Practical course: Instrumental bioanalysis

Fluorescence Resonance Energy Transfer (FRET)

In this experiment, the fluorescence quenching of human serum albumin (HSA) by 3- hydroxyflavones (3-HF) is to be determined in order to be able to determine the distance of the FRET pair 3-HF/HSA. Several fluorescence spectra with different 3-HF concentrations are measured to obtain information about the fluorescence intensity.

The decrease in fluorescence intensity of a fluorophore without its destruction is generally referred as quenching. A distinction is made between dynamic, static and quenching by fluorescence/Förster resonance energy transfer (FRET). In the former, the energy of the excited fluorophore is transferred to a quencher molecule through collisions. The energy is lost in the form of heat. In static fluorescence quenching, a non-fluorescent or weakly fluorescent complex is formed between the fluorophore and the quencher molecule. FRET, on the other hand, is mediated by dipolar interactions between two fluorophores. A FRET pair consists of a donor and an acceptor. An important condition for efficient transfer is a high overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor.

Important information

A passed colloquium before the attempt is a condition for the execution of the attempt. If the colloquium is not passed, there will be a make-up date. The protocol must be handed in at the latest by 9:00 a.m. on the fourth day following the attempt.

1 Theoretical foundation

Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (also known as Förster resonance energy transfer) is an important physical process of energy transfer, whereby energy is transferred from a donor to an acceptor. A prerequisite for the energy transfer is that the amount of energy transferred by the donor is within the range of the possible energy absorption of the acceptor.

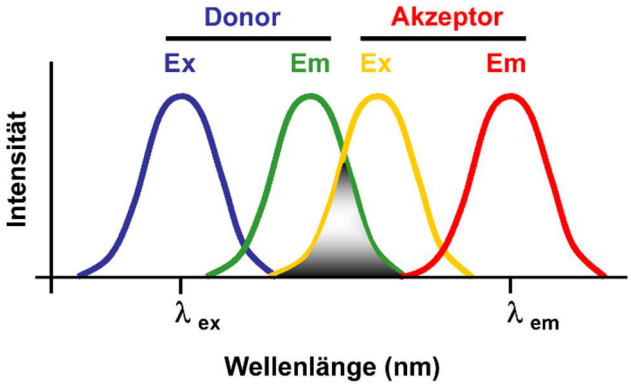

Fig. 1 Excitation (Ex) and emission spectrum (Em) of donor and acceptor, respectively. The overlapping area (marked grey) of the emission spectrum of the donor and the excitation spectrum of the acceptor shows the possibility of a radiationless energy transfer.

This means that the overlap integral J must not be equal to 0.

$$J = \varepsilon_A(\lambda_{Em}^D)\lambda_{Em}^{D\ 4}$$

$\varepsilon_A(\lambda_{Em}^D)$ is the extinction coefficient ($\varepsilon_A(\lambda_{Em}^D)$= 9064*$10^{17}$ ± 10*10^{17}) of the solution to be investigated at the emission wavelength $\lambda_{Em}^D$of the donor. This formula is valid under the assumption that the emission spectrum of the donor has an infinitesimal line width. The consequence of an efficient transfer is the

observation of fluorescence of the acceptor at irradiation of the absorption wavelength of the donor.

This can be observed in fluorescence spectroscopy with a fluorescence spectrometer. Lambert-Beer's law describes the attenuation of the initial intensity I_0 when passing through a medium with an absorbing substance. The decrease depends on the layer thickness d and the concentration c.

$$A = log_{10}\left(\frac{I_0}{I_1}\right) = \varepsilon_\alpha * c * d$$

In contrast to absorption spectroscopy, in which detection takes place directly behind the cuvette, the detector in fluorescence spectroscopy is offset by 90°. This ensures that as little direct light as possible from the light source reaches the detector. Figure 2 shows the general, highly simplified structure of a fluorescence spectrometer. The light source emits white light, the monochromator can be used to select the irradiation wavelength that hits the cuvette and thus the sample solution.

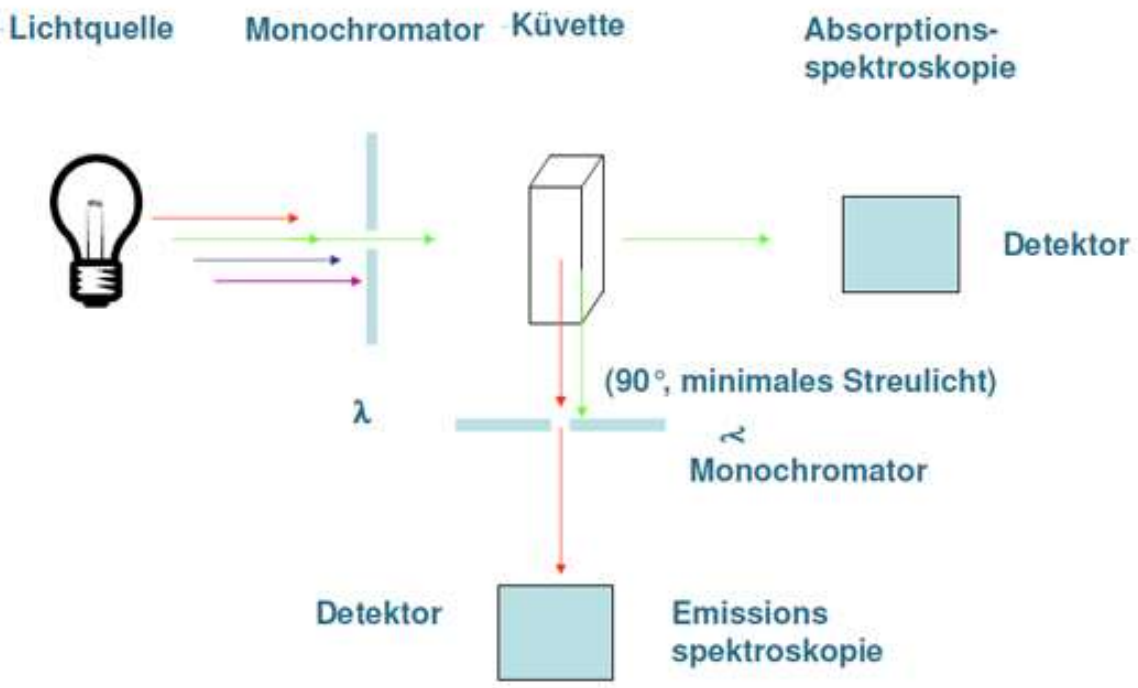

Fig. 2 Sketched structure of a fluorescence spectrometer

Different energy transfer mechanisms

Energy transfer processes can be divided into intermolecular and intramolecular energy transfers. In an intermolecular transfer, the excitation energy is transferred from one molecule to another. In an intramolecular transfer, on the other hand, the energy is transferred within a molecule. Electronic energy transfer in general is the transfer of the excitation energy of an excited donor molecule D^* to an acceptor molecule A. This represents a two-step process:

1) $D^* \rightarrow D + hv$
2) $hv + A \rightarrow A^*$

In the process, the excited molecule D* emits a photon which is absorbed by A.

Radiationless energy transmission

There are two forms of non-radiative energy transfer

a) Collision or exchange interaction (Dexter energy transfer)
b) Coulomb interaction (Förster energy transfer)

During the energy transfer according to Dexter, the orbitals involved "collide", resulting in the exchange of the excited electron of the donor with an unexcited electron of the acceptor.

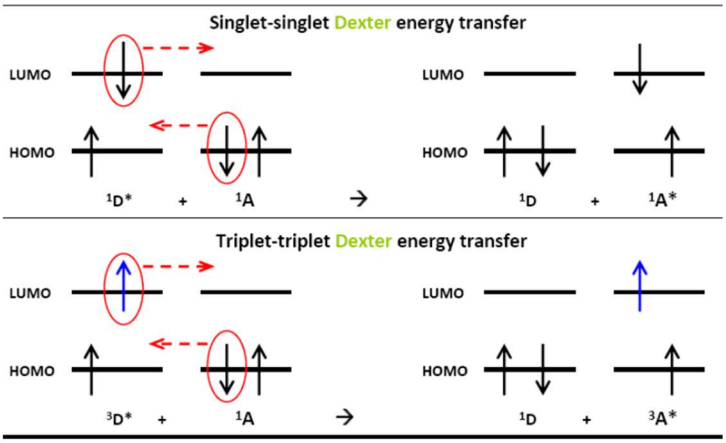

Fig. 3 Schematic representation of the Dexter energy transfer with the two possible transitions

$$^3D^* + ^1A \rightarrow ^1D + ^3A^*$$

The spin interactions decrease exponentially with distance and are only effective at r < 1 nm. In the Dexter energy transfer, singlet-singlet transitions as well as triplet-triplet transitions are possible.

Fig. 4 schematically shows the processes in the Förster mechanism:

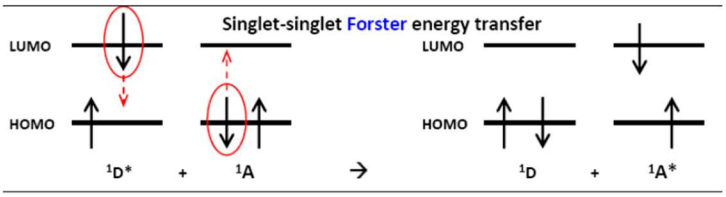

Fig. 4 Schematic representation of the Förster energy transfer

The deactivation of D* and excitation of A are coupled with each other. The coupling occurs through a Coulomb dipole-dipole interaction. It should be noted that no spatial contact (as in the impact mechanism) between the molecules is required for this form of energy transfer to be possible

The transition probability for a radiative transition between an excited state and the ground state is determined by the transition dipole moment. The transition dipole moment $\langle\mu\rangle$ is defined by

$$\langle\mu\rangle = \int \varphi_2^*(-er)\,\varphi_1 dV$$

φ1 the electronic wave function of the molecule in the excited state
φ2 the conjugate complex electronic wave function of the molecule in the ground state
e the electric charge
r the location coordinate.

The integral reflects the displacement of the charge cloud between the excited and ground states during an induced transition by the electromagnetic alternating field of light. The transition dipole moments of the donor and acceptor can now transfer the excitation energy without radiation.

FRET efficiency

An important quantity is the FRET efficiency *E*, which can assume values between 0 and 1. This reflects the efficiency of the energy transfer between donor and acceptor and is defined as follows:

$$E = \frac{Anzahl\ der\ Energietransfers\ pro\ Zeitintervall}{Anzahl\ der\ Donoranregungen} = \frac{k_{ET}}{k_f + k_{ET} + \sum_i k_i}$$

k_{ET} is the rate of transitions caused by energy transfer; this is set in relation to the sum of all possible transition types.

k_f represents the fluorescence transition rate, k_i the transition rate caused by all other mechanisms.

To enable an effective transfer, the donor and acceptor may only be a few nm apart. The transfer rate k_{ET} shows the following distance dependence, which is sketched in Fig. 5.

$$k_{ET} = k_D \frac{R_0^{\ 6}}{r^6}$$

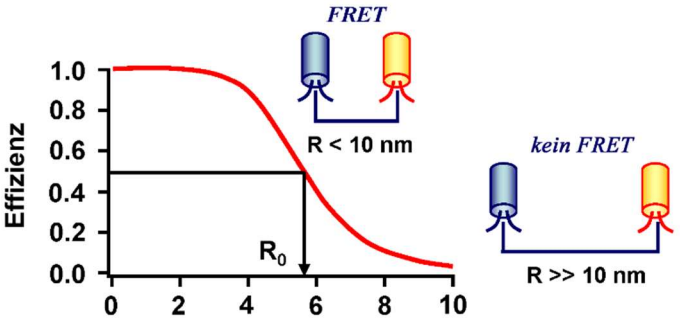

Fig.5 The red curve shows the course of the distance dependence of the transfer efficiency E for a Förster radius R_0 around 6 nm. In the range around the Förster radius, the distance can be determined particularly precisely, since the slope of the curve is largest in terms of magnitude here.

k_D is the radiation emission rate of the donor dye, R_0 is the so-called Förster radius of the donor-acceptor pair. This represents the distance between donor and acceptor at which 50% energy transfer occurs. The Förster radius can be calculated using the following formula:

$$R_0^6 = 8{,}8 * 10^{-28} * \kappa^2 n^{-4} Q_0 J$$

κ^2 is the orientation factor, n the refractive index and Q_0 the quantum yield of the donor without energy transfer.

The orientation factor depends on the angles between the transition dipole moments of the molecules, which is a measure of the molecule's ability to absorb or emit electromagnetic radiation.

The orientation factor can assume values between 0 and 4. The interaction of both transition dipole moments is most effective if the dipoles are arranged collinearly behind each other, i.e. κ 2 = 4 (see Figure 5b). If they are aligned perpendicular to each other, κ 2 = 0 and no energy transfer takes place. For most FRET experiments, an orientation factor of 2/3 is assumed, since it is a dynamic equilibrium in solutions.

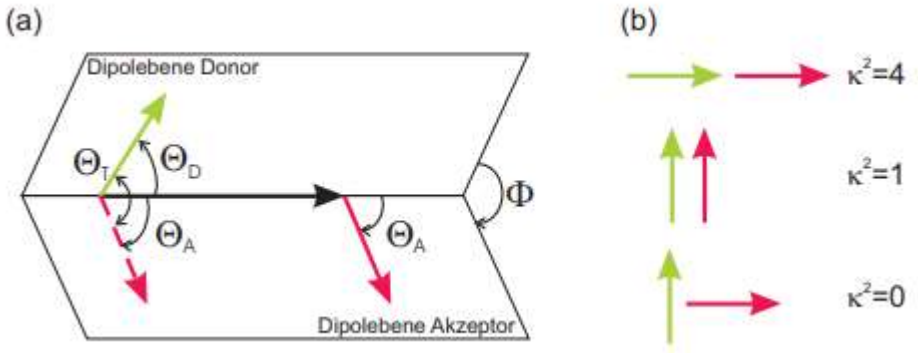

Fig. 6 Orientation of the transition dipole moments; transition dipole moments of the donor (green) and acceptor (red); a) shows the angles of the transition dipole moments. b) The values of κ2 as a function of the position of the dipole moments in relation to each other.

The course of the dependence shows in which range the distance can be determined particularly precisely with the help of this method or it reacts particularly sensitively to changes in it. The FRET efficiency can consequently also be expressed in the following way

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} = 1 - \frac{F}{F_0}$$

F represents the intensity of the donor fluorescence in the presence, F_0 in the absence of the acceptor. By measuring these two values, the transfer efficiency E and thus the distance between the two dyes can be determined.

Stern-Volmer equation and quenching

The Stern-Volmer equation describes the dependence of the fluorescence of a dye on the concentration [Q] of a quencher in the environment, which in this case is the acceptor of the FRET pair. It reads:

$$\frac{F_0}{F} = 1 + K_{SV} * [Q].$$

With the Stern-Volmer constant K_{SV}. The prerequisite for the validity of this formula is the equal accessibility of all fluorophores by the quencher. K_{SV} can be regarded here as an association constant and thus the dissociation constant K_D can be determined.

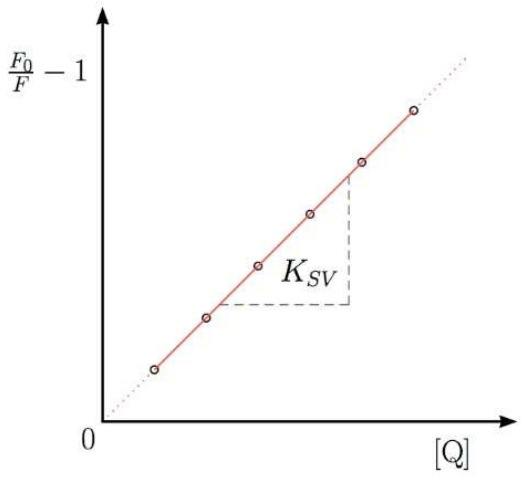

Fig. 7 Stern-Volmer plot

The FRET pair in this experiment is the tryptophan of the protein human albumin (HSA) as donor and the ligand molecule 3-hydroxyflavone (3-HF) as acceptor. HSA consists of 585 amino acids and has a molecular mass of about 66,470 kDa. It has exactly one tryptophan located near a major binding site.

In addition, the natural dye quercetin can be used as an acceptor, which will be compared with 3-HF in the protocol.

2 Implementation

In the solutions with HSA, increase the 3-HF concentration in several steps (0.1-50 μM, min. 15 points, non-linear, e.g. 1 µL, 2 µL, 5 µL, 8 µL...) and examines the fluorescence intensity of the donor or the acceptor. How do these changes? In the cuvette for the fluorescence measurement, the starting sample quantity should be 2 ml (pure HSA solution). By adding the 3-HF solution, not only its concentration but also the total volume increases. What error is made in the measurement as a result? Estimate it.

A concentration is to be measured 5 times to obtain the error of the device. Likewise, any concentration is measured again to determine the error. A measuring point is randomly selected and directly prepared to check the operation of direct sample preparation with slow addition.

Solutions to be produced

5 ml of an HSA solution (protein concentration 6 µM). NaPh buffer (pH 7) is provided as solvent. The solvent is extrapolated to a defined weight (~ 5-10 mL).

1 ml 3-HF solution in ethanol with the concentration 400 µM. The solvent is extrapolated to a reasonable weight.

Addition: 1 ml quercetin solution in ethanol with the concentration 400 µM. Solvent is extrapolated to a reasonable weight.

Protocol

- Sizes to be used:

Refractive index $n = 1{,}4 \pm 0{,}05$

Fluorescence quantum yield of the donor tryptophan: $Q_0 = 0{,}11 \pm 0{,}005$

Orientation factor: $\kappa^2 = 2/3$

Extinction coefficient $\varepsilon_A(\lambda_{Em}^D)$ = 9064 * 10^{17} (nm^2 /mol) ± 10 * 10^{17} (nm^2 /mol)

M(3-HF) = 238.34 g/mol

M(HSA) = 66,470 kDa

M(quercetin) = 302.24 g/mol

Quantities without error are estimated with an error of 0.1%!

- FRET is possible for different pH values (example: pH 3 and pH 7, see appendix). Justification and comparison of the figures

- Include table of used concentrations with error and error of weighed-in quantity in evaluation

- Calculate the overlap integral J and thus the Förster radius of the FRET pair at pH value 7 from the available measurement graphs and data. To do this, calculate the integrals of the individual spectra using Origin. Determine the error of the calculation.

- Calculates the average wavelength from the multiple measurement of the HSA

- Plots the measured variables

- Carry the relative donor fluorescence $\frac{I_0}{I} - 1$ (I_0 Donor fluorescence without acceptor, I donor fluorescence with acceptor) as a function of the acceptor concentration (Stern-Volmer plot). Determine the Stern-Volmer constants from this. Give these as well as the dissociation constants with error and the relative donor fluorescence.

- Plots the FRET efficiency as a function of the acceptor concentration and explains the course of the curves. Calculates the error

- Calculates the distance between donor tryptophan and acceptor 3-HF and for quercetin including error calculation.

- Discussing errors, looking at critical steps in implementation

General comment:

- An error calculation is to be carried out for each calculated quantity.

- For all calculated errors, the corresponding error calculation must be included in the protocol!

- Insert the table with all calculated values incl. errors in the appendix as well as the measured data!

Control questions for the colloquium

- Name some fluorescent substances, which substances fluoresce and why

(life span, examples from daily life, atomic phosphorescent markers,....)

- Information content of fluorescence spectra

- Repetition of fluorescence including Jablonski diagram, Franck-Condon principle and its effects, quench types.

- At which wavelength does tryptophan absorb and at which wavelength does it fluoresce?

- What conditions must a FRET pair fulfil

- Lambert-Beer's law

- Fermi's golden rule

- Dipole-dipole interactions, transition dipole moment, dependence of the orientation factor κ on the dipole moment

- What are the possible applications in science and industry

- Meaning of important terms in the experiment such as overlap integral, FRET efficiency, etc.

- What is meant by mean, median, quantile, variance, standard deviation, regression line, meaning of R^2 (see Excel), error propagation?

- What is meant by accuracy, scatter and precession in analytics?

Appendix

Preset measurement data for evaluation:

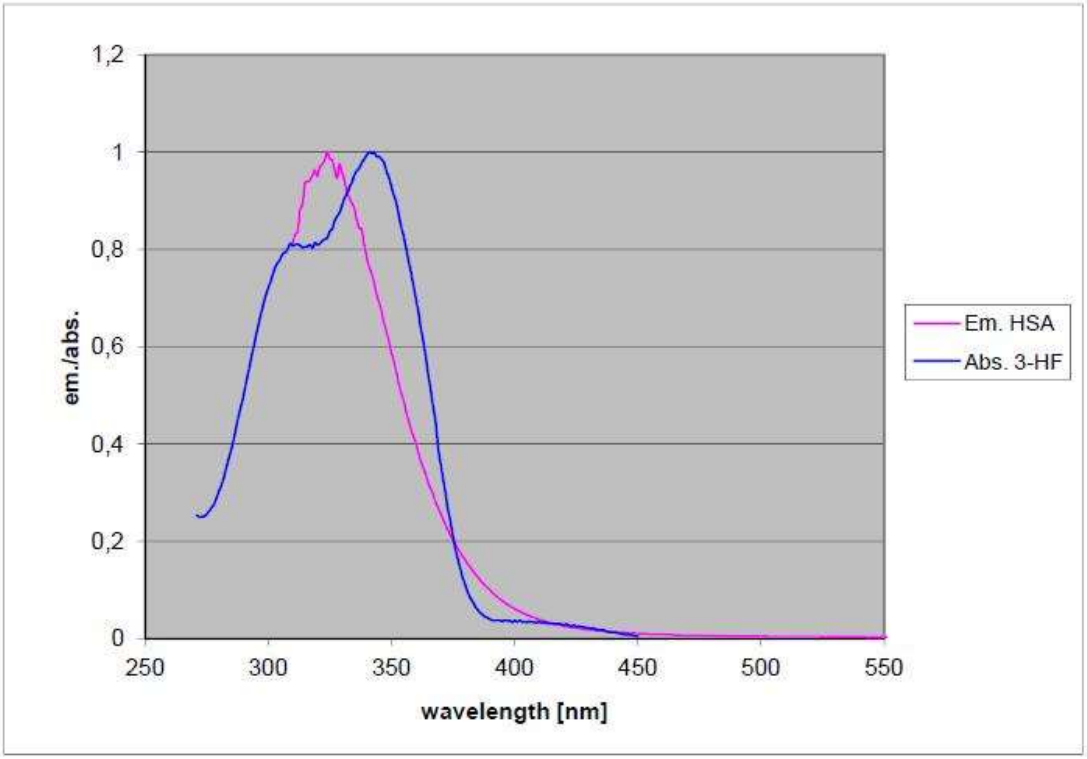

Fig. 8 Emission spectrum of HSA and absorption spectrum of 3-HF at pH=3.

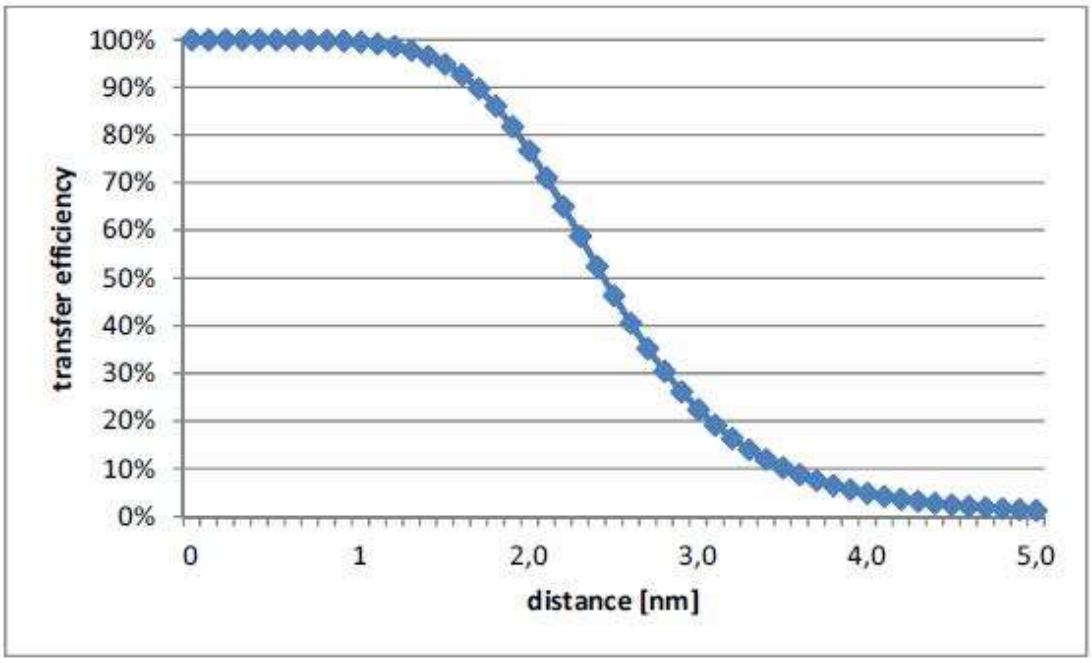

Fig. 9 Distance dependence of the transfer efficiency of the FRET pair at pH=3.

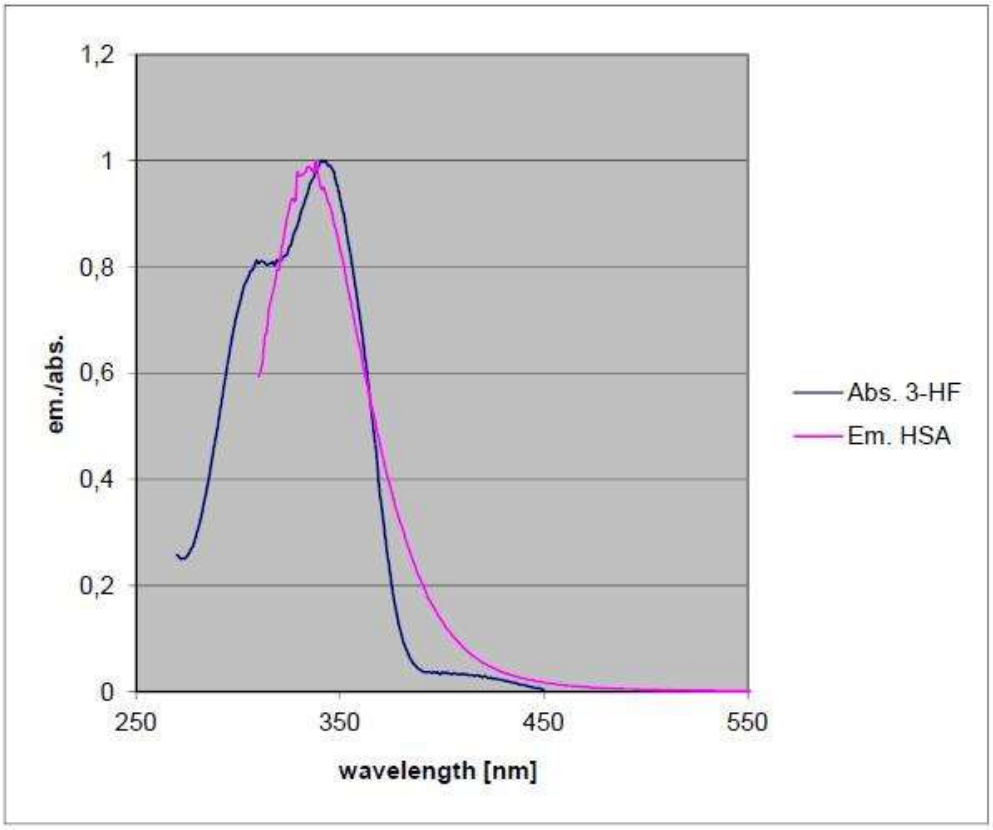

Fig. 10 Emission spectrum of HSA and absorption spectrum of 3-HF at pH=7.

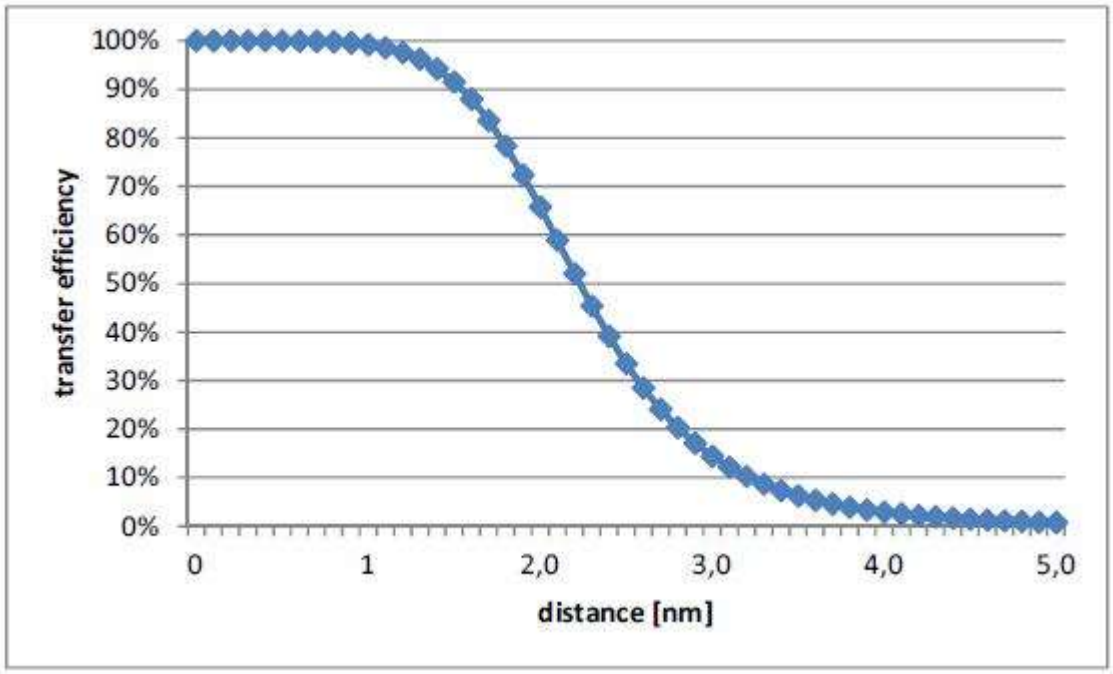

Fig. 11 Distance dependence of the transfer efficiency of the FRET pair at pH=7.

Literature

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