Photo-induced dark states in fluorescence spectroscopy – investigations & applications

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Abstract

This thesis focuses on investigations of transient dark states of fluorescent molecules using spectroscopic techniques. The main purpose is to show and convince the reader that transient dark states are not always a nuisance, but also represent an additional source of information. Several studies with fluorescence correlation spectroscopy were performed, all related to non-fluorescent states such as triplet state or isomerized states.

Photobleaching is one of the main problems in virtually all of the fluorescence techniques. In this thesis, mechanisms that retard photobleaching are characterized. Several compounds, antioxidants and triplet state quenchers, which decrease photobleaching, are studied, and guidelines for achieving optimal fluorescence brightness using these compounds are presented.

Triplet state quenching by several compounds was studied. Detailed investigations of the fluorescence quencher potassium iodide demonstrated that for some of fluorophores, except of quenching, there is fluorescence enhancement mechanism present. In agreement with the first publication in this thesis, antioxidative properties were found to play an important role in the fluorescence enhancement. Quenching of the triplet state is proposed as a tool for monitoring diffusion mediated reactions over a wide range of frequencies.

Specially designed fluorophores combining high triplet yields with reasonable fluorescence brightness and photostability were characterized for possible applications in novel super-resolution imaging techniques based on fluorescence photoswitching. Except of benefits for imaging techniques, photoinduced switching to non-fluorescent states could be used for monitoring molecular diffusion, which was also demonstrated in this thesis.

Studies of the triplet state kinetics of fluorophores close to dielectric interfaces were performed using fluorescence spectroscopy. The analysis of the triplet state kinetic can provide information about the local microenvironment and electrostatic interactions near dielectric interfaces.

Keywords: fluorescence correlation spectroscopy, triplet state, isomerisation, photobleaching, quenching, diffusion, total internal reflection, interface
Papers

This thesis is based on the following papers (reprinted with permissions by the publications):

**Paper I**  
*Strategies to Improve Photostabilities in Ultrasensitive Fluorescence Spectroscopy*  
Jerker Widengren, Andriy Chmyrov, Christian Eggeling, Per-Åke Löfdahl, and Claus A. M. Seidel  
DOI: 10.1021/jp0646325  
Contribution by author: The author performed part of the measurements and data analysis.

**Paper II**  
*Iodide as a triplet state promoter and quencher – mechanisms and possible implications*  
Andriy Chmyrov, Tor Sandén and Jerker Widengren  
Manuscript  
Contribution by author: The author performed all of the measurements and data analysis. The author worked out a major part of the theoretical framework. TS assisted with initial measurements and data analysis.

**Paper III**  
*Nitroxide spin-label quenching of fluorophore’s triplet state as a tool for studying diffusion mediated reactions in lipid membranes*  
Johan Strömqvist, Andriy Chmyrov, Sofia Johansson, August Andersson, Lena Mäler and Jerker Widengren  
Manuscript  
Contribution by author: The author performed the characterization of the quencher and the dye and performed solution measurements.

**Paper IV**  
*Characterization of new fluorescent labels for ultra-high resolution microscopy*  
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Recovery of photo-induced reversible dark states utilized for molecular diffusion measurements
Andriy Chmyrov, Tor Sandén and Jerker Widengren
Manuscript
Contribution by author: The author performed all of the instrument and experiment design, measurements, data acquisition and analysis. The author worked out a major part of the theoretical framework. TS assisted with discussions, simulations and preliminary measurements.

Paper VI
Triplet-State Investigations of Fluorescent Dyes at Dielectric Interfaces Using Total Internal Reflection Fluorescence Correlation Spectroscopy
Hans Blom, Andriy Chmyrov, Kai Haßler, Lloyd M. Davis and Jerker Widengren
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Contribution by author: The author together with HB conducted the measurements. The author performed reference measurements on a confocal setup, and computer simulations.

Paper VII
Electrostatic Interactions of Fluorescent Molecules with Dielectric Interfaces Studied by Total Internal Reflection Fluorescence Correlation Spectroscopy
Hans Blom, Kai Haßler, Andriy Chmyrov and Jerker Widengren
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Contribution by author: The author together with HB performed a large part of the measurements and data analysis.

Related publications by author not included in the thesis:

Paper VIII
Maximizing the Fluorescence Signal and Photostability of Fluorophores by Quenching Triplet and Radical States
Daniela Pfiﬁ, Stanislav Kalinin, Denis Dörr, Ralf Kühnemuth, Sebastian Overmann, Brigittre A. Bier, Andriy Chmyrov, Jerker Widengren, Thomas J. J. Müller, Klaus Schaper and Claus A. M. Seidel
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Chapter 1

INTRODUCTION

Fluorescence is a wonderful tool which provides great specificity and sensitivity for the investigation, analysis, control and diagnostics in many fields relevant to physical, chemical, biological and medical sciences. During the past 20 years there has been a remarkable increase in the use of fluorescence especially in the biological sciences. Fluorescence right now is a dominant tool used extensively in biotechnology, flow cytometry, medical diagnostics, DNA sequencing, forensic and genetic analysis, just to name a few [Lakowicz 2006]. Utilization of fluorescence for cellular and molecular imaging has also increased dramatically, especially with development of fluorescent proteins [Tsien 1998], which enable genetic encoding of a fluorescent label into the proteins of interest. New imaging techniques based on fluorescence offer single molecule sensitivity and are approaching single molecule resolution [Hell 2009a]. Fluorescence correlation spectroscopy (FCS) was developed for the characterization of the dynamics of molecular processes in systems at thermodynamic equilibrium [Magde et al. 1972]. With proper averaging of stochastic single molecule fluctuations, it provides an insight into single molecule dynamics by measuring statistical fluorescence intensity fluctuations on a macroscopic scale. Being used most often for measuring diffusion coefficients and concentrations, it can access any kinetics originating from a molecular process that manifests itself as a change in the fluorescence intensity [Krizevsky and Bonnet 2002]. Not only fluorescence emission per se provides useful information, but also its absence constitutes a complimentary or even major source of data. This thesis deals with a non-
Chapter 1. Introduction

fluorescent part of the fluorescence process – photoinduced transient dark states, such as triplet, isomerised or radical states of fluorescent molecules.

The thesis starts with an extended introduction to different aspects of fluorescence and some of the fluorescence techniques: photophysics, fluorescence microscopy and correlation spectroscopy, focusing mainly on features relevant for the following chapters. Further four chapters present the work behind the seven papers. In the last chapter the work is summarized and concluded.

Some of the fluorescence techniques were invented too early to be widely applied immediately – both confocal microscopy and fluorescence correlation spectroscopy belong to them. Several decades of technological advances were needed, but now these techniques represent the standard tools for molecular imaging and monitoring of molecular interactions. Nonetheless, further improvements, both in terms of technology and methodology, are persisting to arise, offering new possibilities and extending the practical applicability of these techniques. This thesis contributes to such everlasting efforts.

Despite almost 140 years since the first chemical synthesis of Fluorescein (one of the first and still one of the most widely used fluorophore) [Pawley 2006], the development of new fluorescent probes is still a necessity. New fluorescence imaging and detection methods desire to obtain higher fluorescence output rates in harsher excitation conditions for longer times. In Chapter 3 (paper I) problems of extension and maximization of fluorescence output are addressed, by the use of carefully selected additives. In this chapter it is shown that a large part of non-fluorescent fluorophore radicals can be regenerated back into active fluorescent emitters. Balancing the amount of additives is crucial for the success of such experiments, since any excess of additives will turn viable fluorophores into undesired dark states.

Fluorescence quenching is widely recognized as a highly versatile tool and applied for studying molecular interactions [Lakowicz 2006]. In Chapter 4 (papers II and III) the behavior of two common quencher molecules – potassium iodide and TEMPO is investigated. In paper II the beneficial effects of fluorescence recovery from transient dark states are observed for several fluorophores in the presence of iodide. A recipe in the sense of fluorophores and concentration range is proposed in order to take advantage of such effects. In paper III a quenching mechanism of triplet states of fluorophores is proposed for monitoring diffusion in lipid membranes. Advantage of the relatively long triplet lifetime is utilized, while still keeping the high sensitivity of fluorescence as a detection mode.

The presence of various long-lived dark transient states of fluorophores has gen-
erally been considered disadvantageous, as they reduce the fluorescence brightness and render the data analysis more complicated [Rasnik et al. 2006]. Chapter 5 (papers IV and V) reveals a different view on such dark states. In paper IV the fluorophores were intentionally developed and characterized for having high triplet yields, while being still reasonably fluorescent and photostable. The combination of such properties meets the requirements of new super-resolution imaging techniques. In paper V a concept is described where the transitions to reversible dark states are monitored for measuring the mobility of the molecules. This method features a simple instrumentation, possible parallelization and a wide range of accessible concentrations.

It is well known that the fluorescence properties can be modified close to interfaces [Hellen and Axelrod 1987]. Chapter 6 (papers VI and VII) address the properties of fluorophores near dielectric interface using the phenomenon of total internal reflection as an excitation mode. Since the use of the triplet states as readout parameter is increasing, paper VI provides an insight on photophysical kinetics of the fluorophore in the proximity of a dielectric interface. In paper VII the electrostatic interactions between dielectric surfaces and fluorophores possessing different electric charges are investigated. Although negligible from macroscopic point of view, these small charges and their interactions are vital for understanding dynamics of molecules close to interfaces.
Chapter 2

FLUORESCENCE

2.1 Fluorescence and photophysics

Fluorescence is a form of luminescence, the physical process of emission of light upon excitation of a molecule. There are several different types of luminescence, categorized according to the mode of excitation – photoluminescence (including fluorescence, phosphorescence and delayed fluorescence), chemoluminescence (resulting of a chemical reaction), bioluminescence (by a living organism), electroluminescence, (in response to an electric current passed through it), mechanoluminescence (resulting from any mechanical action on a solid), sonoluminescence (in response to ultrasound) and others [Valeur 2002]. In this thesis we will be dealing with photoluminescence – the process of emitting a photon caused by absorption of a photon(s).

There are two types of photoluminescence: fluorescence and phosphorescence. Fluorescence is typically a fast process (nanoseconds), involving the radiative relaxation of a molecule from a state with a paired spin to the ground state. Phosphorescence is normally a slow process (milliseconds), which involves the radiative relaxation from a state of higher spin multiplicity, usually a triplet state.

2.1.1. Historical background

The first reported observation of fluorescence was made by the Spanish physician
Nicolas Monardes in 1565. He described the wonderful peculiar blue color of an infusion of the wood called Lignum Nephriticum. This wood was further investigated by Boyle, Newton and others, but the phenomenon was not understood. The first reported observation of phosphorescence was made in 1602 by an Italian cobbler, Vincenzo Cascariolo, whose hobby was alchemy [Valeur 2002]. One day he went for a walk in the Monte Paterno area and he picked up some strange heavy stones. After calcination with coal, he observed that these stones glowed in the dark after exposure to light. It was recognized later that the stones contained barium sulfate, which, upon reduction by coal, led to barium sulfide – a phosphorescent compound.

Despite the fact that first recorded observation of phosphorescence was made later than that of fluorescence, the term “phosphorescence” is much older (meaning that this phenomenon was observed earlier, but the knowledge about that did not pass through time). It comes from the Greek word φωσφορός [fōsforos] = light carrying, (φῶ [fō] = light, φέρω [ferō] = to carry). The term phosphor has indeed been assigned since the Middle Ages to materials that glow in the dark after exposure to light [Valeur 2002]. The term “fluorescence” was introduced by Sir George Gabriel Stokes, a physicist and professor of mathematics at Cambridge in the middle of the nineteenth century. In his paper from 1853 he invented the term fluorescence, from the name of the fluorspar (mineral containing calcium fluoride: fluorite), which was known to emit light when exposed to solar light beyond the violet part of the spectrum. Interestingly, fluorescence in this mineral is due to the presence of small amounts of impurities (mainly europium ions, yttrium and dysprosium), because fluorite itself is not fluorescent.

The year earlier, Stokes reported about the phenomenon of emitting a light following absorption of light [Stokes 1852]. He formed the solar spectrum using a prism, and while moving a tube filled with a solution of quinine sulfate through the spectrum he observed a blue glow of the solution in the non-visible part of the spectrum corresponding to UV excitation. Stokes stated that the emitted light is always of longer wavelength than the exciting light, today known as Stokes’ law. However, already 10 years before that, the French physicist Edmond Becquerel published a paper where he reported about wavelength shifts for light emitted by calcium sulfide, which is phosphorescent. The term “luminescence” (coming from the Latin lumen = light) was introduced first as luminescenz by the physicist and light historian Eilhardt Wiedemann in 1888, to describe ‘all those phenomena of light which are not solely conditioned by the rise in temperature’, as opposed to incandescence (the emission of light, typically red and infrared, from a hot body due to its high temperature) [Valeur 2002].
The process of fluorescence can be divided into three main events, all of which occur at different timescales, separated by several orders of magnitude. First, the excitation of a molecule occurs in femtoseconds ($10^{-15}$ s) by absorption of an incoming photon of suitable energy. As a result, an electron gets promoted from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). Since the mass of an electron is at least three orders of magnitude lower than the mass of the nuclei, the transition time is too short for any significant displacement of nuclei. As a consequence, all electronic transitions in the energy-distance plot are vertical [Condon 1926; Franck and Dymond 1926], as it is described by the Franck-Condon principle. This means that an electronic transition out of the lowest vibrational state of the ground state $S_0$ of the molecule, which is mainly populated at room temperature conditions according to the Boltzmann distribution, will take place into a higher vibrational level of the first electronic excited state. Franck-Condon principle is applied equally to absorption and to fluorescence emission. This is illustrated in figure 2.1.

After excitation, the vibrational relaxation (typically on picoseconds, $10^{12}$ s, time scale) occurs to the lowest vibrational state of the excited state [Kasha 1950]

![Figure 2.1: Franck-Condon principle energy diagram. The potential wells are shown favoring transitions between the vibrational states $\nu = 0$ and $\nu = 2$.](image)
Chapter 2. Fluorescence

(Kasha’s rule – after the American scientist of Ukrainian origin Michael Kasha). Finally, emission of a longer wavelength photon and return of the molecule to the ground state occurs in the relatively long time period of nanoseconds (10^{-9} s).

Several important deviations from this simple scheme can occur, most of which are presented on Jablonski diagram [Jabłoński 1935] of fluorescence, named after polish physicist of Ukrainian origin Alexander Jablonski (1898-1980). One of the many possible variants of this diagram is presented in figure 2.2.

The electronic states are arranged vertically by energy and grouped horizontally by spin multiplicity (described later). The vibrational ground states of each electronic state are indicated with thick lines, the higher vibrational states with thinner lines.

Typically, a fluorescent molecule at the lowest ground state (S_0) is in a singlet state, which means that molecular orbital is occupied by two electrons with an opposite spin direction. Multiplicity is the quantification of the amount of unpaired electron spin and is calculated as 2s+1, where s is the number of singly occupied electrons multiplied by the electron spin projection quantum number m_s = -1/2 or +1/2 [Banwell and McCash 1994]. In the singlet state all of the electrons occupying molecular orbital are paired (have opposite spins) and the multiplicity is 1. This arrangement is strictly required by the Pauli Exclusion Principle. Upon excitation the electron preserves its spin and because of that the multiplicity of higher excited states (S_1, S_2) is also 1. The process of excitation in fluorophores is always a singlet-
singlet transition. After excitation to higher vibrational sublevels of a higher excited singlet state (typically \(S_1\)) a molecule quickly relaxes to the lowest vibrational level of the higher excited singlet state. Due to a short time (few nanoseconds) for which the electron resides in \(S_1\) state, and because of the energy mismatch for \(S_0-S_1\) and \(S_1-S_2\) transitions, there is typically no further excitation to higher singlet states. From the lowest vibrational sublevel of \(S_1\) state there are different de-excitation pathways to return to the ground state, \(S_0\). The most common one is a transition to one of higher vibrational sublevels of the ground singlet state. This transition can be radiative (accompanied by emission of a photon) – which is called fluorescence, or non-radiative (no photon will be emitted, excess of energy is released as heat) – which is called internal conversion. There are a number of other possible non-radiative transitions – intersystem crossing, intramolecular charge transfer, conformational change, and different pathways due to intermolecular interactions such as electron transfer, proton transfer, energy transfer, excimer/exciplex (excited state complex) formation, photochemical transformations [Turro et al. 2009].

### 2.1.3 Absorption and emission spectra

Since electronic transitions in fluorophore molecules occur between discrete energy levels, one may expect absorption and emission spectra to be a series of sharp lines, as it is for atoms (see figure 2.3). However, a peculiarity of the spectra of organic fluorophores as opposed to atomic and ionic spectra is the width of the absorption and emission bands, which usually covers several tens of nanometers [Schäfer 1990]. This becomes clear if one recalls that a typical dye molecule may have at least fifty (and usually more) atoms, giving rise to at least 150 normal vibrations of the molecular skeleton. Many of these vibrations are closely coupled to the electronic transitions by the change in electron densities over the bonds constituting the conjugated chain. After the electronic excitation has occurred, there is a change in bond length (typically 1%-2%) due to the change in electron density. Since the bond is lengthened in the excited molecule, atoms will start to oscillate, classically speaking, around this new position. A molecular skeletal vibration is excited this way. In general case of a large fluorophore molecule, many normal vibrations are coupled to an electronic transition. Additionally, collisional and electrostatic perturbations, caused by surrounding solvent molecules, broaden the individual lines of vibrational states. As a further complication, every vibronic sublevel of every electronic state, including a ground state, has superimposed on it a ladder of rotationally excited sublevels. These are extremely broadened because of the frequent collisions with solvent molecules which hinder the rotational movement so that there is quasi-continuum of states superimposed on every electronic level. The population of these
levels in contact with thermalized solvent molecules is determined by a Boltzmann distribution. After an electronic transition which leads to a non-equilibrium Franck-Condon state, the approach to thermal equilibrium is very fast in liquid solutions at room temperature. The reason is that a large molecule experiences at least $10^{12}$ collisions/s with solvent molecules, so that equilibrium is reached in time on the order of one picosecond [Schäfer 1990].

Because of all the mentioned reasons, a typical absorption spectrum is practically continuous all over the absorption band (see figure 2.3). The same is true for the fluorescence emission corresponding to the transition from the electronically excited state of the molecule to the ground state. Thus, the emission spectrum is a mirror image of the absorption spectrum displaced towards longer wavelength [Stokes 1852] (due to partial lost of the energy in vibrational relaxation – Stokes shift) by reflection at the wavelength of the purely electronic transition. In some cases, excitation by high energy photons leads to the population of higher electronic and vibrational levels ($S_2, S_3, \ldots$), which quickly dissipate the excess of energy by internal conversion as the fluorophore relaxes to the lowest vibrational level of the first excited state (see figure 2.2). Because of this rapid relaxation process, emission spectra are generally independent of the excitation wavelength, which is referred as the Vavilov’s rule [Wawilow 1927] (some fluorophores emit from higher energy states, but such activity is rare). For this reason, emission is the mirror image of the ground state to lowest excited state transitions, but not of the entire absorption spectrum, which may include transitions to higher energy levels [Lakowicz 2006]. In addition, one needs to compare the symmetry of excitation and emission spectra in a linear

**Figure 2.3:** A. Schematic representation of the absorption and fluorescence spectra corresponding to the energy diagram in figure 2.1. Electronic transitions between the lowest vibrational levels of the electronic states (the 0-0 transition) have the same energy in both absorption and fluorescence. B. Spectrum of a commonly used fluorescent dye, Rhodamine 6G.
plot having wavenumber (the reciprocal of wavelength or the number of waves per centimeter) on the abscissa axis, because wavenumber is directly proportional to the frequency and quantum energy.

In addition, the polarity of a solvent and the local environment of the fluorophore molecule will generally influence it’s the fluorescence emission spectra. This property is known, when talking about spectral effects, as solvatochromism or, in a more general way, as perichromism (peri: around). This dependence is due to the fact that fluorescence lifetimes (1-10 nanoseconds) are usually much longer than the time required for relaxation of solvent molecules (10-100 picoseconds). Since absorption of light occurs on much faster timescale (femtoseconds), the absorption spectrum is much less dependent from polarity. Solvents of higher polarity shift emission spectra to longer wavelengths [Lakowicz 2006].

2.1.4 Fluorophore structure

Electronic transitions are characterized by their energies. Working with fluorescence, one is usually interested in transitions which energy differences fall into the visible region of the electromagnetic spectrum (380-750 nm). Fluorescent molecules are typically organic unsaturated compounds, consisting of hydrocarbons and their derivatives and possessing at least one double or triple chemical bond. Double and triple bonds consist of one $\sigma$ (sigma) and one or two $\pi$ (pi) covalent bonds. Sigma bonds are the strongest type of covalent chemical bonds and are characterized by the rotational symmetry of their wavefunctions in respect to the bond direction [Banwell and McCash 1994]. Sigma bond is formed by two electrons in atomic s orbitals, one s and one p$_z$ orbital or two p$_z$ orbital ($z$ is defined as the axis of the bond). Pi bonds are usually weaker than sigma bonds, and they are formed by two electrons of atomic p orbitals which are overlapping laterally. Pi bonds are rigid and do not allow rotation around the bond axis. Organic compounds without double or triple bonds usually absorb at wavelengths below 160 nm, which not only fall out of visible region but constitute energies that are higher than dissociation energy of most chemical bonds. Therefore photochemical decomposition is likely to occur upon absorption of such high energy photons. Carbon-carbon double $\text{–C=\text{C–}$ (or triple $\text{–C≡\text{C–}$) bonds absorb photons with wavelength of 170 nm, which is still far from the visible region. If two double bonds are separated by a single bond $\text{–C=\text{C–C=\text{C–}$, the two double bonds are called conjugated. Two conjugated double bonds absorb at 220 nm, three conjugated double bonds – at 260 nm [Yadav 2004]. By increasing the number of the conjugated bonds, absorption and emission wavelengths increase. Thus, fluorophore molecules absorbing and emitting in the visible
Chapter 2. Fluorescence

range of the spectra possess several conjugated double bonds. For this molecules the highest occupied molecular orbital (HOMO) is typically a bonding π orbital, and the lowest unoccupied molecular orbital (LUMO) is typically antibonding π* orbital. Absorption of a photon typically induces π→π* transition in fluorophores [Schäfer 1990]. In conjugated systems π orbitals typically extend over the whole system and allow free electron movement around it, which is called delocalization of electrons. In most cases the more delocalized π orbitals are, the lower the energy of the transition is, and the longer the wavelength. In benzene (figure 2.4A, chemical formula C₆H₆) π orbitals are completely delocalized above and below of the plane of carbons, so on the structure figures three alternating double bonds are typically substituted by one ring inside. Compounds with delocalization of electrons in a ring (as benzene) are characterized by increased chemical stability and called aromatic due to historical reasons (however only some of them have notable aromas) [Birks 1970].

A single aromatic ring (benzene) absorbs light strongly around 180 nm with weaker band at 200 nm, the added conjugation of three rings in anthracene (figure 2.4B) shifts absorption to around 250 nm. Substitution of one of the central carbons in anthracene by oxygen makes xanthene, which is the basis of a large family of dyes which includes Fluorescein, Eosins, and Rhodamines. Xanthene dyes absorb in the blue to yellow region of the spectra and fluoresce from green to red region, covering most of the visible range. Introducing additional groups to the xanthene unit makes Rhodamine 6G (figure 2.4C), which is one of the most well studied fluorophores because of its use in dye lasers [Duarte and Hillman 1990].

2.1.5 Triplet state

De-excitation via the intersystem crossing pathway is of particular interest in the context of this thesis. Intersystem crossing is a non-radiative transition between two iso-energetic vibrational sublevels belonging to electronic states of different multiplicities (see figure 2.2). Typically, intersystem crossing occurs from a singlet state
to a triplet state, however, opposite transitions (reverse intersystem crossing) are also possible, especially from higher excited states [Widengren and Seidel 2000; Ringemann et al. 2008]. Because the triplet state has a higher multiplicity, it has a lower energy level than the excited single state, this is referred as Hund’s rule [Banthwell and McCash 1994]. Therefore, from the energy point of view this transition can be possible, because it is dissipative. However, these transitions are inevitably accompanied by a spin flip and are classically “forbidden” by quantum mechanics, just as a direct excitation from the ground state $S_0$ into the triplet state $T_1$ is forbidden. Due to spin-orbit coupling (coupling between the orbital magnetic moment and the spin magnetic moment) there is a mixing of vibrational sublevels of singlet and triplet states (see figure 2.5). Under these conditions classical prohibition is relaxed and intersystem crossing transition becomes possible. Presence of heavy atoms [Kasha 1952; McGlynn et al. 1969; Ketsle et al. 1976] (with large atomic number, like Br, I, Pb) or paramagnetic species [Hoijtink 1960] (including oxygen [Stracke et al. 1999]) in the structure of the molecule or its surroundings can influence the efficiency of intersystem crossing.

For some fluorescent molecules this de-excitation is very efficient and occurs faster than fluorescence. For example in benzophenone the rate of intersystem crossing $k_{\text{ISC}}$ is $10^{10}$ s$^{-1}$, whereas the rate of fluorescence $k_{10}$ is $10^6$ s$^{-1}$, meaning that intersystem crossing occurs at a rate which is $10^4$ faster than fluorescence, making fluorescence improbable. After intersystem crossing and following vibrational relaxation, the molecule cannot easily return to the excited singlet state because of the energy difference. Nor can it easily return to the ground state (which is a singlet state), as

![Figure 2.5: Left: intersystem crossing is strictly forbidden for lowest vibrational singlet (S) and triplet (T) state. Right: intersystem crossing is partially allowed when spin-mixing mechanism is available near the crossing point of the energy curves for the S and T states.](image-url)
this transition requires another “forbidden” spin flip. Hence, the triplet excited state usually has a long lifetime (compare to lifetimes of the other excited states), because it has generally nowhere to which it can easily go. Because of this long lifetime, at suitable conditions (2-color excitation or high irradiances) there can be an excitation to higher triplet states if photon(s) with the matching energy will be absorbed [English et al. 2000; Widengren and Seidel 2000; Ringemann et al. 2008]. The molecule will eventually relax back from the excited triplet state T1 to the ground singlet state S0 either radiationless (via interactions with surrounding molecules by different mechanisms - dissipating energy as heat, energy transfer to another molecule in triplet state, e.g. oxygen) or by emitting a photon (phosphorescence). Although this process is again classically “forbidden”, it nevertheless occurs when there is no other open pathway by which the molecule can dissipate its excess of energy.

2.1.6 Isomerised state

Fluorescent molecules containing double bond between carbon atoms in the chemical structure typically have an additional de-excitation mechanism involving cis- trans- isomerisation [McCartin 1965]. The terms cis and trans are from Latin, in which cis means “on the same side” and trans means “on the other side” or “across”. Double chemical bonds are rigid and do not allow free rotation around their axis, but there is a possibility to make a 180° twist around it. This transition changes the structure of the molecule and, strictly speaking, makes a new entity with different physical properties. Since photoisomerisation takes place in the conjugated hydrocarbon chain, this signposts that fluorophores belonging to cyanine (carboxycyanine) family will exhibit this relaxation pathway. Cyanine is a non-systematic name of a synthetic dye family belonging to polymethine group. They were first synthesized over a century ago, and there are a large number of cyanines reported in the literature.

Cyanines were originally used to increase the sensitivity range of photographic emulsions, also as passive modelockers in lasers, as media in CD-R and DVD-R discs. Cyanine dyes are generally characterized by high extinction coefficients, short fluorescence lifetimes and relatively low fluorescence quantum yield <0.30 (due to the large flexibility of long conjugated chain). They cover green to red span of the visible spectrum and are among the few fluorophores that absorb at far red wavelengths, which define their popularity in biomedical applications due to low autofluorescence and reduced damage in cells. A variety of low-cost, energy efficient, rugged diode lasers and highly sensitive detectors in the visible-near-IR region also facilitates the extensive use of long wavelength cyanine dyes. The importance of cyanine dyes has therefore motivated a large amount of scientific work concerning
their photophysical properties. Among the most popular fluorophores belonging to cyanine family are Cy3, Cy5 (figure 2.6A) and Alexa Fluor 647.

Absorption and emission spectra of cis- and trans-isomers are usually shifted in relation to each other, with cis isomer being shifted more to the red region the visible spectrum [McCartin 1965; Chibisov 1966; Tinnefeld et al. 2001; Huang et al. 2005]. Except of the difference in absorption/emission spectra, there is significant difference also in fluorescence brightness. Cis-isomer is considered to be a “dim”, non-fluorescent state. This is due to a larger flexibility of the conjugated chain, which makes non-radiative internal conversion decays to be the predominant pathways of de-excitation. Usually, trans-isomers are more stable than the cis-isomers. This is partly due to their shape; the straighter shape of the trans-isomer leads
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to hydrogen intermolecular forces that make the isomer more stable (see figure 2.6).

Transitions between trans- and cis- state are driven by excitation, and shift of the absorption spectrum leads to difference in excitation rate for different photoisomers. This defines the steady state proportion between trans- and cis- isomers during constant excitation (which is about 50% in each state for Cy5 at excitation with 594 nm or 633 nm light). Figure 2.6B presents the Jablonski diagram normally adopted to model the photophysical behavior of cyanine dyes [Widengren and Schwille 2000].

Triplet state population and intersystem crossing are rather inefficient for most of cyanines, due to the competition with effective deexcitation via photoisomerisation. A direct populating of the triplet level of cyanine fluorophores occurs under conditions preventing or limiting the process of trans-cis isomerisation [Chibisov 1976]. Therefore, the triplet energy levels are omitted in figure 2.6B for simplicity. Different environmental factors influence the rate of isomerisation, among others – viscosity of the surrounding medium, temperature, solvent polarity and absence or presence of sterical hindrances [Korobov and Chibisov 1983; Aramendia et al. 1994; Noukakis et al. 1995].

2.1.7 Fluorescence quenching

The term quenching refers to any process which decreases the emitted fluorescence of a given substance. A variety of processes can result in quenching, such as excited state reactions, energy transfer, complex-formation and quenching due to collisions with other molecules [Lakowicz 2006]. Typically fluorescence quenching is perceived as an undesirable process, because it reduces the fluorescence output. However, it can be taken advantage of as a valuable source of information about the interactions of a fluorescent molecule with a quencher. There are countless examples of such applications, to name a few – to study structure and dynamics of proteins [Eftink and Ghiron 1981; Zhuang et al. 2000; Yang et al. 2003; Chattopadhyay et al. 2005; Nettels et al. 2007] and interactions with nucleotides [Seidel 1991; Seidel et al. 1996; Widengren et al. 1997; Bonnet et al. 1998; Eggeling et al. 1998a; Zhu et al. 2005].

It has been known for a long time that fluorescence is quenched by certain anions [Pringsheim 1949]. The quenching ability strongly depends on the chemical nature of the anion, with quenching ability being stronger for iodide (I-), bromide (Br-) and less stronger for chloride (Cl-). As one of the mechanisms of quenching by anions, a charge transfer reaction could be considered [Drexhage 1990].

Another mechanism – resonance energy transfer [Förster 1948] (often referred as
FRET – Fluorescence or Förster Resonance Energy Transfer) occurs over distances up to 10 nm through nonradiative dipole–dipole coupling. FRET efficiency depends on the distance between the donor and the acceptor, the spectral overlap of the energy donor emission spectrum and the energy acceptor absorption spectrum, the relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment. This mechanism is being extensively used in many biophysical fields and there are many reviews of various applications published [Clegg 1992; Selvin 2000; Jares-Erijman and Jovin 2003; Roy et al. 2008; Schuler and Eaton 2008; Clegg 2009; Matthews et al. 2010].

Quenching by the so called heavy atom effect originates due to enhancement of the intersystem crossing rate to the triplet state. There can be quenching due to internal [Chandra et al. 1978; Solov’ev and Borisevich 2005] (when quencher is a part of a molecule itself) or external [Ketsle et al. 1976; Bryukhanov et al. 1992; Rae et al. 2003] (with quencher being present in the solution) heavy atom effect. Mechanistically, it responds to a spin-orbit coupling enhancement produced by a heavy atom, which decreases the energy difference and facilitates transitions from the singlet to the triplet state.

Further more, at high concentrations of fluorophores there can be fluorescence quenching due to aggregation [Jelley 1936; Levshin and Nizamov 1966; Drexhage 1990; Kelkar et al. 1990; Yuzhakov 1992; Bergström et al. 2001; Marmé et al. 2005] (dimerisation), which is most pronounced in solutions where the solvent consists of small, highly polar molecules – notably water. Dispersive forces between the large fluorophore molecules tend to bring them together, which is slightly counteracted by repulsive Coulomb forces if the fluorophores are charged. Upon aggregation fluorescence intensity is reduced and absorption spectrum radically changes its shape, with an enhancement of the short-wavelength part at the expense of the long-wavelength part.

Excited state reactions also contribute to quenching of fluorescence [Tomin 2008]. For example, the absorption spectrum of fluorophore Rhodamine 6G in ethanol is unchanged even at concentrations as high as 10 mM, which suggests that there is no dimerisation. However, fluorescence at such concentrations is strongly reduced due to collisions of the excited state molecules with those in the ground state [Baranova 1965].

2.1.8 Photobleaching

Photobleaching is usually defined as the irreversible decomposition (formation of non-fluorescent product) of fluorescent molecules in the excited state because
of their interaction with molecular oxygen (or impurities in the solution) before fluorescence may occur. Because of its influence as a limiting factor for the performance of, basically, all fluorescence techniques, photobleaching has been extensively studied by various means [Widengren and Rigler 1996; Eggeling et al. 1998b; Dittrich and Schwille 2001; Eggeling et al. 2005; Eggeling et al. 2006; Yeow et al. 2006; Vogelsang et al. 2008]. The average number of excitation and emission cycles that occur for a particular fluorophore before photobleaching is dependent upon its molecular structure and the local environment. Some fluorophores bleach quickly after emitting only a few photons, while others that are more robust can undergo thousands or millions of cycles before bleaching occur.

Several mechanisms could lead to photobleaching of a fluorophore, typically involving triplet states and/or radical states. The triplet state is relatively long-lived with respect to the singlet state, thus allowing excited molecules a much longer timeframe for interaction with the environment. The triplet state has two unpaired electrons and thus has a radical character, reacting with impurities, dissolved oxygen, solvent molecules or other fluorescent molecules to yield various decomposition products. Especially the oxygen concentration is a very important factor in photobleaching dynamics, because of its involvement both in quenching of the triplet state [Kawaoka et al. 1967; Wilkinson 1997; Hubner et al. 2001] and in the production of non-fluorescent compounds. The quenching of the triplet state of the fluorophore facilitates the relaxation from the excited triplet to the ground singlet state, resulting in the formation of the higher energy singlet oxygen species. Such singlet oxygen reacts rapidly with exposed chemical groups in organic fluorophores; amino acids such as cysteine, histidine, tyrosine, and tryptophan [Davies 2004]; and guanosine in DNA [Sies and Menck 1992]. The oxidized dyes are thereafter no longer fluorescent, and such oxidative damage impairs the folding and function of biomolecules. At different experimental conditions oxygen can therefore either enhance or reduce photobleaching affecting the equilibrium between triplet quenching and radical formation. Substituting the oxygen in the solution by another efficient triplet quencher decreases photobleaching and extends the observation times of the fluorophores [Rasnik et al. 2006; Widengren et al. 2007; Aitken et al. 2008; Vogelsang et al. 2008].

Under certain circumstances, the photobleaching effect can also be utilized to obtain specific information that would not otherwise be available. For example, in fluorescence recovery after photobleaching (FRAP) experiments [Axelrod et al. 1976; Meyvis et al. 1999; White and Stelzer 1999; Lippincott-Schwartz et al. 2001], fluorophores within a target region are intentionally bleached with excessive levels
of irradiation. As new fluorophore molecules diffuse into the bleached region of the specimen (recovery), the fluorescence emission intensity is monitored to determine the lateral diffusion rates of the target fluorophore. In this manner, the translational mobility of fluorescently labeled molecules can be ascertained within small (few micrometers) region of a single cell or section of living tissue.

2.2 Fluorescence microscopy

The most popular application of fluorescence is in optical microscopy. Among the advantages of fluorescence in microscopy are its specificity and sensitivity, high spatial and temporal resolution and compatibility with live biological systems. The high specificity is due to the fact that detected signal (fluorescence) originates only from the introduced fluorescent labels, with most of the background being efficiently suppressed – ‘you see only what you want to see’. The sensitivity comes from high brightness of fluorophores and very efficient detectors, sensitive to single photons. The spatial resolution of optical systems is limited by diffraction of light, however several microscopy techniques has successfully circumvented this limitation [Hell and Wichmann 1994; Betzig et al. 2006; Rust et al. 2006; Hell 2007]. Temporal resolution on the fast time scale is limited so far only by electronics and instrumental development is advancing rapidly. Temporal resolution on the slow time scale is limited by photobleaching, which can be reduced with various additives. Furthermore, development of fluorescent proteins [Tsien 1998] has greatly facilitated the use of fluorescence in live cell imaging due to the possibility of genetically encoding of a fluorescent label into the proteins of interest.

2.2.1 Confocal microscopy

A distinctive characteristic of confocal microscopy is the presence of a pinhole in the light detection path (see figure 2.7), which reduces out-of-focus background and decreases depth of field of view, which by-turn makes possible to do optical sectioning and 3D image recording.

The basic concept of confocal microscopy was originally developed by Marvin Lee Minsky in the mid-1950s (patented in 1957) when he was a postdoctoral student at Harvard University, working on imaging of neural networks in unstained preparations of brain tissue [Minsky 1957]. This invention was severely ahead of it’s time due to lack of intense light sources (lasers were not yet invented) necessary for fluorescence imaging and the computer data-processing power required to handle large amounts of data. Unintentionally, shortly after Minsky’s patent had expired, practical laser scanning confocal microscope designs were translated into working
Instruments by several investigators. But it was not until almost 30 years after its invention that the first 3-dimensional images of fluorescently labeled biological samples were demonstrated by the use of laser scanning confocal microscopes [Carlsson et al. 1985; Carlsson and Åslund 1987; White et al. 1987]. The first commercial instruments appeared in 1987. During the 1990s, advances in optics and electronics afforded more stable and powerful lasers, high-efficiency scanning mirror units, high-throughput fiber optics, better thin film dielectric coatings, and detectors having reduced noise characteristics. In addition, improved fluorescent labels started to be synthesized. Coupled to the rapidly advancing computer processing speeds, enhanced displays, and large-volume storage technology that emerged in the late 1990s, all prerequisites were created for a virtual explosion in the number of applications that could be targeted with laser scanning confocal microscopy [Pawley 2006].

Even though confocal microscopy provides only a marginal improvement in both axial and lateral optical resolution compared to conventional widefield optical epifluorescence microscopy, but is able to exclude from the resulting images secondary fluorescence in areas of the focal plane. The principal sketch of a typical confocal microscope arrangement is presented in figure 2.7.
As seen from the sketch in figure 2.7, in confocal microscopy only a single point of a specimen is illuminated at a time. To receive an image of the whole specimen, some scanning arrangement needs to be introduced. In the original instrument built by Minsky the laser beam was kept stationary and the specimen itself was moved on a scanning stage. This arrangement has the advantage of uniform imaging properties over the entire image area, which can eliminate most lens defects (like off-axis aberrations and vignetting) that would affect the image. For soft biological specimens, however, movement of the specimen can cause wobble and distortion, resulting in a loss of resolution in the image. The speed and accuracy of the movements of the scanning stage would thus contribute as limiting factors for temporal and lateral resolution.

Another possibility is to scan the laser beam with a help of a system of mirrors. This is done typically with computer-controlled galvanometer mirrors, which oscillate around a central position [Paddock 2000]. One mirror oscillates faster, scanning excitation and detection pathway around fast axis (in either lateral or axial direction). Another mirror oscillates slowly, forming two-dimensional image in a raster way. A third dimension is added by scanning the objective with stepper motor (typically in axial direction). Several sophisticated methods of scanning has been developed with time [Saggau 2006], with mechanisms without moving parts for example using liquid crystal optics [Khan and Riza 2006] or arrays of laser emitting diodes [Poher et al. 2007]. Compare to specimen scanning, beam scanning is much less demanding concerning mechanical precision, because the scanning mechanism can be placed on the image side of the microscope objective. The specimen is not subjected to any mechanical influence, and its size and weight are of no relevance to the scanning process. The disadvantage with beam scanning is that the imaging properties will not be uniform over the image area due to off-axis aberrations and vignetting.

As early as in the middle of the 19th century, fundamental works of the German physicist Ernst Karl Abbe laid the foundations of light microscopy. He developed many important concepts, among them a mathematical description for the resolution limit of the microscope, often referred as Abbe’s resolution limit.

Optical resolution describes the ability of an imaging system to resolve details in the object that is being imaged. The ability of a lens to resolve details is usually determined by the quality of the lens but is ultimately limited by diffraction [Hecht 2001]. The point spread function (PSF) describes the response of an imaging system to a point source. Light coming from a point in the object is diffracted by the lens aperture and thus forms a diffraction pattern in the image plane, which has a central
spot and surrounding bright rings, separated by dark nulls. This pattern is known as the Airy pattern (see figure 2.8A), and the central bright lobe as the Airy disk. The intensity of the Airy pattern (the Fraunhofer diffraction pattern of a circular aperture) is mathematically given by:

\[
\text{PSF}(r) = \left( \frac{2J_1(\rho)}{\rho} \right)^2
\]  
(1)

where \( \rho = \frac{2\pi}{\lambda} r NA \), \( \theta \) is the half-focusing angle of the objective lens, \( r \) is a radial space coordinate, \( \lambda \) is wavelength of light, \( NA = n \sin \theta \) is the Numerical Aperture of the objective, which characterizes the ability of the objective lens to focus/collection light, \( n \) is the index of refraction of the medium in which the lens is working, \( J_1 \) is the Bessel function of the first kind of order one. The airy pattern can be successfully approximated with the Gaussian function (figure 2.8B).

According to the empirical diffraction limit, known as a Rayleigh criterion, the images of two different points are regarded as just resolved when the principal diffraction maximum of one image coincides with the first minimum of the other (figure 2.8C-D). If the distance is greater, the two points are well resolved and if it is smaller, they are not resolved [Hecht 2001]. Mathematically, this corresponds to an intensity dip of 26.4 % between the peaks. The radius of the first zero-intensity fringe of the Airy disc, Rayleigh lateral resolution, is given by:

\[
\Delta r_{\text{widefield}} = \frac{0.61\lambda}{NA}
\]  
(2)

These considerations for resolution assume that the object is viewed in conventional wide-field microscopy. Likewise, due to reciprocity (illumination and detection is done via the same objective lens) this formula is valid for the minimum size of diffraction-limited focus of the excitation volume (Figure 2.8E).

In the case of confocal microscopy, the point spread function will be squared: \( \text{PSF}_\text{microscope} = (\text{PSF}_\text{lens})^2 \), because the diffraction of illumination source will be combined with the diffraction of the pinhole in the image plane. In the limit of an infinitely small pinhole, its image will be identical to PSF. Defining the resolution according to the same Rayleigh criterion, one obtains:
Figure 2.8: A. Airy pattern. B. A radial cross-section through the Airy pattern (solid curve) and its Gaussian profile approximation (dashed curve) for $NA=1.2$. C. The Rayleigh criterion for resolution. D. Detected intensity distribution from two point-sources just resolved by the Rayleigh criterion. E. The axial intensity distributions for a typical widefield fluorescence microscope, approximated as the Gaussian function – the excitation volume. F. The axial intensity distributions for a typical confocal fluorescence microscope – the detection volume.

$$\Delta r_{\text{confocal}} = \frac{0.44\lambda}{NA} \quad (3)$$
The axial resolution, i.e. the resolution along the optical axis of the microscope or $z$-axis, is defined using the three-dimensional diffraction image of a point source that is formed near the focal plane [Born and Wolf 1999]. The PSF along the optical axis has the following mathematical form:

$$\text{PSF}(z) = \text{sinc}^2 \left( \frac{\zeta}{4} \right) = \left( \frac{\sin \frac{\zeta}{4}}{\frac{\zeta}{4}} \right)^2$$

where $\zeta = \frac{2\pi}{n\lambda} NA^2 z$, and $z$ is the coordinate in the axial direction with its origin in the focus.

Using Rayleigh criterion for widefield imaging, one obtains axial resolution limit:

$$\Delta z_{\text{widefield}} = \frac{2n\lambda}{NA^2}$$

For confocal case:

$$\Delta z_{\text{confocal}} = \frac{1.41 n\lambda}{NA^2}$$

In contrast to the lateral resolution, the axial resolution decreases with the inverse square of the numerical aperture of the objective, $NA$. The ratio of axial-to-lateral resolution is substantially larger than one and is inversely proportional to the $NA$ of the objective.

### 2.2.2 TIR microscopy

In conventional widefield and confocal microscopy illumination is done with a broad cone of light. This is disadvantageous due to inefficiency of light utilization, photobleaching and undesirable excitation and scattering from out-of-focus regions – referred as background signal. The introduction of a confocal pinhole significantly restricts some of mentioned effects, but does not eliminate them completely. Multiphoton excitation microscopy [Denk et al. 1990] goes a step further by restricting the excitation area to an ellipsoid having sub-micron dimensions owing to the very low probability of the near-simultaneous absorption of multiple photons. This
reduces the amount of out of focus photobleaching and secondary fluorescence produced by fluorophores in the cone above and below of the excitation volume. Both confocal and multiphoton fluorescence microscopy produce optical sections of similar size. If there is a need to produce an excitation/detection volume in far-field from the objective lens, there is no alternative to focusing of light with a lens. Oppositely, if one is interested in studying processes by fluorescence which occurs directly at the interface of lens-specimen – in near-field, the phenomenon of total internal reflection could be taken advantage of.

Total internal reflection (TIR) fluorescence microscopy employs the unique properties of an induced evanescent wave to selectively illuminate and excite fluorophores in a restricted region immediately adjacent to a glass-water interface [Axelrod et al. 1984]. The basic concept of TIR is simple, requiring only an excitation light beam traveling at a high incident angle through the solid glass coverslip (see figure 2.9). Refractive index differences between the glass and water regulate how light is refracted or reflected at the interface as a function of incident angle. Refraction at the interface is described by Snell’s law:

\[ n_1 \sin \theta_1 = n_2 \sin \theta_2 \]  

(7)

For a plane wave that is incident from the optically denser medium at the interface, \((n_1 > n_2)\), at a specific critical angle, the beam of light is totally reflected from the glass/water interface, rather than passing through and refracting in accordance with Snell’s Law. This corresponds to refraction at 90 degrees \((\theta_2=90^\circ, \sin\theta_2=1)\), and
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the condition for the critical incidence angle arises from Snell’s Law:

\[ \sin \theta_c = \frac{n_2}{n_1} \]  

(8)

Total internal reflection does not occur suddenly as a new phenomenon at the critical angle, but a continuous transition is followed from predominant refraction with a small amount of reflection (at low incidence angles), to total reflection when the critical angle is exceeded. As the incident angle increases toward the critical angle value, the transmitted (refracted) beam diminishes in intensity while the reflected beam grows stronger. At all angles greater than the critical angle \( (\theta_i > \theta_c) \), total internal reflection takes place, in which essentially all of the light is reflected back into the first medium (this effect builds the basis for light guidance in optical fibers and waveguides). Even though the light no longer propagates into the second medium, there is a small amount of penetration of light across the interface, which then propagates parallel to the surface, creating an electromagnetic field in the second medium immediately adjacent to the interface [Hecht 2001]. Solving the Maxwell equations for this case shows that the \( z \) component of the wave vector for the refracted beam becomes imaginary. The intensity is therefore not zero inside the medium with refractive index \( n_2 \) but decays exponentially with the distance, \( z \), to the interface.

\[ I(z) = I(0) \exp(-z/d) \]  

(9)

The penetration depth \( (d) \), at which the intensity decays by a factor 1/e, is dependent upon the wavelength of the incident illumination \( \lambda_0 \), the angle of incidence \( \theta \), and the refractive indices of the media at the interface \( n_1 \) and \( n_2 \), according to the equation:

\[ d = \frac{\lambda_0}{4\pi} \left( \frac{n_1^2 \sin^2 \theta - n_2^2}{n_2^2} \right)^{-1/2} \]  

(10)

At small incidence angles, light waves propagating through the interface to the lower-refractive index medium are sinusoidal, and have a characteristic period. At increasing angle, approaching the critical value, the period of refracted rays becomes longer and the propagation direction becomes more nearly parallel to the interface.
When the critical angle is achieved, the wave period becomes infinite and the refracted light wavefronts are aligned perpendicular to the interfacial surface. This field is termed the evanescent field, or evanescent wave, and within a limited region near the interface, usually less than 200 nanometers, it is capable of exciting fluorophores. In fact, the excitation of fluorescence was used as an early proof for the existence of the evanescent field [Woods 1934].

The evanescent wave intensity at the surface \( I(0) \) is a function of both the incident angle and the polarization components of the light beam. It is composed of contributions from parallel (P-pol, \( I_0^p \)) and perpendicularly (S-pol, \( I_0^s \)) polarized fields, which are

\[
I_0^p = I^p \frac{4 \cos^2 \theta (2 \sin^2 \theta - n^2)}{n^4 \cos^2 \theta + \sin^2 \theta - n^2}
\]

\[
I_0^s = I^s \frac{4 \cos^2 \theta}{1 - n^2}
\]

where \( n = n_2/n_1 \), \( I^p \) and \( I^s \) denote incident intensities. Up to a factor of 5 in enhancement of the excitation intensity compare to the incident intensity is possible using TIR excitation on a glass/water interface. This enhancement factor can be increased even further on metal films [Burghardt and Thompson 1984; Hellen and Axelrod 1987]. The maximum enhancement is reached when the beam is incident at the critical angle and the axial extent of the evanescent field approaches very large values. Figure 2.10A shows the resulting levels of irradiance due to the evanescent field for different angles of incidence for P- and S-polarized incident light.

Fluorophores existing in the close proximity of an interface do not emit light isotropically, unlike those dispersed in bulk solution [Lukosz and Kunz 1977a; Lukosz and Kunz 1977b; Lukosz 1979]. Instead, fluorescence emission is produced in a complex spatial pattern that is highly dependent upon the orientation of the fluorophore transition dipoles with respect to the interface geometry [Hellen and Axelrod 1987; Enderlein et al. 1999]. Characteristics of fluorescence emission close to an interface between dielectric media can be derived by modeling the fluorophore by a classical dipole. For a randomly oriented dipole near an interface separating two dielectric half-spaces the radiated power is canalized along the critical angle and emission into the medium with higher refractive index is favored. This is illustrated in Figure 2.10B, where the emission profiles were calculated using a formalism proposed by Mertz [Mertz 2000].
From technical instrumental point, two main configurations of total internal reflection fluorescence microscopy (TIRFM) exist [Axelrod 2001], called prism-type TIRFM and objective-type TIRFM. In prism-type TIRFM a glass prism is used to achieve the evanescent excitation at the sample. It is placed above the sample, which is sandwiched between two coverglasses. A water immersion objective lens is placed below the lower coverglass and immersion oil is used on the interface between the prism and the upper coverglass to eliminate refractive index change. A laser beam traveling inside the prism is used to generate an evanescent wave at the interface between the upper glass-slide and the sample.

In objective type TIRFM, an oil-immersion objective with high \( NA \) (\( \geq 1.4 \)) is used for the excitation and the collection of emitted light. A laser beam is focused onto the back focal plane of the objective, which causes the beam to emerge collimated from the objective. This beam is focused off-axis onto the back focal plane, and therefore it emerges under a certain angle \( \theta > 0 \) out of the objective. If the angle is large enough, the beam is totally reflected at the coverglass/water interface and an evanescent field builds up at the surface.

Several variations of setups with these configurations exist, including configurations that allow using a conventional light source instead of a laser, or a waveguide instead of a prism to generate an evanescent field. Both setups have their advantages.

Figure 2.10: A. Normalized enhancement factor of the evanescent field for P-polarization \( I_o^P/I^P \) and for S-polarization \( I_o^S/I^S \) of the incident light at a glass \( (n_1 = 1.52) \) and water \( (n_2 = 1.33) \) interface as a function of the angle of incidence. B. Normalized radiated power displayed as a polar plot assuming a randomly oriented dipole located at the water/glass interface, \( z = 0 \). The right half-circle (\( \pm 90^\circ \)) shows the highly anisotropic emission into the glass, with a maximum around the critical angle \( (\theta_C = 61^\circ) \), which can be collected by high numerical-aperture microscope objective. The normalization is such that radiated power is scaled to that of the same dipole in the absence of the interface (which has a value of 1 on this polar graph).
and disadvantages. Both of these most common configurations were successfully applied to image single fluorescent molecules. The prism-type TIRFM benefits from decoupling of excitation and emission paths, which offers more flexibility regarding selection of incidence angle and significantly reduces the background due to the fact that excitation light does not travels into emission pathway. One major disadvantage of the prism-type excitation is the lower collection efficiency. In objective-type TIRFM a high \( NA \) objective is used that collects light efficiently. Moreover, fluorescence emission in close vicinity of the interface is highly favored into the direction of the interface (Figure 2.10B). Larger part of this anisotropic emission goes into the glass and propagates at an angle close to \( \theta_c \). This fluorescence is mostly collected by high \( NA \) objectives in objective-type TIRFM, but totally missed in prism-type TIRFM (because it goes in the direction opposite to objective lens). It was shown [Enderlein et al. 1999] that the fraction of collected fluorescence can exceed 60% for objective-type TIRFM. The presence of a relatively large prism above the sample also hinders access and hampers sample manipulation in prism-type TIRFM.

### 2.2.3 Super-resolution microscopy

The resolving power of a microscope is the most important feature of that optical system and it influences the ability to distinguish between fine details within specimen. As discussed in previous chapters, the fundamental limit of resolution in light microscopy is imposed by diffraction. An instant glance on the equation 2, defining the resolution limit, immediately suggests two obvious ways to decrease it by reducing the wavelength or increasing the numerical aperture of the objective lens. Unfortunately, many problems arise with following these suggestions. The light with wavelength below 350 nanometers is often phototoxic to live biological specimens and is strongly absorbed by common optics used for visible light microscopy. Increase of the numerical aperture of the objective lens is presently limited to the largest technical achievable semi-aperture angle of about 70°, which in combination of immersion oil with high refractive index (\( n=1.52 \)) gives highest \( NA \) values of about 1.5.

Many different sophisticated techniques were developed to circumvent the diffraction limit in light microscopy. If there is an interest only in surface-bound studies, techniques based on TIR (previous section) significantly reduce axial resolution. Reducing lateral resolution in surface-bound studies is well achieved in the scanning near field optical microscope (SNOM) [Synge 1928; Hecht et al. 2000]. It uses a small sub-diffraction aperture or small tip to illuminate the object, which is scanned over the sample. Although resolution down to 10 nm is achievable [Hosaka and Saiki 2006] with SNOM, it is ultimately a surface-bound technique unfeasible for
most of biological applications, except of membrane studies.

Many ideas have been worked out to address the resolution problem in the far-field as well [Hell 2009b]. In 1956, Toraldo di Francia suggested shrinking the central focal spot by applying an elaborate phase pattern in the entrance pupil of the objective lens [Di Francia 1952]. However, the creation of smaller central spot is accompanied with giant sidelobes making this concept impractical. A redistribution of the focal energy will always suffer from such side lobe effects.

As early as in 1966 ideas of using different gratings [Lukosz 1966] for illumination and/or the detection pathway for improving the resolution in reflection imaging were expressed. Later, many techniques were developed based on this principle, like laterally modulated excitation microscopy [Heintzmann and Cremer 1999], structured illumination microscopy [Gustafsson 2000], objective-launched standing-wave total internal reflection fluorescence microscopy [Chung et al. 2006]. Nonlinear relationship between the illumination intensity and the excitation probability in structural illumination were taken advantage of in saturated patterned excitation microscopy (SPEM) [Heintzmann et al. 2002] and saturated structured-illumination microscopy (SSIM) [Gustafsson 2005].

In addition, some techniques take to the extreme both the illumination aperture and the detection aperture, like 4Pi microscopy [Hell 1990; Hell and Stelzer 1992] and 15M microscopy [Gustafsson et al. 1995]. As the name 4Pi suggests (a full sphere has a solid angle of 4π), the idea is to get as close as possible to illumination (and detection) from all sides of the sample. This is achieved by combining two objective lenses opposed to each other along the optical axis direction. 15M microscopy uses similar method combined with incoherent illumination. However, these techniques significantly improve resolution only in axial direction.

Furthermore, several techniques are able to produce super-resolution images without breaking the diffraction limit per se – using localization of emitters. The resolution problem arises when there are several fluorescent molecules in close proximity to each other. If there is only one emitter, it is possible to localize it with very high accuracy by fitting the obtained fluorescence PSF with an appropriate analytical function and determining the center of it [Thompson et al. 2002]. If the fluorescence from a single molecule is distributed in a Gaussian profile and the background noise is small compared to the molecular signal, the error in the fitted position is inversely proportional to the number of detected photons from that molecule. To decrease the error of localization by two, one needs to detect 4 times more photons – which inevitably relates to the image acquisition time and limits performance of such techniques when it comes to imaging of dynamical processes. For localization
techniques it is therefore crucial to avoid the presence of several emitters inside of one PSF. This is done differently in various realizations. In photoactivation light microscopy (PALM) [Betzig et al. 2006] this is done by sparse activation of fluorescent proteins and subsequent intentional bleaching in the end of localization. Stochastic optical reconstruction microscopy (STORM) [Rust et al. 2006] exploits the unique property of switching of the cyanine dye, Cy5, between a fluorescent and a dark state in a controlled and reversible manner by light of different wavelengths [Bates et al. 2005]. Fluorescence photoactivation localization microscopy (FPALM) [Hess et al. 2006] relies on the low probability of simultaneous excitation of several molecules under very low excitation irradiances. Photoactivation localization microscopy with independently running acquisition (PALMIRA) [Geisler et al. 2007] benefits from the un-synchronization of excitation pulses with the detection read out, which decreases the possibility of detection fluorescence from several close emitters under sparse excitation. In points accumulation for imaging in nanoscale topography (PAINT) [Sharonov and Hochstrasser 2006] fluorescence is generated by the binding of fluorescent probes to the structure to be imaged, and switches off by free diffusion or another dark (bleached) state. Ground-state depletion and single-molecule return microscopy (GSDIM) [Fölling et al. 2008] stochastically shelves fluorophores into the triplet state and uses unsynchronized camera as a read-out mechanism. As a general feature, localization techniques typically produce very nice, sharp, background-free images (because the background is deliberately rejected if it does not pass predefined threshold), but suffer from long acquisition times (from hours to several minutes per image) and sample drifts during imaging.

The most successful super-resolution method so far is stimulated emission depletion (STED) microscopy [Hell and Wichmann 1994]. In STED the fluorescence, which is created by a focused beam of excitation light, is reduced in space by simultaneously applying a second spot of light shaped into a doughnut that features a central zero, for molecular de-excitation (see figure 2.11). The role of the de-excitation (STED) beam is to effectively confine molecules to the ground state, thus, effectively switching off the ability of the fluorophore to fluoresce. Stimulated de-excitation occurs within the nanosecond lifetime of the fluorescent state. Because no de-excitation occurs at the central zero, the excited state is allowed to spontaneously decay only in the central region close to the zero. STED microscopy is technically somewhat complex, demanding precise alignment of optical components for overlapping two beams with high accuracy and stability. Initially it required a system of several complex and expensive laser sources, however with time variants of STED microscopy with one pulsed supercontinuum fiber laser [Wildanger et al. 2008] or continuous lasers [Willig et al. 2007; Moneron et al. 2010] have been developed.
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Combination of STED and 4Pi microscopy [Dyba and Hell 2002] allows simultaneous increase of resolution in all dimensions, and viability of two-photon excitation in STED microscopy was demonstrated [Moneron and Hell 2009]. Simultaneous dual color imaging with STED microscopy was first implemented [Donnert et al. 2007; Meyer et al. 2008] using complex laser system for excitation, and later significantly simplified by using supercontinuum laser source. An ultimate resolution of 5.8 nm have been demonstrated [Rittweger et al. 2009] by imaging fluorescent nitrogen vacancies in diamonds. Besides high demands regarding the experimental setup and the excitation sources (which tends to be soften upon recent technological developments), STED microscopy requires high irradiances for stimulated emission – on the order of GW/cm² (given in part from the short fluorescence lifetime and the low stimulated emission cross-section). Decreasing either the fluorescence lifetime or utilization of long lived non-fluorescent states will decrease irradiances needed for de-excitation of fluorophores outside the central zero point.

To circumvent the necessity of high irradiances for de-excitation, ground state depletion (GSD) microscopy [Hell and Kroug 1995] was suggested. GSD microscopy is somewhat similar to STED microscopy, although it uses an entirely different mechanism for switching off fluorescence. To allow for much lower intensities, the fluorophore is switched off by transiently shelving it into the metastable triplet state, T₁ (see figure 2.2). Due to the long triplet lifetime (about 3 to 6 orders of magnitude longer than fluorescence lifetime), de-excitation irradiances are reduced by the same factor. Other suitable non-fluorescent state for GSD microscopy is

![Figure 2.11: Effective molecule detection efficiency (MDE) function in STED/GSD techniques. Black line represents Gaussian MDE without switching doughnut. Gray profiles represent MDE after illumination with a doughnut with local zero at radial coordinate 0 for peak intensities I₀ = 10 (light gray), 50 (gray), 500 (dark gray) times over saturation limit Iₜ₉, at which 50% of the molecules are in a fluorescent state. Left axis represents intensity distribution within MDE. Right axis represents magnitude of switching intensity.](image-url)
photo-isomerised state in cyanine dyes or in photoactivatable fluorescent proteins [Hell et al. 2003]. Experiments with the asFP595 protein demonstrated [Hofmann et al. 2005] sub-diffraction resolution imaging with switching intensities as low as 10W/cm², which are by 6 orders of magnitude lower than those required for STED. Various variants of utilizing reversible saturable optical (fluorescence) transitions were generalized under term RESOLFT microscopy [Hell et al. 2004].

2.3 Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a fluctuation spectroscopy technique, which makes use of temporal fluctuations in the detected fluorescence signal under equilibrium conditions in order to obtain information about the processes that give rise to these fluctuations. Statistical analysis is performed on fluctuations in the form of a correlation function, which provides information about the characteristic times and the relative weights of different processes which give rise to fluctuations.

FCS is closely related to other optical fluctuation method – dynamic light scattering (DLS), also known as quasi-elastic light scattering. DLS characterizes molecular motion in terms of optical interference effects from measurements of scattered coherent laser light [Berne and Pecora 1976]. It is an excellent method for studying molecular transport in highly resolved and fairly concentrated systems, or in mixtures where one species dominates the scattering. However, it is somewhat limited to diffusion studies and relatively high concentrations, and is not useful for measuring the progress of chemical reactions or following the dynamics of specific molecule at low concentration given the presence of other molecular species at high concentrations.

In contrast to the other popular fluorescence technique for studying diffusion and binding kinetics – fluorescence recovery after photobleaching (FRAP) [Axelrod et al. 1976], FCS is not relying on external perturbation of the system under study, but instead it harnesses the deviations from the equilibrium. Macro-equilibrium states often appear to be very dynamic on the molecular level. For example, when studying diffusion, Brownian motion of the molecules will cause deviations in number of molecules inside the observation volume and smaller the observation volume – larger these deviations will be. By labeling molecules of interest with a fluorophore, fluctuations in number of molecules will be translated to fluctuations in emitted fluorescence signal. Proper detection and analysis of these fluctuations will provide information about the diffusion properties of the molecule of interest. In fact, all of the processes which effect fluorescence emission are feasible for monitoring and
investigation with FCS, for example – translational diffusion [Magde et al. 1972],
diffusion coupled ligand-receptor interactions [Elson and Magde 1974], rotational
motion [Ehrenberg and Rigler 1974], uniform translation and laminar flow [Magde
et al. 1978], singlet-triplet transitions dynamic [Widengren et al. 1994], cis-trans
isomerisation dynamic [Widengren and Schwille 2000], FRET [Widengren et al.
2001], antibunching [Mets et al. 1997], charge transfer [Widengren et al. 1997],
oxidation-reduction [Widengren et al. 2007], just to name a few.

FCS was developed in early 1970s and the first published application was dif-
fusion and binding studies of DNA-ethidium bromide interaction [Magde et al.
1972]. However, the technical difficulties imposed by immaturity of equipment
(excitation sources, fluorescence filters, detectors and electronics) resulted in large
excitation/detection volumes containing many molecules, low relative fluctuations
of a signal which needed to be compensated by very long measurement times (up
to 24 hours) [Magde et al. 1974]. Due to the mentioned shortcomings the tech-
nique did not really get exploited up to 1990s, when Rudolf Rigler and co-workers
first used a confocal microscope arrangement combined with FCS to detect single
molecules [Rigler and Widengren 1990]. By reducing the focal volume of excitation
and applying the confocal principle, background is reduced considerably, number
of molecules inside detection volume is reduced, generating larger relative fluctua-
tions, collected and analyzed by more high-performance detectors and electronics.

Besides its confocal implementation, FCS have been combined with many other
modes of excitation. Many experimental variations evolved, like scanning FCS [Pe-
tersen 1986], two-photon excitation FCS [Berland et al. 1995], two-color cross
correlation [Schwille et al. 1997], TIR-FCS [Thompson et al. 1981], STED-FCS
[Kastrup et al. 2005], sub-wavelength sized apertures FCS [Leutenegger et al. 2006],
two-focus FCS [Dertinger et al. 2007], inverse FCS [Wennmalm et al. 2009] and
others.

2.3.1 Confocal FCS

A typical confocal FCS setup (figure 2.12) consists of a confocal microscope, in
which fluorescence is directed to a highly sensitive photodetector with high tempo-
ral resolution. A laser beam is expanded by a telescope with a pair of lenses, reflected
by a dicroic mirror and directed into a high numerical aperture microscope objective.
Different degrees of laser beam expansion are used in order to achieve differ-
tent sizes of the excitation volume. The objective then focuses the laser beam into a
sample, where fluorophores are excited. The emitted fluorescence is partly collected
by the same objective, transmitted through a dicroic mirror and focused onto a
pinhole. The size of the observation volume is defined by the size of the confocal pinhole and the excitation volume, which can be as low as few femtoliters if the laser beam is focused to a diffraction limited spot. An important technical constrain is to match the size of the pinhole and the excitation volume in order to achieve desirable (Gaussian) properties of the detection volume [Hess and Webb 2002]. After the pinhole, fluorescence is directed to a detector, normally avalanche photodiode (APD) or photomultiplier tube (PMT). Due to a presence of the undesirable effects of afterpulsing (that is generating a false count after detecting a photon) and dead-time (inability to detect a second photon immediately after detecting a first photon) in detectors, typically two detectors are used in FCS. Fluorescence after the pinhole is re-collimated with a lens, split by a beam splitter and focused onto the sensitive area of each detector. In order to minimize reflected and Rayleigh and Raman scattered laser light, thin film interference filters are placed in the beam path.

Figure 2.12: Sketch of a typical confocal FCS setup. Characteristic FCS curve exhibiting correlation component due to diffusion and relaxation term for singlet-triplet transitions included in the lower left corner.
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before detectors. The electrical signal from the detectors is analyzed by a specialized hardware correlator or recorded by a data collection card.

Fluorescence intensity (number of photons) collected from the detection volume at certain time \( t \) will be proportional to the molecular detection efficiency (MDE) function \( E(\vec{r}) \) and the concentration of the emitting molecules \( C(\vec{r},t) \):

\[
I(t) = q \int E(\vec{r}) Q C(\vec{r},t) d^3 \vec{r}
\]  

(13)

where the brightness factor \( Q \) depends on the absorption cross section, the fluorescence quantum yield and the fluorescence lifetime and \( q \) is a proportionality constant accounting for the overall instrument detection efficiency.

Here an ideal solution of chemical compounds consisting of only one fluorescent species is considered. Its local concentration is \( C(\vec{r},t) \), the space-and-time ensemble averaged concentration \( \langle C \rangle = \langle C(\vec{r},t) \rangle \) and the local deviation \( \delta C(\vec{r},t) = C(\vec{r},t) - \langle C \rangle \). In equation (13) photon shot noise is not included, since it is not correlated for different emitters and does not contribute to correlation function \( G(\tau) \).

The deviation of the detected photon counts from the mean \( \langle I \rangle = \langle I(t) \rangle \) is:

\[
\delta I(t) = I(t) - \langle I \rangle = q \int E(\vec{r}) Q \delta C(\vec{r},t) d^3 \vec{r}
\]  

(14)

In FCS experiments, the fluorescence correlation function \( G(\tau) \) is defined as time averaged product of the intensity \( I(t) \) at time \( t \) multiplied by intensity \( I(t+\tau) \) at time \( t+\tau \), normalized by the square of average intensity

\[
G(\tau) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2}
\]  

(15)

This expression can be rewritten as

\[
\frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{\langle (\bar{I} + \delta I(t)) \cdot (\bar{I} + \delta I(t+\tau)) \rangle}{\langle I \rangle^2} = 1 + \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I \rangle^2}
\]
Due to ergodicity of the systems studied by FCS, it’s possible to replace the time average with the ensemble average:

\[
G(\tau) = 1 + \frac{\langle \delta I(0) \cdot \delta I(\tau) \rangle}{\langle I \rangle^2}
\]  

(16)

By substituting equation (14) into equation (16) we obtain

\[
G(\tau) = 1 + \frac{\eta^2}{\langle I \rangle^2} \int \int E(\vec{r}) E(\vec{r}') Q^2 \langle \delta C(\vec{r},0) \delta C(\vec{r}',\tau) \rangle d^3\vec{r} d^3\vec{r}'
\]  

(17)

The observation volume is mathematically represented by the MDE function \( E(\vec{r}) \), which is proportional to the intensity detected from a single emitter as a function of its position \( \vec{r} \) in the sample volume. It is calculated by multiplying the excitation intensity \( I_{\text{exc}} \) by the collection efficiency function (CEF) [Rigler et al. 1993].

\[
E(\vec{r}) = I_{\text{exc}}(\vec{r}) \cdot \text{CEF}(\vec{r})
\]  

(18)

The CEF describes the probability of detection a photon as a function of the position of the emitter [Qian and Elson 1991], and may be expressed as:

\[
\text{CEF}(\vec{r}) = \frac{1}{\Delta} \int \text{circ}(\vec{r}'/s_0) \text{PSF}(\vec{r}', \vec{r}) d\vec{r}'
\]  

(19)

where PSF denotes the point spread function of the confocal microscope (Airy pattern) and \( \Delta \) is a normalization function. Integration is carried out in the sample space. The disk function, circ, represents the transfer function of the pinhole projected into the sample space, and the pinhole projection, \( s_0 \), is the radius of the confocal pinhole divided by the magnification of the detection system.

In order to simplify analytical expression, the MDE function for confocal detection is frequently approximated by 3-dimensional Gauss function:

\[
E(\vec{r}) = E(0) \cdot \exp \left( -2 \frac{x^2 + y^2}{\omega_{xy}^2} - 2 \frac{z^2}{\omega_z^2} \right)
\]  

(20)
where \( \omega_{xy} \) and \( \omega_z \) are the lateral and axial radii of the detection volume. This approximation is rather accurate under most typical experimental conditions and it produces mathematically simple analytical expression for the correlation function. Another even more accurate approximation assumes a Gauss-Lorentzian [Marrocco 2007; Blom and Björk 2009] volume, however this analytical expression it is not so mathematically straightforward in comparison to simpler 3D-Gauss approximation.

In order to obtain a simpler analytical expression for the correlation function in equation (17), the concentration fluctuation function have to be obtained by solving the differential equations that govern the molecular dynamics – either diffusion or reaction-diffusion equation (the later for the case of binding, singlet-triplet transitions, isomerisation or others). Here, an expression of the correlation function for only pure diffusion will be derived. The derivation of the correlation function for reaction-diffusion systems is well described in [Elson and Magde 1974].

The diffusion equation is a partial differential equation which is usually written as:

\[
\frac{\partial C(\vec{r},t)}{\partial t} = D \nabla^2 C(\vec{r},t)
\]  

(21)

where \( D \) is the diffusion coefficient of a molecule in aqueous solution, defined according to Stokes-Einstein relationship:

\[
D = \frac{k_BT}{6\pi\eta R_b}
\]  

(22)

with \( \eta \) representing the viscosity of the solution, \( k_B \) is Boltzmann’s constant, \( T \) is the absolute temperature and \( R_b \) is the hydrodynamic radius, which could be used to estimate the molecular weight of a particle. For spherical particles with molecular weight \( M \) more than 1000 Da, \( R_b \propto M^{1/3} \) and consequently \( D \propto M^{2/3} \).

The diffusion equation (21) can be easily solved by applying a Fourier transform, which yields:

\[
\frac{d\tilde{C}(\vec{q},t)}{dt} = -q^2D \tilde{C}(\vec{q},t)
\]  

(23)
where \( \tilde{C}(\vec{q}, t) = (2\pi)^{-3/2} \int e^{-i \vec{q} \cdot \vec{r}} C(\vec{r}, t) d^3 \vec{r} \) is a Fourier transform of \( C(\vec{r}, t) \).

The solution of equation (23) is:

\[
\tilde{C}(\vec{q}, t) = \tilde{C}(\vec{q}, 0) \exp(-D\vec{q}^2 t)
\]  

(24)

In the following derivations, the condition of ideality of the chemical solution will be used: the correlation length is much smaller than the distances between the molecules. The positions of different molecules of the same species are therefore uncorrelated, which mathematically may be written as:

\[
\langle \delta C(\vec{r}, 0) \cdot \delta C(\vec{r}''', 0) \rangle = \langle C \rangle \delta(\vec{r} - \vec{r}''')
\]  

(25)

where \( \langle C \rangle \) is the mean square fluctuation of the concentration of the molecules \( C(\vec{r}, t) \) in a unit volume, for Poisson statistics being equal to its average \( \langle C(\vec{r}, t) \rangle \)

Using equations (24), (25) and taking into account the fact that the Fourier transform and the ensemble averaging are independent linear operators and thus can be applied in any order, we can evaluate

\[
\langle \delta C(\vec{r}, 0) \delta C(\vec{r}', \tau) \rangle = (2\pi)^{-3/2} \int e^{i \vec{q} \cdot \vec{r}} \langle \delta C(\vec{r}, 0) \delta \tilde{C}(\vec{q}, \tau) \rangle d^3 \vec{q} =
\]

\[
= (2\pi)^{-3/2} \int e^{i \vec{q} \cdot \vec{r}} \exp(-D\vec{q}^2 \tau) \langle \delta C(\vec{r}, 0) \delta \tilde{C}(\vec{q}, 0) \rangle d^3 \vec{q} =
\]

\[
= (2\pi)^{-3/2} \int e^{i \vec{q} \cdot \vec{r}} \exp(-D\vec{q}^2 \tau) \times (2\pi)^{-3/2} \int e^{-i \vec{q} \cdot \vec{r}} \langle \delta C(\vec{r}, 0) \delta C(\vec{r}''', 0) \rangle d^3 \vec{r}''' =
\]

\[
= (2\pi)^{-3} \langle C \rangle \int e^{-i \vec{q} \cdot (\vec{r} - \vec{r}''')} \exp(-D\vec{q}^2 \tau) d^3 \vec{q}
\]  

(26)

Substitution of equation (26) into equation (17) gives

\[
G(\tau) = 1 + \frac{\vec{q}^2}{\langle I \rangle^2} \int \int d^3 \vec{r} d^3 \vec{r}'' E(\vec{r}) E(\vec{r}'') Q^2 \times
\]

\[
\times \langle C \rangle \int e^{-i \vec{q} \cdot (\vec{r} - \vec{r}'')} \exp(-D\vec{q}^2 \tau) d^3 \vec{q}^\prime
\]  

(27)

By changing the order of integration – first on \( \vec{r} \) and \( \vec{r}'' \) and using the fact that
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Fourier transform of an even function is even, we can write

\[ G(\tau) = 1 + \frac{q^2}{\langle I \rangle^2} \int |\tilde{E}(\vec{q})|^2 Q^2 \langle C \rangle \exp(-Dq^2\tau)d^3\vec{q} \]  \hspace{1cm} (28)

where \( \tilde{E}(\vec{q}) = (2\pi)^{-3/2} \int e^{-i\vec{q}\cdot\vec{r}} E(\vec{r})d^3\vec{r} \) is the Fourier transform of \( E(\vec{r}) \).

Note that \( \tilde{E}(0) = (2\pi)^{-3/2} \int E(\vec{r})d^3\vec{r} \), then obviously \( \int E(\vec{r})d^3\vec{r} = (2\pi)^{3/2} \tilde{E}(0) \).

The average fluorescence intensity \( \langle I \rangle \) in equation (28) is determined from equation (13):

\[ \langle I \rangle = q \int E(\vec{r}) Q \langle C \rangle d^3\vec{r} = (2\pi)^{3/2} \tilde{E}(0)qQ \langle C \rangle \]  \hspace{1cm} (29)

From equation (20), the Fourier transform of MDE is

\[ \tilde{E}(\vec{q}) = E(0) \frac{\omega_{zy}^2 \omega_z}{8} \exp \left\{ -\frac{\omega_{zy}^2}{8} (q_x^2 + q_y^2) - \frac{\omega_z^2}{8} q_z^2 \right\} \] \hspace{1cm} (30)

\[ \tilde{E}(0) = E(0) \frac{\omega_{zy}^2 \omega_z}{8} \] \hspace{1cm} (31)

Combining equations (29), (30) and (31) into equation (28) gives:

\[ G(\tau) = 1 + \frac{(2\pi)^{-3}}{\langle C \rangle} \int \exp \left\{ -\frac{\omega_{zy}^2}{4} (q_x^2 + q_y^2) - \frac{\omega_z^2}{4} q_z^2 - Dq^2\tau \right\}d^3\vec{q} \] \hspace{1cm} (32)

This expression can be easily integrated, which results in:

\[ G(\tau) = 1 + \frac{1}{\pi^{3/2} \omega_{zy}^2 \omega_z \langle C \rangle} \left( 1 + \frac{4D\tau}{\omega_{zy}^2} \right)^{-1} \left( 1 + \frac{4D\tau}{\omega_z^2} \right)^{-1/2} \] \hspace{1cm} (33)

In order to have the ability of measuring concentrations, the size of observation volume needs to be defined. This size is normally defined by the effective volume \( V_{\text{eff}} \).
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[Thompson 1992]:

\[ V_{\text{eff}} \equiv \frac{W_1^2}{W_2} \]  \tag{34}

where \( W_n \equiv \frac{1}{E_n'(0)} \int E_n'(\vec{r}) d^3\vec{r} \)

Using equation (20) we get \( V_{\text{eff}} = \pi^{3/2} \omega^2 \omega_z \). Defining the average number of molecules within observation volume as \( N = V_{\text{eff}} \langle C \rangle \), the characteristic diffusion time across the detection region as \( \tau_D = \omega^2 / 4D \) and the axial to radial aspect ratio of the sampling volume as \( \omega = \omega_z / \omega_y \), the following expression is deduced:

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \left( 1 + \frac{\tau}{\omega^2 \tau_D} \right)^{-1/2} + 1 = \frac{1}{N} G_D(\tau) + 1 \]  \tag{35}

Due to the presence of singlet-triplet transitions in almost all fluorophores and the fact that triplet state is not fluorescent, these transitions will have an influence on the correlation curve and need to be taken into account. Singlet-triplet transitions can be treated as a unimolecular reaction, and such reaction-diffusion systems are described by modifying the diffusion equation (21) into:

\[ \frac{\partial C_i(\vec{r},t)}{\partial t} = D_i \nabla^2 C_i(\vec{r},t) + \sum_{k=1}^{m} K_{ik} C_k(\vec{r},t) \]  \tag{36}

where the first term accounts for diffusion and the second term describes chemical changes. \( C_i(\vec{r},t) \) is the fractional concentration of the molecules in state \( \Sigma_i \) (singlet \( S_0, S_1 \) or triplet \( T_1 \)). Coefficients \( K_{ik} \) are combined from the chemical rate constants and the equilibrium concentrations of the species.

Equation (36) can be solved by taking Fourier transform in the similar fashion as equation (21), taking into account modified variant of equation (25) for the case of several species, the positions of which are uncorrelated is space:

\[ \langle \delta C_i(\vec{r},0) \cdot \delta C_k(\vec{r}''',0) \rangle = \langle C_i \rangle \delta_{ik} \delta(\vec{r} - \vec{r''}) \]  \tag{37}

The full derivation of the correlation function for chemical reactions is present-
ed in [Elson and Magde 1974], for singlet-triplet transitions in [Widengren et al. 1995] and for trans-cis isomerisation in [Widengren and Schwille 2000].

In the general case of chemical reactions, brightnesses of the states should be considered [Thompson 1992], but the triplet state is assumed to be totally dark, and FCS function describing combining Brownian diffusion and triplet kinetics thus becomes:

\[
G_N(T) = \frac{1}{N(1-T)} G_D(T) \left[ 1 - T + T \exp \left( -\frac{\tau}{\tau_T} \right) \right] + 1
\]

where \( T \) is the space and time averaged fraction of the molecules inside the observation volume being in the triplet state and \( \tau_T \) is the relaxation time of singlet-triplet transitions, defined by the rates from figure 2.2 (if only excitations to the first excited states are considered) [Widengren et al. 1995]:

\[
T = \frac{k_{01} k_{\text{ISC}}}{k_{01} (k_{\text{ISC}} + k_T) + k_{10} k_T}
\]

\[
\tau_T = \left( k_T + \frac{k_{01} k_{\text{ISC}}}{k_{01} + k_{10}} \right)^{-1}
\]

### 2.3.2 TIR-FCS

Total internal reflection fluorescence correlation spectroscopy (TIR-FCS) was introduced in 1981 [Thompson and Axelrod 1981; Thompson et al. 1981]. Before the implementation of the confocal principle to FCS, evanescent field was the natural way of confining the observation volume. Initial experimental realizations were done with the adaptation of prism-type TIRFM and were used for absorption studies of Rhodamine labeled immunoglobulin on quartz [Thompson and Axelrod 1981]. Although the theoretical framework was established for TIR-FCS, the technique was rarely used in the following years, presumably due to the large background and the low detection efficiency, implying small signal-to-noise ratio and poor reliability of the measurements.

Introduction of modern objective based TIR-FCS [Hassler et al. 2005a; Hassler et al. 2005b] increased significantly the countrates per molecule and signal-to-noise ratio, facilitating the applicability of the technique. A principal sketch of a typical objective-based TIR-FCS setup is presented in figure 2.13.
Derivation of the correlation function for TIR excitation is done analogously to the confocal case, the only difference being the MDE function. In the following derivation of the correlation function for Brownian diffusion, the results from the previous section will be used. MDE function for TIR detection volume could be approximated by:

\[
E(r) = E(0) \cdot \exp\left(-2 \frac{x^2 + y^2}{\omega_{xy}^2}\right) \cdot \exp\left(-\frac{z}{h}\right)
\]  

(41)

Figure 2.13: Sketch of a typical objective-based TIR-FCS setup. Compare to a confocal FCS setup, the main difference is in the presence of the third lens in the laser path before the dicroic mirror. This lens focuses the beam off-axis onto the back focal plane of the objective lens. The beam emerges collimated from the objective, totally reflects from the interface, returns off-axis into objective and is finally blocked. The focusing lens and the dicroic mirror are moved in one block by a linear translator with micrometer screws to adjust the lateral position of the laser beam entering the objective. In this way, the excitation angle can be adjusted without altering the optical path length between the focusing lens and the objective.
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where $\omega_{xy}$ is the lateral radius of the excitation volume and $h$ is the detected penetration depth of the evanescent field.

Since MDE is technically defined only in the positive half-space ($z > 0$), for the purpose of the analytical derivation of Fourier-transform equation (41) will be complemented, for negative half-space, by introducing modulus of axial coordinate $z$.

\[
E_{\mathbf{r}} = E(0) \cdot \exp \left( -2 \frac{x^2 + y^2}{\omega_{xy}^2} \right) \cdot \exp \left( -\frac{|z|}{h} \right) \tag{42}
\]

According to equation (28), we need to calculate the Fourier transform of MDE:

\[
\tilde{E}_{\mathbf{q}} = E(0) \cdot \frac{\omega_{xy}^2}{2\sqrt{2\pi}} \cdot \frac{h}{(1 + h^2 q_z^2)} \cdot \exp \left( \frac{-\omega_{xy}^2}{8} (q_z^2 + q_y^2) \right) \tag{43}
\]

\[
\tilde{E}_0 = E(0) \cdot \frac{\omega_{xy}^2}{2\sqrt{2\pi}} \cdot h \tag{44}
\]

Combining equations (29), (43) and (44) into equation (28) finally gives:

\[
G(\tau) = 1 + \frac{(2\pi)^{-3}}{\langle C \rangle} \int (1 + h^2 q_z^2)^{-2} \cdot \exp \left( \frac{-\omega_{xy}^2}{4} (q_z^2 + q_y^2) - Dq^2 \tau \right) d^3q \tag{45}
\]

This expression can be easily integrated, resulting in:

\[
G(\tau) = 1 + \frac{1}{4\pi \omega_{xy}^2 h \langle C \rangle} \left( 1 + \frac{4D\tau}{\omega_{xy}^2} \right)^{-1} \left( \frac{2\sqrt{D\tau}}{h\sqrt{\pi}} + \left( 1 - \frac{2D\tau}{h^2} \right) w \left( i\frac{\sqrt{D\tau}}{h} \right) \right) \tag{46}
\]

where $w$ is the complex error function, also known as the Faddeeva function:

\[
w(x) = \exp(-x^2) \text{erfc}(-ix) \tag{47}
\]

and erfc is the complimentary error function. By making substitutions such as
structure parameter $\omega = \hbar/\omega_{xy}$, axial diffusion time $\tau_z = \hbar^2/4D$, and calculating effective volume from equations (42) and (34) to be $V_{\text{eff}} = 4\pi \omega_{xy}^2 h$, we can rewrite equation for the correlation function for diffusion in TIR:

$$G(\tau) = \frac{1}{N} \left( 1 + \frac{\omega^2 \tau}{\tau_z} \right)^{-1} \left( \sqrt{\frac{\tau}{\pi \tau_z}} + \left( 1 - \frac{\tau}{2 \tau_z} \right) w \left( i \sqrt{\frac{\tau}{4 \tau_z}} \right) \right) + 1 \quad (48)$$

Taking singlet-triplet transitions into account by substituting the diffusion equation (21) with the reaction-diffusion equation (36), one can derive the autocorrelation function for diffusion+triplet kinetics (full derivation presented in [Hassler 2006]):

$$G(\tau) = 1 + \frac{1}{N} \left( 1 + \frac{T}{1 - T} \exp \left( - \frac{\tau}{T_T} \right) \right) \times$$

$$\times \left( 1 + \frac{\omega^2 \tau}{\tau_z} \right)^{-1} \left( \sqrt{\frac{\tau}{\pi \tau_z}} + \left( 1 - \frac{\tau}{2 \tau_z} \right) w \left( i \sqrt{\frac{\tau}{4 \tau_z}} \right) \right) \quad (49)$$

### 2.3.3 TRAST spectroscopy

Transient state (TRAST) spectroscopy is a recently developed technique [Sandén et al. 2007; Sandén et al. 2008] which uses temporal modulation of the excitation in order to obtain information about photoinduced dark transient states, like the triplet state. It relies on the fact that fluorescence emission, after excitation, is not a stochastic but rather a deterministic process (on the timescale of µs), governed by the kinetic rates of transitions between the electronic states and well described by a system of differential equations (from figure 2.2, considering only ground singlet and first excited singlet and triplet states):

$$\frac{\partial}{\partial t} \begin{bmatrix} S_0(\vec{r},t) \\ S_1(\vec{r},t) \\ T_1(\vec{r},t) \end{bmatrix} = \begin{bmatrix} -k_{01}(\vec{r},t) & k_{10} & k_T \\ k_{01}(\vec{r},t) & -(k_{10} + k_{\text{ISC}}) & 0 \\ 0 & k_{\text{ISC}} & -k_T \end{bmatrix} \begin{bmatrix} S_0(\vec{r},t) \\ S_1(\vec{r},t) \\ T_1(\vec{r},t) \end{bmatrix} \quad (50)$$

In the differential equation system, the excitation rate $k_{01}$ is given by $k_{01}(\vec{r},t) = \sigma_{01} I_{\text{exc}}(\vec{r},t)$, where $\sigma_{01}$ is the excitation cross-section (calculated from the molar extinction coefficient) and $k_{01}(\vec{r},t) = \sigma_{01} I_{\text{exc}}(\vec{r},t)$ denotes the excitation
irradiance at a location \( \vec{r} \) within the detection volume. \( S_0(\vec{r}, t) \) is the probability for the molecule to reside in the ground singlet state, \( S_1(\vec{r}, t) \) and \( T_1(\vec{r}, t) \) – in the first excited singlet and triplet state, respectively. Assuming that, the fluorophore concentration can be written as:

\[
C(\vec{r}, t) = C(\vec{r}, t) \left[ S_0(\vec{r}, t) + S_1(\vec{r}, t) + T_1(\vec{r}, t) \right]
\]

(51)

so that \( S_0(\vec{r}, t) + S_1(\vec{r}, t) + T_1(\vec{r}, t) = 1 \).

The initial conditions to equation (50) originate from the fact that before the excitation at time \( t = 0 \) all of the fluorophores are regarded to be in ground singlet state:

\[
\begin{bmatrix}
S_0(\vec{r}, 0) \\
S_1(\vec{r}, 0) \\
T_1(\vec{r}, 0)
\end{bmatrix} = \begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix}
\]

(52)

In order to obtain a simpler expression for the solution, one can assume that the decay of the singlet state by fluorescence or internal conversion is much faster than either of the processes of intersystem crossing or triplet state decay:

\[
k_{10} \gg k_{\text{ISC}}, k_T
\]

(53)

Applying the boundary conditions (52) to differential equation (50), taking into account simplification (53) and assuming the excitation rate to be stationary in time, the probabilities of occupying the different electronic states for a fluorophore in space \( \vec{r} \) as a function of time [Widengren et al. 1995] become:

\[
S_0(\vec{r}, t) = \frac{k_{10}k_T}{k_{01}^p(k_{\text{ISC}} + k_T) + k_{10}k_T} e^{\lambda_1^p t} + \frac{k_{01}^p}{k_{01} + k_{10}} e^{\lambda_2^p t} + \frac{k_{01}^p k_{10} k_{\text{ISC}}}{k_{01} (k_{\text{ISC}} + k_T) + k_{10} k_T} e^{\lambda_3^p t}
\]

(54)

\[
S_1(\vec{r}, t) = \frac{k_{01}^p}{k_{01} + k_{10}} e^{\lambda_2^p t} + \frac{(k_{01}^p)^2 k_{\text{ISC}}}{k_{01} (k_{\text{ISC}} + k_T) + k_{10} k_T} e^{\lambda_3^p t}
\]

\[
T_1(\vec{r}, t) = \frac{k_{01}^p k_{\text{ISC}}}{k_{01} (k_{\text{ISC}} + k_T) + k_{10} k_T} e^{\lambda_3^p t} - \frac{k_{01}^p k_{\text{ISC}}}{k_{01} (k_{\text{ISC}} + k_T) + k_{10} k_T} e^{\lambda_3^p t}
\]
with the eigenvalues related to the relaxation modes of the population kinetics:

\[
\begin{align*}
\lambda_1^{(r)} &= 0 \\
\lambda_2^{(r)} &= -\left( k_{01}^{(r)} + k_{10} \right) \\
\lambda_3^{(r)} &= -\left[ k_T + \frac{k_{01}^{(r)} k_{\text{ISC}}}{k_{01}^{(r)} + k_{10}} \right]
\end{align*}
\]  

\[(55)\]

In (55), the first eigenvalue \( \lambda_1 \) is zero indicating that the population will approach steady-state as \( t \to \infty \). The second eigenvalue \( \lambda_2 \) represents the so called antibunching term (\( \tau_{\text{AB}} = \lambda_2 \)) [Mets et al. 1997], reflecting the time it takes to establish equilibrium between singlet states. The third eigenvalue \( \lambda_3 \) is related to the rate at which buildup of the triplet state population takes place. Visualization of the dynamics of the states defined by equation (54) is presented in figure 2.14A.

In the results of the simulations presented in figure 2.14, it is important to note that population of first excited state \( S_1 \) after onset of the excitation initially increases, and then decreases due to triplet state build up. Since state \( S_1 \) is the state from which fluorescence is generated, it can be expected that fluorescence intensity immediately after the excitation will be higher than during the steady-state equilibrium. However, to detect this extra-fluorescence, high temporal resolution is needed. To circumvent this problem, pulsed excitation is applied and the excitation is switched off after certain time – pulse width \( w \). Obviously, for the very short pulses with
The initial implementation of TRAST was realized on a confocal FCS setup using APDs with high temporal resolution as detectors. However high temporal resolution is superfluous for this technique and it was demonstrated that detection with ordinary of-the-shelf CCD camera works equally good for detection, decreasing instrumental demands and allowing parallel, large-scale detection [Sandén et al. 2007]. TRAST is easily implemented on an ordinary laser scanning confocal microscope, because scanning is equivalent to temporal modulation, which was successfully demonstrated in [Sandén et al. 2008].

Furthermore, TRAST have been applied for measuring the triplet state rates of the fluorophores in liposomes [Sandén et al. 2008], for imaging oxygen consumptions during the contractions of a single smooth muscle cell [Geissbühler et al. 2010] and for monitoring oxidation-reduction reactions on surfaces [Spielmann et al. 2010].
Chapter 3

PHOTOBLEACHING & ADDITIVES

Photophysical and photochemical properties of fluorescent molecules are of great importance for all applications of fluorescence spectroscopy where a high read-out rate or a high sensitivity is required. In particular, this is important for applications regarding single molecule detection (SMD) or fluorescence correlation spectroscopy (FCS), where a low fluorescence signal can not be compensated by an increased fluorophore concentration. In this respect, there is a need to optimize the fluorescence, both with respect to the total number of photons that can be detected per molecule, as well as with respect to the fluorescence emission rate itself.

In paper I, various strategies are proposed for improving the fluorescence rate and decreasing photobleaching of fluorophores. In particular, the effects of the addition of two main categories of anti-fading compounds (see figure 3.1): anti-oxidants (n-propyl gallate, nPG, and ascorbic acid, AA) and triplet state quenchers (mercaptoethylamine, MEA, and cyclooctatetraene, COT) were investigated and the relevant rate parameters involved were determined for Rhodamine 6G (Rh6G). Addition of each of the compound categories yielded significant improvements in the fluorescence brightness of the monitored fluorescent molecules in FCS measurements. For anti-oxidants, we identify the balance between reduction of photionized fluorophores on the one hand, and that of intact fluorophores on the other, as an important guideline for what concentrations to add for optimal fluorescence generation in FCS and SMD experiments. For nPG/AA this optimal concentration
was found to be in the lower micromolar range, which is considerably less than what has previously been suggested [Longin et al. 1993]. Also, for MEA, a compound known as a triplet state quencher, it is in the end its anti-oxidative properties, and the balance between reduction of fluorophore cation radicals and that of intact fluorophores, that defines the optimal added concentration. Interestingly, in this optimal concentration range, the triplet state quenching is still far from sufficient to fully minimize the triplet populations.

### 3.1 Antioxidants *n*-propyl gallate and ascorbic acid

**nPG as an antioxidant in mM concentrations**

According to previous reports on the photobleaching retarding effects of nPG, relatively high nPG concentrations of around 10 mM should be necessary in order to obtain a significant effect [Giloh and Sedat 1982; Longin et al. 1993; Gaigalas et al. 2004]. However, from our FCS measurements nPG was found to strongly quench the Rh6G molecules at these concentrations. To analyze the nature of the quenching more closely a series of correlation curves was measured at varying nPG concentrations and excitation irradiances (figure 3.2A). The measured autocorrelation curves showed a characteristic fluctuation process in the time range of a few hundred nanoseconds or shorter, which very much resembled that obtained from the electron transfer induced quenching of deoxy guanosine triphosphate (dGTP)
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in a previous study [Widengren et al. 1997].

The time-dependent part of the correlation curves was well fitted to the expression of equation (38) with an additional exponential process:

\[
G(\tau) = \frac{G_D(\tau)}{N(1-T-C)}[1-T-C+T\exp\left(-\tau/\tau_T\right)+C\exp\left(-\tau/\tau_C\right)]+1
\]

(56)

where \( G_D(\tau) \) is defined in the same way as in equation (35).

Similar to dGTP, and because nPG is known as a reducing agent, this additional exponential process can be attributed to nPG acting as an electron donor with charge-transfer taking place upon hydrophobic interaction between the nPG and the Rh6G molecules. As for the charge transfer observed for Rh6G and dGTP [Widengren et al. 1997], a rate scheme as outlined in the inset of figure 3.2A was used to describe the observed exponential relaxations in the FCS curves. Except of usual states \( S_0 \), \( S_1 \), and \( T_1 \) (figure 2.2), this scheme includes the possibility of complex formation between Rh6G and nPG and subsequent electron transfer from nPG to Rh6G with different rates depending on wherever Rh6G is in its \( S_0 \) or \( S_1 \) state.

The rate scheme can be reduced to a three state model, consisting of a Rh6G singlet state, \( S \) (\( S_0 \) and \( S_1 \)), the lowest triplet state, \( T_1 \), and a complexed state with nPG, \( C \) (\( S_{C0} \) and \( S_{C1} \), see inset figure 3.2A), by recognizing that the intra-singlet state transitions (i.e., those between \( S_0 \) and \( S_1 \), and between \( S_{C0} \) and \( S_{C1} \), respectively) take

---

**Figure 3.2:**

**A:** FCS curves of Rh6G in aqueous solution containing 1, 3 and 10 mM of nPG. Inset: Rate scheme describing the relaxation behavior of the two exponential processes of the recorded correlation curves, involving singlet-triplet transitions and charge transfer following hydrophobic complex formation (Rh6G-nPG).

**B:** Extracted rates of association (\( k_{ass} \)) and dissociation (\( k_{diss} \)) of Rh6G and nPG.
place on a time scale much faster than any of the other dynamic processes involved:

\[
C \xleftarrow{k_{\text{ass}0}} S \xleftarrow{k_{\text{ISC}0}} T_1
\]

where \(k_{\text{ass}0}\) and \(k_{\text{ISC}0}\) denote the effective rates of complex formation between Rh6G and nPG and of intersystem crossing within the Rh6G molecules, respectively. Due to very fast deactivation of the excited state of Rh6G-nPG complex, dissociation from this state (rate \(k_{\text{diss}1}\)) can be disregarded in equation (57). The measured amplitudes (\(T\) and \(C\)) and relaxation times (\(\tau_T\) and \(\tau_C\)) from the correlation curves are given from the corresponding to equation (57) system of coupled first order differential equations. This system was solved in a similar way as was done in [Widengren et al. 1997]. The obtained rate constants of \(k_{\text{ass}0}\), \(k_{\text{ass}1}\) and \(k_{\text{diss}0}\) are plotted versus the concentration of nPG in figure 3.2B. Note the increase of the association rates \(k_{\text{ass}0}\) and \(k_{\text{ass}1}\).

From the results presented in figure 3.2B, it can be concluded, that at millimolar concentrations nPG does not show an overall beneficial effect on the Rh6G fluorescence due to its strong contribution to both static (\(k_{\text{ass}0}\)) as well as dynamic (\(k_{\text{ass}1}\)) fluorescence quenching. Although the photobleaching measurements undertaken at those concentrations indeed show a retardation of the photobleaching. Also under our experimental conditions we observe no significant shortening of the overall decay times of the correlation curves. However, the fluorescence quenching is much more pronounced. Addition of nPG in millimolar concentrations therefore does not seem to provide a useful strategy to reduce photobleaching under FCS conditions.

**nPG as an antioxidant in µM concentrations**

In contrast, at concentration levels of nPG in the µM range, at which most of the static and dynamic quenching effects observed at millimolar concentration are no longer traceable, the retarding effect of nPG was still pronounced in the sense that addition of increasing amounts of nPG increased the overall decay times of the correlation curves. In particular, this effect could clearly be seen from the FCS measurements with the expanded sample volume element, so that the passage time of the Rh6G molecules are in the order of a few milliseconds (see figure 3.3A).

These correlation curves recorded with the expanded sample volume were well fitted to the expression used to describe photobleaching [Eggeling et al. 1998b]:

\[
G_B(\tau) = G(\tau)\left[1 - B + B \exp(-k_B\tau)\right]
\]

\[\text{(58)}\]
where \( G(\tau) \) is defined as in equation (38).

With increasing concentrations of nPG, the amplitude \( B \) and was found to continuously decrease, while the relaxation rate \( k_B \) was found to increase (figure 3.3A). Taking into account the ability of nPG to act as an electron donor, as shown above, this behavior is well in agreement with a bimolecular reaction involving the nPG and the fluorophore molecules. In this reaction, the fluorescence emission of the fluorophores can be “switched off” as a consequence of laser irradiation and photoionization. The photoionized Rh6G molecules can then be “switched on” again by receiving an electron, following a collisional encounter with an nPG molecule. The major features of this photoionization/reduction process are outlined in figure 3.4A.

From previous studies on photobleaching it was known that photobleaching quantum yield \( \Phi_B \) deviates from linear dependence at high excitation irradiances [Eggeling et al. 1998a]. This increase of \( \Phi_B \) was found to be taken well into account by including excitation to higher excited singlet and triplet states from which a subsequent photobleaching reaction may occur. For this reason, the scheme outlined in figure 3.4A was extended to take into account photoionization from higher excited states as well. This modified 5-level model is presented in figure 3.4B.

Defining and solving the correspondent system of rate equations and fitting the experimentally obtained values of \( B \) and \( k_B \) from equation (58) measured at various concentrations of nPG and various excitation irradiances, one can extract the corresponding oxidation (\( k_{\text{ox1}} \) and \( k_{\text{ox2}} \)) and reduction (\( k_{\text{red}} \)) rates, which are presented in figure 3.3B. While \( k_{\text{ox1}} \) and \( k_{\text{ox2}} \) remain close to constant and independent of the nPG concentration, \( k_{\text{red}}' \) (defined as \( k_{\text{red}X[nPG]} \)) shows a linear dependence.
The dependence of $k_{\text{ox1}}$, $k_{\text{oxn}}$, and $k_{\text{red}}$ on nPG concentration (figure 3.3B) well supports the hypothesized model of photo-oxidation and subsequent restoring reduction of the photo-oxidized fluorophores by nPG, taking place via collisional interactions.

**AA as an antioxidant in μM concentrations**

To strengthen the support of the proposed model further, we also investigated in the same manner the well-known antioxidant AA. As for the investigation involving nPG, a series of correlation curves of Rh6G in water were recorded at various AA concentrations and excitation intensities using an expanded volume element. The results are very similar to those obtained after addition of nPG. As shown in figure 3.5, addition of increasing amounts of AA in the sub-millimolar concentration range to the Rh6G solution increases the overall correlation decay time (compare figures 3.5 and 3.3A).

From the effects of nPG and AA on the fluorescence of Rh6G, it seems as if the concentration of antioxidants has to be balanced. The antioxidants then have to be present at high enough concentrations that the effective rate of reduction can compete with the effective photo-oxidation rate. On the other hand, if present at too high concentrations, the extent of reduction of fluorescent fluorophore molecules by the antioxidants can get significant and can generate a strong static as well as dynamic quenching.
Figure 3.5: FCS curves of Rh6G in aqueous solution with addition of various concentrations of ascorbic acid (0-80 µM). Excitation irradiance is 0.7 MW/cm².

### 3.2 Triplet state quenchers – mercaptoethylamine and cyclooctatetraene

Adding MEA into an aqueous solution of Rh6G led to a clear reduction of the triplet state contribution in the correlation curves (figure 3.6A). Moreover, in addition to the triplet state relaxation (~1 µs range), an additional relaxation process in the ~10 µs range could be observed (figure 3.6A) with an amplitude that increased

Figure 3.6: A. FCS curves of Rh6G in aqueous solution with addition of MEA at various concentrations (0-8 mM). Excitation intensity 2.9 MW/cm² for all correlation curves. B. The MEA concentration dependence of $k_{ISC}$ and $k_T$, extracted from FCS measurements and the $\tau_T$ and $T$, measured at different excitation intensities (10 kW/cm²-3MW/cm²). C. FCS curves of Rh6G in deoxygenated aqueous solution with addition of 2 mM MEA at different excitation intensities.
with the applied excitation intensities. By fitting the correlation curves measured at different excitation intensities to equation (56) and then fitting all the obtained $T$ and $\tau_T$ parameters to equations (39) and (40), respectively, the rates for intersystem crossing $k_{\text{ISC}}$ and triplet state decay $k_T$ could be determined [Widengren et al. 1995]. The triplet state rate parameters determined for different concentrations of MEA are plotted in the figure 3.6B. It can be seen that $k_T$ increases linearly with the MEA concentration while $k_{\text{ISC}}$ remains essentially unaffected. In addition, a strong increase of the countrate per molecule (CPM) was observed upon addition of MEA.

However, by increasing the concentrations of MEA above 2-6 mM, no significant further increase in the fluorescence output could be noticed. We attribute this to the process underlying the additional exponential relaxation process that could be observed in the FCS curves upon addition of MEA (figure 3.6A). The amplitude of this additional process was increasing for concentrations above 6 mM, with simultaneous decrease of fluorescence rate (CPM). Therefore, this can be contributed to MEA-induced reduction of the Rh6G molecules.

In order to strengthen this assumption, measurements with MEA were performed under deoxygenated conditions. Molecular oxygen plays a double role for fluorophores. It is an efficient triplet state quencher, and collisional triplet state quenching by oxygen is the main contribution to $k_T$ under air saturated conditions [Korobov and Chibisov 1983]. On the other hand, this triplet quenching process results in an excited singlet state of oxygen (so called singlet oxygen), which is very reactive and contributes to the photodegradation of fluorophores [Singh et al. 1992]. As can be seen from figure 3.6C – without oxygen a strong accumulation of the fluorophores in their triplet states occurs. Upon addition of MEA the triplet relaxation term is significantly reduced. However, the second relaxation process is clearly seen, with much higher amplitude than under air-saturated conditions, at similar excitation irradiances and MEA concentrations. This is well in agreement with previous reports stating that the major decay pathway of the reduced (non-fluorescent) form of Rh6G is by collisional interactions with the dissolved molecular oxygen. From this point of view, addition of MEA to deoxygenated solutions has a stronger relative effect on the triplet state population. However, MEA also promotes formation of reduced Rh6G, which in the absence of oxygen can be strongly accumulated. Therefore, in deoxygenated as well as in air-saturated solutions, there is a tradeoff between triplet quenching on the one hand and fluorophore reduction on the other upon addition of MEA.

Also, FCS measurements on Rh6G with various concentrations of COT were performed in ethanol. The beneficial effect in terms of reduction the triplet state
fraction is visible in figure 3.7A. Performing the same analysis as for MEA, linear dependence of the triplet deactivation rate $k_T$ versus COT concentration was observed, with triplet intersystem crossing rate $k_{ISC}$ being unchanged. At COT concentrations higher than 3 mM the triplet quenching effect was diminished, probably because of micelle formation of COT molecules. Below this limit, the addition of COT enhanced the fluorescence output from the Rh6G molecules. These results support the view of COT as a potent triplet state quencher of Rh6G, as put forth by investigators since its use in dye lasers [Pappalardo et al. 1970].

3.3 Optimization of fluorescence output

A summary of the achieved fluorescence countrate per molecule (CPM) of Rh6G is plotted versus excitation irradiance in figure 3.8A-B. In agreement with previous findings, addition of the antioxidant nPG in micromolar concentrations has a strongly beneficial effect on the recorded CPM. However, at increased concentrations the extent of electron donation and quenching of intact fluorophores by the antioxidants becomes increasingly dominant and reduces the fluorescence brightness of the fluorophores. With MEA, higher countrates could be achieved than with addition of AA or nPG, which reflects the double impact of MEA – as a triplet quencher and as antioxidant at the same time. In figure 3.8C CPM values are plotted versus MEA concentration at the excitation intensity where maximum CPM values were achieved. It is interesting to note that the optimum MEA concentration is considerably lower than that required for a full triplet quenching (see figure 3.6A). This indicates that for MEA, its antioxidative properties and the balance between electron donation to photo-oxidized fluorophores (promoting fluorescence) and the
Chapter 3: Photobleaching & additives

reduction of non photo-oxidized fluorophores (quenching fluorescence) is at least as important as the extent of triplet quenching.

Figure 3.8: CPM of Rh6G measured by FCS with an expanded volume element versus excitation irradiance and after addition of various concentrations of nPG (A) and MEA (B). C. CPM of Rh6G as a function of added concentration of MEA.
Chapter 4

TRIPLET STATE & QUENCHERS

Fluorescence quenching can be used profitably as a valuable source of information about interactions of a fluorescent molecule with a quencher. Among different varieties of quenching mechanisms (Section 2.1.7) and fluorophore states involved, the triplet state is especially beneficial due to its long lifetime (microseconds). This longer lifetime allows a longer time for interactions with local environment, thus offering greater sensitivity. In this chapter fluorescence quenching by two quenchers – potassium iodide (paper II) and TEMPO (paper III) is studied in detail.

As demonstrated in Chapter 3, balancing the concentration of additives influencing the fluorescence is of crucial importance. Compounds which are known to act as fluorescence promoters in certain conditions (like nPG and AA in µM concentrations) can turn out to be the fluorescence quenchers if applied inappropriately (meaning – in mM concentrations). In paper II, FCS was used to investigate the effect of potassium iodide (KI) on a set of fluorophores. Iodide is well known for enhancing intersystem crossing, in particular by the so-called heavy atom effect [Birks 1970; Turro et al. 2009]. Previously, it has been shown for several fluorophores that addition of KI lead to a strong enhancement of the triplet state population [Widengren et al. 1995]. However, in paper II we show that for many fluorophores absorbing/emitting in the blue/green range, addition of KI instead leads to a reduction of the triplet state population. Apart from a heavy atom effect, triplet state deactivation was also found to take place by electron transfer between the fluorophore and KI.
Chapter 4: Triplet state & quenchers

This electron transfer was found not only to affect the triplet state, but also photooxidized as well as fluorescently viable fluorophore molecules in their singlet states.

In paper III, an approach to study bimolecular interactions in model lipid bilayers and biological membranes based on the triplet quenching mechanism is introduced. It exploits the influence of membrane-associated electron spin resonance labels TEMPO on the fluorescence signal of a likewise membrane-bound fluorophore marker. Using fluorescence as readout maintains a high detection sensitivity, without sacrificing the benefit of the long lifetime and environmental sensitivity of the triplet state. The quenching mechanisms were investigated first in aqueous solution. Then the TEMPO-induced quenching of Lissamine Rhodamine B (LRB) was analyzed in unilamellar liposomes, with the fluorophores covalently linked to lipid headgroups. A two-dimensional model for a diffusion-controlled, bi-molecular quenching process was applied and found to predict well the diffusion behavior and the collisional encounter frequencies between the LRB- and TEMPO-labeled lipids. The proposed approach can be applied to a wide range of molecular interaction studies in membranes.

4.1 Iodide as a triplet state promoter and quencher

A range of fluorophores was investigated with respect to the effect of KI on their triplet state population dynamics. For the various fluorophores, KI was added in concentrations from 0.5 mM up to 50 mM, and their triplet state kinetics was studied at different excitation irradiances (from 10 to 1000 kW/cm²). From this initial investigation, it was possible to identify two categories of fluorophores. For the first group (in order of increase in absorption wavelength: Rhodamine 6G, TMR, Lissamine Rhodamine B (LRB), ATTO 590, Alexa 594, ATTO 610, Alexa 610 and Alexa 633), a prominent increase of triplet state fraction was observed with increasing KI concentrations. This response is well in agreement with that previously observed for KI on Rh6G [Widengren et al. 1995]. For the second group of fluorophores (in order of decrease in absorption wavelength: Rhodamine 123, Rhodamine Green (RhGr), ATTO 488 and Alexa 488) the effect of KI was the opposite – with increasing KI concentrations a distinct decrease of the triplet state fraction was observed. To investigate the underlying mechanisms behind the different response to KI, one fluorophore from each category, Rh6G from the first group and RhGr from the second group, were selected and investigated more in detail.

A set of FCS curves recorded at various KI concentrations for these two fluorophores was investigated with respect to the effect of KI on their triplet state population dynamics. For the various fluorophores, KI was added in concentrations from 0.5 mM up to 50 mM, and their triplet state kinetics was studied at different excitation irradiances (from 10 to 1000 kW/cm²). From this initial investigation, it was possible to identify two categories of fluorophores. For the first group (in order of increase in absorption wavelength: Rhodamine 6G, TMR, Lissamine Rhodamine B (LRB), ATTO 590, Alexa 594, ATTO 610, Alexa 610 and Alexa 633), a prominent increase of triplet state fraction was observed with increasing KI concentrations. This response is well in agreement with that previously observed for KI on Rh6G [Widengren et al. 1995]. For the second group of fluorophores (in order of decrease in absorption wavelength: Rhodamine 123, Rhodamine Green (RhGr), ATTO 488 and Alexa 488) the effect of KI was the opposite – with increasing KI concentrations a distinct decrease of the triplet state fraction was observed. To investigate the underlying mechanisms behind the different response to KI, one fluorophore from each category, Rh6G from the first group and RhGr from the second group, were selected and investigated more in detail.

A set of FCS curves recorded at various KI concentrations for these two fluoro-
phores is shown in figure 4.1. From this figure, the different response can be clearly seen, with a significant increase of the triplet state population, \( T \), of Rh6G and a corresponding decrease of \( T \) for RhGr. For both fluorophores the triplet relaxation time was observed to significantly decrease with increasing KI concentrations. For RhGr, a second relaxation process in the time range 5-10 µs could be observed in the FCS curves at KI concentrations above 5 mM. Increase of the amplitude and decrease of the relaxation time of this relaxation process versus the applied excitation irradiance was observed in the FCS curves, in a similar way as for the triplet relaxation term. This suggests that except of an external heavy atom effect influencing the intersystem crossing rate from the singlet to the triplet manifold, there is, at least for the second group of fluorophores, an additional triplet state deactivation mechanism present. Such appearance of the second exponential process occurring in the FCS curves of RhGr upon addition of KI indicates that an enhanced deactivation of the lowest triplet state is accompanied by the build-up of another, more long-lived non-fluorescent state. As a possible additional deactivation mechanism of the triplet state of RhGr in the presence of KI, a charge transfer reaction could be considered [Korobov and Chibisov 1983].

To further investigate the underlying mechanisms of the deactivation of the RhGr triplet state by KI, and what possible additional effects KI may have on the fluorescence properties of RhGr, FCS measurements on RhGr were performed systematically over a broad range of KI concentrations (1 µM-50 mM) on the setup with an expanded excitation/detection volume. The outcome is presented in figure 4.2.
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This behavior is similar to that found for the well known antioxidants n-propyl gallate (nPG) and ascorbic acid (AA) (see Section 3.1). Interestingly, KI is not primarily known as an antioxidant and antifading compound, as it appears to be for the fluorophores of the second group, but rather the opposite – as a fluorescence quencher. However, when dissociated in aqueous solution into potassium and iodide ions, it is known to act as a mild reducing agent [Drexhage 1990]. As an electron donor, it participates in a bimolecular reaction with the fluorophore molecules, which thereby return to the normal state by receiving an electron from the iodide ions. The expanded volume measurements were analyzed in the same way as described in Section 3.1. The obtained values of the oxidation and reduction rates for each concentration of KI are plotted in figure 4.2B. While the oxidation rates \( k_{ox1} \) and \( k_{oxn} \) remain virtually constant and independent of KI concentration, the reduction rate, \( k_{red} \), shows a linear dependence.

When gradually increasing the KI concentration from 0.5 mM to 50 mM, it was observed (figure 4.1B) that the triplet relaxation term in the FCS curves was decreased and shifted to faster relaxation times (tens of nanoseconds). At the same time, at KI concentrations above 10 mM an additional relaxation process in the time range of several microseconds could be observed. In analogy to the behavior observed for other investigated antioxidants (nPG and AA), applied in similar concentrations (see Section 3.1) this latter process can be attributed to reduction of non-oxidized fluorophores. Also decrease of the excited state lifetime of RhGr was observed in presence of mM concentrations of KI. A modified kinetic scheme incorporating these transitions is presented in figure 4.3.

**Figure 4.2:** A. FCS curves of RhGr measured in aqueous solution using an expanded excitation volume. Excitation irradiance 240 kW/cm². With increasing KI concentration, the amplitude of the second relaxation process \( R \) is decreasing from 0.42 to 0.09, while the relaxation time \( \tau_R \) is decreasing from 378 µs to 117 µs. B. The parameters of \( k_{ox1} \), \( k_{oxn} \) and \( k_{red} \) obtained after fitting the experimental parameters from FCS curves obtained for each concentration of KI in µM range.
In order to reduce the complexity of the system, FCS measurements with high concentrations of KI were again recorded from a diffraction limited observation volume, with resulting dwell times of the fluorophore molecules in the order of 30 µs. At this fast passage times and such high concentrations of KI, photo-oxidation of the fluorophores is relatively low and is fully recovered by iodide, therefore the fraction of fluorophores being in $\textit{R}^+$ approaches to zero. Consequently, transitions to and from the photo-oxidized state, $\textit{R}^+$, can be disregarded in the correlation curves,

Figure 4.3: A modified kinetic scheme of photoinduced processes in presence of KI at mM concentrations. Compare to figure 3.4B, the influence on intersystem crossing rate due to external heavy atom effect is included by the rate $k_{\text{ISC-KI}}$. Triplet quenching effects is included by the rate $k_{\text{T-KI}}$. Singlet quenching effect is indicated by $k_{\text{10-KI}}$. Reduction of intact viable fluorophores by iodide is included by the rate $k_{\text{red}}$, and KI-independent oxidation of fluorophore anions represented by the rate $k_{\text{ox*}}$. $\textit{R}$ indicates cationic fluorophore radical. $\textit{R}^-$ indicates anionic fluorophore radical.

Figure 4.4: A. The dependence of fluorescence deactivation rate from KI concentration extracted from TCSPC measurements. B. KI concentration dependence of $k_{\text{ISC}}$ and $k_{\text{T}}$, extracted from FCS measurements. C. The parameters of iodide-induced reduction of fluorophores $k_{\text{red}}$ ($=k_{\text{red}}[\text{KI}]$) and iodide-independent oxidation of fluorophore anions $k_{\text{ox*}}$, obtained after fitting the experimental parameters from FCS curves obtained for each concentration of KI in mM range.
Chapter 4: Triplet state & quenchers

and only transitions to and from the reduced radical state \( R^- \) and the triplet dynamics remain to be considered. The outcome of the analysis is presented in figure 4.4.

From the investigations of the effects of KI on RhGr, we observe that dissolved I\(^-\) not only promotes the intersystem crossing to the triplet state by an external “heavy atom” effect, but can also deactivate the \( T_1 \) state, as well as the excited singlet states by a charge transfer reaction. In order to demonstrate and more specifically investigate the isolated “heavy atom” effect for the fluorophores of the second group (Rhodamine Green - alike), measurements with Iodobenzene (IB) were performed in ethanol. A set of FCS curves, recorded at various IB concentrations are shown in figure 4.5A, together with the determined \( k_{\text{ISC}} \) and \( k_T \) rates in figure 4.5B.

When IB is dissolved into ethanol, Iodine is not present as an ion, but rather in a coupled form. This excludes charge transfer reactions and fluorophores present in the solution are only expected to be influenced by a pure external heavy atom effect. Indeed, as can be seen from figure 4.5A, there is a clear increase of the triplet state fraction with increasing IB concentrations. The intersystem crossing rate was found to increase linearly with the IB concentration, while the triplet relaxation rate is close to constant (figure 4.5B).

Given the different effects of KI on the transition rates between the electronic states of RhGr, which can influence the fluorescence yield in different and opposing directions, we investigated effects of I\(^-\) on the fluorescence countrate per molecule (CPM) of RhGr by adding KI at different concentrations. The outcome is shown in figure 4.6.
As previously found for MEA, the optimum concentration of KI is considerably lower than that required for a full triplet quenching of RhGr fluorophore (figure 4.1B). It is rather the balance between antioxidative properties of KI and its strength of triplet quenching which defines the concentration at which a maximum CPM can be reached.

**Figure 4.6:** CPM of RhGr without and with KI measured by FCS versus laser power. CPM is increasing significantly for KI concentrations up to 5 mM, but at higher concentrations effect of fluorescence quenching and KI-induced oxidation is prevailing.

**Figure 4.7:** FCS curves of RhGr (A) and LRB (B) measured in aqueous solution with and without TEMPO. Excitation irradiance 450 kW/cm². With addition of TEMPO, for RhGr the amplitude of triplet relaxation term $T$ decreases from 0.48 to 0.07, and the triplet relaxation time $\tau_T$ decreases from 1.4 µs to 0.12 µs. Also, the second relaxation process is observed with the amplitude 0.23 and relaxation time 1.7 µs. For LRB, addition of TEMPO results in the increased $T$ from 0.32 to 0.57, and the decreased $\tau_T$ from 1.7 µs to 0.1 µs.
A similar double-sided effect of both triplet quenching and induction, as observed for KI on the fluorophores of the second category, can also be found for other compounds. Similar to molecular oxygen, transitions to and from the triplet state of fluorophores can also be enhanced by other paramagnetic species [Korobov and Chibisov 1983]. Figure 4.7 shows FCS curves monitoring the triplet state population dynamics of the fluorophores RhGr and LRB in the presence of TEMPO choline. TEMPO choline is a widely used label compound in electron spin resonance spectroscopy [Banwell and McCash 1994]. For fluorophores with excitation wavelength below 560 nm, like TMR, Rh6G and RhGr, a decrease of the triplet state population is observed upon addition of TEMPO. For fluorophores with excitation wavelength above 560 nm (such as LRB, ATTO590 and Alexa594) TEMPO induces triplet state build-up. This effect will be used in the following section.

4.2 Triplet state quenching for monitoring diffusion mediated reactions

Time-correlated single photon counting (TCSPC) measurements of LRB in aqueous solution show that upon addition of TEMPO in mM concentrations a small decrease in the fluorescence lifetime of LRB can be observed. The enhancement of \( k_{10} \) by the addition of TEMPO follows to a first approximation a linear relationship \( k_{10} = (0.70 \pm 0.001) \times 10^9 + (8.8 \pm 0.8) \times 10^9 \) [TEMPO] M\(^{-1}\)s\(^{-1}\). The presence of 1 mM TEMPO thus yields a relative change of \( k_{10} \) of LRB of about 1-2 percent. A similar relative change in the fluorescence intensity can be expected, when recorded under conventional excitation conditions (non-saturating excitation intensities). Given the paramagnetic properties of TEMPO, a considerably stronger relative effect can be expected on the transition rates to and from the triplet state of LRB.

To investigate the quenching properties of TEMPO on the triplet states of LRB, a series of FCS measurements was performed in aqueous solution, both under air-saturated and deoxygenated conditions. Under air-saturated conditions, FCS curves were recorded from LRB sample solutions with varying concentrations of TEMPO (0 mM to 5 mM), and for each TEMPO concentration, the excitation irradiance was varied from 20 to 500 kW/cm\(^2\). Upon addition of TEMPO an increase in the triplet amplitude, \( T \), and a drastic shortening of the triplet relaxation time, \( \tau_T \), could be observed in the correlation curves (see figure 4.8A). The correlation curves could be well fitted to equation (38) without the need to add any additional exponential relaxation terms in the fitting process.

For each concentration of TEMPO, the intersystem crossing rate, \( k_{\text{ISC}} \), could
then be obtained as a global fitting parameter from the excitation irradiance dependence of $T$ and $t_T$ using equations 39 and 40. Results of such fit are presented in figure 4.8. It can be noted that both $k_T$ and $k_{ISC}$ are influenced by the concentration of added TEMPO, [TEMPO], displaying a close to linear dependence. Considering the influence of TEMPO on $k_T$ and $k_{ISC}$ to be a bimolecular reaction, molar quenching rates coefficients $k_QISC = 1.6 \times 10^9$ M$^{-1}$s$^{-1}$ and $k_{QT} = 4.0 \times 10^8$ M$^{-1}$s$^{-1}$, were determined.

From the quenching coefficients one can see that the effect of TEMPO is about 4 times stronger on the $k_{ISC}$ rate, than on the $k_T$, which results in the observed increase of the triplet state fraction under air-saturated conditions. The opposite effect was observed in a previous study [Heupel et al. 2000], where the effect of TEMPO on the triplet state parameters of Rhodamine 6G (Rh6G) was investigated. However, Rh6G has a lower maximum absorption wavelength than LRB, i.e. a higher lying first excited singlet state. Since the energy levels of $S_1$ and $T_1$ are typically closely related, also the $T_1$ state of Rh6G can be expected to lie higher in energy than that of LRB. Because of this, charge transfer reactions to the ground triplet state of TEMPO are more likely to occur from the $T_1$ state of Rh6G than from the $T_1$ state of LRB. This additional de-activation channel of the $T_1$ state should then be reflected in the FCS measurements as a decreased steady-state triplet populations and a shortening of the triplet relaxation times. Indeed, FCS measurements on Rh6G and TEMPO showed not only a decrease of the triplet state fraction (in agreement with results reported in [Heupel et al. 2000]), but also the presence of a second relaxation component in the correlation curves. This additional term can be attributed

**Figure 4.8:** A. FCS curves of LRB measured in aqueous solution with and without TEMPO in concentrations 0-5 mM. Excitation irradiance $370$ kW/cm$^2$. With increasing TEMPO concentration the triplet fraction $T$ is increasing from 0.37 to 0.65, while the triplet relaxation time $t_T$ is decreasing from 1.6 $\mu$s to 0.07 $\mu$s. B. TEMPO concentration dependence of $k_{ISC}$ and $k_T$, extracted from FCS measurements and $T$ and $t_T$ parameters.
to formation of Rh6G radicals via their T₁ states [Becker et al. 1998]. However, for LRB the FCS measurements give no evidence of a strong charge-transfer-mediated quenching of T₁. This effect was therefore not included in the analysis.

The FCS measurements were repeated on the same samples under deoxygenized conditions. Similar to the observation in air-saturated measurements, the triplet relaxation times were found to decrease upon addition of TEMPO. However, in contrast to the air-saturated measurements, the triplet state amplitudes, T, decreased with increasing [TEMPO]. In air-saturated aqueous solutions most of the deactivation of T₁ is due to quenching by molecular oxygen. In the absence of oxygen, k_T is on the order 10³ s⁻¹. Addition of TEMPO then leads to a much stronger relative increase of k_T, compared to the relative increase it would generate in an air-saturated solution. With this in mind, and with reference to equation 40, it is clear that the decrease of T with increasing [TEMPO] found in deoxygenated solutions reflects the same influence of TEMPO on the k_T and kISC rates of LRB, as found under air-saturated conditions.

Also FCS measurements were performed on LRB-labeled liposomes in aqueous solution, with varying fractions of TEMPO labeled lipids included, both under air-saturated and under de-oxygenized conditions. The fraction of TEMPO-labeled lipids in the liposomes, L, was varied from 0 to 8% for the air-saturated measurements, and from 0 to 2% under the de-oxygenized conditions. Following the same type of analysis as for the measurements in solution, the triplet rates k_T and kISC were determined.

The number of reactions per second (ϕ) between molecules, B, and a single molecule, A, is equivalent to the flow of molecules B through a volume within a reaction radius, s, around A. For the reactions taking place in a spherical shell, i.e. for reactions in a membrane, Berg and Purcell provided an approximate expression for ϕ [Berg and Purcell 1977]. In the special case when all molecules occupy the same average area, A, then ϕ may be written as:

\[
ϕ = \frac{4\pi(D_A + D_B)P}{1.1A} \ln \left[ \frac{1.2A}{4\pi Lt^2} \right]^{-1}
\]

(59)

Here, D_A and D_B are the diffusion coefficients of A and B, respectively, and P is the probability of a reaction to occur when the reaction partners are within the reaction distance, s, from each other. L is the fraction of B molecules relative the total number of molecules in the membrane. In this context, A means lipids in the liposome without TEMPO and B means lipids with covalently bound TEMPO.
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From the \( k_T \) and \( k_{ISC} \) rate parameters, determined from FCS measurements, the average time between two quenching reactions could be determined for each fraction \( L \) from the difference of the inverse rates, in the presence and absence of TEMPO-labeled lipids:

\[
\frac{1}{\varphi_T} = \frac{1}{k_T(L) - k_T(0)} \tag{60}
\]

\[
\frac{1}{\varphi_{ISC}} = \frac{1}{k_{ISC}(L) - k_{ISC}(0)} \tag{61}
\]

Here, \( \varphi_T \) and \( \varphi_{ISC} \) denote the molecular encounter rates as given by equation (59), for triplet deactivation from \( T_1 \) and intersystem crossing to \( T_1 \), respectively. \( k_T(L) \) and \( k_{ISC}(L) \) denote the rates of \( k_T \) and \( k_{ISC} \) at a lipid fraction of \( L \), and \( k_T(0) \) and \( k_{ISC}(0) \) are the same rates in the absence of TEMPO-labeled lipids (\( L=0 \)).

Results of the analysis are presented in figure 4.9 for the de-oxygenated liposomes measurements. It can be noted, that collision rates \( \varphi_T \), determined via the triplet relaxation rate \( k_T \) give much better correspondence to the model, which reflects higher sensitivity of the triplet deactivation rate to TEMPO in the absence of oxygen. Another advantage of the de-oxygenation for the proposed approach is that in the absence of oxygen, the triplet state lifetimes get significantly longer and even slower molecular interaction frequencies can be followed.
Chapter 5

TRIPLET STATE & APPLICATIONS

Photoinduced dark states in fluorescence are still largely considered to be a nuisance, because they are always associated with a loss of fluorescence signal. In this chapter two possible applications are described, which make use of transient states – as labels for optical nanoscopy (paper IV) and as readout for monitoring molecular diffusion (paper V).

Photo-induced switching of fluorophores into dark, long-lived states, such as the triplet state, has recently gained increasing interest, as a means to achieve ultra-high optical resolution. Out of the multitude of super-resolution techniques (see Section 2.2.3), GSD is especially promising technique for biological live cell application, owing to lower irradiances required for the switching mechanism. For optimal performance of the GSD strategy, the fluorophores used should be readily photoswitchable into dark states. High triplet state populations of most fluorophores can be easily achieved by use of certain additives that enhance photo-induced triplet state generation, or by removal of dissolved oxygen. However, addition of these chemical compounds or de-oxygenation is not always compatible with biological applications. Also, dark states often appear as precursor states for photobleaching (see Section 2.1.8 and Chapter 3), which potentially can limit their usefulness. In paper IV, a set of fluorophores was investigated with respect to their triplet state dynamics and photostabilities, under conditions relevant for super-resolution microscopy. Out of the investigation, several fluorophores were found to meet the
requirements for super-resolution microscopy, combining a prominent triplet state yield with reasonable photostability.

In paper V, recovery of photoinduced reversible dark states is utilized as a read-out parameter for molecular diffusion investigations. In TRAST techniques (see Section 2.3.3) transitions of these states were monitored via the response in the time-averaged fluorescence to a time-modulated excitation. In the initial TRAST realizations typically information about the population dynamics of photo-induced transient states was obtained. In a similar way it is also possible to get information about diffusion in and out of the detection volume, because these transitions are superimposed on a modulation of the excitation and transitions to dark states – both of which are well-defined.

## 5.1 Novel fluorescent labels for super-resolution microscopy

To achieve a combination of a high triplet yield in biologically relevant environments (like water, buffers) combined with a low photobleaching quantum yield, two chemical strategies were devised. The first of them, substitution by sulfur, has been known to promote triplet state build-up due to the heavy atom effect (see Section 2.1.7). First, a pyronin fluorophore was used as the starting point for chemical modification, where Oxygen was substituted by Sulfur. However, the resulting Thiopyronin (figure 5.1A) has no coupling functionality. Also it is not stable at pH-values beyond 8, since it is prone to nucleophilic attack by hydroxyl ions at

![Chemical structures of investigated fluorophores.](image)

**Figure 5.1:** Chemical structures of investigated fluorophores. **A.** Thiopyronin. **B.** ATTO 465. **C.** Rhodamine derivatives, for Rhodamine 6G: R2 = CO₂C₂H₅, R4 = CH₃, R5 = H, R6 = C₂H₅, X = O; for TMR: R2 = CO₂C₂H₅, R4 = H, R5 = CH₃, R6 = CH₃, X = O; for Thiorhodamine: R2 = COOH, R4 = H, R5 = CH₃, R6 = CH₃, X = S; for ATTO Thio12: R2 = CON(CH₃)(CH₂)₃COOH, R4 = H, R5 = CH₃, R6 = CH₃, X = S. **D.** Halogenated rhodamine derivatives, for DR25: R1 = H, R2 = CO₂C₂H₅; for MR71: R1 = F, R2 = CO₂C₂H₅; for JA4: R1 = Cl, R2 = CO₂H; for JA98: R1 = Br, R2 = CO₂H.
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the central carbon atom of the chromophore. To render this position inaccessible, a carboxyphenyl group was introduced. The synthesized thiorhodamine (figure 5.1C) was found to be much more pH stable. Finally, a NHS-ester coupling group was introduced to permit coupling to biomolecules of interest, resulting in ATTO Thio12.

The second strategy applied, halogenation, is well known to promote triplet state build-up, as seen for instance in Eosin and Erythrosin. In these fluorophores the halogen atoms are linked directly to the chromophore unit xanthene body of the fluorophore, very close to the fluorescently active π-orbital region. However, this leads to a triplet induction too strong for our purpose. Therefore we investigated instead a set of Rhodamine derivatives with halogenated carboxyphenyl groups. The molecular structures for all investigated fluorophores are presented in figure 5.1.

Absorption and emission maxima as well as fluorescence lifetimes are presented in figure 5.2.

The triplet state properties of the fluorophores were investigated in detail by FCS. In figure 5.3, FCS curves are shown for Thiopyronin (TP), Thiorhodamine (TR) and ATTO Thio12 (AT12). A set of FCS curves for Rhodamine 6G (Rh6G), recorded at comparable excitation intensities, are shown for reference. Comparing the correlation curves for TP, TR and AT12 with those of Rh6G it is evident that
Chapter 5: Triplet state & applications

The triplet formation of these dyes is much more prominent. For TP, TR as well as AT12, the fraction of fluorophores within the excitation volume that are in the triplet states reaches 90% and more, already at irradiances of 30–40 kW/cm². The recorded correlation curves could be well fitted to a model accounting for diffusion and singlet–triplet transitions – equation (38). CPM of all the fluorophore molecules, being in the fluorescent and non-fluorescent (triplet) states, is significantly lower for TP, TR and AT12 than for the reference (Rh6G, TMR) fluorophores. On the other hand, when correcting for the non-fluorescent fraction of the fluorophores being in their triplet states, the fluorophores show a (singlet state) CPM which is well comparable to fluorophores such as Fluorescein.

The halogenated rhodamine derivatives (MR71, JA4, JA98 and DR25—a rhodamine analogue without halogens for reference) were studied by FCS in the same fashion. For these fluorophores no significant effects could be directly observed in the individual correlation curves on the triplet state parameters \( T \) and \( \tau_T \) arising from the halogenation in the phenyl ring.

**Figure 5.3:** FCS curves recorded in aqueous solution at different excitation irradiances. **A.** Thiopyronin, triplet fraction \( T \) is increasing from 0.53 to 0.87, relaxation time \( \tau_T \) 0.68-0.14 µs. **B.** Thiorhodamine, \( T \): 0.65-0.92, \( \tau_T \): 0.72-0.14 µs. **C.** ATTO Thio 12, \( T \): 0.67-0.90, \( \tau_T \): 0.78-0.19 µs. **D.** Rhodamine 6G, \( T \): 0.04-0.21, \( \tau_T \): 2.95-1.54 µs.
In the same manner ATTO 465 (figure 5.1B) was studied as a possible candidate for having relatively high triplet quantum yield. For this fluorophore and for irradiances up to 200 kW/cm² the fraction of the fluorophores being in the triplet state increased up to 75%, but then dropped to about 40% for higher irradiances applied. This drop in triplet fraction could be attributed to a high photobleaching quantum yield $\Phi_B$ of ATTO 465 and photodestruction of fluorophores at high excitation irradiances. The relative contribution of fluorescence from the periphery of the detection volume is increased, where lower excitation irradiances are experienced, and where the relative triplet state population of the fluorophores is lower. This drop in the observed triplet state population can be reversed by adding an antioxidant, 30 µM of ascorbic acid in this particular case. This indicates that the photodestruction mainly is due to photooxidation.

In order to extract the rate coefficients for intersystem crossing, $k_{ISC}$, and triplet state decay, $k_T$, FCS measurements were performed at different excitation irradiances. Extracted parameter values of $T$ and $\tau_T$ were fitted to equations (39)-(40).
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Table 5.1: Fluorescence parameters for studied fluorophores: fluorescence lifetime $\tau_{fl}$, fluorescence quantum yield in EtOH $q$, intersystem crossing rate $k_{ISC}$ and triplet relaxation rate $k_T$.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>$\tau_{fl}$ (ns)</th>
<th>$k_{ISC}$ ($\mu$s$^{-1}$)</th>
<th>$k_T$ ($\mu$s$^{-1}$)</th>
<th>$q$ in EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiopyronin</td>
<td>2.0</td>
<td>99</td>
<td>0.9</td>
<td>0.45</td>
</tr>
<tr>
<td>Thiorhodamine</td>
<td>2.1</td>
<td>125</td>
<td>0.5</td>
<td>0.34</td>
</tr>
<tr>
<td>ATTO Thio12</td>
<td>2.0</td>
<td>118</td>
<td>0.5</td>
<td>0.34</td>
</tr>
<tr>
<td>ATTO 465</td>
<td>2.2</td>
<td>56</td>
<td>1.3</td>
<td>0.55</td>
</tr>
<tr>
<td>Rhodamine 6G</td>
<td>3.9</td>
<td>1.1</td>
<td>0.5</td>
<td>0.95</td>
</tr>
<tr>
<td>TMR</td>
<td>2.1</td>
<td>0.9</td>
<td>0.5</td>
<td>0.90</td>
</tr>
<tr>
<td>DR25</td>
<td>4.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.97</td>
</tr>
<tr>
<td>MR71</td>
<td>4.0</td>
<td>0.5</td>
<td>0.3</td>
<td>0.90</td>
</tr>
<tr>
<td>JA4</td>
<td>4.0</td>
<td>0.6</td>
<td>0.4</td>
<td>0.90</td>
</tr>
<tr>
<td>JA98</td>
<td>4.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Results are presented in figure 5.4.

The triplet state parameters extracted from the FCS curves for all fluorophores studied in this section are presented in Table 5.1. From the fitted parameters it is evident that the high triplet population for the fluorophores TP, TR, AT12 and ATTO 465 is mainly due to the increase of the intersystem crossing rate $k_{ISC}$. The rate $k_T$ does not seem to be influenced significantly by the chemical modifications and is believed to be dependent mainly on the concentration of dissolved oxygen.

As a possible explanation for this strong increase of the $k_{ISC}$ rate in TP, TR and AT12 compared to similar traditional rhodamines (Rh6G, TMR), the intramolecular heavy-atom effect may be considered (Section 2.1.7). As a result, spin–orbit coupling will be stronger, promoting transitions from $S_1$ to $T_1$. This explanation would be in agreement with the literature and observations for TP, TR and AT12 where the central oxygen atom is replaced by sulfur, whose atomic mass is twice as large. However, this reasoning does not explain the case of ATTO 465, where the central oxygen atom is replaced by nitrogen, which has an atomic mass even smaller than oxygen. Although a convincing theoretical description for the increase in triplet yield cannot be given, a simple empirical rule can explain both situations. This rule states that in chromophore unit where the $\pi$-electrons can make a loop when oscillating between the end groups, the triplet yield will be higher than in a related compound where this loop is partially blocked [Drexhage 1990]. In the acridine fluorophore ATTO 465 the central nitrogen atom participates strongly in the conjugation of the chromophoric system making $\pi$-electron looping possible and thus giving rise to a high triplet yield. This effect is relatively small in common rhodamines as the oxygen atom in the center of the chromophore essentially prevents looping of $\pi$-electrons. A heuristic argument for this effect is that the circulating electrons create an orbital magnetic moment which couples with the spin of the electron. This
increased spin–orbit coupling is believed to enhance the rate of intersystem crossing, thus giving rise to a higher triplet yield.

In case of the halogenated fluorophores, a minor trend can be observed, indicating that halogenation by heavy halogen atoms causes a slight increase in $k_{\text{ISC}}$. The reason why this effect is not more prominent is most likely that the halogenated sites are not in direct contact with the chromophore unit.

Photobleaching properties of the fluorophores were studied with FCS. The fluorophores TP, TR and AT12 are similar in structure and were also found to show very similar photostabilities, when compared to each other. The photobleaching quantum yield $\Phi_B$ of TP was estimated to be $1.3 \times 10^{-4}$. It can be noted that the photostability of TP is thus not as good as that of stable rhodamine fluorophores such as TMR or Rh6G, but still better than that of, for example, coumarins and well comparable to that of Fluorescein, an extensively used in fluorescence microscopy studies.

### 5.2 TRAST spectroscopy for measuring diffusion

As shown recently [Sandén et al. 2007; Sandén et al. 2008], the dependence of the time-averaged fluorescence on the modulation characteristics of the excitation (e.g. duration, power and separation of the pulses within an excitation pulse train) can be used to extract information about the population dynamics of photo-induced, long-lived transient states of fluorophores. Likewise, if the transient state transition properties of the fluorophore are known it is also possible to get information about the modulation characteristics from the fluorescence response. This concept to determine molecular dwell times or diffusion properties can in principle be realized, by utilizing the kinetic properties of a range of transient photo-induced states. However, trans-cis isomerisation (see Section 2.1.6) offers an additional advantage, because it is light driven in both directions. Apart from a slow thermal deactivation by $k_{\text{PN}}$ (figure 2.6B), in the absence of excitation the cis-isomer tends to remain photo-isomerised. The extent of build-up of cis-isomers thus reflects the amount of excitation irradiation that the molecules have been exposed to, or in other words how long time they on average have spent in the excitation volume. By modulation, the distribution of the excitation irradiation in time can be adapted so that the extent of the build-up of the transient state, in our case the cis isomer, is maximally sensitive to changes in the dwell times of the molecules in the excitation volume.

In order to incorporate diffusion to the kinetic scheme of isomerisation presented in figure 2.6B, some simplifications were applied. Transitions between the ground ($N_0$ and $P_0$) and the first excited states ($N_1$ and $P_1$) were disregarded, because
they occur on a very fast timescale of a couple of nanoseconds, and compare to utilized excitation pulse duration of several µs, the steady-state populations are already achieved. The diffusion process is simplified by the chemical reaction with the rate of $k_D$ of molecular exchange between the molecules inside of the excitation volume (in Trans and Cis states) and outside of the volume (“Bulk” state). With these simplifications applied, the kinetic scheme could be reduced to the presented in figure 5.5.

By defining and solving the system of linear differential equations corresponding to the model of Figure 5.5, it is possible to calculate the time-dependence of the fluorescence and population levels of the isomerised states for square wave excitation pulse trains with different pulse characteristics. Representative outcomes of the calculated time development of the fluorescence and the population levels of the isomerised states are shown in figure 5.6.

As can be seen in figure 5.6, the time required for pulse trains with short excitation pulse durations, to generate a steady-state ratio between the trans- and cis- isomers of the fluorescent molecules is longer than that required for longer pulses with the same repetition rate. Fluorescence intensity $F$ within the pulse is thus higher for the shorter pulses, if the number of pulses per molecular passage through the excitation volume is limited. Since fluorescence emission rate is rigorously defined by the excitation and relaxation rates, it will be only the diffusion time which will lead to deviations from the predicted averaged fluorescence.

In figure 5.7 simulations of normalized time-averaged fluorescence intensity ($F_{EXC} = F / \eta$, where $\eta = w / T$) are presented for several values of the pulse width and varying pulse period for stationary and diffusing fluorophore molecules. It can be seen that for molecules diffusing faster through the excitation volume (shorter diffusion times) $F_{EXC}$ is higher, because the molecules experience lower number of excitations per passage. By measuring the average fluorescence within the pulse for different pulse widths and different pulse periods, it should be possible to extract diffusion rate $k_D$ from such decays.
In order to test the proposed model in detail, it is required to perform modulated measurements on fluorescent molecules with different excitation volume residence times. Different residence times can either be achieved by coupling fluorophore Cy5 to molecules of different sizes. However, a more convenient and tunable approach is to vary the residence times by applying different flow speeds in a microchannel using a syringe pump. The flow speeds were analyzed with FCS using the expression

\[ F = k_D \cdot \frac{N_1}{T} \]

**Figure 5.6:** A realization of the solution to the differential equations corresponding to figure 5.5 for relative populations of Trans (subplot B) and Cis (subplot C) isomers, and emitted fluorescence \( F \) (subplot A), which assumed to be proportional to \( N_1 \) population of Trans isomer. During the pulses, a cis-isomer population is built up, which then decays marginally due to thermal deactivation in-between the pulses. Solid lines represent stationary fluorophore and dotted lines - the diffusion-mediated exchange of the molecules into and out of the excitation/detection volume to \( k_D=1/50 \, \mu s^{-1} \).

**Figure 5.7:** Calculated plots, with the time-averaged fluorescence plotted versus the pulse period \( T \), with all pulses having the same pulse width \( w \) of 1.0 µs (blue), 3.0 µs (green) and 5.0 µs (red). Solid lines are calculated for diffusion rate \( k_D=0 \, \mu s^{-1} \), dash lines for \( k_D=1/300 \, \mu s^{-1} \), dash-dot lines for \( k_D=1/200 \, \mu s^{-1} \) and dot lines for \( k_D=1/100 \, \mu s^{-1} \).
Chapter 5: Triplet state & applications

The results are presented in figure 5.8. At low flow rates (few µl/min), the characteristic flow times $t_{flow}$, as obtained from the FCS measurements are lower than the diffusion time $t_D$. $t_{flow}$ is then not representative for the molecular residence times. To estimate the residence times at different flow rates, we therefore instead used the integral of the area of the correlation curve representing diffusion and/or flow:

$$\tau_{residence} = \int_0^\infty G_D(\tau)G_{flow}(\tau)d\tau$$  \hspace{1cm} (62)

Figure 5.8: A. FCS curves for Cy5 in presence of laminar flow in microchannel with fits. Excitation irradiance is 16 kW/cm². Diffusion time $\tau_D = 70\ \mu s$, flow time $t_{flow}$ varies from 230 µs at flow rate 50 µs/min to 10 µs at flow rate 1200 µl/min. B. Observed linear dependence of the flow speed $V = \tau_{flow} / \omega_0$ versus applied flow rate with the syringe pump.

Figure 5.9: A. Measured time-averaged fluorescence $F_{exc}$ from measurement with time-modulated excitation on Cy5 for various flow rates 0-300 µl/min. Pulse width $\nu=2.0\ \mu s$, pulse period $T$ is varied from 5.1 to 300 µs. Curves are normalized to 1 for the longest pulse period of 300 µs. Dotted lines represent the fit according to the proposed model B. Inverse of the diffusion rate $k_D$ obtained as a result of the fit of the data represented in plot A.
where \( G_D(\tau) = \left(1 + \frac{\tau}{t_D} \right)^{-1} \left(1 + \frac{\tau}{(z_0 / \omega_0)^2 \cdot \tau_D} \right)^{-1/2} \)

and \( G_{\text{flow}}(\tau) = \exp \left\{ -\left( \frac{\tau}{\tau_{\text{flow}}} \right)^2 \cdot \left[1 + \frac{\tau}{\tau_D} \right]^{-1} \right\} \)

A series of excitation-modulated measurements was performed on Cy5 in aqueous solution in a microchannel applying different flow rates. The residence times of the fluorophore molecules were varied from 130 µs to ~40 µs, measured by FCS in the same setup, but without excitation modulation, before and after each series of excitation-modulated measurements. For each adjusted flow rate, \( F_{\text{EXC}} \) was measured by excitation-modulated measurements, applying different pulse trains varying the pulse widths, \( w \) (1-5 µs), and the pulse periods, \( T \) (5-300 µs) of the different pulse trains. A significant increase in \( F_{\text{EXC}} \) could be observed with increasing \( T \) for pulse widths shorter than the residence times of the fluorophores (figure 5.9A). The peak power of the pulses was kept constant, corresponding to an excitation irradiance of 24 kW/cm² in the detection volume, for each pulse train series.

The measured \( F_{\text{EXC}} \) and its variation with \( T \) and \( w \) were analyzed by the model displayed in figure 5.5, having all of the rates fixed to values obtained from FCS measurements of freely diffusing fluorophore. Only the diffusion rate (\( k_D \)) was entered as a free parameter. In figure 5.9B, the resulting inverse diffusion rates, \( 1/k_D \), are plotted versus the residence times, \( \tau_{\text{residence}} \), as determined from the corresponding FCS measurements, displaying a linear relationship between \( 1/k_D \) and \( \tau_{\text{residence}} \).

Excitation-modulated measurements were also performed in the same manner on the fluorescent protein DsRed, which shows a prominent dark state relaxation component in FCS curves with relative amplitude of about 30% and with relaxation time in the sub-ms time range. The isomerisation dynamics in DsRed is significantly more complex than in Cy5. As for many fluorescent proteins a multitude of states can be expected, with relaxation times found over a broad time range. We therefore did not apply the model from figure 5.5 to evaluate the DsRed measurements. Nonetheless, the normalized average fluorescence \( F_{\text{EXC}} \) versus the pulse train period was found to follow the same increase as in Cy5 measurements. Consequently, this supports the applicability of the proposed method for protein mobility studies in live cells using fluorescent proteins.
Chapter 6

TRIPLET STATE & SURFACE EFFECTS

In the majority of fluorescence techniques one is interested in maximizing the fluorescence output per emitter. This can be done in various ways, like using antioxidative additives in order to decrease photobleaching (Chapter 3), decreasing excitation irradiances in order to decrease triplet state build up, introducing modulated excitation with low duty cycle to allow the fluorophores to relax from the triplet state before the next excitation will occur. The last two approaches lead to increased measurement times, which is disadvantageous. As the triplet state plays an important role in the saturation of the fluorescence emission, the finite photodegradation lifetime of the fluorophore, and the production of highly reactive oxygen species, it is important to investigate triplet state kinetics under different experimental conditions. In TIR-microscopy the excitation field is inevitably confined to an interface of two media with different refractive indexes, and the presence of this interface can influence the photophysical processes of the fluorophore – resulting in a decreased or an increased fluorescence rate, depending on the type of the interface. Paper VI addresses specifically the influence of the interface on triplet state rates.

Another property of the interface – electrical charge – is of primary importance for better understanding of interfacial dynamics when it comes to interactions of charged particles. Since many of the commonly used fluorophores carry electrical charge (positive, negative or both), electrostatic interactions will inevitably influence their diffusion, and therefore fluorescence. This effect, which may be consid-
Chapter 6: Triplet state & surface effects

...erated undesirable, can be taken advantage of and used for probing the electrostatic properties of a charged surface. TIR-FCS is a well suitable technique for analysis of diffusion in the vicinity of the interface. Results of such investigations and analysis, published in paper VII, provide an increased understanding of how fluorophores are influenced by the microenvironment of a dielectric surface, and show a promising approach for characterizing electrostatic interactions at interfaces in general.

6.1 Triplet state kinetic rates at dielectric interfaces

To determine the rate constants for intersystem crossing, \( k_{\text{ISC}} \), and the decay of the triplet-state, \( k_T \), the TIR-FCS autocorrelation function is collected at different excitation intensities – so called “power series”. In figure 6.1, the resulting TIR-FCS autocorrelation functions for Fluorescein measured at three different excitation intensities are shown. The measurements show contributions from triplet-state kinetics and free diffusion. To highlight the kinetics of the triplet state, \( G(\tau) \) has been normalized to one at short lag times, \( \tau \). This makes it clearly visible that the triplet fraction \( T \) increases and triplet relaxation time \( \tau_T \) decreases with increasing excitation intensities.

![Figure 6.1](image)

Figure 6.1: Normalized correlation curves of Fluorescein in TRIS buffer (pH = 8.2 and 150 mM NaCl) at three different laser excitation powers and the corresponding TIR-FCS fits using equation (49.)

The data from figure 6.1 are fitted with the correlation function given by expression (49) to extract the experimental values of \( T \) and \( \tau_T \). By plotting the experimentally determined \( T \) and \( \tau_T \) values as a function of excitation irradiances and simultaneously making nonlinear least square fits to equations (39) and (40), the unknown \( k_{\text{ISC}} \) and \( k_T \) rates are determined (shown in figure 6.2).
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To be able to compare the intersystem crossing and the triplet decay rates deduced by TIR-FCS, with and without the glass surfaces, FCS measurements of the same sample were also performed with a confocal setup, using the original approach introduced in [Widengren et al. 1995]. Obtained rates for the investigated fluorophores are presented in table 6.1

From table 6.1 it can be seen that the triplet rates $k_{\text{ISC}}$ and $k_T$ measured with TIR-FCS are slightly higher than the reference rates determined by confocal FCS.

**Table 6.1:** Obtained values of the triplet rates for Fluorescein (in TRIS buffer, pH=8.2 150 mM NaCl) and ATTO 488, Rhodamine 110, and Rhodamine 123 (in PBS buffer, pH=7.4).

<table>
<thead>
<tr>
<th>fluorophore</th>
<th>TIR-FCS</th>
<th>confocal FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{ISC}}$ ($\mu$s$^{-1}$)</td>
<td>$k_T$ ($\mu$s$^{-1}$)</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>13.5 ± 1.0</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>ATTO 488</td>
<td>1.7 ± 0.1</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Rhodamine 110</td>
<td>1.3 ± 0.1</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>Rhodamine 123</td>
<td>1.2 ± 0.1</td>
<td>0.58 ± 0.04</td>
</tr>
</tbody>
</table>

**Figure 6.2:** The measured triplet-state parameters, $\tau_T$ (filled squares) and $T$ (filled circles), and the corresponding weighted and spatially averaged global fits for: Fluorescein (A), ATTO 488 (B), Rhodamine 110 (C) and Rhodamine 123 (D).
A reason for the higher rates at the surface could be the underestimation of the axial extent of the excitation, \( h \). Also an underestimation of the magnitude of the evanescent field would lead to an overestimation of the rates. In addition, the values used for the excitation intensity distribution also depend on the saturation properties of the dye. In confocal FCS, high laser powers often generate distortion of the emission profile [Hess and Webb 2002], which may lead to underestimation of the \( k_{\text{ISC}} \) rates. Furthermore, high excitation irradiance opens up additional relaxation channels, like reverse intersystem crossing that may lower the \( k_T \) values [Ringemann et al. 2008]. The confocal reference rates might therefore be slightly underestimated.

The relatively low irradiances used in TIR-FCS do not introduce the problems just mentioned. However, the fluorescence lifetime, \( \tau_F \), used in calculating the triplet rates should probably be modified slightly. The reason for this is that the interface can modify the electromagnetic decay channels of the fluorophore [Fort and Grésillon 2008]. For a bare dielectric surface this can lead to a decrease in fluorescence lifetime by 5-10% compared to that in bulk solution. To take this into account, the determined rates for \( k_{\text{ISC}} \) of Fluorescein, ATTO 488, Rhodamine 110, and Rhodamine 123 should be increased about five to ten percent.

When the corresponding measurements and analysis were done for Rhodamine 6G in concentration 50 nM, much higher triplet rates in TIR-FCS were obtained (\( k_{\text{ISC}} = 1.6 \pm 0.2 \mu s^{-1} \) and \( k_T = 1.2 \pm 0.04 \mu s^{-1} \)), compare to reference confocal FCS values of \( k_{\text{ISC}} = 1.1 \pm 0.2 \mu s^{-1} \) and \( k_T = 0.49 \pm 0.05 \mu s^{-1} \). This indicates that there is an interface-induced effect which influence the triplet-state kinetics of Rhodamine 6G. To investigate this further we decided to change the concentration of the Rh6G sample. Figure 6.3 shows a comparison of the TIR-FCS triplet-state rate analysis of \( \tau_T \) and \( T \) at sample concentrations of 1 nM and 50 nM in PBS. As it is clearly visible, lower concentration generates longer triplet relaxation times and higher triplet amplitudes.

Among possible reasons for the concentration-dependence of the triplet rates for Rh6G, hydrophobicity-induced interactions with the surface opening new efficient deactivation channels may be considered [Mialocq et al. 1991]. Formation of aggregates is often accompanied by a change in the radiative and radiation-less transition probabilities. Furthermore, aggregates in the form of adsorbed monomers, dimers, and trimers have been shown to play an important role in long range energy and electron-transfer mechanisms for freely diffusing Rh6G molecules [López Arbeloa et al. 1988]. The strong spin-orbit coupling induced by aggregates through spreading in their singlet energy levels can actually increase the intersystem crossing rates, as seen above. Shortening of the triplet relaxation times, via triplet-triplet annihila-
tion, may additionally be enhanced by surface adsorbed aggregates [Bryukhanov et al. 1978].

Another dye-solvent effect that is of interest is the well-known property of fluorescence quenching by potassium iodide (see Chapter 4). The effect is shown in figure 6.4. Addition of 5 mM iodide give rise to large triplet populations and fast triplet relaxation times, which transform into increased measured triplet rates.

In contrast to the bulk solution case, the extracted $k_{\text{ISC}}$ and $k_{\text{T}}$ rates do not show a linear dependence over the whole concentration range. The trend of getting similar rates with TIR-FCS and confocal FCS decreased as the iodide concentration is lowered. This can be attributed to influence from aggregates that enhance the triplet rates and to changes in saturation and bleaching properties of Rh6G at the surface.

Here it was generally assumed that all Rh6G fluorophores are photochemically intact and no photobleaching may occur. The saturation and photobleaching properties of the fluorophore under investigation can differ considerably with different environmental parameters. The ratio of the intersystem crossing rate and the triplet relaxation rate actually gives an estimate on how prone the dye is to photobleaching. Addition of potassium iodide greatly increases this ratio, which leads to increased
Chapter 6: Triplet state & surface effects

photodestruction probabilities. Computer simulations of how the concentration of Rh6G molecules in the sample volume is depleted in the presence of bleaching are shown in figure 6.4C. A strong depletion of molecules within the observation volume and its surroundings is predicted.

In the vicinity of the surface, high triplet-state buildup of Rh6G due to very high excitation irradiances is further increased upon addition of potassium iodide. This inevitably leads to promoted photodestruction of fluorophores at the interface, which makes the overall concentration depletion even larger. Figure 6.4D shows the saturation of detected fluorescence in the observation volume when adding potassium iodide. When all of these effects are taken into account, there is a resulting reduction in the relative fluorescence contributions from Rh6G molecules close to

Figure 6.4: **A.** normalized correlation curves of Rh6G in PBS buffer without and with addition of 5 mM KI, and the corresponding TIR-FCS fits to equation at maximum laser excitation power. **B.** triplet state parameters for 50 nM Rh6G + 5 mM KI with the corresponding weighted and spatially averaged global fits, yielding $k_{ISC} = 24.4 \pm 1.5 \mu s^{-1}$ and $k_T = 0.71 \pm 0.04 \mu s^{-1}$. **C.** Simulations of concentration changes of Rh6G molecules in the axial direction without and with addition of KI (2 mM and 5 mM) at maximum laser excitation power. **D.** The axial detected fluorescence profiles in the observation volume. Note the large fluorescence saturation effects in the vicinity of the interface with high potassium iodide concentrations.
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Table 6.2: Obtained values of the intersystem crossing rates, \( k_{\text{ISC}} \), and the triplet relaxation rate, \( k_T \), for Rhodamine 6G with and without KI.

<table>
<thead>
<tr>
<th>[KI] (mM)</th>
<th>( \tau_f^{-1} ) (( \mu \text{s}^{-1} ))</th>
<th>( k_{\text{ISC}} ) (( \mu \text{s}^{-1} ))</th>
<th>( k_T ) (( \mu \text{s}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>253</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>0.2</td>
<td>254</td>
<td>1.6 ± 0.1</td>
<td>0.92 ± 0.06</td>
</tr>
<tr>
<td>0.5</td>
<td>257</td>
<td>3.5 ± 0.3</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>267</td>
<td>10.8 ± 0.7</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>284</td>
<td>24.4 ± 1.5</td>
<td>0.71 ± 0.04</td>
</tr>
</tbody>
</table>

the surface compared to those further away. This leads to a bias in the correlation analysis towards non-interfacial Rh6G molecules, which may explain the similar triplet rates seen with TIR-FCS and confocal FCS at high potassium iodide concentrations (table 6.2).

6.2 Electrostatic interactions at dielectric interfaces

When a solid material is in contact with an aqueous solution, a thin interfacial charge layer is formed, which consist of surface charges and charge balancing counterions. The presence of the surface charges causes a rearrangement of the ions in the vicinity of the surface. This arrangement of a nonzero net charge at the solid-liquid interface is usually referred to as the electric double layer. In the electric double layer, two regions of charge distributions may be identified. Immediately next to the charged surface, counterions are bound to the surface due to strong electrostatic attraction. Outside of this immobile layer counterions may move, meaning that they can diffuse in the potential set up by the partially screened surface. The immobile region is often referred to as the compact layer or the Stern layer, and the mobile region – as the diffusive layer. The electrostatic potential at the boundary dividing the compact layer and the diffusive layer is the so-called zeta (\( \zeta \)) potential [Delgado et al. 2005]. A schematic model of the electric double layer on a glass surface is shown in figure 6.5.

On uncoated glass surface, acidic silanol groups acquire a negative charge (SiO\(^-\)) at neutral pH. This negative surface charge density is balanced by counterions attracted to the surface and diffusive ions counterbalancing the potential set up by the partially screened surface. On uncoated hydrophilic surfaces such as glass, the surface potential has been shown to be indistinguishable from the zeta potential [Bouzigues et al. 2008].
How rapid the surface potential drops is connected to the Debye-Hückel parameter [Ohshima 2006], which for dilute electrolytes can be expressed as \( \kappa^2 = \frac{2\chi e^2}{\varepsilon \varepsilon_0 k_B T} \). Here \( \chi \) is the ionic strength of the solution, \( e \) is the elementary charge and the parameters \( \varepsilon \) and \( \varepsilon_0 \) are the permittivity of the electrolyte and that of vacuum, \( k_B \) is the Boltzmann constant and \( T \) is the absolute temperature. The

**Figure 6.5:** Schematic model of electric double layer. The negative surface charges (−) are counterbalanced by bound and free ions (+). The electrostatic surface potential is plotted for salt concentrations of 100 mM, 10 mM, 1 mM, and 0.1 mM, which gives Debye lengths of 0.95 nm (black curve), 3 nm (blue curve), 9.5 nm (green curve), and 30 nm (orange curve), respectively. The evanescent penetration depth is for comparison plotted in the red curve.

**Figure 6.6:** A. Autocorrelation curves for the cationic Rhodamine 123 at ionic strengths of 100 mM, 10 mM and 0.1 mM. To visualize the effect of the electrostatic interactions on the diffusion time, the curves have been normalized. B. The unnormalized TIR-FCS curves used to visualize changes in concentration of molecules in the vicinity of the glass surface.
reciprocal of $\kappa$ correspond to the fundamental length scale of the surface potential, or in other words to the thickness of the electric double layer. This so-called Debye screening length, $z_D$, depends on the ionic strength of the solution, $\chi$, and can at room temperature be expressed as $\kappa^{-1} = z_D = 0.3 / \sqrt{\chi}$ in units of nanometer. Figure 6.5 shows the surface potential at the different ionic strengths, together with the evanescent excitation profile given by the $z$-dependent part of equation (41). The electrostatic surface potentials are plotted according to Gouy-Chapman theory that assumes it to decay exponentially with distance to the interface [Ohshima 2006]:

$$\Psi(z) = \Psi_0 \exp\left(-z/z_D\right).$$

The influence of the surface potential on the molecular motion is illustrated in figure 6.6, which shows the TIR-FCS autocorrelation curves for cationic Rhodamine 123 interacting with the negative glass surfaces at different ionic strengths. Note an increased decay-time (figure 6.6A) with decreasing ionic strength (meaning a less screened surface potential). Figure 6.6B shows how the concentration of molecules, in the vicinity of the surfaces, increases as the ionic strength decreases from 100 mM to 0.1 mM. This is reflected in a decrease of the amplitude of the correlation curve. Both the shift in amplitude, as well as the shift in decay-time of the correlation curves, clearly shows that the positively charged rhodamine 123 is attracted to the negatively charged dielectric surface.

Figure 6.7 shows all the parameters extracted from TIR-FCS measurements for Rhodamine 123. At low ionic strength cationic fluorophore molecules are attracted towards the negatively charged surface, which results in increased local concentration (measured via number of molecules $N$), increased axial passage times – the fluorophore spends more time closer to the surface, and increased CPMs and triplet amplitude – due to on average higher excitation irradiances experienced.

Assuming a Poisson-Boltzmann description for the number of fluorophores (at thermal equilibrium) in the vicinity of the surface, the one-dimensional concentration distribution at the surface may be approximated as:

$$C(r) = C(z) = C_B \times \exp(-qe\Psi(z)/k_B T).$$

Here $q$ is the charge value of the fluorescent molecule probing the screened glass surface ($q = +1, \pm 0, -1, -2$) and $C_B$ is the concentration in the bulk (that is far away from the surface). In TIR-FCS, the number of molecules is determined exclusively by the concentration profile, $C(z)$, and the dimension and shape of the detection volume. While there are no (well-defined) border of the detection volume, its ab-
Figure 6.7: Parameters extracted from TIR-FCS measurements on Rhodamine 123 as a function of ionic strength. A. The averaged number of molecules, $N$ (triangles) and the ratio of molecules, $N_x = N/N_B$ (pentagons). B. The deduced mean surface potential of the electric double layer. C. The axial passage-time, $\tau_z$. D. The counts-rate-per-molecule, CPM. E. The triplet amplitude, $T$. F. The triplet relaxation time, $\tau_T$.

Solute size is exactly defined and corresponds to the volume containing $N$ molecules as defined via equation (49), that is $C'(z) \propto N = [G(0) \times (1 - T)]^{-1}$. Similarly, the bulk values $N_B \propto C_B$ can be approximated with the number of molecules found in the volume at a “fully” screened surface.
Assuming negatively charged glass surfaces, depending on the charge of the fluorescent molecule, three cases of concentration variations can thus be identified and presented in figure 6.8. No concentration changes at all (\(N = N_B\)) should be generated when \(q = 0\). Fluorescent molecules having a positive \(q\)-value should be attracted towards the surface (\(N > N_B\)), whereas a negative \(q\)-value would...
lead to repelling of molecules by the surface ($N$ smaller than $N_B$). By measuring the excess (or deficit) of molecules with TIR-FCS, a mean surface potential (figure 6.7B) may thus be evaluated for each ionic strength via equation (63).
CONCLUDING REMARKS

This thesis focuses on investigations of transient dark states in fluorescent molecules using spectroscopic techniques. The main purpose is to show and convince the reader that transient dark states are not always a nuisance, but also represent an additional valuable source of information. Knowledge about origins, properties and dynamics of transient states could be used as a mean to either utilize these states, or eliminate them.

In paper I, it was shown that with the right strategy and using on-shelf additives, it is possible to significantly reduce photodegradation of fluorophores. Thus, higher signal and longer observation times in fluorescence imaging and spectroscopy can be achieved. The balance between reduction of ionized fluorophores and reduction of intact fluorophores should be an important guideline for selecting the concentration ranges to be applied. Adjusting the properties of potent available additives by introducing new chemical groups with desired effects is the way for improving performance of the anti-bleaching agents. This would not be possible without the detailed characterization of the process of fluorescence blinking.

In paper II triplet state quenching was investigated. Potassium iodide, a compound commonly used as a fluorescence quencher, turned out to be a fluorescence promoter for several fluorophores. The underlying mechanisms were investigated, and apart from the heavy atom effect, an electron transfer reaction was identified. This electron transfer reaction was found not only to affect the triplet state, but also the photo-oxidized as well as the fluorescently viable fluorophore molecules in their singlet states. The kinetic transition rates and their KI concentration dependence
Concluding remarks

were determined, providing information for which fluorophores and in what concentrations KI can be added such that primarily triplet state and other non-fluorescent states of the fluorophores are deactivated, rather than the fluorescently viable forms of the fluorophores. Based on this information, and when added in balanced concentrations, KI can be used as a fluorescence promoter and anti-fading compound. In addition, the fact that KI acts differently on different fluorophores and fluorophore states suggests that KI can be used as a contrast enhancement mechanism in biomolecular dynamics and interaction studies.

In paper III quenching of fluorescence with the nitroxide radical TEMPO was investigated in aqueous solution. This resulted in the use of this quencher for monitoring bimolecular reactions in lipid membranes. The high environmental sensitivity of the triplet state combined with the excellent detection sensitivity of the fluorescence read-out meets all prerequisites for exploitation of triplet quenching for monitoring different processes.

In paper IV special purpose fluorophores were developed and characterized for possible use in super-resolution imaging techniques based on photoswitching. Due to the involvement of the triplet state in the photobleaching mechanisms, the fluorophores were required to combine high triplet yield with reasonable photostability. Of the dyes investigated, the rhodamine and the pyronin dyes with sulfur atom replacing the central oxygen atom in the xanthene unit were found to meet these requirements. Thiorhodamine with a coupling functionality became available commercially as ATTO Thio 12.

In paper V photoinduced switching to non-fluorescent states was used for monitoring molecular diffusion. Since fluorescence emission is rigorously defined (on a timescale of $\mu$s) by the kinetic excitation and relaxation rates, factors influencing fluorescence emission in a systematic way (like Brownian diffusion) could be monitored and measured, given the right model of the underlying kinetics. The proposed approach provide advantages such as simple instrumentation, scaling possibility and wide range of accessible concentrations. Feasibility of the proposed method for protein mobility studies in live cells using fluorescent proteins was demonstrated.

In papers VI and VII studies of the triplet state kinetics of fluorophores at dielectric interfaces were performed. These studies are important due to the involvement of the triplet state in the saturation of fluorescence emission and photodegradation of the fluorophore. Better understanding of the fundamental photophysical processes is a prerequisite for optimization of conditions for ultrasensitive fluorescence microscopy. For all of the fluorophores studied, higher triplet rates were found. This was attributed to possible modifications of photophysical properties, occurring at
dielectric interfaces. Specific interface-induced effects were observed for fluorophore Rhodamine 6G, dependent on the concentration of the fluorophore and the ionic strength of the solvent. Also, the analysis of the triplet state kinetic can provide information about local microenvironment and electrostatic interactions near dielectric interfaces.
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