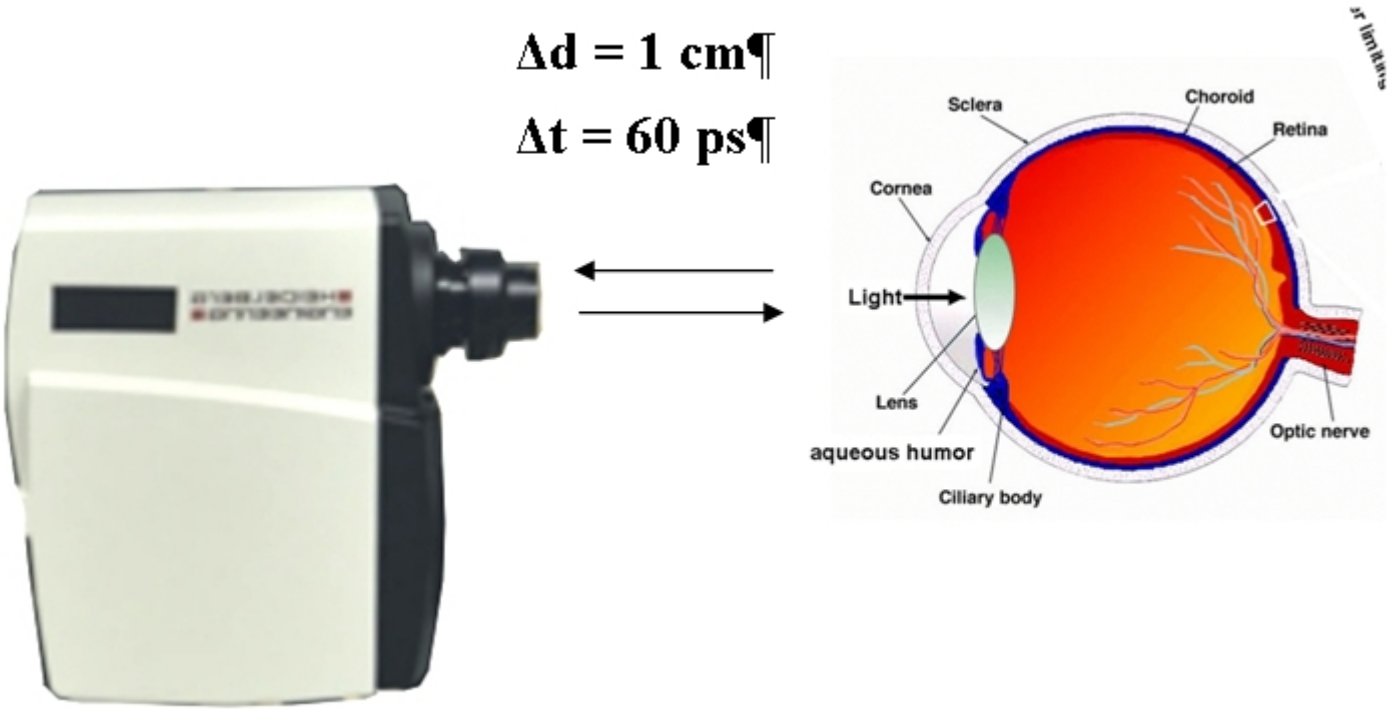
a) Photon numbers are Poisson-distributed. The signal-to-noise ratio for the photon number, N and the lifetime, τ, is

$$SNR_N = \sqrt{N} \qquad SNR_\tau = \sqrt{N}$$

b) Unwanted signals cannot simply be subtracted. Even if you know the shape and amplitude you can only subtract the average, not the noise from the Poisson distribution!

1.3. FLIO Data are Temporal Data

The fluorescence decay data shift with the optical path length.

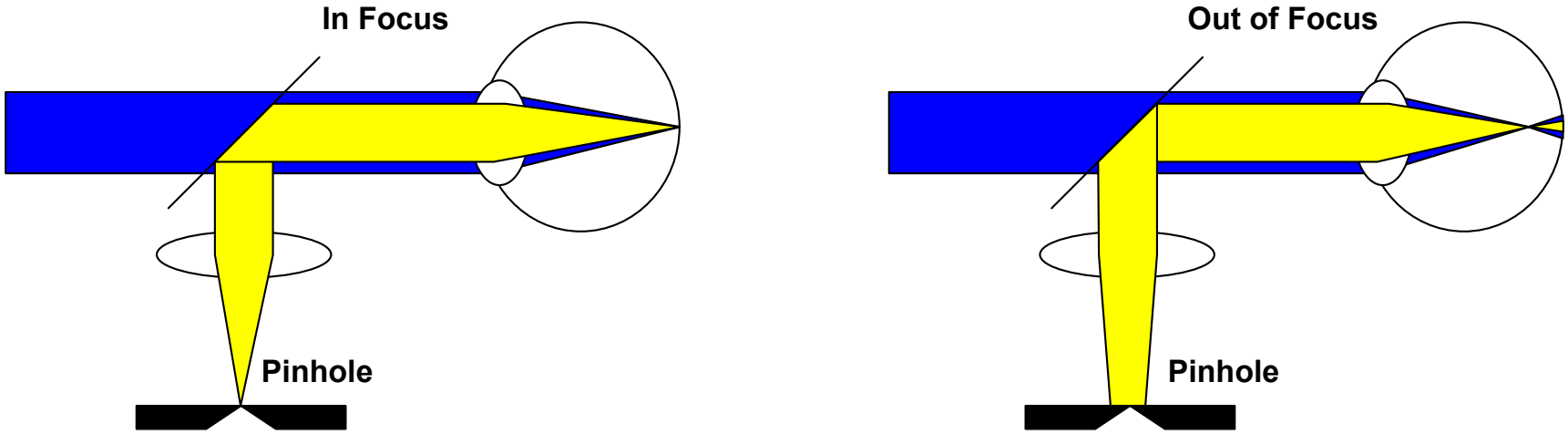


FLIO data analysis can account for different path length of different patients, but not for variable path length during the data acquisition.

2. Recording the Best Possible Data

2.1. Maximise the number of photons

Focus correctly. The FLIO is a confocal system. Only light from the focal plane passes the pinhole efficiently.



Out-of focus images are not only blurry, they also contain substantially less photons.
More than that, the ratio of the good signal and unwanted signals decreases.
Therefore the drop in the lifetime accuracy is larger than the drop in SQRT (N).

>>> Focus correctly!

2.2. Avoid Recording of Unwanted Signals.

Background from daylight:

Background has an enormous influence on the lifetime accuracy. 10% of background light reduces the lifetime accuracy by about 50%

Reason: The timing noise for the background photons is larger than timing variation of the fluorescence photons.

>>> Make sure that no background is recorded from daylight, computer screens, and instrument indicator lights.

>>> Make sure that the intensity of the fundus signal is as high as possible.

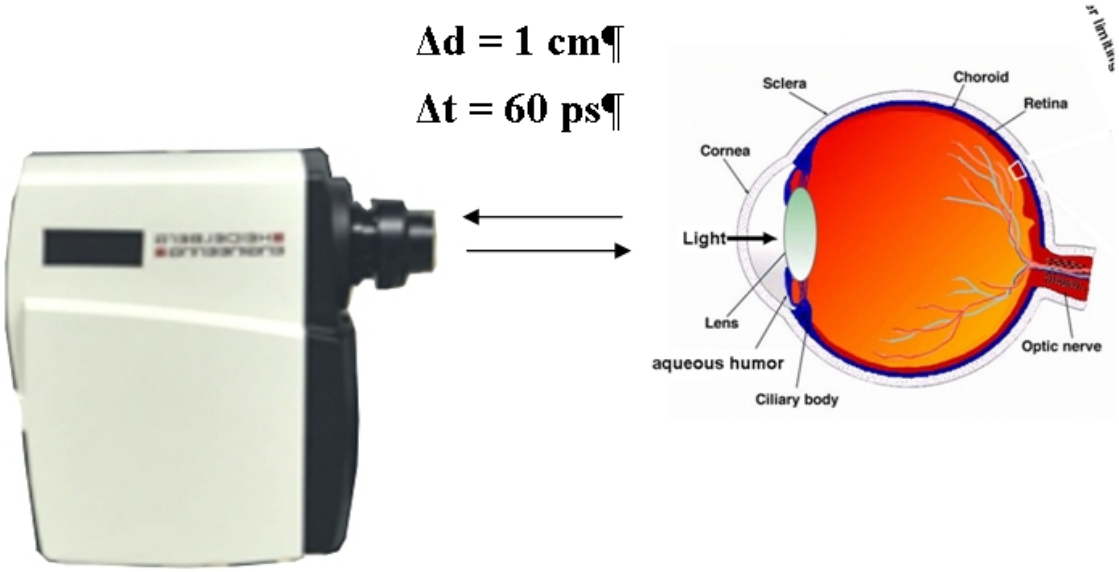
Focus correctly!

Fluorescence of the lens of the eye:

The lens of the eye is fluorescent. Some of the lens fluorescence passes the pinhole. Poor focusing reduces the signal from the fundus, so that the relative contribution of the lens fluorescence increases.

>>>> Focus correctly!

2.3. Keep the distance between the scanner and the patient constant.



More than 1cm variation during the data acquisition cannot be tolerated. The effect would be a broadening of the IRF (the temporal response function of the system). Data analysis can deliver correct results only if the IRF is correct.

3. Understanding the Data Analysis

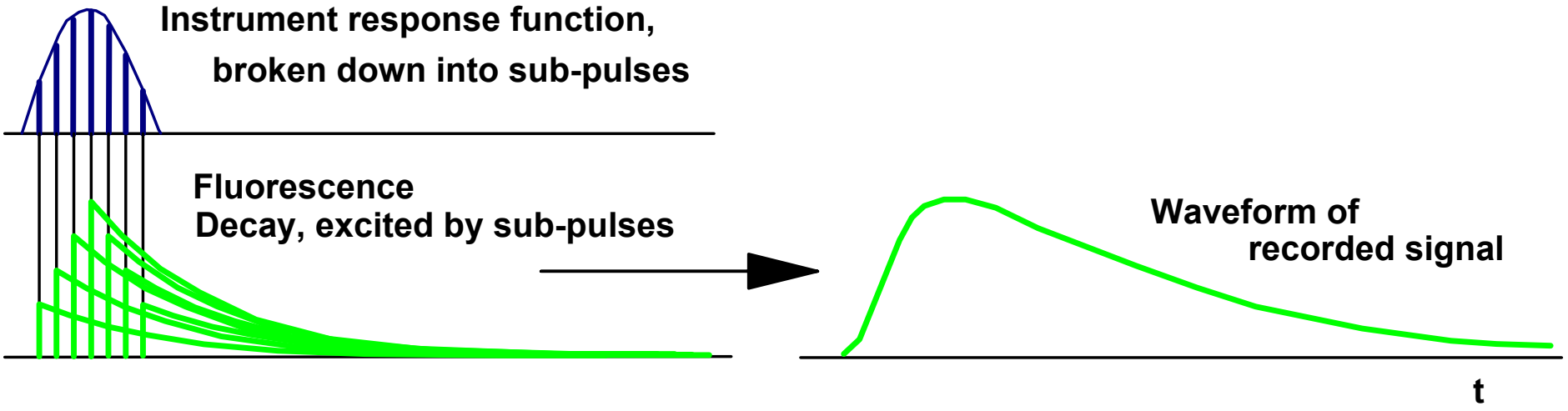
3.1. The recorded signal is a convolution of the fluorescence decay profile with the 'instrument-response function' (IRF):

$$f_m(t) = \int_{\tau=0}^{t} f(\tau) IRF(t-\tau) d\tau$$

$f_m(t)$ = measured fluorescence function, $f(t)$ = true fluorescence decay function.

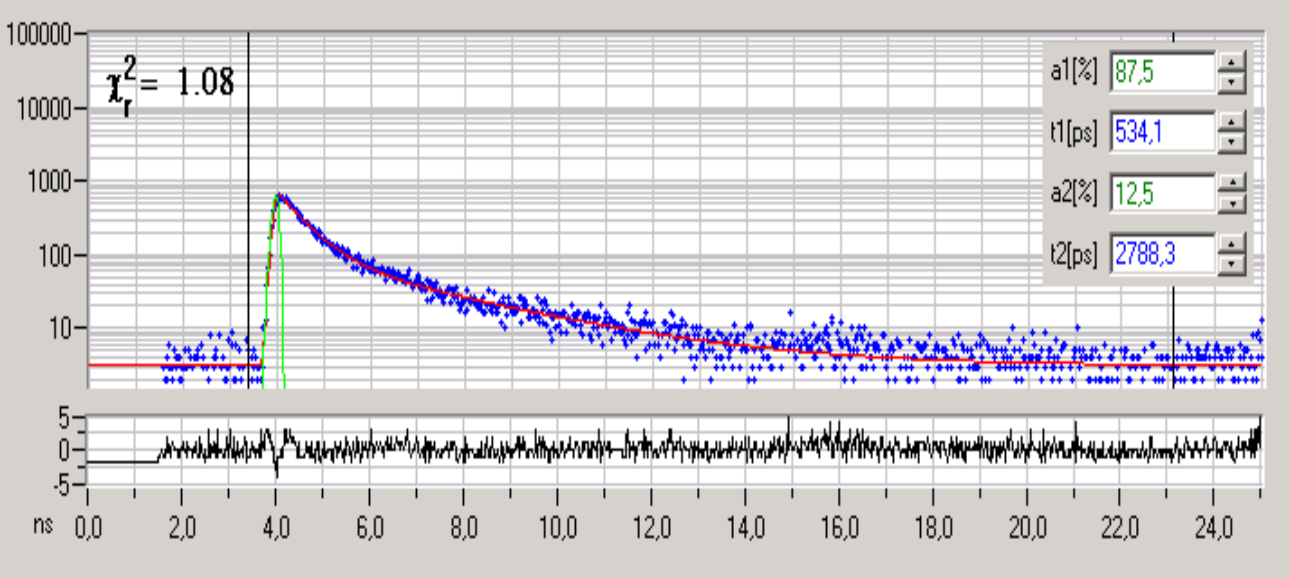
Understanding the Convolution Integral:

- Break down the IRF into a sequence of infinitely short sub-pulses of different amplitude
- Construct the recorded signal shape from a sequence of fluorescence decay functions excited by these sub-pulses
- For an infinite number of sub-pulses the result becomes the convolution integral



Convolution cannot be reversed analytically

An 'iterative convolution' and fit procedure must be used to derive the real decay function.



Deconvolution Procedure:

- **Convolute the model function,** $f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$ **with the IRF**
- **Compare result with measured fluorescence data**
- **Change model parameters until best fit is obtained**
- **Repeat the procedure for all pixels of the FLIM data set**

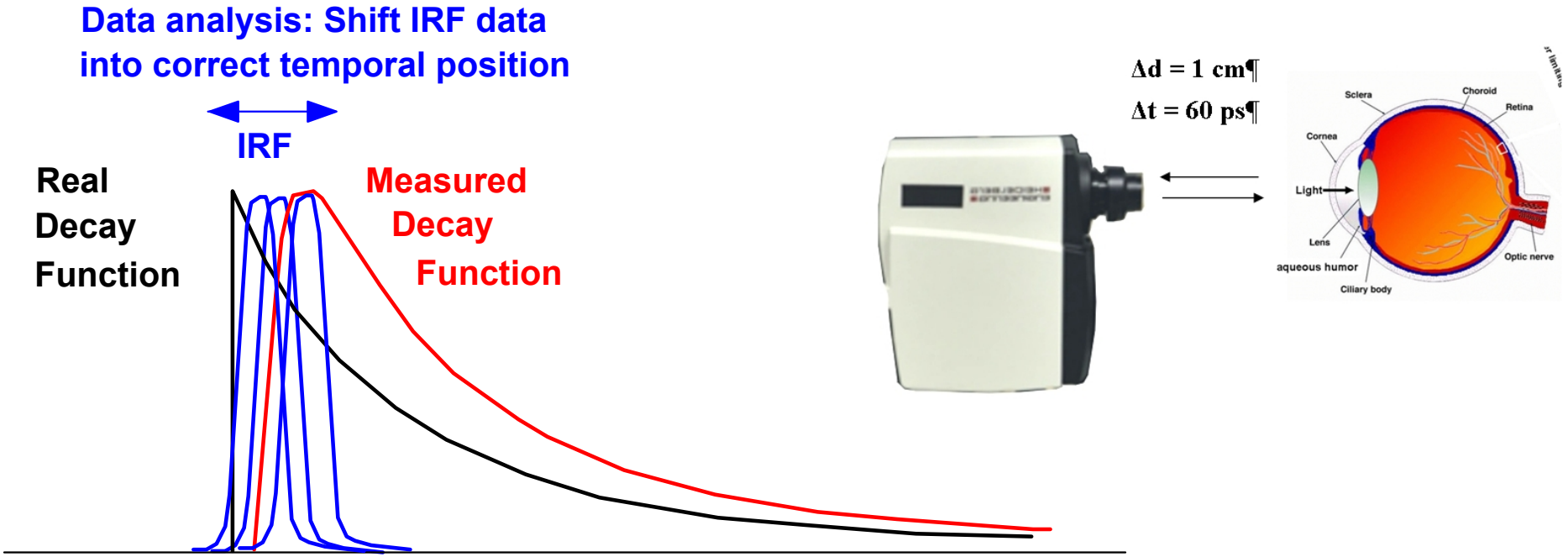
Correct data analysis requires that the correct IRF and the correct model function be used!

3.1. Problems FLIO Data Analysis: IRF shape and position

1. We cannot exactly measure the IRF. Dichroic mirrors and filters in the beam path prevent us from doing so.
2. The optical path length varies from patient to patient. The IRF shifts.

Solution:

1. A synthetic IRF is used.
2. The data analysis shifts the IRF in a position where it fits the data best.



But there are other pitfalls:

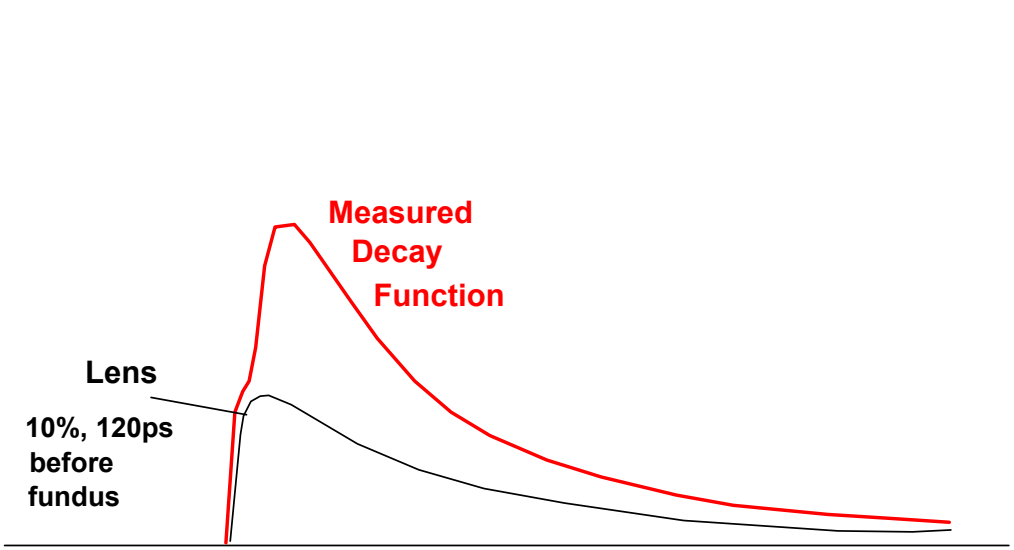
3.2. Problems of FLIO Analysis: The Commonly Used Model Function is Wrong!

Commonly-used model: Triple-exponential decay

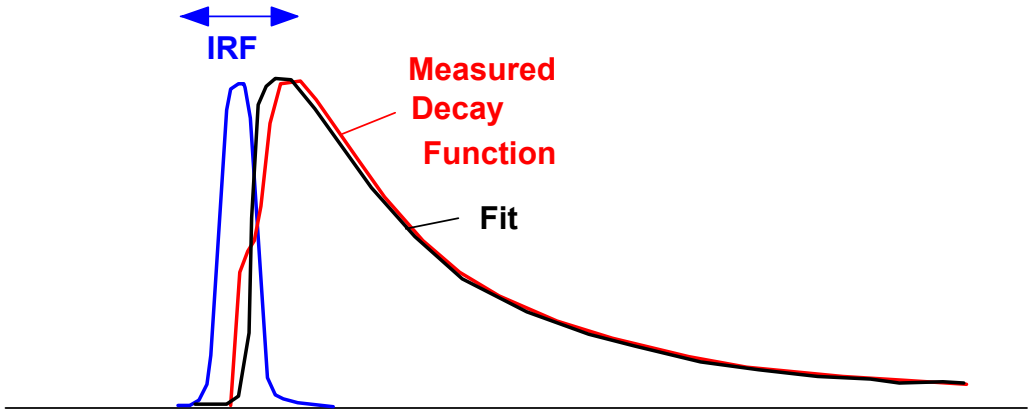
$$f(t) = a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2} + a_3 e^{-t/\tau_3}$$

This is wrong!

The fluorescence from the fundus is overlaid by fluorescence from the lens:



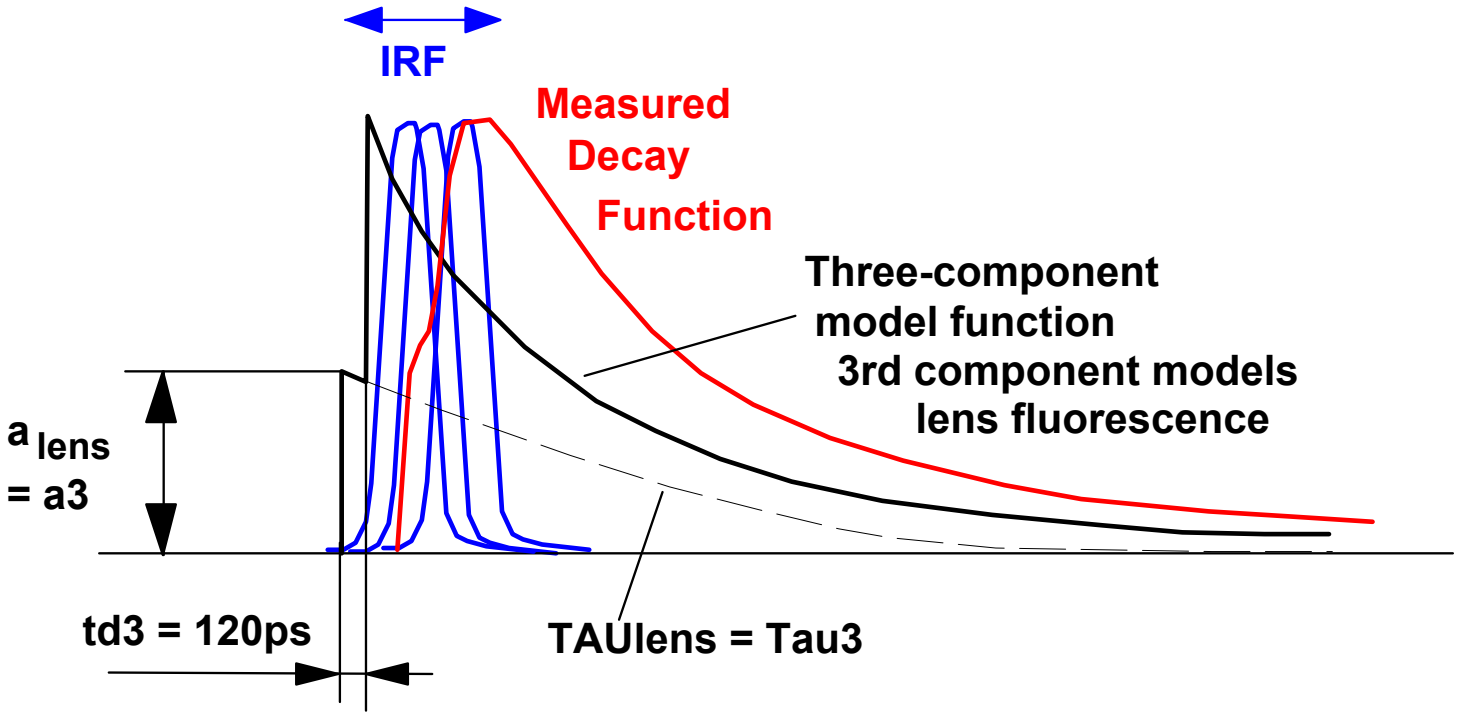
Fit with traditional 3-exp model:
Where ever you shift the IRF, the model does not fit the rising edge
IRF positioning does not work



Consequences:

- **poor fit of rising edge of fluorescence**
- **instability of the fit**
- **inability of the fit routine to find the correct IRF position**
- **result depends on relative amount of lens fluorescence**

3.3. Correct Model Function for FLIO Data: The Shifted-Component Model



Model Function: $f(t) = a_1 e^{-t/\tau 1} + a_2 e^{-t/\tau 2} + a_3 e^{(-t+td3)/\tau 3}$

a1, a2, a3, τ1, τ2, τ3 are fit parameters

t_{d3} is assumed to be constant. In reality, t_{d3} may vary with the length of the eye.

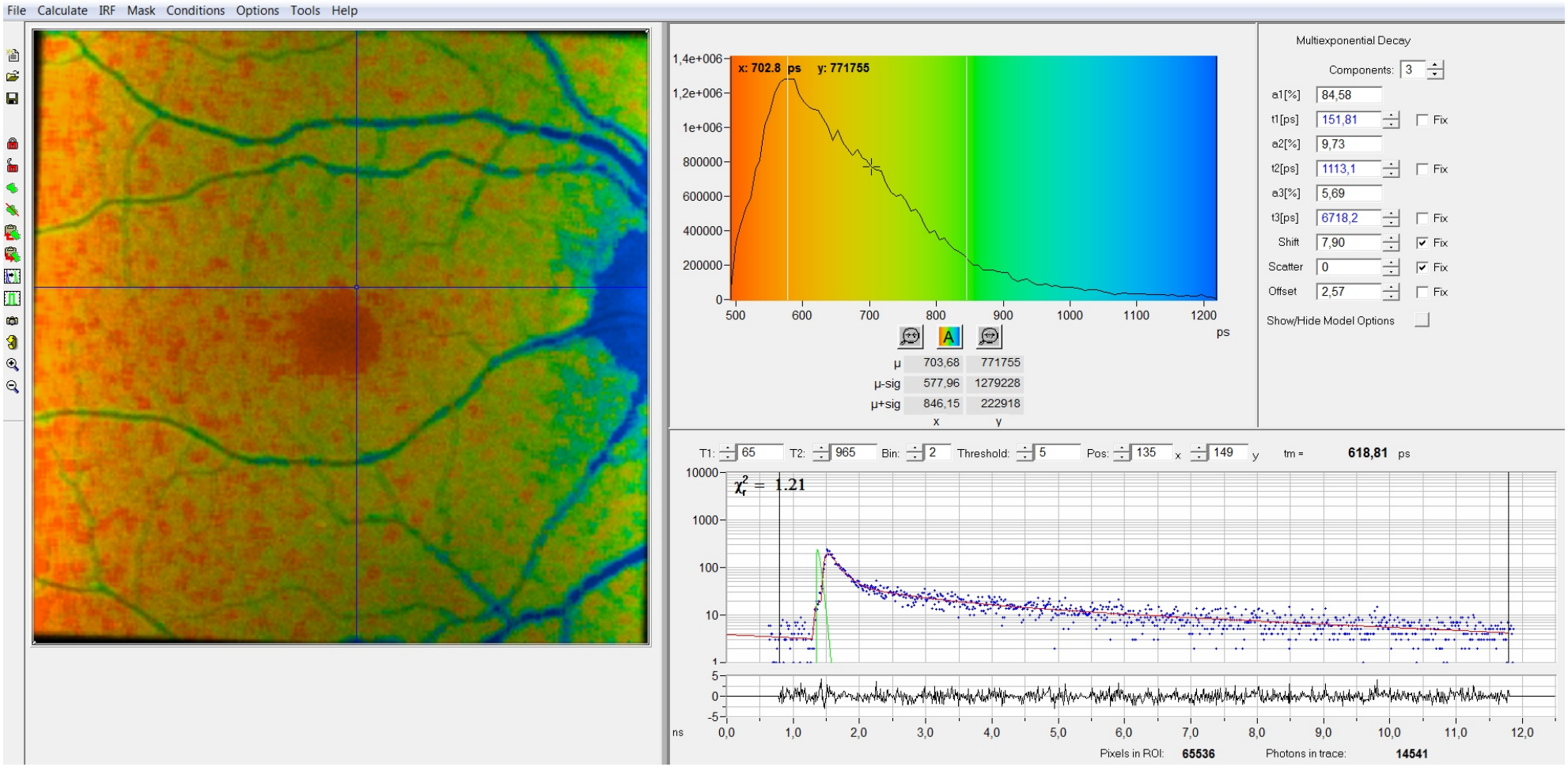
Our tests have shown that a td3 of -120ps to -150ps works well for adult humans.

The correct model function with the synthetic IRF fits the data perfectly

The figure below shows test data recorded with an ultra-fast detector

- The delayed-component model fits the rising edge perfectly

- Result: The temporal position of the IRF is determined correctly



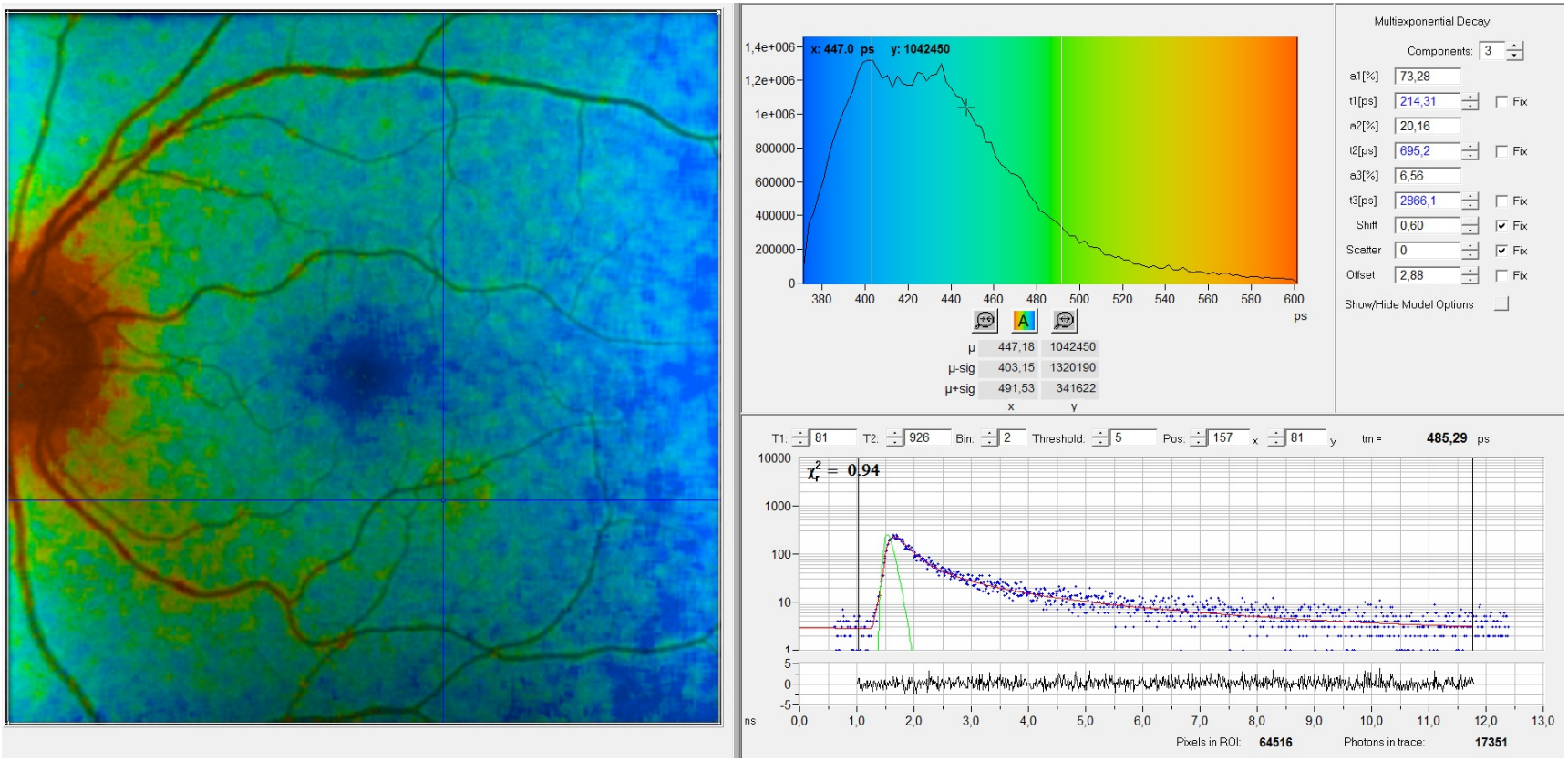
The correct model function with the synthetic IRF fits the data perfectly

The figure below shows data recorded with the standard HPM-100-40 detector of the FLIO

- **Due to the slower IRF the distortion of the rising edge is less pronounced**
- **Nevertheless, the delayed-component model fits the rising edge perfectly**

Result:

- **The fit routine is able to reliably determine the temporal position of the IRF**
- **Reproducible lifetimes and amplitudes of the decay components are obtained**



3.4. Summary of Analysis with Delayed-Component Model

The problem of the conventional data analysis was that

- **the exact shape and position of the IRF are unknown**
- **the model function did not correctly describe the rising edge of the fluorescence pulse**
- **consequently, the correct position of the IRF was not determined reliably**
- **the lifetimes and amplitudes of the decay components were not determined reliably**

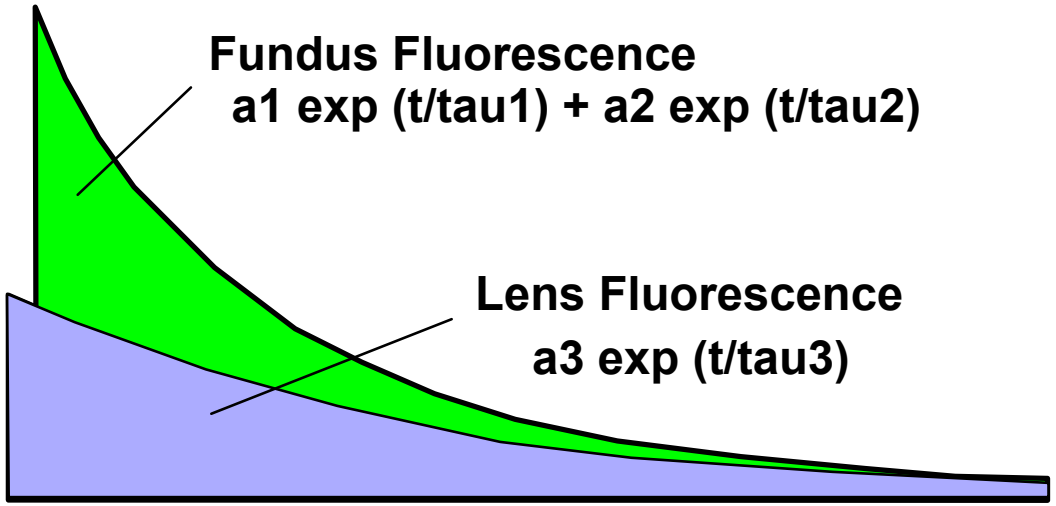
The Delayed-Component Model solves these problems.

- **the model function correctly describes the rising edge of the fluorescence pulse**
- **the correct position of the IRF is determined**
- **the lifetimes and amplitudes of the decay components are determined reliably**
- **the data analysis runs without the need of tricky user interactions**

Please see: W. Becker, Axel Bergmann, Lydia Sauer, Shifted-Component Model Improves FLIO Data Analysis, Application note, available on www.becker-hickl.com

4. Separating the Fundus and from the Lens

4.1. The lens fluorescence and the fundus fluorescence start at different times after the excitation pulse



- Fit the data with the delayed-component model
- The fundus fluorescence is: $a_1 \exp(t/tau_1) + a_2 \exp(t/tau_2)$
- Calculate amplitude-weighted lifetime for components 1 and 2:

$$t_{m12} = a_1\, tau_1 + a_2\, tau_2 / (a_1 + a_2)$$

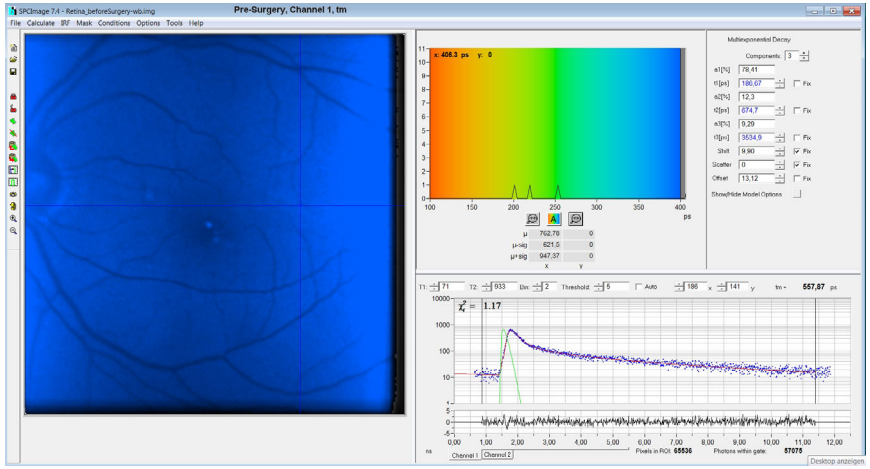
- t_{m12} is equivalent to former t_m, but free of lens fluorescence

Please see: W. Becker, Axel Bergmann, Lydia Sauer, Shifted-Component Model Improves FLIO Data Analysis, Application note, available on www.becker-hickl.com

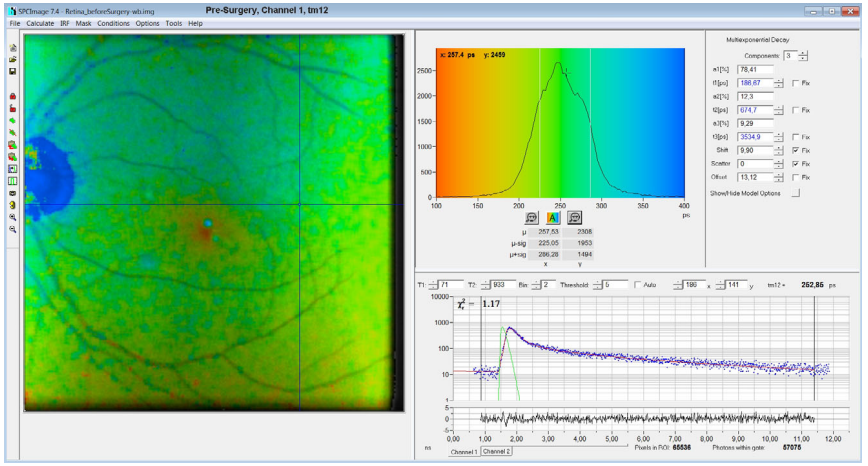
4.1. Experiment: FLIO before and after Cataract Surgery

Pre-Surgery Images. Strong Lens Fluorescence.

tm image, contains lens fluorescence, totally out of normal range.

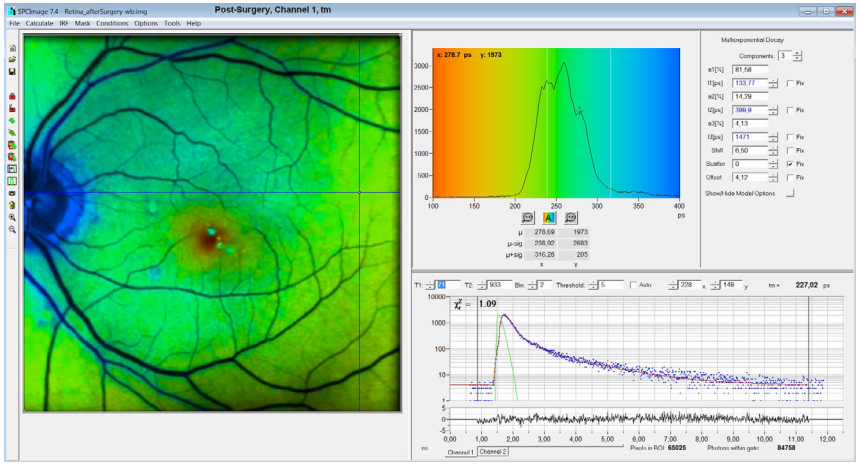


tm12 image, free of lens fluorescence, tm12 in normal range.

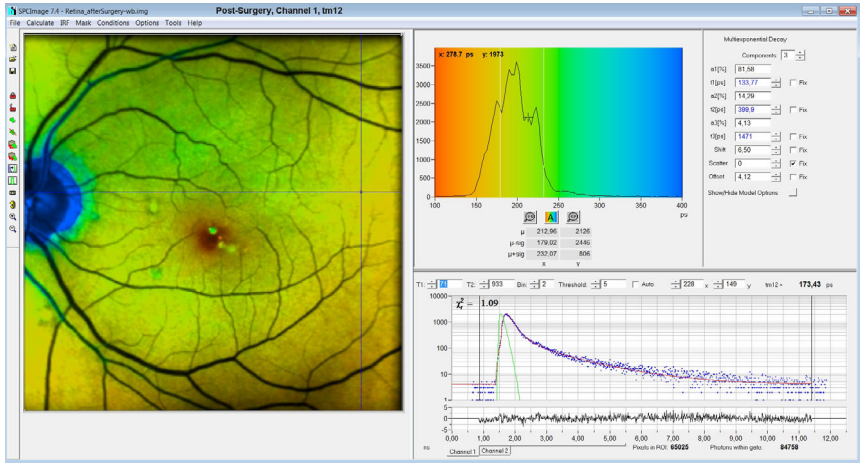


Post-Surgery Images. Artificial Lens. Weak Lens Fluorescence

tm image is in correct range.



tm12 is in correct range.



Data courtesy of Lydia Sauer, University of Utah

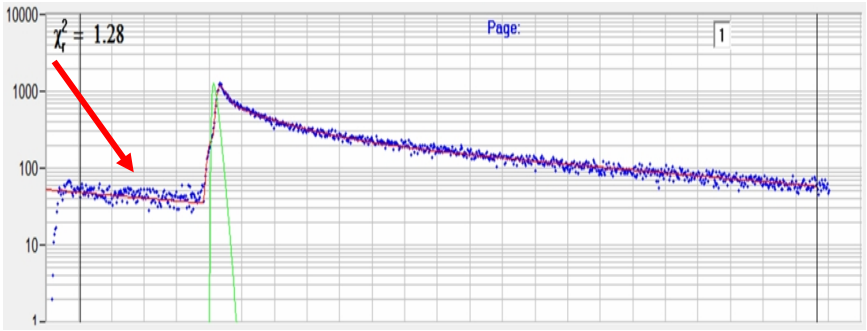
Delayed-Component model delivers correct fundus lifetimes in presence of lens fluorescence

5. Other Model Considerations

Incomplete Decay

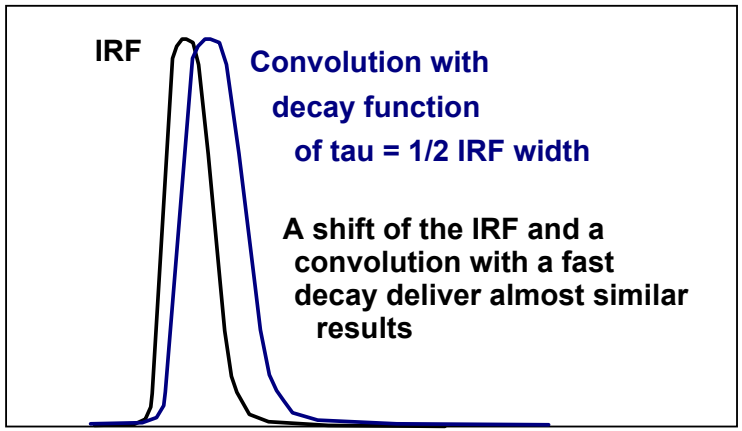
FLIO data contain decay components with long lifetimes. These components do not completely decay within the excitation pulse period.

Use the 'Incomplete Multiexponentials' model of SPCImage to account for this effect.



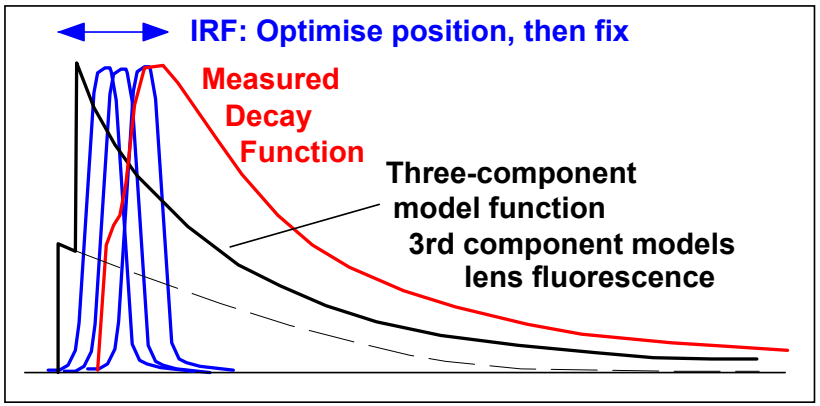
Floating IRF

SPCImage can leave the IRF position floating. In principle, a floating IRF solves the problem of the undefined IFR position. However, a shift in the IRF has almost the same effect on the convolution integral as a convolution with a fast decay. As a result, the fit of fast decay components with a floating IRF becomes ambiguous. A floating IRF should therefore not be used for FLIO analysis.



Fix IRF shift before calculation

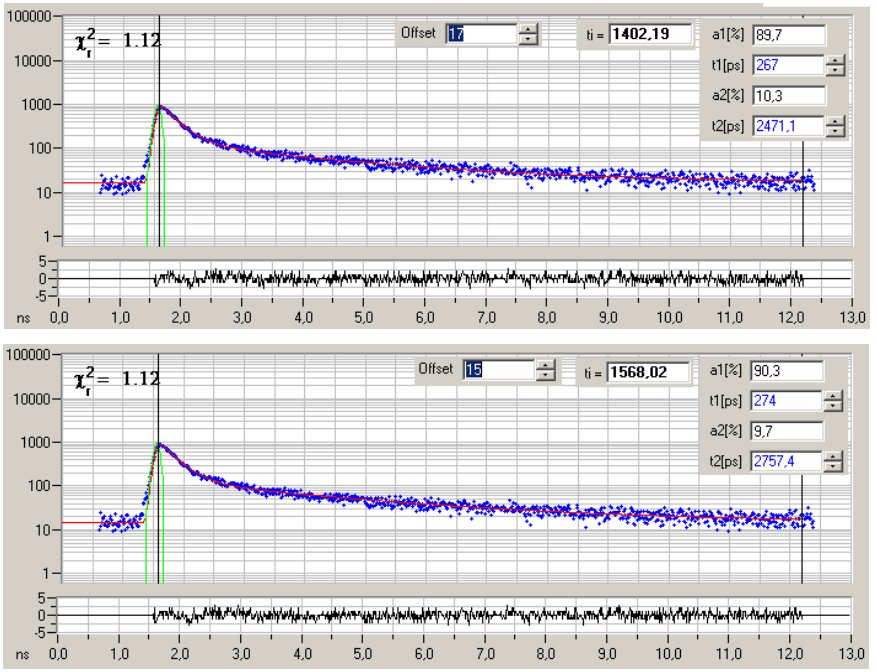
In the model parameters, use the option 'Fix IRF shift before calculation'. The fit procedure then determines the best IRF position, and then runs the deconvolution procedure with an IRF in the optimised position.



Baseline Offset

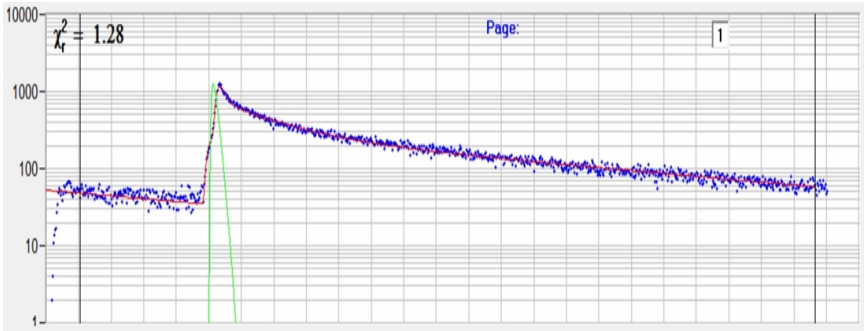
SPCImage has a fit parameter for a possible baseline offset. However, a change in the baseline offset has closely the same effect on the fit as a change in the lifetime of a slow decay component. The problem is enhanced when incomplete decay is present. Unfortunately, baseline offset by pickup of environment light is not entirely avoidable with the FLIO. Therefore, there is currently no way around using the baseline offset parameter. All that can be done is to keep daylight pickup as low as possible.

Example on the right: A fit with offset = 17 and t2 = 2471 ps and a fit with offset = 15 and t2= 2757 ps deliver the same quality of the fit, $\chi^2 = 1.12$.



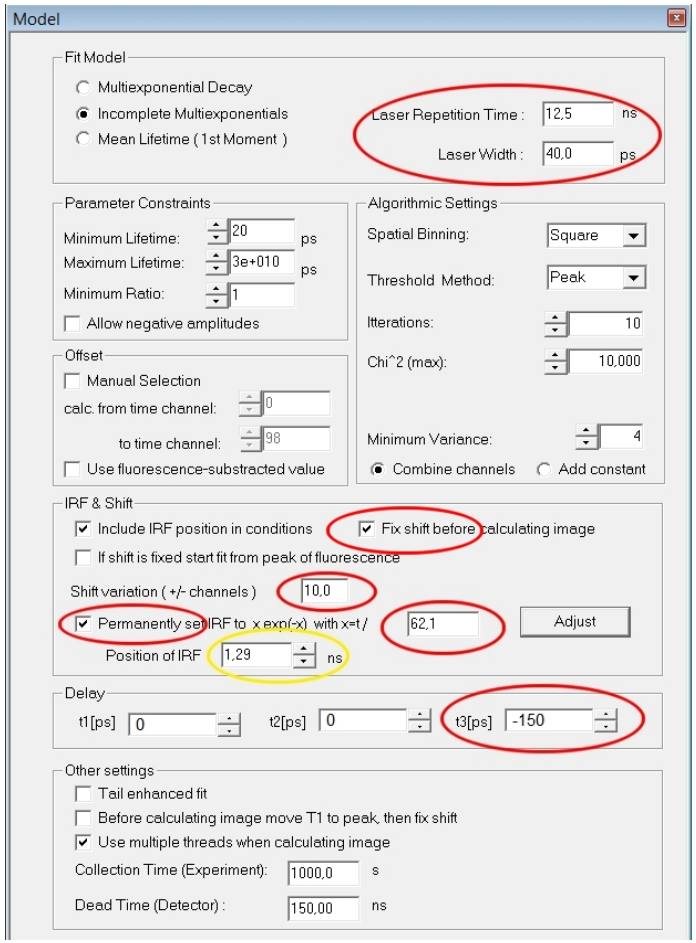
Fit Interval

The time interval where the decay data are fitted is defined by the cursors in the decay curve window. In the past, there have been contradicting suggestions of where to put the decay cursors. Usually, the fit interval was set to exclude the rising edge of the fluorescence from the fit. With the delayed-component model, set the cursors so that the fit range includes the entire decay curve.



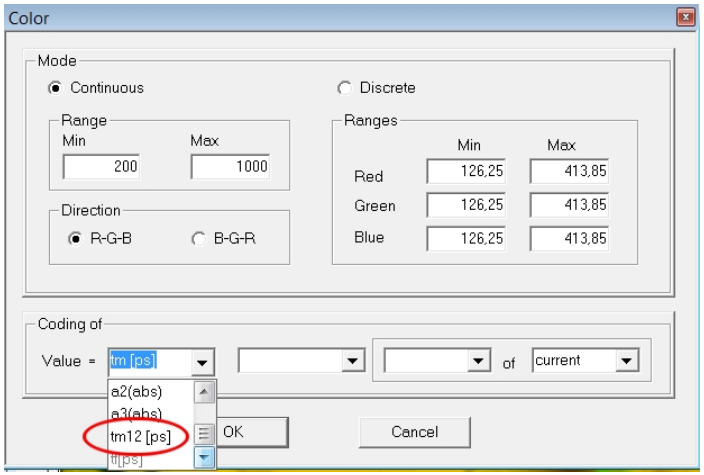
6. Model-Parameter Definitions in SPCImage 7.4

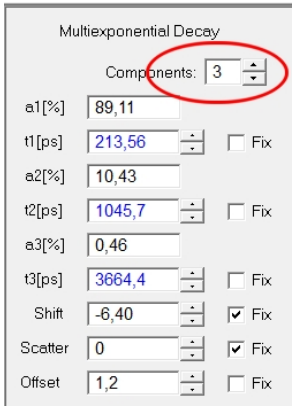
6.1. The model parameters for FLIO analysis with the Delayed-Component Model are shown below. Parameters marked red are identical for all instruments. Parameters marked yellow are instrument-specific.



Permanent setting, all instruments

Permanent, but instrument-dependent





Please note:

The parameter definitions may change in later SPCImage versions. Please check www.becker-hickl.com for later versions of this document.

6.2. Permanent Configuration of SPCImage for Analysis with the Shifted-Component Model and the Synthetic IRF

Users of Heidelberg-Engineering FLIO Systems

- **Start SPCImage from Desktop or Windows Start Menu**
- **Set IRF parameters as shown in the previous section**
- **Click on ‘Store Conditions’ in the head bar of SPCImage**
- **To reverse the changes, load an earlier .img file, and click on ‘Store Conditions’**

Other SPCImage Users

- **Start SPCImage from Desktop or Windows Start Menu**
- **Set IRF parameters as shown in the previous section**
- **Click on ‘Store Conditions’ in the head bar of SPCImage**
- **To retrieve the changes at next SPCImage start, click on ‘Load Conditions’**

If you require help please send us a .img file with good FLIO data. We will set the model parameters and send the file back to you. Load this file and click on ‘Store Conditions’.

7. Summary

FLIO data are recorded by a three-dimension TCSPC technique

FLIO data are photon distributions over x, y, and t

The FLIO photon distribution can be considered an image with a decay curve in each pixel

FLIO analysis is based on a iterative convolution and fit procedure

The traditional triple-exponential decay model has been replaced with triple-exponential shifted-component model

In the new model, the fluorescence of the eye lens is modelled by third component shifted left by 150 ps

A synthetic IRF is used to define the temporal response of the FLIO instrument

Compared with the traditional model the fit stability of the new model is much better

Data analysis with the new model runs with permanently fixed parameters, and without user interaction

The delayed-component model is able to separate the fundus fluorescence from the lens fluorescence

This work has been part of the Meta-Net project, funded by German BMBF.