

# Fluorescence Quenching

## Summary

The emission of light from the excited state of a molecule (fluorescence or phosphorescence) can be quenched by interaction with another molecule. The stationary and time-dependent observation of such processes reveals insight into the deactivation mechanisms of the excited molecule and can be used for monitoring distance and orientation changes between different parts of biomolecules. In this experiment you will record fluorescence spectra of different dyes and measure the fluorescence intensity after adding quencher molecules at different concentrations. Fluorescence lifetimes are derived from a Stern-Volmer analysis of this data.

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# 1 Introduction

## 1.1 Fluorescence

When a molecule absorbs light in the visible or ultraviolet range of the spectrum, it is excited from the electronic ground state to an excited state. From there it can return to the ground state by releasing the absorbed energy in the form of heat and by radiation in the visible or near-infrared spectral range. The emitted light is called fluorescence (or phosphorescence if the excited state is a triplet state, see below). Fluorescence can be detected with very high sensitivity even from single molecules and it is used in a large number of chemical and biochemical applications. Sensitive fluorescence detection relies on the fact that the emitted light usually has a longer wavelength than the intense light used for excitation, which can therefore be suppressed by filters or monochromators. This difference between absorption and fluorescence wavelength (maxima) is also known as Stokes shift and can be understood in the following way: in addition to the change of electronic structure absorption can also lead to the excitation of vibrational levels, which requires more energy or light of shorter wavelength. In some molecules like benzene, this leads to a distinct pattern (vibrational progression) in the absorption spectrum, as shown on the left hand side of Figure 1. In solution, the vibrational energy is very quickly dissipated by collisions with the solvent and the molecule adopts a new equilibrium configuration from where emission takes place. Emission can again populate excited vibrational states, this time however, in the electronic ground state (right hand side of Figure 1). In contrast to the excitation process, the energy gaps are now smaller, leading to a shift of the fluorescence to longer wavelength.

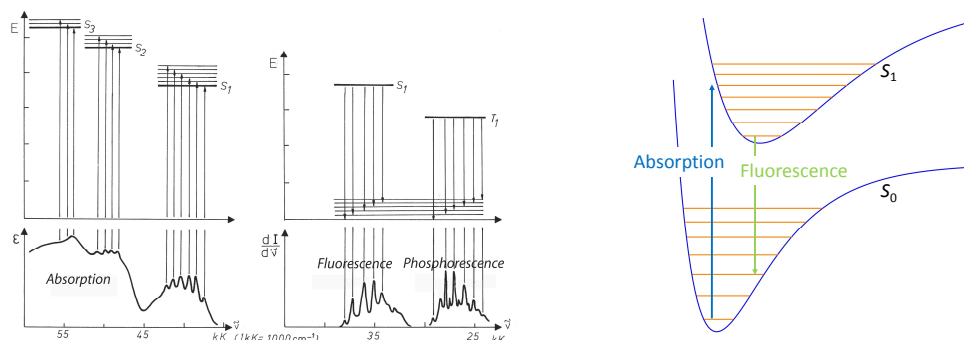


Figure 1: Absorption and emission of light in the case of benzene (left) and schematically for two shifted potential energy surfaces (right). The excitation of vibrational levels leads to a blue shift in absorption and a red shift in emission.

## 1.2 Singlet and Triplet States

If we describe the electronic states of a molecule using simple molecular orbital theory, absorption of light at longest wavelength corresponds to a transition of an electron from the highest occupied orbital to the lowest unoccupied orbital (HOMO → LUMO transition). There are two different possibilities for this excitation: The two electrons, which

had opposite spin in the HOMO can also have opposite spin when in two different orbitals. The corresponding excited state is then called a singlet state  $S_1$ . If the spin of the two electrons points in the same direction in LUMO and HOMO, the molecule is in a triplet state  $T_1$ . Because electrons with opposite spin can stay further apart (Hund's rule) the triplet state is usually lower in energy than the corresponding singlet state. This situation is depicted at the left hand side of Figure 2.

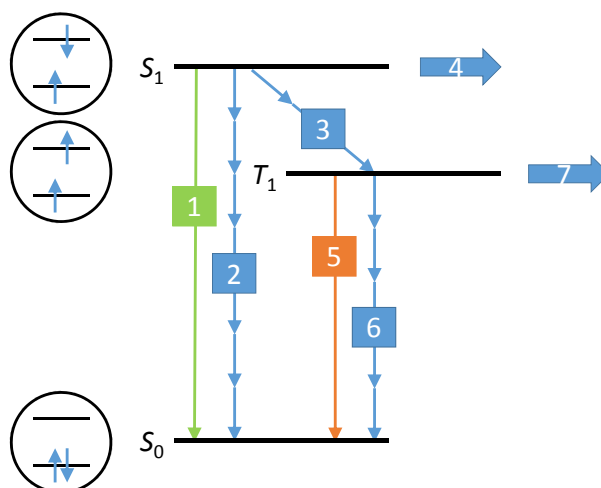


Figure 2: Ground state  $S_0$  and first excited singlet and triplet states  $S_1$  and  $T_1$  of a molecule. The corresponding spin configurations in the HOMO and LUMO are shown schematically on the left. Arrows illustrate radiative, non-radiative and reactive deactivation processes as explained in the text.

### 1.3 Deactivation Processes

Because of the large excess energy (more than 100 times the typical thermal energy  $kT$ ), many things can happen with a molecule after electronic excitation. The most important processes of deactivation for a polyatomic molecule are illustrated in Figure 2:

1. Radiative decay  $S_1 \rightarrow S_0$  (Fluorescence): Usually after very fast vibrational relaxation in  $S_1$ . Rate constant  $k_f \leq 10^9 s^{-1}$ .
2. Non-radiative deactivation  $S_1 \rightarrow S_0$ : After a fast vibrational relaxation in  $S_1$  energy is transferred to highly excited vibrational states of the electronic ground state  $S_0$ . Via collisions with solvent molecules as well as through emission of infrared radiation, the molecule finally reaches its vibrational ground state in  $S_0$ . Rate constant  $k_{nf}$ .
3. Non-radiative deactivation  $S_1 \rightarrow T_1$  (Intersystem Crossing): This is a radiationless process as above, which however includes a spin change and is therefore very slow in the absence of heavier elements. Rate constant  $k_{isc}$ .

4. Photo reactive channel  $S_1 \rightarrow$  photoproduct: This is usually a reaction of first order with rate constant  $k_r$ . Sometimes, however, this can be a second order (bimolecular) reaction.

After an intersystem crossing process (ISC) the molecule reaches the triplet state  $T_1$ , with similar deactivation channels:

5. Radiative deactivation  $T_1 \rightarrow S_0$  (Phosphorescence): This transition is spin-forbidden, which results in small rate constants:  $k_p$  is usually around  $10^{-1} - 10^0 s^{-1}$ .
6. Non-radiative deactivation  $T_1 \rightarrow S_0$  (Intersystem Crossing): In contrast to the singlet state, radiationless deactivation of  $T_1$  can often compete with the radiative decay. Rate constant  $k_{nrT}$ .
7. Photo reactive channel  $T_1 \rightarrow$  photoproduct: Bimolecular reactions are more likely than in the singlet state because of the much longer lifetime of the triplet states. Reaction rate constant  $k_{rT}$ .

## 1.4 Fluorescence Decay

The most direct way to observe the deactivation of the excited state of a molecule is to monitor the fluorescence intensity as a function of time after the excitation light has been switched off. The fluorescence will then decay exponentially with the excited state population at the rate:

$$k_f + k_{nf} + k_{isc} + k_r = k_f + k_{nf} = 1/\tau \quad (1)$$

where we have introduced  $k_{nf} = k_{nr} + k_{isc} + k_r$  as the sum of all rates of first order processes that do not lead to fluorescence. The inverse of this rate  $\tau$  is the time it takes until the detected intensity has reached  $1/e$  of its original value (see Figure 3). In order to

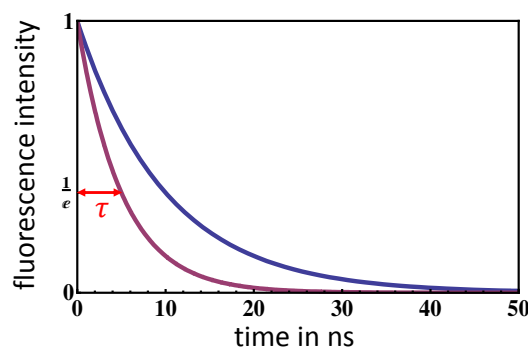


Figure 3: Fluorescence intensity as a function of time after the excitation light has been switched off. The decay time  $\tau$  is the time at which only  $1/e$  of the initial fluorescence is seen. Blue:  $\tau = 10$  ns, Red  $\tau = 5$  ns.

record a fast fluorescence decay directly, however, we need very short light pulses (usually pulsed lasers), a fast detector and fast electronics. When  $k_{nf}$  is very large and fluorescence

lifetimes are on the sub-nanosecond timescale, even more involved experimental methods are needed. In this practical course you will use an indirect method for determining nanosecond lifetimes, which relies on a further deactivation process which is discussed in detail below:

## 1.5 Energy transfer and assisted relaxation

Excited state deactivation by energy transfer is illustrated in Figure 4, depicting the HOMO and LUMO spin configurations. The photo excited molecule, called **donor**, starts in the  $S_1$  configuration and has a larger gap between HOMO and LUMO than the **acceptor** molecule, which is initially in the  $S_0$  ground state. As the donor returns to the ground state, the acceptor is promoted to the excited state. There are two different mechanisms by which this **energy transfer** can take place:

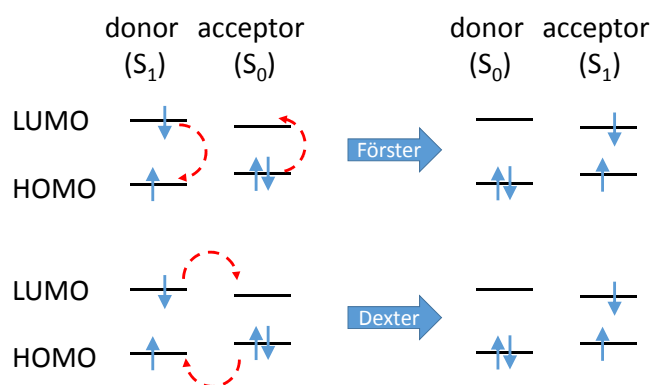


Figure 4: Changes in spin configuration of HOMO and LUMO during energy transfer.

**Förster mechanism:** Charge fluctuations in donor and acceptor can influence each other over distances of the order of 10 nm if they occur near resonance of an electronic transition in both molecules (transition dipole interaction). The probability of energy transfer in this case is proportional to the overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor and decreases with donor-acceptor distance  $R$  like  $1/R^6$ . This mechanism is responsible for the transfer of energy from the light-collecting antenna complexes to the reaction centre in natural photosynthesis.

**Dexter mechanism:** When donor and acceptor come sufficiently close for their orbitals to overlap, the excited electron of the donor can be transferred to an unoccupied orbital of the acceptor. At the same time, an electron of the acceptor moves to the HOMO of the donor. This process is only effective for donor-acceptor distances smaller than 15 Å. A common variant of this process is triplet quenching, when the donor is initially in the  $T_1$  state.

The excited states of typical quenchers like  $I^-$  are usually too high in energy for efficient resonant excitation transfer from dyes that emit in the visible, however, there can still be directed **electron transfer** from one molecule to another.<sup>[4]</sup> During reductive quenching the quencher transfers an electron to the excited molecule, which stops to fluoresce. This

is a very important step in many photocatalytic reactions, for example for solar fuel production.<sup>[2]</sup>

Heavy elements can also quench fluorescence by strongly enhancing the rate of intersystem crossing (i.e. the change from triplet to singlet states or vice versa) and this relaxation mechanism is therefore known as the **External heavy atom effect**. The nearby heavy atom only favors the spin flip in the originally excited molecule but is not excited itself as in the Förster or Dexter mechanisms.

Apart from the reactive channels, which may require a second molecule as a reaction partner, all the deactivation processes shown in Figure 2 are first order processes, meaning that they are independent of concentration. Transfer of excitation or electrons to another molecule or the external heavy atom effect, on the other hand, are strongly concentration-dependent second order processes. They are not only very important deactivation mechanisms in many biological systems, the quenching of the excited state by another molecule can also be used for the determination of short fluorescence lifetimes by relatively simple means.

## 1.6 Stern-Volmer Method

We can gain information about the fluorescence lifetime and excited state deactivation by introducing quenchers (heavy ions or acceptor molecules) and observing the fluorescence intensity as a function of their concentration. To see how this is possible we build a rate model for the concentration of molecules in the fluorescing excited state  $S_1$  and use the following notations:

$[M]$	Concentration of the fluorescing molecule in the ground state
$[M^*]$	Concentration of the molecule (donor) in the fluorescing $S_1$ state
$[Q]$	Concentration of the quenching molecule (acceptor in the ground state)

Neglecting the possibility photo chemical reactions, the following processes contribute to a change of  $[M^*]$  (compare Figure 2):

Photo excitation	$M \rightarrow M^*$	$k_{\text{abs}}[M]$
Fluorescence	$M^* \rightarrow M$	$k_f[M^*]$
Non-fluorescent relaxation (intramolecular)	$M^* \rightarrow M$ (or other excited state)	$k_{\text{nf}}[M^*]$
Quenching (intermolecular)	$M^* + Q \rightarrow M + Q$ (or $Q^*$ )	$k_q[M^*][Q]$

The fraction of excited molecules at any time is usually very small (unless intense pulsed light sources are used), so  $[M] \approx \text{const.}$  Defining  $I_{\text{abs}} = k_{\text{abs}}[M]$  we obtain the following differential equation for the excited state population:

$$\frac{\partial[M^*]}{\partial t} = I_{\text{abs}} - (k_f + k_{\text{nf}} + k_q[Q])[M^*] \quad (2)$$

Without quencher molecules ( $[Q] = 0$ ) the equation becomes even simpler:

$$\frac{\partial[M^*]}{\partial t} = I_{\text{abs}} - (k_f + k_{\text{nf}})[M^*] \quad (3)$$

Under continuous irradiation a stationary state is quickly established and the excited state population  $[M^*]$  is constant ( $\partial[M^*]/\partial t = 0$ ).

An important quantity for the determination of different reaction rate constants is the **fluorescence quantum yield**  $\Phi$ :

$$\Phi = \frac{\text{number emitted photons}}{\text{number absorbed photons}} = \frac{\text{rate of emission}}{\text{rate of absorption}} \quad (4)$$

In our notation, the rate of absorption is  $I_{\text{abs}} = k_{\text{abs}}[M]$  and the rate of emission is  $k_{\text{f}}[M^*]$ . With the help of equations 2 and 3 under stationary conditions ( $\partial[M^*]/\partial t = 0$ ), we obtain for the quantum efficiencies with and without quencher molecules:

$$\Phi_Q = \frac{k_{\text{f}}}{k_{\text{f}} + k_{\text{nf}} + k_{\text{q}}[Q]} \quad (5)$$

and

$$\Phi_0 = \frac{k_{\text{f}}}{k_{\text{f}} + k_{\text{nf}}} \quad (6)$$

The ratio of the quantum yields is equal to the ratio of the observed emission intensities without and with the quencher molecules:

$$\frac{I_0}{I_Q} = \frac{\Phi_0}{\Phi_Q} = \frac{k_{\text{f}} + k_{\text{nf}} + k_{\text{q}}[Q]}{k_{\text{f}} + k_{\text{nf}}} = 1 + \frac{1}{k_{\text{f}} + k_{\text{nf}}} k_{\text{q}}[Q] \quad (7)$$

Inserting the fluorescence lifetime in the absence of the quencher molecules (equation 1) we obtain the final result (also known as Stern-Volmer equation):

$$\frac{I_0}{I_Q} = 1 + \tau_{\text{f}} k_{\text{q}}[Q] \quad (8)$$

## 1.7 Estimating the quenching rate

The Stern-Volmer equation allows us to determine the product  $\tau_{\text{f}} k_{\text{q}}$  from the slope of a plot of  $I_0/I_Q - 1$  against the quencher concentration  $[Q]$ . In order to extract the fluorescence lifetime of the excited molecules we thus need to know the quenching rate  $k_{\text{q}}$ . To estimate it, we assume that the quenching process is **diffusion-limited**. This means that it is much less likely that molecule and quencher come close to each other than that they interact (or exchange electrons as a donor/acceptor pair) once they actually meet. In other words, quenching (by deactivation or electron transfer) would take place much more often, if quencher and molecule were to meet more frequently.  $k_{\text{q}}$  is then given by the rate at which  $M^*$  and  $Q$  meet. For two (equal) solid spheres in a solution of viscosity  $\eta$  this second order diffusion rate is given by (see e.g. [3])

$$k_{\text{diff}} = \frac{8RT}{3\eta} \quad (9)$$

where  $R=8.314 \text{ JK}^{-1}\text{mol}^{-1}$  is the gas constant,  $T$  is the temperature in Kelvin and  $\eta$  is the viscosity in  $\text{kg m}^{-1} \text{ s}^{-1}$ . If quenching is diffusion-limited,  $k_{\text{q}} \approx k_{\text{diff}}$  should be inversely proportional to the viscosity of the solution. Note that usually only the heavy atom effect or excitation transfer of the Dexter type (electron exchange) are diffusion limited because Förster energy transfer can take place over a much longer distance.

## 2 Experiment

### 2.1 Fluorescence Spectrometers

For fluorescence measurements we need to selectively excite molecules to the desired excited state, requiring light at a certain wavelength or in a limited wavelength range. For this purpose, commercial fluorescence spectrometers often use the same kind of monochromator arrangement that is found in absorption spectrometers (see the UV-vis experiment in this course). Emitted fluorescence is then again frequency selected (by a second monochromator) and can be recorded as a function of emission wavelength. In this way, emission from different excited states can be separated and, most importantly, the excitation light can be suppressed. You will have the opportunity to measure emission spectra of your dye solutions in this way.

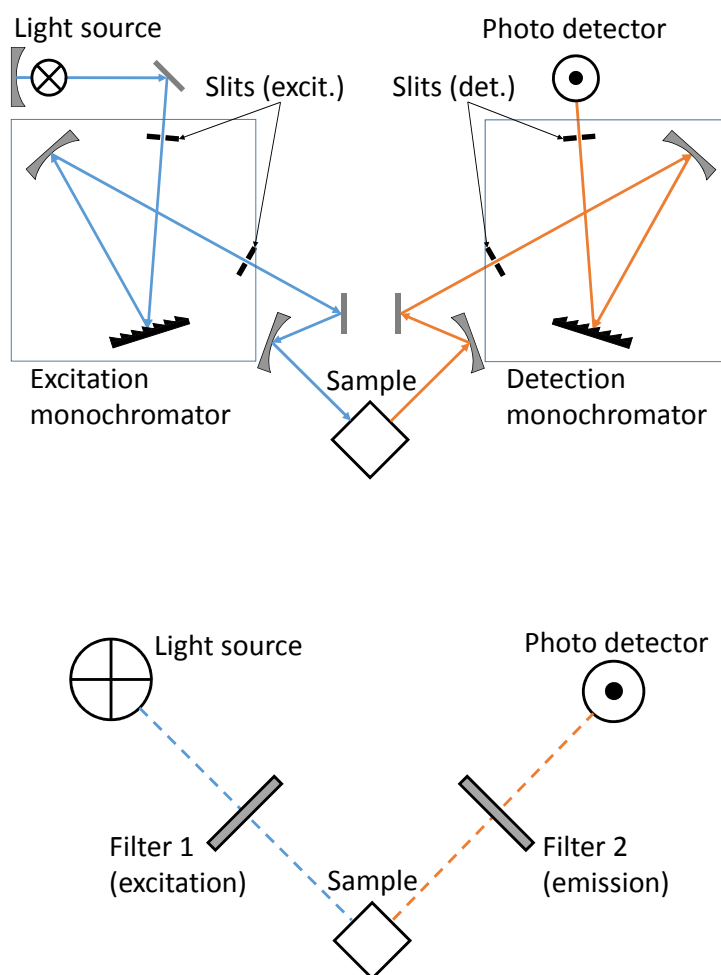


Figure 5: Schematic view of a fluorescence spectrometer (top) and a simple setup for Stern-Volmer and lifetime analysis (bottom). In a commercial spectrometer, excitation and emission wavelengths are selected by monochromators instead of filters

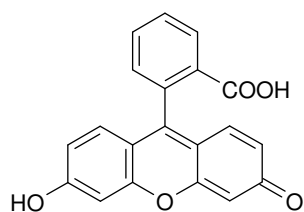
The fluorescence intensity data for the Stern-Volmer analysis can, on the other hand,



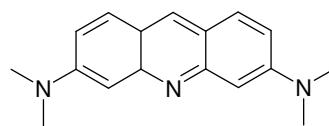
be measured with a much simpler setup as shown in Fig. 5. Here the selection of excitation and detection wavelength is achieved with different filters, and/or by using a narrow-band light source, such as an LED. If we modulate the LED light source intensity at high frequency (tens of kHz), we can also measure the phase shift of a long-lived fluorescence signal ( $>100$  ns) and determine the lifetime without the Stern-Volmer analysis. This is explained in more detail in the Appendix.

## 2.2 Dye Molecules

Like many organic dye molecules, acridinorange is an aromatic compounds, which intercalates well with DNA and can be mutagenic. In the high dilutions in which it may be used in this experiment, however, it is not very dangerous. Fluorescein, on the other hand, is rather harmless and is sometimes added to color rivers for hydrological studies. Nonetheless, carefully pay attention to cleanliness and safety. **Use protective gloves and change them regularly.** Remove spilled substance immediately. In case of skin contact wash thoroughly with water. The safety sheets for these compounds can be found in the laboratory.



Fluorescein



Acridinorange

## 2.3 Experimental Tasks

The instructions are for the dye fluorescein. An analogous procedure can be followed for acridinorange with  $\text{CrSO}_4^-$  quencher in pure water.

Fluorescein can serve as an indicator, because its fluorescence quantum efficiency drops strongly at acidic pH ( $\text{p}K_a=6.4$ ). Record the absorption and fluorescence spectra of fluorescein in basic aqueous solution and measure the integrated fluorescence intensity as a function of quencher concentration (KI). Deduce the fluorescence lifetime from a Stern-Volmer plot and repeat the measurements in a water/glycerol mixture with higher viscosity to test if fluorescence quenching is diffusion limited. Proceed as follows:

- Prepare a dilution series with 5 different quencher concentrations by adding different amounts of the stock quencher solutions to 25 ml flasks as detailed in the table in the appendix. Use a burette to add quencher solution to the empty flasks and weigh the flasks to determine the exact amount of quencher added. Add exactly 10 ml of dye stock solution (provided) to all 5 flasks and fill them with 0.1M KOH solution to 25 ml.
- Record the absorption spectra of the solution without quencher and with the highest quencher concentration, using the spectrometers of the UV-vis experiment.

- From the absorption data, deduce the range of possible excitation wavelengths and record the fluorescence spectra of the same solutions with the commercial fluorescence spectrometer. Adjust the slit width of the excitation and detection monochromators to achieve good signal to noise without saturation (see Appendix).
- Use the simpler setup (with appropriate filters if necessary) to record the fluorescence intensities  $I_Q$  of the samples with different quencher concentration. There is a turning knob and a switch with two positions at the detector. In one position of the switch, the knob can be adjusted until the output voltage is minimal (0). Flip the switch after adjustment and do not touch the knob anymore. The voltage you read is now proportional to the fluorescence intensity. Before and after each measurement of a quencher solution, also record the fluorescence intensity of the dye-only solution to obtain  $I_0$  under identical conditions for normalization.
- Repeat each measurement 2-3 times. Estimate the range of the signal fluctuations and try to obtain a mean value.
- Prepare three solutions with identical dye concentrations and increasing amounts of glycerol. Then prepare identical solutions all with the same quencher concentration (see the second table in the appendix).
- Measure the fluorescence intensity ratios of the water/glycerol solutions with and without quencher (average over several readings).
- Dispose of all dye solutions in the aqueous waste container. First collect all the alkaline fluorescein solutions in a beaker and neutralize them.

### 3 Data Analysis

- Generate combined plots of normalized absorption and fluorescence spectra.
- Calculate the exact quencher concentrations in all solutions.
- Form the ratios of quenched and unquenched fluorescence intensity ( $I_0/I_Q - 1$ ) and plot them against the quencher concentrations  $[Q]$  (Stern-Volmer equation 8).
- Determine the product  $\tau_f k_q$  from the gradient of these plots (with errors and units).
- Assuming that quenching is diffusion-controlled ( $k_q = k_{\text{diff}}$ ) calculate the quenching rate constants  $k_q$  using equation 9 ( $\eta_{H_2O} \approx 10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$ ).
- Compute the fluorescence lifetime  $\tau_f$  and the experimental uncertainty.
- Plot  $(I_0/I_Q - 1)$  for the three water/glycerol mixtures as a function of viscosity. The viscosity of water/glycerol mixtures<sup>[4]</sup> can be read off Figure 7 in the appendix or calculated with the corresponding [Mathematica CDF program](#). Do your results support the assumption that quenching is diffusion controlled?

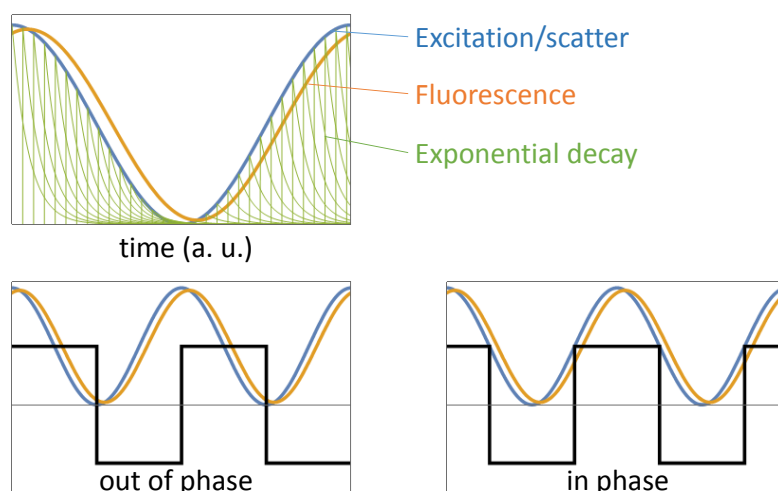


Figure 6: Time modulation of the excitation light (blue) and the fluorescence signal (orange). When the lifetime  $\tau$  is a significant fraction of the modulation period  $2\pi/\omega$ , the fluorescence signal is shifted in time. Bottom: the detector multiplies the signal with a reference signal (black) and yields a time-integrated output.

## 4 Appendix

### A Lifetime determination via phase shift measurements

Unless we make use of a pulsed laser, it is very difficult to turn off the excitation light fast enough to observe the fluorescence decay directly. It is, however, possible to modulate a light source - in particular modern LEDs - at 10-50 kHz frequencies. As illustrated in Fig. 6, this is slower than a typical decay time. Since the detector sees the sum of all the fluorescence signals excited at different moments in time, the fluorescence light is slightly time-shifted with respect to the excitation (or scattered) light. This shift becomes larger with increasing fluorescence lifetime. Mathematically, the detected intensity is the convolution of the cosine-modulated excitation (blue) and the exponential fluorescence decay (green):

$$S(t, \omega, \tau) = \int_{-\infty}^{\infty} (1 + \cos \omega t_0) \frac{\Theta[t - t_0]}{\tau} \exp\left[-\frac{(t - t_0)}{\tau}\right] dt_0 = \frac{1}{1 + \tau^2 \omega^2} (\cos \omega t + \tau \omega \sin \omega t) \quad (10)$$

A very sensitive way of measuring the time-shift is to multiply the fluorescence signal  $S$  with a reference signal which is changing between positive and negative at the modulation frequency, as shown by the black lines in Fig 6. When the sign change of this reference occurs at the maxima and minima of the modulation cosine (bottom left in Fig. 6), integration yields the *out of phase* signal

$$I_{\text{out of phase}} = \int_0^{\frac{\pi}{\omega}} S(t, \omega, \tau) - \int_{\frac{\pi}{\omega}}^{\frac{2\pi}{\omega}} S(t, \omega, \tau) = \tau \frac{4}{1 + \tau^2 \omega^2} \quad (11)$$

This signal should be zero when  $\tau = 0$ , so we can use scattered excitation light to properly adjust the delay of the reference signal. When we now change the switch on the detector, the reference signal is time-shifted by exactly a quarter period (bottom right in Fig. 6) and we record the *in phase signal*:

$$I_{\text{in phase}} = - \int_0^{\frac{\pi}{2\omega}} S(t, \omega, \tau) + \int_{\frac{\pi}{2\omega}}^{\frac{3\pi}{2\omega}} S(t, \omega, \tau) - \int_{\frac{3\pi}{2\omega}}^{\frac{2\pi}{\omega}} S(t, \omega, \tau) = \frac{1}{\omega} \frac{4}{1 + \tau^2 \omega^2} \quad (12)$$

The ratio of these two signals is simply

$$\frac{I_{\text{out of phase}}}{I_{\text{in phase}}} = \omega\tau = 2\pi f\tau \quad (13)$$

where  $f$  is the frequency at which the light source is modulated. The procedure for measuring the fluorescence lifetime of a dye molecule is thus rather straightforward:

- Remove any filters and replace the sample with a piece of paper in order to record the scattered excitation light.
- Turn the potentiometer knob until the *out of phase* detector output is zero.
- Measure the *in phase* and the *out of phase* signals of your sample.
- Measure the modulation frequency  $f = \omega/(2\pi)$  by connecting an oscilloscope or frequency meter to the BNC output of the detector.
- Calculate  $\omega\tau = I_{\text{out of phase}}/I_{\text{in phase}}$  and hence determine  $\tau$ .

## B Sample Preparation

### B.1 For Stern-Volmer plot (25 ml flasks, all values in ml):

Sample label	1a	1b	1c	1d	1e	
0.1 M KI (in 0.1M KOH)	0	2	5	7	10	burette and weigh pipette fill to level
Fluorescein (in 0.1M KOH)	10	10	10	10	10	
0.1M KOH	15	13	10	8	5	

### B.2 For viscosity dependent measurements (25 ml flasks, all values in ml):

sample	2a	2b	2c	3a	3b	3c	
Fluorescein (in 0.1M KOH)	10	10	10	10	10	10	pipette pipette burette fill to level
0.1 M KI (in water)	0	0	0	5	5	5	
Glycerol	0	5	10	0	5	10	
Water	15	10	5	10	5	0	

Note that a neutral quencher solution is used here instead of the 0.1 M KOH solution above in order to simplify the sample preparation. The final OH concentration is thus 0.04 M, which is still more than enough to maintain the fluorescence characteristics of fluorescein.

## C The FL Winlab Software

- After starting the software check that the program saves ASCII format data: Go to Utilities  
Configuration  
and set Default spectral format to ASCII. This is rather old software. Note that you cannot use directory- or filenames with spaces or more than 8 characters!
- Use an existing SCAN method or go to Application and select Scan.
- Select Emission, enter the start and stop wavelength for the emission spectrum you want to record and enter a suitable excitation wavelength.
- Adjust the slit width of the excitation and detection monochromators to get sufficient signal without saturation (1000 units).
- Add suitable filters for excitation or detection if necessary.

## D Viscosity of Water Glycerol Mixtures and other useful values

Acridinorange	438.10 g/mol
Fluorescein	332.32 g/mol
Gas constant R	8.314 JK <sup>-1</sup> mol <sup>-1</sup>
Unit centiPoise	1 cP = 10 <sup>-3</sup> kg m <sup>-1</sup> s <sup>-1</sup>
	1 mol/m <sup>3</sup> = 10 <sup>-3</sup> mol/l

[5–7]

## References

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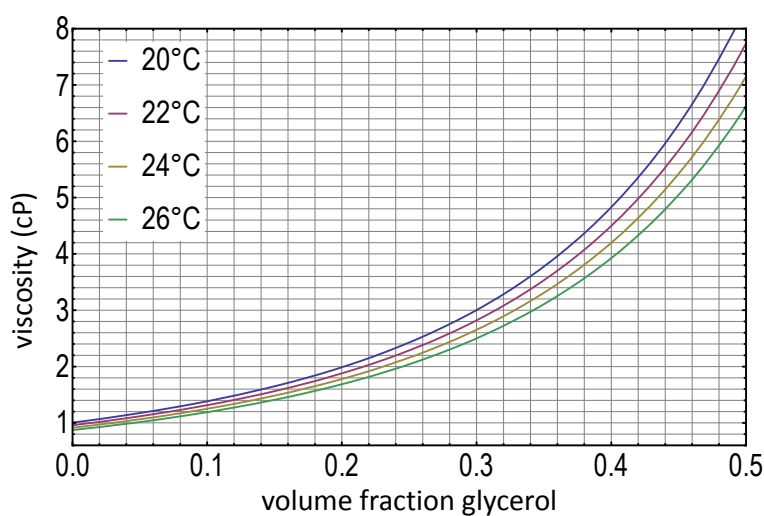


Figure 7: Viscosity of glycerol/water mixtures at different temperatures as a function of the volume fraction of glycerol (according to the empirical formula given by Nian-Sheng Cheng, *Ind. Eng. Chem. Res.* 2008, 47, 3285-3288).

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