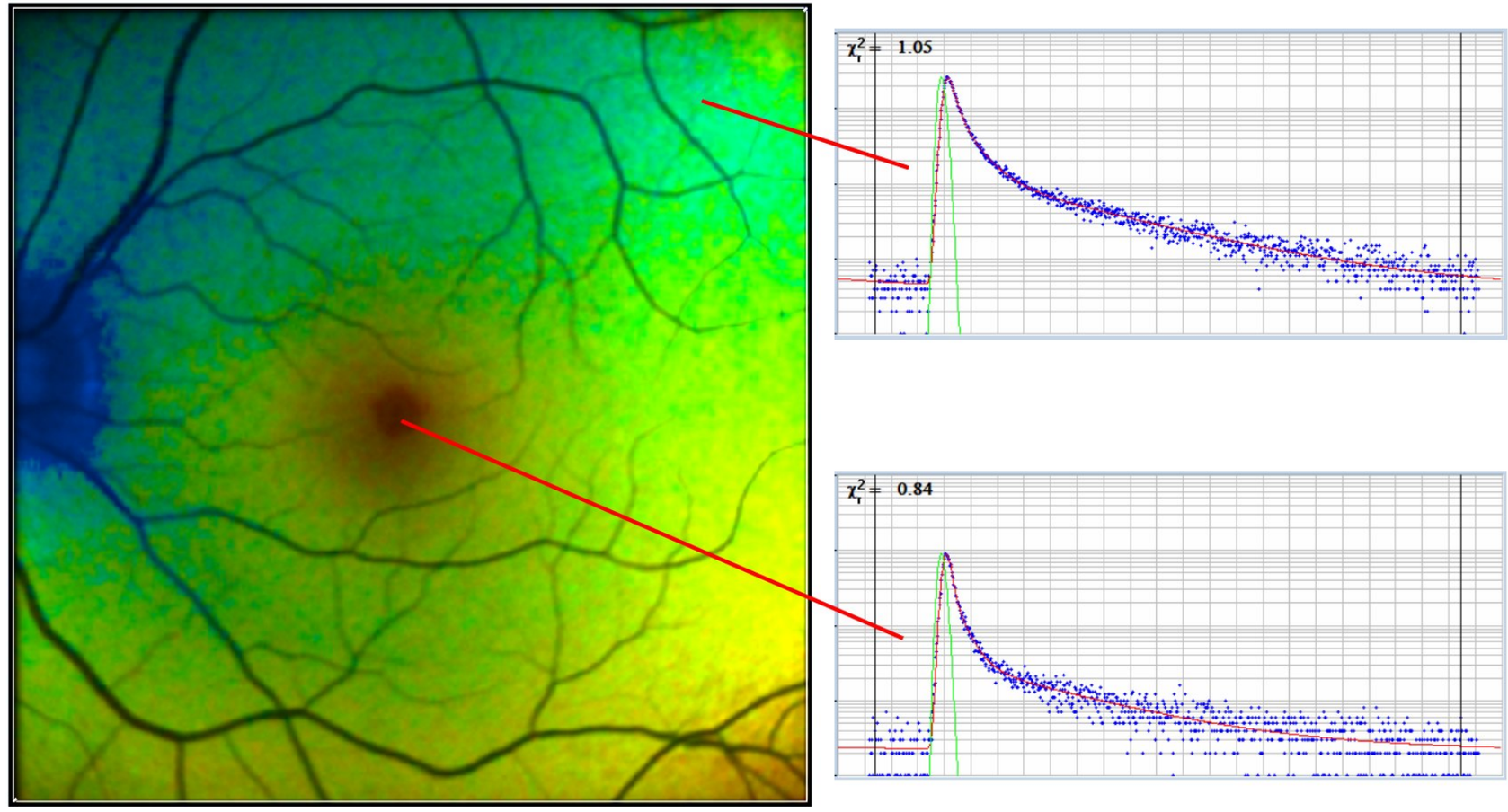
Fluorescence-Lifetime Imaging Ophthalmoscopy

Principles, Challenges, Solutions, and Applications

Wolfgang Becker, Becker & Hickl GmbH

A Guide to Beautiful FLIO Results

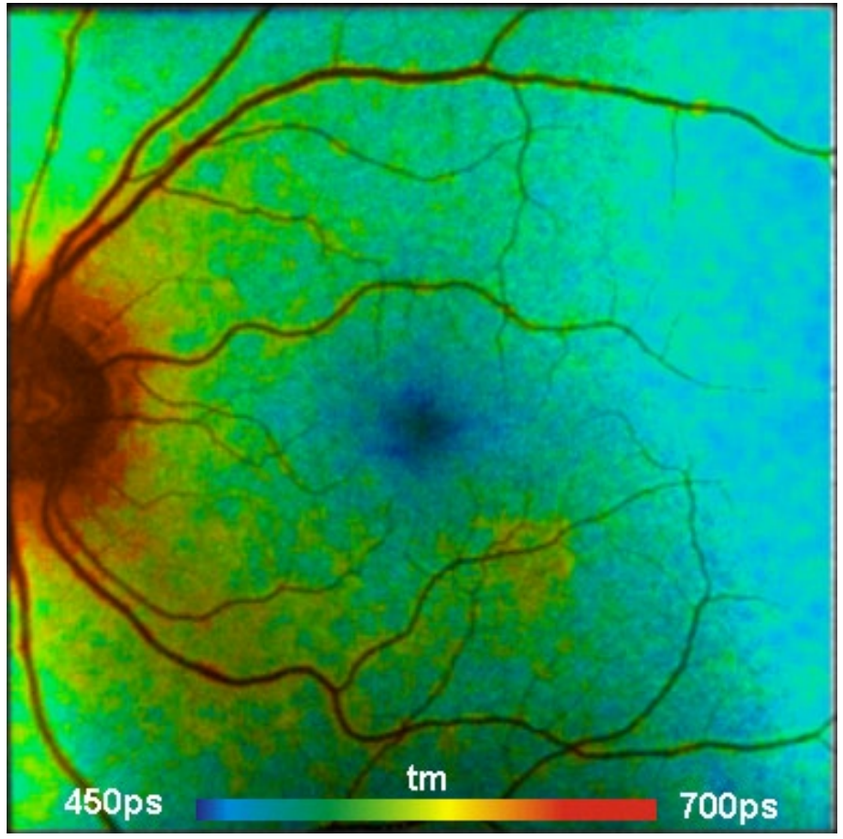


Why Base Clinical Diagnosis on Fluorescence Lifetimes?

Fluorescence lifetimes change with the molecular environment of the fluorophore

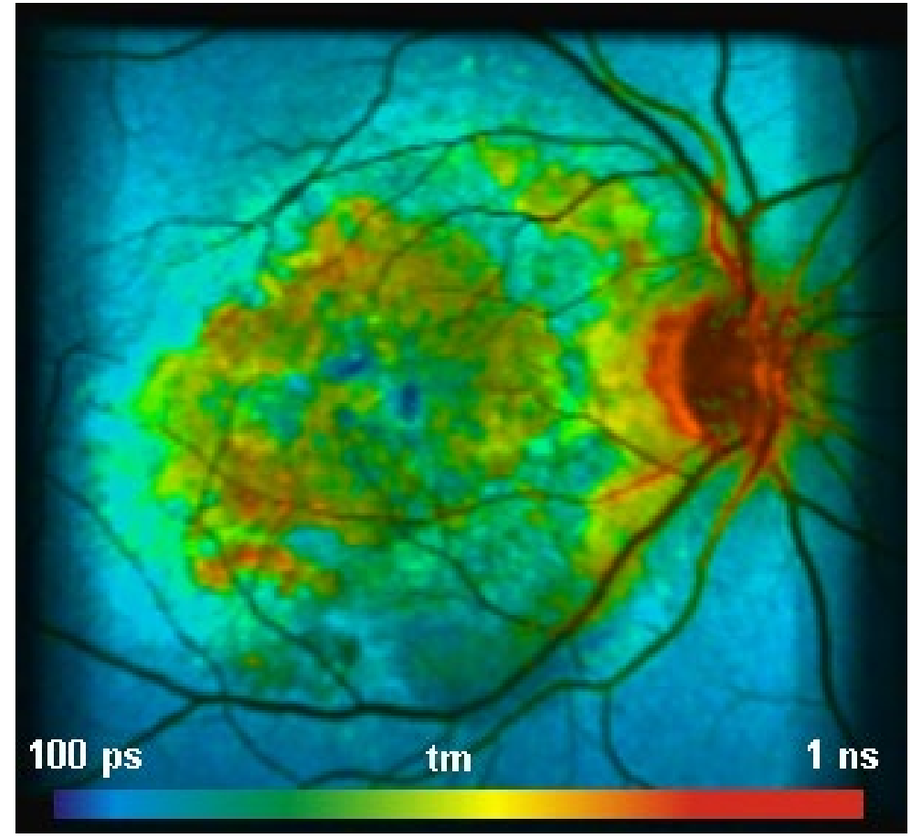
Degeneration in metabolic function manifests in lifetime changes of endogenous fluorophores

Early stages of degeneration can be detected before they have caused irreversible damage to cells and tissues

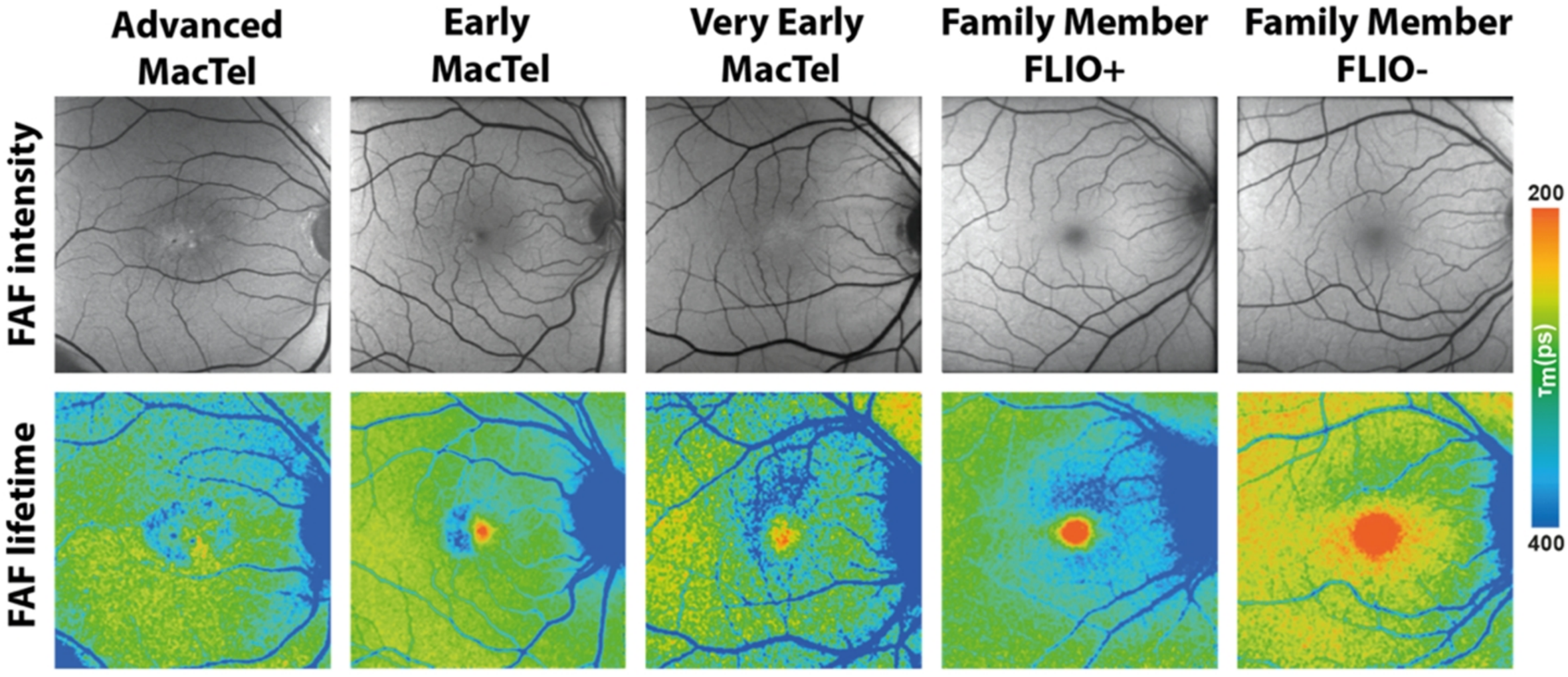


STOP

DISEASE!



MacTel Desease



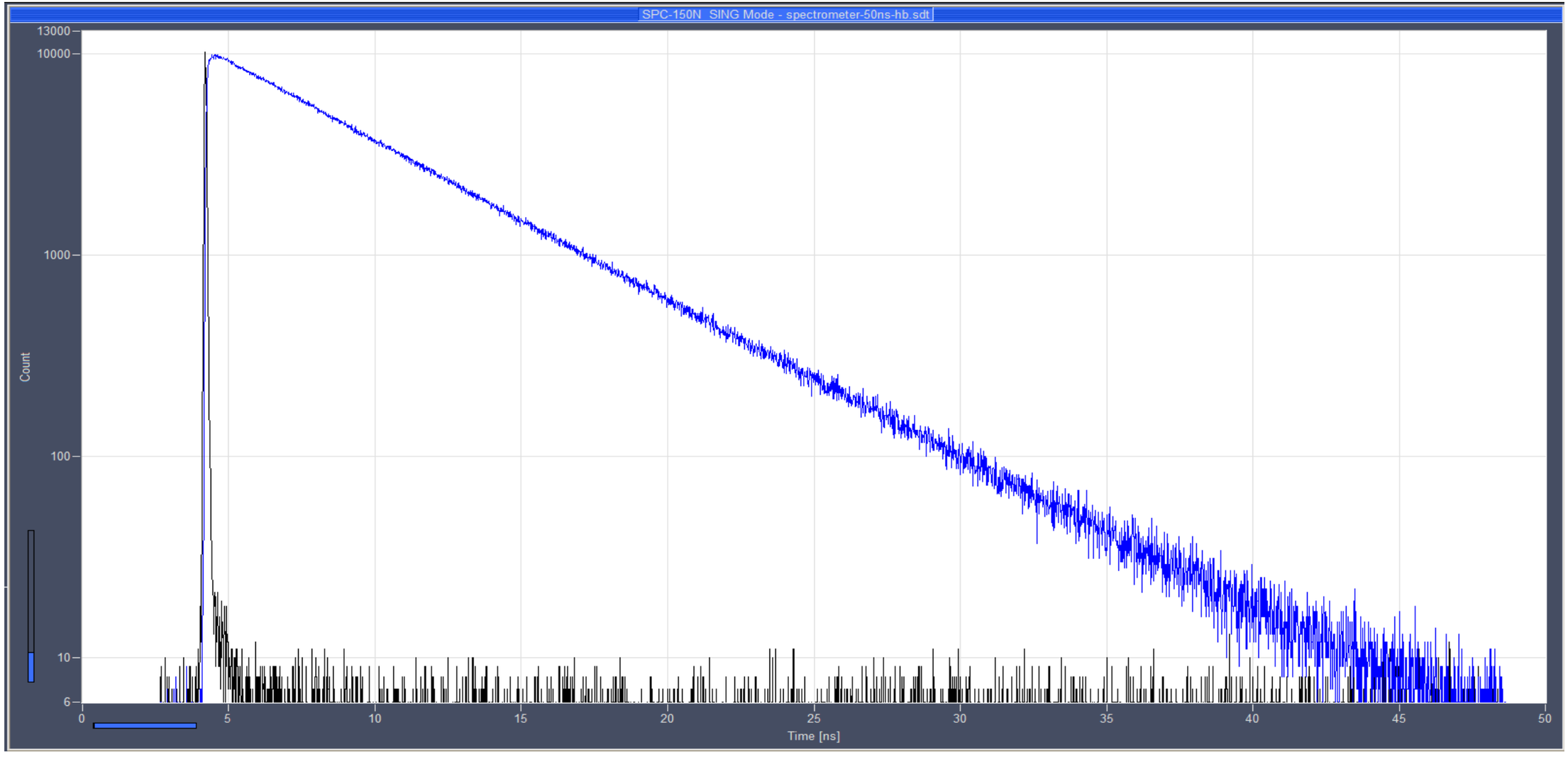
Lydia Sauer, University of Utah

Contents

Time for Questions will be provided at the end of the sections and at the end of of the lecture.

The Fluorescence Decay Function

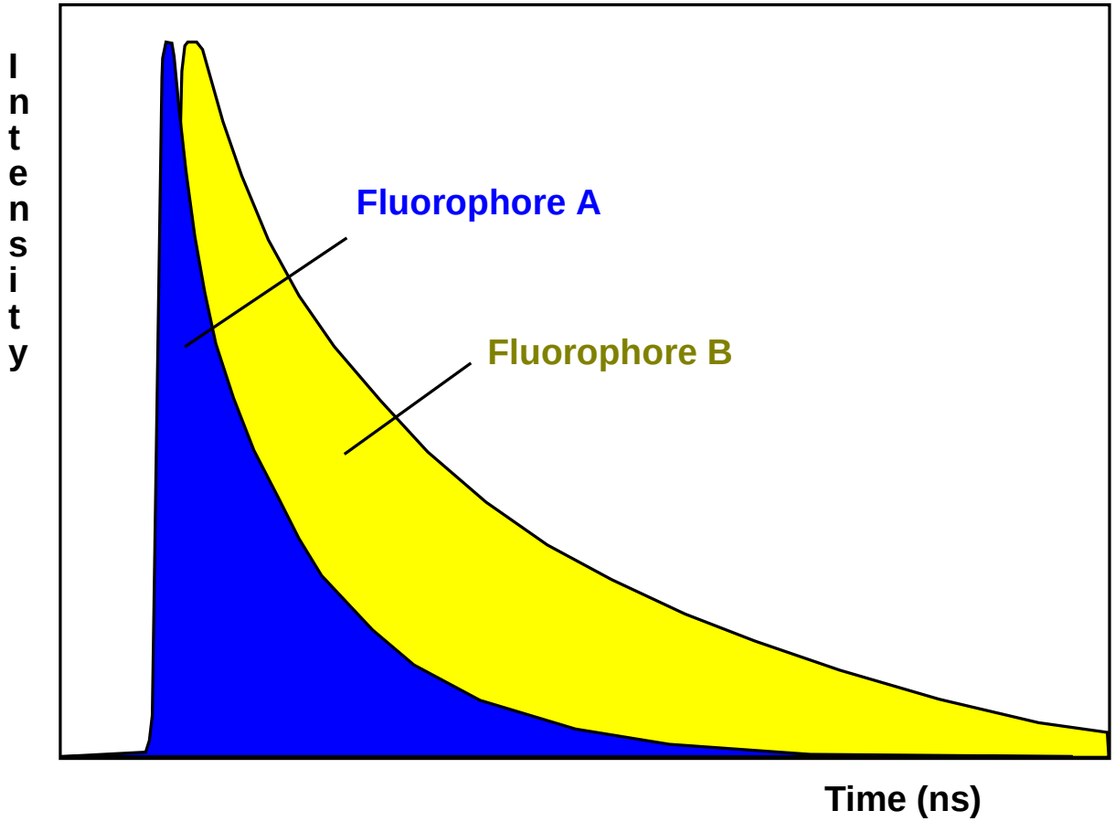
Log Scale



The decay function of a single fluorophore in a homogeneous environment is single-exponential

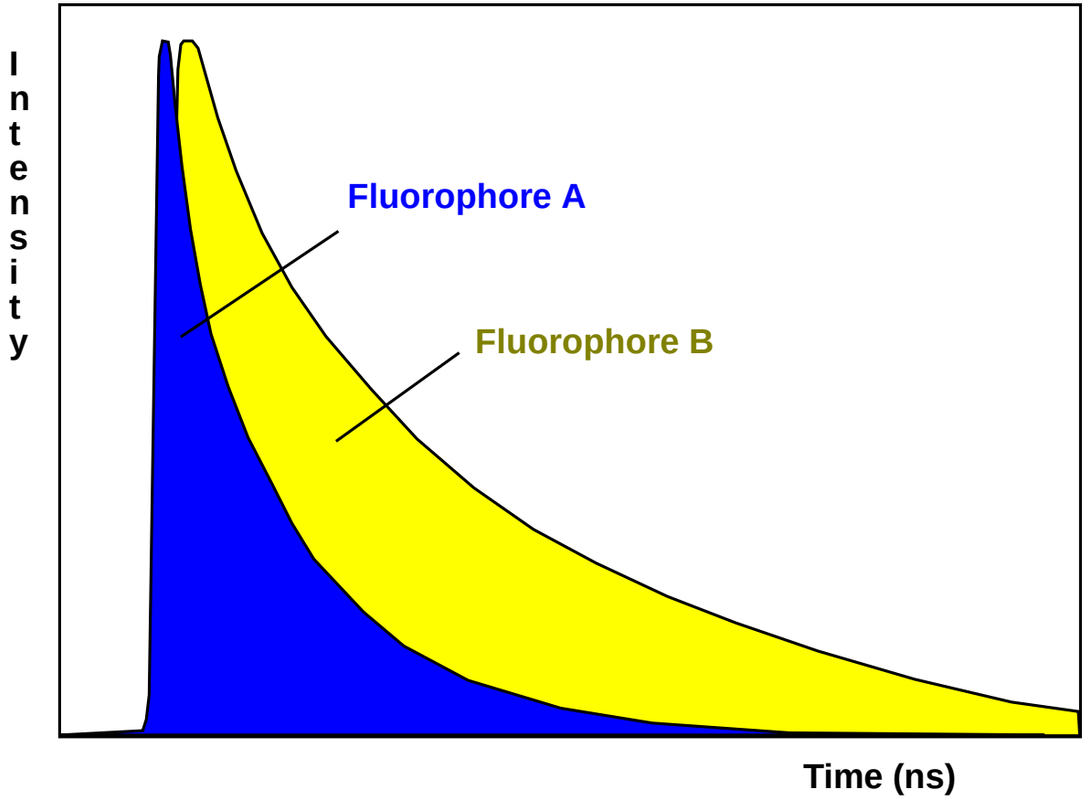
Probability to return from the excited state is time-invariant

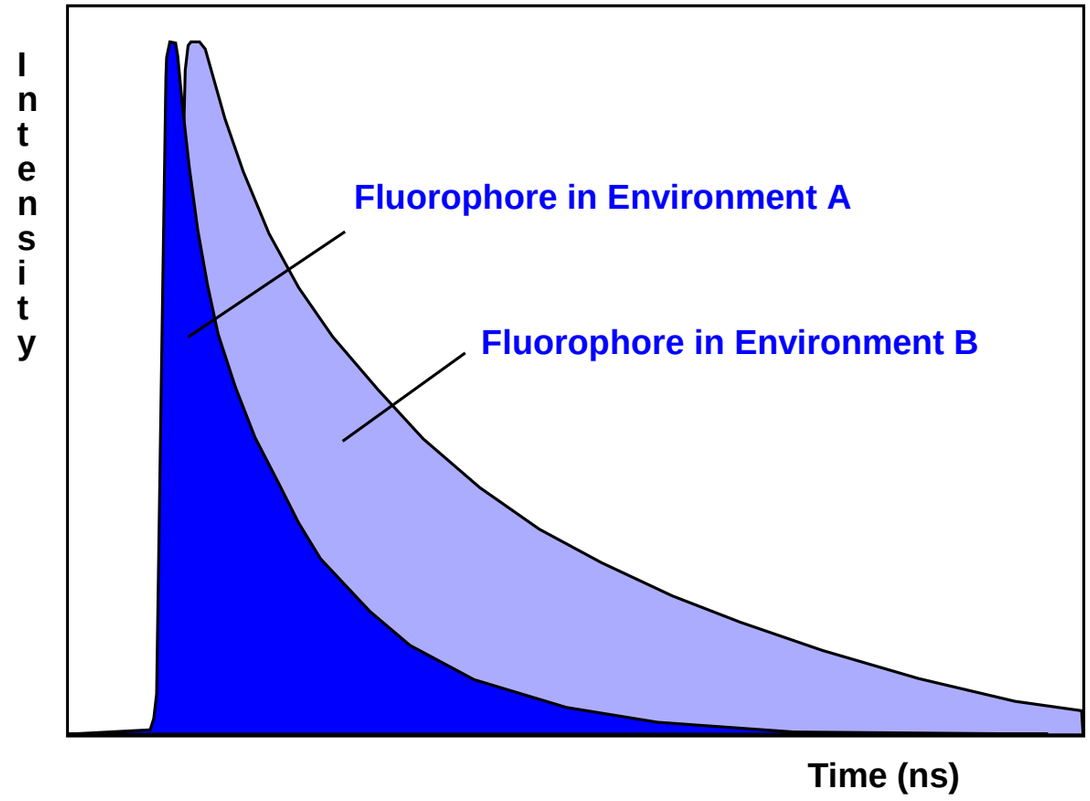
The Fluorescence Decay Function



Lifetime is different for different fluorophores
'Great: I can use it to distinguish different fluorophores'

The Fluorescence Decay Function





The fluorescence Lifetime is an Indicator of the Molecular Environment:

- **Concentration of biologically relevant ions**
- **Binding to proteins**
- **Conformation and interaction of proteins**

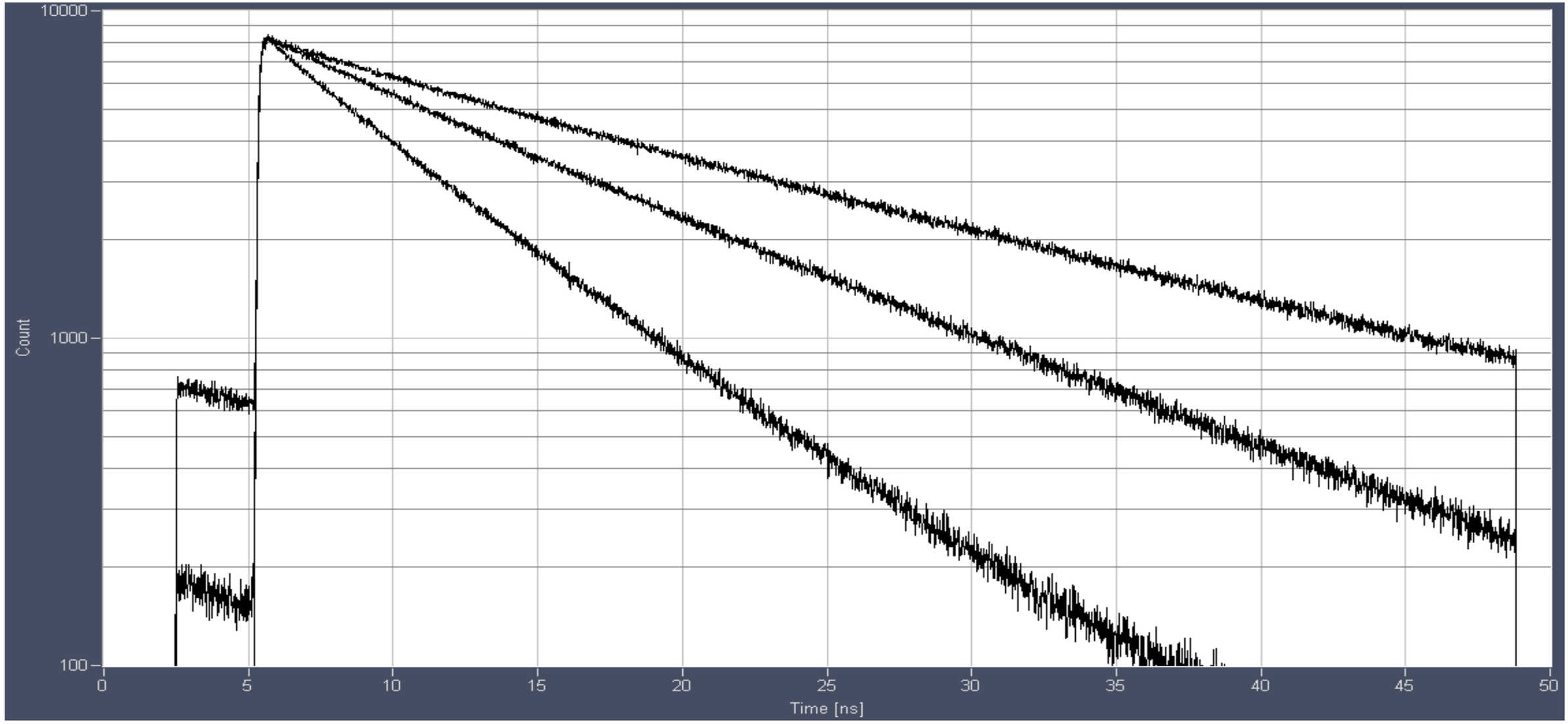
Endogenous compounds / enzymes may be fluorescent themselves

- **Conformation of enzymes**
- **Interaction of enzymes with proteins**
- **Metabolic state**

The Fluorescence Lifetime ins an indicator of the Molecular Environment

The classic example: Quinine Sulphate, different concentration of Cl^-

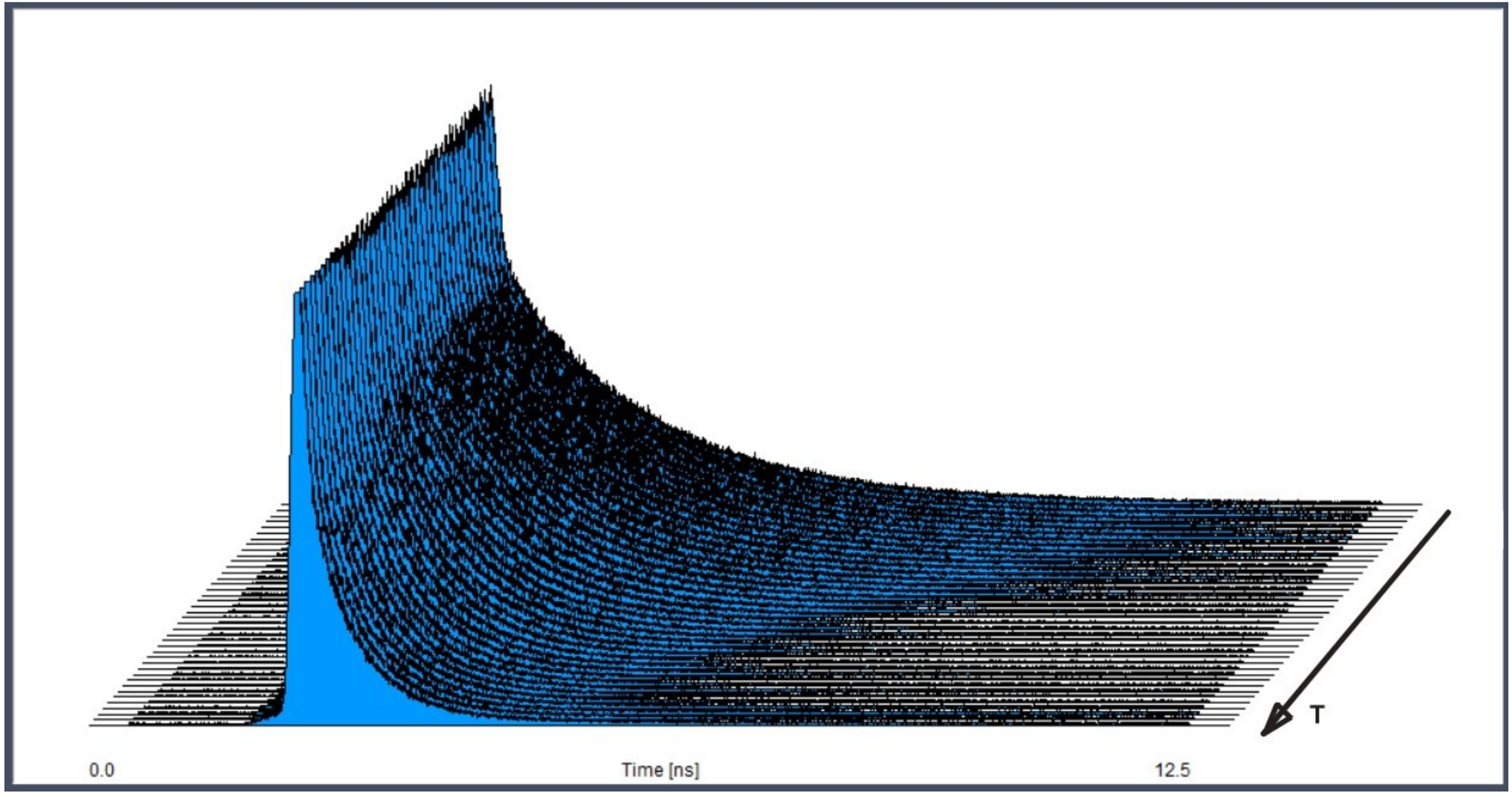
'Collisional Quenching'



Rhodamine B

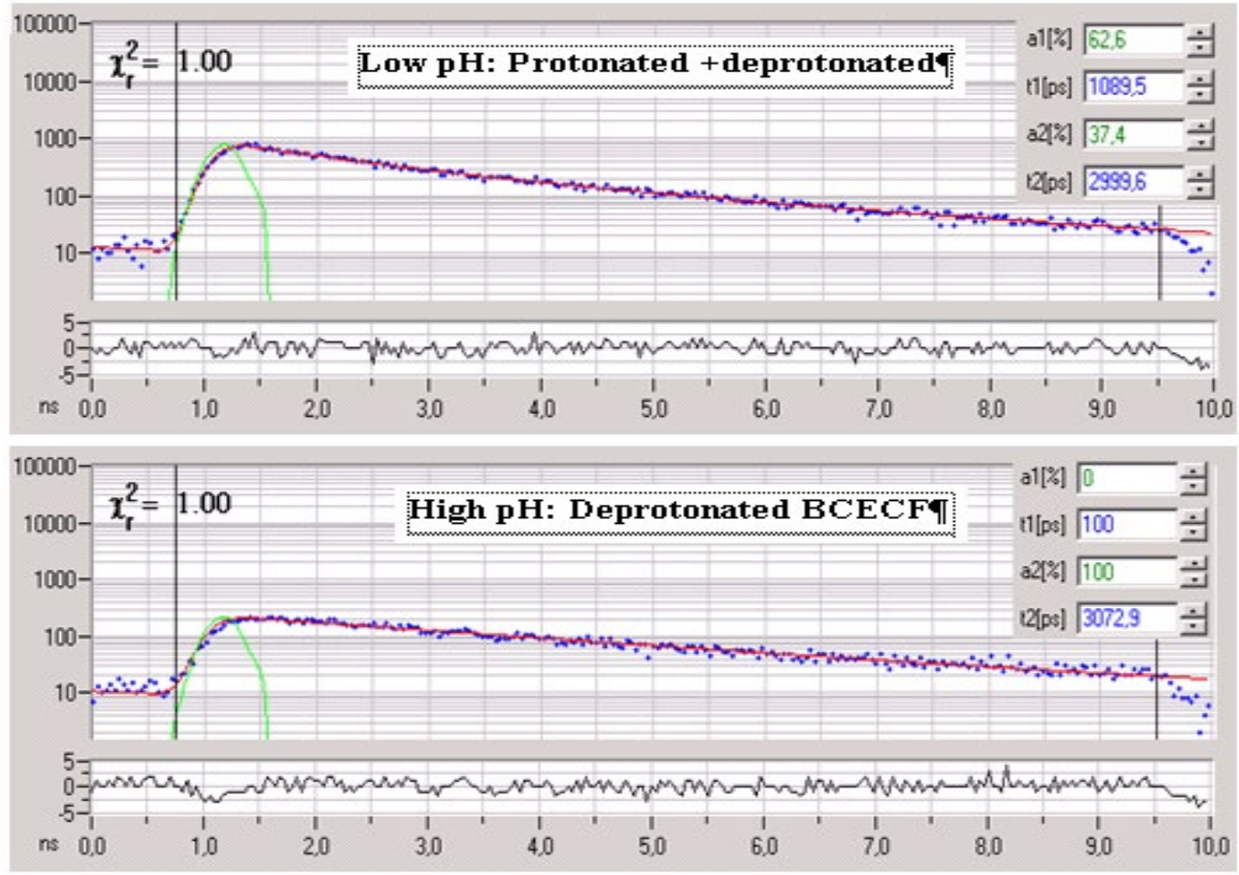
Dependence on Temperature

Solvatation: Solvent molecules arrange around the fluorophore molecule



BCECF, a Fluorescein Derivate

Protonation, Lifetime Depends on pH



Other Fffects of Lifetime Changes:

Folding State of Fluorophore
Binding to Proteins

Local Viscosity
Solvent Polarity

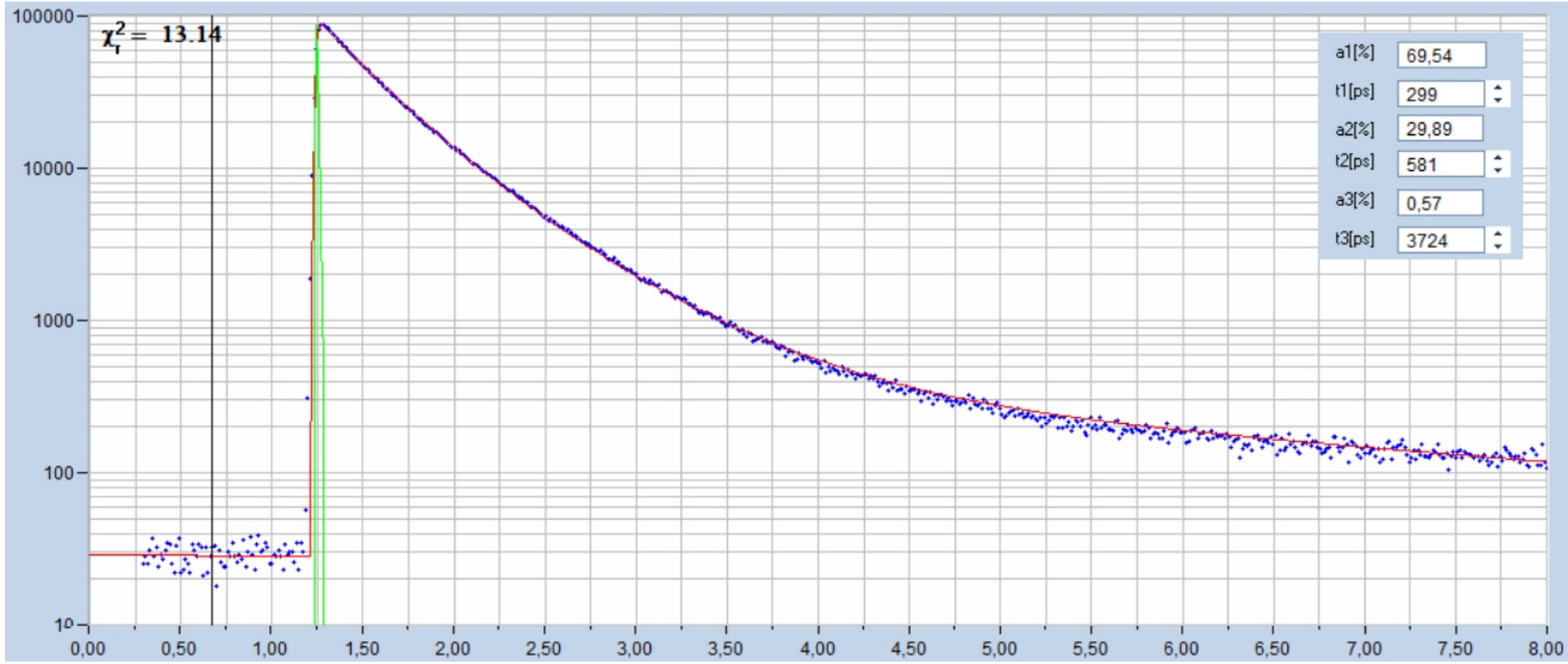
Energy Transfer
Electron Transfer

Redox Potential
or: 'Mechanism Unknown'

Multi-Exponential Decay Functions

The rule rather than the exception in biological systems

NADH in Water



Why are decay functions multi-exponential?

Are there fluorophores with intrinsically multi-exponential fluorescence decays?

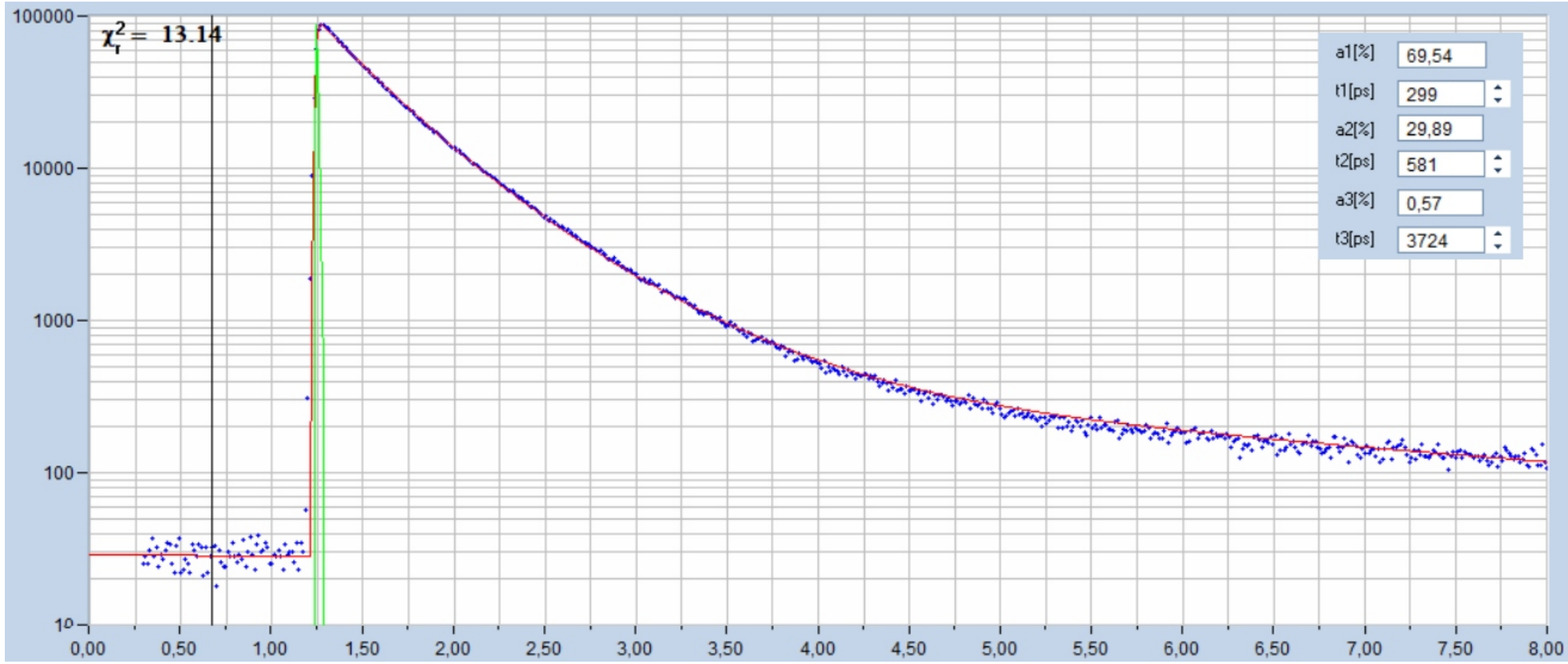
No! Transition rate back to ground state is time-invariant.

So, what's the reason?

Multi-Exponential Decay Functions

The rule rather than the exception in biological systems

NADH in Water



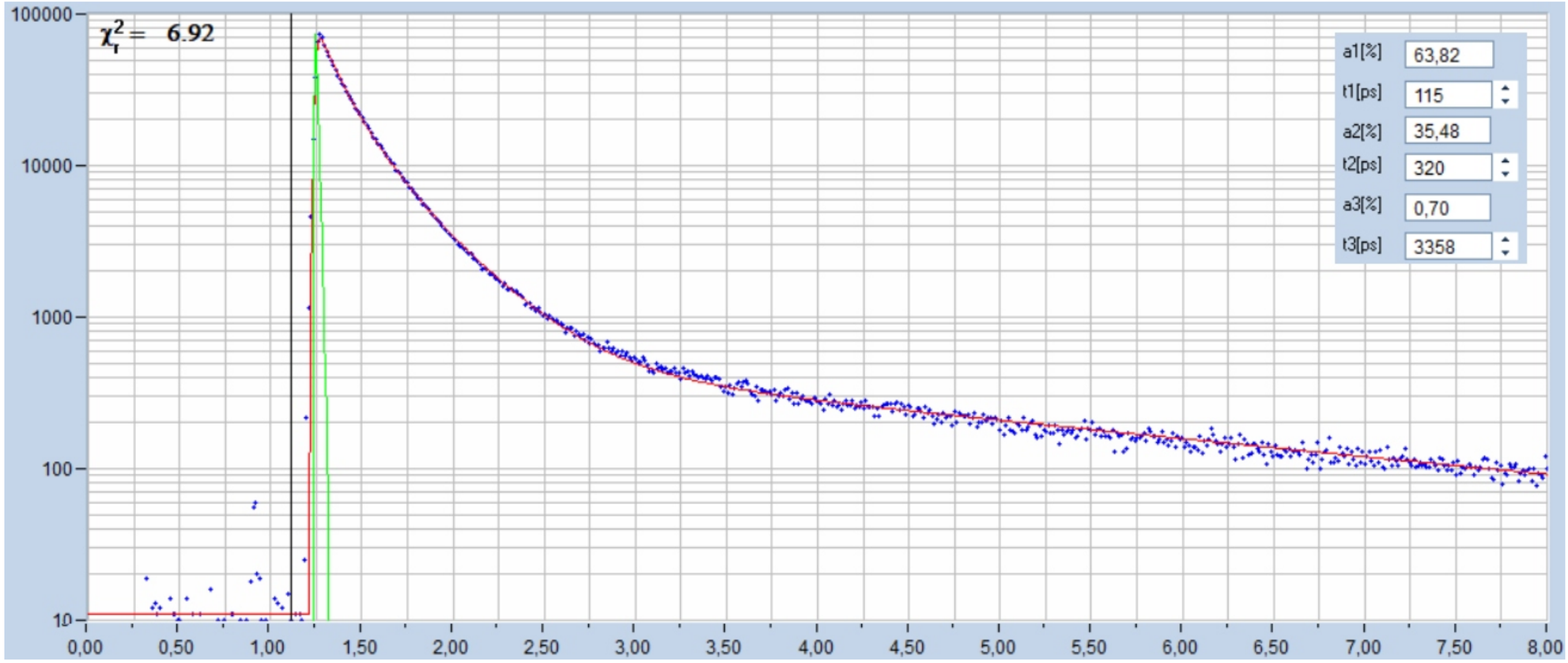
There are mixtures

- **mixture of different fluorophores. FLIO: FAD, Lipofuscin, AGEs**
- **mixtures of different geometric configurations of the molecule: Stretched, folded**
- **mixtures of protonated / deprotonated forms, free / protein-bound forms etc.**
- **The shape of the decay function changes with the molecular environment, see next.**

Multi-Exponential Decay Functions

The rule rather than the exception in biological systems

NADH in Water + Citric Acid, pH = 4



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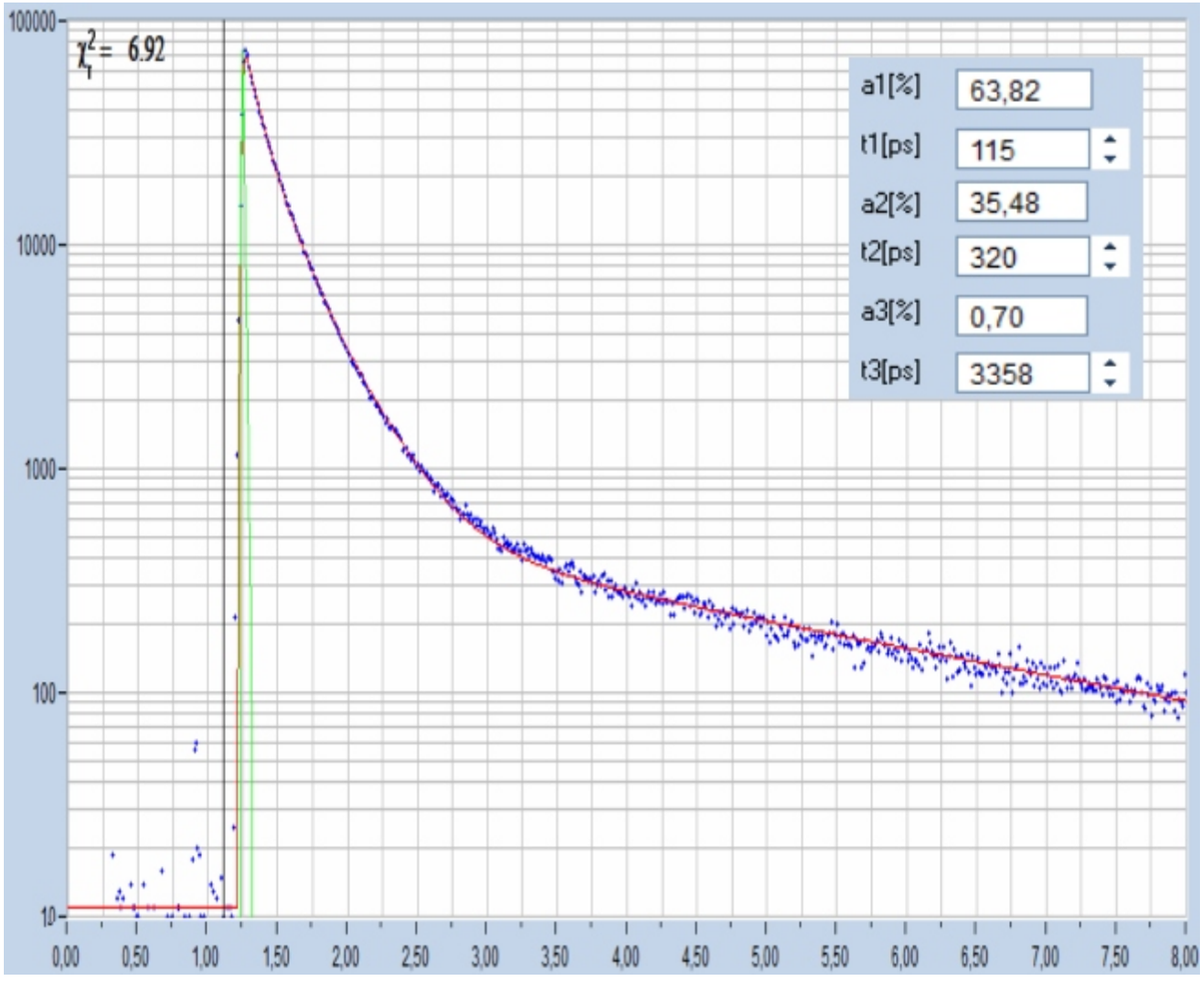
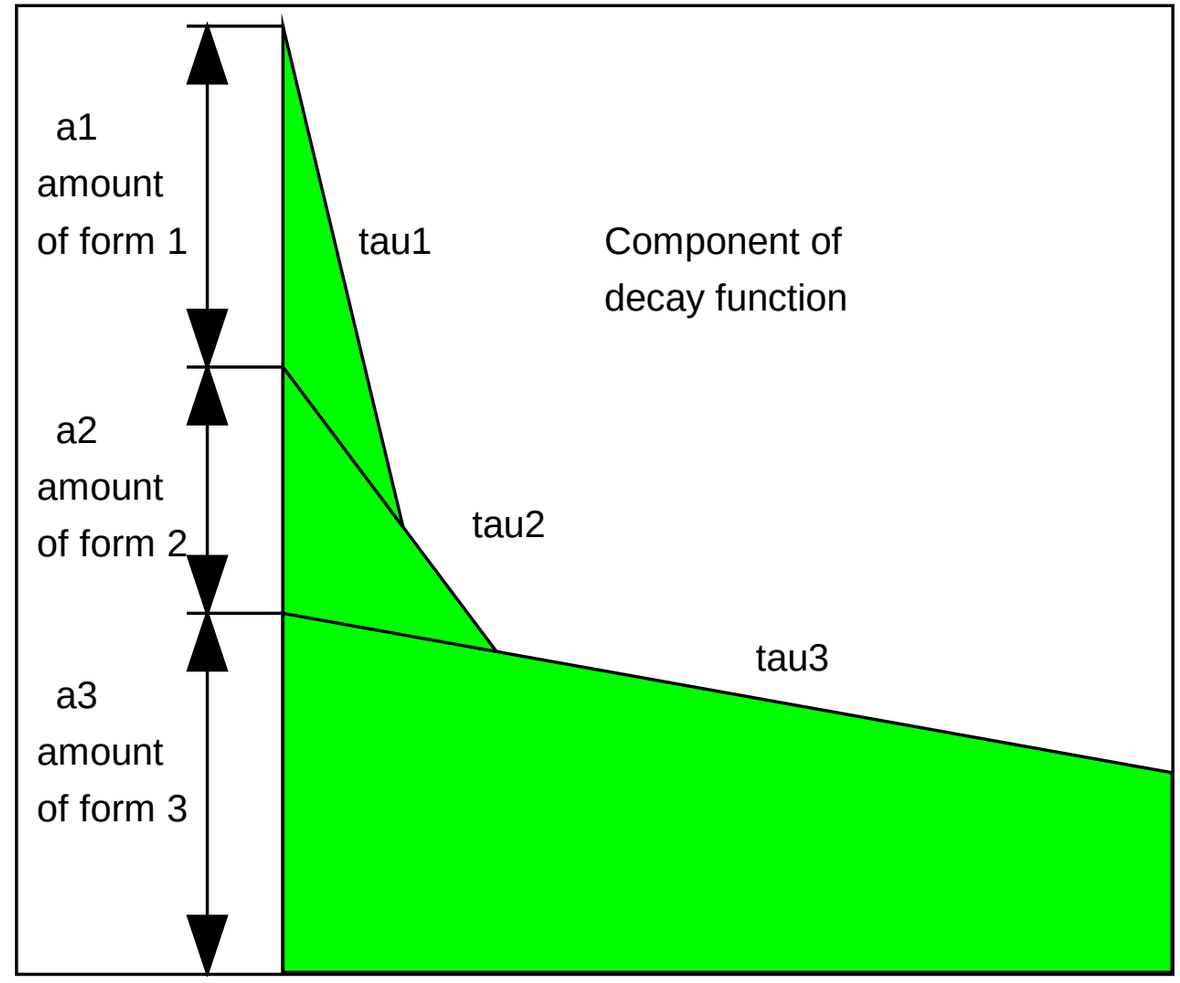
- **The shape of the decay function changes with the molecular environment**

Information from Multi-Exponential Decay Functions





a1, a2, a3: Amounts of fluorophore forms 1,2,3 (How much is there?)

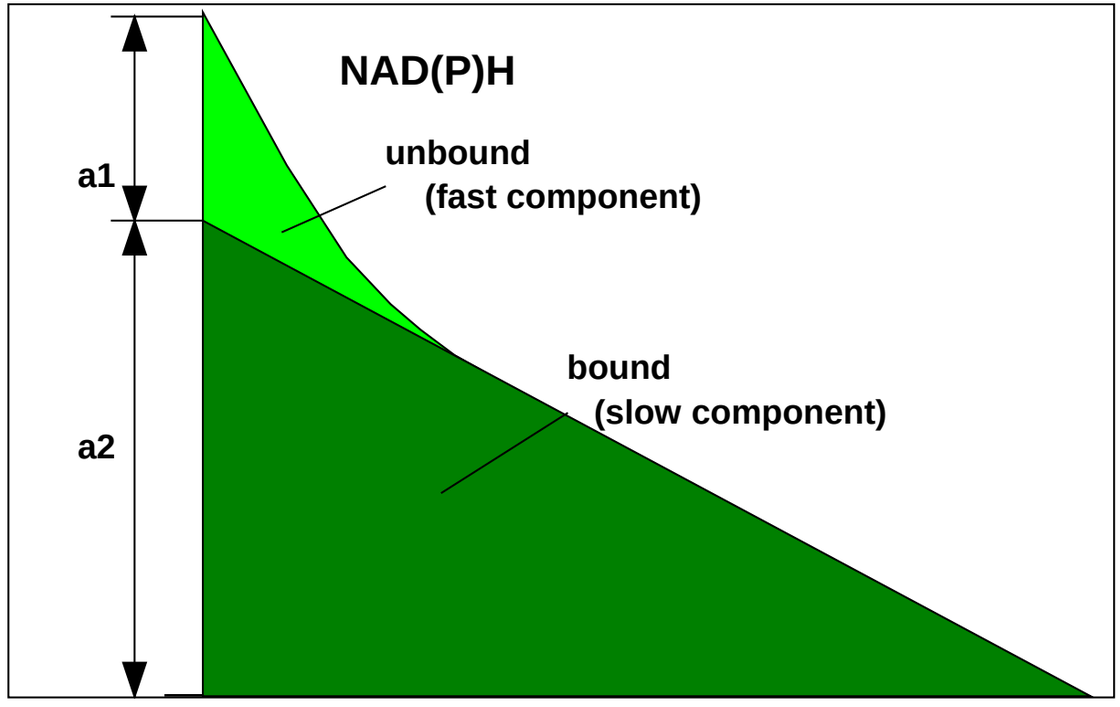
τ1, τ2, τ3: Lifetimes of fluorophore forms 1,2,3 (What does it do there?)

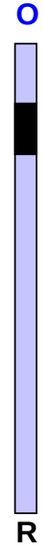
Note: Changes in these parameters cause a change also in the average lifetime.

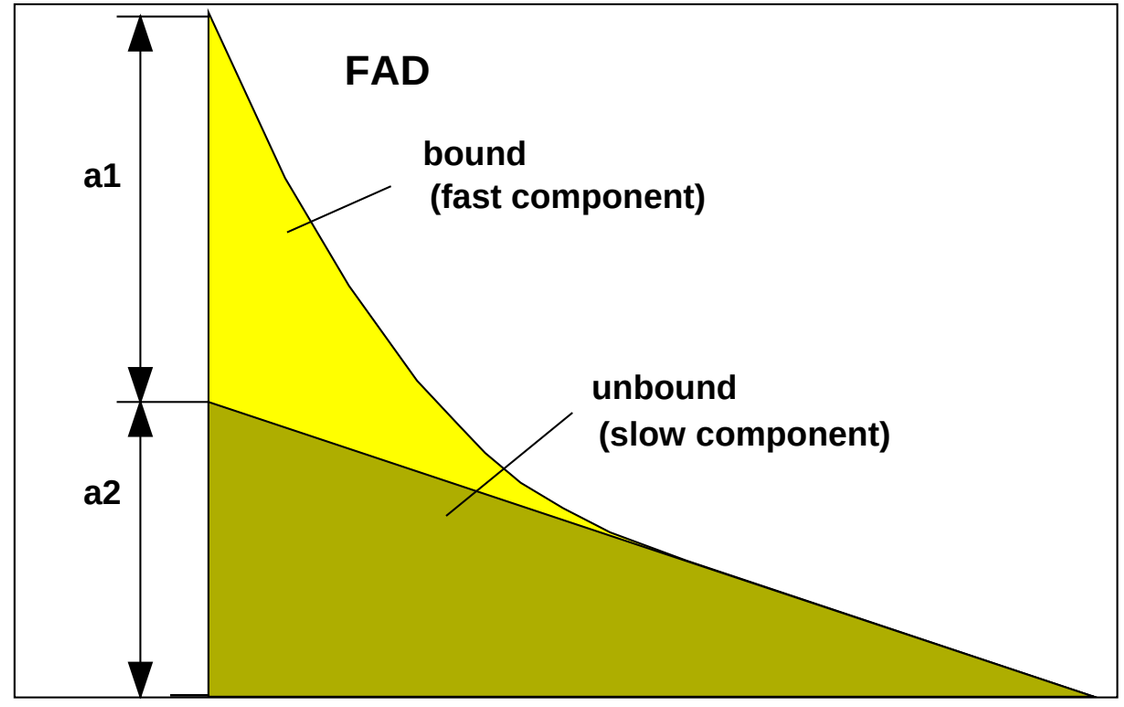
But from the lifetime you can only see that something changed, you can't tell what it is.

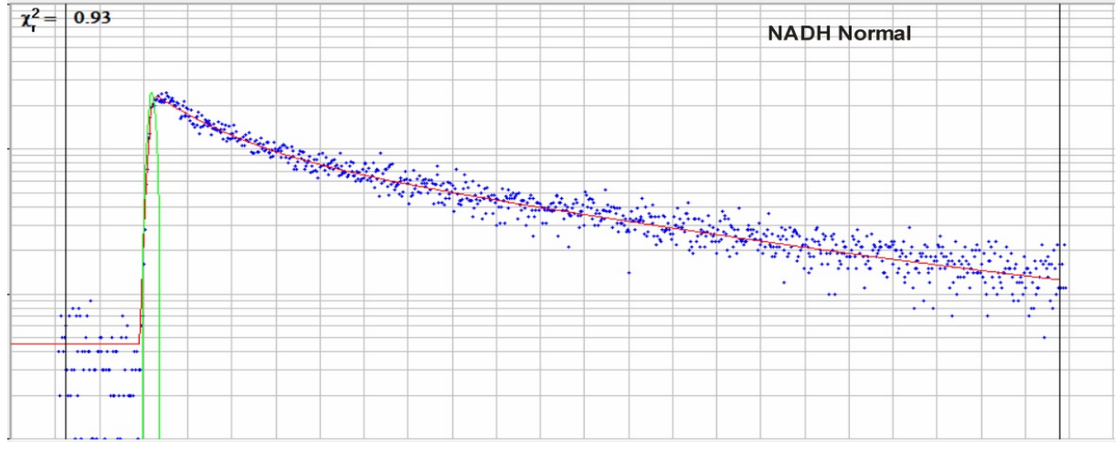
Look at the Amplitudes! NADH and FAD

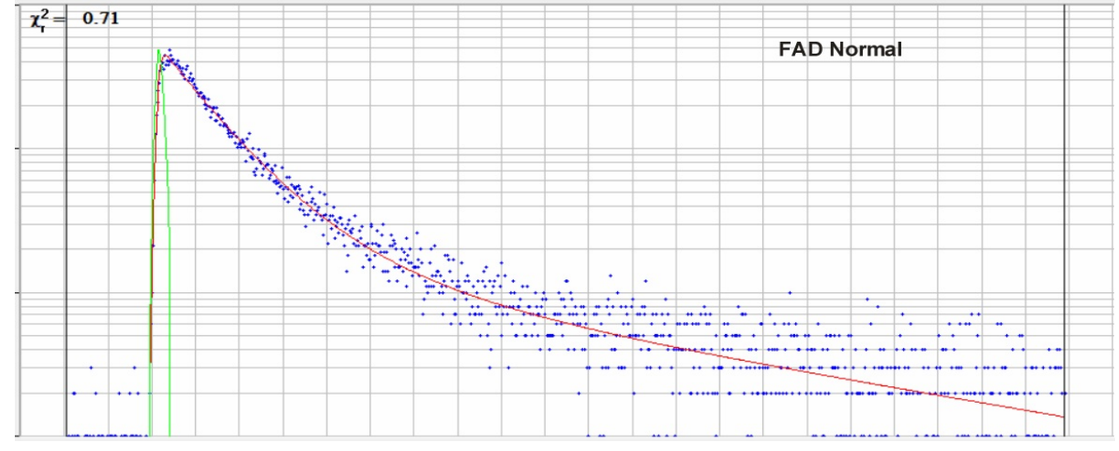
Free-Bound Ratio Depends on Metabolic State: Normal Cells





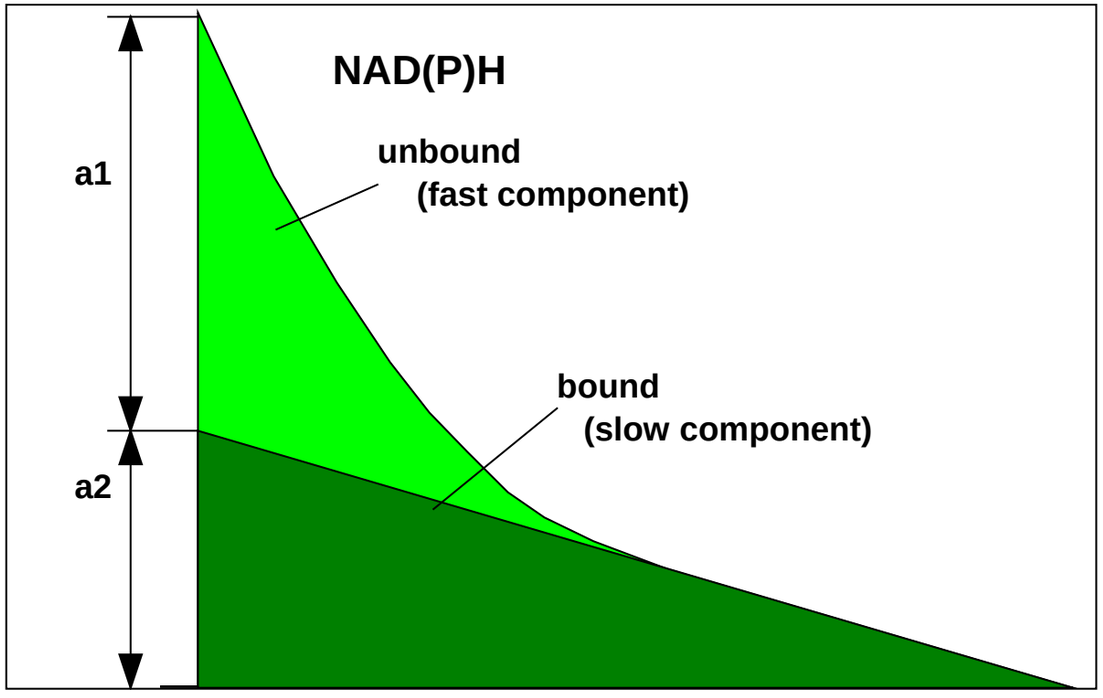


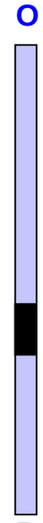


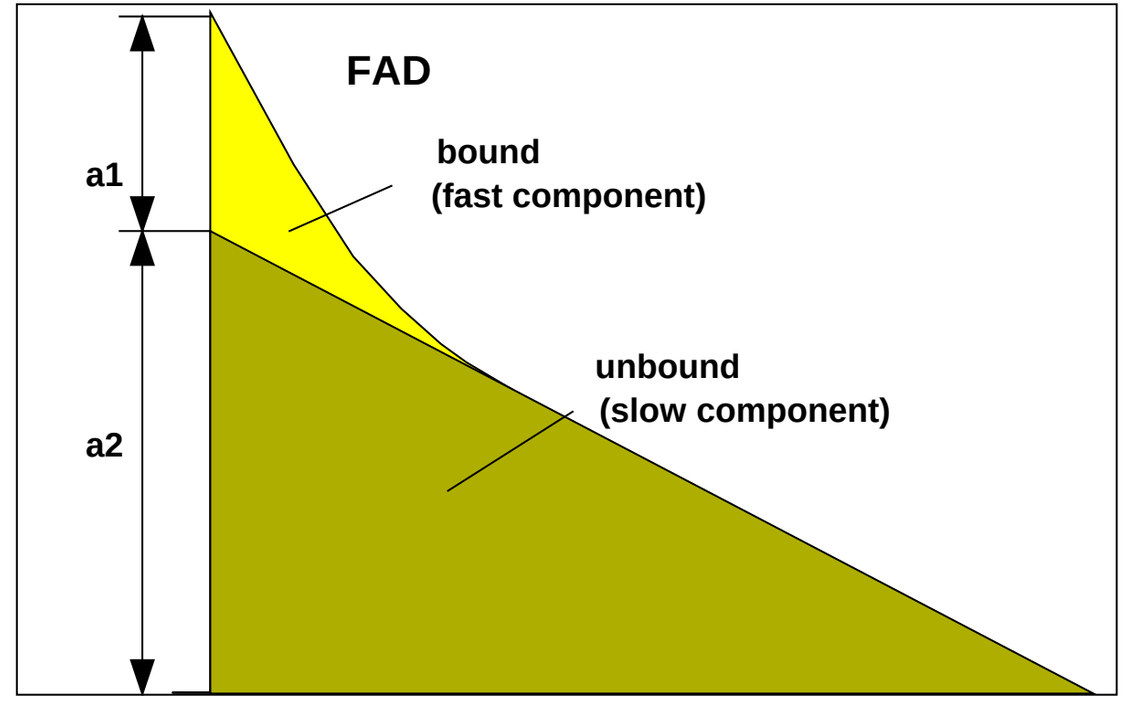


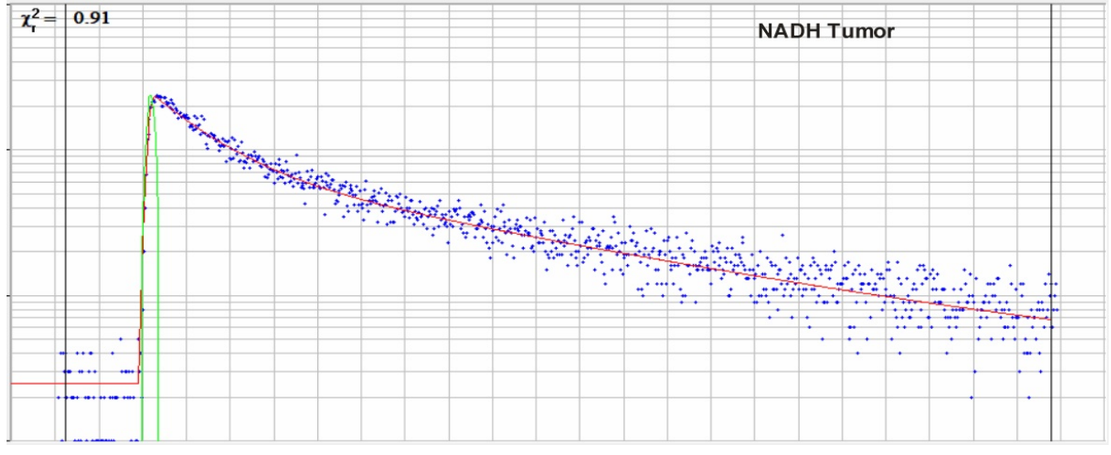
Example: NADH and FAD

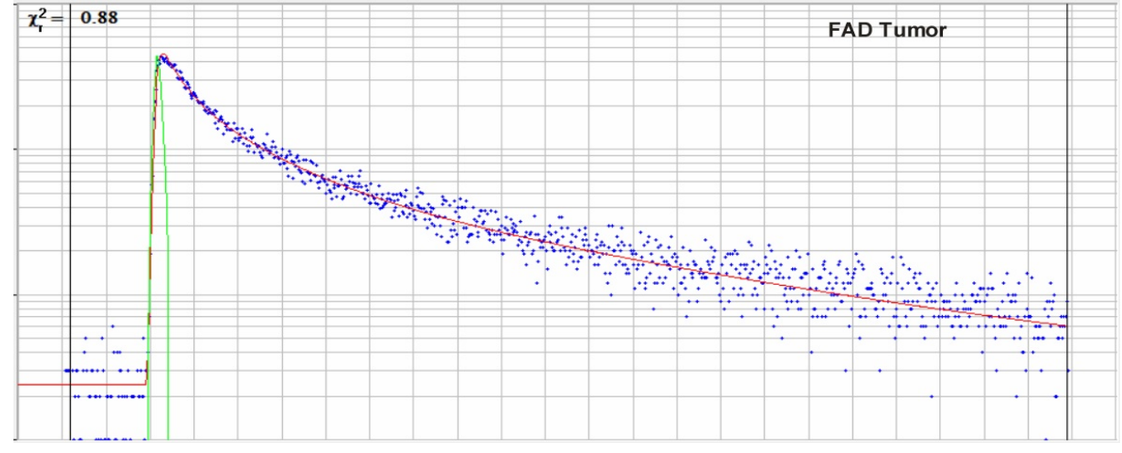
Free-Bound Ratio Depends on Metabolic State: Tumor Cells



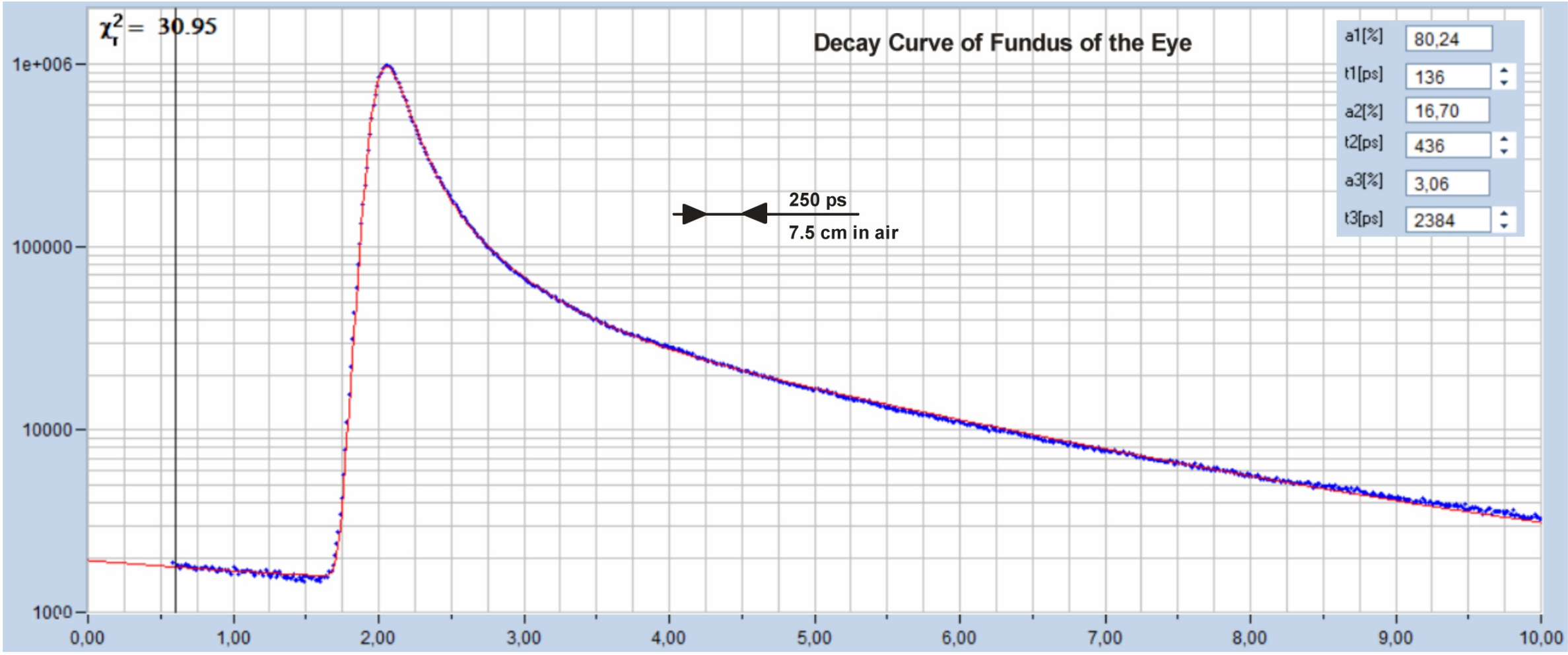








Decay Curve of the Fundus of the Eye



Multi-exponential decay

Extremely fast decay components

Fastest component 136 ps

Extremely high time resolution needed

Extremely high timing stability needed

Optical path length matters (250 ps = 7.5 cm)

Out-of-focus suppression needed

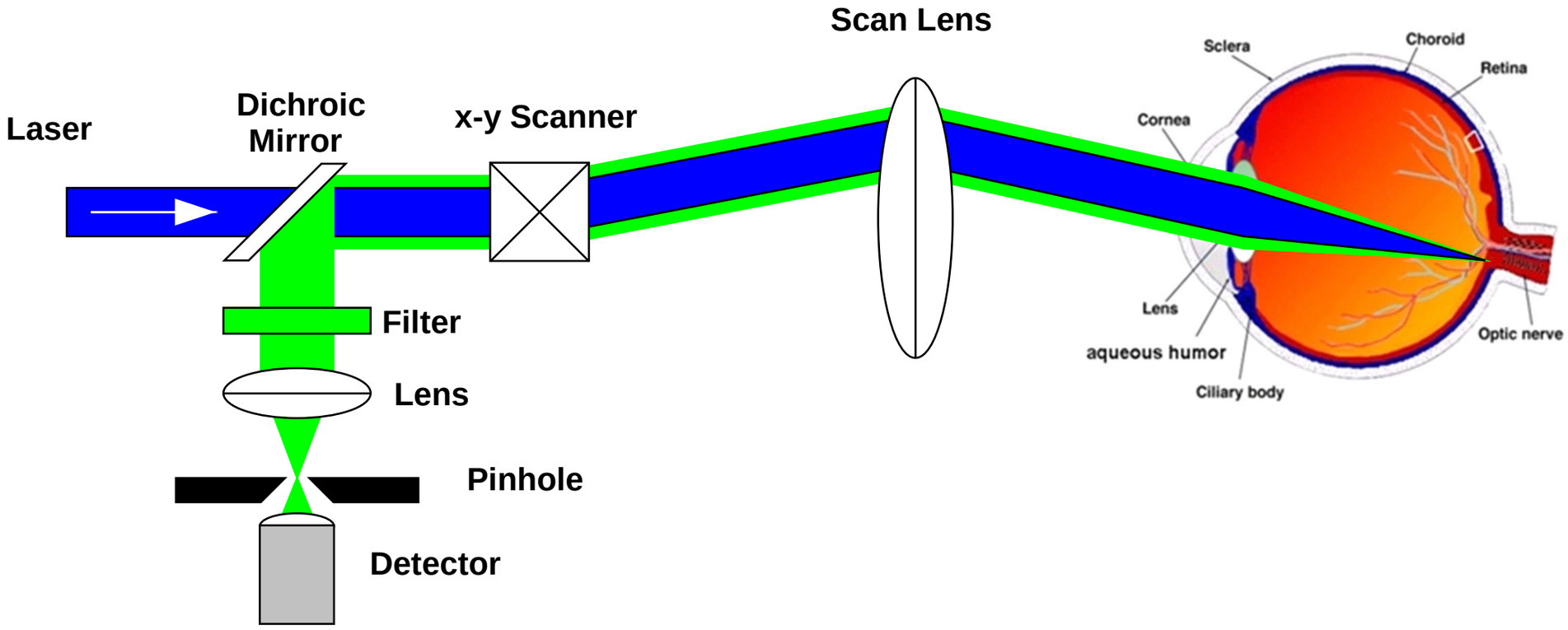
Suppression of scattered signals needed

High sensitivity needed

Questions?

How are FLIO Data Recorded?

Principle of Scanner



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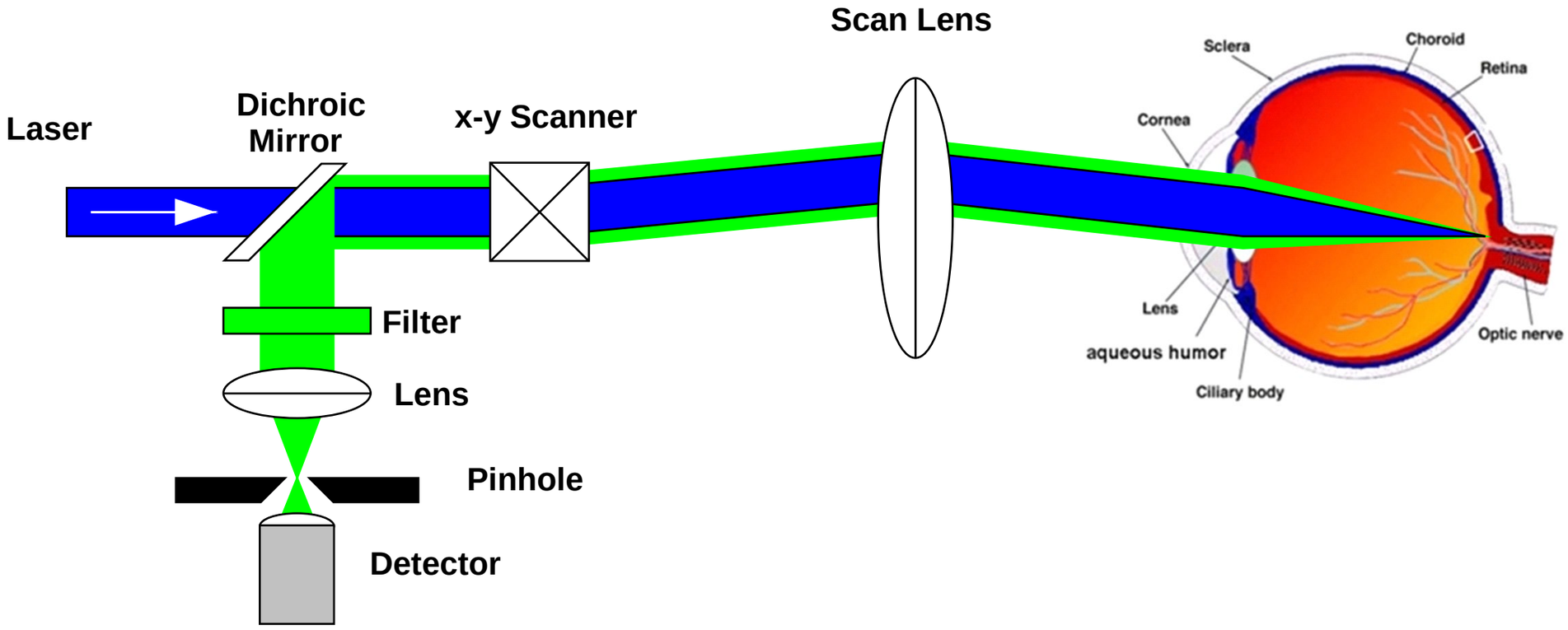

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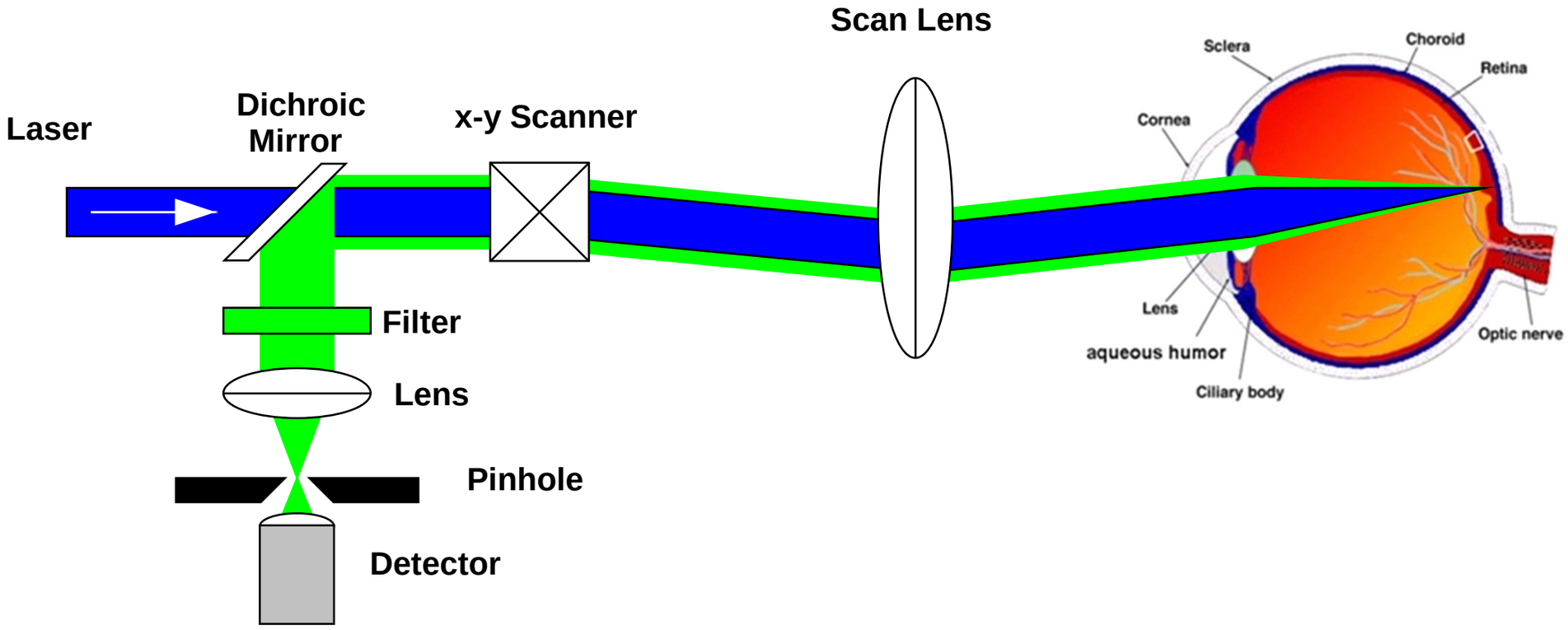

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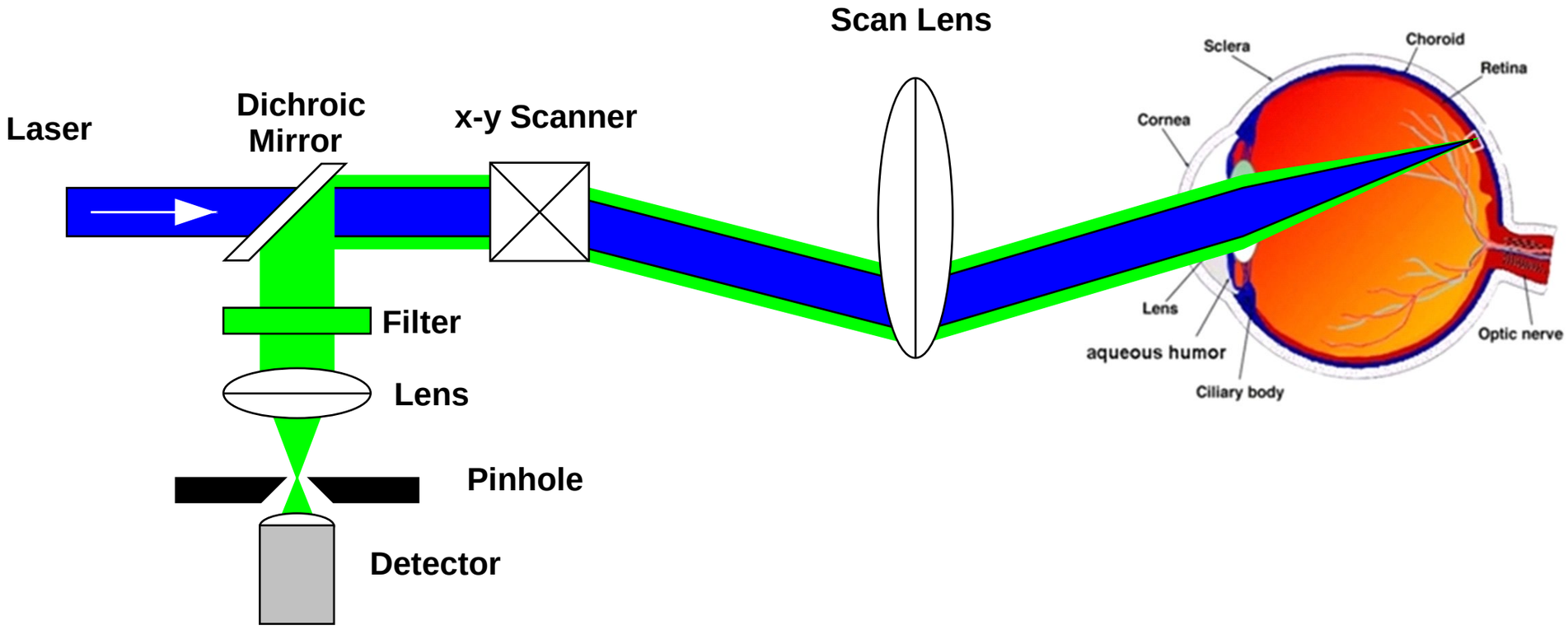


Why Scanning?

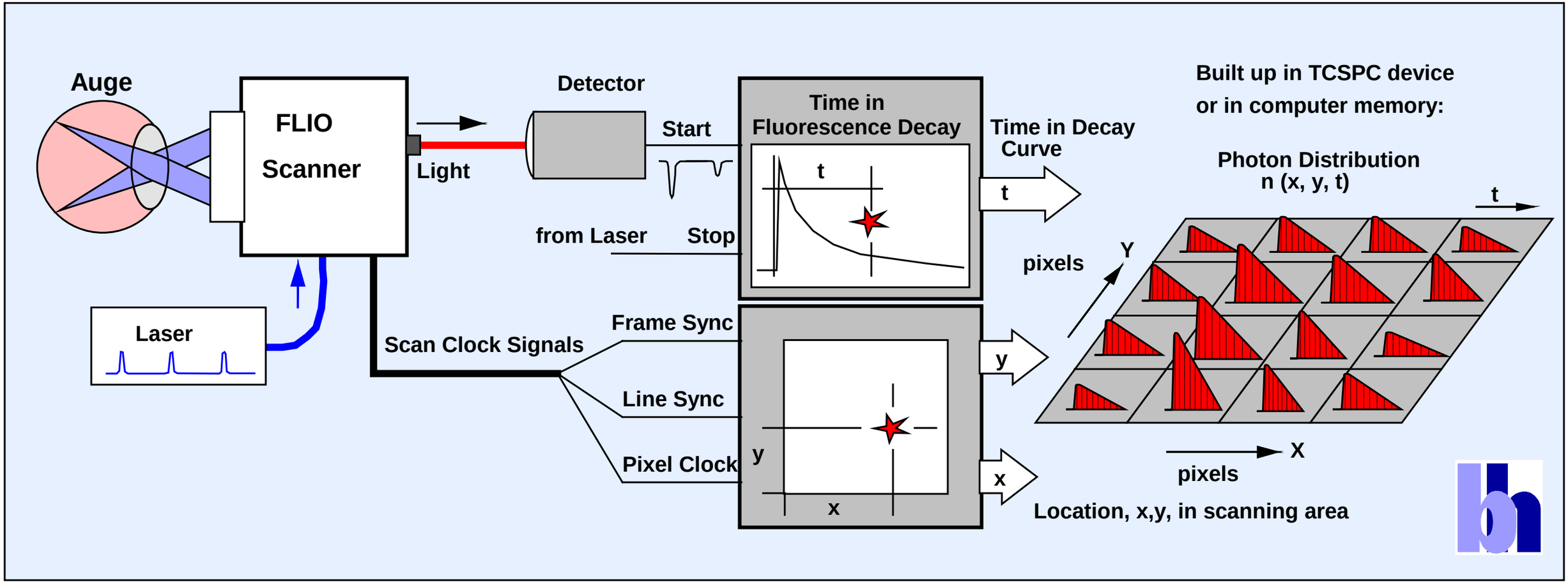
Suppresses out-of-focus light

Suppresses lateral scattering

Perfectly compatible with bh's multi-dimesnsional TCSPC Process

Recording Principle of FLIO

Combination of Multi-Dimensional TCSPC with a Laser Scanning Ophthalmoscope



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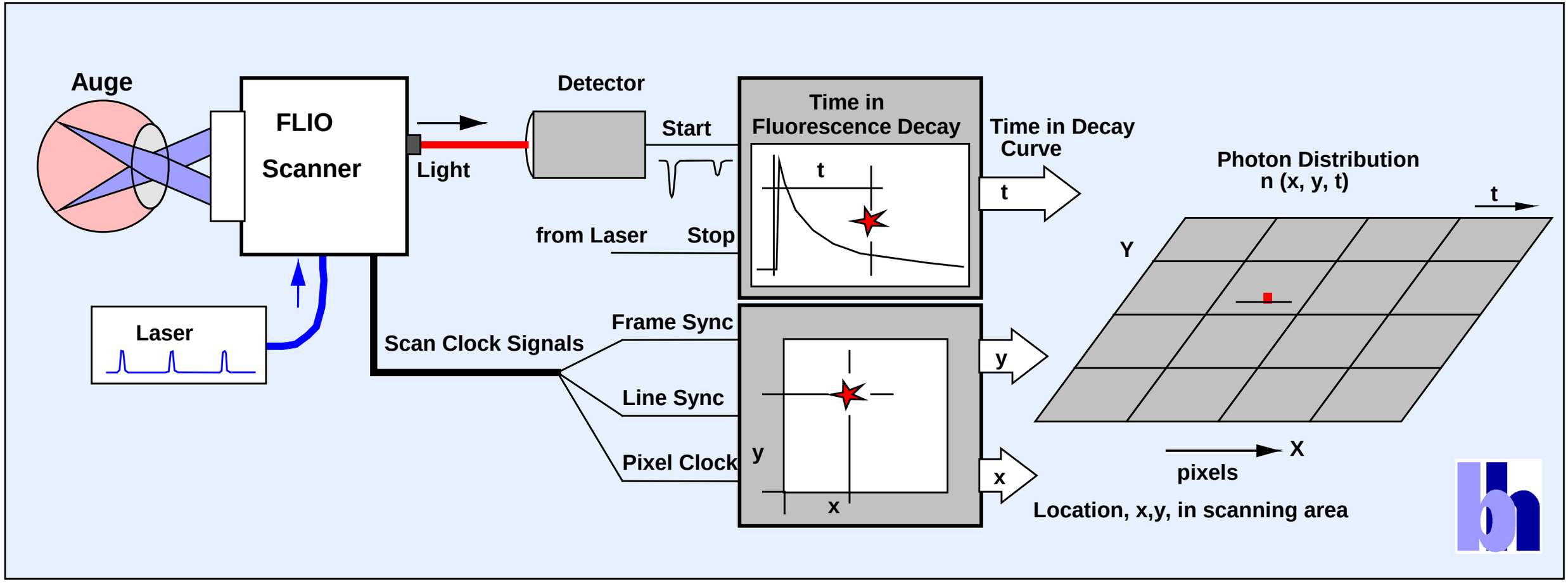

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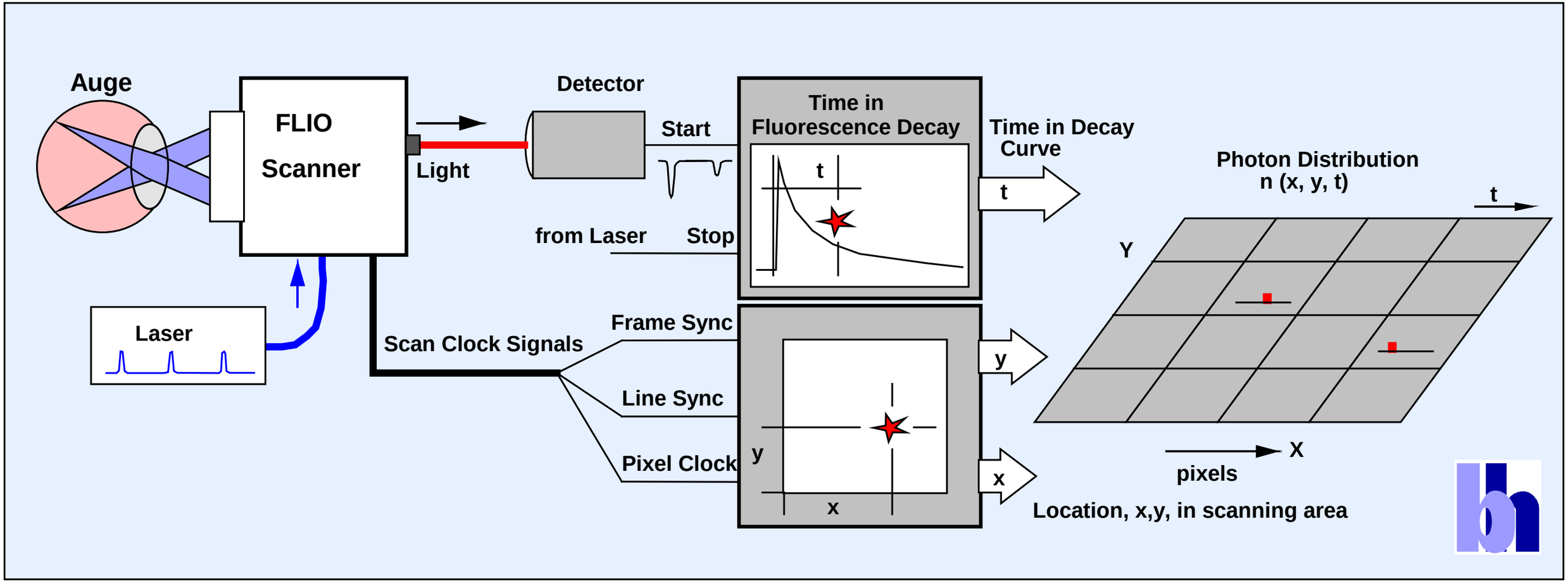

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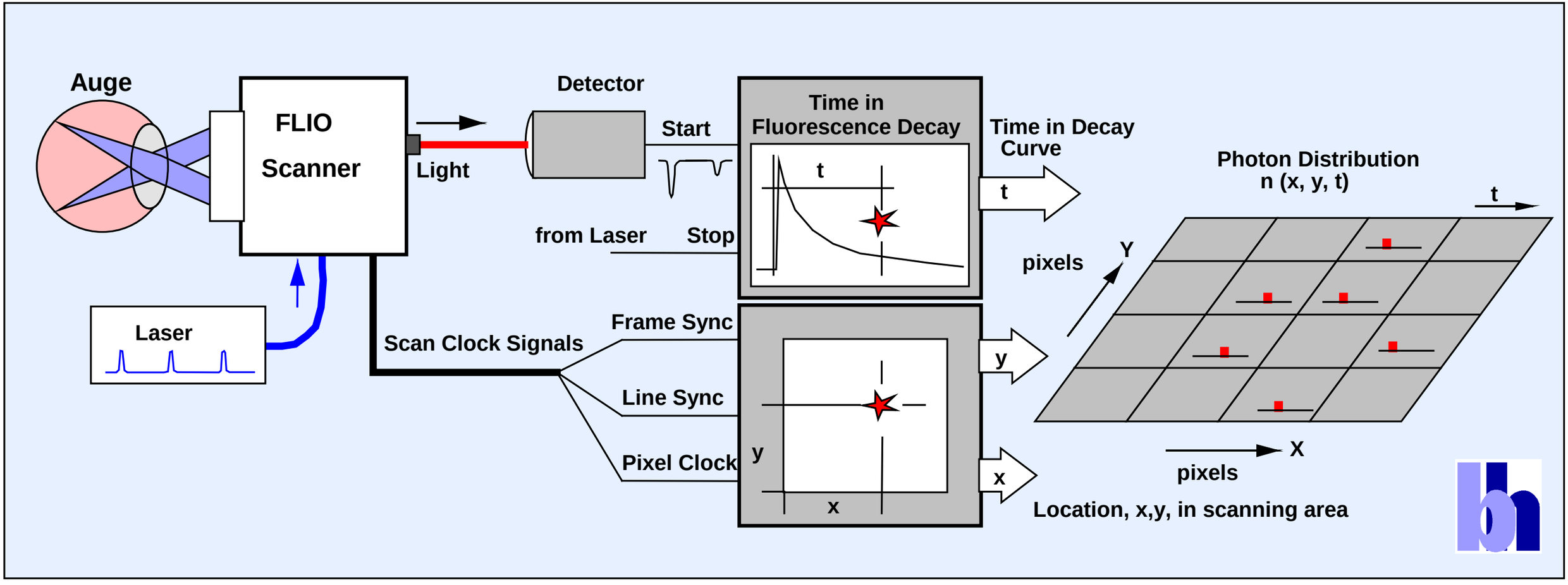

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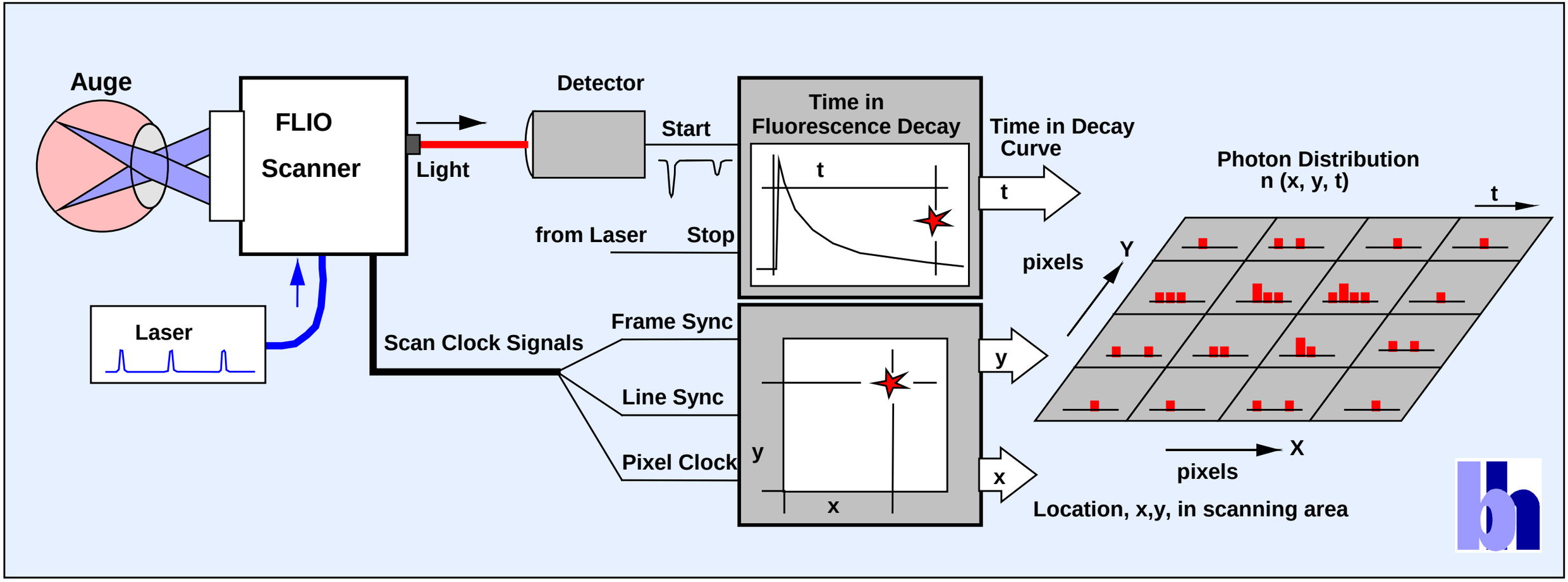

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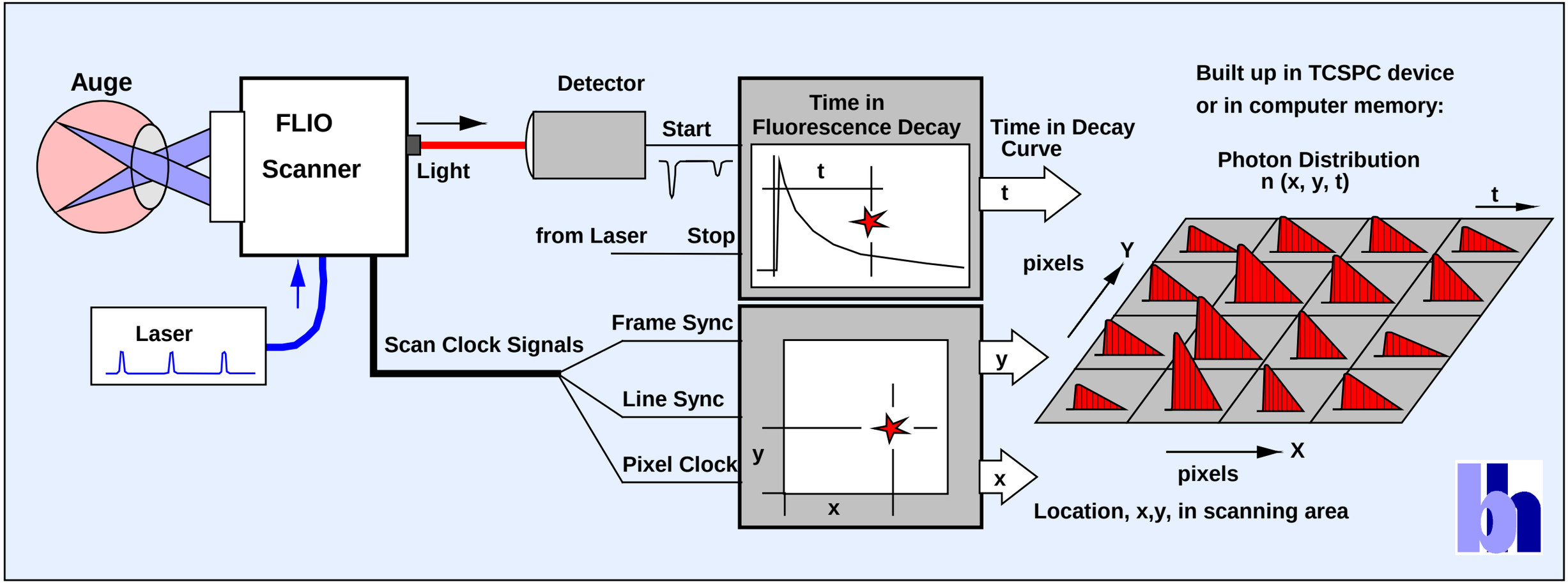

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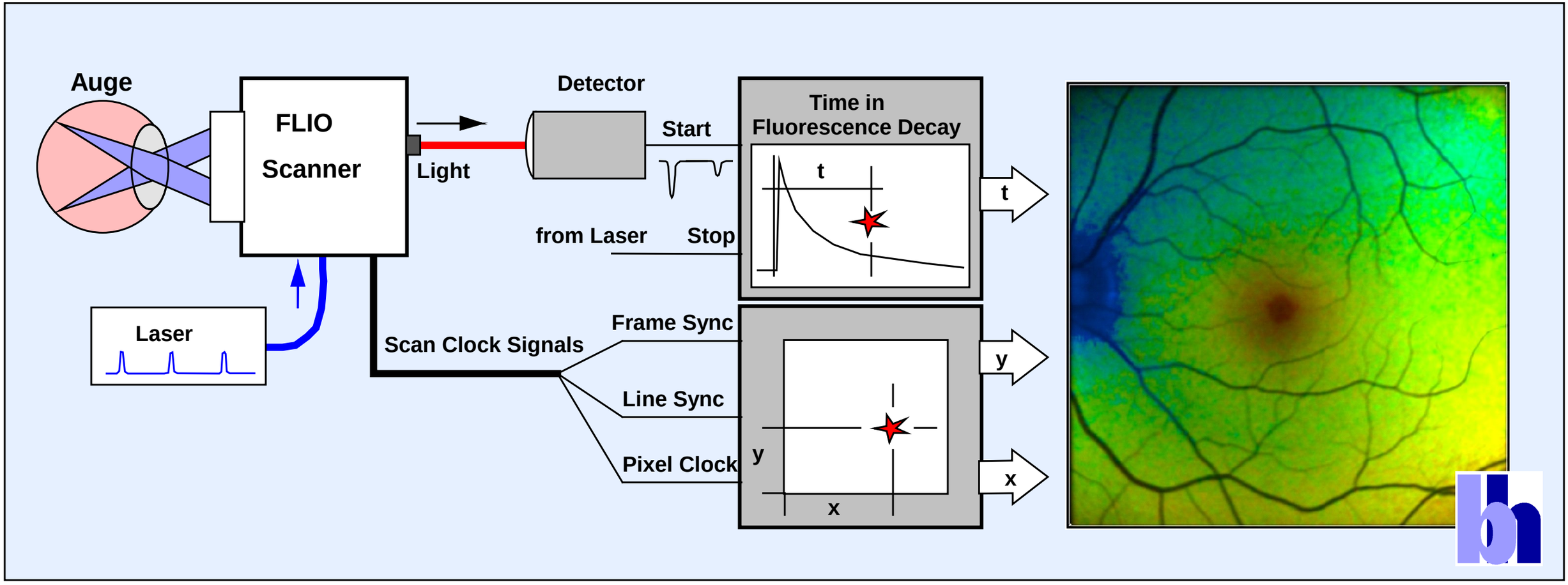
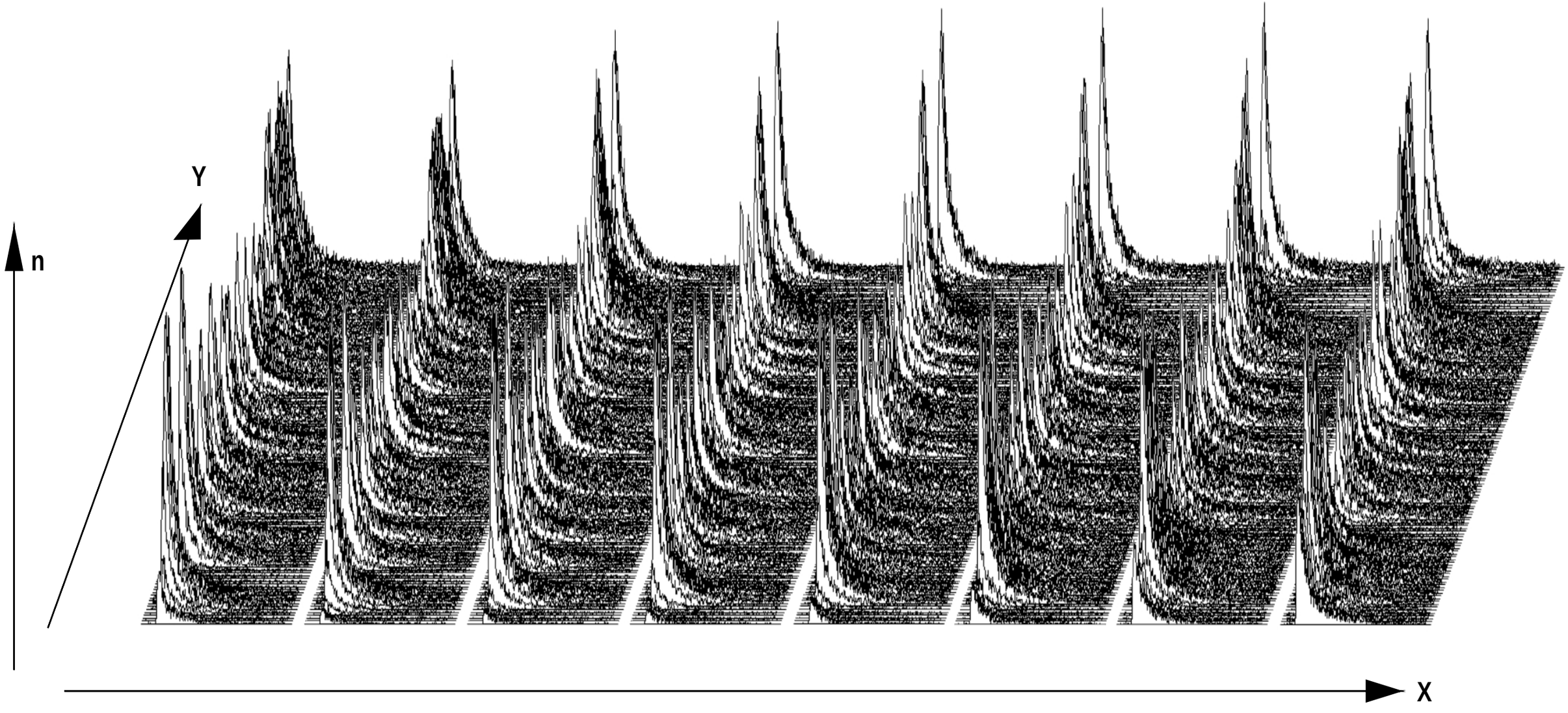


What are FLIO Data?

Photon Distribution over the Image Coordinates and the Time in the Fluorescence Decay



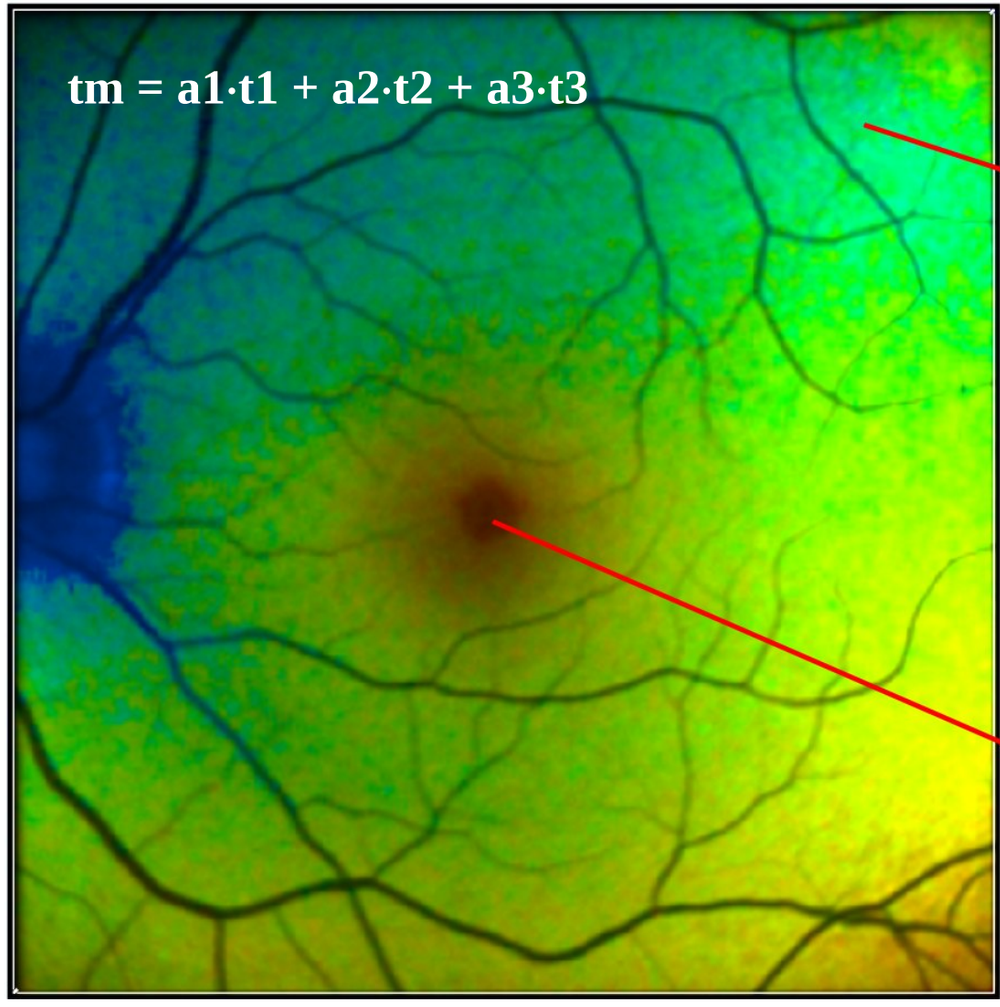
The Task of Data Analysis

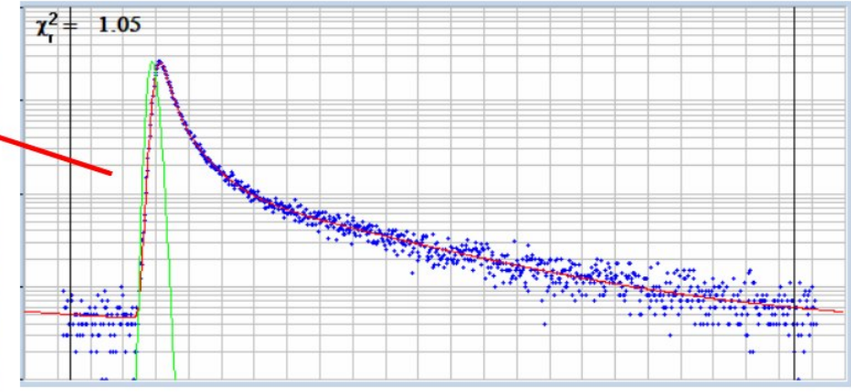
Determine decay parameters in the individual pixels

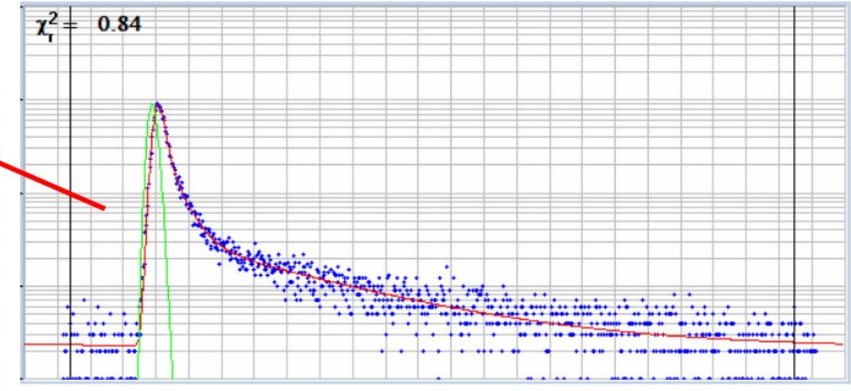
Create an image which displays the desired decay parameters as colour

Which decay parameter? We have multi-component decays!

Average (amplitude-weighted) liftime

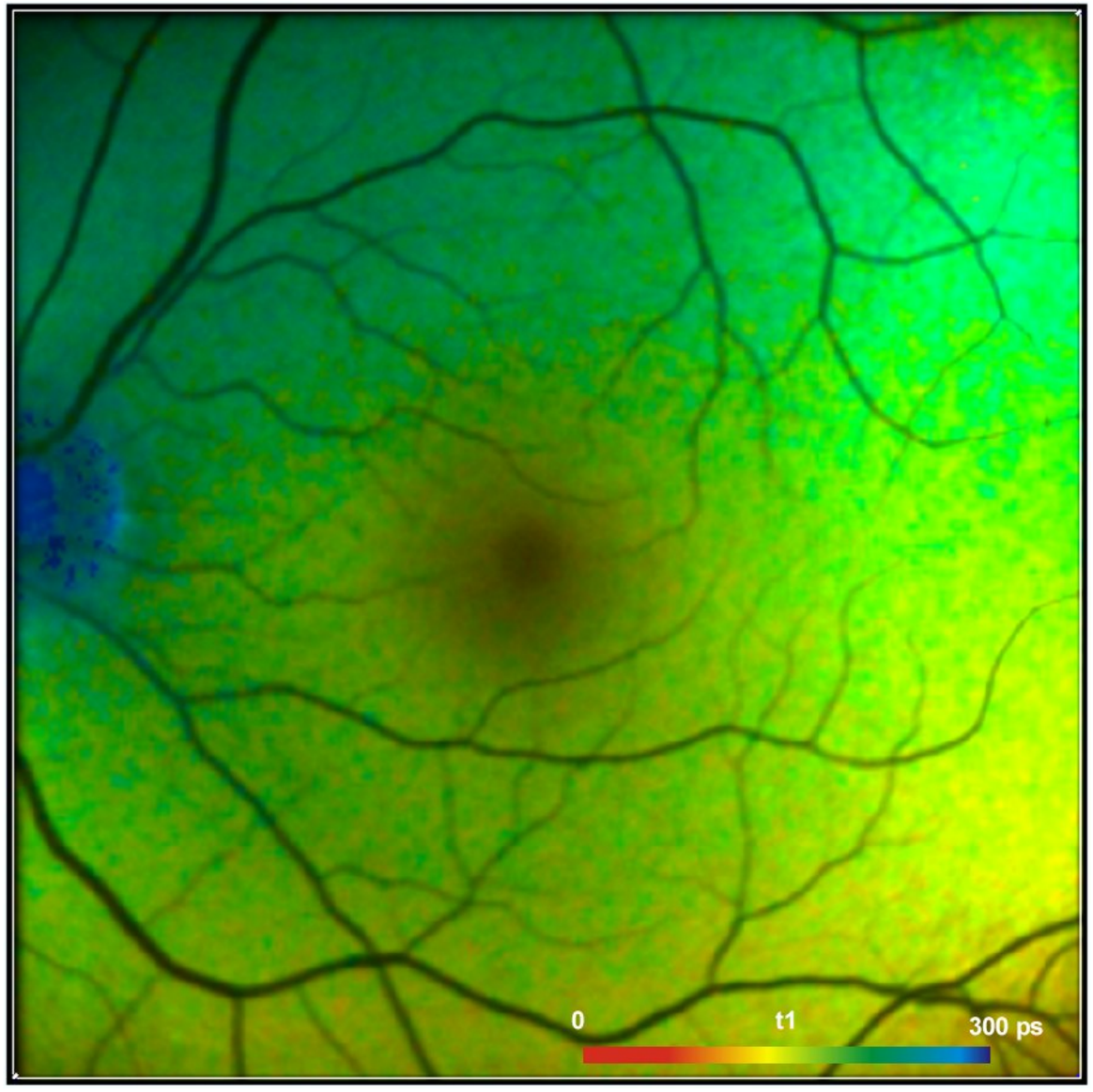




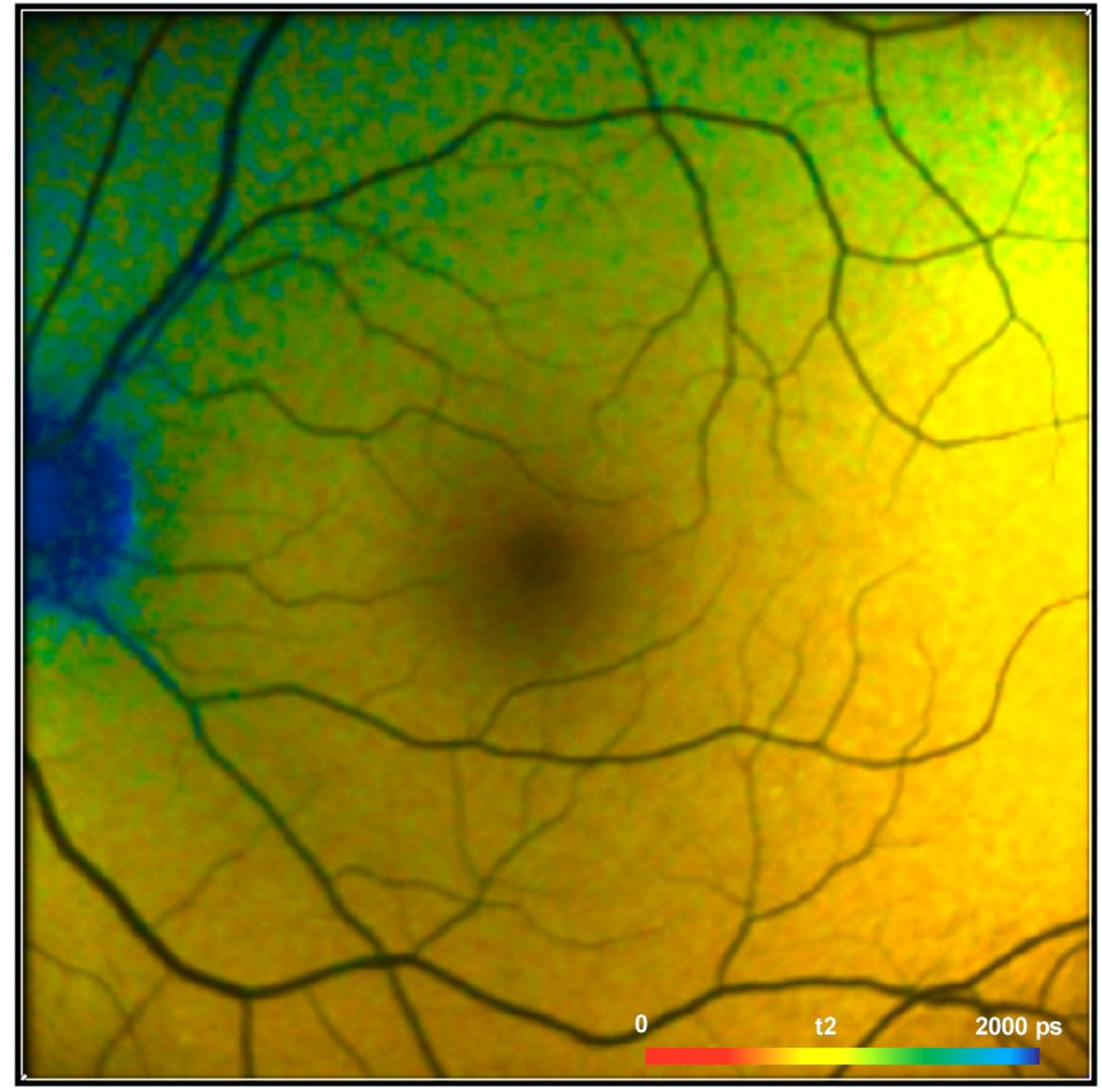


Lifetimes of Decay Components

Lifetime of fast component, t1

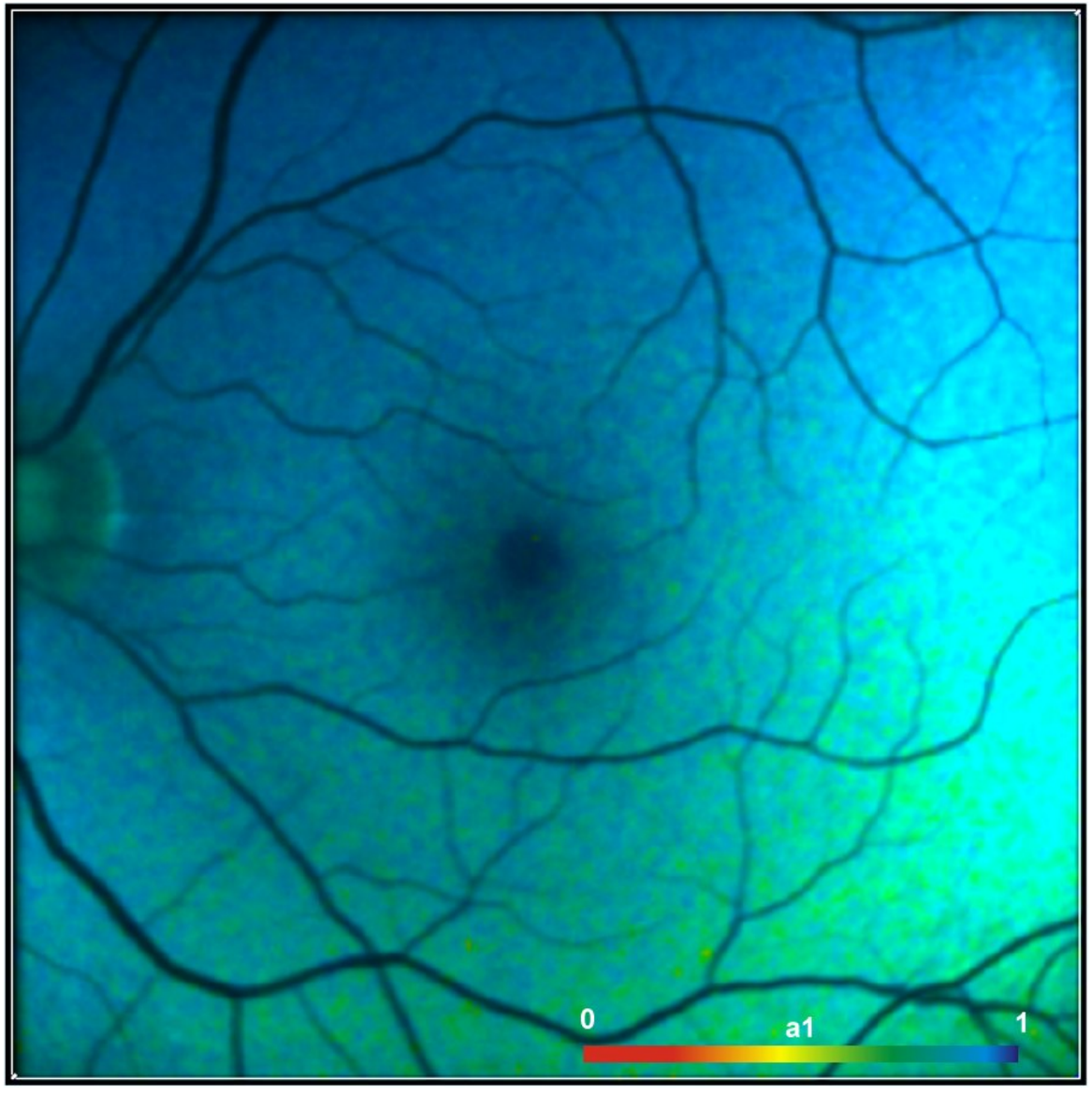


Lifetime of slow component, t2

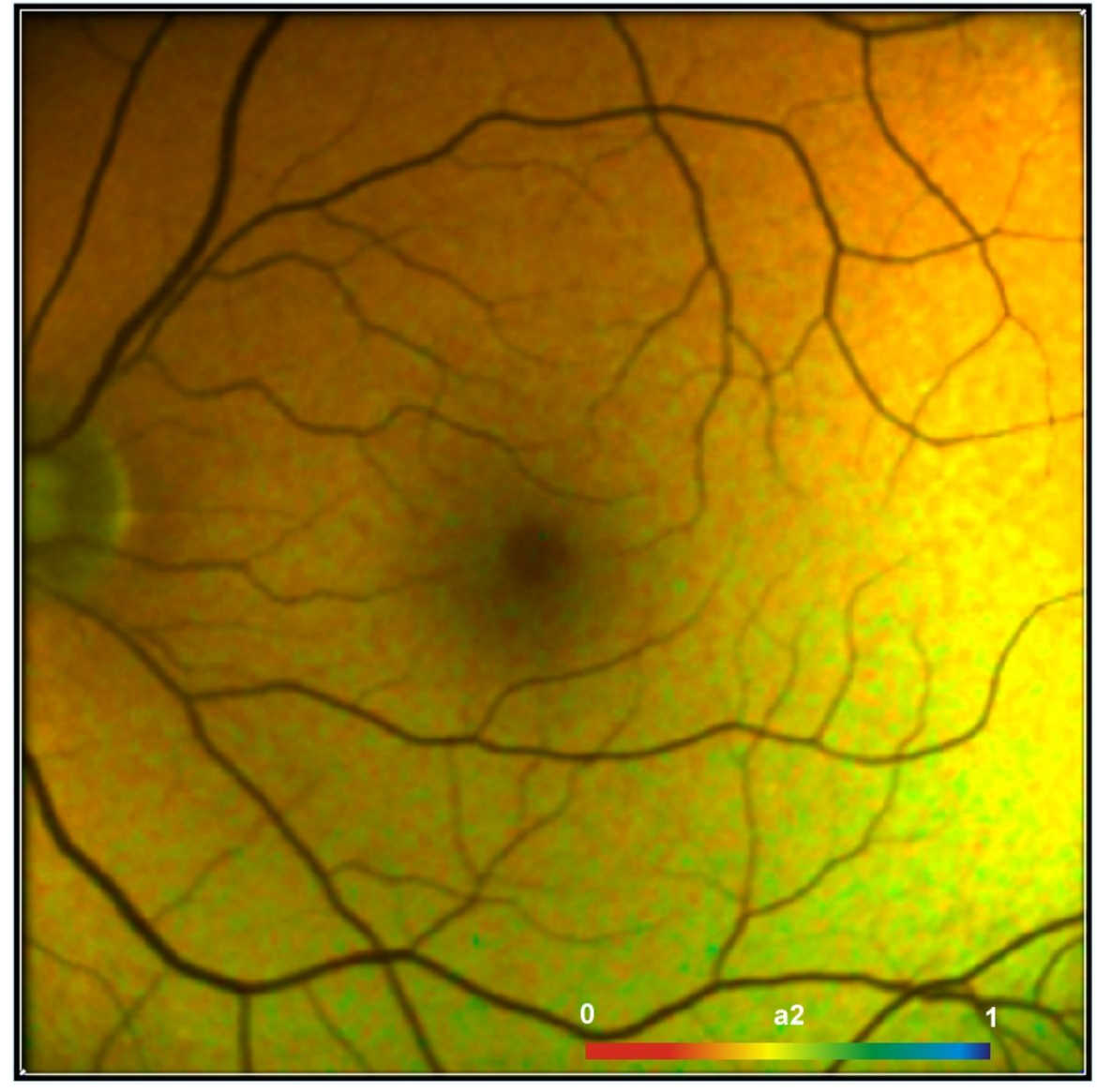


Amplitudes of Components

Amplitude of fast component, a1



Amplitude of slow component, a2

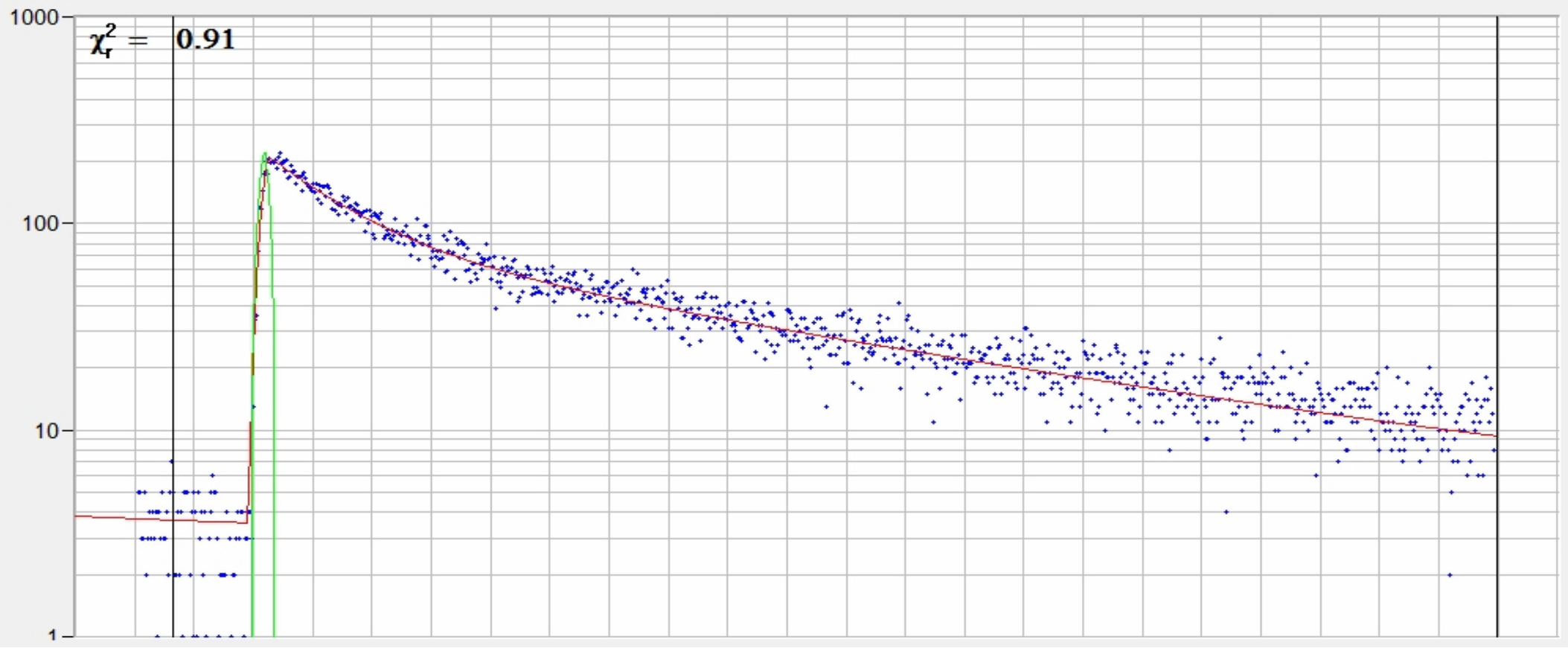


How are the Decay Paramters Determined?

The shape of the photon distribution does not exactly represent the fluorescence decay function

Fluorescence is excited by laser pulses of non-zero width

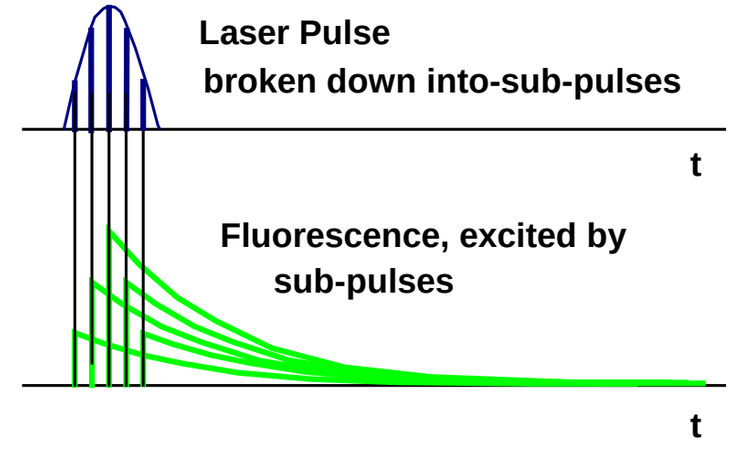
Fluorescence is detected by a detector of finite speed

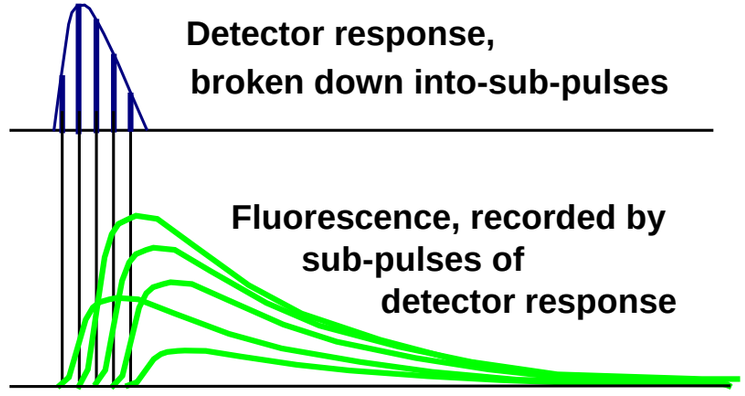


The measured waveform is a convolution of the real decay curve with the Instrument-Response Function

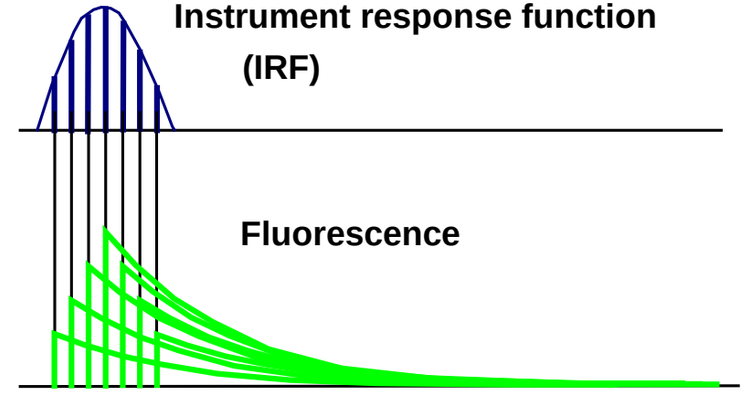
The Convolution Integral

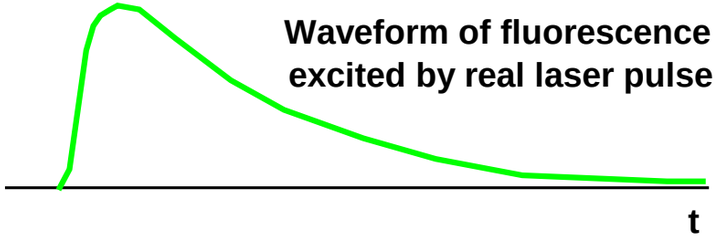
$$f_m(t) = \int_{\tau=0}^{t} f(\tau) IRF(t-\tau) d\tau$$

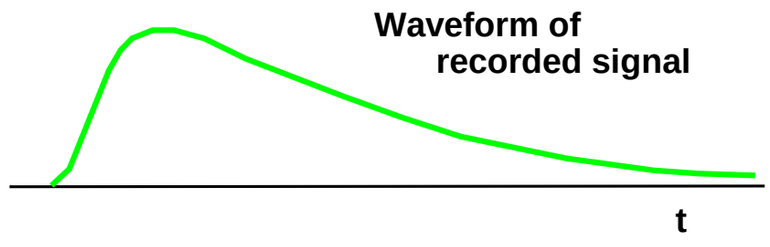


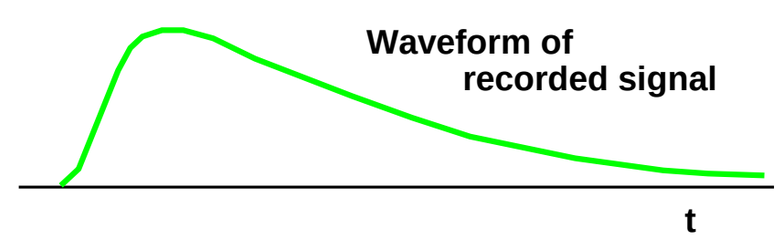


Or:

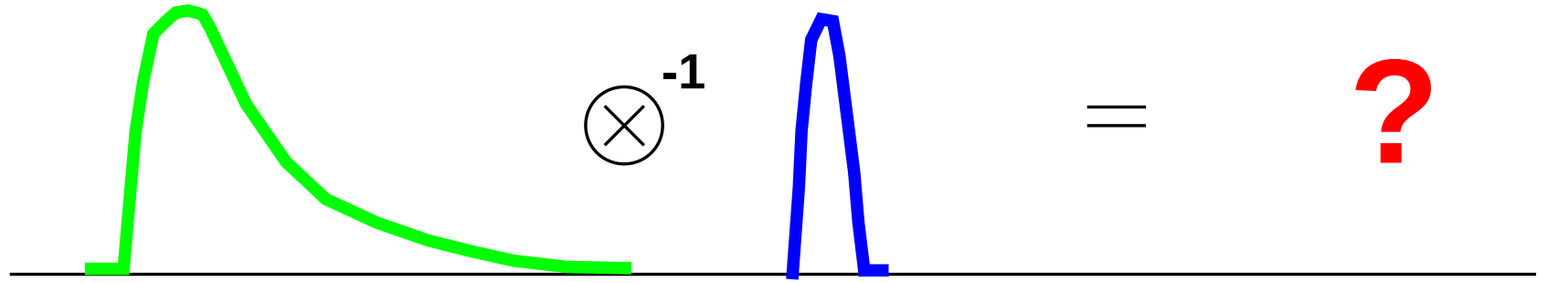








De-Convolution



The Convolution Integral

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

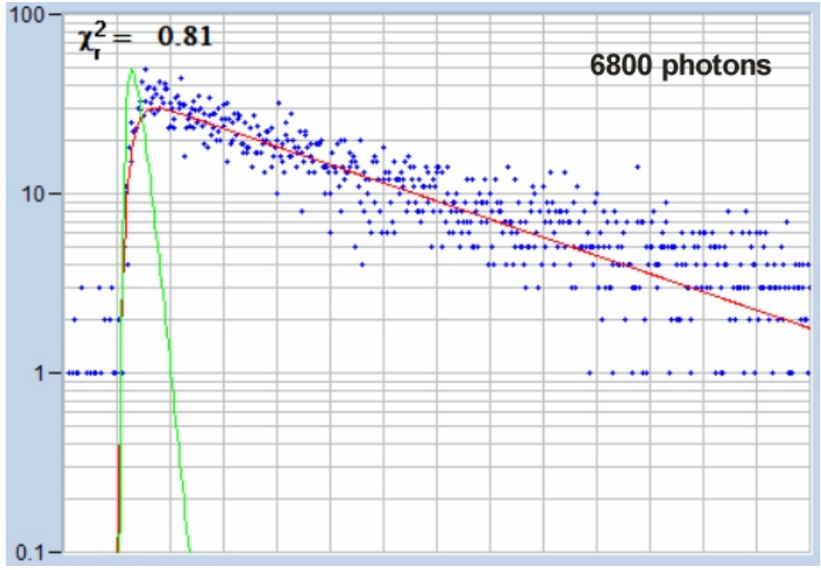
cannot be reversed.

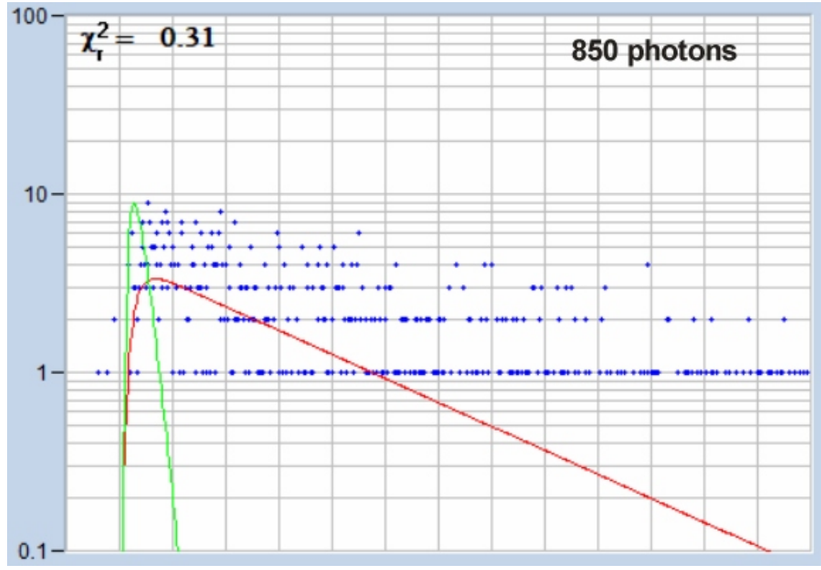
Except for a few special cases there is no analytical solution for *f (t)* as a function of *fm(t)* and *IRF(t)*.

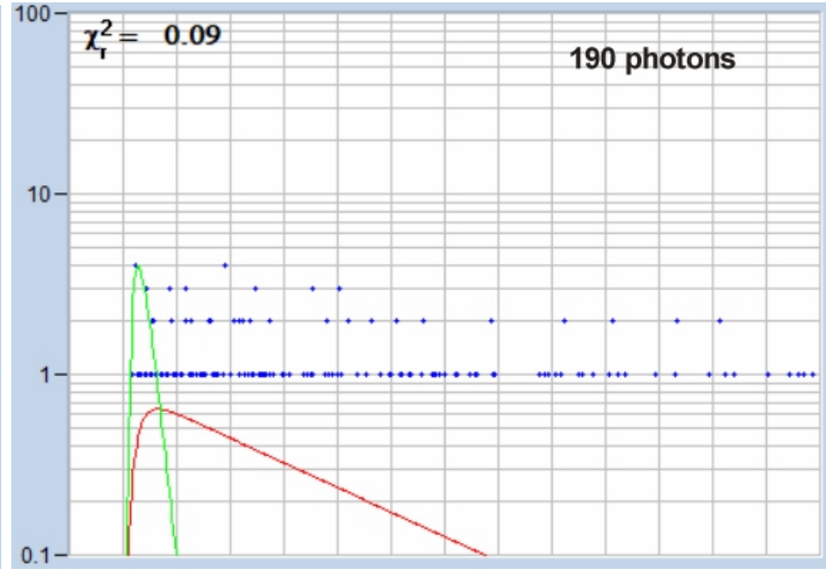
Fit Procedures: Least-Sqare Fit

Fine at high photon number

At low N the result is biased towards lower vlues







τ = 1950 ps **1820 ps** **1590 ps**

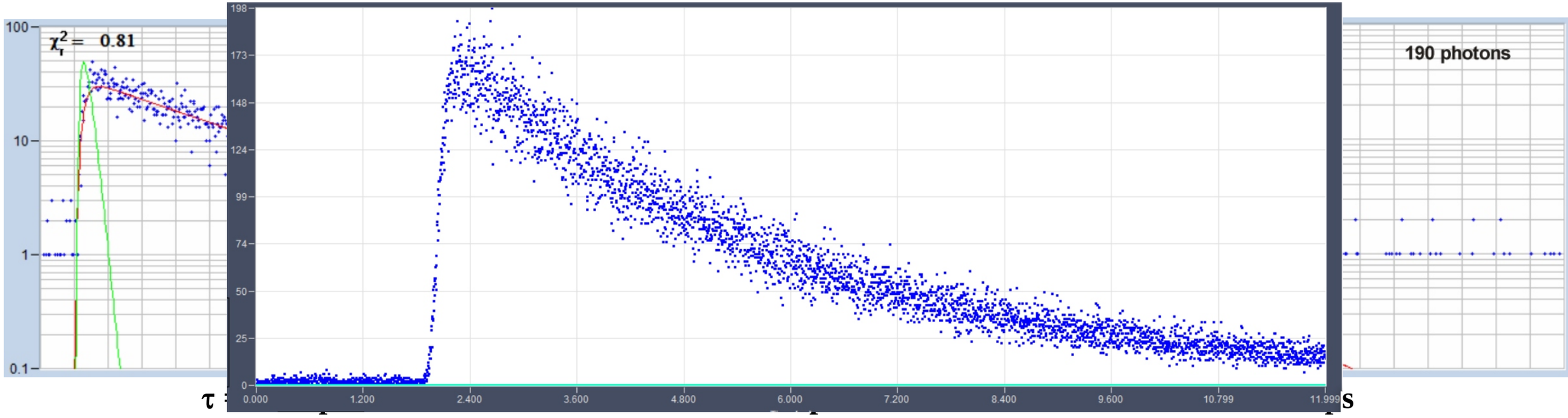
A dependence of τ on the photon number is the last thing we want!

What's the reason of the different τs?

Fit Procedures: Least-Sqare Fit

Fine at high photon number

Problems if number of photons is low



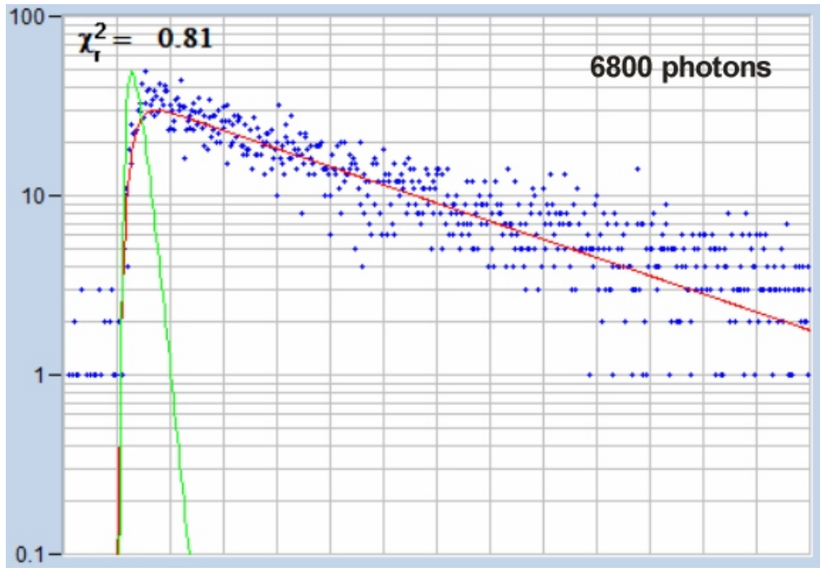
τ = … s

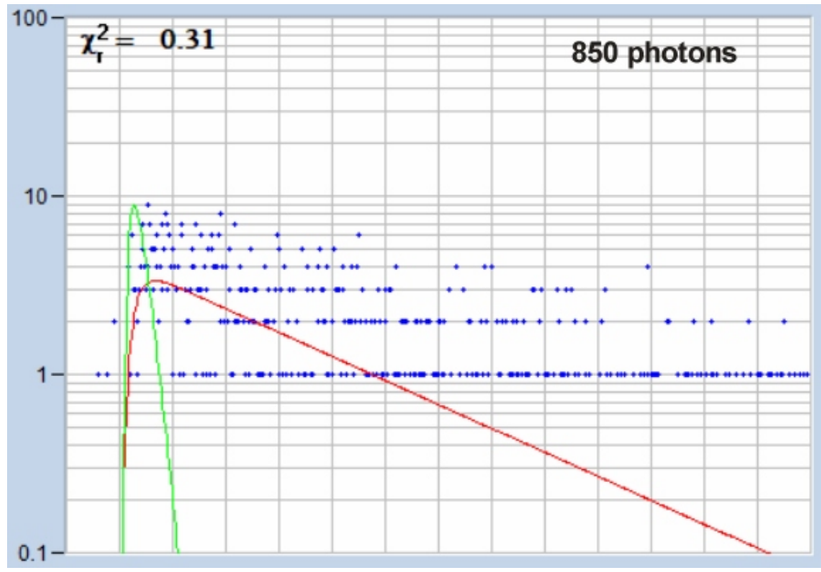
- **WLS minimises the the square error sum,** $\sum (n - f(t))^2$
- **But the photon numbers, n, in the time channels are Poisson-distributed**
- **Noise in n depends on n itself:** $\sigma = \sqrt{n}$
- **Weighting of the errors required. Weight for channels with lower n must be higher.**
- **Correct weighting factor would be** $w = 1/\sqrt{n}$ **. Impossible for N=0!**

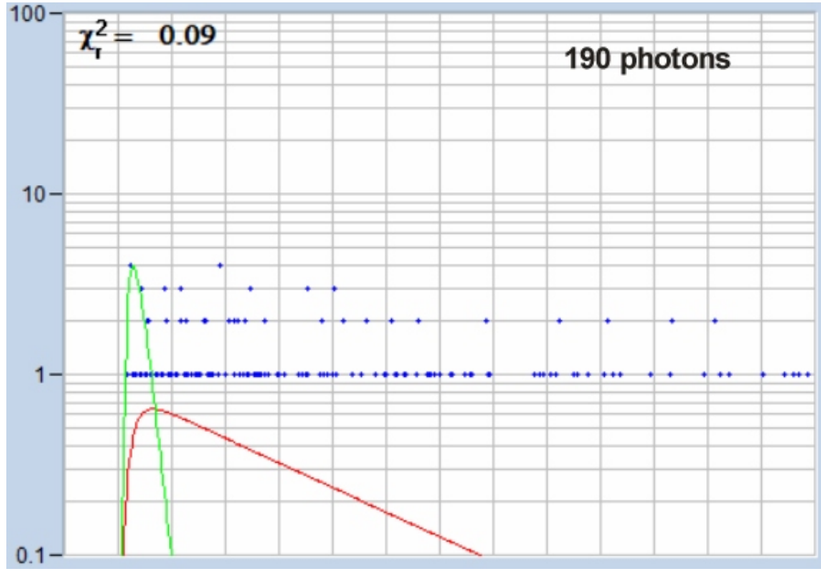
Fit Procedures: Least-Sqare Fit

Fine at high photon number

Problems if number of photons is low







τ = 1950 ps **1820 ps** **1590 ps**

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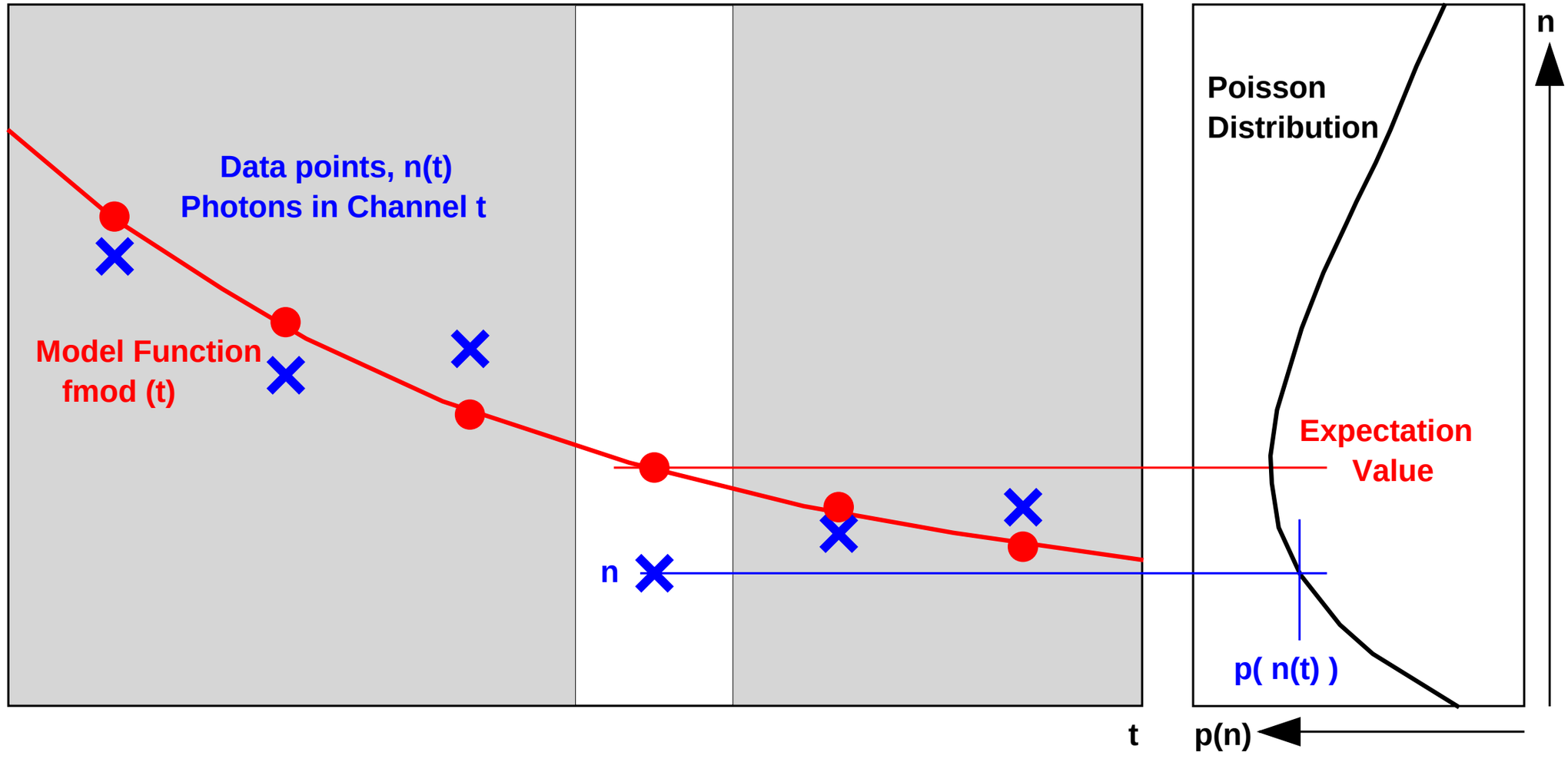
- Correct weighting factor would be $w = 1/\sqrt{n}$ **. Impossible for N=0!**

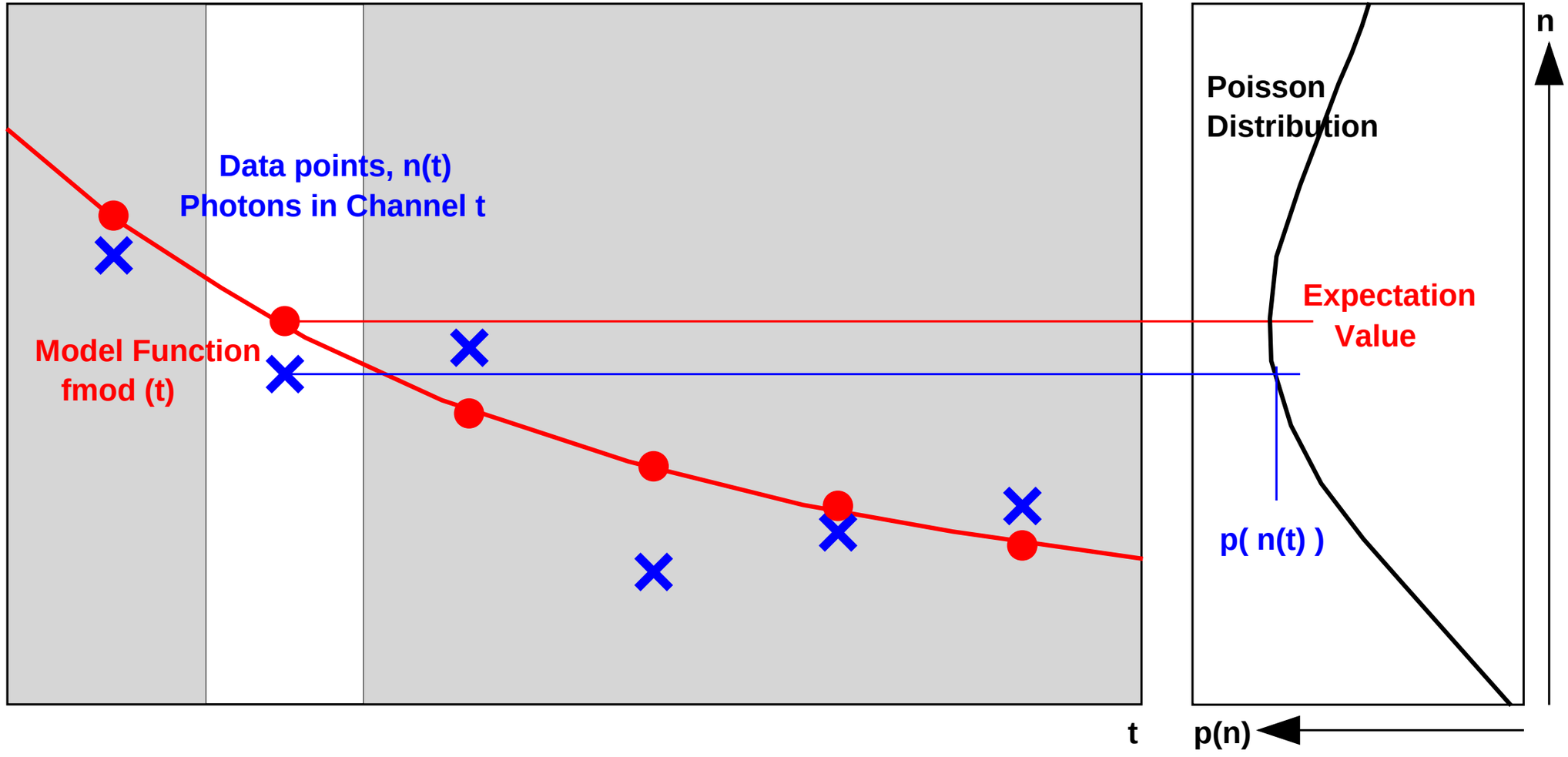
We need a better fit algorithm!

SPCIMage NG: Maximum-Likelihood Estimation (MLE)

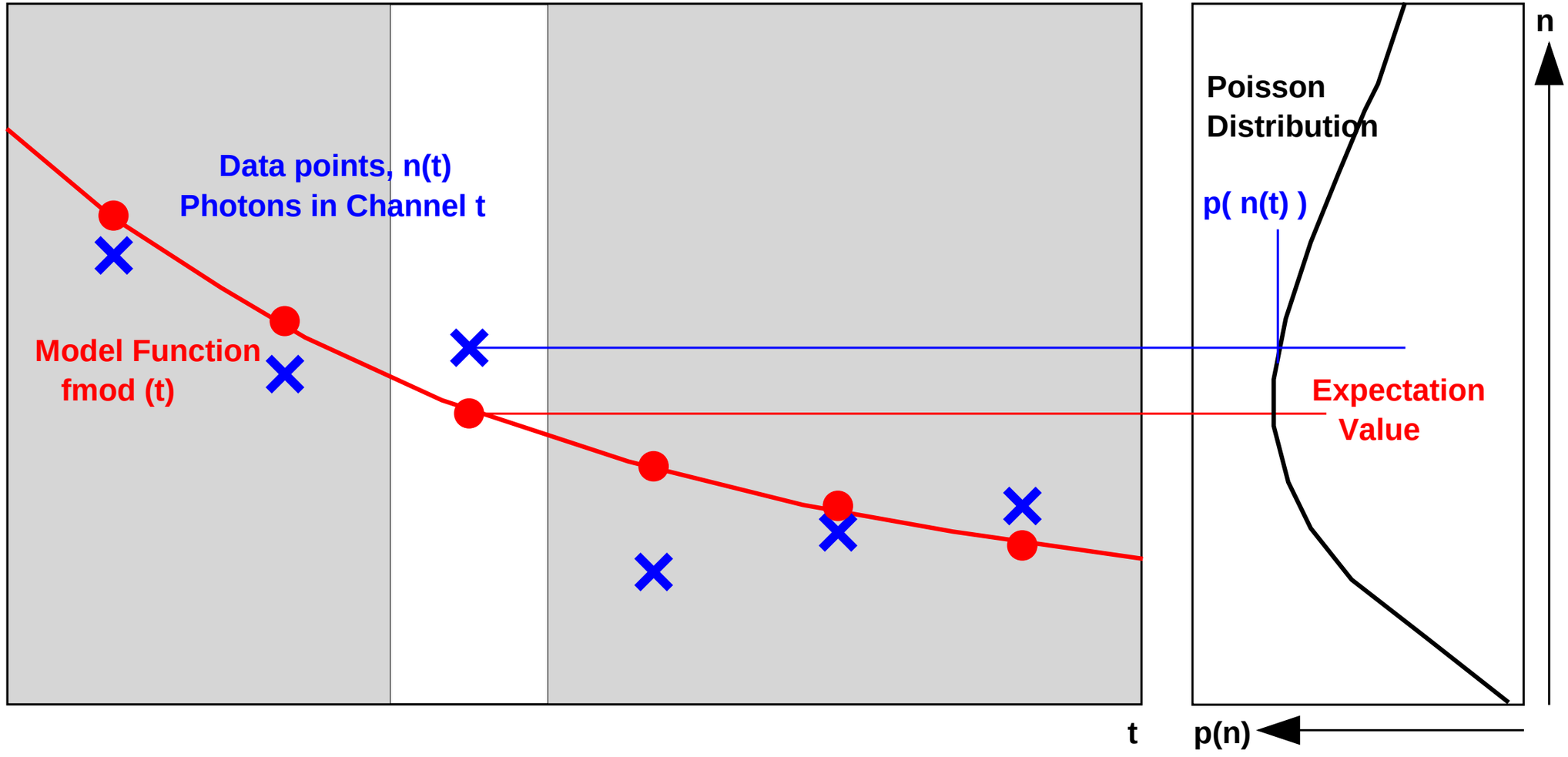


- MLE calculates the probability that a particular value of n appears in a particlar time channel

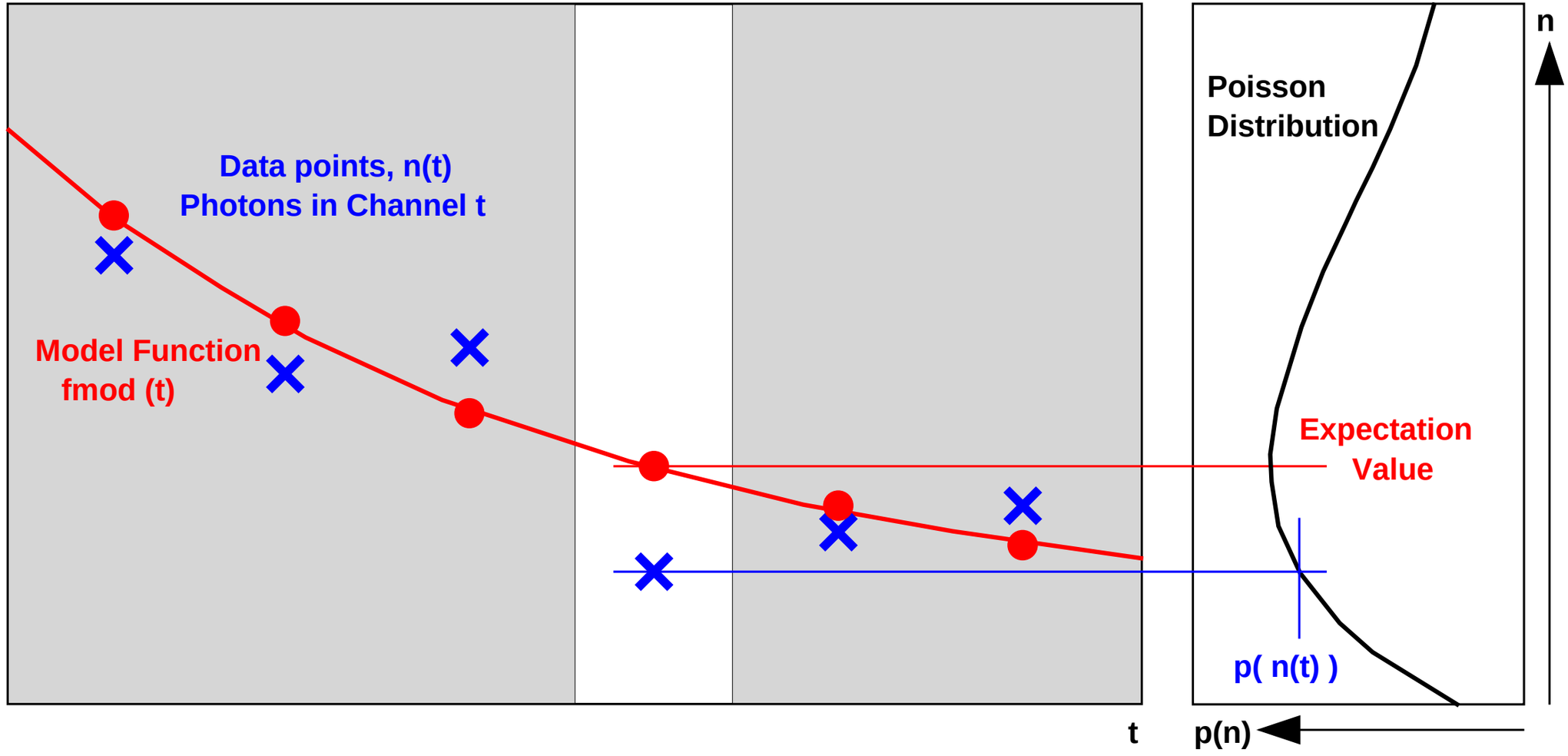
SPCIMage NG: Maximum-Likelihood Estimation (MLE)



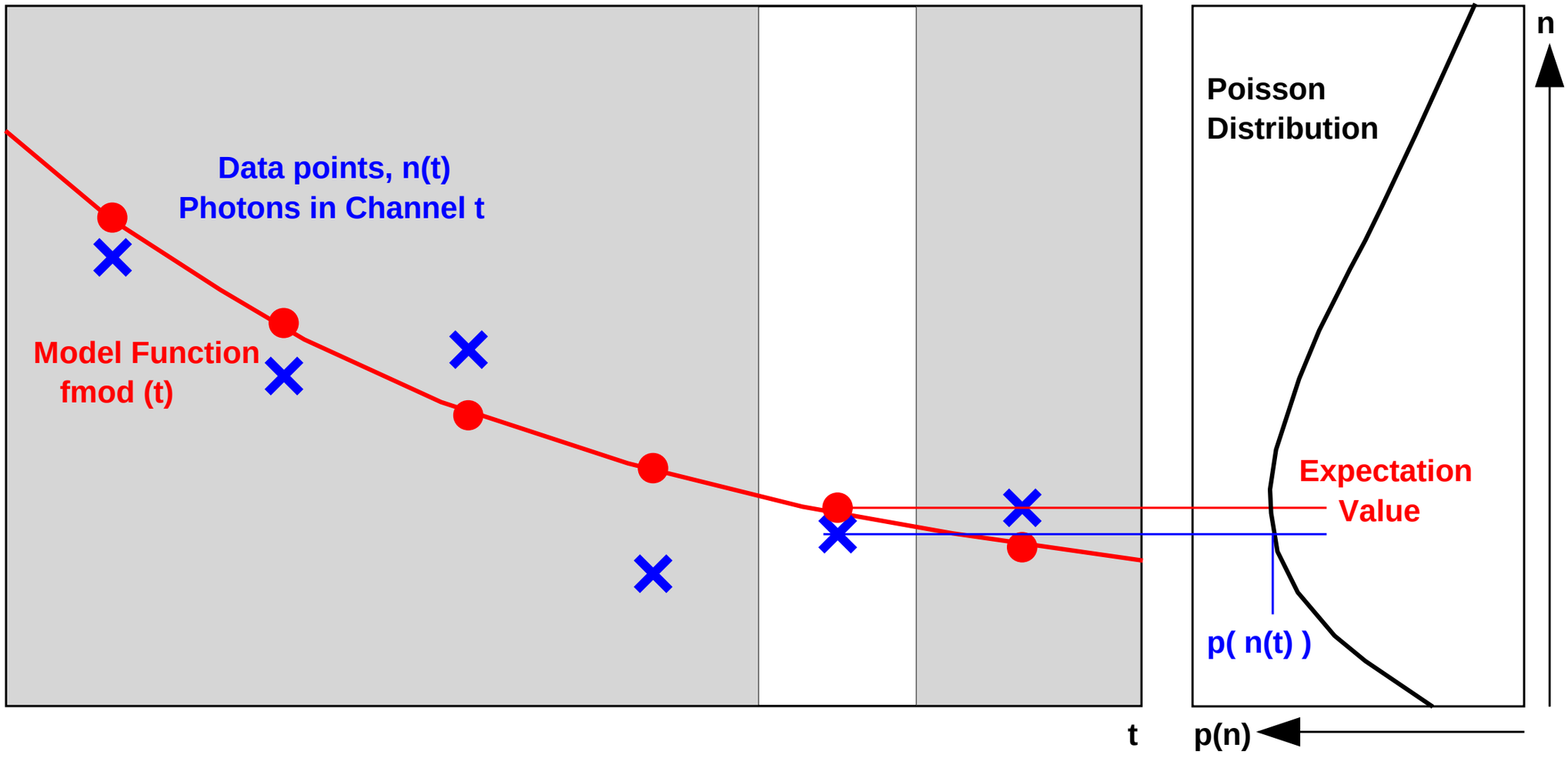
SPCIMage NG: Maximum-Likelihood Estimation (MLE)



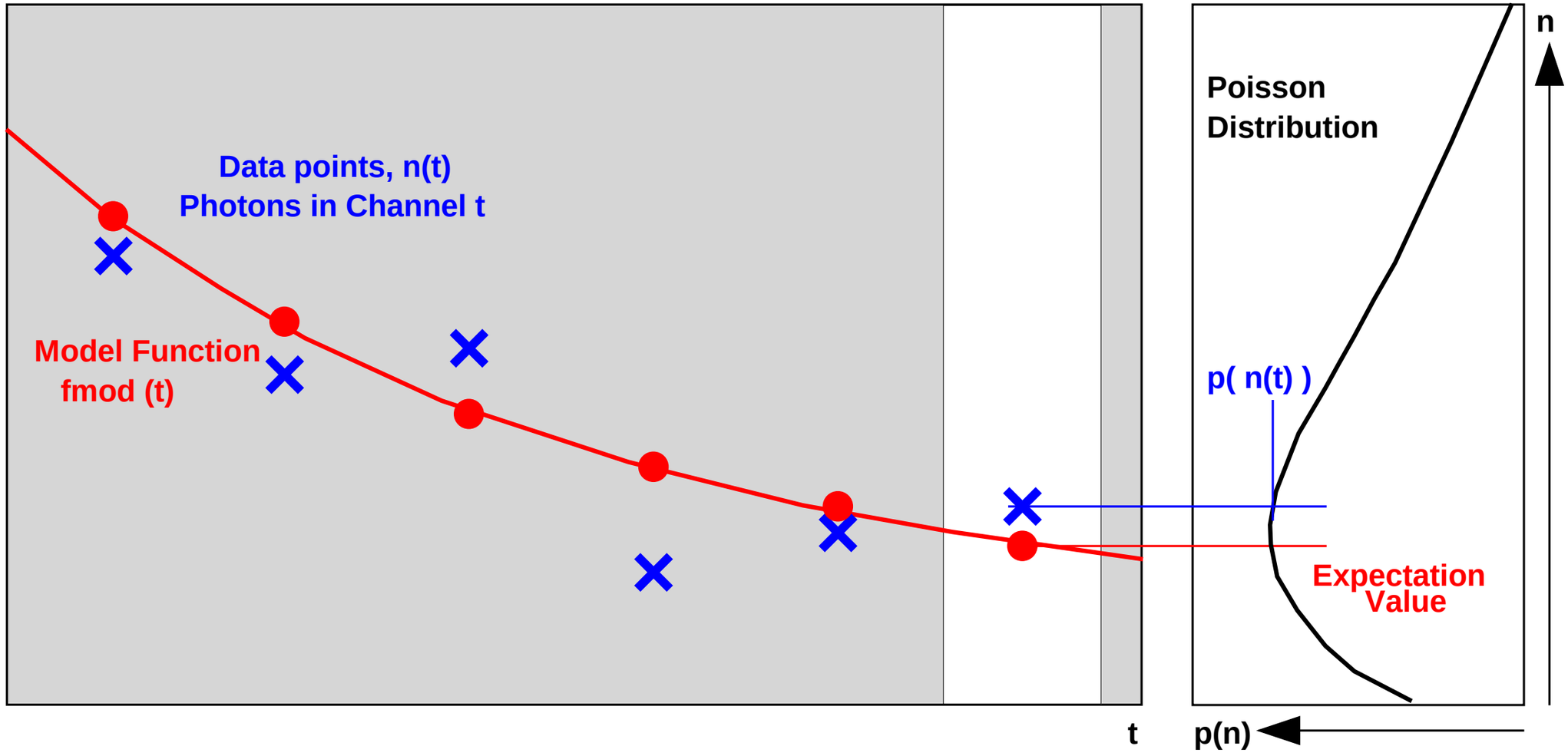
SPCIMage NG: Maximum-Likelihood Estimation (MLE)



SPCIMage NG: Maximum-Likelihood Estimation (MLE)



SPCIMage NG: Maximum-Likelihood Estimation (MLE)

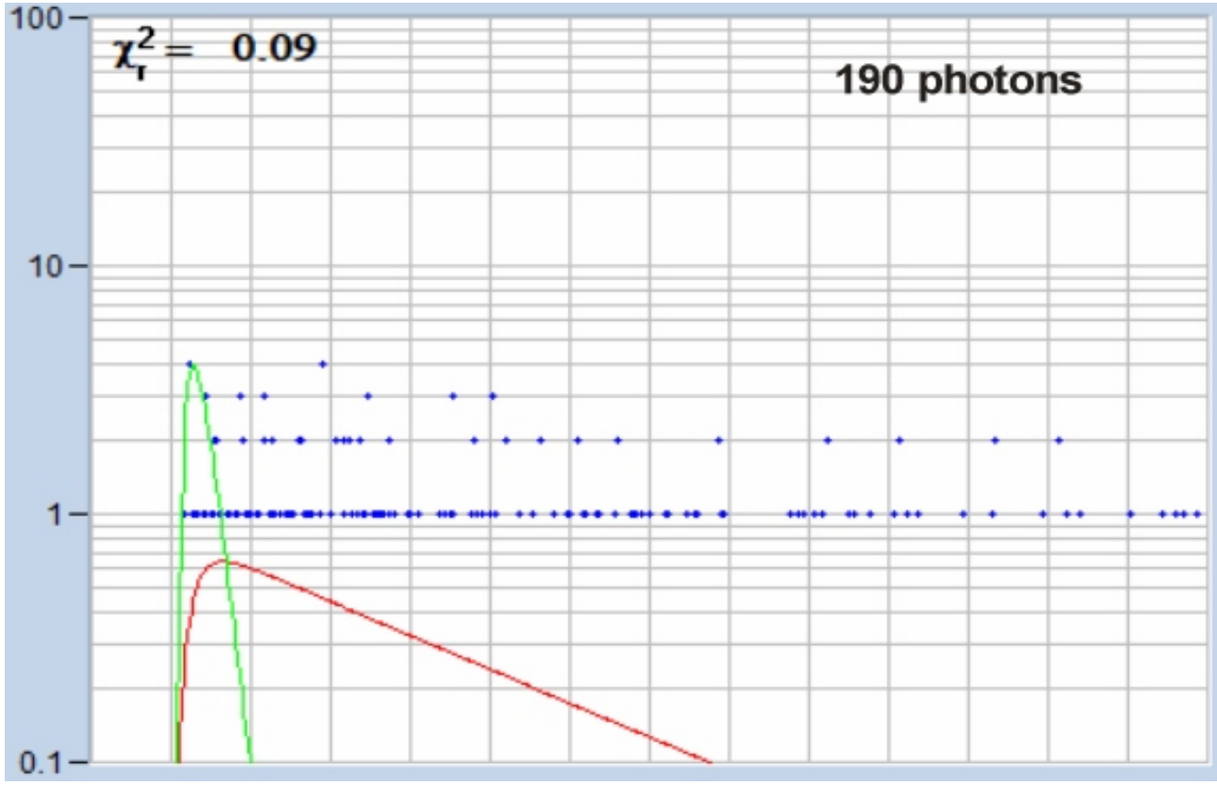


- Optimise model parameters until product of probabilities is at maximum
- MLE correctly takes into account the poisson distribution of the photon numbers
- MLE has no problem problem if the number of photons in some channels is zero

MLE Delivers Correct Lifetimes at Low Photon Number

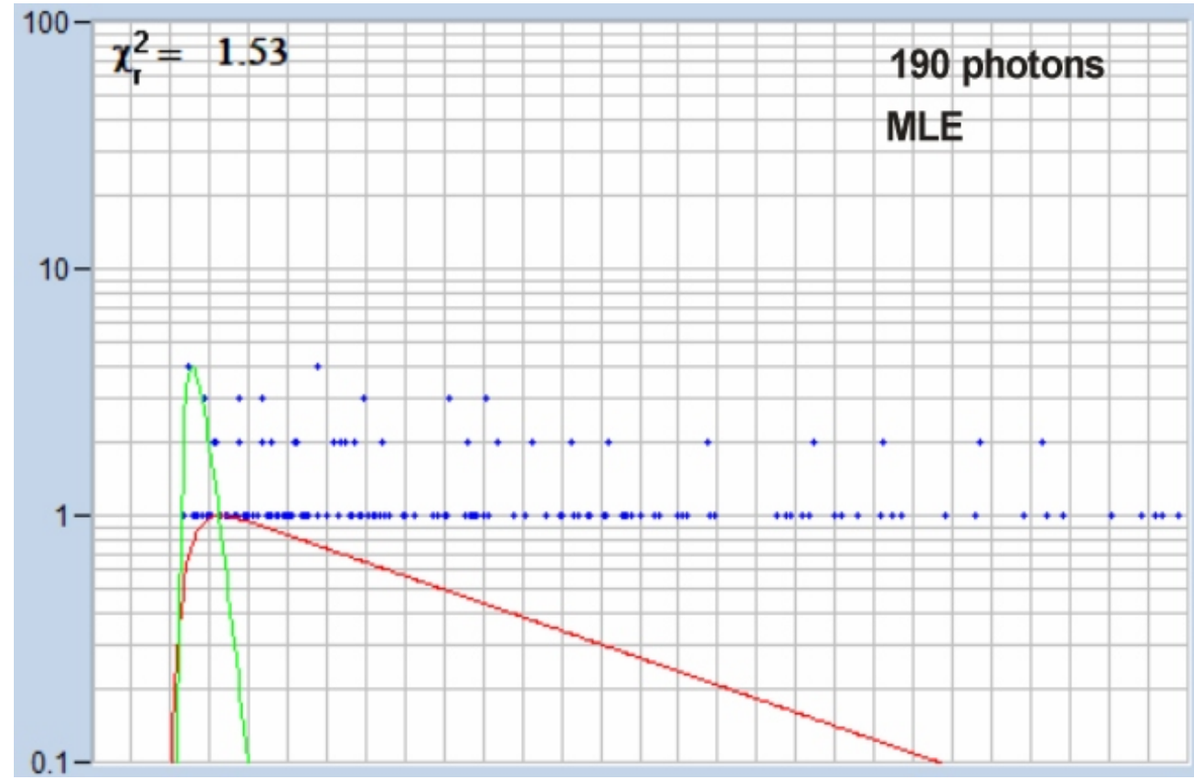
Weighted Least-Square Fit

τ = 1590 ps



MLE Fit

τ = 1960 ps

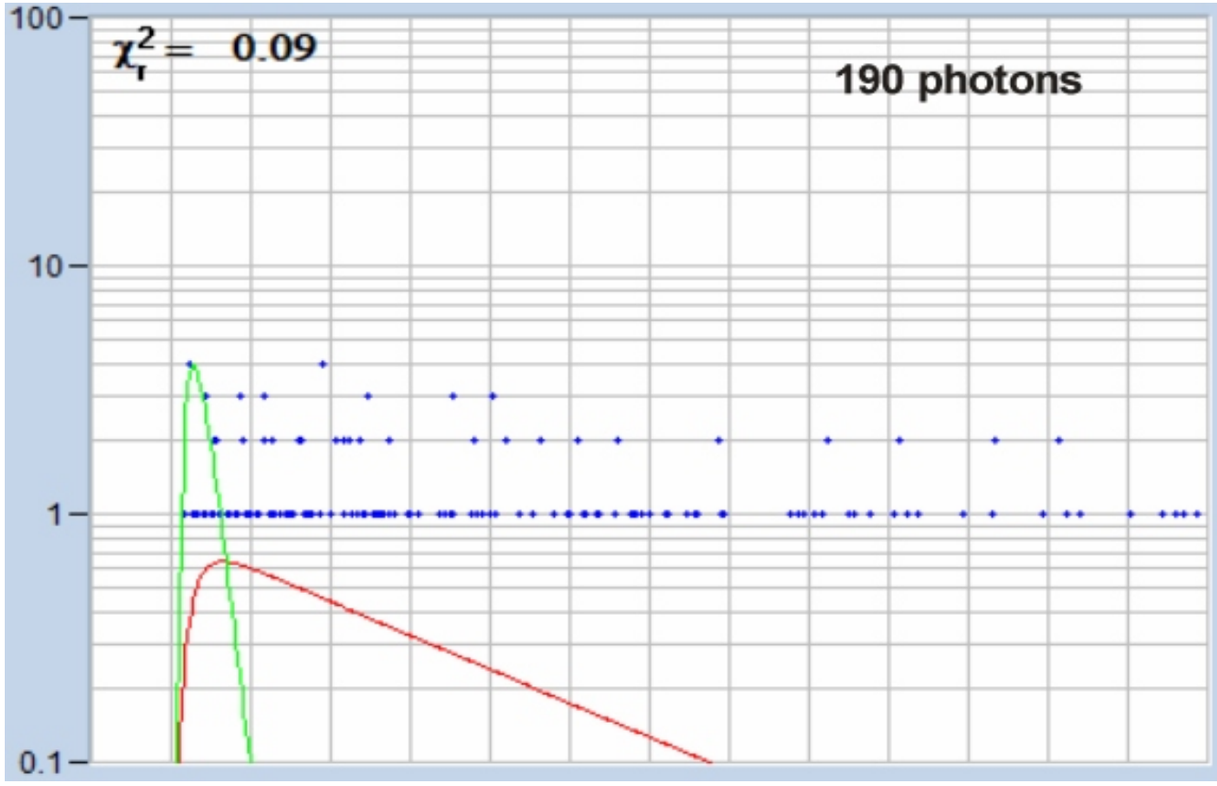


file name: bpae-63x-pixels-512-01.img

MLE Delivers Correct Lifetimes at Low Photon Number

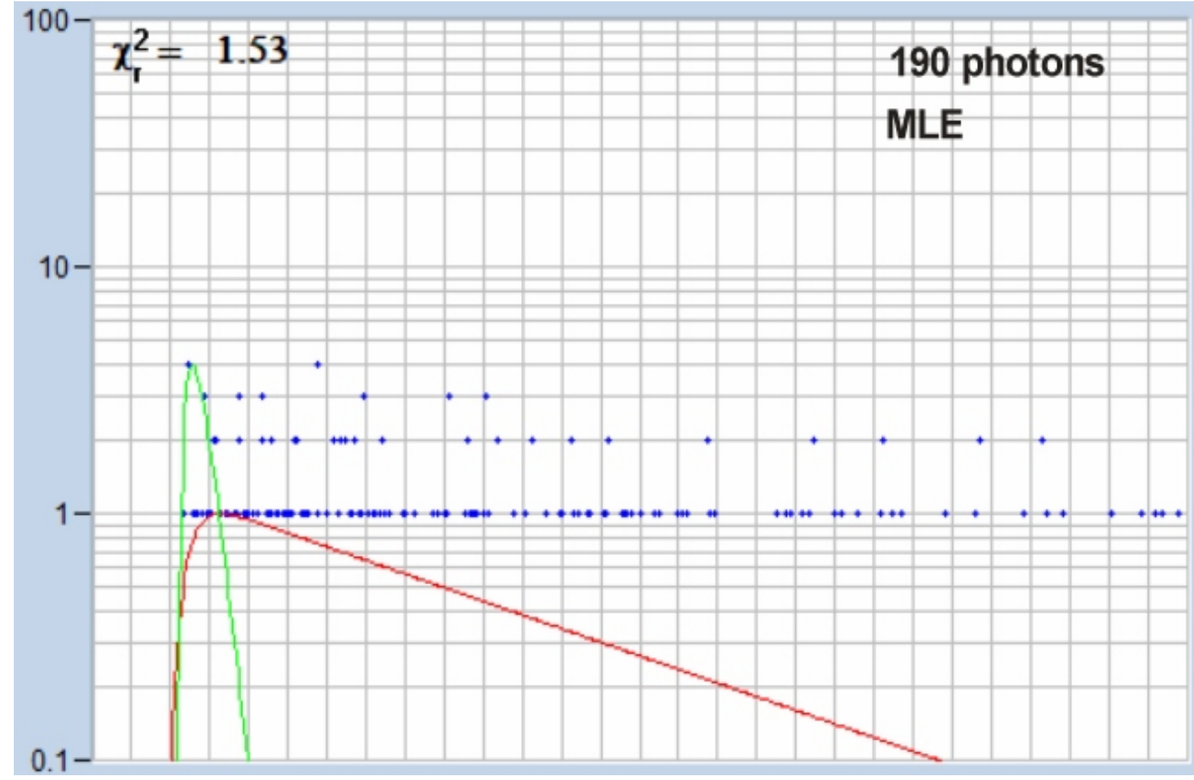
Weighted Least-Square Fit

τ = 1590 ps



MLE Fit

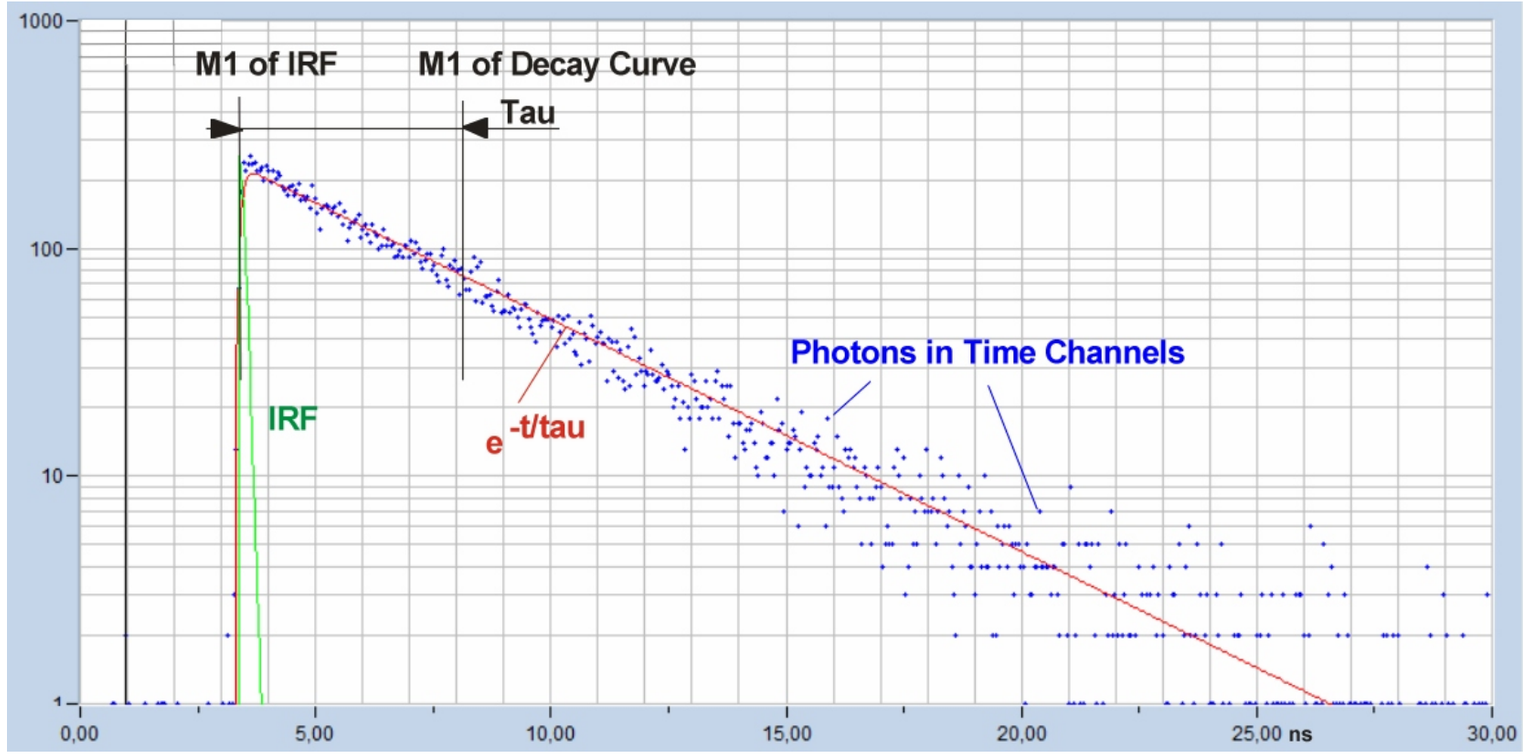
τ = 1960 ps



file name: bpae-63x-pixels-512-01.img

What's the statistical accuracy of a lifetime calculated from decay data?

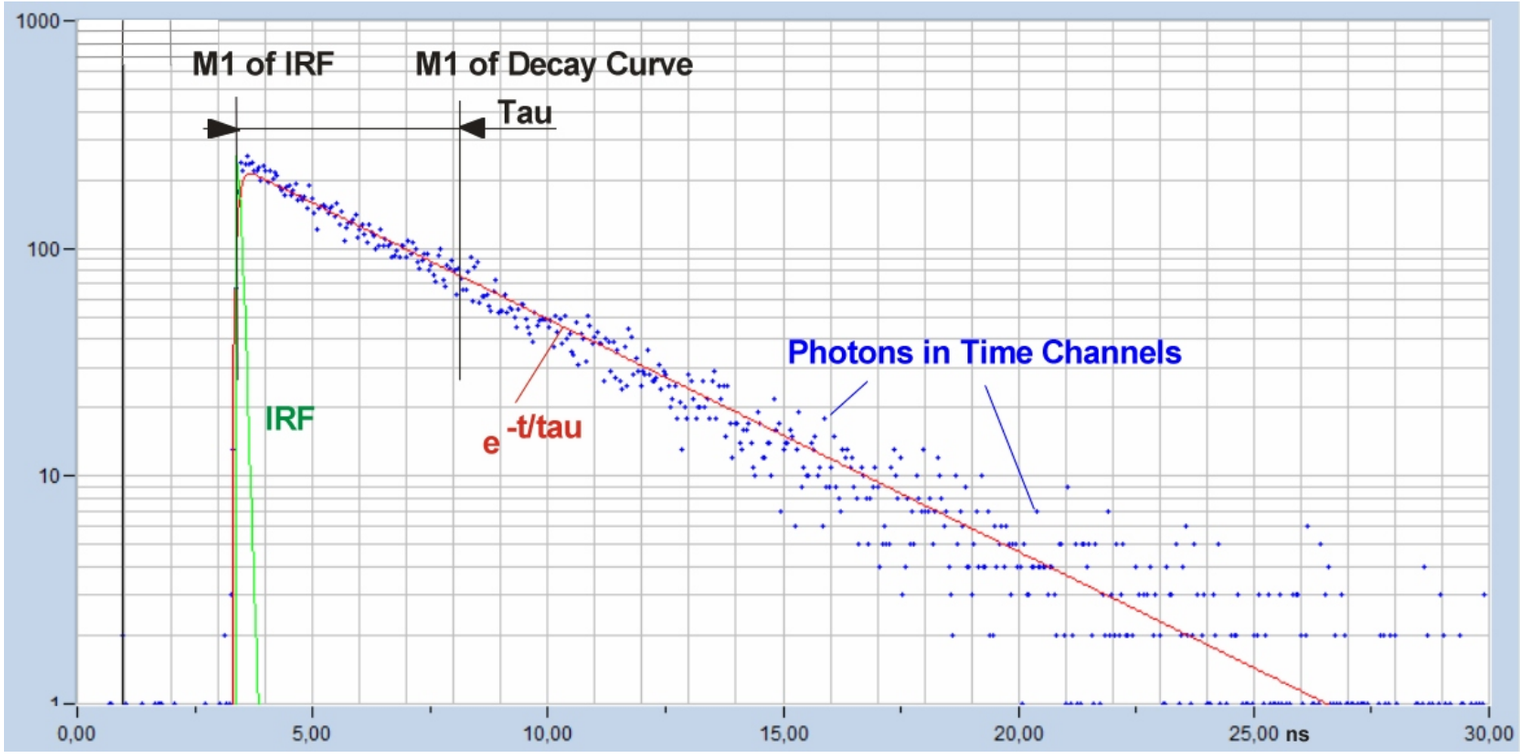
Accuracy of the Lifetime: Single-Exponential Decay



Average Arrival Time: $M1 = \frac{1}{N}\sum tn(t)$ $\tau = M1_{fluorescence} - M1_{IRF}$

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

Accuracy of the Lifetime: Single-Exponential Decay



$$M1 = \frac{1}{N}\sum tn(t) \qquad \tau = M1_{fluorescence} - M1_{IRF}$$

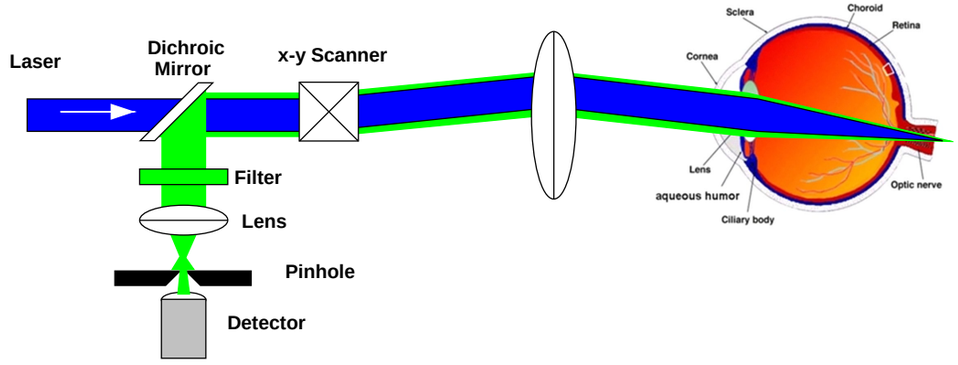
$$SNR_{\tau} = \sqrt{N}$$

Maximise N

Focus correctly!

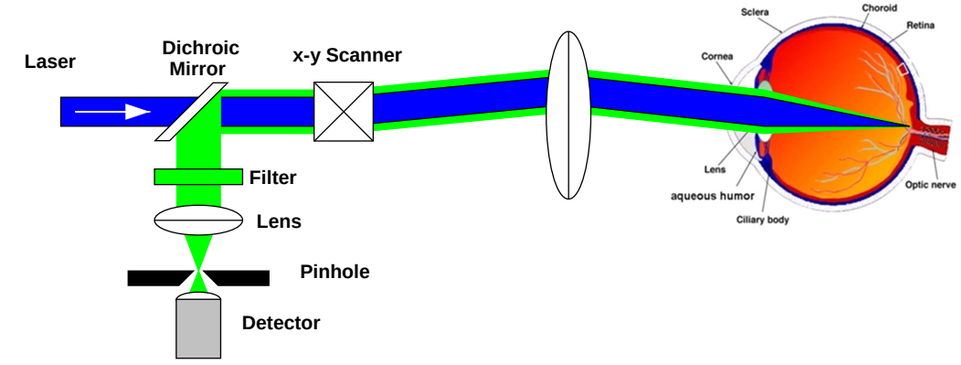
Poorly focused

Only a small part of the light passes the pinhole

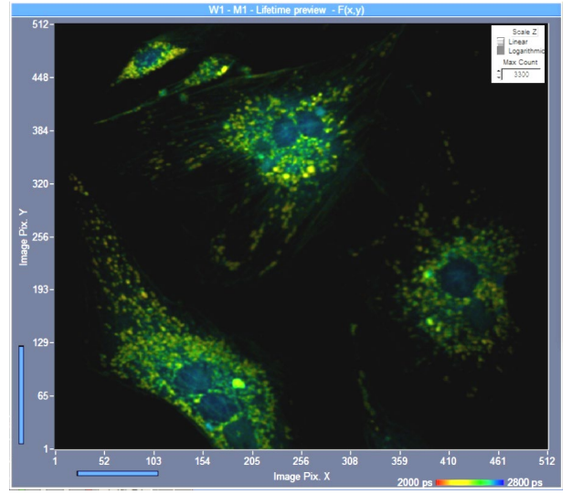


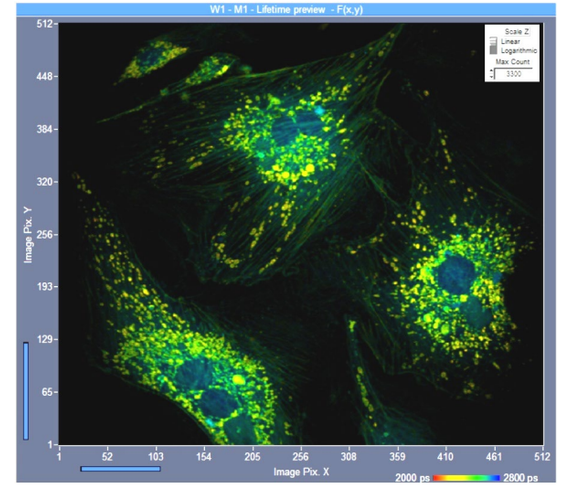
Correctly focused

All light from the focal plane passes the pinhole



Resolution may be only slightly impaired, but the loss in sensitivity may be large



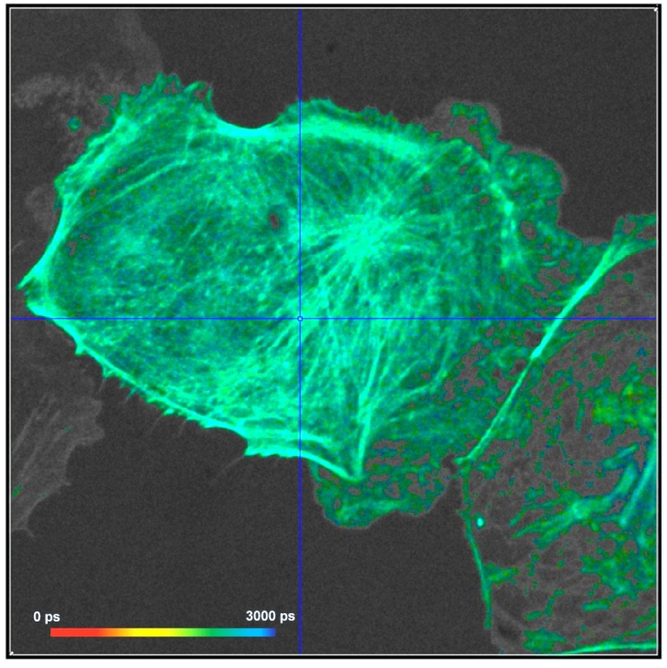


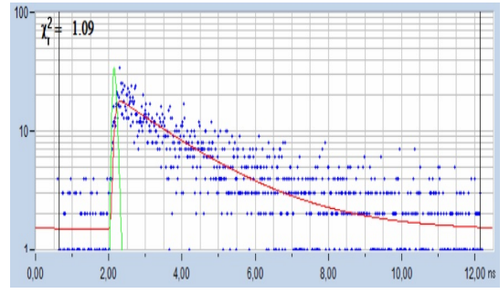
(Example from microscopy: 50% loss in photon number by poor focusing!)

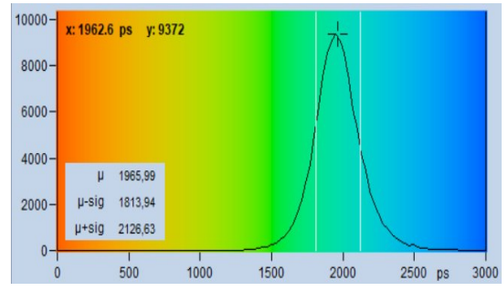
Get as Close as Possible to SQRT(N)

Don't Record Background Counts!

10% Background

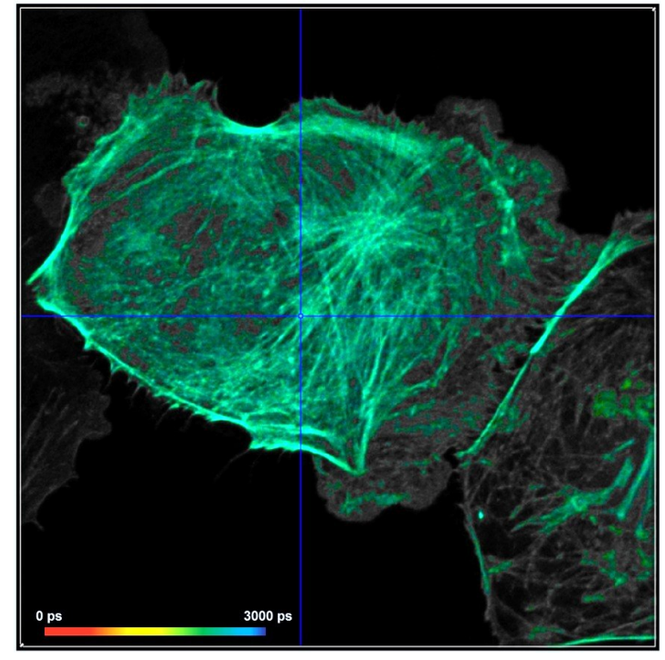


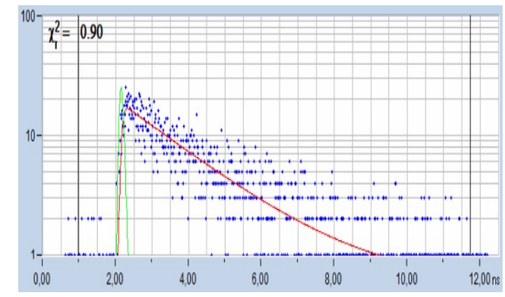


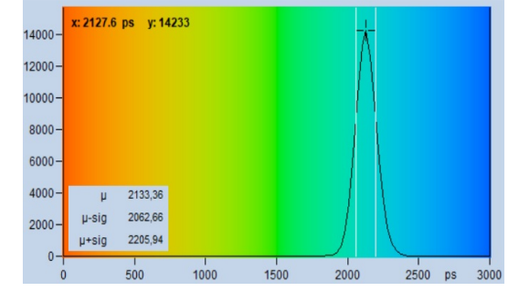


$$SNR_\tau < \sqrt{N}$$

No Background







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

313 ps **Width of lifetime distribution** **144 ps**

Equivalent to a loss of 75% of the photons!

Why such dramatic effect?

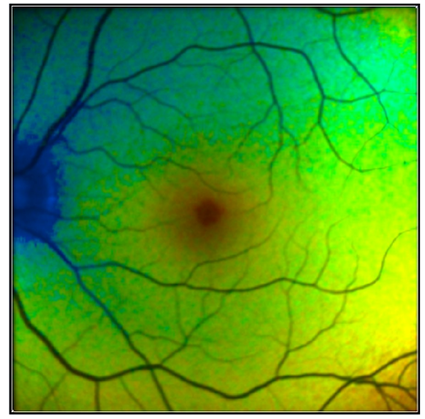
The timing variance for the background photons is much larger than for the fluorescence photons

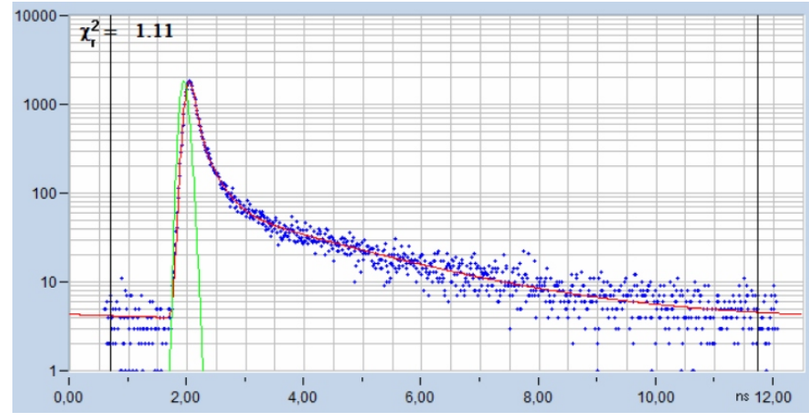
Questions?

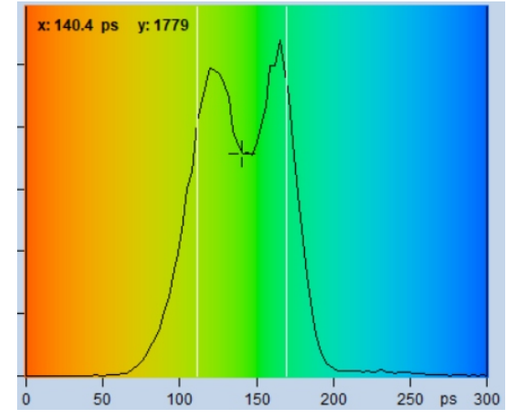
Analysis of FLIO Data: The Challenges of FLIO Analysis

Extremely fast

Decay Components

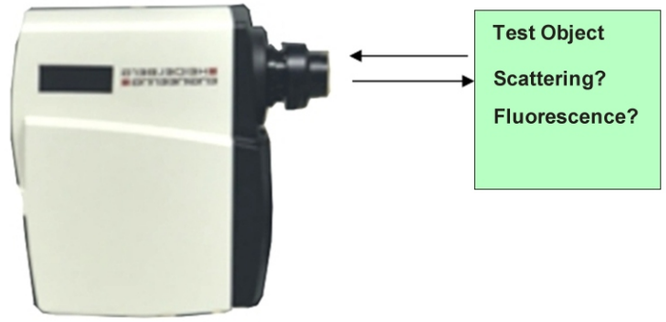


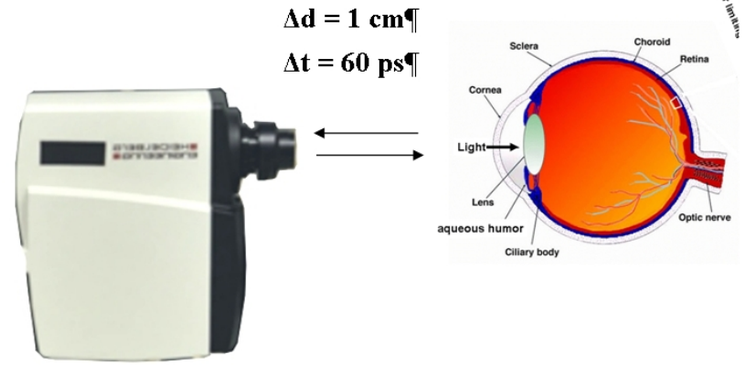




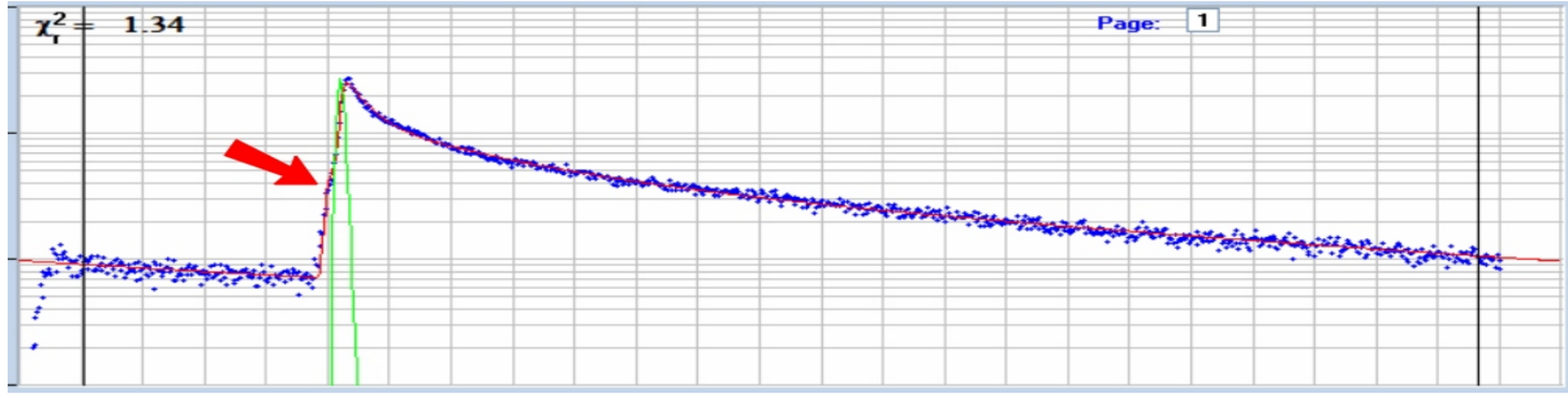
Don't know what the IRF is

Don't know where the IRF is





Don't know what the decay model is



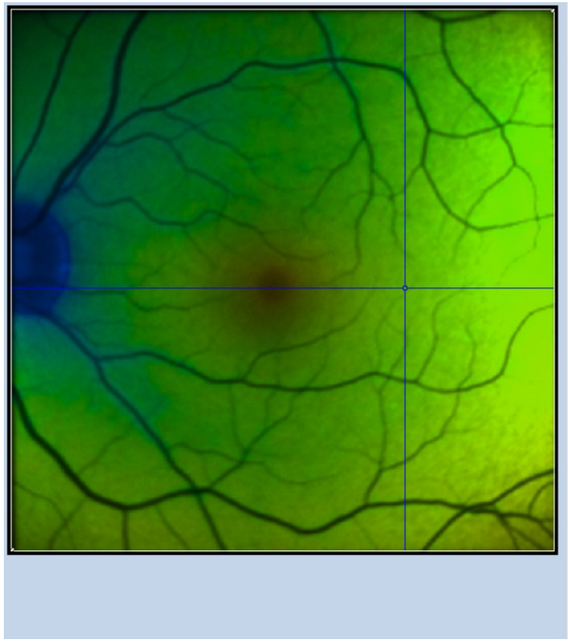
Extremely fast Decays

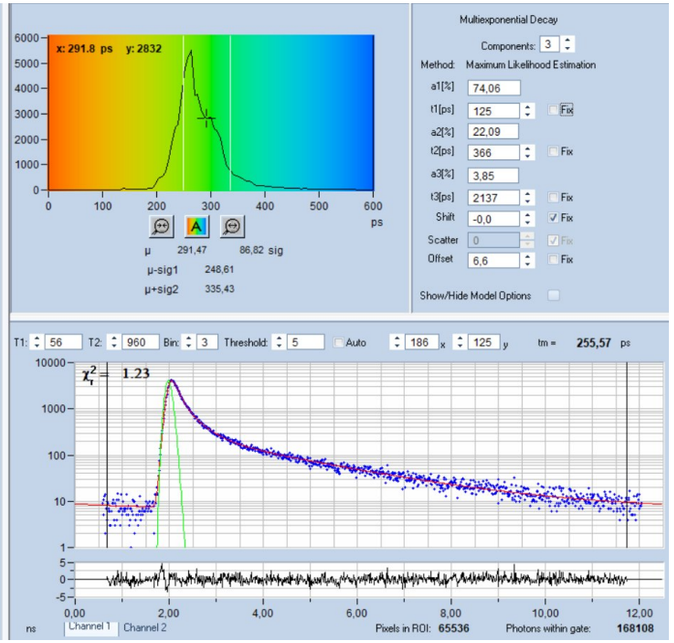
No problem down to lifetimes and lifetime components of about 50 ps

.... if an IRF of the correct shape is used. And if we know where it is.

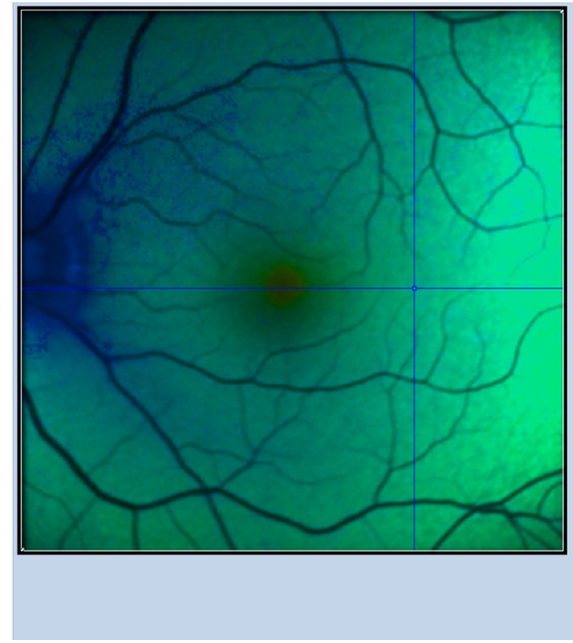
But we don't!

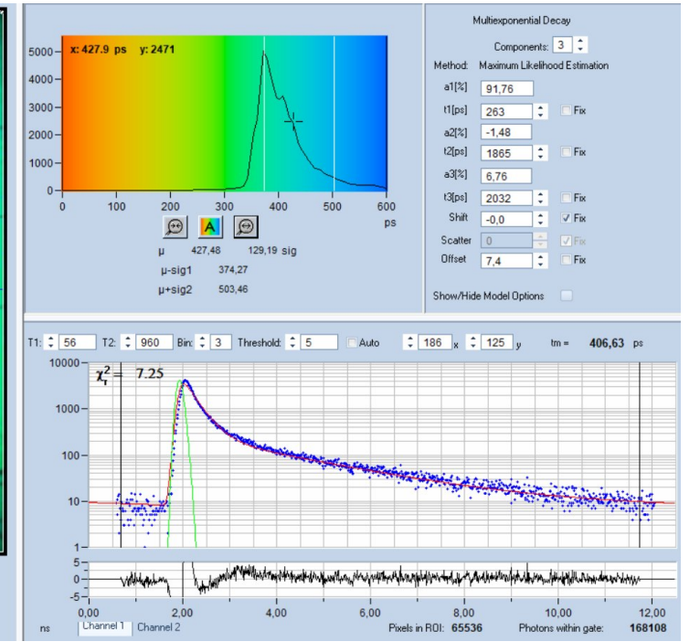
Correct IRF





IRF shifted left by 50 ps

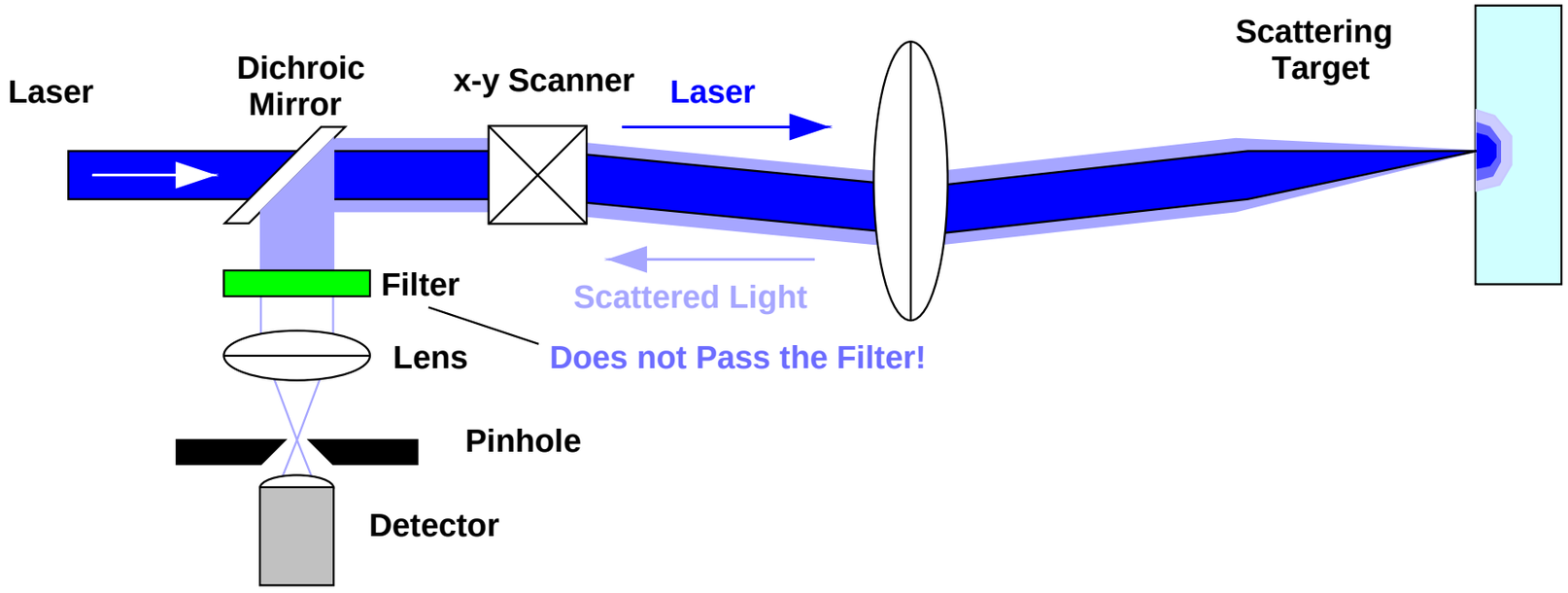




Maximum of tm Distribution: **260 ps** **380 ps**

Difference in tm: 120 ps

Can We Measure the IRF?



Back-scattered light does not pass the filter

Take out the filter for IRF Recording?

Multiple scattering in target increases IRF width

Use fluorescence of extremely short lifetime?

We'd need a fluorophore with a lifetime of <10ps. It doesn't exist.

And:

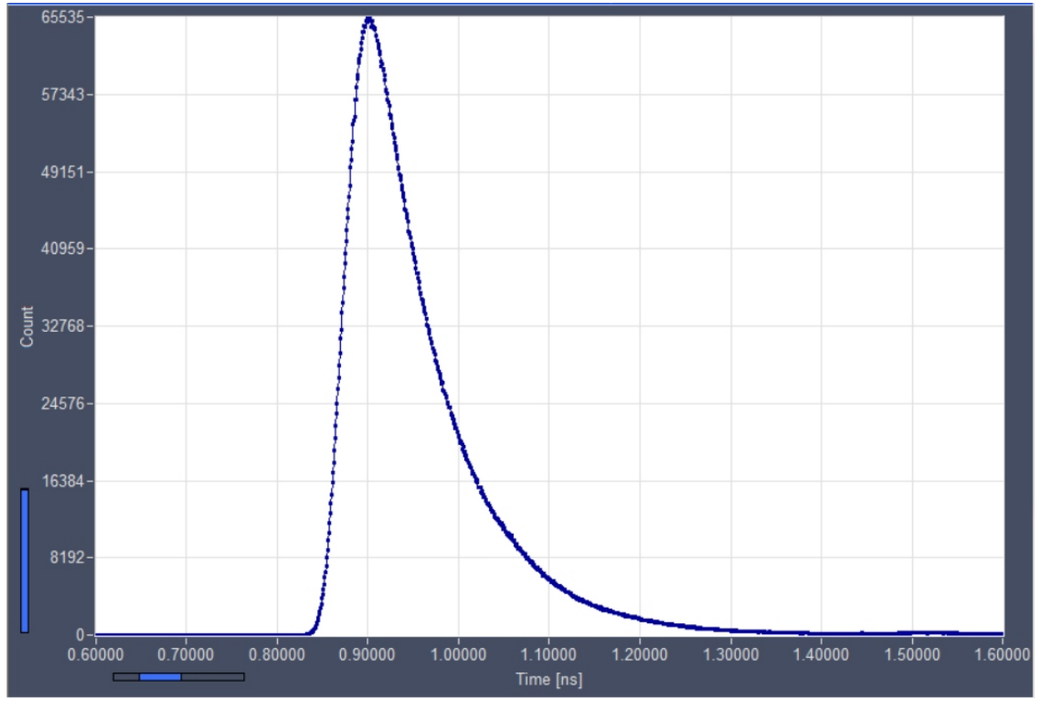
Measuring the IRF does not solve the problem of the unknown IRF position!

From Where Can Get a Correct IRF?

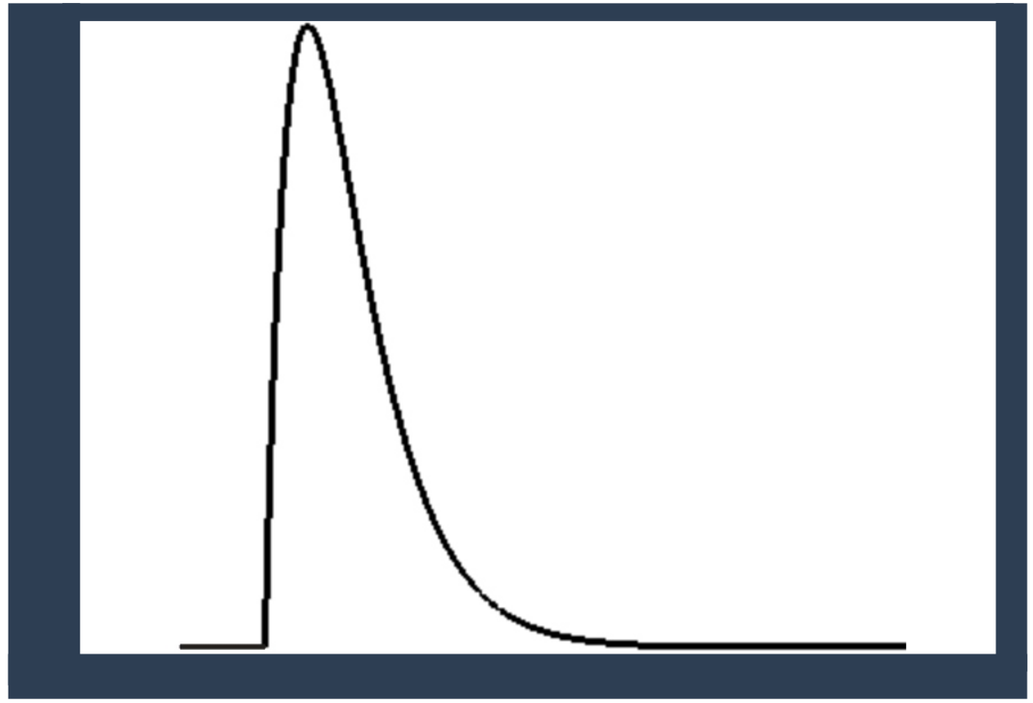
IRF modelling: Generate a syntethic IRF and use it instead of a measured one

Task: Find a simple function that resembles the general shape of the real IRF. Characterised by *one* parameter.

IRF of bh FLIM system with GaAsP Hybrid detector



Function $x \cdot e^{-x}$



Model the IRF by: $t/t_w \cdot e^{-t/tw}$

Run a normal fit of the decay data using this IRF. Include the IRF width parameter, t_w, in the fit.

When we know t_w we have the effective IRF

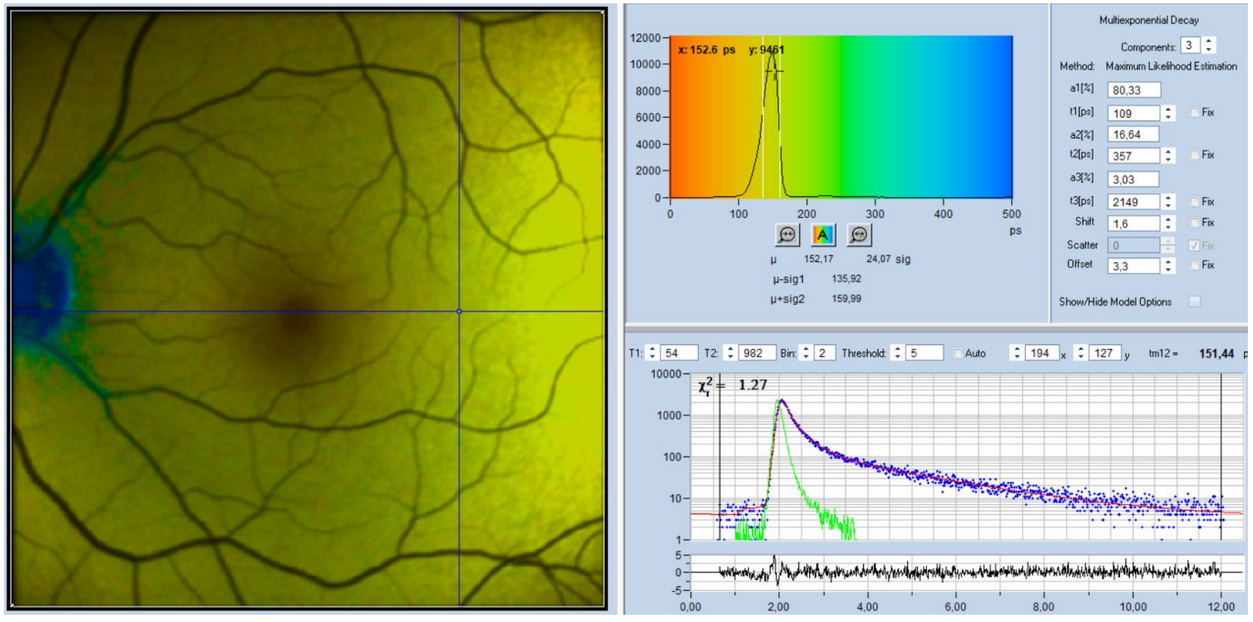
We then use this IRF for further data analysis

Measured IRF Versus Syntethic IRF

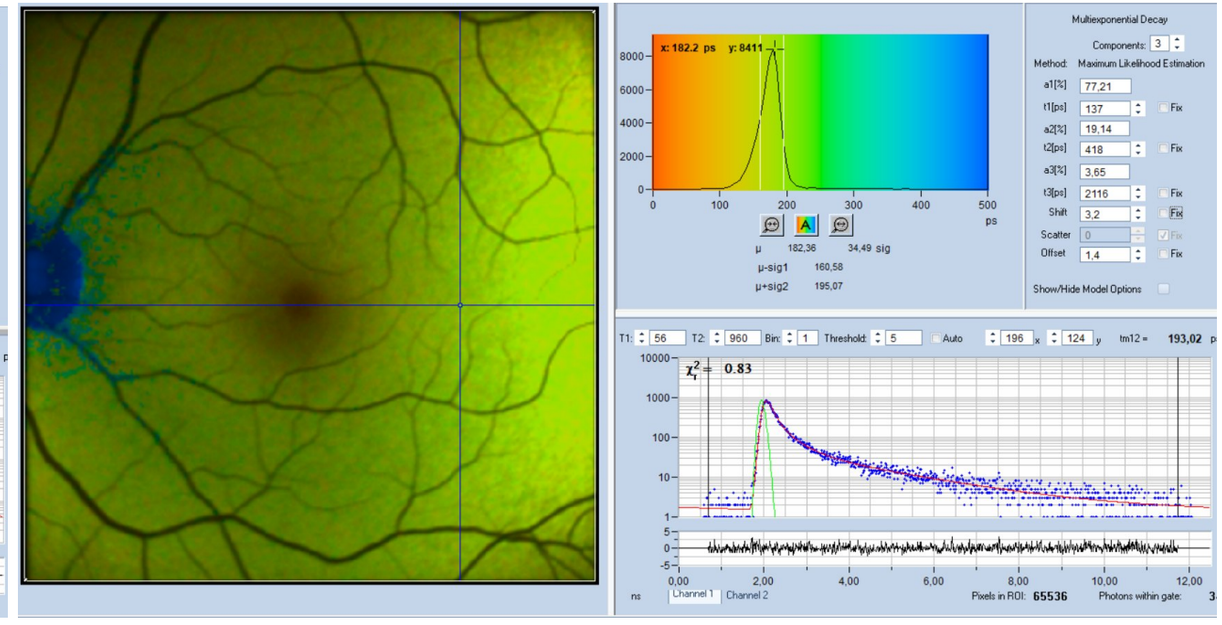
tm12 Images of shifted-component model (see later)

MLE, shift parameter floating

Measured IRF



Syntethic IRF



Max of tm12 distribution: 150 ps

Imperfect fit of rising edge

Measured IRF too broad, lifetime too short

Max of tm12 distribution: 180 ps

(Almost) perfect fit of rising edge

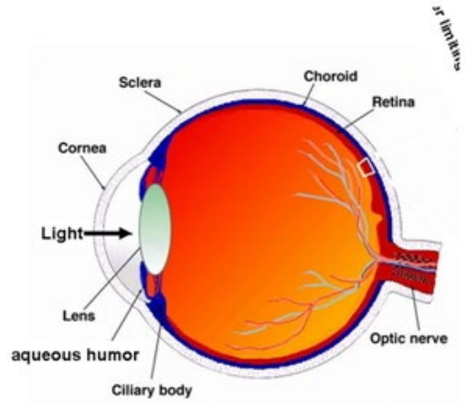
Synthethic IRF correct, lifetime correct

Difference is small but syntethic IRF yields better fit and better lifetimes

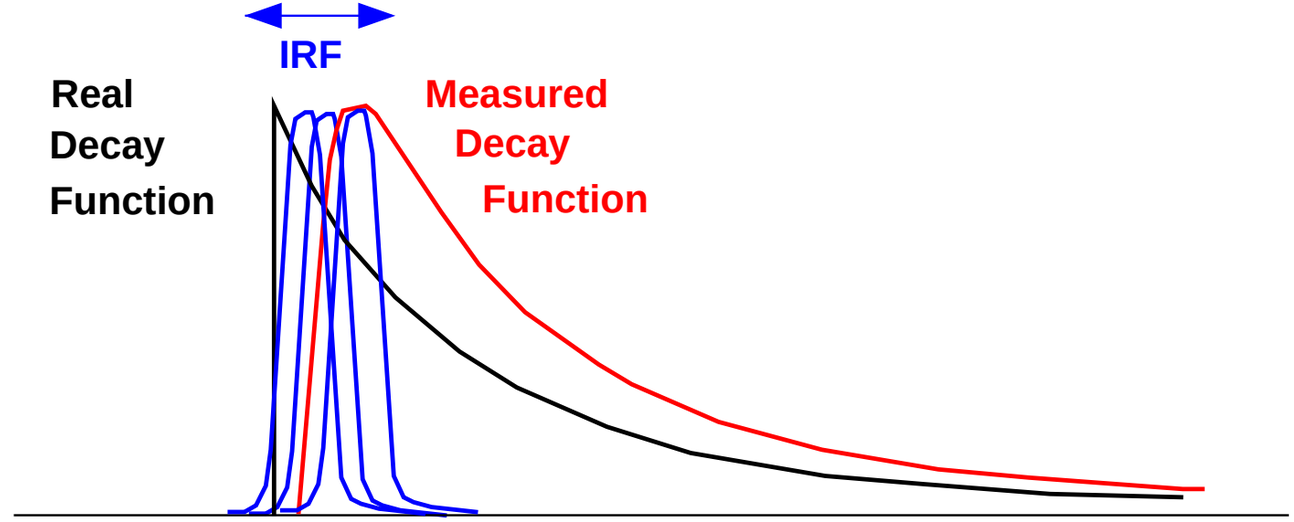
What is the Correct Position of IRF on the Time Axis?

Δd = 1 cm¶

Δt = 60 ps¶



Data analysis: Shift IRF data into correct temporal position



Fit the data with the correct model, the correct IRF shape, and a 'Shift' parameter.

Important: Use the correct model.

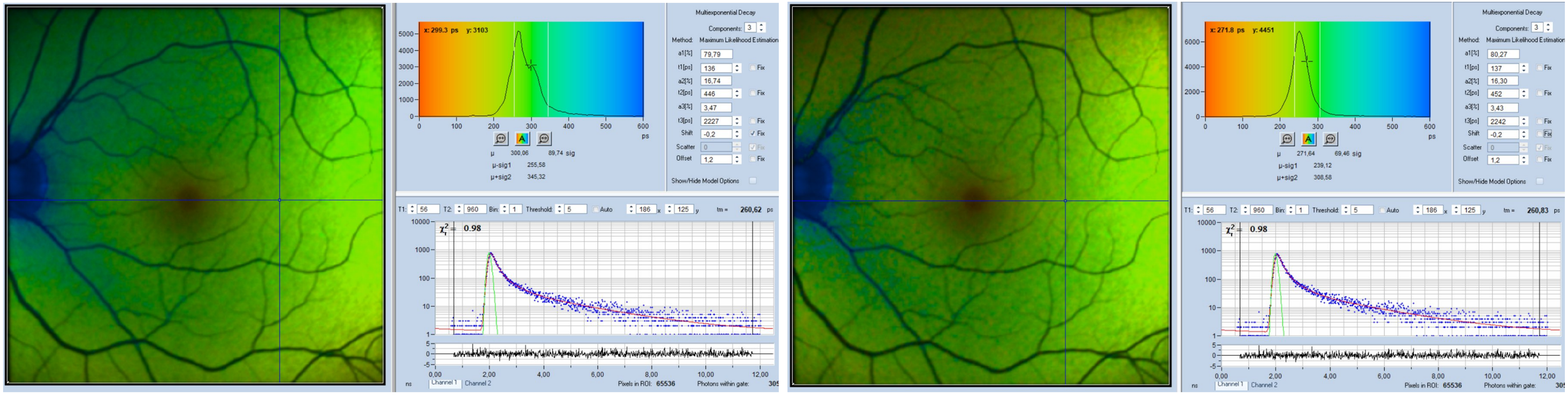
Determine IRF Position once and fix it before starting the fit in all pixels?

Or leave the IRF position floating pixel by pixel?

Fix IRF Position Before Calculation or Leave it Floating?

MLE

IRF Position Fixed **Mean Lifetime, tm** **IRF Position Floating**



There is a diagonal shift in the signal transit time, caused by mechanical effect in the scanner

Floating IRF gives narrower lifetime distribution

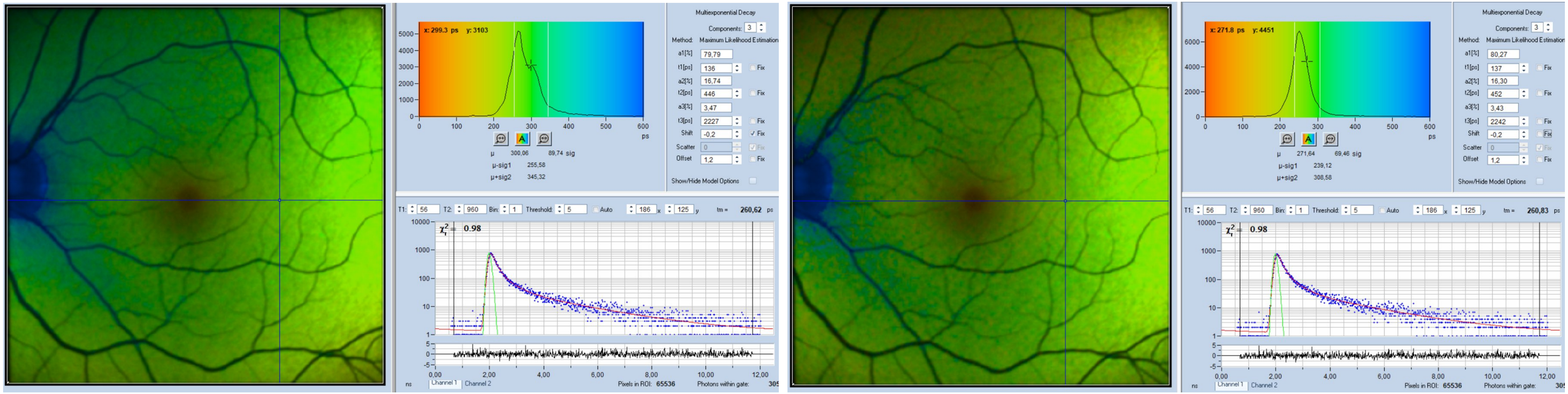
MLE gives better result for floating IRF than WLS

(And it is *not* slower)

Fix IRF Position or Leave it Floating?

MLE

IRF Position Fixed **Mean Lifetime, tm** **IRF Position Floating**



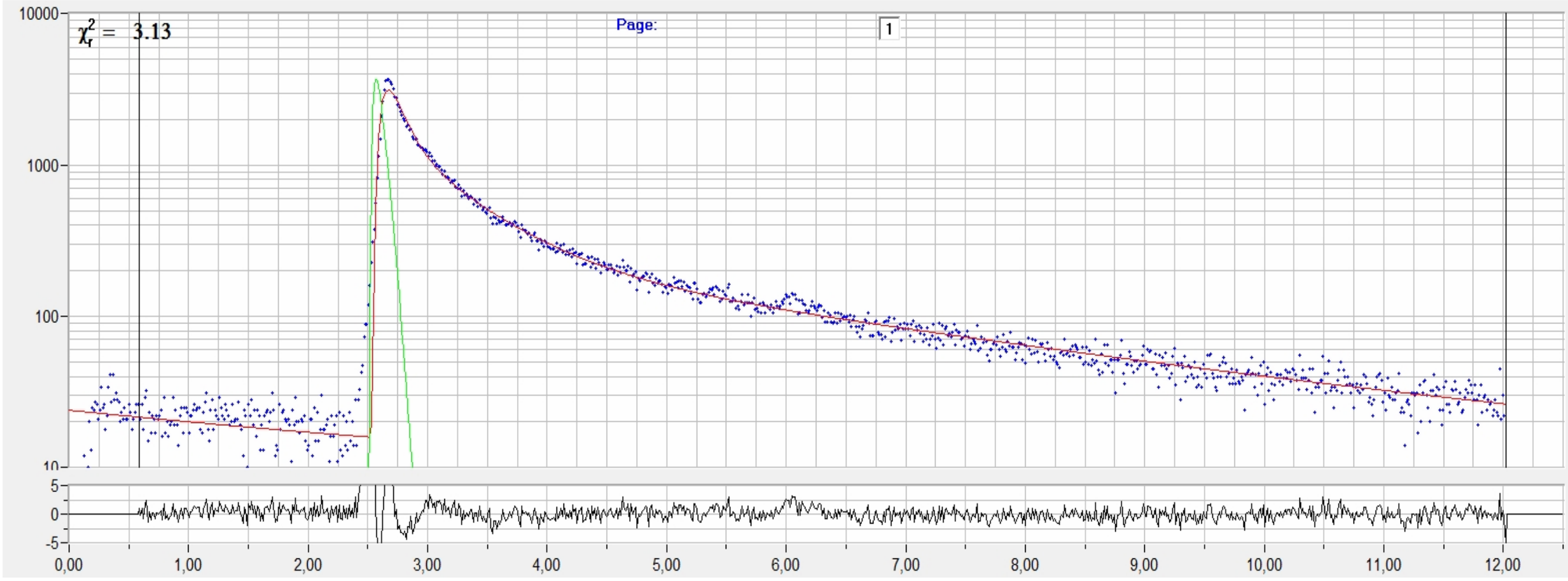
All Problems Solved?

Not Quite.

Some Mysteries remain.

Poor fit of rising edge and peak of the fluorescence decay curve

Looks like a distorted rising edge

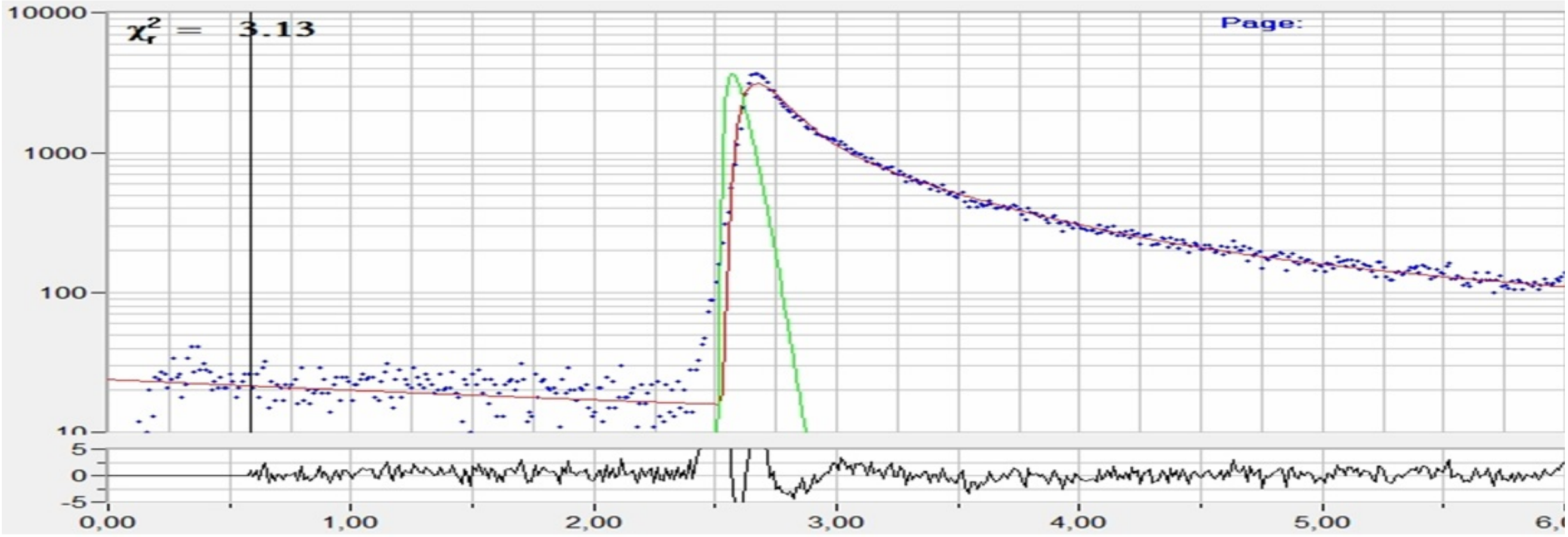


Not Quite.

Some Mysteries remain.

Poor fit of rising edge and peak of the fluorescence decay curve

Looks like a distorted rising edge

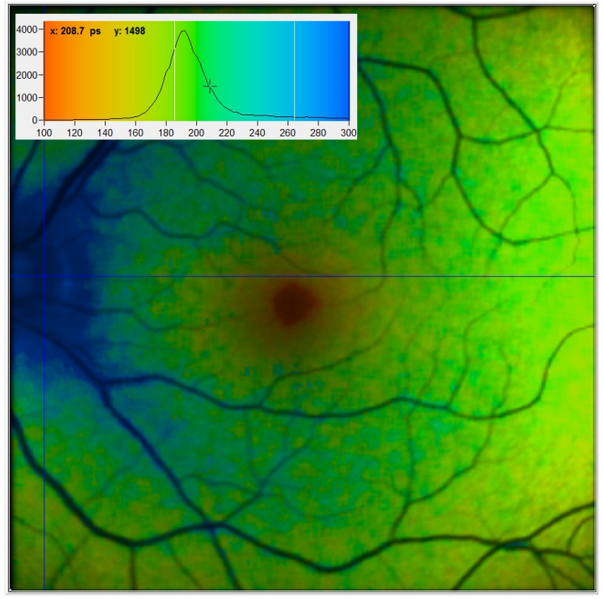


Note: If we have an inaccurate fit we will never get an accurate IRF position.

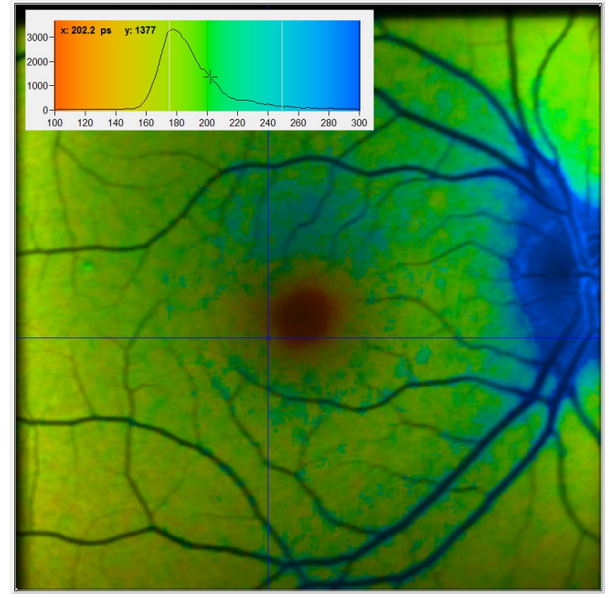
If we have an inaccurate IRF position we will never get accurate lifetimes!

And: What's that!

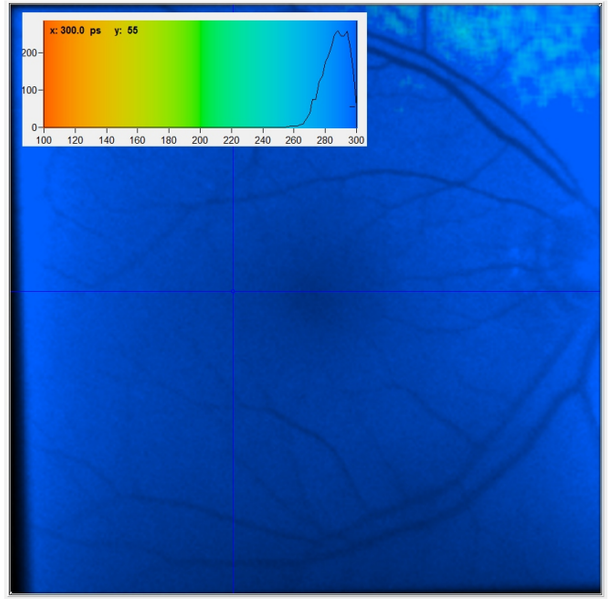
Normal



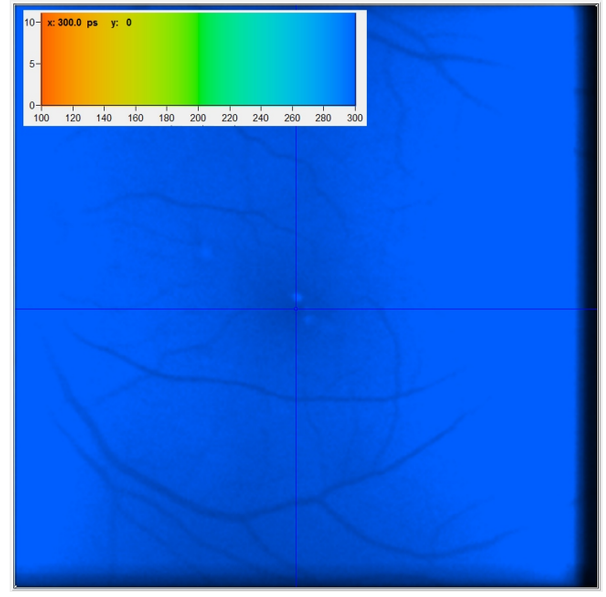
Normal



Poorly Focused



Cataract Patient



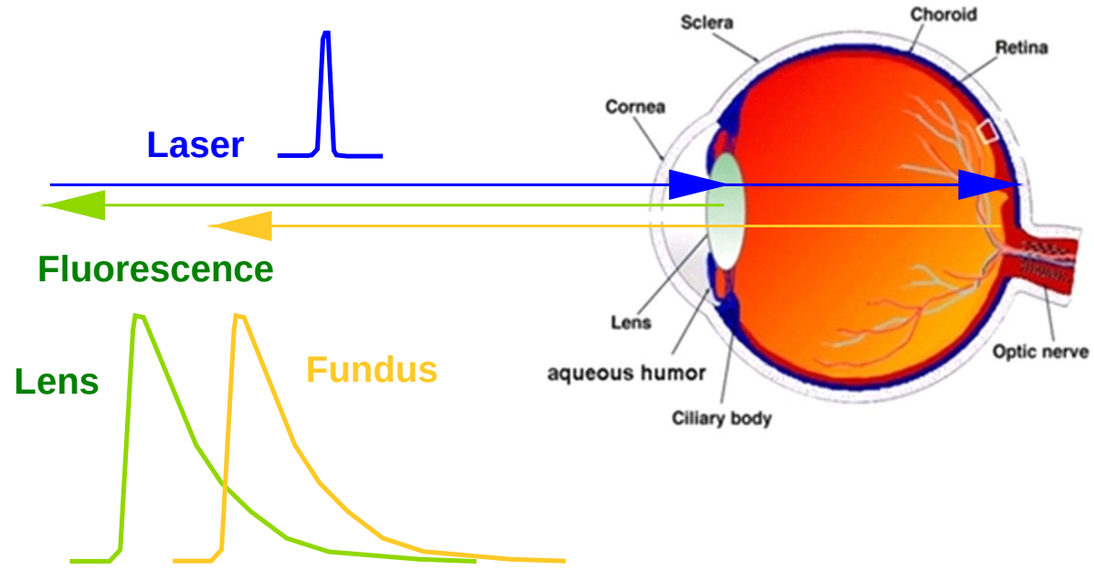
What has a Cataract Patient in Common with a Defocused Image?

More Fluorescence from the Lens!

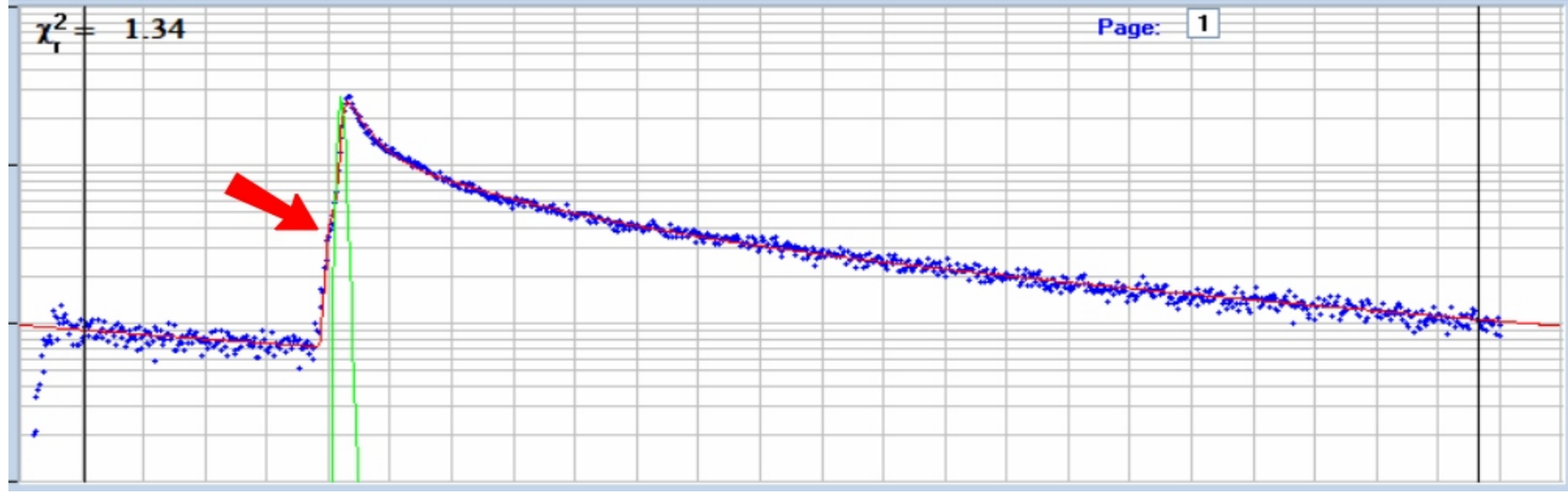
What can Distort the Rising Edge?

Fluorescence from the Lens!

The Fluorescence from the Fundus is Overlaid by Fluorescence from the Lens

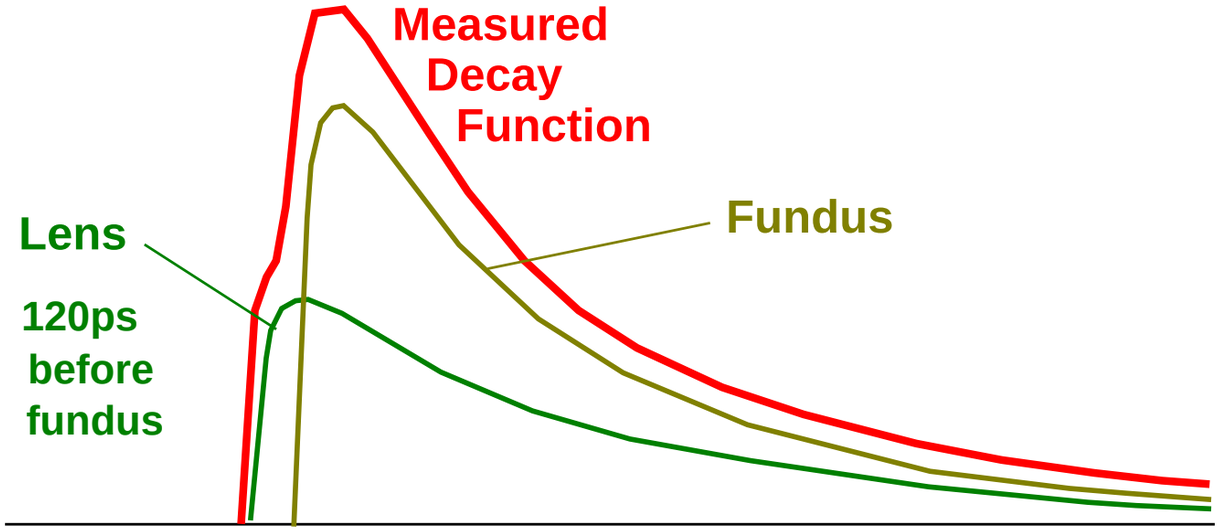


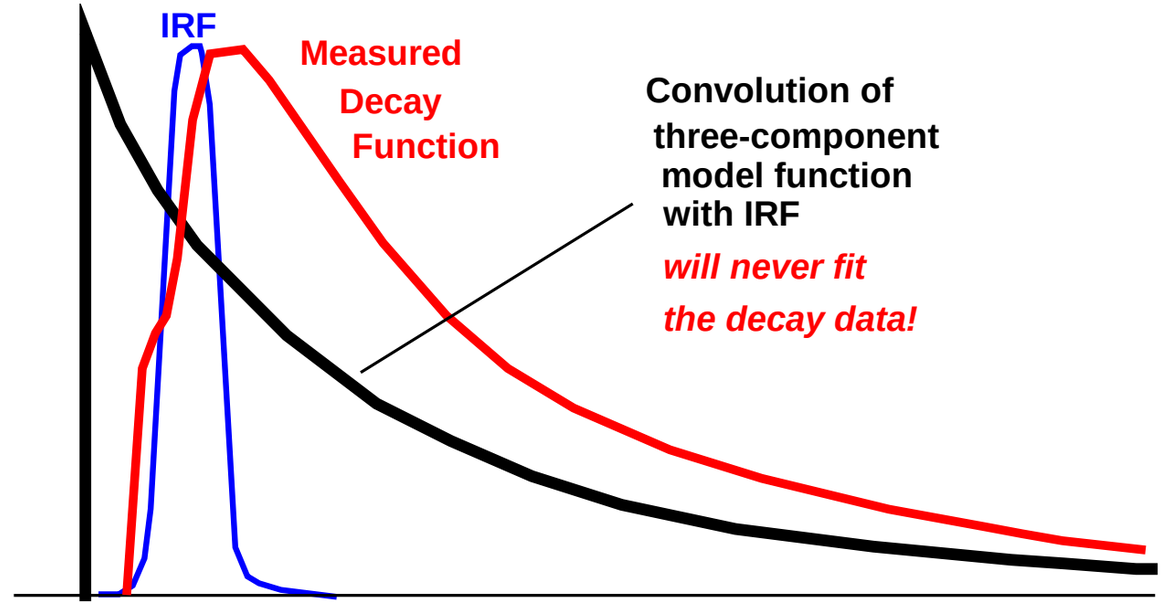
The lens fluorescence comes 120 to 180 ps earlier than the fundus fluorescence!



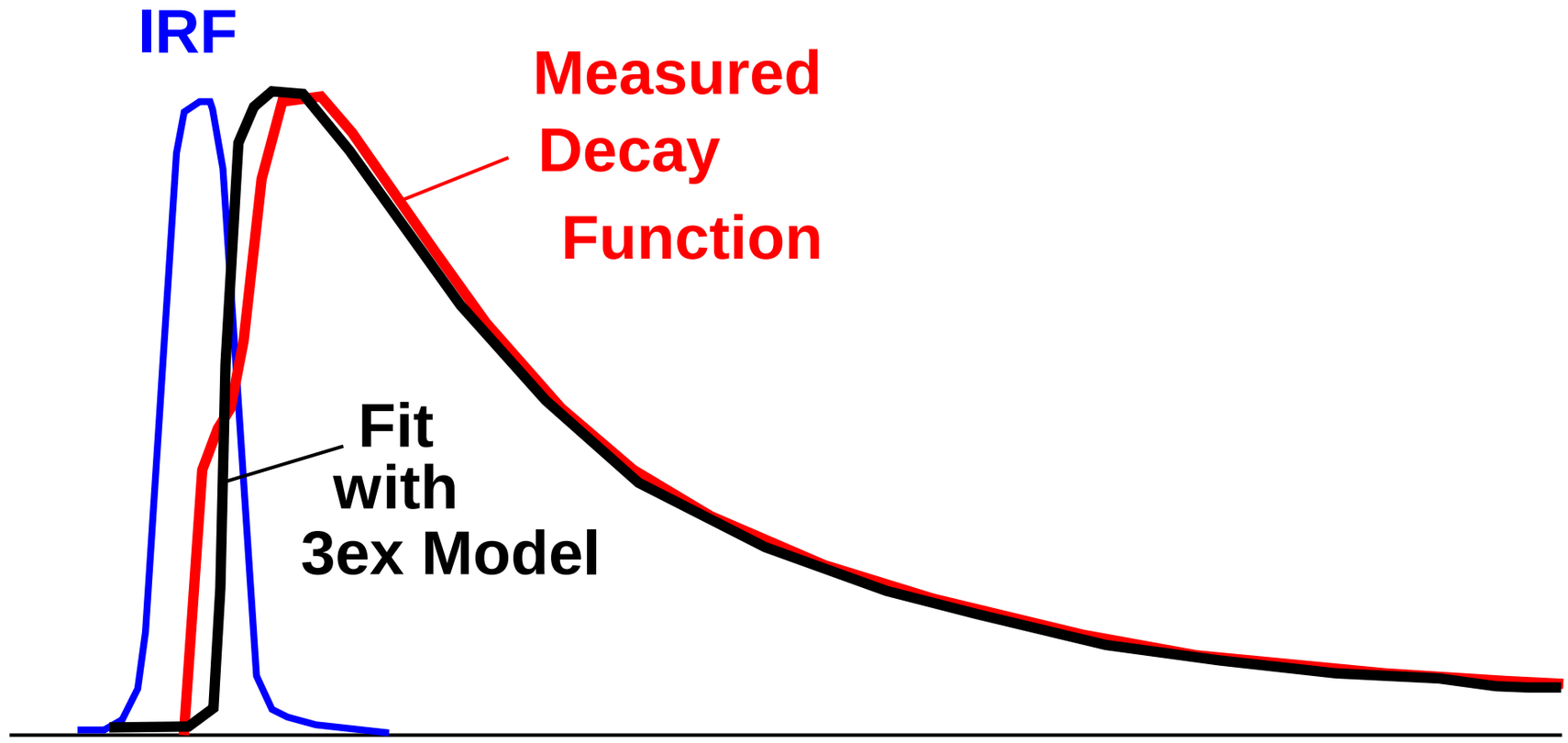
(D. Schweitzer, W. Becker, many years ago. But nobody believed us.)

The Conventional 2- and 3-Exponential Model is Unable to Fit the Decay Function!





The Conventional 3-exp. Model is Unable to Fit the Decay Function!



The conventional 3-exp. model does not fit the data correctly

Result: Unreliable fit, unreliable decay parameters

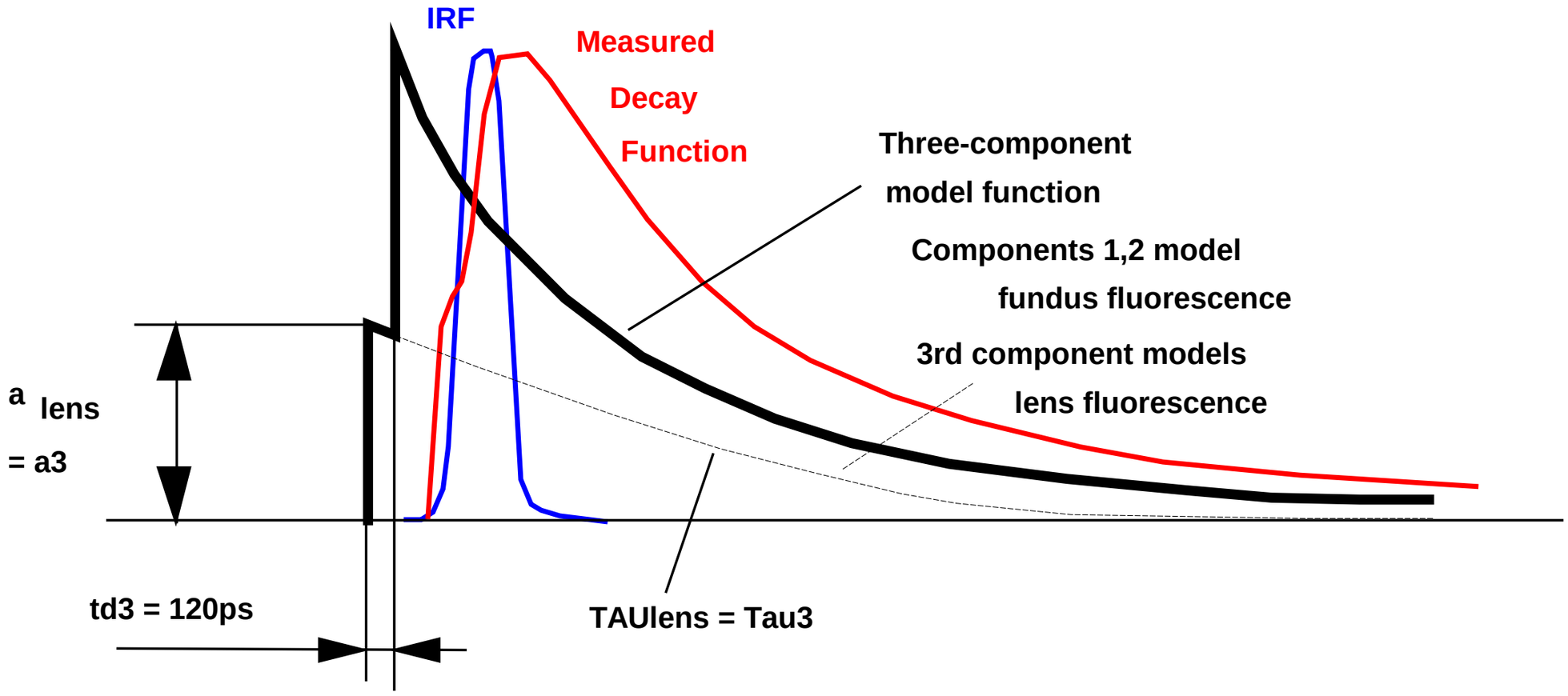
Worse:

Fit compensates wrong shape of model function by wrong position of IRF

IRF position depends systematically on amount of lens fluorescence

Decay times are determined wrong

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



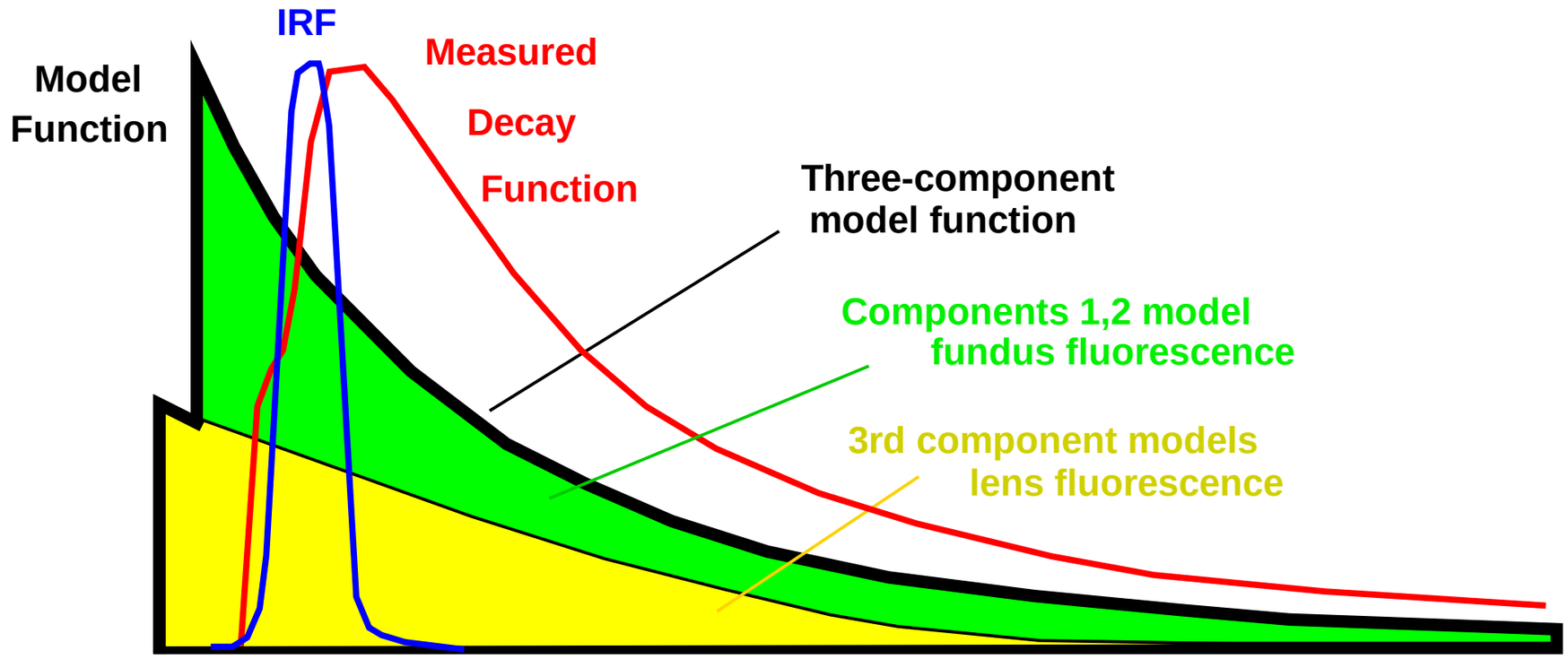
Luckily, it turns out that the slow component, τ3, comes from the lens

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

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

But t_{d3} is not critical. t_{d3} = -120ps to -150ps works well for adult humans.

A Beautiful Byproduct: tm12 is the Fundus Lifetime



$f_{12}(t) = a_1 e^{-t/\tau 1} + a_2 e^{-t/\tau 2}$ is decay function of fundus

$t_{m12} = (a_1\tau_1 + a_2\tau_2) / (a_1+a_2)$ is mean lifetime of fundus - excluding decay component from lens

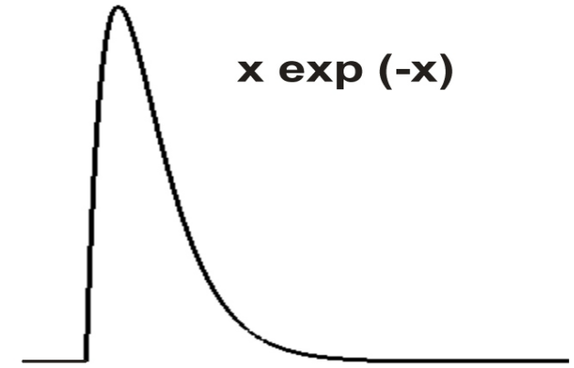
Let's replace former t_m with t_{m12} !

So, Whats's New?

Syntethic IRF Replaces Measured One

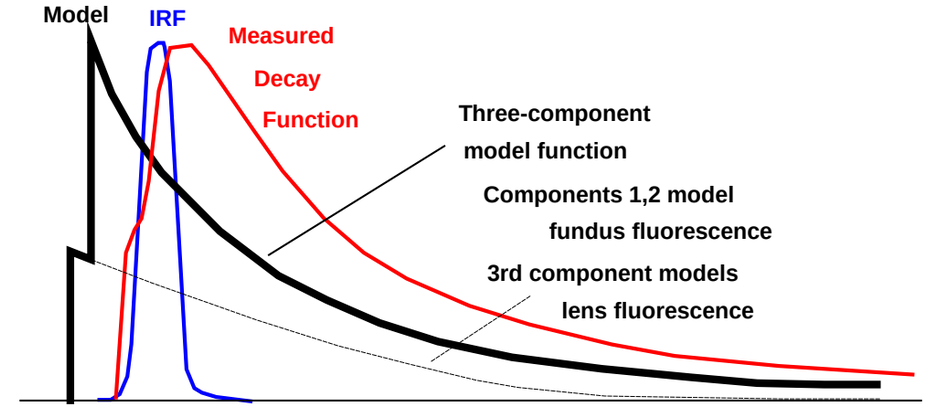
IRF position is determined by fitting x together

with the decay model to the fluorescence-decay data



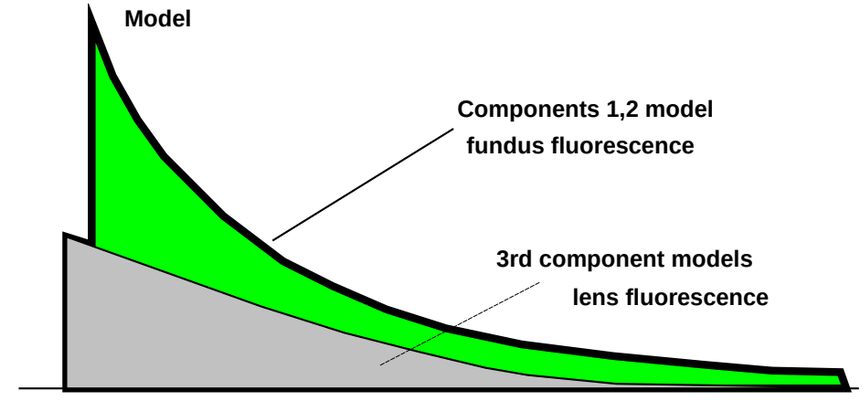
New Model Function Includes Early Arrival of Lens Fluorescence

3rd component models lens fluorescence



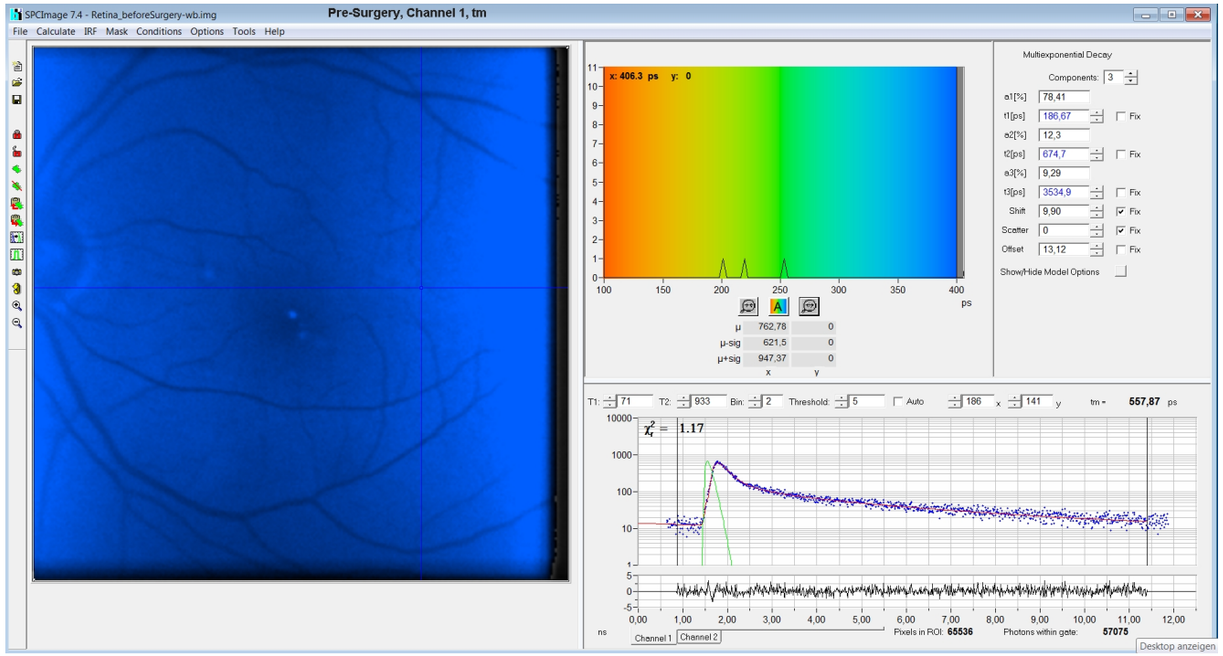
tm12 Extracts Fundus Lifetime from Decay Data

Lens fluorescence is rejected from lifetime images



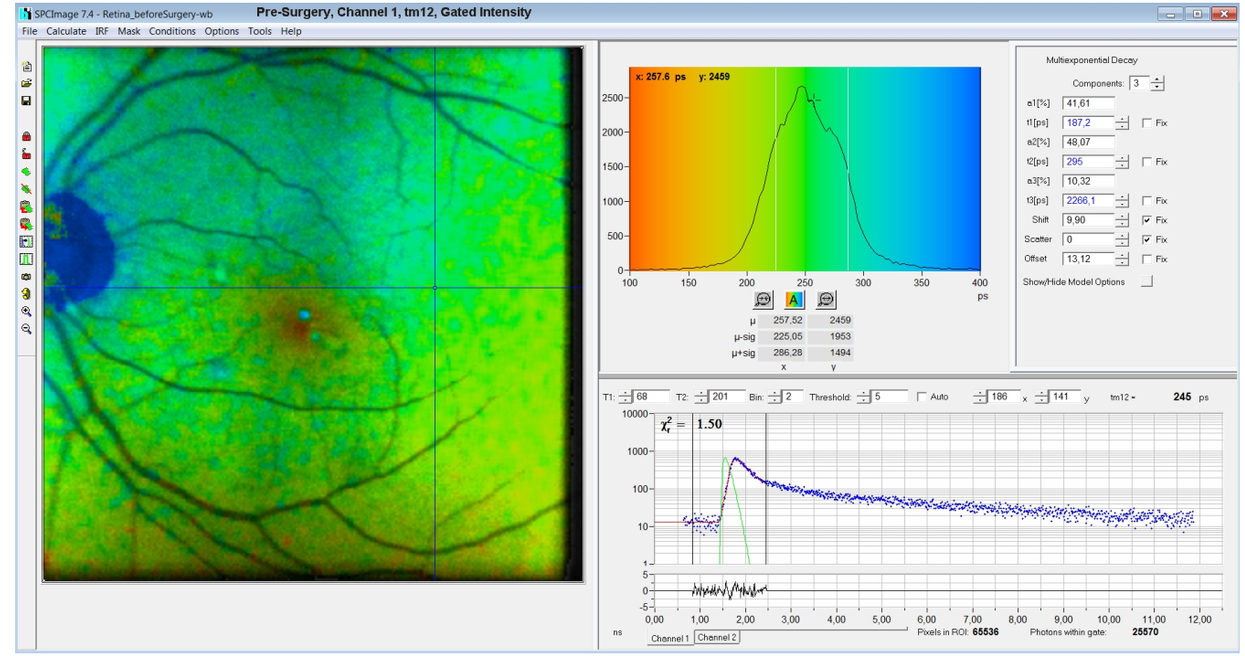
t_{m12} of Shifted-Component Modell Extracts Fundus Lifetimes

Cataract patient, tm, traditional model



Totally off range

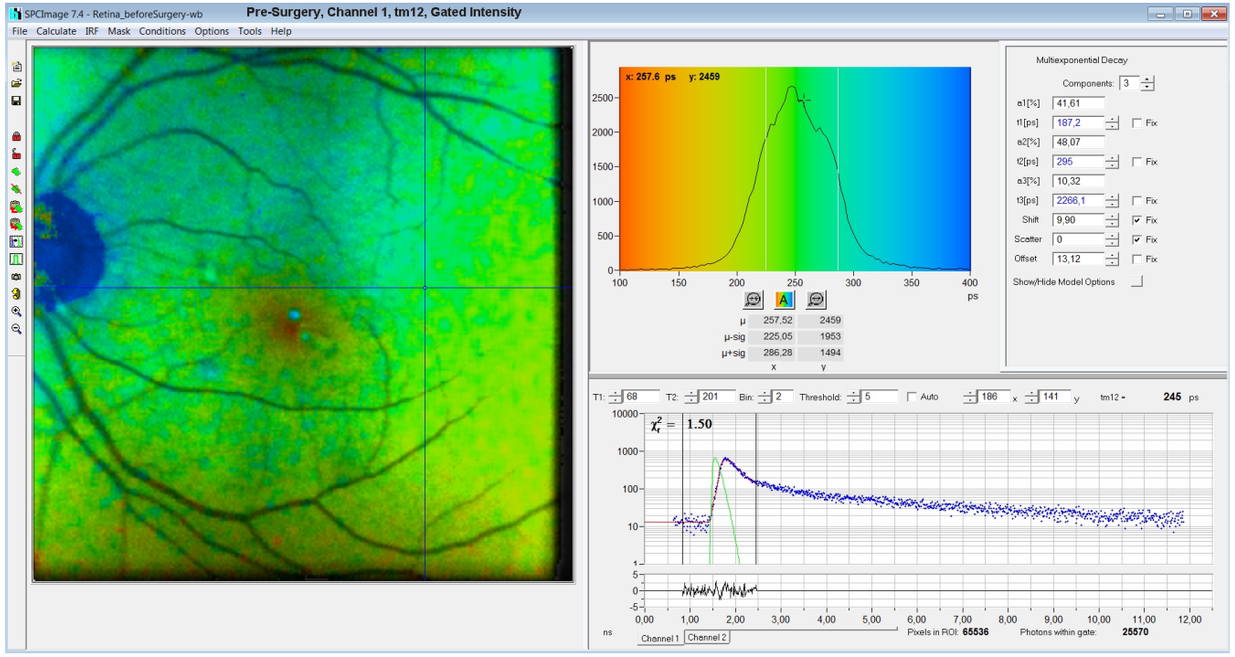
Cataract patient, tm12, shifted-component model



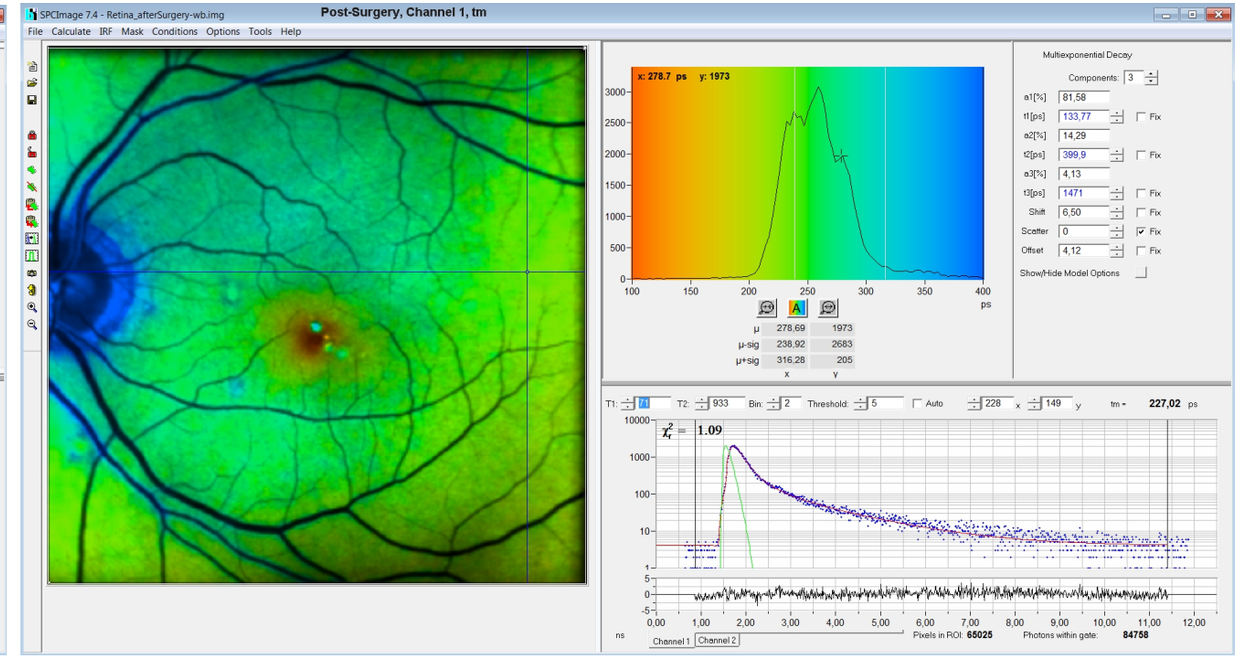
Reasonable fundus lifetimes

t_{m12} of Shifted-Component Modell Extracts Fundus Lifetimes

Cataract patient, tm12, shifted-component model



Cataract patient, post-surgery, tm, traditional model



Pre-surgery tm12 coincides with post-surgery tm

Data from Lydia Sauer. Thank you, Lydia!

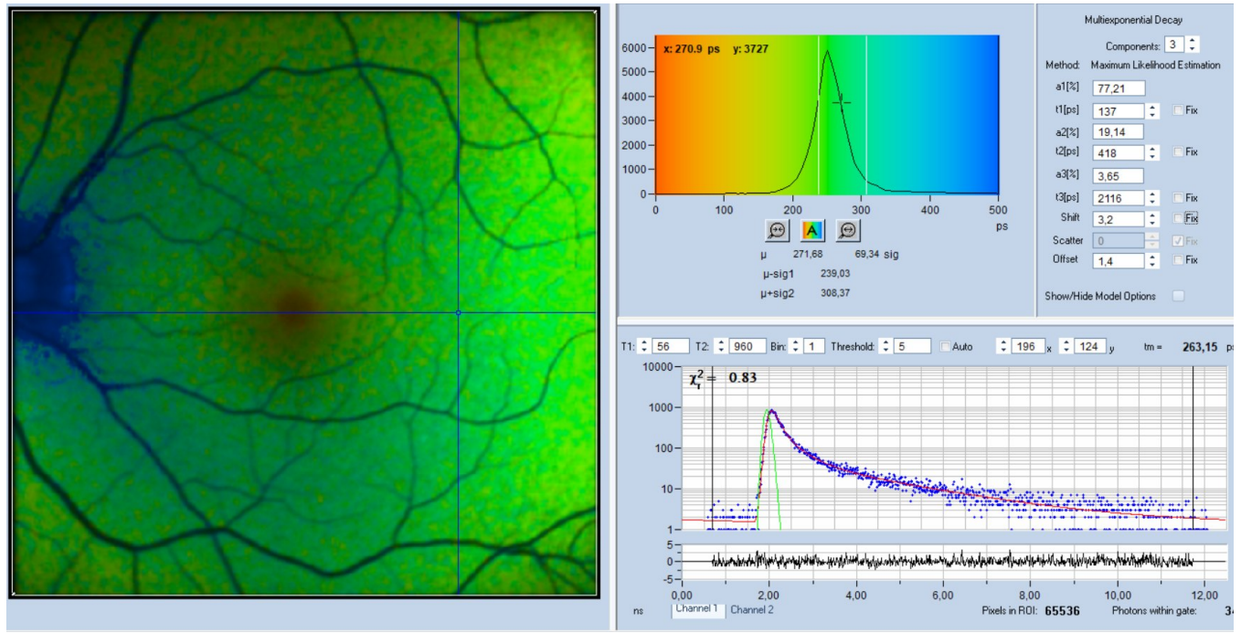
Shifted-Component Model, tm versus tm12

Healthy Patient, 25 Years Old

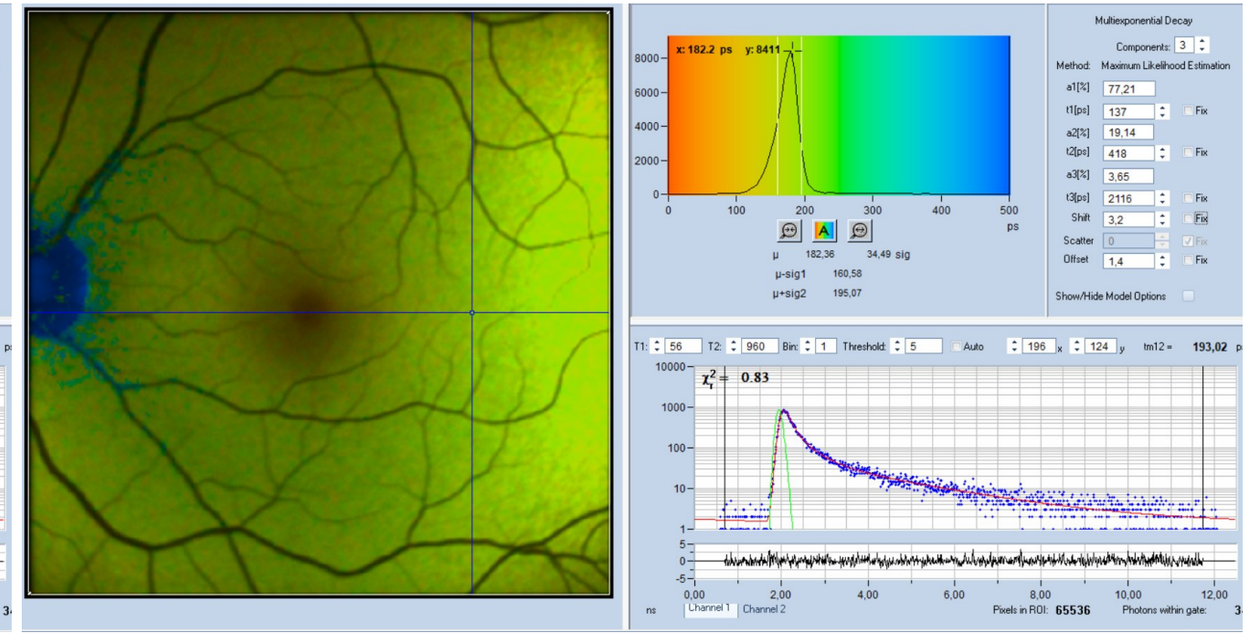
Lens fluorescence at this age is weak

Shifted-component model, syntethic IRF, shift parameter floating

tm



tm12



tm: Max of tm distribution: 250 ps

tm contains lens fluorescence

tm12: Max of tm distribution: 180 ps

tm12 does not contain lens fluorescence

Former Fundus-Lifetimes of healthy patients are 20-40% too long.

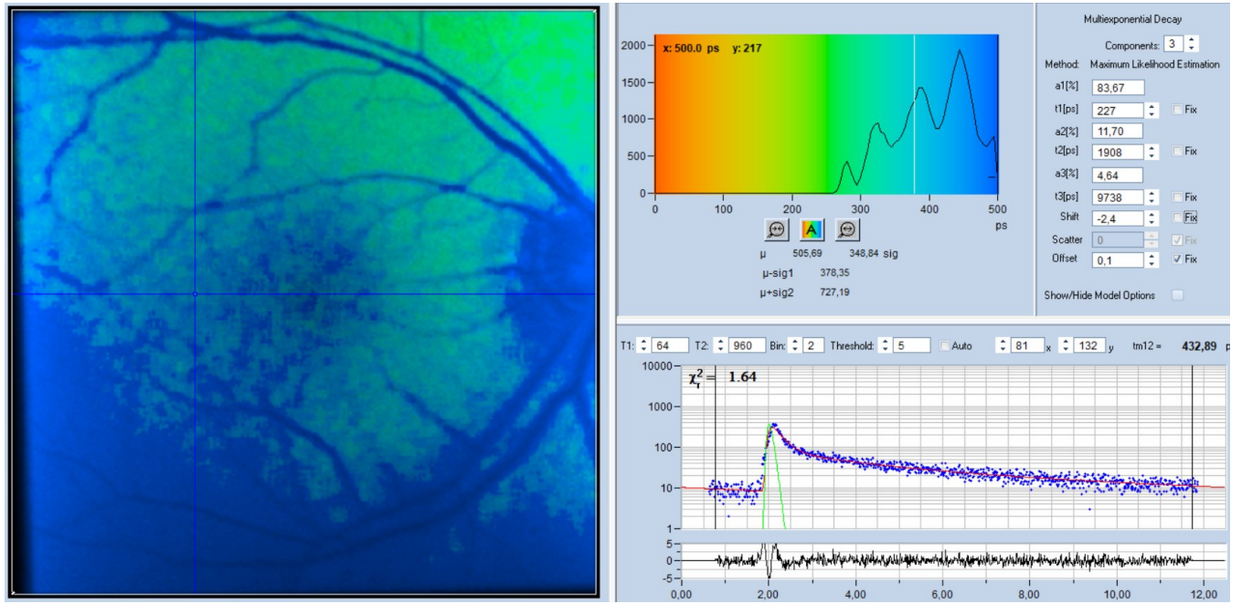
Observed inscrease of FLIO lifetime with age may in part be caused by increased amount of lens fluorescence

Re-evaluate old data with new model!

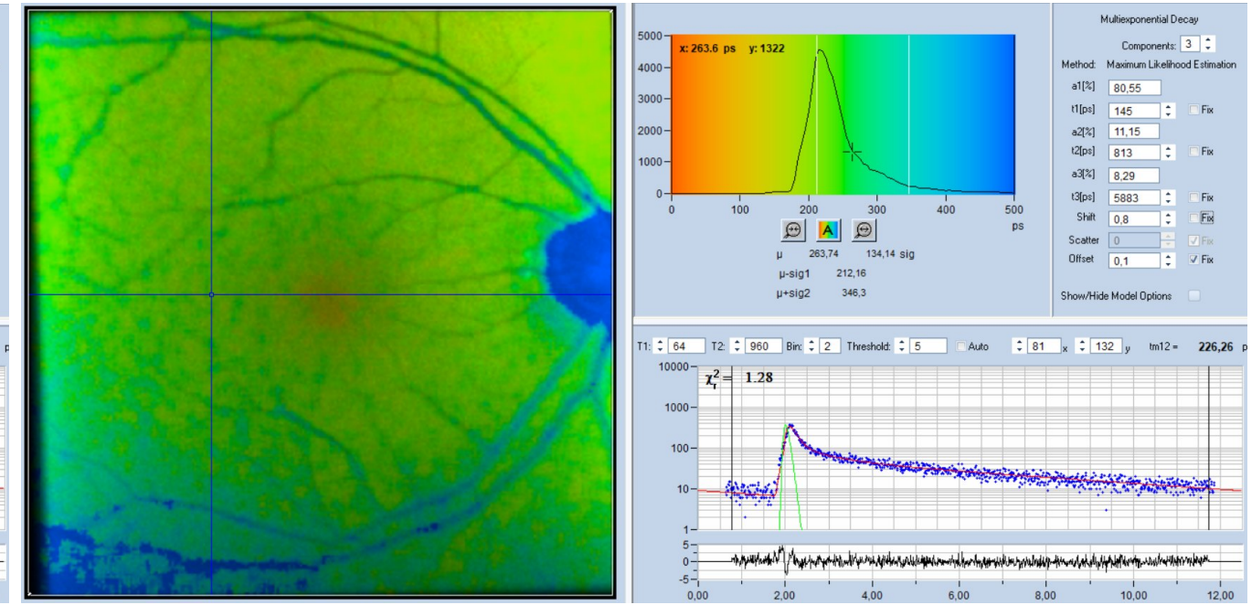
Conventional 3-exp Model vs. Shifted-Component Model

Cataract Patient, 70 Years, Position of IRF Floating

Conventional 3-exp. Model



Shifted Component Model, td3 = -150ps



Max of tm12 distribution: approx. 450 ps

Imperfect fit of rising edge

IRF position undefined and too early

Lifetime undefined and too large

Max of tm12 distribution: 220 ps

Good fit of rising edge

Correct IRF position

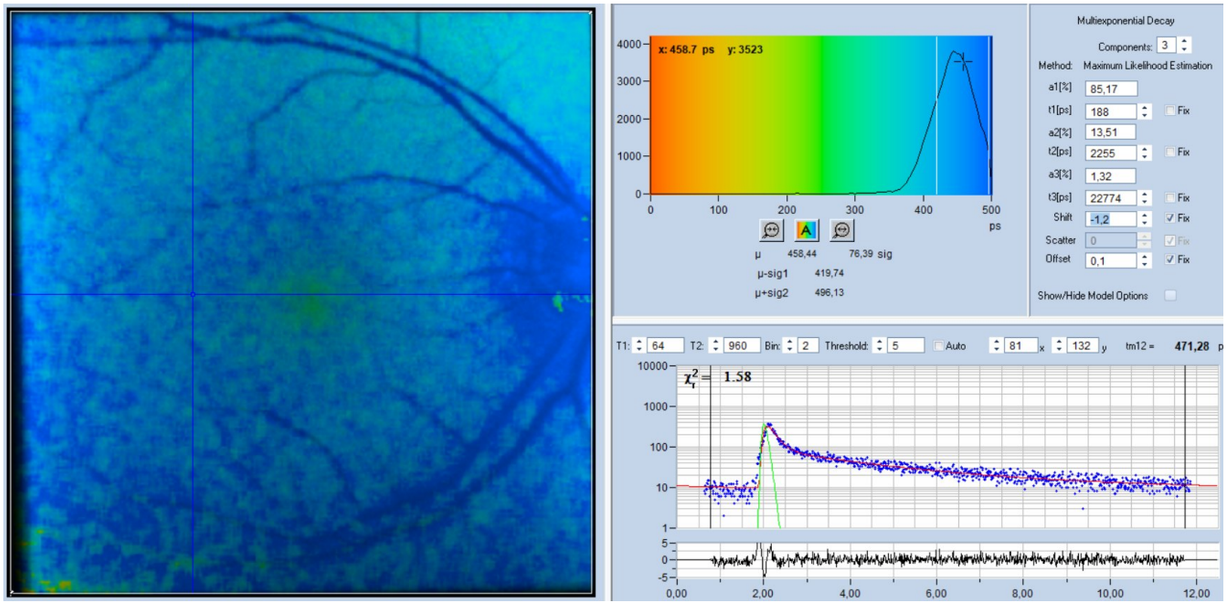
Lifetime correct

Former Fundus Lifetimes of cataract patients can be 400% too long!

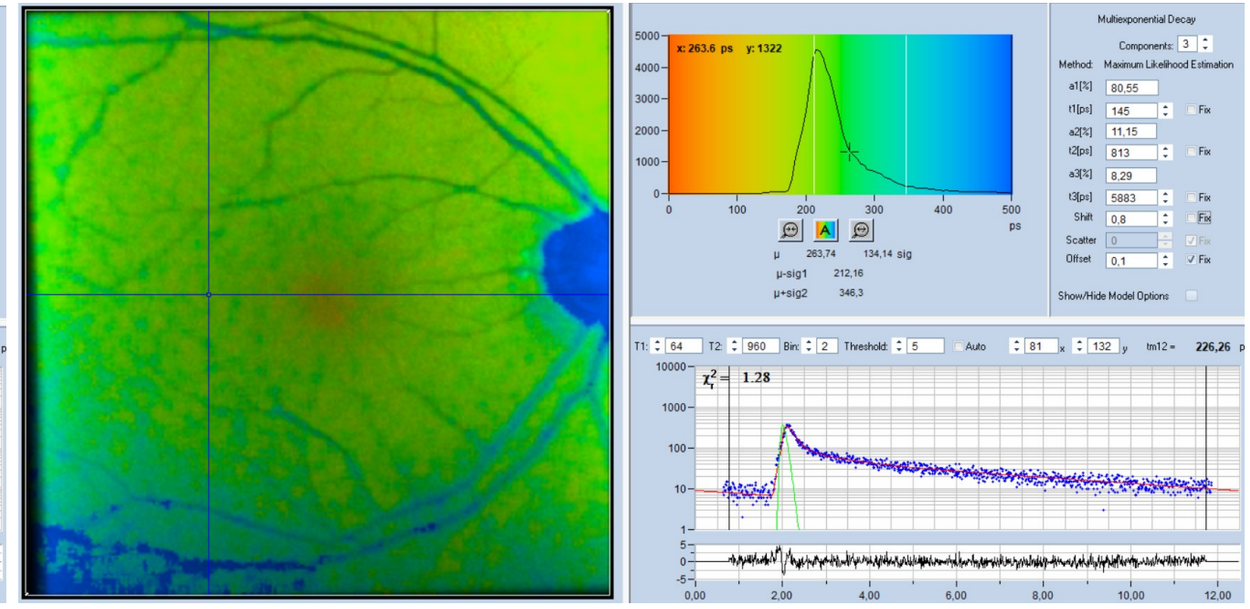
Conventional 3-exp Model vs. Shifted-Component Model

Cataract Patient, 70 Years

Conventional 3-exp. Model, fixed shift



Shifted Component Model, td3 = -150ps



Max of tm12 distribution: 460 ps

Imperfect fit of rising edge

IRF position too early

Lifetime too large

Max of tm12 distribution: 220 ps

Reasonable fit of rising edge

Correct IRF position

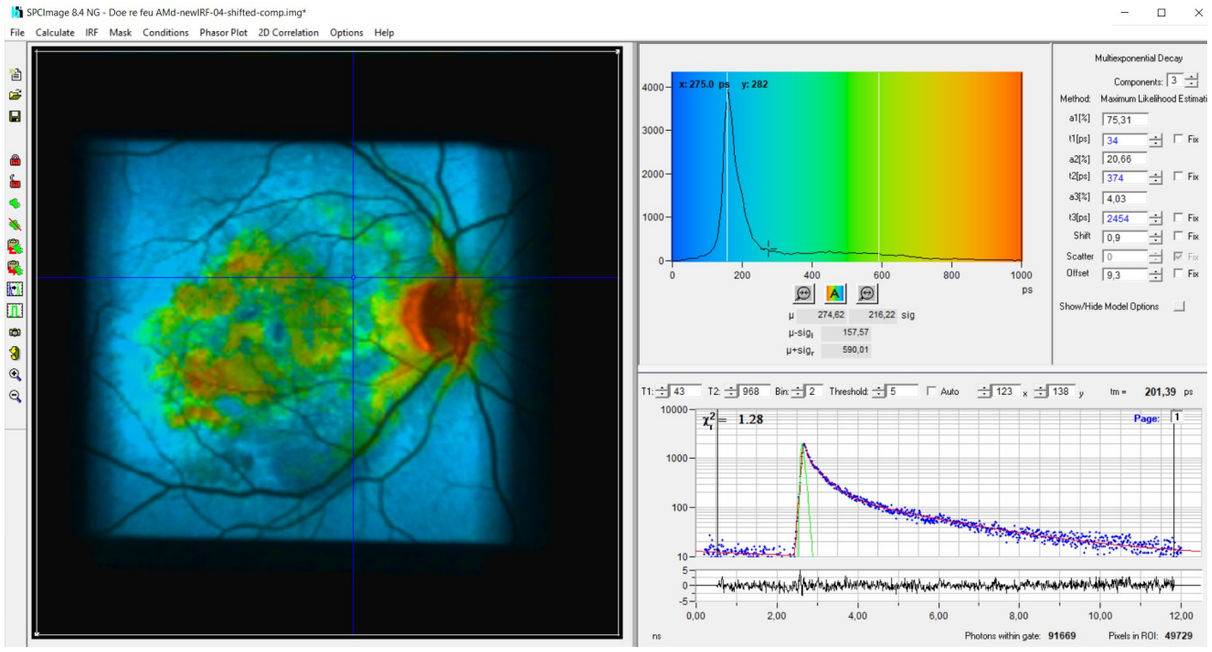
Lifetime correct

Former Fundus Lifetimes of cataract patients can be 400% too long!

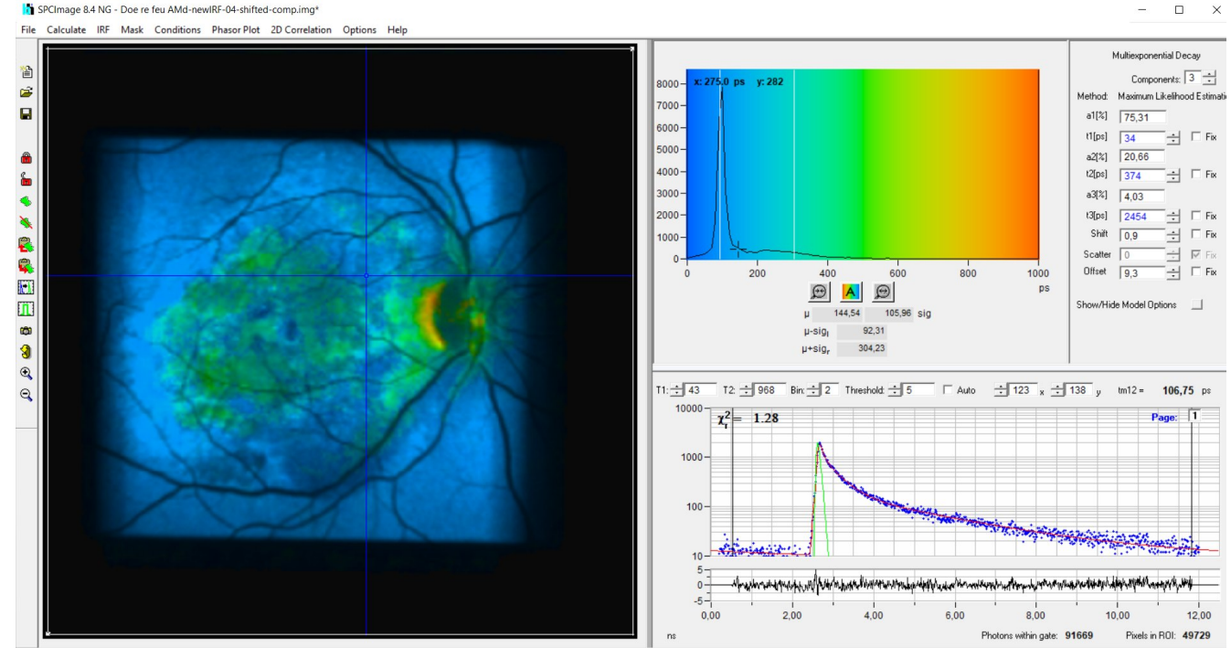
New Analysis Procedures Work on Old Data

AMD Patient, Data from Dietrich Schweitzer and Martin Hammer (2012)

tm Image
0 ... 1000 ps



tm12 Image
0 ... 1000 ps



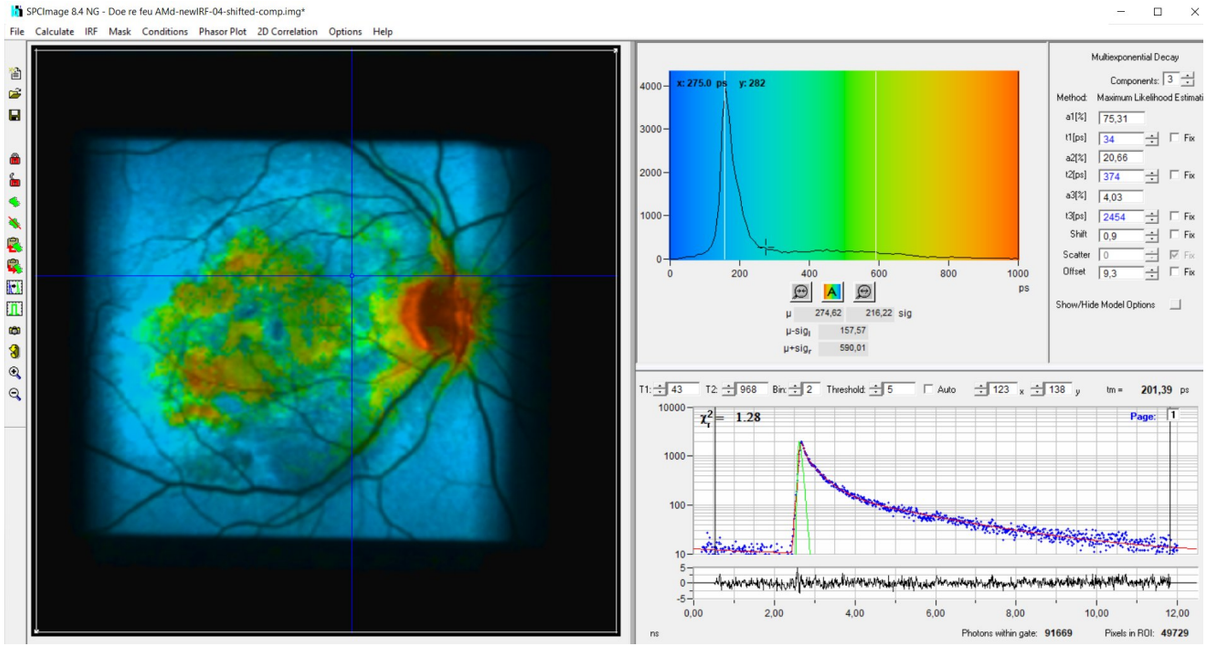
Average tm = 180 ps

Average tm12 = 100 ps

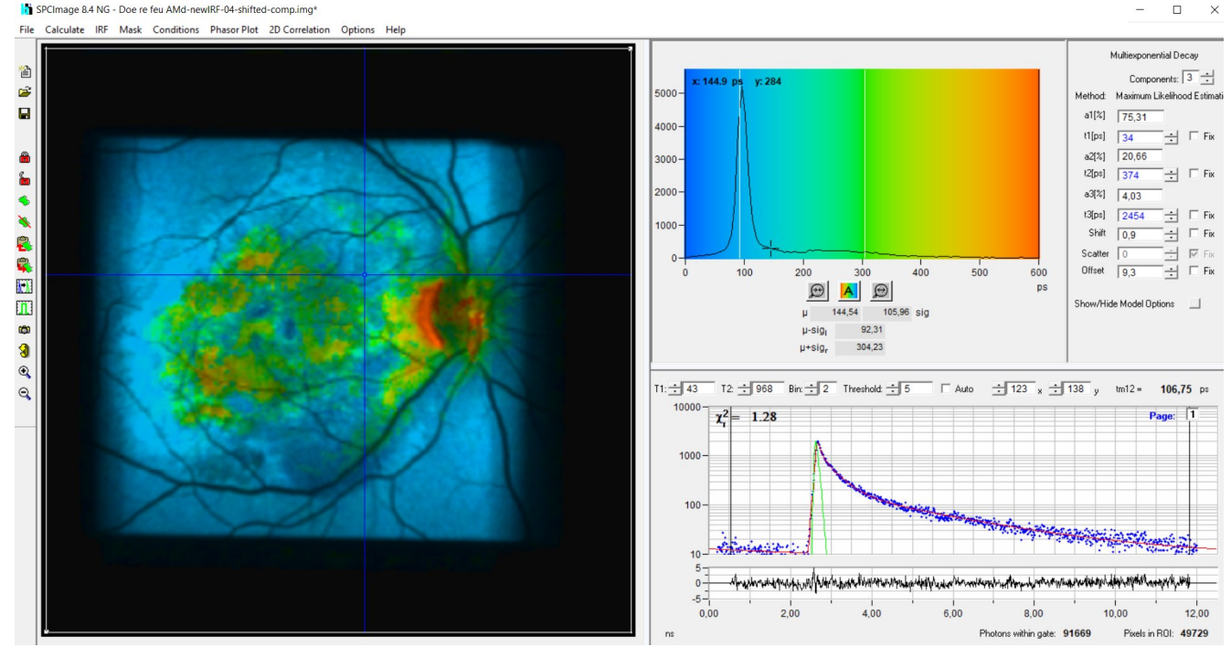
New Analysis Procedure Works on Old Data, Different Time Scales

AMD Patient, Data from Dietrich Schweitzer and Martin Hammer (2012)

tm Image
0 ... 1000 ps



tm12 Image
0 ... 600 ps

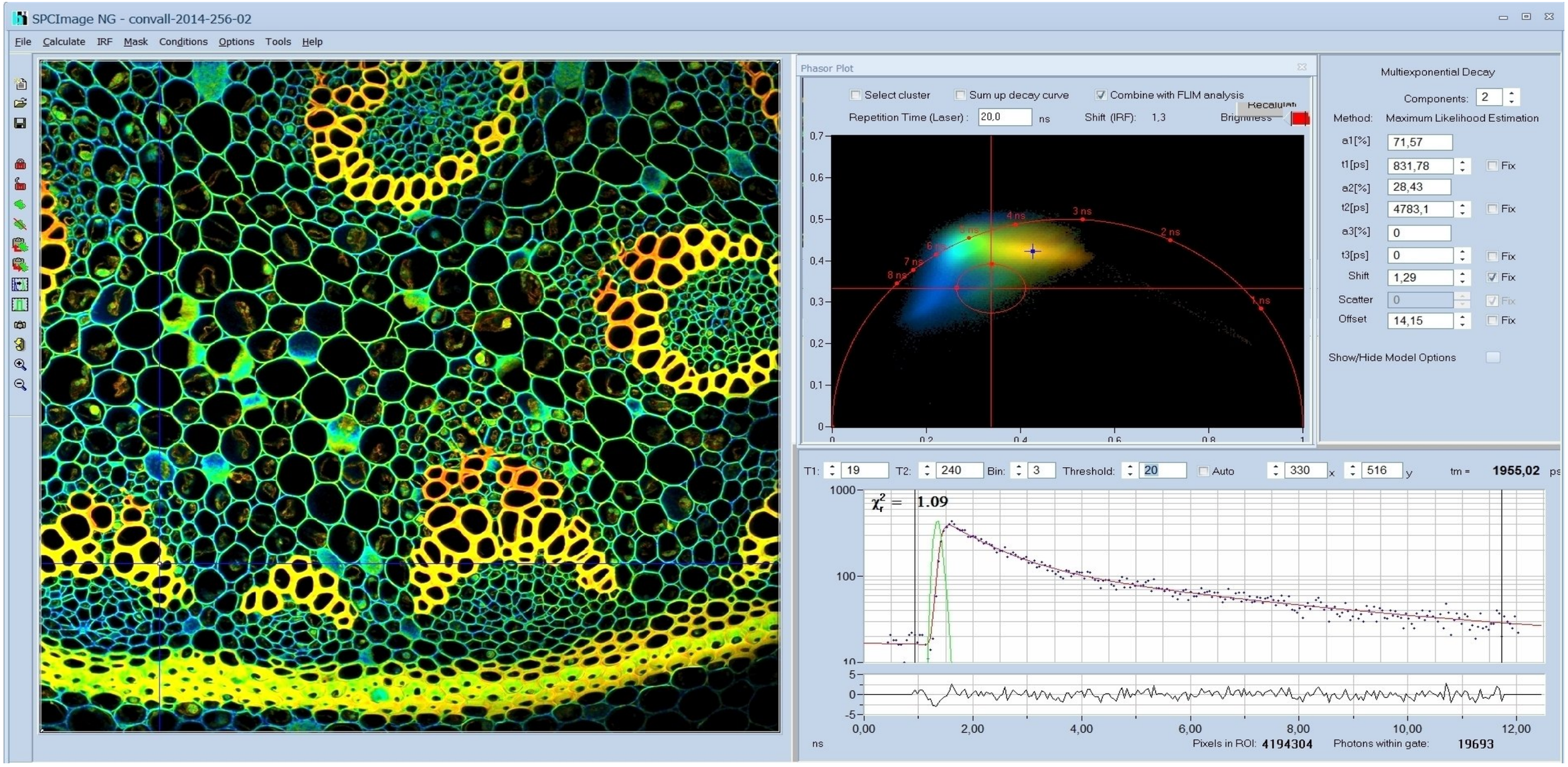


Average tm = 180 ps

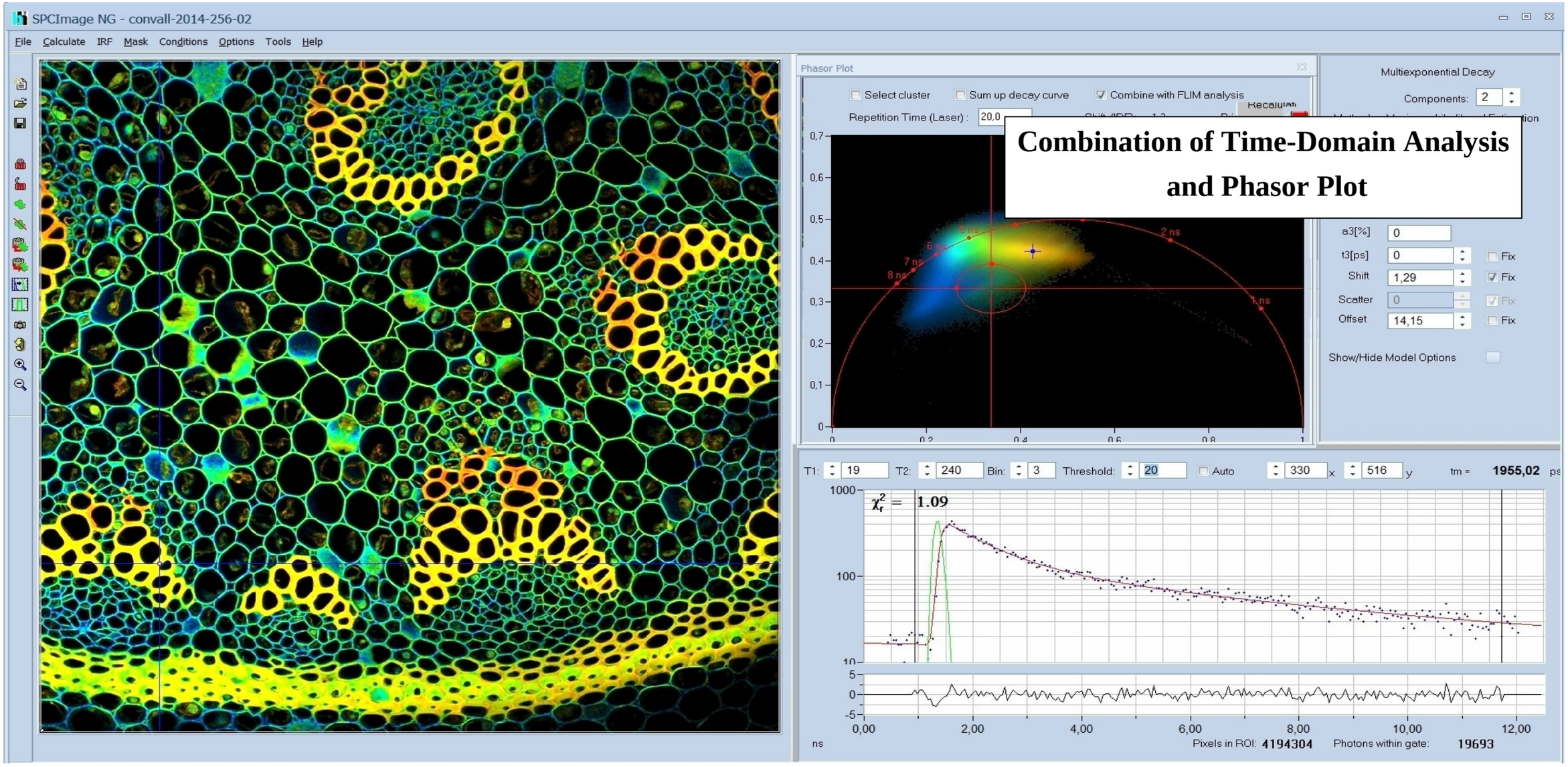
Average tm12 = 100 ps

Questions?

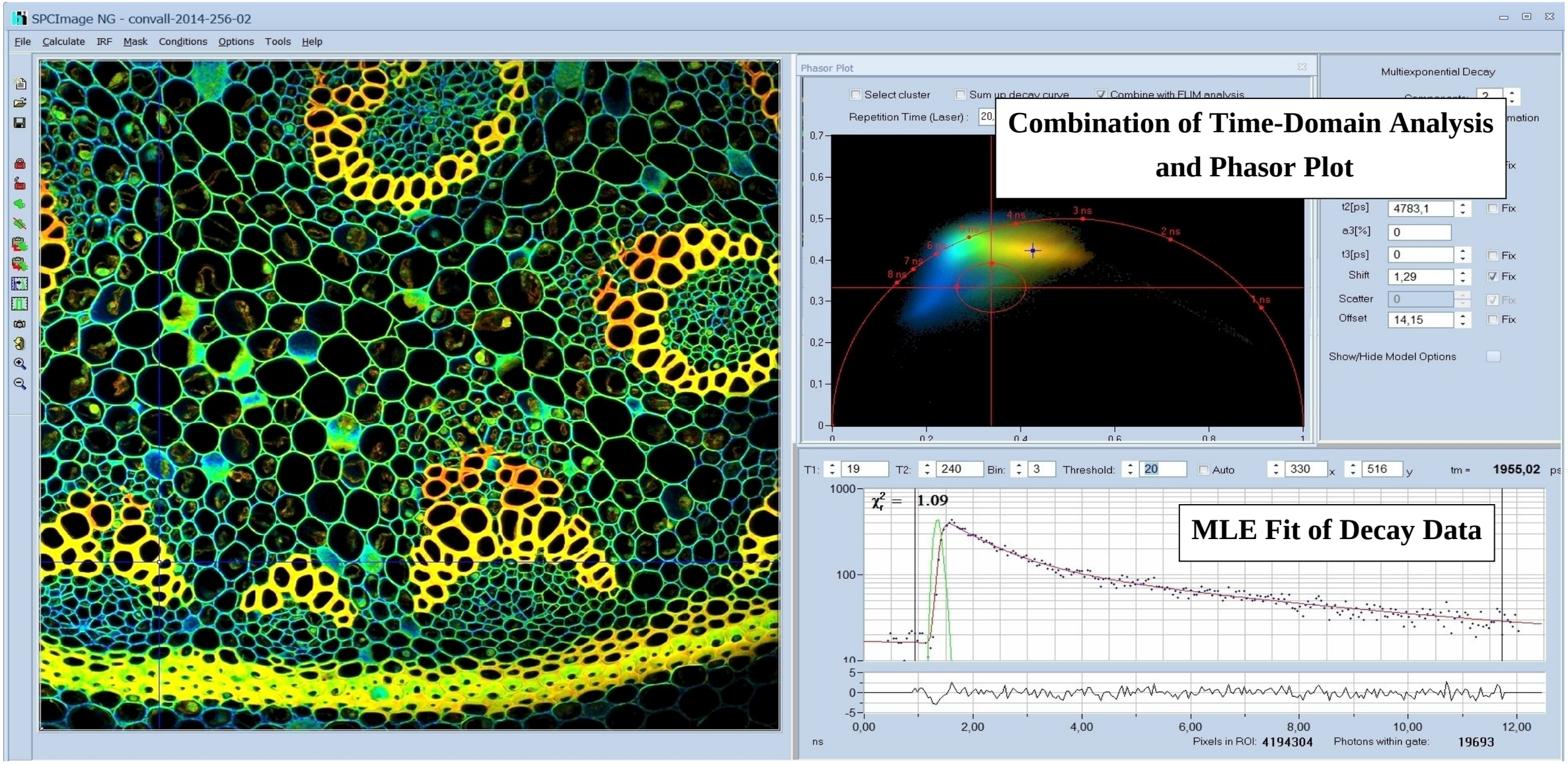
SPCImage NG Data Analysis



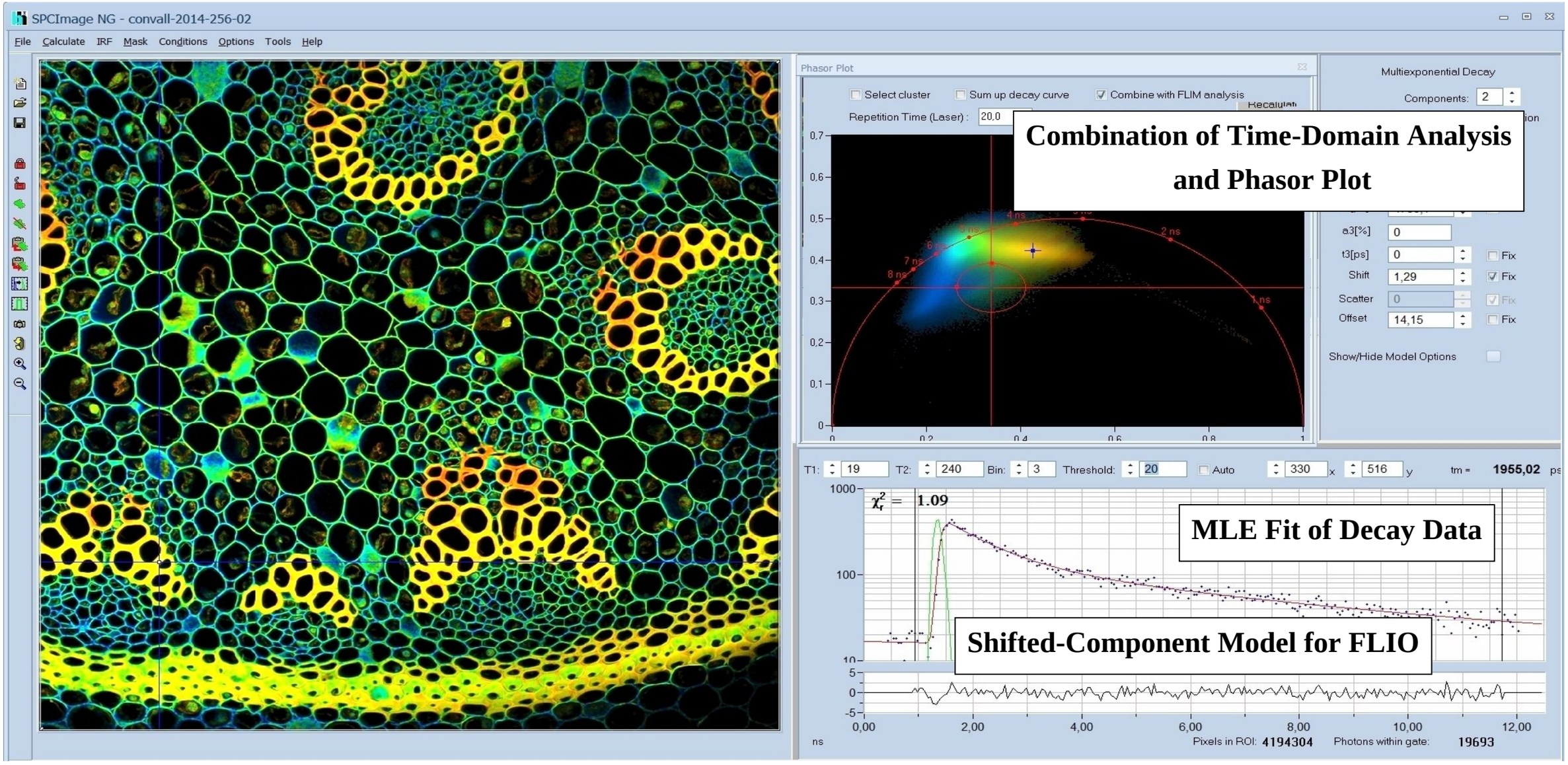
SPCImage NG Data Analysis



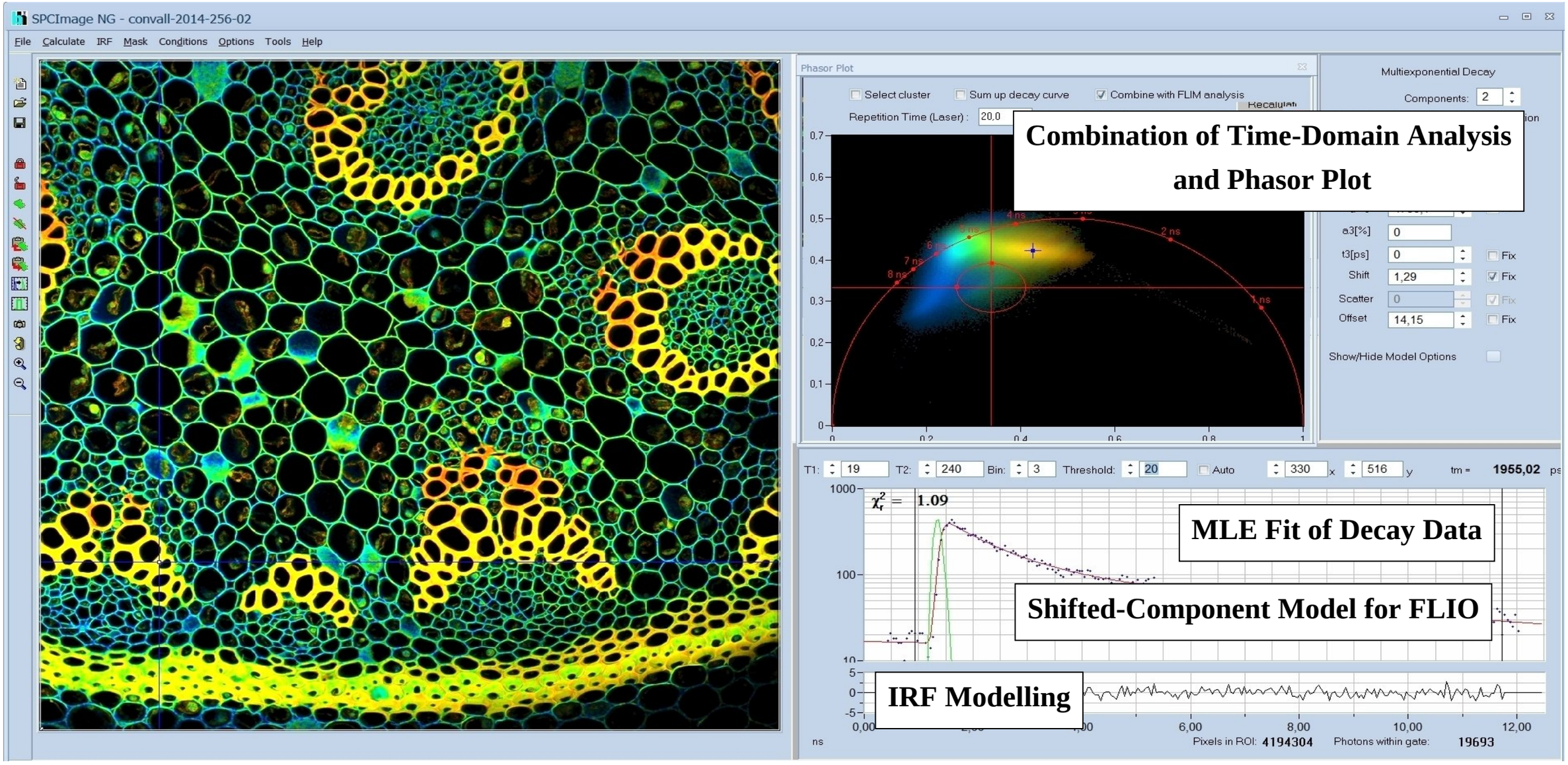
SPCImage NG Data Analysis



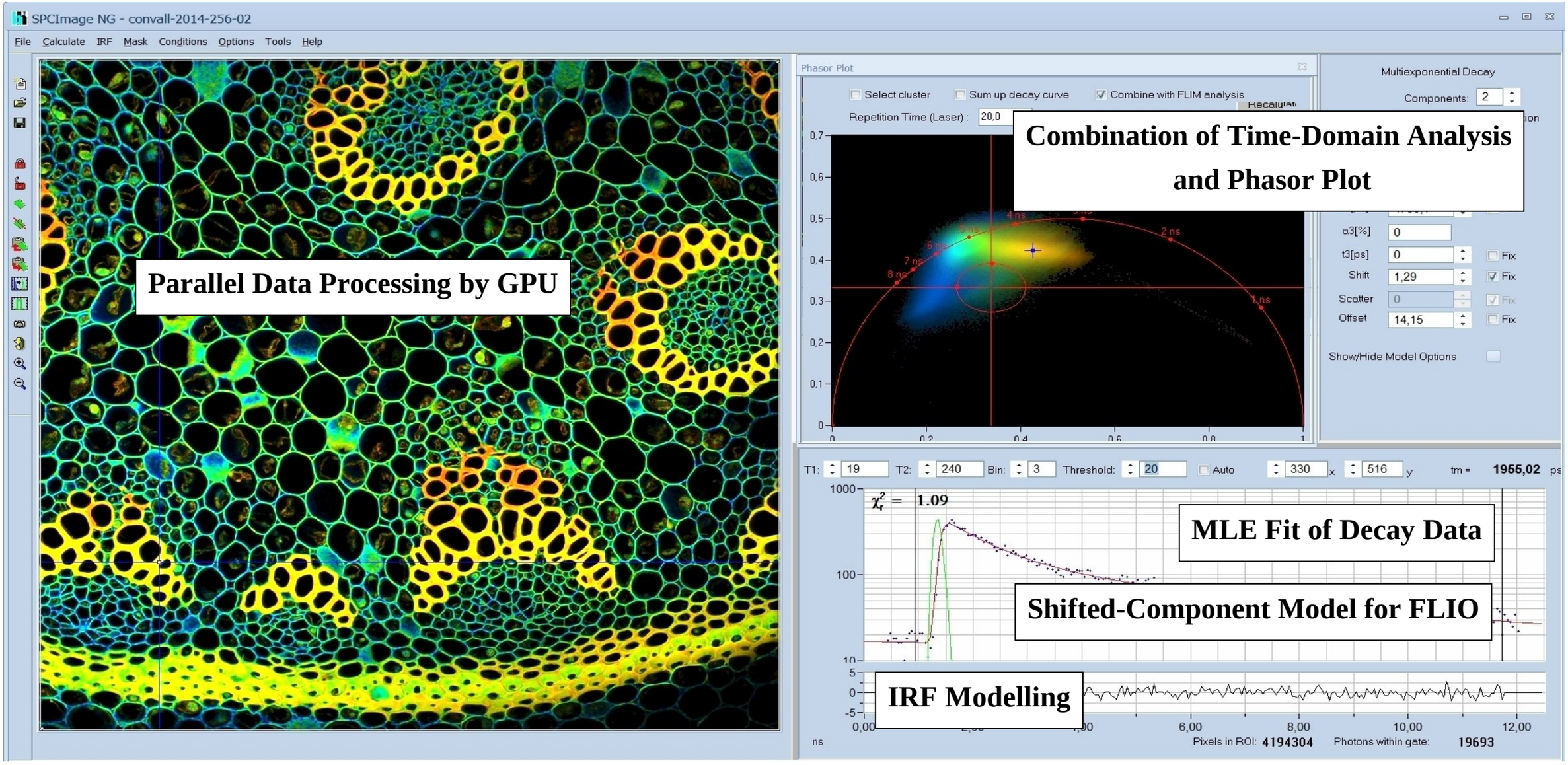
SPCImage NG Data Analysis



SPCImage NG Data Analysis

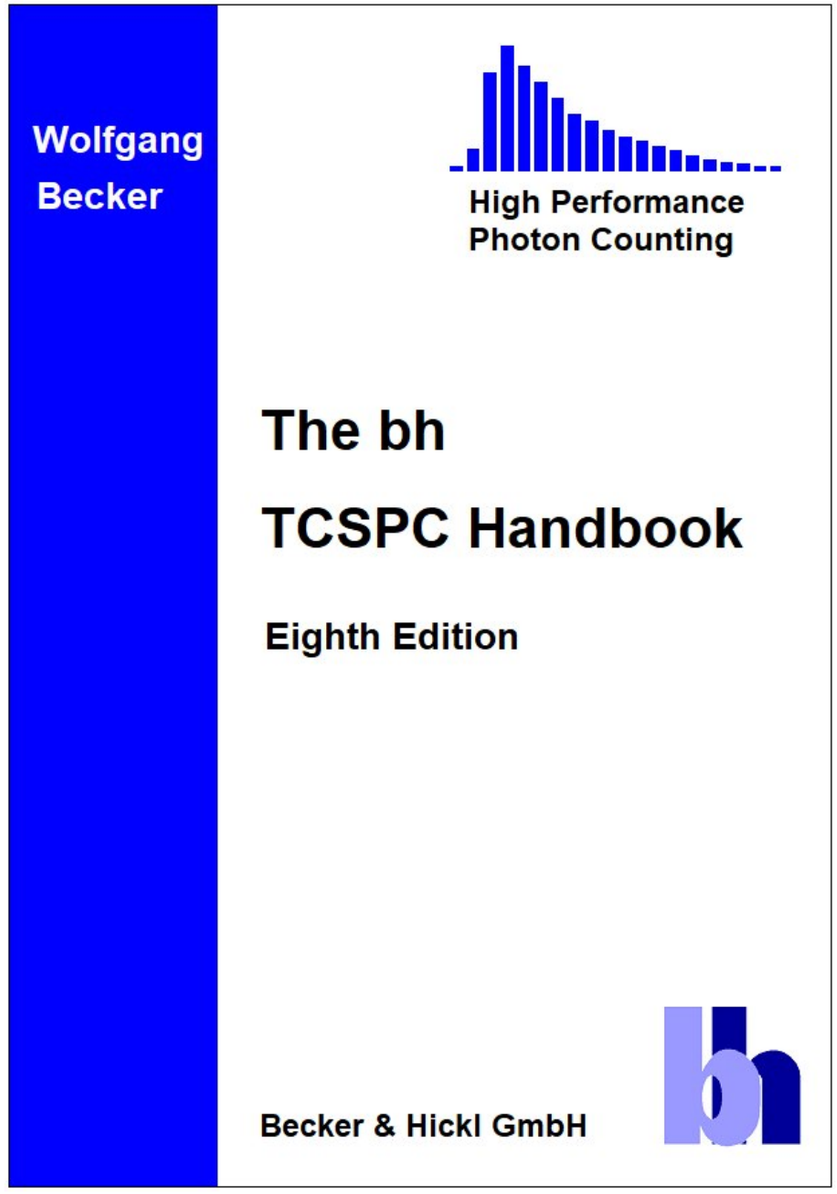


SPCImage NG Data Analysis



Please See 'bh TCSPC Handbook', Chapter 'SPCImage NG Data Analysis Software'

Download from https://www.becker-hickl.com or contact bh for printed copy



SPCImage NG Data Analysis Software¶

SPCImage NG is a new generation of bh's TCSPC-FLIM data analysis software. It combines time-domain and frequency-domain analysis, uses a maximum-likelihood algorithm to calculate the parameters of the decay functions in the individual pixels, and accelerates the analysis procedure by GPU processing. 1D and 2D parameter histograms are available to display the distribution of the decay parameters over the pixels of the image or over selectable ROIs. Image segmentation can be performed via a phasor plot, and pixels with similar signature can be combined for high-accuracy time-domain analysis. SPCImage NG provides decay models with one, two, or three exponential components, incomplete-decay models, and shifted-component models. Another important feature is advanced IRF modelling, making it unnecessary to record IRFs for the individual FLIM data sets.¶

Overview¶

Images of Decay Parameters¶

SPCImage NG produces images of fluorescence lifetimes and other fluorescence decay parameters from TCSPC FLIM data. It runs an iterative fit and de-convolution procedure on the decay data of the individual pixels of the FLIM images. In the simplest case, the result is the lifetime of the decay functions in the individual pixels. For complex decay functions the fit procedure delivers the lifetimes and amplitudes of the decay components. SPCImage then creates colour-coded images of the amplitude- or intensity-weighted lifetimes in the pixels, images of the lifetimes or amplitudes of the decay components, images of lifetime or amplitude ratios, and images of other combinations of decay parameters, such as FRET intensities, FRET distances, bound-unbound ratios, or the fluorescence-lifetime redox ratio, FLIRR. A few examples are shown in Fig. 1044 through Fig. 1047.¶

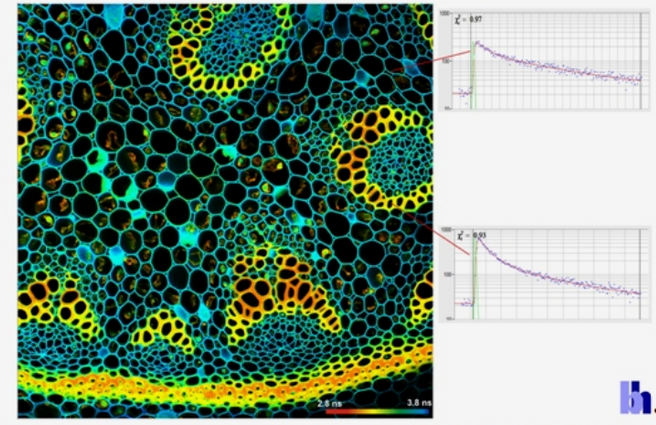
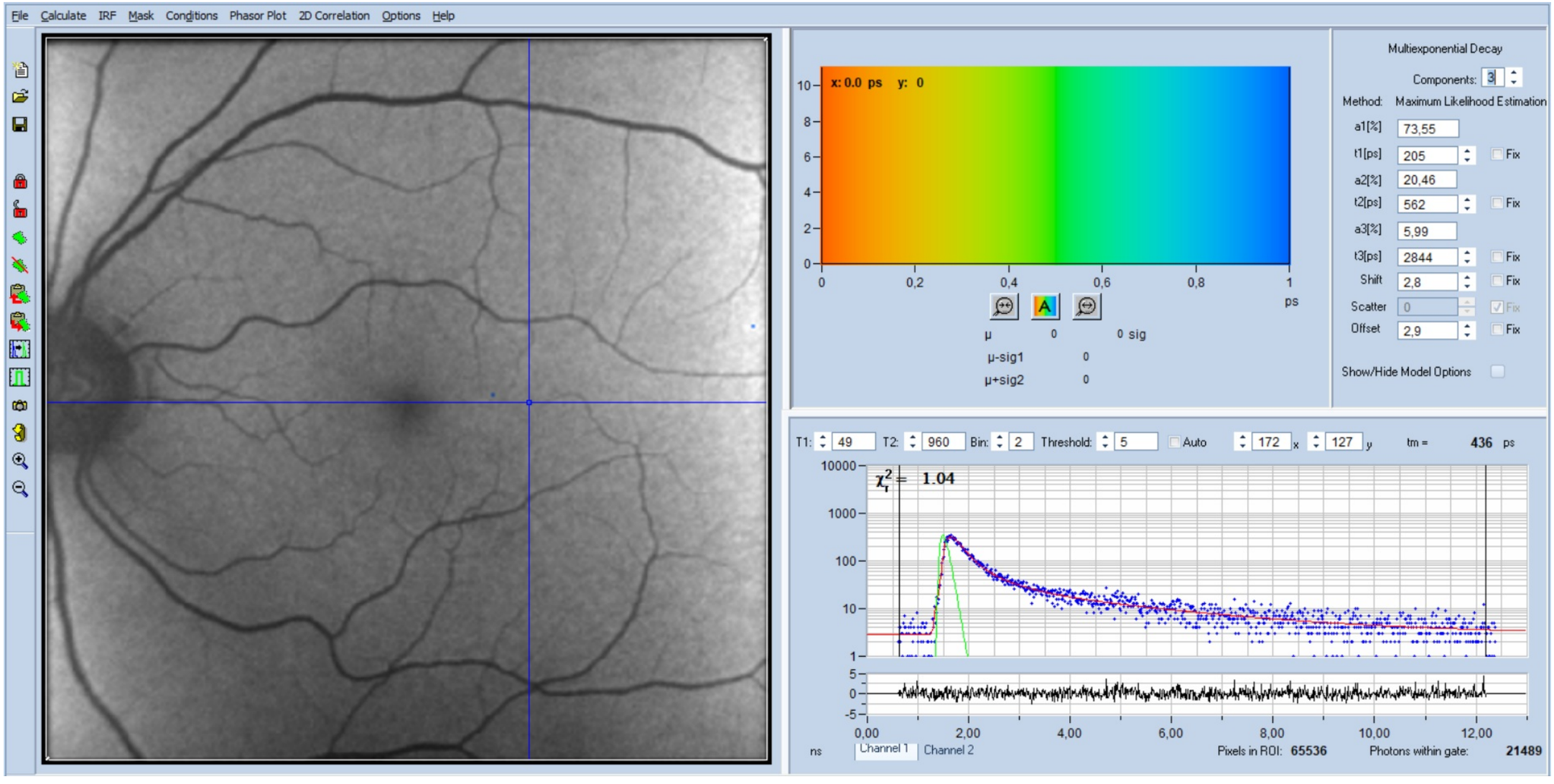
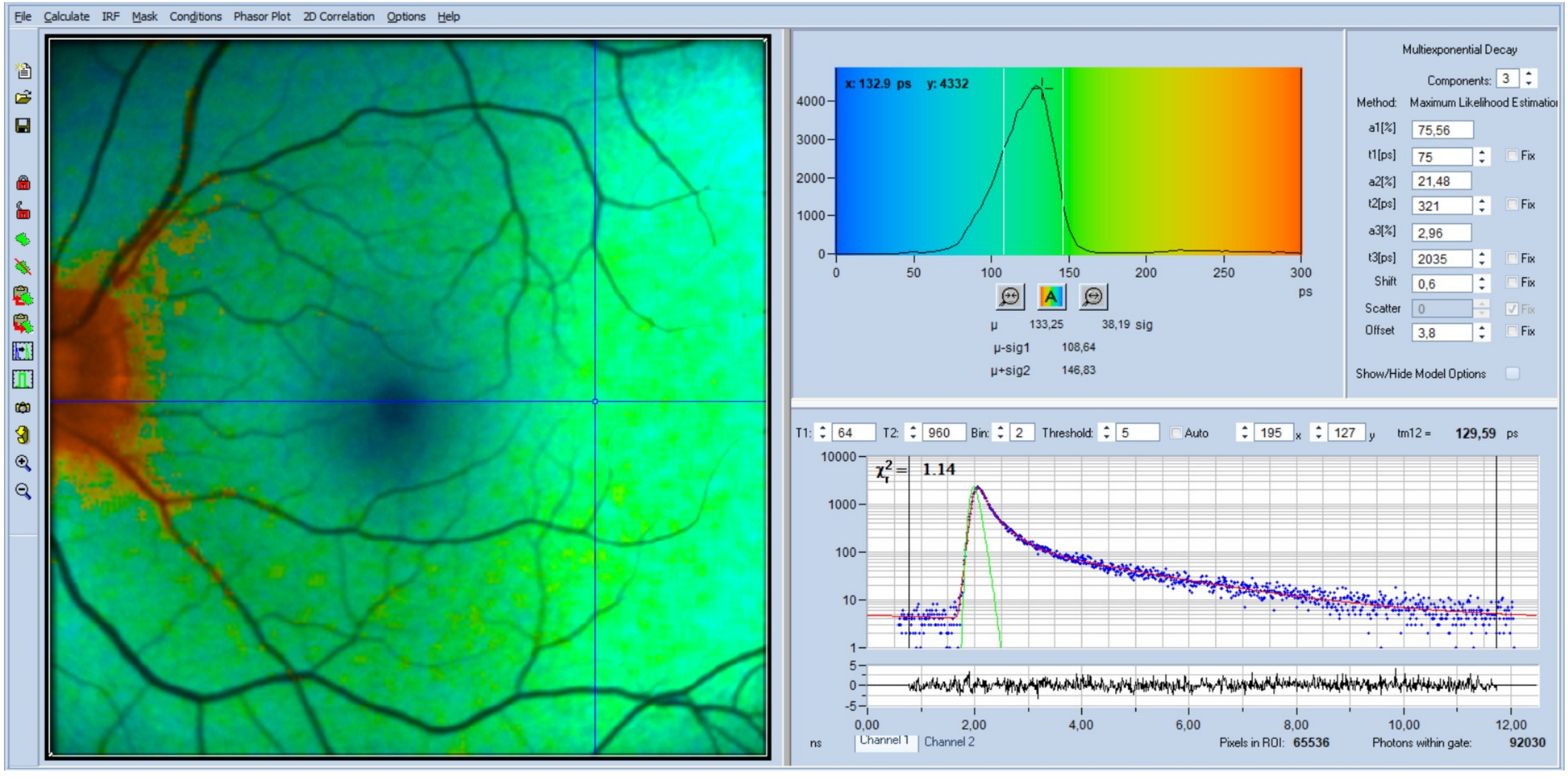


Fig. 1044: Image of the amplitude-weighted lifetime, τm, of a double-exponential decay. Right: Fluorescence decay curves in selected pixels.¶

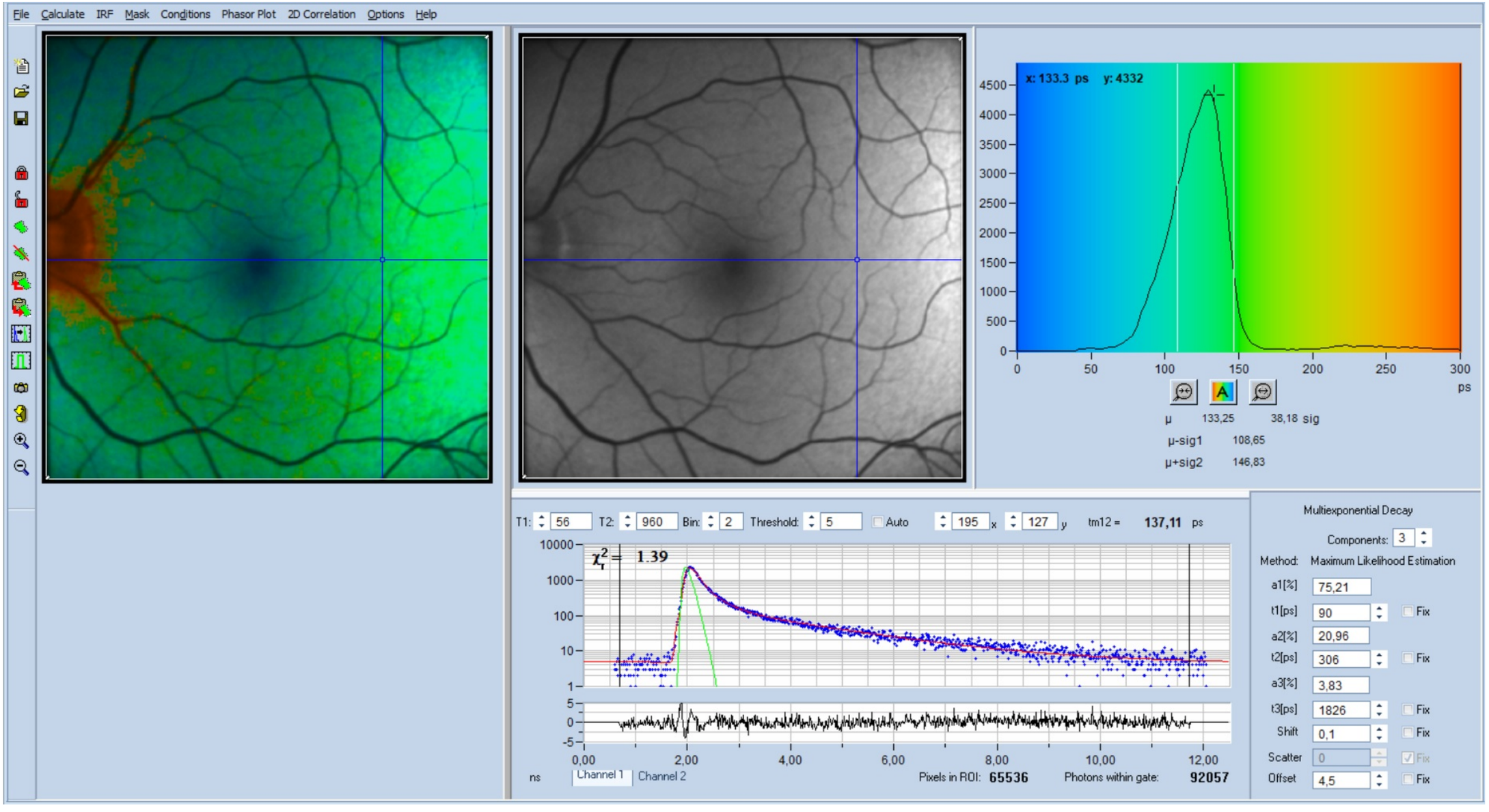
Different Appearances



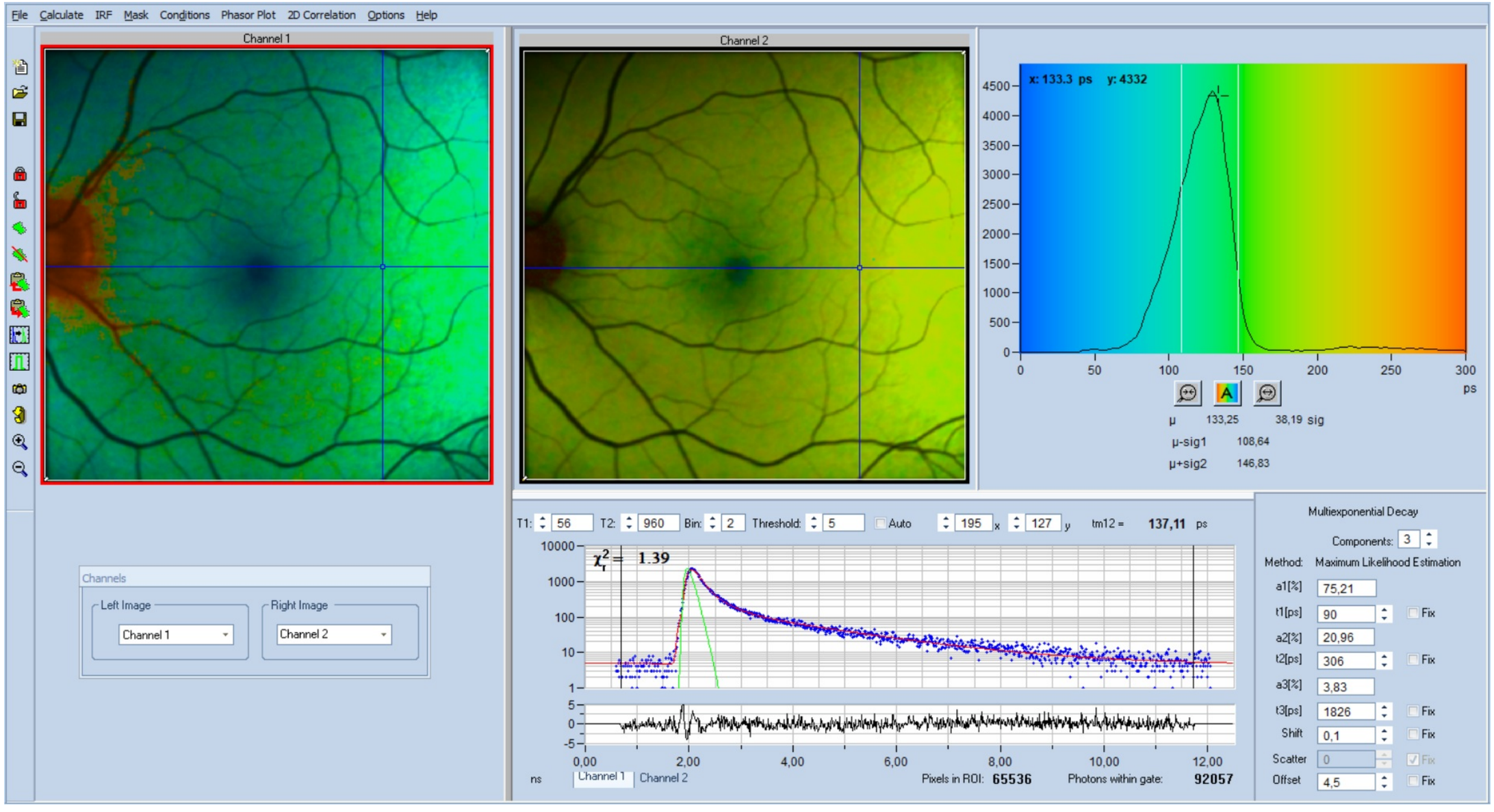
Different Appearances



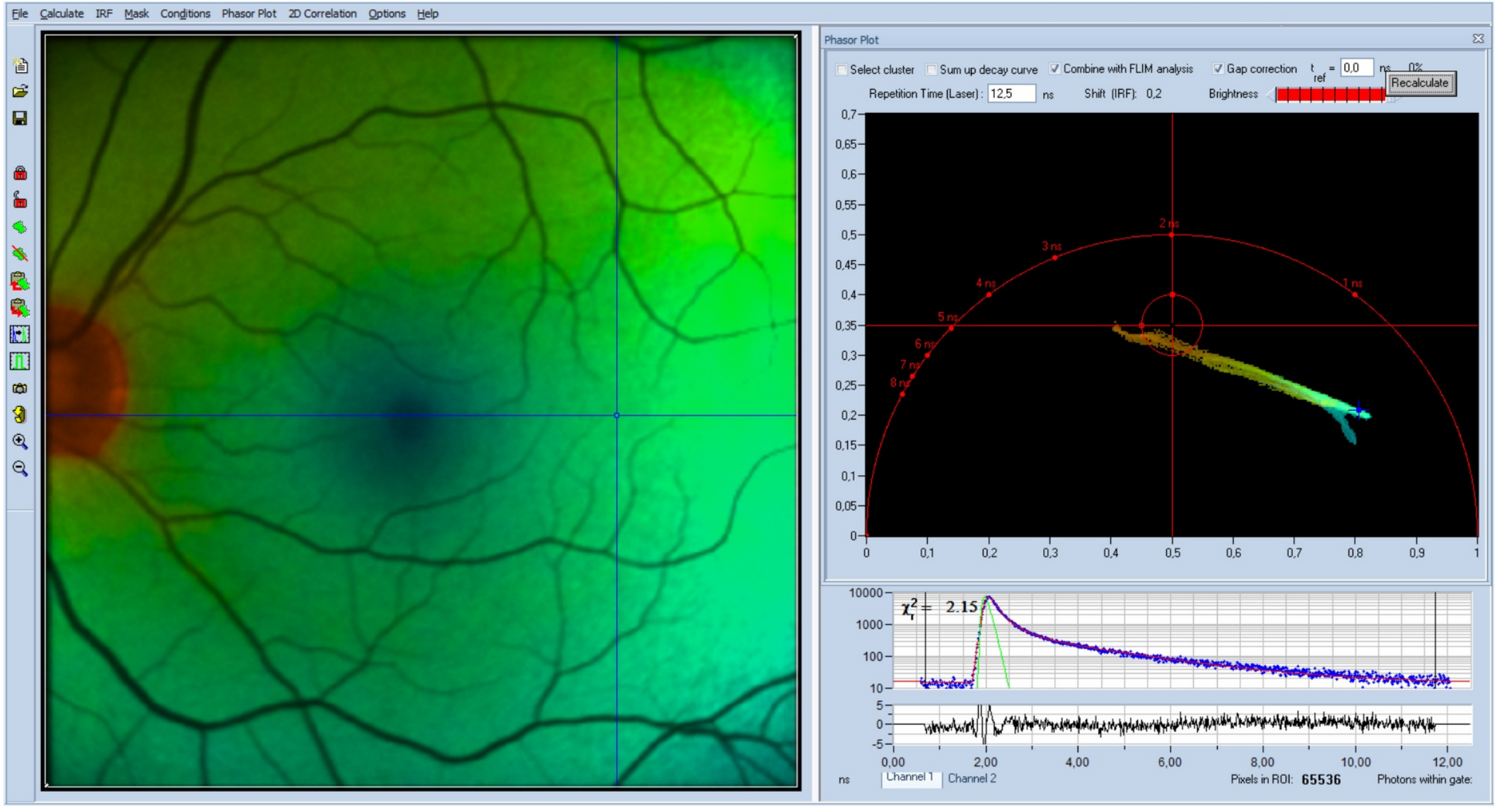
Different Appearances



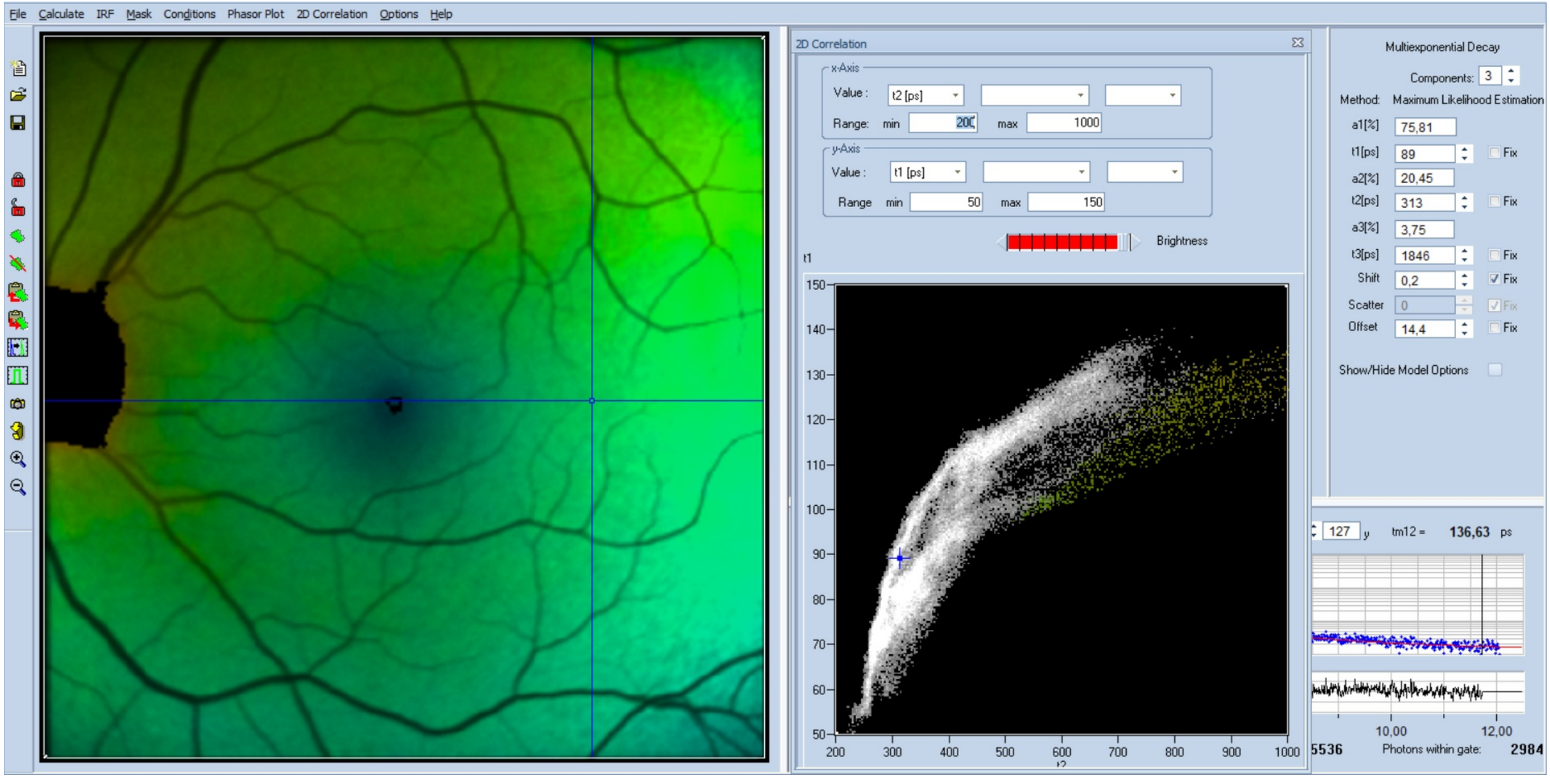
Different Appearances



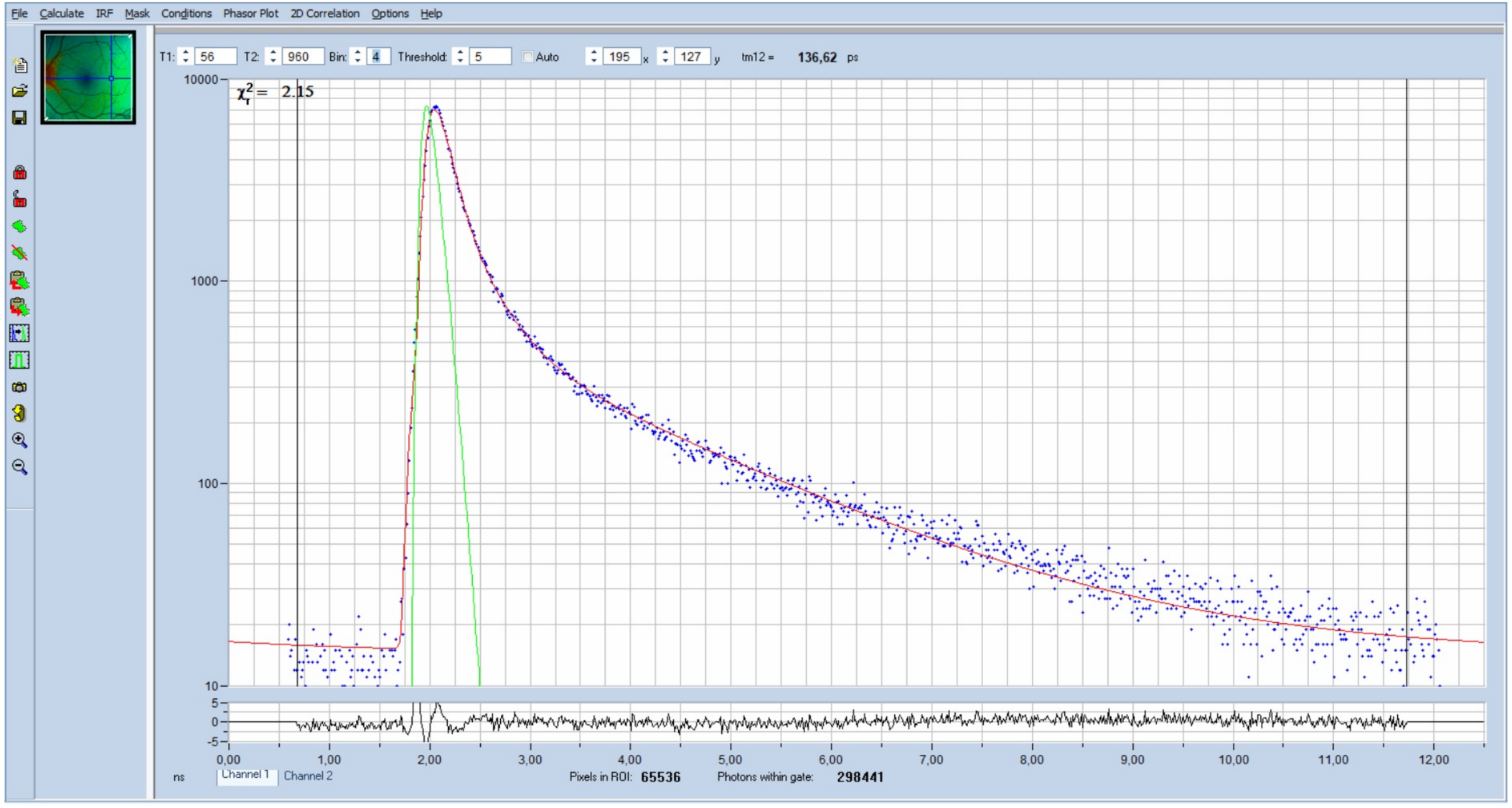
Different Appearances



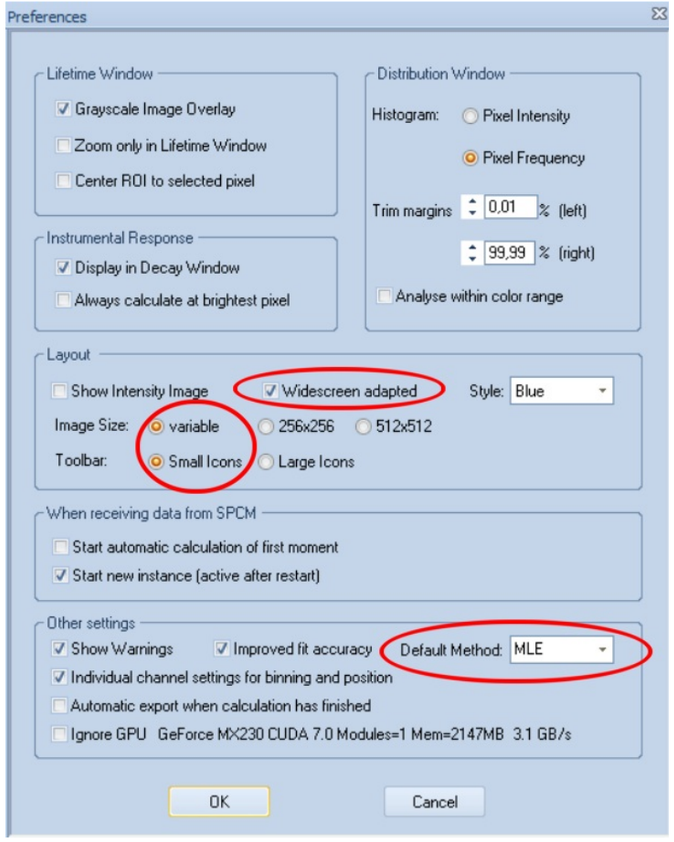
Different Appearances

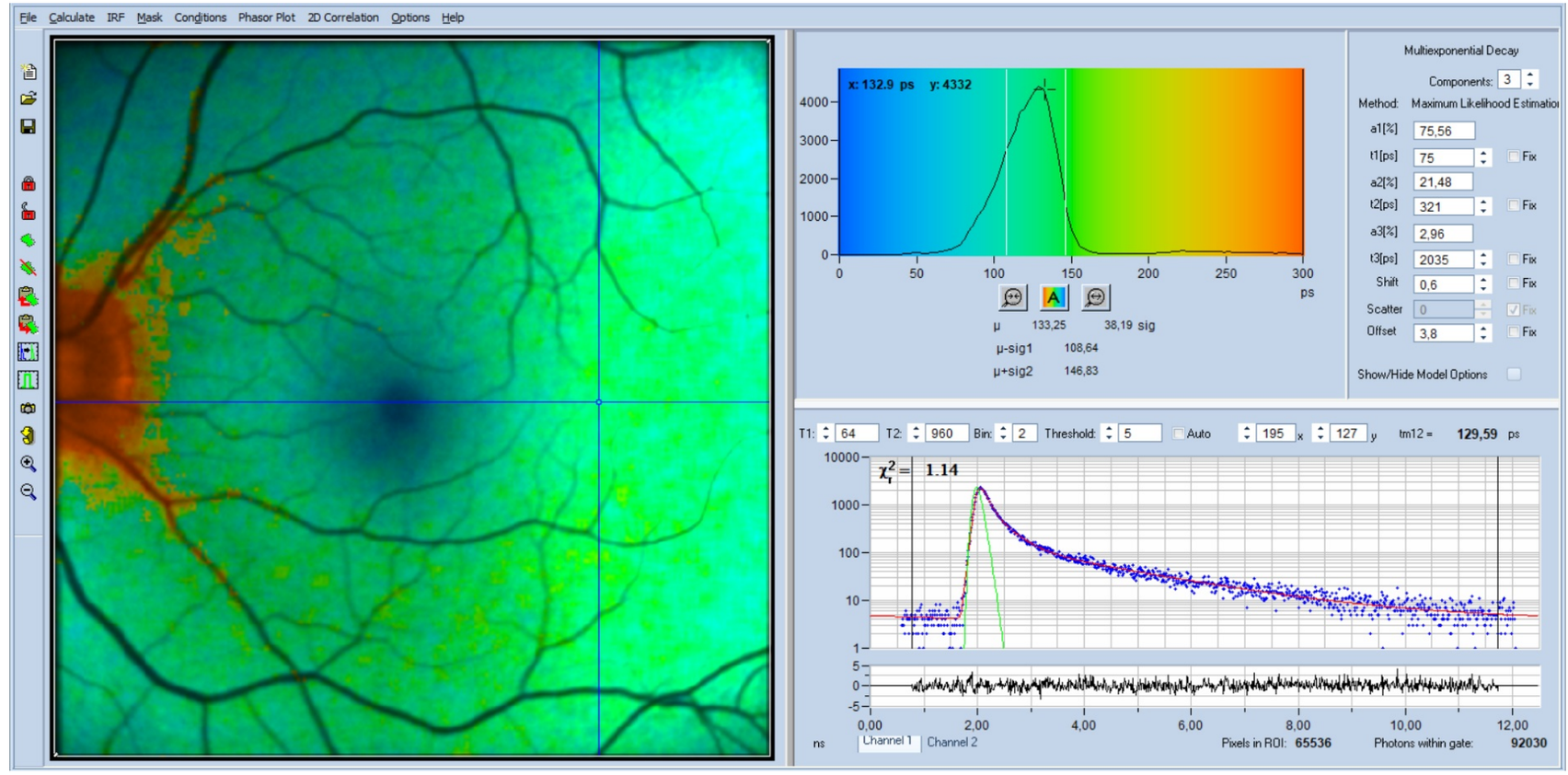


Different Appearances

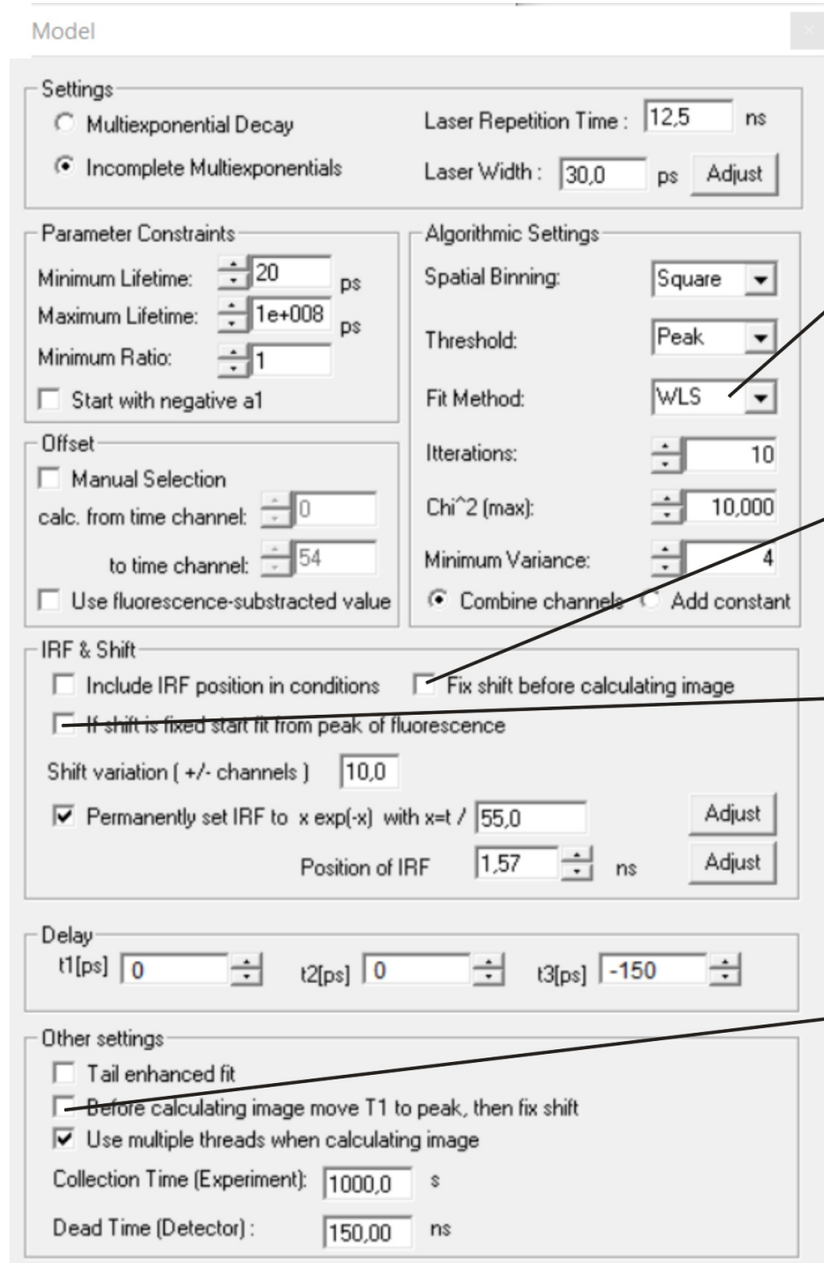


Recommended 'Preferences' Setup for FLIO





Settings NOT to be used for FLIO analysis



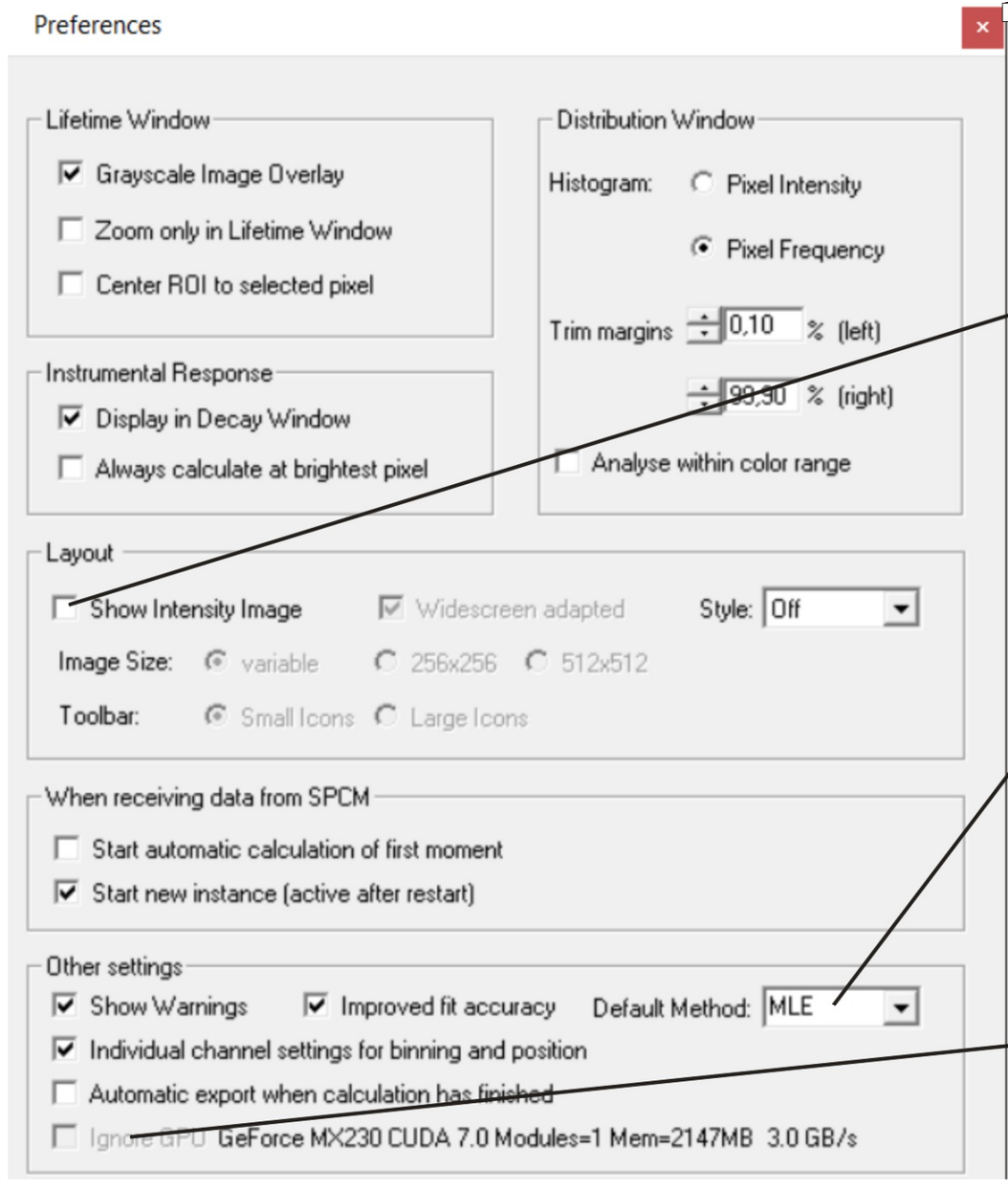
Old Fit Procedure
Use MLE instead
Runs on GPU, is faster, and performs better with floating IRF

Runs fit with same IRF position in all pixels
No compensation of diagonal lifetime shift

Fits decay curves only after maximum.
Used with old models to deal with distortion of leading edge by lens fluorescence

Fits decay curves only after maximum.
Used with old models to deal with distortion of leading edge by lens fluorescence

Settings NOT to be used for FLIO analysis

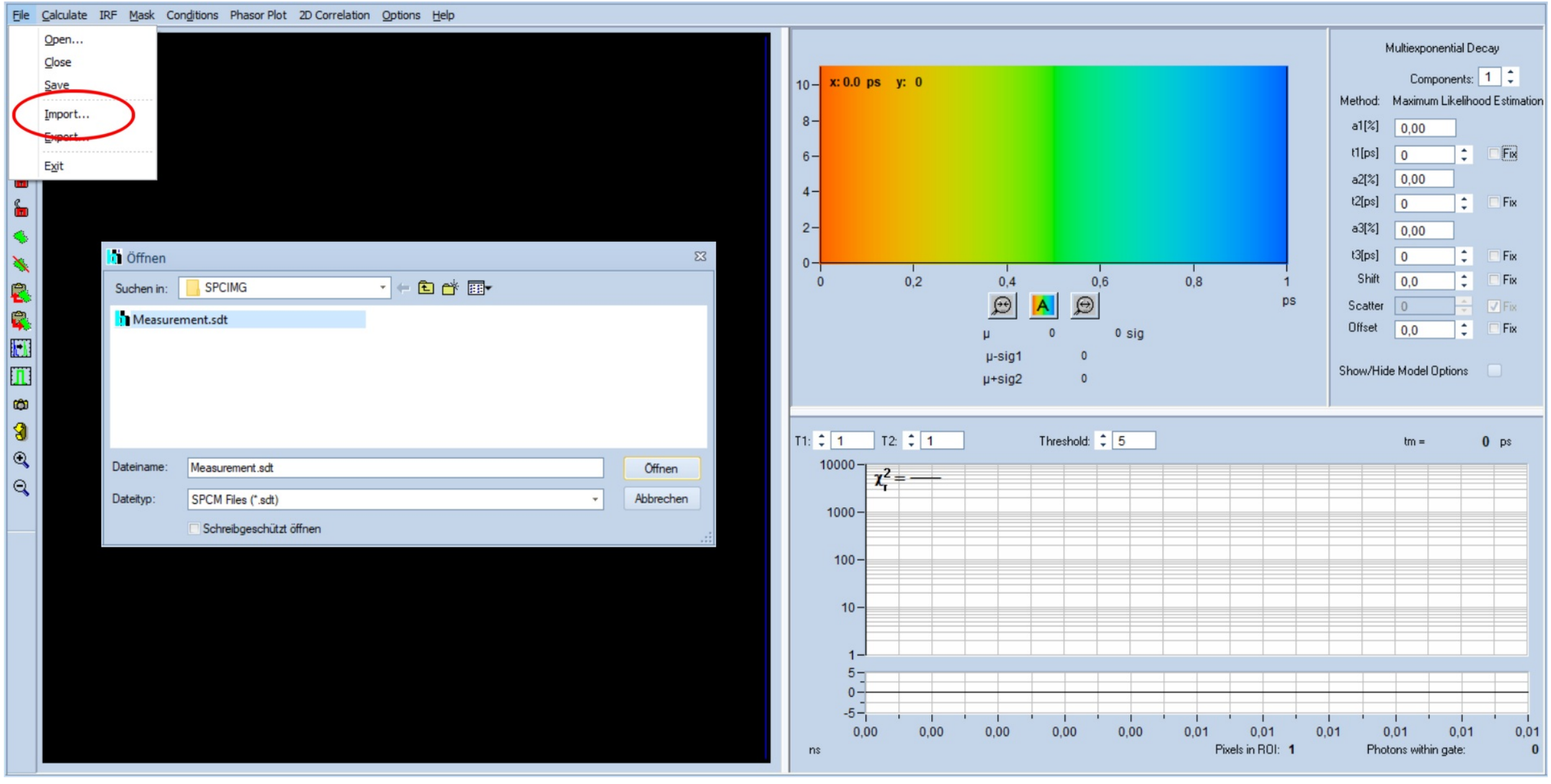


Intensity image in parallel with lifetime image
No FLIO-relevant information

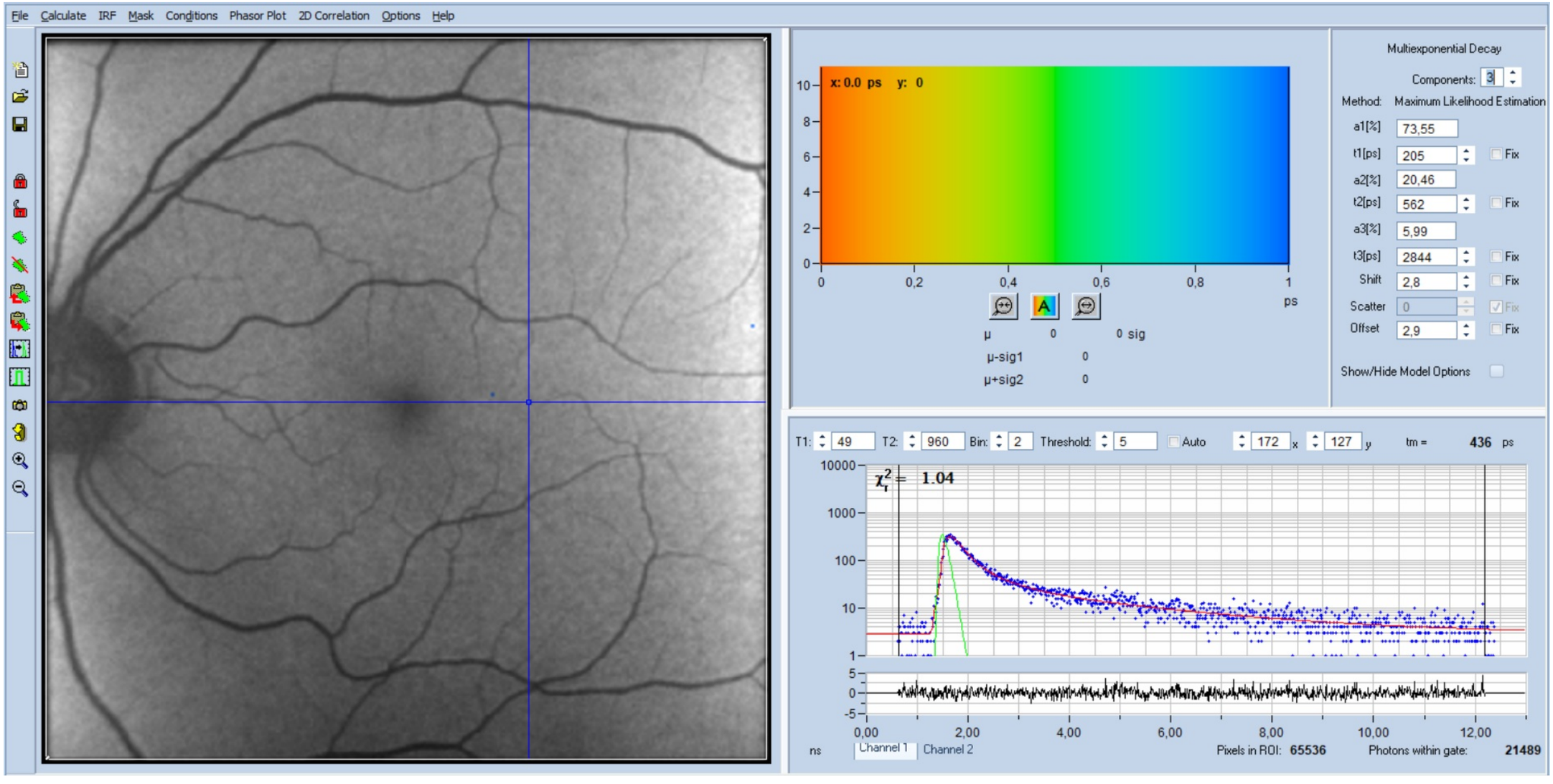
Don't use WLS
Use MLE as a default
MLE runs on GPU and performs better with floating IRF

Disables GPU in case of compatibility problems

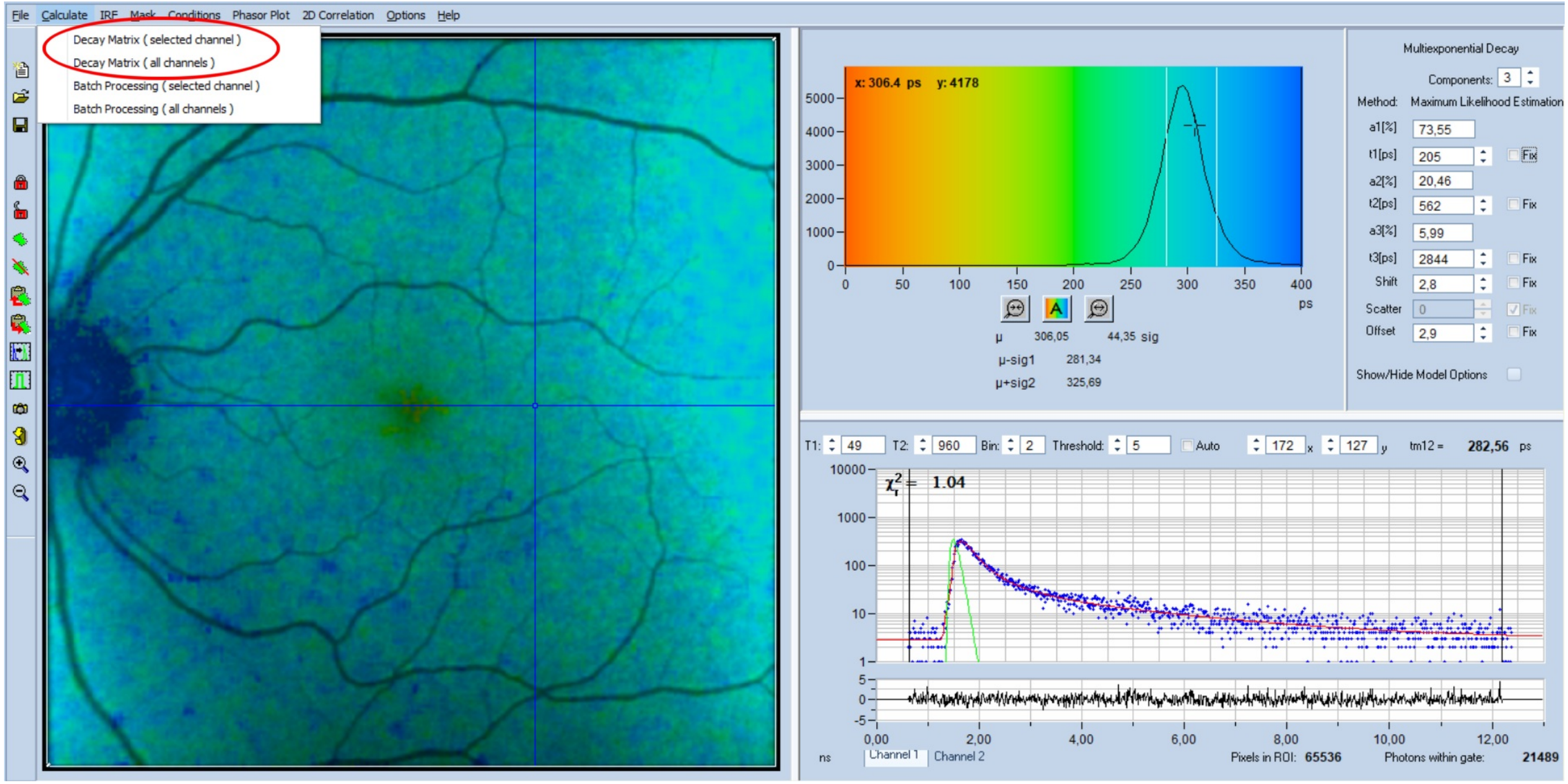
Importing the Data



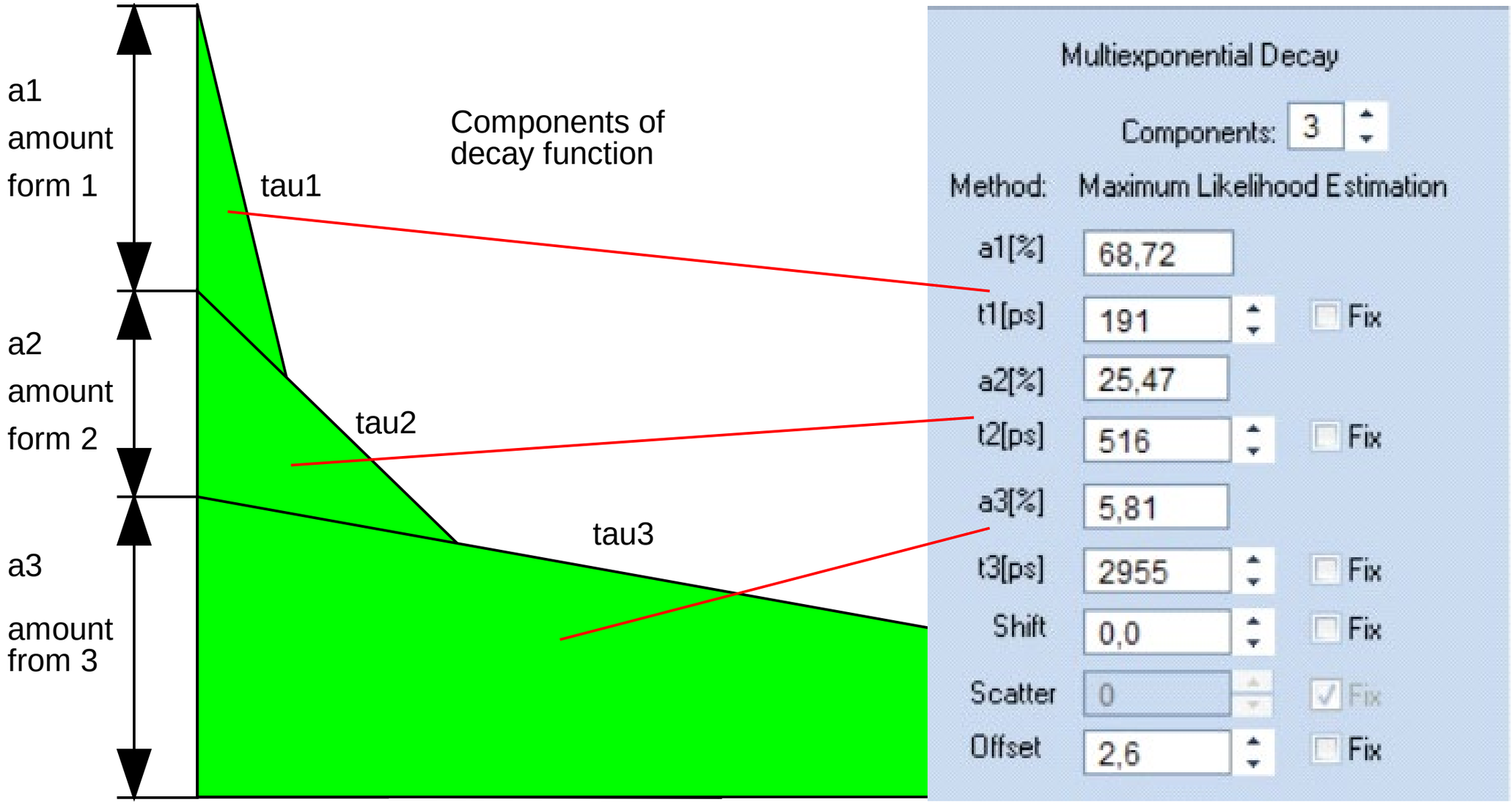
Data Imported but not yet Analysed



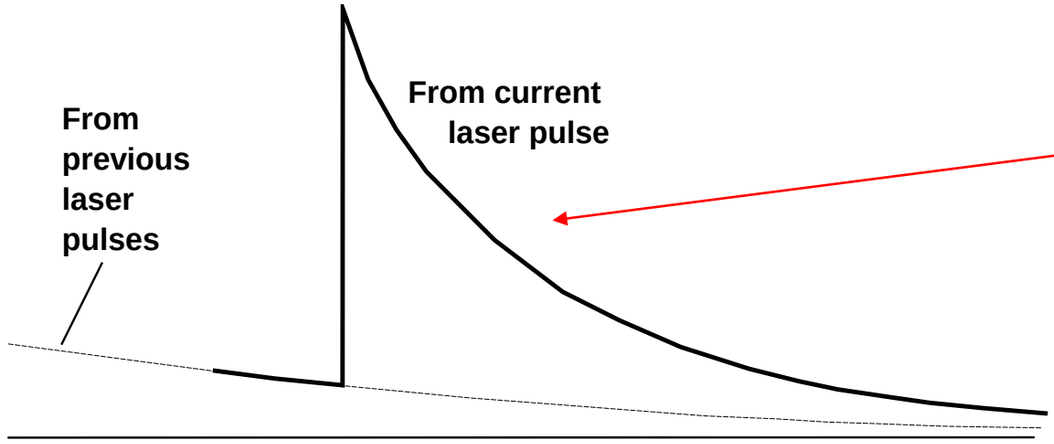
Calculate Lifetime Image

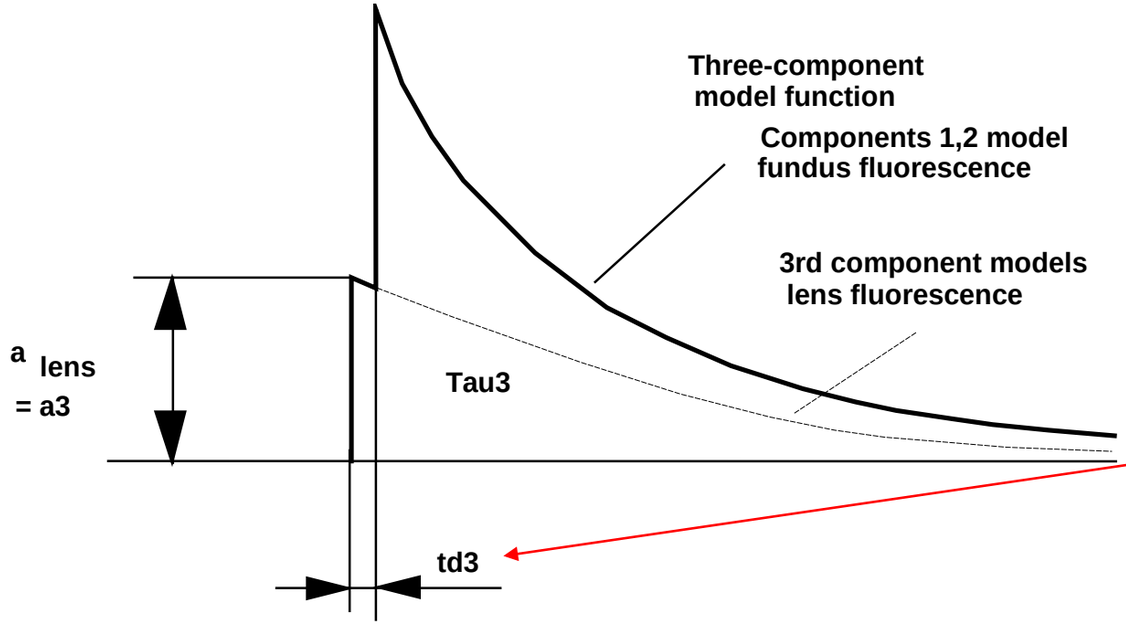


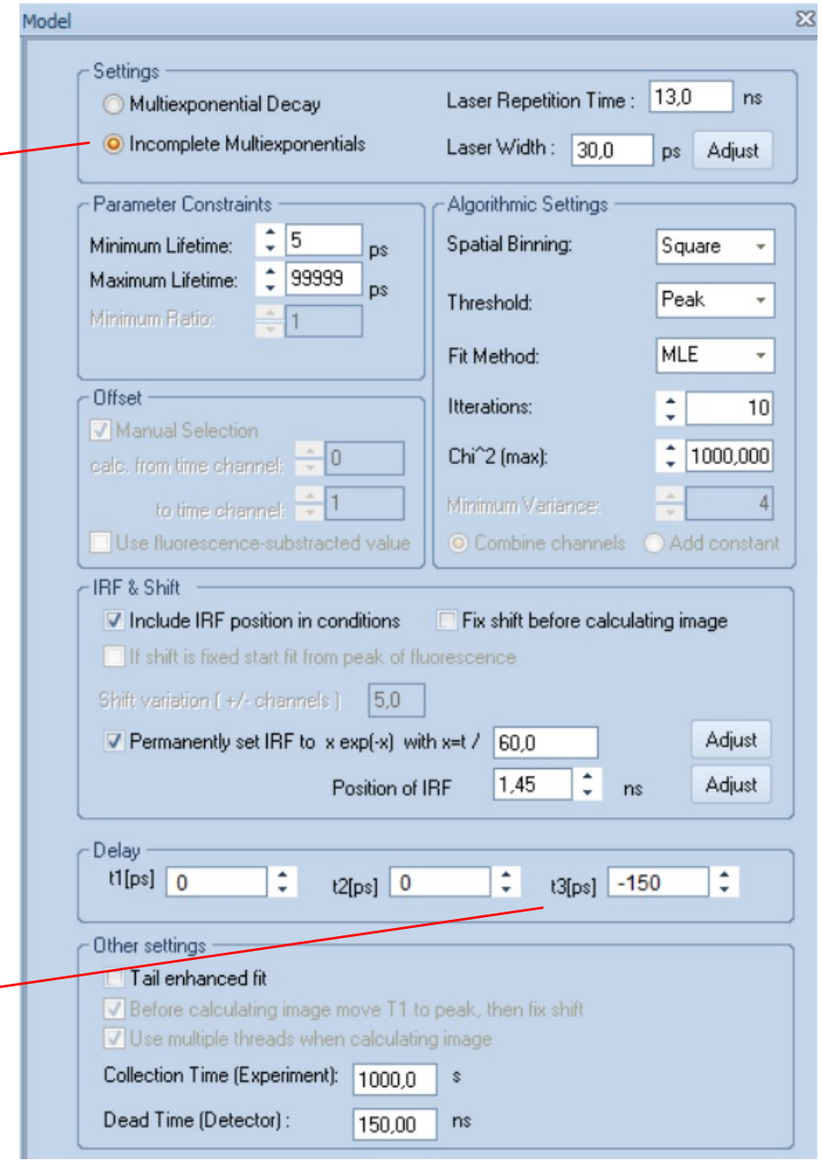
Basic Model Definitions



Advanced Model Definitions

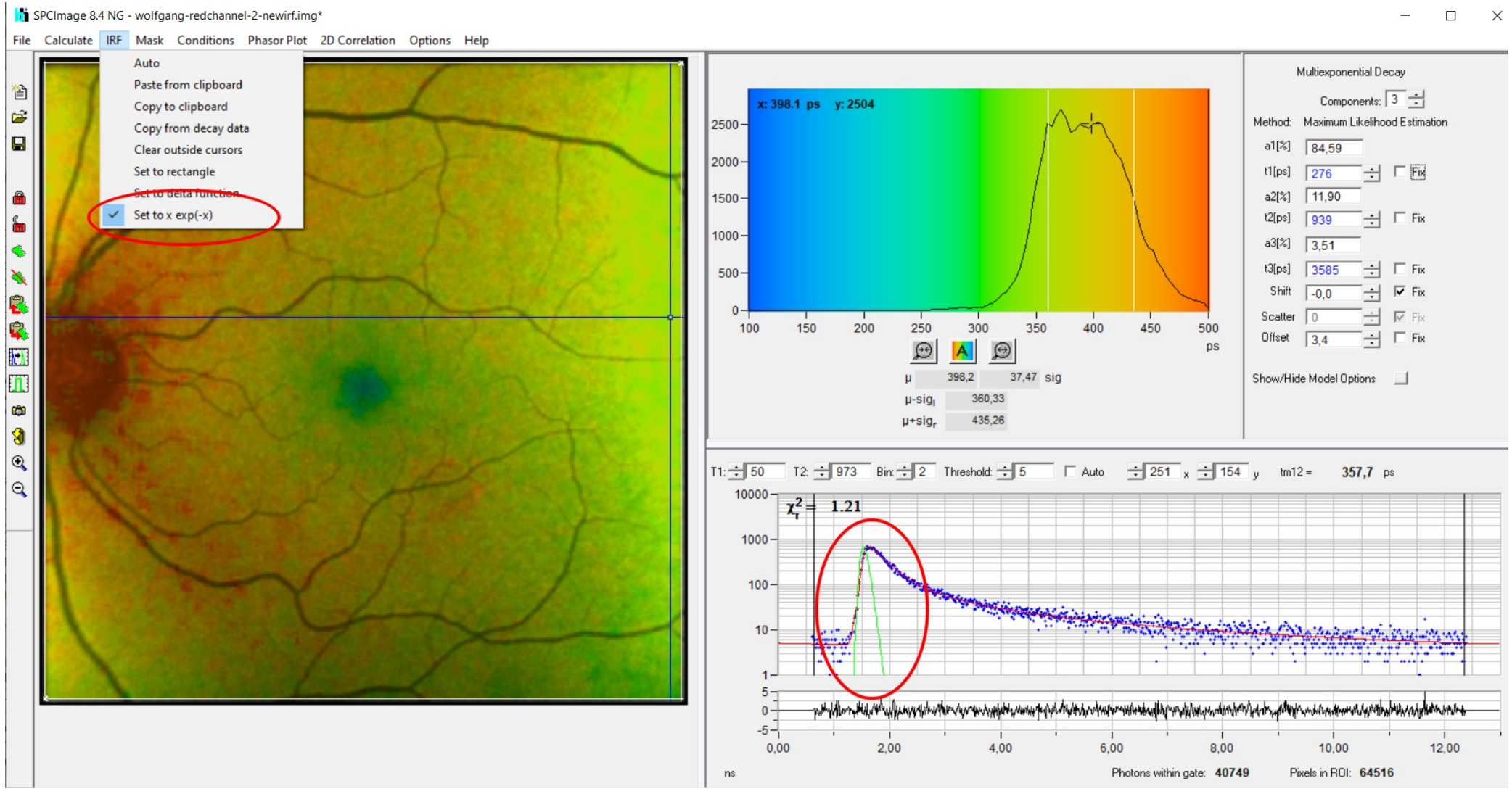






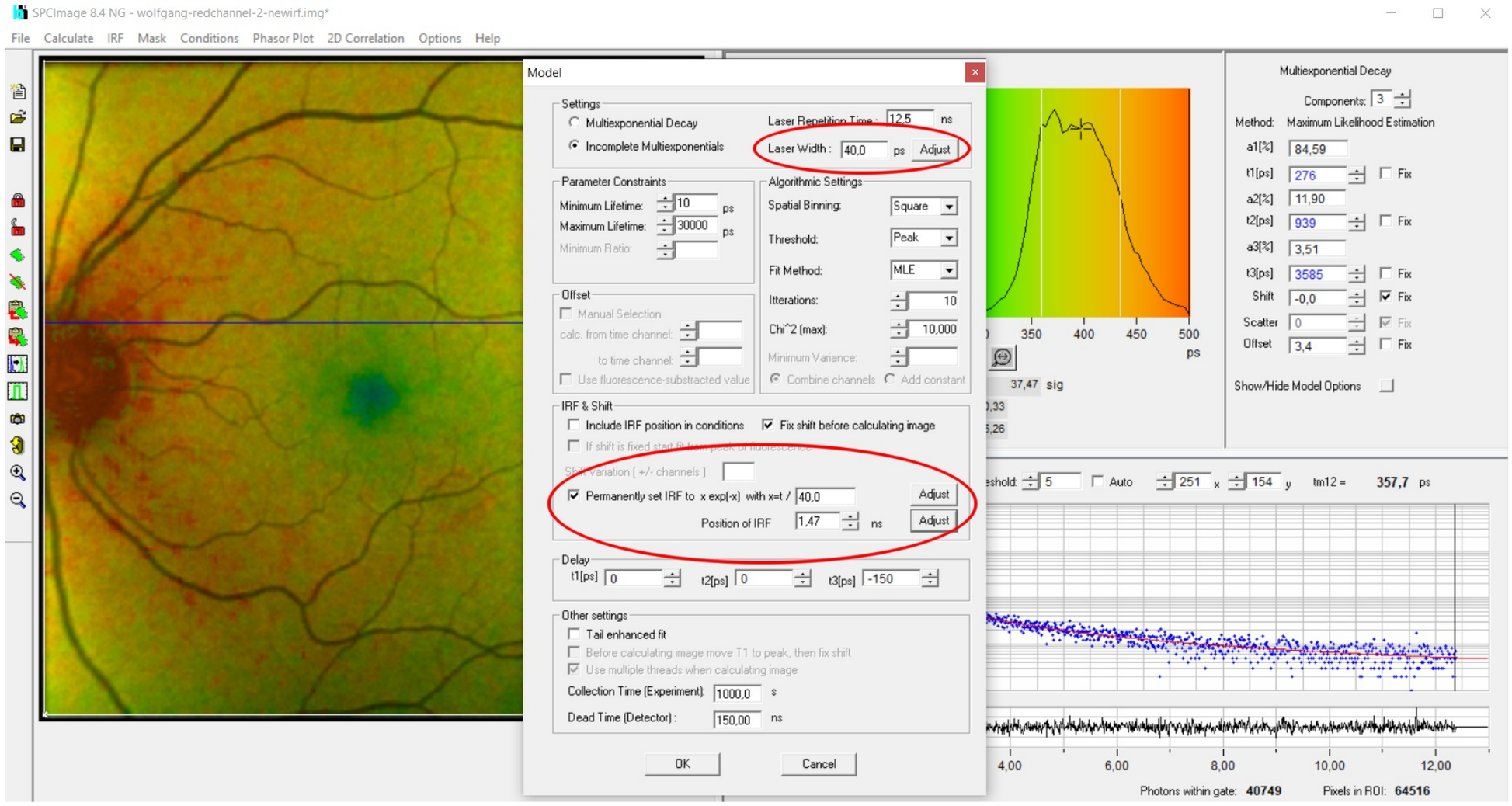
IRF Definition

Selection of IRF Type



IRF Definition

Definition of IRF



Use this IRF 'Permanently'

When both wavelength channels are loaded: IRF definition is separate for the two wavelength channels

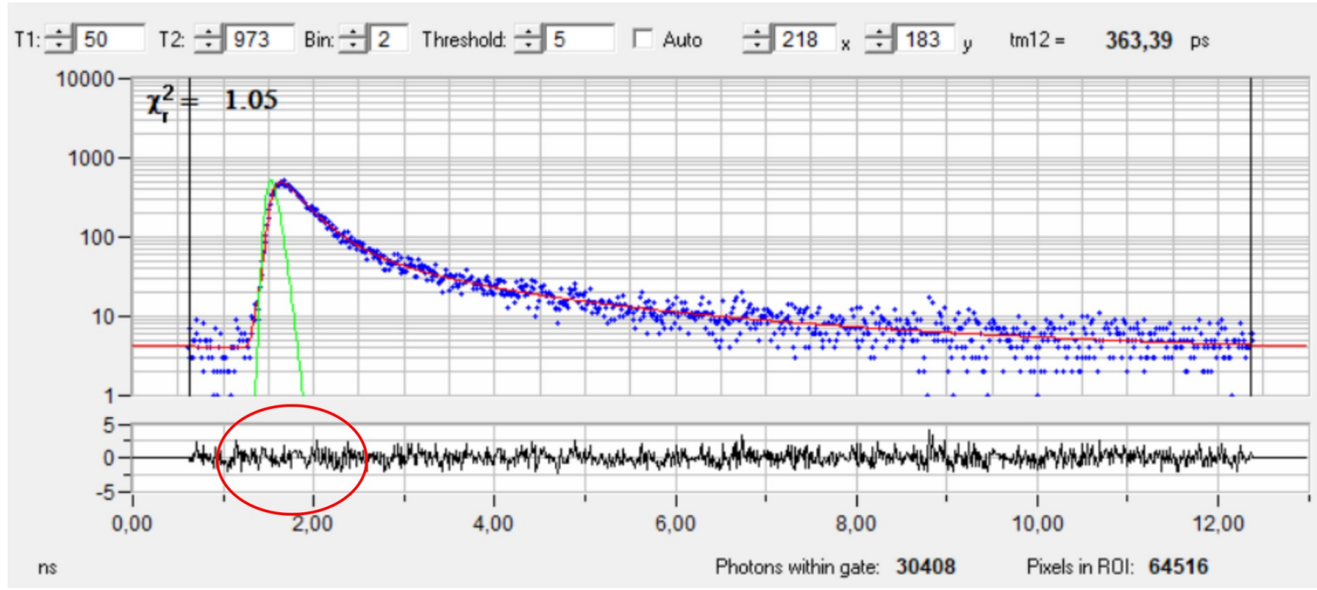
Everything OK?

Before running 'Calculate' take a look at the residuals!

Smooth Residuals:

Perfect Fit

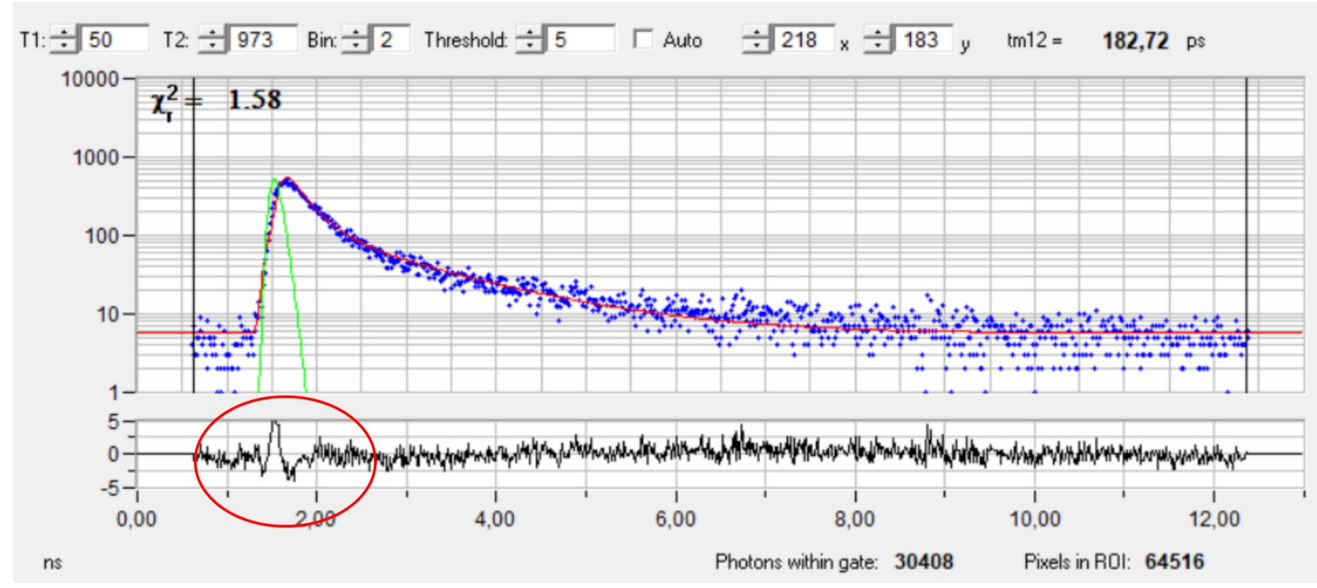
- **Model paramters correct**
- **IRF parameters correct**
- **Calculation will deliver accurate results**



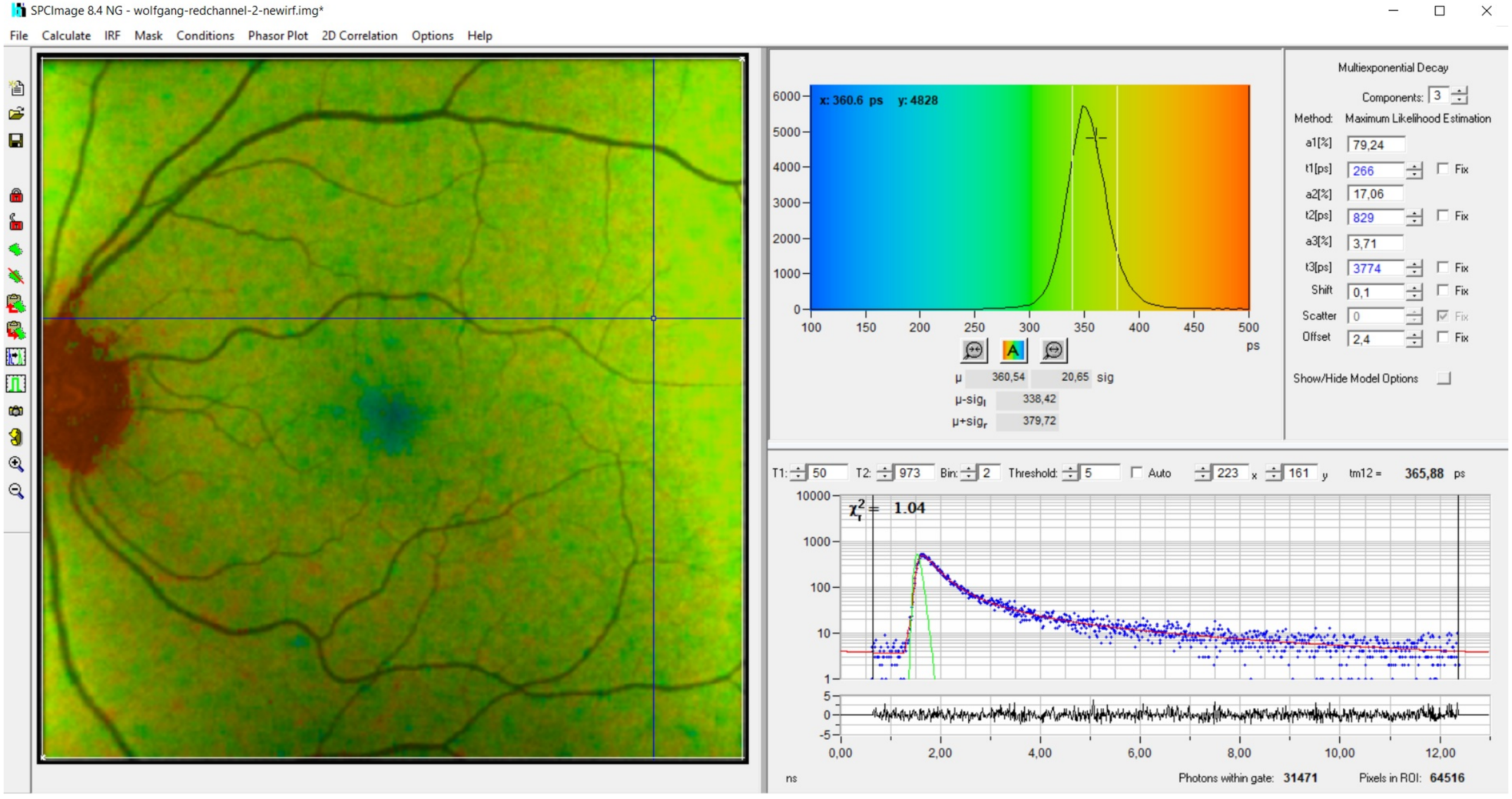
Bumps in Residuals:

Poor FIT

- **Model parameters incorrect?**
- **IRF parameters incorrect?**
- **Check parameters, or calculation may deliver inaccurate results!**



Everything OK! Let's run 'Calculate'!



And be no fool. Run it on a GPU!

It's just a $200 investment.

And it processes 512 pixels in parallel.

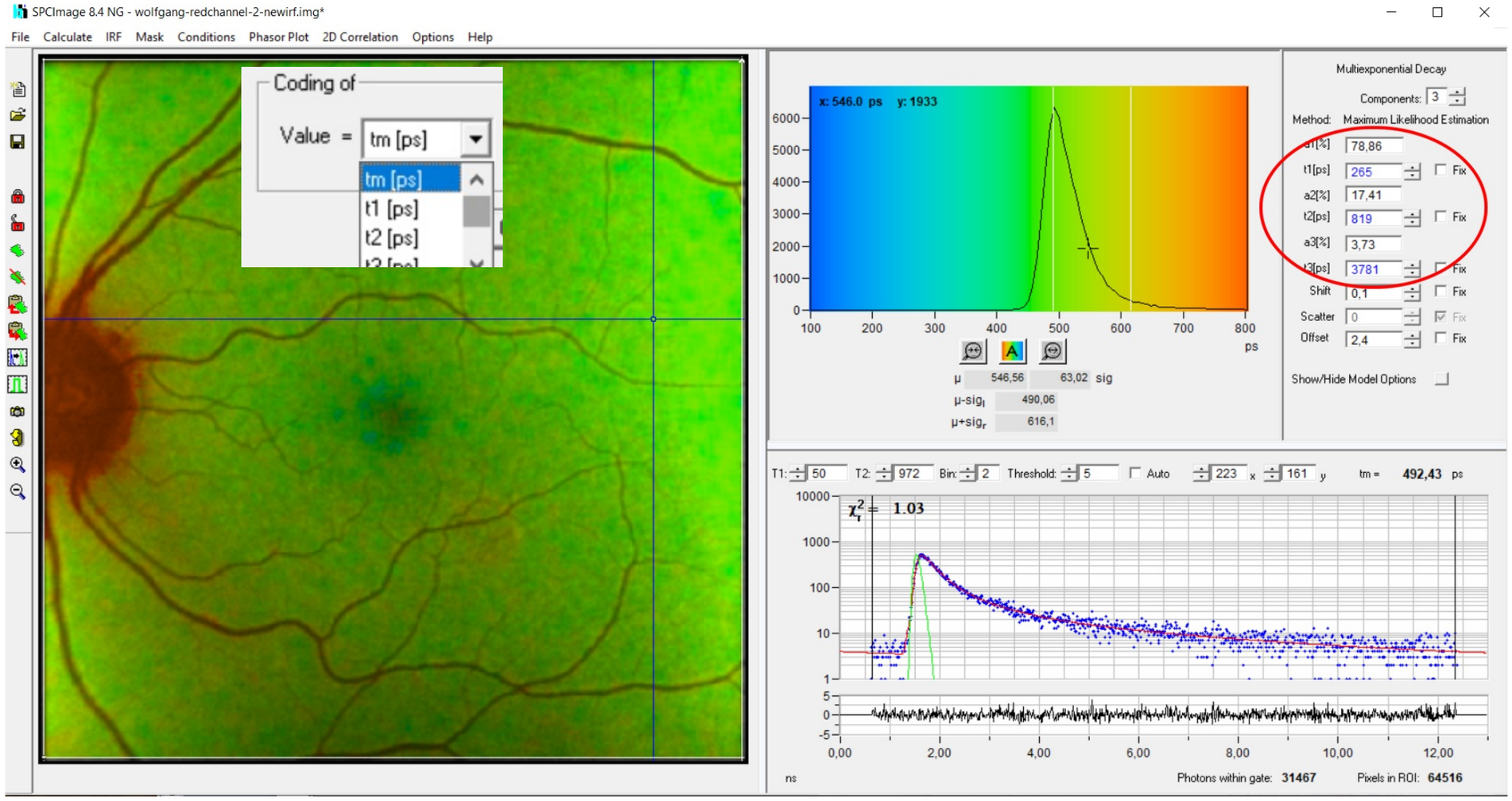
The Speed Difference is > 1:100

Which Lifetime Do We Want to Display?

tm: The traditional FLIO lifetime. Amplitude-weighted lifetime of all components

Does not separate between Lens and fundus

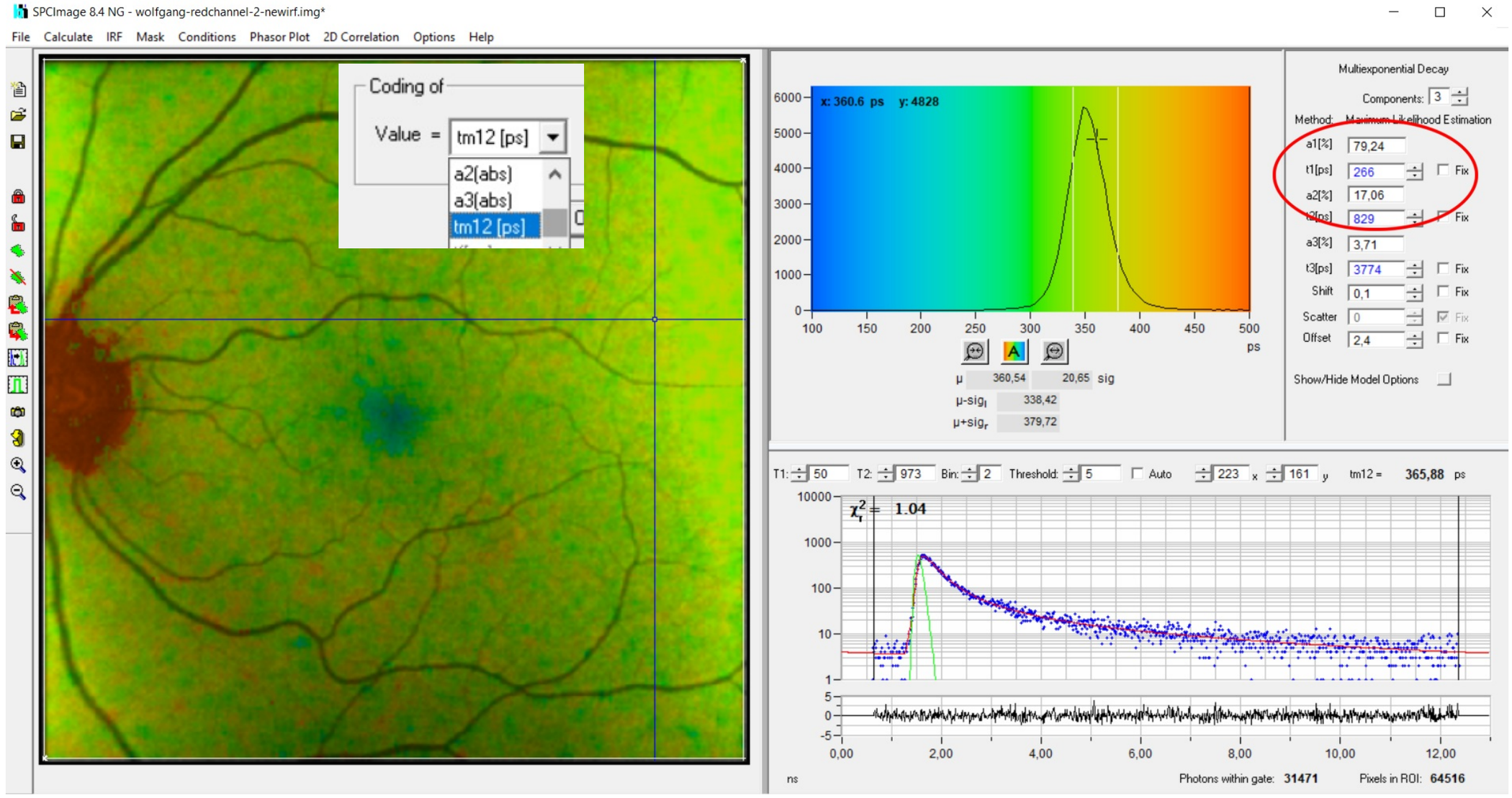
Equivalent to Pre-MetaNetz era lifetimes



Which Lifetime Do We Want to Display?

tm12: Amplitude-weighted lifetime of components 1 and 2

Fundus Lifetime (Note different time scale)



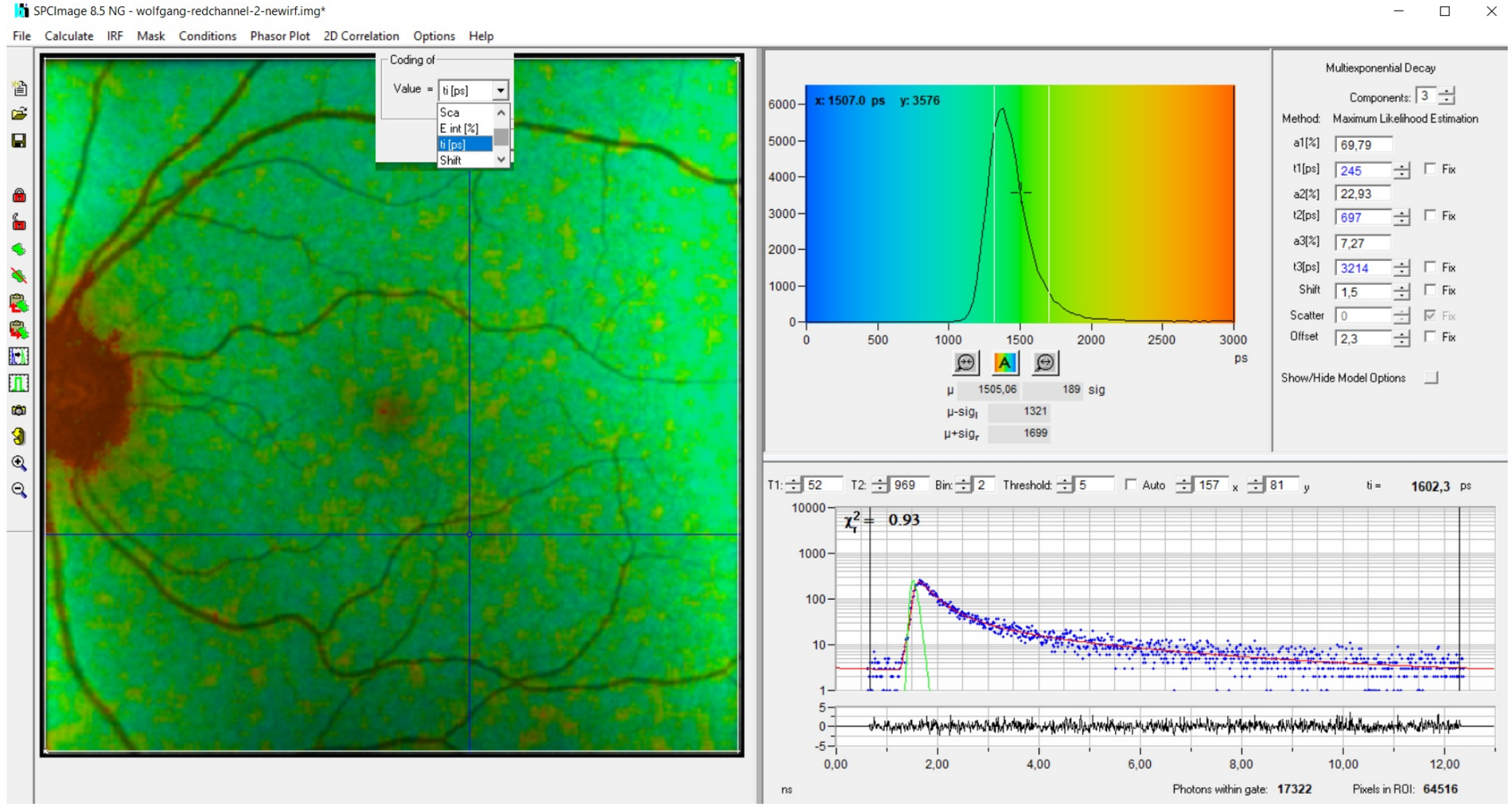
Which Lifetime Do We Want to Display?

ti: Intensity-weighted lifetime of all components

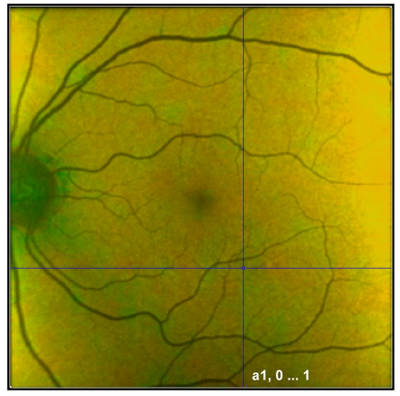
Dominated by long-lifetime components

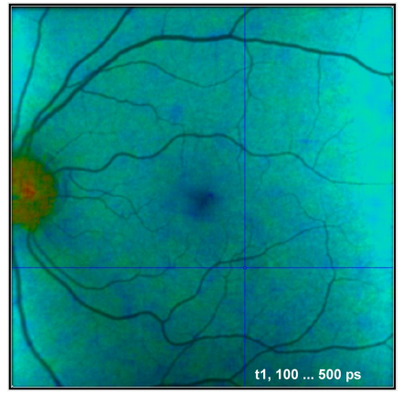
Lens fluorescence!

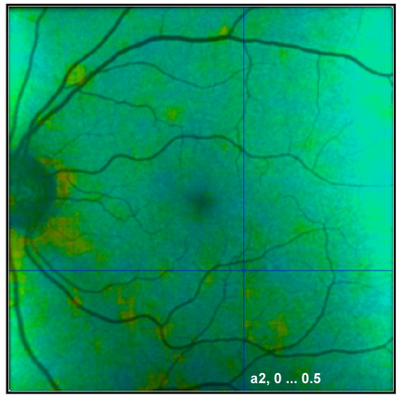
Don't use ti for FLIO

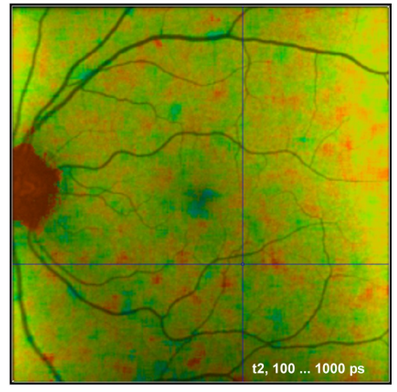


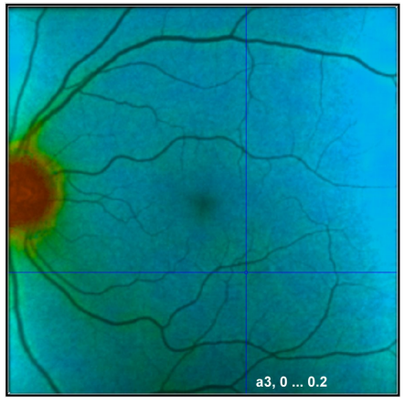
Component Lifetimes and Amplitudes

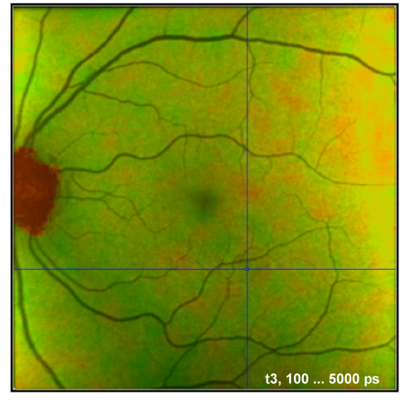


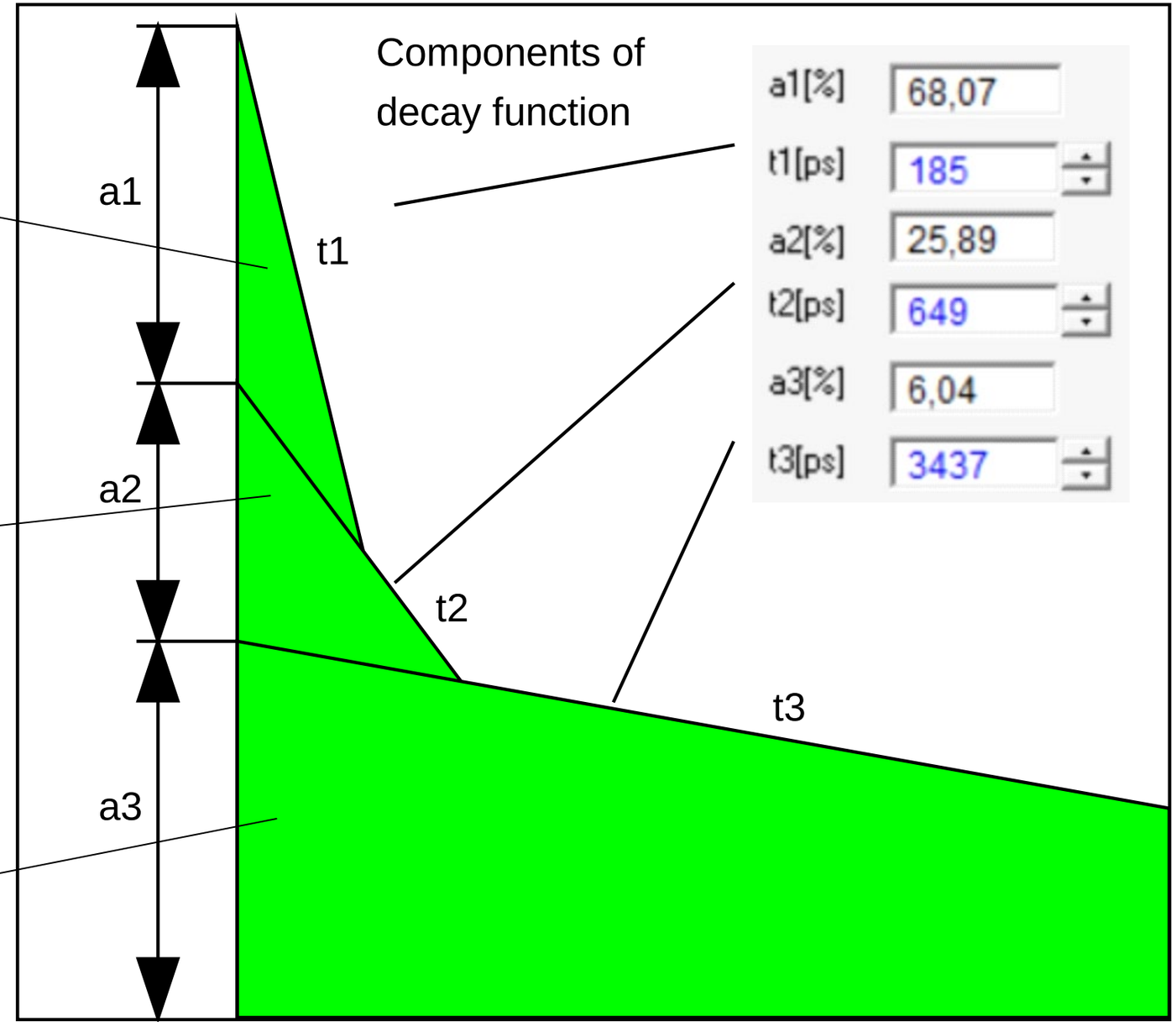






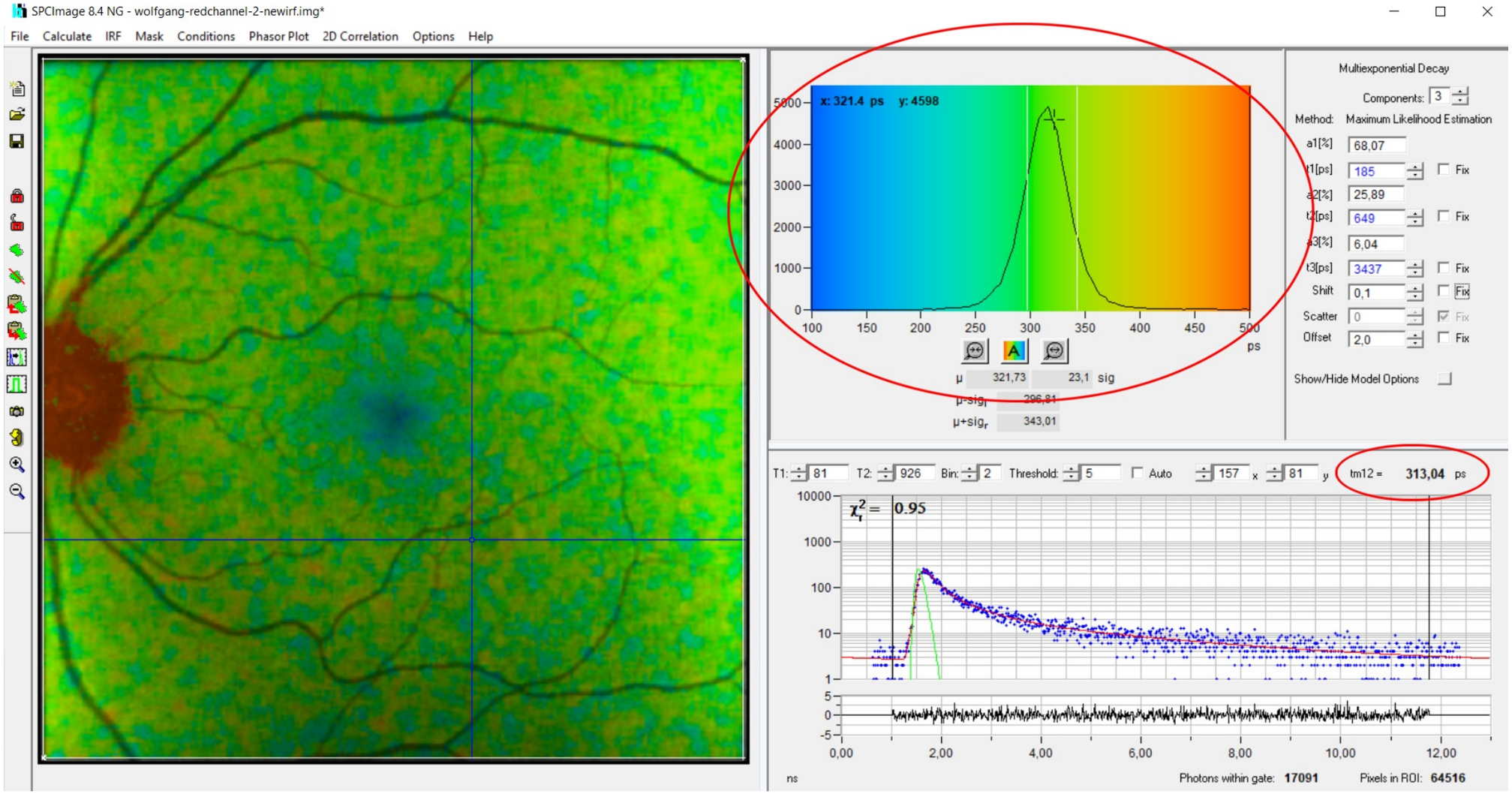






The Parameter Histogram

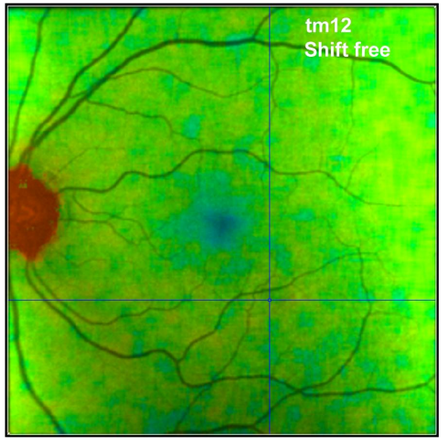
How frequently does a given paramter value appear in the image?

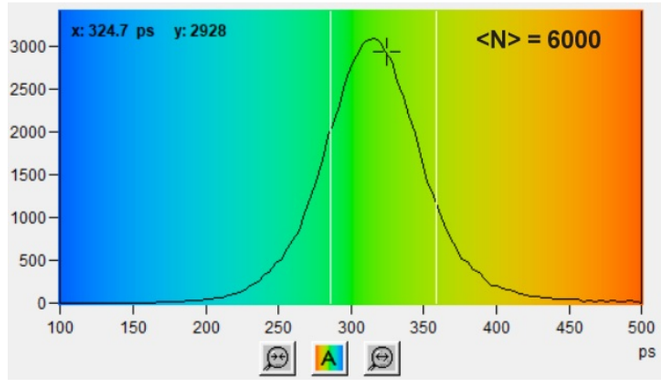


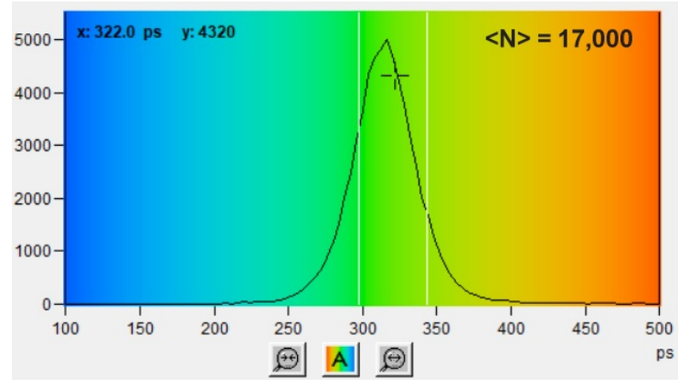
Width of the Histogram

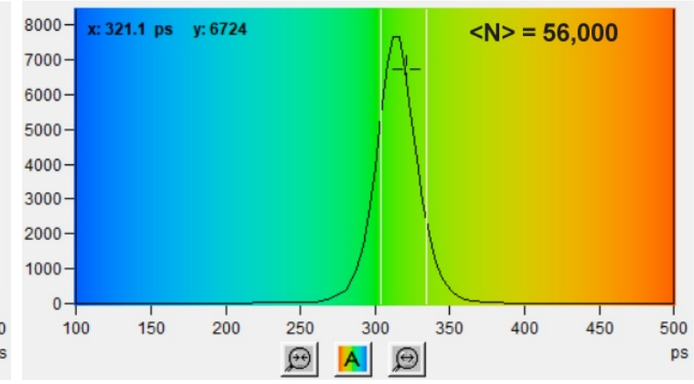
Determined by

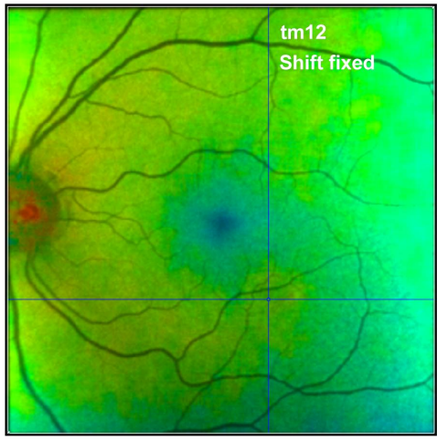
- **Noise from the photon statistics. Width decreases with photon number.**
- **Real heterogeneity in the image. Width is constant.**

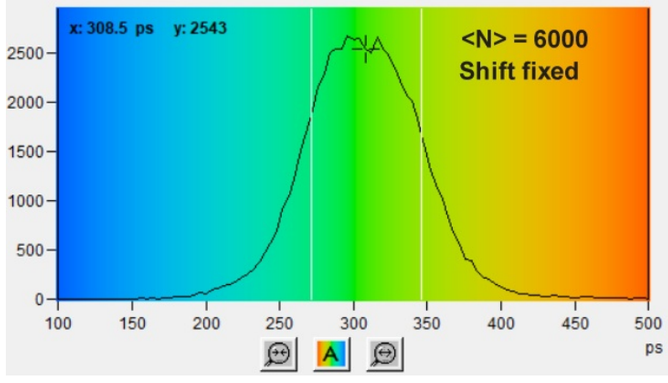


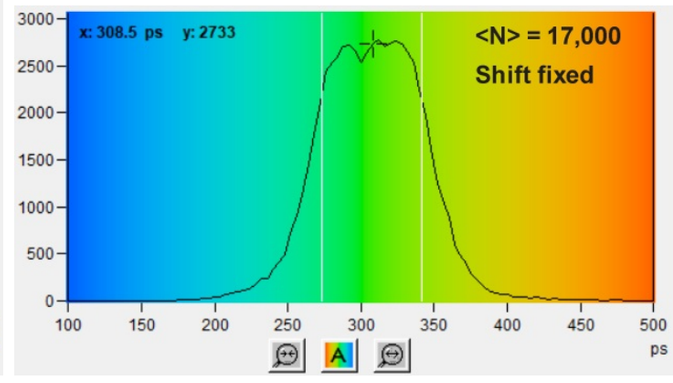


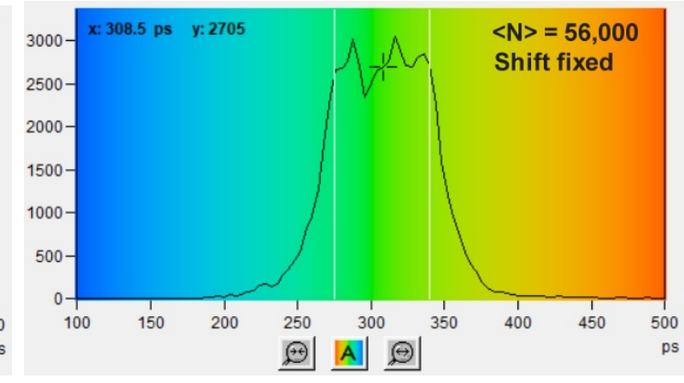








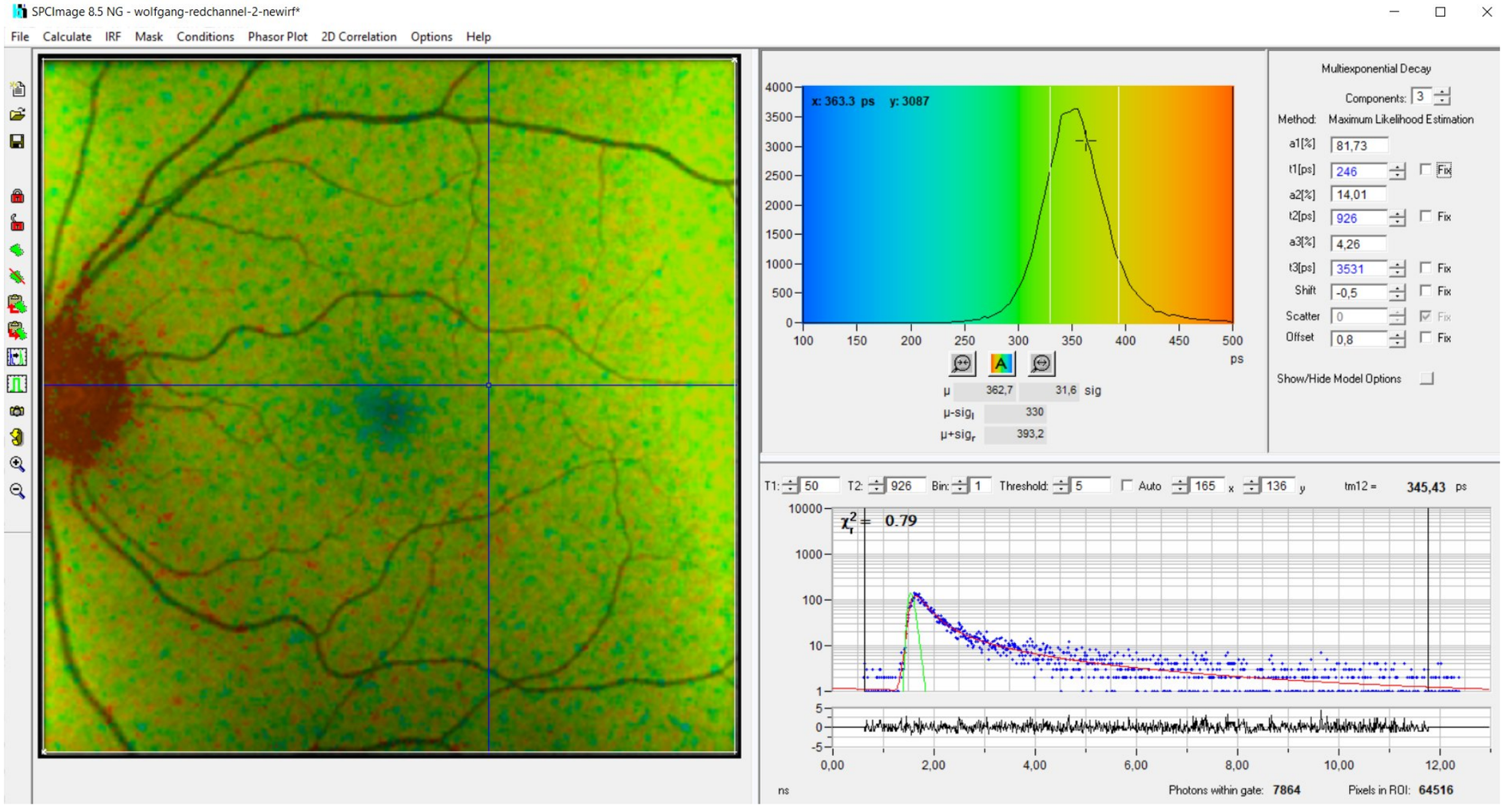




The width of the histogram is a quality indicator!

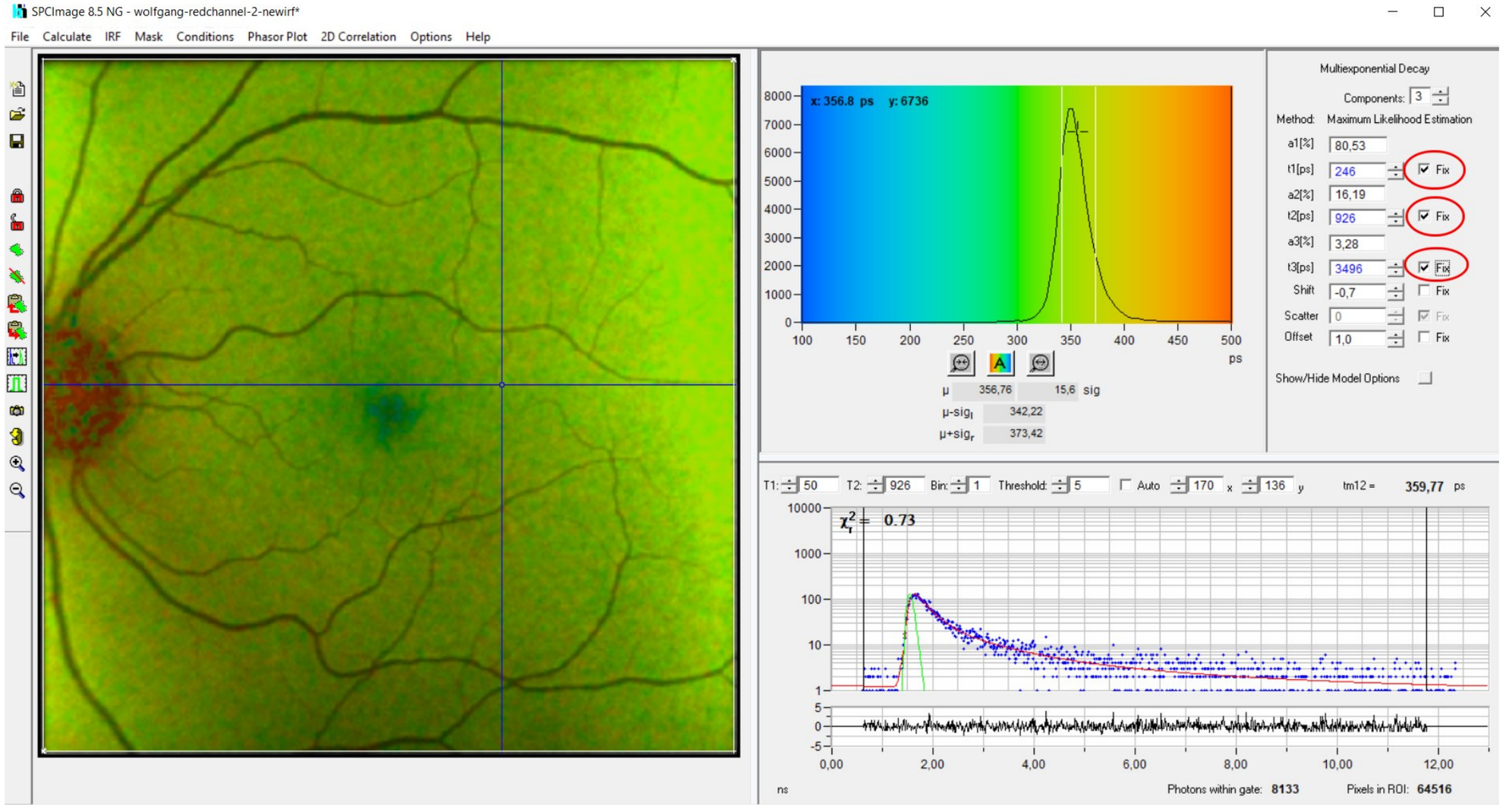
Analysis with Fixed Component Lifetimes

Free component lifetimes



Analysis with Fixed Component Lifetimes

Fixed component lifetimes, taken from decay data at cursor position

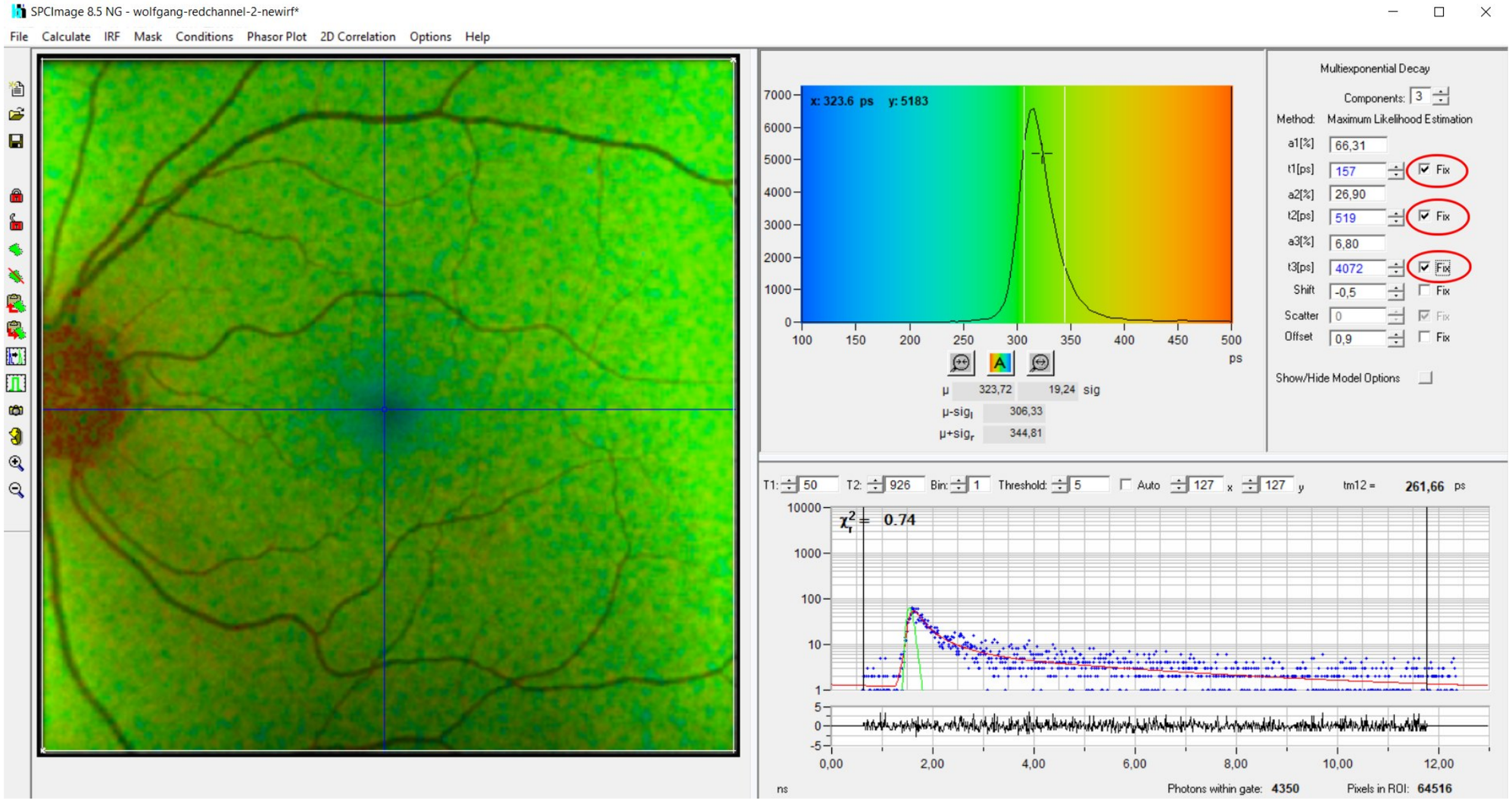


Narrower Distribution. Better signal-to-noise ratio.

But caution:

Analysis with Fixed Component Lifetimes

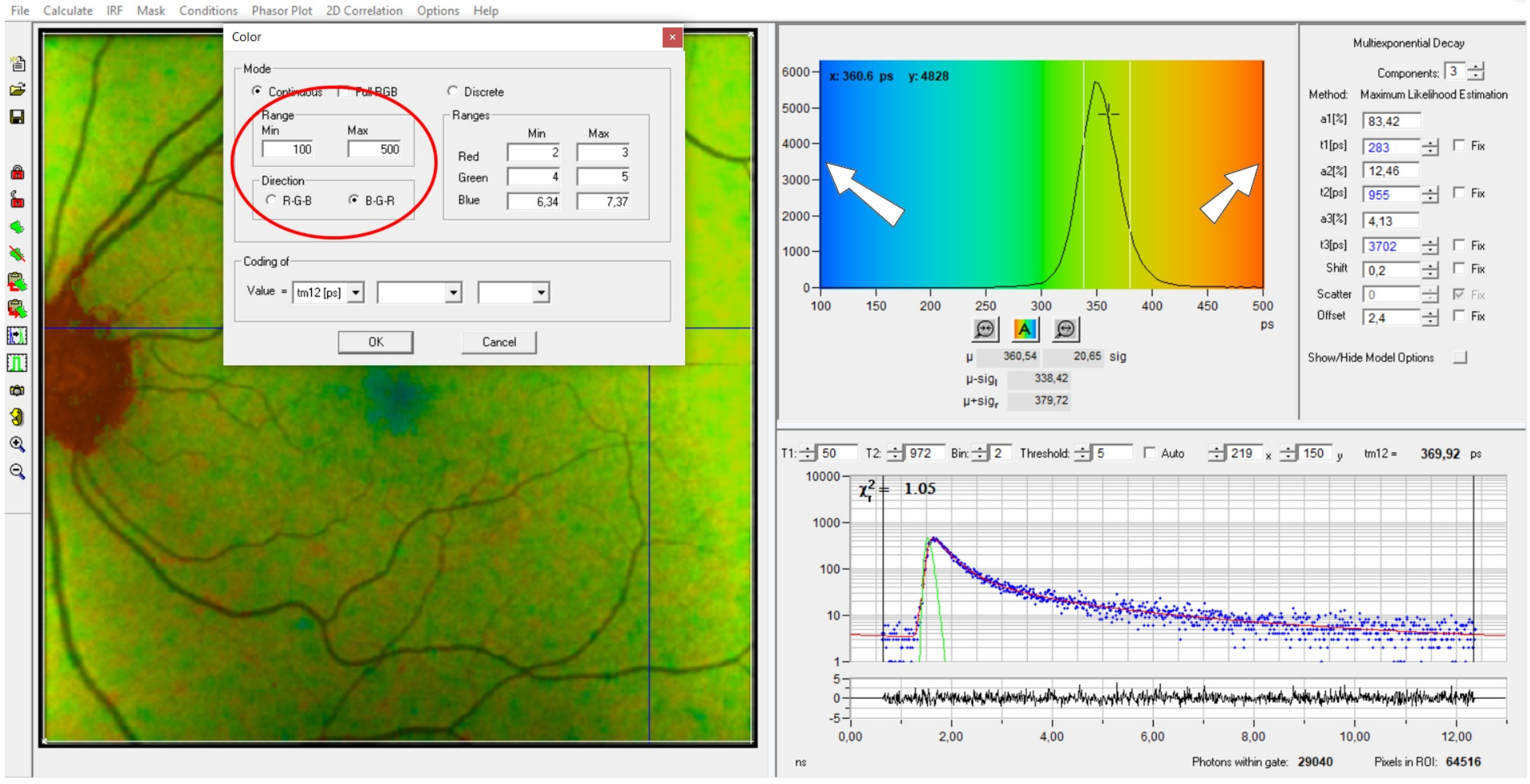
Fixed component lifetimes taken from another image position



Correct only in the area around the position from which the component liftimes have been taken!

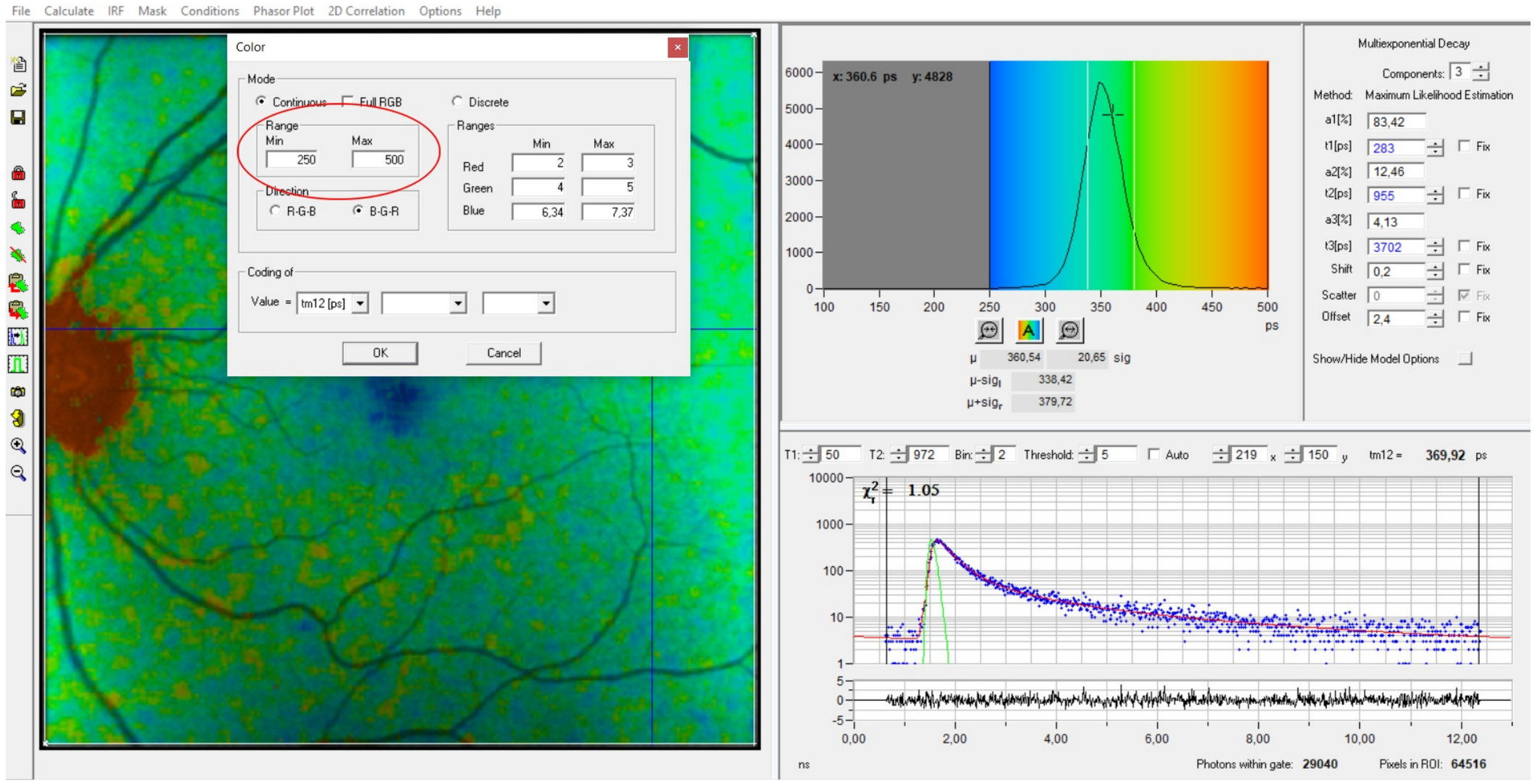
Display of the Images

Parameter Range



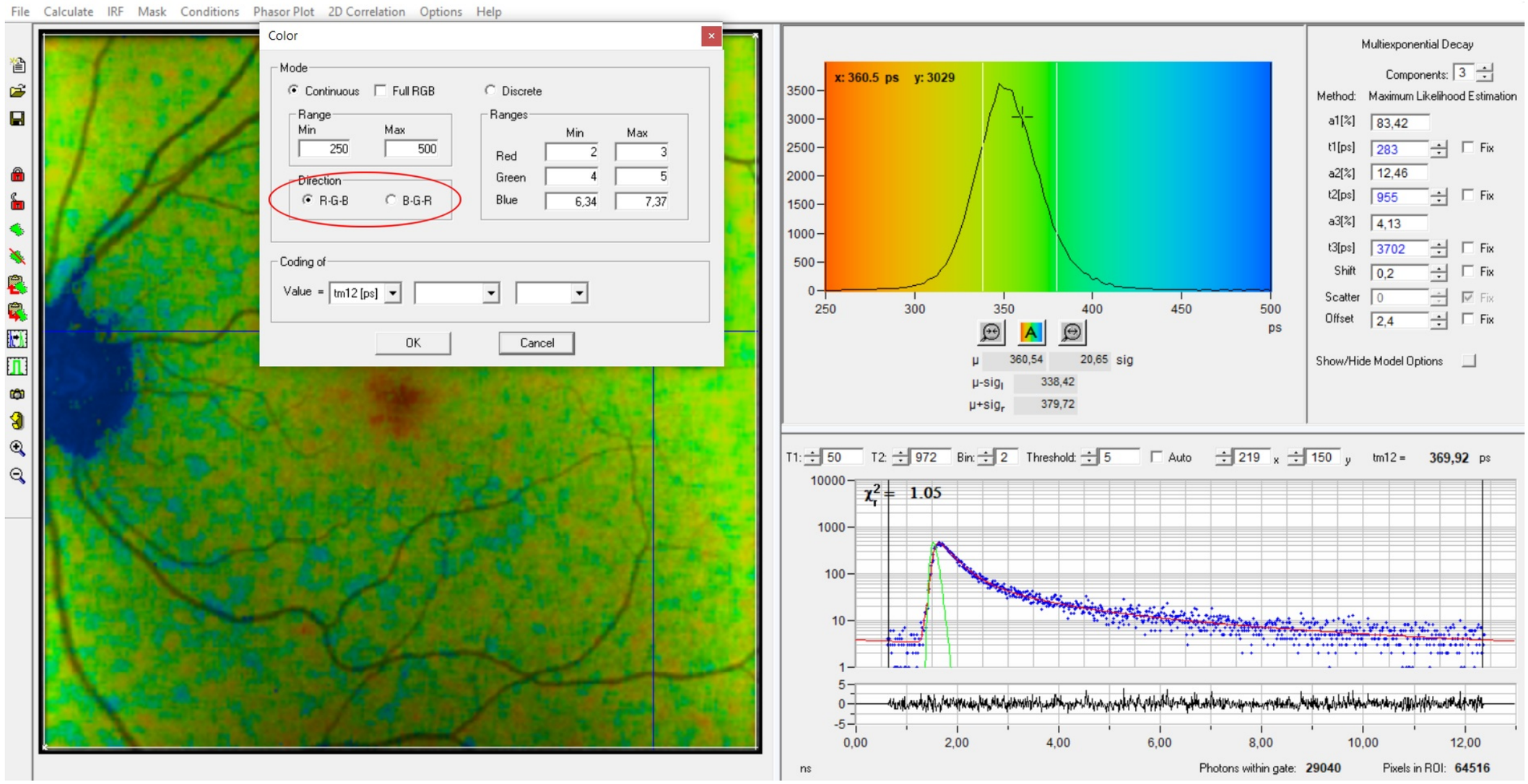
Display of the Images

Parameter Range



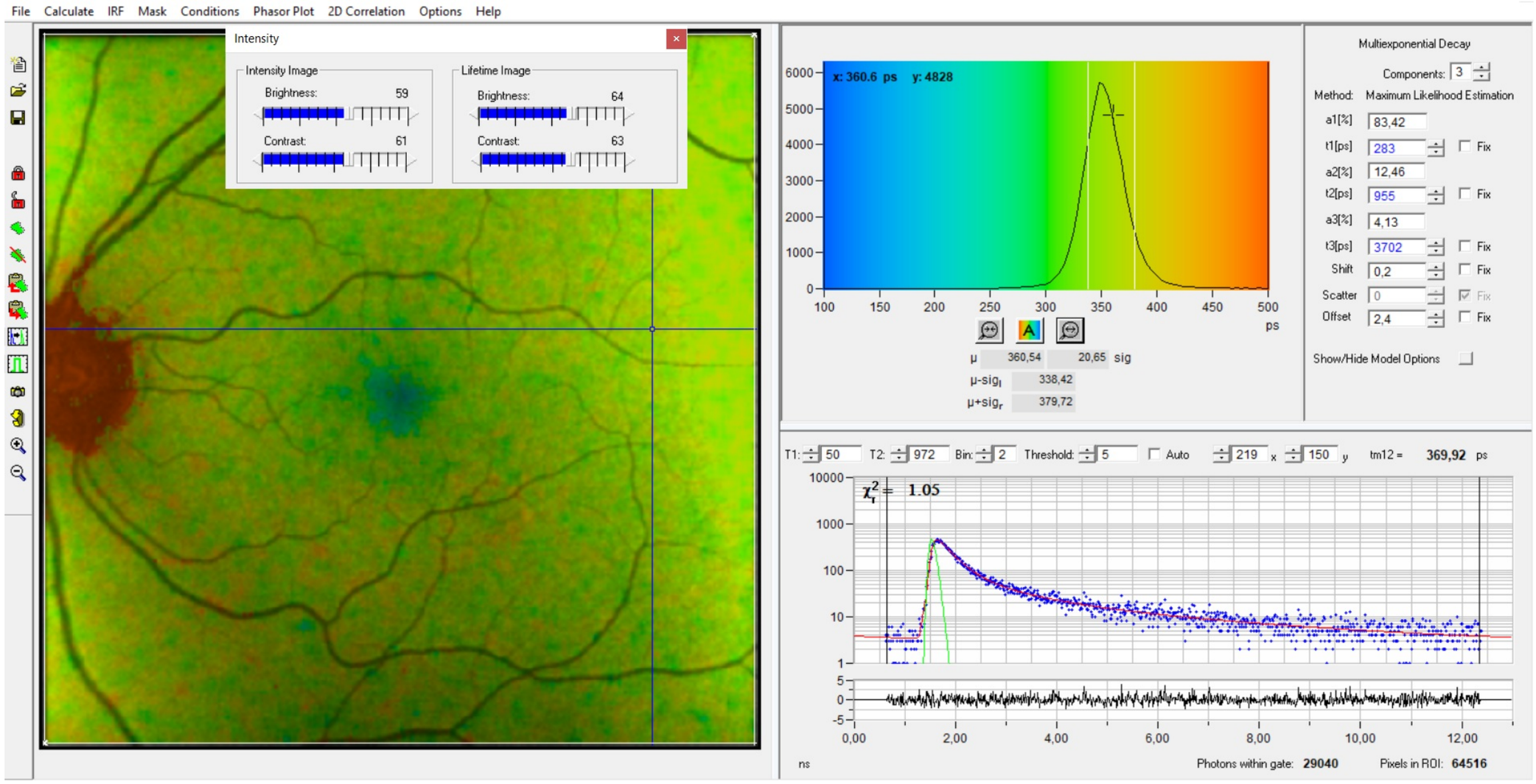
Display of the Images

Direction of Lifetime Scale



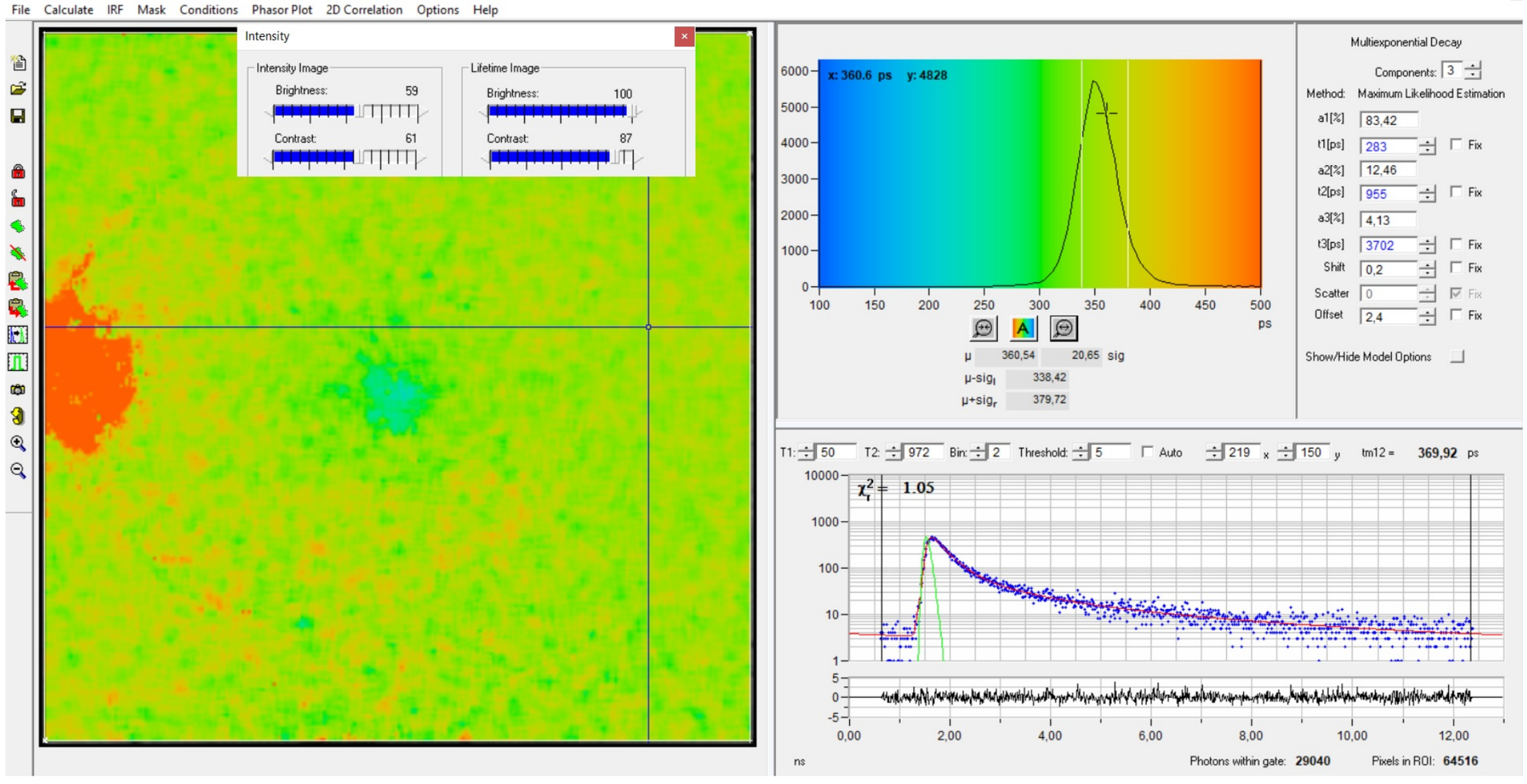
Display of the Images

Brightness and Contrast: Medium



Display of the Images

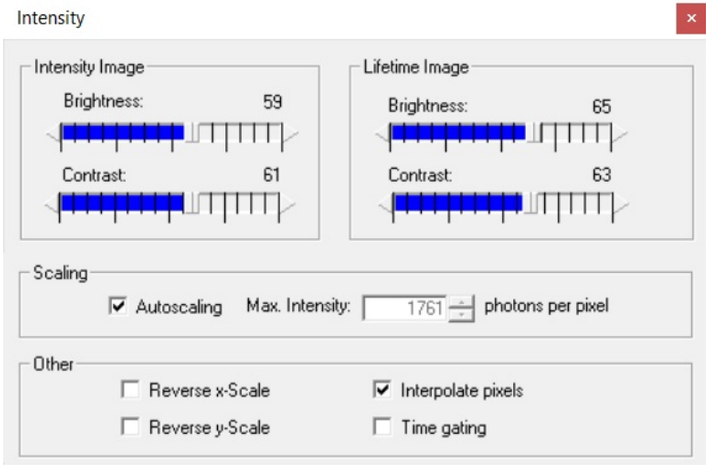
Brightness and Contrast: Fully pulled up

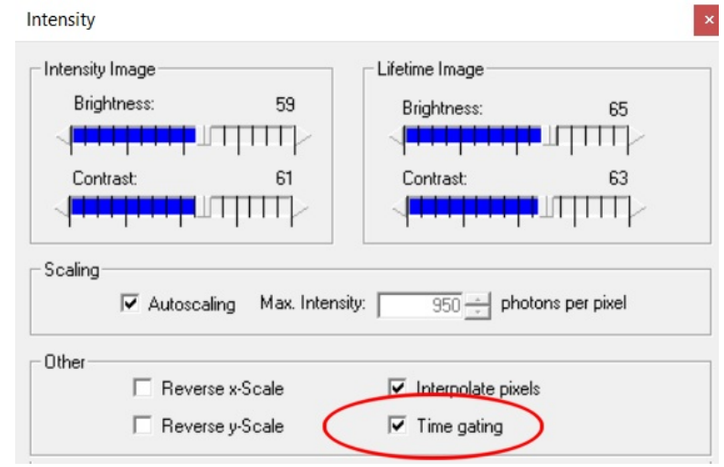


Ophthalmology Style

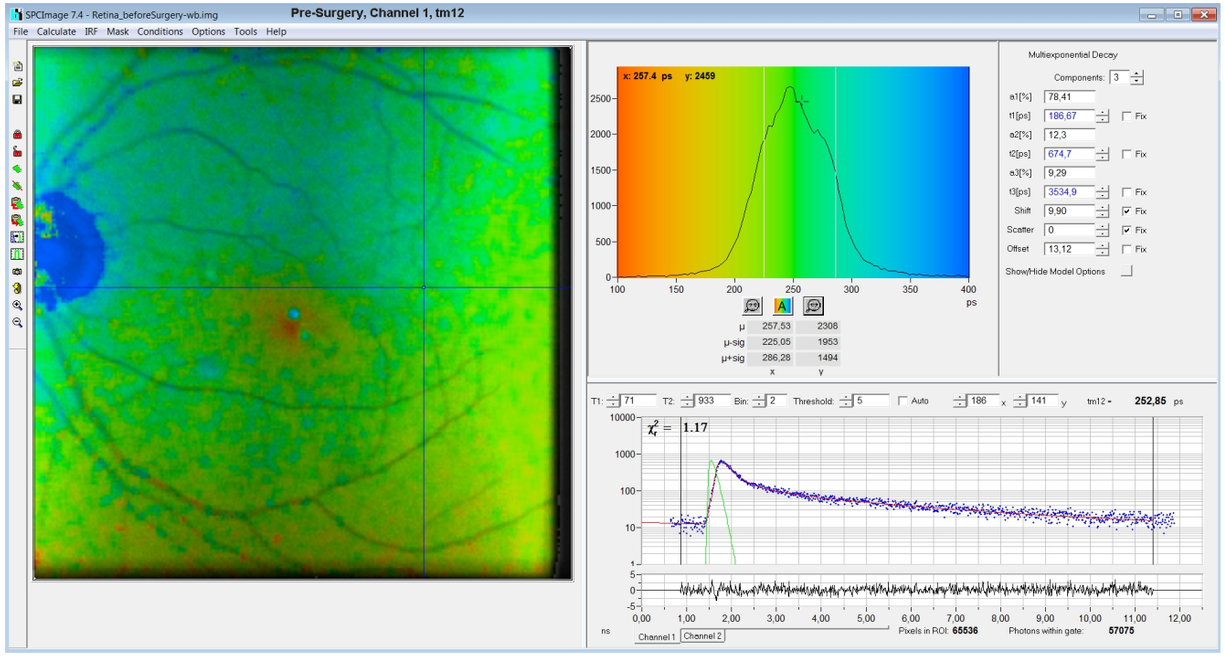
Anatomic structures entirely obscured. Is this desireable?

Gated Intensity

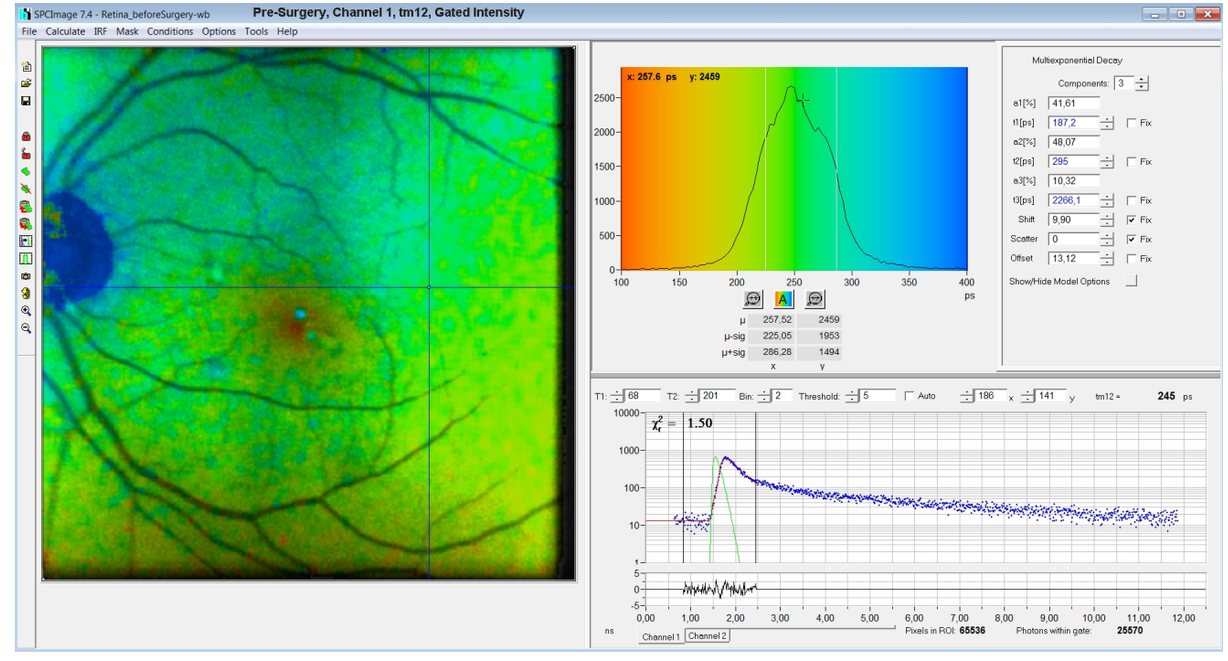




Intensity from entire time range



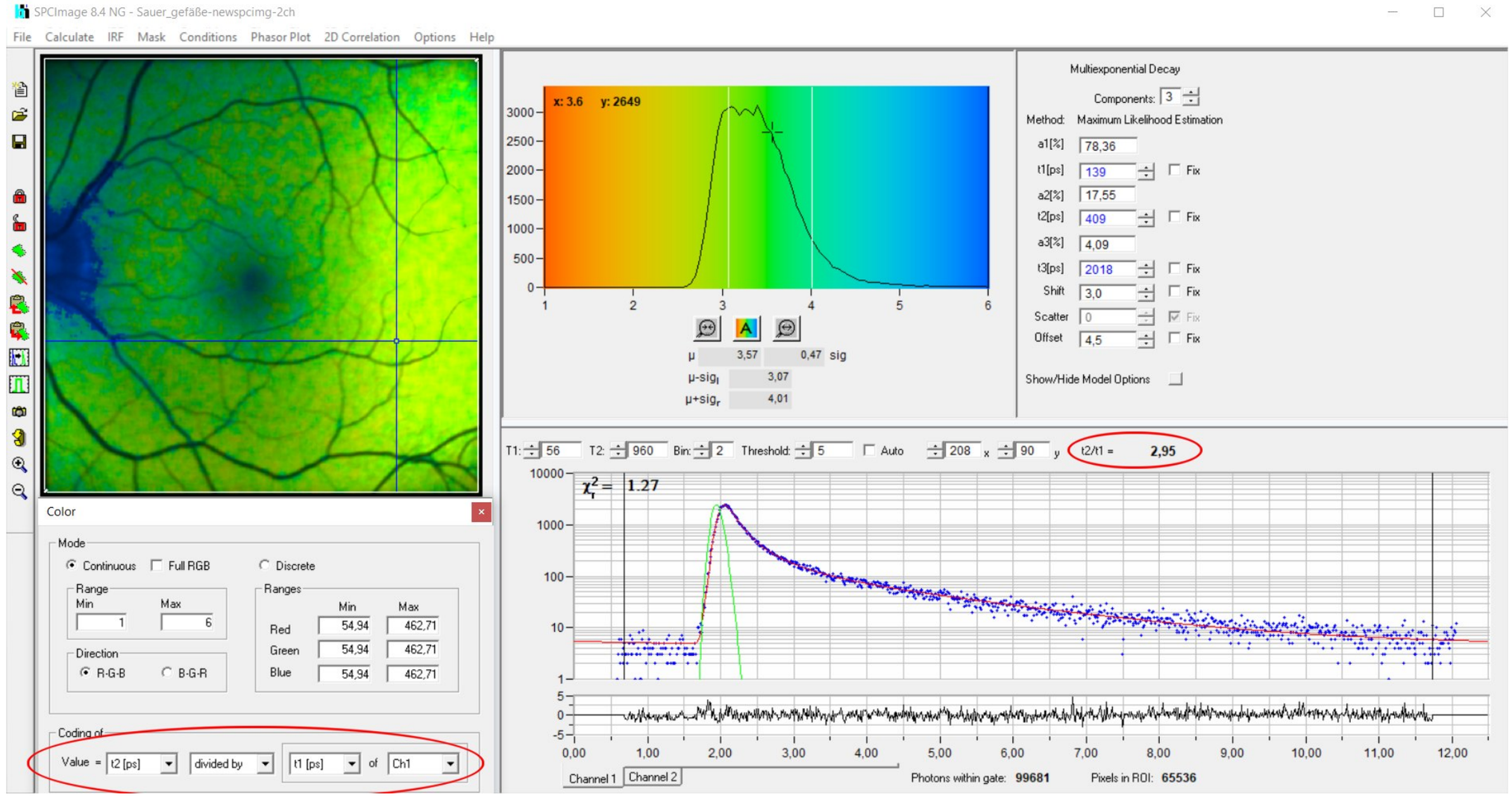
Intensity from time interval of fundus fluorescence only



Questions?

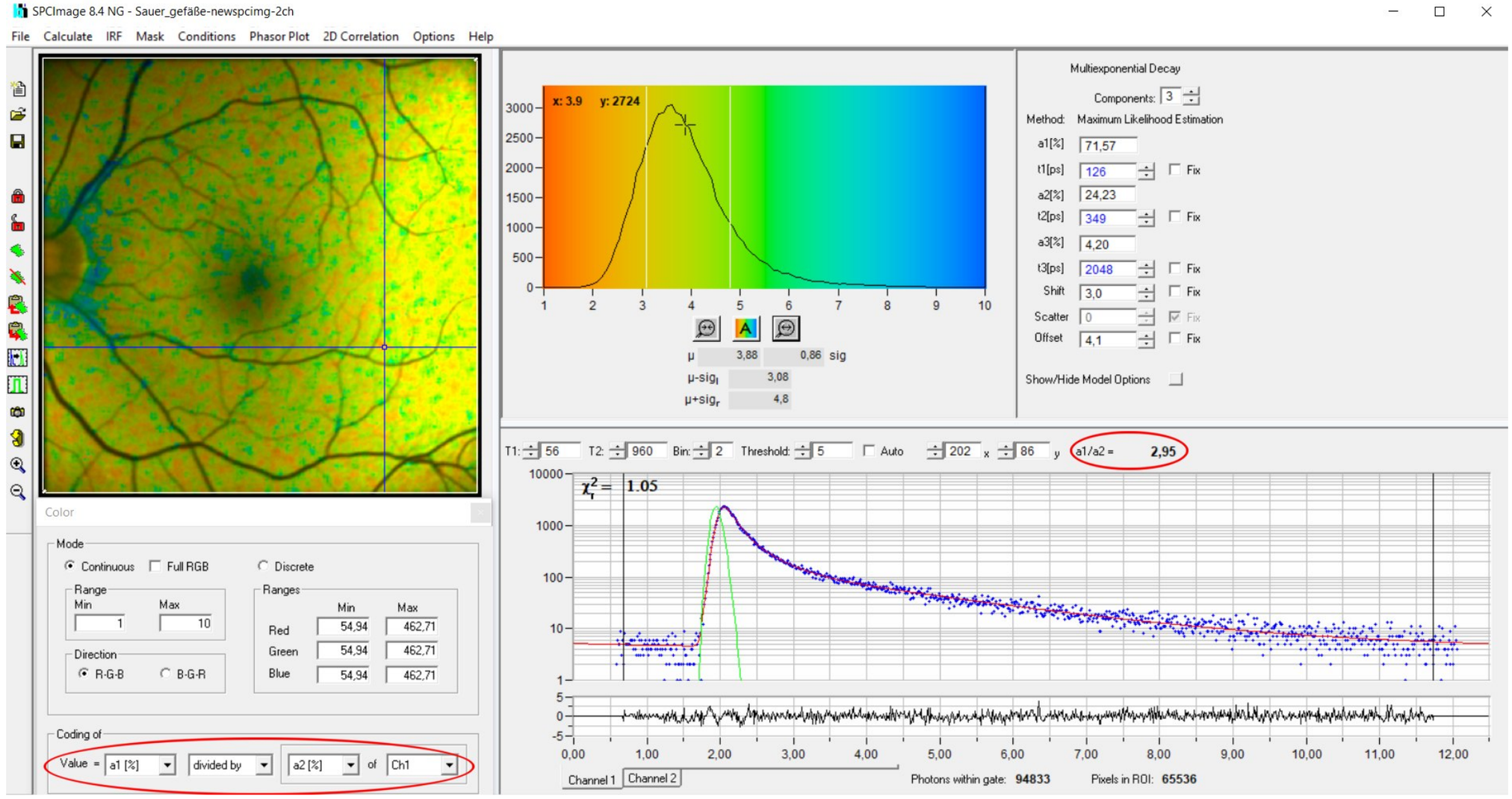
Ratios of Decay Parameters

Ratio t2/t1



Ratios of Decay Parameters

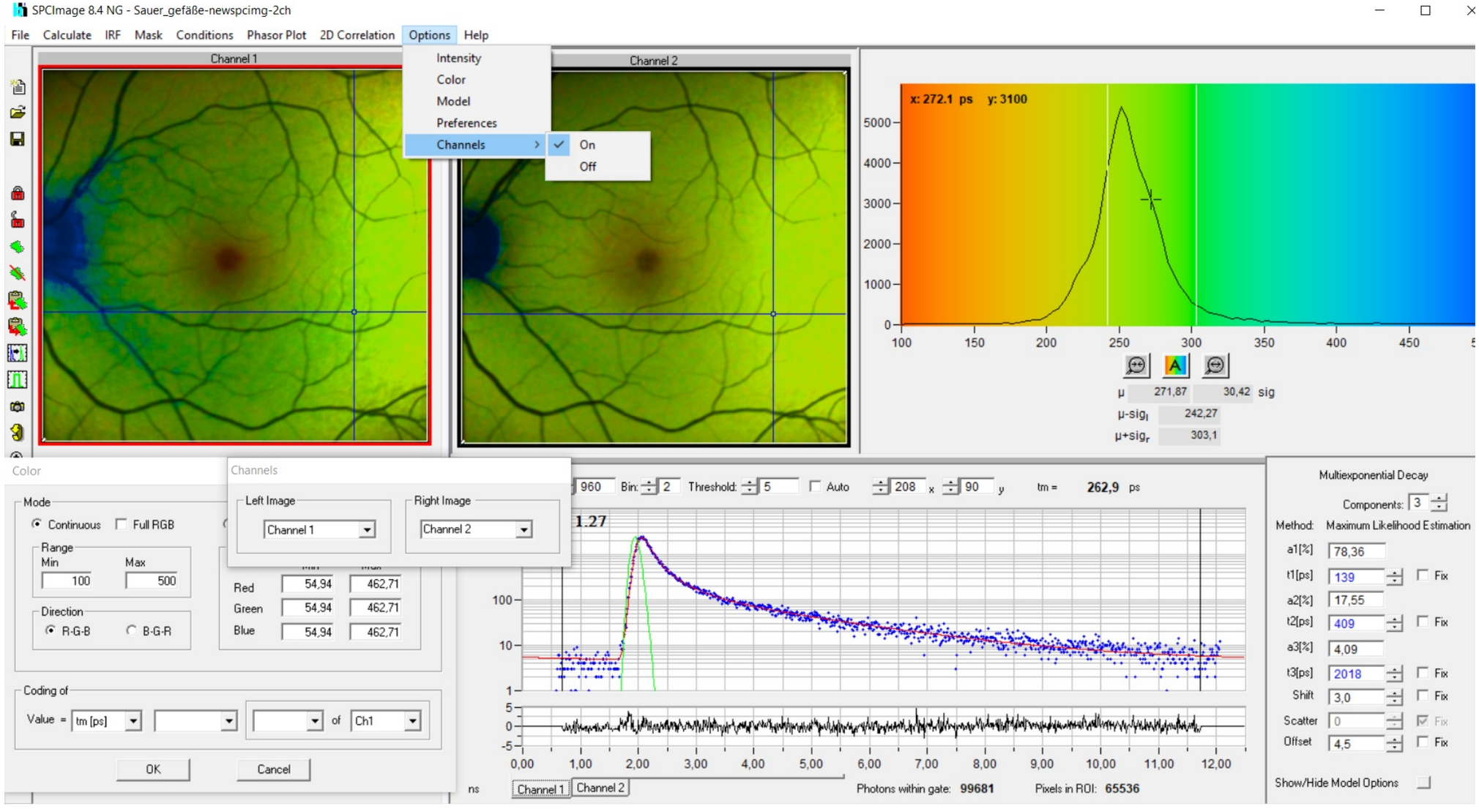
Ratio a1/a2



SPCImage: Dual-Channel Configuration

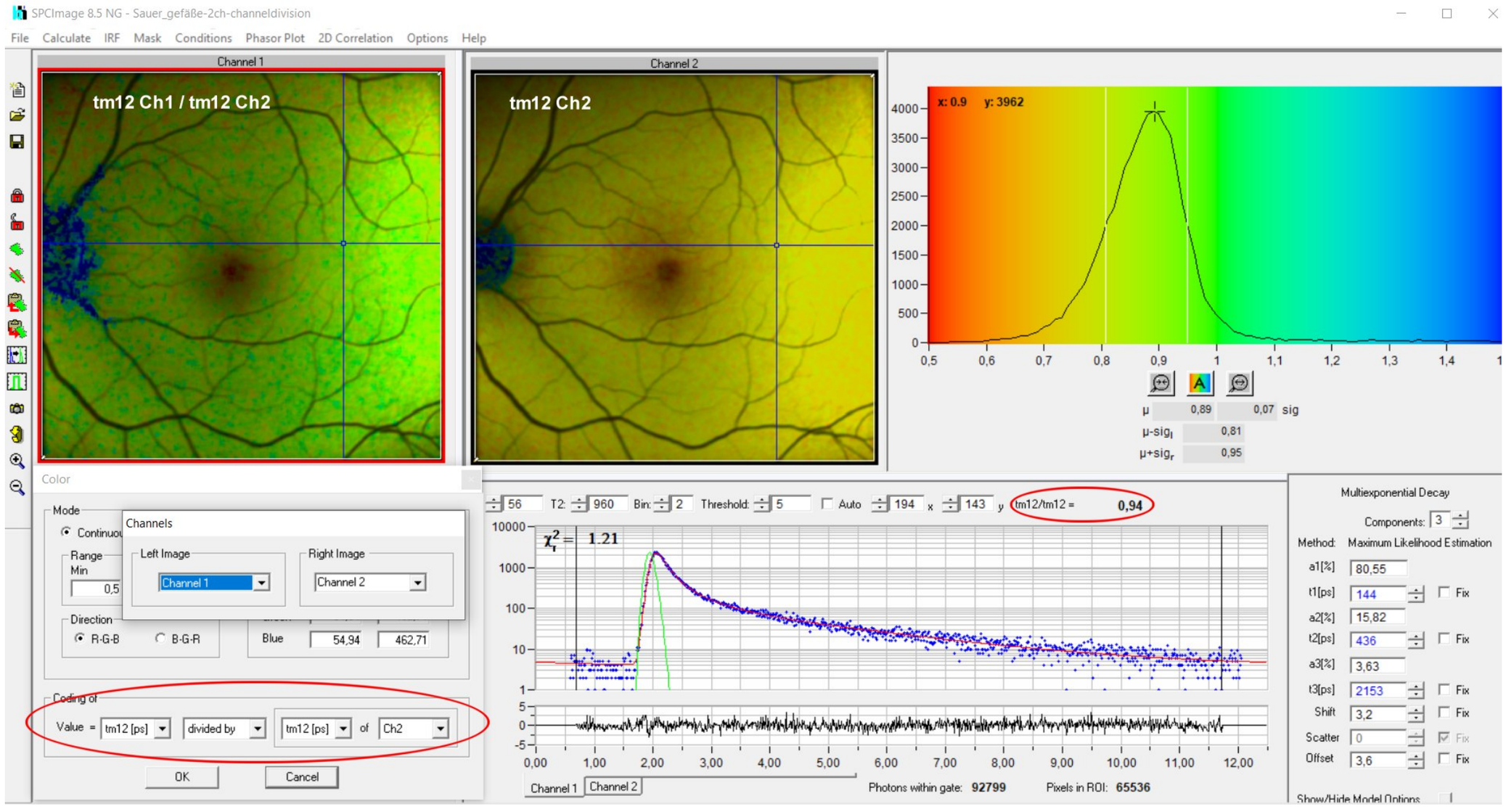
Two Channels, displayed tm_{ch1} and tm_{ch2}

Click into 'Options', 'Channels', select channels for left and right image



SPCImage: Cross-Calculation from Different Channels

Displayed: tm_{ch1} / tm_{ch2} and tm_{ch2}



The Struggle for High Number of Photons

The best lifetime accuracy you can get is

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

Note this is the SNR of the apparent lifteime, τ

Is this all we want?

No!

We may want to determine lifetimes and amplitudes of decay components.

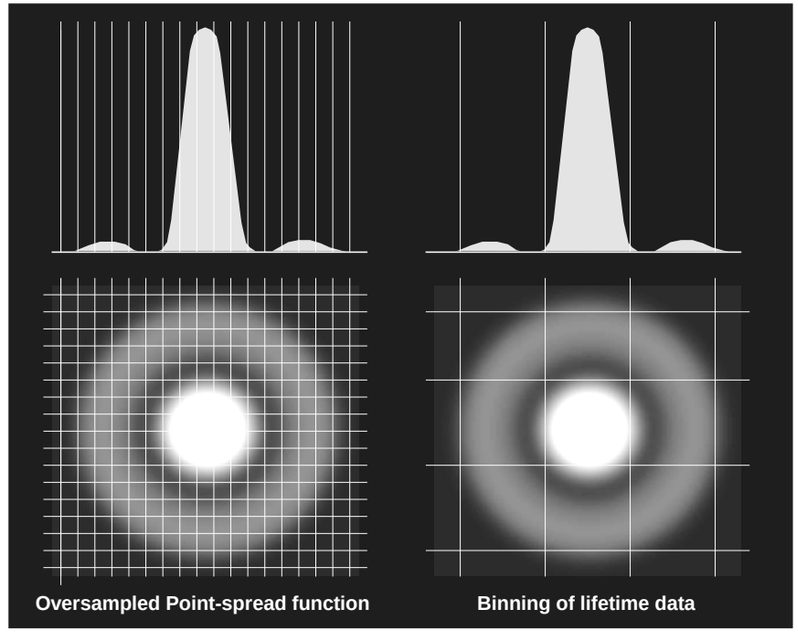
We may want to calculate ratios of these parameters.

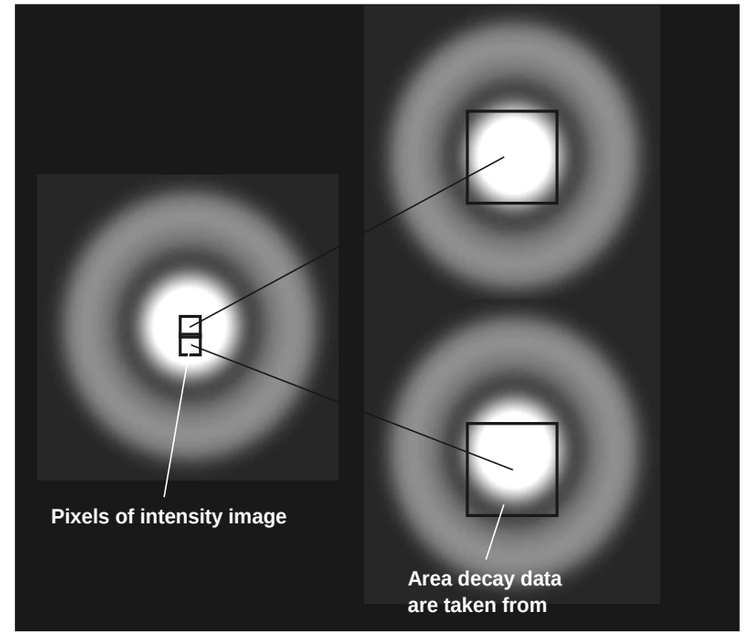
And we want to do so with low noise and high accuracy.

For that we need even more photons!

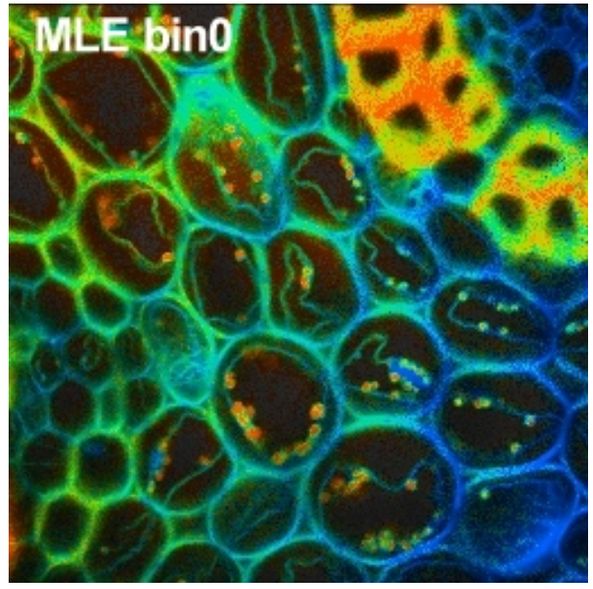
How can we get them without exceedingly long acquisition time?

The SQRT(N) Problem: Get Higher N by Spatial Binning

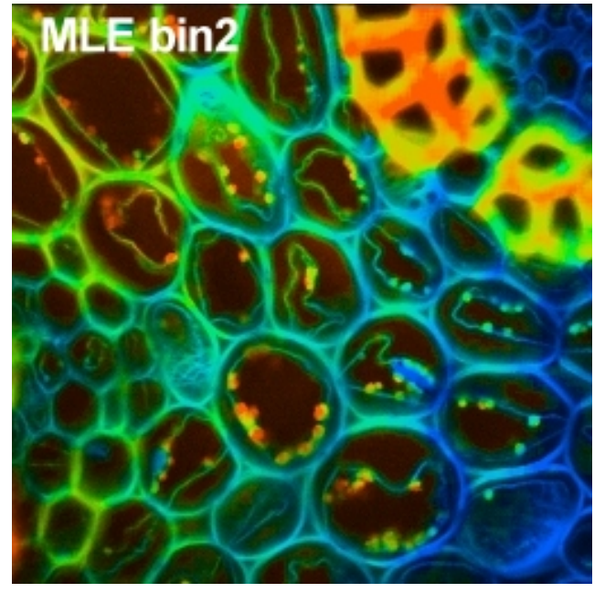




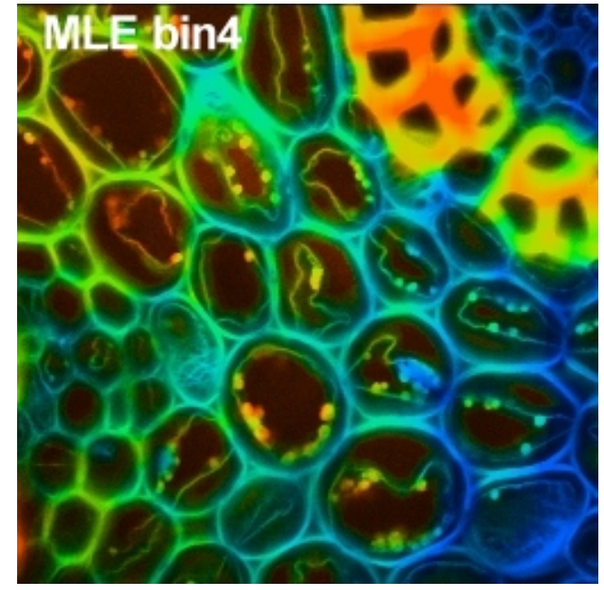
No binning



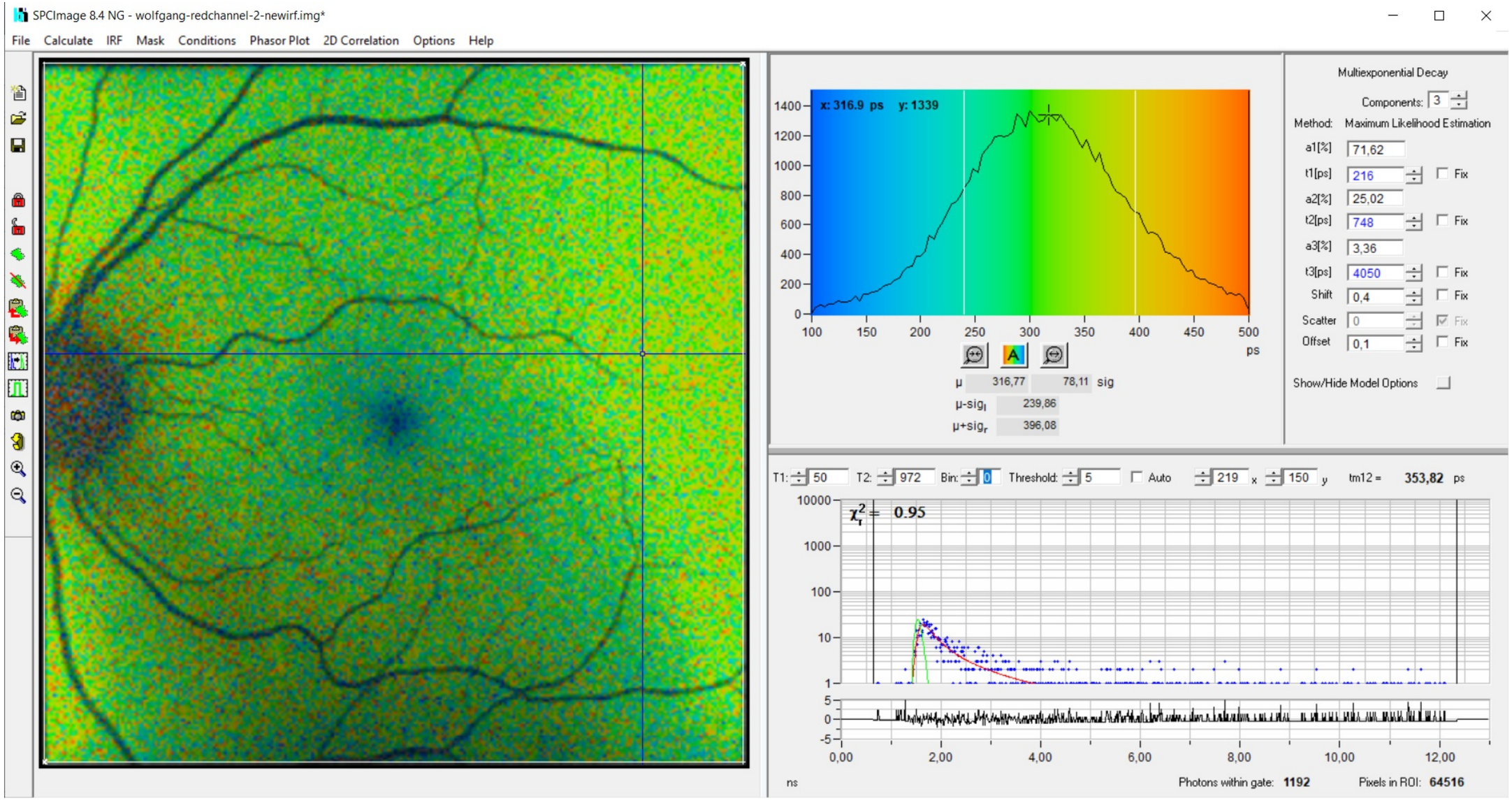
Binning = 2 (5x5)



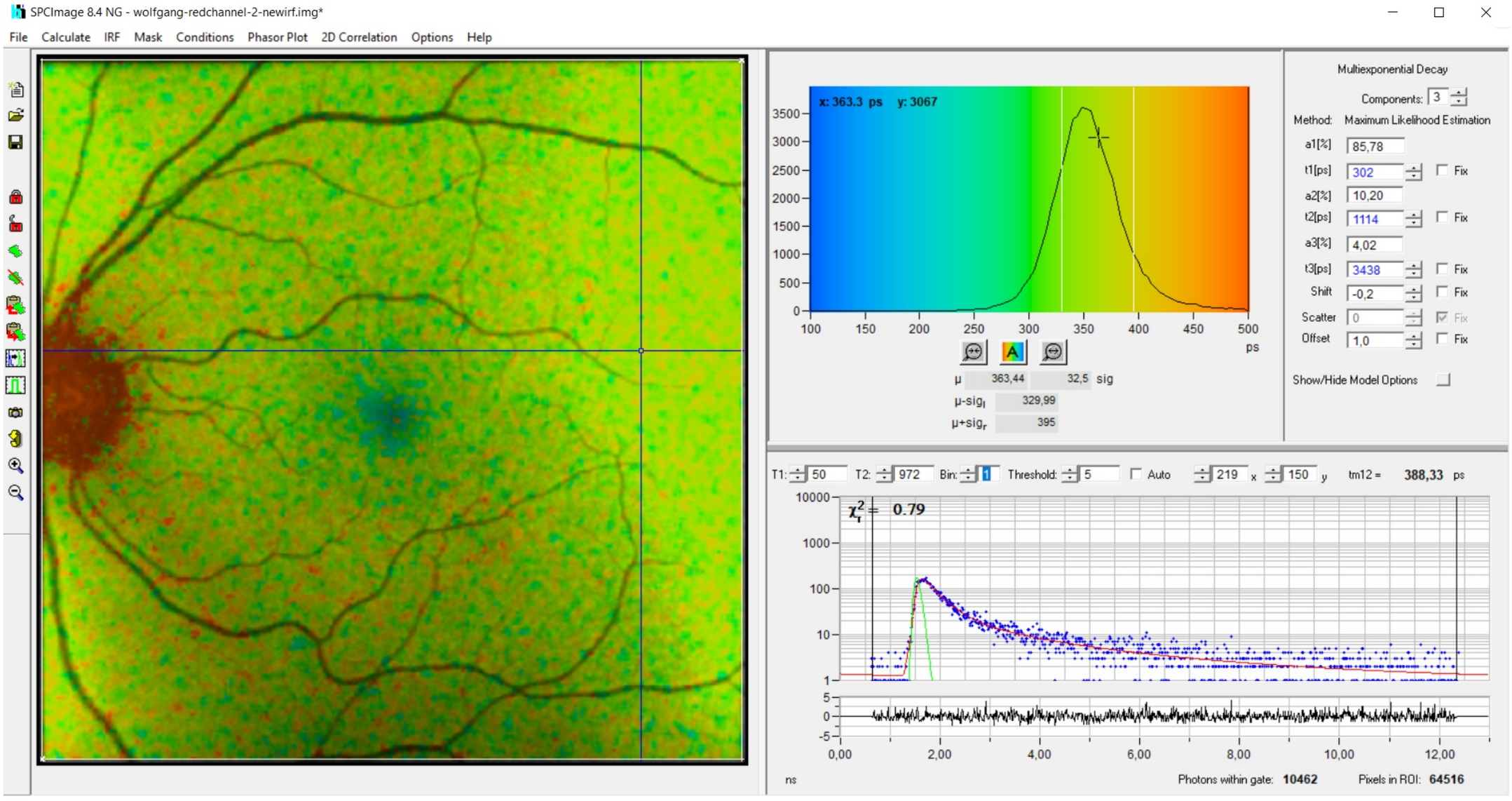
Binning = 4 (9x9)



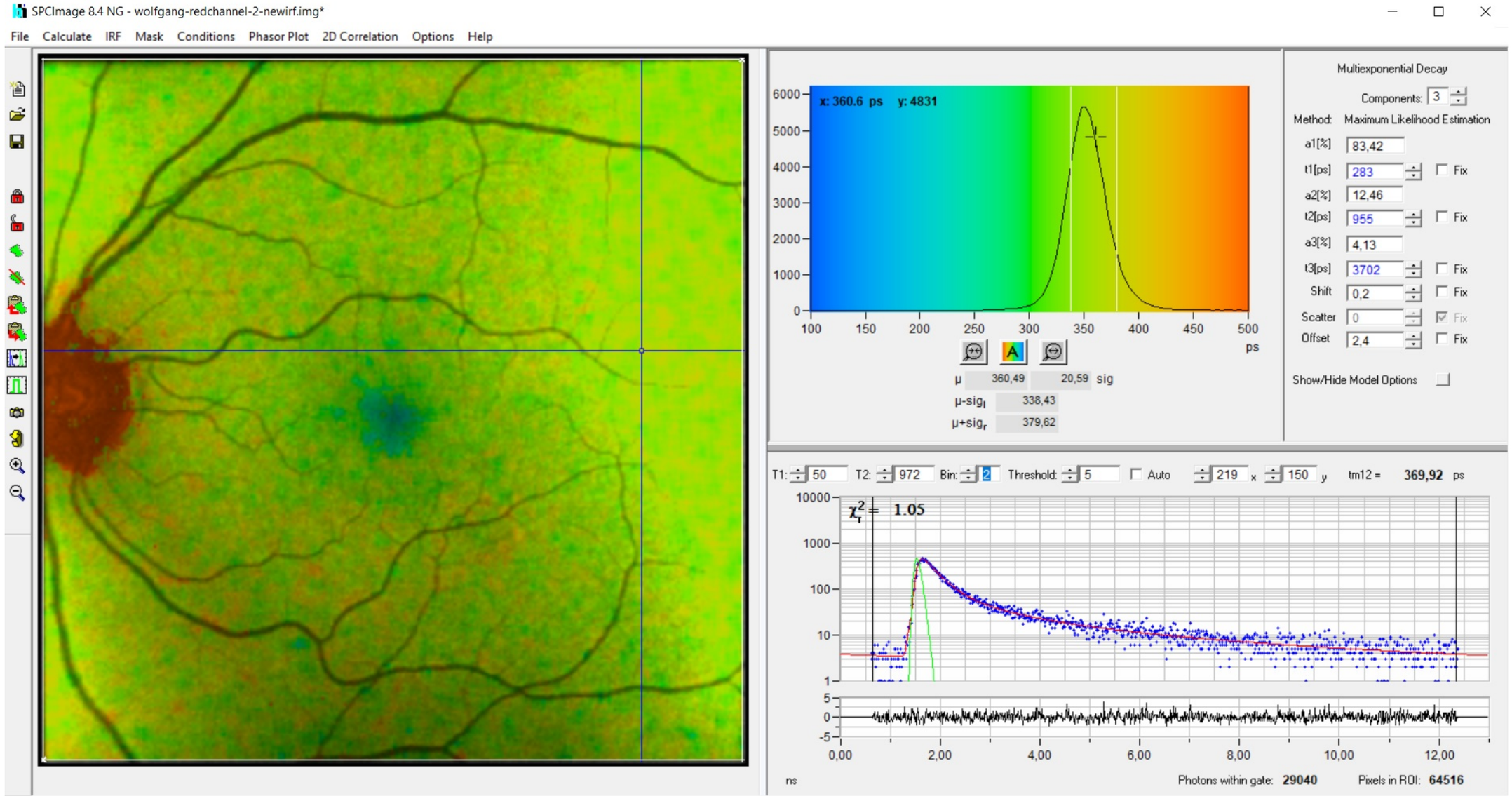
bin = 0: No Binning



bin = 1: Binning 3x3 Pixels

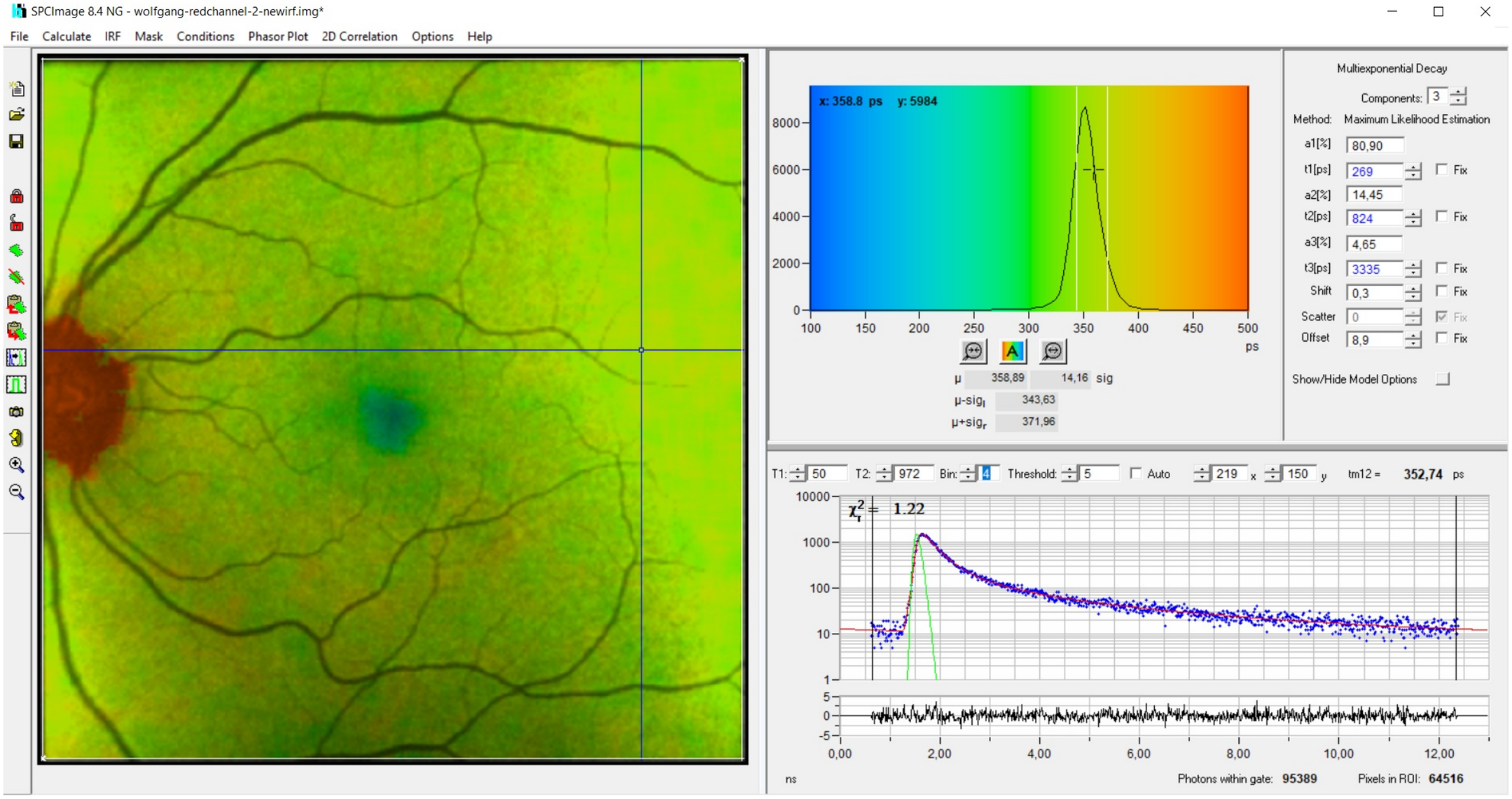


bin = 2: Binning 5x5 Pixels



bin=2 is a good binning factor to start with

Bin = 4: Binning 9x9 Pixels



How far can we go with binning?

How Far Can We Go with Binning?

Many diseases are associated with large-area FLIO signatures
bin = 4 .. 5 may be appropriate in these cases
This is an increase of N by a factor of 100!

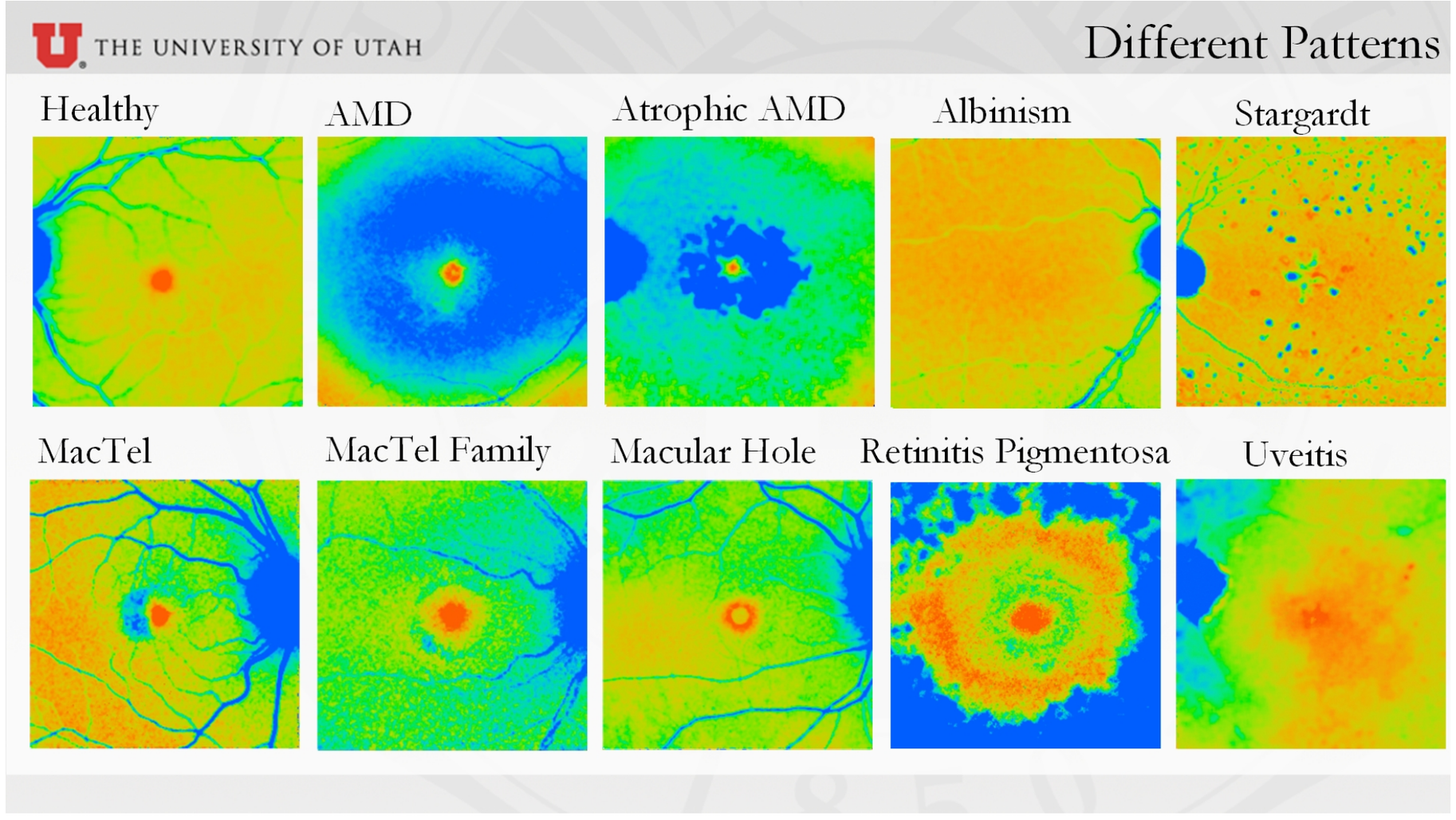
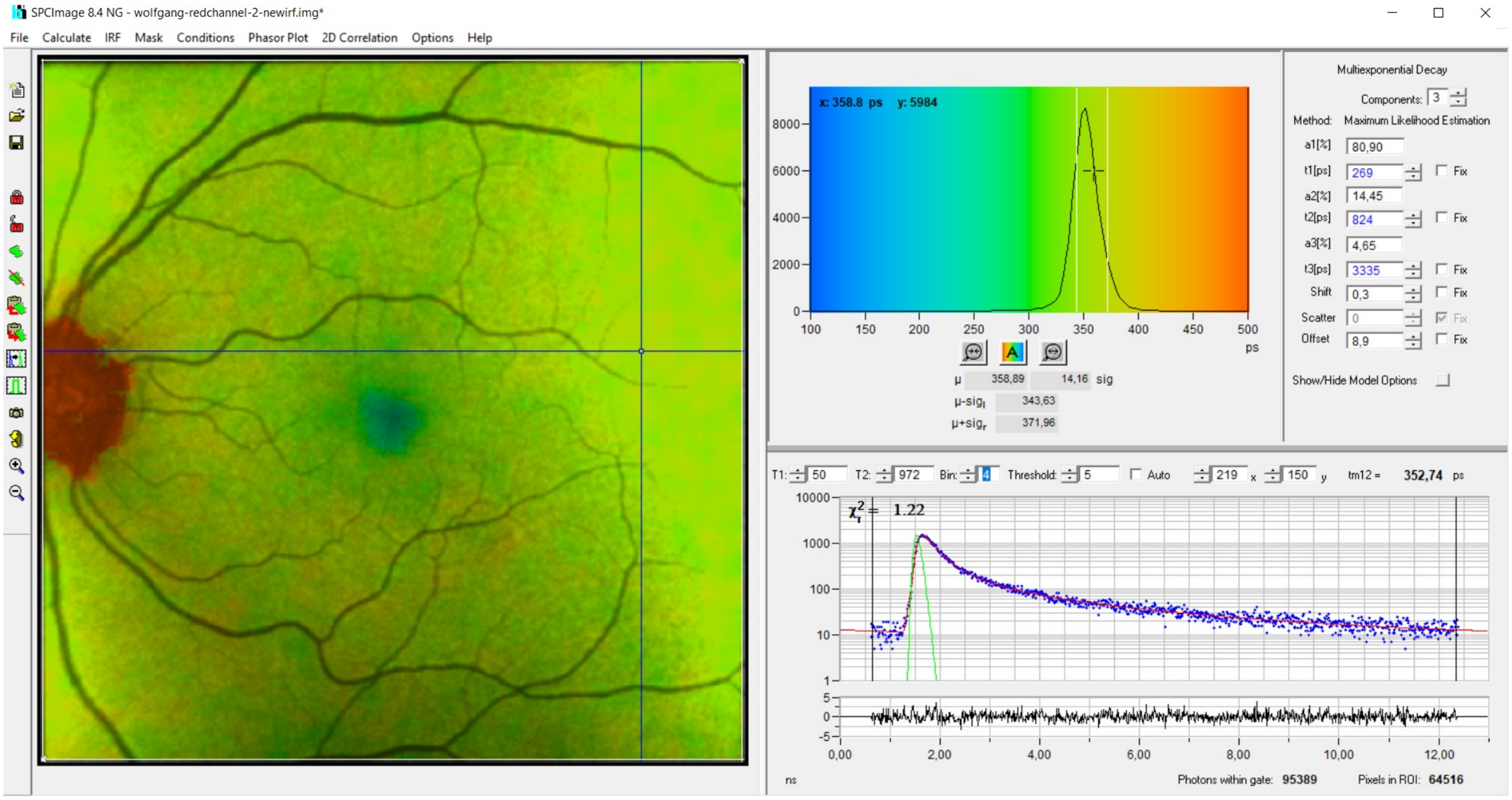


Figure courtesy of Lydia Sauer, Univ. of Utah

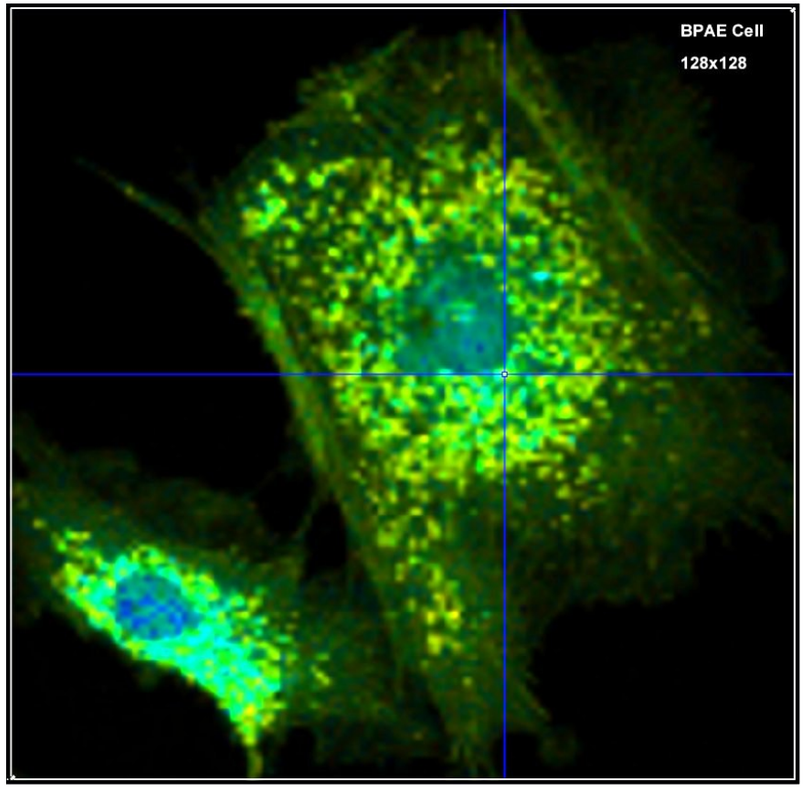
Question: Can we record with higher pixel number and instead use binning in SPCImage?

Bin = 4: Binning 9x9 Pixels



An Example from Microscopy

Image with 128 x 128 Pixels, no Binning



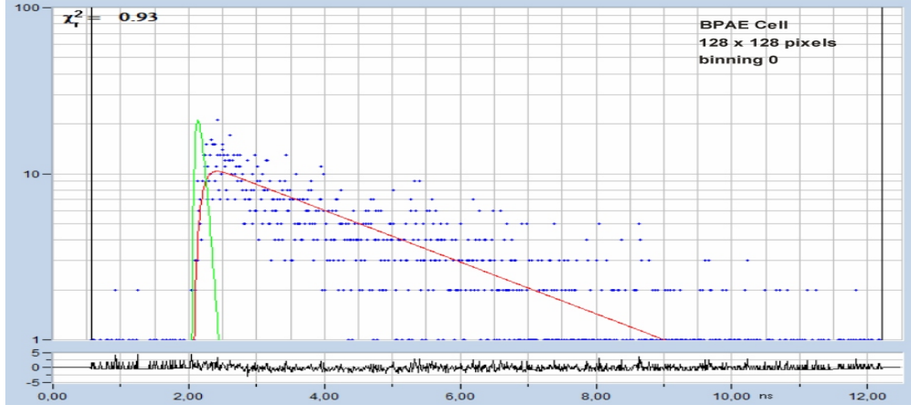
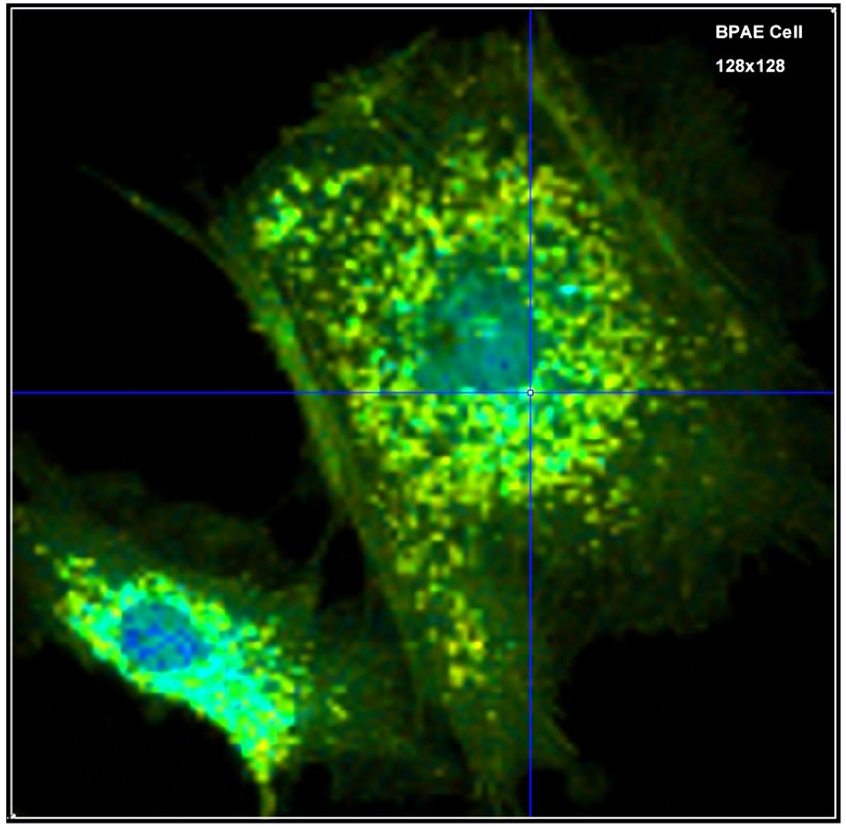


Image with 128 x 128 Pixels, no Binning



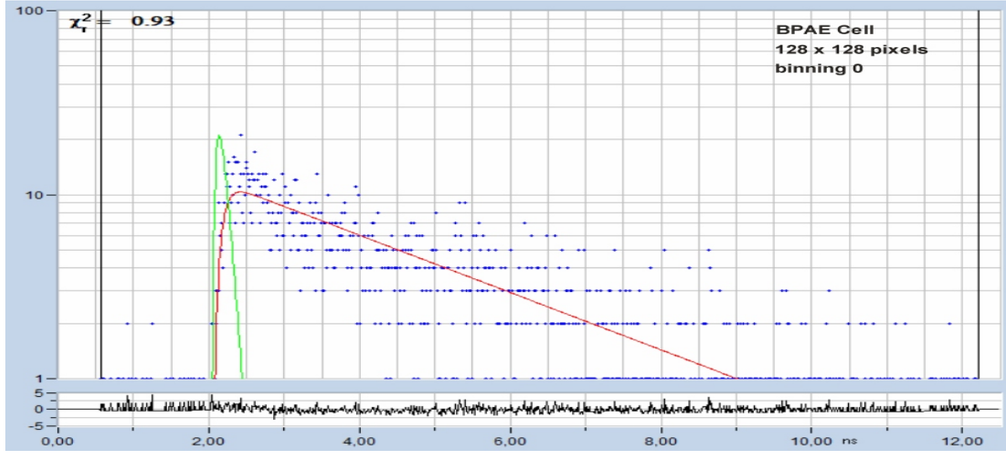
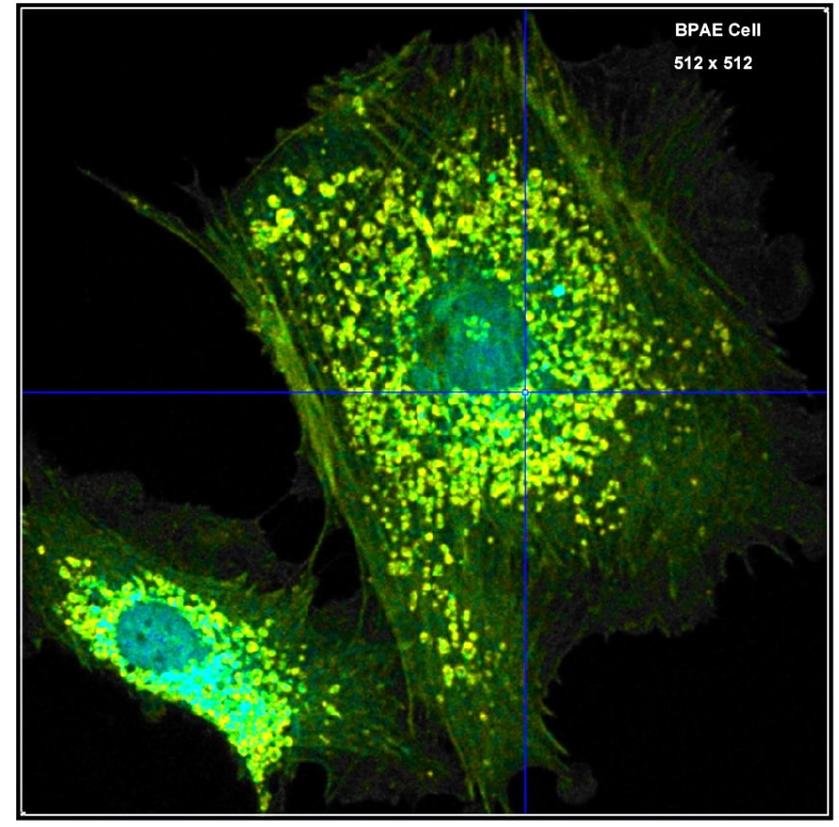
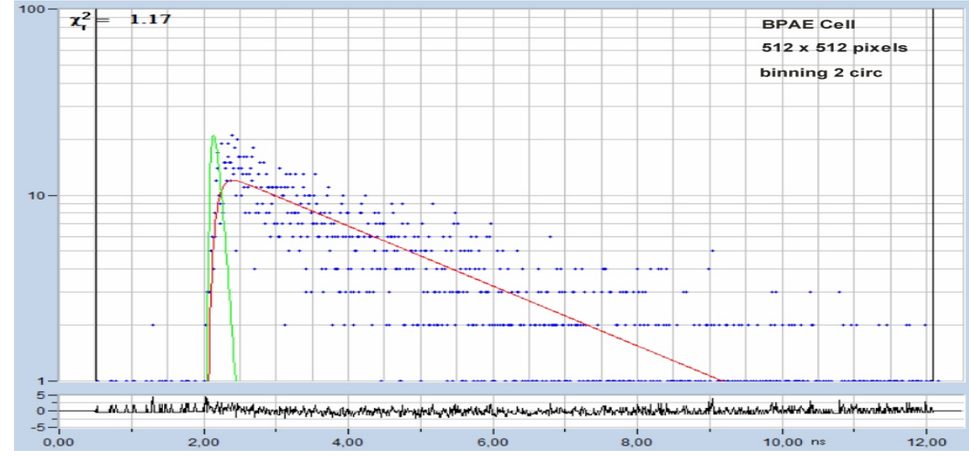
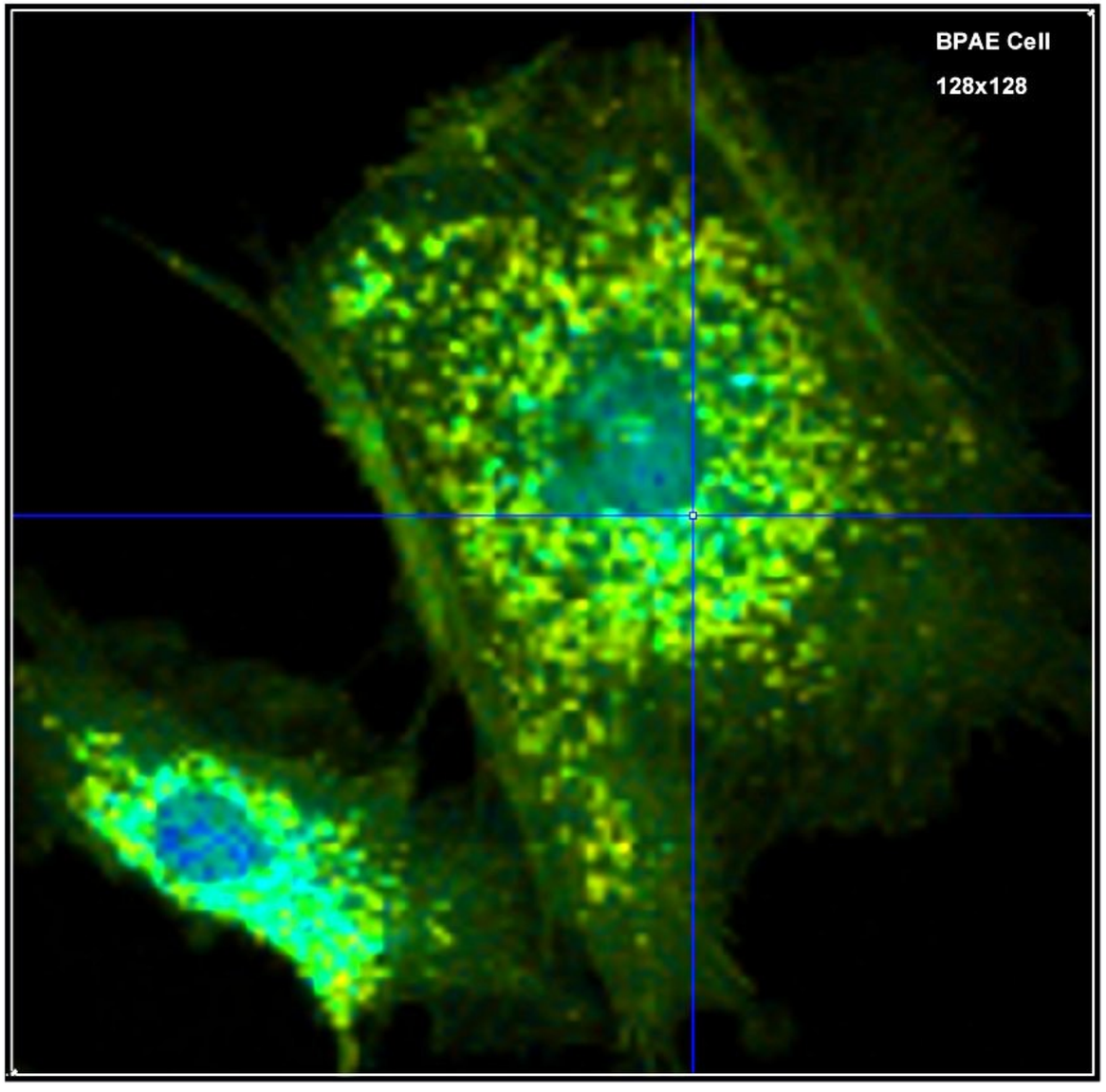


Image with 512 x 512 Pixels and Binning

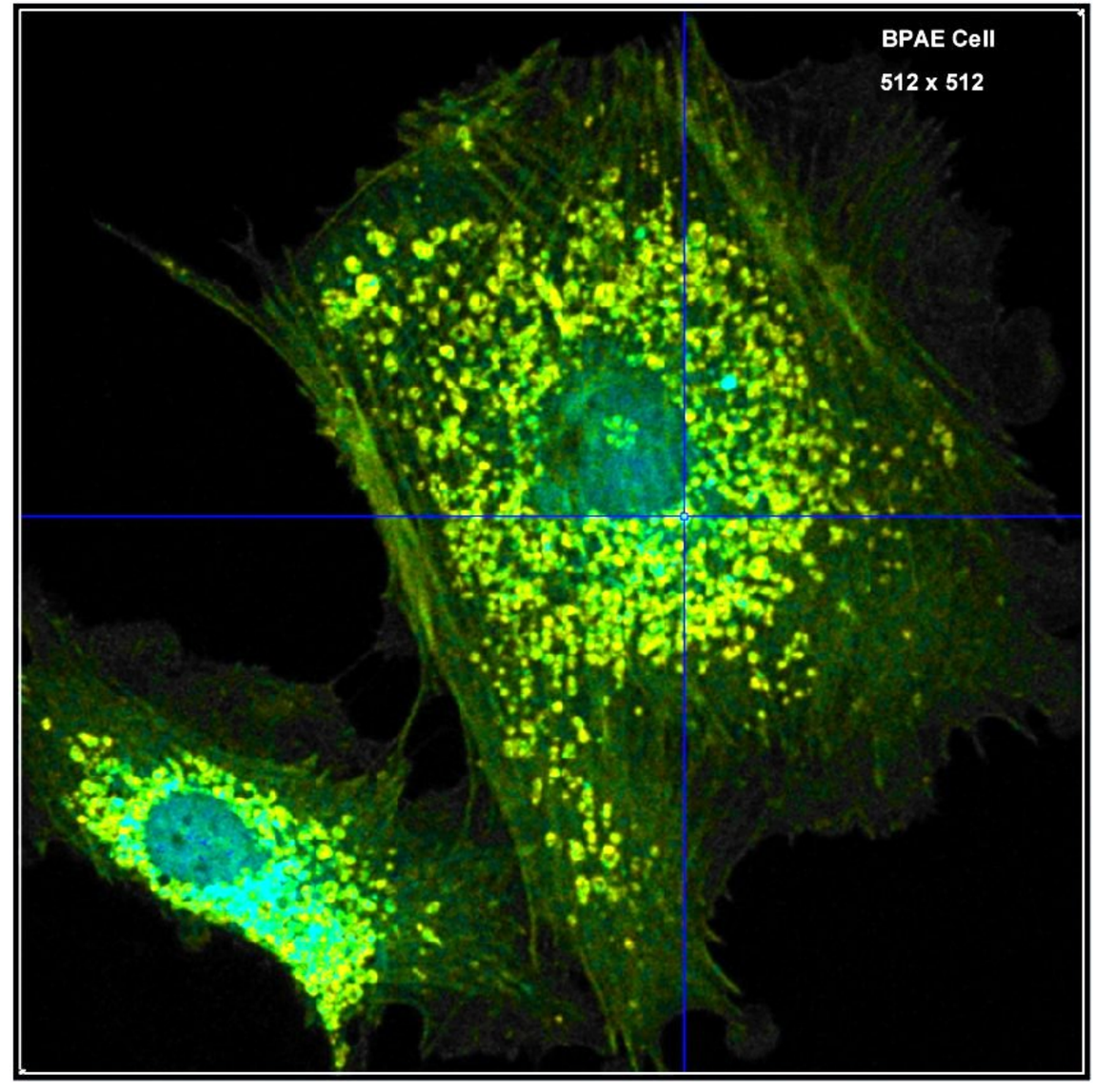




128 x 128 Pixels, no Binning



512 x 512 Pixels, Binning 21 pixels



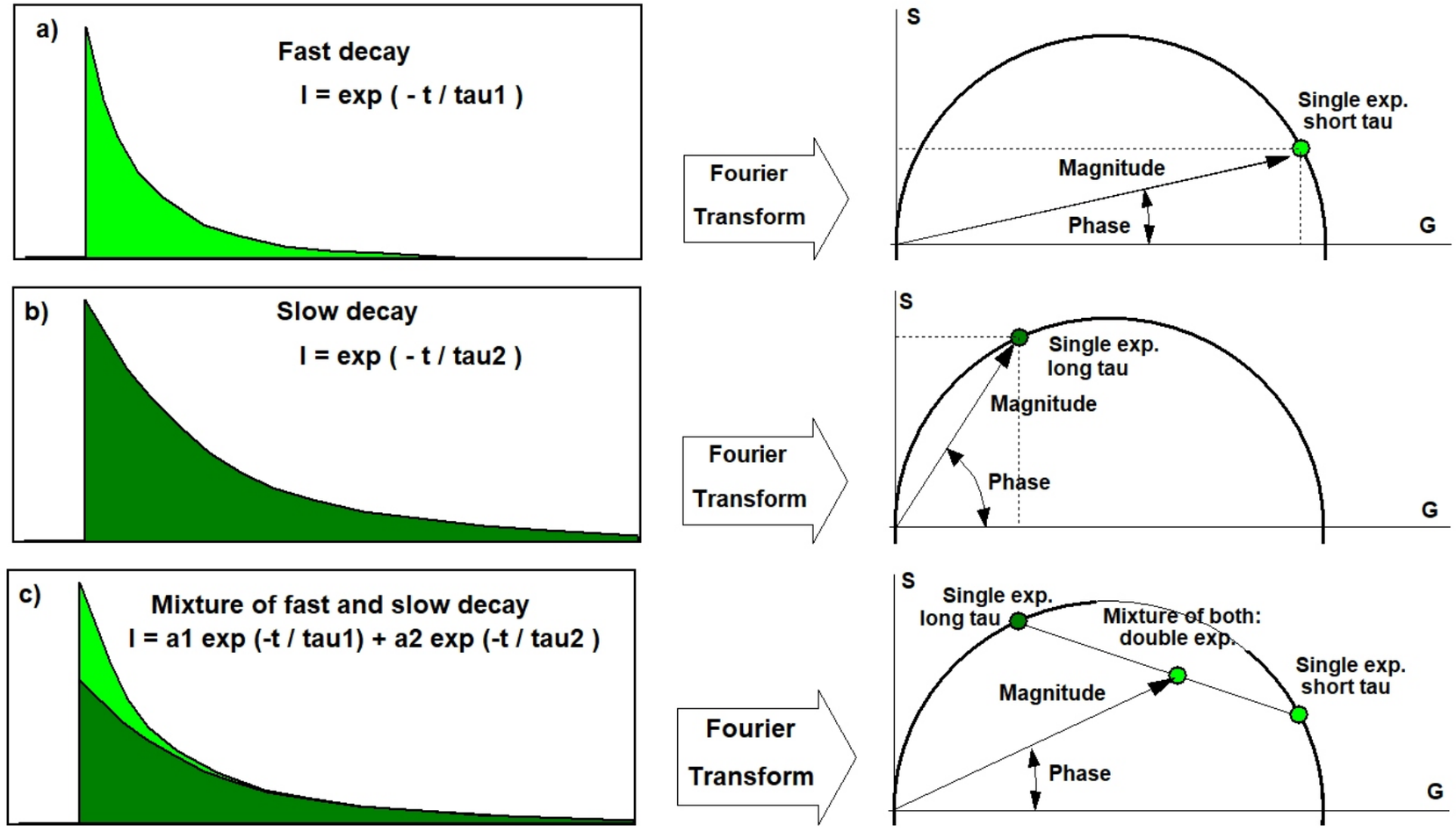
Should we go for 512x512-pixel FLIM images?

Can we get more photons than by binning?

The Phasor Plot

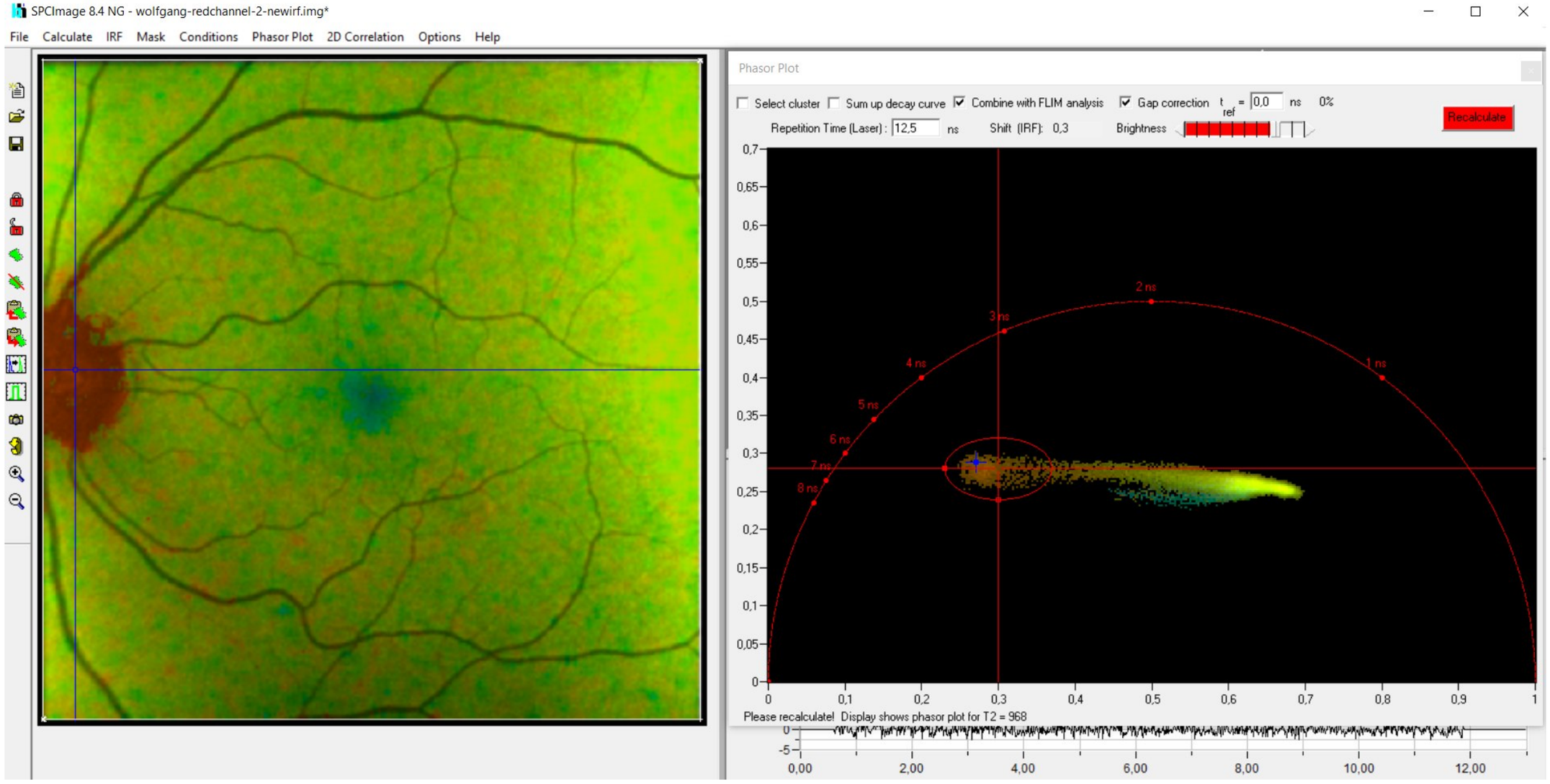
Transformation from time-domain into frequency domain

The shape of a decay curve is represented by magnitude and phase



Phasor Plot

Phasors for all pixels of an image



Every pixel of the image forms a dot in the phasor plot

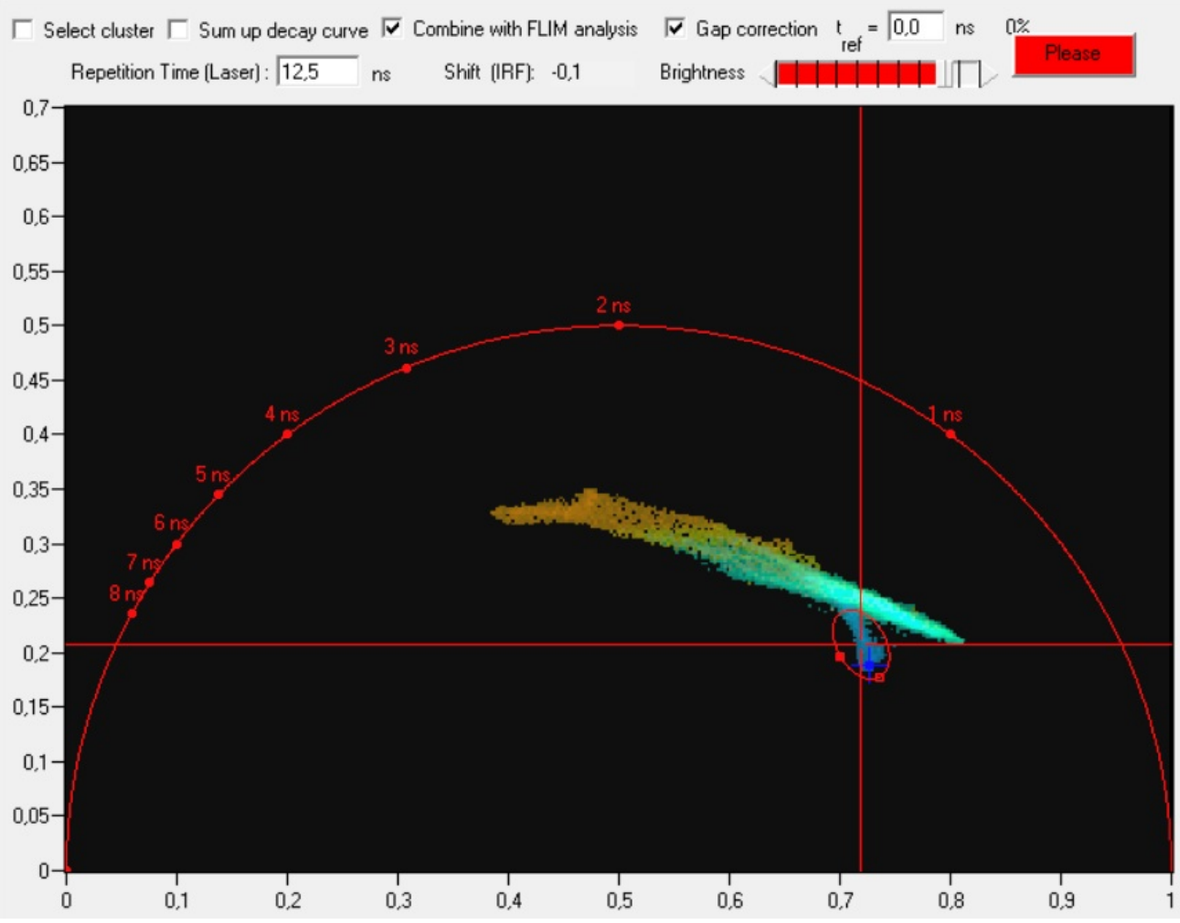
The location of this dot depends on the shape of the decay function in this pixel

Is there clinical information directly visible in the phasor plot?

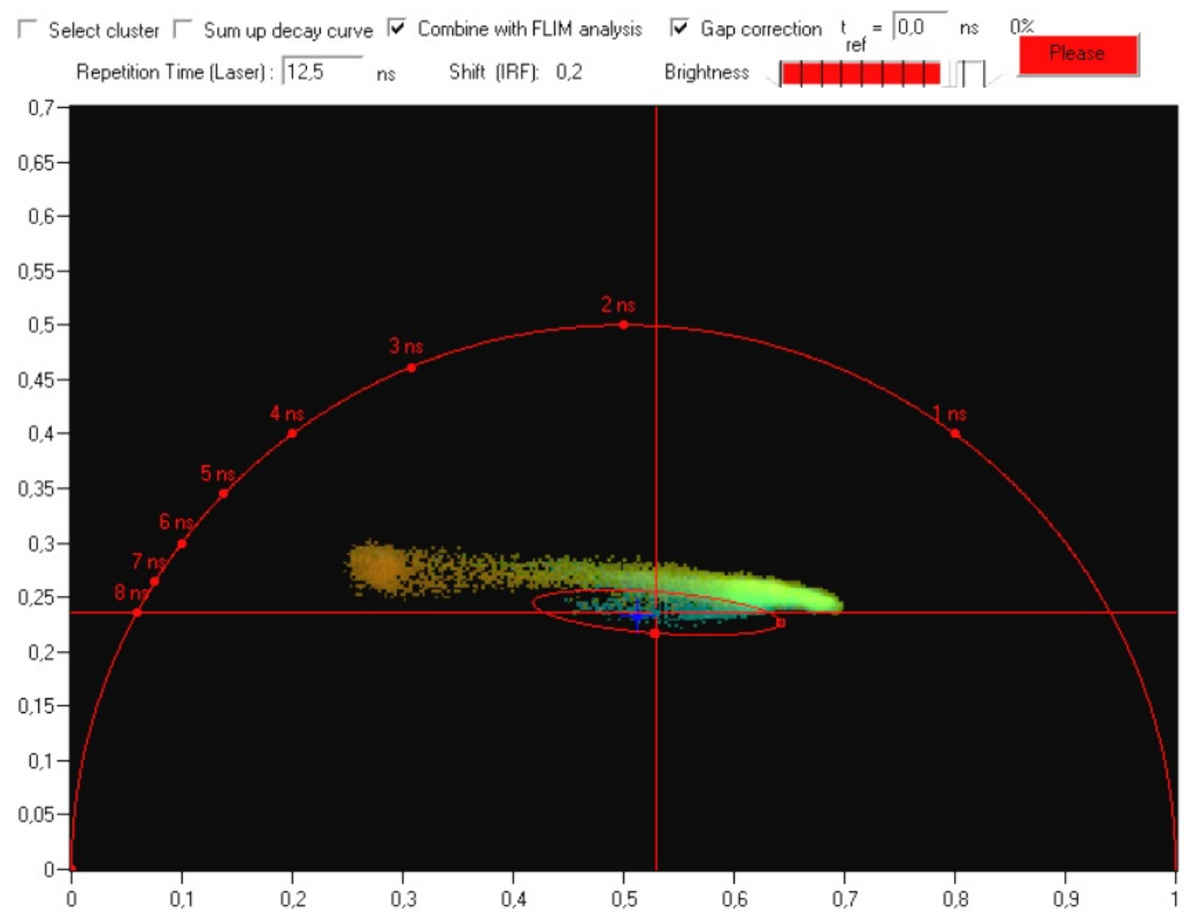
Different patients, fovea marked by red ellipse

Why are the phasor clusters of the fovea different?

Healthy 25 years



Healthy 62 years

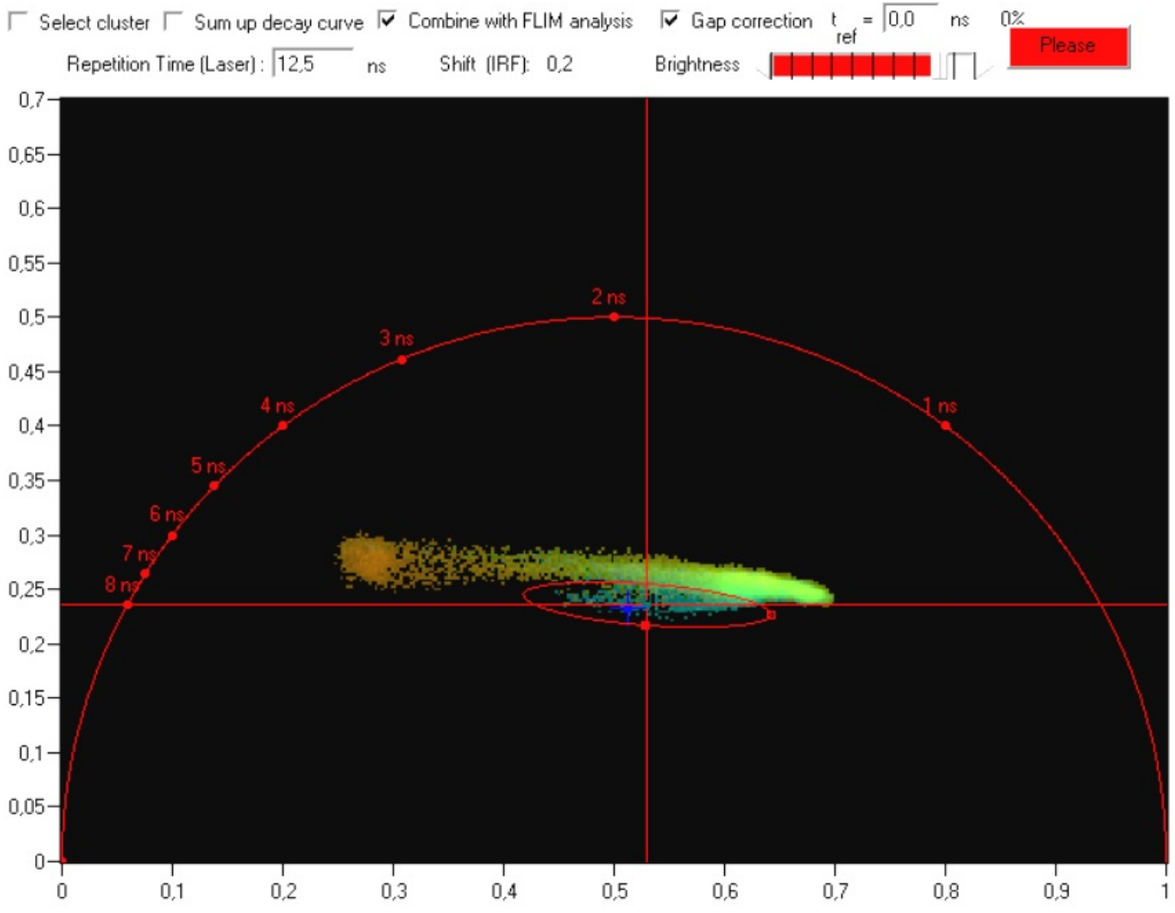


Is there clinical information directly visible in the phasor plot?

Different patients, fovea marked by red ellipse

Comparison with AMD Patient

62 Years Patient



AMD Patient

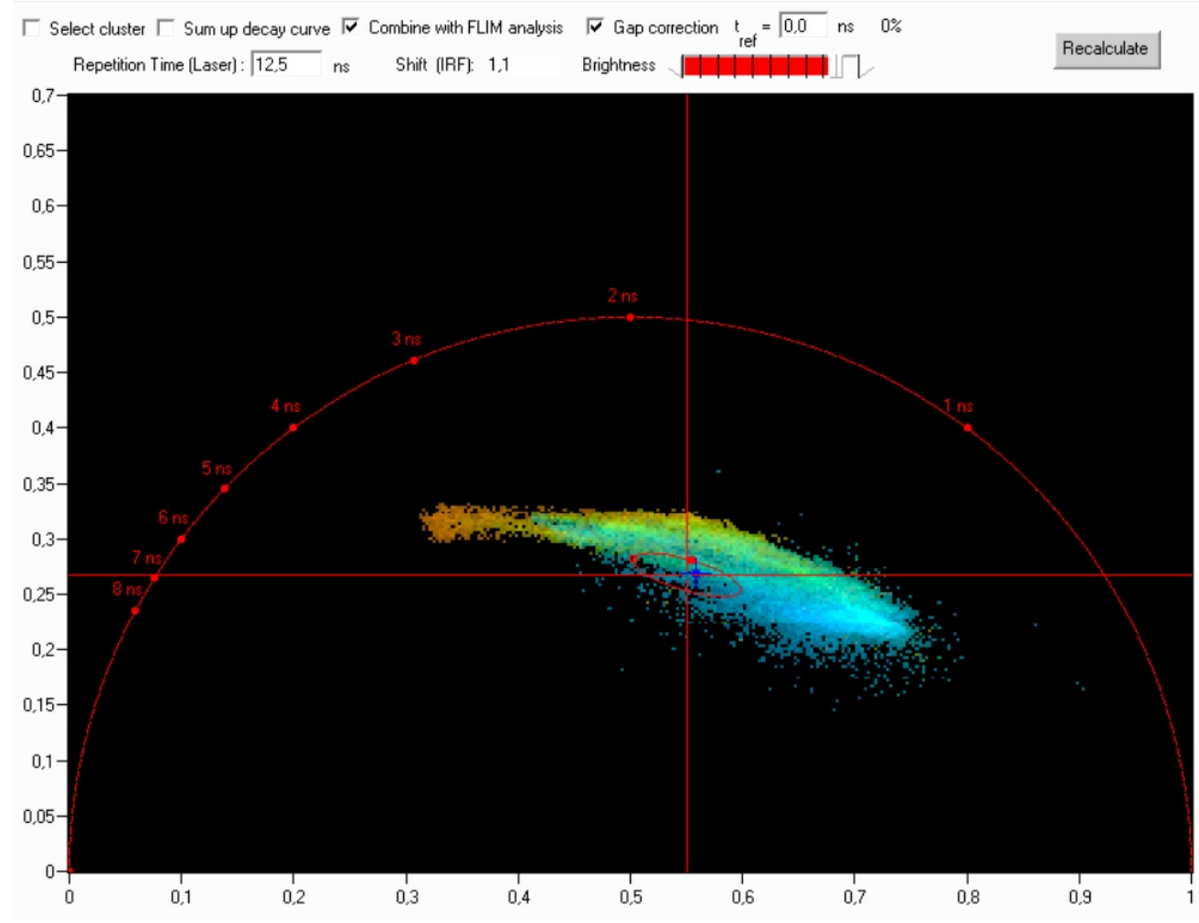


Image Segmentation by Phasor Plot

Select Cluster in Phasor Plot - Optical Disc

Back-annotate in lifetime image

Sum up decay data in selected region - obtain high-N decay function

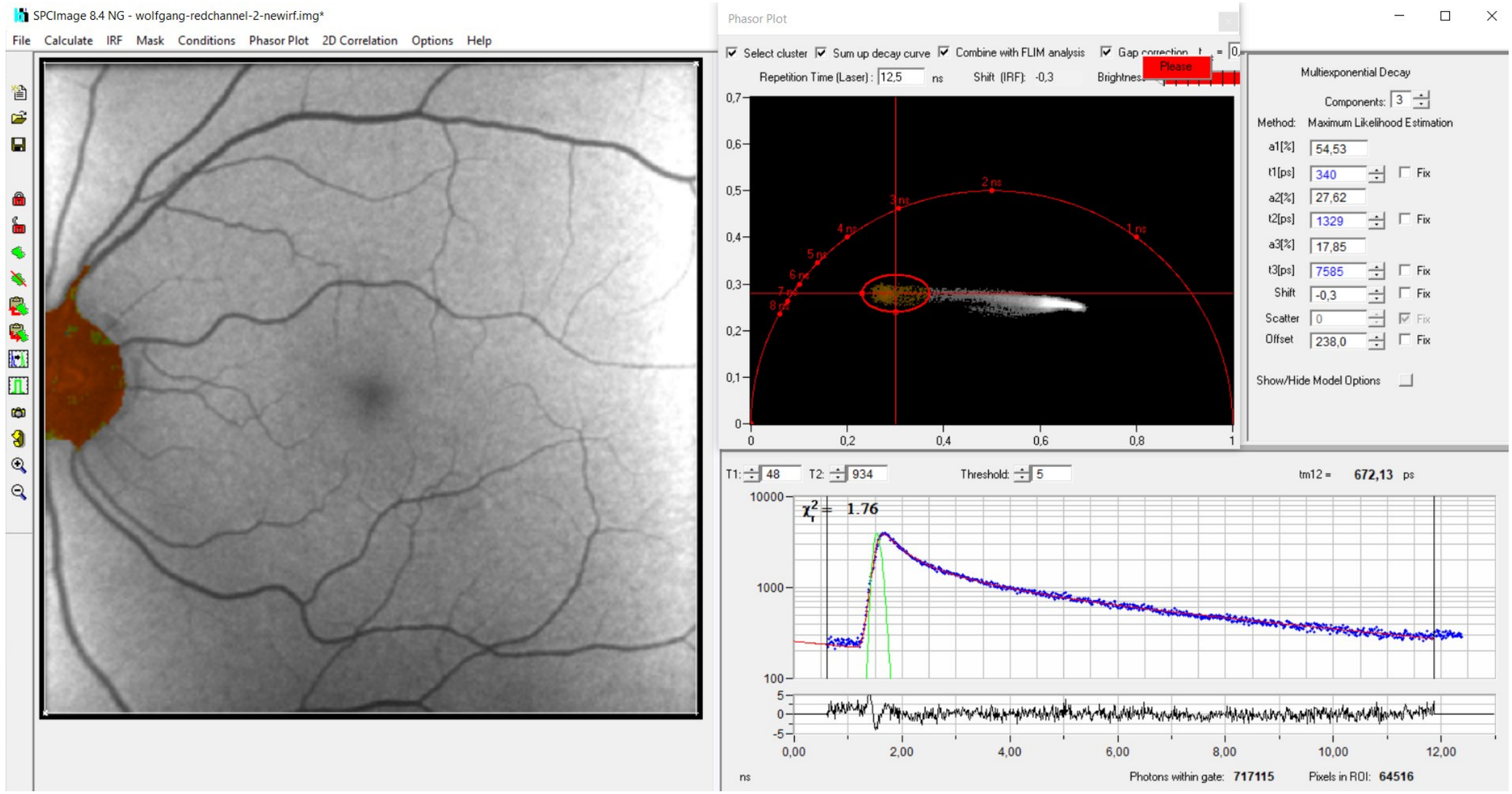


Image Segmentation by Phasor Plot

Select Cluster in Phasor Plot - Fovea

Back-annotate in lifetime image

Sum up decay data in selected region - obtain high-N decay function

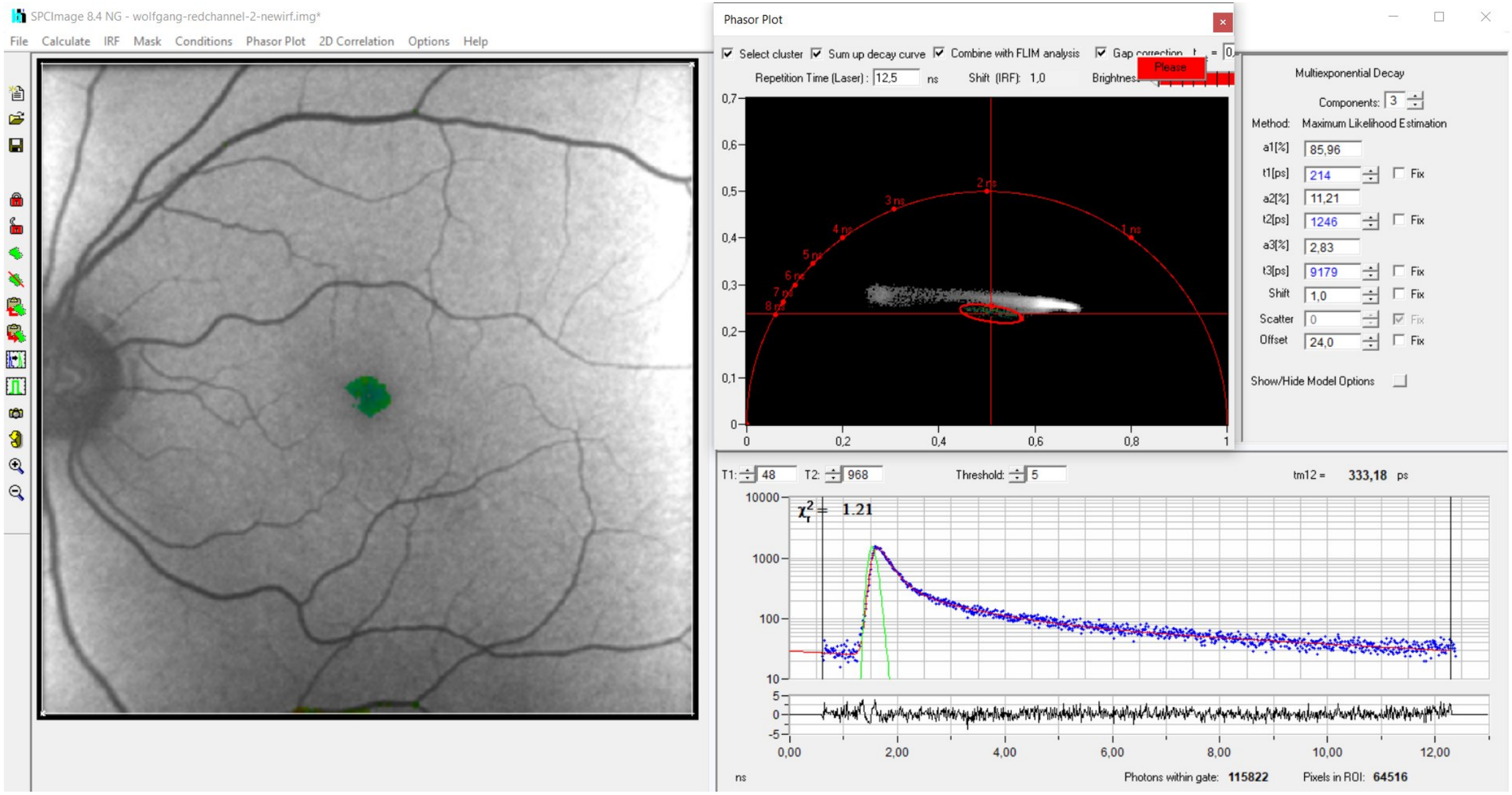


Image Segmentation by Phasor Plot

Selection of deposits along blood vessels

Back-annotate in lifetime image

Sum up decay data in selected region - obtain high-N decay function

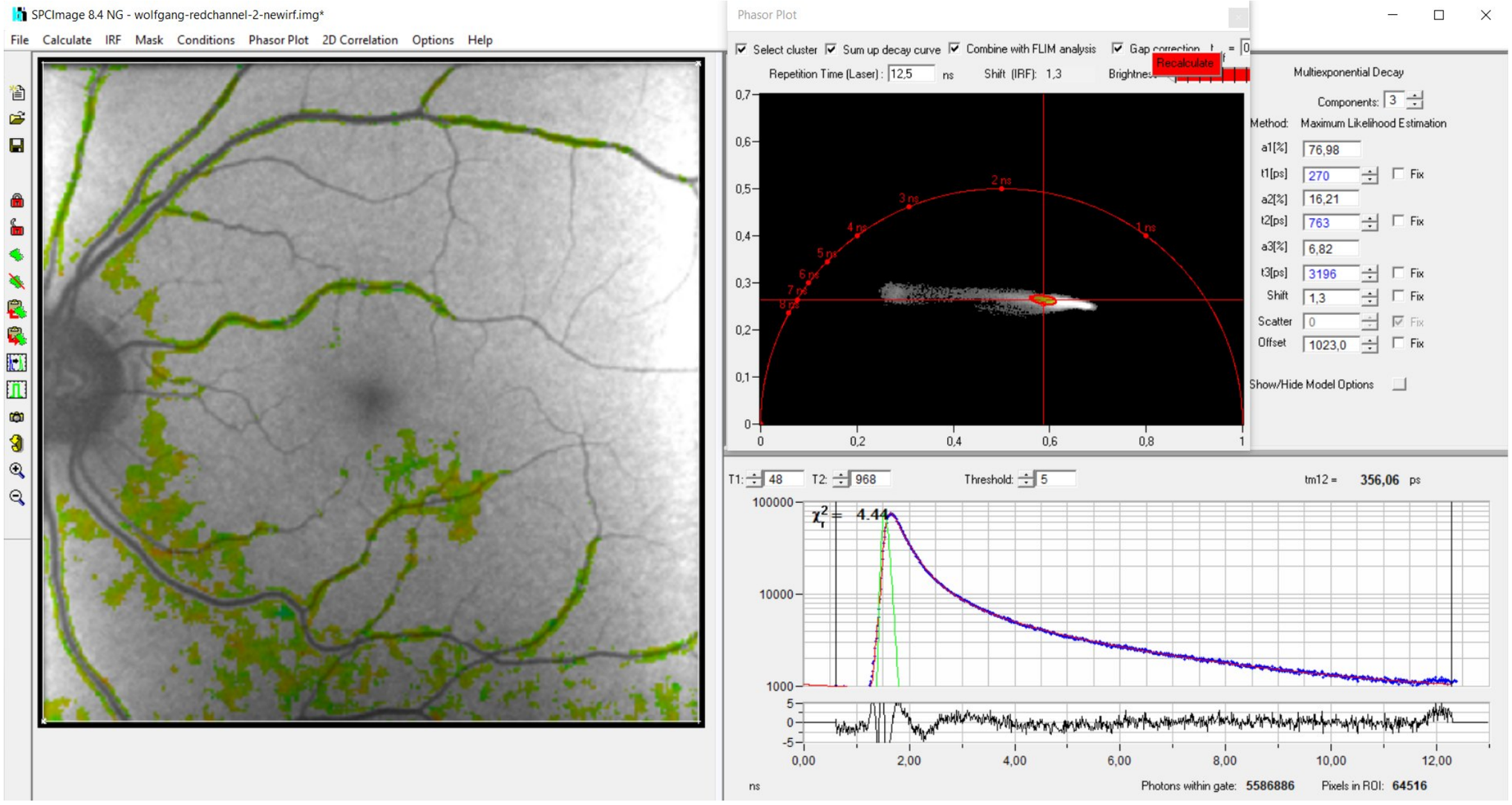
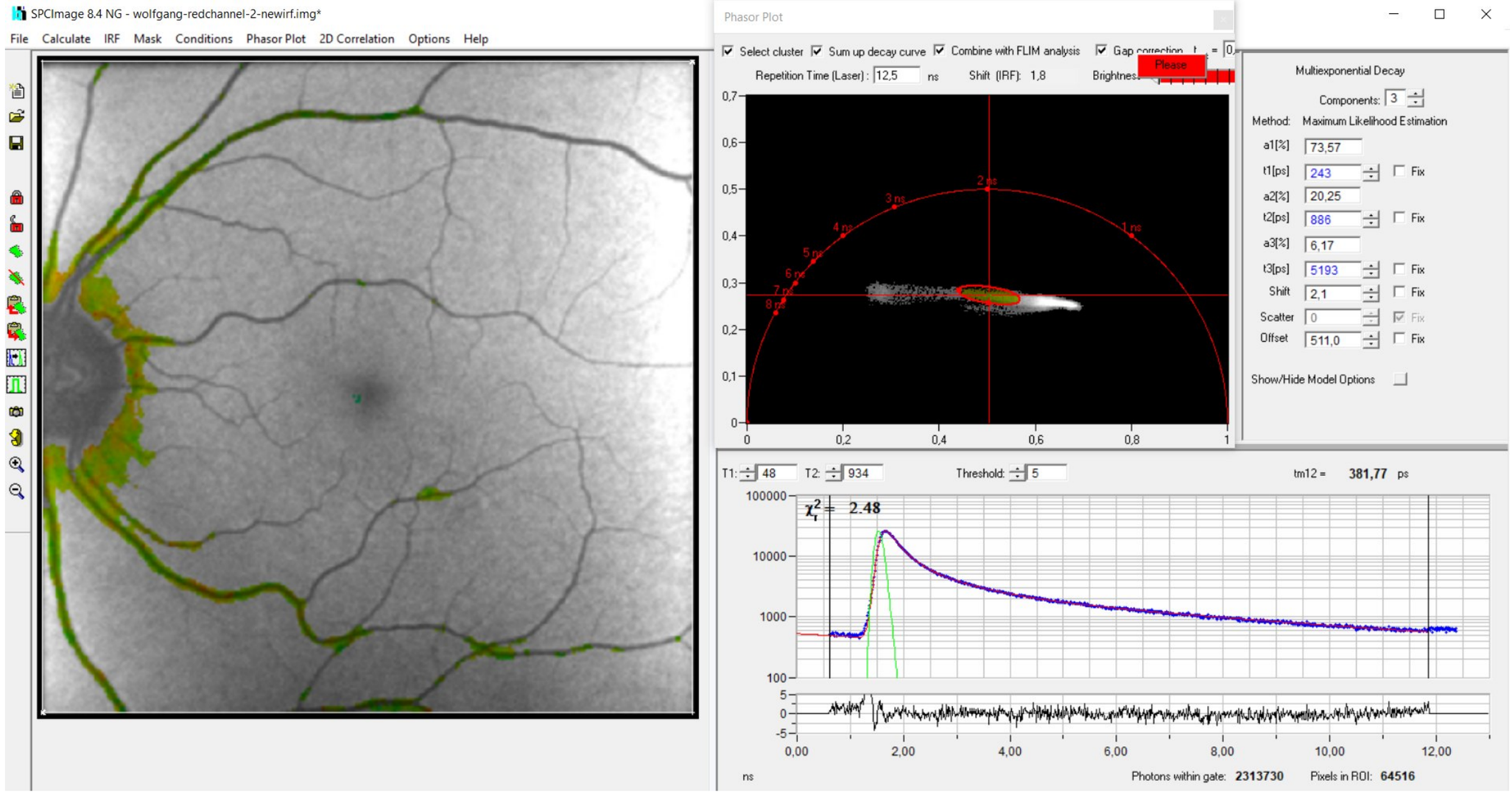


Image Segmentation by Phasor Plot

Selection of area around optical disc

Back-annotate in lifetime image

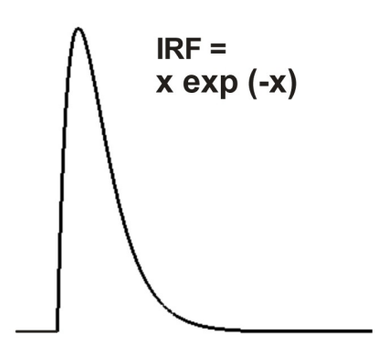
Sum up decay data in selected region - obtain high-N decay function

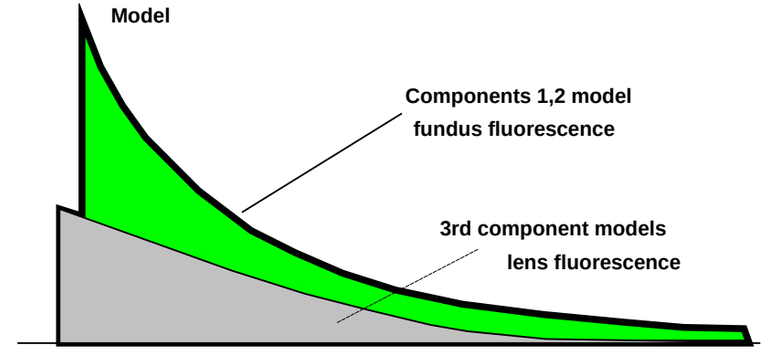


What Should be the Plan for the Near Future?

Use the new data-analysis principles!

Profite from the better reproducibility!



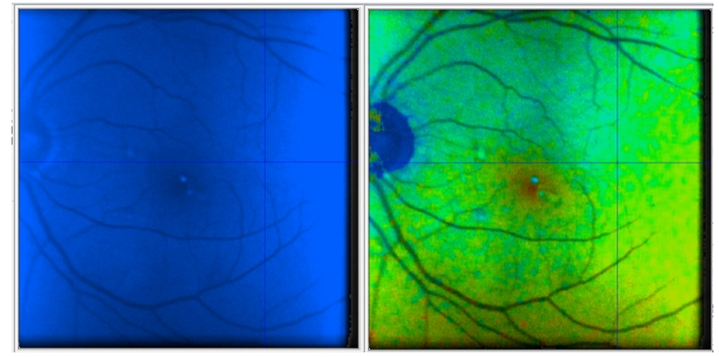


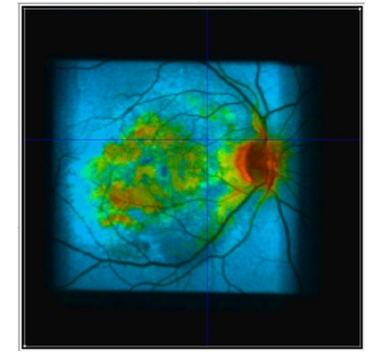
Re-evaluate existing data!

Squeeze out more information from them!

Separate the fundus from the lens!

Look at component data!

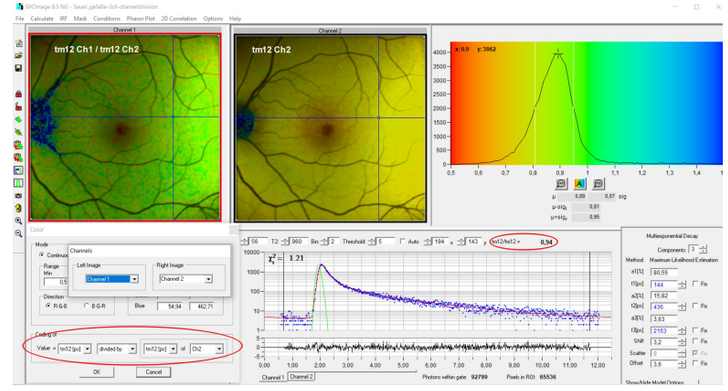


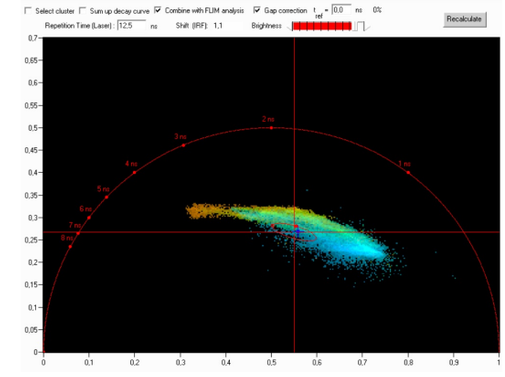


Get data for early stages of diseases!

Try the new approaches on them!

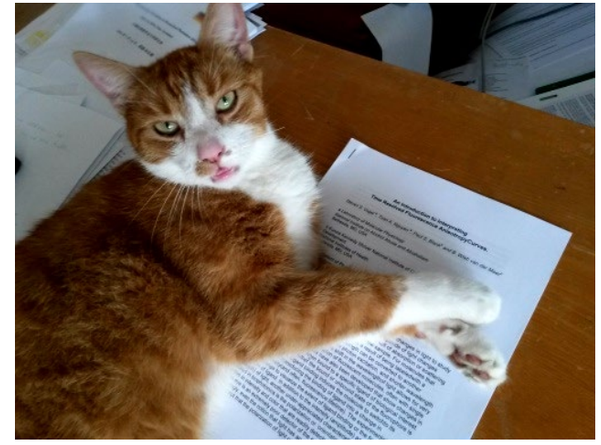
Find out which approach shows the signs of a particular disease best!





Get a GPU for that! A $200 device is enough!

Keep us in the line when new results, new data and manuscripts for new papers are available! This helps us refine the new analysis procedures.



Provide a fast and easy way to exchange data!