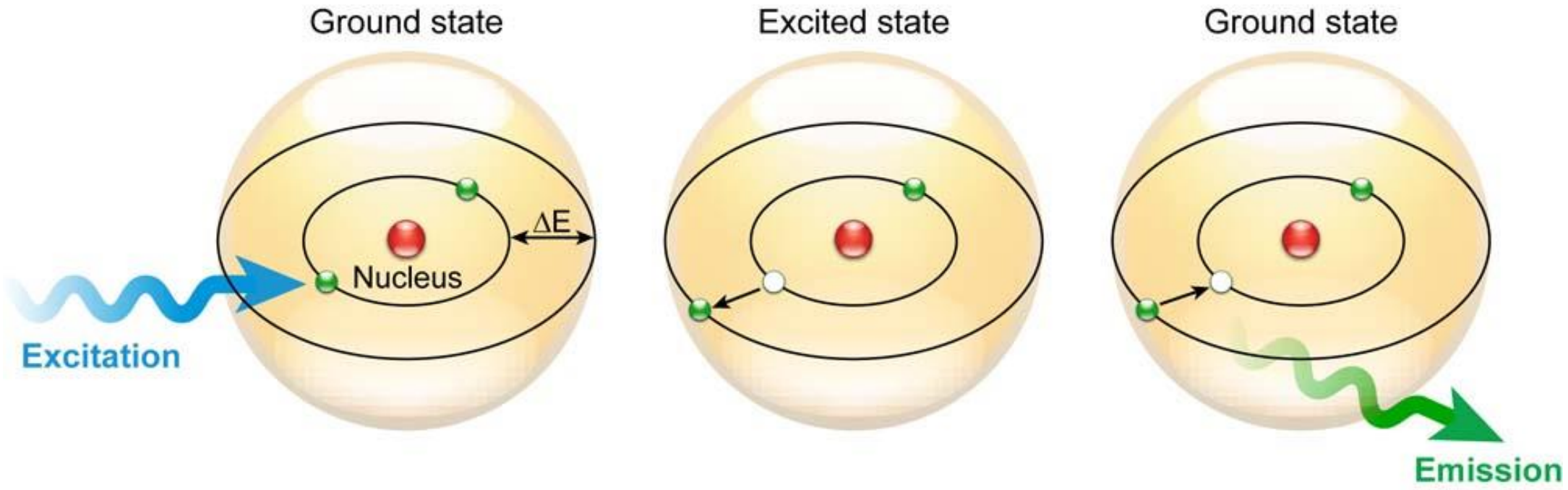
F* techniques: FRAP, FLIP, FRET, FLIM, FCS

Antonia Göhler

March 2015

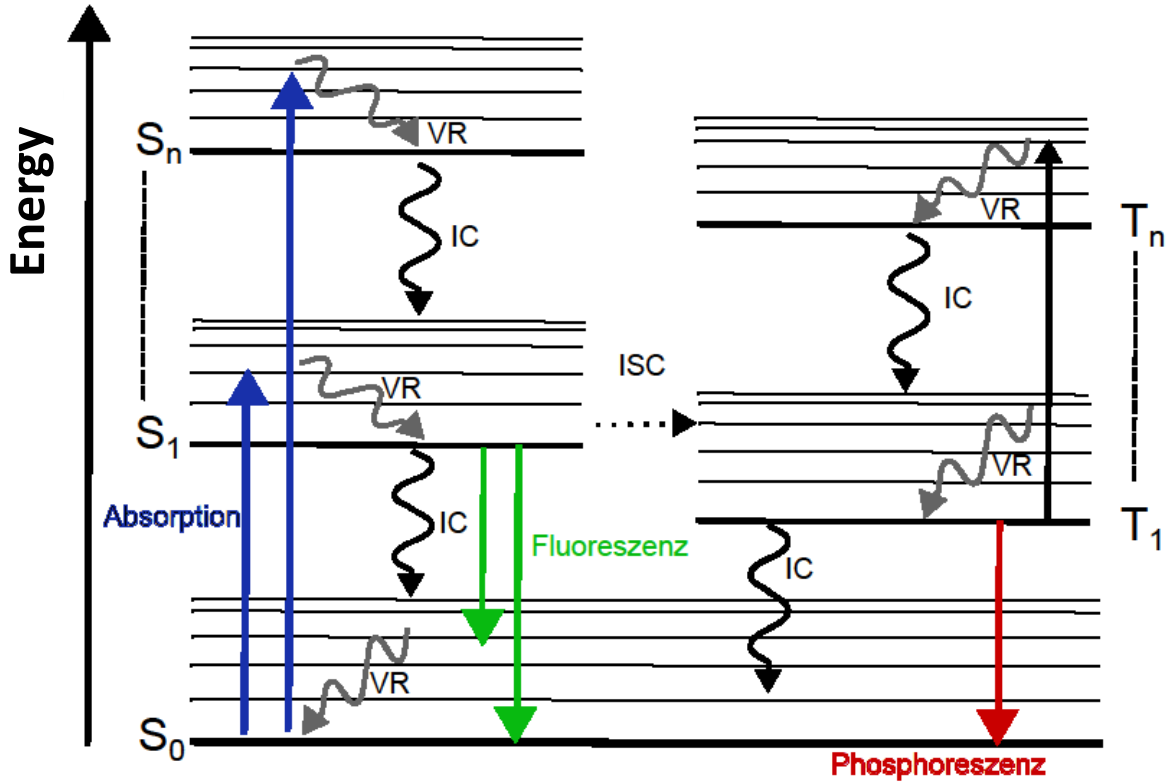
Fluorescence explained in the Bohr model

Absorption of light (blue) causes an electron to move to a higher energy orbit. After a particular time in the excited state (fluorescence lifetime) the electron returns to its ground state and the fluorophore dissipates the excess energy by emitting a photon (green).

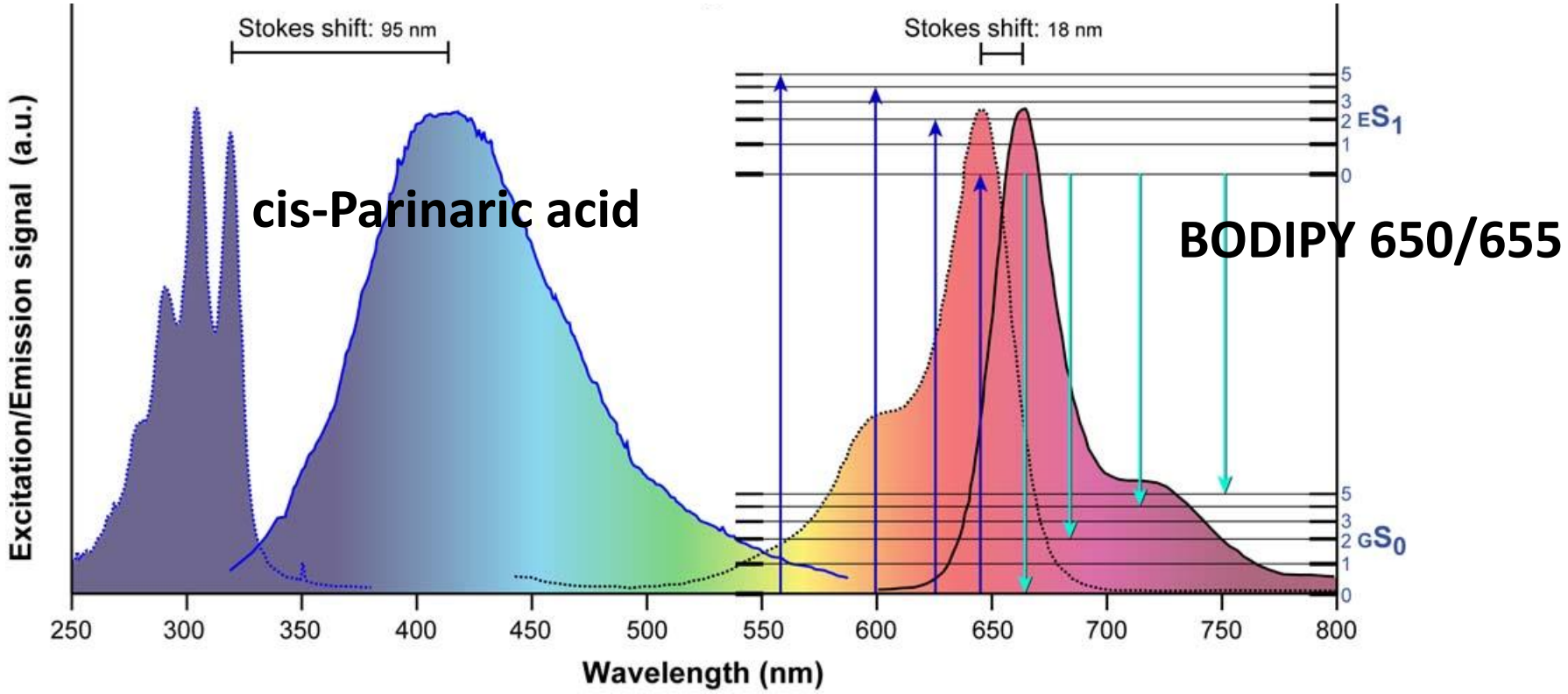


Jablonski-Diagram

- molecules have electronic, vibrational and rotational energy levels
- absorption of light = electron leaves ground state and moves to a higher excited state
- relaxes quickly to a lower vibrational excited state (grey line) and thereby looses energy (Stokes-Shift)
- return to the ground state: dissipation of the remaining energy by emitting a photon with a longer wavelength (fluorescence, phosphorescence)



Stokes-Shift and Mirror-Rule



- difference between excitation and emission maxima caused by a quick electron relaxation/ intramolecular vibrational energy loss (red-shifted)
- near-mirror image spectra: same electronic transitions are involved in both excitation and emission

Overview of Fluorescence Characteristics

- advanced fluorescence techniques can be used to measure or molecular interactions such as binding processes or conformational dynamics
- most important characteristics of fluorophores
    - Quantum yield Q = # emitted photons /# absorbed photons
    - Fluorescence lifetime τ = 1/relaxation rates k
    - Quenching: interactions between molecules lead to a reduction of the quantum yield or the fluorescence lifetime (dynamic, static, self-quenching)

- Q depends on solvent polarization, pH, fluorophore concentration, oxygen
- fluorescence lifetime measurements are not influenced by internal settings of the instrument like laser intensity or detector gain

Photobleaching

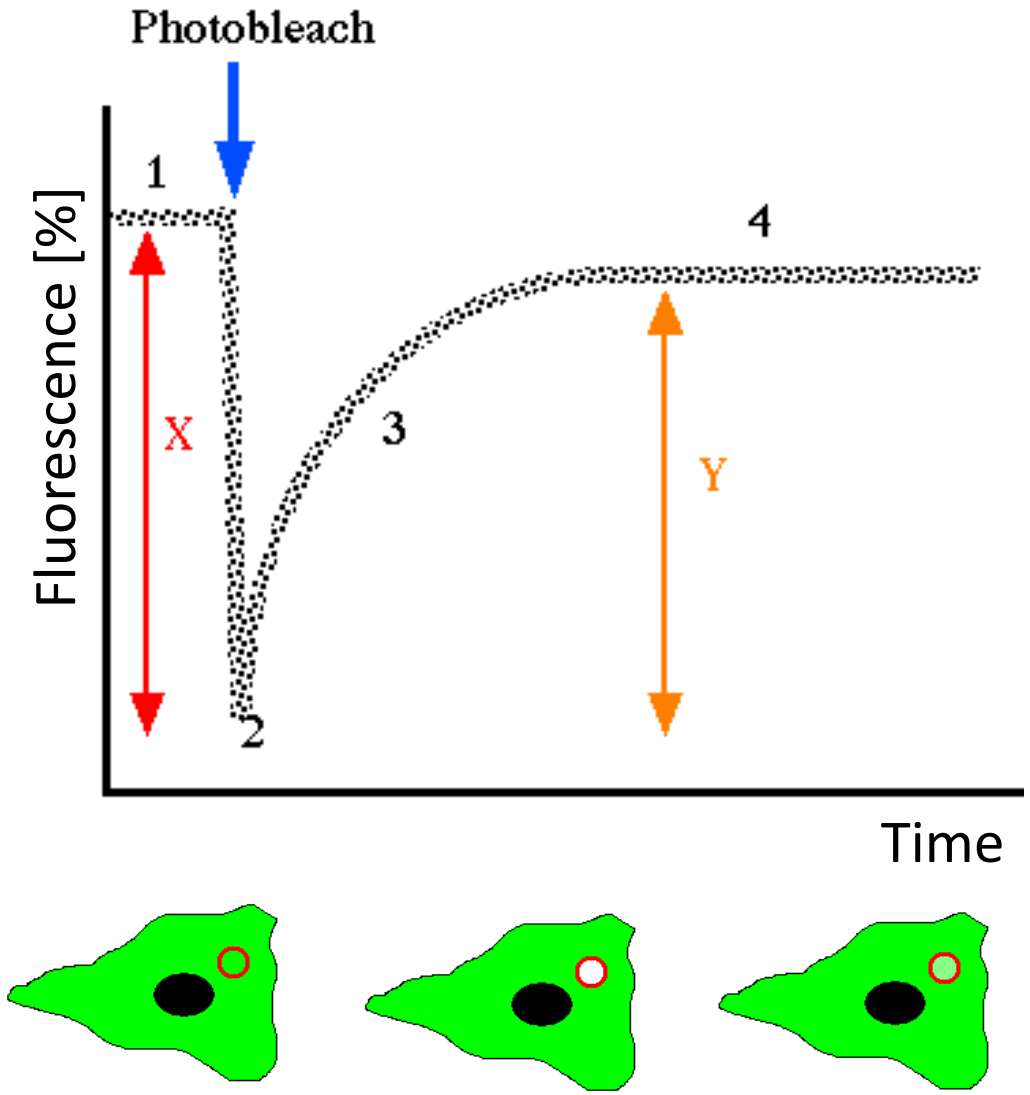
- photochemical process
- the fluorophore’s ability to enter repetitive excitation/emission cycles is permanently interrupted by destruction or irreversible covalent modification of the fluorophore

- usually an unwanted effect, microscopy images need correction for photobleaching

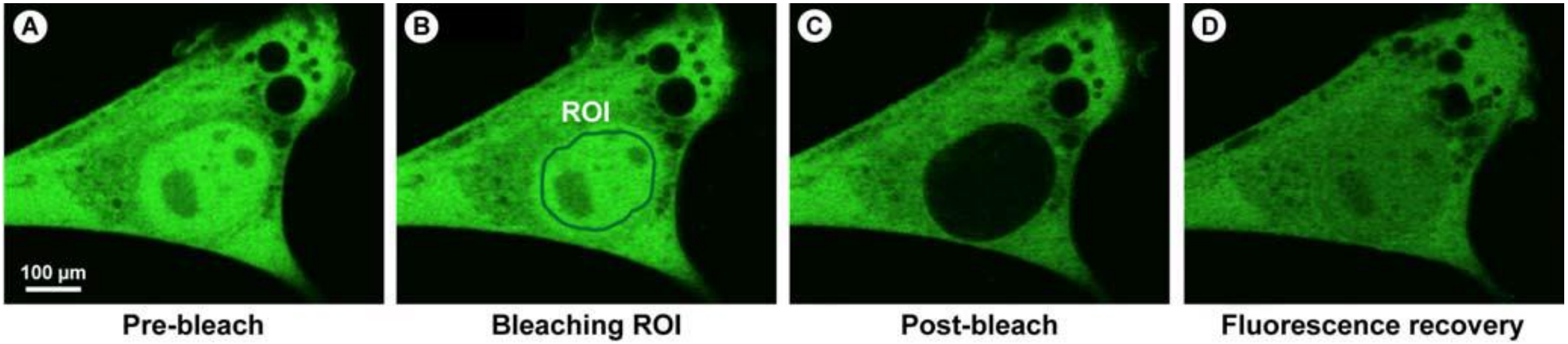
- Photobleaching-based techniques for assessing cellular dynamics: FRAP, FLIP, FLAP
- LSMs are good for photobleaching techniques due to their high intensity laser light sources and their flexible illumination control. They allow selective bleaching of arbitrary regions within the filed of view and can then rapidly switch between bleaching and imaging.

FRAP – Fluorescence Recovery after Photobleaching

- optical technique to quantify 2D lateral diffusion of fluorophores, diffusion rates, protein dynamics and interactions with other cellular components
- recovery occurs by replacement of fluorophores in the bleached region by lateral transport from the surrounding surface
- different recovery profiles classifying the proteins' mobility (highly, intermediate with an immobile fraction, or immobile)



FRAP: monomeric GFP can pass the nuclear membrane



(A) Myoblast cell line is homogenously expressing GFP-Myosin III

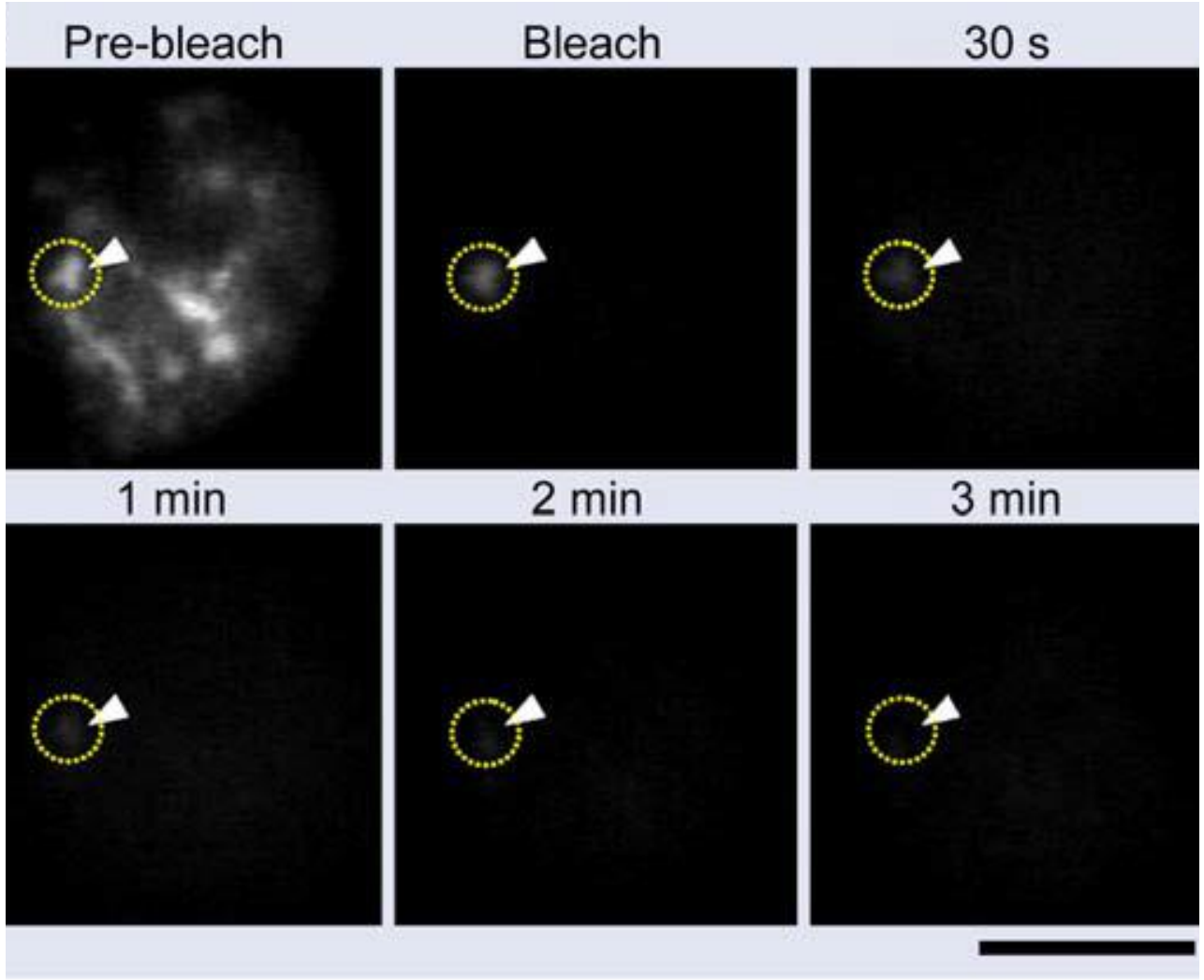
(B) Region of interest (ROI) is bleached

(C) Dark area: fluorophores were permanently damaged (no emission)

(D) Fluorescence in the photobleached region recovers

Inverse FRAP (iFRAP)

- initially developed to study the mobility of molecules in small areas of the nucleus and their exchange with the surrounding nucleoplasm
- the entire population of fluorophores in the cell is bleached, except the accumulated fluorophores in a small part of the organelle
- subsequently, the loss in fluorescence in the accumulation is recorded over time

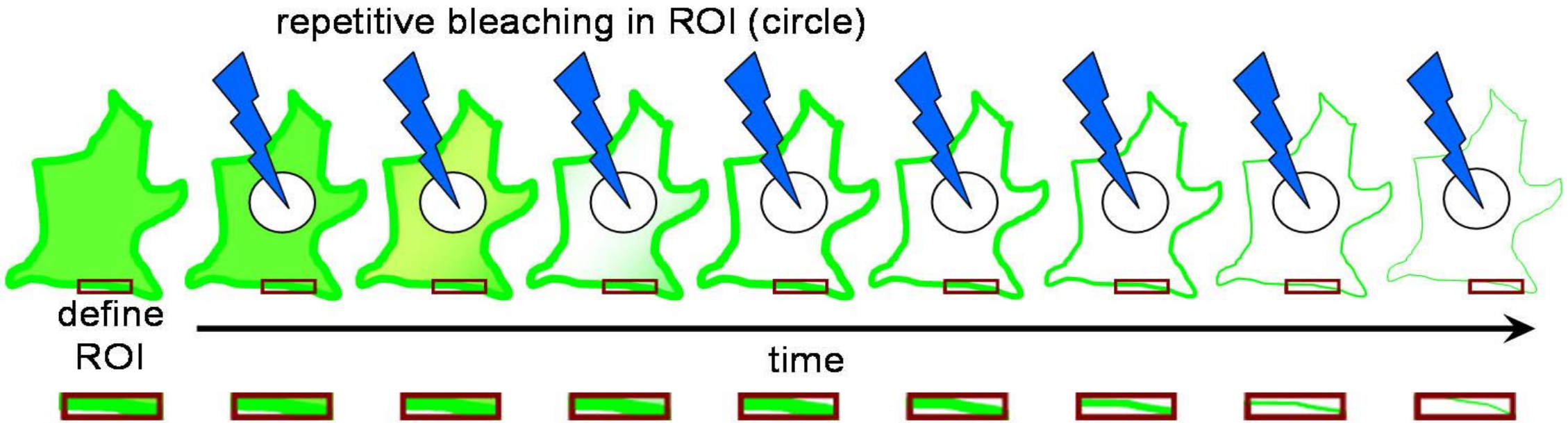


Potentiell Complications

- data modelling and processing, Operator errors
- living cells move during experiment (compensation via alignment algorithm)
- correction for overall loss of fluorescence
- 3D samples: bleached structure is far more complex than visible
- blinking or reversible photobleaching may cause flawed FRAP results
- photo-induced cross-linking may occur (free radical induced cross-linking reactions) which may lead to different bleaching intensities

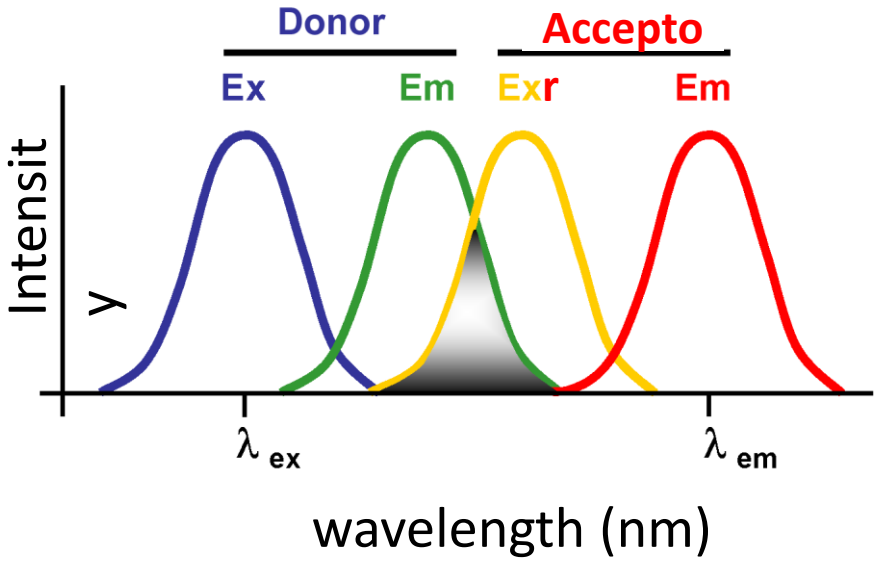
FLIP – Fluorescence Loss in Photobleaching

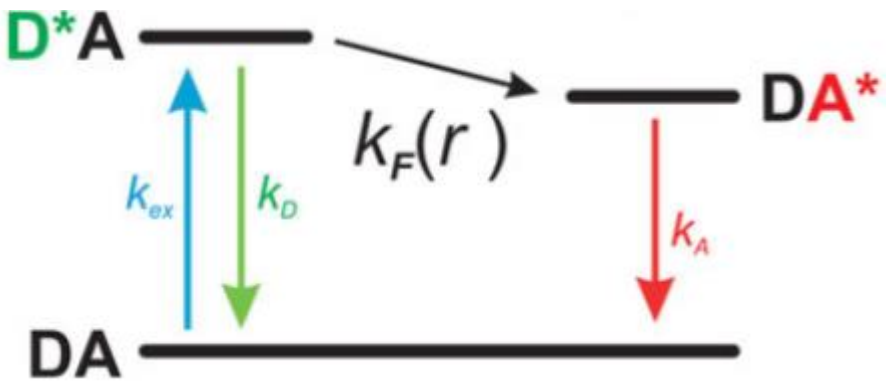
- related to FRAP, a small region is repeatedly bleached and the loss of fluorescence in another region is measured.
- useful to show connectivity of compartments or for measuring turnover of molecules between different compartments



FRET – Förster Resonance Energy Transfer

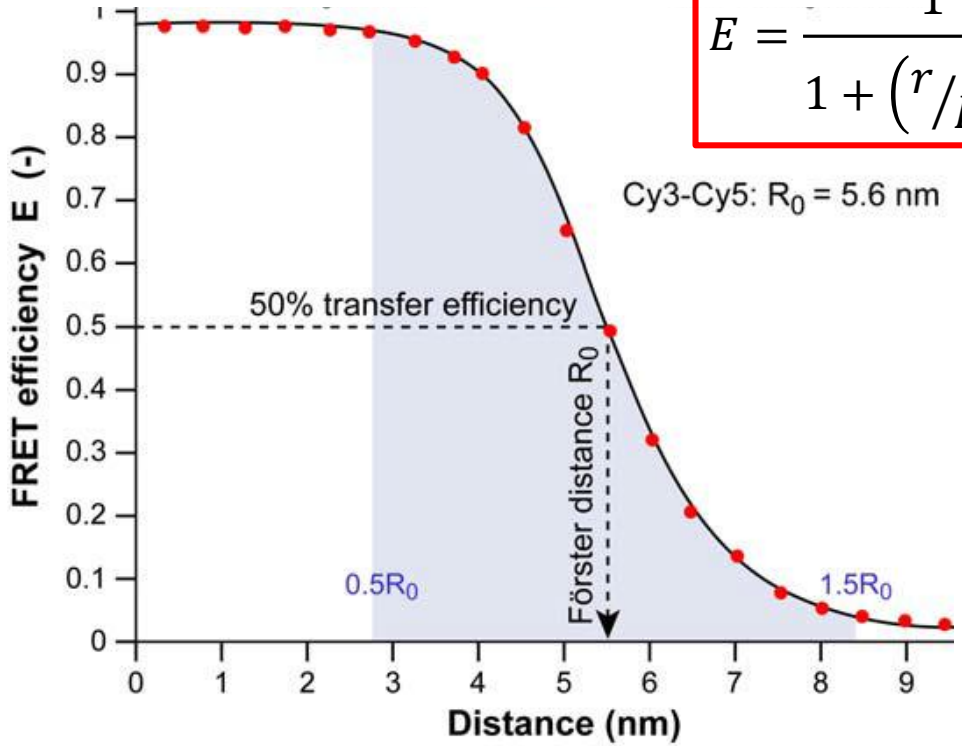
- energy is transferred non-radiatively (via long-range dipole-dipole coupling)
- relies on the close physical interaction of the two molecules (0.5 – 10nm)
- can be used to determine molecular interaction/molecular proximity beyond the resolution limits of the classical light microscope

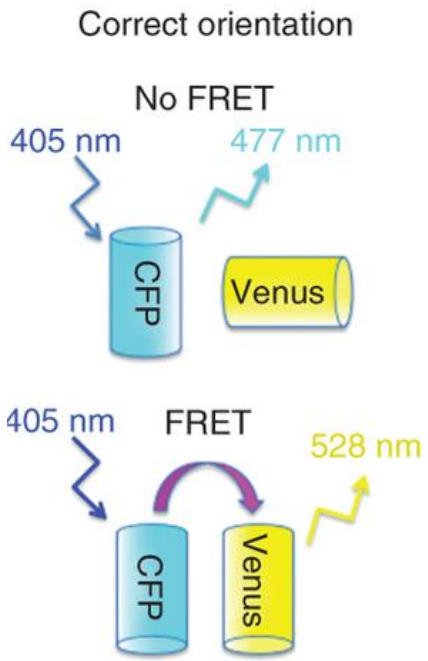


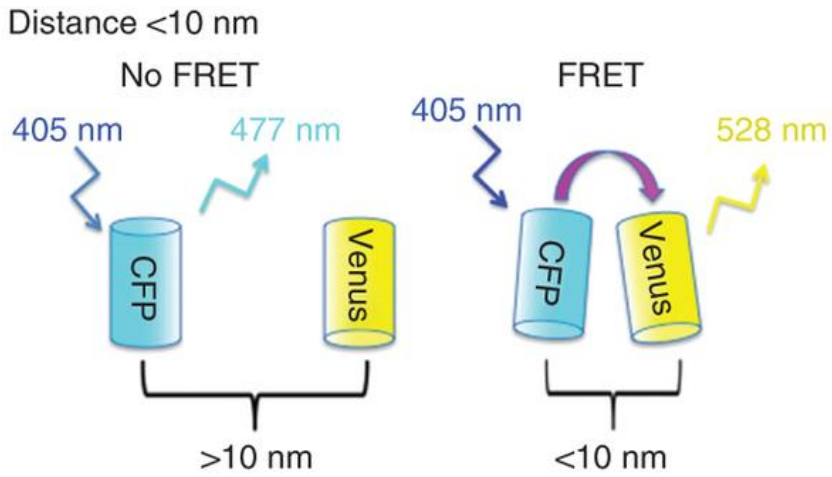


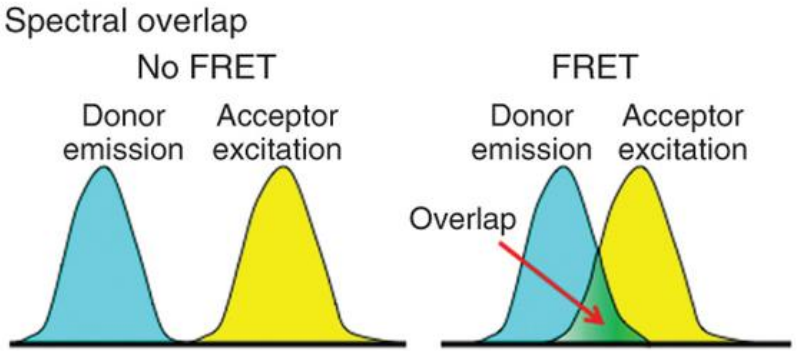
- distance between two molecules
- spectral overlap
- the quantum yield of the donor
- the relative orientation of the transition dipoles

$$E = \frac{1}{1 + \left({}^{r}/_{R_0}\right)^6}$$

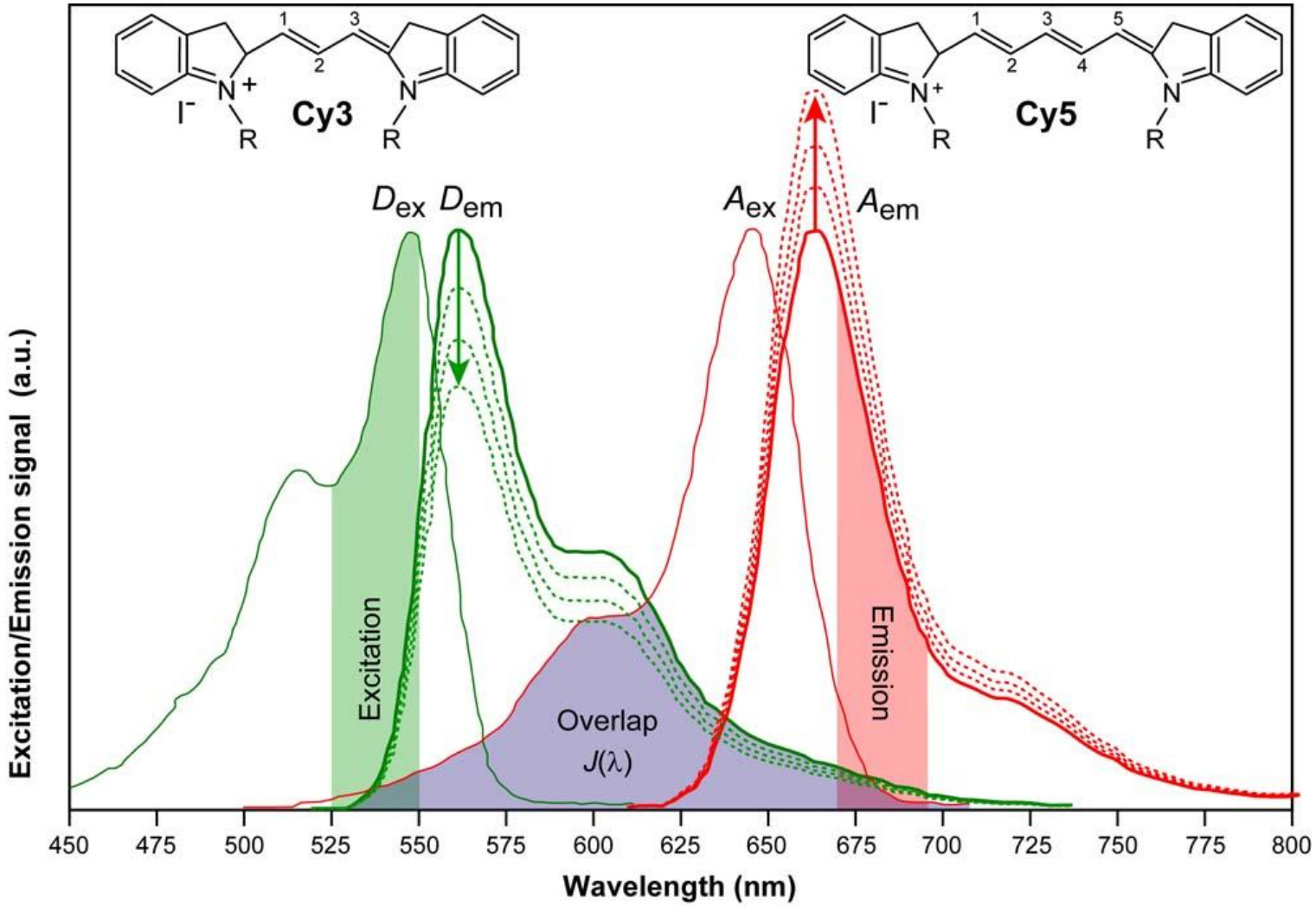








NatProt, 8, 265-281, 2013

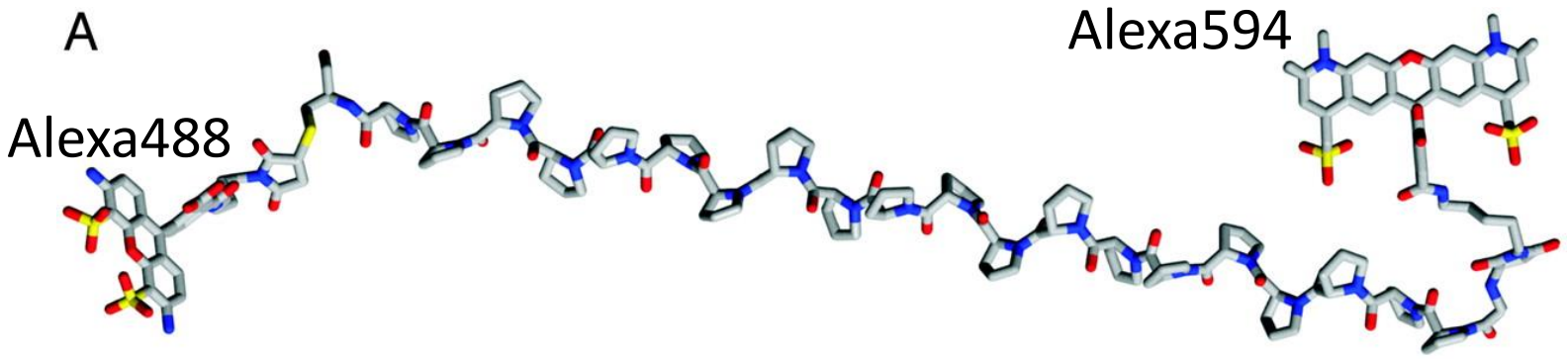


Overlap integral of the Cy3 emission spectrum and the excitation spectrum of Cy5. As a result of FRET, the donor emission (D_{em}) is reduced while the acceptor emission (A_{em}) increases.

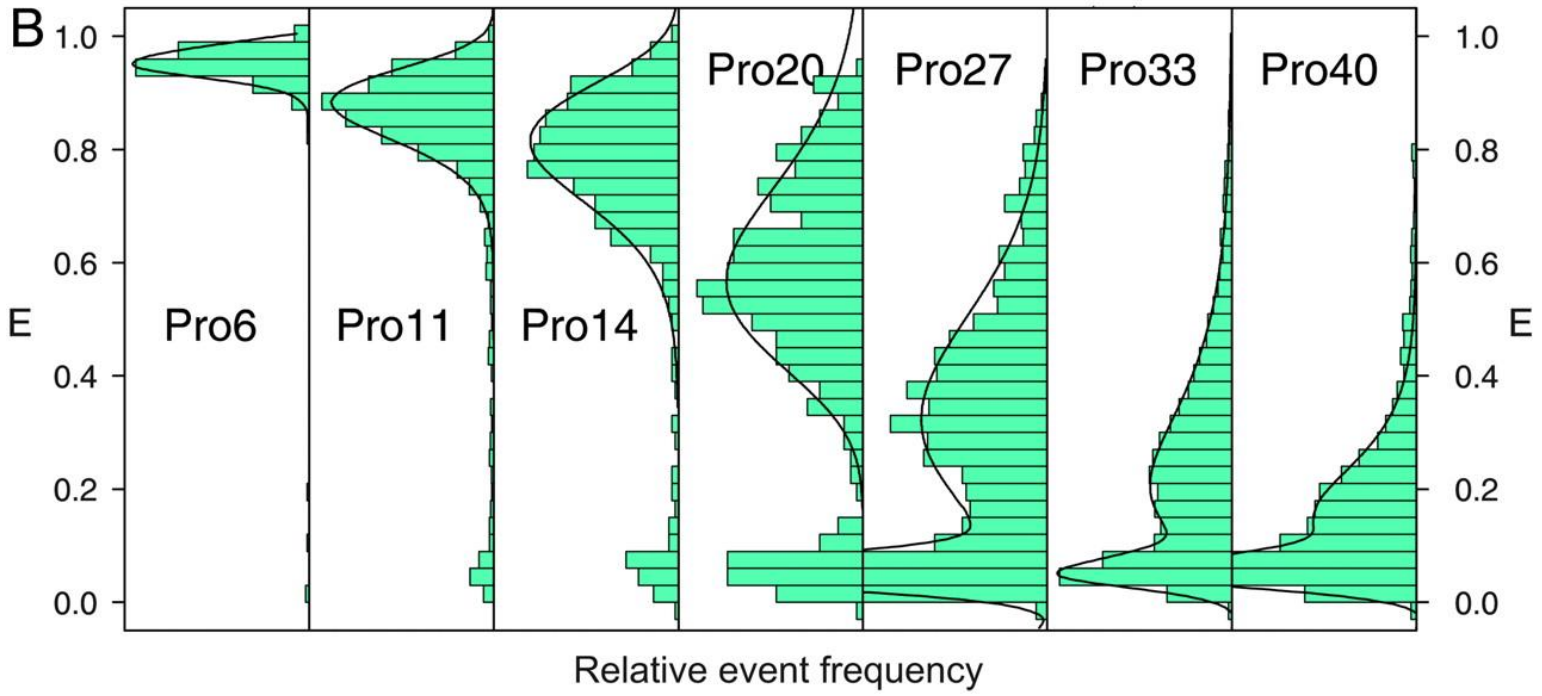
FRET-Couples

Donor	Acceptor	Donor Excitation λ_{max} (nm)	Acceptor Emission λ_{max} (nm)	Donor QY (–)	Acceptor ε ($M^{-1}cm^{-1}$)	Förster Distance (nm)
Fluorescent protein FRET pairs						
BFP	DsRFP	380	586	0.18	72,500	3.1–3.3
EBFP2	mEGFP	383	507	0.56	57,500	4.8
CFP	GFP	433	509	0.40	21,000	4.7–4.9
CFP	YFP	433	526	0.40	77,000	~ 5.0
Cerulean	YFP	440	526	0.62	77,000	–
ECFP	EYFP	440	527	0.40	83,400	4.9
Cerulean	Venus	440	528	0.62	92,200	5.4
MiCy	mKO	472	559	0.90	51,600	5.3
GFP	YFP	475	526	0.77	77,000	5.5–5.7
GFP	mRFP	475	579	0.77	50,000	~ 4.7
CyPet	YPet	477	530	0.51	104,000	5.1
TFP1	mVenus	492	528	0.85	92,200	5.1
EGFP	mCherry	507	510	0.60	72,000	5.1
Venus	mCherry	528	610	0.57	72,000	5.7
Venus	tdTomato	528	581	0.57	138,000	5.9
Venus	mPlum	528	649	0.57	41,000	5.2
Fluorescent protein-dye FRET pairs						
EGFP	Alexa Fluor 555	484	568	0.6	155,000	6.3
EGFP	Alexa Fluor 546	484	573	0.6	112,000	5.7
EGFP	Alexa Fluor 594	484	618	0.6	92,000	5.3
EGFP	Alexa Fluor 568	484	603	0.6	88,000	5.4
Dye and dye-biofluorochrome FRET pairs						
Cy2	Cy3	489	570	> 0.12	150,000	5.0–6.0
Cy3	Cy5	550	670	> 0.15	250,000	>5.0
Cy5	Cy5.5	649	694	> 0.28	250,000	>8.0
Fluorescein	Tetramethylrhodamine	487 [1]	574	0.93 [2]	~ 87,000	4.9–5.5
FITC	TRITC	494	572	0.92	100,000	5.4
Phycoerythrin	APC [3]	(546), 565	660	0.98	700,000	4.0–11.0
Europium	APC	340	660	–	700,000	9.0
Tryptophan	Dansyl	280	525	0.01–0.35 [4]	4050	2.1
Dansyl	FITC	335	519	< 0.035 [5]	77,000	3.3–4.1
Dansyl	Octadecylrhodamine	335	625	< 0.035	106,000	4.3
Europium	Cy5	340	670	–	250,000	7.0
Atto 488	Atto 647N	501	670	0.8	150,000	5.1
Atto 488	Atto 590	501	621	0.8	120,000	6.0
Atto 550	Atto 647N	554	670	0.8	150,000	6.5
Atto 550	Atto 655	554	684	0.8	125,000	6.4
Atto 590	Atto 655	594	684	0.8	125,000	7.3
Alexa 405	Alexa 430	401	541	–	16,000	–
Alexa 488	Alexa 514	495	542	0.92	80,000	–
Alexa 488	Alexa 532	495	554	0.92	81,000	–
Alexa 488	Alexa 546	495	573	0.92	104,000	6.4
Alexa 488	Alexa 610	495	628	0.92	138,000	–
Alexa 647	Alexa 680	650	702	0.33	184,000	–
Alexa 647	Alexa 700	650	723	0.33	192,000	–
Alexa 647	Alexa 750	650	780	0.33	240,000	–
Non-fluorescent acceptor pairs						
Rhodamine 6G	Malachite Green (Abs 628 nm)	526	NF	0.95	76,000	6.1
Alexa 488	QSY 35 (Abs 475 nm)	495	NF	0.92	23,000	4.4
Alexa 488	Dabcyl (Abs 453 nm)	495	NF	0.92	32,000	4.9
Alexa 647	QSY 21 (Abs 661 nm)	650	NF	0.33	90,000	6.9

FRET – traditionally used as Molecular Ruler



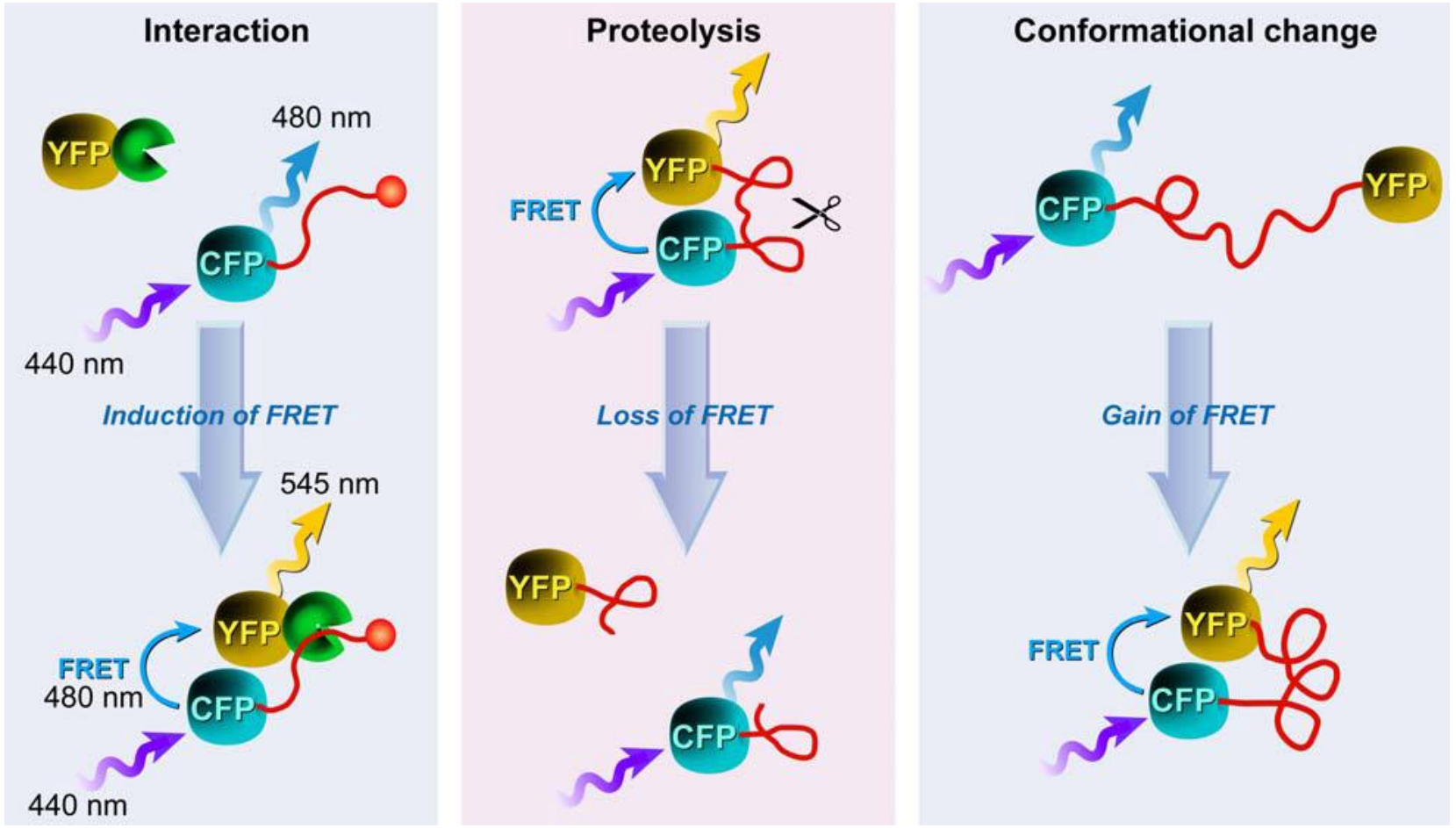
molecular model:
polyproline peptide



PNAS,102,2754-2759, 2005

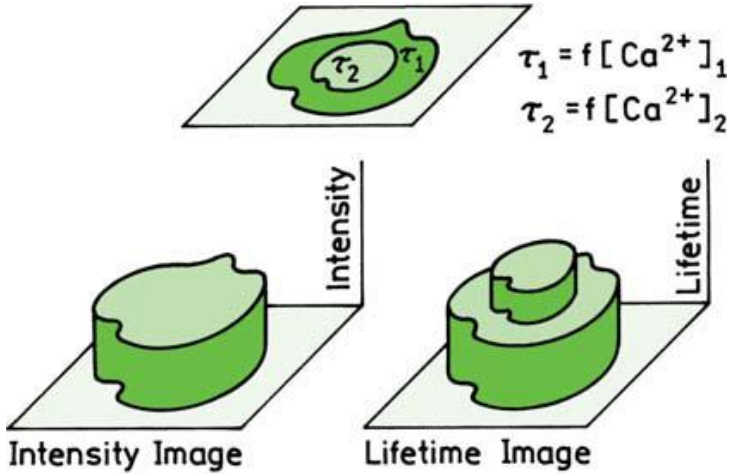
Fundamental cellular FRET-Approaches

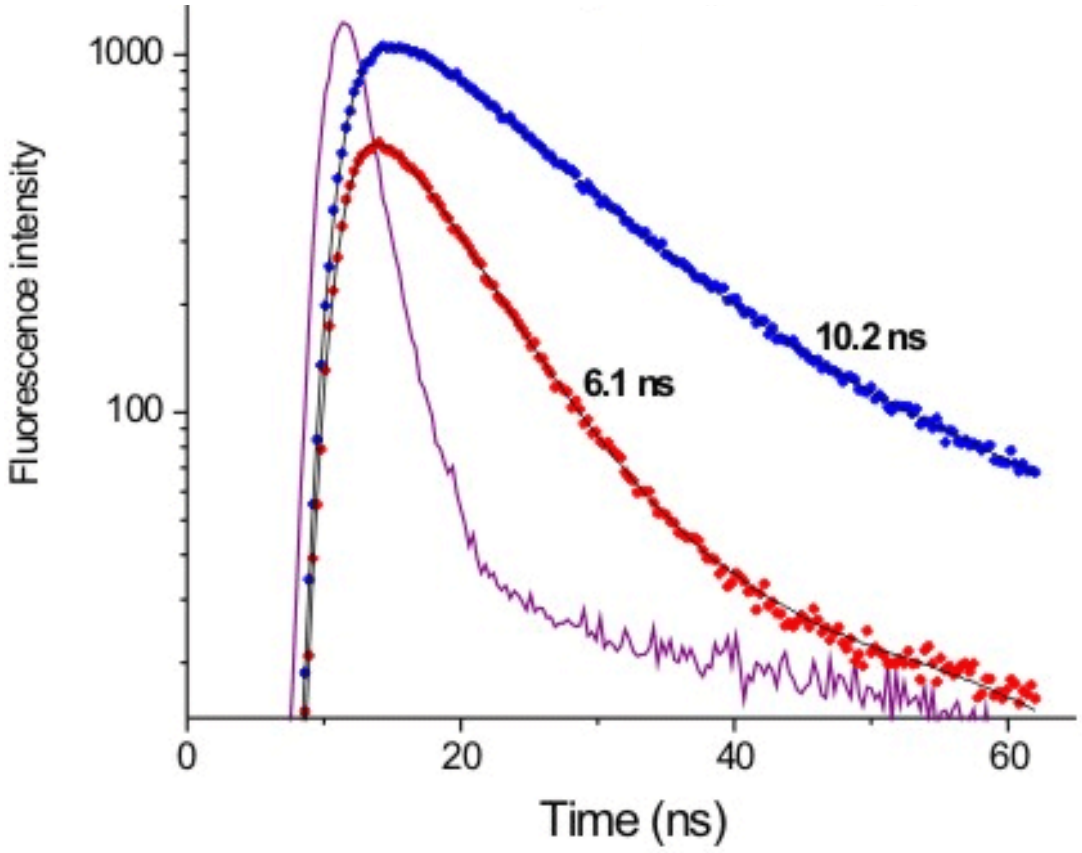
- FRET-biosensors = fusion proteins of ECFP/EYFP or other appropriate pairs EGFP/mRFP linked by a sensory domain
- monitoring of donor/ acceptor channels and detection of changes in the FRET signal as a result of biological activity



FLIM – Fluorescence Lifetime Imaging

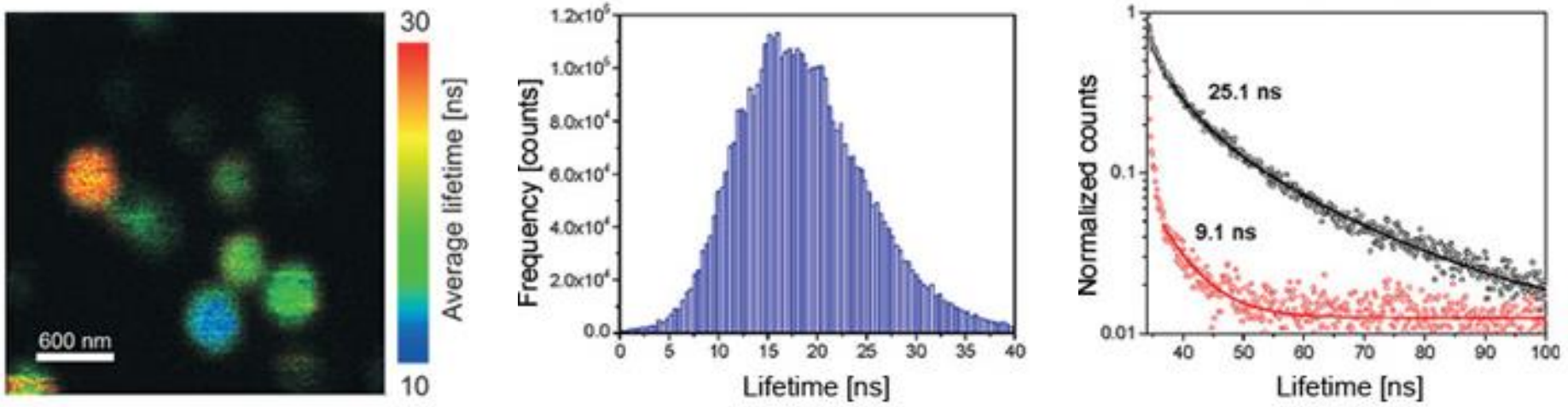
- **…**is an imaging technique for producing an image based on the differences in the exponential decay rate of fluorophores.
- the lifetime of the fluorophore signal, rather than its intensity, is used to create the image in FLIM. This has the advantage of minimizing the effect of photon scattering in thick layers of sample.





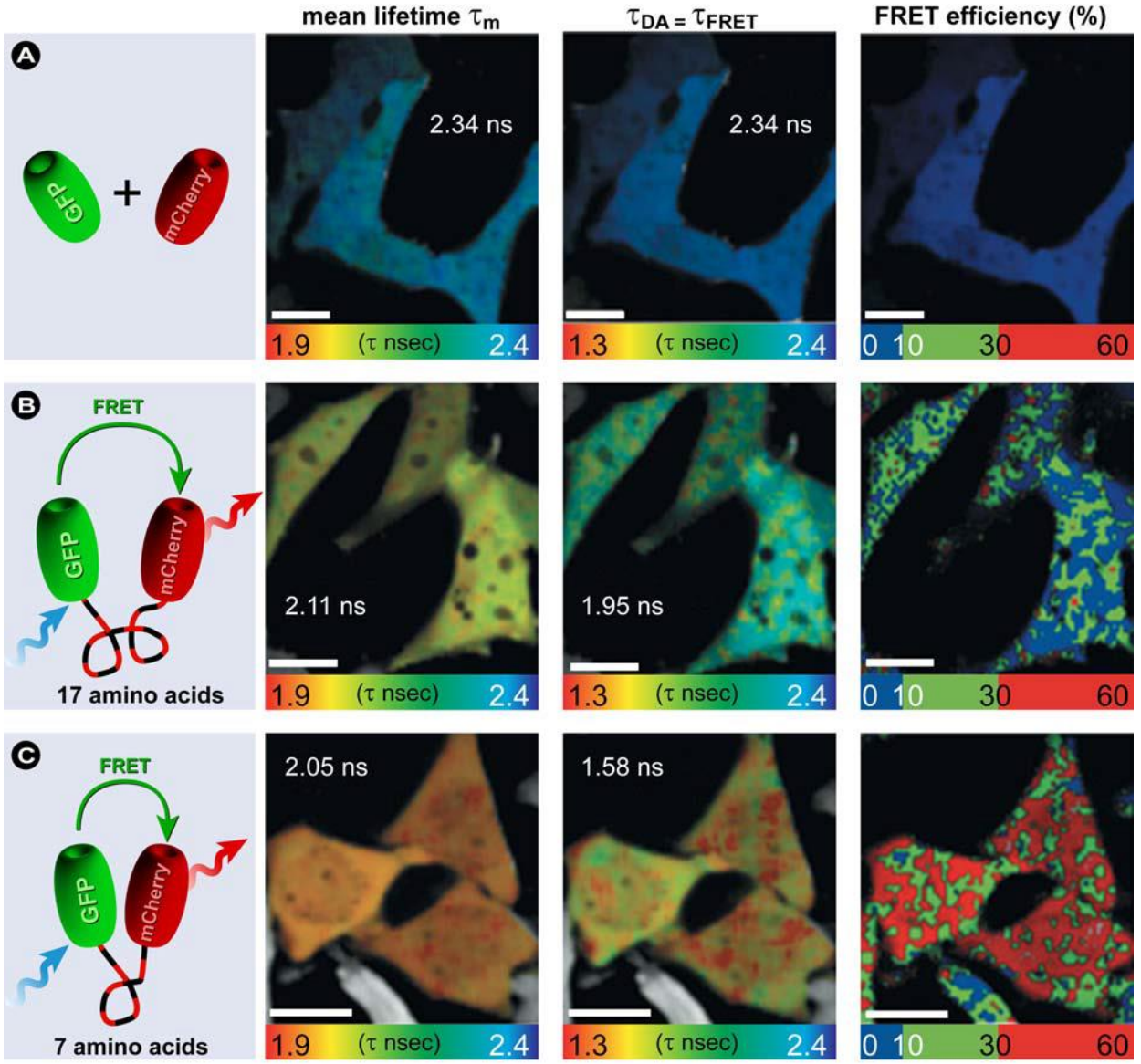
FLIM Measurements

- essential components of a FLIM set-up: pulsed laser source, detector (APD or PMT), dichroic mirror (to separate fluorescence and excitation), objective (to focus the excitation light into the sample and collect fluorescence signal) and a TCSPC unit to measure the time between excitation and fluorescence emission
- the delay times are sorted into a histogram that plots the occurrence of emission over time after the excitation pulse
- line and frame marker signals from the scanner (confocal microscope) are additionally recorded in order to sort the time stream of photons into the different pixels.



Combined FRET - FLIM

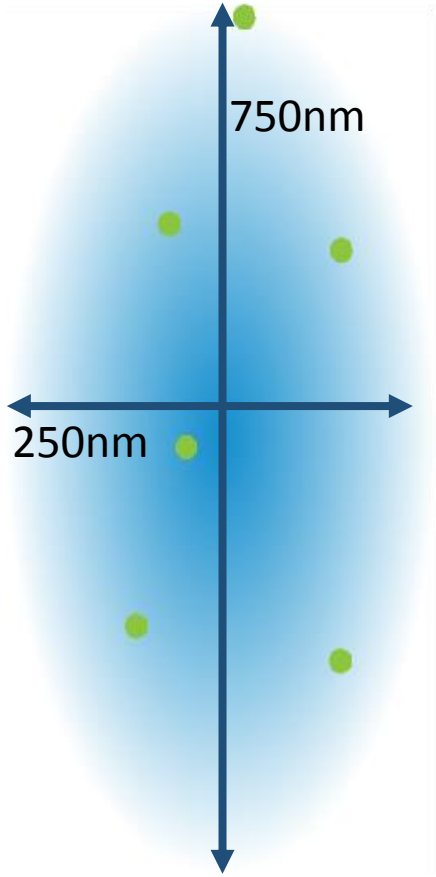
- allows the measurement of lifetime dynamics pixel-by-pixel
- mapping of spatial distributions to indirectly measure biomolecule concentrations, interactions between biomolecules, and conformational changes with a much higher accuracy than conventional FRET methods
- FLIM-FRET + TPE = advantage of less scattering, increased spatial resolution and depth-sectioning
- The shorter the linker the larger the change in mean flurescence lifetime/FRET-efficiency



FCS – Fluorescence Correlation Spectroscopy

- FCS is used to get time-resolved information about an ensemble with single molecule sensitivity in a small confocal volume

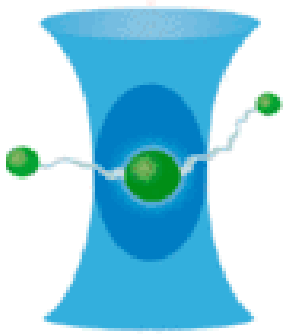
- Widely used technique to obtain quantitative information such as
  - diffusion coefficients
  - hydrodynamic radii
  - average concentrations
  - kinetic chemical reaction rates
  - singlet-triplet dynamics

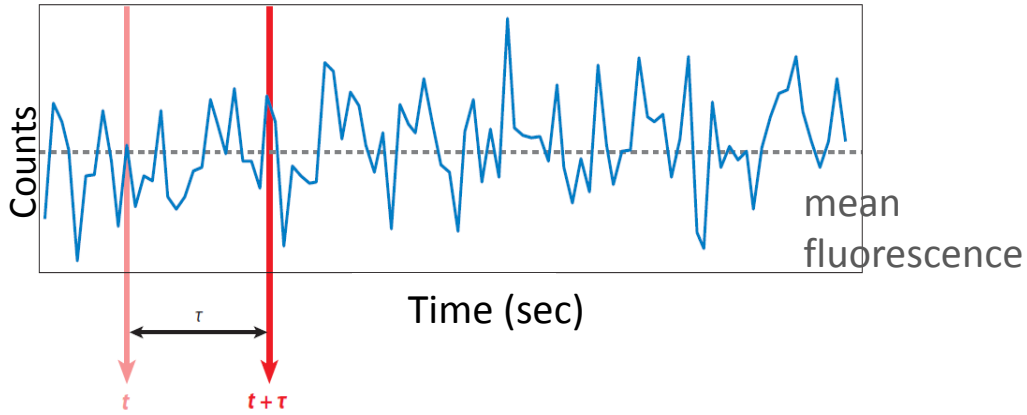


Volume ≈ 10^{-15}l

FCS – Autocorrelation of Fluorecensce Fluctuations

confocal Volume

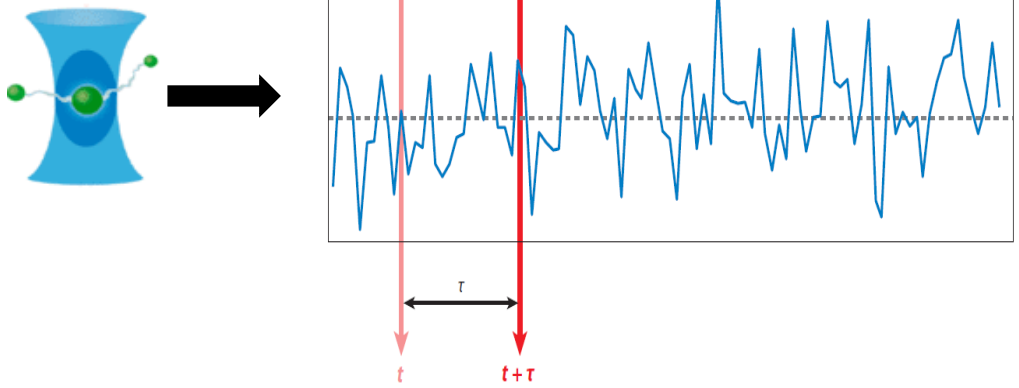


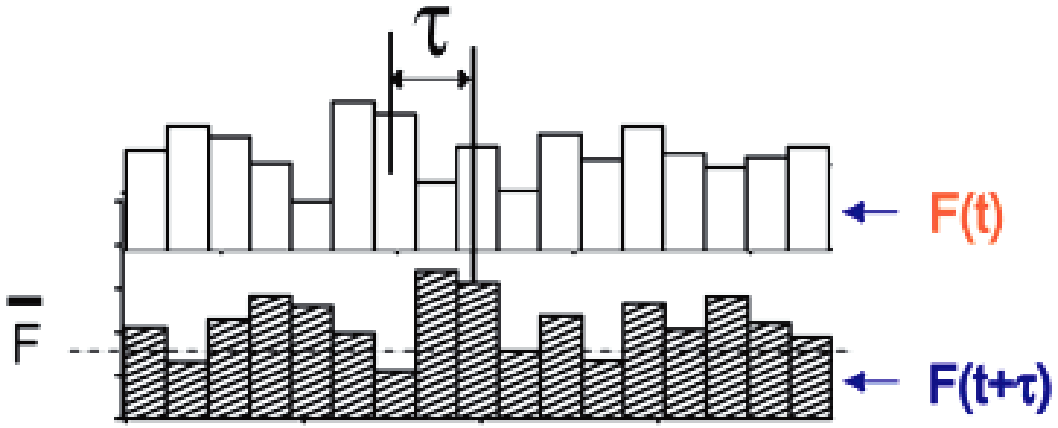


What causes fluctuations in intensity? a) Poison noise, b) diffusion of molecules

Bursts of photons are seen as single fluorophores diffuse in and out of the laser beam

FCS – Autocorrelation of Fluorecensce Fluctuations





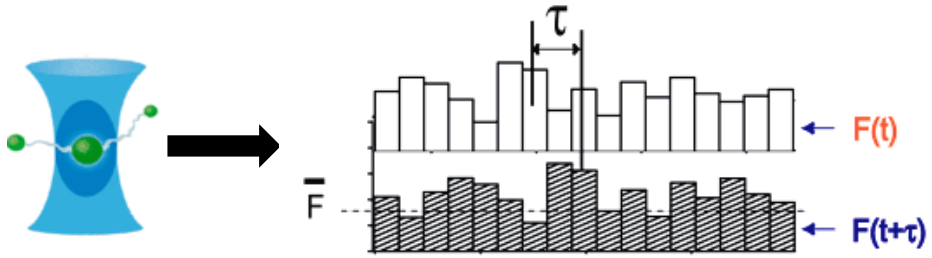
autocorrelation of fluorescence alterations

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle \delta F(t) \rangle^2}$$

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

Autocorrelation describes the probability of finding the molecule still in the confocal volume at later times T

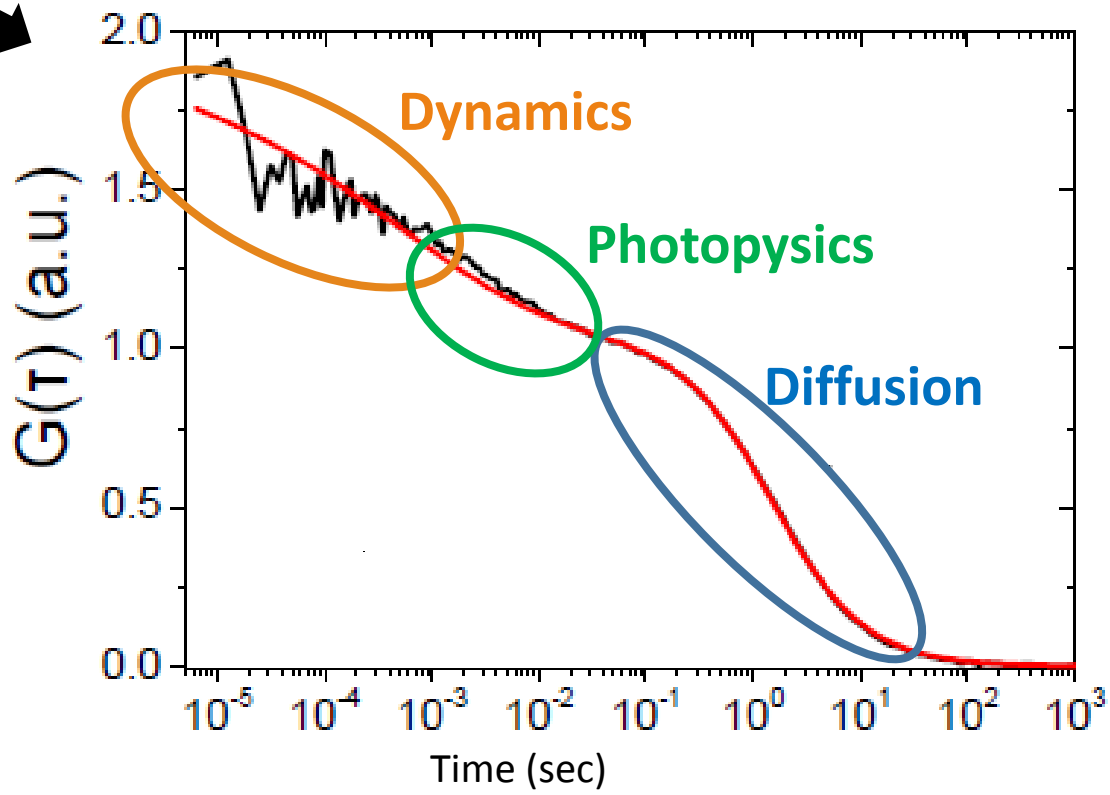
FCS – Autocorrelation of Fluorecensce Fluctuations



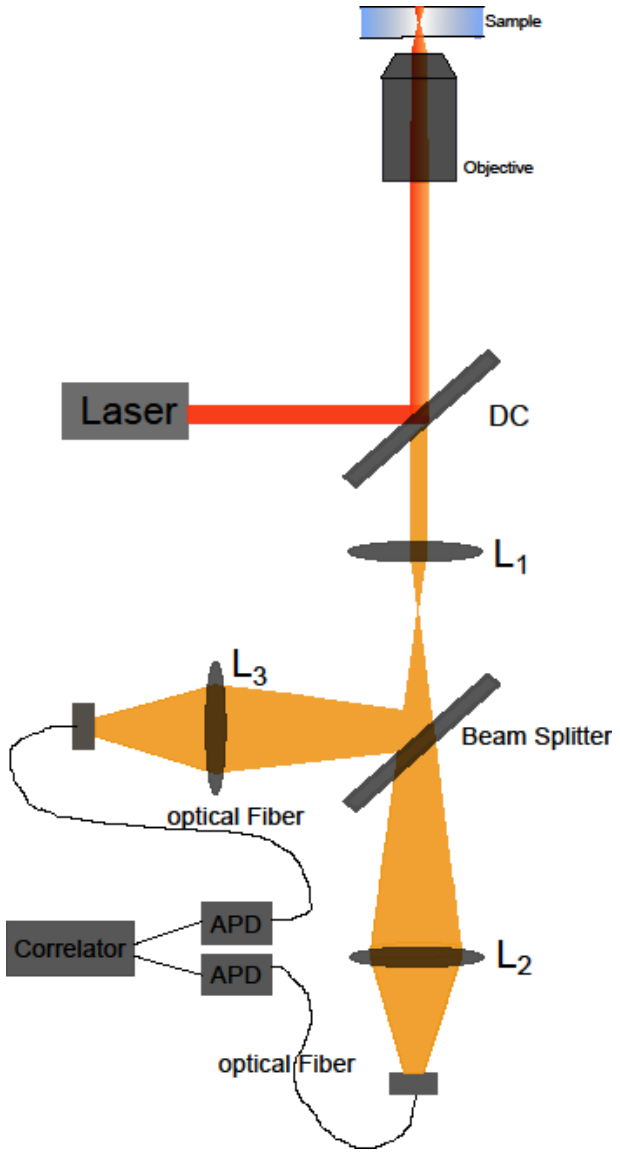
$$G(\tau) = \frac{1}{N}(1+\frac{\tau}{\tau_D})^{-1}(1+A\exp(-k\tau)^{\beta})$$

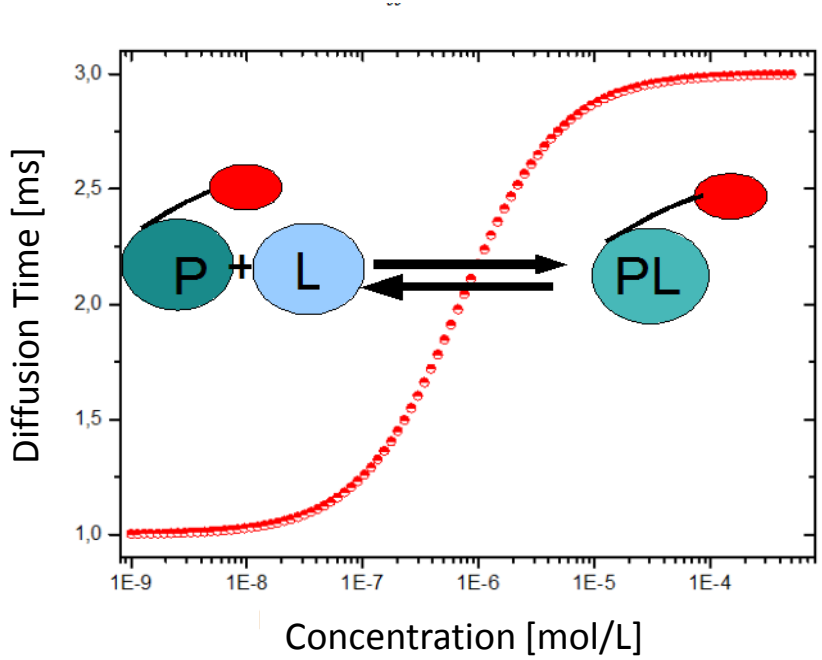
2D Gaussian Model

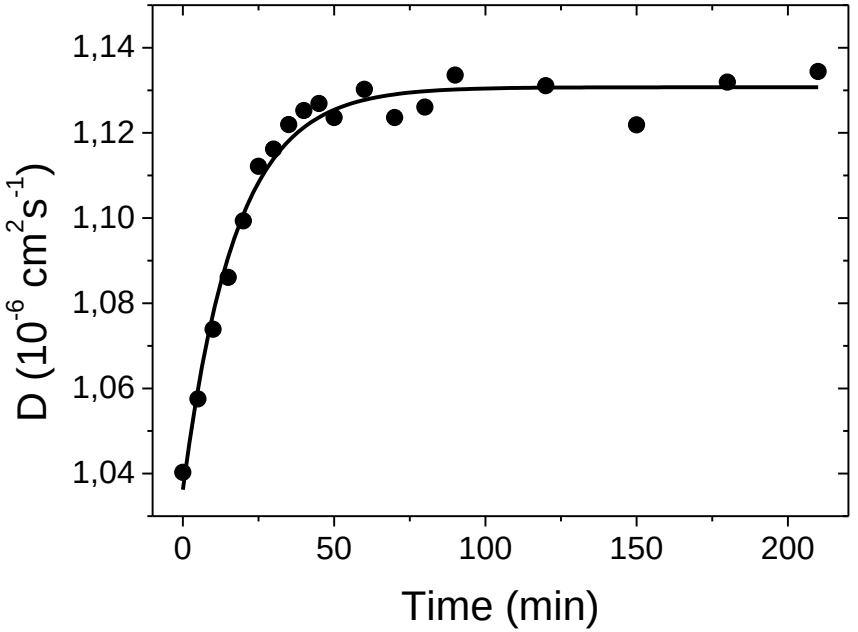
- What does FCS tell us?
  - diffusion rates - $D \propto m^{1/3}$.
  - the number of fluorescent objects in volume and hence concentration.
  - Relaxation rates and folding times



FCS – Binding Kinetics







FCS – Conformational Dynamics

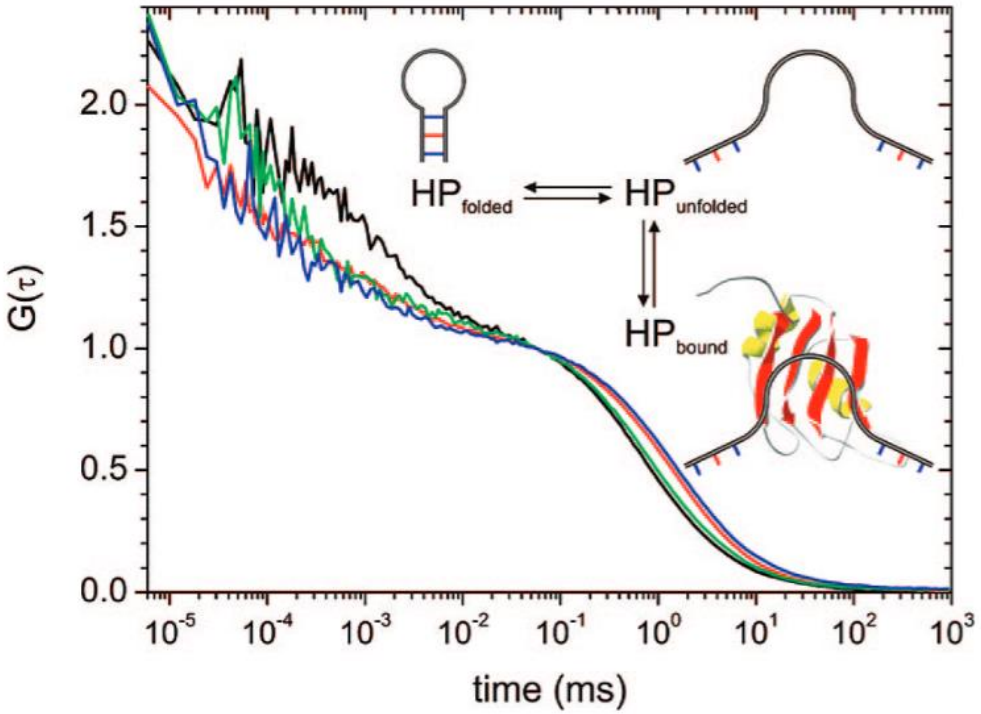
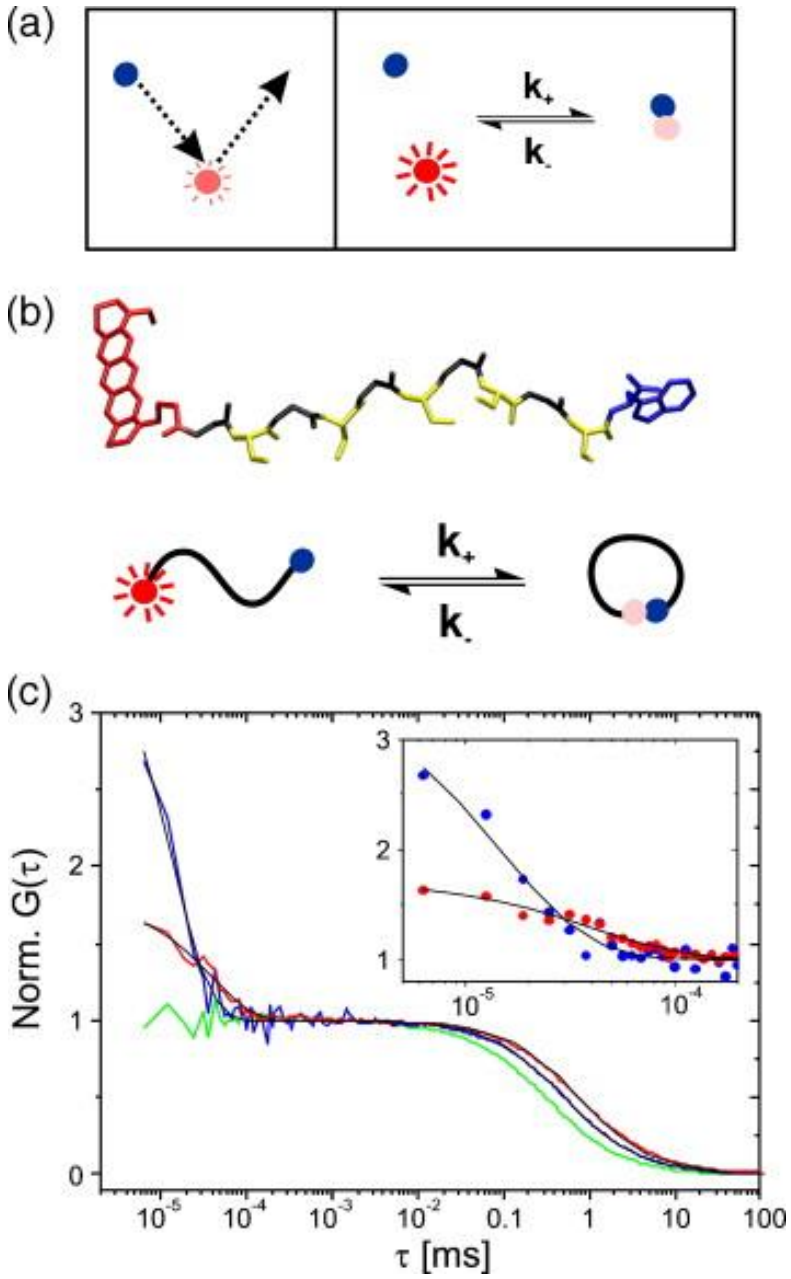


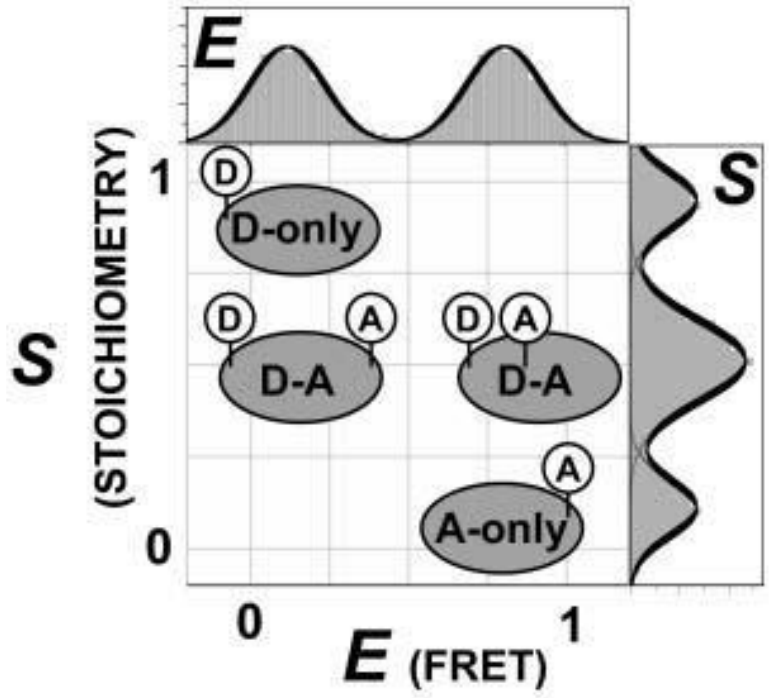
Figure 3. Normalized FCS curves measured for 10^{-9} M solutions of the RNA hairpin HP and the native ORN NT in the absence and presence of *At*GRP7 protein. The fluorescence correlation functions of free HP and NT are shown in black and green, respectively. The curve for HP bound to *At*GRP7 is shown in red and that for NT bound to *At*GRP7 in blue. The proposed model of a two-state hairpin-folding equilibrium whose unfolded state can be bound by the protein[33] is illustrated in the inset.

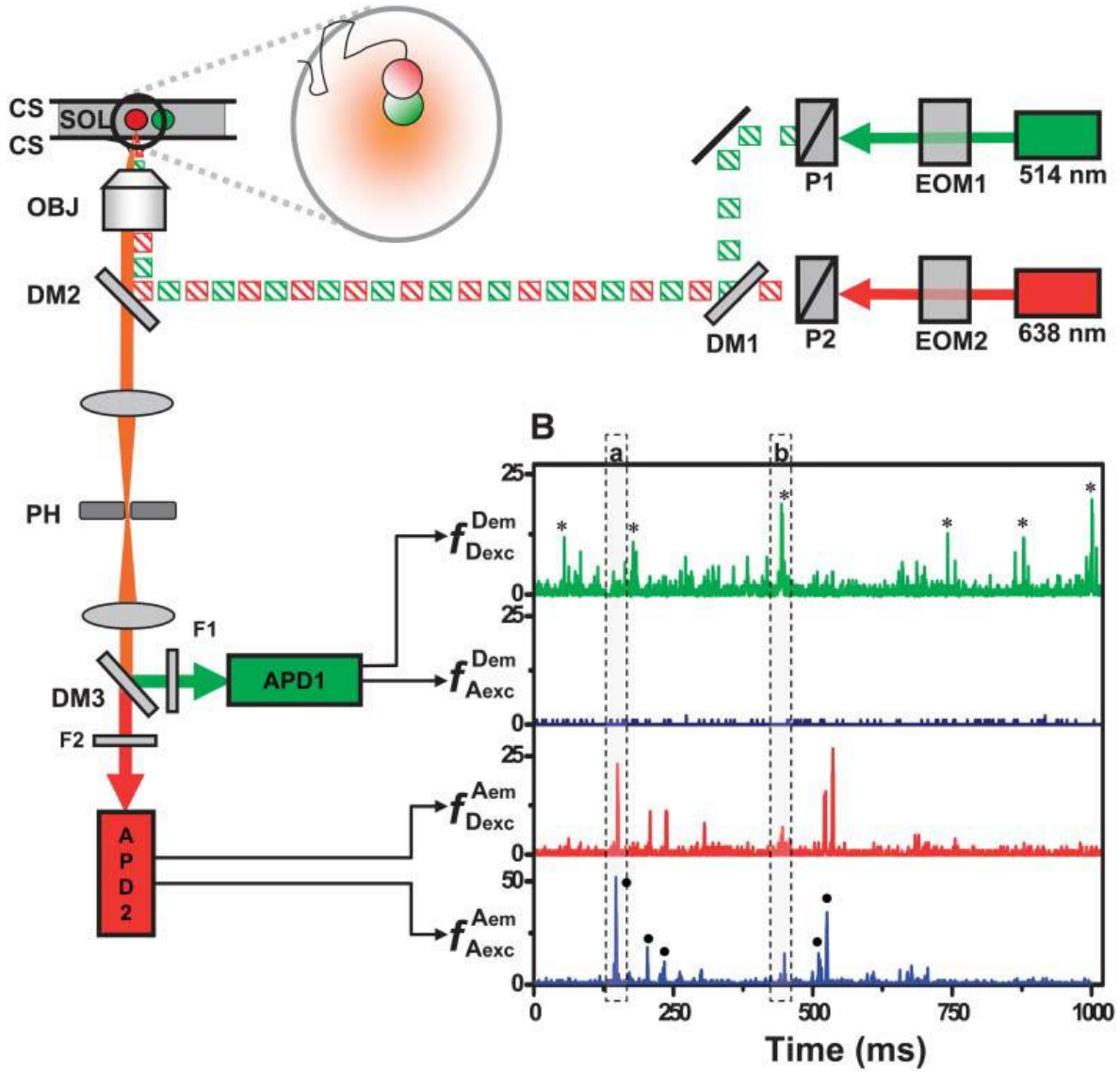


JACS., 2008, 130 (29)

ALEX – FCS

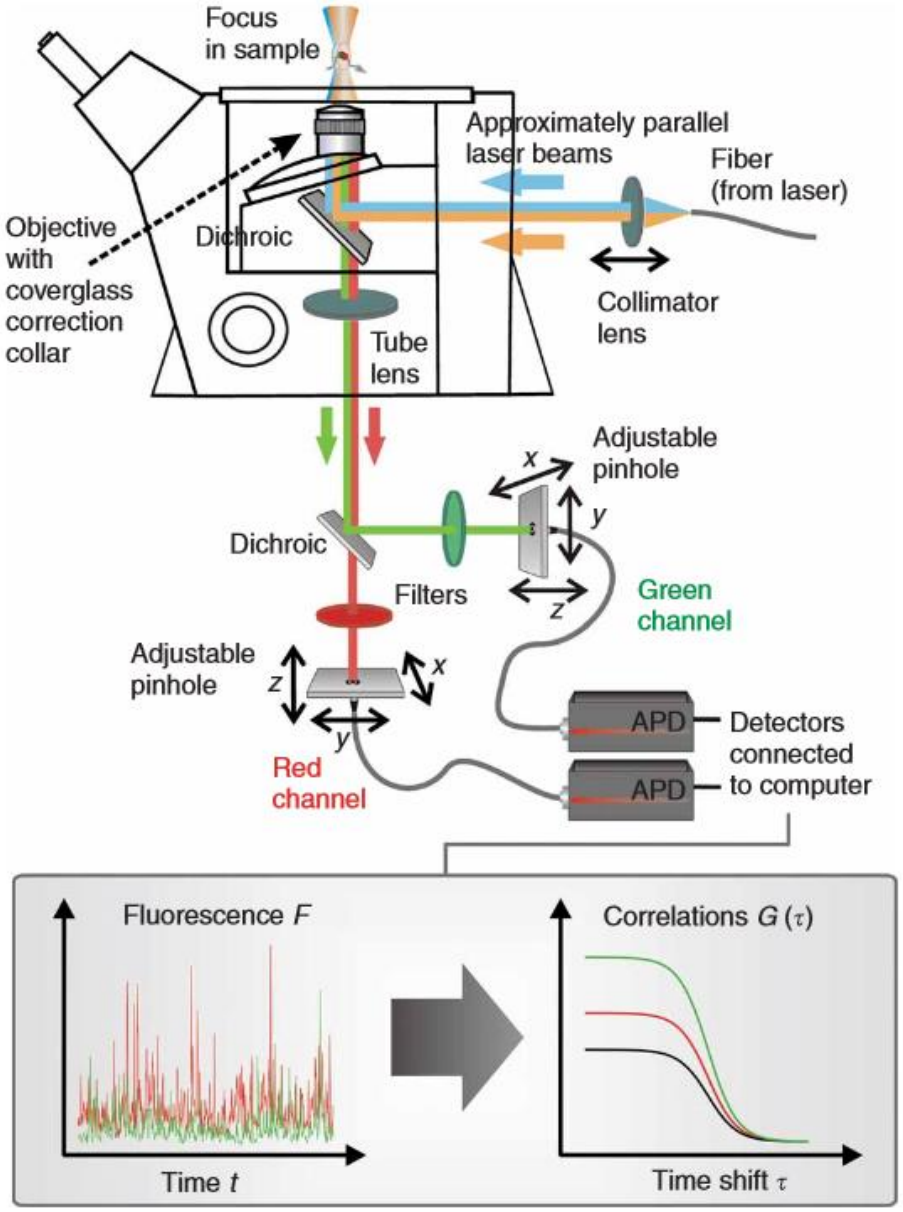
FCS with Alternating Laser Excitation and FRET-pairs





FCCS – Dual-Colour Extension of FCS

- probes the interaction of two differently labeled molecular species with higher precision than single-color FCS
- correlating the fluorescence signal of both two spectrally distinct detection channels and with each other (‘cross’-correlation)
- high specificity: FCCS curve is only formed if the differently labeled molecules are bound (co-diffuse). Only then their fluorescence fluctuations correlate in time
- From amplitude and decay time: binding constants, mobility of the bound complex, concentrations of all of the species



Summary

- Fluorescence Microscopy offers efficient and unique approaches to study fixed and living cells.
- Fluorescence characteristics can be used to visualize and analyse complex dynamics and molecular interactions in cells, organelles and sub-cellular components.
- All techniques take advantage of particular aspects of the fluorescence process (emission, excitation, damage, non-radiative decays)
- They have different strengths and weaknesses.
- All of them require careful experiment design and data analysis.

Literature

- great overview: Molecules 2012, 17(4), 4047-4132
- best book ever about fluorescence: J. Lakowicz, Principles of Fluorescence Spectroscopy
- also the webpages of Olympus, Zeiss and Nikon have really good introductions to the different techniques

- FRET paper:
  - Nature Protocols, 8, 265-281, 2013
  - PNA,102,2754-2759, 2005
  - J Cell Biol, 3, 2003; 160(5): 629–633
  - FRET and FLIM Techniques, edited by Theodorus W. J. Gadella
- FRAP
  - Biophys J., 1976; 16(9): 1055–1069.
  - Biophys J., 2004; 86(6): 3473–3495
  - J Biomed Opt. Jan 2012; 17(1): 011008
- FCS:
  - J. Am. Chem. Soc., 2008, 130 (29), pp 9507–9513
  - P. Schwille, E. Haustein, FCS- An introduction to its concepts and applications
  - Nature Methods, 4, 963 - 973 (2007)

The End!