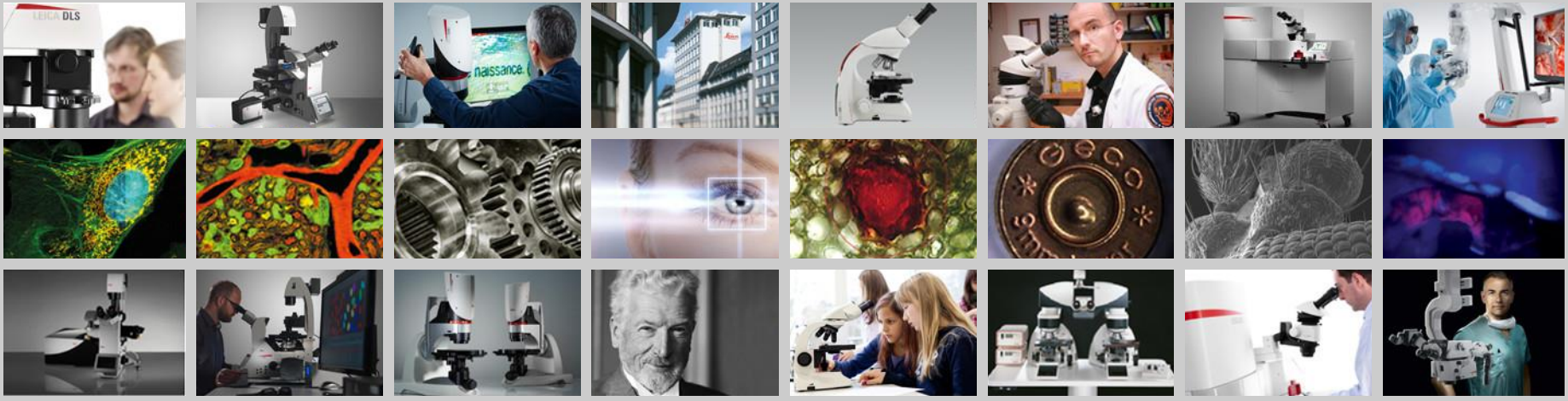
From Eye to Insight

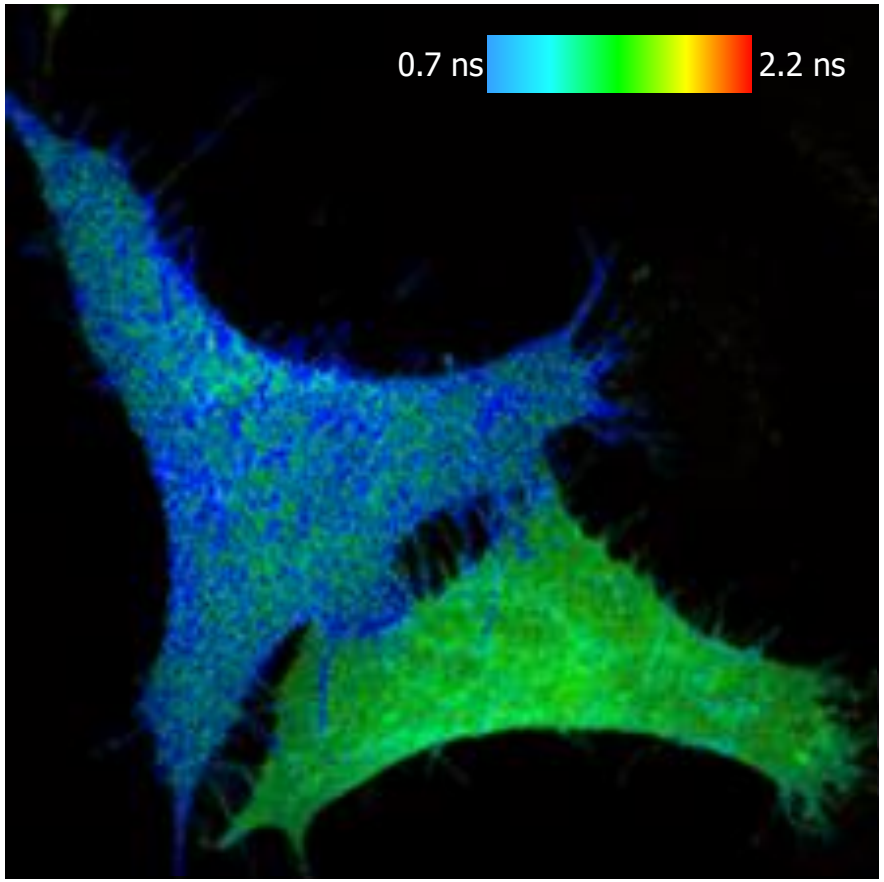


# FLIM Tutorial – Leica TCS SMD with SPT64

Heike Glauner – Leica Microsystems

Science Lab

# FLIM – Adding a new Dimension to Imaging




GFP in HeLa cells
right cell: only GFP expressed; left cell: GFP-mCherry FRET construct
Courtesy: Corentin Spriet, Imaging Center Lille, France

- Fluorescence Intensity Imaging

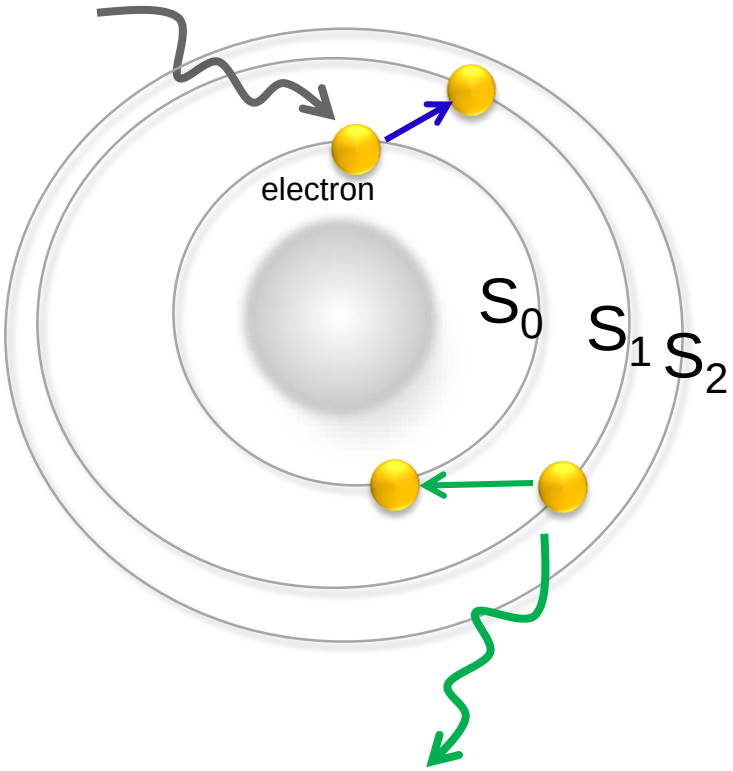
  Where is the fluorescence located in my specimen?

- Fluorescence Lifetime Imaging Microscopy „FLIM“

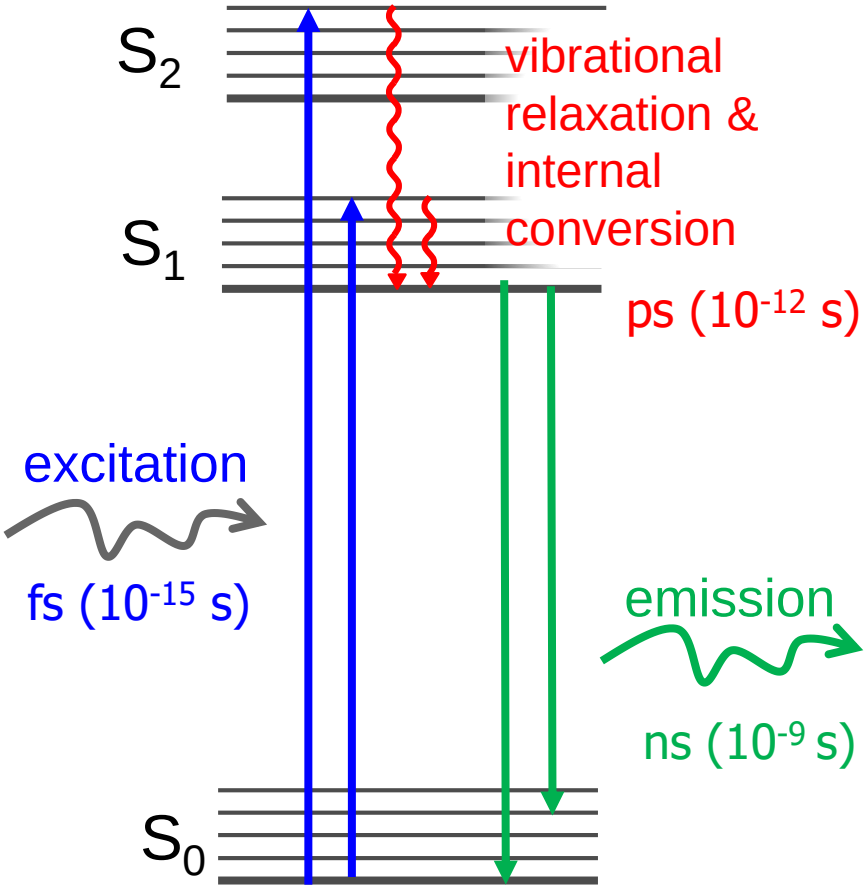
  At what time is the fluorescence emitted in my specimen?

# What is the „Fluorescence Lifetime“?

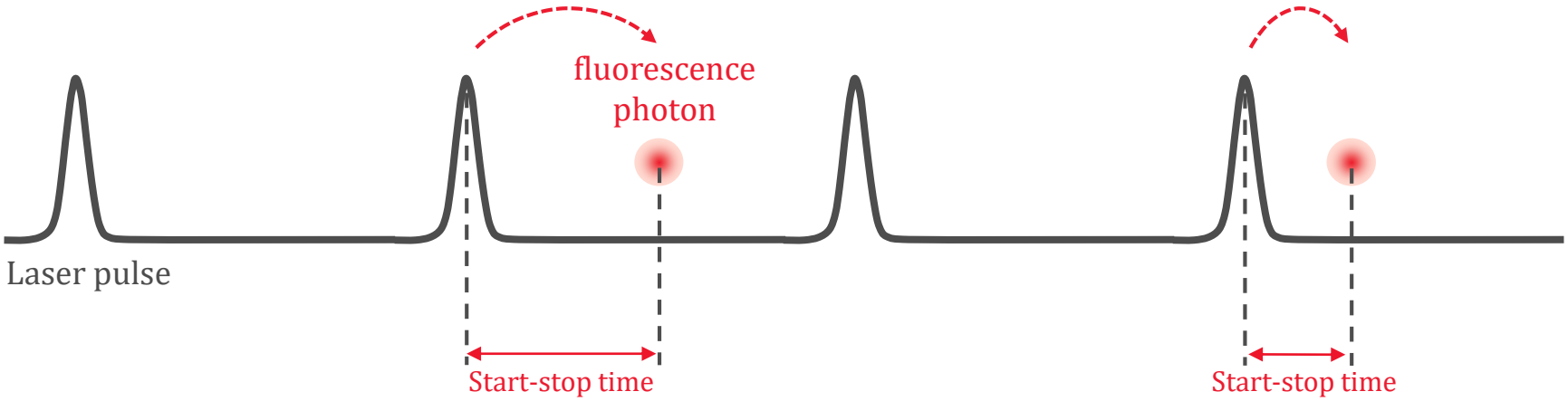
Fluorophore atom




Jablonski diagram



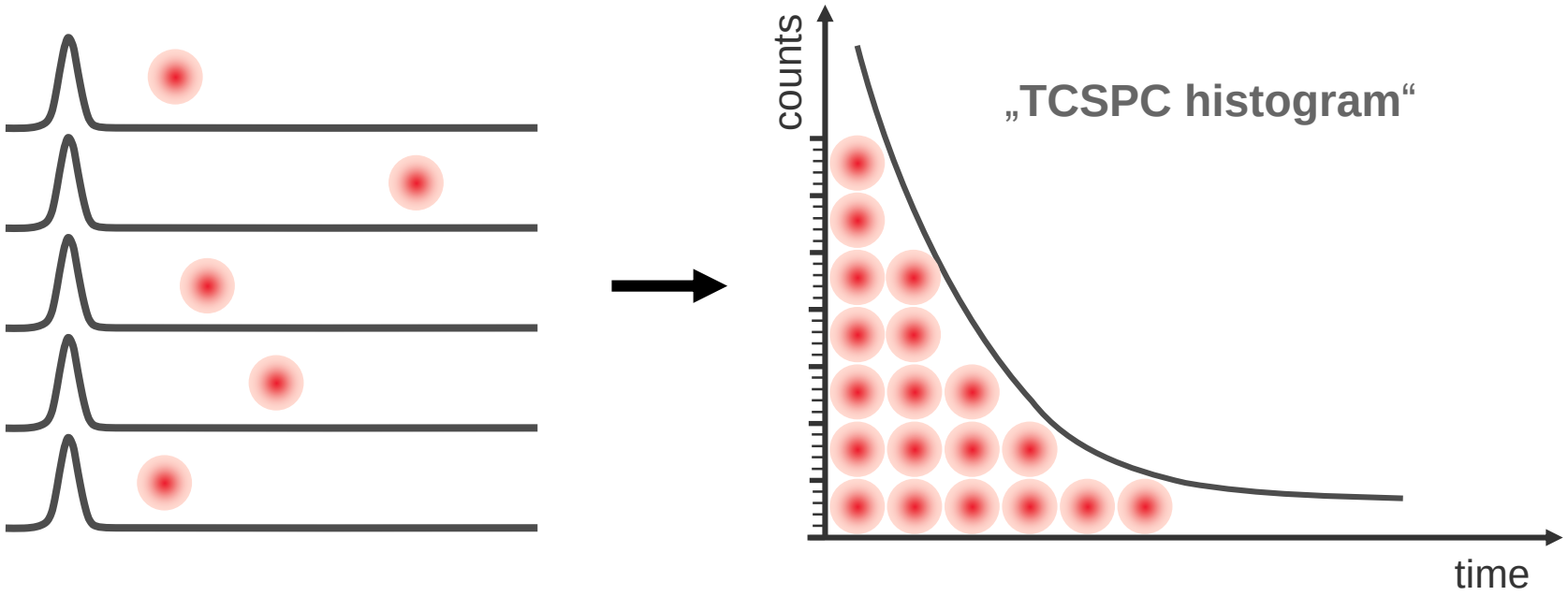
# TCSPC Method for Lifetime Determination




- stop watch principle :
  1. Start the clock with a laser pulse
  2. Stop the clock with the first photon that arrives at the detector
  3. Reset the clock and wait for next start signal

- TCSPC: Time Correlated Single Photon Counting

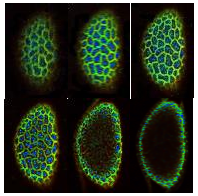
# Lifetime Analysis Based on Decay Curves




- many excitation pulse cycles are monitored

- accumulated photons from multiple cycles are used to reconstruct the single cycle decay profile
- Curve fitting for read-out parameters

# Types of Fluorescent Markers for FLIM

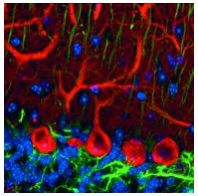


- Autofluorescence

e.g. NADH, Flavins, chlorophyll, melanin, collagen, elastin, lipofuscin, lignin, …

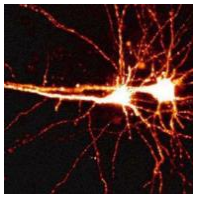
- Fluorescent proteins

e.g. eCFP, eGFP, eYFP, mCherry, …



- Fluorescent markers bound to antibodies

e.g. FITC, Alexa, Atto, Cy, ...



- Ion indicators

e.g. Calcium-Green for Ca2+, Sodium-Green for Na+, BCECF for pH …

# Fluorescence Lifetime

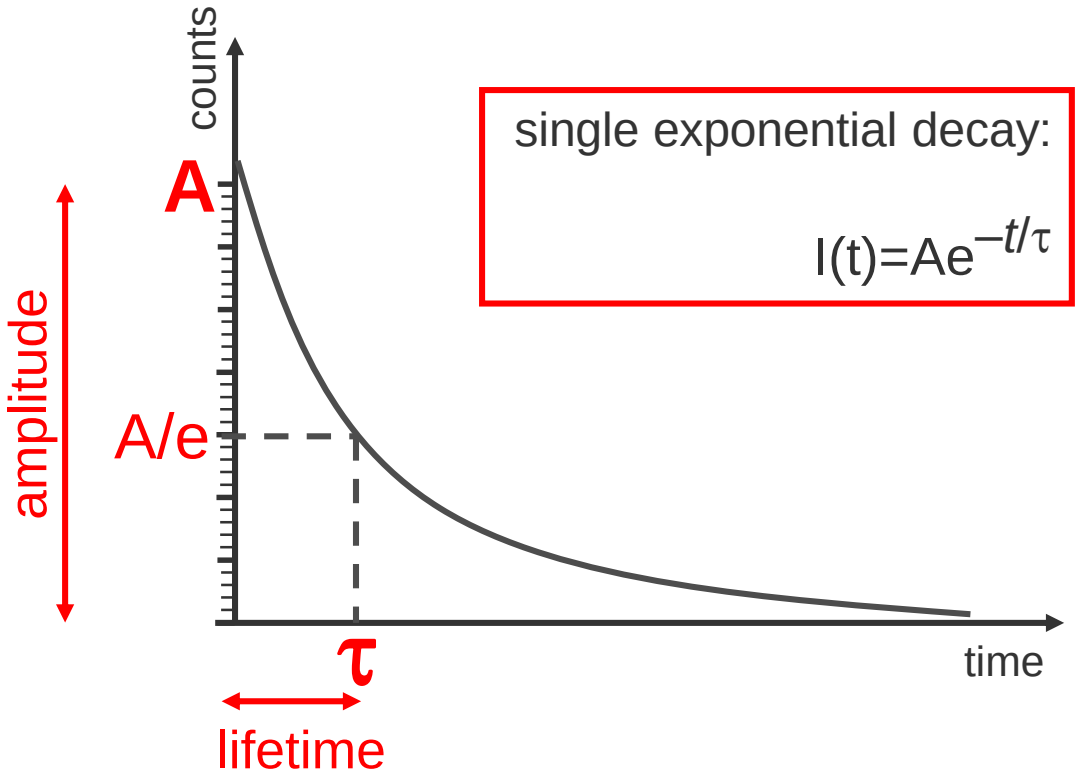
fluorescence lifetime depends on:

- intrinsic dye property
  - Isomerisation
  - Protonation
  - Folding
  - …
- micro-surrounding
  - pH
  - ion concentration
  - Water concentration
  - oxygen concentration
  - lipophilic surrounding
  - ….
- molecule binding

fluorescence lifetime is independent of:

- fluorophore concentration limit: aggregation & quenching at very high concentrations
- photobleaching artifacts
- specimen thickness (shading)
- excitation intensity
- light source noise

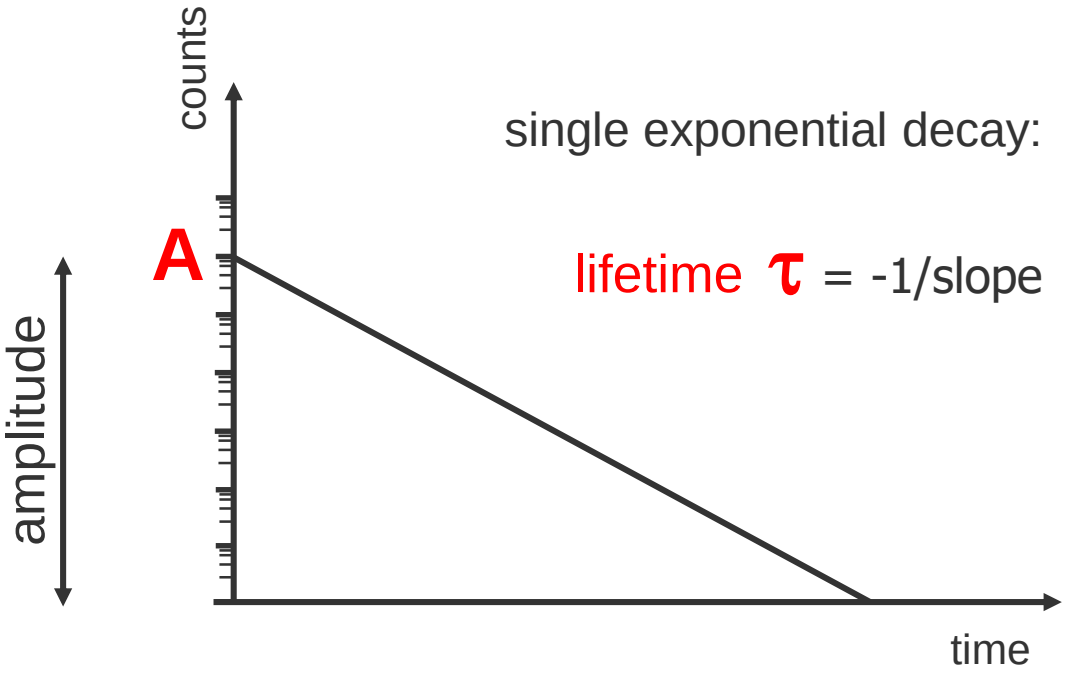
# Analysis of TCSPC Histograms




Readout parameters after curve fitting with monoexponential decay model:

- amplitude **A**: number of photons at t=0
- fluorescence lifetime **τ**: time at which amplitude **A** of the fit curve decays to **A**/e (e≅2.7)

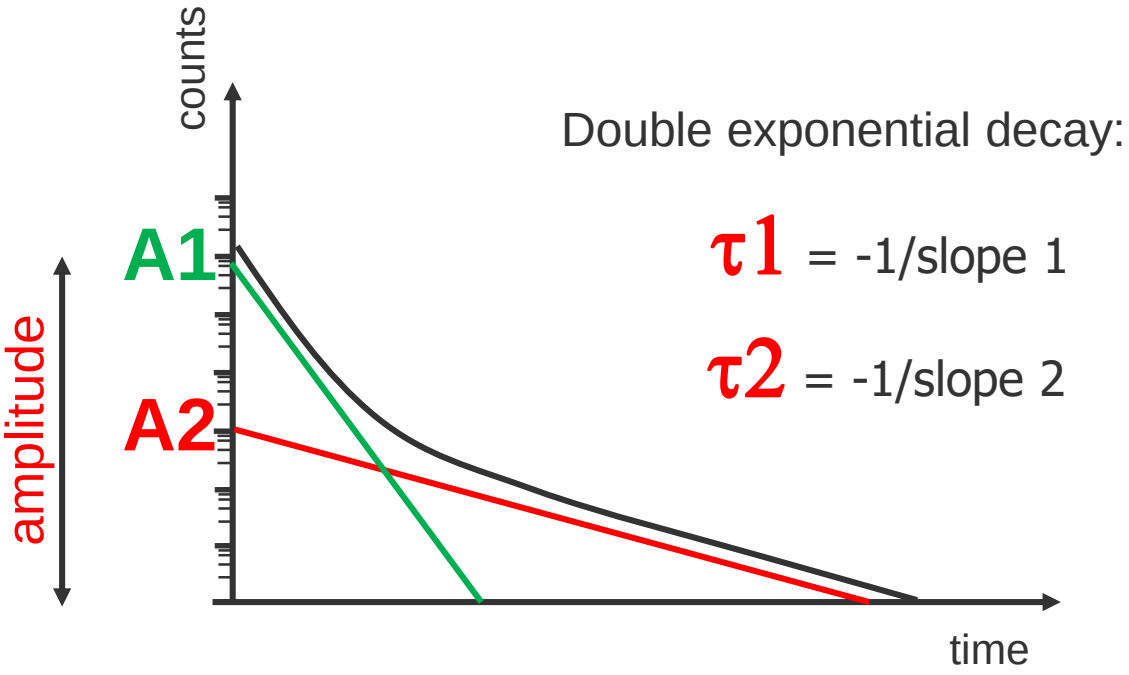
# Analysis of TCSPC Histograms




Semi-logarithmic scale

- fluorescence lifetime τ can be read out from the slope

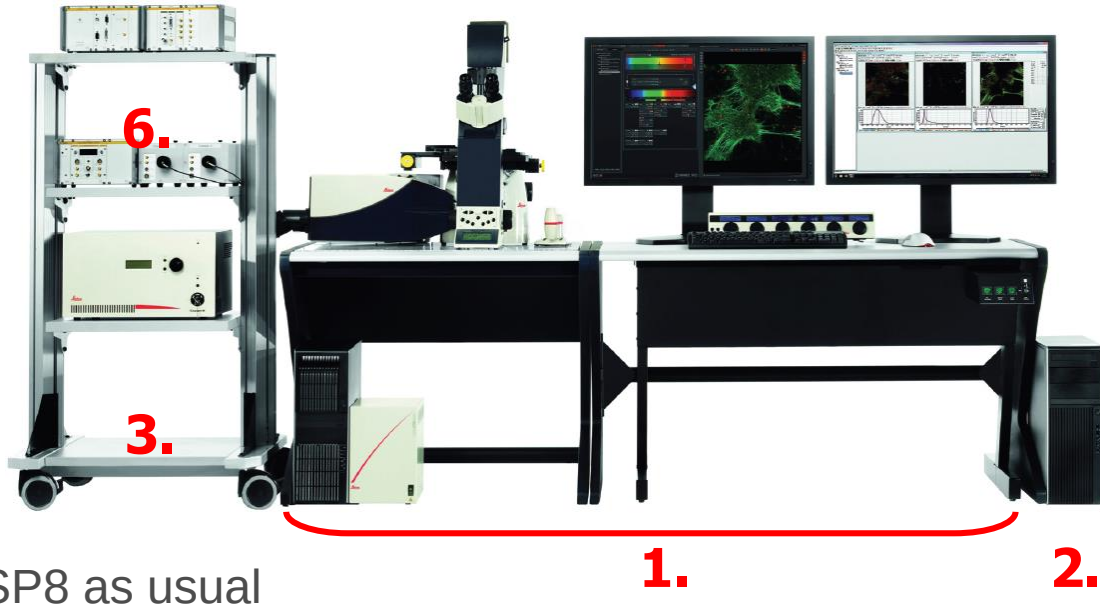
# Analysis of TCSPC Histograms




Semi-logarithmic scale

- The measured curve is a sum of two decays
- Fitting with bi-exponential curve mode reveals 2 amplitudes and 2 lifetimes

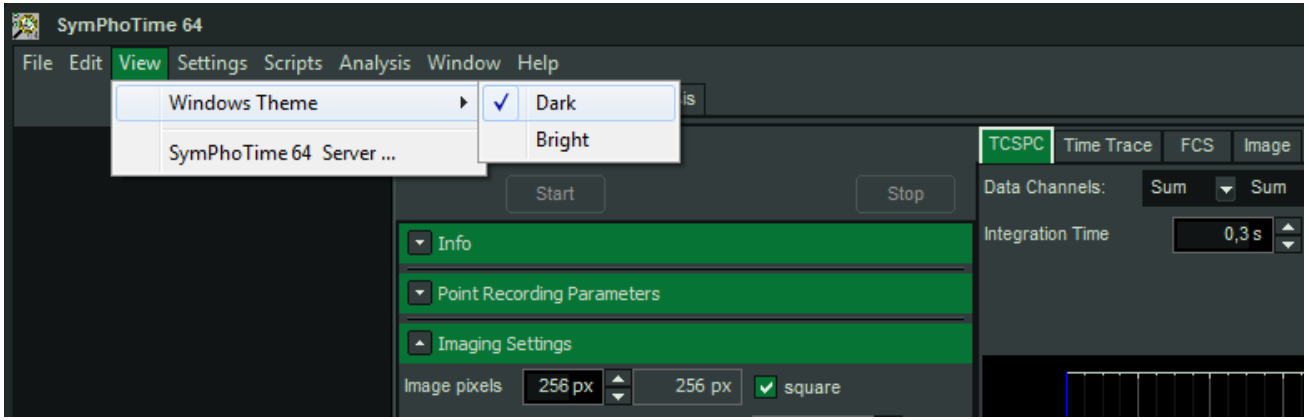
# Order of Switching on Components

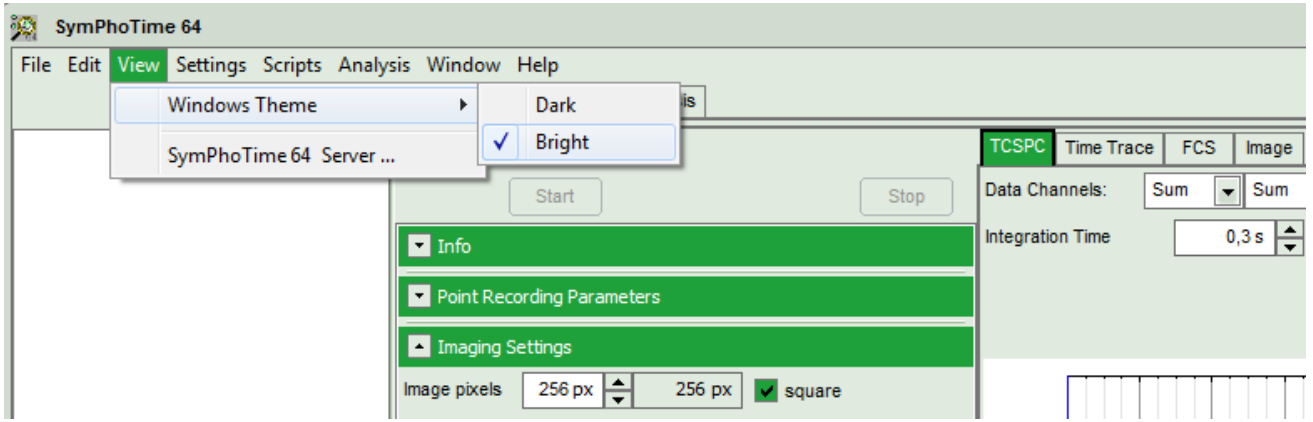



1. start the SP8 as usual
2. start the SMD workstation
3. switch on the Picoquant rack via the multiple socket outlet
4. start the laser driver and switch the laser(s) on. Set any present key switch to the „ON“ position. Prior to data acquisition, the laser head should be operated for approx. 20 minutes to stabilize
5. start the SPT64 software on the SMD workstation
6. if you are using SPADs or APDs you need to switch them on as well. This can be done right before the measurement

# SPT64 Software: Windows Theme

- Symphotime has a dark and bright GUI theme which can be selected by the user via View > Windows Theme > Dark/Bright:



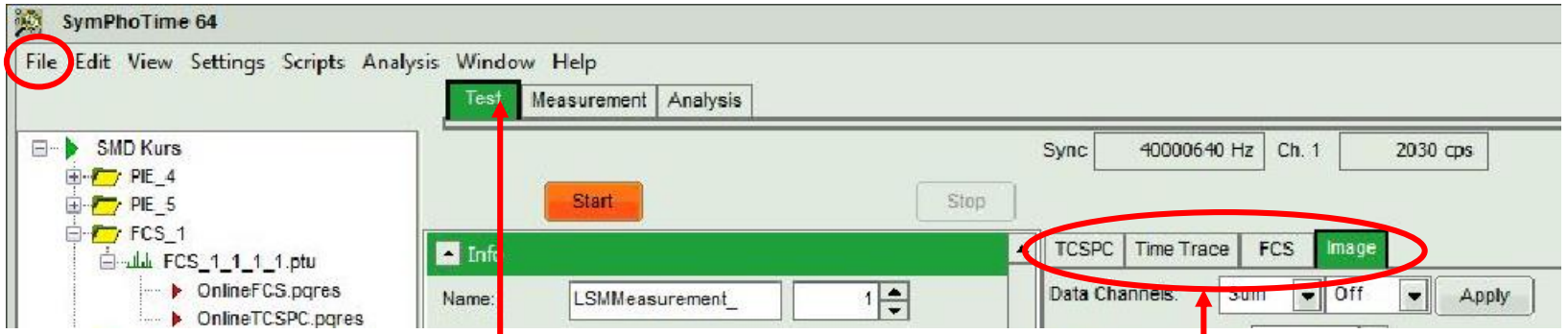



# SPT64 Software: Workspace and GUI concept

- To be able to start a SMD measurement in SymPhoTime a workspace has to be created:

  1. File menu > New Workspace. The workspaces in SymPhoTime have the ".sptw" file extension
  2. define storage location & name > click Open.

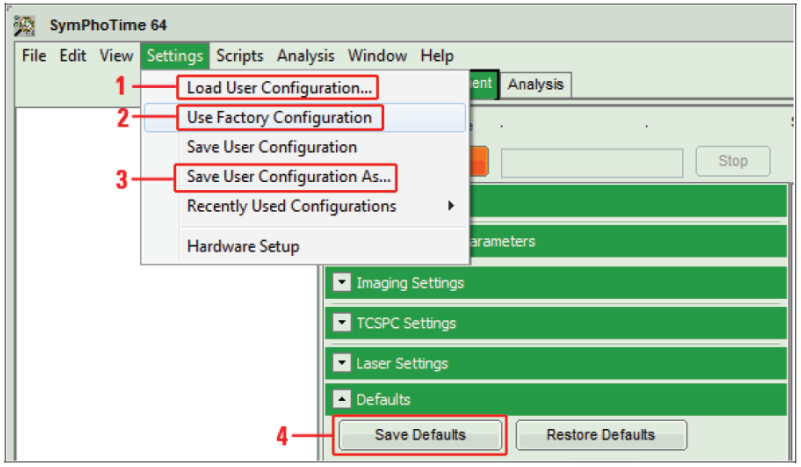
- After a workspace has been created, the SymPhoTime software automatically starts recording once a measurement has been started via LAS AF.




- the „Test“ tab will automatically be active

- the user can jump between the TCSPC / time trace / image tab manually

# Factory vs. User Settings




There are two important configuration files for the user in SPT64:

- Factory configuration
- User configuration

The configuration files control hardware and software GUI settings

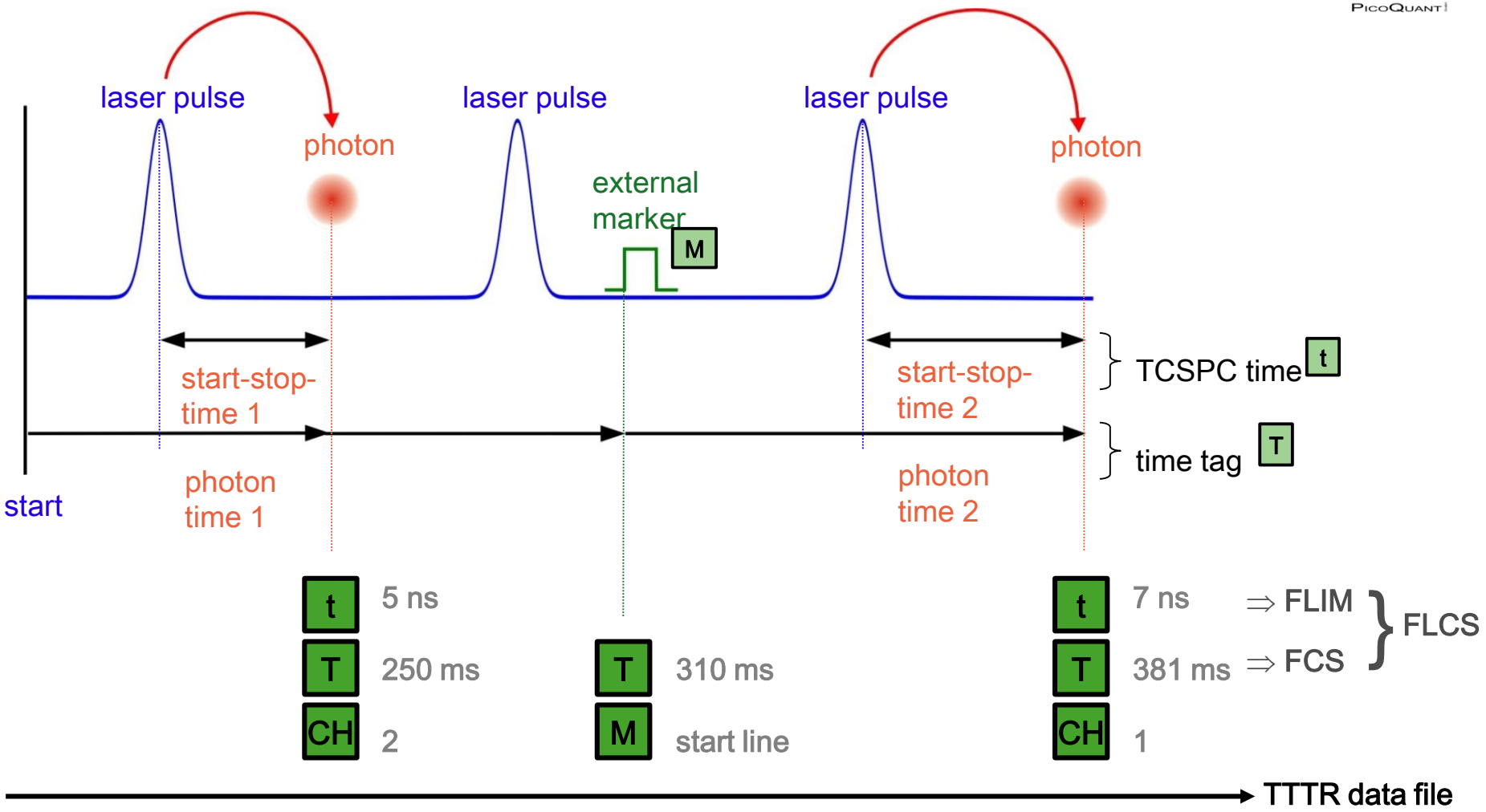
- Factory configuration

This configuration has been defined by Picoquant and Leica. It is stored in the „Settings.pfs“ file. Factory settings can be modified if Symphotime is run in Configure mode and will affect the SPT settings in all Window accounts. Factory settings will override user settings. But be careful: any modification or relocation of this file could cause SPT to stop functioning properly.

- User configuration

The factory configuration can be adapted and saved as user-specific configuration which can only be seen within the respective windows account. User configurations have the „.pus“ file extension. They can be saved at any location and can be exchanged between users.

# Time-Tagged Time-Resolved (TTTR) Single Photon Detection



# Practical Aspects

„Setup Corr-ring“ menu
- Adjusting the correction collar of immersion objectives

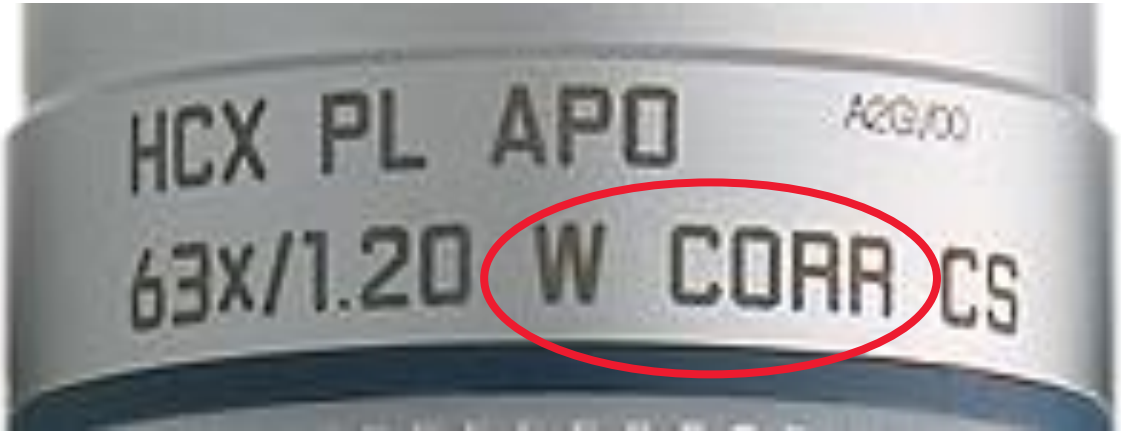
„Setup FLIM“ menu
- defining image parameters for FLIM
- optimization of photon count rates
- selecting the correct laser repetition rate

„FLIM measurement“ menu
- duration of image acquisition

FLIM analysis
- „fast FLIM“ vs. curve fitting
- FLIM-FRET

# Which Objectives have a Correction Collar?

- Water immersion objectives „W CORR“
- Glycerol immersion objectives „GLYC CORR“
- Multi immersion objectives (oil / water / glycerol) „IMM CORR“



# How does a Correction Collar look like?

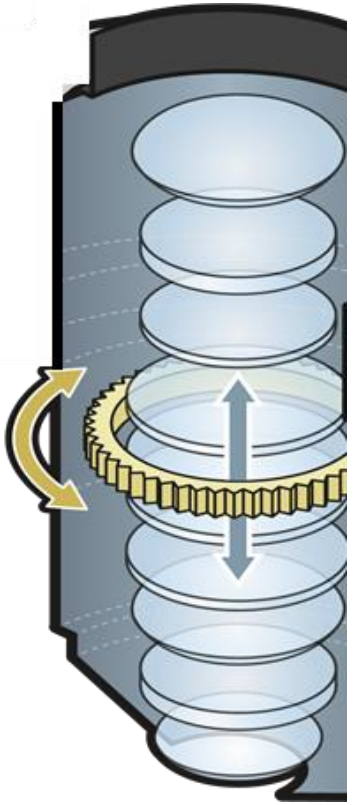
- Manual version


- Motorized „motCORR“ version

# What is the Correction Collar Good For?

It adjusts for ….

- Differences in coverslip thickness
- Refractive Index (RI) mismatch
- Deviations in temperature



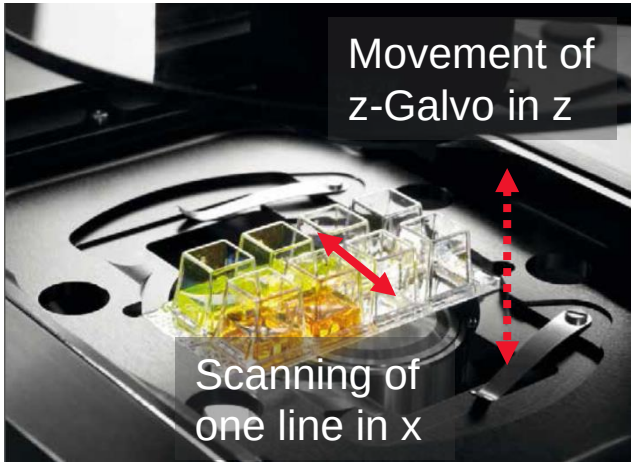
# Coverslip Thickness Variations

- objectives are usually designed to be used with coverslips of 170 µm thickness
- commercially available coverslips are sold in thickness classes - actual glass thickness varies within those classes:

| class | ideal thickness | range |
|---|---|---|
| #0 | 100 µm | 80 - 130 µm |
| #1 | 150 µm | 130 - 170 µm |
| #1.5 | 170 µm | 160 - 190 µm |
| #2.0 | 220 µm | 190 - 250 µm |

# Correction Collar Adjustment

1. Activate „Setup CORR-ring“ menu in FLIM wizard
2. Activate live scan
3. Search for coverslip surface reflection using the coarse focus drive
   - lower line for inverse stands
   - upper line for upright stands
4. Sharpen the reflection line by turning the correction collar



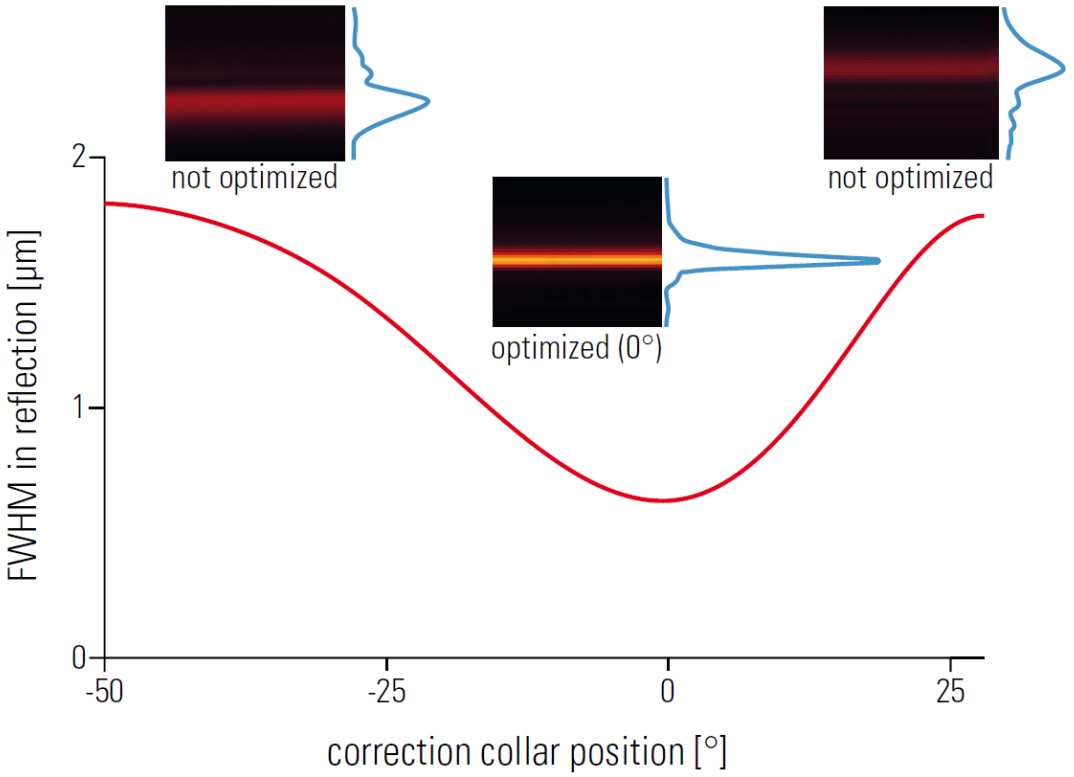

How is the scan configured in „Setup CORR-ring“?

- Xz scan mode (one line is scanned and the z-galvo focus drive moves up and down quickly to generate the second image dimension)
- Detection window is underneath the laser line
- AOBS is programmed to reflection
- No notch-filter is in the light path

→ Reflections on glass surfaces can be seen

# Correction Collar Adjustment

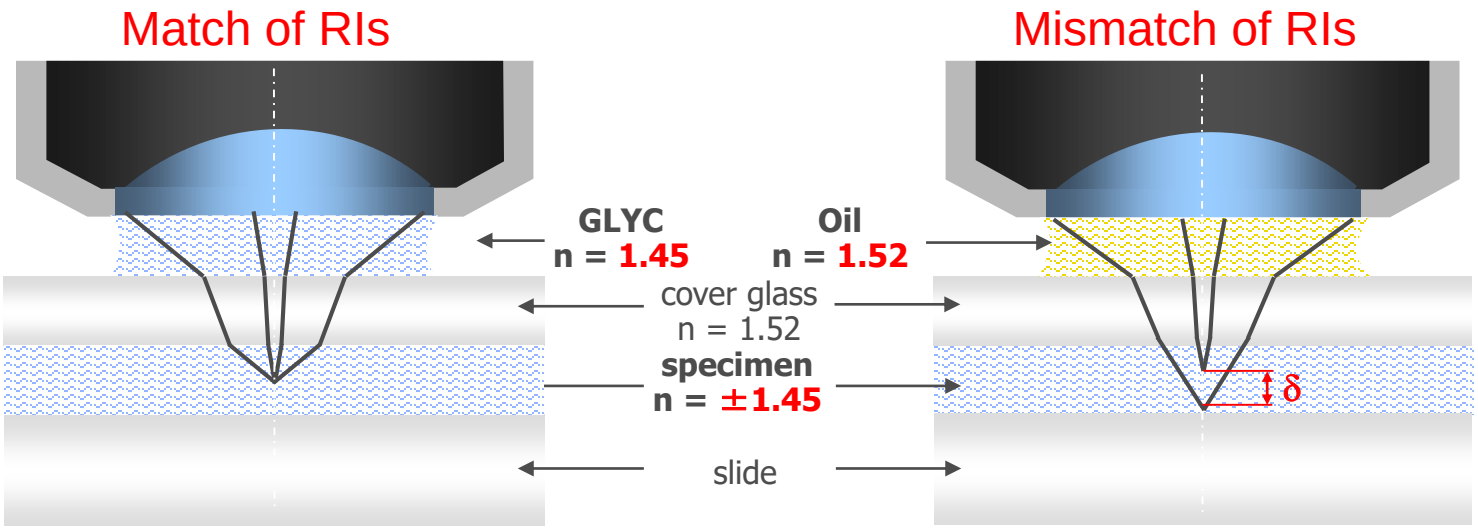
- the FWHM of the coverslip reflection in xz visualizes distortions of the PSF
- Optimized, sharpest reflection of coverslip surface → objective is optimally adapted → best imaging result

# Matching Refractive Indices to Prevent Spherical Aberrations

Optimal conditions for imaging:

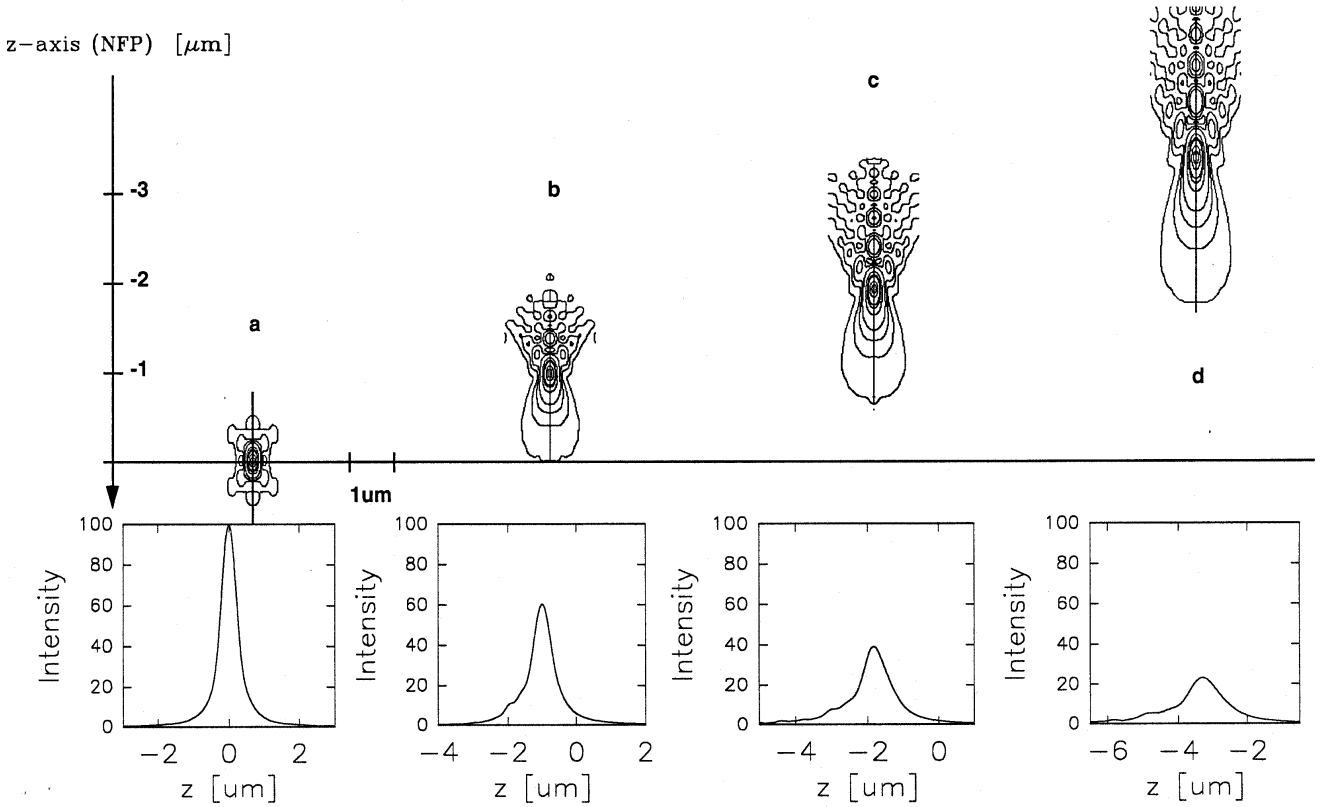
- refractive index of immersion medium & sample / mounting medium is the same




Matching RI is crucial for confocal microscopy !

- spherical + chromatic aberrations in z
- spread of focus spot
- distortion of structures
- loss of resolution
- loss of intensity

# Mounting Medium and Immersion: Aberrations caused by Mismatch of RI




- FWHM ↑, thus Resolution ↓
- Intensity ↓

S.Hell et al., „Aberrations in Confocal Fluorescence Microscopy induced by Mismatch in Refractive Index“, J. Microscopy 169, 391-405 (1993)

# Practical Aspects

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- Adjusting the correction collar of immersion objectives

„Setup FLIM“ menu
- defining image parameters for FLIM
- optimization of photon count rates
- selecting the correct laser repetition rate

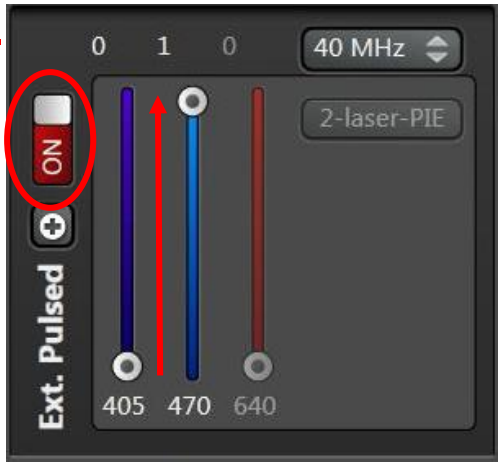
„FLIM measurement“ menu
- duration of image acquisition

FLIM analysis
- „fast FLIM“ vs. curve fitting
- FLIM-FRET

# Defining Image Parameters in „Setup FLIM“

- remember to switch to pulsed lasers

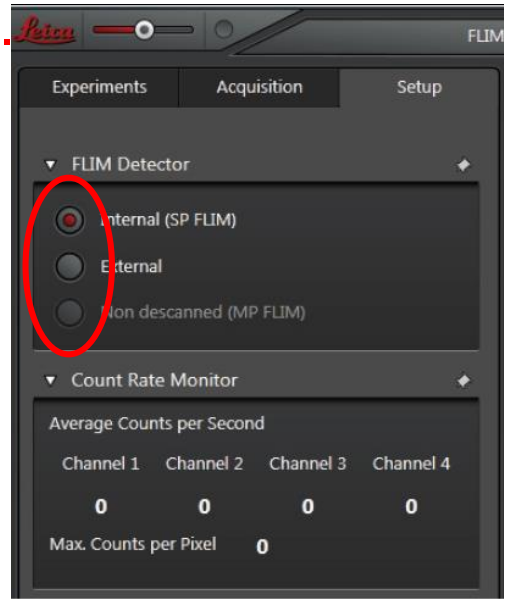
1.




Picoquant Diodes
or MP
or WLL

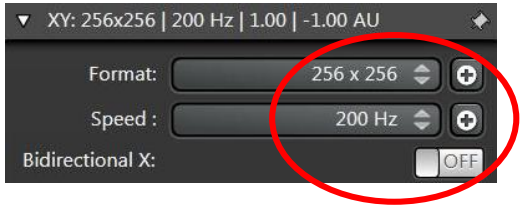
- select FLIM capable detectors

2.



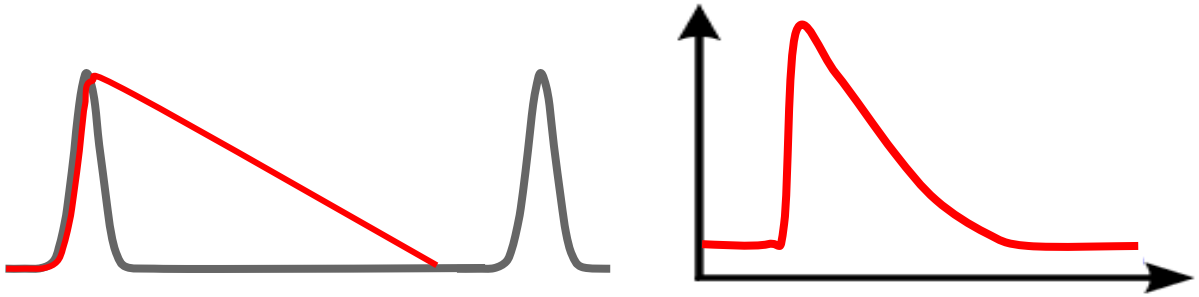

- pixel format: 256 x 256 or smaller
- scan speed: adjust to obtain a pixel dwell time of 20 µs for correct calculation of Maximum Count Rates (e.g. 50 Hz @ 256², 100 Hz @ 128²)
- monodirectional scan (bidir only for zoom >6 with separate phase corr)

3.

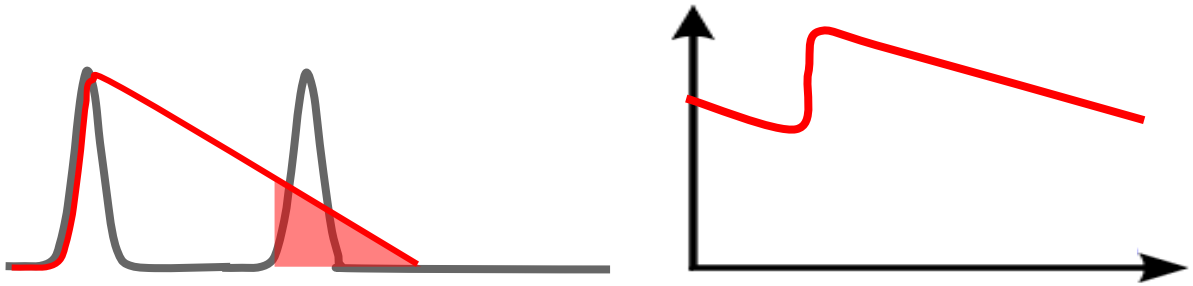


# What is the Correct Laser Repetition Rate?

- Fluorescence has decay to background before the next laser pulse starts for correct analysis
- Rule of thumb: pulse interval 4-5x as long as average lifetime



- If the time interval between two pulses is too short for a complete decay then the „wrap around“ effect will be occur

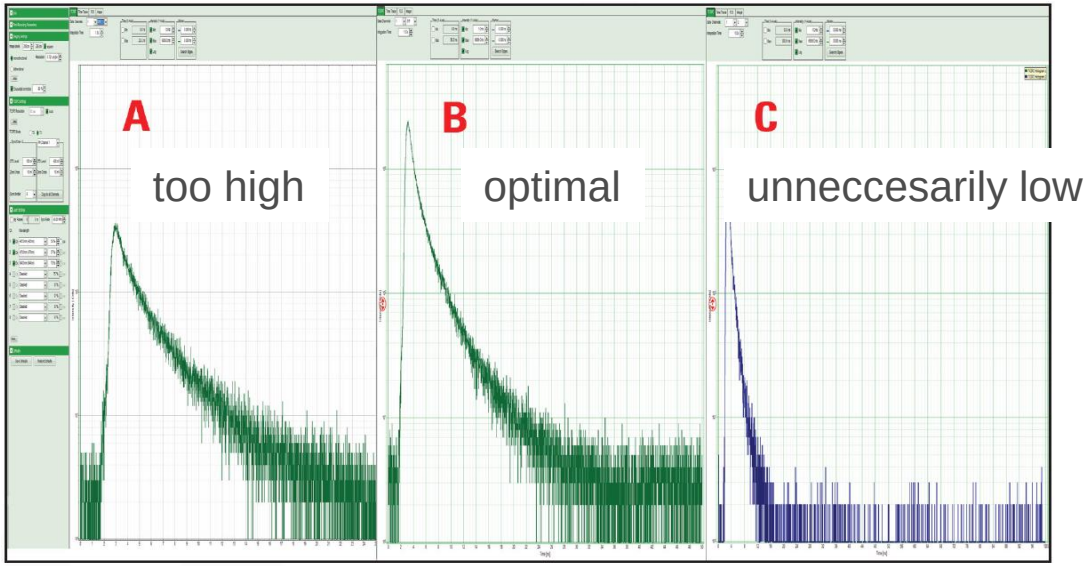


- decay tail appears as onset
- high background

# What is the Correct Laser Repetition Rate?

- control whether FLIM curve decays to background level within acquisition window:

1. start FLIM test
2. look at online TCSPC histogram
3. adjust the laser repetition rate in LASX so that complete decay to background can be seen

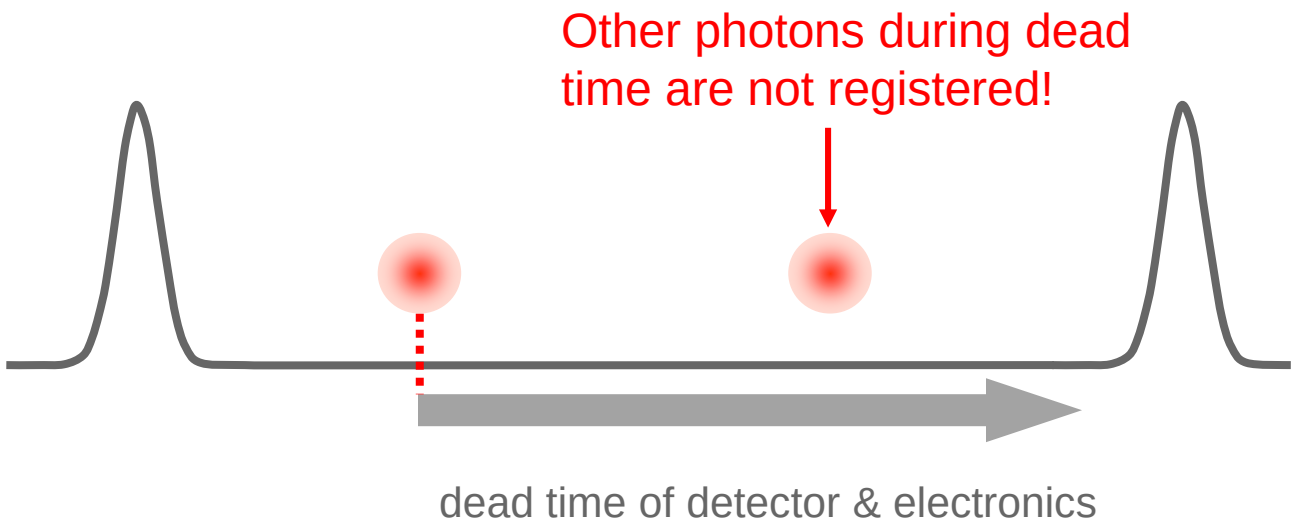



- rule of thumb for FLIM: the acquisition window for a FLIM curve needs to be 4-5 x as long as the lifetimes to be analyzed

| laser frequency [MHz] | time interval between pulses [ns] |
|---|---|
| 80 | 12.5 |
| 40 | 25 |
| 20 | 50 |
| 10 | 100 |
| 5 | 200 |

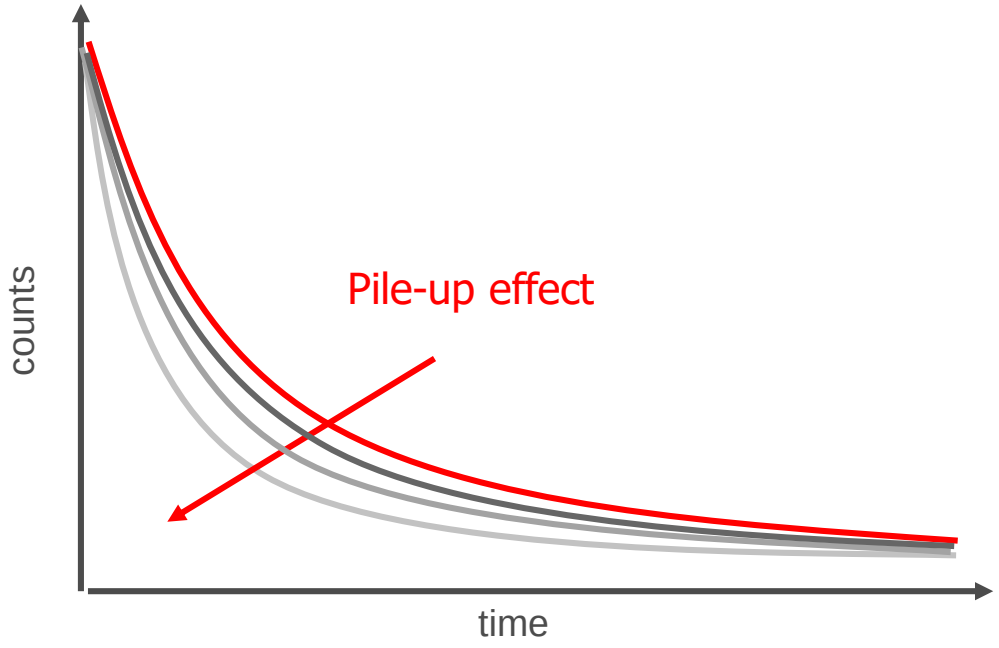
# Pile-up effect

- only one photon per excitation/emission event can be registered due to the dead time of detector and electronics
- if the number of photons per excitation cycle is >1 → first photon is registered, the following ones are missed
- = „Pile-up Effect“



# Pile-up effect in TCSPC

- Consequence of Pile-up in TCSPC histograms:
- over-representation of early photons as the later photons are missed
  → shorter apparent lifetimes



# Prevention of Pile-up Effect

- Adjust laser intensity to such a low level so that statistically seen no photons are emitted during the dead time of the detector / electronics

> Recommendation by Picoquant:
> count rate should be at maximum 10 % of laser frequency

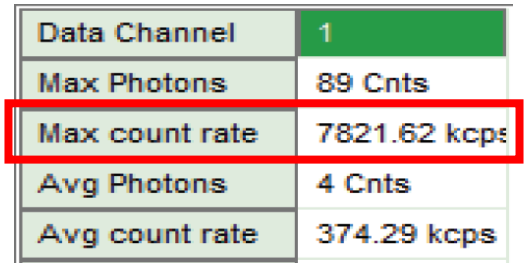
- Recommendation mostly read in literature:
  maximum count rate should be at most 1-4% of the excitation rate
- Reason why Picoquant finds 10% justifiable:
  if max. count rate = 4 % of laser frequency → theoretical pile-up error ~ 1 %
  if max. count rate = 10 % of laser frequency → theoretical pile-up error ≤ 5 %
- If pile-up is present then it gives rise to a fast, short-lived component which, however, is only visible if photons from this component represent more than 10 % of the total photons

# Optimization of Photon Count Rates

- adjust laser intensity so that the „Max count rate“ is not exceeding the following values while using a pixel dwell time of 20 µs:

not for MP or WLL without pulse picker

| Laser repetition rate | Upper count rate limit for 20 µs pixel dwell time |
|---|---|
| 80 MHz | 8000 |
| 40 MHz | 4000 |
| 20 MHz | 2000 |
| 10 MHz | 1000 |
| 5.0 MHz | 500 |
| … | always 10% of rep. rate |

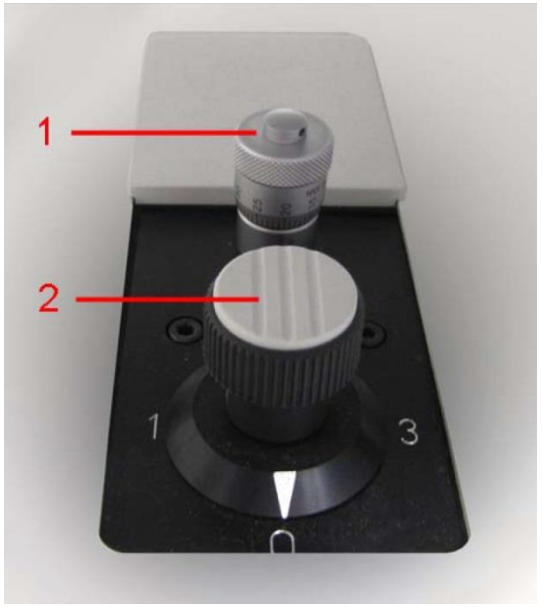


| Data Channel | 1 |
|---|---|
| Max Photons | 89 Cnts |
| Max count rate | 7821.62 kcps |
| Avg Photons | 4 Cnts |
| Avg count rate | 374.29 kcps |

- if **several detectors** are used simultaneously: the **sum** of „Max count rates“ for all channels is critical

for SPT32 the upper count rate limits were smaller (≤ 1000 kcts/s) and more or less independant of pixel dwell time due to binning of the maximal count rates

# Attenuation of Pulsed Diode Lasers if >1 diode is present




For systems equipped with >1 pulsed diodes:

There are two knobs on the Laser Coupling Unit to control laser intensity

- Large knob = Filter Wheel (2)

  coarse adjustment with neutral density filters

  | | | |
  |---|---|---|
  | 0 | = | 100 % Transmission |
  | 1 | = | 10 % Transmission |
  | 2 | = | 1 % Transmission |
  | 3 | = | 0.1 % Transmission |

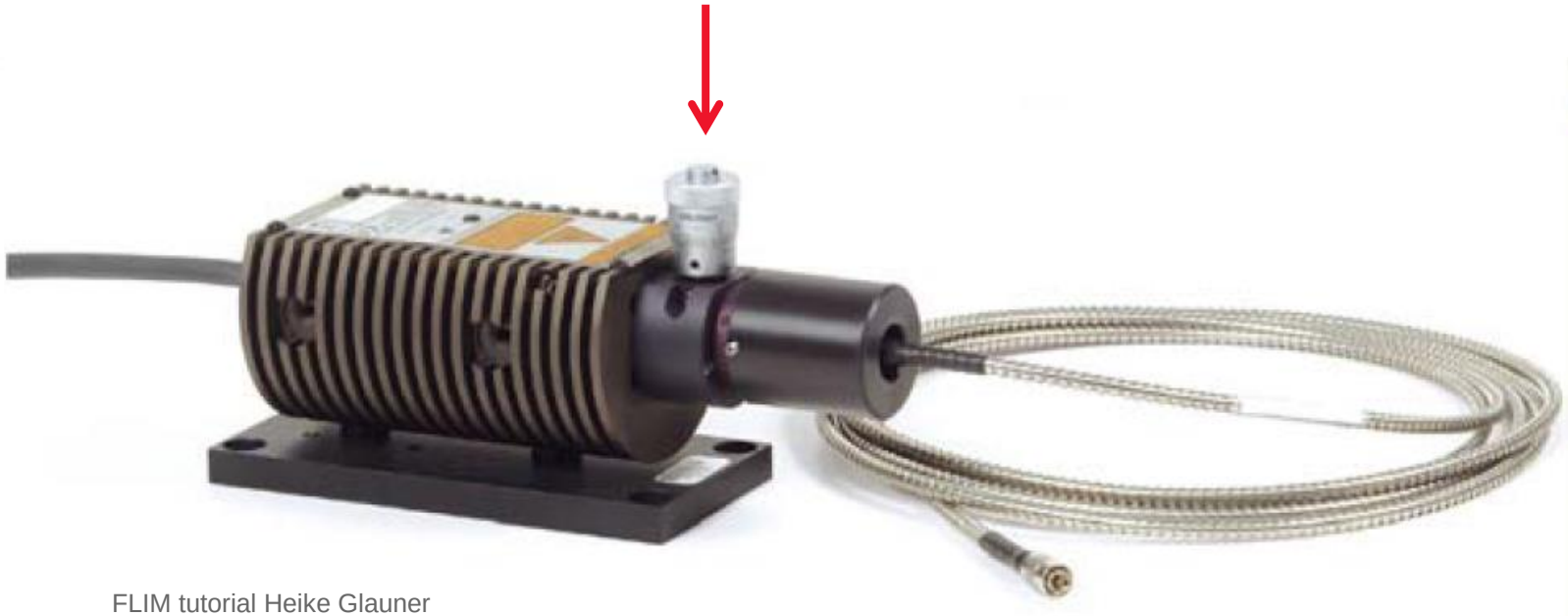
- Small knob = Scaffold (1)

  fine adjustment via a metal plate cutting of some of the laser beam

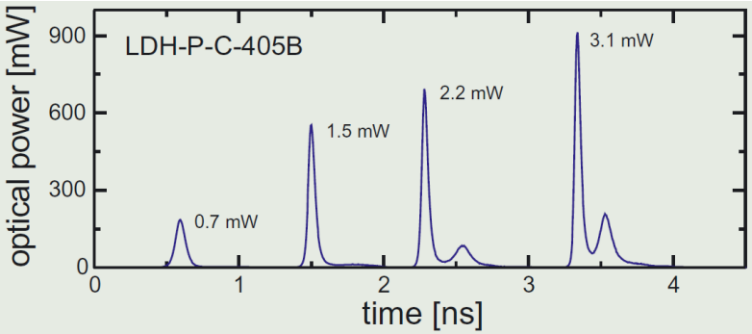
# Attenuation of Pulsed Diode Lasers if one single diode is present

For systems equipped with one single pulsed diode:
There is one knob in front of the laser head to control laser intensity

- = Scaffold
  fine adjustment via a metal plate cutting of some of the laser beam



# Changing Power of Pulsed Lasers will Change Pulse Shape & Width




pulsed 405 PQ diode as example

(for longer wavelengths the side peak becomes more prominent)

- PicoQuant diodes have a power threshold at which they start to pulse
- Above this threshold the the laser pulse will broaden with increasing power and therefore the time resolution of the system will go down

  → keep laser power as close as possible to threshold for best time resolution

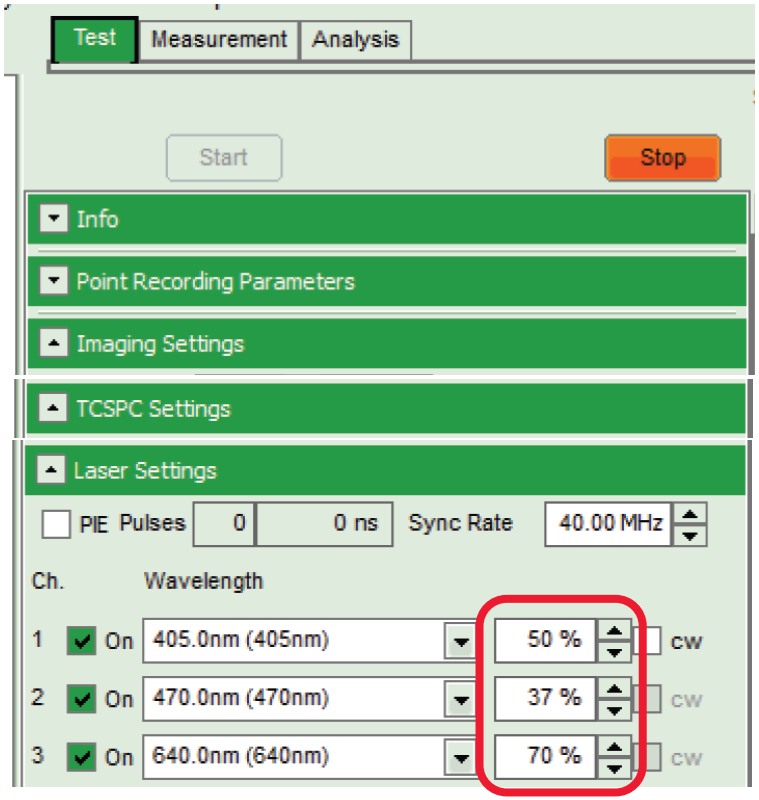
  → do not change laser power between measurements to be compared

- changes regarding AOTF/EOM/grey filters do not have an effect on laser peak width and can be changed between measurements to be compared

# How to Change Power of Pulsed PQ Diodes

- PDL 800-B laser driver:


- PDL 828 „SepiaII“ laser driver: control via SPT64 GUI



# Practical Aspects

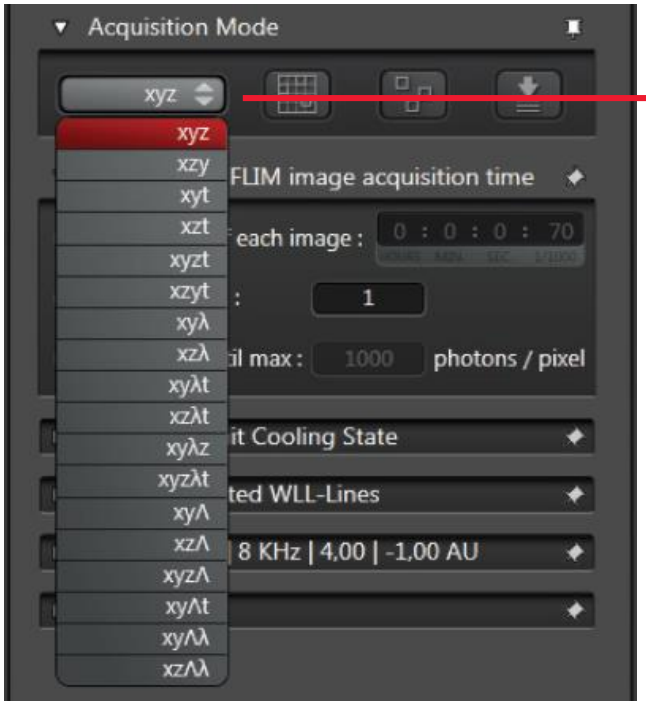
„Setup Corr-ring“ menu
- Adjusting the correction collar of immersion objectives

„Setup FLIM“ menu
- defining image parameters for FLIM
- optimization of photon count rates
- selecting the correct laser repetition rate

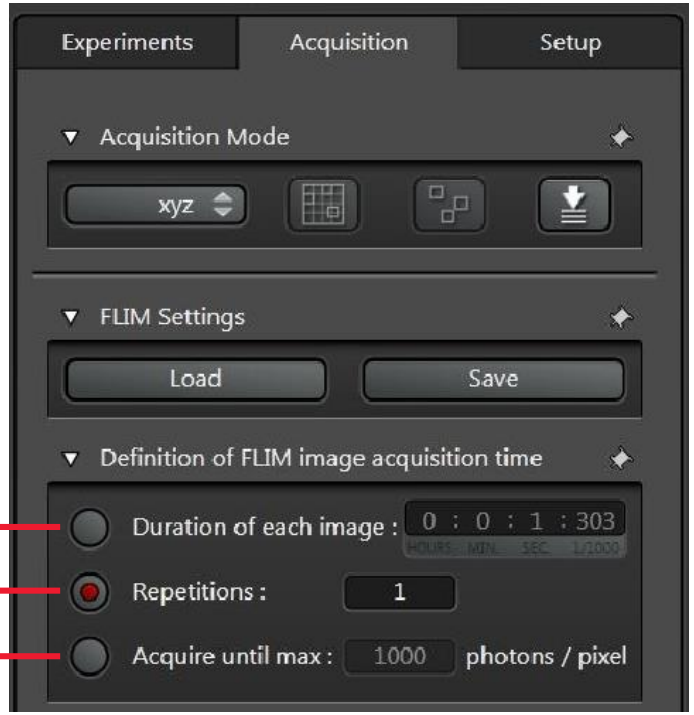
„FLIM measurement“ menu
- duration of image acquisition

FLIM analysis
- „fast FLIM“ vs. curve fitting
- FLIM-FRET

# Control of FLIM Data Acquisition




- scanning modes: z-stacks, time series and λ-scans are possible (Λ for WLL or MP only)




- acquisition until defined time is over
- how many frames are to be scanned
- acquisition until x photons are counted in the brightest image pixel

# How many Photons Do I Need for Proper Analyses of Lifetimes?

- I want to analyze fluorescence decays on pixel-wise basis

- one needs at least 500 counts per pixel for a reasonable two-component fit if the two lifetimes are clearly separated
- if more components are to be fitted or the expected lifetimes are close together then considerably more photons will be needed!

- I want to analyze fluorescence decays in ROIs

- for ROIs all photons will be added up for one fluorescence decay curve
- therefore also less than 500 counts per pixel are acceptable
- the criterion mainly determining how many photons are needed is the size of the ROIs

Use a reference system (for instance mixture of dyes of known lifetimes) to test out limits if in doubt

# Practical Aspects

„Setup Corr-ring“ menu
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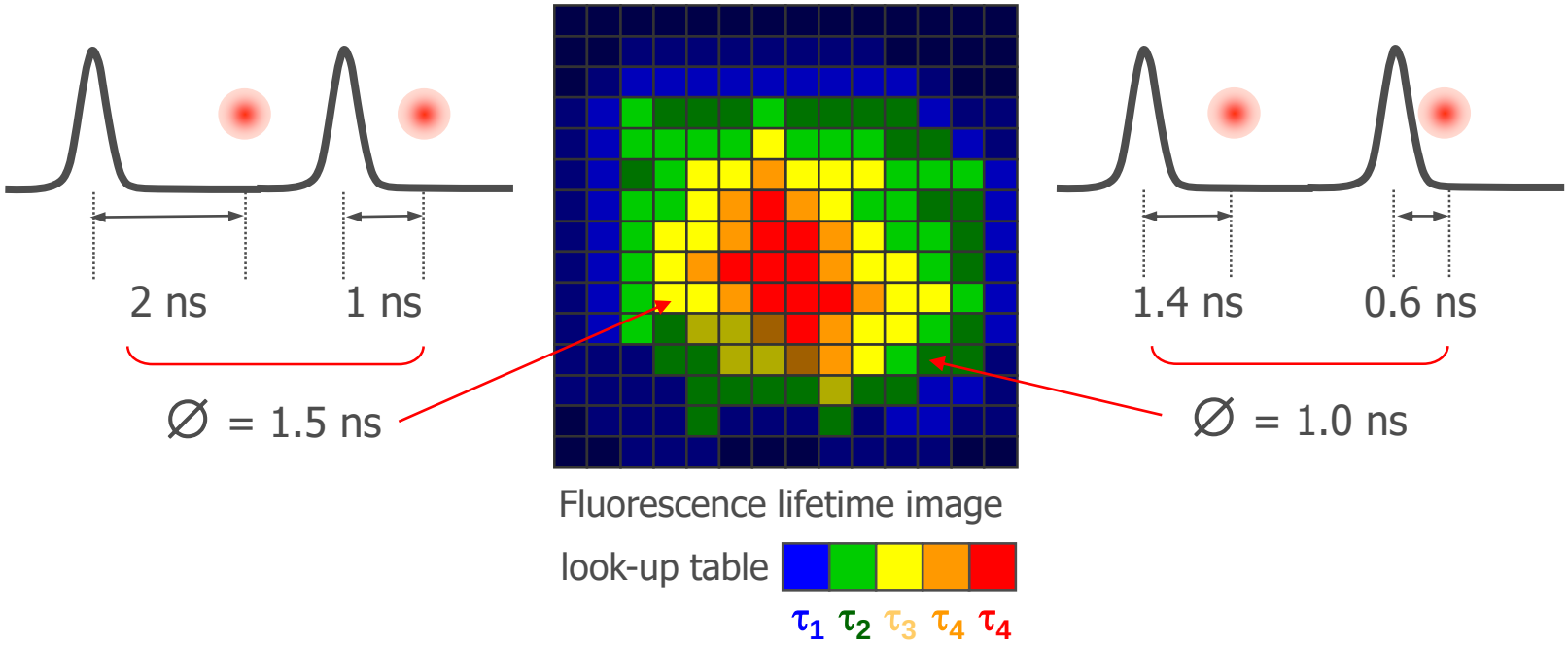
- selecting the correct laser repetition rate

„FLIM measurement“ menu
- duration of image acquisition

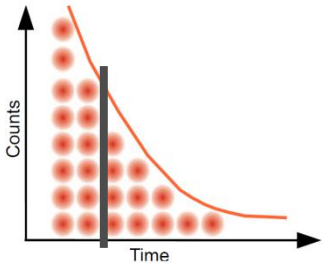
FLIM analysis
- „fast FLIM“ vs. curve fitting
- FLIM-FRET

# Display of Average Arrival Times: “fast FLIM”

- the „ fast FLIM“ average arrival time does not involve any fitting of the fluorescence decay curves yet. It merely displays average arrival times of photons.



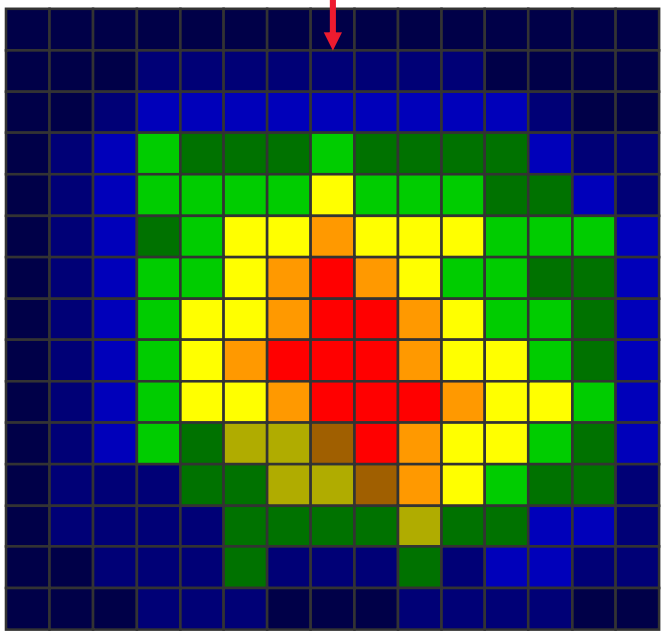

- for comparison with decay curves: the „fast FLIM“ time corresponds to the center of mass of the pixel‘s decay



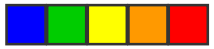
# Display of “Fast FLIM”

display of average photon arrival times as overlay on intensity image

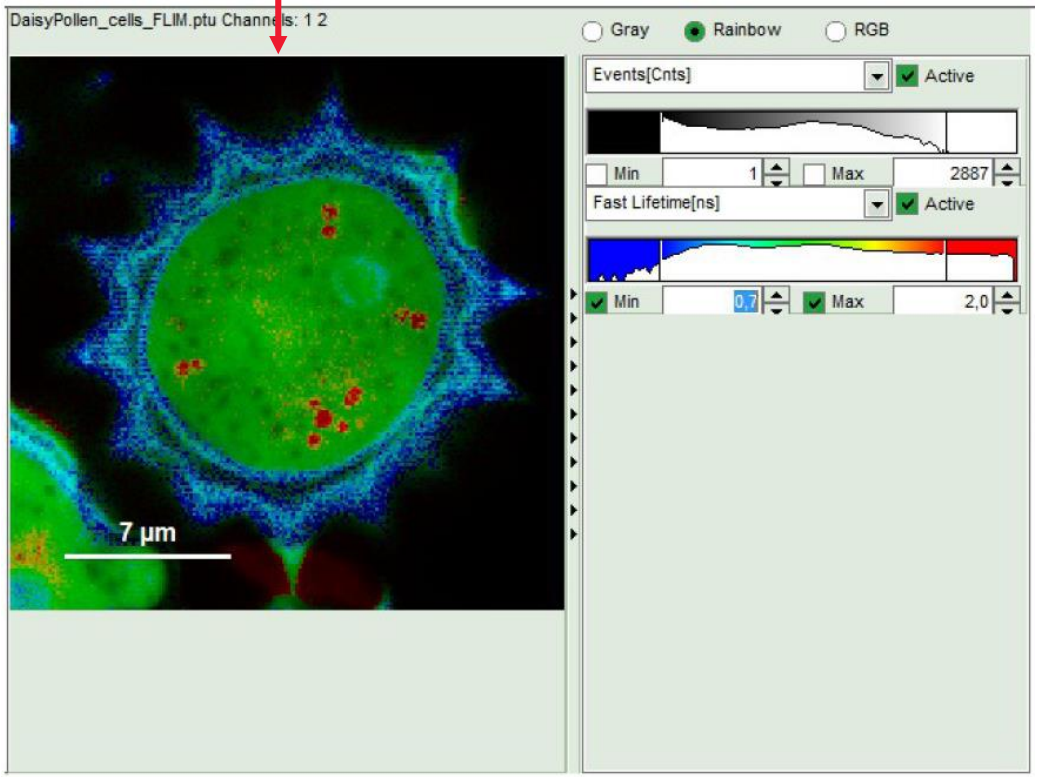
modulation of brightness and lifetime colour contrast possible



look-up table

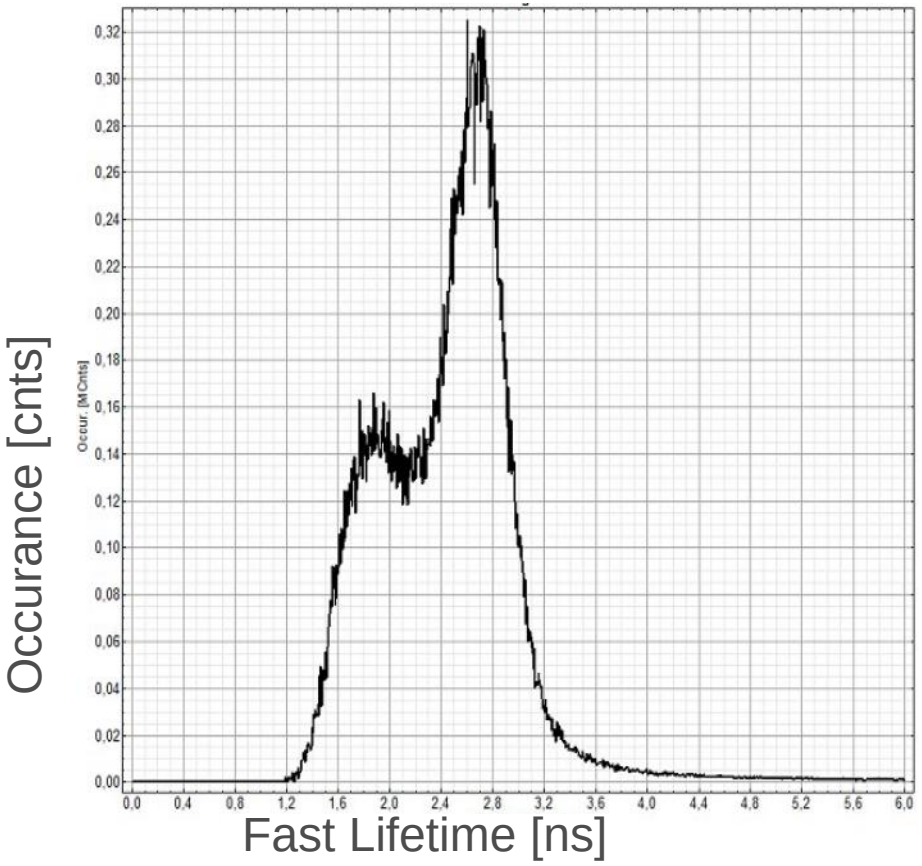


$\tau_1$ $\tau_2$ $\tau_3$ $\tau_4$ $\tau_5$



# Display of Lifetime Histogram

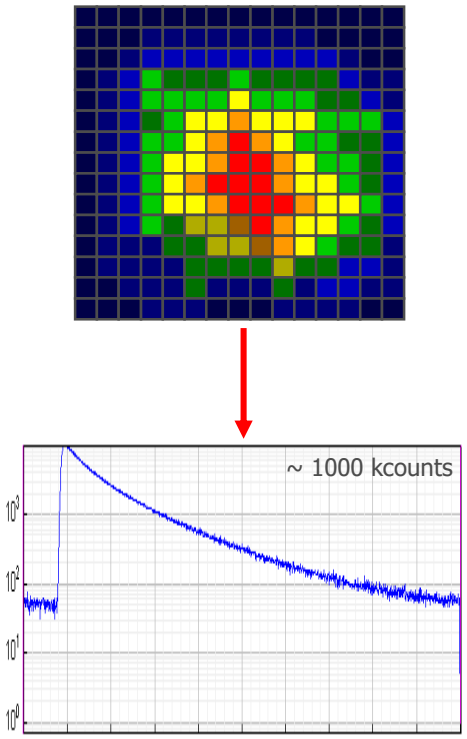
- The plot of the lifetime histogram shows how often each lifetime occurs in the image or ROI
- The lifetime histogram is intensity weighted. For example, a pixel with the amplitudes A1 and A2, which correspond to the lifetimes τ1 and τ2 will increase the frequency of the lifetime histogram channels corresponding to τ1 resp. τ2 by a value of A1*τ1 resp. A2*τ2.



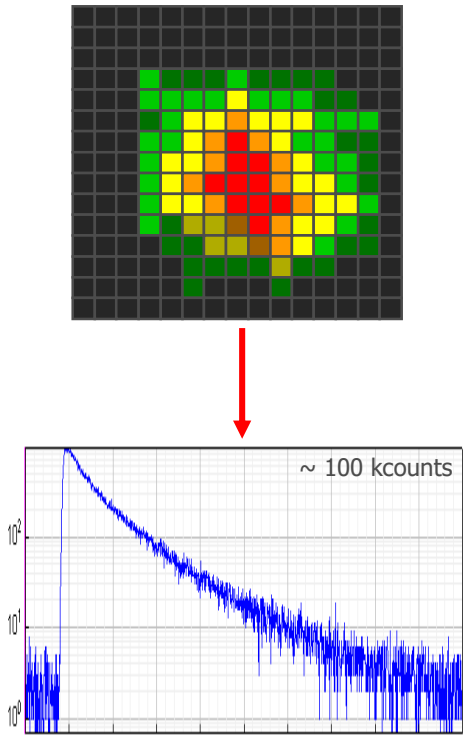
# Analysis of Fluorescence Decay Curves

- Fluorescence decay curves are also referred to as „TCSPC histograms“.
- They can be analysed...

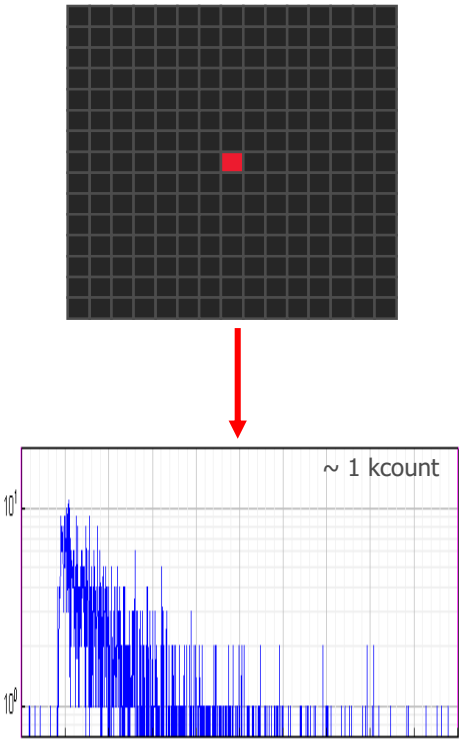
...for whole images



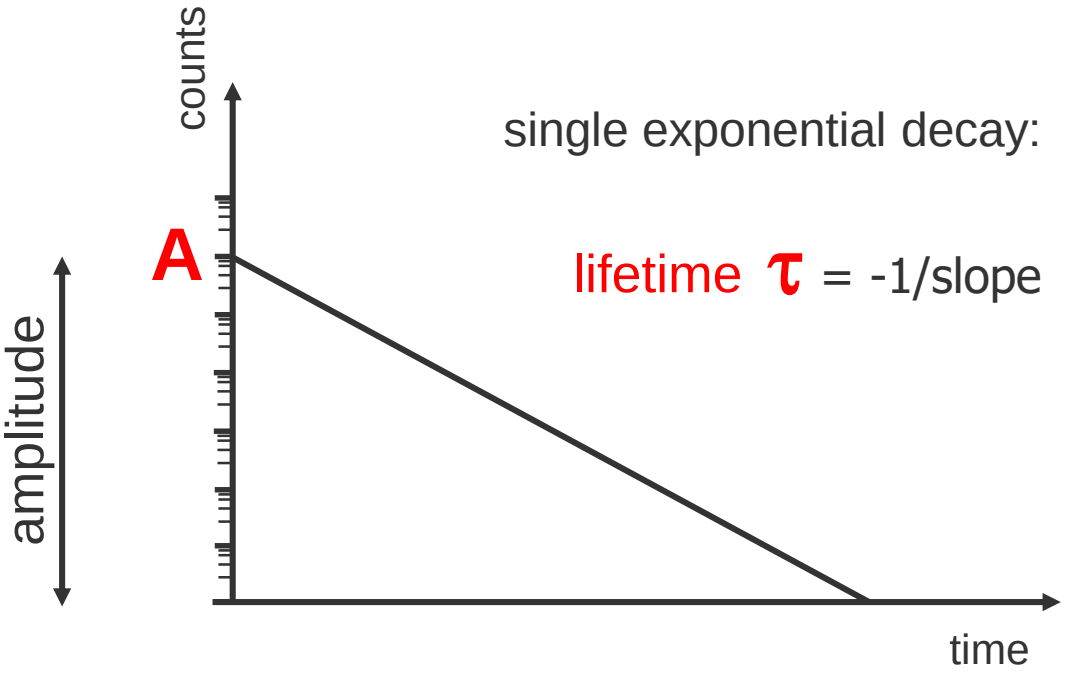

...for region of interests




...for individual pixels



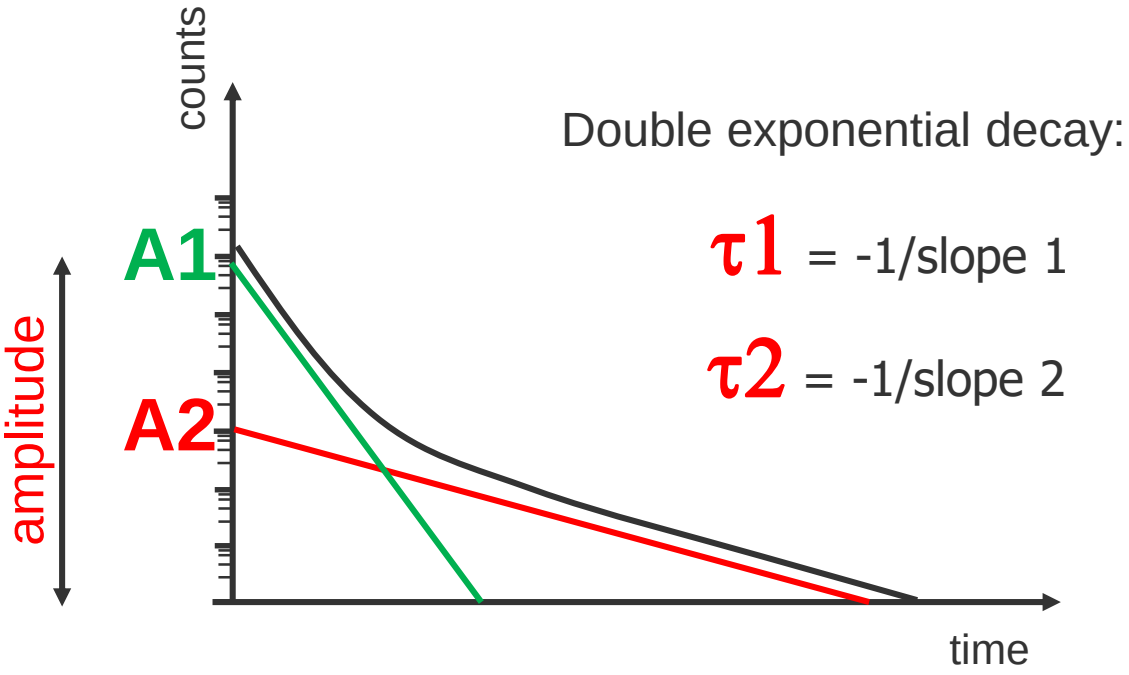
# Analysis of TCSPC Histograms




Semi-logarithmic scale

- fluorescence lifetime τ can be read out from the slope

# Analysis of TCSPC Histograms




Semi-logarithmic scale

- The measured curve is a sum of two decays
- Fitting with bi-exponential curve mode reveals 2 amplitudes and 2 lifetimes

# Origin of Multiple Lifetimes

- presence of multiple fluorophores / fluorescent proteins
- multiple micro-environments in sample
- several conformational states of fluorophore / protein
- tautomers of fluorophores
- several maturation stages of fluorescent proteins
- energy transfer between interacting molecules

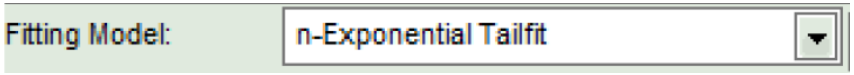
- impurities in sample
- scattered light
- systematic errors in measurement

# Tail-Fitting (1)

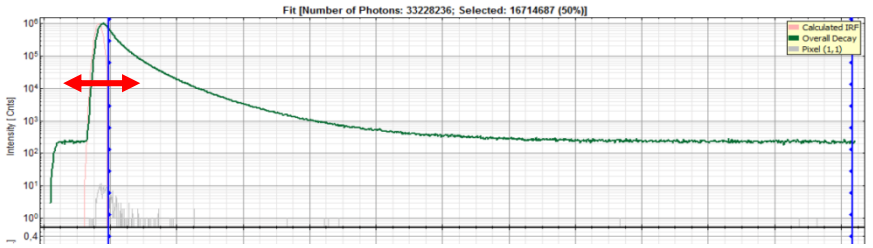
- the rising flank of the decay histogram is ignored for fitting
- timepoint zero is estimated
- + fast way of fitting
- - low precision especially for short lifetimes

- How to proceed practically:

1. Select „n-Exponential Tailfit“ as fitting model left of the TCSPC histogram



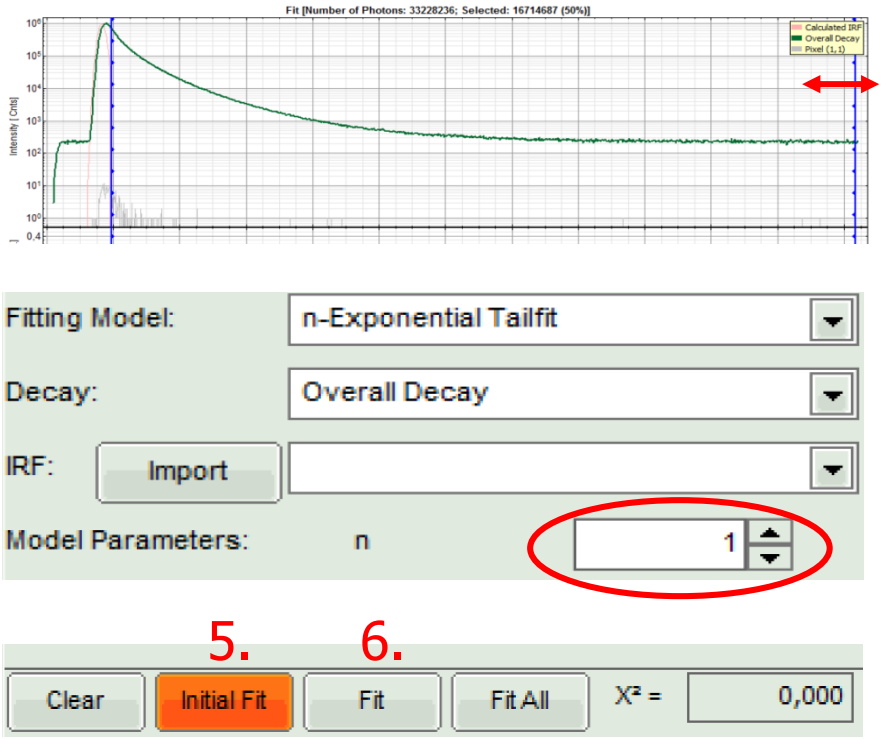

2. Start and end of fitting range are proposed by system. Usually, this fitting range is unnecessarily small: adjust the left fitting range slider to be as close to the peak without getting into the IRF region



# Tail-Fitting (2)

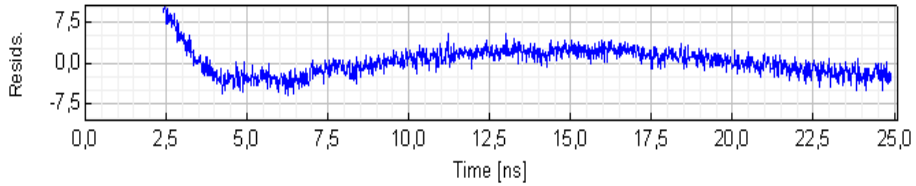
- How to proceed practically continued:

3. adjust the right fitting range slider to include a bit of the background that follows the decay tail if possible
4. In cases of unknown sample: start with the simplest appropriate (usually mono-exponential) model
5. press “Initial Fit”
6. press “Fit” to make the fitting more precise
7. evaluate fitting result (see next page)



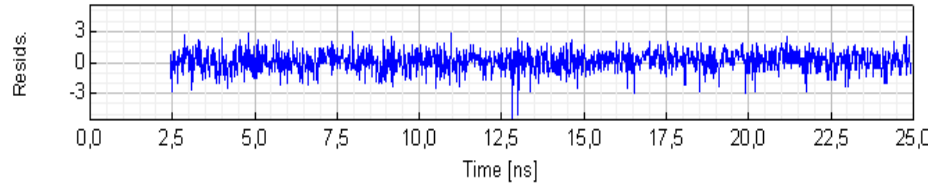
# Tail-Fitting (3)

7. Have a look at the weighted residuals below the graph. Ideally, they fluctuate randomly around zero without any obvious trend. In practice, you will often find spikes or oscillations at early times (around the rising edge of the IRF / decay trace), which are due to various instrument artefacts. If you observe major deviations from zero also at later times then the fit is most likely not appropriate.




residues after mono-exponential fit;
fit is not appropriate

8. If the fit is not appropriate press the *Clear* button and proceed step-by-step to more complicated models with more exponentials.




residues after double-exponential fit;
fit is appropriate

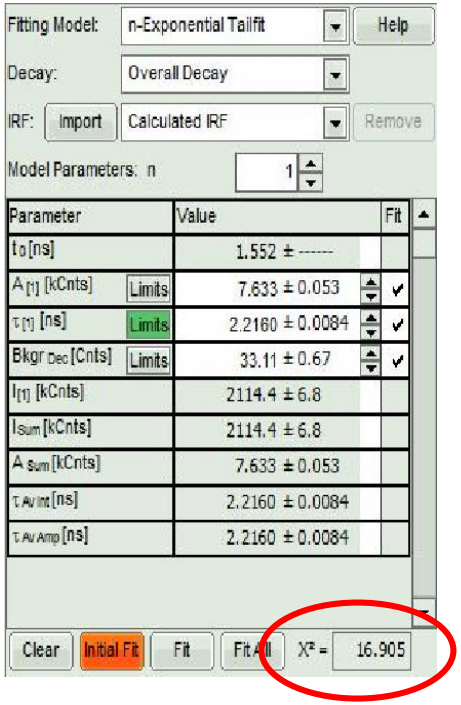
# Optimizing the Goodness-of-fit

- Increasing the number of exponential terms always improves the quality of the fit. However, this does not necessarily mean that the model is more appropriate!

- Critically evaluate whether the more complicated model (e.g. higher order exponential) leads to a significant improvement. Three- or four-exponential fit results are often mathematical representations of decay curve artefacts rather than real kinetic parameters.

- The basic rule is: use the simplest one consistent with the physics of your sample and experiment

# $\chi^2R$ („chi squared reduced“): Another Measure for the Goodness-of-fit




- $\chi^2_R$ is the error function used by certain fitting procedures, e.g. Monte Carlo Simulation
- For a good fit $\chi^2_R$ is 1
- As an exclusive measure of the goodness-of-fit it is insufficient, however. In addition, residuals should always be observed

$$\chi^2 = \frac{\text{measured value} - \text{calculated value}}{\text{standard deviation}}$$

$$\chi^2_R = \frac{\chi^2}{\text{number of data points} - \text{number of floating parameters}}$$

$$= \frac{\chi^2}{\text{number of degrees of freedom}}$$

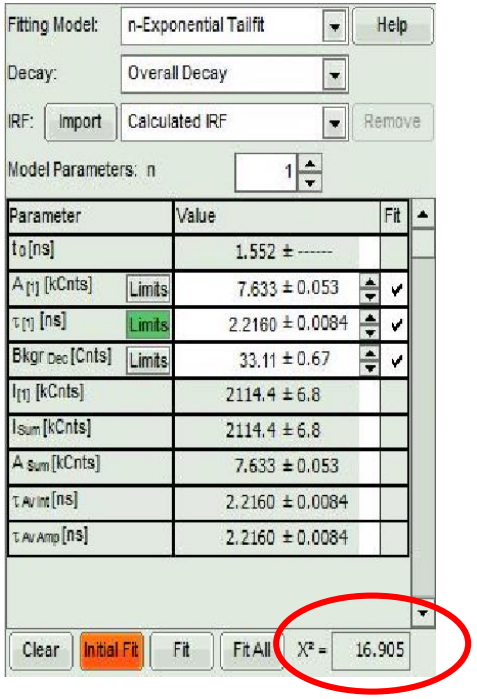
# What if $\chi^2$R is below 1?!

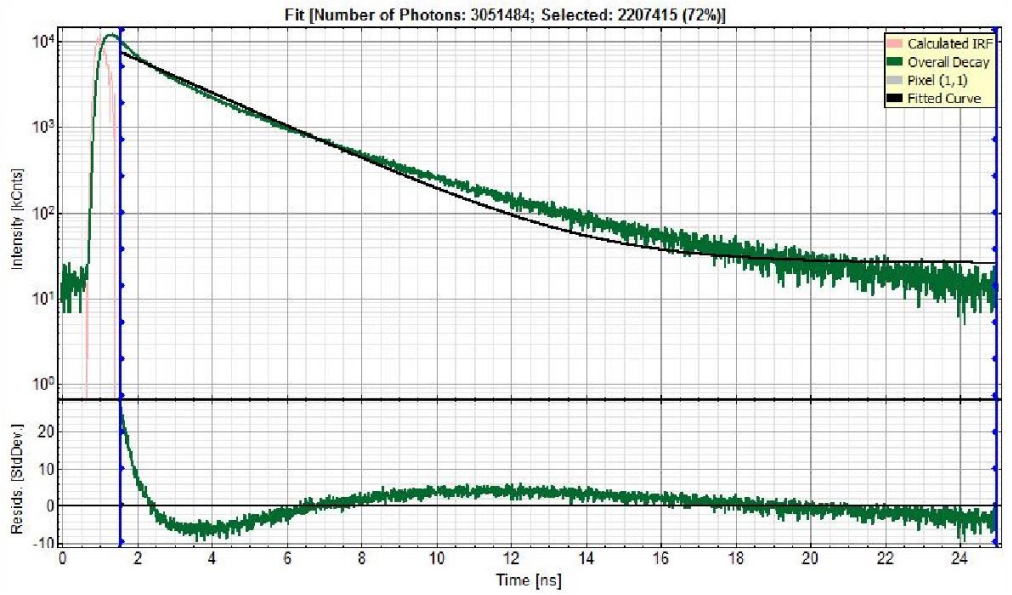
- There are some situations, where a $\chi^2$ value below 1 may occur. This behaviour is always an indication that one or several preconditions of least squares fitting are violated

- For FLIM images, this effect originates mostly from dark time bins or image pixels, which contain very few counts. Consequentially, the noise of the corresponding TCSPC curve cannot be regarded as Gaussian anymore. It is rather Poissonian. Make sure to apply MLE fitting by pressing the „Fit“ button. MLE is suited for Poissonian noise.

# Practical Example: Estimating the Goodness-of-fit

- the black fitted curve does not overlay well with the decay curve
- the residuals below the decay show large deviations from zero
- $\chi^2 R$ („chi squared reduced“) is at ~ 17



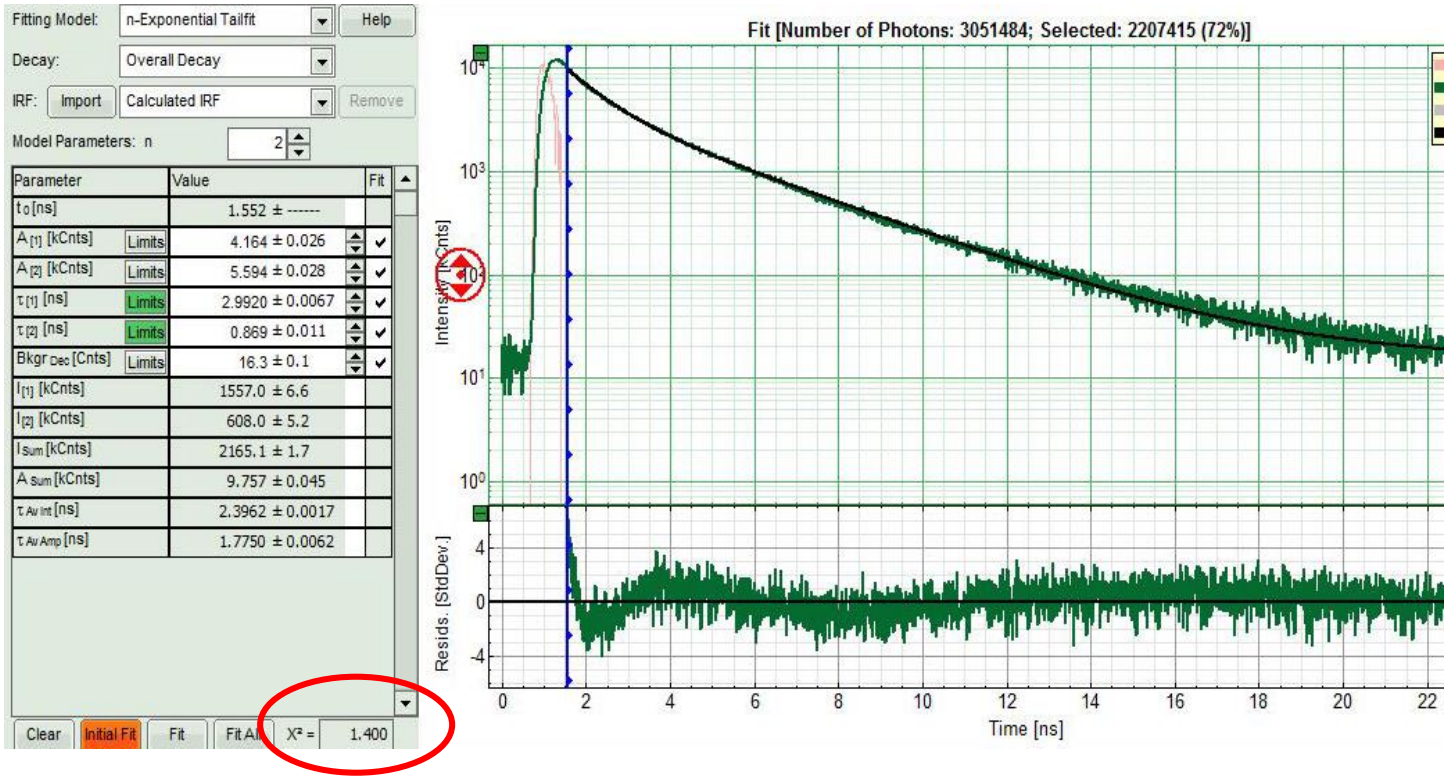




Result for this example: fit is not appropriate > increase # of components

# Practical Example: Estimating the Goodness-of-fit

Double-exponential fit:

- residuals look a lot better, $X^2R$ is relatively close to 1

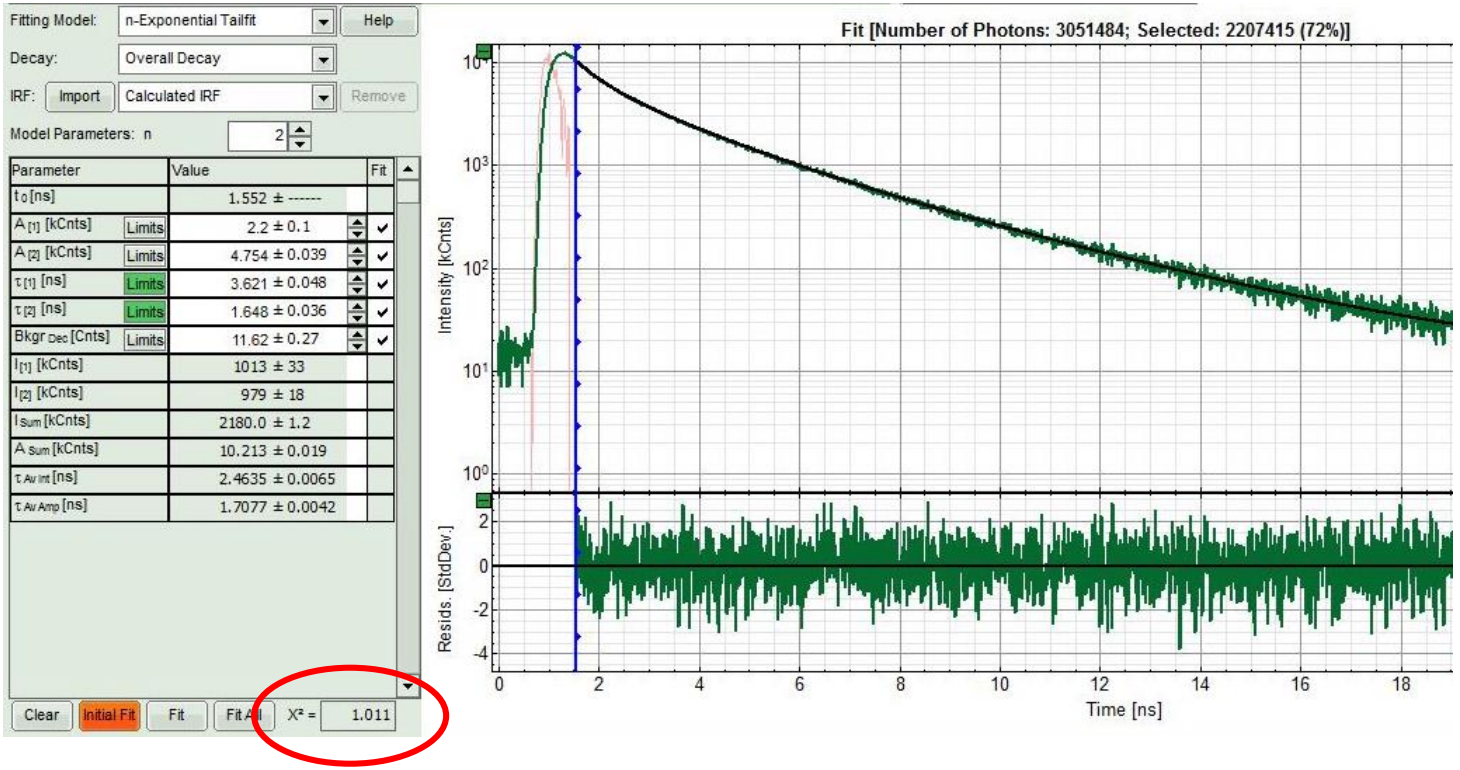



Result for this example: fit is most likely appropriate for 2 lifetime components

# Practical Example: Estimating the Goodness-of-fit

Double-exponential fit using only a ROI and excluding background pixels:

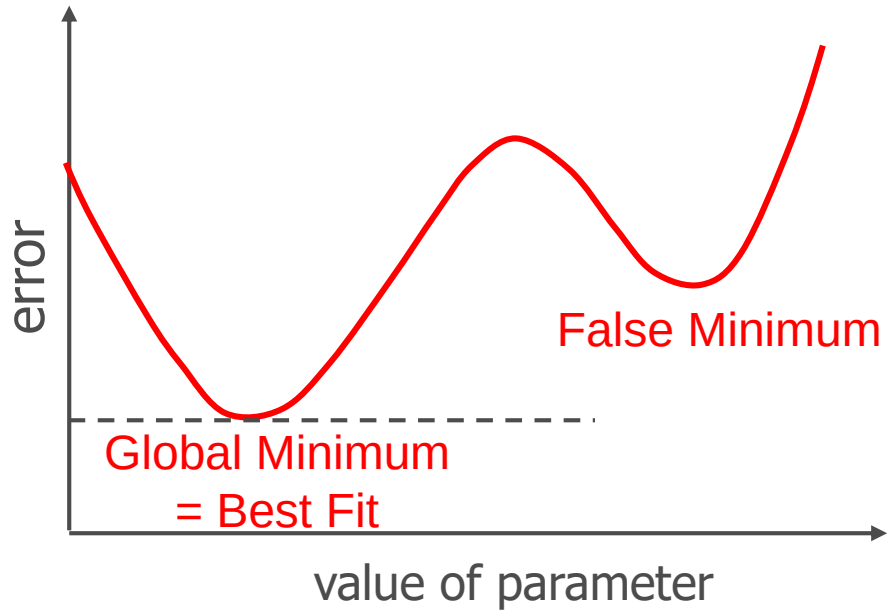
- residuals even better, $X^2R$ practically 1




Result for this example: 2-component fit is definitely appropriate, previous deviations came from background

# What Happens When I Press the „Initial Fit“ or the „Fit“ Buttons

- Fitting Algorithms need to find correct values for various parameters in order to obtain curves that resemble the data
- They assume different values and determine how much the fitted result deviates from the measured curve, i.e. they calculate the error
- They do so repeatedly until they approach the optimum
- But how can the optimum be found?



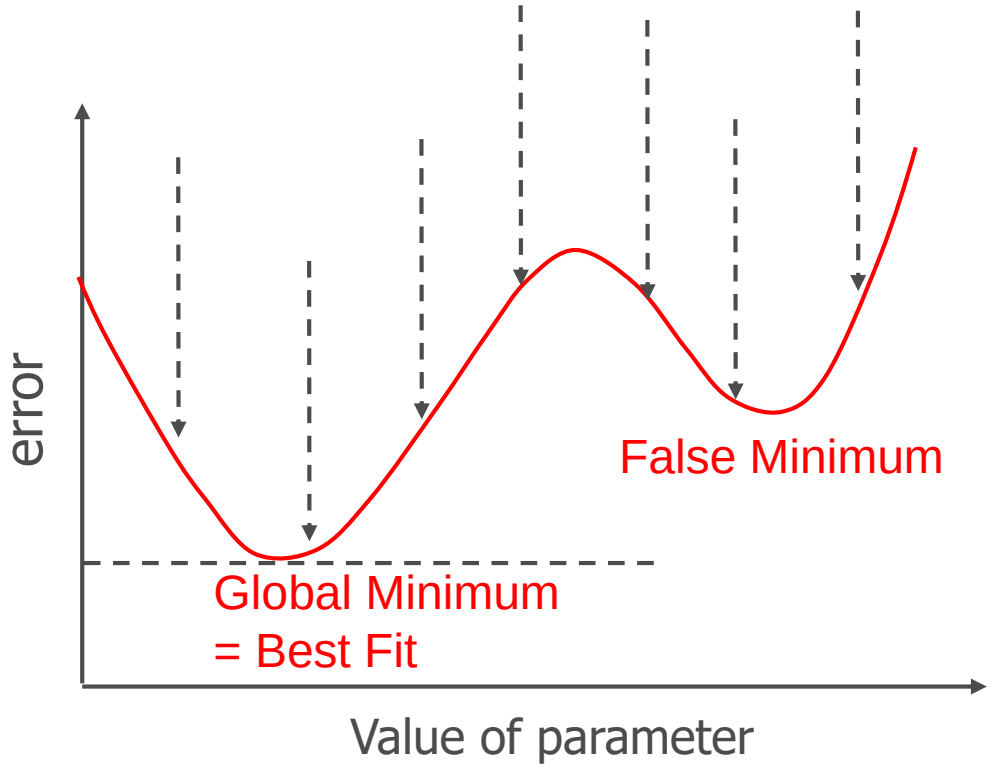

Goal in fitting:

to find the parameter value producing the smallest error

Challenge:

not to get stuck in false minima

# What Happens When I Press the „Initial Fit“ or the „Fit“ Buttons



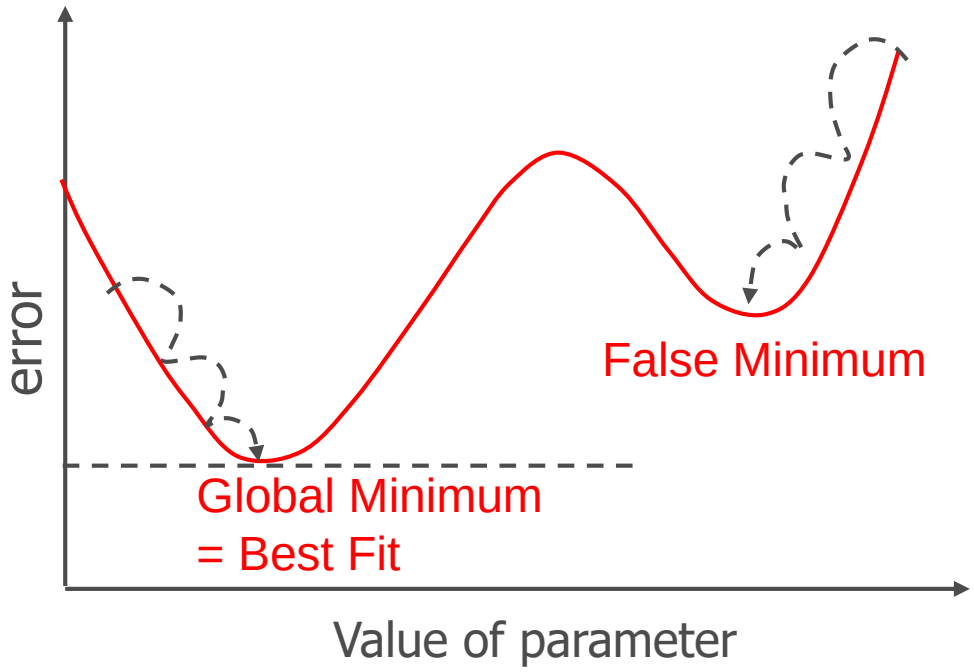


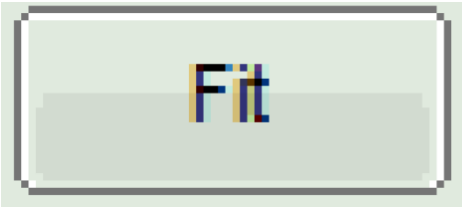
- Monte Carlo Searches assume widely distributed parameter values thereby covering very much of the error curve

➢ high probability to get close to the global minimum / best fit

➢ due to large gaps they might, however, miss the absolute minimum slightly

# Finding the Best Fit: Maximum Likelihood Estimation (MLE)



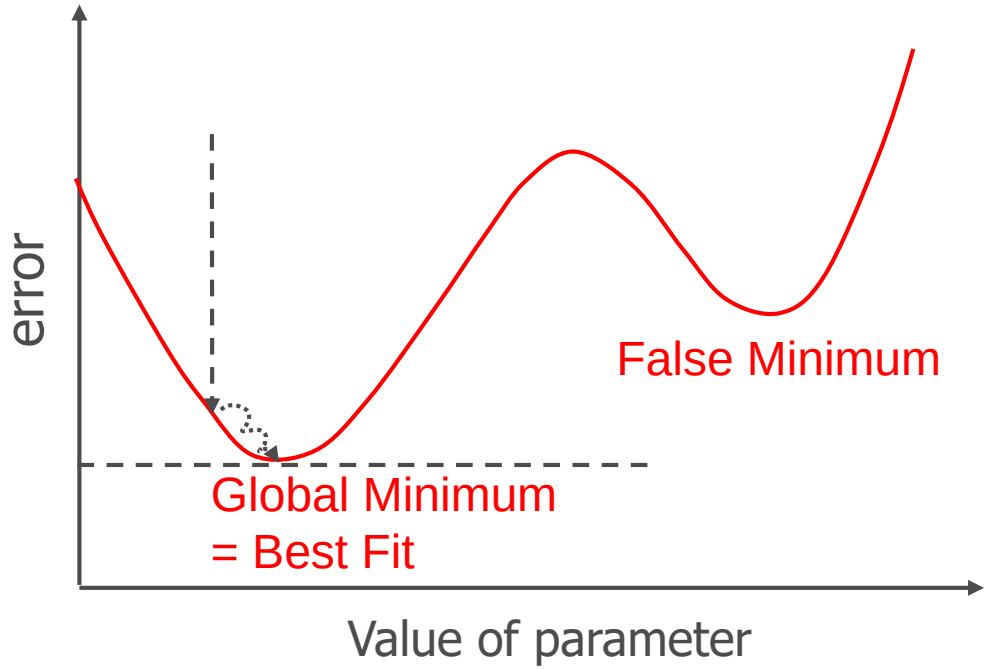

- MLE looks in the vicinity of the assumed starting values and checks whether the error decreases or not
- = „hopping to the minimum“
- BUT: if starting point is wrong, algorithm might find false minimum!

# Finding the Best Fit: Combination of Monte Carlo and MLE

- both fitting approaches have limitations
- → combine them:

First: Monte Carlo search to find the approximate right range for parameter

Second: MLE to improve further



# Tips and Tricks on Analysis (1)

- When you fit a **multi-exponential** model to the decay of a sample with several different fluorophores, try to fix the lifetime parameters to their expected values. They can often be obtained from independent measurements of single fluorophores under the same conditions.

- Very **short apparent decay components** (with lifetimes on the order of magnitude of the IRF may be an artefact that results from insufficient suppression of the scattered excitation light. Scattered light can be taken into account in deconvolution models as an additional parameter. However, it is always preferable to remove the scattered excitation light (or similar artefacts, such as back-reflections) during the experiment, instead of including them in the reconvolution fit.

- Anisotopy effects (e.g. due to rotational relaxation) often manifest themselves as fast decaying or rising components. Rising components result in negative amplitudes.

# Tips and Tricks on Analysis (2)

- **Regular oscillations of weighted residuals** are indicative of RF noise pick-up. Spikes and/or trends in residuals may be due to different decay law (most probably higher exponential order than in your model). They may indicate the presence of scattered IRF light or may result from polarization effects.

- Sometimes a multi-exponential fit yields **two or more identical decay times**. Set one of them to a small value or decrease the exponential order.

- If the **fitting parameters for one component** are **zero** then the exponential order is most likely too large.

- **Negative amplitudes** can be a result of low photon numbers. Try MLE, pixel-binning, or fixing to expected lifetimes. In specific cases, negative amplitudes are meaningful: due to anisotropy effects, exciplex kinetics, or for FRET-acceptor decays.

# Advanced Concepts for Curve Fitting

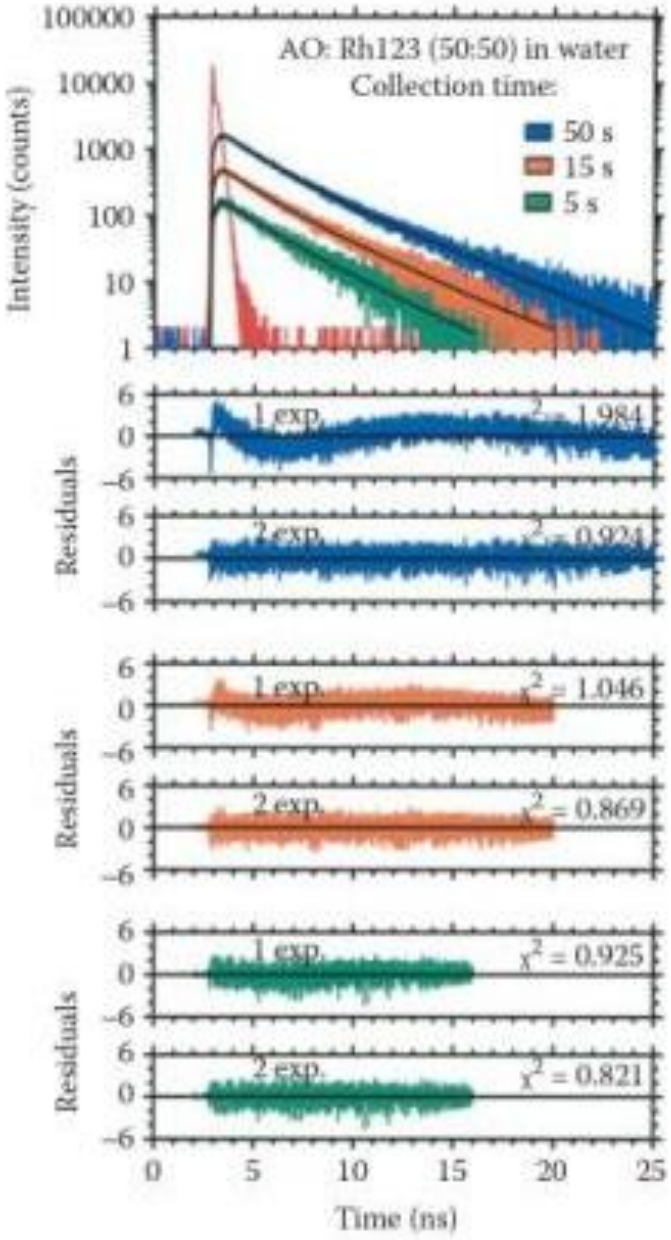
- **Applying limits for model parameters**

In some cases it might be useful to limit certain model parameters to a given interval. However, it must be stated that setting the parameter limits will (of course) bias the lifetime histogram. Because any limit will bring back all parameter values that violate this limit, a sharp peak, which is located at the limit will be visible in the lifetime histogram. Furthermore, setting limits will reduce the ability of the fit to converge, because pathways for convergence can be blocked even if the final parameter set would not have violated any limits.

- **Setting intensity thresholds**

Unphysically long lifetimes on some pixels indicate that there are not enough photons to define a lifetime. You can set an intensity threshold in the FLIM menu in order to "skip" these dark pixels. Also you may want to apply some pixel binning to improve photon statistics. Note that a "lifetime" or "decay curve" of a pixel which is much smaller than the confocal resolution is not really meaningful from physics point of view.

# Photon Statistics Influence whether Multiple Lifetimes can be Distinguished




- Joseph R. Lakowicz:
- Principles of Fluorescence Spectroscopy,
- 3rd Edition, Springer

FIGURE 12.7 Intensity decays for mixture of AO and Rh123 and residual distribution for different data collection times.

# How many Photons Do I Need for Proper Analyses of Lifetimes?

- I want to analyze fluorescence decays on pixel-wise basis

- one needs at least 500 counts per pixel for a reasonable two-component fit if the two lifetimes are clearly separated
- if more components are to be fitted or the expected lifetimes are close together then considerably more photons will be needed!

- I want to analyze fluorescence decays in ROIs

- for ROIs all photons will be added up for one fluorescence decay curve
- therefore also less than 500 counts per pixel are acceptable
- the criterion mainly determining how many photons are needed is the size of the ROIs

Use a reference system (for instance mixture of dyes of known lifetimes) to test out limits if in doubt

# FLIM Fitting: Readout Parameters

| Parameter | Significance |
|---|---|
| $A_n$ [n] | amplitude of the n-th exponential |
| $\tau_n$ [ms] | lifetime of the n-th exponential |
| Bkgr Dec | background of the decay |
| Shift_IRF | IRF shift (for reconvolution fit only) |
| Bkgr_IRF | IRF background (for reconvolution fit only) |
| $I_n$ | intensity of the n-th exponential |
| I_Sum | sum of all intensities |
| A_Sum | sum of all amplitudes |
| tau_Av_Int | intensity based average lifetime (explanation see next slide) |
| tau_Av_Amp | amplitude based average lifetime (explanation see next slide) |
| $Xi^2$ | chi squared value (measure of the goodness-of-fit) |

# Intensity- vs. Amplitude-weighted Average Lifetime

The average lifetimes can be defined in two ways. They differ in the way the single decay times are weighted in the averaging:

$$\tau_{int} = \frac{\sum_i Ampl_i \cdot \tau_i^2}{\sum_i Ampl_i \cdot \tau_i} \qquad \tau_{amp} = \frac{\sum_i Ampl_i \cdot \tau_i}{\sum_i Ampl_i}$$

$\tau_{int}$ equals the average amount of time for which the emitters (e.g. fluorophores) remain in their excited state after the onset of excitation.

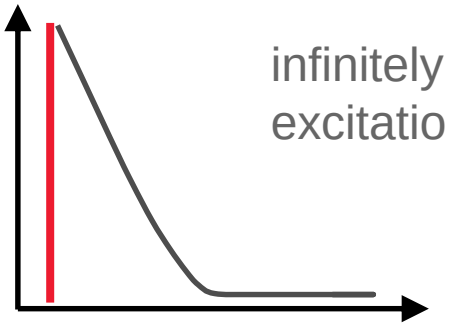
$\tau_{amp}$ is proportional to the steady state intensity. It is applicable when the FRET efficiency is calculated based on the lifetimes of the donor in the presence and absence of the acceptor molecule.

**If not otherwise stated, for the SymPhoTime software the term average lifetime always refers to the definition of $\tau_{int}$.**

For a mono-exponential decay $\tau 1 = \tau_{int} = \tau_{amp}$ holds. Care should be taken when you interpret these values if rising components (i.e. components with a negative amplitude) are present in the decay.

# Convolution of Decay with the Instrument Response Function (IRF)

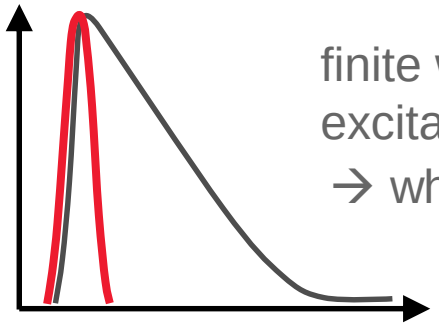
- **ideal world**



infinitely short excitation pulse

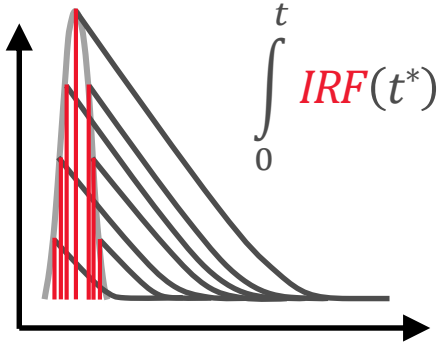
1. onset of fluorescence from 0 to 100% at t=0
2. physically real decay

- **real world**



finite width excitation pulse
→ when is t=0 ?

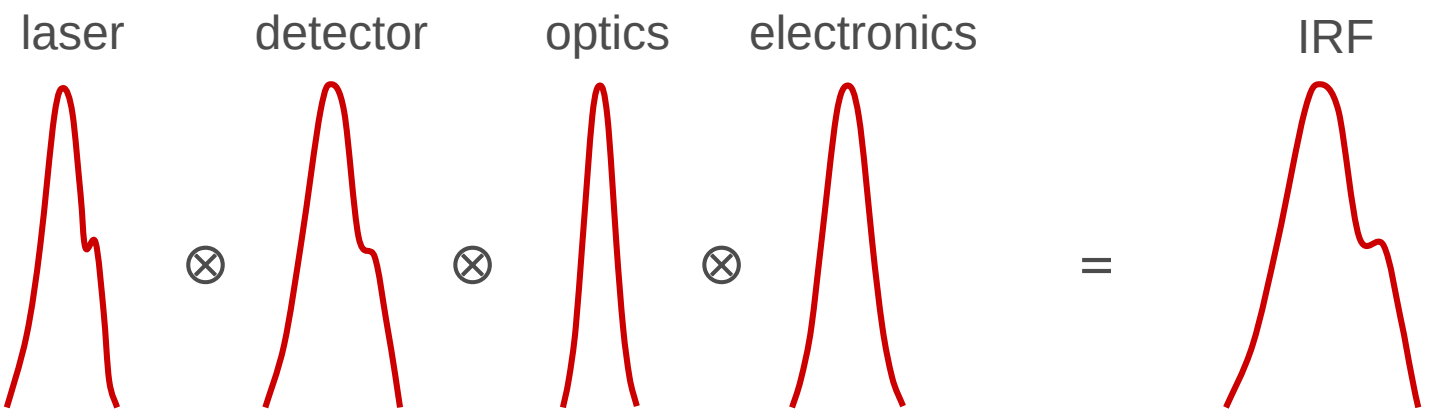
1. gradual onset of fluorescence
2. decay shape „distorted“ by excitation pulse



$$\int_0^t IRF(t^*)\,Decay(t-t^*)\,dt^*$$

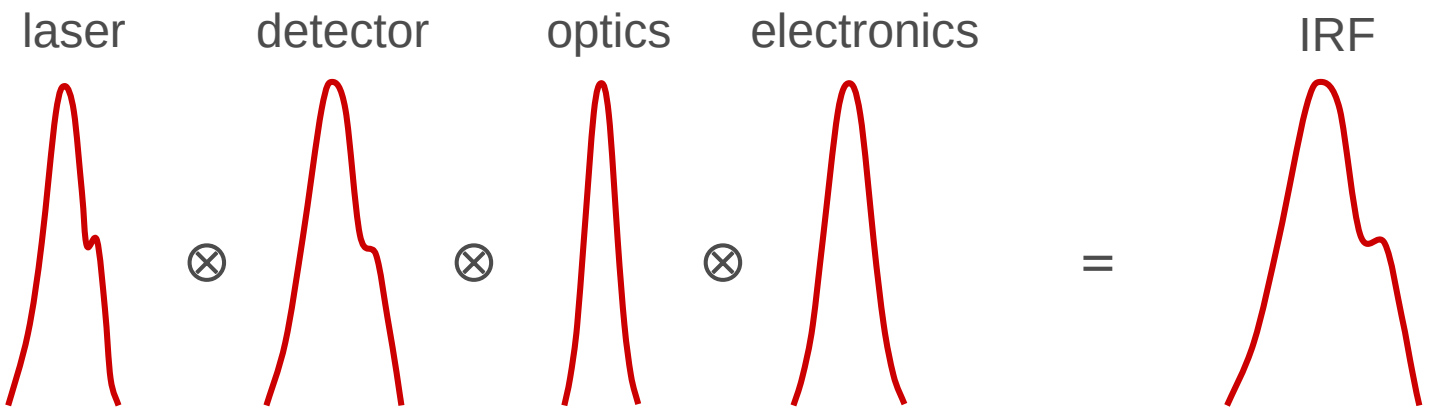
# Instrument Response Function (IRF)

- All components of an instrument have a limited time resolution and contribute to the behaviour of the instrument
- the Instrument Response Function (IRF) is a convolution of all of these contributions




- IRF = TCSPC histogram of the mere excitation pulse as seen by the instrument

# Instrument Response Function (IRF)

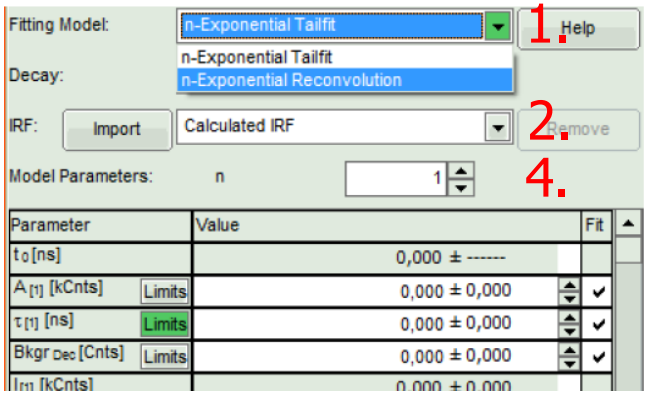


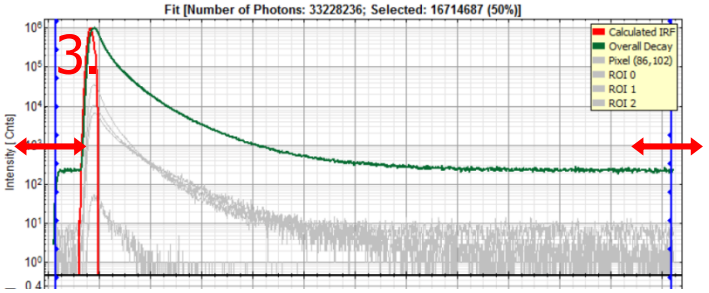

- between measurements to be compared the time resolution of the factors shown must be constant!

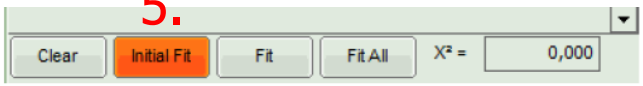
  - do not change laser output power (changes in laser transmission via AOTF / EOM / grey filters / scaffolds are fine)
  - do not change detectors / detector settings influencing time resolution such as (a) voltage gain for PMTs or (b) detection window
  - do not change optical components, e.g. objective
  - (do not use different electronics)

# How to do „Reconvolution“ Fitting Using the IRF

1. In the fitting parameter table, change the fitting model to "n-Exponential Reconvolution Fit".
2. Select which IRF you would like to use: a software estimated IRF or a previously measured IRF.
3. Edit the fitting range sliders: the fitting range should start on the left of the onset of the decay curve. On the right, the fitting range should also include a bit of the background that follows the decay tail.
4. Select a mono-exponential model to start with.
5. Press the Initial Fit button.
6. For optimizing the fit, proceed as for tail fitting.



# Determining the IRF Experimentally

**material:**

- use special dyes with very fast lifetime (lower ps range) and same/similar emission range as your sample
- check usage of saturated Potassium Iodide solution (KI) as collisional quencher to shorten lifetimes of other, long-lived dyes (1.4-1.5 g PI for 1 ml H2O)
- if no appropriate fluorophore is available: use scatterer

**measurement:**

- use same laser repetition rates as for your “normal” measurements
- during IRF measurements, the count rate should be less than 0.2%
- it is recommended to collect the sample decay and the IRF up to a similar peak count level, in order to ensure that they are of comparable statistical precision
- But not higher than 10-50 thousand counts at the TCSPC peak. Above non-linearities in the electronics will influence recorded histograms

# Fast Decaying Dyes for IRF Measurements

LDS 798 dye in aqueous solution: lifetime ~ 24 ps, independent of excitation wavelength.

→ Luchowski R., Gryczynski Z., Sarkar P., Borejdo J., Szabelski M.,Kapusta P., Gryczynski I., "Instrument response standard in time-resolved fluorescence", Review of Scientific Instruments, Vol.80, 033109 (2009), http://dx.doi.org/10.1063/1.3095677

Rose Bengal water solutions quenched by potassium iodide: lifetime ~ 115 ps

→ Szabelski M., Luchowski R., Gryczynski Z., Kapusta P., Ortmann U., Gryczynski I., "Evaluation of instrument response functions for lifetime imaging detectors using quenched Rose Bengal solutions", Chemical Physics Letters, Vol.471, p.153-159 (2009), http://dx.doi.org/10.1016/j.cplett.2009.02.001

Erythrosine B quenched by potassium iodide: lifetime ~ 24 ps

→ Szabelski M., Iliev D., Sarkar P., Luchowski R., Gryczynski Z., Kapusta P., Erdmann R., Gryczinski I., "Collisional quenching of Erythrosine B as a potential reference dye for impulse response function evaluation", Applied Spectroscopy, Vol.63, p.0363-0368 (2009), http://www.ingentaconnect.com/content/sas/sas/2009/00000063/00000003/art00017

Pinacyanol iodide in methanol ~ 6ps lifetime. Very broad emission spectrum (starting at around 550nm).
Pinacyanol iodide is also known as Quinaldine blue, 1,1' -Diethyl-2,2' -carbocyanine iodide, CAS Number:605-91-4. Pinacyanol bromide, CAS Number:2670-67-9 or Pinacyanol chloride CAS Number:2768-90-3 should work too, however, the dye must be pure, otherwise you may end up with measuring (long lifetime) impurity emission.

→ van Oort B., Amunts A., Borst J. W., van Hoek A., Nelson N., van Amerongen H., Croce R., "Picosecond fluorescence of intact and dissolved PSI-LHCI crystals", Biophysical Journal, Vol.95, p.5851-5861 (2008), http://dx.doi.org/10.1529/biophysj.108.140467

# Fast Decaying Dyes for IRF Measurements

Allura Red: lifetime ~ 10 ps. Emission spectrum 550 – 750 nm.

Allura Red is also known as Disodium 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonate, CAS Number:25956-17-6.

→ Chib R., Shah S., Gryczynski Z., Fudala R., Borejdo J., Zelent B., Corradini M., Ludescher R., Gryczynski I., "Standard reference for instrument response function in fluorescence lifetime measurements in visible and near infrared Measurement", Science and Technology, Vol.27, 027001 (2016) http://dx.doi.org/10.1088/0957-0233/27/2/027001

Pyridine 1, FRET-quenched by interactor 60 mM Rhodamine 800 in a poly(vinyl) alcohol matrix: lifetime ~ 20 ps.

→ Luchowski R., Kapusta P., Szabelski M., Sarkar P., Borejdo J., Gryczynski Z., Gryczynski I., "Förster resonance energy transfer (FRET)-based picosecond lifetime reference for instrument response evaluation", Measurement Science and Technology, Vol.20, 095601 (2009), http://dx.doi.org/10.1088/0957-0233/20/9/095601

5-carboxyfluorescein, FRET-quenched by BHQ2-Acceptor: lifetime ~ 18 ps.

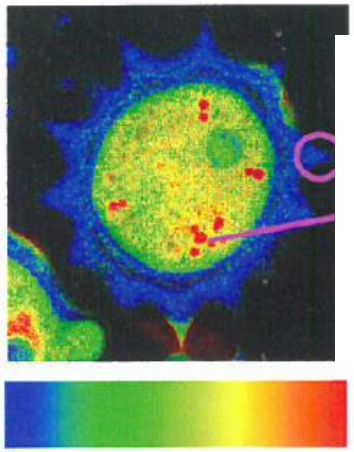
→ Szabelski M., Gryczynski Z., Gryczynski I.
Photophysical properties of novel fluorescein derivative and its applications for time-resolved fluorescence spectroscopy
Chemical Physics Letters, Vol.493, p.399-403 (2010)
http://dx.doi.org/10.1016/j.cplett.2010.05.061

… or try any fluorophore solution and quench it with a saturated solution of Potassium Iodide

# Applying the Results of ROI Curve Fitting to Single Pixel TCSPC Histograms

- TCSPC histogram fitting results can also be applied to FLIM image → „fast FLIM“ is replaced by „pixel-by-pixel FLIM“ / „FLIM fit“
- the decay of every pixel is fitted with the lifetimes found in the decay curve of a whole image / ROI

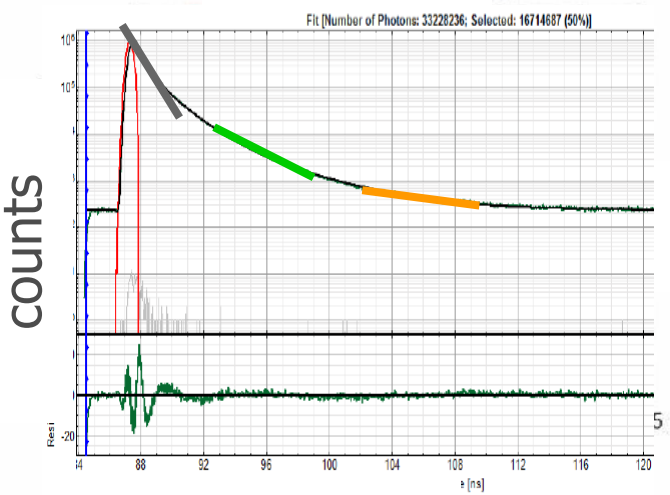
**Fast FLIM**



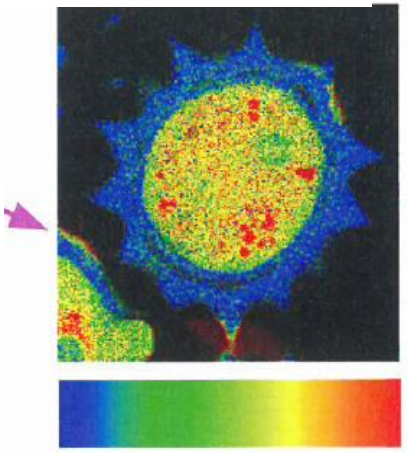
1 ns 1.8 ns

- Fast
- Good lifetime contrast
- Less lifetime - noise

**TCSPC histogram**




**pixel-by-pixel FLIM**

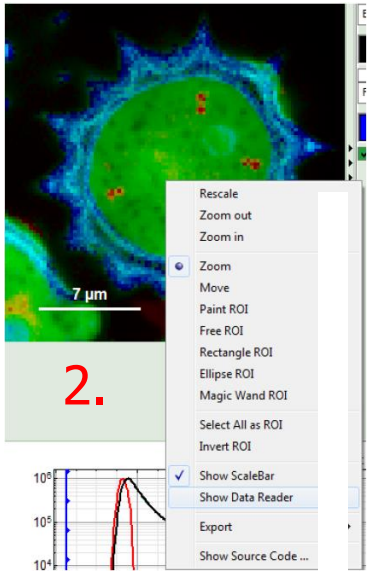


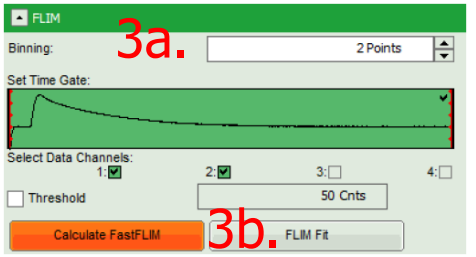
1 ns 1.8 ns

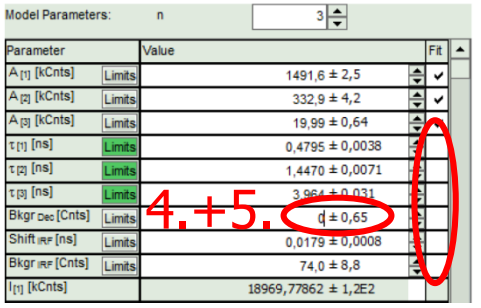
- More accurate results for
  - → very short lifetimes
  - → complex dye mixtures
- lifetime noise is usually increased in images

# How to do Lifetime Fitting („pixel-by-pixel FLIM“) of Every Image Pixel

1. fit the TCSPC histogram of a whole image / a ROI
2. make sure that each pixel contains enough counts for pixel-wise fitting: place the cursor over the image, activate „show data reader“ in the right-click context menu. The number of counts will be shown when the cursor is moved over the image.
3. If there are too little counts then (a) increase pixel binning and (b) press the Calculate fastFLIM button
4. Fix the parameters „Shift IRF“ and „Bkgr IRF“ to their present values by removing the checkmark in the last column of the fitting table. Fix „Bkgr Dec“ to zero.
5. Optional: fix the lifetimes if no further variation of the lifetimes is desirable.
6. press the FLIM Fit button
7. the result will be visible in both the image and in the lifetime histogram (see next page)



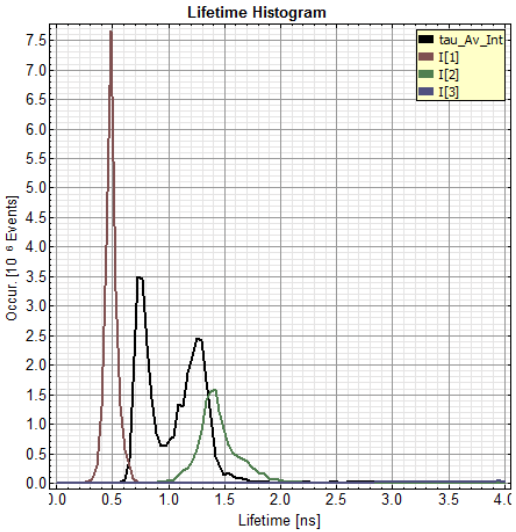



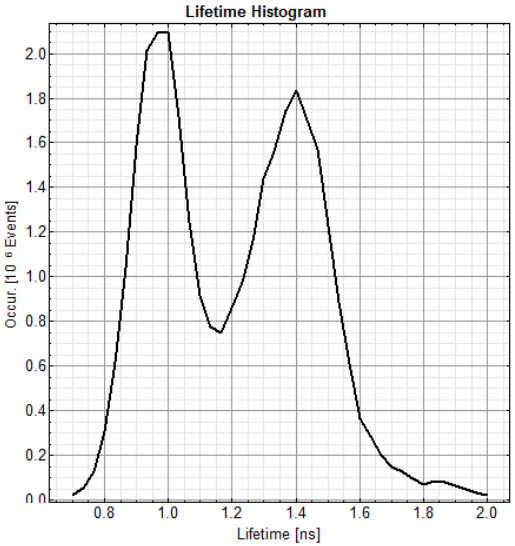



# Lifetime Histogram after Lifetime Fitting

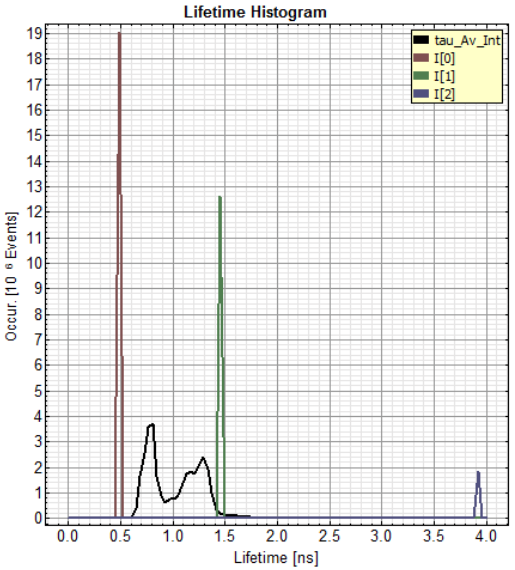
- In addition to the average lifetime (black line), the lifetime distribution of each exponential component is plotted (coloured).

lifetimes variable



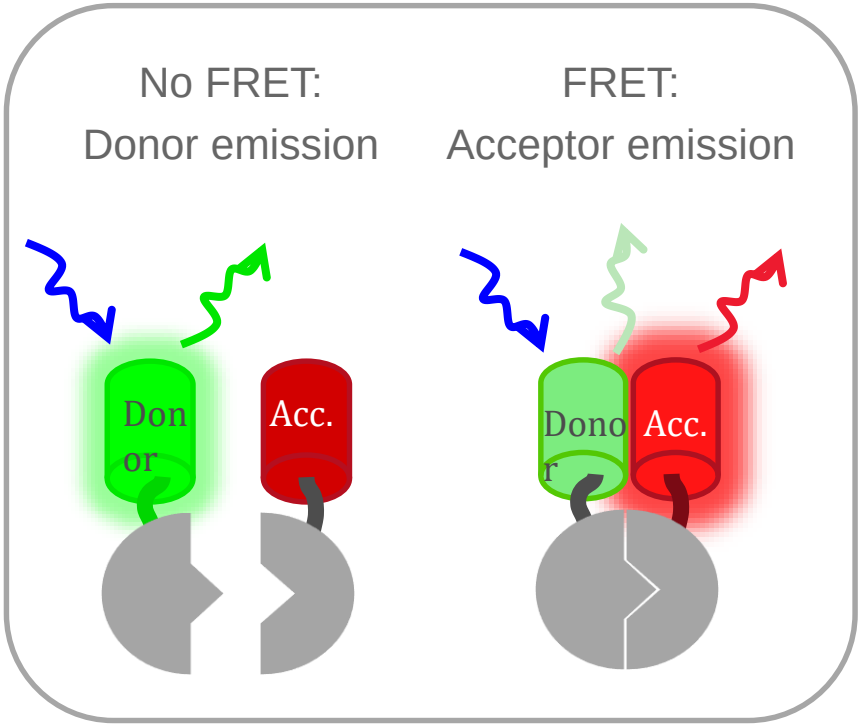

lifetimes fixed

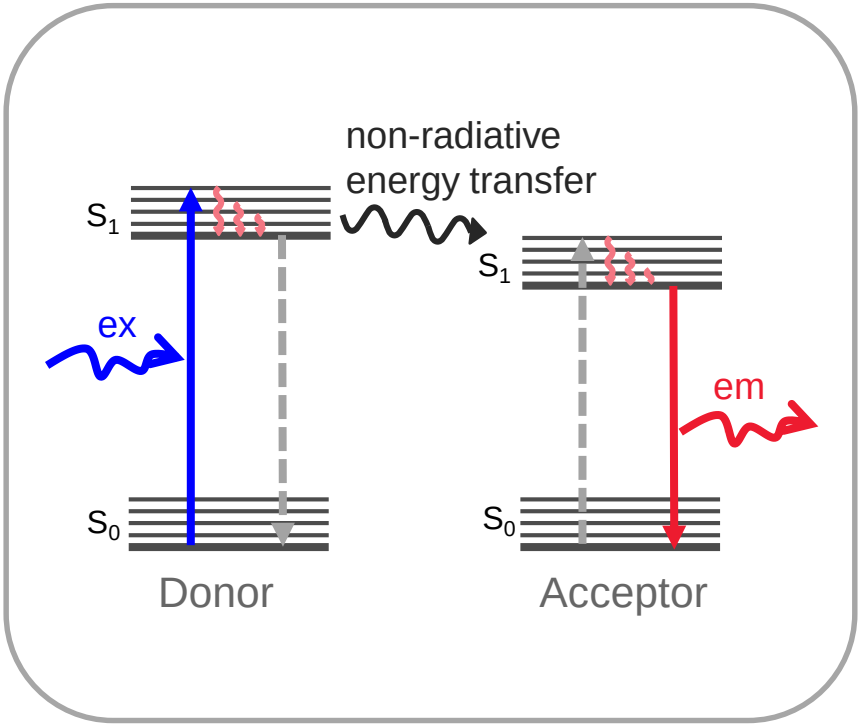



As a rule of thumb, the stronger the resemblance of the average lifetime and the sum of all contri-butions, the less correlated are the exponentials.

# Detecting Interactions with FRET

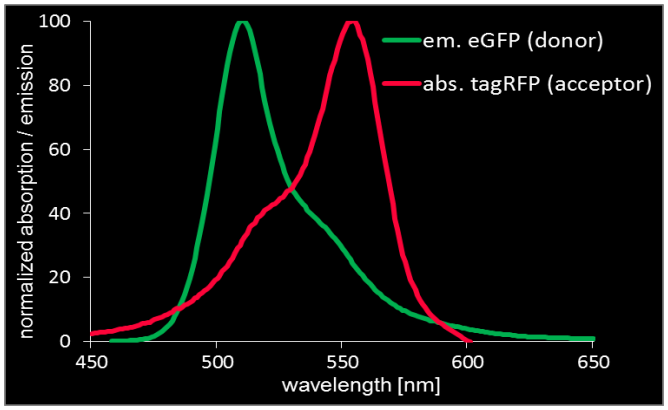
- Probing whether fluorescently-labelled molecules are very close to each other (and most likely interact)
- FRET: non-radiative energy transfer from an excited donor molecule to an acceptor in close proximity



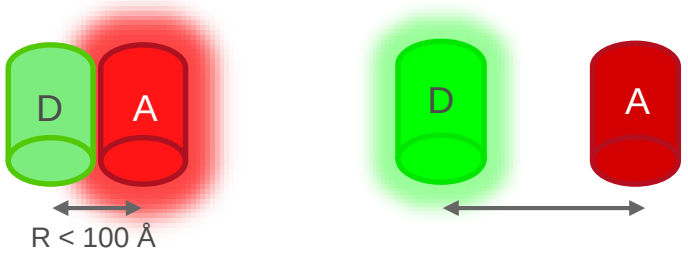



# Prerequisites for FRET

1. overlapping spectra for donor emission & acceptor excitation



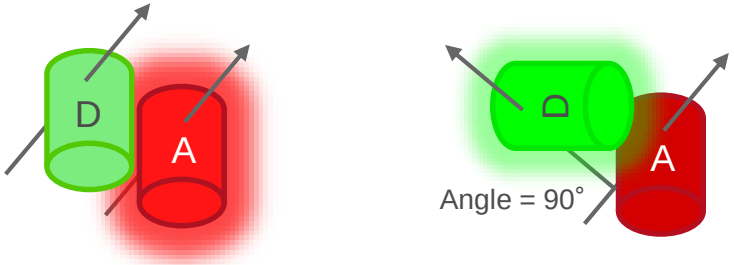

2. Molecules must be in close proximity on an Angstrom scale



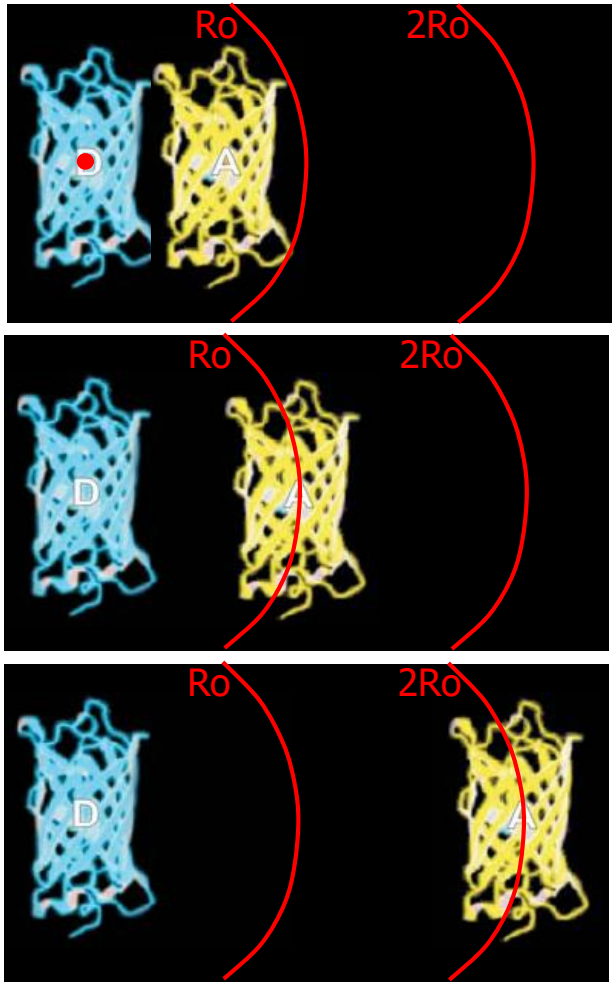

FRET

No FRET

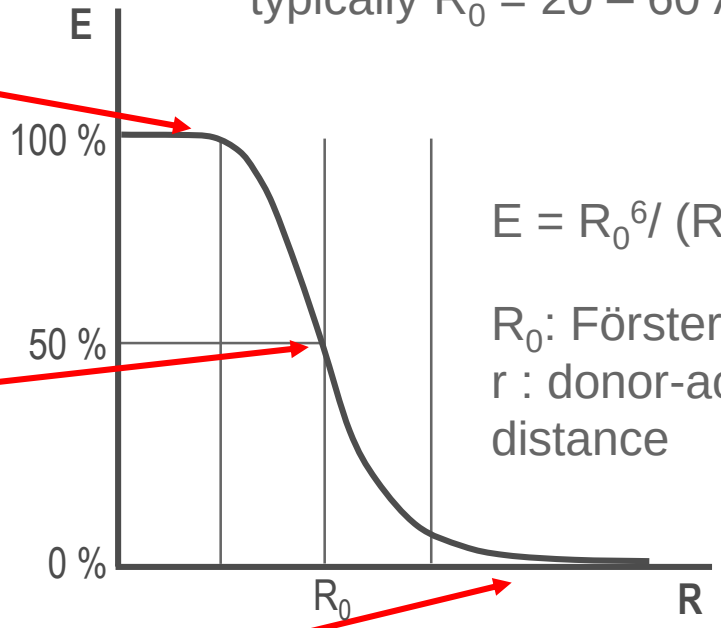
3. Molecules must have the appropriate relative orientation.



# Distance Dependence of FRET Efficiency




$R_0$ = 50 % FRET Efficiency
typically $R_0$ = 20 – 60 Å




$E = R_0^6/(R_0^6+r^6)$

$R_0$: Förster radius
r : donor-acceptor distance

Ref: Truong et al., Structural Biology 2001 &
Timo Zimmermann, ALMU, CRG Barcelona

# Intensity Based FRET Detection Methods

- Acceptor Photobleaching
- Donor Photobleaching

fixed samples

- Ratio Imaging (intramolecular FRET)
- Sensitized Emission

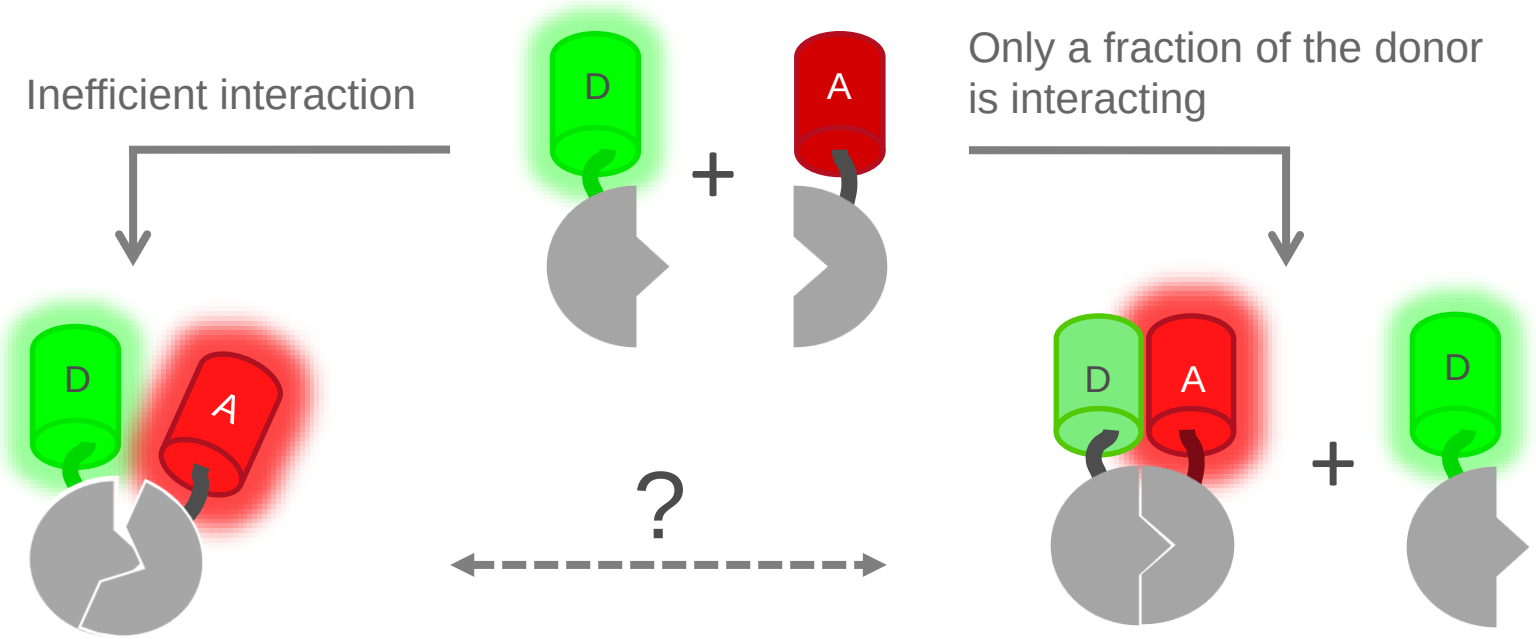
living samples

Result = always „apparent“ FRET Efficiencies

# What a Low Apparent FRET Efficiency Can Mean With Intensity Based FRET

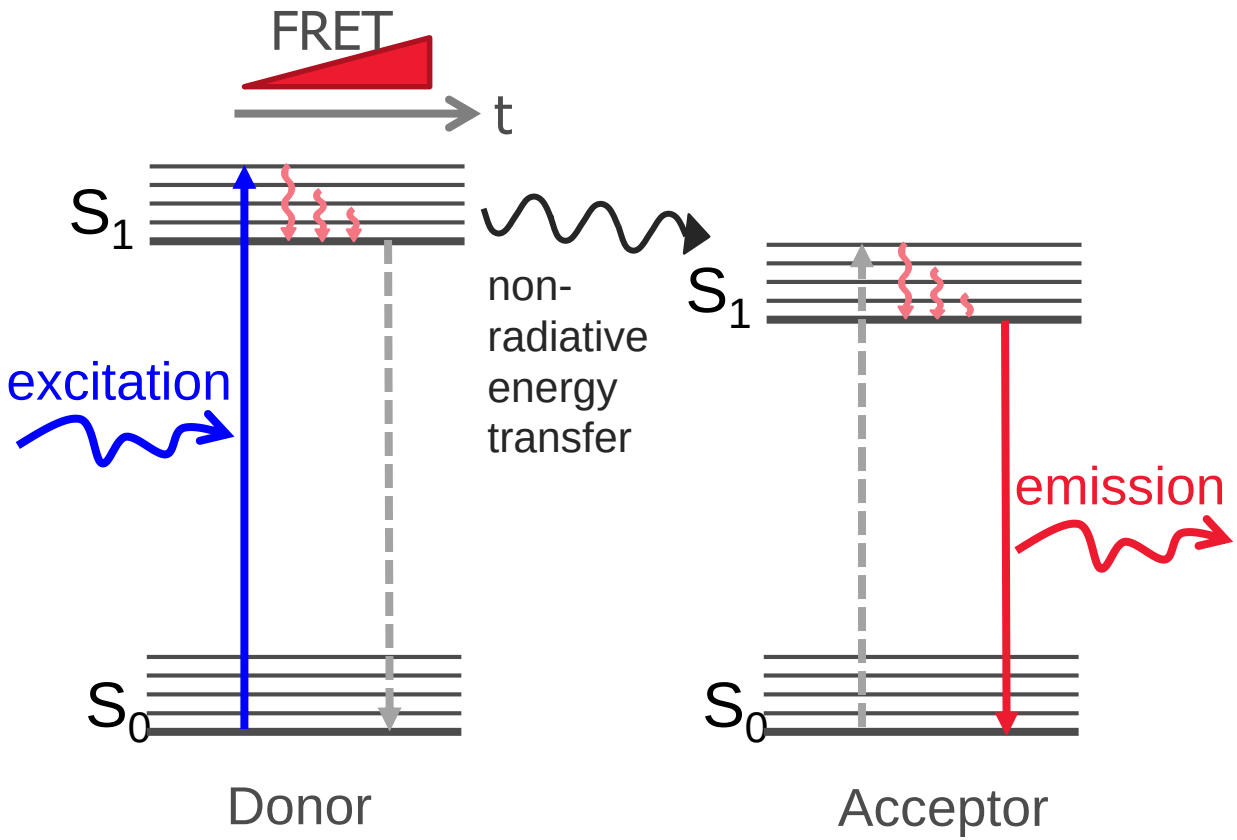
Resulting „apparent“ FRET Efficiencies are always a product of

(a) the interaction efficiency and
(b) the amount of interacting donor



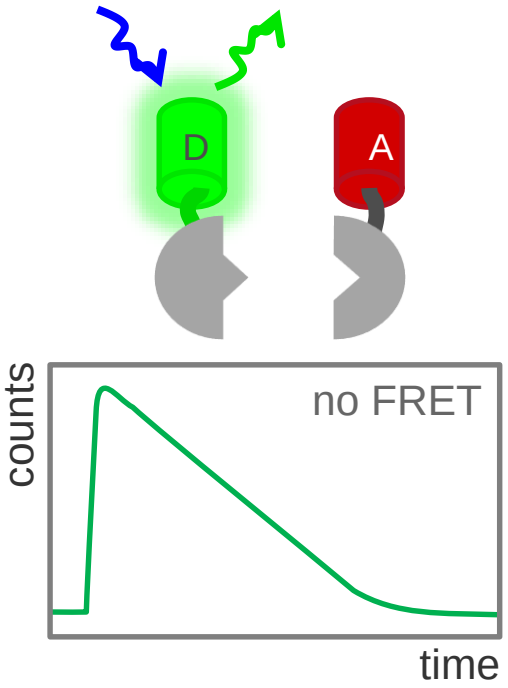
# FRET Measurements based on Lifetime

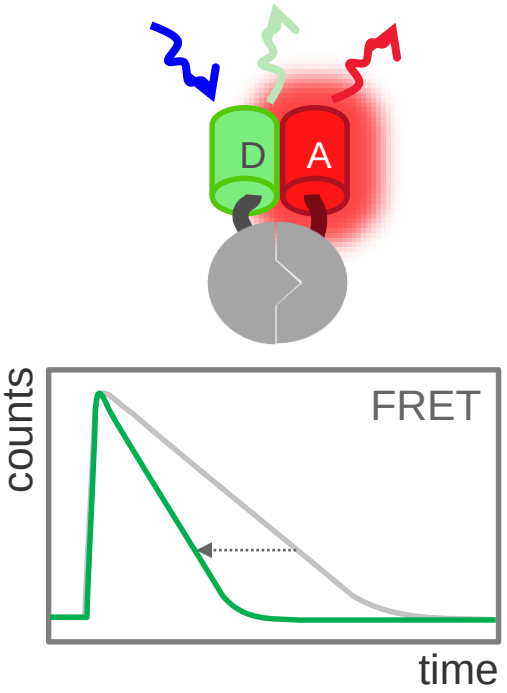
- Donor electrons residing longer in S1 state have a higher probability of transferring their energy to the acceptor



# Analysis of FRET Efficiency via FLIM-FRET

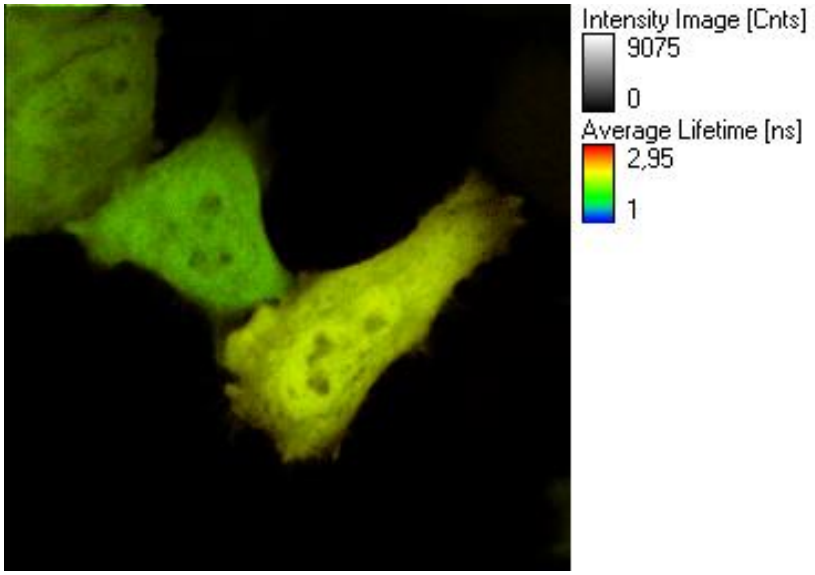
- Analysis of donor fluorescence lifetime

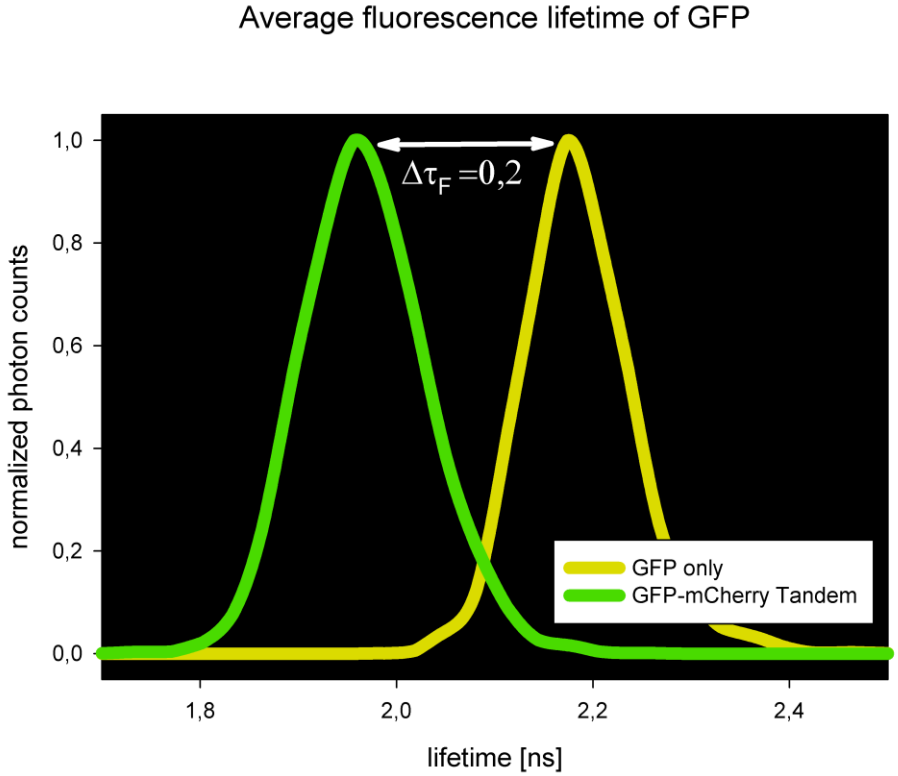



Calculation of FRET efficiency: $E = \frac{\tau_{\text{no FRET}} - \tau_{\text{FRET}}}{\tau_{\text{no FRET}}}$

# FLIM-FRET using Fluorescent Proteins

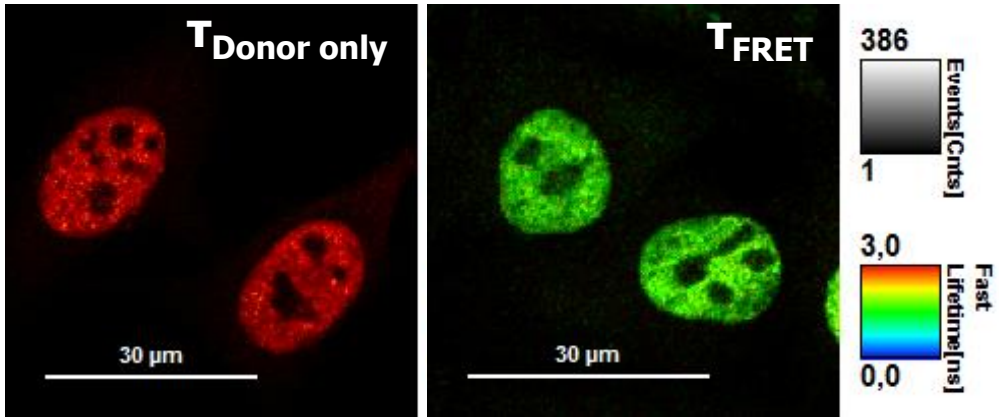


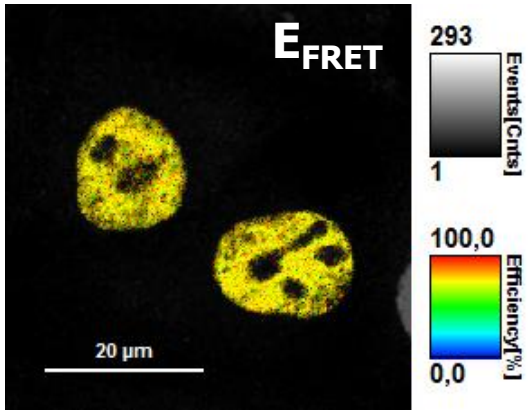



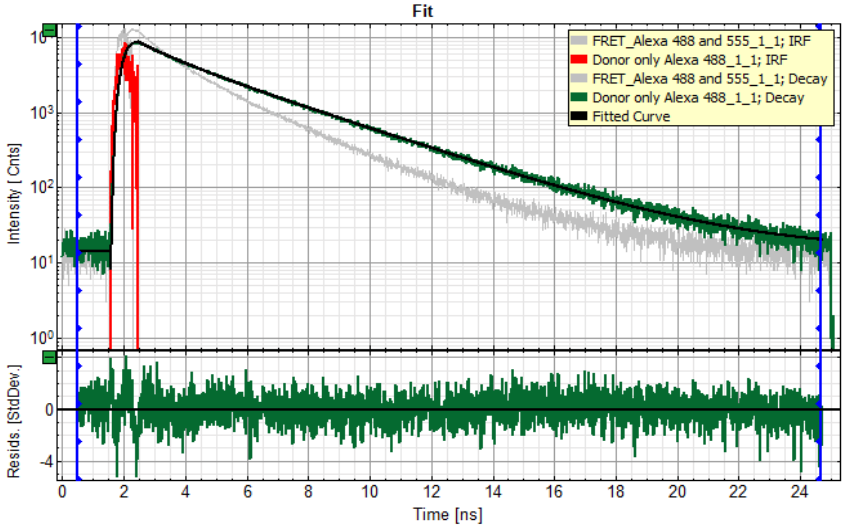

- GFP-only cell (yellow) and cell with GFP-mCherry tandem construct (green)
- FRET between GFP and mCherry: drop of the average fluorescence lifetime by 0.2 ns.

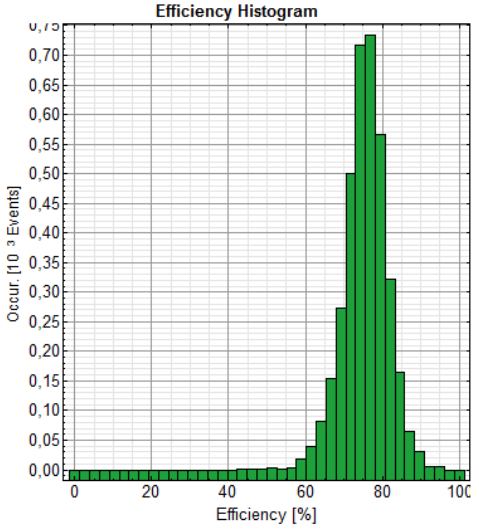
# FLIM FRET using Alexa 488 and Alexa 555

- Sample: fixed cells, with labelled nuclear proteins
- Donor label: Alexa 488, Acceptor label: Alexa 555, Ratio donor:acceptor = 1:1
- Courtesy: Pascal Kessler, Yves Lutz, Imaging Center of IGBMC, Strasbourg, France



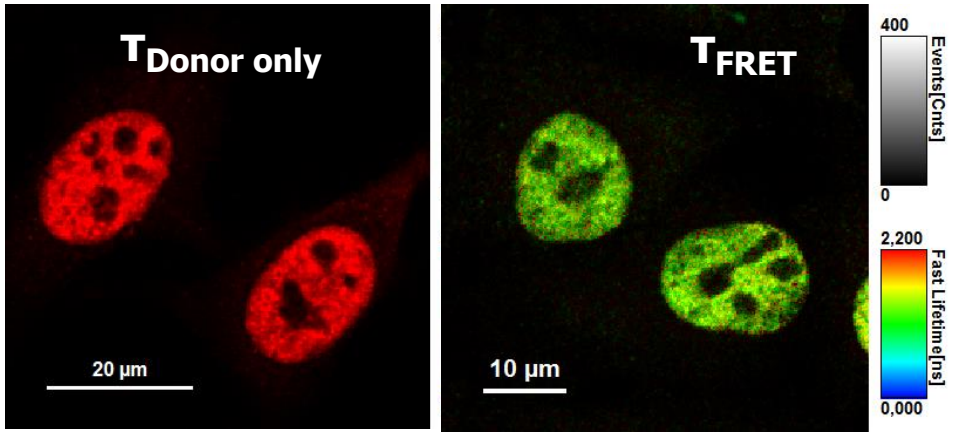



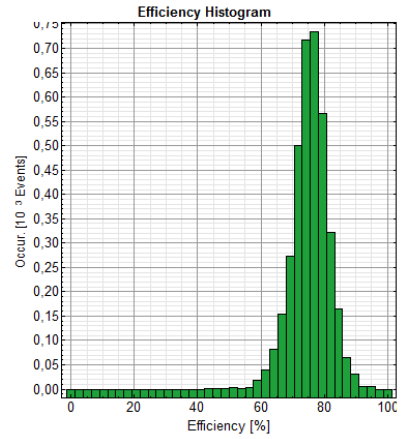



# FLIM FRET using Alexa 488 and Alexa 555

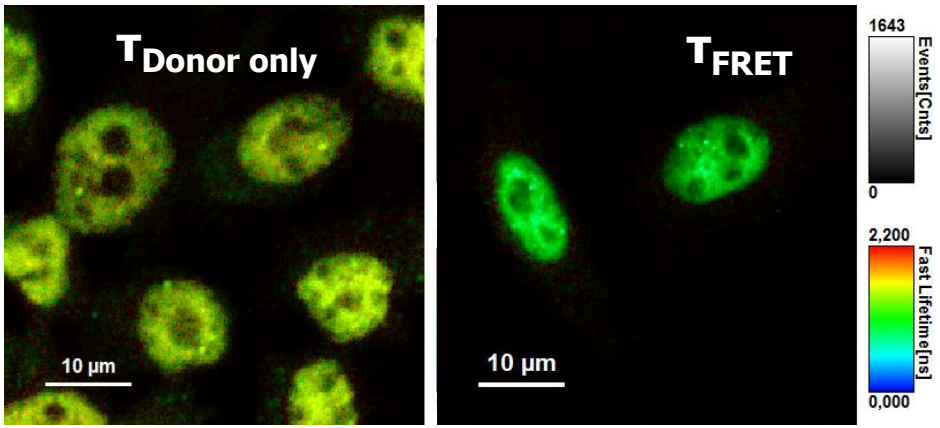
Sample: cells fixed with different media
Donor: Alexa 488, Acceptor: Alexa 555,
Courtesy: Pascal Kessler, Yves Lutz, Imaging Center of IGBMC, Strasbourg, France

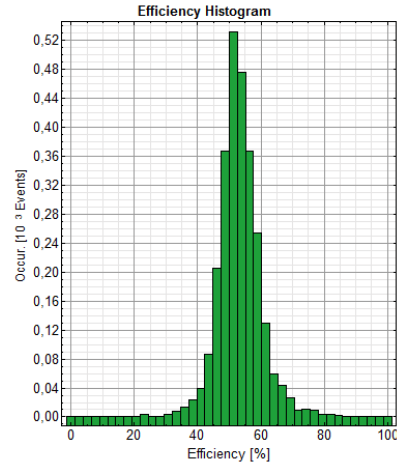
Embedding medium 1



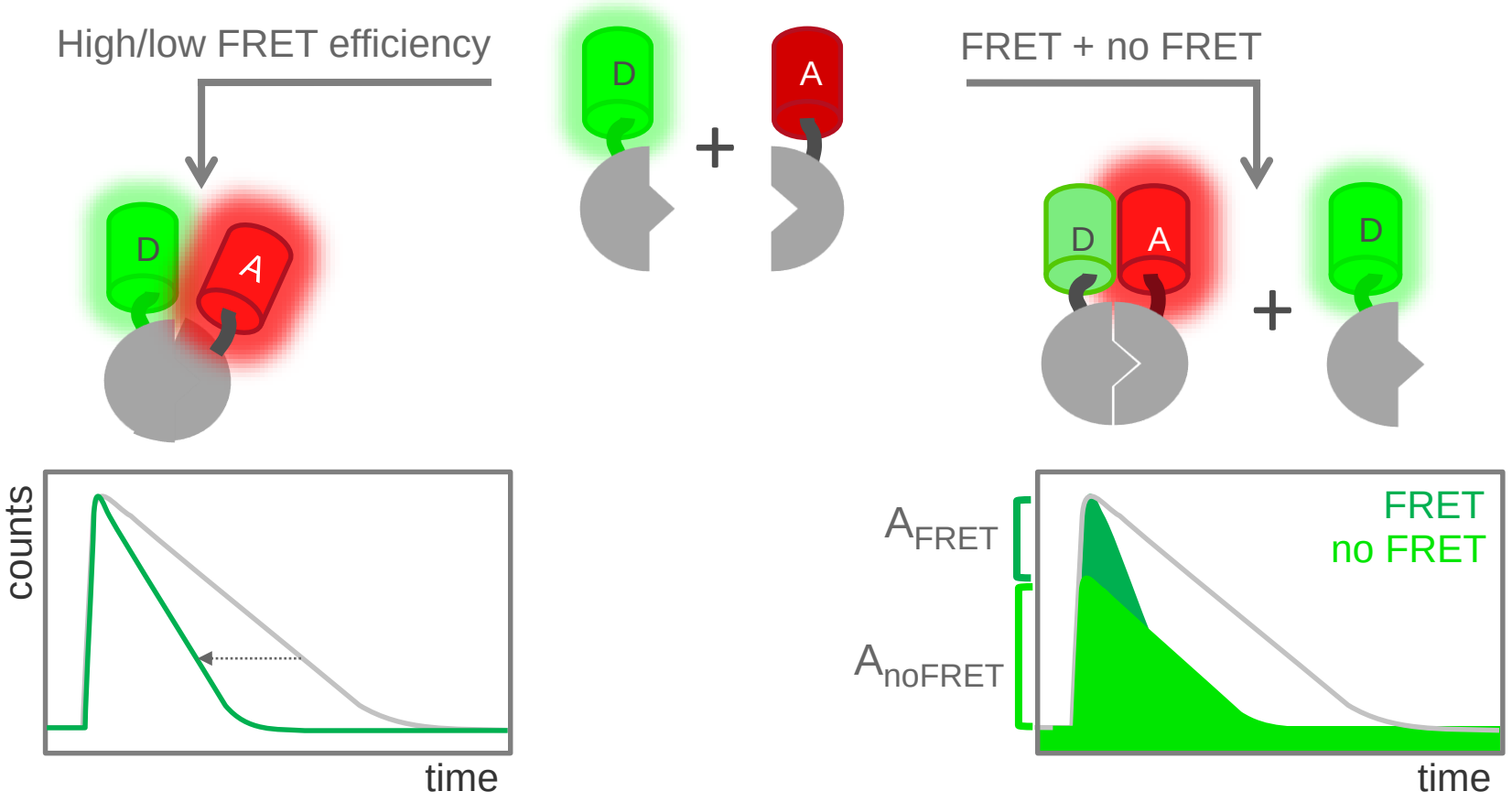




Embedding medium 2



# FLIM-FRET can Resolve Subpopulations




Fraction of bound donor: $\text{Binding} = \frac{A_{FRET}}{A_{no\ FRET} + A_{FRET}}$

# Analysis of the Bound Donor Population

Prerequisite: Mono-exponentially decaying donors!

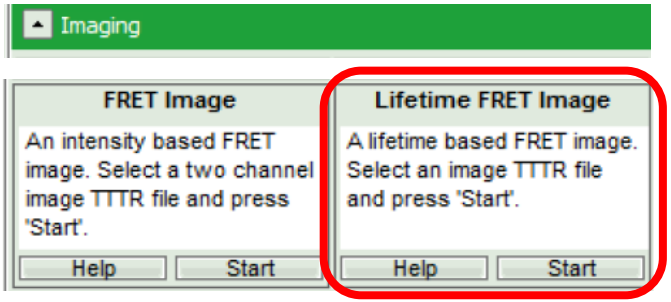
- mTurquoise (2)
- mTFP1
- T-Sapphire
- Citrine
- EYFP
- TagGFP

# Summary FLIM-FRET

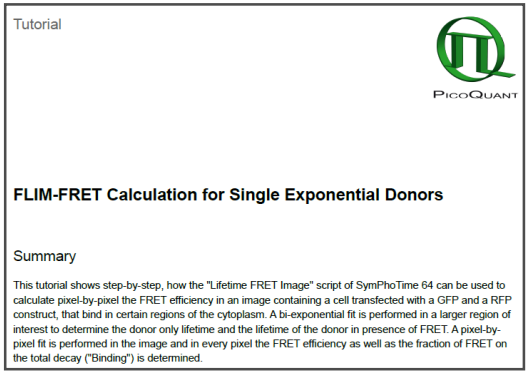
- In most cases, FRET is assessed by looking at the lifetime of the donor
- FRET shortens the fluorescence lifetime of the donor
- The FRET-Efficiency obtained by this technique is the „true“ FRET efficiency measuring the interaction efficiency of the interacting donor molecules with their acceptor
- Additional information is obtained if the donor has one lifetime of its own: the percentage of binding
- The percentage of binding reveals how many of the donor molecules are bound to an acceptor and how many are free

# FLIM-FRET Scripts in SPT64

- Select the „Lifetime FRET Image“ Analysis Script




- for **donor** fluorophores with **mono-exponential** decay two lifetimes can be discriminated, the unbound bound donor
- Besides FRET efficiency the degree of binding can be calculated


Tutorial

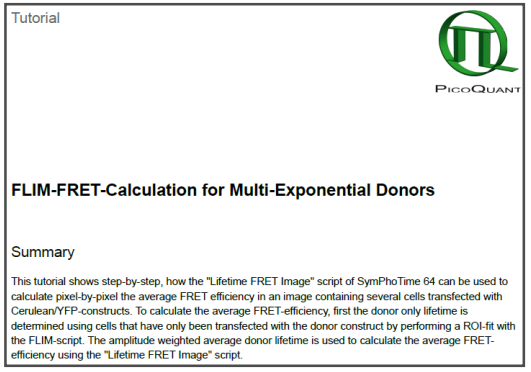
PicoQuant

**FLIM-FRET Calculation for Single Exponential Donors**

Summary

This tutorial shows step-by-step, how the "Lifetime FRET Image" script of SymPhoTime 64 can be used to calculate pixel-by-pixel the FRET efficiency in an image containing a cell transfected with a GFP and a RFP construct, that bind in certain regions of the cytoplasm. A bi-exponential fit is performed in a larger region of interest to determine the donor only lifetime and the lifetime of the donor in presence of FRET. A pixel-by-pixel fit is performed in the image and in every pixel the FRET efficiency as well as the fraction of FRET on the total decay ("Binding") is determined.

- for **donor** fluorophores with **double-exponential** decay as a result only one lifetime can be measured, i.e. the average shortened lifetime when quenching occurs
- No degree of binding can be calculated


Tutorial

PicoQuant

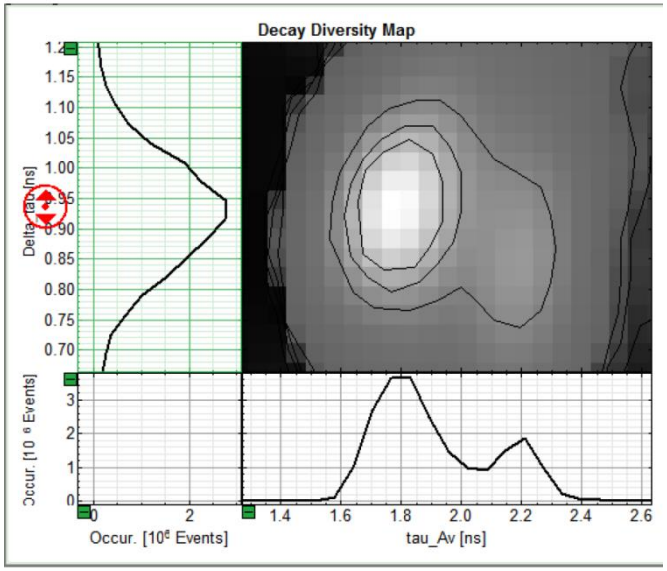
**FLIM-FRET-Calculation for Multi-Exponential Donors**

Summary

This tutorial shows step-by-step, how the "Lifetime FRET Image" script of SymPhoTime 64 can be used to calculate pixel-by-pixel the average FRET efficiency in an image containing several cells transfected with Cerulean/YFP-constructs. To calculate the average FRET-efficiency, first the donor only lifetime is determined using cells that have only been transfected with the donor construct by performing a ROI-fit with the FLIM-script. The amplitude weighted average donor lifetime is used to calculate the average FRET-efficiency using the "Lifetime FRET Image" script.

# Pattern Matching: Decay Diversity Map

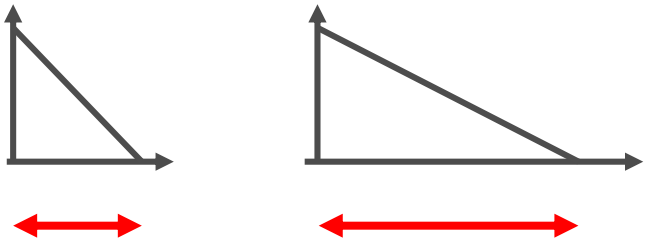
- The Decay Diversity Map tells you more about the different populations in the FLIM image.
- The x-axis shows a histogram of the average lifetimes of the pixels in the image.
- The y-axis shows a histogram of the Delta-Tau values of each pixel in the image. This quantity relates to the deviation of the fluorescence decay from a mono-exponential decay. While a small value depicts a mono-exponential decay, a bigger Delta-Tau value indicates a multi-exponential one.




$$\text{Delta-Tau} = \sqrt{\text{Std.dev}^2 - \tau_{av}^2}$$

= variance around average value

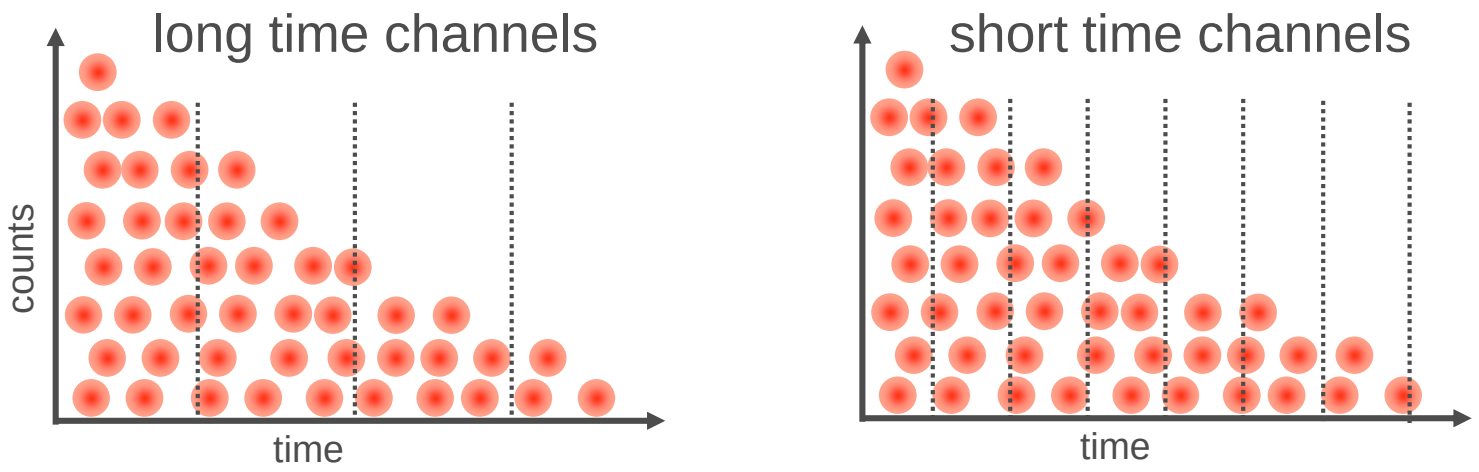
1 comp. ≥ 2 comp.



small vs large variance

# Appendix

# Defining the Resolution of Time Channels

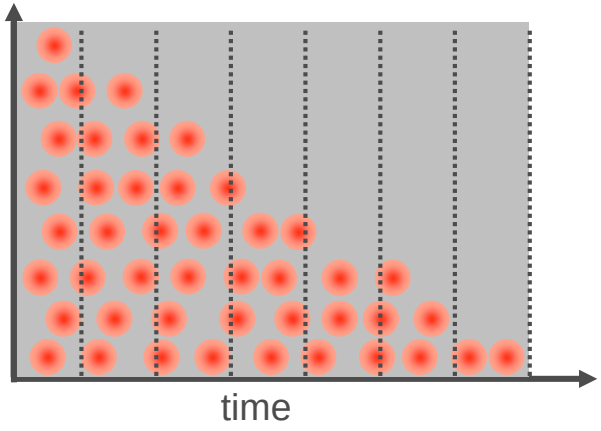



- the time channel resolution determines how much the arrival time of photons can differ so that they are electronically considered to have arrived at the same time point (i.e. are sorted into the same time bin)

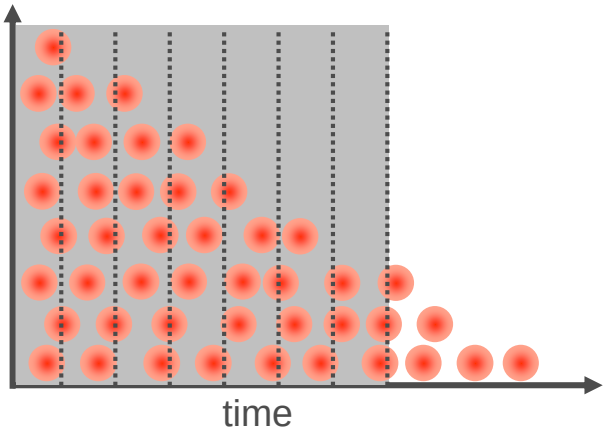
- available time channel resolutions:  4 ps – 512 ps
- available number of time channels for recording: 4096

# Defining the Resolution of Time Channels

**Example:**
imagine only 7 time channels were available...




**long time channels**
→ long recording window sufficient to monitor whole decay



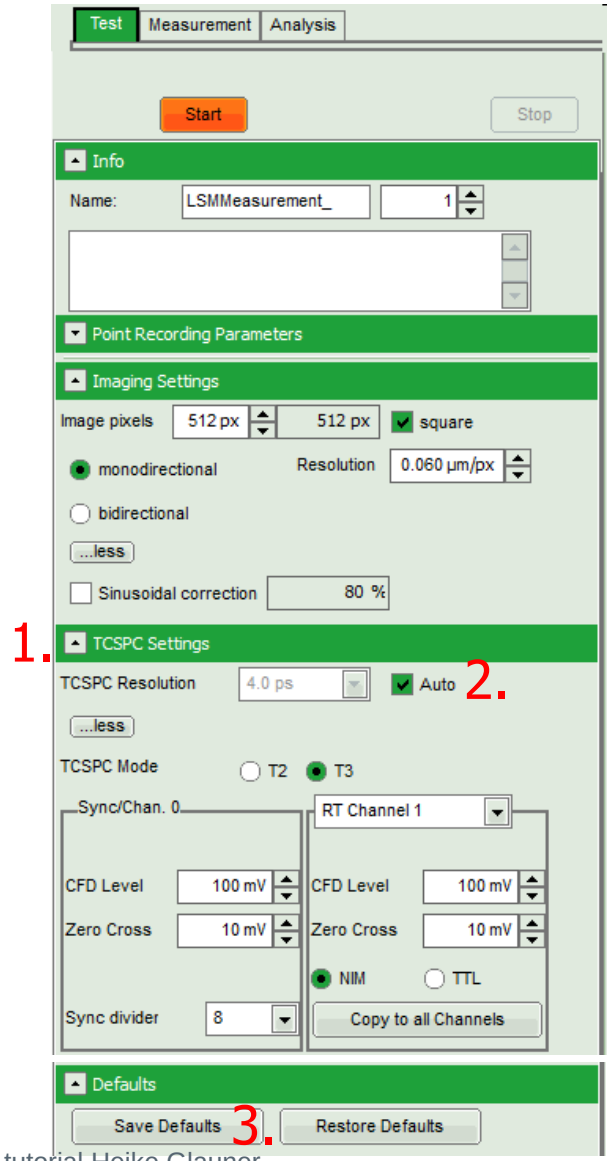

**short time channels**
→ short recording window not sufficient to monitor whole decay

- SPT64 adjusts the channel resolution automatically so that the whole time window between two laser pulses can be displayed. The user can override the automatic settings within SPT64.

| laser frequency | time between laser pulses | "resolution" in SPT | time channels | | acquisition window |
|---|---|---|---|---|---|
| 80 MHz | 12.5 ns | 4 ps | x 4096 | = | 16 ns |
| 40 MHz | 25 ns | 8 ps | x 4096 | = | 33 ns |
| 20 MHz | 50 ns | 16 ps | x 4096 | = | 65 ns |
| 10 MHz | 100 ns | 32 ps | x 4096 | = | 131 ns |
| 5 MHz | 200 ns | 64 ps | x 4096 | = | 262 ns |

4096 x width of time channel = length of acquisition window

# Defining the Resolution of Time Channels

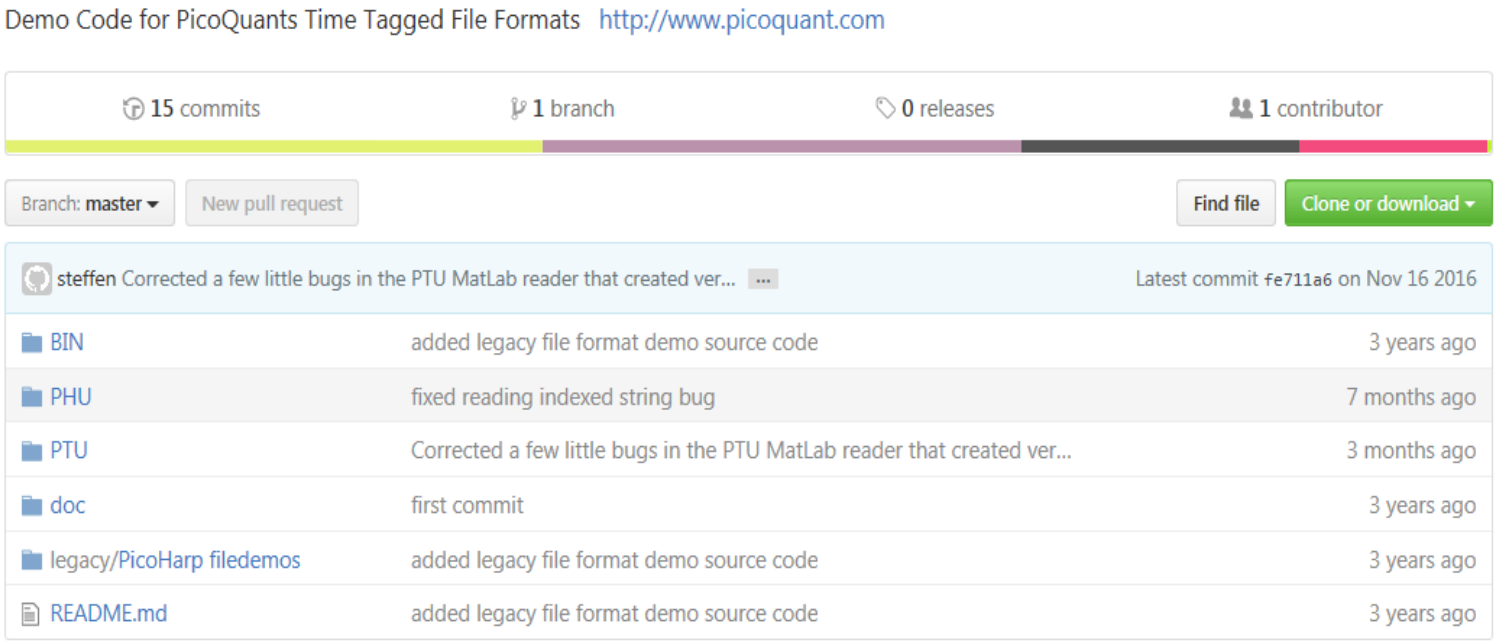



1. select the „TCSPC Settings“ menu inside the „Test“ tab
2. uncheck the „Auto“ field and adjust TCSPC resolution as desired
3. press „Save Defaults“ button at bottom of window to apply the changes

# Exporting TTTR Raw Data

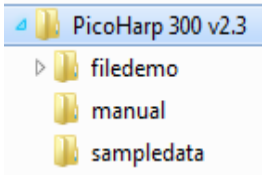
- Primary TTTR data can be exported for analysis with home-made software.
- For some demo codes please refer to the following homepage:

- https://github.com/PicoQuant/PicoQuant-Time-Tagged-File-Format-Demos



# Exporting TTTR Raw Data

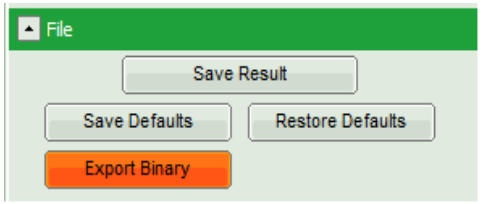
- For more information about how this export works please check the „PicoHarp 300 v2.3“ folder on the PicoHarp-CD delivered with every system




- Folder „manual“:
  - Chapter 5.4 → pages 29-31: general intro about the T3 measurement mode of the counting card
  - Page 33: Using TTTR Mode Data Files
  - Chapter 8.2.3 → pages 61-62: structure of the T3 File Format
- Folder „filedemo“:
  - Pt3-Folder: contains some example codes for TTTR data import into certain programming languages

# Export of Pre-Histogrammed Image Data

- Use the „Export Binary“ Button within the FLIM analysis GUI:




- Structure of the pre-histogrammed image data file:

| Data Item | Type | Description |
|---|---|---|
| **PixX** | int32 | pixels in X-direction |
| **PixY** | int32 | pixels in Y-direction |
| **PixResol** | float32 | spatial pixel resolution in µm |
| **TCSPCChannels** | int32 | number of TCSPC channels per pixel |
| **TimeResol** | float32 | time resolution of the TCSPC histograms in ns |
| **The following block will appear in the file for each y = 1 to <PixY>** | | |
| The following block will appear in block (y) for each x = 1 to <PixX> | | |
| The following data will appear in the block (x,y) for each t = 1 to <TCSPCChannels> | | |
| HistogramData [x,y,t] | int32 | counts of the TCSPC channel t of pixel (x,y) |
| end of block | | |
| end of block | | |
| **end of block** | | |

- For an example in python see here:
  https://github.com/PicoQuant/pre-histogrammed-Image-Data-File

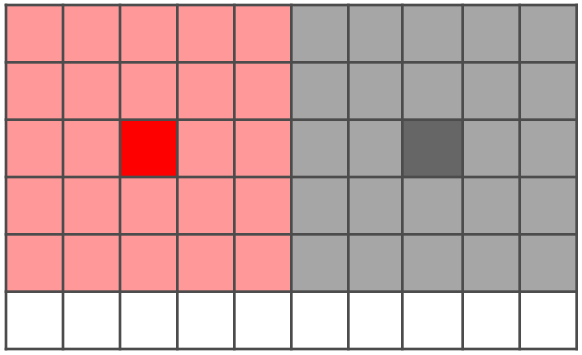
# Hidden Pixel Binning in Becker&Hickl Systems

| binning @ PQ | binning N @ B&H |
|---|---|
| 1x1 | 0 |
| 3x3 | 1 |
| 5x5 | 2 |
| 9x9 | 3 |
| 16X16 | 4 |
| 25x25 | 5 |

- the standard setting in PQ systems is a binning of 1x1 and in B&H N=1. This results in a difference of photon numbers by a factor of 9
- B&H only bins the lifetime data and not the grey level data. Thus, images still look very detailed. Additionally, a sliding average with the lifetime data is calculated creating an even more detailed image.
- In contrary, PQ makes a real binning in both, lifetime and intensity image, without averaging if binning is set to higher than 1x1.

# Supplier Dependant Binning Approaches

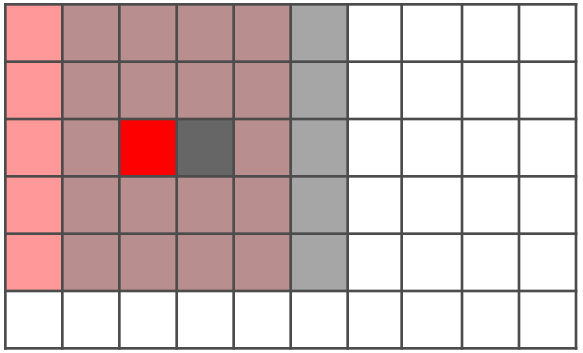
## Picoquant: 5x5 Binning



- 5x5 = 25 pixels are merged into one pixel
- binning leads to a reduction of pixel format

■/■ = central pixel to be recalculated

## Becker & Hickl: Binning of "2"



- not classical binning but rather lowpass filter function
- 2 pixels on each side of a pixel are taken into consideration when recalculating the central pixel value
- the total pixel number is thus not reduced
- 4/5 of the original data points are the same for neighboring pixels in the binned image → lifetime data looks very smooth