Fluorescence Lifetime Imaging Microscopy (FLIM)

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Optical Technology Development

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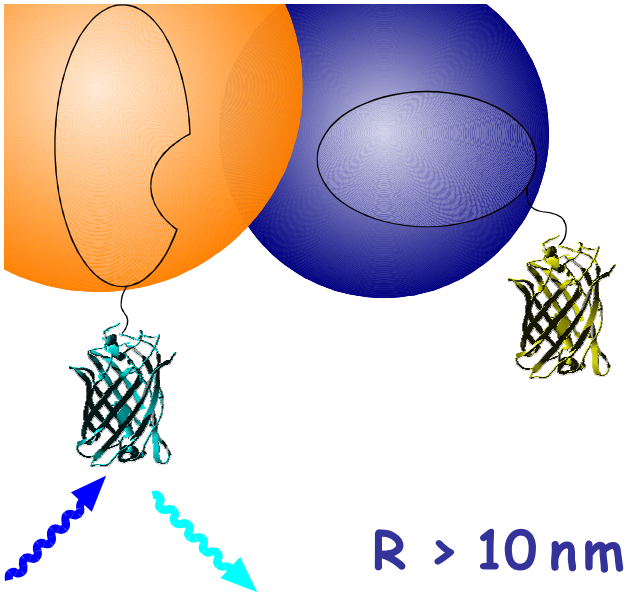
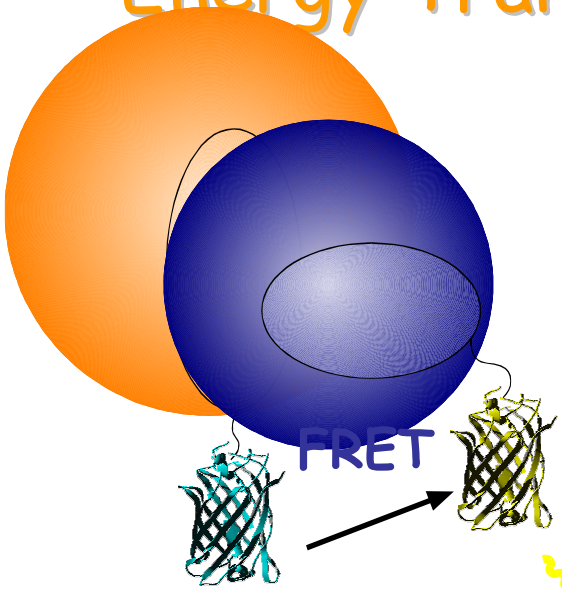
FRET-FLIM course, May 2009

Fluorescence Resonance Energy Transfer (FRET) Microscopy



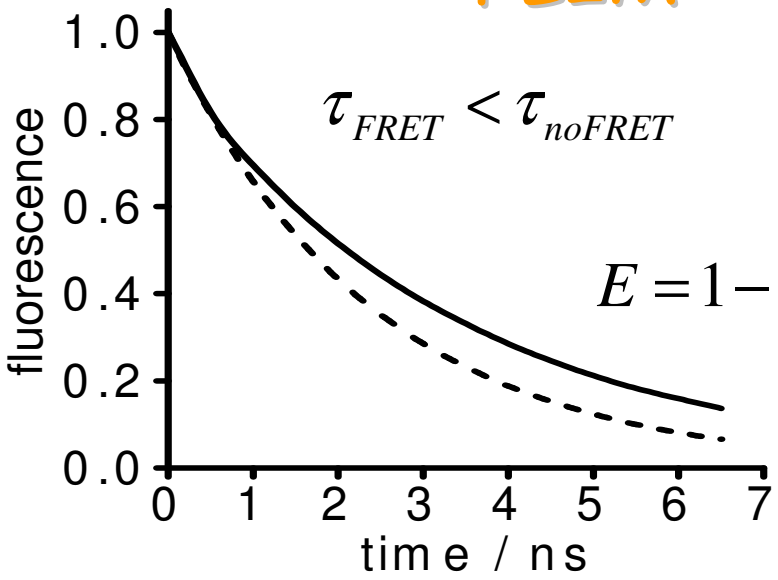
R > 10 nm



FRET

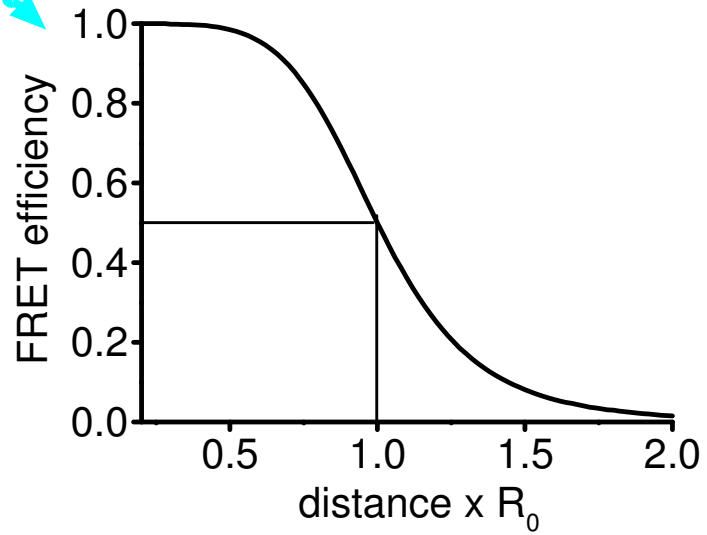
R ~ 2-8 nm

FLIM



$$\tau_{FRET} < \tau_{noFRET}$$

$$E = 1 - \frac{\tau_{FRET}}{\tau_{noFRET}}$$



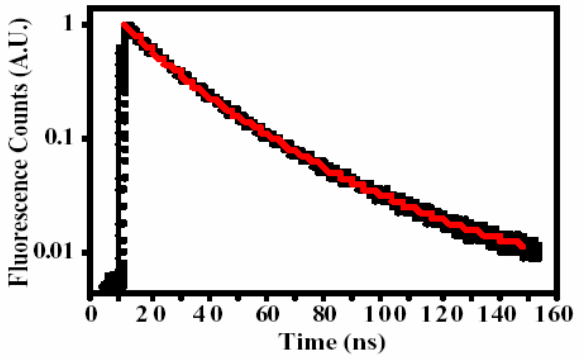
Fluorescence lifetime

Question: how quickly do excited molecules relax back to the ground state?

Since emission is a spontaneous process, its rate is proportional to the concentration of molecules in the excited state [N*]:

$$\frac{d[N^*]}{dt} = -(k_f + k_x)[N^*] \quad \Rightarrow \quad N(t) = N_0 \exp(-t/\tau_{ex})$$

Exponential decay:



intrinsic or natural lifetime $\tau_f = \frac{1}{k_{fluor}}$

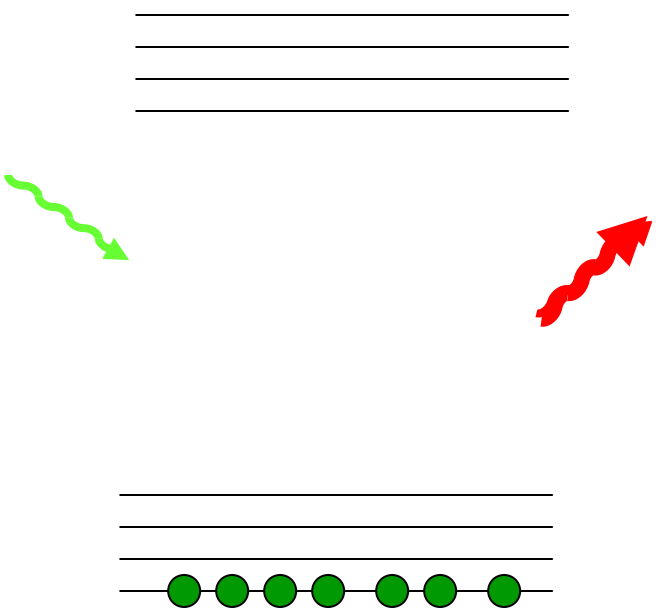
experimental lifetime $\tau_{ex} = \frac{1}{k_{fluor} + k_{nonfluor}}$

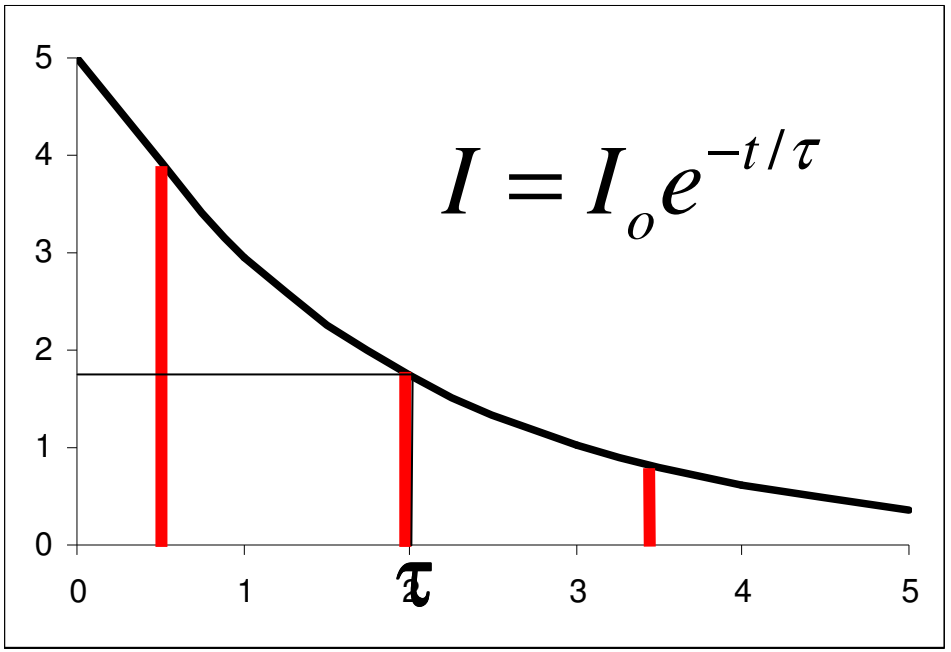
$$\tau_{ex} = \tau_f \cdot Q$$

The experimentally determined excited state lifetime is always smaller then the theoretical one:

The larger the quantum yield, the longer τ_{ex}

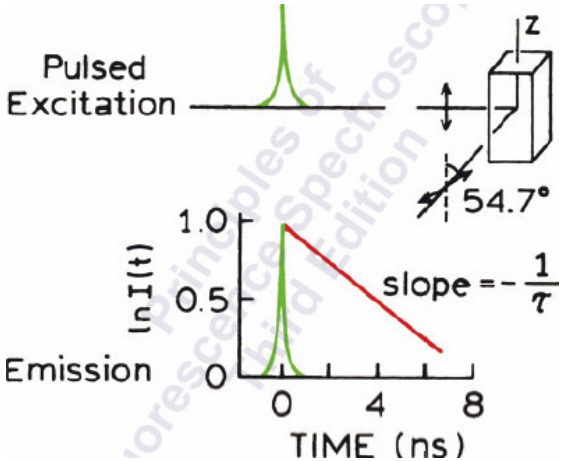
Fluorescence lifetime





Lifetime measurements

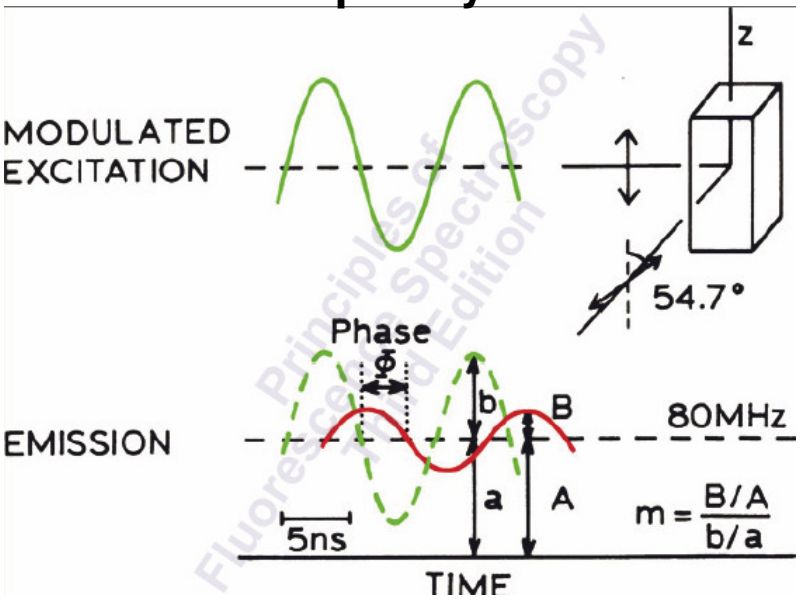
Pulse or Time-Domain



$$\frac{dN}{dt} = -(k_f + k_x)N = -\frac{1}{\tau}N$$

$$I(t) = I_0 e^{-t/\tau}$$

Phase-modulation or Frequency-Domain



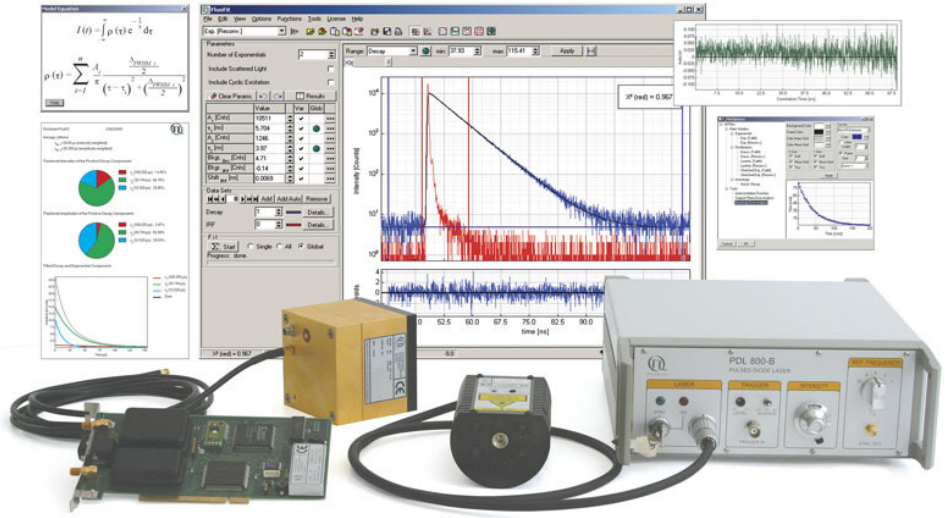
$$\tan\Phi = \omega\tau_p \qquad m = \left[1 + \omega^2\tau_m^2\right]^{-1/2}$$

$$\tau_P = \frac{\tan\Phi}{\omega} \qquad \tau_m = \frac{\left[\left(1/m^2\right) - 1\right]^{1/2}}{\omega}$$

$\tau_P = \tau_m$ single exponential decay

$\tau_P \neq \tau_m$ multi-exponential decay

2 Approaches to Measure FLIM in Time-Domain



Time Correlated Single Photon Counting (TCSPC)

- Upgrade for scanning micsocopes
- High temporal and spatial resolution
- Slow (30-60 s per FLIM image)

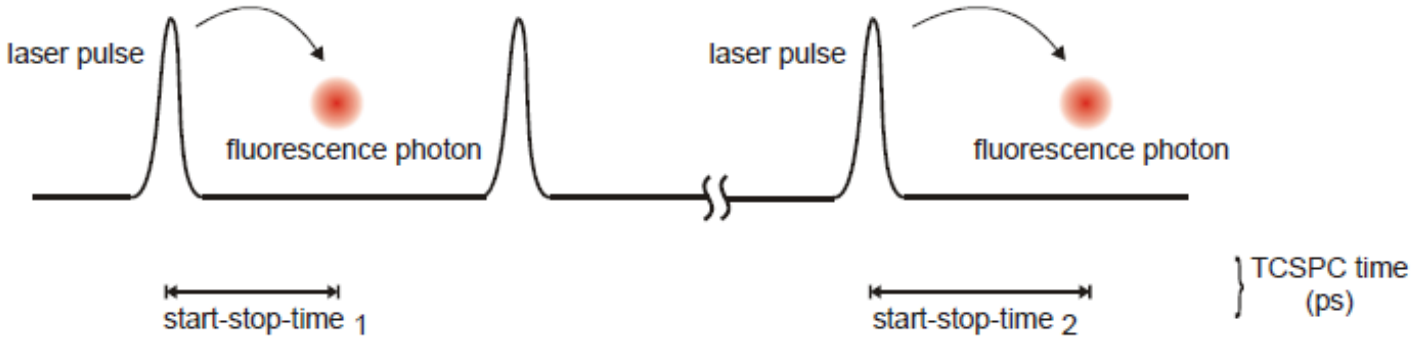
=> useful for fixed samples

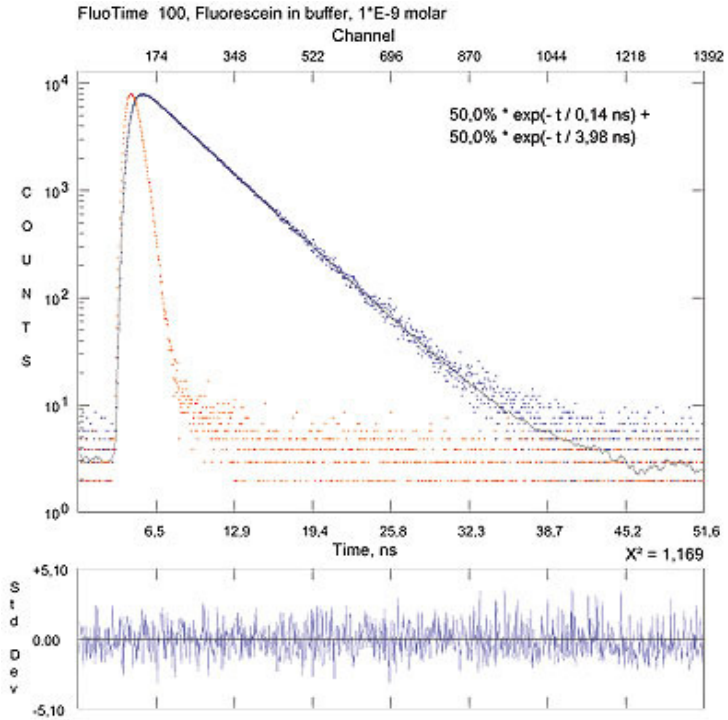
Timegated Intensified CCD Camera

- Upgrade for all camera based microscope (wide-field, TIRF, spinning-disc, 2-photon)
- Lower spatial resolution due to intensifier
- Fast (0.5 – 2 s per FLIM image)

=> suitable for live cell imaging

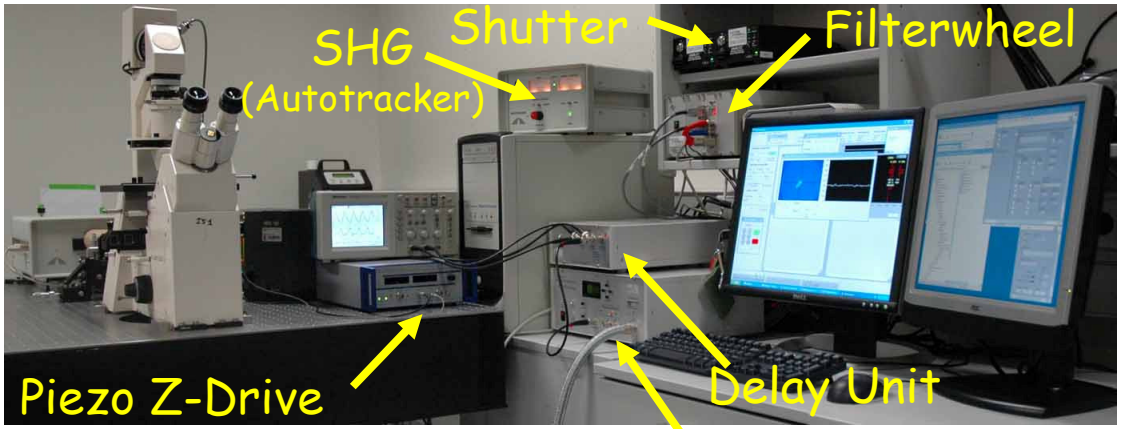
Measurement of fluorescence lifetimes: TCSPC

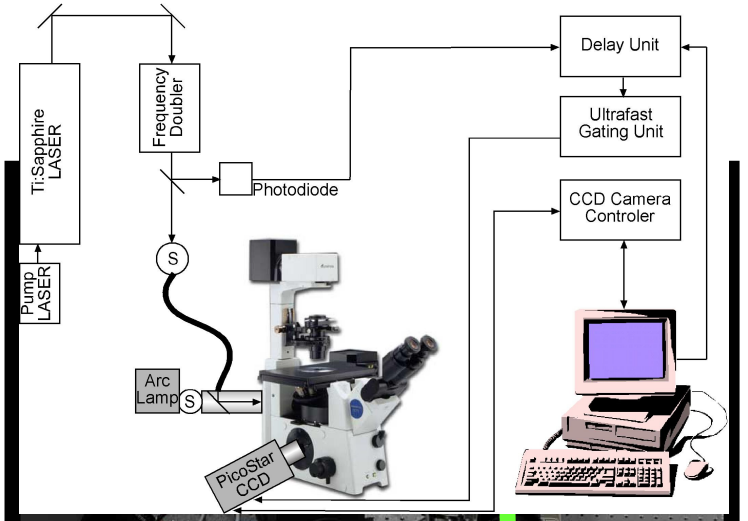


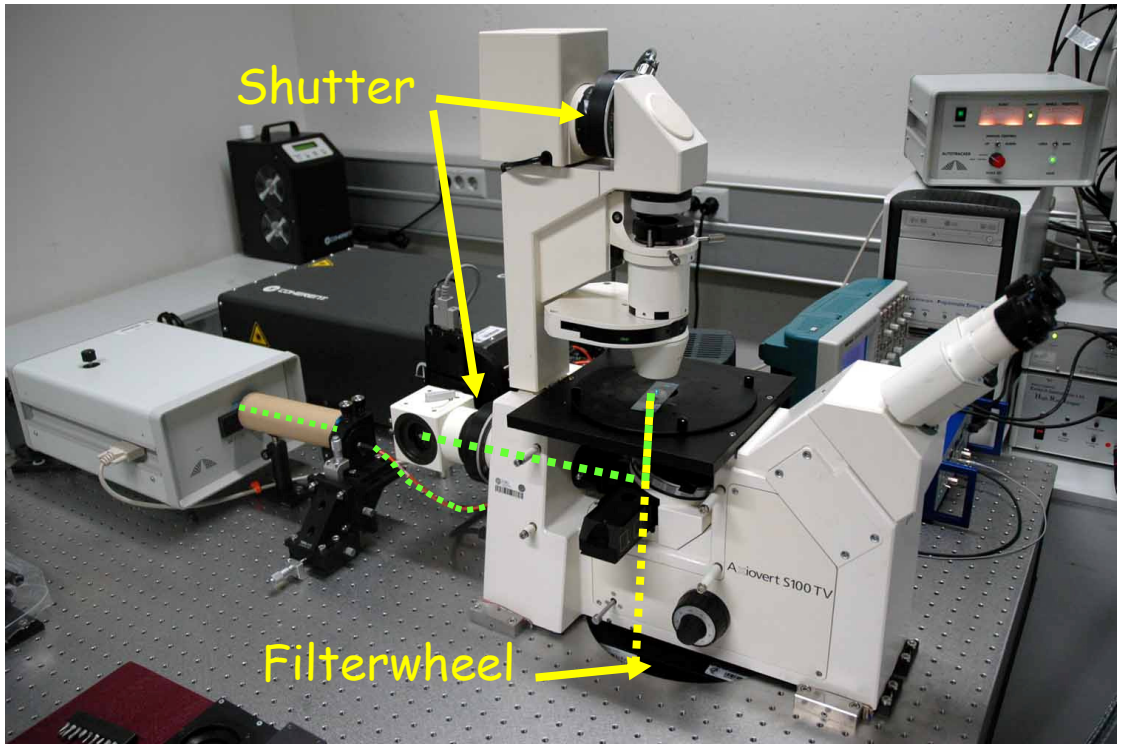


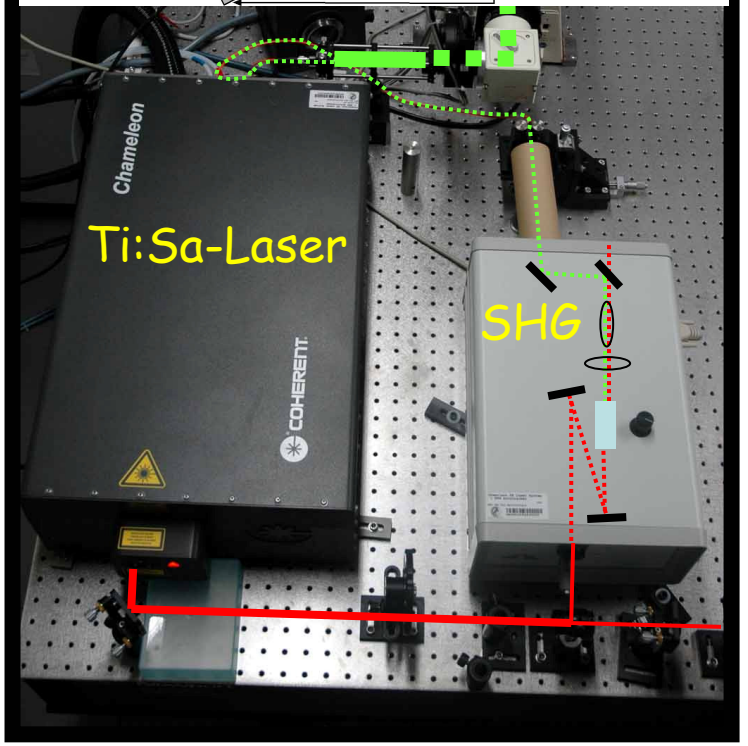
- Measurement of delay times between absorption and fluorescence of a photon.
- Plot in logarithmic scale to yield a histogram, which is in the simplest case fitted by a straight line.
- Caution! Limited to 1 photon per pulse! Doesn't work at high emission signals.
- Usually 1 emitted photon per 50 - 100 excitation pulses.

Fluorescence lifetime imaging microscopy (FLIM) with a time-gated CCD camera

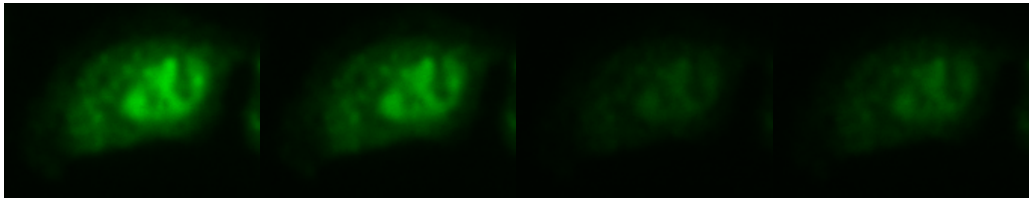


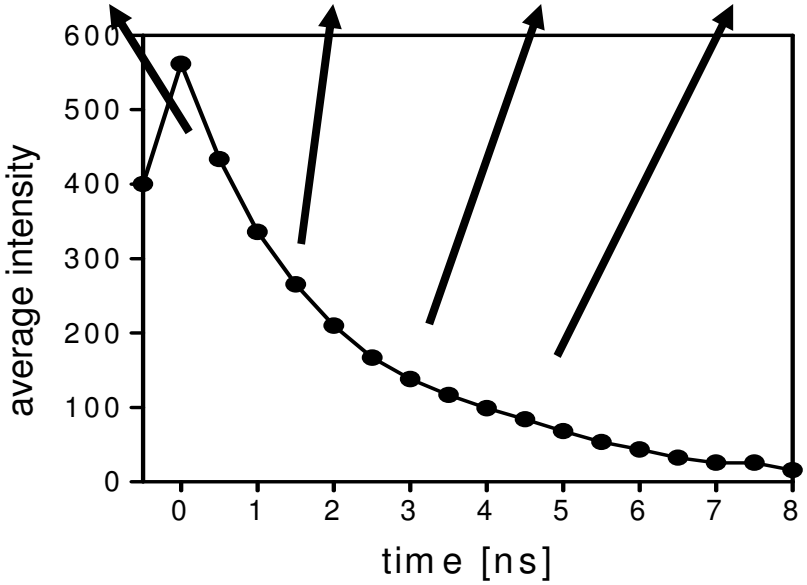


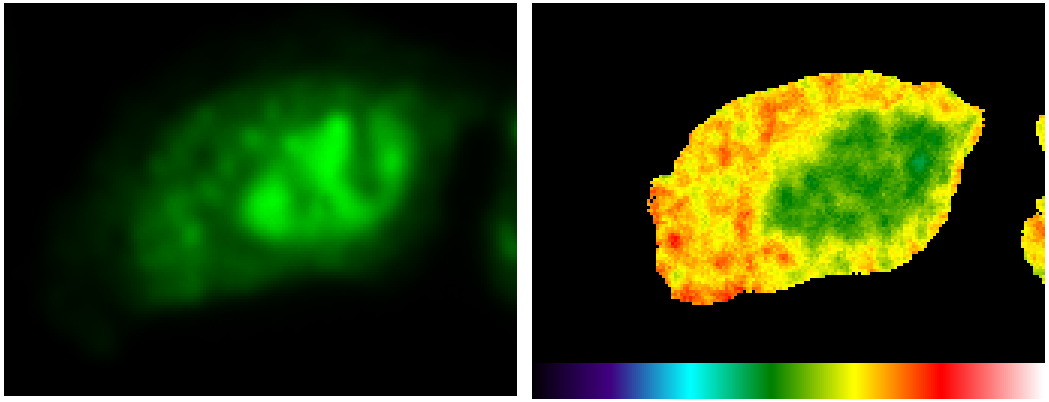




Measurement of fluorescence lifetimes: Time-gated CCD camera



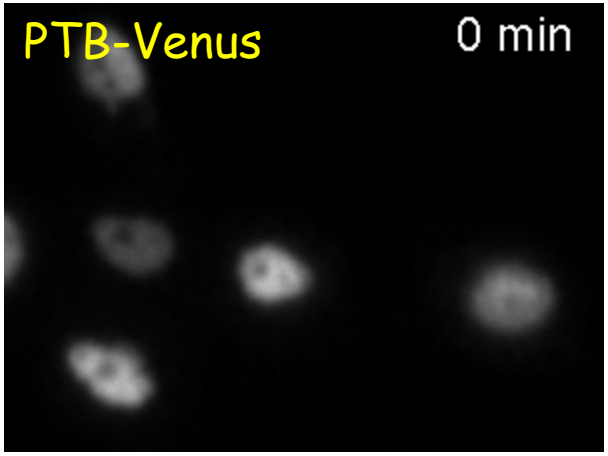


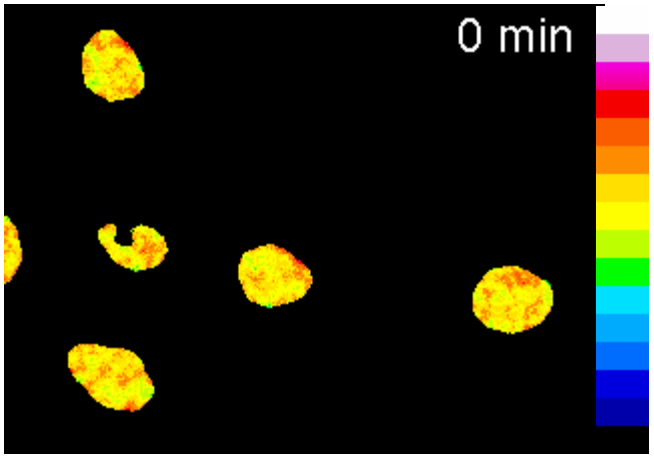


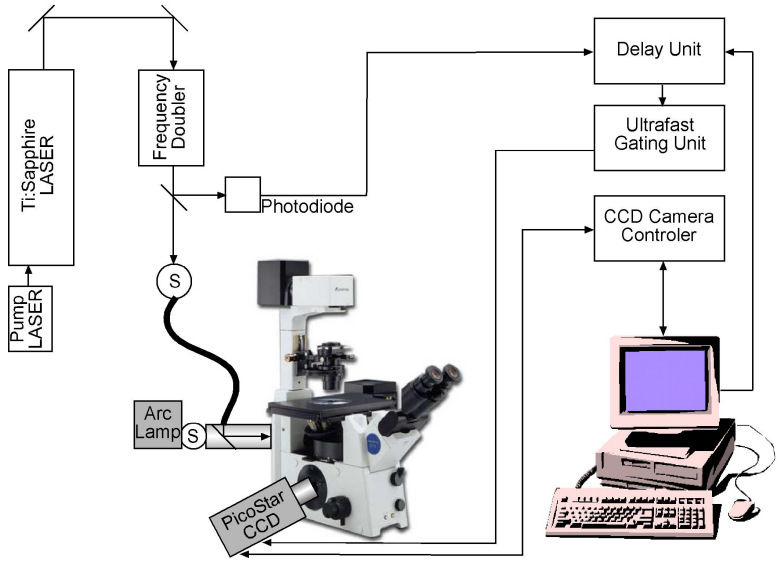
Fluorescence lifetime can be extracted from only 2 images (timepoints)

$$\tau = \frac{\Delta t}{\ln(I_{t1} / I_{t2})}$$

Dynamics and interaction by live-cell FLIM



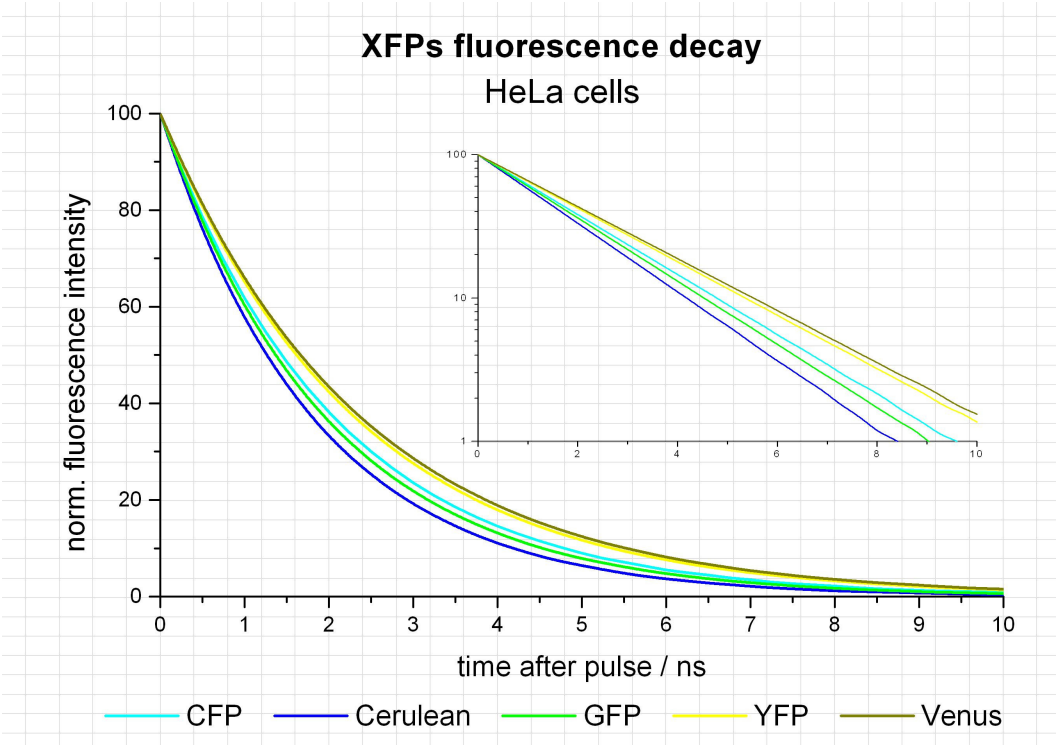


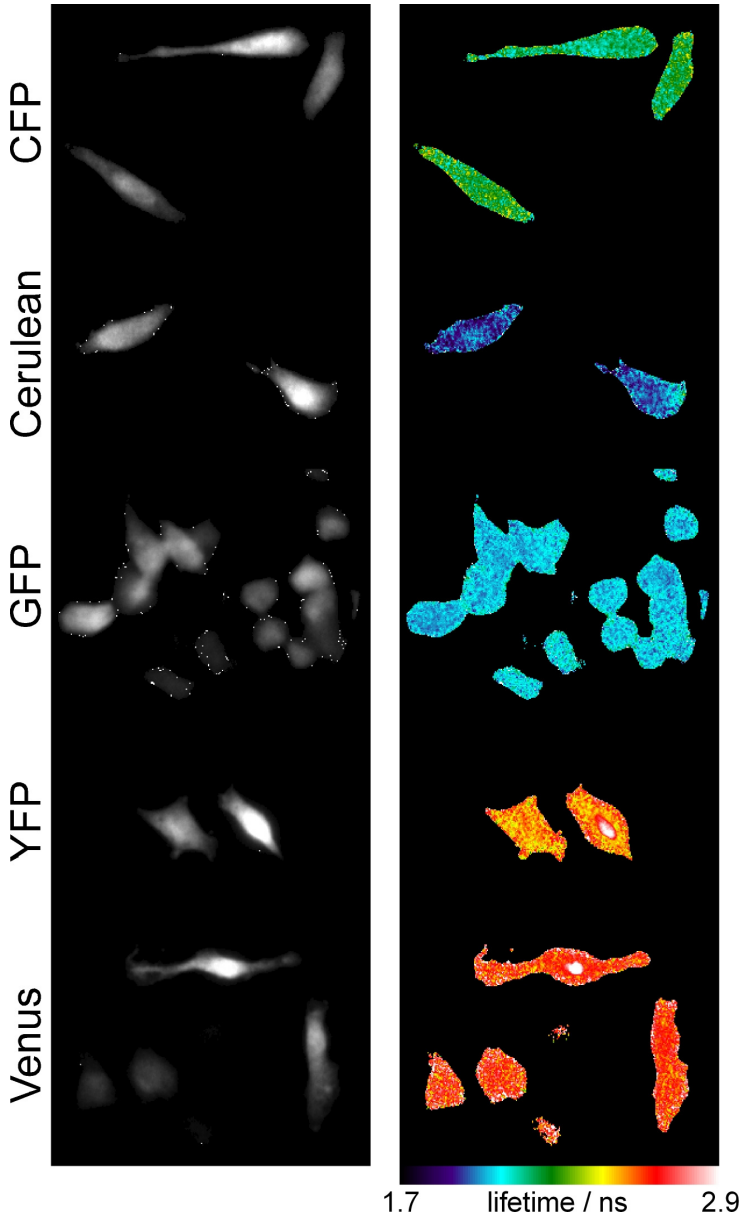


- Hela cells imaged every 5 min for 10 h @ 37°C
- FLIM stack acquired in approx. 1 s

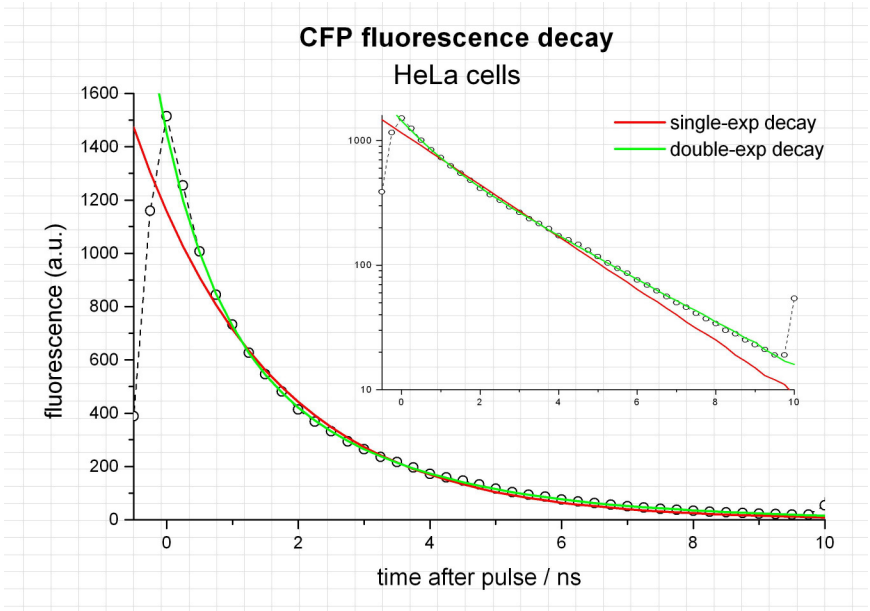
Fluorescence Lifetime of XFPs

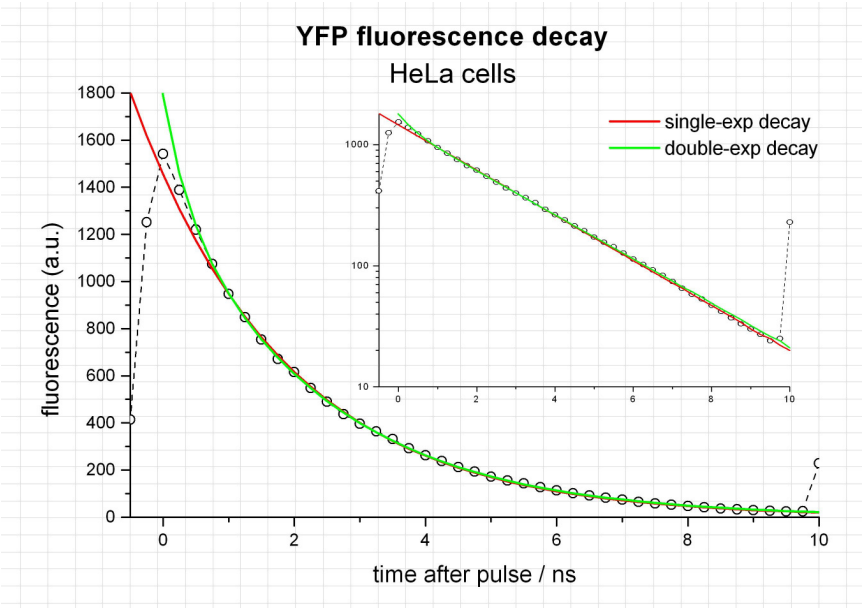
	FLIM		Curve Fit	
	tau / ns	sd	tau /ns	sd
CFP	2.22	0.03	2.20	0.02
Cerulean	1.93	0.02	1.88	0.05
GFP	2.05	0.13	2.02	0.12
YFP	2.38	0.15	2.37	0.16
Venus	2.47	0.13	2.45	0.13

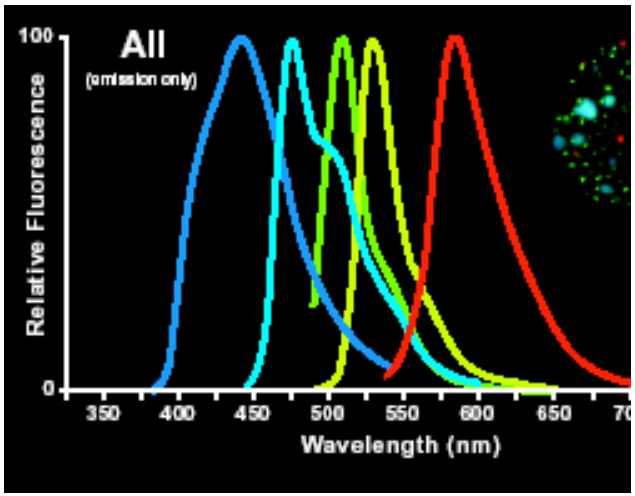




CFP and Cerulean show a multi-exponential decay





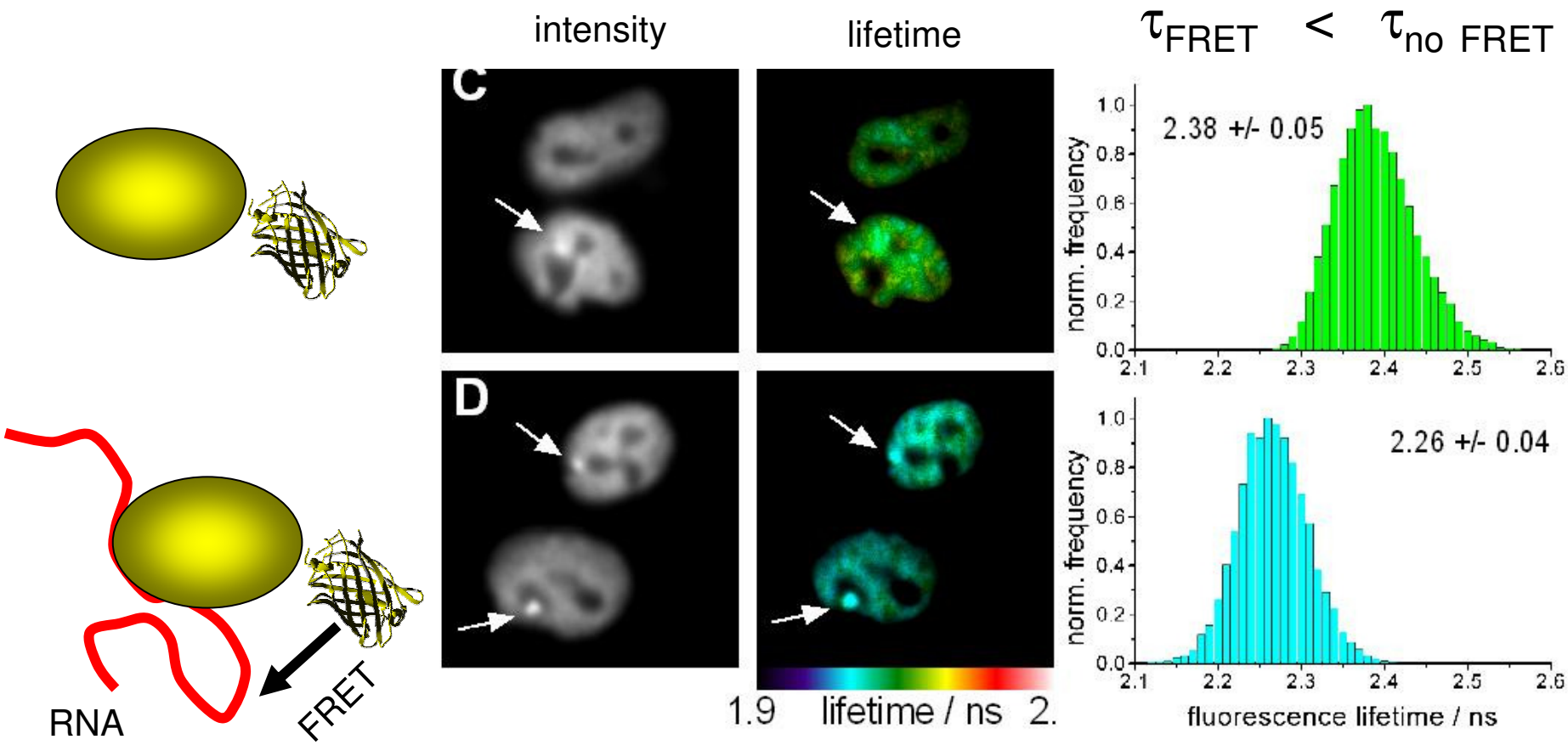


	FLIM		Curve Fit - 1exp		Curve Fit - 2exp		
	tau / ns	sd	tau /ns	sd	tau1 / ns	tau2 / ns	x1
CFP	2.22	0.03	2.20	0.02	0.53	2.63	0.73
Cerulean	1.93	0.02	1.88	0.05	0.56	2.44	0.78
GFP	2.05	0.13	2.02	0.12			
YFP	2.38	0.15	2.37	0.16			
Venus	2.47	0.13	2.45	0.13			

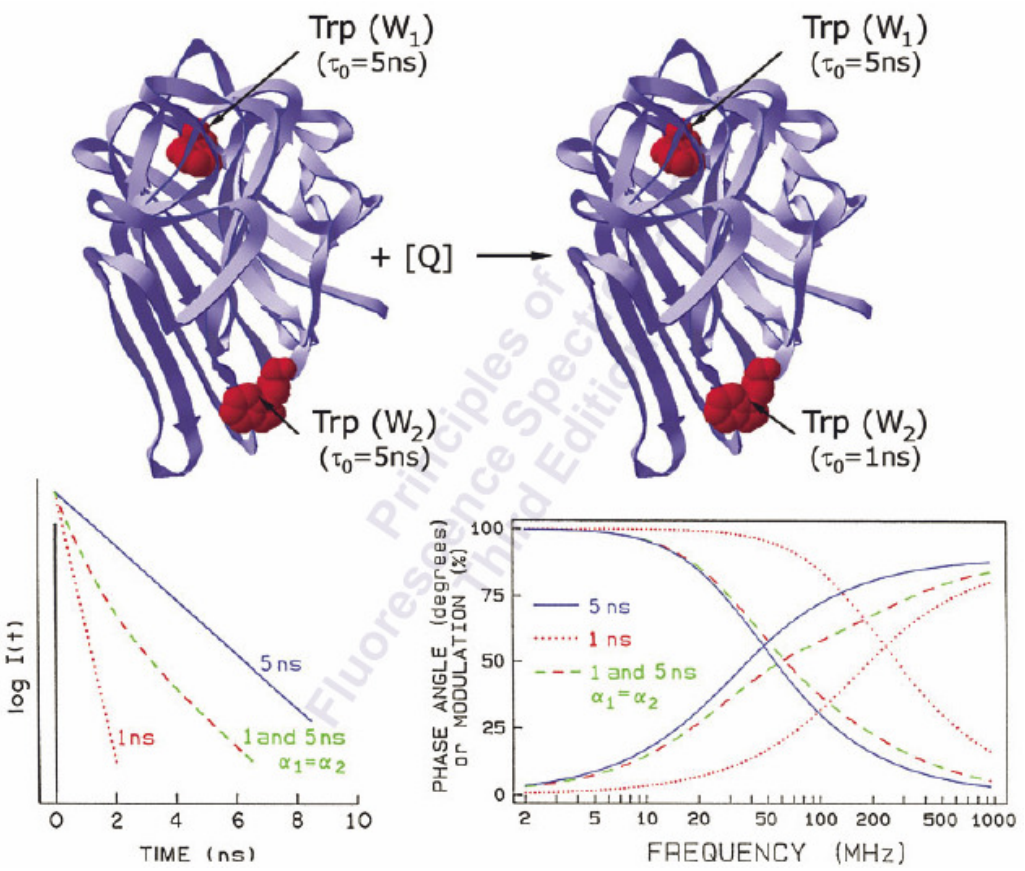
Fluorescence lifetime imaging microscopy (FLIM)

Observing protein-RNA interaction via FRET-FLIM inside cells

- Protein is tagged with a yellow version of GFP
- RNA is stained with a red intercalator dye



Examples of lifetime measurements

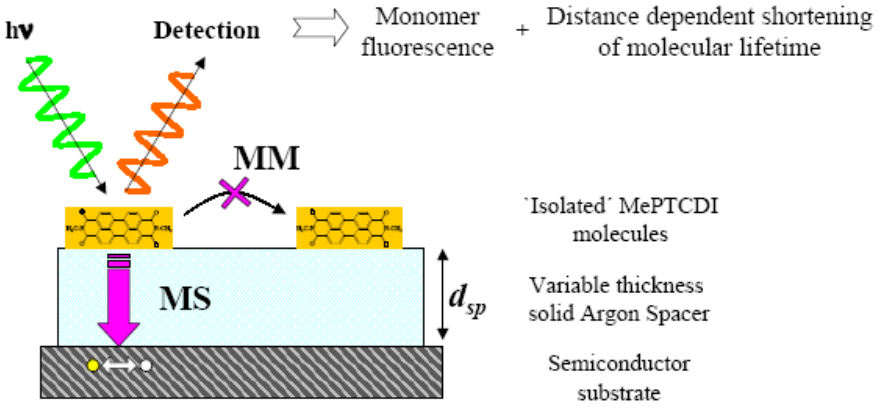


A protein contains two tryptophan residues, each with a distinct lifetime. Because of spectral overlap of the absorption and emission, it is not possible to resolve the emission from the two residues from steady-state data. However, time-resolved data can distinguish between both of them indicating a quenching of one of them (shorter lifetime).

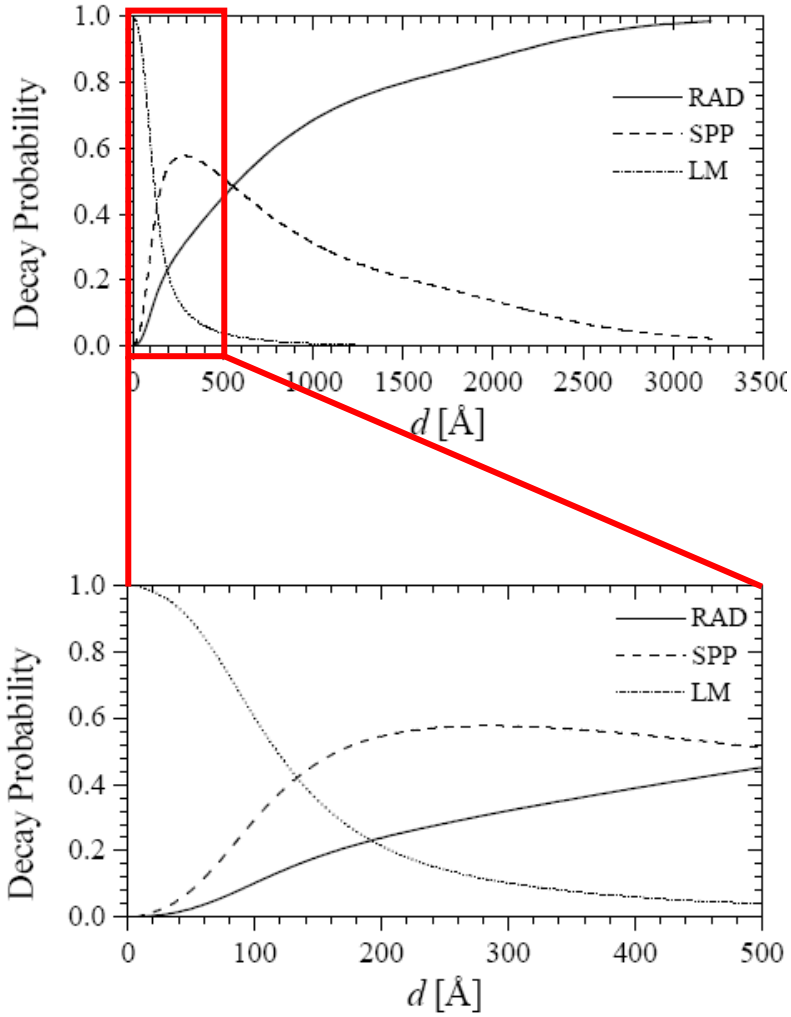
Time-resolved data can provide information not available from steady-state fluorescence measurements.

- Distinguish static and dynamic quenching
- Separate dyes with similar spectral properties by their lifetime
- Distinguish population of dyes rather than a average value (e.g in FRET)

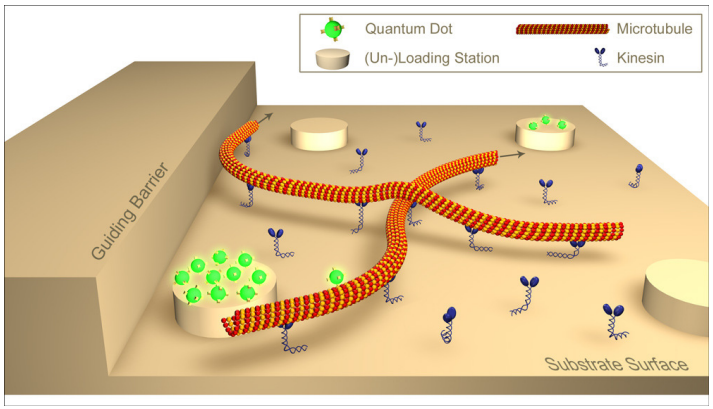
Surface Plasmon Resonance:
Reduction of the fluorescence intensity and lifetime close to metallic surfaces

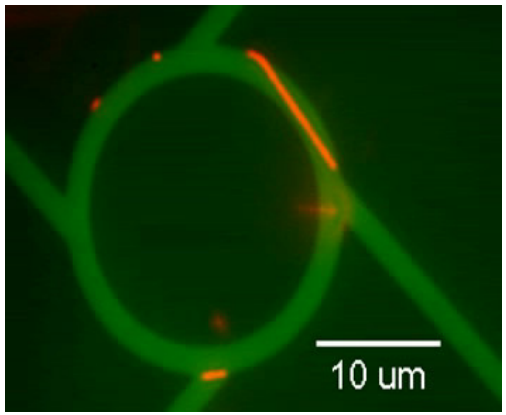


$$\tau = \frac{1}{k_{rad} + k_{SPP} + k_{LM}}$$

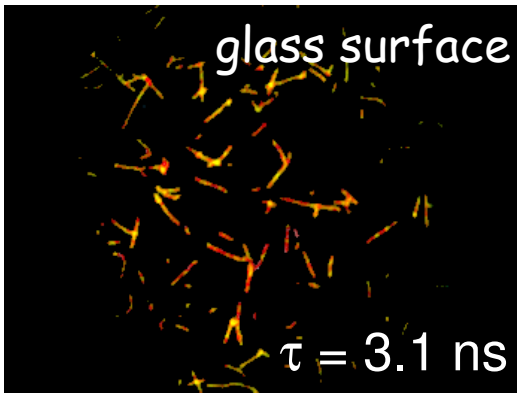


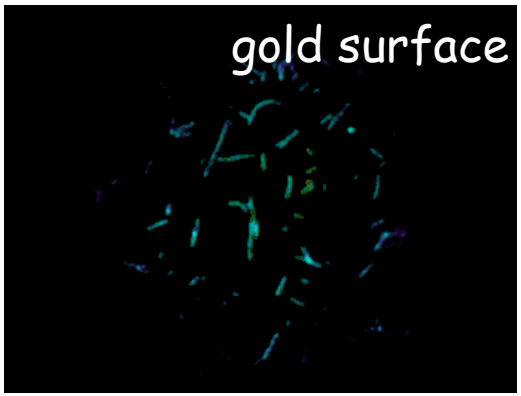
Measuring the height of microtubules by SPR-FLIM

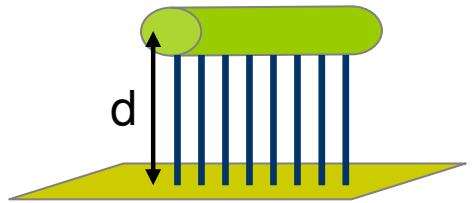


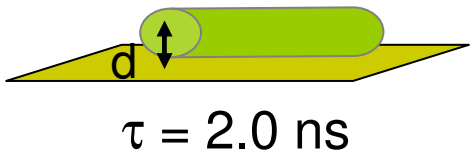


Lifetime of Alexa488-labeled mictrotubules





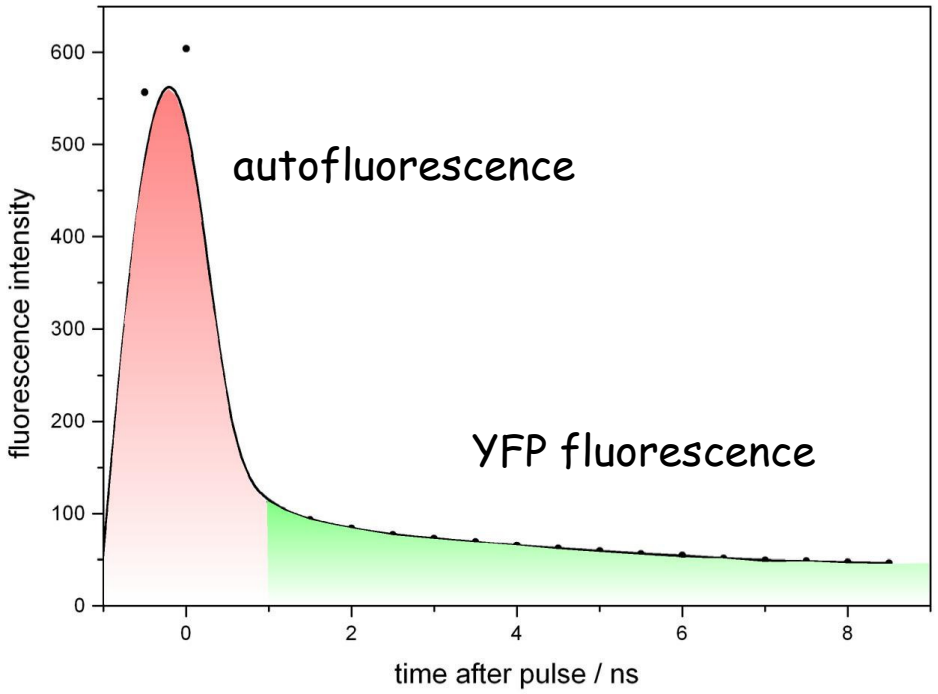




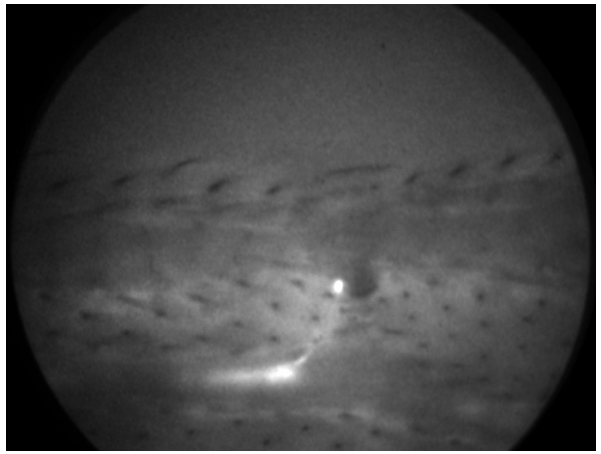
(collaboration with M. Berndt & S. Diez)

Timegated imaging decreases autoflurescence and improves S/N

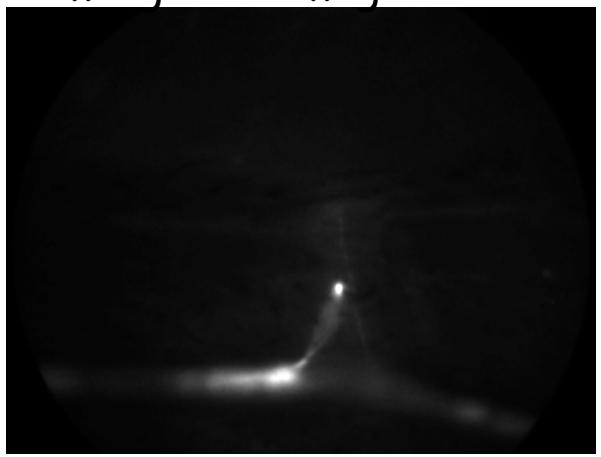
Autofluorescence has typically a shorter lifetime (<0.5-1 ns) than fluorescence dyes or fluorescent proteins (> 2 ns).



"normal" fluorescence image

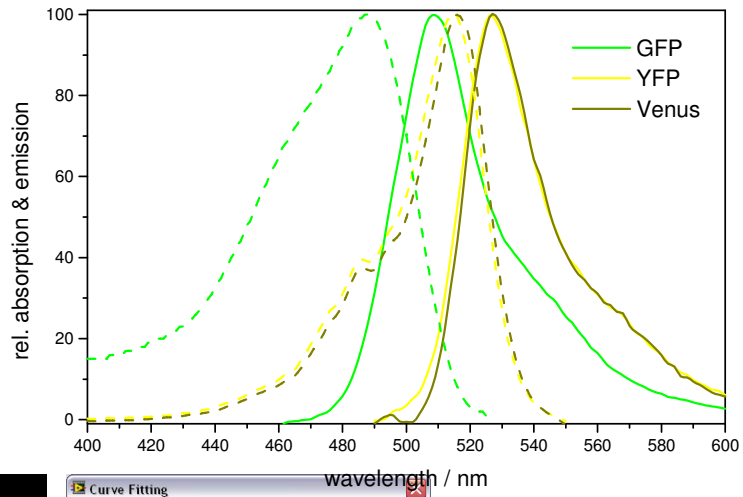


Time-gated image



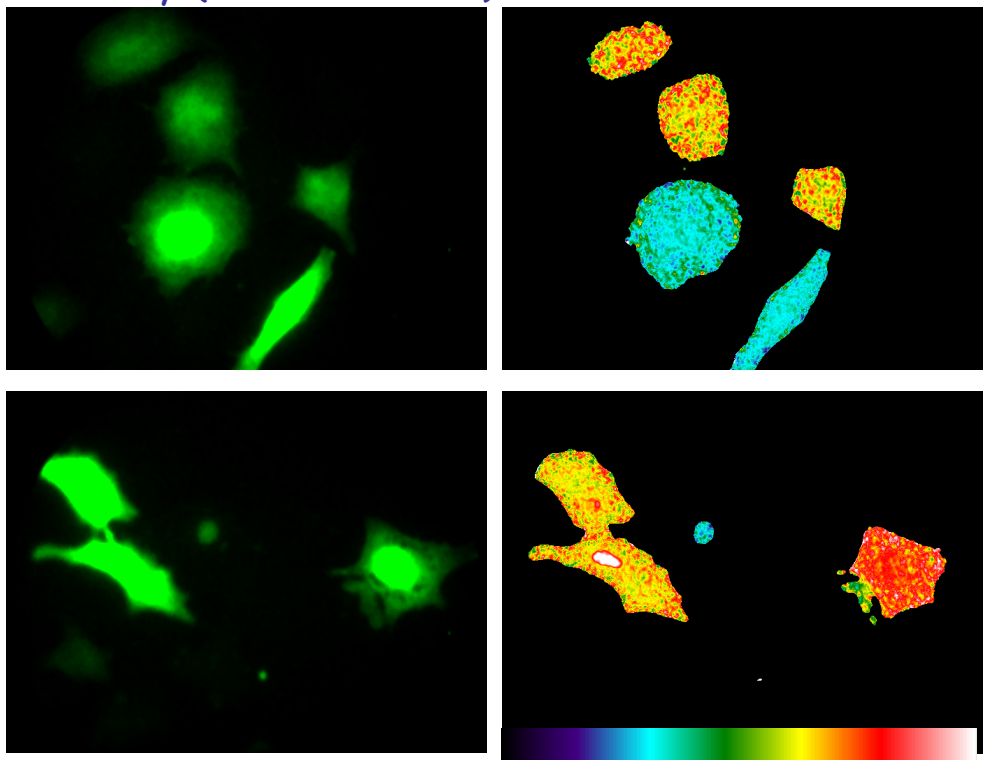
YFP image of a fly wing expressing a Ca^{2+}-FRET sensor

Unmixing of GFP, YFP, and Venus expressing cells by fluorescence lifetime imaging microscopy



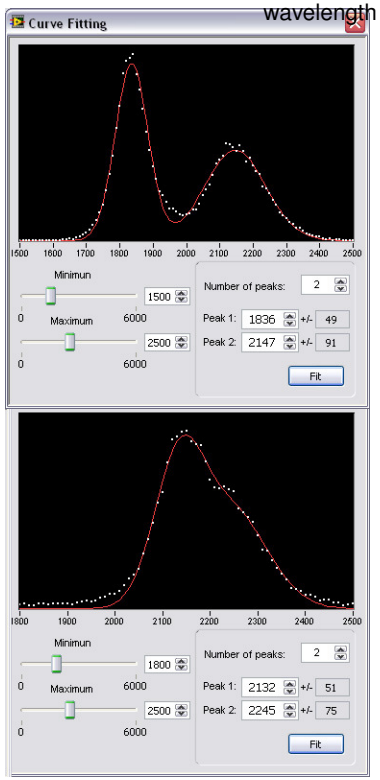
Intensity (FITC-Channel)

FLIM



1.5

2.5



FLIM microscope can be used for …

… lifetime measurements
- observe environment (e.g. pH, membrane lipids composition)
- ion imaging (e.g. Ca^{2+}, Zn^{2+}, Na^{+}, K^{+})
- separation of spectral similar fluorophores (e.g. GFP & YFP)

… FRET
- protein-protein interactions
- protein activity due to conformational changes
- DNA-protein interactions
- RNA-protein association
- Several interaction in parallel (???)

… time-resolved fluorescence microscopy
- separation of spectral similar fluorophores
- reduction of autofluorescence (tau < 0.5ns)

… time-resolved anisotropy measurements

… ???

Literature

Review
Bastiaens & Squire, Trends Cell Biol (1999), 48.
Wouters et al., Trends Cell Biol (2001), 203.
Piston & Kremers, Trends Biochem Sci (2007), 407.

Widefield-FLIM setup
Elangovan et al., J Micros (2002), 3.
Lorenz, RNA (2009), 97.