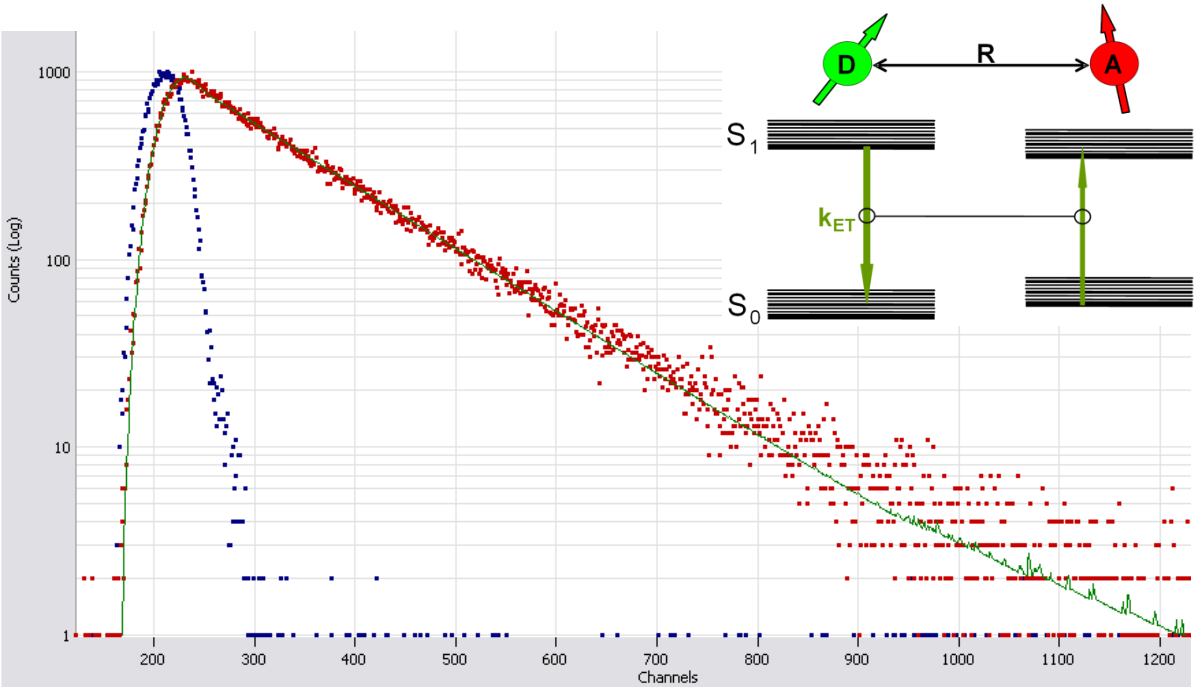
Biophysics Lab Course

ulm university universität ulm

Fluorescence lifetime

Fluorescence lifetime measurements for probing local environments of DNA-bound dye molecules and for distance determinations using FRET

Subjects: Fluorescence lifetime and quantum yield, fluorescence quenching, Förster resonance energy transfer (FRET) for distance measurements, Time-correlated single photon counting (TCSPC), Instrument response function (IRF)



Experiments:

→ λ_{ex} = 554 , λ_{em} = 568

The local environment of a fluorescent dye (Cy3) is probed by studying the fluorescence lifetime with time-correlated single photon counting. Changes in fluorescence lifetime due to constrained intramolecular dynamics caused by intramolecular bridges (Cy3B) or attachment to single-stranded or double-standed DNA are investigated. The distance between two fluorophores attached to complementary DNA strands is determined by FRET, utilizing the decrease of the donor lifetime in presence of the acceptor dye.

dye-dye
dye - ssdna
dye - ds dna

in fluorescence?

→ Förster Resonance Energy Transfer.

→ do we record Acceptor emission?

Introduction

The excited state lifetime of a fluorescent molecule (often also termed as 'fluorescence lifetime') is the average time which a dye molecule spends in the excited state before it returns to the ground state. The return to the ground state is a stochastic process, which may occur by emitting a photon (fluorescence) or without photon emission (nonradiative processes). Typically, the fluorescence lifetime of a dye (typ. some ns) is shorter, the more possibilities for returning to the ground state exist.

A prominent example for nonradiative processes are intramolecular rotations, which lead to a short fluorescence lifetime in case of the fluorescent dye Cy3 (Fig. 1). Hindering the rotational degrees of freedom by intramolecular bridges, as shown for Cy3B, increases the fluorescence lifetime.

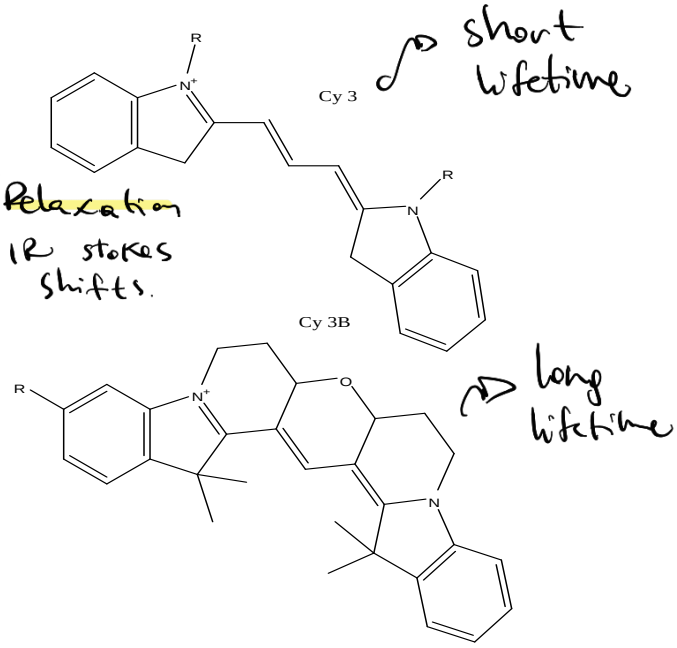


Fig. 1: Chemical structure of the fluorescent marker dyes Cy3 and Cy3B

Rotational motions can also be hindered by the environment of a dye molecule and thus fluorescence lifetime measurements can be used for sensing of local interactions.

Förster resonance energy transfer (FRET) is a further competition process, which leads to nonradiative transfer of the excitation energy to a second (acceptor) molecule and thus reduces the fluorescence lifetime of the donor molecule. Due to the strong dependence of FRET from the intermolecular distance of the molecules, the technique can be used as a 'spectroscopic ruler' on the nm scale.

Here, we will investigate Cy3 and Cy3B as well as Cy3- and Cy3B-labeled DNA oligonucleotides in order to probe the change in the local environment upon DNA-attachment and hybridization with a complementary DNA-strand. Furthermore, we will study FRET to an acceptor dye functionalized to the complementary strand and determine the distance of the two labels in the double-stranded DNA.

1. Fluorescence

1.1 Rate coefficients, fluorescence lifetime, fluorescence quantum yield

After reaching the excited singlet state S_1 by photon absorption, a fluorophore may return to the electronic ground state S_0 by emitting a photon (fluorescence) or via nonradiative processes (internal conversion).

Fluorescence: $S_1 \xrightarrow{k_F} S_0 + h\nu_F$

Nonradiative: $S_1 \xrightarrow{k_{nr}} S_0$

The rate coefficients k for the processes involved are governing the measurable fluorescent properties. The depopulation of the excited state S_1 is described by the differential equation:

$$\frac{d}{dt}N_1 = -k_F \cdot N_1 - k_{nr} \cdot N_1$$

with the solution

$$N_1(t) = N_1(0) \cdot \exp\left(\frac{-t}{\tau_F}\right),$$

where the excited state lifetime τ_F, which is also called 'fluorescence lifetime', is given by the inverse sum of all rate coefficients:

$$\tau_F = \frac{1}{k_F + k_{nr}}$$

The fluorescence quantum yield is the ratio of fluorescent transitions as compared to all possible deactivation processes (including fluorescence). It is given by the ratio of rate coefficients of the corresponding processes:

$$\phi_F = \frac{k_F}{k_F + k_{nr}}$$

The fluorescence intensity measured in a fluorometer is proportional to the fluorescence quantum yield as well as to the number of molecules in the measured volume. In contrast, measuring the fluorescence lifetime yields a molecular property.

depends on n° of Molecles & ϕ_F

. Independent of Moles & Q_f

. dependant of Environment.

1.2 Fluorescence quenching

The reversible reduction of fluorescence due to an additional nonradiative process caused by interaction with a second molecule is called 'quenching' (in contrast to photobleaching, which is a irreversible photochemical process, leaving a nonfluorescent product).

→ Collisions

In case of dynamic quenching, the excited fluorophores eventually collide with a ‚quencher' molecule and thus return nonradiatively to the ground state. This additional pathway leads to a shortening of the fluorescence lifetime and a reduction in fluorescence quantum yield

how sensible is your instrument

→ more often

$$\tau = \frac{1}{k_F + k_{nr} + k_Q \cdot [Q]} \qquad \phi = \frac{k_F}{k_F + k_{nr} + k_Q \cdot [Q]},$$

→ less events

which depend on the quencher concentration [Q].

The ratios of lifetimes and quantum yields without and with quencher are given by:

→ without Quencher / → with Quencher

$$\frac{\tau_0}{\tau} = 1 + \tau_0 k_Q [Q] \qquad \frac{\phi_0}{\phi} = 1 + \tau_0 k_Q [Q] = 1 + K_{SV}[Q],$$

the Stern-Volmer equation, with the Stern-Volmer constant K_{SV}, which describes the concentration dependence of quenching. The bimolecular rate coefficient k_Q contains the probability for collisions as well as the probability for quenching in case of collision.

→ COMPLEX FORMATION

In case of static quenching an increasing number of fluorophores is captured in nonfluorescing complexes with increasing quencher concentration. Thus, there are two distinct species of fluorophores: (1) nonfluorescent fluorophore/quencher complexes and (2) free fluorophores with unaffected fluorescence. The increasing number of complexes leads to a decreasing fluorescence signal of the sample due to the decreasing average quantum yield of the ensemble of fluorophores. Again a Stern-Volmer relation is valid, but now caused by the binding equilibrium for the complexes:

$$K = \frac{[FQ]}{[F][Q]} = \frac{[F_0]-[F]}{[F][Q]} = \frac{[F_0]}{[F][Q]} - \frac{1}{[Q]} \quad \Rightarrow \quad \frac{\phi_0}{\langle\phi\rangle} = \frac{I_0}{\langle I\rangle} = \frac{[F_0]}{[F]} = 1 + K_{SV}[Q]$$

In contrast to the average quantum yield, the fluorescence lifetime does not change in case of static quenching. Due to the heterogeneous ensemble of free (unaffected) and bound (fully quenched) fluorophores, only the free fluorophores will contribute to the lifetime measurement, leaving the lifetime unchanged despite the presence of quencher molecules.

↖ because the fluorescence measured is only from ~~FREE~~ Dye Molecules

Thus, fluorescence lifetime measurements allow to distinguish between the cases of static and dynamic quenching.

1.3 Förster resonance energy transfer (FRET)

A special case of fluorescence quenching is FRET, the radiationless transfer of energy from an excited molecule (donor) to a second molecule (acceptor) by dipole-dipole coupling.

$$D^{*} + A \xrightarrow{k_{FRET}} D + A^{*}$$

Important conditions for a high rate coefficient k_{ET} are the spectral overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the two transition dipoles and the distance between the two dye molecules (Fig. 2).

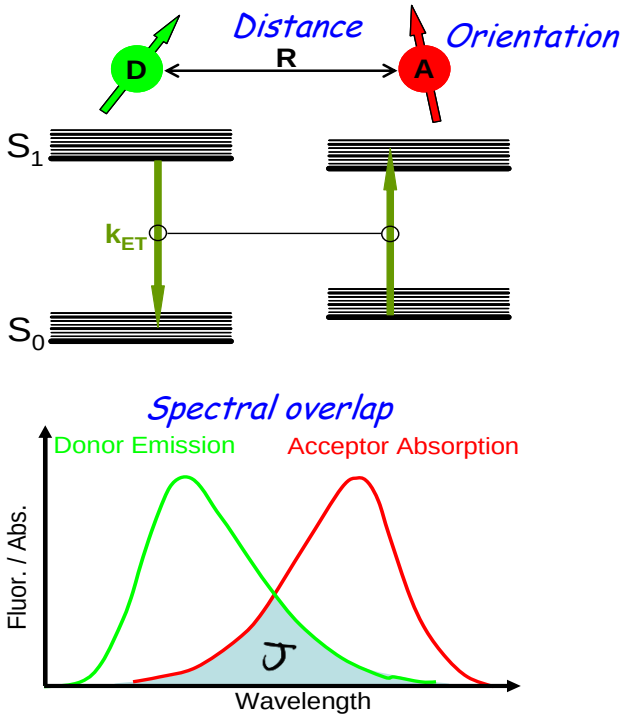


Fig. 2: Steric und energetic conditions for efficient Förster transfer

The distance dependence of the rate coefficient for energy transfer is found to be:

$$k_{FRET}(R) = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6 \qquad \text{mit} \qquad R_0^6 = \left(\frac{9\ln(10)\kappa^2\phi_D}{128\pi^5 N_A n^4}\right)\cdot J\,,$$

where the characteristics of the FRET pair are collected in the ‚Förster-Radius' R_0. Besides the fluorescence quantum yield of the donor ϕ_D, the refractive index n and the orientation factor κ^2, the overlap integral J is important:

$$J = \int \varepsilon_A(\lambda)\, f_D(\lambda)\lambda^4 d\lambda$$

For practical calculations of the Förster radius, a formula is used, where the constants are combined and units are converted:

$$R_0 = 0.0211 \cdot (J\kappa^2 n^{-4} \phi_D)^{1/6} \quad .$$

Here, the förster radius R_0 is calculated in units of nm if in the overlap integral J the absorption is given in $(M \cdot cm)^{-1}$ and the wavelength in nm. For typical FRET pairs, the Förster radius is in the range of 3 - 8 nm.

If the distance R between the fluorophores equals R_0, in half of the cases the excitation is transferred to the acceptor. This becomes obvious from the definition of the energy transfer efficiency E, which relates the transferrate to the sum of rates for all possible processes:

a kind of Quantum Yield. used because usually you don't have Radius.

$$E = \frac{k_{FRET}(R)}{k_{FRET}(R) + k_F + k_{nr}} = \frac{1}{1 + (R/R_0)^6}$$

k_{nr}: relaxation

Due to the strong distance dependence (sixth power!) of the transfer efficiency, FRET can be used as a sensitive tool for distance determinations on the nanometer scale around R_0 of the dye pair.

A prominent example for FRET as a 'spectroscopic ruler' for distance determinations on the nm scale is the study of Stryer and Haugland [2], where the theory of Theodor Förster was verified. Here, a series of molecules was used, where a donor (naphtyl) and an acceptor (dansyl) were kept at a defined distance (Fig. 3). The transfer efficiency was determined as a function of the donor-acceptor distance, which was varied by the number n of prolyl-residues between the dyes.

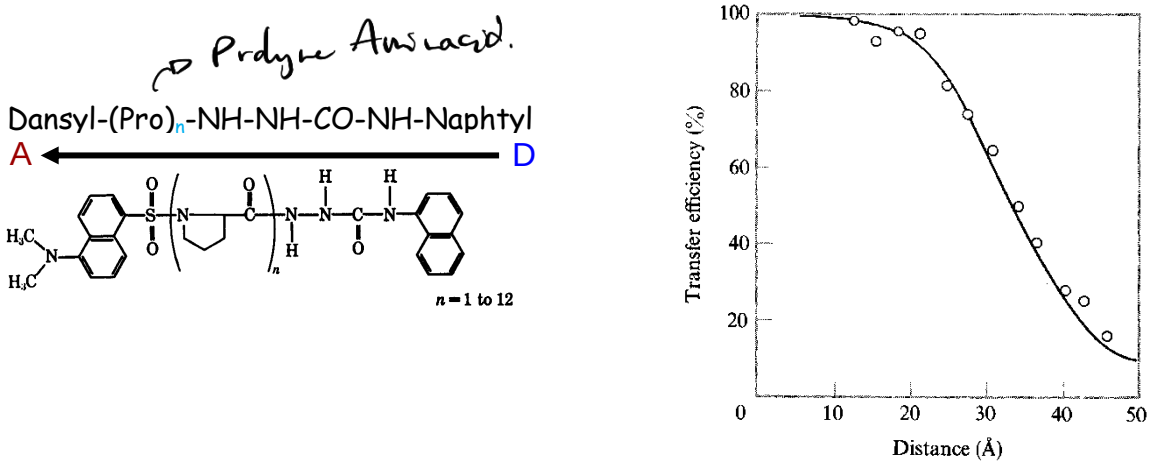
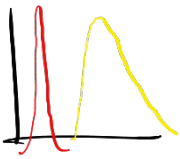


Fig. 3: Experimental verification of Förster resonance energy transfer (Stryer & Haugland 1967).

1.4 Time-correlated single photon counting

The most common method for fluorescence lifetime studies is time-correlated single photon counting (TCSPC), where the arrival time of a photon at the detector is determined in relation to the exciting (short) laser pulse for each detected fluorescence photon.

Due to the stochastic nature of photon emission, the individual arrival times are strongly varying and a single or a few detected photon arrival times are not conclusive. If, however, an arrival time histogram is collected for typically millions of photons, this histogram resembles the probability for staying in the excited state for a certain time. This 'fluorescence decay curve' is a single exponential function in the simplest case, where the decay constant equals the fluorescence lifetime.



by the law of BIG NUMBERS you get closer to TRUE Histogram

In practice, the exciting laser pulse is not infinitely short and the timing is not infinitely precise. Thus, an instrumental response function (IRF) has to be used in order to interpret the results correctly (Fig. 4). The IRF is experimentally determined by measuring a nonfluorescent solution, where the photons are only scattered so that no sample-induced time delay occurs. For analysis of the fluorescence lifetime, the exponential model function has to be convoluted with the IRF with iterative parameter optimization in order to fit the measured fluorescence decay.

SIGNAL ⊗ IRF

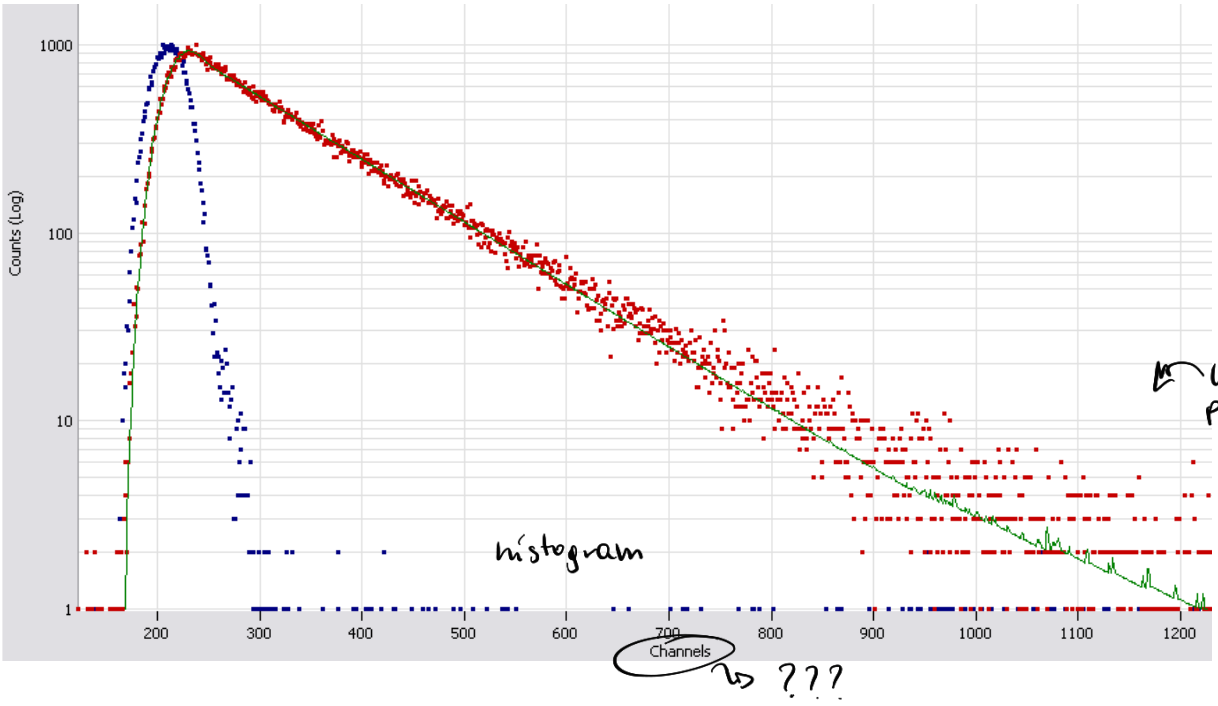


Fig. 4: Experimental example for a fluorescence decay (red) and an IRF (blue) on a logarithmic scale.

2. Problems

2.1 Preliminaries

Find on the internet the excitation and emission spectrum of Cy3 and suggest which wavelengths should be used for the experiment. How can the IRF be determined?

2.2 IRF and dye measurements

Measure the IRF using PBS buffer and an emission wavelength of 500 nm.

Determine the fluorescence lifetime for the dyes Cy3 and Cy3B in PBS.

2.3 Fluorescence lifetime for structure sensing

Perform TCSPC measurements and analysis on a 5'-Cy3-labeled single-stranded DNA and compare with the same construct hybridized to a complementary DNA.

Is a single exponential function sufficient to fit the decay?

Repeat the experiments with a DNA-construct which is labeled with Cy3B.

Interpret and discuss the experiments with the two dyes in all different environments.

2.4 FRET measurements

Perform TCSPC measurements on dsDNA which consists of a Cy3B-labeled strand (like in 2.3) and a complementary strand which is labeled with the FRET acceptor dye Alexa647 (FRET-A sample).

What is an appropriate model function, if you assume that there is some unpaired DNA and/or bleached acceptor dye in your sample?

Determine the fraction of the 'Donor-only' species from the relative amplitudes.

Determine the fluorescence lifetime of Cy3B in presence of the acceptor τ_{DA}.

Calculate the FRET efficiency for the construct using the relation:

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

Which of the previously measured lifetimes τ_D has to be used for the calculation?

Determine the distance between the dyes, assuming a Förster radius of 6.8 nm.

Repeat the experiment using a DNA construct where the acceptor dye is at a different position (FRET-B sample).

Calculate the expected distances between donor and acceptor for both samples with a simple structure model, using the DNA-sequences shown in the 'Materials' section.

Compare the results of your FRET measurements to the expectation from the model and discuss possible reasons for deviations.

Restriction enzyme BamHI

Prepare a new FRET-B sample, using now 'cutsmart' buffer instead of PBS and perform a TCSPC measurement. Add 1 µl of BamHI-HF solution to the sample and do TCSPC measurements immediately and also 5, 10, 20 and 30 minutes after addition of the enzyme.

Analyze the relative amplitude of the Donor-only species as a function of time. Find the restriction site of BamHI in the sequence of the oligonucleotide and explain your finding.

3. Experimental Details

3.1 Instrumental settings and procedures

After switching on the Fluorohub controller, the TBX power supply and the PC, start the 'Datastation' software, choose 'TCSPC lifetime' and select 'R'everse data from the 'tools' menu. Choose the setting of the emission monochromator ('Optics' panel on the left) according to the spectral properties of the dye. Before starting a measurement, switch the LED output to 'on' (NanoLED panel on the left). Select an IRF measurement (called 'prompt' in the software), or lifetime measurement (called 'decay') and push the start button.

For all experiments, at least 3 decays should be recorded and analyzed to achieve a more robust result. For data analysis the software 'DAS6' is used.

First try a single-exponential model for the analysis of all samples. If the residuals show strong systematic deviations and $\chi^2 >> 1$, use a double exponential model and characterize the lifetimes and relative amplitudes of both species. The initial values for the lifetime(s) are suggested by the software upon request.

For the analysis of FRET data a double-exponential decay model is used where one of the lifetimes is fixed to a value that corresponds to the donor-only lifetime in order to account for unpaired DNA or bleached acceptor dye.

3.2 Materials

For almost all experiments PBS is used as dilution medium. Before loading a new sample, the cuvette is flushed with ultraclean water at least three times. Each measurement has to be done at least twice. Samples are prepared by adding 3 µl of a stock solution (typically 1µM) to 72 µl PBS in the cuvette. For the digestion study with the restriction enzyme BamHI 'cutsmart' buffer is used instead of PBS for diluting the FRET-B stock solution.

DNA oligonucleotides used:

Forward Cy3 / Cy3B:

5’ **X** TGG ATC CAT AGT AGC GTA GCG TAG CGT AGC GTA GCG TAG C
X = Cy3 / Cy3B bound to a T

Reverse:

5’ G CTA CGC TAC GCT ACG CTA CGC TAC GCT ACT ATG GAT CCA A

Reverse FRET-A sample:

5’ G CTA CGC TAC GCT ACG CTA CGC **Y**AC GCT ACT ATG GAT CCA A
Y = Alexa 647 bound to a T

Reverse FRET-B sample:

5’ G CTA CGC TAC GCT ACG CTA CGC TAC GC**Y** ACT ATG GAT CCA A
Y = Alexa 647 bound to a T

Literature:

Lakowicz, J.R., 'Principles of Fluorescence Spectroscopy', Springer 2006

Stryer, L., Haugland, R.P., 'Energy Transfer: A Spectroscopic Ruler', Proc. Natl. Acad. Sci. USA **58** (1967) 719-726

at Beginning

To Repeat for each measurement

Variations for FRET

Variations for Restriction

We record 1 IRF for each Sample of PBS

We add 3 µL of Sample.

* We will get

Determine the Donor Only Fraction

$$\frac{B_1}{B_1 + B_2} = e^{-t/\tau_1}$$

Fix the #

LAB REPORT

1 Week

All data

Show all formulas used

↳ only formula

↳

Don't overdo the introduction

↳ We took only 1 Measurement of Cy3 or because it Cy3B PROBABLY

↳ We did not know because it was not written, but the one with longer lifetime is Cy3B

↳ show the screenshots.

Sample 1 = Cy3B
Sample 2 = Cy3

★ IRF is recorded at 500 nm

★ lifetime is recorded at 570 nm

★ Sample ssDNA Cy3B
↳ 1° Experiment was FAST (data 1)
↳ 2° Experiment was SLOW. (data 2)

1.2 nSecond Pulse length

1 MHz Freqency