Manual: Single Molecule Förster-Resonance-Energy-Transfer

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1 Introduction

'During the past 20 years there has been a remarkable growth in the use of fluorescence in the biological sciences. Fluorescence spectroscopy and time-resolved fluorescence are considered to be primarily research tools in biochemistry and biophysics. This emphasis has changed, and the use of fluorescence has expanded. Fluorescence is now a dominant methodology used extensively in biotechnology, flow cytometry, medical diagnostics, DNA sequencing, forensics, and genetic analysis, to name a few. Fluorescence detection is highly sensitive, and there is no longer the need for the expense and difficulties of handling radioactive tracers for most biochemical measurements. There has been dramatic growth in the use of fluorescence for cellular and molecular imaging. Fluorescence imaging can reveal the localization and measurements of intracellular molecules, sometimes at the level of single-molecule detection.' [1]

2 Theory

2.1 Fluorescence

By absorption of electromagnetic radiation orbital electrons of molecules can be excited to states with higher energy. Excited states relax to the ground state via different pathways. One process is the emission of a photon during relaxation from the first excited singlet state (S1) to the ground state (S0). This phenomenon is called fluorescence. These transitions occur with a typical lifetime of 10ns. Relaxation of the first excited triplet state (T1) has much longer lifetimes and is called phosphorescence. Photons can also excite higher singlet states like S2. They can relax via radiationless de-excitation called internal conversion to the S1 state. During excitation by or emission of photons preferentially excited vibrational

states are populated. These discrete states relax to the vibrational ground state within 10ps. These processes can be summarized in a Jablonski diagram shown in figure 1.

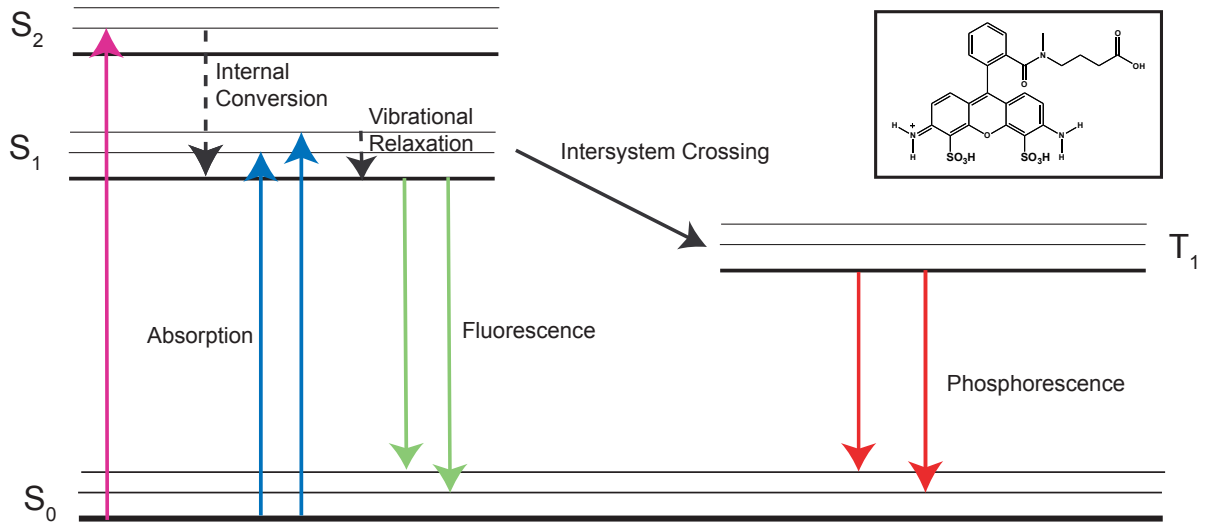


Figure 1: Jablonski diagram: schematical illustration of fluorescence.

Fluorophores (fluorescent molecules) are typically aromatic molecules that exhibit an individual, characteristic absorption and fluorescence spectrum between 350 and 750nm. In this experiment, we use high performance fluorophores Atto 550 and Atto 647N, their different spectra are shown in figure 2. A typical spectroscopical feature is the Stokes Shift between absorption and fluorescence maximum. The shift to higher wavelength is due to rapid decay of excited vibrational states and is often used in experiments to seperate excitation from emission light. This is essential for successful single molecule experiments because excitation power is much larger than the fluorescence signal. Another common feature that can be seen in these spectra is that absorption and fluorescence spectrum are mirrored. This can be explained by the Franck-Condon principle which concludes that the same vibrational states are populated with the same probability during the absorption and the fluorescence process. Consult [2] for more detailed information on quantum molecule physics.

2.2 FRET

Förster Resonance Energy Transfer (FRET) is an electrodynamical phenomenon that can be explained by classical physics. It is an induced dipole-dipole interaction between two different fluorophores. First, the fluorophore with lower absorption wavelength, called donor, is excited. If a fluorophore with higher absorption wavelength, called acceptor, is in close vicinity, a radiationless energy transfer can occur leaving an unexcited donor and an excited acceptor. This acceptor fluorophore can now emit fluorescence light as though it was excited in its own absorption spectrum.

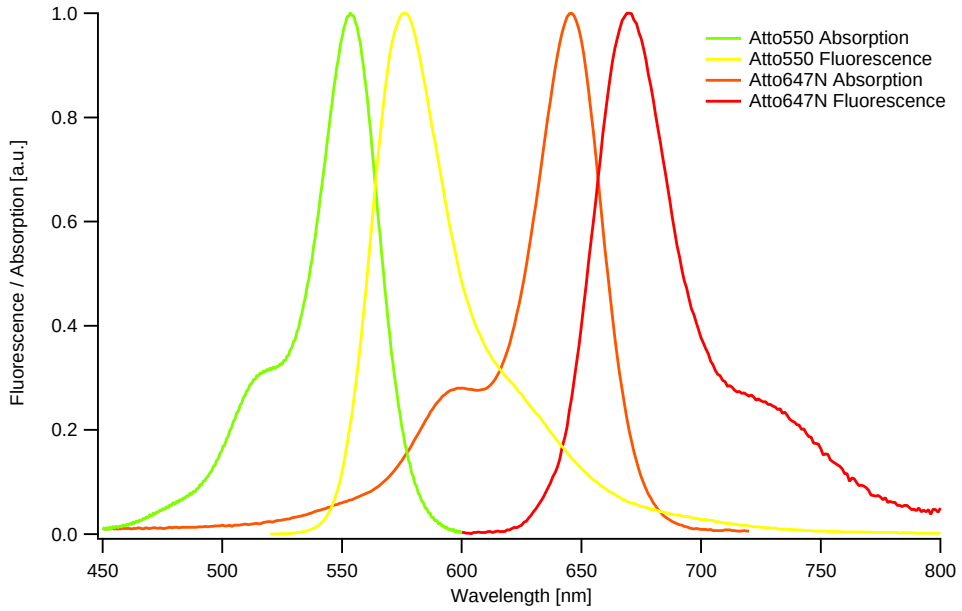


Figure 2: Absorption and fluorescence spectra of Atto 550 and Atto 647N (ATTO-Tec, Siegen). Numbers in fluorophore names specify the absorption maximum.

FRET can only occur if the donor's emission spectrum overlaps with the acceptor's absorption spectrum (cf. figure 2). The efficiency of the energy transfer decreases with donor-acceptor (FRET pair) distance to the power of 6, based on the dipole character of both fluorophores.

$$E = \frac{1}{1 + (\frac{R}{R_0})^6}. \tag{1}$$

The constant R_0 is called Förster radius and can be calculated by

$$R_0 = \frac{9000 \cdot (ln10) \cdot \kappa^2 \cdot Q_D}{128 \cdot \pi^2 \cdot N \cdot n^4} \int_0^\infty F_D(\lambda) \cdot \epsilon_A(\lambda) \cdot \lambda^4 \cdot d\lambda \tag{2}$$

with refractive index of the surrounding medium n, quantum efficiency Q_D, Avogadro constant N, corrected fluorescence intensity $F_D(\lambda)$ and extinction coefficient $\epsilon(\lambda)$. The factor κ^2 accounts for the orientation of the dipoles to each other and is 2/3 for freely rotating fluorophores. Since the FRET efficiency strongly depends on the distance, it is an excellent tool to measure lengths and length changes in the order of the Förster radius. Förster radii are typically in the range of 35-75 Å and therefore suited to investigate single biomacromolecules.

FRET is also used to study inter- and intramolecular distances [3, 4, 5]. For this application it has also to be taken into account that the FRET distance measured represents the effective distance between the two dyes and not the two attachment points of those. Therefore, accessible volume (AV) calculations are used as a tool to improve the resolution of FRET based structures [5].

Figure 3 shows an example of the FRET efficiency as a function of donor-acceptor distance of Atto 550 and Atto 647N. Highlighted is the small distance change in the linear regime from 4.6 nm to 7.9 nm that results in a FRET efficiency change of about 55%. Experimentally, the FRET efficiency is calculated from the intensities in the donor and acceptor channel (cf. equation 3). The γ factor corrects for different quantum yields of the fluorophores and different detector efficiencies at different wavelengths.

$$E = \frac{I_A}{I_A + \gamma I_D} \tag{3}$$

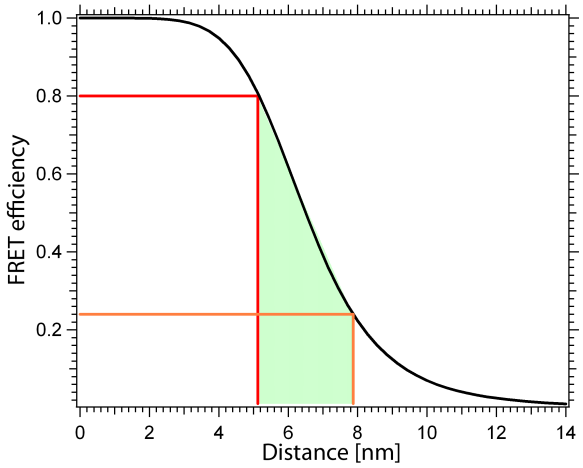


Figure 3: Distance dependence of FRET efficiency calculated according to equation 1 for fluorophores ATTO 550 and ATTO 647N with a Förster radius of 6.5 nm, derived from equation 2.

3 Heat shock protein 90

The 90 kDa heat shock protein Hsp90 is an ubiquitious molecular chaperone overexpressed upon stress (such as heat). Chaperones are proteins that control others for correct folding and are able to prevent aggregation of misfolded proteins, dissolve aggregates or guide proteins to their correct folding. In eukaryotes, Hsp90 is essential even under physiological conditions. It is involved in several cellular processes such as proliferation, signal transduction and transcription. The interaction with various oncoproteins makes Hsp90 an attractive drug target. Hsp90 is a dimer and a slow ATPase that undergoes large conformational changes during its interaction with nucleotides, cochaperones and substrates with at least two distinct conformations, referred to as 'open' and 'closed' state in the literature. The crystal structure of the eukaryotic yeast Hsp90 in the closed state has been solved[6]. Even without interaction partners, Hsp90 is able to undergo conformational changes at equilibrium [7].

These conformational dynamics will be studied in this experiment using FRET on single Hsp90 molecules, where on each protomer one residue is mutated to a cysteine and labelled with a fluorophore by maleiimide chemistry.

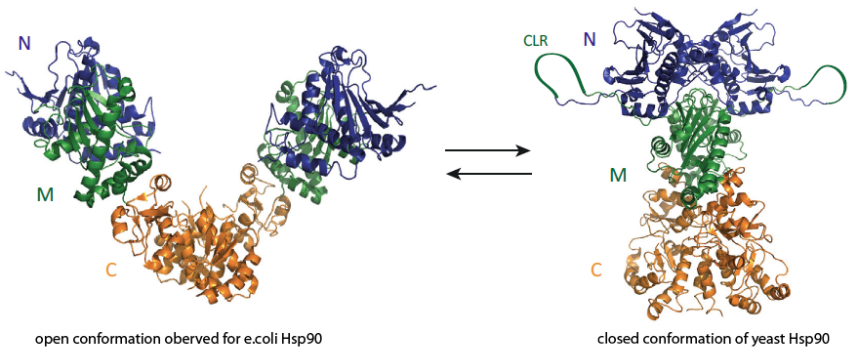


Figure 4: Coformational dynamics of Hsp90 [8]

4 Methods

To detect fluorescence of single molecules we use a custom-built prism-type TIRF microscope. It consists of three main parts: an excitation and a detection pathway and the sample chamber (with the prism and a quarz slide that carries the labeled biomolecules). The exitation pathway is used to excite fluorophores with lasers at different wavelengths in the measurement chamber (in our case we will just use one green laser to excite Atto 550). The detection pathway is used to collect fluorescence light. This light is split up by a dichroic mirror into different wavelengths and focused on a CCD camera with single photon sensitivity to detect the fluorescence signal of the according fluorophores in the sample. With this setup, shown in figure 5, we can follow different fluorescence signals at one spot over time.

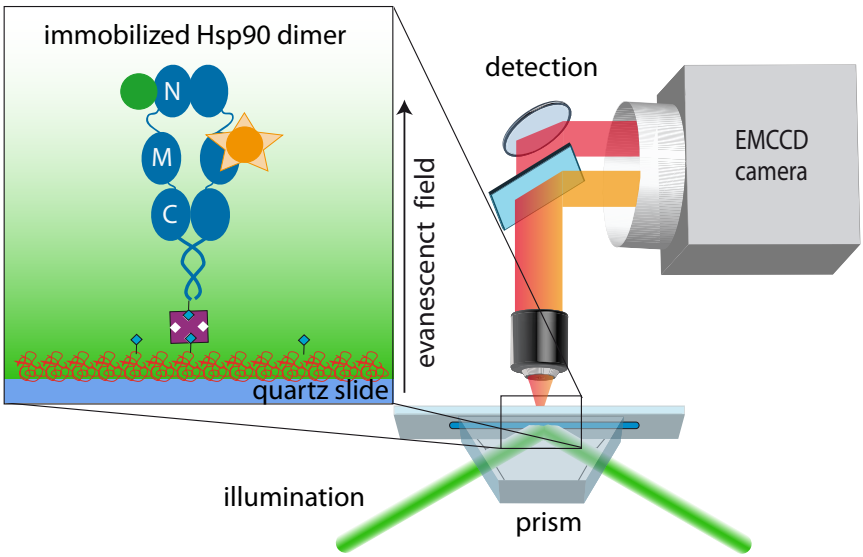


Figure 5: Single molecule prism-type TIRF setup.

In single molecule experiments the sample volume has to be very small (ideally in the

order of femtoliter) to maximize the signal to noise ratio. For instance noise can occur from Raman scattering on water molecules at (550 nm and 610 nm) or impurities. In our setup we achieve this by Total Internal Reflection Fluorescence (TIRF). As shown in figure 5 the angle of the excitation beam is above the critical angle for this medium boundary and therefore is totally internally reflected. Nonetheless, fluorophores that are attached to the surface can be excited, by the so called evanescent field, that decays exponentially within a few nanometers in respect to surface distance. The reason for this field is found in the continuity in the solution of Maxwell's equations. The mean penetration depths can be calculated by

$$d_0 = \frac{\lambda_0}{4\pi} \left(n_1^2 sin^2\theta - n_2^2 \right)^{-\frac{1}{2}} . \tag{4}$$

During the experiment we monitor the fluorescence intensities of the two fluorophores at single spots. This leads to two "intensity versus time" traces of the single fluorophores. The FRET efficiency is calculated out of the two anticorrelated fluorescence traces.

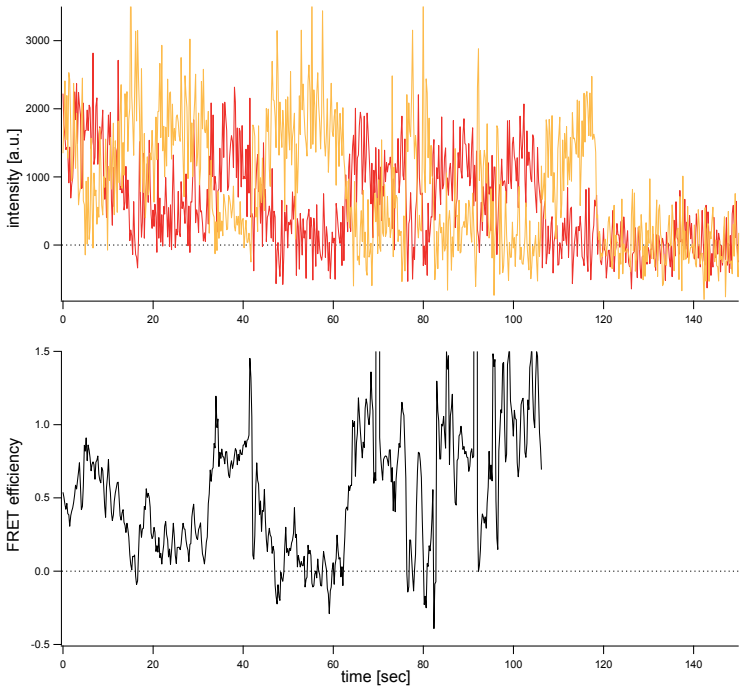


Figure 6: Data evaluation in FRET experiment: Top chart shows fluorescence signals of 2 fluorophores versus time (donor orange, acceptor 1 red). Below the resulting FRET trace is shown.

To identify conformational states of the sample we can plot the FRET efficiencies in a histogram. This approach is pictured in figure 6. Moreover, we can determine transition rates by measuring dwell times in the FRET traces. A dwell time is the period of time that a system remains in a given state. The distribution of dwell times is given by the rates of the underlying chemical reaction.

Consider the simple equilibrium

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B.$$

In a single molecule experiment, the only process changing the population of state A once it is reached is $A \xrightarrow{k_1} B$. Thus

$$\begin{aligned} \frac{\mathrm{d}[A]_t}{\mathrm{d}t} &= -k_1 \cdot [A]_t \\ \frac{\mathrm{d}[A]_t}{[A]_t} &= -k_1 \,\mathrm{d}t \\ \ln[A]_t &= \ln[A]_0 - k_1 \cdot t \\ [A]_t &= [A]_0 \cdot \exp(-k_1 \cdot t). \end{aligned}$$

5 Tasks and Experimental Procedures

In this experiment we will investigate the structure and conformational changes in apo Hsp90. One sample of Hsp90 is labeld with Atto 647N in the middle domain and is functionalized with biotin to bind it to our measurement chamber. The other sample is labeled with Atto 550 at the N-terminal domain. These samples are mixed and incubated at 47°C (monomer exchange). Afterwards, 50% of the dimers are heterodimers and only these will be visible during the measurement. Why?

Preparation:

- Make Hsp90 Dilutions to 20, 50, 100 and 200 pM final concentration from a provided stock solution of 1 µM, each aliquot 500 µL
- Dilute Neutravidin 1:1 with measurement buffer

Measurement:

- Mount measurement chamber, adjust lasers and start software
- Flush Neutravidin into measurement chamber, incubate for 5 min, flush with measurement buffer
- Flush the protein into the chamber, beginning with the highest dilution until an appropriate number of fluorescent spots are visible on the CCD. Flush unbound protein out of the chamber with measurement buffer. Now start measurement and record 5 movies of 3 min length.

Evaluation:

- Find fluorescence traces with FRET events (more than 10)
- Derive FRET efficiency traces

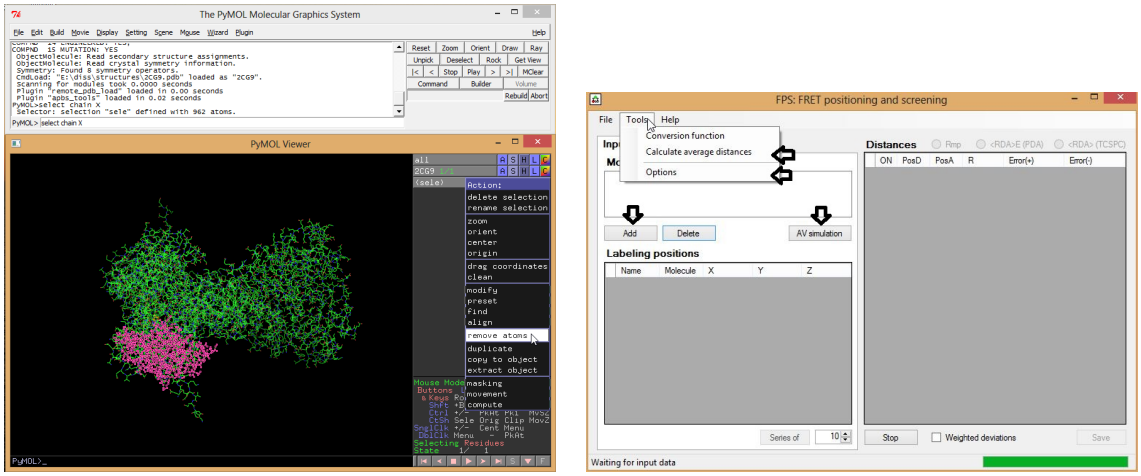
- Plot them in a histogram
- Measure dwell times in FRET efficiency traces and plot them in histograms (more than 30 dwell times per state)
- Fit these histogram with an appropriate function (justify!) and determine kinetic rates

6 Report

- Write an introduction that explains the difference between single molecule and ensemble measurements
- Show an example fluorescence and resulting FRET trace and discuss it
- Show the FRET histogram and discuss it with respect to the conformational flexibility of Hsp90
- Show dwell time histograms and discuss the corresponding rates
- Compare the measured FRET efficiencies measured with the closed conformation of Hsp90 as observed in the crystal structure. Be aware that the measured FRET efficiency and the effective distance is measured between the two fluorophores and not the two anchoring positiong for the fluorophores. To calculate the expected FRET efficiency from the crystal structure with the used fluorophore pair, use the Fluorescence Positioning Software (FPS) by the Group of Claus Seidel [5] (http://www.mpc.uni-duesseldorf.de/seidel/):

  1. Download the crystal structure of Hsp90 from the PDB (http://www.pdb.org/), ID: 2CG9
  2. Edit the structure as preparation for the accessible volume calculation with PyMOL (http://www.pymol.org/). Now remove the p23 molecules and the residue atoms that do not exist due to cysteine mutation from the structure: Type 'select chain X', left-click the A next to the ◁ sele ▷ in the PyMOL viewer and chose 'remove atoms', same for chain Y. Type 'select resi 61+385 and not(name CA+C+N+O+CB)' and remove the atoms of the selection. Save the structure
  3. Get The ID of the attachment points. The dyes are attached to the C_β of the amino acid 61 (for the donor dye) and 385 (for the acceptor). To get the atom id, open the structure in PyMOL and type 'id_atom chain A and name CB and resi 61'; equivalent for the acceptor position in the opponent chain

4. Calculate the Accessible Volumes (AV) for the donor and the acceptor dye: Open FPSgui.exe. 'Add' the structure. Click 'AV simulation'. Set Simulation type to 'Simple AV', Length to 12, Width to 3.5 and dye radius to 6. Insert the Atom ID of the attachement point of the donor dye, click 'calculate'. Save the xyz-File and repeat for the acceptor
5. Calculate the expected FRET efficiency: Open FPSgui.exe. Chose the menu 'Tools ▷ Options'. Set the Foerster radius to 65 A. Choose 'Tools ▷ Calculate average distances' and get the calculated FRET efficiency.



(a) HowTo PyMOL **(b)** HowTo FPS

Figure 7: 7(a) and 7(b) show where you have to click

Discuss differences between the measured and the expected FRET efficiency.

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