Quantitative *in vivo* imaging of molecular distances using FLIM-FRET

S. Orthaus, V. Buschmann, B. Krämer, M. König, F. Koberling,
U. Ortmann and R. Erdmann

EMBO Practical Course about Quantitative FRET, FRAP and FCS
Heidelberg, 25th September 2009

PicoQuant GmbH

Our location

Technology Park Adlershof

The Brandenburg Gate

The PicoQuant Team

PicoQuant

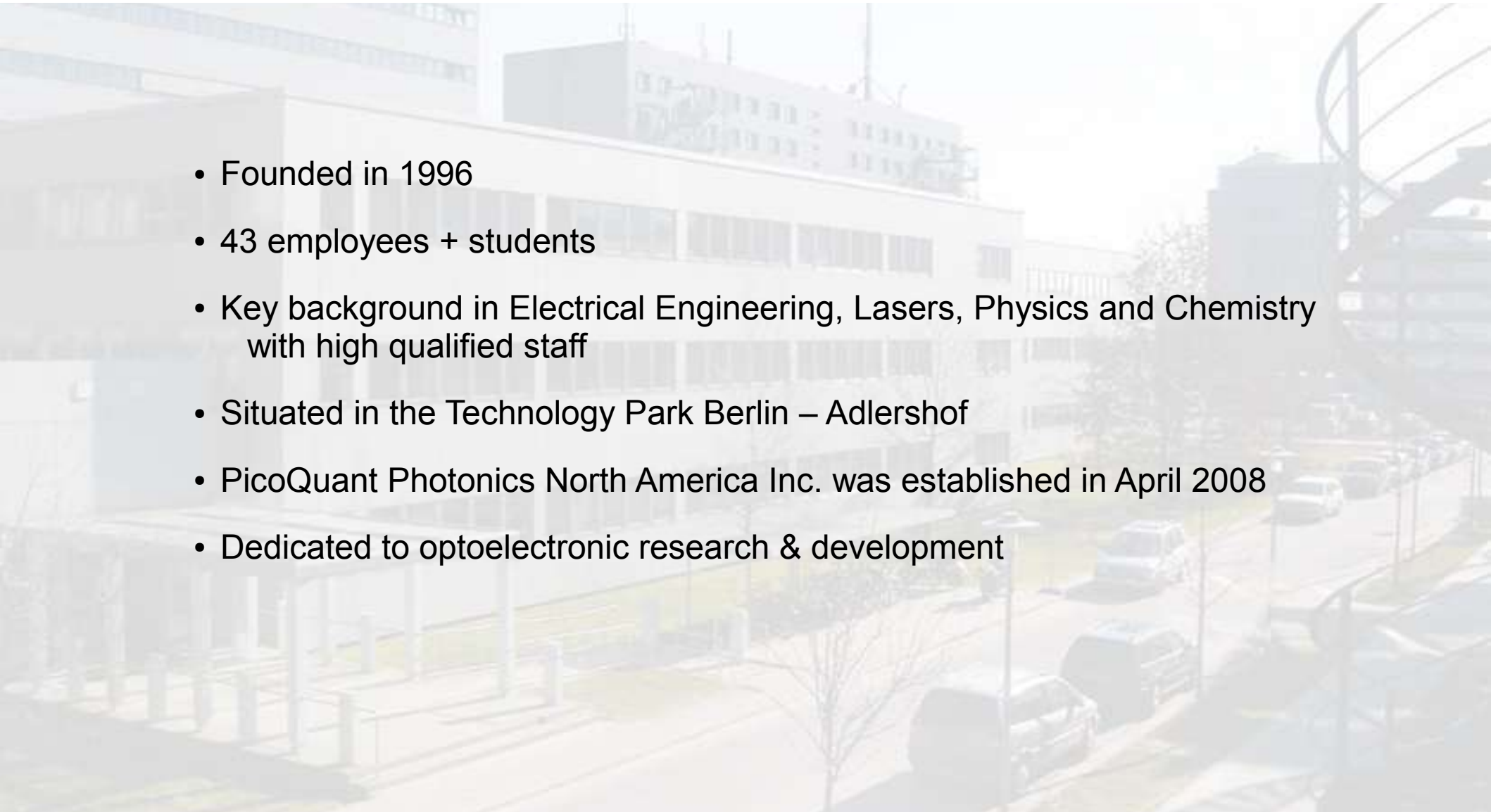
Pulsed Diode Lasers

Time-resolved Confocal Microscopes & LSM upgrade kits

Fluorescence Lifetime Spectrometer

Photon Counting Instrumentation

PicoQuant GmbH



- Founded in 1996
- 43 employees + students
- Key background in Electrical Engineering, Lasers, Physics and Chemistry with high qualified staff
- Situated in the Technology Park Berlin – Adlershof
- PicoQuant Photonics North America Inc. was established in April 2008
- Dedicated to optoelectronic research & development

FLIM in Life Sciences

FLIM
- Time-domain analog to multicolor image
- New parameters independent of system settings and fluorophore concentration

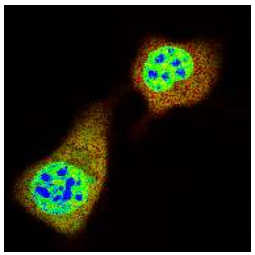
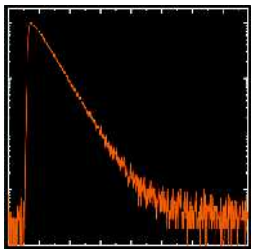
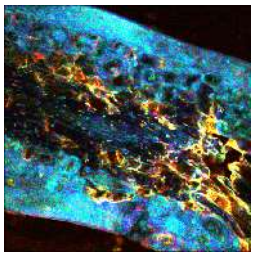
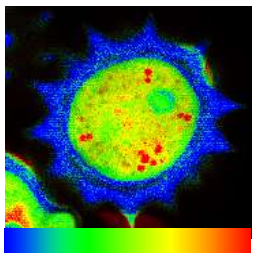
Multi-Staining
- Imaging of multiple dyes with similar emission but different lifetimes
- Discrimination of autofluorescence

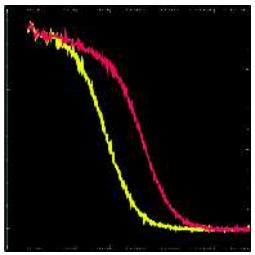
Local environment sensing
- Viscosity
- Lipophilic/Hydrophilic environment
- pH sensing
- Oxygen, water or ion concentration

FRET
- Distance measurements (nm range)
- Intra- and intermolecular interactions
- In fixed as well as in living cells and organisms
- Time lapse analysis

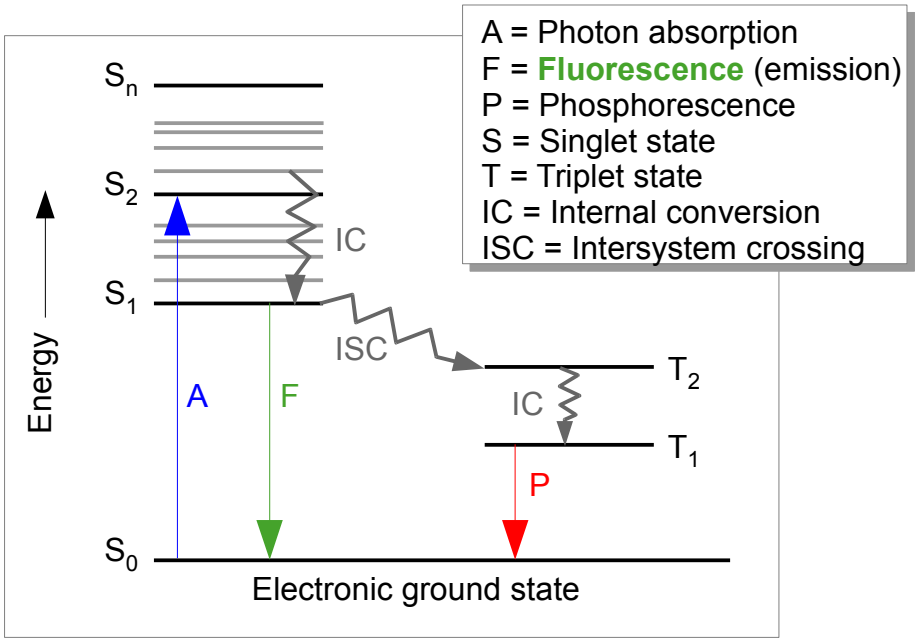
Quenching and Anisotropy
- Accessibility and conformational studies (protein folding)
- Molecular Rotation

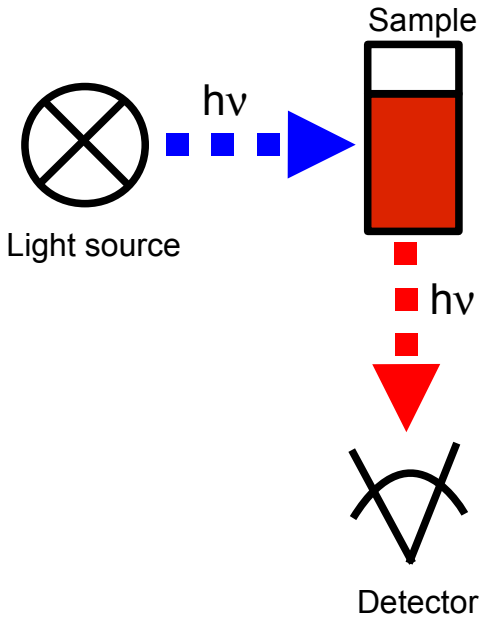
FLCS / FLCCS
- Correction for background, detector artifacts and spectral bleed trough

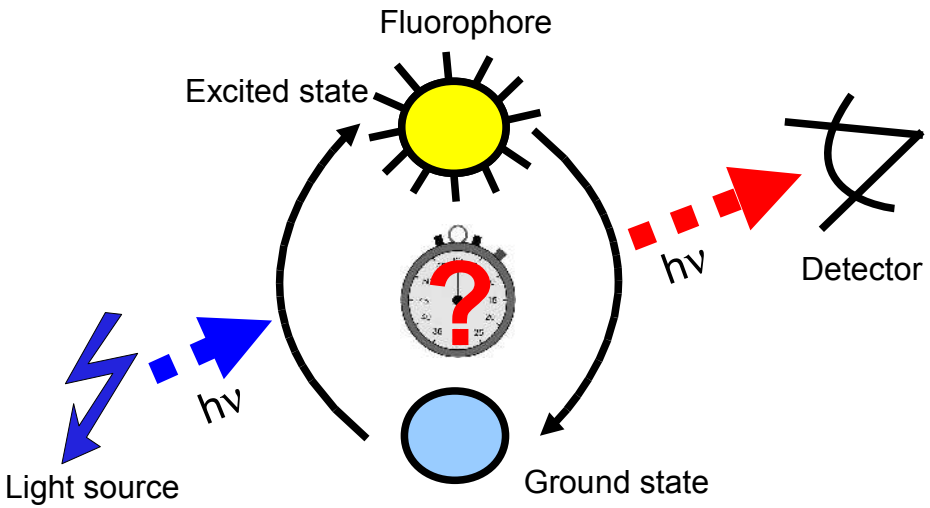
Fluorescence Photocycle





Observables

- Fluorescence intensity
- Color or wavelength
- Polarization
- Fluorescence lifetime

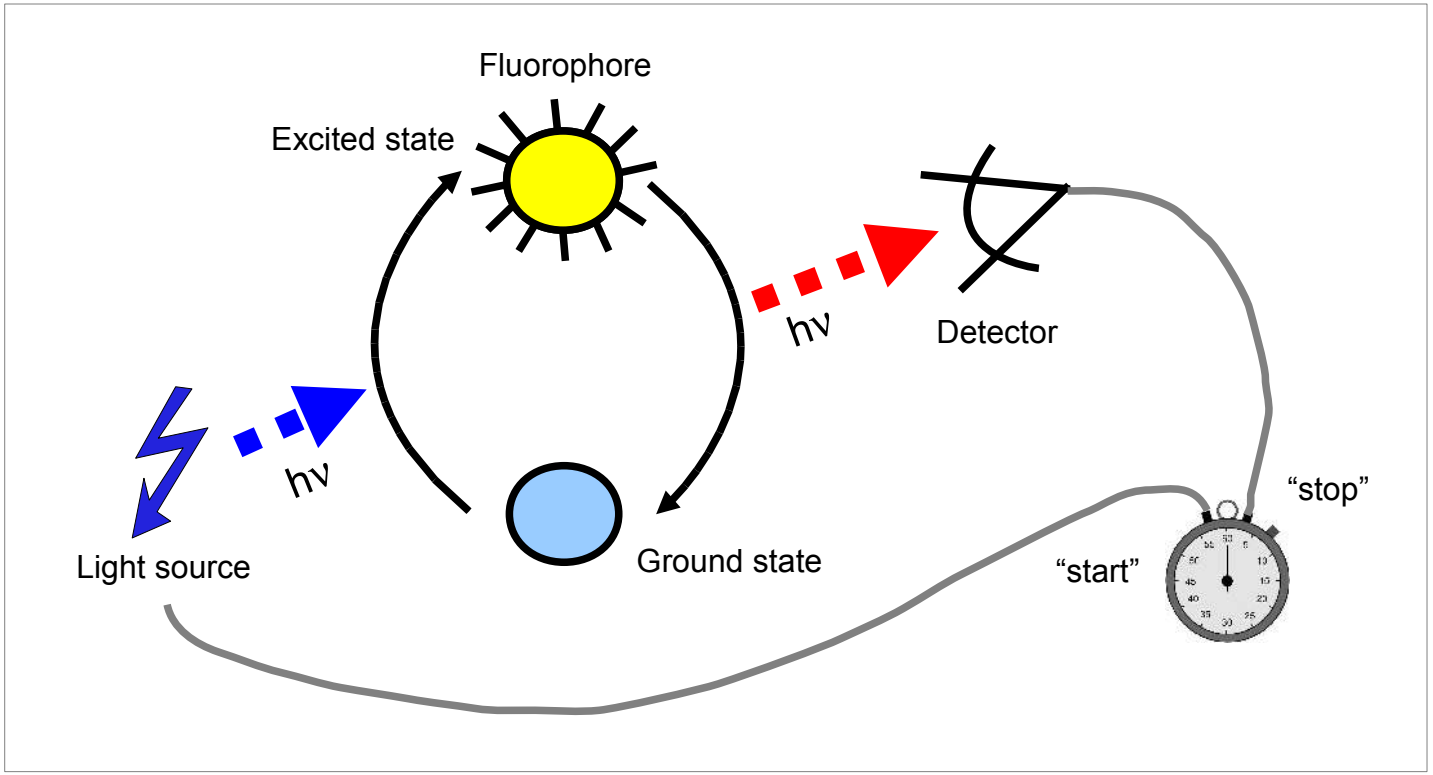


Fluorescence Lifetime = average time that a molecule remains in the excited state prior to returning to the ground state by emitting a photon

How fast is the photocycle?

➔ typ. ps [10^{-12} s] to ns [10^{-9} s]

How to Measure the Fluorescence Lifetime?



One needs:

a defined "start" of the experiment → pulsed excitation; each laser pulse is a new "start"

a defined "stop" of the experiment → single photon sensitive detector; photon arrival at the detector is the "stop"

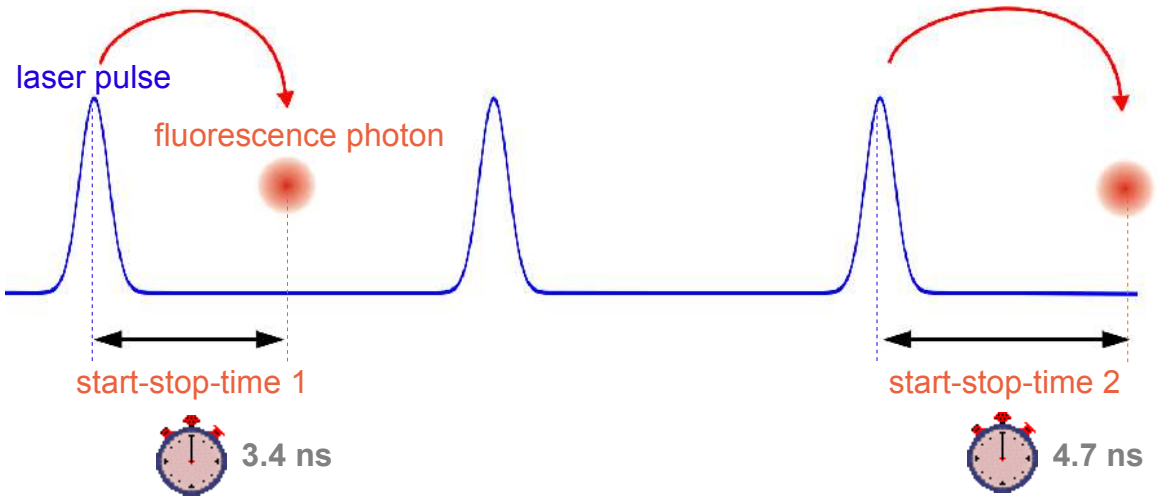
a fast "stopwatch" to measure the time difference between "start" and "stop"

Time-Correlated Single Photon Counting (TCSPC) to Measure the Fluorescence Lifetime

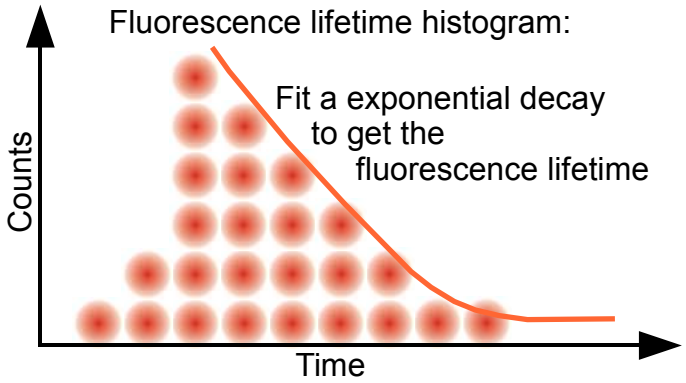
→ In principle with a stop watch:

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

A statistical process!

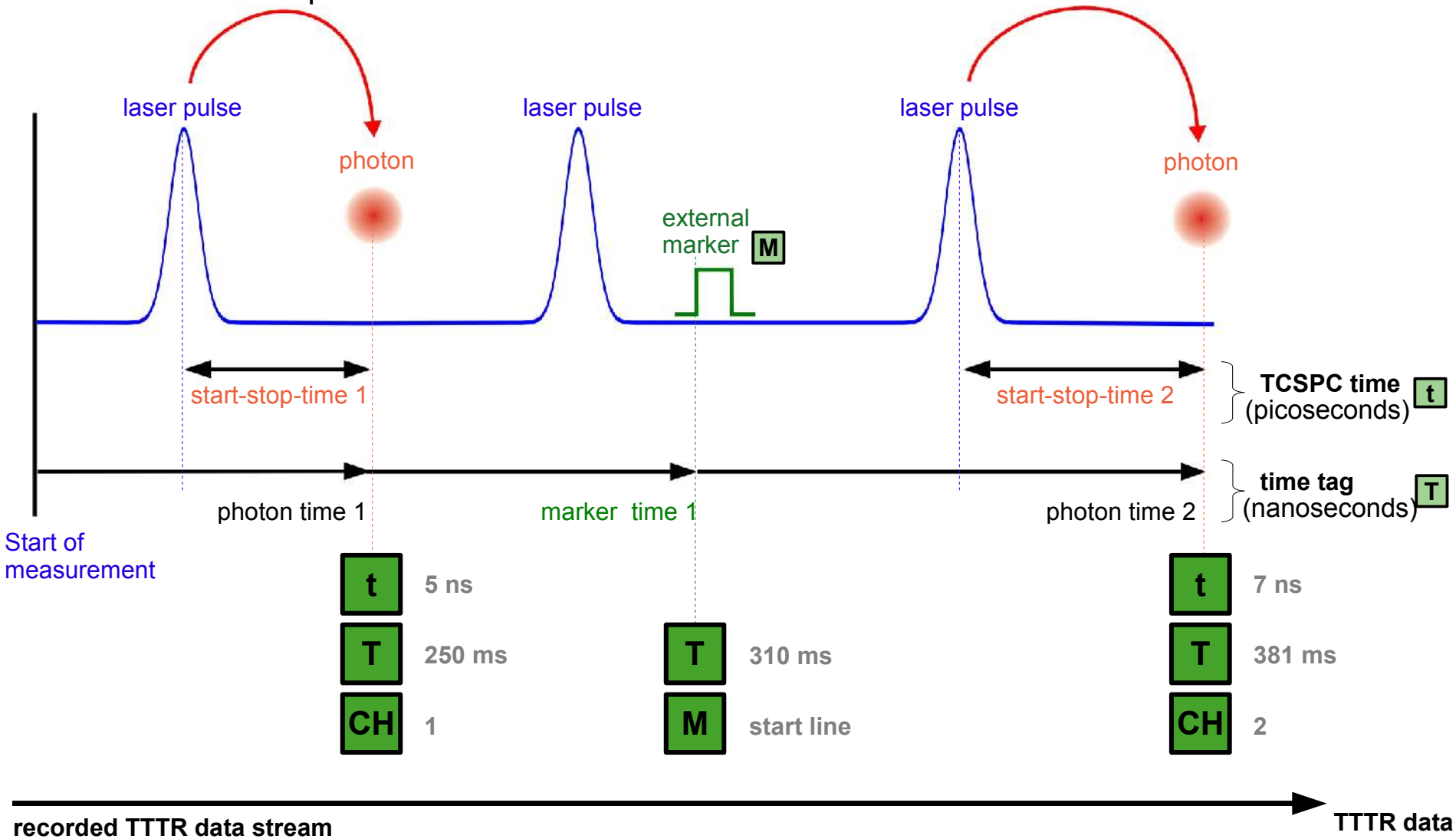


- Repeat this time measurement very often and count "how many photons have arrived after what time"
- Sort the photons within a histogram into time bins according to their arrival times



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

PicoQuant data acquisition mode:



The photon records (t, T, CH) are collected continuously. The data stream is recorded to disk. It can be processed immediately for display and analysis. ALL temporal information is preserved!

TTTR File: four pieces of information

- TCSPC time:
  - ➔ Start-stop photon time
  - ➔ Time difference between the excitation and the arrival of the first photon at the detector
  - ➔ Measured by a “stop watch” (picosecond resolution)

- Time tag:
  - ➔ Represents the global arrival time of each photon relative to the beginning of the experiment
  - ➔ Measured with nanosecond resolution

- Marker signal:
  - ➔ External synchronization signal from the LSM scanner given at the beginning and the end of each line and start of each frame with the corresponding global time tag
  - ➔ Spatial information of each photon to rebuild the FLIM image

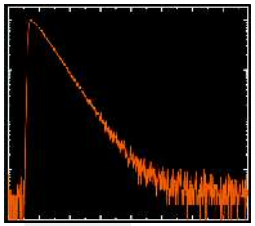
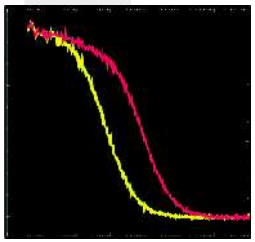
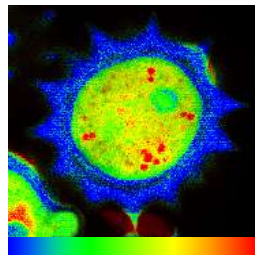
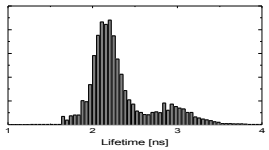
- Channel information:
  - ➔ In case of a multi-channel detector setup
  - ➔ Add a channel identifier to each measured TCSPC time to get the information, on which detector the photon was detected

Time-Tagged Time-Resolved (TTTR) - Data Display and Analysis Possibilities

evaluated data
ignored data

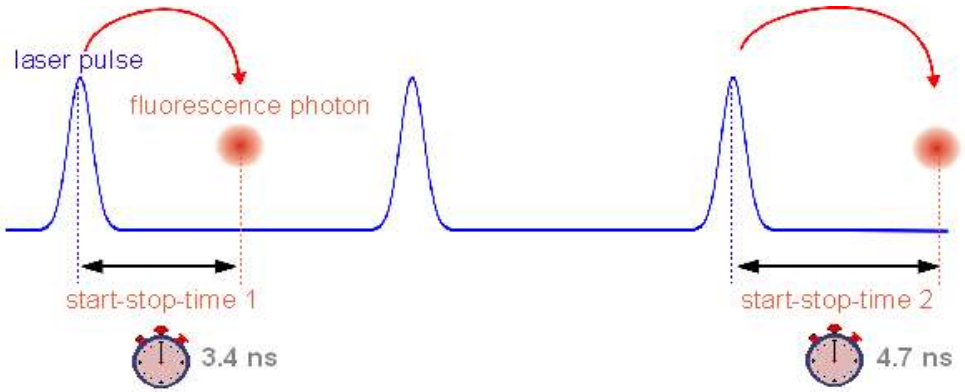
t TCSPC time
T time tag (global arrival time)
CH detection channel indicator
M marker indicator

	marker event	photon event	
point		t T CH	**fluorescence lifetime**, time-gated analysis, **PIE**, coincidence correlation, antibunching
		t T CH	temporal intensity fluctuations (blinking, bursts), **FCS**
		t T CH	spectral splitting (**FRET**), cross correlation
		t T CH	temporal lifetime fluctuations (lifetime trace), **FLCS, PIE-FRET, lifetime FRET**
image	T M	t T CH	intensity imaging
	T M	t T CH	... + spectral splitting (**FRET**)
	T M	t T CH	time-gated imaging, **PIE-FRET,** fluorescence lifetime imaging (**FLIM**), **FLIM-FRET**

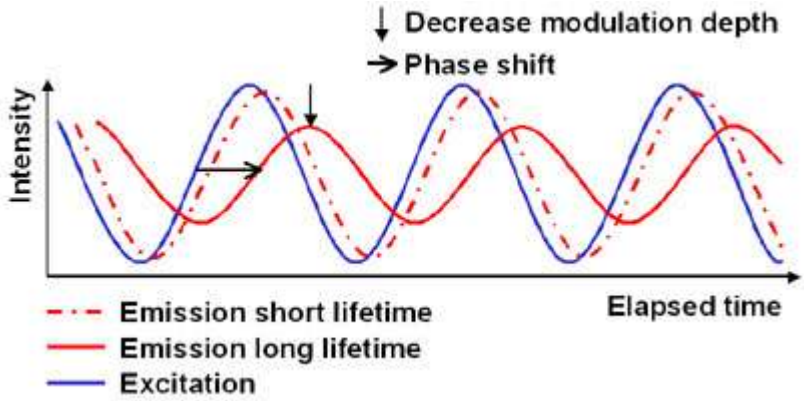





Advantages of time-domain versus frequency-domain FLIM

time-domain



frequency-domain



- Upgrade of confocal LSM
- Very intuitive approach
- Higher sensitivity: counting single photons is much better suited for biological samples with often relative low fluorescence intensities due to e.g. moderate expression levels that are comparable to endogenous concentrations
- Better timing resolution
- Higher accuracy of multi-exponential decay analysis that is essential for FLIM analysis in the heterogeneous cellular environment
- Possibility of single molecule studies (e.g. FCS)

FLIM & FCS Upgrade Kit for Laser Scanning Microscopes

FLIM & FCS Upgrade Kit for Laser Scanning Microscopes: Components

Single photon counting detector unit:
2 Single Photon Avalanche Detectors (SPAD)

emission

Router

"stop"

"Stop watch"
Time-Correlated Single Photon Counting (TCSPC) unit

Synchronization (Line and Frame clock)

LSM

"start"

Pulsed diode laser driver

Fiber Coupling Unit (FCU II) with pulsed diode laser heads of LDH series

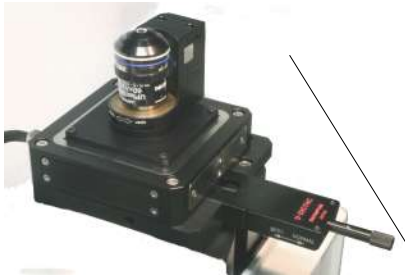
excitation

Pulsed laser system

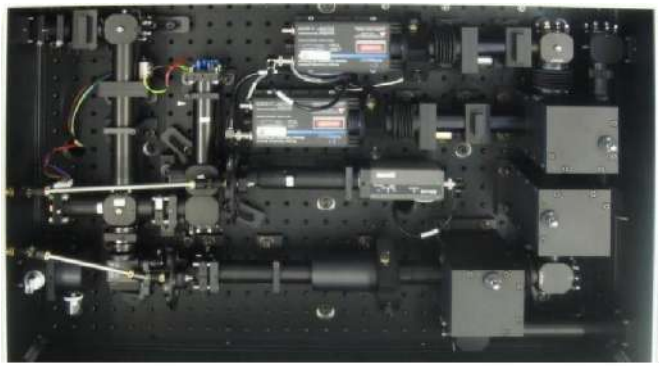
Single Molecule Sensitivity in a Complete System: MicroTime 200

Excitation subsystem

Objective scanning and
DIC prism for two focus FCS

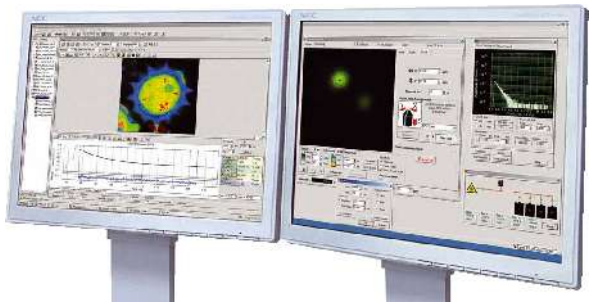


Confocal excitation and detection optics



Computer controlled laser driver

Advanced system and analysis software

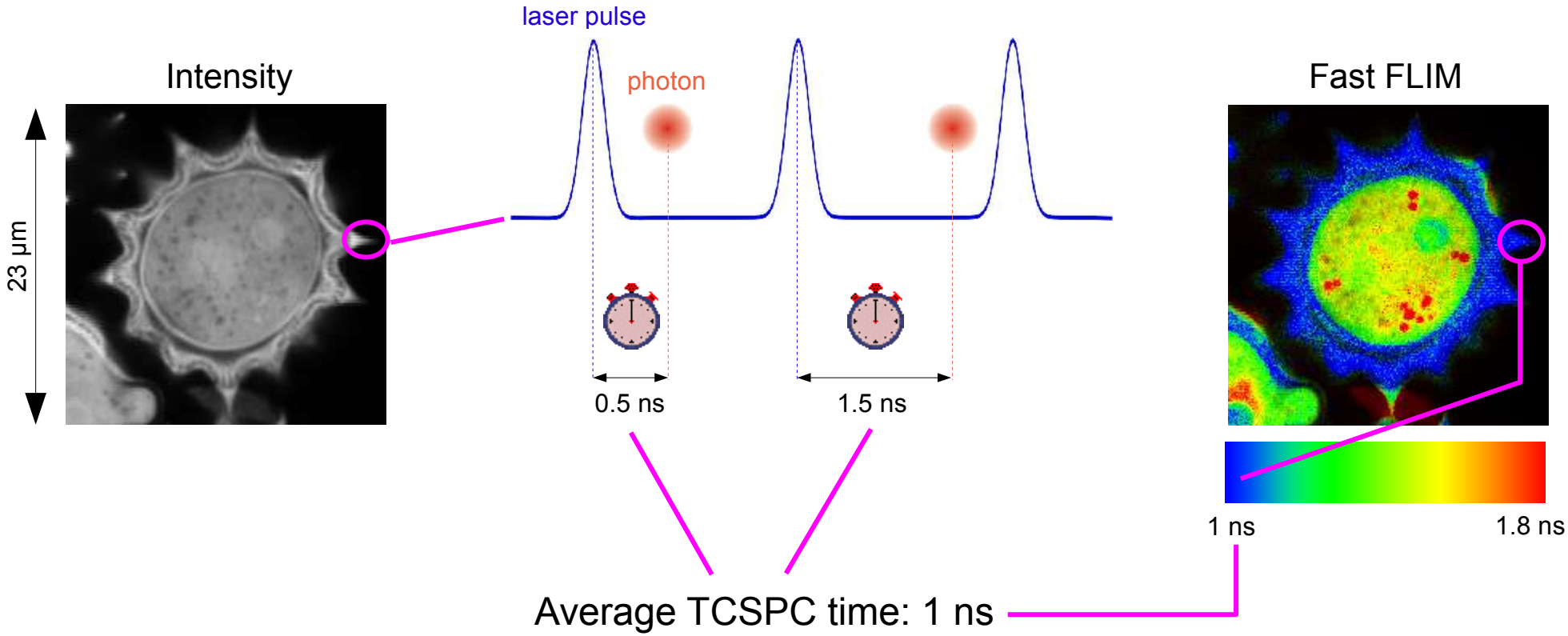


Time-correlated single
photon counting unit

Fast Fluorescence Lifetime Imaging (Fast FLIM)

- Online display of the image during data acquisition
- Fast FLIM displays the average photon arrival time
- Facilitates data acquisition and pre-selection photon by photon

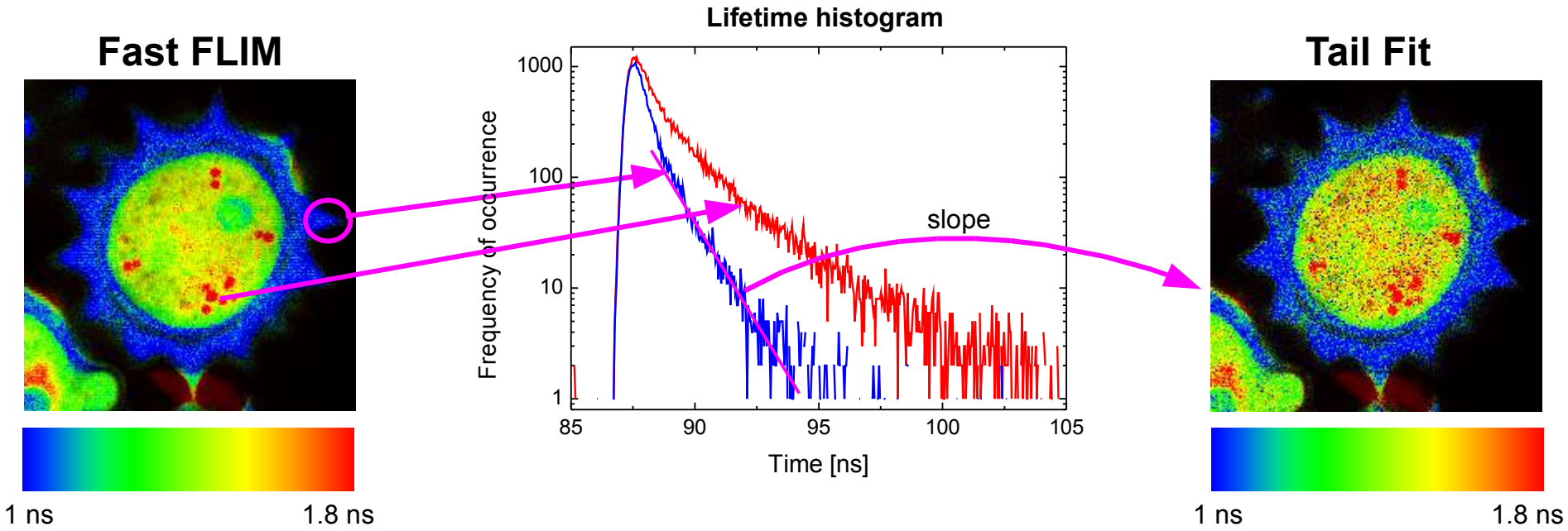
Daisy pollen, measured with
MicroTime 200 confocal microscope



Lifetime Histogram: Tail Fit

- Display of the photon arrival times in a histogram
- Tail fit for lifetime analysis

Daisy pollen, measured with
MicroTime 200 confocal microscope



- Fast
- Good lifetime contrast
- Less lifetime - noise

- More accurate results for
  ➔ very short lifetimes
  ➔ complex dye mixtures

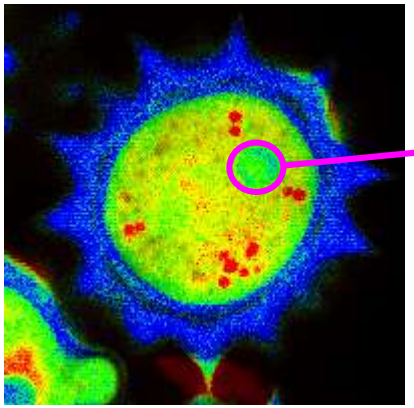
Multi-exponential Decay

More than one fluorophore with different lifetimes present in sample

Tail fit with multi-exponential decay

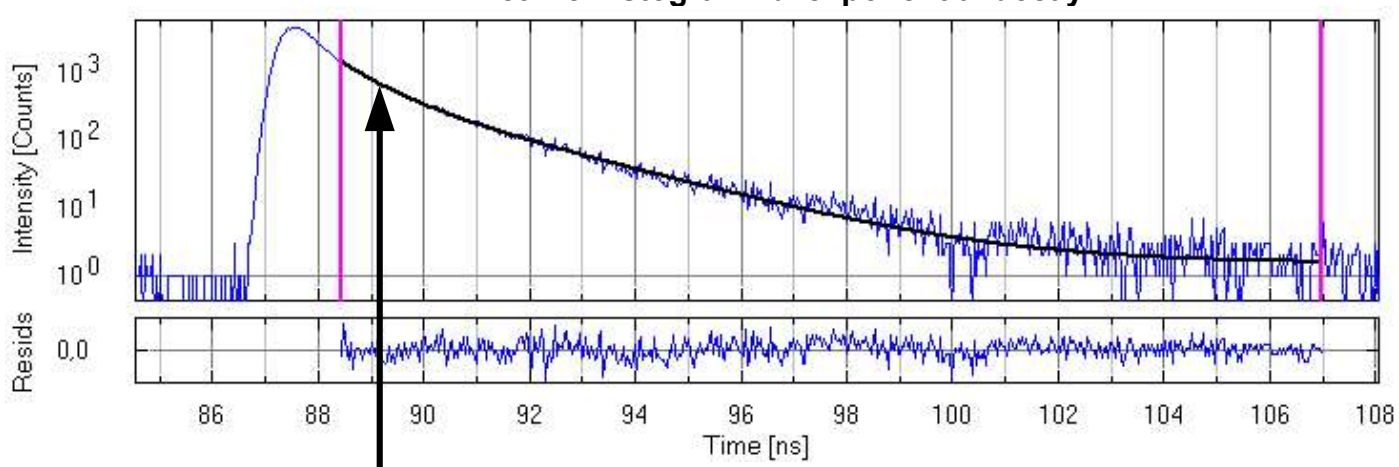
Daisy pollen, measured with MicroTime 200 confocal microscope

FLIM



1 ns 1.8 ns

Lifetime histogram: bi-exponential decay



Short component: 0.8 ns

Long component: 2.4 ns

Environmental sensing by FLIM

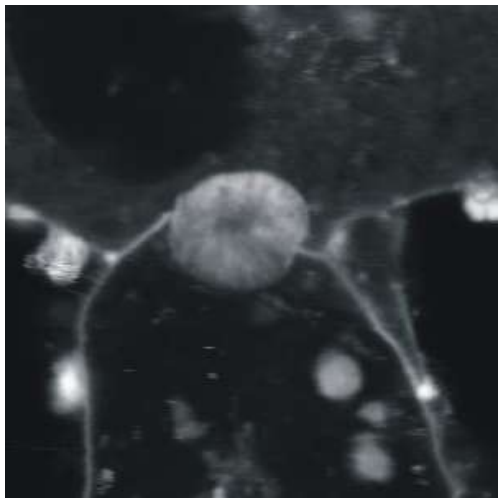
Living hepatocyte (liver cell) containing a canalicular vacuole, stained with NBD (7-nitrobenz-2-oxa-1,3-diazole).

The FLIM image visualizes the different hydrophobicities and their local variations within the cell.

→ Canalicular vacuole is very likely of bilayer type at the rim (membrane) and of micellar type in the center

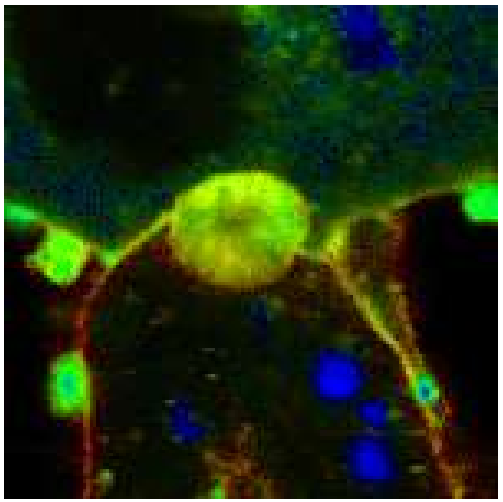
λ_{exc} = 467 nm
100x, 1.3 N.A. oil immersion
filter: LP500
300 × 300 pixels
acquisition time: 3 min.

Fluorescence intensity



0 kcps Intensity 1.3 Mcps

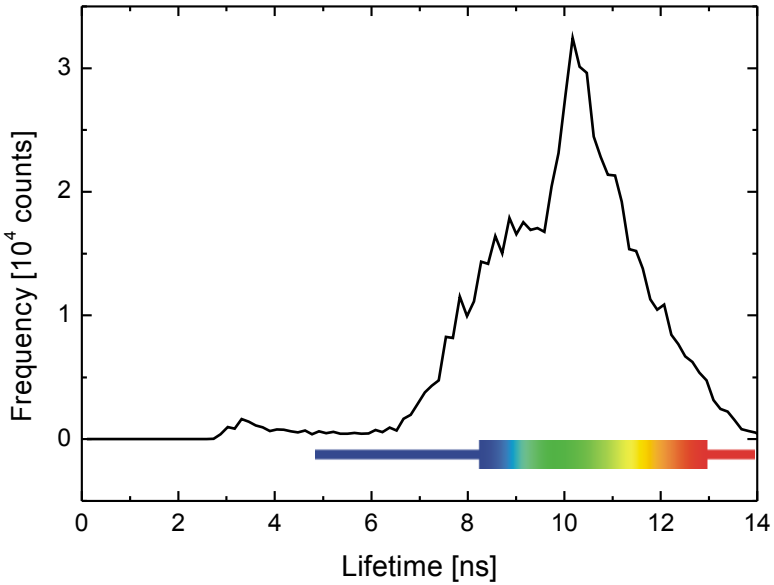
Fluorescence lifetime



8 ns Lifetime 13 ns

H_2O conc.

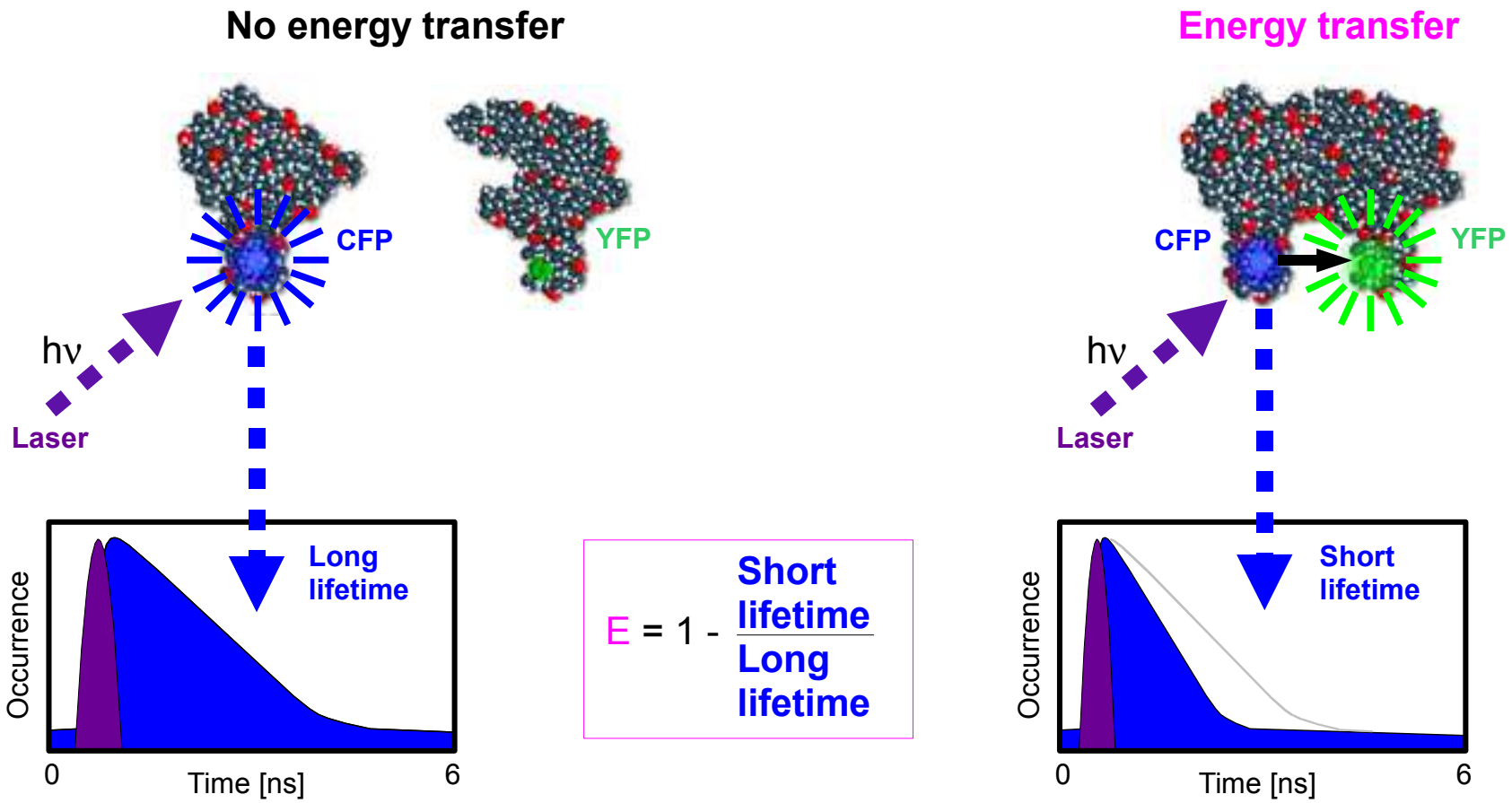
Lifetime distribution



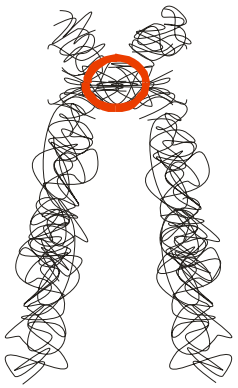
Sample courtesy of Astrid Tannert, Thomas Korte, Humboldt University Berlin

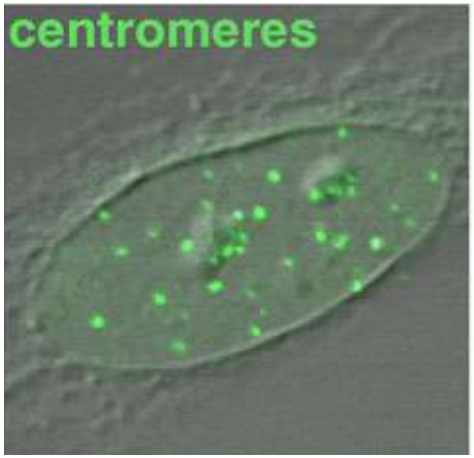
Förster Resonance Energy Transfer (FRET)

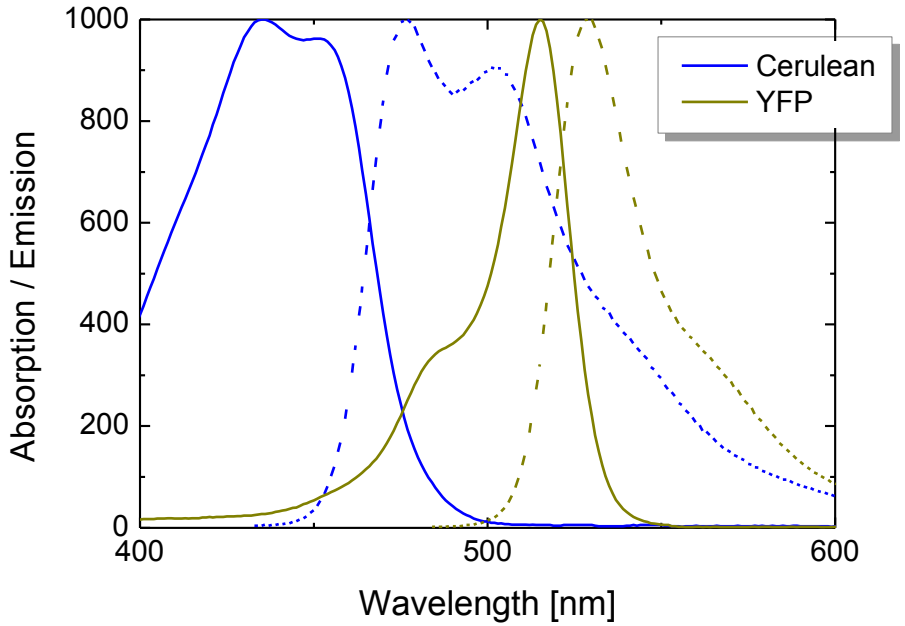
- Detection of protein interaction
- Both proteins labeled with donor and acceptor fluorophores, e.g. CFP and YFP
- (Donor) Fluorescence Lifetime measurement



Protein Interactions of CENP-A and CENP-B via FLIM-FRET







Human centromere kinetochore complex

- ensures correct chromosome segregation during cell division
- located at the primary constriction of each chromosome
- ~50 kinetochore proteins (CENPs) and underlying DNA (centromere)

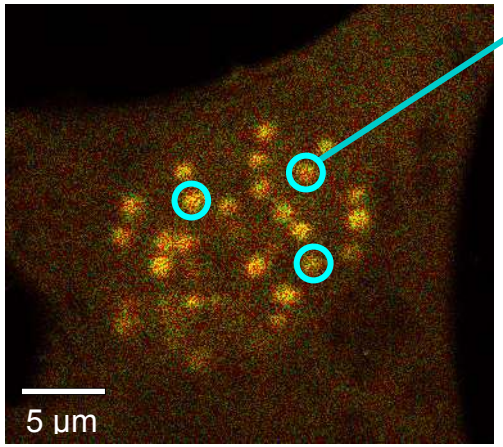
➔ Determination of neighbourhood relations of kinetochore proteins by FLIM-FRET *in vivo*

- Example: CENP-A and CENP-B
- Fluorophores: Cerulean / EYFP
  ➔ Well suited for FRET studies
  ➔ Donor excitation: 405 nm or 440 nm

Sample courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI), Jena
Dye spectra taken from: http://www.tsienlab.ucsd.edu/Documents.htm

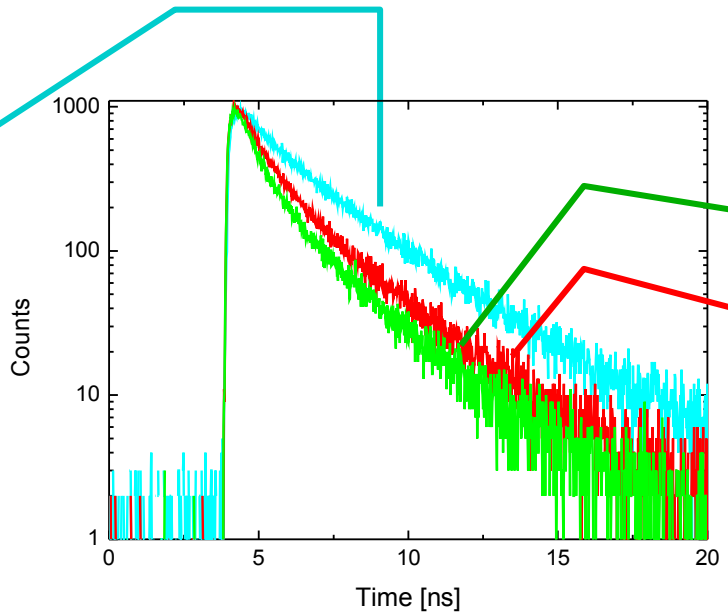
Protein Interactions of CENP-A and CENP-B via FLIM-FRET

U2OS cell transfected with CENP-B-Cerulean (donor)

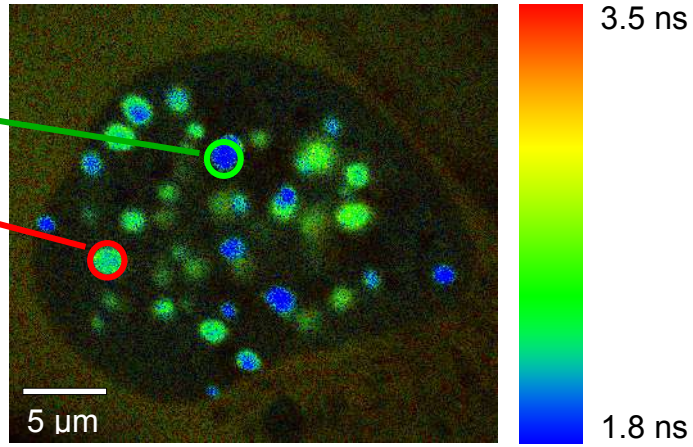


similar fluorescence lifetimes in all centromeres

τ_{av} ~ 2.94 ns



U2OS cell transfected with CENP-B-Cerulean (donor) & YFP-CENP-A (acceptor)



every centromere shows a specific fluorescence lifetime

τ_{av} between ~ 1.8 ns and 2.2 ns

FRET

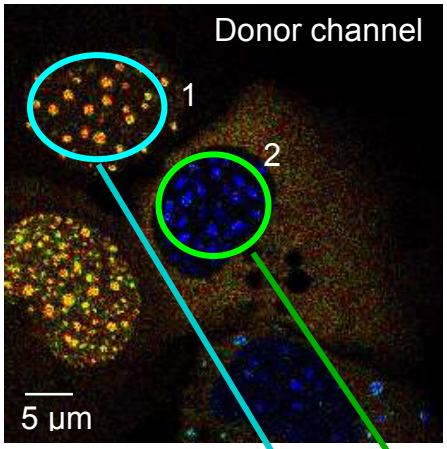
CENP-A and CENP-B are in direct vicinity at human centromers

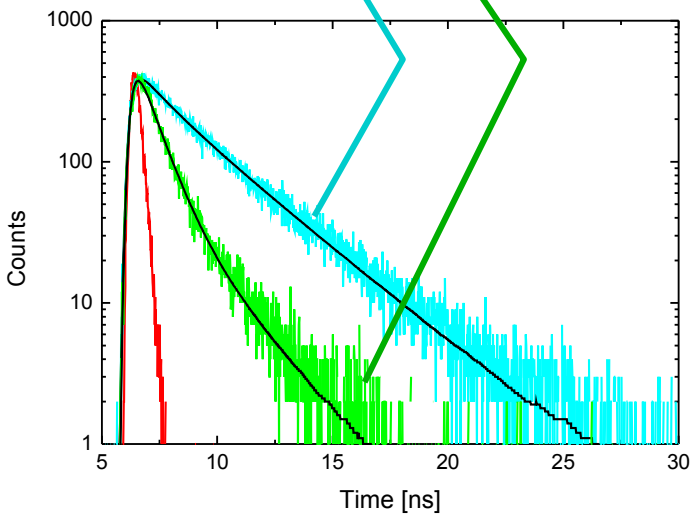
Excitation: 440 nm, 20 MHz
Emission: 480 / 40 bandpass-filter
objective: UPLSAPO 60x O NA1.35
LSM Upgrade Kit

Sample courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI), Jena

Protein Interactions of CENP-A and CENP-B via FLIM-FRET: Dual Channel detection

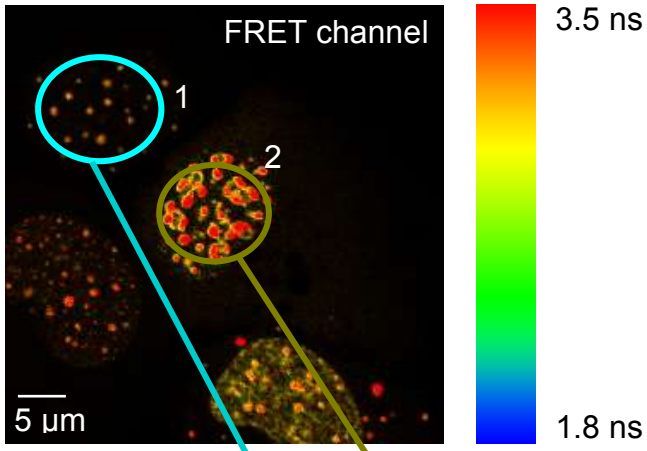
Dual Channel Detection

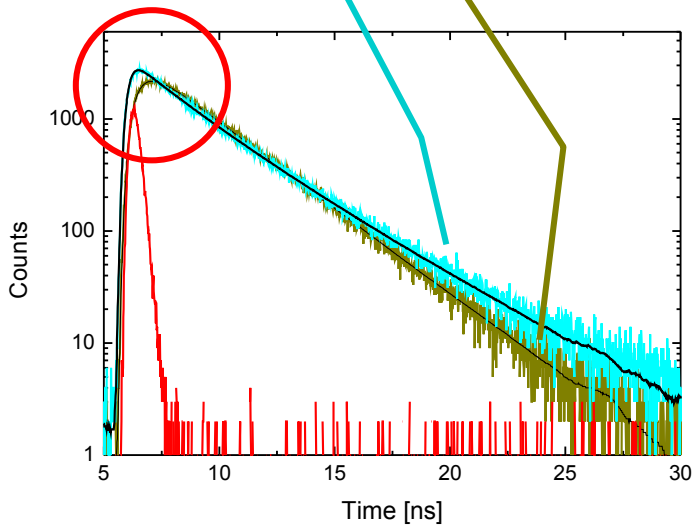




Cell 1:
contains only the donor CENP-B-Cerulean
Donor and Acceptor channel:
τ_{av} = ~3 ns
(= CENP-B-Cerulean)

Cell 2:
transfected with both YFP-CENP-A and CENP-B-Cerulean
Donor channel:
τ_{av} = ~1.2 ns
E_{FRET} = 60%
Acceptor channel:
τ = ~2.8 ns
+ rise time of
τ = ~0.5 ns
(all fits including IRF)

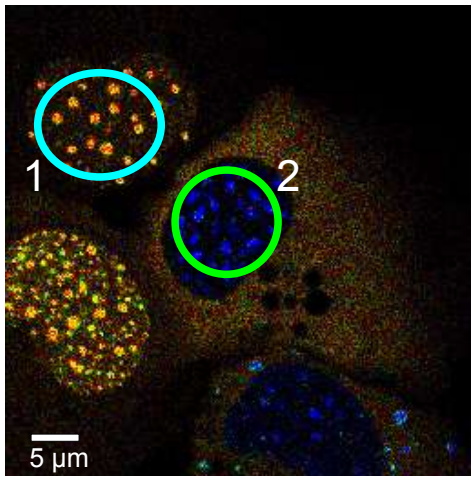


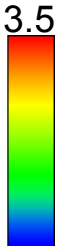


Sample courtesy of Sandra Orthaus, former member of Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI), Jena

Protein Interactions of CENP-A and CENP-B via FLIM-FRET

Donor Channel





Cell 2:
transfected with both YFP-CENP-A and CENP-B-Cerulean

Donor channel:

τ_{av} = ~1.2 ns

E_{FRET} = 60%

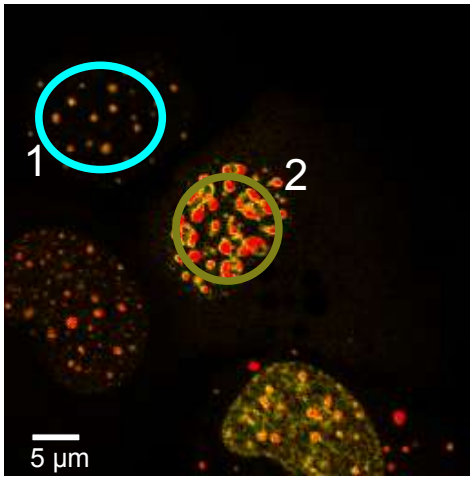
Acceptor channel:

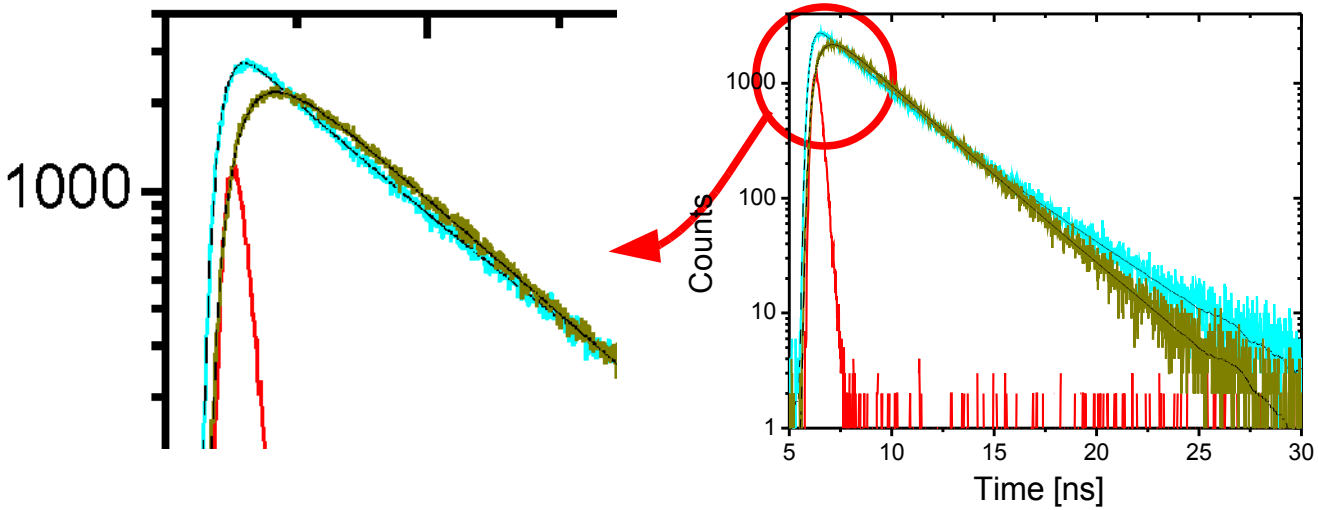
τ = ~2.8 ns

+ rise time of

τ = ~0.5 ns

Acceptor Channel





Sample courtesy of Sandra Orthaus, Fritz Lipmann Institute (FLI), Jena

FLIM-FRET measurements: 2-photon excitation and acceptor photo-bleaching

EGFP-RFP fusion construct expressed in living cells

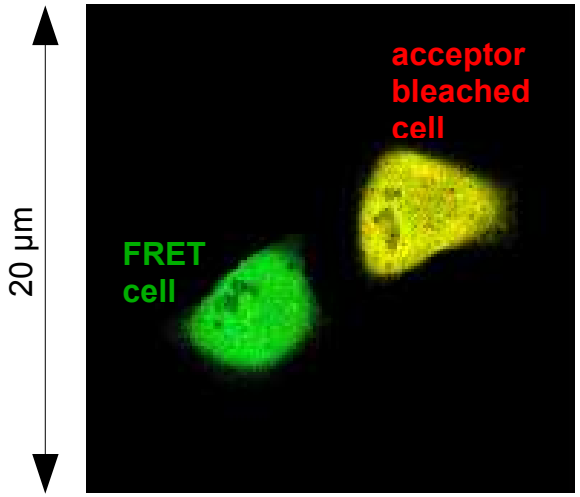
= POSITIVE CONTROL

	FRET (D+A)	Bleach (D)
Lifetime 1	2.4 ns	2.4 ns
Lifetime 1	1.2 ns	1.2 ns
Amp. 1	51%	85%
Amp. 2	49%	15%

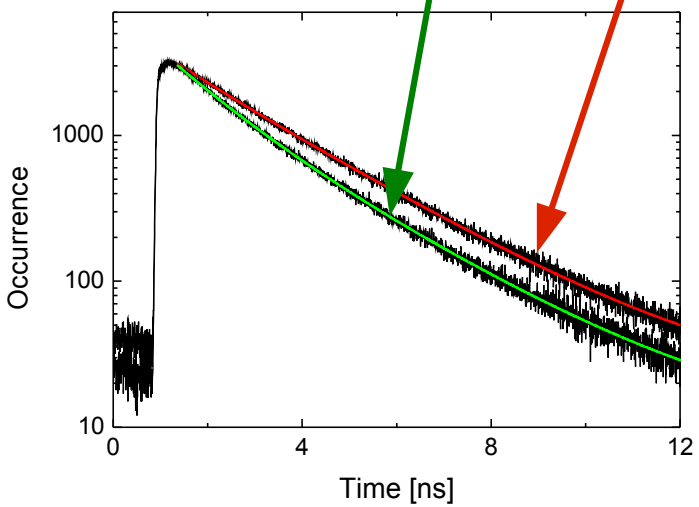
Leica SP5
Two photon excitation:
λ_{exc} = 850 nm, 80 MHz
filter: BP (500-540) nm

Lifetime of EGFP alone: 2.4 ns

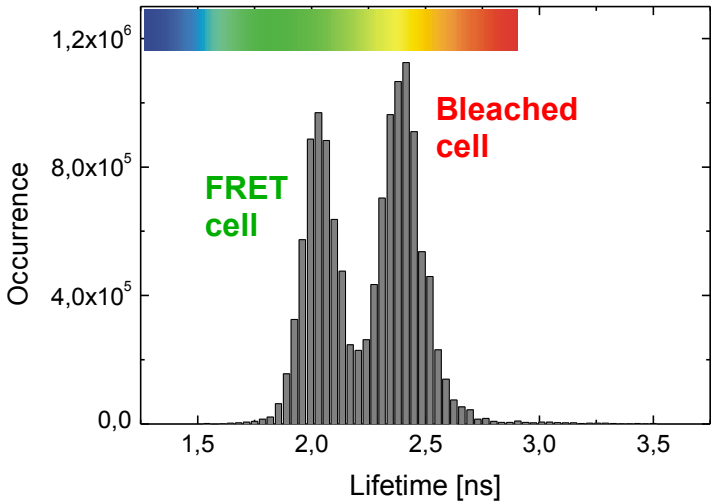
Fluorescence lifetime image (FLIM)



Fluorescence decays



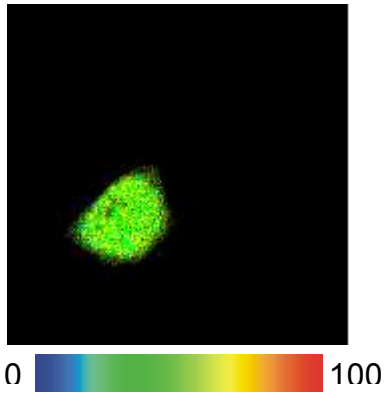
Lifetime histogram



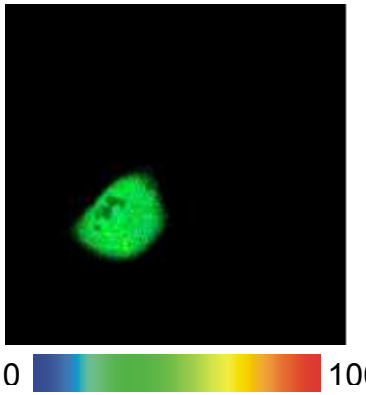
Sample courtesy of Dirk Daelemans, Thomas Vercruysse, Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium

FLIM-FRET Analysis with Scripting

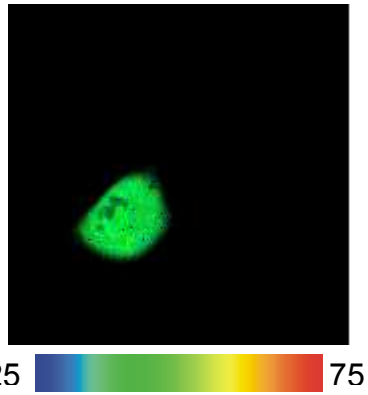
$$E_{FRET} = 1 - \frac{\tau_{D(A)}}{\tau_D}$$

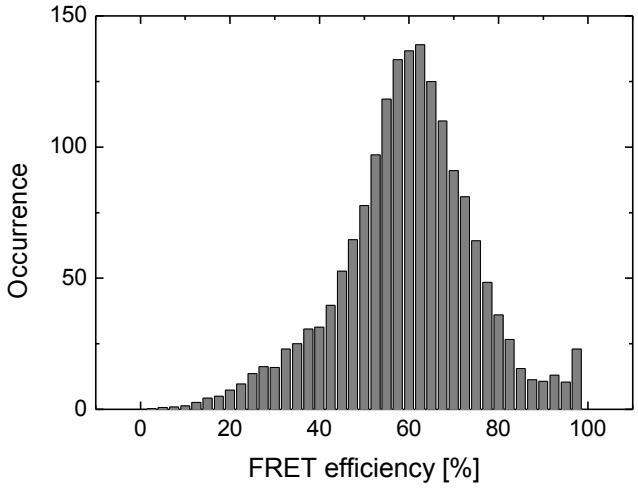


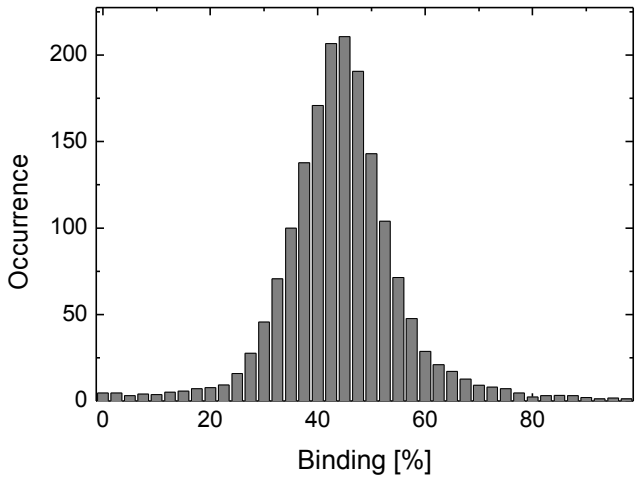
Binding

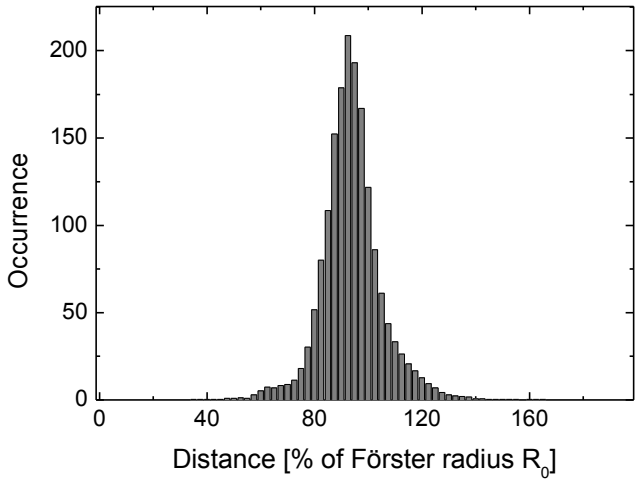


Distance



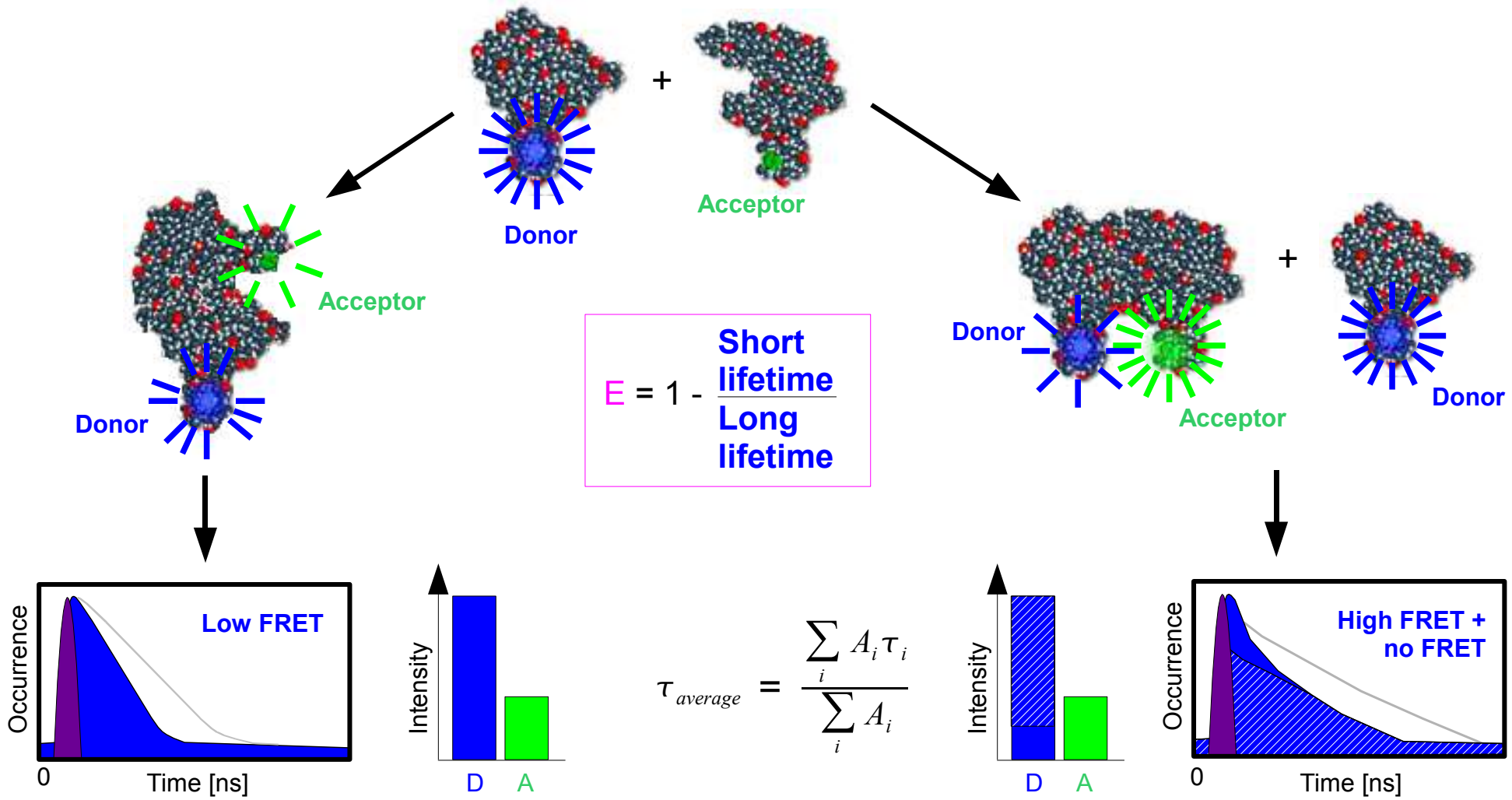






Sample courtesy of Dirk Daelemans, Thomas Vercruysse,
Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium

FLIM-FRET Can Resolve Subpopulations



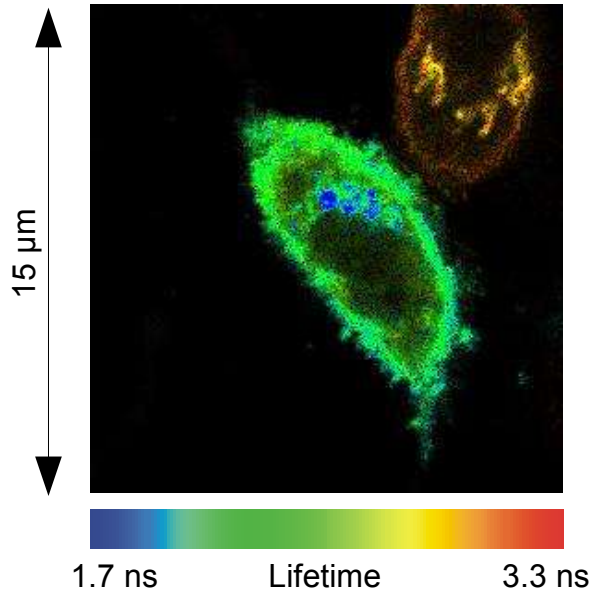
FRET Analysis via FLIM

Interactions of fluorescent proteins in inside living cells (12V HC Red cells) labeled with EGFP and RFP attached to each other

→ After acceptor bleaching the quenching of the donor is strongly reduced

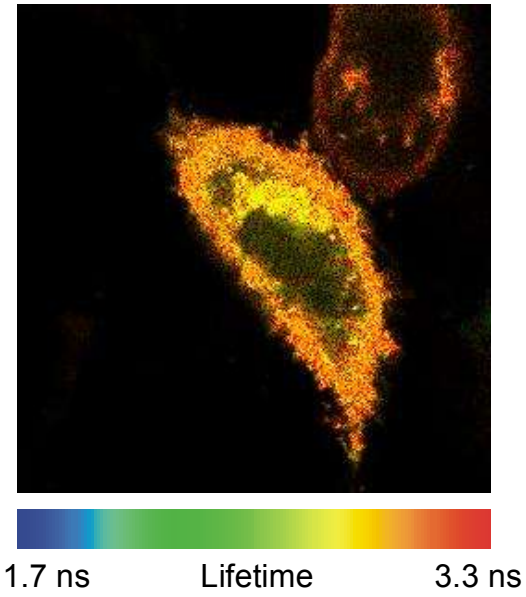
Olympus FV1000
excitation: λ_{exc} = 470 nm, 40 MHz
Bleaching: λ_{exc} = 568 nm
Apo 60x, 1.4 N.A. oil immersion
filter: BP (500-540) nm
256 × 256 pixels

Fluorescence lifetime before bleach



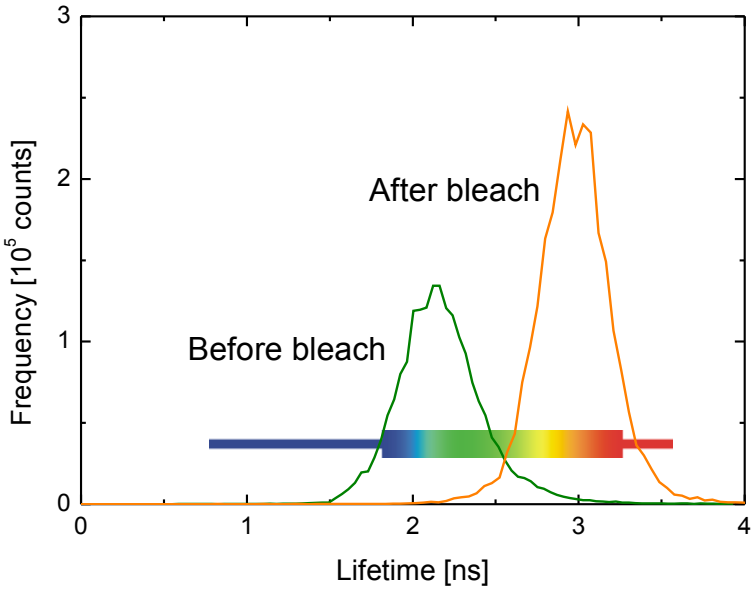
Mean lifetime: 2.2 ns

Fluorescence lifetime after bleach



Mean lifetime: 2.9 ns

Lifetime distribution

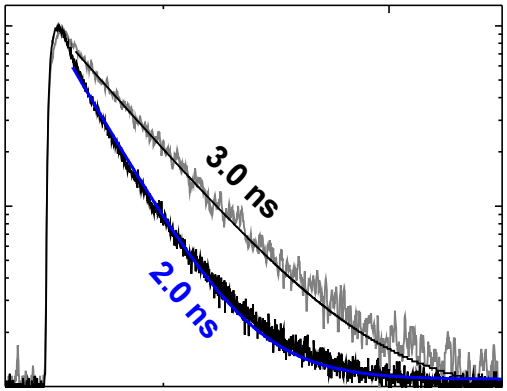


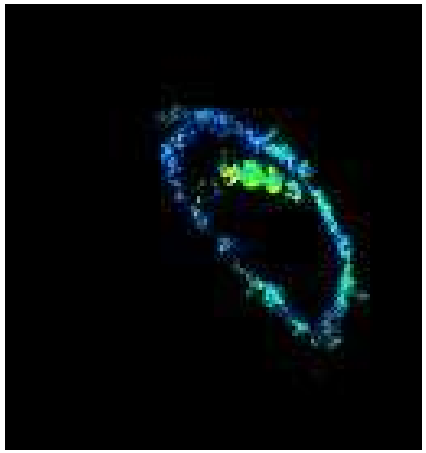
Sample courtesy of Philippe Bastiaens, Max Planck Institute for Molecular Physiology, Dortmund, Germany

FLIM-FRET - Separating Quenched from Unquenched Donor Species

$$E_{FRET} = 1 - \frac{\tau_{D(A)}}{\tau_D}$$

Single exp. analysis

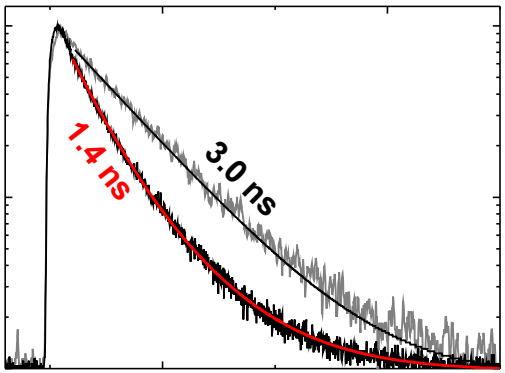




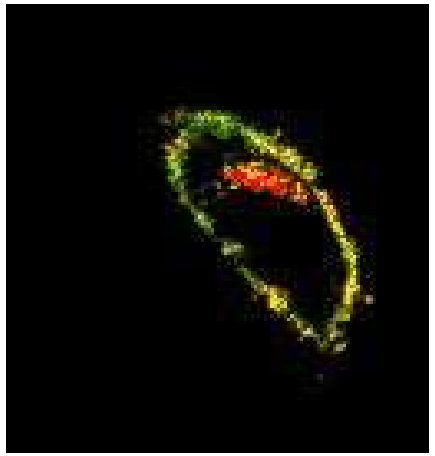
? different FRET efficiencies (GFP-RFP distances)?
→ **wrong**

? different ratios between quenched (GFP-RFP) and unquenched (GFP) species?
→ **correct**

Double exp. analysis



3.0 ns
(no FRET)



25  50

FRET efficiency [%]

25  75

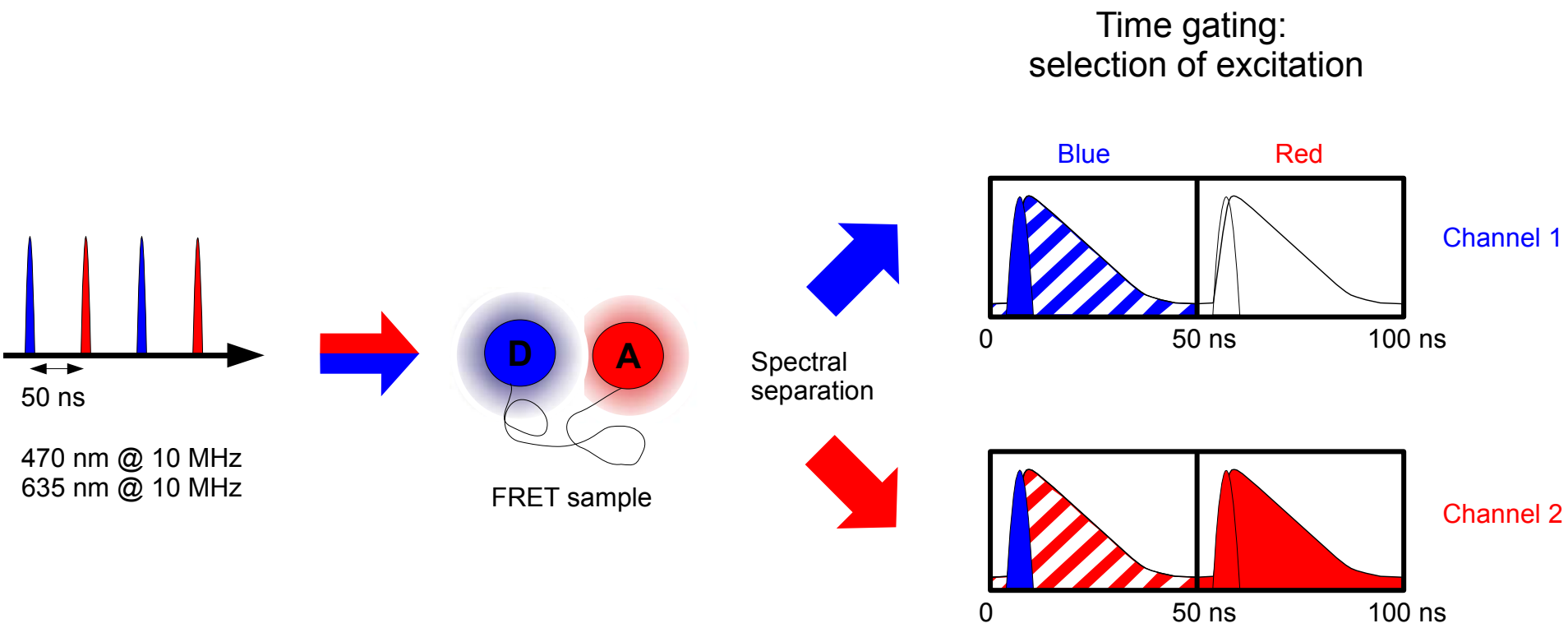
Ampl. (FRET) / Σ Ampl. [%]
(% of binding)

$$\%\,Binding_i = \frac{A_i}{\sum_i A_i}$$

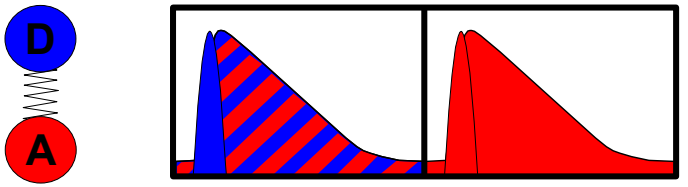
Sample courtesy of Philippe Bastiaens, Max Planck Institute for Molecular Physiology, Dortmund, Germany

Time-Gated Analysis: Pulsed Interleaved Excitation (PIE)

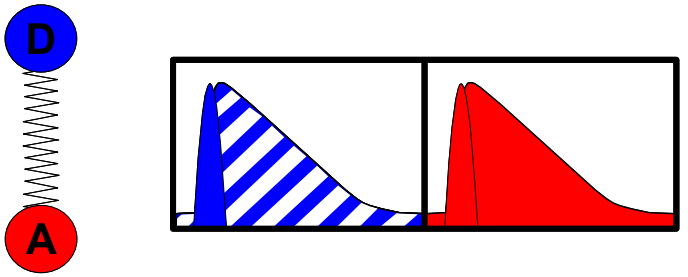
Dual colour Pulsed Interleaved Excitation (PIE) to identify FRET artifacts
(effectively only possible at the single molecule level)



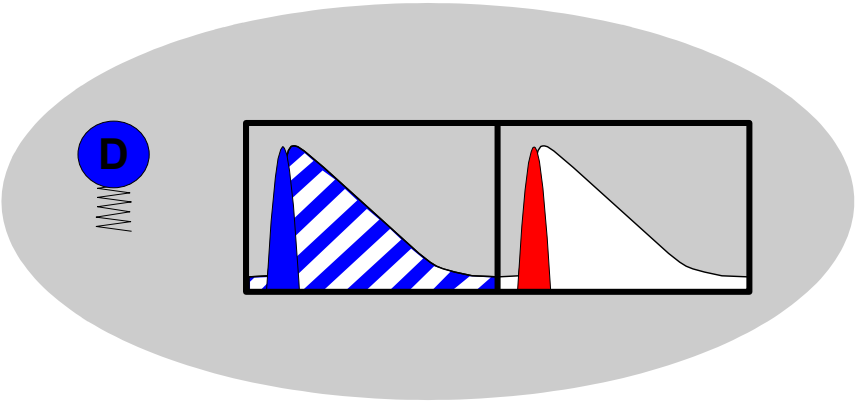
Time-Gated Analysis: PIE-FRET



Intact pair
→ FRET

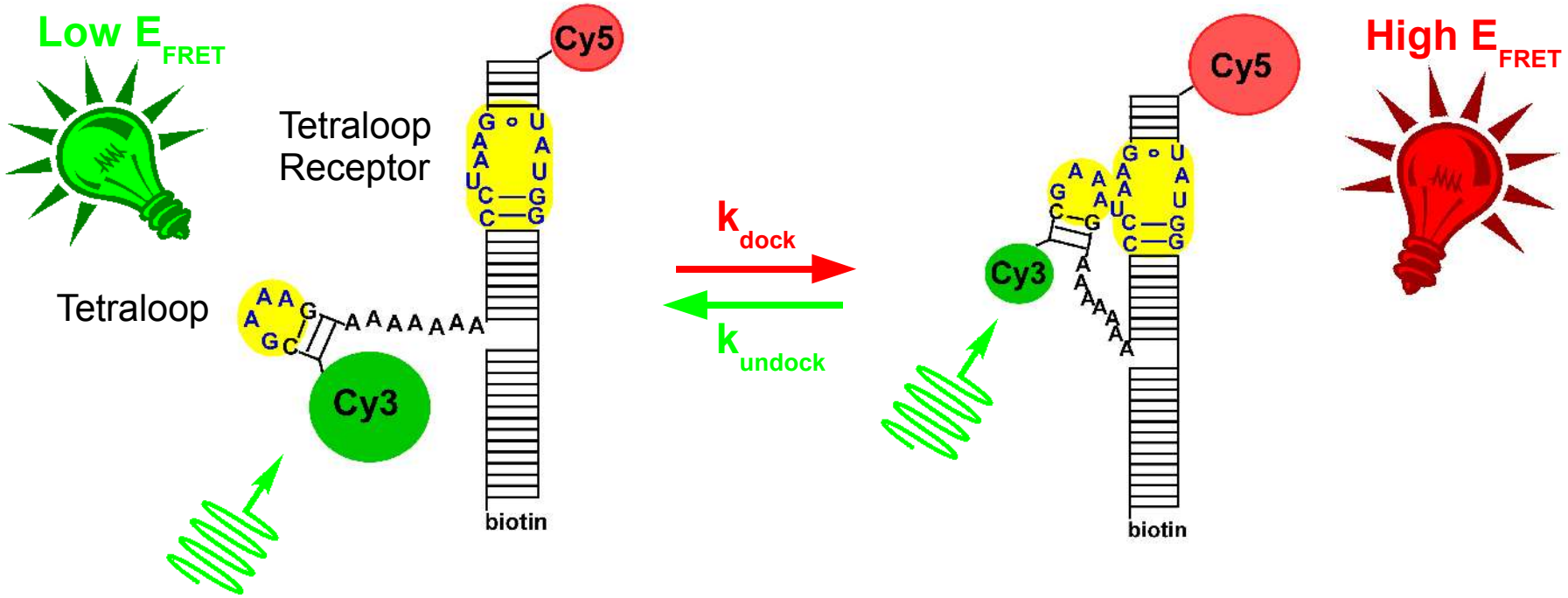


Intact pair
→ no FRET



Non fluorescing
acceptor

PIE-FRET in RNA Folding Studies

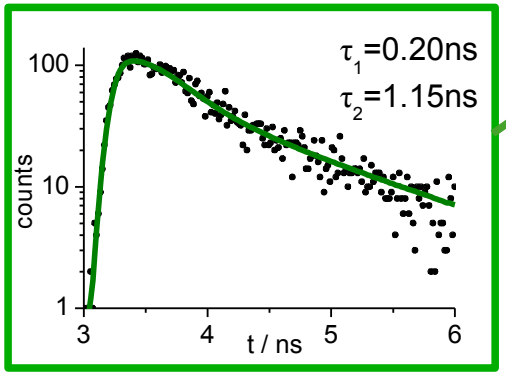


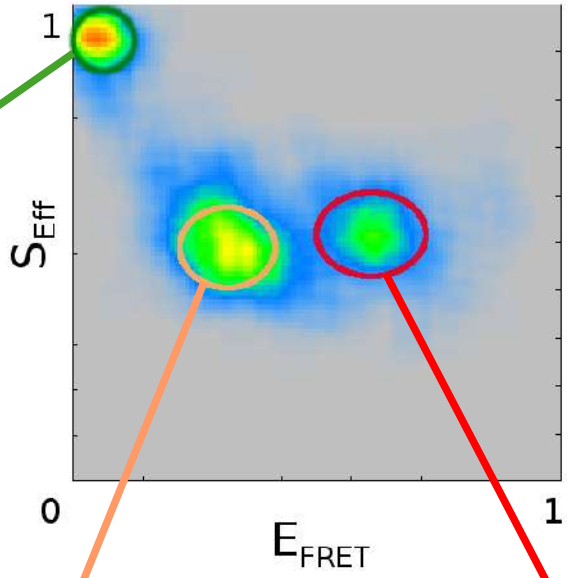
- Folding and unfolding monitored by FRET
- Mg^{2+} driven
- Important RNA folding motif
- Excitation: 532nm

in collaboration with J. Fiore and David Nesbitt (JILA, Univ. of Colorado, Boulder)

PIE-FRET: Analysis of Sub-Populations

donor only





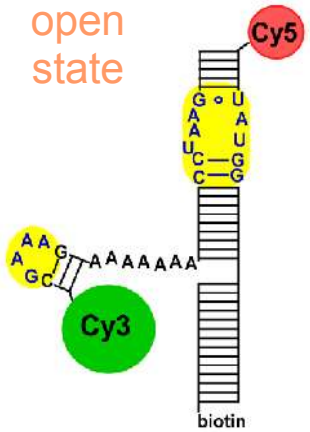
Photon Stoichiometry

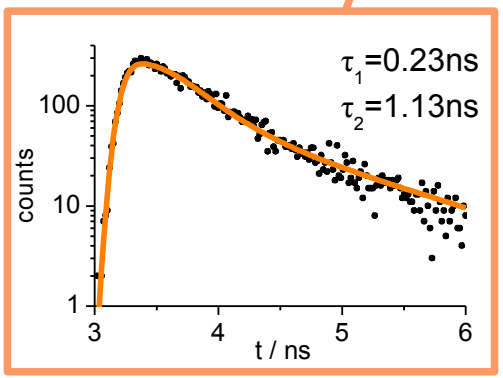
$$S_{Eff} = \frac{n_{A\text{-}Fret} + n_D}{n_{A\text{-}Fret} + n_D + n_{A\text{-}Direct}}$$

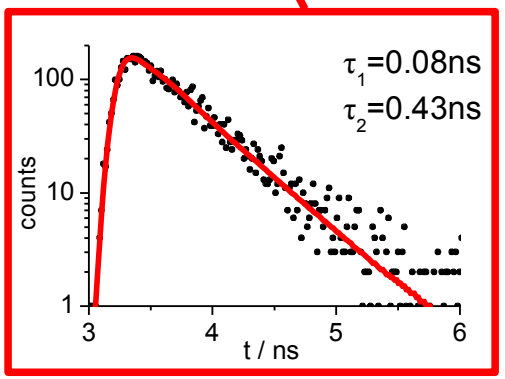
FRET Efficiency

$$E_{FRET} = n_{A\text{-}Fret} / (n_{A\text{-}Fret} + n_D)$$

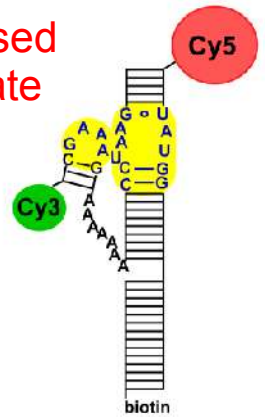
open state







closed state

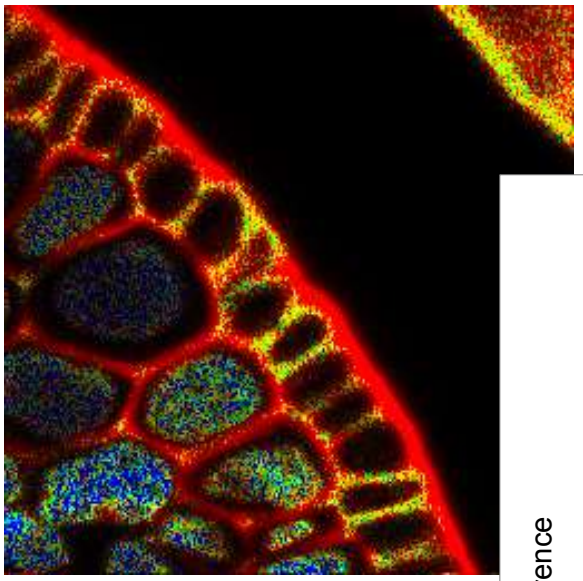


Sample courtesy of Julie Fiore and David Nesbitt, University of Colorado, Boulder

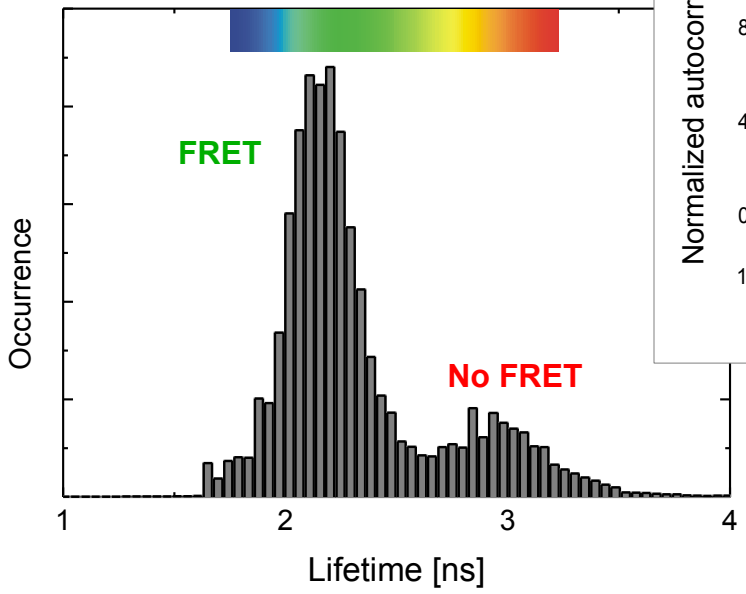
Summary

LSM Upgrade kit / MicroTime 200 enable for...

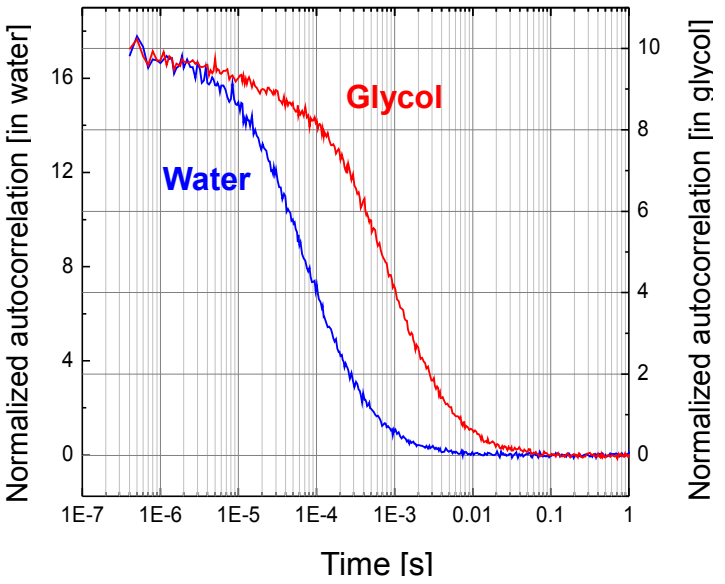
Fluorescence Lifetime Imaging



Förster Resonance Energy Transfer



Fluorescence Correlation Spectroscopy



… and much more ...

Acknowledgement

Astrid Tannert and Thomas Korte
Humboldt University Berlin, Germany

Philippe Bastiaens
Max Planck Institute for Molecular Physiology, Dortmund, Germany

Dirk Daelemans and Thomas Vercruysse
Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium

Julie Fiore and David Nesbitt
University of Colorado, Boulder, USA

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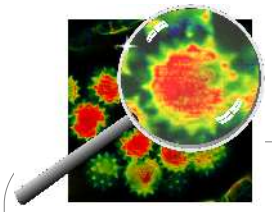
- BMBF Biophotonics III program, project code 13N9271 (“3D Tissue”)
- BMBF Biophotonics III program, project code 13N8850 (“Fluoplex”)
- BMWi, grant MNPQ 12/06

Forschungsschwerpunkt Biophotonik
LICHT FÜR DIE GESUNDHEIT

Federal Ministry of Education and Research

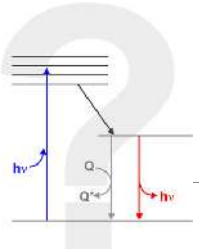
PicoQuant Events



2nd European Short Course on "Time-Resolved Microscopy and Correlation Spectroscopy"

16 – 18 February 2010, Berlin-Adlershof, Germany

- Topics: Introduction to Microscopy, Hardware for Time-Resolved Microscopy, FCS, FLIM, FRET, Steady-State Microscopy Techniques
- Course instructors: Jörg Enderlein, Paul French, Johan Hofkens, Fred Wouters
- Hands-On experimentation and lab demonstration by: Leica, Nikon, Olympus and PicoQuant
- www.picoquant.com/_mic-course.htm



7th European Short Course on "Principles & Applications of Time-Resolved Fluorescence Spectroscopy"

9 – 12 November 2009, Berlin-Adlershof, Germany

Topics: Steady state and time-resolved fluorescence spectroscopy and instrumentation, time- and frequency domain measurements, anisotropy, solvent effects, quenching and Förster energy transfer, data analysis, ...

Course instructors: Joseph R. Lakowicz, Karol Gryczynski, Rainer Erdmann, Matthias Patting, Michael Wahl

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Förster Resonance Energy Transfer (FRET)

Interactions of protein partners in their natural environment inside living cells can be studied with time-resolved FRET microscopy

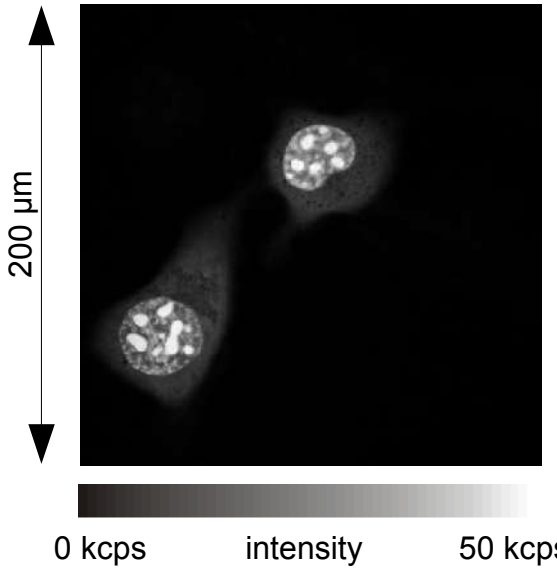
→ Characterization of intra-nuclear dimer formation for the transcription factor C/EBP a in living pituitary GHFT1-5 cells of mice

Members of the C/EBP family of transcription factors are critical determinants of cell differentiation

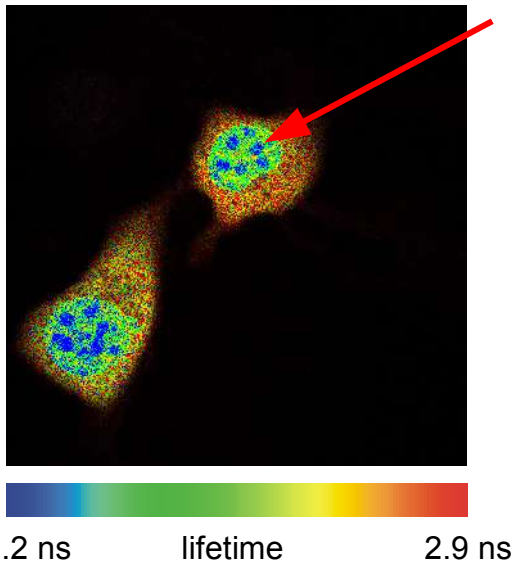
Olympus FV1000
λ_{exc} = 440 nm, 40 MHz
Apo 60x, 1.4 N.A. oil immersion
filter: LP460
512 × 512 pixels

Lifetime of CFP alone: 2.7 ns

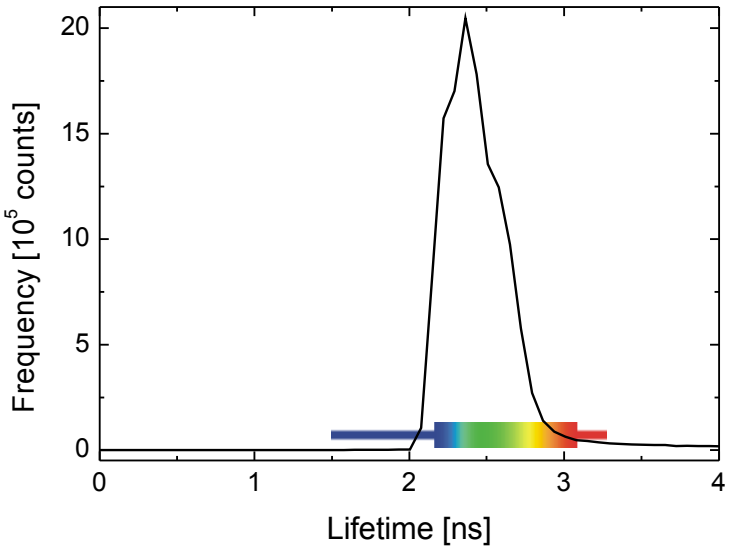
Fluorescence intensity



Fluorescence lifetime



Lifetime distribution

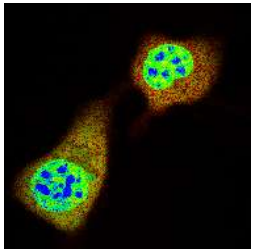


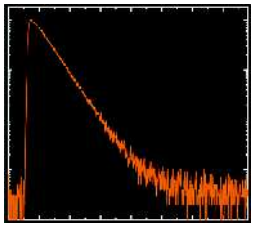
Sample courtesy of Ammasi Periasamy, University of Virginia, USA

Why Fluorescence Lifetime Imaging (FLIM)?

Fluorescence Lifetime Imaging (FLIM) gives you new parameters

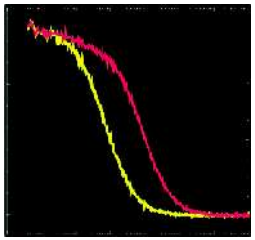
- Independent of system settings, fluorophore concentration
- Discrimination between fluorophores with similar excitation spectra (e.g. EGFP and EYFP) and from autofluorescence
- Measurements of environmental parameters
  - ➔ hydrophobicity
  - ➔ pH value
  - ➔ Oxygen, water or ion - concentrations





Förster Resonance Energy Transfer (FRET)

- Distance measurements in the nanometer range
- Can be measured down to the single molecule level
  - ➔ Intra- and intermolecular interaction studies
  - ➔ Protein folding
  - ➔ Moving of molecular motors



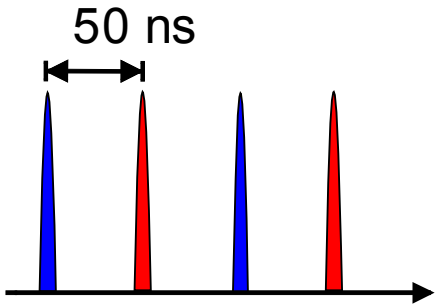
Fluorescence Correlation Spectroscopy (FCS)

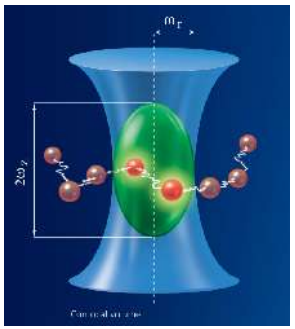
- Mobility, dynamics and concentration
  - ➔ Fluorescence Lifetime Correlation Spectroscopy (FLCS)
  - ➔ Time-gated FCS

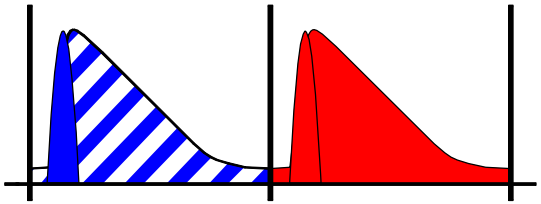
Advanced Excitation Schemes

Pulsed Interleaved Excitation (PIE)

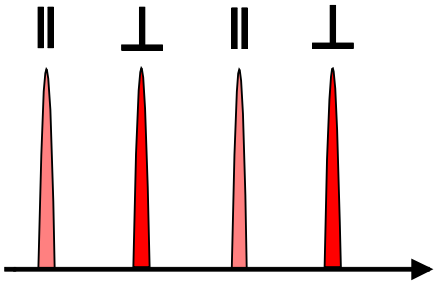
- coding spectral information in time

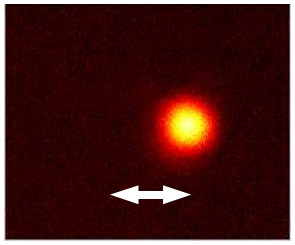






- coding spatial information in time





350
nm

absolute
diffusion coefficient

Laser heads with pulsed and cw Excitation

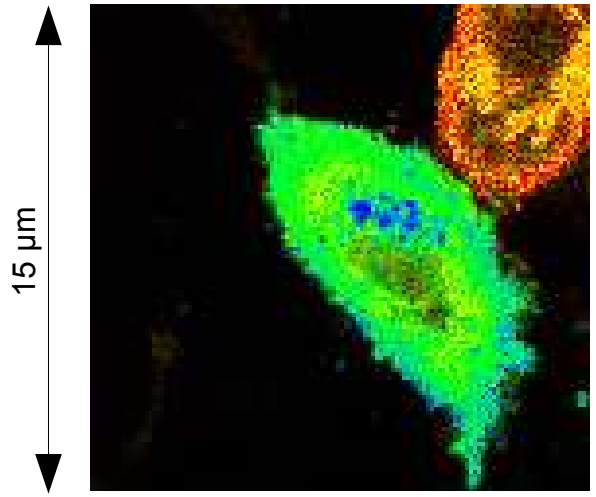
- Antibunching
- Total correlation from ps to seconds

FRET Analysis via FLIM

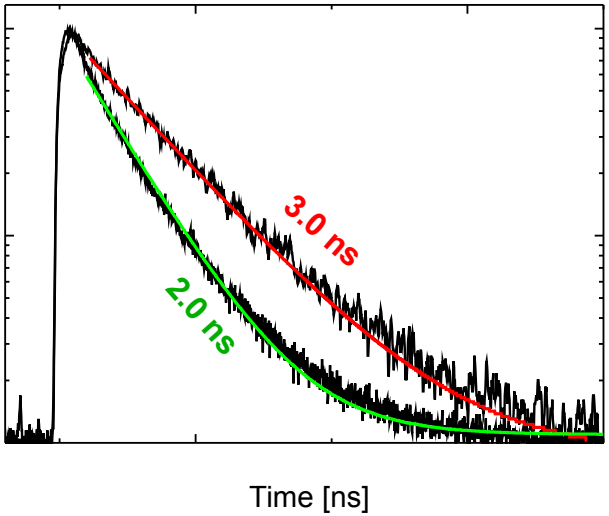
EGFP-RFP fusion construct expressed
in living cells (12V HC Red cells)

Olympus FV1000
excitation: λ_{exc} = 470 nm, 40 MHz
Apo 60x, 1.4 N.A. oil
filter: BP (500-540) nm
256 × 256 pixels

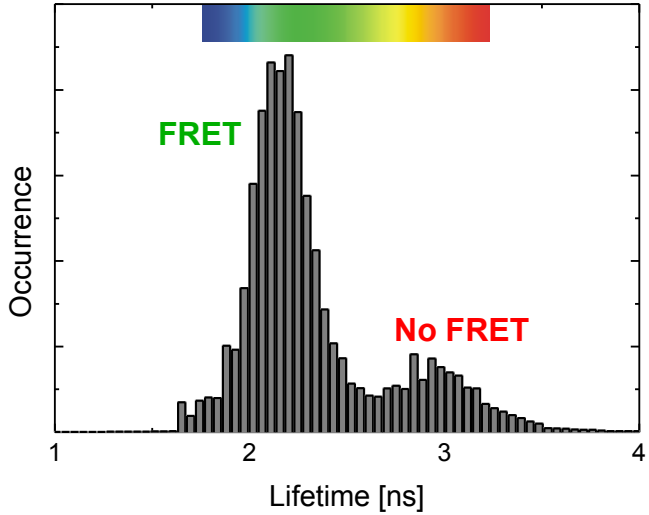
Fluorescence lifetime image (FLIM)



Fluorescence decays



Lifetime histogram



Sample courtesy of Philippe Bastiaens, Max Planck Institute for Molecular Physiology, Dortmund, Germany

Conclusion

LSM Upgrade kit / MicroTime 200 enable for:

- Time-Correlated Single Photon Counting with up to two/four detectors (PMT or SPAD) and five laser wavelengths simultaneously
- Spatial, spectral and timing information for every photon
  ➔ Universal data pre-selection photon by photon

- Fluorescence Lifetime Imaging (FLIM) with online visualization for increased information:
  ➔ Distance measurements, molecular interactions (FRET)
  ➔ Environmental parameters

- Fluorescence Correlation Spectroscopy (FCS) with online visualization for measurements of:
  ➔ Diffusion coefficients
  ➔ Concentration of molecules
  ➔ FLCS measurements
    - more realistic concentrations at high dilutions
    - afterpulsing removal