Faculty of Science

University of South Bohemia in České Budějovice

Bachelor thesis

Characterisation of quenching of far red organic fluorescent probe by DNA nucleobases

Barbora Emmerová

Tutor: Doc. RNDr. Frantisek Vacha, Ph.D.

Tutor specialist: Mgr. Tomas Fessl

České Budějovice, 2010

Bachelor thesis

Emmerova, B., 2010. Characterisation of quenching of far red organic fluorescent probe by DNA nucleobases,
Faculty of Science, The University of South Bohemia, České Budějovice, Czech Republic.

Annotation: This work reports the fluorescence quenching of the zwitterionic fluorescent dye ATTO 680 by oligonucleotides of adenine, guanine, cytosine, and thymine.

Anotace: Tato práce podává zprávu o zhášení fluorescence zwitterionického fluorescenčního barviva ATTO 680 oligonukleotidy adeninu, guaninu, cytosinu a thyminu.

Annotation: Die vorliegende Arbeit befasst sich mit der Fluoreszenzlöschung des zitterionischen fluoreszierenden Farbstoffes ATTO 680 durch die Oligonukleotide von Adenin, Guanin, Cytosin und Thymin.

Affirmation:

I hereby declare under oath that the submitted bachelor thesis has been written solely by me without any third-party assistance. Additional sources or aids are fully documented in this paper, and sources for literal or paraphrased quotes are accurately credited.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my dissertation thesis, in full / in shortened form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.
In České Budějovice, 20 May 2010

……

Barbora Emmerová

Acknowledgment

Herein I would like to to give great thanks to my tutor Tomas Fessl for his patience, support and qualified leading during this work. Special thanks to Frantisek Vacha.

I also want to give great thanks to my parents for a great moral and financial support. Last but not least, I want to thank a lot my sister Jaroslava, brother Jiri, my dear friends and Pavel for help with carrying of the heavy burden of academic studies.

This project is supported by the grant GAAV - KAN200100801 of the Academy of Sciences of the Czech Republic.

Abstract:

This work reports the fluorescence quenching of the zwitterionic fluorescent dye ATTO 680 by DNA bases. The aim of our effort was to acquire knowledge of the quenching of the fluorophore ATTO 680 by oligonucleotides of adenine, guanosine, cytosine, and thymine, in order to use it for the suggestion of a new method to investigate the DNA structure. Generally it is supposed, that guanine is the only quenching nucleobase. The Stern-Volmer analysis was performed to examine the quenching efficiency of different DNA bases. Quenching activity of other bases than guanine was proved and quantified. Additionaly, the fluorescence lifetime of all four bases was measured to distinguish the mechanism of quenching. The measurement suggests the nucleobases quenching is static.

Key words: fluorescence quenching, zwitterionic dye, nucleobasis fluorescence quenching, Stern-Volmer analysis

I. CONTENTS

II. ABBREVIATIONS

A	adenine
C	cytosine
CD	circular dichroism
ELISA	enzyme-linked immunoassays
FISH	fluorescence in situ hybridization
FRET	Förster resonance energy transfer
FTIR	Fourier transform infrared (spectroscopy)
G	guanine
Hb	hemoglobin
HCV	hepatitis C virus
ODF	oligodeoxynuceloside fluorophore
PET	photoinduced electron transfer
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
S-V	Stern-Volmer plot
T	thymine
Trp	tryptophan

III. FIGURES

IV. AIMS

In my bachelor thesis, I would like to explore the quenching efficiency of DNA nucleobases. According to general knowledge, only guanine is thought to have a quenching activity. In this work I would like to quantify quenching activity of guanine and other nucleobases. The final goal is to find out, if our fluorescence probe is suitable for development of a new structural assay based on different quenching efficiency of particular dye by specific DNA nucleobases. I solved following subquestions: what is the possible mechanism of nucleobases quenching, and what is the quenching activity of particular nucleobases; this second task was solved by the Stern-Volmer analysis.

The main motivation for my experiments was the need of suitable fluorescent probe for development of a new structural assay based on quenching phenomena. This assay will scan the interaction between fluorescent probe and its nanoenviroment, in our case between probe and particular nucleobases. When we will have a suitable fluorescent probe differently reacting to particular nucleobases, we can move the position of the probe and hence create the matrix of the quenching efficiency of local nanoenviroments for particular position of probe. From this matrix we gain structural information. Compared to today widely used FRET method, this method has several advantages. First, it has a higher sensitivity and resolution as it occurs at shorter distances than FRET, and last but not least it is a cheaper method than FRET as only one dye is required for an experiment.

V. INTRODUCTION

Fluorescence methods are thanks to their nonivasivity and quickness a useful and popular tool. In recent years the fluorescence plays role in fields such as environmental monitoring, clinical chemistry, DNA sequencing, and genetic analysis by fluorescence in situ hybridization (FISH). Generally the phenomenon of fluorescence is used for cell identification or cellular imaging to discover the localization and movement of intracellular substances by the fluorescence microscopy. In medical tests the fluorescence is still preferred because of its costs and easy handling compared to radioactive assays. Moreover, these tests are already being widely used as enzyme-linked immunoassays (ELISA) or fluorescence polarization immunoassays.

V. a) Fluorescence principles

Fluorescence phenomena is nicely described by the Jablonski diagram (see Figure X). When a fluorophore absorbs light energy, it is usually excited to a higher vibrational energy level in the first excited state (S(1)) followed by the rapid relaxing to the lowest S(1) energy level. This event, depicted in the figure as a "stair-step" transition from the upper to lower bars in S(1), is termed vibrational relaxation or internal conversion and occurs in about a picosecond or less. Fluorescence lifetimes are typically four orders of magnitude slower than vibrational relaxation, giving the molecules sufficient time to achieve a thermally equilibrated lowest-energy excited state prior to the fluorescence emission. Phosphorescence decay is similar to fluorescence, except the electron undergoes a spin conversion into a so-called "forbidden" triplet state (T(1)) instead of the lowest singlet excited state, a process known as intersystem crossing. Emission from the triplet state occurs with lower energy relative to fluorescence, hence emitted photons have longer wavelengths. With delayed fluorescence, the electron first decays into the triplet state, and then crosses back over into the lowest singlet excited state before returning to the ground state.The thermally relaxed excited electronic state can also result in following processes (also called deexcitation processes). Excitation transfer - donor to acceptor energy transfer (also called FRET – Förster Resonance Energy Transfer). If the transfer occurs or how fast it goes depends on the donor-acceptor spectral energy overlap, the donor-acceptor distance (about 1.5 – 10 nm) and their orientation towards each other, and the donor fluorescence lifetime. Quenching - the excited state lifetime decrease by the processes

such as excited state reactions, energy transfer, complex-formation and collisional quenching. Quenching will be described in more detail.

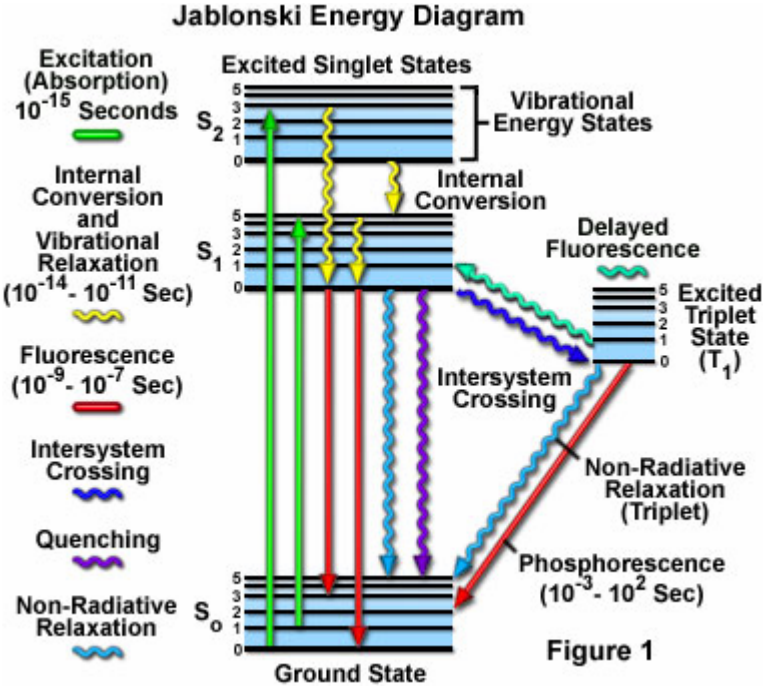


Fig.1: Jablonski diagram describing electronic states of a molecule and the transitions between them. (the picture is taken from Olympus Microscopy, Fluorescence Microscopy Interactive Java Tutorials, 2010)

This image illustrates the singlet ground state (S(0)), in addition to the first (S(1)) and second (S(2)) excited singlet states as a stack of horizontal lines. In the diagram, the thicker lines represent *electronic energy levels*, while the thinner lines denote the various *vibrational energy states*. *Transitions between the states* are illustrated as straight or wavy arrows, depending upon whether the transition is associated with *absorption or emission* of a photon (straight arrow) or results from a molecular internal conversion or non-radiative relaxation process (wavy arrows). Vertical upward arrows indicate the instantaneous nature of *excitation processes*, while the wavy arrows are reserved for those events that occur on a much longer timescale.

Immediately following the absorption of a photon, various processes may occur with different probabilities. The most likely is the *relaxation to the lowest vibrational energy level* of the

first excited state (S(1)). The relaxation process is known as *internal conversion* or *vibrational relaxation* (loss of energy in the absence of light emission) and generally occurs in a picosecond or less.

An excited molecule exists in the lowest excited singlet state (S(1)) for periods on the order of nanoseconds (the fluorescence process is the longest time period) before finally relaxing to the ground state. If relaxation from this long-lived state is accompanied by emission of a photon, the process is called the *fluorescence*.

Several other relaxation pathways that have varying degrees of probability compete with the fluorescence emission process. The excited state energy can be dissipated *non-radiatively* as *heat* (illustrated by the cyan wavy arrow in Figure 1), the excited fluorophore can collide with another molecule to transfer energy in a second type of non-radiative process (for example, *quenching*, as indicated by the purple wavy arrow in Figure 1), or a phenomenon known as *intersystem crossing* to the lowest excited triplet state can occur (the blue wavy arrow in Figure 1). The latter event is relatively rare, but ultimately results either in emission of a photon through *phosphorescence* or a transition back to the excited singlet state that yields *delayed fluorescence*. Transitions from the triplet excited state to the singlet ground state are "forbidden", which results in rate constants for triplet emission that are several orders of magnitude lower than those for fluorescence. (I.D.Johnson and M.W.Davidson, 2010)

Fluorescence lifetime

The fluorescence lifetime refers to the average time the molecule stays in its excited state before emitting a photon. The original fluorescence lifetimes (usually in ns) are often decreased by the competing processes. The intrinsic fluorescence lifetime originates from the transition dipole moment of spontaneous emission. Fluorescence typically follows first-order kinetics:

$$[S_1] = [S_1]_0 0e^{-\Gamma t}, \tag{1.1}$$

where [S1] is the concentration of excited state molecules at time t, $[S1]_0$ is the initial concentration and Γ is the decay rate or the inverse of the fluorescence lifetime. This is an

instance of exponential decay. Various radiative and non-radiative processes can de-populate the excited state. Than we can express the total decay rate is the sum over all rates:

$$\left[\Gamma_{tot}\right] = \Gamma_{rad} + \Gamma_{nonrad}, \tag{1.2}$$

where Γ_{tot} is the total decay rate, Γ_{rad} the radiative decay rate, and Γ_{nrad} the non-radiative decay rate. (Kenneth Sauer and Martin Debreczeny, 1996; Lakowicz, 1999)

Quantum yield

The *quantum yield* Q_y is the ratio of the photons emitted relative to the photons absorbed. In other words, the compound with high quantum yield shows the bright emission. (Lakowicz, 1999)

$$Qy = \frac{(\#\,\text{photons emited})}{(\#\,\text{photons absorbed})} \tag{1.4}$$

The maximum fluorescence quantum yield is 1.0; every photon absorbed results in a photon emitted. The quantum yield approaches 1 when the non-radiative decay is much slower than the radiative decay. (Lakowicz, 1999)

V. b) The Fluorescence Quenching, Collisional and Static Quenching

Many kinds of the fluorescence quenching can be distinguished due to the cause of the fluorescence decrease. Generally, we can distinguish two cases of quenching, static and dynamic quenching. (Lakowicz, 1999)

Static quenching (or *contact quenching*) is caused by the non-fluorescent complex formation in the ground state. E.g. Zn^{2+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} .

Dynamic quenching (or *collisional quenching*) is caused by the collision of fluorophore and the quencher molecule. E.g. oxygen, amines, halogens, or an electron-deficient molecule (e.g. acrylamide).

The *collisional quenching*, is caused by the crash of another molecule that deactivates the fluorophore excited-state. The calculation may be done following the Stern – Volmer equation: (Lakowicz, 1999)

$$\frac{F_0}{F} = 1 + K[Q] = 1 + k_q \tau_0 [Q], \quad (1.5)$$

Where K is the Stern – Volmer quenching constant, k_q is the bimolecular quenching constant, τ_0 is the unquenched lifetime, [Q] denotes the quencher concentration, and F_0/F denotes fluorescence intensities without/with the quencher.

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q], \quad (1.6)$$

Where K_D means the Stern – Volmer quenching constant in the case of dynamic quenching.

$$K_D = k_q \tau_0, \quad (1.7)$$

F_0/F is linearly dependent upon the quencher concentration

The plot of the dependence of F_0/F upon [Q] intercepts value 1 on the y-axis. The slope is equivalent to K_D.

K_D^{-1} = [Q] quencher concentration at which $F_0/F = 2$ which means that 50% of the intensity is quenched. (Lakowicz, 1999)

$$\frac{F_0}{F} = \frac{\tau_0}{\tau}, \quad (1.8)$$

Where F_0/F denotes fluorescence intensities without/with the quencher and τ_0/τ means the lifetime without/with the quencher.

In the case of collisional quenching the decrease is equivalent in fluorescence intensity F and in lifetime τ. The static quenching does not decrease the lifetime, in this case only the

fluorescent molecule is seen and the fluorophore that is not bound in the complex has the unquenched lifetime τ_0. (Lakowicz, 1999)

Static quenching is caused by the nonfluorescent fluorophore-quencher complex formation. (Lakowicz, 1999)

The relation between fluorescence intensity and quencher concentration:

$$K_S = \frac{[F-Q]}{[F][Q]}, \tag{1.9}$$

Where [F-Q] is the concentration of the complex, [F] is the uncomplexed fluorophore, and [Q] is the concentration of the quencher, K_s is the equilibrium constant.

$$[F_0] = [F] + [F-Q], \tag{1.10}$$

Where $[F_0]$ is the fluorophore total concentration. Therefore by the substitution we obtain the equation (1.11).

$$K_S = \frac{[F_0]-[F]}{[F][Q]} = \frac{[F_0]}{[F][Q]} - \frac{1}{[Q]} \tag{1.11}$$

By the rearrangement of equation (1.11) one can obtain equation (1.12).

$$\frac{F_0}{F} = 1 + K_S[Q] \tag{1.12}$$

The fluorescence intensity (F_0/F) is linearly dependent on [Q] – as in the case of dynamic quenching (Lakowicz, 1999)

The static and dynamic quenching differs in their dependence on temperature and viscosity and the lifetime measurement.

a) increased temperature speeds up the diffusion resulting in increased number of collisional quenching. Increased temperature leads to the dissociation of the weakly-bound complexes decreasing the number of static quenching. (Lakowicz, 1999)

b) the static and dynamic quenching can be distinguished by the absorption spectra of the fluorophore. The dynamic quenching does not affect the absorption spectra, it changes only the excited states of the fluorophore. In the static quenching the complex formed in the ground state leads to the perturbation of the absorption spectrum of the fluorophore. (Lakowicz, 1999)

c) the main difference between static and dynamic quenching is in the fluorescence lifetime τ (Lakowicz, 1999):

- for **static quenching:** $\tau_0/\tau = 1$

- for **dynamic quenching:** $\tau_0/\tau = F_0/F$

V. c) Applications of quenching

Basically, any action decreasing the fluorescence intensity of a sample substance can be understood as a quenching process. A numerous processes such as **excited state reactions**, **energy transfer**, **complex-formation**, and **molecular collisions** can result in a **quenching**. Quenching offers itself as an indicator of processes which takes place at the molecular level. Nowadays, quenching is being used of in various types of sensors; for example the quenching effect of oxygen on certain rubidium complexes enables the measurement of oxygen saturation in solution. Quenching can be understood as the basis for fluorescence resonance energy transfer (**FRET**) analyses (Peng et al., 2006; Peng et al., 2009; Osterman et al., 2009). In addition, quenching and dequenching upon interaction with a particular molecular biological target is the base for activatable optical contrast agents for **molecular imaging**. (Blum et al., 2009; Weissleder et al., 1999) Fluorescence quenching reports the

conformational dynamics of macromolecules. (Sauer et al.,2009) According to Sauer et al. (2009) the photoinduced electron transfer (**PET**) between suitable fluorophore and electron donating substances, such as tryptophan Trp or the nucleobase guanine, quenches the fluorescence upon van der Waals contact and therefore reports on molecular contact. As far as we focus on dynamic and static quenching only, the dynamic quenching transiently alters the fluorescence lifetime of the fluorophore and the quantum yield, whereas the static quenching in π-stacked complexes efficiently diminishes fluorescence emission over time scales longer than the fluorescence lifetime. Thus, the static quenching provides sufficient contrast for the detection at a single-molelcule level. (Sauer et al.,2009)

Kool et al. (2009) employed a fluorescence quencher dabcyl with oligodeoxynuceloside fluorophores ODFs revealing a strong sequence dependence of **quenching of ODFs**. They suggest a high importance of delocalized excited states of the π-stack of fluorophore dyes in the fluorescence quenching. (Kool et al., 2009)

Ning Xu et al. (2009) utilized a nucleobase-quenched probe for polymerase chain reaction (PCR) genotyping to achieve a **detection of single nucleotide polymorphisms (SNPs)**.

According to Noda et al. (2009) a high-throughput **assay of hepatitis C virus (HCV) helicase** inhibitors can be performed using the fluorescence quenching. The helicase unwounds dsDNA releasing the dye from the guanine bases, which is evident as the dye emits fluorescence.

In addition, a guanine quenched fluorophore can be employed for the fluorescence assay for prion protein. (Geng Fu Xiao et al., 2009) According to Geng Fu Xiao et al. (2009) the aptamer-mediated turn-on fluorescence assay is well suited for the **protein analysis**. (Aptamer is an expression for an oligonucleic acid or peptide molecule that binds to a specific target molecule.)

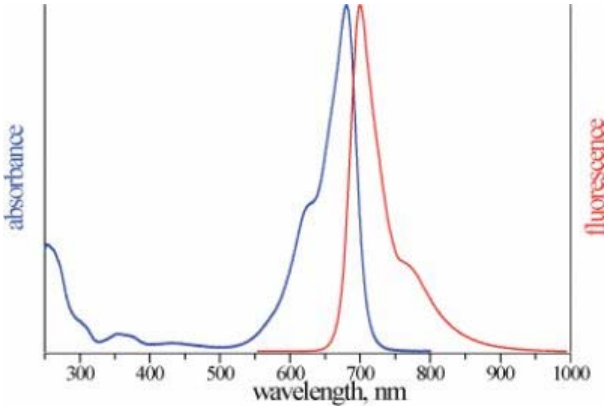
Mahato et al. (2010) investigated the formation of a **bioconjugate of human hemoglobin (Hb) with silver (Ag)** as well as the extent of interaction using different spectroscopic and microscopic techniques. Their fluorescence study showed that the Tryptophan (Trp) residues of Hb are in a more polar environment after conjugation. Initial fluorescence enhancement upon the addition of silver is caused by a metal-enhanced fluorescence. In addition, the fluorescence quenching after the formation of the Hb-Ag bioconjugate follows the altered

Stern-Volmer (S-V) plot. The S-V plot and the time-resolved fluorescence study suggest the presence of both static and dynamic types of quenching. Moreover, the reduction potential values of the substances (Hb-heme, Ag(+), and Trp) could have been caused by electron transfer. The secondary structure determination from CD and FTIR spectra suggests alpha-helix to beta-sheet conversion, and besides that the unfolding of Hb also influences the bioconjugate formation. The overall data indicate a change in both the secondary and the tertiary structure of hemoglobin after conjugation with silver. (Mahato et al., 2010)

VI. MATERIALS AND METHODS

The fluorescent dye

The fluorescent dye atto 680 was selected. Atto 680 is a zwitterionic dye falling into a new generation of fluorescent labels. This label can be characterised by strong absorption, high fluorescence quantum yield, good water solubility, and high thermal and photo-stability. Atto 680 is also a strong electron acceptor. Atto 680 fluorescence can be, according to general knowledge, quenched by electron donors like guanine, tryptophan, etc. Therefore it is suitable for quenching assays in the area of life science. The fluorescence of atto 680 is excited most efficiently in the range of wavelength 645 - 695 nm.



λ_{abs} = 680 nm

ε_{max} = 1.25 x 10^5 M^{-1} cm^{-1}

λ_{fl} = 700 nm

η_{fl} = 30 %

τ_{fl} = 1.8 ns

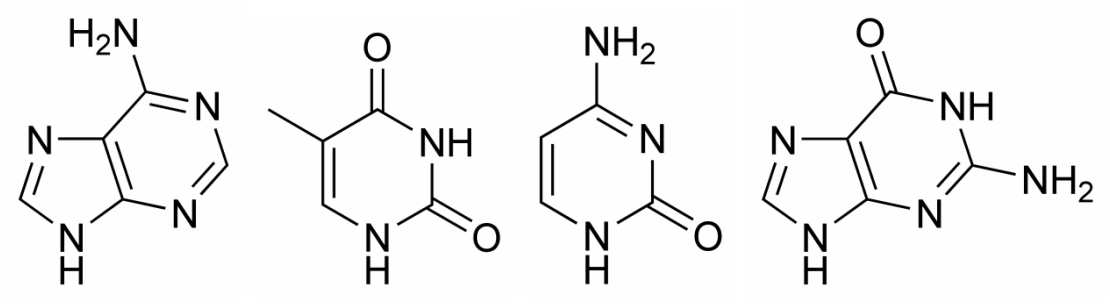
CF_{260} = 0.30

CF_{280} = 0.17

Fig. 2: Absorption (blue) and emission (red) spectra of the fluorescent dye ATTO 680. (ATTO-TEC, ATTO 680 Product Description, 2010)

The Quencher

The DNA nucleobases were chosen to be probed as the fluorescence quenching agens. For this purpose 14-mer of deoxyadenosine, deoxyguanosine, deoxycytidine and deoxythymidine were used. (Sigma-Aldrich, Inc.) Chemical formulas are shown in figures 3 - 6. For simplification, only bases of particular deoxynucleotides are depicted.



Adenine Thymine Cytosine Guanine

Fig. 3 - 6: The structural formulas of nucleobases adenine, thymine, cytosine, and guanine.

Used oligonucleotide sequences:

1) 5'-AAAAAAAAAAAAAA-3'

2) 5'-TTTTTTTTTTTTTT-3'

3) 5'-CCCCCCCCCCCCCC-3'

4) 5'-GGGGGGGGGGGGGG-3'

V. a) The fluorescence quenching measurement:

The apparatus

Emission and absorption spectra Emission spectra were measured at room temperature using a Fluorolog spectrofluorometer (SPEX, USA), filtered by Raman emitter RS 664 LP (679.3 - 1497.7 nm). The room temperature absorption spectra were recorded with an UV300 spectrophotometer (Spectronic Unicam,UK)

Each measurement was three times repeated under unaltered conditions for the sake of reproducibility of the measurement.

For the fluorescence measurement 1 cm diameter quartz cuvette was filled with 1 ml of the dye solution. Concentrations of oligonucleotides and fluorescence dye in the saples were adjusted as follows:

Sample	Concentration of quencher (μM)	Concentration of ATTO 680
Blank	0 μM	5 μM
$Sample_1$	7,5 μM	5 μM
$Sample_2$	3,75 μM	5 μM
$Sample_3$	1,875 μM	5 μM
$Sample_4$	0,9275 μM	5 μM
$Sample_5$	0,46875 μM	5 μM

V. b) The fluorescence lifetime measurement:

The apparatus

The microscope configuration consists of: inverted Olympus IX70 microscope, Triax 320 imaging spectrograph with back illuminated liquid nitrogen-cooled CCD camera (Spectrum One, Jobin Yvon, 2048x512 pixels, pixel size 13,5 x 13,5 μm) and picosecond-pulse laser diode module (PicoQuant LDH-D-C-640, 640 nm, lin. polar) as an excitation source. We used objective Olympus 100x, NA of 1.35, UplanApo, and the samples were excited at 640 nm by prism total internal reflection. The microscope is equipped with an Olympus filter cube (Olympus, Japan) containing Raman emitter RS 664 LP (679.3 - 1497.7 nm), HC-Laser Clean-up MaxDiode 640/8 (Semrock, Germany). Two single photon counting avalanche photodiodes (Perkin Elmer, SPCM-AQR-16) are attached to the side exit of the Triax monochromator for measurement of the fluorescence lifetime.

Composition of a sample buffer was chosen to minimize the dye photobleaching with a respect to the DNA properties. The buffer contained 0,01% Tween 20, 200 μM sodium L-ascorbate, 0.3 mM EDTA, and 10 mM TRIS at pH 7,4.

A mixture of the buffer with ATTO 680 and a nucleobase with the final volume of 150 μl was used for the fluorescence lifetime measurement. The concentration of pure ATTO 680 was 1 μM. The solutions of 1 μM ATTO 680 with the 0,1 μM quencher were mixed with the final concentration of 0.667 μM ATTO 680 and 3.33 μM quencher.

VII. RESULTS

VII. a) The fluorescence quenching measurement:

Stern-Volmer diagram in the figure 7 shows us the comparison of quenching measurements done with all four types of oligonucleotides. The intensity of quenching of nucleobases is linearly dependent on the quencher concentration. The quenching efficiency of each quencher can be concluded from the slope of each line shown in the table 1.

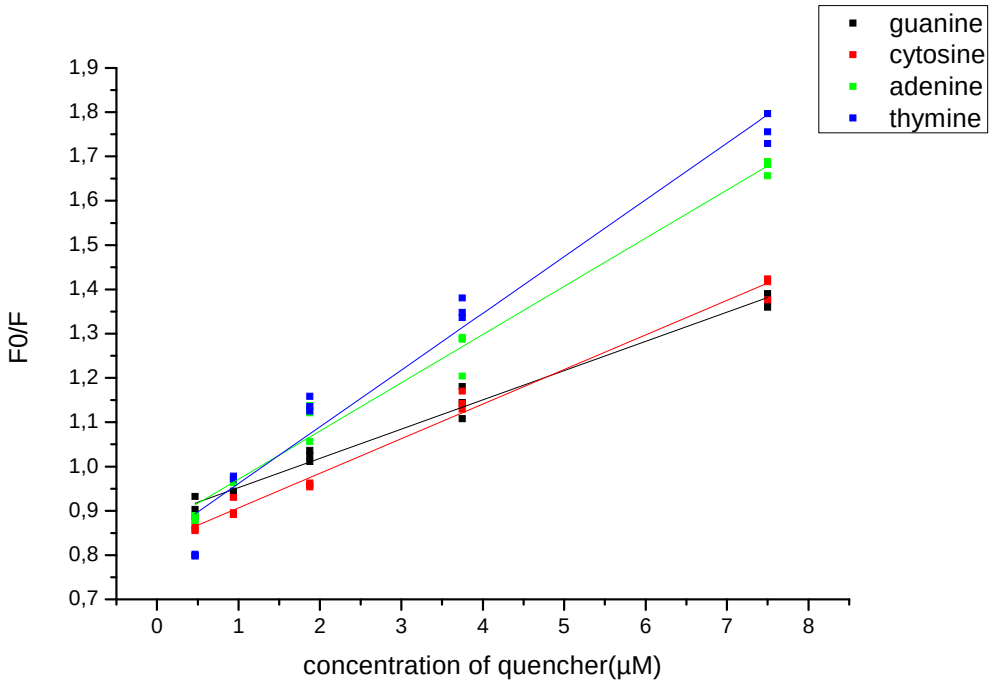


Fig. 7: Stern-Volmer diagram of all four quenchers. F_0/F indicates fluorescence intensities without/with the quencher. The slope of the linear fit shows us the efficiency of the quenching. (see Tab.1) Three measurements of each quencher has been performed.

It was found that not only guanin is capable of fluorescence quenching. In the case of fluorescence dye ATTO 680 all four bases exhibit quencing properties with oligoadenin and oligothyamine as the most efficient quenchers. Table 1 sumarizes the quenching parameters of the oligonucleotides used in theis study. The slope of each line represents the efficiency of

the quenching of the base, the intercept is the point of crossing the y-axis, and the standard error and the R-square evaluate the accuracy of the fit.

Linear fit		**Value**	**Standard Error**	**R-Square**
guanine	intercept	0,88618	0,02237	0,95125
	slope	0,06608	0,00578	
cytosine	intercept	0,82829	0,00866	0,98864
	slope	0,07816	0,00224	
adenine	intercept	0,86235	0,01395	0,98488
	slope	0,1089	0,0036	
thymine	intercept	0,83352	0,02501	0,96552
	slope	0,12811	0,00646	

Tab. 1: The table of values obtained from the linear fit of our measured data.

VII. b) The fluorescence lifetime measurement:

In order to define which of the fluorescence quenching mechanisms is responsible for the quencing of ATTO 680 fluorescence, we have performed series of fluorescence lifetime measurements with all four base oligonucleotide samples. The result of these experiments is in the figure 8 showing practically constant fluorescence lifetimes for all samples with the value of about 1.8 ns

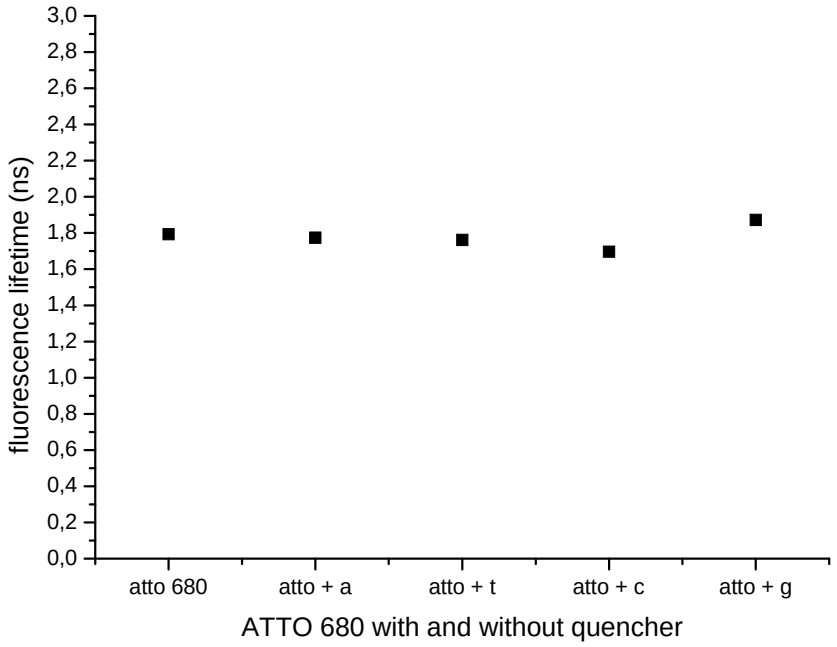


Fig. 8: The fluorescence lifetime measurement. In this diagram "atto + a" means ATTO 680 with the addition of adenine quencher, "atto + t" means ATTO 680 with the addition of thymine, "atto + c" means ATTO 680 with the addition of cytosine, and "atto + g" means ATTO 680 with the addition of guanine.

VIII. CONCLUSION AND DISCUSSION

In my bachelor thesis I have investigated the quenching activity of DNA nucleobases. According to general knowledge, only guanine is thought to have a quenching activity. In this work I tried to quantify quenching activity of guanine and other nucleobases, cytosine, adenine, and thymine. The aim of my effort was to find out, if our fluorescence probe ATTO 680 is suitable for development of a new structural assay based on different quenching efficiency of the particular dye by specific DNA nucleobases. I have solved following subquestions: what is the quenching activity of particular nucleobases and. what is the possible mechanism of nucleobases quenching.

First, I have performed the fluorescence intensity measurements in order to quantify the nucleobases quenching, and then I measured the fluorescence lifetimes of the fluorophore with and without the quencher in order to confirm whether the quenching is static or dynamic.

The fluorescence intensity measurements described us the nucleobases quenching. As we have found out, not only guanine quenches the fluorescence. All four investigated nucleobases have a significant quenching activity, especially adenine and thymine. In the Stern-Volmer analysis the intercept of a linear fit with y-axis should be 1, as it is the fluorescence intensity without/with quencher at zero concentration of the quencher. In our case it approaches 0,9. This difference is caused by the measurement error. As the handling with the sample solution is not automated, it is not easy to avoid such an error. The measurement error in the range of 0,1 has been found in the literature as well. (Kool, 2009)

Fluorescence lifetimes of mixture of ATTO 680 with certain DNA nucleobases did not significantly differ. These results of the fluorescence lifetime measurements suggest that the quenching of the dye by nucleobases is static. We assume that the fluorescent probe forms a complex with the nucleobases, such complex has then decreased fluorescence compared to the pure ATTO 680.

In the beginning of our experiments our results were not quite constant. We figured out that the lamp needs to run for a few hours in advance in order to stabilise the light source and thus obtain completely constant signal. The second issue was the reproducibility of our

measurements based on the unaltered position of the cuvette during the sample exchange. Therefore, for the refilling of the cuvette the 250 µl "hamilton" glass microsyringe was carefully used without touching the cuvette in the holder because of the importance of completely unaltered position of the cuvette with the sample in the fluorescence set-up. Even small changes appeared to be highly significant. Finally, this method was found to be optimal.

This method can be understood as a new tool that opens the possibility to study and detect nucleic acid structures. It has also several advantages compared to widely used FRET method, such as higher sensitivity and lower costs.

Our measurements confirmed that the fluorescent dye ATTO 680 is well suited for this kind of experiments. Since ATTO 680 belongs to far red dyes, it appears to be a good candidate for *in vivo* applications.

IX. REFERENCES

ATTO-TEC, ATTO 680 Product Description, 2010. https://www.atto-tec.com/attotecshop/product_info.php?info=p116_ATTO-680.html

Blum G, Weimer RM, Edgington LE, Adams W, Bogyo M, 2009. Comparative Assessment of Substrates and Activity Based Probes as Tools for Non- Invasive Optical Imaging of Cysteine Protease Activity. PLoS ONE 4(7): e6374.

Doose S, Neuweiler H, Sauer M., 2009. Fluorescence quenching by photoinduced electron transfer: a reporter for conformational dynamics of macromolecules. Chemphyschem. 10(9-10): 1389-1398. Review.

Johnson I.D. and Davidson M.W., 2010. National High Magnetic Field Laboratory, 1800 East Paul Dirac Dr., The Florida State University, Tallahassee, Florida, 32310, http://www.microscopy.olympus.eu/microscopes/39_9231.htm

Lakowicz J. R., 1999. Principles of Fluorescence Spectroscopy 2nd Ed. Kluwer Academic/ Plenum Publishers, New York

Luo G, Zheng L, Zhang X, Zhang J, Nilsson-Ehle P, Xu N., 2009. Genotyping of single nucleotide polymorphisms using base-quenched probe: a method does not invariably depend on the deoxyguanosine nucleotide. Anal Biochem. 386(2): 161-166.

Mahato M, Pal P, Kamilya T, Sarkar R, Chaudhuri A, Talapatra GB., 2010. Hemoglobin-Silver Interaction and Bioconjugate Formation: A Spectroscopic Study. J Phys Chem B *in press*

Olympus Microscopy, Fluorescence Microscopy Interactive Java Tutorials, 2010. http://www.microscopy.olympus.eu/microscopes/39_9231.htm

Osterman, H., 2009. The Next Step in Near Infrared Fluorescence: IRDye QC-1 Dark Quencher. LI-COR Biosciences, Lincoln, NE 68504

Peng, X., Draney, D.R., Volcheck, W.M., 2006. Quenched near-infrared fluorescent peptide substrate for HIV-1 protease assay. Proc. SPIE 6097, 60970F

Peng, X., Chen, H., Draney, D.R., Volcheck, W.M., 2009. A Non-fluorescent, Broad Range Quencher Dye for FRET Assays. Anal Biochem 388: 220–228.

Sauer K. and Debreczeny M., 1996. Biophysical Techniques in Photosynthesis. Jan Amesz & Arnold J. Hoff (eds), Chapter 3 Fluorescence, Kluwer Academic Publisher, The Netherlands, pp.41-61

Tani H, Akimitsu N, Fujita O, Matsuda Y, Miyata R, Tsuneda S, Igarashi M, Sekiguchi Y, Noda N., 2009. High-throughput screening assay of hepatitis C virus helicase inhibitors using fluorescence-quenching phenomenon. Biochem Biophys Res Commun 379(4): 1054-1059.

Teo YN, Wilson JN, Kool ET, 2009. Polyfluorophore labels on DNA: dramatic sequence dependence of quenching. Chemistry 15(43): 11551-11558.

Weissleder R, Tung CH, Mahmood U, Bogdanov A, 1999. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. Nat Biotechnol 17 (4): 375–378.

Xiao SJ, Hu PP, Li YF, Huang CZ, Huang T, Xiao GF, 2009. Aptamer-mediated turn-on fluorescence assay for prion protein based on guanine quenched fluophor. Talanta 79(5): 1283-1286.